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JOINT DANUBE SURVEY 4

JOINT DANUBE SURVEY 4 SCIENTIFIC REPORT:

A SHARED ANALYSIS OF THE DANUBE RIVER

ICPDR IKSD

International Commission for the Protection of the Danube River

Internationale Kommission zum Schutz der Donau

Editors

Igor Liška, Franz Wagner, Manfred Sengl, Karin Deutsch, Jaroslav Slobodník and Momir Paunović

This report contains an overview of the scientific findings of the Joint Danube Survey 4 (JDS4).

For a number of chapters, more detailed information and data is available via the full extended report to be found on *www.danubesurvey.org/jds4/full-report*.

A map showing the locations of all JDS4 sampling sites can be found at the end of this report.

The authors wish to thank all those who made JDS4 possible and carried out this unique international survey – including national delegations to the ICPDR from throughout the Danube River Basin, core team members, national coordinators and national teams, supporting experts and laboratories, as well as donors and sponsors.

Although it's not possible to list every individual who contributed to JDS4, they will recognise themselves within the pages of this report, and we acknowledge their efforts and expertise.

Made possible with the generous financial support of

Umwelt Bundesamt

Federal Ministry Republic of Austria Agriculture, Regions and Tourism

Life

ICPDR Contracting Parties

Austria, Bosnia & Herzegovina, Bulgaria, Croatia, Czech Republic, European Union, Germany, Hungary, Moldova, Montenegro, Romania, Serbia, Slovakia, Slovenia, Ukraine

Many thanks also to

viadonau, Coca-Cola Hellenic Bottling Company, patagonia, PURE WATER FOR GENERATIONS

Imprint

Published by: ICPDR – International Commission for the Protection of the Danube River
Publishing Coordination: Hélène Masliah-Gilkarov
Language Editing: Tristan Bath
Layout & Design: Ivo Monnerjahn, www.lmp.wien
Illustration: Büro X, www.buerox.at, Tobias Held, www.thisisteam.com
Photo credits: ICPDR/JDS4

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01 Together for a cleaner and healthier Danube: Joint Danube Survey 4

Igor Liška (ICPDR, Vienna, Austria)

The TransNational Monitoring Network is an important tool under the Danube River Protection Convention (DRPC), whose Contracting Parties are committed to co-operate in the field of monitoring and assessment of water quality. Formally launched in 1996, the TNMN aims to provide a well-balanced overall view of pollution and long-term trends in water quality and pollution loads in the Danube and its major tributaries.

The EU Water Framework Directive (WFD) requires that countries in the Danube River Basin periodically assess certain water characteristics in their territory. The DRPC sets out processes, requirements and goals for cooperation throughout this assessment process.

With the view to obtaining a complex picture of the water quality in the Danube and its major tributaries, the yearly assessment of water quality published in TNMN Yearbooks has been supplemented by periodic investigative surveys, which are carried out every six years in sync with the river basin management planning period according to the EU WFD.

The first Joint Danube Survey was carried out in 2001. For the first time, comparable data about the entire course of the river was provided covering over 140 different biological, chemical and bacteriological parameters. This data was used as an essential information source for the first analysis of the Danube River Basin District according to Art. 5 of the EU WFD. Six years later, the second Joint Danube Survey (JDS2) created a comprehensive and homogeneous database on the status of the aquatic ecosystem of the Danube and its major tributaries. For the first time, the fish survey was carried out along the entire Danube River, bringing a unique dataset and also contributing to methodological harmonization between EU and non-EU countries. The findings of JDS2 contributed to the first Danube River Basin Management Plan and were used in the EU intercalibration process of large rivers.

The third Joint Danube Survey (JDS3), which took place in 2013, provided the largest ever amount of knowledge about the Danube water pollution collected within a single scientific exercise. It reconfirmed that the Danube flora and fauna show a high degree of biodiversity. During JDS3, the depth of information on hydromorphological conditions was significantly improved, as in-situ measurements of hydrological, morphological and hydraulic characteristics were performed for the first time along the entire Danube and its tributaries. The first complex testing of antibiotic resistance was carried out along the entire stretch of the Danube River. Several new analytical techniques and strategies were applied targeting hundreds of organic substances, resulting in the most comprehensive information ever acquired on this topic for the Danube River. The analysis of such a large amount of organic substances enabled the first suggestions for the update and prioritization of Danube River Basin Specific Pollutants.

As a result, the signatories of the Danube Declaration (adopted at the 2016 ICPDR Ministerial Meeting) appreciated the very valuable scientific results of the third Joint Danube Survey in 2013 as well as its considerable effect on awareness raising for the ICPDR, requested the ICPDR to prepare, based on an evaluation of the previous surveys, a fourth Joint Danube Survey to be held in 2019, and committed to secure the necessary funding.

Joint Danube Surveys are planned and supervised by the ICPDR Monitoring and Assessment Expert Group (MA EG). When the MA EG experts evaluated the previous three Joint Danube Surveys, a common pattern was discerned: a Core Team of leading experts was responsible for the completion of all sampling jobs also undertaking analysis of samples in the case of biology, microbiology and hydromorphology. National experts only played a supporting role during this process, joining the Core Team in an observer role only when being in their respective countries (sometimes also providing assistance to the Core Team). Following reassessment of the previous approach, the ICPDR decided that JDS4 should be based on more active participation from countries. It was decided that most fieldwork and sampling should be carried out by national experts while the Core Team should have a coordinating and advisory role to ensure coherence between the approaches used by the national experts. This more active deployment of national experts put a higher burden on countries but resulted in a very intense monitoring exercise, which not only generated another huge amount of data but also significantly strengthened both cooperation and coordination between the countries in the Danube River Basin.

To make sure that the methods used by the national experts in biology would provide comparable results, training workshops for each biological quality element were organized prior to JDS4. The national experts responsible for sampling and assessment of the EU WFD biological quality elements (BQEs) took part, together with the respective Core Team members. This was the first time ever when the experts on all EU WFD BQEs from all ICPDR Contracting Parties met to discuss monitoring and assessment harmonization issues. It was already this overture to JDS4, which demonstrated the significant benefits of the new JDS concept.

As before, the key objectives of JDS4 were decided to include producing comparable and reliable information on a wide range of water quality elements for the whole of the length of the Danube River including the major tributaries on a short-term basis. The other key objectives were to provide an opportunity for harmonization and training in WFD-related monitoring and to cover the information gaps for the Danube River Basin Management Plan Update 2021.

JDS4 has provided a great deal of added value to the current monitoring practices in the Danube River Basin. The following benefits can be highlighted:

- Independent basin-wide platform for improving national surface water monitoring practices;
- Practical joint testing and comparison of national methodologies for biological and hydromorphological quality elements leading to their future harmonization;
- Interactive platform for hands-on training in sampling and assessment of biological quality elements;
- A unique source of data for a number of quality elements (especially for emerging substances) for the whole Danube;
- Knowledge transfer between EU and non-EU member states.

The key advantages of the new approach used for JDS4 are:

- Reaching a higher level of cooperation in the Danube River Basin. A shift from country experts watching how the leading experts do the job towards the job being done by the countries;
- An excellent opportunity for all ICPDR Contracting Parties to demonstrate in practical terms the cooperation towards better water quality;
- ICPDR Contracting Parties, which are not sharing the Danube main course (Czech Republic, Slovenia, Bosnia and Herzegovina) were given the opportunity to be fully-fledged participants in JDS4;
- This new concept did not require an expensive ship deployment. Monitoring by cars and boats enabled more cost-effective sampling in the whole Danube River Basin as well as more flexible sampling patterns allowing to choose optimal conditions for sample collection. Substantially increased flexibility of the survey logistics helped to solve the logistical problems concerning sampling under bad weather conditions, which caused dangerous situations during previous surveys. The flexible set-up enabled sampling of groundwater and wastewater as well;
- Strengthened ownership: carrying out the significant part of sampling activities and of biological analysis increased the ownership of JDS4 results by the ICPDR countries;
- Strong training, educational and harmonization value of the new concept: JDS4 provided an additional contribution to the intercalibration exercise as defined by the EU WFD;
- Establishing close links between national and international monitoring programs;
- Active involvement of all participants led to a high spirit of cooperation, which engaged more people, being an important mobilizing factor for the ICPDR Contracting Parties to put more support into the project;
- The new concept enabled linking of JDS4 monitoring to national surveillance monitoring, which is obligatory for each EU Member State once every 6 years. The countries had the possibility to synchronize their national surveillance monitoring with JDS4 and to therefore provide a significant in-kind contribution to JDS4 at no extra cost;
- It conveyed a very strong message that the Danube countries had entered a higher level of international cooperation and were ready to carry out ground-breaking special JDS4 monitoring by themselves using harmonized methods.

Post-JDS4 discussions among ICPDR experts saw overall positive feedback on the new JDS4 concept. The new approach was found successful in terms of national and international exchange of experiences and harmonization in sampling methods. The training and harmonization workshops were found to have been very helpful. The new JDS4 spirit created much stronger national activities and engagement amongst concerned authorities and their staff. All standard operating procedures were found to be detailed and effective reference documents for the sampling procedure.

As with previous surveys, JDS4 was not only an important source of information on Danube water quality for the ICPDR, but also presented an excellent opportunity for public awareness-raising for a healthier and cleaner Danube among the people who live in the Danube River Basin and beyond. The Communication Strategy for JDS4 was carefully prepared by the ICPDR's Public Participation Expert Group (PP EG), including graphic design, unique branding and a new logo. This graphic identity was deployed online and presented visibly at public events relating to JDS4. This helped to give a sense of purpose amongst the various teams working on JDS4 by unifying them behind a single graphic identity regardless of their role or location. The JDS4 motto 'Discover Danube', designed as a call to action, was also utilized as a key part of the branding, positioned readably in text, and re-used online in social media and elsewhere whenever possible to underline

the message. A set of fish cards to be used by both experts and the interested public and schoolchildren alike was designed and produced as a streamlined and field-ready resource to assist in the identification of fish species in the Danube River. A special animated JDS4 video also contributed to enhancing the public perception[1]. The massive use of social media for promoting JDS4 as the ICPDR's flagship activity helped to increase the public visibility of this monitoring exercise substantially. Furthermore, Joint Danube Surveys have a dedicated website (www.danubesurvey.org).

JDS4 was significantly affected by the pandemic of coronavirus disease in Europe in 2020. The COVID-19 lockdown had fortunately no impact on sampling activities but it affected the laboratory work leading in many cases to delayed delivery of draft manuscripts. The ICPDR recognized the special efforts made by the authors of the JDS4 Final Report, in analysing JDS4 samples and evaluating and discussing the generated data under COVID-19 restrictions, and appreciated their enthusiasm in trying to minimize effects on the reporting plan.

It is important to note that the enhanced fourth Joint Danube Survey was only made possible thanks to the joint commitment and enthusiasm of all ICPDR Contracting Parties. The financial support of Germany, Austria and the EU as well as the numerous in-kind contributions by the ICPDR Contracting Parties in terms of sample collection and laboratory analyses of physico-chemical parameters and biological quality elements are highly appreciated. Significant scientific and laboratory support was also provided by the EC Joint Research Center (JRC) in Ispra, the German Federal Environment Agency (Umweltbundesamt), the NORMAN Association (Network of reference laboratories, research centers and related organizations for monitoring of emerging environmental substances), Medical Universities in Vienna and Graz, Technical University of Vienna and University of Vienna. A substantial part of (e)DNA-based analyses were provided as an in-kind contribution by DNAqua-Net. In-kind analyses of target pollutants were provided by Water Research Institute in Bratislava; National Laboratory of Health, Environment and Food in Maribor; Bavarian Environment Agency in Augsburg and Povodi Moravy in Brno. Additional financial support was received from viadonau, Coca-Cola Hellenic, Pure Water for Generations (PWG) and Patagonia.

Our gratitude goes to all ICPDR Contracting Parties, institutions, governmental officials, experts, stakeholders and other "friends of the Danube" for their commitment, enthusiasm and contributions, without which JDS4 would not have been such a successful adventure.

[1] *https://youtu.be/il1Xw58kQ94*

02 Survey logistics

Jaroslav Slobodnik (Environmental Institute, Kos, Slovakia)
Alexander Höbart (ICPDR, Vienna, Austria)

Sampling sites

JDS4 was organized on the Danube River including its major tributaries, with a sampling programme focused on 51 sites nominated by the MA EG. The sites comprised TNMN sites, JDS3 sites and sites for national surveillance monitoring in 2019. Seven additional groundwater sites and 11 urban wastewater treatment plants (WWTPs) were nominated by the GW TG and PM EG, respectively. For locations of the sampling sites, see the JDS4 Overview Map in this report.

A set of 24 so-called 'super sites' was selected for special chemical analyses of large volume (LV) water samples collected by Special Longitudinal Survey Teams (SLSTs). Additional sample volumes were collected from three of these 'super sites' (JDS4-6, JDS4-24, JDS4-47) for collaborative trials on testing of performance of nontarget screening techniques and effect-based monitoring tools. The details are available in Chapters 26, 28, 29 and 30.

The Microbiology Team collected samples from 36 sites, some of which did not fully match the 51 JDS4 sites. The specific reason was that their programme required highly polluted sites, with preference to be as close as possible to the sources of pollution or to maintain continuity with sampling from JDS3. For more details, see Chapters 19 and 20.

The microplastics suspended particulate matter (SPM) samples were collected by National Teams from 15 sites selected from the main JDS4 sampling programme (51 sites) with additional samples from three special locations (Brno, CZ; Tisza Uzh, UA; Sava, RS). For more details, see Chapter 42.

The Passive Sampling Team installed a battery of samplers at nine sites for 100 days, with the consideration that at the same sites also fish samples will be analysed. For more details, see Chapter 32.

Subsets of fish and molluscs samples were collected for chemical and microplastics analyses. The details are described in Chapters 24, 29, 30 (chemical analyses) and 44 (microplastics).

A list of the JDS4 sampling sites with an overview of samples actually taken for analyses of various parameters described in this report is available in Annex 1. In total, more than 1700 individual samples were collected for the follow-up analyses.

Technical programme

During the sample preparation, detailed information on each sampling site was provided in the JDS4 site information sheets, with a basic description of each site (Name, Sharing countries, Latitude, Longitude, River, River Kilometres) as well as information on planned sampling date, sampling location and sample matrix for each JDS4 sample to be collected at that particular site. All sampling teams were encouraged to record the exact information on any sample already taken in the field using a mobile application developed for JDS4 (see Chapter 3).

Sampling containers, chemicals and materials needed for sampling of samples to be analysed in the JDS4 reference laboratories were purchased by the ICPDR and delivered in a box to the National Teams prior to each part of the survey (surface, groundwater, wastewater). Each sample vessel was pre-labelled using a harmonised JDS4 coding system (see Annex 2). As an example, an infographic of the content of one of such boxes is in Figure 1. The box contained also Standard Operating Procedures (SOPs) on how to sample each of the different sample types and how to transport the samples to the reference laboratories by a centrally organised courier service, or how to store the samples prior to the transport by a car shuttle service. Special care was taken to keep the samples under temperature-controlled (cooled, frozen) conditions during the storage and transport to avoid their degradation. A fleet of cars equipped with cooling facilities was employed for long-distance transport and special microchips were used for monitoring temperature during transportation.

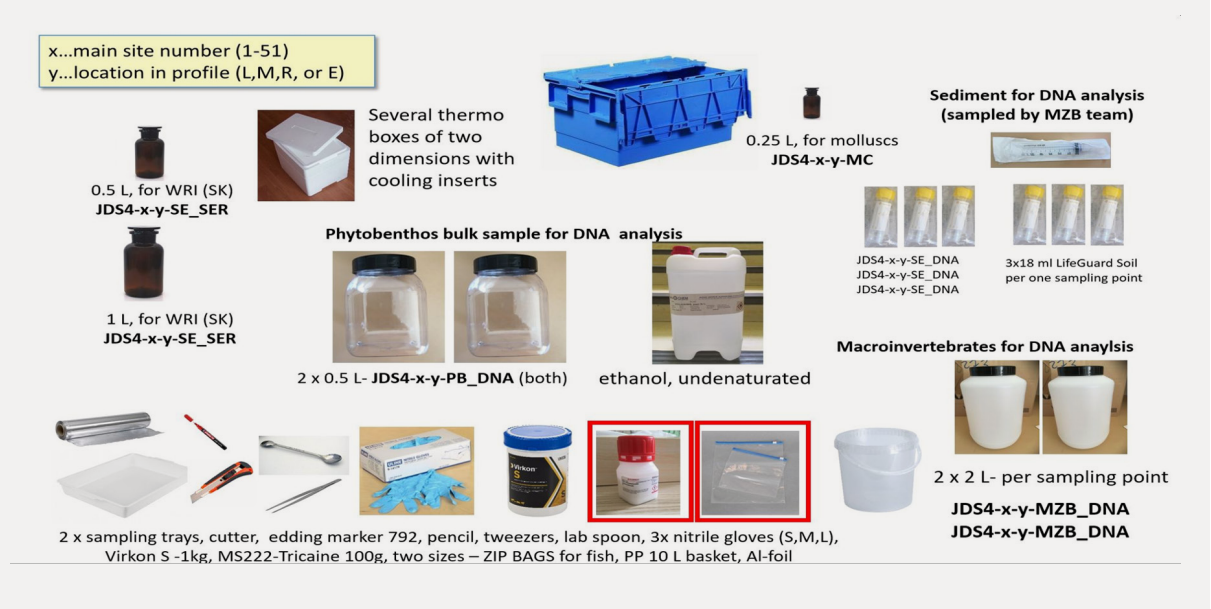

Figure 1: An example of infographic explaining the content of the box with sampling vessels, chemicals and materials distributed to the National Teams prior to the survey.

JDS4 Teams

JDS4 was organized in a different way when compared to previous surveys. The major part of the sampling during JDS4 was accomplished by the national experts while the Biology Core Team and Chemistry Experts focused on methodological coordination and advisory to ensure coherence between the approaches used by the national experts. The Management and Support and Administrative Teams took care of the project management, political backup, data collection and public awareness. The National Coordinators organised the national sampling activities. The involvement of the ICPDR Expert and Task Groups ensured wide participation of Danube experts in planning and reporting on JDS4.

JDS4 Management

JDS4 Manager	Igor Liška	JDS4 project management
Technical Coordinator	Jaroslav Slobodnik	Logistical support and sampling coordination

JDS4 Support and Administrative Team

ICPDR Executive Secretary	Ivan Zavadsky	Political backup of the JDS4 project
Information Management Expert	Alexander Höbart	Data collection and data management
Public Awareness Expert	Hélène Masliah-Gilkarov	Public awareness
GIS Expert	Zoran Major	Map preparation
Financial Officer	Martina Noitzmüller	Financial accounting support
Editorial Support	Tristan Bath Ivo Monnerjahn	
Administration Support	Jelena Krstajic Olexandra Lohunova	

JDS4 Biology Core Team

JDS4 Core Team leader for biology	Momir Paunović
Fish expert	Vinzenz Bammer (supported by Predrag Simonovic as the Lower Danube expert)
Macrozoobenthos expert	Miroslav Očadlík
Phytobenthos expert	Dana Fidlerová and Jarmila Makovinská
Phytoplankton expert	Igor Stankovič
Macrophytes expert	Kateřina Bubíková and Igor Stanković
IAS expert	Béla Csányi
eDNA	Jonas Astrin and Alexander Weigand
Microbiology	Alexander Kirschner

JDS4 Chemistry Experts

Manfred Sengl, Karin Deutsch, Carmen Hamchevic, Zoran Stojanović, István György Tóth, Peter Tarábek, Hana Hudcová

JDS4 National Coordinators

The ICPDR Heads of Delegations nominated the following JDS4 National Coordinators:

Country	National Coordinator	Deputy National Coordinator
Germany	Manfred Sengl Benno Kügel	
Austria	Karin Deutsch	Helena Mühlmann
Czech Republic	Ivana Beděrková	
Slovakia	Emília Mišíková Elexová	Soňa Ščerbáková
Hungary	Tünde Andrea Zagyva György Istvan Tóth	
Slovenia	Irena Cvitanič Tjaša Zimšek Muc	
Croatia	Draženka Stipaničev	
Serbia	Marta Mihailović	
Romania	Monica Mainerici	Florentina Soare
Bulgaria	Mina Assenova Valeriya Gyosheva	
Ukraine	Iurii Nabyvanets	Sergiy Afanasiev
Moldova	Arcadie Leahu Petru Prodan Victor Bujac	

Supporting ICPDR Expert and Task Groups

Group	Chairperson
Monitoring and Assessment Expert Group (JDS4 organiser)	Franz Wagner
Groundwater Task Group	Andreas Scheidleder
Hydromorphology Task Group	Petra Repnik-Mah
Public Participation Expert Group	Susanne Brandstetter
Information Management and GIS Expert Group	Dragana Ninković
Pressures and Measures Expert Group	Elena Tuchiu

Special Longitudinal Survey Teams (SLST)

SLST 1	Peter Oswald, Zoran Stojanović
SLST 2	Nikiforos Alygizakis, Jörg Ahlheim
SLST 3	Michal Kirchner, Martin Hanuska

eDNA Survey Teams

eDNA Team 1	Didier Pont, Michael Schabuss
eDNA Team 2	Emre Keskin, Aysegul Er, Esra Mine Unal, Elena Stoica, Mihaela Tanase

Microbiology Team

Alexander Kirchner, Clemens Kittinger, Gernot Zarfel, Michael Koller, Daniela Toplitsch, Rita Baumert, Stefan Jakwerth, Erika Toth, Stoimir Kolarević, Mary Craciun, Cristina Dumitru

Passive Sampling Team

Branislav Vrana, Roman Prokeš, Jakub Vinkler

JDS4 reference laboratories

In total, more than 140 laboratories from all over Europe participated in the JDS4 analytical programme. For details, see affiliations in each chapter. Next to national laboratories directly involved in the ICPDR activities, there was also a significant contribution from numerous specialised laboratories contributing specific analyses:

1. Biological Quality Elements: Hungarian Academy of Sciences, Centre for Ecological Research, Danube Research Institute, Budapest, Hungary; Institute for Biological Research "Siniša Stanković", University of Belgrade, Serbia; National Museum of Natural History Luxembourg; EC Joint Research Centre, Ispra, Italy; WWF Slovakia, Bratislava, Slovakia; Nature Conservation Agency of the Czech Republic, Prague, Czech Republic; Hrvatske vode, Zagreb, Croatia; Danube Research Institute, Budapest, Hungary; Agrint Ltd., Gödöllő, Hungary; University of Zagreb, Croatia; Danube Research Institute, Debrecen, Hungary; Technical University Zvolen, Slovakia; Water Research Institute, Slovak National Water Reference Laboratory, Bratislava, Slovakia
2. DNAquaNet COST Action (CA15219): Université de Genève, Geneva, Switzerland; IDGene ecodiagnostics, Geneva, Switzerland; ECOSSA (Ecological Sediment & Soil Assessment), Starnberg, Germany; Institute of Oceanology, Polish Academy of Sciences, Sopot, Poland; National Museum of Natural History Luxembourg; Botanischer Garten und Botanisches Museum Berlin, Freie Universität Berlin, Germany; Center for Natural Science, University of Pannonia, Veszprém, Hungary; Hungarian Academy of Sciences, Budapest, Hungary; UMR CARRTEL, INRAE, Université de Savoie MontBlanc, ThononlesBains, France; INRA, UMR CARRTEL, Thonon les Bains cedex, France; Water Research Institute, Slovak National Water Reference Laboratory, Bratislava, Slovakia; Aquatic Ecosystem Research, University of DuisburgEssen, Germany; Slovak Academy of Sciences, Bratislava, Slovakia; University of Belgrade, Serbia; Danube Research Institute, Budapest, Hungary; University of Natural Resources and Life Sciences, Vienna; Evolutionary Genetics Laboratory (eGL), Ankara University, Ankara, Turkey; Bundesamt für Wasserwirtschaft, Institut für Gewässerökologie und Fischereiwirtschaft, Abteilung Gewässerökologie, Scharfling, Austria; SPYGEN, Le Bourget du Lac, France; Centre for Ecological Research, Tihany, Hungary; Technical University of Munich, Germany; Trnava University, Slovakia; PRO FISCH OG Ecological Consultants, Vienna, Austria; National Institute for Marine Research and Development "Grigore Antipa", Constanţa, Romania; Zoological Research Museum Alexander Koenig (ZFMK), Bonn, Germany

3. NORMAN network: UFZ Leipzig, Germany; University of Athens, Greece; Environmental Institute, Kos, Slovakia; RECETOX, Brno, Czech Republic; University of Lorraine, CNRS, France; TU Munich, Germany; Water Research Institute, Slovak National Water Reference Laboratory, Bratislava, Slovakia
4. Widescope target and suspect screening survey and bioassays: LW Langenau, Germany
5. Polarity-extended non-target screening: AFINTS, Augsburg, Germany
6. Target analyses of chemical parameters: EC Joint Research Centre, Ispra, Italy; NLZOH, Maribor, Slovenia; PM, Brno, Czech Republic; Umweltbundesamt GmbH, Vienna, Austria; WRI, Bratislava, Slovakia
7. Bioassays survey: BDS, Amsterdam, the Netherlands; University of Belgrade, Serbia; National Institute of Biology, Ljubljana, Slovenia
8. Microbiology survey: EC Joint Research Centre, Ispra, Italy; Karl Landsteiner University of Health Sciences, Krems, Austria; Technical University Vienna, Austria; Medical University Vienna, Austria; Medical University Graz, Austria; University of Insubria, Varese, Italy; Interuniversity Cooperation Centre Water & Health, Austria; Eötvös Loránd University, Budapest, Hungary; Institute for Biological Research "Siniša Stanković", University of Belgrade, Serbia
9. Microplastics survey: German Federal Environment Agency and BAM, Berlin, Germany; Institute for Biological Research "Siniša Stanković", University of Belgrade, Serbia; University of Comenius, Bratislava, Slovakia
10. Stable isotopes of water and nitrate and radiology survey: IAEA Vienna and BOKU, Vienna, Austria
11. Nanoparticles survey: University of Vienna, Austria

An interlaboratory study to increase capacities of Danube laboratories in widescope target, suspect and nontarget screening was organised by UFZ Leipzig with involvement of Croatian Waters, Zagreb, Croatia, WRI Bratislava, Slovakia, SEPA Belgrade, Serbia, University of Athens, Greece, Environmental Institute, Kos, Slovakia, LfU Augsburg and BfG Koblenz, Germany.

JDS4 National laboratories

Regional office for water management, Donauwörth, Germany; Regional office for water management, Ingolstadt, Germany; Regional office for water management, Landshut, Germany; Regional office for water management, Deggendorf, Germany; State Office for Water Management, section biology, Donauwörth, Germany; State Office for Water Management section biology, Ingolstadt, Germany; State Office for Water Management, section biology, Landshut, Germany; State Office for Water Management, section biology, Deggendorf; Bavarian Environment Agency, unit 83, Ecology of Rivers and Lakes, Hof, Germany; Bavarian Environment Agency, unit 54, Fish and Freshwater Ecology Wielenbach, Germany; DWS Hydro-Ökologie GmbH, Vienna, Austria; Systema, Bio-Management Consulting GmbH, Vienna, Austria; Institute of Hydrobiology and Aquatic Ecosystem Management, Vienna, Austria; Environmental Agency, Vienna, Austria; Institut für Gewässerökologie und Fischereiwirtschaft, BAW, Scharfling, Mondsee, Austria; Fa. Synlab Analytics & Services Austria GmbH / Eurofins Umwelt Österreich GmbH, Vienna, Austria; ESW Consulting Wruss ZT GmbH, Vienna, Austria; National Water Reference Laboratory, Water Research Institute, Bratislava, Slovakia; Budapest Waterworks, Budapest, Hungary; DMRV Danubian Regional Waterworks Corporation, Vác, Hungary; Pest County Government Office, Érd, Hungary; Wessling Hungary Ltd., Budapest, Hungary; Hrvatske vode, Central Water Management Laboratory, Zagreb, Croatia; Department of Biology, University

of J. J. Strossmayer, Osijek, Croatia; Department of Biology, Faculty of Science, University of Zagreb, Croatia; Eurofins Croatiakontrola d.o.o., Zagreb, Croatia; Slovenian Environment Agency, Ljubljana, Slovenia; National laboratory of Health, Environment and Food, Novo mesto, Slovenia; Institute for Biological Research "Siniša Stanković" – National Institute of the Republic of Serbia, University of Belgrade; University of Belgrade, Faculty of Biology, Serbia; Faculty of Sciences, University of Novi Sad, Department of Biology and Ecology, Serbia; Serbian Environmental Protection Agency; Department of Biology and Ecology, Faculty of Sciences and Mathematics, University of Niš, Serbia; Institute of Chemistry, Technology and Metallurgy, National Institute, Belgrade, Serbia; Jaroslav Černi Water Institute, Belgrade, Serbia; University of Kragujevac, Faculty of Science in Kragujevac, Department of Biology and Ecology, Serbia; Institute of Public Health of Serbia "Dr Milan Jovanović Batut"; Regional Laboratory Montana, Executive Environment Agency, Sofia, Bulgaria; Regional Laboratory Pleven, Executive Environment Agency, Sofia, Bulgaria; Regional Laboratory Ruse, Executive Environment Agency, Sofia, Bulgaria; Regional Laboratory Varna, Executive Environment Agency, Sofia, Bulgaria; Institute of Biodiversity and Ecosystem Research at the Bulgarian Academy of Science, Sofia, Bulgaria; Faculty of Biology, Plovdiv University, Bulgaria; Water Quality Laboratory SGA Mehedinti, Turnu Severin, Romania; Water Quality Laboratory ABA Jiu, Craiova, Romania; Water Quality Laboratory SGA, Tulcea, Romania; Water Quality Laboratory ABADL, Constanta, Romania; National Water Quality Laboratory, Bucharest, Romania; Water Quality Laboratory SGA, Calarasi, Romania; Water Quality Laboratory SGA, Giurgiu, Romania; Water Quality Laboratory SGA Mehedinti, Turnu Severin, Romania; Water Quality Laboratory ABA Jiu, Craiova, Romania; Water Quality Laboratory SGA, Tulcea, Romania; Water Quality Laboratory ABADL, Constanta, Romania; National Water Quality Laboratory, Bucharest, Romania; Water Quality Laboratory SGA Ialomita, Slobozia, Romania; Regional Water Quality Laboratory ABAST, Cluj Napoca, Romania; Water Quality Laboratory ABA Buzău-Ialomita, Romania; Water Quality Laboratory SGA, Arad, Romania; Water Quality Laboratory SGA, Bucharest, Romania; Water Quality Laboratory SGA Caras-Severin, Resita, Romania; Water Quality Laboratory SGA Gorj - Tg. Jiu, Romania; Water Quality Laboratory ABA Banat, Timisoara, Romania; Water Quality Laboratory ABA Siret, Bacau, Romania; Water Quality Laboratory SGA, Calarasi, Romania; Water Quality Laboratory ABAPB, Iasi, Romania; Water Quality Laboratory ABAC, Oradea, Romania; Water Quality Laboratory SGA, Braila, Romania; Water Quality Laboratory SGA Vrancea - Focsani, Romania, Monitoring Department ABA Jiu - Craiova, Romania; Monitoring Department ABA Siret -Bacau, Romania; Executive Environment Agency, Water Basin Administration Arges-Vedea, Water Quality Laboratory, Giurgiu, Romania; „IWA": „Institut für Wasseraufbereitung, Abwasserreinigung und –forschung", Austria; Vodovody a kanalizace Hodonín a.s., Czech Republic; Laboratory of Bratislavská vodárenská spoločnosť, a.s., Slovakia; PANNON-VÍZ Zrt. Minőségvizsgáló Laboratórium, Hungary; Komunala Novo mesto d.o.o., Laboratorij na CČN Novo mesto, Slovenia; Internal laboratory of the WWTP Županja, Croatia; Plant laboratory at the Central Wastewater Treatment Plant of Sabac, PUC "Vodovod Sabac", Serbia; Stația de Epurare Giurgiu (SC APA SERVICE SA GIURGIU), Romania; "Regional Laboratory Vratsa", Directorate "Laboratory and Analytical Activity" at the Executive Environmental Agency, Bulgaria; Wastewater control laboratory of the Uzhorod utility company "Vodokanal", Ukraine.

Reporting

The JDS4 report is also available on the JDS website of the ICPDR (http://www.danubesurvey.org/jds4), where also the long versions of selected chapters can be found. The data management issues are addressed in Chapter 3.

Annex 1

List of samples collected during JDS4. For explanation of abbreviations, see Annex 2.

Site No.	Site Name	Countries	FI	MZB_MHS	MZB_KAS	MZB_LNT	MZB_AMS	MZB_DNA	PP	PB	PB_DNA	MP	ZP	SE_SER	SE_DNA	SW	FC	FM	MC	MPL	SWD	SWLS_GRB	SWLS_LMR	SWLS_LMX	SPMLS_LMX	SWLS_LHR	SWP	SWM	GW	WW
1	Böfinger Halde	DE	x	x				x	x	x	x	x	x	x	x	x	x	x		x	x	x	x	x	x					
2	Bittenbrunn	DE	x	x				x	x	x	x	x	x	x	x	x	x	x	x		x	x	x	x	x					
3	Above Klösterl Kelheim	DE	x	x			x	x	x	x	x	x	x	x	x	x	x	x	x		x	x	x	x	x	x		x		
4	Niederalteich Mühlau	DE	x	x			x	x	x	x	x	x	x			x	x	x			x	x	x	x	x			x		
5	Passau Ingling	DE		x				x		x	x			x	x	x						x	x	x	x	x		x		
6	Jochenstein	DE/AT	x	x	x	x		x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x		
7	Enghagen	AT		x	x			x	x	x	x	x	x	x	x	x					x	x	x	x	x			x		
8	Oberloiben	AT	x	x	x			x	x	x	x	x	x	x	x	x	x	x	x		x	x	x	x	x	x		x		
9	Klosterneuburg	AT					x							x					x	x		x	x	x	x	x		x		
9m	Downstream Vienna	AT																										x		
10	Hainburg, upstream Morava	AT	x	x	x			x	x	x	x	x	x	x	x	x	x	x		x	x	x	x	x	x					
11	Pohansko	CZ		x	x		x	x	x	x	x		x	x	x	x	x	x	x	x		x	x	x	x					
12	Lanžhot	CZ		x	x		x	x	x	x	x		x	x	x	x	x	x	x	x		x	x	x	x	x				
13	Devín	SK	x	x	x		x	x	x	x	x			x	x	x	x		x			x	x	x	x					
14	Bratislava	SK	x	x	x			x	x	x	x		x	x	x	x	x	x		x		x	x	x	x	x		x		
15	Čunovo, Gabčíkovo resevoir	SK	x									x		x			x	x	x			x	x	x	x		x			
16	Medveďov / Medve	SK/HU	x	x	x			x	x	x	x		x	x	x	x	x	x	x			x	x	x	x					
17	Vének	HU	x	x	x	x		x	x	x	x	x	x	x	x	x	x	x	x			x	x	x	x			x		
18	Gönyű	HU	x	x	x	x		x	x	x	x	x	x	x	x	x	x	x			x	x	x	x	x			x		
19	Komárno	SK	x	x	x			x	x	x	x			x	x	x	x		x			x	x	x	x					
20	Kamenica	SK	x	x	x		x	x	x	x	x			x	x	x	x					x	x	x	x					
21	Salka	SK	x	x	x			x	x	x	x	x		x	x	x	x		x			x	x	x	x					
22	Szob	HU/SK	x	x	x	x		x	x	x	x	x	x	x	x	x	x		x			x	x	x	x	x				
23	Budapest upstream (Megyeri Bridge)	HU	x	x	x	x		x	x	x	x	x	x	x	x	x	x	x		x	x	x	x	x	x	x		x		
24	Budapest downstream (M0 bridge)	HU	x	x	x	x		x	x	x	x	x	x	x	x	x	x	x	x	x		x	x	x	x	x	x	x		
25	Tass	HU	x	x	x	x		x	x	x	x	x	x	x	x	x	x	x				x	x	x	x					
26	Dunaföldvár	HU	x	x	x	x		x	x	x	x	x	x	x	x	x	x	x			x	x	x	x	x			x		
27	Paks	HU	x	x	x	x		x	x	x	x	x	x	x	x	x	x	x	x			x	x	x	x					
28	Baja	HU	x	x	x	x		x	x	x	x	x	x	x	x	x	x	x				x	x	x	x					
29	Hercegszántó / Batina / Bezdan	HU/HR/RS	x	x	x	x		x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x			
30	Drava mouth (rkm 5.0)	HR	x	x	x				x	x			x	x		x	x					x	x	x	x	x		x		
30m1	Upstream Drava	HR																										x		
30m2	Downstream Drava	RS																										x		
31	Ilok / Bačka Palanka	HR/RS	x	x	x	x		x	x	x	x	x	x	x	x	x	x	x	x		x	x	x	x	x	x				
32	Tiszasziget / Martonoš	HU/RS	x	x	x	x		x	x	x	x	x	x	x	x	x	x	x	x			x	x	x	x					

Site No.	Site Name	Countries	FI	MZB_MHS	MZB_KAS	MZB_LNT	MZB_AMS	MZB_DNA	PP	PB	PB_DNA	MP	ZP	SE_SER	SE_DNA	SW	FC	FM	MC	MPL	SWD	SWLS_GRB	SWLS_LMR	SWLS_LMX	SPMLS_LMX	SWLS_LHR	SWP	SWM	GW	WW
33	Tisza mouth (rkm 1.0)	RS	x	x	x	x		x	x	x	x	x	x	x	x	x	x	x	x			x	x	x	x	x		x		
33m1	Downstream Novi Sad	RS																										x		
33m2	Downstream Tisza / Upstream Sava	RS																										x		
34	Jesenice na Dolenjskem	SI		x	x			x		x	x				x	x	x					x	x	x	x	x				
35	Jamena	RS/BA	x	x	x	x		x	x	x	x	x	x	x	x	x	x	x	x			x	x	x	x	x				
36	Sava mouth (rkm 7.0)	RS	x	x	x	x		x	x	x	x	x	x	x	x	x	x	x	x	x		x	x	x	x	x		x		
37	Downstream Pančevo	RS	x	x	x	x		x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x		
38	Varvarin	RS	x	x	x	x		x		x	x	x	x	x	x	x	x	x	x			x	x	x	x					
39	Velika Morava mouth	RS	x	x	x	x		x	x	x	x	x	x	x	x	x	x	x				x	x	x	x					
40	Banatska Palanka / Bazias	RS/RO	x	x	x	x		x	xx	x	x	x	x	x	x	x	x	x	x		x	x	x	x	x	x		x		
41	Upstream Timok (Rudujevac / Gruia)	RS/RO	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x		x		
41m1	IGR Tekija/Orsova	RS																										x		
41m2	Vrbica/Simijan	RO																										x		
41p	Kladovo	RS				x																					x			
42	Timok mouth (rkm 0.2)	RS/BG	x		x	x				x	x	x		x	x	x	x	x				x	x	x	x			x		
43	Pristol / Novo Selo Harbour	RO/BG	x	x	x		x	x	xx	x	x	x	x	x	x	x	x					x	x	x	x	x		x		
43p	Vidin	BG																									x			
44	Iskar mouth (rkm 0.3)	BG	x													x	x	x				x	x	x	x					
45	Jantra mouth (rkm 1.0)	BG	x													x	x	x				x	x	x	x					
45m1	Downstream Zimnicea/ Svistov	BG																										x		
46	Russenski Lom mouth	BG	x													x	x	x				x	x	x	x			x		
46p	Ruse	BG																		x							x			
47	Downstream Ruse / Giurgiu (Marten)	BG/RO	x	x	x			x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x		x		
47m1	Arges (tributary)	RO																										x		
47m2	Downstream Arges	RO																										x		
48	Chiciu / Silistra	RO/BG	x	x	x	x	x	x	xx	x	x	x	x	x	x	x	x		x		x	x	x	x	x	x				
49	Giurgiulesti	MD/RO	x	x	x			x	x	x	x	x	x	x		x	x					x	x	x	x					
49m	Giurgeni	RO																										x		
50	Reni	RO/UA	x	x	x	x		x	x	x	x	x	x	x	x	x	x	x			x	x	x	x	x	x		x		
50m	Tulcea, St. George branch	RO																										x		
50p	Galati	RO																									x			
51	Vilkove Chilia / Kilia arm	RO/UA		x					x	x		x	x		x	x				x	x	x	x	x	x					
GW1	Vienna	AT																											x	

Site No.	Site Name	Countries	FI	MZB_MHS	MZB_KAS	MZB_LNT	MZB_AMS	MZB_DNA	PP	PB	PB_DNA	MP	ZP	SE_SER	SE_DNA	SW	FC	FM	MC	MPL	SWD	SWLS_GRB	SWLS_LMR	SWLS_LMX	SPMLS_LMX	SWLS_LHR	SWP	SWM	GW	WW
GW2	Šamorín Kalinkovo	SK																											x	
GW3	Surány	HU																											x	
GW4	Topolje	HR																											x	
GW5	Novi Sad	RS																											x	
GW6	Slobozia	RO																											x	
GW7	Slivo pole	BG																											x	
WW1	Donauwörth	DE																												x
WW2	LinzAsten	AT																												x
WW3	Hodonín	CZ																												x
WW4	Vrakuňa (Bratislava)	SK																												x
WW5	Győr	HU																												x
WW6	Novo mesto (Ločna)	SI																												x
WW7	Županja	HR																												x
WW8	Šabac	RS																												x
WW9	Giurgiu	RO																												x
WW10	Vratsa	BG																												x
WW11	Uzhgorod	UA																												x

Annex 2

Coding of samples collected within JDS4. Explanation of abbreviations from Annex 1.

Sample code	Matrix (code)	Sampling method (code)	Sampling/analysis activity	Sampled by/remark
FI	Fish – ichthyology (FI)		Fish	National Teams
MZB_MHS	Macrozoobenthos (MZB)	Multihabitat sampling (MHS)	Macrozoobenthos	National Teams
MZB_KAS	Macrozoobenthos (MZB)	Kick & Sweep (KAS)	Macrozoobenthos	National Teams
MZB_LNT	Macrozoobenthos (MZB)	LiNi Traps (LNT)	Macrozoobenthos	National Teams
MZB_AMS	Macrozoobenthos (MZB)	Additional molluscs sample (AMS)	Macrozoobenthos	National Teams
PP	Phytoplankton (PP)		Phytoplankton	National Teams
PB	Phytobenthos (PB)	Phytobenthos brush (PBB)	Phytobenthos	National Teams
MP	Macrophytes (MP)		Macrophytes	National Teams
ZP	Zooplankton (ZP)		Zooplankton	National Teams
SWD	Surface Water eDNA (SWD)		eDNA special survey: fish & MZB	eDNA Teams

Sample code	Matrix (code)	Sampling method (code)	Sampling/analysis activity	Sampled by/remark
MZB_DNA	Macrozoobenthos (MZB)	(e)DNA sample (DNA)	MZB eDNA bulk sample used for DNA analysis	National Teams
PB_DNA	Phytobenthos (PB)	(e)DNA sample (DNA)	Phytobenthos brush bulk sample for DNA analysis	National Teams
SE_DNA	Sediment (SE)	(e)DNA sample (DNA)	Sediment sample for (e)DNA extraction	National Teams
FC	Fish chemical analyses (FC)		Target analysis: metals	National Teams
FC	Fish chemical analyses (FC)		Target analysis: organic substances	National Teams
FC	Fish chemical analyses (FC)		Wide-scope target and suspect screening	National Teams
MC	Molluscs chemical analyses (MC)		Target analysis: molluscs	National Teams
SW	Surface Water (SW)	Grab sample (GRB)	Physico-chemical parameters	National Teams
SW-LS_GRB	Surface Water (SWLS)	Grab sample (GRB)	Target analysis: organic substances	SLS Teams. The sample further split into subsamples for different labs.
SWLS_GRB	Surface Water (SWLS)	Grab sample (GRB)	1,4-dioxane in water	SLS Teams
SWLS_GRB	Surface Water (SWLS)	Grab sample (GRB)	Direct injection LC-HRMS; wide-scope target and non-target screening	SLS Teams
SWLS_GRB	Surface Water (SWLS)	Grab sample (GRB)	Direct injection LC-HRMS; screening of very polar compounds	SLS Teams
SWLS_GRB	Surface Water (SWLS)	Grab sample (GRB)	On-line SPE-LC-HRMS; wide-scope target screening; special focus on pesticides and their TPs	SLS Teams
SWLS_GRB	Surface Water (SWLS)	Grab sample (GRB)	SPE-LC-HRMS; wide-scope target screening and four bioassays	SLS Teams
SWLS_GRB	Surface Water (SWLS)	Grab sample (GRB)	Radioactivity	SLS Teams
SWLS_GRB	Surface Water (SWLS)	Grab sample (GRB)	Analysis of DOM and REE	SLS Teams
SWLS_GRB	Surface Water (SWLS)	Grab sample (GRB)	Analysis by fluorescence spectroscopy	SLS Teams
SWLS_GRB	Surface Water (SWLS)	Grab sample (GRB)	Water samples stable isotopes	SLS Teams
SWLS_GRF	Surface Water (SWLS)	Grab sample filtered (GRF)	Water samples stable isotopes of nitrate	SLS Teams
SWLS_GRB	Surface Water (SWLS)	Grab sample (GRB)	Water samples for HCO3	SLS Teams
SWLS_LMR	Surface Water (SWLS)	LVSPE Mariani Box (LMR)	LVSPE water samples, wide-scope target screening	SLS Teams
SWLS_LMX	Surface Water (SWLS)	LVSPE MAXX Sampler (LMX)	LVSPE water samples, wide-scope target and non-target screening	SLS Teams
SPMLS_LMX	Suspended Particulate Matter (SPMLS)	LVSPE MAXX Sampler (LMX)	SPM samples, wide-scope target and non-target screening; bioassays	SLS Teams

Sample code	Matrix (code)	Sampling method (code)	Sampling/analysis activity	Sampled by/remark
SWLS_LHR	Surface Water (SWLS)	LVSPE Horizon Field Sampler (LHR)	LVSPE water samples, wide-scope target and suspect screening; bioassays	SLS Teams
SWP	Surface Water Passive sampling (SWP)		Passive sampling, extracts analysed for target substances, wide-scope target and suspect screening; bioassays	Passive Sampling Team; also sampled on additional sites with suffix "p"
SE_SER	Sediment (SE)	Sediment – raw (SER)	Sediment samples for wide-scope target and suspect screening	National Teams
SE_SER	Sediment (SE)	Sediment – raw (SER)	Radioactivity	National Teams
SE_SEC	Sediment (SE)	Core sediment (SEC)	Nanoparticles: sediment core 1077 rkm (Ram – Stara Palanka), fly ash samples Kostolac, sediment grab sample 1097 rkm	Serbian National Team
MPL	Microplastics SPM (MPL)		Sampling of SPM including microplastic in sedimentation boxes	National Teams
SPM_MPL	Suspended Particulate Matter (SPM)	MPL	1 l of water for determination of SPM content sampled on the first and last day of sampling; accompanying information for MPL analysis	National Teams
FM	Fish microplastics analyses (FM)		Fish sample for microplastics analysis	National Teams
SWM	Surface Water Microbiology (SWM)		Microbiology	Microbiology Team
GW	Groundwater		Water samples; wide-scope target and suspect screening	National Contact Points
WW	Wastewater		24 hours composite influent and effluent samples; wide-scope target and suspect screening	National Contact Points

03 Data management

Alexander Höbart (ICPDR Vienna, Austria)

Abstract

The setup of JDS4 as a collaborative activity by many independent teams of different kinds of experts in 13 countries called for an extra effort to facilitate a coordinated data collection approach. The ICPDR Secretariat, under the guidance of the Monitoring and Assessment Expert Group (MA EG) and in cooperation with the Core Team experts, developed a common sample coding standard and a specific tool set for data collection. This tool set included a mobile application for data entry of samples taken in the field and templates for bulk data upload. Data collected included basic physico-chemical parameters, target substances and species lists of biological quality elements.

The JDS4 Data Collection Portal, accessible to all experts involved in the survey, was the central platform to collect, validate and access data of the survey. The portal will be further developed, integrated and maintained as part of the ICPDR information system.

3.1 Introduction

The ICPDR maintains and operates the TNMN database of water quality data which contains national data, compiled yearly since 1996 by the SHMU Bratislava and published on the web. Data from Joint Danube Surveys 1, 2 and 3 were also added into this database, although the collection of the vast amount of data and integration into the simplistic TNMN data structure became more challenging from survey to survey.

For JDS4, the ICPDR Secretariat developed a new database and new tools and provided them in the JDS4 Data Collection Portal specifically for the experts involved in the survey. This database was still based on, but extended the data structure of the previous TNMN database, to allow supporting the specific needs of JDS4 data.

The following types of data were collected using the JDS4 Data collection portal:

- **Main sites** nominated by countries during preparation phase of JDS4: this base dataset was managed by the ICPDR Secretariat based on agreements of the MA EG and inputs from the countries. It was later during the preparation extended with additional sites for passive sampling (4), microbiology (12), groundwater (7), and wastewater (11).
- **Sampling data** recorded during the survey – including exact coordinates, matrices, sampling dates and methods, plus accompanying photos. This data was collected via 3 alternative tools. It was up to the country or survey team to select the most appropriate tool:
  - **ODK Collect** app for Android mobile devices – primarily for usage in the field as coordinates are recorded using the device's GPS sensor
  - **Web form** usable in a modern web browser on any platform and device
  - **Excel template** which allowed batch upload of sample data collected by other means
- **Analysis result data** and species lists of Biological Quality Elements (BQE) collected via specific Excel templates.
- **Hydromorphological assessment update** via online forms – this is described in the related Chapter 4 "Recording and assessment of hydromorphological changes 2013-2019".

3.2 Methods

3.2.1 Software

The JDS4 data collection portal was built on open source software components, some of which have already been used in the information technology infrastructure of the ICPDR, that enabled rapid development of tools in a resource-limited setting. The content management system Drupal (http://www.drupal.org), using some community contributed and some custom developed modules, served as the backend for managing user access, content in the form of templates and datasets, forms and database import, validation and retrieval functions.

The mobile application for Android was built using the open source Open Data Kit, simply called ODK (http://getodk.org). ODK Collect is a generic app that can be installed from the Google Play store. It can load customized forms to replace paper forms used in survey-based data gathering and is designed to work well without network connectivity. Users can save their data at any point on their devices. Finalized submissions are sent to (and new forms downloaded from) an ODK Aggregate server. The connection to the server must be configured by the user with URL, username and password.

Enketo Express (https://enketo.org/) was used to provide the same ODK form as a web form as an alternative data entry tool.

ODK Collect was used in JDS4 to collect location and sample data in the field. The ODK Aggregate server was integrated into the JDS4 Data Collection Portal by custom Drupal modules. These modules synchronised the user accounts and their access rights from Drupal to ODK Aggregate and imported data submission from ODK Aggregate into the main JDS4 database accessible via Drupal Views.

Microsoft Excel files were used to provide templates for all types of data. One Excel template provided the same fields as the ODK Collect app, so it was appropriate to submit multiple records of sampling data when the coordinates had been collected by other means. Other Excel files were developed as templates for flow data, basic physico-chemical parameters, target substances and species lists of biological quality elements.

Custom Drupal modules were developed to read the Excel templates uploaded to the portal by users, validate the data and import valid datasets into the main JDS4 database. The data retrieval, presentation, visualisation on maps and graphs, as well as export functions were realised mostly with Drupal community modules and a few customisations.

3.2.2 Coding of sites, locations and samples

The common approach to coding of sites and samples aimed to ensure the proper labelling of sample containers to support logistics and eventually to be able to subsequently link the sampling data with the results data of analysis and determination.

To achieve this, it was essential that each sampling location and each sample have a code which uniquely identifies them. The code structure was designed in a pragmatic approach to be as short as possible, but also human readable and applicable even beyond JDS4 purposes. The appropriate code for a sample could be derived easily by entering or selecting the appropriate elements using one of the three data collection tools provided within the survey.

The codes have a common structure with the following elements:

1. **Main site** code consists of these elements:
   a. Survey prefix "JDS4"
   b. Main site number 1 - 51; other sites for the specific groundwater (GW) and wastewater (WW) sampling were added later with a prefix with their own numbering.
   c. Optional suffix for off-site sampling points in-between main sites used for specific passive (p) and microbiology (m) sampling
2. **Location** code is added to the main site code to jointly provide a unique **Site code** for each sampling activity – it consists of these elements:
   a. Location in profile (L, M, R, P, E)[1]
   b. Sampling matrix (SW, SE, SPM, MZB, PP, PB, MP, ZP, FI, etc.)[2]
   c. Optional sequence code for more locations sampled at same profile and of same matrix e.g. the Special Longitudinal Survey team used "LS" to distinguish their data from national sampling
3. Optional **Sample** suffix is added to the site code only if more than one sample is taken with the same site code to jointly provide a unique **Sample code** – it can consist of the following elements:
   a. Method code (for matrices with defined distinguishing methods)[3]
   b. Date (or appropriate distinguishing part, e.g. month and day for phytoplankton samples taken every 4 weeks in JDS4)
   c. Numeric sequence for specific cases

[1] *L = left bank, m = middle of river, R = right bank, P = pooled (mixed), E = entire profile*

[2] *Matrix codes as mentioned in Chapter 2 "Survey logistics"*

[3] *Sampling method codes as mentioned in Chapter 2 "Survey logistics"*

The elements of the main site and location codes are joined with hyphen ("-"), the optional sample code elements are joined with an underscore ("_") to form the complete code. If only a single sample is taken for a matrix at a location, the site code is also used as the sample code as it identifies both the site and the sample.

Examples (for illustration of the concept of the coding):

JDS4-1-L-SW_GRB: grab sample of surface water taken from the left bank at JDS4 main site 1

JDS4-11-M-ZP: zooplankton sample in the middle of the river at JDS4 main site 11

JDS4-1-L-MZB_MHS: macrozoobenthos sample taken using MHS method on the left bank of JDS4 main site 1

JDS4-1-L-MZB_KAS: macrozoobenthos sample taken using Kick & Sweep method on the same location as above example

JDS4-13-M-PP_0416: Phytoplankton sample taken on 16 April (Month 04) in the middle of the river at JDS4 main site 13

JDS4-32-R-MP-2: second of a sequence of macrophytes samples/locations on the right bank at JDS4 main site 32

3.2.3 Data description

The following data elements were collected for sampling data. Some elements are also used for the coding and explained in more detail in the previous section:

- For each sampling location:
  - JDS4 Main Site
  - Location in profile
  - Sample matrix
  - Sequence
  - Latitude
  - Longitude
  - Altitude and accuracy (only in ODK Collect app as taken from GPS sensor)
  - Remarks (optional)
- For each sample:
  - Date (and optionally time) of sampling
  - End date and time of sampling (optional – for long-term sampling)
  - Sampling method (only for specific matrices, if multiple methods were used)
  - National sample code (optional) as a reference to the national sampling programme
  - Sequence

Figure 1 shows a data model of sampling data in light blue – Main sites having multiple Sampling locations with one or more Samples. The main attributes of the entities are listed, indicating their obligation, as well as primary, unique and foreign keys which define the relationships. The results (in light yellow) are always linked to that data via the sample code. The Analysis and Species entities are given as simplified examples for illustration (e.g. determinands and analytical methods are actually defined with more detail attributes in separate entities). Further result types with different structures can be linked and thus integrated in the same way.

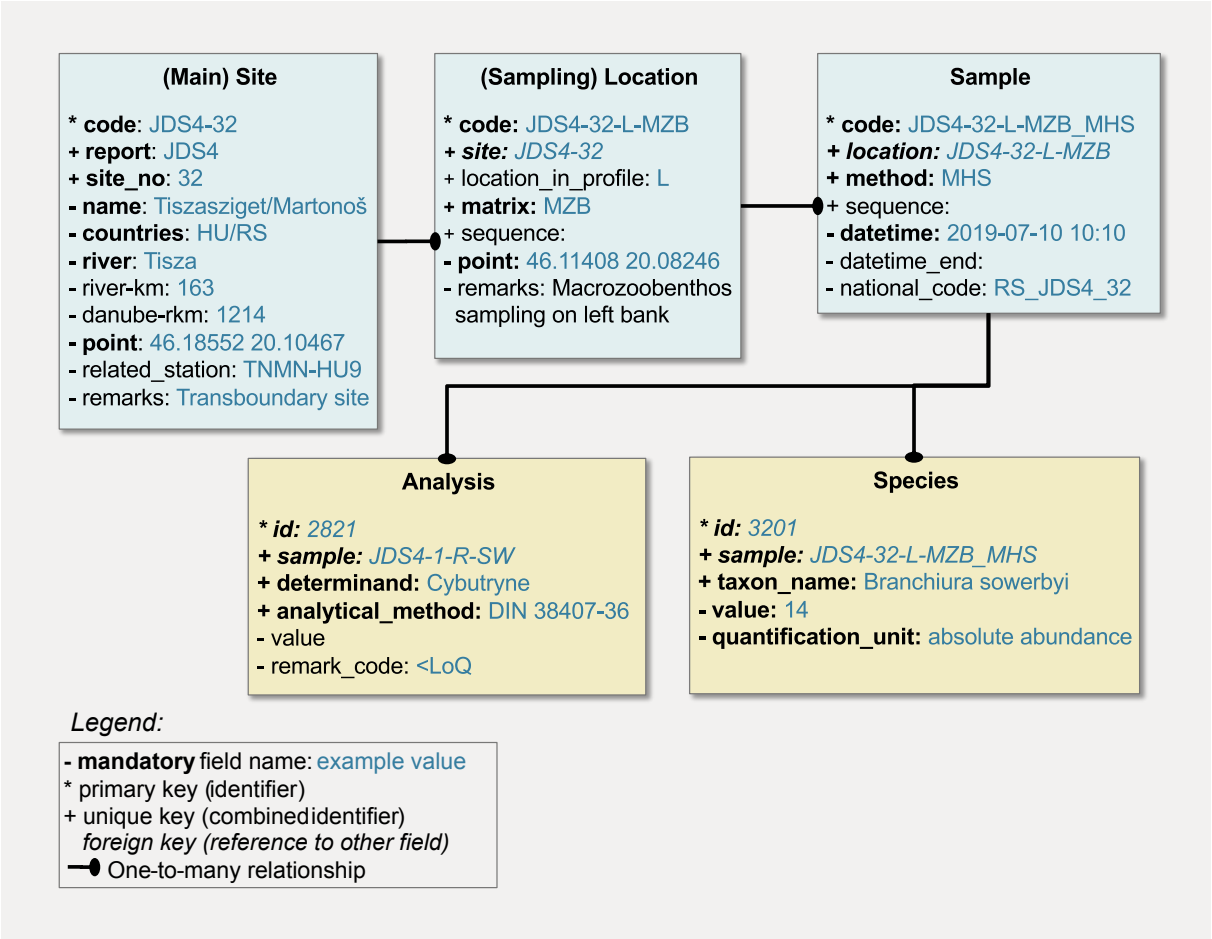

Figure 1: Simplified data model of JDS4 sampling data and linked results.

In addition to data as such, it was possible to upload documenting photos of sampling, either via the ODK app or direct upload into the portal.

For survey results, the portal made available specifically structured templates, in particular for flow data, concentrations of basic physico-chemical parameters (including description of analytical methods, which were pre-filled from the ICPDR TNMN database for national data provisions), target substances and metals (provided with specific list of determinands for specific laboratories), as well as a base template for determined species. Later this was slightly adapted, i.e. contained more or fewer fields, depending on the needs of specific BQE data processing, e.g. growth form for macrophytes.

3.2.4 Usage of data collection tools

The ICPDR Secretariat provided a JDS4 Data Collection Manual to all experts involved. This document described the coding for sites and samples and provided step by step instructions for using the tools used for data collection.

The ODK Collect (and web) app was targeted for data entry use in the field, as it provided easier data entry of single sampling records and automatic recording of location data. The crucial advantage of ODK was the automatic entry of coordinates for sampling sites via the mobile phone's GPS sensor and the ability to use it offline and submit collected data any time later when Internet connectivity could be established.

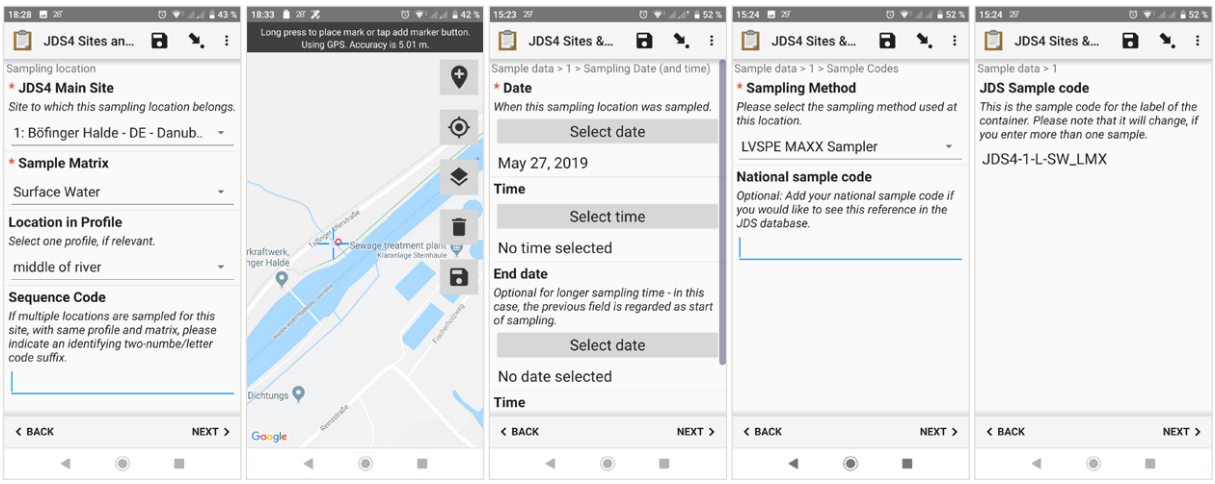

Figure 2: Screenshots of the site and sampling data entry form in ODK Collect.

The Excel template, targeted for desk use and integration of data collected in other ways, allowed bulk data provision, but the sampling sites and samples had to be linked correctly by the data input user.

If sampling data were submitted again for an already previously submitted sampling location (i.e. with the same site code), the previous data were overwritten. This allowed for corrections of submitted data.

Out of 998 sampling locations collected for JDS4, 589 were provided via ODK (app and web form) and 409 via Excel sheets; 772 were submitted during the survey in the period April to October 2019, the rest of 226 records afterwards and only up to a year later.

At these locations, experts reported collection of 1745 samples to the database. 679 photos were uploaded to document the sampling locations, sampling activities and samples.

Various laboratories and experts used the templates for different types of result data to directly upload and import their data into the JDS4 database: 245 records of flow data, 779 records of basic parameters analysis, 1527 of target substances analysis, 680 of metals analysis, and 6852 species.

All submitted data was listed and visualised on maps immediately. National or laboratory-specific datasets import were thus integrated immediately and the survey-wide data could be viewed, compared and exported by all experts involved.

The portal was used by 170 users during and after the survey, not only for data collection and review, but also for coordination of the report writing.

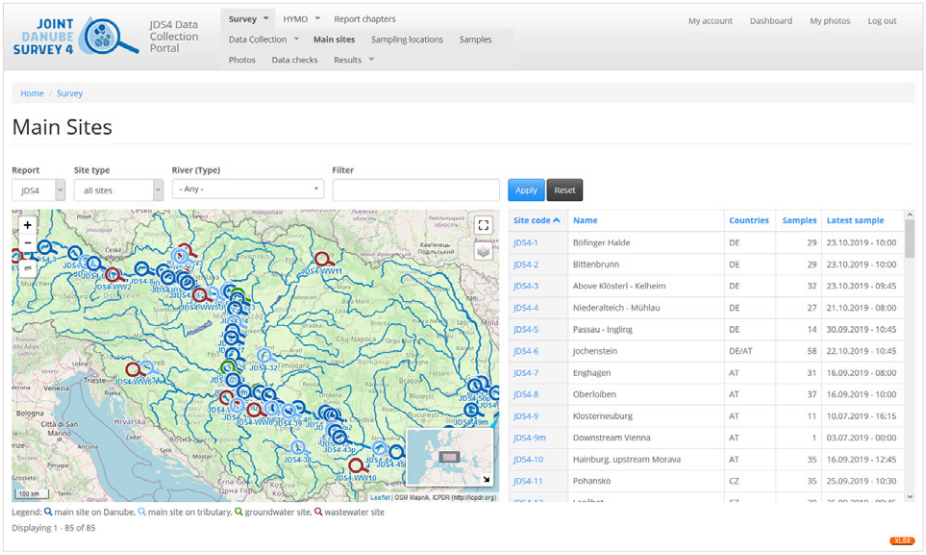

Figure 3: Screenshot of the JDS4 data collection portal with an interactive map of sampling sites.

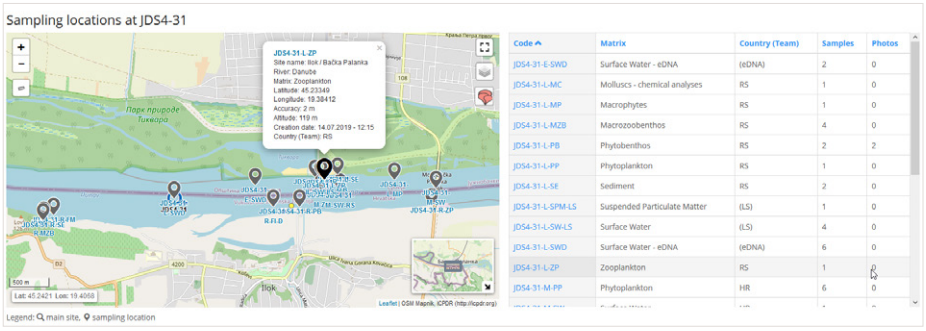

Figure 4: Screenshot of an interactive map of multiple sampling locations at one main site.

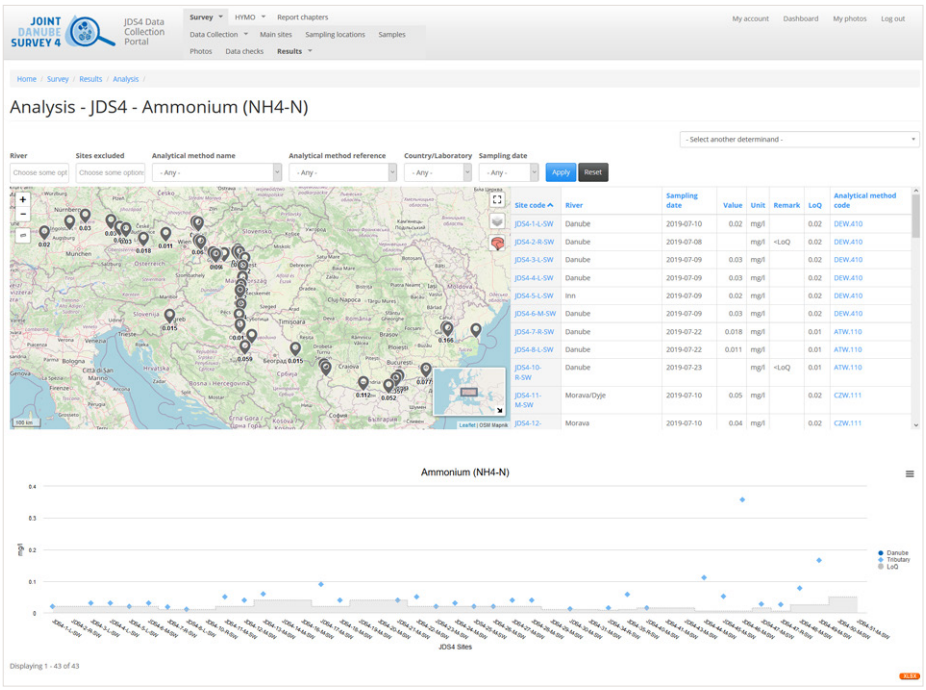

Figure 5: Screenshot of results of a selected determinand including an interactive map, table, graph and download option.

3.3 Results and discussion

Compared with previous JDSs, the collection of basic survey data, particularly sampling data and basic results, was much faster and available data is more extensive due to the tools provided and despite the higher number of experts involved. In previous surveys, exact coordinates of specific samples were hardly provided and post processing of various data formats was much more time intensive.

The common coding of samples was difficult to plan as every other sampling activity had a different perspective on their way of sampling and some of those needs only became clear at a very late stage of planning. Thus, the original idea of a simple code structure was adapted several times and in the end the code structure became relatively complicated again. Still, the main goal of having a common way to identify samples was achieved and would just need some refinements in any future application.

3.4 Conclusions

The general approach in JDS4 data collection of using a common coding for sampling sites and samples, building up on existing TNMN data structures and extending them, providing tools for in-field data entry and bulk data upload and providing an online working space for data validation, visualisation and retrieval seems to have worked in most aspects and was welcomed and actively used by JDS4 experts.

The elaborated coding and database structures could be used as a model for similar activities. More specifically, the whole tool set could be used, with some refinements, in a future Joint Danube Survey.

04 Recording and assessment of hydromorphological changes 2013 - 2019

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Alexander Höbart (ICPDR, Vienna, Austria)

Abstract

Based on the results of JDS3 for the continuous overall and WFD 3-digit hydromorphological assessments of 10-rkm sections of the Danube, JDS4 delivered hydromorphological data for changes (improvements/ deteriorations) for channel, banks and floodplain. For the first time, countries uploaded harmonized data via the JDS4 data tool. The centralised evaluation of changes and finally the reassessment of segments resulted in 73 changes (54 improvements and 19 deteriorations) within 55 segments. The reassessment of JDS3 shows several improvements on the still strongly altered Upper and Middle Danube and slight deteriorations on the Lower Danube. In most cases the changes lead only to the reassessment of individual parameters, but not to the shift of overall assessment classes for entire segments.

4.1 Introduction

Hydromorphology can be understood as the natural structure of river channels, banks and floodplains built under dynamic processes in space and time and is a fundamental pre-requisite to understand different river types, to define reference conditions and to estimate human induced pressures and impacts on the entire riparian ecosystems, in particular for aquatic habitats, as defined in the WFD (EC 2000). The WFD considers the morphology, hydrology and river continuum to be assessed for the determination of the high ecological status, but only supportive for all other classes indicating the deviation of the reference conditions or for the definition of heavily modified water bodies. Based on the JDS3 assessment (Schwarz et.al. 2015) the Danube can be characterised by the absence of class one, as over long stretches being moderately altered (39% of all segments), including longer slightly altered reaches on the Lower Danube (21%) while the Upper Danube and Iron Gate reach fall in the extensively and severely altered classes (together 40%) including all major dams with impoundments.

The update of JDS3 concerns the continuous survey of 241 sections of 10 km length, according to the agreed methodology (CEN Standards from 2004 and 2010) and comprises the overall and WFD 3-digit assessment of the hydromorphological features for the navigable Danube from Kelheim (rkm 2,415) to the delta (rkm 0 at Sulina branch).

Under the changed JDS4 framework conditions, with a more active role for national authorities and individual countries, the continuous assessment focused on the update of the HYMO assessment of the predefined

10-rkm-segments with regard to changes (deteriorations[1], improvements) of channel, banks and floodplain. The data collection and assessment was performed by national experts doing investigations (deskwork) supported by a consultant and the ICPDR Secretariat. For this task, an online data collection tool for the changes and projects was integral part of the JDS4 data collection portal.

4.2 Methods

For the JDS HYMO assessment 2013, the Danube was divided into 10-rkm-segments assessing channel, banks and floodplains individually before generating the overall assessment for each segment (compare Figure 1). The usage of the segmentation of JDS3 was mainly a technical step to precisely locate changes – deteriorations and improvements – and does not interfere with the definition of river section types as required to define the reference conditions for the assessment according to CEN standard. For JDS4 it was decided to update the HYMO parameters based on the same segments and to shift the assessment only to those segments with significant changes.

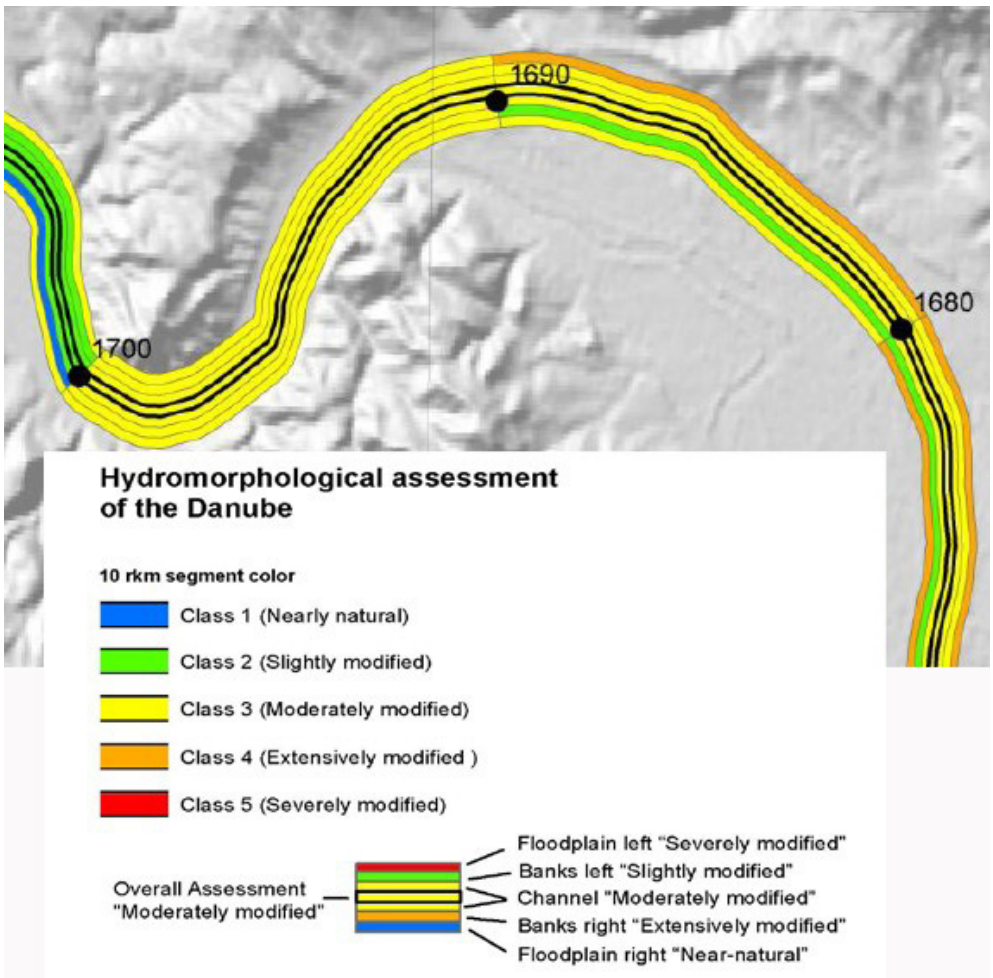


Figure 1: 10-rkm assessment segments of the JDS3 as base for JDS4 (Schwarz 2014).

[1] *The term "deterioration" reflects only the hydromorphological conditions within the 10-rkm river segments and is not to be used according to WFD, which refers to ecological status/potential assessed on water body scale.*

The JDS4 data collection and assessment covered all relevant HYMO changes compared to JDS3 for the period from summer 2013 to summer 2019. The task included the following steps:

- Setup of an online HYMO "change" database under the roof of the JDS4 data collection portal by the ICPDR Secretariat, considering the agreed HYMO parameters and significance criteria as based on the previous JDS3 assessment which need to be updated for the 10-rkm-segments.
- Collection of hydromorphological alterations for the 10-rkm-segments by the Danube countries (coordinated by the national expert of the ICPDR HYMO Task Group).
- Collection of information on relevant Danube River restoration projects and infrastructure projects within the period 2013-2019 by the countries, as integral inventories of the changes database.
- Analysis and assessment of the data and visualisation of individual changes of segments and finally of changed assessment, performed by a consultant in co-operation with the countries.

The changed parameters for overall and WFD 3-digit assessment (morphology, hydrology and river continuity) have been collected in the data collection tool:

- Channel, hydrology, river continuity: Planform (1), substrate (2), erosion/deposition character (3), artificial in-channel features (4) (dams with impoundments and changes in discharge, groynes), continuity (5) (biota/sediment)
- Banks and riparian zone: Extent of reach affected by artificial bank material (6), land cover in riparian zone (7)
- Floodplain: Land cover beyond the riparian zone (8), degree of lateral connectivity of river and floodplain (9), Degree of lateral movement of river channel (10)

Significant new alterations (occurring for the first time between summer 2013 and summer 2019), as well as restoration activities listed below had to be considered if the level of significance exceeded within one of the 241 10-rkm-segments, namely 0.5 km changes in lengths or 5% change of floodplain areas:

- Channel, including hydrology and continuity: Closure of side-channels, groyne construction/ removal, specific, intensive dredging, ongoing, raising or decreasing channel incision, flow regime changes (impoundment length, hydropeaking, water abstraction, particular exposure to ship waves (no thresholds defined), restoration/widening/reconnection of Danube main and side-channels, construction of fish passes or measures to improve sediment transport (gravel feeding, sediment management).
- Banks: New riprap, bank reinforcements, change of land use in riparian zone, restoration of riverbanks (removal of rip-rap).
- Floodplain: Further reduction of floodplain areas by cut-off, change in land use or reconnection of floodplains/retention areas.

Pressure data generated under the DanubeSediment Project (Habersack et al. 2019 and 2020) have been considered as reference by the countries as far as available during the project phase.

Finally, the inventory table for infrastructure and restoration projects is based on the data entries made by the national experts of Danube countries for the JDS4.

After the collection and analysis of changes (improvements and deteriorations) the two assessments of 10-rkm-segments as of JDS3, the overall continuous assessment and the WFD 3-digit assessment had to be revised for the reported 10-rkm-segments with changes (compare JDS3 report, Schwarz et al. 2015).

The overall CEN assessment is based on individual parameters for channel, banks and floodplain and allows an assessment into five classes based on arithmetic mean values for each parameter group and the overall assessment. For channel, the parameters 2-5 are assessed only in three classes (1, 3 or 5).

According to the assessment methods used for the JDS3, the threshold for changes in the assessment for the individual parameters was set for most of the parameters to >5% of affected assessment segment or with other words, if 500 m out of the 10-km assessment segment was altered within the monitoring period, it must be recorded for the update (for areas to be assessed 5% of floodplain area respectively). The "significance" of changes was approved by entire "class changes", but all sub-classes were considered for cumulative effects (e.g. if the sum of changes in the sub-parameters 1-5 for "Channel" exceeded together the 500 m, the assessment for the "Channel" might be changed). Not in all cases did changes necessarily lead to a shift in the assessment class.

4.2.1 The HYMO data collection tool

As part of the JDS4 online data collection portal developed by ICPDR, the module for HYMO allows the seamless data entry, review and update, including upload of accompanying photos and documents, directly by all experts. The tool thus facilitates the strong involvement of national expertise and provides a good basis to receive harmonised results. It can also serve as a reference or even used as it is for the next update.

The database and data entry forms are based on the three entities segments, changes and projects:

- Segments: the spatial data (lines) and base attributes (rkm from-to, country, overall assessment result 2013) of the 10-rkm sections were imported from JDS3 data and served as a reference for the other entities. The tool provides a map and list of segments as an entry point for users to search and select segments for entry of change records.
- Changes: data was entered by users into a web form; each record includes a reference to one or multiple segments, change (improvement, deterioration, no change), assessment group (based on parameters 1-10 of table 1), type (with main options *infrastructure project, maintenance, restoration project, natural process* and respective sub-options), length in km, area in % (for floodplains), optional reference to a project (see below), description, optional photos and files for documentation.
- Projects: data was also entered into a web form; each project record can be related to one or more change records and includes the project type and purpose (based on sub-options for change type of Changes), optional project code, title, implementation year or year range, and optional description.

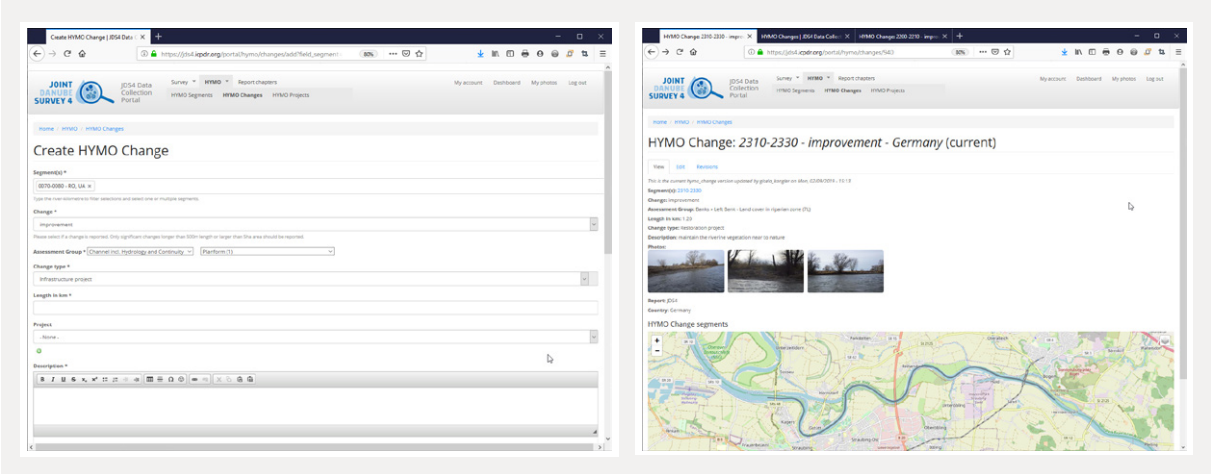


Figure 2: Each different change had to be recorded using the online form. However, as each change record could be related to multiple segments and projects could be referenced to multiple changes, the overall data entry work was minimised.

The entered changes and projects can be listed and filtered by various criteria. All data can be exported as Excel (XLSX) files. The further analysis of data was done externally by the consultant.

4.3 Results and discussion

Based on the 241 10-rkm JDS3 segments (navigable Danube downstream of Kelheim, including only the Sulina branch in the Delta), countries recorded changes of the three main assessment groups (channel, banks and floodplains) for the period 2013-2019.

All riparian countries participated using the data collection tool provided by the ICPDR to record all relevant changes. While for the Upper Danube and SK-HU reach of the Middle Danube reported changes are frequent, long reaches on the Lower and Middle Danube segments have no change. Transboundary reaches were collected independently for each country, but analysed jointly for the whole segment. Reference projects and documentation were not available in all cases.

4.3.1 Analysis of recorded changes

In total, the recorded changes comprise 54 improvements and 19 deteriorations (73). However, several changes occurred in the same 10-rkm-segments for individual parameters, transboundary changes were reported twice (as planned), changes were recorded for two neighbouring segments at once or being recorded for one and the same segment as deterioration and improvement, which is possible. Therefore, only 56 main segments (entire 10-km-segment including all sub segments for channel, banks right/left and floodplain right/left, compare Figure 1) have been subject to individual changes. Nine further changes below the threshold of 0.5 km in length have to be allocated with other changes in the same segment (possible aggregation to 0.5 km) or to be excluded from the segment assessment, which are five segments (three improvements and two deteriorations). Finally, changes as required by the methodology can be assumed for only 55 main segments or 23% of all segments.

Aside of many segments with no changes (186 or 77%), most records are improvements falling into 43 main segments or 18% covering mostly the Upper and Middle Danube in DE, AT, SK and HU, while the 12 segments with deteriorations (5%) can be found in HU, RS, BG, RO and UA (Figure 3).

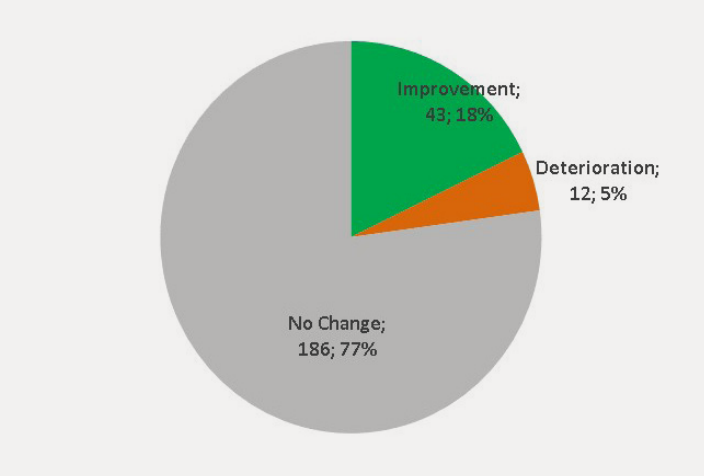


Figure 3: Distribution of changes by number of continuous 10-rkm-segments.

The analysis of changes (Figures 4-6) is based on the total number of recorded changes (73) to keep transparently all records sent by the countries (from the data collection tool).

River bank changes (restoration or construction) prevail with 46% followed by changes of the floodplain (29%) including the reconnection of side-channels and 25% for the channel (Figure 4).

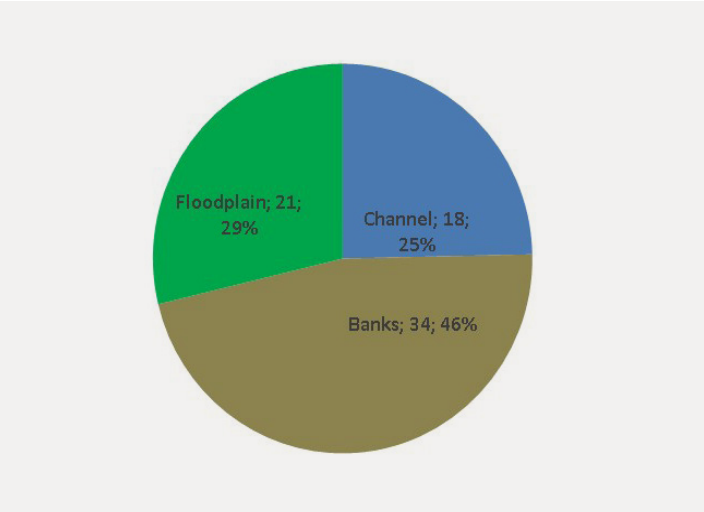


Figure 4: Distribution of prevailing changes for the main categories.

The total length of all changes (73) cumulatively sums up to 159.69 km. Regarding the length of the changes, rather "short and small" projects predominate. The exception are fish passes opening entire 10-rkm-segments for migration of biota. Short measures < 2 km comprise 64% of all changes, but only 37.99 km or 24% of all changes by total length (Figure 5). The average length of changes is about 2.2 km, but excluding the full length of 10-km-sections for continuum restoration by fish passes, the average length dropped to 1.7 km.

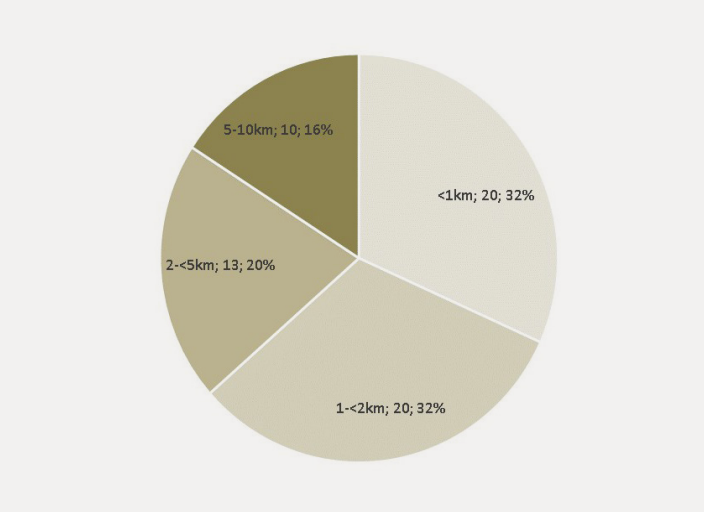


Figure 5: Length classes distribution in km for changes.

Most of the changes are related to river bank development [parameters 6 & 7] with in total 34 changes (Figure 6). The removal of rip-rap clearly prevails with 23 cases. Side channel connections [9] as mainly improvements are rather frequent (8 times) followed by channel changes [1], which are recorded in junction to side-channel connections on the Middle Danube (five times), but also as deterioration (four times due to infrastructure and dredging activities on the Lower Danube). As already mentioned, parameter [5] for continuum improvements are realised entirely in the Upper Danube. Merely the parameter [4] on changed flow conditions and regime by structures (groynes, dams with impoundments) was not reported at all.

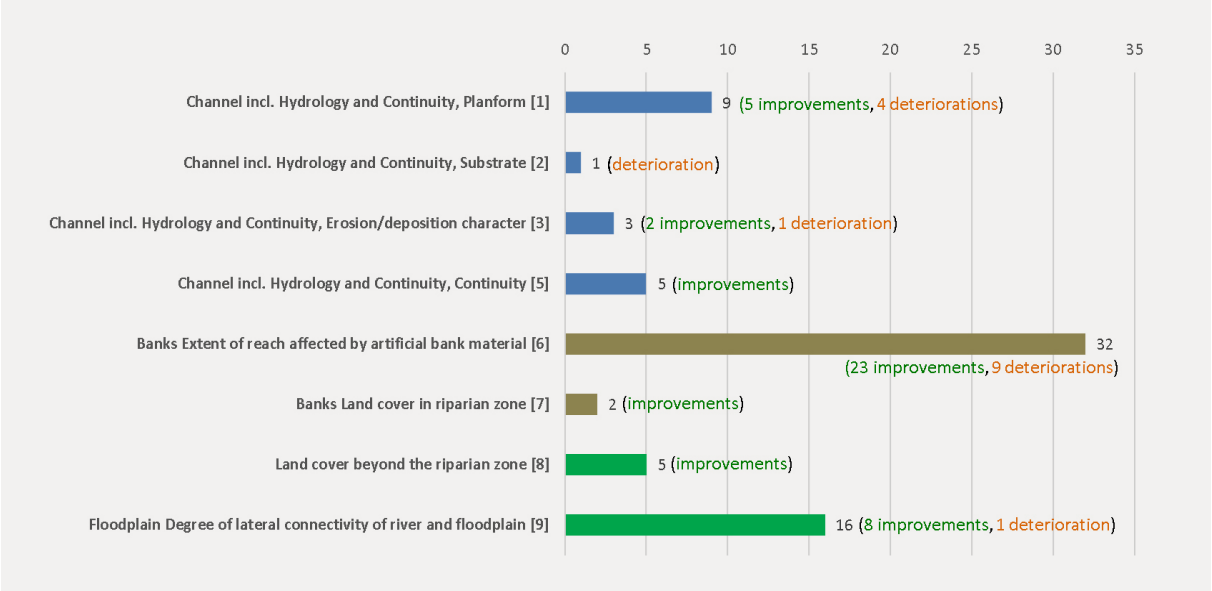


Figure 6: Types of restoration/alteration per all individual changes (blue for "Channel", brown for "banks" and green for "floodplains") and number of improvements/deteriorations per type.

4.3.2 Updated overall assessment

Most of the observed changes cover bank and floodplain segments and show the ambitions of many countries to improve the hydromorphological conditions. However, the length and extent of changes (for structural measures the mean length is 1.7 km) did not lead in all cases to a shift of assessment classes. This has two reasons, firstly the "small size" of changes in relation to the 10-rkm-segment and secondly the previous nearest assessment class boundary.

This lead in total to the class shift of individual assessments for channel, banks and floodplain of 22 out of 55 segments with changes.

After screening and comparing the changes in detail (starting with major changes > 1 km length and by overlaying changes within one and the same segment, e.g. for the transboundary reach of the Danube downstream of Gabčikovo improvements and deterioration reported by both countries neutralize each other), only two segments changed in overall assessment, two in the worse direction, but already having been close to poor assessments before. Those are the segments just downstream of Iron Gate II in Serbia (the bank assessment was reduced from class three to four leading to an overall shift from 3 to 4, however the bank and flood dike construction for Radujevac affect only a small new stretch, in total 2.8 km) and the Danube near Reni in Ukraine (due to recorded dredging in and close to the harbour affecting planform and substrates of channel from 3 to 4 leading to a shift in overall assessment, however the reach of 1.2 km and the amount of dredged material is limited and the dredging started in early 2019, at the end of the monitoring period).

Further several overall assessments for segments (arithmetic mean of classes for channel, banks and floodplain) fail to shift in a better class due to close boundaries, but are strong candidates for the next cycle of restoration measures (e.g. two segments in the AT reach east of Vienna).

Regarding the fish bypasses in the Austrian Danube, the four related segments didn't shift in assessment as for the 3-digit assessment due to the numbers of sub-parameters for the channel group remaining in the worst class: If planform, flow character, sediment grain size, sedimentation/deposition character are untouched from the measure the segment remains in the worst class 5, even the barrier is assessed as class "3" for "partial passable" (for fish but not for sediment).

Considering the reported changes only a few 10-rkm-segments changed for overall class: In two cases the assessment dropped from class three to four in already strongly altered reaches (Figure 7).

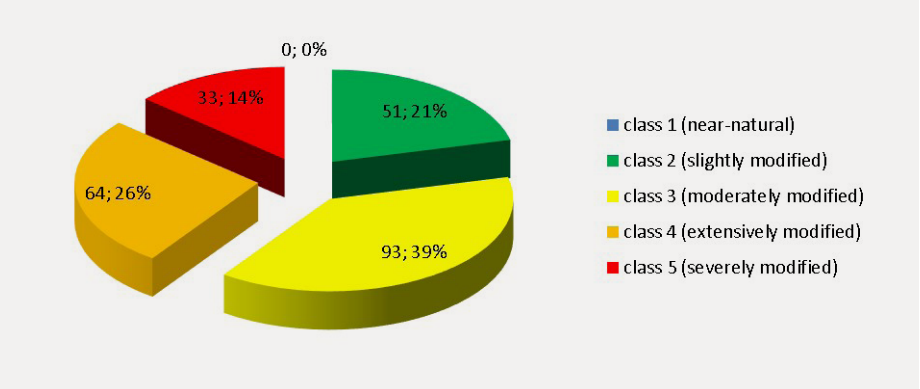


Figure 7: Overall assessment of JDS4 as based on JDS3 with only slight changes (shift of two segments from class 3 to 4, no change in percentage).

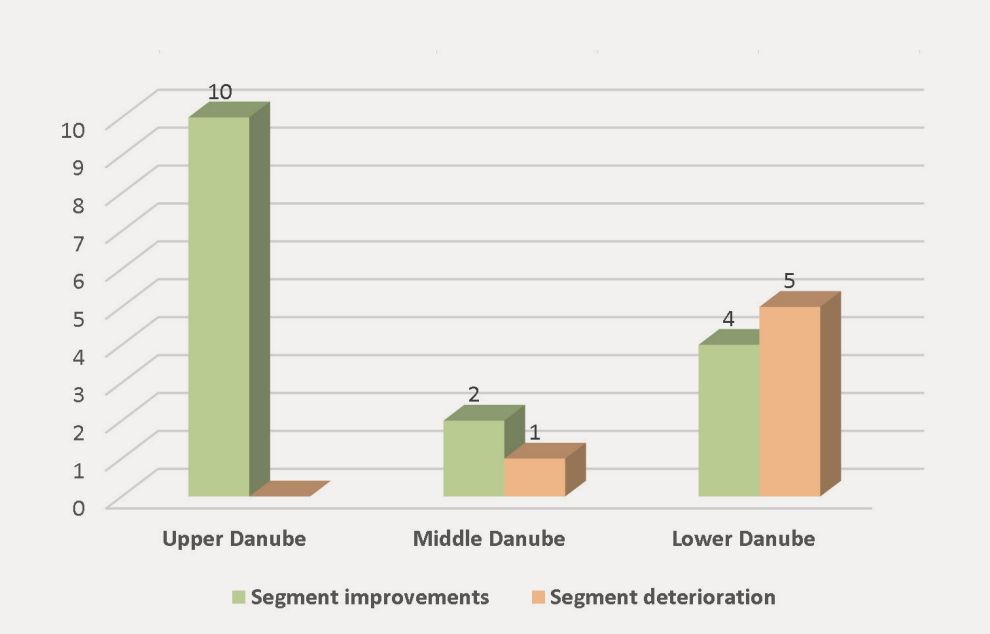


Figure 8: Overview of segments with changes for at least one parameter group (channel, banks, floodplain) along the three main section of Danube.

In general, the recorded changes imply many improvements in the strongly altered Upper and partially the Middle Danube while on the Lower Danube a few deteriorations prevail (Figure 8), however, based on the much better original JDS3 assessment for the Lower Danube in comparison with the Middle and Upper Danube and the deteriorations are spatially limited. In the total perspective, the positive aspects predominate, regarding the fish continuum the construction of bypass solutions for Austrian dams is an important step. Several side-channel connections including SK and HU are good examples for the proceeding restoration. The reason why more segments on the Upper Danube improved in comparison to the Middle Danube, can be explained with the worse situation before in DE and AT, while the free-flowing SK and HU reach assessment in the third moderate class was closer to class four rather than two.

4.3.3 Updated WFD 3-digit assessment

The WFD 3-digit analysis for the entire Danube (Figure 9) indicates the general alteration similar to the overall assessment (prevailing classes 3-5 for the 241 10-km-segement), in particular for the best documented parameter group "Morphology", but also the "Hydrology". The longitudinal continuity is interrupted by 18 dams (segments). In 2013 for two dams with functionning fish passes and partial sediment feeding (Wien-Freudenau and Melk) the value was "3" according to CEN standard.

The biggest difference now is the restoration of partial continuum (for fish) in the Austrian Danube reach. Four additional hydropower dams are in the meantime equipped with fish bypasses, the ecologically most efficient way to restore fish passability. For the Austrian reach therefore only the dam in Altenwörth remains, but will be equipped in 2020, which will expand the passability towards Wachau and even up to Aschach. For bedload sediment (gravel) the dams are still a considerable obstacle (compare outcomes of the Danube Sediment Project, Habersack et al. 2019 & 2020).

For most of the other changes, mainly improvements like the removal of rip-rap for short stretches only on the left or rigth side respectively, the 3-digit evaluation is not as sensitive as the overall assessment, due to the integration of assessment values for both banks and floodplains. For example, if the bank was improved from class 5 to 4 only on the right side, the integrative "Bank" indicator (arithmetic mean) remains class 5. Only in case of improvements on both sides does the assessment value shift. Regarding these major changes within two of the three assessment groups ("Morphology" and "Continuum") a total of seven segments shifted to a better class, including four fish bypasses all located in the Upper Danube, while two deteriorations on the Lower Danube were recorded.

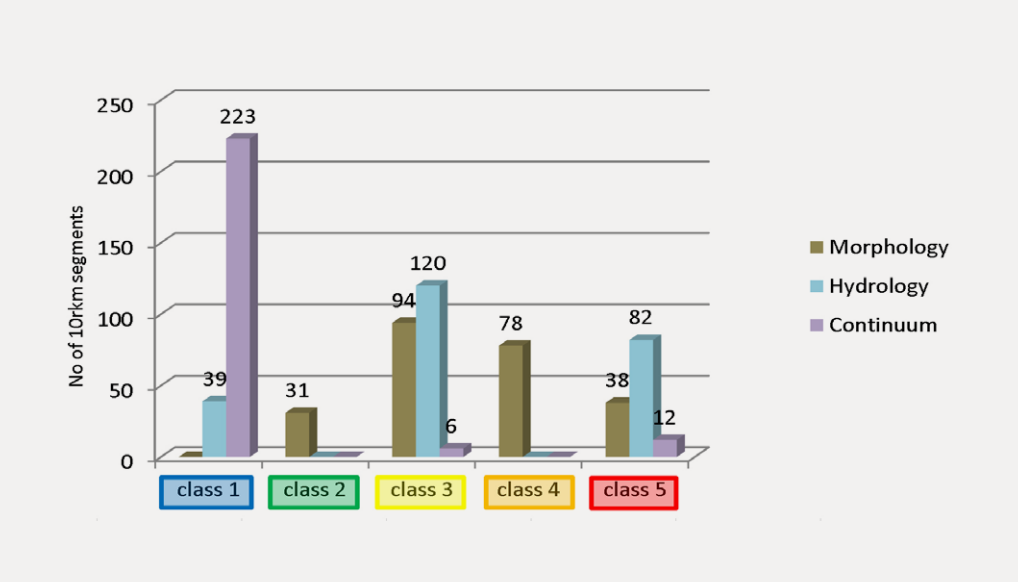


Figure 9: WFD 3-digit assessment as based on JDS3, mainly changed for the continuity for fish by the construction of fish passes in AT (hydrology and continuum were assessed only in classes 1, 3 or 5).

4.4 Conclusion

- All significant changes regarding pressures and restoration along the 241 10-km-segments of the Danube were collected for the time period 2013-2019 and it was possible to update the hydromorphological assessment of JDS3. Even before the start of JDS4, a draft documentation of changes was submitted to the national BQE teams, supporting their assessment of the JDS4 sites.
- In total 55 main 10-km-segments have been recorded to be subject of changes (43 improvements, 12 deteriorations). Finally, only 22 changes lead to shifts in the individual assessment groups (channel, banks, floodplain), while only two segments on the Lower Danube shift in overall assessment, from class 3 to class 4. Regarding the WFD 3-digit assessment four segments profit from fish passes in Austria, reconnecting in total seven segments (70 km) for fish migration.
- In general, improvements prevail on the Upper and Middle Danube, while on the Lower Danube, with exception of some improvements in Bulgaria, slight deteriorations have been recorded (two segments shift in overall assessment). This trend is understandable looking at the previous assessments, indicating many more alterations along the Upper and Middle Danube, while the Lower Danube keeps over long distances a character of fewer alterations (less stabilized banks and rectification of channel, more bars and islands). A general clear trend for the entire Danube cannot be observed for the given period, however the intensified restoration activity on the Upper and Middle Danube and the slight deterioration of the Lower Danube suggest a positive outlook.
- The pressure and restoration update should encourage further detailed in-situ measurement and assessment work (which has to be applied according to WFD finally on waterbody level). It serves as a general estimation of trends along entire Danube. To document the changes and having a monitoring tool for the six-year WFD cycle, the approach is feasible and affordable.
- To scope and fulfil the requirements as under the new CEN Standard (CEN 2018) the methodology has to be further developed to keep previous assessments and to apply the new topics, namely the process-based assessment of fluvial systems. The DanubeSediment project delivered many extremely valuable quantitative hydromorphological data and made first technical proposals as how to assess sediment transport, to improve monitoring, both essential parts of future hydromorphological assessment.
- The outcomes of the DanubeSediment Project (Habersack et al. 2019 & 2020) point towards necessary monitoring and assessments including morphology and quantitative sediment aspects. One out of more potential applications and synthesis of the descriptive and pressure- oriented CEN analysis of JDS HYMO on the basis of 10-km-segments and the quantitative and process- based continuous analysis of the river within the DanubeSediment projects, in particular regarding the longitudinal profile and channel development could be the German ValMorph approach, as applied to the Lower Rhine river (Quick 2019).
- It is recommended to take into consideration the Interreg Danube Transnational Programme DanubeFloodplain project outcomes and related solutions for the improvement of floodplain connectivity with the river.

4.5 References

CEN (2018): prEN 14614:2018, Water quality – Draft Guidance standard for assessing the hydromorphological features of rivers.

CEN (2010): EN 15843:2010, Water quality - Guidance standard on determining the degree of modification of river morphology.

CEN (2004): EN 14614:2004, Water quality - Guidance standard for assessing the hydromorphological features of rivers.

EUROPEAN COMMISSION (2000) Directive 2000/60/ EC of the European Parliament and the Council of 23 October 2000 Establishing A Framework for Community Action in the Field of Water Policy. Off J Eur Union L 327:1–73

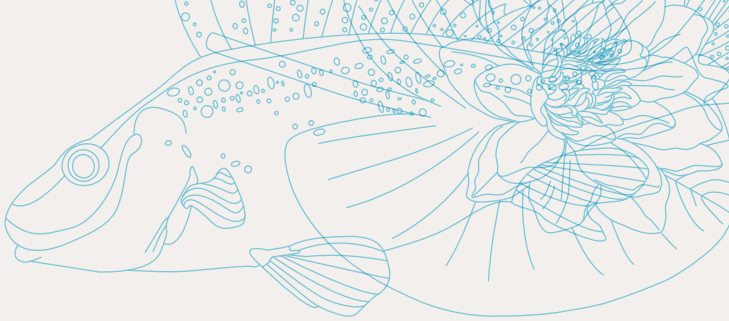
HABERSACK H., Baranya S., Holubova K., Vartolomei F., Skiba H., Schwarz U., Krapesch M., Gmeiner Ph., Haimann M. (2020). Sediment Manual for Stakeholder. Output 6.2 of the Interreg Danube Transnational Project DanubeSediment co-funded by the European Commission, Vienna.

HABERSACK H., Baranya S., Holubova K., Vartolomei F., Skiba H., Babic-Mladenovic M., Cibilic A., Schwarz U., Krapesch M., Gmeiner Ph., Haimann M. (2019). Danube Sediment Management Guidance. Output 6.1 of the Interreg Danube Transnational Project DanubeSediment co-funded by the European Commission, Vienna.

QUICK I.; König F.; Baulig Y.; Schriever S.; Vollmer S. (2019): Evaluation of depth erosion as a major issue along regulated rivers using the classification tool Valmorph for the case study of the Lower Rhine, International Journal of River Basin Management, Volume 18, 2020, Issue 2, pages 191-206.

SCHWARZ U., Holubova K., Cuban, R., Matok, P., Busovsky, J. (2015): JDS 3 Hydromorphological survey, pp. 40-71. In: ICPDR Joint Danube Survey 3. A comprehensive analysis of Danube water quality. Vienna, 369 pp.

SCHWARZ U. (2014): Danube Atlas Hydromorphology. Joint Danube Survey 3. Prepared for ICPDR. Vienna, 40 pp.



05 Fish

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Abstract

During JDS4, fish sampling was conducted using a standardised procedure at 43 sampling sites, which led to a total number of sampling sets of 51, due to parallel activities by the national teams at bilateral sites. In total 76,265 specimens out of 72 fish and three jawless species could be detected, which underlines the importance of the Danube as substantial source of fish biodiversity in Europe. As the composition of fish communities is used worldwide to evaluate the ecological status of rivers, the according national Water Framework Directive methods for the assessment were applied to 32 data sets to get an indication of possible impacts on the fish fauna. The results indicate, that the fish community is threatened along the whole river course but on the other hand, that the diversity of fish taxa still lies on a high level, which raises hope, that effective restoration measures can help to improve the ecological status in order to finally meet the WFD goals.

5.1 Introduction

In total about 100 species of freshwater fish inhabit the Danube along its entire course, covering various ecological and functional guilds (Schiemer et al. 2004, Eros et al. 2005). This comparatively high number is a result of its remarkable importance as an east-west migration route after the end of the last ice age (Balon et al., 1986), which led to the genesis of many endemic species. Danubian fish stocks are declining (Schiemer, 2003) and many species are on the edge of extinction or even beyond that point (Spindler, 1997), nevertheless, fish are still of great economic importance, as an important food source and a valuable target of recreational fishery. Beside this importance, fish communities are a good indicator for human pressures on rivers, in particular for hydromorphological alterations, which are the main cause of declining fish stocks in the Upper Danube (Spindler, 1997). Various studies (e.g. Wiesner et al., 2007) have

shown, that the loss of connectivity due to the extensive use of hydropower and the resulting deterioration of habitat quality can be seen as the main reason for ecological deficits of the fish fauna in the Upper Danube, whereas bad water quality and the exploitation of fish stocks both by legal fishery and poaching are the most considerable causes in the middle and lower course (Schmall & Friedrich, 2014). In order to investigate the current situation of the Danubian fish fauna along the whole river course again, like during the last two surveys (2007 and 2013), standardised fish sampling was undertaken as part of the Joint Danube Survey 4 in 2019.

5.2 Methods and sampling strategy

Deviating from the fish sampling procedure for JDS2 and JDS3, each JDS4 fish site was sampled by the corresponding national team, following the standardised procedure ("JDS4 method") that was agreed on and is defined in the standard operation procedure (SOP) and is based on electric fishing solely. To ensure the use of the correct sampling technique by the national teams, two workshops were held in summer 2018. On selected locations in the border areas between two countries, the JDS4 sites were sampled by both teams independently. Focus was set on the main channel, whereas only some tributaries were sampled as well. The main sampling in the field took place from July 1 to August 28 and acted on the basis of the EU Water Framework Directive and the European Standard "Water Analysis – Fishing with Electricity (EN 14011; CEN, 2003) for wadable and non-wadable rivers. The procedure followed the habitat specific approach (strip fishing method) published by Schmutz et al. (2001) in the litoral area only. As fish assemblages in large rivers show different spatial distribution in the course of day and night (Erős et al, 2017), the standardized sampling effort was 2500 meters at day and 2500 meters at night, whereas depending on the sampled type of habitat, either a boom or hand-held anode was used. In general fish sampling in the Danube was conducted from boats. For sampling purposes the electric field was activated by activating the dead man's switch at irregular intervals. All fish showing electro-tactic movement towards the anode or paralysis were sampled with dip nets, put in a fish tank and afterwards determined to species level, measured (+/- 0.5 cm total length TL) and released alive immediately afterwards. In cases where bulks of specimens had been attracted, a representative subsample was taken and the percentage of caught individuals was estimated by the sampling team.

In the first week the water in the Upper Danube section was a little turbid as a consequence of heavy rainfalls in the Inn catchment area. The German sites were only sampled during day-time as a consequence of staff shortage. The Bulgarian sites JDS43, JDS47 and JDS48 were sampled by beach seine only, the same was done with the Moldovan fish sampling on site JDS49, as electric fishing in general is legally not allowed in the republic of Moldova. The Bulgarian tributary sites JDS44, JDS45 and JDS46 were sampled using back-pack generators. Data for sampling site JDS21 (Ipel-mouth) derive from a sampling session by the Slovakian national team on October 13th, 2018 as this site could not be fished in 2019. All in all the JDS4 fish data contains 51 sampling sets from 43 sampling sites (see Annex A), as at bilateral sites parallel sampling activities were undertaken from the corresponding national teams. In the upper and especially in the Middle Danube sections, the sampling sites were quite dense, whereas in the Upper Danube fish sampling sites were less frequent than in the previous surveys.

5.3 Data processing

Field sampling data were transferred into standardised MS Excel sheets by national teams and sent to the Federal Agency for Water Management (BAW) in Austria. After validation and short analysis of fish data, they were imported into the Danufishbase, which was developed for JDS2 purposes and also used for JDS3 fish data processing. For calculations of abundance and biomass values the same procedure as for JDS2 and JDS3 data was used, following the requirements of the strip fishing method (Schmutz et al, 2001). Beside the JDS site code, a sampling code was generated to differentiate between samplings done at the same JDS site. As electric-fishing at night is not allowed in all participating countries and only some use additional sampling techniques for their national Water Framework Directive (WFD) assessment methods, for calculations of the quantitative stock parameters abundance and biomass, exclusively data from daylight fishing effort were used. Night fishing data delivered additional data for the species composition and age structure at sampling sites. The finalized data base queries were sent out to all national team leaders who were asked to calculate their WFD related assessment index for the ecological status based on the standardized sampling data sets for their national sites.

According to the requirements of the WFD, all EU-member states have to establish a monitoring network and develop assessment methods for all four biological quality elements (BQEs) in all natural water bodies. As the methodological approach for small and medium-sized rivers is well known and widely used, there are lots of reliable datasets which provide a sound basis for the development of appropriate assessment tools. WFD assessment methods for the BQE fish are already intercalibrated for small and medium-sized waterbodies, but for very large rivers with a catchment larger than 10.000km^2 the process has not been completed yet. For the evaluation of potential impacts on the fish fauna at the corresponding JDS4 sampling sites, the applied fish indices, as well as the editors who calculated them are shown in the following table 1.

*Table 1: national assessment methods and editors; * = not WFD compliant.*

Country	Fish assessment method	Editor
Germany	Fisch basiertes Bewertungssystem (fiBs)	Michael Effenberger
Austria	Fisch Index Austria (FIA)	Vinzenz Bammer
Slovakia	Fish Index Slovakia (FIS)	Vladimír Kováč
Hungary	Hungarian Multimetric Fish Index (HMMFI)	Tibor Eros
Croatia	Croatian Quantitative Index of Biotic Integrity IBIHR	Perica Mustafić
Serbia	European Fish Index (EFI)*	Vinzenz Bammer
Romania	European Fish Index (EFI)*	Vinzenz Bammer
Bulgaria	Type specific Bulgarian Fish Index (TsBRI)	Apostolos Apostolou

In addition to this, for sites in the Upper Danube stretch (Germany, Austria, Slovakia, Hungary), FIA and FIS were generated for a direct comparison of results delivered by these methods (see table 5).The proper use of each national WFD assessment method requires their specific, standardized sampling strategy and they only deliver reliable and accurate results based on these. The JDS4 sampling approach was chosen as a kind of minimum effort, in order to be able to use the sampling data from national samplings.

Nevertheless the ecological quality ratio (EQR) values deriving from the national methods build a suitable basis for comparison of the reaction of the fish fauna to different stressors at the JDS4 sampling sites. However, due to differing national methods for determination the ecological status (e.g. three samplings in one WFD-period in Germany, use of additional methods in Austria, night fishing based assessment in Hungary, …), the presented values do not correspond to the official, national WFD results! For comparisons of all different indices, ecological quality ratios deriving from the national assessment methods were used.

5.4 Results

In total 76.265 specimens out of 72 fish and three jawless species could be caught, with the most species (33) detected at the site JDS41, Ilok- Backa Palanka. Most frequent species in all catches by far with a mean relative proportion of the total abundances of 52,28 % was bleak (*Alburnus alburnus*) followed by round goby (*Neogobius melanostomus*) with 9.49 % and chub (*Squalius cephalus*) with 3.90 %. The high abundance value of the allochthonous silver carp (*Hypothalmichthys nobilis*), which was mainly caught in tributaries beginning downstream the river Tisa (JDS32 and JDS33) but also in the Main Danube channel at Pristol (JDS43) is alarming. Detailed analysis of alien fish data will be given in the corresponding IAS Chapter. Figure 1 shows the 20 most abundant species and their relative proportion of total abundance on basis of the complete JDS4 data set, containing data from electric fishing only.

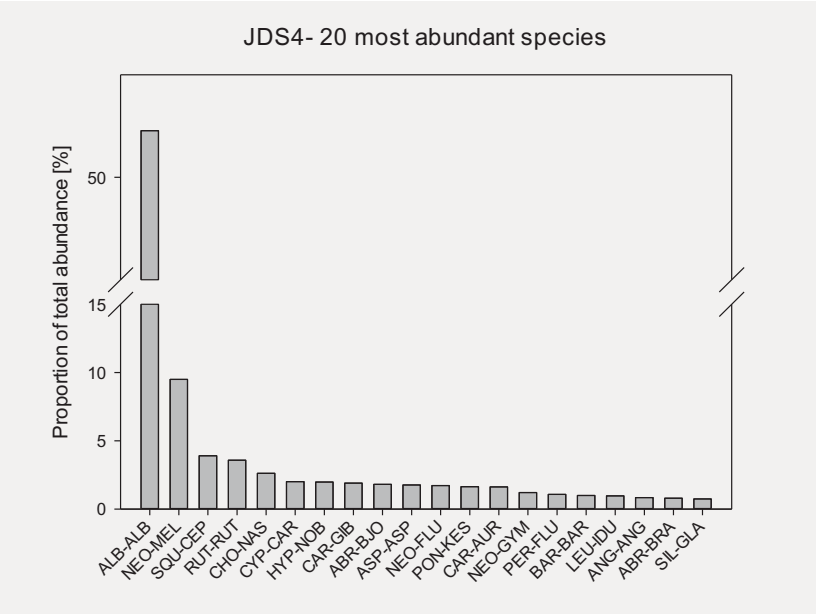

Figure 1: Proportional abundance of the 20 most abundant species, detected by JDS4 sampling; ABR-BJO Blicca bjoerkna, ABR-BRA Abramis brama, ALB-ALB Alburnus alburnus, ANG-ANG Anguilla anguilla, ASP-ASP Aspius aspius, BAR-BAR Barbus barbus, CAR-AUR Carassius auratus, CAR-GIB Carassius gibelio, CHO-NAS Chondrostoma nasus, CPR-CAR Cyprinus carpio, HYP-NOB Hypothalmichtys nobilis, LEU-IDU Leuciscus idus, NEO-FLU Neogobius fluviatilis, NEO-GYM Neogobius gymnotrachelus, NEO-MEL Neogobius melanostomus, PER-FLU Perca fluviatilis, PON-KES Ponticola kessleri, RUT-RUT Rutilus rutilus, SIL-GLA Silurus glanis, SQU-CEP Squalius cephalus.

A comparison with results of JDS2 and JDS3 shows a similar picture with a strongly dominating proportion of bleak in both previous surveys. Between JDS2 and JDS3 a shift in the second most abundant species from Prussian carp (*Carassius gibelio*) to round goby (*Neogobius melanostomus*) could be detected, with round goby still being highly abundant in JDS4 catches.

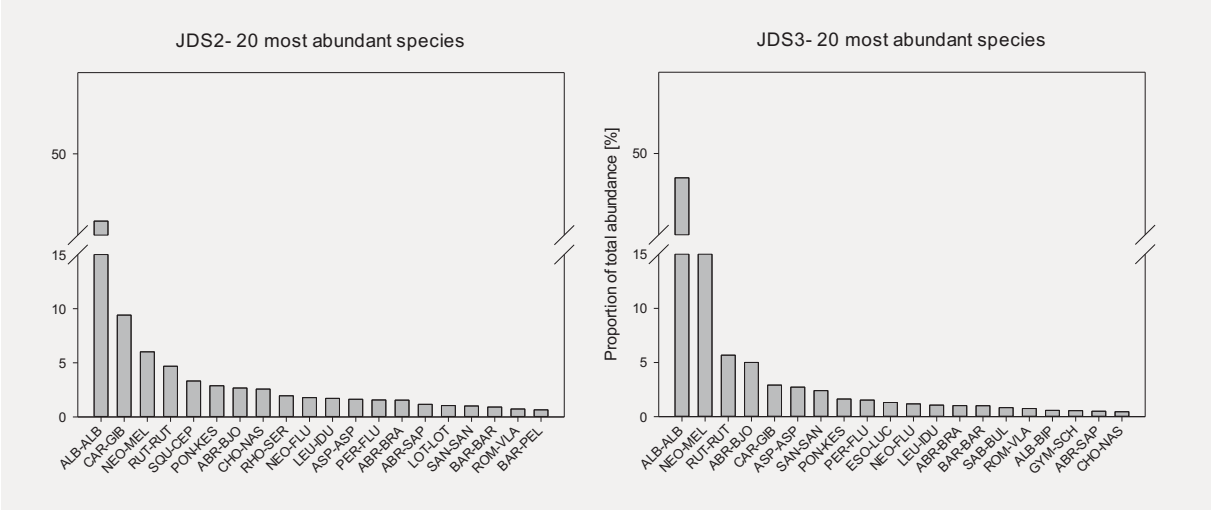

Figure 2: Proportional abundance of the 20 most abundant species, detected by JDS2 and JDS3 sampling, ABR-BJO Blicca bjoerkna, ABR-BRA Abramis brama, ABR-SAP Ballerus sapa, ALB-ALB Alburnus alburnus, ALB-BIP Alburnus bipunctatus, ASP-ASP Aspius aspius, BAR-BAR Barbus barbus, BAR-PEL Barbartula balcanica, CAR-GIB Carassius gibelio, CHO-NAS Chondrostoma nasus, ESO-LUC Esox lucius, GYM-SCH Gymnocephalus schraetser, LEU-IDU Leuciscus idus, LOT-LOT Lota lota, NEO-FLU Neogobius fluviatilis, NEO-GYM Neogobius gymnotrachelus, NEO-MEL Neogobius melanostomus, PER-FLU Perca fluviatilis, PON-KES Ponticola kessleri, RHO-SER Rhodeus amarus, ROM-VLA Romanogobio vladykovi, RUT-RUT Rutilus rutilus, SAB-BUL Sabanejewia bulgarica, SAN-SAN Sander sander, SQU-CEP Squalius cephalus.

Fish abundance –as displayed in figure 3- shows varying values in the middle section of the Danube between 261 and 3.651 individuals per hectare but a clear peak at the Upper Danube site Kelheim (rkm 2.420- JDS03) with 14.873 and a sharp rise in the Romanian section with a maximum value of 59.497 indivuals per hectare at the site Reni (rkm 36- JDS50).

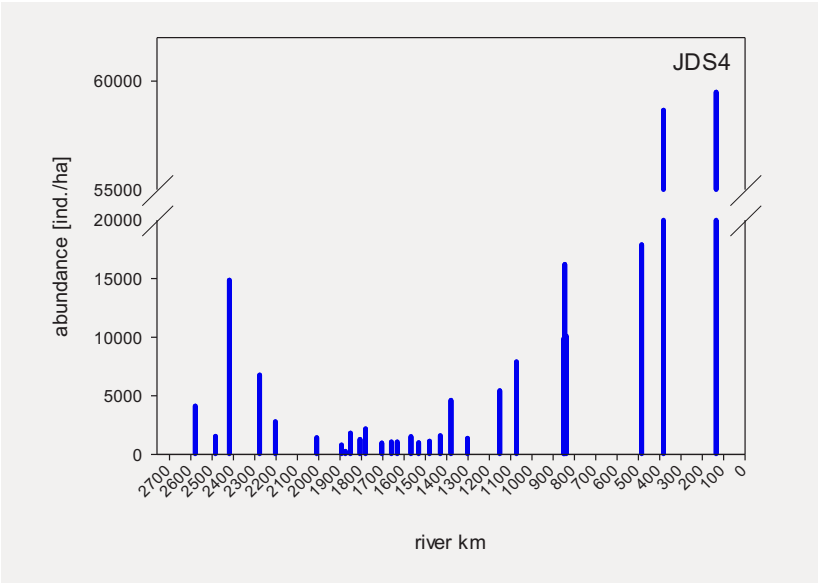

Figure 3: Fish abundance for JDS4 sampling data along the river course.

Regarding fish biomass, a similar effect can be seen (see figure 4): relatively high values in the most upper section with a maximum of 676,37 kg/ha in the German site Niederalteich (JDS04) and peaking values in the Lower Danube starting from the sampling site downstream Ruse/ Giurgiu (rkm 485 -JDS47) to Reni (rkm 136 -JDS50). The biomass from sampling sites in between fluctuated between 18,08 kg/ha (Ilok, Backa Planaka, rkm 1.303 -JDS31) and 106,20 kg/ha (Upstream Timok, rkm 846- JDS41).

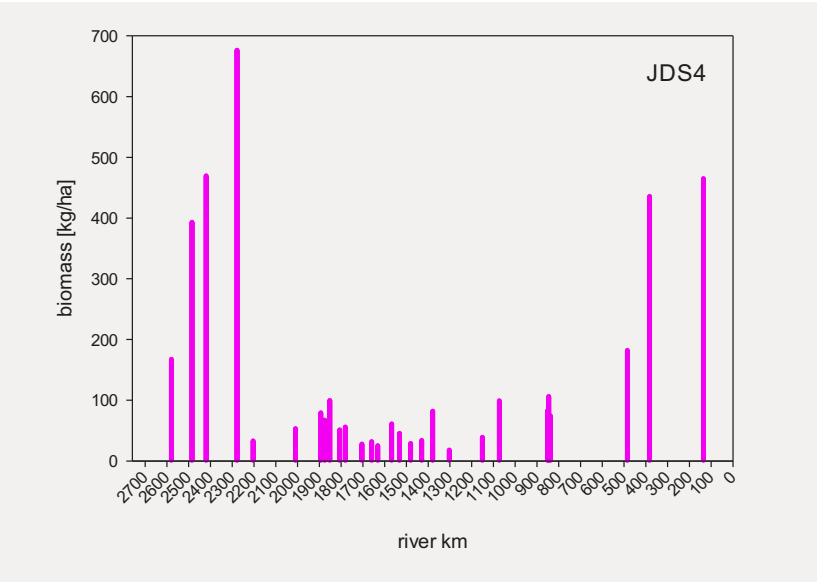

Figure 4: Fish biomass for JDS4 sampling data along the river course.

A comparison of these two quantitative parameters between the three joint sampling surveys shows a decline of both in the area of the middle section of the Danube between 2013 and 2019 at first sight (see figure 5). The values for data from JDS2 and JDS3 showed little fluctuations except the peak value of more than 20.000 specimens per hectare at the site Ilok / Bačka Palanka (rkm 1303 -JDS31) in 2013. Unfortunately the number of the sampling sites during the different surveys as well as their position were not identical in the Middle and Lower Danube sections (between river kilometer 1400 and 1000), which does not allow a sound comparison for that area.

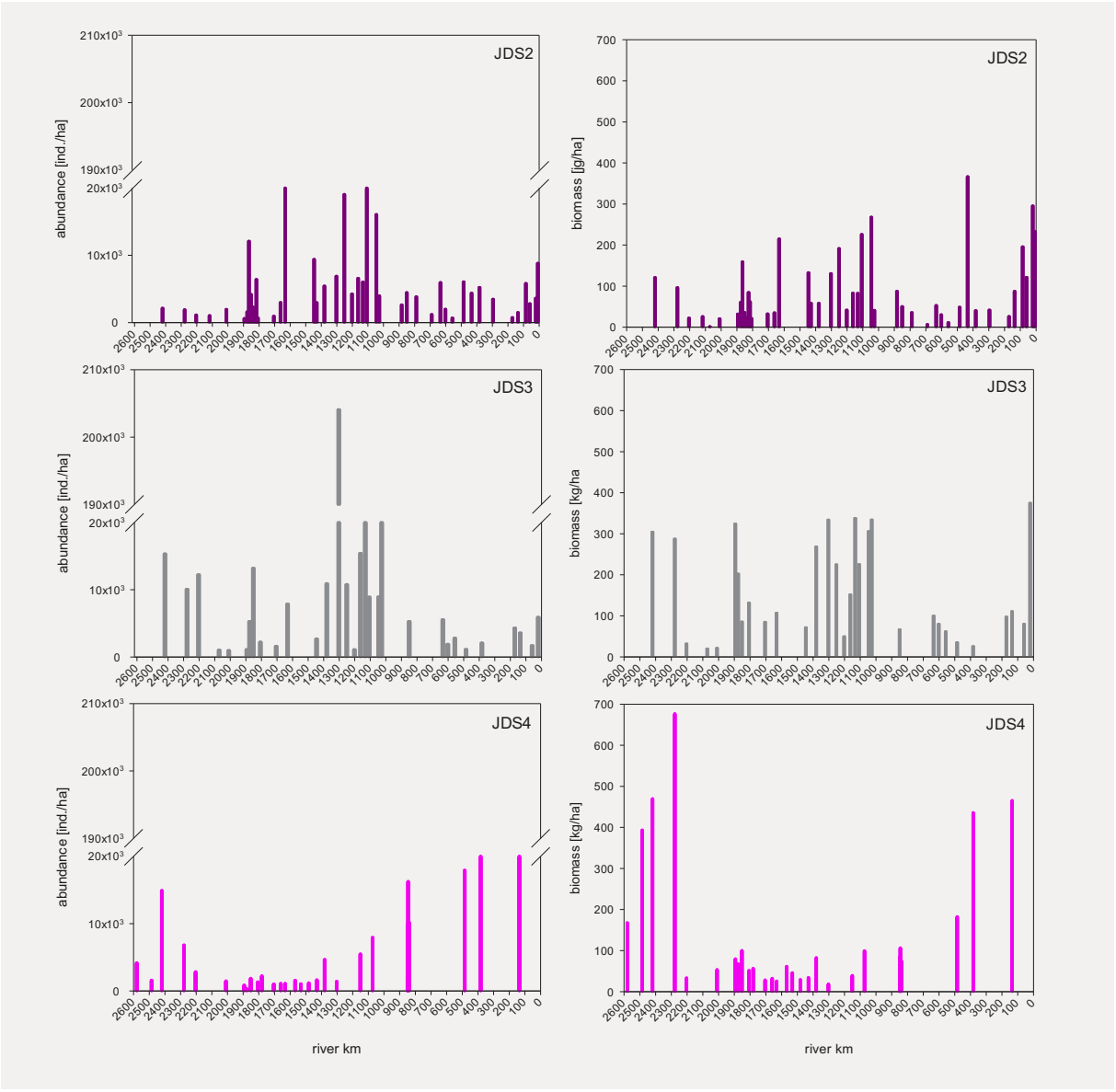

Figure 5: Comparison of abundance and biomass values for all three surveys.

For the German sites in the Upper Danube, a steady increase of biomass between JDS2 and JDS4 can be seen, whereas the abundance values showed a larger variability in the same period with higher levels in 2013 and 2019.

5.5 Indication of the ecological status

As mentioned above, on the basis of JDS4 method data only indications of the ecological status can be given. The online assessment tool for calculating the adapted EFI (European Fish Index) version EFI+ that was used for assessing Romanian sites, has not been available for some years. Therefore it was decided to calculate the EFI for these sites purpose. Same goes for the Serbian stretches, as the FIS, which was proposed to be used first, turned out not be suitable due to the absence of proper reference communities. The German sites JDS01 and JDS06 are declared as heavily modified waterbodies (HMWB) and therefore the basis for the calculation of the national index fiBS is the potential fish coenosis, instead of the reference

assemblage, indicated as "fiBs (pot.)". Hungary delivered two values for their national index: one based on night-fishing data, the other for daylight results. It was agreed with their national team leader to use the mean of both for this report. The Hungarian site JDS25 is categorised as HMWB, for which the national method does not fit. As a consequence it was decided to calculate the EFI for this report. As the sampling strategy for all fish indices solely relies on electric fishing, for those sites which were sampled with beach seine only, no sound and reliable index can be given.

*Table 2: Indicative assessment of the ecological status at JDS4 Danube sampling sites; EQR = ecological quality ratio, *= given value calculated as mean HMMFI based on day- and night fishing; **= EFI score does not accurately correspond to EQR values; editors: AA = Apostolos Apostolou. ME = Michael Effenberger, PM = Perica Mustafić, TE = Tibor Eros, VB = Vinzenz Bammer, VK = Vladimir Kovac.*

JDS4_ID	sampling_ID	EQR/ EFI score***	Ecological Status	Index	Editor
JDS01	DE_JDS4_01	0.440	Good	fiBS (pot.)	ME
JDS02	DE_JDS4_02	0.350	Moderate	fiBS	ME
JDS03	DE_JDS4_03	0.290	Moderate	fiBS	ME
JDS04	DE_JDS4_04	0.360	Moderate	fiBS	ME
JDS06	DE_JDS4_06a	0.250	Moderate	fiBS (pot.)	ME
JDS06	AT_JDS4_06b	0.000	Bad	FIA	VB
JDS08	AT_JDS4_08	0.250	Poor	FIA	VB
JDS10	AT_JDS4_10	0.540	Moderate	FIA	VB
JDS13	SK_JDS4_13	0.147	Bad	FIS	VK
JDS14	SK_JDS4_14	0.165	Bad	FIS	VK
JDS15	SK_JDS4_15	0.133	Bad	FIS	VK
JDS16	SK_JDS4_16	0.145	Bad	FIS	VK
JDS17	HU_JDS4_17	0.352	Poor	HMMFI*	TE
JDS18	HU_JDS4_18	0.566	Moderate	HMMFI*	TE
JDS19	SK_JDS4_19	0.463	Moderate	FIS	VK
JDS21	SK_JDS4_21	0.606	Good	FIS	VK
JDS22	SK_JDS4_22a	0.167	Bad	FIS	VK
JDS22	HU_JDS4_22b	0.498	Moderate	HMMFI*	TE
JDS23	HU_JDS4_23	0.535	Moderate	HMMFI*	TE
JDS24	HU_JDS4_24	0.537	Moderate	HMMFI*	TE
JDS25	HU_JDS4_25	0.205	Poor	EFI**	VB
JDS26	HU_JDS4_26	0.545	Moderate	HMMFI*	TE
JDS27	HU_JDS4_27	0.628	Good	HMMFI*	TE
JDS28	HU_JDS4_28	0.568	Moderate	HMMFI*	TE
JDS29	HR_JDS4_29	0.815	Good	IBIHR	PM
JDS30	HR_JDS4_30	0.826	Good	IBIHR	PM
JDS31	HR_JDS3_31	0.769	Good	IBIHR	PM
JDS32	RS_JDS4_32	0.342	Moderate	EFI**	VB
JDS33	RS_JDS4_33	0.220	Poor	EFI**	VB

JDS4_ID	sampling_ID	EQR/ EFI score***	Ecological Status	Index	Editor
JDS35	RS_JDS4_35	0.171	Bad	EFI**	VB
JDS36	RS_JDS4_36	0.232	Poor	EFI**	VB
JDS37	RS_JDS4_37	0.227	Poor	EFI**	VB
JDS38	RS_JDS4_38	0.372	Moderate	EFI**	VB
JDS39	RS_JDS4_39	0.357	Moderate	EFI**	VB
JDS40	RS_JDS4_40a	0.148	Bad	EFI**	VB
JDS40	RO_JDS4_40b	0.238	Poor	EFI**	VB
JDS41	RS_JDS4_41b	0.210	Poor	EFI**	VB
JDS41	RO_JDS4_41a	0.286	Moderate	EFI**	VB
JDS42	RS_JDS4_42	0.159	Bad	EFI**	VB
JDS44	BG_JDS4_44	0.680	Good	TsBRI	AA
JDS45	BG_JDS4_45	0.800	Good	TsBRI	AA
JDS46	BG_JDS4_46	0.340	Poor	TsBRI	AA
JDS47	RO JDS4_47a	0.340	Moderate	EFI**	VB
JDS48	RO_JDS4_48b	0.314	Moderate	EFI**	VB
JDS49	RO_JDS4_49a	0.301	Moderate	EFI**	VB
JDS50	RO_JDS4_50	0.374	Moderate	EFI**	VB

Based on JDS4 data and as seen in table 2, 17.02 % (8 out of 47 datasets) deliver a good ecological status, as demanded by the WFD latest by end of 2027. A good ecological status in the Danube has only been detected at the German site Boefinger Halde (JDS01), the Hungarian site Paks (JDS27) and the stretch of the Danube shared by Serbia and Croatia Bezdan-Batina (JDS29) and Ilok / Bačka Palanka (JDS31). The other sites in this assessment class are situated in the tributaries Ipel (JDS21), Drava (JDS30), Iskar (JDS44) and Jantra (JDS45). For most sites (42.55 %) only a moderate status could be observed and a poor or bad status is indicated for 19.15 % respectively (see table 3).

Table 3: Indicative ecological status classes for all JDS4 sampling sites.

	High	Good	Moderate	Poor	Bad
sampling data sets (n)		8	20	9	9
Relative proportion (%)		**17.02**	**42.55**	**19.15**	**19.15**

Regarding the indications for the ecological status at sampling sites in the Danube itself (without sites in the tributaries), an even worse situation is obvious: for only four sites or 8.51 % of the sampling sites, the WFD target class "good status" is reached yet, most sites (46.88 %) are classified as moderate, 21.88 % as poor and 18.75 % as bad. (see table 4)

Table 4: Indicative ecological status classes for Danube JDS4 sampling sites.

	High	Good	Moderate	Poor	Bad
sampling data sets (n)		4	15	7	6
Relative proportion (%)		**12.5**	**46.88**	**21.88**	**18.75**

In comparison to the results of the previous two surveys, the latest data are indications for slight deteriorations in the Upper and most sampling sites in the Middle Danube section concerning the ecological status based on the BQE fish. Obvious fish ecological improvements are indicated at the Croatian sites Batina (JDS29) and Ilok, Backa Palanka (JDS31) using JDS4 methods, whereas the recent results indicate a mostly unchanged situation in the Lower Danube. As mentioned above, for the Upper Danube section the indices fiBs and FIS were calculated additionally to FIA and EFI which were applied on all data sets.

*Table 5: Comparison of indications for the ecological status between JDS2, JDS3 and JDS4; * = insufficient data set.*

Site name	**rkm**	**JDS 2**		**JDS 3**			**JDS4**			
		Status FIA	Status EFI	Status FIA	Status EFI	Status FIS	Status JDS4	Status FIA	Status FIS	Status EFI
Boefinger Halde	2,580						Good	Good	Good	high
Bittenbrunn	2,485						Moderate	Good	high	high
Kelheim	2,420	Good	Good	Good	Good	Poor	Moderate	Good		Good
Niederalteich	2,278	Good	Good	Good	Good	Bad	Moderate	Good	Bad	Moderate
Jochenstein	2,215	Poor	Good	Bad	Good	Bad	Moderate	Moderate	Bad	Good
Jochenstein	2,215						Bad	Bad		Good
Ybbs	2,072	Bad	Moderate	Bad	Good	Poor				
Oberloiben	2,010	Poor	Good	Bad	Good	Good	Poor	Poor	Bad	Good
Wildungsmauer - Hainburg	1,894	Good	Good	Moderate	Moderate	Moderate	Moderate	Moderate	Poor	Moderate
Bratislava	1,876	Moderate	Moderate	Good	Moderate	Moderate	Bad	Moderate	Bad	Moderate
Cunovo	1,852	Bad	Poor	Moderate	Poor	Bad	Bad	Moderate	Bad	Poor
Medvedov	1,807	Bad	Good	Moderate	Moderate	Moderate	Bad	Poor	Bad	Moderate
Gönyu	1,781						Moderate	Moderate	Bad	Good
Szob	1,705	Moderate	Good	Good	Moderate	Moderate	Bad	Poor	Bad	Moderate
Szob	1,706						Moderate	Bad		Moderate
Budapest upstream	1,660						Moderate	Poor	Bad	Moderate
Budapest downstream	1,632	Good	Good	Good	Moderate	Poor	Moderate	Bad	Bad	Moderate
Dunafoldvar	1,568						Moderate	Moderate	Poor	Moderate
Paks	1,532						Good	Moderate	Poor	Moderate
Baja	1,481						Moderate	Good	Bad	Moderate
Mohacs Hercegszanto	1,446	Good	Good	Good	Moderate	Moderate				Moderate
Batina	1,434						Good	Poor	Poor	Moderate
Upstream Drava, Aljmas	1,380	Moderate	Moderate	Good	Moderate	Moderate				
Ilok, Backa Palanka	1,303	Moderate	Moderate	Moderate	Moderate	Bad	Good	Bad	Poor	Moderate
Novi Sad downstream	1,252	Moderate	Moderate	Moderate	Moderate	Poor				
Belegish	1,202	Moderate	Moderate	Poor	Moderate	Moderate				

Site name	rkm	JDS 2		JDS 3			JDS4			
		Status FIA	Status EFI	Status FIA	Status EFI	Status FIS	Status JDS4	Status FIA	Status FIS	Status EFI
Downstream Sava,	1,163	Moderate	Moderate	Moderate	Bad	Poor				
Pancevo downstream	1,151						Poor	Poor	Poor	Poor
Grocka	1,132	Moderate	Moderate	Moderate	Poor	Bad				
Velika Morava downstream	1,107	Good	Moderate	Good	Moderate	Bad				
Golubak Koronin	1,046	Moderate	Bad	Good	Poor					
Banatska Palanka / Bazias	1,073						Bad			Bad
Banatska Palanka/Bazias	1,073						Poor			Poor
Vrbica, Simijan	1,027			Good	Moderate					
Upstream Timok	850		Moderate	Moderate	Poor		Poor			Poor
Upstream Timok	851						Moderate			Poor
Timok mouth	0,2						Bad			Bad
Pristol-Novo Selo Harbour	839						Good			Poor
Downstream Kozloduy	690		Poor	*	*					
Downstream Iskar	634		Poor	*	*		Good			Moderate
Downstream Olt	602		Moderate	Moderate	Poor					
Jantra, before estuary	537						Good			Good
Russenski Lom	498						Poor			Moderate
Downstream Ruse - Giurgiu	485		Moderate	*	*		Moderate			Moderate
Chiciu, Silistra	383	Bad	Poor	Poor	Moderate		Moderate			Moderate
Downstream Braila	172		Moderate	Good	Moderate					
Reni	136		Moderate	Good	Moderate		Moderate			Moderate
Chilia Arm-Valcov	60		Moderate	Good	Moderate					
Sulina - Sulina Arm	21		Moderate	Good	Moderate					

5.6 Conclusive Discussion

The original Danubian fish assemblage is well documented from historical studies and the total number of species between the rithral Upper section and the potamal Lower area is in the order of 100 species (Schiemer et al. 2004). Our results show, that still most species of the reference communities can be found at nearly all sites. This is even true for strongly altered hydromorphological stretches in the Upper Danube section. The species compositions at the different sampling sites reflect the wide range of aquatic habitats in this large stream and the combination of rhitral and potamal elements. As a consequence, the number of 72 fish and jawless taxa that could be detected during JDS4 is still remarkable and even higher than was found in 2013 (67). Concerning the historic diversity of the Danubian fish fauna, Schiemer et at al. (2004) refer to the work of Marsilius (1726) and Heckel & Kner (1858), who indicated a total number along the whole river course of around 100. According to a review by Balon (1964), the highest species diversity (approximately 60) was found in the Lower Danube as a consequence of the influence of migratory species from the Black Sea. A second diversity hotspot with about 50 fish taxa was found downstream of the alluvial plains of the Austrian Donau-Auen National Park, which can be explained by a sharp increase of habitat diversity in the area of the transition between foothills and lowlands, which leads to suitable conditions for many different kinds of aquatic species. Although the Danubian fish community is under threat along the whole river course, the fact, that most species of the historic ichthyofauna still can be found in the Danube raises hope, that effective restoration measures can help to improve the ecological status in order to finally meet the WFD goals.

As was observed in the previous two Joint Danube Surveys (JDS2 and JDS3), there was an extraordinary dominance of bleak (*Alburnus alburnus*), a typical swarm fish, which prefers the upper water column close to the surface and of round gobies (*Neogobius melanostomus*), which hide in cavities of the litoral rip-rap structures, was detected. This must be seen in context with the species selectivity of electric fishing, as both species can be collected quite easily with electric fishing in relatively high abundances. At the moment the anode is dipped into water, an electric field is built up imediately between the electric poles, which attracts close-by fish first, whereas more distant individuals have more time to escape. Round gobies which hide in structures like in cavities of the rip-rap at the shore line, when disturbed, can be collected in large numbers during sampling with hand-held anode close to such refuges. This explains the high abundances of these species at least to a certain extent and must be kept in mind when using these data for assessment purpose. Taking this into consideration, alternative calculations of FIS were performed for the Slovak stretch of the Danube (Bratislava, Čunovo, Medveďov, Szob) by the national team leader. When the numbers of bleak in the samples were reduced to 30-50 % (difference in the efficiency of the sampling method between bleak and other species, estimated by the national sampling team), the resulting values of FIS improved the indication for the ecological status of these sites from bad to moderate, which matches the indicative status of FIA better. Nevertheless the recent JDS4 dataset is a solid basis for evaluations of the Danubian fish assemblage, as electric fishing was the method of choice in the quantitative analysis for the previous fish sampling for JDS2 and JDS3.

The uncertain decline in biomass values at Lower Danubian JDS4 sampling sites has to be confirmed/ falsified by national monitoring data and should at least be a call for selecting an appropriate number of sampling sites and for monitoring them continuously.

Based on the recent JDS4 sampling, data national assessment methods indicate a moderate to worse ecological status for most sampling sites in the Danube. As mentioned above, each national WFD compliant method requires a standardised sampling procedure as well as effort which both vary between single countries due to different hydrological and biotic requirements. Some use night fishing data only,

others day light sampling only and also a combined effort with additional methods is in use. In a literature review Potyó and Guti (2012) indicated the methodological challenges for quantitative fish sampling in large rivers. With a closer look at the national WFD assessment results, a similar variability can be seen, which makes the comparison of those national indices difficult. For JDS2 and JDS3 data all sites had been assessed using FIA and EFI. Both are not adequate for evaluating fish communities along the whole stretch of the Danube: FIA delivers sound results for the ichtyofauna in the Upper Danube and detects structural/ hydromorphological deficits solidly, whereas EFI scores are not reliable for the Danube at all and were only used when no national assessment method was available. Nevertheless the fact that nearly all national methods in the Upper Danube (DE, AT, SK, HU), indicate a status worse than required to meet the WFD targets, the need for action at least in this area is evident. JDS4 data for the stretch of the Danube shared by Croatia and Serbia Danube sampling sites showed a good status whereas for the Lower Danube section the JDS4 sampling sites had a comparable low density, which in combination with the fact that in contrast to 2007 and 2013 no sound assessment was available, does not allow a reliable classification for 2019.

5.7 Acknowledgement

The research of the Slovak team was partially supported by the project APVV-16-0253.

5.8 References

BALON E.K. (1964): Verzeichnis und ökologische Charakteristik der Fische der Donau, Hyrobiologica, 24: 441-52

BALON E.K., CRAWFORD, S.S., LELEK A. (1986): Fish communities of the Upper Danube Germany, Austria prior to the new Rhein-Main-Donau connection. Env. Biol. Fish., 15: 243-71

CEN, European Committee for Standardization (2003): EN 14011, Water quality - Sampling of fish with electricity

EROS, T. (2005): Life-history diversification in the Middle Danubian fish fauna - a conservation perspective. Arch. Hydrobiol., Suppl. Large Rivers 16,1-2: 289-305.

ERŐS, T., BAMMER, V., GYÖRGY, Á. I., PEHLIVANOV, L., SCHABUSS, M., ZORNIG, H., WEIPERTH, A., SZALÓKY, Z. (2017): Typology of a Great River Using Fish Assemblages: Implications for the Bioassessment of the Danube River. River Research and Applications, 33(1) 37-49. DOI: 10.1002/rra.3060

HECKEL R., KNER R. (1858): Die Süßwasserfische der Österreichischen Monarchie mit Rücksicht an die angrenzenden Länder, Leipzig, W. Engelmann, 338 pp.

MARSILIUSA.F.(1726):Danubiuspannonico-mysicus,observationibusgeographicis,astronomicis,hydrographicis,historicis,physicisperlustratus et in sex Tomos digestus. Tomus IV. De piscibus in aquis Danubii viventibus. Hagae Comitum, Amstelodami. 89 pp

POTYÓ I., GUTI G. (2012): Requirements for representative sampling for fluvial fish assemblages – literary review; Opusc. Zool. Budapest, 2012, 43(2): 203–209

SCHIEMER, F. (2003): Ecological status and problems of the Danube River and its fish fauna: a review; in Proceedings of the second International Symposium on the Management of Large Rivers for Fisheries. Vol. I; Sustaining livelihoods and biodiversity in the new millennium ; RAP Publication (FAO) , no. 2004/16; International Symposium on the Management of Large Rivers for Fisheries, 2, Phnom Penh (Cambodia), 11-14 Feb 2003.

SCHIEMER, F., GUTI, G., KECKEIS, H., STARAS, M., (2004): Ecological status and problems of the Danube and its fish fauna. A review- Proceedings of the second international Symposium on the management of large rivers for fisheries

SCHMUTZ, S., ZAUNER, G., EBERSTALLER, J., JUNGWIRTH, M. (2001): Die Streifenbefischungsmethode: eine Methode zur Quantifizierung von Fischbeständen mittelgroßer Fließgewässer; Österreichs Fischerei JG 54/2001, pp 14-27

SCHMALL B., FRIEDRICH T. (2014): Das Schicksal der großen Störarten in der oberen Donau, Denisia 0033, 423-442

SPINDLER, T. (1997): Fischfauna in Österreich. Ökologie - Gefährdung - Bioindikation - Fischerei - Gesetzgebung. Monographien des Umweltbundesamtes, Band 87. Wien: Umweltbundesamt.

WIESNER, C., SCHOTZKO, N., CERNY, J., GUTI, G., DAVIDEANU, G., JEPSEN, N., (2007): TechnicalReport with Results from the Fish Sampling and Analyses from the Joint Danube Survey 2007. International Commission for the Protection of the Danube River, Vienna, 73 pp.

Annex A: Sampling Effort

Site ID	Sampling ID	Electric fishing- day		Electric fishing- night		Beach seine	
		Nuber of strips [n]	Sampled area [m²]	Nuber of strips [n]	Sampled area [m²]	Nuber of strips [n]	Sampled area [m²]
JDS01	DE_JDS4_01	10	7500				
JDS02	DE_JDS4_02	6	6750				
JDS03	DE_JDS4_03	8	9500				
JDS04	DE_JDS4_04	8	9500				
JDS06	AT_JDS4_06b	11	11430	9	10647		
JDS06	DE_JDS4_06a	9	8500				
JDS08	AT_JDS4_08	12	11998	11	12600		
JDS10	AT_JDS4_10	14	15094	13	13318		
JDS13	SK_JDS4_13	1	840				
JDS14	SK_JDS4_14	5	9510	4	5300		
JDS15	SK_JDS4_15	5	6000	4	5500		
JDS16	SK_JDS4_16	6	7270	5	6750		
JDS17	HU_JDS4_17	10	3750	10	3750		
JDS18	HU_JDS4_18	10	3750	10	3750		
JDS19	SK_JDS4_19	1	1050				
JDS21	SK_JDS4_21	1	300				
JDS22	HU_JDS4_22b	10	3750	10	3750		
JDS22	SK_JDS4_22a	3	4750	1	1500		
JDS23	HU_JDS4_23	10	3750	10	3750		
JDS24	HU_JDS4_24	10	3750	10	3750		
JDS25	HU_JDS4_25	10	3750	10	3750		
JDS26	HU_JDS4_26	10	3750	10	3750		
JDS27	HU_JDS4_27	10	3750	10	3750		
JDS28	HU_JDS4_28	10	3750	10	3750		
JDS29	HR_JDS4_29	5	7500	1	1500		
JDS30	HR_JDS4_30	5	7500	4	6000		
JDS31	HR_JDS3_31	5	7500	5	7500		
JDS32	RS_JDS4_32	10	3750	10	3750		
JDS33	RS_JDS4_33	10	3750	10	3750		
JDS35	RS_JDS4_35	10	3750				
JDS36	RS_JDS4_36	10	3750	9	3375		
JDS37	RS_JDS4_37	10	3750	10	3750		
JDS38	RS_JDS4_38	10	3750	10	3750		
JDS39	RS_JDS4_39	10	3750	10	3750		
JDS40	RO_JDS4_40b	19	6900				
JDS40	RS_JDS4_40a	10	3750	10	3750		
JDS41	RO_JDS4_41a	13	4950				
JDS41	RS_JDS4_41b	10	3750	10	3750		
JDS42	RS_JDS4_42	10	3750	10	3750		
JDS43	BG_JDS4_43a					1	840
JDS43	RO_JDS4_43b	5	2025				
JDS44	BG_JDS4_44	1	1500				
JDS45	BG_JDS4_45	1	2250				
JDS46	BG_JDS4_46	1	500				
JDS47	BG JDS4_47a					1	300
JDS47	RO JDS4_47b	15	5625				
JDS48	BG_JDS4_48a					1	300
JDS48	RO_JDS4_48b	9	3150				
JDS49	MD_JDS4_49b					3	750
JDS49	RO_JDS4_49a	7	2625				
JDS50	RO_JDS4_50	11	4050				

06 Aquatic macroinvertebrates

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Abstract

Benthic macroinvertebrates were sampled by national experts during the JDS4 campaign in the first weeks of July with five different sampling approaches. Samples from Multi-Habitat Sampling (MHS) were completely analysed and used for Indicative Status Assessment (ISA). National experts with help of external experts processed and identified MHS samples according to JDS4 MZB Methodology. In the majority of cases, only one side of the river was selected for sampling, though at transboundary sites, both sides were usually sampled. In total, 484 taxa were found belonging to 19 higher taxonomical groups, 394 taxa were found in the Danube River and 287 taxa in tributaries. For definition of water quality, the Saprobic index and Slovak Multi-metric Index were used for indication of responds of macroinvertebrates assemblage to both effects of pollution and changes in hydromorphology.

6.1 Introduction

Benthic macroinvertebrates are the most widely used indicator group for lotic systems (Moog et al., 2018). These organisms, when used in such investigations, offer several benefits including easy identification at high taxonomic levels by non-specialists, high sensitivity of a great number of species to environmental stress, a wide distribution in various freshwater habitats and a relatively sedentary behaviour and short life cycle, in comparison to fish, which facilitate the detection of changes over time (Johnson et al. 1993).

The following subchapters describe the methods applied; the characteristics of the macroinvertebrate community along the Danube River and its tributaries and show resulting ISA and Saprobic index compared with previous JDS2 (2007), JDS3 (2013) and national assessment results.

6.2 Methods

6.2.1 Sampling Methods

The JDS4 monitoring campaign for benthic macroinvertebrates was carried out by national teams while the Core team of international experts had a coordinating and advisory role to ensure the coherence between the approaches used by the national experts.

Based on the experiences from the previous Joint Danube Surveys, five different approaches were applied:

Main approach: **Multi-Habitat-Sampling (MHS)** – used as a standardized WFD sampling method for the ecological status assessment (AQEM Consortium, 2002) was effective for ecological status assessment of wadable rivers – or large rivers at lower water period (Graf et al. 2015).

Additional approaches: i) **Kick and sweep (K&S)**, ii) **Deep Water Sampling – dredging (DWD)**, iii) **Specific sampling for molluscs (AMS** – Additional Molluscs Sampling) and iv) **Specific sampling for crayfish (LiNi)**.

Methods are described in detail in full report and Standard Operational Procedures (SOP) for MZB and Invasive Alien Species (available on www.danubesurvey.org/jds4).

A total number of 46 JDS4 sampling sites were planned for macroinvertebrates sampling. Due to high water levels, sampling was postponed (to end of September) in the case of the River Inn at Passau-Ingling (JDS4-5-L) below the power station. Sampling site Timok mouth (JDS4-42; 0.2 r. km) was sampled but no living organisms were found. From all five sampling approaches, only MHS was used for the diversity overview and ISA, samples from other approaches were processed partially and used for neozoa and molluscs study. Out of 45 JDS4 sites, 35 sites were sampled at one river side/bank and 10 at both sides/banks (explained in paragraph 2.2). Hence, 55 samples were collected in total.

6.2.2 Metrics and Indicative Status Assessment (ISA) Method

Only one river side was selected for sampling. In case of transboundary sites, both river sides were usually sampled. Sampling sides were agreed on bilateral negotiations. Each side (left or right bank) was considered and assessed as a separate sample.

Multi-metric Index (MMI) Slovak national method for large rivers (Makovinská et al. 2015) was used for the ISA and already tested with prior Austrian Danube data providing reasonable results (Leitner, 2013). Relevant metrics were selected for rivers in altitude below 200 m a.s.l. and between 200 – 500 m a.s.l.. Internal Water Research Institute software INFOSYS based on ASTERICS ver. 4.0.4 was used for calculation of metrics and Indicative status final evaluation.

Saprobic indices (SI) were calculated based on available national method, using ASTERICS 4.04 and EcoProf 5.0 software. For the indication of quality classes, threshold values according to Buijs (2006) were applied.

6.2.3 Statistical Method

Ordination and classification methods were used to gain insight into variability of invertebrate communities along the Danube River. Principal coordinate analysis (PCoA) using matrix of Hellinger distances was employed to extract main compositional gradients. Longitudinal zones across which the invertebrate communities changed markedly were identified using stratigraphically constrained incremental sum of

squares cluster analysis (CONISS, Grimm, 1987). Broken-stick model was used to determine significant number of zones in the cluster analysis (Bennett, 1996). For the multivariate analyses, data from left and right bank of the river were pooled within sites (Fig. 1).

PCoA was also used to visualize differences in community composition between communities sampled at left and right banks. Only the sampling sites with both banks sampled were used in this analysis.

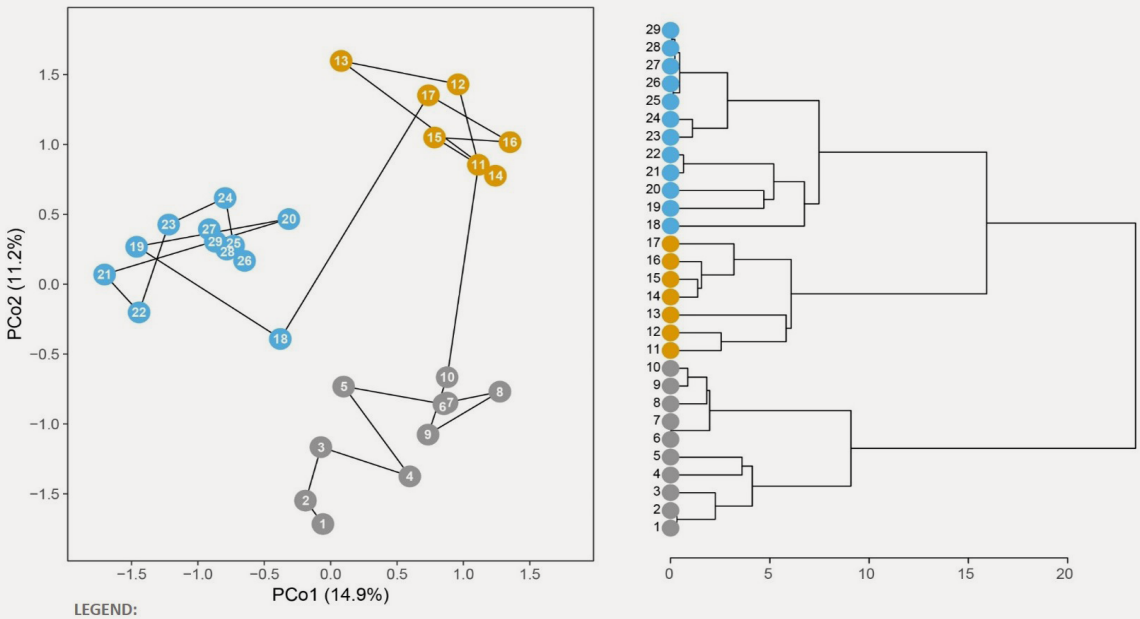
6.3 Results and Discussion

6.3.1 Diversity and density from Multi Habitat Sampling (MHS)

During the JDS4 sampling campaign, in total, 484 aquatic macroinvertebrate taxa were found in 55 samples. Altogether 394 taxa were found in the Danube River and 287 taxa in tributaries (Inn, Dyje, Morava, Moson Danube,Vah, Hron, Ipel, Ráckevei, Drava, Tizsa, Sava, Velika Morava and Prut).

The most diverse groups were Diptera (160 taxa) and Oligochaeta (53), followed by Trichoptera (42) and Gastropoda (41) then Crustacea (32), Ephemeroptera (30), Bivalvia (28), Coleoptera (25) and Odonata (22). Heteroptera (12), Hirudinea (9) and Turbellaria (5) are less heterogeneous groups. Other groups were even less diverse. Nematodes were only well identified by Bulgarian national experts at the species level (11 taxa) and were excluded from diversity and statistical analyses as they are not considered as a typical benthic macroinvertebrates (often categorized as microinvertebrates) and also for comparison purpose.

Focusing only at the Danube River reaches (Upper Danube River: from source to rkm 1790, Middle Danube River: from rkm 1790 – 943, Lower Danube River from rkm 943 to mouth; Tab. 6), most diverse groups are as follows: Diptera (130 taxa), Oligochaeta (40), Trichoptera (37), Mollusca (Gastropoda 36 taxa, Bivalvia 23 taxa), Crustacea (29), Ephemeroptera (23), Coleoptera (20) and Odonata (13). Along the Danube River reaches, EPT (Ephemeroptera, Plecoptera & Trichoptera), Coleoptera and Bivalvia taxa are decreasing in diversity. On the contrary, Oligochaeta together with Gastropoda were increasing in heterogeneity (Fig. 2). Other groups are constant. Less than 10 taxa were recorded on sampling sites 29-L and 41-R and less than 6 taxa were examined on sites 23-L and 28-R in total. Cluster analysis of Danube River samples shows MZB assemblage changes in longitudinal gradient (Fig. 1). As the slope of the river determines the flow velocity, the bed sediment and benthic communities gradually change. Analysis indicates 3 separate sections, and the boundary between upper and middle section (16-R Medveďov / 18-R Gönyű) is similar to the pre-defined Upper and Middle Danube River reaches and where the boundary between Danubian and Pontocaspian fauna could be found (Brtek, 1953). However, the boundary between the middle and lower section has shifted upstream in comparison to the pre-defined Middle/Lower Danube reach, which already includes the bigger part of the Hungarian stretch.



Number	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
JDS4 site	1-L	2-R	3-R	4-R	6-L+R	7-R	8-L	10-R	14-R	16-R	18-R	22-R	23-L	24-L	26-R
Number	16	17	18	19	20	21	22	23	24	25	26	27	28	29	
JDS4 site	27-L	28-R	29-L+R	31-L	31-R	37-L	37-R	40-L+R	41-L+R	43-L+R	47-L+R	48-L+R	50-R	51-R	

Figure 1: PCoA ordination plot (left) and CONISS dendrogram (right) of invertebrate communities (only Crustacea, Diptera, Ephemeroptera, Mollusca, Oligochaeta and Trichoptera could be used; data from left and right bank were merged). Significant zones are highlighted in different colours. Variance explained by the ordination axes is given in parentheses.

Differences in invertebrate community composition between left and right banks of the river were sometimes as large as differences among the sampling sites (Fig. 3, right). The variation within sites could be attributable to different habitat composition and/or to influence of tributaries.

When compared to the results from JDS3, a similar diversity pattern occurred, however, the number of taxa of Gastropoda groups found during JDS4 has doubled. On the other hand, several Ponto-Caspian species native to the Lower Danube River stretch found during JDS3 were now seen to be missing. In addition, species from genus *Pisidium* sp. are completely missing in the taxalists from the middle and lower reaches.

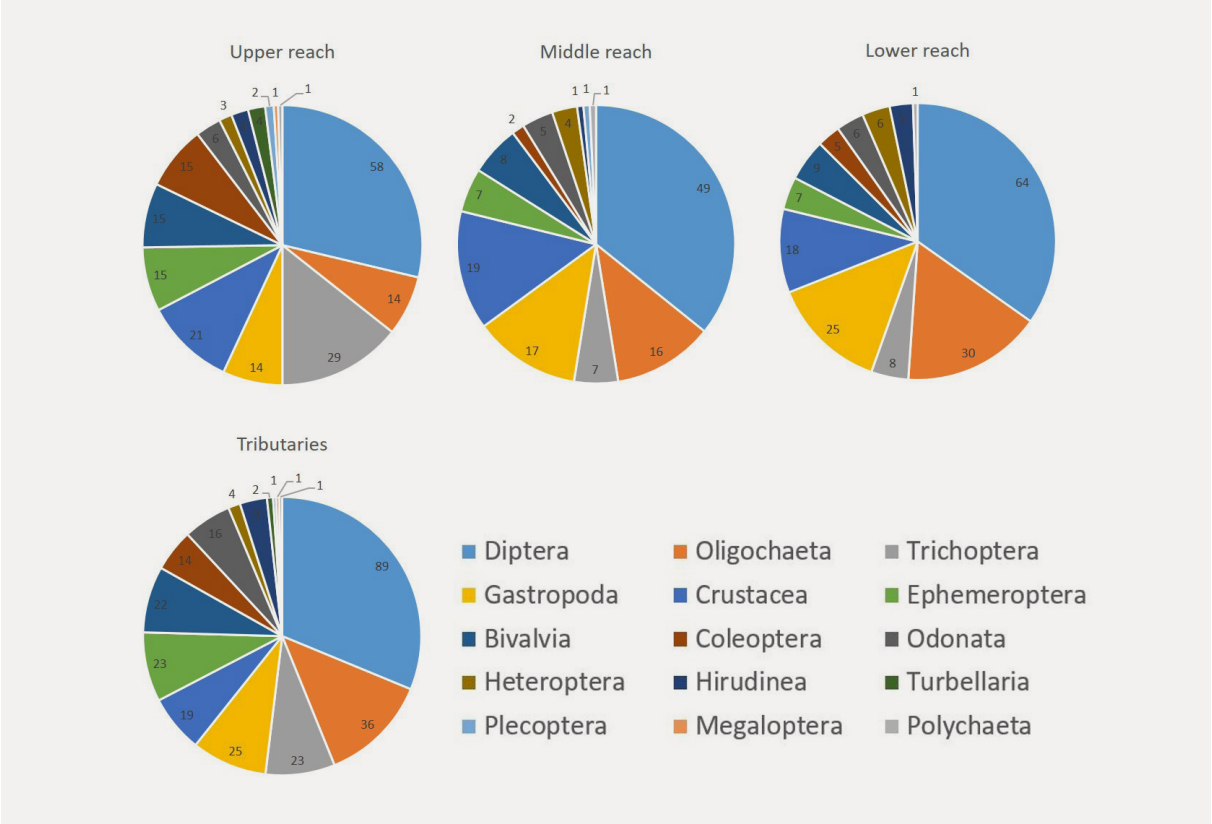


Figure 2: Number of taxa per taxagroup in upper, middle and lower reach of the Danube River and its tributaries.

In terms of total density (number of ind./1.25 m^2), groups Crustacea and Gastropoda followed by Oligochaeta and Diptera (mostly Chironomidae) (Fig. 3, left) are the most dominant part of the benthic macroinvertebrates assemblage.

Along the Danube River longitudinal profile, density of Coleoptera, Ephemeroptera, Trichoptera, Gastropoda and Polychaeta is decreasing. Large rivers are one of the freshwater ecosystems most affected by hydrologic alternation, bank modification, pollution and navigation. EPT taxa in particular, are highly sensitive. However, in the case of JDS4, the diversity of these particular taxa could be affected also by the sampling season (late summer). Some National experts noticed a higher water level before and during the sampling campaign. This could affect the density and diversity of the benthic macroinvertebrates assemblage as flood flow was referred to decrease of Annelida, Ephemeroptera, Trichoptera, Coleoptera and Plecoptera groups in general (McMullen & Lytle, 2012).

Polychaeta represented only by *Hypania invalida* occurred mostly in the upper reach. On the contrary, Heteroptera increased in density from the Upper to Lower Danube River. Taxa of Gastropoda and Oligochaeta that suits flat banks with sandy and muddy sediments show a peak in the middle reach.

Crustacean *Chelicorophium chelicorne* was not found during JDS1/2/3 campaigns, and it is surprising that it had been present in such high numbers during JDS4 as reported in 50-R and 51-R sites. The rare species, *Theodoxus transversalis* was reported on in JDS2 and JDS3, with occurrences in very restricted areas on the Lower Danube River recorded only at site 48 (Chiciu/Silistra, rkm 375).

In tributaries, Gastropoda is the most dominant group, followed by Diptera and Oligochaeta group. Compared to the Danube River reaches, Diptera represent principal part of the community, represented mainly by the family Chironomidae.

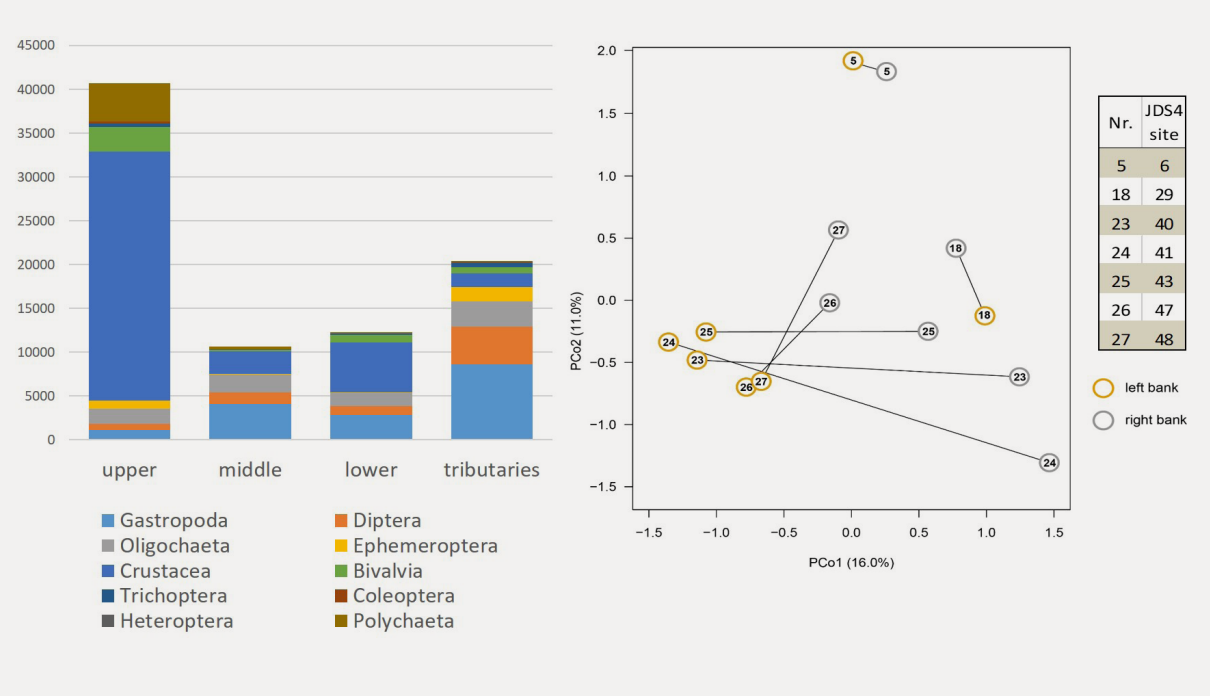


Figure 3: In left: Density per taxagroup (ind./1.25 m²) in upper, middle and lower reach of the Danube River and in tributaries (only most abundant groups); Right: PCoA ordination plot showing differences in community composition between left and right sides (banks) of the same sampling sites. Variance explained by the ordination axes is given in parentheses.

6.3.2 Comparison of sampling efficiency based on K&S, DWD, AMS: mussels and crustaceans

The detected results of two different taxonomic groups are illustrated briefly in order to show the effectiveness of different sampling methods during the JDS4 campaign. Across the whole investigated Danube River, Unionidae mussels were detected only at 10 sites where 4 species and 64 individuals were detected (Fig. 4).

MHS indicates that *Sinanodonta woodiana* is the dominant species on the entire Danube River similarly to the result of the K&S method. Both of these methods detected only four species in the river. However, DWD and AMS carried out on the Middle and the beginning of Lower Danube River stretch (Hungarian and Serbian Danube River) illustrate more even occurrence of the four species, together with the detection of a fifth species (*Unio crassus*) that has very low abundance with rare occurrence and limited distribution along the Danube River. The dominant species is *Unio tumidus* by both methods.

In the case of searching for mussels that were always regarded as relatively rare organisms in several Danube River sections, a careful sampling procedure is necessary due to the limited availability of the special habitats in which they can live. The existence of "quasi-stationary" environments - principally concerning abiotic components such as bed load, transport, erosion and sedimentation - is necessary during juvenile and adult age for their successful colonization, growth and long-term survival.

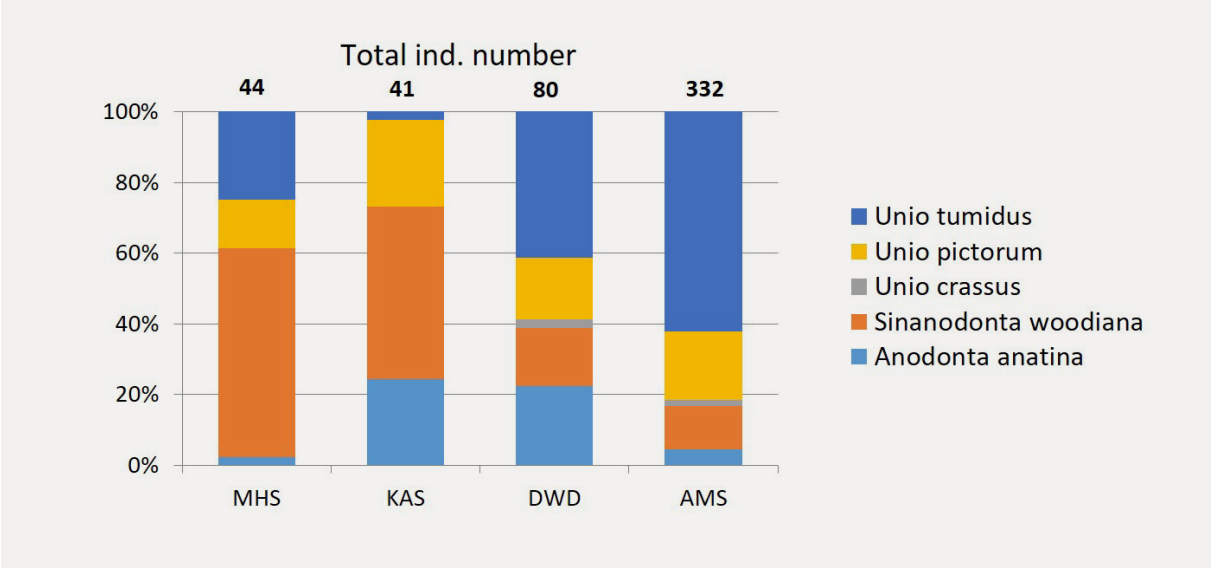


Figure 4: Species composition and abundance of detected Unionidae stock by different sampling procedures along the Danube River during JDS4. Note that MHS and KAS refer to the entire Danube whereas DWD and AMS was done only on the HU-RS section (Total ind. number = total caught animals per method).

Looking at the dataset referring to the Malacostraca group some remarkable phenomena are evident. K&S and MHS sampling resulted in very similar species composition and abundance of crustaceans. However, DWD surveyed these organisms in shorter Danube River section (approx. 1000 rkm in HU and RS) and in a smaller number of sites but in higher abundance. The explanation is clear: the larger individual number of these actively moving animals illustrates that faster flowing habitats situated in deep water regions are quite optimal for them. Dredging is carried out in deeper habitats that are not available for sampling techniques of shallow (wadable) waters.

Results are described in detail in the full report, to be found on www.danubesurvey.org/jds4/full-report.

6.3.3 Indicative Status Assessment (ISA) based on Multi-metric Index (MMI) and Saprobic Index (SI)

The saprobic system takes into account the varying sensitivity of the macrozoobenthos species to oxygen depletion in particular. Water quality class expressed by SI is derived from the individual saprobic values assigned to bioindicators occurring in assessed water environment.

Indicative status assessment (ISA) is assessment based on one sampling event only, and results are neither aimed to replace nor influence national assessment, but rather to serve to compare situations along the investigated stretch of the Danube River and its tributaries.

Along the Danube River reaches (36 samples in total), 24 samples (67%) can be classified into good status, 5 samples (14%) into high status, 4 samples (11%) to moderate and 3 samples (8%) fall into the poor status. Compared to the JDS3 and JDS2, results are similar, however Graf et al. (2015) note the differences between Airlift and MHS results. Besides that, at the banks the conditions can be different and can even vary between right and left bank, which can be seen at sites 37, 40, 41 and 48 (Tab. 4; Fig. 3, right).

In the case of samples from tributaries (19 samples), the situation is as follows: 13 (68%) samples can be classified into good status, 4 (21%) to moderate and 2 samples to poor status (Tab. 2).

Results from the Danube River using MMI show good indicative class in 13 samples, moderate class in 11 samples and poor class in 10 samples (Tab. 1).

In two sites, high status was indicated: 2-R Bittenbrunn, where the highest diversity was documented and 29-L Hercegszanto/Batina/Bezdan, where surprisingly only 8 taxa were found (status based on BMWP index was 4) and therefore the overall indicative status for this site cannot be considered as fully reliable.

From the tributaries, 8 samples fall in moderate class, 5 samples into poor class, 4 samples to good class and 2 samples achieved high class (Tab. 2). These results are not plausible and lead us to conclusion that the Slovak method should not be used for the ISA in tributaries, as seen especially in the cases of the Velika Morava and Sava Rivers, with high variance of classes within their longitudinal stretches (Tab. 2).

*Table 1: **Indicative status assessment**: Saprobic index class (SI) and Slovak MMI status class (SK) for the Danube River sites with results from JDS2 (only Saprobic index class, Airlift sampling method) and JDS3 (MHS method) – Saprobic index class and Slovak MMI compared to **National assessment**: DE – national intercalibrated MZB assessment tool Perlodes; AT, SK, HU, HR, RO and BG – national methods applied on JDS4 data (* samples were not taken under the best possible conditions).*

JDS4 site no.	JDS4 rkm	River		Sampling site	JDS2	JDS3		JDS4				National assesment	
					SI	SI	SK	SI		SK			
					Airlift	MHS		Right side	Left side	Right side	Left side	Class	Country
					Class	Class		Class		Class			
1	2581	Danube		Böfinger Halde		II	2		**II**		**2**	2	DE
2	2479	Danube		Bittenbrunn 700m below P. station				**II**		**1**		2	DE
3	2417	Danube		Above Klösterl - Kelheim	II	II	2	**II**		**3**		3	DE
4	2258	Danube		Niederalteich - Mühlau		II	2	**II**		**3**		3	DE
6	2204	Danube	Upper Reach	Jochenstein	III	III	**4**	**III**	**III**	**4**	**4**	4	AT
7	2113	Danube		Enghagen				**II**		**4**		3	AT
8	2008	Danube		Oberloiben	II	II	**3**		**II**		**4**	3	AT
10	1878	Danube		Hainburg, upstream Morava	I	II	2	**I**		**2**		3	AT
14	1871	Danube		Bratislava	II	II	2	**II**		**2**		2	SK
16	1806	Danube		Medveďov / Medve	II	II	2	**II**		**3**		3	SK
18	1791	Danube		Gönyű		II	2	**II**		**2**		4	HU*
22	1707	Danube		Szob	II	II	2	**II**		**4**		4	HU*
23	1666	Danube		Budapest upstream - Megyeri bridge	II	II	**3**		**II**		**4**	4	HU*
24	1632	Danube		Budapest downstream - M0 bridge	I	III	**3**		**I**		**2**	4	HU*
26	1560	Danube	Middle Reach	Dunafoldvar	II	II	2	**II**		**3**		4	HU*
27	1532	Danube		Paks	II	II	2		**I**		**3**	3	HU*
28	1480	Danube		Baja	II	II	2	**I**		**4**		4	HU*
29	1425	Danube		Hercegszanto / Batina / Bezdan	II			**II**	**II**	**2**	**1**	1	HR
31	1300	Danube		Ilok / Backa Palanka	II	II	**3**	**II**	**II**	**3**	**3**	4	HR*
37	1150	Danube		Downstream Pancevo	IV			**IV**	**II**	**3**	**4**		
40	1075	Danube		Banatska Palanka / Bazias	II	II	2	**IV**	**III**	**4**	**2**	1	RO
41	850	Danube		Upstream Timok (Rudujevac / Gruia)	II	II	**3**	**III**	**II**	**4**	**2**	1	RO
43	836	Danube	Lower Reach	Pristol / Novo Selo Harbour	II	II	2	**II**	**II**	**2**	**2**	2 1	BG RO
47	488	Danube		Downstream Ruse/Giurgiu (Marten)	II	I	**3**	**II**	**I**	**2**	**2**	3 2	BG RO
48	375	Danube		Chiciu/Silistra	III	II	**3**	**IV**	**II**	**3**	**3**	3 2	BG RO
50	132	Danube		Reni	II	II	**3**	**II**		**3**		2	RO
51	17	Danube		Vilkova - Chilia arm/Kilia arm	II	III	**3**	**II**		**2**		1	RO

*Table 2: **Indicative status assessment**: Saprobic index class (SI) and Slovak MMI status class (SK) for the Danube tributaries with results from JDS2 (only Saprobic index class – Airlift sampling method) compared to **National assessment**: CZ – intercalibrated MZB assessment; SK, HR, SI and RO - national methods applied on JDS4 data.*

JDS4 site no.	JDS4 rkm	River	Sampling site	JDS2	JDS3		JDS4				National assesment	
				SI	SI	SK	SI		SK			
				Airlift	MHS		Right side	Left side	Right side	Left side	Class	Country
				Class	Class		Class		Class			
5	4	Inn	Inn at Passau - Ingling below PS				II			2		
11	17	Dyje	Pohansko				II		3		3	CZ
12	79	Morava	Lanžhot				II		2		2	CZ
13	1	Morava	Devín				III			4	4	SK
17	2	Moson Danube	Vének	IV			II			3		
19	2.8	Vah	Komárno				III		3		3	SK
20	1.7	Hron	Kamenica				II		2		2	SK
21	12	Ipeľ	Salka				II		3		3	SK
25	1	Ráckevei	Tass	II			II			3		
30	5	Drava	5 km upstream Danube confluence				II		2		2	HR
32	155	Tisza	Tiszasziget / Martonoš				IV		4			
33	1	Tisza	Tisza mouth				III			3		
34	729	Sava	Jesenice na Dolenjskem				II		1		2	SI
35	205	Sava	Jamena				II			3		
36	12	Sava	Sava mouth (rkm 7.0)				II		4			
38	154	Velika Morava	Varvarin				II		1			
39	0.5	Velika Morava	Velika Morava mouth				IV		4			
49	0.5	Prut	Giurgiulesti				II	III	3	4	1	RO

6.4 Conclusions

Change in substrate composition of the Danube River induce gradual benthic community shifts from rheophilous to potamophilous in longitudinal profile. Based on cluster analysis of MZB assemblage from the Danube River samples, three sections have been identified: Upper/Middle section between sampling sites 16 (Medveďov, rkm 1806) and 18 (Gönyű, rkm 1791) and for Middle/Lower section with boundary between sites 28 (Baja, rkm 1480) and 29 (Batina, rkm 1425).

The saprobity of the Danube River and its tributaries varied between water quality class I, II, III and even IV. However, in some cases, the number of bioindicators found was too small for valid interpretation or conclusions.

Despite the assessment approach being very similar, the indicative status shows generally worse conditions (roughly by one class) when compared to JDS3 results. This could be caused by different sampling methodology (sampling from one river bank was preferred) which reduced the number of sensitive taxa and, in some cases, the higher water level increased bed load movement and could affect benthic communities, leading the recolonization of habitats to take longer.

Slovak Multi-metric index seems not to be suitable for the tributaries' assessment. Hence, the large tributaries along the Danube River deserve their own particular approach. For the next JDS, assessment methods should be tested on JDS4 data from main channel and tributaries separately.

For ensuring best results, both river banks should be sampled. The application of different sampling methods always provide better data in several aspects, however from a practical point of view, national teams should focus only at one main sampling technique (e.g. MHS or DWS in the lower Danube River reach). Assistance of external experts with most problematic groups, e.g. Oligochaeta and Chironomidae (Diptera), could be recommended for each participating country. This will ensure data comparability (especially for statistical methods) of the most abundant groups.

6.5 Acknowledgement

The research of the Slovak team was partially supported by the project APVV-16-0253.

6.6 References

AQEM CONSORTIUM (2002). Manual for the application of the AQEM method. A comprehensive method to assess European streams using benthic macroinvertebrates, developed for the purpose of the Water Framework Directive. Version 1.0, February 2002.

ASTERICS/PERLODES SOFTWARE (2008). Deutsches Bewertungssystem auf Grundlage des Makrozoobenthos. Version 4.0.4.

BENNETT, K. D. (1996). Determination of the number of zones in a biostratigraphical sequence. New Phytologist 132: 155-170.

BRTEK, J. (1953). Príspevok k poznaniu rozsírenia niektorých pre faunu ČSR novych alebo málo známych pontokaspickych druhov živočíchov v Dunaji. Biológia (Bratislava), 9 (4) (1953), pp. 297-302.

BUIJS., P. (2006). Development of operational tools for monitoring, laboratory and information Management. Objective 3: Options for developing WFD type specific quality nutrient standards in the Danube River. Draft final report. UNDP/GEF, Danube Regional Report.

GRAF, W., LEITNER, P., & PLETTERBAUER, F. (2015). Short overview on the benthic macroinvertebrate fauna of the Danube River. In I. Liska (Ed.), The Danube River Basin, Springer Berlin Heidelberg, (pp. 287–315), ISBN: 978-3-662-47738-0.

GRIMM, E. C. (1987). CONISS: a FORTRAN 77 program for stratigraphically constrained cluster analysis by the method of incremental sum of squares. Computers & Geosciences 13: 13-35.

JOHNSON, R. K., WIEDERHOLM, T. & ROSENBERG, D. M. (1993). Freshwater biomonitoring using individual organisms, populations, and species assemblages of benthic macroinvertebrates. Pp. 40-158. In: Rosenberg, D. M. & Resh, V. H. (Eds.). Freshwater biomonitoring and benthic macroinvertebrates. Chapman & Hall, New York, 487 p.

LEITNER, P. (2013). Anwendung der Slowakischen Bewertungsmethode für Große Fließgewässer an der Österreichischen Donau. Bundesministerium Für Land- Und Forstwirtschaft, Umwelt und Wasserwirtschaft, 16 pp.

MAKOVINSKÁ, J., MIŠÍKOVÁ ELEXOVÁ, E., RAJCZYKOVÁ, E., BALÁŽI, P., PLACHÁ, M., KOVÁČ, V., FIDLEROVÁ, D., ŠČERBÁKOVÁ, S., LEŠŤÁKOVÁ, M., OČADLÍK, M., VELICKÁ Z., HORVÁTHOVÁ, G. & VELEGOVÁ, V. (2015). Methods of monitoring and assessment of surface water bodies in Slovakia, Výskumný ústav vodného hospodárstva, Bratislava. ISBN: 978- 80- 89740-02- 4

McMULLEN, L., & LYTLE, D. (2012). Quantifying invertebrate resistance to floods: A global-scale meta-analysis. Ecological Applications, vol. 22, no. 8, 2012, pp. 2164–2175.

MOOG, O., SCHMUTZ, S. & SCHWARZINGER, I. (2018). Biomonitoring and Bioassessment. In: Schmutz, S. & Sendzimir, J. (eds). Riverine Ecosystem Management. Aquatic Ecology Series, vol. 8. Springer, Cham, pp. 371-390.

07 Phytobenthos

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Abstract

Benthic diatom data together with environmental variables obtained during JDS4 in summer 2019 were analysed. The main aim of this study was to describe the structure of benthic diatom communities and to evaluate the indicative status in the Danube and selected tributaries. The performed survey and statistical analyses revealed the following facts: (i) diatom communities differed between the different Danubian types and Danubian reaches from the upper to the Lower Danube; (ii) diatom species structure reflected the diversity of environmental conditions, ranged from oligotraphentic to hypereutraphentic and from oligosaprobous to polysaprobous. (iii); the environmental variables, which most significantly influenced diatom species composition and diatom metrics (diatom indices, diatom life-forms and partly diatom ecological guilds) were general descriptors (e.g. geographical coordinates), followed by physico-chemical variables (e.g. concentrations of nutrients and parameters indicating the level of organic pollution); (iv) indicative status of Danubian samples was generally getting worse from the Upper Danube towards the mouth; in general, indicative status of tributaries was found to be better in comparison to the Danube.

7.1 Introduction

Benthic algae (phytobenthos) are found in nearly all running waters and often are important in fluvial food webs (Allan and Castillo, 2007). Their assemblages are usually attached to substrate and their growing and prospering can respond directly and sensitively to physical, chemical and biological variables occurring in the river reach (Moog et al., 2018). Furthermore, aquatic plants (phytobenthos and macrophytes) are one of the biological quality elements required by the Water Framework Directive (The European Parliament and European Council, 2000) to be monitored for the identification of anthropogenic impacts on aquatic habitats. For these reasons, phytobenthos communities were investigated in all conducted Joint Danube Surveys (JDS). Unlike the previous JDS, the phytobenthos was collected and analysed in JDS4 by national experts at their stretches of the Danube and selected tributaries. Only benthic diatoms were chosen from the community of phytobenthos as its representative part for this purpose. Most European countries use benthic diatoms as a representative assemblage for phytobenthos in the WFD-compliant ecological status assessment (Kelly et al., 2009) and their suitability in the bioassessment was widely demonstrated (e.g. Rimet, 2012).

7.2 Methods

7.2.1 Sampling and laboratory analysis

Benthic diatoms were sampled from 29 June 2019 for up to 2 weeks (except for JDS4-5L, which was sampled in September due to flood conditions) following the European standard (CEN, 2014a). Diatoms were collected separately from both river banks at all sampling sites where it was possible and indicated. The length of the selected sampling stretch was at least 10 m long. Samples were brushed from the upper surface of substrate, usually from at least five stones occurring in the euphotic zone from an area of minimum 10 cm^2. Each sample was divided into three bottles and preserved according to purpose of use (1 – check of the physiological status of diatom cells, 2 – microscopic analysis, 3 – molecular analysis). The hot hydrogen peroxide method was usually applied to remove organic material from samples, and treated diatom suspensions were mounted on permanent slides usually using Naphrax©. The range of 300-500 diatom valves were counted and identified on each permanent slide under a light microscope (1000 × magnification) to the lowest possible level according to CEN (2014b). Identifications were primarily based on Hofmann et al. (2013).

7.2.2 Data treatment

Diatom taxa list and abundance were processed with OMNIDIA 6.0 (omnidia/fr/en), a software for calculation of 18 diatom water quality indices.

The diatom community structure was described by calculating the proportion of species belonging to ecological guilds (low profile, high profile and motile guild) according to Passy (2007) and Berthon et al. (2011) and to two life forms (planktonic, benthic) according to Rimet & Bouchez (2011; 2012).

7.2.3 Statistical methods

The abundance of species was expressed as relative counts (in %). Only species with a relative abundance above 3% in at least one sample were included into the statistical analyses.

Sixteen environmental variables were included in the statistical analysis, such as physico-chemical variables [water temperature (temp), conductivity (cond), pH, dissolved oxygen (O_2), total phosphorus (TP), orthophosphate phosphorus (PO_4-P), nitrate nitrogen (NO_3-N), nitrite nitrogen (NO_2-N), ammonium nitrogen (NH_4-N), biological oxygen demand after 5 days (BOD_5) and suspended solids (susp)], hydromorphological variables [daily average flow (flow)] and general descriptors [latitude, longitude, altitude and river kilometre (rkm)].

Danubian sites were separated into the Danubian types according to Moog et al. (2004) as follows Type 1: 2581 rkm (site 1), type 2: 2479,3-2258 rkm (sites 2-4), type 3: 2204-2008 rkm (sites 6-8), type 4: 1878-1791 rkm (sites 10, 14, 16, 18), type 5: 1707-1532 rkm (sites 22-24, 26, 27), type 6: 1480-1073 rkm (site 28, 29, 31, 37, 40), type 7: was lacking of sampling sites, type 8: 852–488 rkm (sites 41, 43, 47), type 9: 375–132 rkm (sites 48, 50), type 10: 17 rkm (site 51). All tributaries were classified into one group (sites 5, 11-13, 17, 19-21, 25, 30, 32-36, 38-39, 42, 49). Mentioned Danubian types could be distributed into traditionally separated major Danubian reaches such as the Upper Danube (types 1-4), the Middle Danube (types 5-6) and the Lower Danube (types 7-10).

Canonical Correspondence Analysis (CCA, ter Braak and Verdonschot, 1995) with forward selection of significant variables was performed to detect changes in diatom species composition to the particular environmental data and gradients. ANOSIM (Clarke, 1993) was used to test significance of differences in diatom species composition among Danubian types and tributaries and SIMPER (Clarke and Gorley, 2006) was performed to choose the diatom species, which contributed the most to the similarity within types.

Relationships among the diatom metrics (diatom indices, diatom guilds and life forms) and environmental variables were assessed with the Spearman correlations. Samples from the right and left bank were treated separately. Kruskal-Wallis H-test was employed to test statistical differences in diatom metrics among different Danubian types and tributaries.

Evaluation of indicative status (IS) was realized based on the IPS index (CEMAGREF, 1982) using ecological status class boundaries according to the Slovak and Bulgarian assessment method: high/good IPS>15.5/15.2, good/moderate IPS>13.1/11.6, moderate/poor IPS>9.7/8.1, poor/bad IPS>6.9/4.5. The first two boundaries were intercalibrated (Birk et al., 2012). For comparison between JDS32, JDS3 and JDS4, the worse value of IPS index was taken when both river banks were sampled.

7.3 Results and discussion

7.3.1 Diatom species composition

385 diatom taxa belonging to 78 genera were identified in 72 samples. 158 diatom taxa reached a relative abundance over 1% in at least one sample. The most abundant and the most frequent species with a mean relative abundance of at least 5% and frequency of at least 10% of samples were *Achnanthidium delmontii* Pérès, Le Cohu & Barthès, *Amphora pediculus* (Kützing) Grunow, *Cocconeis euglypta* Ehrenberg, *Cyclotella meneghiniana* Kützing, *Navicula recens* (Lange-Bertalot) Lange-Bertalot, *Nitzschia dissipata* (Kützing) Grunow and *Skeletonema potamos* (C. I. Weber) Hasle.

7.3.2 Analyses of relationships of diatoms with environmental parameters

Results of CCA analysis revealed 11 environmental parameters significant ($p<0.05$) in explaining the variance of species data and they altogether explained 32.5% of the species data variance (Fig. 1). The diagram shows a tendency of grouping of diatom samples according to Danubian types however, distinct overlap can be found in samples from all types. Geographical coordinates (longitude, latitude) reflecting natural direction of the flow of the Danube influenced diatom species composition at most. Besides them the diatoms were affected by several physico-chemical variables e.g. concentrations of nutrients (nitrate, nitrite, ammonium nitrogen and total phosphorus), organic pollution variable (biological oxygen demand), pH, concentration of dissolved oxygen and hydromorphological variable (daily average flow). ANOSIM confirmed these results and showed that differences in diatom assemblages between Danubian types are significant but groups can overlap markedly (Global $R=0.114$, $p<0.05$). The overlaps observed between the neighbouring types are caused by the natural connectivity of investigated sites.

For Danubian types and tributaries, the indicator species were identified, however they were more or less shared in particular types. Such diatom species structure generally reflected the diversity of environmental conditions of JDS4 sampling sites from oligotraphentic to hypereutraphentic regarding inorganic pollution by nutrients and from oligosaprobous to polysaprobous regarding organic pollution.

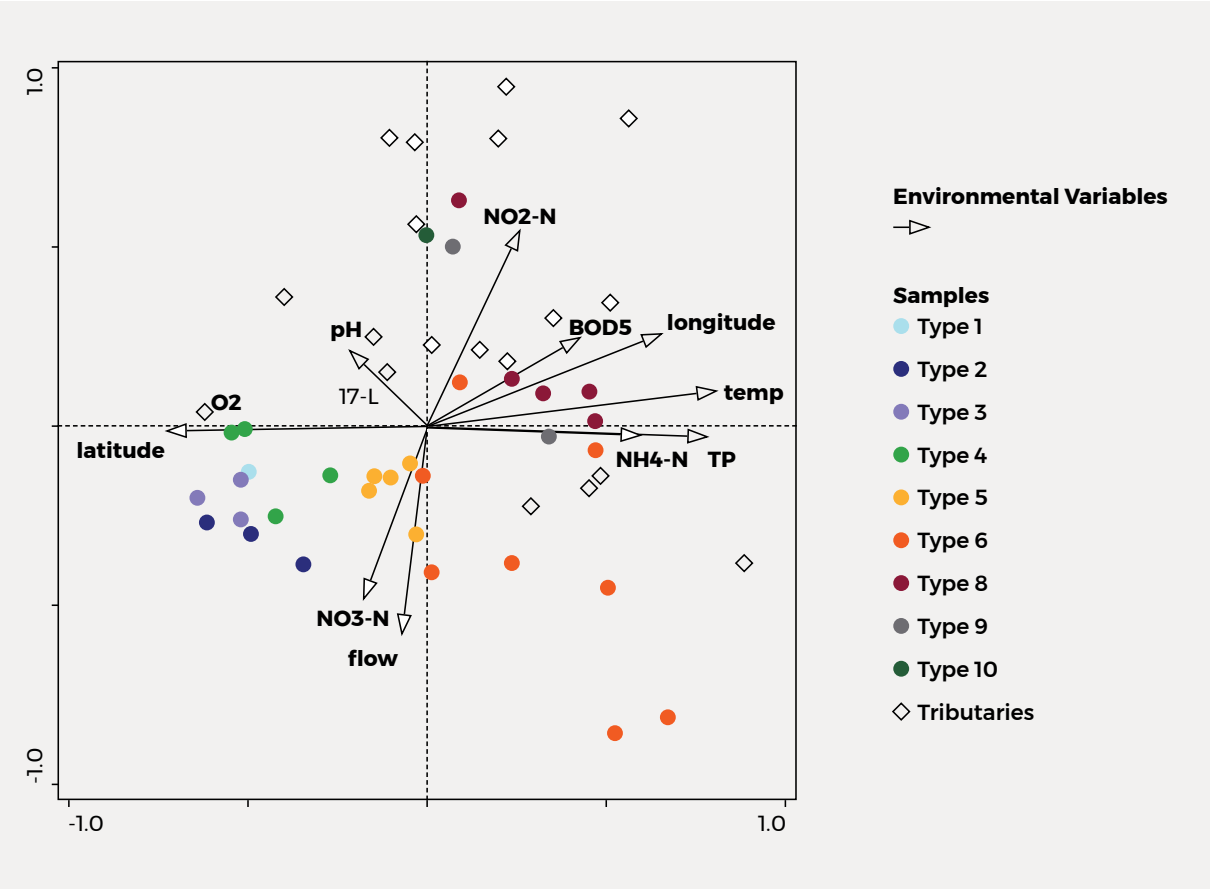

Figure 1: Canonical Correspondence Analysis (CCA) ordination diagram for the first two axes designated based on the diatom species composition and environmental variables. Samples are distinguished according to the Danubian types, tributaries are comprehended in one group.

7.3.3 Diatom indices, diatom guilds and life-forms

The most significant correlations among calculated diatom indices and environmental variables were found for general descriptors (latitude, longitude, altitude and especially with river kilometre), which underlined that indices decrease longitudinally from the Upper Danube down to the mouth. The highest correlations of indices with physico-chemical parameters were determined for dissolved oxygen, water temperature and total phosphorus. Among 18 indices tested the IPS index (CEMAGREF, 1982) achieved the highest correlations with environmental variables and one of the best distinctiveness among different Danubian types and tributaries.

In the Danube, the motile guild reached the highest proportion (48.9%), followed with the low-profile guild (40.4%) and high-profile guild (10.8%). In tributaries the low-profile guild reached the highest proportion (49.8%), followed with the motile guild (39.3%) and high-profile guild (11%). The ecological guilds showed inconclusive results to change significantly in the longitudinal profile (Fig. 2A, B) and much lower sensitivity on environmental variables comparing to diatom indices. This could be caused by unstable hydrological conditions due to the high-water levels before and during JDS4 sampling, which probably influenced stability of the diatom communities.

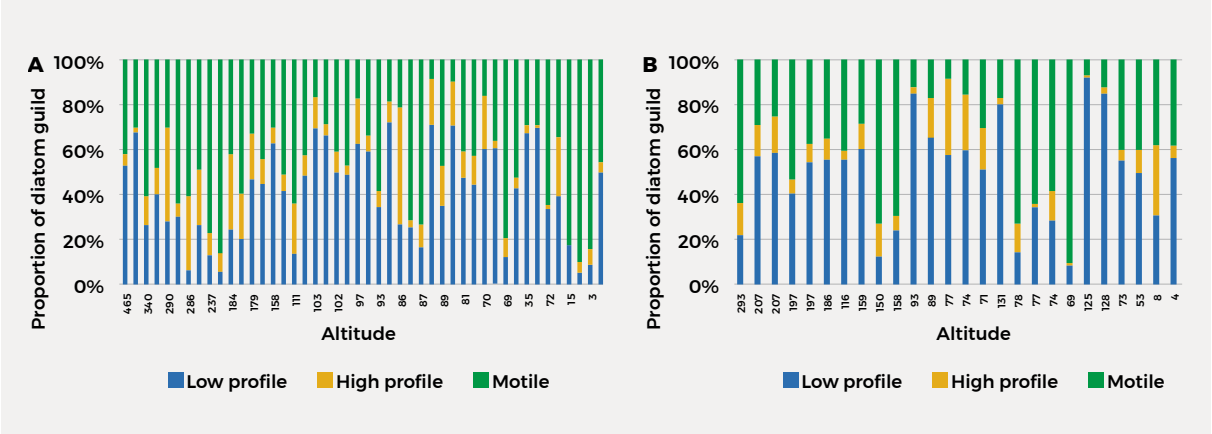

Figure 2: Distribution of diatom guilds among the examined samples: A – Danube; B – Tributaries.

With respect to diatom life forms, proportion of benthic diatoms in the Danube reached 91.1% and proportion of planktonic diatoms reached 8.9%. In tributaries the benthic diatoms reached also the prevailing proportion (78.8%), the planktonic reached 21.2%. Proportion of both planktonic ($p<0.001$) and benthic diatoms ($p<0.01$) proved to differ significantly among the different Danubian types and tributaries and showed to change significantly in the longitudinal profile (Fig. 3A, B). The close relations were observed between diatom life forms and general descriptors, e.g. latitude, longitude, altitude and river kilometre. However, high correlation coefficients were calculated also for physico-chemical parameters, e.g. water temperature, nutrient concentrations and biological oxygen demand.

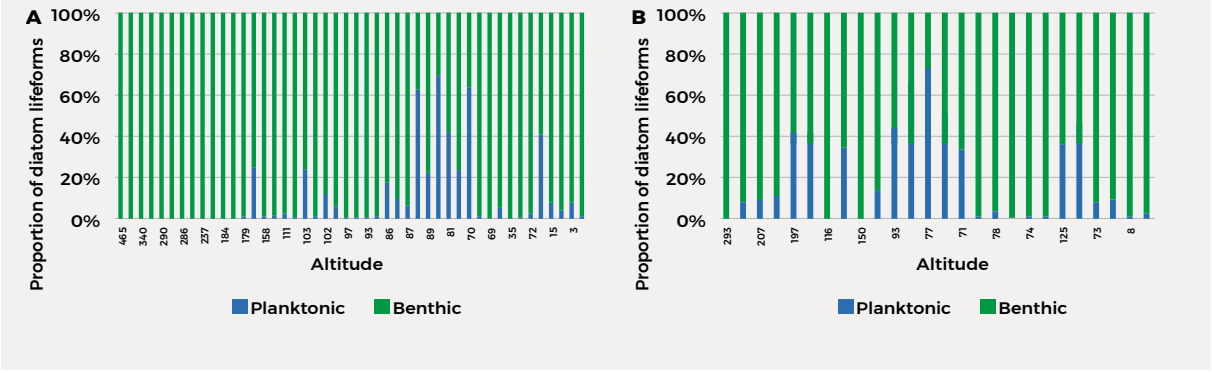

Figure 3: Distribution of planktonic and benthic diatoms among the examined samples: A – Danube; B – Tributaries.

7.3.4 Indication of the status assessment

The boundaries of individual classes for IPS index from Slovak (SK) and Bulgarian (BG) status assessment methods were used for illustration of changes in water quality in the whole stretch of the Danube and selected tributaries (Table 1). Originally, the boundaries were designed for national purpose (Upper and Lower Danube respectively) and here they are only used for indication of the status. It must be said that **indication of status means** in case of **benthic diatoms** in particular **water quality**.

Table 1: Indicative status assessment in all JDS4 sampling sites calculated based on IPS index. Boundaries of indicative status classes are those used in Slovak method for ecological status assessment in the Danube.

Site no.	River	Sampling site name	JDS4 left bank					JDS4 right bank					JDS Comparison		
			IPS	Indication of status		rkm	Processing country	IPS	Indication of status		rkm	Processing country	JDS2	JDS3	JDS4
				SK	BG				SK	BG					
1	Danube	Böfinger Halde	15.0	G	G	2581.0	DE							15.5	15.0
2	Danube	Bittenbrunn 700 m below power station						15.5	G	H	2479.3	DE			15.5
3	Danube	above Klösterl						14.9	G	G	2417.0	DE	13.9	14.3	14.9
4	Danube	Mühlau						13.4	G	G	2258.0	DE		14.1	13.4
5	Inn	Passau - Ingling below power station	13.4	G	G	4.0	DE						15.0		13.1
6	Danube	Jochenstein	15.5	G	G	2204.0	DE	14.8	G	G	2203.7	AT	14.8	15.2	14.8
7	Danube	Enghagen	13.7	G	G	2113.0	AT	14.1	G	G	2113.0	AT	15.4[b]	15.4[b]	13.7
8	Danube	Oberloiben	14.4	G	G	2008.0	AT	13.6	G	G	2008.0	AT	15.3	15.9	13.6
10	Danube	Hainburg	14.2	G	G	1878.0	AT	12.8	M	G	1878.0	AT	11.1	14.3	12.8
11	Dyje	Pohansko	12.4	M	G	17.0	CZ	12.8	M	G	17.0	CZ			12.4
12	Morava	Lanžhot	10.4	M	M	79.0	CZ	9.8	M	M	79.0	CZ			9.8
13	Morava	Devín	9.7	P	M	1.0	SK						10.7	11.7	9.1
14	Danube	Bratislava						13.7	G	G	1871.0	SK	12.2	15.3	13.7
16	Danube	Medveďov/Medve	12.5	M	G	1806.0	SK	14.7	G	G	1806.0	SK	9.5	11.7	14.7
17	Mosoni-Danube	Vének	13.5	G	G	2.0	HU							10.1	13,5
18	Danube	Gönyü	11.3	M	M	1791.0	HU	9.0	P	M	1791.0	HU		12.5	9.0
19	Váh	Komárno						12.0	M	G	2.8	SK	10.2	10.9	12.0
20	Hron	Kamenica nad Hronom	9.0	P[a]	M	1.7	SK						11.7		9.0
21	Ipeľ	Salka	8.5	P[a]	M	12.0	SK						4.8		8.5
22	Danube	Szob	9.5	P	M	1707.0	HU	14.3	G	G	1707.0	HU	10.2	10.8	9.5
23	Danube	upstream to Budapest	14.1	G	G	1666.0	HU	13.9	G	G	1666.0	HU	12.6	12.4	13.9
24	Danube	downstream to Budapest	13.3	G	G	1632.0	HU	13.6	G	G	1632.0	HU	10.2	10.3	13.3
25	Ráckevei-Soroksári-Danube	Tass	13.0	M	G	1.0	HU						12.9		13.0
26	Danube	Dunaföldvár	14.0	G	G	1560.0	HU	12.0	M	G	1560.0	HU	11.9	7.3	12.0
27	Danube	Paks	14.3	G	G	1532.0	HU	12.6	M	G	1532.0	HU	9.4	9.0	12.6
28	Danube	Baja	12.4	M	G	1480.0	HU	10.1	M	M	1480.0	HU	11.9	7.3	10.1
29	Danube	Bezdan/Batina	11.4	M	M	1425.0	RS	12.2	M	G	1434.0	HR	13.1[c]	8.4[c]	11.4
30	Drava	5 km upstream Danube confluence						12.2	M	G	5.0	HR	12.2[d]	12.8	12.2
31	Danube	Bačka Palanka/Ilok	11.8	M	M	1300.0	RS	10.9	M	M	1300.0	HR	10.4	9.1	10.9
32	Tisza	Martonoš	11.3	M	M	155.0	RS								11.3
33	Tisza	Tisza mouth	10.9	M	M	8.7	RS	10.6	M	M	8.7	RS	9.5	10.7	10.9
34	Sava	Jesenice na Dolenjskem						14.2	G	G	729.0	SI			14.2
35	Sava	Jamena	9.7	P	M	205.0	RS	9.7	P	M	205.0	BiH			9.7
36	Sava	Sava mouth	10.1	M	M	12.0	RS	9.5	P	M	11.0	RS	9.4	12.5	9.5
37	Danube	Downstream Pančevo	12.1	M	G	1150.0	RS	10.6	M	M	1150.0	RS	10.2	9.1	10.6
38	Velika Morava	Varvarin	11.0	M	M	154.0	RS	9.3	P	M	154.0	RS			9.3
39	Velika Morava	Velika Morava mouth						10.7	M	M	0.5	RS	6.6	8.0	10.7
40	Danube	Banatska Palanka/Bazias	14.2	G	G	1073.0	RO	10.7	M	M	1077.0	RS	10.8	8.6	10.7
41	Danube	Upstream Timok (Rudujevac/Gruia)	9.3	P	M	847.0	RO	12.3	M	G	852.0	RS	13.3	9.5	9.3
42	Timok	Upstream Timok mouth	11.0	M	M	36.0	RS						8.1	8.5	11.0
43	Danube	Pristol/Novo Selo Harbour	13.1	M	G	837.0	RO	9.1	P	M	834.0	BG	11.0	9.3	9.1
47	Danube	Downstream Ruse/Giurgiu (Marten)	9.2	P	M	488.0	RO	9.6	P	M	488.0	BG	13.6	9.8	9.2
48	Danube	Chiciu/Silistra	9.6	P	M	375.0	RO						18.6	9.3	9.6
49	Prut	Giurgiulesti	13.8	G	G	0.5	MD	11.6	M	G	0.5	RO	12.6	10.9	11.6
50	Danube	Reni	9.8	M	M	132.0	RO						10.1	9.3	9.8
51	Danube	Valkov						12.9	M	G	17.0	RO	8.4[e]		12.9

Note: [a] whole profile, [b] rkm 2120, [c] rkm 1434, [d] 1,4 km upstream confluence, [e] rkm 18 (Vilkova)

Based on the Slovak boundaries of IPS index, in the Upper Danube (types 1-4) more than 76% Danubian samples indicate good status. Most of the Upper Danube tributaries shows moderate indicative status (57%), 28% samples reached good and the rest indicated poor status. The indication of the status in the Middle Danube varied between good and moderate. More than 56% samples of the tributaries of the Middle Danube referred to moderate and more than 37% to poor status indication. In case of the Lower Danube (types 8-10) most of samples (more than 55%) indicated poor situation and more than 44% moderate one. Tributaries shows better results comparing to the Danube, more than 66% of them achieved moderate status indication and more than 33% samples indicated good situation.

Table 1 illustrates evaluation of IPS index based on SK and BG boundaries, where in case of 19 sites of the Danube and tributaries less stringent evaluation can be seen using BG boundaries as opposed to SK ones.

Additionally, Table 1 shows the comparison of the IPS index of the Danube and the tributaries in the period of 2007 (JDS2), 2013 (JDS3) and 2019 (JDS4). The same results were observed at eight sampling sites on the Danube and at five stations on the tributaries. Increasing of IPS index, respective improvement in water quality in time appears in four Danube sites and five tributaries, while deterioration occurs at six stations.

7.4 Conclusions

Diatom samples differed between the individual Danubian types, but groups were also seen to overlap. The high similarity between the neighbouring types was probably caused by the natural connectivity of investigated sites.

A lot of indicator species were shared in more than one of the Danubian reaches and in several Danubian types. Diatom structure generally reflects the diversity of environmental conditions of JDS4 sampling sites from oligotraphentic to hypereutraphentic and from oligosaprobous to polysaprobous.

General descriptors such as geographical coordinates (longitude, latitude) reflecting natural direction of the flow of the Danube seemed to have the most important influence on diatom species composition. Besides them, the diatom species composition was influenced by several physico-chemical variables, e.g. concentrations of nutrients and organic pollution variables.

Also, diatom indices and diatom life forms (planktonic and benthic diatoms) differed among the individual Danubian types and tributaries and showed changes in the longitudinal profile. Both groups of metrics reflected several environmental variables – mainly general descriptors (latitude, longitude, altitude, river kilometre), but also physico-chemical parameters. It suggested that these diatom metrics were more closely related to parameters which change naturally longitudinally, than to physico-chemical variables.

Ecological guilds (high profile, low profile and motile guild) showed inconclusive results to change in the longitudinal profile. Only the proportion of the low-profile guild differed between individual Danubian types and tributaries and ecological guilds seem to have much more lower sensitivity towards environmental variables.

The IPS index was selected for indication of the status for JDS4, in spite of the diatom community being a good indicator for water quality. The values of the IPS index generally decrease downstream indicating a longitudinal increase in pollution.

Using boundaries of the Slovak and Bulgarian status assessment method for IPS metric a simple indication was used to illustrate longitudinal profile of the Danube including tributaries and to compare results of JDS2, JDS3 and JDS4 data. Results of JDS4 indicated that the Danube and the tributaries fall into three of the five classes (good, moderate or poor). The indication of status of the Danube was generally getting worse from the Upper Danube towards the mouth while the situation of the tributaries was diverse. Based on comparison of IPS index of three periods (2007, 2013, 2019), it can be stated that situation did not change in the case of 13 monitoring stations (28%), the improvement of water quality occurred in 9 stations (19.6%) and the deterioration occurred in 6 monitored stations (13%), while the other stations (21) showed no trend.

Besides convincing results shown by phytobenthos it should be added that the sampling in the summer season was not appropriate for majority of sampling sites. Therefore, it would be appropriate to consider the shifting of the activities focusing on phytobenthos sampling in the next JDSs to another season, which would be as most as possible in accordance with national methodologies of participating countries, e.g. spring or autumn. Due to the comparability with previous JDSs results, the autumn would be probably more

suitable for sampling since JDS1 – JDS3 always started in the second half of August and finished at the end of September. However, it should be discussed with national experts from all participating countries.

Despite many issues, which needed to be solved due to the implementation of JDS4 activities at the national level the new JDS4 approach seems to be successful. This approach based on execution of collection and analyses of phytobenthos samples via national experts at their stretches of the Danube and selected tributaries allowed participation of many researchers from different European regions and provided many interesting and useful results.

For more details of used methods, achieved results and discussion please see the full report, available at www.danubesurvey.org/jds4/full-report.

7.5 References

ALLAN J. D. & CASTILLO M. M. (2007). Primary producers. In: Stream Ecology. Springer, Dordrecht, pp. 105–134.

BERTHON V., BOUCHEZ A. & RIMET F. (2011). Using diatom life forms and ecological guilds to assess organic pollution and trophic level in rivers: a case study of rivers in south-eastern France. Hydrobiologia 673: 259–271.

BIRK S., SCHÖLL F. & BÖHMER J. (2012). XGIG Large River Intercalibration Exercise – WFD Intercalibration Phase 2: Milestone 6 Report. 73 p.

CEN 13946 (2014a). Water quality. Guidance for the routine sampling and preparation of benthic diatoms form rivers and lakes. European Committee for Standardization, Brussels.

CEN 14407 (2014b). Water quality. Guidance for the identification and enumeration of benthic diatom samples from rivers and lakes. European Committee for Standardization, Brussels.

CEMAGREF (1982). Etude des méthodes biologiques d'appréciation quantitative de la qualité des eaux., Vol. Rapport Q.E.Lyon-A.F.Bassin Rhône-Méditeranée-Corse. 218 p.

CLARKE K. R. (1993). Non-parametric multivariate analyses of changes in community structure. Aust. J. Ecol. 18: 117–143.

CLARKE K. R. & GORLEY R. N. (2006). PRIMER v6: User Manual/Tutorial. PRIMER-E, Plymouth, UK, 192 p.

HOFMANN G., LANGE-BERTALOT H. & WERUM M. (2013). Diatomeen in Süsswasser-Benthos von Mitteleuropa. In: LANGE-BERTALOT H. (eds). Bestimmungsflora Kieselalgen für die ökologische Praxis Über 700 der häufigsten Arten und ihre Ökologie, Koeltz Scientific Books, 908 p.

KELLY M. G., BENNETT C., COSTE M., DELGADO C., DELMAS F., DENYS L., ECTOR L., FAUVILLE C., FERREOL M., GOLUB M., JARLMAN A., KAHLERT M., LUCEY J., CHATHAIN B. N., PARDO I., PFISTER P., PICINSKA-FALTYNOWICZ J., ROSEBERY J., SCHRANZ C., SCHAUMBURG J., VAN DAM H. & VILBASTE S. (2009). Comparison of national approaches to setting ecological status boundaries in phytobenthos assessment for the European Water Framework Directive: results of an intercalibration exercise. Hydrobiologia 621: 169–182.

MOOG O., SOMMERHÄUSER M., ROBERT S., BATTISTI T., BIRK S., HERING D., OFENBÖCK T., SCHMEDTJE U., SCHMIDT-KLOIBER A. & VOGEL B. (2004). Typology of the Danube River based on „top-down" and „bottom-up" approaches. http://www.oen-iad.org/conference/docs/6_invertebrates/moog_et_al.pdf.

MOOG O., SCHMUTZ S. & SCHWARZINGER I. (2018). Biomonitoring and Bioassessment. In: SCHMUTZ S. & SENDZIMIR J. (eds). Riverine Ecosystem Management. Aquatic Ecology Series, vol 8. Springer, Cham, pp. 371–390.

Nariadenie vlády Slovenskej republiky č. 398/2012 z 28. novembra 2012, ktorým sa mení a dopĺňa nariadenie vlády Slovenskej republiky č. 269/2010 Z. z., ktorým sa ustanovujú požiadavky na dosiahnutie dobrého stavu vôd. https://www.slov-lex.sk/pravne-predpisy/SK/ZZ/2012/398/20130101

PASSY S. I. (2007). Diatom ecological guilds display distinct and predictable behavior along nutrient and disturbance gradients in running waters. Aquat Bot 86: 171–178.

RIMET F. (2012). Recent views on river pollution and diatoms. Hydrobiologia 683: 1–24.

RIMET F. & BOUCHEZ A. (2011). Use of diatom life-forms and ecological guilds to assess pesticide contamination in rivers: lotic mesocosm approaches. Ecol. Indic. 11: 489–499.

RIMET F. & BOUCHEZ A. (2012). Life-forms, cell-sizes and ecological guilds of diatoms in European rivers. Knowl Manag Aquat Ecosyst 406: 01.

TER BRAAK C. J. F. & VERDONSCHOT P. F. M. (1995). Canonical correspondence analysis and related multivariate methods in aquatic ecology. Aquat Sci 57: 255–289.

THE EUROPEAN PARLIAMENT AND EUROPEAN COUNCIL (2000). Directive 2000/60/EC of the European Parliament and of the Council establishing a framework for Community action in the field of water policy. Official J.L. 327: 1–73.

08 Phytoplankton

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Abstract

Within the framework of the 4th Joint Danube Survey (JDS4) we analysed phytoplankton at 26 sampling sites in the Danube River and 10 sampling sites in the tributaries. Samples were collected monthly from April to September in 2019. A total of 682 taxa were identified, amongst diatoms were dominant taxonomic group, mostly represented with planktic taxa like *Stephanodiscus hantzschii, Cyclostephanos dubius, Cyclotella meneghiniana, Skeletonema potamos,* or benthic ones like *Diatoma vulgaris*. The application of functional groups revealed more detailed composition and dynamics. Dominant functional groups in the Upper reach of the Danube River and in the tributaries were TB, A, C and D, in the Middle reach those were A and D, while in the Lower reach D, A and C were the dominant ones indicating a shift in trophic conditions. Functional group approach was proven once more to be an excellent tool for interpretation of the phytoplankton composition, and in the case of the Danube River, it precisely reflects existing hydrological and trophic conditions. The concentration of chlorophyll *a* and total biomass of phytoplankton showed temporal and longitudinal dynamics. The highest chlorophyll *a* (55.7 μgL^{-1}) and biomass (21.4 mgL^{-1}) values were measured in the Middle reach of the Danube River. Among the tributaries the Morava, Ipeľ and Rackevei-Soroksari Danube Arm had the highest values. The peak of chlorophyll *a* was characteristic in late spring for the Upper reach, and in mid-summer in the rest of the Danube and the tributaries. Environmental parameters that highly influenced the phytoplankton in the Danube River were water temperature and flow conditions, while water temperature, total phosphorous and BOD influenced most the phytoplankton in the tributaries. Phytoplankton based ecological status assessment indicated low to high status.

8.1 Introduction

Rivers, during their course, change significantly from naturally heterotrophic systems (Dodds and Cole, 2007) to naturally autotrophic ones, where they become large in low discharge periods (Wehr and Descy, 1998). In the middle and downstream reaches of the rivers, potamoplankton thrives and plays an important role in providing organic carbon towards higher trophic levels (Ramaraj et al., 2014). This was recognized by the EU Water Framework Directive when phytoplankton was proposed as one of the five biological quality elements for ecological status assessment, not only in lakes, but in rivers as well (WFD, 2000).

Before practical application in ecological status assessment, potamoplankton has been studied for decades by phytoplankton ecologists (reviewed by Abonyi et al. (2020)), and these studies provided many information on the taxonomical, structural and functional properties of large rivers' phytoplankton. Recent studies (Abonyi et al., 2018; Abonyi et al., 2012; Bórics et al., 2007; Nagy-László et al., 2020; Stanković et al., 2012) demonstrated, that by aggregating species into the functional groups, systems' complexity can be successfully reduced, which helps our understanding of how potamoplankton performs under various environmental constraints. Besides helping to better understand the processes in the riverine ecosystems, the functional group approach has also been applied for the calculation of metrics for ecological status assessment (Bórics et al., 2007). This approach has been successfully intercalibrated with others at the European level (Mischke et al., 2018), and together with the species-based German metric (Mischke et al., 2011) served as part of the intercalibration common metric.

Under the framework of the 4th Joint Danube Survey (JDS4, 2019) we investigated phytoplankton in the Danube River and its chosen tributaries. The main objectives of this study were to: determine the seasonal and longitudinal composition of river phytoplankton, describe environmental factors that affect the composition and biomass of phytoplankton, describe functional group composition along the Danube River and in its tributaries and to indicate the phytoplankton ecological status.

8.2 Methods

Sampling and sample analysis

Phytoplankton samples and samples of water for physical and chemical analysis were collected monthly from April to September and analysed in the national laboratories. Samples were taken from the middle of the river (thalweg) on most of the sampling sites, preserved with Lugol's solution, and before analysis stored in the dark at a temperature between 4 and 8°C (CEN - EN 16698, 2015). Phytoplankton samples were counted by Utermöhl's method (CEN - EN 15204, 2006). Biovolumes were calculated by determining an average individual size of up to 30 randomly chosen cells of each taxon, and then multiplying by the observed species abundance, or they were obtained from the national database. Biomass (freshweight) was derived from biovolumes and used for further analyses, where 1 mm^3L^{-1} = 1 mgL^{-1} (CEN - EN 16695, 2015).

Phytoplankton taxa were assigned to functional groups according to Bórics et al. (2007); Padisák et al. (2009) and Reynolds et al. (2002). EQR's for ecological status assessment based on the phytoplankton were calculated and provided by the countries.

Samples were successfully collected and analysed on 36 out of 40 planned sampling sites with 26 sampling sites in the Danube River and 10 sampling sites in the tributaries. A full list of sampling sites is presented in Chapter 2 and they are shown on the Overview Map. Because of technical problems, the following sampling sites differ in sampling dynamics: JDS4-3 (five samples, July x2, September missing), JDS4-11 (five samples, August x2, May and June missing), JDS4-12 (August x2, June missing), JDS4-40 and JDS4-49 (May x2, April missing), JDS4-51 (five samples, April missing).

Flow data were obtained from national hydrological services.

Data analysis

A one-way SIMPER analysis based on Bray-Curtis similarity was performed on the taxonomic composition of the phytoplankton where characteristic taxa and functional groups were analysed in the Primer 6 software (Clarke and Gorley, 2006). A canonical correspondence analysis (CCA) was used to ordinate taxonomic group composition with environmental variables which was done in CANOCO 5 (ter Braak and Šmilauer, 2012). The CCA analysis was performed separately for the Danube River and for the tributaries, using data for all taxonomic groups, 36 sampling sites and 11 environmental variables. Phytoplankton biomass data were log-transformed. Environmental data were normalised prior to analyses and Draftman's plot was conducted to eliminate variables with significant autocorrelation. A Box-Whisker plot of concentration of chlorophyll *a* and total biomass was done in Grapher™ (Grapher™, 2019), while the proportion of functional groups along the Danube River and the tributaries was displayed using Microsoft Excel 365.

Samples and sampling sites were grouped for better understanding and visualisation according to Moog et al. (2006) where the Danube River is divided into 10 types. Types were grouped into river reaches: Upper (types 1-4), Middle (types 5-7) and Lower (types 8-10).

8.3 Results and discussion

Taxonomic composition of phytoplankton

A total of 682 taxa were identified in 213 samples. They belonged to nine major taxonomic groups (Phylum): Bacillariophyta (249), Charophyta (23), Chlorophyta (224), Choanozoa (1), Cryptophyta (17), Cyanobacteria (77), Euglenozoa (35), Myzozoa (10) and Ochrophyta (46).

Bacillariophyta was the dominant taxonomic group contributing to the total biomass in most of the samples, both in the Danube River (24.4 - 99.3%) and in the tributaries (12.2 - 99.8%). Cyanobacteria, Chlorophyta and Cryptophyta were taxonomic groups that were occasionally dominant or co-dominant with Bacillariophyta. Cyanobacteria contributed to the total biomass up to 64.3% in the Danube River and up to 82.4% in the tributaries. Maximum values of Cyanobacterial biovolumes were identified on sampling sites JDS4-2 in the Danube River and in the Morava River (JDS4-11). On the latter sampling site, potentially toxic *Raphidiopsis raciborskii* (Woloszynska) Aguilera, Berrendero Gómez, Kastovsky, Echenique & Salerno was the dominant species. Chlorophyta contributed to the total biomass almost equally in both the Danube River (0.2 - 47.1%) and the tributaries (0.0 - 59.9%). Cryptophyta occurred sporadically in most samples, but occasionally co-dominated with others.

A one-way SIMPER analysis based on Bray-Curtis similarity performed on phytoplankton assemblage showed characteristic taxa for each reach of the Danube River, as well as the tributaries. The SIMPER analysis brought up about 20 different taxa that mostly contributed to the similarity between samples in the Upper and Lower reaches, while only 10 of them were characteristic for the Middle reach. Characteristic taxa and those that contributed most to the similarity between samples for the Upper reach were *Stephanodiscus hantzschii* Grunow, *Cyclotella meneghiniana* Kützing and *Diatoma vulgaris* Bory de Saint. The Middle reach was characterised with *Cyclostephanos invisitatus* (Hohn & Hellermann) Theriot, Stoermer & Håkasson, *S. hantzschii, Skeletonema potamos* (C.I.Weber) Hasle, *C. meneghiniana, Cyclostephanos delicatus* (Genkel) S.J.Casper & W.Scheffler, *Plagioselmis nannoplanctica* (H.Skuja) G.Novarino, I.A.N.Lucas & S.Morrall and *Stephanodiscus minutulus* (Kützing) Cleve & Möller. The Lower reach was characterised with centric diatoms composed of dominantly *C. meneghiniana, Actinocyclus normanii* (W.Gregory ex Greville) Hustedt, *S. potamos* and *Stephanodiscus neoastraea* Håkansson & Hickel. Dominant taxa that contributed most to the similarity between samples in the tributaries was a mixture of planktic and benthic diatoms: *C. meneghiniana, S. hantzschii, Melosira varians* C.Agardh, *C. invisitatus, Ulnaria ulna* (Nitzsch) P.Compère, *Navicula lanceolata* Ehrenberg and *D. vulgaris*.

Composition of phytoplankton functional groups

A total of 29 phytoplankton functional groups were identified and they showed spatial and temporal dynamics (Fig. 1). Bacillariophyta were the dominant taxonomic group, therefore functional groups representing diatoms were dominant in the Danube River and its tributaries. The Upper reach of the Danube River was represented with co-dominance of benthic (TB) and planktic diatoms (A, C and D). According to the one-way SIMPER analysis based on Bray-Curtis similarity, non-diatom functional groups that contributed significantly to the similarity between samples in the Upper reach were X2 (9.9%), J (5.4%) and Y (5.2%). The Middle and Lower reaches of the Danube River were mostly represented with planktic diatoms. Functional groups A and D were co-dominant in the Middle reach while the Lower reach had dominance of functional group D and co-dominance of functional groups A and C. Besides planktic diatoms, the SIMPER analysis showed that functional groups X2 and TB contributed to the similarity between samples with 5.7% and 5.6% in the Middle reach, as well as functional groups TB and J with 15.7% and 2.2% in the Lower reach. It is hard to

generalize all tributaries because of their differences in the catchment size and geographical position, but in most samples diatom functional groups were dominant there as well. Functional group composition in the tributaries was quite like that in the Upper reach of the Danube River. Dominance or co-dominance was usually with functional groups TB, C and D, while the SIMPER analysis also indicated that functional groups J (4.4%), X2 (3.4%) and Y (3.2%) contributed to the similarity between samples.

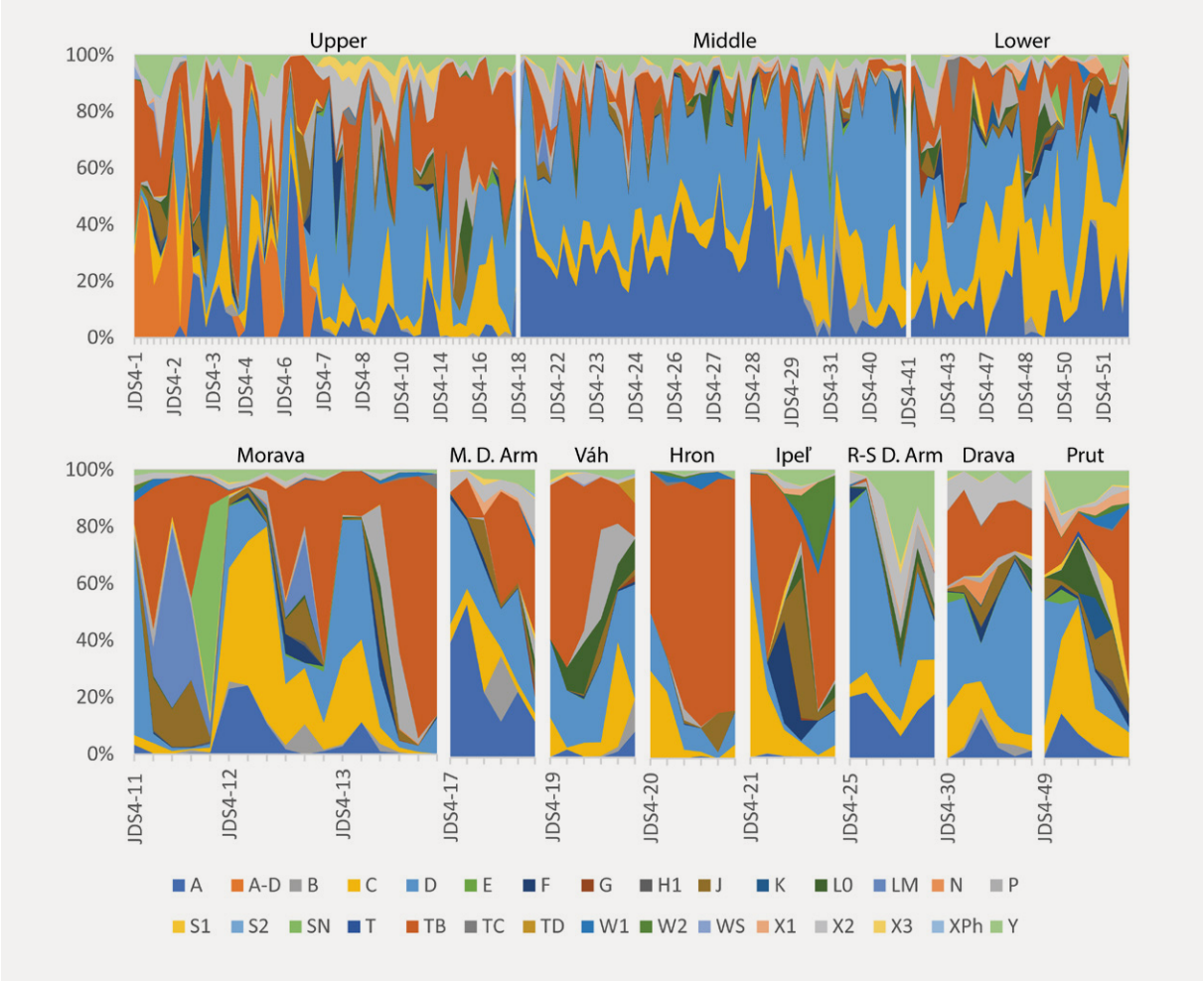

Figure 1: Relative biomass of phytoplankton functional groups in the Danube River (up) and tributaries (down) on all sampling sites during the study period. Thick marks on the x-axis represent the months of sampling, starting with April from the left side.

Chlorophyll *a* and phytoplankton total biomass

The chlorophyll *a* concentration and total biomass are presented in Figure 2. Their values in the Danube River ranged from 0.8 to 55.7 μgL^{-1} and from 0.1 to 19.5 mgL^{-1}, respectively. Generally highest values were measured in the Middle reach. The concentration of chlorophyll *a* was two times larger in the tributaries and ranged between 1.0 and 112.5 μgL^{-1}, while total biomass was very similar to the Danube values ranging between 0.2 and 21.4 mgL^{-1}. The highest values of both parameters were measured on sampling sites JDS4-13 (Morava), JDS4-21 (Ipeľ) and JDS4-25 (Rackevei-Soroksari Danube Arm). Besides longitudinal patterns, temporal patterns of chlorophyll *a* and total biomass were observed. The peak of chlorophyll *a* and total biomass was the highest in late spring (May) in the Upper Danube reach, while it shifted to mid-summer period in the Middle and Lower Danube reaches. The tributaries showed similar trends like the Lower Danube reach, having the highest chlorophyll *a* and total biomass values in mid-summer.

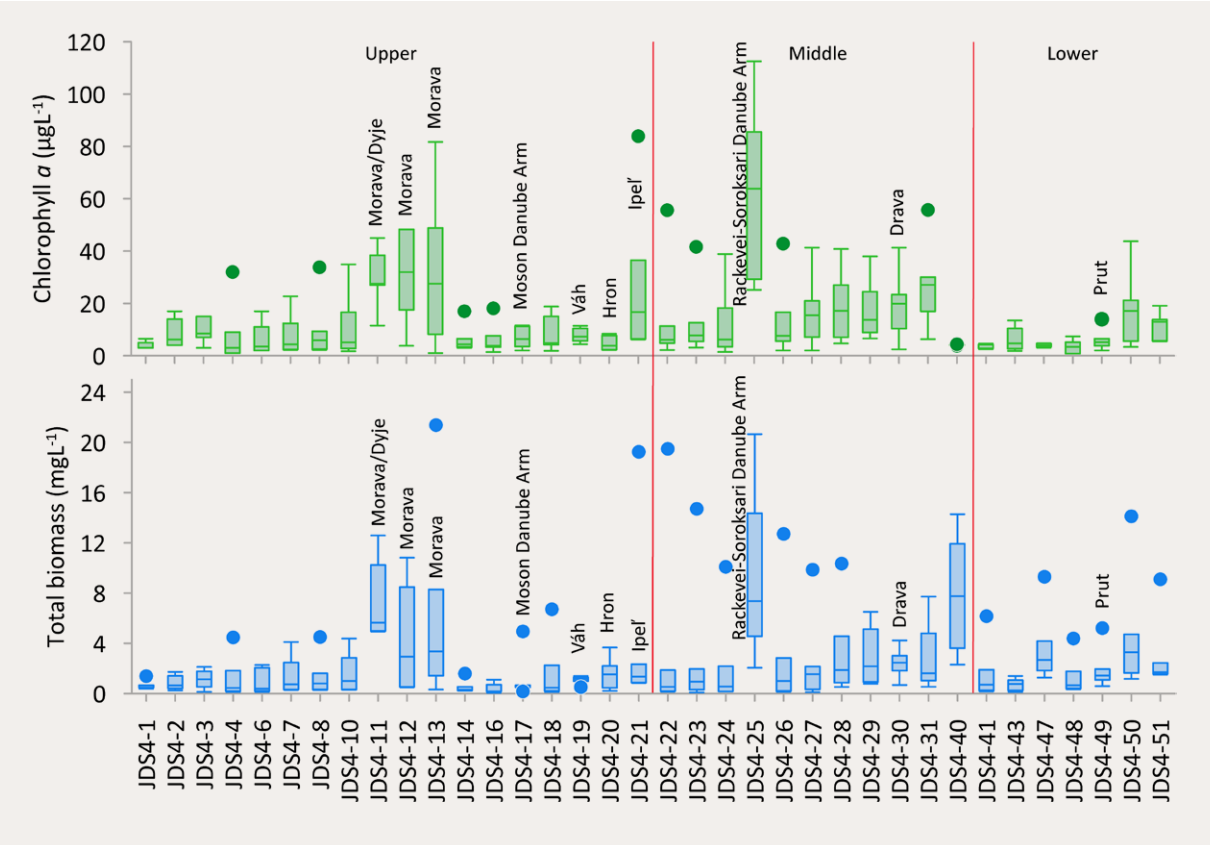

Figure 2: Box-Whiskers plot of chlorophyll a concentration and total biomass of phytoplankton at each sampling site in the Danube River and its tributaries during JDS4. The centre line stands for median value, while outliers are indicated as dots.

Relationship of phytoplankton with environmental parameters

The following physical and chemical parameters were selected for further analyses: water temperature, pH, conductivity, alkalinity, oxygen concentration, concentration of ammonia, nitrites, nitrates, total phosphorous, BOD and total suspended solids. Water discharge or flow was selected as hydrological parameter.

The ordination results of phytoplankton taxonomic groups and environmental data of the CCA in the Danube River are presented on the F1×F2 ordination plot (Fig. 3, left). Eigenvalues of the first two axes are 0.041 and 0.017 and they explain 68.1% of the variance of phytoplankton and environmental data. Axis 1

had the highest correlation with water temperature (R=0.402), while axis 2 had the highest correlation with the flow (R=0.268). Strong differences in the phytoplankton community and between the Danube reaches can be observed on the diagram. The Lower reach was influenced by high flow, temperature and ammonia and appeared to be the most optimal habitat for Myzozoa and Euglenozoa. Bacillariophyta, Chlorophyta and Cyanobacteria positioned in the centre of the diagram indicating their general occurrence through all the Danube reaches. The Upper reach was characterised with higher alkalinity and nitrates, while the Middle reach has higher TP and BOD indicating a higher organic load in that reach, possibly brought by the tributaries.

The ordination results of phytoplankton taxonomic groups and environmental data of the CCA in the tributaries are presented on the F1×F2 ordination plot (Fig. 3, right). Eigenvalues of the first two axes are 0.312 and 0.109 and they explained 80.8% of the variance of phytoplankton and environmental data. Axis 1 had the highest correlation with total phosphorous (R=0.829), while axis 2 had the highest correlation with water temperature (R=0.561) and BOD (R=0.456). Although phytoplankton samples of certain tributaries are scattered around the diagram, still there are quite indicative results. In the Ipel' River, Euglenozoa positively correlates with turbidity, ammonia and pH. BOD had the highest correlation with Cryptophyta and Myzozoa in the Rackevei-Soroksari Danube Arm and few summer samples (high temperature) from the Mosoni Danube Arm, Drava and Prut rivers which indicates the highest organic pollution. Bacillariophyta preferred the highest concentration of nitrates but positioned in the centre of the diagram indicating dominance in most of the samples of all the tributaries. Cyanobacteria showed the highest correlation with TP in the Morava River.

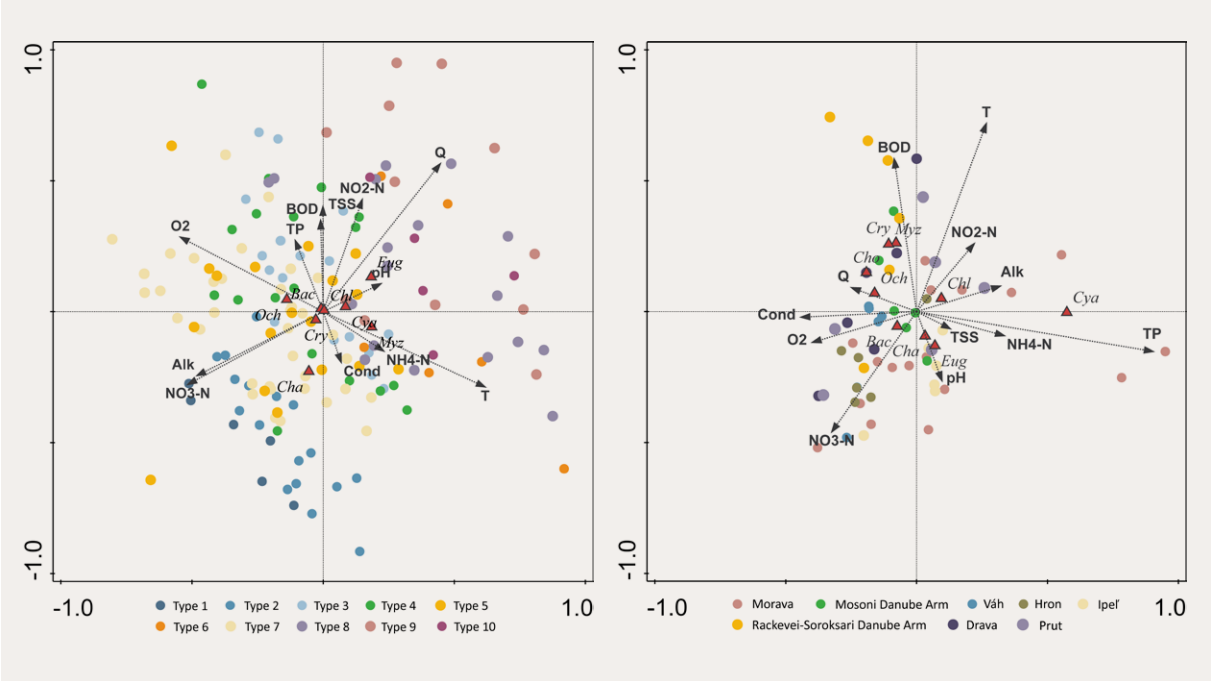

Figure 3: Canonical Correspondence Analysis (CCA) triplot of phytoplankton taxonomic groups, environmental scores and samples (coloured dots). The left diagram presents analysis in the Danube River and the right diagram presents analysis in the tributaries. Environmental variables are abbreviated as: T – temperature, pH – pH, O2 – oxygen concentration, Cond – conductivity, Alk – alkalinity, NH4-N – ammonia, NO2-N - nitrites, NO3-N – nitrates, TP – total phosphorous, BOD – biological oxygen demand, TSS – Total suspended solids and Q – daily flow. Taxonomic groups are abbreviated as: Bac – Bacillariophyta, Cha – Charophyta, Chl – Chlorophyta, Cho – Choanozoa, Cry – Cryptophyta, Cya – Cyanobacteria, Eug – Euglenozoa, Myz – Myzozoa and Och – Ochrophyta.

Indication of phytoplankton based ecological status assessment

Classification of phytoplankton based ecological status assessment is presented in Figure 4. All countries that provided the data have intercalibrated their methods (Mischke et al., 2018). Phytoplankton indicated four status classes. Good ecological status was on most of the sampling sites (23), high status was indicated on four sampling sites in the Danube River and on two in the tributaries. Moderate status was indicated on two sampling sites in the Danube River and two in the tributaries, while poor status was indicated only on one sampling site in Rackevei-Soroksari Danube Arm (JDS4-25).

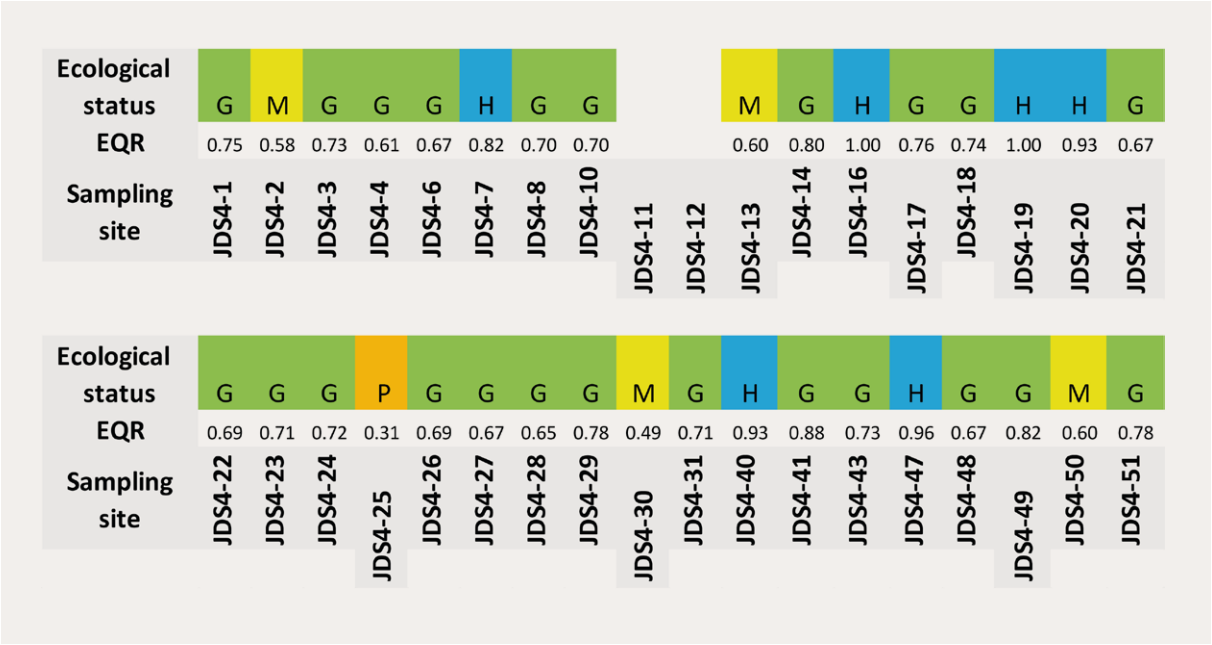

Figure 4: Results of phytoplankton based classification of ecological status assessment. Tributaries codes are lowered.

8.4 Conclusions

In this study, we present the results of the first seasonal and longitudinal investigation of phytoplankton carried out in the Joint Danube Survey concept, that used the widely accepted Utermöhl method for quantitative analysis. Since all previous phytoplankton investigations had only one-time summer sampling and different approaches to the analysis of phytoplankton, it is quite hard to compare the present and previous results. Despite that, it can be concluded that in this study we found the same tendency as it had been found during the JDS3 (2013), where the concentration of chlorophyll *a* and total biomass was the highest in the Middle reach of the Danube River.

Diatoms were the dominant group of algae in the phytoplankton community of the Danube River and its tributaries with occasional occurrence of dominance or co-dominance of Cyanobacteria, Chlorophyta or Cyptophyta. The Upper reach of the Danube and tributaries had a higher proportion of benthic diatoms, while the Middle and Lower reaches had a higher proportion of planktic diatoms.

The functional group approach was successfully applied, and these results revealed clear seasonal and temporal dynamics in the phytoplankton community.

The highest phytoplankton production as equivalent of the chlorophyll *a* concentration and total biomass was in the tributaries Rackevei-Soroksari Danube Arm (which is an artificially isolated lake-like reach of the Danube), Morava and Ipel' rivers in general. When the Danube River is observed separately, both parameters showed the highest values in the Middle reach.

Environmental factors that influenced most the phytoplankton in the Danube River were water temperature and flow regime. Water temperature, total phosphorous and BOD influenced most the phytoplankton assemblages in the tributaries.

Phytoplankton based classification of ecological status assessment changed from low to high along the Danube River and in the tributaries, but good on most of the sampling sites.

8.5 References

ABONYI, A., ÁCS, É., HIDAS, A., GRIGORSZKY, I., VÁRBÍRÓ, G., BORICS, G. & KISS, K. T. (2018). Functional diversity of phytoplankton highlights long-term gradual regime shift in the middle section of the Danube River due to global warming, human impacts and oligotrophication. Freshwater Biol 63(5): 456-472.

ABONYI, A., DESCY, J.-P., BORICS, G. & SMETI, E. (2020). From historical backgrounds towards the functional classification of river phytoplankton sensu Colin S. Reynolds: what future merits the approach may hold? Hydrobiologia, 23 May 2020.

ABONYI, A., LEITÃO, M., LANÇON, A. M. & PADISÁK, J. (2012). Phytoplankton functional groups as indicators of human impacts along the River Loire (France). Hydrobiologia 698(1): 233-249.

BÓRICS, G., VÁRBIRÓ, G., GRIGORSZKY, I., KRASZNAI, E., SZABÓ, S. & KISS KEVE, T. (2007). A new evaluation technique of potamo-plankton for the assessment of the ecological status of rivers. Archiv für Hydrobiologie, Supplementband Large rivers 161(3-4): 465-486.

CEN - EN 15204, (2006). Water quality - Guidance standard on the enumeration of phytoplankton using inverted microscopy (Utermohl technique) (EN 15204:2006). European Committee for Standardization (CEN) Brussels, Belgium.

CEN - EN 16695, (2015). Water quality - Guidance on the estimation of microalgal biovolume (EN 16695:2015). European Committee for Standardization (CEN) Brussels, Belgium.

CEN - EN 16698, (2015). Water quality - Guidance on quantitative and qualitative sampling of phytoplankton from inland waters (EN 16698:2015). European Committee for Standardization (CEN) Brussels, Belgium.

CLARKE, K. R. & GORLEY, R. N. (2006). PRIMER v6: User Manual/Tutorial. PRIMER-E, Plymouth.

DODDS, W. K. & COLE, J. J. (2007). Expanding the concept of trophic state in aquatic ecosystems: It's not just the autotrophs. Aquat Sci 69(4): 427-439.

GRAPHER™, (2019). Golden Software, LCC. 15 edn. Golden Software, Inc., 809 14th Street, Golden, Colorado 80401.

MISCHKE, U., BELKINOVA, D., BIRK, S., BORICS, G., GARBEA, R., HLÚBIKOVÁ, D., JEKABSONE, J., OPATRILOVA, L., PANEK, P., PICIŃSKA-FAŁTYNOWICZ, J., PIIRSOO, K., PLACHA, M., ROTARU, N., STANKEVICIENE, J., STANKOVIĆ, I., VAN WICHELEN, J., VARBIRO, G., VIRBICKAS, T. & WOLFRAM, G., (2018). Intercalibrating the national classifications of ecological status for very large rivers in Europe: Biological Quality Element: Phytoplankton. EUR 29337 EN, Publications Office of the European Union, Luxemburg.

MISCHKE, U., VENOHR, M. & BEHRENDT, H. (2011). Using Phytoplankton to Assess the Trophic Status of German Rivers. Int Rev Hydrobiol 96(5): 578-598.

MOOG, O., SOMMERHÄUSER, M., ROBERT, S., BATTISTI, T., BIRK, S., HERING, D., OFENBÖCK, T., SCHMEDTJE, U., SCHMIDT-KLOIBER, A. & VOGEL, B., (Year) Published. Typology of the Danube River base d on „top-down" and „bottom-up" approaches In: 36th International Conference AC-IAD, Vienna, 2006. p 5.

NAGY-LÁSZLÓ, Z., PADISÁK, J., BORICS, G., ABONYI, A., B-BÉRES, V. & VÁRBÍRÓ, G. (2020). Analysis of niche characteristics of phytoplankton functional groups in fluvial ecosystems. J Plankton Res 42(3): 355-367.

PADISÁK, J., CROSSETTI, L. & NASELLI-FLORES, L. (2009). Use and misuse in the application of the phytoplankton functional classification: a critical review with updates. Hydrobiologia 621(1): 1-19.

RAMARAJ, R., TSAI, D. D.-W. & CHEN, P. H. (2014). Freshwater microalgae niche of air carbon dioxide mitigation. Ecol Eng 68: 47-52.

REYNOLDS, C. S., HUSZAR, V., KRUK, C., NASELLI-FLORES, L. & MELO, S. (2002). Towards a functional classification of the freshwater phytoplankton. J Plankton Res 24(5): 417-428.

STANKOVIĆ, I., VLAHOVIĆ, T., GLIGORA UDOVIČ, M., VÁRBÍRÓ, G. & BÓRICS, G. (2012). Phytoplankton functional and morpho-functional approach in large floodplain rivers. Hydrobiologia 698(1): 217-231.

TER BRAAK, C. & ŠMILAUER, P. (2012). Canoco reference manual and user's guide: software of ordination (version 5.0). Microcomputer Power (Ithaca, NY, USA).

WEHR, J. D. & DESCY, J.-P. (1998). Use of phytoplankton in large river management. J Phycol 34(5): 741-749.

WFD (2000). Directive 2000/60/ec of the European Parliament and of the Council 22.12.2000. Official Journal of the European Communities L327: 1-72.

09 Macrophytes

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Abstract

Macrophytes, or aquatic plants visible to the naked eye, are an important part of the aquatic ecosystems. Within the framework of the 4th Joint Danube Survey (JDS4), national experts sampled and analysed macrophytes in 38 official sampling sites altogether during July 2019. The Danube River was covered by 27 sampling sites, with 11 additional sampling sites in selected tributaries. A total of 132 taxa of bryophytes, pteridophytes and angiosperms were identified. Bryophytes were a dominant plant group in the Upper Reach of the Danube River, while angiosperms prevailed in the Middle and Lower Reach, as well as in the tributaries. Hydrophytes were a dominant life form in the Danube River and the tributaries, but helophytes also showed their dominance at a few locations. Analysis of similarities between Danube River sections showed a clear separation of the River sections belonging to the Upper Reach, while the River sections of Middle and Lower Reach were partially grouped, although still showing river continuum and connection to the hydromorphological features of the river. Environmental parameters that highly influenced hydrophytes in the Danube River, a group of aquatic plants constantly immersed into the water, were water temperature, dissolved oxygen, nitrates, and conductivity. The analysis clearly showed that bryophytes preferred colder and oxygenated water, rich in nitrates while floating or rooted angiosperms and pteridophytes preferred warmer, nutrient, and organically rich water. Based on the comparison of outcomes from previous Joint Danube Surveys, the composition of macrophytes has been stable in terms of richness and diversity over the course of several years.

9.1 Introduction

All aquatic plants that live directly in the littoral zone of lakes and rivers, or are exclusive inhabitants of the occasionally flooded riverbanks, are named macrophytes (Haslam, 2006). They comprise macroalgae, mosses, liverworts and vascular plants that live in permanently wet places and are visible to the naked eye (Lacoul and Freedman, 2006). Macrophytes can be free-floating, submerged or rooted. Based on their dependency on the water column, different life forms are known. True aquatic plants permanently living in the water are hydrophytes. Helophytes have submerged basal sections, while amphibious plants or amphiphytes are capable of living both in the water and ashore.

Macrophytes are an important element for both running and standing waters because they provide shelter, feeding and breeding place for aquatic animals. Besides, their roots that are holding sediments, absorption of the wave energy helps to reduce shoreline erosion (Kalff, 2001). Because of their direct connection to

the aquatic environment, macrophytes are one of five biological quality elements for assessment of the ecological status of water bodies within the Water Framework Directive (WFD, 2000), as they are proven to be excellent indicators for eutrophication and hydromorphological degradation.

The investigation of macrophytes in the Danube River (Rath, 1995; Rath, 1997), its tributaries, sidearms and nearby lakes (Pall et al., 1996; Sârbu et al., 2011) has a long tradition, but on short stretches. Being the second largest river in Europe, the Danube faces constant human pressure (ICPDR, 2015; Tockner et al., 2009). Therefore, the Joint Danube Survey framework enables extensive and detailed data collection, better knowledge of the pressure-impact relationship and its reflection on the living organisms, as well as the river itself and the whole catchment area. The main objectives of this study were to: determine taxonomic, plant group and life form composition of macrophytes in the Danube River and selected tributaries, compare the composition of macrophytes along the Danube River sections for better understanding of river continuity and biotic response to alterations, describe environmental factors that affect the structure and composition of hydrophytes.

9.2 Methods

Sampling and sample analysis

The sampling of macrophytes was done on 27 sampling sites in the Danube River and 11 sampling sites in the tributaries. A full list of sampling sites is presented in Chapter 2 and they are shown on the Overview Map. The sampling was done in July 2019 by the national experts, according to the methodology agreed at the workshop organized for national experts that follows *Guidance for the surveying of aquatic macrophytes in running waters* (CEN – EN 14184, 2014). Whenever it was possible, sampling was conducted from a small boat on six survey units of one-kilometre length, three on the left and three on the right side on each sampling site. In each survey unit of one-kilometre length, the plants were recorded while slowly passing along the banks and at least two full stops were made (usually at 200 and 700 m) to collect macrophytes. Collection of macrophytes was done with the help of a rake on a rope or a telescope stick and for measurement and recording of survey units, a portable GPS device was used. In shallow wadable sites, the whole watercourse stretch was done without separation to the left and the right bank.

The abundance of macrophytes was estimated according to the 5-level Kohler scale (Kohler, 1978). Taxa were identified in the field when it was possible. Others were collected for later identification that was carried out in the national laboratories with the help of up-to-date literature for identification of aquatic bryophytes, pteridophytes and angiosperms. Species names were updated according to The Plant List (2013).

Descriptive parameters of habitat were assessed regarding bank structure, sediment type, flow class, and transparency according to the record sheets used during JDS3.

JDS4 sampling sites that were not sampled for macrophytes are JDS4-9, JDS4-11 to JDS4-14, JDS4-16, JDS4-19, JDS4-20, JDS4-30, JDS4-34, and JDS4-44 to JDS4-46. Sampling sites that were sampled partially are JDS4-5, JDS4-29, JDS4-31, JDS4-36, JDS4-36, and JDS4-42 only left side, JDS4-14, JDS4-49, and JDS4-51 only right side, JDS4-38 one left and one right km, and JDS4-21 three km equally. At German sites only 100 metres long stretches were sampled (sites JDS4-1 to JDS4-5). All other sites were sampled according to the standard procedure with three kilometres long stretches on the left and right side.

Data analysis

The 5-level Kohler scale was transformed into the metric Relative Plant Mass (RPM) (Kohler and Janauer, 1995; Pall and Janauer, 1995). RPM was used for all other calculations and statistical analysis.

A one-way SIMPER analysis based on Bray-Curtis similarity was performed on the RPM data of taxonomic composition of the macrophytes, where characteristic taxa were analysed in the Primer 6 software (Clarke and Gorley, 2006). RPM data were log-transformed before analysis.

Taxa were grouped in plant groups (bryophytes, pteridophytes and angiosperms) and to the life forms (hydrophytes, helophytes, amphiphytes, water related species and chance species) (Stanković et al., 2014). The proportion of plant groups and life forms along the Danube River and the tributaries was displayed using Microsoft Excel 365.

A canonical correspondence analysis (CCA) was used to ordinate the composition of hydrophytes with environmental variables which was done in CANOCO 5 (ter Braak and Šmilauer, 2012). The CCA analysis was performed using average RPM per sampling site, 24 sampling sites and 12 environmental variables. Environmental data were normalised before analysis and Draftsman's plot was conducted to eliminate variables with significant autocorrelation.

Samples and sampling sites were grouped for better understanding and visualisation according to Moog et al. (2006) where the Danube River is divided into ten River sections. River sections were grouped into River Reaches: Upper (types 1-4), Middle (types 5-7) and Lower (types 8-10).

9.3 Results and Discussion

Species composition

A total of 132 taxa were identified in 38 sampling sites, in 173 subsamples. The Majority of the taxa were identified in the Danube River (120), while in the tributaries only 53 taxa were identified, with 12 unique taxa for the tributaries and not found in the Danube River.

The proportion of plant groups in the Danube River sections and tributaries based on the Relative Plant Mass is shown in Figure 1. Angiosperms, with 97 taxa were dominant plant group in most of the Danube River sections, as well as in the tributaries. Bryophytes, with 32 taxa, were characteristic for Upper Reach. They were dominant in the River sections S1 and S3, while co-dominant with angiosperms in S2 and S4. Pteridophytes were represented with three taxa, and they were found occasionally with a small proportion in the total Relative Plant Mass.

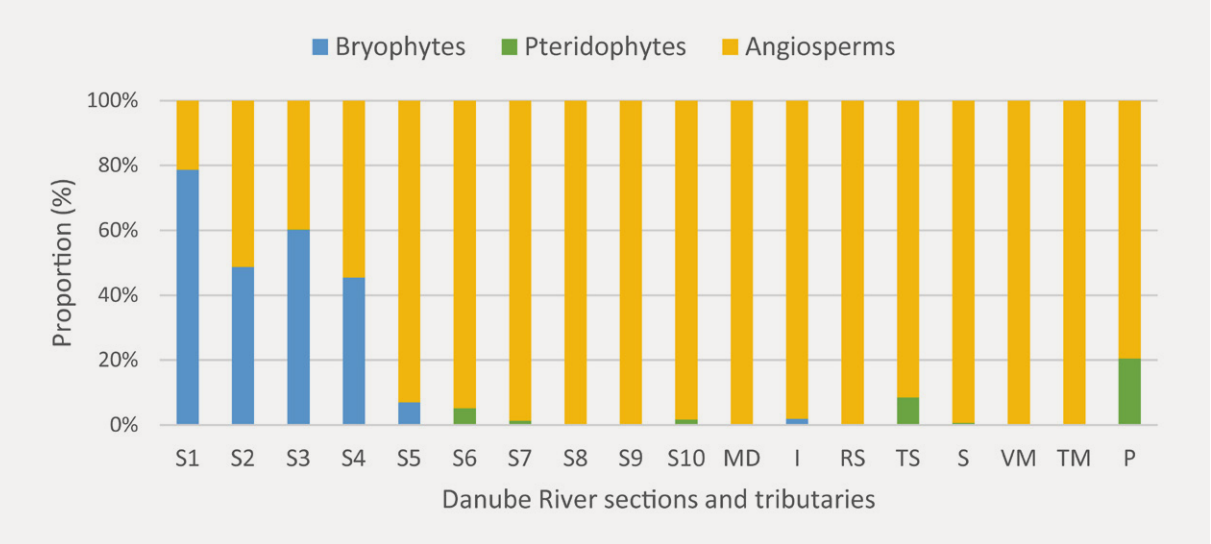


Figure 1: The proportion of plant groups in all Danube River sections (S1 – S10) and tributaries (MD – Mosoni Danube Arm, I – Ipeľ, RS – Rackevei-Soroksari Danube Arm, TS – Tisza, S – Sava, VM – Velika Morava, TM – Timok, P – Prut).

Macrophytes with highest Relative Plant Mass, most dominant life form in the Danube River and tributaries, were hydrophytes (49 taxa), especially in all River sections of the Upper Reach (S1 – S4), in River sections S6 – S8, S10, and tributaries Tisza, Sava and Prut rivers (Fig. 2). Helophytes (25 taxa) dominated with Relative Plant Mass in the Danube River section S5 and co-dominated with hydrophytes in S10. In the tributaries Ipeľ, Rackevei-Soroksari Danube Arm and Timok, helophytes were the dominant life form. The highest proportion of Relative Plant Mass of amphiphytes (33 taxa) was identified in the River section S9 as well as in the Mosoni Danube Arm and Timok River. Water related plants (19 taxa) and chance species (six taxa) were occasionally identified in the Danube River as well as in the tributaries, but never with a high proportion of the Relative Plant Mass.

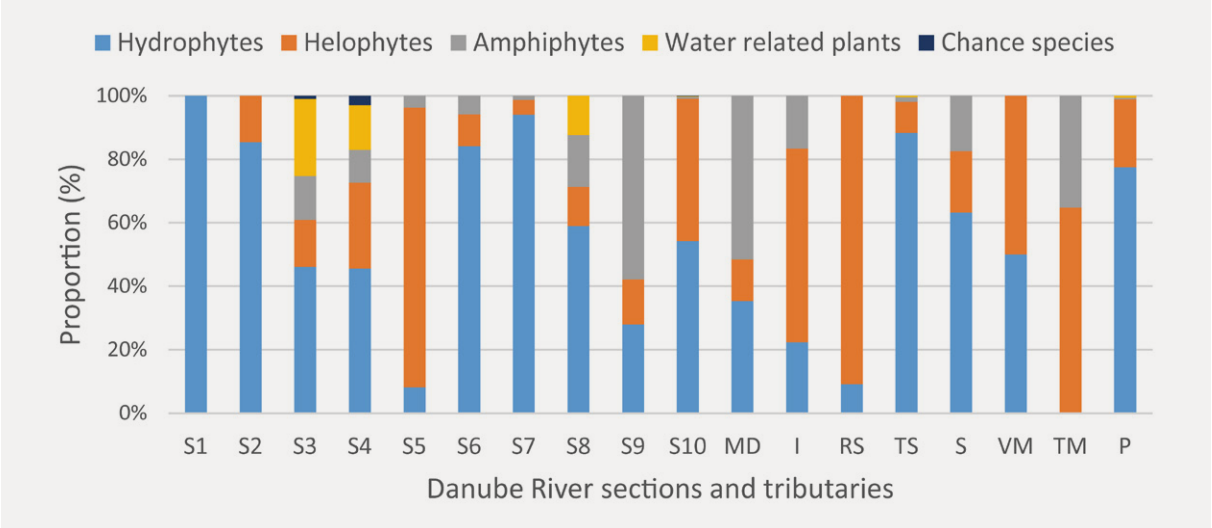


Figure 2: The proportion of life forms in all Danube River sections (S1 – S10) and tributaries (MD – Mosoni Danube Arm, I – Ipeľ, RS – Rackevei-Soroksari Danube Arm, TS – Tisza, S – Sava, VM – Velika Morava, TM – Timok, P – Prut).

A one-way SIMPER analysis based on the Bray-Curtis similarity performed on the composition of macrophytes showed characteristic taxa for each Danube River section and tributaries. In the first half of the Upper Reach, where hydrophytes were dominant, *Cinclidotus riparius* (Host ex Brid.) Arn. and *Fontinalis antipyretica* Hedw. were dominant taxa in the River section S1, while only *F. antipyretica* was dominant in S2. River sections S3 and S4 had almost identical dominant taxa composed of *C. riparius, Phalaris arundinacea* L. and *Solidago gigantea* Aiton with addition of *F. antipyretica* in River section S3.

The Middle and Lower Reaches of the Danube River were quite diverse. According to the SIMPER analysis, River sections S5 had two dominant taxa, *P. arundinacea* and *Phragmites australis* (Cav.) Trin. ex Steud. River section S6 was characterised by the dominance of unrooted and floating taxa *Ceratophyllum demersum* L, *Spirodela polyrrhiza* (L.) Schleid., *Lemna minor* L., as well as *P. arundinacea*, more likely to prevail on the riverbanks than in the watercourse. River sections S7 and S8 were dominated only by large, submersed plants, most of them well-rooted in the soft sediments. *Potamogeton perfoliatus* L., *C. demersum, Potamogeton gramineus* L. and *Stuckenia pectinata* (L.) Bubani are dominant in the River section S7, while *C. demersum, Vallisneria spiralis* L. and *Myriophyllum spicatum* L. are dominant in the River section S8. Dominant taxa of River section S9 were *Berula erecta* (Huds.) Coville and *Butomus umbellatus* L. In River section S10, in comparison to other River sections, SIMPER analysis revealed the most diverse group of characteristic taxa, where they should rather be classified as co-dominant. Those were *P. australis, P. perfoliatus, C. demersum, Hydrocharis morsus-ranae* L., *Stratiotes aloides* L. and *Trapa natans* L., showing great diversity of life forms in mosaic habitats, such as a large river delta.

Species composition in the tributaries was quite diverse and each river had its own special composition of aquatic plants. According to the SIMPER analysis, *Rorippa amphibia* (L.) Besser, together with floating *L. minor* and *S. polyrrhiza* were dominant taxa in the samples of the Mosoni Danube Arm. Characteristic and dominant taxa in the Ipeľ River were *P. arundinacea, Carex riparia* Curtis and *Leersia oryzoides* (L.) Sw. *Phragmites australis* and *M. spicatum* were dominant taxa in the Rackevei-Soroksari Danube Arm while floating taxa *S. polyrrhiza, Salvinia natans* (L.) All., *L. minor* and *C. demersum* were dominant taxa in the Tisza River. In the Sava River, most represented taxa were *S. pectinata, B. umbellatus, S. polyrrhiza* and *Sagittaria sagittifolia* L., while only *P. australis* was dominant in the Velika Morava River. Helophytes *Schoenoplectus lacustris* (L.) Palla and *P. australis* were found as dominant in the Timok River, while unrooted taxa *C. demersum, S. natans* and *L. minor* were dominant in the Prut River.

The similarity of Danube River sections

NMDS analysis of River sections, based on Bray-Curtis similarity performed on Relative Plant Mass of macrophytes, and overlaid with cluster analysis, showed separation of a few groups of River sections (Fig. 3). Upper Reach of the Danube River was separated into two groups. River sections S1 and S2 had 60%, while River sections S3 and S4 had only 40% similarity. Those two subgroups showed <20% of mutual similarity. The whole River section S5, except sampling site JDS4-28, represented with all Hungarian sampling sites was separated from all other sections. This separation was expected due to the domination of helophytes in that section (88%). Largest cluster was formed with most of the sampling sites from Middle Reach and all from Lower Reach. River sections S6, S7 and S10 showed 40% similarity, while River sections S8 and S9 showed 20% similarity between themselves and with other Sections in the group.

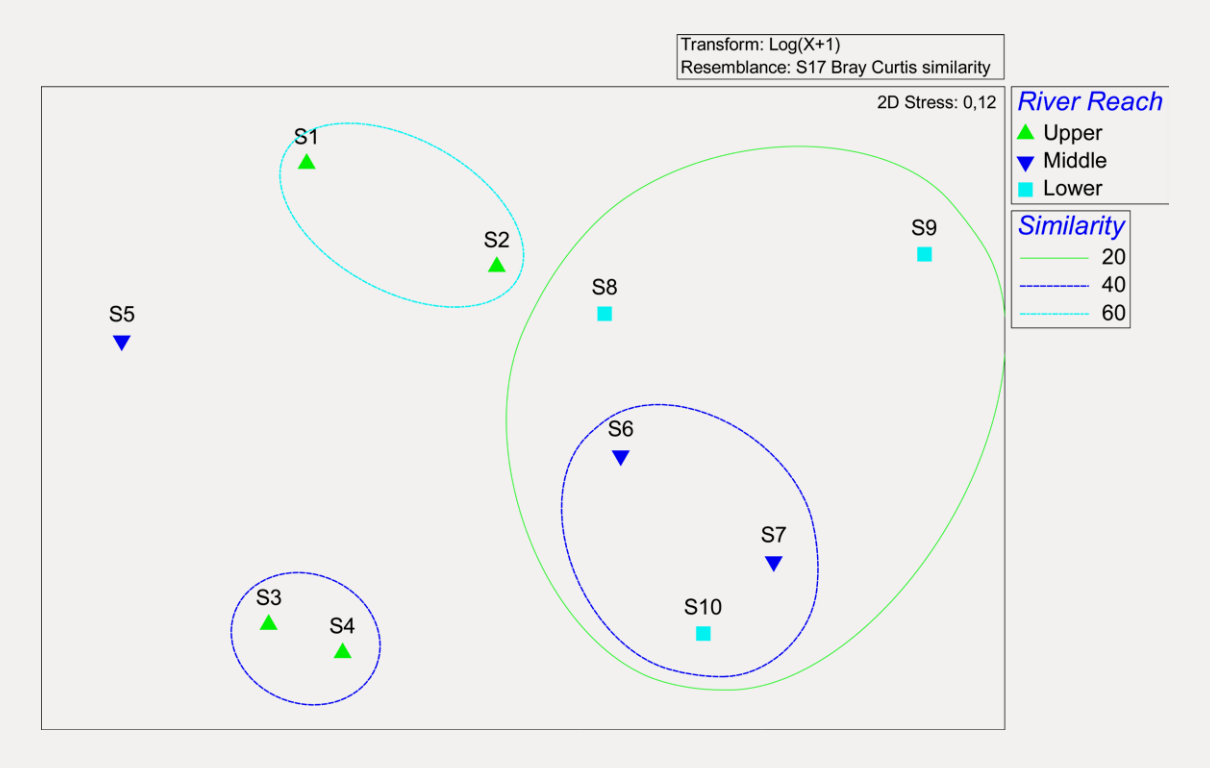


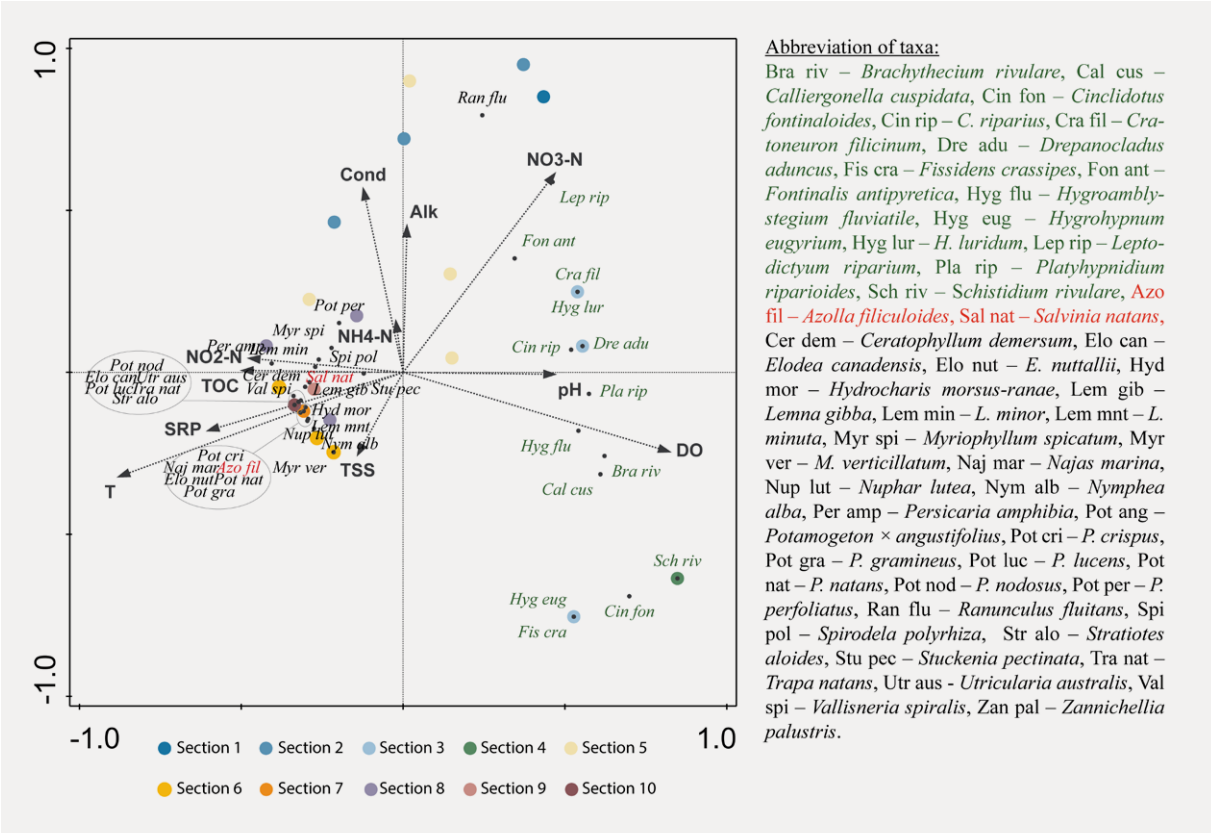
Figure 3: NMDS analysis of River sections performed after Bray-Curtis similarity of macrophytes based on the Relative Plant Mass and overlaid with cluster analysis.

Ecological features of hydrophytes

Ecological features of hydrophytes were analysed with Canonical Correspondence Analysis (CCA), where the following physical and chemical parameters were selected: water temperature, pH, conductivity, alkalinity, dissolved oxygen, the concentration of ammonia, nitrites, nitrates, soluble reactive phosphorus, total organic carbon and total suspended solids.

The ordination results of hydrophytes and environmental data of the CCA in the Danube River are presented on the F1×F2 ordination plot (Fig. 4). Eigenvalues of the first two axes are 0.861 and 0.358 and they explain 48.5% of the variance of macrophytes and environmental data. Axis 1 had the highest correlation with water temperature (R=−0.863) and dissolved oxygen (R=0.808), while axis 2 had the highest correlation with nitrates (R=0.599) and conductivity (R=0.553). Roughly the same grouping pattern of the River sections here based only on hydrophytes is very similar to the one in NMDS analysis, where the whole macrophyte

community was used for the analysis. Sampling sites of River sections from the Upper Reach grouped on the upper and right side of the plot. They were influenced by higher DO, pH, nitrates, alkalinity, and conductivity, which appeared to be the most optimal habitat for bryophytes. Sampling sites of the River section 5 again showed separation from other sections and a low number of hydrophytes. Sampling sites of River sections belonging to the Middle and Lower Reach grouped and were influenced by high-water temperature, nutrients (ammonia, nitrites, SRP) and rich in organic matter (TOC, TSS). Such habitat preferred most of the angiosperms, rooted or floating ones, and pteridophytes.



Abbreviation of taxa:
Bra riv – *Brachythecium rivulare*, Cal cus – *Calliergonella cuspidata*, Cin fon – *Cinclidotus fontinaloides*, Cin rip – *C. riparius*, Cra fil – *Cratoneuron filicinum*, Dre adu – *Drepanocladus aduncus*, Fis cra – *Fissidens crassipes*, Fon ant – *Fontinalis antipyretica*, Hyg flu – *Hygroambly-stegium fluviatile*, Hyg eug – *Hygrohypnum eugyrium*, Hyg lur – *H. luridum*, Lep rip – *Lepto-dictyum riparium*, Pla rip – *Platyhypnidium riparioides*, Sch riv – *Schistidium rivulare*, Azo fil – *Azolla filiculoides*, Sal nat – *Salvinia natans*, Cer dem – *Ceratophyllum demersum*, Elo can – *Elodea canadensis*, Elo nut – *E. nuttallii*, Hyd mor – *Hydrocharis morsus-ranae*, Lem gib – *Lemna gibba*, Lem min – *L. minor*, Lem mnt – *L. minuta*, Myr spi – *Myriophyllum spicatum*, Myr ver – *M. verticillatum*, Naj mar – *Najas marina*, Nup lut – *Nuphar lutea*, Nym alb – *Nymphea alba*, Per amp – *Persicaria amphibia*, Pot ang – *Potamogeton × angustifolius*, Pot cri – *P. crispus*, Pot gra – *P. gramineus*, Pot luc – *P. lucens*, Pot nat – *P. natans*, Pot nod – *P. nodosus*, Pot per – *P. perfoliatus*, Ran flu – *Ranunculus fluitans*, Spi pol – *Spirodela polyrhiza*, Str alo – *Stratiotes aloides*, Stu pec – *Stuckenia pectinata*, Tra nat – *Trapa natans*, Utr aus - *Utricularia australis*, Val spi – *Vallisneria spiralis*, Zan pal – *Zannichellia palustris*.

Figure 4: Canonical Correspondence Analysis (CCA) triplot of hydrophytes, environmental scores, and samples (coloured dots) in the Danube River. Environmental variables are abbreviated as: T – temperature, pH – pH, DO – dissolved oxygen, Cond – conductivity, Alk – alkalinity, NH4-N – ammonia, NO2-N - nitrites, NO3-N – nitrates, SRP – soluble reactive phosphorus, TOC – total organic carbon and SS – suspended solids. Letters of bryophytes are in green, pteridophytes in red and angiosperms in black colour.

Detailed analysis of the whole macrophyte community and hydromorphological features of the habitat within the JDS3, showed a strong correlation between taxa and general description of the habitat (Stanković et al., 2014). Besides colder and oxygenated water, rich in nitrates shown by the results of the JDS4, results of the JDS3 demonstrated that bryophytes also preferred shaded habitat, with the hard substrate and fast-flowing water. Current results showed that floating or rooted angiosperms and pteridophytes preferred warmer, nutrient, and organically rich water, which consisted of the habitat preferences from the JDS3 which are open and sunny littoral water with soft sediments, small bottom slope and slow water current. The appearance of floating macrophytes in the very large rivers is mostly an indicator of the existence of backwaters and its connection to the main channel, indicating good lateral connectivity (Ecke et al., 2016).

Indication of macrophytes based ecological status assessment

Classification of macrophytes based ecological status assessment is presented in Figure 5. Countries AT, BG and RO have used macrophytes for assessment of ecological status, while all other countries have reported that macrophytes were not assessed during the JDS4 campaign due to instead of several reasons (not relevant BQE, metrics are not yet developed, macrophytes are not present or rare). On sampling sites in the Upper Reach ecological status was indicated as good, while sampling sites in the Lower Reach were indicated as good to moderate.

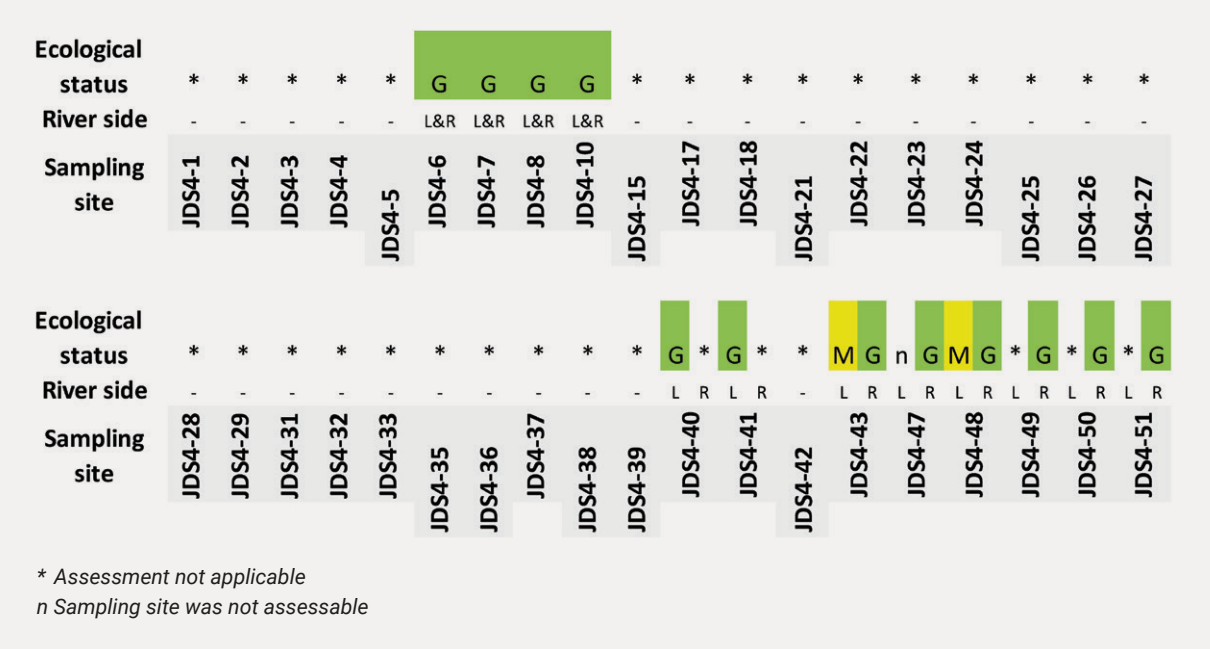


Figure 5: Results of macrophytes based classification of ecological status assessment. Tributaries codes are lowered. River side is indicated where indication of ecological status is different on left or right side.

Comparison with outcomes from JDS1, JDS2 and JDS3

JDS3 provided the richest dataset in the term of total species counts (198 taxa) and JDS1 the poorest (48 taxa). Species counts obtained in JDS3 were slightly different due to the higher number of species in both life-form and taxa groups. JDS4 was more focused on aquatic plants and hence bank vegetation was omitted on several sampling sites. A little disadvantage of such approach is a loss of information about the distribution of invasive alien species growing on banks like *Reynoutria* spp. or *Solidago* spp. since watercourses serve as an ideal migration corridor for invasive species.

In the list of 132 taxa identified during this research, 79% and 73% of taxa are identical to JDS2 and JDS3, respectively. Similarity with taxa of JDS1 is only 57%. Cumulatively, the number of taxa of macrophytes and other plants related to river habitat, identified in all four Joint Danube Surveys, is now 289 taxa.

The proportion of plant groups, as well as life forms in JDS3 and JDS4, can be easily compared because the data were processed and presented in the same way. The proportion of life forms in the Danube River sections is almost identical. The exception is with macroalgae that are not presented within JDS4 dataset because of low sampling effort. Also, charophytes were not identified at all during this sampling campaign, and they were identified during JDS2 and JDS3. Charophytes are very rare in the Danube River and can easily be overlooked.

9.4 Conclusions

This study brings a complete and representative overview of Danube macrophytes carried out for the fourth time within the Joint Danube Survey concept. The exception was that this time it was done by multiple national experts, instead of one Core Team expert. The survey was successful.

Bryophytes were most commonly found in the Danube River sections of Upper Reach, while angiosperms rooted or floating, dominated in the Middle and Lower Reach of the Danube River. The proportion of plant groups was different in different tributaries, unique for each one.

Hydrophytes or macrophytes that are permanently living in the water, were the dominant life form in most of the Danube River sections and tributaries. Helophytes were dominant in the Danube River section S5 as well as in the Ipel' River, Rackevei-Soroksari Danube Arm and Timok River.

Statistical analysis demonstrated clear separation of Danube River sections into a few subgroups. The Upper Reach was separated from the Middle and Lower Reaches, where the latter two also showed specific grouping, roughly following the River Continuum Concept (Vannote et al., 1980).

Canonical correspondence analysis revealed that environmental parameters which highly influenced hydrophytes in the Danube River were water temperature, dissolved oxygen, nitrates, and conductivity. Bryophytes preferred colder and oxygenated water, rich in nitrates that run in the Upper Reach of the fast-flowing Danube River that has shaded banks with the hard substrate. Floating or rooted angiosperms and pteridophytes preferred warmer, nutrient, and organically rich water in the Middle and Lower Reach of the slow-flowing Danube River, with small bank slope and soft sediments.

The abundance of floating macrophytes in the Middle and Lower Reach of the Danube River suggests good lateral connectivity to backwaters which imply the good status of one aspect of hydromorphological conditions.

This research, just like three Joint Danube Surveys before, demonstrated that in certain river stretches there is a natural lack of microhabitats with the proper conditions for the successful growth of macrophytes. This causes almost plant-free river parts with none macrophytes or with insignificant abundance. Therefore, their usage in the assessment of ecological status in very large rivers is disputable, but certainly gives additional information on hydromorphological condition.

Macrophytes are used only in several countries for ecological status assessment where the results indicated good to moderate ecological status. However, the majority of the Danube River and tributaries are left without ecological status classification based on macrophytes because they are not relevant BQE, metrics have not yet been developed, and they are not present or rare in the river.

Based on comparison with the outcomes of previous Joint Danube Surveys, the composition of macrophytes has remained stable in terms of richness and diversity over the course of several years.

9.5 References

CLARKE, K. R. & GORLEY, R. N. (2006). PRIMER v6: User Manual/Tutorial. PRIMER-E, Plymouth.

ECKE, F., HELLSTEN, S., KÖHLER, J., LORENZ, A. W., RÄÄPYSJÄRVI, J., SCHEUNIG, S., SEGERSTEN, J. & BAATTRUP-PEDERSEN, A. (2016). The response of hydrophyte growth forms and plant strategies to river restoration. Hydrobiologia 769(1): 41-54.

EN14184, (2014). Water quality -- Guidance standard for the surveying of aquatic macrophytes in running waters (EN 14184:2014). 15.

ICPDR (2015). The Danube River Basin District. Update 2015. ICPDR Secretariat, Vienna, 164 p.

KOHLER A. (1978). Methoden der Kartierung von Flora und Vegetation von Süsswasser Biotopen. Landschaft und Stadt 10: 73–85.

KOHLER, A. & JANAUER, G. A., (1995). Zur Methodik der Untersuchung von aquatischen Makrophyten in Fließgewässern. In Steinberg, C., H. Bernhard & K. H. (eds) Handbuch der angewandten Limnologie VIII-1.1.3. Ecomed, Landsberg/Lech., 1-22.

HASLAM, S. M., (2006). River Plants: The Macrophytic Vegetation of Watercourses, Second edn. Forrest Text, Cardigan, UK.

KALFF, J., (2001). Limnology. Prentice Hall, New Jersey.

KOHLER, A. & JANAUER, G.A. (1995). Zur Methodik der Untersuchung von Fließgewässern. In: Steinberg, Ch., Bernhardt, H. & Klapper, H. (Hrsg.): Handbuch Angewandte Limnologie, Ecomed Vlg., Landsberg/Lech, VIII-1.1.3, 1-22.

LACOUL, P. & FREEDMAN, B. (2006). Environmental influences on aquatic plants in freshwater ecosystems. Environmental Reviews 14: 89–136.

MOOG, O., SOMMERHÄUSER, M., ROBERT, S., BATTISTI, T., BIRK, S., HERING, D., OFENBÖCK, T., SCHMEDTJE, U., SCHMIDT-KLOIBER, A. & VOGEL, B., (Year) Published. Typology of the Danube River base d on „top-down" and „bottom-up" approaches In: 36th International Conference AC-IAD, Vienna, 2006. p 5.

PALL, K. & JANAUER, G. A. (1995). Die Makrophytenvegetation von Flussstauen am Beispiel der Donau zwischen Fluß-km 2552,0 und 2511,8 in der Bundesrepublik Deutschland. Archiv für Hydrobiologie Supplementband 101, Large Rivers 9/2: 91-109.

PALL, K., RATH, B., JANAUER, G. A. (1996). Die Makrophyten in dynamischen und abgedämmten Gewässersystemen der Kleinen Schüttinsel (Donau-Fluss-km 1848 bis 1806) in Ungarn. Limnologica 26: 105-115.

RATH, B. (1995). Potamogeton pectinatus - Bestände als Bio-Indikatoren der Schwermetallbelastung im Hauptarm der Donau. Opuscula Zoologica 27-28: 167-174.

RATH, B., (1997). Verbreitung von aquatischen Makrophytenbeständen im Flussbett des früheren Hauptarmes der Donau (Szigetköz, Stromkm (1826-1843)). In: Conference of the International Association for Danube Research, Vienna, 1997. vol 32. p 227-232.

SÂRBU, A., JANAUER, G., SCHMIDT-MUMM, U., FILZMOSER, P., SMARANDACHE, D. & PASCALE, G. (2011). Characterisation of the potamal Danube River and the Delta: connectivity determines indicative macrophyte assemblages. Hydrobiologia 671(1): 75-93.

STANKOVIĆ, I., PALL, K. & JANAUER, G., (2014). Long report on Macrophytes. Joint Danube Survey 3. ICPDR, Vienna.

TER BRAAK, C. & ŠMILAUER, P. (2012). Canoco reference manual and user's guide: software of ordination (version 5.0). Microcomputer Power (Ithaca, NY, USA).

THE PLANT LIST (2013). Version 1.1. Published on the Internet; http://www.theplantlist.org/ (accessed 24th October 2020).

TOCKNER, K., UEHLINGER, U. & ROBINSON, C. T., (2009). Rivers of Europe. Elsevier, Amsterdam.

VANNOTE, R. L., MINSHALL, G. W., CUMMINS, K. W., SEDELL, J. R. & CUSHING, C. E. (1980). The River Continuum Concept. Canadian Journal of Fisheries and Aquatic Sciences 37(1): 130-137.

WFD (2000). Directive 2000/60/ec of the European Parliament and of the Council 22.12.2000. Official Journal of the European Communities L327: 1-72.

10 Invasive alien species

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Abstract

The study of non-indigenous species, with emphasis on invasive alien species (IAS) within JDS4 was performed in order to collect comparable data along the Danube and its main tributaries, with the aim to assess current status in respect to bioinvasions within the Danube River Basin (DRB) and to evaluate stress caused by this important pressure. The study was realized along with other analyses on 51 JDS4 sites, as well as on additional sites (mainly in the Middle and Lower Reaches of the Danube: 35 sites on the Danube, 26 on tributaries, 9 on adjacent canals and lakes, 7 on reservoirs). The data comprised results of screening of biological quality elements performed by the JDS4 national teams, but also additional information specifically collected for the analyses of the distribution of the IAS using more detailed sampling and DNA-based approaches. In addition, the Smartphone application 'Invasive Alien Species in Europe' developed by the European Commission's Joint Research Centre (JRC), and specifically updated to be effectively used within the DRB, was used to collect information on the IAS within JDS4. A total of 6 aquatic macrophyte, 35 aquatic macroinvertebrate and 17 non-native fish species were recorded during JDS4. Number of recorded alien species, as well as values of indices indicating the pressure caused by biological invasions (SBC and BAI index), revealed a better situation in the Lower Danube in comparison to the Upper and Middle reaches, mainly since the Lower Danube could be considered as the native area of distribution for Ponto-Caspian taxa, that are non-indigenous in the Middle and Upper Danube. The (e)DNA-based detection of aquatic IAS was approved as being effective. All results pointed again to the importance of IAS for the DRB.

Key words: Biological invasions, alien aquatic species, non-indigenous taxa, Danube River Basin

10.1 Introduction

Historical changes in the environment led to changes in the distribution of organisms and those alterations have been accelerated by human influence. Pollution, hydromorphological degradation, aquaculture, aquaristics, navigation, as well as other human activities strongly affect the aquatic ecosystems.

The influence of Invasive Alien Species (IAS) has been recognised as one of the major threats to native biodiversity for the Danube River Basin (ICPDR, 2015; Paunović et al., 2015). Invaders can alter fundamental ecological features such as dominant species in a community, productivity, nutrient cycling and thus can alter the structure and function of the ecosystems.

The term 'alien species' refers to any live specimen of a species, subspecies or lower taxon of animals, plants, fungi or micro-organisms introduced outside its natural range; it includes any part, gametes, seeds, eggs or propagules of such species, as well as any hybrids, varieties or breeds that might survive and subsequently reproduce. Other terms are extensively used in the literature to qualify taxa as alien: non-native, or non-indigenous species, neozoa, neobiota and neophyta. IAS means an alien species whose introduction or spread has been found to threaten or adversely impact upon biodiversity and related ecosystem services (EU, 2014).

Following the construction of the Rhine-Main-Danube channel, the Danube became an important invasion route. Canals can provide conduits for species to spread between previously separate biogeographic regions either by active movement, drift and/or as a result of ship transport (Bij de Vaate et al., 2002). The spread of non-indigenous species along the Danube (in both directions, upstream and downstream), as well as the expansion of neobiota from the Danube to its tributaries has been repeatedly recorded. The Danube River is characterised as a part of the "Southern Invasion Corridor" and a branch of the European Invasion Network (Panov et al., 2009; Panov et al., 2010).

The assessment of the ecological and economical/societal impacts of the introduction of non-indigenous species (NIS) became one of the primary focus areas of bioinvasion or biopollution science (Olenin et al., 2007; Panov et al., 2009).

The European Commission's Joint Research Centre (JRC) has developed a smartphone application 'Invasive Alien Species in Europe' (Tsiamis et al., 2017, Figure 3). The aim of the application is to enable the general public (amateurs), but also professionals, to contribute to the detection, monitoring and management of invasive alien species that are found to be of interest for Europe (IAS of EU concern, and/or alien taxa of specific interest for particular region of Europe in wider geographical scale – such as the DRB). For the purposes of the JDS4, the JRC in collaboration with the ICPDR developed an extended list with IAS of regional concern for the DRB, factsheets for each species on the list, and updated the smartphone application 'Invasive Alien Species in Europe' with the developed species DRB catalogue. The list includes 64 species – 29 fish and 44 aquatic invertebrates. The app facilitates sightings for each species, and the collection of at least one picture, sighting location, species coverage, and the related habitat, and has been used for the collection of additional information on the IAS of the DRB during the JDS4 and testing the new DRB application.

The aim of this work is to present the state of the art in respect to the presence of non-native aquatic species (aquatic macrophytes, aquatic macroinvertebrates and fish) in the Danube River Basin based on the results of the Joint Danube Survey 4 (JDS4). Also, the present state is compared with prior situations inferred from previous Joint Danube Surveys.

10.2 Methods

The same dataset related for each Biological Quality Element (BQE) from 51 JDS4 sites was used for collection of comparable information on the IAS during the JDS4. Information from additional sites, located mainly in the middle and lower reach of the Danube was also considered: 35 sites on the Danube, 26 on tributaries, 9 on adjacent canals and lakes, 7 on reservoirs).

It is important to mention that all data from JDS4 was collected via the JDS4 collection portal specifically developed by the ICPDR, which enabled, beside collection, data check and validation.

Details on sampling methodology are provided in Chapters 5-9 and 12-16 of this report. Basic sectioning of the Danube River was defined to Upper, Middle and Lower Danube, according to Liška et al., (2008) and Literáthy et al., (2002), as follow – Upper Danube River: from source to 1,790 river kilometre (rkm), Middle Danube River: from rkm 1,790 – 943 and Lower Danube River from rkm 943 to the mouth.

For the JDS4 an additional effort to collect high quality data on alien species has been applied. For that purpose, Kick and Sweep sample collection and LiNi crayfish traps were applied.

For supplementary collection of crayfish species, LiNi crayfish traps (Figure 1) with appropriate bite (small fish, wet cat food or fresh liver, etc.) were used at 27 sites (from site JDS4-6, Jochenstein, river km 132, to JDS4-50, Reni, river km 2204), covering 2,072 kilometers of the Danube, as well as six tributaries and one side arm (Figure 2). All together 71 traps (in average 3 per site) were positioned for approximately 5 hours during the late evening hours or night at different depths and bottom types, thus covering the majority of possible habitat types (activity areas).

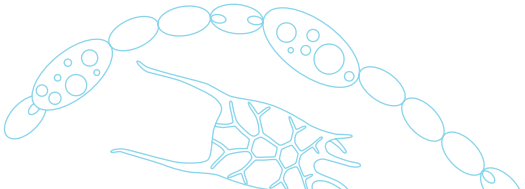
At the additional sites, dredging, dip net and sieves, beach seine nets and gill nets were also applied.

During JDS4, the three Biological Quality Elements - Fish, Macrozoobenthos (MZB) and Phytobenthos, as well as the sediment fauna, were assessed by (e)DNA-based tools for the first time (see chapters 12 and 13-16).

The total number of alien species and mean percentage participation of alien taxa in the total communities have been considered as a strong indicator of the state of communities within the investigated river stretches and have been assessed based on the results of surveys of BQEs described in detail in Chapters 5-9 of this report.

In order to estimate the level of biological invasions we used Site-specific Biological Contamination (SBC) Index (Arbačiauskas et al., 2008) and Bioinvasion Assessment (BAI) Index (Paunović and Csányi, 2018) were used.

SBC and BAI were calculated using macroinvertebrate and fish data collected using conventional methods (details in chapters 5 and 6; data collected by additional methods are excluded, e.g. dredging, LiNi traps, detail mussel survey), in order to provide coherence of the information and comparability along the Danube and over the time. The (e)DNA data is also excluded from the calculation due to species abundance data.



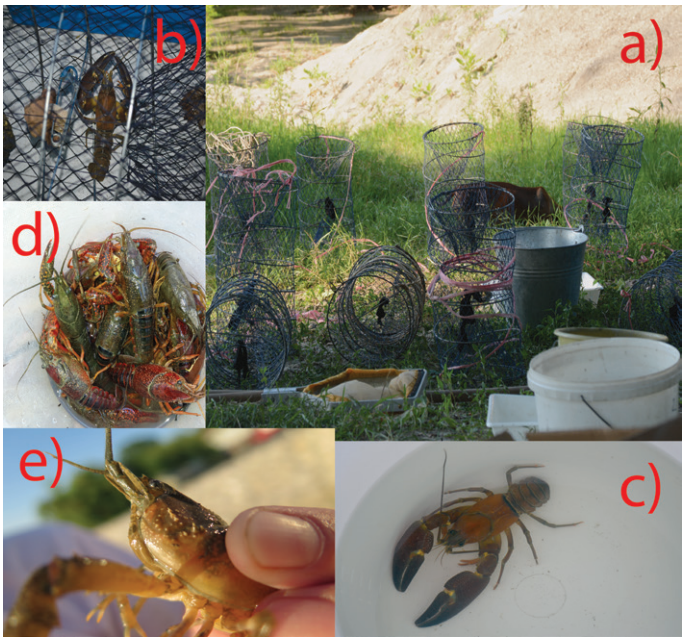


Figure 1: LiNi crayfish trap sampling; a) Preparation of LiNi traps for sampling; b) and c) Pacifastacus leniusculus (Dana, 1852), d) Procambarus clarkii (Girard, 1852) and Faxonius limosus (Rafinesque, 1817) individuals collected.

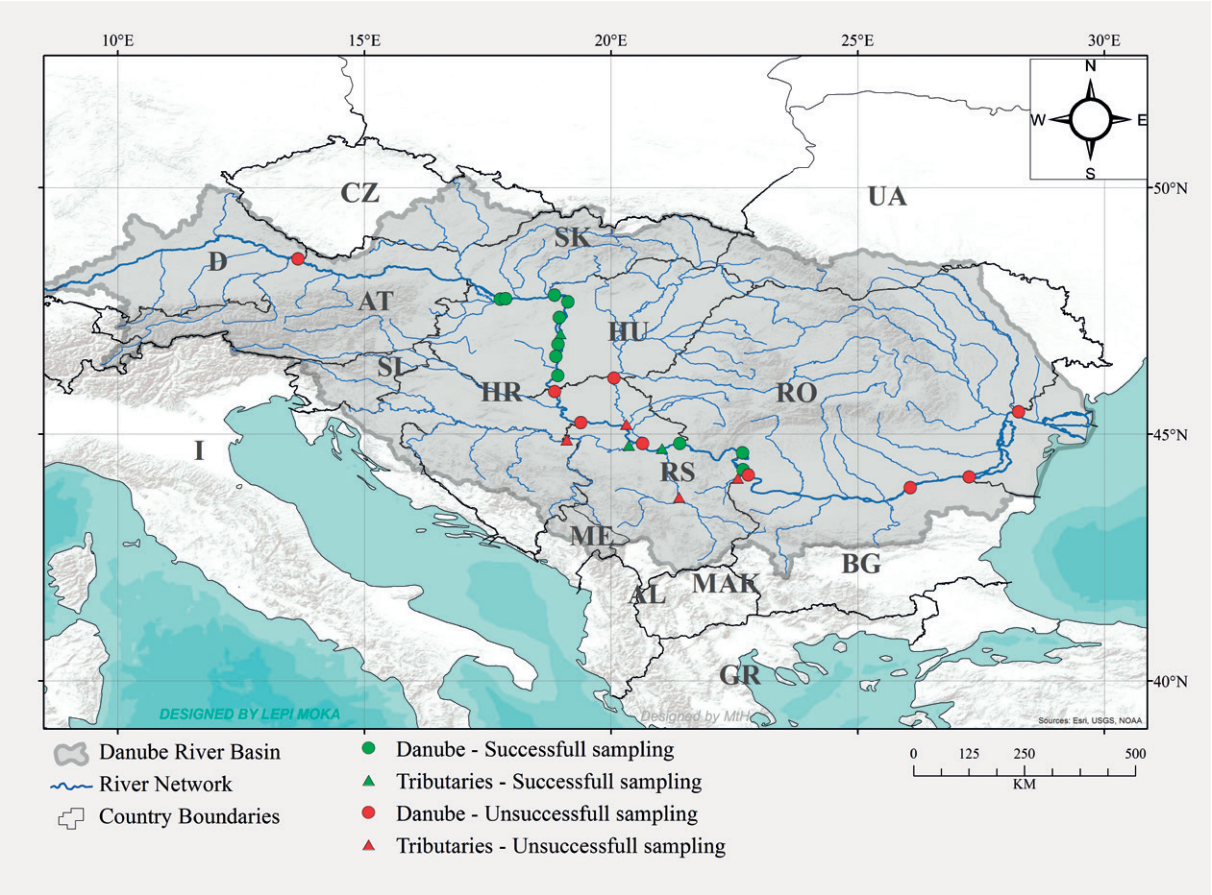


Figure 2: Location of sampling with LiNi crayfish traps.

SBC index estimates biological contamination of the specific sites and it can be used for comparison of biological contamination of different locations and for estimation. Site-specific Biological Contamination (SBC) involves both the specific value of number of alien species and the specific value of an abundance of alien species in the total fish community by using the formula:

$$SBC = (n_a / n_{sum} + \log N_a / \log N_{sum}) / 2,$$

where n_a is a number of alien species, n_{sum} a number of all species in the sample, N_a abundance of alien species and N_{sum} total abundance of species in the sample.

For the calculation of SBC, the results of macroinvertebrate and fish JDS3 surveys were used. JDS2 datasets on macroinvertebrates (Liška et al., 2008) were also used to calculate SBC and compare the level of biological contamination over time.

The index range from 0 to 4 and the following classification scale was used (modified original scale proposed by Arbačiauskas et al. 2008): 0 (no biocontamination, no pressures caused by biological invasions), 1 (low biocontamination, minor pressures caused by biological invasions), 2 (moderate biocontamination, moderate pressures caused by biological invasions), 3 (high biocontamination, high pressures caused by biological invasions) and 4 (severe biocontamination, high pressures caused by biological invasions).

The Bioinvasion Assessment Index (BAI) – the final score for each species assessed by the Risk Assessment Procedure for evaluation of the invasiveness of non-indigenous species relevant for the Danube River – IAS-RAP-Danube (Paunović and Csányi, 2018), is linked with the abundance of non-indigenous taxa by applying the following calculation:

$$BAI = (N1*P1 + N2*P2 \ldots Nn*Pn) / A,$$

where N is the abundance of each recorded alien species, P is the "relative contribution – Pondering Value" from IAS-RAP-Danube for each recorded alien species, and A is the total abundance of the assessed community.

The described BAI index takes into the consideration both, abundance and characteristics of each particular taxa (by involving the "Relative contribution – Pondering Value").

The index range is between 0 and 1, with assessed state high 1 (class 1) BAI=0; good (class 2)

BAI=0.01-0.1; moderate-3 (class 3) BAI=0.11-0.2, poor (class 4) BAI=0.21-0.5 and bad (class 5) BAI>0.50.

The analyses was done in respect to basic sectioning of the Danube River to Upper, Middle and Lower Danube, as defined in Liška et al., (2008) and Literáthy et al., (2002).

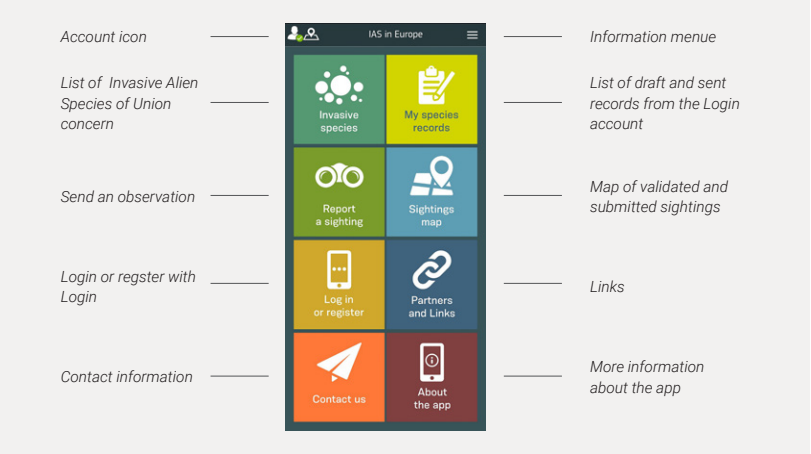


Figure 3: Invasive Alien Species mobile application – screen view.

10.3 Results and discussion

Traditional taxonomic IAS survey

The IAS survey is based on traditional survey of Biological Quality Elements: phytoplankton, macrophytes, phytobenthos, macroinvertebrate fauna and fish. A list of non-indigenous species of macrophytes, macroinvertebrates and fish registered during JDS4 survey is given in Table 1.

*Table 1: Alien species detected during JDS4; *Species recorded only with additional methods than MHS (LiNi traps, K&S, hand collection), dredging, beach seine, gill nets, hand collection) and additional sites.*

Macroinvertebrates	Fish
Bryozoa *Pectinatella magnifica* (Leidy, 1851)* **Hirudinea** *Piscicola haranti* (Jarry, 1960) **Turbellaria** *Gerardia tigrina* (Girard, 1850) **Polychaeta** *Hypania invalida* (Grube, 1860) **Oligochaeta** *Branchiura sowerbyi* (Beddard, 1892) *Potamothrix moldaviensis* (Vejdovsky and Mrazek, 1902) **Bivalvia** *Corbicula fluminea* (O. F. Müller, 1774) *Sinanodonta woodiana* (Lea, 1834) *Dreissena polymorpha* (Pallas, 1771) *Dreissena rostriformis bugensis* (Andrusov, 1897) **Gastropoda** *Physella acuta* (Draparnaud, 1805) *Potamopyrgus antipodarum* (J. E. Gray, 1853) *Borysthenia naticina* (Menke, 1845) **Decapoda** *Faxonius limosus* (Rafinesque, 1817) *Pacifastacus leniusculus* (Dana, 1852)* *Procambarus clarkii* (Girard, 1852)* **Amphipoda** *Chelicorophium robustum* (G. O. Sars, 1895) *Chelicorophium curvispinum* (G. O. Sars, 1895) *Chelicorophium sowinskyi* (Martynov, 1924) *Echinogammarus ischnus* (Stebbing, 1899) *Obesogammarus obesus* (G. O. Sars, 1894) *Dikerogammarus villosus* (Sowinsky, 1894) *Dikerogammarus haemobaphes* (Eichwald, 1841) *Dikerogammarus bispinosus* (Martynov, 1925) *Synurella ambulans* (O. F. Müller, 1846) **Mysida** *Limnomysis benedenii* (Czerniavsky, 1882) *Paramysis (Serrapalpisis) lacustris* (Czerniavsky, 1882) **Isopoda** *Jaera istri* (Vieuille, 1979)	*Carassius gibelio* (Bloch, 1783) *Gasterosteus aculeatus* (Linnaeus, 1758) *Rhodeus sericeus* (Pallas, 1776) *Lepomis gibbosus* (Linnaeus, 1758) *Babka gymnotrachelus* (Kessler, 1857) *Ponticola kessleri* (Günther, 1861) *Neogobius melanostomus* (Pallas, 1814) *Neogobius fluviatilis* (Pallas, 1814) *Proterorhinus marmoraus* (Heckel, 1837) *Pseudorasbora parva* (Temminck et Schlaegel, 1842) *Ameiurus melas* (Rafinesque, 1820) *Ameiurus nebulosus* (Lesueur, 1819) *Ctenopharyngodon idella* (Valenciennes, 1844) *Hypophthalmichthys molitrix* (Valenciennes, 1844) *Hypophthalmichthys nobilis* (Richardson, 1845) *Percottus glenii* (Dybowski, 1877) *Oncorhynchus mykkis* (Walbaum, 1792) **Macrophytes** *Azolla filiculoides* Lam. *Elodea canadensis* Michx. *Elodea nuttallii* (Planch.) H.St.John *Lemna minuta* Kunth *Paspalum distichum* L. G.L.Nesom *Vallisneria spiralis* L.

The total number of non-indigenous species per taxa group recorded during JDS4 is presented in Table 2. For comparison, the number of non-indigenous taxa per quality element recorded during previous surveys (Paunović et al., 2015) is presented in the same table.

Table 2: Number of alien species per taxa group recorded during JDS4 and previous Danube Surveys; For JDS4 first number represent total number of alien taxa detected by all methods; Numbers in brackets represent no. of species detected by traditional methods and by (e)DNA IAS based detection, respectively.

Quality element	JDS1 (2001)	ADS (2004)	JDS2 (2007)	JDS3 (2013)	JDS4 (2019)
Aquatic macrophytes	3	-	6	4	6
Macroinvertebrates	12	13	20	34	35 (27/29)
Fish	-	-	14	12	17 (17/12)

In general, a rise in the no. of identified alien species was recorded on three occasions (Table 2).

The number of alien taxa of aquatic macrophytes, macroinvertebrates and fish recorded during JDS4 in the main Danube stretches is presented at Figure 4.

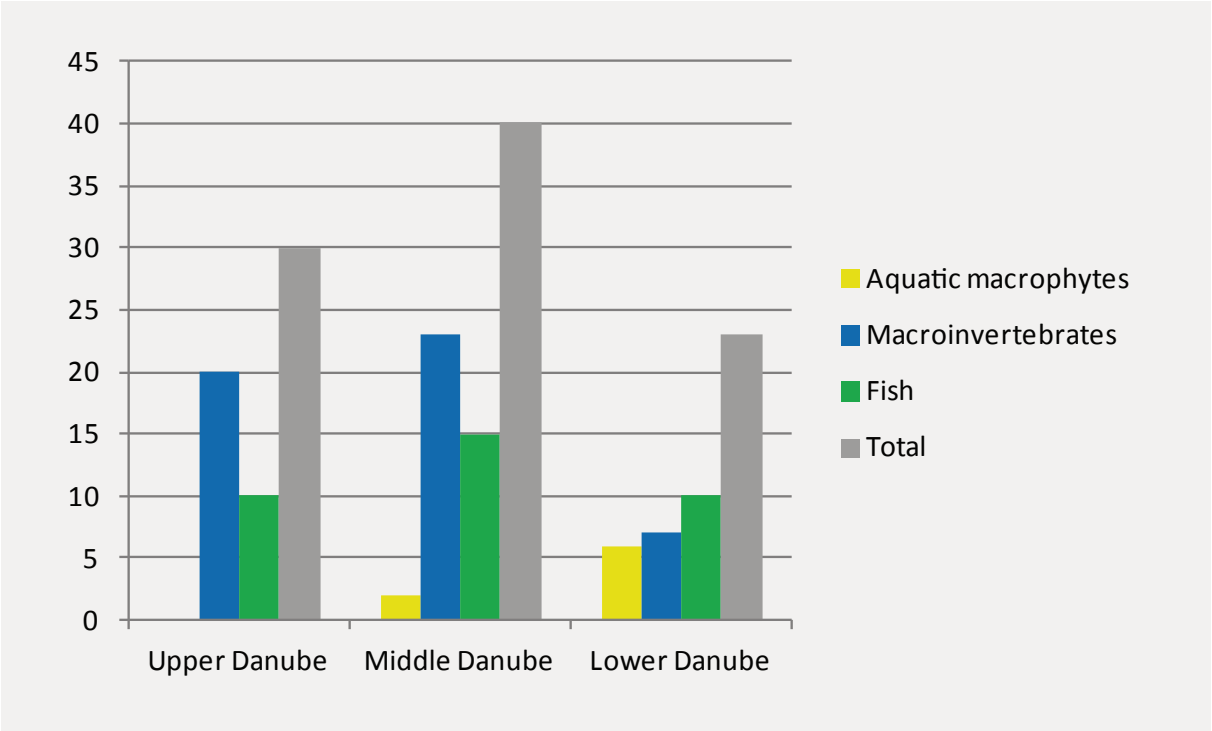


Figure 4: The number of alien taxa of aquatic macrophytes, macroinvertebrates and fish recorded during JDS4 in the main Danube stretches.

During JDS4 S. *woodiana* proved to be abundant, particularly on the Iron Gate stretch.

A significant change can be observed comparing the JDS4 data to the results of the last two JDS missions (JDS2 and JDS3) in respect to distribution of *C. fluminea*. JDS4 revealed considerable decline in the abundance of this species in part of the Middle and Lower Danube.

LiNi Crayfish trapping

LiNi Crayfish trapping revealed presence of three invasive crayfish species – *Faxonius limosus, Pacifastacus leniusculus* and *Procambarus clarkii. P. clarkii* was not detected by MHS and (e)DNA based IAS detection.

The distribution of non-native crayfish species revealed by the LiNi Crayfish collection is presented in Figure 5.

Based on data at hand, *F. limosus* is the most widespread species and is present along the entire Danube, with larger abundance in Lower Danube, while other species are limited to the Upper and Middle Danube.

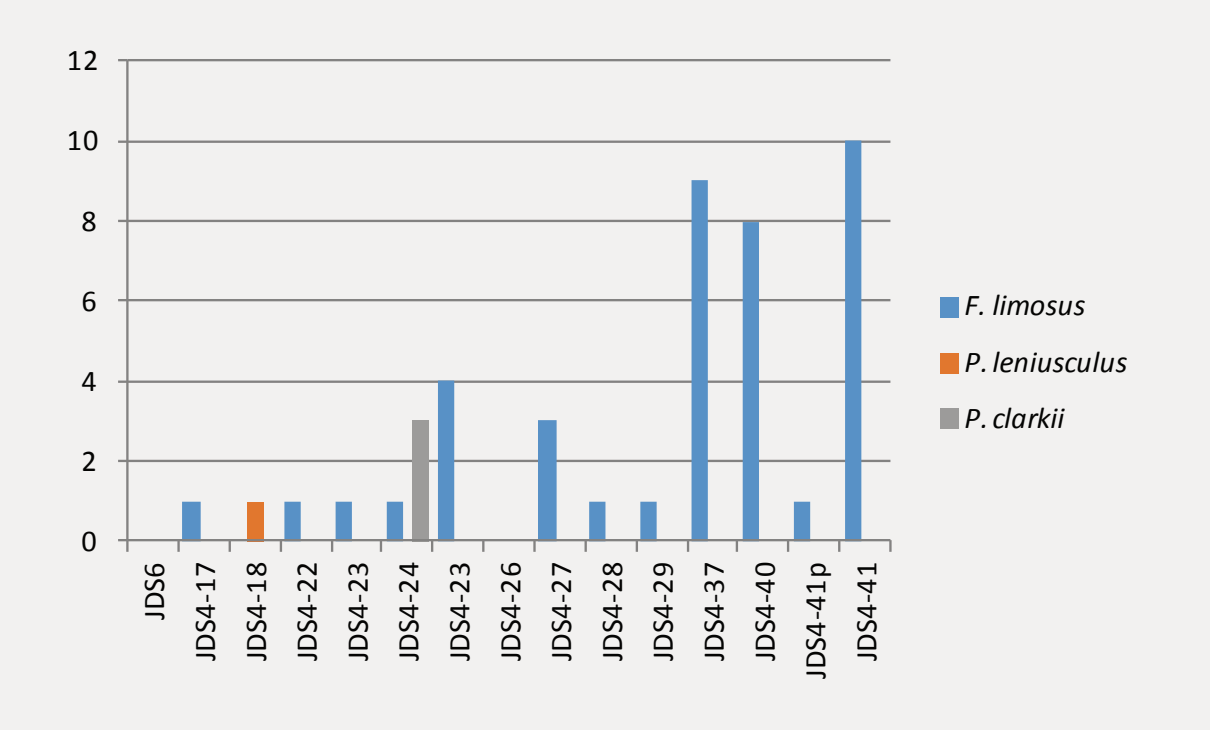


Figure 5: The distribution of non-native crayfish species.

Based on data at hand, *F. limosus* is the most widespread species and is present along the entire Danube, with larger abundance in Lower Danube, while other species are limited to the Upper and Middle Danube.

(e)DNA-based IAS detection

A total of 42 alien species have been molecularly revealed, either by their direct presence or by traces of environmental DNA - deriving from water, sediment or gut content (Table 3). Thereby, some species are identified by several sample types (sediment, bulk, eDNA water, eDNA fixative) and / or by more than a single barcode marker (COI, 12S, 18S), whereas others rely on a single report.

The most frequently observed and most widely distributed groups were fish (12 species), amphipods (11) and molluscs (6). Some rarer findings are also notable, such as the discovery of *Pacifastacus leniusculus* at site JDS4-1 or of *Katamysis warpachowskyi* at three sites.

From a methodological perspective it must be highlighted, that a few species were only identified by means of the macroinvertebrate (MZB) bulk sample (and not detected in eDNA of water or the fixative), and that some invasive alien fish species cannot reliably resolved down to the species level by all applied 12S barcode markers (e.g., *Ctenopharyngodon idella* and *Hypophthalmichthys molitrix*).

Table 3: Overview of invasive alien species discovered by molecular tools during JDS4. Except for more mobile fish species, the JDS4 sampling sites of their discovery are indicated. S = sediment analysis; B = bulk sample; eDNA-W = eDNA from water; eDNA-F = eDNA from ethanol fixative. 1 = species cannot be unambiguously identified by the applied eDNA marker at those sites, but presence is likely; UD – Upper Danube, MD – Middle Danube, LD – Lower Danube.

Group	Taxon	UD	MD	LD	Trib.	JDS4 sampling sites	marker	method
Bryozoa	Pectinatella magnifica	x	x			1, 7, 8, 11, 12, 14, 16, 19, 23, 25, 27, 29, 32, 33, 35, 37	COI, 18S	S, eDNA-W, B
Cnidaria	Cordylophora caspia		x		x	14, 16, 19, 49	COI	S, eDNA-W
Cnidaria	Craspedacusta sowerbii		x		x	11, 13, 14, 16, 20, 21, 32	COI	eDNA-W, B
Oligochaeta	Branchiura sowerbyi	x	x	x	x	6, 10, 13, 14, 17, 20, 23, 25-27, 31-33, 35-41, 48, 49	COI, 18S	S, eDNA-F, eDNA-W, B
Oligochaeta	Potamothrix moldaviensis	x	x	x	x	3, 5-8, 10, 11, 13, 14, 16-20, 22-27, 29-33, 35-41, 43, 47, 48	COI	S, eDNA-F, eDNA-W
Polychaeta	Hypania invalida	x	x	x	x	1-4, 6-8, 13, 16, 18, 19, 22-24, 26-31, 41, 48	COI	B
Bivalvia	Sinanodonta woodiana	x	x	x	x	10, 12-14, 16, 19-21, 31, 32, 35, 39, 47	COI	S, eDNA-F, eDNA-W, B
Bivalvia	Dreissena rostriformis bugensis	x	x		x	7, 10, 13, 14, 16, 19, 20	COI	S, eDNA-W
Bivalvia	Dreissena polymorpha	x	x	x	x	2, 4, 6-8, 10-14, 16, 19, 23-29, 31-34, 37, 49, 50	COI, 18S	S, eDNA-W, B
Bivalvia	Corbicula fluminea	x	x	x	x	3, 4, 6-8, 10-14, 16, 17, 19-33, 35-41, 47-49	COI, 18S	S, eDNA-W, B
Gastropoda	Physella acuta		x		x	5, 11, 13, 17, 25, 29	COI	eDNA-F, B
Gastropoda	Potamopyrgus antipodarum	x	x			1-4, 6, 10, 14, 16, 26	COI	S, eDNA-W, B
Gastropoda	Bulinus umbilicatus			x		47	COI	S, B
Mysida	Paramysis lacustris		x	x	x	16, 18, 19, 22-24, 26-28, 30, 31, 39, 40, 43, 48	COI	S, B
Mysida	Katamysis warpachowskyi		x		x	18, 19, 25	COI	B
Mysida	Limnomysis benedeni	x	x	x	x	2, 4, 7, 13, 16-19, 22, 24-31, 36, 37, 39, 40, 43, 47-50	COI	eDNA-F, B
Amphipoda	Chelicorophium curvispinum	x	x	x	x	2, 3, 6, 7, 13, 18, 19, 24, 27, 29-33, 37, 40, 43, 47, 48, 50	COI	B
Amphipoda	Chelicorophium robustum	x	x	x	x	3, 7, 8, 14, 16, 27, 29, 32, 47, 50	COI	eDNA-W, B

Group	Taxon	UD	MD	LD	Trib.	JDS4 sampling sites	marker	method
Amphipoda	Chelicorophium sowinskyi	x	x	x	x	3, 5-8, 10, 14, 16, 18, 22, 29, 32, 35, 40, 43, 47, 48	COI	eDNA-W, B
Amphipoda	Dikerogammarus bispinosus	x	x			4, 6-8, 10, 14, 16, 18, 24, 26, 27, 29	COI	eDNA-F, eDNA-W, B
Amphipoda	Dikerogammarus haemobaphes	x	x	x	x	3, 4, 6-8, 10, 16, 18, 19, 22, 24-27, 29, 32, 35, 37, 43, 47-49	COI	S, eDNA-W, B
Amphipoda	Dikerogammarus villosus	x	x	x	x	1-8, 10, 13, 14, 16-20, 22, 24-33, 37, 39, 40, 43, 47, 48, 50	COI	S, eDNA-F, eDNA-W, B
Amphipoda	Echinogammarus ischnus	x	x	x	x	4, 6-8, 10, 14, 16, 18, 22, 24, 26, 27, 29, 35, 37, 43, 47-49	COI	eDNA-F, eDNA-W, B
Amphipoda	Niphargus hrabei				x	20	COI	B
Amphipoda	Obesogammarus obesus	x	x	x	x	6-8, 16, 18, 19, 22, 24, 26, 27, 29, 30, 31, 37, 47, 48, 50	COI	eDNA-F, B
Amphipoda	Pontogammarus robustoides		x		x	40, 49	COI	B
Amphipoda	Synurella ambulans				x	34	COI	B
Decapoda	Faxonius limosus		x		x	19, 21, 25, 29, 39	COI	eDNA-F, eDNA-W, B
Decapoda	Pacifastacus leniusculus	x				1	COI	eDNA-F
Chordata	Pseudorasbora parva	x	x	x			12S	eDNA-W
Chordata	Ctenopharyngodon idella	x[1]	x	x	x[1]		12S, COI	S, eDNA-W
Chordata	Hypophthalmichthys molitrix	x[1]	x	x	x[1]		12S	eDNA-W
Chordata	Carassius gibelio	x[1]	x	x	x[1]		12S	eDNA-W
Chordata	Ameiurus nebulosus			x			12S	eDNA-W
Chordata	Ameiurus melas	x	x	x	x		12S	eDNA-W
Chordata	Lepomis gibbosus	x	x	x	x		12S	eDNA-W
Chordata	Neogobius melanostomus	x	x	x	x		12S	eDNA-W
Chordata	Neogobius fluviatilis	x	x	x		12S, COI	S, eDNA-W	
Chordata	Ponticola kessleri	x	x	x	x		12S, COI	S, eDNA-W
Chordata	Babka gymnotrachelus	x	x	x	x		12S, COI	S, eDNA-W
Chordata	Perccottus glenii		x	x	x		12S	eDNA-W
Heterocontophyta	Discostella woltereckii	x	x	x	x	1-3, 5-8, 10-14, 16, 19, 21-29, 31-33, 36, 37, 43, 47-49	18S	S

Assessment of pressures caused by biological invasions

As underlined in section Material and Methods, SBC and BAI indices were calculated using macroinvertebrate and fish data collected using conventional methods, in order to provide coherence of the information and comparability along the Danube and over the time.

According to the results of the JDS4 macroinvertebrate and fish surveys, the SBC Index indicated that majority of the sites could be characterized as highly to severely contaminated (SBC=4 and 3), while fewer sites have been characterized as moderately biocontaminated (SBC=2) or with a low level of biocontamination (SBC=1).

Mean values of the SBC Index calculated from JDS4 dataset for macroivertebrates and fish are presented in Table 4. For comparison, the SBC class values for JDS2 (2007, only for macroinvertebrates) and JDS3 (2013) are provided in the same table (Table 4). JDS3 dataset on macroinvertebrates provided SBC values of 1.53, 3.18 and 3.07, respectively (Paunović et al., 2015). JDS3 dataset on the fish data provided SBC values of 1.86, 2.17 and 3.2, respectively (Paunović et al., 2015).

SBC data shows that the pressure caused by biological invasions is generally the same if comparing the situation 2019 (JDS4) and 2013 (JDS3), but improvement is evident in comparison to data on macroinvertebrates from 2007 (JDS2).

Mean values of BAI index per the Danube main stretches are presented in Table 5.

Table 4: Mean values of SBC index for the Danube main stretches recorded during JDS4 (2019), JDS3 (2013) and JDS2 (2007); MZB refers to macroinvertebrates.

Stretch	JDS4 SBC Fish	JDS4 SBC Fish class	JDS4 SBC MZB	JDS4 SBC MZB class	SBC Class Fish 2013 – JDS3	SBC Class MZB 2013 – JDS3	SBC Class MZB 2007 – JDS2
Upper	2.56	3	3	3	4	3	4
Middle	2.56	3	2.56	3	2	3	4
Lower	1.9	2	0.86	1	1	1	3

Table 5: Mean values of BAI index for the Danube main stretches recorded during JDS4.

Stretch	BAI Fish	BAI class	BAI Macroinvertebrates	BAI class
Upper	0.38	4	0.15	3
Middle	0.14	3	0.11	3
Lower	0.02	1	0.06	1

As in the case of SBC, BAI index revealed considerable pressures caused by biological invasions, indicating moderate to poor state for the Upper and Middle Danube and good state for the Lower Danube.

Based on SBC and BAI indexes, the level of biocontamination of the Danube River was estimated as moderate to high, with higher levels for the Upper (high to severe biocontamination) and Middle Danube (moderate to high biocontamination), in comparison to the Lower Danube (low biocontamination).

The reduced pressure caused by bioinvasion recorded for the Lower Danube in comparison to the Middle and Upper sections recording during JDS4, but also during previous Danube surveys could be explained by the fact that Ponto-Caspic species are considered as native in this stretch.

The integrated biocontamination by type of water bodies and by different taxonomic groups and methods of sampling at the JDS4 and additional sites in Bulgaria ranged from moderate in the shoreline zone of the Danube River, moderate to high in the canals and lakes adjacent to the Danube River, to severe in the Danube tributaries and studied reservoirs (Table 6).

Table 6: Biocantamination of studied JDS4 and additional sites in Bulgaria during JDS4. SBC – site-specific biocontamination index; IBC – integrated biocantamination index (after Arbačiauskas et al. 2008, Panov et al. 2009); Methods applied (1) Hand net; (2) Dip net, length 100 m; (3) Dredging, (4) Beach seine, length 100m and (5) Gill nets.

Site	SBC Decapoda (1)	SBC Decapoda (2)	SBC Mollusca (3) up to 2 m	SBC Mollusca (3) 2–4.5 m	SBC Mollusca (3) 10 m^2	SBC Fish (4)	SBC Fish (2)	SBC Fish (5)	IBC
Danube River	1		2	4		1		2	2
Danube tributaries		4			4		2		4
Lakes and canals					1		4		2/3
Reservoirs					4		4		4

It would be of the great importance to design the procedure of use of the (e)DNA data in calculation of SBC and BAI indices, specifically to provide quantitative input (species relative abundance data).

Results of using 'Invasive Alien Species in Europe' mobile phone application

The results of IAS application use for the purpose of collection of the data on IAS for the DRB are presented in Trichkova (Trichkova et al., 2019). The species records, after validation, were shared through the European Alien Species Information Network (EASIN, https://easin.jrc.ec.europa.eu/easin), which is the information system in support of the implementation of the Regulation on IAS (EU Regulation no. 1143/2014; EU, 2014), becoming as such available for IAS assessments and management in Europe. For example, a total of 56 non-native specimens were recorded with the smartphone application 'Invasive Alien Species Europe' in Bulgaria.

10.4 Conclusions

As in previous surveys, JDS4 showed that the Danube River and the main tributaries are under considerable influence from biological invasions. The number of recorded alien species, values of SBC and BAI index, revealed a better situation in the Lower Danube when compared to Upper and Middle reaches, mainly since the Lower Danube can be considered as native area of distribution of Ponto-Caspian taxa, that are considered as alien in the Middle and Upper Danube.

In general, a rise in the number of identified alien species was recorded on three occasions, 2007 –>2013 –> 2019. From the other side, the SBC data show that the pressure caused by biological invasions is generally similar if comparing the situations 2019 (JDS4) and 2013 (JDS3). JDS4 and JDS3 SBC data indicates improvement in bioinvasion pressure when compared to JDS2 (2007) macroinvertebrate data. This, at first glance contrasting information, in fact indicates that many alien species are not at the same time invasive and, which is of significant importance, that assessment of bioinvasion pressure should be done on comprehensive way and should involve different data and approaches.

Although the biocontamination in some sectors of the Danube (Lower Danube) was classified from moderate to low, the IAS pressure in the Danube tributaries and the adjacent standing water bodies was much higher as some of the species find suitable habitats and establish abundant populations in these water bodies.

The (e)DNA-based detection of aquatic IAS was approved as effective and revealed the presence of a non-indigenous snail species that was not detected earlier for the Danube – *Bulinus umbilicatus*. Moreover, this method discovered the presence of four additional non-native aquatic macroinvertebrate species that were not detected by other methods during JDS4. Furthermore, it would be of great significance to use the (e)DNA data for the assessment of bioinvasion pressure based on quantitative approach.

For the first time, a smartphone application for invasive species detection was used in JDS4. The application was found to be a helpful tool that greatly facilitates the access and update of records on invasive species. It has a very broad usage, not only for public users, but also for researchers. Its broader usage may contribute to IAS awareness raising in the Danube countries and involve citizens actively in future surveys.

10.5 Acknowledgement

The Authors wish to thank Eugenio Gervasini, Kostas Tsiamis, Sven Schade, Irena Mitton and Fabiano Spinelli for collaboration in the development of the application. This collaboration was carried out in the context of the JRC proof of concept project "IAS Europe app: engaging citizens in alien species surveillance "performed in complementarity with the Institutional resources of work packages ASPEN (JRC.D.2) and CSData (JRC.B.6).

10.6 References

ARBAČIAUSKAS, K., SEMENCHENKO, V., GRABOWSKI, M., LEUVEN, R.S.E.W., PAUNOVIĆ, M., SON, M.O., CSÁNYI, B., GUMULIAUSKAITE, S., KONOPACKA, A., NEHRING, S., VAN DER VELDE, G., VEZHNOVETZ, V., PANOV, V.E., 2008. Assessment of biocontamination of benthic macroinvertebrate communities in European inland waterways. Aquat. Invasions 3. https://doi.org/10.3391/ai.2008.3.2.12

BIJ DE VAATE, A., JAZDZEWSKI, K., KETELAARS, H.A.., GOLLASCH, S., VAN DER VELDE, G., VELDE, G. VAN DER, 2002. Geographical patterns in range extension of Ponto-Caspian macroinvertebrate species in Europe. Can. J. Fish. Aquat. Sci. 59, 1159–1174. https://doi.org/10.1139/f02-098

EU, 2014. Regulation of the European Parliament and of the Council on the prevention and management of the introduction and spread of invasive alien species.

ICPDR, 2015. The Danube River Basin District Management Plan: Part A – Basin-wide overview, Update 2015. Vienna.

LIŠKA, I., WAGNER, F., SLOBODNIK, J., 2008. Joint Danube Survey 2 - Final Scientific Report.

LITERÁTHY, P., KOLLER-KREIMEL, V., LISKA, I., 2002. Joint Danube Survey Technical Report. International Commission for the Protection of the Danube River - ICPDR, Vienna.

OLENIN, S., MINCHIN, D., DAUNYS, D., 2007. Assessment of biopollution in aquatic ecosystems. Mar. Pollut. Bull. 55, 379–394. https://doi.org/10.1016/j.marpolbul.2007.01.010

PANOV, V.E., ALEXANDROV, B., ARBACIAUSKAS, K., BINIMELIS, R., COPP, G.H., GRABOWSKI, M., LUCY, F., LEUVEN, R., NEHRING, S., PAUNOVIC, M., 2010. Risk Assessment of Aquatic Invasive Species' Introductions via European Inland Waterways. Atlas Biodivers. risk. Pensoft, sofia. ISBN 954–978.

PANOV, V.E., ALEXANDROV, B., ARBAČIAUSKAS, K., BINIMELIS, R., COPP, G.H., GRABOWSKI, M., LUCY, F., LEUVEN, R.S., NEHRING, S., PAUNOVIĆ, M., SEMENCHENKO, V., SON, M.O., 2009. Assessing the risks of aquatic species invasions via european inland waterways: From concepts to environmental indicators. Integr. Environ. Assess. Manag. 5. https://doi.org/10.1897/IEAM_2008-034.1

PAUNOVIĆ, M., CSÁNYI, B., 2018. Guidance document on Invasive Alien Species (IAS) in the Danube River Basin, ICPDR.

PAUNOVIĆ, M., CSÁNYI, B., SIMONOVIĆ, P., ZORIĆ, K., 2015. Invasive Alien Species in the Danube, Handbook of Environmental Chemistry. https://doi.org/10.1007/698-2015-376

PAUNOVIĆ, M., CSÁNYI, B., STANKOVIĆ, I., GRAF, W., LEITNER, P., BRAMMER, V., HUBER, T., SZEKERES, J., BORZA, P., 2015. Invasive Species, in: Liška, I., Wagner, F., Sengl, M., Deutsch, K., Slobodník, J. (Eds.), Joint Danube Survey 3: A Comprehensive Analysis of Danube Water Quality. International Commission for the Protection of the Danube River - ICPDR, Vienna, pp. 140–147.

TRICHKOVA, T., TODOROV, M., BOTEV, I., KENDEROV, M., HUBENOV, Z., 2019. Study and assessment of ecological status of the Bulgarian sector of the Danube River in the frame of the Joint Danube Survey (JDS4) in 2019 Task: Invasive Alien Species. Sofia.

TSIAMIS, K., GERVASINI, E., D'AMICO, F., IVAN, D., ROGLIA, E., SHADE, S., MASSIMO, C., CARDOSO, A.C., 2017. Citizen science application, Invasive Alien Species in Europe. Ispra. https://doi.org/10.2760/043856

11 Zooplankton

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Abstract

Zooplankton (Rotifera, Cladocera, Copepoda) assemblages collected from JDS4 sampling sites in summer 2019 were identified and analysed. The study and the analyses of the samples from the Danube and selected tributaries revealed: (i) differences in the composition and density of zooplankton assemblages from Upper to Lower Danube; (ii) density differences in the river profile and differences between the Danube and selected tributaries; (iii) current status of the allochthonous zooplankton species in the Danube area; (iv) comparison of the results with the zooplankton results of former JDS expeditions.

157 taxa /118 Rotifera, 21 Cladocera, 18 Copepoda/ were identified from 39 JDS4 sites (27 from the Danube and 12 from tributaries). Opposite to previous JDS results, high Rotifera species richness was observed at the Upper Danube and there was no longitudinal trend of zooplankton abundance along the Danube. During the four JDS the species richness of Rotifera gradually increased indicating the importance of cross-sectional sampling and the improving ecological conditions of the Danube River. Compared to JDS1-3, the density of zooplankton in JDS4 sites was very low (3.29 ind. l^{-1}), and the maximum values were observed in the Upper Danube.

11.1 Introduction

Zooplankton includes a wide range of animals, especially microscopic, from primitive protozoans to the larvae of more complex animals. This group plays an essential role in aquatic ecosystems. These organisms serve as intermediary species in the food chain, transferring energy from planktonic algae (primary producers) to larger invertebrate predators and fish who in turn feed on them. Zooplankton species commonly consume phytoplankton, other zooplankton and detritus and control algae blooms by increasing zooplankton grazing.

Many studies show that zooplankton (sampled from water and sediment) is of strong value as an indicator and is useful as an indicator of ecological status (Jeppesen et. al. 2011). Effects of environmental disturbances can be detected through changes in species composition, abundance and body size distribution.

There are many investigations (e. g. review paper of Naidenow 1998) which deal with the zooplankton assemblages in different section of the Danube. Naidenow (1998) summarized the qualitative and quantitative aspects of Danube zooplankton in a comprehensive work, based on the results of 164 studies and 343 Rotifera and 145 Crustacea species were mentioned from the whole section of the Danube. According to these results the typical zooplankton communities of the Danube consist of mainly rotifers and high proportion of nauplius and copepodit larvae and the most frequent species are *Brachionus calyciflorus, Keratella spp., Synchaeta spp., Bosmina longirostris, Thermocyclops crassus* and *Acanthocyclops robustus*. These taxa are typical of still or slow-flowing eutrophic waters. The number of the approximately

simultaneous investigations in the whole Danube River, are limited (Bothár 1974, Pujin 1990; Naidenow & Schewzowa 1991 and within the framework of JDS: Gulyás, 2002, Zsuga 2008, 2014).

11.2 Methods

The three main characteristic groups of zooplankton, Rotifera, Cladocera and Copepoda were investigated in detail. During the sampling campaign 85 zooplankton samples were collected from 39 JDS4 sites (27 from the Danube and 12 from tributaries /JDS4-11: Dyje, JDS4-12: Morava, JDS4-17: Mosoni Danube Arm, JDS4-25: Ráckevei-Soroksári Danube, JDS4-30: Drava, JDS4-32: Tisza, JDS4-33: Tisza mouth, JDS4-35: Sava, JDS4-36: Sava mouth, JDS4-38: Velika Morava, JDS4-39: Velika Morava mouth, JDS4-49: Prut). 18 river profile samples (left, middle and right side of the Danube) were investigated to explore the differences in the river profile with reference to zooplankton. The method of sampling was similar to the previous JDS expeditions, a total of 100 litres water was filtered through plankton net (50 or 40 µm mesh size) and the samples were preserved with formaldehyde to 4-5 % concentration. The quantity and qualitative composition of zooplankton was determined with both light- and stereomicroscopes, and density was estimated in ind./L unit. For the exact identification of some Rotifera species their trophi were prepared using sodium hypoclorite solution. The developmental stages of Crustaceas were also counted and included to total density.

11.3 Results and Discussion

11.3.1 Zooplankton species composition

During the survey 157 taxa /118 Rotifera, 21 Cladocera, 18 Copepoda/ were identified. Most of the species were of planktonic life form, but a number of tychoplanktonic elements were also identified. They were washed to the plankton from aquatic plant environment or from the surface of the sediment through mud-mixing. Most of the taxa (49.5 %) occurred only in one or two sampling sites and in case of 31 taxa only one specimen was found in the JDS4 sites.

Similar to previous JDS results, the characteristic planktonic species of the Danube were the most abundant with high relative frequencies: *Brachionus angularis* (54.11 %), *Brachionus calyciflorus* (57.64 %), complex of *Synchaeta oblonga/tremula* (63.52 %), *Keratella* spp. (49.41%), as well as among Crustacea zooplankton, *Acanthocyclops robustus* (69.41 %) and *Thermocyclops crassus* (49.41 %). A limited number of studies is dealing with the occurrence of *Bdelloidea* family in the Danube, nonetheless their relative frequency was high (63.52 %) in JDS4 sites.

The majority of the rare species examined in the study area were found close to the riverbank or tributaries. The rare *Encentrum wisniewskii* prefers sandy habitats and some taxa are phytophilous, they prefer macrophyte beds (*Dicranophorus, Lecane, Trichocerca, Graptoleberis testudinaria, Eucyclops macrurus, Macrocyclops albidus*). Some specimens of the largest planktonic cladoceran, *Leptodora kindtii* and one specimen of the very rare *Halicyclops* taxon were found in the Serbian stretch of the Danube, as well as the rare cladoceran, *Pleuroxus trigonellus* in Danube Delta (JDS4-51).

Compared to previous JDS results, the density of *Brachionus forficula* increased in the Danube, it was collected at 16 JDS4 sites. *Brachionus bidentata* has never been found in JDS sites in the past, but now it was collected from nine JDS4 sites. Both rotifers are warm stenothermic species, typical of subtropical and tropical regions. The density and relative frequency of the thermophilous *Moina brachiata* (Cladocera) and *Thermocyclops crassus* (Copepoda) increased as well in the whole Danube River basin. The occurrence of those species indicates climatic changes and increased temperature in the catchment area of the Danube.

The zooplankton species richness varied between 0 (JDS4-18-L, JDS4-23-M, JDS4-23-R) and 21 (JDS4-2) in the Danube and between 2 (JDS4-25-M) and 34 (JDS4-17-R) in the tributaries. The average number of taxa was 9.65 for rotifers and very low for crustaceans (Cladocera: 1.64, Copepoda: 1.96). The species richness was high (more than 20 taxa) at seven JDS4 sites: there are four in the Danube (JDS4-2 /Bittenbrunn/, JDS4-3 / above Klösterl/, JDS4-6 / Jochenstein/, JDS4-40 /Banatska Palanka/Bazias) and three in tributaries (Mosoni Danube Arm, Ráckevei-Soroksári Danube, Velika Morava mouth (JDS4-39-M).

Opposite to previous JDS results, high Rotifera species richness was observed in the Lower Danube (JDS4-2: Bittenbrunn 700m below power station, JDS4-3: above Klösterl – Kelheim, JDS4-6: Jochenstein). The rotifer taxon richness decreased significantly in the lower part of the Danube basin from the JDS4-41 site. In the case of planktonic Crustaceans, there was no longitudinal trend in the number of taxa. The selected tributaries had no effects on the species richness in the Danube.

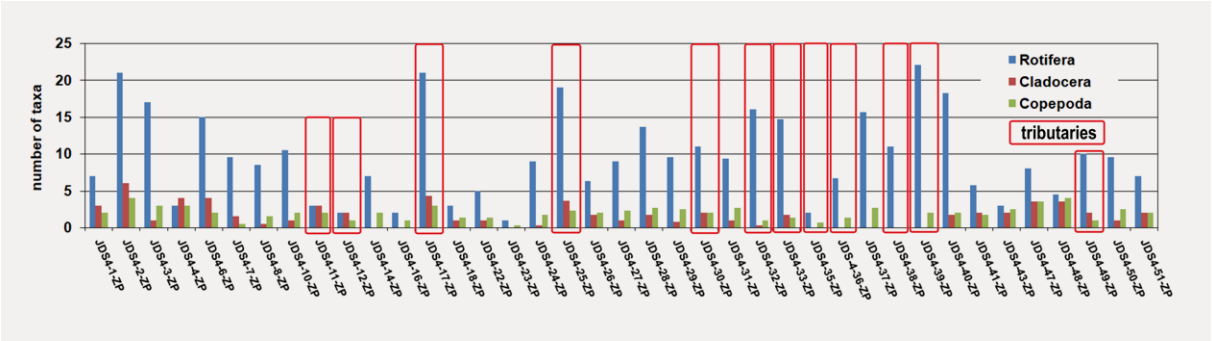

Figure 1: The species richness in the Danube and the investigated tributaries (in red rectangles) /in case of river profile samples average values were indicated/.

11.3.2 Density of zooplankton assemblages in the Danube and the selected tributaries

The density of assemblages varied between 0 (JDS4-18-L, JDS4-23-M, JDS4-23-R) and 46.27 (JDS4-2-M) ind. l^{-1} in the Danube and 0.02 (JDS4-35-M) and 93.10 (JDS4-25-R) ind. l^{-1} in the tributaries. In most of the sampling sites the zooplankton density was low, less than 3 ind. l^{-1}. In three JDS4 sites relatively high density values (but less than in previous JDS investigations) were observed, because of massive occurrence of the rotifer species: *Synchaeta oblonga/tremula* complex in Bittenbrunn, below the power station, *Lecane bulla* in downstream Budapest and three *Synchaeta* species (*S. longipes, S. oblonga/tremula, S. pectinata*) in the cross-section of Tass (JDS4-25). In the rotifers community, a high ratio of *Synchaeta tremula* and *S. oblonga* or *Lecane bulla* indicates eutrophic conditions of the river.

The average density of zooplankton was very low (3.29 ind. l^{-1}) at JDS4 sites and the density of the two groups were nearly similar and very low in the Lower Danube. The density values of assemblages were also low in the tributaries, except the Ráckevei-Soroksári Danube.

Opposite to previous JDS results there was no longitudinal trend of zooplankton abundance along the Danube. Unexpectedly, the peak densities in the Danube were recorded in the Upper Danube, from the first three JDS4 sites. This irregular trend in the density pattern of assemblages could have been caused by unstable hydrological conditions due to the high water-levels before and during sampling in JDS4 sites (see also in Phytobenthos chapter).

There was strong positive relationship between the density of rotifers and crustaceans ($y = 0.037x + 14.11$, $R^2 = 0.717$) indicating absence of direct competitive effects between the two groups. This relationship is usually negative in freshwater ecosystems, possible explanations of this pattern could be the unstable hydrological conditions during the JDS4 and the low-density values in the whole river.

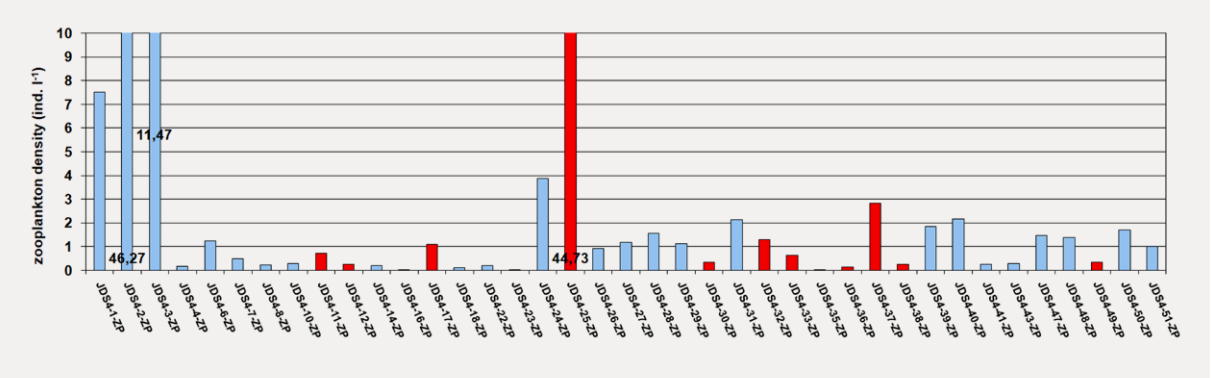

Figure 2: Density of zooplankton assemblages in the longitudinal profile of the Danube (blue) and the tributaries (red) /in case of river profile samples average values were indicated/.

11.3.3 Differences in the river profile

18 river cross-section samples (left, middle and right side of the Danube) were investigated to explore the possible zooplankton differences in the river profile.

The taxon richness of rotifers was a little bit higher in the middle of the Danube and showed a similar range in the tributaries. The number of taxa was significantly lower in the Sava River (JDS4-35) in all three profiles indicating the inadequate ecological status of the river (see also in Phytobenthos chapter). At the Sava mouth (JDS4-36) the species richness was notably lower on the right side compared to the left side presumably because of the urbanization effects of Belgrade. The species richness of Crustaceans was low and approximately similar in all three profiles in the Danube and there were more species in the left and right side of the tributaries owing to the better habitat conditions in the riverbank.

Regard to density values, rotifers were more abundant in the middle of the Danube and the right side of the tributaries. These difference are especially significant in the Ráckevei-Soroksári-Danube (L: 10.7, M: 2.9, R: 89.6 ind. l^{-1}). The Crustacean density was very low in all three cross-section of the Danube and the density was higher in the right side of the tributaries.

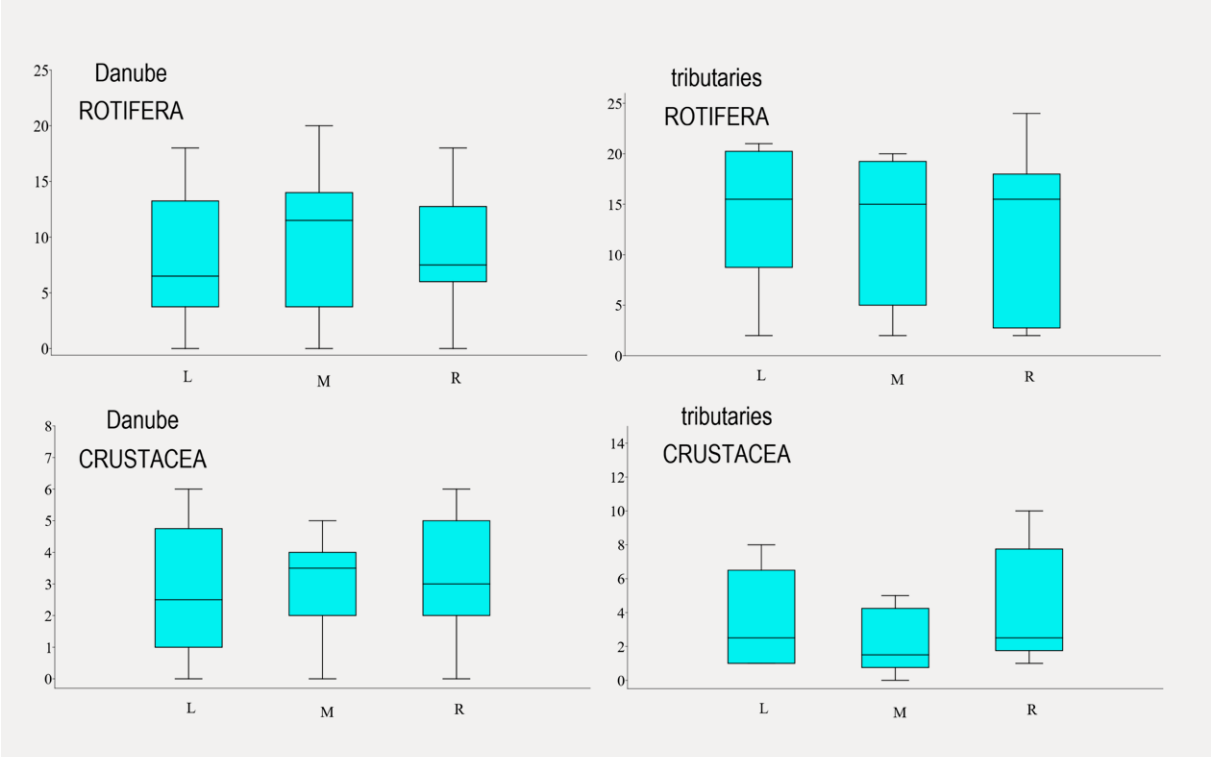

Figure 3: The species richness of Rotifera and Crustacea in the river profile, left, middle and right side of the Danube and the tributaries.

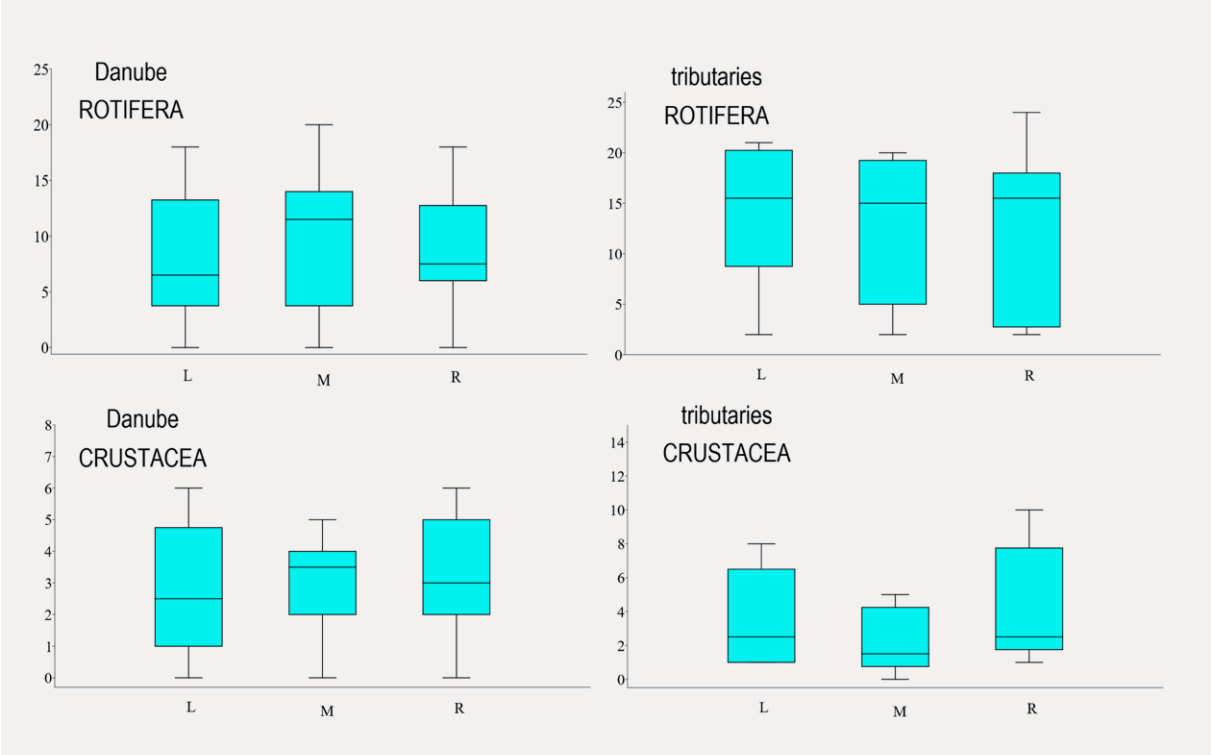

Figure 4: The density (ind. l^{-1}) of Rotifera and Crustacea in the river profile, left, middle and right side of the Danube and the tributaries.

11.3.4 Alien zooplankton species

During the JDS4, three allochtonous species were detected: *Pleuroxus denticulatus* (Cladocera), *Eurytemora affinis* and *Eurytemora velox* (Copepoda). Compared to previous JDS results the relative frequency increased in case of all three species. *Pleuroxus denticulatus* was introduced from North-America, and has occurred in Europe since the 1970s. Its occurrence in the Danube probably corresponded with the junction of the Danube and Rhine Rivers (Hudec & Illyová, 1998). Now, this species is widely distributed in the catchment area of the Danube.

The euryhaline *Eurytemora velox* and *affinis* are originally saltwater species migrated from the estuaries of the North Sea upstream of many rivers and from the Black Sea upstream in the Danube River (Tollinger, 1911). The freshwater occurrence of *E. velox* is common in the catchment area, during JDS4 E. *affinis* was detected exclusively in the Lower Danube, from the JDS4-40 to the Black Sea.

11.3.5 Comparison of the results with the previous JDS zooplankton results

Differences between the four JDS investigations are summarized in Table 1. The recorded taxon richness of Rotifera was gradually increased in spite of the decreasing number of sampling sites. During all four JDS the assemblages were dominated by rotifers and copepods, Cladocera populations were less abundant. The ratio of copepod nauplii and copepodites larvae was high, except during JDS4. The number and relative frequency of alien species were highest in JDS4.

The longitudinal changes in species richness and density were about similar in the previous JDS investigations, but completely different in JDS4. The richness and abundance of species were the highest in the Hungarian and Serbian stretch in JDS1-JDS3 and in the Upper Danube in JDS4.

The peak densities were significantly higher (around 1000 ind. l^{-1}) in JDS1, the very high-density values in the Serbian section were indicating the eutrophic, polytrophic condition of the Danube. The average and maximum density decreased equally in JDS4 and these parameters were the highest in the Upper Danube.

The trends were similar in the river profile in JDS3 and JDS4 indicating significantly higher density of rotifers in the middle sections. Opposite to JDS1 there were no detectable effects of tributaries on species richness and density of zooplankton assemblages in the Danube.

Table 1: Summary of the zooplankton results of JDS1 (Gulyás 2002), JDS2 (Zsuga 2008), JDS3 (Zsuga 2014) and JDS4.

	JDS1	JDS2	JDS3	JDS4
sites	98	96	53	39
samples	98	96	159	85
species richness	**120 taxa** 79 Rotifera 27 Cladocera 14 Copepoda	**126 taxa** 87 Rotifera 30 Cladocera 9 Copepoda	**149 taxa** 107 Rotifera 33 Cladocera 9 Copepoda	**157 taxa** 118 Rotifera 21 Cladocera 18 Copepoda
alien species	*Eurytemora velox*	*Pleuroxus denticulatus* *Eurytemora velox*	*Eurytemora velox*	*Pleuroxus denticulatus* *Eurytemora velox* *Eurytemora affinis*
density /ind. l^{-1}) minimum and maximum values	Danube: 0.28-1383.6 tributaries: 1.14-799.6	Danube: 0-341.2 tributaries: 0-296.2	Danube: 0.33-353.5 tributaries: 0-54.44	Danube: 0-46.27 tributaries: 0.02-93.10
longitudinal trends	1. species richness and density: gradual increase in the downstream direction, 2. density peak at Serbian reach, 3. from 1161 rkm (downstream Pancevo) the zooplankton density drastically decreased	1. density peak in the Serbian stretch, 2. low density in the Upper and Lower Danube sections	1. density and taxon maxima at Serbian section, 2. high density values, approximately four times higher than JDS2, 3. low density in the Upper and Lower Danube sections	1. there was no longitudinal trend, 2. irregularly high species richness at the Upper Danube, 3. peak densities were recorded in the Upper Danube, 4. low density in the Lower Danube
river profile	not investigated	not investigated	higher rotifer density in the middle section	higher rotifer density in the middle section
effects of tributaries	yes, especially the Tisza	no effects, except the Morava	no significant effects	no significant effects

11.4 Conclusions

157 taxa - 118 Rotifera, 21 Cladocera, 18 Copepoda - were identified in the zooplankton sampling sites of JDS4. Most of the species are of planktonic life form, but a number of tychoplanktonic elements were also identified. A significant part of the taxa (49.5 %) occurred only in one or two sampling sites. Some of them are rare species in the Danube catchment like *Encentrum wisniewski, Brachionus bidentata, Leydigia leydigi, Pleuroxus trigonellus* and *Halicyclops* sp.

The abundance of the three detected alien zooplankton species gradually increased during the four JDS. Compared to previous JDS results *Eurytemora affinis* occurred in the Lower Danube. The increased relative frequency of thermophilous species could be linked to climatic changes in the catchment area. The recorded taxon richness of Rotifera gradually increased indicating the importance of cross-sectional sampling and the improving ecological conditions of the Danube River. Similar to JDS3, there were differences in the river profile indicating significantly higher density of rotifers in the middle sections and there were no detectable effects of tributaries on species richness, nor the density of zooplankton assemblages in the Danube.

The average density of zooplankton in JDS4 sites was very low (3.29 ind. l^{-1}), the average density of rotifers more than ten times higher than the density of crustaceans. The longitudinal changes of the species richness and abundance of planktonic zooplankton were different in the JDS4 when compared to JDS1-3, the maximum values were observed in the Upper Danube. These results could be explained by the unstable hydrological conditions due to the high water-levels before and during sampling. These trends also indicate that the Danube, as a large river with its tributaries and floodplains is a very heterogeneous system with highly variable hydrological dynamics, flood events, floodplains, water level fluctuations and hydrological connectivity.

The results of JDS4 confirm that zooplankton species richness and abundance can be a good indicator of trophic status and river conditions (see nutrients data in General physico-chemical determinands and nutrients chapter).

11.5 References

BOTHÁR, A. (1974): Horizontale Planktonuntersuchungen an der Donau von Rajka bis Turnu Severin. (Stormkm 1850-930). (Danubialia Hungarica LXVIII). Annal. Unic. Sci. Budapest., 16: 157-162.

GULYÁS, P. (2002): Qualitative and quantitative investigations of Rotatoria and Crustacea plankton in the River Danube. (In Hungarian.) Vízügyi Közlemények, 84: 601–620.

HUDEC I. & ILLYOVÁ M. (1998): Pleuroxus denticulatus (Crustacea: Anomopoda: Chydoridae): a new invader in the Danube Basin. Hydrobiologia, 368: 65-73.

JEPPESEN, E., NÔGES, P., DAVIDSON, T. A, HABERMAN, J,. BLANK, K., LAURIDSEN, T. L. et al. (2011): Zooplankton as indicators in lakes: a scientific-based plea for including zooplankton in the ecological quality assessment of lakes according to the European Water Framework Directive (WFD). Hydrobiologia, 676: 279-297.

NAIDENOW, 1998: Das Zooplankton der Donau. In: Kusel-Feztmann R., W. Naidenow, B. Russev: Plankton und Benthos der Donau – IAD Ergebnisse der Donau-Forschung. Band 4.

NAIDENOW,W. & SCHEWZOWA, L. (1991): Über die Verteilung des Metazoenplanktons der Donau zwischen Str. km 20 und 1928. Hydrobiology (Sofia), 37: 15-23.

PUJIN, V. (1990): Zur Kenntnis des Donauplanktons aufgrund der Untersuchung bei der Internationalen Donauexpedition 1988. Ergebnisse der Donauexpedition 1988, Eigenverlag der IAD, Wien, pp. 199–201.

TOLLINGER M. A. (1911): Die geographische Verbreitung der Diaptomiden und anderer Süss- und Brackwasser-Gattungen aus der Familie der Centropagiden. Zoologische Jahrbücher, Abteilung für Systematik, Ökologie und Geographie der Tiere, 30: 1–302

ZSUGA K. (2008): Zooplankton In: Liska, I., Wagner F. and Slobodník J (eds.): Joint Danube Survey 2 Final Scientific Report, ICPDR – International Commission for the Protection of the Danube River, 82-85. http://www.danubesurvey.org/jds2/files/ICPDR_Technical_Report_for_web_low_corrected.pdf

ZSUGA, K (2014): Zooplankton. In: Igor Liška, Franz Wagner, Manfred Sengl, Karin Deutsch and Jaroslav Slobodník (eds): Joint Danube Survey 3. A Comprehensive Analysis of Danube Water Quality. ICPDR – International Commission for the Protection of the Danube River,

12 Introduction: (e)DNA-based activities

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Abstract

With JDS4, genetic methods were included in the extensive analytical program of the Joint Danube Survey for the first time. The fish community of the Danube, along with its macrozoobenthos, phytobenthos and sediment community, were investigated using different DNA- or environmental DNA (eDNA)-based metabarcoding approaches. The aim was to test the different (e)DNA-based approaches in a real-world, international and highly integrative setting. This chapter introduces the basics of (e)DNA metabarcoding and provides an overview of the individual organism group-specific approaches pursued during JDS4. It concludes by highlighting the importance of well-curated barcode sequence reference libraries and the potential of biobanking samples.

12.1 Introduction

With JDS4, for the first time, (e)DNA-based approaches were integrated into the program of the JDS, focussing on the three biological quality elements (BQEs) of fish, macrozoobenthos and phytobenthos, and additionally sediment fauna (including macrozoobenthos and meiofauna) (Figure 1). Some of the reasons for introducing such genetic methods into the survey programme are:

a) Organisms can be identified down to species level. If reference sequences are available, this identification links to a classical Linnaean name. If no reference sequences are available, still identifications can be made to higher taxonomic levels like genus or family, or in any case, unique molecular species pendants created (so-called MOTUs, see below). Although the latter have no Linnaean species annotation, they are unique and can be analysed within and compared between datasets.

b) Taxonomic information can be unlocked even in cases where morphotaxonomic knowledge and expertise are limited,

c) All developmental stages (e.g. larvae, eggs), cryptic species, indeterminable sexes as well as body fragments (roots, legs, exuviae) can be readily identified; using metabarcoding, one can also analyse the invisible eDNA traces left by organisms in their environment,

d) Taxalists relying on sequence information are objective, reproducible and comparable (aspects, which are particularly important for a longitudinal survey involving many countries), and,

e) Overall, this additional line of taxonomic evidence helps to get a more precise and comprehensive picture of the Danubian biota.

In JDS4, the methodology of DNA metabarcoding was applied for the three aforementioned BQEs and the sediment fauna. In this approach, DNA is isolated from a sample and an organism group-specific but universal primer pair used to mass-amplify a target barcode marker during polymerase chain reaction (PCR). As such, the final PCR output volume contains the parallel amplified barcode marker templates present in the sample. In a subsequent step, those DNA molecules are subjected to high-throughput sequencing (HTS), rendering them bioinformatically readable. The generated sequences are quality-filtered, clustered into Molecular Operational Taxonomic Units (MOTUs) based upon sequence identity and those MOTUs compared to taxonomically annotated reference sequences stored in barcode databases. Usually, validated taxonomic annotation for the deposited reference sequence has been previously achieved by individually sequencing a morphologically identified voucher specimen, which is then stored in museum or institutional collections for future reference. In the final step, sequence-based taxalists are produced by comparing all MOTUs with the reference database. Thereby, species-, genus-, family- and order-level matches rely upon pre-defined genetic similarity thresholds (Taberlet et al. 2012). DNA extracts resulting from the analysis are then stored for future reference.

Although all the applied DNA metabarcoding approaches share the common element of compiling sequence-based taxonomic lists for a collected sample by matching genetic sequences of MOTUs with reference databases, it is noteworthy to further introduce some central terms and conceptual decisions in detail as they can vary between laboratories and the different organism groups / BQEs analysed (whose details are outlined in the respective chapters).

12.1.1 Sample type

A sample is defined as the material from which the DNA for subsequent genetic analysis is extracted. If, e.g., brushed phytobenthos samples or macrozoobenthos kick-net samples are collected for simultaneous DNA isolation, the sample is called a "bulk sample", which comprises many specimens of mixed identity ("BS", Figure 1). In contrast, DNA can also be directly collected and isolated from the environment without the need for individual specimen or bulk sample collection (so called environmental DNA, or eDNA). If, for example, water is collected and filtered for the analysis of fish, the sample type is commonly referred to as "eDNA water" ("eDNA", Figure 1). For the approach of eDNA metabarcoding water samples, fish community composition is reconstructed based on analysing the intra- and extracellular (floating and particular-bound) DNA molecules shed by the fish community into the water body. Likewise, when taking sediment cores for the analysis of the benthic fauna, this refers to the sample type "eDNA sediment". A more complicated scenario emerges when bulk samples are collected, but the preservation liquid or fixative (often >95% ethanol) is analysed, without homogenizing the bulk sample. This sample type might be defined as "eDNA ethanol", either originating from the collection (1st phase; or fixative) or storage ethanol (2nd phase; or preservative) ("PL", Figure 1).

12.1.2 Barcode marker

The standardised genetic fragment used for molecular species identification in a DNA metabarcoding context is commonly referred to as the barcode marker or barcode fragment (Kress et al. 2015, Taberlet et al. 2012). Sometimes the term marker gene is used, but strictly speaking, the barcode marker for metabarcoding a) usually is too short to cover a full gene and b) does not have to have the characteristics of a gene, i.e. protein or RNA encoding. Furthermore, even when the same marker gene is indicated (e.g. COI, 18S), the selection of different primer pairs can result in barcode markers of different lengths, with different areas of coverage of the marker gene and hence can produce variation in taxonomic resolution. The selection of an appropriate barcode marker is therefore of great importance.

12.1.3 Primer selection

The barcode marker is amplified by a specific primer pair. As important as the choice of a taxonomically informative barcode marker is the selection of a suitable primer pair generating the barcode marker. Thereby, a balance must be achieved between barcode marker coverage (=are enough reference sequences available? Are they public?), primer pair efficiency (=are target taxa sufficiently well amplified?) and diagnostic resolution (=are the generated barcodes able to discriminate the target species?). As such, the same marker gene (e.g. COI) can encompass multiple barcode markers, which are amplified by group-specific primer pairs. In case specific target groups are systematically over- or underrepresented in the metabarcoding sequence read output, or do not amplify at all, this is referred to as "primer bias" (Elbrecht & Leese 2015).

12.1.4 MOTU

A Molecular Operational Taxonomic Unit (MOTU) can be considered as an alternative operational classification system to a Linnaean species. A MOTU is generated when similar genetic sequences are bioinformatically clustered. Depending on a percentage similarity threshold, only almost identical (e.g. >99%) or very similar sequences (e.g. >97%) are clustered into a single MOTU. Alternatively, MOTUs can be more flexibly clustered based on the frequency distribution of closely related sequences in the dataset (Mahé et al. 2015), or, in special cases, each unique sequence can be considered a separate MOTU. The latter are also known as zero-centroid MOTUs or Exact / Amplicon Sequence Variants (ESVs / ASVs) (Callahan et al. 2017). Similar to Linnaean species, also MOTUs can be regarded as a stand-alone taxonomic classification system, but most often the consensus sequence of each MOTU is compared to a barcode reference library using Linnaean classification as a backbone. This results in a taxonomic list including Linnaean species, genera and families, but inferred from genetic data and thus sequence-based.

12.2 Overview of (e)DNA-based activities in JDS4

The (e)DNA-based activities during JDS4 were coordinated by the DNAqua-Net consortium (Leese et al. 2016) targeting the BQEs fish, MZB and diatoms based on different sample types collected by the national teams and two mobile eDNA teams. Furthermore, the benthic fauna from sediment samples was molecularly investigated (Figure 1).

The BQE fish was molecularly surveyed via eDNA water, collected by two eDNA teams. The first eDNA team travelled in downstream direction and sampled in the main channel of the Danube as well as in various tributaries. Since the sampling campaign lasted several weeks, a second eDNA team was installed ensuring the temporal sampling overlap with the parallel activity of effect-based tools / non-target analytics in the Lower Danube. The second eDNA team started at site JDS51 and travelled in upstream direction sampling at eight sites. Additional eDNA samples were collected by cooperating projects (Interreg MEASURES, IAD and VigiLIFE) before and after the JDS4 eDNA survey. For the selection of JDS4 eDNA sampling sites, special emphasis was laid to cover as many of the nominated JDS4 sites as possible, therefore increasing the benefit of the integrative JDS4 setting and additional environmental parameters to be collected. However, eDNA sampling sites within the main channel of the Danube also needed to respect a certain longitudinal distance (~100 km) and had to avoid a proximity too close to major confluences. The sampling design was further coordinated with the microbiology team. Species lists were generated by sequencing 12S barcode markers.

It was planned to molecularly assess the BQE macrozoobenthos using three sample types: eDNA water, DNA from bulk samples and eDNA from the ethanol used as a preservation liquid. Although eDNA water samples were successfully collected by the 1st eDNA longitudinal special sampling team and their DNA isolated, their timely analysis was not possible due to the emerging COVID-19 pandemic situation in early 2020, which led to the closing of laboratories and very restricted working environments. The bulk samples for molecular analyses were obtained by Multi-Habitat Sampling (MHS) of 20+1 subsamples, carried out by the national teams in parallel to the traditional MHS for morphological investigation. If present, underrepresented habitats were sampled with an additional 21st sample. Further genetic sequence information was integrated as a result of an additional eDNA water sampling taking place in Slovakia. Species lists were generated by sequencing different COI barcode markers.

Benthic diatoms were molecularly investigated by taking two brushed samples for each JDS4 site (left and right riverbank), and for all sites where sampling was possible (i.e. presence of suitable substrate, safe entrance to the river, etc.). Species lists were generated by sequencing a 18S as well as a rbcL barcode marker.

The diversity of benthic organism groups (e.g. meiofauna) traditionally not included in biomonitoring was the primary focus of the (e)DNA sediment analyses. Such groups include, among others, nematodes, water mites, ciliates and other protists. The diversity of these groups was molecularly targeted by studying the hypervariable regions (e.g. V1-V2, V4, V9) of the 18S gene and COI. Furthermore, sediments also include (e)DNA signals for other, traditionally used groups such as Crustacea, Insecta, Oligochaeta – so that further species could be added to the site-specific and overall taxa lists. The majority of the official JDS4 sampling sites were investigated for their benthic community.

Finally, a small ring test was performed on the (e)DNA samples available originating from Austrian and Slovenian sites where (e)DNA samples were taken. Those comprised eDNA water samples analysed for fish and MZB, MZB multi-habitat samples analysed as bulk samples and as preservation liquids, as well as sediment samples. As stated above, sample processing in the ring test was also impacted by the COVID-19 outbreak.

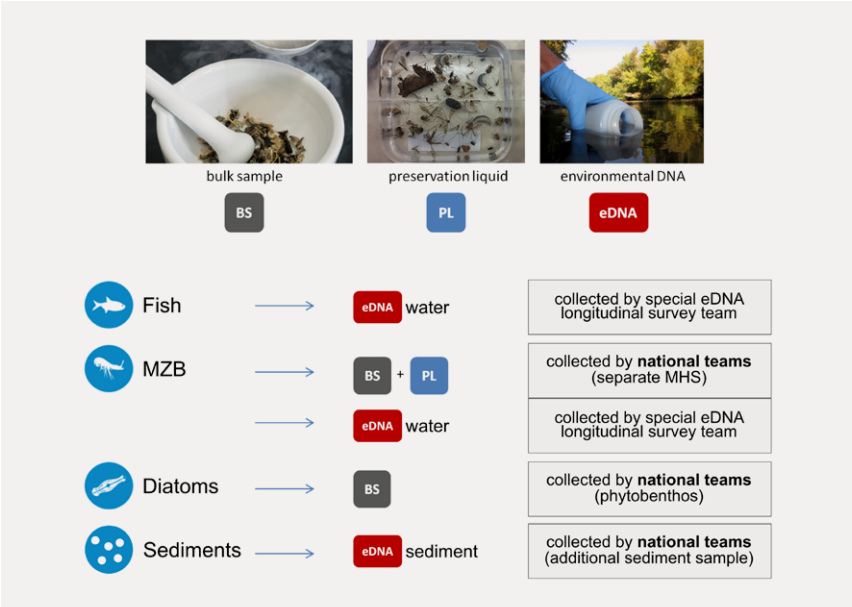

Figure 1: Overview of (e)DNA-based activities of the three BQEs fish, MZB and diatoms, and the sediment fauna, during JDS4. Sample types refer to bulk sample (BS), preservation liquid (PL) and environmental DNA (eDNA) taken from water and sediment. MHS = Multi Habitat Sample; MZB = Macrozoobenthos.

12.3 Reference library development in JDS4

As stated above, a high proportion of species-level identifications can be achieved only when validated barcode sequences are available in reference databases. Weigand et al. (2019) showed that this is generally the case for most MZB groups and for abundant phytobenthos species, and especially for fish. However, on a European scale, some taxa are less represented in barcode reference databases, in particular Plathelminthes, Annelida, and Mollusca, but also Ephemeroptera and some groups of Diptera. In the specific case of the Danubian biota, an independent barcode coverage analysis was performed for fish, MZB and phytobenthos, relying on JDS3 taxalists and the respective barcode markers used in the BQE-specific DNA metabarcoding protocols (Table 1). This analysis was meant to flag potential a priori gaps in taxalists, as a result of missing barcode sequences (i.e. the scenario when a species cannot be identified because of lacking reference sequence information, although its genetic sequence has been amplified from the sample). Yet, all three BQEs showed very high (>90% for fish) or high (84% for MZB and 88% for abundant phytobenthos) coverage values, so that the (e)DNA-based approaches can be expected to be implemented effectively from this perspective.

In addition, the Zoological Research Museum Alexander Koenig (ZFMK, Bonn, Germany) offered free reference barcoding through the German Barcode of Life (GBOL; Geiger et al. 2016) project for animals (i.e. fish and MZB) prior to JDS4. After the end of GBOL II (June 2019), DNAqua-Net and ZFMK offered reference barcoding through dedicated workshops and targeted sampling, reducing the remaining barcode reference gaps. ZFMK will also archive all JDS4 samples in its biobank.

12.4 Biobanking in JDS4

During JDS4 and its preparatory phase, several types of molecular samples were produced: mixed DNA (eDNA or bulk DNA) from samples analyzed via metabarcoding, as well as DNA and tissue from individual specimens (used to construct the reference database). After concluding molecular analysis, these samples will be archived in frozen form and using standardized operating procedures at the Biobank of the ZFMK, Bonn, Germany.

Thus, JDS4 constitutes the beginning of building a molecular repository for Danube samples. Biobanking warrants reproducibility of results at all times and holds the potential to later expand the original results (Astrin et al. 2013). Furthermore, biobanking increases the visibility of collected samples (and thereby of the project itself) when these are made available to the scientific community. Samples from periodically repeated surveys constitute an important time series documenting environmental change. When we accompany this process through proper biobanking, we keep open a window in time that will also allow for such a comparative perspective at the genetic level.

Table 1: DNA barcode coverage reports for the Danubian biota of the three BQEs and the sediment fauna molecularly investigated during JDS4.

Organism group	Fish	Macrozoobenthos	Phytobenthos	Sediment community
Date of analysis	09.06.2020	09.06.2020	12.06.2020	No JDS3 checklist available; but coverage very variable for individual groups and the barcode markers analysed (18S and COI)
Species in JDS3 taxalist	72	385	307, of which 52 are abundant	
Investigated barcode marker(s)	12S	COI	18S-V4 and rbcL combined	
coverage	>90%, but depending on reference database	84%, but variable for individual groups	69%, but 88% for abundant taxa	

12.5 References

ASTRIN, J. J., ZHOU, X., & MISOF, B. (2013). The importance of biobanking in molecular taxonomy, with proposed definitions for vouchers in a molecular context. Zookeys, 365, 67-70.

CALLAHAN, B. J., MCMURDIE, P. J., & HOLMES, S. P. (2017). Exact sequence variants should replace operational taxonomic units in marker-gene data analysis. The ISME journal, 11(12), 2639.

ELBRECHT, V., & LEESE, F. (2015). Can DNA-based ecosystem assessments quantify species abundance? Testing primer bias and biomass-sequence relationships with an innovative metabarcoding protocol. PLOS ONE, 10(7).

GEIGER, M. F., ASTRIN, J. J., BORSCH, T., BURKHARDT, U., GROBE, P., HAND, R., HAUSMANN, A., HOHBERG, K., KROGMANN, L., LUTZ, M., MONJE, C., MISOF, B., MORINIÈRE, J., MÜLLER, K., PIETSCH, S., QUANDT, D., RULIK, B., SCHOLLER, M., TRAUNSPURGER, W., HASZPRUNAR, G., & WÄGELE, W. (2016). How to tackle the molecular species inventory for an industrialized nation – lessons from the first phase of the German Barcode Of Life initiative GBOL (2012-2015). Genome, 59, 661-670.

KRESS, W. J., GARCÍA-ROBLEDO, C., URIARTE, M., & ERICKSON, D. L. (2015). DNA barcodes for ecology, evolution, and conservation. Trends in Ecology & Evolution, 30(1), 25-35.

LEESE, F., ALTERMATT, F., BOUCHEZ, A., EKREM, T., HERING, D., MEISSNER, K., ... & STEINKE, D. (2016). DNAqua-Net: Developing new genetic tools for bioassessment and monitoring of aquatic ecosystems in Europe. Research Ideas and Outcomes, 2, e11321.

MAHÉ, F., ROGNES, T., QUINCE, C., DE VARGAS, C., & DUNTHORN, M. (2015). Swarm v2: highly-scalable and high-resolution amplicon clustering. PeerJ, 3, e1420.

TABERLET, P., COISSAC, E., POMPANON, F., BROCHMANN, C., & WILLERSLEV, E. (2012). Towards next-generation biodiversity assessment using DNA metabarcoding. Molecular Ecology, 21(8), 2045-2050.

WEIGAND, H., BEERMANN, A.J., ČIAMPOR, F., COSTA, F.O., CSABAI, Z., DUARTE, S., GEIGER, M.F., GRABOWSKI, M., RIMET, F., RULIK, B., STRAND, M., SZUCSICH, N., WEIGAND, A.M., WILLASSEN, E., WYLER, S.A., BOUCHEZ, A., BORJA, A., ČIAMPOROVÁ-ZAŤOVIČOVÁ, Z., FERREIRA, S., DIJKSTRA, K.D., EISENDLE, U., FREYHOF, J., GADAWSKI, P., GRAF, W., HAEGERBAEUMER, A., HOORN, B.B. VAN DER, JAPOSHVILI, B., KERESZTES, L., KESKIN, E., LEESE, F., MACHER, J., MAMOS, T., PAZ, G., PEŠIĆ, V., PFANNKUCHEN, D.M., PFANNKUCHEN, M.A., PRICE, B.W., RINKEVICH, B., TEIXEIRA, M.A.L., VÁRBÍRÓ, G., EKREM, T. (2019). DNA barcode reference libraries for the monitoring of aquatic biota in Europe: gap-analysis and recommendations for future work. Science of the Total Environment, 678, 499-524.

13

Metabarcoding of macrozoobenthos samples

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Abstract

DNA metabarcoding of homogenised macrozoobenthos bulk samples collected by multi-habitat sampling (MHS) from 46 JDS4 sampling sites, as well as DNA metabarcoding of their preservation liquid (fixative), was performed. Both metabarcoding approaches detected more species (333 and 321) than morphological identification (275) across all 46 shared Danube and tributary samples. This increase can be mainly attributed to detection of additional insect species. The most dominant group at all sites was Diptera with Chironomidae being the most dominant dipteran family in terms of richness. While reaches showed only little variation on higher taxonomic level, a high number of exclusive species was detected for each reach. Comparisons between the two DNA-based and the morphological identification results revealed a high number of exclusive species for all three approaches (16-20%) and only a low overlap in detected species (18-33%).

For seven JDS4 sites mainly situated in Slovakia, a comparison of four assessment methods (morphology, bulk sample, fixative and eDNA water metabarcoding) was performed. Environmental DNA water analysis detected the highest number of families, fixative metabarcoding most of the species. Bulk sample metabarcoding showed the highest overlap on family and species level with morphology. Yet, each method added a specific proportion of families and species to the overall biodiversity detected in the Danube and its tributaries.

While all DNA metabarcoding approaches significantly increased the number of detected species in JDS4 individually, the overall number of detected species can be maximised by combining several identification methods. In this context, it must be also highlighted that MHS of MZB was installed as an effective monitoring approach, and not meant to detect as much MZB biodiversity as possible.

13.1 Introduction

The aim of identifying macrozoobenthos samples using DNA metabarcoding in JDS4 was to investigate the potential of DNA-based identification methods for assessing Danube's invertebrate diversity. Different DNA metabarcoding methods were used (homogenised bulk sample metabarcoding, preservation liquid (fixative) metabarcoding and water eDNA metabarcoding) and compared to each other as well as to morphological results. As a more detailed biodiversity assessment, additional water samples were collected and analysed via eDNA metabarcoding for seven JDS4 sites.

Since DNA metabarcoding analyses of eDNA water were postponed due to the COVID-19 outbreak in early 2020, the present chapter only reports on the results of the DNA metabarcoding of homogenised bulk samples, preservation liquid and additional eDNA samples from the Slovakian survey.

13.2 Methods

A total of 46 samples were collected for MZB metabarcoding analyses, of which 29 belonged to Danube sites (upper reach: 9, middle reach: 15, lower reach: 5) and 17 to tributaries. All samples contained organisms as well as varying amounts of substrate.

13.2.1 DNA metabarcoding of MZB preservation liquid samples

For all bulk samples, 250 mL preservation liquid (first phase ethanol, i.e. the initial ethanol that was used in the field to preserve specimens) was filtered and DNA captured on a 0.45 µm cellulose nitrate membrane-filter in the process. Subsequently, DNA was extracted from the filters using a modified salt precipitation protocol and amplified in a two-step PCR protocol using the degenerate PCR primer pair fwh2n & EPTDr2n, which are optimised for insect taxa (Leese et al., 2020). Every sample was amplified in two PCR replicates in the first step and pooled prior to the second PCR reaction. All steps subsequent to filtration were carried out in a separate lab room designated to processing eDNA samples including UV light exposure between work shifts. After sequencing, quality filtering of the retained sequencing reads and clustering into Molecular Operational Taxonomic Units (MOTUs) of 97% sequence similarity, as well as the initial taxonomic assignment of MOTUs was carried out using BOLDigger (Buchner and Leese, 2020) and the Barcode of Life reference sequence database (BOLD). Linnaean species information was assigned to MOTUs in case at least one published barcode sequence with a similarity of ≥97% was present in the database. In case identical Linnaean species information was assigned to several MOTUs, the respective MOTUs were combined to a single Linnaean species in all downstream analyses. TaxonTableTools (Macher et al., 2020) was used for data analysis as well as creating Venn diagrams and Krona charts (Ondov et al., 2011).

13.2.2 DNA metabarcoding of MZB bulk samples

From 39 bulk samples, up to 1,000 specimens were randomly subsampled and homogenised to fine powder before extracting DNA using a magnetic bead-based extraction protocol. The remaining seven samples contained fewer specimens and were fully processed. In the process of subsampling specimens, molluscs were sorted from other invertebrates for separate downstream processing. A two-step PCR was carried out using the primer pair BF3 and BR2 (Elbrecht et al., 2019). For molluscs a modified version of the BF3/BR2 primer pair with higher primer degeneracy was used to minimise the potential of false negative results as

a result of primer mismatches. In contrast to the preservation liquid samples, two extraction replicates per sample and invertebrate/mollusc fraction were used, separately amplified and sequenced. Bioinformatic processing, including taxonomic annotation of MOTUs, was carried out following the same procedure as described above.

13.2.3 DNA metabarcoding of eDNA water samples (Slovakian survey)

For a subset of 11 sampling sites located on the Slovakian stretch of the Danube (n = 5), at a nearby site in Austria (1) and in the main tributaries of this part of the Danube (5), 1 litre of filtered water per site was analysed for eDNA, and MZB diversity assessed by using the primer pair BF3/BR2 targeting COI. eDNA water samples were collected within five days, and a team of four people performed the complete bioinformatic analysis within ~2 weeks. In total, comparative data for four methods was available for seven JDS4 sites (Table 1).

Table 1: Sampling sites of the Slovakian eDNA survey used for methodological comparison.

Site	Locality name	River	Methods compared
JDS4-10	Hainburg	Danube	eDNA water, eDNA fixative, bulk, morphology
JDS4-13	Devín	Morava	eDNA water, eDNA fixative, bulk, morphology
JDS4-14	Pečnianska lúka	Danube	eDNA water, eDNA fixative, bulk, morphology
JDS4-16	Medveďov	Danube	eDNA water, eDNA fixative, bulk, morphology
JDS4-19	Komárno	Váh	eDNA water, eDNA fixative, bulk, morphology
JDS4-20	Kamenica nad Hronom	Hron	eDNA water, eDNA fixative, bulk, morphology
JDS4-21	Salka	Ipeľ	eDNA water, eDNA fixative, bulk, morphology

13.3 Results and discussion

13.3.1 DNA metabarcoding of homogenized MZB bulk samples and their preservation liquid

While DNA metabarcoding of homogenised bulk samples resulted in 833 Molecular Operational Taxonomic Units (MOTUs) and 333 detected species across all 46 samples, 1,147 MOTUs and 321 species were detected by metabarcoding of the sample fixative. In 163 (bulk) and 491 cases (fixative) MOTUs with the same taxonomic information were collapsed into one respective entry. While this is appropriate in many cases to not over split species into molecular derived operational taxonomic entities, it has to be noted that by doing so, simultaneously information on potential cryptic diversity is omitted. In 12 (bulk) and 18 cases (fixative) comparing DNA barcodes to reference sequence information resulted in conflicting taxonomic assignments with more than one species being assigned to a single MOTU. This either indicates reference sequences of misidentified voucher specimens in the database, taxonomic (yet unknown) synonyms, species with recent speciation events or hybridization of the respective species in question. In the latter two, rare cases, DNA metabarcoding is inappropriate to distinguish between respective species. All other MOTUs lacking annotation of species information reflect a gap in reference libraries, but simultaneously highlight the further potential of metabarcoding in detecting species once databases are further complemented.

With both methods, Insecta showed the highest species richness (bulk: 142 species, fixative: 170) across all 29 Danube sites followed by Oligochaeta (bulk: 33, fixative: 42) (Figure 1). While fixative metabarcoding detected more dipteran species (136, including 106 Chironomidae species) than bulk metabarcoding (90, including 80 Chironomidae species), with the latter approach more Trichoptera (19 vs. 2), Amphipoda (10 vs. 4) and Bivalvia species (13 vs. 5) were detected. This deviation can be attributed to the primers used, as the fixative primers have a negative bias towards the underrepresented taxa, and the source of DNA, as soft-bodied taxa like dipterans are often overrepresented and sclerotised or hard-shelled taxa underrepresented in fixative approaches.

In contrast to the different number of samples taken per stream reach, the number of detected species was equal between upper reach (UR; 151 species) and middle reach (MR; 152 species) in the fixative approach or even higher in the UR (152 species) than the MR (124) when analysing bulk samples, indicating a general higher species richness in Danube's upper reach. Both methods returned the lowest number of species in the lower reach (LR; bulk: 89, fixative: 81).

The taxonomic composition on a higher taxonomic level varied between Danubian reaches and to a smaller degree between the two applied methods (Figure 2). While a decline in Insecta species was observed from the UR (bulk: 65% of all detected species; fixative: 78%) to the MR (51%/59%) and LR (47%/58%), there was an increase in Oligochaeta species from upstream to downstream (UR: 11%/15%, MR: 22%/25%, LR: 24%/27%). In addition to the differences of taxonomic composition patterns on a higher taxonomic level, a comparison of shared and exclusive taxa per reach on MOTU level revealed a high number of exclusive species for each stream reach (Figure 3) with the UR having the highest number of exclusive species. While this comparison is not independent of the number of samples taken per reach and thus explaining the lowest number of exclusive species in the LR, it underlines the importance of high taxonomic resolution in this survey, and the value of upper reaches for aquatic biodiversity in general.

13.3.2 Comparison of morphological and DNA metabarcoding results

Both homogenised bulk sample and fixative metabarcoding detected more species (333 and 321) than morphological identification (275) across all 46 shared Danube and tributary samples (Table 2), which can be mainly attributed to an increase in detected insect species (bulk: +70 species; fixative: +82). In contrast, morphological identification performed better at determining Gastropoda (28) than both other approaches (bulk: 17; fixative: 12). The comparison of all three methods showed a high number of exclusively detected species per method (Figure 4) rendering a complementary approach ideal for capturing more of Danube's macroinvertebrate diversity. It has to be noted that morphological identification shared more detected species with bulk (143) than fixative (106) metabarcoding, but that the two DNA-based methods shared significantly more detected species (191) and that the total number of detected species was increased to 463 when combining both metabarcoding approaches. While the number of species detected with DNA-based identification methods will further increase with complementing reference databases, in which on a European scale specific taxa are underrepresented (e.g. Plathelminthes, Annelida, Mollusca; Weigand et al. 2019), DNA-based identification methods will remain uninformative on specimen sex or life stage. Finally, it has to be noted that samples for morphological and DNA-based identification were separately taken but originate from the same sites and sampling events. Nevertheless, some differences in sample community composition are expected independently of the applied identification method.

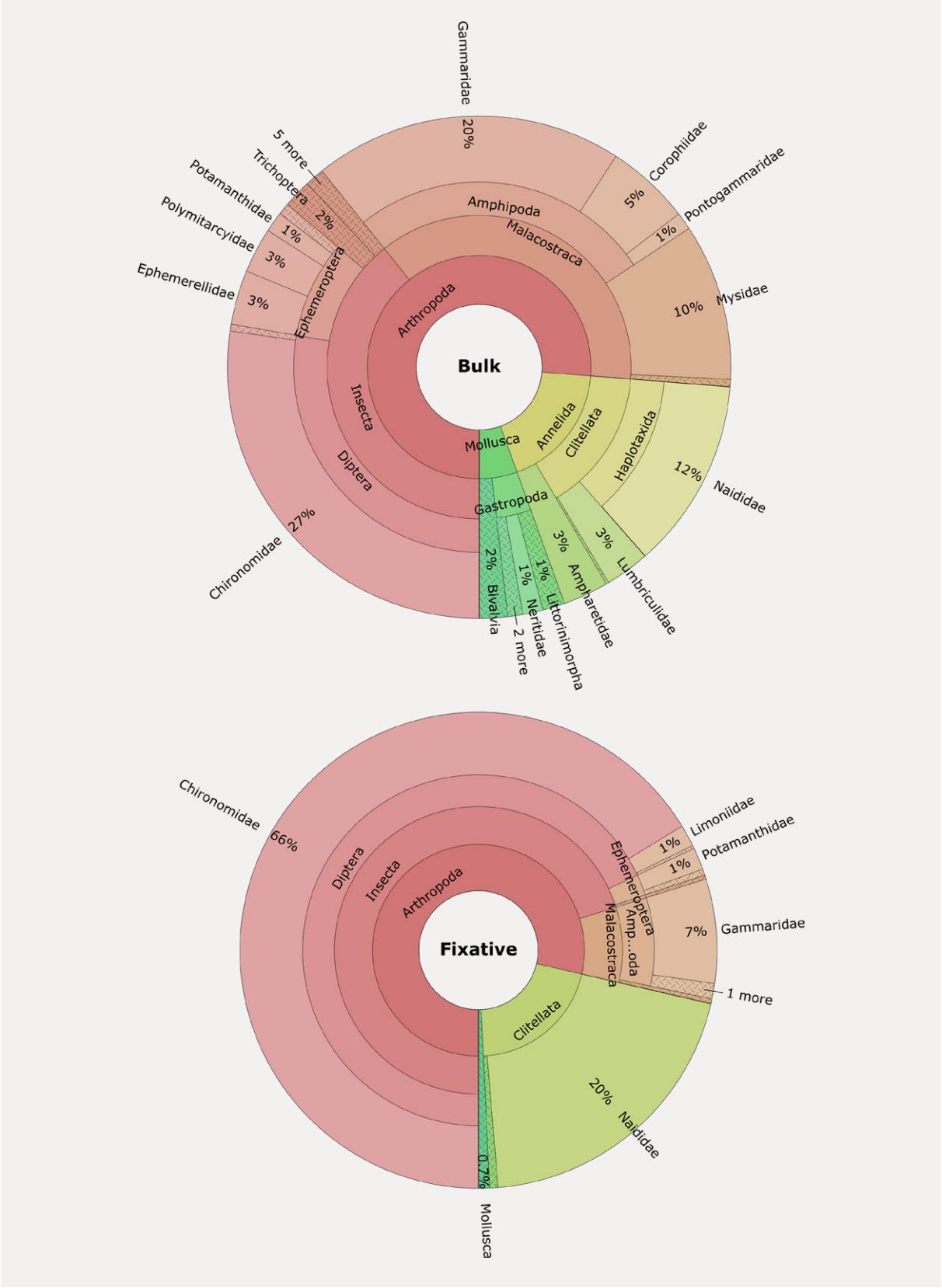


Figure 1: Taxonomic composition of JDS4 macrozoobenthos communities of 29 Danube sites (tributaries not included) derived by DNA metabarcoding of the homogenised bulk samples (top) and the preservation liquid (bottom). The figure is based only on MOTUs with available species-level information. Higher taxonomic levels are collapsed to the lowest taxonomic level containing more than one sub-taxon (e.g., in the bottom chart Annelida is collapsed to Clitellata since all Annelida OTUs are assigned to Clitellata).

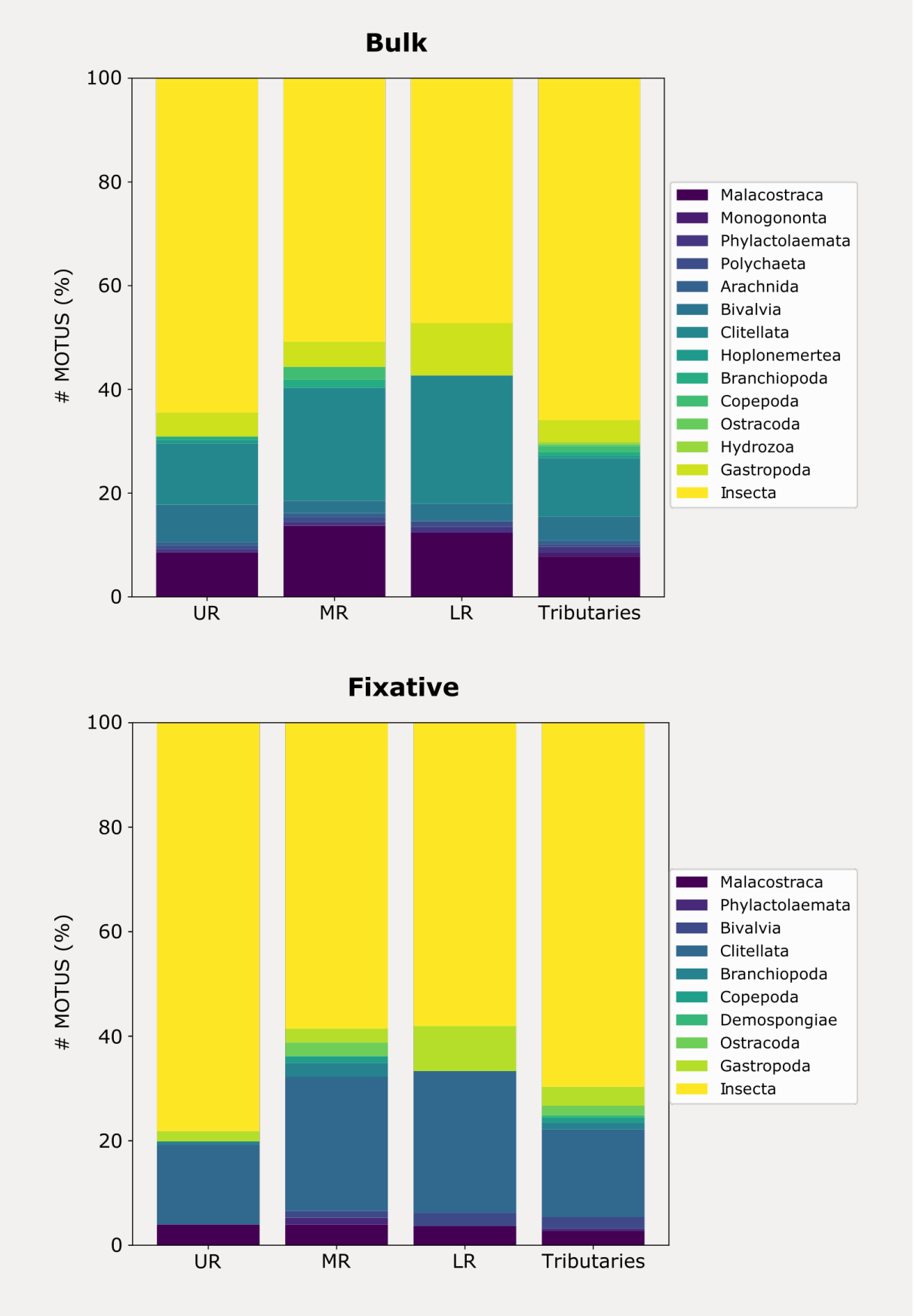


Figure 2: Taxonomic composition of JDS4 macrozoobenthos communities derived by DNA metabarcoding of homogenised bulk samples (top) and the preservation liquid (bottom) separated into upper reach (UR), middle reach (MR) and lower reach (LR) as well as tributaries.

Figure 3: Shared and exclusive number of species between the upper reach (9 sites), middle reach (15) and lower reach (5) of all 29 Danube sites.

Table 2: Number of detected species per method in JDS4 across all 46 shared sites (29 Danube, 17 tributaries).

Higher Taxon	Taxon	Morphology	Bulk sample metabarcoding*	Fixative metabarcoding	Bulk+fixative metabarcoding
Annelida	Hirudinea	7	5	2	5
Annelida	Oligochaeta	44	37	46	52
Annelida	Polychaeta	1	1	0	1
Arthropoda	Acari	0	2	0	2
Arthropoda	Branchiopoda	0	4	8	10
Arthropoda	Copepoda	0	4	3	6
Arthropoda - Insecta	Coleoptera	11	20	14	24
Arthropoda - Insecta	Diptera	68	118	174	199
Arthropoda - Insecta	Ephemeroptera	21	23	21	29
Arthropoda - Insecta	Hemiptera	5	6	3	7
Arthropoda - Insecta	Megaloptera	1	2	2	2
Arthropoda - Insecta	Neuroptera	0	3	2	4
Arthropoda - Insecta	Odonata	11	6	2	6
Arthropoda - Insecta	Plecoptera	2	2	6	7
Arthropoda - Insecta	Thysanoptera	0	1	0	1
Arthropoda - Insecta	Trichoptera	28	36	5	36
Arthropoda - Crustacea	Amphipoda	14	14	5	14
Arthropoda - Crustacea	Decapoda	2	2	2	3
Arthropoda - Crustacea	Isopoda	3	3	0	3
Arthropoda - Crustacea	Mysida	2	3	1	3
Arthropoda	Ostracoda	0	1	5	5
Bryozoa		0	3	2	4
Cnidaria		0	1	0	1
Mollusca	Bivalvia	17	16	5	16
Mollusca	Gastropoda	28	17	12	19
Nematoda		6	0	0	0
Nemertea		0	1	0	1
Platyhelminthes		4	0	0	0
Porifera		0	0	1	1
Rotifera		0	2	0	2
Total		**275**	**333**	**321**	**463**

** For bulk sample metabarcoding 1,000 specimens per sample were used (7 samples contained less than 1,000 specimens).*

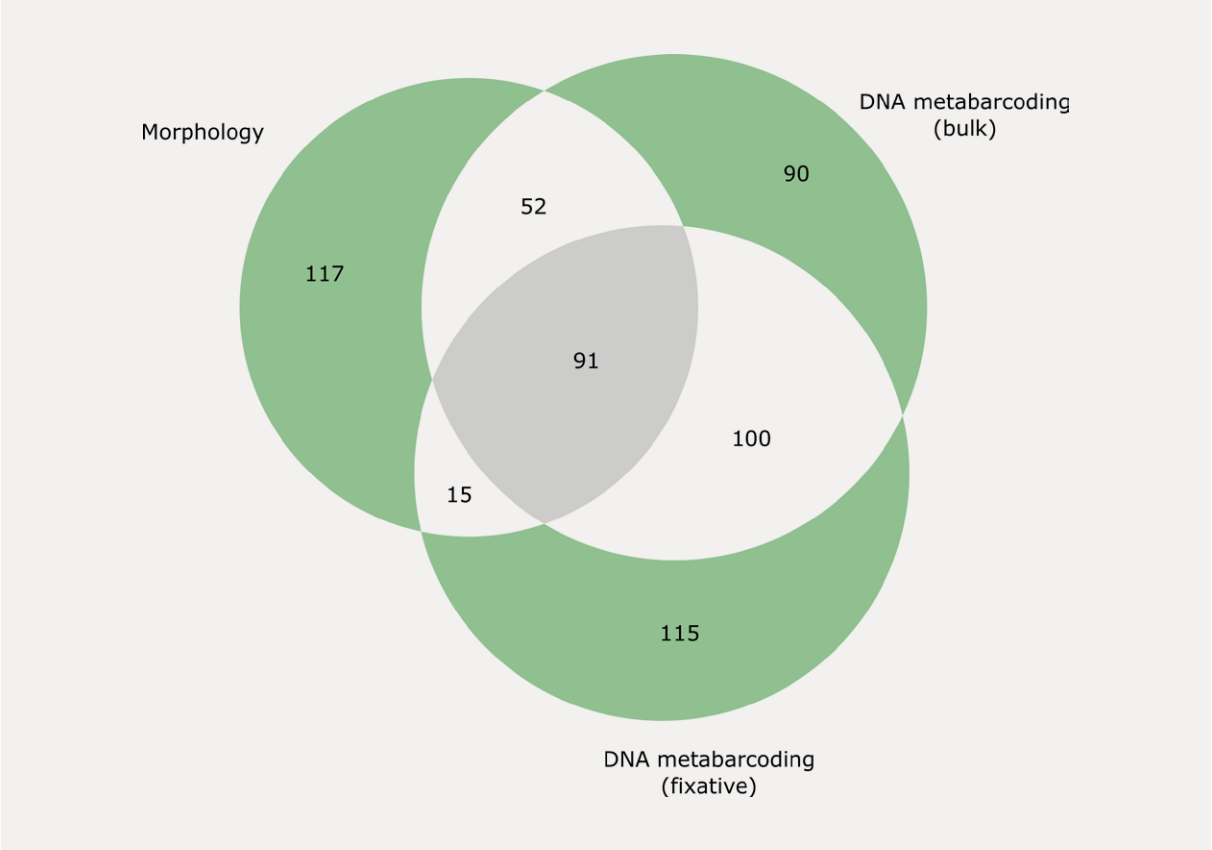


Figure 4: Shared and exclusive number of species identified by morphology, homogenised bulk metabarcoding and fixative metabarcoding across 29 Danube and 17 tributary samples.

13.3.3 Slovakian eDNA survey

The four-method comparison at seven sites revealed a total of 353 Linnaean taxa in 95 families, of which 278 (79%) were assigned to species level. The eDNA water analysis detected the highest number of families (n = 56), followed by morphological identification and bulk sample metabarcoding (both 53), less so by the eDNA fixative approach (35). In particular, several families of Branchiopoda, Cnidaria, Plathelminthes and Bryozoa have been almost exclusively added by eDNA metabarcoding of water samples. The malperformance of the fixative approach on family level can be best attributed to a strong primer bias, also negatively influencing the molecular discovery of gastropods, bivalves and caddisflies, which all were much underrepresented in the fixative dataset. On the contrary, eDNA metabarcoding of the fixative generated the most species level hits (n = 139), closely followed by bulk sample metabarcoding (133). Fewer species were detected by eDNA water metabarcoding (101) and morphological identification (98). Each method added a very high proportion of exclusive taxa to the overall detected biodiversity of the seven investigated sites (max. 35 families for eDNA metabarcoding of water and 49 species for fixative metabarcoding), but likewise missed some MZB diversity present in the Danube and its reaches (Figure 5). The increased number of species detected by fixative and bulk metabarcoding can be primarily attributed to additional species detected within Diptera (particularly Chironomidae) and Oligochaeta (particularly Naididae), as well as Ephemeroptera and Trichoptera. On the other hand, morphological identification particularly added species of Odonata, Bivalvia and Coleoptera as well as some Diptera (Chironomidae, Simuliidae) and Trichoptera to the overall taxalist. However, it must be highlighted that MHS was not designed to capture most of the MZB diversity, but to provide robust ecological assessment data.

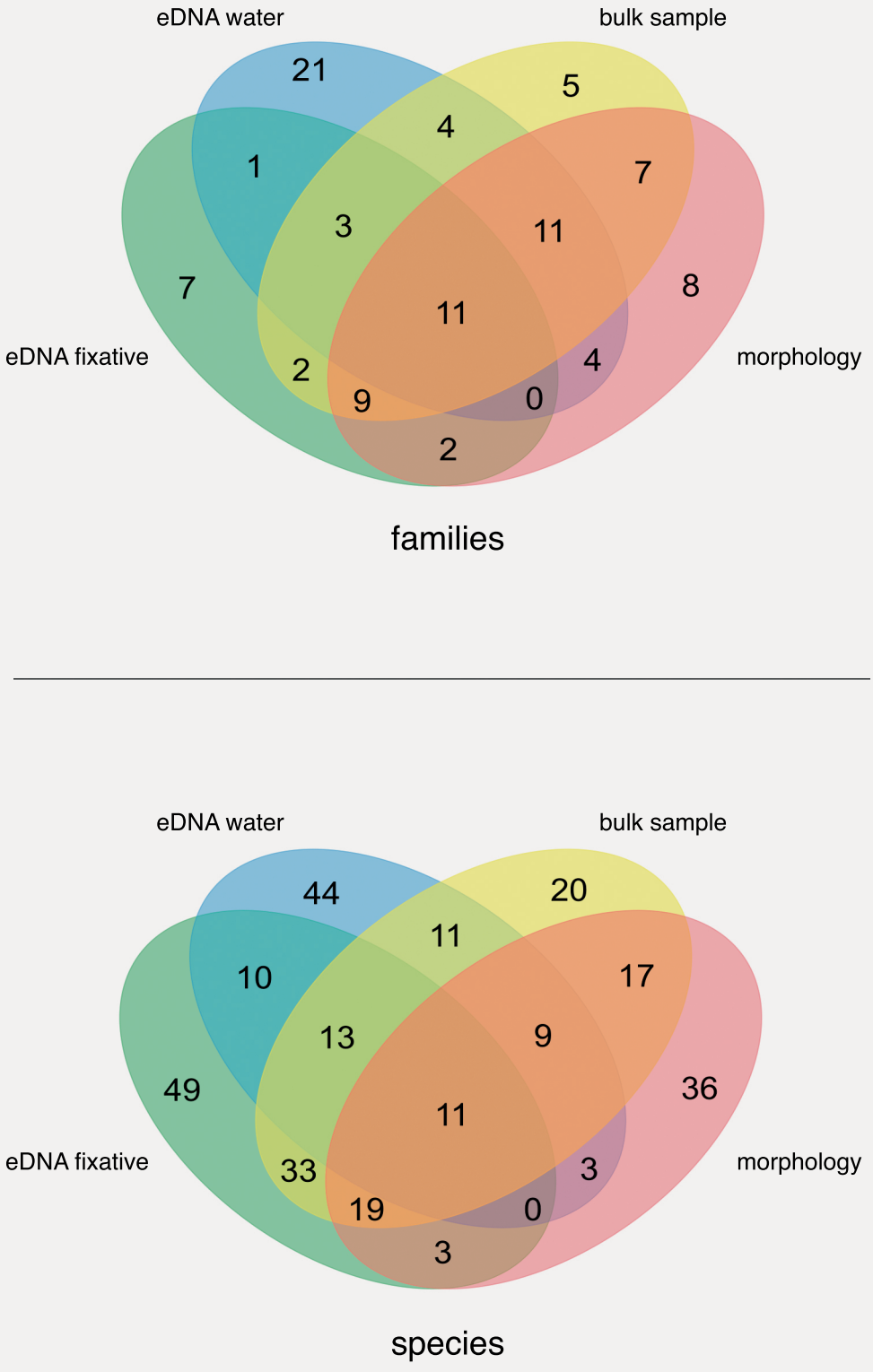
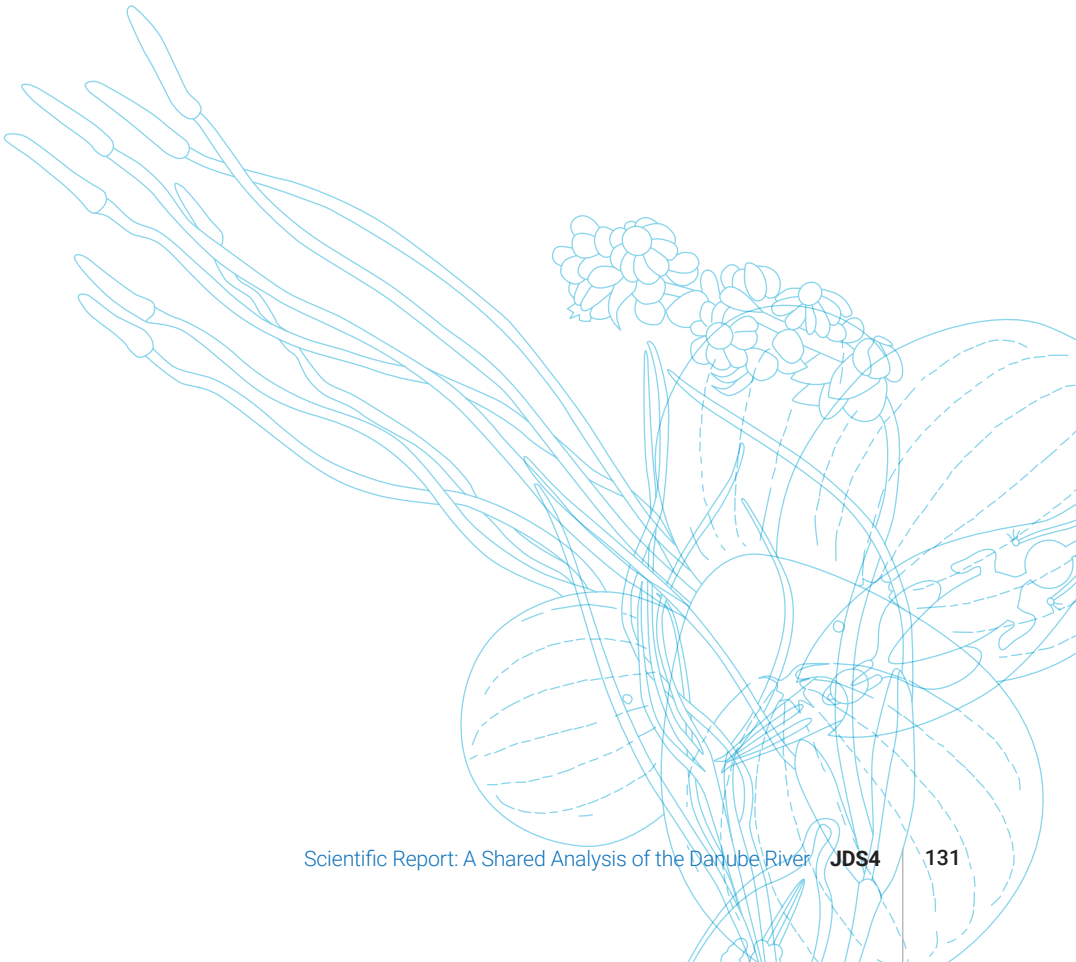


Figure 5: Venn diagrams of the four-method comparison at seven JDS4 sites for the taxonomic levels family and species.

13.4 Conclusions

- Both metabarcoding methods detected a high number of Linnaean species (bulk sample: 333; fixative: 321), in particular additional oligochaetes and chironomids, but partly also additional caddisflies, stoneflies and mayflies were detected
- Analysis of eDNA in water particularly added further meiofaunal species
- When compared to morphological identification, gastropod and odonate species were underrepresented in DNA-based taxalists
- Morphological identification, bulk sample, fixative and eDNA water metabarcoding all detected a large proportion of methodologically exclusive families and species
- Bulk sample metabarcoding results had the highest species-level overlap with morphology-based results
- Conflicting taxonomic results can provide effective feedback loops and mark the start for further taxonomic investigations
- Methodological restrictions for DNA-based assessments (such as primer bias and availability of barcode references) have to be understood and taken into account for upcoming surveys



13.5 References

BUCHNER, D., & LEESE, F. (2020). BOLDigger – a Python package to identify and organise sequences with the Barcode of Life Data systems. Metabarcoding and Metagenomics, 4, e53535.

ELBRECHT, V., BRAUKMANN, T. W., IVANOVA, N. V., PROSSER, S. W., HAJIBABAEI, M., WRIGHT, M., ... & STEINKE, D. (2019). Validation of COI metabarcoding primers for terrestrial arthropods. PeerJ, 7, e7745.

LEESE, F., SANDER, M., BUCHNER, D., ELBRECHT, V., HAASE, P., & ZIZKA, V.M. (2020). Improved freshwater macroinvertebrate detection from eDNA through minimized non-target amplification. bioRxiv.

MACHER, T. H., BEERMANN, A. J., & LEESE, F. (2020). TaxonTableTools – A comprehensive, platform-independent graphical user interface software to explore and visualise DNA metabarcoding data. bioRxiv.

ONDOV, B. D., BERGMAN, N. H., & PHILLIPPY, A. M. (2011). Interactive metagenomic visualization in a Web browser. BMC bioinformatics, 12(1), 385.

WEIGAND, H., BEERMANN, A.J., ČIAMPOR, F., COSTA, F.O., CSABAI, Z., DUARTE, S., GEIGER, M.F., GRABOWSKI, M., RIMET, F., RULIK, B., STRAND, M., SZUCSICH, N., WEIGAND, A.M., WILLASSEN, E., WYLER, S.A., BOUCHEZ, A., BORJA, A., ČIAMPOROVÁ-ZAŤOVIČOVÁ, Z., FERREIRA, S., DIJKSTRA, K.D., EISENDLE, U., FREYHOF, J., GADAWSKI, P., GRAF, W., HAEGERBAEUMER, A., HOORN, B.B. VAN DER, JAPOSHVILI, B., KERESZTES, L., KESKIN, E., LEESE, F., MACHER, J., MAMOS, T., PAZ, G., PEŠIĆ, V., PFANNKUCHEN, D.M., PFANNKUCHEN, M.A., PRICE, B.W., RINKEVICH, B., TEIXEIRA, M.A.L., VÁRBÍRÓ, G., & EKREM, T. (2019). DNA barcode reference libraries for the monitoring of aquatic biota in Europe: Gap-analysis and recommendations for future work. Science of the total environment, 678, 499-524.

14 Metabarcoding of fish eDNA samples

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Abstract

Water samples were collected at 29 Danubian River sites and 18 tributaries, and their fish environmental DNA (eDNA) contents were analysed by DNA metabarcoding. In total, 80 taxa were detected, of which 19 corresponded mainly to farmed fish or food fish due to eDNA release in waste waters. Of the remaining 61 taxa, 50 taxa were identified at the species level, six taxa comprised two to three species of the same genus, and five taxa two to three species of different genera. From the Danube River, 50 taxa were detected both by eDNA and traditional fish surveys (TFS), nine only by TFS and eight only by eDNA – notably including several sturgeon species. The relative abundance of sequence reads per site allowed to describe the longitudinal structure of the fish community efficiently. The calculation of a fish index, based on the common metrics used to intercalibrate national fish-assessment methods at the European scale, classified most sites as of moderate ecological status.

14.1 Introduction

In complement to the traditional fish survey along the Danube, a fish eDNA metabarcoding-based survey was implemented along the Danube River at 20 sites within the framework of the monitoring programme organised by DNAqua-Net. In addition, a collaboration with the Interreg "MEASURES" program (DTP2-038-2.3) coordinated by BOKU University (Institute of Hydrobiology and Aquatic Ecosystem Management, Vienna) and with support from the Austrian Federal Ministry of Agriculture, Regions and Tourism (BMLRT) and the ÖK-IAD (Österreichisches Komitee der Internationalen Arbeitsgemeinschaft Donauforschung) allowed sampling to take place at 9 and 17 additional sites on the Danube and its main tributaries, respectively (see legend Fig. 1).

14.2 Methods

The 29 sampling sites on the Danube were chosen in such a manner that the average distance between sites was 99.2 km (standard error: 26.0 km; range: 38-149 km). This distance is sufficient to avoid potential influence of eDNA transported downstream from one site to the next (Pont et al., 2018). For the same reason, sampling sites were not located within several tens of km downstream of the confluence of a major tributary. Sites were sampled between June 29 and July 19, 2019, except for one site near Vienna (August 6). During the same period, 18 tributaries were sampled 5-10 km upstream of their confluence with the Danube. Due to absence or low DNA amplification obtained from some samples, the Inn River site was re-sampled in May 2020 and samples collected by us at JDS4-10 in July 2017 were used. Two water samples were collected at each site using a peristaltic pump and the water filtered *in situ* (VigiDNA 0.45 µm crossflow filtration capsule, SPYGEN), with disposable sterile tubing. The mean filtration time per sample and the mean water volume filtered were 22.34 min and 28.73 L (3 to 40 L), respectively, depending on the clogging speed of the filtration capsule. At the end of each filtration, the water in the capsule was drained and the capsule was refilled with 80 mL of conservation buffer CL1 (SPYGEN) to prevent eDNA degradation. DNA extraction, amplification using teleo primers (Valentini et al., 2016), high-throughput sequencing and bioinformatic analysis were performed following the protocol described in Pont et al. (2018) except for filters applied to rare species. Twelve PCR replicates were performed per sample. To monitor possible contaminants, negative extraction controls and negative PCR controls (ultrapure water) were amplified and sequenced in parallel to the samples. Library preparation and sequencing were performed at Fasteris (www.fasteris.com) and sequence reads analysed using OBITools package (Valentini et al., 2016, Milhau et al., 2020). The local marker reference database used for taxa identification included most of European freshwater fish species (Valentini et al., 2016, and complementary data to be published). This database is freely accessible for scientific purposes and licensed for commercial purposes. The taxonomical nomenclature refers to Kottelat and Freyhof (2007). The total number of sequence reads per sample were standardized to allow a comparison between sites in terms of relative abundance (Pont et al., 2018).

The comparison of the list of species/taxa detected by TFS (mainly electrofishing, Bammer et al., JDS4 data) and eDNA-based method considered all the samples collected along the Danube River itself. The comparison between the species relative abundance obtained by both methods considered the 13 common Danubian sites (i.e. distance between TFS and eDNA sites no more than three kilometres) (see legend Fig. 1).

As a preliminary attempt to assess Danubian sites on the basis of eDNA samples, the mean value of the two common metrics used to intercalibrate the eight national fish assessment methods in the Danubian

and Lowland-Midland Geographic Intercalibration Group (Pont et al., 2011) were used to compute a fish index based on eDNA data for the Danube River and its tributaries (except the Inn River), according to the European Water Framework Directive (Council of the European Communities, 2000). These two metrics, issued from the European fish Index (EFI, Pont et al., 2009), were the density of oxygen depletion intolerant species and the number of species requiring a rheophilic reproduction habitat. A correspondence was noted between the list of species belonging to these two ecological guilds and the list of eDNA taxa (Pont et al., 2019). The thresholds between High/Good and Good/Moderate ecological classes were the median values of the official threshold values used to check comparability between the national assessment methods in the intercalibration process (Pont et al., 2011). The indication of the ecological status based on TFS data was calculated at the 13 sites in common with eDNA sites, using the same assessment method. All statistical analyses were conducted in R, version 3.3.3 (R Core Team, 2018).

14.3 Results and discussion

14.3.1 Species inventory

No DNA amplification could be obtained from the Inn river samples, although additional eDNA testing was re-run to ensure no inhibition. Sites downstream of its confluence in Austria (in particular JDS4-6 and JDS4-10) also showed a very low number of detections compared to other sites. At its confluence, the Inn has a mean discharge normally comparable to that of the Danube, and probably much higher at the sampling period due to an exceptional flood (end June 2019) in association with the high loads of suspended solids owing from melting water from snow and glaciers. Such a dilution effect probably led to a decrease in eDNA concentration at the downstream sites. Inversely the samples collected at the Inn River site in May 2020 and at site JDS4-10 (Hainburg) in August 2017 allowed for the detection of a number of taxa comparable to the other Danubian sites.

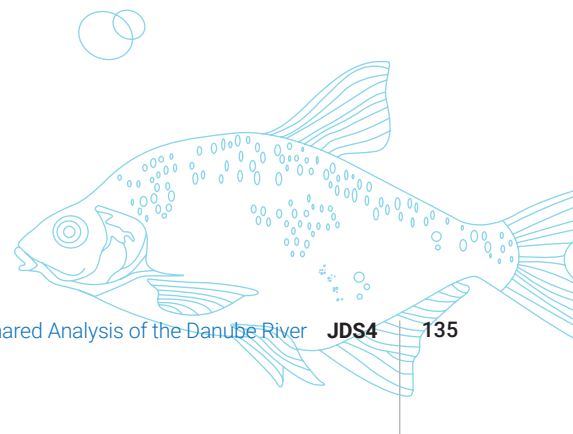

*Table 1: List of taxa detected. *: Species absent from the Danube catchment are excluded.*

Species Names	Abbreviations	Species	Abbreviations
List of taxa corresponding to a single species			
Abramis brama	Abr_bra	*Neogobius fluviatilis*	Neo_flu
Acipenser ruthenus	Aci_rut	*Neogobius melanostomus*	Neo_mel
Acipenser stellatus	Aci_ste	*Oncorhynchus mykiss*	Onc_spp
Alburnoides bipunctatus	Alb_bip	*Perca fluviatilis*	Per_flu
Alburnus alburnus	Alb_alb	*Perccottus glenii*	Per_gle
Ameiurus melas	Ame_spp	*Phoxinus phoxinus*	Pho_pho
Anguilla anguilla	Ang_ang	*Ponticola kessleri*	Pon_kes
Aspius aspius	Asp_asp	*Proterorhinus semilunaris*	Pro_sem
Babka gymnotrachelus	Bab_gym	*Pseudorasbora parva*	Pse_par
Barbatula barbatula	Bar_bar	*Pungitius platygaster*	Pun_pla
Barbus barbus	Bar_bab	*Rhodeus amarus*	Rho_ama
Benthophiloides brauneri	Ben_sp	*Romanogobio uranoscopus*	Rom_ura
Cobitis elongatoides	Cob_elo	*Rutilus rutilus*	Rut_rut
Cottus gobio	Cot_sp	*Rutilus virgo*	Rut_vir
Cyprinus carpio	Cyp_car	*Sabanejewia balcanica*	Sab_bal
Esox lucius	Eso_luc	*Salmo trutta*	Sal_tru
Gambusia holbrooki	Gam_hol	*Scardinius erythrophtalmus*	Sca_ery
Gasterosteus aculeatus	Gas_acu	*Silurus glanis*	Sil_gla
Hucho hucho	Huc_huc	*Squalius cephalus*	Squ_cep
Hypophthalmichthys nobilis	Hyp_nob	*Syngnathus abaster*	Syn_sp
Lampetra planeri	Lam_spp	*Thymallus thymallus*	Thy_thy
Lepomis gibbosus	Lep_gib	*Tinca tinca*	Tin_tin
Lota lota	Lot_lot	*Umbra krameri*	Umb_kra
Misgurnus fossilis	Mis_fos	*Zingel streber*	Zin_str
Mugil cephalus	Mug_cep	*Zingel zingel*	Zin_zin
List of taxa corresponding to several species from the same genus			
Acipenser gueldenstaedtii / A. naccarii			Aci_1
Alosa immaculata / A. tanaica			Alos_2
Carassius carassius / C. auratus / C. gibelio			Car_spp
Gymnocephalus baloni / G. cernua / G. schraetser			Gym_spp
Salvelinus alpinus / S. fontinalis / S. namaycush			Sal_spp
Sander lucioperca / S. volgensis			San_spp
List of taxa corresponding to several species from different genera *			
Telestes souffia / Chondrostoma nasus			Cypr_1
Hypophthalmichthys molitrix / Ctenopharyngodon idella			Cypr_2
Ballerus sapa / Blicca bjoerkna / Vimba vimba			Cypr_3
Gobio gobio / Romanogobio albipinnatus / R. kesslerii / R. vladykovi			Cypr_4
Leuciscus idus / L. leuciscus / Pelecus cultratus			Cypr_5

80 taxa were detected from a total of 35,060,453 sequence reads. At nine sites, 19 taxa (4.7% of the total number of reads), unknown in the Danube and its tributaries, were food or farmed fish (15 species of marine fish, *Salmo salar*, *Coregonus* sp., *Clarias gariepinus*) and one species of tropical gobiid *Sicydium altum* belonging to a genus used in aquaria). Only three from these nine sites receiving wastewater from large cities had more than one of these taxa: Arges and Russenski Lom tributaries, Vienna site (respectively six, six and seven taxa). *Salvelinus* species and *Oncorhynchus mykiss* are food fish but also stocked in many water bodies within the upper Danube catchment. One occurrence of *Alosa* spp. on the Upper Danube (Oberloiben site) had been also omitted. Of the remaining 61 taxa, 50 taxa were identified at the species level, six taxa corresponded to two to three species of the same genus, and five taxa two to three species of different genera (Table 1). For the Danubian study sites, we considered four taxa (Lam_spp, Cot_sp, Syn_sp and Ben_sp) as only representative of *Lampetra planeri, Cottus gobio, Syngnathus abaster* and *Benthophiloides brauneri* because of the fish fauna composition in the Danube catchment. A total of 61 taxa were detected, corresponding to 61 to 79 species (i.e. some taxa group several species known to be present in the Danube River). In comparison, the total species richness in the Danube catchment and the Danube River itself were estimated as 115 and 79 species, respectively (Sommerwerk et al., 2009, Kottelat and Freyhof, 2007). 55 of the 61 taxa were common to the Danube and all the 17 sampled tributaries.

14.3.2 Longitudinal organisation of fish communities

The longitudinal distribution of fish species (Fig. 1 and 2) showed a succession of species from upstream to downstream. For example, *B. barbatula, C. gobio, H. hucho, L. planeri, P. phoxinus* and *T. thymallus*, were restricted to the Upper Danube whereas *A. ruthenus, N. fluviatilis, S. ballerus, S. erythrophtalmus,* were detected from Vienna to the Danube River mouth. *Abramis brama, A. alburnus, C. carpio, S. glanis, S. sp, Z. streber* were detected all along the river course; *Alosa* spp. and *S. abaster* downstream from the Iron Gate; A. *stellatus* and *U. krameri* only on the most downstream site (Danube delta). The species richness tended to increase from upstream to downstream whereas the diversity showed a sharp decrease from downstream Pancevo (rkm 1151) to upstream Timok (rkm 849), including the Velika Morava River (Fig. 3).

According to eigenvalues associated with a principal component analysis (Fig. 4), the first principal component explained 28.8% of the total inertia and allowed to distinguish three sections along the Danube: from the source to Ulm (site JDS1), the next 706 km to Hainburg-Upstream Morava (site JDS4-10, limit of the Upper Danube), and the Lower Danube with a gradual change in fish assemblages towards the delta. These results confirm the main change in fish community between Upper and Middle Danube reaches (Erős et al., 2017).

The coordinates of the tributaries on the first principal component, as additional individuals, followed a longitudinal pattern like that of the Danube itself (Fig. 4). Nevertheless, fish communities of the Traun and Enns rivers in Austria were closer to the fish assemblage of the Danube further Upstream. The Arges and Russenski Lom tributaries were quite distant from the Lower Danubian sites.

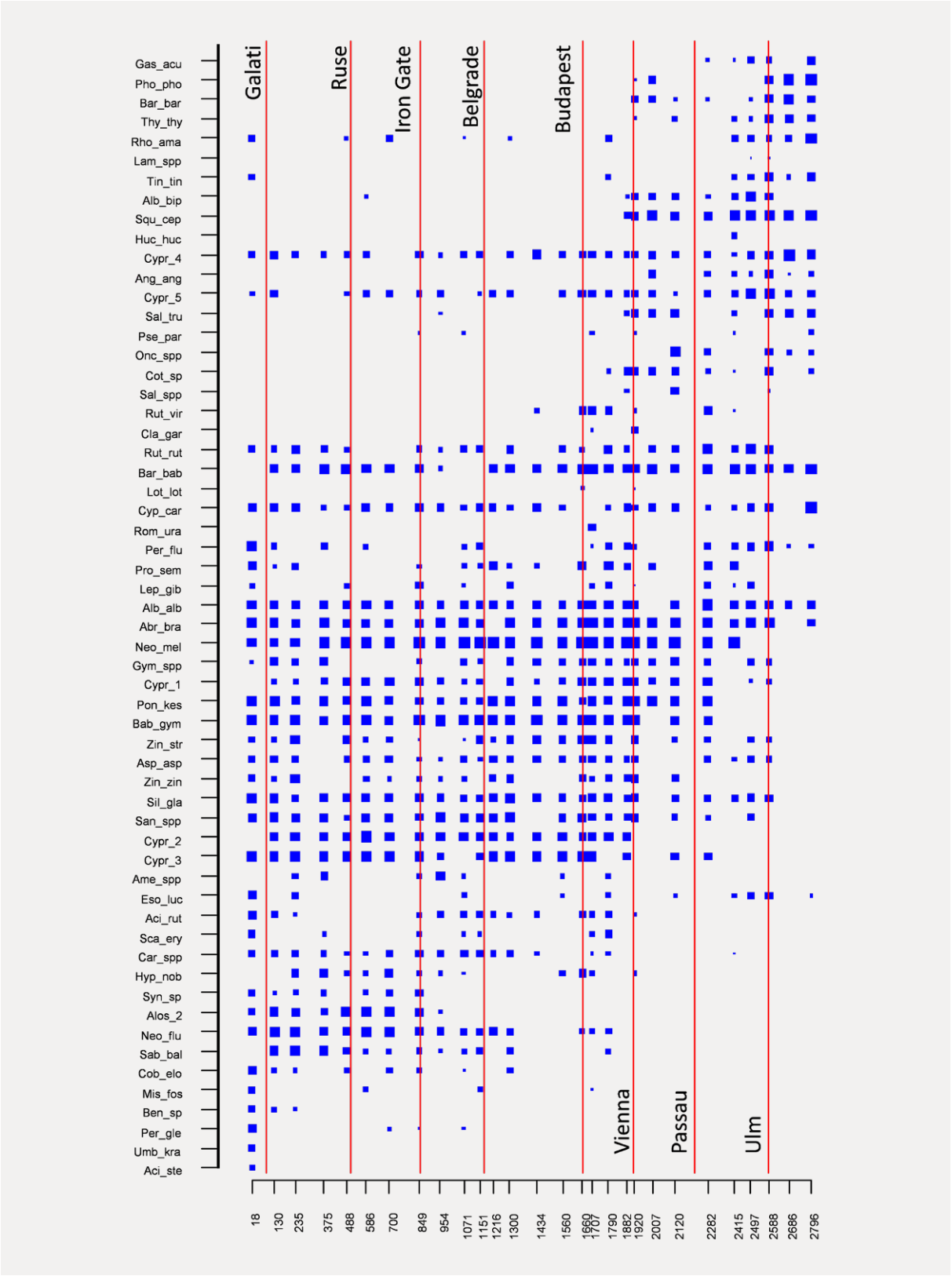

Figure 1: Relative abundance of the 57 taxa detected along the Danube River, from rkm 18 to rkm 2796. The size of the square is a function of the relative abundance of the corresponding taxa in the sample at a given site (see Table 1 for corresponding taxa names). The sites are located at rkm: 2796, 2686, 2588 (JDS4-1), 2497 (JDS4-2), 2415 (JDS4-3), 2282 (JDS4-4), 2120 (JDS4-7), 2007 (JDS4-8*), 1920, 1882 (JDS4-10), 1790 (JDS4-18*), 1707 (JDS4-22*), 1660 (JDS4-23*), 1560 (JDS4-26), 1434 (JDS4-29*), 1300 (JDS4-31*), 1216, 1151 (JDS4-37*), 1071 (JDS4-40*), 954, 849 (JDS4-41*), 700, 586, 488 (JDS4-47*), 375 (JDS4-48*), 235, 130 (JDS4-50*), 18 (JDS4-51). * JDS sites in common with TFS.*

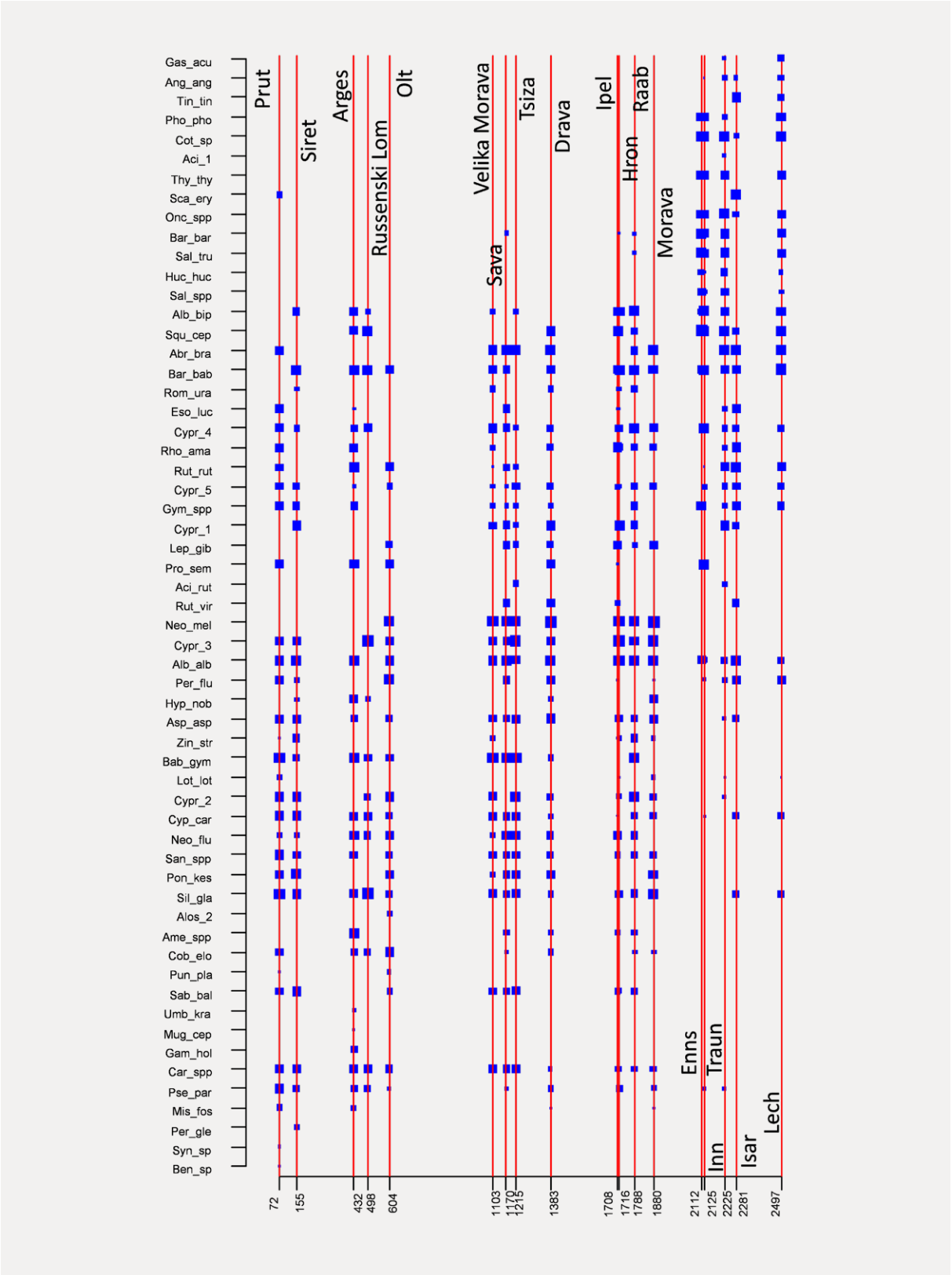

Figure 2: Relative abundance of the 59 taxa detected along the 18 tributaries of the Danube River (rkm 72 to rkm 2497). The size of the square is a function of the relative abundance of the corresponding taxa in the sample (see Table 1 for corresponding taxa names).

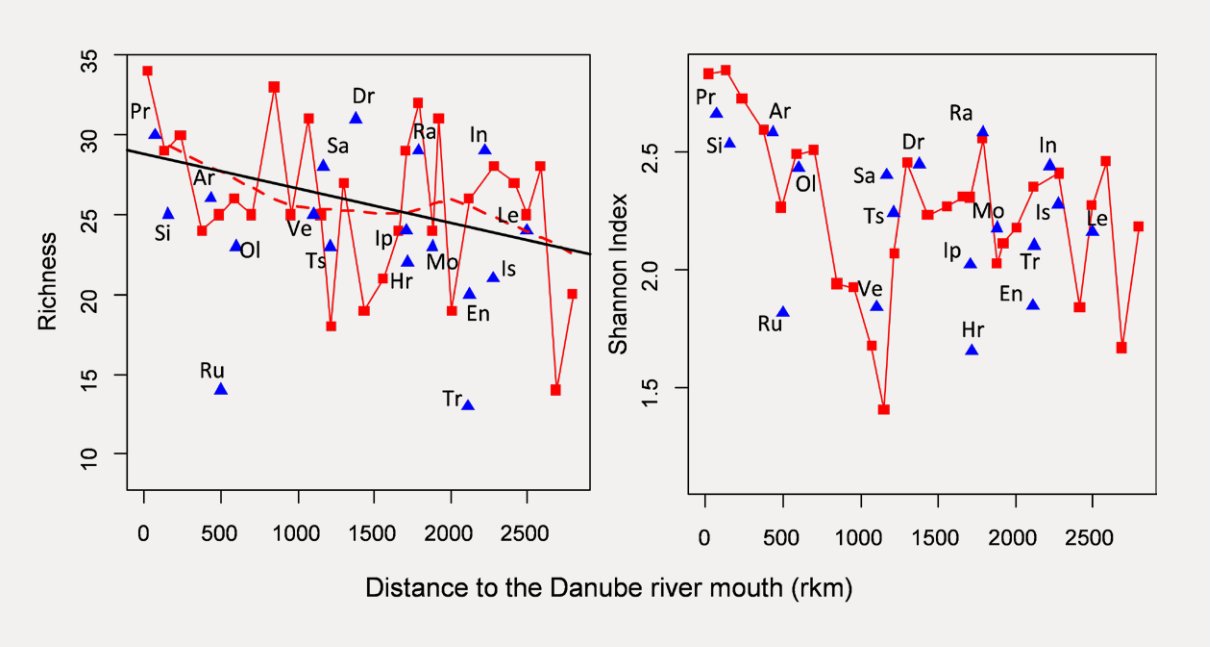

Figure 3: Changes in species richness and diversity (Shannon Index) along the Danube (red) and in major tributaries (blue). Tributary names from upstream to downstream: Lech (Le), Isar (Is), Inn (In), Traun (Tr), Enns (En), Morava (Mo), Raab (Ra), Hron (Hr), Ipel (Ip), Drava (Dr), Tsiza (Ts), Sava (Sa), Velika_Morava (Ve), Olt (Ol), Russenski_Lom (Ru), Arges (Ar), Siret (Si), Prut (Pr).

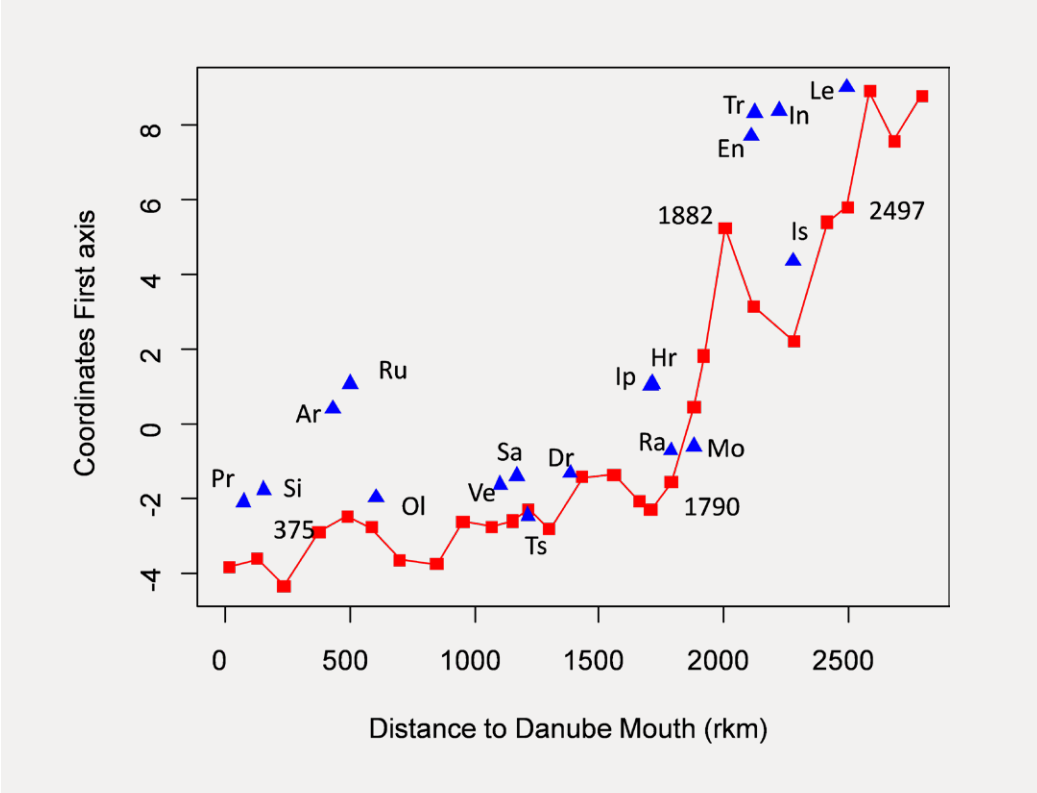

Figure 4: Longitudinal changes in site coordinates on the first axis of a principal component analysis (log-transformed standardized number of reads per taxa).

14.3.3 Comparison with JDS4 traditional fish survey (TFS)

69 and 57 species/markers were detected along the Danube River by the TFS and eDNA surveys, respectively, and 50 of these taxa were detected by both methods. The eDNA method identified 39 of them at the species level, and the remaining 11 at a higher taxonomic level (mainly genus, see Table 1). Nine species were captured by TFS alone: except for *Ballerus ballerus, Barbus peloponnesius* and *Ameiurus nebulosus*, no eDNA markers were available in the utilised reference library for the six remaining species (*Alburnus chalcoides, Clupeonella cultriventris, Eudontomyzon danfordi, Eudontomyzon mariae, Neogobius eurycephalus, Sabanejewia bulgarica*) – hence a detection on species level was methodologically not possible. At the opposite, eight species were only detected by eDNA. Except for the Salvelinus group, these were all benthic species, which are difficult to catch by electrofishing in large rivers (*Acipenser ruthenus, Acipenser stellatus, Benthophilus sp., Romanogobio uranoscopus, Sabanejewia balcanica, Umbra krameri*).

Comparing the relative abundance (based on individuals or biomass, respectively, sequence reads) of several dominant fish taxa at the 13 common sites differed between TFS and eDNA methods (Fig. 5). While *A. alburnus* was the dominant species from TFS samples, both in terms of abundance (58.7%) and biomass (40.3%), this sub-surface species represented only 3.3% of the total number of eDNA reads. At the opposite, benthic species such as *N. melanostomus, B. gymnocephalus, P. kessleri* and *Z. streber* were more abundant in eDNA samples (respectively 31.2%, 10.5%, 4.2% and 1.7%). Other species (e.g. *Abramis brama, Alosa* spp.) showed a similar pattern.

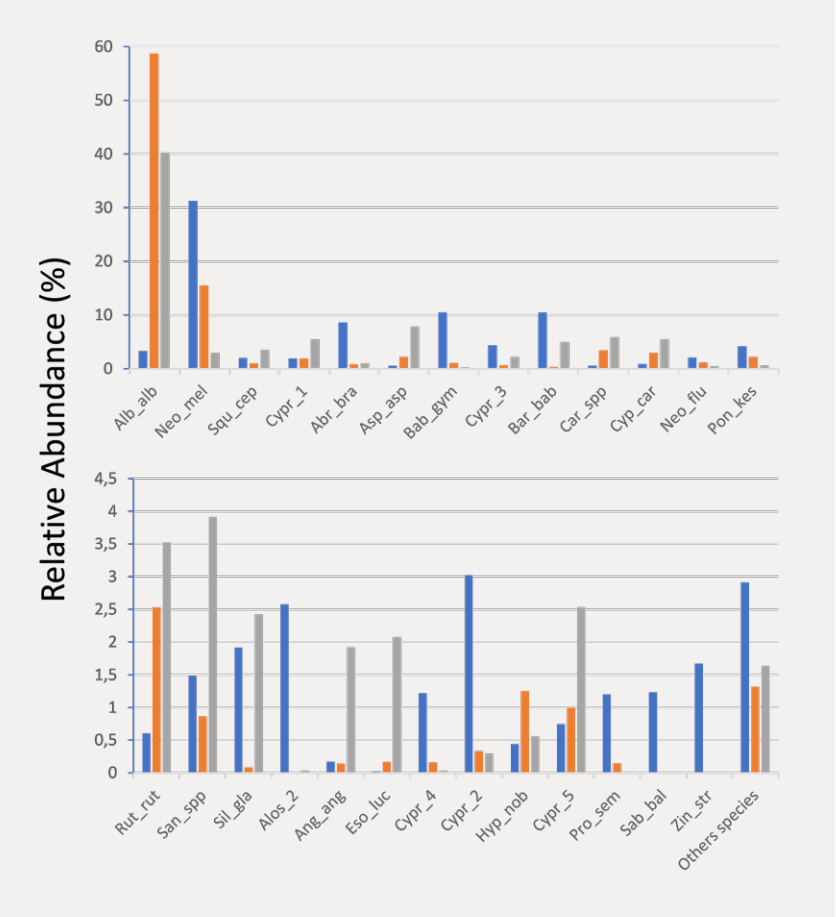

Figure 5: Mean relative abundance of taxa detected by eDNA (blue).
Mean relative abundance (orange) and mean relative biomass (grey) of species caught by TFM.
Only the 26 most abundant species (> 1%) detected among the 13 common Danube sites are individually represented.

14.3.4 Fish-based assessment using eDNA data

The indicative ecological status of the Upper Danube, calculated with eDNA data, was always moderate (Fig. 6). It improved in the Middle Danube (Slovak border to upstream Belgrade) with 3 of the 5 sites classified as Good. From downstream Belgrade to the Iron Gate, the situation deteriorated with sites classified as Moderate or Poor. The situation remained similar downstream but improved significantly in the last 300 river km. All tributaries were classified as moderate, except for the Raab River (Good), the Isar river (Poor) and the Russenki Lom River (Poor). Three sites are ranked in good status (High, Good) by eDNA instead of degraded (Moderate, Poor), due to the highest relative abundance of benthic oxygen intolerant species (Z. streber, P. marmoratus). Comparison of indicative ecological status calculated using the same assessment method from TFS and eDNA data at the common Danube sites showed a similar classification for six of the 13 sites and a difference of one class for the remaining seven sites (Table 2).

Table 2: Comparison of ecological status calculated using the same method from TFS and eDNA data at the 13 common Danube sites.

			TFS		eDNA	
Site_code	**Site**	**River_km**	**Index value**	**Class**	**Index value**	**Class**
JDS4-3	Kelheim	2415	0.445	4_poor	0.628	3_moderate
JDS4-8	Oberloiben	2007	0.542	3_moderate	0.646	3_moderate
JDS4-18	Gonyu	1790	0.639	3_moderate	0.726	3_moderate
JDS4-22	Szob	1707	0.631	3_moderate	0.768	3_moderate
JDS4-23	US_Budapest	1660	0.684	3_moderate	0.792	2_good
JDS4-29	Hercegszanto	1434	0.733	3_moderate	0.829	2_good
JDS4-31	Ilok_Backa_Palanka	1300	0.668	3_moderate	0.733	3_moderate
JDS4-37	Downstream_Pancevo	1151	0.842	2_good	0.594	3_moderate
JDS4-40	Banatska_Palanka	1071	0.723	3_moderate	0.598	3_moderate
JDS4-41	Upstream_Timok	849	0.617	3_moderate	0.471	4_poor
JDS4-47	Downstream_Ruse	488	0.769	3_moderate	0.637	3_moderate
JDS4-48	Chiciu_Silistra	375	0.726	3_moderate	0.403	4_poor
JDS4-50	Reni	130	0.69	3_moderate	0.844	2_good

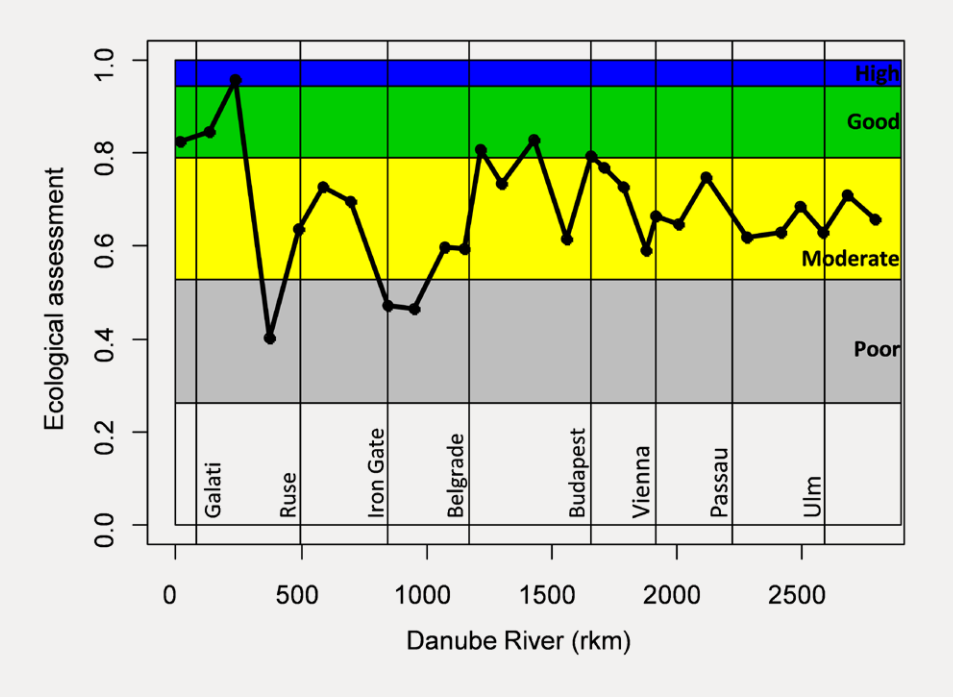

Figure 6: eDNA fish-based ecological assessment of the Danube River.

14.3.5 Comparison of eDNA markers and reference libraries for fish

A second eDNA survey was performed at eight sites in the Lower Reach of the Danube. Environmental DNA water samples were taken as two site replicates and the fish community investigated via the 12S marker gene using the Teleo primers (as in the first eDNA survey), respectively, MiFish primers (Miya et al., 2015). Taxa were taxonomically annotated using the EMBL vertebrate v144 database, respectively, a local European freshwater fish 12S MiFish database. A species match was accepted in case a taxon had ≥ 97% sequence identity and more than 0.01% of reads per sample and within the overall dataset.

The results clearly demonstrate that the choice of primer and reference database are important aspects for the interpretation of the eDNA-based ecological assessment. In the optimal case, all species detections are congruent between different primer combinations and reference databases used. However, in reality, different primer pairs can taxonomically resolve or amplify species differently. As such, *Tinca tinca, Umbra krameri, Sicydium altum, Benthophilus sp., Cobitis elongatoides, Acipenser ruthenus, A. stellatus, Perccottus glenii, Neogobius fluviatilis, Zingel streber* and *Z. zingel* were only detected using Teleo-primers and the local reference database of the first eDNA survey, whereas e.g. *Atherina pontica, Carassius auratus, C. gibelio, Hypophthalmichthys molitrix, Gymnocephalus cernua, G. baloni, Ballerus sapa, Blicca bjoerkna, Leuciscus idus, Rutilus virgo, Sander lucioperca* and *S. volgensis* were only resolved on species level or detected at all by the MiFish primers. Furthermore, different reference libraries can contain synonyms (e.g. *Aspius aspius / Leuciscus aspius, Syngnathus caspicus / S. abaster*) or outdated taxonomic annotations (e.g. *Proterorhinus semilunaris / P. marmoratus, Rhodeus sericeus amarus / R. amarus*) leading to initially conflicting results. Finally, the MiFish primers in combination with the 12S EU reference library suggested a larger number of currently unknown fish species for the Danube catchment, whose taxonomic annotation and origin (i.e. eDNA trace) has to be checked further.

Thus, to increase data robustness, results should be (and were) compared with traditional fish surveys (former and present data) to check for their plausibility.

14.4 Conclusions

- eDNA metabarcoding produced similar results and ecological status assessments when compared to traditional electrofishing data
- eDNA-based assessment was particularly suitable for benthic fish species difficult to catch by electrofishing in large rivers
- Traditional abundance data and relative abundances inferred from eDNA sequence reads were not comparable, but both produced plausible longitudinal successions of fish communities along the Danube River
- eDNA traces originating from wastewater treatment plants, farming or gaming fish species artificially increased the list of fish species detected in the Danube catchment
- occasional flooding events or high pollution levels (via inhibition) can (locally) prohibit successful eDNA metabarcoding application
- eDNA metabarcoding surveys for fish based on different primer pairs and reference databases can lead to contrasting species list. A harmonized eDNA approach and completed fish reference library must be envisaged for JDS5

14.5 References

COUNCIL OF THE EUROPEAN COMMUNITIES (2000). Directive 2000/60/EC of the European Parliament and of the Council of 23 October 2000 establishing a framework for Community action in the field of water policy. Q15 Official Journal of the European Communities, L327, 1–73.

KOTTELAT, M. & FREYHOF, J. (2007). Handbook of European freshwater fishes. Kottelat, Comol, Switzerland and Freyhof, Berlin, Germany.

MILHAU, T., VALENTINI, A., POULET, N., ROSET, N., JEAN, P., GABORIAUD, C. & T. DEJEAN (2020). Seasonal dynamics of riverine fish communities using eDNA. Journal of fish biology. DOI: 10.1111/jfb.14190.

MIYA, M., SATO, Y., FUKUNAGA, T., SADO, T., POULSEN, J. Y., SATO, K., ... & M. KONDOH (2015). MiFish, a set of universal PCR primers for metabarcoding environmental DNA from fishes: detection of more than 230 subtropical marine species. Royal Society open science, 2(7), 150088.

PONT, D. (coordinator) (2011). Water Framework Directive. Intercalibration Phase 2. River Fish European Intercalibration Group. Final Report to ECOSTAT.

PONT D., P. BADY, M. LOGEZ & J. VESLOT (2009). Final development and validation of the new European Fish Index and method, including a complete technical description of the new method. EFI+ Project. Improvement and spatial extension of the European Fish Index., 6th Framework Programme Priority FP6-2005-SSP-5-A. N° 0044096, 179 pages.

PONT, D., ROCLE, M., VALENTINI, A., CIVADE, R., JEAN, P., MAIRE, A. ROSET N., SCHABUSS M., ZORNIG H. & T. DEJEAN (2018). Environmental DNA reveals quantitative patterns of fish biodiversity in large rivers despite its downstream transportation. Sci Rep, 8, 13.

PONT, D., VALENTINI, A., ROCLE, M., MAIRE, A., DELAIGUE, O., JEAN, P. & T. DEJEAN (2019). The future of fish-based ecological assessment of European rivers: from traditional EU Water Framework Directive compliant methods to eDNA metabarcoding-based approaches. Journal of Fish Biology. DOI: 10.1111/jfb.14176.

R CORE TEAM (2018). A language and environment for statistical computing. R foundation for statistical Computing (Vienna, Austria)

SOMMERWERK, N., BAUMGARTNER, C., BLÖSCH, J., HEIN, T., OSTOJIC, A., PAUNOVIC, M., SCHNEIDER-JAKOBY, M., SIBER, R. & TOCKNER, K. (2009). The Danube River Basin. In: Tockner, K., Uehlinger, und & Robinson, C. T. (Hrsg.), Rivers of Europe. Elsevier Academic Press, Amsterdam, S. 59-112

ERŐS, T., BAMMER, V., GYÖRGY, A.I., PEHLIVANOV, L., SCHABUSS, M., ZORNIG, H., WEIPERTH, A. & SZALÓKY, Z. (2017). Typology of a Great River Using Fish Assemblages: Implications for the Bioassessment of the Danube River. River Research and Applications, 33(1), 37-49.

VALENTINI, A., TABERLET, P., MIAUD, C., CIVADE, R., HERDER, J., THOMSEN, P.F. et al. (2016). Next-generation monitoring of aquatic biodiversity using environmental DNA metabarcoding. Molecular Ecology, 25, 929-942.

15

Metabarcoding of phytobenthos samples

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Abstract

Phytobenthos samples were collected at 69 sites during JDS4. Whenever possible, samples were obtained from both riverbanks and analysed by DNA metabarcoding with two markers, 18SV4 and rbcL. The genera with the most Sequence Units were Nitzschia, Navicula, Sellaphora and Amphora. The most abundant species were Navicula cf. ramosissima, N. tripunctata and Melosira varians. Community composition shows only a weak longitudinal pattern when sorted by reach or river typology, but correlated well with temperature, dissolved oxygen, total organic carbon and conductivity. In several cases, communities obtained from the right and left riverbank at a given JDS4 site were quite different. In conclusion, although DNA metabarcoding identified less taxa at species level than classical light microscopy during JDS4 because of gaps in barcode reference libraries, the unassigned and hidden diversity present in the DNA-based datasets (i.e. intraspecific genetic diversity or morphologically cryptic lineages) help to better understand the impact of environmental variables and to describe community composition. So far, the metabarcoding approach was able to reveal 78% of all the most abundant diatom species identified in JDS2, 3 and 4, as well as six of the seven dominant taxa identified by light microscopy in JDS4. However, more complete reference databases and adjusted sampling designs will allow higher proportions of species-level matches in the future.

15.1 Introduction

This chapter shows the results of the metabarcoding analysis for the 18SV4 and *rbc*L markers. The metabarcoding approach could potentially provide a more objective, faster and less expensive way of identifying species at a higher taxonomic resolution for more refined diatom diversity assessments than possible by only using the traditional method. So far, no DNA metabarcoding studies have been conducted across the largest river systems in Europe, such as in the Danube or the Rhine, despite the fact that this method can potentially provide a faster and more reproducible way in characterizing diatom communities for their use in ecological assessment, complementing the microscopy-based method.

15.2 Methods

Benthic diatoms were sampled from 29 June 2019 up to 2 weeks (except for JDS4-5L, which was sampled in September) following the European standard (EN 13946:2014, CEN, 2014). For details on the sampling procedure, please refer to Chapter 7. The 69 bulk DNA metabarcoding samples were preserved with 97% ethanol (final concentration approximately 70%) as well as deep-frozen as fast as possible to protect the DNA from degradation in long term storage (Stein et al., 2013).

Samples were defrosted and pellets of biofilms were prepared by centrifuging between 2 and 4 ml of the initial biofilm suspension at 3x g for 30 min. DNA extraction was performed using the Macherey & Nagel NucleoSpin® Soil kit (MN-Soil) following the manufacturer's instructions. Two molecular markers were amplified by PCR, the nuclear-encoded V4 region of the 18S rRNA gene according to Visco et al. (2015) and a fragment of the plastid *rbc*L gene according to Vasselon et al. (2017). Three PCR replicates were performed for each sample. Library preparation was performed following Mora et al. (2019). The libraries were quantified with the Qubit® dsDNA HS (High Sensitivity) Assay Kit, quality checked on the Agilent Tapestation and sequenced on a MiSeq machine using paired-end sequencing for 600 cycles with Standard kit v3. Sequencing was performed at the BGBM in the BeGenDiv consortium on the Illumina MiSeq platform.

Illumina sequence reads were processed with the MetBaN pipeline (Proft et al., 2017, Bailet et al., 2019) for the 18SV4 marker, clustering reads into Molecular Operational Taxonomic Units (MOTUs) at a 3-bp (base-pair) distance, equivalent to a 1% dissimilarity threshold. Sequences were considered assigned to species level if they showed genetic similarities of ≥99% to a reference sequence. For *rbc*L, the software package DADA2 (Callahan et al., 2016), which generates Amplicon Sequence Variants (ASVs), was adapted to process HTS diatom data as used in Tapolczai et al. (2019) and is available on Github (https://github.com/fkeck/DADA2_diatoms_pipeline). MOTUs and ASVs obtained from both markers are referred to as Sequence Units (SUs) (see Bailet et al., 2020).

Non-metric multidimensional scaling (NMDS) was performed on the molecular dataset, using Bray–Curtis dissimilarity index to study the pattern of diatom assemblages. Sites were grouped by river typology and analysis of similarities (ANOSIM) was conducted. It tests whether the similarity between groups is different from the similarity within the groups. The test statistic R varies between -1 and 1 where higher values indicate higher similarity within sites than between sites.

15.3 Results and discussion

15.3.1 DNA-based species and genus level annotations

The 69 phytobenthos samples analysed resulted in 11,748,888 quality-filtered reads for the 18SV4 marker from a total of 179 replicate-samples from one sequencing run, with diatoms being the dominant algal group, representing 63.7% of those sequence reads. These reads were clustered into 22,246 SUs of diatoms. From those, 5,539 SUs were identified at the species level corresponding to 162 individual diatom species within 60 genera (Table 1).

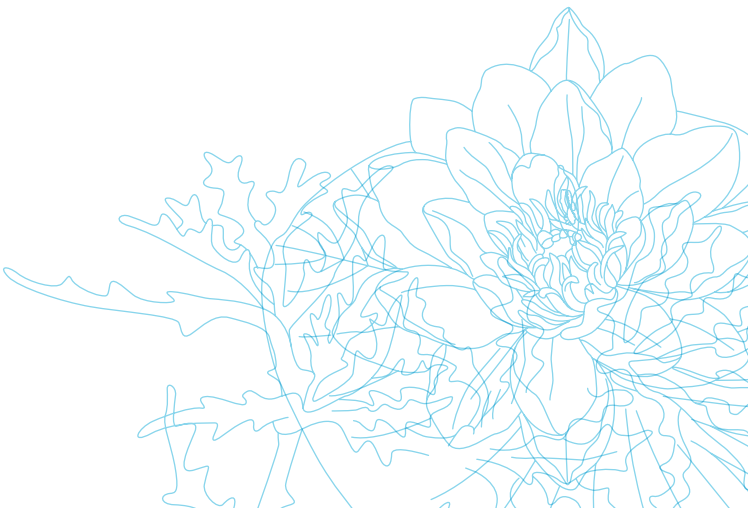
Two sequencing runs of 309 replicate-samples for the *rbc*L marker resulted in 12,051,653 high-quality reads corresponding to 2,694 SUs. 2% of the reads could not be affiliated to diatom classes. After post-bioinformatic treatment (taxonomy, SU read number and length control, rarefaction), the number of reads was reduced to 11,741,533 (1,617 SUs), with 33 replicate-samples excluded from the analyses.

For 18SV4, the genera with most SUs per genus assigned to species level were *Nitzschia, Navicula* and *Amphora* (Table 1). Regarding *rbc*L, the genera with the most SUs were also *Nitzschia* and *Navicula*, but also *Gomphonema*. Some genera were detected by a single marker only, but most often consisted of a single species each. Two notable exceptions refer to *Fallacia* (3 species) and *Iconella* (4), which were only detected by *rbc*L. The majority of genera revealed by both markers are predominantly benthic, e.g. *Achnanthidium, Cocconeis, Nitzschia, Navicula,* and *Planothidium*, which is in accordance with the sampled environment.

One of the limitations that hinders the taxonomic assignment of more SUs down to species level are gaps in DNA barcode reference libraries. These gaps or lack of reference sequences are particularly large for diatoms, compared to fish or plants. Only 15% of the diatom species present in Europe have at least one sequence of the barcoding markers *rbc*L or 18SV4. However, the most common benthic species in freshwater monitoring are better represented in reference libraries, reaching 70% by both markers (Weigand et al., 2019). For the Danube catchment and based on the JDS3 diatom species list, 69% of the 307 taxa and 88% of the 52 most abundant taxa have available 18S or *rbc*L barcode reference data (see Chapter 12).

Table 1: Number of species per genus after taxonomic assignment of Sequence Units with 18SV4 reference sequences from the Nucleotide Sequence Database of EMBL, as well as rbcL Sequence Units with Diat.barcode (Rimet et al., 2019), a curated barcode library for diatoms. Species number based on 18SV4 is given first, followed by rbcL.

Genus	Species	Genus	Species	Genus	Species	Genus	Species
Acanthoceras	1 | 1	*Diadesmis*	0 | 1	*Hannaea*	0 | 1	*Pleurosigma*	0 | 1
Achnanthes	1 | 1	*Diatoma*	2 | 3	*Hantzschia*	2 | 2	*Pleurosira*	2 | 1
Achnanthidium	3 | 6	*Diploneis*	0 | 1	*Haslea*	1 | 0	*Psammodictyon*	0 | 1
Adlafia	0 | 1	*Discostella*	2 | 3	*Hippodonta*	1 | 1	*Psammothidium*	1 | 2
Amphipleura	1 | 0	*Ellerbeckia*	0 | 1	*Iconella*	0 | 4	*Punctastriata*	1 | 0
Amphora	8 | 4	*Encyonema*	3 | 6	*Karayevia*	0 | 1	*Reimeria*	1 | 1
Anomoeoneis	1 | 0	*Encyonopsis*	0 | 3	*Lemnicola*	1 | 1	*Rhoicosphenia*	1 | 1
Asterionella	0 | 1	*Entomoneis*	0 | 1	*Lindavia*	0 | 1	*Rossithidium*	1 | 0
Aulacoseira	1 | 3	*Epithemia*	1 | 2	*Luticola*	1 | 2	*Sellaphora*	6 | 7
Brachysira	0 | 1	*Eunotia*	2 | 4	*Mayamaea*	1 | 3	*Skeletonema*	2 | 2
Brebissonia	1 | 0	*Fallacia*	0 | 3	*Melosira*	1 | 1	*Stauroneis*	2 | 1
Caloneis	2 | 3	*Fistulifera*	4 | 1	*Meridion*	0 | 1	*Staurosira*	2 | 4
Campylodiscus	1 | 0	*Fragilaria*	6 | 4	*Nanofrustulum*	0 | 1	*Stephanodiscus*	3 | 4
Cocconeis	2 | 3	*Fragilariforma*	1 | 0	*Navicula*	12 | 19	*Surirella*	6 | 3
Conticribra	2 | 1	*Fragilariopsis*	0 | 1	*Neidium*	1 | 2	*Tabularia*	1 | 1
Craticula	5 | 4	*Frustulia*	1 | 1	*Nitzschia*	19 | 30	*Thalassiosira*	4 | 4
Cyclostephanos	1 | 4	*Gedaniella*	1 | 0	*Parlibellus*	0 | 1	*Tryblionella*	2 | 3
Cyclotella	3 | 3	*Geissleria*	1 | 2	*Pauliella*	2 | 0	*Ulnaria*	2 | 2
Cymbella	3 | 5	*Gomphonema*	7 | 13	*Pinnularia*	5 | 7	*Urosolenia*	0 | 1
Cymbopleura	0 | 2	*Gyrosigma*	1 | 1	*Placoneis*	2 | 1		
Denticula	0 | 1	*Halamphora*	2 | 3	*Planothidium*	4 | 5		



15.3.2 Longitudinal variation of community composition and relative frequencies of species

In order to analyse general changes in the phytobenthos community structure, metabarcoding data were Hellinger transformed and non-metric multidimensional scaling (NMDS) was performed using the Bray-Curtis dissimilarity index. Sites were then grouped based on being situated in similar river typologies (type 1-9). Analyses of similarities (ANOSIM) were conducted based on the 18SV4 and *rbc*L datasets. In both cases, similarities within river type groups are significantly greater ($p<0.01$) than similarities between groups (Figure 1). The R-value is however higher for the *rbc*L data set (R=0.23) than for 18SV4 (R=0.17).

According to 18SV4, the most abundant species in the Danube main channel were *Navicula* sp. 1 (according to assignment data most likely N. cf. *ramosissima* with some uncertainty), *N. tripunctata, N. antonii* and *Gomphonema minutum*, reaching relative abundances ≥ 5% in at least one of the reaches (Table 2). In the tributaries, *Navicula* cf. *ramosissima* and *N. tripunctata* were also found in high relative abundance, together with *Achnanthidium minutissimum, Cyclotella meneghiniana, Cocconeis pediculus, C. placentula, and Gomphonema sp.* and *Nitzschia palea*, all of them reaching relative abundances of ≥ 5% in any of the three groups of tributaries (Upper, Middle and Lower).

Based on *rbc*L, the most abundant species in the Danube were *Navicula* cf. *ramosissima, Melosira varians, Diatoma vulgaris, Nitzschia palea* and *Amphora pediculus* (Table 3). In the tributaries, *M. varians, N.* cf. *ramosissima* and *N. palea* were also the most abundant species along with *Pleurosira laevis* and *Achnanthidium delmontii* (Table 3).

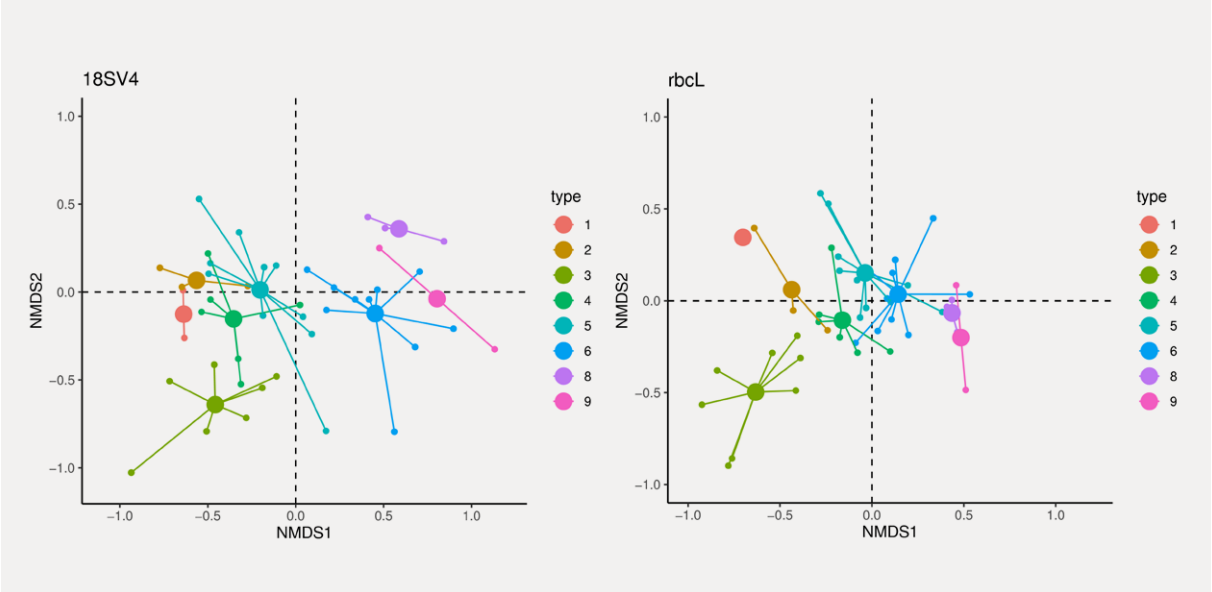


Figure 1: NMDS plots indicating similarities of community composition for both markers according to river type groups.

Table 2: List of the most abundant (relative abundance in %, 18S data) taxa in the Danube (Upper, Middle and Lower reaches), as well as in the Danubian tributaries, also grouped according to which of the three reaches they join in the Danube. Relative abundances for taxa higher than 1% in a reach or tributary group are marked in bold.

Taxon name	**Danubian reach**			**Danubian tributary**		
	Upper	Middle	Lower	Upper	Middle	Lower
Navicula cf. *ramosissima*	**2.90**	**23.58**	**42.48**	0.28	**17.48**	**5.94**
Navicula tripunctata	**13.70**	**4.41**	0.33	**3.96**	**2.83**	**7.23**
Cocconeis placentula	**1.02**	**4.15**	**4.31**	**4.09**	**5.60**	**1.31**
Navicula antonii	**3.05**	**5.03**	**1.19**	**1.57**	**1.31**	0.14
Gomphonema minutum	**5.60**	**3.13**	0.13	**2.10**	0.59	0.46
Gomphonema parvulum	**4.41**	**2.08**	0.49	0.94	0.70	0.86
Diatoma vulgaris	**4.82**	0.02	0.00	0.01	**1.24**	0.00
Navicula cryptotenella	**3.03**	0.69	0.59	**2.01**	**1.11**	**3.17**
Rossithidium anastasiae	**3.60**	0.33	0.00	0.56	0.87	0.00
Cocconeis cf. *placentula*	**1.05**	**2.65**	0.05	0.27	**1.53**	0.00
Skeletonema potamos	0.08	0.98	**2.39**	0.21	0.77	0.02
Melosira varians	**2.73**	0.39	0.31	**4.32**	**1.95**	0.45
Nitzschia palea	0.43	**1.48**	**1.47**	**3.61**	**2.22**	**2.06**
Gomphonema pumilum	**1.10**	**1.41**	0.17	**1.01**	0.23	0.03
Navicula sp. 2	0.58	0.88	0.96	0.63	0.74	**3.15**
Navicula sp. 3	**1.44**	0.95	0.00	0.05	0.00	0.00
Amphora sp.	0.31	**1.59**	0.15	0.36	0.06	0.06
Navicula radiosa	0.44	0.76	0.79	0.39	0.19	**1.90**
Navicula gregaria	**1.15**	0.75	0.03	0.39	0.04	0.20
Surirella librile	0.32	0.90	0.69	0.09	0.17	0.00
Surirella cf. *minuta*	0.61	**1.06**	0.21	**2.10**	0.27	**1.55**
Cyclotella meneghiniana	0.28	**1.37**	0.02	**1.72**	**7.73**	0.04
Rhoicosphenia abbreviata	0.67	0.27	0.08	**1.84**	0.70	0.39
Gomphonema sp.	0.71	0.23	0.08	0.05	0.05	**6.59**
Navicula veneta	0.08	0.41	0.25	0.35	0.82	**1.64**
Cocconeis pediculus	0.51	0.03	0.01	**7.94**	**1.19**	0.13
Amphora berolinensis	0.17	0.29	0.05	**1.35**	0.46	0.28
Achnanthidium minutissimum	0.00	0.01	0.01	0.00	0.00	**12.24**
Pleurosira laevis	0.00	0.00	0.00	**2.13**	0.00	0.00
∑	54.77	59.83	57.24	44.32	50.86	49.84

Table 3: List of the most abundant (relative abundance in %, rbcL data) taxa in the Danube (Upper, Middle and Lower reaches), as well as in the Danubian tributaries, also grouped according to which of the three reaches they join in the Danube. Relative abundances for taxa higher than 1% in a reach or tributary group are marked in bold.

Taxon name	Danubian reach			Danubian tributary		
	Upper	Middle	Lower	Upper	Middle	Lower
Achnanthidium delmontii	**5.37**	0.82	0.02	**1.34**	**1.17**	0.01
Achnanthidium minutissimum	**1.35**	0.17	0.23	0.97	0.16	**18.16**
Amphora ovalis	**1.69**	**2.73**	**1.41**	**5.07**	**4.20**	**1.12**
Amphora pediculus	**4.82**	**8.88**	**1.61**	**4.73**	**3.56**	**3.18**
Cocconeis placentula	0.59	**1.83**	**1.41**	**1.42**	**1.71**	0.44
Craticula subminuscula	0.76	0.98	**4.97**	**1.49**	**1.86**	0.02
Cyclotella meneghiniana	**1.19**	**2.30**	0.06	**1.09**	**11.13**	0.43
Diatoma vulgaris	**15.79**	0.12	0.01	0.24	**3.29**	0.10
Discostella sp.	0.68	0.26	0.00	0.62	**1.02**	0.01
Fistulifera saprophila	**2.38**	**3.27**	0.54	0.51	**1.20**	0.06
Gomphonema rosenstockianum	**2.25**	0.77	0.01	0.03	0.00	0.00
Gomphonema saprophilum	**3.42**	**2.67**	0.89	**1.01**	**1.00**	**1.04**
Gomphonema tergestinum	**3.30**	**3.65**	0.04	0.04	0.03	0.00
Mayamaea permitis	0.84	**1.50**	**1.45**	0.65	**1.63**	0.71
Melosira varians	**8.25**	**2.50**	**6.90**	**8.39**	**5.50**	**7.97**
Navicula antonii	**1.32**	**3.11**	**1.26**	0.62	0.98	0.09
Navicula cryptotenella	**1.87**	**1.31**	**1.01**	**1.65**	**1.23**	**3.75**
Navicula gregaria	0.81	0.71	0.03	0.61	0.03	0.24
Navicula lanceolata	**2.31**	0.35	0.00	0.61	0.01	0.00
Navicula cf. *ramosissima*	**2.16**	**20.48**	**36.98**	0.27	**16.10**	**5.37**
Navicula tripunctata	**3.86**	**1.41**	0.18	0.53	0.69	**4.66**
Nitzschia dissipata var. *media*	**7.96**	**4.05**	**1.03**	**1.12**	0.62	**2.86**
Nitzschia inconspicua	0.16	0.74	**1.71**	0.36	**1.00**	0.11
Nitzschia palea	**1.27**	**6.09**	**8.24**	**4.95**	**6.66**	**11.68**
Nitzschia paleacea	**2.20**	0.60	0.14	0.22	0.13	0.03
Pleurosira laevis	0.05	0.10	0.72	**27.67**	0.71	0.14
Sellaphora minima	0.19	0.32	**1.70**	**1.79**	**1.35**	**7.02**
Skeletonema potamos	0.06	**1.37**	**1.64**	0.20	**1.04**	0.02
Ulnaria ulna	**2.03**	0.37	**1.57**	**2.30**	**1.53**	**5.98**
∑	78.93	73.46	75.76	70.5	69.54	75.2

15.3.3 Correlation of community composition with environmental variables

In order to correlate the 18S-based SU data with environmental variables obtained during JDS4, the two datasets had to be harmonized, which led to a loss of eleven sampling sites (i.e. 58 remaining). This slight reduction of samples also led to a decrease of SUs by eliminating those that were present only in the removed samples. A total of 21,980 SUs remained in the final dataset. Environmental variables used in further analyses were: Alkalinity, BOD_5, chlorophyll-*a*, COD, Conductivity, DO, DOC, NH_4-N, NO_2-N, NO_3-N, O_2 saturation, pH, PO_4-P, Suspended solids, temperature, TN, TOC, TP.

Temperature (R^2 = 0.70; p = 0.001), dissolved oxygen (DO; R^2 = 0.47; p = 0.001), organic carbon (TOC; R^2 = 0.43; p = 0.001) and conductivity (R^2 = 0.41; p = 0.001) correlated best with changes in community composition.

15.3.4 Comparison of taxa detected by DNA metabarcoding and morphology

Within JDS4, DNA metabarcoding was applied in parallel to classical light microscopy to detect diatom species (see Chapter 7). Both approaches resulted in different numbers of taxa identified to species level, with 385 taxa for the morphological approach, whereas with metabarcoding 221 (*rbc*L) and 160 (18SV4) taxa were identified, respectively. However, significantly more SUs could be assigned as diatom taxa on the genus level, showing the potential to measure the hidden diversity. As such, and even though the number of species-level identifications is lower in both metabarcoding datasets, the high amount of additional biological information detected by metabarcoding (e.g. intraspecific diversity, morphologically cryptic lineages) helps us better understand community changes along environmental gradients.

The contrasting numbers of species-level identifications can be mainly explained by gaps in and the taxonomic inconsistencies of reference libraries. For example, most of the species in the genus *Achnanthidium* given in the morphological results of JDS2, JDS3 and JDS4 are not present in any reference database, and, unfortunately, this is applicable for several other species-rich genera as well. In these and other genera (e.g. *Achnanthidium, Navicula, Nitzschia*) a broad list of SUs could be assigned to the genus level representing the genetic diversity detected. Still, the metabarcoding approach was able to reveal 78% of all the most abundant diatom species identified in JDS2, JDS3 and JDS4, as well as six of the seven dominant taxa identified by light microscopy in JDS4 (Table 4).

*Table 4: Comparative table of the most abundant species in the Joint Danube Surveys 2-4. Only species that reached at least 2% relative abundance on average are included and marked in bold. For JDS4 samples that were analysed molecularly (metabarcoding data for both 18SV4, rbcL), refer to Table 3 and 4 for relative abundances per reach and tributary type. * refers to taxa that were formerly part of the Cocconeis placentula species complex and were lumped into this taxon in the molecular analysis. Species in light grey mark taxa for which no barcode reference data (18SV4, rbcL) is available.*

Taxon name	**Morphology**		**Metabarcoding**	
	JDS2	JDS3	JDS4	JDS4
Achnanthidium atomoides		**3.95**		
Achnanthidium catenatum		**2.13**		X
Achnanthidium delmontii			**≥5**	**X**
Achnanthidium eutrophilum		**2.78**		
Achnanthidium minutissimum		**3.17**		X
Amphora pediculus	**3.55**	**7.3**	**≥5**	X
Cocconeis euglypta		1.83	**≥5**	**X***
Cocconeis pediculus	1.08	1.4		**X**
Cocconeis placentula var. *lineata*	**4.34**			**X***
Craticula subminuscula		**2.15**		X
Cyclotella atomus		**3.96**		X
Cyclotella meneghiniana	**4.28**	**8.49**	**≥5**	**X**
Diatoma vulgaris	1.63			**X**
Discostella pseudostelligera		**6.66**		X
Gomphonema minutum		**2.62**		**X**
Gomphonema tergestinum		**3.61**		**X**
Luticola goeppertiana	1.79	**3.03**		X
Luticola hlubikovae		**2.7**		
Mayamaea permitis		**2.33**		X
Navicula antonii	1.28	1.24		**X**
Navicula cryptotenella	**2.96**	**5.68**		**X**
Navicula erifuga	1.61	**2.27**		
Navicula recens	**22.9**	**6.32**	**≥5**	
Navicula tripunctata	**7.53**	1.97		**X**
Navicula rostellata	**3.41**	1.68		X
Nitzschia amphibia	1.02	1.5		X
Nitzschia clausii		**4.97**		
Nitzschia dissipata		**5.4**	**≥5**	X
Nitzschia inconspicua	**3.53**	**19.22**		X
Nitzschia palea	1.15	1.82		**X**
Nitzschia palea var. *debilis*		**2.41**		
Nitzschia sociabilis		**2.88**		
Rhoicosphenia abbreviata	**2.81**	1.23		**X**
Sellaphora nigri ≡ *Eolimna minima*	1.35	**5.72**		X
Sellaphora seminulum		**2.06**		X
Skeletonema potamos			**≥5**	**X**
Ulnaria ulna	0.59			X

15.3.5 Comparison of communities obtained from left and right riverbanks

Comparison of the relative abundances of the most abundant taxa showed large differences between the left and right banks for both markers (Fig. 2). For example, *Cocconeis placentula* was barely detected in the left bank at site JDS4-35 (0.1%), but reached 20% at the right bank; in the same sampling site, *Navicula* cf. *ramosissima*, the most abundant diatom across the Danube reached an abundance of 84% in the left bank but only 13% in the right bank. The reason for this large difference in relative abundances of several taxa might be due to the varying microhabitat conditions, e.g. light exposure and flow within the transects on the two banks. As already shown in Chapter 7, these deviations in abundance data between left and right bank can result in different ecological indicative status. Based on the results obtained, we argue that standard protocols such as sampling within a 10 m long transect should be modified to include longer transects in large rivers like the Danube, to get a more representative sample, as well as sampling both banks whenever possible.

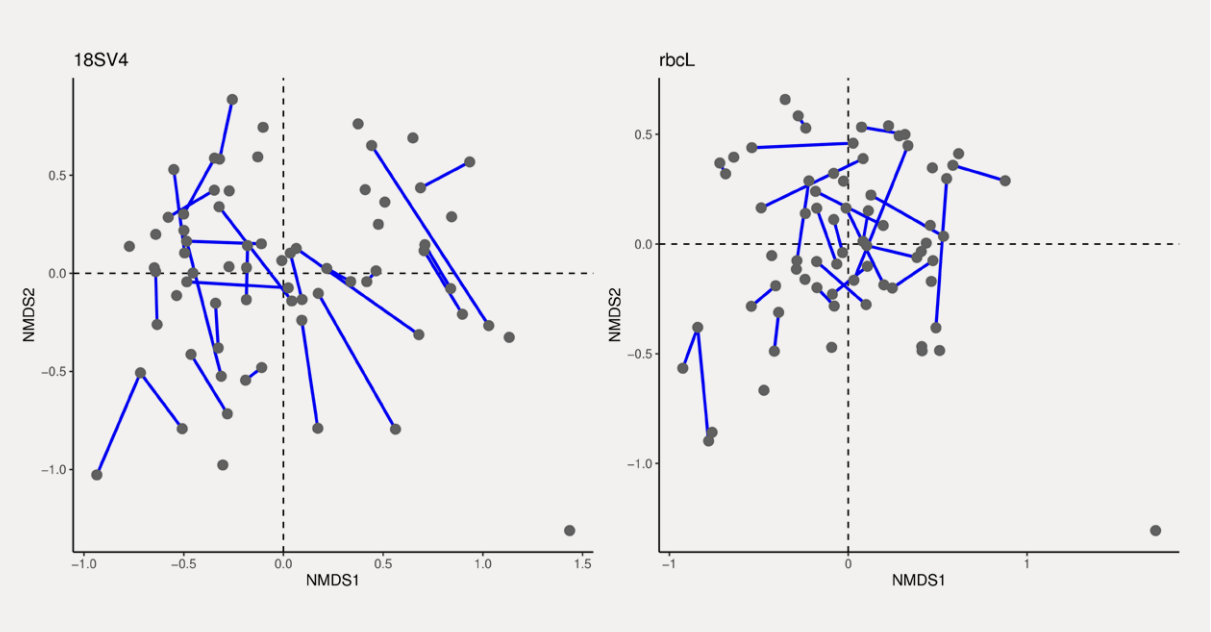


Figure 2: NMDS plots for both metabarcoding markers (18SV4, rbcL) indicating differences in diatom relative abundance data between the right and left river bank sample at a given JDS4 site. The longer the line connecting two points, the more different are two samples.

15.3.6 Index calculation

IPS index calculation was performed with the software OMNIDIA (see Chapter 7 for details). Three different data sets were used for IPS index calculation and their correlations tested by Pearson's r statistic: morphological data (originating from Chapter 7), *rbc*L metabarcoding data as well as 18SV4 metabarcoding data (this chapter). Although the results have to be interpreted with some caution, all three pairwise comparisons indicate a good to strong positive linear correlation (Figure 3), highlighting the potential of DNA-based index calculations.

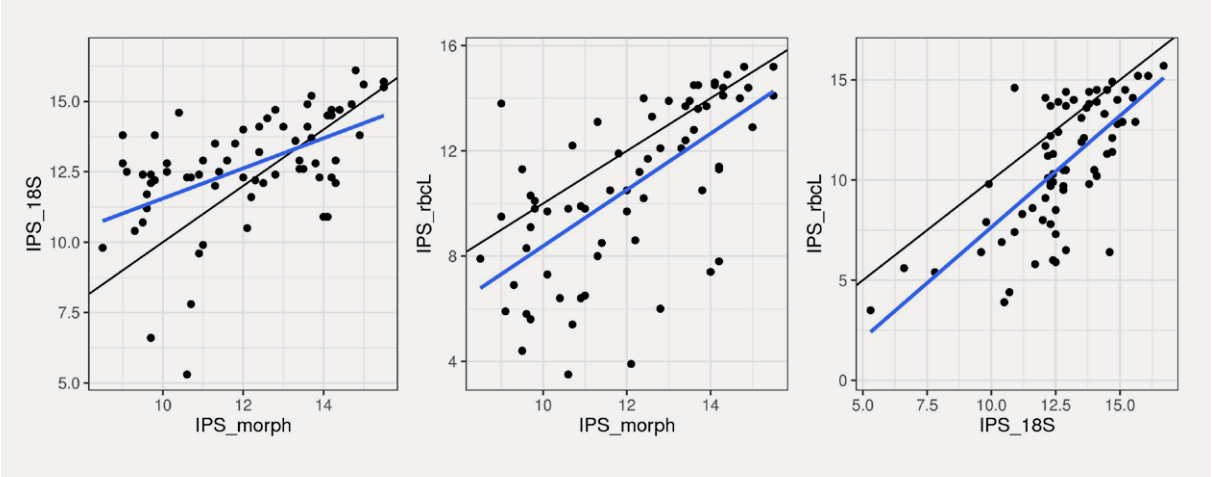


Figure 3: Correlation of IPS index calculations based on morphological, rbcL and 18SV4 metabarcoding data. Pearson's r values are 0.52 (morph-18S), 0.65 (morph-rbcL) and 0.70 (18S-rbcL).

15.4 Conclusions

- 221 diatom species in 72 genera were identified by metabarcoding with *rbc*L; for 18SV4 160 species within 60 genera were identified
- DNA metabarcoding (18SV4 and *rbc*L combined) identified 78% of all the abundant diatom species revealed by light microscopy during JDS2, JDS3 and JDS4
- generally, the number of species detected by light microscopy was higher, since non-living taxa can be identified and are integrated; DNA metabarcoding can not reveal those taxa because of lacking suitable DNA concentrations
- DNA metabarcoding detected many further sequence units which could not be assigned a Linnaean species name 1) due to gaps in reference databases, 2) corresponding to cryptic and semi-cryptic taxa that cannot be differentiated by light microscopy, and 3) also corresponding to intraspecific genetic diversity within a species.
- this hidden diversity can help to better correlate community composition with environmental variables
- temperature, dissolved oxygen, total organic carbon and conductivity have been identified as the best correlating environmental variables describing community composition
- more comprehensive and taxonomically curated DNA barcode reference libraries will constantly increase the proportion of species-level detections in the future
- a complemented approach of morphology- and DNA-based identification promises to provide a comprehensive biodiversity assessment

15.5 References

BAILET, B., APOTHÉLOZ-PERRET-GENTIL, L., BARIČEVIĆ, A., CHONOVA, T., FRANC, A., FRIGERIO, J. M., ... & RAMON, M. (2020). Diatom DNA metabarcoding for ecological assessment: Comparison among bioinformatics pipelines used in six European countries reveals the need for standardization. Science of The Total Environment 745:140948.

CALLAHAN, B. J., MCMURDIE, P. J., ROSEN, M. J., HAN, A. W., JOHNSON, A. J. A., & HOLMES, S. P. (2016). DADA2: High-resolution sample inference from Illumina amplicon data. Nature Methods 13:581–583.

CEN 13946 (2014). Water quality. Guidance for the routine sampling and preparation of benthic diatoms form rivers and lakes. European Committee for Standardization, Brussels.

MORA, D., ABARCA, N., PROFT, S., GRAU, J. H., ENKE, N., CARMONA, J., ... & ZIMMERMANN, J. (2019). Morphology and metabarcoding: a test with stream diatoms from Mexico highlights the complementarity of identification methods. Freshwater Science 38:448–464.

PROFT, S., GRAU, J., CASWARA, C., MAZZONI, C., MORA, D., & ZIMMERMANN, J. (2020). MetBaN automated pipeline for metabarcoding data using taxonomical/phylogenetical classification of organisms. https://doi.org/10.5281/zenodo.1035193

RIMET, F., GUSEV, E., KAHLERT, M., KELLY, M. G., KULIKOVSKIY, M., MALTSEV, Y., ... & ZIMMERMANN, J. (2019). Diat.barcode, an open-access curated barcode library for diatoms. Scientific Reports 9:15116.

STEIN, E. D., WHITE, B. P., MAZOR, R. D., MILLER, P. E., & PILGRIM, E. M. (2013). Evaluating Ethanol-based Sample Preservation to Facilitate Use of DNA Barcoding in Routine Freshwater Biomonitoring Programs Using Benthic Macroinvertebrates. PLOS ONE 8:e51273.

TAPOLCZAI, K., KECK, F., BOUCHEZ, A., RIMET, F., & VASSELON, V. (2019). Diatom DNA Metabarcoding for Biomonitoring: Strategies to Avoid Major Taxonomical and Bioinformatical Biases Limiting Molecular Indices Capacities. Frontiers in Ecology Evolution 7:409.

VASSELON, V., RIMET, F., TAPOLCZAI, K., & BOUCHEZ, A. (2017b). Assessing ecological status with diatoms DNA metabarcoding: Scaling-upon a WFD monitoring network (Mayotte Island, France). Ecological Indicators, 82, 1–12.

VISCO, J. A., APOTHÉLOZ-PERRET-GENTIL, L., CORDONIER, A., ESLING, P., PILLET, L., & PAWLOWSKI, J. (2015). Environmental Monitoring: Inferring the Diatom Index from Next-Generation Sequencing Data. Environmental Science & Technology 49:7597-7605.

WEIGAND, H., BEERMANN, A. J., ČIAMPOR, F., COSTA, F. O., CSABAI, Z., DUARTE, S., ... & EKREM, T. (2019). DNA barcode reference libraries for the monitoring of aquatic biota in Europe: Gap-analysis and recommendations for future work. Science of the Total Environment 678:499-524.

ZIMMERMANN, J., ABARCA, N., ENK, N., SKIBBE, O., KUSBER, W. H., & JAHN, R. (2014). Taxonomic Reference Libraries for Environmental Barcoding: A Best Practice Example from Diatom Research. PLoS ONE 9(9):e108793.

16 Metabarcoding of sediment communities

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Abstract

Community composition of sediment samples was analysed by DNA metabarcoding. Two molecular markers (18S V1V2 and COI) were analysed to generate an overview of community composition on a higher taxonomic level (18S) and to access metazoan species diversity at a closer detail (COI). Three independent sediment cores were analysed at 44 JDS4 sites.

Community composition between the three cores taken at a single site were similar but not identical, indicating that each core added further species to the overall biodiversity present in the Danube – most likely due to microhabitat differences. The 18S marker revealed 12,780 individual genetic sequences, of which the majority could be assigned to Metazoa (198 species). A total of 47 nematode species were detected within the 18S dataset, which were used to calculate a pollution-index based on local nematode community structure. The COI marker revealed 29,494 individual sequences and detected 261 metazoan species. In terms of species richness, the Danubian sediment fauna was dominated by chironomids, oligochaetes, rotifers and mayflies. Additionally, eDNA traces of semi-aquatic and terrestrial taxa were revealed (e.g. beaver, beetles, butterflies, birds, livestock animals).

Both genetic markers contributed specific proportions of the Danubian biodiversity to the overall bioassessment. Primer selectivity, taxonomic resolution of the marker and the variation in reference database completeness might best explain those discrepancies in detection.

16.1 Introduction

Partly due to difficulties in identifying taxa to the level of species, organisms living in sediments are often neglected in biodiversity assessments. However, they comprise some of the most species-rich and ecologically indicative hololimnic groups, such as nematodes, ostracods, oligochaetes or copepods. Furthermore, sediments serve as a habitat for numerous macroinvertebrate species during part of their lives (e.g. caddisflies, chironomids) and as a spawning area for fish. At the same time, sediments can be highly polluted as they accumulate inorganic and organic substances, influencing local community compositions (Landrum & Robbins 1990). The aim of this molecular survey was to characterise the community composition of sediments in the Danube catchment by means of DNA metabarcoding, which has been demonstrated to be an effective method for the bioassessment of in particular hard-to-identify meiofaunal groups (Beermann et al. 2018, Weigand & Macher 2018, Vivien et al. 2019, Schenk et al. 2020).

16.2 Methods

Three independent sediment cores were collected at 44 JDS4 sites, thus to allow for the detection of a larger number of species due to microhabitat differences. The sediments of three sites were analysed from both riverbanks (JDS4-6, -29, -31). Two barcode markers, the mitochondrial cytochrome c oxidase subunit 1 gene (COI) and the hypervariable regions of nuclear 18S rRNA gene (18S V1V2) were investigated. The 18S marker was analysed by the primer pair F04mod/R22mod to generate patterns of community composition on a higher taxonomic level. COI was targeted with the primer pair mlCOIintF/dgHCO2198, enabling a higher number of metazoan species-level identifications, except for nematodes, which were primarily identified by means of 18S.

Sediment samples were extracted using the DNeasy PowerMax Soil Kit (Qiagen). For each extraction, 10 ml of sediment were treated according to the manufacturer's instructions and then precipitated and resuspended in 600 µl Tris 10mM solution. DNA extracts were stored at −20 °C until PCR amplification. PCR reactions were performed in three replicates and a negative control for each DNA extract included to identify potential cross-contaminations. PCR replicates of each sample were combined. Taxonomic annotations were done using the vsearch toolkit (Rognes et al. 2016), searching for up to three candidate reference sequences in the MIDORI (Leray et al. 2018) and SILVA (Quast et al. 2012) databases. The annotation was done by using the Lowest Common Ancestor approach and when the query sequence had at least 95% similarity with any sequence in the database. For Metazoan species, a match to a Linnaean species was accepted in case of at least 97% sequence identity in the COI marker and 99.5% in the 18S V1V2 marker. Bray-Curtis dissimilarity matrices were used to calculate the Beta dispersion of samples at two spatial scales (within sampling sites, and within each river) using the betadisper function in the vegan package. Non-Metric multidimensional scaling analysis was performed on the Bray-Curtis dissimilarity matrices to explore the compositional variation of communities between tributaries and between reaches of the Danube (Upper, Middle and Lower).

16.3 Results and discussion

16.3.1 Community composition on higher taxonomic levels

Only a small fraction of the generated sequences remained unassigned within the 18S dataset. Holozoa (mainly metazoans) and diatoms (Diatomea) dominated the community composition in all three reaches, followed by Nucletmycea (mainly fungi), and Phragmoplastophyta (plants and Characeae) in the middle reach (MR) and lower reach (LR) only *(Figure 1)*. Chlorophyceae (green algae) were strongly represented in the upper reach (UR), less so in the MR and LR. Conversely, the taxon Thecofilosea (amoeboid protists) only reached a higher read proportion in the LR.

For COI, however, a large proportion of sequences remained unassigned. This is probably due to a lower specificity of the metabarcoding primers to metazoans only, and indicates that the majority of the sequences produced likely belong to co-amplified Bacteria *(Figure 1)*. This non-target amplification was highest in the LR. An optimised primer pair can reduce this bias in the future. In all three reaches, dipterans and oligochaetes (Haplotaxida) dominated the proportion of metazoan sequence reads. Ostracods (Podocopida), branchiopods (Diplostraca) and rotifers (Ploima) were also strongly represented.

16.3.2 Changes in community composition between site replicates

Three independent sediment samples were analysed for each sampling site, thus to account for variation in communities due to microhabitat characteristics. Comparison of Bray-Curtis dissimilarities, indicating changes in community composition, show that communities of replicates of the same site are more similar than between all samples analysed for a river *(Figure 2)*. Although this was expected, the analysis also points to the fact that each site replicate harboured a certain amount of taxa specific to an individual sediment core, underlining the importance of the conceptual approach taken in detecting a high amount of biodiversity present in the sediments of the Danube and its tributaries.

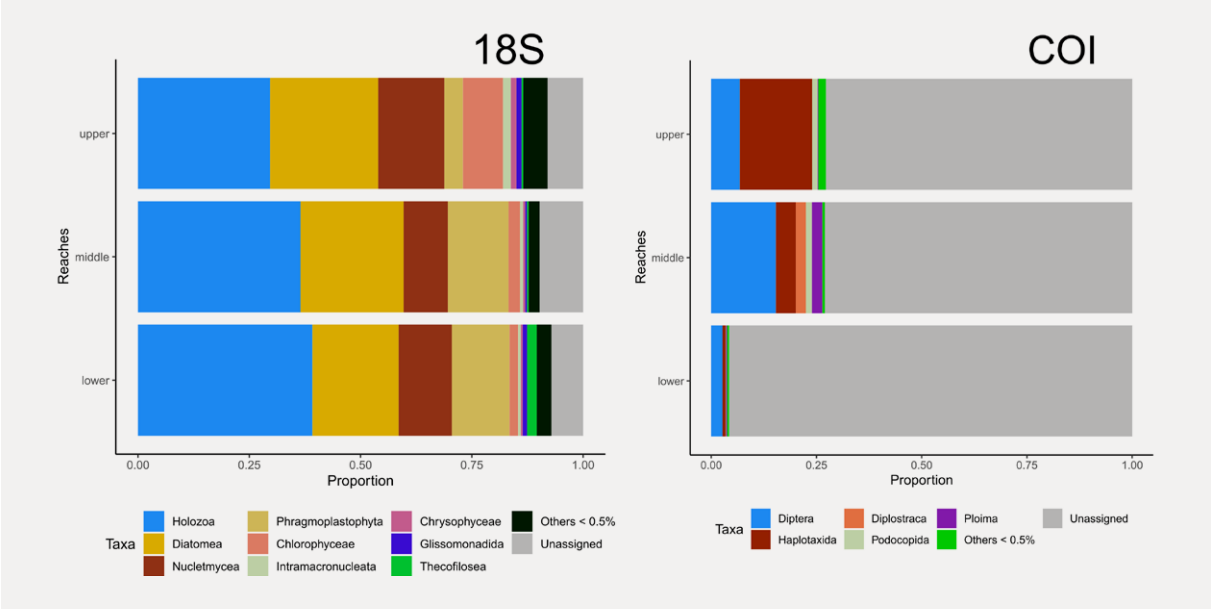

Figure 1: Community composition of Danubian reaches for the COI and 18S V1V2 markers.

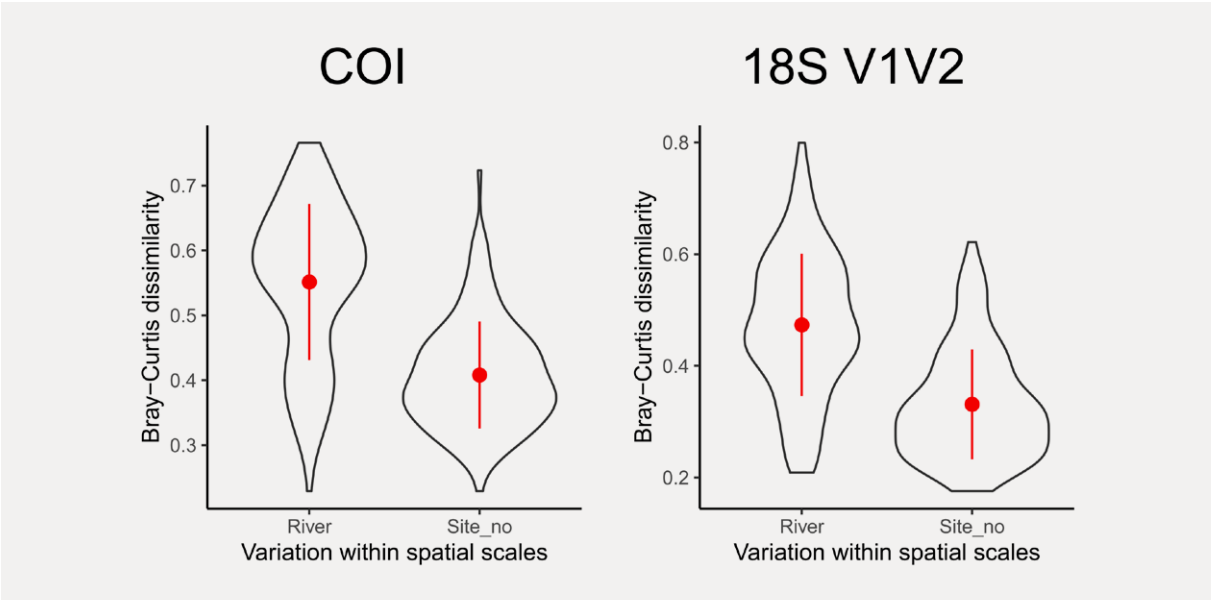

Figure 2: Community changes between sites within the same river vs. replicates at the same site.
Indicated are violin plots which show the taxonomic similarity of sediment samples within the same river (i.e. Upper, Middle or Lower Reach of the Danube, respectively, tributary) or between the three sediment cores taken at the same sampling site.

16.3.3 Changes in community composition between Danubian reaches and tributaries

NMDS plots based on Bray-Curtis dissimilarities indicate that communities retrieved from sites (and site replicates) within the same Danubian reach were generally more similar than between reaches *(Figure 3a, b)*. In the 18S V1V2 NMDS plot, sediment samples from tributaries form a distinct point cloud compared to sediment samples from the Danube main channel *(Figure 3d)*. This distinction is not recovered in the COI-based analysis *(Figure 3c)* and might be a result of the 18S V1V2 marker in additionally incorporating signals of e.g. fungi, plants, diatoms and green algae in community composition *(Figure 1)*. Together, those taxa play a prominent role in shaping community structures in Danubian tributaries, and the main channel. Additionally, the majority of COI reads had to be excluded due to non-target amplification (i.e. Bacteria), thereby also losing resolution power. Stress-values for all four NMDS plots suggest a good to moderate representation of patterns in reduced dimensions.

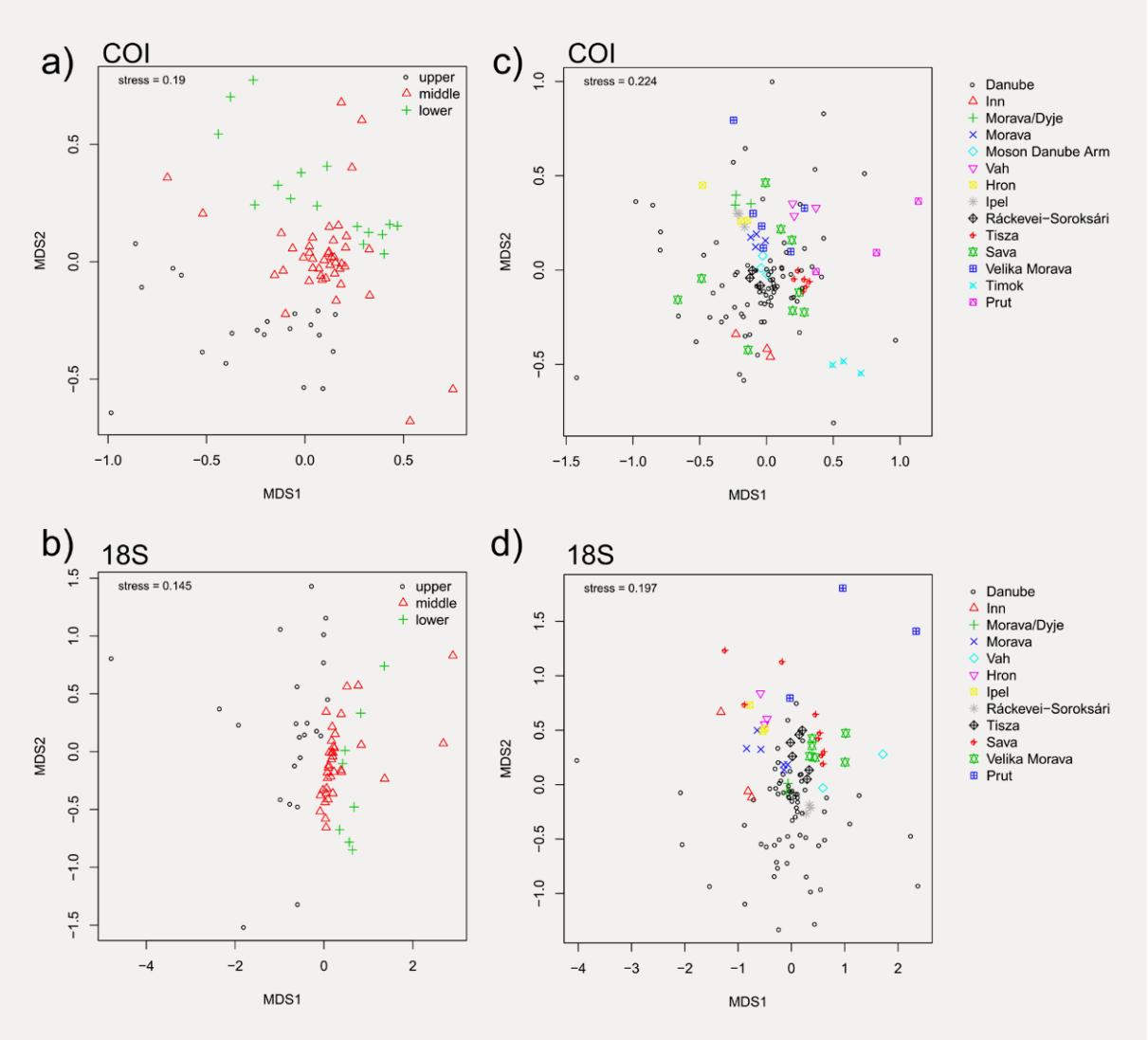

Figure 3: NMDS plots based on the community composition detected by two molecular markers and three sediment replicates per site. a) COI dataset for the three reaches of the Danube; b) COI dataset for the Danube and its tributaries; c) 18S dataset for the three reaches of the Danube; d) 18S dataset for the Danube and its tributaries.

16.3.4 Metazoan biodiversity based on COI

A total of 29,494 Amplicon Sequence Variants (ASVs, see Chapter 12) were produced, of which 979 were assigned to Metazoa resulting in 261 identified Linnaean species. Another 21 taxa were only assigned to genus level. The DNA-based sediment analysis primarily detected fully aquatic species living in the sediment (e.g. meiofaunal organisms such as ostracods, copepods and oligochaetes) or developmental stages of otherwise non-benthic adult taxa living in the sediment (e.g. fish, mayflies, chironomids) but likewise eDNA signals of terrestrial (butterflies, vertebrates, beetles), amphibic (e.g. *Castor fiber*) and freshwater species. Sediments of the sampling sites JDS4-17-L (48 species), JDS4-26-R (40 species), JDS4-40-R (42 species) and JDS4-41-R (40 species) were particularly biodiverse. The three JDS4 sampling sites at which sediments were investigated on both river sites highlight that this sampling strategy detects a larger number of species, potentially due to microhabitat differences between river banks: JDS4-6-L and -R detected 9 and 17 species, respectively, and 22 when combined; JDS4-29-L and -R detected 33 and 17 species (combined 42); and JDS4-31-L and -R detected 26 and 22 species (combined 39). The following groups were detected: Diptera (59 taxa on species level), Oligochaeta (37), Ephemeroptera (23), Rotifera (22), Gastropoda (11), Acari (11), Branchiopoda (10), Copepoda (9), Bivalvia (9), Mammalia (8), Coleoptera (6), Plecoptera (5), Ostracoda (5), Amphipoda (4), Nematoda (4), Cnidaria (4), Gobiiformes (4), Trichoptera (3), Odonata (3), Lepidoptera (3), Collembola (2), Aves (2), Cypriniformes (2), Hemiptera (2), Isopoda (1), Protura (1), Diplopoda (1), Aranea (1), Mysida (1), Hymenoptera (1), Bryozoa (1), Amphibia (1), Gastrotricha (1), Plathelminthes (1), Porifera (1) and Tardigrada (1). Overall, Chironomidae comprised the most abundant family with 42 species, of which 11 were assigned to the genus *Tanytarsus*.

The most abundant species based on occupancy were *Limnodrilus hoffmeisteri* (at 33/44 sites; 75%), *L. claparedianus* (25/44; 57%), *Cladotanytarsus mancus* (27/44; 61%), *Tanytarsus ejuncidus* (29/44; 66%), *T. volgensis* (27/44; 61%), *Cypridopsis vidua* (26/44; 59%), *Limnocythere inopinata* (41/44; 93%) and *Brachionus calyciflorus* (33/44; 75%). Abundant species can often comprise a high intraspecific genetic diversity or even can form complexes of cryptic species. As such, it can be expected that several ASVs (or here, COI haplotypes) are produced for a single Linnaean species. This was the case for the oligochaetes *Limnodrilus claparedianus* (with 13 ASVs), *L. hoffmeisteri* (29), the beetle *Prionus insularis* (18), the chironomids *Cladotarnytarsus mancus* (23), *Tanytarsus brundini* (61), *T. volgensis* (34), the ostracods *Cypridopsis vidua* (56), *Limnocythere inopinata* (27) and the rotifers *Brachionus calyciflorus* (31), *B. quadridentatus* (14) and *Euchlanis dilatata* (12). It becomes obvious that six of the eight most abundant Linnaean species also have a high number of ASVs, harboring a high level of genetic diversity. In some cases, the different ASVs of a single species were primarily detected in a single reach each, furthermore pinpointing to a high degree of spatio-genetic structuring within those species.

The number of taxa was highest in the MR (161), a bit lower for tributaries (158) and lowest for the LR (101) and UR (75) *(Table 1, Figure 4)*. Yet, relative sampling densities have to be taken into account (tributaries: 17 sites; MR: 13 sites; UR and LR: 7 sites each). All reaches showed a large proportion of reach-specific taxa, ranging from 37% for the MR to 22% for the LR. Only 25 taxa were shared between all three reaches and tributaries (9%). Oligochaetes + Dipterans constituted between 47% (MR) to 40% (LR and Trib.) of all taxa detected. The relative proportion of chironomids among dipteran taxa detected in the sediment increased from the UR to the LR (76% to 86%), and was lowest in the tributaries (74%). On the contrary, EPT taxa showed a remarkable decrease from the UR+MR (11%, respectively, 10%) to the LR (2%), being equally high in the tributaries (11%). This pattern was mainly driven by Ephemeroptera, constituting 7 and 12 species of five families for the UR and MR, respectively, whereas only *Baetis rhodani* and *Rhithrogena germanica* were detected in sediments of the LR. Likewise, the relative proportions of oligochaete and mollusk species declined from the UR (25%, respectively, 12%) to the LR (18%, respectively, 6%).

Table 1: Relative proportion of selected taxonomic groups between reaches of the Danube in % (based on COI). UR: Upper Reach; MR: Middle Reach; LR: Lower Reach; Trib. = Tributaries.

Taxon	UR n = 75	MR n = 161	LR n = 101	Trib. n = 158
Oligochaeta	25	17	18	17
only Naididae	19	12	14	14
EPT	11	10	2	11
only Ephemeroptera	9	7	2	9
Copepoda	1	1	3	4
Ostracoda	5	1	2	3
Branchiopoda	0	4	3	4
Diptera	21	30	22	23
only Chironomidae	16	23	19	17
Rotifera	11	12	12	11
Mollusca	12	4	6	6

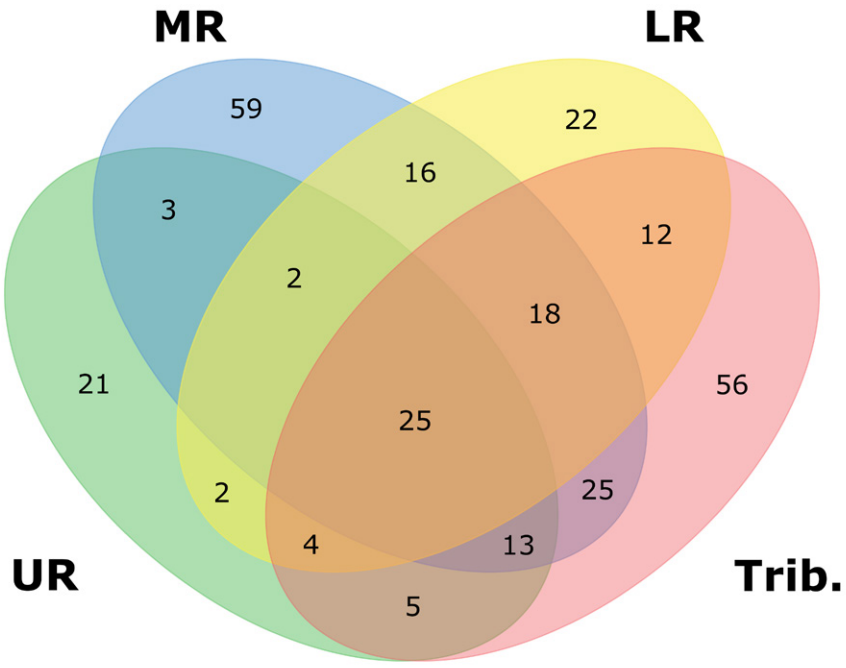

Figure 4: Venn diagram indicating the number of exclusive and shared taxa for each reach of the Danube (based on COI) and the tributaries. UR: Upper Reach; MR: Middle Reach; LR: Lower Reach; Trib.: Tributaries.

16.3.5 Metazoan biodiversity based on 18S

A total of 12,780 ASVs were detected, including 1,434 Metazoan ASVs of which 198 were assigned to distinct Linnaean species. For some species, and similar to COI, several equally annotated ASVs were detected and collapsed into a single taxonomic entry. The following groups were detected: Nematoda (47), Plathelminthes (30), Arachnida (23, of which 22 Acari), Gastrotricha (16), Insecta (15, of which 6 Ephemeroptera, 3 Diptera, 2 Odonata, 2 Plecoptera, 2 Coleoptera), Annelida (12, of which 11 Oligochaeta), Rotifera (12), Ostracoda (9), Branchiopoda (8), Mollusca (8, of which 5 Gastropoda, 3 Bivalvia), Collembola (6), Tardigrada (5), Bryozoa (3), Vertebrata (2), Nematomorpha (1) and Micrognathozoa (1).

The majority of species detected by this marker belongs to the meiofauna and must be regarded as complementary to the species detected by the COI metabarcoding approach. In particular, the 47 species of nematodes in 37 genera (compared to 4 species by COI), 30 species of Plathelminthes in 26 genera (compared to 1 species) and 16 species of Gastrotricha in 5 genera (compared to 1 species) must be highlighted. On the contrary, the 18S marker only detected / resolved 15 insect (compared to 105 species by COI), 12 rotifer (compared to 22 species) and 12 annelid species (compared to 37 species).

16.3.6 Ecological quality of fine sediments based on nematode community structure

The comparatively large diversity of nematode taxa detected by the 18S analysis allowed the calculation of a pollution index for fine sediments based on nematode community structure. At each sampling site, the NemaSPEAR[%]-index takes the relative proportion of vulnerable **Nema**tode **spe**cies **a**t **r**isk into account, which preferably are present at less polluted or unpolluted sites (Höss et al. 2017, Schenck et al. 2020). From a management perspective, the 30% value indicates the threshold between an acceptable and non-acceptable quality status of fine sediments (i.e. the higher the %-value, the higher the frequency of observed vulnerable nematode species, and the better the assumed ecological quality of the sediment). The majority of JDS4 sampling sites was analysed, but some sites had to be excluded due to too low numbers of 18S sequence reads. The sites JDS4-7, -13, -20 and -32 received the highest quality scores (= high/good ecological status), whereas values for the sites JDS4-2, -3, -8 and -34 were particularly low (= bad/poor ecological status) *(Figure 5)*. The pattern was strongly influenced by the vulnerable species *Eumonhystera vulgaris*.

Since the index relies on relative abundance estimates of nematode species (inferred from the relative proportion of sequence reads, which can be biased by biomass differences and primer selectivity), classification results should be only treated as a further line of evidence in a multiple evidence framework.

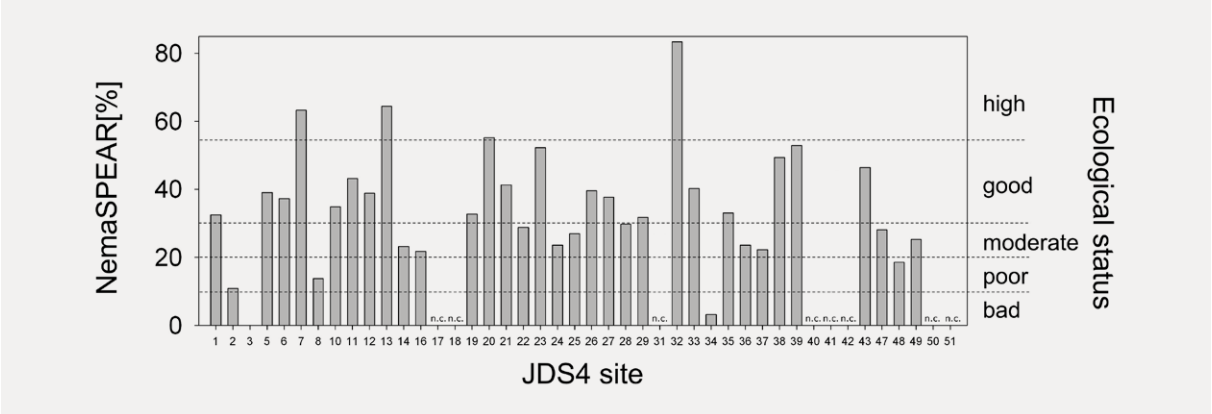

Figure 5: Results of the NemaSPEAR[%]-index for fine sediments based on 18S sequence data. n.c. = index not calculated due to a low number of sequence reads available for the respective site.

16.4 Conclusions

- *DNA metabarcoding of sediments detected a high number of amplicon sequence variants (COI: 29,494; 18S: 12,780), including a high number of metazoan species (COI: 261; 18S: 198)*
- *The COI and 18S molecular marker each detected a distinct set of taxa and were able to characterise the community composition on higher taxonomic levels, thus together corroborating to the overall detection of biodiversity present in the Danube*
- *The COI dataset to a large extent consisted of unassigned sequence reads, likely originating from co-amplified bacteria; optimised primers will likely reduce this bias*
- *Primer selectivity, taxonomic resolution of the marker and the variation in reference database completeness, might best explain discrepancies in detection*
- *Overall, community composition of the three sediment replicates was comparable, but each sediment core added further taxa to the overall biodiversity at a site; likely due to microhabitat differences and sampling effect*
- *47 nematode taxa were detected by 18S; nematode community structure was used to calculate a pollution index for fine sediments (NemaSPEAR[%])*

16.5 References

BEERMANN, A. J., ZIZKA, V. M., ELBRECHT, V., BARANOV, V., & LEESE, F. (2018). DNA metabarcoding reveals the complex and hidden responses of chironomids to multiple stressors. Environmental Sciences Europe, 30(1), 26.

HÖSS, S., HEININGER, P., CLAUS, E., MÖHLENKAMP, C., BRINKE, M., & TRAUNSPURGER, W. (2017). Validating the NemaSPEAR [%]-index for assessing sediment quality regarding chemical-induced effects on benthic communities in rivers. Ecological Indicators, 73, 52-60.

LANDRUM, P. F., & ROBBINS, J. A. (1990). Bioavailability of sediment-associated contaminants to benthic invertebrates. Sediments: chemistry and toxicity of in-place pollutants, 8, 237-263.

LERAY, M., HO, S. L., LIN, I. J., & MACHIDA, R. J. (2018). MIDORI server: a webserver for taxonomic assignment of unknown metazoan mitochondrial-encoded sequences using a curated database. Bioinformatics, 34(21), 3753-3754.

QUAST, C., PRUESSE, E., YILMAZ, P., GERKEN, J., SCHWEER, T., YARZA, P., PEPLIES, J. & GLÖCKNER, F. O. (2012). The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic acids research, 41(D1), D590-D596.

ROGNES, T., FLOURI, T., NICHOLS, B., QUINCE, C., & MAHÉ, F. (2016). VSEARCH: a versatile open source tool for metagenomics. PeerJ, 4, e2584.

SCHENK, J., HÖSS, S., BRINKE, M., KLEINBÖLTING, N., BRÜCHNER-HÜTTEMANN, H., & TRAUNSPURGER, W. (2020). Nematodes as bioindicators of polluted sediments using metabarcoding and microscopic taxonomy. Environment International, 143, 105922.

VIVIEN, R., APOTHÉLOZ-PERRET-GENTIL, L., PAWLOWSKI, J., WERNER, I., & FERRARI, B. J. (2019). Testing different (e) DNA metabarcoding approaches to assess aquatic oligochaete diversity and the biological quality of sediments. Ecological Indicators, 106, 105453.

WEIGAND, A. M., & MACHER, J. N. (2018). A DNA metabarcoding protocol for hyporheic freshwater meiofauna: Evaluating highly degenerate COI primers and replication strategy. Metabarcoding and Metagenomics, 2, e26869.

17 Summary: performance of (e)DNA-based activities

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Abstract

JDS4 provided the excellent opportunity to evaluate (e)DNA-based approaches in an applied, international and highly integrative setting. The fish community of the Danube, its macrozoobenthos (MZB), phytobenthos and sediment fauna were assessed using group-specific metabarcoding approaches. Although still a certain degree of methodological variation exists, the outcomes clearly demonstrate the huge potential of DNA and environmental DNA-based approaches for biodiversity and ecological status class assessments: eDNA water analysis of fish revealed most of the taxa also detected by the traditional fish survey, and was particularly effective in detecting the hard to capture benthic taxa (including endangered sturgeon species). The (e)DNA-based taxalists of the MZB likewise covered many of the traditionally assigned species, but also included a plethora of additional chironomid and oligochaete species. Molecular ecological status class assessments based on presence-absence values of MZB species were also largely congruent to traditional abundance or presence-absence-based outcomes. Although the molecular assessment of the phytobenthos revealed less species than traditional light microscopy, much more taxa were detected, which await a species-level taxonomic annotation in the future. Metabarcoding of the sediment community enabled the comprehensive assessment of the meiofaunal community (i.e. an often neglected but ecologically highly sensitive component of the Danubian biodiversity) and the molecular inference of fine sediment quality based on local community structures of vulnerable nematode species. Finally, all (eDNA)-based taxalists were compiled to effectively inform invasive alien species detection in the Danube River Basin.

In summary, (e)DNA-based methods can be seen as a highly effective, complementary tool to provide consolidated results for biodiversity and ecological status class assessments in a multiple evidence framework, as pursued during the JDS4. Yet, and despite their already very promising performance and large coherence with traditional outcomes, the full potential of (e)DNA-based approaches in the context of larger environmental surveys might be released by:

- *a) developing and curating a DNA barcode reference library specifically designed for the targeted catchments,*
- *b) further standardizing (e)DNA-based approaches to a small set of good practice setups,*
- *c) more explicitly integrating genetic diversity (and spatiotemporal changes thereof) in ecological status class assessments,*
- *d) installing a dense, large-scale environmental DNA-based screening for biological quality elements or invasive alien species, based on which traditional surveys can be performed at conspicuous sites,*
- *e) specifically educating and training national authorities in state-of-the-art molecular tools.*

The strong international and capable of acting network of the ICPDR, consisting of stakeholders, water managers, politicians and scientists, thereby represents the ideal framework to cooperatively address those issues.

17.1 Introduction

One of the aims of JDS4 was to test the performance of (e)DNA-based approaches in an applied, international and highly integrative setting. This chapter focuses on the outcomes of the diverse molecular activities pursued during JDS4 (see Chapter 12). The fish community of the Danube, its macrozoobenthos (MZB), phytobenthos and sediment fauna, were investigated using different DNA- and environmental DNA (eDNA)-based metabarcoding approaches. Furthermore, all molecular data were merged to inform invasive alien species detection in the Danube River Basin. The following subchapters provide an overview of infrastructural issues faced before individually summarising the performance of (e)DNA-tools for the different target groups. The chapter ends with a conclusion and states some potential roadmap items, which can help to increase the effectiveness of (e)DNA-based tools for large-scale environmental surveys in the future.

17.2 Infrastructural issues

Since (e)DNA-based activities were conducted during JDS4 for the first time, the (e)DNA survey plan (Chapter 12) was discussed at several meetings with the ICPDR experts on monitoring and assessment. Additionally, dedicated training lessons were installed during the preparatory phase of JDS4. The content trained and the specific sampling guidelines prepared (e.g. drilling sediment cores) helped all involved parties, but especially the national teams, to collect the samples (i.e. MZB multi-habitat sample, phytobenthos brushed sample and sediment cores) as appropriate as possible for subsequent DNA-based analysis. In the large majority of cases, samples were optimally preserved.

Still, a few issues must be mentioned. In some cases, the labelling of MZB containers was imprecise or wrong, or the label partly bleached by (the evaporating) ethanol. Yet, a correct labelling of samples is of paramount importance to later on connect the multitude of abiotic and biotic results obtained for a given JDS4 site. Some sampling teams have not respected the indicated ratio of preservative volume to biomass. Since a too low e.g. ethanol (for MZB and phytobenthos) or LifeGuard solution concentration (for sediment cores) leads to the degradation of DNA, it was not possible to generate DNA-based taxalists for all anticipated sampling sites in the sediment analysis, for MZB and for phytobenthos. Finally, most of the samples arrived in the respective analytical laboratory after a few days, which was expected. However, the German custom control retained some MZB containers for several days, which likewise must have led to a degradation of the DNA as sampling containers very likely were not properly stored. Even so, in the large majority of cases, collection, handling, shipping and labelling of samples was very effective and appropriate across all (e)DNA-based approaches.

The emerging COVID-19 pandemic situation in early 2020 has pinpointed one of the major drawbacks of (e)DNA-based activities. While traditional taxonomic results were prepared by morphological experts mostly unaffected by the restricted situation, the to-be-established hygiene conditions and social distancing measures led to a strong reduction of molecular laboratory capacities in almost all (e)DNA-based approaches, as well as a temporal unavailability of some chemicals – or increased prices, such as for ethanol. This unforeseen situation further postponed the generation of some molecular contents (e.g. an anticipated ring test). On another note, the MZB laboratory in Essen (Germany) noted that a much larger than expected freezing capacity was needed to adequately store sampling containers. This infrastructural requirement has to be kept in mind for future surveys.

17.3 Evaluation of (e)DNA-based assessments

The overall pattern of results obtained from (e)DNA-based tools indicates a very good performance of the pursued molecular approaches for Danubian biodiversity assessment – and were executed, for ecological status class assessment – despite considerable methodological variation.

As expected, the complementary implementation of (e)DNA-based approaches during JDS4 has:

a) led to a great increase in taxonomic resolution for multiple taxonomic groups,

b) enabled the detection of hard to observe species (e.g. benthic fish, bivalves),

c) allowed the identification of taxonomically heterogeneous (=invasive alien species) or difficult to identify organism groups (=meiofauna) and developmental stages (e.g. chironomid larvae),

d) revealed as-yet-unknown species for the Danube catchment, and

e) produced widely coherent results when compared to traditional taxalists and assessment results.

However, and before results of the organism group-specific surveys are presented, one overarching beneficial aspect of (e)DNA-based tools must be emphasized. The sequence-based datasets generated within an (e)DNA-based survey are comparable 'inside' and 'outside' the environmental program – an aspect that seems particularly important for a repeated and very comprehensive longitudinal survey involving many countries, such as the Joint Danube Survey. Because sequence-based taxalists for individual biological quality elements are generated by a single authority and are available as FAIR raw data (Wilkinson et al. 2016), the definition of molecular operational taxonomic units (MOTUs), their Linnaean taxonomic annotation and site-to-site as well as survey-to-survey comparisons are repeatable and transparent for any other user. As such, there exists "a single identifier" for all longitudinal samples of a taxonomic group or biological quality element, as well as a unique DNA sequence for each MOTU. This inherent nature of sequence-based data creates a high level of sustainability, with the outcomes being easily comparable between sites and surveys – or across space and time.

17.3.1 Fish

The fish community was targeted by three separate environmental DNA sampling campaigns (Chapter 12, 14 and 21). The most comprehensive eDNA survey visited 29 Danubian River sites and 18 tributaries (Chapter 14), detecting a total of 80 taxa, of which 19 corresponded mainly to farmed / food fish due to eDNA release of wastewater treatment plants in urban areas. Of the remaining 61 taxa, 50 were identified down to the species level. For six, respectively, five taxa, the analysed barcode marker was not able to methodologically resolve congeneric taxa or species of different genera. Compared to the results of the traditional fish survey (TFS), it can be stated that 50 taxa were detected by both methodologies. Nine taxa were only detected by TFS, and eight taxa only by eDNA – particularly hard-to-catch benthic taxa including several sturgeon species. The proportion of species-level hits and the nature of the fish community detected, thereby largely depend on the barcode marker investigated and the reference library consulted (but see Chapter 14). Furthermore, and although no direct 1:1 inference of abundance data or biomass values for fish species is possible from eDNA signals obtained from water samples, the relative abundance of sequence reads per site produced plausible longitudinal patterns (and shifts) of fish community composition (see also Pont et al. 2019). Ethically speaking, it means that no specimens had to be caught or even sacrificed, but still informative taxalists generated. The eDNA-based ecological status class assessment of 13 JDS4 sites common with the TFS inferred most of the sites as being 'moderate'. TFS- and eDNA-based assessment

results were similar but sometimes varied by ±1 status class. From a more technical point of view, it must be highlighted that some eDNA metabarcoding samples were compromised, either by inhibition due to high levels of pollutants, or by too low DNA concentrations in the filtered water samples due to flooding events. As such, it was also not possible to totally streamline the eDNA water sampling for fish with all JDS4 sites, as otherwise eDNA traces of fish taxa originating from confluences and wastewater treatment plants as well as increased levels of inhibitors would have biased results.

17.3.2 Macrozoobenthos

The MZB was genetically analysed at 46 JDS4 sites (Chapter 13). Two metabarcoding approaches were pursued, either generating DNA-based taxalists from the homogenised multi-habitat sample (=bulk sample) or from the preservation liquid in which bulk samples had been stored. For seven JDS4 sites mainly situated in Slovakia, a comparison of four assessment methods was performed (i.e. morphology, bulk sample, preservation liquid and eDNA water metabarcoding). All three metabarcoding approaches targeted the COI gene, but applied different primer combinations. They consistently produced more comprehensive, species-level taxalists for each site and throughout the whole survey. As an example, the morphology-based assessment at the 46 investigated JDS4 sites revealed 275 species, whereas bulk sample and preservation liquid metabarcoding detected 333, respectively, 321 species. When taxalists of both metabarcoding approaches were combined, they included 463 distinct species. This increase of species-level identifications in DNA-based taxalists can be mainly attributed to additionally detected chironomid dipterans, but also to mayflies, stoneflies and caddisflies (=EPT) as well as to aquatic oligochaetes. Coincidentally, in the conclusion of the traditional MZB survey (Chapter 6), dipterans and oligochaetes are explicitly highlighted and a request for further 'external' taxonomic expertise formulated. On the contrary, only half of all odonate species and approximately 2/3 of the traditionally observed gastropod species were detected by metabarcoding.

The assessment of the MZB community by means of analysing their environmental DNA from water revealed three interesting patterns. First, eDNA-based taxalists comprised the highest number of families. Second, many additional meiofaunal species were detected. Third, a large proportion of the listed species / taxa was exclusively found on the eDNA-derived taxalist. The results can be best explained by that fact that riverine networks act as collectors of the biodiversity present in the water and adjacent environments. As such, a water sample will not only comprise the eDNA of MZB and meiofaunal species present at a given site, but also partly will contain eDNA traces that have been washed in from upstream sites and the terrestrial realm. This circumstance has to be considered when conclusions have to be drawn that must be spatially explicit. Otherwise, eDNA sampling designs very effectively allow the characterisation and the assessment of biodiversity on the level of larger reaches and whole catchments.

Finally, in this subchapter, ecological status class assessments based on MZB were generated from DNA metabarcoding datasets (from Chapter 13, based on presence-absence values), and compared to the traditionally derived multi-habitat sample assessment results (from Chapter 6, based on abundance data and presence-absence values). Saprobic Index (SI) and Multi-Metric Index (MMI) calculations were executed by Patrick Leitner (BOKU, Vienna, Austria). Pairwise comparisons were evaluated by the Pearson r-statistic (Tab. 1). All correlations were significant and strong, i.e. >0.4 after Cohen (1988), and at the large majority of sites a higher number of classified taxa entered DNA-based index calculations. The outcomes further highlight the potential of DNA-based ecological status class assessments based on presence-absence values for MZB. As such, JDS4 results are in line with recently published scientific studies and recommendations (e.g. Beentjes et al. 2018, Hering et al. 2018, Pawlowski et al. 2018, Buchner et al. 2019, Zizka et al. 2020).

Table 1: Pearson's r-values for pairwise correlations of index calculations derived from traditional multi-habitat samples and DNA samples of the macrozoobenthos.

SI = Saprobic Index; MMI = Multi-Metric Index; MHS_{Abs} = Multi-habitat sample calculation based on abundance values; $MHS_{P/A}$ = Multi-habitat sample calculation based on presence-absence values; DNA_{bulk} = Calculation from DNA metabarcoding bulk sample and presence-absence values; $DNA_{fixative}$ = Calculation from DNA metabarcoding fixative sample and presence-absence values; for SI, calculations contain sites in the Danube River and its tributaries; for MMI, calculations were separately performed for the Danube River (values below the diagonal) and tributaries (values above the diagonal), since the Austrian MMI method seems particularly questionable for tributaries and the lower reach of the Danube. All p-values for Pearson's r correlations are significant.

Saprobic Index (SI)		MHS_{Abs}	$MHS_{P/A}$	DNA_{bulk}
	MHS_{Abs}	–		
	$MHS_{P/A}$	0.776	–	
	DNA_{bulk}	0.439	0.497	–
	$DNA_{fixative}$	0.594	0.581	0.759
Multi-Metric Index (MMI)		MHS_{Abs}	DNA_{bulk}	$DNA_{fixative}$
	MHS_{Abs}	–	0.792	0.764
	DNA_{bulk}	0.788	–	0.987
	$DNA_{fixative}$	0.571	0.715	–

17.3.3 Phytobenthos

The phytobenthos community was analysed from 69 JDS4 sites (Chapter 15). Whenever possible, samples were taken from both riverbanks thus, to account for microhabitat differences due to variable light exposure and flow velocity – among others. Species composition was assessed by performing two complementary DNA metabarcoding approaches (based on 18SV4 and *rbc*L). The sequence-based taxalists comprised 160 (for 18SV4), respectively, 221 species (for *rbc*L), compared to 385 taxa found on the taxalist retrieved from classical light microscopy during JDS4. These contrasting numbers of species-level identifications on the one hand must be attributed to the current sampling design. Light microscopy identifies phytobenthos species by the presence of their frustules in a sample, regardless of them being alive or dead during the sampling campaign (e.g. originating from only temporary submerged habitats as a result of strong water level fluctuations), whereas DNA metabarcoding depends on a sufficiently high DNA concentration obtained from living specimens. On the other hand, differences between taxalists obtained from light microscopy and DNA metabarcoding must be attributed to the lack of available DNA barcode references for the one or the other barcode marker (but see Chapter 12 and 15), rendering a sequence-based identification impossible for certain species, although they were likely present in the sample. This is further exemplified by the fact that both metabarcoding approaches detected a multitude of additional MOTUs, whose species-level taxonomic annotation so far was not possible (e.g. at genus-level only). Still, the metabarcoding approaches were able to reveal 78% of all the most abundant diatom species identified during JDS2, JDS3 and JDS4, as well as six of the seven most dominant taxa identified by light microscopy in JDS4. Total genetic diversity (i.e. Linnaean species + above species-level MOTUs) was used to describe community composition and to analyse shifts thereof in correlation to environmental parameters. Temperature, dissolved oxygen, total organic carbon and conductivity were identified as the most explanatory variables. Finally, light microscopy-based index

calculations were compared with the results generated by both metabarcoding approaches. Although the outcomes have to be interpreted with some caution, all three pairwise comparisons indicate a good to strong positive linear correlation (Chapter 15).

17.3.4 Sediment community

Sediment-dwelling organisms are frequently neglected in environmental surveys, often simply due to their minute nature and difficulty of identification. However, at the same time, sediments harbor some of the ecologically most sensitive communities such as the meiofauna (including e.g. nematodes, oligochaetes, dipteran chironomids, copepods, rotifers). During JDS4, the sediment community of the Danube River and its tributaries was assessed by taking three sediment cores at 44 JDS4 sites, and by metabarcoding two markers (COI and 18S, but see Chapter 16). The (e)DNA-based approaches allowed the characterization of the sediment community at different taxonomic levels. A high species-level resolution was achieved for some hard-to-identify but dominant groups of organisms, i.e. oligochaetes, dipteran chironomids (both by COI), nematodes and plathelminths (both by 18S). One of the major outcomes was the finding that the three sediment cores taken at an individual site – or at both riversides at the same JDS4 site – were generally comparable, but each to a certain degree comprised an exclusive proportion of taxa. This highlights the need to account for local variation in available microhabitats by collecting more than a single sediment core at a site.

The total number of 47 nematode species detected by 18S metabarcoding and their site-specific community composition were used to calculate a molecular index for vulnerable *nema*tode *spe*cies *a*t *r*isk, which preferably are present at less polluted or unpolluted sites (NemaSPEAR[%], Höss et al. 2017). Overall, the index produced very plausible results for the local quality of fine sediments. Nevertheless, since the calculation relies on relative abundance estimates of nematode species (inferred from the relative proportion of sequence reads, which can be biased by biomass differences and primer selectivity), classification results should be only treated as a further line of evidence in a multiple evidence framework. Finally, the (e)DNA-based analysis of the sediment community revealed a multitude of terrestrial and semi-aquatic taxa, whose eDNA traces very likely were washed in from the terrestrial realm into the submerged sediments.

17.3.5 Invasive alien species

The target community of invasive alien species (IAS) comprises a taxonomically quite heterogeneous group or organisms. As such, many taxonomists and different collection methods have to be involved to compile comprehensive lists of IAS. During JDS4, the detection of aquatic IAS was supplemented by (e)DNA-based tools (see Chapter 10). A total of 41 animal IAS were molecularly revealed, either by their direct presence in a sample, or by traces of their environmental DNA. The traditional assessment detected 44 animal IAS. When both assessment lists were combined, this resulted in a total of 52 animal IAS detected during JDS4 (35 macroinvertebrates, 17 fish). Notably, the medically important gastropod species *Bulinus umbilicatus* was detected by means of (e)DNA metabarcoding in the Danube River Basin for the first time. In summary, the application of (e)DNA-based tools was approved as an effective method for the detection of aquatic IAS during JDS4.

17.4 Conclusion and outlook

The application of (e)DNA-based tools during JDS4 can be considered very effective for a comprehensive assessment of the Danubian biodiversity (i.e. fish, macrozoobenthos, phytobenthos, sediment community and invasive alien species detection) and showed very promising potential for ecological status class assessments. For the time being, a complementary approach of traditional assessment techniques and (e)DNA-based tools holds great promise. One could imagine a dense, (e)DNA-based survey of biological quality elements or invasive alien species throughout the Danube River Basin. Traditional assessments could be then explicitly performed at conspicuous sites or where more integrated data are needed. Yet, to streamline future activities and to benefit even more so from the molecular data generated, the following roadmap items might be considered:

- A higher proportion of species-level annotations can be achieved for all organism groups investigated when gaps in DNA barcode reference libraries are specifically addressed for Danubian biota. A gold standard here would be a *well curated DNA barcode reference library for Danubian biota.*
- Besides the focus on classical biological quality elements, (e)DNA-based approaches enable us to *integrate additional ecologically sensitive target groups into environmental assessments* (e.g. nematodes).
- When generating biodiversity patterns and investigating correlations to environmental parameters, analyses could *more explicitly focus on patterns of genetic diversity* (e.g. occupancies of MOTUs and ASVs) additional to Linnaean species. As such, the full potential of (e)DNA-based approaches can be released, and Danubian biodiversity more fully accessed (i.e. by integrating cryptic lineages and intraspecific genetic diversity as one pillar of biodiversity).
- Furthermore, a *metadata analysis of (e)DNA patterns combined with the outcomes of non-target analytics / effect-based tools* holds great promise to understand the ecological drivers of habitat changes and shifts in community composition.
- To reduce the plethora of methodological variation, *standardisation work has to be conducted and a limited set of well-performing and praxis-oriented (e)DNA-based approaches selected.* A good framework for such standardisation work might be the newly installed CEN working group WG28 "DNA and eDNA methods".
- New or adapted *(e)DNA-based biotic indices for ecological status assessment should be more explicitly tested* and intercalibration experiments performed (see also Hering et al. 2018, Pawlowski et al. 2018).
- Last but not least, *national authorities should be educated and trained in state-of-the-art molecular tools,* fostering the development of a strong collaborative international network between all parties involved (Leese et al. 2018).

17.5 References

BEENTJES, K. K., SPEKSNIJDER, A. G., SCHILTHUIZEN, M., SCHAUB, B. E., & VAN DER HOORN, B. B. (2018). The influence of macroinvertebrate abundance on the assessment of freshwater quality in The Netherlands. Metabarcoding and Metagenomics, 2, e26744.

BUCHNER, D., BEERMANN, A. J., LAINI, A., ROLAUFFS, P., VITECEK, S., HERING, D., & LEESE, F. (2019). Analysis of 13,312 benthic invertebrate samples from German streams reveals minor deviations in ecological status class between abundance and presence/absence data. PloS one, 14(12), e0226547.

COHEN, J. (1988). Statistical power analysis for the behavioral sciences (2nd ed.). Hillsdale, N.J.: L. Erlbaum Associates.

HERING, D., BORJA, A., JONES, J. I., PONT, D., BOETS, P., BOUCHEZ, A., … & LEESE, F. (2018). Implementation options for DNA-based identification into ecological status assessment under the European Water Framework Directive. Water Research, 138, 192-205.

HÖSS, S., HEININGER, P., CLAUS, E., MÖHLENKAMP, C., BRINKE, M., & TRAUNSPURGER, W. (2017). Validating the NemaSPEAR [%]-index for assessing sediment quality regarding chemical-induced effects on benthic communities in rivers. Ecological Indicators, 73, 52-60.

LEESE, F., BOUCHEZ, A., ABARENKOV, K., ALTERMATT, F., BORJA, Á., BRUCE, K., … & WEIGAND, A.M. (2018). Chapter Two – Why We Need Sustainable Networks Bridging Countries, Disciplines, Cultures and Generations for Aquatic Biomonitoring 2.0: A Perspective Derived From the DNAqua-Net COST Action, in: Bohan, D.A., Dumbrell, A.J., Woodward, G., Jackson, M. (Eds.), Advances in Ecological Research, Next Generation Biomonitoring: Part 1. Academic Press, pp. 63–99.

PAWLOWSKI, J., KELLY-QUINN, M., ALTERMATT, F., APOTHÉLOZ-PERRET-GENTIL, L., BEJA, P., BOGGERO, A., … & KAHLERT, M. (2018). The future of biotic indices in the ecogenomic era: Integrating (e) DNA metabarcoding in biological assessment of aquatic ecosystems. Science of the Total Environment, 637, 1295-1310.

PONT, D., VALENTINI, A., ROCLE, M., MAIRE, A., DELAIGUE, O., JEAN, P., & DEJEAN, T. (2019). The future of fish-based ecological assessment of European rivers: from traditional EU Water Framework Directive compliant methods to eDNA metabarcoding-based approaches. Journal of Fish Biology. https://doi.org/10.1111/jfb.14176

WILKINSON, M. D., DUMONTIER, M., AALBERSBERG, I. J., APPLETON, G., AXTON, M., BAAK, A., … & BOUWMAN, J. (2016). The FAIR Guiding Principles for scientific data management and stewardship. Scientific data, 3(1), 1-9.

ZIZKA, V. M., GEIGER, M. F., & LEESE, F. (2020). DNA metabarcoding of stream invertebrates reveals spatio-temporal variation but consistent status class assessments in a natural and urban river. Ecological Indicators, 115, 106383.

18 Overview chapter on ecology and biology

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Abstract

Assessment of the ecological status (and designation of Heavily Modified Water Bodies) for each water body is a task of the EU member states and was not done during Joint Danube Survey 4. The ambition of JDS4 was to provide a scientific snapshot of the whole Danube, a homogeneous internationally coordinated fingerprint at a certain time – this included also an indication of the ecological status for the sites using a harmonized approach regardless if these sites were located in natural or Heavily Modified Water Bodies. Biological quality elements indicating pressure from nutrients and oxygen depletion by biodegradable substances – Phytoplankton, Macrophytes, Phytobenthos, partly Macrozoobenthos – indicated a good status at many sites and point at local pressure only. Fish and Macrozoobenthos, however, indicated impacts induced by hydromorphological pressures at a majority of sites. A general improvement of the ecological status along the whole length of the Danube over the last years is not visible except for some sites. Climate change phenomena and increasing pressure from invasive alien species may additionally influence the ecological status. The applicability of molecular methods using DNA and environmental DNA for status assessment proved to be promising and delivered sound results for a majority of sites.

18.1 Introduction

The European Water Framework Directive (WFD, 2000) is constructed around the assessment of the ecological (and chemical – dealt with in Chapter 39) status. Failing the objective of reaching good status in a waterbody triggers the necessity of mitigation measures, may lead to the designation of a Heavily Modified Water Body (HMWB) and has diverse consequences for water management. For the Danube catchment area, this is described in detail in the River Basin Management Plans of the ICPDR[1]. As the Joint Danube Survey 4 (JDS4) was collecting an extensive amount of data and assessing the quality of the Danube with uniform indices for all sampling sites, it is dealing – beside other objectives (see Chapter 1) – with the ecological status as an important aspect of the data analysis.

Why only "indication" of ecological status?

The assessment of the ecological status is a national task of EU member states regulated in detail in Annex V of the Water Framework Directive and specified in various Common Implementation Strategy Guidance Papers of the European Commission[2]. The ecological status is established for each water body based on

[1] *more information: https://www.icpdr.org/main/activities-projects/river-basin-management*

[2] *more information: https://ec.europa.eu/environment/water/water-framework/facts_figures/guidance_docs_en.htm*

data from one or several representative monitoring sites by applying sampling and assessment methods that were designed with scientific principles for each biological quality element. Those principles include the degree of deviation from natural reference conditions and the correlation between pressure and impact, and depend on typological, seasonal, hydrological and other criteria – a complex and exactly applicable system.

For obvious reasons JDS4 could not obey all the necessary instructions regarding representative site selection, choice of sampling time (in relation to season and discharge), selection of assessment indices suitable for the whole Danube and all tributaries. The ambition of JDS4 is to provide a scientific snapshot of the whole Danube, a homogeneous internationally coordinated fingerprint at a certain point in time. Its valuable results gathered with harmonized and uniform methods allow statements about the condition of the river along its whole course and over time when compared to earlier Joint Danube Surveys. Thus, the strength of this data is comparability; the results are not on national level approved status assessments for water bodies as not all required WFD criteria could be met by the JDS design. Therefore, the approach taken in this report refers to ***the indication of ecological status* for sites** (and not status assessment of the water bodies).

From the legal point of view of member states, even more important is that many of the water bodies in the Danube were designated as Heavily Modified Water Bodies. The WFD allows water bodies that are substantially changed in character due to physical alterations by human activity and where restoration measures necessary to achieve the good ecological status would have significant adverse effects on the wider environment or the "specified uses" to be designated as Heavily Modified Water Bodies (Art. 4(3)(a), WFD, 2000). For them, other legal objectives – summarized in the ecological potential – come into place and replace the ecological status.

18.2 What is the (indication of) ecological status of the Danube?

A complicated question to provide just a simple answer by JDS monitoring! From what we stated above, it follows that a legally relevant statement concerning the ecological status is not possible from JDS4 data. Nevertheless, we put all the available information together to draw a picture of the conditions the scientists involved in the JDS4 observed in the Danube (Table 1). The following remarks and analyses correspond to the Danube River only, because the involved indices and methods for indication of status have limited explanatory power for tributaries (see BQE chapters for detailed explanations). Disclaimer: For this overview considerations data were simplified and aggregated – left and right bank of Danube were summarized by worst case value, the various indices available were used, interpretation may serve as evaluation of trends without claiming to be complete or representative for national point of view.

Aquatic macroinvertebrates

The sediment inhabiting animals of the biological quality element macrozoobenthos, the aquatic macroinvertebrates, are indicators for oxygen depletion due to pollution by degradable organic substances (Index: SI, saprobic index) as well as for general habitat degradation (index: SK MMI, multi-metric index used in Slovakia). The results of saprobic index analyses show that organic pollution is a local problem, 81% of sites (67% of samples) show an indication of good or high status. As also known from past surveys and TNMN[3] data the indication of good and high status decreases downstream – 91% of sites in the Upper

[3] *Transnational Monitoring Network of the ICPDR: https://www.icpdr.org/main/activities-projects/tnmn-transnational-monitoring-network*

Table 1: Indication of status assessment of sites located in the Danube (without tributaries) – overview of results from JDS2, 3 and 4: Based on national assessment methods for JDS4 and on harmonized indices applied by the experts during JDS2, 3 and 4. Worst case value is given when data was available separately for left and right side of Danube. For Phytobenthos, Macrozoobenthos, and Macrozoobenthos in several cases differing values are given by border sharing countries (separated by /, order of countries can be seen in column Countries).

	JDS 4 Site	JDS 3 Site	JDS 2 Site	Site Name	Countries	Rkm	Phytoplankton			Phytobenthos					Macrophytes					Macrozoobenthos						Fish								
							Status Class JDS4 - National Method [a]	Status Class JDS2: Chl.a - TNMN Classification	Status Class JDS3: Chl.a - TNMN Classification	**Status Class JDS4 - National Method**	Status Class JDS4-Method:-SK IPS	Status Class JDS4-Method:-BG IPS	Status Class JDS3: SK-IPS	Status Class JDS2: SK-IPS	**Status Class JDS4 - National Method** [a]	Status Class JDS3: AT Method	Status Class JDS3: SK Method	Status Class JDS3: HU Method	Status Class JDS2: AT Method	**Status Class JDS4 - National Method** [a]	Status Class JDS4-Method: SI	Status Class JDS4-Method: SK-Method	Status Class JDS3: SI - diverse national SI	Status Class JDS3: SK-Method	Status Class JDS2: SI - diverse national SI	**Status Class JDS4 - National Method** [a]	Status Class JDS4-Method: EFI	Status Class JDS4-Method: FIA	Status Class JDS4-Method: FIS	Status Class JDS3: FIS	Status Class JDS3: FIA	Status Class JDS3: EFI	Status Class JDS2: EFI	Status Class JDS2: FIA
Upper Danube	**1**	1		Böfinger Halde	DE	2581	2		1	**2**	2	2	2		**f)**	2	1	2		**2**	2	2	2	2		**2**	1	2	2					
	2			Bittenbrunn 700m below power st.	DE	2480	3			**2**	2	1			**f)**					**2**	2	1				**3**	1	2	1					
	3	2	2	Kelheim	DE	2415	2	1	1	**3**	2	2	2	2	**f)**	2	1	1	2	**3**	2	3	2	2	2	**3**	2	2		4	2	2	2	2
	4		5	Niederalteich - Mühlau	DE	2276	2	1		**2**	2	2	2		**f)**				2	**3**	2	3	2	2		**3**	3	2	5	5	2	2	2	2
	6	6	7	Jochenstein	DE/AT	2204	2	1	1	**3/2**	2	2	2	2	**-/2**	2	1	2	2	**4**	3	4	3	4	3	**5**	2	5	5	5	5	2	2	4
	7			Enghagen	AT	2113	1			**2**	2	2	2	2	**2**					**3**	2	4												
	8	8	10	Oberloiben	AT	2008	2	1	1	**3**	2	2	2	2	**2**	2	1	1	f)	**3**	2	4	2	3	2	**4**	2	4	5	2	5	2	2	4
	9	9	12	Klosterneuburg	AT	1942		1	1							3	1	2	f)															
	10	11 [b]	14 [b]	Hainburg, upstream Morava	AT	1879	2	1	1	**2**	3	2	2	3	**2**	3	2	2	f)	**3**	1	2	2	2	1	**3**	3	3	4	3	3	3	2	2
	14	13	16	Bratislava	SK	1868	2	1	1	**2**	2	2	2	3		3	1	2	3	**2**	2	2	2	2	2	**5**	3	3	5	3	2	3	3	3
	15	14	17	Čunovo, Gabcikovo resevoir	SK	1855		1	1						**f)**	3	2	3	3							**5**	4	3	5	5	3	4	4	5
	16	15	18	Medvedov / Medve	SK/HU	1806	1	1	1	**3/f**	3	2	3	4		3	1	2	2	**3**	2	3	2	2	2	**5**	3	4	5	3	3	3	2	5
	18			Gönyű	HU	1790	2			**3**	4	3	3		**f)**					**4**	2	2	2	2		**3**	2	3	5					
Middle Danube	**22**	20	26	Szob	HU/SK	1707	2	1	1	**3/-**	4	3	3	3	**f)**	3	2	3	2	**4**	2	4	2	2	2	**5**	3	5	5	3	2	3	2	3
	23	21	29	Budapest upstream - Megyeri Bridge	HU	1660	2	1	1	**2**	2	2	3	3	**f)**	3	3	3	2	**4**	2	4	2	3	2	**3**	3	4	5					
	24	22	32	Budapest downstream - M0 bridge	HU	1630	2	1	1	**2**	2	2	3	3	**f)**	3	3	3	2	**4**	1	2	3	3	1	**3**	3	5	5	4	2	3	2	2
	26	24	35	Dunafoldvar	HU	1560	2	1	1	**3**	3	2	4	3	**f)**	3	2	2	2	**4**	2	3	2	2	2	**3**	3	3	4					
	27	25	36	Paks	HU	1532	2	1	1	**3**	3	2	3	3	**f)**	3	2	2	f)	**3**	1	3	2	2	2	**2**	3	3	4					
	28	26	38	Baja	HU	1481	2	2	1	**3**	3	3	4	3	**f)**	3	2	3	2	**4**	1	4	2	2	2	**3**	3	2	5					
	29	27	39	Hercegszanto / Batina / Bezdan	HU/HR/RS	1434	2	2	1	**f/2/2**	3	3	4	2	**f)**	3	3	2	2	**-/1/4**	2	2	2	2	2	**2**	3	4	4					
	31	31	45	Ilok / Backa Palanka	HR/RS	1300	2	3	2	**2/2**	3	3	3	3	**f)**	3	2	2	2	**4/3**	2	3	2	3	2	**2**	3	5	4	5	3	3	3	3
	37	39	53	Downstream Pancevo	RS	1151		2	1	**3**	3	3	4	3	**f)**	4	3	3	2	**3**	4	4	2	2	4	**4**	4	4	4					
	40	43	59	Banatska Palanka / Bazias	RS/RO	1073	1	1	1	**3/2**	3	3	4	3	**f)/2**	4	3	3	2	**4/1**	4	4	2	2	2	**5**	5							
Lower Danube	**41**	47	65	Upstream Timok (Rudujevac / Gruia)	RS/RO	847	2	1	1	**4/2**	4	3	4	2	**f)/2**	3	3	3	2	**4/1**	3	4	2	3	2	**4**	4				3	4	3	
	43	49	67	Pristol / Novo Selo Harbour	RO/BG	837	2	1	1	**2/3**	4	3	4	3	**3/2**	3	3	2	2	**2/3**	2	2	2	2	2		4							
	47	57	82	Downstream Ruse/Giurgiu (Marten)	BG/RO	488	1	1	1	**3/2**	4	3	3	2	**2/e**	3	4	2	2	**3/2**	2	2	1	3	2	**3**	3				c)	c)	3	
	48	60	86	Chiciu/Silistra	RO/BG	375	2	1	1	**2/c)**	4	3	4	1	**3/2**	4	4	2	2	**2/3**	4	3	2	3	3	**3**	3				4	3	4	5
	50	65	92	Reni	RO/UA	132	3	1	1	**2/d)**	3	3	4	3	**2/d)**	4	4	4	2	**2/d)**	2	3	2	3	2	**3**	3				2	3	3	
	51	66	93	Vilkova - Chilia arm/Kilia arm	RO/UA	18	2	1	1	**2/d)**	3	2		4	**2/d)**	3	3	2	2	**1/d)**	2	2	3	3	2						2	3	3	

a taken from chapters, here partially corrected by national representatives (not fully identical with data in chapters)
b site not identical to JDS4 sampling location but close
c not calculated due to poor data
d no method available yet
e site not accessible
f not applicable

Danube, 80% in the Middle Danube and 67% in the Lower Danube. The multi-metric index shows a different picture: only 37% of the sites reach an indication of good status. The situation is better in the Upper Danube (45%) and in the Lower Danube (50%) compared to the Middle Danube (20%). The MMI is an indicator for habitat degradation and these results show hydromorphological deficits caused by a variety of pressures.

Fish

The good news is that still most species of the reference communities can be found at nearly all sites, even at strongly altered hydromorphological stretches. Hence, the diversity of aquatic habitats is still present in an extent to allow species to survive. However, the indication of ecological status for fish is pointing towards a failing status for a majority of the sites in the Danube. Several indices were used by the experts and all of them show the deficits of the fish community caused by hydromorphological pressures (good status according to FIS: 11% of sites, EFI: 23%, FIA: 25%). Those indices were not developed and are not suitable for the whole length of the Danube, however, the national assessments also show corresponding low 17% of the sites reaching the objective of good status. Two thirds of the sites classified worse than good show the same indication of status by using the MMI for benthic invertebrates. On the other hand, 50% of the remaining good sites are classified as failing the objectives regarding the quality element macrozoobenthos. This illustrates the differing indicative power of those two groups – although partly they show the same or interlinked habitat quality aspects.

Phytobenthos

The indicative status of benthic diatoms (index: Slovakian IPS) decreased from the Upper Danube towards the mouth. In the Upper Danube 61% of the sites indicate good status, in the middle section of the Danube 20% of the sites and in the Lower Danube none. However, it should be noted that results from national assessment of the JDS4 data differs essentially from this indicative assessment, especially for the Lower Danube (see Table 1). Additionally, nutrient levels do not reflect the differences in phytobenthos assessment – diatoms are used particularly as indicators for nutrient pollution. Scientists concluded that general descriptors (longitude, latitude, typology) reflecting natural direction of the flow of the Danube had the most important influence on diatom species composition.

Macrophytes

The results show that environmental parameters, which highly influence water plants in the Danube, were water temperature, dissolved oxygen, nitrates, and conductivity. Mosses preferred colder and more oxygenated water, rich in nitrates in the Upper Reach that has shaded banks with hard substrate. Beside these relationships, the water plants are well known indicators for hydromorphological alterations. The abundance of floating macrophytes in the Middle and Lower Reach of the Danube River suggests good lateral connectivity to backwaters. Just like three Joint Danube Surveys before, the results demonstrate that in certain river stretches there is naturally a lack of microhabitats with proper conditions for the successful growth of macrophytes. This causes almost plant-free river parts without macrophytes or with insignificant abundance – making the assessment difficult to impossible. Based on the comparison of outcomes of previous Joint Danube Surveys, the composition of macrophytes is stable in terms of richness and diversity over several years.

Phytoplankton

In contrast to previous Joint Danube Surveys, when only one sample per site was taken, during JDS4 samples were collected monthly from April to September enabling an assessment of the ecological status according to the methodology guidelines of the member states. Thus, instead of Chlorophyll *a* (after TNMN methodology) this time the national indication of the status was used. However, the results are similar to

previous investigations – 92% of sites show high or good ecological status, only 2 sites were classified as indication for moderate status.

How different is the national assessment of the status compared to JDS4 indication?

For some sites, very different! Reasons for national assessment to often deviate quite a lot are mentioned above and the comparison can be seen in Table 1. For example, the indication of ecological status for macrozoobenthos with national methods (using the same data) is in accordance with results from the Saprobic Index at 31% of the sites, but when including the results from the Slovakian MMI for 73% of the sites. This is not astonishing as the two indices are indicators for different pressures – SI for oxygen depletion due to pollution by degradable organic substances, MMI for general habitat degradation including hydromorphological pressures affecting streambed where the invertebrates live. Some national methods cover both aspects (e.g. the Austrian method is a worst-case combination of the national SI and MMI), others cover just a part of the pressures indicated by SI and MMI, depending on the national requirements. Thus the results using different national methods may vary.

Is the ecological status of Danube improving? Can we see an effect of past measures?

Another very difficult question – but most interesting. The explanatory power of the comparison between data from JDS2, 3 and 4 is somehow limited as the sampling designs were not completely in line with the requirements for the used indices – concerning sampling season, hydrological conditions and sampling methodology/effort. Even though, the comparison still can show general trends and hints at how the Danube is doing. Figure 1 shows how indication of the status changed at sites from JDS2 to 4 and from JDS3 to 4. Both progressions are shown to reduce by comparison the interpretation artefacts caused by exceptional water level or other events during or prior to one of the surveys. For macrozoobenthos (SI) and phytobenthos (both are indicators mainly for organic pollution and nutrients) some fluctuations are visible, sites improved and deteriorated but the status of a majority of sites was stable. The status fluctuations do not seem to indicate a long term trend as they are pointing to different directions when comparing changes from JDS2 to 4 and from JDS3 to 4 (Figure 1). For fish and macrozoobenthos (MMI) (both are indicators mainly for hydromorphological pressures) a number of sampled sites show a deterioration but for a low number of sites also improvements are visible. For the MMI the experts explained the "deterioration" as consequence of high water level before JDS4 sampling – this acted like a hydromorphological pressure and was reflected in the assessment.

The deterioration of indication of ecological status for fish is a warning sign, even if it was partly caused by methodological reasons. It could point to an increasing impact of hydromorphological pressures. However, this can be the case even if the pressure itself is constant – this phenomenon has been well known all over Europe in recent years and may be interlinked or intensified by effects coming from environmental and climate change and invasive alien species.

For phytoplankton and macrophytes, the comparison of status changes was not done due to changes of the applied methodology between the Joint Danube Surveys. However, both biological groups are indicators for pressures caused by nutrients and other general physico-chemical elements, indicating a quite good status for most of the Danubian sites pointing at the quite good situation of the Danube in this respect.

From the biological results of JDS4 we have the impression that the ecological status of the Danube is at least at some locations improving, which might be a consequence of mitigation measures of the past years. However, also deterioration can be observed. This is in line with the findings of hydromorphology experts who pointed out that both improvements but also slight deteriorations took place in recent years.

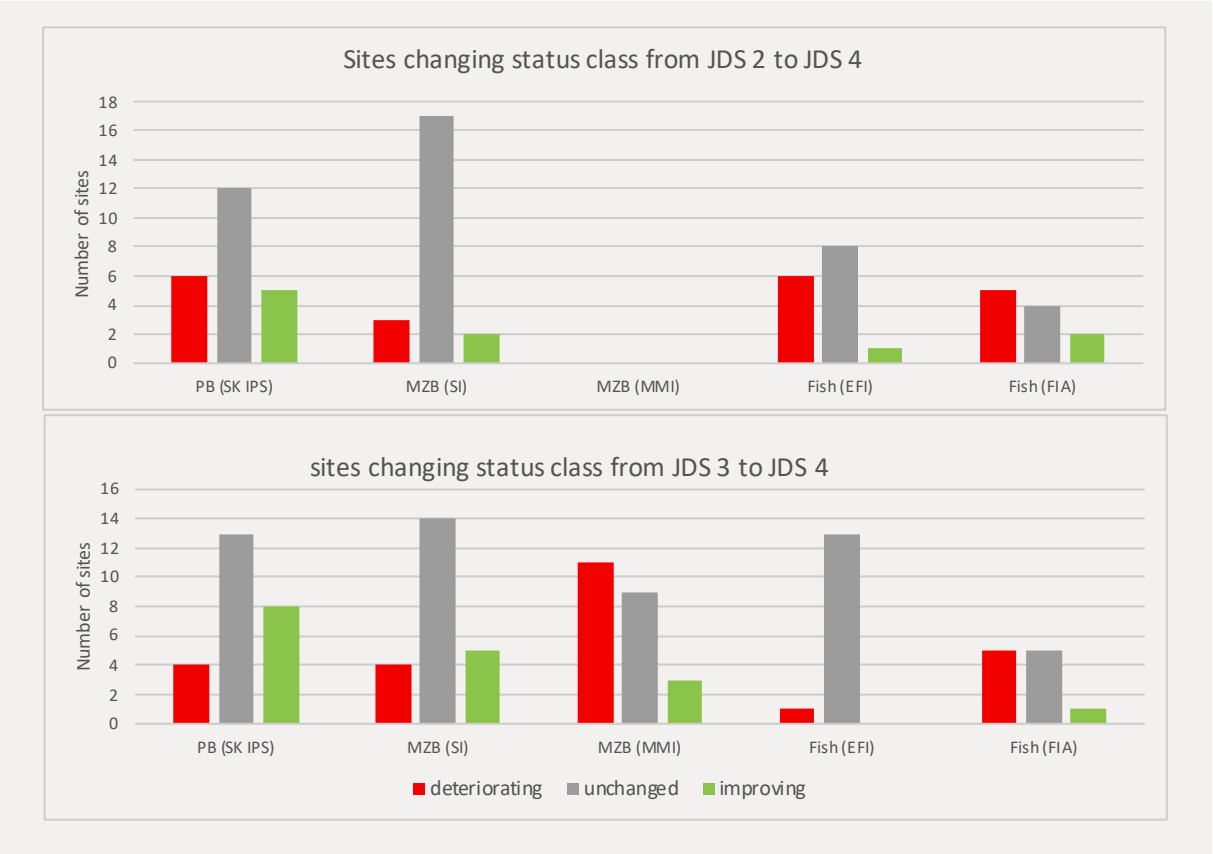

Figure 1: Number of sites changing status class from JDS 2 to JDS 4 (above) and from JDS3 to JDS4. Indices are explained in detail in the respective chapters for the biological quality elements. Slovakian MMI was not calculated during JDS2.

Can we detect an impact of climate change?

Effects of climate change on temperature and interlinked environmental variables are altering the habitat conditions for the biological quality elements and thus changing the conditions for animals and plants. Hence other species than those of the original reference community have the opportunity to conquer the habitat leading to a shift in communities and to successful establishment of highly competitive invasive alien species. Consequently, in future, assessment systems will have to be adapted to the new conditions.

Data from the zooplankton investigation indicate that increased frequencies of species preferring higher temperatures could be linked to climatic changes in the catchment area. Likewise, the high abundance and species diversity of invasive alien macrozoobenthic species at many sites may have been supported by climate change effects and decreasing fish abundances and disturbed age distributions could be partly linked to changes in the temperature regime. In general, significant statements and analysis of climate change effects have to be based on long-term data series. It is obvious that the JDS4 data will be a valuable basis for further investigations in this field.

What is the impact of invasive alien species to the ecosystem?

The Danube River and the main tributaries are under considerable influence of biological invasions. Data from the biological groups demonstrate that the number of recorded alien species revealed is lower in the Lower Danube in comparison to Upper and Middle Danube, since the Lower Danube can be considered as native habitat of some animals and plants that are classified as aliens in the more upstream located areas. The comparison with JDS3 data reveals that the rise of the invasive alien species is progressing.

In the assessment methods, the increasing influence of invasive alien species is more and more a problem. They not only replace native species but also influence the performance of assessment indices that are not designed for the application with these organisms. Regarding macrozoobenthos at some sampling sites invasive alien species reach extremely high abundances - e.g. 99% at site JDS4-10. For the future, a critical adaptation of indicator values for some of those species is therefore necessary

However, like all biological systems, the distribution and abundance patterns of alien species are also highly dynamic. For example, the Asian clam *Corbicula fluminea*, first found in the lower Hungarian Danube in 1998, was detected in high densities during JDS3 (Liska et al., 2015), but was detected only in low densities during JDS4.

18.3 Future of ecological assessment: (e)DNA-based tools

Within the scientific program of JDS4 molecular methods using DNA and environmental DNA (eDNA) for the identification of species (and higher taxonomical groups) were applied for the first time at the scale of an international river basin. A variety of different sample types was used for testing scientific approaches and to evaluate the applied performance of the molecular methods, but also a comparison concerning the applicability of (e)DNA methods for WFD status assessment was done.

Performance of the (e)DNA-methods – biodiversity

78% of all the most abundant diatom species (Phytobenthos) identified in JDS2, 3 and 4, and six of the seven dominant taxa identified morphologically (by light microscopy) in JDS4 were identified by molecular methods. Within the benthic invertebrates, three types of DNA samples were processed and compared with the results from classical Multi-habitat Sampling (MHS): MHS bulk samples (mixture of animals and sediment), preservation liquid and water samples from the river. The comparison of the results revealed a high number of exclusive species for all four types of samples (up to 20%) and only a low overlap in detected species (up to 33%). Concerning fish 75% of the species taxa were detected both by eDNA in water and traditional fish surveys, about 13% and 12% only by each single method. Several benthic fish species were detected only by eDNA: the sturgeons *Acipenser ruthenus* from the Black Sea to Vienna downstream and *Acipenser stellatus* in the Danube delta, shad species (*Alosa immaculata* / *A. tanaica*) downstream of the Iron Gate, and the two Zingel species all along the Danube. The entire longitudinal structure of fish communities along the Danube and its tributaries was convincingly described.

Kind of funny: Salmon and Tuna in the Danube!

Environmental DNA surveys detect any fragments of DNA that are floating in water and sometimes this can reveal unexpected findings. The experts detected DNA from a number of exotic species, some of that presumably from aquaculture or released aquarium fish, most of them originating from sewage coming from wastewater treatment downstream of large cities – DNA from fish that is consumed in the basin area, like Atlantic salmon, sardines, ocean perches, tuna and herring!

Potential of (e)DNA for ecological assessment

Methods for ecological assessment should be robust enough for reproducible results in space and time – e.g. different samples that are taken at the same site but at consecutive days and at different spots will vary in number and abundance of species and thus also in terms of index values, but they should be comparable in terms of ecological status class. Thus, we compared results for status assessment obtained by molecular

methods with classical results. This should give some overview hints of the potential of molecular methods for status assessment without claiming scientific precision. A more detailed analysis is given in Chapter 17.

Fish experts used intercalibration common metrics for ecological assessment of sites with data from classical fish survey and from eDNA analysis. For 46% of the sites they found the same status class and for 70% of the sites the final classification of reaching or failing the WFD objective of good status was identical. The indication of ecological status based on fish eDNA is mostly moderate, but with improvement in the Middle Danube and the delta, and degradation between Belgrade and the Iron Gate (Figure 6 in Chapter 14, Metabarcoding of fish eDNA samples).

For benthic invertebrates, the sites were compared by using the Austrian SI and MMI[4]. Both indices were calculated with species data originating from a) classical MHS sampling, b) DNA from bulk samples (like classical samples – all material mixed together), c) DNA from preservation liquid (alcohol extracted from the bulk samples). A comparison was done by using abundance data but also presence and absence of species for classical samples (DNA methods do not deliver abundance estimates but presence/absence-values – differences between abundance and presence/absence results may indicate the importance of abundance values for ecological assessment). Results are presented in Table 2. Accordance of the status class assessment is high for the SI between classical samples and preservation liquid (62%) and even higher between classical samples and bulk samples (66%). The accordance increases over 80% when using presence/absence data for classical samples. This difference shows that the use of exact abundance data may account for information that is not given when using presence/absence information. It will be one of the future challenges for the use of DNA methods for ecological assessment to either incorporate this quantitative aspect by methodological adaptations or to find alternative approaches.

For the MMI the identical status classes identified by the three different methods is few percent lower (Table 2) but follows the same pattern as described above for the SI.

Table 2: Number of sampling sites with identical results for SI and MMI. MHS (abu): classical samples with abundance values; MHS (P/A): classical samples with presence/absence values; DNA bulk: DNA from bulk samples taken similar to classical samples (invertebrates and sediment mixed together), DNA liquid: liquid from DNA bulk samples was analysed.

status class identical

	SI		MMI	
	MHS (abu)	MHS (P/A)	MHS (abu)	MHS (P/A)
DNA bulk	66%	83%	59%	79%
DNA liquid	62%	83%	55%	59%

status class identical

	SI	MMI
	DNA liquid	DNA liquid
DNA bulk	79%	66%

reaching/failure of objective identical

	SI		MMI	
	MHS (abu)	MHS (P/A)	MHS (abu)	MHS (P/A)
DNA bulk	83%	86%	93%	86%
DNA liquid	83%	86%	86%	79%

reaching/failure of objective identical

	SI	MMI
	DNA liquid	DNA liquid
DNA bulk	79%	86%

Table 2 also shows the comparison between status classes from bulk samples and from preservation liquid (79% accordance for SI and 66% for MMI) to demonstrate that the accordance between the two molecular methods is not higher than with classical methods.

[4] *Calculated by the Austrian experts Patrick Leitner and Wolfram Graf, University of Natural Resources and Life Science (BOKU), Vienna. Results may differ from Chapter 6 - Aquatic Macroinvertebrates.*

The same analysis was done (instead of status classes) for the information if the site reaches or fails the quality objective of the WFD, the good status. This is a relevant information, because failing the objective is provoking the need for restoration measures in natural water bodies. Here the accordance between classical sampling and molecular methods is even higher and reaches up to 93% (Table 2).

These results demonstrate the high potential of DNA-methods for ecological assessment – especially taking into consideration that this was a test only and for sound status assessment adaptations of the assessment method would be necessary (e.g. reference values, performance of metrics).

For a smaller number of three sampling sites the indicative status for benthic invertebrates based on the Austrian indices SI and MMI was calculated for the above mentioned sample types and additionally for eDNA from water samples (Table 3). The results are astonishingly close together and when looking at the index values (that are not presented here, but can be downloaded from the JDS 4 website. Please see the full report, available at www.danubesurvey.org/jds4/full-report) they are even closer. This shows the potential of using eDNA from water for ecological assessment.

Table 3: Indicative status class calculated with MZB data from: MHS (abu): classical samples with abundance values; MHS (P/A): classical samples with presence/absence values; DNA bulk: bulk samples taken similar to classical samples (invertebrates and sediment mixed together), DNA liquid: liquid from DNA bulk samples was analysed, eDNA: DNA taken from water sample.

	SI					MMI				
Site	**MHS abu**	MHS (P/A)	DNA bulk	DNA liquid	**eDNA**	**MHS abu**	MHS (P/A)	DNA bulk	DNA liquid	**eDNA**
10	**1**	2	2	2	**2**	**3**	3	2	3	**3**
14	**1**	2	2	2	**2**	**2**	3	2	2	**3**
16	**2**	2	2	2	**2**	**3**	3	3	3	**3**

What is the advantage of eDNA and DNA-based methods for ecological assessment?

The results of JDS4 show that the use of molecular methods can increase the detected biodiversity. Species that are either rare or difficult to sample with classical methods can be detected – e.g. benthic fish. For example, sturgeons were only detected with the eDNA method. Additionally, some organismic groups are difficult to identify and only few experts are available. This is the case for chironomids, oligochaetes and nematodes that were found in high species numbers and diversity by molecular methods. In this case, the modern methods use the knowledge of traditional taxonomists that is preserved in genetic reference databases. This increase in exactness and coverage of taxonomical identification opens possibilities for future development of indices and metrics for ecological assessment. Ecological status assessment may become cheaper and faster by use of (e)DNA-based methods and it could become possible to get more information in shorter time, which is of advantage for water management.

Gaps and future challenges

Results from the DNA-based methods show also that molecular methods are only as good as the information contained in the databases and still there are gaps of missing species (macrozoobenthos) or genetic markers not enough specific to distinguish between several taxonomically related species (fish). All the more the work of classically trained taxonomists is important to fill those gaps and to ensure constant quality assurance.

The (e)DNA-based methods used during JDS4 did not describe quantitative information regarding community structure. High abundances of certain taxa, (e.g. invasive species reaching up to 99% of the total macrozoobenthos abundance) was not reflected by this approach but could be complemented in future study designs (e.g. by occupancy patterns of replicates or subsamples).

The traditionally applied sampling designs and assessment systems were scientifically designed in accordance to each other and are not automatically suitable for the (e)DNA-based approaches. For the practical application of molecular methods for ecological assessment, a further development has to take place – including definition of reference conditions, and assessment metrics and indices. For eDNA methods it has to be taken especially into account that classical methods assess the environmental variables at a certain spot or stretch and eDNA from river water somehow integrates information coming from a longer distance. This may be seen both as advantage or disadvantage, depending on the study design.

18.4 Acknowledgements

We would like to thank Patrick Leitner and Wolfram Graf, University of Natural Resources and Life Science (BOKU), Vienna for data analysis and valuable expert input to the discussion. Didier Pont, Alexander Weigand, Igor Liška and other JDS4 experts improved the chapter with their essential comments.

18.5 References

LIŠKA, I., WAGNER, F., SENGL, M., DEUTSCH, K., SLOBODNÍK, J. (eds.) (2015). Joint Danube Survey 3 A Comprehensive Analysis of Danube Water Quality. International Commission for the Protection of the Danube.

WFD (2000). Directive 2000/60/EC of the European Parliament and of the Council 22.12.2000. Official Journal of the European Communities L327: 1-72.

19 Microbial faecal pollution and source tracking

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Abstract

The extent and origin of microbial faecal pollution along the Danube and its most important tributaries was determined based on the standard faecal indicator bacterium E. coli and genetic microbial source tracking markers. In total, 72 samples were collected at 36 sites, with 18 sites where samples were collected from the middle and from the left and right side of the river. 56 samples (78%) displayed little or moderate pollution levels as it can be expected for rivers with state-of-the-art wastewater management. 14 samples (19%) showed critical and 2 samples (3%) strong pollution levels. No site with excessive pollution level was observed during JDS4. Hotspots of microbial faecal pollution were identified in the middle and lower section of the Danube and in the tributaries Arges, Rusenski Lom and Drava. At many sampling sites, the influence of a wastewater input (from a point source or a tributary) could only be detected at one of the two river sides. A slight yet statistically insignificant trend towards lower values in comparison to JDS3 was observed. Corresponding to earlier investigations, human-associated genetic faecal markers were detected in a high percentage of samples showing that human faecal contamination is the major source of microbial faecal pollution in the Danube River basin. Only at very few sites, low concentrations of ruminant- and pig-associated source tracking markers were found. The future implementation of genetic faecal markers for bird-associated faecal pollution to cover a potential impact of poultry industry is recommended.

19.1 Introduction

19.1.1 Background

Escherichia coli is used worldwide as sensitive indicator for the assessment of faecal pollution in the aquatic environment. Faecal indicators are excreted by humans and warm-blooded animals in high concentrations and survive for a certain time in aquatic systems. Faecal pollution in rivers can be caused by point sources

like discharges of sewage from human sources or livestock enterprises and by non-point sources like pasture, urban and agricultural run-off or water fowl. Faeces frequently contain pathogenic microorganisms like bacteria, viruses or parasites. Therefore, intestinal indicator bacteria like *E. coli* indicate the potential presence of pathogens and are especially well appropriate to indicate faecal pollution in surface waters. The usefulness of *E. coli* as faecal indicator was shown repeatedly for assessing the microbiological water quality of the Danube during previous JDS (KIRSCHNER ET AL 2008, KIRSCHNER ET AL 2015).

Faecal pollution and microbial contamination from anthropogenic sources via treatment plant effluents or untreated sewage have been shown to be a crucial problem throughout the Danube River Basin leading to serious debasement of water quality (KIRSCHNER ET AL 2009, KIRSCHNER ET AL 2017). Moreover, the river and its tributaries receive faecal polluted run-off from animal farms and agricultural areas. Thus, detailed knowledge on the extent and the origin of microbiological faecal pollution is crucial for watershed management activities in order to maintain safe waters according to established quality targets (EU Bathing Water Directive, EU Drinking Water Directive).

Microbial faecal source tracking (MST) methods were developed to provide information on the origin of faecal pollution. During the last two decades, methods for the molecular detection of source-associated bacterial and viral indicators of faecal pollution have been established as the methods of choice to identify the responsible sources of environmental contamination (HAGEDORN ET AL 2011). Most prominent and widely used among these approaches is the detection and quantification of genetic faecal markers targeting source-associated bacterial faecal populations from the phylum Bacteroidetes (WUERTZ ET AL 2011, FARNLEITNER ET AL 2011). Usually these markers are detected by applying quantitative polymerase chain reaction (qPCR) on DNA extracted from water samples. Extracted DNA can be stored at -80°C before further molecular analysis is performed, supporting the collection of large DNA sample libraries.

Ample MST investigations were conducted on the Danube in the past. The human-associated marker BacH (REISCHER ET AL 2007) was evaluated on samples from JDS2 (REISCHER ET AL 2008). It was shown that the marker was detectable in Danube and tributary samples throughout the catchment. Investigations on samples from JDS2 showed that faecal pollution in the tributaries was dominated by human sources as demonstrated by a clear relationship between the standard faecal indicator *E. coli* and the BacH parameter (KIRSCHNER ET AL 2008; KIRSCHNER ET AL 2015). For JDS3, human- (BacH, HF183II) ruminant- (BacR) and pig-associated (Pig2Bac) markers were determined together for the first time, corroborating that human faecal pollution is the dominant source of faecal pollution in the Danube River basin (KIRSCHNER ET AL 2017).

19.1.2 Aims of the study

Data of microbial faecal pollution were collected during the Joint Danube Survey 4 (2019) along the longitudinal stretch of the River Danube from the upper section (rkm 2415) to the Delta (rkm 104) for the following aims:

- analysis of the extent and variation of faecal pollution on the basis of the standard bacterial faecal indicator bacterium *E. coli* along the longitudinal stretch of the River Danube and main tributaries
- identifying hotspots of faecal pollution of the Danube River basin
- classification of faecal pollution according to a classification scheme developed in KAVKA ET AL (2006) and KIRSCHNER ET AL (2009)
- Quantification of microbial source tracking markers for human-associated, ruminant-associated and pig-associated faecal pollution based on quantitative PCR (qPCR)

- Determining the relationship of the MST markers to the *E. coli* concentrations
- Comparison of *E. coli* and MST marker data with results from JDS3

19.2 Methods

19.2.1 Survey logistics

In contrast to the official JDS4 logistics where samples were taken by the national teams, the microbiological sampling campaign was done with cars using a small rubber boat for taking the samples. The trip down the Danube started in Kelheim, Germany on June 30, 2019 and lasted until July 19 in Tulcea, Romania. Samples were processed in partner laboratories in Regensburg (Germany), Vienna (Austria), Budapest (Hungary), Belgrade (Serbia), Turnu-Severin and Calarasi (both Romania). Processed samples were stored at 4°C or frozen at -22°C and delivered to the home laboratories in Austria (Vienna and Graz) within 96 hours.

19.2.2 Sampling and storage

Water samples were collected by hand from small boats at a water depth of approx. 30 cm in two sterile 500-mL plastic bottles from 36 sampling sites. 28 sites were located on the Danube River, 8 were situated in the mouth of tributaries. At 18 Danube sites, samples were taken from the left, middle and right sides of the river. At 10 Danube sites and all tributaries samples were taken either in the middle, or at the left or right side of the river. All samples were immediately cooled in a cooling box and brought to the partner laboratory within a maximum of 4 hours, where they were subsequently processed.

19.2.3 *Escherichia coli*

E. coli concentrations were determined according to ISO 9308-2 with Colilert 18 (IDEXX, Ludwigsburg, Germany), a most probable number (MPN) technique, using two volumes (100 ml, 1 ml). Samples were incubated at 36 ± 2°C for 18 - 22 hours and analysed in a UV-cabinet. Quantitative values were obtained by comparison with the MPN table provided by the manufacturer. For the first 4 sampling points in Germany, parallel samples were taken for comparative measurements via ISO 9308-2 and ISO 9308-3, performed at Bayerisches Landesamt für Umwelt in Augsburg (Dr. Margit Schade). Due to the fact that intestinal Enterococci data did not add significant additional information to the faecal pollution situation during the previous JDS, only *E. coli* was determined in JDS4.

19.2.4 Classification system

To enable the assessment of faecal pollution levels, faecal indicators were classified by a system of 5 microbiological water quality categories after KAVKA ET AL (2006) and KIRSCHNER ET AL (2009) (Table 1). For setting up this scheme, one concentration derived from the EU Bathing Water Quality Directive 2006 was used as anchor point (1000 MPN / 100 ml). Faecal pollution levels of quality class I and II are below, quality classes III, IV, and V exceed these values. The EU Bathing Water Directive and the assessment of bathing water quality could not be applied for the JDS data set since the data of bacterial indicators of faecal pollution generated during the Joint Danube Survey are single measurements. It can thus be considered only as a snapshot analysis of faecal pollution. According to the EU Bathing Water Directive the assessment of bathing water quality shall always comprise at least 16 samples compiled in relation to that bathing

season and the three preceding bathing seasons, based upon a 95-percentile and 90-percentile evaluation, respectively.

Table 1: Microbiologically based classification system of water quality according to faecal pollution (KAVKA ET AL 2006; KIRSCHNER ET AL 2009). Faecal indicator concentrations are given in most probable numbers (MPN) per 100 ml.

Classification of faecal pollution		Class				
		I	II	III	IV	V
Parameter	Faecal pollution	little	moderate	critical	strong	excessive
Escherichia coli	in 100ml water	≤ 100	> 100 - 1 000	> 1 000 - 10 000	> 10 000 - 100 000	> 100 000

19.2.5 DNA extraction

Duplicate subsamples with a volume of 300 ml were filtered through 0.2 µm polycarbonate filters. Filters were immediately frozen at -20°C and within 4 days all filters were transferred to a -80°C freezer after transport of the filters to the home laboratory. Clean filters were frozen and stored alongside the sample filters as filter controls. DNA was extracted by a phenol-chloroform extraction combined with bead-beating (REISCHER ET AL 2008). DNA was dissolved in 100 µl of 10 mmol L-1 Tris buffer. Extraction controls were routinely run alongside each extraction batch.

19.2.6 Microbial source tracking markers

qPCR quality assurance and inhibition control

The sample DNA was diluted 1:4 and 1:16 and the AllBac assay (LAYTON ET AL 2006) was applied to ensure the presence of amplifiable bacterial DNA and the absence of inhibition.

Microbial faecal source tracking assays

The human-associated faecal marker BacHum (KILDARE ET AL 2007) and a modified version of the HF183II (GREEN ET AL 2014) - which was recently renamed as HF183/BacR287 (USEPA 2019) - were determined by qPCR indicating human-associated faecal pollution. The ruminant-associated BacR qPCR assay (REISCHER ET AL 2007) and the pig-associated Pig2Bac qPCR assay (MIESZKIN ET AL 2009) were included as methods for detecting animal faecal pollution sources. All these qPCR assays were adapted to run on the Rotor-Gene Q thermocycler with the Rotor-Gene Multiplex PCR mastermix (Qiagen Inc.). Quantification was achieved by running plasmid standard dilution series of known concentration. No-template controls were applied at all instrument runs.

Data analysis

The recovered qPCR data were log10 +1 transformed. Graphs were produced using Microsoft Excel and IBM-SPSS, version 24. Statistical analysis was performed using IBM SPSS version 24 for Windows.

19.3 Results

19.3.1 Variation in *E. coli* concentrations

E. coli concentrations are shown in Figure 1 and expressed in most probable numbers (MPN) per 100 ml. In the upper part of the Danube until Dunaföldvar (rkm 1560; Hungary) all *E. coli* concentrations corresponded to class I and II (little to moderate pollution) with only one exception directly downstream the wastewater treatment plant effluent of Linz/Asten (Enghagen, Austria), where the limit value of moderate pollution was exceeded only slightly (1.050 MPN per 100 ml). The comparative measurements performed for the first 4 sampling sites were in perfect agreement with these observations (data not shown). In the middle part of the Danube, starting with the tributary Drava, many sampling sites showed critical pollution levels, specifically downstream (ds) Drava (rkm 1377 right), ds Novi Sad (rkm 1252 left/middle/right), ds Tisza (rkm 1212 middle), ds Belgrade/Pancevo (rkm 1151, right/middle), and at the Iron Gate reservoir in Tekija (rkm 954 right). The large tributaries Tisza and Sava exhibited little and moderate pollution, respectively.

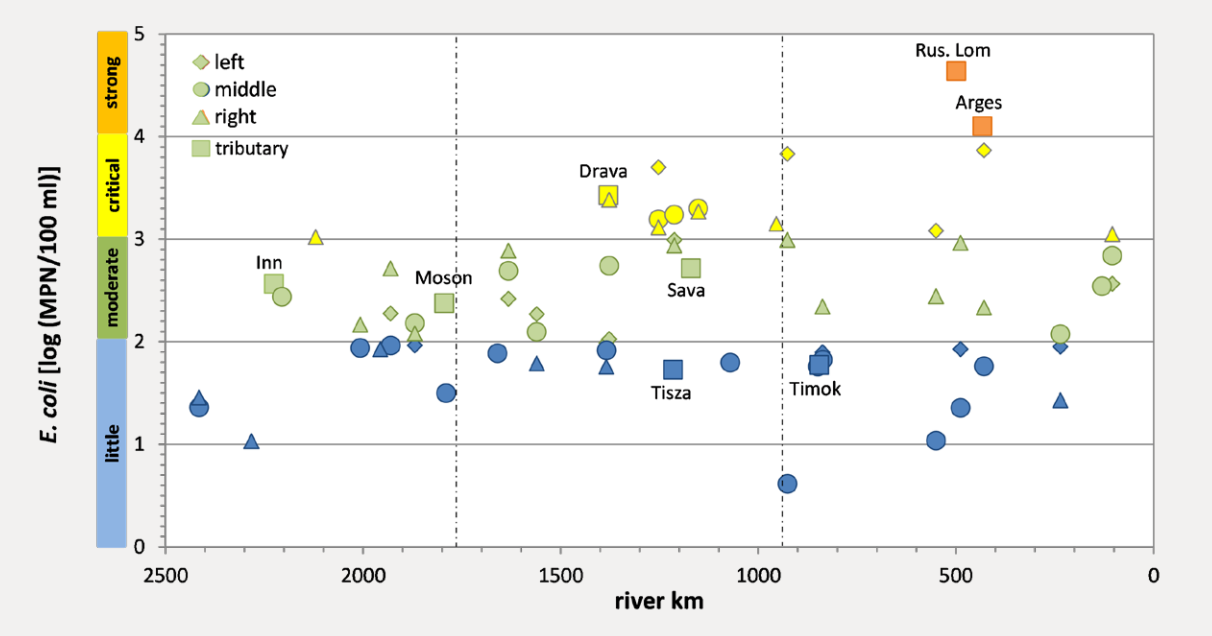

Figure 1: E. coli concentrations along the Danube and in selected tributaries (squares). Data were log – transformed: 1 = 10 MPN per 100 ml, 2 = 100 MPN per 100 ml, 3 = 1.000 MPN per 100 ml, 4 = 10.000 MPN per 100 ml, 5 = 100.000 MPN per 100 ml. Samples were taken left (diamonds), middle (large circles) and right (triangles) at 18 Danube stations. From the other Danube stations and the tributaries Inn, Moson Danube, Drava, Tisza, Sava, Timok, Rusenski Lom and Arges samples were taken only on one position (left, middle or right). Coloured bars along the y-axis indicate the pollution status according to Table 1, from little (blue) to strong (orange) pollution. Dashed vertical lines: borders between Upper, Middle and Lower Danube.

In the lowest part of the Danube, critical pollution levels were observed at four Danube sampling sites, at Simijan (rkm 926 left), downstream Zimnicea/Svistov (rkm 550 left), downstream the strongly polluted tributary Arges (rkm 432, left) and at Tulcea (rkm 104, right). The two investigated tributaries showed both strong pollution levels with a maximum of 43,500 MPN per 100 ml.

Summing up, 56 of 72 investigated samples displayed little or moderate pollution as it can be expected for rivers with state-of-the-art WWTP influents. With one exception, critical pollution levels only occurred in the middle and lower stretch of the Danube and its tributaries. Strong pollution levels only occurred in two tributaries in the lower stretch, of all 72 investigated samples no sample indicating excessive pollution was recorded.

19.3.2 Comparison with JDS3

A direct comparison of the *E. coli* concentrations of 2019 with those from 2013 revealed no overall significant difference between the two JDS. A paired T-test with all 72 corresponding samples resulted in a p-value of > 0.1. Table 2 depicts the key data of both surveys.

Table 2: Median, minimum and maximum E. coli concentrations of all 72 corresponding samples from JDS3 and JDS4.

E. coli	2013		2019	
	[MPN/100ml]	log[MPN/100 ml]	[MPN/100ml]	log[MPN/100 ml]
Median	335	2.52	201	2.30
minimum	2	0,3	3	0,49
maximum	393,000	5,59	43,500	4,64

A 1:1 analysis of log-normalized data by scatterplot, however, showed a trend towards lower values in 2019. The linear correlation line markedly deviated from the 1:1 line and 6 samples were identified that showed by 1 log lower values in 2019 than in 2013 (Figure 2). Specifically, at Oberloiben and Kelheim, where ships had been suspected to be responsible for the high values in 2013, moderate pollution levels were observed in 2019. In addition, the tributary Arges, the receiving water of the wastewater discharges from Bucharest, showed by > 1 log lower results in 2019. At Dunaföldvar, 2-log lower values were observed in 2019 and downstream Zimnicea/Svistov and in the tributary Timok, pollution levels were about 1-log lower than in 2013. In contrast, at the Iron Gate reservoir near Tekija and ds the WWTP effluent Linz/Asten the pollution levels in 2019 were 2.1 and 1.4 log higher than in 2013.

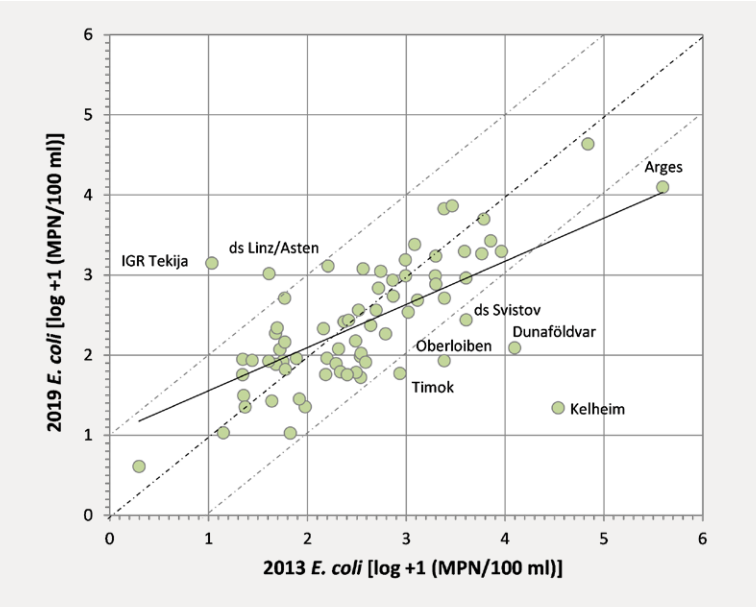

Figure 2: Comparison of E. coli concentrations at 72 corresponding samples of the Danube and selected tributaries. The stations where the concentrations differed by more than 1 log are named. The middle dashed line indicates the 1:1 curve, the outer dashed lines indicate 1 log difference.

19.3.3 Microbial Source Tracking

Occurrence of source-associated genetic faecal markers during JDS4

The concentrations of the human-associated genetic faecal markers HF183 II and BacHum were determined using quantitative PCR. These genetic markers were designed to be specific indicators of human faecal influence originating from untreated and treated sewage discharges into the environment. They could be found in more than 69% of the investigated samples. From in total 72 samples, 58 samples passed the quality control of the qPCR process. From these 58 samples, 40 and 41 samples were positive for HF183 II and BacHum, respectively. The concentrations of the BacHum marker were of similar magnitude as HF183 II (Figure 3).

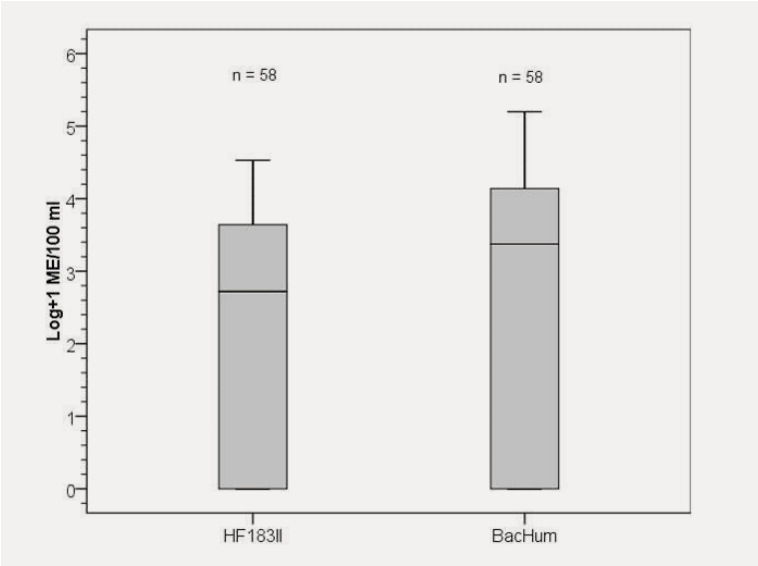

Figure 3: Distribution of HF183 II and BacHum marker concentrations in Danube and tributary samples (ME, marker equivalents; Boxes, 25th and 75th percentile; lines within the boxes, median; whiskers, 10th and 90th percentile, respectively; n, number of samples).

Highest marker concentrations were found in the Drava tributary, downstream (ds) of its confluence with the Danube, ds Novi Sad, ds Belgrade/Pancevo, at Simijan, ds Ruse, in the Arges tributary and ds of its confluence with the Danube. In comparison to JDS3, the percentage of samples that were positive for the human-associated markers was slightly lower. During JDS3, more than 90% positive samples were found. As well, the number of samples that passed quality control in JDS4 was lower than in JDS3. One major reason was that in the upper section of the Danube the high water of the Inn resulted in a significant decrease in DNA extraction efficiency and false-negative qPCR results, most likely due to the high load of inorganic particles capturing the DNA. This influence of the Inn (rkm 2225) was detectable until ds Bratislava (rkm 1869), affecting in total 13 samples.

In order to detect the possible presence of animal faecal pollution, two additional MST markers were included. The BacR marker targets Bacteroidetes populations associated with ruminant animal faeces, while the Pig2Bac marker is targeting pig-associated Bacteroidetes populations. In significant contrast to the human-associated markers, the animal-associated markers were rarely detected in the investigated JDS4 samples. The BacR marker was detected in only 1 out of 58 samples (1.7%), the Pig2Bac assay only 7 out of 58 samples (12.0%). In both cases the detected concentrations were very low and close to the limit of detection (results not shown).

Summing up, as in JDS3 (KIRSCHNER ET AL 2017), human faecal pollution was the dominant source of faecal pollution in the Danube and its most important tributaries during JDS4, while animal faecal pollution only plays a minor role along the whole Danube with a few exceptions at specific locations.

Correlation and regression analysis of genetic markers with faecal indicators

In order to investigate for relationships between the levels of source-associated genetic faecal markers and the bacterial standard indicator of faecal pollution (*E. coli*), non-parametric Spearman rank correlations were calculated for all samples where a positive marker result was obtained by qPCR. Both human-associated MST markers were highly correlated with each other (rho=0.94, p<0.001, n=34) strongly supporting the reliability of the two markers as indicators of human faecal pollution. Remarkably, there were high correlations of the human-associated genetic faecal markers with the *E. coli* concentrations with rho = 0.67 and p < 0.001 for HF183 II and with rho = 0.69 and p < 0.001 for BacHum, respectively. Without the outlier (Rusenski Lom, high *E. coli* but relatively low marker concentrations, see Figure 4), correlation coefficients would be markedly higher (rho = 0.81 for both BacHum and HF183 II). In sharp contrast to the human associated markers, there were no correlations between *E. coli* concentrations and the ruminant- and pig-associated genetic faecal markers BacR and Pig2Bac, due to the few positive results.

Linear regression analysis showed that for all samples from the Danube River and its tributaries where a positive qPCR signal was obtained, 51% and 41% of the variation in *E. coli* concentrations could be explained by the respective levels of the human-associated genetic faecal markers HF183 II and BacHum, respectively (Figure 4). In comparison to the data from JDS3 (REISCHER ET AL 2015, KIRSCHNER ET AL 2017), the correlation between the human-associated MST markers and *E. coli* was less pronounced in JDS4. If the outlier mentioned above would have been eliminated from the data set, comparable values of 60 to 65% would have been achieved.

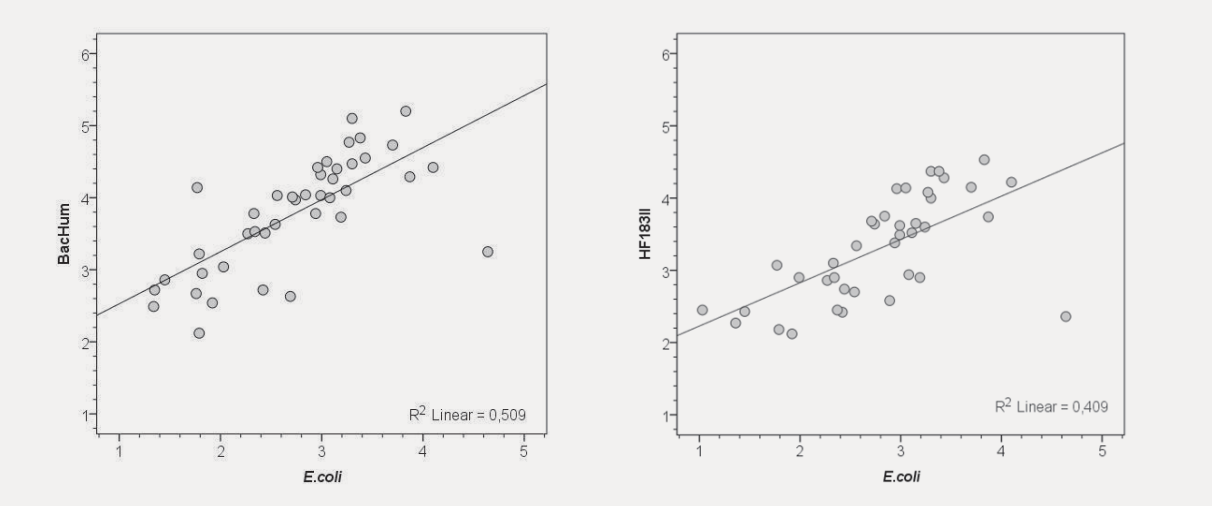

Figure 4: Regression analysis of human-associated Bacteroidetes genetic faecal marker versus E. coli levels (as indicator for total faecal pollution) based on linear regression models.

19.4 Conclusions

- The longitudinal study along the Danube River and its major tributaries by applying uniform methods in the partner laboratories allowed for a reliable quantitative estimation of the presence of the faecal indicator bacterium *E. coli* and thus faecal pollution levels.
- Through the application of a "5-level" classification system, the assessment of the microbiological water quality regarding faecal pollution based on a single event sampling was possible. However, a classification according to the EU Bathing Water Directive is not directly possible since the bathing water quality assessments comprise at least 16 samples and a percentile evaluation.

- Sixteen sampling points (13 Danube samples and 3 tributaries) out of 72 (22%) were classified either as critically (14) or as strongly (2) polluted. As hotspots of strong pollution the tributaries Arges and Rusenski Lom were identified. The highest contamination in the Danube with critical pollution levels was measured downstream the confluence with the Arges, as well as ds Novi Sad (RS) and Simijan (RO), in the middle stretch, with generally critical faecal pollution levels. Another hotspot of faecal pollution was observed in the Drava River and downstream of its confluence with the Danube.
- Sampling at the left, middle and right river sides enabled a deep view into the microbial faecal pollution patterns of the Danube. At many JDS sampling sites the influence of a wastewater input (from a point source or a tributary) could only be detected at one of the two river sides, most prominently downstream Rusenski Lom (BG), downstream Arges (RO) or after the Iron gates at Vrbica/Simijan (RS/RO). Thus, sampling at both river sides in addition to the midstream is a prerequisite for assessing the microbiological-faecal status of the river.
- A comparison with data from 2013 revealed similar median values for the faecal indicator *E. coli*. Although a slight tendency towards lower values was observed in the Danube and specific tributaries (Arges, Timok), a general improvement of the microbiological water quality cannot be deduced from the data, because of the fact that the microbiological analysis is based on two snapshots only. Stretches in the middle section between Novi Sad and Vrbica/Simijan kept being hotspots of faecal pollution since JDS1. However, stations in Germany, Austria and Hungary (Kelheim, Oberloiben and Dunaföldvar), where hotspots of faecal pollution were observed only in 2013, were inconspicuous in 2019 indicating that local short term effects (e.g. from shipping industry) were responsible for the observed high pollution levels in 2013.
- Microbial source tracking data from JDS4 corroborated that human faecal contamination is the main driver of faecal pollution levels in the Danube and its major tributaries. Human-associated genetic faecal marker levels could be predicted by the bacterial standard indicator variations, such as E.coli, to a high extent.
- In contrast to human-associated genetic faecal markers, ruminant- and pig-associated genetic faecal markers could very infrequently be detected and showed very low levels (close to the detection limit of the method). This indicates that faecal pollution from ruminant and pig contamination sources did not play a significant role for faecal pollution in the Danube River and its major tributaries at their confluence sites.
- One valuable addition in the future would be the application of genetic faecal markers for bird-associated faecal pollution, but unfortunately up to date there are no such methods available that have been tested in the Central European region.

19.5 Acknowledgements

Besides funding from ICPDR, the study was financed by the FWF-project P32464 granted to AK, AF and GZ. The authors want to express their gratitude to Dr. Michael Schabuss and Dr. Horst Zornig from ProFisch for their great help in sampling and all responsible and supporting persons in the partner laboratories at University of Regensburg (Dr. Sebastien Ferreira-Cerca), at ELTE University Budapest (Dr. Zsuzsa Keki), at University of Belgrade (Prof. Momir Paunovic), and at Romanian Water Laboratories in Turnu-Severin and Calarsi (Dr. Florentina Soare, Mary Craciun, Cristina Dumitru, Geanina Aldea). Moreover, the authors thank Dr. Margit Schade (LFU Bayern) for the parallel investigation of faecal indicators of the German water samples and Dr. Jaroslav Slobodnik and Peter Mazan for organizing and performing the transport of cooled samples to the main laboratories.

19.6 References

EU BATHING WATER QUALITY DIRECTIVE (2006) Directive 2006/7/EC concerning the management of bathing water quality.

EU DRINKING WATER DIRECTIVE (1998) Council Directive 98/83/EC of 3 November 1998 on the quality of water intended for human consumption. Official Journal L 330, 32-54

FARNLEITNER AH, REISCHER GH, STADLER H, KOLLANUR D, SOMMER R, ZEROBIN W, BLÖSCHL G, BARRELLA KM, TRUESDALE JA, CASAREZ EA & DI GIOVANNI GD (2011) Microbial Source Tracking: Methods, Applications and Case Studies. Chapter 18 - Agricultural and Rural Watersheds, in: Hagedorn C, Haarwood J, Blanch A (ed) Springer – New York, pp. 399-432

GREEN HC, HAUGLAND RA, VARMA M, MILLEN HT, BORCHARDT MA, FIELD KG, WALTERS WA, KNIGHT R, SIVAGANESAN M, KELTY CA & SHANKS OC (2014) Improved HF183 Quantitative Real-Time PCR Assay for Characterization of Human Fecal Pollution in Ambient Surface Water Samples. Appl Environ Microbiol. 80, 3086-3094

HAGEDORN C, HARWOOD VJ & BLANCH A (2011) Microbial Source Tracking: Methods, Applications, and Case Studies, Springer, New York, USA.

ISO 9308-2 (2012) Water quality — Enumeration of Escherichia coli and coliform bacteria — Part 2: Most probable number method.

ISO 9308-3 (1998) Water quality — Detection and enumeration of Escherichia coli and coliform bacteria — Part 3: Miniaturized method (Most Probable Number) for the detection and enumeration of E. coli in surface and waste water

KAVKA GG, KASIMIR GD & FARNLEITNER AH (2006) Microbiological water quality of the River Danube (km 2581 – km 15): Longitudinal variation of pollution as determined by standard parameters. In: Proceedings 36th International Conference of IAD. Austrian Committee for Danube Research / IAD, Vienna. ISBN 13: 978-3-9500723-2-7, pp 415-421

KILDARE BJ, LEUTENEGGER CM, MCSWAIN BS, BAMBIC DG, RAJAL VB & WUERTZ S (2007) 16S rRNA-based assays for quantitative detection of universal, human-, cow-, and dog-specific fecal Bacteroidales: A Bayesian approach. Water Res. 41, 3701-3715

KIRSCHNER AKT, KAVKA GG, VELIMIROV B, REISCHER GH, MACH RL & FARNLEITNER AH (2008) Microbiological water quality and DNA based quantitative microbial source tracking. In: Liska I, Wagner F, Slobodnik J (eds) Joint Danube Survey 2, Final scientific report. ICPDR Vienna, Austria; pp 86-95

KIRSCHNER AKT, KAVKA GG, VELIMIROV B, MACH RL, SOMMER R & FARNLEITNER AH (2009) Microbiological water quality along the Danube River: Integrating data from two whole-river surveys and a transnational monitoring network. Water Research 43: 3673-3684

KIRSCHNER AKT, JAKWERTH S, KOLAREVIC S, SOMMER R, BLASCHKE AP, KAVKA G, REISCHER G & FARNLEITNER AH (2015) Microbiology – Bacterial Faecal Indicators. In: Joint Danube Survey 3, Final scientific report. Liska I, Wagner F, Slobodnik J (eds.) ICPDR Vienna, Austria

KIRSCHNER AKT, REISCHER GH, JAKWERTH S, SAVIO D, IXENMAIER S, TOTH E, SOMMER R, MACH RL, LINKE R, EILER A, KOLAREVIC S & FARNLEITNER AH (2017) Multiparametric monitoring of microbial faecal pollution reveals the dominance of human contamination along the whole Danube River. Water Res 124:543-555

LAYTON A, MCKAY L, WILLIAMS D, GARRETT V, GENTRY R & SAYLER G (2006) Development of Bacteroides 16S rRNA gene TaqMan-based real-time PCR assays for estimation of total, human, and bovine fecal pollution in water. Appl. Environ. Microbiol. 72, 4214-4224

MIESZKIN S, FURET JP, CORTHIER G & GOURMELON M (2009) Estimation of Pig Fecal Contamination in a River Catchment by Real-Time PCR Using Two Pig-Specific Bacteroidales 16S rRNA Genetic Markers. Appl. Environ. Microbiol. 75, 3045-3054

REISCHER GH, KASPER DC, STEINBORN R, FARNLEITNER AH & MACH RL (2007) A quantitative real-time PCR assay for the highly sensitive and specific detection of human faecal influence in spring water from a large alpine catchment area. Lett Appl Microbiol. 44, 351-356

REISCHER GH, KAVKA GG, KASPER DC, WINTER C, MACH RL, FARNLEITNER AH (2008) Applicability of DNA based quantitative microbial source tracking (QMST) evaluated on a large scale in the Danube River and its important tributaries. Fundam Appl Limnol/Arch Hydrobiol Suppl. 166, 117-125

REISCHER G, KIRSCHNER AKT, SCHNITZER G, SAVIO D, MACH RL, BAHLMANN A, SCHULZE T, BRACK W & FARNLEITNER AH (2015) Microbiology – Microbial Faecal Source Tracking. In: Joint Danube Survey 3, Final scientific report. Liska I, Wagner F, Slobodnik J (eds.) ICPDR Vienna, Austria

USEPA (2019) Method 1696: Characterization of Human Fecal Pollution in Water by HF183/BacR287 TaqMan® Quantitative Polymerase Chain Reaction (qPCR) Assay.

WUERTZ S, WANG D, REISCHER GH & FARNLEITNER AH (2011) Library-Independent Source Tracking Methods. In: Hagedorn C, Blanch AR, Harwood VJ (Eds), Microbial Source Tracking: Methods, Applications, and Case Studies, Springer, New York, USA, pp. 61-113

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Occurrence of non-wild type antibiotic resistant Escherichia coli in the River Danube

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Abstract

The occurrence of human-induced antibiotic resistant bacteria (ARB) is not only limited to clinical surroundings, they can also be found in the human population, animals and the water environment. Particularly large river systems are of great concern regarding the spreading of ARB. Thus, the aim of this study was to analyze the Escherichia coli population of Europe´s second largest river, the Danube, for presence of human-induced resistances. Furthermore, the obtained data were compared with the occurrence of ARB that were isolated in 2013 from the River Danube during JDS3.

The results show a significant increase in multi-resistance (acquired resistances to antibiotics from three or more tested antibiotic classes) and extended spectrum beta-lactamase (ESBL) phenotype. This indicated that the accumulation of resistance mechanisms in the River Danube E. coli population has continued over the last six years. From 797 E. coli isolates, 110 (13.8 %) were multi-resistant, 198 (24.8 %) showed resistances to one or two classes of antibiotics and 489 (61.4 %) revealed no acquired resistance to the antibiotics tested. 18 isolates (2.26 %) expressed the ESBL phenotype. The most common resistances were those to ampicillin (198 isolates, 24.8 %) and tetracycline (192 isolates, 24.1 %), respectively. No resistances were detected to imipenem, meropenem, tigecycline, amikacin and colistin.

20.1 Introduction

In the last decades, the number of human-induced antibiotic resistant bacteria (ARB) has risen not only in the clinical setting but also in the natural environment. One main reason for this is the extensive use of antibiotics in animal breeding and human therapy. Antibiotics and ARBs originate from hospitals, industry and farming and their residues are excreted or discharged via the drain. Flushed to the sewage, and passing

sewage treatment plants, they finally end up in surface waters. Surface waters, especially rivers seem to play an important role in the spread of ARB because they serve as habitats and as a transport media for microorganisms (Allocati et al. 2013).

In the course of JDS4 (Joint Danube Survey 4), Escherichia coli were isolated in a quantitative approach from surface water samples of the Danube River, collected at 36 locations, and examined for non-wild type (multi-)resistances to antibiotics.

E. coli is a Gram-negative bacterium within the family of Enterobacteriaceae. It colonizes the intestinal tract of humans and warm blooded animals and can cause pathogenic diarrhoea and urogenital infections (Allocati et al. 2013). The rising number of new resistances, especially due to their capability to develop or receive genes for extended spectrum β-lactamases (ESBL), makes them insensitive to a larger group of antibacterial agents (Kittinger et al. 2016). ESBLs are enzymes produced by a great variety of bacteria and hydrolyze beta-lactam antibiotics such as penicillins, cephalosporins and monobactams (Okai et al. 2019).

20.2 Methods

Water samples were taken from 18 sampling points along the Danube from the left, middle and right side of the river at a depth of 30 cm. The samples were spread in portions of 500 µL on Chromocult® Coliform agar (Merck, Austria) and CHROMagar Orientation (Becton Dickinson Austria GmbH, Austria) immediately and transferred to Graz within 48 hours at 4°C where the resulting isolates were stored at -70°C in 50 % glycerol.

The isolates were thawed and incubated on LB-agar overnight for subsequent confirmation via mass spectrometry MALDI-TOF VITEK MS™ Assurance (Biomerieux, Austria). For all confirmed E. coli isolates, the antibiotic susceptibility testing (AST) was performed according to the guidelines of the European Committee on Antimicrobial Susceptibility Testing (EUCAST 2020). For tetracycline, chloramphenicol and colistin, there are no criteria available for this test. Therefore, tetracycline and chloramphenicol testing was carried out according to the Clinical Laboratory Standards Institute (CLSI 2020). Protocols of Boyen et al. were used for the determination of resistance to colistin (Boyen et al. 2010). The isolates were tested for the antibiotics ampicillin (10µg), amoxicillin/clavulanate (20 µg/10 µg), piperacillin/tazobactam (100 µg/ 10 µg), cefalexin (30 µg), cefuroxime (30 µg), cefoxitin (30 µg), cefotaxime (5 µg), ceftazidime (10 µg), cefepime (30 µg), meropenem (10 µg), imipenem (10 µg), ciprofloxacin (5 µg), moxifloxacin (5 µg), colistin (10 µg), amikacin (30 µg), gentamicin (10 µg), chloramphenicol (30 µg), trimethoprim/sulfamethoxazole (1.25 µg/23.75 µg), tetracycline (30 µg) and tigecycline (15 µg). Figure 1, panel A to C, displays petri dishes used to perform AST: the isolate expresses an ESBL phenotype.

CLSI standards were also used to perform confirmation tests for E. coli displaying an ESBL-like resistance pattern after the preceded AST (CLSI 2020). This double disc test comprises ceftazidime (30µg), cefepime (30µg), ceftazidime-clavulanic acid (30/10µg) and cefepime-clavulanic acid (30/10µg). As an example an ESBL-positive double disc test of a single isolate is shown in Figure 1, panel D.

Both – AST and conformation of ESBL – were performed using BD BBL™ Sensi-Disc™ antimicrobial susceptibility test discs (Becton Dickinson Austria GmbH, Austria).

Statistical significance was calculated using Fisher´s exact test. P-values below 0.05 were assessed as significant.

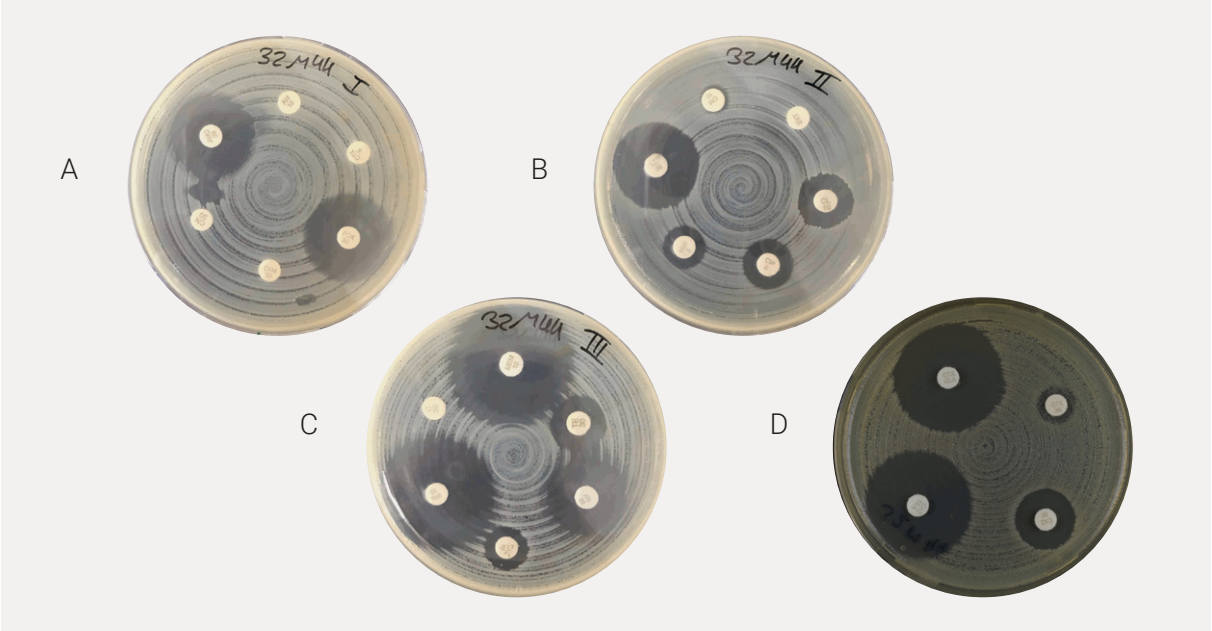

Figure 1: Disc diffusion tests performed for antibiotic susceptibility testing. An E. coli isolate was tested for antibiotic susceptibility using the disc diffusion method according to guidelines of EUCAST, CLSI and Boyen et al. displaying an ESBL resistance pattern (panel A - C). A subsequent ESBL confirmation test according to CLSI standards shows an affirmative result (panel D). Clear circular spaces in the discs´ periphery are inhibition zones without bacterial growth due to the antibiotics diffusing into the surrounding medium. The diameters of these inhibition zones are compared with the specific corresponding breakpoints given in the guidelines and indicate the isolate´s susceptibility or resistance to each antibiotic.

20.3 Results and Discussion

797 E. coli isolates have been tested so far. 110 (13.8 %) E. coli were multi-resistant (resistances to three or more of the tested antibiotic classes), 198 (24.8 %) showed resistances to one or two classes of antibiotics and 489 (61.4 %) revealed a wild type resistance pattern (Figure 1). These findings indicate a significant increase by 42 % (4.1 percentage points, p=0.021) in the number of multi-resistant E. coli when compared with data obtained during JDS3 when multi-resistance occurred in 9.7 % (61 of 629) of the isolates (Kittinger et al. 2016).

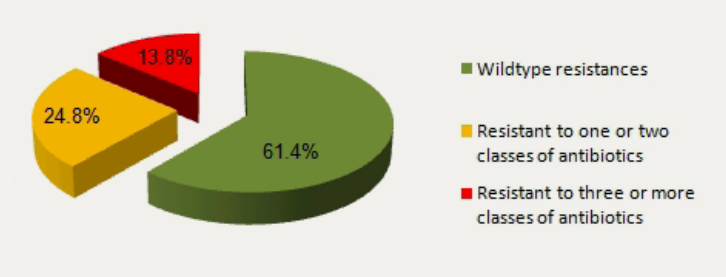

Figure 2: Classification and frequency of antibiotic resistances. The total number of isolates tested was 797. Multi-resistance (resistance to 3 or more classes of antibiotics) occurred for 13.8 % (indicated in red). Resistance to 1 or 2 classes of antibiotics was shown by 24.8 % of the isolates (indicated in orange) and 61.4 % displayed wildtype resistances (green).

The most frequently detected resistances were against ampicillin and tetracycline with 198 isolates, 24.8 %, and 192 isolates, 24.0 %, respectively (Figure 2). These findings are similar to the results of JDS3 with 21.8 % of isolates resistant to ampicillin and 24.0 % to tetracycline, respectively (Kittinger et al. 2016). All isolates were susceptible to meropenem, imipenem, amikacin, colistin and tigecycline. This is in concordance with the findings in 2013. Therefore, resistances to last line antibiotics are still not detectable in the River Danube E. coli population.

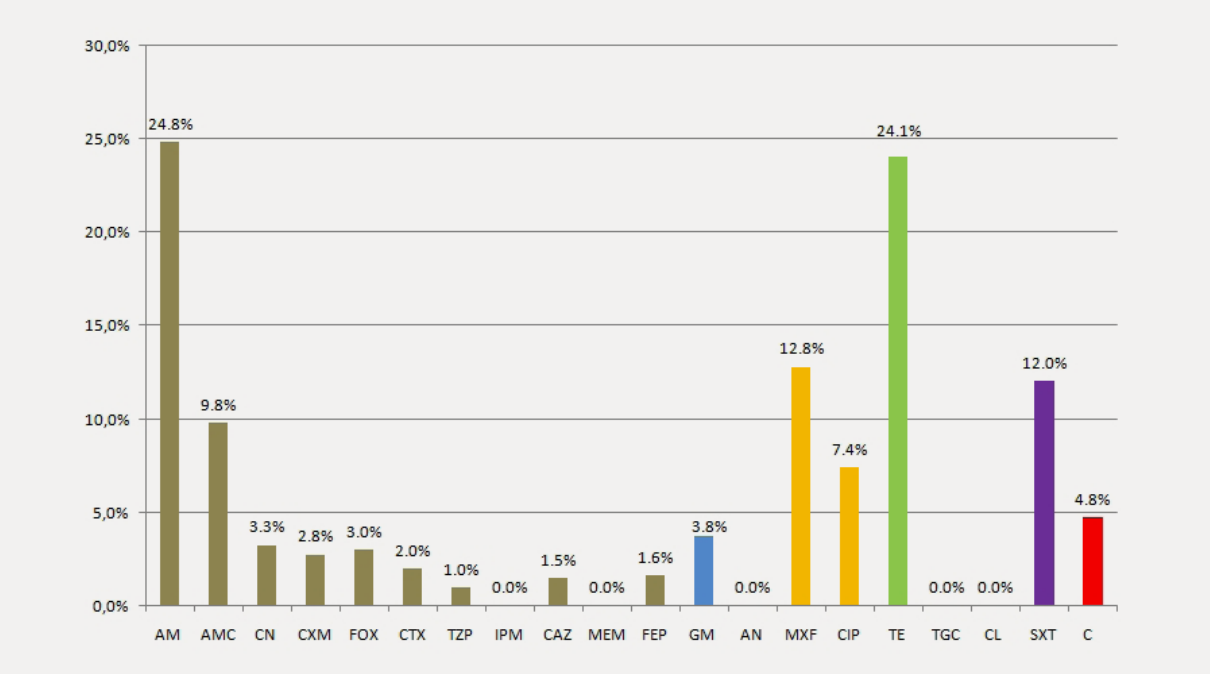

Figure 3: Antibiotic-specific frequencies of resistances. The most frequent resistances within the 797 tested isolates occurred to ampicillin (198 isolates, 24.8 %) and tetracycline (192 isolates, 24.0 %). Antibiotics and abbreviations: ampicillin (AM), amoxicillin/clavulanate (AMC), cefalexin (CN), cefuroxime (CXM), cefoxitin (FOX), cefotaxime (CTX), piperacillin/ tazobactam (TZP), imipenem (IPM), ceftazidime (CAZ), meropenem (MEM), cefepime (FEP), gentamicin (GM), amikacin (AN), moxifloxacin (MXF), ciprofloxacin (CIP), tetracycline (TE), tigecycline (TGC), colistin (CL), trimethoprim/ sulfamethoxazole (SXT), chloramphenicol (C). Classes of antibiotics are indicated by colours: olive green – beta-lactams, blue – aminoglycosides, orange – chinolones, light green – tetracyclines, violet – antagonists of folic acids, red – chloramphenicol.

Resistance patterns indicating ESBL after AST were detected in 18 (2.26 %) isolates and confirmed by performing a subsequent confirmation test according to CLSI standards (Table 1). During JDS3 4 ESBL producing E. coli (0.6 %) were isolated (Kittinger et al. 2016). Therefore, the presence of ESBL phenotype in 2019 was nearly five times higher (increase by 1.66 percentage points, p=0.016) than in 2013. The reasons for this increase could be a stronger entry of resistant bacteria from the human population or from domestic and farm animals. It remains unclear whether the establishment of resistance will continue in the upcoming years and how much it is influenced by a constant input of resistant bacteria from the above-mentioned sources. Even more critical is the question to what extent the observed resistances in large surface waters contribute to the establishment and stabilisation of resistances in the human and animal population.

LOCATION	AM	AMC	CN	CXM	FOX	CTX	GM	TZP	MXF	CIP	CL	SXT	IPM	CAZ	AN	TE	MEM	C	FEP	TGC	ESBL TEST CLSI
Oberloiben (rkm 2007)	R	R	R	R	S	R	R	S	R	R	S	S	S	R	S	S	S	S	R	S	POSITIVE
Vienna, downstream (rkm 1930)	R	S	R	R	S	R	S	S	R	S	S	R	S	R	S	R	S	S	S	S	POSITIVE
Pancevo, downstream (rkm 1151)	R	S	R	R	S	R	R	S	R	R	S	S	S	R	S	S	S	S	S	S	POSITIVE
Pancevo, downstream (rkm 1151)	R	R	R	R	S	R	R	S	R	R	S	S	S	R	S	S	S	S	R	S	POSITIVE
Pancevo, downstream (rkm 1151)	R	S	R	R	S	R	S	S	R	R	S	S	S	R	S	S	S	S	R	S	POSITIVE
Pancevo, downstream (rkm 1151)	R	S	R	R	S	R	S	S	S	S	S	S	S	R	S	S	S	S	R	S	POSITIVE
Pancevo, downstream (rkm 1151)	R	S	R	R	R	R	S	S	R	R	S	S	S	R	S	R	S	R	R	S	POSITIVE
Vrbica/ Simijan (rkm 926)	R	R	R	R	S	R	R	S	S	S	S	R	S	S	S	R	S	S	R	S	POSITIVE
Pristol/ Novo Salo (rkm 837)	R	R	R	R	S	R	R	S	R	R	S	S	S	R	S	R	S	R	R	S	POSITIVE
Russenski Lom (tributary, rkm 498)	R	S	R	R	S	R	R	S	R	R	S	R	S	R	S	S	S	R	R	S	POSITIVE
Ruse/Giurgiu (Marten), downstream (rkm 488)	R	S	R	R	S	R	R	S	R	R	S	S	S	R	S	S	S	S	R	S	POSITIVE
Arges (tributary, rkm 432)	R	S	S	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	POSITIVE
Arges, downstream (rkm 429)	R	S	R	R	S	R	S	S	S	S	S	S	S	S	S	S	S	S	R	S	POSITIVE
Giurgeni (rkm 235)	R	S	R	R	S	R	S	S	S	S	S	S	S	S	S	S	S	S	R	S	POSITIVE
Reni (rkm 130)	R	S	R	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	R	S	POSITIVE
Reni (rkm 130)	R	R	R	R	S	R	R	S	R	R	S	S	S	S	S	S	S	S	S	S	POSITIVE
Reni (rkm 130)	R	R	R	R	S	R	R	S	R	R	S	R	S	R	S	R	S	S	R	S	POSITIVE
Reni (rkm 130)	R	R	R	R	R	R	R	S	R	R	S	R	S	R	S	S	S	S	R	S	POSITIVE

Table 1: Antibiograms of ESBL-producing E. coli. The displayed ESBL-producing isolates represent a percentage of 2.26 % (18 of all 797 E. coli tested). Each line shows the test results of a single isolate. The first column shows the geographic location of isolation. From the second up to the second to last column each table element displays the inhibition zone diameters for the current antibiotic. A red colour indicates resistance and white a sensitive test result. In the last column, the results of the ESBL confirmation tests are displayed. Antibiotics and abbreviations: ampicillin (AM), amoxicillin/ clavulanate (AMC), cefalexin (CN), cefuroxime (CXM), cefoxitin (FOX), cefotaxime (CTX), piperacillin/ tazobactam (TZP), imipenem (IPM), ceftazidime (CAZ), meropenem (MEM), cefepime (FEP), gentamicin (GM), amikacin (AN), moxifloxacin (MXF), ciprofloxacin (CIP), tetracycline (TE), tigecycline (TGC), colistin (CL), trimethoprim/ sulfamethoxazole (SXT), chloramphenicol (C). rkm: river kilometre.

20.4 Conclusions

- From 797 Escherichia coli isolates 110 (13.8 %) were multi-resistant, 198 (24.8 %) showed resistances to one or two classes of antibiotics and 489 (61.4 %) revealed no acquired resistance to tested antibiotics.
- 18 (2.26 %) isolates were affirmatively tested for the ESBL phenotype.
- The six-year-comparison with data from JDS3 shows a significant increase in multi-resistance and ESBL phenotype for the E. coli population of the River Danube.

20.5 Acknowledgements

The study was financed by the Austrian Science Fund (FWF), project number P32464, and funded by the ICPDR. We highly appreciate the support of the partnering teams and institutes during sampling: Dr. Michael Schabuss and Dr. Horst Zornig (ProFisch), Dr. Sebastien Ferreira-Cerca and Prof. Herbert Tschochner (University of Regensburg, Germany), Dr. Zsuzsa Keki (ELTE University Budapest, Hungary), Prof. Momir Paunovic (University of Belgrade, Serbia), Dr. Florentina Soare, Mary Craciun, Cristina Dumitru (Romanian Water Laboratories in Turnu-Severin and Calarsi, Romania). Furthermore, we thank Dr. Jaroslav Slobodnik and Peter Mazur for their support in sample transport logistics.

20.6 References

ALLOCATI, N. ET AL., 2013. Escherichia coli in Europe: an overview. International journal of environmental research and public health, 10(12), pp.6235–6254.

BOYEN, F. ET AL., 2010. Disk prediffusion is a reliable method for testing colistin susceptibility in porcine E. coli strains. Veterinary microbiology, 144(3-4), pp.359–362.

CLSI, 2020. CLSI M100-ED30:2020 Performance Standards for Antimicrobial Susceptibility Testing, 30th Edition, Clinical and Laboratory Standards Institute, Available at: https://clsi.org/.

EUCAST, 2020. European Committee on Antimicrobial Susceptibility Testing, Available at: https://www.eucast.org/.

KITTINGER, C. ET AL., 2016. Enterobacteriaceae isolated from the river Danube: antibiotic resistances, with a focus on the presence of ESBL and carbapenemases. PloS one, 11(11), p.e0165820.

OKAI, M. ET AL., 2019. Antibiotic-resistance of Fecal Coliforms at the Bottom of the Tama River, Tokyo. Biocontrol science, 24(3), pp.173–178.

21 Microbiome: Microbial community and environmental DNA analysis

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Abstract

Microbial communities in natural ecosystems are rapidly responsive to environmental changes by activating or inhibiting specific metabolic pathways which may cause variations in the overall community composition and functionality, then having an impact on the entire ecosystem. Moreover, among the main threats to the water environment, anthropogenic pressure and climate changes have negative impacts on water quality and biodiversity. Here, we investigated changes in the microbial community composition in eight selected sampling points along the Danube River by using a 16S rDNA sequencing approach. In accordance with the data reported during the Joint Danube Survey 2 (JDS2), we observed that Proteobacteria, Actinobacteria and Bacteroidota were the most dominant phyla detected in the river. We also identified antibiotic resistant genes (ARG) against antibiotics belonging to β-lactams (Bla_{TEM}), sulfonamides (Sul1) and quinolones (qnrS1), which are among the main used in human and veterinary medicine. Due to the increasing use of antibiotics, their concentration in waterbodies is indeed increasing and can contribute to the spread of the antimicrobial resistance (AMR). In fact, our results showed that these ARG were present in at least one sampling point. On the other hand, the environmental DNA (eDNA) analysis (mitochondrial 12S rDNA sequencing) was instead used to detect and identify fish populations along the Danube, at least to an order level. Here, we were able to detect ten fish orders along five selected sampling points. These orders had representative native and invasive species reported during the Joint Danube Survey 3 (JDS3).

21.1 Introduction

High quality freshwater ecosystems are essential and critical natural resources which, in the last decades, have been threatened by anthropogenic activities.

The assessment of water quality and biodiversity in aquatic ecosystems traditionally has relied on microscopy analysis, traps, electrofishing and active sampling. These methodologies are frequently invasive, destructive and dependent on a skilled operator to identify species found in the environment, as well as labour intensive and time consuming (Beng & Corlett, 2020). Advanced DNA sequencing techniques with their modest cost, now offer the opportunity, as an alternative to the traditional survey, to perform monitoring research on the complex and often unknown biodiversity in the ecosystems. The use of metagenomics has indeed become a common technique to explore the effects of anthropogenic pollution in river ecosystems by assessing changes in microbial communities and microbiome (Bai et al., 2014; Saccà et al., 2019).

Microorganisms are important players in the biogeochemical cycling of nutrients, biodegradation of pollutants and maintenance of ecosystem health (Holguin et al., 2001), however, the diversity and community structure could be affected by changes in the environment, and can be therefore used as an indicator of environmental conditions. Among others, antibiotics are considered emerging pollutants in the environment. The consumption of antibiotics in both human and veterinary medicine is increasing in many countries, resulting in their detection in waterbodies. Moreover, wastewater treatment plants (WWTP) are not suitable to completely remove antibiotics during the treatment processes, and consequently, these substances are released directly in the environment. The amount of antibiotics in water has become a serious threat, principally because they represent a driving force behind the increasing occurrence of the antimicrobial resistance (AMR) and the spread of antibiotic resistant genes (ARG).

Ultimately, fish are very sensitive to anthropogenic impact and climate changes, and studies on their population have been used as indicators of human pollution. For fish surveys, the environmental DNA metabarcoding, or environmental DNA (eDNA), has been recently used to analyse the genetic material which is present in environmental samples such as sediment or water. This methodology is based on the principle that all living organisms shed DNA into the environment via, for example, skin or excrement (Ruppert et al., 2019) (see Chapter 12). The eDNA analysis allows the simultaneous detection and identification of organisms across different trophic levels, providing relevant information about the complex biotic interactions related to ecosystem populations and changes. Although the use of eDNA is a relatively new method of screening, it has proven its potential in ecological monitoring without the need of disturbing or even destructing the habitat, contrary to what happened often with conventional methods (Djurhuus et al., 2020; Hajibabaei et al., 2011; Thomsen & Willerslev, 2015).

In this chapter, we performed a sequencing analysis for monitoring the microbial community (16S rDNA sequencing) and fish populations (mitochondrial 12S rDNA sequencing) in eight and five different sampling points respectively, in order to study the water quality status of the Danube River. Finally, to gain an insight into the antibiotic pollution in the river, the expression of ARG belonging to the antibiotic classes β-lactams, sulfonamides and quinolones was analysed through polymerase chain reaction (PCR).

21.2 Methods

Sampling points and filtration

The water sampling campaign was carried out at eight different stations along the Danube River from 30th of June to 20th of July 2019. The sites were classified as reported in Table 1. For each sampling station, two 5L samples were collected in distinct bottles, acid-washed and rinsed with river water, representing true biological replicates. All sampling sites were used for investigating the microbial community composition by 16S rDNA sequencing and for antibiotic resistance genes (ARG) identification through polymerase chain reaction (PCR), whereas five sampling sites were selected for environmental DNA (eDNA) studies by the mitochondrial 12S rDNA sequencing (Table 1). The 16S is part of the 30S subunit of the ribosomes in bacteria and even though this gene is conservative, sequence differences (polymorphisms) in the hypervariable regions allow the taxonomic classification and the phylogenetic analysis of the microbial populations. For vertebrates, mitochondrial DNA (mtDNA) is predominantly maternally inherited, and its rapid mutation rate, together with its high number of copies in each cell, make it suitable for vertebrate identification (Cawthorn et al., 2012).

For DNA extraction, 1L samples from each replicate were filtered using MF-Millipore membrane filters, 0.22µm pore size (Millipore), except for the sampling site Joint Danube Survey4-4 (JDS4-4) where the volume filtered was 400mL. For eDNA extraction, 1L samples from each replicate were filtered using nitrocellulose NC45 membranes, 0.45µm pore size (Whatman).

Water samples were filtered upon arrival to the laboratory, and all filters were stored at -20°C until further analyses.

Table 1: List of Danube River sampling sites analysed for 16S rDNA, mitochondrial 12S rDNA and antibiotic resistance genes (ARG).

Sampling site	Collection area in the river	Place	Country	River Km	Coordinates		Analysis
JDS4-4	Right	Niederaltaich-Mühlau	DE	2282	48.763617	13.017867	16S/12S/ ARG
JDS4-9m	Middle	Downstream Vienna	AT	1930	48.162879	16.503526	16S/12S/ ARG
JDS4-14	Middle	Bratislava	SK	1869	48.075482	17.156551	16S/12S/ ARG
JDS4-40	Left	Banatska Palanka	RS/RO	1071	44.825379	21.343977	16S/ARG
JDS4-41m2	Middle	Vrbica/Simijan	RS/RO	926	44.601839	22.709641	16S/12S/ ARG
JDS4-41	Middle	Upstream Timok (Rudujevac / Gruia)	RS/RO	849	44.232340	22.673430	16S/ARG
JDS4-47	Middle	Downstream Ruse/Giurgiu (Marten)	BG/RO	488	43.928989	26.073169	16S/ARG
JDS4-50	Middle	Reni	RO/UA	130	45.45484	28.25887	16S/12S/ ARG

Laboratory environment and controls

DNA extraction, library preparation and sequencing were conducted in three different dedicated and physically separated rooms to prevent sample contamination. Controls during DNA extraction and library preparation were used to monitor possible DNA contamination in reagents or sample manipulation. Benchtops were cleaned with RNase Away (Invitrogen) and then wiped with ethanol 70%. PCR were prepared in a designed DNA-free hood and pipettes were wiped also with RNase Away and UV-irradiated for at least 20 minutes.

DNA extraction

For DNA extraction, filters were incubated overnight in 50 mM KH_2PO_4 buffer and then sonicated for 15 minutes at 60°C as described in Kisand et al. (Kisand et al., 2012). Enzymatic digestion using lysozyme (100mg/ml, Sigma) and β-mercaptoethanol (14 mM, Sigma) was also performed prior to column-based DNA extraction with DNease Blood and Tissue Extraction Kit (Qiagen), according to supplier instructions. DNA was extracted from two biological replicates/sampling site (1L/sample) and mixed in equal volume ratio before performing further analyses.

DNA extraction for environmental DNA (eDNA) was performed using the PowerWater Kit (Qiagen) in accordance with supplier instructions, but was also slightly modified. Briefly, the incubation time with the beads was increased to 30 minutes and the incubation with IRS solution to 10 minutes. The total DNA was recovered in 20µl Tris buffer and replicates were pooled to reduce variations between sampling and extraction efficiencies.

DNA and eDNA concentration was checked at Nanodrop (Thermofisher Scientific) and quantified by Qubit dsDNA HS assay kit (Invitrogen).

Library preparation and DNA sequencing

Amplification of 16S rDNA (variable regions V3-V4) and 12S rDNA (mitochondrial conserved region) was performed using primer pairs shown in Table 2. PCR was carried out using 25ng of high-quality genomic DNA (for 16S rDNA) and 10µL, 1:10 or 1:100 dilutions of environmental DNA (eDNA, for 12S rDNA). For 16S rDNA, PCR conditions were: 1 cycle of 3 minutes at 95°C followed by 25 cycles of 40 seconds at 95°C, 2 minutes at 55°C, 1 minute at 72°C and 7 minutes incubation at 72°C. For eDNA, the PCR samples were denatured at 95°C for 10 minutes, followed by 50 cycles of 30 seconds at 95°C, 30 seconds at 55°C and 1 minute at 72°C, followed by a final elongation at 72°C for 7 minutes.

PCR amplicons were used to prepare amplicon libraries with the Ion Plus Fragment Library Kit (Thermo Fisher Scientific) according to manufacturer's instructions. Libraries were amplified and pooled at an equimolar ratio (100pM) based on concentrations assessed with the 2100 Bioanalyzer instrument using the Agilent High Sensitivity Kit (Agilent). Sequencing was performed on the IonS5 Instrument (Thermo Fisher Scientific) at the Joint Research Centre (JRC) in Ispra (VA, Italy). All 16S rDNA and 12S rDNA samples were sequenced as 400bp and 200bp reads, respectively, using chips 520 (Thermo Fisher Scientific).

Table 2: List of primers used for amplifying 16S rDNA and 12S rDNA.

Gene	Forward	Reverse	Amplicon size (bp)	Ref
16S	5'-CCTACGGGNGGCWGCAG-3'	5'-GACTACHVGGGTATCTAATCC-3'	464	(Klindworth et al., 2013)
12S	5'-ACACCGCCCGTCACTCT-3'	5'-CTTCCGGTACACTTACCATG-3'	126	(Valentini et al., 2016)

Antibiotic resistance genes detection by Polymerase Chain reaction (PCR)

The presence of genes encoding resistance to the antibiotic classes sulfonamides (*Sul1*), β-lactams (Bla_{TEM}) and quinolones (*qnrS1*) were monitored by PCR using primer pairs listed in Table 3. Amplifications were performed in a final volume of 25µL, using 5µL of a 1:10 dilution of the pooled DNA from two biological replicates. The amplicons were analysed with the 2100 Bioanalyzer instrument (Agilent) to ensure the correct size of the product.

Table 3: List of primers used for amplifying antibiotic resistance genes (ARG).

Antibiotic class	Gene	Forward	Reverse	Amplicon size (bp)	Ref
β-lactams	Bla_{TEM}	5'-GCKGCCAACTTACTTCTGACAACG-3'	5'-CTTTATCCGCCTCCATCCAGTCTA-3'	247	(Xi et al., 2009)
Sulfonamides	Sul1	5'-CGCACCGGAAACATCGCTGCAC-3'	5'-TGAAGTTCCGCCGCAAGGCTCG-3'	163	(Pei et al., 2006)
Quinolones	qnrS1	5'-GACGTGCTAACTTGCGTG-3'	5'-TGGCATTGTTGGAAACTT-3'	118	(Marti & Balcázar, 2013)

16S rDNA and 12S rDNA sequencing data analysis

All 16S rDNA V3-V4 amplicon reads were initially converted into reads with the same (forward) sense by a custom-written Perl script. Reads shorter than 300 nucleotides were removed. In order to obtain an equal number of reads per sample, 295,000 reads were randomly selected from each sample. The data was then combined into one datafile and Operational Taxonomic Units (OTU) (Dadheech et al., 2013) clustered with USEARCH (https://www.drive5.com/usearch) applying a 97% sequence identity cutoff. Taxonomic classification of OTU was performed using the SINTAX algorithm of USEARCH against the 16S rDNA reference database from GTDB (https://gtdb.ecogenomic.org/). Heatmap clustering of OTU abundance across samples was performed in R using custom-written R scripts.

For 12S rDNA analysis, *in silico* PCR simulations were performed by using ThermonucleotideBLAST (Gans & Wolinsky, 2008), with the primers listed in the Table 2 tested on all GenBank vertebrate assembled genomes available at NCBI (2,280 at time of writing). ThermonucleotideBLAST was run on each assembled genome with default parameters, except for the following ones: -e 30 -E 40.

Putative not redundant amplicons were then extracted by in-house developed scripts and used to build a dataset of sequences (called 12S-Kraken-DB) in the format suitable the Kraken 2 software (Wood et al., 2019). Each NGS read dataset was screened by using Kraken 2 and the built 12S-Kraken-DB, in order to assign taxonomic labels to reads.

21.3 Results and Discussion

Microbial community structure (16S rDNA)

A metagenomic approach was used to analyse the microbial community in eight Danube samples which were selected based on differences in anthropogenic pressures. The sites were classified as shown in Table 4.

Table 4: List of Danube River sampling sites and their anthropogenic pressure.

Sampling site	Anthropogenic pressure
JDS4-4	Little polluted reference site
JDS4-9m	Directly after inflow of WWTP Vienna
JDS4-14	Important capital with expected wastewater
JDS4-40	Moderate pollution reference site
JDS4-41m2	High pollution levels during JDS3-left and right
JDS4-41	Low pollution reference site
JDS4-47	High pollution levels-right river side
JDS4-50	After Siret/Prut tributaries

To obtain an overview of the microbial community complexity, 16S rDNA reads were clustered in Operational Taxonomic Units (OTU) (Dadheech et al., 2013) and six main clusters were detected as shown in Figure 1. We could observe a 16S rDNA sequence similarity at the OTU level for sampling sites JDS4-41m2, JDS4-41, JDS-4-50, JD4-47 and for JDS4-14 and JDS-9m. Instead, the low polluted (JDS4-4) and moderate polluted (JDS4-40) sites exhibited distinct OTU profiles when compared to the other sampling points (Figure 1).

Metagenomic data analysed at the phylum level revealed that the microbial community was dominated by Proteobacteria, Actinobacteria and Bacteroidota (Figure 2). Dominance of these phyla in freshwater and in the Danube River JDS2 campaign has also been observed in previous studies (Liu et al., 2012; Newton et al., 2011; Savio et al., 2015). In particular, in the current study, Proteobacteria was the most abundant phylum in most of the samples analysed, with the exception of two sites, JDS4-41m2 and JDS4-41, which were instead dominated by Actinobacteria (Figure 2). The highest relative abundance of Proteobacteria was detected in sampling sites JDS4-47 and JDS4-14, while lowest levels were found in JDS4-41 and JDS4-41m2 (Figure 2). Bacteroidota was the third most abundant microbial population in the majority of samples, followed by Cyanobacteria, Planctomycetota, Verrucomicrobiota, and Patescibacteria (Figure 2). Reported changes in bacterial community composition were apparently not directly influenced by physico-chemical parameters (pH, temperature, conductivity, O_2 (%) and O_2 (mg/L)) or nutrient content (total phosphorus, total nitrogen and total organic carbon) as no marked variations of their values were observed across sites. According to Savio et al. (Savio et al., 2015), modulations in the bacterial community along the Danube River could be due to an environmental-condition-based sorting ("species-sorting"), intended as species selection caused by differences in environmental local conditions. Further analysis will be needed to better understand the reported differences in our results.

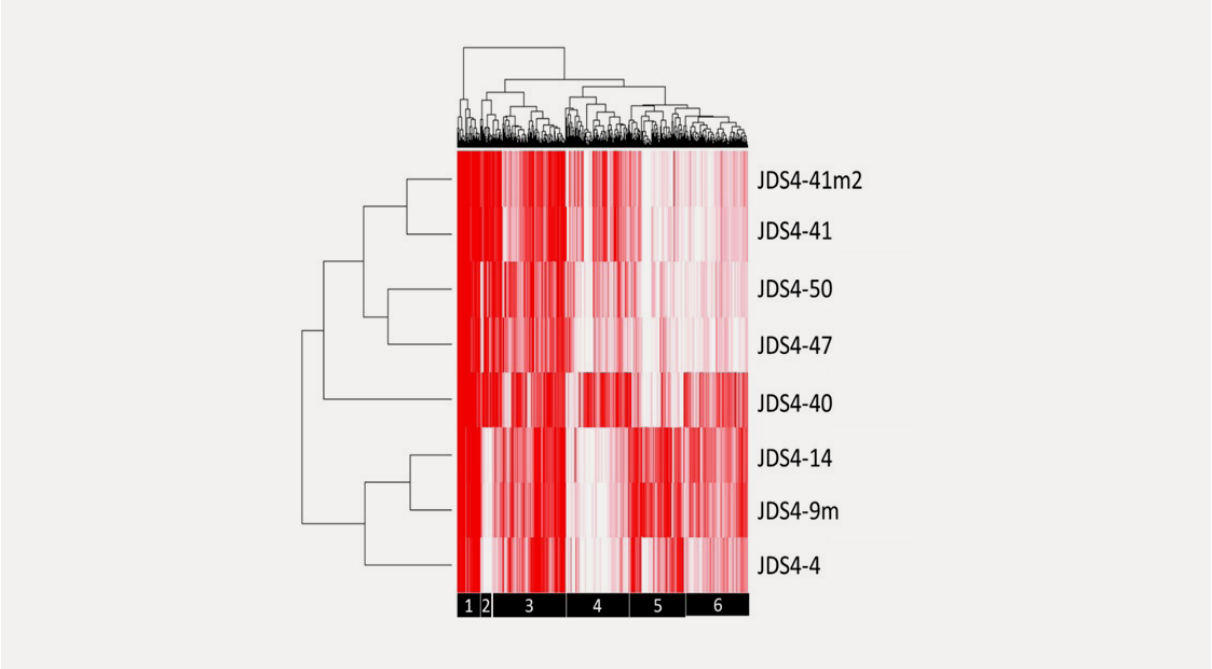

Figure 1: Metagenomic data analysed at the Operational Taxonomic Unit (OTU) (Dadheech et al., 2013) level. The 16S rDNA metagenomic data was clustered using the OTU abundance levels. For clustering, abundance values were log2 converted setting zero counts to 1. Only OTUs present more than 100 times in total across all samples were included. Six different OTU clusters were identified as indicated by the numbers shown at the bottom of the figure.

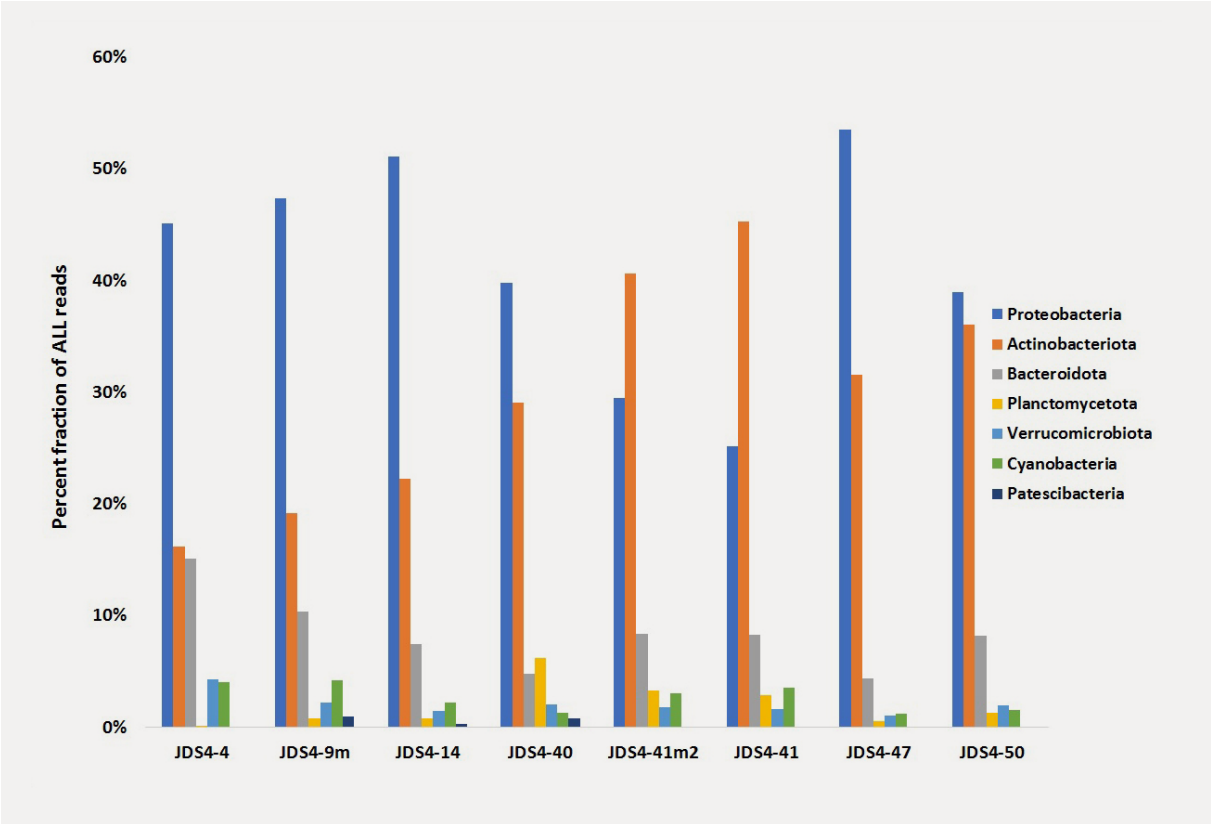

Figure 2: Bacterial community at the phylum level. The microbial community composition at each sampling point (x axis) was determined by 16S rDNA sequencing. 16S rDNA sequencing reads were analysed at the phylum level and results expressed as the percent fraction of all 16S rDNA reads in the sample (y axis). Only dominant phyla (> 1% fraction) are shown.

Fish population using environmental DNA (eDNA)

Environmental DNA (eDNA) analysis was used to investigate the fish population at five sampling points along the Danube River. This analysis was carried out as a complementary study to Chapter 14. Among the fifteen orders of fish detected by Kraken2 in a customized Teleostei 12S database (see Materials and Methods), four belonged to marine fish orders (Beryciformes, Gadiformes, Spariformes, Pleuronectiformes) and one did not belong to the Danube area (Pristiformes/Rhiniformes).

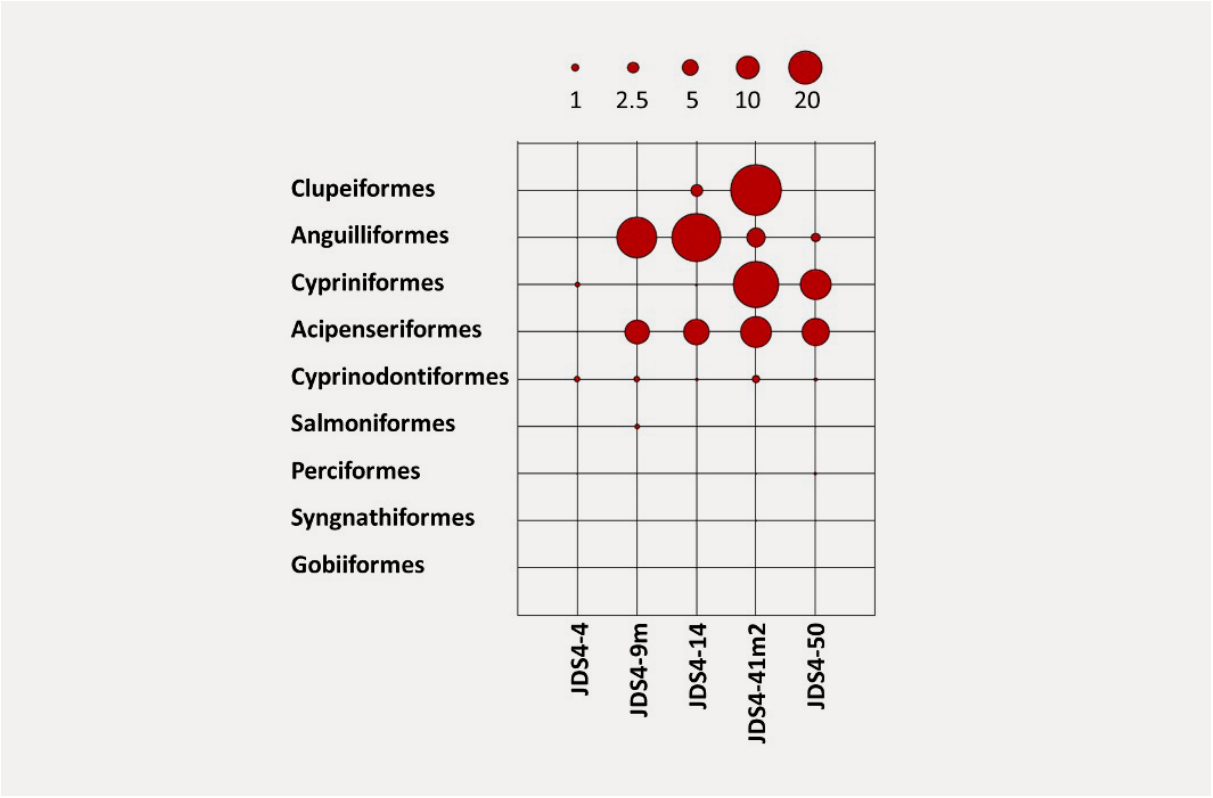

Figure 3: Fish population at the order level. Fish population detected in five sampling points along the Danube River by 12S rDNA sequencing. Samples were analysed at the order level and results expressed as percentage fraction of 12S rDNA reads per each sampling point.

Table 5: List of fish orders detected by 12S rDNA sequencing in five sampling points along the Danube River. Results of the analysis are expressed as percentage fraction of reads per each sampling point.

Fish Orders	JDS4-4	JDS4-9m	JDS4-14	JDS4-41m2	JDS4-50
Clupeiformes	0	0	2.578	43.134	0
Anguilliformes	0.002	27.333	39.411	6.232	1.483
Cypriniformes	0.449	0.056	0.110	35.665	15.526
Acipenseriformes	0.006	10.270	10.874	15.984	13.167
Cyprinodontiformes	0.554	0.525	0.222	0.970	0.239
Salmoniformes	0	0.512	0	0	0.033
Perciformes	0.013	0.031	0.034	0.064	0.205
Syngnathiformes	0.064	0.033	0.033	0.058	0.033
Gobiiformes	0.002	0.021	0.014	0	0
Gadiformes	0	0.012	0	0	0
Beryciformes	0.006	0	0	0.004	0.009
Spariformes	0	0.008	0	0.005	0
Pristiformes/Rhiniformes	0	0.003	0.003	0	0
Pleuronectiformes	0.001	0	0	0	0
Siluriformes	0.001	0	0	0	0

The 12S rDNA analysis showed that the most detected orders along the Danube belonged to the Clupeiformes, Anguilliformes, Cypriniformes and Acipenseriformes, followed by Cyprinodontiformes and Salmoniformes (Figure 3 and Table 5).

Clupeiformes is an order with mostly marine fish species, however, some species native to the Black Sea, migrates up in the Danube to spawn, and in the case of the Pontic shad (*Alosa immaculata*) this migration happens between April and August.

The genus *Alosa* was identified in Chapter 14 and, in our study, Clupeiformes were detected in two different sampling points: Slovakia (JDS4-14) and Romania (JDS14-41m2) (Figure 3 and Table 5).

The order Anguilliformes was detected in all sampling points analysed in the JRC laboratories (Figure 3 and Table 5). Although we did not go through the analysis at species level, we supposed that the most abundant species is represented by *Anguilla anguilla*. This species was identified during the last campaign in Danube River (see Chapter 14) and it was also observed during Joint Danube Survey 3 (JDS3). In this latter, it was detected in the upstream section of the Iron Gate Dam (located in Romania), whereas Wiesner et al. (Wiesned, 2007) found *Anguilla anguilla* along the entire course of the river.

The order Cypriniformes, was the third having higher number of reads along the Danube stream, particularly in the lower section of the river (JDS4-41m2 and JDS4-50 sampling points) (Figure 3 and Table 5). Indeed, different species of Cypriniformes have been identified along the Danube, such as *Abramis brama, Alburnus alburnus* and *Cyprinus carpio*, as reported in Chapter 14. During JDS3, it was observed that the catches were dominated by two different species, *Neogobius melanostomus* (order Gobiiformes) and *Alburnus alburnus*, a small cyprinid native from European freshwaters. The presence of non-native cyprinids such as *Hypophthalmichthys molitrix* (downstream the Iron Gate Damn in Romania) and *Pseudorasbora parva* have also been reported during the JDS3 and JDS4 campaigns (see Chapter 14).

As described already for the other orders, Acipenseriformes, which includes 6 species native in the Danube River, was also detected in all sampling points analysed (Figure 3 and Table 5). The higher number of

reads was found in the middle-lower course of the river (JDS4-9m, JDS4-14, JDS4-41m2 and JDS4-50). Indeed, species such as *Acipenser ruthenus* were reported in Chapter 14. During JDS3, the species *Acipenser ruthenus* was also caught by electrofishing at three different sampling points: Belegis (close to our sampling point JDS4-41m2), Reni (same sampling point corresponding to JDS4-50) and Valcov in the Chilia Arm (downstream Reni). However, the eDNA approach also spotted Acipenseriformes at sampling points located in the upper part of Danube River, where it is known that low number of this species can still be found (Friedrich, 2018).

Cyprinodontiformes, Perciformes and Syngnathiformes were also detected at all five sampling points (Figure 3 and Table 5). For Syngnathiformes, Wiesner et al. (Wiesned, 2007) detected the introduced genus Syngnatus in the upper stream of the Iron Gates dam, although our data also showed the presence of fish populations belonging to this order in the lower section of the river. The invasive species *Lepomis gibbosus*, order Perciformes, was identified (see Chapter 14) and during JDS3, it was also observed throughout the entire course of the river. Further analyses will be performed to verify if the same species was also present in all our samples.

In our study, Gobiiformes were confined to the upper part of Danube River (JDS4-4, JDS4-9m and JDS4-14) (Table 5). Species from this order have been identified along the Danube, as showed in Chapter 14, and similar results were found during the JDS3, with the species *Neogobius melanostomus*, being the most detected.

Finally, the Salmoniformes were observed in two sampling points located in Austria (JDS4-9m) and Reni (JDS4-50) (Figure 3 and Table 5). Also in this case, a species belonging to this order, the Oncorhynchus mykiss (an invasive species from Pacific Ocean in Asia and North America) was reported in the Danube during the JDS3 campaign, and species such as *Hucho hucho* and *Thymallus thymallus* were reported in Chapter 14.

Overall, our results are in accordance with the ones shown in Chapter 14. In order to compare different methodologies, further 12S analysis of our samples should be done at the species level. In this way, it will be possible to correlate our results with species identified by the electrofishing survey and other eDNA approaches.

Antibiotic resistance genes detection

In order to detect antibiotic resistance genes (ARG) in water collected at eight different sampling sites of the Danube River, PCR analysis was carried out using the specific primer pairs listed in Table 3. Although this is not a quantitative method, it can be adopted to perform a first screening for presence/absence of ARG. The genes selected confer resistance to β-lactams (*BlaTEM*) (i.e., ampicillin), sulfonamides (Sul1) (i.e. sulfamethoxazole), and quinolones (qnrS1) (i.e. ciprofloxacin) and they were detected in at least one sample. The resistance genes Sul1 and BlaTEM, were observed in three of the eight sampling points, while qnrS1 was reported in one sampling site (see Table 6). *BlaTEM*) was observed in sites expected to have an anthropogenic impact (JDS4-14 and JDS4-47) as well as in an area which was classified as low polluted (JDS4-41). A similar situation was reported for *Sul1*. This gene was detected in an area close to a waste water treatment plant (WWTP) as well as in moderately polluted (JDS4-9m and JDS4-40) and low polluted (JDS4-4) sites. The gene *qnrS1* was only detected in a low polluted area (JDS4-40). A study performed in a WWTP discharging water in the Danube River showed a wide spread occurrence of *Sul1* in the effluent. Other genes, such as *qnrS* and the β-lactams gene *Blashv* were detected sporadically in the samples (Alygizakis et al., 2019). These findings are in accordance with the results obtained during our study, although in our case, *Sul1* was observed in all samples. *Escherichia coli* (*E. coli*) isolates from water samples collected in the Joint Danube Survey 4 (JDS4) campaign were found to be resistant mainly to ampicillins (β-lactams)

and tetracycline (see Chapter 20). These results were also observed during JDS3, where more than 50% of *E. coli* showed resistance to the antibiotics tested (e.g. tetracycline), with a higher proportion to amoxicillin (β-lactam), while the genera Pseudomonas was found to be susceptible to the aminoglycosides only. It was therefore concluded that the water from Danube River represented a reservoir for antibiotic resistant bacteria (ARB), as also supported by our results.

Table 6: Antibiotic resistance genes (ARG) detected by polymerase chain reaction (PCR) in Danube River water samples (+ detected, - not detected).

Sampling site	Bla_{TEM}	*Sul1*	*qnrS1*
JDS4-4	—	+	—
JDS4-9m	—	+	—
JDS4-14	+	—	—
JDS4-40	—	+	—
JDS4-41m2	—	—	—
JDS4-41	+	—	+
JDS4-47	+	—	—
JDS4-50	—	—	—

21.4 Conclusions

During this study, a metagenomic analysis was performed to investigate the taxonomic composition of the microbial community in the Danube River. The eight selected sites showed a similar bacterial distribution, with main phyla belonging to Proteobacteria, Actinobacteria and Bacteroidota. Interestingly, a shift from the Proteobacteria-dominated community to the prevalence of Actinobacteria was observed at two sampling points (JDS4-41m2 and JDS4-41) located south of the stretch of river that crosses the Đjerdap National Park (Serbia). Considering physico-chemical parameters and nutrients content did not seem to be directly involved in the microbial community variations, the "species-sorting" could influence the community structure along the Danube River as reported by Savio et al. (Savio et al., 2015). A shotgun analysis with a concomitant metabolic pathway investigation will be performed to obtain an in depth-analysis of the results.

In order to determine the fish populations residing in the selected sampling points, we performed a 12S rDNA analysis and we were able to detect ten different fish orders for which, some native and alien species have been detected in the Danube River. Using this approach, we observed the fish order Acipenseriformes in all sampling points, while during the Joint Danube Survey 3 (JDS3) it was only reported in the lower section of the river. Acipenseriformes is an ancient order of fish with high relevant economic importance, but overfishing and the alteration of its habitat caused the population to collapse, with many species considered extinct, highly endangered, or vulnerable. We were also able to detect Gobiiformes, an order which includes some invasive species. Although further analyses at the species level are needed, our results showed the added value of the environmental DNA (eDNA) analysis as a promising method for the detection of fish populations in a river basin.

In addition, during this study, we assessed the presence of three genes conferring resistance to β-lactams *(Bla_{TEM})*, sulfonamides *(Sul1)* and quinolones *(qnrS1)* in eight selected sampling points. Our results showed the detection of the genes analysed in at least one sampling point. In recent years, antibiotic resistance has become a serious global health problem. Indeed, the aquatic environment can contribute to the dissemination of the antimicrobial resistance (AMR) as well as act as a reservoir of antibiotic resistance genes (ARG) (Baquero et al., 2008). The European Commission (EC) recognised the importance in addressing the AMR issue since 2011, when the first "Action Plan" against AMR was adopted (EC, 2011). Subsequently, in 2017, the "One Health Action Plan" reinforced the previous document by encompassing the environmental contribution to the spread of AMR (EC, 2017). Moreover, three antibiotics (azithromycin, clarithromycin and erythromycin) were included in the 1st surface water Watch List (WL) under the European Water Framework Directive (WFD) in 2015 (EC, 2015), and two additional antibiotics (ciprofloxacin and amoxicillin) were added in the following WL exercise (2018) (EC, 2018). WL is a list of emerging pollutants for which monitoring data at Union level were not sufficient to establish the risk they may pose to or via the aquatic environment. Recently, sulfamethoxazole and trimethoprim were identified as suitable substances to be included in the last WL (2020), thus confirming the European attention towards the problems posed by antibiotics to the environment (Gomez Cortes et al., 2020).

21.5 Acknowledgements

We would like to thank the teams who kindly took the water samples and shipped them to the JRC.

21.6 References

EC (European Commission). Commission Implementing Decision (EU) 2015/495 of 20 March 2015 establishing a watch list of substances for Union-wide monitoring in the field of water policy pursuant to Directive 2008/105/EC of the European Parliament and of the Council. Official Journal of the European Union, L 78/40-42, 24.3.2015. (2015).

EC (European Commission). Commission Implementing Decision (EU) 2018/840 of 5 June 2018 establishing a watch list of substances for Union-wide monitoring in the field of water policy pursuant to Directive 2008/105/EC of the European Parliament and of the Council and repealing Commission Implementing Decision (EU) 2015/495 (notified under document C(2018) 3362). (2018).

EC (European Commission). Communication from the Commission to the Council and the European Parliament. A European One Health Action Plan against Antimicrobial Resistance (AMR). Brussels, COM (2017) 339 final.

EC (European Commission). Communication from the Commission to the Council and the European Parliament. Action Plan against the rising threats from Antimicrobial Resistance. Brussels, COM (2011) 748 final.

ALYGIZAKIS, N. A., BESSELINK, H., PAULUS, G. K., OSWALD, P., HORNSTRA, L. M., OSWALDOVA, M., MEDEMA, G., THOMAIDIS, N. S., BEHNISCH, P. A. & SLOBODNIK, J. (2019). Characterization of wastewater effluents in the Danube River Basin with chemical screening, in vitro bioassays and antibiotic resistant genes analysis. Environment International, 127, 420-429.

BAI, Y., QI, W., LIANG, J. & QU, J. (2014). Using high-throughput sequencing to assess the impacts of treated and untreated wastewater discharge on prokaryotic communities in an urban river. Appl Microbiol Biotechnol, 98(4), 1841-1851.

BAQUERO, F., MARTÍNEZ, J.-L. & CANTÓN, R. (2008). Antibiotics and antibiotic resistance in water environments. Current Opinion in Biotechnology, 19(3), 260-265.

BENG, K. C. & CORLETT, R. T. (2020). Applications of environmental DNA (eDNA) in ecology and conservation: opportunities, challenges and prospects. Biodiversity and Conservation, 29, 2089 - 2121.

CAWTHORN, D.-M., STEINMAN, H. A. & WITTHUHN, R. C. (2012). Evaluation of the 16S and 12S rRNA genes as universal markers for the identification of commercial fish species in South Africa. Gene, 491(1), 40-48.

DADHEECH, P. K., GLOCKNER, G., CASPER, P., KOTUT, K., MAZZONI, C. J., MBEDI, S. & KRIENITZ, L. (2013). Cyanobacterial diversity in the hot spring, pelagic and benthic habitats of a tropical soda lake. FEMS Microbiol Ecol, 85(2), 389-401.

DJURHUUS, A., CLOSEK, C. J., KELLY, R. P., PITZ, K. J., MICHISAKI, R. P., STARKS, H. A., WALZ, K. R., ANDRUSZKIEWICZ, E. A., OLESIN, E., HUBBARD, K., MONTES, E., OTIS, D., MULLER-KARGER, F. E., CHAVEZ, F. P., BOEHM, A. B. & BREITBART, M. (2020). Environmental DNA reveals seasonal shifts and potential interactions in a marine community. Nature Communications, 11(1), 254.

FRIEDRICH, T. (2018). Danube Sturgeons: Past and Future. In Riverine Ecosystem Management: Science for Governing Towards a Sustainable Future eds. S. Schmutz and J. Sendzimir), pp. 507-518. Cham: Springer International Publishing.

GANS, J. D. & WOLINSKY, M. (2008). Improved assay-dependent searching of nucleic acid sequence databases. Nucleic Acids Research, 36(12), e74.

GOMEZ CORTES, L., MARINOV, D., SANSEVERINO, I., NAVARRO CUENCA, A., NIEGOWSKA, M., PORCEL RODRIGUEZ, E., LETTIERI, T. & (2020). Selection of substances for the 3rd Watch List under the Water Framework Directive.

HAJIBABAEI, M., SHOKRALLA, S., ZHOU, X., SINGER, G. A. C. & BAIRD, D. J. (2011). Environmental Barcoding: A Next-Generation Sequencing Approach for Biomonitoring Applications Using River Benthos. PLOS ONE, 6(4), e17497.

HOLGUIN, G., VAZQUEZ, P. & BASHAN, Y. (2001). The role of sediment microorganisms in the productivity, conservation, and rehabilitation of mangrove ecosystems: an overview. Biology and Fertility of Soils, 33(4), 265-278.

KISAND, V., VALENTE, A., LAHM, A., TANET, G. & LETTIERI, T. (2012). Phylogenetic and Functional Metagenomic Profiling for Assessing Microbial Biodiversity in Environmental Monitoring. PLOS ONE, 7(8), e43630.

KLINDWORTH, A., PRUESSE, E., SCHWEER, T., PEPLIES, J., QUAST, C., HORN, M. & GLÖCKNER, F. O. (2013). Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. Nucleic acids research, 41(1), e1-e1.

LIU, Z., HUANG, S., SUN, G., XU, Z. & XU, M. (2012). Phylogenetic diversity, composition and distribution of bacterioplankton community in the Dongjiang River, China. FEMS Microbiology Ecology, 80(1), 30-44.

MARTI, E. & BALCÁZAR, J. L. (2013). Real-Time PCR assays for quantification of qnr genes in environmental water samples and chicken feces. Applied and Environmental Microbiology, 79(5), 1743-1745.

NEWTON, R. J., JONES, S. E., EILER, A., MCMAHON, K. D. & BERTILSSON, S. (2011). A Guide to the Natural History of Freshwater Lake Bacteria. Microbiology and Molecular Biology Reviews, 75(1), 14-49.

PEI, R., KIM, S. C., CARLSON, K. H. & PRUDEN, A. (2006). Effect of river landscape on the sediment concentrations of antibiotics and corresponding antibiotic resistance genes (ARG). Water Res, 40(12), 2427-2435.

RUPPERT, K. M., KLINE, R. J. & RAHMAN, M. S. (2019). Past, present, and future perspectives of environmental DNA (eDNA) metabarcoding: A systematic review in methods, monitoring, and applications of global eDNA. Global Ecology and Conservation, 17, e00547.

SACCÀ, M. L., FERRERO, V. E. V., LOOS, R., DI LENOLA, M., TAVAZZI, S., GRENNI, P., ADEMOLLO, N., PATROLECCO, L., HUGGETT, J., CARACCIOLO, A. B. & LETTIERI, T. (2019). Chemical mixtures and fluorescence in situ hybridization analysis of natural microbial community in the Tiber river. Science of The Total Environment, 673, 7-19.

SAVIO, D., SINCLAIR, L., IJAZ, U. Z., PARAJKA, J., REISCHER, G. H., STADLER, P., BLASCHKE, A. P., BLÖSCHL, G., MACH, R. L., KIRSCHNER, A. K. T., FARNLEITNER, A. H. & EILER, A. (2015). Bacterial diversity along a 2600 km river continuum. Environmental Microbiology, 17(12), 4994-5007.

THOMSEN, P. F. & WILLERSLEV, E. (2015). Environmental DNA – An emerging tool in conservation for monitoring past and present biodiversity. Biological Conservation, 183, 4-18.

VALENTINI, A., TABERLET, P., MIAUD, C., CIVADE, R., HERDER, J., THOMSEN, P. F., BELLEMAIN, E., BESNARD, A., COISSAC, E., BOYER, F., GABORIAUD, C., JEAN, P., POULET, N., ROSET, N., COPP, G. H., GENIEZ, P., PONT, D., ARGILLIER, C., BAUDOIN, J.-M., PEROUX, T., CRIVELLI, A. J., OLIVIER, A., ACQUEBERGE, M., LE BRUN, M., MØLLER, P. R., WILLERSLEV, E. & DEJEAN, T. (2016). Next-generation monitoring of aquatic biodiversity using environmental DNA metabarcoding. Molecular Ecology, 25(4), 929-942.

WIESNED, C., SCHOTZKO, N., CERNY, J., GUTI, G., DAVIDEANU, G., JEPSEN, N. (2007). Technical Report with Results from the Fish Sampling and Analyses from the Joint DanubeSurvey 2007. International Commission for the Protection of the Danube River, Vienna, International Commission for the Protection of the Danube River, Vienna(73), pp.

WOOD, D. E., LU, J. & LANGMEAD, B. (2019). Improved metagenomic analysis with Kraken 2. Genome Biology, 20(1), 257.

XI, C., ZHANG, Y., MARRS, C. F., YE, W., SIMON, C., FOXMAN, B. & NRIAGU, J. (2009). Prevalence of Antibiotic Resistance in Drinking Water Treatment and Distribution Systems. Applied and Environmental Microbiology, 75(17), 5714-5718.

22 General physico-chemical determinands and nutrients

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Abstract

JDS4 offered a different approach to the three previous surveys, in which sampling, analysis and data providing were shifted from a core team of leading experts to national teams from the Danube basin. As far as regards selected general physico-chemical parameters and nutrients forms, the results obtained during JDS4 confirmed the main findings of both investigative monitoring of the JDS type and long-term surveillance monitoring type carried out within the Trans-National Monitoring Network (TNMN). Thus, comparison of the nutrients data produced within the four surveys organised so far and between nitrates data produced by TNMN laboratories and stable nitrate isotopes data provided by two different laboratories – belonging to the International Atomic Energy Agency (IAEA) and the University of Lorraine (UoL) respectively shows high degree of comparability, despite the fact that the samples were completely different (different sampling date, teams and sampling recipients).

22.1 Introduction

General physico-chemical parameters and as well as Nitrogen and Phosphorous nutrients forms play a major role within the monitoring strategy carried out under the ICPDR coordination. In recent years, the nutrients issue has been highlighted starting with the Danube Basin Analysis–Roof Report (http://icpdr.org/main/dba-2013), in which the results of the analysis showed that relatively significant parts of the course of the Danube River were subject to the risk of not meeting the environmental objectives of the Water Framework Directive due to four types of pressure, among which nutrient pollution made that 65% of the length of the Danube River to be classified at risk (especially the lower section of the river). Subsequently, according to the results presented in the Basin Management Plan at district level – Update 2015 (http://icpdr.org/main/activities-projects/river-basin-management-plan-update-2015), the four types of pressures from Roof Report become Significant Water Management Issues (SWMI). What is important to highlight in this analysis is that a distinction is made between pressures considered continuous (persisting in the past and still present today) and pressures that may occur in the future due to long-term evolutionary trends and future directions. This is one of the major considerations that determined the nutrients to be included in the list of parameters analysed during JDS4.

This chapter aims to present the results obtained for several physico-general parameters and nutrients forms in JDS4 water samples.

22.2 Methods

Unlike previous Joint Danube Surveys, in which the data sets for general parameters and nutrients were produced either on-board or by a single designated laboratory, JDS4 offered a different and effective alternative, focused entirely on national teams, which were responsible for sampling, samples transport, analyses and data delivery to the ICPDR JDS4 portal. Since the general parameters and nutrients are routinely TNMN water quality indicators annually monitored with monthly and bi-weekly frequency, analytical methods used by the national laboratories are standardised or fully validated methods, whose performances are systematically checked within the QUALCO Danube analytical quality control testing scheme organised by the ICPDR at basin level.

Data analysis of the JDS4 results from this chapter were compiled using Excel graphs from the JDS4 data portal and STATISTICA software version 12.0. In box-plots used for comparison of the nutrients data between the four JDSs, outliers and extreme values were identified by applying the criterion known as *Tukey one-sided upper*. (http://www.statsoft.com/Products/STATISTICA-Features).

22.3 Results and Discussion

22.3.1 General physico-chemical parameters

Water temperature variation pattern was typical of the summer sampling period (July) and the geographical region, with increasing spatial profile from Upper to Lower Danube: in the main course of the river, water temperature ranged between 17.4 °C at 1-L/*Böfinger Halde* and 27.3 °C at 48-M/*Chiciu/Silistra*, while in tributaries it varied between 15.4 °C (5-L/*Inn*) and 28.6 °C (44-M/*Iskar*).

Conductivity showed a decreasing profile in the Upper Danube, from more than 400 $\mu S.cm^{-1}$ along 1-L/*Böfinger Halde* – 4-L/*Niederalteich–Mühlau* to 275 $\mu S.cm^{-1}$ at 6-M/*Jochenstein* due to the influence of the *Inn* tributary (low salt content of the tributary – 220 $\mu S.cm^{-1}$) and the difference in flow discharges (427 $m^3.s^{-1}$ at 4-L/*Niederalteich–Mühlau* vs. 1012.3 $m^3.s^{-1}$ at 5-L/*Inn*). A constant profile was noticed along the Middle and Lower Danube (250 – 350 $\mu S.cm^{-1}$). In tributaries, conductivity ranged between 220 $\mu S.cm^{-1}$ (5-L/*Inn*) and 716 $\mu S.cm^{-1}$ (46-M/*Russenski Lom*).

The good buffer capacity of the Danube water is sustained by the 72% of **pH** values above 8.00 as well as the small variation range between 6.80 at 29-M/*Hercegszanto/Batina/Bezdan* and 8.32 at 47-M/*Downstream Ruse/Giurgiu*. Similarly with the previous surveys, a slight decreasing profile (from 8.10-8.20 to 7.70-7.90) was recorded in the *Iron Gates area*, caused by the decomposition of the organic matter in slow flowing water stretches (according to the JDS3 Final Scientific Report – 2015, in this area maximum velocity is not higher than 0.7 m/s and in downstream direction decreases to 0.4 m/s). In tributaries, pH varied between 7.29 (45-M/*Jantra*) and 8.40 (12-M/*Morava/Lanzhot*), the latter value being a direct consequence of biological activity illustrated by the chlorophyll "a" concentration (46.4 $\mu g.L^{-1}$) and dissolved oxygen saturation (103%).

Except for two values, all **dissolved oxygen saturation** values measured in the Danube River ranged between 80% and 110%, demonstrating that oxygen content consumed by decomposition of organic matter and respiration and oxygen released as a by-product of aquatic plant photosynthesis and physically transferred from the atmosphere are in good equilibrium. The excepted values from this spatial profile

were measured at 17-M/*Moson Danube Arm* (71.8%) and 50-M/*Reni* (78.9%), probably caused by organic pollution (sustained by the DOC and TOC concentrations: in the *Moson Danube Arm*, the maximum DOC and TOC concentrations from the Upper Danube stretch were measured – 2.9 $mg.L^{-1}$ and 3.9 $mg.L^{-1}$ respectively). Tributaries presented relatively similar oxygen content with the Danube itself, except for the oversaturation (118%) from 34-R/*Sava-Jesenice* and depletion (48%) from 44-M/*Iskar*, the low measured oxygen content from *Iskar* being favoured by the high water temperature (28.6 °C).

The spatial distribution of **biodegradable organic matter measured by BOD_5** showed a scattered profile along the Danube River, with low values in the upper stretch (ranging between 0.49 $mg.L^{-1}$ at 18-M/*Gönyű* and 1.70 $mg.L^{-1}$ at 8-L/*Oberloiben* and three values below the LOQ), followed by an elevated profile in the middle stretch, with BOD_5 concentrations between 0.60 $mg.L^{-1}$ at 29-M/*Hercegszanto*/*Batina*/*Bezdan* and 3.6 $mg.L^{-1}$ at 25-M/*Baja*. The lower stretch was characterized by values between the lab's LOQ (1.5 $mg.L^{-1}$ at 50-M/*Reni* and 51-M/*Valkova-Chilia arm*) and 3.3 $mg.L^{-1}$ at 47-M/*Downstream Ruse*/*Giurgiu*. In tributaries, BOD_5 values were relatively similar to the ones from the Danube, with three values below the LOQ (0.7 $mg.L^{-1}$ in 19-M/*Vah* and 1.0 $mg.L^{-1}$ in 5-L/*Inn* and 44-M/*Iskar*). However, it has to be mentioned that the low concentration from the *Iskar* was, most likely, caused by the low oxygen capital from this tributary (4.3 $mg.L^{-1}$ and 48% oxygen saturation respectively). The maximum concentration (4.5 $mg.L^{-1}$) was measured in 25-M/*Ráckevei-Soroksári*.

Chemical Oxygen Demand (COD) presented a slight increasing profile from upper to middle and lower stretches of the Danube. Three COD values from the Danube River and one value from tributaries were reported below the LOQ (10 or 15 $mg.L^{-1}$, relatively to the performance parameters of the analytical method used). Unlike BOD, the values in tributaries were slightly higher than those in the main course of Danube, with few exceptions: 5-L/*Inn* (below the LOQ of 15 $mg.L^{-1}$), 35-R/*Sava* and 44-M/*Iskar*. The maximum COD concentration (27.8 $mg.L^{-1}$) was measured in 49-M/*Prut* tributary.

Similar to COD, both **DOC** and **TOC** spatial profiles showed slight increasing profiles from upper to middle and lower stretch of the Danube, with minimum values (1.20 and 1.50 $mg.L^{-1}$) measured at 6-M/*Jochenstein* and maximum values (5.68 and 5.85 $mg.L^{-1}$) at 51-M/*Vilkova-Chilia* arm. Except for three sites (5-L/*Inn*, 30-M/*Drava* and 34-R/*Sava*/*Jesenice*, where low organic carbon content was found), all tributaries presented slightly higher concentrations than the Danube itself, ranging between 2.60 (19-M/*Vah*) and 7.96 $mg.L^{-1}$ (11-M/*Morava*/ *Dyje*).

22.3.2 Nutrients

In the main course of the river, half of the **N-Ammonium** concentrations were below or equal to lab's LOQs (values comprised between 0.005 and 0.050 $mg.L^{-1}$ N). All quantifiable concentrations were below 0.100 $mg.L^{-1}$ N, with the minimum value (0.011 $mg.L^{-1}$ N) at 8-L/*Oberloiben* and the maximum one (0.090 $mg.L^{-1}$ N) at 17-M/*Moson Danube Arm*. Correlated with oxygen saturation value (71.8%), DOC and TOC concentrations (2.9 and 3.9 $mg.L^{-1}$ respectively), it can be concluded that *Moson Danube Arm* is impacted by organic pollution combined with secondary pollution from decomposition of organic matter. In tributaries, 28% of the measured concentrations were below or equal to lab's LOQ, ranging between 0.005 and 0.040 $mg.L^{-1}$ N. If the majority of the tributaries presented concentrations similar to the ones from the Danube itself, slightly high values were measured in 44-M/*Iskar* (0.112 $mg.L^{-1}$ N) and 49-M/*Prut* (0.166 $mg.L^{-1}$ N), while the maximum concentration was found in 46-M/*Russenski Lom* (0.357 $mg.L^{-1}$ N).

N-Nitrites showed a scattered spatial profile along the Danube: a decreasing line was noticed in the upper stretch, from 0.011 – 0.012 $mg.L^{-1}$ N at 1-L/*Böfinger Halde* and 3-L/*Above Klösterl-Kelheim* to 0.004 – 0.005 $mg.L^{-1}$ N at 8-L/*Oberloiben* and 10-R/*Hainburg*, followed by an increasing line, reaching 0.018 $mg.L^{-1}$

N at 17-M/*Moson-Danube arm*. In the Middle and Lower Danube, a relative stable profile was recorded, with several peaks ranging between 0.012 and 0.014 mg.L^{-1} N. One of the tributaries (5-L/*Inn*) presented N-nitrites concentration below the LOQ (0.005 mg.L^{-1} N) and most of them showed similar levels to the ones from the Danube itself, with few exceptions: 0.026 and 0.023 mg.L^{-1} N in 25-M/*Ráckevei-Soroksári* and 46-M/*Russenski Lom* while relative high values were measured in 11-M/*Morava/Dyje* (0.058 mg.L^{-1} N) and 35-R/*Sava/Jamena* (0.061 mg.L^{-1} N).

A slight decreasing profile of **N-Nitrates** concentrations from Upper to Middle and Lower Danube was present, with maximum concentration (2.40 mg.L^{-1} N) measured at 1-L/*Böfinger Halde* and minimum (0.58 mg.L^{-1} N) at 50-M/*Reni*. Most of the tributaries showed a comparable profile with the main course of the river, with several fluctuations: low concentrations (0.22–0.23 mg.L^{-1} N) in 49-M/*Prut* and 12-M/*Morava/Lanzhot* and high concentration (6.05 mg.L^{-1} N) in 46-M/*Russenski Lom*. The minimum value of N-Nitrates for the Danube River as well as the maximum one in tributaries came in good agreement with the information presented in the TNMN Yearbook 2018, in which the minimum 90th percentile in the Danube River (1.42 mg.L^{-1} N) belonged to TNMN-RO5, the same as JDS4-50-M site and the maximum 90th percentile in tributaries (8.27 mg.L^{-1} N) belonged to 46-M/*Russenski Lom*.

Total Nitrogen concentrations (Figure 1) showed a similar profile to the N-nitrates, since the nitrates were the major component of the Total Nitrogen content ($r=0.915$, $N=25$, $p<0.05$ for the Danube River data and $r=0.910$, $N=15$, $p<0.05$ for tributaries); thus, decreasing spatial profile from Upper to Middle and Lower Danube was detected, with maximum concentration (2.40 mg.L^{-1} N) measured at 1-L/*Böfinger Halde*, minimum (0.93 mg.L^{-1} N) at 47-M/*Downstream Ruse/Giurgiu* and one value below the lab's LOQ (1.0 mg.L^{-1} N) at 40-M/*Banatska Palanka/Bazias*. In tributaries, low levels (0.70 and 0.77 mg.L^{-1} N) were measured in 5-L/*Inn* and 12-M/*Morava/Lanzhot*, while high concentration level (7.25 mg.L^{-1} N) was recorded in 46-M/*Russenski Lom*. One data inconsistency occurred for 44-M/*Iskar* tributary, for which Total Nitrogen concentration below the labs' LOQ (0.1 mg.L^{-1} N) was reported, while Total Inorganic Nitrogen content was 2.01 mg.L^{-1} N.

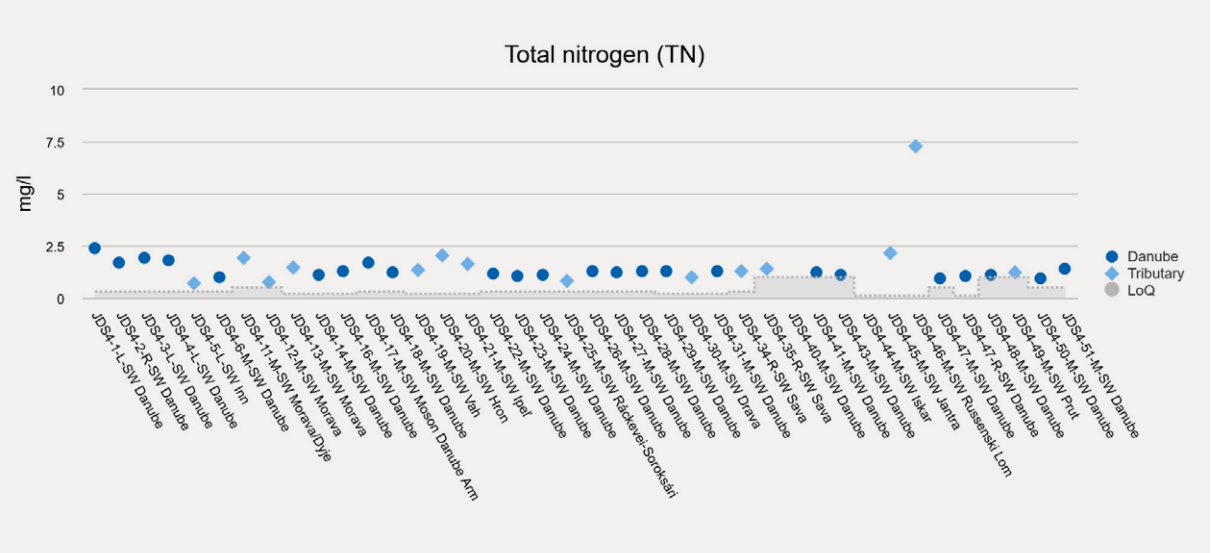

Figure 1: Total Nitrogen concentrations in the Danube River and tributaries during JDS4 (all values in mg.L^{-1} N).

In the **P-ortho-phosphates** data set, three concentrations in the Danube River (at 2-R/*Bittenbrunn*, 3-L/*Above Klösterl-Kelheim* and 48-M/*Chiciu/Silistra*) and one in tributaries (44-M/*Iskar*) were below the LOQ (0.005 mg.L^{-1} P). The maximum concentration in the main course of the river (0.082 mg.L^{-1} P) was measured at 17-M/*Moson Danube arm*. Spatial profile presented an increasing line from upper to middle and lower stretches of the Danube, with several peaks above the level of 0.050 mg.L^{-1} P: 0.052 at 41-M/*Upstream Timok* and 47-M/*Downstream Ruse/Giurgiu*, 0.060 at 43-M/*Pristol/Novo Selo* and 0.062 mg.L^{-1} P at 4-L/*Niederalteich-Mühlau* and 4-M/*Banatska Palanka/Bazias*. Two tributaries (5-L/*Inn* and 34-R/*Sava/Jesenice*) presented concentrations below the lab's LOQs (0.005 and 0.002 mg.L^{-1} P respectively), while the maximum value (0.349 mg.L^{-1} P) was measured in 11-M/*Morava/Dyje*. This latter value came in line with data from TNMN Yearbook 2018, according to which, at TNMN-CZ2 site (*Morava/Dyje-Pohansko*), the 90th percentile of P-ortho-phosphates concentrations was 0.510 mg.L^{-1} P. During JDS4, relatively high concentrations were also measured in 46-M/*Russenski Lom* (0.192 mg.L^{-1} P), 13-M/*Morava/Devin* (0.210 mg.L^{-1} P) and 21-M/*Ipel'* (0.220 mg.L^{-1} P).

Similarly to P-ortho-phosphates, increasing spatial profile from Upper to Middle and Lower Danube was noticed in the case of **Total Phosphorous** (Figure 2), but decreasing tendency was present downstream the *Iron Gates Reservoir*, from 0.113 mg.L^{-1} P at 31-M/*Ilok/Backa Palanka* to 0.085 mg.L^{-1} P at 43-M/*Pristol/Novo Selo Harbour*, as a confirmation of the previously findings according to which the *Iron Gates Reservoir* acts as a retention area for Total Phosphorous (daNUbs, 2005; Schreiber et al., 2005). In the main course of the Danube River, the minimum concentration (0.023 mg.L^{-1} P) was measured at 8-L/*Oberloiben* and the maximum one (0.150 mg.L^{-1} P) at the same site as the maximum for P-ortho-phosphates was found, at 17-M/*Moson Danube arm*. In tributaries, the minimum concentration (0.019 mg.L^{-1} P) was found in 34-R/*Sava/Jesenice* and the maximum one (0.573 mg.L^{-1} P) in 46-M/*Russenski Lom*, correlated with very high concentration of Suspended Matters (0.568 mg.L^{-1}). Similarly to P-ortho-phosphates, also relatively high Total P concentrations were measured in 13-M/*Morava/Devin* (0.280 mg.L^{-1} P), 21-M/*Ipel'* (0.320 mg.L^{-1} P-PO4) and 11-M-*Morava/Dyje* (0.373 mg.L^{-1} P).

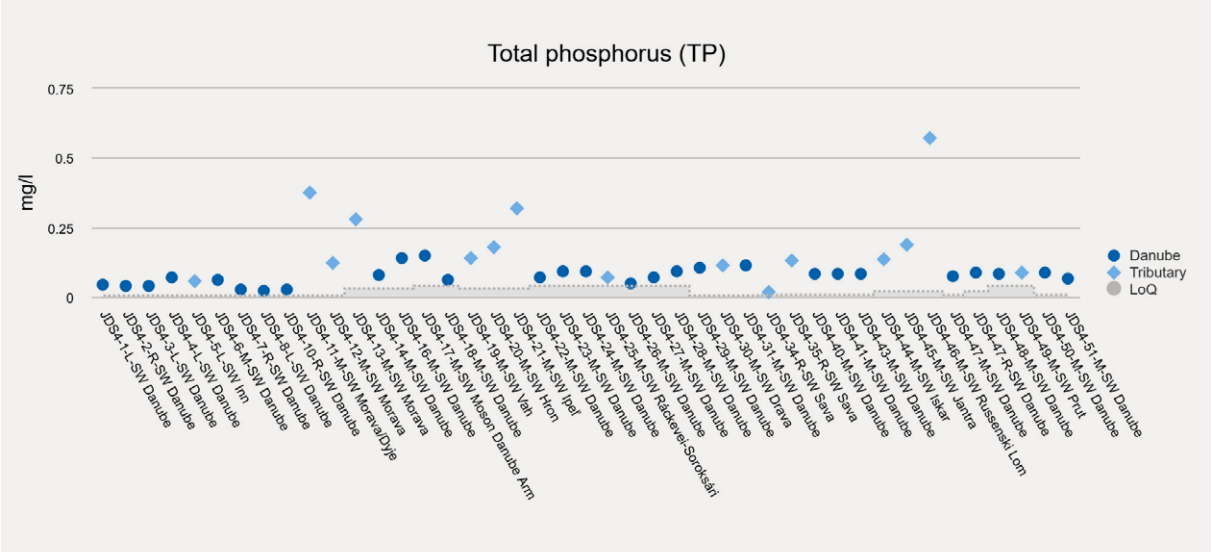

Figure 2: Total Phosphorous concentrations in the Danube River and tributaries during JDS4 (all values in mg.L^{-1} P).

22.3.3. Comparison between the four JDSs

In Figure 3 and Figure 4 comparison between the four JDSs in respect to **Total Nitrogen** concentration in the Danube River and tributaries is presented. Lowering trend in variation ranges as well as in descriptive statistics can be noticed: for the main course of the river, the median value systematically decreased from 1.83 mg.L^{-1} N in 2001 to 1.80 in 2007, 1.60 in 2013 and to 1.20 in 2019, the latest survey being a confirmation of the decreasing tendency previously identified. Improved situation is also illustrated by the less and less concentration values "falling" into the outliers and extreme categories according to Tukey criterion: if in 2001 the threshold above which outliers and extremes values were identified was 2.82 mg.L^{-1} N, in 2019 this threshold went down to 1.65 mg.L^{-1} N. Even if not so clearly as in the case of the Danube itself, decreasing trend is visible also for tributaries: median value decreased from 2.17 mg.L^{-1} N in 2001 to 1.47 and 1.50 in 2007 and 2013 respectively, reaching 1.38 mg.L^{-1} N in 2019. However, one of the so-called "hot spots" identified at the basin-wide level in previous surveys was confirmed in the current one (*Russenski Lom* tributary)[1].

Total Phosphorous concentration in the Danube River during the four JDSs (Figure 5) shows high data comparability and a slight decreasing trend in median values in the last two surveys compared to the first ones. What stands out is the decrease of the extreme values and the thresholds above which they are identified: if in 2001 concentrations above 0.205 mg.L^{-1} P and above 0.410 mg.L^{-1} P were calculated as being outliers and extreme values respectively, in last surveys (2013 and 2019) these thresholds dropped to 0.125 and 0.250 mg.L^{-1} P. In tributaries (Figure 6) no significant trend of variation could be noticed, the median values being in the range 0.155 in 2001 and 0.139 in 2019. Still, the situation is better for *Iskar* and *Jantra*, but relatively unchanged for *Russenski Lom*.

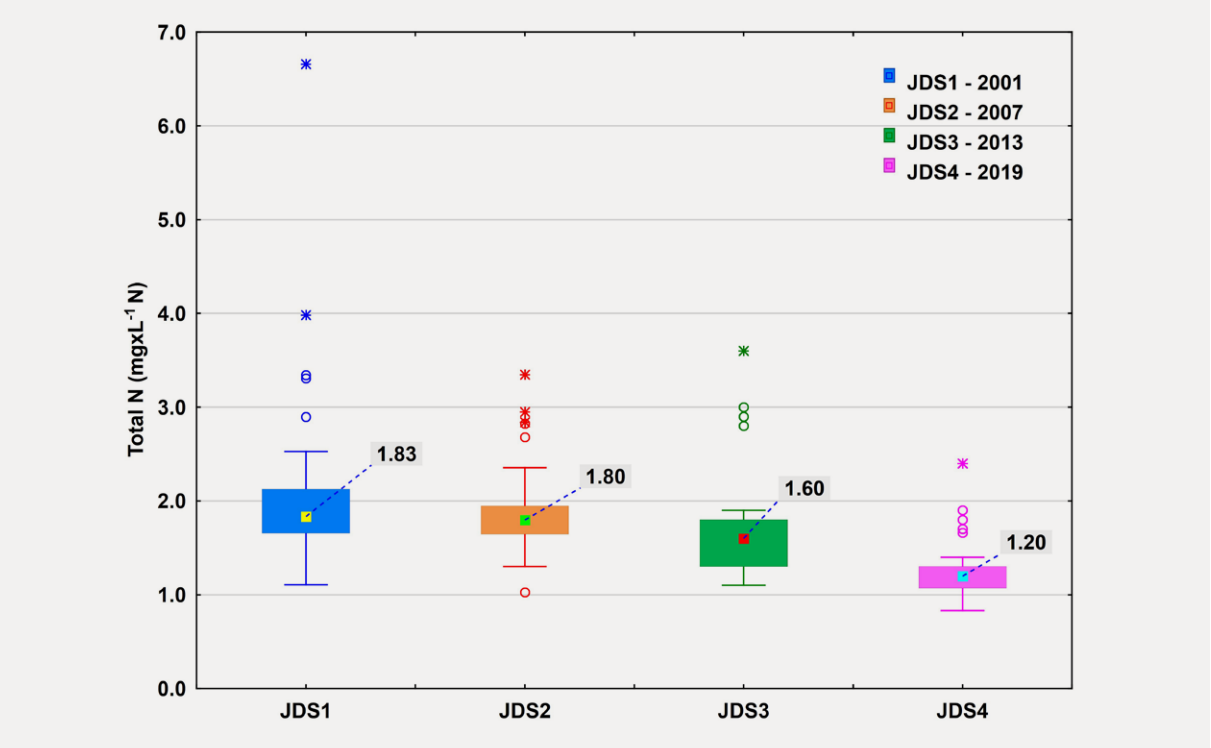

Figure 3: Comparison between Total Nitrogen concentrations in the Danube River during the four JDSs (all values in mg.L^{-1} N). Middle point: median; box values: quartile range (25th percentile - 75th percentile); whisker value: non-outlier range (1.5 x quartile range); circles: outliers; stars: extremes.

[1] *During JDS4, Arges tributary was not included in the sampling programme.*

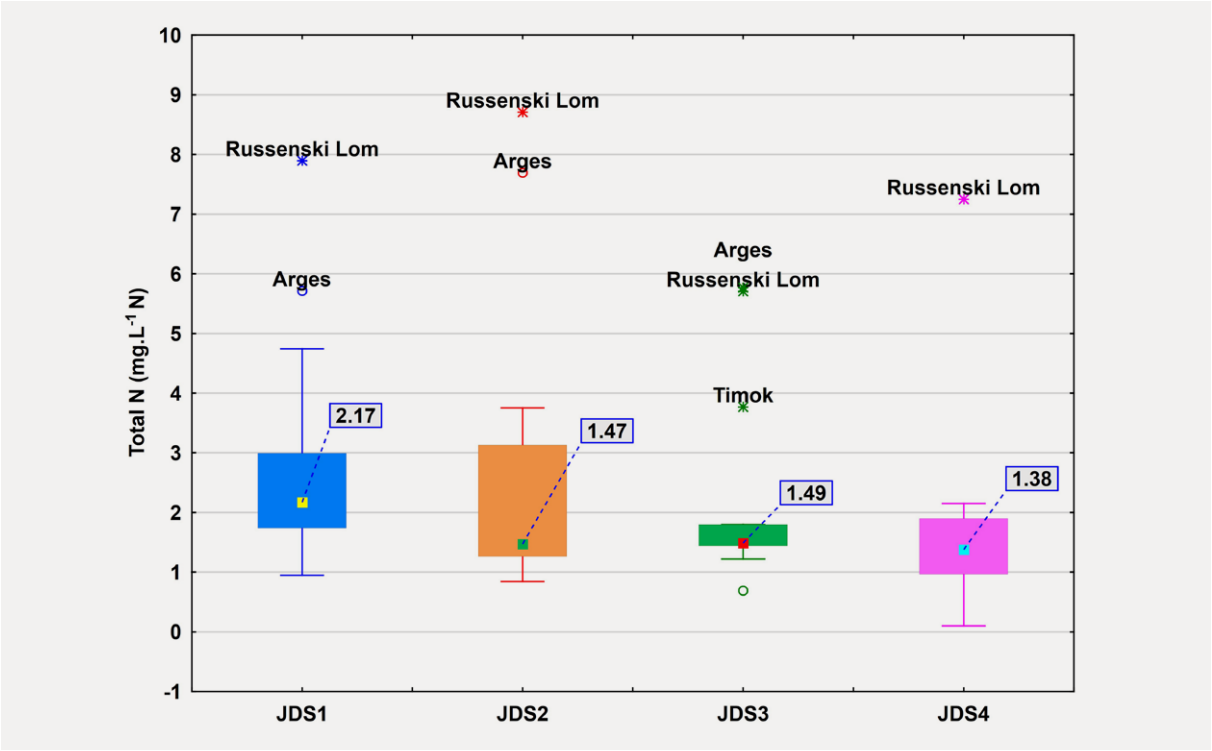

Figure 4: Comparison between Total Nitrogen concentrations in tributaries during the four JDSs (all values in mg.L^{-1} N). Middle point: median; box values: quartile range (25th percentile - 75th percentile); whisker value: non-outlier range (1.5 x quartile range); circles: outliers; stars: extremes.

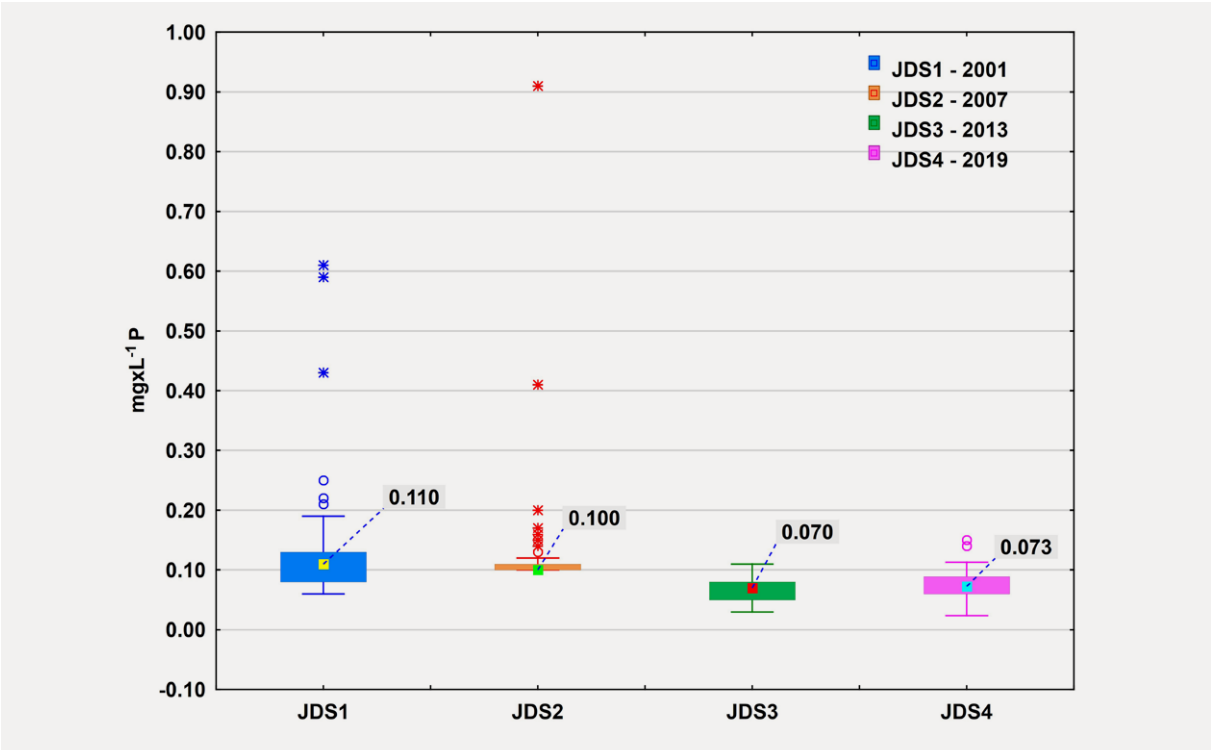

Figure 5: Comparison between Total Phosphorous concentrations in the Danube River during the four JDSs (all values in mg.L^{-1} P). Middle point: median; box values: quartile range (25th percentile - 75th percentile); whisker value: non-outlier range (1.5 x quartile range); circles: outliers; stars: extremes.

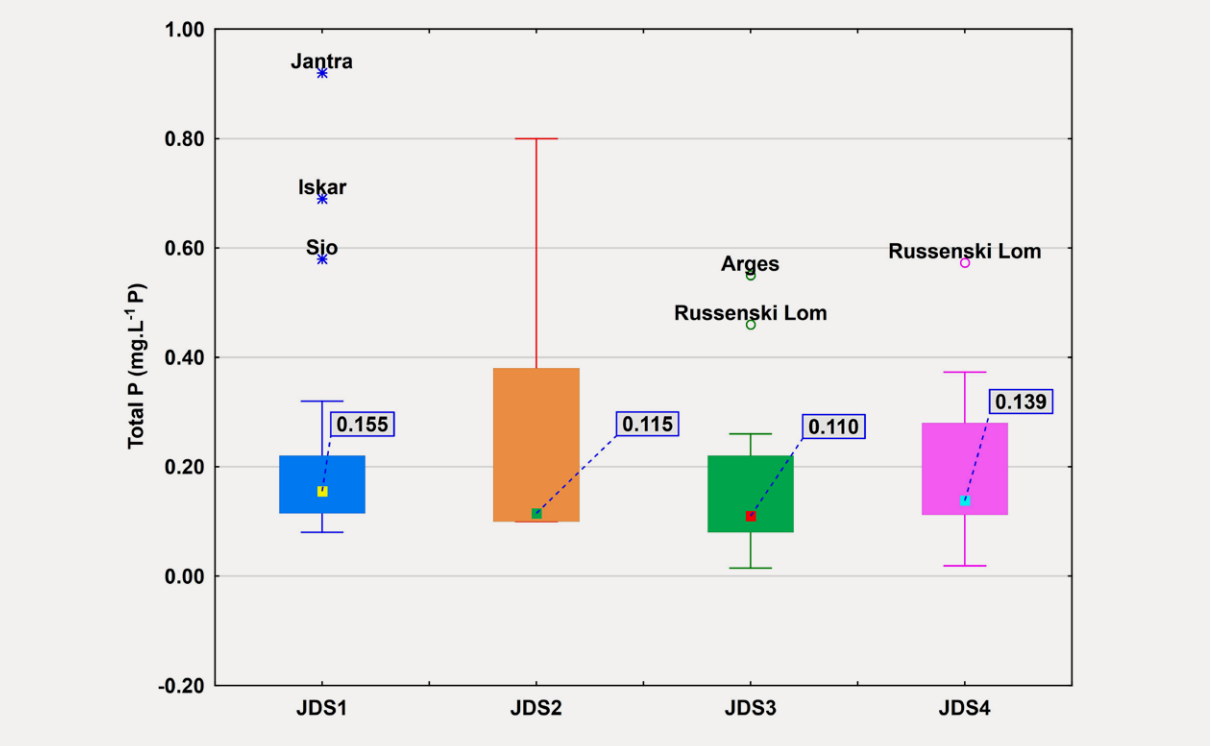

Figure 6: Comparison between Total Phosphorous concentrations in tributaries during the four JDSs (all values in mg.L^{-1} P). Middle point: median; box values: quartile range (25th percentile - 75th percentile); whisker value: non-outlier range (1.5 x quartile range); circles: outliers; stars: extremes.

22.3.4. Comparison N-Nitrates concentrations

Given the new approach of the JDS4 survey, in which data for basic chemical parameters were provided by national laboratories involved in TNMN Surveillance programme and given the fact that one of the objectives of the investigative monitoring surveys refers to increasing the data comparability, comparison between the nitrate data delivered by the Danube national laboratories with nitrate data reported by two different laboratories, the International Atomic Energy Agency (IAEA) and the University of Lorraine (UoL), might bring an illustrative view in this respect. As it can be seen from Figure 7, high comparability is highlighted by the values of coefficient of determination (R^2) 0.9328 and 0.9338 between national laboratories and IAEA and UoL on one hand and 0.9915 between IAEA and UoL on the other hand. This level of comparability is even more satisfactory because the samples differed significantly (taken at different days, by different teams). This can be explained by the similarity in analytical methods used: IAEA used spectrophotometric detection as did 53.5% of the national labs, while UoL used ion-chromatography as did 46.5% of the national labs. Much more information on nitrates content in Danube Basin, its sources and stability can be found in Chapter 46.

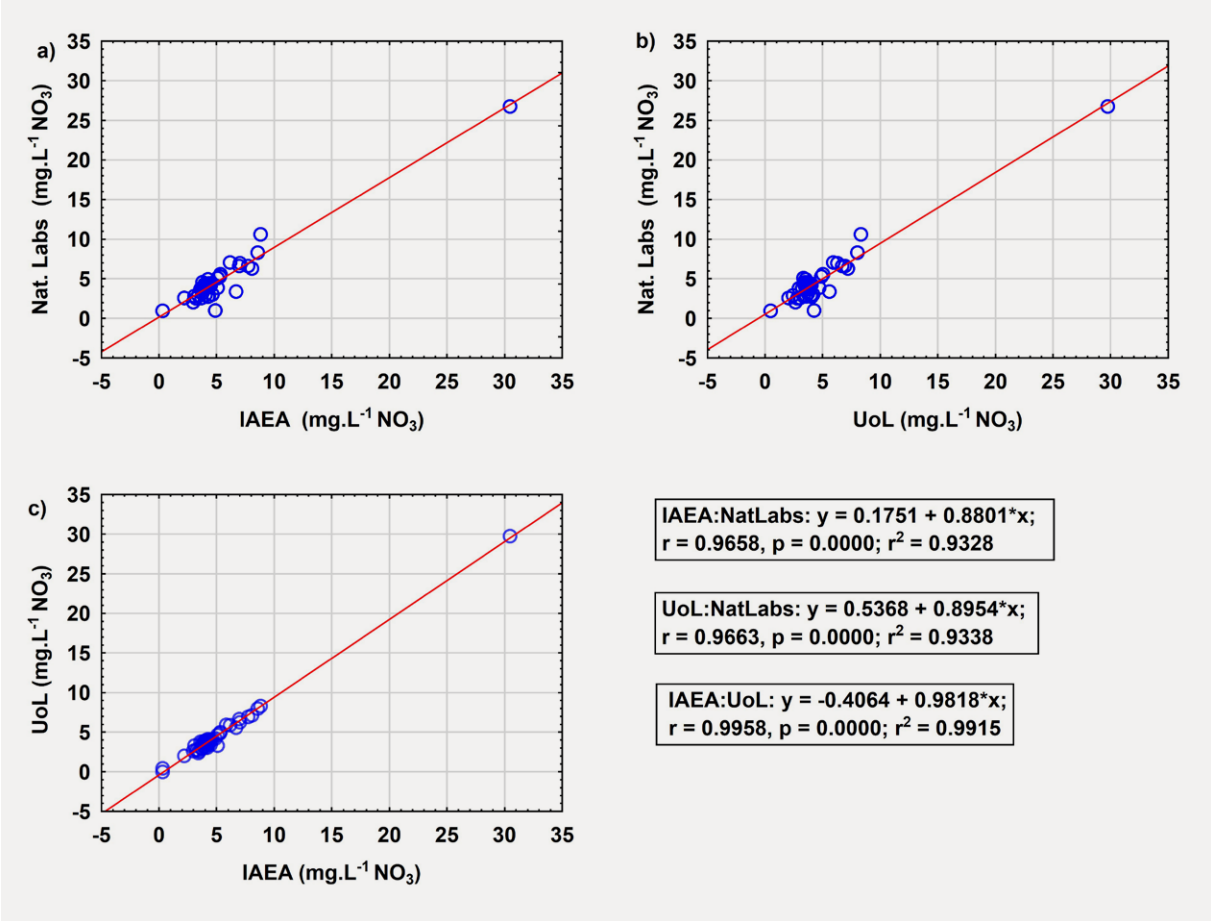

Figure 7: Comparison between nitrate data from TNMN laboratories and nitrate data provided by IAEA and UoL (all values in mg.L^{-1} NO3).

22.4 Conclusions

- General physico-chemical parameters measured were typical to the survey time (July) and geographical area;
- Spatial patterns previously identified were confirmed during JDS4: decreasing of Total Nitrogen and increasing of Total Phosphorous profiles from Upper to Middle and Lower Danube respectively;
- A decreasing trend in Total N concentrations in the Danube River and several tributaries was also confirmed during JDS4; no significant temporal variation could be noticed for Total Phosphorous;
- Some of the *"hot-spots"* in tributaries noticed in previous surveys were confirmed in JDS4 (*Russenski Lom*) whereas some of them showed an improved situation (*Iskar* and *Jantra*);
- The overall view showed high comparability of data produced by the new approach of the JDS with similar previous data (JDS1, JDS2, JDS3);
- A high level of comparability between nitrates data provided by the Danube TNMN laboratories and stable isotopes of nitrates data reported by IAEA and UoL is also shown;
- Variation of nutrients concentration in the Danube Basin during the period covered by the four expeditions carried out so far comes as a confirmation of those underlined in the DRBMP 2015, according to which continuous nutrient pollution poses a risk of failure to meet the quality objective for 20% of the length of surface water bodies in the basin, which is a considerable improvement over the situation identified in the year 2004 and future pollution with nutrients would induce a practically zero risk, reduced to only 128 km of surface water bodies at the basin-wide level;
- Since the JDS data gathered every six years constitute just a *'snap-shot'* specific to the summer-autumn sampling time, integration and analysis of these data into the TNMN comprehensive dataset would certainly be a useful tool to watch the entire *'movie'*.

22.5 References

LIŠKA I., WAGNER, F., SENGL, M., DEUTSCH, K., SLOBODNIK, J. (eds.) (2015) Joint Danube Survey 3 – A Comprehensive Analysis of Danube Water Quality, ICPDR – International Commission for the Protection of the Danube, http://www.icpdr.org/main/activities-projects/joint-danube-survey-3.

LIŠKA I., WAGNER, F., SLOBODNIK, J. (eds.) (2008) Joint Danube Survey 2, Final Scientific Report. ICPDR – International Commission for the Protection of the Danube River, pp. 118-131, http://www.icpdr.org/main/activities-projects/joint-danube-survey-2.

NUTRIENT MANAGEMENT IN THE DANUBE BASIN AND ITS IMPACT ON THE BLACK SEA (2005), daNUbs Final Report.

SCHREIBER, H., BEHRENDT, H., CONSTANTINESCU, L. T., CVITANIC, I. DRUMEA, D., JABUCAR, D., JURAN, S., PATAKI, B., SNISHKO, S., ZESSNER, M. (2005): Point and diffuse nutrient emissions and loads in the transboundary Danube River basin – a modelling approach. Arch. Hydrobiol. Suppl. Large Rivers.

WATER QUALITY IN THE DANUBE RIVER BASIN – TNMN Yearbooks 2018, http://www.icpdr.org.

DANUBE BASIN ANALYSIS - WFD Roof Report (2005). Vienna: ICPDR.

DANUBE BASIN ANALYSIS (2013), https://www.icpdr.org/main/dba-2013

THE DANUBE RIVER BASIN DISTRICT MANAGEMENT PLAN – Part A, Basin-wide overview, Update 2015, https://www.icpdr.org/main/management-plans-danube-river-basin-published

23 Target analysis of organic substances in water

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Abstract

Nineteen priority substances regulated in the European Water Framework Directive were analysed in Danube River Basin waters. Only for cypermethrin and cybutryne concentrations above the Environmental Quality Standards (EQS) were observed at a few sampling sites. All other priority pollutants showed concentrations below the respective EQS.

From the new list of Danube River Basin specific pollutants one pharmaceutical, four pesticides and one metabolite were found in relevant concentrations at a few sampling sites. Ten substances from the EU Watch List were analysed and elevated concentrations could be detected for the pharmaceutical diclofenac, the natural hormone 17-beta-estradiol and the insecticide imidacloprid.

In addition, very low concentrations of 1,4-dioxane and 14 flame retardant substances were found to be present in waters, thus posing no risk to the Danube River Basin.

The overall results of target analysis of organic substances show a satisfactory situation. Only for a few substances at a few sampling sites were effect thresholds found to be exceeded. Often the highest concentrations were found in tributaries, whereas in the Danube itself, dilution leads to significantly lower values. The results provide valuable information for the next update of the WFD-list of priority pollutants and the list of River Basin Specific Pollutants.

23.1 Introduction

EU member states are obliged to report data for priority substances according to Directive 2008/105/EEC which was amended by Directive 2013/39/EU. In total 45 substances or groups of substances together with 5 additional substances originally selected according to Directive 74/464/EEC are listed together with EQS for water and/or biota. An EQS comparison of average values for at least 12 measurements within one year enables the assessment of the chemical status of water bodies. It has to be stressed that a single value gathered through JDS4 samples cannot be used for chemical status assessment but provides valuable hints for surface water contaminations.

According to the WFD the list of priority substances should be revised every 4 years. As a result of the prioritisation process for the update of the list of priority substances 17 substances are listed in a "Watch List" published by Commission decision 2015/495 of 20 March 2015 (notified under document C(2015) 1756). The proposed PNEC values defined in 2015 used for the assessment were updated (lowered)

in the meantime for 9 single substances based on additional information from Switzerland and 3 new substances were added.

A list of Danube-specific substances was derived within the EU-project SOLUTIONS taking into regard monitoring data and using ecotoxicological data taken from literature or modelling. Nineteen substances – mostly pharmaceuticals and pesticides – were analysed.

In addition 1,4-dioxan was analysed as an in-kind contribution to check for possible point sources of this substance mainly used as a solvent. Also 14 common organophosphorus compounds – in use as flame retardants – were analysed at trace concentrations.

23.2 Methods

For trace analysis of target substances the following methods were applied (mostly according to international standards):

- Solid-phase extraction (SPE) in combination with LC-MS
- Stir-bar-sorptive-extraction, thermodesorption, GC-MS/MS
- LC-MS/MS after direct injection of the water sample
- Liquid-liquid-extraction in combination with GC-ECD
- Head-space analysis in combination with GC-MS for 1,4-dioxane
- Large-volume SPE (5L), GC-MS and LC-MS.

The laboratories are accredited according to ISO 17025 for most of the methods.

23.3 Results

23.3.1 Priority pollutants

Pesticides are an important group of priority substances. Most of the pesticides listed as priority pollutants could be analysed at concentrations below the annual-average environmental quality standards (AA-EQS).

Alachlor, chlorfenvinphos, chlorpyriphos, trifluraline, dicofol, quinoxyfen, aclonifen, dichlorvos and bifenox were absent in all JDS4 samples. Isoproturon, diuron and terbutryne and cybutryne were found at few sampling sites in low concentrations (table 24-1). The concentrations for cybutryne at the stations JDS4-19 and JDS4-36 (0.0027 µg/L and 0.0052 µg/L) are above the AA-EQS of 0.0025 µg/L. The pesticides terbutryne and cybutryne are also registered as biocides, so surface water contaminations may also come from run-off from building fronts or other sources where biocides are applied.

Table 1: Positive results of selected priority pesticides in water.

sampling site	terbutryne µg/l	isoproturon µg/l	diuron µg/l	cybutryne µg/l
JDS4-19	n.d.	n.d.	n.d.	0,0027
JDS4-32	n.d.	n.d.	0,0099	n.d.
JDS4-33	0,0064	n.d.	0,033	n.d.
JDS4-36	n.d.	n.d.	n.d.	0,0052
JDS4-38	n.d.	0,0059	n.d.	n.d.
JDS4-39	n.d.	0,0060	n.d.	n.d.
JDS4-44	0,0065	n.d.	n.d.	n.d.
AA-EQS	0,065	0,3	0,2	0,0025
n.d. = not detected				

Cypermethrin was found at four sampling stations surpassing the very low AA-EQS of 0.00008 µg/L at the stations JDS4-24 (0.00015 µg/L), JDS4-41 (0.00009 µg/L) and JDS4-43 (0.00013 µg/L).

As atrazine and simazine could be analysed at sub-ng/l concentrations, positive results were found at all stations for atrazine (maximum 0.01 µg/L at JDS4-33) and at 42 stations for simazine (maximum 0.002 µg/L at JDS4-22), but the results fall well below the AA-EQS for both herbicides. As both substances have been banned in all European countries for many years, their presence in surface waters stems from groundwater influence.

For the plasticiser Di(ethylhexyl)-phthalate (DEHP) the limit of quantification of 0.2 µg/L was not surpassed at any sampling site. This result confirms the outcome of JDS3 whereas in the JDS2 campaign the AA-EQS of 1.3 µg/L was often exceeded.

From the group of organochlorine pesticides 4,4-DDT and metabolites as well as hexachlorocyclohexane (HCH) isomers were analysed in very low concentrations (LOQ 0.000009 µg/L for 4,4-DDT and derivatives and 0.000012 µg/L for HCHs). These substances were found at up to 50 sampling sites but the summed concentrations as well as the concentration of 4,4-DDT were below the respective AA-EQS values by a factor of 10 or more.

23.3.2 River Basin Specific Pollutants (RBSP)

Within the EC-project SOLUTIONS a list of Danube RBSP was derived from monitoring data and risk assessment data following a prioritisation scheme developed by the NORMAN laboratory framework. 19 substances were selected and the lowest PNEC defined the concentration level to be reached by the analytical methods. The analysis of caffeine was disturbed by high blanks in the laboratory and ibuprofen could not be analysed. Amoxicillin is also listed as a Watch List substance and discussed there.

Bisphenol A, chloroxuron, bromacil, dicamba and fipronil were not found in any sample, whereas the other RSBP could be detected at 8 up to 51 sampling sites. The maximum values of diazinon, carbamazepine, metolachlor, metazachlor, terbuthylazine and desethylterbuthylazine exceeded the lowest PNEC values indicating a risk to the aquatic environment.

Table 2: Results for River Basin Specific Pollutants.

Substance	CAS No.	Lowest PNEC µg/l	LOQ µg/l	Number of positive results	Min µg/l	Max µg/l	Number of positive results above PNEC
Chloroxuron	1982-47-4	0,0024	0,0001	0	0	0	0
Caffeine	58-08-2	0,1	high blanks				
Bromacil	314-40-9	0,01	0,0074	0	0	0	0
Diazinon	333-41-5	0,001	0,0007	12	0,00076	**0,0028**	9
Carbamazepine	298-46-4	0,05	0,0002	47	0,00023	**0,058**	4
Metolachlor	51218-45-2	0,07	0,00005	51	0,00044	**0,11**	5
Metazachlor	67129-08-2	0,02	0,0008	35	0,00076	**0,029**	3
Terbuthylazine	5915-41-3	0,06	0,0006	51	0,0032	**0,087**	4
Desethylterbuthylazine	30125-63-4	0,03	0,0008	51	0,00099	**0,16**	8
Linuron	330-55-2	0,26	0,0006	8	0,00070	0,0024	0
Tebuconazole	107534-96-3	0,24	0,00013	51	0,0019	0,075	0
Bisphenol A	80-05-7	0,2	0,1	0	0	0	0
Chlorothalonil	1897-45-6	0,06	0,00002	27	0,00002	0,0005	0
Dicamba	1918-00-9	0,13	0,0017	0	0	0	0
Dimethenamid	87674-68-8	0,2	0,00004	48	0,00006	0,014	0
2-Phenylphenol	90-43-7	0,36	0,00008	51	0,00009	0,0065	0
Fipronil	120068-37-3	0,00077	0,0008	0	0	0	0
Ibuprofen	15687-27-1	0,011	no data				
Amoxicillin	26787-78-0	0,078	see table 3				

Desethylterbuthylazine – a metabolite of the herbicide terbuthylazine – exceeds the proposed PNEC especially in the Upper Danube starting at JDS4-4 (Niederalteich) with 0.061 µg/L down to JDS4-10 (Hainburg) showing a short decrease at JDS4-6 (Jochenstein) because of dilution with water from the Inn River. The maximum values of 0.13 µg/L and 0.16 µg/L were found in the Morava tributary at sampling sites JDS4-11 and JDS4-12. Also, Russenski Lom showed an elevated concentration of desethylterbuthylazine with 0.032 µg/L.

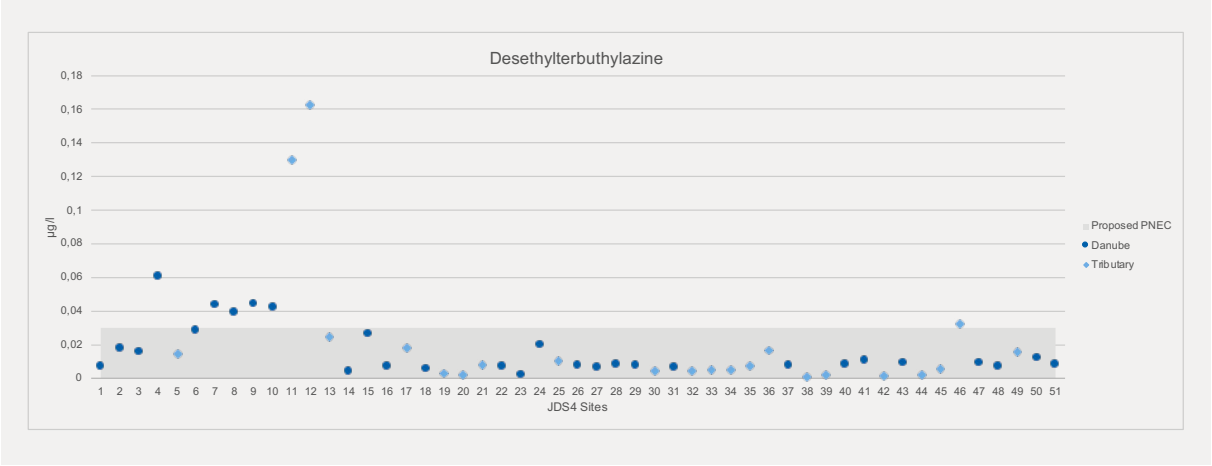


Figure 1: Concentration of desethylterbuthylazine (µg/L) in the Danube and the tributaries.

Terbuthylazine was found slightly above the proposed PNEC in the Morava at sampling sites JDS4-11 and JDS4-13 and shortly downstream in the Danube at JDS4-15. Also Russenski Lom (JDS4-46) had an elevated concentration of 0.07 µg/L. The herbicide metolachlor – often applied together with terbuthylazine – shows a maximum concentration of 0.11 µg/L in the Sava River at sampling site JDS4-36 clearly above the proposed PNEC of 0.07 µg/L and shortly downstream in the Danube at JDS4-37 (downstream Pancevo). Exceedances of the PNEC were found also in the tributaries Tisza, Russensiki Lom and Prut at sampling sites JDS4-33, JDS4-46 and JDS4-49.

For the herbicide metazachlor three minor exceedances of the proposed PNEC were reported in the Morava at sampling sites JDS4-12 (0.029 µg/L) and JDS4-13 (0.029 µg/L) as well as in the Lower Danube at sampling site JDS4-47 (0.028 µg/L). The insecticide diazinon was found above the proposed PNEC at nine sampling sites – mainly in tributaries of the Middle and Lower Danube section with a maximum concentration of 0.0028 µg/L in the Tisza River (JDS4-32).

The pharmaceutical carbamazepine was found above the proposed PNEC at the sampling sites JDS4-13 (Morava), JDS4-15 (Danube, Čunova), JDS4-21 (Ipel) and JDS4-22 (Danube, Szob).

23.3.3 Watch List substances

10 Watch List substances were selected for analysis including amoxicillin, ciprofloxazin and metaflumizone from the first Watch List update. The LOQs corresponding to the proposed PNEC values for 17-beta-estradiole (E2, 0.0004 µg/L) and 17-alpha-ethinylestradiole (EE2, 0.000035 µg/L) were missed by a factor of 2 and 100, respectively as these substances need a specific highly-sensitive method.

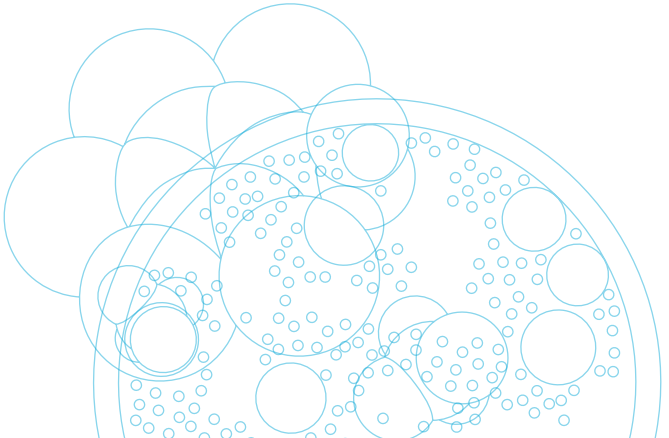
The limit of quantification of E2 of 0.0007 µg/L was exceeded only at the three sampling sites Jochenstein (JDS4-6), Morava (JDS4-13) and Ipel (JDS4-21). At three sampling sites (JDS4-34, JDS4-35 and JDS4-47) E2 was found in concentrations below LOQ indicating estrogene activity in the water. Estrone (E1) was detected in 28 samples in the upper and middle section of the Danube always below the proposed PNEC value of 0.0036 µg/L, whereas EE2 was not found above the LOQ of 0.0036 µg/L.

Table 3: Results for Watch List substances.

Substance	CAS No. / PNEC values water	PNEC value updated µg/l	LOQ µg/l	Number of positive results	Min µg/l	Max µg/l	Number of positive results above PNEC
Diclofenac	15307-79-6	0,05	0,0015	48	0,0021	**0,063**	2
17-Beta-estradiol (E2)	50-28-2	0,0004	0,00070	3	**0,0008**	**0,0021**	3
Estrone (E1)	53-16-7	0,0036	0,00005	28	0,0002	0,0031	0
17-Alpha-ethinylestradiol (EE2)	57-63-3	0,000035	0,00360	0	0	0	0
Imidacloprid	105827-78-9/ 138261-41-3	0,0083	0,00033	50	0,0003	**0,040**	7
Clarithromycin	81103-11-9	0,12	0,000003	51	0,000013	0,0050	0
Azithromycin	83905-01-5	0,019	0,00003	28	0,00004	0,0052	0
Amoxicillin	26787-78-0	0,078	0,0013	41	0,0015	0,052	0
Ciprofloxazin	85721-33-1	0,089	0,016	4	0,017	0,025	0
Metaflumizone	139968-49-3	0,0654	0,00007	0	0	0	0

Metaflumizone, a pesticide registered only in some European countries, was not found in any sample. The antibiotics clarithromycin, azithromycin, amoxicillin and ciprofloxazin were detectable at 4-51 sampling sites, but all concentrations were below the PNEC values. Some results for amoxicillin and ciprofloxazin close to the PNEC values indicate that during the winter period, assuming a higher consumption of antibiotics, these values might be exceeded.

Imidacloprid is an insecticide from the neonicotinoic group with broad application in horticulture and agriculture and obviously in wide-spread use in the Danube Basin. It was detected in 50 out of 51 samples with 7 sampling sites (JDS4-13, JDS4-23, JDS4-25, JDS4-33, JDS4-45, JDS4-46, JDS4-49) surpassing the proposed PNEC value of 0.0083 µg/L. These elevated concentrations were mostly found in tributaries with a maximum of 0.040 µg/L in Russenski Lom. In general imidacloprid concentrations in the Danube increases from the upper to the lower section of the Danube.



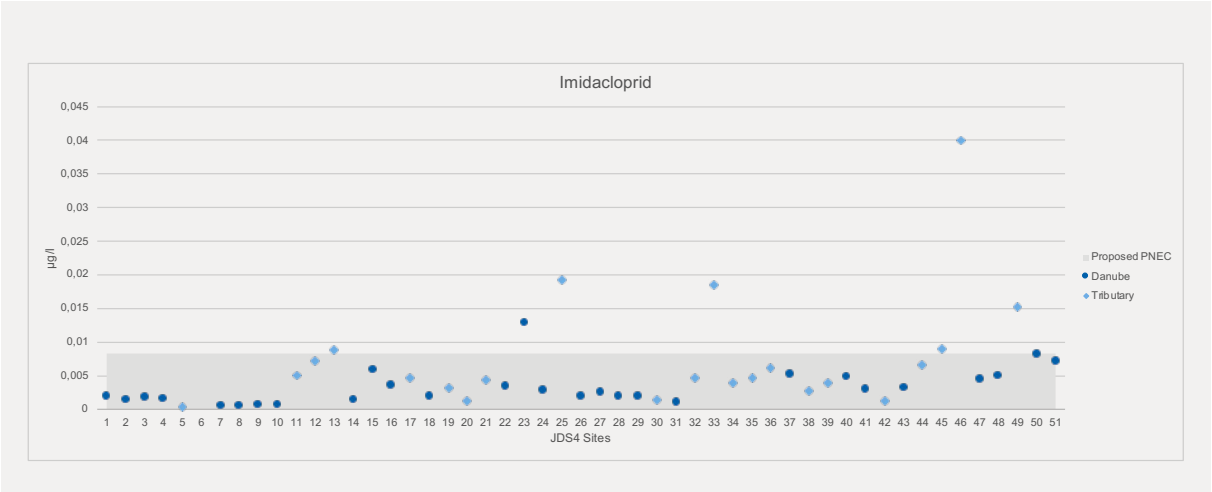


Figure 2: Concentration of imidacloprid (µg/L) in the Danube and the tributaries.

The widely used pharmaceutical diclofenac was detectable at 48 sampling sites, but the PNEC was exceeded only at sampling stations JDS4-15 (Danube Čunovo, 0.063 µg/L) and JDS4-46 (Russenski Lom, 0.051 µg/L).

23.3.4 1,4-Dioxane

1,4-Dioxan is an inert and water-soluble chemical. It is used as a solvent for the production of adhesives, colorants, cleaning agents or paper. It is also a by-product of the synthesis of polyester materials and certain non-ionic tensides. 1,4-Dioxane is not degraded in WWTPs and it is not eliminated during bank filtration. According to the REACH registration dossier (ECHA 2018) 1000 tons per year are used within the EU.

1,4-Dioxane was found in 31 samples with a median value of 0,20 µg/L and a maximum concentration of 0.53 µg/L (JDS4-5, Inn). In the Lower Danube no 1,4-dioxane could be detected.

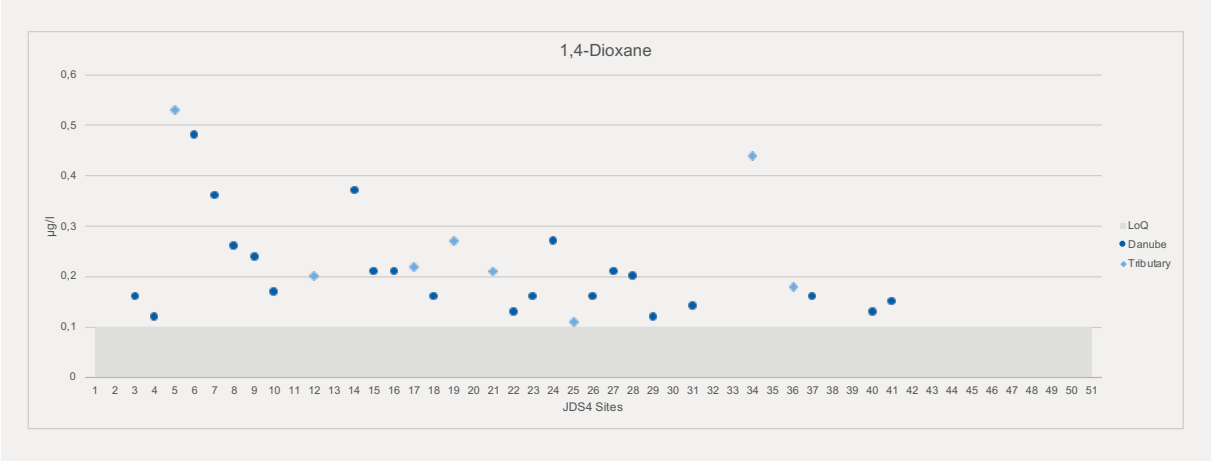


Figure 3: Concentration of 1,4-dioxane (µg/L) in the Danube and the tributaries.

In Germany an AA-EQS of 57.5 mg/L for surface water was proposed by LfU/UBA in 2019. For drinking water, a preliminary guidance value of 5 µg/L is used for lifetime consumption.

1,4-Dioxane concentrations found in the Danube and the tributaries pose no risk, neither to the aquatic biocenosis nor to drinking water produced via bank filtrated water.

23.3.5 Organophosphorus compounds (OPCs)

Chlorinated and non-chlorinated OPCs are widely used as flame retardants in a great variety of products like rubbers, textiles or electronic equipment. 14 OPCs were analysed mostly in sub-nanogram per litre concentrations. During JDS3 13 out of these 14 OPCs were analysed in the dissolved fraction of the surface water whereas in JDS4 whole water samples were used. Although the data are not directly comparable in JDS4 tris(1-chloro-2-propyl)phosphate (TCPP) again is the OPC showing the highest concentrations (maximum 0.45 µg/L) followed by triethylphosphate (TEP, maximum 0,37 µg/L) and tris(isobutyl)phosphate (TiBP, maximum 0.25 µg/L).

Table 4: Target analysis of organic substances in water.

Substance	CAS nr	LOQ	n >LOQ	Min	Max	Median	Lowest PNEC*
		ng/L		ng/L	ng/L	ng/L	µg/l
TEP	78-40-0	2,8	47	3,8	373	24	632
TnPP	513-08-6	0,67	2	1,7	2,0	1,9	2,32
TiBP	126-71-6	8,8	35	9,1	246	20	11
TnBP	126-73-8	0,99	50	0,83	32	3,9	no data
TCEP	115-96-8	0,71	50	0,70	37	5,2	4
TCPP	13674-84-5	0,93	50	24	449	67	120
TDCPP**	13674-87-8 and 78-43-3	0,75	49	2,2	61	11	1,1 and 0,011
TBOEP	78-51-3	0,78	44	1,2	60	11	0,14
TPhP	115-86-6	0,08	50	0,49	36	2,0	0,36
EHDP	1241-94-7	0,17	50	0,41	54	2,4	0,018
TEHP	78-42-2	0,01	50	0,26	25	1,4	0,039
TMPP	1330-78-5	0,14	28	0,15	3,5	0,32	no data
TiPPP	64532-95-2	0,11	10	0,13	1,4	0,23	no data
T35DMPP	25653-16-1	0,02	10	0,02	0,60	0,06	no data

* data from NORMAN ECOTOX Database
** Tris(1,3-dichloropropyl)phosphate and Tris(2,3-dichloropropyl)phosphate

According to JDS4 data the broad commercial use of OCPs has not diminished during recent years. All measured concentrations are well below the lowest PNEC concentrations available in the NORMAN ECOTOX database except 6 values for 2-ethylhexyl diphenylphosphate (EHDP) which are above the PNEC value of 0.018 µg/L up to a factor of 3.

23.4 Conclusions

- 19 out of 45 WFD priority pollutants were analysed in water samples;
- Concentrations of cypermethrin at four, as well as cybutryne at two sampling stations were above the AA-EQS whereas all other priority substances were below the EQS values;
- Pesticides could be identified only in very low concentrations although – in comparison to JDS1 to JDS3 – the sampling period at the beginning of July was much closer to the normal application period;
- 4,4-DDT and its derivatives and HCH isomers are still present in the surface water in low concentration probably as a result of re-solution processes from sediment burdens;
- For 6 out of suggested 16 Danube River Basin specific pollutants – diazinon, carbamazepine, metolachlor, metazachlor, terbuthylazine and desethylterbuthylazine – the maximum values exceeded the lowest PNEC according to the SOLUTIONS project proposal list – thus confirming the relevance of the prioritisation process for the Danube Basin;
- 10 Watch List substances were analysed showing few exceedances of the defined PNEC values for diclofenac, 17-beta-estradiol and imidacloprid. As the limit of quantification for 17-alpha-ethinylestradiol was a hundred times higher than the PNEC, this substance with very high estrogenic effects to the aquatic environment could not be reasonably checked;
- 1,4-dioxane – analysed for the first time within a Danube survey – was present at 31 sampling sites with a maximum concentration of 0.53 µg/L. This maximum is far below proposed PNEC values or national guidance values;
- 14 organophosphate flame retardants were analysed in ultra-trace concentrations. The highest concentrations were found for TCPP, TEP and TiBP. The values for 2-ethylhexyl diphenylphosphate at six sampling sites exceeded slightly the lowest PNEC derived for the NORMAN ECOTOX database, whereas all other concentrations were well below the PNEC values.

23.5 References

Umlauf G., Suurkuusk G., Mariani G., Tavazzi S., Parachhini B., Joint Danube Survey 3, 2015, ISBN 978-3-200-03795-3, Chapter 24 "Organophosphorus compounds (OCPs) in surface waters of the Danube und selected tributaries"

NORMAN ECOTOX database, https://www.norman-network.com/nds/ecotox/lowestPnecsIndex.php, visited 01.07.2020

24 Target analysis of organic substances and metals in biota

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Abstract

Directive 2013/39/EU lists EQS in biota for 11 compounds. 9 of these compounds were analysed during JDS4 – in fish at 44 sites, and in mussels at 26 sites. Hexachlorobenzene and hexchlorobutadiene were not analysed, as compounds data from JDS2 and JDS3 did not show an exceedance in fish muscle and liver samples for either.

The results of the monitoring show a quite satisfactory picture for most of the parameters. For the parameters dicofol, HCBDD, PFOS and benzo(a)pyrene all sites show concentrations below the EQS. For dioxins and dioxin-like compounds, heptachlor and fluoranthene only at single sites are concentrations higher than the biota EQS.

The results for mercury and BDE are different with all sites showing concentrations higher than the EQS. Exceedance for these parameters are reported from many countries. Both compounds are considered as ubiquitous persistent, bioaccumulative and toxic substances (uPBTs). Whether the existing mitigation measures for these compounds are effective has to be shown in future monitoring programs.

Additional results for 20 metals not regulated Europe-wide are given.

24.1 Introduction

The Water Framework Directive requires EU member states to monitor priority substances for the assessment of the chemical status. The assessment is based on the Environmental Quality Standards (EQS) from Directive 2008/105/EC in the revision of Directive 2013/39/EU. In light of the protection of predators from risk of secondary poisoning, the protection of human health via the consumption of fish and analytical reasons, Environmental Quality Standards (EQS) in biota were also included. Directive EQS 2008/105/EC includes biota standards for 3 compounds. With Directive 2013/39/EU biota standards become increasingly important and biota standards for 8 additional compounds were implemented.

For all of these substances periodic monitoring data for a long-term trend analysis are necessary.

According to Directive 2013/39/EU, 7 of these 11 substances behave like ubiquitous persistent, bioaccumulative and toxic substances (u PBTs), the chemical status can be reported in separate maps.

In the past Joint Danube Surveys biota samples were only partly analysed. Via the Trans National Monitoring Network (TNMN), biota data have been available for mercury since 2014/2015 (ICPDR, 2018). To obtain a comparable data set for the whole Danube and the major tributaries, in JDS4 most of the compounds with biota standards were analysed at almost all sites. Problems with the availability of mussels at some sampling sites led to a reduced number of results for fluoranthene and benzo(a)pyrene.

Hexachlorobenzene and hexchlorobutadiene were not analysed as for these compounds data from JDS2 and JDS3 never showed an exceedance in fish muscle and liver samples.

An overview of all analysed compounds and additional information regarding the EQS, the protection goal and the analysed tissue/organism is shown in Table 1.

Table 1: Overview of the analysed priority substances with EQS in biota.

	EQS* µg/kg.ww	Protection goal ***,****	Ubiquitous PBT	Analysed in	Number of sites
Mercury	20	sec pois	x	whole fish	44
Brominated diphenylethers (BDE)*****	0.0085	hh	x	whole fish	44
Perfluorooctane sulfonic acid and its derivates (PFOS)	9.1	hh	x	whole fish	44
Dioxins and dioxin-like compounds (Sum of PCDD+PCDF+ PCB-DL)******	0.0065 µg/kgTEQ**	hh	x	whole fish	44
Hexabromocyclododecane (HBCDD)	167	sec pois	x	whole fish	44
Dicofol	33	sec pois		whole fish	44
Heptachlor and heptachlorepoxide	0.0067	hh	x	whole fish	44
Fluoranthene	30	hh		mussels	26
Benzo(a)pyrene	5	hh	x	mussels	26

** Directive 2013/39/EU as µg/kg wet weight*
*** Toxic equivalents according to WHO 2005 Toxic Equivalence Factors*
**** sec pois: Protection of predators from risk of secondary poisoning*
***** hh – human health: Protection of humans from adverse effects resulting from food-consumption*
****** EQS refers to the technical mixture of pentabromodiphenylehters, characterised by the sum of the concentrations of the congeners number 28, 47, 99, 100, 153 and 154*
******* EQS refers to 7 PCDDs, 10 PCDFs and 12 PCB-DL*

In addition, 20 metals were analysed in fish samples. These data allow a first overview of the distribution of not EU wide regulated metals.

24.2 Methods

24.2.1 Biota

The tendency of pollutants to accumulate in fish and mussels is influenced by various factors. Age, feeding and habitat preferences of the different species as well as the fate (depuration or transformation) of the chemical of interest can lead to differences in the detected concentrations for the same sites. In order to obtain comparable data for a longitudinal comparison in JDS4, the use of the same species and a normalisation of the measured concentrations was aspired. Based on the recommendations of Guidance Document No. 32 on Biota Monitoring (EU, 2014) substances that accumulate in lipids of organisms (e.g. dioxins) were normalised to 5% lipid content. Substances that do not accumulate in lipids but via other mechanism of accumulation (e.g. mercury, PFOS) were normalised to 26% dry weight.

Table 2 gives an overview of the analysed species at the different sites and the concentration ranges for dry matter and lipid content.

Table 2: Description of analysed biota-samples.

Species	Number of sites	JDS4 – site number	Samples from	Number of fish/ site	Dry matter (%)			Lipid content in % ww			Trophic level (based on fishbase)*
					min	max	mean	min	max	mean	
Fish											
Alburnus alburnus (bleak)	34	1,2,4,6,8,10-12,17, 18,22-41,43,47-49	Danube, Tributary	23-38	23.7	33.7	28.3	4	17	9.2	2.7 +- 0.29
Leuciscus aspius (asp)	4	14-16,22	Danube	1-3	21.6	26.2	24.3	1.8	4.7	3.2	4.5+- 0.8
Leuciscus cephalus (chub)	7	13,19-21,44-46	Tributary	3-10	24.8	29.8	26.8	3.9	9.5	5.6	2.7 +- 0.1
Mussel											
Corbicula sp., Unio sp.	26	6,11,12,15-17,19, 21,22,24,27,29, 31-33,35-41,47,48			7.4	22.2	15.7				

**www.fishbase.org*

Bleak were analysed at 77% of the sites, the lipid content of the different fish samples varies between 4-17%. Chub was analysed in only 7 tributaries, the lipid content varies between 3.9 - 9.5%. Asp was analysed at 4 sites; the lipid content varies between 1.8-4.7%.

The position of the organism in the food web is described by the trophic level (TL) and can vary for each species, between and within ecosystems. Published values (www.fishbase.org) for bleak and chub show quite similar trophic levels, whereas the carnivorous asp, which feeds mainly on fish, especially on bleak, has a TL of approx. 4.5.

The moisture content of the analysed mussels varies between 78.8 – 92.6%.

24.2.2 Chemical analysis

Sample preparation and chemical analysis was performed by Water Research Institute, Bratislava; National Laboratory of Health, Environment and Food, Maribor; Bavarian Environment Agency, Augsburg; Environment Agency, Vienna.

Lipid content (in % of wet weight)

For the gravimetric fat estimation, the lyophilised samples were extracted with toluene/ethanol (1/2).

Mercury

Atomic adsorption method after thermal decomposition – based on EPA 7473.

BDE

BDE (congener numbers 28, 47, 99, 100, 153 and 154) were analysed according to EPA 1614 but were detected by GC-MS/MS.

Perfluorooctane sulfonate

Perfluorooctane sulfonate was detected using LC-MS/MS following extraction with acetonitrile and cleaning with graphitized carbon adsorbent. The samples were spiked with isotope labelled standards prior to extraction.

Dioxins and dioxin-like compounds

Method complies with US EPA 1613 and EPA 1668 methods.

After extraction with a mixture of dichlormethane-hexane (1:1, v/v) and clean up, the samples were fractionated using Florisil columns. The final extract is spiked with syringe internal standard and injected to HRGC-HRMS instrument.

HBCDD

The method used for analysis of HBCDD was extraction with acetonitril after spiking with isotope labelled standards and clean up with dispersive SPE (QuEChERS). For determination LC-MS/MS was used.

Dicofol

Dicofol was analysed by GC-MS/MS based on „Amtliche Sammlung von Untersuchungsverfahren nach §64 LFGB L 00.00-115". Dicofol-d8 was used as an injection standard.

Heptachlor and heptachlor epoxide

Heptachlor and heptachlor epoxide were analysed according to DIN ISO 10382. For determination GC-MS/MS was used. 13C labelled heptachlor and heptachlorepoxide were used as surrogates.

Fluoranthene and benzo(a)pyrene

In-house method with dispersive solid phase extraction (d-SPE) using octadecyl bonded silica (C18).

Metals

ICP-MS according DIN EN ISO 17294-2:2017-01 after microwave-assisted digestion.

24.3 Results

24.3.1 Mercury

Mercury was detected in all fish-samples (LOQ 10 µg/kg.ww). The concentrations vary between 18 – 1300 µg/kg.ww, after normalisation (on 26% dry weight) between 16 – 1566 µg/kg ww. Additional results from a second laboratory with normalised concentration ranges between 27 – 1320 µg/kg.ww confirm these results.

The results for all sites are shown in Figure 1.

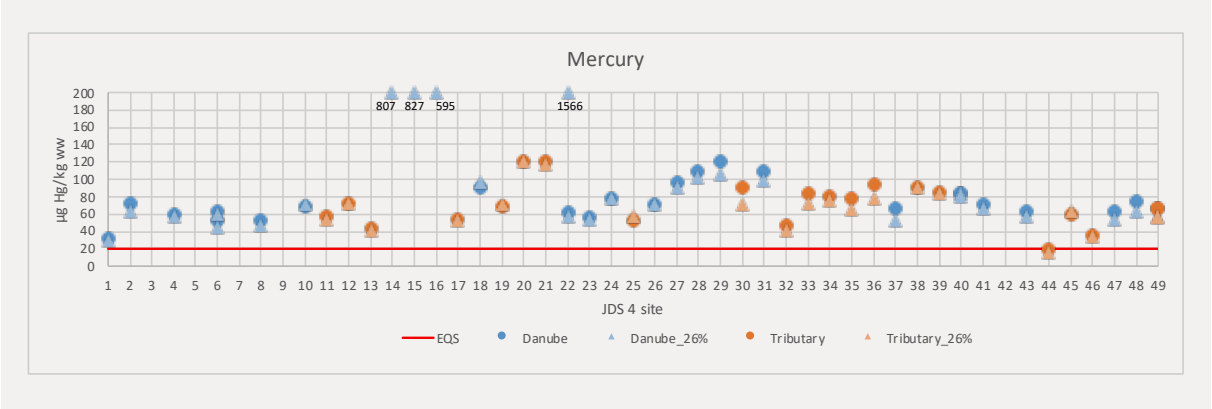

Figure 1: Mercury in fish in the Danube River and tributaries during JDS4 (concentration values in µg/kg.wet weight – measured values and normalised to 26% dry weight content).

The Biota-EQS is 20 µg/kg.ww. Most of the 44 sites show concentrations higher than the EQS. Only one site (JDS 4-44, Iskar mouth) shows concentrations below or slightly higher than the EQS (18 respectively 27 µg/kg.ww). The observed ubiquitous exceedances in biota are reported from many European countries (EEA, 2018) and highlighted in the DRBMP-Update 2015 (ICPDR, 2015).

The concentration at all sites, where leak or chub were analysed, are in the range of 18-120 µg/kg.ww and are comparable with the results from the TNMN (ICPDR, 2018). The 37 TNMN sites (whole fish of comparable trophic level) show concentrations in the range of 15 – 200 µg/kg.ww. In JDS 3 mercury was analysed in muscles samples from Abramis brama (trophic level comparable to leak and chub) only at six sites, the results ranged between 210 and 440 µg/kg.ww. A recalculation from the concentrations in muscle to whole fish by using a factor of 0.7 (Fliedner et al., 2018) results in concentration for Abramis (total fish) between 147 and 308 µg/kg.ww. Whether the lower values in JDS4 are a result of substantial reduction or only based on the variability of biological data has to be ascertained by future monitoring programs.

The influence of species selection and the aspect of biomagnification might be illustrated by the results of sites JDS4-14-16 and especially JDS4-22, where considerably higher concentrations were found. The analysed fish species (asp) is mainly carnivorous and therefore on a higher trophic level. Additional results for bleak at site JDS4-22 show significant lower concentrations, similar to the adjacent sites. Other factors (especially age) may also play a role.

24.3.2 Brominated diphenylethers (BDE)

BDE were used as flame-retardants in different kinds of plastic. The technical mixtures of penta- and octabromodiphenylethers have been prohibited since May 2009 under the regulations of the Stockholm Convention.

The EQS for biota (fish) refers to the technical mixture of pentabromodiphenylethers, characterised by the 6 congener numbers BDE 28, BDE 47, BDE 99, BDE 100, BDE 153 and BDE 154.

In JDS4, most of the 6 congeners could be quantified at all sites. The highest concentrations were found for congener 47 (concentration range 0.09 – 4.8 µg/kg.ww), the lowest for congener 99 (< 0.015-0.14 µg/kg.ww). All three analysed fish species (bleak, asp and chub) show mostly the same distribution pattern of congeners.

The concentration for the sum of BDE varies between 0.17 – 7.24 µg/kg.ww.

The results for all sites are shown in Figure 2. For increasing the longitudinal comparability, the data were normalised to a lipid content of 5%.

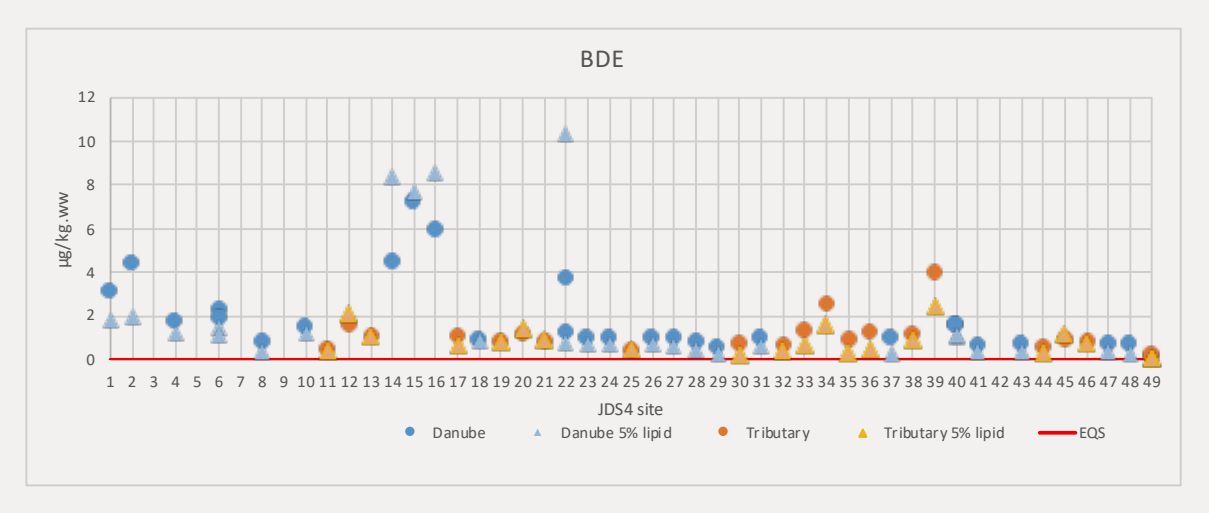

Figure 2: BDE in fish in the Danube River and tributaries during JDS4 (concentration values in µg/kg.wet weight - measured values and normalised to 5% lipid content in fish).

The Biota-EQS is 0,0085 µg/kg.ww. All 44 sites show concentrations higher than the EQS with a minimum exceedance ratio of approx. 20. The observed exceedances in biota are reported from many European countries.

A longitudinal comparison of the normalised concentrations (5% lipid content) of all sites where leak or chub were analysed, shows slightly higher concentrations in the upper part of the Danube. In the tributaries, the highest concentration was found at JDS4-39 (Velika Morava).

The high concentrations at JDS4-14-16 and especially JDS4-22 might be mainly related to the difference in the analysed fish species (asp). Additional results for bleak at site JDS4-22 show significant lower concentrations, similar to the adjacent sites.

During JDS3 only BDE-209 was analysed, a comparison is therefore not possible. Monitoring results from fish filet of the Elbe (2018) shows concentration ranges between 0,06-1.7 µg/kg.ww

24.3.3 Perfluorooctane sulfonic acid and its derivates (PFOS)

Perfluorooctanesulfonic acid was used as a component of fire-fighting foams, in galvanic baths, in some impregnation agents for textiles, paper, and leather; in photolithographic chemicals and in hydraulic fluids. PFOS has been prohibited since May 2009 under the regulations of the Stockholm Convention.

PFOS was detected in fish-samples at 39 out of 44 sites (LOQ 2 µg/kg.ww). The concentrations vary between < 1 – 8 µg/kg.ww, after normalisation (on 26% dry weight) between < 1 – 6,9 µg/kg ww

The results for all sites are shown in Figure 3. Concentrations lower than LOQ are shown with LOQ/2 and lower LOD as zero. To increase longitudinal comparability, the data were normalised to a dry weight content of 26%.

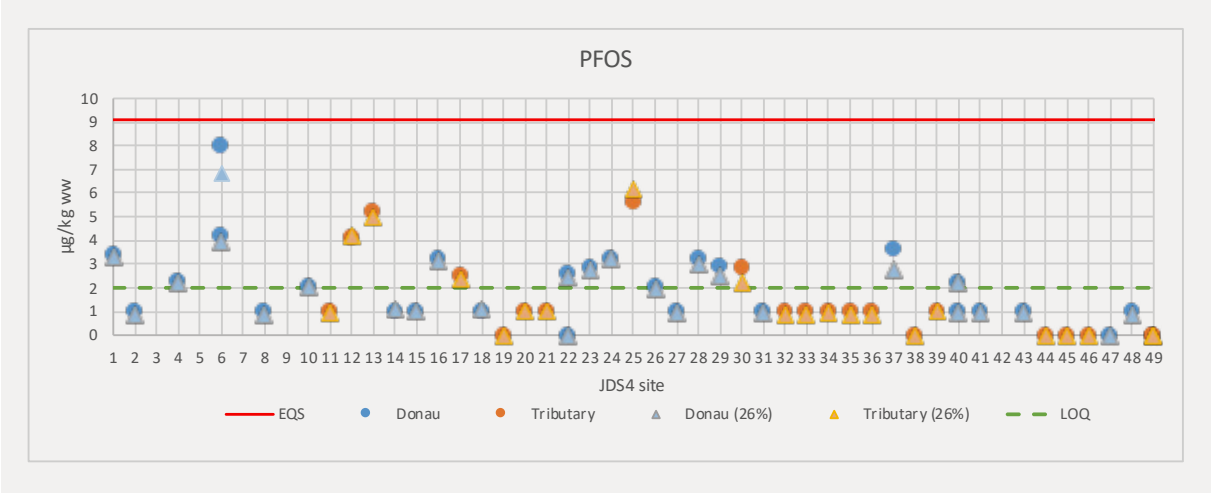

Figure 3: PFOS in fish in the Danube River and tributaries during JDS4 (concentration values in µg/kg.wet weight – measured values and normalised to 26% dry weight).

The biota EQS is 9,1 µg/kg.ww. All sites show concentrations lower than the EQS.

A longitudinal comparison of the normalised concentrations (26% dry weight) of all sites where leak or chub were analysed, shows slightly higher concentrations in the upper and middle part of the Danube. The highest concentration was found at JDS4-6.

The influence of the analysed fish species is not clearly visible as for the other parameters, the normalised concentrations of the analysed asp at sites JDS4-14-16 and JDS4-22 are similar to the adjacent sites.

In JDS4 PFOS was also analysed at all sites in water samples (see Chapter 29). These results show a different picture concerning assessment and longitudinal distribution. Most of the sites show concentrations higher than the water EQS (0.00065 µg/l). This observation highlights, that biota and water EQS do not represent the same level of protection, but the water EQS is more protective than the biota EQS. This might be due to the fact, that the water EQS was derived from the biota EQS using "worst case" bioaccumulation and biomagnification factors. The observed higher concentrations in biota in the upper part of the Danube, which reflect more the concentration pattern in water in JDS3 (2013) than in JDS4 (2019), point to the more time integrated way of sampling by using biota.

In JDS2 and JDS3 only for a small number of sites (4) PFOS monitoring results are available from fish liver (one site filet) but not for whole fish. A direct comparison is therefore not possible.

24.3.4 Dioxins and dioxin-like compounds

Dioxins and dioxin-like compounds are by-products of imperfect combustion involving organic matter and chlorine or chemical reactions.

The assessment for "dioxins and dioxin like compounds" refers to a sum of 7 polychlorinated dibenzo-p-dioxins (PCDDs), 10 polychlorinated dibenzofurans (PCDFs) and 12 dioxin-like polychlorinated biphenyls (DL-PCBs). Due to their similar toxicological behaviour but different potencies, the concentrations of the individual compounds/congeners are converted into toxic equivalents (according to WHO 2005 Toxic Equivalence Factors – TEF) and summed up. Single results below LOQ were set to zero.

All compounds/congeners were analysed in fish. The 17 PCDDs and PCDFs were quantified only partly, mainly TCDF and PeCDF (concentration range < LOQ – 0.0013 µg/kgTEQ.ww). The dominating substances are PCB DL, all congeners were quantified in all samples (concentration range 0.0005 – 0.0091 µg/kgTEQ.ww). The sum PCDD/Fs and PCB-DL varies between 0.0006 and 0.010 µg/kgTEQ.ww.

The results for all sites are shown in Figure 4. For increasing the longitudinal comparability, the data were also normalised to a lipid content of 5%.

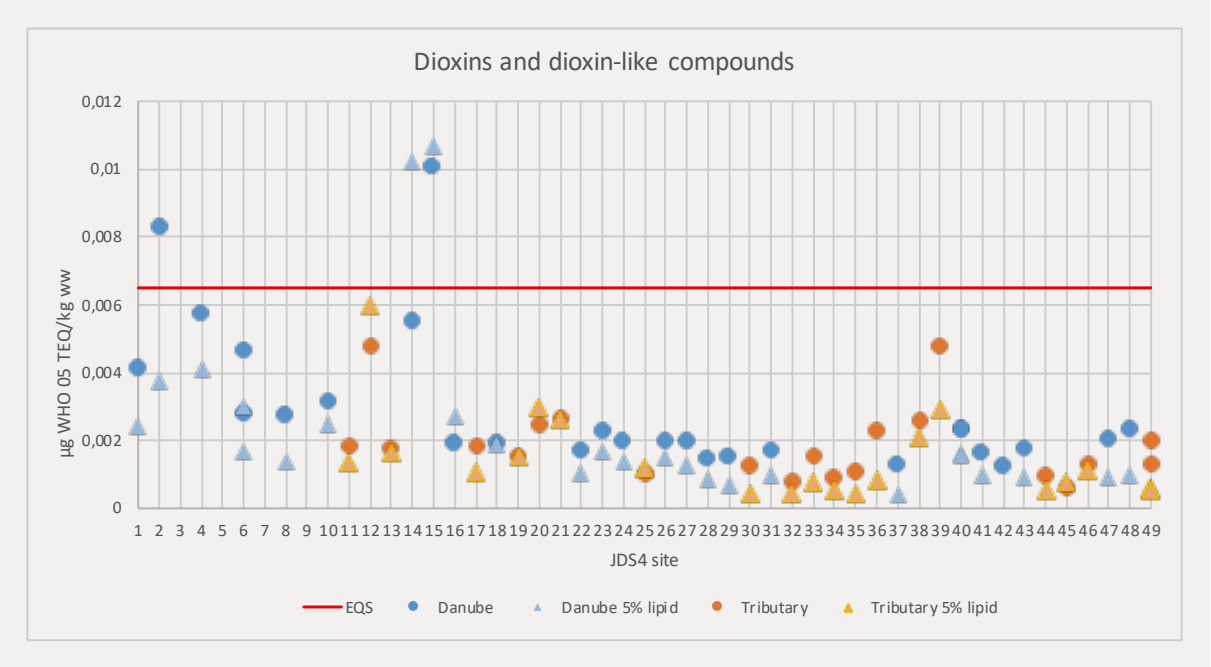

Figure 4: Dioxins and dioxin-like compounds in fish in the Danube River and tributaries during JDS4 (concentration values in µg/kg.wet weight – measured values and normalised to 5% lipid content in fish).

The biota EQS is 0.0065 µg/kgTEQ.ww. Most of the sites show concentrations lower than the EQS. At two sites (JDS4-2 and JDS4-15) higher concentrations were observed. A longitudinal comparison of the normalised concentrations (5% lipid content) shows comparable concentrations with slightly higher concentrations in the upper part of the Danube. The highest concentrations are found at site JDS4-15 (analysed fish asp) and show again the influence of the feeding pattern of the analysed fish species.

In JDS2 and JDS3 only for a small number of sites (5 respectively 7) monitoring results are available from fish (bream) filet, a direct comparison is therefore difficult. In JDS3 the concentration ranges of PCDD/Fs in filet were 0.00019 – 0.0010 µg/kg.ww, for PCB-DL 0.0005-0.0034 µg/kg.ww.

24.3.5 Hexabromocyclododecane (HBCDD)

Hexabromocyclododecane (HBCDD) is used as flame retardant and for thermal insulation in the building industry.

All 44 sites show values below LOD (LOQ for the single congener is 30 µg/kg.ww) and therefore below the EQS (167 µg/kg.ww). The same results were found in JDS3. Fish analysis in biota (bream filet) on 7 sampling sites show values below the LOD and therefore below the EQS.

24.3.6 Dicofol, Heptachlor

Dicofol is an acaricide and used in plant protection products and as a biocide. The usage has been prohibited since 2009 in the European Union, and worldwide since December 2020. Heptachlor was used as an insecticide. The usage and its production have been prohibited since 2004.

Dicofol was not detectable at all 44 sites (analysis in fish) and therefore below the EQS. The LOQ (4 µg/kg.ww) was low enough to assess the biota EQS (33 µg/kg.ww). The same results were found in JDS3 where all 7 sampling sites (analysis in bream filet) showed values below the LOD and therefore below the EQS.

For Heptachlor (and heptachlor epoxide) most of the sites (fish analysis) show values below LOD (LOQ 0.5 µg/kg.ww) with the restriction that for assessing the EQS in biota (0.0067 µg/kg.ww) the required LOQ could not be reached. Detectable concentrations for heptachlor epoxide, and therefore values higher than the EQS, were only found in the tributary Russenski Lom (site JDS4-46).

In JDS3 all 7 sampling sites (analysis in bream filet) showed values below the LOD (LOQ 0.4µg/kg.ww)

24.3.7 Fluoranthene, Benzo(a)pyrene

The polyaromatic hydrocarbons fluoranthene and benzo(a)pyrene are by-products of the combustion of organic compounds.

Fluoranthene and benzo(a)pyrene were analysed in mussels at 26 sites. Detectable concentrations were found for fluoranthene at 12 sites and for benzo(a)pyrene at 2 sites (see Table 3).

Table 3: Fluoranthene and benzo(a)pyrene in mussels.

	LOQ in µg/kg.ww	EQS in biota in µg/kg.ww	number of samples > LOQ	number of samples > EQS	maximum concentration in µg/kg.ww	Sampling site with maximum
Fluoranthene	1.5	30	12	1	34.5	JDS4-12
Benzo(a)pyrene	1.5	5	2	0	3.7	JDS4-12

For fluoranthene most of the sites show concentrations below the biota EQS, only site JDS4-12 (Morava) shows higher concentrations.

For benzo(a)pyrene all sites have concentrations below the biota EQS.

Comparable data from former JDS are not available, but the assessment of the water samples in JDS3 shows quite similar results for fluoranthene. Only one site showed a higher concentration than the water EQS (JDS3-24). The results of benzo(a)pyrene could not be compared with the water EQS (0.00017 µg/l) as the LOQ (0.002 µg/l) was not sufficient, but 95% of the sites showed concentrations below the LOQ.

24.3.8 Metals in fish

Additional to the parameters regulated in Directive 2013/39/EU in JDS4 20 metals were analysed by the Bavarian Environment Agency, Augsburg in the fish samples from all 44 biota sites. 10 metals could be quantified at all sites, further 5 at most of the sites. 2 metals could be quantified in none of the sites. The concentration ranges, median and 90% percentiles are given in table 4.

Table 4: Metals in fish.

		LOQ	Site number > LOQ	Min mg/kg.ww	Median mg/kg.ww	90% Percent. mg/kg.ww	Max mg/kg.ww
Silver	Ag	< 0.01	0	< 0.01	< 0.01	< 0.01	< 0.01
Arsenic	As	< 0.05	42	< 0.05	0.0925	0.133	0.21
Bismuth	Bi	< 0.005	2	< 0.005	< 0.005	< 0.005	0.0054
Cadmium	Cd		44	0.0023	0.011	0.025	0.034
Cobalt	Co		44	0.0053	0.013	0.0316	0.045
Chromium	Cr-total		44	0.069	0.185	0.794	2.1
Copper	Cu		44	0.48	0.855	1.2	1.9
Manganese	Mn		44	0.54	3.3	5.53	8
Molybdenum	Mo		44	0.016	0.035	0.109	0.19
Nickel	Ni		44	0.042	0.13	0.646	0.95
Lead	Pb		43	0.0063	0.0225	0.0633	0.4
Rubidium	Rb		44	0.72	3.25	5.55	7.9
Antimony	Sb	< 0.01	1	< 0.01	< 0.01	< 0.01	0.39
Selenium	Se		44	0.19	0.335	0.5	1.2
Tin	Sn	< 0.01	2	< 0.01	< 0.01	< 0.01	2.5
Thallium	Tl	< 0.02	0	< 0.02	< 0.02	< 0.02	< 0.02
Uranium	U	< 0.001	40	< 0.001	0.0021	0.00456	0.0088
Vanadium	V	< 0.01	38	< 0.01	0.0295	0.0774	0.12
Tungsten	W	< 0.002	38	< 0.002	0.0028	0.00744	0.027
Zinc	Zn		44	10	33	37	44

These data allow a first overview of the distribution of EU non-regulated metals in biota. For some metals, as tin and antimony, high concentrations could be observed only on one site (JDS4-48). A further assessment is difficult as for most metals, biota EQS are not available.

In former JDS, metals were analysed only in water, sediments or suspended matter, a comparison is therefore not possible.

24.4 Conclusions

- During JDS4 9 of 11 substances regulated in Directive 2013/39/EU with EQS in biota were analysed in fish at 44 sites and in mussels at 26 sites. Problems with the availability of mussels at some sampling locations led to the reduced number of sites for mussels. The limit of quantification did meet the requirements (for all compounds except heptachlor).
- The analysed fish species were Alburnus alburnus (bleak) at 34 sites, which supports a longitudinal comparison of the results. Leuciscus cephalus (chub) and Leuciscus aspius (asp) were analysed on 7 and 4 sites respectively. On one site (JDS4-22) results for asp and bleak are available.
- For analysis, the whole fish was used. The aspect, that for most of the compounds higher concentration were found in whole fish than in muscle (filet) might be considered in the further interpretation and assessment of the results. For compounds, where the EQS is mainly derived to protect wildlife from secondary poisoning a direct comparison of the results with the EQS is appropriate. For compounds as PBDE and Dioxins and DL-compounds, where the protection goal is human health from consuming fish products, the risks might be overestimated (see also EU, 2014).
- Mercury:
  Apart from one (JDS4-44) all sites show concentrations higher than the EQS. Notably higher concentrations were observed at the 4 sites were asp was analysed. This fish species (asp) is mainly carnivorous and on a higher trophic level than bleak and chub. Additional results for bleak at site JDS4-22 show significant lower concentrations, similar to the adjacent sites.
- BDE:
  All 44 sites show concentrations higher than the EQS with a minimum exceedance ratio of approx. 20. A longitudinal comparison of the normalised concentrations (5% lipid content) of all sites where bleak or chub were analysed, shows slightly higher concentrations in the upper part of the Danube. In the tributaries, the highest concentration was found in JDS4-39 (Velika Morava). Clearly higher than EQS values were detected for BDE also at those four sites where asp was analysed. Additional results for bleak at site JDS4-22 show significant lower concentrations, similar to the adjacent sites.
- PFOS:
  All sites show concentrations below the biota EQS. A longitudinal comparison of the normalised data (26% dry weight) shows slightly higher concentrations in the upper and middle part of the Danube. The difference of the results for asp to bleak and chub are not as clearly visible as for the other compounds.
- Dioxins and dioxins-like compounds:
  Most of the sites show concentrations lower than the EQS. At two sites (JDS4-2 and JDS4-15) concentrations above the EQS were observed. A longitudinal comparison of the normalised concentrations (5% lipid content) shows comparable concentrations with slightly higher values in the upper part of the Danube. The highest concentration was found at site JDS4-15 (analysed fish, asp).

- HBCDD:
  All sites show concentrations below LOQ und below the EQS.
- Dicofol:
  All sites show concentrations below LOD und below the EQS.
- Heptachlor and heptachlor epoxide:
  Most of the sites show values below LOQ, for assessing the EQS in biota (0.0067 µg/kg.ww) the required LOQ could not be reached. Detectable concentrations for heptachlor epoxide, and therefore values higher than the EQS, were found only in the Russenski Lom tributary (site JDS4-46).
- Fluoranthene, benzo(a)pyrene:
  For fluoranthene most of the sites show concentrations below the biota EQS. Only site JDS4-12 (Morava) shows higher concentrations.
  For benzo(a)pyrene all sites show concentrations below the biota EQS.
- Except fluoranthene, all parameters exceeding the EQS are ubiquitous persistent, bioaccumulative and toxic substances (uPBTs) according to Directive 2013/39/EU. They can be found for decades even if mitigation measures were taken and some of them are subjected to long-term transport. Whether the existing mitigation measures are successful and effective has to be proven in future monitoring programs.

24.5 References

EEA (2018): Chemicals in European waters. European Environmental Agency – Report 18/2018.

EU (2014): Common Implementation Strategy for the Water Framework Directive (2000/60/EC). Guidance Document No. 32 on Biota Monitoring

FGG ELBE (2018): Schadstoffuntersuchungen in Biota

FLIEDNER A., RÜDEL H., LOHMANN N., BUCHMEIER G., KOSCHORRECK J. (2018): Biota monitoring under the Water Framework Directive: On tissue choice and fish species selection. Environmental Pollution 235:129-140.

ICPDR (2015): Danube River Basin District Management Plan – Update 2015 (http://icpdr.org/main/activities-projects/river-basin-management-plan-update-2015)

ICPDR (2018): TNMN – Yearbook 2016 (http://icpdr.org/main/publications/tnmn-yearbooks)

25 Groundwater screening

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Abstract

Seven groundwater monitoring sites (GW-sites) along the Danube River were sampled and the results were compared to the concentrations detected at the closest Danube sites to identify any kind of interaction. The seven GW-sites are supposed to be more or less interconnected with the water from the Danube River through bank-filtration.

In total 286 pesticide substances, pharmaceuticals, drugs, artificial sweeteners, industrial substances, isotopes, dissolved organic matter and rare earth elements, which are usually not monitored within standard monitoring programs, were detected in either groundwater or in a Danube monitoring site closest to a monitored GW-site.

The analysis showed that in many cases the bank-filtration process contributes to a smaller number of substances and lower concentrations being detected in groundwater than in the Danube River. Nevertheless, this effect cannot be generalised and is compound- and site-specific. For many of the detected substances the situation is opposite and the concentration in groundwater is often higher than in the Danube. Even so, a considerable number of substances (23%) were only detected in a groundwater site and not found in any of the adjacent Danube sites, which indicates that pollution of groundwater is being caused by local or regional polluting activities.

A broad range of chemical substances is widely used in industrial, medical and agricultural activities and thus many of those compounds were also present in the groundwater samples. The goal of JDS4 was to perform a comprehensive target and nontarget screening of substances which are normally not analysed during ongoing monitoring at the most extreme limits of detection and quantification. Hence, a lot of substances were detected, but it should be kept in mind, that most of the findings are at a concentration range of few ng/L (=0.001 µg/L) or even pg/L (=0.001 ng/L). This is owed to the extreme sensitivity of detection of the applied methods – 1 pg/L = 1/2 sugar cube (= 2 g) in Lake Balaton with 1.9 km³ (1.9 trillion L) of water – which means that many of these substances would have never been detected with standard laboratory methods and their concentrations are far below any currently existing European quality standard. Nevertheless, it has to be considered that certain substances have adverse (e.g. endocrine) effects at such low concentration levels.

25.1 Introduction

Seven members of the ICPDR Groundwater Task Group (GW TG) nominated one groundwater site to be sampled during JDS4. The selection criteria were: 1. Danube bank-filtered water aquifers monitored during JDS3; 2. Other Danube bank-filtered water aquifers; 3. Other relevant aquifers (relevant from the perspective of the nominating country).

For each of the seven selected JDS4 groundwater sites (GW-sites) the closest Danube site was considered in the summary and interpretation of the results. Whenever this chapter refers to 'Danube sites', only the listed seven Danube sites adjacent to the seven GW-sites are meant. Table 1 gives an overview of the considered sites, their codes, their names and the distance between the GW-site and the corresponding Danube site.

Table 1: JDS4 GW-sites and JDS4 Danube sites considered in the interpretation of the groundwater data.

country	JDS4 GW-site		Closest JDS4 Danube site			Approximate distance between GW and Danube sampling sites
	Code	Name	Code	Name	Danube km	
AT	GW1	Vienna	9	Klosterneuburg	km 1942	GW1 ~20 km downstream 9
SK	GW2	Šamorín - Kalinkovo	15	Čunovo, Gabčíkovo reservoir	km 1855	GW2 ~1 km downstream 15
HU	GW3	Surány 6. radial well	23	Budapest upstream - Megyeri Bridge	km 1660	GW3 ~12.5 km upstream 23
HR	GW4	Topolje (26741)	29	Hercegszántó / Batina / Bezdan (HU/HR/RS)	km 1434	GW4 ~6.5 km inland 29
RS	GW5	Novi Sad	31	Ilok / Bačka Palanka (HR/RS)	km 1300	GW5 ~41 km downstream 31
RO	GW6	Drill F1, Slobozia, Giurgiu County	46	Ruse (BG)	km 494	GW6 ~3 km inland 46
BG	GW7	Slivo pole, shaft well P8 - pumping station	47	Downstream Ruse/ Giurgiu (Marten) (BG/RO)	km 488	GW7 ~13 km downstream 47

25.2 Methods

Groundwater sampling was done by national sampling teams between 8 and 30 July 2019. Beforehand, the national teams had received a box with cooling elements, eleven bottles (from 20 mL to 10 L), two cellulose filters (0.45 µm), one nylon filter (0.45 µm), one Leuer tip syringe, one vial with nitric acid, one with sulfonic acid and clear instructions.

The groundwater samples were grabbed or pumped below the water surface (about 20-50 cm) and all parameters were taken from the same water fraction (same bucket). Bottles and syringes were rinsed with sample water (if applicable filtered water). The samples for the stable isotopes of nitrate were filtered with nylon filters and acidified with 1 mL sulfonic acid, the samples for anions were filtered with cellulose filters and the samples for the cations were filtered with cellulose filters and acidified with 5 drops of nitric acid.

In addition, in-situ physico-chemical data (pH, temperature, O_2, electrical conductivity) and discharge were measured.

For all other parameters, the bottles were filled up to the top (no headspace) without filtering or preservation. All samples were stored at cool or at room temperatures (no freezing!). After sampling, the boxes with the cooled bottles were sent to WRI by courier for further distribution to the responsible laboratories.

The groundwater samples were analysed by the International Atomic Energy Agency (IAEA), Joint Research Centre (JRC), Bavarian Environment Agency (LfU), University of Athens (UoA), Université de Lorraine (UoL) and Helmholtz Centre for Environmental Research GmbH (UFZ). The samples for the basic parameters were taken according to the national procedures and analysed by national laboratories. The methods of sample preparation and analyses in the laboratories are precisely described in the respective chapters dealing with the interpretation of results in the Danube. This concerns Chapter 23 for the target analysis of organic substances in water, Chapter 27 on wide-scope target and non-target screening, Chapter 29 on wide-scope target and suspect screening of emerging substances, Chapter 30 on drugs, antibiotics and their metabolites, Chapter 40 on rare earth elements, Chapter 41 on dissolved organic matter and Chapter 46 on stable isotopes of water and nitrate.

Data on organic substances were taken from the above-mentioned chapters for the selected Danube sites to compare them with the groundwater data. Nevertheless, data on organic substances in surface water are also presented in other chapters.

25.3 Results and discussion

In total 286 pesticide substances, pharmaceuticals, drugs, artificial sweeteners, industrial substances, isotopes, dissolved organic matter and rare earth elements which are usually not monitored within standard monitoring programmes were detected in either groundwater or in a Danube monitoring site closest to a monitored GW-site (see Table 1).

- 92 substances (32%) were found in both a GW-site and a corresponding Danube site. No general pattern could be identified concerning a relationship between the concentrations in groundwater and in the Danube. For 18 substances all 51 measured concentrations in groundwater were above the concentrations in the corresponding Danube sites, for 23 substances the opposite was the case for 74 detections. For the remaining 51 substances, concentrations in groundwater were both higher and lower than in a corresponding Danube-site – in 116 monitoring data pairs the groundwater concentration was higher and in 125 cases the concentration in groundwater was lower than in surface water.
- 118 substances (41%) were only found in an adjacent Danube site but not in any of the seven GW-sites and this shows that the filtering effect between the Danube and the groundwater site is quite effective.
- 66 substances (23%) were only found in groundwater and not in any of the seven adjacent Danube sites, which indicates that the pollution of groundwater is due to local or regional activities apart from the Danube.
- Finally, 10 substances (4%) were either found in a GW-site or in a Danube site but not in monitoring site pairs.

The groups of substances are described and discussed in closer detail within the following sub chapters. When comparing the detections in groundwater and in the Danube, it has to be considered, that the distances between some corresponding monitoring sites (see Table 1) are significant and it cannot be excluded that there are discharges of polluting substances into the Danube in between.

25.3.1 Water stable isotopes and isotopes of nitrate in groundwater

In total six groundwater samples were analysed for water stable isotopes and isotopes of nitrate. Water stable isotope compositions in groundwater (see Figure 1) had a range of -11.3 to -8.4 ‰ for $\delta^{18}O$ and -81.2 to -60.1 ‰ for $\delta^{2}H$. All groundwater samples had more positive δ-values than average Danube River water measured within the section of the same country during JDS4. This indicates that the groundwater was not recharged with Danube River water during the period of sampling and is rather recharged by precipitation. An exception is GW5, where the isotopic compositions of groundwater and Danube River water were similar. GW7 also had isotopic compositions relatively close to the Danube River water. GW1 and GW4 showed a clear shift to the right on the Global Meteoric Water Line, indicating that the water went through an important evaporation process (e.g. in the soil column or land surface).

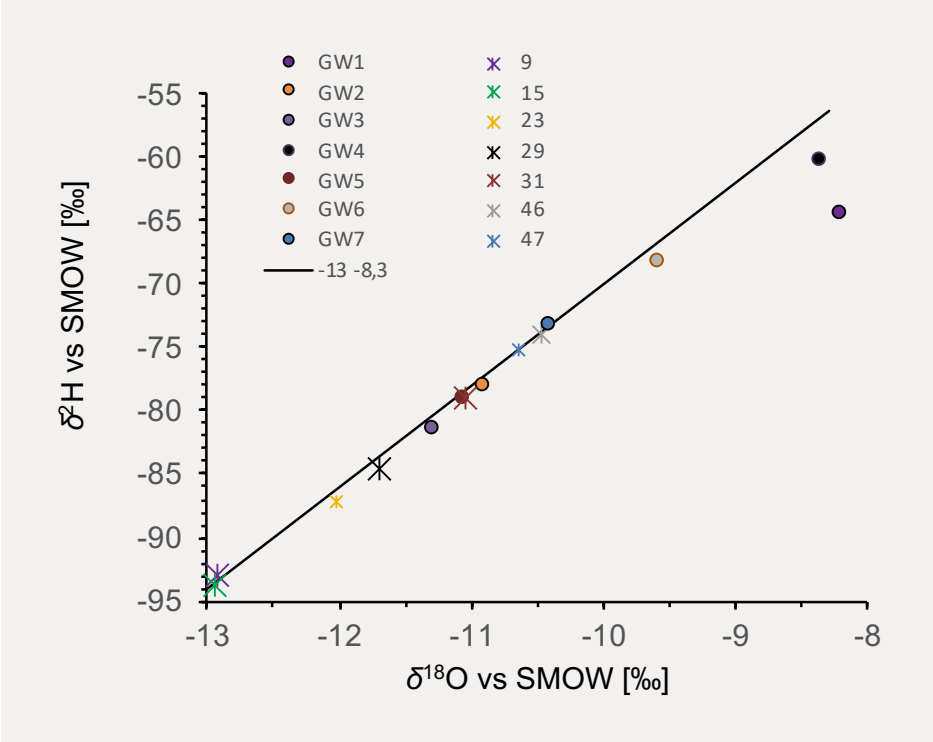

Figure 1: Water stable isotpes in groundwater and in the adjacent Danube sites.

The isotopes of nitrate had a range of 8.4 to 17.9 ‰ for $\delta^{15}N\text{-}NO_3$ and 1.8 to 11.1 ‰ for $\delta^{18}O\text{-}NO_3$. The isotopic compositions were not measured for the site pair 29/GW4 and 31/GW5, as the nitrate concentration in the samples was too low. The analyzed groundwater samples had higher $\delta^{15}N\text{-}NO_3$ values than common for natural soil derived NO_3 and nitrate may be added by wastewater or manure, however not mineral fertilizers. The relatively high δ-values and low NO_3^- concentrations in GW1 indicate important denitrification processes.

25.3.2 Pesticides

A total of 91 individual pesticide active substances or metabolites and 2 groups of substances were quantified in groundwater and/or in an adjacent Danube site:

- 50 individual substances and 2 groups of substances in groundwater,
  - 27 substances and 2 groups in sampling site pairs, in a GW-site and an adjacent Danube site,
  - 7 substances in a GW-site or in an adjacent Danube site, but not in sampling site pairs, and
  - 16 substances were found in groundwater only and not in any adjacent Danube site,
- 41 substances were only found in an adjacent Danube site, but not in groundwater.

Quality standards for pesticides and metabolites are given in the EU Groundwater Directive with 0.1 µg/L for individual pesticide substances and relevant metabolites and 0.5 µg/L for the total concentration of all quantified pesticide substances and relevant metabolites. The EU Drinking Water Directive lists the same drinking water standards (parametric values) but as maximum permissible values.

Figure 2 depicts an overview of the sum concentrations and the number of detected pesticide substances and metabolites per monitoring site (GW-site and adjacent Danube site). By far the highest sum concentration of 1.99 µg/L is recorded at Danube site 46. The sum concentration at corresponding GW6 is significantly lower (0.24 µg/L), but still higher than at the upstream GW-sites. The highest sum concentration in groundwater was recorded at GW7 with 0.33 µg/L, with remarkable concentrations for the insecticide cyromazine (0.07 µg/L) and the metabolite chloridazon-desphenyl (0.09 µg/L).

The GW-site with the most pesticide substances >LOQ was GW2 with 26 quantified detections, but all of the substances were found in very low concentrations.

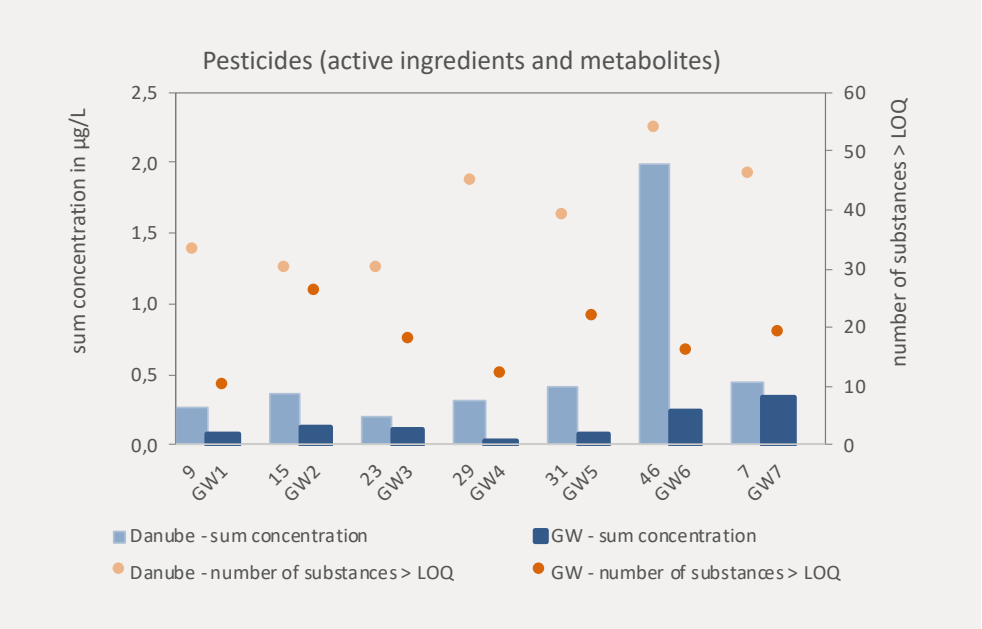

Figure 2: Sum concentration (in µg/L) and number of substances > LOQ for the corresponding monitoring sites Danube/groundwater.

Individual pesticide concentrations in groundwater were generally lower than 0.03 µg/L, except chloridazon-desphenyl (0.09 µg/L), cyromazine (0.07 µg/L), atrazine-2-hydroxy (0.04 µg/L) and metolachlor ESA (0.04 µg/L).

The legacy herbicide atrazine and the metabolite metolachlor ESA were quantified at all seven GW-sites and adjacent Danube sites. Various triazine herbicides and their metabolites were also frequently found in groundwater and in the Danube, such as atrazine-2-hydroxy, atrazine-desethyl, simazine and terbuthylazine-2-hydroxy.

Pyrethrin I was found at all GW-sites in extremely low concentrations of 0.0005 to 0.002 µg/L, but not in the Danube. Cyromazine was found at six GW-sites, but not in the Danube.

Figure 3 presents the detected concentrations of the most frequently found pesticide substances and metabolites in groundwater. It should be considered, that the approval of atrazine at EU level expired in 2004, but the substance is very persistent, and the approval of cyromazine and chloridazon also expired but the substances still can be used up.

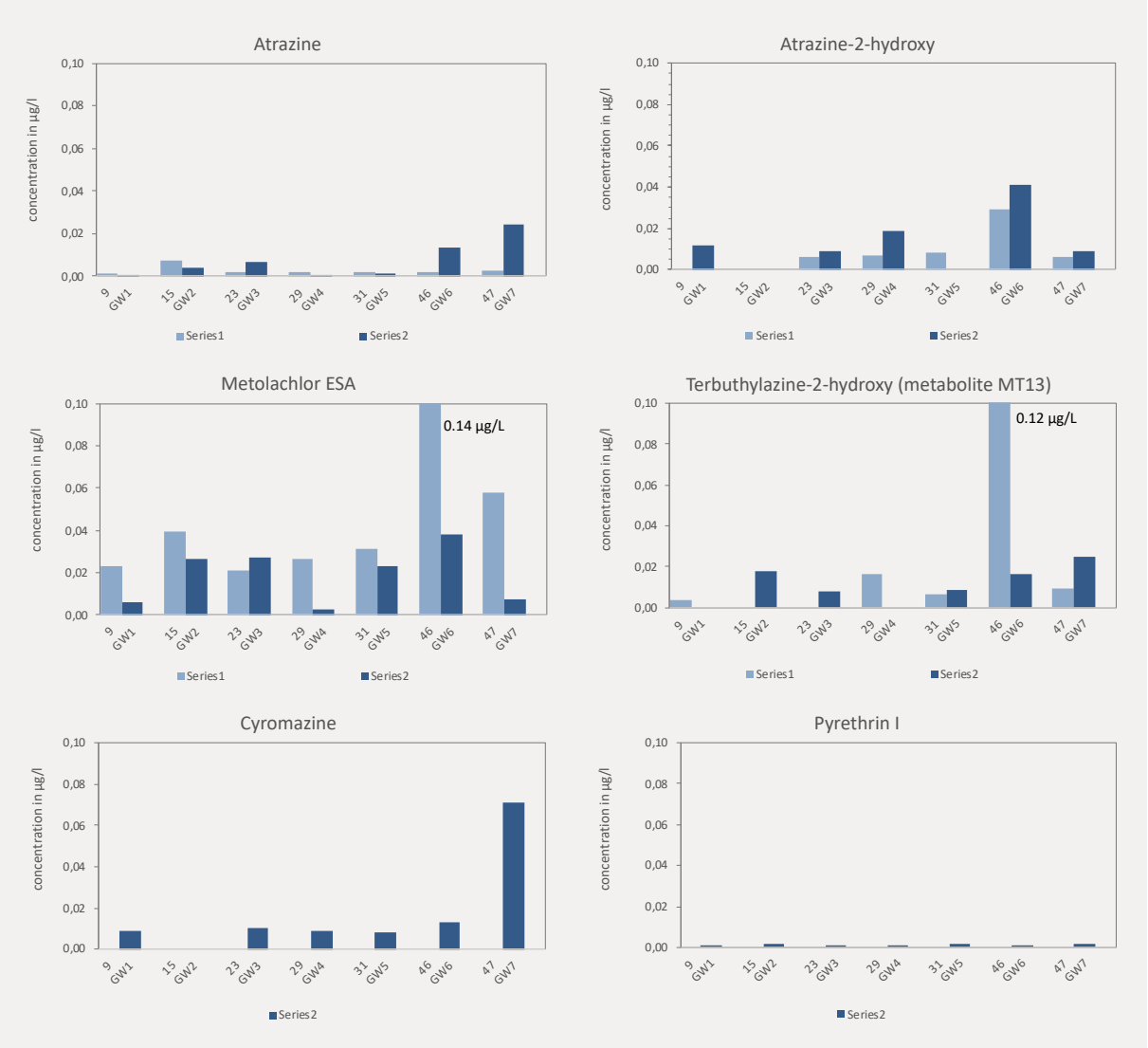

Figure 3: Concentrations (in µg/L) of most frequently found pesticides and metabolites in groundwater and in the corresponding Danube sites.

For most pesticides, concentrations in groundwater tend to be lower than in the Danube, e.g. for metolachlor ESA. However, there are exceptions: atrazine concentrations were much higher in groundwater than in the corresponding Danube sites 23, 46 and 47 and also for atrazine-2-hydroxy, when found in groundwater, its concentration is higher than in the Danube.

Also, substances such as hexachlorocyclohexane (HCH), not in use since 2008, and DDT along with some of its related compounds, banned since 1983, were found in groundwater and in the Danube, though concentrations usually did not exceed 0.0003 µg/L. At GW2, all HCH compounds were quantified in concentrations up to 0.005 µg/L, whereas the Danube showed much less pollution by HCH.

The results indicate two effects that influence pesticide concentrations in the investigated GW-sites. On the one hand, substances are retained in subsoil during bank filtration, so that the concentrations in groundwater are correspondingly lower than in the Danube. However, it can be seen that not all substances are eliminated during subsoil passage. On the other hand, at some GW-sites the pollution by pesticide substances and metabolites seems to be influenced locally or regionally from groundwater moving towards the Danube. This is particularly evident with regard to those pesticide substances which are no longer in use.

Finally, none of the detected pesticide concentrations in groundwater, neither for individual substances nor for their sum, exceeded a quality standard set under the EU Groundwater and the EU Drinking Water Directive.

25.3.3 Pharmaceuticals

A total of 83 active pharmaceutical ingredients or metabolites were quantified in groundwater and/or in adjacent Danube sites:

- 38 substances in groundwater,
  - 15 substances in sampling site pairs – in a GW-site and in an adjacent Danube site,
  - 2 substances in a GW-site or in an adjacent Danube site, but not in sampling site pairs, and
  - 21 substances were found in groundwater only and not in any adjacent Danube site,
- 45 substances were only found in an adjacent Danube site, but not in groundwater.

Figure 4 gives an overview of the sum concentrations and the number of detected pharmaceutical substances and metabolites per monitoring site (GW-site and adjacent Danube site).

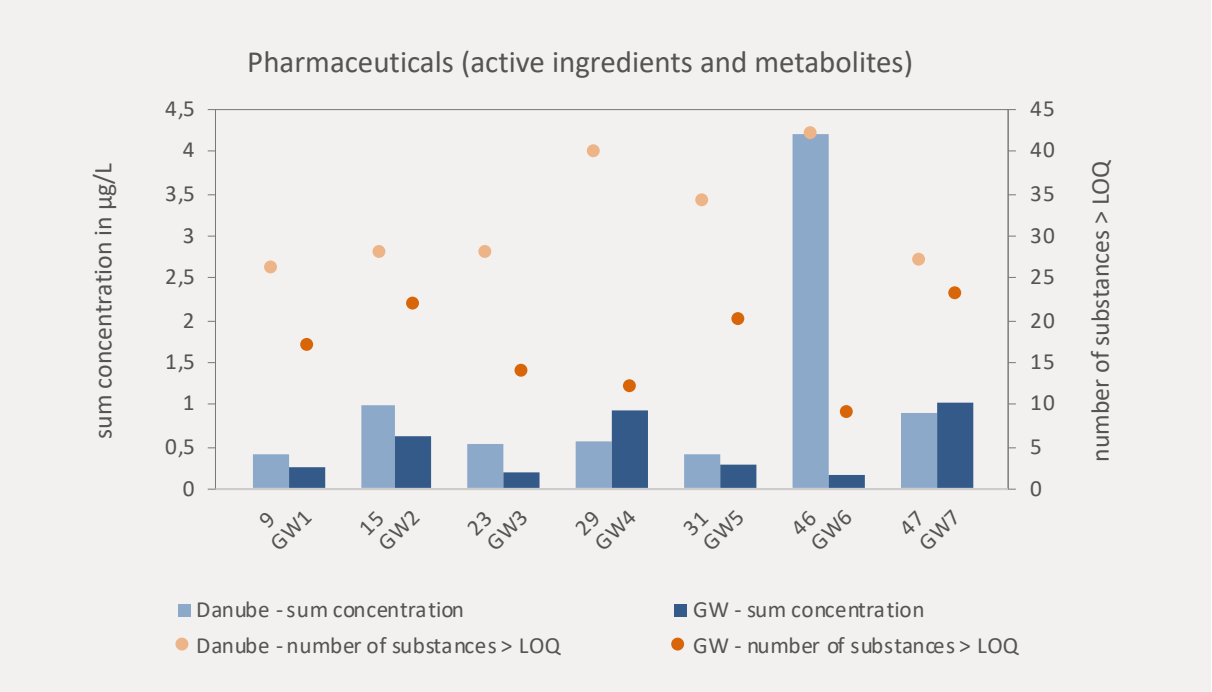

Figure 4: Sum concentration (in µg/L) and number of substances > LOQ for the corresponding monitoring sites Danube/groundwater.

By far the highest sum concentration of 4.19 µg/L was recorded at the Danube site 46 and in parallel, the sum concentration at corresponding GW6 (0.17 µg/L) is the lowest of all GW-sites investigated. Comparatively similar sum concentrations for the Danube and in groundwater exist for the monitoring site pair in 47/GW7. The highest sum concentration in groundwater was recorded at GW7 with 1.01 µg/L. A gabapentin concentration of 0.53 µg/L contributes significantly to this high sum value. The number of pharmaceuticals found in the Danube and at the GW-site is similar for the site pair 47/GW7.

At GW4, the sum concentration in groundwater was significantly higher than in the Danube. Only a few substances could be quantified in groundwater, but in some cases in very high concentrations, notably a vigabatrin concentration of 0.52 µg/L.

The anti-inflammatory drug diclofenac was found in all GW-sites and all adjacent Danube sites except for site 47 in Bulgaria. The highest concentration in groundwater was found in GW1with 0.06 µg/L, while the remaining GW-sites show values below 0.02 µg/L. Also, at the Danube sites 9, 15 and 46 concentrations are elevated (0.04–0.06 µg/L). During JDS3 diclofenac concentrations in the Danube and its tributaries were generally below 0.04 µg/L. Diclofenac is known to act as an ideal tracer, since concentrations in the environment are mainly reduced by dilution and UV radiation. Therefore, the findings in groundwater are quite plausible.

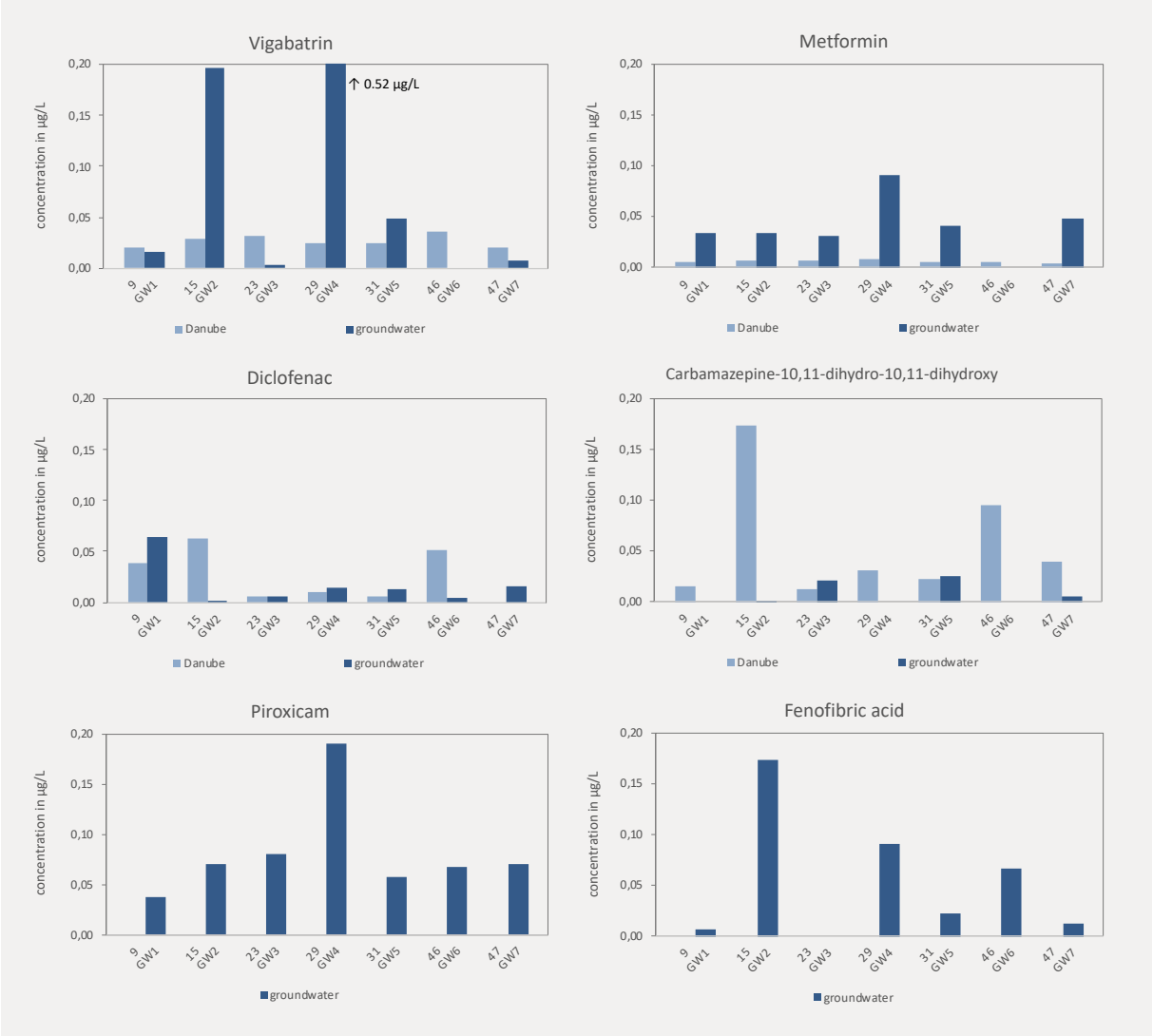

Figure 5: Concentrations (in µg/L) of selected active pharmaceutical ingredients and metabolites in groundwater and in the closest Danube sites.

The antiepileptic drug vigabatrin was quantified at all GW-sites except for GW6 and at all adjacent Danube sites. Concentrations were generally below 0.05 µg/L in groundwater and in the Danube. Exceptions were GW6 (0.52 µg/L) and GW2 (0.2 µg/L). Besides the antiepileptic drug gabapentin, vigabatrin had the highest concentration of all drugs found in groundwater.

Metformin, an antidiabetic drug, was detected in all GW-sites except for GW6. Generally, concentration levels were rather similar and ranged from 0.03 to 0.05 µg/L. The only exception was GW4 (0.09 µg/L). Metformin concentrations in the Danube were very low (< 0.008 µg/L) and similar at all sites.

Fenofibric acid is a lipid-lowering drug and concentrations in groundwater ranged from 0.006 µg/L to 0.17 µg/L. The pharmaceutical was not detected in the Danube. Also, the anti-inflammatory drug piroxicam was quantified at all GW-sites but not in the Danube. Concentrations were generally below 0.08 µg/L. The only exception was GW4 (0.19 µg/L).

The antiepileptic carbamazepine was detected at five GW-sites in concentrations < 0.0004 µg/L and at all adjacent Danube sites. In JDS3 traces of carbamazepine up to 0.023 µg/L were found at three GW-sites. Concentrations in the Danube were generally < 0.011 µg/L, with the exception of the Danube site 23 (0.058 µg/L) and the Danube site 46 (0.027 µg/L). The metabolite carbamazepine-10,11-dihydro-10,11-dihydroxy was detected at four GW-sites in concentrations up to 0.025 µg/L. Concentrations in the Danube were below 0.04 µg/L except for the Danube site 23 (0.17 µg/L) and Danube site 46 (0.095 µg/L). Comparably to diclofenac the substance carbamazepine acts as an ideal tracer in the aquatic environment. Therefore, the findings are plausible.

Four active substances or metabolites were quantified only at site GW7, but in concentrations > 0.05 µg/L: gabapentin (0.53 µg/L), 5-carboline (0.1 µg/L), carboline (0.09 µg/L) and 4-hydroxyquinoline (0.06 µg/L). None of these substances was detected in the adjacent Danube.

For most pharmaceuticals, groundwater concentrations were detected below 0.04 µg/L. Exceptions were described above. However, there were local differences in concentrations and patterns of substances, both in the Danube and in the groundwater. In comparison with the GW-sites, the Danube mostly showed a higher pollution with pharmaceuticals, both in terms of substance concentrations and number of substances. It is striking that the particularly high pollution of the Danube at site 46 in Romania is not reflected in the groundwater. This indicates that the groundwater was largely unaffected by bank filtrate during the period of sampling or the filter effect was quite effective for pharmaceuticals. Notably GW4 in Croatia and GW7 in Bulgaria seem to be strongly affected locally or regionally by outflowing groundwater moving towards the Danube. With regard to the substances found both in the Danube and in groundwater, the pair of monitoring sites in Serbia (31/GW5) showed the highest similarity.

There are no quality standards for pharmaceuticals in groundwater and drinking water. Environment Agency Austria together with the Austrian Agency for Health and Food Safety derived tolerable concentrations in drinking water for a set of substances, based on toxicological key figures. The values for babies for some of the detected substances range between 0.3 and 10 µg/L. The tolerable concentrations for adults are usually about 4.5-times higher (Hartmann 2017). None of the detected substances exceed any of the tolerable concentrations proposed in Austria.

25.3.4 Drugs of abuse, steroids and tobacco ingredients

Three such substances were detected in groundwater. 4-Androsten-11-beta-ol-3,17-dione is a steroid hormone and was detected in four GW-sites at concentrations between 0.21 and 0.76 ng/L, but not in an adjacent Danube site.

Galaxolidone, a metabolite of galaxolide (HHCB) which is used as a musk fragrance in cosmetics, was found in GW5 (166 ng/L) but not in the adjacent Danube site 31 and it was detected in three other Danube sites between 2.54 and 13.83 ng/L but not in groundwater.

Cotinine is found in tobacco and is the predominant metabolite of nicotine. It was detected in all seven Danube sites at levels between 8 and 32 ng/L and it was found in five GW-sites at levels between 14 and 1545 ng/L. The maximum was found in GW4 which is 77-times higher than the concentration in the adjacent Danube site (20 ng/L)[1].

Benzoylecgonine, a metabolite of cocaine, was found in four adjacent Danube sites at very low concentrations (0.11–0.24 ng/L) but not in groundwater.

For none of these substances quality standards in groundwater or drinking water are established.

25.3.5 Caffeine, artificial sweeteners

Caffeine is easily biodegradable but nevertheless it was detected in all seven GW-sites and at all adjacent Danube sites. The concentration level was low in groundwater, between 0.0004 and 0.004 µg/L. The measured concentrations in groundwater reached about 2% to 80% of the concentrations in the adjacent Danube sites (ranging from 0.0013 to 0.023 µg/L). At JDS3 caffeine was detected in only one of eleven GW-sites.

Six artificial sweetener substances were detected in at least one of the seven groundwater adjacent Danube sites, but only sucralose was found in groundwater - at GW3 and GW5 only - in quite low concentrations of 0,021 and 0.023 µg/L, very similar to the values in the Danube (0.013 and 0.026 µg/L).

Sucralose persists during sewage treatment and is also known to be long-term persistent in groundwater. It can be used as tracer of wastewater and for age dating as has been used in Europe since 2003 (W.D. Robertson et al. 2015).

Neither acesulfame, which was detected in almost every of the ten bank filtration wells during JDS3, nor cyclamate, which was detected in two GW-sites during JDS3, were detected during JDS4. It is also remarkable that although the Danube site 46 showed all six artificial sweeteners at concentrations between 0.009 µg/L (sucralose) and 3.218 µg/L (cyclamate), nothing was detected at the adjacent GW6.

For none of these substances quality standards in groundwater or drinking water are established.

[1] *The high cotinine concentration could be explained by the fact that the people involved in sampling are smokers.*

25.3.6 Industrial substances

About 72 industrial substances were detected either in groundwater and/or in an adjacent Danube site:

- 46 substances in groundwater,
  - 20 substances in sampling site pairs – in a GW-site and in an adjacent Danube site,
  - 26 substances were found in groundwater only and not in an adjacent Danube site, and
- 26 substances were only found in an adjacent Danube site, but not in groundwater.

The number of substances per GW-site ranges from 14 to 26 and the sum concentrations range from 0.41–5.83 µg/L.

In parallel, the number of substances in the adjacent Danube sites is higher with 29 to 38 substance and also the sum concentrations are higher, ranging from 3.17–6.27 µg/L. The highest sum concentrations for groundwater and the Danube were found at GW1 and the lowest sum concentrations were found at GW6.

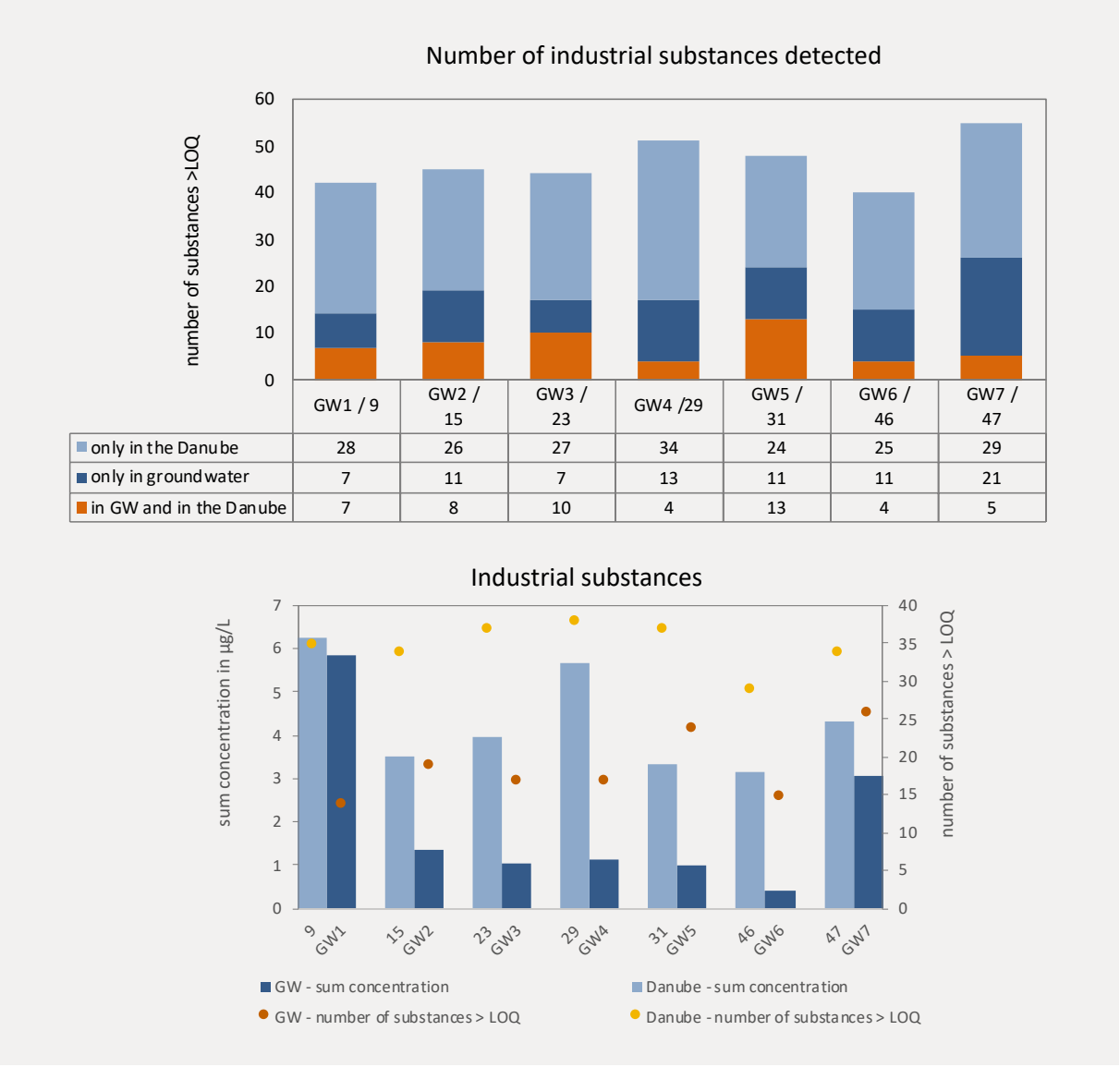

Figure 6: Industrial substances in groundwater and in the Danube (in µg/L).

The detected industrial substances are further distinguished into the following groups:

- Phosphate flame retardants (PFRs) – 7 detected in groundwater
- Poly- and perfluorinated substances (PFAS) – 4 detected in groundwater
- Benzotriazoles and benzothiazoles – 5 detected in groundwater
- Phenolic substances – 3 detected in groundwater
- Other industrial substances – 27 detected in groundwater

Phosphate Flame Retardants (PFRs)

14 phosphate flame retardants (PFR) were analysed in JDS4. Seven substances (EHDP, TCPP, TDCPP, TEP, TIBP, TNBP and TPhP) were found in both, groundwater and in the closest Danube site. Five substances (TBOEP, TCEP, TEHP, TIPPP and TMPP) were detected in the seven Danube sites which are closest to the GW-sites, but they were not detected in groundwater.

PFR were used for several decades in many industries, including the production of dyes, varnishes, adhesives, synthetic resins, polyvinyl chloride, hydraulic fluids, plastics and textiles and as flame retarding additives in polyurethane foam, plastic materials and hydraulic fluids. Some of these PFRs are carcinogenic (the Cl-containing PFRs TCPP, TCEP, TDCPP), toxic to (aquatic) organisms (TPhP, DCP and TCEP) and very persistent (Diethylphosphinic acid, TCEP) (Van der Veen and de Boer 2012). No European quality standards for groundwater or drinking water are established for these substances.

TIBP was quantified in six of the seven GW-sites in a range of 1.4–23.9 ng/L. TNBP, TDCPP and TPhP were found in two GW-sites and TEP, TCPP and EHDP in only one site. The maximum concentration was detected for TCPP with 65.7 ng/L at GW5.

It has to be considered, that LODs and LOQs are varying considerably between surface water and groundwater and by substance - between 0.004 (TEHP) and 14.53 ng/L (TCPP).

Each of the seven GW-sites contains between one and six PFR substances. Six substances were found at GW5 with a sum concentration of 126 ng/L. The sum concentration is between 1.4 and 15.5 ng/L for the remaining six GW-sites. At GW4 three substances and at GW3 two PFRs were detected.

When comparing the detected concentrations in groundwater with the corresponding concentrations in the most adjacent (mainly upstream) Danube site, the values in six GW-sites are quite similar or below the concentrations in the Danube. At GW5 all concentrations of the six detected PFRs in groundwater are 1.6 to 8.4 times higher than in the Danube.

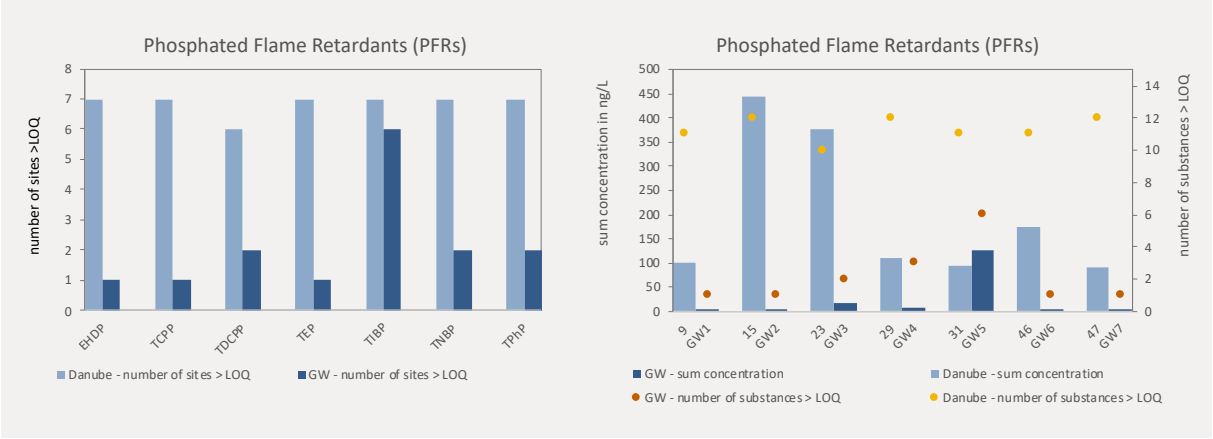

Figure 7: Phosphated Flame Retardants (PFRs) in groundwater and in the Danube (in ng/L).

Poly- and perfluoroalkyl substances (PFAS)

PFAS are a group of more than 4700 substances, they are widely used (they are stable under intense heat, used as surfactants, water and grease repellents etc.) and they are highly persistent in the environment, longer than any other man-made substance. PFAS have toxic effects, some are toxic for reproduction and can harm the development of foetuses, and several cause cancer (https://echa.europa.eu).

Four perfluoroalkyl substances (PFOA, PFOS, PFBuS and PFHxA) were detected in three (PFOS) to five (PFOA) GW-sites at very low concentrations ranging between 0.005 and 0.018 µg/L. The parallel concentrations in the Danube vary between 0.0004 and 0.0044 µg/L.

In addition, also PFHxS and PFHpA were detected in adjacent Danube sites only, at very low levels, PFHxS at Danube-sites 9, 15, 23, 29, 31 and 47 (range: 0.001–0.0014 µg/L) and PFHpA at site 23 with 0.00073 µg/L.

The sum of detected concentrations in groundwater varies between 'not detected' (GW4) and 0.03 µg/L (GW3). The sum concentrations in the adjacent Danube sites varies between 0.0007 and 0.0114 µg/L.

At GW2, GW3 and GW5 all four above mentioned substances were found, both in groundwater and in the Danube. The concentration levels in the GW-sites and the closest Danube sites are very similar except for PFHxA at GW6 where the concentration in groundwater is about 11-times higher than in the Danube. But it has to be considered that the detected concentration levels are very low in the range of few ng/L which does not allow for drawing conclusions on the influence of the Danube on GW and vice versa.

Within the revision of the EU Drinking Water Directive a quality standard of 0.1 µg/L is discussed for the sum of a list of selected 20 PFAS substances which includes three of the substances found in groundwater. A further limit value for 'PFAS total' is proposed with 0.5 µg/L. Considering the detected concentrations in the GW-sites along the Danube, none of the discussed quality standards would have been exceeded.

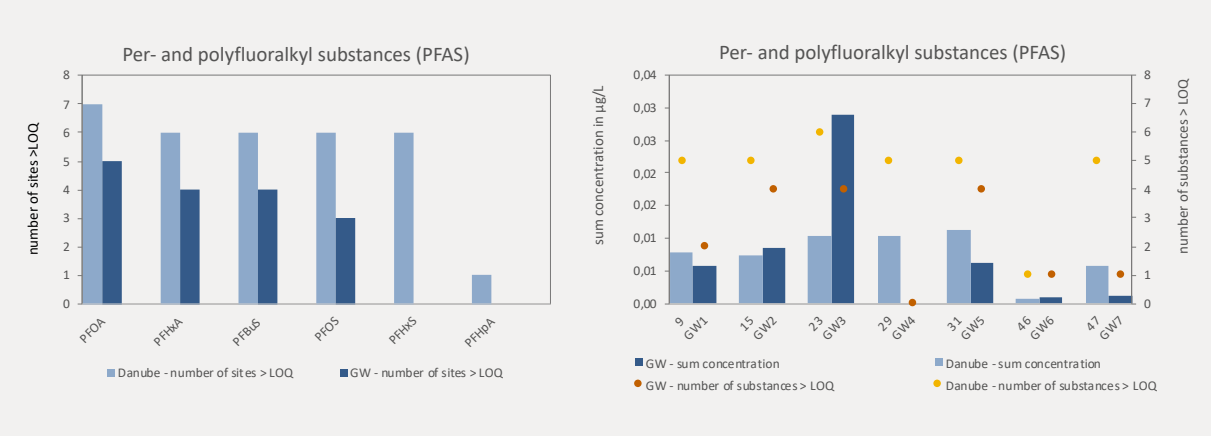

Figure 8: Per- and polyfluoralkyl substances (PFAS) in groundwater and in the Danube (in µg/L).

Benzotriazoles and benzothiazoles

Benzotriazoles (BT) and benzothiazoles (BTH) are water soluble chemicals that are produced in high volumes, mainly used as corrosion inhibitors and widely distributed in the environment.

BT derivatives are found in plastics, dishwasher detergents, dry cleaning equipment, and de-icing/anti-icing fluids. BTH are used in the manufacture of rubber and they have wide applications as fungicides, herbicides, accelerators for vulcanization of rubber and for dye, lumber and leather production. (Calvo-Flores et al. 2018)

Five BT and BTH substances were detected in groundwater in five of seven GW-sites at sum concentrations ranging between 0.14 (GW7) and 5.54 µg/L (GW1). The five substances are: benzotriazole and its derivates 4-Me-BT + 5-Me-BT, diMe-BT and benzothiazole and its derivate 2-Hydroxybenzothiazole (2-OHBT).

The high total sum concentration at GW1 of 5.54 µg/L is basically caused by benzothiazole with a concentration of 5.51 µg/L which is not detected in the adjacent Danube site 9.

In the adjacent Danube sites only three substances were found: benzotriazole, 4-Me-BT + 5-Me-BT and benzothiazole-2-sulfonic acid (not detected in groundwater) and the sum concentrations are at levels of 0.19–0.75 µg/L, very similar to groundwater except for Danube site 9.

BT and BTH substances were not found in GW4 and GW6 although they were found at the related Danube sites in sum concentrations of 0.34 respectively 0.19 µg/L.

For BT and BTH there are no European quality standards, neither for groundwater nor for drinking water.

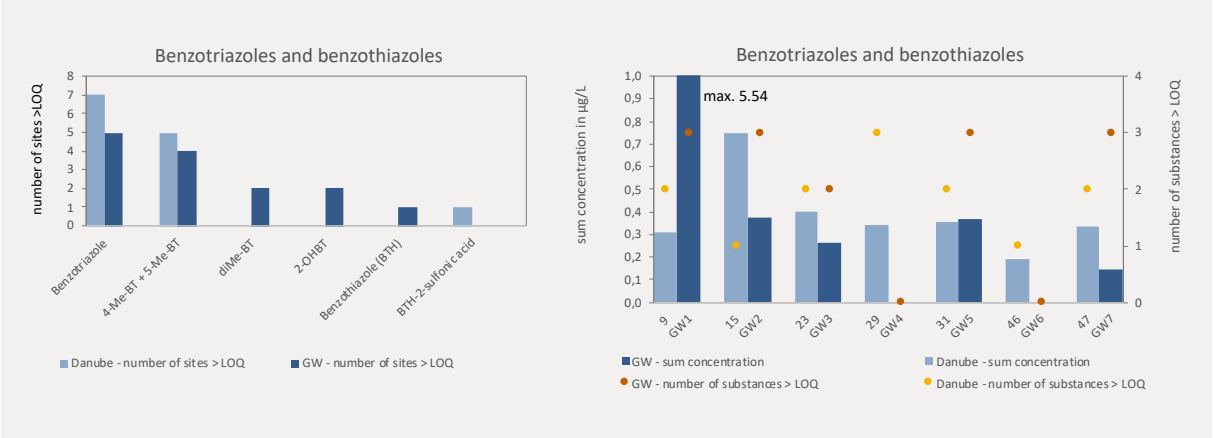

Figure 9: Benzotriazoles and benzothiazoles in groundwater and in the Danube (in µg/L).

Phenolic substances

Bisphenol A was found at all seven GW-sites but not in any of the adjacent Danube sites. The concentrations range between 0.093 (GW4) and 0.16 µg/L (GW6). Within the current revision of the EU Drinking Water Directive the inclusion of a drinking water standard for bisphenol A of 0.01 µg/L is under discussion. All the seven detected concentrations would have exceeded the discussed drinking water quality standard.

Octylphenol-4-tert (4-t-OP) was found in three GW-sites (GW1, GW4 and GW6) at concentrations of 0.07–0.11 µg/L and it was found in all seven adjacent Danube sites at concentrations between 0.009 and 0.12 µg/L. At all three GW-sites with positive detections, the concentrations are higher than in the adjacent Danube site.

Bisphenol S was found only in GW4 with 0.005 µg/L and but not in any of the adjacent Danube sites and 2,4-dichlorophenol was only detected in one adjacent Danube site but not in a GW-site.

All four phenolic substances are categorised as endocrine disruptive.

Other industrial substances

About 27 other industrial substances (except PFRs, PFAS, phenolic substances, BT and BTH), were found in groundwater, 12 substances in more than two GW-sites (see Table 2), 15 in only 1 site. About 17 other industrial substances were detected in one to seven adjacent Danube sites but not in groundwater.

The number of substances per GW-site ranges from 6–20 and the sum concentrations range from 0.08–2.7 µg/L.

Except for GW7, the number of substances in the adjacent Danube sites is higher with 14–18 substance and also the sum concentrations in the Danube are higher, ranging from 2.2–5.8 µg/L. The highest sum concentration in the Danube (5.8 µg/L) was found at Danube site 9, most adjacent to GW1, where also the lowest sum concentration for groundwater was registered (0.08 µg/L).

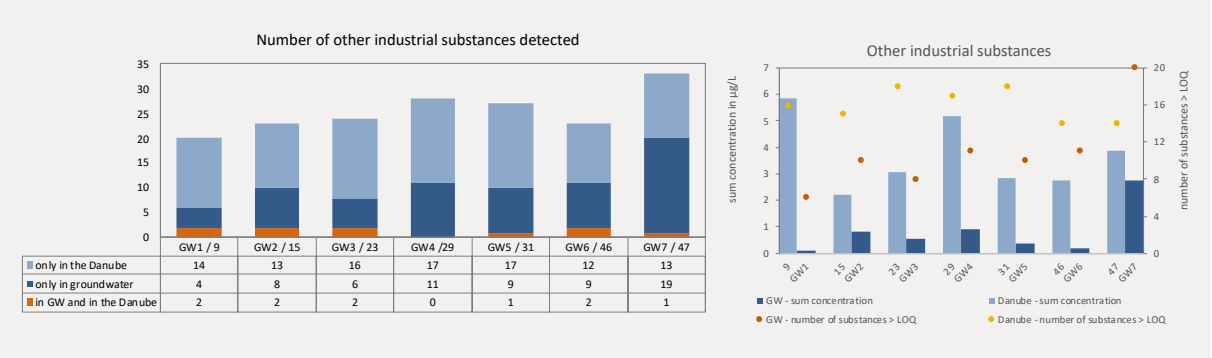

Figure 10: Occurrence of all other industrial substances present in groundwater (in µg/L).

The following Table 2 shows all other industrial substances which were found in at least one GW-site and the identified concentration ranges in groundwater and the adjacent Danube sites.

Table 2: Occurrence of all other industrial substances detected in groundwater.

Substances	GW-sites		Adjacent Danube sites	
	Number of GW-sites	Concentration range in µg/L	Number of Danube sites	Concentration range in µg/L
Benzoic acid	7	0.058–0.151	5	0.045–0.115
Benzododecinium	4	0.0005–0.016	7	0.72–4.37
Tetraglyme	2	0.009–0.025	5	0.055–0.185
4'-Aminoacetanilide Benzophenone-4	1	0.072 0.003	7	0.013–0.042 0.021–0.054
Tri(butoxyethyl)phosphate	1	0.028	3	0.032-0.073
Diglyme, Lauryl diethanolamide and Tetraethylene glycol monododecyl ether	7	0.0001–0.012	0	
DINCH N,N-Dimethyldodecylamine N-oxide	6	0.037–0.623 0.0009–0.005	0	
Phthalate-Di-n-butyl	5	0.0003–0.003	0	
Cyclohexylamine N,N Dimethyltetradecylamine-N-oxide	4	0.2–0.364 0.0003–0.005	0	
Perfluorooctanoic acid	2	0.003–0.008	0	
12 substances (9 of these substances in GW7): (3-Chloro-2-hydroxypropyl)trimethylammonium, 1-Butyl-3-methyl-imidazolium, 2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine | PhIP, 7-Amino-4-methylcoumarin, N,N-Dimethyldecylamine, N-Phenyl-1-naphthylamine, Octyl-methoxycinnamate, o-Toluidine, Perfluorooctanesulfonic acid, Quinoline, Trimethyloctylammonium, Tris(2-ethylhexl)phosphate	1	0.003–0.731	0	

Other industrial substances detected in groundwater at concentrations above 0.1 µg/L

Benzoic acid is produced at very high volumes and used as an important precursor for the industrial synthesis of many organic substances. It was found in GW4 at a concentration of 0.15 µg/L whereas nothing was detected in the adjacent Danube site 29.

DINCH is a plasticizer, produced in high volumes, and found in four GW-sites (GW2, GW3, GW4 and GW7) at values between 0.33–0.62 µg/L, but nothing was detected in any adjacent Danube site.

Cyclohexylamine is an intermediate in the synthesis of other organic compounds and a metabolite of cyclamate. It was detected in all four GW-sites (GW2, GW4, GW and GW7) in concentrations above 0.1 µg/L, between 0.2–0.36 µg/L. Nothing was detected in any adjacent Danube site

Twelve substances were only found once in groundwater, nine substances only in GW7, in a range of 0.003–0.73 µg/L.

The following four substances, only found in GW7 show concentrations above 0.1 µgL: PhIP (in cooked meat) with 0.73 µg/L, o-Toluidine (intermediate in the synthesis of pesticides and dyes etc.) with 0.37 µg/L, N,N-Dimethyldecylamine (stabiliser, foam maker) with 0.26 µg/L and Octyl-methoxycinnamate (sun screen) with 0.11 µg/L.

Benzododecinium is a disinfectant compound and found in considerably high concentrations of 0.72–4.37 µg/L in all adjacent Danube sites but it was found at very low concentrations in groundwater.

Isophorone is a solvent and found only in adjacent Danube sites at concentrations between 0.51–3.9 µg/L, but nothing was found in any GW-site.

25.3.7 Rare Earth Elements in groundwater

Rare Earth Elements (REEs) usually refer to the lanthanide's series (from lanthanum (La) atomic number Z=57 to lutetium (Lu) Z=71), including scandium (Z=21) and yttrium (Z=39). They are naturally found in water systems because of rocks weathering, and they are commonly used in geochemistry as processes and/or sources tracers, as they behave in a coherent way. However, REEs have wide and growing applications in new technologies, industries, medicine and agriculture. Those anthropogenic uses disrupt the geochemical and biological cycles of REEs and lead to enrichment of some REEs in waters. The first enrichment observed was that in gadolinium (Gd) (Bau and Dulski, 1996), reported in surface waters. Indeed, Gd is used as a contrast agent for Magnetic Resonance Imaging (MRI) analyses, in highly stable Gd-organic complex forms (as Gd^{+3} is toxic for human body). After injection in the human body, the Gd contrast agent is excreted within a few hours by urine and ends up in wastewater. Because of their stability, Gd-complexes are not removed by conventional wastewater treatment plants (WWTPs), and WWTP effluents are now recognized as the principal source of anthropogenic Gd (Gd_{anth}) in waters (Bau and Dulski, 1996; Kümmerer and Helmers, 2000; Verplanck et al., 2005). As, so far, no proven toxicity of the Gd-complex has been shown, some studies suggest using this complex as tracer for wastewater-derived contamination in natural waters (Gd is easier and less expensive to measure than other micro pollutants discharged from WWTP effluents).

There are no European quality standards established for these substances, neither for groundwater nor for drinking water.

JDS4 was the first campaign on the Danube River during which REEs concentrations and distributions were investigated, both in river waters and groundwater. Such investigation can inform about WWTP effluents or untreated sewage or non-collective sanitation units effluents leakage into groundwater.

Results and discussion

REEs patterns in groundwater are shown in Figure 11. All patterns display a cerium (Ce) negative anomaly (Ce/Ce* from 0.07 to 0.21), except for GW3 (Ce/Ce* 0.95). This negative anomaly is a natural anomaly, which occurs due to the redox behavior of Ce. Globally, this Ce anomaly is more important in groundwater samples than in surface water samples. That indicates some redox conditions changes during the flow or inside the wells.

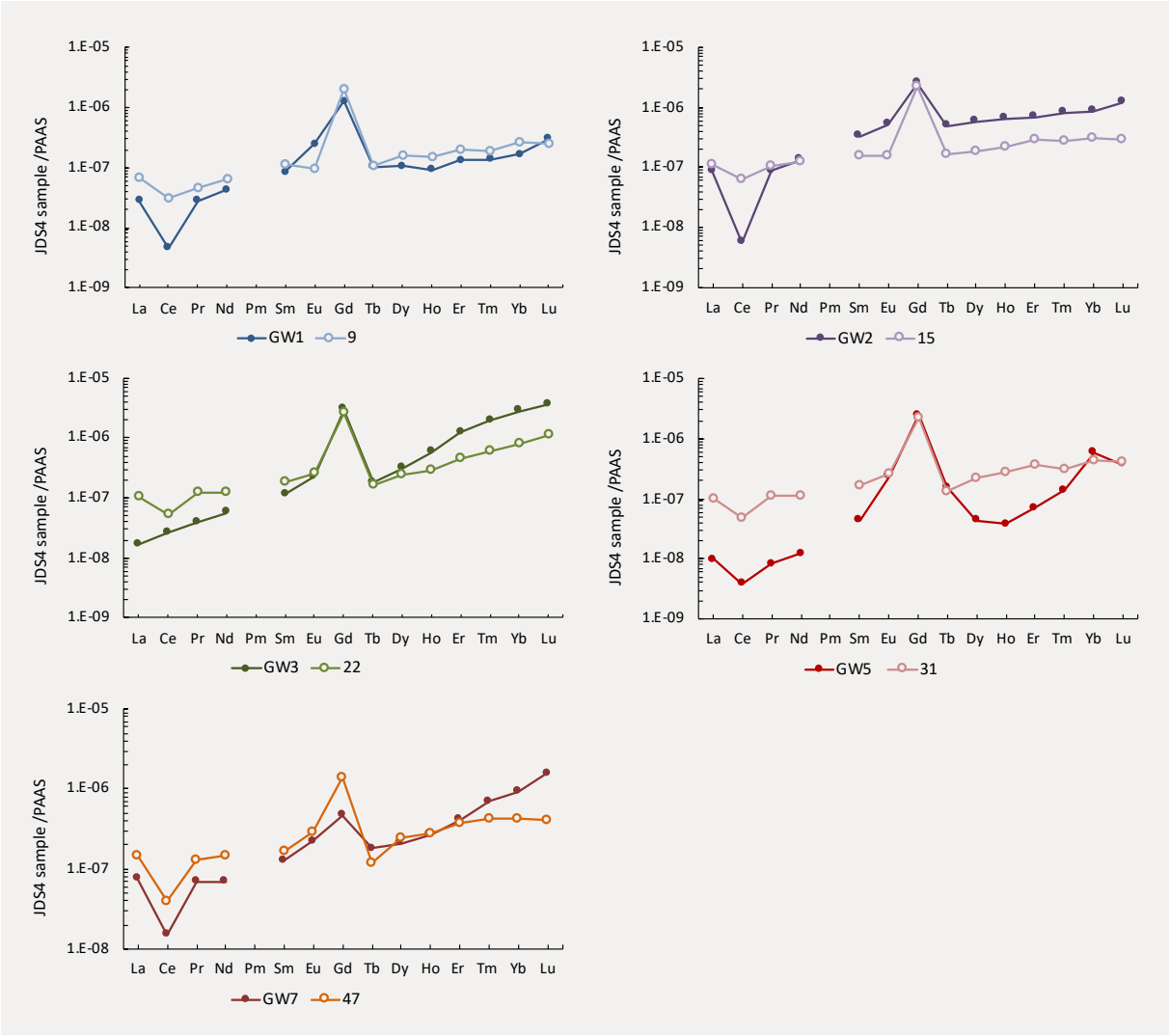

Figure 11: REE patterns in groundwater (dark colour) and the closest Danube water samples (light colour) (PAAS=Post Archean Australian Shale).

The Gd positive anomaly is detectable in all the groundwater samples (Gd/Gd* value from 3.02 to 80.82). To define a Gd anomaly as an anthropogenic anomaly, the threshold value of Gd/Gd* is set to 1.5 (Bau et al., 2006). In the case of groundwater, the visible Gd anomalies are always above this threshold value: that allows the calculation of Gd_{anth} concentrations. In groundwater samples, Gd_{anth} represents from 66 to 94% of total gadolinium. Presence of Gd anomaly from anthropogenic origin reveals leakage from WWTP effluents, or untreated sewage or non-collective sanitations unit effluents in groundwater. Up to now, there is no proven toxicity of Gd_{anth} and its presence in some waters can therefore be used as a wastewater-derived contamination tracer. Compared to their closest Danube samples, GW2, GW3 and GW5 have similar Gd anomaly and Gd_{anth} concentration. It could be explained by the proximity between the two kind of samples and by the nature of groundwater (alluvial groundwater, mainly loaded by the Danube River). Anthropogenic Gadolinium is not affected during the flow from the Danube to the groundwater, unlike other REEs. GW1 shows values slightly lower than Danube site 9. This GW-site is located further from the Danube River, and is surrounded by other water bodies. For GW7 and Danube site 47, the Danube site is located just downstream a WWTP whereas the GW-site is around 13 km further down.

Table 3: Total Gd (ng/L), Gd anomaly (Gd/Gd), Gd_{anth} (ng/L) and % of Gd_{anth} in groundwater samples and the closest surface water samples.*

Country	JDS4-sites	Total Gd	Gd anomaly	Gd_{anth}	Gd_{anth} %
AT	GW1	6.03	15.91	4.91	93.72
	9	9.78	16.89	9.21	94.08
SK	GW2	11.65	6.24	8.36	83.98
	15	10.63	14.35	9.89	93.03
HU	GW3	13.82	14.35	11.27	93.03
	22	12.24	13.45	11.33	92.57
RS	GW5	11.76	80.82	9.14	98.76
	31	10.72	12.92	9.89	92.26
BG	GW7	2.17	3.02	1.17	66.89
	47	6.63	6.80	5.66	85.29

An ytterbium (Yb) positive anomaly was detected in GW5. So far, there is no mention of such anomaly in natural water in the literature. It is unclear whether this anomaly is natural or due to anthropogenic inputs. This is the first REEs investigation on the Danube River within the Joint Danube Survey project and there is no possibility to compare data from previous analyses.

Conclusions

Rare Earth Elements were monitored in groundwater along the Danube River. The concentrations found were normalized to a reference rock type (Post Archean Australian Shale, PAAS) to detect potential anomalies. A negative Ce anomaly was observed in most of the GW-sites: it is natural and related to the redox behaviour of Ce. A positive Gd anomaly was observed in all GW-sites. Given the wells positions, anthropogenic gadolinium found in groundwater must certainly come from the Danube, and is not affected during water infiltration to alluvial groundwater.

25.3.8 Dissolved organic matter in groundwater

Dissolved organic matter (DOM) is a complex mixture of thousands of organic compounds and it is a small fraction of the decomposition products in soil; however, it is highly mobile and reactive. DOM is leaching to groundwater and influences water quality and serves as a source of carbon and energy for microbial metabolism and drives the bioremediation of many contaminants. Concentrations of dissolved organic carbon (DOC) can reflect the likelihood of contamination by synthetic organic compounds (Barcelona 1984). Humic and fulvic acids in DOM affect the solubility of organic pollutants in groundwater and can contribute to the long-range transport of harmful chemicals (Chiou et al. 1986).

Spectrophotometric methods were applied to characterize DOM in groundwater and the information provided by synchronous fluorescence spectroscopy and UV-visible spectroscopy of seven groundwater samples collected during JDS4 is hereby discussed and compared to Danube water samples collected nearby.

Results and discussion

Figure 12 compares the DOC concentration and the specific ultraviolet absorbance at 254 nm (SUVA254) of the groundwater samples and of the corresponding Danube samples. A decrease of the DOC concentration was observed between the surface water and the groundwater at all seven GW-sites (Figure 12a), indicating a positive effect of the natural soil filtration between the Danube and the groundwater. The aromaticity of most samples is not affected by the bank filtration, except for GW4 and GW7 for which it increases strongly and for GW2 to a lower extent. For these GW-sites, there is an apparent selectivity of the bank filtration which retains the DOM with the lowest aromaticity.

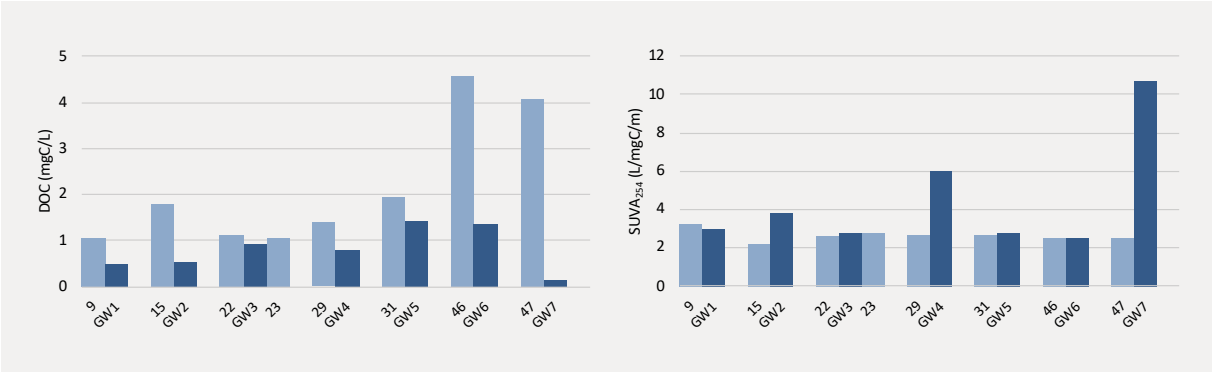

Figure 12: DOC (a) and $SUVA_{254}$ (b) for groundwater (dark blue) and for the closest Danube sites (light blue).

The spectral slope is inversely related to the molecular weight. The average spectral slope is 0.14 nm^{-1}, with a coefficient of variation of 28%, but this average value should be considered with care as only seven groundwater samples were analysed. This is slightly less than the average spectral slope observed over all the JDS4 surface water samples (51 samples). For five groundwater samples the spectral slope of the groundwater samples is lower than the spectral slope of the corresponding surface water samples (Figure 13): a decrease of the spectral slope indicates an increase of the average molecular weight of DOM. This would indicate that substances with a low molecular weight are retained preferably during bank filtration.

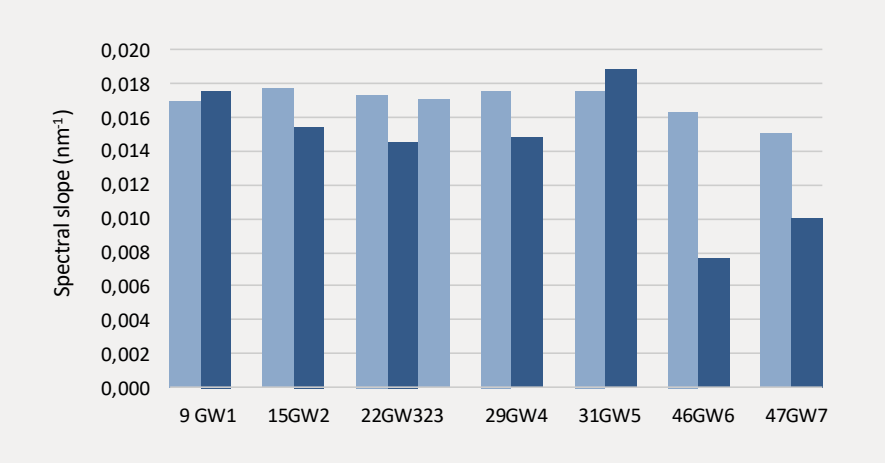

Figure 13: Spectral slope for groundwater (dark blue) and for the corresponding Danube samples (light blue).

Figure 14 presents the synchronous fluorescence spectra of the seven groundwater samples. The 50 nm gap provides information on both the fluorescence due to protein-like substances (excitation around 280 nm) and humic substances (excitation between 300 nm and 400 nm). Protein-like fluorophores are related to biological reactions, but also to the run-off of biological substances from the watershed and to the discharge of untreated or not sufficiently treated urban sewage. Biological reactions can occur in the surface water itself but also in the biofilm developing on the filtration support (i.e. soil) (Hoffmann and Gunkel, 2011). Humic substances are the main organic components of humus and their presence in surface water is essentially due to soil run-off from the watershed.

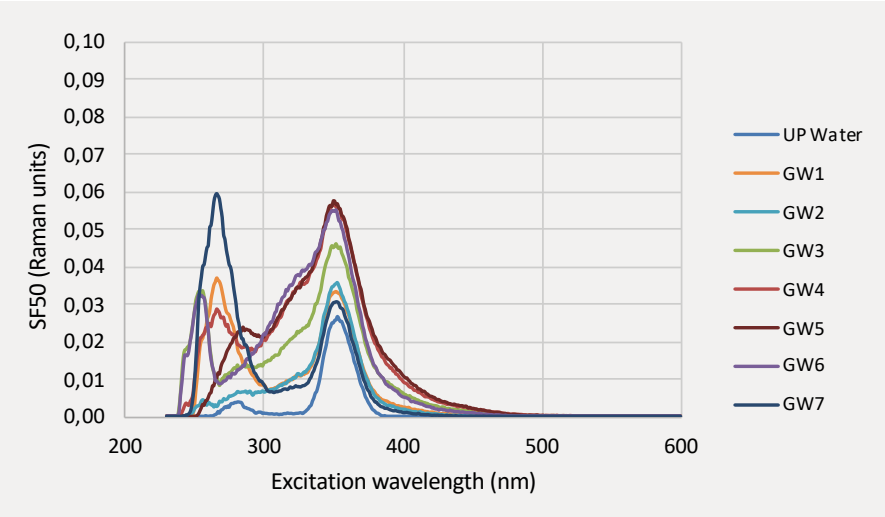

Figure 14: SF50 spectra for groundwater samples.

Figure 15 compares both types of samples after the decomposition into five fluorophores. The fluorescence of the groundwater samples is due on one hand to protein-like substances (F(280) and F(310)) and on the other hand to humic substances (F(330), F355) and F(370)). There is no general behaviour for the groundwater samples that can be compared to surface water samples. The fluorescence of GW2 and GW6 samples is much lower than the fluorescence of the corresponding Danube samples. In the case of GW6, it brings back to the water quality, in terms of fluorescent substances, to the general quality of Danube water. For GW2, the groundwater is the less fluorescent one from the analysed set.

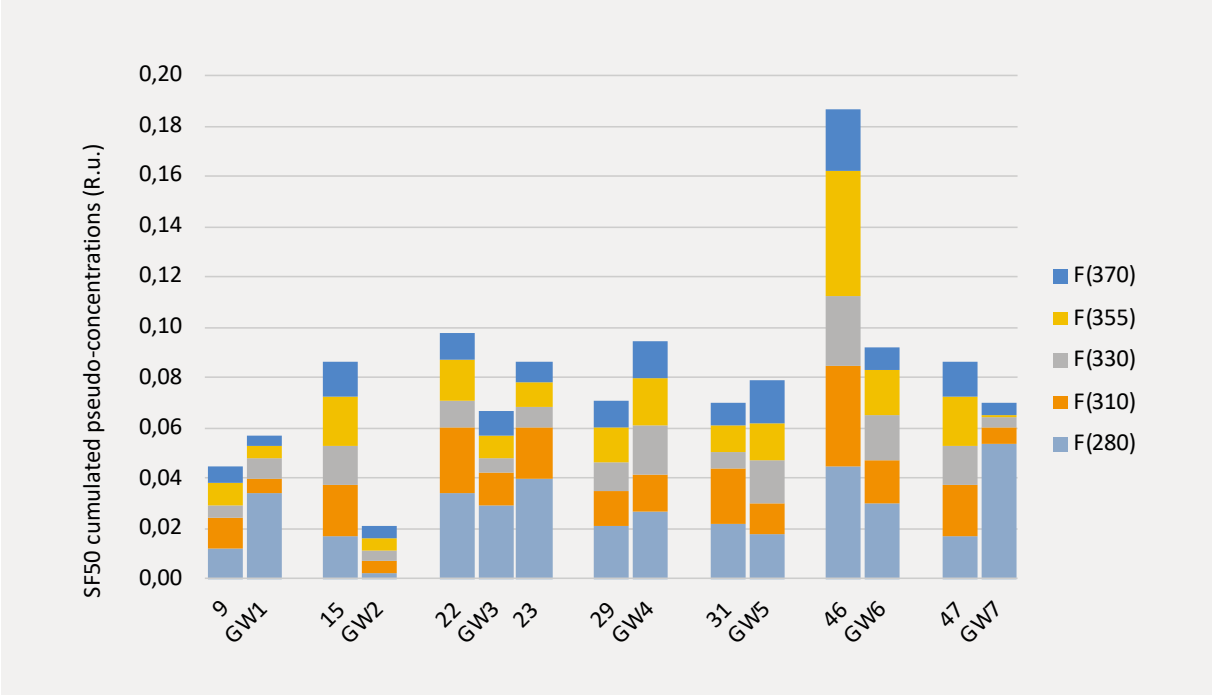

Figure 15: SF50 spectra decomposition for groundwater samples and the corresponding surface water samples.

Conclusions

The characteristics of the DOM of the GW-sites were compared to the closest Danube sites by easy-to-perform optical methods. It is difficult to conclude for the whole Danube River as the number of groundwater samples was limited (seven). Furthermore for some GW-sites, the nearest Danube site was not in the immediate vicinity. It may happen that WWTPs discharge between the Danube site and the GW-site.

No general behaviour could be observed in terms of DOM characteristics (Table 4). However there are hints that bank filtration induces mostly a decrease of the DOC and an increase of the DOM aromaticity and molecular weight. An important decrease of the fluorescence was observed for GW6 and GW2.

The quantity and structure of dissolved organic matter (DOM) in groundwater and surface water used to produce drinking water is a key issue: depending upon the type of process applied, the degradation of DOM can lead to the formation of harmful disinfection by-products (DBP) such as trihalomethanes, dihaloacetic acids, etc. (Evlampidou et al. 2020).

Table 4: Summary of the groundwater characteristics with respect to the characteristics of the closest Danube site.

	9 / GW1	15 / GW2	22 / GW3 / 23	29 / GW4	31 / GW5	46 / GW6	47 / GW7
DOC	-	--	=	-	-	--	--
DOM aromaticity	=	+	=	+	=	=	++
DOM molecular weight	=	+	+	+	=	++	+
Protein-like fluorescence	+	--	-	=	=	--	+
Humic substances fluorescence	=	--	-	++	+	--	--

++ … large increase, + … increase, = … no change, - … decrease, --… large decrease

25.3.9 Parallel monitoring in Hungary

In parallel to JDS4, Hungary analysed a comprehensive set of polluting substances at a cross section with ten groundwater sites, three groundwater abstraction wells at bank-filtered aquifers, four groundwater monitoring wells in drinking water protection zones and a triplet well-group outside the protection zones near GW3 to observe the quality of ambient groundwater.

Results in line with JDS4 observations show higher concentrations for many substances in the adjacent Danube site 23 than those detected in bank-filtered abstraction and monitoring wells. In the triplet wells indicating the impact of groundwater, concentrations for several substances exceed concentrations detected in the Danube River water (Imidacloprid, caffeine) or it shows the mixing of Danube River water versus groundwater (in case of bisphenol A).

Despite the limits of the survey as well as the lack of knowledge on bank filtration processes, the high quality of bank-filtrated water draws attention to the importance of protecting riverbed formations to maintain its purification capacity and to take necessary measures in protection zones for protecting groundwater quality from ambient pollution.

25.4 Conclusions

Due to the selection criteria, all seven GW-sites are supposed to be interconnected with the water from the Danube River through bank-filtration. The analyses showed that the bank-filtration process is in many cases contributing to both, a smaller number of substances detected in groundwater than in the Danube River and lower concentrations in groundwater than in the Danube, but this is not a general pattern, as it differs from substance to substance and from site to site. Furthermore, when comparing the detections in groundwater and in the Danube, it has to be considered, that the distance between some corresponding monitoring sites are significant and it cannot be precluded that there are discharges of pollutants into the Danube in between.

Around half of the detected substances were either found in an adjacent Danube site only or in both groundwater and the Danube and all detected concentrations of these substances in groundwater are lower than in the corresponding Danube site. This shows that the filtering effect between the Danube and the groundwater site is quite effective. Almost one third of substances was only found in groundwater or if detected both then the concentration in groundwater was higher than in any adjacent Danube site, which means that pollution of groundwater is in parallel also caused by local or regional polluting activities apart from the Danube or there were substantial discharges of pollutants between the Danube site and the corresponding downstream GW-site.

The analysis illustrates the legacy of 'old substances' like certain pesticides and industrial substances, which are no longer used but still found and it proves the presence of a broad range of emerging substances, which are widely used for various purposes and finally end up in groundwater, although at very low concentrations.

As a broad range of chemical substances is widely used in industrial, medical and agricultural activities, it should not be a surprise that many of these substances are also present in groundwater. The goal of JDS4 was to perform comprehensive screening of substances which are normally not analysed during ongoing monitoring by applying the most sophisticated target and non-target screening methods at the most extreme limits of detection and quantification. Hence, a lot of substances were detected, but it should be kept in mind, that most of the findings are at a range of few ng/L (=0.001 µg/L) or even pg/L (=0.001 ng/L). This is owed to the extreme sensitivity of detection of the applied methods – 1 pg/L = 1/2 sugar cube (= 2 g) in Lake Balaton with 1.9 km³ (1.9 trillion L) of water – which means that many of these substances would have never been detected with standard laboratory methods and their concentrations are far below any quality standard. Nevertheless, certain substances have adverse (e.g. endocrine) effects at such low concentration levels.

None of the pesticide substances and metabolites for which European quality standards for groundwater and drinking water exist, have exceeded these standards. For the majority of substances, no quality standards exist, for some (PFAS and bisphenol A) drinking water standards are under discussion. For PFAS the discussed standards would not have been compromised, whereas for bisphenol A all the seven detected concentrations in groundwater would have exceeded the discussed drinking water quality standard of 0.01 µg/L by 9- to 16-times.

Acknoledgements

We thank the Members of the ICPDR Groundwater Task Group for their support in promoting groundwater to be part of JDS4, for taking the groundwater samples, for analyzing the basic parameters and for commenting on this chapter.

25.5 References

Barcelona M.J. (1984) TOC determinations in ground water. Groundwater 22(1): 18–24

Bau M. & Dulski P. (1996) Anthropogenic origin of positive gadolinium anomalies in river waters. Earth and Planetary Science Letters 143: 245–255.

Bau M., Knappe A. & Dulski P. (2006) Anthropogenic gadolinium as a micropollutant in river waters in Pennsylvania and in Lake Erie, northeastern United States. Chemie der Erde – Geochemistry, 66: 143–152.

Chiou C.T., Malcolm R.L., Brinton T.I. & Kile D.E. (1986) Water solubility enhancement of some organic pollutants and pesticides by dissolved humic and fulvic acids. Environ Sci Technol 20(5):502–508

Calvo-Flores F.G., Isac-Garcia J. & Dobado J.A. (2018): Emerging Pollutants: Origin, Structure, and Properties. Wiley-VCH

Hartmann Ch. (2017): Abgeleitete Toleranzwerte für ausgewählte Arzneimittelwirkstoffe in Trinkwasser. REP-0623. Umweltbundesamt. Wien.

Hoffmann A. & Gunkel G. (2011) Bank filtration in the sandy littoral zone of Lake Tegel (Berlin): Structure and dynamics of the biological active filter zone and clogging processes. Limnologica 41:10–19

Kümmerer K. & Helmers, E. (2000) Hospital effluents as a source of gadolinium in the aquatic environment. Environmental Science & Technology 34:573–577.

Robertson W.D., Van Stempvoort D.R., Spoelstra J., Brown S.J. & Schiff S.L. (2015): Degradation of sucralose in groundwater and implications for age dating contaminated groundwater. Water Research 88 653–660.

Van der Veen I. & de Boer J. (2012) Phosphorus flame retardants: Properties, production, environmental occurrence, toxicity and analysis. Chemosphere – Environmental Chemistry. Volume 88, Issue 10: 1119–1153.

Verplanck P.L., Taylor H.E., Nordstrom D.K. & Barber L.B. (2005) Aqueous stability of gadolinium in surface waters receiving sewage treatment plant effluent, Boulder Creek, Colorado. Environmental Science & Technology 39: 6923–6929.

26 Wide-scope target and non-target screening of MAXX large-volume samples of the Danube River with LC-ESI-HRMS and GC-EI-HR-MS

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Abstract

This chapter reports the concentrations and occurrences of organic chemicals dissolved in the Danube River water. The water samples were collected using an automated sampling device, the so-called MAXX large volume solid phase extraction (LVSPE). The SPE technique utilises specific polymers, which adsorb the chemicals dissolved in the water. This device was used during JDS3 for the first time in a larger survey. The purpose of the LVSPE technique is the on-site (on shore) extraction of larger water volumes without need to bring the water sample to the laboratory. The latter is a logistical challenge and may cause alteration of the sample or a secondary contamination of the samples. The extracts of the water samples were subjected to gas and liquid chromatography, high-resolution mass spectrometry analysis, and a set of different bioassays. The main goal was the assessment of the chemical content of the water samples using target and non-target screening. Data from LC-HRMS non-target screening were evaluated using frequency and rarity scores, which combine signal intensity of a peak in a dataset with its frequency of occurrence in that dataset in one single number and allow for a rapid assessment of frequently occurring and site-specific contamination. Based on this approach, mainly PEG-based surfactants could be identified as the predominant, ubiquitous compounds in the Danube River Basin, with higher concentration levels at the lower stretches and adjacent tributaries.

26.1 Introduction

The anthropogenic pollution of water resources with organic and inorganic chemicals is a major global societal and ecological challenge. More than 350 000 chemicals are already in commerce and thousands of new chemicals enter the marketplace annually many of them are expected to be found in the environment due to their emissions to air, water and soil. Key problems are the missing or inadequate abatement options (e.g. green chemistry, closed production cycles, enhanced treatment technologies) on the emission site and the current water quality protection, assessment and management to foster a clean aquatic environment (Brack, 2019; Posthuma et al., 2019; Munthe et al., 2017). The weakness of the current chemical water quality is the focus on a few legacy chemicals and a few emerging chemicals on the EU WFD surface water Watch List, while many more anthropogenic chemicals can be detected simultaneously in our aquatic resources (Altenburger et al., 2019; Arle et al., 2016; Loos, Robert et al., 2018). However, an emission of a chemical or mixtures thereof into aquatic systems and the exposure of organisms to those mixtures

does not always cause adverse biological effects or indicates mitigation measures (Altenburger et al., 2019). Hence, a better understanding of the present mixtures and their associated combined effects are required to specify suitable strategies for monitoring and assessment of the aquatic environment (Faust et al., 2019; Altenburger et al., 2019). The strategy to overcome the limits of pure target analysis are wide-scope chemical target screening and non-target screening approaches utilising high performance liquid- and gas-chromatography coupled to high resolution mass spectrometers (HRMS). The advantage is that the HRMS technology allows a measurement and thus digital archiving of signals of all compounds in a sample, while target analysis only records specific signals for selected compounds. This allows for the detection and identification of new contaminants of emerging concern (CECs) and retrospective monitoring strategies (Brack et al., 2019; Hollender et al., 2019). Retrospective monitoring is the assessment of stored samples with modern technologies to unravel e.g. newly identified CECs. Environmental specimen banks (ESBs) are physical archives of environmental samples to preserve them for the future analysis with advanced analytical technologies. However, water samples are likely impossible to preserve in the long-term in ESBs because large volumes of water need to be homogenated, sub-sampled and stored. The latter is a major challenge because of possible secondary contamination, inhomogeneity, degradation and finite storage capacities in the archives. Digital repositories of the measured data are the state of the art to preserve chemical signals from HRMS measurement of finite samples. They are well established in life sciences as repositories and virtual research environments (Haug et al., 2012; Salek et al., 2013; Wang et al., 2016). The NORMAN Digital Freezing Platform (DSFP) was developed as a repository, suspect screening and retrospective monitoring tool for the storage and analysis of the raw data of HRMS measurements of environmental samples (Alygizakis et al., 2019). While the integrity of real samples is finite, the digital storage of measured samples in repositories is infinite (if the raw data files are stored in an open data format and the repository is always updated to recent software technologies including data transformation of existing dataset into new enhanced formats). With respect to the chemical mixtures, wide-scope target screening and non-target screening and retrospective monitoring will be used in future for a better assessment of environmental mixtures. A better knowledge of chemical occurrence will help to derive combined effect estimates as a basis for an advanced development of environmental quality standards (Altenburger et al., 2019). Complementary, an adequate set of bioanalytical tools is required to gain information about bioactivity of the environmental mixtures and to understand the potential adverse outcomes of toxicity drivers and their mixtures and of mixture toxicity. The 5-year EU-funded project SOLUTIONS put major efforts into the development of new concepts and tools for holistic and solutions-oriented monitoring and impact assessment of complex environmental mixtures to cope with the weaknesses of the current European water monitoring strategy (Brack, 2019). Last, but no least, every analytical process starts with a sampling technology and approach that is able to cover a broad range of water contaminants with different physico-chemical properties. In the best case, the coverage and the analytical recoveries of the chemicals and their effects is known in detail. Effect-based monitoring (EBM) requires larger volumes of samples than chemical monitoring for proper dosing of the samples in the bioassays (Schulze et al., 2017). MAXX large-volume solid phase extraction (MAXX LVSPE) (Schulze et al., 2017) is a well assessed and well performing sampling approach especially developed for the purpose of EBM (Neale et al., 2018; König et al., 2017; Neale et al., 2015). The goal of this chapter is to demonstrate the feasibility of the application of target and non-target screening as a future holistic monitoring of environmental samples.

26.2 Methods

On-site large-volume solid phase extraction (LVSPE, MAXX, Rangendingen, Germany) was performed at all 51 surface water sampling sites of JDS4. Briefly, 20 or 50 litres of water were pumped through a glass fibre filter (0.7 µm nominal pore size, Sartorius) and extracted using 10 g of HR-X material (Macherey-Nagel). In general, a volume of 20 L was sampled. The sites JDS4-6 (Jochenstein), JDS4-24 (Budapest, downstream M0 bridge) and JDS4-47 (Downstream Ruse) were selected for an intercomparison of sampling techniques and analytical methods. Thus, a volume of 50 L was collected to gain sufficient extract for all purposes. The SPE cartridges were eluted with a mixture of ethyl acetate and methanol (50:50) and subsequently with methanol (1 % formic acid) and methanol (2 % 7 N ammonia in methanol). The details on the LVSPE, the elution method and the evaluation of the performance of the sampling approach are available in Schulze et al. (2017), Välitalo et al. (2017) and Neale et al. (2018).

For analysis of LVSPE extracts by LC-HRMS 100 µL of extract (EF1000) were transferred into a 2 mL autosampler vial with an insert and 10 µL of an internal standard mixture containing 40 isotope-labelled compounds (1 µg mL−1), 30 µl of methanol and 60 µL of water were added. Chemical analysis was done as for the direct injection of water samples. An injection volume of 5 µL was used. Matrix- and method-matched calibration standards were prepared by spiking 1 L of water from a pristine stream in the Upper Harz mountains with 15 concentration levels (0.1, 0.2, 0.5, 1, 2, 5, 10, 20, 50, 100, 200, 500, 1000, 2000 and 5000 ng/L) and processing these by laboratory-scale SPE using a procedure downscaled from the LVSPE method. For data evaluation the same methods as for the water samples were used. For analysis of LVSPE extracts by GC-HRMS, 100 µl of extract (EF1000) were transferred into a 2 mL autosampler vial with an insert and 10 µL of an internal standard mixture containing isotope-labelled compounds (1 µg/mL) were added. GC-HRMS analysis was done using a Q Exactive GC hybrid quadrupole-Orbitrap GC-MS/MS system (Thermo Scientific). Chromatographic separation was performed using a DB-5MS capillary column (30 m × 250 µm, 0.25 µm; Agilent Technologies). Helium with a flow rate of 1 mL/min was used as carrier gas. 2 µL of the extracts were injected into a Thermal Desorption Unit (TDU-2; Gerstel), containing disposable desorption tubes with glass inserts for liquid injection. During thermal desorption the sample was trapped in a cooled injection system (CIS; Gerstel) at 10 °C and subsequently the injector temperature was raised at 720 °C/min to 300 °C. During the HRMS measurements, the temperature of the ion source was 250 °C, and ionization was performed by electron ionization at an emission current of 50 µA and an electron energy of 70 eV. The measurement was performed in full scan mode in the mass range of m/z 50-650 at a resolving power of 60,000 (referenced to m/z 200). Data evaluation was done the same way as for the LC-HRMS screening samples using MZmine and MZquant. The raw files were converted to mzML format and centroided with ProteoWizard (http://proteowizard.sourceforge.net) using the build in vendor's library. The mzML files were processed with MZmine 2.52 (http://mzmine.github.io) for peak picking, alignment, gap filling and peak annotation (Beckers et al., 2020; Hu et al., 2016). The resulting annotated peak list was further analysed using an in-house R-package "MZquant" for automated quantification. Compounds with very broad peaks or high backgrounds were analysed with Tracefinder 4.1 (Thermo Scientific). The aligned peak table exported from MZmine was further processed for the prioritisation of site-specific peaks using the rarity score (RS) proposed by Krauss et al. (2019) and for frequently occurring peaks using a frequency

score (FS). For each peak *i*, RS and FS were calculated according to:

$$RS_i = \frac{maximum\ intensity_i}{median\ intensity_i} \times \frac{total\ number\ of\ samples}{number\ of\ positive\ detects\ above\ the\ threshold\ intensity\ of\ i} \quad (1)$$

$$FS_i = \frac{average\ intensity_i \quad \times \quad number\ of\ positive\ detects\ above\ the\ threshold\ intesity\ of\ i}{threshold\ intensity_i} \quad (2)$$

The threshold intensity was calculated from injection, solvent and extraction blanks intensities according to equation 3 or set to the peak detection threshold level of 10,000 if no peaks were present in the blanks.

$$threshold\ intensity_i = average_{i,j} + Qt_i(p, df) \times s_{i,j} \quad (3)$$

Where the *threshold intensity*$_i$ of each peak *i* is calculated from the *average* of the peak *i* in all blank samples *j*, Qt_i is quantile of the t-distribution for each peak *i* with the probability *p* and the degree of freedom *df* is N_j -1, where N_i is the number of blank peaks and s_j is the standard deviation of peak *i* in all blank samples *j*. In cases of $N_j \leq 4$, Qt_i was replaced by a fixed factor of 2.

26.3 Results and Discussion

26.3.1 Target screening analysis

In the JDS4 MAXX LVSPE surface water samples, 298 organic substances out of 519 targeted compounds have been detected with a frequency of detection (FoD) of at least one sampling site. The sampling site JDS4-8 did not contain any compounds of interest. The cause could not be clarified. The analysis and detailed assessment of steroids and other endocrine disruptors is included in Chapter 28.

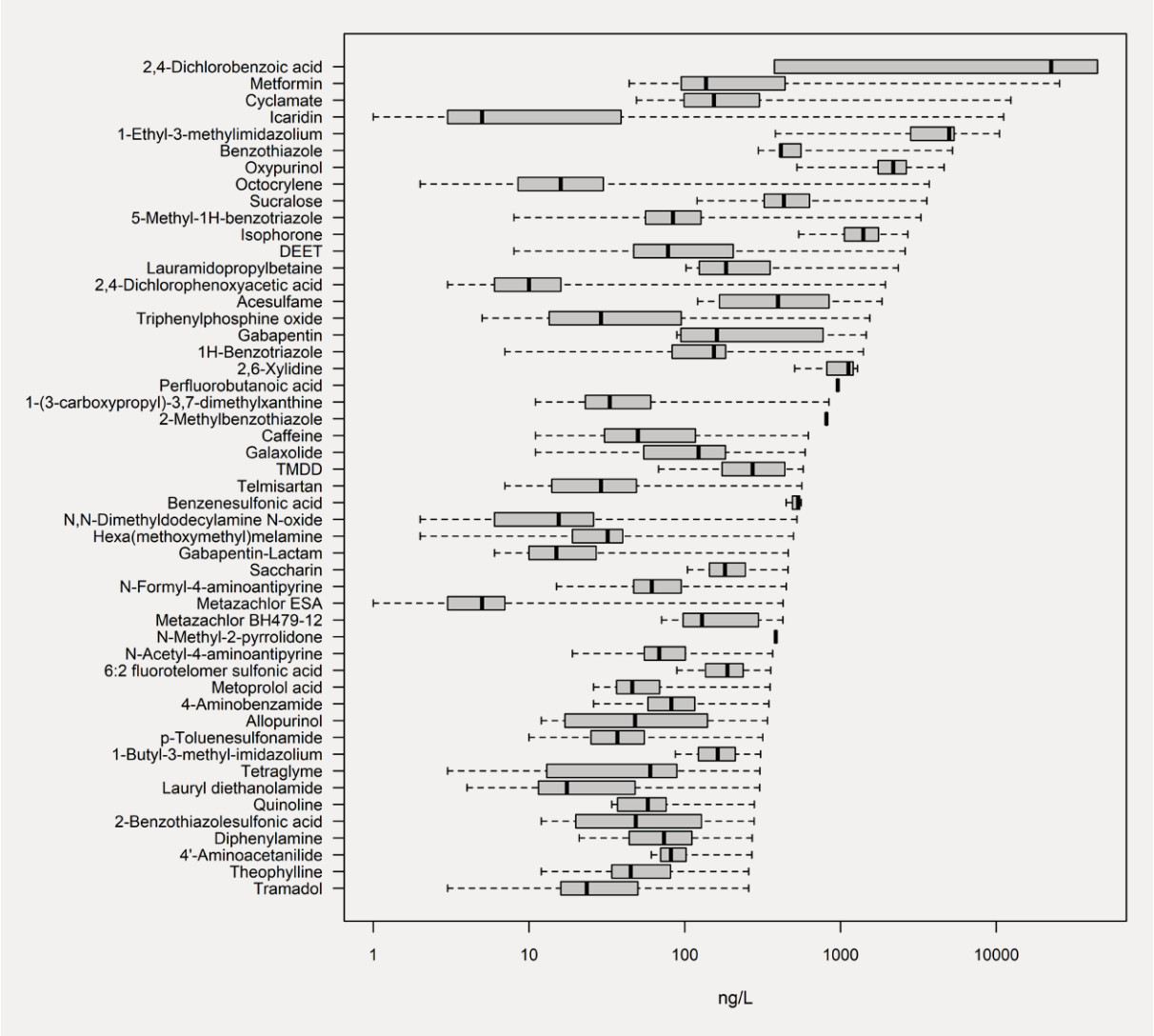

Figure 1: Ranges of concentrations of frequently determined organic compounds in Danube River surface water samples extracted by MAXX LVSPE (rank 1-50). The compounds are ordered by their maximum concentrations monitored.

Figures 1-3 show the boxplots of the concentrations of the 150 highest ranked substances. The compounds were ordered by their maximum concentration value. The concentration levels span over 2-3 orders of magnitude. The compound with the maximum concentrations was 2,4-dichlorobenzoic acid, but it was found only at two sites (site JDS4-3 (Kelheim) with 44,000 ng/L and site JDS4-46 (Russenski Lom tributary) with 376 ng/L). Metformin, a type 2 diabetes drug, was detected at 50 sites with a maximum concentration of 25,000 ng/L at JDS4-42 (Timok tributary). Further pharmaceuticals found in several of the MAXX LVSPE samples were the xanthine oxidase inhibitors allopurinol (which is used to treat gout and Leishmaniasis)

and its metabolite / transformation product oxypurinol, which is also an active drug ingredient itself, with maximum concentrations of 340 and 4600 ng/L, respectively. The angiotensin II receptor blockers valsartan, candesartan, losartan and telmisartan were also present in many of the samples with concentrations up to 565 ng/L (telmisartan). Furthermore, the anticonvulsants gabapentin-lactam (in 42 samples with a maximum of 462 ng/L) and lamotrigine (in 50 samples with a maximum of 224 ng/L) and the beta blocker metoprolol and its TP metoprolol acid at 46 and 23 sampling sites with concentrations up to 38 and 353 ng/L, respectively. N-formyl-4-aminoantipyrine and N-acetyl-4-aminoantipyrine, the urine metabolites of the non-opioid analgesic pharmaceutical aminopyrine were detected as well as the TPs 10,11-dihydro-10,11-dihydroxycarbamazepine, 10,11-dihydro-10-hydroxycarbamazepine and 2-hydroxycarbamazepine of the anticonvulsant drug carbamazepine.

Other compounds from the groups of food ingredients, industrial substances and other urban chemicals were the sweeteners cyclamate, sucralose, acesulfame and saccharin (cyclamate up to 12,400 ng/L), the sun screen octocrylene (in 48 samples with up to 3700 ng/L), the polymer precursor isophorone (in 21 samples with up to 2700 ng/L), the vulcanisation accelerator benzothiazole (at 5 sites up to 5200 ng/L) and the corrosion inhibitors 1H-benzotriazole and 4+5-methyl-1H-benzotriazole.

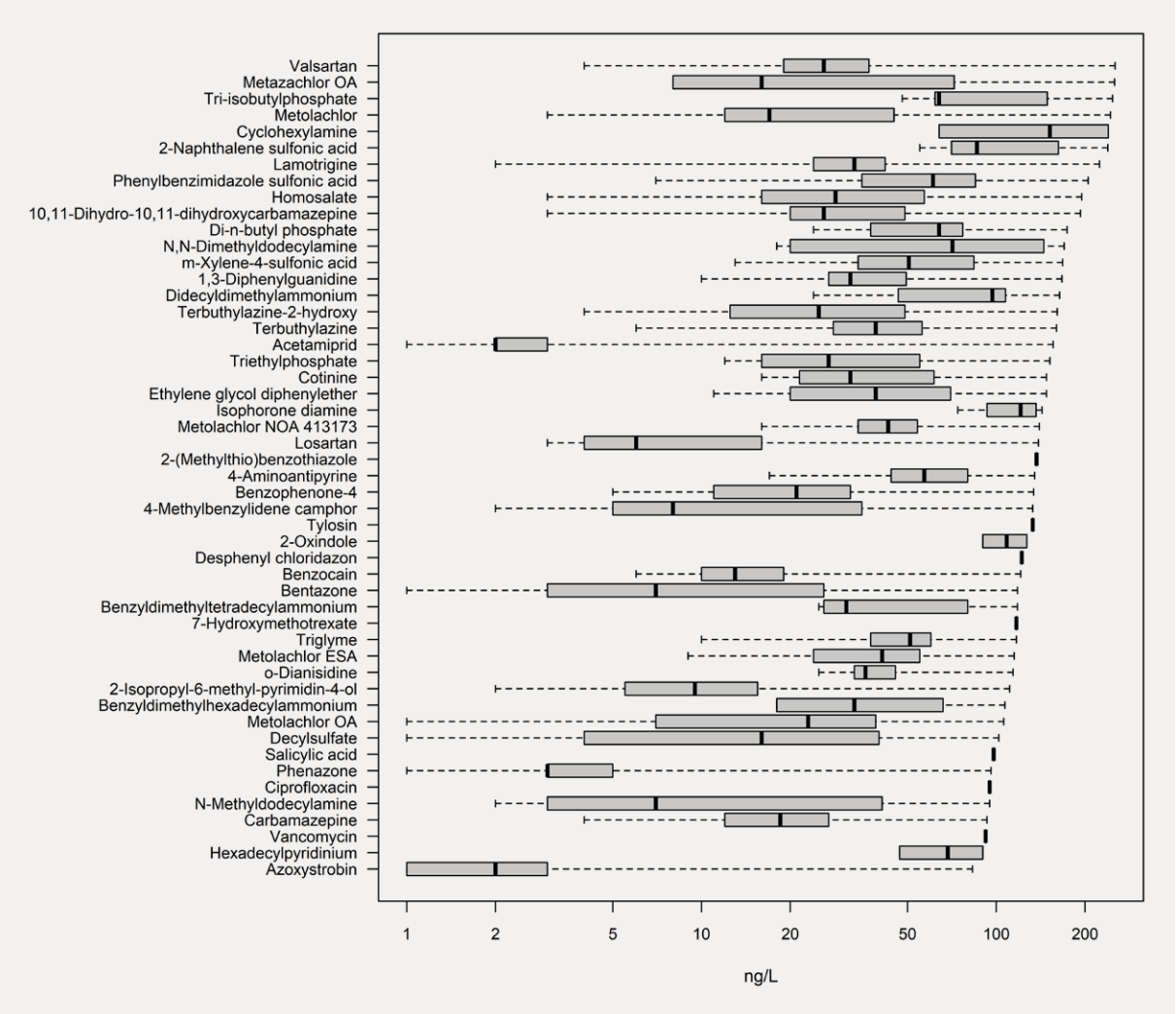

Figure 2: Ranges of concentrations of frequently determined organic compounds in Danube River surface water samples extracted by MAXX LVSPE (rank 51-100). The compounds are ordered by their maximum concentrations monitored.

In comparison to the grab water samples (Chapter 27), the chemicals used in farming were lower ranking in the MAXX LVSPE samples. The enrichment of some compounds, which are not found in the grab water samples can result in lower detection limits (Chapter 37). The dominating pesticide group are the herbicides such as 2,4-dichlorophenoxyacetic acid, terbuthylazine, bentazone, metolachlor and its TPs metazachlor ESA, S-metolachlor NOA 413173 and metolachlor OA. 2,4-dichlorophenoxyacetic acid was detected at 25 sites with concentrations up to 1944 ng/L.

The potential impact of some of the above chemicals are discussed in Chapter 35. For example, the herbicides did also rank in higher positions in the risk estimation in Chapter 35, while other compounds such as 2-dichlorobenzoic acid not. The reason is that pesticides are used in the environment for pest control and thus they have a high intrinsic toxicity.

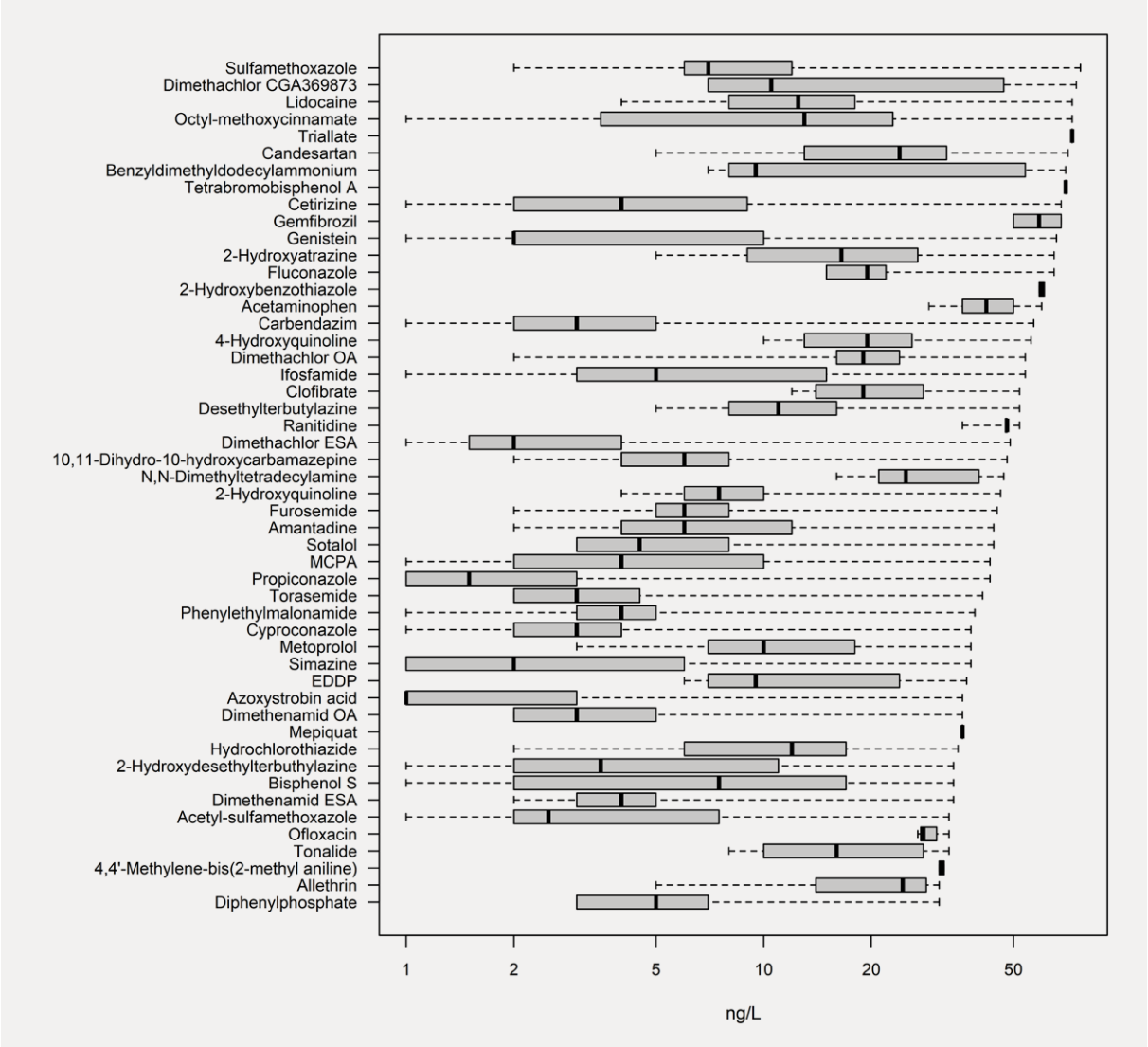

Figure 3: Ranges of concentrations of frequently determined organic compounds in Danube River surface water samples extracted by MAXX LVSPE (rank 101-150). The compounds are ordered by their maximum concentrations monitored.

26.3.2 Non-target screening analysis

By non-target screening, altogether 95,669 peaks could be detected in ESI+ mode, and 31,083 in ESI- mode, which was reduced to 91,419 peaks (ESI+) and 27,239 (ESI-) peaks above the threshold through the blank correction procedure. Table 1 gives a breakdown of the numbers of detected peaks with high FS and RS values in the whole dataset and across the individual samples. Each peak (or feature) in non-target analysis represents an ionised molecule measured in the mass spectrometer. The molecules include a chemical in the sample (natural or artificial) measured as its molecule ion (M, typically measured as M+H or M-H in electrospray ionisation), but also its isotopes (e.g. C13 isotopes) and / or so called adducts thereof. Adducts can be ions with different ionisations (e.g. M+) or containing additional atoms (e.g. sodium: M+Na). Non-target workflows may include therefore steps to annotate isotopes and adducts in order to clean up the peak list for further analysis.

Table 1: Total numbers of detected peaks with FS and FS values >500 in the whole dataset and across individual samples.

	ESI+			ESI-		
	Whole dataset	**Median in samples**	**Range in samples (min-max)**	**Whole dataset**	**Median in samples**	**Range in samples (min-max)**
FS > 5000	692	688	622-692	4	4	3-4
5000 > FS > 1000	2804	2704	1467-2782	96	92	61-96
1000 > FS > 500	3389	2458	840-2620	212	204	88-212
RS > 5000	191	7	0-124	14		0-13
5000 > RS > 1000	2078	141	51-1546	118		
1000 > RS > 500	2413	290	91-1317	253		

For ESI+ mode data, about 60 % of the peaks with FS > 5000 had retention times (RT) between 14 and 16 minutes and m/z values between 450 and 1400 (Figure 4) and could be assigned to alkyl-polyethylene glycol ether (PEG) surfactants showing predominantly C_{10}- to C_{16}-alkyl chains and 5 to 30 ethylene-oxide units. These technical surfactant mixtures showed complex mass spectra with dominating $M+NH_4^+$, and lower intensity $M+Na^+$ and $M+H^+$ adducts, as well as doubly charged ions (mainly $[M+2\ NH_4]^{2+}$), as shown for one example in Figure 5. While the retention times of these compounds increased with increasing alkyl chain length, the retention time was only minimally affected by the length of the polyethylene glycol chain. Further homologue series were evident in the data, such as PEG, RTs 6.5-10 min and another substituted PEG, RTs between 12 and 14 min. Overall, more than 85% of the peaks with FS > 5000 in ESI+ mode were contained in homologue series, pointing to the huge importance of surfactants in the inventory of high-intensity and frequently occurring peaks in the dataset. In ESI+ mode, the peaks with highest FS values

showed a trend for higher intensities at the lower stretches of the Danube and adjacent tributaries than at the upper stretches, suggesting raw wastewater as main input pathway of the associated surfactants (Figure 6). The two peaks of polyethylene glycol (m/z 592.3892, RT 9.3 min and m/z 636.4152, RT 9.5 min) were present in all samples, but showed distinct highest intensities in tributaries of the lower Danube, particularly site JDS4-44, Iskar. The overall low intensities of peaks in sample JDS4-8 coincides with the findings of the target screening. Among the peaks not related to surfactants most notably were a peak m/z 387.1924, RT 12.4 min, $[C_{19}H_{31}O_6P+H]^+$, for which the MS^2 spectrum suggest a so far unknown aryl-alkyl-phosphate, and three isomer peaks at m/z 288.2891, RT 10.9, 11.1 and 11.2 ($[C_{17}H_{38}O_2N+H]^+$), with identical MS^2 fragments.

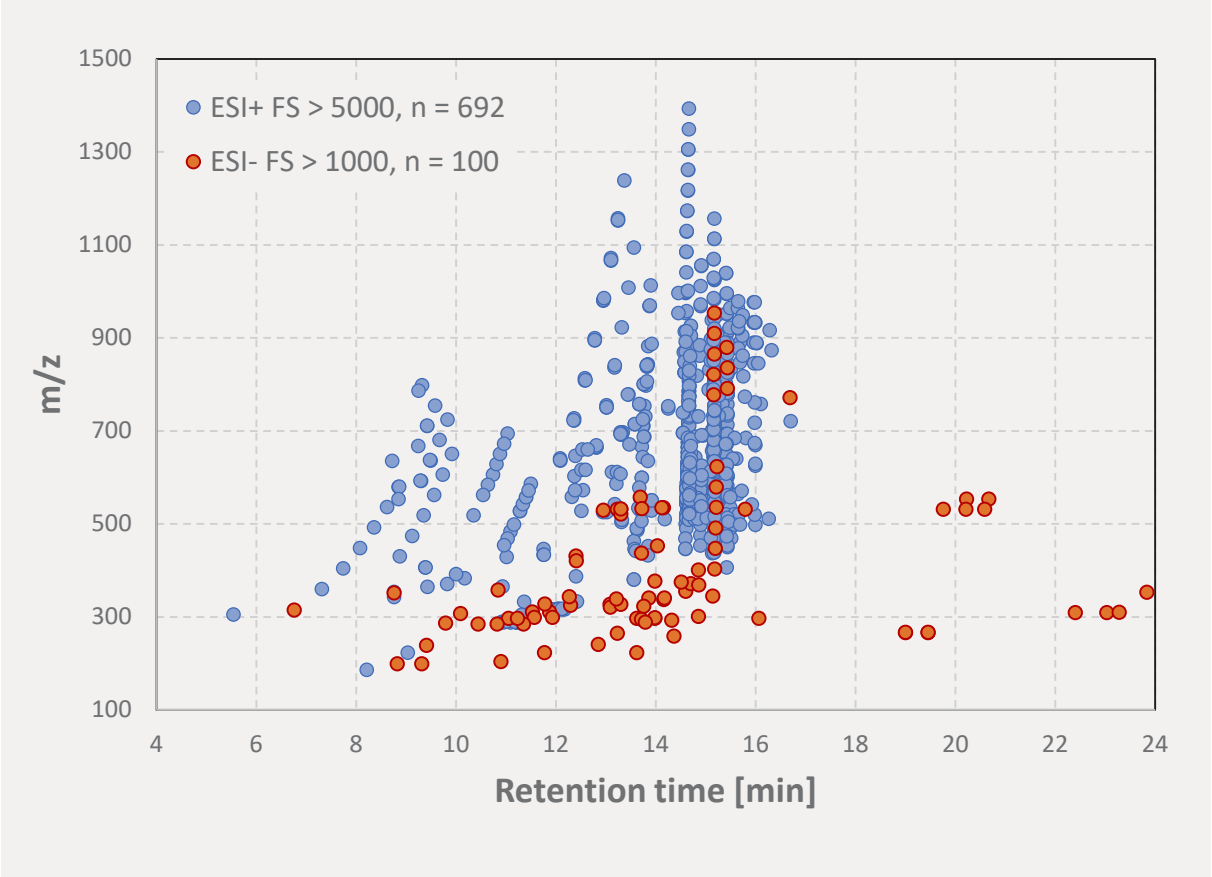

Figure 4: m/z vs. retention time diagram of peaks with high frequency scores in the JDS4 LVSPE samples.

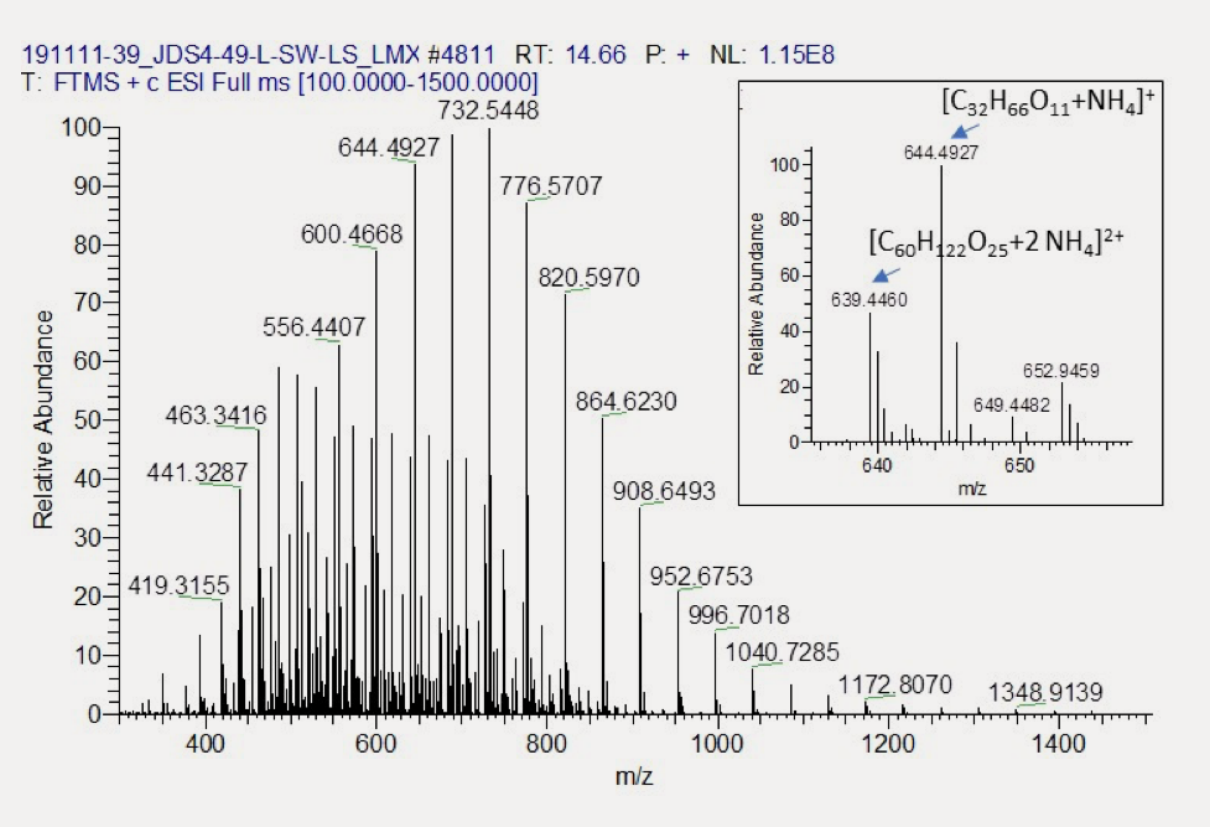

Figure 5: Mass spectrum of Dodecyl-PEG ethers $C_{12}H_{25}$-$(C_2H_4O)_n$-OH in sample JDS49 showing predominantly singly and doubly charged ammonium adducts.

For ESI- mode data, about 30% of the peaks with FS > 1000 could be attributed to surfactants, mainly alkyl sulfates, and sulfophenyl alkly carboxylic acids or sulfotetralin-alkyl carboxylic acids (SPACs). The latter two are degradation products of commercial linear alkylbenzene sulfonate surfactants, all three groups have been described as widely occurring wastewater-derived contaminants (Gago-Ferrero et al., 2015). Among the peaks not related to surfactants an isopropylbenzenesulfonic acid isomer (m/z 199.0436, RT 9.3 min could be identified, which was previously reported for the Danube in Schymanski et al. (2015). Several peaks with retention times close to the column dead time (around 0.5-0.6 min) and strong negative mass defects (m/z 260.7718, 262.7688, 316.7392) were likely artefacts from the ionisation, as they co-occurred with $[FeCl4]^-$ complexes resulting from trace chloride and FE from the electrospray capillary. In ESI- mode, the peaks with highest FS values showed no clear trends along the Danube or its tributaries (Figure 7).

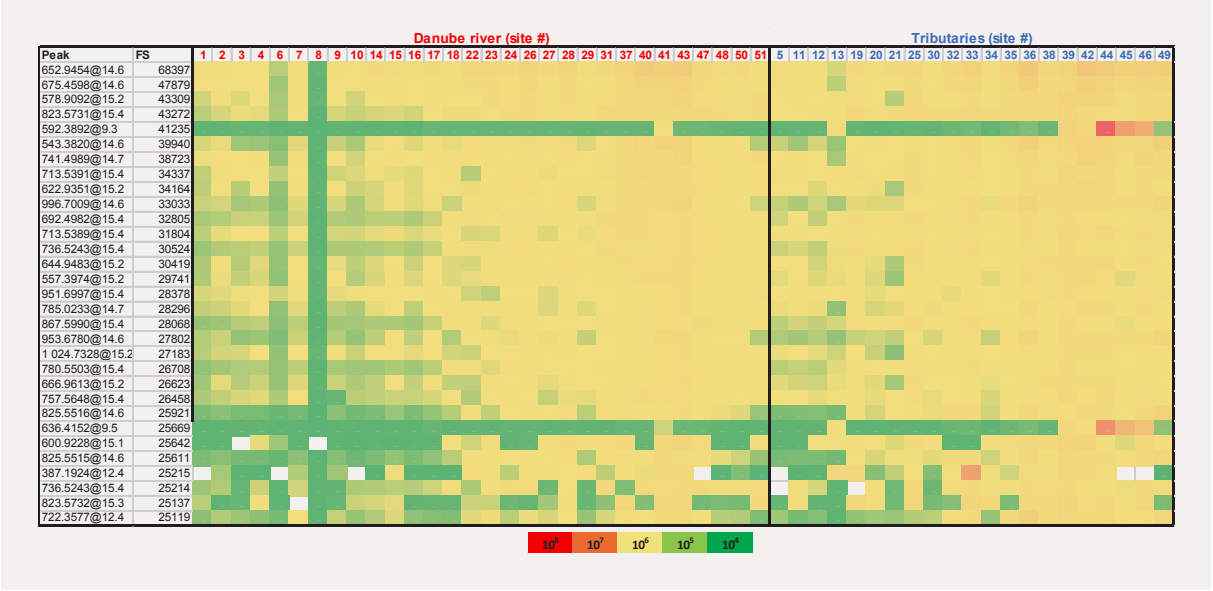

Figure 6: Occurrence and intensities of peaks in ESI+ mode with FS values > 25000 at all studied sites.

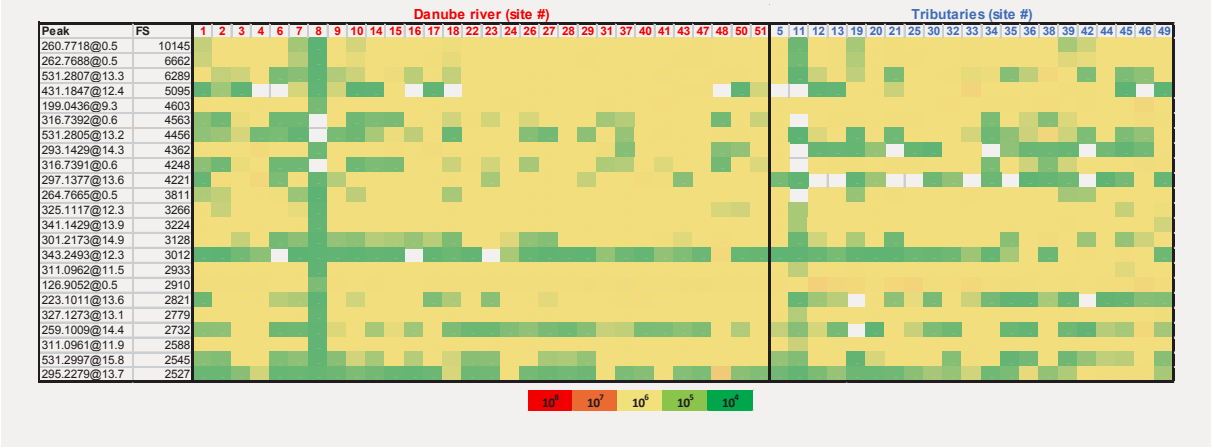

Figure 7: Occurrence and intensities of peaks in ESI- mode with FS values > 2500 at all studied sites.

Peaks with high rarity scores (RS) indicating site-specific contamination were predominantly present in sample JDS4-49 (Prut) in ESI+ and ESI- mode, and in samples JDS4-31 (Danube at Ilok) and the Tisza and Sava tributaries (JDS4-32, -33, -34; Figure 8 and 9). Many of these peaks could be identified as surfactants, showing high retention times > 20 minutes and m/z above 400, indicating that also site-specific surfactants contamination might occur besides the ubiquitous one. At site JDS4-34, the peak m/z 330.1265 (RT 8.3 min, $[C_{17}H_{19}O_2N_3S+H]^+$) could be tentatively identified as omeprazole sulfide, based on a good MS/MS match with literature (Shin et al., 2020), and the peak m/z 354.0878 (RT 8.8 min, $[C_{16}H_{14}F_3N_3OS+H]$) as lansoprazole sulfide. Both compounds are metabolites (or synthesis impurities) of the proton pump inhibitor drugs omeprazole and lansoprazole, respectively and have so far not been reported in surface water and the finding suggests a site-specific source in the Sava River.

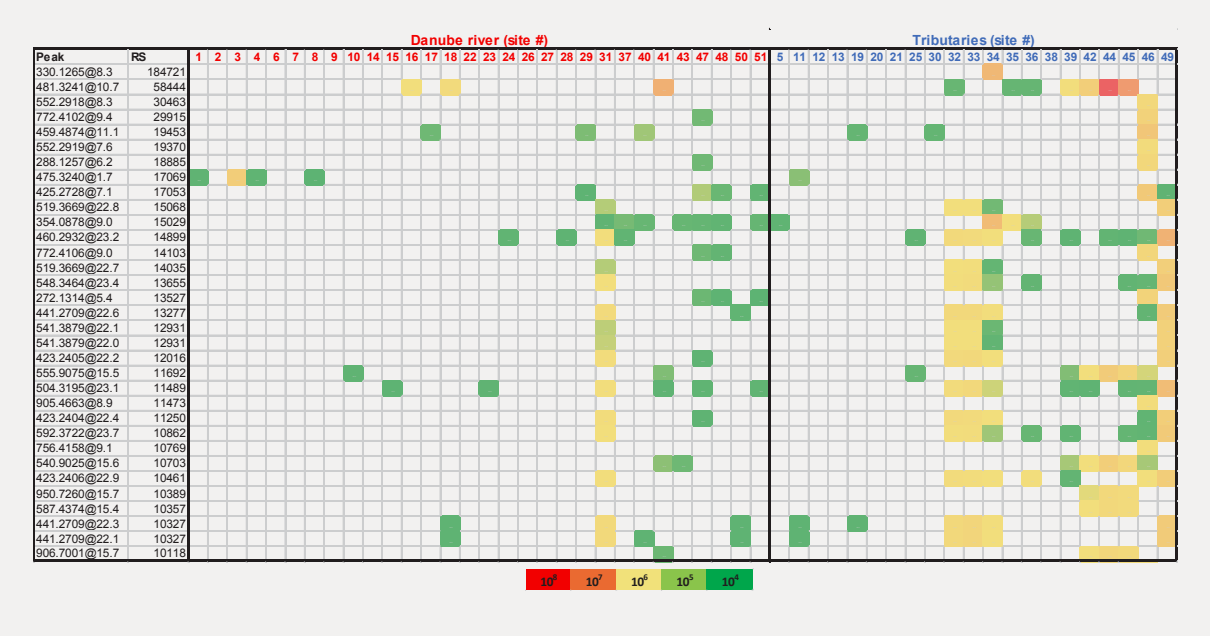

Figure 8: Occurrence and intensities of peaks in ESI+ mode with RS values > 10000 at all studied sites.

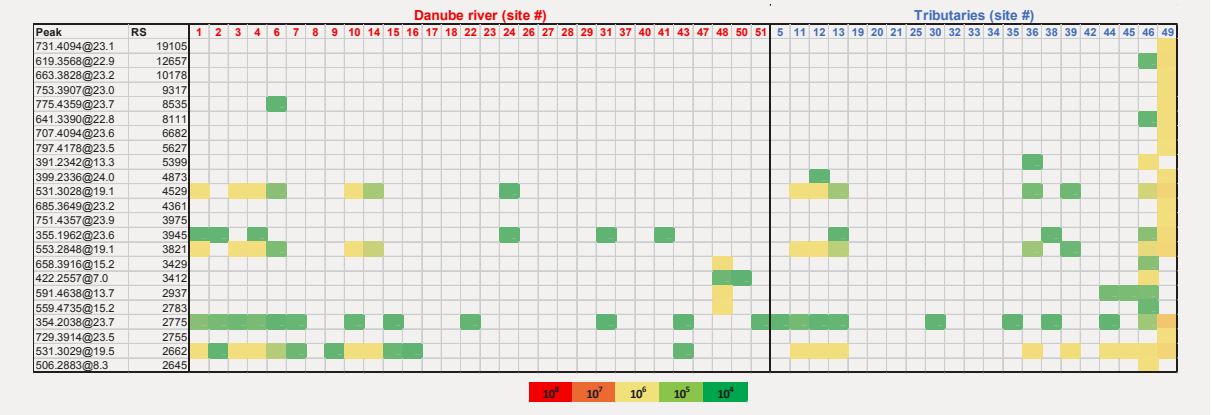

Figure 9: Occurrence and intensities of peaks in ESI- mode with RS values > 2500 at all studied sites.

26.4 Conclusions

The sampling of 51 sites of the Joint Danube Survey 4 was performed successfully using the MAXX LVSPE sampling device. All chemical analysis was finalised, but unfortunately the effect-based analysis was not included in this report due to the COVID-19 pandemic.

In the MAXX LVSPE samples, the most abundant compounds were pharmaceuticals and their transformation products and other compounds from the groups of food ingredients, industrial substances and other urban chemicals.

The frequency and rarity scores used, provided a simple and robust measure to prioritize site-specific and frequently occurring compounds, as they combine frequency of occurrence and peak intensities into a single value. Non-target screening revealed the dominance of a range of surfactants as the most frequently occurring compounds in the Danube River basin and points to sites where a site-specific contamination occurs. Overall, the data provide a basis for further identification efforts.

26.5 References

ALTENBURGER R., BRACK W., BURGESS R. M., BUSCH W., ESCHER B. I., FOCKS A., MARK HEWITT L., JACOBSEN B. N., DE ALDA, M. L., AIT-AISSA S., ET AL. (2019): Future water quality monitoring: improving the balance between exposure and toxicity assessments of real-world pollutant mixtures. Environmental Sciences Europe, 31: 12.

ALYGIZAKIS N. A., OSWALD P., THOMAIDIS N. S., SCHYMANSKI E. L., AALIZADEH R., SCHULZE T., OSWALDOVA M., SLOBODNIK, J. (2019): NORMAN digital sample freezing platform: A European virtual platform to exchange liquid chromatography high resolution-mass spectrometry data and screen suspects in "digitally frozen" environmental samples. TrAC Trends in Analytical Chemistry 115: 129–137.

ARLE J., MOHAUPT V., KIRST I. (2016): Monitoring of Surface Waters in Germany under the Water Framework Directive—A Review of Approaches, Methods and Results. Water 8: 217.

BECKERS L.-M., BRACK W., DANN J. P., KRAUSS M., MÜLLER E., SCHULZE T. (2020): Unraveling longitudinal pollution patterns of organic micropollutants in a river by non-target screening and cluster analysis. Science of The Total Environment 727: 138388.

BRACK W. (2019): Solutions for present and future emerging pollutants in land and water resources management. Policy briefs summarizing scientific project results for decision makers. Environmental Sciences Europe 31: 74.

BRACK W., HOLLENDER J., DE ALDA M. L., MÜLLER C., SCHULZE T., SCHYMANSKI E., SLOBODNIK J., KRAUSS M. (2019). High-resolution mass spectrometry to complement monitoring and track emerging chemicals and pollution trends in European water resources. Environmental Sciences Europe 31: 62.

FAUST M., BACKHAUS T., ALTENBURGER R., DULIO V., VAN GILS J., GINEBREDA A., KORTENKAMP A., MUNTHE J., POSTHUMA L., SLOBODNIK J., ET AL. (2019): Prioritisation of water pollutants: the EU Project SOLUTIONS proposes a methodological framework for the integration of mixture risk assessments into prioritisation procedures under the European Water Framework Directive. Environmental Sciences Europe 31: 66.

GAGO-FERRERO P., SCHYMANSKI E. L., BLETSOU A. A., AALIZADEH R., HOLLENDER J. THOMAIDIS N. S. (2015): Extended suspect and non-target strategies to characterize emerging polar organic contaminants in raw wastewater with LC-HRMS/MS. Environmental Science & Technology 49: 12333-12341.

HAUG K., SALEK R. M., CONESA P., HASTINGS J., DE MATOS P., RIJNBEEK M., MAHENDRAKER T., WILLIAMS M., NEUMANN S., ROCCA-SERRA P., ET AL. (2012): MetaboLights—an open-access general-purpose repository for metabolomics studies and associated meta-data. Nucleic Acids Research 41: 781-786.

HOLLENDER J., VAN BAVEL B., DULIO V., FARMEN E., FURTMANN K., KOSCHORRECK J., KUNKEL U., KRAUSS M., MUNTHE J., SCHLABACH M., ET AL. (2019): High resolution mass spectrometry-based non-target screening can support regulatory environmental monitoring and chemicals management. Environmental Sciences Europe 31: 42.

HU M., KRAUSS M., BRACK W., SCHULZE T. (2016). Optimization of LC-Orbitrap-HRMS acquisition and MZmine 2 data processing for non-target screening of environmental samples using design of experiments. 2016/10/22 ed. Analytical and Bioanalytical Chemistry 408: 7905–7915.

KÖNIG M., ESCHER B. I., NEALE P. A., KRAUSS M., HILSCHEROVÁ K., NOVÁK J., TEODOROVIĆ I., SCHULZE T., SEIDENSTICKER S., KAMAL HASHMI M. A., ET AL. (2017): Impact of untreated wastewater on a major European river evaluated with a combination of in vitro bioassays and chemical analysis. Environmental Pollution 220: 1220–1230.

KRAUSS M., HUG C., BLOCH R., SCHULZE T., BRACK W. (2019): Prioritising site-specific micropollutants in surface water from LC-HRMS non-target screening data using a rarity score. Environmental Sciences Europe 31: 45.

LOOS R., MARINOV D., NAPIERSKA D., LETTIERI T. (2018): Review of the 1st Watch List under the Water Framework Directive and recommendations for the 2nd Watch List. JRC. Available at: https://doi.org/10.2760/614367.

MUNTHE J., BRORSTRÖM-LUNDÉN E., RAHMBERG M., POSTHUMA L., ALTENBURGER R., BRACK W., BUNKE D., ENGELEN,G., GAWLIK B. M., VAN GILS J., ET AL. (2017): An expanded conceptual framework for solution-focused management of chemical pollution in European waters. 2017/03/25 ed. Environmental Sciences Europe 29: 13.

NEALE P. A., AIT-AISSA S., BRACK W., CREUSOT N., DENISON M. S., DEUTSCHMANN B., HILSCHEROVA K., HOLLERT H., KRAUSS M., NOVÁK J., ET AL. (2015): Linking in vitro effects and detected organic micropollutants in surface water using mixture toxicity modeling. Environmental Science & Technology 49: 14614–14624.

NEALE P. A., BRACK W., AIT-AISSA S., BUSCH W., HOLLENDER J., KRAUSS M., MAILLOT-MARECHAL E., MUNZ N., SCHLICHTING R., SCHULZE T., ET AL. (2018): Solid-phase extraction as sample preparation of water samples for cell-based and other in vitro bioassays. Environmental Science: Processes & Impacts, 20: 493–504.

POSTHUMA L., MUNTHE J., VAN GILS J., ALTENBURGER R., MÜLLER C., SLOBODNIK J., BRACK, W. (2019): A holistic approach is key to protect water quality and monitor, assess and manage chemical pollution of European surface waters. Environmental Sciences Europe, 31: 67.

SALEK R. M., HAUG K., CONESA P., HASTINGS J., WILLIAMS M., MAHENDRAKER T., MAGUIRE E., GONZÁLEZ-BELTRÁN A. N., ROCCA-SERRA P., SANSONE S.-A., ET AL. (2013): The MetaboLights repository: curation challenges in metabolomics. Database 2013: bat029–bat029.

SCHULZE T., AHEL M., AHLHEIM J., AÏT-AÏSSA S., BRION F., DI PAOLO C., FROMENT J., HIDASI A. O., HOLLENDER J., HOLLERT H., ET AL. (2017): Assessment of a novel device for onsite integrative large-volume solid phase extraction of water samples to enable a comprehensive chemical and effect-based analysis. Science of The Total Environment 581–582: 350–358.

SCHYMANSKI E., SINGER H., SLOBODNIK J., IPOLYI I., OSWALD P., KRAUSS M., SCHULZE T., HAGLUND P., LETZEL T., GROSSE S., THOMAIDIS N., BLETSOU A., ZWIENER C., IBÁÑEZ M., PORTOLÉS T., DE BOER R., REID M., ONGHENA M., KUNKEL U., SCHULZ W., GUILLON A., NOYON N., LEROY G., BADOS P., BOGIALLI S., STIPANIČEV D., ROSTKOWSKI P., HOLLENDER J. (2015): Non-target screening with high-resolution mass spectrometry: critical review using a collaborative trial on water analysis. Analytical and Bioanalytical Chemistry 407: 6237-6255.

SHIN S.-H., PARK Y., PARK M.-H., BYEON J.-J., ILL L. B., CHOI, J., SHIN Y.G. (2020): Profiling and dentification of omeprazole metabolites in mouse brain and plasma by isotope ratio-monitoring liquid chromatography-mass spectrometric method. Life 10: 115.

VÄLITALO P., MASSEI R., HEISKANEN I., BEHNISCH P., BRACK W., TINDALL A. J., DU PASQUIER D., KÜSTER E., MIKOLA A., SCHULZE T., ET AL. (2017): Effect-based assessment of toxicity removal during wastewater treatment. Water Research 126: 153–163.

WANG M., CARVER J. J., PHELAN V. V., SANCHEZ L. M., GARG N., PENG Y., NGUYEN D. D., WATROUS J., KAPONO C. A., LUZZATTO-KNAAN T., ET AL. (2016): Sharing and community curation of mass spectrometry data with Global Natural Products Social Molecular Networking. Nature Biotechnology 34: 828–837.

27 Wide-scope target and non-target screening of surface water samples by direct injection LC-HRMS techniques

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Abstract

This chapter reports the concentrations and occurrences of organic chemicals dissolved in Danube River water. The water samples were directly subjected to chemical analysis with liquid chromatography and high-resolution mass spectrometry without prior treatment or enrichment. This approach has the advantage that the samples could be analysed as soon as possible after sampling and that the so-called matrix effect is minimised. The main findings of the target analysis were a wide use of herbicides during the sampling period. At almost all sites different herbicides or their transformation products (TPs) were identified such as azines, bentazone, metolachlor and nicoforone. A second prominent group of compounds were pharmaceuticals such as the TPs of the analgesic drug aminopyrine, N-formyl-4-aminoantipyrine and N-acetyl-4-aminoantipyrine, the anticonvulsants carbamazepine (and its TPs), lamotrigine and gabapentin-lactam as well as the angiotensin II receptor (alpha) blockers valsartan, candesartan, losartan and telmisartan, the beta-blocker metoprolol, the antidiabetic metformin and the pain drug tramadol. An important industrial chemical identified was isophorone, a precursor in the production of polymers also used as a solvent. Chemicals of daily use found in the Danube River and its tributaries were the corrosion inhibitors 1H-benzotriazole and 4- as well as 5-methyl-benzotriazole.

27.1 Introduction

The pollution of water resources with organic and inorganic chemicals caused by human activity is a major global societal and ecological challenge. More than 100,000 chemicals are of daily use in industrial (all kind of chemicals), food industry (including packaging), farming, house and personal care products and from other urban activities and many of them are expected to be found in the environment due to their emissions to air, water and soil. Key problems are the missing or too weak abatement options (e.g. green chemistry, closed production cycles, enhanced treatment technologies) on the emission site and the current water quality protection, assessment and management to foster a clean aquatic environment (Brack, 2019; Posthuma et al., 2019; Munthe et al., 2017). The weakness of the current chemical water quality is the focus on a few legacy chemicals and a few current chemicals on the Watch List, while many more anthropogenic chemicals can be detected simultaneously in our aquatic resources (Altenburger et al., 2019; Arle et al., 2016; Loos, et al., 2018). The strategy to overcome the limits of target analysis are wide-scope chemical target and non-target screening approaches utilising high performance liquid chromatography (HPLC) coupled to high resolution mass spectrometers (HRMS). The samples were injected directly in the

HPLC system without prior treatment or enrichment. This approach has the advantage that the samples could be analysed as soon as possible after sampling and that the so-called matrix effect is minimised. The matrix effect is the interference of the chemicals of interest with co-eluting compounds during the measurement. This effect could result in a suppression or an enhancement of peaks and thus causing under- or overestimation of concentrations, respectively. The disadvantage of the direct injection method are higher detection limits and thus a lower number of detectable compounds compared to enrichment methods such as solid phase extraction. Direct injection and measurement of SPE enriched samples are complementary to cover a broader range of compounds to improve risk estimation.

The goal of this chapter is to demonstrate feasibility of the application of rapid wise-scope target screening with minimum sample preparation effort.

27.2 METHODS

The samples were taken during JDS4 by the special sampling teams. Grab samples were transferred to a clean glass beaker and 1 mL of the water sample was transferred on-site into an amber glass vial (1.5 mL, VEREX, Phenomenex) using polypropylene transfer tips. At each site, five sub-samples were collected. For quality control, a vial with 1 mL LCMS grade water was transported with the sample vials as sampling blank. The LCMS water in the vial was drawn in the pipette and released back to the vial to account for possible blank peaks. All samples and blanks were stored at -20 °C until analysis. Samples were prepared for direct large volume injection (100 µL). To this end, 10 µL of a 2 M ammonium formate buffer, 25 µL of methanol and 25 µL of an internal standard mixture containing 40 isotope-labelled compounds (40 ng/mL) were added to 1 mL of sample. Chemical analysis was performed on an UltiMate 3000 LC system (Thermo Scientific) coupled to a quadrupole-Orbitrap MS (Q Exactive Plus, Thermo Scientific) with a heated electrospray ionization (HESI) source. Chromatographic separation was performed on a Kinetex 2.6 µm EVO C18 (50 × 2.1mm, Phenomenex) column equipped with a pre-column (C18 EVO 5 × 2.1 mm) and an inline filter. A gradient elution with water and methanol (both containing 0.1 % of formic acid) was used. Separate runs were conducted in positive and negative ion mode. The nominal resolving power in the full scan experiments was 70,000 (referenced to 200 m/z). For data independent (DIA)-MS2 experiments covering 12 m/z windows of 50 (up to m/z 475) and 260 mass units, the nominal resolving power was 35,000 (referenced to m/z 200) and higher energy collisional dissociation (HCD) was operated at normalised collision energies specific for each m/z window. Calibration standards were run at 12 levels (1, 2, 5, 10, 20, 50, 100, 200, 500, 1000, 2000 and 5000 ng/L) to check for mass accuracy, intensity changes during the run and as a quality control during peak picking. Solvent blanks (95 % H_2O / 5% methanol) were analysed to account for background contamination. The raw files were converted to mzML format and centroided with ProteoWizard (http://proteowizard.sourceforge.net) using the build in vendor's library. The mzML files were processed with MZmine 2.52 (http://mzmine.github.io) for peak picking, alignment, gap filling and peak annotation (Beckers et al., 2020; Hu et al., 2016). The resulting annotated peak list was further analysed using an in-house R-package "MZquant" for automated quantification. Compounds with very broad peaks or high backgrounds were analysed with Tracefinder 4.1 (Thermo Scientific). For details of the analytical methods see Beckers et al. (2020).

27.3 Results and Discussion

27.3.1 Target screening analysis

In the JDS4 grab surface water samples, 157 organic pollutants out of 534 targeted compounds were detected with a frequency of detection (FoD) of at least one sampling site. The analysis of steroids and other endocrine disruptors is not included. Steroid and phenol analysis was only performed in the LVSPE samples but not in the direct injected surface water samples (Chapter 26), because the sample volume of 1 mL is too low to process and enrichment on solid phases is required. A detailed assessment of the occurrences of these substances in the JDS4 MAXX LVSPE samples and their potential impact is presented in Chapter 28.

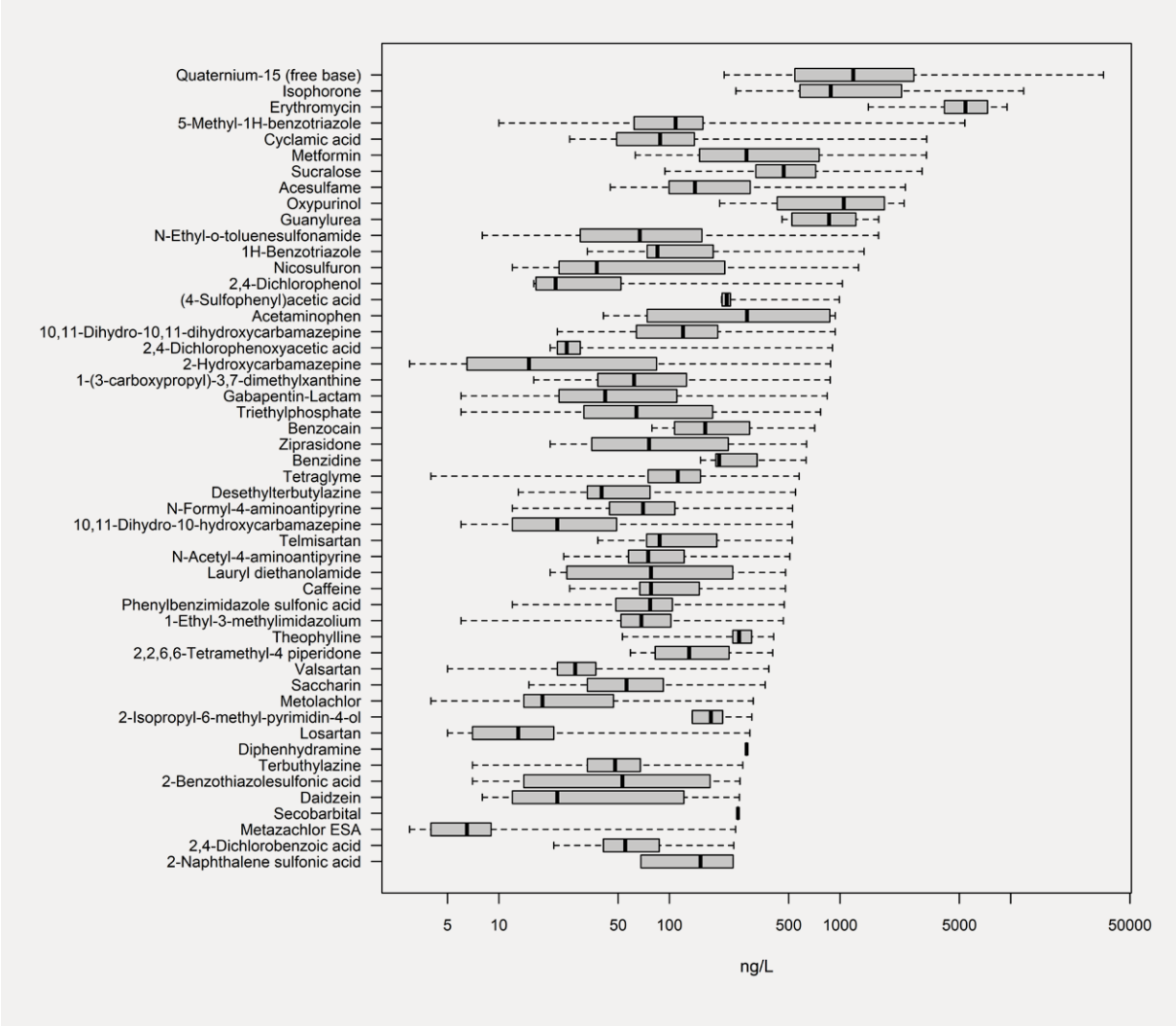

Figure 1: Ranges of concentrations of frequently determined organic compounds in Danube River surface water samples. The compounds are ordered by their maximum concentrations monitored.

Figures 1-3 show the boxplots of the concentrations of almost all detected substances. The compounds are ordered by their maximum concentration value. The concentration levels span over 2-3 orders of magnitude. Among the chemicals found in all samples were the both urine metabolites N-formyl-4-aminoantipyrine and N-acetyl-4-aminoantipyrine of the non-opioid analgesic pharmaceutical aminopyrine with levels of more

than 500 ng/L. Further pharmaceuticals detected in almost all samples were carbamazepine (CBZ) and its transformation products (TPs) 10,11-dihydro-10,11-dihydroxycarbamazepine, 10,11-dihydro-10-hydroxycarbamazepine and 2-hydroxycarbamazepine. Carbamazepine and caffeine are considered as markers for untreated wastewater and the three TPs of CBZ are markers for treated wastewater. The angiotensin II receptor blockers valsartan, candesartan, losartan and telmisartan were also present in many of the samples with concentrations up to 524 ng/L (telmisartan). The pain medication tramadol was found in values up to 90 ng/L in 47 samples, the anticonvulsants lamotrigine (in 47 samples with up to 112 ng/L) and gabapentin-lactam (in 48 samples with a maximum of 841 ng/L), the antidiabetic metformin in 42 samples with a peak concentration of 3207 ng/L and the beta blocker TP metoprolol acid at 37 sampling sites with up to 107 ng/L.

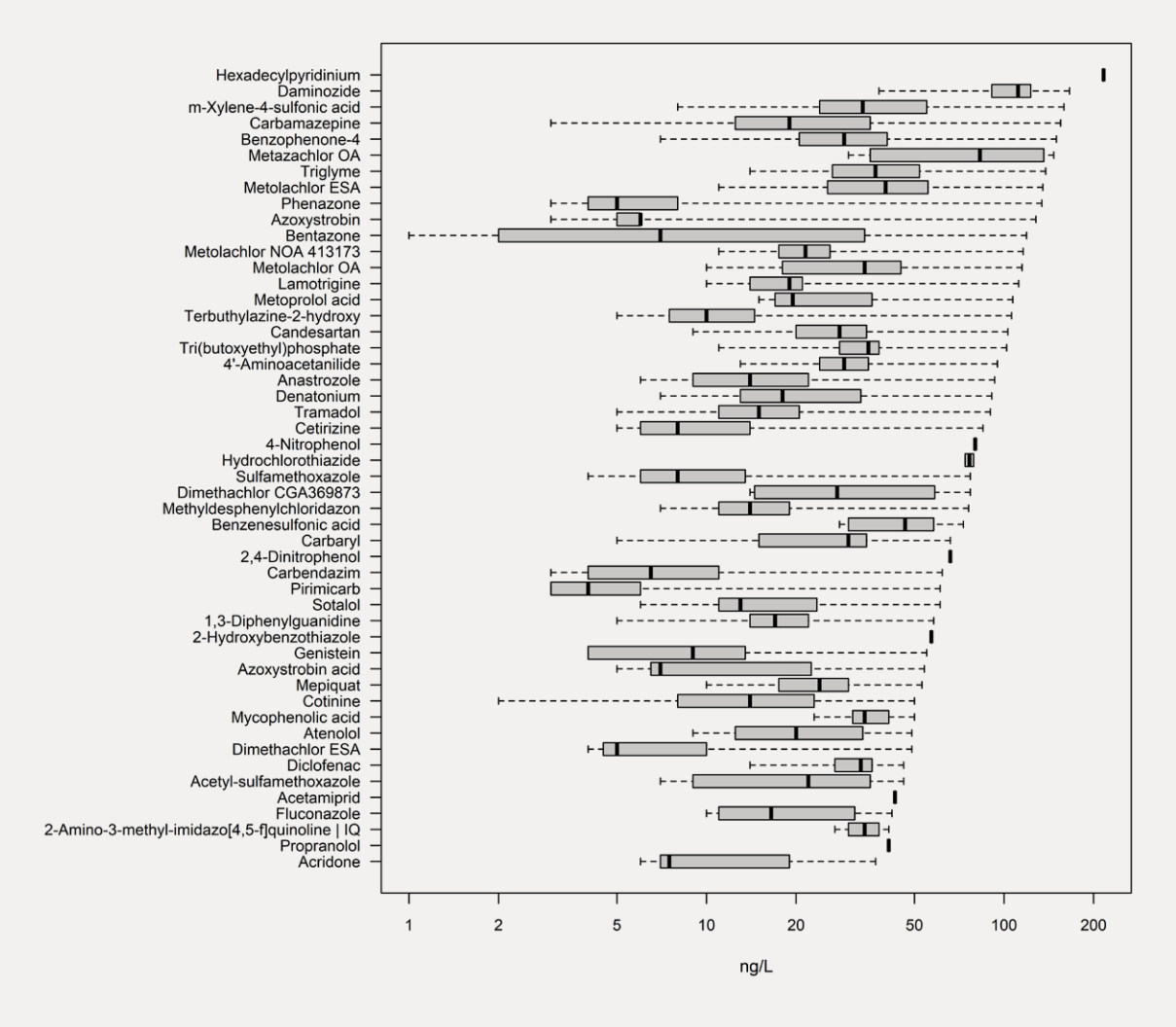

Figure 2: Ranges of concentrations of frequently determined organic compounds in Danube River surface water samples. The compounds are ordered by their maximum concentrations monitored.

The proxies for agricultural impact on the ecosystem of the Danube River are for example the herbicides terbuthylazine and other azines (maximum 548 ng/L at up to 50 sites, including also the legacy herbicide atrazine), metolachlor (in 48 samples with a peak concentration of 310 ng/L) and its TPs metolachlor ESA, metolachlor OA and metolachlor NOA 413173 (at all sites with a maximum value of 131 ng/L, at 40 site with 65 ng/L and in 40 samples with a peak of 116 ng/L, respectively), bentazone (at 41 sites with a maximum

of 119 ng/L), the chloridazon TP methyldesphenylchloridazon (at 38 sites with a maximum concentration of 76 ng/L). The herbicide nicosulforon was identified at 23 sites with a peak concentration of 1135 ng/L. Among other pesticides were the insecticide carbaryl (at 33 sites with up to 66 ng/L) and at 18 sites, the growth regulator daminozide with values of up to 166 ng/L.

Further important chemicals found in many samples were the surfactant quarternium-15 (found in 30 samples with a maximum value of 33000 ng/L), the industrial chemical isophorone (a polymer precursor and solvent) was detected at 32 sites with a peak concentration of 12000 ng/L detected at 32 sites, the antibiotic erythromycin was found only in 11 samples, but also with a maximum concentration of 9500 ng/L. Additional well known chemicals detected were corrosion inhibitors (1H-benzotriazole and 4- as well as 5-methyl-1H-benzotriazole at 47 and 48 sites with up to 5387 ng/l and 1382 ng/L, respectively) and sweeteners (sucralose, acesulfam and cyclamic acid).

The potential impact of some of the above-discussed chemicals on the aquatic biological quality elements fish, crustacean and algae will be discussed in Chapter 35.

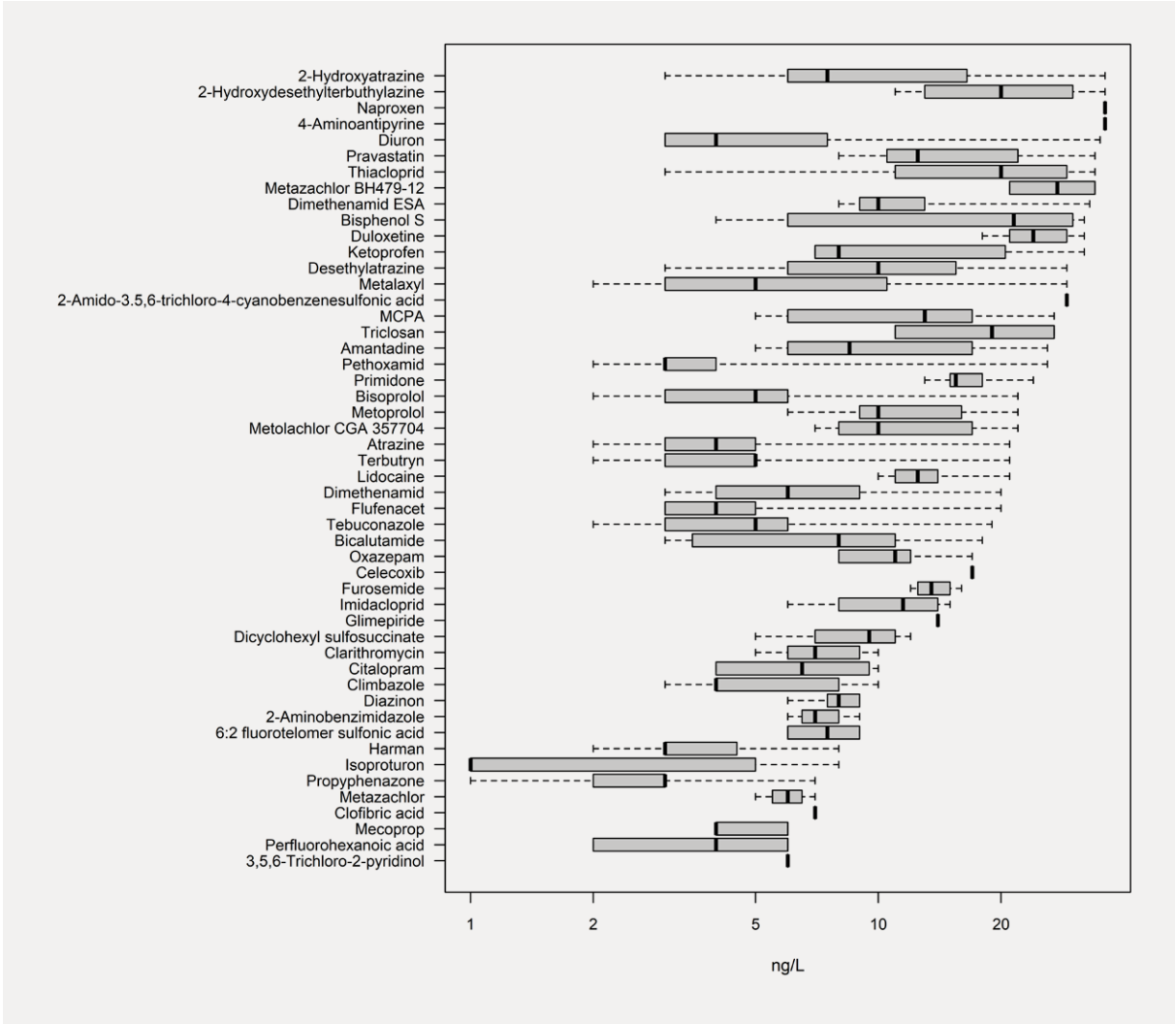

Figure 3: Ranges of concentrations of frequently determined organic compounds in Danube River surface water samples.

27.3.2 Non-target screening analysis

The samples of all sampling sites were successfully uploaded in the NORMAN Digital Sample Freezing Platform (https://norman-data.net). The samples were subjected to analysis with MZmine and processed with the prioritisation approach described in Chapter 26. The results revealed higher occurrences of metal complexes in samples measured in the negative mode which might be related to in source fragments and / or occurrences in the river water samples. Compared to the results of the MAXX LVSPE samples, the non-targeted analysis of the grab water samples did not did not reveal presence of additional substances of interest and thus the results of MAXX LVSPE samples are also valid for the grab water samples.

27.4 Conclusions

Grab water samples were collected at the 51 JDS4 sampling sites and subjected to direct water injection liquid-chromatography high-resolution mass spectrometry analysis. The method has demonstrated its potential as a simple way to perform wide-scope target screening of Danube water samples.

27.5 References

ALTENBURGER R., BRACK W., BURGESS R. M., BUSCH W., ESCHER B. I., FOCKS A., MARK HEWITT L., JACOBSEN B. N., DE ALDA, M. L., AIT-AISSA S., ET AL. (2019): Future water quality monitoring: improving the balance between exposure and toxicity assessments of real-world pollutant mixtures. Environmental Sciences Europe, 31: 12.

ARLE J., MOHAUPT V., KIRST I. (2016): Monitoring of Surface Waters in Germany under the Water Framework Directive — A Review of Approaches, Methods and Results. Water 8: 217.

BECKERS L.-M., BRACK W., DANN J. P., KRAUSS M., MÜLLER E., SCHULZE T. (2020): Unraveling longitudinal pollution patterns of organic micropollutants in a river by non-target screening and cluster analysis. Science of The Total Environment 727: 138388.

BRACK W. (2019): Solutions for present and future emerging pollutants in land and water resources management. Policy briefs summarizing scientific project results for decision makers. Environmental Sciences Europe 31: 74.

HU M., KRAUSS M., BRACK W., SCHULZE T. (2016). Optimization of LC-Orbitrap-HRMS acquisition and MZmine 2 data processing for non-target screening of environmental samples using design of experiments. Analytical and Bioanalytical Chemistry 408: 7905–7915.

LOOS R., MARINOV D., NAPIERSKA D., LETTIERI T. (2018): Review of the 1st Watch List under the Water Framework Directive and recommendations for the 2nd Watch List. JRC. Available at: https://doi.org/10.2760/614367.

MUNTHE J., BRORSTRÖM-LUNDÉN E., RAHMBERG M., POSTHUMA L., ALTENBURGER R., BRACK W., BUNKE D., ENGELEN,G., GAWLIK B. M., VAN GILS J., ET AL. (2017): An expanded conceptual framework for solution-focused management of chemical pollution in European waters. 2017/03/25 ed. Environmental Sciences Europe 29: 13.

POSTHUMA L., MUNTHE J., VAN GILS J., ALTENBURGER R., MÜLLER C., SLOBODNIK J., BRACK, W. (2019): A holistic approach is key to protect water quality and monitor, assess and manage chemical pollution of European surface waters. Environmental Sciences Europe, 31: 67.

28 Screening of endocrine substances in combination with in vitro assays in the Danube River by MAXX large volume solid-phase extraction and LC-HRMS

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Abstract

To provide a comprehensive picture on the presence of endocrine disrupting compounds, LVSPE extracts were analysed for 85 compounds (mainly natural and synthetic estrogens, androgens, glucocorticoids, progestagens as well as phenolic xenoestrogens) by LC-MS/MS and LC-HRMS, and tested using the Yeast Estrogen Screen assay combined with high-performance thin-layer chromatography and high-throughput reporter gene assays for estrogen receptor α and glucocorticoid receptor activity. Chemical analysis showed the presence of low levels of estrogens (estrone and estriol, up to 3 ng/L) and androgens (androsterone, epiandrosterone and androstenedione, up to 7.5 ng/L) in most samples, while progestagens (progesterone and different synthetic ones, up to 2 ng/L) and several glucocorticoids (up to 3 ng/L) were present only in a few samples. The concentrations of phenolic xenoestrogens were typically higher, bisphenol A and methylparaben (both ranging from 1 up to several hundred ng/L) showed the highest frequency of occurrence. The YES-HPLTC approach suggests that 17ß-estradiol and estrone were mainly responsible for the observed estrogenic effects, while the high-throughput reporter gene assays for ERα *and GR did not detect any effects due to a masking by cytotoxicity of the extracts.*

28.1 Introduction

Endocrine disruption can be considered as one of the most severe sub-lethal adverse effects on aquatic organisms. In particular feminisation and to a lesser extent masculinisation of fish has often been described for wastewater-impacted aquatic ecosystems (e.g., Huang et al., 2016), having strong impacts on the development and fitness of fish populations. Natural hormones, their synthetic analogues used as pharmaceuticals as well as endocrine active industrial chemicals such as bisphenol A or nonylphenol have been identified as endocrine disrupting chemicals in surface waters (e.g., Neale et al., 2015).

The chemical analysis of endocrine disrupting compounds is challenging due to low concentration levels of potent natural estrogens. In-vitro bioassays targeting endocrine disruption are very sensitive and allow a detection of the cumulative effect of all causative compounds, some of which might be missed by chemical analysis alone (Könemann et al., 2018). However, they lack the selectivity for individual compounds and effects might be masked by cytotoxicity in samples with a high overall compound load (Hashmi et al., 2020). The combination of bioassays with high-performance thin-layer chromatography (HPTLC) offers a possibility to mitigate these limitations, as the separation might remove interfering compounds, and individual compounds or groups can be associated with effects based on their specific retardation factors on the TLC plate (Spira et al., 2013).

In this chapter, the application of targeted chemical analysis, high-throughput in-vitro bioassays and a bioassay-HPTLC approach are used to characterize the presence of endocrine disruption and the associated compounds in the JDS4 samples.

28.2 Methods

28.2.1 Chemical analysis

For the chemical analysis of steroids and endocrine disrupting phenols, 200 µL aliquots of LVSPE extracts (see Chapter 26) were cleaned-up using an aminopropyl column similar to the method of Labadie and Budzinski (2005). Half of the cleaned-up extract was subjected to a derivatisation procedure with dansyl chloride based on the method by Backe et al. (2015) for the analysis of phenolic estrogens and some endocrine phenols. The final extracts were concentrated 1000-fold for analysis as compared to the original water sample. Calibration standards were prepared in methanol at level corresponding to 0.1 to 1000 ng/L in the original water sample for phenols and 0.01 to 100 ng/L for all steroids. These were processed the same way as the samples starting from the aminopropyl column clean-up. The obtained concentrations thus do not reflect those of the original water samples, but those of the LVSPE extracts and were corrected for the LVSPE recoveries of the respective compound.

The derivatives of five phenolic estrogens and 17 phenols were analysed by LC-HRMS using a LC-QExactive Plus system. Compounds were separated using a phenylhexyl column (100x3 mm, 2.6 µm, Thermo Scientific) and 1 mM NH4F / methanol for gradient elution after injection of 10 µL of extract. For ionisation, ESI in positive mode was used. The MS was operated in full scan mode at a nominal resolving power of 35,000 (referenced to m/z 200) and a m/z range from 280 to 1200. For data evaluation and quantification of compounds the TraceFinder 4.1 software was used.

Ketosteroids (50 synthetic and natural compounds, among them nine androgens, one antiandrogen, eleven progestagens, three mineralcorticoids, 25 glucocorticoids, and one aromatase inhibitor) as well as ten phenols were analysed by LC-MS/MS using an Agilent 1290 LC system coupled to a Sciex QTrap 6500 using a TurboV ESI source. Ketosteroids were separated using a Kinetex C18 column (100x3.0 mm, 2.6 µm, Phenomenex), a water-methanol gradient (both containing 0.1% of formic acid) and an injection volume of 10 µL. The ion source was operated in positive mode and 2-3 MRM transitions were recorded per compound in scheduled multiple reaction monitoring (sMRM). Phenols were separated using a Kinetex XB-C18 column (100x3.0 mm, 2.6 µm, Phenomenex). The eluents used for gradient separation were 1 mM NH4F in water and 1 mM NH4F in methanol. The injection volume was 5 µL. The ion source was operated in negative mode and 2-3 MRM transitions were recorded per compound in scheduled multiple reaction monitoring

(sMRM). The Multiquant 3.0.3 software was used for identification and quantification of compounds for both ketosteroids and phenols.

28.2.2. YES combined with high-performance thin-layer chromatography (HPTLC)

Aliquots of the sample extracts were applied to the HPTLC silica gel F_{254} plate (Merck) and separation was performed with the AMD2 (Automated multiple development 2, Camag) with a gradient consisting of seven steps. Afterwards the plates were adjusted to a neutral pH value by use of ammonia vapor before the Yeast Estrogen Screen (YES) assay was directly applied on the TLC plate. To this end the HPTLC plate was immersed in a yeast cell suspension (genetically modified Saccharomyces cerevisiae BJ3505) and incubated. If estrogenic substances bind to the ER-α receptor of the yeast cells, a signalling cascade starts at the end of which the enzyme ß-galactosidase is formed. After incubation, methylumbelliferyl-ß-D-galactopyranosid was added, which is cleaved by the ß-galactosidase to the blue fluorescent product 4-methyllumbelliferone (4-MU), the estrogenic effect was detected by photographing the TLC plate irradiated at 366 nm wavelength (Spira et al., 2013). The pictures were evaluated and an effect chromatogram was received through plotting of the effect against the retardation factor R_F of the HPTLC separation.

28.2.3. High-throughput reporter gene assays for ERα and GR

Prior to the bioassays, 1 mL aliquots of the extracts were cleaned-up using aminopropyl columns as for chemical analysis to reduce the load of potentially cytotoxic matrix ticonstituents. To test the extracts for agonistic hormonal activities of the estrogen alpha receptor (ERα) and the glucocorticoid receptor (GR) the GeneBLAzer reporter gene assays (ERα UAS-bla GripTite and GR-AUS-bla HEK 293Tcell lines, respectively) were used as described by König et al. (2017) with some modifications. In all bioassays, serial dilutions of samples were tested to derive the inhibitory concentration for cytotoxicity (IC10). Only relative enrichment factors (REFs) lower than IC10 were included in the concentration-effect modelling of the activation of the reporter gene to avoid the false positive results due to the cytotoxicity burst (Escher et al., 2020). The effect concentrations (EC10) of environmental samples were expressed in relative enrichment factors (REFs) to the original water samples and were derived from linear concentration-response curves (Escher et al., 2018).

The evaluation of concentration-effect curves was done by using Microsoft Excel and Graph Pad Prism (version 6.05, Graph Pad Software) and equations for data evaluation were used as described by Escher et al. (2018).

28.3 Results and Discussion

28.3.1 Chemical analysis

Out of the 25 analysed phenols, eight could not be detected in any sample (p-chlorocresol, chlorophene, dichlorophene, 3,4,5-trichlorophenol, 4-bromophenol, bisphenol Z, bisphenol BP and bisphenol C), while two out of the five analysed estrogens (17ß-estradiol and 17α-ethinylestradiol) were not detected above the method detection limits (MDLs) of 0.021 and 0,030 ng/L, respectively. The concentrations of the detected compounds are shown in Figure 1.

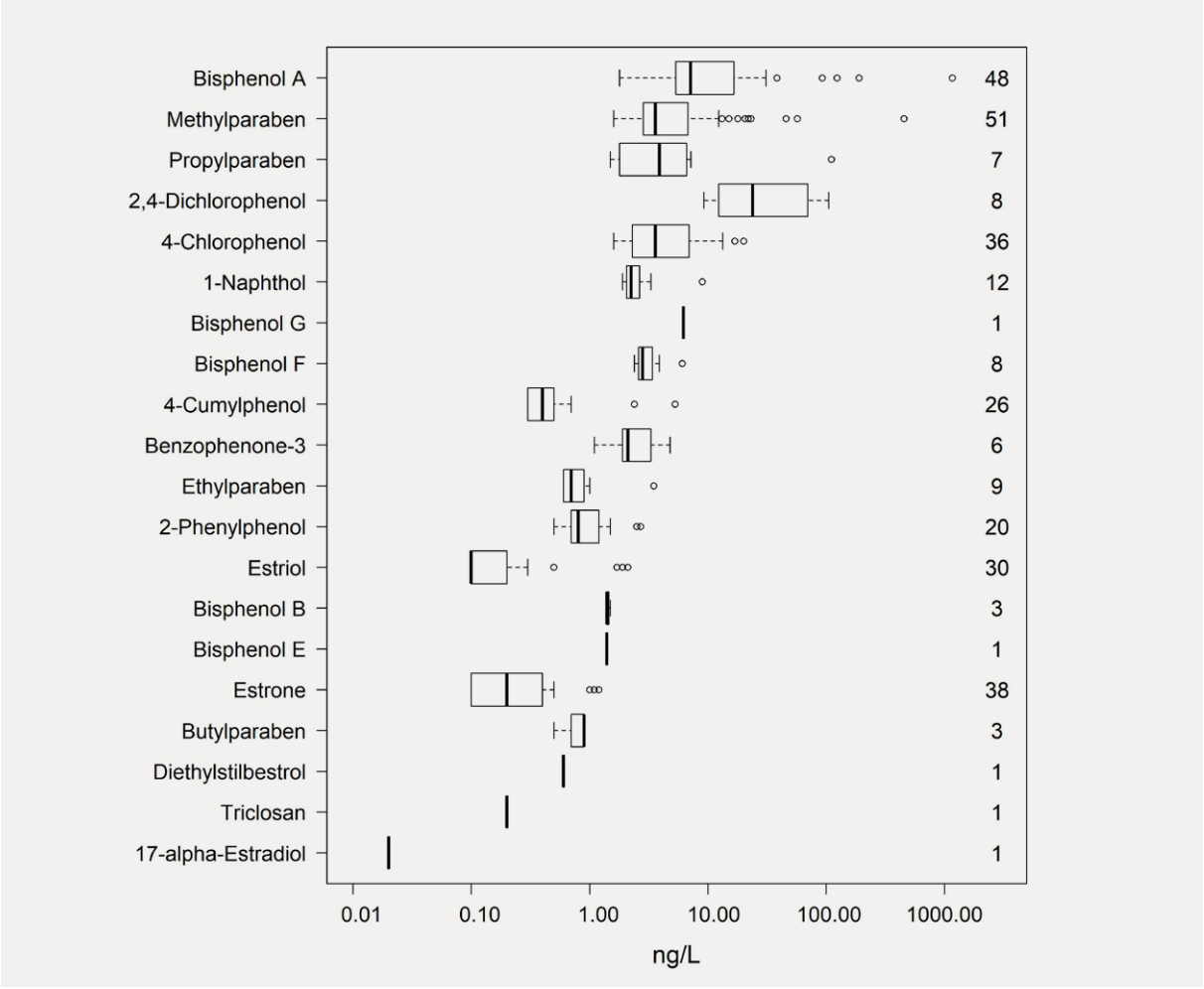

Figure 1: Boxplots of the concentrations of detected phenols and estrogens in the JDS4 samples shown as median values, 25-75-percentile interval (boxes), non-outlier range (1.5 x 25-75 percentile; whiskers) and outliers as individual points. Note the logarithmic scale. On the right the number of detections is given.

Methylparaben and bisphenol A were detected in almost all samples, with concentrations varying by one order of magnitude, indicating some specific sources in the catchment. More than 1 µg/L bisphenol A was found at site 46 (Russenski Lom) and > 400 ng/L methylparaben at site 41 (Danube upstream Timok) while the median concentrations were below 10 ng/L. 2,4-Dichlorophenol was detected in only eight samples, in concentrations from above the method detection limit of 9 ng/L to 100 ng/L. All other phenols showed concentrations below 10 ng/L, mostly around 1 ng/L.

The estrogens estrone and estriol were detected at 38 and 30 sites, respectively, with concentrations peaking at about 1 ng/L, while 17α-estradiol had one detection at site 11 (Pohansko). In no case were the WFD Watch List PNEC values of 3.6 ng/L for estrone (MDL: 0.026 ng/L), 0.4 ng/L for 17ß-estradiol and 0.035 ng/L for 17α-ethinylestradiol exceeded. In general, detection frequencies and concentration ranges were comparable in the Danube and the tributaries and no trends along the course of the Danube could be observed.

Out of the 50 analysed ketosteroids, 20 could be detected in at least one sample above the respective MDL, which was for most compounds between 0.05 and 0.2 ng/L (Figure 2). The androgens androstenedione, androsterone and epiandrosterone were detected in most of the samples and were also the compounds with the highest concentrations reaching up to 7.5 ng/L at site 46. Androsterone and epiandrosterone two

are metabolites of testosterone, which was detected in about tenfold lower concentrations at only 13 sites. Natural (cortisone and hydrocortisone) and synthetic glucocorticoids were found only at a few individual sites at levels around 2 or below 1 ng/l, respectively. The natural progestagen progesterone and synthetic progestogens mainly used as contraceptives were detected at levels up to above 1 ng/L at a few sites. Detection frequencies and concentrations were somewhat higher in tributaries as compared to the main river, probably due to higher wastewater fractions .

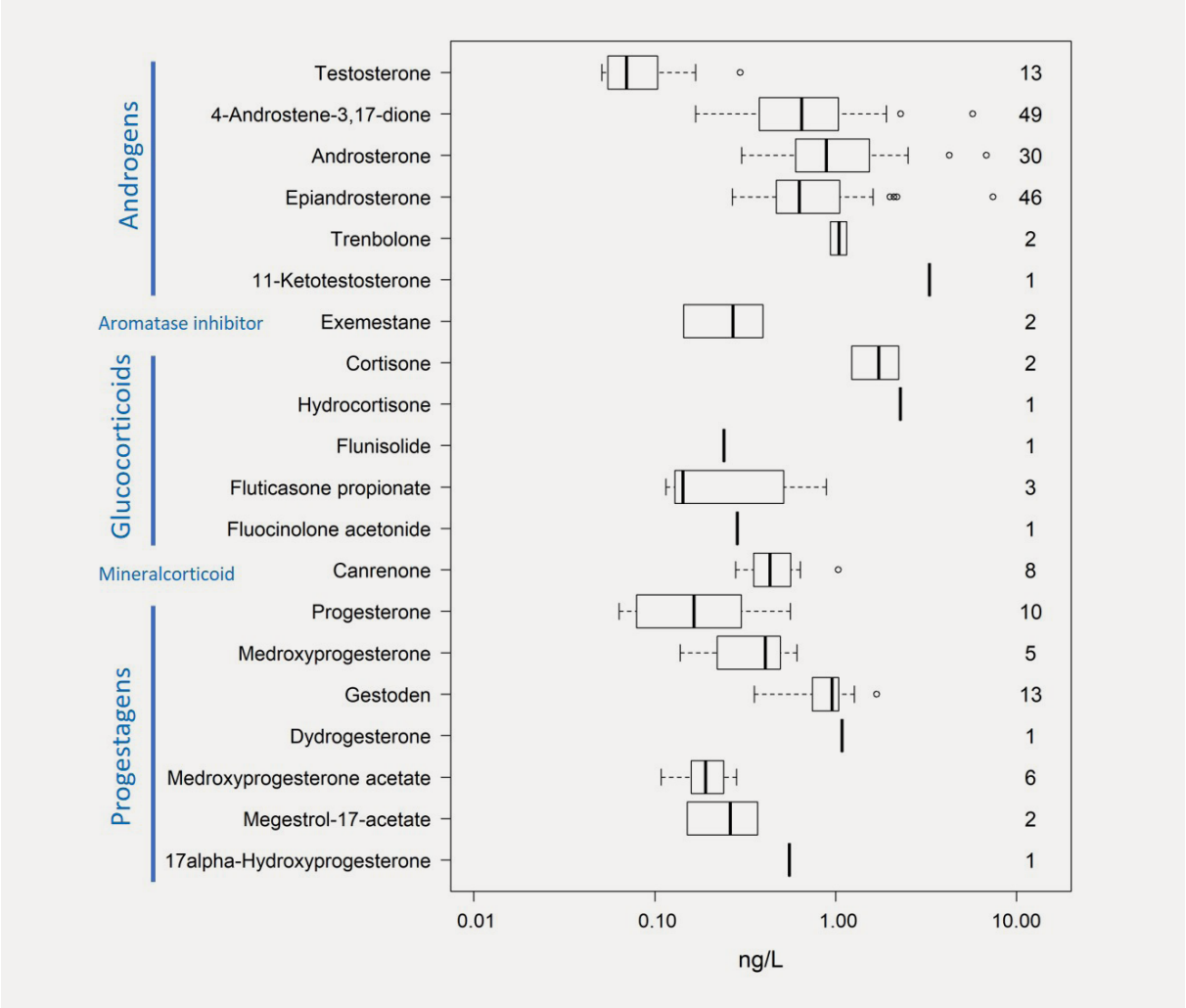

Figure 2: Boxplots of the concentrations of detected ketosteroids in the JDS4 samples shown as median values, 25-75-percentile interval (boxes), non-outlier range (1.5 x 25-75 percentile; whiskers) and outliers as individual points. Note the logarithmic scale. On the right the number of detections is given.

28.3.2. YES combined with HPTLC

After the separation of the extracts with TLC, two effective zones (RF = 59 and RF = 0.66) with varying intensity occurred in almost all samples (Figure 2). Due to their retardation factors, it was suspected that the estrogens estrone and 17ß-estradiol were responsible for the effects observed in these zones. Additionally, an effect with the retardation factor of the substance estriol (RF = 0.44) occurred in sample 46 (Russenski). Two further estrogenic effects (RF = 0.52 and RF = 0.72) could not be assigned to any estrogen based on their retardation factors. The fluorescence on the application zone of the samples is caused by fluorescent substances in the matrix of the samples and is therefore not considered as a relevant estrogenic effect.

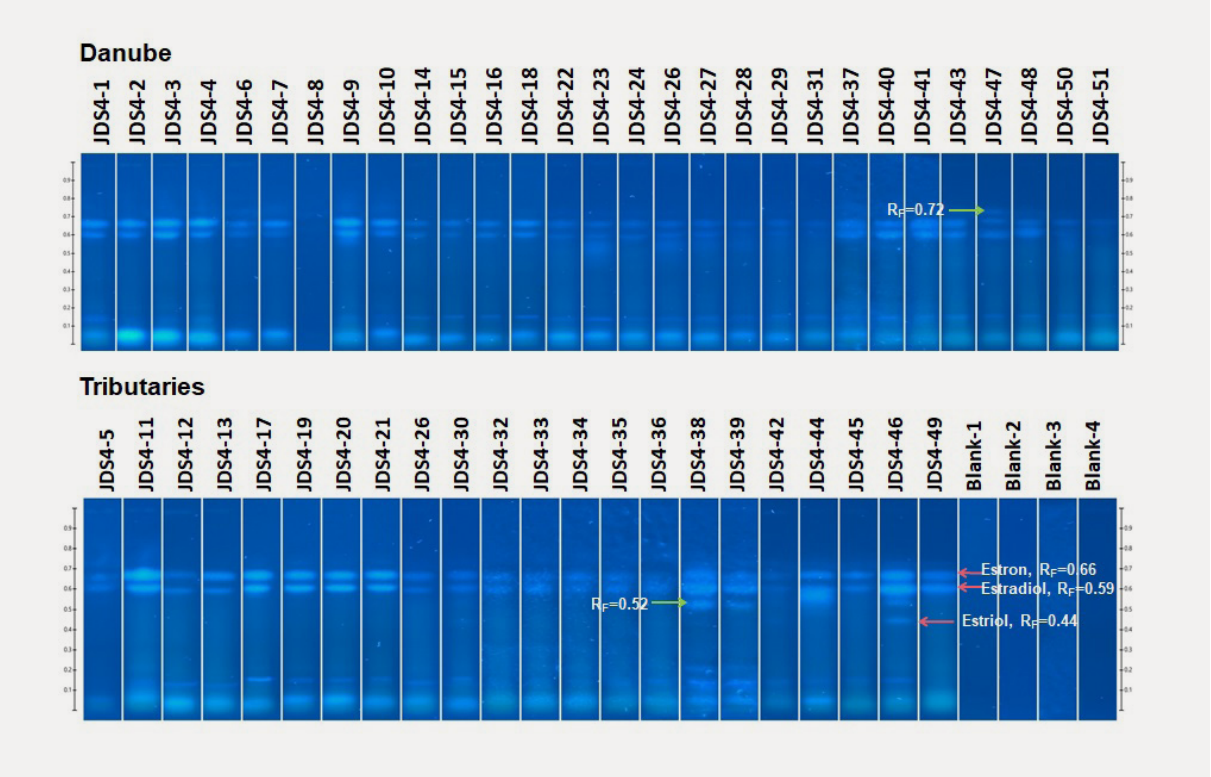

Figure 3: Overview of the YES results (red arrows indicate effects with retardation factors of known estrogens; green arrows indicate unknown estrogenic effects) for the Danube River and its tributaries.

To confirm the assumption that the two dominant effects in the samples were caused by the two substances estrone and 17ß-estradiol, sample extracts showing highest fluorescent signals were analysed by LC-MS target analysis for five estrogens (17ß-estradiol, 17α-estradiol, 17α-ethinylestradiol, estriol and estrone). In most of the extracts 17ß-estradiol, 17α-estradiol and/or estrone could be detected in the ng/L range. For three selected samples (11, 38 and 46), a confirmation experiment was performed based on the detected estrogen concentrations. It was investigated whether the effect in the samples can be explained by the detected concentrations. In samples 11 and 38, the effect of estrone and 17ß-estradiol could be explained almost completely by the concentrations of estrone and 17ß-estradiol determined. In sample 46, the effect of estrone could be explained with 59% and the effect of estriol with 21%. Only the effect at RF = 0.59 could not be explained, because no estradiol could be detected in sample 46 via target-analysis. Presumably the effect is caused by a previously unknown substance, as are the effects in samples 38 and 46 with an RF = 0.52.

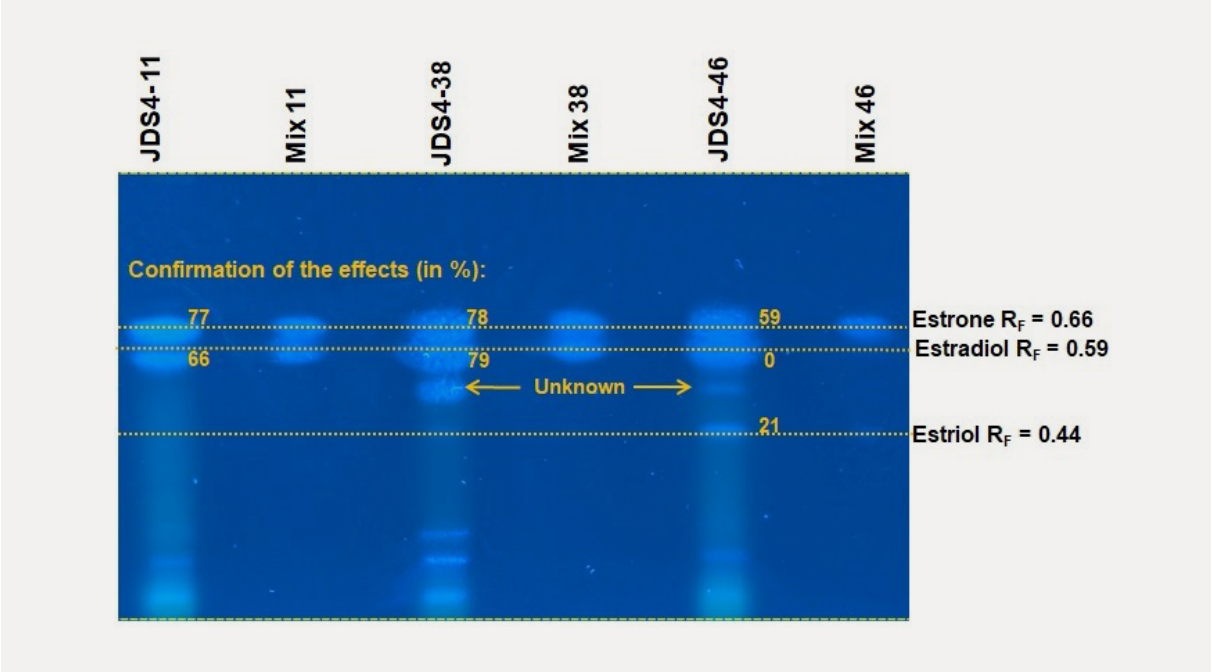

Figure 4: Confirmation experiment of the detected estrogenic effects (mix: mixtures of estrogen concentrations determined via target-analysis) in the YES.

The prevalence of estrone and 17ß-estradiol as drivers of estrogenic effect in surface waters confirms previous finding on rivers and stems from the input of treated and particularly untreated wastewater (Hashmi et al., 2018).

28.3.3 High-throughput reporter gene assays for ERα and GR

The tested river water extracts showed considerable cytotoxicity in both the ERα and GR assays (the inhibitory concentration for 10% reduced cell viability IC10 was at a relative enrichment factor REF 5-74 for the ERα and REF 3-28 for the GR) despite the additional clean-up step. Considering these IC10 values, no estrogenic or glucocorticoid activity could be detected in any of the samples. While estrogenic effects could be regularly detected in wastewater treatment plant effluents using the same assays it is unclear why all the samples of this study showed a cytotoxic masking of the estrogenic or glucocorticoid activity. Such a dominant masking of effects could not be observed in other screening campaigns to this extent (Könemann et al., 2018, Müller et al., 2018). During JDS3, 16 from 22 samples had estrogenic effects with EC10 ranging from REF 0.5 to 145 (Neale et al.,2015), which is in the same range or potent than we find now as IC10 in JDS 4 samples. No cytotoxicity data had been reported in Neale et al. (2015). Danube River samples from Novi Sad that were heavily impacted by untreated wastewater also showed strong cytotoxicity in the acidic and basic fraction of the extract impeding detection of some endocrine effects but estrogenicity could be detected very well in the neutral extracts using three ER assays, among them the one applied here (König et al., 2017). In LVSPE extracts from JDS3, Serra et al. (2020) detected a low estrogenic activity below 0.1 ng/L 17β-estradiol equivalents in most samples using one zebrafish- and one human-based *in-vitro* assay.

28.4 Conclusions

The YES-HPLTC approach suggests that 17ß-estradiol (which could not be detected by chemical analysis) and estrone (detected by chemical analysis in nearly all samples) were mainly responsible for the observed estrogenic effects in extracts from the Danube and its tributaries. In contrast, the impact of phenolic xenoestrogens on the observed effects was likely low given their much lower potencies, although a few sites showed unexplained signals in the YES-HPLTC approach. A range of androgens and progestagens as well as occasionally a few glucocorticoids could be detected at levels below 7.5 ng/L, but mostly below 1 ng/L in extracts from the Danube and its tributaries. The high-throughput reporter gene assays for ERα and GR did not detect any effects, as these were masked by the cytotoxicity levels of the extracts despite and additional clean-up step. These findings show that the detection of endocrine disruption by chemical and biological analysis at the low levels occurring in the Danube is challenging and requires detection limits in the sub-ng/L range and some fractionation of the extract (as done in the YES-HPLTC approach) is necessary to overcome the cytotoxicity impeding the detection.

28.5 References

BACKE, W. J. (2015). An Ultrasensitive (Parts-Per-Quadrillion) and SPE-Free Method for the Quantitative Analysis of Estrogens in Surface Water. Environmental Science & Technology 49, 14311-14318.

ESCHER, B., P.A. NEALE AND D. VILLENEUVE (2018): The advantages of linear concentration-response curves for in vitro bioassays with environmental samples. Environ. Toxicol. Chem. 37, 2273–2280.

ESCHER, B.I., L. HENNEBERGER, R. SCHLICHTING and F.C. FISCHER (2020). Cytotoxicity burst or baseline toxicity? Differentiating specific from nonspecific effects in reporter gene assays. Environ. Health Perspect., provisionally accepted.

HASHMI, M.A.K., M. KRAUSS, B. ESCHER, I. TEODOROVIC and W. BRACK (2020): Effect-directed analysis of progestogens and glucocorticoids at trace concentrations in river water. Environ. Toxicol. Chem. 39, 189-199.

HUANG, G.-Y., LIU, Y.-S., CHEN, X.-W., LIANG, Y.-Q., LIU, S.-S., YANG, Y.-Y., … YING, G.-G. (2016): Feminization and masculinization of western mosquitofish (Gambusia affinis) observed in rivers impacted by municipal wastewaters. Scientific Reports, 6, 20884.

KÖNEMANN, S., R. KASE, E. SIMON, K. SWART, S. BUCHINGER, M. SCHLÜSSENER, H. HOLLERT, B. ESCHER, I. WERNER, S. AIT-ASSA, E. VERMEIRSSEN, V. DULIO, S. VALSECCHI, S. POLESELLO, P. BEHNISCH, B. JAVURKOVA, O. PERCEVAL, C. DI PAOLO, D. OLBRICH, E. SYCHROVA, R. SCHLICHTING, L. LEBORGNE, M. CLARA, C. SCHEFFKNECHT, Y. MARNEFFE, C. CHALON, P. TUSIL, P. SOLDAN, B. VON DANWITZ, J. SCHWAIGER, M. I. SANMARTIN BECARES, F. BERSANI, K. HILSCHEROVA, G. REIFFERSCHEID, T. TERNES and M. CARERE (2018): Effect-based and chemical analytical methods to monitor estrogens under the European Water Framework Directive. Trends Analyt. Chem. 102: 225-235.

KÖNIG, M., B.I. ESCHER, P.A. NEALE, M. KRAUSS, K. HILSCHEROVÁ, J NOVÁK, I. TEODOROVIĆ, T. SCHULZE, S. SEIDENSTICKER, M.A.K. HASHMI, J. AHLHEIM and W. BRACK (2017): Impact of untreated wastewater on a major European river evaluated with a combination of in vitro bioassays and chemical analysis. Environ. Pollut. 220, 1220-1230.

LABADIE, P. and H. BUDZINSKI (2005). Determination of Steroidal Hormone Profiles along the Jalle d'Eysines River (near Bordeaux, France). Environ. Sci. Technol. 39, 5113-5120.

MÜLLER, M.E., B.I. ESCHER, M. SCHWIENTEK, M. WERNEBURG, C. ZARFL and C. ZWIENER (2018): Combining in vitro reporter gene bioassays with chemical analysis to assess changes in the water quality along the Ammer River, Southwestern Germany. Environ. Sci. Eur. 30, 20.

NEALE, P.A., S. AIT-AISSA, W. BRACK, N. CREUSOT, M.S. DENISON, B. DEUTSCHMANN, K. HILSCHEROVÁ, H. HOLLERT, M. KRAUSS, J. NOVÁK, T. SCHULZE, T.-B. SEILER, H. SERRA, Y. SHAO and B.I. ESCHER (2015): Linking in vitro effects and detected organic micropollutants in surface water using mixture toxicity modelling. Environ. Sci. Technol. 49, 14614-14624.

SERRA, H, F. BRION, C. CHARDON, H. BUDZINSKI, T. SCHULZE, W. BRACK & S. AIT-AISSA (2020): Estrogenic activity of surface waters using zebrafish- and human-based in vitro assays: The Danube as a case-study. Environ Toxicol Pharmacol 78, 103401.

SPIRA, D., G. REIFFERSCHEID and S. BUCHINGER (2013): Combination of high-performance thin-layer chromatography with a specific bioassay - A tool for effect-directed analysis. J. Planar Chromatogr.--Mod. TLC 26: 395-401.

29

Wide-scope target screening of industrial chemicals and plant protection products in wastewater, groundwater, river water, sediments and biota by liquid and gas chromatography coupled with high-resolution mass spectrometry

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Abstract

A state-of-the-art wide scope target screening of more than 2,400 chemicals and their transformation products was carried out in samples collected within JDS4. The analysed contaminants of emerging concern (CECs) were divided into five main categories based on their use: plant protection products (PPPs), industrial chemicals, pharmaceuticals (including antibiotics), drugs of abuse (including tobacco ingredients) and miscellaneous chemicals. This chapter provides an overview of the occurrence of industrial chemicals, PPPs and miscellaneous chemicals in samples of influent and effluent wastewater, groundwater, river water, sediments and biota, aggregating results obtained by three reference laboratories of the NORMAN network (UoA, EI, LfU; www.norman-network.net) and the EC JRC laboratory in Ispra, Italy. The overview of the occurrence of pharmaceuticals and drugs of abuse is provided in the complementary Chapter 30.

In total, 580 CECs were detected in the samples. As expected, influent wastewater samples were the most contaminated in terms of both number of compounds and concentration levels. Next came treated wastewater followed by surface water, biota and finally groundwater. Up to 55% of the PPPs, industrial and miscellaneous chemicals determined in the influent wastewater samples were efficiently removed (removal rate ≥80 %) by the wastewater treatment plants (WWTPs). However, numerous specific contaminants passed the WWTPs unaltered to significant extent and were detected at elevated concentration levels in the effluents and subsequently in the Danube River waters and sediments. Furthermore, some of them appear to accumulate to a small extent in biota. Besides the pollution coming from the WWTPs, the results indicated that the Danube River also receives significant loads of PPPs from diffuse sources. However, due to dilution, transformation and other processes governed by

their physico-chemical properties leading to retention in soils, only a few contaminants reach the groundwater used for the production of drinking water or accumulate in biota. Concentrations of eight industrial chemicals and nineteen PPPs exceeded their ecotoxicological thresholds (lowest Predicted No-Effect Concentration (PNEC) values) in one or more of the investigated environmental compartments, indicating that their occurrence in the environment might be of concern at the river basin scale. All wide-scope target screening data presented in this chapter and in Chapter 30 were stored in an on-line database and can be viewed via an interactive map at https://norman-data.eu/JDS4.

29.1 Introduction

29.2.1 Sampling, extraction, and instrumental analysis

The analytical program covered samples of 51 river water, 11 influent wastewater, 11 effluent wastewater, 7 groundwater, 11 biota (bleak muscle and one asp muscle) and 4 sediment samples, all obtained within the JDS4 in June and July 2019 (see Chapter 2).

A group of 67 polar and hydrophobic compounds including WFD priority substances, Watch List compounds and Danube RBSPs identified as an outcome of JDS3 (Brack et al., 2019) was analysed by the JRC. River water, effluent wastewater and groundwater samples were obtained using a large volume solid phase extraction (LVSPE) device termed "MARIANI-Box" (Mariani et al., 2017). The device is designed to perform sampling and sample extraction in the field. The procedure involves filtering of a 7 L, 5 L and 0.5 L respectively for groundwater, river water and wastewater samples, spiked with a mix of labelled internal standards, and extracted on an OASIS HLB disk mounted in the MARIANI-Box. The OASIS HLB disks were stored refrigerated until they were extracted in the laboratory by means of Solid Phase Extraction (SPE) (J2 Scientific). The extracts were evaporated under a gentle nitrogen stream before the analysis. LC-MS/MS (QTrap 5500, Sciex) and GC-HRMS (DFS, Thermo) were used for the determination of polar and non-polar compounds, respectively.

A group of 139 pesticides and their TPs were analysed in 51 river water and 7 groundwater samples by the Bavarian Environmental Agency (LfU). An on-line solid-phase extraction (SPE) using C18 material combined with LC-HRMS (QExactive, Thermo) methodology was applied.

A group of 2,316 CECs and their TPs frequently found in the environment was analysed by the UoA. All river water, WWTP influent and effluent water, and groundwater samples were extracted in the laboratory of Environmental Institute (EI) using HORIZON SPE-DEX 4790 device (USA). The samples were concentrated on Atlantic HLB-M Disk with 47 mm disk holder according to an automated extraction program (Alygizakis et al., 2020). The extracts were evaporated using a gentle stream of nitrogen and reconstituted in 50:50 methanol:water (500 uL total volume extract). The extracts were then shipped to the UoA and subjected to analysis of illicit drugs and pharmaceuticals (including many antibiotics) by UHPLC-ESI-QqQ (Thomaidis et al., 2016). Especially for illicit drugs, the method was tested in annual collaborative trials organized by SCORE COST action ES1307 for more than 9 years (Gonzalez-Marino et al., 2020). In addition, the extracts were analysed for 2,316 compounds by an in-house UHPLC-ESI-QTOF method (Dionex UltiMate 3000 RSLC from Thermo Fisher Scientific coupled to a Maxis Impact QTOF from Bruker) (Gago-Ferrero et al., 2020).

Sediment samples were extracted at UoA using a validated protocol (Gago-Ferrero et al., 2015). Briefly, 0.2 g of freeze-dried sediment sample were placed into a 15 mL centrifuge tube and the analytes were extracted

with 2 mL methanol–Milli-Q water (pH 2.5, formic acid 0.5% and 0.1% EDTA), 50:50 (v/v) by vortex (1 min), followed by ultrasonic extraction at 50°C for 15 min. After the extraction, the extract was centrifuged, and the supernatant was collected in a glass test tube. The extraction was repeated two more times and the total extract of 6 mL was collected and evaporated to dryness under a gentle steam of nitrogen at 40°C. The dried extract was reconstituted with 0.2 mL methanol/Milli-Q water, 50:50 (v/v).

In addition, the extraction of biota (fish muscle) was performed at the UoA using an optimized multi-residue method for fish tissues (Dasenaki and Thomaidis, 2015). Briefly, 0.2 g freeze-dried biota sample was placed into a 15 mL centrifuge tube and extracted with 2 mL of Milli-Q water containing 0.1% formic acid (v/v) and 0.1% EDTA (w/v), 2 mL of methanol, and 2 mL of acetonitrile sequentially, using a vortex mixer (30 sec) and ultrasonic bath at 60°C for 20 min. The samples were centrifuged and the supernatants transferred to new plastic centrifuge tubes to precipitate lipids and remaining proteins at -20 °C for 12 h. After an additional defatting step by liquid-liquid extraction with hexane (5 mL), the extract was collected in a glass tube, evaporated to dryness under a gentle steam of nitrogen at 40 °C and reconstituted in 0.2 mL methanol/Milli-Q water, 50:50 (v/v). The extract was filtered through a 0.22 μm RC syringe filter of 4 mm diameter (Phenomenex, USA) and transferred into a glass vial for liquid chromatography tandem mass spectrometry (LC-MS/MS using a Thermo UHPLC Accela system connected to a TSQ Quantum Access triple-200 quadrupole mass spectrometer) and LC-HRMS analysis.

A thorough quality assurance and quality control (QA/QC) was applied in all sample preparation and instrumental methods. A mix of internal standards was added into each sample prior to extraction to assure satisfactory recovery of the target compounds. Moreover, procedural blank and field blank samples were prepared to assess any external contamination which might have been brought in during the sampling campaign, sample preparation of the extracts and analysis. More details about QA/QC protocols can be found in Chapter 37.

29.2.2 Risk assessment

To assess the risk of the detected substances, the concentrations of the contaminants were evaluated in relation to the respective PNEC values. The occurrence of CECs with detected concentrations above PNEC were considered to represent a potential risk for the impacted ecosystem. PNEC values for all detected substances were extracted from the NORMAN Ecotoxicology Database (https://www.norman-network.com/nds/ecotox/; a part of the NORMAN Database System (Dulio et al., 2020)) for river water, sediments and biota samples. For compounds where no experimental toxicity data was available, predicted PNECs (P-PNECs) were derived by QSAR models (Aalizadeh et al., 2017). For risk assessment purposes, the lowest PNEC was selected in the order of (a) environmental quality standard (EQS) values; (b) experimental PNEC values from reference laboratories; (c) in-silico predicted P-PNEC. Steroids in biota were considered as natural occurring compounds and were not considered for the risk assessment.

29.3 Results and Discussion

All results were collected in the pre-programmed spreadsheets termed Data Collection Templates (DCTs) gathering all necessary metadata (e.g. sampling site name, date, coordinates, sample matrix etc.) and information to judge the quality of the results (e.g. Limit of Detection/Quantification, level of the validation of the used methods and accreditation of the laboratory etc.). The DCTs were so far uploaded into the NORMAN Database System (NDS; https://www.norman-network.com/nds/) and its EMPODAT module (https://www.norman-network.com/nds/empodat/). All results can be interactively visualized in an on-line map (https://norman-data.eu/JDS4/). The results for groundwater samples were included and were discussed in detail in Chapter 25.

Most frequently detected compounds and compounds with the highest concentration levels for river water samples, sediments, biota and wastewater (influent and effluent) are discussed in sections 29.3.1, 29.3.2, 29.3.3 and 29.3.4 respectively. Cooccurring contaminants in various environmental matrices are discussed in section 29.3.5. Finally, top-ranked contaminants based on risk assessment are discussed in section 29.3.6.

29.3.1 Danube River water samples

Industrial chemicals and PPPs were the dominant use categories of emerging contaminants in the JDS4 river water samples as regards the concentration levels (Figure 1). The data of the surfactant benzododecinium was not included in Figure 1, since this substance was determined at extremely high concentration levels with an average concentration of 2.1 µg L^{-1} and a peak concentration of 11.3 µg L^{-1} at the station of Budapest JDS4-24. Benzododecinium is a widely used cationic surfactant that is used as biocide, fabric softening, wetting agent by the textile industry, road construction and cosmetics industry (Jardak et al., 2016). The concentration levels found in river water and wastewater samples indicate multiple input sources in the Danube.

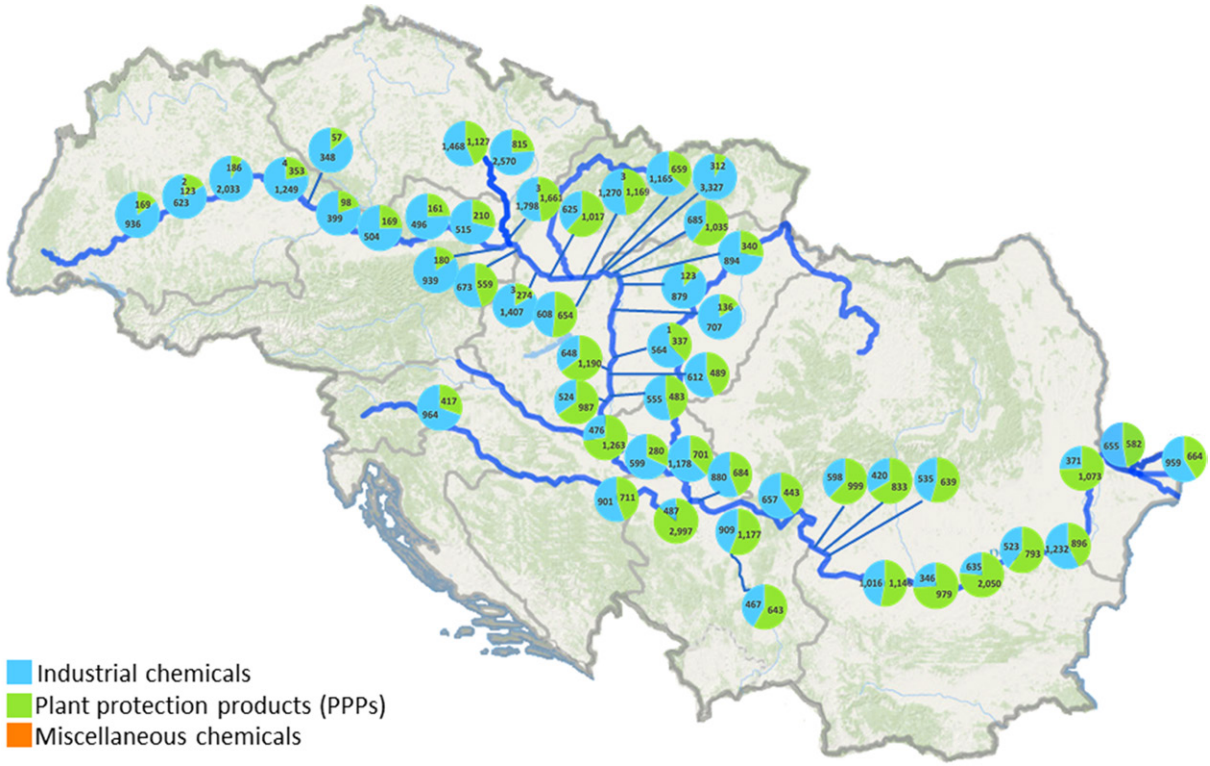

Figure 1: Cumulative concentrations of industrial chemicals, plant protection products (PPPs) and miscellaneous chemicals at the 51 JDS4 river water stations. Numbers in the pie charts represent sum of concentrations of individual substances expressed in ng L^{-1}. Contribution of the surfactant benzododecinium was excluded.

In general, cumulative concentrations of industrial chemicals seemed to dominate in the samples of the Upper and Middle Danube whereas their concentration decreased in the Lower Danube and, instead, the contribution by PPPs increased. Despite the lower number of detected industrial chemicals (40 compounds) in comparison to PPPs (120 compounds), the concentration of industrial chemicals was remarkably higher than PPPs. Concentration of industrial chemicals covered on average 42.5 % of the total concentration of all detected compounds. The sampling site with the highest concentration of industrial chemicals was JDS4-20 (3.3 µg L^{-1}, 81% of the total concentration of pollutants in the sampling station) followed by JDS4-3 (2.0 µg L^{-1}, 74% of the total concentration of pollutants in the sampling station).

The most frequently detected industrial chemicals were benzotriazole (BTR) and 4/5-methyl-benzotriazole (4/5-Me-BTR), whereas low frequency of appearance (FoA) was found for 4,5-di-Me-BTR. BTR was detected in all river water samples (FoA 100%) with an average concentration in river water of 350 ng L^{-1}, whereas 4/5-Me-BTR was detected with FoA 96.1% and an average concentration of 145 ng L^{-1}. From the class of benzothiazoles, 2-amino-benzothiazole was detected with remarkably high FoA (98 %) but in much lower concentration levels (average concentration 3.7 ng L^{-1}). Similarly, low concentration but high FoA were observed for six perfluorinated substances according to results provided by UoA, i.e. perfluorooctanoic acid (PFOA, FoA 100%, average concentration 2.1 ng L^{-1}), perfluorooctanesulfonic acid (PFOS, FoA 96.1%, average concentration 2.1 ng L^{-1}), perfluorohexanoic acid (PFHxA, FoA 94.1%, average concentration 3.2 ng L^{-1}), perfluorohexanesulfonic acid (PFHxS, FoA 90.2%, average concentration 1.5 ng L^{-1}), perfluorobutanesulfonic acid (PFBS, FoA 86.3%, average concentration 0.6 ng L^{-1}) and perfluoroheptanoic acid (PFHpA, FoA 56.9%, average concentration 1.1 ng L^{-1}). Despite the low concentration levels, the frequent (almost ubiquitous) occurrence of perfluorinated substances is of concern as they possess a high bioaccumulation potential.

Another important class of detected industrial chemicals was phenols, specifically 2,4-dinitrophenol (FoA 100%, average concentration 3.6 ng L^{-1}), the Water Framework Directive (WFD) priority substance (PS) 4-tert-octylphenol (FoA 88.2%, average concentration 55.2 ng L^{-1}) as well as four novel bisphenol A related compounds: bisphenol A diglycidyl ether, bisphenol A (3-chloro-2-hydroxypropyl) glycidyl ether, bisphenol A (2,3-dihydroxypropyl) glycidyl ether, and bisphenol A bis(3-chloro-2-hydroxypropyl) ether. A distinct spatial distribution was observed for these four Bisphenol A-related chemicals, which were detected in the Upper Danube (Germany, Austria, Slovakia) but not in the rest JDS4 river samples. Their concentrations were remarkably high in the Upper Danube ranging from 55 up to 271 ng L^{-1}.

Out of the group of organophosphates, nine organophosphates were detected in almost all river water samples (FoA>96%). The highest concentration was observed for phosphate-tris(2-chloro-1-methylethyl) (TCPP), which was present with an average concentration of 107 ng L^{-1} in the river water samples. The rest of organophosphates were detected at lower concentrations (most of them below 10 ng L^{-1}). However, their widespread occurrence might be of concern. Similarly ubiquitously, the plasticizer diethyl phthalate was detected in 98% of the river water samples and at average concentration 81 ng L^{-1}. The high FoA of organophosphates and phthalates is known from the previous JDS sampling campaigns and their widespread occurrence was not of surprise since these substances are commonly used as plasticizers.

PPPs were detected in river water samples at much lower concentration levels than industrial chemicals. However, many PPPs were detected with high FoA. 28 compounds were detected in many river water samples with FoA>80%. The average concentration of PPPs in the Danube River was below 10 ng L^{-1} for most of the substances with a very few exceptions.

One of those exceptions was diethyltoluamide (DEET) detected in all river water samples with the average concentration of 218 ng L^{-1}. DEET showed a clear spatial distribution pattern with the highest concentration levels being revealed at the JDS4 sampling sites located in Hungary (up to 1065 ng L^{-1}). Another exception was metolachlor and its TP metolachlor-ESA. Both compounds were detected in all samples (FoA 100%) at average concentration levels of 24 and 42 ng L^{-1}, respectively. Other TPs of metolachlor such as metolachlor-OXA and metolachlor-morpholinon were also detected but with much lower FoA (47 and 12%, respectively). Another PPP that stood out because of its relatively high concentration levels was carbendazim. Carbendazim which is also used as biocide was detected in 92.2% of the investigated samples at concentration levels of 151 ng L^{-1} on average. It is worth noting carbendazim has been banned as PPPs in the EU in the beginning of 2019. Therefore, its concentration levels in the aquatic environment of Danube are expected to decrease in the near future. High FoA (>70%) and concentration above 10 ng L^{-1} was also proved for terbuthylazine (FoA 100%, average concentration 22 ng L^{-1}) and its two major TPs desethyl-terbuthylazine (FoA 100%, average concentration 19 ng L^{-1}) and 2-hydroxy-terbuthylazine (FoA 72.5%, average concentration 18 ng L^{-1}). Pesticides which were detected in all analysed river water samples (FoA 100%) but very low concentration levels (average concentration below 5 ng L^{-1}) were tebuconazole, imidacloprid, terbutryn, o-hydroxybiphenyl and simazine.

29.3.2 Sediments

River sediments were less contaminated in comparison with other investigated matrices. Nineteen industrial chemicals and seven PPPs were detected. Most of the substances that were detected were semi-polar and non-polar ($\log K_{ow} \geq 3$), leading to accumulation in river sediments. Surfactants was the dominant use category of chemicals in river sediments with diglyme, benzododecinium, didecyldimethylammonium and triglyme being detected in all samples and lauryldiethanolamide, tetraethyleneglycol-monododecyl ether, N,N-dimethyltetradecylamine, N,N-dimethyltetradecylamine-N-oxide, N,N-dimethyldodecylamine being

detected with FoA≥ 50%. Three phthalates, bis-(2-ethylhexyl) phthalate (DEHP; WFD PS), diethyl phthalate and di-n-butyl phthalate were also detected in all sediment samples. DEHP was the compound with the highest concentration up to 1342 ng g^{-1} dry weight.

From the use category of PPPs, the pesticide barban was detected in 75% of the samples with average concentration 67 ng g^{-1} dry weight, while the rest of PPPs were detected with lower FoA and/or at concentration levels below the limits of quantification (LOQ). Such compounds were oxfendazole, aramite and desisopropyl-atrazine. Methiocarb and chlordimeform were detected just above their LOQs at concentrations 5 and 6 ng g^{-1} dry weight respectively.

29.3.3 Biota

In the analysed 11 biota fish samples (muscle), 8 industrial chemicals, 17 PPPs and 2 unclassified emerging contaminants were determined. This indicates a potential for their persistence and bioaccumulation (P and B criteria) according to the REACH legislation.

N-Methyl-2-pyrrolidone and PFOS were detected in all samples at average concentration of 22 and 13 ng g^{-1} wet weight, respectively, followed by 4-tert-octylphenol, which was detected with lower FoA (91%) but at similar average concentration levels. Regarding the sub-group of perfluorinated substances, two additional compounds were detected in biota that were not detected in river water: perfluorodecanoic acid and perfluoroundecanoic acid, which have a very high bioaccumulation potential. Both perfluorinated substances were detected with much lower FoA of 27.3% and at low concentrations of 1.1 ng g^{-1} wet weight.

The PPPs barban, methoprene and 3-hydroxy-carbofuran were detected in all biota samples. The highest average concentration was observed for barban (25 ng g^{-1} wet weight), whereas lower concentration levels were found for methoprene (5.3 ng g^{-1} wet weight) and 3-hydroxy-carbofuran (3.6 ng g^{-1} wet weight). The fourth most frequently detected agricultural chemical was alachlor-OXA, which was detected with relatively high FoA (72.7%) and at average concentration 11 ng g^{-1} wet weight, while all the other PPPs were detected in the samples with FoA≤28%.

29.3.4 Influent and effluent wastewater

Influent and effluent wastewater (24h-composite reflecting the hydraulic retention time of each WWTP) are discussed together since the purpose of the sampling was also to indicate the removal rates for CECs in the various WWTPs in the catchment. Therefore, the analysis was narrowed down to substances (PPPs and industrial chemicals) detected before and after treatment and thus reliable removal rates could be obtained. It must be noted that PPPs originate mainly from agricultural activities. However, the introduction of PPPs from WWTPs into the aquatic environment is not always insignificant. In order to draw as robust results as possible given the generated dataset, the analysis was restricted to substances that were detected in at least six out of the 22 (11 influent and 11 effluent) wastewater samples. The result of the analysis is summarized in Table 1.

Table 1: Concentrations of industrial chemicals, PPPs and miscellaneous chemicals that were measured in both influent and effluent wastewater. The table presents only substances that were detected in at least six out of the 22 wastewater samples (11 influent and 11 effluent).

Compound	Influent wastewater		Effluent wastewater		Removal
	No. of samples	Concentration range (ng L^{-1})	No. of samples	Concentration range (ng L^{-1})	
1,2,3,6-cis-Tetrahydrophthalimide	11	5.6-56	0	<LOD	Efficient
2-Benzothiazolesulfonic acid	10	38-111	11	40-147	Poor
4-tert-Octylphenol (4-t-OP)	11	74-284	11	41-236	Poor
Azoxystrobin	1	1.5-1.5	8	1.7-3.3	Poor
Benzododecinium (Benzyl-dimethyl-dodecylammonium)	11	28-186	8	0.61-21	Efficient
Benzoic acid	10	16-2292	8	11-106	Efficient
Benzothiazole-2-OH	9	11-267	3	7.7-21	Efficient
Benzotriazole (BTR)	11	240-9240	11	329-25923	Poor
Benzotriazole-5-methyl	8	34-4930	11	18-3123	Poor
Bisphenol A	8	5.9-81	4	6.5-118	Poor
Bisphenol S	6	5.8-13	0	<LOD	Efficient
Climbazole	11	9.3-28	10	9.6-21	Poor
Cyclamic acid	11	15-110	0	<LOD	Efficient
Daidzein	11	11-103	0	<LOD	Efficient
DEET (Diethyltoluamide)	10	17-104	11	1.7-29.3	Efficient
Didecyldimethylammonium (DADMAC (C10:C10))	10	0.57-22	9	0.55-1.2	Efficient
Diglyme	11	29-136	11	3.0-14	Efficient
Diuron	3	7.2-8.4	7	1.2-10	Poor
Dodecyl-benzenesulfonate	11	90.8-1325	11	5.67-110	Efficient
Endothal	10	17-192	0	<LOD	Efficient
Fipronil	4	7.7-30	9	1.62-59.7	Poor
Fludioxonil	4	0.16-1.5	7	0.19-0.64	Poor
Indole-3-acetic acid	7	30-284	0	<LOD	Efficient
Lauryl diethanolamide	11	1.5-803	11	0.61-6	Efficient
Methoprene	0	<LOD	6	1-5.5	Poor
Methoxyphenamine	0	<LOD	6	2.7-36	Poor
N,N-Dimethyldodecylamine	11	0.18-7.8	0	<LOD	Efficient
N,N-Dimethyldodecylamine-N-oxide	11	60-275	11	0.66-12	Efficient
N,N-Dimethyltetradecylamine-N-oxide	11	6-141	11	0.76-18	Efficient
N-Methyl-2-pyrrolidone	11	14-444	8	1.1-4.5	Efficient
N-Methyldodecylamine	10	40-763	0	<LOD	Efficient
Oxfendazole	1	8.2-8.2	7	0.63-18	Poor

Compound	Influent wastewater		Effluent wastewater		Removal
	No. of samples	Concentration range (ng L^{-1})	No. of samples	Concentration range (ng L^{-1})	
PFHxA	6	1.9-1.9	5	1.7-10	Poor
Phthalate-diethyl	10	0.68-36	1	<0.6-<0.6	Efficient
Phthalate-di-n-butyl	10	0.9-21	9	3.1-11	Poor
Picaridin (Icaridin)	10	1.4-129	4	0.35-1.2	Efficient
Propazine-2-hydroxy (Prometon-hydroxy)	1	3.3-3.3	7	0.87-3.8	Poor
Sethoxydim	11	15-52	1	19-19	Efficient
Tetraethylene glycol monododecyl ether	10	11-280	10	1.2-9.4	Efficient
Thiabendazole	0	<LOD	7	0.69-2.6	Poor
Thiamethoxam	3	2.4-4.7	4	2.3-13	Poor
Toluenesulfonamide	8	10-464	5	121-171	Poor

WWTPs removed efficiently 23 out of the 42 PPPs and industrial chemicals (55%). Efficient removal was considered if the WWTP reduced the concentration of the contaminant by 80%. Therefore, efficient removal did not indicate complete removal of the substance. Many substances that were detected in the river water originated at least partially from wastewater effluents (e.g. benzotriazole, 4-tert-octylphenol, benzododecinium, didecyldimethylammonium etc.) caused by the low efficiency of the WWTPs to eliminate them. In some cases, the concentration in the effluent wastewater was higher than in the influent wastewater, which can be attributed to the cleavage of conjugated substances happening during the various processes of the WWTPs (Brown and Wong, 2018). Despite the low average removal efficiency (55%) for the studied chemicals, WWTPs proved to significantly reduce the loads of contaminants that are discharged into the Danube River and thus help to protect the river and its ecosystems, but more efficient treatment techniques should be established.

29.3.5 Commonly detected compounds in different environmental matrices

Substances that are not eliminated by the WWTPs are being continuously introduced in the Danube River ecosystem. Depending on their physico-chemical properties, substances may barely undergo transformation or adsorption to particulate matter and therefore persist in the water phase. Examples of such compounds were PFOS, 4-tert-octylphenol, benzododecinium, didecyldimethylammonium, phosphate-triphenyl (TPhP), phosphate-triethyl (TEP) and phthalate-diethyl. There are also compounds that are at least partially transformed to new TPs (e.g. cases of benzotriazole, atrazine, metolachlor etc.) which may be more toxic than their parent compounds. Persistent substances may enter the groundwater used for the production of drinking water (e.g. DEET, benzotriazole etc.) or accumulate in the biota (e.g. 4-tert-octylphenol, PFOS etc.) thus putting human health at risk. Table 2 summarizes the observed occurrence of selected PPPs and industrial chemicals in the catchment.

Table 2: Commonly detected PPPs and industrial chemicals in JDS4 samples.

Compound	Detected in JDS4 matrices*	Compound	Commonly detected matrices*
4-tert-Octylphenol (4-t-OP)	R, WW, G, S, B	Metalaxyl	R, WW, B
Benzododecinium (Benzyl-dimethyl-dodecylammonium)	R, WW, G, S, B	Atrazine-desisopropyl	R, G, S
Didecyldimethylammonium (DADMAC (C10:C10))	R, WW, S, B	Clothiandin	R, G, B
Phosphate-Triphenyl (TPhP)	R, WW, G, S	Bis-(2-ethylhexyl)-phthalate (DEHP)	G, S, B
Phosphate-triethyl (TEP)	R, WW, G, S	3,3-pentamethylene-4-butyrolactam	WW, G
Phthalate-diethyl	R, WW, G, S	Bisphenol A	WW, G
PFOS	R, WW, G, B	N,N-Dimethyldodecylamine N-oxide	WW, G
Benzotriazole (BTR)	R, WW, G	Atrazine-2-hydroxy	WW, G
N-Methyl-2-pyrrolidone	WW, S, B	PFDA	WW, B
Phthalate-di-n-butyl	WW, G, S	Propoxur	WW, B
Diglyme	WW, G, S	Barban	S, B
Lauryl diethanolamide	WW, G, S	Chlordimeform	S, B
Tetraethylene glycol monododecyl ether	WW, G, S	Benzothiazole -2-OH	R, WW
N,N-Dimethyltetradecylamine-N-oxide	WW, G, S	Toluenesulfonamide	R, WW
Oxfendazole	WW, G, S	Benzothiazole-2-Amino	R, WW
Benzotriazole-5-methyl	R, WW, G	Melamine	R, WW
Benzoic acid	R, WW, G	PFHxS	R, WW
PFHxA	R, WW, G	PFHpA	R, WW
PFBS	R, WW, G	2-4-Dinitrophenol (DNP)	R, WW
PFOA	R, WW, G	Phosphate-tris(2-ethylhexyl) (TEHP)	R, WW
Phosphate-tri-n-butyl (TNBP)	R, WW, G	Phosphate-tris(3,5-dimethylphenyl) (T35DMPP)	R, WW
Phopshate-triisobutyl (TIBP)	R, WW, G	Phosphate-tris(2-chloroethyl) (TCEP)	R, WW
Phosphate-2-ethylhexyl-diphenyl (EHDP)	R, WW, G	Phosphate-tris(2-butoxyethyl) (TBOEP)	R, WW
Phosphate-tris(1,3-dichloro-2-propyl) (TDCPP)	R, WW, G	Phosphate-tris(methylphenyl) (TMPP)	R, WW
Phopshate-tris(2-chloro-1-methylethyl) (TCPP)	R, WW, G	Phosphite-(nonylphenyl) (TNPP)	R, WW
DEET (Diethyltoluamide)	R, WW, G	Phosphate-triisopropyl (TIPPP)	R, WW
Pyrethrin I	R, WW, G	Climbazole	R, WW
Chloridazone	R, WW, G	Fludioxonil	R, WW
Atrazine	R, WW, G	Azoxystrobin	R, WW

Compound	Detected in JDS4 matrices*	Compound	Commonly detected matrices*
Propazine-2-hydroxy (Prometon-Hydroxy)	R, WW, G	Diuron	R, WW
Metolachlor	R, WW, G	Thiamethoxam	R, WW
Picaridin (Icaridin)	R, WW, G	MCPA (4-chloro-2-methylphenoxyacetic acid)	R, WW
Chlorotoluron	R, WW, G	Boscalid	R, WW
Atrazine-desethyl	R, WW, G	Imidacloprid-urea	R, WW
Bentazone	R, WW, G	Diazinon	R, WW
Carbendazim	R, WW, G	Azoxystrobin acid	R, WW
Metazachlor	R, WW, G	Chloridazone-methyl-desphenyl	R, WW
Dinoseb	R, WW, G	Pethoxamide	R, WW
Imidacloprid	R, WW, G	Acetamiprid	R, WW
Terbutryn	R, WW, G	op-DDE	R, WW
Tebuconazole	R, WW, G	op-DDD	R, WW
Terbuthylazine-desethyl	R, WW, G	op-DDT	R, WW
Terbuthylazine	R, WW, G	O-Hydroxybiphenyl	R, WW
Dimethenamide	R, WW, G	Cypermethrin	R, WW
Chlorpyriphos	R, WW, G	Benzotriazole-5 6-di-methyl	R, G
Triallate	R, WW, G	Phthalate-Dimethyl	R, G
pp-DDE	R, WW, G	Metolachlor-ESA	R, G
pp-DDD	R, WW, G	Dazomet	R, G
pp-DDT	R, WW, G	Simazine	R, G
Total DDTs	R, WW, G	Propiconazole Metabolite SYN 547889	R, G
Chlorothalonil	R, WW, G	Terbuthylazin-2-hydroxy	R, G
a-HCH	R, WW, G	Metazachlor metabolite (Metazachlor ESA, 479M008; 291634)	R, G
b-HCH	R, WW, G	Metalaxyl metabolite (Metalaxyl acid, CGA 62826, NOA 409045)	R, G
d-HCH	R, WW, G	Terbuthylazin metabolite (SYN545666 /LM6)	R, G
e-HCH	R, WW, G	Metolachlor, S-	R, G
g-HCH	R, WW, G	4-Piperidinecarboxamide	R, B
Sum-HCHs	R, WW, G	Metolachlor metabolite (Metolachlor OA, CGA 351916/CGA 51202)	R, B
Methoprene	R, WW, B	Imazamox	R, B

** R: river water, WW: wastewater, G: groundwater, S: sediments, B: biota*

29.3.6 Risk assessment

In an effort to prioritize the substances based on their potential to pose a threat for the aquatic ecosystem, concentration levels of the detected contaminants were compared to their PNEC values. In Table 3, a list of potentially toxic PPPs and industrial chemicals is presented. In total, nineteen PPPs and eight industrial chemicals exceeded their respective ecotoxicological threshold in at least one site during JDS4.

Table 3 also summarizes the number of samples that exceeded the PNEC. This information indicates whether the exceedance was local or of a basin-wide importance. All these substances need further attention of the regulators and the researchers. A prioritisation of the substances using the above results and the NORMAN methodology (Dulio and von der Ohe, 2013) will be presented in Chapter 36.

Table 3: PPPs and industrial chemicals that exceeded their PNECs in JDS4 surface river water, sediment and biota samples.

Surface waters				
Compound	**PNECfw (ng/L)**	**Samples >PNEC**	**Range of concentrations >PNEC (ng/L)**	**Sample with highest exceedance**
Benzododecinium	62	49	364-11279	JDS4-24
PFOS	0.65*	46	0.71-11.5	JDS4-12
Pethoxamide	0.49	35	1-16.5	JDS4-12
Terbuthylazin-2-hydroxy	7.3	23	7.9-122	JDS4-46
Carbendazim	150	10	168-1523	JDS4-36
Methoprene	1.4	8	10.5-40.4	JDS4-37
Imidacloprid	8.3*	7	8.8-39.9	JDS4-46
Phosphate-2-ethylhexyl-diphenyl (EHDP)	18	6	18.6-53.6	JDS4-51
4-tert-Octylphenol (4-t-OP)	100*	5	101-124	JDS4-27
Terbuthylazine	60	4	61.9-87.1	JDS4-11
pp-DDE	0.4	4	0.58-2.7	JDS4-46
Nicosulfuron	9	4	9.6-47.1	JDS4-49
Metazachlor	20	3	27.9-29.3	JDS4-12
2,4-D	20	2	56.5-943	JDS4-36
Imazamox	11	1	26.0	JDS4-46
Pyrethrin I	1.4	1	3.0	JDS4-34
Dazomet	38	1	38.2	JDS4-48
Bisphenol A-bis(3-chloro-2-hydroxypropyl) ether	340	1	536	JDS4-04
pp-DDD	0.5	1	0.82	JDS4-13

Sediments				
Compound	**PNECsed (µg/kg d.w.)**	**Samples >PNEC**	**Range of concentrations >PNEC (µg/kg d.w.)**	**Sample with highest exceedance**
Bis-(2-ethylhexyl)-phthalate (DEHP)	0.0077	4	469-1342	JDS4-24
Benzododecinium	0.1	4	3.5-18.9	JDS4-24
N-Methyldodecylamine	9.04	2	297-540	JDS4-24
Methiocarb (Mercaptodimethur)	0.12	2	3.1-6.1	JDS4-51
4-tert-Octylphenol (4-t-OP)	12.3	1	25.7	JDS4-51
N,N-Dimethyltetradecylamine	6.11	1	17.5	JDS4-24
Cadusafos	0.031	1	0.61	JDS4-24
Biota				
Compound	**PNECbio (µg/kg w.w.)**	**Samples >PNEC**	**Range of concentrations >PNEC (µg/kg w.w.)**	**Sample with highest exceedance**
PFOS	9.1*	6	9.7-22.1	JDS4-6.2-Y-FC
Methoprene	0.1	11	2.0-6.9	JDS4-29-R-FC
4-tert-Octylphenol (4-t-OP)	19.9	7	23.5-78.9	JDS4-23-Y-FC
Bis-(2-ethylhexyl)-phthalate (DEHP)	1.33	4	6.6-134	JDS4-6.2-Y-FC
Imazamox	0.064	4	0.72-1.8	JDS4-6.2-Y-FC
Imazapyr	0.061	3	5.6-31.4	JDS4-6.2-Y-FC
Propoxur	0.046	1	1.0	JDS4-49-R-FC
Indole-3-acetic acid	42.1	1	115	JDS4-6.2-Y-FC

** Environmental quality standard (EQS)*

29.4 Conclusions

This chapter summarises the occurrence of industrial chemicals and PPPs in river, wastewater, groundwater, sediment and biota (fish muscle) samples collected within JDS4. The removal of industrial chemical and PPPs by the WWTPs was investigated, their fate in the catchment was reported, and attention was drawn to nineteen PPPs and eight industrial chemicals that exceeded their respective ecotoxicological thresholds in various matrices and thus qualifiers as possible Danube RBSPs. WWTPs proved partially unable to effectively remove industrial chemicals and PPPs. However, they nonetheless managed to significantly reduce concentration levels of the vast majority of studied contaminants. Overall, the concentration levels of PPPs were at significantly lower concentration levels than those of industrial chemicals.

29.5 References

AALIZADEH R, VON DER OHE PC, THOMAIDIS NS. Prediction of acute toxicity of emerging contaminants on the water flea Daphnia magna by Ant Colony Optimization-Support Vector Machine QSTR models. Environ Sci Process Impacts 2017; 19: 438-448.

ALYGIZAKIS NA, BESSELINK H, PAULUS GK, OSWALD P, HORNSTRA LM, OSWALDOVA M, et al. Characterization of wastewater effluents in the Danube River Basin with chemical screening, in vitro bioassays and antibiotic resistant genes analysis. Environment International 2019; 127: 420-429.

ALYGIZAKIS NA, URIK J, BERETSOU VG, KAMPOURIS I, GALANI A, OSWALDOVA M, et al. Evaluation of chemical and biological contaminants of emerging concern in treated wastewater intended for agricultural reuse. Environ Int 2020; 138: 105597.

BRACK W, AISSA SA, BACKHAUS T, DULIO V, ESCHER BI, FAUST M, et al. Effect-based methods are key. The European Collaborative Project SOLUTIONS recommends integrating effect-based methods for diagnosis and monitoring of water quality. Environmental Sciences Europe 2019; 31.

BROWN AK, WONG CS. Distribution and fate of pharmaceuticals and their metabolite conjugates in a municipal wastewater treatment plant. Water Research 2018; 144: 774-783.

DASENAKI ME, THOMAIDIS NS. Multi-residue determination of 115 veterinary drugs and pharmaceutical residues in milk powder, butter, fish tissue and eggs using liquid chromatography-tandem mass spectrometry. Anal Chim Acta 2015; 880: 103-21.

DIAMANTIKS, ALYGIZAKIS NA, NIKA MC, OSWALDOVA M, OSWALD P, THOMAIDIS NS, et al. Assessment of the chemical pollution status of the Dniester River Basin by wide-scope target and suspect screening using mass spectrometric techniques. Anal Bioanal Chem 2020; 412: 4893-4907.

DULIO V, KOSCHORRECK J, VAN BAVEL B, VAN DEN BRINK P, HOLLENDER J, MUNTHE J, et al. The NORMAN Association and the European Partnership for Chemicals Risk Assessment (PARC): let's cooperate! Environmental Sciences Europe 2020; 32.

DULIO V, VAN BAVEL B, BRORSTROM-LUNDEN E, HARMSEN J, HOLLENDER J, SCHLABACH M, et al. Emerging pollutants in the EU: 10 years of NORMAN in support of environmental policies and regulations. Environ Sci Eur 2018; 30: 5.

DULIO V, VON DER OHE PC. NORMAN prioritisation framework for emerging substances, http://www.norman-network.net/sites/default/files/norman_prioritisation_manual_15%20April2013_final_for_website.pdf, (Last accessed 21 August 2020).

ENAULT J, ROBERT S, SCHLOSSER O, DE THE C, LORET JF. Drinking water, diet, indoor air: Comparison of the contribution to environmental micropollutants exposure. Int J Hyg Environ Health 2015; 218: 723-30.

GAGO-FERRERO P, BLETSOU AA, DAMALAS DE, AALIZADEH R, ALYGIZAKIS NA, SINGER HP, et al. Wide-scope target screening of >2000 emerging contaminants in wastewater samples with UPLC-Q-ToF-HRMS/MS and smart evaluation of its performance through the validation of 195 selected representative analytes. J Hazard Mater 2020; 387: 121712.

GAGO-FERRERO P, BOROVA V, DASENAKI ME, TAUHOMAIDIS NUS. Simultaneous determination of 148 pharmaceuticals and illicit drugs in sewage sludge based on ultrasound-assisted extraction and liquid chromatography-tandem mass spectrometry. Anal Bioanal Chem 2015; 407: 4287-97.

GONZALEZ-MARINO I, BAZ-LOMBA JA, ALYGIZAKIS NA, ANDRES-COSTA MJ, BADER, BANNWARTH, et al. Spatio-temporal assessment of illicit drug use at large scale: evidence from 7 years of international wastewater monitoring. Addiction 2020; 115: 109-120.

JARDAK K, DROGUI P, DAGHRIR R. Surfactants in aquatic and terrestrial environment: occurrence, behavior, and treatment processes. Environmental Science and Pollution Research 2016; 23: 3195-3216

MARIANI G, TAVAZZI S, COMERO S, BUTTIGLIERI G, PARACCHINI B, SKEJO H, et al. Short-term isochronous stability study of contaminants of emerging concern in environmental waters samples, JRC Technical Report 2017; (https://publications.jrc.ec.europa.eu/repository/bitstream/JRC99966/kj-na-28425-en-n.pdf, Last accessed 21 August 2020).

THOMAIDIS NS, GAGO-FERRERO P, ORT C, MARAGOU NC, ALYGIZAKIS NA, BOROVA VL, et al. Reflection of Socioeconomic Changes in Wastewater: Licit and Illicit Drug Use Patterns. Environ Sci Technol 2016; 50: 10065-72.

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Wide-scope target screening of illicit drugs, pharmaceuticals, antibiotics and personal care products in wastewater, groundwater, river water, sediments and biota by liquid chromatography coupled with high resolution mass spectrometry

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Abstract

This chapter provides an overview on the determination of more than 1,300 pharmaceuticals, illicit drugs, antibiotics, personal care products (PCPs) and their transformation products (TPs) in samples collected within JDS4. In total, residues of 287 of these substances were detected in wastewater, 140 were detected in surface water samples, 41 were found in biota and 31 in river sediments. The measured concentration levels of individual substances were benchmarked against their Predicted No-effect Concentration (PNEC) values retrieved from the NORMAN Ecotoxicology Database (https://www.norman-network.com/nds/ecotox/). The most prominent from the group of illicit drugs was benzoylecgonine – metabolite of cocaine, detected in all 11 tested wastewater samples with the highest concentrations in influent samples from WWTPs Novo Mesto (Locna, SI; 0.666 µg L^{-1}), Vrakuňa (Bratislava, SK; 0.513 µg L^{-1}) and Šabac (RS; 0.360 µg L^{-1}). Norbuprenorphine, an opiate often used as a substitute for heroin, was present in wastewater samples in Győr (HU), Šabac (RS), Vrakuňa (SK) and Giurgiu (RO) and in biota samples at Jochenstein (DE/AT) and upstream Timok (RS/BG). Concentrations of amphetamine and metamphetamine were highest in influent wastewater samples in Uzhgorod (UA), Vrakuňa (Bratislava, SK), Hodonín (CZ) and Győr (HU). In general, concentrations of illicit drugs represented only ca. 1% of the overall load of studied substances and were reduced significantly during the treatment at WWTPs. The trace levels in river water, biota and sediments were typically several orders of magnitude below their respective toxicity threshold values and thus presenting no threat to ecosystems and human health. Antibiotics were the most frequently detected class of compounds in water matrices, whereas several pharmaceuticals were detected with remarkably high frequency of appearance (FoA) in all samples. In total, 17 compounds exceeded their PNECs indicating potential adverse effects on the impacted ecosystem. The antipsychotic drug sulpiride exceeded its provisional PNEC value of 5.87 mg Kg^{-1} ww in 8 biota samples with highest concentrations in Hercegszántó/Batina/Bezdan (HU/HR/RS; 52.4 mg Kg^{-1} ww) and

Cunovo (SK; 36.9 mg Kg^{-1} ww). All wide-scope target screening data presented in this chapter and in Chapter 29 were stored in a non-line NORMAN Database System (https://www.norman-network.com/nds/empodat/) and can be viewed via an interactive map at https://norman-data.eu/JDS4.

30.1 Introduction

Pharmaceuticals and their TPs are nowadays well recognised as emerging contaminants, since they are being continuously introduced into the environment and detected, sometimes at alarming concentration levels, in all environmental compartments at the global scale. It has already been demonstrated that they may have an adverse impact on the river fauna and flora (Miller et al. 2018, Brain et al. 2008). The terms "illicit drugs" and "drugs of abuse" are often interchangeable in the literature. Illicit drugs, including opioids, cocaine, cannabis, amphetamine-type and ecstasy-group compounds, are highly addictive substances for which nonmedical use is prohibited by national or international laws and they are illegal to make, sell and/or use. Drugs' abuse refers to the inappropriate or excessive use of any drug, or the use of prescription or over-the-counter drugs for recreational or pleasure purposes, or to affect one's mode, consciousness or a body function unnecessarily. Personal care products (PCPs), pharmaceuticals, illicit drugs and their metabolites, may enter the environment primarily through wastewater treatment plants (WWTPs) effluent discharges, since they are not fully removed during the treatment processes. Despite the ubiquitous presence of pharmaceuticals and PCPs, their regular monitoring is not requested by the current EU water legislation. Obviously, there is a need to gather critical mass of monitoring data in support of future regulations at the EU and basin scale. A presence of numerous pharmaceuticals and PCPs has already been reported in the Danube River Basin (DRB) (Liška et al. 2015), and some of them have been proposed as Danube River Basin Specific Pollutants (RBSPs). The JDS3 results indicated also an occurrence of several illicit drugs and their metabolites in surface water. This became an issue of high public concern and JDS4 followed up on this to provide a thorough overview on their distribution and potential effects at the basin scale.

The aim of this chapter is to report on the occurrence and fate of pharmaceuticals, illicit drugs, PCPs and their metabolites and transformation products (TPs) in river water, wastewater, groundwater, sediments and biota (fish) matrices in the DRB. The presence of illicit drugs at the DRB scale is reported for the first time.

Wide-scope target screening methodologies were complemented with the 'suspect screening' by LC-HRMS providing an insight on presence/absence and semi-quantification of more than 65,000 chemicals and their TPs in each single sample. Results of suspect screening are reported in the Chapter 34 and 36. All samples analysed by HRMS are stored in the NORMAN Database System (https://www.norman-network.com/nds/) (Dulio et al. 2020) and its Digital Sample Freezing Platform (DSFP; https://norman-data.net/) (Alygizakis et al. 2019) and thus available for retrospective screening of any detected compound, even those labelled as 'unknown' today, using their unique 'fingerprints' (mass spectra).

30.2 Methods

30.2.1. Sample preparation methods

The analytical programme covered samples of 51 river water, 11 influent wastewater, 11 effluent wastewater, 7 groundwater, 11 biota samples (mainly bleak muscle, one asp muscle) and 4 sediment samples (<63 μm) sampled within the JDS4 in June and July 2019 (see Chapter 2).

River water, groundwater and wastewater samples were extracted by HORIZON SPE-DEX device. The samples were also processed using MARIANI box (Mariani et al. 2017) for the follow-up determination of 13 pre-selected pharmaceuticals of increasing concern in the DRB (carbamazepine, sulfamethoxazole, 10,11-dihydro-10,11-dihydroxy-carbamazepine, azithromycin, clarithromycin, amoxicillin, diclofenac, naproxen, bezafibrate, ibuprofen, ciprofloxacin, 17beta-estradiol and estrone (E1)). Extraction of sediments was carried out based on a validated protocol for the determination of pharmaceuticals and illicit drugs in sewage sludge (Gago-Ferrero et al. 2015), whereas biota extraction was performed following a multi-residue optimized method for the determination of veterinary drugs and pharmaceuticals (Dasenaki and Thomaidis 2015). The methods are described in more detail in Chapter 29.

30.2.2 Instrumental methods

Two complementary instrumental methods were used for the screening of 1,301 pharmaceuticals, PCPs, illicit drugs and their TPs in the JDS4 samples' extracts. A highly sensitive LC-MS/MS method using multiple reaction monitoring (MRM) scan mode was used for the determination of 158 illicit drugs, drugs of abuse, commonly consumed pharmaceuticals, antibiotics, and their TPs at trace-level concentrations (Alygizakis et al. 2016). Furthermore, high-resolution mass spectrometric analysis by LC-electrospray (ESI)-QTOFMS, through full-scan MS and MS/MS acquisition, enabled the screening of additional >65,000 substances including thousands of pharmaceuticals, PCPs and their TPs. The analytical description of the chromatographic separation, mass spectrometric detection and data treatment workflows of these methodologies are available in previously published studies (Dasenaki and Thomaidis 2015, Diamanti et al. 2020) and described in Chapter 29.

30.3 Results

The results of determination of all studied contaminants were collected in the pre-programmed spreadsheets termed 'Data Collection Templates' (DCTs) gathering all necessary metadata (e.g. sampling site name, date, coordinates, sample matrix etc.) and information to judge the quality of the results (e.g. Limit of Detection/Quantification, level of the validation of the used methods and accreditation of the laboratory etc.). The DCTs were uploaded into the NORMAN Database System (NDS) and its EMPODAT module (https://www.norman-network.com/nds/empodat/). All results can be interactively visualized in an on-line map (https://norman-data.eu/JDS4/). The results for groundwater samples were discussed in Chapter 25. Although most of the pharmaceuticals have broad uses and pharmacological actions, for the statistical treatment and presentation of the results, a classification was attributed to the detected compounds, based on their main use, application or therapeutic action. When the contaminants were detected above their LOD but below their respective LOQ, LOQ/2 values were used for the calculation of cumulative concentrations per class for reporting purposes in this chapter, as indicated by the Directive 2009/90/EC. The results describing the occurrence of pharmaceuticals, PCPs and illicit drugs per environmental matrix are presented below.

30.3.1 Danube surface river water samples

140 pharmaceuticals, PCPs, illicit drugs and their TPs were detected in JDS4 river water samples and categorized into 10 main sub-classes. The most frequently detected sub-classes were antibiotics (32 compounds), antipsychotic drugs (17), illicit drugs and stimulants (14) and analgesics (13), whereas "other pharmaceuticals" consisted of 29 pharmaceuticals with various clinical uses.

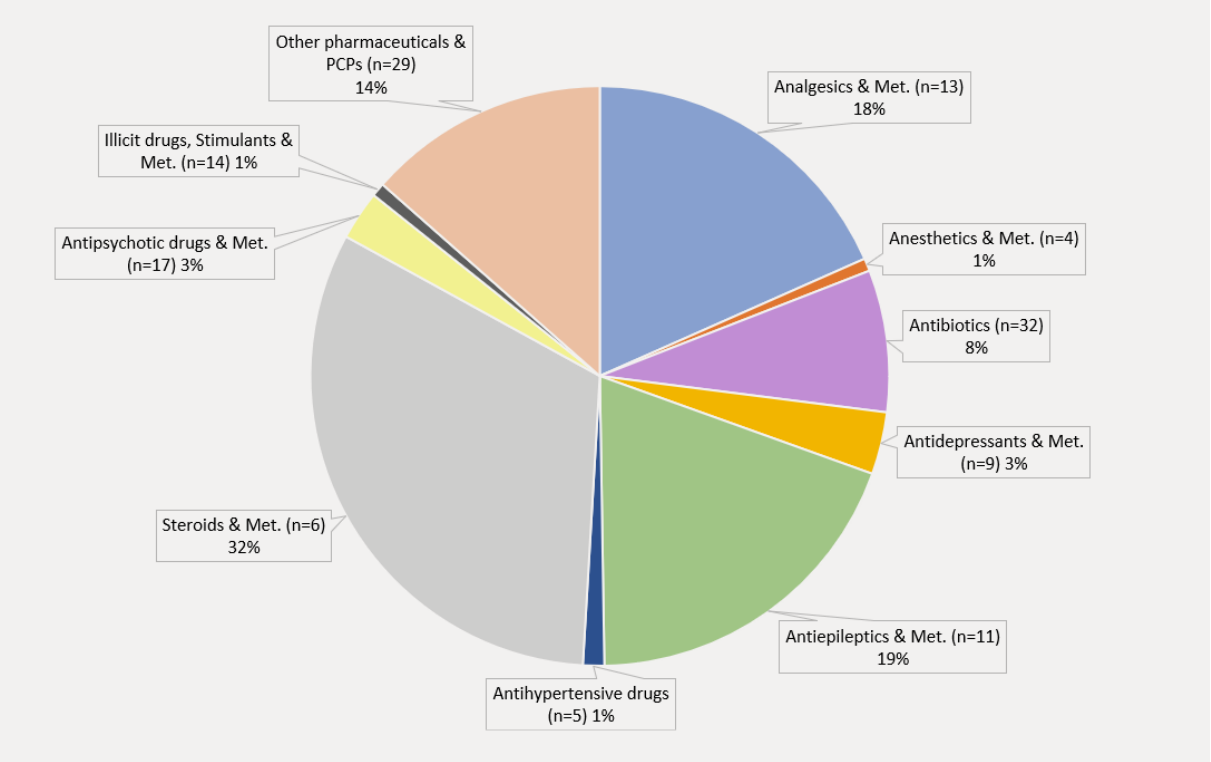

Figure 1: %Contribution of the different classes of pharmaceuticals and illicit drugs to the overall cumulative contamination of the JDS4 surface water samples (expressed as concentration).

As illustrated in Figure 1, mostly steroids and their metabolites contributed to the overall pollution of surface water (32%), expressed as a total concentration in all tested samples. Antiepileptics and analgesics followed with a contribution of 19 and 18%, respectively. Among the detected compounds, caffeine, clarithromycin, sulfamethoxazole, carbamazepine and its metabolite 10,11-dihydro-10,11-dihydroxy-carbamazepine, metformin and 19-norandrosterone were present in all tested samples. 58 compounds were detected in less than 10% of the analysed samples. In total, 24 TPs of pharmaceuticals and illicit drugs were detected in the analysed samples. In most cases, both parent compound and characteristic TPs were detected (including e.g. mirtazapine and its TPs: 8-OH-mirtazapine and normirtazapine, amisulpride and amisulpride-N-oxide, tramadol and nortramadol and lidocaine and lidocaine-N-oxide). For nine compounds (cotinine, cetirizine-N-oxide, galaxolidone, nortilidine, 4-acetamidoantiyrine-benzoylecgonine, norclozapine, nordiazepam and 7-amino-flunitrazepam), only the TPs were detected. JDS4-13, JDS4-15 and JDS4-44 were the samples that presented the highest total levels of TPs (ranging from 191 to 200 ng L^{-1}). Several compounds, including amoxicillin, ciprofloxacin, sulfamethoxazole, trimethoprim, venlafaxine and fluconazole that are listed in the most recent EU Watch List (EU 2020/1161), were detected in JDS4 river water samples. All compounds were detected typically at concentration levels up to tens of ng L^{-1}, only the maximum detected concentrations for 4-acetamido-antipyrine, 10,11-dihydro-10,11-dihydroxy carbamazepine, tenofovir, corticosterone and 19-norandrosterone, ranged from 114 ng L^{-1} (tenofovir) to 1,171 ng L^{-1} (19-norandrosterone). JDS4-12 was

the most polluted sample, with the total cumulative concentration of detected pharmaceuticals at 1,330 ng L^{-1}, mainly due to the detection of high concentration of 19-norandrosterone. The highest number of the studied compounds (65) was detected in JDS4-1. Since the detection of the steroid 19-norandrosterone (concentration range: 4.69-1,171 ng L^{-1}), significantly affected the overall concentration profile of the river water samples, it was not considered in **Figure A (Annex)** illustrating the total cumulative concentrations per class and the total number of detected compounds.

Samples JDS4-44 (BG), JDS4-13 (SK) and JDS4-46 (BG) from the Danube tributaries revealed the maximum cumulative concentrations of pharmaceuticals and illicit drugs at 790, 760 and 758 ng L^{-1}, respectively. The highest cumulative concentration of antibiotics was detected in JDS4-24 (65.7 ng L^{-1}), while JDS4-46 was the most polluted surface water sample in terms of analgesics (342 ng L^{-1}), illicit drugs and stimulants (24.3 ng L^{-1}). The maximum cumulative concentrations for antiepileptics, other pharmaceuticals and PCPs (278 and 246 ng L^{-1}) were determined in JDS4-13 and JDS4-30, respectively. Anaesthetics were detected at significantly lower levels compared to the rest of the analysed target compounds, reaching a maximum of 14.3 ng L^{-1} at JDS4-37 in Serbia. The maximum cumulative concentrations for antihypertensives (116 ng L^{-1}), antidepressants (53.5 ng L^{-1}) and antipsychotics (69.9 ng L^{-1}) were detected in samples from JDS4-34, JDS4-19 and JDS4-51, respectively.

When comparing the concentration ranges from all discussed substance categories in the Danube surface water (29 samples) and its tributaries (22 samples), as shown in Figure 2, a clear trend of increasing median cumulative concentration and higher deviation can be seen in the samples collected from the tributaries. This profile was not similar in the case of anaesthetics. An almost equal distribution between the pollution of the main stream and the tributaries was recorded. In contrast, antipsychotic drugs were observed with a 2-fold higher median concentration levels in the Danube River samples compared to its tributaries. The surface waters collected from the tributaries, especially JDS4-11, JDS-12 and JDS4-13 from the Morava river, and JDS4-44 and JDS4-46 from the Iskar and Russenski Lom, were among the most polluted samples, considering the total concentration and number of detected compounds. Moreover, JDS4-17 (Mosoni, Hungary) and JDS4-21(Ipel river, Slovakia), presented high cumulative concentrations compared to all JDS4 surface samples (Figure A).

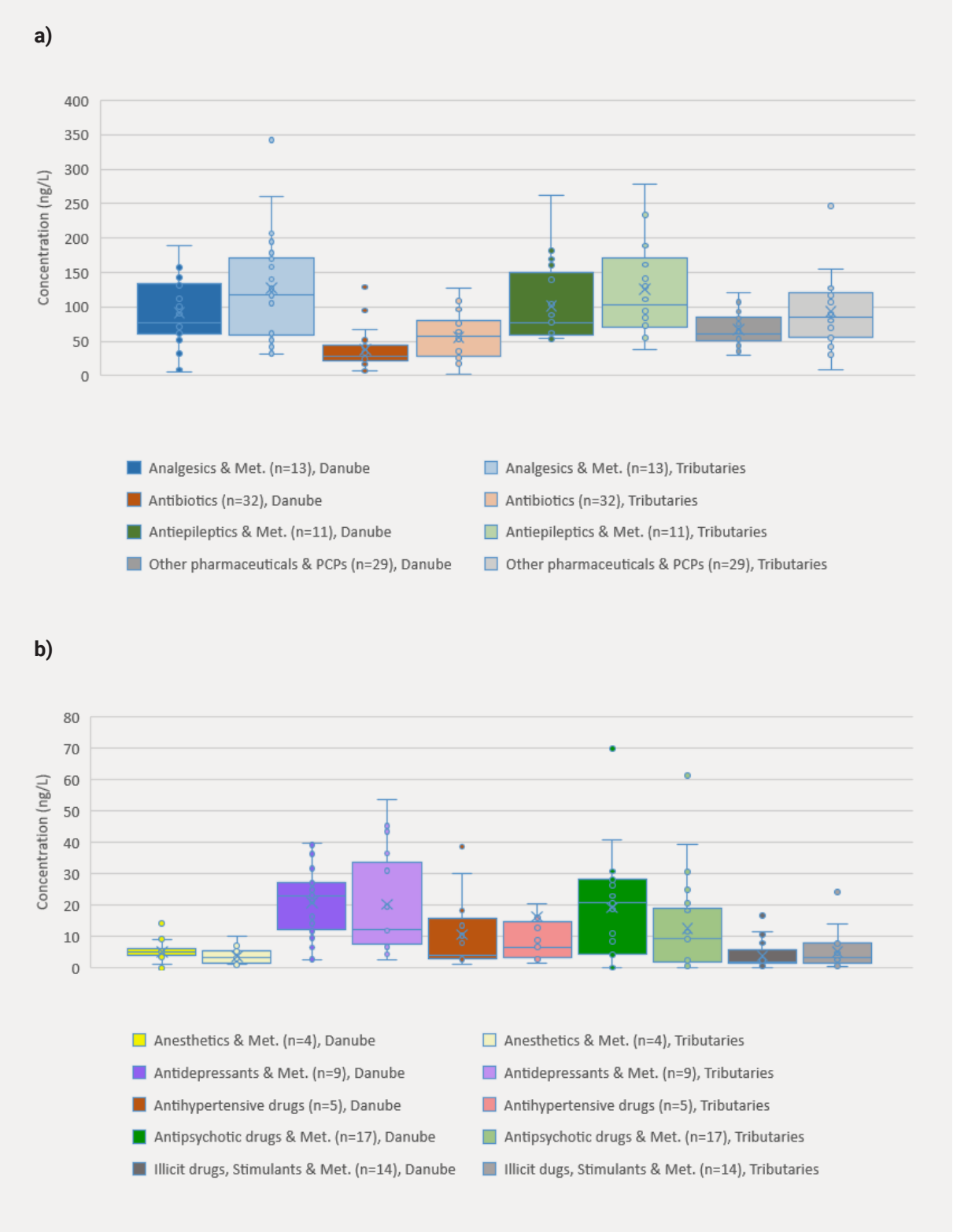

Figure 2: Box plots of different classes of pharmaceuticals and illicit drugs in the Danube River (No. of sites, 29) and its tributaries (No. of sites, 22).

30.3.2 Sediments

Overall, 31 pharmaceuticals were detected in the four analysed samples, with antibiotics being the most frequently detected class of compounds (n=10). Seven antidepressants, antipsychotic drugs, and their metabolites, four antiepileptics and antihypertensive drugs, three analgesics, two PCPs and their metabolites, four other pharmaceuticals and the metabolite of nicotine, nornicotine, were determined. Most of the detected compounds were found at concentration levels up to tens of μg Kg^{-1} dry weight, while clarithromycin, sulfadoxine, mirtazapine, sulpride, chlordiazepoxide, chlorpromazine, propafenone and oxfendazole, were present only below their respective LOQs. The antibiotic sulfadiazine, the antiepileptic carbamazepine and the UV-filter octocrylene were the most abundant compounds found at maximum concentrations of 120, 213 and 162 μg Kg^{-1}, in JDS4-47, JDS4-6 and JDS4-47, respectively. Amisulpride, citalopram, bisoprolol, apophedrin, methocarbamol and galaxolidone, were the most ubiquitous compounds, as they were detected in all tested samples. Nine out of 31 compounds were detected only in one sediment sample.

Figure B (Annex) illustrates the total cumulative concentration of pharmaceutical and PCPs, detected in the analyzed samples. Nornicotine was not included in the graph, as it was the only representative from the stimulants class, and it was detected only in JDS4-24 (1.71 μg Kg^{-1}). JDS4-47 (BG/RO) was the most contaminated sample, reaching a total concentration of 600 μg Kg^{-1}, while JDS4-24 (HU) and JDS4-51 (RO/UA) presented the lowest total concentration levels of 212 and 209 μg Kg^{-1}, respectively. The maximum total cumulative concentrations of antidepressants, antipsychotics and their metabolites (50.3 μg Kg^{-1}), as well as other pharmaceuticals (243 μg Kg^{-1}), were detected in JDS4-6, while JDS4-47 was the most contaminated sample for antibiotics (171 μg Kg^{-1}), antiepileptics and antihypertensive drugs (24.7 μg Kg^{-1}), PCPs and their metabolites (184 μg Kg^{-1}). The highest levels of analgesics were observed in JDS4-51 (10.5 μg Kg^{-1}). Concerning the total number of detected compounds, at JDS4-6 site 23 compounds were detected compared to ≤16 for the other three sediment samples. Only three TPs were among the detected compounds, norvenlafaxine (metabolite of venlafaxine), nornicotine (metabolite of nicotine) and galaxolidone (metabolite of galaxolide).

30.3.3 Biota

The analysis of 11 biota samples (fish muscle) revealed the presence of 41 pharmaceuticals, illicit drugs and PCPs. Among them were six antidepressants, antipsychotic drugs and illicit drugs, five analgesics, four antibiotics and NSAIDs, four antiepileptics, four antihypertensive drugs, three anesthetics, two PCPs and twenty-three 'other' pharmaceuticals with a broad range of clinical uses, along with their metabolites. Steroids were not reported for biota samples, as they are naturally occurring compounds in such matrices and therefore their detection cannot be linked to potential contamination. The most frequently detected compounds were 4-acetamido-antipyrine, salicylic acid, epinephrine cytarabin and galaxolidone, being present in 10 out of 11 analyzed samples, whereas 4-formyl-antipyrine, simvastatin, ephedrine, allopurinol and acamprosate were detected in all samples. On the other hand, 12 compounds were detected in only one biota sample. 88% of the detected compounds were found at concentration levels below 40 μg Kg^{-1} wet weight, whereas apophedrine, ibuprofen and sulpiride were the most abundant compounds, reaching maximum detected concentrations of 113, 57.2 and 52.4 μg Kg^{-1}, respectively. Nine of the detected compounds were metabolites of pharmaceuticals, including 4-formyl-antipyrine, 4-acetamido-antipyrine, nortramadol, normirtazapine, norbuprenophine, gabapentin-lactam, N-acetyl-mesalazine and galaxolidone.

As shown in **Figure C (Annex)**, no significant variation in the cumulative concentrations and number of detected pharmaceuticals and PCPs could be seen among the tested biota samples. JDS4-43 (RO/BG), was the least contaminated sample (157 µg Kg^{-1}), whereas the highest total cumulative concentration (335 µg Kg^{-1}) was detected in JDS4-29 (HU/HR/RS). When comparing the cumulative concentrations among JDS4-6 and JDS4-6.2 (DE/AT), and JDS4-23 and JDS4-24 (HU), quite similar contamination profile (distribution per class and total cumulative concentration) was noticed. This indicates that biota samples collected within the same country and at close sampling points may have similar contamination profiles. The highest levels of antibiotics and NSAIDs (67.8 µg Kg^{-1}), antidepressants, antipsychotic drugs and illicit drugs (53.8 µg Kg^{-1}) and antihypertensive drugs (18.4 µg Kg^{-1}) were detected in JDS4-29, while anesthetics and antiepileptics were detected at maximum levels of 21.3 and 86.9 µg Kg^{-1} in sample from JDS4-6.2, respectively. Up to 33.4, 204.9 and 21.2 µg Kg^{-1} of analgesics, other pharmaceuticals, and PCPs, were detected in JDS4-37, JDS4-49 and JDS4-6, respectively.

30.3.4 Influent and effluent wastewater

In total, 287 compounds from the studied list of 1,301 substances and their TPs were detected in wastewater samples. Among them, 239 pharmaceuticals and illicit drugs were present in at least one influent wastewater sample and 202 in at least one effluent wastewater sample. Parent compounds and metabolites from all classes were among the most ubiquitous contaminants, as they were detected in all wastewater samples, both influent and effluent, indicating that parent compounds were not efficiently removed through the processes that are applied in the wastewater treatments plants (WWTPs) and that TPs must not be underestimated in monitoring studies. Among these compounds were widely used pharmaceuticals such as caffeine, telmisartan, sulfamethoxazole, sulfapyridine, metformin, diclofenac, meclofenamic acid, norfentanyl (the main metabolite of fentanyl), hydrochlorothiazide, two main metabolites of metamizole: 4-formylamino antipyrine and 4-acetamido-antipyrine, phenoxybenzamide, valsartan, lamotrigine, oxcarbazepine, carbamazepine and its main metabolites 10,11-epoxide carbamazepine and 10,11-dihydro-10,11 dihydroxy carbamazepine, galaxolide (metabolite of the synthetic musk galaxolide), as well as benzoylecgonine, the main metabolite of cocaine, and the antidepressant doxepin. Although most of the detected contaminants were detected at low-ng L^{-1} concentration levels, caffeine and its metabolite theophylline, tramadol, telmisartan, cloxacillin, sulfamethoxazole, valsartan, valproic acid, 10,11-dihydro-10,11 dihydroxy carbamazepine, lidocaine-N-oxide, hydrochlorothiazide, diclofenac, naproxen, galaxolidone and prednisolone, were the most abundant compounds with their maximum detected concentration ranging from 1.11 to 9.88 µg L^{-1}. 17beta-estradiol, included in the Watch List established by the Commission Implementing Decision (EU) 2018/840, was detected in five wastewater effluent samples, at concentration levels from 2.02 to 4.04 ng L^{-1}. Amoxicillin, ciprofloxacin, sulfamethoxazole, trimethoprim, O-desmethyl-venlafaxine and fluconazole that are included in the updated Watch List of 2020 (EU 2020/1161), were also detected in wastewater samples. To visualize the results in graphs, the detected compounds were grouped into 17 main classes of pharmaceuticals, illicit drugs and their metabolites. Most of the detected compounds were antibiotics (41 compounds), antihypertensive drugs and their metabolites (33), antipsychotic drugs and their metabolites (31), whereas 38 compounds having diverse uses were classified as other pharmaceuticals.

Results presented in Figure 3 show that although the number of detected compounds was significantly higher in influents (average 109) compared to effluents (81), the total cumulative concentration of the compounds did not vary remarkably between the two tested matrices, presenting average total cumulative concentrations of 1.28 and 1.06 µg L^{-1}, in influents and effluents, respectively.

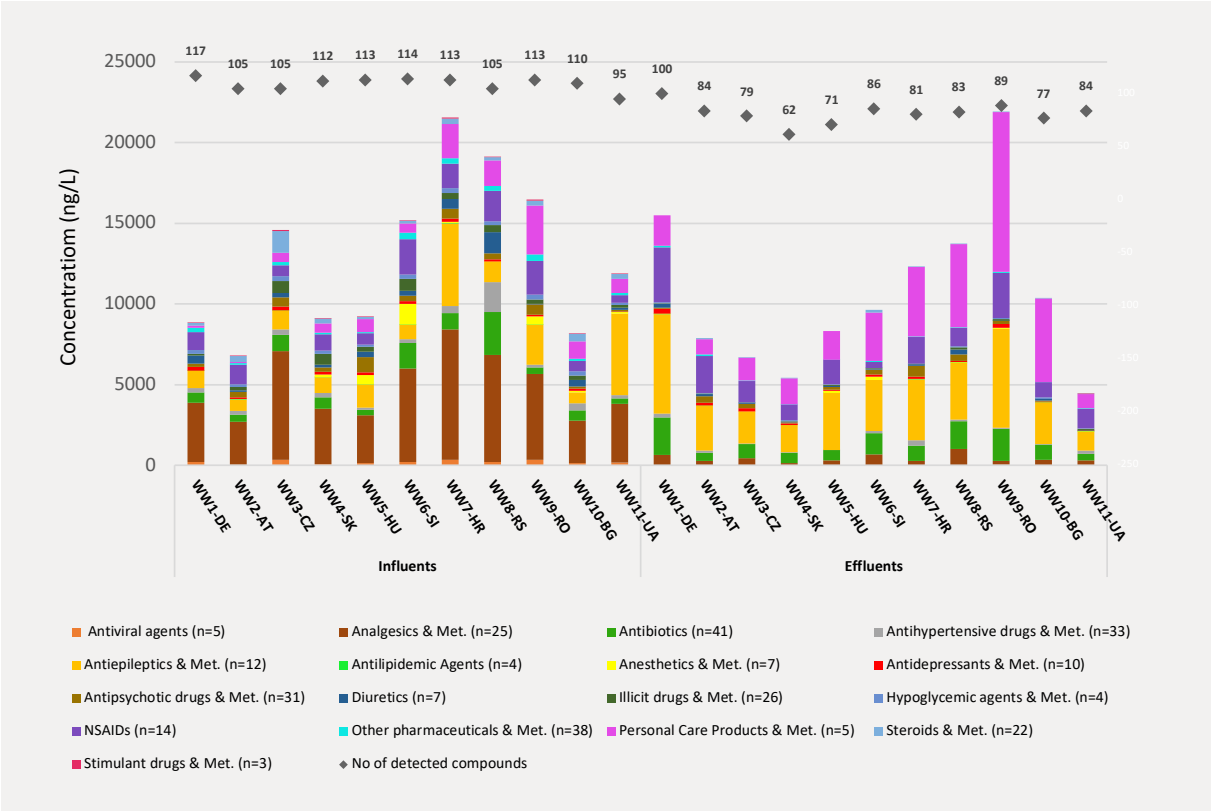

Figure 3: Overall detected cumulative concentrations of pharmaceuticals, illicit drugs and their TPs in the influent and effluent JDS4 wastewater samples.

Concerning the influent wastewater samples, the highest total cumulative concentration of pharmaceuticals, illicit drugs and their metabolites was noticed in the influent WW7-HR, reaching up to 6.78 μg L^{-1}, whereas the sample from Austria (WW2-AT) was the least contaminated (2.16 μg L^{-1}). Overall, analgesics and their metabolites were the class that contributed most to the total detected concentration in influent samples, ranging from 30% (WW11-UA) to 46% (WW3-CZ). Antiepileptic drugs and their metabolites was the class that dominated the sample WW11-UA (40%) and contributed significantly (24%) to WW7-HR contamination. The highest total cumulative concentration of antibiotics (2.69 μg L^{-1}), antihypertensive drugs and their metabolites (1.84 μg L^{-1}), as well as diuretics (1.33 μg L^{-1}), were detected in the WW8-RS influent sample. Cumulative concentrations of analgesics and antiepileptics, and their metabolites, were up to 3 and 7 times higher at WW7-HR (8.09 and 5.15 μg L^{-1}, respectively) compared to the rest of influent samples. Antilipidemic drugs and stimulants concentration levels reached up to tens of ng L^{-1} levels. Interestingly, hypoglycemic agents presented the lowest relative standard deviation of cumulative concentrations across all the samples (24%). The maximum concentrations for antiviral drugs (352 ng L^{-1}) and steroids (1.35 μg L^{-1}) were detected at WW3-CZ, whereas NSAIDs and other pharmaceuticals reached up to 2.16 μg L^{-1} and 418 ng L^{-1}, respectively, in WW6-SI. Concentration levels up to 50 times higher compared to the rest of influent extracts, were measured for total pharmaceuticals and PCPs in WW9-RO. Significantly lower concentrations of total antidepressants (32 ng L^{-1}) and antipsychotic drugs (71 ng L^{-1}) were detected in WW11-UA, compared to the maximum concentrations detected in WW3-CZ (240 ng L^{-1}) and WW5-HU (928 ng L^{-1}), respectively. The sum of up to 26 detected illicit drugs, resulted in total cumulative concentrations that ranged from 102 (WW1-DE) to 759 ng L^{-1} (WW3-CZ).

Analgesics, antipsychotics, and antibiotics were the most frequently detected classes in the effluent wastewater, while antiepileptics, NSAIDs and PCPs were the most abundant compounds with median concentrations equal to 3.18, 1.32 and 1.87 μg L^{-1}, respectively. However, the high concentration levels observed for these classes were mainly attributed to the high concentration of one compound per class: 10,11-dihydro-10,11 dihydroxy carbamazepine for antiepileptics, diclofenac for NSAIDs and galaxolidone for PCPs. The maximum cumulative concentrations for several classes, including antibiotics (2.32 μg L^{-1}), antiepileptics (6.16 μg L^{-1}), antidepressants (275 ng L^{-1}), NSAIDs (3.41 μg L^{-1}), and other pharmaceuticals (113 ng L^{-1}), were detected in WW1-DE. Stimulant drugs and antiviral agents were found less frequently and at lower concentration levels, with a maximum of 1.50 and 32.3 ng L^{-1} at WW11-UA and WW9-RO, respectively. The highest concentration levels of analgesics (999 ng L^{-1}), diuretics (285 ng L^{-1}) and illicit drugs (138 ng L^{-1}) were measured in the effluent sample of Serbia (WW8). The concentration levels of antipsychotics ranged from 18 ng L^{-1} (in WW11-UA) to 673 ng L^{-1} (in WW7-HR), whereas the highest concentration of PCPs was detected in the sample from Romania (WW9), following the same profile of the influent samples. Influent wastewater samples were composed of both parent compounds and TPs of pharmaceuticals and illicit drugs. During the processes applied in WWTPs, parent compounds may be (bio) transformed into several TPs. In total 50 TPs, including metabolites, were detected in the JDS4 wastewater samples, underlying the significance of collecting wide-scope target monitoring and suspect screening data to acquire a holistic view of the chemical fingerprint in the environmental samples. The parent compounds and TPs of pharmaceuticals and illicit drugs that were detected in the JDS4 wastewater samples, along with their frequency of detection and concentration range, are presented in **Table A (Annex)** (steroids were excluded from the table).

The most frequently detected compounds were caffeine and its main metabolites theophylline and theobromine; metamizole and its metabolites 4-formylamino-, 4-acetamido- and 4-amino-antipyrine; nicotine's metabolites cotinine and cotinine-hydroxy; carbamazepine and 10,11-epoxide-, 10,11-dihydro-10,11 dihydroxy- and 10-hydroxy- carbamazepine, as well as cocaine and its metabolite benzoylecgonine. Metformin was detected in all influent and effluent samples, whereas its main biotransformation product guanylurea was detected in most effluent samples, indicating the incomplete removal and transformation of metformin during the treatment processes in the investigated WWTPs. There were also cases when only the TPs and not the parent compounds were detected; e.g. galaxolidone – metabolite of galaxolide was detected in all samples, whereas its parent compound was not detected in any of the samples. The same was observed for citalopram-N-oxide, a TP of citalopram. Three main metabolites of the antipsychotic drug venlafaxine were detected in the effluent samples, whereas the parent compound remained undetected.

30.3.5 Presence of illicit drugs and drugs of abuse in the Danube River Basin

The occurrence of illicit drugs, drugs of abuse and their metabolites in the Danube River Basin samples was significant, considering that overall, 87 compounds were reported in at least one JDS4 environmental matrix (Table 1).

Table 1: Detected drugs of abuse, illicit drugs and their metabolites in the Danube River Basin.

Classification	Compounds	Matrices
Anesthetics & Met.	Fentanyl, Norfentanyl	R, WW, G, B
	Lidocaine, Lidocaine-N-oxide	R, WW
	Tolycaine	WW, B
	Norketamine, Propipocaine	WW
Antidepressants & Met.	8-Hydroxy-Mirtazapine, Clomipramine	R, WW, G
	Normirtazapine	R, WW, G, B
	Mirtazapine	R, WW, S
	Doxepin, Imipramine, Zolpidem	R, WW
	Amitriptyline, Quetiapine	R
	Citalopram	S
	AMT (Alpha-Methyltryptamine), Mazindol, GHB (Gamma-Hydroxybutyric acid)	WW
Antipsychotic drugs & Met.	Amisulpride-N-Oxide, Venlafaxine-N-oxide, 9-Hydroxy-Risperidone, Alprazolam, Bromazepam, Chlorpromazine, Fluoxetine, Nordiazepam, Risperidone, Sertraline	R, WW
	7-amino-flunitrazepam, Oxazepam	R, WW, G
	Amisulpride	R, WW, G, S
	Sulpiride	R, WW, G, S, B
	Norvenlafaxine	R, WW, S
	Midazolam, Temazepam	WW, B
	Chlodiazepoxide, Medazepam, D,L-N,O-Didesmethyl-venlafaxine	WW, G
	Norclozapine, Venlafaxine	R
	Chlordiazepoxide, Clorpromazine	S
	3-OH-Bromazepam, Citalopram N-oxide, D,L-N,N-Didesmethyl-Venlafaxine, Flurazepam-Desalkyl, 1-OH-Midazolam, (2-) Phenethylamine, Tiapride, Venlafaxine-O-Desmethyl (Desvenlafaxine), Lorazepam, Tetrazepam	WW

Classification	Compounds	Matrices
Illicit drugs & Met.	Cannabidiol, MDAI (5,6-methylenedioxy-2-aminoindane), Methadone, Methenolone	R
	6-O-Monoacetylmorphine (6-MAM), Cathine, Codeine, EDDP, Heroin (3,6-diacetylmorphine), MDMA, Morphine, Remifentanil	R, WW
	Norbuprenorphine	R, WW, B
	Benzoylecgonine	R, WW, G
	3,4-Methylenedioxyamphetamine (MDA), AB-PINACA 5Cl, alpha-Pyrrolidinopentiophenone, Barbital-Pheno, Deschloroetizolam, DMPEA, MDPPP (3-4-Methylenedioxy-a-pyrrolidinopropiophenone), Mepirapim, 2,5-diethoxy-4-methoxyamphetamine, Amphetamine, Cocaine, 2-Oxo-3-hydroxy-LSD, Mephedrone, Methamphetamine (MA), Oxycodone	WW
Sympathomimemtics	Ephedrine	R, WW, B
	Norephedrine	WW
	Methylephedrine	B

** R: river water, WW: wastewater, G: groundwater, S: sediments, B: biota*

The main contributors were antipsychotic drugs (40%) and illicit drugs (33%), whereas several antidepressants (15%), anaesthetics (8%) and sympathomimetics (3%), along with their metabolites, were also detected. 43 compounds that were present in surface river water samples, groundwater, sediments and/or biota, were also detected in wastewater, indicating that WWTPs-derived loads may remarkably affect the quality of the Danube River Basin. Among them 10 compounds, including the antidepressants clomipramine and two metabolites of mirtazapine (normirtazapine and 8-hydroxy-mirtazapine), the antipsychotics amisulpride, sulpiride and oxazepam, and benzoylecgonine, the main metabolite of cocaine, were present in at least one sample of all tested JDS4 water matrices (wastewater, river water and groundwater).

30.3.6 Comparison of illicit drugs and drugs of abuse occurrence data in JDS3 and JDS4

The first evidence of illicit drugs in the Danube River samples in 2013 showed that more emphasis should be placed in their future monitoring using bigger targeted databases and extend their investigation to other environmental matrices, like groundwater, sediments and biota. For this reason, in JSD4, additional analysis was performed, and advanced methodologies were followed for the screening of more than 250 illicit drugs, antidepressants, antipsychotic drugs and their TPs in all tested matrices. The results were compared to those obtained during the previous campaign during JDS3 in 2013 (Liška et al. 2015), in order to get an insight on their trend over the years. Overall, 23 anesthetics, antidepressants, antipsychotic drugs and illicit drugs and their TPs were detected in JDS4 samples. Their average detected concentration and % frequency of appearance (FoA) during the two campaigns are listed in **Table B (Annex).**

Most of the analytes, presented a decreasing occurrence trend in the most recent campaign. In particular, lidocaine, imipramine, mirtazapine, nordiazepam, alprazolam, risperidone, norclozapine, amisulpride, sulpride, sertaline, MDMA, EDDP, benzoylecgonine, codeine and 6-MAM were detected with both significantly lower frequency and in considerably lower average concentration levels in JDS4 samples. Furthermore, cocaine, that was detected in high frequency in JDS3 river samples was detected in the most recent campaign only in wastewater. On the other hand, the monitoring data revealed that the antidepressant doxepin presented an increasing trend of occurrence in the Danube River during the two campaigns, reaching 92% FoA. The average detected concentrations for the antidepressant amitriptyline and the antipsychotic drug oxazepam were more than 50% higher in JDS4 samples, compared to those of JDS3. Additionally, 14 compounds that were not investigated during JDS3, were detected in JDS4 surface river water samples. Among them, the N-oxides of lidocaine, venlafaxine and amisulpride, were detected with remarkably high %FoA of 90, 78 and 41 in JDS4 surface river waters, respectively. Moreover, as presented in Table 1, 42 additional illicit drugs and drugs of abuse were detected in wastewater, groundwater, sediments and/or biota. Several compounds including the antidepressants 8-hydroxy-mirtazapine, normirtazapine, clomipramine, the antipsychotic drugs amisulpride, sulpiride and medazepam and the metabolite of cocaine, benzoylecgonine were present in groundwater samples.

30.3.7 Commonly detected compounds in different environmental matrices

Overall, by comparing the pharmaceuticals and illicit drugs that were detected in river water, wastewater, groundwater, sediments and biota, 140 compounds were detected in more than one of the JDS4 environmental matrices. **Table C (Annex)** summarizes these compounds, along with the matrix in which they have been detected. Among them, 26 compounds were commonly detected in river water, wastewater and groundwater, while additional 82 and 17 compounds were commonly detected in river water/wastewater and wastewater/groundwater, respectively, indicating that there might be a link in contamination profiles between these environmental compartments. The most ubiquitous class of compounds in all analysed water matrices were antibiotics (23 compounds), whereas the antipsychotic drug sulpiride and the metabolite of the synthetic musk galaxolide, galaxolidone were omnipresent in all analyzed matrices within JDS4.

30.3.8 Risk assessment

In order to assess the potential ecotoxicological threat of pharmaceuticals and illicit drugs, the measured concentrations of all the detected compounds were compared to their lowest PNEC values retrieved from the NORMAN Ecotoxicology database (Dulio et al. 2020). The Ecotoxicology database contains PNECs for freshwater, marine waters, sediments and biota. Risk assessment of groundwater samples is discussed in Chapter 25. In the NORMAN Prioritisation framework (Dulio and von der Ohe 2013) effluent wastewater findings are used for risk assessment by converting the individual concentrations to freshwater concentrations using a factor of 5 (optionally 2 or 10). An outcome of such prioritization is presented in Chapter 36. Table 2 summarizes the list of compounds that exceeded their PNECs in the JDS4 surface waters, sediments and biota, the extent of exceedance of PNEC and the most polluted samples.

Table 2: Compounds that exceeded their PNECs in JDS4 surface water, sediments and biota.

Surface waters				
Compound	**PNECfw (ng/L)**	**Samples >PNEC**	**Range of concentrations >PNEC (ng/L)**	**Sample with highest exceedance**
Candesartan	3.10	8	4.2-31.2	JDS4-34
17beta-Estradiol	0.4*	5	0.473-2.10	JDS4-13
Carbamazepine	50.0*	4	50.9-57.6	JDS4-15
Diclofenac	50.0*	2	51.2-63.1	JDS4-15
Dicloxacillin	5.10	1	5.49	JDS4-13
Sediments				
Compound	**PNECsed (µg/kg d.w.)**	**Samples >PNEC**	**Range of concentrations >PNEC (µg/kg d.w.)**	**Sample with highest exceedance**
Octocrylene	52.4	3	74.5-162	JDS4-47
Fenbendazole	8.40	2	23.3-26.5	JDS4-6
Sulfadiazine	7.29	2	15.6-120	JDS4-47
Sulfaclozine	17.0	2	11.5-31.8	JDS4-47
Carbamazepine	1.70	1	3.90	JDS4-6
Apophedrin	205	1	213	JDS4-6
Biota				
Compound	**PNECbio (µg/kg w.w.)**	**Samples >PNEC**	**Range of concentrations >PNEC (µg/kg w.w.)**	**Sample with highest exceedance**
Sulpiride	5.87	8	12.4-52.4	JDS4-29
Cytarabin	16.0	7	16.2-41.6	JDS4-24
Lovastatin	4.52	6	5.34-17.5	JDS4-29
Niflumic acid	1.65	2	1.67-1.99	JDS4-6
Sulfamethoxazole	15.9	1	28.9	JDS4-23
Temazepam	2.9	1	3.70	JDS4-23
Reproterol	0.200	1	0.519	JDS4-23

** Environmental Quality Standard (EQS)/ EQS proposal*

Only five out of 140 substances detected in the JDS4 Danube surface river water samples exceeded their respective PNECs values. Dicloxacillin, carbamazepine and diclofenac were detected slightly above their ecotoxicological threshold values (up to 1.3-fold), in one, four and two river samples, respectively. The antihypertensive drug candesartan exceeded its PNEC in eight samples, mainly from Germany, Czech Republic, and Hungary (maximum concentration 10-fold above the PNEC at JDS4-34). The steroid 17beta-estradiol was detected in five samples in concentrations up to 5.3-fold higher than the respective PNEC. Maximum concentrations of cefazolin and estrone (E1) were detected close to their PNECs. Diclofenac was on the EU 2015/4951 Watch List, whereas estrone and 17beta-estradiol were among the updated EU 2018/840 Watch List compounds. The outcomes of JDS4 justify further regulatory monitoring of these three compounds.

Concerning sediments, six compounds exceeded the respective PNEC values calculated from PNECs for freshwater according to the NORMAN Prioritisation Framework (Dulio and von der Ohe 2013). The maximum detected concentrations were in most cases up to 3-fold higher than their respective PNECs, except for sulfadiazine, which was detected at the level exceeding 17-fold its PNEC in the JDS4-47 sample. Although most of the compounds exceeded their PNECs in one sediment sample, sulfadiazine and octocrylene were above the PNEC values in 2 and 3 samples, respectively. Overall, JDS4-47 (BG/RO) was the sample in which the highest frequency and extent of PNECs exceedances were observed.

Seven compounds exceeded the PNEC values in biota (fish muscle). The maximum detected concentrations for sulfamethoxazole, niflumic acid, temazepam and reproterol were up to 2.6-fold higher than their PNECs in one biota sample, while lovastatin and cytarabin values exceeded their PNEC threshold up to 3.9 and 2.6-fold in 6 and 7 samples, respectively. Sulpiride was the compound that most frequently exceeded its PNEC (73%), reaching up to almost 9-fold higher concentrations in the tested samples. The highest total exceedance of PNECs was observed at JDS4-29.

30.4 Conclusions

A novel approach was presented demonstrating usefulness of wide-scope target screening of 1,301 pharmaceuticals, illicit drugs, PCPs and their TPs in surface water, wastewater, groundwater, sediments and biota samples. The application of HRMS screening methodology revealed the presence of hundreds of pharmaceuticals and 87 illicit drugs, drugs of abuse and their TPs in the JDS4 samples. The occurrence of several illicit drugs and drugs of abuse was reported for the first time on basin-wide scale. Although more than 300 compounds were detected in the samples, only ca. 5% exceeded their ecotoxicological threshold values. These substances were included among the potential Danube RBSPs (see Chapter 36). The detected concentration levels of illicit drugs and their TPs seem to pose no environmental risk. The antipsychotic drugs sulpiride and temazepam exceeded the respective PNECs in biota. The majority of illicit drugs and drugs of abuse that were detected in surface water in JDS3 were determined at significantly lower concentration levels in JDS4 samples. The findings of this chapter are complementary to those reported in Chapter 29.

30.5 References

ALYGIZAKIS, N.A., GAGO-FERRERO, P., BOROVA, V.L., PAVLIDOU, A., HATZIANESTIS, I. AND THOMAIDIS, N.S. (2016) Occurrence and spatial distribution of 158 pharmaceuticals, drugs of abuse and related metabolites in offshore seawater. Sci Total Environ 541, 1097-1105.

ALYGIZAKIS, N.A., OSWALD, P., THOMAIDIS, N.S., SCHYMANSKI, E.L., AALIZADEH, R., SCHULZE, T., OSWALDOVA, M. AND SLOBODNIK, J. (2019) NORMAN digital sample freezing platform: A European virtual platform to exchange liquid chromatography high resolution-mass spectrometry data and screen suspects in "digitally frozen" environmental samples. TrAC Trends in Analytical Chemistry 115, 129-137.

BRAIN, R.A., HANSON, M.L., SOLOMON, K.R. AND BROOKS, B.W. (2008) Reviews of Environmental Contamination and Toxicology. Whitacre, D.M. (ed), pp. 67-115, Springer New York, New York, NY.

DASENAKI, M.E. AND THOMAIDIS, N.S. (2015) Multi-residue determination of 115 veterinary drugs and pharmaceutical residues in milk powder, butter, fish tissue and eggs using liquid chromatography–tandem mass spectrometry. Analytica Chimica Acta 880, 103-121.

DIAMANTI, K.S., ALYGIZAKIS, N.A., NIKA, M.-C., OSWALDOVA, M., OSWALD, P., THOMAIDIS, N.S. AND SLOBODNIK, J. (2020) Assessment of the chemical pollution status of the Dniester River Basin by wide-scope target and suspect screening using mass spectrometric techniques. Analytical and Bioanalytical Chemistry 412, 4893–4907.

DULIO, V. AND VON DER OHE, P.C. (2013) NORMAN Prioritisation framework for emerging substances. NORMAN Association, https://www.norman-network.net/sites/default/files/files/Publications/NORMAN_prioritisation_Manual_15%20April2013_final%2020for%2020website-f.pdf.

DULIO, V., KOSCHORRECK, J., VAN BAVEL, B., VAN DEN BRINK, P., HOLLENDER, J., MUNTHE, J., SCHLABACH, M., AALIZADEH, R., AGERSTRAND, M., AHRENS, L., ALLAN, I., ALYGIZAKIS, N., BARCELO', D., BOHLIN-NIZZETTO, P., BOUTROUP, S., BRACK, W., BRESSY, A., CHRISTENSEN, J.H., CIRKA, L., COVACI, A., DERKSEN, A., DEVILLER, G., DINGEMANS, M.M.L., ENGWALL, M., FATTA-KASSINOS, D., GAGO-FERRERO, P., HERNÁNDEZ, F., HERZKE, D., HILSCHEROVÁ, K., HOLLERT, H., JUNGHANS, M., KASPRZYK-HORDERN, B., KEITER, S., KOOLS, S.A.E., KRUVE, A., LAMBROPOULOU, D., LAMOREE, M., LEONARDS, P., LOPEZ, B., LÓPEZ DE ALDA, M., LUNDY, L., MAKOVINSKÁ, J., MARIGÓMEZ, I., MARTIN, J.W., MCHUGH, B., MIÈGE, C., O'TOOLE, S., PERKOLA, N., POLESELLO, S., POSTHUMA, L., RODRIGUEZ-MOZAZ, S., ROESSINK, I., ROSTKOWSKI, P., RUEDEL, H., SAMANIPOUR, S., SCHULZE, T., SCHYMANSKI, E.L., SENGL, M., TARÁBEK, P., TEN HULSCHER, D., THOMAIDIS, N., TOGOLA, A., VALSECCHI, S., VAN LEEUWEN, S., VON DER OHE, P., VORKAMP, K., VRANA, B. AND SLOBODNIK, J. (2020) The NORMAN Association and the European Partnership for Chemicals Risk Assessment (PARC): let's cooperate! Environmental Sciences Europe 32(1), 100.

EUROPEAN COMMISSION (2015) Commission implementing decision (EU) 2015/495 of 20 March 2015 establishing a watch list of substances for Union-wide monitoring in the field of water policy pursuant to Directive 2008/105/EC of the European Parliament and of the Council. Official Journal of the European Union L78:40-42.

EUROPEAN COMMISSION (2018) Commission implementing decision(EU) 2018/840 of 5 June 2018 establishing a watch list of substances for Union-wide monitoring in the field of water policy pursuant to Directive 2008/105/EC of the European Parliament and of the Council and repealing Commission Implementing Decision (EU) 2015/495. Official Journal of the European Union L141:9-12.

EUROPEAN COMMISSION (2020) Commission implementing decision (EU) 2020/1161 of 4 August 2020 establishing a watch list of substances for Union-wide monitoring in the field of water policy pursuant to Directive 2008/105/EC of the European Parliament and of the Council. . Official Journal of the European Union L 257:32-35.

GAGO-FERRERO, P., BOROVA, V., DASENAKI, M.E. AND THOMAIDIS, N.S. (2015) Simultaneous determination of 148 pharmaceuticals and illicit drugs in sewage sludge based on ultrasound-assisted extraction and liquid chromatography–tandem mass spectrometry. Analytical and Bioanalytical Chemistry 407(15), 4287-4297.

LIŠKA, I., WAGNER, F., SENGL, M., DEUTSCH, K. AND SLOBODNÍK, J. (2015) Joint Danube Survey 3: A Comprehensive Analysis of Danube Water Quality. International Commission for the Protection of the Danube River.

MARIANI, G., TAVAZZI, S., COMERO, S., BUTTIGLIERI, G., PARACCHINI, B., SKEJO, H., ALCANDE SANZ, L. AND GAWLIK, B.M. (2017) Short-term isochronous stability study of contaminants of emerging concern in environmental waters samples, JRC Technical Report.

MILLER, T.H., BURY, N.R., OWEN, S.F., MACRAE, J.I. AND BARRON, L.P. (2018) A review of the pharmaceutical exposome in aquatic fauna. Environmental Pollution 239, 129-146.

Annex

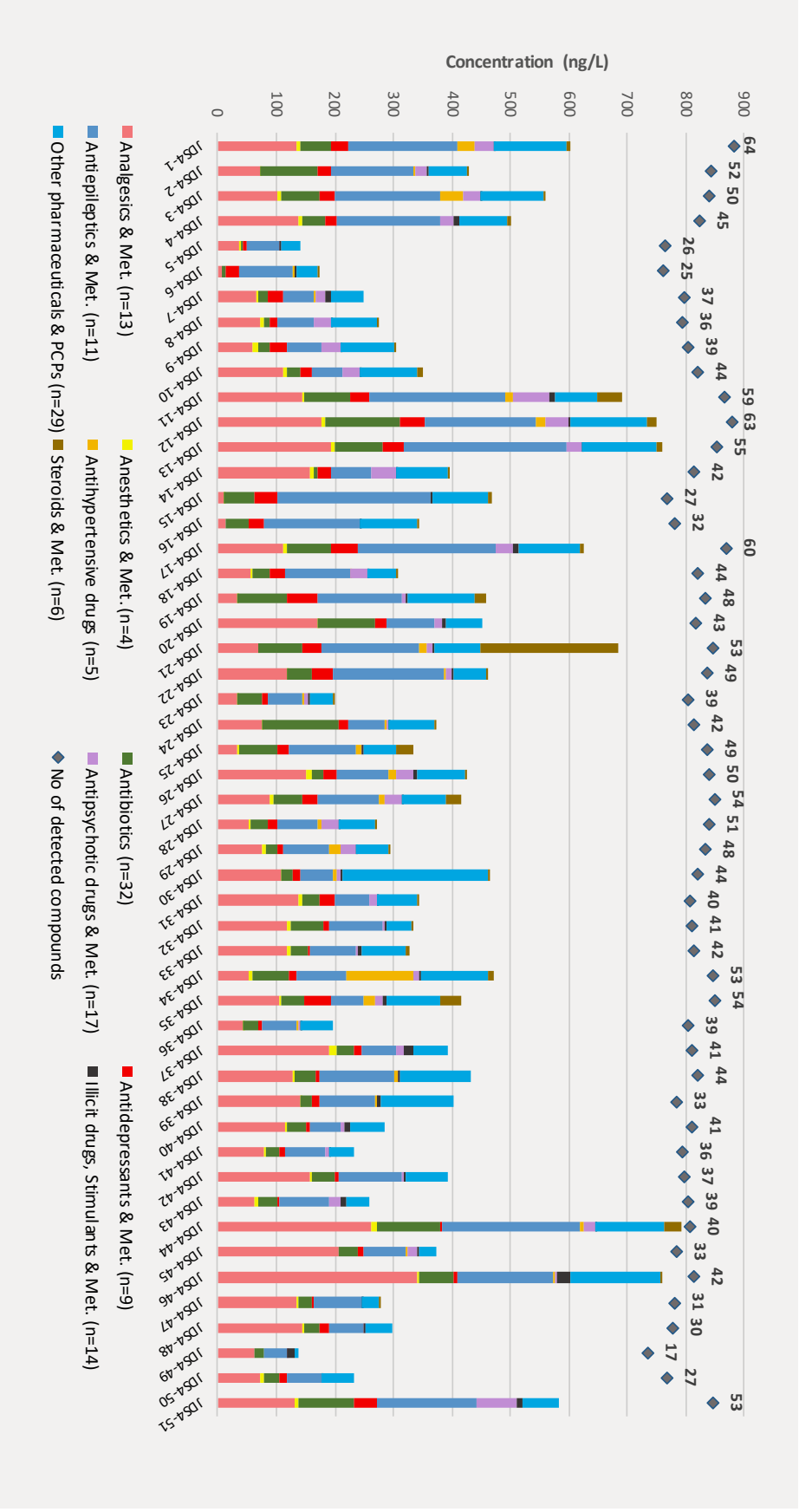

Figure A: Overall detected cumulative concentrations of pharmaceuticals, PCPs, illicit drugs and their TPs in JDS4 surface water samples.

** 19-norandrosterone was not considered.*

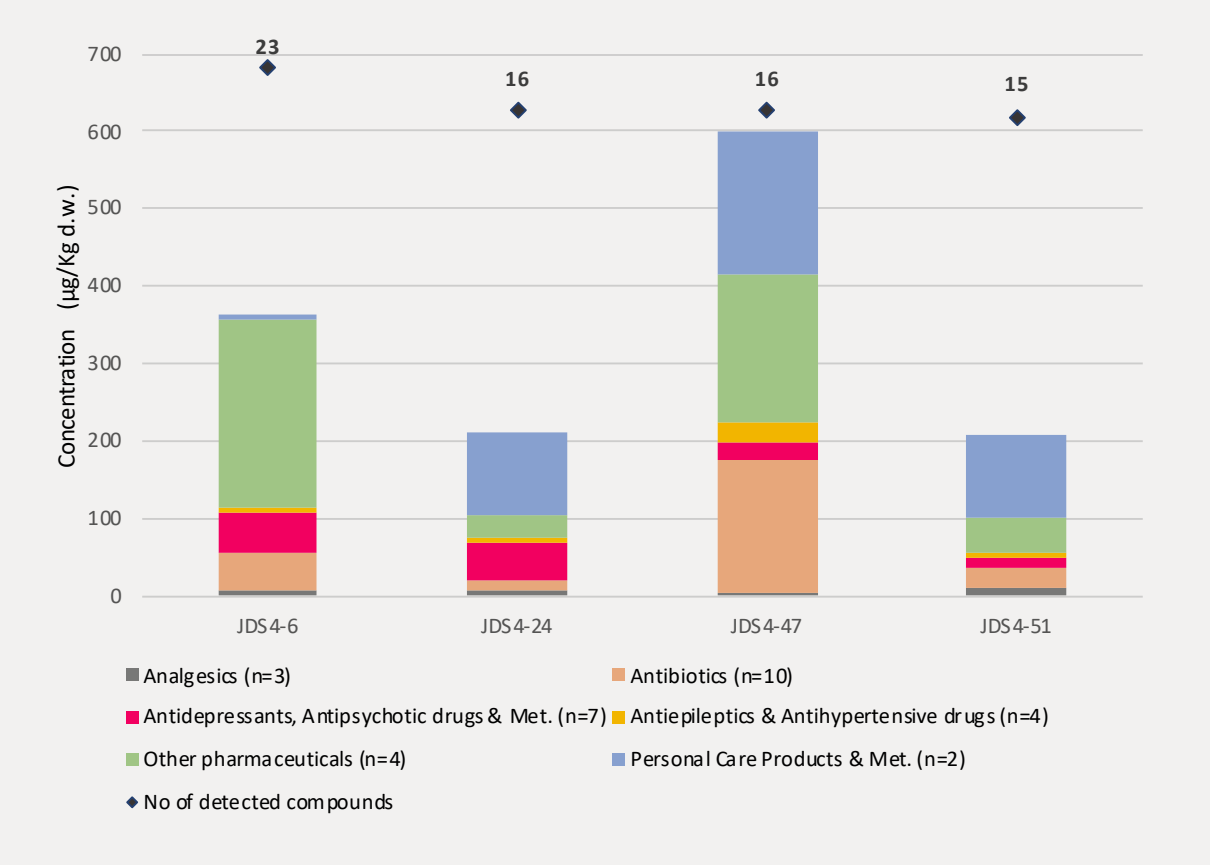

Figure B: Overall detected cumulative concentrations of pharmaceuticals and their TPs in the JDS4 sediments.

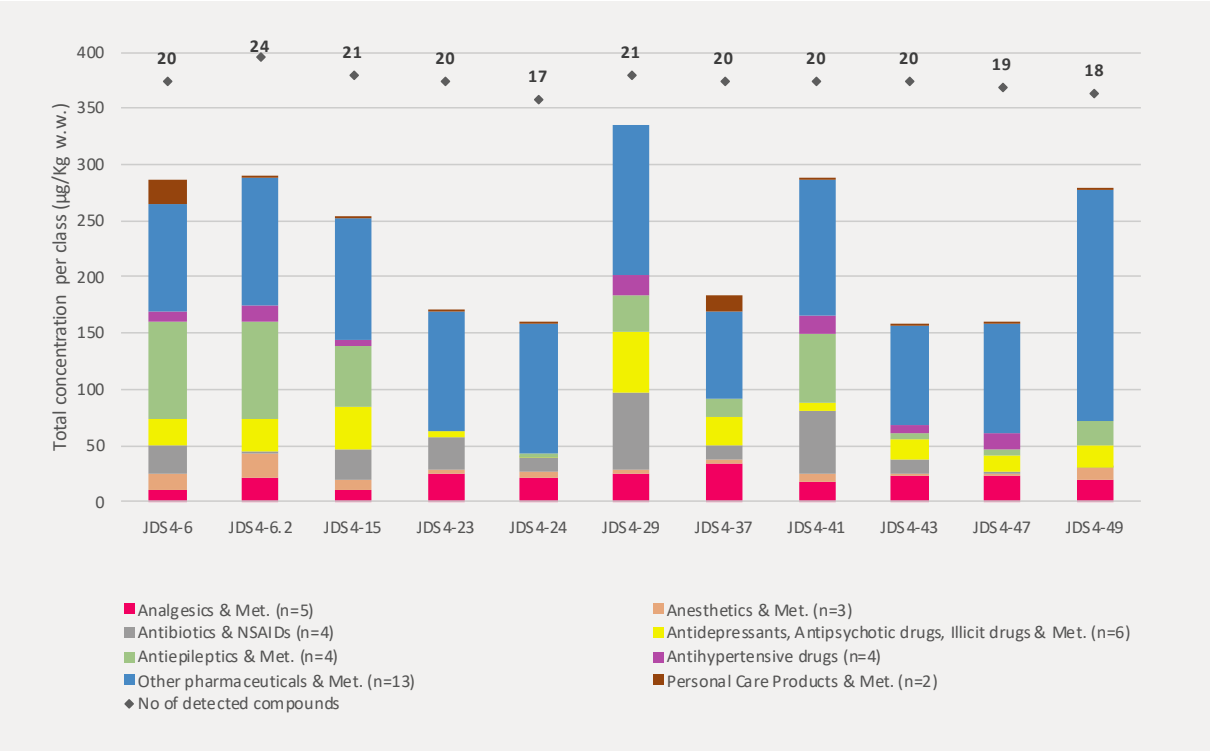

Figure C: Overall detected cumulative concentrations of pharmaceuticals, illicit drugs and their TPs in the JDS4 biota samples.

Table A: Pairs of parent compounds and metabolites of pharmaceutical and illicit drugs detected in JDS4 wastewater samples.

Classification	Parent compounds & metabolites	Influent samples		Effluent samples	
		No of samples	Concentration range (ng/L)	No of samples	Concentration range (ng/L)
Analgesics & Met.	**Caffeine**	11	986-3944	11	6.50-345
	Theophylline	11	310-1296	10	4.51-28.0
	Theobromine	11	116-378	3	3.51-8.40
	Buprenorphine	1	4.10	1	1.00
	Norbuprenorphine	1	79.0	4	32.03-74
	Dipyron (Metamizol)	1	1.60	0	<0.200
	Antipyrine-4-Formylamino	11	21-145	11	13.5-120
	Antipyrine-4-Acetamido	11	37-330	11	19.5-60.0
	Antipyrine-4-Amino (4-AAP)	2	2.00-4.00	0	<0.300
	Tramadol-O-Desmethylnor	6	3.50-14.0	4	3.51-6.50
	Tramadol-O-Desmethyl	3	3.50-6.90	2	3.51-3.60
	Tramadol-N-oxide	10	0.42-4.50	11	0.100-8.60
Anesthetics & Met.	**Fentanyl**	1	0.0450	1	0.0450
	Norfentanyl	11	0.045-2.10	11	0.300-1.30
	Norketamine	5	1.35	1	1.35
	Lidocaine	9	7.00-380	3	4.00-12.0
	Lidocaine-N-oxide	6	74.0-1106	10	6.00-199
Antibiotics	**Sulfamethoxazole**	11	10.5-410	11	103-1638
	Sulfamethoxazole-N4-Acetyl	10	3.20-32.0	2	2.50-2.80
Antihypertensive drugs & Met.	**Atenolol**	6	8.40-15.0	0	<0.800
	Atenolol acid (Metoprolol acid)	3	3.00-11.0	0	<0.800
	Clopidogrel	8	1.80-55.0	2	1.60-2.50
	Clopidogrel Carboxylic acid	1	3.80	0	<1.20
	Pheniramine N-Oxide	0	<0.0100	5	0.300-0.800
	D617 (met. of verapamil)	0	<0.200	1	0.700
Hypoglycemic agents & Met.	**Metformin**	11	139-297	11	4.10-40.0
	Guanylurea	0	<0.200	9	25.0-64.0
Other pharmaceuticals & Met.	**Cetirizine**	1	1.60	0	<0.400
	Cetirizine-N-Oxide	6	1.30-6.20	9	1.60-6.50
	Ephedrine	9	8.10-114	0	<0.400
	Norephedrine	1	21.0	0	<02.20
Stimulant drugs & Met.	**Nicotine**	3	13.0-25.0	0	<0.400
	Cotinine	11	5.90-25.0	1	1.5
	Cotinine-Hydroxy	11	7.50-53.0	0	<0.700
Personal Care Products & Met.	Galaxolidone	11	20.0-2947	11	859-9884

Classification	Parent compounds & metabolites	Influent samples		Effluent samples	
		No of samples	Concentration range (ng/L)	No of samples	Concentration range (ng/L)
Antiepileptics & Met.	**Carbamazepine**	11	21.0-181	11	3.07-10.4
	Carbamazepine-10,11-epoxide	11	1.40-5.40	11	2.00-11.0
	Carbamazepine-10,11-dihydro-10,11 dihydroxy	11	270-4950	11	1042-5726
	Carbamazepine -10-Hydroxy	0	<0.700	5	4.20-21.0
Antidepressants & Met.	**Mirtazapine**	2	0.300	5	0.300
	Normirtazapine	5	4.40-15.0	6	16.00-54
	8-Hydroxy-Mirtazapine	9	9.70-134	5	4.70-25.0
Antipsychotic drugs & Met.	Citalopram N-oxide	2	1.20-5.10	10	1.20-13.0
	7-amino-flunitrazepam	0	<0.900	1	0.800
	Bromazepam	1	35.0	2	38.0
	Bromazepam-3-OH	1	15.0	1	20.0
	Midazolam	1	0.470	1	0.470
	Midazolam-1-Hydroxy	1	0.460	3	0.400-2.10
	Diazepam	1	4.20	3	4.21-4.91
	Nordiazepam	4	1.00-3.10	4	2.60-4.20
	Flurazepam-Desalkyl	1	1.80	2	2.00-3.70
	Amisulpride	10	28.0-850	7	15.0-72.0
	Amisulpride-N-Oxide	9	0.620-4.00	9	0.600-8.40
	Risperidone	1	0.290	7	0.300-0.800
	9-Hydroxy-Risperidone	3	0.150	5	0.300-0.600
	Venlafaxine-D,L-N,O-Didesmethyl	4	0.430-0.750	2	0.300-0.800
	Norvenlafaxine	0	<0.0100	10	17.50-212
	Venlafaxine-N-oxide	10	0.950-9.20	10	2.00-21.0
	D,L-N,N-Didesmethyl-Venlafaxine	0	<0.0100	1	0.500
	Venlafaxine-O-Desmethyl	3	2.70-9.60	0	<0.800
Illicit drugs & Met., metabolite of ethanol	**Cocaine**	11	0.300-9.60	0	<0.200
	Benzoylecgonine	11	9.90-666	11	0.600-12.0
	Ethyl sulfate	8	0.440-5.80	5	0.630-1.50
	2-Oxo-3-hydroxy-LSD	1	2.80	2	0.760-4.20
	Heroin (HER)	1	1.65	2	6.51-7.80
	6-Monoacetylmorphine (6-MAM)	5	0.700-1.10	0	<0.600

** detected compounds <LOQ are expressed as LOQ/2*

Table B: Occurrence data of drugs of abuse, illicit drugs and their metabolites in JDS3 and JDS4 surface river water samples.

		JDS3		JDS4	
Classification	**Compounds**	**%FoA**	**Average Concentration (ng/L)**	**%FoA**	**Average Concentration (ng/L)**
Anaesthetics & Met.	Fentanyl	26	3.78	22	0.0450
	Lidocaine	96	5.09	9.8	0.0275
	Lidocaine-N-oxide		NI	90	4.43
	Norfentanyl	69	1.60	47	0.249
Antidepressants & Met.	8-Hydroxy-Mirtazapine		NI	18	9.44
	Amitriptyline	82	4.68	76	9.81
	Clomipramine	ND	<3.00	2.0	0.050
	Doxepin	40	2.45	92	11.4
	Imipramine	72	10.1	9.8	0.058
	Mirtazapine	49	5.04	5.9	0.300
	Normirtazapine		NI	33	0.659
	Quetiapine	51	0.41	22	0.624
	Zolpidem	51	1.74	25	0.304
Antipsychotic drugs & Met.	7-amino-flunitrazepam	65	0.888	59	0.551
	9-Hydroxy-Risperidone	37	0.886	3.9	0.340
	Alprazolam	84	4.23	35	0.207
	Amisulpride	89	3.61	7.8	0.195
	Amisulpride-N-Oxide		NI	41	16.9
	Bromazepam	31	13.1	27	1.80
	Clorpromazine		NI	2.0	0.606
	Fluoxetine		NI	3.9	0.634
	Norclozapine	46	26.8	2.0	1.65
	Nordiazepam	100	18.4	2.0	0.450
	Norvenlafaxine		NI	45	0.180
	Oxazepam	57	3.74	47	11.8
	Risperidone	43	4.42	2.0	0.897
	Sertraline	28	10.0	7.8	0.391
	Sulpiride	85	17.0	9.8	0.268
	Venlafaxine	91	3.36	27	4.64
	Venlafaxine-N-oxide		NI	78	1.28
Illicit drugs & Met.	6-O-Monoacetylmorphine (6-MAM)	52	28.3	31	1.52
	Benzoylecgonine	100	3.61	73	0.329
	Cannabidiol		NI	7.8	5.30
	Cathine		NI	7.8	1.07
	Codeine	92	43.3	12	0.180
	EDDP	83	5.41	25	0.0300
	Heroin (3,6-diacetylmorphine)		NI	12	2.00
	MDAI (5,6-Methylenedioxy-2-aminoindan)		NI	9.8	7.64
	MDMA	98	11.8	9.8	0.150
	Methadone		NI	7.8	0.300
	Morphine	68	2.90	3.9	1.21
	Norbuprenorphine	83	11.7	2.0	18.5
	Remifentanil		NI	20	1.32

** FoA: Frequency of Appearance, NI: Not investigated, LOQ/2 values were used for compounds detected below LOQ levels.*

Table C: Commonly detected pharmaceuticals, illicit drugs and their metabolites in JDS4 samples.

Compounds	Matrices	Compounds	Matrices
Acetylsalicylic acid	R, WW	Buprenorphine	R,WW, G
Lidocaine-N-oxide		Cefaclor	
Ciprofloxacin		Cefazolin	
Florfenicol		Sulfapyridine	
Fluconazole		8-Hydroxy-Mirtazapine	
Candesartan		Clomipramine	
Irbesartan		Carbamazepine-10,11-dihydro-10,11 dihydroxy	
Losartan		Lamotrigine	
Gemfibrozil		Oxcarbazepine	
Amisulpride-N-Oxide		Valproic acid	
Venlafaxine-N-oxide		7-amino-flunitrazepam	
Hydrochlorothiazide		Oxazepam	
Meclofenamic Acid		Benzoylecgonine	
Mefenamic acid		Metformin	
Naproxen		Tiagabine	
Amantadine		Salicylic acid	R,WW, G, B
Cetirizine-N-Oxide		Fentanyl	
17beta-Estradiol		Norfentanyl	
Androsterone-19-nor		Sulfamethoxazole	
E1 (estrone)		Normirtazapine	
Methenolone		Caffeine	R,WW, G, S
Cotinine		Sulfadiazine	
Tenofovir		Carbamazepine	
Nalorphine		Amisulpride	
Pentoxifylline		Galaxolidone	R,WW, G, S, B
Theobromine		Sulpiride	
Theophylline		Tramadol	R,WW, S
Lidocaine		Azithromycin	
Amoxicillin		Clarithromycin	
Ampicillin		Sulfaclozine	
Dicloxacillin		Sulfadimethoxine	
Oxytetracycline		Sulfathiazole	
Sulfadimidine		Trimethoprim	
Sulfamerazine		Mirtazapine	
Sulfamethoxazole-N4-Acetyl		Norvenlafaxine	
Sulfamonomethoxine		Tramadol-Nor (Tramadol-N-desmethyl)	R, B
Doxepin		Rivastigmine	
Imipramine		Ranitidine	R, G
Zolpidem		Vigabatrin	R, G, B
Lacosamide		Tolycaine	WW, B
Primidone		Lincomycin	
Topiramate		Lovastatin	
Valsartan		Sotalol	
Bezafibrate		Midazolam	
9-Hydroxy-Risperidone		Temazepam	

Compounds	Matrices	Compounds	Matrices
Alprazolam	R, WW	Allopurinol	WW, B
Bromazepam		Antipyrine-4-Formylamino	WW, G
Chlorpromazine		Tramadol-N-oxide	
Fluoxetine		Carbamazepine-10,11-epoxide	
Nordiazepam		Carboxin	
Risperidone		Carbuterol	
Sertraline		Fenofibric acid	
Triamterene		Phenoxybenzamide	
6-O-Monoacetylmorphine (6-MAM)		Chlodiazepoxide	
Cathine		Medazepam	
Codeine		Venlafaxine-D,L-N,O-Didesmethyl	
EDDP		Piroxicam	
Heroin (3,6-diacetylmorphine)		Norephedrine	
MDMA		Benzophenon 3 (=2-Hydroxy-4-methoxybenzophenon)	
Morphine		4-Androsten-11beta-ol-3,17-dione	
Remifentanil		Oxfendazole	WW, G, S
Sitagliptin		Octocrylene	
Diclofenac		Levetiracetam	WW, S, B
Omeprazol		Apophedrin (Phenylethanolamine)	
Zopiclone		Propyphenazone	WW, S
Cortisole		Propafenone	
Antipyrine-4-Acetamido	R, WW, B	Methocarbamol	
Ibuprofen			
Ephedrine			
Paracetamol			
Norbuprenorphine			
Niflumic acid			
Phenylbenzimidazole sulfonic acid			

31 Characterization of wastewaters in the Danube River Basin with chemical screening and a battery of *in vitro* bioassays

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Abstract

The feasibility of implementing a methodology for an ongoing update of the Urban Waste water Treatment Directive (UWWTD; 91/271/EEC) as proposed by the NORMAN Association and Water Europe was tested during JDS4. For this purpose, influent and effluent samples from 11 wastewater treatment plants (WWTPs) in 11 countries in the Danube River Basin (DRB) were collected. To assess the performance of the wastewater abatement process in the selected WWTPs, the actual removal rates of the initial list of 11 proposed indicator substances by NORMAN and Water Europe were determined. The removal rates of 12 additional indicator substances used to evaluate the effectiveness of wastewater treatment in WWTPs which have implemented advanced treatment with either ozone or activated carbon (AC) in Switzerland, were also calculated. Rather alarmingly, eight out of the 20 indicator substances (two above lists put together, 3 common substances) were eliminated with a removal rate below 50%. Moreover, in order to address mixture toxicity (combined adverse effect of multiple contaminants) as a 'safety net', the effluent wastewater samples were also analysed with a battery of seven NORMAN/SOLUTIONS in vitro bioassays covering a wide-spectrum effect endpoints. The results, including those obtained in the previous surveys in the DRB, indicate that the current water treatment technologies used in the studied WWTPs are unable to remove efficiently groups of contaminants of emerging concern (CECs) that cause specific effects such as estrogenicity, PAH activity, xenobiotic metabolism and oxidative stress. Furthermore, risk assessment has been performed by comparing measured concentrations (>2,400 target chemical substances) to their toxicity threshold values (lowest PNECs) with the aim to establish a 'sub-list' of the Danube River Basin Specific Pollutants (DRBSPs) that clearly originate from wastewater. The top 17 substances that potentially pose a risk for the Danube and originate from wastewater accompanied with their respective Emission Limit Values (ELVs) were proposed to be considered for inclusion in the monitoring plans of the WWTPs in the DRB.

31.1 Introduction

The joint position paper of the NORMAN Association and Water Europe argues that the current version of the UWWTD is not 'in phase' with the substantial evidence base that the occurrence of CECs in the environment is an issue of concern for exposed ecosystems and human health (NORMAN network and Water Europe, 2019). CECs are released into the environment as a result of anthropogenic activities, with a trend of increasing loads and types of pollutants due to population growth and the escalating introduction of new chemicals to the market (Alygizakis et al., 2018). Not all CECs are persistent, but due to their continuous use and discharge into the environment, many of them are regularly found in the environment and can accumulate in food webs (Movalli et al., 2019; Diamanti et al., 2020). Moreover, although many chemicals are only used in small quantities which may be considered harmless, there is increasing concern about mixture – or cocktail – effects arising from the multitude of chemicals present in our environment (Brack et al., 2016; Alygizakis et al., 2019a). Improved pollution prevention measures should be promoted as a priority. Discharges from WWTPs are major points of release of CECs into the environment and their mitigation has an important role in pollution prevention.

A prerequisite to ensure that the target objectives for abatement of CECs are met is the definition of a set of performance indicators which facilitate the systematic assessment of the performance of a wastewater abatement process. Based on current experience, the compounds to be selected as performance indicators should be compounds which:

- are continuously discharged and regularly found in the influent of WWTPs,
- occur in most WWTP effluents at measurable concentrations,
- can be easily and routinely measured by as few as possible (optimally one) analytical methods,
- broadly cover the range of physico-chemical properties and biodegradability affecting their removal by the various treatment processes,
- broadly represent the range of treatability features; from "biodegradable during conventional activated sludge treatment or biofiltration", to "not degradable during conventional activated sludge treatment or biofiltration, but amenable to chemical oxidation or sorption to activated carbon (AC)", and "not degradable during conventional activated sludge treatment or biofiltration, and not amenable to chemical oxidation or sorption to AC",
- undergo a similar degree of abatement in advanced treatment technologies (e.g., ozonation or sorption to AC).

In one of the most progressive legislations worldwide, the target for the reduction of CECs in WWTP effluents applied in the new Swiss Water Protection Act is an abatement by 80%, to be evaluated as average abatement of selected indicator substances over the whole treatment (Eggen et al., 2014; Bourgin et al., 2018). Another more stringent requirement in addition to the list of performance indicators mentioned above is to use a battery of bioassays (*in vitro* and *in vivo*) (Coppens et al., 2015) and associated Effect-based Trigger Values (EBTs) (Escher et al., 2018) as a "safety net" at the outlet of the WWTP. If one or more EBTs are exceeded, it is proposed to the WWTP operator to take actions to identify both the pollutants (toxicity drivers) responsible for the observed effects (Brack et al., 2019) and their sources, and adopt measures in line with the 'polluter pays' principle. To complete the list of wastewater-relevant CECs it is also necessary to define the ubiquitous WWTP-related pollutants as candidates for the list of RBSPs and the threshold levels (ELVs) at which they should be monitored.

This chapter is addressing the feasibility of applying the concept of monitoring wastewater for (a) 20 performance indicators to evaluate the wastewater treatment efficiency; (b) effect-based methods (seven bioassays) to capture unforeseen and/or mixture toxicity effects and (c) a short (manageable) list of wastewater-related RBSPs. Action plans at the WWTP operator level are proposed when the ELVs, derived from the toxicity threshold values, are exceeded.

31.2 Methods

31.2.1 Sampling

The selected 11 WWTPs were nominated by the ICPDR Pressures and Measures Expert Group based on countries' dominant technology and the number of served population with the aim to get a representative and holistic view of the pollution status. 24h-composite influent and effluent wastewater samples were collected in certified clean polycarbonate bottles during dry weather and under normal operating conditions (sampling date: 26 August 2019). Samples for analyses of CECs remained in the freezer at -20 °C in the WWTP and frozen during transport. 2 L sample aliquots were processed for chemical analyses and 1 L aliquots were processed for the analysis by *in vitro* bioassays. All samples were processed immediately after arrival to the laboratory.

31.2.2 Chemical analysis

Samples for chemical analysis were cleaned-up and pre-concentrated 4000-fold on an Atlantic HLB-M Disk using HORIZON SPE-DEX 4790 with 47 mm I.D. disk holder according to an automated extraction program following the same procedure as described elsewhere (Alygizakis et al., 2019b). The extracts were evaporated using a gentle stream of nitrogen, reconstituted with 500 µl of 50:50 methanol:water and filtered through RC syringe filters of 4 mm I.D. and 0.2 µm pore size (Phenomenex, USA). Instrumental analysis was performed by UHPLC apparatus (Dionex UltiMate 3000 RSLC, Thermo Fisher Scientific, Dreieich, Germany) coupled to a QTOF-MS mass analyzer (Maxis Impact, Bruker Daltonics, Bremen, Germany). Chromatographic separation was performed on an Acclaim RSLC C18 column (2.1 x 100 mm, 2.2 µm) from Thermo Fisher Scientific preceded by a guard column of the same packaging material. Gradient programme and instrumental parameters are described in detail elsewhere (Gago-Ferrero et al., 2020). Removal rates were calculated for the target indicator substances to evaluate the performance of WWTPs.

In order to propose contaminants that are potentially harmful and originate from the WWTPs, NORMAN Prioritization workflow (NORMAN network, 2013) was used to produce three lists of compounds: i) CECs that exceeded their ecotoxicology threshold values (Predicted No-effect Concentrations (PNECs)) in the Danube River and its tributaries, ii) CECs that exceeded their PNECs in effluent wastewater (using dilution factor (DF) 10 to convert concentrations from wastewater to surface water) and iii) CECs that exceeded their PNECs in effluent wastewater (DL 5) (Link et al., 2017; NORMAN network and Water Europe, 2019). The PNEC values were retrieved from the NORMAN Ecotoxicology Database (NORMAN network, 2020), which is part of the NORMAN Database System (Dulio et al., 2020). The proposed ELVs and their background PNECs (Table 1, Table 2 and Table 4) should be subjected to a further detailed scrutiny by ecotoxicology experts. Rather unexpectedly, four pesticides (pethoxamid, nicosulfuron, metazachlor, 4-((1,1-dimethylethyl) amino)-6-(ethylamino)-1,3,5-triazin-2(1H)-one) fulfilled the above criteria (i, ii and iii).

Despite one would expect agricultural activities as their main source, it became obvious that they enter environment in significant amounts also via WWTPs' effluents.

Table 1: Removal efficiencies of the initial 11 performance indicator CECs in the selected 11 WWTPs in the DRB and their proposed emission limit values (ELVs) – calculated as predicted no-effect concentration in surface water (PNECfw) multiplied by the dilution factor (DF), which is 10 for large rivers, default 5.

NORMAN ID	Indicator chemical	Use category / Chemical functional use	ELV (DF5) (µg L^{-1})	ELV (DF10) (µg L^{-1})	Removal efficiency (%)
Biodegradable[1]					
NS00010261	Benzotriazole	Corrosion inhibitor	38.9	77.7	61±12
NS00000212	Diclofenac	Pharmaceutical (anti-inflammatory / antirheumatic)	0.3	0.5	27±8
NS00000335	Gabapentin	Pharmaceutical (antiepileptic)	50	100	64±7
NS00000211	Trimethoprim	Pharmaceutical (other antibacterial)	600	1200	64±14
NS00000268	Sulfamethoxazole	Pharmaceutical (sulfonamide antibacterial)	3.0	6.0	67±9
NS00000459	Valsartanic acid	Pharmaceutical (antihypertensive agent)	0.8	1.5	-16±28
NS00006655	Oxypurinol	Pharmaceutical (inhibitor of xanthine oxidase)	288	576	98±2
Not biodegradable, but oxidizable[2]					
NS00000381	Acesulfam	Sweetener	362	724	97±1
NS00000207	Carbamazepine	Pharmaceutical (antiepileptic)	0.3	0.5	-57±16
Difficult to degrade biologically; not amendable to chemical oxidation[3]					
NS00010387	Tris (2-carboxyethyl) phosphine (TCEP)	Industrial chemical (Phosphate)	20	40	40±10
NS00000320	Sucralose	Sweetener	149	297	5±13

1 Biodegradable during conventional activated sludge treatment or biofiltration.
2 Not degradable during conventional activated sludge treatment or biofiltration, but amendable to chemical oxidation.
3 Not degradable during conventional activated sludge treatment or biofiltration, not amendable to chemical oxidation.

Table 2: Removal efficiencies of the 12 performance indicator CECs (Switzerland; ensuring the efficiency of the upgraded wastewater treatment plants) in the selected 11 WWTPs in the DRB.

NORMAN ID	Indicator chemical	Use category / Chemical functional use	ELV (DF5) (µg L^{-1})	ELV (DF10) (µg L^{-1})	Removal efficiency (%)
NS00000416	Amisulpride	Pharmaceutical (antipsychotic)	7.2	14.3	85±6
NS00000207	Carbamazepine	Pharmaceutical (antiepileptic)	0.3	0.5	-57±16
NS00000035	Citalopram	Pharmaceutical (antidepressant)	80	160	88±12
NS00098550	Clarithromycin	Pharmaceutical (macrolide antibacterial)	0.6	1.2	88±8
NS00000212	Diclofenac	Pharmaceutical (anti-inflammatory / antirheumatic)	0.3	0.5	27±8
NS00000343	Hydrochlorothiazide	Pharmaceutical (diuretic)	42	84	75±4
NS00000197	Metoprolol	Pharmaceutical (beta blocking agent)	43	86	51±26
NS00000031	Venlafaxine	Pharmaceutical (antidepressant)	0.2	0.4	98±2
NS00010261	Benzotriazole	Corrosion inhibitor	38.9	77.7	61±12
NS00008943	Methylbenzotriazole	Corrosion inhibitor	750	1500	-20±25
NS00009281	Candesartan	Pharmaceutical (antihypertensive agent, angiotensin II antagonist)	0.016	0.03	-59±28
NS00000387	Irbesartan	Pharmaceutical (antihypertensive agent, angiotensin II antagonist)	3520	7040	-71±25

31.2.3 Bioassays

CALUX® bioassays (Chemical Activated Luciferase eXpression; BioDetection Systems BV, Amsterdam, the Netherlands) were applied for analysis of wastewater effluent samples. A detailed sample preparation protocol using fully validated methods and standard operational procedures are described elsewhere (Alygizakis et al., 2019b). Briefly, the samples were extracted using an optimized solid-phase extraction (SPE) and the genetically modified cell lines were exposed to the mixture of compounds being present in the enriched extract. CALUX® bioassays utilise cell lines, incorporating the firefly luciferase gene. The luciferase gene is coupled to Responsive Elements (REs) such as a reporter gene. The reporter gene is activated in the presence of specific compounds. Cells that were exposed to the compounds of interest trigger the activation of REs, the creation of luciferase, which emits light in the presence of appropriate substrate. The amount of light produced is proportional to the amount of ligand-specific receptor binding, which is benchmarked against the relevant reference compounds.

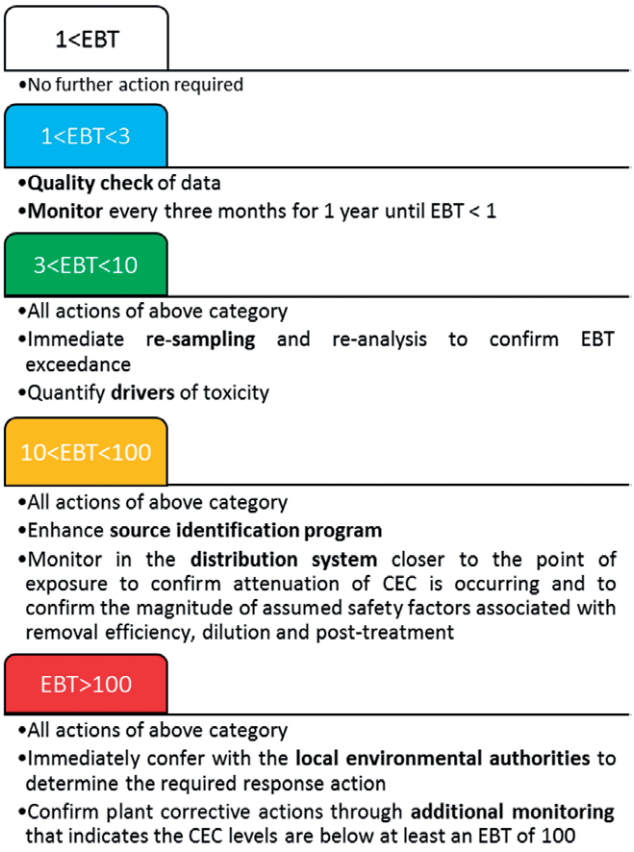

Figure 1: Proposed actions in case of exceedance of EBTs.

Extracts were subjected to the investigation of seven out of eight bioassays proposed by the joint position paper (NORMAN network and Water Europe, 2019): Estrogenic (ERα), Anti-androgenic (anti-AR), Glucocorticoid (GR), PPARγ receptor (PPARγ), PAHs (PAH), Oxidative stress (Nrf2) and Pregnane X receptor (PXR). The results of the CALUX® bioassays are reported in the following units: 17ß-estradiol for ERα, Flutamide for anti-AR, Dexamethasone, for GR, Rosiglitazone for PPARγ, B[a]P for PAHs, Curcumine for Nrf2, and Nicardipine for PXR. The responses from the bioassays were benchmarked against the lowest EBTs reported in the literature (van der Oost et al., 2017; Escher et al., 2018). The applied battery of bioassays and the EBT values can be found in Table 3.

Table 3: Performance indicator bioassays and their effect-based trigger values (EBTs).

Activity	EBT
Estrogenic (ER_α)	0.1 ng 17ß-Estradiol-eq/L
Anti-androgenic (anti-AR)	14 µg Flutamide-eq/L
Glucocorticoid (GR)	100 ng Dexamethasone-eq/L
PPARγ receptor (PPARγ)	10 ng Rosiglitazone-eq/L
PAHs (PAH)	6.2 ng B[a]P-eq/L
Oxidative stress (Nrf2)	10 µg Curcumine-eq/L
Pregnane X receptor (PXR)	3 µg Nicardipine-eq/L

Based on the exceedance of the EBT, a putative action plan (Figure 1) is suggested (Alygizakis et al., 2019b; NORMAN network and Water Europe, 2019). This scheme is a proposal for actions to be taken at the level of the WWTP operators.

31.3 Results and discussion

31.3.1 Removal rates of indicator chemicals

The removal rates of the 20 indicator substances (combined list from Tables 1 and 2) were investigated by comparing the concentration levels in influent and effluent wastewater of all investigated WWTPs (Figure 2). Six out of the 20 indicator substances (venlafaxine, acesulfame, oxypurinol, clarithromycin, and amisulpride) showed high average removal rates (> 80%). Medium removal rates (27-75%) were observed for eight substances (hydrochlorothiazide, sulfamethoxazole, gabapentin, trimethoprim, benzotriazole, metoprolol, 2-carboxyethyl)phosphine, and diclofenac). Poor elimination (<5%) or even negative removal rates were observed for six substances: sucralose, valsartanic acid, methylbenzotriazole, carbamazepine, candesartan and irbesartan. Negative removal rates indicate that the concentration levels in effluent wastewater were higher than in influent wastewater. This phenomenon is sometimes observed for transformation products (e.g., methylbenzotriazole is a transformation product of benzotriazole) or compounds for which cleavage of the substances takes place. Carbamazepine is excreted also as metabolites, which are re-transformed into carbamazepine during biological treatment, therefore negative elimination is often reported. Candesartan and irbesartan are corresponding to the expected “no removal” observations from other studies, which typically means the interval from ca. -20 to +20%. Outliers are usually due to sampling, e.g., time-offset between influent and effluent sampling when influent concentration is fluctuating. All of the above observations were confirmed by results from two independent laboratories.

31.3.2 Potential RBSPs originating from wastewater

Prioritisation and the process of gradual update of the DRBSPs is described in Chapter 36. Here, an approach of selecting only RBSPs obviously originating from WWTPs and exceeding ecotoxicology

threshold values in the river water at the basin scale are discussed. Similar to the Tables 1 and 2, we have sought a short list of substances, which are 'released in high concentrations causing adverse effects to environment', 'always present' and 'easy to analyse'. An initial list of 17 substances based strictly on the results of the JDS4 (no historical data) is presented in Table 4.

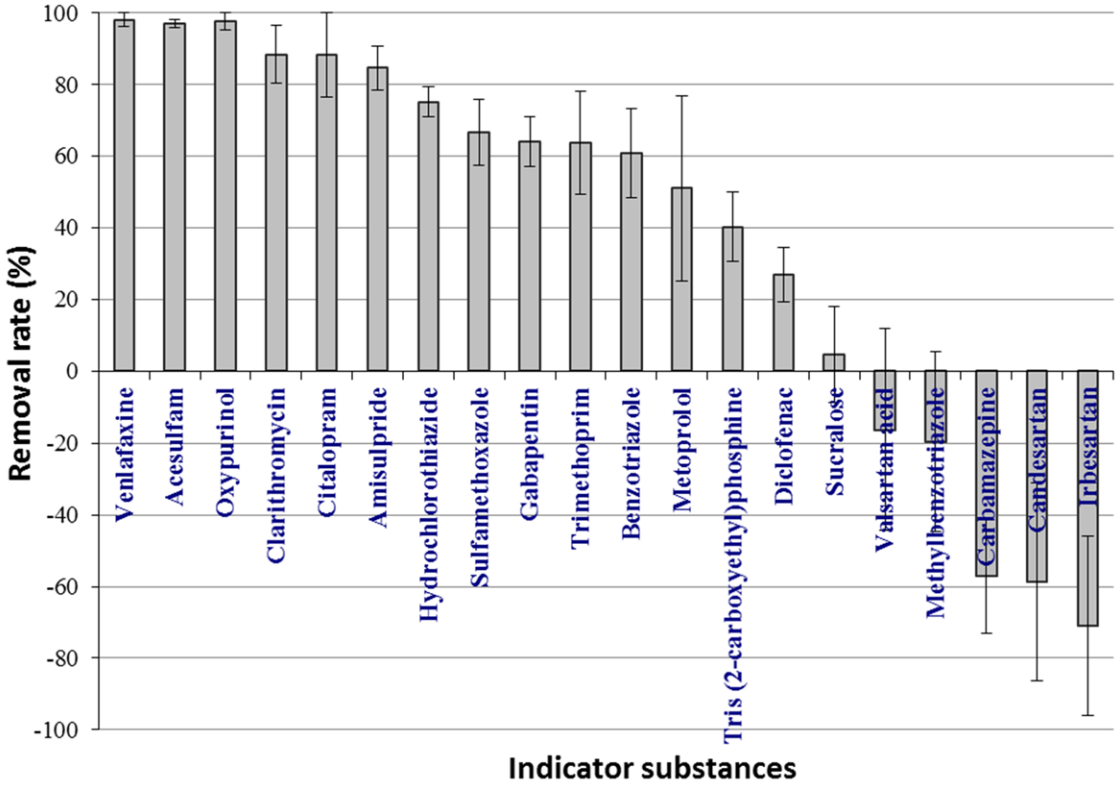

Figure 2: Removal rates of indicator chemicals from the studied 11 WWTPs in the DRB.

Four of these substances (perfluorooctanesulfonic acid (PFOS), 17β-estradiol, fipronil, diclofenac) have already been assigned as Danube RBSPs (Liška et al., 2015). The automated NORMAN Prioritisation methodology tool shows that the PNECs for five 'new' substances (telmisartan, benzododecinium, candesartan, hexa(methoxymethyl)melamine and spinosyn A) should be experimentally verified before any final conclusions. Carbamazepine, imidacloprid, EHDP and ciprofloxacin are obvious candidates to extend the list of DRBSPs. The KEMI exposure score in Table 4 indicates production volume, widespread of use and ease of release into the environment of a chemical as a number between 0 and 1. This allows to convert confidential information from REACH registry into a useful indicator. It should be noted that 12 out of 17 prioritized compounds have the KEMI Exposure score higher than 0.3, which indicates their high production volumes and use. Substances with lower KEMI Exposure score were three pharmaceuticals (telmisartan, candesartan and diclofenac). This could be caused by the fact that data on pharmaceuticals (as well as pesticides and biocides) are not systematically collected in the REACH registry.

Table 4: A list of CECs which originate from WWTPs and exceed their PNECs in river water (RW) at the DRB scale. The information on 'Lowest PNEC' and 'Type PNEC' is taken from the NORMAN Ecotoxicology Database (NORMAN network, 2020). The Final Risk Score is a sum of Frequency of Exceedance (FoE) and Extent of Exceedance (EoE) of the lowest PNEC (NORMAN network, 2013). Concentrations of substances in wastewater are converted into concentrations in surface water by using 'dilution factor' 10 (large rivers) and 5 (default).

Compound	CAS No.	Lowest PNEC (µg L^{-1})	PNEC type	Risk score Dilution 10/ Dilution 5/RW	Exposure score KEMI
Telmisartan	144701-48-4	0.00055	predicted PNEC*	0.86/0.86/1.5	0.04
Perfluorooctanesulfonic acid (PFOS)	1763-23-1	0.00065	EQS chronic water (=AA-EQS)	0.24/0.33/1.2	0.34
Benzododecinium	139-07-1	0.063	predicted PNEC*	0.045/0.045/1.5	0.66
Candesartan	139481-59-7	0.0031	predicted PNEC*	0.7/0.89/1.2	0.13
Carbamazepine	298-46-4	0.05	PNEC chronic	0.33/0.6/0.28	0.30
Imidacloprid	138261-41-3	0.0083	EQS-proposal	0.24/0.42/0.24	0.53
Hexa(methoxymethyl) melamine	68002-20-0	0.057	predicted PNEC*	1.2/1.5/0.22	0.55
2-Ethylhexyl diphenyl phosphate (EHDP)	1241-94-7	0.018	PNEC acute	0.28/0.22	0.46
Spinosyn A	131929-60-7	0.0027	predicted PNEC*	0.19/0.19/0.2	0.38
17β-Estradiol	50-28-2	0.0004	EQS chronic water (=AA-EQS)	0.045/0.33/0.2	0.37
Fipronil	120068-37-3	0.00077	RAC	0.37/0.57/0.18	0.45
Diclofenac	15307-86-5	0.05	EQS-proposal	0.74/1.2/0.039	0.13
Ciprofloxacin	85721-33-1	0.089	EQS-proposal	0.045/0.02	0.31
Pethoxamid	106700-29-2	0.0005	PNEC chronic	0.091/0.091/0.69	0.37
Nicosulfuron	111991-09-4	0.009	JD-UQN	0.091/0.14/0.47	0.21
Metazachlor	67129-08-2	0.02	AA-EQS	0.045/0.045/0.059	0.34
4-((1,1-dimethylethyl) amino)-6-(ethylamino)-1,3,5-triazin-2(1H)-one	66753-07-9	0.0073	PNEC chronic	0.045/0.14/0.75	0.02

**All predicted PNECs should be verified.*

31.3.3 Bioassays

The results of bioassays are presented in Table 5 as fold change of the limit of quantification (LOQ). In other words, Table 5 presents the results normalized to the LOQ, indicating how many times higher were the observed signals than LOQ. The results were compared with the findings acquired using the same methodology for effluent wastewater in the sampling campaign of 2017 (Alygizakis et al., 2019b). Effluent wastewater of WWTP in Šabac (Serbia) was monitored in both campaigns and the results were consistent between the two campaigns. Overall, the findings indicate that the investigated plants are unable to remove efficiently groups of CECs that cause specific adverse effects. In both sampling campaigns (22 WWTPs) the most frequently detected effects were estrogenicity, PAH activity, CECs causing xenobiotic metabolism (PXR) and oxidative stress (Nrf2). The GR and PPARγ2 effects were low in both campaigns. The PAH activity effects were the highest followed by the ERα. One extreme value of PAH CALUX® was observed for wastewater from Donauwörth (more than 30000-fold times higher than LOQ).

Table 5: Results of the analysis of effluent wastewater samples collected within JDS4 by a battery of NORMAN/SOLUTIONS bioassays. The values represent the fold-induction of each analysis relative to its respective LOQ. Results that were below LOQ are presented as 0.5-fold LOQ (0.5).

Sampling 2019 (JDS4)	Cytotox CALUX	anti-AR CALUX	ERα CALUX	GR CALUX	PPARγ CALUX	PAH CALUX	PXR CALUX	Nrf2
EWW Hodonín CZ	2.5	2.5	48	0.5	0.5	96	0.5	7.6
EWW Asten AT	1.2	2.6	26	1.5	0.5	115	10	9.5
EWW Novo Mesto SI	0.5	0.5	5.0	0.5	0.5	58	4.4	1.9
EWW Šabac SRB	1.4	1.3	32	0.5	0.5	76	3.6	3.0
EWW Győr HU	1.2	1.5	17	0.5	1.3	90	24	2.0
EWW Županja HR	1.3	0.5	30	0.5	0.5	92	27	4.4
EWW Vratsa BG	6.2	3.0	58	0.5	0.5	71	0.5	0.5
EWW Uzhgorod UA	0.5	1.3	38	0.5	0.5	104	5.0	2.0
EWW Giurgiu RO	0.5	0.5	44	2.4	0.5	36	5.9	2.2
EWW Donauwörth DE	0.5	3.6	30	5.0	1.0	32292	44	3.7
EWW Bratislava SK	0.5	0.5	12	0.5	0.5	177	4.7	2.5

The results were compared with the EBT values (Table 3) and based on the exceedance of the EBT, translated into an action plan (Table 6) using the colour-coding scheme proposed in Figure 1. Cases with EBT exceedance between 10 and 100 are highlighted in yellow, e.g., PXR in all plants except for the WWTPs of Asten and Vratsa. These results suggest the need for initiation of source identification and monitoring of the distribution system in addition to the actions proposed for the category with EBT exceedances between 3 and 10 (highlighted in green colour; Figure 1). In cases with EBT exceedances between 3 and 10 (e.g., Nrf2

activity in the WWTPs of Asten, Giurgiu, Bratislava and Uzhgorod) the operators are proposed to quality check data, set-up monitoring plan every three months for one year, re-sample and re-analyse to confirm EBT exceedance and quantify toxicity drivers. Cases with EBT exceedance between 1 and 3 are highlighted in blue, see e.g., anti-AR activity in the WWTPs of Asten, Vratsa, Hodonín, and Donauwörth. Here it is proposed to quality check data and organize a monitoring plan every three months for one year.

Table 6: Proposed action plan based on the signals of in vitro bioassays from analysis of JDS4 effluent wastewater samples. For the interpretation of colours, see Figure 1.

	EWW Asten AT	EWW Vratsa BG	EWW Hodonín CZ	EWW Donauwörth DE	EWW Županja HR	EWW Győr HU	EWW Giurgiu RO	EWW Šabac RS	EWW Novo Mesto SI	EWW Bratislava SK	EWW Uzhgorod UA
PAH CALUX	17.7	11.0	14.8	5000	14.2	13.9	5.6	11.8	9.0	27.4	16.1
ERα CALUX	13.0	29.0	24.0	15.0	15.0	8.5	22.0	16.0	2.5	6.2	19.0
Nrf2	6.8	<LOD	<LOD	29.0	18.0	16.0	3.9	2.4	2.9	3.1	3.3
PXR CALUX	<LOD	<LOD	85.7	41.7	49.3	23.0	25.0	34.3	21.0	28.7	22.7
anti-AR CALUX	1.6	1.9	1.6	2.2	<LOD	0.9	<LOD	0.8	<LOD	<LOD	0.8
PPARγ CALUX	<LOD	<LOD	<LOD	63.0	<LOD	82.0	<LOD	<LOD	<LOD	<LOD	<LOD
GR CALUX	0.4	<LOD	<LOD	1.2	<LOD	<LOD	0.6	<LOD	<LOD	<LOD	<LOD

Concentration units for the different bioassays are as following: PAHs CALUX® [ng B[a]P-eq/L]; ERα CALUX® [ng 17ß-Estradiol-eq/L]; Nrf2 CALUX® [µg Curcumine-eq/L]; PXR CALUX® [µg Nicardipine-eq/L]; anti-AR CALUX® [µg Flutamide-eq/L]; PPARγ CALUX® [ng Rosiglitazone-eq/L]; CALUX® [ng Dexamethasone-eq/L].

31.4 Conclusions

Daily composite wastewater influent and effluent samples from 11 WWTPs nominated by the ICPDR PMEG were collected and analysed for a target list of more than 2,400 chemicals and their transformation products together with a battery of seven NORMAN/SOLUTIONS bioassays. The removal rates of WWTP technology related performance indicators were calculated for 11 substances intended for use in standard WWTPs and 12 substances intended for use in upgraded WWTPs. The results showed that eight out of the (sum total) 20 indicator contaminants were eliminated with a removal rate below 50%. The effect-based analyses indicated that the currently used water treatment technologies in the DRB are unable to remove efficiently groups of CECs causing estrogenicity, PAH activity, CECs causing xenobiotic metabolism and oxidative stress. A list of RBSPs specifically originating from wastewater and causing exceedances of ecotoxicology threshold values in the river water, with provisional emission limit values, was proposed for regular monitoring in the DRB. Overall, a monitoring of wastewater effluents by a short (10-12) list of water treatment technology related substances, battery of bioassays (once in six months) and a short list (max. 10) of wastewater originating substances causing exceedances of their PNECs in the DRB are proposed.

31.5 References

ALYGIZAKIS NA, BESSELINK H, PAULUSG K, OSWALD P, HORNSTRA LM, OSWALDOVA M, et al. Characterization of wastewater effluents in the Danube River Basin with chemical screening, in vitro bioassays and antibiotic resistant genes analysis. Environment International 2019; 127: 420-429.

ALYGIZAKIS NA, SAMANIPOUR S, HOLLENDER J, IBANEZ M, KASERZON S, KOKKALI V, et al. Exploring the Potential of a Global Emerging Contaminant Early Warning Network through the Use of Retrospective Suspect Screening with High-Resolution Mass Spectrometry. Environ Sci Technol 2018; 52: 5135-5144.

BOURGIN M, BECK B, BOEHLER M, BOROWSKA E, FLEINER J, SALHI E, et al. Evaluation of a full-scale wastewater treatment plant upgraded with ozonation and biological post-treatments: Abatement of micropollutants, formation of transformation products and oxidation by-products. Water Res 2018; 129: 486-498.

BRACK W, AISSA SA, BACKHAUS T, DULIO V, ESCHER BI, FAUST M, et al. Effect-based methods are key. The European Collaborative Project SOLUTIONS recommends integrating effect-based methods for diagnosis and monitoring of water quality. Environmental Sciences Europe 2019; 31.

BRACK W, AIT-AISSA S, BURGESS RM, BUSCH W, CREUSOT N, DI PAOLO C, et al. Effect-directed analysis supporting monitoring of aquatic environments--An in-depth overview. Sci Total Environ 2016; 544: 1073-118.

COPPENS LJ, VAN GILS JA, TER LAAK TL, RATERMAN BW, VAN WEZEL AP. Towards spatially smart abatement of human pharmaceuticals in surface waters: Defining impact of sewage treatment plants on susceptible functions. Water Res 2015; 81: 356-65.

DIAMANTI KS, ALYGIZAKIS NA, NIKA MC, OSWALDOVA M, OSWALD P, THOMAIDIS NS, et al. Assessment of the chemical pollution status of the Dniester River Basin by wide-scope target and suspect screening using mass spectrometric techniques. Anal Bioanal Chem 2020; 412: 4893-4907.

DULIO V, KOSCHORRECK J, VAN BAVEL B, VAN DEN BRINK P, HOLLENDER J, MUNTHE J, et al. The NORMAN Association and the European Partnership for Chemicals Risk Assessment (PARC): let's cooperate! Environmental Sciences Europe 2020; 32.

EGGEN RI, HOLLENDER J, JOSS A, SCHARER M, STAMM C. Reducing the discharge of micropollutants in the aquatic environment: the benefits of upgrading wastewater treatment plants. Environ Sci Technol 2014; 48: 7683-9.

ESCHER BI, ASMALL YI U-AYUS, BEHNISCH PA, BRACK W, BRION F, BROUWER A, et al. Effect-based trigger values for in vitro and in vivo bioassays performed on surface water extracts supporting the environmental quality standards (EQS) of the European Water Framework Directive. Sci Total Environ 2018; 628-629: 748-765.

GAGO-FERRERO P, BLETSOU AA, DAMALAS DE, AALIZADEH R, ALYGIZAKIS NA, SINGER HP, et al. Wide-scope target screening of >2000 emerging contaminants in wastewater samples with UPLC-Q-ToF-HRMS/MS and smart evaluation of its performance through the validation of 195 selected representative analytes. J Hazard Mater 2020; 387: 121712.

LINK M, VON DER OHE PC, VOSS K, SCHAFER RB. Comparison of dilution factors for German wastewater treatment plant effluents in receiving streams to the fixed dilution factor from chemical risk assessment. Sci Total Environ 2017; 598: 805-813.

LIŠKA I, WAGNER F, SENGL M, DEUTSCH K, SLOBODNIK J. Joint Danube Survey 3 - A Comprehensive Analysis of Danube Water Quality (http://www.danubesurvey.org/jds3/jds3-files/nodes/documents/jds3_final_scientific_report_1.pdf, Last accessed 12 September 2020). 2015.

MOVALLI P, DUKE G, RAMELLO G, DEKKER R, VREZEC A, SHORE RF, et al. Progress on bringing together raptor collections in Europe for contaminant research and monitoring in relation to chemicals regulation. Environ Sci Pollut Res Int 2019; 26: 20132-20136.

NORMAN network. NORMAN Prioritisation framework for emerging substances, ISBN: 978-2-9545254-0-2 (Available at http://www.norman-network.net/sites/default/files/files/Publications/NORMAN_prioritisation_Manual_15%20April2013_final%20for%20website-f.pdf, Last accessed 19 September 2020).

NORMAN network. NORMAN Ecotoxicology, Module of the NORMAN Database System (https://www.norman-network.com/nds/ecotox/, Last accessed 26 August 2020).

NORMAN network, Water Europe. Contaminants of Emerging Concern in Urban Wastewater - Joint NORMAN and Water Europe Position Paper. (https://www.normandata.eu/sites/default/files/files/Publications/Position%20paper_CECs%20UWW_NORMAN_WE_2019_Final_20190910_public.pdf, Last accessed 26 August 2020).

VAN DER OOST R, SILENO G, JANSE T, NGUYEN MT, BESSELINK H, BROUWER A. SIMONI (Smart Integrated Monitoring) as a novel bioanalytical strategy for water quality assessment: Part II-field feasibility survey. Environ Toxicol Chem 2017; 36: 2400-2416.

32 Analysis of organic substances in the Danube River surface water by passive sampling

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Abstract

This chapter reports the concentration levels and occurrence of organic chemicals dissolved in surface water of the Danube River. The scope of the campaign was the screening of trace organic contaminants in the water column of the Danube, the assessment of their spatial distribution along the river, evaluation of their temporal trends (demonstration at a single site JDS4-15), and comparison of levels of selected hydrophobic organic pollutants in surface water and fish. The sampling was performed using stationary passive samplers deployed for 100 days at nine sites close to sites where fish were also caught for analysis. Passive samplers provide a time-integrated image of pollution in the aqueous phase over an extended time period, providing a representative picture of the surface water quality in summer 2019. The results show that the spatial variability of investigated hydrophobic priority substances in surface water of the Danube is low. No deterioration of Danube surface water contamination by hydrophobic priority substances was observed in JDS4 in comparison with the results from JDS3. Among investigated organochlorine compounds and PAHs at JDS4-15, a significant concentration decreasing trend was observed for hexachlorobenzene, PCB 28, PCB 52 and p,p'-DDE, whereas no significant temporal trend was found for PCBs with a higher degree of chlorination or for priority PAHs. Passive sampling of hydrophobic substances in surface water provides a worst-case scenario of fish exposure to those substances and should be considered as a viable alternative to biota monitoring. In the Upper and Middle Danube stretches, the occurrence of polar organic contaminants is associated with the discharge of municipal wastewaters to the river. In the Danube stretch downstream of the Iron Gates dam, the contaminant pattern and concentrations in surface water reveals application of pesticides in agriculture as the main contamination source.

32.1 Introduction

Organic pollutants are often present in the water column at trace concentrations that are difficult to detect when conventional low volume spot sampling of water is applied. The scope of the sampling campaign performed using passive samplers was the screening of freely dissolved trace organic pollutants in the water column of the Danube, as well as the assessment of their spatial distribution along the river. Freely dissolved concentrations of priority substances in the water phase (C_{free}) can be derived from the uptake of these

substances by passive samplers, and because accumulated contaminants represent a large water volume, low limits of quantification can be obtained. C_{free} is a more stable parameter than a concentration measured in whole water as the level is not influenced by variable amounts of the substance bound to dissolved and suspended particulate organic matter. Thus, it is very suitable for assessment of contamination trends. C_{free} is further considered to play a key role in chemical uptake by aquatic organisms. It is proportional to the chemical activity (Mayer et al., 2003) and if in equilibrium with surrounding environmental compartments, it also represents chemical activity of those environmental compartments, relevant for assessment of exposure of aquatic organisms (Reichenberg and Mayer, 2006). The application of temporal integrative passive sampling approach resulted in samples that provide a representative picture of the pollution situation by a wide range of organic contaminants at nine sites in the Danube River.

32.2 Methods

Sampling

Passive sampling was performed between mid-May and end of August/early September 2019 at 9 sites along the Danube River. Sites were selected to match sites where fish were collected for chemical analysis (supersites; Table 1). At each site, samplers were deployed in surface water using open wire frame holders at a depth of approximately 1 m below the water surface. Samplers were deployed from bridges, buoys or jetties hanging on ropes using buoys to keep the sampler holders floating.

Table 1: Passive sampling sites in Danube surface water – deployment from May till August 2019.

Site name	Lattitude	Longitude	River km	JDS4 site	River bank	Exposure (days)	Water volume sampled by SR (L)[a]
Jochenstein, water dam	48° 31.240'N	13° 42.122'E	2205	JDS 6	Left	101	2257
Čunovo, dam of the water reservoir	48° 1.807'N	17° 13.485'E	1855	JDS 15	Right	103	11541
DS Budapest, M0 bridge	47° 23.230'N	18° 59.460'E	1632	JDS 24	Middle	105	5971
Batina, bridge	45° 50.632'N	18° 51.315'E	1434	JDS 29	Right	104	2017
Pančevo, bridge	44° 49.877'N	20° 29.671'E	1154	JDS 37	Left	104	2886
Kladovo, jetty	44° 36.784'N	22° 36.820'E	926	JDS 41p[b]	Right	104	4917
Vidin-Calafat, bridge	44° 0.293'N	22° 56.840'E	796	JDS 43p	Middle	104	3128
Ruse, harbor	43° 51.555'N	25° 57.508'E	490	JDS 46p	Right	104	4690
Galati, water company	45° 22.650'N	28° 1.417'E	152	JDS 50p	Left	102	5059

[a] Volume of water extracted by the SR sampler with a surface area 1000 cm^2; it is calculated for a model compound with a molecular mass of 300. [b] The index 'p' means that the passive sampling site was in vicinity but not exactly a the position of the JDS4 site.

Passive samplers, sample processing and analysis

Two types of passive samplers were applied: partitioning samplers for hydrophobic compounds (silicone rubber (SR) and adsorption samplers for polar compounds based on styrene-divinylbenzene with hydrophilic moieties (HLB disks) sorbent solid phase extraction disks. Samplers accumulated organic compounds from the dissolved phase during exposure to Danube water.

Passive sampling of hydrophobic compounds

Passive samplers were made from silicone elastomer Altesil (Altec, UK) and applied as sheets of 9.5 × 5.5 cm of 500 µm thickness. In each of these sheets two holes are punched which allows them to be fixed to the frame using cable ties. Before exposure, they were Soxhlet extracted in ethyl acetate and spiked according to the procedure described in Booij and Smedes (2010) with 14 performance reference compounds (PRC: D_{10}-biphenyl and 13 polychlorinated biphenyl (PCB) congeners that do not occur in technical mixtures. A silicone (SR) passive sampler consisted of 10 silicone sheets per sampling site, with a total surface area of 1,000 cm^2.

Exposed, field blank, and control samplers, were spiked with recovery internal standards and Soxhlet extracted for 8 h with acetonitrile. The extract was concentrated by Kuderna-Danish (KD) apparatus to 2 mL. An aliquot representing 30% of the total extract in hexane was further cleaned-up over a silica gel column (non-destructive clean-up) by elution with dichloromethane. The volume was reduced by KD, 100 µL of nonane was added, quantitatively transferred to a vial (1 mL), instrumental internal standards were added and the samples were analyzed for a range of target substances by gas chromatography coupled to mass spectrometry (GC/MS). The remaining 70% was purified using activated silica gel modified with sulphuric acid. Sample was eluted with dichloromethane:hexane (1:1, v/v), the volume was reduced to 1 ml, 50 µL of nonane was added and transferred to a vial. After addition of instrumental internal standards and volume reduction extracts were analysed by GC/MS for PCBs, PRCs, heptachlor and PBDEs by instrumental methods described in Vrana et al., (2018).

The extracts were measured for other emerging compounds including pyrethroids, musk fragrances, UV filters and other industrial precursors by a Thermo Scientific Trace 1300 gas chromatography coupled to a high-resolution Orbitrap mass spectrometer (Q Exactive GC) with a resolving power of 60,000 and electron ionisation at 70 eV. An Agilent HP-5MS column (30 m x 0.25 mm x 0.25 µm) was used for separation. An aliquot of 2 µL was injected into a thermodesorption unit connected to cold injection system (Gerstel). The extracts were solvent exchanged to ethyl acetate and 22 isotope-labelled internal standards were added to each aliquot before analysis to have a concentration of 50 ng/ml.

Passive sampling of polar compounds

The HLB disk sampler consisted of ten solid phase extraction Affinisep AttractSPE®Disks HLB with 47 mm diameter. The surface area exposed to water was 113 cm^2. Before exposure samplers were pre-conditioned and kept immersed in MilliQ water until exposure. These samplers were not spiked with any PRCs.

Following exposure, all HLB disk samplers for chemical analysis were spiked with recovery internal standards (stable isotope labelled currently used pesticides, PFAS and pharmaceuticals). All samplers were then freeze dried for 24 hours. The disks were extracted three times overnight (12 h) by slow shaking at room temperature with 50 ml acetone. Combined extracts were reduced by vacuum rotary evaporation. After removal of particles by filtration through a layer of anhydrous Na_2SO_4 the extract was further reduced in volume to 1 ml. The acetone extract was transferred to methanol by addition of methanol (20 ml) and subsequent evaporation and a nitrogen flow to further reduce in volume to 2 ml. Aliquots of the extract were divided into vials for different types of analysis.

The targeted analysis of HLB disk extracts was performed for 154 compounds including pharmaceuticals, pesticides, benzotriazoles, illicit drugs and their metabolites. For analysis, 50 µL of sample extract was diluted by 50 µL of ultra-pure water and the mixture of isotopically labelled internal standards was added. The samples were analysed by liquid chromatography with tandem mass spectrometry (TSQ Quantum, Thermo Fisher Scientific) using heated electrospray (338 °C) in both positive (+ 3.5 kV) and negative (- 2.5 kV)

ionization modes. For separation of analytes, Hypersil Gold aQ column (50 × 2.1 mm; 5 µm particles, Thermo Fisher Scientific) was used in gradient elution of ultra-pure water and acetonitrile, both acidified with 0.1 % formic acid. The system was operated by Xcalibur Software, the data were processed by TraceFinder 3.3 (both software from Thermo Fisher Scientific). The eight-points calibration curve was prepared in range 0.1-100 ng/mL and used for method linearity evaluation and for determination of limit of quantification. For calculation of concentration of pesticides and pharmaceuticals in HLB disks, the method of matrix matching standard and isotopically labelled internal standard was used.

QA/QC

Quality assurance monitoring comprised a series of quality control measures. These included the analysis of the reagents and solvents (reagent blanks), preparation controls, field controls and recovery spikes. The meaning and application of individual control samples is defined in the technical norm Water quality - sampling - part 23: Guidance on passive sampling in surface waters, EN ISO 5667-23 (ISO, 2011).

Data analysis

Dissolved aqueous concentrations (C_{free}) were calculated from analyte amounts accumulated in SR samplers as follows. Amounts of analytes absorbed by the samplers follow a first-order approach to equilibrium. Aqueous concentrations were calculated from the mass absorbed by the samplers, the *in situ* sampling rate (R_S) of the compounds and their sampler-water partition coefficients (Smedes, 2019; Smedes et al., 2009) as described in Smedes and Booij (2012). Sampling rates were estimated from dissipation of PRCs from samplers during exposure using nonlinear least squares method by Booij and Smedes (2010), considering the fraction of individual PRCs that remains in the SR after the exposure as a continuous function of their sampler-water partition coefficient and their molecular mass, with adjustable parameter *F*. R_s for a compound accumulated under water boundary layer control was calculated as a function of its molecular mass $R_s=F\times M^{-0.47}$ (Rusina et al., 2010).

For HLB disk sampler calibration data are not available so far. For compounds under investigation, we assumed a sorption distribution equilibrium after 100 days contact between sampler and water. Identification of pollutant gradients along the Danube was performed based on the amount of a compound sampled by the HLB disk sampler at individual sites.

32.3 Results

32.3.1. Hydrophobic compounds

SR samplers were deployed at 9 sampling sites to characterise the spatial variability of hydrophobic compounds in the water column of the Danube River.

Polybrominated diphenyl ethers (PBDEs). C_{free} of dissolved PBDEs (referring to the sum of the concentrations of congener numbers 28, 47, 99, 100, 153 and 154) ranged between 3 and 11 pg L^{-1} at sites JDS 50p and JDS 29, respectively. Comparable concentrations of 12 pg L^{-1} were measured by SR passive samplers during JDS3 in 2013 in the stretch Passau to Bratislava (between sites JDS 6 and JDS 15). At all sites, BDE 47 and BDE 99 were the dominant BDE congeners. This corresponds with the composition of widely used flame-retardants in consumer products, containing BDE 47 as the dominant congener.

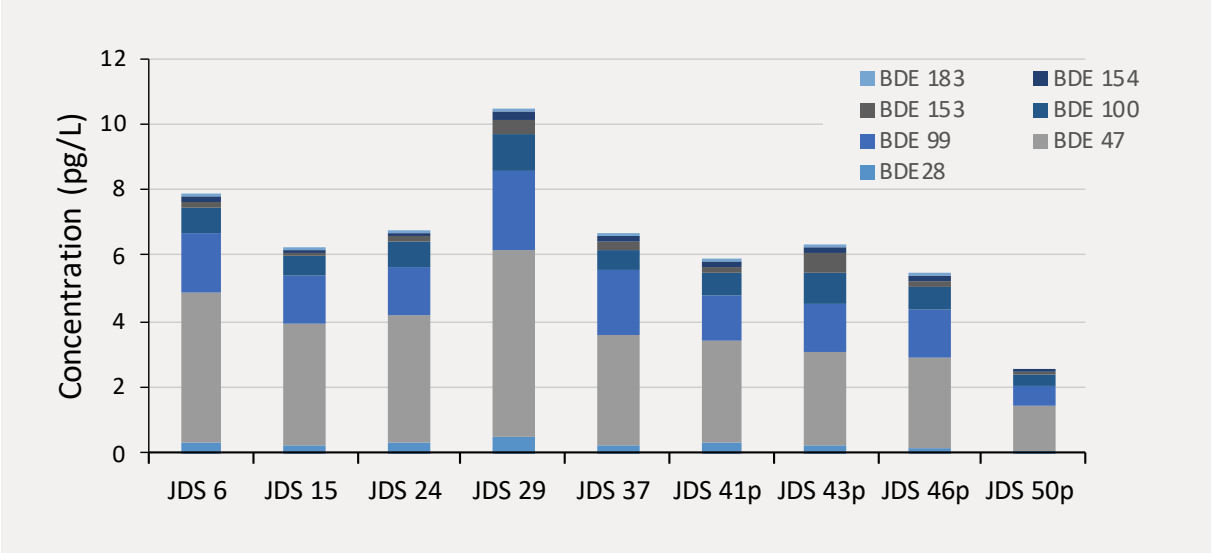

Figure 1: Longitudinal profile of C_{free} concentrations of PBDEs along the Danube.

Polychlorinated biphenyls, penta and hexachlorobenzene. The C_{free} pattern of individual PCB congeners showed lower values for PCBs of higher hydrophobicity at all sites. The sum C_{free} of the seven analyzed indicator PCB congeners ranged between 64 and 264 pg L^{-1}. The C_{free} median concentration measured by passive sampling during JDS3 in 2013 was 2.4-times higher than during JDS4 in 2019. Since the comparison is based on two sampling campaigns only, and the sampler deployment duration was not the same, the observed decrease should only be considered as an indication of declining PCB trend in the Danube. However, a significant decreasing trend of PCB 28 and PCB 52 was observed at the site JDS 15, where C_{free} measurements by passive sampling were performed annually (Figure 10). However, at JDS 15 no significant temporal trend was found for PCB congeners with a higher degree of chlorination. For penta- and hexachlorobenzene (PeCB and HCB) C_{free} ranged from 6 to 32 (PeCB) and from 9 to 24 (HCB) pg L^{-1}, respectively. The average of measured HCB concentrations was approximately 4-times lower than that measured by passive sampling in JDS3 and also lower than in JDS 2, when the median and maximum aqueous concentrations of HCB in the water column were 18 and 74 pg L^{-1}, respectively (Umlauf et al., 2008). At the site JDS 15 a significant decreasing trend was observed for HCB with the estimated halftime of 2 years (Figure 10).

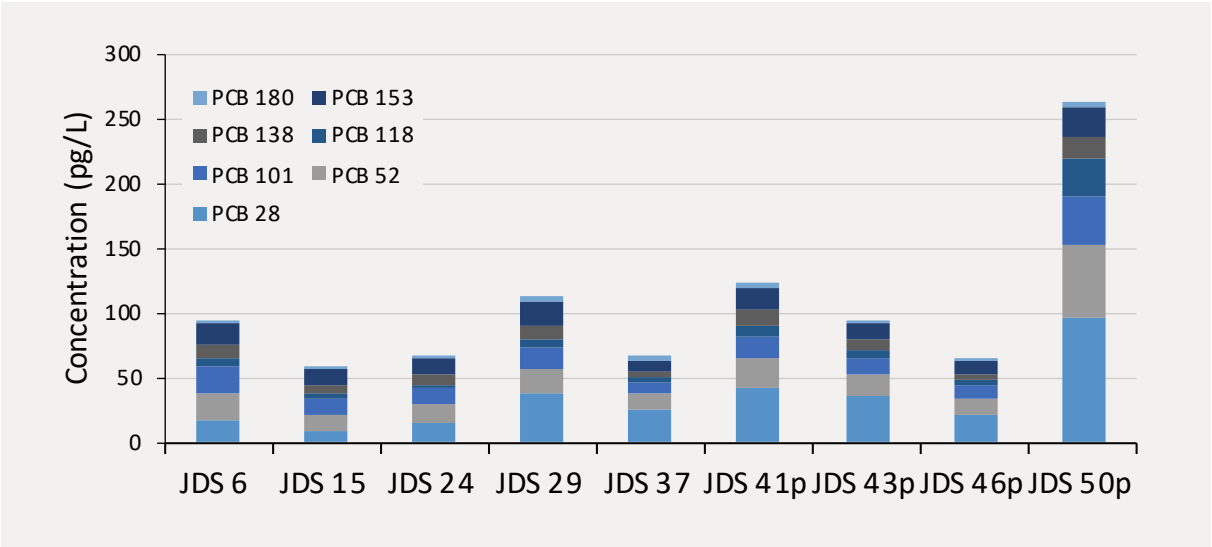

Figure 2: Longitudinal profile of C_{free} concentrations of PCBs along the Danube.

Cyclodiene pesticides. In the cyclodiene pesticide pattern present in the Danube water column, dieldrin and endosulfan sulfate dominated. The latter is the main endosulfan metabolite. C_{free} concentrations were mostly in units to tens pg L^{-1} without any clear extremes along the river.

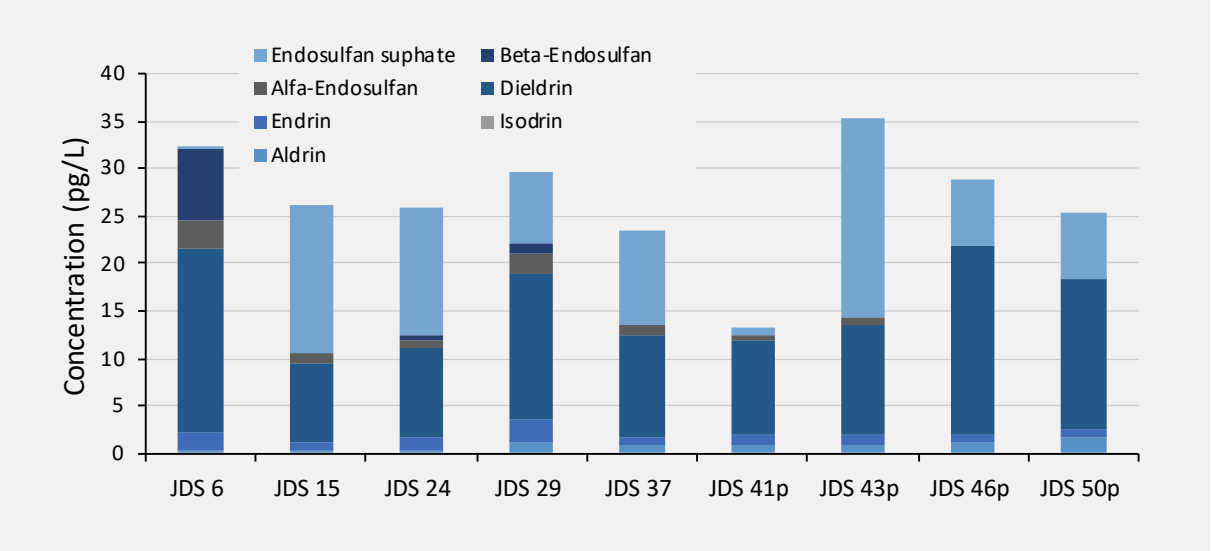

Figure 3: Longitudinal profile of C_{free} concentrations of cyclodiene pesticides along the Danube.

DDT and its metabolites (DDx). The C_{free} pattern of DDx was dominated by metabolites p,p'-DDE and p,p'-DDD. C_{free} of p,p'-DDT (2-13 pg L^{-1}) comprised only 5-7% of the total DDT, which indicates no current use of DDT in the monitored Danube stretch. Unfortunately, in JDS4 passive samplers were not deployed in the Danube delta area, where the highest DDT concentrations were detected in JDS2 and JDS3 (Vrana et al., 2015). At the site JDS 15 a significant decreasing temporal trend was observed for p,p'-DDE with the estimated halftime of 2 years (Figure 10).

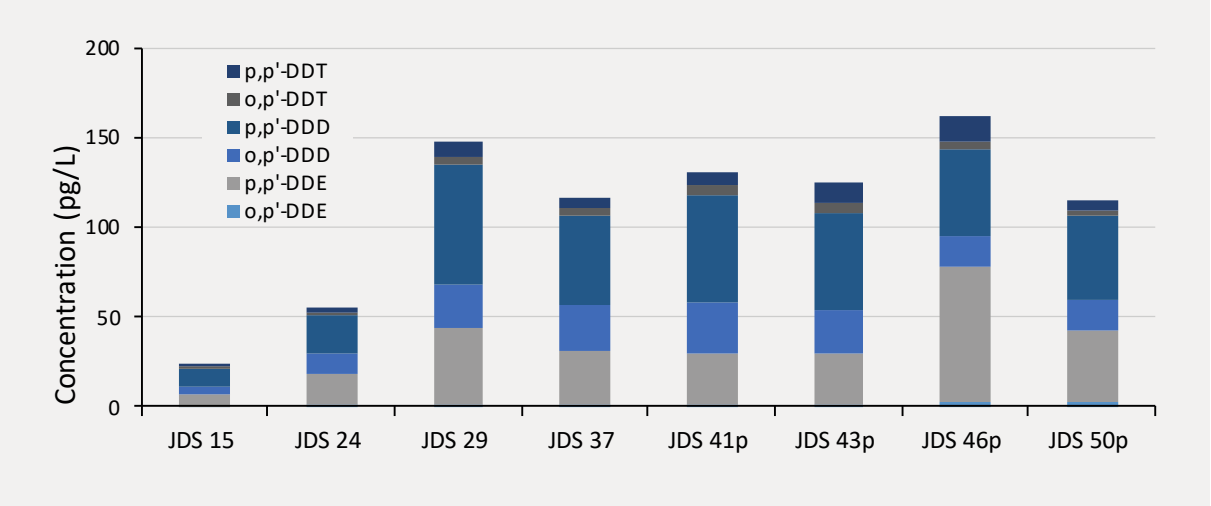

Figure 4: Longitudinal profile of C_{free} concentrations of DDT and its metabolites along the Danube.

Heptachlor. At most sites C_{free} concentrations were mostly in units of pg L^{-1} and heptachlor epoxide, the heptachlor metabolite, dominated in the aqueous phase. Presence of heptachlor and elevated concentrations at the site in Ruse indicates recent application of this pesticide in the area.

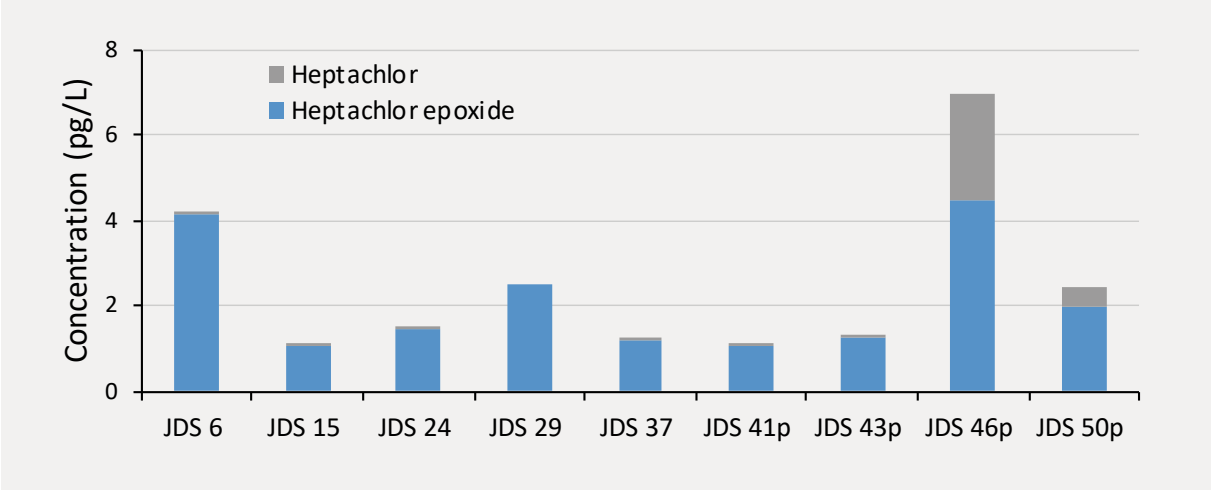

Figure 5: Longitudinal profile of C_{free} concentrations of heptachlor and heptachlor epoxide along the Danube.

Polycyclic aromatic hydrocarbons (PAHs). Among priority PAHs, naphthalene was not monitored because of QA/QC issues. As for PCBs there is a strong decrease of free dissolved concentration with increasing compound hydrophobicity. In agreement with observations made in JDS3, elevated PAH concentrations were observed in the middle stretch of Danube (JDS 29 and JDS 37). At the site JDS 15 no significant temporal trend was found for any of the priority PAHs. This indicates that free dissolved PAH concentrations and their patterns remain in the same range over a period of several years.

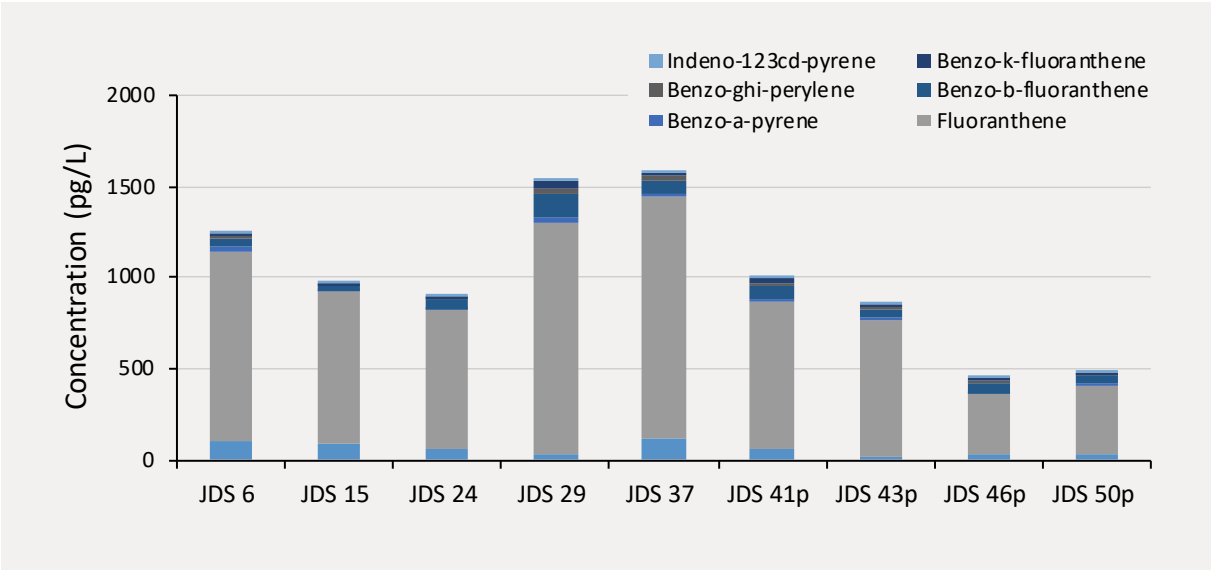

Figure 6: Longitudinal profile of C_{free} concentrations priority PAHs along the Danube.

Pyrethroids. The pyrethroids insecticide concentrations ranged between 0.3 and 196 pg L^{-1} along the river. The three highest concentrations were recorded for cyhalothrin, permethrin and etofenprox at site JDS 37, downstream of Pančevo. The sites JDS 41p (Kladovo) and JDS 43p (Vidin) were found to be the sites with lowest pyrethroids' load.

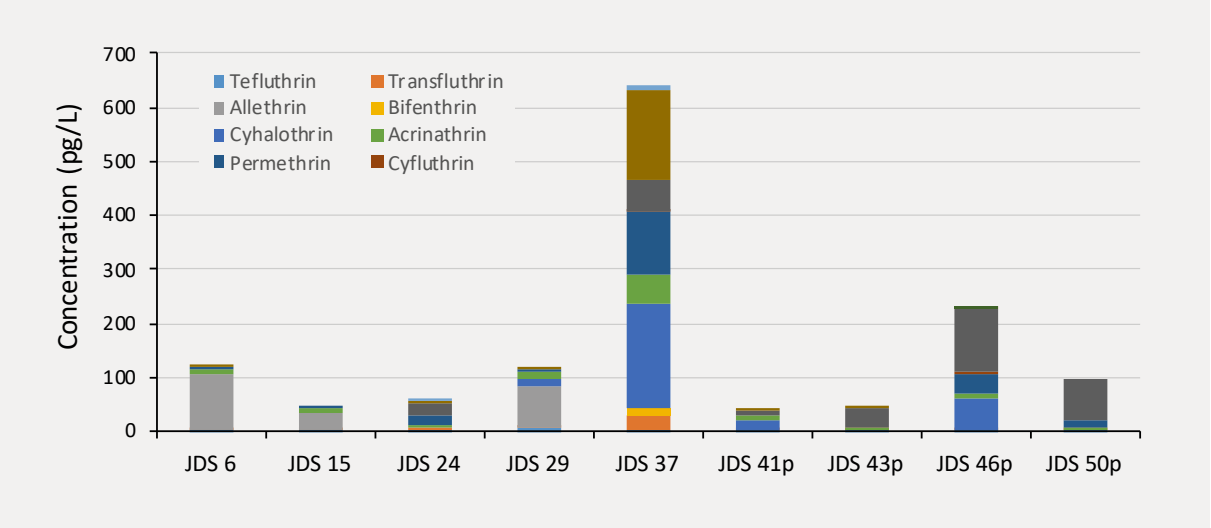

Figure 7: Longitudinal profile of C_{free} concentrations of detected pyrethroids along the Danube.

Chlorpyrifos. The organophosphate pesticide chlorpyrifos was detected in 10-fold higher concentrations compared to the pyrethroids along the river except for the site Jochenstein. The calculated freely dissolved concentrations ranged between 40-7400.

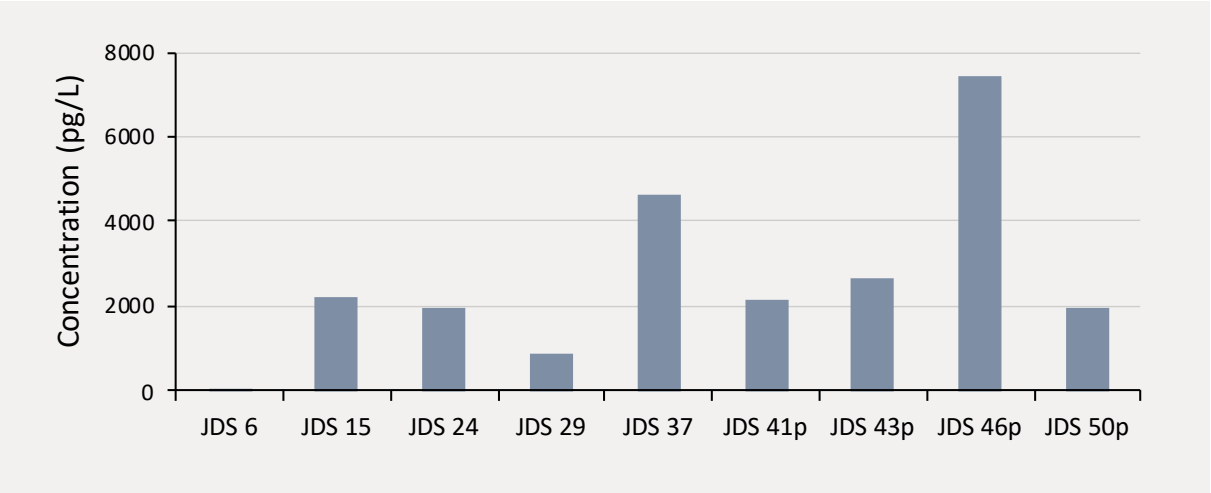

Figure 8: Longitudinal profile of C_{free} concentrations of chlorpyrifos along the Danube.

Fragrances. Among the measured fragrances the synthetic polycyclic musk galaxolide dominated the detected fragrance profile along the river. The galaxolide concentration ranged between 18 and 67 ng L^{-1}. The other synthetic polycyclic musk fragrances ambrettolide, tonalide and velvion were detected at 10-fold lower concentrations compared to galaxolide. Besides the polycyclic musk fragrances, the musk like fragrance cashmeran was detected in all sites along the river having a concentration range between 170 and 650 pg L^{-1}.

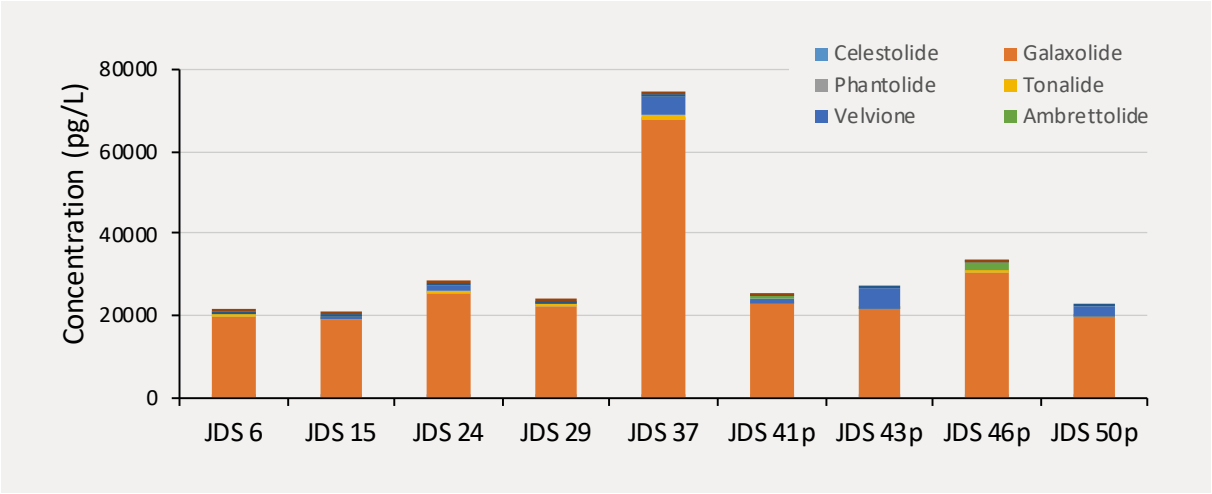

Figure 9: Longitudinal profile of C_{free} concentrations of fragrances along the Danube.

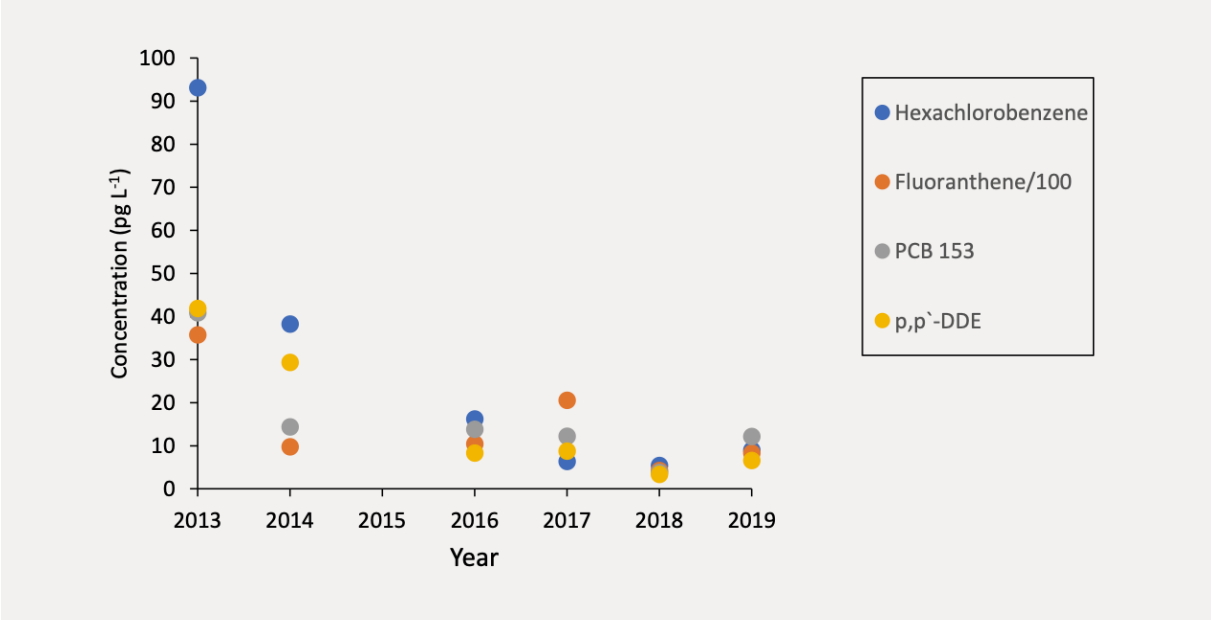

Figure 10: An example of temporal variability of freely dissolved aqueous concentrations of hydrophobic organic compounds (HOCs) at the site JDS 15 in Čunovo. The concentrations were derived from stationary passive sampling in summer or autumn every displayed year. In 2013 and 2019, samplers were deployed within JDS 3 and JDS 4, respectively. Among analysed organochlorine compounds and PAHs, a significant concentration decreasing trend was observed e.g. for hexachlorobenzene, PCB 28, PCB 52 and p,p'-DDE, with estimated halftime of 2 years. No significant temporal trend was found for PCBs with a higher degree of chlorination or for priority PAHs. Values for fluoranthene were divided by hundred to fit into the scale of other displayed compounds.

Hydrophobic organic compounds: thermodynamic level difference between fish and the water phase

For a meaningful comparison of levels in water with those in fish it is necessary to convert their concentrations into equal units. In biota concentrations of hydrophobic compounds (HOC) are associated with the lipid content and therefore their levels are commonly compared as lipid–based concentrations. In aqueous passive sampling the HOC concentrations in the sampler are typically converted to aqueous free dissolved concentrations but can also be expressed on lipid basis. To do this, C_{free} derived from SR passive sampling was first multiplied by polymer/water partition coefficient, which gives the concentration in the sampler

polymer at equilibrium with water. The result is then multiplied with the lipid-polymer partition coefficient to provide the lipid–based concentration at equilibrium with the water phase ($C_{L\text{-water}}$). This $C_{L\text{-water}}$ is the concentration the abiotic neutral lipid (triacylglyceride) would have if it was at equilibrium with the water phase. $C_{L\text{-water}}$ is essentially the exposure pressure the water phase has on organisms living in it. Comparing $C_{L\text{-water}}$ with $C_{L\text{-fish}}$ enables to compare on the same scale the level of HOC bioaccumulation in lipid of these organisms relative to the level in neutral lipid at thermodynamic equilibrium with the water phase (Figure 11). The general assumption is that bioaccumulation and trophic magnification cause build-up of HOC levels in aquatic organisms unless the compounds are actively transformed by their metabolism. Recently, Smedes et al. (2020), in addition observed that HOC's $C_{L\text{-fish}}$ become increasingly lower than $C_{L\text{-water}}$, as hydrophobicity increases, a phenomenon that has also been observed at the nine sites investigated in JDS4.

At all investigated sites except one, the common bleak (*Alburnus alburnus*) was taken for HOC level comparison with the water phase. Bleak has an estimated trophic level (TL) of 2.7±0.3 (www.fishbase.se). At a single site JDS 15, the comparison was performed for asp (*Leuciscus aspius*), a predatory fish with estimated TL=4.5±0.8 (www.fishbase.se). Smedes et al. (2020) demonstrated at several freshwater sites in central Europe, that for fish species with trophic level around 3, $C_{L\text{-fish}}$ negatively deviated from the thermodynamic equilibrium with the water phase up to several orders of magnitude for HOCs with very high K_{ow} (log K_{ow} > 6).

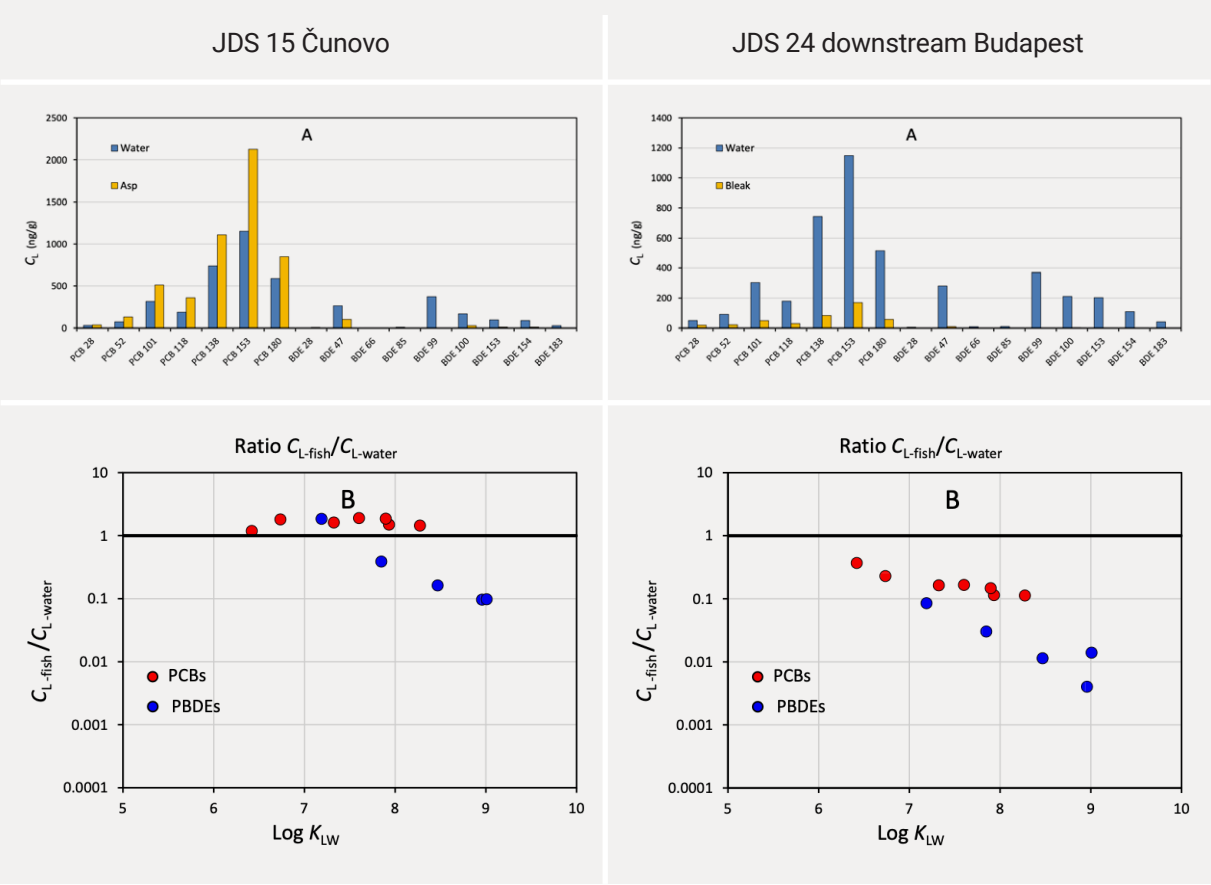

Figure 11: Thermodynamic level difference between fish and the surface water, both expressed as lipid-based concentrations C_L (upper row graphs A). The example is given for two fish species with different trophic level: asp Leuciscus aspius (TL= 4.5) at JDS 15 in Čunovo and common bleak Alburnus alburnus (TL=2.7) at JDS 24 downstream Budapest, respectively. In the lower row graphs B the ratio $C_{L\text{-fish}}/C_{L\text{-water}}$ is plotted versus the lipid-water partition coefficient K_{LW} for PCBs and PBDEs.

With increasing TL, $C_{L\text{-}fish}$ increased, getting to about the same level as $C_{L\text{-}water}$ at TL=4. The data from JDS4 perfectly match this observation (Figure 11). JDS4 thus provides more experimental evidence that partitioning passive sampling indicates a realistic worst case (the highest expected) estimate of HOC level in fish at TL=4. Passive sampling may find future application in a tiered approach in compliance monitoring, as suggested by Miège et al. (2015). This is an important finding, especially in view of current technical difficulties with the implementation of the Directive 2013/39/EU due to inherent variability of biota data (lipid content, trophic level, age etc.) and limitation of methods applicable for biota data normalisation (EU, 2013). For checking compliance with EQSbiota according to 2013/39/EU, data from fish species with low trophic level (such as in JDS4) may be converted to equivalent values at TL=4 (EC, 2014). To perform such conversion, substance and site-specific trophic magnification factors are required, which are rarely available and associated with a high uncertainty.

32.3.2. Polar compounds

The targeted analysis of HLB disk passive samplers provided information on occurrence of 154 polar contaminants at 9 JDS sites. We assume that after 100 days of exposure, the analysed compounds reached distribution equilibrium with Danube water, and that sorbent/water distribution did not significantly differ among sampling sites. In such case compound amounts sorbed on HLB disk (ng/disk) can be used to investigate differences in contaminant patterns between sites and visualise longitudinal profiles of contaminant concentrations in the Danube.

We compared the contaminant patterns in HLB disk extracts at different sites by principal component analysis (PCA). PCA revealed that 63% of the observed data variance can be projected in a two-dimensional factor plane (Figure 12). The score plot (Figure 12 left) shows the separation of the sites along the principal components (factors). As can be seen in the loading plot (Figure 12, right), the 154 compounds (variables) are separated on the principal component plane according to their covariance with the two main factors. The most important trend (factor 1) accounts for 46% of the total variance and along the factor 1 sites in the Upper Danube stretch (JDS 6, JDS 15 and JDS 24) have negative scores, whereas those in the Lower Danube have positive scores. In addition, the second most important trend (factor 2) shows a positive score for sites downstream the Iron Gates dam (JDS 41p, JDS 43p,JDS 46p) with the exception of the site at Galati (JDS 50p).

Most of the analysed pharmaceuticals, illicit drugs, benzotriazoles and some pesticides (atrazine desethyl, chloridazon methyl desphenyl, DEET, diuron, diuron desmethyl, fenuron, isoproturon, flusilazole, methabenzthiazuron, monolinuron, picloram, pirimicarb, propiconazole, terbutryn, triallat, and triticonazole) show a significant negative correlation with the factor 1. Factor 1 most likely reflects the wastewater effluent dilution in the Danube since most of the above-named pesticides are used for maintaining of roofs etc., not in agriculture. In contrast, compounds showing a positive correlation with both factor 1 and factor 2 comprise mostly pesticides actively applied in agriculture, such as dimethachlor, terbutylazine, simazine, chlorantraniliprole, linuron, carbendazim, sulfamethazine, metolachlor, desethyl-terbuthylazine, azoxystrobin, tebuconazole, metazachlor, epoxiconazole, triadimenol, alachlor, acetochlor, metobromuron, propazine, meclozine, desamino-metribuzin, chlorpyrifos, metribuzin, atrazine and epoxy carbamazepine (metabolite of carbamazepine – a human drug).

When grouping the analysed compounds according to their use (pharmaceuticals, pesticides, benzotriazoles) two distinct longitudinal concentration profiles are revealed (Figure 13). Whereas concentrations of pharmaceuticals and benzotriazole continuously decrease at sites further downstream the river, there is an apparent increase of concentrations of currently used pesticides in the Lower Danube downstream the Iron Gates dam.

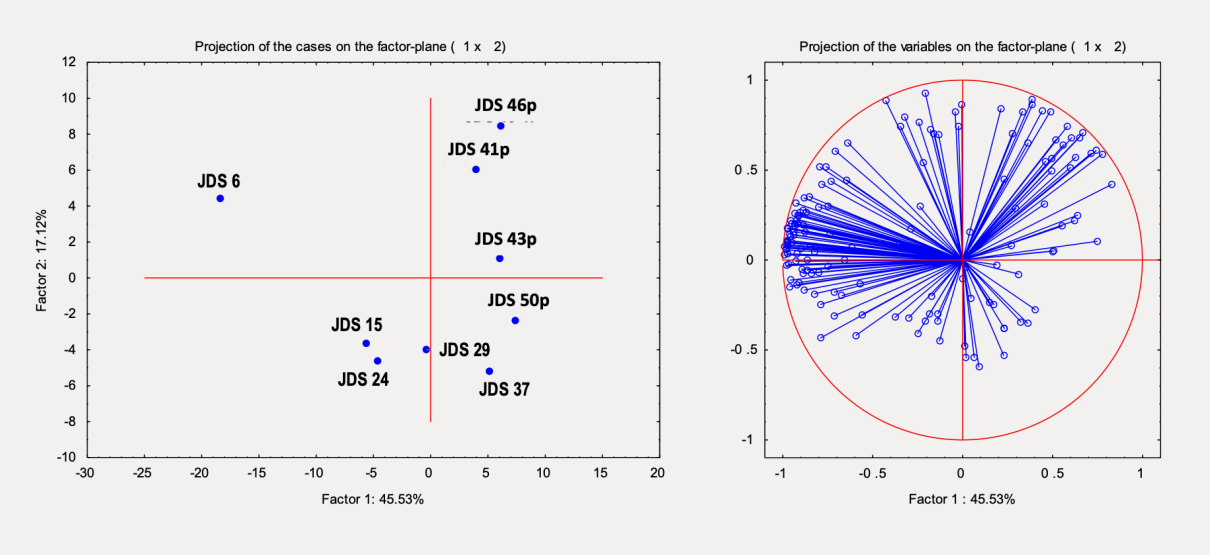

Figure 12: Principal component analysis (PCA) based on targeted analysis of HLB-disk extracts by LC/MS/MS, comprising 154 compounds including pharmaceuticals, pesticides, benzotriazoles, illicit drugs and their metabolites.

Based on the above analysis we conclude that the main sources of the found contaminants in the upper and middle Danube are mainly the municipal wastewaters discharged to Danube. These are diluted by the increasing water volume due to tributaries in the Lower Danube. Moreover, the emissions of compounds associated with municipal wastewater are likely lower in the Lower Danube because of lower population density in the area. In the Lower Danube stretch, the contaminant pattern reveals active application of pesticides in agriculture as the main contamination source.

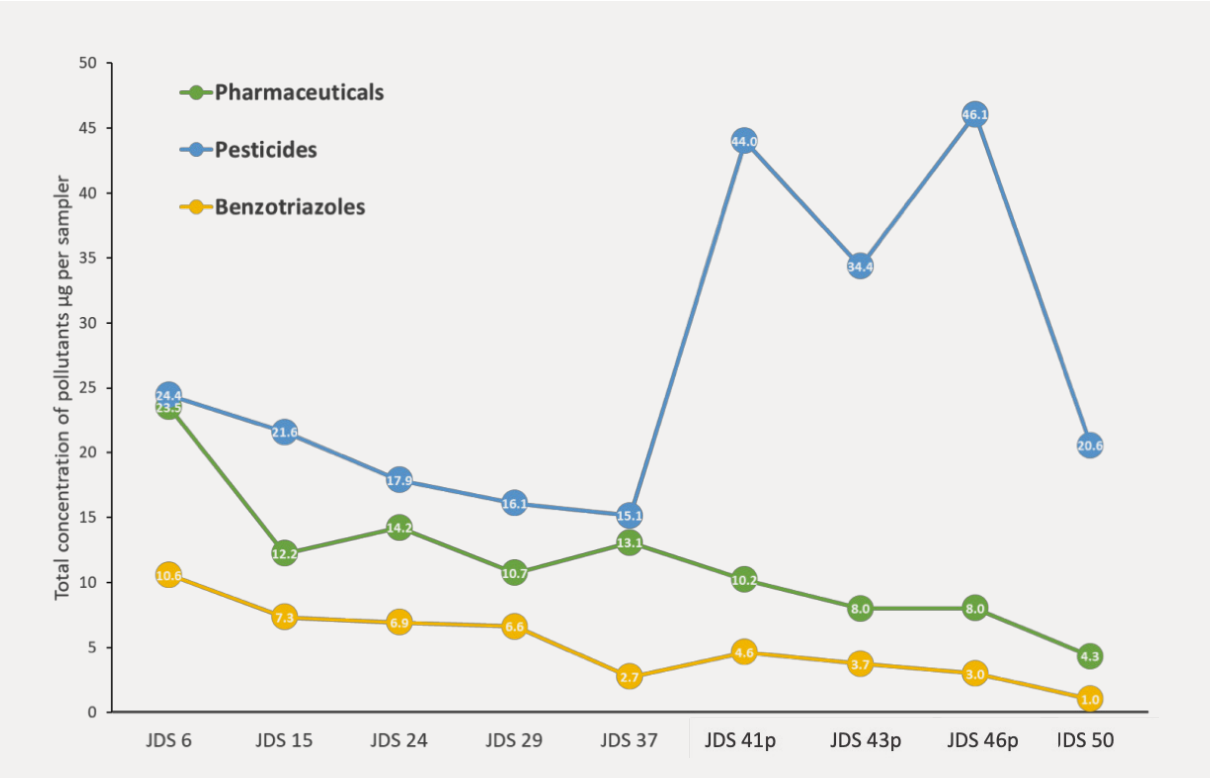

Figure 13: Longitudinal profile of concentrations of 154 polar compounds (divided into three groups: pharmaceuticals, pesticides and benzotriazols) along the Danube, based on amounts accumulated in HLB disks.

32.4 Conclusions

- Spatial variability of investigated hydrophobic priority substances in surface water of the Danube is low.
- No deterioration of Danube surface water contamination by hydrophobic priority substances was observed in JDS4 in comparison with the results from JDS3.
- Among investigated organochlorine compounds and PAHs at JDS 15, a significant concentration decreasing trend was observed for hexachlorobenzene, PCB 28, PCB 52 and p,p'-DDE, with estimated clearance halftime of 2 years. No significant temporal trend was found for PCBs with a higher degree of chlorination or for priority PAHs.
- Passive sampling of hydrophobic substances in surface water provides a worst-case scenario of fish exposure to those substances and should be considered as a viable alternative to biota monitoring.
- In the upper and middle Danube stretches, the occurrence of polar organic contaminants is associated with the discharge of municipal wastewaters to the river.
- In the Danube stretch downstream the Iron Gates dam, the contaminant pattern and concentrations in surface water reveals application of pesticides in agriculture as the main contamination source.

32.5 Acknowledgments

We acknowledge the NORMAN association www.normannetwork.net and the Czech Science Foundation grant No. GACR 20-04676X “Holistic exposure and effect potential assessment of complex chemical mixtures in the aquatic environment” for the financial support.

32.6 References

BOOIJ, K., SMEDES, F., 2010. An improved method for estimating in situ sampling rates of nonpolar passive samplers. Environ. Sci. Technol. 44, 6789–94. https://doi.org/10.1021/es101321v

EC, 2014. Guidance document No. 32 on Biota Monitoring (The implementation of EQSbiota) under the Water Framework Directive. Common Implementation Strategy for the Water Framework Directive (2000/60/EC). European Commission, Brussels. https://doi.org/doi: 10.2779/833200

EU, 2013. Directive 2013/39/EU of the European Parlament and of the Council of 12 August 2013 amending Directives 2000/60/EC and 2008/105/EC as regards priority substances in the field of water policy. Off. J. Eur. Union L226, 1–17.

ISO, 2011. ISO 5667-23:2011. Water quality - sampling - part 23: Guidance on passive sampling in surface waters.

MAYER, P., TOLLS, J., HERMENS, J., MACKAY, D., 2003. Peer reviewed: Equilibrium sampling devices. Environ. Sci. Technol. 37, 184A-191A. https://doi.org/10.1016/S0889-8561(03)00030-4

MIÈGE, C., MAZZELLA, N., ALLAN, I., DULIO, V., SMEDES, F., TIXIER, C., VERMEIRSSEN, E., BRANT, J., O'TOOLE, S., BUDZINSKI, H., GHESTEM, J.P., STAUB, P.F., LARDY-FONTAN, S., GONZALEZ, J.L., COQUERY, M., VRANA, B., 2015. Position paper on passive sampling techniques for the monitoring of contaminants in the aquatic environment - Achievements to date and perspectives. Trends Environ. Anal. Chem. 8, 20–26. https://doi.org/10.1016/j.teac.2015.07.001

REICHENBERG, F., MAYER, P., 2006. Two Complementary Sides of Bioavailability : Accessibility and ... Environ. Toxicol. Chem. 25, 1239–1245.

RUSINA, T., SMEDES, F., KOBLIZKOVA, M., KLANOVA, J., 2010. Calibration of silicone rubber passive samplers: experimental and modeled relations between sampling rate and compound properties. Environ. Sci. Technol. 44, 362–7. https://doi.org/10.1021/es900938r

SMEDES, F., 2019. SSP silicone–, lipid– and SPMD–water partition coefficients of seventy hydrophobic organic contaminants and evaluation of the water concentration calculator for SPMD. Chemosphere 223, 748–757. https://doi.org/10.1016/J.CHEMOSPHERE.2019.01.164

SMEDES, F., BOOIJ, K., 2012. Guidelines for passive sampling of hydrophobic contaminants in water using silicone rubber samplers. https://dx.doi.org/10.17895/ices.pub.5077. International Council for the Exploration of the Sea, Copenhagen.

SMEDES, F., GEERTSMA, R.W., VAN DER ZANDE, T., BOOIJ, K., 2009. Polymer-water partition coefficients of hydrophobic compounds for passive sampling: Application of cosolvent models for validation. Environ. Sci. Technol. 43, 7047–7054. https://pubs.acs.org/doi/10.1021/es9009376

SMEDES, F., SOBOTKA, J., RUSINA, T.P., FIALOVÁ, P., CARLSSON, P., KOPP, R., VRANA, B., 2020. Unraveling the Relationship between the Concentrations of Hydrophobic Organic Contaminants in Freshwater Fish of Different Trophic Levels and Water Using Passive Sampling. Environ. Sci. Technol. acs.est.9b07821. https://doi.org/10.1021/acs.est.9b07821

UMLAUF, G., CHRISTOPH, E., HUBER, T., MARIANI, G., MUELLER, A., SKEJO, H., WOLLGAST, J., 2008. Cross matrix inter-comparison of semi-volatile organic compounds in water, suspended particulate matter, sediments and biota, in: Liska, I., Wagner, F., Slobodnik, J. (Eds.), Joint Danube Survey 2 - Final Scientific Report. ICPDR-International Commission for the Protection of the Danube River, Vienna, pp. 174–191.

VRANA, B., SMEDES, F., ALLAN, I., RUSINA, T., OKONSKI, K., HILSCHEROVÁ, K., NOVÁK, J., TARÁBEK, P., SLOBODNÍK, J., 2018. Mobile dynamic passive sampling of trace organic compounds: Evaluation of sampler performance in the Danube River. Sci. Total Environ. 636, 1597–1607. https://doi.org/10.1016/j.scitotenv.2018.03.242

VRANA, B., SMEDES, F., RUSINA, T., OKONSKI, K., ALLAN, I., GRUNG, M., HILSCHEROVA, K., NOVÁK, J., TARÁBEK, P., SLOBODNÍK, J., 2015. Passive sampling: chemical analysis and toxicological profiling, in: Liška, I., Wagner, F., Sengl, M., Deutsch, K., Slobodník, J. (Eds.), Joint Danube Survey 3. ICPDR – International Commission for the Protection of the Danube River, Vienna, pp. 304–315.

33 Polarity-extended non-target screening using RPLC-HILIC-ESI-QToF-MS/MS and tailor-made data handling

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Abstract

Very polar organic molecules are increasingly becoming emerging compounds of interest while studying aqueous environmental samples. These highly water-soluble molecules need to be analyzed (together with polar and non-polar compounds) with so-called polarity-extended chromatography, for example the well-established serial RPLC-HILIC coupling. The method, the data handling strategy and data processing workflow on a JDS4 sample using RPLC-HILIC-ESI-QToF-MS/MS monitoring are presented. The non-target screening (NTS) measurements and the consequent data handling, regarding mass spectrometry (including tandem mass spectrometry), polarity filter (like RTI and negative log D) and the application of the FOR-IDENT platform and the STOFF-IDENT database as well as the MetFrag tool help to analyze novel molecules outside of the current 'identification' box. Detailed results regarding the suggested identification and suspects will be published in a research article.

33.1 Introduction

Trace organic compounds of anthropogenic origin are generally known to be present in surface waters such as the Danube River Basin (Liška et al., 2015). Especially a group of compounds that are persistent in the environment, mobile in the aquatic environment, and toxic (PMTs) or very persistent in the environment and very mobile in the aquatic environment (vPvMs) raises environmental concerns (Berger et al., 2018). Despite the advances in reversed-phase liquid chromatography (RPLC) hyphenated with high-resolution mass spectrometry (HRMS), PMTs and vPvMs constitute a so-called "analytical gap" (Reemtsma et al., 2016). One technique able to close this gap is hydrophilic interaction liquid chromatography (HILIC) since its retention mechanism is complementary to that of RPLC (Bieber et al., 2017; Bieber & Letzel, 2020; Chalcraft & McCarry, 2013) and is suitable for the separation of very polar compounds (Minkus et al., 2020; Tang et al., 2016). Using a serial coupling of RPLC and HILIC is significantly extending the polarity range of molecules separated in a single experimental run (Greco et al., 2013). In combination with HRMS detection in full scan mode, non-targeted data can be acquired which may include encoded information on environmentally relevant molecules.

In the following, the application of such a polarity extending separation technique in combination with a so-called hidden target data evaluation workflow is presented. This data processing strategy (Letzel et al., 2015; Minkus et al., 2020) incorporates and connects accurate molecular mass and MS/MS information of detected features to compound candidates with information on their physicochemical properties such as polarity or hydrophilicity, in order to identify "probable" structures as communicated by Schymanski et al. (Schymanski et al., 2014) and others (Letzel et al., 2014).

33.2 Methods

51 surface water samples and seven groundwater samples were used in the hidden-target workflow for the polarity-extended monitoring. In this chapter, the workflow is illustrated on one exemplary environmental sample, namely the JDS4-7 sample. It was collected in Enghagen (Austria; rkm 2113), approximately 8 km downstream of the wastewater treatment plant Asten (Austria). In this study it was compared to a laboratory blank sample consisting of pure water (LC-MS grade) as described in the 'NTS-Guideline for water analysis' (Schulz, 2019). In addition, 177 reference standards were measured to assess the stability and repeatability of the instrumental setup as well as facilitate the development of the data evaluation method. The reference standards covered a polarity range of -5.6 ≤ log D (pH 7) ≤ 4.9. All samples were measured in triplicates.

The liquid chromatographic separation was performed on a system that connects two orthogonal separation mechanisms in series in order to retain very polar, polar and nonpolar compounds (classification according to Bieber et al., 2017): Hydrophilic liquid interaction chromatography (HILIC) and reversed-phase liquid chromatography (RPLC). A schematic presentation of the instrumental setup is shown in Figure 1.

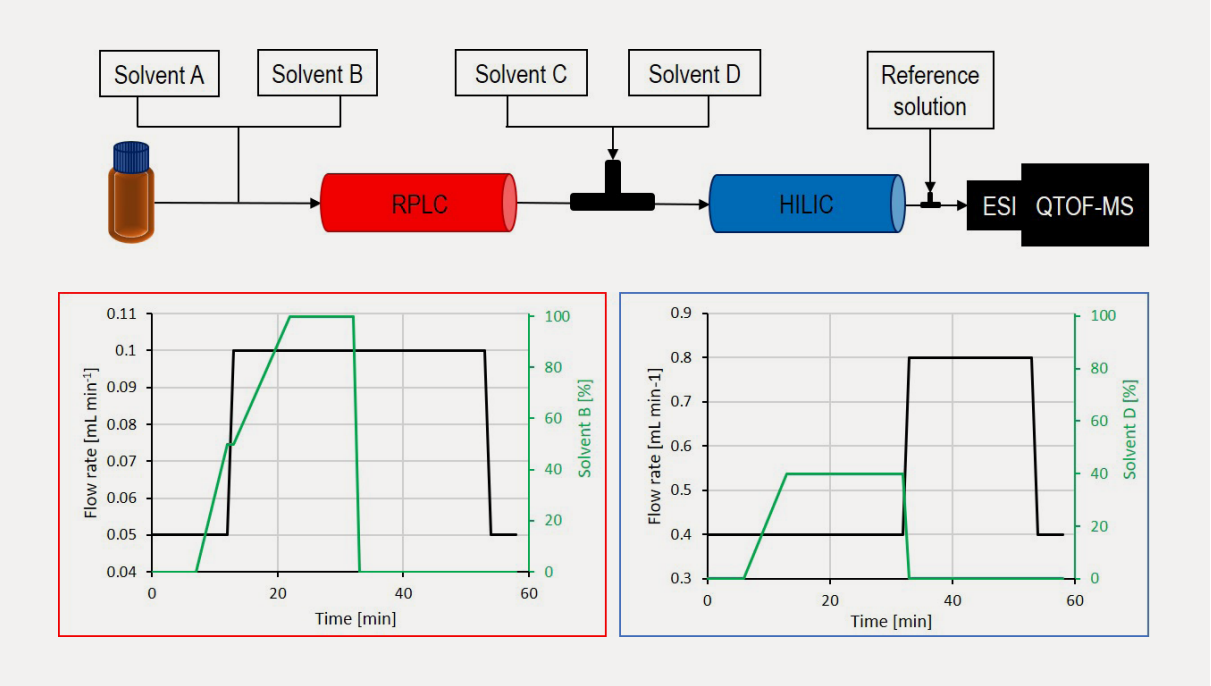

Figure 1: Schematic presentation of the instrumental setup with flow rates and gradients of RPLC (bottom left) and HILIC (bottom right).

As initially described by Greco and co-workers (Greco et al., 2013), the set-up comprised two binary pumps, two online degassers, two mixing chambers, an isocratic pump, a column oven, an autosampler and a diode array detector (Agilent Technologies, Waldbronn, Germany). The HILIC separation was performed on a ZIC-HILIC column (150 × 2.1 mm, 5 µm, 200 Å; Merck Sequant, Umea, Sweden) and the reversed-phase separation on a Poroshell 120 EC-C18 (50.0 × 3.0, 2.7 µm; Agilent Technologies). The two columns were coupled in series through a T-piece including a mixing frit (Upchurch, IDEX Europe GmbH, Erlangen, Germany). Via the third port of the T-piece the organic solvent content was increased in order to counteract the opposing eluent strengths on the two columns. For the HILIC mobile phase acetonitrile (solvent C) and water (solvent D) were used. The RPLC mobile phase consisted of 10 mM ammonium acetate in water/ acetonitrile 90/10, v/v; solvent A) and 10 mM ammonium acetate in water/ acetonitrile 10/90, v/v; solvent B). The gradients are graphically presented in Figure 1. The total run time was 58 min including an equilibration time of 20 min. The injection volume was 10 µL. A reference solution including 125 nM purine and 6.25 nM HP-921 MS tuning mix (Agilent Technologies, Waldbronn) was continuously pumped at 0.05 mL min^{-1} into the main flow before entering the ionization source. The chromatographic system was controlled by a MassHunter Workstation LC/MS Data Acquisition software (version B.05.01; Agilent Technologies).

After separating the organic compounds of the JDS4-7 sample on the RPLC-HILIC coupling, their masses were analysed on a TripleTOF® 4600 mass spectrometer equipped with a DuoSpray® ion source (AB Sciex, Darmstadt, Germany). A full scan experiment was conducted covering a mass range from 65 to 1000 Da at a scan time of 1.10 s and an accumulation time of 0.25 s. In eight parallel data-dependent MS/MS experiments, fragmentation information was gathered at a collision energy of 40 ± 20 eV and an accumulation time of 0.10 s. The electrospray ionization probe was operated solely in positive mode and the spray parameters were optimized in a preceding study to the following values: Curtain gas 29 psi, nebulizer gas 44 psi, heater gas 50 psi, ion spray voltage floating 2000 V, temperature 650 °C and declustering potential 46 V. HRMS raw data was acquired using Analyst TF software (version 1.7.1; AB Sciex).

The data processing workflow from non-targeted raw data to a list of polar as well as nonpolar candidates present in the JDS4-7 sample, is depicted in Figure 2. First off, chromatographic peaks were picked from the HRMS full scan data in a non-targeted manner. They were extracted separately from the retention interval of the HILIC separation (5 to 17 min) and the RPLC separation (16 to 34 min). The intention of overlapping extraction windows was to avoid peak losses due to slight variations in retention time or peak width within the replicates. The five peak picking parameters were set with respect to optimizing the recovery of 51 standard compounds in a mixture, as a subset of the 177 compounds which were analysed in a separate run. As a result, the subtraction offset was set to 15 scans, the subtraction multiplication factor to 1.0, the noise threshold to 50 and the minimum spectral peak width to 2 ppm for both. The minimum RT peak width was set to 10 scans for HILIC features and 5 scans for RPLC features. Subsequently, the features were aligned across the technical replicates, considering only those features, which were detected in all replicates. Features present in the blank sample were excluded during peak recognition in the real sample data set. Peak picking and alignment were done by MarkerView software (version 1.3.1, AB Sciex).

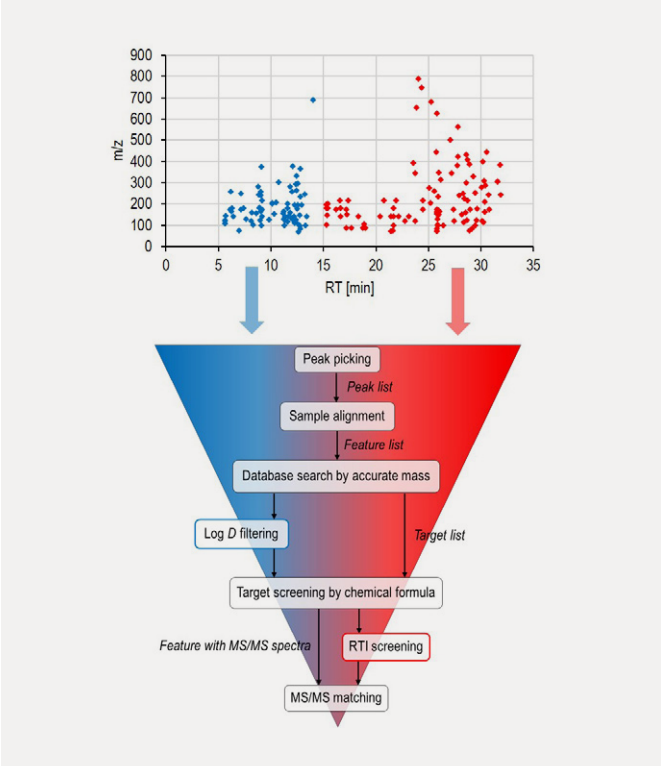

Figure 2: Data processing workflow from nontargeted raw data to a list of polar as well as nonpolar candidates present in the JDS4-7 sample.

The remaining features were uploaded to the FOR-IDENT platform (Letzel & Sengl, 2016; https://water.for-ident.org/#!home; FI) and compared to the compound database STOFF-IDENT (Letzel et al., 2015; https://www.lfu.bayern.de/stoffident/#!home; SI). The search parameters in molecular screening mode included a pH value of 7, a maximum mass deviation of 5 ppm and an intensity threshold for MS/MS data of 3 counts per second (cps). HILIC features were matched with STOFF-IDENT entries by accurate mass and a maximum accepted mass deviation of 5 ppm and then filtered for those with a negative log D value.

For RPLC features the retention time index (RTI) was also considered as described in the FI manual (Grosse & Letzel, 2017). The RTI of an RPLC feature allows estimating a log D value which is then matched with database entries of potential candidate compounds. For this purpose, the log D values of nine standard compounds (as part of the 177 reference standards) were related to their normalized RTs by linear calibration.

Via the first database inquiry chemical formulae were derived from the initial feature list. Those were implemented into a target screening meant for post-hoc extraction of features' ion chromatograms (EICs) and integration of the underlying peaks in order to gain information on isotopes as well as MS/MS spectra. Targeted analysis and integration were achieved by SCIEX OS (version 1.4.0.18067; AB Sciex). Chromatographic peaks were integrated using the MQ4 algorithm. The EICs were evaluated manually and a feature of the JDS4-7 sample was accepted if it exhibited:

a) a mass error ≤ 5 ppm and
b) a plausible isotopic pattern and
c) an area larger than the one of the blank sample by a factor of at least 10 and
d) an approximate gaussian shape.

If for at least one out of three peaks MS/MS information was available, the spectrum was uploaded once again to the FI platform. The fragment peaks were matched with those predicted by MetFrag, a tool for in silico fragmentation (Ruttkies et al., 2016; Wolf et al., 2010).

33.3 Results

In the following each step of the data processing workflow, as depicted in Figure 2, is discussed. Each individual step can be interpreted as a filter and the feature reduction for the HILIC and the RPLC pass is presented in Figure 3.

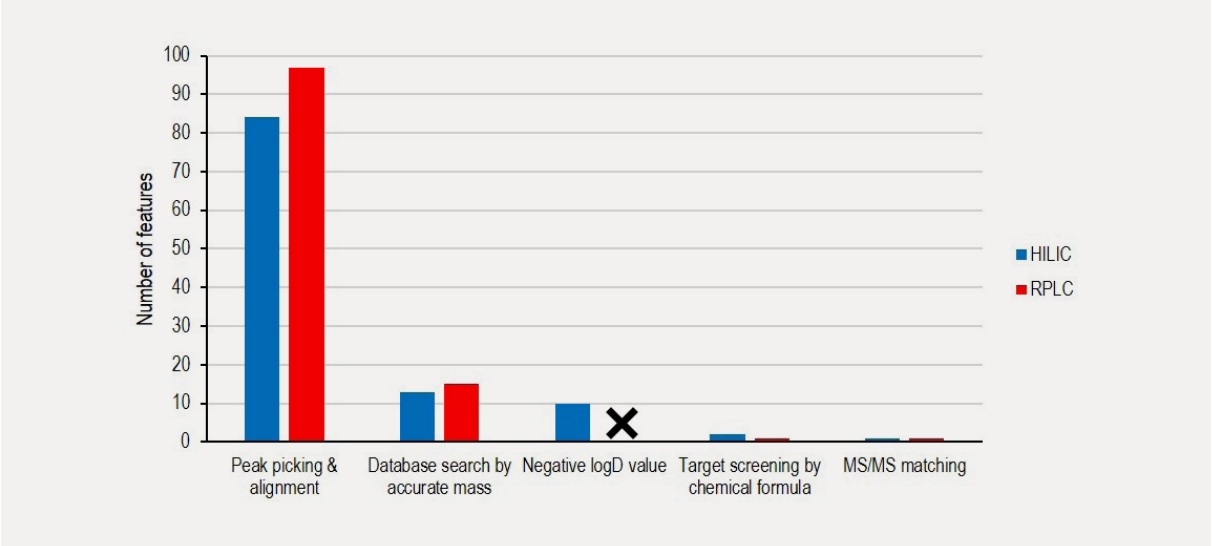

Figure 3: Feature reduction for the HILIC and the RPLC pass.

The stability and repeatability of the instrumental setup was evaluated on the stated 177 target analytes. The maximum mass precision calculated for compounds retained by the HILIC column was 3 % and for RPLC compounds 5 %. The RTs shifted by ≤ 6 % for HILIC and ≤ 3 % for RPLC retained compounds, respectively. The tolerances for aligning chromatographic peaks across all three replicates were set accordingly.

More detailed information on stability data of very polar, polar and nonpolar targets is summarized in Table 1.

Table 1: Stability data of the 177 standard compounds. Each compound was measured three times. The targets were categorized in very polar, polar and nonpolar compounds. For each group information is given on RT stability, mass precision and mass accuracy.

	Number of reference standards	Maximum RT shift [%]	Maximum deviation from average mass [%]	Maximum deviation from target mass [ppm]
Very polar (log D < -2.5)	29	6.2	2.4	3.8
Polar (-2.5 < log D < 2.0)	117	3.0	5.0	5.0
Nonpolar (log D > 2.0)	31	2.3	1.6	4.2

Peak picking

The individual chromatographic peaks were previously picked from raw HRMS full scan data. The settings of the peak picking parameter were optimized on the target compounds. The requirement was a recovery of at least 75 % of the contained known compounds in the peak picking. These settings were then used for all investigated samples.

Alignment

The features of all replicates were aligned, within a mass tolerance of 5 ppm as well as an RT tolerance of 6 % and 4 % for the HILIC and for the RPLC retention interval, respectively. Features found in the blank sample were excluded. As a result, a total of 84 features was extracted from the HILIC retention interval (5 - 17 min) and 97 features from the RPLC retention interval (16 – 34 min).

Database search by accurate mass

The retrieved features were then uploaded to FOR-IDENT and the integrated anthropogenic compound database SI was then queried for the exact masses of the pseudo-molecular ions. As before, the m/z tolerance was set to 5 ppm. Each one of 13 HILIC features was successfully allocated to at least one or to a maximum of four potential candidate compounds in SI by accurate mass. The list of all candidates proposed by the database was filtered by log D. Only compounds with a negative log D (pH 7) were further considered, suggesting they are polar or very polar as expected from HILIC retained analytes, as demonstrated elsewhere (Bieber et al., 2017; Minkus et al., 2020). For the feature 10.2 min/201.1728 Da, which had retrieved three candidate compounds by mass search in SI, two of the three candidates were eliminated by this filtering step.

Database search by accurate mass and RTI

For RPLC features, the RTI was used in addition to prioritise candidate molecules suggested by the platform. Therefore, the RTs of nine standard compounds were linearly correlated with their log D values as presented in Figure 4.

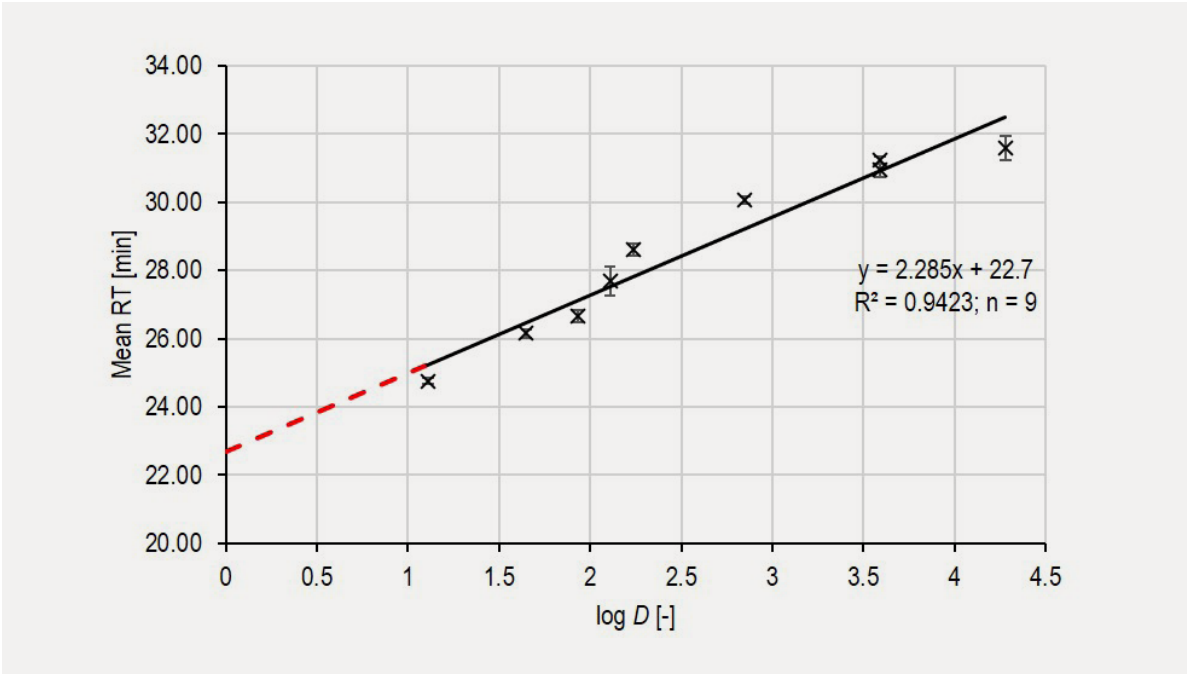

Figure 4: Database search filter RTI (shown in a normalization plot with the log D values of reference compounds).

By extrapolating the regression line to a log D of 0 prior to the inquiry, an RT window from 22.7 to 31.6 min could be covered. For charged molecules the log D values were corrected as explained in the FI manual (Grosse & Letzel, 2017). A score from 0 to 1 was calculated for each database hit, expressing the difference Δ log D between the log D stored in the database and the value determined by means of RTI normalization. The considered pH level for the log D values can be chosen at the beginning of an inquiry and in this case was set to 7. 15 RPLC features matched with at least one and at most six tentative compounds stored in the database. All database hits for each feature were ranked according to the RTI screening score, which expresses the difference of the estimated log D value (calculated from RTI) and the log D value of the candidate compound. For the feature 31.6 min/301.1768 Da the inquiry yielded two structural isomers at a mass deviation of 3.9 ppm: Dapoxetine (CAS 119356-77-3) and 5-(1-methylpiperidin-4-yl)-5H-dibenzo[a,d][7]annulen-5-ol (CAS 3967-32-6). An unambiguous allocation by accurate mass only was not possible at this point. However, the Δ log D was lower for dapoxetine by 0.62 points. During a collaborative trial on the untargeted analysis of house dust samples, an acceptance interval of Δ log D ± 0.7 was defined (Rostkowski et al., 2019). With a Δ log D of 1.19, 5-(1-methylpiperidin-4-yl)-5H-dibenzo[a,d][7]annulen-5-ol fell outside the specified range and was therefore ranked at second position.

Target screening by chemical formula

Afterwards, the HRMS raw data were retrospectively screened, using two targeted methods. These were built by using the chemical formulae proposed for the HILIC as well as for the RPLC features and their retention times. The chromatographic peaks and isotopic patterns were evaluated manually based on the criteria a) – d) listed in the Methods section. Thereby, eight features were eliminated from the HILIC list. After applying the target screening filtering step on the RPLC feature list, only the feature (31.6 min/301.1768 Da) was left.

MS/MS matching

Since information was available for the two remaining features fragmentation, they were uploaded once again to the FI platform. However, solely 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-ethanol, that was proposed for the accurate mass of the HILIC feature (10.2 min/201.1728 Da), could be validated by MS/MS matching with MetFrag. A search for the candidate in the FI-included mass spectrometric database Mass Bank gave no hit. The remaining feature from the RPLC separation resulted in two FI hits. Comparing the observed fragments with the predicted spectra, 5-(1-methylpiperidin-4-yl)-5H-dibenzo[a,d][7]annulen-5-ol (Figure 5a) was prioritized over dapoxetine (Figure 5b). However, this result is inconsistent with the one suggested by the RTI screening which favours dapoxetine and has to be studied further.

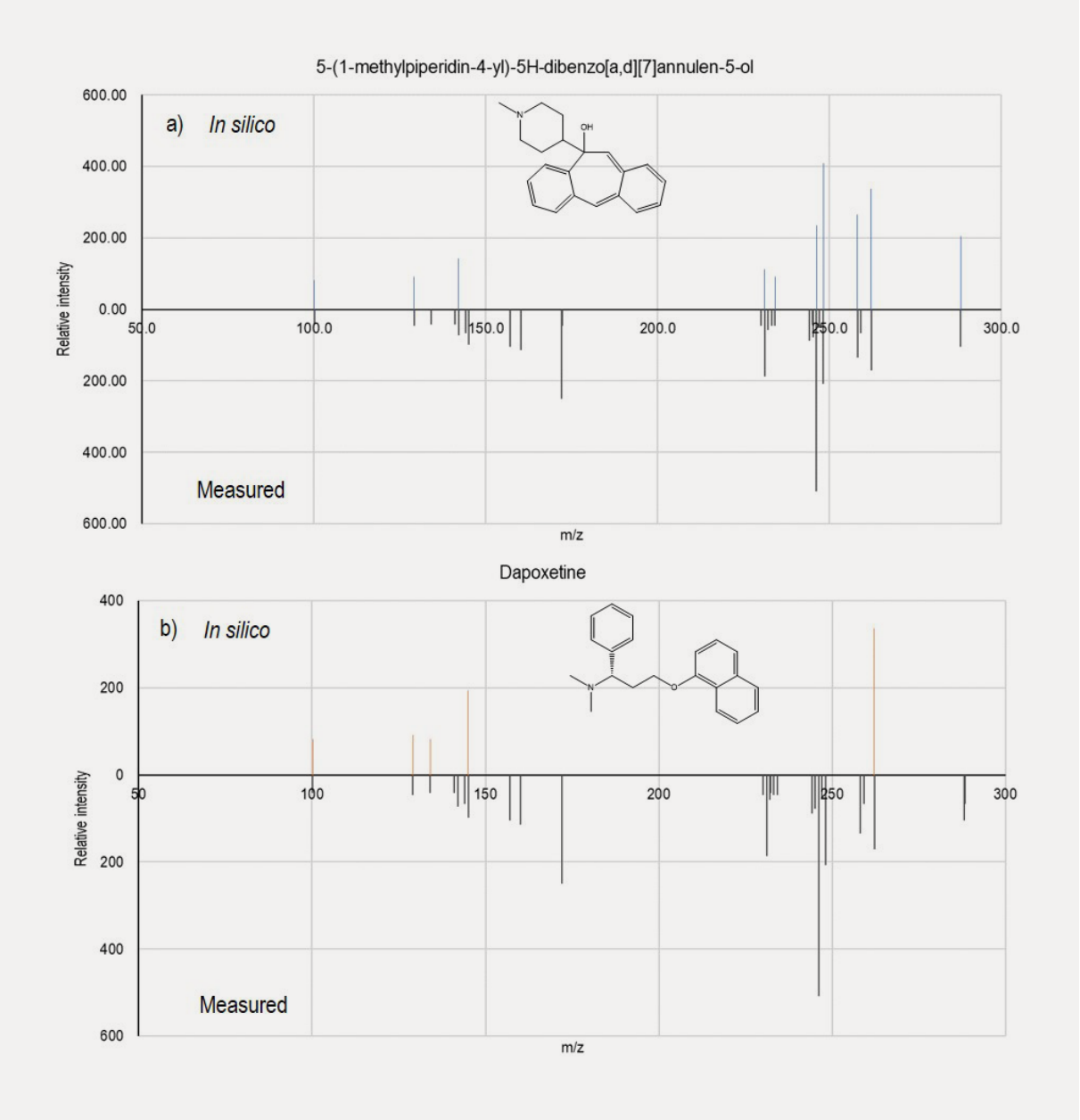

Figure 5: MS/MS matching.

These findings emphasize the need for an unequivocal confirmation via comparison with a reference standard. Neither candidate derived from the HILIC side and the RPLC side of the workflow, thus far have been validated. Other hits and proposals for identification using non-target screening can be found in corresponding chapters of this publication.

33.4 Conclusions

The strategy and workflow on one example of the entire JDS4 sample set using polarity-extended chromatography in non-target screening is presented. A few very polar substances are proposed after application of the workflow, but validation using reference standards still has to be performed.

A publication is currently in progress including data and results from the RPLC-HILIC-ESI-QToF monitoring of the complete set of samples and using the HILIC data processing workflow as described above. Thus, here only a detailed view about the method and strategy could be given rather than the identification of novel molecules.

33.5 References

BERGER, U., OST, N., SÄTTLER, SCHLIEBNER, I., KÜHNE, R., SCHÜÜRMANN, G., NEUMANN, N., & REEMTSMA, T. (2018). Assessment of persistence, mobility and toxicity (PMT) of 167 REACH registered substances. Report No. (UBA-FB) 002595. Umweltbundesamt, 09(2).

BIEBER, S., GRECO, G., GROSSE, S., & LETZEL, T. (2017). RPLC-HILIC and SFC with Mass Spectrometry: Polarity-Extended Organic Molecule Screening in Environmental (Water) Samples. Analytical Chemistry, 89(15), 7907–7914. https://doi.org/10.1021/acs.analchem.7b00859

BIEBER, S., & LETZEL, T. (2020). Polarity-Extended Chromatography: A holistic solution for the analysis of organic molecules in the aqueous environment. AFIN-TS Forum. https://afin-ts.de/wp-content/uploads/2020/04/AFIN-TS_01_2020_Pol_Ex.pdf

CHALCRAFT, K. R., & MCCARRY, B. E. (2013). Tandem LC columns for the simultaneous retention of polar and nonpolar molecules in comprehensive metabolomics analysis. Journal of Separation Science, 36(21–22), 3478–3485. https://doi.org/10.1002/jssc.201300779

GRECO, G., GROSSE, S., & LETZEL, T. (2013). Serial coupling of reversed-phase and zwitterionic hydrophilic interaction LC/MS for the analysis of polar and nonpolar phenols in wine. Journal of Separation Science, 36(8), 1379–1388. https://doi.org/10.1002/jssc.201200920

GROSSE, S., & LETZEL, T. (2017). User Manual for FOR-IDENT Database. https://water.for-ident.org/download/manual.pdf

LETZEL, T., BAYER, A., SCHULZ, W., HEERMANN, A., LUCKE, T., GRECO, G., GROSSE, S., SCHÜSSLER, W., SENGL, M., & LETZEL, M. (2015). LC – MS screening techniques for wastewater analysis and analytical data handling strategies : Sartans and their transformation products as an example. Chemosphere, 137, 198–206. https://doi.org/10.1016/j.chemosphere.2015.06.083

LETZEL, T., LUCKE, T., SCHULZ, W., SENGL, M., & LETZEL, M. (2014). In a class of its own – OMI (Organic Molecule Identification) in water using LC-MS(/MS): Steps from "unknown" to "identified": a contribution to the discussion. Lab & More International, 4, 24–28.

LETZEL, T., & SENGL, M. (2016). FOR-IDENT - Platform International hunt for unknown molecules combining international workflows and software tools. In NORMAN Bulletin (Issue 5, pp. 20–21).

LIŠKA, I., WAGNER, F., SENGL, M., DEUTSCH, K., & SLOBODNÍK, J. (2015). Joint Danube Survey 3: A Comprehensive Analysis of Danube Water Quality. http://www.danubesurvey.org/results

MINKUS, S., GROSSE, S., BIEBER, S., VELOUTSOU, S., & LETZEL, T. (2020). Optimized hidden target screening for very polar molecules in surface waters including a compound database inquiry. Analytical and Bioanalytical Chemistry, 412(20), 4953–4966. https://doi.org/10.1007/s00216-020-02743-0

REEMTSMA, T., BERGER, U., ARP, H. P. H., GALLARD, H., KNEPPER, T. P., NEUMANN, M., QUINTANA, J. B., & VOOGT, P. DE. (2016). Mind the Gap: Persistent and Mobile Organic Compounds - Water Contaminants That Slip Through. Environmental Science and Technology, 50(19), 10308–10315. https://doi.org/10.1021/acs.est.6b03338

ROSTKOWSKI, P., HAGLUND, P., AALIZADEH, R., ALYGIZAKIS, N., THOMAIDIS, N., ARANDES, J. B., NIZZETTO, P. B., BOOIJ, P., BUDZINSKI, H., BRUNSWICK, P., COVACI, A., GALLAMPOIS, C., GROSSE, S., HINDLE, R., IPOLYI, I., JOBST, K., KASERZON, S. L., LEONARDS, P., LESTREMAU, F., … YANG, C. (2019). The strength in numbers: comprehensive characterization of house dust using complementary mass spectrometric techniques. Analytical and Bioanalytical Chemistry. https://doi.org/10.1007/s00216-019-01615-6

RUTTKIES, C., SCHYMANSKI, E. L., WOLF, S., HOLLENDER, J., & NEUMANN, S. (2016). MetFrag relaunched: Incorporating strategies beyond in silico fragmentation. Journal of Cheminformatics, 8(1), 1–16. https://doi.org/10.1186/s13321-016-0115-9

SCHULZ, W. (2019). Use of non-target screening by means of LC-ESI-HRMS in water analysis (1.0). https://www.wasserchemische-gesellschaft.de/images/HAIII/NTS-Guidline_EN_s.pdf

SCHYMANSKI, E. L., JEON, J., GULDE, R., FENNER, K., RUFF, M., SINGER, H. P., & HOLLENDER, J. (2014). Identifying small molecules via high resolution mass spectrometry: Communicating confidence. Environmental Science and Technology, 48(4), 2097–2098. https://doi.org/10.1021/es5002105

TANG, Y., XU, Y., LI, F., JMAIFF, L., HRUDEY, S. E., & LI, X. (2016). Nontargeted identification of peptides and disinfection byproducts in water. Journal of Environmental Sciences, 42, 259–266.

WOLF, S., SCHMIDT, S., MÜLLER-HANNEMANN, M., & NEUMANN, S. (2010). In silico fragmentation for computer assisted identification of metabolite mass spectra. BMC Bioinformatics, 11. https://doi.org/10.1186/1471-2105-11-148

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Comparability of data obtained by suspect and non-target screening and by NORMAN panel of *in vitro* and *in vivo* bioassays: results of an interlaboratory study

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Abstract

This chapter reports on the results of the NORMAN/ICPDR interlaboratory trial for non-target screening and effect-based methods (EBM). A training for scientists from the laboratories in the ICPDR countries was organised to harmonise and enhance the knowledge on non-target screening principles and techniques. The goal was to provide the training before starting the non-target screening analysis of JDS4 samples with modern high-resolution mass spectrometers in participating laboratories. Capacity building in the field of non-target screening is an important pre-requisite for the implementation of a future holistic chemical and effect-based monitoring in the Danube River Basin. Analytical and data assessment methods used by the participants were compared using a spiked water sample. In order to compare samples with expected different contamination levels, on-site large volume solid phase extraction (LVSPE) was applied at three JDS4 sampling sites (Jochenstein, Budapest downstream M0

bridge and downstream Ruse/Giurgiu). The MAXX LVSPE device and the HORIZON LVSPE field sampler were used. The samples were subjected to non-target and suspect screening and bioanalytical assessment. The goal was the intercomparison of different sampling, analytical and data exploration techniques in order to show the present state and inter-comparability of the tools available in the participating laboratories.

34.1 Introduction

The objective of JDS4 was to produce comparable and reliable information on selected water quality elements for the whole length of the Danube River including the major tributaries on a short-term basis and to provide an opportunity for harmonisation and training in WFD-related monitoring. Non-target screening (NTS) is a powerful tool to detect thousands of chemical pollutants with state-of-the-art high-resolution mass spectrometry techniques, which are currently on the market, however, available only in a few laboratories in the Danube River Basin. The goal of the NORMAN / ICPDR interlaboratory study (ILS) on NTS was to train participants from these laboratories on the use of NTS and different analytical tools to be tested and compared. The ILS should demonstrate the practical use of the new analytical techniques in the current regulatory framework to overcome shortcomings of the chemical monitoring in the WFD. The ILS was an opportunity for the participants to use their own laboratory instrumentation, procedures and software in combination with additional open source and open access software tools and web services to perform NTS. The samples were taken with the two large volume solid phase extraction techniques (MAXX LVSPE, HORIZON field sampler) and subjected to liquid-chromatography high-resolution mass spectrometry (LC-HRMS) analysis. The samples' extracts were distributed also to different bioanalytical laboratories to compare the signals obtained by a panel of SOLUTIONS / NORMAN bioassays.

34.2 Methods

34.2.1 Samples and sample preparation

Three sampling sites were selected for the ILS from Upper, Middle and Lower Danube (JDS4-6: Jochenstein, JDS4-24: Budapest, downstream M0 bridge, JDS4-47: downstream Ruse/Giurgiu). The samples were taken with two different devices (MAXX LVSPE, UFZ and HORIZON LVSPE field sampler, EI). In the ICPDR coding system, the abbreviations of the samples are LMX for MAXX LVSPE and LHR for the HORIZON LVSPE. The MAXX LVSPE is a well-established sampling device for the chemical and effect-based analysis of water samples (Schulze et al., 2017; Välitalo et al., 2017; Tousova et al., 2017). The HORIZON LVSPE field sampler is a new development (Oswald 2020, Chapter 38). For each laboratory and analysis an equivalent of 1 L real water sample was provided for chemical analysis, in addition to two laboratory machine blank samples (extracted LC-MS water without matrix), one solvent blank (methanol without matrix) as well as a MAXX LVSPE extract of a natural stream (with matrix very low contamination). The raw extract of the natural stream used as a test matrix was spiked with a set of liquid chromatography accessible analytes (Table 1). The composition of the spiked analytes, i.e. their number and identity, was unknown to the participants of the trial and included 81 compounds suitable for electrospray ionisation in positive mode (ESI positive).

Table 1: Compounds spiked in a natural matrix sample suitable for analysis in electrospray positive mode.

Compound	InChIKey	NORMAN SusDat ID
1,2-Benzisothiazolinone	DMSMPAJRVJJAGA-UHFFFAOYSA-N	NS00000271
1H-Benzotriazole	QRUDEWIWKLJBPS-UHFFFAOYSA-N	NS00010261
2(4-morpholinyl)benzothiazole	VVUVJGRVEYHIHC-UHFFFAOYSA-N	NS00014431
2-(Methylthio)benzothiazole	UTBVIMLZIRIFFR-UHFFFAOYSA-N	NS00007832
2,6-Di-tert-butyl-1,4-benzoquinone	RDQSIADLBQFVMY-UHFFFAOYSA-N	NS00010801
2-Acetonaphthone	XSAYZAUNJMRRIR-UHFFFAOYSA-N	NS00004934
2-Hydroxyatrazine	NFMIMWNQWAWNDW-UHFFFAOYSA-N	NS00000255
2-Isopropyl-6-methyl-pyrimidin-4-ol	AJPIUNPJBFBUKK-UHFFFAOYSA-N	NS00000359
2-Methylbenzothiazole	DXYYSGDWQCSKKO-UHFFFAOYSA-N	NS00010908
2-Morpholinothiobenzothiazole	MHKLKWCYGIBEQF-UHFFFAOYSA-N	NS00004147
2-Octyl-4-isothiazolin-3-one	JPMIIZHYYWMHDT-UHFFFAOYSA-N	NS00000270
3,4-Dichlorophenylurea	CYESCLHCWJKRKM-UHFFFAOYSA-N	NS00000263
3-Iodopropynyl butylcarbamate	WYVVKGNFXHOCQV-UHFFFAOYSA-N	NS00000275
4-Methylbenzylidene camphor	HEOCBCNFKCOKBX-UHFFFAOYSA-N	NS00093626
5-Carboline	RDMFHRSPDKWERA-UHFFFAOYSA-N	NS00067038
5-Methyl-1H-benzotriazole	LRUDIIUSNGCQKF-UHFFFAOYSA-N	NS00008943
Allethrin	ZCVAOQKBXKSDMS-UHFFFAOYSA-N	NS00008969
Ambrettolide	NVIPUOMWGQAOIT-RQOWECAXSA-N	NS00012531
Amidosulfuron	CTTHWASMBLQOFR-UHFFFAOYSA-N	NS00000555
Atrazine	MXWJVTOOROXGIU-UHFFFAOYSA-N	NS00000262
Benzothiazole	IOJUPLGTWVMSFF-UHFFFAOYSA-N	NS00000291
Bis(4-chlorophenyl)sulfone	GPAPPPVRLPGFEQ-UHFFFAOYSA-N	NS00003990
Carbendazim	TWFZGCMQGLPBSX-UHFFFAOYSA-N	NS00010265
Carboline	BPMFPOGUJAAYHL-UHFFFAOYSA-N	NS00027675
Cashmeran	MIZGSAALSYARKU-UHFFFAOYSA-N	NS00005325
Celestolide	IKTHMQYJOWTSJO-UHFFFAOYSA-N	NS00001394
Chloridazon	WYKYKTKDBLFHCY-UHFFFAOYSA-N	NS00008895
Chlorotoluron	JXCGFZXSOMJFOA-UHFFFAOYSA-N	NS00000251
Clomazone	KIEDNEWSYUYDSN-UHFFFAOYSA-N	NS00010041
Daidzein	ZQSIJRDFPHDXIC-UHFFFAOYSA-N	NS00010903
DCOIT	PORQOHRXAJJKGK-UHFFFAOYSA-N	NS00000292
DEET	MMOXZBCLCQITDF-UHFFFAOYSA-N	NS00000221
Denatonium	ZFQMTVNLDNXRNQ-UHFFFAOYSA-O	NS00006955
Desethylatrazine	DFWFIQKMSFGDCQ-UHFFFAOYSA-N	NS00008490
Desethylterbutylazine	LMKQNTMFZLAJDV-UHFFFAOYSA-N	NS00007860
Desisopropylatrazine	IVENSCMCQBJAKW-UHFFFAOYSA-N	NS00000261
Diazinon	FHIVAFMUCKRCQO-UHFFFAOYSA-N	NS00008266
Diflufenican	WYEHFWKAOXOVJD-UHFFFAOYSA-N	NS00008837
Dimethachlor	SCCDDNKJYDZXMM-UHFFFAOYSA-N	NS00007718
Dimethenamid	JLYFCTQDENRSOL-UHFFFAOYSA-N	NS00000237

Compound	InChIKey	NORMAN SusDat ID
Diuron	XMTQQYYKAHVGBJ-UHFFFAOYSA-N	NS00000265
Ethofumesate	IRCMYGHHKLLGHV-UHFFFAOYSA-N	NS00010438
Etofenprox	YREQHYQNNWYQCJ-UHFFFAOYSA-N	NS00002113
Flufenacet	IANUJLZYFUDJIH-UHFFFAOYSA-N	NS00000324
Flurtamone	NYRMIJKDBAQCHC-UHFFFAOYSA-N	NS00009620
Genistein	TZBJGXHYKVUXJN-UHFFFAOYSA-N	NS00009870
Harman	PSFDQSOCUJVVGF-UHFFFAOYSA-N	NS00014584
Harmine	BXNJHAXVSOCGBA-UHFFFAOYSA-N	NS00015429
Icaridin	QLHULAHOXSSASE-UHFFFAOYSA-N	NS00008107
Imazalil	PZBPKYOVPCNPJY-UHFFFAOYSA-N	NS00009564
Irgarol	HDHLIWCXDDZUFH-UHFFFAOYSA-N	NS00000272
Lenacil	ZTMKADLOSYKWCA-UHFFFAOYSA-N	NS00000444
Metamitron	VHCNQEUWZYOAEV-UHFFFAOYSA-N	NS00010410
Metazachlor	STEPQTYSZVCJPV-UHFFFAOYSA-N	NS00000249
Methylchloroisothiazolinone	DHNRXBZYEKSXIM-UHFFFAOYSA-N	NS00000256
Metolachlor	WVQBLGZPHOPPFO-UHFFFAOYSA-N	NS00000248
Metribuzin	FOXFZRUHNHCZPX-UHFFFAOYSA-N	NS00001636
Musk ketone	WXCMHFPAUCOJIG-UHFFFAOYSA-N	NS00001194
N-Cyclohexyl-2-benzothiazole-amine	UPWPIFMHSFSVLE-UHFFFAOYSA-N	NS00014432
N-Cyclohexyl-2-benzothiazole-sulfenamide	DEQZTKGFXNUBJL-UHFFFAOYSA-N	NS00006793
Nicosulfuron	RTCOGUMHFFWOJV-UHFFFAOYSA-N	NS00008411
Norharmane	AIFRHYZBTHREPW-UHFFFAOYSA-N	NS00010572
Pendimethalin	CHIFOSRWCNZCFN-UHFFFAOYSA-N	NS00000529
Pethoxamid	CSWIKHNSBZVWNQ-UHFFFAOYSA-N	NS00000317
Phantolide	VDBHOHJWUDKDRW-UHFFFAOYSA-N	NS00004430
Picolinafen	CWKFPEBMTGKLKX-UHFFFAOYSA-N	NS00009710
Piperonal butoxide	FIPWRIJSWJWJAI-UHFFFAOYSA-N	NS00011484
Propachlor	MFOUDYKPLGXPGO-UHFFFAOYSA-N	NS00000323
Propoxycarbazone	JTHMVYBOQLDDIY-UHFFFAOYSA-N	NS00008432
Propyzamide	PHNUZKMIPFFYSO-UHFFFAOYSA-N	NS00000560
p-Toluenesulfonamide	LMYRWZFENFIFIT-UHFFFAOYSA-N	NS00010636
Quinmerac	ALZOLUNSQWINIR-UHFFFAOYSA-N	NS00000708
Quinoxyfen	WRPIRSINYZBGPK-UHFFFAOYSA-N	NS00010580
Terbuthylazine	FZXISNSWEXTPMF-UHFFFAOYSA-N	NS00000258
Terbuthylazine-2-hydroxy	OYTCZOJKXCTBHG-UHFFFAOYSA-N	NS00000294
Terbutryn	IROINLKCQGIITA-UHFFFAOYSA-N	NS00000276
Thiabendazole	WJCNZQLZVWNLKY-UHFFFAOYSA-N	NS00007958
Tonalide	DNRJTBAOUJJKDY-UHFFFAOYSA-N	NS00003324
Triethylcitrate	DOOTYTYQINUNNV-UHFFFAOYSA-N	NS00008140
Velvione	ABRIMXGLNHCLIP-UHFFFAOYSA-N	
Warfarin	PJVWKTKQMONHTI-UHFFFAOYSA-N	NS00010884

34.2.2 Instrumental methods for chemical analysis

The instruments and experimental methods applied by the participants for LC-HRMS are listed in Table 2. The list of instruments included two QTOF systems of Agilent, two QTOF system of Sciex, one QTOF system of Bruker and two Thermo Fisher Q Exactive (Plus) Orbitrap systems. All systems were coupled to high performance liquid chromatography systems equipped with reversed phase C18 columns for separation. All laboratories acquired MS1 spectra in full scan mode and performed MS2 experiments with data dependent and / or data independent acquisition.

Table 2: Instrumentation and acquisition settings (ESI: electro spray ionisation; HRMS; high resolution mass spectrometry; DDA: data-dependent acquisition; DIA: data-independent acquisition; IDA: information-dependent acquisition; HCD: higher collision dissociation; CID: collision induced dissociation).

Laboratory	Instrument / column	Acquisition mode	References
Croatian Waters (CW)	Agilent Technologies 6550 iFunnel Q-TOF LC/HRMS system with a column ACQUITY UPLC HSS T3 (1,8 µm, 150 × 2.1 mm)	ESI positive mode. MS1: Full Scan MS with a resolving power of 45,000 @ 922 m/z and 23,000@118 and a scan range 100-1200 m/z MS2: DIA using HCD fragmentation with a resolving power of 45,000 @ 922 m/z and 23,000@118 m/z and a collision energy of 0,20,40	Schymanski et al. (2015)
Bavarian Environment Agency (LfU)	Thermo Fisher Q Exactive HRMS coupled to Thermo Scientific Ultimate3000 UHPLC system equipped with Agilent Zorbax Eclipse Plus C18 (3.5 µm, 150 x 2.1 mm)	ESI positive mode. MS1: Full Scan MS with a resolving power of 140,000 @ m/z 200 and a scan range m/z 100-1050. MS2: DDA using HCD fragmentation with a resolving power of 17,500 @ m/z 200, a scan range m/z 100-1050 and collision energy of 20, 40, 60	
Federal Institute for Hydrology (BfG)	TripleTOF 6600 HRMS (Sciex) coupled to an ESI (IonDrive) source and to a binary HPLC instrument (1260 Infinity, Agilent) equipped with a reversed phase (C18) column (Zorbax Eclipse Plus, 2.1 mm x 150 mm, 3.5 µm, Agilent)	ESI positive mode. MS1: Full Scan MS with a scan range m/z 100-1200. MS2: IDA acquisition of the 8 most intensive MS 1 precursors. Fragmentation with a collision energy of 40 V and a collision spread set with potential 15 V. MS1 and IDA acquisition time were 150 ms and 30 ms respectively, resulting in a cycle time of 440 ms. Dynamic exclusion after three repetitions for 3 s.	Köppe et al. (2020)

Laboratory	Instrument / column	Acquisition mode	References
Helmholtz Centre for Environmental Research (UFZ)	Thermo Fisher Q Exactive Plus HRMS coupled with liquid chromatography system equipped with a column Kinetex EVO C18 column (2.6 µm, 50 × 2.1 mm)	ESI positive mode. MS1: Full Scan MS with a resolving power of 70,000 @ 200 m/z and a scan range 100-1500 m/z. MS2: DIA using HCD fragmentation with a resolving power of 35,000 @ 200 m/z, a scan range 100-1500 m/z and a normalised collision energy of 30	Beckers et al. (2020). Chapter 27
Landeswasserversorgung (LW)	Sciex X500R HRMS coupled with liquid chromatography system equipped with a column Zorbax Eclipse Plus C18 column (3.5 µm, 150 x 2.1 mm)	ESI positive mode. MS1: Full Scan MS with a resolving power of 30,000 @ 200 m/z and a scan range of 100-1200 m/z. MS2: Acquisition in triplicate (2x DDA, 1x DIA). DDA selecting 12 precursors with the highest intensity using CID. DIA (SWATH) using 16 fragmentation windows. Resolving power of 35,000 @ 200 m/z, scan range 30-1200, collision energy spread 30-50	Bader et al. (2017, 2016). Stütz et al. (2019)
University of Athens (UoA)	Bruker maXis Impact QTOF coupled with liquid chromatography system (Thermo Fisher Scientific Dionex Ultimate 3000) equipped with a column Thermo Acclaim RSLC C18 (2.2 µm, 100 × 2,1mm)	MS1/MS2 data-independent: Full Scan MS with a resolving power 30,000 @ 500 m/z and a scan range 50-1000 m/z. High collision energy at 25 eV without prior mass isolation. MS1/MS2 data dependent: Full Scan MS with a resolving power 30,000 @ 500 m/z and a scan range 50-1000 m/z. High collision energy at ramp 10-50 eV with prior isolation of five most abundant ions per scan	Gago-Ferrero (2020). Chapter 29. Chapter 31
Water Research Institute (WRI)	Agilent 6545 QTOF coupled with 1290 Infinity II LC system equipped with guard column EC C18 3x5mm, 2.7µm + ZORBAX Eclipse Plus C18 RRHT 2.1×100mm, 1.8µm LC column	ESI positive mode. MS2: DIA and DDA using CID fragmentation (N2) with CE 20 eV and 40 eV, resolving power of 9800@118 m/z and 13700@922 m/z, scan range 50-1000 m/z	-

34.2.3 Effect-based methods

The effect-based methods (EBM, Altenburger et al., 2019) applied in the NORMAN ICPDR interlaboratory study are listed in Table 3. The panel of EBM tools involved *in vitro* and *vivo* bioassays representing different receptor based and whole organism endpoints. The cell-based *in vitro* bioassays included one anti-androgen receptor assay (anti-AR), two estrogenic receptor (ERα) assays, one anti-ERα assay, one glucocorticoid receptor (GR) assay and one dioxin like activity assay (aryl hydrocarbon receptor, AhR). The algae growth inhibition assay with *Scenedesmus vacuolatus*, the daphnia immobility assay with *Daphnia magna* and the fish embryo assay with *Danio rerio* represented the apical endpoints and *in vivo* bioassays. The high-performance thin layer chromatography (HPTLC) based bioassays (Stütz et al., 2019) are specific assays for a quick screening of environmental samples. Today, they do not provide full concentration effect relationships and thus quantitative results, but a semi-quantitative estimation of the effect strength.

Table 3: In vitro and in vivo bioassays used for analysis and evaluation of toxicity of Danube samples; ERα: estrogen receptor, AhR: aryl hydrocarbon receptor, AR: androgen receptor, GR: glucocorticoid receptor; HPTLC: high performance thin layer chromatography; YES: yeast estrogen screen; AChE: acetylcholinesterase inhibition assay; BS: Bacillus subtilis inhibition assay.

Bioassay	Laboratory	Cell line / species	Positive control	References
algae growth inhibition 24 h	UFZ	Scenedesmus vacuolatus	diuron	Gawel et al. (2020)
anti-AR	RECETOX	MDA-kb2	dihydrotestosterone (DHT). flutamide (FLU)	Wilson et al. (2002)
ERα	UFZ	ERα-UAS-bla GripTite	17β-estradiol (E2)	König et al. (2017)
ERα	RECETOX	ERα-HeLa-9903	17β-estradiol (E2)	Procházková et al. (2017)
anti-ERα	RECETOX	ERα-HeLa-9903	17β-estradiol (E2). fulvestrant (ICI)	Procházková et al. (2017)
Daphnia immobilisation assay 48 h (screening at REF 25 and REF 50)	UFZ	Daphnia magna	potassium dichromate (K2Cr2O7)	OECD 202 (2004) with UFZ test adaptations
dioxin like activity AhR	RECETOX	H4G1.1c2 (CAFLUX)	2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)	Nagy et al. (2002)
Fish embryo assay 96 h (screening at REF 25 and REF 50)	UFZ	Danio rerio	3,4-dichloro aniline (3,4-DCA)	OECD 236 (2013) with UFZ test adaptations
GR	UFZ	GR-AUS-bla HEK 293T	dexamethasone (DEX)	König et al. (2017)
HPTLC-YES	LW	Saccharomyces cerevisiae BJ3505	17ß-estradiol (E2)	Stütz et al. (2019)
HPTLC-AChE	LW	Enzyme acetylcholinesterase	paraoxon-ethyl	Stütz et al. (2019)
HPTLC-BS	LW	Bacillus subtilis	azithromycin	Stütz et al. (2019)

34.2.4 Data processing of mass spectral data

One goal of this study was the intercomparison of different analytical methods, instrumentation and data processing workflows for non-target and suspect screening. The utilised data processing software and peak annotation strategies are listed in Table 4. The tools are diverse and include commercial and open source / in-house software. Three out of six laboratories applied vendors' software tools and the other half made use of in house and / or open source software tools. The submitted raw mass spectral files were assessed with the R package patRoon (Helmus et al., 2020) in order to gain information about the peak inventory after conversion to mzML format with ProteoWizard (Kessner et al., 2008).

Table 4: Software tools utilised for the processing and analysis of mass spectral data for suspect and non-target screening.

Laboratory	Mass spectral data processing	Peak annotation	References
Croatian Water (CW)	Agilent Mass Hunter NORMAN Digital Sample Freezing Platform	Agilent PCDL library search NORMAN Digital Sample Freezing Platform	Schymanski et al. (2015) Alygizakis et al., (2019)
Federal Institute for Hydrology (BfG)	In-house workflow	In-house workflow with custom library search based on MS2 spectra	Jewell et al. (2020) Köppe et al. (2020)
Helmholtz Centre for Environmental Research (UFZ)	ProteoWizard 3.0.19246 MZmine 2.51 patRoon 1.0.4	MZmine 2.51 with custom library annotation based on MS1 (m/z and retention time)	Beckers et al. (2020) Krauss et al. (2019) Kessner et al. (2008) Pluskal et al. (2010) Helmus et al. (2020)
Landesamt für Umwelt (LfU)	Thermo Fisher Compound Discoverer 3.0	mzCloud and custom library search	
Landeswasserversorgung (LW)	Sciex Marker View Sciex OS MatLab	Matlab script with custom library search based on MS1 (m/z and retention time)	Bader et al. (2016) Bader et al. (2017) Stütz et al. (2019)
University of Athens (UoA)	Bruker CompassXport 3.0.9.2. NORMAN Digital Sample Freezing Platform	NORMAN Digital Sample Freezing Platform	Alygizakis et al., (2019)
Water Research Institute (WRI)	Agilent Mass Hunter NIST MS Search 2.0	Agilent PCDL, NIST 14 msms and MassBank library search	https://massbank.eu/MassBank

The participants provided mass spectral files (raw or open mzML / mzXML format) and spreadsheets with their suspect annotations to the study convenor UFZ. In addition, the spectral data were uploaded to the NORMAN Digital Sample Freezing Platform (DSFP, https://norman-data.net).

The suspect annotation lists were manually inspected and compound annotations were standardised. The standardised suspect lists were merged and finally processed for downstream data analysis using a spreadsheet software. The data were post-processed using R 4.0.3 (R Core Team, 2020) and the R packages data.table 1.13.0 (Dowle and Srinivasan, 2020) and VennDiagram 1.6.20 (Chen, 2018) to plot the Venn diagrams. The scatter charts in Figure 4 were plotted using a Matlab script (Bader et al., 2017).

In order to compare the raw mass spectral files, all submitted data were processed with the R package patRoon (Helmus et al., 2020; https://github.com/rickhelmus/patRoon). All single batches per laboratory were optimised for peak picking and peak grouping with the R package xcms 3.12.0 (Benton et al., 2008; Smith et al., 2006) utilising functions implemented in patRoon based on the R package IPO 1.16.0 (Libiseller et al., 2015). After optimisation, the data was processed with patRoon to compute the number of peaks and aligned peak groups in the single batches in a standardised way independent from the findings of the participants.

34.3 Results and Discussion

34.3.1 Training on non-target screening

The training on NTS was performed in November 2019 in Leipzig (Germany). Participants from five laboratories of the ICPDR countries and other guests attended the three days training event. The topics of the training were NTS and the identification of unknown compounds, the demonstration of data tools for the assessment of mass spectral data and practical exercises with open software tools (e.g. MZmine). On the last day, Nikiforos Alygizakis (Environmental Institute) joined the training to demonstrate the NORMAN Digital Sampling Freezing Platform (DSFP). The overall feedback of the participants on the training event was very positive.

34.3.2 Participation in the interlaboratory study

In the interlaboratory study, eleven chemical analytical and six bioanalytical laboratories committed to receive, measure and process samples. In the group of chemical analytical laboratories, six out of eleven laboratories were located in Danube riparian countries. Two bioanalytical laboratories were located in ICDPR riparian countries. Finally, seven chemical analytical and three bioanalytical laboratories submitted completed results. In two bioanalytical laboratories, the analysis is still in progress due to lockdowns during the COVID-19 pandemic.

34.3.3 Results of non-target screening

Processing of raw mass spectral data includes peak picking (the identification of real chromatographic peaks showing a Gaussian or Laurentian shape), peak alignment (the grouping of peaks across samples by their exact mass and retention time) and noise filtering (low level cut-off and elimination of high level blank or background peaks). The cleaned and aligned data are then subjected to target, suspect or non-target (screening) analysis (Altenburger et al., 2019). Non-target analysis is an approach with no *a priori* knowledge on the identity of the peaks. Only, the exact mass, the retention time and the ionisation behaviour (positive or negative) is known. In order to prioritise peaks for identification, they could be ordered by their signal intensities, frequencies of occurrence, time series trends or other scoring (Altenburger et al., 2019; Krauss et al., 2019; Hollender et al., 2019). An example for prioritisation and further identification is shown in Chapter 27.

The results show that the total numbers of aligned peak groups and peaks per sample measured in different laboratories were variable depending on sample characteristics, chromatographic systems, mass spectrometers, experimental settings and data processing. While the instrumental and experimental settings are variable, a mutual data processing is possible using vendors' independent software packages

such as xcms (Smith et al., 2006; Tautenhahn et al., 2008; Benton et al., 2008), patron (Helmus et al., 2020), or MZmine (Pluskal et al., 2010). Xcms is a basic R command line suite for the processing of mass spectral data. MZmine is a software suite integrating build in and external algorithms for the processing of mass spectral data; patRoon is a so-called wrapper software basically build on xcms algorithms to perform non-target and suspect screening and to prepare the data for annotation in e.g. Sirius (Dührkop et al., 2019), GenForm (Meringer et al., 2011) or non-target (Loos, 2015).

In Table 5, the results of the assessment using patRoon are shown. The total numbers of peak groups assigned were between 15766 and 36940. Besides the experimental settings, and chromatographic conditions, the differences are caused by the variable resolutions and sensitivities of the mass spectrometers. HRMS enables the differentiation between isobaric compounds, *i.e.* compounds with the same nominal mass but different exact masses. The sensitivity is the capability to register low intensity peaks and separate them from the background noise. The resolution of Orbitrap mass spectrometers is by technology higher than the resolution of the QTOF mass spectrometers. The sensitivity of Orbitraps and QTOFs are similar, but newer or more expensive instruments are more sensitive due to enhanced measurement and filtering technologies. The operation mode is an additional factor. For example, the measurement of UFZ and LfU derived average number of MS1 peaks of 10665 and 15650, respectively. The HRMS system of LfU was operated with a higher resolving power of 140,000 compared to a resolving power of 70,000 applied by the UFZ. Therefore, it was possible to derive a larger number of peaks with the LfU approach. In general, the LMX extracts tended to exhibit a higher number of peaks.

Table 5: Results of non-target data processing with patRoon. The raw mass spectral were peak picked, grouped and finally filtered to remove noise peaks. The peak groups were retrieved during alignment of the peaks by their exact mass and retention times over all samples.

Laboratory	UFZ	UoA	LfU	WRI	BfG	CW
Technology	Thermo Q Exactive Plus	Bruker QTOF	Thermo Q Exactive	Agilent QTOF	Sciex QTOF	Agilent QTOF
Number of peak groups	23751	30691	36940	29846	32873	15766
Number of peaks in each sample						
JDS4-6 LMX	12716	12578	17593	7096	12435	8923
JDS4-24 LMX	14432	13767	18536	9527	15417	8097
JDS4-47 LMX	12800	5650	17645	8900	15532	7489
JDS4-6 LHR	7924	2943	17977	3177	3119	7551
JDS4-24 LHR	8875	4561	13549	10813	6785	8998
JDS4-47 LHR	7245	4907	8602	3594	3471	5531

For the inspection of the peak inventories derived with both sampling technologies, intensity-fold change scatterplots were used (Figure 1) for all sampling locations (A: JDS4-6, Jochenstein; B: JDS4-24, Budapest, downstream M0 bridge; C: JDS4-47, downstream Ruse/Giurgiu). The green dots indicate higher peak intensities in the LMX samples (fold change <0.2), red dots higher peak intensities in the LHR samples (fold change >5). The light green, white and yellow dots cover the fold change range between 0.2 ≤ fold change ≤ 5 and thus peaks overlapping between both sampling technologies. The overall overlap of peaks between

both methods is quite poor, namely between 15 % and 35 %. Except for JDS4-24 (Figure 1B), higher peak numbers were obtained in the LMX samples. A detailed view on the peak inventory of the two samples collected at JDS4-24 (Budapest, downstream M0 bridge) is depicted in Figure 2. While the LMX-sample contained a higher number and intensity of single small compounds, the LHR-sample was dominated by a higher number and intensity of homologues series. The samples were taken at the same time and location, but LMX cartridges were eluted using ethyl acetate, methanol and basic and acidic methanol while the LHR cartridges were eluted with ethyl acetate and methylene chloride. We assume that in particular some more polar compounds were not extracted from the disks of the latter one.

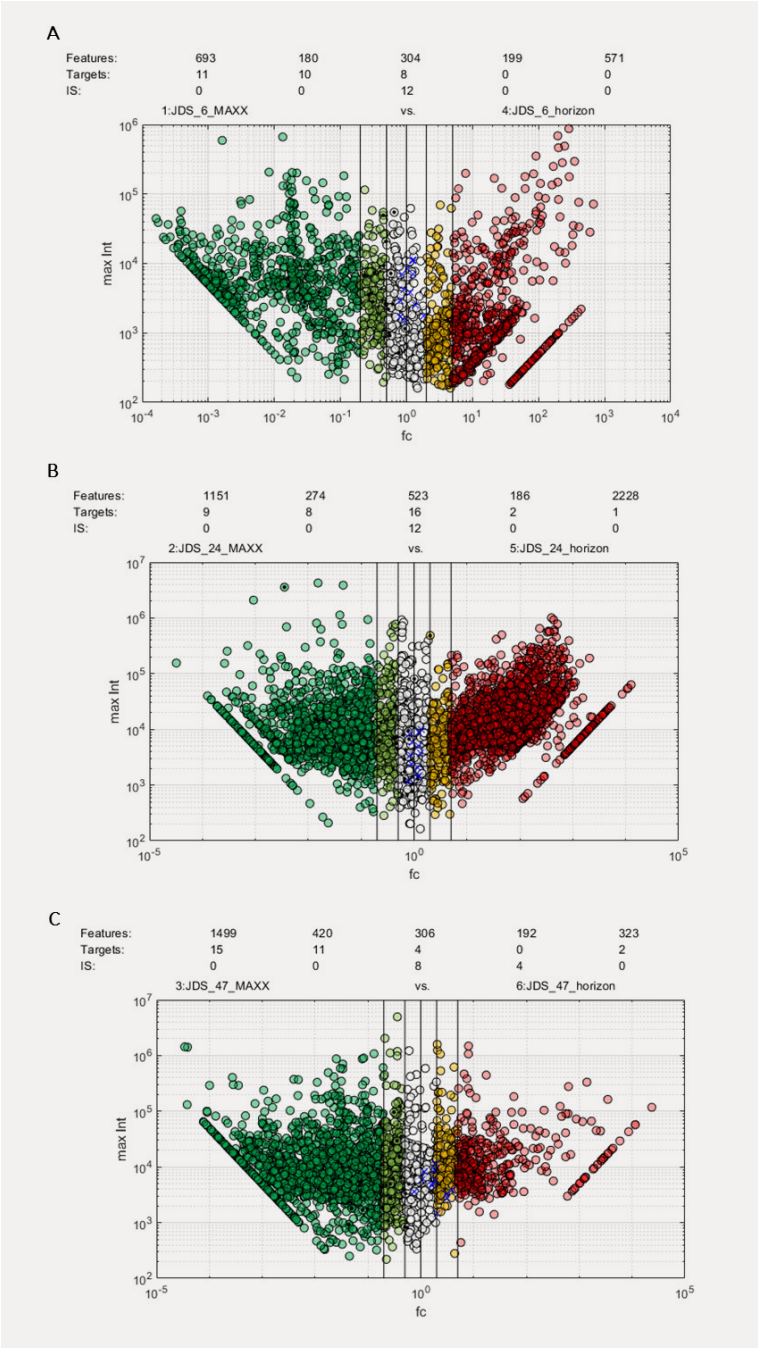

Figure 1: Intensity-fold change scatterplot to compare peak inventories of LVSPE extracts of samples derived with LMX (MAXX) LVSPE and LHR (Horizon) LVSPE at JDS4-6 (Jochenstein), JDS4-24 (Budapest, downstream M0 bridge) and JDS4-47 (downstream Ruse/Giurgiu). Data shown for the samples measured by LW.

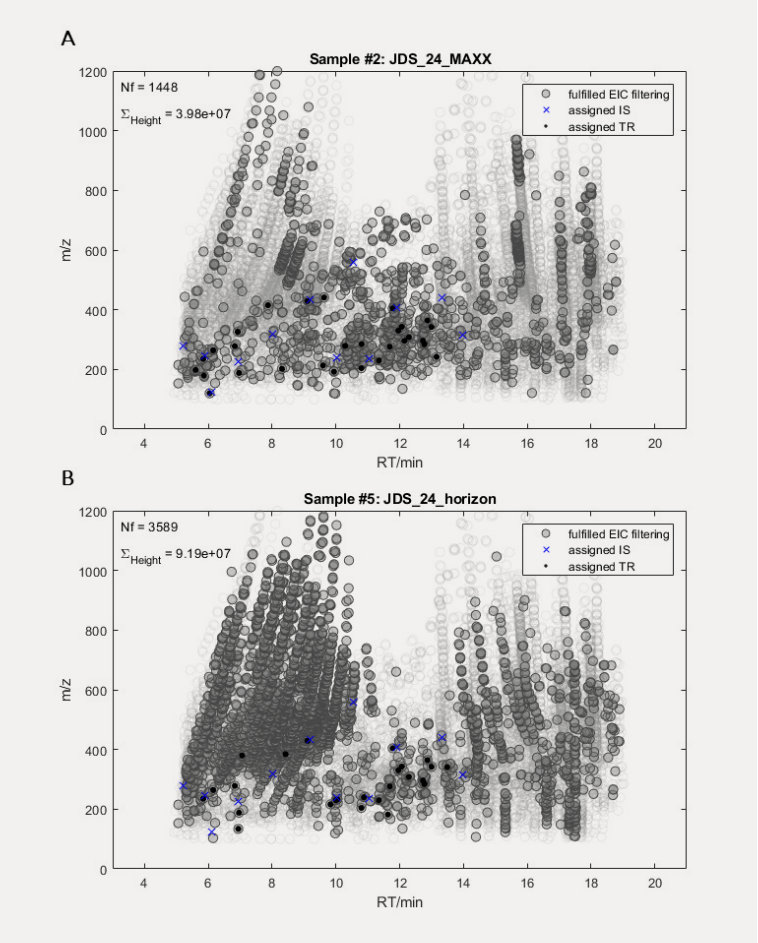

Figure 2: Mass-retention time scatterplot to compare peak inventories of LVSPE extracts of samples derived with LMX (MAXX) LVSPE and LHR (Horizon) LVSPE at JDS4-24 (Budapest, downstream M0 bridge). Data shown for the samples measured by LW.

34.3.4 Results of suspect screening

Suspect screening is the assessment of the aligned peak lists using a suspect list containing a number of compounds, the m/z of the expected ion (MS ready) and a known or predicted retention time. The software tools annotate the peaks if they are in a within predefined m/z and retention time ranges, for example ± 5 ppm m/z and ± 0.6 min, respectively. The search in a mass spectral library is also considered as suspect screening. However, the annotations are only indications and require further assessment by additional evidences (e.g. MS2 spectra) and finally confirmation by real reference standards (Altenburger et al., 2019; Schymanski et al., 2014).

The participants in the chemical analytical part of this ILS received an extract of a water sample spiked with a mixture of 81 compounds. The sample was analysed by each laboratory using their in-house instrumentation, settings and data processing workflows. Details of the data processing are listed in Table 3. Figures 3A and 3B depict the results of the suspect screening using commercial software (including combination with in-house R or Matlab scripts) and open source software and in-house scripts, respectively. In both groups, the results of UFZ were used as a reference. The data of UFZ was processed automatically in MZmine and checked for duplicate annotations or false negative findings. The sample was compared with a solvent standard as reference. Duplicates were removed and false negatives were annotated manually.

The Venn diagrams are based on contingency tables. If a compound was found, the finding was marked by one in the table. The Venn technology counts the intersected and complemented observations based on this table. Thus, the total number of compounds in the diagrams are less than the number of spiked compounds, because some compounds were not annotated by any laboratory.

In the group A, processing with vendors' software and (commercial) library search, a common set of 47 compounds (out of 81 spiked chemicals) was found by all laboratories. This substance group included for example atrazine, cybutryne, DEET, diuron and 1H-benzotriazole which are known to ionise without artefacts in ESI positive mode. Five compounds occurred only in the reference measurement of UFZ. Among those are compounds which are unstable at low pHs (e.g. 2-morpholinothiobenzothiazole, N-cyclohexyl-2-benzothiazole-sulfenamide), have high background concentrations (e.g. 2-methylbenzothiazole, cashmeran), show low peak intensities (e.g. benzothiazole) or tend to be exclusively detected as adducts and not by the M+H+ ion (e.g. bis(4-chlorophenyl)sulfone (Na+ or NH4+), piperonal butoxide (M+Na+, M+NH4+)). A reason might be that in all cases of vendors' software usage a library search was applied. The commercial libraries may lack in content, especially of adducts. The updates of commercial libraries are an effort of several years and many laboratories do not update to newer versions due to monetary reasons. Therefore, the permanently increasing numbers of mass spectra in open access libraries (e.g. MassBank, https://massbank.eu/MassBank) and in open access suspect screening lists (e.g. NORMAN Suspect Exchange, https://www.norman-network.com/?q=suspect-list-exchange) will enhance vendor and open source tools (e.g. DSFP) to derive correctly assigned annotations in future.

In the second group (Figure 3B), two compounds were only annotated by UFZ and two were only annotated by CW. However, only 7 compounds were found by all methods in group B. The results of UFZ processed with MZmine and UoA processed with DSFP were similar. The annotation applying the approach of BfG is based on a MS2 library search. The lower number of findings can be explained by missing MS2 information in the data dependent mass spectral data or in missing entries in the linked MS2 library.

In conclusion, the overall performance of both groups, commercial software and in-house / open source workflows, was satisfactory. Many of the spiked compounds were annotated and identified correctly. Most of the non-identified compounds were adducts or challenging compounds due to low ionisation efficiency, high background concentrations and stability issues at low pHs. While the low ionisation efficiency and pH issues could be mitigated by different experimental settings, background concentrations or ion suppressions are often impossible to manage. With respect to the adducts, there might be a great demand to include more adducts in the commercial and non-commercial libraries with regards to better matches in library search and to enhance the awareness of the mass spectrometry experts to consider adducts in their identification and annotation strategies. Furthermore, more efforts in increasing content of mass spectral libraries is required to improve library aided peak annotation.

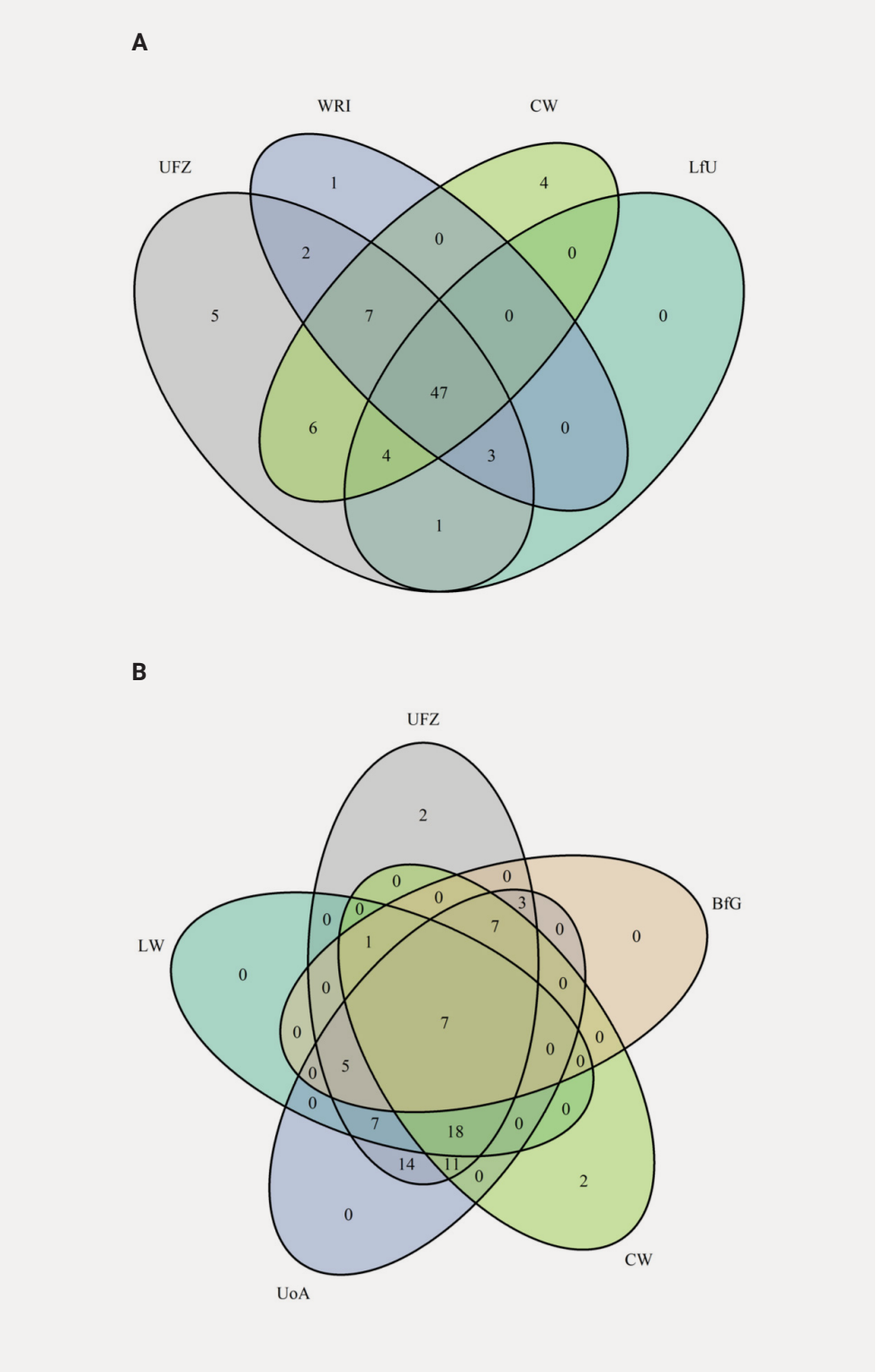

Figure 3: Venn diagrams showing the performance of correct annotations of compounds spiked in a natural matrix sample; A: reference laboratory (UFZ: MZmine processing and annotation based on MS1 (m/z and retention time) with user library (Beckers et al., 2020; Krauss et al., 2019)) and laboratories using vendor tools (CW: Agilent Mass Profiler with PCDL library search (Schymanski et al., 2015)), LfU: Thermo Scientific Compound Discoverer 3.0 and annotation with mzCloud and user libraries; WRI: Agilent MassHunter processing and user annotation with PCDL library and NIST MS Search 2.2 with NIST 14 msms and MassBank library; B: reference laboratory (UFZ) and laboratories using in-house or online tools (BfG: processing and processing with an in-house workflow and custom library annotation based on MS2 mass spectra (Köppe et al., 2020; Jewell et al., 2020), CW and UoA: processing and annotation with DSFP (Alygizakis et al., 2019);) LW: Sciex Marker View 1.2.1 mass spectral data processing and Matlab script for annotation with user library based on MS1 (m/z and retention time).

34.3.5 Results of effect-based analysis

Effect-based methods (i.e. *in vitro, in vivo* and *in situ* bioassays) are considered as a part of current and future environmental water quality monitoring (Altenburger et al., 2019). The combination of non-targeted analytical methods and EBM may provide comprehensive information for advanced water quality monitoring. EBM represent a complementary approach to chemical monitoring on targeted chemical analysis. Targeted chemical analysis of hundreds of thousands of chemicals is impossible and also suspect and non-target chemical screening analysis is still impeded by measurement and data science obstacles. In addition, information on (eco-)toxicity is missing or limited for many detected anthropogenic chemicals and natural compounds. This knowledge gap hinders estimation or modelling of ecotoxicological risks of environmental mixtures solely based on targeted chemical monitoring data. This becomes even more a challenge, if the effects of environmental mixtures in bioassays cannot be explained comprehensively by the targeted chemical analysis (Neale et al., 2015).

Therefore, EBM is important to assess the intrinsic toxicity of a sample including all chemicals which are unknown or occurring at concentrations below the detection limits of the chemical analytical instruments. Known and unknown chemicals and compounds may cause and or contribute to effects even at these low concentrations such as steroids and by joint effects of even dissimilar acting compounds (Kortenkamp et al., 2019; Brack et al., 2019; Silva et al., 2002).

Table 6: Results of the testing of the LMX and LHR samples in different in vitro and in vivo bioassays; REF: relative enrichment factor, ERα: estrogen receptor, AhR: aryl hydrocarbon receptor, AR: androgen receptor, GR: glucocorticoid receptor, EC: effect concentration, (B) EQ: (bioanalytical) equivalent; ICI: fulvestrant, DHT: dihydrotestosterone, FLU: flutamide, TCDD: 2,3,7,8-tetrachlorodibenzo-p-dioxin; HPTLC: high performance thin layer chromatography; YES: yeast estrogen screen; AChE: acetylcholinesterase inhibition assay; BS: Bacillus subtilis inhibition assay; n.a.; not analysed; values marked with < are results below the limit of quantification.

Bioassay	Number of bioassays	JDS4-6-LMX	JDS4-24-LMX	JDS4-47-LMX	JDS4-6-LHR	JDS4-24-LHR	JDS4-47-LHR
Algae growth inhibition 24 h (EC50 REF)	1	52.1	29.3	37.3	No effect	No effect	No effect
anti-AR (BEQ-EC50 in µg FLU-EQ/L)	1	[23.1 ± 8.4]	63 ± 43	194 ± 106	<9.21	<9.21	<9.21
ERα	UFZ	No effect	No effect	No effect	No effect	No effect	No effect
ERα (BEQ-EC10 in pg E2-EQ/L)	RECETOX	<18.9	<19	<62.4	<17	<17	<17
anti-ERα (BEQ-ICI EC50 in ng ICI-EQ/L)	1	5.5 ± 2.9	8.1 ± 5.0	53.1 ± 22.4	<2.9	<2.9	<2.9
GR	1	No effect	No effect	No effect	No effect	No effect	No effect
Fish embryo assay 96 h screening (% at REF 25)	1	No effect	40	100	n.a	n.a.	n.a.
Daphnia immobilisation assay 48 h screening (% at REF 25)	1	No effect	No effect	100	n.a.	n.a.	n.a.
Dioxin like activity AhR (BEQ-EC10 in pg TCDD-EQ/L)	1	16.4 ± 5.9	27.4 ± 27.0	<15.1	<4.13	5.9 ± 3.6	<4.13
HPTLC-YES	1	+	+++	+++	-	-/+	-
HPTLC-AChE	1	+	++	+++	-	-/+	+
HPTLC-BS	1	+	-/+	+++++	-	-	+++

34.3.5.1 Results of the *in vitro* bioassays

The results of the assessment of the LMX and LHR samples are listed in Table 6. None of the samples was active (above the limit of quantification) in the ERα or GR assays due to masking effects of cytotoxicity (UFZ) (Chapter 28).

The LMX and JDS4-24-LHR showed anti-estrogenicity, however, the potency of JDS4-24-LHR did not reach EC50 and therefore the BEQ based on EC50 could not be quantified. No cytotoxicity was observed in either agonist or antagonist mode of the ERα assay performed at the RECETOX laboratory. The LMX samples were active in the anti-AR assay and the highest tested REF caused cytotoxicity. The samples caused a pH decrease in the used L-15 medium and thus it had to be adjusted with NaOH before the cell exposure. In addition, certain pH decreases of the L-15 medium could be observed in the two highest tested concentrations of the LMX samples over the 24h exposure even after the pH adjustment prior to exposure. This could be possibly attributed to metabolization of contained compounds. The LMX blank sample (JDS4-Blank_4-SW-LS_LMX) elicited an effect of 33.9 ± 8.9 BEQ EC50 µg FLU-EQ/L and thus the anti-androgenic potential in sample JDS4-6-LMX is questionable.

In the AhR assay to test for dioxin like activity, none of the samples reached the effect level of 20 % of the highest level of reference compound. Samples JDS4-6-LMX, JDS4-24-LMX and JDS4-47-LHR showed minor dioxin-like activity, which was quantified using the EC10 values. The effect in JDS4-47-LHR was near the limit of quantification and the variance considerable. Sample JDS4-24-LMX showed cytotoxicity at the highest tested concentration, which was excluded from the calculation of effect concentrations.

34.3.5.2 Results of the *in vivo* bioassays

The LMX extracts showed concentration-dependent toxicity in the green algae assay with *Scenedesmus vacuolatus*. The effects in the green algae assay are supported by high contents of herbicides in the samples (Chapter 27). The effect of the blank sample (JDS4-Blank_4-SW-LS_LMX) with an EC50 of REF 87 was above the results and thus not critical. We observed a dose-dependent increase of fluorescence in the LHR samples which might be related to organic macro molecules contained in the samples. The extraction disks used in LHR are made from polytetrafluoroethylene (PTFE) membranes. PTFE is known to be instable in contact with methylene chloride causing a swelling of the PTFE and assumable enhanced passing of larger molecules. The screening of the LMX samples in the daphnia immobilisation assay with *Daphnia magna* and the fish embryo assay with *Danio rerio* at REF 25 and REF 50 resulted in a daphnia immobility of 100 % at REF 25 in sample JDS4-47-LMX (downstream Ruse/Giurgiu) and a fish embryo mortality of 40 % at REF 25 in sample JDS4-24-LMX (Budapest, downstream M0 bridge) and 100 % at REF 25 in sample JDS4-47-LMX. The blank sample was not active in the daphnia assay and very low active in the fish embryo assay (20 % mortality at REF 25).

34.3.5.3 Results of the HPTLC based bioassays

The results of the assessment of the samples in the HPTLC assays are not quantitative compared to the other assays, but they facilitate some insights in the samples which are not possible with the other assays. Separation of the samples was performed using HPTLC gradient development. After the separation a multiple-wavelength scan and additionally photos of the HPTLC plate were taken. The fluorescence image (366 nm) of the investigated ILS samples is shown in Figure 4. When comparing the fluorescence of the ILS samples, it is noticeable that significantly more fluorescent substances are enriched with the LMX samples compared to the LHR samples. However, in the LHR samples, a red bar was observed with RF = 0.7. The red fluorescent substances often arise from red chlorophylls in algae. The weak observations of fluorescence in the LHR samples support the findings of low or even no effects in the other bioassays. Not all compounds are fluorescent, but the absence of stronger blue bends is a supporting evidence.

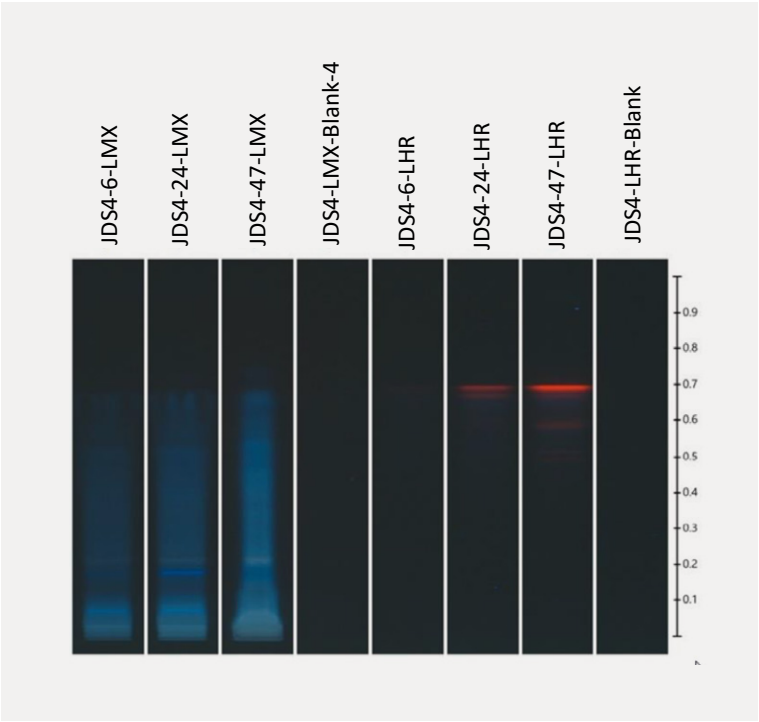

Figure 4: Fluorescence (366 nm) image of the ILS samples measured with the HPTLC method.

The yeast estrogen screen (YES) was used to detect estrogenic effects. Estrogen effects were detected in the LMX samples (Figure 5). Very low estrogen effects were detected in sample JDS4-24-LHR. Two estrogenic effects with RF = 0.59 and RF = 0.66 occurred in the Danube samples that coincided with the RF-values of estrone and estradiol (Chapter 28).

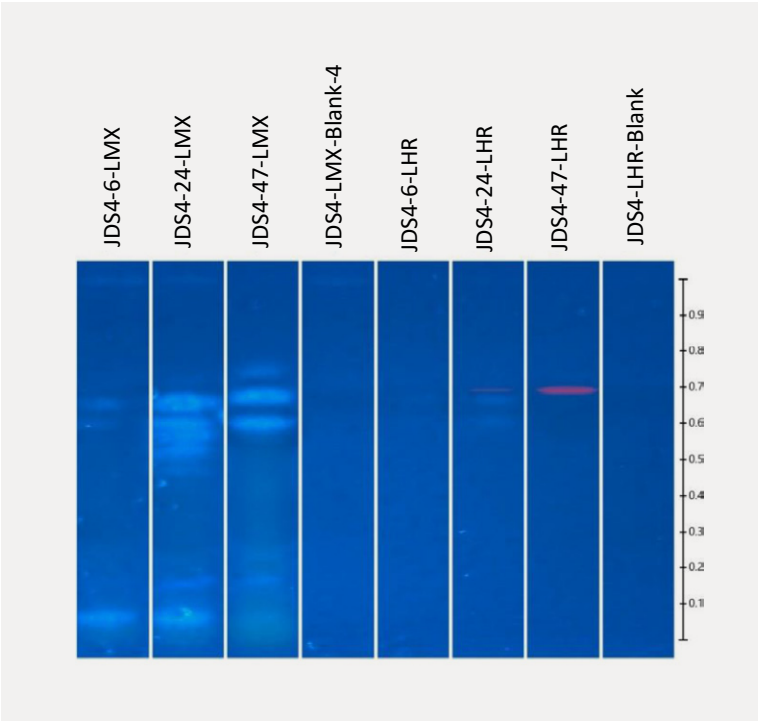

Figure 5: Overview of the yeast estrogen screen (YES) results of the ILS samples measured with the HPTLC method.

The acetylcholinesterase assay (AChE) was used for the detection of potential neurotoxic compounds. It should be noted that the assay is non-specific to matrix compounds in the sample extracts. Therefore, background inhibitions occur more frequently, which cannot be assigned to specific substances. At the application area (RF = 0.0), this assay showed a disturbance in the LMX samples (Figure 6), which was also detected in the machine blank. Therefore, this effect was not considered for further evaluation. In the LHR samples on the other hand, no matrix interferences could be detected. Overall, the AChE assay showed few effects in the samples. In the LMX samples, some weakly effective zones occurred in the middle RF-range, while those were not observed in the LHR samples.

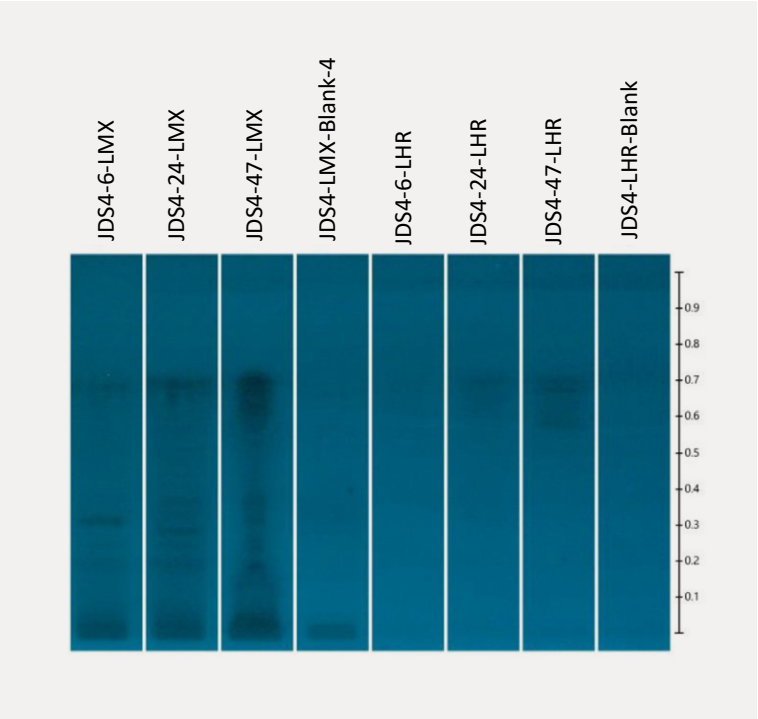

Figure 6: Overview of the acetylcholinesterase assay (AChE) results of the ILS samples measured with the HPTLC method.

The *Bacillus subtilis* assay is used to detect cytotoxic effects. The investigation of the JDS4 samples extracts revealed a cytotoxic effect at the application area of the HPTLC plate (RF = 0.0) also occurring in the blank samples examined. This effect was therefore classified as false positive and was not further considered for evaluation. Surprisingly, no more disturbances through cytotoxicity could be detected during the examination of the ILS samples (Figure 7). In the JDS4-6-LMX and JDS4-47-LMX samples, an effect at RF = 0.33 was detected. In the sample JDS4-24-LMX also an effect occurred, but the RF-value was slightly lower at 0.30, so it is probably caused by another substance than in the both other LMX samples. In addition, sample JDS4-47-LHR showed a very strong effect with RF = 0.60. This is the only effect also detected in LHR, because the effects with RF = 0.30 and 0.33 could not be detected with this enrichment technique.

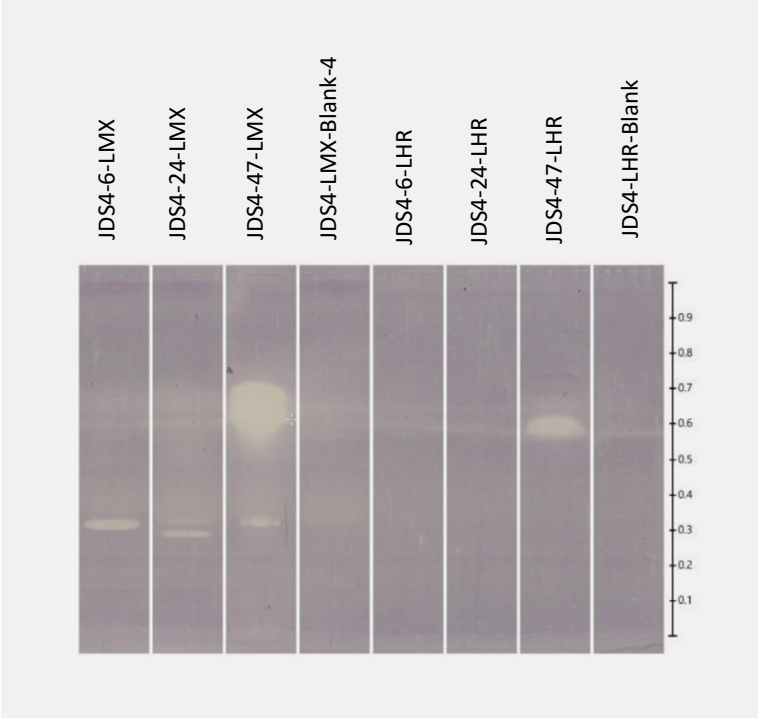

Figure 7: Overview of Bacillus subtilis assay results of the ILS samples measured with the HPTLC method.

The HPTLC technology elicited valuable additional information. The method does not derive quantitative data and full concentration effect relationships such as the other assays, but it is a promising and quick screening tool which includes a separation step and the possibility to apply the bioassays directly on the plates (not in all cases). Several effects were observed at quantifiable level, while there were also non-effect observations in some of the employed assay. A possible reason is the high dilution of anthropogenic chemicals in the Danube River in a way that even a REF of 100 is not sufficient rather to detect effects in short-term toxicity tests. With given materials, sorbents and solvents there are limits of possible enrichment due to co-enrichment of impurities. These impurities do not interfere with chemical analysis but may cause blank toxicities above REFs of 100 even if the cleanest materials on the market are used and extensively pre-cleaned before use.

34.4 Conclusions

In this chapter, the outcomes of a training to improve capacities of laboratories in the Danube River Basin in suspect and non-target screening technologies was reported. Four out of seven chemical-analytical laboratories participating in the interlaboratory study were located in the Danube riparian states, including one environmental agency, two water companies and one national laboratory. The other participants from outside of the basin were a university and federal laboratory and a research institute. An extract of a natural water sample was spiked with 81 compounds suitable for analysis with electrospray ionisation positive mode to perform a common suspect screening exercise. The results of the suspect screening of compounds spiked in an extract of a reference natural water sample were quite promising. The participants identified many of the spiked compounds, at least the most important water contaminants such as 1H-benzotriazole, terbuthylazine or metolachlor. We showed that vendors' software is not necessarily better than in-house or open source software tools to assess mass spectral data. In comparison to the vendors' tools, the in-house and open software tools were able to identify also more challenging compounds such as in-source adducts. With regards to EBM, four laboratories with nine *in vitro* and three *in vivo* bioassays reported their results. Two of the laboratories were based in the Danube riparian states. Thus, currently EBM are powerful tools to discriminate low-toxicity from more toxic samples.

34.5 Acknowledgement

The research at RECETOX was supported by the Czech Science Foundation Grant No. GACR 20-04676X.

34.6 References

ALTENBURGER R., BRACK, W., BURGESS R. M., BUSCH W., ESCHER B. I., FOCKS A., HEWITT L. M., JACOBSEN B. N., DE ALDA M. L., AIT-AISSA S., ET AL. (2019): Future water quality monitoring: improving the balance between exposure and toxicity assessments of real-world pollutant mixtures. Environmental Sciences Europe 31: 12.

ALYGIZAKIS N. A., OSWALD P., THOMAIDIS N. S., SCHYMANSKI E. L., AALIZADEH R., SCHULZE T., OSWALDOVA M., SLOBODNIK J. (2019): NORMAN digital sample freezing platform: A European virtual platform to exchange liquid chromatography high resolution-mass spectrometry data and screen suspects in "digitally frozen" environmental samples. TrAC Trends in Analytical Chemistry 115: 129–137.

BADER T., SCHULZ W., KÜMMERER K., WINZENBACHER R. (2016): General strategies to increase the repeatability in non-target screening by liquid chromatography-high resolution mass spectrometry. Analytica Chimica Acta 935: 173–186.

BADER T., SCHULZ W., KÜMMERER K., WINZENBACHER R. (2017): LC-HRMS Data processing strategy for reliable sample comparison exemplified by the assessment of water treatment processes. Analytical Chemistry 89: 13219–13226.

BECKERS L.-M., BRACK W., DANN J. P., KRAUSS M., MÜLLER E., SCHULZE, T. (2020): Unravelling longitudinal pollution patterns of organic micropollutants in a river by non-target screening and cluster analysis. Science of The Total Environment 727: 138388.

BENTON H. P., WONG D. M., TRAUGER S. A., SIUZDAK G. (2008): XCMS2: processing tandem mass spectrometry data for metabolite identification and structural characterization. Analytical Chemistry 80: 6382–6389.

BRACK W., AIT-AISSA S., ALTENBURGER R., COUSINS I., DULIO V., ESCHER B., FOCKS A., GINEBREDA A., HERING D., HILSCHEROVÁ K., ET AL. (2019): Let us empower the WFD to prevent risks of chemical pollution in European rivers and lakes. Environmental Sciences Europe 31: 47.

CHEN H. (2018): VennDiagram: Generate High-Resolution Venn and Euler Plots. Available at: https://CRAN.R-project.org/package=VennDiagram.

DOWLE M. and Srinivasan A. (2020): data.table: Extension of `data.frame`. Available at: https://CRAN.R-project.org/package=data.table.

DÜHRKOP K., FLEISCHAUER M., LUDWIG M., AKSENOV A. A., MELNIK A. V., MEUSEL M., DORRESTEIN P. C., ROUSU J., BÖCKER S. (2019). SIRIUS 4: a rapid tool for turning tandem mass spectra into metabolite structure information. Nature Methods 16: 299–302.

GAGO-FERRERO P., BLETSOU A. A., DAMALAS D. E., AALIZADEH R., ALYGIZAKIS N. A., SINGER H. P., HOLLENDER J., THOMAIDIS N. S. (2020): Wide-scope target screening of >2000 emerging contaminants in wastewater samples with UPLC-Q-ToF-HRMS/MS and smart evaluation of its performance through the validation of 195 selected representative analytes. Journal of Hazardous Materials 387: 121712.

GAWEL, A., SEIWERT, B., SÜHNHOLZ, S., SCHMITT-JANSEN, M. AND MACKENZIE, K. (2020). In-situ treatment of herbicide-contaminated groundwater–Feasibility study for the cases atrazine and bromacil using two novel nanoremediation-type materials. Journal of Hazardous Materials 393: 122470.

HELMUS R., LAAK T., DE VOOGT P., WEZEL A., SCHYMANSKI E. (2020): Patroon: open source software platform for environmental mass spectrometry based non-target screening. Available at: doi:10.21203/rs.3.rs-36675/v1.

HOLLENDER J., VAN BAVEL B., DULIO V., FARMEN E., FURTMANN K., KOSCHORRECK J., KUNKEL U., KRAUSS M., MUNTHE J., SCHLABACH M., et al. (2019): High resolution mass spectrometry-based non-target screening can support regulatory environmental monitoring and chemicals management. Environmental Sciences Europe 31: 42.

JEWELL K. S., KUNKEL U., EHLIG B., THRON F., SCHLÜSENER M., DIETRICH C., WICK A., TERNES T. A. (2020): Comparing mass, retention time and tandem mass spectra as criteria for the automated screening of small molecules in aqueous environmental samples analyzed by liquid chromatography/quadrupole time-of-flight tandem mass spectrometry. Rapid Communications in Mass Spectrometry 34

KESSNER D., CHAMBERS M., BURKE R., AGUS D., MALLICK P. (2008): ProteoWizard: open source software for rapid proteomics tools development. Bioinformatics, 24: 2534–2536.

KÖNIG, M., ESCHER, B. I., NEALE, P. A., KRAUSS, M., HILSCHEROVÁ, K., NOVÁK, J., TEODOROVIĆ, I., SCHULZE, T., SEIDENSTICKER, S., KAMAL HASHMI, M. A., ET AL. (2017). Impact of untreated wastewater on a major European river evaluated with a combination of in vitro bioassays and chemical analysis. Environmental Pollution 220: 1220–1230.

KÖPPE T., JEWELL K. S., DIETRICH C., WICK A., TERNES T. A. (2020): Application of a non-target workflow for the identification of specific contaminants using the example of the Nidda river basin. Water Research 178: 115703.

KORTENKAMP A., FAUST M., BACKHAUS T., ALTENBURGER R., SCHOLZE M., MÜLLER C., ERMLER S., POSTHUMA L., BRACK W. (2019): Mixture risks threaten water quality: the European Collaborative Project SOLUTIONS recommends changes to the WFD and better coordination across all pieces of European chemicals legislation to improve protection from exposure of the aquatic environment to multiple pollutants. Environmental Sciences Europe, 31: 69.

KRAUSS M., HUG C., BLOCH R., SCHULZE T., BRACK W. (2019): Prioritising site-specific micropollutants in surface water from LC-HRMS non-target screening data using a rarity score. Environmental Sciences Europe 31: 45.

LIBISELLER G., DVORZAK M., KLEB U., GANDER E., EISENBERG T., MADEO F., NEUMANN S., TRAUSINGER G., SINNER F., PIEBER T., ET AL. (2015): IPO: a tool for automated optimization of XCMS parameters. BMC Bioinformatics 16: 118.

LOOS M. (2015): nontarget: detecting, combining and filtering isotope, adduct and homologue series relations in high-resolution mass spectrometry (HRMS) data. Available at: http://CRAN.R-project.org/package=nontarget.

MERINGER M., REINKER S., ZHANG J., MULLER, A. (2011): MS/MS data improves automated determination of molecular formulas by mass spectrometry. MATCH Commun. Math. Comput. Chem 65: 259–290.

NAGY S. R., SANBORN J. R., HAMMOCK B. D., DENISON, M. S. (2002): Development of a green fluorescent protein-based cell bioassay for the rapid and inexpensive detection and characterization of Ah receptor agonists. Toxicological Sciences, 65: 200–210.

NEALE P. A., AIT-AISSA S., BRACK W., CREUSOT N., DENISON M. S., DEUTSCHMANN B., HILSCHEROVA K., HOLLERT H., KRAUSS M., NOVÁK J., ET AL. (2015): Linking in vitro effects and detected organic micropollutants in surface water using mixture toxicity modeling. Environmental Science & Technology 49: 14614–14624.

OECD (2004): Test No. 202: Daphnia sp., acute immobilisation test. OECD Publishing.

OECD (2013): Test No. 236: Fish embryo acute toxicity (FET) test. OECD Publishing.

OSWALD,P.(2020):HORIZONLVSPEfieldsampler.Availableat:https://norman-data.eu/JDS4/HORIZON%20LVSPE%20field%20sampler.pdf

PLUSKAL T., CASTILLO S., VILLAR-BRIONES A., ORESIC M. (2010): MZmine 2: modular framework for processing, visualizing, and analyzing mass spectrometry-based molecular profile data. BMC Bioinformatics 11: 395.

PROCHÁZKOVÁ T., SYCHROVÁ E., JAVŮRKOVÁ B., VEČERKOVÁ J., KOHOUTEK J., LEPŠOVÁ-SKÁCELOVÁ O., BLÁHA L., HILSCHEROVÁ K. (2017): Phytoestrogens and sterols in waters with cyanobacterial blooms - analytical methods and estrogenic potencies. Chemosphere 170: 104–112.

R CORE TEAM (2020): R: a language and environment for statistical computing. Available at: http://www.r-project.org.

SCHULZE T., AHEL M., AHLHEIM J., AÏT-AÏSSA S., BRION F., DI PAOLO C., FROMENT J., HIDASI A. O., HOLLENDER J., HOLLERT H., ET AL. (2017): Assessment of a novel device for onsite integrative large-volume solid phase extraction of water samples to enable a comprehensive chemical and effect-based analysis. Science of The Total Environment 581–582: 350–358.

SCHYMANSKI E. L., JEON J., GULDE R., FENNER K., RUFF M., SINGER H. P., HOLLENDER J. (2014): Identifying small molecules via high resolution mass spectrometry: communicating confidence. Environmental Science & Technology, 48: 2097–2098.

SCHYMANSKI E., SINGER H., SLOBODNIK J., IPOLYI I., OSWALD P., KRAUSS M., SCHULZE T., HAGLUND P., LETZEL T., GROSSE S., ET AL. (2015): Non-target screening with high-resolution mass spectrometry: critical review using a collaborative trial on water analysis. Analytical and Bioanalytical Chemistry 407: 6237–6255.

SILVA E., RAJAPAKSE N., KORTENKAMP A. (2002): Something from "Nothing" - Eight weak estrogenic chemicals combined at concentrations below NOECs produce significant mixture effects. Environmental Science & Technology 36: 1751–1756.

SMITH C., WANT E., O'MAILLE G., ABAGYAN R., SIUZDAK G. (2006): XCMS: processing mass spectrometry data for metabolite profiling using nonlinear peak alignment, matching, and identification. Analytical Chemistry 78: 779–787.

STÜTZ L., LEITNER P., SCHULZ W., WINZENBACHER R. (2019): Identification of genotoxic transformation products by effect-directed analysis with high-performance thin-layer chromatography and non-target screening. JPC - Journal of Planar Chromatography - Modern TLC 32: 173–182.

TAUTENHAHN R., BOTTCHER C., NEUMANN S. (2008): Highly sensitive feature detection for high resolution LC/MS. BMC Bioinformatics, 9: 504.

TOUSOVA Z., OSWALD P., SLOBODNIK J., BLAHA L., MUZ M., HU M., BRACK W., KRAUSS M., DI PAOLO C., TARCAI Z., ET AL. (2017): European demonstration program on the effect-based and chemical identification and monitoring of organic pollutants in European surface waters. Science of The Total Environment: 601–602: 1849–1868.

VÄLITALO P., MASSEI R., HEISKANEN I., BEHNISCH P., BRACK W., TINDALL A. J., DU PASQUIER D., KÜSTER E., MIKOLA A., SCHULZE T., ET AL. (2017): Effect-based assessment of toxicity removal during wastewater treatment. Water Research 126: 153–163.

WILSON V. S., BOBSEINE K., LAMBRIGHT C. R., GRAY L. E. (2002): A novel cell line, MDA-kb2, that stably expresses an androgen- and glucocorticoid-responsive reporter for the detection of hormone receptor agonists and antagonists. Toxicological Sciences: an official journal of the Society of Toxicology 66: 69–81.

35

Comparison of novel and current approaches for the target- and non-target screening, effect-based monitoring and prioritisation of river basin specific pollutants to improve future water quality monitoring

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Abstract

The aim of this chapter was the comparison of novel and current approaches for the target- and non-target screening and effect-based methods applied by the Helmholtz Centre for Environmental Research – UFZ during JDS4. The chapter reflects the feasibility of the tools to be used to address effects in the aquatic environment and to be applied in the routine monitoring according to the Water Framework Directive. An analysis of possible gaps in the approaches and strategies was included. Proposals and recommendations for the improvement and further needs of implementation are made.

35.1 Introduction

In European water policy, prioritisation and monitoring approaches focus mainly on widely occurring priority, river basin specific pollutants and pollutants on the EU Watch List. The basis for the monitoring of water resources by chemical pollution is the Water Framework Directive (WFD, Directive 2000/60/EU) and its daughter directives. The WFD defines pollution as the human-caused introduction of substances into the air, water or land concerning impact on humans and ecology by negatively affecting the goods and services of water resources (WFD, Article 2.33). However, water quality can be threatened by an infinite number of chemicals and site-specific mixtures of these (Posthuma et al., 2019b; Carvalho et al., 2014; Faust et al., 2019). The current strategy for the prioritisation and establishment of environmental quality standards (EQS, 2013/39/EU) is focused on single priority compounds (Loos et al., 2018) and country or river basin specific pollutants (Arle et al., 2016). These approaches do not account for risks of chemical mixtures (Faust et al., 2019) or site-specific pollution (Krauss et al., 2019). The single substance monitoring and assessment strategy of the WFD is not sufficient and protective for the water quality, because a complex chemical mixture poses a higher risk than any individual compounds alone due to mixture toxicity effects and this risk scenario can be very site-specific (Silva et al., 2002; Faust et al., 2019; Kortenkamp et al., 2019; Carvalho et al., 2014; Brack, 2019; Krauss et al., 2019). Thus, one of the goals of the Joint Danube Survey 4 (JDS4) was the verification of the use of alternative methods for pollutant analysis with the view of a better effort-cost-benefit relation than the present monitoring strategy under the WFD.

The aim of this chapter was the comparison of current approaches for chemical and effect-based monitoring to address the following questions:

- How can the tools be used to address effects of chemicals in the aquatic environment?
- What is the status and reliability of the tools to be used in routine monitoring?
- What are the gaps and further needs for implementation?

35.2 Methods

35.2.1 Sampling techniques

35.2.1.1 MAXX large volume solid-phase extraction device

The MAXX large volume solid-phase extraction (LVSPE) device has been developed since 2010 with MAXX Mess- und Probenahmetechnik GmbH (Rangendingen, Germany) and a previous version was applied in JDS3 (Schulze et al., 2017). The performance and the applicability of the MAXX LVSPE as an appropriate and reliable sampling technology was proven in several studies including chemical analysis, effect-based methods (EBM) and effect-directed analysis (Hashmi et al., 2018; König et al., 2017; Neale et al., 2015, 2018; Schulze et al., 2017; Serra et al., 2020; Tousova et al., 2017, 2018; Välitalo et al., 2017; Mijangos et al., 2020). In JDS4, MAXX LVSPE was used to collect samples at all 51 sampling sites (Chapter 26). For quality control, three travelling blanks and one machine blank were obtained. The travelling blanks were routinely prepared MAXX LVSPE extraction cartridges, which were sent to the three special sampling teams together with the cartridges for the surface water samples. After the sampling campaign, the travelling blanks were transported back to the UFZ laboratory and treated in same procedure than the samples. The machine blank was a specific blank prepared in the laboratory according to the procedure described in Schulze et al. (2017).

35.2.1.2 Grab samples for direct water injection analysis

During JDS4 the samples were taken by the special sampling teams. Grab samples were transferred to a clean glass beaker and 1 mL of the water sample was transferred on-site into an amber glass vial (1.5 mL, VEREX, Phenomenex) using polypropylene transfer tips. At each site, five sub-samples were collected. For quality control, a vial with 1 mL LCMS grade water was transported with the sample vials as sampling blank. The LCMS water in the vial was drawn in the pipette and released back to the vial to account for possible blank peaks. All samples and blanks were stored at -20 °C until analysis.

35.2.2 Methods for target- and non-target screening analysis

The methods for wide scope target- and non-target screening analysis applied by UFZ are listed in Table 1. In order to analyse the extracts of the MAXX large volume solid phase extraction (MAXX LVSPE) samples, a general liquid chromatography high-resolution mass spectrometry (LC-HRMS) was applied for combined target and non-target screening (Chapters 27 and 35). The grab surface water samples were analysed with a similar instrumental method with the difference that the water samples were injected directly in the LC-system (Chapter 28). The difference of the reliable detectable target compounds (Table 1) between the enriched and direct injected water samples is regarded to different ionisation efficiencies, matrix effects and detection limits. The analysis of endocrine substances was performed with specific target methods

which include clean-up with using aminopropyl columns and derivatisation and instrumental analysis with LC-HRMS und liquid-chromatography tandem mass spectrometry (LC-MSMS) (Chapter 28).

Table 1: Overview on target- and non-target screening methods applied by UFZ in JDS4; LC: liquid-chromatography; HRMS: high-resolution mass spectrometry; MSMS: tandem mass spectrometry.

Type of screening	Type of samples Extraction	Number of target compounds	Applied instrumental technology	Data assessment	References / chapter
Wide scope target screening	MAXX LVSPE surface water samples Extraction onsite	519	Thermo LC-Q Exactive Plus HRMS	MZmine In house R script Tracefinder 4.1	Chapter 26
Wide scope target screening	Grab surface water samples Direct injection (UFZ-DI)	534	Thermo LC-Q Exactive Plus HRMS	MZmine In house R script Tracefinder 4.1	Chapter 27
Target analysis of endocrine substances	MAXX LVSPE surface water samples	75	Thermo LC-Q Exactive Plus HRMS SCIEX LC-QTRAP-MSMS	MZmine In house R script Thermo Tracefinder 4.1 SCIEX MultiQuant	Chapter 28
Non-target screening	MAXX LVSPE surface water samples	n.a.	Thermo Q Exactive Plus HRMS - LC	MZmine In house R script	Chapter 26 Chapter 34

35.2.3 Effect-based methods

Aliquots of the MAXX LVSPE extracts were subjected to bioanalytical assessment in *in vitro* and *in vivo* bioassays at UFZ laboratories (Table 2). Prior to analysis in the *in vitro* assays, the extracts were cleaned using aminopropyl columns to remove sample matrix in order to lower detection limits and interferences. In all *in vitro* bioassays, serial dilutions of samples were tested to derive the inhibitory concentration for cytotoxicity (IC10). Only relative enrichment factors (REFs) lower than IC10 were included in the concentration-effect modelling of the activation of the reporter gene to avoid the false positive results due to the cytotoxicity burst (Escher et al., 2020). The effect concentrations (EC10) of environmental samples were expressed in relative enrichment factors (REFs) to the original water samples and were derived from linear concentration-response curves (Escher et al., 2018).

In the *in vivo*, the raw MAXX LVSPE extracts were examined without prior clean-up. The extracts were tested in a microtiter-plates based pre-screening in the fish embryo assay with *Danio rerio* and the daphnia immobilisation test with *Daphnia magna* only at REF 25 and REF 50. The final assessment according to OECD 202 and OECD 236 is still in progress due to the laboratory shutdown and backlog issues during the COVID-19 pandemic. In the algae growth inhibition assay with *Scenedesmus vacuolatus*, full concentration effect relationships could be derived with a maximum test concentration of REF 100.

Table 2: Overview on effect-based methods applied by UFZ in JDS4.

Bioassay	Cell line / species	Application	References / chapters
In vitro assays			
Estrogenic receptor (ERα)	ERα-UAS-bla GripTite	Screening of surface water samples	König et al. (2017) Chapters 2 and 34
Glucocorticoid receptor (GR)	GR-AUS-bla HEK 293T	Screening of surface water samples	König et al. (2017) Chapters 28 and 34
In vivo assays			
Algae growth inhibition assay (24 h)	Scenedesmus vacuolatus	Screening of surface water samples	Gawel et al. (2020) Chapter 34
Daphnia immobilisation assay (48 h)	Daphnia magna	Screening of surface water samples	OECD 202 (2004) with UFZ test adaptations Chapter 34
Fish embryo assay (96 h)	Danio rerio	Screening of surface water samples	OECD 236 (2013) with UFZ test adaptations Chapter 34

35.2.4 Prioritisation of non-target screening results

Non-target screening (NTS) can be applied without any prior knowledge of the compounds present solely starting from the analytical data and result often in a large number of peaks before data assessment (Krauss et al., 2010). The mass spectral data of environmental samples contain up to tens of thousands of peaks (Chapter 34) and many advanced tools have been developed for the identification (Altenburger et al., 2019; Alygizakis et al., 2019; Dührkop et al., 2019; Helmus et al., 2020). Nevertheless, NTS has been successfully applied to prioritise chemicals for identification based on time series analysis (Albergamo et al., 2019; Carpenter et al., 2019; Hollender et al., 2017), their spatial trends in river courses (Beckers et al., 2020; Ruff et al., 2015) or in the context of a known toxic pressure in a river (Peter et al., 2018).

However, an exhaustive and reliable identification of all chemicals is still a challenge (Krauss et al., 2019; Ludwig et al., 2020) and thus the development and application of rapid methods for the prioritisation of relevant peaks is demanded. In two mentioned studies, promising k-means clustering methods were applied to work on trajectories in longitudinal data (Genolini et al., 2015) to prioritise non-target peaks in a bank filtration situation (Albergamo et al., 2019) and in a small river basin (Beckers et al., 2020). The sheer size of the Danube Basin and the indispensable limitations of a big river survey unfortunately does not allow the application of the trajectory approach. The collection of adequate samples is not affordable. Therefore, we applied a robust method for the prioritisation of site-specific contamination from local emission sources based on LC-HRMS data (Krauss et al., 2019) for the demonstration of the applicability of this prioritisation technique in a large river system such as the Danube (Chapter 26).

35.2.5 Risk-based prioritisation of target screening results

In order to investigate the potential risks of the compounds detected by the target screening of UFZ, the results were compared by applying three different prioritisation approaches: 1) toxic units (TUs), 2) the multi-substance potentially affected fraction (msPAF) expressed as hazard units (HU) and 3) and hazard quotients (HQ) based on lowest predicted non-effect concentrations (PNEC) as depicted in Figure 1. This

prioritisation approach was discussed as an outcome of the SOLUTIONS project (Brack et al., 2018; Faust et al., 2019).

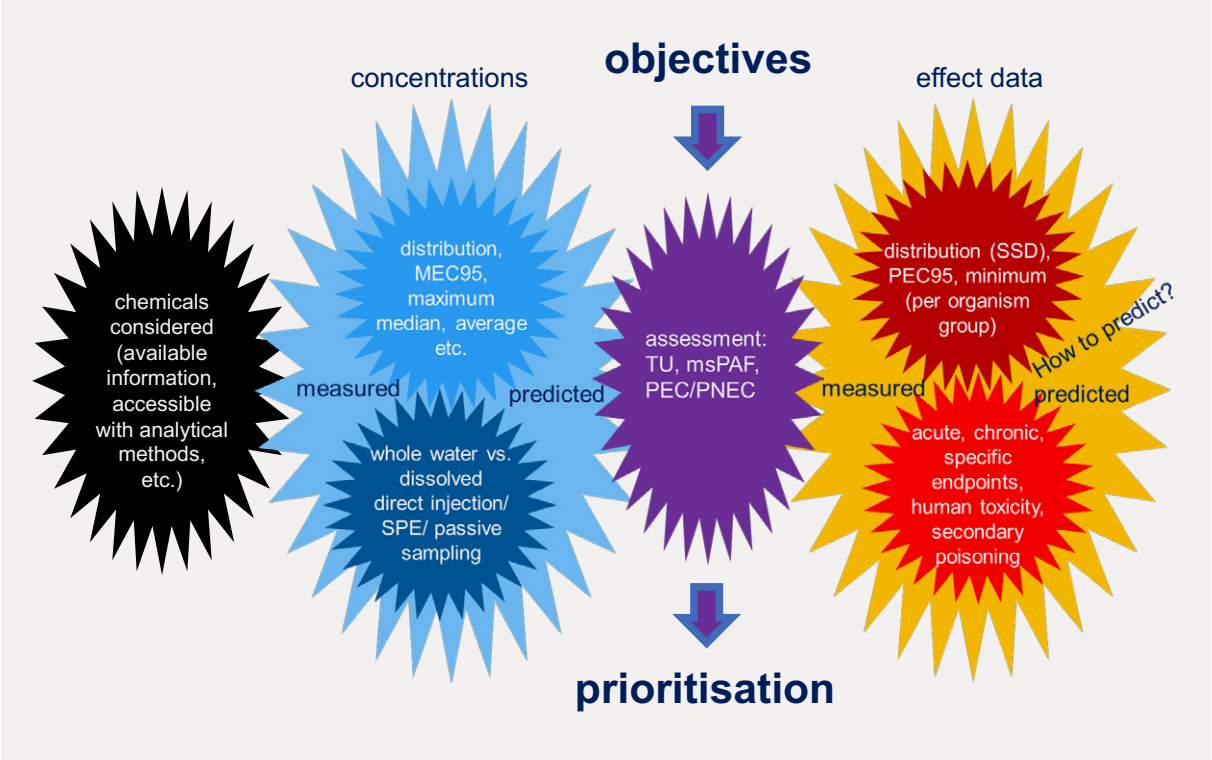

Figure 1: Framework of possible input values for the combination of chemical monitoring and effect-based methods for the prioritisation of chemicals. MEC95: measured environmental concentration (95th percentile), SPE: solid-phase extraction, TU: toxic units, msPAF: multi-substance potentially affected fraction, PEC: predicted effect-concentration, PNEC: predicted non-effect concentration.

35.2.5.1 Toxic units

The toxic unit (TU) approach is a measure of the intrinsic toxicity of a compound towards an environmental concentration to compare and add the toxicities of different substances for a biological quality element (BQE) to each other (Sprague, 1970; von der Ohe et al., 2011). TUs were calculated for each chemical and biological quality element by normalising the measured environmental concentration (MEC) in the water c_w (in mg/L) to the lethal (LC_x) or effect concentrations (EC_x) or the lethal doses LD_x causing x % lethality, immobility or growth inhibition according to equation 1 (Sprague, 1970).

$$TU = \frac{c_w}{LC_x} or \frac{c_w}{EC_x} or \frac{c_w}{LD_x} or \frac{c_w}{LOEL} or \frac{c_w}{NOEL} \qquad (1)$$

The *LOEL* and *NOEL* represent the observable effect level and the non-observable effect level, respectively. Comparable to LC, EC and LD values, they are statistically derived from the concentration-effect-relationships (CRC) in comparison with the control samples while the lowest effect concentration (LOEC) and the non-observable effect concentration (NOEC) are not and thus they are prone to under- or overestimation of risks. The reason is that the LOEC and NOEC are derived from fixed concentration levels. Hence, if the concentration range of the CRC is large or the density of concentration levels in the area of the LOEC / NOEC is low, the result is fuzzy.

Acute toxicity data was selected in the order: (1) Experimental data retrieved from US EPA ECOTOX Knowledgebase (US EPA, 2020) and (2) predicted data using the ECOSAR type baseline model for the BQE fish, daphnia and green algae in ChemProp 6.7.1 (UFZ Department of Ecological Chemistry, 2019).

The US EPA ECOTOX Knowledgebase was retrieved in text file format (exotox_ascii_15_09_2020) and the 5th percentile of all effect concentrations of one group (EC/LC/LD $_{[10\text{-}90]}$, LOEL, NOEL) were calculated for each biological quality element (BQE) group (fish, crustacean, algae [Chromista, Plantae, Monera]). This predicted effect concentration could be named EC05$_{est}$, because it represents real measurements, but it is a statistical value. The calculations include all available data for all BQEs independent from freshwater or marine habitats in order to retrieve a more robust data base. The responses of the organisms to chemical pressures are assumable independent from the salinity of the environment because the organisms are adopted to their habitat.

In brief, the data was filtered and transformed as follows:

- include only mortality, growth inhibition, population and movement inhibition data
- select only data for active ingredients and exclude formulations
- include only datasets of "short-term effects" (<= 120 hours or <= 5 days)
- transform all units to mg/L including recalculation of molar based units using the molecular weight

Finally, the 5th percentiles for all effect values and the count of included studies for each BQE were calculated by a pivot query. In cases, the measured or predicted ecotoxicological data exceeded more than a half log unit above the predicted water solubility, the measured or predicted ecotoxicological value was replaced by the predicted water solubility to account for problems with often reported nominal concentrations in ecotoxicological studies. The solubility was estimated as a consensus solubility by computing the geometrical mean of quantity-structure relationship (QSAR) estimation of the solubility using OPERA 2.6 (Mansouri et al., 2018), ChemAxon JChem (academic license kindly provided by ChemAxon, Budapest, Hungary) and ACD/Percepta 2014 (ACD Labs). QSAR-ready structures (Gadaleta et al., 2018) were calculated using OPERA 2.6.

35.2.5.2 msPAF

The multi-substance potentially affected fraction (msPAF) model was developed to assess the toxicity risk of complex mixtures using a two-step, mixed-model approach (de Zwart and Posthuma, 2005). In the first step, concentration addition (CA) is applied to calculate a risk value for substances that have a common toxic mode of action (TMoA), for example baseline toxicity, by modelling species sensitivity distributions (SSD). In the second step, a response addition (RA) model is applied to compounds with outlying SSDs, assuming a specific TMoA for those compounds. The msPAF estimation mandatory requires the input of some additional parameters to model the water condition (e.g. pH, contents of ammonia, sodium, chloride, magnesium and calcium, water temperature). This data was retrieved from the JDS4 internal database of ICPDR (if available). Missing data points at some sites were imputed by building the geometrical mean. For parameters which were not measured during JDS4, data was obtained from the Danube River Basin Water Quality Database (ICPDR, 2020) and the geometrical mean was used for all sites. The mPAF hazard units *HU* were calculated according to equation 2:

$$\sum_{i=1}^{n} HU_i, \text{ where } HU_i \text{ is } \frac{PEC_i}{SSD\ midpoint\ mu_i} \text{ or } \frac{MEC_i}{SSD\ midpoint\ mu_i} \qquad (2)$$

The SSD midpoint mu_i of compounds detected in the Danube samples were retrieved from the Netherlands National Institute for Public Health and Environment (RIVM) database (Posthuma et al., 2019a) with support of Jaap Slootweg and Leo Posthuma (RIVM).

35.2.5.3 Predicted non-effect concentrations

In order to compare the results of JDS4 in a regulatory context, the measured environmental concentrations (MEC) were compared with the predicted non-effect concentrations (PNEC) or the EQS (if existing) to derive a hazard quotient by equation 3:

$$HQ = \frac{PEC}{PNEC} \; or \; \frac{MEC}{PNEC} \; or \; \frac{PEC}{EQS} \; or \; \frac{MEC}{EQS} \qquad (3)$$

where *HQ* is hazard quotient, *PEC* is the predicted (modelled) environmental concentration. The PEC/PNEC based hazard quotient is used to decide whether a compound is discharged in significant quantities because it can be referred to the EQS or to the regulatory accepted PNECs. The disadvantage is that the PNEC are often only available for the most sensitive species by definition and assessment factors are applied to account for uncertainties in the data bases of the PNECs. The former is a hurdle for a holistic assessment of all three BQE (fish, crustacean, algae). The latter makes the PEC/PNEC approach less valuable for the evaluation of the acute risk of chemicals for the aquatic environment because the data cannot be directly compared to acute toxicity derived from effect-based methods. An added value of the approach is that the monitoring data can be assessed by the frequency of exceedance (equation 3) and extent of exceedance (equation 4) (von der Ohe et al., 2011).

$$Frequency\ of\ exceedance = \frac{\sum n}{N} \qquad (4)$$

$$Extent\ of\ exceedance = \frac{MEC_{95}}{lowest\ PNEC} \qquad (5)$$

Where *n* is the number of sites with a MEC_{site} / *lowest PNEC* HQ greater than one, *N* is the total numbers of sites considered and MEC_{95} is the 95th percentile of all MEC_{sites}. The extent of exceedance ranks the compounds according to their local importance in the context of all considered sites. If the compound occurs only in low concentrations near to the PNEC, it will rank down in the global assessment. The extent of exceedance was only calculated for chemicals with more than 20 findings according to von der Ohe et al. (2011). Details on the application of this approach can be found in Chapter 36. Predicted PNEC values were retrieved from the NORMAN SusDat Database (NORMAN Network 2020).

35.3 Results and Discussion

35.3.1 Wide scope target screening and targeted analysis of endocrine compounds

35.3.1.1 General comparison of the applied methods for target-screening

A first major difference between the enrichment and direct injection methods used was in the handling of suspended particulate matter (SPM) in the samples (Table 1). The MAXX LVSPE device uses a glass fibre filter cartridge (1 µm) prior to the SPE cartridge, thus SPM-bound micropollutants are removed before (Schulze et al., 2017). The direct injection method (UFZ-DI) allows for a settling of SPM prior to sample injection. The most severe systematic effects on quantification performance are typically compound losses during sample processing and matrix effects (in most cases ion suppression by co-eluting matrix constituents) during LC-MS/MS and LC-HRMS ionisation. To compensate for these effects, isotope-labelled internal standards (ILIS) are typically used for organic trace analysis, which is also the case for the presented methods. In an ideal case, for each compound an isotope-labelled surrogate standard is used (i.e., the same compound, but isotope-labelled), which is nearly the case for the JRC method with 43 ILIS for 67 target compounds, all of them being a surrogate standard for one analyte and closely related compounds for the remaining ones (Chapter 37). The MAXX LVSPE (Chapter 28) method does not allow the addition of ILIS prior to extraction, as samples are directly taken from the surface water and the same extracts are foreseen for a simultaneous use in biotesting, which does not allow the addition of an ILIS. UFZ thus also used a method-matched calibration using 13 levels, but this was done by a laboratory scale SPE procedure down-scaled from the LVSPE as a more cost and time-efficient approach. Furthermore, water from a pristine stream from the Upper Harz mountains (Germany) was used as a surface water matrix. Although composition and chemistry of this stream water differs from that of a large lowland river as the Danube, a more appropriate surrogate matrix without anthropogenic contamination is not available. For the direct injection method (Chapter 27), the ILIS has solely the aim to compensate for matrix effects, as no further sample processing, possibly resulting in compound losses, was done. The calibration was also prepared matrix-matched in pristine stream water at 12 levels.

35.3.1.2 Comparison of quantification in target screening

In the JDS4 MAXX LVSPE surface water samples, 298 organic substances out of 519 targeted compounds were detected with a frequency of detection (FoD) of at least one sampling site. (Chapter 26). In the JDS4 grab surface water samples analysed by direct injection (UFZ-DI), 157 organic pollutants out of 534 targeted compounds were detected with a FoD of at least one sampling site (Chapter 27). Either sampling techniques have been proven to be robust to detect compounds over two to three orders of magnitude (Chapters 26 and 27). The comparison of the quantified concentrations was done for ten compounds analysed with the MAXX LVSPE and UFZ-DI method. It should be noted that we cannot rule out differences in concentrations based on the different sampling approaches. However, all samples were taken within the same period of time within up to three hours from large rivers at sites with well-mixed waters (i.e., not directly downstream of influents). Thus, we do not expect strong concentration changes within that sampling periods. A look at the limits of quantification (LOQs) – and method detection limits (MDL) according to US EPA (2011) in case of the MAXX LVSPE and direct injection method, respectively. The direct injection method showed somewhat higher MDLs than the SPE-based methods, as the water volume injected was just 100 µL as compared to much higher corresponding water volumes of the enriched extracts. A pairwise comparison

of measured concentrations between the JRC method and the UoA and MAXX LVSPE methods (Figure 2A) shows that about 73 % of the MAXX LVSPE data and 68 % of the UoA data was within a factor of 3 from the JRC data (Chapter 37). However, for both screening methods concentrations of some compounds in some samples were much higher or lower as measured by JRC, as shown in Figure 2B.

In contrast, for 8 out of 51 sites the carbamazepine concentrations measured by the UoA and UFZ-LVSPE methods were about 10 times higher than those measured by JRC. A closer look at the individual sites reveals that the carbamazepine concentrations measured by the different methods show often common trends for the samples taken by sampling teams 1 and 2, but larger deviations for sampling team 3 Sites 31-51, Figure 2A and 2B). The samples of the lower stretch of the Danube were taken by Sampling team 3 from the mouth upstream and show fairly uniform carbamazepine concentrations in the UFZ-DI and MAXX LVSPE data (Figure 2).

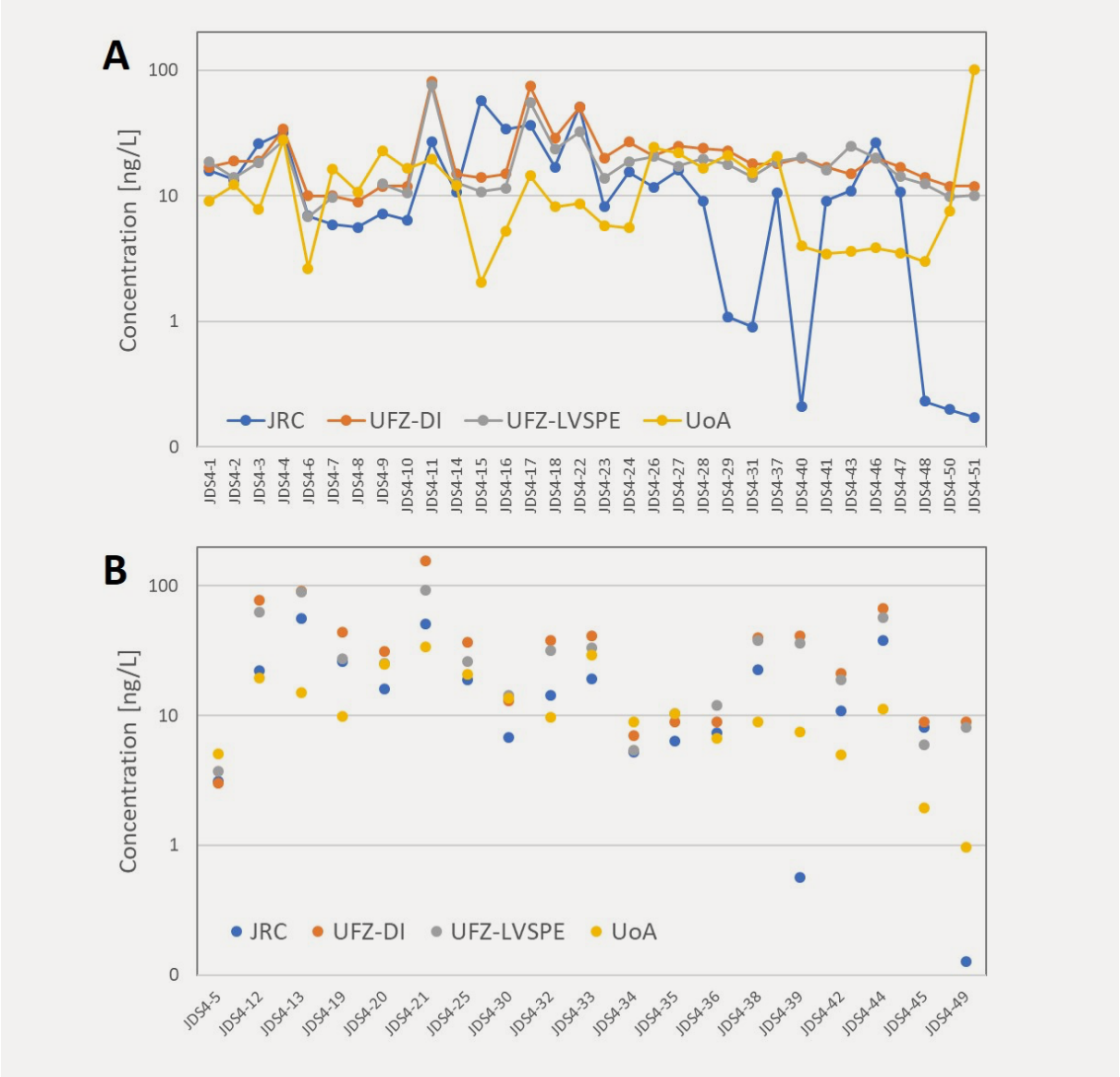

Figure 2: Concentrations of carbamazepine measured by the JRC, UFZ-DI, MAXX LVSPE and UoA methods in (A) the Danube main river and (B) the Danube tributaries.

35.3.1.3 Specific targeted analysis of endocrine compounds

The chemical analysis of endocrine disrupting compounds is challenging due to low concentration levels of potent natural estrogens. *In vitro* bioassays targeting endocrine disruption are very sensitive and allow a detection of the cumulative effect of all causative compounds, some of which might be missed by chemical analysis alone (Könemann et al., 2018). However, they lack the selectivity for individual compounds and effects might be masked by cytotoxicity in samples with a high overall compound load (Hashmi et al., 2020).

Out of the 25 analysed phenols (Chapter 28), eight could not be detected in any sample (p-chlorocresol, chlorophene, dichlorophene, 3,4,5-trichlorophenol, 4-bromophenol, bisphenol Z, bisphenol BP and bisphenol C), while two out of the five analysed estrogens (17ß-estradiol and 17α-ethinylestradiol) were not detected above MDLs of 0.021 and 0.030 ng/L, respectively. Methylparaben and bisphenol A were detected in almost all samples, with concentrations varying by one order of magnitude, indicating some specific sources in the catchment. More than 1 µg/L bisphenol A was found at site JDS4-46 (Russenski Lom) and >400 ng/L methylparaben at site 41 (Danube upstream Timok) while the median concentrations were below 10 ng/L. 2,4-Dichlorophenol was detected in only eight samples, in concentrations from above the method detection limit of 9 ng/L to 100 ng/L. All other phenols showed concentrations below 10 ng/L, mostly around 1 ng/L. The estrogens estrone and estriol were detected at sites JDS4-38 and JDS4-30, respectively, with concentrations peaking at about 1 ng/L, while 17α-estradiol had one detection at site 11 (Pohansko). In no case were the WFD watch list PNEC values of 3.6 ng/L for estrone (MDL: 0.026 ng/L), 0.4 ng/L for 17ß-estradiol and 0.035 ng/L for 17α-ethinylestradiol exceeded. In general, detection frequencies and concentration ranges were comparable in the Danube and the tributaries and no trends along the course of the Danube could be observed.

Out of the 50 analysed ketosteroids, 20 could be detected in at least one sample above the respective MDL, which was for most compounds between 0.05 and 0.2 ng/L (Chapter 28). The androgens androstenedione, androsterone and epiandrosterone were detected in most of the samples and were also the compounds with the highest concentrations reaching up to 7.5 ng/L at site JDS4-46. Androsterone and epiandrosterone two are metabolites of testosterone, which was detected in about tenfold lower concentrations at only 13 sites. Natural (cortisone and hydrocortisone) and synthetic glucocorticoids were found only at a few individual sites at levels around 2 or below 1 ng/l, respectively. The natural progestagen progesterone and synthetic progestogens mainly used as contraceptives were detected at levels up to above 1 ng/L at a few sites. Detection frequencies and concentrations were somewhat higher in tributaries as compared to the main river, probably due to higher wastewater fractions.

35.3.2 Performance of effect-based methods

35.3.2.1 Effects in *in vitro* bioassays

The tested river water extracts showed considerable cytotoxicity in both the ERα and GR assays (the inhibitory concentration for 10 % reduced cell viability IC10 was at a relative enrichment factor REF 5-74 for the ERα and REF 3-28 for the GR) despite the clean-up step (Chapter 28). Considering these IC10 values, no estrogenic or glucocorticoid activity could be detected in any of the samples. While estrogenic effects could be regularly detected in wastewater treatment plant effluents using the same assays it is unclear why all the samples of this study showed a cytotoxic masking of the estrogenic or glucocorticoid activity. Such a dominant masking of effects could not be observed in other screening campaigns to this extent (Könemann et al., 2018; Müller et al., 2018). During JDS3, 16 from 22 samples had estrogenic effects with EC10 ranging from REF 0.5 to 145 (Neale et al., 2015), which is in the same range or potent than we find now as IC10 in JDS 4 samples. No cytotoxicity data were reported in Neale et al. (2015). In the Danube River samples from Novi Sad that were heavily impacted by untreated wastewater also showed strong cytotoxicity in the acidic

and basic fraction of the extract impeding detection of some endocrine effects but estrogenicity could be detected very well in the neutral extracts using three ER assays, among them the one applied here (König et al., 2017). In LVSPE extracts from JDS3, Serra et al. (2020) detected a low estrogenic activity below 0.1 ng/L 17β-estradiol equivalents in most samples using one zebrafish- and one human-based *in vitro* assay.

35.3.2.2 Effects in *in vivo* bioassays

The MAXX LVSPE resulted in an overall low inhibition in the green algae assay with *Scenedesmus vacuolatus* (Figure 4 and Figure 5). The tributary samples revealed a slightly higher toxicity than the Danube River samples (Figure 4), but the two observation groups were not significantly different (unpaired t-test, α = 0.05, P = 0.5656 with a probability correctness of 72.4 %, two-tailed). The toxicity of the blanks samples was low with an average EC50 fluorescence of 87.7 REF. The effects of the median and 25 % of most toxic samples were two- to three-fold greater than the blank samples. While the traveling blank of team 1 the machine blank showed low effects, the travelling blanks of team 2 and 3 did not inhibit algae growth. The used solid phases and all high-quality solvents were obtained from the same charges and thus the differences are caused by a maybe random secondary contamination which cannot be evaluated in retrospect. In cases of samples with low effects, the distance to the blanks could be too small to distinct between effects of the blanks and the samples. In JDS4, this could be the case at site JDS4-10 (Hainburg), JDS4-18 (Gönyű), JDS4-26 (Dunafoldvar) in the Danube River and at site JDS4-5 (Inn) in the tributaries.

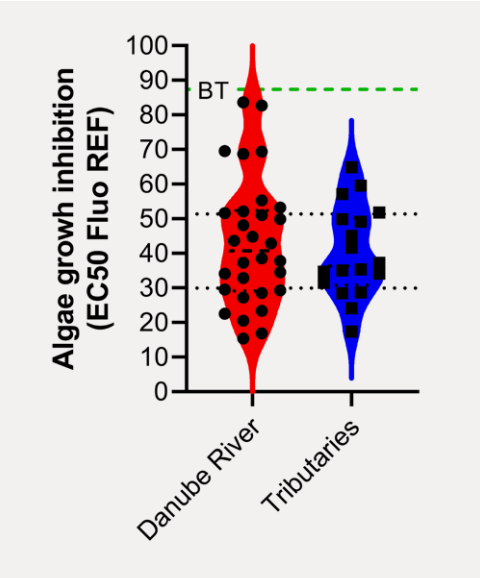

Figure 3: Violin plot (red: Danube River samples, blue: tributary samples) of the effects expressed as EC50 fluorescence (Fluo) REF observed in the MAXX LVSPE samples in the green algae growth inhibition assay with Scenedesmus vacuolatus (24 h) based on chlorophyll fluorescence measured with a MAXI-Imaging PAM-fluorometer (Walz); the green dashed indicates the blank threshold (BT) value of 86.7 [EC50 Fluo REF] derived of the measurement of machine and travelling blanks; the dots indicate the single results (black circles: Danube River samples, black square: tributary samples; the black doted lines represent the 25th- and 75th-percentiles REF: relative enrichment factor.

Higher toxicities (below the 25th percentile) were observed in the samples JDS4-3 (Kelheim), JDS4-16 (Medvedov), JDS4-22 (Szob), JDS4-24 (Budapest, downstream M0 bridge), JDS4-27 (Paks), JDS4-40 (Banatska Palanka / Bazias), JDS4-48 (Chiciu / Silistra) and JDS4-50 (Reni) in the Danube River and JDS4-25 (Tass), JDS4-33 (Tisza mouth, rkm 1.0), JDS4-39 (Varvarin) and JDS4-44 (Iskar mouth, rm 0.3) in the tributaries (Figure 4). In two samples (Szob and Tass), the UV-filter octocrylene might play an important role as the toxicity driver in algae growth inhibition. In the other samples, a herbicide driven mixture toxicity could be the cause of the effects.

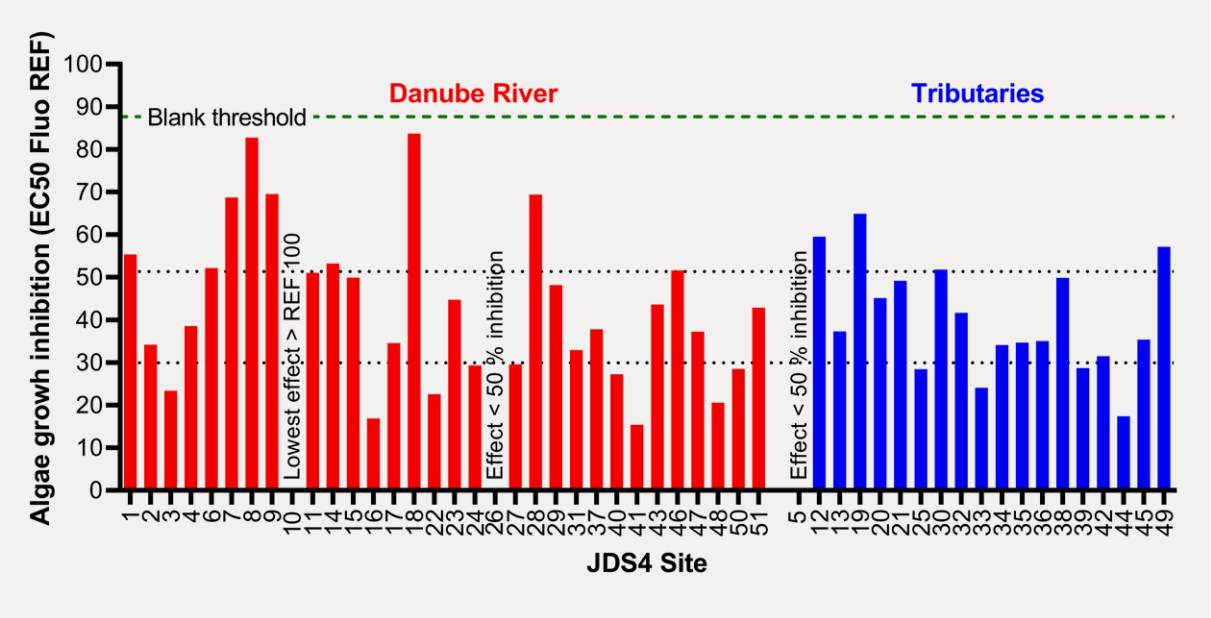

Figure 4: Bar plot of the effects expressed as EC50 fluorescence (Fluo) REF observed in the MAXX LVSPE samples in the green algae growth inhibition assay with Scenedesmus vacuolatus (24 h) based on chlorophyll fluorescence measured with a MAXI-Imaging PAM-fluorometer (Walz); the green dashed line indicates the blank threshold value of 86.7 [EC50 Fluo REF] derived of the measurement of machine and travelling blanks; the black doted lines represent the 25th- and 75th-percentiles; REF: relative enrichment factor.

Unfortunately, the assessment of the samples with the fish embryo test and the daphnia immobilisation test is not finalised at the time of writing. A delay in the delivery of the data until August 2020 was proposed before the start of the project and acknowledged by the ICPDR and the Federal German Environmental Agency. The delay was amplified by the COVID-19 pandemic due to a laboratory shutdown causing a backlog in the laboratory work.

Preliminary results of the pre-screening at REF 25 and REF 50 showed fish embryo mortalities (96 h exposure) between 0 % and 100 % at REF 25 and between 20 % and 100 % at REF 50. Regrettably, high effects were also observed in the blanks. In the daphnia immobilisation test (48 h), effects between 0 % and 100 % at REF 25 and effects between 0 % and 100 % at REF 100 were detected, respectively. A refinement of the results is in progress.

35.3.3 Prioritisation of non-target screening results

For the prioritisation of non-target peaks, the frequency scores (FS) and rarity scores (RS) (Krauss et al., 2019) for each peak were calculated. The FS estimates the average intensity of most frequent peaks above the blank or detection threshold. The RS represent a low frequency of occurrence of a peak in a dataset and its maximum signal intensity in relation to the median intensity in one single number. The non-target screening applied to the MAXX LVSPE samples resulted in altogether 95,996 single peaks detected in electrospray positive (ESI+) mode, and 31,083 in ESI negative (ESI-) mode, which was reduced to 91,419 peaks (ESI+) and 27,239 peaks (ESI-) above the threshold through the blank correction procedure. The Table 3 gives a breakdown of the numbers of detected peaks with high frequency scores (FG) and rarity scores (RS) in the whole datasets and across the individual samples. The reduction was more than 99 % for the peaks with high FS and RS. In the further text, we exemplify the most evident results, for details we refer to Chapter 26.

Table 3: Total numbers of detected peaks with frequency scores (FS) and rarity scores (RS) in the whole dataset and across individual samples.

	ESI+			ESI-		
	Whole dataset	**Median in samples**	**Range in samples (min-max)**	**Whole dataset**	**Median in samples**	**Range in samples (min-max)**
FS > 5000	692	688	622-692	4	4	3-4
5000 > FS > 1000	2804	2704	1467-2782	96	92	61-96
1000 > FS > 500	3389	2458	840-2620	212	204	88-212
RS > 5000	191	7	0-124	14		0-13
5000 > RS > 1000	2078	141	51-1546	118		
1000 > RS > 500	2413	290	91-1317	253		

For ESI positive mode data, about 60 % of the peaks could be assigned to alkyl-polyethylene glycol ether (PEG) surfactants showing predominantly C_{10}- to C_{16}-alkyl chains and 5 to 30 ethylene-oxide units. Further homologue series were evident in the data and thus overall, more than 85 % of the peaks with FS > 5000 in ESI+ mode was contained in homologue series, pointing to the huge importance of surfactants in the inventory of high-intensity and frequently occurring peaks in the dataset. In ESI+ mode, the peaks with highest FS values showed a trend for higher intensities at the lower stretches of the Danube and adjacent tributaries than at the upper stretches, suggesting raw wastewater as main input pathway of the associated surfactants (Figure 5). The two peaks of polyethylene glycol (m/z 592.3892, RT 9.3 min and m/z 636.4152, RT 9.5 min) were present in all samples, but showed distinct highest intensities in tributaries of the lower Danube, particularly at site JDS4-44, Iskar. The overall low intensities of peaks in sample JDS4-8 coincides with the findings of the target screening.

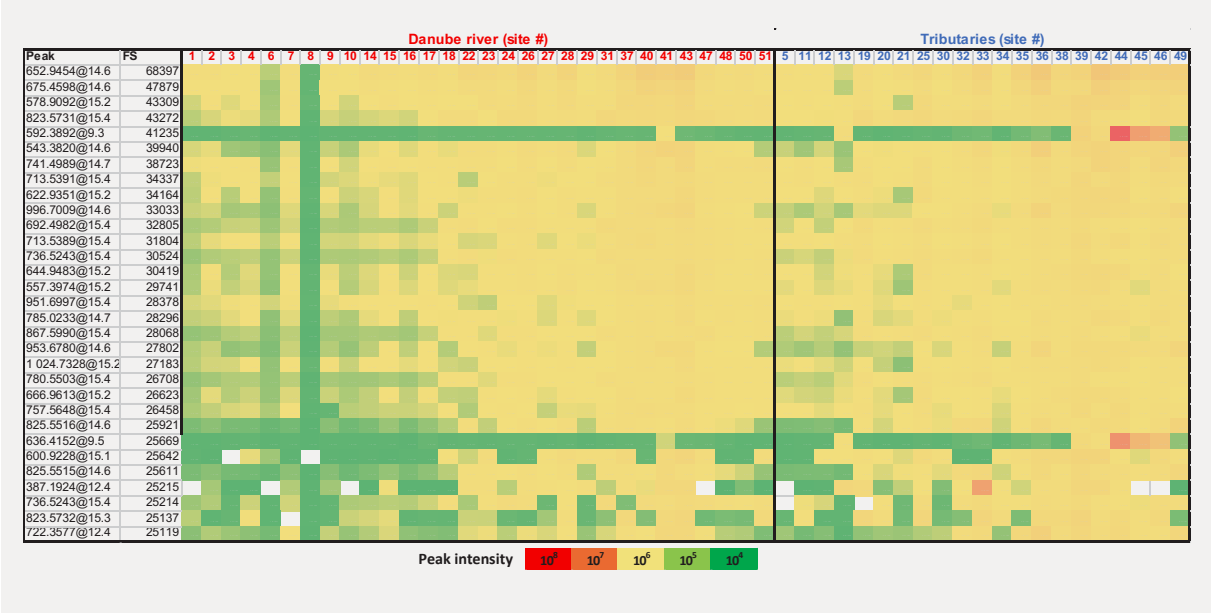

Figure 5: Occurrence and intensities of peaks in ESI positive mode with FS values >25,000 at all study sites, separated in Danube River and tributaries.

Peaks with high rarity scores (RS) indicating site-specific contamination were predominantly present in sample JDS4-49 (Prut) in ESI+ mode, and in samples JDS4-31 (Danube at Ilok) and the Tisza and Sava tributaries (JDS4-32, JDS4-33, JDS4-34; Figure 6).

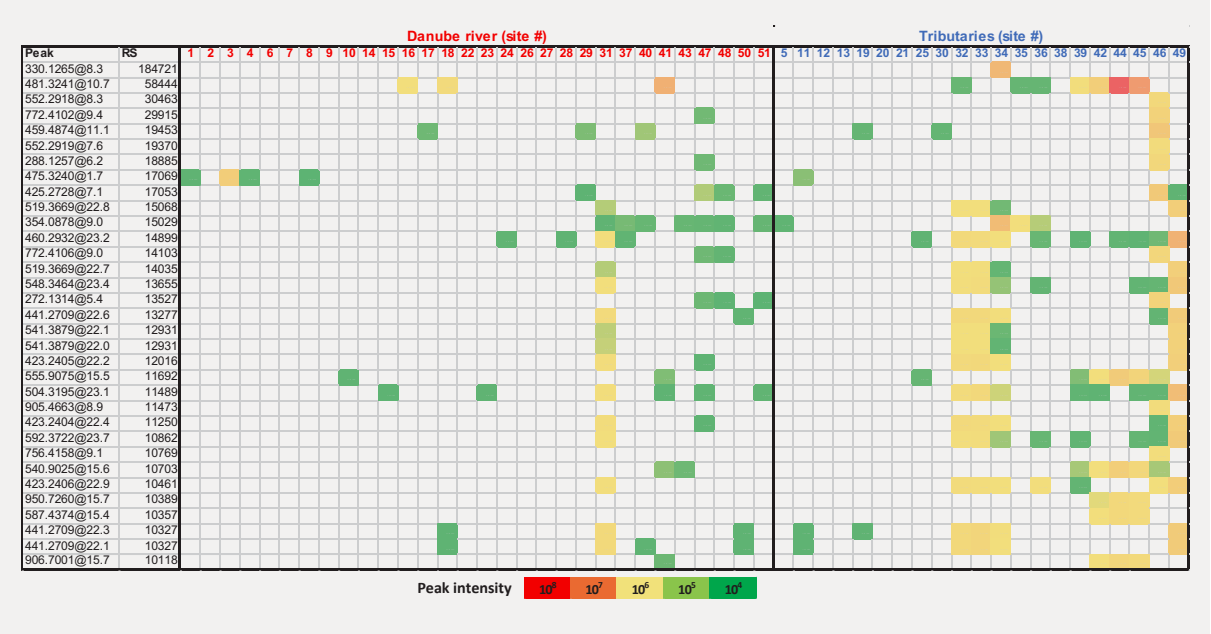

Figure 6: Occurrence and intensities of peaks in ESI positive mode with RS values >10,000 at all study sites, separated in Danube River and tributaries.

Many of these peaks could be identified as surfactants, showing high retention times, indicating that also site-specific surfactants contamination might occur besides the ubiquitous one. At site JDS4-34, the peak m/z 330.1265 (RT 8.3 min) could be tentatively identified as omeprazole sulphide (InChIKey XURCIPRUUASYLR-UHFFFAOYSA-N), based on a good MS/MS match with literature (Shin et al., 2020), and the peak m/z 354.0878 (RT 8.8 min) as lansoprazole sulphide (InChIKey: CCHLMSUZHFPSFC-UHFFFAOYSA-N). Both compounds are metabolites (or synthesis impurities) of the proton pump inhibitor drugs omeprazole (InChIKey: SUBDBMMJDZJVOS-UHFFFAOYSA-N) and lansoprazole (InChIKey: MJIHNNLFOKEZEW-UHFFFAOYSA-N), respectively and have so far not been reported in surface water and the finding suggests a site-specific source in the Sava River.

The frequency and rarity scores used, provided a simple and robust measure to prioritise site-specific and frequently occurring compounds in MAXX LVSPE samples, as they combine frequency of occurrence and peak intensities into a single value. The scores were applied to distinguish important peaks out of a matrix of several thousands peaks for identification. Especially, the rarity score unravelled single peaks in Danube and tributary samples which might have been overlooked in other multivariate statistical approach as for example principal component analysis. Non-target screening revealed the dominance of a range of surfactants as the most frequently occurring compounds in the Danube River basin and points to sites where a site-specific contamination occurs.

35.3.4 Risk-based prioritisation of target screening results

35.3.4.1 Toxic units

In Figure 7, the ranked TUs are depicted for the BQE fish for MAXX LVSPE samples and the UFZ DI samples. The figures show only the 10 % high ranking TUs. The summarised TUs for all detected compounds is 0.23 (MAXX LVSPE) and 0.07 (UFZ DI). In the MAXX LVSPE samples, the UV-filter octocrylene ranked first with a TU of 0.1 mg/L ($EC05_{est}$). In both sample types, the surfactant hexadecylpyridinium (among other surfactants in the LMX samples) and the fungicide carbendazim was dominant due to a high effect concentration of up to 0.035 mg/L ($EC05_{est}$). In the MAXX LVSPE samples thefragrance galaxolide ranked on the fifth position, followed by the pyrethroids allethrin and etofenprox, the sun screen homosolate and the antioxidant diphenylamine. In the UFZ DI samples, the overall ranking is different due to a different chemical domain coverage of the both sampling techniques. The coverage is based on the physico-chemical properties of the chemicals. A very water solulable polar compound may be found in the direct water samples, but not trapped on the solid phase used in MAXX LVSPE. Thus, the fungicide azoxystrobin, the herbicide terbutylazine, the organophosphate insecticide diazinon and the carbamate carbaryl dominate the most toxic compounds.

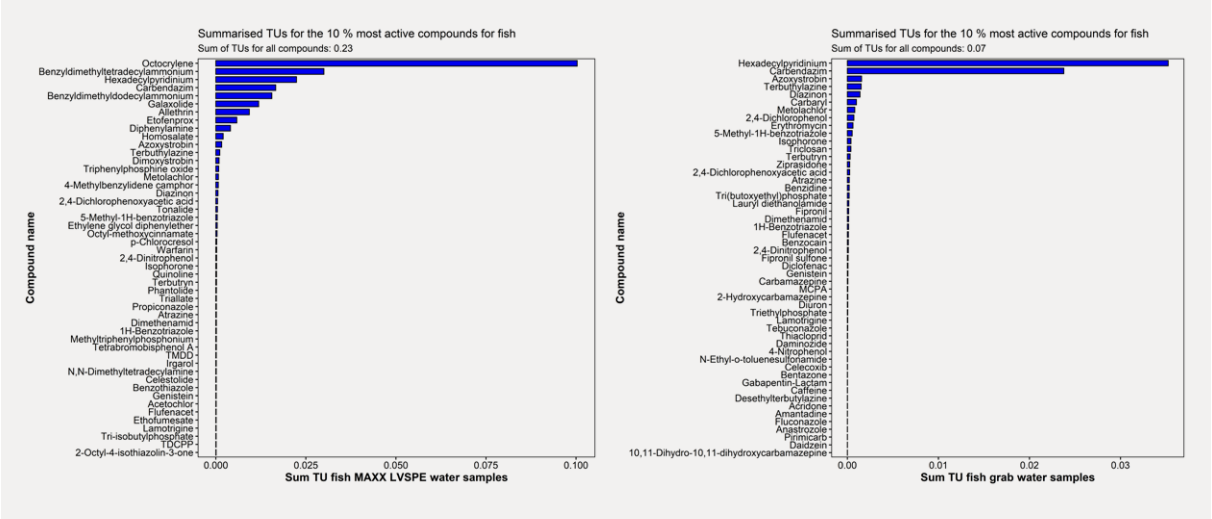

Figure 7: Toxic units derived from assessment of MAXX LVSPE (left) and UFZ DI (right) samples for fish (10 % most active compounds).

In Figure 8, the ranked TUs are depicted for the BQE crustacean for MAXX LVSPE samples and UFZ DI samples. The figure shows only the 10 % high ranking TUs. The summarised TU for all detected compounds is 3.86 (MAXX LVSPE) and 6.3 (UFZ DI). In both sample types, diazinon was the toxic driver. Diazinon has an $EC05_{est}$ of 10^{-5} mg/L. Diazinon is neurotoxic and banned in the European Union except for the usage in harnesses for pets to counteract ticks and other parasites. In the MAXX LVSPE samples, the organophosphate pesticide fenthion, galaxolide, etofenprox, terbutylazine and octocrylene ranked next. Fenthion is not approved for usage in the European Union, but it was identified in sample JDS4-37 at concentration level of 31 ng/L. However, it is a very site specific compound which can dominate the ranking of compounds based on TUs. In the UFZ DI samples, the next ranked analytes were 2,4-dichlorophenol and carbaryl. The prevalence of the sum of all TUs was controlled by a few compounds in both sample types. However, compared to both other BQEs - fish and algae, crustaceans are affected by less compounds, but those few remaining compounds pose a high risk to crustaceans.

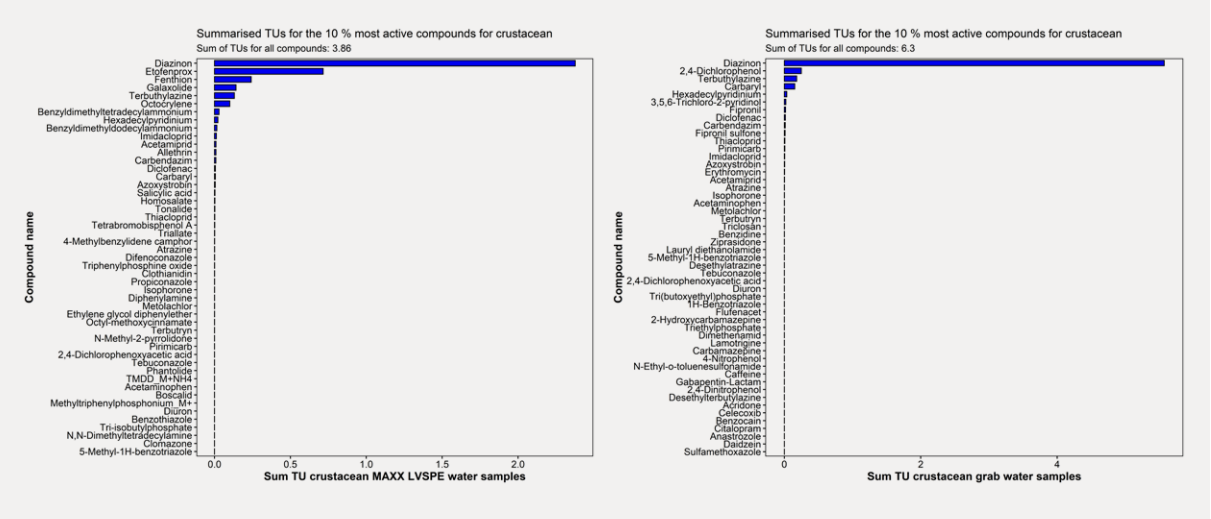

Figure 8: Toxic units derived from assessment of MAXX LVSPE (left) and UFZ DI (right) samples for crustaceans (10 % most active compounds).

The toxic units derivation for algae is shown in Figure 9. The summarised TUs of 2.7 and 5.2 of the MAXX LVSPE samples and the UFZ DI samples, respectively, are in the middle range between fish and crustaceans. In the UFZ DI samples, the antibiotic erythromycin was ranked on the first position with a TU of 2.7 mg/L ($EC05_{est}$). Erythromycin was only found in the Lower Danube samples. The toxicity drivers with risks to algae are almost call herbicides such as MCPA, cybutryne, diuron, metolachlor or nicosulforon and a few other compounds found frequently in environmental samples, such as 1H-benzotriazole and galaxolide.

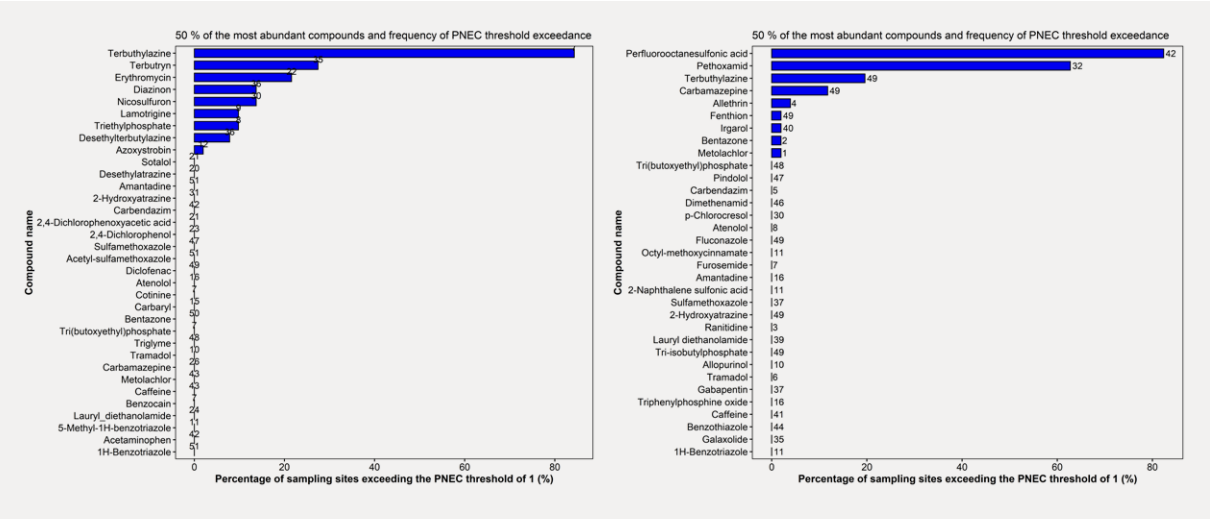

Figure 9: Toxic units derived from assessment of MAXX LVSPE (left) and UFZ DI (right) samples for algae (10 % most active compounds).

35.3.4.2 msPAF

The results of the risk estimation using msPAF hazard units (TU) in comparison to aggregated toxic units of the MAXX LVSPE and grab water samples of JDS4 are shown in Figures 10 and 11, respectively. While the toxic units are calculated for each single BQE (e.g. fish, algae and crustacean), the msPAF hazard units are derived from sensitivity species distributions (SSDs). In the SSDs, multiple species of plants and animals are included. Thus, msPAF is a proxy related to an ecological risk compared to the TU approach

which estimates the risk of each single species (e.g. *Daphnia magna* or rainbow trout or any green algae). In order to compare both methods, the geometrical mean of all three BQEs algae, crustaceans and fish were calculated and the HU and TU datasets were joined by their matching compounds (grab water samples: 90 compounds; MAXX LVSPE water samples: 99 compounds). The calculation of the geometrical mean cannot replace a real SSD which is the basis of the msPAF HUs, but it is an approximation for a basic comparison of both methods. It has to be mentioned that the HU and TU values cannot be compared directly. HUs below 1 account for a low risk of hazard and HUs above 1 for a higher risk. The TUs are open scaled, the higher the TU of a compound is compared to the TU of another substance, the higher is the risk of the first compound.

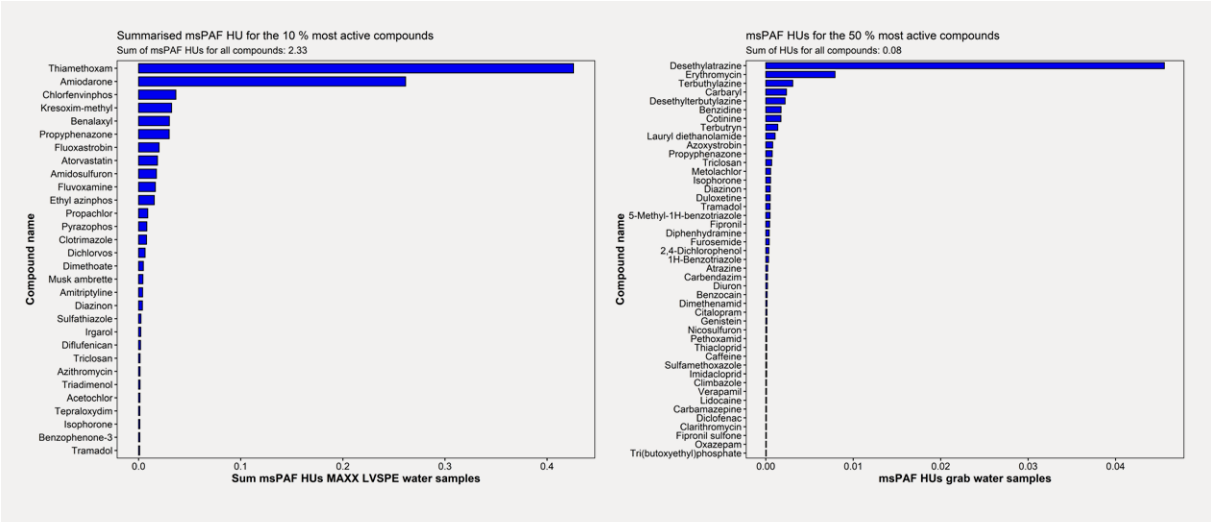

Figure 10: msPAF hazard units (HU) (left) and aggregated toxic units (TU) (right) of MAXX LVSPE water samples (50 % most active compounds).

In the MAXX LVSPE water samples (Figure 10), pesticides such as thiamethoxam, benalaxyl and diazion and pharmaceuticals, e.g. propyphenazone and fluvoxamine, are highly ranked by the msPAF HUs. In the TU based assessment, with exception of the fragrance galaxolide, herbicides and other pesticides are the top-ranking compounds.

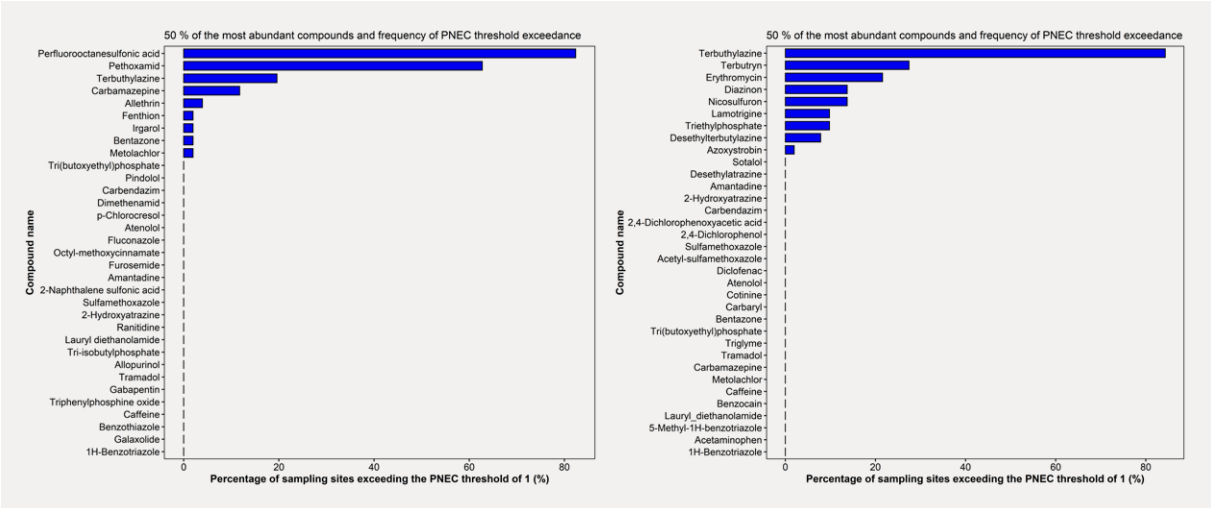

Figure 11: msPAF hazard units (HU) (left) and aggregated toxic units (TU) (right) of JDS4 UFZ DI samples (50 % most active compounds).

At the comparison of the UFZ DI samples ranked by the msPAF HUs and the TUs (Figure 11), the overall rankings of the compounds are quite similar. With some exceptions (e.g. cotinine, lauryl diethanolamide, propyphenazone and isophorene), the high-ranking compounds are herbicides, biocides, antibiotics and insecticides.

35.3.4.3 PNEC

In a more regulatory context, the exceedance of a threshold (e.g. the predicted non-effect concentration, PNEC) or environmental quality standard (EQS) is of importance. A frequently used approach is the comparison with the measured environmental concentrations (MEC). In order to assess the most abundant concentration levels, the 95th percentile of the values is calculated and used for the assessment (MEC95). In comparison to the TU and msPAF HU methods, the PNEC approach is considering the PNEC value of the most sensitive organism or biological quality element. Hence, the PNECs are complementary to the TU and msPAF procedures.

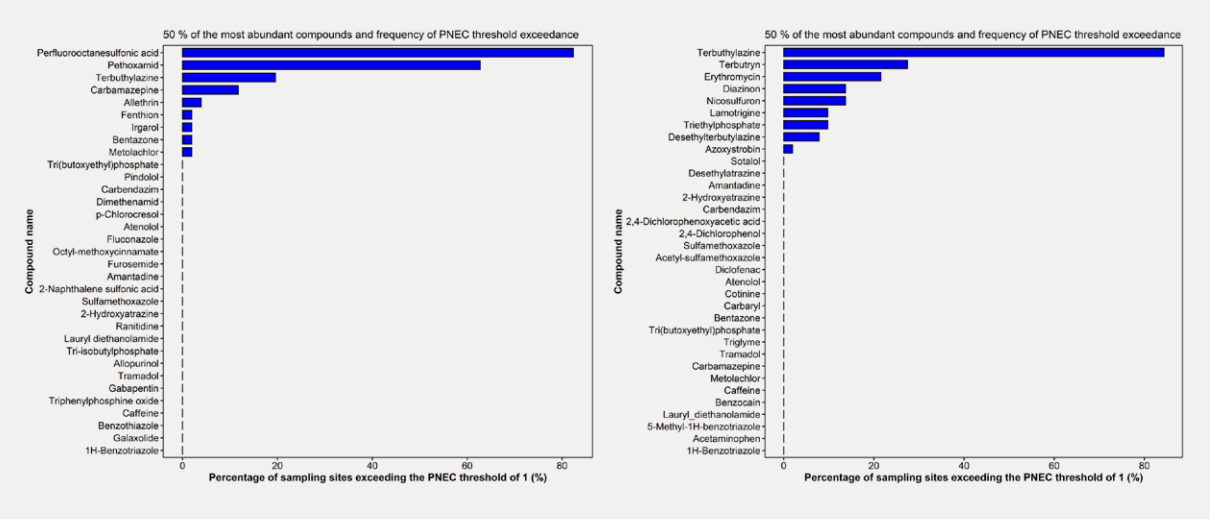

Figure 12: Frequencies of PNEC threshold exceedances of the 50 % most abundant compounds in the JDS4 MAXX LVSPE samples (left) and grab water samples (right). The compounds are ordered by their frequency exceedance and the MEC95.

In Figure 12, the frequencies of exceedance and the 50 % of most abundant compounds (ranked by the frequency of exceedance and the MEC95) are depicted. In the MAXX LVSPE water samples dataset, 66 compounds were matching compounds with TUs and msPAF HUs available, and in the grab water samples dataset, 68 compounds were overlapping. This is caused by missing values in the PNEC database. For the comparability of the approaches, the risk estimation was focussed on the matching compounds. In the MAXX LVSPE and grab water samples, once again, the herbicides, other pesticides and some pharmaceuticals dominated the top-ranking compounds. Interestingly, the herbicide pethoxamid ranked highly in the MAXX LVSPE samples, while it was not relevant in the TU based ranking for algae (Figure 5) and less relevant in the msPAF HU based assessment. The results of the grab water samples were also comparable to the former findings in the TU and msPAF HU based evaluations. The most frequent sensitive group has been algae, followed by daphnids and fish.

35.4 Conclusions

35.4.1 Sampling technologies

The sampling technologies applied by UFZ were proven to address the requirements of current environmental monitoring. The collection and on-site storage in small vials with subsequent cooling or refrigeration of grab water samples with the goal to inject them directly in liquid chromatography high-resolution mass spectrometry is a promising concept. The possibilities of sample loss, alteration and secondary contamination are minimised, because the samples are handled in only few steps from sampling to analysis. The collection of larger volumes of water and enrichment using the MAXX LVSPE (or other solid-phase-extraction based approaches) is appropriate and required for the purpose of the chemical analysis of low abundant contaminants (e.g. steroids) and for the application of the extracts in effect-based analysis. It has to be mentioned that the MAXX LVSPE separates suspended particulate matter and the water phase with glass fibre filters (pore size: 0.63 µm). The MAXX LVSPE technology has been long term developed since 2010. It was optimised and proven for its performance in several studies scaling from small farmland creeks to large rivers such as the Danube or Rhine Rivers and also marine applications. It is a ready to go technology for the current and future environmental monitoring and commercially available.

35.4.2 Target and non-target screening and targeted analysis

The wide-scope target and the non-target screening applications are current and at a high level of quality and reliability. The general results of JDS4 suggest that LC-HRMS-based screening methods are able to provide similar results as targeted LC-MS/MS methods and thus hold the potential to be applied in WFD monitoring if a larger set of compounds should be considered. The methods could be applied to compounds occurring from low ng/L levels up to three- to four-fold concentration ranges without the necessity of sample dilutions. For the analysis of endocrine compounds, a specific developed target analysis method was applied to a larger river for the first time. A range of androgens and progestagens as well as occasionally a few glucocorticoids could be detected at levels below 7.5 ng/L, but mostly below 1 ng/L in extracts from the Danube and its tributaries.

We observed some systematic deviations of the measured concentrations while comparing the results of UFZ with the results of other analytical groups involved in JDS4. Those deviations occurred especially at low levels, which might be related to calibration errors or specific matrix interferences. In selected cases, high and unsystematic deviations were observed among the methods, which require more in-depth investigations in causes and mitigation measures. Also, an improvement and harmonization of QA/QC measures for screening methods and the reporting of data quality is recommended to improve the comparability of different methods and to judge the reliability for individual compounds, as different methods will not perform equally well on a specific compound.

Regardless of the advancements in wide scope target and non-target screening, the analysis of challenging compounds such as steroids will require specifically developed targeted methods for the near future until more sensitive and selective analytical solutions are developed and implemented.

35.4.3 Risk based prioritisation

In order to investigate the potential risks of the compounds detected by the target screening, we the results were compared by applying three different prioritisation approaches discussed as a building block of the SOLUTIONS prioritisation: 1) toxic units (TUs), 2) the multi-substance potentially affected fraction (msPAF) expressed as hazard units (HU) and 3) and hazard quotients (HQ) based on lowest predicted non-effect concentrations (PNEC). The TU-based risk estimation is the approach, which derives the most comprehensive results for different BQEs. It can unravel mixtures of compounds with similar modes of toxic actions. The msPAF HU approach delivers the risk of chemical or a mixture of chemicals for a broader range of organisms and thus indicates the risk or communities. The lowest PNEC method is biased to the most sensitive species or BQE, while TUs and msPAF HUs are related to mixture toxicity. The PNEC is used in a regulatory context, which demands a conservative and threshold-based approach for single chemicals. The assessment of the MAXX LVSPE and direct injected grab water samples using the TU, msPAF HU and PNEC approaches, showed that all three methods can derive rather similar sets of priority compounds. Thus, the combination of these three methods is promising with respect to a comprehensive risk-based prioritisation.

35.4.4 Effect-based methods

The assessment of the MAXX LVSPE samples with EBM proved to be challenging. We observed some issues with blanks and levels of cytotoxicity interfering with some of the bioanalytical tools. Furthermore, some of the bioassays could not be finalised because of the COVID-19 pandemic, i.e. the fish embryo assay with *Danio rerio* and the daphnia immobilisation assay with *Daphnia magna*. The high-throughput reporter gene assays for ERα and GR did not detect any effects, as these were masked by the cytotoxicity levels of the extracts despite and additional clean-up step. These findings show that the detection of endocrine disruption by chemical and biological analysis at the low levels occurring in the Danube is challenging and requires detection limits in the sub-ng/L range and some fractionation of the extract is necessary to overcome the cytotoxicity impeding the detection. The investigation of the samples with the green algae growth inhibition assay with *Scenedesmus vacuolatus* resulted in overall low effects in the bioassay. However, it was possible to distinct different levels of toxicity between the samples. The effects in the tributaries were slightly, but not significantly higher than in the Danube River samples. Despite the observation of toxicity in one travelling and in the machine blank, it was possible to calculate full concentration-effect relationships for the majority of the samples without an assumed interference with background contamination.

It is recommended to implement rigorous quality measures for future larger sampling campaigns and surveys. In order to unravel possible background toxicity issues (blank toxicity), all required solid-phase materials, solvents, reagents and other materials which get in contact with the samples should be assessed in advance and issues should be mitigated by more cleaning or by use of other charges of materials. If possible, only materials of one charge should be used, enough materials should be obtained and stored only for the purpose of the survey or sampling campaign. In a routine monitoring, single failures might be acceptable, but they are not in a laborious survey.

The EBM revealed to be applicable for the assessment of large river systems such as the Danube River reflecting the contamination and risk levels. The Danube River is a very diluted system with a relatively flat variability and the EBM showed exactly that: middle or low effects close to the limit of detection which is defined as the maximum relative extraction factor (REF) that can be achieved before blank toxicity. With the recommended measures for mitigation, they can be minimized but never completely excluded. This is similar to noise in chemical analysis. The result of a “no effect” below a specific REF is a valuable

result, because there is no potential risk. The limited variability of contamination is also reflected by limited variability of the bioassay results.

Nevertheless, it requires more efforts to finally demonstrate the discriminative and diagnostic power of EBTs because of the flat variability of the Danube River. For the demonstration of the power of EBM, the setting of the study should consider site with high or specific contamination and such with low, other or less specific contaminated. The contaminations patterns should be detectable and mapped by the EBM in order to promote them as valuable and diagnostic monitoring tools. In future studies, smaller catchments with as much diversity as possible should be selected to demonstrate the applicability of EBM. This does clearly not hold for the Danube River. Last, but not least, the low effects, for example shown in the algae assays, also indicate a good status despite the fact that especially algae are the most sensitive group unravelled by the risk-based prioritisation of target screening data.

35.5 References

ALBERGAMO V., SCHOLLÉE J. E., SCHYMANSKI E. L., HELMUS R., TIMMER H., HOLLENDER J., DE VOOGT P. (2019): Nontarget screening reveals time trends of polar micropollutants in a riverbank filtration system. Environmental Science & Technology 53: 7584–7594.

ALTENBURGER R., BRACK, W., BURGESS R. M., BUSCH W., ESCHER B. I., FOCKS A., HEWITT L. M., JACOBSEN B. N., DE ALDA M. L., AIT-AISSA S., ET AL. (2019): Future water quality monitoring: improving the balance between exposure and toxicity assessments of real-world pollutant mixtures. Environmental Sciences Europe 31: 12.

ALYGIZAKIS N. A., OSWALD P., THOMAIDIS N. S., SCHYMANSKI E. L., AALIZADEH R., SCHULZE T., OSWALDOVA M., SLOBODNIK J. (2019): NORMAN digital sample freezing platform: A European virtual platform to exchange liquid chromatography high resolution-mass spectrometry data and screen suspects in "digitally frozen" environmental samples. TrAC Trends in Analytical Chemistry 115: 129–137.

ARLE J., MOHAUPT V., KIRST I. (2016): Monitoring of Surface Waters in Germany under the Water Framework Directive—A Review of Approaches, Methods and Results. Water, 8: 217.

BECKERS L.-M., BRACK W., DANN J. P., KRAUSS M., MÜLLER E., SCHULZE, T. (2020): Unravelling longitudinal pollution patterns of organic micropollutants in a river by non-target screening and cluster analysis. Science of The Total Environment 727: 138388.

BRACK W. (2019): Solutions for present and future emerging pollutants in land and water resources management. Policy briefs summarizing scientific project results for decision makers. Environmental Sciences Europe 31: 74.

BRACK, W., ESCHER, B. I., MÜLLER, E., SCHMITT-JANSEN, M., SCHULZE, T., SLOBODNIK, J., HOLLERT, H. (2018): Towards a holistic and solution-oriented monitoring of chemical status of European water bodies: how to support the EU strategy for a non-toxic environment? Environmental Sciences Europe 30: 33.

CARVALHO R. N., ARUKWE A., AIT-AISSA S., BADO-NILLES A., BALZAMO S., BAUN A., BELKIN S., BLAHA L., BRION F., CONTI D., ET AL. (2014): Mixtures of Chemical Pollutants at European Legislation Safety Concentrations: How Safe Are They? Toxicological Sciences 141: 218–233.

CARPENTER C. M. G., WONG L. Y. J., GUTEMA D. L., HELBLING D. E. (2019): Fall creek monitoring station: using environmental covariates to predict micropollutant dynamics and peak events in surface water systems. Environmental Science & Technology 53: 8599–8610.

DE ZWART, D., POSTHUMA, L. (2005). Complex mixture toxicity for single and multiple species: Proposed methodologies. Environmental Toxicology and Chemistry 24: 2665–2676.

DÜHRKOP K., FLEISCHAUER M., LUDWIG M., AKSENOV A. A., MELNIK A. V., MEUSEL M., DORRESTEIN P. C., ROUSU J., BÖCKER S. (2019). SIRIUS 4: a rapid tool for turning tandem mass spectra into metabolite structure information. Nature Methods 16: 299–302.

ESCHER B. I., NEALE P.A., VILLENEUVE D. (2018): The advantages of linear concentration-response curves for in vitro bioassays with environmental samples. Environmental Toxicology and Chemistry 37: 2273–2280.

EUROPEAN UNION (2000): Directive 2000/60/EC of the European Parliament and of the Council of 23 October 2000 establishing a framework for community action in the field of water policy.

EUROPEAN UNION (2013): Directive 2013/39/EU of the European Parliament and the Council amending Directives of 12 August 2013 amending Directives 2000/60/EC and 2008/105/EC as regards priority substances in the field of water policy.

FAUST M., BACKHAUS T., ALTENBURGER R., DULIO V., VAN GILS J., GINEBREDA A., KORTENKAMP A., MUNTHE J., POSTHUMA L., SLOBODNIK J., ET AL. (2019): Prioritisation of water pollutants: the EU Project SOLUTIONS proposes a methodological framework for the integration of mixture risk assessments into prioritisation procedures under the European Water Framework Directive. Environmental Sciences Europe 31: 66.

GADALETA, D., LOMBARDO, A., TOMA, C., BENFENATI, E. (2018): A new semi-automated workflow for chemical data retrieval and quality checking for modeling applications. Journal of Cheminformatics 10: 60.

GAWEL, A., SEIWERT, B., SÜHNHOLZ, S., SCHMITT-JANSEN, M. AND MACKENZIE, K. (2020). In-situ treatment of herbicide-contaminated groundwater–Feasibility study for the cases atrazine and bromacil using two novel nanoremediation-type materials. Journal of Hazardous Materials 393: 122470.

GENOLINI C., ALACOQUE X., SENTENAC M., ARNAUD C. (2015): kml and kml3d: R Packages to cluster longitudinal data. Journal of Statistical Software 65: 1–34.

HASHMI M.A.K., KRAUSS M., ESCHER E. I., TEODOROVIC I., BRACK W. (2020): Effect-directed analysis of progestogens and glucocorticoids at trace concentrations in river water. Environmental Toxicology and Chemistry 39: 189-199.

HELMUS R., LAAK T., DE VOOGT P., WEZEL A., SCHYMANSKI E. (2020): Patroon: open source software platform for environmental mass spectrometry based non-target screening. Available at: doi:10.21203/rs.3.rs-36675/v1.

HOLLENDER J., SCHYMANSKI E. L., SINGER H. P., FERGUSON P. L. (2017): nontarget screening with high resolution mass spectrometry in the environment: ready to go? Environmental Science & Technology 51: 11505–11512.

ICPDR (2020): Danube River Basin Water Quality Database. Available at: https://www.icpdr.org/wq-db [Accessed 17 June 2020].

KÖNEMANN S., KASE R., SIMON E., SWART K., BUCHINGER S., SCHLÜSENER M., HOLLERT H., ESCHER B. I., WERNER I., AÏT-AÏSSA S., ET AL. (2018): Effect-based and chemical analytical methods to monitor estrogens under the European Water Framework Directive. TrAC Trends in Analytical Chemistry 102: 225–235.

KÖNIG, M., ESCHER, B. I., NEALE, P. A., KRAUSS, M., HILSCHEROVÁ, K., NOVÁK, J., TEODOROVIĆ, I., SCHULZE, T., SEIDENSTICKER, S., KAMAL HASHMI, M. A., ET AL. (2017). Impact of untreated wastewater on a major European river evaluated with a combination of in vitro bioassays and chemical analysis. Environmental Pollution 220: 1220–1230.

KORTENKAMP A., FAUST M., BACKHAUS T., ALTENBURGER R., SCHOLZE M., MÜLLER C., ERMLER S., POSTHUMA L., BRACK W. (2019): Mixture risks threaten water quality: the European Collaborative Project SOLUTIONS recommends changes to the WFD and better coordination across all pieces of European chemicals legislation to improve protection from exposure of the aquatic environment to multiple pollutants. Environmental Sciences Europe, 31: 69.

KRAUSS M., HUG C., BLOCH R., SCHULZE T., BRACK W. (2019): Prioritising site-specific micropollutants in surface water from LC-HRMS non-target screening data using a rarity score. Environmental Sciences Europe 31: 45.

LOOS R., MARINOV D., NAPIERSKA D., LETTIERI T. (2018): Review of the 1st Watch List under the Water Framework Directive and recommendations for the 2nd Watch List. JRC. Available at: https://doi.org/10.2760/614367.

LUDWIG M., NOTHIAS L.-F., DÜHRKOP K., KOESTER I., FLEISCHAUER M., HOFFMANN M. A., PETRAS D., VARGAS F., MORSY M., ALUWIHARE L., ET AL. (2020): Database-independent molecular formula annotation using Gibbs sampling through ZODIAC. Nature Machine Intelligence 2: 629–641.

MANSOURI K., GRULKE C. M., JUDSON R. S., WILLIAMS A. J. (2018): OPERA models for predicting physicochemical properties and environmental fate endpoints. Journal of Cheminformatics 10: 10

MIJANGOS L., KRAUSS M., DE MIGUEL L., ZIARRUSTA H., OLIVARES M., ZULOAGA O., IZAGIRRE U., SCHULZE T., BRACK W., PRIETO A., ET AL. (2020): Application of the Sea Urchin embryo test in toxicity evaluation and effect-directed analysis of wastewater treatment plant effluents. Environmental Science & Technology 54: 8890–8899.

MÜLLER M. E., ESCHER B. I., SCHWIENTEK M., WERNEBURG M., ZARFL C., ZWIENER C. (2018): Combining in vitro reporter gene bioassays with chemical analysis to assess changes in the water quality along the Ammer River, Southwestern Germany. Environmental Sciences Europe 30: 20.

NEALE P. A., AIT-AISSA S., BRACK W., CREUSOT N., DENISON M. S., DEUTSCHMANN B., HILSCHEROVA K., HOLLERT H., KRAUSS M., NOVÁK J., ET AL. (2015): Linking in vitro effects and detected organic micropollutants in surface water using mixture toxicity modeling. Environmental Science & Technology 49: 14614–14624.

NEALE P. A., BRACK W., AIT-AISSA S., BUSCH W., HOLLENDER J., KRAUSS M., MAILLOT-MARECHAL E., MUNZ N., SCHLICHTING R., SCHULZE T., ET AL. (2018): Solid-phase extraction as sample preparation of water samples for cell-based and other in vitro bioassays. Environmental Science: Processes & Impacts 20: 493–504.

NORMAN NETWORK (2020): NORMAN Substance Database. Available at: https://www.norman-network.com/nds/susdat/ [Accessed 10 October 2020].

OECD (2004): Test No. 202: Daphnia sp., acute immobilisation test. OECD Publishing.

OECD (2013): Test No. 236: Fish embryo acute toxicity (FET) test. OECD Publishing.

PETER K. T., TIAN Z., WU C., LIN P., WHITE S., DU B., MCINTYRE J. K., SCHOLZ N. L., KOLODZIEJ E. P. (2018): Using high-resolution mass spectrometry to identify organic contaminants linked to urban stormwater mortality syndrome in Coho Salmon. Environmental Science & Technology 52: 10317–10327.

POSTHUMA L., GILS J. VAN ZIJP, M. C., VAN DE MEENT D., DE ZWART, D. (2019a): Species sensitivity distributions for use in environmental protection, assessment, and management of aquatic ecosystems for 12 386 chemicals. Environmental Toxicology and Chemistry 38: 905–917.

POSTHUMA L., MUNTHE J., VAN GILS J., ALTENBURGER R., MÜLLER C., SLOBODNIK J., BRACK W. (2019b): A holistic approach is key to protect water quality and monitor, assess and manage chemical pollution of European surface waters. Environmental Sciences Europe, 31: 67.

RUFF M., MUELLER M. S., LOOS M., SINGER H. P. (2015): Quantitative target and systematic non-target analysis of polar organic micro-pollutants along the river Rhine using high-resolution mass-spectrometry – identification of unknown sources and compounds. Water Research 87: 145–154.

SCHULZE T., AHEL M., AHLHEIM J., AÏT-AÏSSA S., BRION F., DI PAOLO C., FROMENT J., HIDASI A. O., HOLLENDER J., HOLLERT H., ET AL. (2017): Assessment of a novel device for onsite integrative large-volume solid phase extraction of water samples to enable a comprehensive chemical and effect-based analysis. Science of The Total Environment 581–582: 350–358.

SERRA H., BRION F., CHARDON C., BUDZINSKI H., SCHULZE T., BRACK W., AÏT-AÏSSA S. (2020). Estrogenic activity of surface waters using zebrafish- and human-based in vitro assays: The Danube as a case-study. Environmental Toxicology and Pharmacology 78: 103401.

SILVA E., RAJAPAKSE N., KORTENKAMP A. (2002): Something from "Nothing" - Eight weak estrogenic chemicals combined at concentrations below NOECs produce significant mixture effects. Environmental Science & Technology 36: 1751–1756.

SPRAGUE J. B. (1970): Measurement of pollutant toxicity to fish, II - utilization and applying bioassays 467 results. Water Research 4: 3–32.

TOUSOVA Z., FROMENT J., OSWALD P., SLOBODNÍK J., HILSCHEROVA K., THOMAS K. V., TOLLEFSEN K. E., REID M., LANGFORD K., BLAHA L. (2018): Identification of algal growth inhibitors in treated waste water using effect-directed analysis based on non-target screening techniques. Journal of Hazardous Materials 358: 494–502.

TOUSOVA Z., OSWALD P., SLOBODNIK J., BLAHA L., MUZ M., HU M., BRACK W., KRAUSS M., DI PAOLO C., TARCAI Z., ET AL. (2017): European demonstration program on the effect-based and chemical identification and monitoring of organic pollutants in European surface waters. Science of The Total Environment: 601–602: 1849–1868.

UFZ DEPARTMENT OF ECOLOGICAL CHEMISTRY (2019): ChemProp. Available at: https://www.ufz.de/index.php?en=34593.

US EPA (2011): 40 CFR Part 136, Appendix B to Part 136 - Definition and Procedure for the Determination of the Method Detection Limit-Revision 1.11.

US EPA (2020): ECOTOXicology knowledgebase (ECOTOX); data release 15 September 2020. Available at https://cfpub.epa.gov/ecotox

VÄLITALO P., MASSEI R., HEISKANEN I., BEHNISCH P., BRACK W., TINDALL A. J., DU PASQUIER D., KÜSTER E., MIKOLA A., SCHULZE T., ET AL. (2017): Effect-based assessment of toxicity removal during wastewater treatment. Water Research 126: 153–163.

VON DER OHE P. C., DULIO V., SLOBODNIK J., DE DECKERE E., KÜHNE R., EBERT R.-U., GINEBREDA A., DE COOMAN W., SCHÜÜRMANN G., BRACK W. (2011): A new risk assessment approach for the prioritization of 500 classical and emerging organic microcontaminants as potential river basin specific pollutants under the European Water Framework Directive. 2011/03/19 ed. Science of the Total Environment 409: 2064–2077.

36 Prioritisation of Danube River Basin Specific Pollutants using the NORMAN Prioritisation Framework

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Abstract

The samples of Danube River water, wastewater, groundwater, sediments and biota obtained during JDS4 were screened for several thousands of organic pollutants and their transformation products by wide-scope target screening (>2,600 substances) and suspect screening (>65,000 substances) techniques. The NORMAN prioritisation scheme was used to identify priority compounds for further actions. According to this scheme the analysed substances are classified into six 'action categories' where, e.g., Category 1 corresponds to substances that need regulatory monitoring, while Category 2 suggests compounds with a need for further monitoring data (Danube Watch List), etc. In addition to the allocation to specific action categories, the substances detected in the samples were prioritised in each matrix separately using exposure, hazard and risk scores in line with the NORMAN prioritisation approach. The risk score, expressing at how many sites and how much the ecotoxicity threshold value of a pollutant is exceeded, was used as a primary indicator to rank substances within each category. From the results of this prioritisation approach on surface water target screening data it appears that only three (PFOS, cybutryne, cypermethrin; out of 45) WFD priority substances are of concern in the Danube River Basin (DRB). Instead, more attention should be paid to the surface water monitoring of six Watch List substances and additional 44 candidate RBSPs. Biota results indicated that monitoring of three legacy substances (BDEs, mercury and PFOS) might be justified, and 16 additional compounds should be recommended as candidate RBSPs. Several substances frequently detected at high concentration levels in wastewater effluents were identified as a clear source of the candidate RBSPs in the surface water. Pollutants present in samples of groundwater used for production of drinking water from seven sites in the DRB do not seem to pose significant risk. Suspect screening revealed numerous substances in each studied matrix, which might be of concern at the DRB level. A wealth of chemical target analysis and screening data obtained during JDS4 make the DRB arguably the best investigated river basin in Europe and globally. The obtained data stored in a well-organised database system could be used by the EC to support its 'zero-pollution policy' and to provide evidence of the need for restrictions / ban on the production, use or import of certain identified priority chemicals in the future.

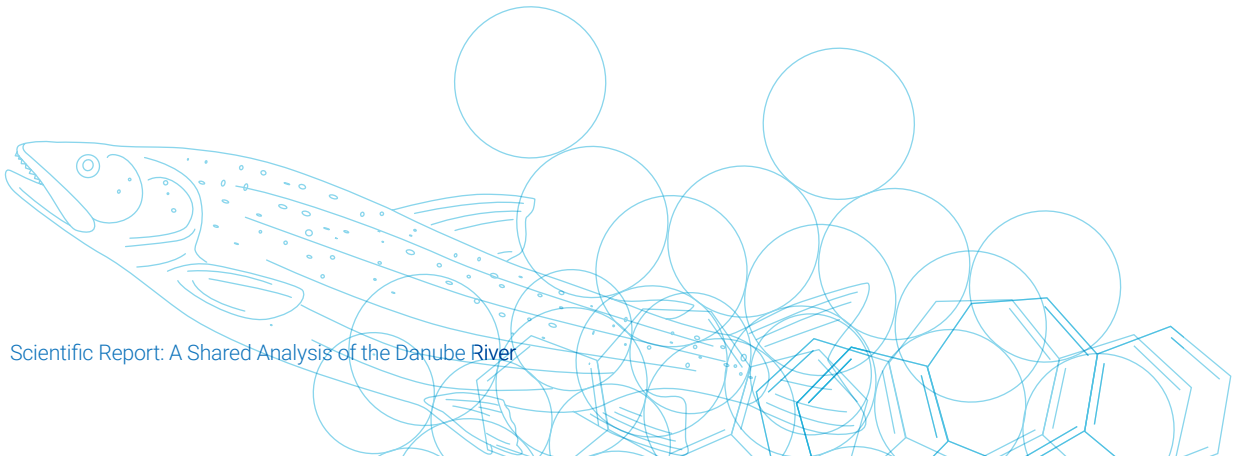
36.1 Introduction

There is increasing evidence that biodiversity and human health can be adversely affected by toxic chemicals present in the environment. More than 350,000 chemicals are manufactured nowadays on an industrial scale with a potential to get into the environment and food chain. Not all of these chemicals are dangerous though, and it is a global challenge to clearly distinguish the toxic chemicals from those which are harmless, and to ban the production, use or import of substances which may threaten the ecosystems and human health.

The NORMAN Association is a network of more than 80 organisations in Europe, North America and Asia dealing with all aspects of contaminants of emerging concern (CECs) in the environment (Dulio et al., 2020) in close collaboration with the EC Services (e.g. JRC, EEA, ECHA). Over the last decade, NORMAN experts have developed a prioritisation methodology (Dulio and von der Ohe et al., 2013), which was applied previously in JDS3 and now in JDS4. The overall prioritisation procedure is carried out in two successive steps. In the first tier, a decision tree classifies chemicals into six categories, considering evidence of exposure and potential risk and existing knowledge gaps, thereby suggesting actions to be taken by the research community and public authorities (Table 1). The second tier entails the prioritisation of the substances within each (action) category, on the basis of criteria / indicators defined for each category (see Fig. 1).

In summary, the criteria and cut-off values applied for the categorisation process at the European level are reported in the Supplementary material, Table 1. The cut-off values can be adapted according the geographical scale of the prioritisation exercise. Subsequently, indicators from three groups: (i) exposure, (ii) hazard and (iii) risk (for more details, see Dulio and van der Ohe et al., 2013) are used to rank the substances.

The prioritisation exercise was performed on the unique dataset of wide-scope target and suspect screening data obtained within JDS4 with the goal to identify Danube RBSPs in water and biota compartments. Additionally, the study aimed at the assessment of chemical pollution risks for sediment and groundwater matrices.



36.2 Methods

Target screening

Each sample was submitted to target screening of more than 2,600 substances, analysed in JDS4 reference laboratories (for a list, see Chapter 2). The list of target substances was selected by the MA EG of the ICPDR prior to the survey. The wide-scope target screening substances were a sub-set of 106,932 compounds registered in the NORMAN Substance Database (SusDat; https://www.norman-network.com/nds/susdat/), identified as relevant pollutants from existing studies or regulations, *i.a.* REACH compounds, pesticides, pharmaceuticals, biocides and their transformation products. Detailed findings are discussed in Chapters. 23 - 31, 33 - 37 and 39. The results, compiled in standardised Data Collection Templates (DCTs), were uploaded into the NORMAN EMPODAT database (https://www.norman-network.com/nds/empodat/). The database is directly feeding into the prioritisation tool, able to perform all steps and related calculations leading to the categorisation & prioritisation of substances as described in the NORMAN Prioritisation Framework. The tool allows for testing various scenarios, e.g. considering different matrices, adjustment of the categorisation / prioritisation criteria to European or regional conditions, etc.

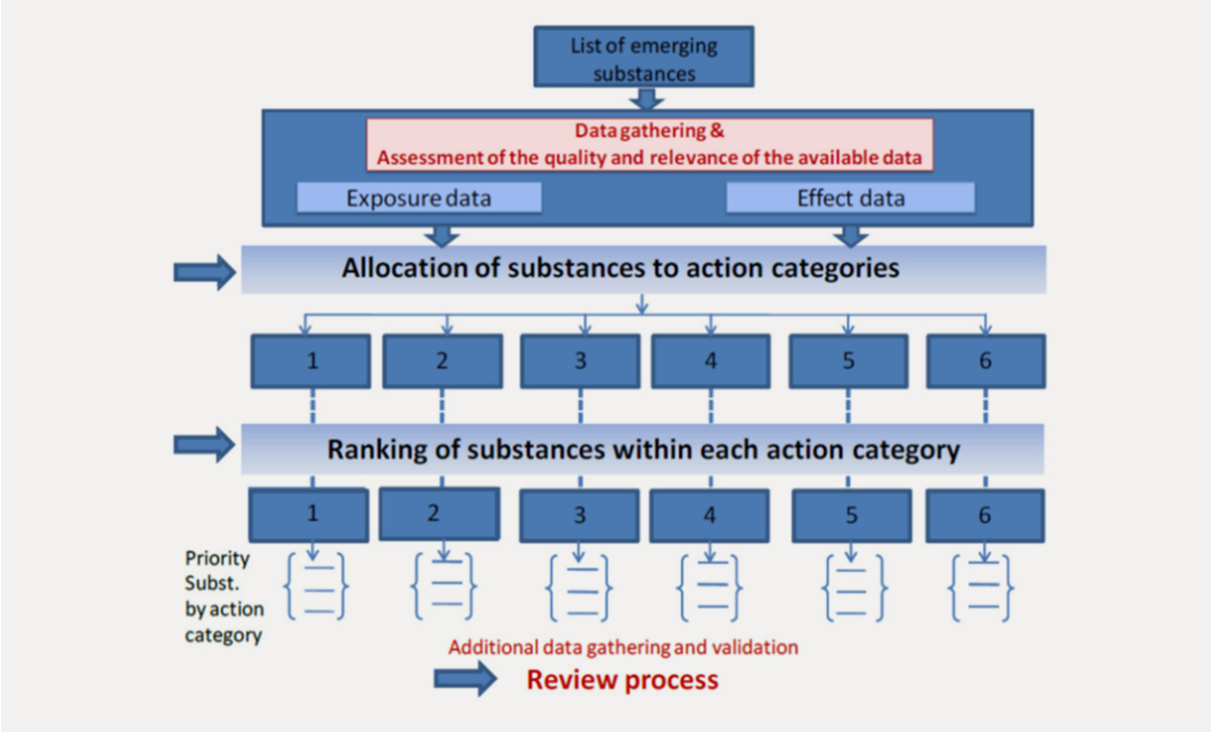


Figure 1: Flow chart of the methodology for categorisation and ranking of emerging substances.

Regarding the initial criteria for categorisation of substances, it has been decided by the MA EG to use criteria allowing for a direct link of assessments made in the DRB with results applicable at the EU scale, *i.e.*: **analyses in a minimum of 4 countries, 100 sites with measurements and with LOQmin < lowest PNEC, as well as 50 sites with measurements >LOQ**, (see Supplementary material, Table 1). It has been decided that only results from JDS4 would be considered in this study in order to avoid bias from the 'old' (>6 years; JDS3, national) monitoring surveys. As a consequence, none of the substances could be allocated to Category 1 (regulatory monitoring needed). All substances appear as substances for which further monitoring is needed to confirm evidence of the identified potential risks.

Suspect screening

Suspect screening of 65,691 compounds from NORMAN SusDat was performed in each of the samples, including their semi-quantification using LC-ESI-HRMS and GC-APCI-HRMS data stored in the NORMAN Digital Sample Freezing Platform (DSFP, http://www.norman-data.eu/; Alygizakis et al., 2019;). DSFP is a novel tool allowing for retrospective screening of suspects and identification of unknown compounds in environmental samples. An overview of the frequency of appearance (FoA) of the screened substances and assessment of their semi-quantified concentration data against the ecotoxicity threshold values (frequency of exceedance of PNEC; FoE)) are presented in the Supplementary material, Tables 5a-d.

Table 1: Six action categories based on evidence of exposure and potential risk and different types of knowledge gaps.

Cat.	Action category	Current situation
1	Integration in **routine monitoring** and **derivation** of legally binding **EQS**	**Sufficient evidence of exposure and adverse effects at environmental concentration**
2	**Screening** studies for information about current exposure	**Hazard assessment** is based on **experimental data** BUT **few monitoring data**
3	**Rigorous hazard assessment**	**Evidence of exposure** BUT **hazard** assessment is based on **predicted toxicity** (P-PNEC)
4	**Improvement of analytical methods** required	**Hazard assessment** is based on **experimental data** BUT **analytical capabilities** not yet satisfactory
5	**Screening** studies AND **rigorous hazard assessment**	No or **few monitoring data** AND **hazard assessment** is based **on predicted toxicity** (P-PNEC)
6	**Monitoring efforts** for these compounds could be **reduced**	Toxicity data are **sufficient for the derivation of an EQS** and there is evidence that the **exposure does not pose a hazard to ecosystems**

Ecotoxicity data

PNEC/EQS values for surface water, sediment and biota were taken from the NORMAN Database System (NDS) – Ecotoxicology database, https://www.norman-network.com/nds/ecotox/. The database also contains legacy EQSs for WFD priority substances and ecotoxicity threshold values for Watch List substances proposed by DG ENV.

The freshwater $PNEC_{fw}$ were derived within the NORMAN network by using a fine-tuned QSAR (Quantitative Structure Activity Relationships) modelling programme (Aalizadeh et al., 2017) for all SusDat substances (available for 64,445 substances as of 20 September 2020), as well as existing experimental toxicity data for ca. 1,100 substances.

PNEC values for biota were transformed from existing PNEC values previously derived for freshwater for almost all NORMAN SusDat compounds, using the equation $PNEC_{fw}$*BCF (fish) and $PNEC_{fw}$*BCF/4 (for molluscs), where $PNEC_{fw}$ is the PNEC for freshwater and BCF is the bioconcentration factor for fish (for more details see Dulio and von der Ohe et al., 2013).

BCF values were retrieved from the US EPA Comptox Chemical Dashboard (https://www.epa.gov/chemical-research/comptox-chemicals-dashboard) and archived in the NORMAN Substance Factsheets database (https://www.norman-network.com/nds/factsheets/). **It should be stressed that the QSAR-predicted P-PNEC values are only an estimate and should be replaced by experimentally-based and commonly agreed values at the regional DRB or EU level before implementation of the identified priority contaminants in the regulation.**

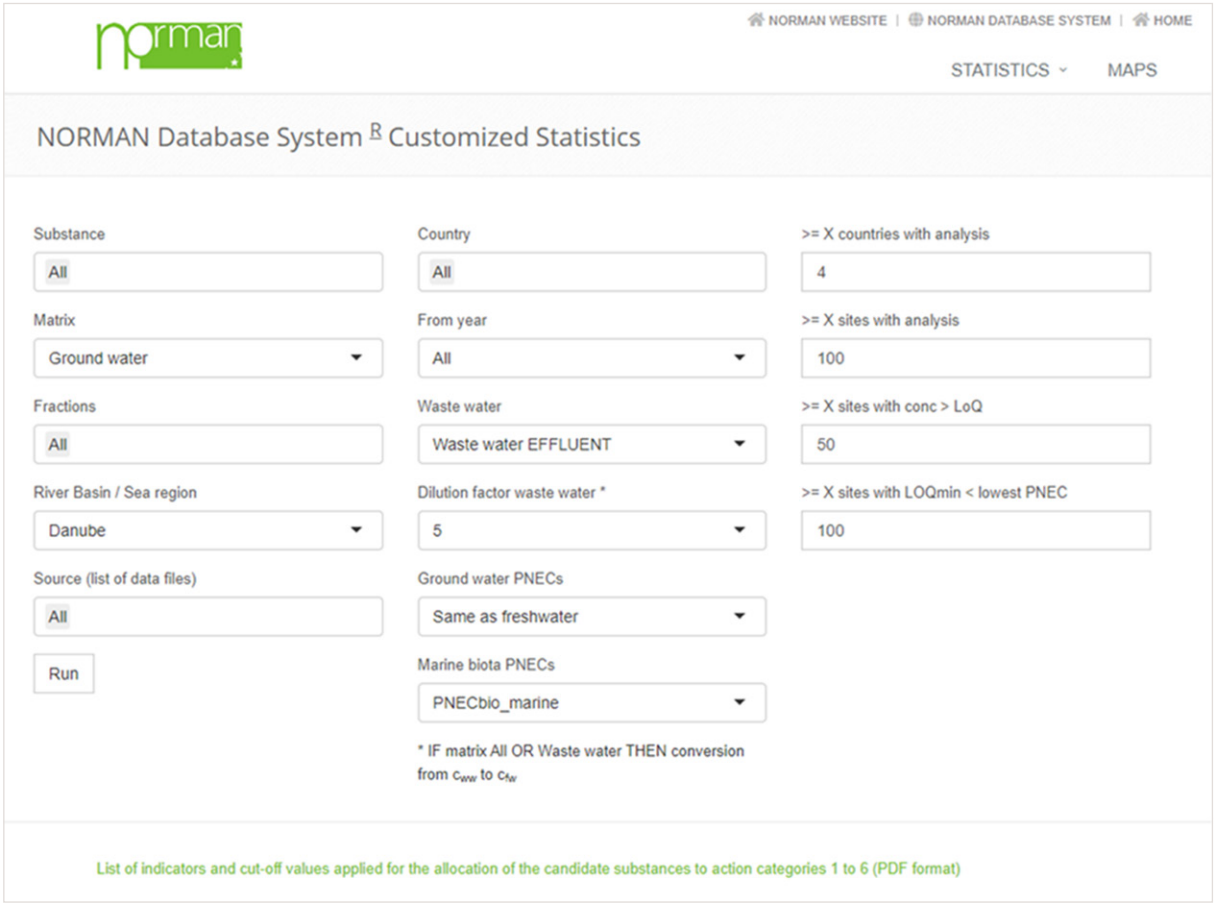


Figure 2: Graphic user interface of the NORMAN on-line prioritisation tool.

Prioritisation process

The prioritisation process was performed on the two datasets (wide-scope target screening data and suspect screening data), following by two separate, complementary workflows.

a) Prioritisation based on wide-scope target screening data

The prioritisation workflow applied to the target screening dataset was as follows:

1. Compilation of JDS4 data on target substances (sub-set of >2,600 compounds from SusDat) and storing them in the NORMAN Database System (NDS; https://www.norman-network.com/nds/) in a harmonised format (DCTs).
2. Collection of additional data including (eco)toxicological data (PNECs, EQSs), physico-chemical properties (K_{ow}, K_{oc}, BCF); PBMT, ED, CMR classifications for the calculation of the Exposure, Hazard and Risk scores.
3. Running of the automated prioritisation workflow as described in the NORMAN Prioritisation Framework (Dulio and von der Ohe, 2013). At the end of this process each substance was allocated to one of six action categories (see Fig. 1 and Table 1) and ranked according to its final score (within the given action category).
4. Expert discussion and common decision on the inclusion (or deleting) of any Danube RBSPs (*to be completed in discussion with the MA EG of the ICPDR*).
5. The overall iterative process involves a periodic revision of the priority substances in each category whenever, e.g., new information / more reliable data become available or feedback from applied reduction measures is available.

The final score within each action category is the sum of the Hazard, Exposure and Risk scores. However, in this exercise, due to a limited number of samples and information, only the **Risk score** based on the two normalised indicators, namely the extent and frequency of exceedance of PNEC values, were used to rank the compounds:

a) Spatial **Frequency of Exceedance** of the Lowest PNEC (**FoE**) = n / N; where n is the number of sites with MEC_{site}/Lowest PNEC ratios above 1 and N is the total number of sites with analytical measurements for the respective compound. MEC_{site} refers to the measured Maximum Environmental Concentration at one site.

b) **Extent of Exceedance** of the Lowest PNEC (**EoE**) = MEC_{95} / Lowest PNEC; MEC_{95} refers to the 95th percentile of all MEC_{site} values, taking into account that data with real concentrations for at least 20 sites, which are needed for calculation of a MEC_{95} with acceptable confidence.

The resulting EoE ratio is then scaled from 0 to 1: 10≥ EoE ≥1: 0.1 point, 100≥ EoE >10: 0.2 point; 1000≥ EoE >100: 0.5 point; EoE >1000: 1 point. The FoE is a ratio and already scaled from 0 to 1. The Final Risk Score is the sum of FoE and EoE.

Finally, the ranking of substances was also aided by the Exposure Index developed by KEMI, Sweden, which is based on normalised values (between 0-1) reflecting (i) the degree of uncontrolled release during use, (ii) annual tonnage and (iii) range of use on the market. The underlying data are confidential, but the index allows use of this information for prioritisation purposes and is available in the NORMAN NDS.

b) Prioritisation based on suspect screening data

In addition to the procedure described above for the prioritisation of substances based on target monitoring data, a prioritisation of the suspect screening data (*i.e.* 65,691 substances screened in each sample and stored in the DSFP) was also performed. Since the concentration of each detected compound can be estimated (semi-quantified) based on structure similarity to a set of internal standards, it was possible to rank them also based on the exceedance of the respective PNEC values (FoE; Alygizakis et al, 2019).

Additionally, Frequency of Appearance (FoA) (n / N; normalised value, where n is no. of samples in which a substance was detected and N is the total no. of all analysed samples; number between 0 - 1) was considered. Compounds which are frequently found in samples and exhibit potential toxicity (*i.e.* having a high FoE) can then make their way to the prioritisation scheme for target substances. The approach is still being tested with various datasets. However, NORMAN WG-1 is already working on integrating the two procedures (prioritisation based on target screening and suspect screening data) into a single workflow. In this way it will be possible to use retrospective suspect screening of samples to anticipate the relevance / level of priority of suspect compounds for which target monitoring data are still scarce or totally missing.

36.3 Results

Altogether, 51 surface water, 11 wastewater effluent, 11 fish, 48 molluscs, 4 sediment and seven groundwater samples were collected by various sampling teams/techniques and analysed in numerous laboratories in Europe (cf. Chapter 2). A dataset of 306,093 data points (measurements) resulting from chemical target and wide-scope target screening analyses were subjected to prioritisation. High resolution mass spectrometry records of each sample were stored for retrospective suspect screening in the NORMAN DSFP. Each of the results reported below is back-traceable in the NORMAN Database System.

36.3.1 Prioritisation of wide-scope target screening data in surface water

Target screening of 2,608 substances was performed on all JDS4 surface water samples. The results of target screening have shown the presence of 495 substances with concentrations above their Limit of Quantification (LOQ) in at least one sample. Out of these, 53 substances exceeded their toxicity threshold value (PNEC or EQS) in at least one sample (see Table 1), with 30, 9 and 14 compounds in Category 2, 4 and 5, respectively. From the WFD PS, only PFOS, cybutryne and cypermethrin were on the list. Due to extremely low EQS of PFOS in water, its analysis is recommended by EQSD to be carried out in biota. Additionally, six WFD surface water Watch List substances (see highlighted in bold in Table 1) were on the list. Among them, diclofenac was frequently found in the majority of European Watch List sites above the threshold values and it has recently been excluded from the new Watch List update to leave the place to other compounds (https://ec.europa.eu/jrc/en/science-update/updated-surface-water-watch-list-adopted-commission). **These six compounds together with the 44 new substances are potential 50 candidate RBSPs in the surface water compartment of the DRB** and their presence in the basin should be carefully monitored. The list was dominated by pesticides (nicosulfuron, terbuthylazine, 2,4-dichlorophenoxyacetic acid, fipronil, metazachlor, allethrin, fenthion, bentazone, metolachlor, cybutryne, imazamox, 2,4-dichlorophenol, dazomet, pethoxamid, methoprene, spinosyn A, pyrethrin I) and their TPs (TPs of terbuthylazine: 1,3,5-triazin-2(1H)-one, 4-((1,1-dimethylethyl)amino)-6-(ethylamino)-; desethylterbuthylazine), pharmaceuticals (anticonvulsant carbamazepine – also a marker of pollution from wastewater, alpha-blocker telmisartan, antipsychotic ziprasidone, immunosuppressive and anti-inflammatory 7-hydroxymethotrexate, candasertan against high blood pressure, antibiotic vancomycin and dicloxacillin; and drug against osteoporosis raloxifene), personal care products (antiseptic cetylpyridinium, antiseptic and disinfectant benzododecinium, fragrance 6-acetyl-1,1,2,4,4,7-hexamethyltetralin), surfactants (N,N-dimethyldodecan-1-amine, cis-1-(3-chloroallyl)-3,5,7-triaza-1-azoniaadamantane), PFAS compounds (perfluorooctanesulfonamide), biocides (antibacterial product benzyl hexadecyl dimethyl ammonium, disinfectant miristalkonium), novel flame retardants (2-ethylhexyl diphenyl phosphate (EHDP), 3,3',5,5'-tetrabromobisphenol A), plasticisers (bisphenol A bis(3-chloro-2-hydroxypropyl) ether (BADGE*2HCl)) and industrial chemicals (4-(1,1,3,3-tetramethylbutyl)phenol, 2-ethylhexyl-2-cyano-3,3-diphenylacrylate, hexa(methoxymethyl)melamine, 2,4-dichlorobenzoic acid).

Out of 495 detected substances, those of low priority for further monitoring are to be identified among those with the lowest score and no risk of exceedance of the PNEC (*i.e.* FoE = 0). However, it should be recalled that because of the criteria applied for categorisation of the compounds (*i.e.*: compounds sufficiently monitored = compounds analysed in at least 4 countries and in at least 100 sites, see the Supplementary material, Table 1) none of the investigated compounds can be considered as sufficiently monitored at this stage (*i.e.* with sufficient evidence of absence of a threat to the Danube ecosystem).

Only nine out of 50 proposed RBSPs also had a high Hazard score (PBMT; (persistence – P; bioaccumulation – B; mobility – M; toxicity – T; value between 0 – 2). A more exhaustive collection of PBMT information on each of the proposed Danube RBSP is recommended in order to use these data as additional ranking indicator.

The NORMAN network (www.norman-network.net) in cooperation with the LIFE APEX project (https://lifeapex.eu/) is currently working on deriving PBT properties with the use of the QSAR-based JANUS model for all substances in SusDat (>106,000 chemicals and their TPs). Once implemented, the score could be used for the retrospective ranking of the Danube RBSPs using JDS4 data.

25 of the 50 candidate RBSP substances show an Exposure score > 0.5 in Table 1 (highlighted in grey colour). The value of 0.5 has been selected arbitrarily; it indicates that these substances are present in samples across the basin and gives them additional priority.

The Exposure Index KEMI proved to be a valuable tool to confirm the relevance of a compound. This index (value ranging from 0 to 1) was lower than 0.3 only for 19 compounds in Table 1, indicating that a majority of top-ranked substances are produced in large annual tonnage with widespread use. The 'low scoring' substances were almost exclusively pesticides and their TPs, pharmaceuticals and biocides, which do not fall under REACH legislation. Therefore, it is expected that their Exposure Index could be underestimated. The NORMAN network is working currently on the development of specific indices for pharmaceuticals and biocides. National water agencies in the DRB should take into account the findings of this JDS4 prioritisation exercise for pesticides and their TPs frequently quantified, with potential risk of exceedance of the PNECs, in view of the application of possible restriction measures.

36.3.2 Prioritisation of wide-scope target screening data in biota

Out of 2,360 substances screened in biota samples, 101 compounds were determined in at least one sample, and 23 (after excluding four naturally occurring substances) exceeded their PNEC or EQS value in at least one sample (Table 2). The prioritised list was dominated by the legacy flame retardants of brominated diphenylethers (BDEs), mercury and PFOS. Tens of BDEs present in mixtures are difficult to distinguish by common analytical techniques and therefore only a sub-selection of 'markers' (congener numbers 28, 47, 99, 100, 153 and 154) are used for WFD regulatory monitoring. **The presence of BDEs in JDS4 samples is overwhelming and therefore further monitoring of the marker compounds, together with mercury and PFOS, is strongly recommended.** WFD PS heptachlor epoxide was determined only in one sample at concentration exceeding its EQS, however, the method LOQ was higher than the EQS and more exceedances could be overlooked. Obviously more evidence is needed to include this substance among the Danube RBSPs.

Interestingly, four substances (PFOS, industrial chemical 4-(1,1,3,3-tetramethylbutyl)phenol, as well as the pesticides imazamox and methoprene) were also responsible for risks in surface water (cf. text above). Regarding the other substances, the list comprised the antibiotic sulfamethoxazole, sleeping pill compound temazepam, herbicide imazapyr, insecticide propoxur, androgen and anabolic steroids norethandrolone, methenolone and 3-hydroxyestran-17-one, antipsychotic drug sulpiride, high blood cholesterol drug lovastatin, chemotherapy medication cytarabine and herbicide barban – already banned for use.

The exposure index by KEMI did not indicate values over 0.3 threshold for pesticides, pharmaceuticals and androgen and anabolic steroids. However, this might be due to the fact that pharmaceuticals and pesticides do not fall under REACH legislation (lack of information on these chemicals in the REACH registry).

36.3.3 Prioritisation of wide-scope target screening data in sediments

In total, 2,317 substances were screened for in sediment samples and 51 compounds were detected in at least one sample. Out of these, 15 substances exceeded their PNEC value (Supplementary material, Table 2). The Lowest PNEC values for sediments were calculated from existing freshwater PNECs, using equation $PNEC_{fw}$*2.6*(0.615+0.019*Koc)' according to the NORMAN Prioritisation Framework. It is important to stress that these PNEC values do not reflect the ecotoxicity for benthic species. They represent the concentration of a given contaminant in sediment, equivalent to its concentration in the water column when the system is at the equilibrium. In general, however, no sediment toxicity threshold values are set at the EU level and only an indication of trends of pollution by individual substances is required by the EQSD.

The highlighted industrial chemicals 2-ethylhexyl-2-cyano-3,3-diphenylacrylate, 4-(1,1,3,3-tetramethylbutyl) phenol, the marker of wastewater pollution – carbamazepine and barban – a banned herbicide, were also prioritised in both the surface water and biota matrices (see text above). The list also comprised the antibiotic sulfadiazine; insecticide methiocarb, adenine 9-beta-D-arabinofuranoside – an antiviral drug which is active against herpes; cadusafos – an insecticide and nematicide that is not approved for use in the EU; novel flame retardant TPHP; surfactants N-methyldodecylamine and N,N-dimethyltetradecylamine; a medication used to treat a variety of parasites in animals – fenbendazole; antibacterial and anticoccidial drug sulfaclozine and the industrial chemical benzenemethanol, .alpha.-(aminomethyl)-.

36.3.4 Prioritisation of wide-scope target screening data in wastewater

Out of 2,516 substances screened for in wastewater effluent samples, 465 were detected above the LOQ in at least one sample and 28 compounds exceeded their ecotoxicity threshold value (Supplementary material, Table 3). Only one WFD PS (PFOS) and five Watch List substances were prioritised as of concern at the basin scale. A 'default' dilution factor 5 was used when converting wastewater into freshwater concentrations, in order to be able to compare them against freshwater PNECs. It should be '10' for large rivers or '2' for small streams, however, in JDS4 most of the studied WWTPs were located on medium size tributaries to the Danube. It is obvious that wastewater is a significant contributor to the surface water pollution – 18 substances from the list (highlighted in yellow) were assigned as potential RBSPs (see also Table 1). The list would be extended or shortened when different dilution factor would be used for calculations. An investigative screening of all 465 'wastewater substances' would therefore be recommended in the longer-term.

36.3.5 Prioritisation of wide-scope target screening data in groundwater

Out of 2,561 screened for substances, 148 were present in at least one sample, but 47 were found at more than 50% of the sites (FoA >0.5 in Supplementary material, Table 4). The list comprised 16 pesticides and their TPs, followed by pharmaceuticals and their TPs, industrial chemicals, surfactants and personal care products. It should be noted that four banned pesticides on the list of WFD PS (atrazine, simazine, lindane, p,p'-DDE) and Watch List substances diclofenac and sulfamethoxazole were frequently detected. Six substances present in groundwater samples (highlighted in yellow) were also among the proposed RBSPs in surface water.

None of the pesticides and their TPs exceeded the legacy threshold value 0.1 µg/l. However, their widespread presence, together with numerous other chemicals, in groundwater used for production of drinking water is of concern. A dedicated study on mixture toxicity, e.g. with the use of a battery of bioassays aiming at revealing human health effects, is recommended.

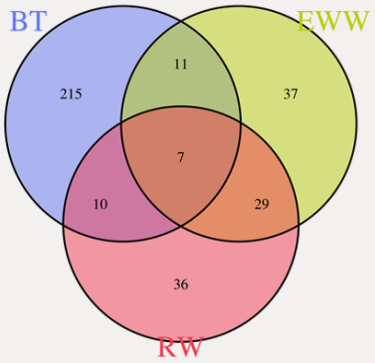
36.3.6 Suspect screening

Suspect screening of all JDS4 samples/all matrices for 65,691 substances from the NORMAN SusDat database revealed the presence of ca. 2,000 compounds and their TPs in at least one sample. Substances also detected by wide-scope target screening were removed, resulting in the final list of 935 additional compounds, accompanied with their semi-quantitative concentration estimates. All raw mass chromatograms allowing to reproduce these results, or even to look for specific compounds of interest retrospectively, are stored in the NORMAN DSFP. For the sake of presenting the results, an arbitrary threshold value of '0.5' was chosen for the Frequency of Exceedance of PNEC value (FoE; with predicted PNEC values for most substances) as an indicator of potential risk to ecosystems. The substances are reported in the Supplementary material, Tables 5a-e; listing 35, 84, 49, 38 and 44 compounds in surface water, biota, sediment, wastewater and groundwater matrices, respectively. The biota list contains several naturally occurring substances (highlighted in green). No effort was made at this stage to remove them, in order not to overlook any important compound. Rather surprisingly, 16 substances were indicated as exceeding threshold value 0.1 µg/l in groundwater and certainly deserve more attention in future investigative screening campaigns.

Overlap of the detected substances among the investigated matrices were examined through Venn diagrams with use of an application (https://norman-data.eu/JDS4_suspect-screening). More specifically, the overlap was investigated in three cases: (i) between river water (RW) and biota (BT) with frequency of appearance (FoA) in selected matrices higher than or equal to 50%, (ii) among RW, BT and effluent wastewater (EWW) with FoA in selected matrices higher than or equal to 50% and (iii) among all investigated matrices: RW, BT, EWW, groundwater (GW) and river sediment (SED) without any restriction for FoA. All presented substances were tentatively identified.

For case (i), seventeen substances that were commonly detected in both RW and BT are shown in the Supplementary material, Fig. 1. It should be noted that most of the substances belong to industrial chemicals, many of which are registered under REACH. The Hazard Index provided by KEMI indicated their high annual production and use in Europe.

For case (ii), seven substances were commonly detected in BT, EWW and RW (see Fig. 3). Finally, in case (iii) 34 compounds were detected in all investigated samples. The substances were presented with decreasing concentration levels in the generated heatmap (see Supplementary material, Fig. 2). In-depth investigation of the sources of these substances and verification of the identity of these substances are recommended as future actions.



NORMAN ID	Compound name	Molecular Formula	CAS number	Chemical structure	Exposure Score (KEMI)	Hazard Score (KEMI)	FoA (All matrices)	FoE (All matrices)
NS00011498	Nonanedioic acid	$C_9H_{16}O_4$	123-99-9		0.51	0.01	84.51	22.86
NS00003596	Acetanilide	C_8H_9NO	103-84-4		0.46	0.02	73.09	5.45
NS00010583	9,10-Dihydroxystearic acid	$C_{18}H_{36}O_4$	120-87-6		0.11	0.02	85.2	85.2
NS00040486	Isodecyl undecyl phthalate	$C_{29}H_{48}O_4$	96507-81-2		0.13	0.13	62.91	62.91
NS00025333	N-Nitroso-N'-methylpiperazine	$C_5H_{11}N_3O$	16339-07-4		0.09	0.13	83.63	0
NS00010316	8-Hydroxychinolin	C_9H_7NO	148-24-3		0.41	0.61	74.65	60.14
NS00019508	1-Propanol, 2-[1-(3,3-dimethylcyclohexyl)ethoxy]-2-methyl-, 1-propanoate	$C_{17}H_{32}O_3$	141773-73-1		0.42	0.09	66.9	46.9

Figure 3: Commonly detected substances in JDS4 biota (BT), effluent wastewater (EWW) and river water (RW) samples. Seven substances were detected in all matrices and observed with frequency of appearance (FoA) higher than 50%.

The results demonstrate that it is feasible (i) to create a baseline of the 'universe of Danube substances' and (ii) to funnel down thousands of substances from suspect screening into a manageable list of primary suspects, which could be included in the follow-up investigative screening studies. The prioritisation procedure, which also takes suspect screening into account, is being currently automated and could be re-applied in the near future to the existing JDS4 dataset. In general, suspect screening provides valuable 'early warning' and 'safety net' information, limiting chances that toxic and ubiquitous substances remain undetected. Here, one should be aware that less polar GC-amenable and highly polar (mobile) compounds were not fully covered by the used techniques and data evaluation workflows.

36.4 Conclusions

A prioritisation scheme applied on the results obtained by wide-scope target screening of JDS4 water, biota, sediments, wastewater and groundwater samples has proven to be practicable and feasible. Using the NORMAN Prioritisation Framework and NORMAN Database System infrastructure, it was possible to propose a list of candidate Danube RBSPs in surface water (50 substances) and biota (19 substances) with their tentative ecotoxicity threshold values. Prioritisation of pollutants in sediment samples was only indicative, since more effort is needed to establish robust ecotoxicity threshold values. Pollutants in groundwater samples were at concentrations not causing health risk concerns according to present legislation. However, it is recommended to analyse them in future with a battery of bioassays to account for mixture toxicity. Wastewater was among the major sources of surface water pollution by candidate RBSPs.

Considering the limited number of samples and that the JDS4 screening was only a 'snapshot' in time, it was recommended that all substances, which exceeded their ecotoxicity threshold values in at least one sample/at one site should be monitored until a critical mass of data is available.

Suspect screening revealed the presence of ca. 2,000 substances in JDS4 samples, out of which 935 were not detected by the wide-scope target screening methods. The comparison of semi-quantitative concentrations against toxicity threshold values showed that 35, 84, 49, 38 and 44 substances were exceeding their PNEC values in more than 50% of the surface water, biota, sediment, wastewater and groundwater samples, respectively. The results were used to create a 'universe of DRB pollutants', and the obtained mass spectrometry information (digitally stored samples) is available for retrospective screening. An on-going work aims at the hazard assessment of these substances by compiling their persistence and bioaccumulation properties.

In summary, prioritisation of wide-scope target and suspect screening has indicated that these novel monitoring techniques have a high potential compared to traditional monitoring approaches focusing on few legacy substances and provide both 'early-warning' and 'safety net' signals needed for a more holistic chemicals management. The current traditional monitoring programmes applied in compliance with the current environmental legislation are not sufficient for exhaustive assessment and management of chemical risks in the DRB.

36.5 References

DULIO V. ET AL., 2020, The NORMAN Association and the European Partnership for Chemicals Risk Assessment (PARC): let's cooperate!, Environ Sci Eur, 32:100, https://doi.org/10.1186/s12302-020-00375-w.

DULIO V., VON DER OHE P.C. AT AL., 2013, NORMAN Framework for prioritisation of emerging substances, ISBN: 978-2-9545254-0-2, http://www.norman-network.net/sites/default/files/files/Publications/NORMAN_prioritisation_Manual_15%20April2013_final%20for%20website-f.pdf.

ALYGIZAKIS N.A. ET AL., 2019, NORMAN digital sample freezing platform: a European virtual platform to exchange liquid chromatography high resolution-mass spectrometry data and screen suspects in "digitally frozen" environmental samples. TrAC Trends Anal Chem 115:129–137, DOI: 10.1016/j.trac.2019.04.008.

AALIZADEH R. ET AL., 2017, Prediction of acute toxicity of emerging contaminants on the water flea Daphnia magna by Ant Colony Optimization-Support Vector Machine QSTR models. Environ. Sci. Processes Impacts 19, 438–448.

More detailed information on results of prioritisation is provided in the Supplementary material to this chapter.

36.6 Supplementary Material

Table 1: List of indicators and cut-off values applied for the allocation of the candidate substances to action categories 1 to 6.

Categories / indicators	Cat. 1		Cat. 2			Cat. 3	Cat. 4			Cat. 5			Cat. 6	
	1A	1B	2A	2B	2F	3	4A	4B	4F	5A	5B	5F	6A	6B
Analyses available in relevant matrix(ces)	Yes	Yes	Yes/No	Yes	No data	Yes	Yes or No data	Yes	Yes OR No data	Yes/No	Yes	No data	Yes	Yes
≥ 4 countries with analysis	Yes	Yes	<4 countries AND/OR <100 sites	Yes	No data	Yes	<4 countries AND/OR <100 sites	Yes	-	<4 countries AND/OR <100 sites	Yes	No data	Yes	Yes
≥100 sites with analysis	Yes	Yes		Yes	No data	Yes		Yes	-		Yes	No data	Yes	Yes
≥ 50 sites with analyses > LOQ (recent data)	Yes	No	-	No	No data	Yes OR No AND LOQ_{max}< PNEC	-	No	-	-	No	No data	Yes	No
More than 100 sites with LOQmin <PNEC	-	Yes	-	No	No data		No or No data	No	No or No data	-	No	No data	-	Yes
LOQ_{min} <PNEC	-	Yes	LOQ_{min} ($_{datasets}$) < PNEC OR $LOQ_{literat}$ <PNEC	LOQ_{min} ($_{datasets}$) < PNEC OR $LOQ_{literat}$ <PNEC	No data	-	No or No data	No	No or No data	LOQ_{min} ($_{datasets}$) < PNEC OR $LOQ_{literat}$ <PNEC	LOQ_{min} ($_{datasets}$) < PNEC OR $LOQ_{literat}$ <PNEC	No data	-	Yes
$LOQ_{literat}$ <PNEC	-	-			Yes	-	No	No	No data			Yes	-	-
Suff. data for hazard assessment	Yes	Yes	Yes	Yes	Yes	No	-	-	-	No	No	No	Yes	Yes
Potential risk identified (MEC_{95} / Lowest PNEC≥1)	Yes	Yes*	-	-	No data	-	-	-	No data	-	-	No data	No	No[9]

** For Category 1B and Category 6B, MECsite_max is used instead of MEC95 to calculate the risk ratio.*

Table 2: Prioritisation of chemicals determined by wide-scope target screening in the JSD4 sediment samples; ranked by the Category and Final Risk score. For abbreviations and details, see text. PNECs from the NORMAN Ecotoxicology database, hazard score and exposure index KEMI from the NORMAN Database System as of 10 October 2020 were used for the risk assessment. WFD PS are highlighted in bold.

No.	Substance	CAS no.	Lowest PNEC [µg/kg dw]	No. of Analyses	No. of Analyses with conc > LoQ	Max. Conc. [µg/kg dw]	Category	FoE score	EoE score	Final RISK score	Final HAZARD score	Final EXPOSURE score	Final EXPOSURE index KEMI
1	2-Ethylhexyl-2-cyano-3,3-diphenylacrylate	6197-30-4	52.4	4	3	162	2A	0.75	0.1	0.85		0.75	0.42
2	Sulfadiazine	68-35-9	7.3	4	2	120	2A	0.5	0.25	0.75		0.5	0.5
3	Methiocarb	2032-65-7	0.1	4	2	6	2A	0.5	0.25	0.75		0.5	0.38
4	Carbamazepine	298-46-4	1.7	4	1	4	2A	0.25	0.1	0.35		0.25	0.3
5	4-(1,1,3,3-Tetramethylbutyl)phenol	140-66-9	12.3	4	1	26	2A	0.25	0.1	0.35		0.25	0.52
6	Adenine 9-beta-D-arabinofuranoside	5536-17-4	5.9	4	4	149	4A	1	0.25	1.2		1	0.13
7	Cadusafos	95465-99-9	0.03	4	1	1	4A	0.25	0.25	0.5		0.25	0.2
8	Adenine*	520-75-2	16.9	4	4	1545	5A	1	0.25	1.2		1	0.02
9	Triphenyl phosphate (TPHP)	115-86-6	6	4	4	9	5A	0.75	0.1	0.85	1.125	1	0.68
10	N-Methyldodecylamine	7311-30-0	9	4	2	540	5A	0.5	0.25	0.75		0.5	0.13
11	Fenbendazole	43210-67-9	8.4	4	2	26	5A	0.5	0.1	0.6		0.5	0.18
12	Sulfaclozine	102-65-8	17	4	2	32	5A	0.25	0.1	0.35		0.5	0.13
13	N,N-Dimethyltetradecylamine	112-75-4	6.1	4	3	17	5A	0.25	0.1	0.35		0.75	0.51
14	Barban	101-27-9	83.1	4	3	84	5A	0.25	0	0.25		0.75	0.19
15	Benzenemethanol, .alpha.-(aminomethyl)-	7568-93-6	205	4	4	213	5A	0.25	0	0.25		1	0.19

** Naturally occurring substance; ecotoxicological relevance to be verified.*

Table 3: Prioritisation of chemicals determined by wide-scope target screening in the JDS4 wastewater effluent samples obtained from 11 WWTPs; ranked by the Category and Final Risk score. Dilution factor 5 was used to convert concentrations of wastewater to surface water in order to make them comparable to freshwater PNECs. For abbreviations and details, see text. PNECs from the NORMAN Ecotoxicology database, hazard score and exposure index KEMI from the NORMAN Database System as of 10 October 2020 were used for the risk assessment. WFD PS and Watch List substances are highlighted in bold.

No.	Substance	CAS no.	Lowest PNEC	No. of Analyses	No. of Analyses with conc > LoQ	Max. Conc. [µg/l]	Category	FoE score	EoE score	Final RISK score	Final HAZARD score	Final EXPOSURE score	Final EXPOSURE index KEMI
1	**Diclofenac****	**15307-86-5**	**0.05**	**33**	**33**	**0.63**	**2A**	**1**	**0.25**	**1.2**		**1**	**0.13**
2	Carbamazepine	298-46-4	0.05	33	33	0.25	2A	0.5	0.1	0.6		1	0.3
3	Fipronil	120068-37-3	0.00077	33	14	0.049	2A	0.32	0.25	0.57		0.57	0.45
4	**Imidacloprid****	**138261-41-3**	**0.0083**	**33**	**29**	**0.065**	**2A**	**0.32**	**0.1**	**0.42**		**0.9**	**0.53**
5	**Perfluorooctanesulfonic acid***	**1763-23-1**	**0.00065**	**22**	**7**	**0.14**	**2A**	**0.23**	**0.1**	**0.33**		**0.36**	**0.34**
6	2-Ethylhexyl diphenyl phosphate (EHDP)	1241-94-7	0.018	11	11	0.026	2A	0.18	0.1	0.28		1	0.46
7	1,3,5-Triazin-2(1H)-one, 4-((1,1-dimethylethyl) amino)-6-(ethylamino)-	66753-07-9	0.0073	22	5	0.018	2A	0.14	0.1	0.24		0.3	0.02
8	Nicosulfuron	111991-09-4	0.009	22	3	0.066	2A	0.14	0.1	0.24		0.18	0.21
9	**Azithromycin****	**83905-01-5**	**0.019**	**22**	**16**	**0.041**	**2A**	**0.09**	**0.1**	**0.19**		**0.82**	**0.23**
10	Metazachlor	67129-08-2	0.02	33	6	0.19	2A	0.05	0	0.05		0.26	0.34
11	Chlorotoluron	15545-48-9	0.1	22	4	0.23	2A	0.05	0	0.05	1.25	0.18	0.4
12	**Ciprofloxacin****	**85721-33-1**	**0.089**	**33**	**11**	**0.12**	**2A**	**0.05**	**0**	**0.05**		**0.61**	**0.31**
13	Diazinon	333-41-5	0.01	33	10	0.061	2A	0.05	0	0.05	1.125	0.51	0.35
14	Telmisartan	144701-48-4	0.00055	22	19	4.63	4A	0.86	0	0.86		0.91	0.04
15	**17beta-Estradiol****	**50-28-2**	**0.0004**	**22**	**5**	**0.0008**	**4A**	**0.23**	**0.1**	**0.33**		**0.3**	**0.37**
16	Pethoxamid	106700-29-2	0.00049	22	3	0.0056	4A	0.09	0.1	0.19		0.15	0.37

17	Spinosyn A	131929-60-7	0.0027	11	1	0.03	4A	0.09	0.1	0.19		0.091	0.38
18	Hexa(methoxymethyl)melamine	68002-20-0	0.057	11	11	9.84	5A	1	0.5	1.5		1	0.55
19	Galaxolidone	256393-37-0	0.1	11	11	1.98	5A	1	0.25	1.2		1	0.02
20	Candesartan	139481-59-7	0.0031	22	15	0.25	5A	0.64	0.25	0.89		0.76	0.13
21	Diisononyl cyclohexane-1,2-dicarboxylate	166412-78-8	0.046	11	11	0.13	5A	0.45	0.1	0.55		1	0.61
22	Fipronil sulfide	120067-83-6	0.012	22	3	0.029	5A	0.09	0.1	0.19		0.15	0.17
23	Ketoconazole	65277-42-1	0.0081	22	5	0.016	5A	0.09	0.1	0.19		0.3	0.25
24	Fendiline	13042-18-7	0.024	11	1	0.034	5A	0.09	0	0.09		0.091	0.11
25	Lorazepam	846-49-1	0.096	22	5	0.15	5A	0.05	0	0.05		0.27	0.18
26	Benzododecinium	139-07-1	0.062	22	17	0.17	5A	0.05	0	0.05		0.85	0.66
27	Celecoxib	169590-42-5	0.09	22	4	0.24	5A	0.05	0	0.05		0.21	0.07
28	Ebastine	90729-43-4	0.0028	22	1	0.11	5A	0.05	0	0.05		0.061	0.04

** WFD priority substances. ** WFD surface water Watch List substances.*

Table 4: Prioritisation of chemicals determined by wide-scope target screening in the seven JDS4 groundwater samples; ranked by the Final Exposure score. For abbreviations and details, see text. PNEC 0.1 µg/l, hazard score and exposure index KEMI from the NORMAN Database System as of 10 October 2020 were used for the risk assessment. Pesticides and their TPs are highlighted in grey shade.

No.	Substance	CAS no.	No. of Analyses	No. of Analyses with conc > LoQ	Max. Conc. [µg/l]	Category	FoE score	EoE score	Final RISK score	Final HAZARD score	Final EXPOSURE score	Final EXPOSURE index KEMI
1	Carboxin	5234-68-4	7	7	0.00002	2A	0	0	0	0.625	1	0.32
2	Benzoic acid	65-85-0	7	7	0.0151	2A	0	0	0		1	0.81
3	Bisphenol A	80-05-7	7	7	0.0159	2A	0	0	0	1	1	0.8
4	Pyrethrin I	121-21-1	7	7	0.00016	2A	0	0	0		1	0.49
5	Tetraethylene glycol monododecyl ether	5274-68-0	7	7	0.00004	5A	0	0	0		1	0.5
6	Piroxicam	36322-90-4	7	7	0.019	5A	0	0	0		1	0.17
7	Metolachlor ESA	171118-09-5	21	17	0.0041	5A	0	0	0		0.94	0.1
8	Benzododecinium	139-07-1	14	11	0.014	5A	0	0	0		0.93	0.66
9	**Atrazine***	**1912-24-9**	**28**	**20**	**0.0029**	**2A**	**0**	**0**	**0**		**0.9**	**0.34**
10	Carbamazepine	298-46-4	21	15	0.0018	2A	0	0	0		0.9	0.3
11	2-Hydroxyatrazine	2163-68-0	14	10	0.0041	2A	0	0	0		0.9	0.09
12	DEET	134-62-3	21	14	0.0024	2A	0	0	0	0.625	0.89	0.52
13	Caffeine	58-08-2	14	9	0.035	2A	0	0	0		0.88	0.56
14	Diisononyl cyclohexane-1,2-dicarboxylate	166412-78-8	7	6	0.062	4A	0	0	0		0.86	0.61
15	N,N-Bis(2-hydroxyethyl)dodecanamide	120-40-1	14	8	0.009	5A	0	0	0		0.86	0.57
16	Fenofibric acid	42017-89-0	7	6	0.017	5A	0	0	0		0.86	0.13
17	Vigabatrin	60643-86-9	7	6	0.052	5A	0	0	0		0.86	0.04
18	N,N-Dimethyldodecylamine-N-oxide	1643-20-5	7	6	0.00051	5A	0	0	0		0.86	0.74
19	Valproic acid	99-66-1	7	6	0.0035	5A	0	0	0		0.86	0.58
20	4-Androsten-11beta-ol-3,17-dione	382-44-5	7	6	0.00008	5A	0	0	0		0.86	
21	Diethylene glycol dimethyl ether	111-96-6	14	7	0.0012	5A	0	0	0		0.83	0.63
22	**Diclofenac****	**15307-86-5**	**21**	**7**	**0.0064**	**2A**	**0**	**0**	**0**		**0.74**	**0.13**

23	Perfluorooctanoic acid	335-67-1	14	7	0.0008	2A	0	0	0		0.74	0.36
24	Metformin	657-24-9	14	6	0.0091	2A	0	0	0		0.71	0.37
25	Dibutyl phthalate	84-74-2	7	5	0.00027	2A	0	0	0	1	0.71	0.67
26	2-Ethylhexyl-2-cyano-3,3-diphenylacrylate	6197-30-4	7	5	0.00059	2A	0	0	0		0.71	0.42
27	Cyromazine	66215-27-8	14	6	0.0071	5A	0	0	0		0.71	0.47
28	Phenoxybenzamide	72084-13-0	14	6	0.00099	5A	0	0	0		0.71	
29	Chloridazon	1698-60-8	21	8	0.0008	2A	0	0	0	0.525	0.7	0.34
30	1,3,5-Triazin-2(1H)-one, 4-((1,1-dimethylethyl) amino)-6-(ethylamino)-	66753-07-9	21	7	0.0025	2A	0	0	0		0.68	0.02
31	Triisobutyl phosphate (TIBP)	126-71-6	14	6	0.0024	2A	0	0	0		0.68	0.67
32	1,2,3-Benzotriazole	95-14-7	21	10	0.024	5A	0	0	0		0.65	0.76
33	**Sulfamethoxazole****	**723-46-6**	**21**	**8**	**0.0023**	**2A**	**0**	**0**	**0**		**0.62**	**0.42**
34	Terbuthylazine	5915-41-3	28	9	0.0009	2A	0	0	0		0.6	0.44
35	Desethylatrazine	6190-65-4	28	9	0.0023	2A	0	0	0		0.6	0.35
36	Cotinine	486-56-6	14	5	0.15	2A	0.14	0.1	0.24		0.6	0.16
37	Lamotrigine	84057-84-1	14	5	0.004	2A	0	0	0		0.6	0.16
38	**Simazine***	**122-34-9**	**28**	**8**	**0.00059**	**2A**	**0**	**0**	**0**		**0.58**	**0.37**
39	**p,p'-DDE***	**72-55-9**	**7**	**4**	**0.00002**	**2A**	**0**	**0**	**0**		**0.57**	**0.27**
40	1,3,5-Triazin-2(1H)-one, 4,6-bis((1-methylethyl)amino)-	7374-53-0	7	4	0.00066	5A	0	0	0		0.57	0.02
41	Cyclohexylamine	108-91-8	7	4	0.036	5A	0	0	0		0.57	0.68
42	Hydroperoxide, 1-phenylethyl	3071-32-7	7	4	0.01	5A	0	0	0		0.57	0.11
43	Oxcarbazepine	28721-07-5	7	4	0.0022	5A	0	0	0		0.57	0.13
44	Perfluorobutanesulfonic acid	375-73-5	7	4	0.00015	5A	0	0	0		0.57	0.22
45	**Lindane***	**58-89-9**	**7**	**4**	**0.00003**	**5A**	**0**	**0**	**0**		**0.57**	
46	10,11-Dihydro-10,11-dihydroxy-carbamazepine		7	4	0.0024	5A	0	0	0		0.57	
47	Desethylterbuthylazine	30125-63-4	28	8	0.00045	2A	0	0	0	1.125	0.54	0.06

** WFD priority substances. ** WFD surface water Watch List substances.*

Table 5a. Substances detected by suspect screening in the JDS4 surface water samples with frequency of exceedance of PNEC (FoE) > 0.5; FoA – frequency of appearance. For more details, see text.

No.	NORMANID	Compounds	PNECfw [µg/l]	FoA	FoE	Final EXPOSURE index KEMI
1	NS00005230	Ximenynic acid	0.11	1.00	1.00	0.15
2	NS00033526	N-Ethyl-1-isopropylcycloheptanecarboxamide	2.62	0.98	0.96	0.09
3	NS00013594	Tridecanedioic acid, dimethyl ester	1	0.94	0.94	0.19
4	NS00028682	4-Ethyl-4-formylhexanenitrile	2.86	0.94	0.94	0.09
5	NS00040486	Isodecyl undecyl phthalate	0.0072	0.92	0.92	0.13
6	NS00008927	Propisochlor	1.95	1.00	0.90	0.11
7	NS00009367	Moxisylyte	2.96	0.96	0.90	0.13
8	NS00019724	1-(2,6,6-Trimethyl-2-cyclohexen-1-yl)-2-butenone	0.11	0.90	0.90	0.49
9	NS00039759	Ethyl 8-(acetoxy)octanoate	7.11	0.88	0.88	0.13
10	NS00000294	1,3,5-Triazin-2(1H)-one, 4-((1,1-dimethylethyl) amino)-6-(ethylamino)-	0.0073	0.86	0.86	0.02
11	NS00056655	Sodium 5-methyl-1H-benzotriazolide	5.9	0.98	0.84	0.32
12	NS00032880	Octyl hydrogen phthalate	0.2	0.84	0.84	0.11
13	NS00010296	Hexa(methoxymethyl)melamine	0.057	0.84	0.84	0.55
14	NS00005146	10-Phenyldecanoic acid	0.38	0.78	0.78	0.02
15	NS00031858	Ammonium octyl sebacate	0.32	0.76	0.76	0.09
16	NS00001491	N-Oleylpalmitic acid amide	0.037	0.75	0.75	0.42
17	NS00022282	Benzoic acid, 2-amino-5-[(4-aminophenyl)methyl]-, methyl ester	0.85	0.78	0.73	0.24
18	NS00010302	Octoxynol-2	0.91	0.73	0.73	0.02
19	NS00002145	Hexa-2,4-dienoic acid	9.26	0.71	0.71	0.67
20	NS00019800	2,3-Benzofuran	5.11	0.94	0.69	0.4
21	NS00019508	1-Propanol, 2-[1-(3,3-dimethylcyclohexyl)ethoxy]-2-methyl-, 1-propanoate	0.27	0.67	0.67	0.42
22	NS00003181	N-(2-Hydroxyethyl)octadecanamide	0.04	0.67	0.67	0.45
23	NS00001445	Benzenepropanal, .beta.-methyl-3-(1-methylethenyl)-	0.5	0.67	0.67	0.19
24	NS00010160	7-Oxa-3,20-diazadispiro[5.1.11.2]heneicosan-21-one, 2,2,4,4-tetramethyl-	0.76	0.65	0.65	0.32
25	NS00039246	1H-Purin-6-amine, N-dodecyl-	0.012	0.63	0.63	0.13
26	NS00005629	Terbutryn sulfoxide	0.045	0.63	0.63	0
27	NS00052280	Sodium 1H-benzotriazolide	7.77	0.98	0.61	0.55
28	NS00013839	Linoleic diethanolamide	0.074	0.57	0.57	0.6
29	NS00003658	Dodecyl(ethylbenzyl)dimethylammonium	0.059	0.57	0.57	0.39
30	NS00013532	CI 75100	0.079	0.57	0.57	0.18
31	NS00023685	[1,1'-Biphenyl]-4-ol, 3-amino-	1.2	0.55	0.53	0.17
32	NS00044882	2-Methyloctadecanoic acid	0.029	0.53	0.53	0.13
33	NS00011466	Isophorone diisocyanate	0.75	0.53	0.51	0.69
34	NS00013874	Tetradecanamide, N-[3-(dimethyloxidoamino)propyl]-	1.78	0.53	0.51	0.41
35	NS00010583	9,10-Dihydroxystearic acid	0.33	0.51	0.51	0.11

Table 5b: Substances detected by suspect screening in the JDS4 biota samples with frequency of exceedance of PNEC (FoE) > 0.5; FoA – frequency of appearance. For more details, see text.

No.	NORMANID	Compounds	PNECbio [µg/kg ww]	FoA	FoE	Final EXPOSURE index KEMI
1	NS00035021	Octanoic anhydride	136.0	1.00	1.00	0.13
2	NS00011498	Nonanedioic acid	136.0	1.00	1.00	0.51
3	NS00010302	Octoxynol-2	256.0	1.00	1.00	0.02
4	NS00005146	10-Phenyldecanoic acid	22.8	1.00	1.00	0.02
5	NS00010828	7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione	1.5	1.00	1.00	0.02
6	NS00029657	Methyl N-ethyl-beta-alaninate	160.0	1.00	1.00	0.11
7	NS00023620	Pyridine, 2-ethenyl-6-methyl-	229.0	1.00	1.00	0.11
8	NS00030859	(2-(Aminomethyl)phenyl)acetic acid	170.0	1.00	1.00	0.13
9	NS00024204	1H-Purine-6,8-dione, 7,9-dihydro-	56.6	1.00	1.00	0.09
10	NS00027511	3-(p-Aminophenyl)propionic acid	67.2	1.00	1.00	0.13
11	NS00009931	Creatine	102.0	1.00	1.00	0.3
12	NS00031858	Ammonium octyl sebacate	11.4	1.00	1.00	0.09
13	NS00010730	Eicosapentaenoic acid	0.7	1.00	1.00	0.19
14	NS00012072	Butyl undec-10-enoate	55.0	1.00	1.00	0.3
15	NS00033286	Phenol, 4-butyl-2,6-bis(1,1-dimethylethyl)-	140.0	1.00	1.00	0.15
16	NS00001041	Epofenonane	13.9	1.00	1.00	0.09
17	NS00036140	1-Eicosanol, hydrogen sulfate	11.8	1.00	1.00	0.13
18	NS00036641	Peroxide, 1-methyl-1-[4-methyl-2(or 3)-(1-methylethyl) phenyl]ethyl 1-methyl-1-phenylethyl	13.7	1.00	1.00	0.11
19	NS00028077	S-Ethyl-l-cysteine	310.0	1.00	1.00	0.11
20	NS00011748	Bacimethrin	71.7	1.00	1.00	0.02
21	NS00025565	N-(3-Aminopropyl)-N'-methylpropane-1,3-diamine	82.7	1.00	1.00	0.09
22	NS00038408	Ethyl cis-4-amino-3-hydroxypiperidine-1-carboxylate	57.5	1.00	1.00	0.09
23	NS00002706	Pleuromutilin	80.2	1.00	1.00	0.22
24	NS00029879	1H,3H,5H-Oxazolo[3,4-c]oxazol-7a(7H)-ylmethyl laurate	48.5	1.00	0.91	0.11
25	NS00039846	(2,2-Bis(hexyloxy)ethyl)benzene	12.0	1.00	0.91	0.09
26	NS00026775	Methyl hydrogen azelate	94.7	0.91	0.91	0.11
27	NS00004924	Cyclooct-4-en-1-yl methyl carbonate	25.0	0.91	0.91	0.43
28	NS00010319	Embelin	1.3	0.91	0.91	0.13
29	NS00029145	Hydroperoxide, (1,4-phenylenebis(1-methylethylidene)) bis-	177.0	0.91	0.91	0.09
30	NS00025758	17alpha-Ethylestradiol 3-methyl ether	161.0	0.91	0.91	0.09
31	NS00030548	Promestriene	38.2	0.91	0.91	0.11
32	NS00012422	6-Ethylideneoctahydro-2H-5,8-methanochromen-2-one	90.7	0.91	0.91	0.36
33	NS00022683	5,9-Pentadecadien-2-one, 6,10,14-trimethyl-	11.6	0.91	0.91	0.19
34	NS00003686	Iloprost	51.2	0.91	0.91	0.02
35	NS00015186	Octanedioic acid	226.0	1.00	0.82	0.42
36	NS00029599	Ethyl 5-methyl-3-oxohexanoate	75.0	1.00	0.82	0.15

No.	NORMANID	Compounds	PNECbio [µg/kg ww]	FoA	FoE	Final EXPOSURE index KEMI
37	NS00039040	11alpha,17,21-Trihydroxy-16beta-methylpregna-1,4-diene-3,20-dione	120.0	0.91	0.82	0.09
38	NS00039532	1-(4-(2-Methoxyethyl)phenyl)ethan-1-one	121.0	0.91	0.82	0.11
39	NS00004343	Eldoral	20.5	0.91	0.82	0.02
40	NS00024727	Methyl 3-oxooctadecanoate	26.1	0.82	0.82	0.11
41	NS00040351	Bis(tert-butyl) 1,4-dihydro-2,6-dimethyl-4-propylpyridine-3,5-dicarboxylate	0.3	0.82	0.82	0.09
42	NS00030835	Methyl (E)-non-6-enoate	164.0	0.82	0.82	0.09
43	NS00012935	Cyclohexanol, 2-methyl-5-(1-methylethenyl)-, (1R,2R,5R)-rel-	35.7	0.82	0.82	0.19
44	NS00002328	Nelarabine	0.6	0.82	0.82	0.02
45	NS00031892	Butyl 5-oxo-L-prolinate	43.3	0.82	0.82	0.09
46	NS00003616	Benzenemethanol, .alpha.,4-dimethyl-	223.0	0.91	0.73	0.25
47	NS00020914	2,2'-[Benzene-1,3-diylbis(oxy)]diethanol	366.0	0.82	0.73	0.28
48	NS00011524	Etienic acid	65.8	0.82	0.73	0.11
49	NS00037296	Diisooctyl isophthalate	0.8	0.73	0.73	0.09
50	NS00010726	Erucamide	1.1	0.73	0.73	0.53
51	NS00037569	Meradine	1.0	0.73	0.73	0.11
52	NS00040183	1-(2,2-Diethoxyethoxy)-4-(1,1-dimethylpropyl)benzene	57.9	0.73	0.73	0.09
53	NS00044950	2,7-Naphthalenedisulfonic acid, 3,6-bis[(4-chloro-2-phosphonophenyl)azo]-4,5-dihydroxy-	0.0	0.73	0.73	0.17
54	NS00049403	2-ethyloctanedioic acid	70.4	1.00	0.64	0.11
55	NS00011498	Nonanedioic acid	136.0	1.00	0.64	0.51
56	NS00010693	Methyl 3,5-bis(tert-butyl)-4-hydroxyhydrocinnamate	262.0	1.00	0.64	0.65
57	NS00026944	N-(o-Tolyl)ethylenediamine	53.3	0.91	0.64	0.09
58	NS00014385	2-Nitrobenzamide	66.0	0.82	0.64	0.13
59	NS00036177	Benzoic acid, 2-[(2-pentyl-2-nonenylidene)amino]-, methyl ester	1.7	0.73	0.64	0.11
60	NS00010307	Acetyl tributyl citrate	22.4	0.64	0.64	0.63
61	NS00003780	3-(2-Amino-2-oxoethyl)-5-methylhexanoic acid	83.9	0.64	0.64	0.09
62	NS00012867	3-(4-Methylcyclohex-3-enyl)but-3-enyl acetate	115.0	0.64	0.64	0.2
63	NS00011977	Benzeneacetic acid, pentyl ester	121.0	0.64	0.64	0.42
64	NS00009175	Tri(2-methyl-2,4-pentanediol)biborate	3.7	0.64	0.64	0.35
65	NS00052824	(4a)-2,15a-dihydroxy-19-norkaur-16-en-18-oic acid	3.8	0.64	0.64	0.09
66	NS00012356	13-Oxabicyclo[10.1.0]tridecane	7.7	0.64	0.64	0.25
67	NS00044426	Linalyl isovalerate	1.3	0.64	0.64	0.27
68	NS00014989	Melezitose	15.6	0.64	0.64	0.15
69	NS00011897	Tigecycline	0.0	0.64	0.64	0.04
70	NS00013126	Phenoxyacetaldehyde	59.7	0.64	0.64	0.32
71	NS00021167	Cyclopropanecarboxylic acid, 2-[1-(3,3-dimethylcyclohexyl)ethoxy]-2-methylpropyl ester	124.0	1.00	0.55	0.19

No.	NORMANID	Compounds	PNECbio [µg/kg ww]	FoA	FoE	Final EXPOSURE index KEMI
72	NS00009556	5'-Uridylic acid	32.6	1.00	0.55	0.15
73	NS00027137	Methyl 3-oxooctanoate	42.4	0.82	0.55	0.11
74	NS00039231	Indoleacetamide	17.5	0.82	0.55	0.13
75	NS00013317	1,3,5-Undecatriene	86.9	0.64	0.55	0.4
76	NS00012524	Furan, 2-heptyl-	256.0	0.64	0.55	0.23
77	NS00040486	Isodecyl undecyl phthalate	0.2	0.55	0.55	0.13
78	NS00022082	Ethyl 2-acetyl-2-allylpent-4-ene-1-oate	11.2	0.55	0.55	0.31
79	NS00026055	Methyl cyclohex-1-ene-1-carboxylate	127.0	0.55	0.55	0.11
80	NS00031551	Dimethyl undecanedioate	159.0	0.55	0.55	0.15
81	NS00038848	3-Phenylpropyl cyclohexanepropionate	23.1	0.55	0.55	0.09
82	NS00005235	Isopropyl lauroyl sarcosinate	30.4	0.55	0.55	0.13
83	NS00022279	Gefarnate	29.4	0.55	0.55	0.24
84	NS00004612	Colfosceril palmitate	4.0	0.55	0.55	0.16

Table 5c: Substances detected by suspect screening in the JDS4 sediment samples with frequency of exceedance of PNEC (FoE) > 0.5; FoA – frequency of appearance. For more details, see text.

No.	NORMANID	Compounds	PNECsed [µg/kg dw]	FoA	FoE	Final EXPOSURE index KEMI
1	NS00035021	Octanoic anhydride	36.4	1.00	1.00	0.13
2	NS00026775	Methyl hydrogen azelate	594	1.00	1.00	0.11
3	NS00009663	Diricinoleate	0.28	1.00	1.00	0.55
4	NS00032880	Octyl hydrogen phthalate	14.5	1.00	1.00	0.11
5	NS00010316	8-Hydroxychinolin	3.18	1.00	1.00	0.41
6	NS00005581	Ibuprofenol acetate	1.01	1.00	1.00	NA
7	NS00002145	Hexa-2,4-dienoic acid	14.8	1.00	1.00	0.67
8	NS00026365	Diethyl (3-oxopropyl)malonate	34.6	1.00	1.00	0.09
9	NS00006955	Denatonium benzoate	2.22	1.00	1.00	0.73
10	NS00040482	Decyl nonyl phthalate	2.83	1.00	1.00	0.15
11	NS00012867	3-(4-Methylcyclohex-3-enyl)but-3-enyl acetate	33.4	1.00	1.00	0.2
12	NS00027559	1-Heptanamine, N,N-diheptyl-	0.67	1.00	1.00	0.15
13	NS00012932	Benzoic acid, 2-[(2-methylundecylidene)amino]-, methyl ester	2.9	1.00	1.00	0.19
14	NS00037296	Diisooctyl isophthalate	4	1.00	1.00	0.09
15	NS00013880	1-Tetradecanol, 1-propanoate	10.5	1.00	1.00	0.32
16	NS00010766	Isopropylphenyl diphenyl phosphate	0.13	1.00	1.00	0.15
17	NS00032359	N-(2-Hydroxy-1,1-dimethylethyl)undecanamide	67.5	1.00	1.00	0.09
18	NS00040887	Bis(2,4-di-tert-butyl-6-methylphenyl)ethyl phosphite	31	1.00	1.00	0.36
19	NS00027471	Benzenemethanol, 4-(phenylamino)-.alpha.,.alpha.-bis[4-(phenylamino)phenyl]-	2.42	1.00	1.00	0.13
20	NS00022691	1-Buten-3-yne	11.4	1.00	1.00	0.28
21	NS00010583	9,10-Dihydroxystearic acid	0.53	0.75	0.75	0.11

No.	NORMANID	Compounds	PNECsed [µg/kg dw]	FoA	FoE	Final EXPOSURE index KEMI
22	NS00013947	PEG-3 Lauramide	1.87	0.75	0.75	0.26
23	NS00010307	Acetyl tributyl citrate	18	0.75	0.75	0.63
24	NS00013839	Linoleic diethanolamide	51.3	0.75	0.75	0.6
25	NS00003181	N-(2-Hydroxyethyl)octadecanamide	25.8	0.75	0.75	0.45
26	NS00022192	3,6,9,12,15-Pentaazaheptadecane-1,17-diamine	36.8	0.75	0.75	0.19
27	NS00039246	1H-Purin-6-amine, N-dodecyl-	2.79	0.75	0.75	0.13
28	NS00019611	Hexadecanamide, N,N'-1,2-ethanediylbis-	50.7	0.75	0.75	0.34
29	NS00014115	Octadecanamide, N-[3-(dimethyloxidoamino)propyl]-	0.35	0.75	0.75	0.15
30	NS00033679	N,N-Dibutyloctanamide	22	0.75	0.75	0.13
31	NS00032044	2-Butene, 1-chloro-3-methyl-	26.2	0.75	0.75	0.2
32	NS00030255	Tetracosyl 5-oxo-L-prolinate	3.68	0.75	0.75	0.09
33	NS00027594	2-Butenedioic acid (2Z)-, mono-2-propenyl ester	22.8	0.75	0.75	0.13
34	NS00016452	6-Amino-3-methyl-4-phenyl-1,4-dihydropyrano[2,3-c] pyrazole-5-carbonitrile	0.29	0.75	0.75	0
35	NS00021167	Cyclopropanecarboxylic acid, 2-[1-(3,3-dimethylcyclohexyl)ethoxy]-2-methylpropyl ester	16	1.00	0.50	0.19
36	NS00010843	1-Decanamine	18.9	0.75	0.50	0.3
37	NS00007746	N-Butylbenzenesulfonamide	124	0.50	0.50	0.35
38	NS00003658	Dodecyl(ethylbenzyl)dimethylammonium	0.095	0.50	0.50	0.39
39	NS00013594	Tridecanedioic acid, dimethyl ester	60.3	0.50	0.50	0.19
40	NS00032233	1-Isopropyl-N,N-dimethylcyclohexanecarboxamide	84.5	0.50	0.50	0.09
41	NS00010726	Erucamide	4.85	0.50	0.50	0.53
42	NS00020277	(Z)-1,2-Dimethylcyclohexane	38.5	0.50	0.50	0.38
43	NS00020914	2,2'-[Benzene-1,3-diylbis(oxy)]diethanol	496	0.50	0.50	0.28
44	NS00007546	cyclohexanediacetic acid	58	0.50	0.50	0.23
45	NS00020684	Benzenetrimethanol, ar-(2-propenyloxy)-	450	0.50	0.50	0.28
46	NS00034112	Laurocapram	30.6	0.50	0.50	0.13
47	NS00039366	Hexanedioic acid, octadecyl ester	8.33	0.50	0.50	0.11
48	NS00030589	3,5-Dimethoxy-alpha,alpha-dimethylbenzyl alcohol	150	0.50	0.50	0.09
49	NS00010360	Felbamate	45	0.50	0.50	0.13

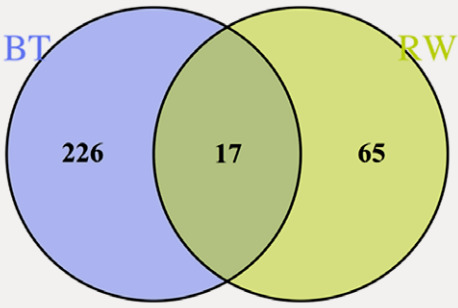
Table 5d: Substances detected by suspect screening in the JDS4 wastewater effluent samples with frequency of exceedance of PNEC (FoE) > 0.5; FoA – frequency of appearance. For more details, see text.

No.	NORMANID	Compounds	PNECfw [µg/l]	FoA	FoE	Final EXPOSURE index KEMI
1	NS00010583	9,10-Dihydroxystearic acid	0.33	1.00	1.00	0.11
2	NS00019508	1-Propanol, 2-[1-(3,3-dimethylcyclohexyl)ethoxy]-2-methyl-, 1-propanoate	0.27	1.00	1.00	0.42
3	NS00040486	Isodecyl undecyl phthalate	0.0072	1.00	1.00	0.13
4	NS00013839	Linoleic diethanolamide	0.074	1.00	1.00	0.6

No.	NORMANID	Compounds	PNECfw [µg/l]	FoA	FoE	Final EXPOSURE index KEMI
5	NS00028682	4-Ethyl-4-formylhexanenitrile	2.86	1.00	1.00	0.09
6	NS00010296	Hexa(methoxymethyl)melamine	0.057	1.00	1.00	0.55
7	NS00011466	Isophorone diisocyanate	0.75	1.00	1.00	0.69
8	NS00009663	Diricinoleate	0.18	0.91	0.91	0.55
9	NS00032880	Octyl hydrogen phthalate	0.2	0.91	0.91	0.11
10	NS00003658	Dodecyl(ethylbenzyl)dimethylammonium	0.059	0.91	0.91	0.39
11	NS00040482	Decyl nonyl phthalate	0.0056	0.91	0.91	0.15
12	NS00000631	4'-Hydroxydiclofenac	0.22	0.91	0.91	0.02
13	NS00035021	Octanoic anhydride	0.32	0.82	0.82	0.13
14	NS00026775	Methyl hydrogen azelate	45.3	0.82	0.82	0.11
15	NS00026812	N-Hexadecylacrylamide	0.026	0.82	0.82	0.09
16	NS00007582	5-Butyl-5-ethyl-2-(2,4,6-tri-tert-butylphenoxy)-1,3,2-dioxaphosphinane	0.014	0.82	0.82	0.39
17	NS00012432	2-Cyclohexene-1-carboxylic acid, 2-methyl-4-oxo-6-pentyl-, ethyl ester	3.53	0.91	0.73	0.41
18	NS00021167	Cyclopropanecarboxylic acid, 2-[1-(3,3-dimethylcyclohexyl)ethoxy]-2-methylpropyl ester	0.38	0.73	0.73	0.19
19	NS00012867	3-(4-Methylcyclohex-3-enyl)but-3-enyl acetate	2.82	0.73	0.73	0.2
20	NS00012932	Benzoic acid, 2-[(2-methylundecylidene)amino]-, methyl ester	0.013	0.73	0.73	0.19
21	NS00031858	Ammonium octyl sebacate	0.32	0.73	0.73	0.09
22	NS00022004	1,4-Benzenedicarboxaldehyde, 2,3,5,6-tetramethyl-, dioxime	1.89	0.73	0.73	0.2
23	NS00000479	Xylometazoline	0.16	0.73	0.73	0.13
24	NS00056655	Sodium 5-methyl-1H-benzotriazolide	5.9	1.00	0.64	0.32
25	NS00009367	Moxisylyte	2.96	1.00	0.64	0.13
26	NS00000400	Bicalutamide	0.52	1.00	0.64	0.11
27	NS00034584	Vinylcyclooctane	0.19	0.73	0.64	0.09
28	NS00005581	Ibuprofenol acetate	0.63	0.64	0.64	NA
29	NS00021325	Carbamic acid, N,N-dimethyl-, 1-ethenyl-1,5-dimethyl-4-hexen-1-yl ester	1.2	0.64	0.64	0.2
30	NS00007544	Degradation product (see structure in the NDS)	0.24	0.64	0.64	NA
31	NS00014214	N-(2,6-Dichlorophenyl)anthranilic acid	0.13	0.64	0.64	0.02
32	NS00006955	Denatonium benzoate	1.39	1.00	0.55	0.73
33	NS00052280	Sodium 1H-benzotriazolide	7.77	1.00	0.55	0.55
34	NS00010447	Norpropoxyphene (Desmethyl propoxyphene)	0.46	0.64	0.55	0.02
35	NS00027559	1-Heptanamine, N,N-diheptyl-	0.0091	0.55	0.55	0.15
36	NS00022626	Phenol, 2,5-dimethyl-4-(1-methylcyclohexyl)-	0.52	0.55	0.55	0.19
37	NS00028755	Tetradecyl heptanoate	0.017	0.55	0.55	0.09
38	NS00032067	9,10-Didehydro-N,6-dimethylergoline-8beta-carboxamide	1.38	0.55	0.55	0.09

Table 5e: Substances detected by suspect screening in the JDS4 groundwater samples with frequency of exceedance of PNEC (FoE) > 0.5; FoA – frequency of appearance. For more details, see text.

No.	NORMANID	Compounds	PNECgw [µg/l]	FoA	FoE	Final EXPOSURE index KEMI
1	NS00010583	9,10-Dihydroxystearic acid	0.1	1.00	1.00	0.11
2	NS00035021	Octanoic anhydride	0.1	1.00	1.00	0.13
3	NS00011498	Nonanedioic acid	0.1	1.00	1.00	0.51
4	NS00026775	Methyl hydrogen azelate	0.1	1.00	1.00	0.11
5	NS00039078	2-Oxononan-1-amide	0.1	1.00	1.00	0.09
6	NS00009663	Diricinoleate	0.1	1.00	1.00	0.55
7	NS00032880	Octyl hydrogen phthalate	0.1	1.00	1.00	0.11
8	NS00001002	Pentaethylene glycol	0.1	1.00	1.00	0.38
9	NS00013947	PEG-3 Lauramide	0.1	1.00	1.00	0.26
10	NS00006548	Embutramide	0.1	1.00	1.00	0.11
11	NS00003780	3-(2-Amino-2-oxoethyl)-5-methylhexanoic acid	0.1	1.00	1.00	0.09
12	NS00013594	Tridecanedioic acid, dimethyl ester	0.1	1.00	1.00	0.19
13	NS00027242	Decyl hexyl adipate	0.1	1.00	1.00	0.17
14	NS00004841	Octinoxate	0.1	1.00	1.00	0.44
15	NS00004924	Cyclooct-4-en-1-yl methyl carbonate	0.1	1.00	1.00	0.43
16	NS00021325	Carbamic acid, N,N-dimethyl-, 1-ethenyl-1,5-dimethyl-4-hexen-1-yl ester	0.1	1.00	1.00	0.2
17	NS00007399	Bisphenol F diglycidyl ether	0.1	1.00	0.86	0.3
18	NS00025333	N-Nitroso-N'-methylpiperazine	0.1	0.86	0.86	0.09
19	NS00003987	Methyl aminolevulinate	0.1	0.86	0.86	0.02
20	NS00010307	Acetyl tributyl citrate	0.1	0.86	0.86	0.63
21	NS00005309	Hexanedioic acid	0.1	0.86	0.86	0.84
22	NS00026365	Diethyl (3-oxopropyl)malonate	0.1	0.86	0.86	0.09
23	NS00003181	N-(2-Hydroxyethyl)octadecanamide	0.1	0.86	0.86	0.45
24	NS00027559	1-Heptanamine, N,N-diheptyl-	0.1	0.86	0.86	0.15
25	NS00039759	Ethyl 8-(acetoxy)octanoate	0.1	0.86	0.86	0.13
26	NS00031858	Ammonium octyl sebacate	0.1	0.86	0.86	0.09
27	NS00023130	Ethyl 3,4-dihydro-6-methyl-2H-pyran-5-carboxylate	0.1	0.86	0.86	0.13
28	NS00026812	N-Hexadecylacrylamide	0.1	0.86	0.86	0.09
29	NS00021167	Cyclopropanecarboxylic acid, 2-[1-(3,3-dimethylcyclohexyl)ethoxy]-2-methylpropyl ester	0.1	0.71	0.71	0.19
30	NS00052280	Sodium 1H-benzotriazolide	0.1	0.71	0.71	0.55
31	NS00027137	Methyl 3-oxooctanoate	0.1	0.71	0.71	0.11
32	NS00013870	Tetradecanamide, N-(2-hydroxyethyl)-	0.1	0.71	0.71	0.2
33	NS00007135	N,N'-Ethylenedi-L-aspartic acid	0.1	0.71	0.71	0.5
34	NS00025715	Sebacamide	0.1	0.71	0.71	0.11
35	NS00013788	Isopropyl methoxy-cinnamate	0.1	0.71	0.71	0.13
36	NS00053360	4-Hydroxyvaleric acid	0.1	0.57	0.57	0.09
37	NS00056655	Sodium 5-methyl-1H-benzotriazolide	0.1	0.57	0.57	0.32
38	NS00003658	Dodecyl(ethylbenzyl)dimethylammonium	0.1	0.57	0.57	0.39
39	NS00012932	Benzoic acid, 2-[(2-methylundecylidene)amino]-, methyl ester	0.1	0.57	0.57	0.19
40	NS00037296	Diisooctyl isophthalate	0.1	0.57	0.57	0.09
41	NS00013880	1-Tetradecanol, 1-propanoate	0.1	0.57	0.57	0.32
42	NS00024727	Methyl 3-oxooctadecanoate	0.1	0.57	0.57	0.11
43	NS00000587	1,3-Benzenedisulfonamide, 4-amino-6-chloro-	0.1	0.57	0.57	0.27
44	NS00010686	1-Bromohexadecane	0.1	0.57	0.57	0.4



NORMAN ID	Compound name	Molecular Formula	CAS number	Chemical structure	Exposure Score (KEMI)	Hazard Score (KEMI)	FoA (All matrices)	FoE (All matrices)
NS00032465	4-(4-Methoxyphenyl)butan-1-ol	$C_{11}H_{16}O_2$	52244-70-9		0.09	0.12	88.8	0.0
NS00010583	9,10-Dihydroxystearic acid	$C_{18}H_{36}O_4$	120-87-6		0.11	0.02	85.2	85.2
NS00011498	Nonanedioic acid	$C_9H_{16}O_4$	123-99-9		0.51	0.01	84.5	22.9
NS00025333	N-Nitroso-N'-methylpiperazine	$C_5H_{11}N_3O$	16339-07-4		0.09	0.13	83.6	0.0
NS00039078	2-Oxononan-1-amide	$C_9H_{17}NO_2$	85866-13-3		0.09	0.12	79.4	7.3
NS00010316	8-Hydroxychinolin	C_9H_7NO	148-24-3		0.41	0.61	74.7	60.1
NS00003596	Acetanilide	C_8H_9NO	103-84-4		0.46	0.02	73.1	5.45
NS00019508	1-Propanol, 2-[1-(3,3-dimethylcyclohexyl)ethoxy]-2-methyl-, 1-propanoate	$C_{17}H_{32}O_3$	141773-73-1		0.42	0.09	66.9	46.9
NS00040486	Isodecyl undecyl phthalate	$C_{29}H_{48}O_4$	96507-81-2		0.13	0.13	62.9	62.9
NS00002145	hexa-2,4-dienoic acid	$C_6H_8O_2$	110-44-1		0.67	0.22	61.7	61.7
NS00010302	OCTOXYNOL-2	$C_{18}H_{30}O_3$	2315-61-9		0.02	0.25	58.2	43.2
NS00024523	Ethyl diethanolamine	$C_6H_{15}NO_2$	139-87-7		0.19	0.03	43.0	1.8
NS00013909	NYLON-6	$C_6H_{13}NO$	25038-54-4		0.51	0.02	42.8	20.0
NS00027389	Tiformin hydrochloride	$C_5H_{12}N_4O$	23256-39-5		0.09	0.08	42.8	0.0
NS00005146	10-Phenyldecanoic acid	$C_{16}H_{24}O_2$	18017-73-7		0.02	0.01	42.2	42.2
NS00002538	N-Vinyl-2-pyrrolidone	C_6H_9NO	88-12-0		0.68	0.25	28.91	0.0
NS00029941	4-Cyclohexylphenylacetic acid	$C_{14}H_{18}O_2$	35889-00-0		0.11	0.13	23.71	0.0

Figure 4: Commonly detected substances in the JDS4 biota (BT) and river water (RW) samples. Seventeen substances were detected in BT, and RW and were observed with frequency of appearance (FoA) higher than 50%.

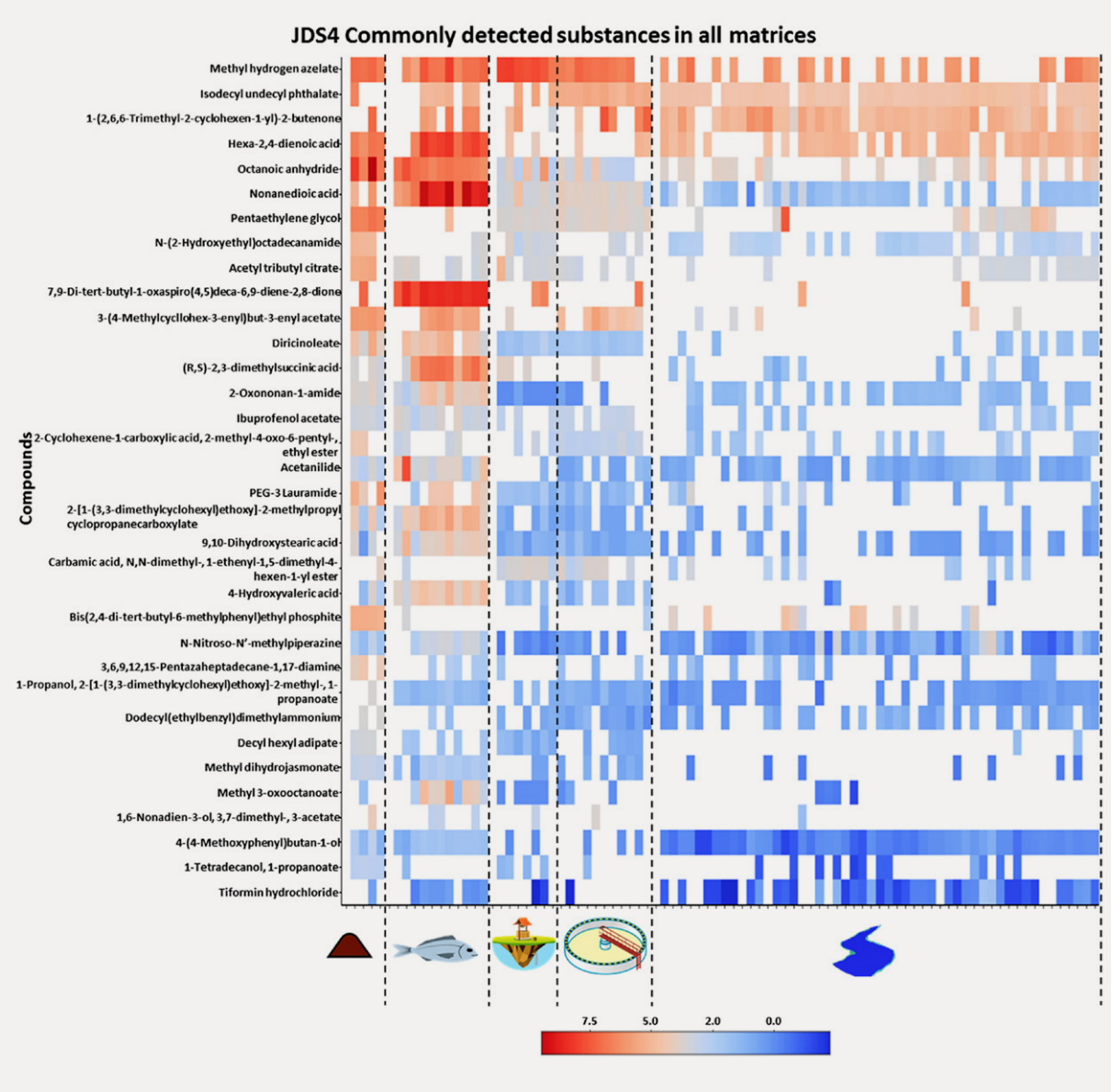


Figure 5: Heatmap presenting 34 commonly detected substances in various matrices from the Danube River Basin. Colours indicate the log10 estimated concentration expressed in ng L^{-1} for water matrices (groundwater, wastewater, river water), ng g^{-1} wet weight for biota and ng g^{-1} dry weight for river sediment.

37 Comparison of target screening and target analysis approaches for surface water samples

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Abstract

We compared altogether five methods for the analysis of organic micropollutants applied to surface water samples in JDS4, one target method based on SPE, LC-MS/MS and GC-HRMS, and four screening methods, of which three were based on SPE and LC-HRMS and one on direct injection LC-HRMS. The different methods were focused either on specific compound classes or aimed at a wide scope screening, and the overlap of all five methods was just 10 compounds. The methods differed considerably in the approaches used for calibration and the number of calibration points. A comparison of concentrations of the 10 compounds analysed by all methods showed in most cases a good agreement within a factor of 3, but in some cases considerable deviations were observed. Some of these deviations are likely related to the different calibration strategies, as they were highest at low concentration levels, but also occurred for certain compounds and sample sub-sets, pointing at specific interferences for one method. This calls for a harmonization of QA/QC measures and a system for reporting the reliability for individual compounds, as different methods will not perform equally well on the same compound.

37.1 Introduction

This chapter critically evaluates the results from different target analysis and target screening methods for organic micropollutants applied within JDS4. As the surface water samples were analysed with the largest number of different approaches, this comparison will focus on this type of sample covering four methods based on solid-phase extraction and one on water direct injection. One method used a state-of-the-art liquid chromatography coupled to triple-quadrupole mass spectrometry (LC-MS/MS) (and gas chromatography

coupled to high resolution mass spectrometry, GC-HRMS) for the quantification of 67 compounds. The other methods can be considered as target screening methods, as these are mainly based on LC-HRMS analysis and cover substantially larger numbers of compounds (Krauss et al., 2010).

The JRC method was considered as a reference for this comparison, and the main objectives were (i) to assess whether HRMS-based target screening approaches yield comparable quantification results as triple-quadrupole-based target methods, and (ii) to compare the screening methods among each other. It should be noted that we cannot make any statements about the accuracy of any of the methods relative to the true concentrations in the samples, as no certified reference materials are available for this kind of matrix-analyte combinations.

37.2 Methods

The individual methods are described in detail in the respective chapters. Here a brief overview and a comparison of main method characteristics (Table 1) is given. The Joint Research Centre (JRC) analysed 67 polar and hydrophobic compounds in extracts obtained by on-site solid-phase extraction (SPE) using a so-called Mariani Box (Mariani et al., 2017). Quantification of polar compounds was done by LC-MS/MS (QTrap 5500, Sciex) and of non-polar compounds by GC-HRMS (DFS, Thermo). The Bavarian Environmental Agency (LfU) analysed 139 pesticides and metabolites by online solid-phase extraction (SPE) on C18 material combined with liquid chromatography-high resolution mass spectrometry (LC-HRMS, QExactive, Thermo) (Chapter 23). The Helmholtz Centre for Environmental Research (UFZ) used a large volume solid phase extraction device (LVSPE, MAXX) for on-site extraction and the extracts were analysed in a wide-scope screening approach for 508 compounds by LC-HRMS on a QExactive Plus instrument and 11 compounds by GC-HRMS on a GC-QExactive (method UFZ-LVSPE, Chapter 26; Schulze et al., 2017). Additionally, UFZ applied a direct injection of water samples with the same LC-HRMS setup (method UFZ-DI, Chapter 27) for the quantification of 534 compounds. The University of Athens (UoA) enriched unfiltered water samples on SPE discs using a Horizon SPE-DEX instrument and analysed the extracts by wide-scope screening for 2290 compounds by LC-HRMS using a Bruker Maxis Impact QToF instrument (chapters 29; Gago-Ferrero et al., 2020).

The data for this comparison was used as received from the Data Collection Templates (DCTs) of JDS4 and compounds among the methods were matched using the InChIkeys and NORMAN Susdat identifiers.

Table 1: Overview of the main characteristics of the methods used (ILIS: isotope-labelled internal standard).

Method	Filtration	Extraction	Relative injection volume[a]	# of analytes	ILIS # / added	Calibration	Type of MS analysis
JRC	None[b]	On-site SPE disc, HLB sorbent, by Mariani box	100 mL (LC-MS/MS 500mL (GC-HRMS)	67	43 / prior to extraction	Solvent calibration, 1 point[c]	LC-ESI-Triple quadrupole MS/MS (32 compounds) + GC-EI-magnetic sector HRMS (35 compounds)
LfU	None (settling only)	Laboratory, online-SPE, C18 cartridge	5 mL	140	40 / prior to extraction	Method-matched, 7 points 1-250 ng/L	LC-ESI-quadrupole-orbitrap MS
UFZ-LVSPE	GFF cartridge	On-site, LVSPE cartridge (HR-X sorbent), MAXX instrument	2.5 mL	519	40 / prior to instrumental analysis	Method and matrix-matched[d,e], 13 points 0.1-1000 ng/L	LC-ESI-quadrupole-orbitrap MS (503 compounds) GC-EI-orbitrap MS (16 compounds)
UFZ-DI	None (settling only)	None	0.1 mL	534	40 / into sample	Method and matrix-matched[e], 12 points, 1-5000 ng/L	LC-ESI-quadrupole-orbitrap MS
UoA	None[b]	Laboratory, SPE disc (HLB sorbent) Horizon SPE-DEX	20 mL	2290	33 / prior to extraction	Method and matrix-matched[f], 5 points (12.5- 250 ng/L)	LC-ESI-quadrupole-time-of-flight MS

[a] injected water volume (in case of UFZ-DI and LfU) or the water volume the injected extract volume corresponds to, respectively.
[b] suspended particulate matter filtered on the SPE-disc were co-extracted during elution of the sorbent
[c] quantification was based on response factors obtained from a standard analyte:ILIS 1:1 prepared in solvent
[d] a manual laboratory SPE procedure downscaled from the LVSPE was used for preparation of calibration standards
[e] water from a small pristine stream was used as surrogate matrix
[f] Calibration was done by standard addition of 5 concentration levels into one of the JDS4 samples

37.3 Results and Discussion

37.3.1 Comparison of analysed compounds and analytical strategies

Altogether 2522 compounds were analysed by the four SPE-based methods, with 10 compounds analysed by all four laboratories and 2140 compounds analysed only by one of the laboratories. Figure 1 provides a summary of the overlap of analysed compounds among the different methods. The UFZ-LVSPE and UFZ-DI method were originally based on the same set of 550 compounds, of which 503 could be analysed with both methods, 16 only after (LV)SPE enrichment with sufficient sensitivity or by GC-HRMS and 31 only with the direct injection method, as an enrichment on the used SPE sorbent was not possible.

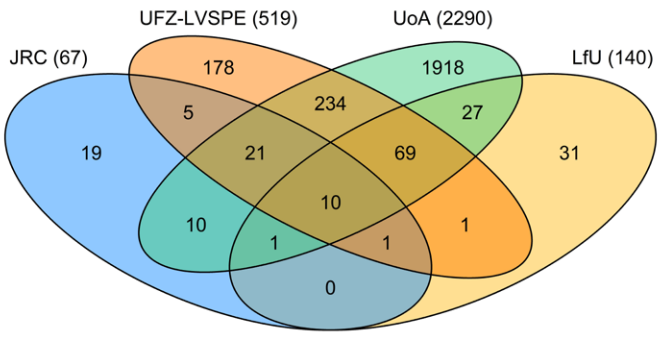

Figure 1: Venn diagram showing the overlap of compounds analysed in JDS4 surface water samples with the four SPE-based methods.

While with the JRC method 61 out of 67 analysed compounds could be detected in at least one sample, the LfU method focusing on pesticides and their transformation products could detect 60 out of 139 targeted compounds. The wide-scope screening method of UoA was able to detect 229 out of 2290 compounds. The UFZ-LVSPE method could detect 298 out of 519 compounds above the MDL, while the detection rate was substantially lower for the UFZ-DI method with 159 out of 534 compounds due to the lower sensitivity.

A first major difference between the methods used was in the handling of suspended particulate matter (SPM) in the samples (Table 1). The UFZ-LVSPE device uses a glass fibre filter cartridge (1 µm) prior to the SPE cartridge, thus SPM-bound micropollutants are removed before. The UFZ-DI and LfU method allow for a settling of SPM prior to sample injection. The JRC and UoA methods include the SPM contained in the sample, which forms a sediment layer on top of the SPE discs during the SPE procedure. In turn, the extraction of the SPE discs with solvents will yield a mixture of compounds dissolved in the water phase and extracted from the SPM. Thus, for more hydrophobic compounds occurring mainly sorbed to SPM, but with low dissolved concentrations, the JRC and UoA methods might report higher concentrations as compared to the other methods addressing only dissolved concentrations.

The most severe systematic effects on quantification performance are typically compound losses during sample processing and matrix effects (in most cases ion suppression by co-eluting matrix constituents) during LC-MS/MS and LC-HRMS ionisation. To compensate for these effects, isotope-labelled internal standards (ILIS) are typically used for organic trace analysis, which is also the case for the presented methods. In an ideal case, for each compound an isotope-labelled surrogate standard is used (i.e., the same compound, but isotope-labelled), which is nearly the case for the JRC method with 43 ILIS for 67 target compounds, all of them being a surrogate standard for one analyte and closely related compounds for the remaining ones. The use of nearly one ILIS per compound is hardly possible for screening methods with a large number of compounds due to the high costs associated with the purchase of ILIS as well as their limited availability. Thus, all screening methods utilize a similarly high number of internal standards as JRC, but for a much larger set of compounds.

With the application of ILIS, two different strategies were used. JRC and UoA spiked the water sample prior to extraction with these ILIS, which aimed at compensating for losses during the SPE and associated processing steps (evaporation, sample transfer) as long as analytes and ILIS show the same behaviour during these procedures. This will be the case for surrogate standards with basically the same physicochemical properties as the corresponding analytes, but deviations might occur for analytes without a well-matching ILIS. To account for compound losses during the SPE procedure, UoA thus employed a method-matched calibration, i.e., the calibration standards were processed the same way as the samples. These were prepared from spiking one native sample from the JDS4 data set at five different levels, thus also obtaining a good match against the matrix. In contrast, JRC prepared a single calibration standard at an analyte:ILIS ratio of 1:1 in solvent. Thus, the JRC method relies solely on the ILIS to compensate for both, SPE losses and matrix effects, and a strong linearity of the response as a function of analyte concentrations.

The UFZ-LVSPE method does not allow the addition of ILIS prior to extraction, as samples are directly taken from the surface water and the same extracts are foreseen for a simultaneous use in biotesting, which does not allow the addition of an ILIS. UFZ thus also used a method-matched calibration using 13 levels, but this was done by a laboratory scale SPE procedure down-scaled from the LVSPE as a more cost and time-efficient approach. Furthermore, water from a pristine stream from the Upper Harz mountains (Germany) was used as a surface water matrix. Although composition and chemistry of this stream water differs from that of a large lowland river as the Danube, a more appropriate surrogate matrix without anthropogenic contamination is not available.

For the LfU method, the ILIS is also spiked into the water sample, due to the direct coupling of online-SPE and LC-HRMS analysis, and calibration standards at seven levels were prepared the same way. For the UFZ-DI method, the ILIS has solely the aim to compensate for matrix effects, as no further sample processing, possibly resulting in compound losses, was done. The calibration was also prepared matrix-matched in pristine stream water at 12 levels.

37.3.2 Comparison of quantification

The comparison of the quantified concentrations was done in a first step for the 10 compounds analysed with all four SPE-based methods and the direct injection method. It should be noted that we cannot rule out differences in concentrations based on the different sampling approaches. However, all samples were taken within the same period of time as grab samples (LfU, UFZ-DI, UoA) or composite samples (UFZ-LVSPE, JRC) within up to three hours from large rivers at sites with well-mixed waters (i.e., not directly downstream of influents). Thus, we do not expect strong concentration changes within that sampling periods.

A look at the limits of quantification (LOQs) – and method detection limits (MDL) according to US EPA (2011) in case of the UFZ-LVSPE and UFZ-DI method, respectively, shows that the JRC method typically had more than an order of magnitude better sensitivity as compared to the SPE-based screening methods, as a rather concentrated extract was analysed. The screening methods had rather comparable LOQs/MDLs, although compound-specific differences could be seen (Table 2). The UFZ-DI method showed somewhat higher MDLs than the SPE-based methods, as the water volume injected was just 100 µL as compared to much higher corresponding water volumes of the enriched extracts.

Overall, the comparison of concentrations measured by different methods, in many cases showed a very good agreement of all four screening methods with the JRC method (Table 2 and Figure 2), with more than two thirds of the measured values less than a factor of three apart, in case of terbutryn and atrazine for more than 90% of the values. A particular high deviation could be seen for simazine and metazachlor, although detection frequencies of the screening methods were low. While in general, detection frequencies were related to different LOQs/MDLs, for some methods a compound was not detected although this could be expected based on the concentrations measured by others and the LOQ. (e.g., the UoA method did not detect atrazine, although concentrations in more than 40 samples should be above its LOQ of 0.7 ng/L).

Table 2: Comparison of LOQs/MDLs, number of dedected compounds, median relative concentrations to those of the JRC method and percentage of values within a factor of 3 from those of the JRC method for 10 compounds analysed in all four methods (nd = not detected).

	Acetamiprid	Atrazine	Desethylterbutylazine	Dimethenamid	Imidacloprid	Metazachlor	Metolachlor	Simazine	Tebuconazole	Terbutryn
Method LOQ or MDL (UFZ-LVSPE and UFZ-DI) in ng/L										
JRC	0.08	0.06	0.84	0.04	0.33	0.76	0.05	0.12	0.13	0.71
LfU	5	1	2	2	25	1	1	2	1	1
UFZ-LVSPE	1	1	1	1	0.5	0.7	0.7	1	0.7	0.7
UFZ-DI	2.5	2	4	2.9	2.5	2.4	1.5	1	2	1.5
UoA	0.2	0.7	1.5	0.9	17.1	7.9	3.8	1.5	12.6	0.4
Number of detections										
JRC	47	51	51	5	51	43	51	51	51	51
LfU	1	48	47	30	0	6	50	2	50	49
UFZ-LVSPE	6	41	43	42	37	14	50	10	42	48
UFZ-DI	1	45	26	31	4	3	49	0	9	42
UoA	7	1	15	19	18	0	36	0	39	14
Median ratio to concentration measured by JRC method										
LfU	0.2	1.3	1.0	1.7	nd	0.2	1.3	26.4	0.7	0.8
UFZ-LVSPE	1.0	1.5	1.3	2.6	0.5	0.3	1.5	4.9	0.3	0.6
UFZ-DI	0.2	1.8	2.6	2.5	0.5	0.2	1.6	nd	0.3	1.2
UoA	0.3	0.1	1.3	1.0	0.3	nd	1.1	nd	0.7	0.6
Percentage of concentrations within a factor of 3 from JRC										
LfU	0	98	72	83	nd	17	78	0	98	90
UFZ-LVSPE	83	95	86	64	86	43	74	30	31	90
UFZ-DI	0	89	50	68	75	0	67	nd	44	95
UoA	29	0	73	79	39	nd	78	nd	97	93

The concentrations measured with the four screening methods often showed the same trends in deviation from the values of the JRC method (particularly visible for dimethenamid), often with generally higher or generally lower values. From Figure 2 it is also evident that the ratios of the concentrations measured by the screening method relative to the JRC method were increasing for low concentrations close to the respective LOQs/MDLs of these methods, suggesting some general differences, maybe related to the fit of the calibration functions at low levels. As the calibration of the JRC method relied on one calibration level and the observation of a linear response, a systematic deviation from methods with a much wider range of calibration levels extending also to low concentrations is likely.

A pairwise comparison of measured concentrations between the JRC method and the UoA and UFZ-LVSPE methods (Figure 3a) shows that about 73% of the UFZ-LVSPE data and 68% of the UoA data was within a factor of 3 from the JRC data. However, for both screening methods concentrations of some compounds in some samples were much higher or lower as measured by JRC, as shown in Figure 3b. While for sulfamethoxazole concentrations measured by UFZ were similar to those measured by JRC, in most samples the UoA method yielded values about ten times lower. In contrast, for 8 out of 51 sites the carbamazepine concentrations measured by the UoA and UFZ-LVSPE methods were about 10 times higher than those measured by the JRC.

A closer look at the individual sites reveals that the carbamazepine concentrations measured by the different methods show often common trends for the samples taken by sampling teams 1 and 2, but larger deviations for sampling team 3 Sites 31-51, Figure 4A and B). The samples of the lower stretch of the Danube were taken by Sampling team 3 from the mouth upstream and show fairly uniform carbamazepine concentrations in the UFZ-DI and UFZ-LVSPE data (Figure 4). A drop of concentrations by a factor of > 10 as seen in the JRC data would mean a dilution of the same factor from one site to a neighbouring one, or a temporal change within a short period of time. Both seem highly unlikely, as the discharge did not change

strongly during the sampling period and a widely used pharmaceutical like carbamazepine is emitted from numerous sources (WWTP or untreated wastewater effluents) at fairly stable levels rather than from a low number of sources with fluctuating concentrations. The reasons for these considerable deviations in some samples are not clear, as both UFZ and JRC had the surrogate ILIS available for quantification, but may be related to calibration errors or specific interferences from the matrix in the JRC method.

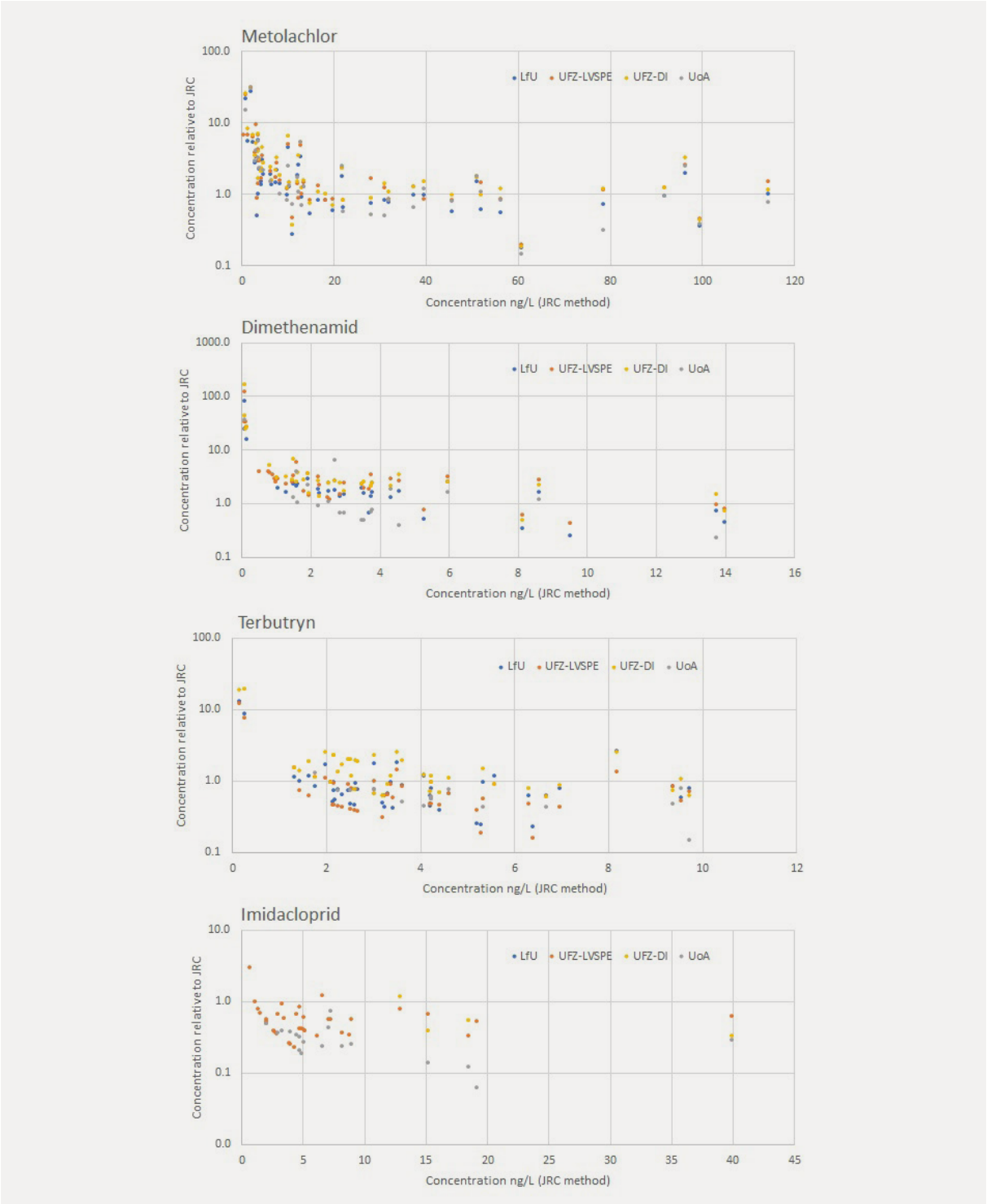

Figure 2: Comparison of measured concentrations relative to those measured by the JRC method (i.e., a perfect match would result in a value of 1) for metolachlor, dimethenamid, terbutryn and imidacloprid in relation to the concentrations measured by the JRC method in the individual samples. Note the logarithmic y axes.

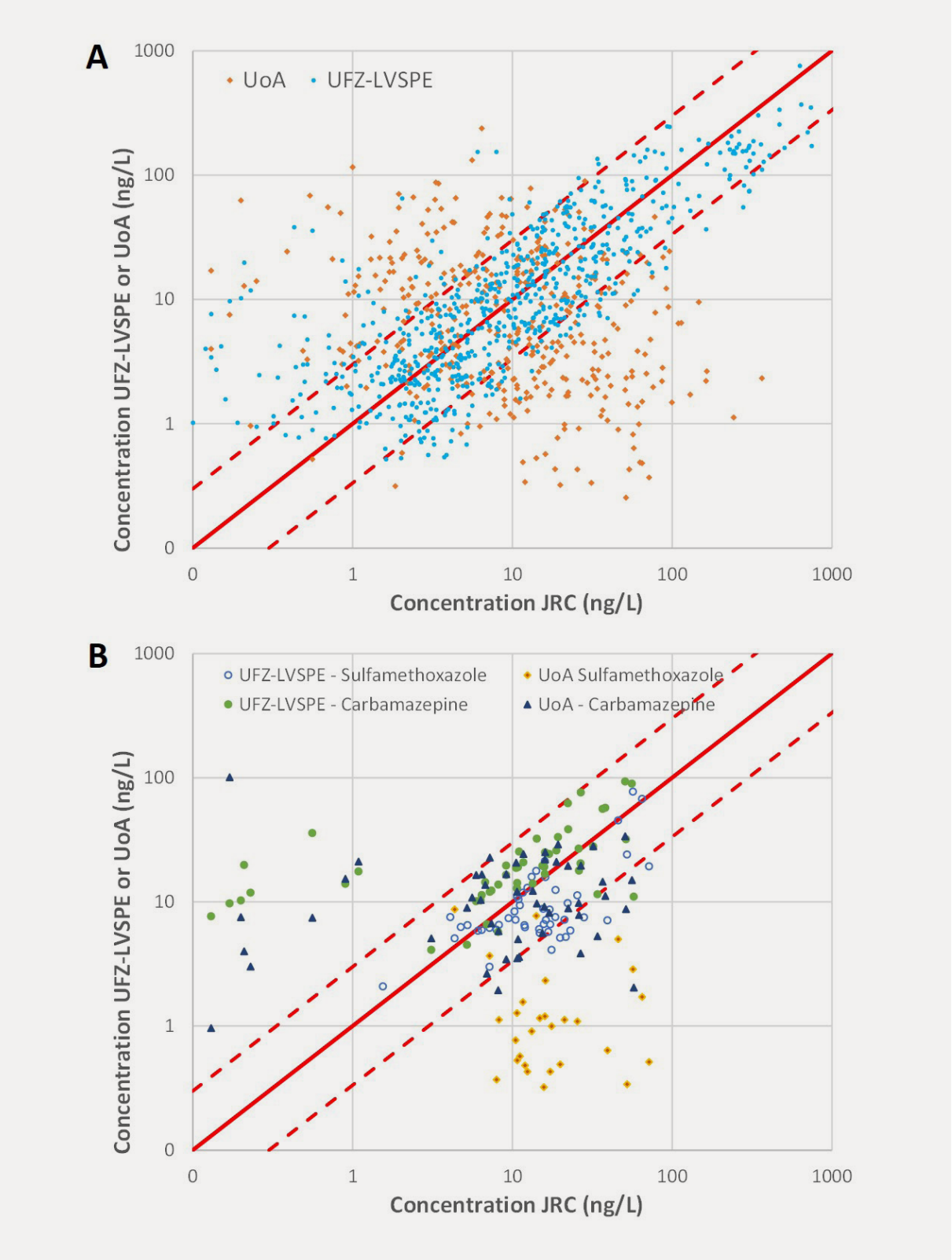

Figure 3: Concentrations of (A) all compounds and (B) carbamazepine and sulfamethoxazole measured by the JRC method and those measured by the UFZ-LVSPE and UoA methods in all samples with the 1:1 line (solid line) and a factor of 3 difference (dashed lines). Icaridin and DEET were removed from the datasets, as a contamination during sampling by the use of insect repellents was likely (see Chapter 26). Note the logarithmic scale of the axes.

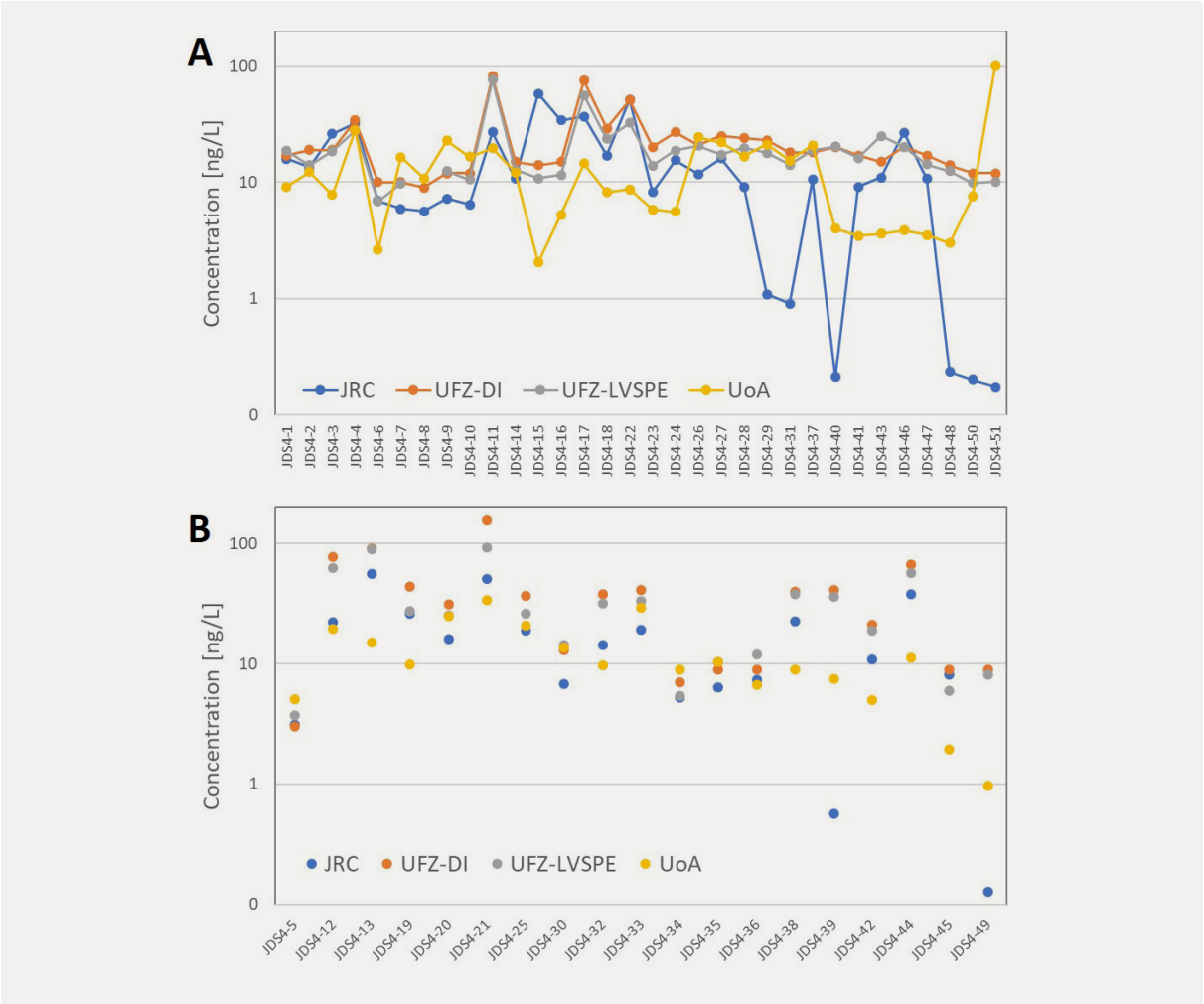

Figure 4: Concentrations of carbamazepine measured by the JRC, UFZ-DI, UFZ-LVSPE and UoA methods in (A) the Danube main river and (B) the Danube tributaries.

37.4 Conclusions

The concentrations measured in JDS4 surface water samples by different target and screening methods showed for many compounds and samples a good agreement within a factor of 3, despite different analytical strategies used. These results suggest that LC-HRMS-based screening methods are able to provide a similar result as targeted LC-MS/MS methods and thus hold the potential to be applied in WFD monitoring if a larger set of compounds should be considered. We observed some systematic deviations of the measured concentrations, especially at low levels, which might be related to calibration errors or specific matrix interferences. In selected cases, high and unsystematic deviations were observed among the methods, which require more in-depth investigations in causes and mitigation measures. Also, an improvement and harmonization of QA/QC measures for screening methods and the reporting of data quality is recommended to improve the comparability of different methods and to judge the reliability for individual compounds, as different methods will not perform equally well on a specific compound.

37.5 References

GAGO-FERRERO P., A.A. BLETSOU, D.E. DAMALAS, R. AALIZADEH, N.A. ALYGIZAKIS, H.P. SINGER, J. HOLLENDER and N.S. THOMAIDIS (2020): Wide-scope target screening of >2000 emerging contaminants in wastewater samples with UPLC-Q-ToF-HRMS/MS and smart evaluation of its performance through the validation of 195 selected representative analytes. J. Hazard. Mater. 387, 121712.

KRAUSS, M., H.P. SINGER & J. HOLLENDER (2010): LC-high resolution MS in environmental analysis: from target screening to the identification of unknowns. Anal. Bioanal. Chem.397, 943-951.

MARIANI G., TAVAZZI S., COMERO S., BUTTIGLIERI G., PARACCHINI B., SKEJO H., ALCALDE S. L., GAWLIK B. (2017): Short-term isochronous stability study of contaminants of emerging concern in environmental water samples. Stabilisation of chemical analytes using a novel sampling device (Text No. EUR 28425 EN), JRC Technical Reports. Joint Research Center (JRC), Ispra.

SCHULZE, T., M. AHEL, J. AHLHEIM, S. AÏT-AÏSSA, F. BRION, C. DI PAOLO, J. FROMENT, A.O. HIDASI, J. HOLLENDER, H. HOLLERT, M. HU, A. KLOSS, S. KOPRIVICA, M. KRAUSS, M. MUZ, P. OSWALD, M. PETRE, J. E. SCHOLLÉE, T.-B. SEILER, Y. SHAO, J. SLOBODNIK, M. SONAVANE, M. J.F. SUTER, K.E. TOLLEFSEN, Z. TOUSOVA, K.-H. WALZ and W. BRACK (2017): Assessment of a novel device for onsite integrative large-volume solid phase extraction of water samples to enable a comprehensive chemical and effect-based analysis. Sci. Tot. Environ. 581-582, 350-358.

US EPA (2011): 40 CFR Part 136, Appendix B to Part 136 - Definition and Procedure for the Determination of the Method Detection Limit-Revision 1.11.

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Analysis of genotoxic activity of the JDS4 surface water samples collected by horizon large volume solid-phase extraction technique

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Abstract

This chapter summarises the results of analyses of cytotoxic and genotoxic activity of surface water samples collected during the JDS4 survey extracted by horizon large volume solid-phase extraction technique. Initial screening in prokaryotic model was performed by SOS/umuC assay while testing in eukaryotic model comprised integrated zebrafish-based battery of bioassays including testing of cytotoxicity, genotoxicity (comet assay) and cell cycle analyses on zebra fish liver (ZFL) cell line and embryotoxicity (zFET). The results demonstrated that about 46% of the extracts were cytotoxic while about 38% of the extracts were found to have DNA-damaging potential to a certain extent. Most of the samples active in applied bioassays were collected from the Middle Danube section. None of the extract had embryotoxic activity at the highest tested REF 100.

38.1 Introduction

Effect Based Methods (EBMs) have been commonly employed in diagnostic and monitoring purposes of the impacts of chemical pollution covering range of different modes of action (MoA). Brack et al. (2019) indicated that among the MoA-specific *in vitro* assays, priority of application should be given to endocrine disruption and mutagenicity. Additionally, it is recommended to complement these assays with apical short-term toxicity bioassays representing at least fish (fish embryo toxicity), invertebrates (immobilization of

daphnia) and algae (inhibition of cell multiplication) as model for biological quality elements (BQEs) for pelagic communities in Water Framework Directive (WFD).

Among the many assays used in genotoxicology, application of prokaryotic test systems is of great interest as they are short-term, very sensitive, simple, cost-effective and play an important role in the screening and legislation of the genotoxic substances (Flegrova et al., 2007). Application of modified strains of *Salmonella typhimurium* is common for detection of mutagenic potential (i.e. the Ames assay) but also for the detection of genotoxicity (i.e. SOS/*umuC* test). Used strains have numerous modifications which multiplies response to the genotoxic agents such as increased cell wall permeability and lack of excision repair system. Apart from prokaryotic models used in ecogenotoxicology, various eukaryotic models have been developed lately, which can mimic the response of the aquatic organisms (i.e. fish derived cell lines) which are of enormous significance especially due to 3R principle (replacement, reduction and refinement). In particular, fish cell lines have been successfully introduced for detection of genotoxic effects of chemicals and can serve as an alternative to animal testing in preliminary eco-/genotoxicological studies. For this purpose, comet assay has been extensively used in fish cell lines for the evaluation of genotoxic potential of chemicals and complex environmental matrices. The most often used fish cell lines in the comet assay are RTG-2, RTgill-W1 and RTL-W1 derived from rainbow trout (*Onchorhynchus mykiss*) gonads, gills and liver, respectively, and ZFL and ZF4 cells established from zebrafish (*Danio rerio*) liver and embryos, respectively (Žegura et al., 2019). Lethal and sub-lethal assays with embryos and early larval stages are available for comprehensive toxic effect evaluation, making use of unobstructed observations of main morphological changes by simply using only low magnification light microscope (Shao et al., 2019).

The major objective of the study was to assess the genotoxic potential of surface water samples collected during JDS4 and extracted by horizon large volume solid-phase extraction technique using prokaryotic and eukaryotic bioassays. Figure 1 shows the scheme of the study experimental design. Initial screening of all samples was performed by SOS/*umuC* assay, with and without metabolic activation, using bacterium *S. typhimurium* TA1535/pSK1002. Testing in eukaryotic model comprised integrated zebrafish-based battery of bioassays including testing of cytotoxicity, genotoxicity (comet assay) and cell cycle analyses on zebra fish liver (ZFL) cell line and embryotoxicity (zFET).

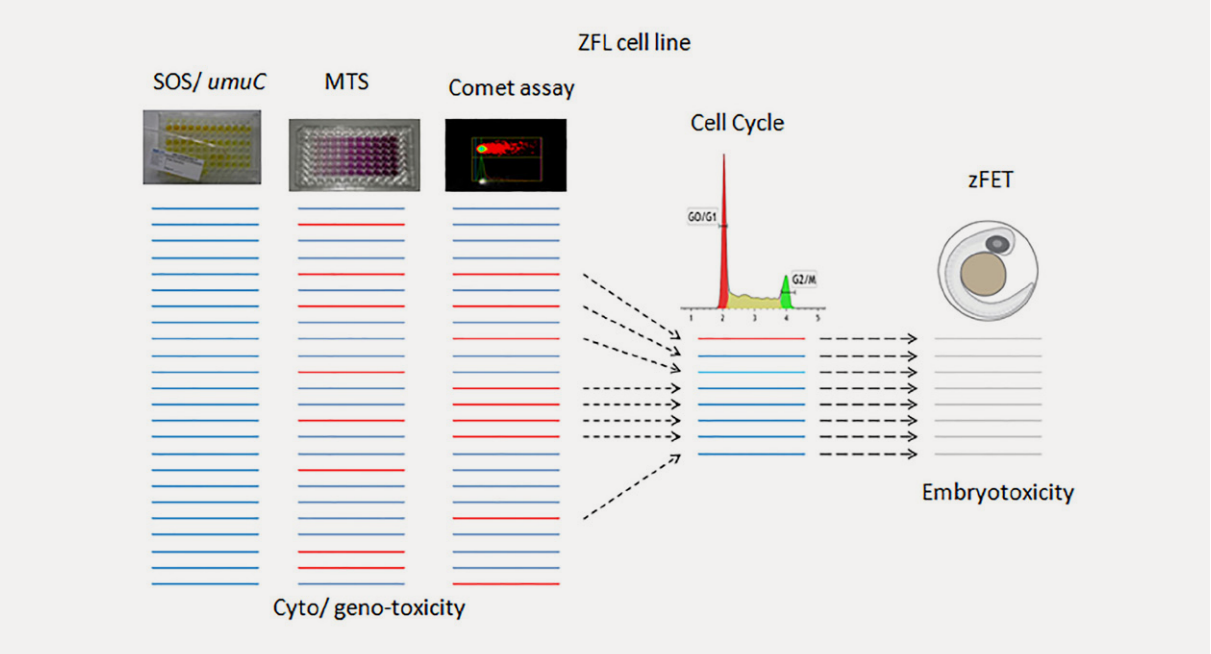

Figure 1: Scheme of the experimental design of the sample processing.

38.2 Methods

38.2.1 Samples

Surface water samples were collected from 24 sites along the Danube River. LVSPE Horizon sampler was used as a field device using Oasis HLB high capacity disk trapping polar and semi-polar compounds providing relative enrichment factor (REF) of the samples of 25,000x. After sampling, the disks were wrapped into aluminium foil and kept at -8 °C during the whole transport. Sample preparation was performed by Environmental Institute Kos (Slovakia). Extracts were prepared in dichloromethane and later transferred in DMSO which was used as vehicle in the experiments. In parallel with the samples, solid phase extraction control (SPEC) was included in experiments and used as a blank.

38.2.2 SOS/*umuC* assay

The SOS/umuC assay was applied using the protocol described in Žegura et al. (2009) with minor modification. Briefly, the treatment was performed for 2 h at 37 °C in incubation mixtures composed of 10 µL of the extract with REF1000 in 1xTGA medium and 90 µL of bacterial culture of S. *typhimurium* TA1535/pSK1002 in exponential phase or in the case of metabolic activation 10 µL of the extract with REF1000 and 90 µL of bacterial culture supplemented with S9 fraction (final REF100). Positive controls included 4-NQO (final concentration 0.5 µg/mL) and BaP (final concentration 10 µg/mL) in cases without and with metabolic activation, respectively. DMSO in a final concentration of 0.4% was used as a vehicle control. After incubation, mixtures were diluted 10 times, incubated for an additional 2 h and the bacterial growth rate was determined by measuring absorbance at 600 nm using spectrofluorimeter (SynergyMx, BioTek,Winooski, USA). ß-Galactosidase activity was determined using ONPG as a substrate after 30 min incubation at 25 °C in dark. Absorption was measured at 420 nm using a reference solution without bacteria. The bacterial growth rate was calculated using the following formula: G = sample OD600/control OD600. A growth ratio less than 0.75 representing 25% inhibition of biomass, was considered to be an indication of cytotoxicity. Induction ratio (IR) was calculated by the formula: sample OD405/control OD420*1/G. An induction ratio of 2 was taken as the threshold above which the sample was considered as genotoxic. All treatments were performed in triplicates in three individual experiments. Statistical significance between samples and control was determined by one-way analysis of variance (1-way ANOVA) and $P < 0.05$ was considered as statistically significant.

38.2.3 ZFL Cell culture

ZFL cells (derived from normal liver of adult zebrafish) were obtained from American Type Culture Collection (ATCC number: CRL-2634) and maintained in conditions as proposed by supplier.

38.2.3.1. Cytotoxicity testing

The viability of ZFL cells was determined with MTS tetrazolium reduction assay (Promega, Madison, USA) according to the manufacturer's instructions. Treatment with the extracts (REF12.5, 25, 50 and 100) was performed for 72 h. Three independent experiments were performed, each time with three replicates per treatment point.

38.2.3.2. Genotoxicity testing

Comet assay was performed following the protocol described by Novak et al. (2017). Treatments were performed in 24-well plates for 72h with maximal REF which showed growth inhibition lesser than 20%. Images of 50 randomly selected nuclei per experimental point were analysed using a fluorescence microscope (Nikon, Eclipse 800) at 400× magnification and the image analysis software (Comet Assay IV, Perceptive Instruments Ltd., UK). Percentage of DNA in comet like shapes – Tail intensity TI% – was used as a measure for genotoxicity. Three independent experiments were performed for each treatment.

38.2.3.3. Cell-cycle analysis

ZFL cells were seeded at a density of 175,000 cells/well into 6-well. After incubation at 28 °C for 24, the growth medium was replaced with fresh medium containing extract in certain REF. For cell-cycle analysis samples which were the most potent in comet assay were chosen at the REFs: JDS4-12 (REF75), JDS4-30 (REF75), JDS4-33 (REF100), JDS4-37 (REF12.5), JDS4-40 (REF50), JDS4-41 (REF12.5), and JDS4-50 (REF100).

Treatment was performed for 72 h. In each experiment, a positive control (1 µg/mL of etoposide) and a vehicle control (0.4% DMSO) were included. After exposure, the cells were harvested and fixed in cold ethanol. Cells were stained with DAPI and flow cytometric analysis was carried out on MACSQuant® Analyzer 10, where 10^4 events were recorded for each sample. The percentage of cells in G0/G1, S, and G2/M phases of the cell cycle were determined using FlowJo (Biosciences, USA). Three independent experiments were performed.

38.2.3.4. zFET

Zebrafish wild type Tübingen were maintained at 28±1 ˚C, 12h light/12h dark photoperiod. Every seven days fish were spawned, and the experiments were conducted if the rate of fertilized eggs was >90%. The experiments were performed in 24-well plates. Ten embryos were placed in each well in 2 ml of ISO water (5.5 mg KCl 294 mg $CaCl_2 \cdot 2H_2O$, 123 mg $MgSO_3 \cdot 7H_2O$ and 62 mg $NaHCO_3$ per L) with sample extracts with maximum REF400. Each sample was tested in triplicate. As a negative control ISO water was used, as solvent control DMSO was applied in a final concentration of 1.6%. Treatments started at 6 hours post fertilization (hpf). The embryos were observed under the stereo microscope Stemi 508 (Carl Zeiss Microscopy, Gottingen, Germany) at 32x and 40x magnification and images were captured with camera Axiocam Erc5s (Carl Zeiss Microscopy, Gottingen, Germany). Biomarkers such as coagulation, non-detached tail and lack of somites were assessed as lethal endpoints at 24 hpf and 48h, while at 72 hpf lack of heartbeat was considered lethal (Lammer et al., 2009). Embryo hatching rates were assessed at 48 and 72 hpf while developmental malformations (head malformation, eye and body pigmentation, tail malformation, scoliosis, yolk morphology, heart malformation, pericardial edema, tail circulation, growth retardation) were assessed at 72 hpf.

38.3 Results and Discussion

Table 1 shows the number of detected substances in each extract and the number of substances with known genotoxic potential based on the available literature. Literature data on all 138 substances detected at the sites was reviewed and the results indicated that 53 of the detected substances are known to be genotoxic (38.4%), 27 have no genotoxic potential (19.6%) while for 58 of the substances there is no data on their genotoxic activity (42%). Oxidative damage was identified as prevalent mechanism of genotoxicity (28 of 53 substances). In the group of genotoxic substances, majority were pharmaceuticals (39.6%) followed by chemicals used in agriculture (herbicides, pesticides and insecticides – 30.2%) and industrial chemicals (11.3%).

The highest number of total and genotoxic substances was detected at site JDS4-12 while sites JDS4-5 and JDS4-6 were characterised by the lowest number of substances. Sites also differ by the concentration of detected substances which was assessed via the toxic units (TU) calculated from the ratio of measured concentration and the lowest Predicted No Effect Concentration (PNEC) obtained from the NORMAN Ecotoxicology Database. Again, the highest SUM TU was detected at the site JDS4-12 and the lowest at sites JDS4-5 and JDS4-6. SUM of TU also pointed to some sites which did not differ evidently by the number of substances (such as JDS4-34), meaning that at these sites substances with high value of TU were recorded.

38.3.1 Prokaryotic model - SOS/*umuC* assay

As shown in Table 1, none of the tested samples has induced SOS response at REF100 with or without metabolic activation. Based on this model, samples in evaluated range had no harmful effect.

38.3.2 Eukaryotic model – ZFL cell line

The result of the MTS assay indicated that 11 of 24 samples had cytotoxicity higher than 20% at REF100. Extracts from the Upper Danube (up to site JDS4-9) had no cytotoxic effect on ZFL cell line. Based on the IC50 value the most cytotoxic samples were JDS41>JDS31>JDS40>JDS37>JDS22>JDS24>JDS36>JDS34>JDS12 in descending order.

Table 1: Overview of the number of substances detected in extracts as well as their cytotoxic and genotoxic activity.

Sample		**No of detected substances**		**SOS/umuC (REF100)**		**Cytotoxicity ZFL**	**Comet assay ZFL**	**SUM of TU**	
Sample	Description	Total	Genotoxic	S9-	S9+	IC50 (REF)	NOEC (REF)	Total	Genotoxic
SPE	SPEC			-	-	>100	100		
JDS4-3	Above Klösterl / Kelheim	46	19	-	-	>100	100	7.29	10.05
JDS4-5	Passau-Ingling	18	6	-	-	>100	100	0.12	0.34
JDS4-6	Jochenstein	19	8	-	-	>100	100	0.39	1.74
JDS4-8	Oberloiben	34	14	-	-	>100	100	1.04	2.37
JDS4-9	Klosterneuburg	39	18	-	-	>100	100	2.01	4.16
JDS4-12	Lanzhot	67	32	-	-	85.6	25	7.28	33.59
JDS4-14	Bratislava	37	16	-	-	>100	100	1.19	2.94
JDS4-22	Szob	43	15	-	-	69.2	12.5	0.86	3.75
JDS4-23	Budapest upstream (Megyeri Bridge)	28	8	-	-	>100	100	0.40	3.14
JDS4-24	Budapest downstream (M0 bridge)	36	14	-	-	72.7	50	0.43	4.17
JDS4-29	Hercegszanto / Batina / Bezdan	49	18	-	-	>100	100	4.64	7.64
JDS4-30	Drava mouth (rkm 5.0)	41	14	-	-	100	25	1.23	2.45
JDS4-31	Ilok / Backa Palanka	44	18	-	-	54.0	25	1.49	4.88
JDS4-33	Tisza mouth (rkm 1.0)	44	15	-	-	>100	75	3.38	4.91
JDS4-34	Jesenice na Dolenjskem	42	19	-	-	79.8	75	11.32	13.83
JDS4-35	Jamena	49	17	-	-	>100	75	2.59	11.59
JDS4-36	Sava mouth (rkm 7.0)	36	11	-	-	74.2	50	3.58	17.79
JDS4-37	Downstream Pancevo	43	15	-	-	62.8	6.25	3.51	13.11
JDS4-40	Banatska Palanka / Bazias	42	13	-	-	59.8	25	1.46	5.28
JDS4-41	Upstream Timok (Rudujevac / Gruia)	41	12	-	-	33.8	6.25	0.83	5.58
JDS4-43	Pristol / Novo Selo Harbour	41	17	-	-	>100	100	2.25	4.92
JDS4-47	Downstream Ruse/Giurgiu (Marten)	36	16	-	-	>100	13	4.36	6.36
JDS4-48	Chiciu/Silistra	39	20	-	-	>100	100	2.21	13.80
JDS4-50	Reni	34	15	-	-	>100	75	2.88	12.99

- no effect

The maximal REFs used for comet assay were the ones that did not reduce cell viability for more than 25%. Induction of 1.5-fold compared to solvent control was considered as positive effect. No observed effect concentrations (NOECs) are indicated in Table 1. Genotoxic effects were observed for 9 of 24 samples (Fig 2). The most active were samples JDS4-37 and JDS4-41. In the Upper Danube only the sample JDS4-12 was genotoxic. In the Middle Danube 6 samples were genotoxic while in the Lower Danube samples JDS4-41 and JDS4-50 induced DNA damage detected with comet assay. Similar observations were found within the previous survey (JDS3) where we identified an increase of genotoxic potential in samples from the Middle Danube using mussels and fish in passive biomonitoring approach (Deutschmann et al. 2016; Kolarević et al. 2015). The cell cycle analysis was performed for 8 samples which were genotoxic. Only sample JDS4-41 resulted in cell arrest in G1 phase of the cell cycle.

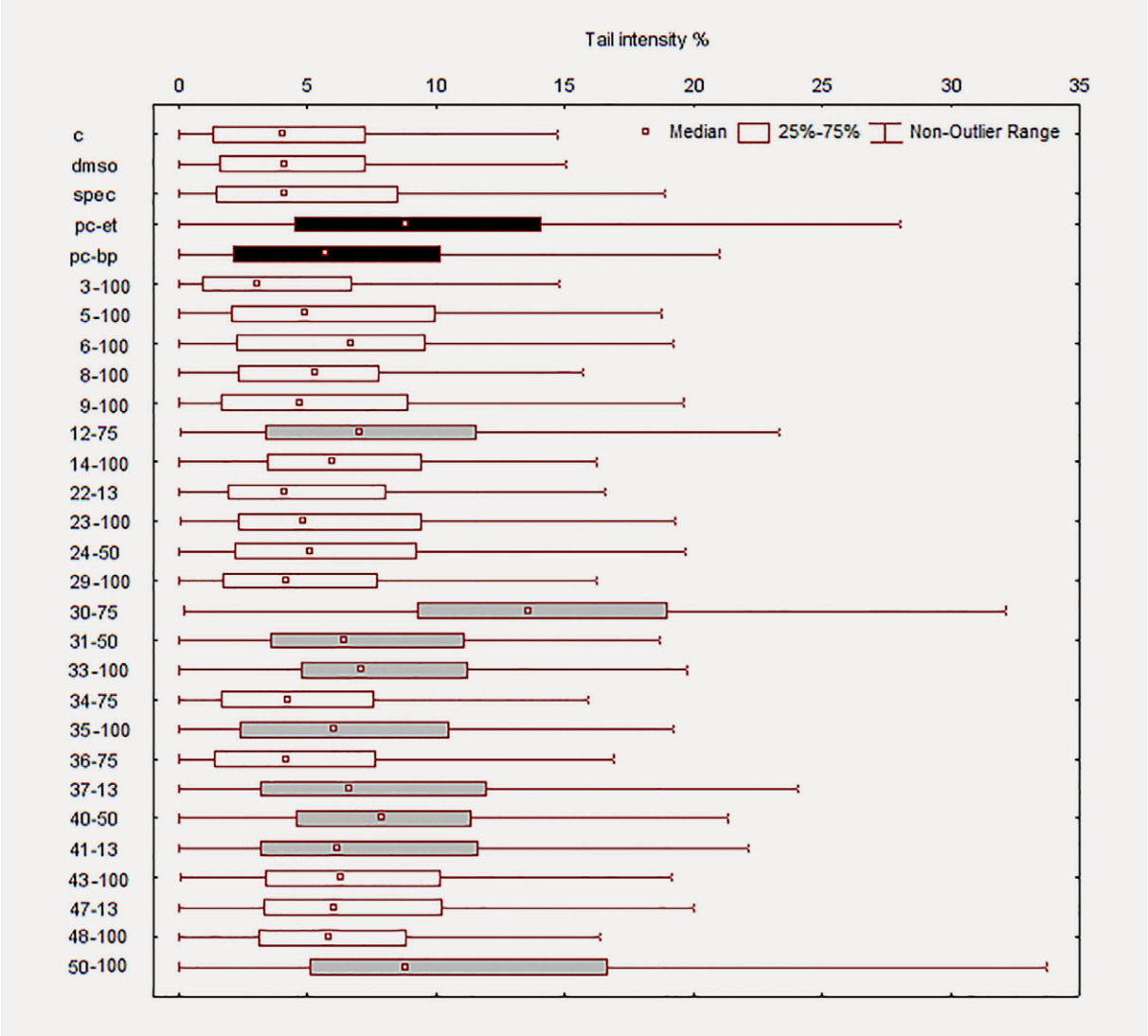

Figure 2: Tail intensity % (percentage of DNA in comet like shapes – Tail intensity (TI% shown at x-axis) was used as a measure for genotoxicity) in the ZFL cell line at the highest tested REF. Plots of the extracts with >1.5-fold DNA damage induction are marked with gray, positive controls (et-etoposide and BaP-benzo(a)pyrene) are marked with black.

38.3.3 zFET

Initial screening of all samples at REF100 was performed and none of the samples had embryo toxic or teratogenic effects. Testing in the higher REF range is planned for further research.

38.4 Conclusions

The results demonstrated that about 46% of the extracts were cytotoxic while about 38% of the extracts were genotoxic to a certain extent. Genotoxic potential was detected only in the eukaryotic model. Most of the samples active in applied bioassays were the ones collected in the Middle Danube section.

38.5 References

BRACK W., AISSA SA., BACKHAUS T., DULIO V., ESCHER B., FAUST M., HILSCHEROVA K., HOLLENDER J., HOLLERT H., MULLER C., MUNTHE J., POSTHUMA L., SEILER TB., SLOBODNIK J., TEODOROVIĆ I., TINDALL A., UMBUZEIRO G., ZHANG X., ALTENBURGER R. (2019): Effect-based methods are key. The European Collaborative Project SOLUTIONS recommends integrating effect-based methods for diagnosis and monitoring of water quality. Environmental Sciences Europe. 31, 1-6.

DEUTSCHMANN B., KOLAREVIC S., BRACK W., KAISAREVIC S., KOSTIC J., KRACUN-KOLAREVIC M., LISKA I., PAUNOVIC M., SEILER TB., SHAO Y., SIPOS S., SLOBODNIK J., TEODOROVIC I., VUKOVIC-GACIC B., HOLLERT H. (2016): Longitudinal profile of the genotoxic potential of the River Danube on erythrocytes of wild common bleak (Alburnus alburnus) assessed using the comet and micronucleus assay. Science of the Total Environment. 573, 1441-1449.

FLEGROVA Z., SKAREK M., BARTOS T., CUPR P., HOLOUBEK I. (2007): Usefulness of three SOS-response tests for genotoxicity detection. Fresenius Environmental Bulletin. 16(11A). 1369.

KOLAREVIĆ S., KRAČUN-KOLAREVIĆ M., KOSTIĆ J., SLOBODNIK J., LIŠKA I., GAČIĆ Z., PAUNOVIĆ M., KNEŽEVIĆ-VUKČEVIĆ J., VUKOVIĆ-GAČIĆ B. (2015): Assessment of the genotoxic potential along the Danube River by application of the comet assay on haemocytes of freshwater mussels: The Joint Danube Survey 3. Science of the Total Environment. 540, 377-385

LAMMER E., CARR GJ., WENDLER K., RAWLINGS JM., BELANGER SE., BRAUNBECK T. (2009): Is the fish embryo toxicity test (FET) with the zebrafish (Danio rerio) a potential alternative for the fish acute toxicity test. Comparative Biochemistry and Physiology. Part C 149, 196-209

NOVAK M., ŽEGURA B., MODIC B., HEATH E., FILIPIČ M. (2017): Cytotoxicity and genotoxicity of anticancer drug residues and their mixtures in experimental model with zebrafish liver cells. Science of The Total Environment. 601. 293-300.

SHAO Y., XIAO H., DI PAOLO C., DEUTSCHMANN B., BRACK W., HOLLERT H., SEILER TB. (2019): Integrated zebrafish-based tests as an investigation strategy for water quality assessment. Water Research 150, 252-260.

ŽEGURA B., HEATH E., ČERNOŠA A., FILIPIČ M. (2009): Combination of in vitro bioassays for the determination of cytotoxic and genotoxic potential of wastewater, surface water and drinking water samples. Chemosphere.75, 1453-1460.

ŽEGURA B., FILIPIČ M. (2019): The application of the Comet assay in fish cell lines. Mutation Research/Genetic Toxicology and Environmental Mutagenesis. 842, 72-84.

39

Overview chapter on chemical pollution

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Abstract

According to the WFD, priority substances causing failure to achieve good chemical status and River Basin Specific Pollutants (RBSPs) adversely impacting ecological status of water bodies should be monitored and eventually phased-out from the environment. An extensive screening of JDS4 surface water, sediment, biota, wastewater and groundwater samples has been performed with target analytical techniques, focused on the determination of legacy pollutants, and novel wide-scope target (>2,600 substances) and suspect (>65,000 substances) screening methodologies. A massive dataset of ca. 310,000 results of target analyses has been compiled. In comparison, 719 substances were screened for, and ca. 47,000 data entries were generated in JDS3. The results have shown that only a handful of WFD priority substances and surface water Watch List substances were posing a threat to Danube fauna and flora. Hundreds of chemicals detected in the samples could be prioritised to several tens of newly proposed candidate Danube RBSPs for each of the studied environmental compartments. This list of candidate substances is also a valuable contribution to the iterative selection process for future Watch List substances at the EU level. Suspect screening demonstrated its feasibility to reveal the presence of toxic substances and their transformation products, which would otherwise stay unnoticed. The raw data with mass spectra ('chemical fingerprints') of all detected pollutants stay stored for future retrospective screening, without the need for additional investments in sampling and analysis campaigns.

Screening of wastewater effluent samples indicated that inefficient treatment in WWTPs across the basin is among the main sources of DRB chemical pollution. Effect-based Monitoring tools have been used for measurements of toxicity effects of mixtures of chemicals and effectiveness of their use was demonstrated for wastewater and more polluted surface water samples. The wastewater monitoring methodology, as proposed by the NORMAN Association and Water Europe, was tested with the JDS4 data and used as an important input in the ongoing discussion on the revision of the Urban Waste Water Treatment Directive (UWWTD; 91/271/EEC). Passive sampling results have shown that the spatial variability of investigated hydrophobic priority substances in the surface water of the Danube is low. No deterioration of Danube surface water contamination by hydrophobic priority substances was observed in JDS4 in comparison with the results from JDS3. Similarly, pollutants in groundwater bodies, connected to the surface water via bank filtration, did not exceed regulatory toxicity threshold values.

It has been concluded that novel monitoring techniques are vastly superior compared to traditional target monitoring of a few legacy substances and provide both 'early-warning' and 'safety net' signals needed for a holistic chemicals management in support to the EU 'zero-pollution policy'. The traditional monitoring applied in compliance with the current environmental legislation does not sufficiently protect the Danube ecosystem.

39.1 Introduction

Article 16 of the WFD sets out the strategy to reduce the chemical pollution of European waters (EU 2000). Thereby, the chemical status assessment is used alongside the ecological status assessment to determine the overall status of a water body and to define management measures. Directive 2013/39/EU (EQSD 2013) establishes environmental quality standards (EQS) for 45 priority substances (PS), expressed as annual average (AA) concentrations and maximum allowable concentrations (MACs) and/or concentration in biota. Compliance with AA-EQSs and MAC-EQSs sets the chemical status of a water body as "good". Under the WFD, Member States must set quality standards (according to Annex V, 1.2.6) for "river basin specific pollutants" (RBSPs; listed in Annex VIII, 1–9) that are "discharged in significant quantities" and take action to meet those quality standards as part of the ecological status (Article 4, 11, and Annex V, 1.3 (EU 2000). EQSs are therefore key tools in assessing and classifying both chemical and ecological status. Whether a compound is "discharged in significant quantities" is commonly decided based on the substance's exposure level, referred to as Predicted Environmental Concentration (PEC). This, in turn is compared to an ecological safety threshold expressed as Predicted No-effect Concentration (PNEC). PEC/ PNEC risk ratios above 1 would trigger the substance's consideration as RBSP and its inclusion in the routine monitoring and the derivation of a legally binding EQS. Given the vast number of chemicals which may be released into the environment, the EC has recognised a need for frequent update of the WFD PS and established a mechanism of surface water Watch List to be analysed by EU MS at reduced frequency at selected sampling sites in order to find out new and relevant emerging chemical threats to the environment.

A list of 20 Danube RBSPs has been established on the basis of results of JDS3 (Liska et al., 2015) within the SOLUTIONS project (https://www.solutions-project.eu/project/) using the methodology developed by the prioritisation working group of the NORMAN network (Dulio et al., 2013). These chemicals, WFD PS and Watch List substances were included in the JDS4 monitoring programme; analysed also by the EC Joint Research Centre laboratory in Ispra, Italy. In total, more than 2,600 substances compiled by the JDS4 reference laboratories were analysed in each sample, selected on the basis of their frequent occurrence in environmental samples and previous risk assessment. JDS4 has been strongly supported by the NORMAN network (Dulio et al., 2020), which strives to establish the 'universe of chemical pollutants in the environment' within its NORMAN Substance Database. At the time of carrying out JDS4, the list of substances, which were accompanied with sufficient information to allow for their screening as 'suspects', contained 65,906 pollutants and transformation products. Presence/absence and semi-quantitative estimate of concentration of each suspect compound could be reported. Importantly, all raw high-resolution mass spectral chromatograms containing 'fingerprints' (mass spectra) of each detected substance in each sample were stored in the NORMAN Digital Sample Freezing Platform (DSFP; Alygizakis et al., 2019). This allows for retrospective screening of any future 'popping up' emerging substances, even those labelled today as 'unknowns', just by re-analysing existing datasets.

The present EU water legislation is focused on the assessment of risks of single chemicals and does not account for effects of 'cocktails of pollutants'. However, specific bioassays can respond to adverse effects of a group of 'similar' pollutants, e.g. those with endocrine disrupting, estrogenic, mutagenic etc. properties. This has been addressed in-depth in the previous SOLUTIONS project and various effect-based methods (EBM) using a recommended battery of NORMAN/SOLUTIONS bioassays were applied in JDS4.

A frequent criticism of spot sampling, even if it is 12 times per year, is that one never knows if the toxic pollutants are released the next day after sampling in a short pollution-wave (e.g. run-off pesticides or overflowing of WWTPs after a storm). This can be tackled by installing passive samplers collecting time-integrated samples for an extended period. A battery of passive samplers was therefore installed at nine JDS4 sites for 100 days. A similar problem is with sampling wastewater, where the concentrations can vary significantly during the day. Here, composite 24 h samples were collected.

The final aim of all chemical screening analyses was to (i) indicate major pollution problems by chemicals in the DRB, (ii) demonstrate the feasibility of using novel wide-scope target and suspect screening techniques in combination with EBM in comparison to the traditional target analyses and (iii) update the list of Danube RBSPs from 2013 (JDS3).

39.2 Chemical and ecological status of the Danube River Basin

No attempt was made to indicate the chemical and ecological status of water bodies in the DRB based on the analysis of chemical pollutants. JDS4 was only a snapshot in assessment of the presence of chemicals in the DRB and for the legal compliance assessment more frequent analyses of surface water would be required (12 times per year for WFD PS; 4 times per year for Danube RBSPs). However, the ICPDR countries could consider to use the results of biota (fish, molluscs) screening for reporting the status assessment of WFD PS requested to be analysed in biota (EQSD, 2013), since it is required only once per year in the RBMP period.

Why aren't WFD priority substances and River Basin Specific Substances assessed together using common standards?

This seems to be a flaw in the WFD and there are already proposals to correct it at its next update. When the WFD came into force two decades ago, there was a need to establish a common European standard to assess the risks coming from chemical pollution, providing an option to EU MS to include RBSPs in their national monitoring programmes. The task proved to be challenging and costly, even today many EU MS are failing to report on all WFD PSs and the analytical methodologies often do not comply with the QA/QC requirements for analysis. e.g. for compounds required to be analysed at extremely low concentration levels. Nationally derived EQSs for RBSPs differ widely, more efforts to harmonise them at the EU level are necessary. This leads to situations when one RBSP in a transboundary river can have two toxicity threshold values approved in the legal systems of two neighbouring countries. The concept of monitoring WFD PSs has been extremely useful and fulfilled its purpose to establish the 'minimum standard' followed by all EU MS. As all concepts, also this one got outdated and is in a need for revision based on the new scientific evidence and progress in environmental research.

In the DRB, it has been observed via national monitoring efforts that only 19 out of 45, WFD PSs might be of risk for surface water. These substances were included in the target analytical programme of JDS4. The outcomes showed that only three WFD PSs (PFOS, cybutryn, cypermethrin) were exceeding their EQS values. Ten substances from the EU Watch List were analysed and elevated concentrations could be detected for the pharmaceutical diclofenac, the natural hormone 17-beta-estradiol and the insecticide imidacloprid. All but two (bromacil, dimefuron) RBSPs identified in JDS3 were present frequently in JDS4 water samples as well. The concentrations of many of JDS3 RBSPs were reported at lower levels in JDS4 and only seven (out of 15) were recommended also for the updated list of Danube RBSPs.

Similarly, the historical data indicated that only 9, out of 11, WFD PSs for biota could be of concern in the DRB. Mercury and flame retardants - brominated diphenyl ethers (BDEs) - were exceeding EQS values in all samples, however, this is a pan-European problem and measures to reduce the pollution should be sought at the EU level. For dioxins and dioxin-like compounds, heptachlor and fluoranthene concentrations were higher than the biota EQS only at single sites and the substances do not seem to be of a basin-wide concern.

The findings of JDS3 (2013) and JDS4 (2019) indicate that WFD-compliant monitoring of all WFD PSs generates a lot of 'expensive zeros' values for compounds not relevant anymore for assessment of chemical and ecological status in the DRB. Instead, newly defined RBSPs are of an immediate environmental concern and an effort should be made to harmonise the methodology for their prioritisation and establishment of legally binding EQS values at the regional (ICPDR) but preferably at EU level. A frequency of re-update of the new lists of RBSPs is at present six years. Based on the scientific evidence, a legal pathway to introduce new RBSP substances into national and regional monitoring programmes in shorter time periods should be sought for. The new lists of RBSPs should also foster the Watch List process for PS.

How can we monitor ever increasing number of chemicals in the environment?

The traditional target analysis techniques were designed to determine a few, or several tens of, substances of concern using laborious sample preparation. Latest analytical instrumentation and novel analytical strategies allow for determination of hundreds of target substances in a single sample for approximately the same or even lower costs. Regarding surface water, the automated large volume solid-phase extraction techniques in combination with ultra-sensitive liquid (LC) and gas chromatography (GC) high-resolution mass spectrometry (HRMS) applied in JDS4 allowed for detection of pollutants at their environmentally relevant ecotoxicity threshold levels (Chapters 26, 28-31). The most advanced instruments even allow for direct analysis of the water samples without any sample pre-treatment (Chapter 27). However, to cover a full chemical space, very polar (mobile) compounds need a special analysis strategy; this has been addressed in JDS4 in Chapter 33.

The HRMS techniques typically detect 2,000-5,000 substances and their TPs in each environmental sample. Even if we do not know what the exact structures/names of the substances are, we have their 'fingerprints' – mass spectra. All HRMS chromatograms of JDS4 samples were stored in the NORMAN DSFP and are available for retrospective analysis. DSFP is fed by the NORMAN Database System (Dulio at al., 2020) hosting a systematic collection of background information on the list of the 'universe of environmental pollutants' (https://www.norman-network.com/nds/susdat/), mass spectral information (https://massbank.eu//MassBank/; in silico predictions Alygizakis et al., 2019), retention time index (RTI; LC), retention index (RI; GC), ecotoxicity threshold values (https://www.norman-network.com/nds/ecotox/), hazard and exposure scores (https://www.norman-network.com/nds/factsheets/). A search for the unique combination of RTI/RI and exact mass spectrum characteristic of a substance greatly reduces the number of false positives. A comparison of signals of suspect substances with those of structurally similar internal standards generates semi-quantitative estimate of their concentrations. At the time of reporting the results of JDS4, the number of substances which could be searched for in each sample was 65,906 and it is expected that the same samples could be screened for more than 106,000 substances in early 2021.

The use of these screening techniques might prevent argumentation of some industries claiming that their products/substances harmful for the environment are safe and cannot be found at ecotoxicologically relevant concentration levels. In such cases, the results can be directly used in support of the REACH regulation and its Substance Evaluation scheme.

Which chemical pollutants are important?

Out of the more than 65,000 substances analysed in JDS4 samples, ca. 2,000 were determined in at least one sample. The NORMAN Prioritisation Framework (Dulio et al., 2013) has been used to 'funnel down' this figure to a manageable number of substances relevant at the basin scale. The NORMAN prioritisation methodology uses a decision tree that first classifies chemicals into six categories depending on the information available. That allows water managers to focus on the next steps to be taken, e.g. (not exhaustive): (1) derivation of EQS for substances already well investigated with sufficient amount of data on their occurrence and toxicity; (2) improvement of analytical methods for substances monitored whose limits of quantification (LOQs) are higher than PNEC values; (3) additional screening when more occurrence data are needed to confirm a basin-wide threat; and, (4) discontinue with monitoring of substances that are already well investigated and proved not to represent a threat to the environment. The priority within each category is then evaluated based on several indicators, including **exposure** (e.g. frequency of observations above LOQs of used methods, annual usage, use pattern, etc.), **hazard** (e.g. Persistence, Bioaccumulation, Toxicity (PBT), Endocrine Disruption (ED) and Carcinogenicity, Mutagenicity and Reprotoxicity (CMR) properties) and **risk** (exceedance of PNECs). For more details, see Chapter 36.

The above approach uses as a basis single substance concentration and its 'lowest PNEC' value from ecotoxicological assessments at three tropic levels (fish, daphnia, algae). However, it has been recognised that a single substance monitoring and assessment strategy of the WFD is not sufficient and protective for the water quality, because a complex chemical mixture poses a higher risk than any individual compound alone due to mixture toxicity effects and this risk scenario can be very site-specific. Therefore, a complementary prioritisation of substances was applied in JDS4, using so-called 'toxic units' (TU). The TU approach is a measure of the intrinsic toxicity of a compound towards an environmental concentration to compare and add (sum together) the toxicities of different substances for a selected biology endpoint: fish, daphnia and green algae (von der Ohe et al., 2011). The methodology pinpoints so-called 'toxicity drivers' - chemicals that are responsible for most (80-90%) of the toxicity in a mixture of chemicals identified at the given site. The TU assessment for surface water showed the importance of pesticides such as pyrethroids, organophosphate and carbamate insecticides and other compounds, e.g., the antioxidant diphenylamine and 5-methyl-1H-benzotriazole for fish. The organophosphate pesticide diazinon was found as the main toxicity driver for daphnia. For algae, different herbicides such as terbutryne, MCPA, cybutryne, diuron, metolachlor or nicosulforon dominated the ranking of compounds based on TU. The findings of the TU-based assessment were confirmed by the multi-substance potentially affected fraction (msPAF) - hazard unit approach developed to assess the toxicity risk of complex mixtures and compared to PNECs (see Chapter 35).

The above two prioritisation approaches are complementary and often bring to attention the same compounds, however, the outcomes of the NORMAN prioritisation methodology was finally used for a proposal of RBSPs, since it provides a basin-wide assessment of pollutants and it is matching the approach used for selection of WFD PS and Watch List substances by the EC.

Surface water and biota

JDS4 surface water samples were subjected to target screening for 2,608 substances, out of which 495 substances were determined in at least one sample and, finally, potential 51 RBSPs were proposed for monitoring in the surface water compartment of the DRB. Starting from 2,360 substances screened for in biota samples, 19 were proposed as candidate RBSPs. The prioritised list was dominated by legacy flame retardants brominated diphenylethers (BDEs), mercury and PFOS. Tables of proposed Danube RBSPs for WFD-relevant matrices surface water and biota are in Chapter 36.

Numerous others besides the above candidate RBSPs were pointed out as important in terms of their frequent presence or local exceedance of toxicity threshold values. Ideally, all substances pinpointed as of concern in Chapters 26-37 should be compiled in a future list of the Danube RBSPs and monitored by a specially developed wide-scope target screening method.

Sediments

Out of 2,317 substances screened for in sediment samples, 15 exceeded their toxicity threshold value. The ranking of substances detected in sediments was based on PNECs derived from surface water according to the NORMAN Prioritisation Framework. Here, one should be aware that there are no toxicity threshold values agreed at the DRB or EU level and only trends in increasing/decreasing concentrations of individual pollutants could be taken into account in compliance with the EQSD.

Wastewater

In total, 2,516 substances were screened for in wastewater samples, 465 were detected in at least one sample and 28 compounds exceeded their ecotoxicity threshold value. Wastewater pollutants were deliberately prioritised aside from those detected in the surface water samples. A dilution factor '5' was used to convert wastewater concentrations into surface water concentrations to allow for comparison with the freshwater PNECs. A factor '10; is recommended for big rivers; a factor '2' for small streams. The Danube WWTPs were mostly positioned on tributaries of the Danube (Chapter 31). The addition of these 'newly calculated concentrations' into the overall prioritisation scheme could bias assessment of the real-world measurements of substances in the surface water.

Groundwater

Out of the 2,561 screened for substances, 47 were present in samples from more than 50% of the sites. The list comprised 16 pesticides and their TPs, followed by pharmaceuticals and their TPs, industrial chemicals, surfactants and personal care products. In groundwater the target and wide-scope target screening analysis showed that in many cases the bank-filtration process contributes to a smaller number of substances and lower concentrations being detected in groundwater than in the Danube River. Nevertheless, this effect cannot be generalised and is compound- and site-specific. For many of the detected substances the situation is opposite and the concentration in groundwater is often higher than in the Danube, which should be more thoroughly examined at well-characterised pairs of Danube/groundwater sampling sites. Even so, a considerable number of substances (23%) were only detected in one groundwater site and not found in any of the adjacent Danube sites, which indicates that pollution of groundwater is being caused by local or regional polluting activities (Chapter 25). Here, the transport of pollutants via air should also be considered.

Rather surprisingly, numerous substances detected and semi-quantified by suspect screening were indicated as exceeding 'safety net threshold value' 0.1 µg/l in groundwater and certainly deserve more attention in future investigative screening campaigns (Chapter 36).

Are the data provided by the novel monitoring techniques robust and comparable?

A comparison of well-established target analysis and novel wide-scope target screening methods has been carried out. The concentrations measured in JDS4 surface water samples showed for many compounds a good agreement within a factor of 3, despite different analytical strategies used. These results suggest that LC-HRMS-based screening methods are able to provide similar result as targeted LC-MS/MS methods and thus hold the potential to be applied in WFD monitoring if a larger set of compounds should be considered. A harmonization of quality assurance/quality control measures for screening methods and the reporting of data quality is recommended to further improve the comparability of different methods and to judge the reliability for individual compounds, as different methods will not perform equally well on a specific

compound (for more details, see Chapter 37). Results obtained by GC-HRMS methods used in JDS4 were not yet systematically compared and the datasets should be re-analysed in future.

There is a concern that non-target screening and EBM are too complex and can be carried out only in a few 'top' European laboratories. An attempt was therefore made to harmonise the current best practices with laboratories in the DRB by organising the NORMAN / ICPDR collaborative trial for non-target screening and effect-based tools (see Chapter 34). Four (out of seven in total) NTS and two (out of four in total) EBM laboratories from the DRB participated. The results of the suspect screening of compounds spiked in an extract of a reference natural water sample were quite promising. Many of the most important spiked compounds were identified by the participants of the chemical analytical part. Regarding EBM, it has been concluded that currently used methods are powerful tools to discriminate low-toxicity from more toxic samples (WWTP effluents, rivers with high wastewater fraction, agriculturally impacted streams etc.) and to quantify their toxic burden, while a quantitative assessment in highly diluted surface waters is currently not possible. A training on NTS organised by UFZ was an important step towards capacity building in the DRB and it was strongly recommended to continue with similar activities in future.

What are the effects of mixtures of chemical pollutants?

Given the ever-increasing number of chemicals in use, there will always be some of them overlooked even by the most sophisticated NTS techniques. Also, the toxicity of chemicals in the mixtures is different, and usually higher than a simple summing up of toxicity contribution by individual chemicals in the mixture. This can be addressed by EBM, where an overall toxicity signal of all chemicals in the mixture with similar toxic mode of action can be measured. A battery of robust and validated in vitro and in vivo bioassays has been defined previously by NORMAN and SOLUTIONS. The in vitro battery was applied on JDS4 wastewater influent and effluent samples (Chapter 31), whereas all bioassays were applied for analysis of surface water samples (not finalised yet at the time of writing this report). Nevertheless, three JDS4 surface water samples were included in the collaborative trial and the results were promising (see Chapter 34). Additionally, a high-throughput HPTLC methodology with four bioassays has been used by LW Langenau as an example of a rapid EBM screening tool (Chapter 28).

Also based on the results of JDS4, EBM has certainly earned its place among the regulatory monitoring techniques. Ideally, it should always be accompanied with NTS in order to be able to identify individual pollutants (or their mixtures) causing the toxicity.

Can we monitor pollutants continuously over a longer period of time?

Passive sampling is a cost-efficient monitoring technique that provides a time-integrated image of pollution in the aqueous phase over an extended time period and gave a representative picture of the surface water quality in summer 2019. The results show that the spatial variability of investigated hydrophobic priority substances in surface water of the Danube is low. No deterioration of Danube surface water contamination by hydrophobic priority substances was observed in JDS4 in comparison with the results from JDS3. Among investigated organochlorine compounds and PAHs at the site selected for a long-term repeated observations (JDS4-15), a significant concentration decreasing trend was observed for hexachlorobenzene, PCB 28, PCB 52 and p,p'-DDE, whereas no significant temporal trend was found for PCBs with a higher degree of chlorination or for priority PAHs.

In the upper and middle Danube stretches, the occurrence of polar organic contaminants is associated with the discharge of municipal wastewaters to the river. In the Danube stretch downstream the Iron Gates dam, the contaminant pattern and concentrations in surface water reveals application of pesticides in agriculture as the main contamination source.

Passive samplers were installed at the same sites from where fish samples were collected for the follow-up chemical analyses. Passive sampling of hydrophobic substances in surface water provides a worst-case scenario of fish exposure to those substances and should be considered as a viable alternative to biota monitoring in the EU regulatory framework.

39.3 Conclusions and future challenges

Each JDS is bigger than the previous one in terms of number of laboratories involved, parameters measured, data produced and state-of-art scientific challenges tackled. Summarising the outcomes, it can be stated with confidence that JDS4 is indisputably the biggest river basin survey ever globally. Regarding only its chemical part, in JDS3, 719 chemical substances were analysed and ca. 47,000 data entries were generated. It took the next four years and power of large ca. 12 million EUR EU-funded project SOLUTIONS to convert the 'data to information'. In JDS4 a massive dataset of more than 309,000 results of target analyses has been compiled, accompanied with suspect screening results for more than 65,000 compounds in each sample. Outcomes of wide-scope target and suspect screening techniques were vastly superior to the classical target analysis of a few WFD-compliance monitoring pollutants. Their potential, together with EBM, to be used in regulatory monitoring has been demonstrated. Chemical screening data were used for drafting a list of candidate RBSPs in surface water and biota. JDS4 provided a possibility to test at a large geographical scale how the revised EU legislation for urban wastewater (UWWTD) would work in practice.

Interlinking chemical screening and EBM data with results of biological monitoring, and especially eDNA remain a challenge. This is directly related to a need for accounting toxicity of chemical mixtures and improved prioritisation of RBSPs. A capacity building of Danube laboratories responsible for regulatory monitoring is needed to be able to carry out NTS and EBM on a routine basis.

39.4 References

WFD (2000). Directive 2000/60/EC of the European Parliament and of the Council 22.12.2000. Official Journal of the European Communities L327: 1-72.

EQSD (2013). Directive 2013/39/EU of the European Parliament and of the Council of 12 August 2013 amending Directives 2000/60/EC and 2008/105/EC as regards priority substances in the field of water policy. Official Journal of the European Communities L 226/1.

Liška, I., Wagner, F., Sengl, M., Deutsch, K., Slobodník, J. (eds.) (2015). Joint Danube Survey 3 A Comprehensive Analysis of Danube Water Quality. International Commission for the Protection of the Danube.

Dulio V. et al. (2020), The NORMAN Association and the European Partnership for Chemicals Risk Assessment (PARC): let's cooperate!, Environ Sci Eur, 32:100, https://doi.org/10.1186/s12302-020-00375-w.

Dulio V., von der Ohe P.C. at al. (2013), NORMAN Framework for prioritisation of emerging substances, ISBN: 978-2-9545254-0-2, http://www.norman-network.net/sites/default/files/files/Publications/NORMAN_prioritisation_Manual_15%20April2013_final%20for%20website-f.pdf.

Alygizakis N.A. et al. (2019), NORMAN digital sample freezing platform: a European virtual platform to exchange liquid chromatography high resolution-mass spectrometry data and screen suspects in "digitally frozen" environmental samples. TrAC Trends Anal Chem 115:129–137, DOI: 10.1016/j.trac.2019.04.008.

Von der Ohe et al. (2011): A new risk assessment approach for the prioritization of 500 classical and emerging organic microcontaminants as potential river basin specific pollutants under the European Water Framework Directive. 2011/03/19 ed. Science of the Total Environment 409: 2064–2077.

40 Rare earth elements

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Abstract

Rare Earth Elements, which include Sc, Y and the lanthanides, have been monitored for the first time along the Danube River and in some of its tributaries. The concentrations found in the surface water samples have been normalized to a reference rock type (Post Archean Australian Shale) to detect potential anomalies. A negative anomaly was observed for cerium (Ce): it is natural and related to the redox behaviour of this element. A large positive anomaly was observed for gadolinium (Gd): it is due to the use of contrast agents incorporating Gd to perform Magnetic Resonance Imaging exams in health facilities. As the contrast agent is not eliminated in wastewater treatment plants, Gd is ultimately disseminated in the aquatic environment.

40.1 Introduction

Rare Earth Elements (REEs) usually refer to the lanthanide's series (from lanthanum (La) (atomic number Z=57 to lutetium (Lu) Z=71), including scandium (Z=21) and yttrium (Z=39). They are naturally found in water systems because of rocks weathering, and they are commonly used in geochemistry as processes and/or sources tracers, as they behave in a coherent way. However, REEs have wide and growing applications in new technologies, industries, medicine and agriculture. Those anthropogenic uses disrupt the geochemical and biological cycles of REEs and lead to enrichment of some REEs in waters. The first enrichment observed was that in gadolinium (Gd) , reported in surface waters. Indeed, Gd is used as contrast agent for Magnetic Resonance Imaging (MRI) analyses, in highly stable Gd-organic complex forms (as Gd^{+3} is toxic for human body). After injection in the human body, the Gd contrast agent is excreted within a few hours by urine and ends up in wastewater. Because of their stability, Gd-complexes are not removed by conventional wastewater treatment plants (WWTPs), and WWTP effluents are now recognized as the principal source of anthropogenic Gd (Gd_{anth}) in waters (Bau and Dulski, 1996; Kümmerer and Helmers, 2000; Verplanck et al., 2005, 2010). As, so far, no proven toxicity of the Gd-complex has been shown, some studies suggest using this complex as tracer for wastewater-derived contamination in natural waters (Gd is easier and less expensive to measure than other micropollutants discharged from WWTP effluents). Some studies highlighted other positive REEs anomalies from anthropogenic origin in natural waters, such as a positive La anomaly in Rhine river, and later a samarium (Sm) positive anomaly, both originating from industrial production of catalysts for petroleum refining.

JDS4 was the first campaign on the Danube River during which REEs concentrations and distributions were investigated, both in river waters and groundwater. Such investigation can inform about anthropogenic pressure on the Danube River, through the detection of the gadolinium anomaly. This anthropogenic pressure could be linked to the population density, the number of WWTPs, the presence of MRI facilities and the number of MRI exams into each country crossed.

40.2 Methods

Water samples were collected in PTFE bottles previously washed with ultrapure HNO_3 acid and rinsed with ultrapure water. Samples were filtrated through 0.45µm pore size regenerated cellulose syringe filters. The fraction below 0.45µm is operationally called "dissolved fraction" (truly dissolved + colloidal fractions). The dissolved fractions were acidified at 1% with ultrapure HNO_3. REEs were determined by Inducted Coupled Plasma Mass Spectrometry (ICP-MS) on a ThermoScientific iCAPQ+prepFast, without pre-concentration. Re and Rh at 50ppb were used as online internal standards. The analytical error was below 5% and the quantification limit 1ng/L for all REEs. SLRS-6 reference material water was used to control the ICP-MS accuracy and reproducibility.

REEs concentrations are commonly normalized by a reference to avoid Oddo-Harkins effect and highlight only geochemical processes (Bau et al., 2018). Then, normalized concentrations of each REE are plotted together, producing a REEs pattern. If there is no anomaly compared to the reference, the pattern will be smooth. If a natural or anthropogenic anomaly appears for a given REE, a "spike" will be visible along the pattern. Here, REEs concentrations are normalized by the Post Archean Australian Shale (PAAS). Gadolinium anomaly and anthropogenic gadolinium concentration were calculated using the following equations

$$Gd/Gd^* = Gd_{PAAS}/(0.4xNd_{PAAS} + 0.6xDy_{PAAS})$$

$$Gd_{anth} = Gd - Gd^*$$

where Gd is the measured concentration in samples, Gd* the calculated geogenic concentration, and Xx_{PAAS} the concentration normalized by the PAAS.

40.3 Results and Discussion

REEs patterns along the Danube River and its tributaries are given in Figures 1 and 2. All patterns display a cerium (Ce) negative anomaly (Ce/Ce* from 0.30 to 0.78). This negative anomaly is a natural anomaly, which occurs due to the redox behavior of Ce. Indeed, Ce can have two oxidation states (Ce^{3+} and Ce^{4+}) in the typical conditions of temperature and pressure of surface water. In natural waters, Ce^{4+} is likely to combine with oxygen to form cerianite. As the cerianite is very insoluble, its formation leads to Ce negative anomaly in water (Seto and Akagi, 2008).

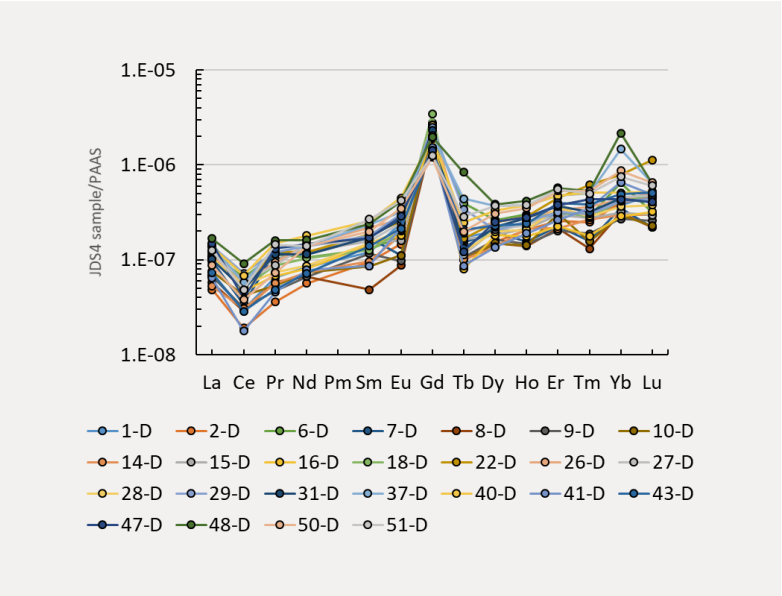

Figure 1: REE patterns along the Danube River.

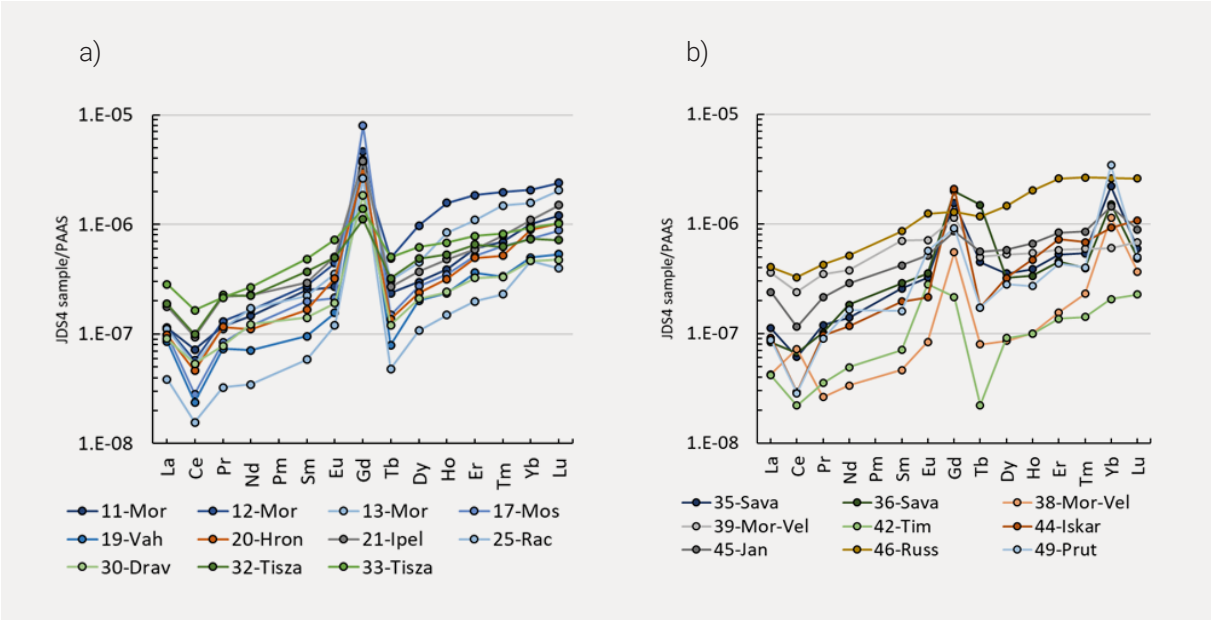

Figure 2: REE patterns in Danube River tributaries.

The Gd positive anomaly is detectable in all the samples (Gd/Gd* value from 1.85 to 37.41), except in sample JDS4-46 Russenski Lom, (tributary in Bulgaria, Gd/Gd*: 1.18). To define a Gd anomaly as an anthropogenic anomaly, the threshold value of Gd/Gd* is set to 1.5 . In the case of the Danube River and its tributaries, the visible Gd anomalies are always above this threshold value: that allows the calculation of Gd_{anth} concentrations. For the Danube River itself, the most important Gd/Gd* value is for the sample JDS4-4 (Gd/Gd*: 25.9). This sampling station is located in Germany, just downstream a WWTP effluent discharge. Figure 3 shows the value of Gd/Gd* and the corresponding calculated Gd_{anth} concentration along the Danube River and its tributaries. Two trends can be identified clearly: from sample JDS4-1 to sample JDS4-31, with a Gd/Gd* globally above 10 and from sample JDS4-32 (Tisza) to sample JDS4-51, with a Gd/Gd* globally below 10. Gd anomaly and Gd_{anth} concentration in these two groups can be related to the number of MRI units and MRI exams in each country crossed.

Figure 4 was built using data on the location and capacity of wastewater treatments plants along the Danube River and its main tributaries (expressed in persons-equivalent served) from the Urban Waste Water Treatment Directive dissemination platform for EU countries (2016 data) and ICPDR data for non-EU countries. For each tributary, the contribution in terms of population to the Danube River watershed was considered, taking into account the eventual sharing of the tributary watershed between different countries.

In the first group, number of MRI exams/year/inhabitants is between 4,000 and 6,000 (Austria, Czech Republic, Slovakia, Hungary, Bosnia and Herzegovina); exceeding 14,000 exams/year/inhabitants in Germany. In the second group, MRI exams/year/inhabitants are below 2,000 (Serbia, Bulgaria, Romania) or data is missing (Moldavia and Ukraine). This lower number of MRI exams in Bulgaria can explain why there is no Gd/Gd* detected in sample JDS4-46.

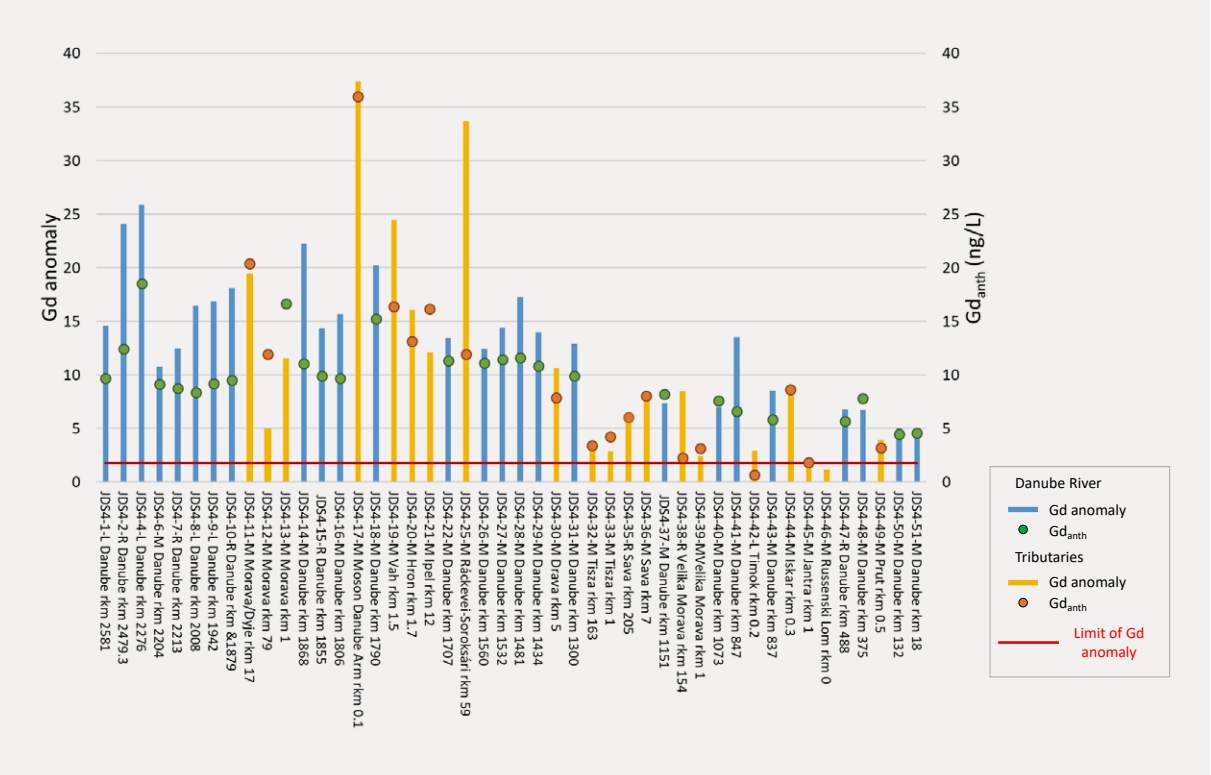

Figure 3: Gd_{anth} concentration and Gd anomaly in the Danube River and its tributaries.

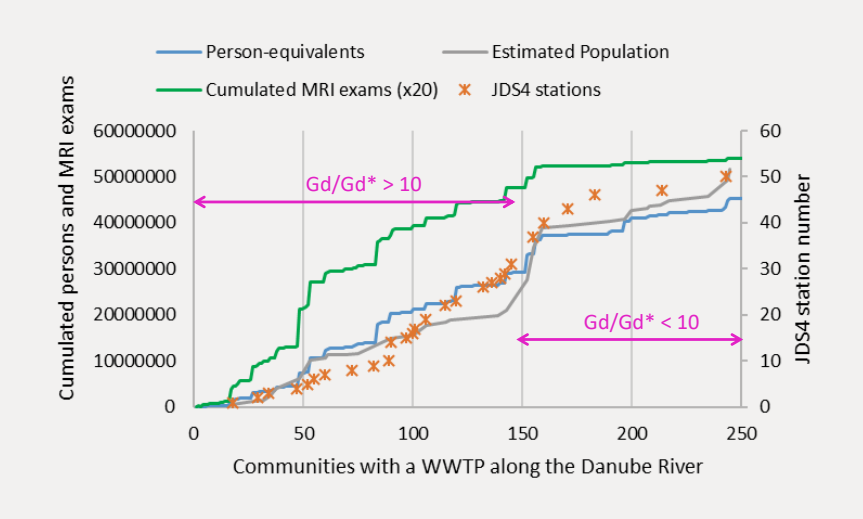

Figure 4: Cumulated persons-equivalent served by WWTPs, cumulated total population, cumulated annual MRI exams and JDS4 sampling stations along the Danube River.

An ytterbium (Yb) positive anomaly has been detected in some samples (JDS4-6, JDS4-35, JDS4-36, JDS4-48, JDS4-49). So far, there is no mention of such an anomaly in natural water in the literature. It could not then be stated with certitude whether this anomaly is natural or from anthropogenic inputs. This is the first REEs investigation on the Danube River within the Joint Danube Survey program and there is no possible data comparison with previous analyses.

40.4 Conclusions

A negative Ce anomaly was found all along the Danube River and its studied tributaries: it is natural, related to the redox behavior of Ce. A positive Gd anomaly was found in all Danube River samples and its tributaries (except for one: Russenski Lom). Its presence reflects the anthropogenic pressure on the river, especially in terms of medical exams and facilities. It is larger in the Upper and Middle Danube sections, where the Danube River is crossing countries and receiving from tributaries in countries where MRI exams are more frequent.

40.5 References

BAU M. & DULSKI P. (1996) Anthropogenic origin of positive gadolinium anomalies in river waters. Earth and Planetary Science Letters 143: 245–255.

BAU M., KNAPPE A. & DULSKI P. (2006) Anthropogenic gadolinium as a micropollutant in river waters in Pennsylvania and in Lake Erie, northeastern United States. Chemie der Erde – Geochemistry, 66: 143–152.

BAU M., SCHMIDT K., PACK A., BENDEL V., KRAEMER D. (2018) The European Shale: An improved data set for normalisation of rare earth element and yttrium concentrations in environmental and biological samples from Europe. Applied Geochemistry 90: 142–149.

Eurostat (2019) Healthcare resource statistics - technical resources and medical technology, https://ec.europa.eu/eurostat/statistics-explained/index.php/Healthcare_resource_statistics_-_technical_resources_and_medical_technology, last accessed on May 30, 2020.

HISSLER C., HOSTACHE R., IFFLY J.F., PFISTER L. & STILLE P. (2015) Anthropogenic rare earth element fluxes into floodplains: Coupling between geochemical monitoring and hydrodynamic sediment transport modelling. Comptes Rendus Geoscience, 347: 294–303.

KULAKSIZ S. & BAU M. (2011) Rare earth elements in the Rhine River, Germany: First case of anthropogenic lanthanum as a dissolved microcontaminant in the hydrosphere. Environment International 37 : 973–979.

KULAKSIZ S. & BAU M. (2013) Anthropogenic dissolved and colloid/nanoparticle-bound samarium, lanthanum and gadolinium in the Rhine River and the impending destruction of the natural rare earth element distribution in rivers. Earth and Planetary Science Letters, 362: 43–50.

KÜMMERER K. & HELMERS, E. (2000) Hospital effluents as a source of gadolinium in the aquatic environment. Environmental Science & Technology 34:573–577.

McLENNAN S.M. (1989) Rare earth elements in sedimentary rocks; influence of provenance and sedimentary processes. Reviews in Mineralogy and Geochemistry 21: 169–200.

PopulationData.net (2020) https://en.populationdata.net/, Last accessed on May 30, 2020

SETO M. & AKAGI T. (2008) Chemical condition for the appearance of a negative Ce anomaly in stream waters and groundwaters. Geochemical Journal, 42: 371-380

Urban Waste Water Treatment Directive: dissemination platform (2019) https://uwwtd.eu/ Last accessed on May 30, 2020

VERPLANCK, P.L., TAYLOR, H.E., NORDSTROM, D.K. & BARBER, L.B. (2005) Aqueous stability of gadolinium in surface waters receiving sewage treatment plant effluent, Boulder Creek, Colorado. Environmental Science & Technology 39: 6923–6929.

WHO (2016) https://gateway.euro.who.int/en/indicators/hlthres_95-magnetic-resonance-imaging-units-per-100-000/, Last accessed on May 30, 2020.

YEGHICHEYAN D., AUBERT D., BOUHNIK-LE COZ M., CHMELEFF J., DELPOUX S., DJOURAEV I., GRANIER G., LACAN, F., PIRO J., ROUSSEAU T., CLOQUET C., MARQUET A., MENNITI C., PRADOUX C., FREYDIER R., VIEIRA DA SILVA-FILHO E. & SUCHORSKI, K. (2019) A new interlaboratory characterisation of silicon, rare earth elements and twenty-two other trace element concentrations in the natural river water certified reference material SLRS -6 (NRC - CNRC). Geostandards and Geoanalytical Research 43, 475–496.

41 Synchronous fluorescence for characterization of dissolved organic matter

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Abstract

The structures of the dissolved organic matter (DOM) of samples collected during JDS4 were characterized by easy-to-perform optical methods, i.e. UV-visible spectroscopy and synchronous fluorescence spectroscopy. As provided by the UV-visible spectroscopy, the DOM aromaticity and molecular weight are moderately variable in the Danube River and its investigated tributaries. The contribution of humic substances to the DOM fluorescence is variable with no specific trend up to the confluence with the Timok River. It increases downstream. The protein-like fluorescence is correlated to the chlorophyll a concentration and to a lesser extent to organic and ammonium nitrogen: it results from a combination of processes: in-water biological reactions, watershed run-off and poorly treated urban sewage.

41.1 Introduction

Part of surface water quality can be assessed through its dissolved organic matter (DOM). Spectrophotometric methods (i.e. UV-visible spectroscopy, fluorescence spectroscopy) have been proposed as fast and easy-to-implement methods to characterize DOM in freshwater and marine water (Coble 1996), where they help to discriminate the DOM autochthonous and allochthonous fractions. By calculating indices from the UV-visible absorption spectrum, the aromaticity of DOM and its molecular weight can be discussed (Weishaar et al. 2003). In terms of fluorescence spectroscopy, excitation-emission matrices (EEM) are often used for DOM characterization. However, synchronous fluorescence (SF), where a constant difference is maintained between excitation and emission wavelengths, provides better defined spectra, easier to interpret (Patra and Mishra, 2002) or offering complementary information (Zhi et al. 2015; Kumar and Mishra 2015).

The spectroscopic information provided by fluorescence spectroscopy and UV-visible spectroscopy is compared to classical pollution markers such as dissolved organic carbon and dissolved total nitrogen nitrogen species.

41.2 Methods

Samples were collected in 250 mL polyethylene bottles previously washed and rinsed with a 10% HNO_3 solution and deionised water. All samples were filtrated with 0.45 µm filters before analysis, kept in the dark at 4°C.

41.2.1 Ancilliary parameters

Dissolved organic carbon (DOC) and dissolved total nitrogen (DTN) were measured with a VCHN system (Shimadzu, Noisiel, France) by catalytic oxidation at 680°C and infrared detection of the produced carbon dioxide. The NOx resulting from the oxidation were reacted with ozone, and the total dissolved nitrogen was quantified via luminescence. Calibration was done with potassium hydrogen phthalate for DOC and potassium nitrate for DTN. Nitrates were analysed by ion chromatography (DIONEX iCS 3000).

41.2.2 UV-vis spectroscopy

UV-vis spectra (200-600 nm) were collected on a Shimadzu UV-2600 spectrophotometer, using a 1cm x 1cm quartz 3.5 mL cuvette. Ultra-pure water was used for blanking. The Specific UV Absorbance at 254 nm ($SUVA_{254}$) was calculated as follows:

$$SUVA_{254} = A_{254}/DOC \cdot 100 \ (in\ L\ mg - C^{-1}m^{-1})$$

The spectral slope (S, nm^{-1}) was derived from the absorption spectra by fitting the data with the following equation (Helms et al., 2013):

$$A_\lambda = A_{\lambda_{ref}} \cdot e^{S(\lambda - \lambda_{ref})}$$

The spectral slope, $S_{275\text{-}295}$, was evaluated between 275 nm (= λ_{ref}) and 295 nm.

41.2.3 Fluorescence spectroscopy

Synchronous fluorescence spectra were collected on a Hitachi F-2500 fluorimeter equipped with a Xenon lamp, by using FL Solution 2.0 software and a 1 cm x 1cm 3.5 mL quartz cuvette. Two series of spectra were collected: one with a gap between excitation and emission of 20 nm (SF20) and one with a gap between excitation and emission of 50 nm (SF50). No correction for the inner-filter effect or the Rayleigh and Raman diffusion was performed, and the spectra were collected at the natural pH of the samples. The blank was performed with ultra-pure water. The fluorescence intensities were expressed in Raman units (R.u.).

To extract quantitative information out of the SF50 spectra, a decomposition procedure was applied (Assaad et al., 2015). In this approach the SF spectrum of a single fluorophore is represented by a Gauss function

$$SF(\lambda) = F(b) \cdot exp(-(\lambda - b)^2/2c^2)$$

Where *F(b)* is the pseudo-concentration of the fluorophore, *b* (in nm) its characteristic excitation wavelength, i.e. the wavelength where the maximal fluorescence is recorded and *c* a parameter related to the width of the Gauss function. Each JDS4 sample SF50 spectrum was decomposed into a number of these functions (i.e., fluorophores) by deconvolution. The same procedure was applied to SF20 spectra, the pseudo-concentrations of the fluorophores being noted *G(b)*. With the 50 nm gap the water Raman scatter interferes with the fluorescence of the fluorophore F(355) and the contribution of water to this fluorophore is further corrected for. With the 20 nm gap the lower end of the decomposition range is fixed at 260 nm so the water Raman scatter does not interfere with the decomposition.

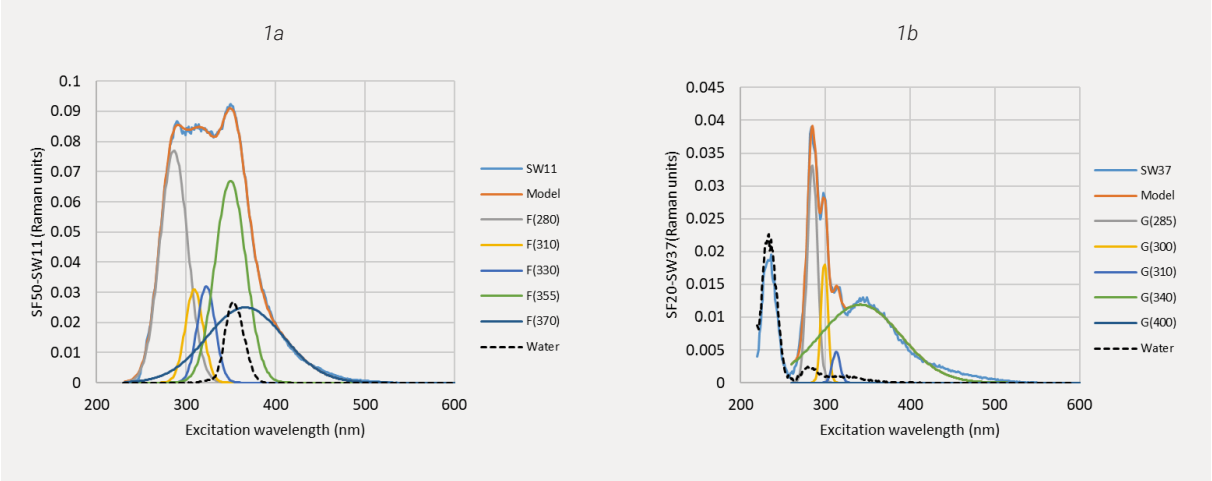

Figure 1: Deconvolution of synchronous fluorescence spectra a) SW11 with a gap of 50 nm. b) SW37 with a gap of 20 nm: the experimental spectrum and it global model are shown as well as the spectrum of each fluorophore.

41.3 Results and Discussion

As shown on Figure 2, a global SUVA can be obtained as the slope of the linear correlation between A_{254} and DOC either on only the samples collected on the Danube River (SUVA = 2.56 L/mgC/m with a coefficient of determination R^2 = 0.86) or on all the samples (Danube River and its tributaries) (SUVA = 2.15 L/mgC/m with R^2 = 0.92). Although SUVA seems a little bit higher in the upper part of the watershed, there is no real trend from upstream to downstream. The lowest SUVA is observed for SW_06 (SUVA = 1.41 L/mgC/m in Jochenstein) and the highest in SW_07 (SUVA = 3.55 L/mgC/m in Enghagen). Based on the individual SUVA values, the average SUVA along the Danube River is 2.84 L/mgC/m with a coefficient of variation (CV) of 14%. An average SUVA of 2.51 L/mgC/m is obtained for the tributaries, with a CV of 14.5%.

The average spectral slope $S_{275\text{-}295}$ is 0.017 nm^{-1} for the Danube River with a CV of 2.7%. The same average spectral slope has been found for the tributaries but with a slightly higher CV (6.3%). Therefore, based on the JDS4 samples, no large variations of the aromaticity and molecular weight of the dissolved organic matter are observed on the Danube River watershed.

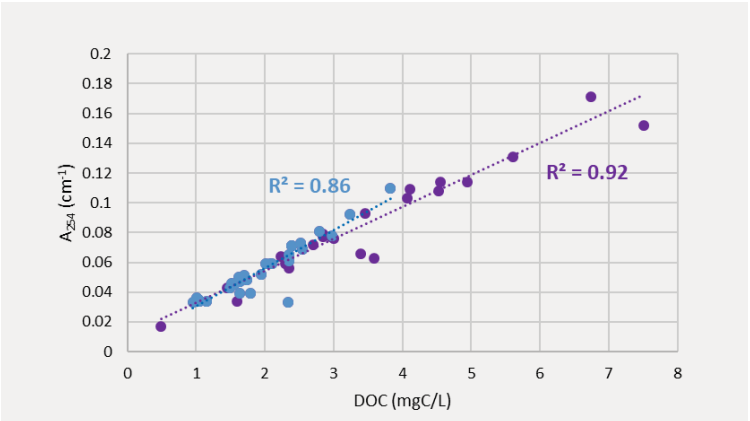

Figure 2: Global linear correlations between A_{254} and DOC.

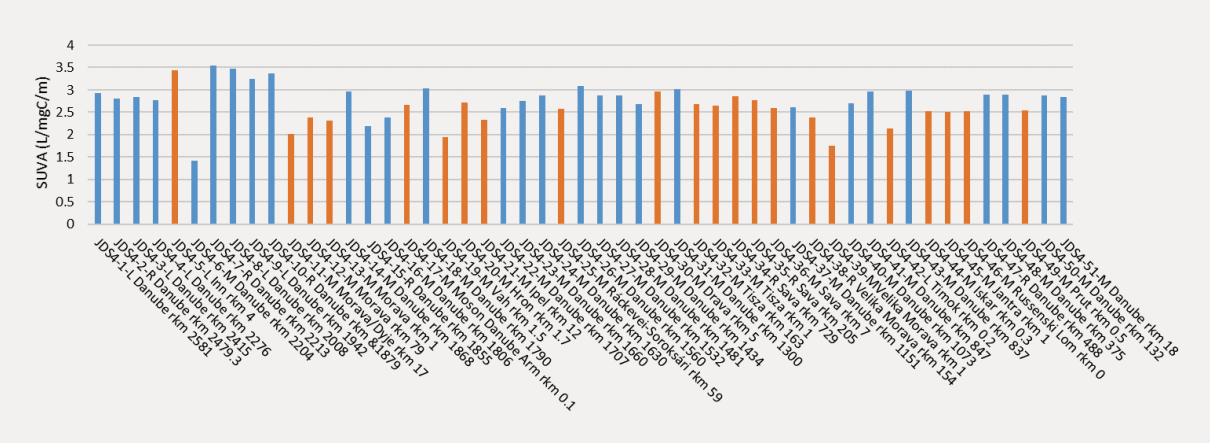

Figure 3: SUVA along the Danube River: in blue the Danube stations and in orange the stations on the Danube River tributaries.

As sample absorbance hinders fluorescence, the absorbance at 254 nm was checked for every sample. A_{254} was lower than 0.1 cm^{-1} for 90% of the samples. The highest A_{254} was recorded for SW_49 (Prut River). The absorbance was considered sufficiently low to record the fluorescence directly, without any correction.

The 50 nm gap provides information on both the fluorescence due to protein-like substances (excitation around 280 nm) and humic substances (excitation between 300 nm and 400 nm). Protein-like fluorophores are related to in-water biological reactions but also to the run-off of biological substances from the watershed and to the discharge of untreated or insufficiently treated urban sewage. Their fluorescence is largely due to the presence of the indole group: this chemical group is present in tryptophan (an essential amino-acid for humans, which is produced only by microorganisms and plants), in auxins (plant hormones), and in acids related to urine. Other substances which fluoresce in the same region as tryptophan in SF50 spectra are threonine (b ≈ 280 nm) (another essential amino-acid for humans) and tyrosin (b ≈ 270 nm), also an amino-acid. Humic substances are the main organic components of humus and their presence in surface water is essentially due to soil run-off from the watershed. The 20 nm gap allows for the increase of the resolution for fluorophores related to protein-like substances.

Figures 4 and 5 present the fluorophores pseudo-concentrations for SF50 and SF20 along the Danube and for its tributaries samples during JDS4. The average total SF50 pseudo-concentration is equal to 0.07 R.u (CV = 22%) along the Upper and Middle Danube River, up the confluence with the Timok River (JDS4-42 at 846 km from the mouth). 44% of the SF50 fluorescence is brought by the humic substances. The variability is higher for the SF20 pseudo-concentration, related to protein-like substances: the average total value is 0.04 R.u. with a CV of 43%.

Some of the main tributaries (Morava, Hron, Ipel, and Sava Rivers) bring highly fluorescent dissolved organic matter into the Danube River the Upper and Middle Danube River: the average total SF50 and SF20 pseudo-concentrations for the tributaries are 0.12 R.u. (CV = 40%) and 0.05 R.u. (CV = 54%), respectively. However, due to dilution, the contributions of these tributaries disappear rapidly. On the contrary, the Inn River brings little fluorescence organic matter to the Danube River.

Downstream the confluence with the Timok River, the fluorescent dissolved organic matter, as estimated by SF50 and SF20 increases in the Danube River, with high inputs from the Russenski Lom River and the Prut River.

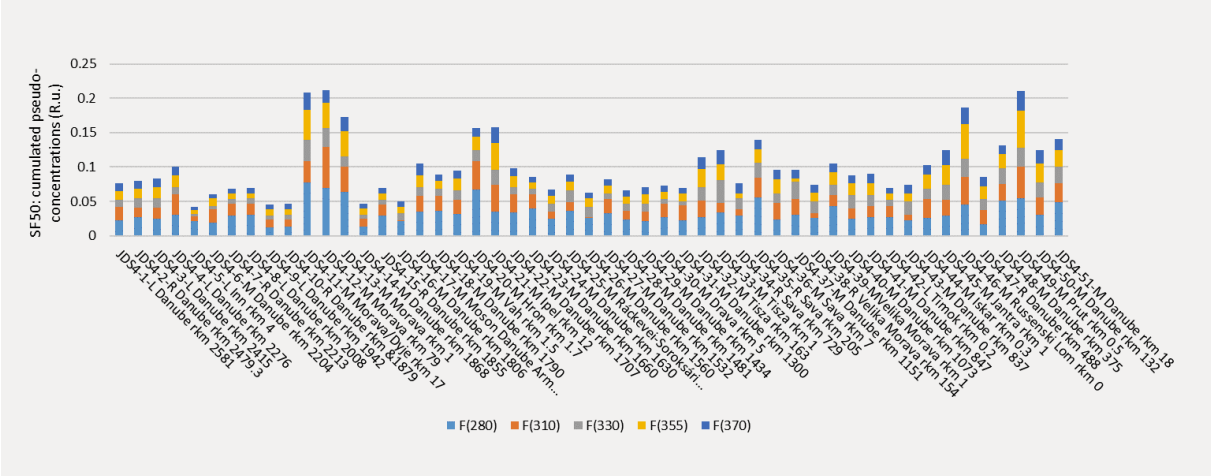

Figure 4: Distributions of pseudo-concentrations of fluorophores extracted from the SF50 spectra along the Danube River.

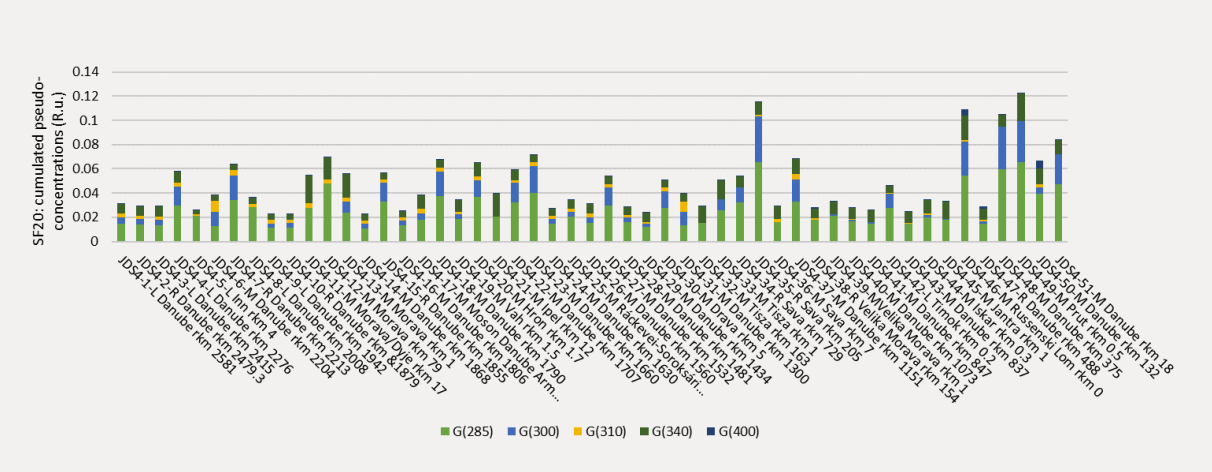

Figure 5: Distributions of pseudo-concentrations of fluorophores extracted from the SF20 spectra along the Danube River.

There is a rather good correlation between the sum of the pseudo-concentrations of fluorophores extracted from the SF50 spectra and the dissolved organic carbon as R^2 is equal to 0.79 (Fig. 6a). The correlation is even better when considering only the fluorophores related to humic substances (i.e. F(330) + F(355) + F(370) with R^2 = 0.87 (Fig 6b): there are the main components of the dissolved organic carbon.

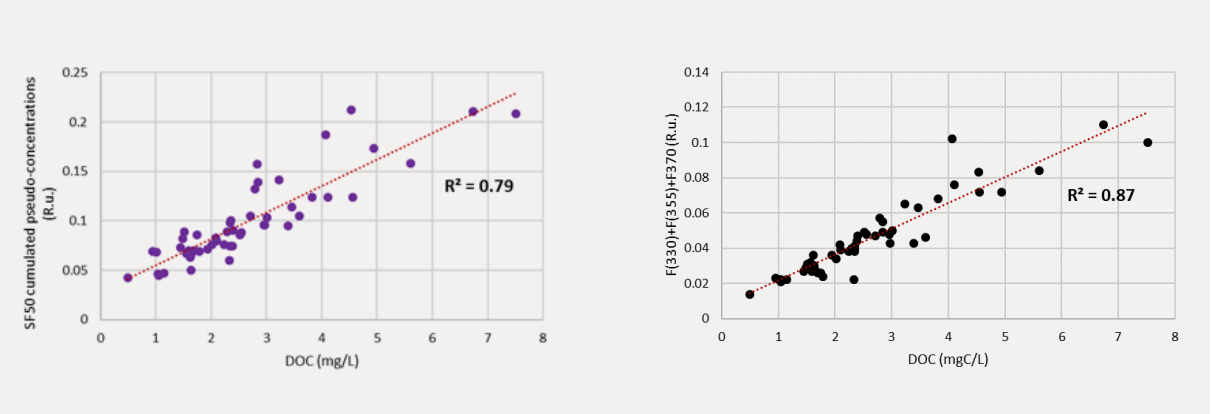

Figure 6: Correlations between DOC and a) the sum of SF50 pseudo-concentrations of fluorophores and b) the sum of SF50. pseudo-concentrations of fluorophores related to humic substances.

To compare SF20 and SF50, correlations have been sought between the sum of SF20 pseudo-concentrations of fluorophores and on one hand F(280) (Figure 7a) and on the other hand F(280)+F(310) (Figure 5b). The data points related to the Morava River and the Hron River have been excluded from the correlations, which enabled to obtain coefficients of determination of 0.70 and 0.71, respectively. So far there is no clue why the Morava and Hron rivers behave differently from the other stations. More sampling on these two tributaries are necessary to understand their behaviour. The correlations indicate the strong relationship between the protein-like fluorophores detected by SF50 and those, more detailed, detected by SF20. It is not possible to further determine the molecular structure of these fluorophores using simply fluorescence spectroscopy.

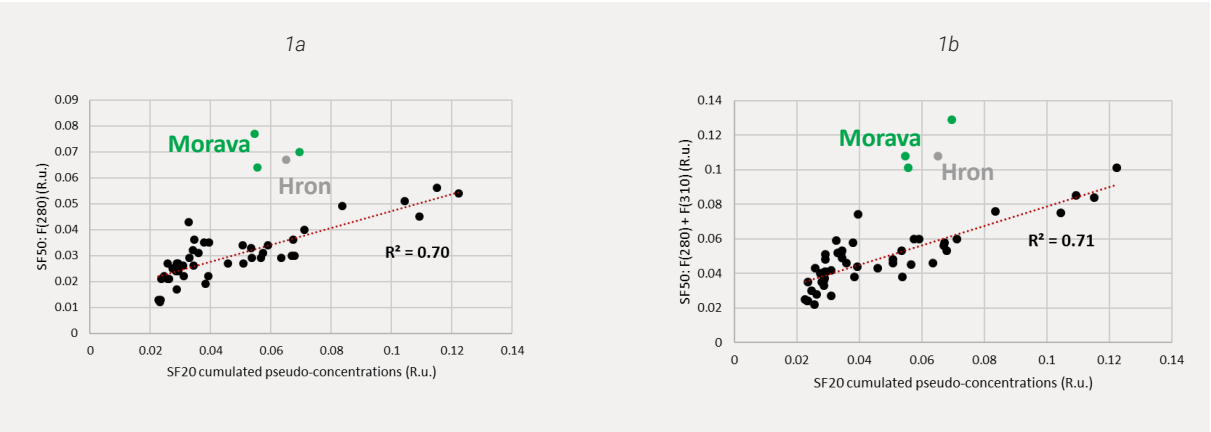

Figure 7: Correlations between the SF20 total pseudo-concentrations and a) F(280) and b) F(280)+F(310).

As proteins contain some nitrogen (between 8 to 14% for tyrosine, threonine and tryptophan) it has been attempted to correlate F(280) with estimated values of Kjeldahl nitrogen. Kjeldahl nitrogen includes organic nitrogen and ammonium, but not the oxidized forms of nitrogen, such as nitrates and nitrites. Kjeldahl nitrogen has been estimated as the difference between total DTN and $N\text{-}NO_3$. Based on the ammonium data recorded during JDS4, ammonium nitrogen represents about 7% of the Kjeldahl nitrogen. The linear correlation between Kjeldahl nitrogen and F(280) is rather poor ($R^2 = 0.16$), as shown in Figure 8. Taking into account F(310) does not improve the correlation.

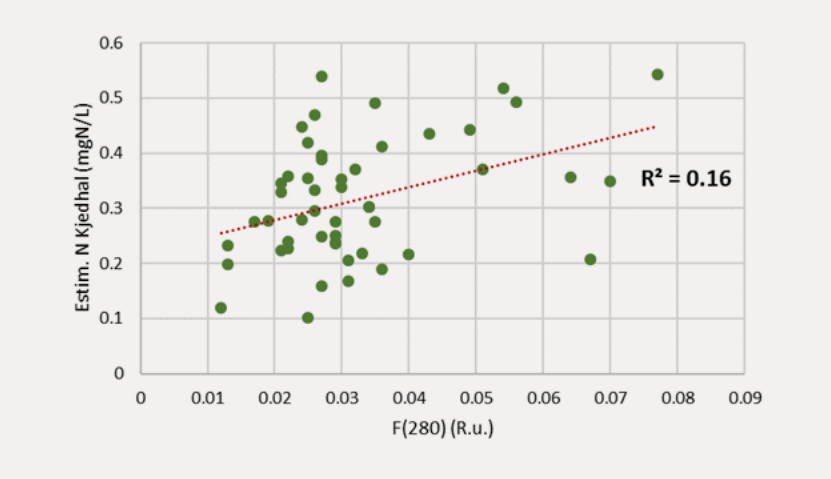

Figure 8: Correlation between F(280) and the estimated Kjeldahl nitrogen concentration.

A better linear correlation (R^2 = 0.5) is observed between the SF50 protein-like fluorophores (i.e. F(280) + F(310) and the chlorophyll-a measured during the JDS4 (Figure 9). Five outliers, corresponding to tributaries, have been removed to establish the correlation: JDS4-11 (Morava/Dyje), JDS4-13 (Morava), JDS4-25 (Ráckevei-Soroksári), JDS4-20 (Hron) and JDS4-49 (Prut). Therefore a part of the protein-like fluorescence is related to in-water biological reactions governed by phytoplankton.

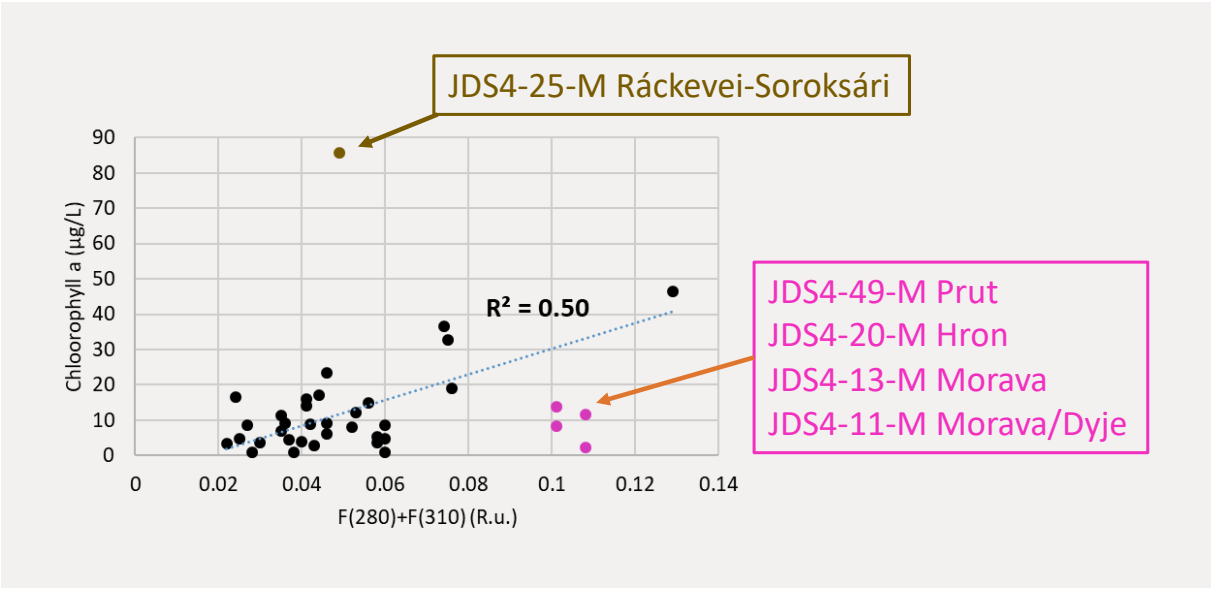

Figure 9: Correlation between F(280)+F(310) and the chlorophyll-a concentration.

41.4 Conclusions

As evaluated by UV-visible spectroscopy the aromaticity and the molecular weight of the DOM does not vary significantly along the Danube River and its sampled tributaries. The DOM composition was further investigated using synchronous fluorescence spectroscopy. The part of DOM which is related to humic substances varies up to the confluence with the Timok River, but with no specific trend. From there it increases up to the Danube River mouth. A similar behaviour is observed for the fluorescence related to the protein-like fluorophores. Some of the tributaries bring highly fluorescent DOM to the Danube River, but it is quickly diluted in the main stream.

DOC is largely correlated to the fluorescence of the humic substances, whose origin is allochtonous. Although the protein-like fluorophores contain some nitrogen atoms, their correlation with Kjeldahl nitrogen is limited. In-water biological reactions cannot be excluded, especially in summer when the photoactivity is high. But run-off of protein-like substances from the watershed or discharge of untreated or not sufficiently treated urban sewage can also contribute to the protein-like fluorescence.

Due to the large distances between the sampling stations, it is difficult to draw conclusions that are more specific: a refined sample collection at different seasons would be useful to go further.

41.5 References

ASSAAD A., PONTVIANNE S., CORRIOU J.P. & PONS M.N. (2015) Spectrophotometric characterization of dissolved organic matter in a rural watershed: the Madon River (N-E France), EMAS, 187:188.

COBLE, P.G. (1996) Characterization of marine and terrestrial DOM in seawater using excitation-emission spectroscopy. Mar. Chem., 51, 325-346.

HELMS J.R., STUBBINS A., PERDUE E.M., GREEN N.W., CHEN H. & MOPPER K. (2013) Photochemical bleaching of oceanic dissolved organic matter and its effect on absorption spectral slope and fluorescence. Mar. Chem. 155: 81–91.

KUMAR K. & MISHRA A.K. (2015) Multivariate curve resolution alternating least square (MCR-ALS) analysis on total synchronous fluorescence spectroscopy (TSFS) data sets: Comparing certain ways of arranging TSFS-based three-way array. Chemometr. Intell. Lab. 147: 66–74.

PATRA D. & MISHRA A.K. (2002) Recent developments in multi-component synchronous fluorescence scan analysis. TrAC, 21: 787-798.

WEISHAAR, J.L., AIKEN G.R., BERGAMASHI B.A., FRAM M.S., FIJII R. & MOPPER K. (2003) Environ. Sci. Technol. 37: 4702-4708.

ZHI E., YU H., DUAN L., HAN L., LIU L. & SONG Y. (2015) Characterization of the composition of water DOM in a surface flow constructed wetland using fluorescence spectroscopy coupled with derivative and PARAFAC. Environ Earth Sci, 73:5 153–5161.

42 Occurrence of microplastics in the Danube River – A first screening

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Abstract

Plastics, and microplastics in particular, are still part of scientific and regulatory discussions. Their inputs from land ultimately end up in the oceans, where they remain for a long time. River systems represent an important path of entry into the oceans. The Danube is the second largest river in Europe and can therefore be an example for the occurrence of plastic in other large river systems. In JDS4 a comprehensive screening of microplastics was carried out over the entire course of the river. Sampling was performed by means of deploying sedimentation boxes into the river for 14 days; followed by thermo-analytical detection (TED-GC/MS) for determination of the total content of various plastic polymers in the collected suspended particulate matter samples. For the first time, a baseline of pollution by microplastics in the Danube River Basin has been established. In all samples almost, all analyzed polymers were detected and quantified, whereas there is no clear trend along the Danube with increasing or decreasing contents. The contents ranged between 0.05 – 22.24, 0.00 – 0.45, 0.00-1.03 and 0.00 – 3.32 for PE, PP, SBR and PS [µg/mg] SPM, respectively.

42.1 Introduction

The first reports on plastic in the environment date back to the 1970´s (Carpenter et al. 1972, Colton et al. 1974). Macro plastic degrades into micro- and nano plastic particles and thus, plastic particles are reported in almost all ecosystem components, even in deep-sea sediments (van Cauwenberghe et al. 2013, Woodall et al. 2014, Yao et al. 2019), remote marine gyres (Law et al. 2010, Maximenko et al. 2012, Pan et al. 2019), on remote islands (Crawford 2017, Schönlau et al. 2019) and in biota (Herzke et al. 2016). Very recently, the USA listed the topic of (micro)plastic at the second position of the most warranted research areas (Fairbrother et al. 2019). Microplastics are defined as plastic particles between 1 µm and 1000 µm (Bannick et al. 2019) and includes plastic polymers, pellets and fibres made out of polyethylene (PE), polypropylene (PP), polystyrene (PS), polyethylene terephthalate (PET), polyamide (PA), natural rubber (NR) and styrene-butadiene rubber (SRB).

Rivers are suspected to be one of the important sources of plastic for marine ecosystems as the final sink (Akdogan & Guven 2019). In recent years, the uncontrolled, increasing occurrence of plastic products, plastic waste (plastic litter) and its fragments in the environment has become a hotly discussed topic of public and political interest (WHO, UNEP, OECD). The ubiquitous presence of plastic in the aquatic environment may lead to adverse impacts on the ecosystems or cause negative economic effects in, e.g., fishery or tourism sectors.

Recent publications deal with inventories of microplastics in large rivers (e.g., Rhine; Mani et al. 2015, Scherer et al. 2020), or their sections (e.g., Danube; Hohenblum et al. 2015, Liebmann et al. 2020). A modelling of the export of plastic from land to sea attempts to identify the sources and the relative contribution to marine pollution (Siegfried et al. 2017, Schmidt et al. 2017, Kawecki et al. 2019). The Danube River is one of the major tributaries to the land-locked Black Sea with a limited potential of transport of the plastics into the Aegean Sea. Recent studies show that the Black Sea is the most polluted European sea in terms of floating marine litter (Slobodnik et al. 2017).

During JDS4, the contents of microplastics (particles < 1 mm) were analyzed for the first time along the entire length of the Danube River to the Black Sea, including selected tributaries, by applying the same sampling technique and detection methods.

The recent work was realised independently of the microplastics monitoring campaign in mussels during JDS 4 (Chapter 44). Regarding the different analytical concept (sampling, sample preparation, detection) the data are not compatible and should not be correlated to each other. Firstly we focus on the complete size range of microplastic particles in water column, secondly, we do not perform an additional sample preparation, which could cause a particle loss or contamination and thirdly we determine an integral result which cannot convert to particle numbers.

42.2 Methods

The sampling locations were nominated by the ICPDR MA EG to represent different stretches of the Danube River (see Figure 1. 15). Several locations were sampled twice (for the list of sampling stations and their coding, see Table 1). An overview map and details about the JDS4 sampling locations are given in the Chapter 2 – Survey logistics. Three 'special samples' (WWTP Brno (CZ), Sava river (RS) and Tisza river (UA) were sampled at sites outside of the official sampling program of JDS4.

Sampling with a sedimentation box on each site was performed within 14 days. A Standard Operation Procedure (SOP) was specifically adapted to the sampling at the Danube River and distributed to the national sampling teams. The SOP was optimised through experience during the sampling campaign. Prior to the sampling, a practical instruction on the handling of the sedimentation box was given on site.

The basic principle of the sedimentation box is the retardation of the flow velocity through blades to induce the sedimentation of particles (Figure 1). Identical models are used in Germany for the collection of suspended particulate matter (SPM) from different water bodies in the course of the investigations of the German Environmental Specimen Bank (Schubert et al. 2012).

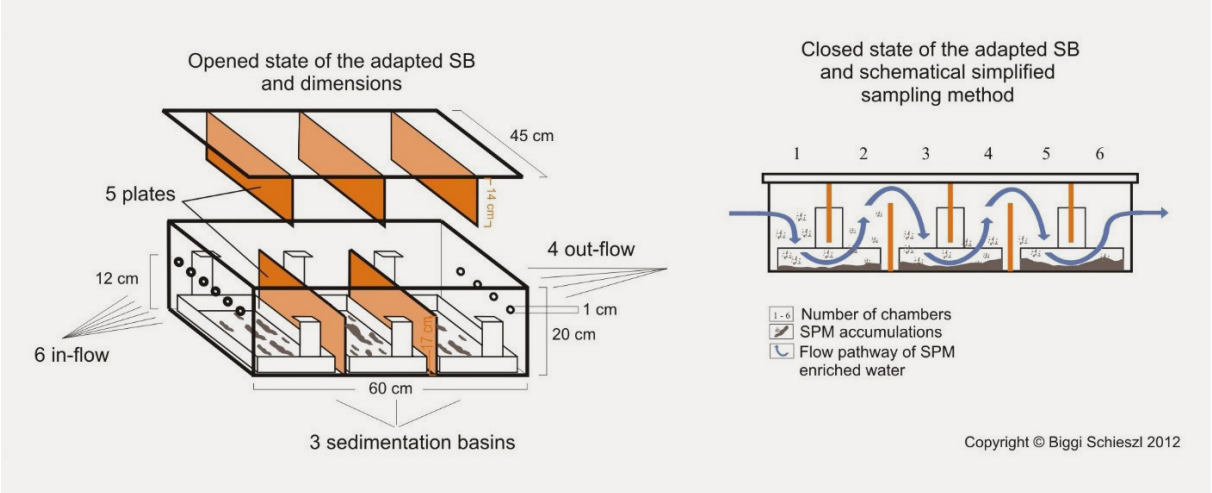

Figure 1: Basic scheme of the sedimentation box used for collection of SPM samples for the German Environmental Specimen Bank (ESB) (Schubert et al. 2012).

The content of the sedimentation boxes was transferred to 50 L stainless steel drums and then immediately cooled and transported to the laboratory of the German Environment Agency for sample preparation. In the laboratory, the samples were fractionated into the following particle size fractions through stainless steel sieves:

- > 1000 µm (upper limit)
- > 500 µm (fraction 500 - 1000 µm)
- > 500 µm which were further fractionated into > 100 µm (fraction 500 - 100 µm) and < 100 µm (fraction 1 - 100 µm).

Details of the preparation scheme are included in the supplementary document (see www.danubesurvey.org/jds4/full-report). The samples were filtered through a 1000 µm and 500 µm mesh and subsamples were further prepared for the fraction > 100 µm and < 100 µm. Further subsamples of the fraction < 500 µm were freeze-dried (2 L) and up to 30 L air-dried for further analysis. Some subsamples were further treated with

NaJ for a density separation. The density preparation was necessary especially for the upstream locations at Jochenstein and Klosterneuburg with almost no organic content and a very high minerogenic fraction of (ca. 98 - 99%).

In total 22 samples were taken, prepared and analyzed. The delivery date and sampling locations are listed in Table 1.

Table 1: List of the samples and countries of the survey.

Delivery	Sampling location	Country	Sample Code
27.06.19	Jochenstein	DE	JDS4-6
	Klosterneuburg	AT	JDS4-9
	Pohansko	CZ	JDS4-11
	Bratislava	SK	JDS4-14
	Ruse	BG	JDS4-46P
	Giurgiu	RO	JDS4-47
15.07.19	Hainburg	AT	JDS4-10
	Lanzhot	CZ	JDS4-12
	Budapest	HU	JDS4-23
	Vilkove - Chilia/Kilia arm	UA	JDS4-51
26.07.19	Budapest	HU	JDS4-24
	Timok	RS	JDS4-41
	Lanzhot	CZ	JDS4-12
22.08.19	Pancevo	RS	JDS4-37
	Brno	CZ	Special sample
23.08.19	Böfinger Halde	DE	JDS4-1
18.11.19	Bratislava	SK	JDS4-14
	Budapest	HU	JDS4-24
	Bezdan	RS	JDS4-29
	Tisza	UA	Special sample
20.12.19	Pancevo	RS	JDS4-37
	Sava	RS	Special sample

For the first screening only, the samples > 100 µm and < 100 µm (fraction < 500 µm) were analyzed. The total content of various plastic polymers was determined by means of TED-GC/MS (Thermo Extraction Desorption-Gas Chromatography-Mass Spectrometry) (Dümichen et al. 2019; Eisentraut et al. 2018).

Sample amounts of 10 – 50 mg were placed in 150 µL alumina crucibles and then measured with a horizontal single-arm thermobalance (Thermo Gravimetric Analysis) TGA/DSC3+ (Mettler Toledo, Columbus, OH, USA). The samples were heated from 25 to 600 °C in an inert nitrogen atmosphere with a nitrogen flow rate of 50 mL min-1 and a heating rate of 10 °C min^{-1}. The characteristic mass loss steps of the moisture content of the samples were analyzed from 25 °C to 180 °C and the content of pyrolysable organic matter from 180 °C to 600 °C.

Subsequent to the TGA results the samples were divided into the three sample mass categories of 10 mg, 20 mg and 50 mg to achieve an optimized Sorbstar™ load for the TED-GC/MS. Due to exchange reactions of the deuterated internal standard in highly minerogenic samples no internal standard was applied. The thermal extraction was performed with a TGA2 (Mettler Toledo) under the same conditions as stated above. and the volatile compounds were trapped on a solid-phase adsorbent (Polydimethylsiloxane. SorbStar, Envea GmbH, Karlsfeld, Germany). Subsequently, the SorbStar was thermodesorbed in a Gerstel TDU-2 connected to a Gerstel CIS 4 cooled injection system. The compounds were separated on a GC-MS System (7890 GC and 5977B MSD, Agilent, Santa Clara, CA, USA) on a HP-5 ms column (30 m x 0.25 mm x 0.25 µm) with an oven program from 40 °C - 300 °C. The MSD was operated in the EI-SCAN mode (70 eV), ramped from (m/z) 35 to 350. Data processing was realized through the Enhanced ChemStation software (version 2015, Agilent) including the NIST-17 database.

42.3 Results and Discussion

42.3.1 Suspended Particular Matter

For the first time suspended particular matter (SPM) samples were taken along the Danube River for the purpose of microplastics analysis applying the same sampling technique. The total SPM content obtained during the sampling period (14 days) varied from 32.88 to 17047 g dry weight (see Table 2). The highest amounts of SPM were sampled in the middle reach of the Danube, downstream the confluence with the Inn River, in Germany and Austria (Jochenstein and Klosterneuburg) and at the Danube arm in Bratislava. As expected, the lowest content was sampled at the most upstream station Böfinger Halde. Most of the SPM consisted of the fine grain fraction < 500 µm; the fraction > 500 µm contributed only around 1 – 2 % of the total SPM content, except for the slightly coarser sample from Pohansko (CZ) with a very low total amount of SPM. In some cases, the samples were very fine grained with the dominating amount of the fraction < 100 µm (see Table 2).

Table 2: Amounts (in g) of total SPM and individual size-fractions collected during JDS4.

Location	Böfinger Halde	Jochenstein	Klosterneuburg	Hainburg	Pohansko	Morava - Lanzhot	Morava - Lanzhot	Bratislava	Bratislava	Budapest Megyen Bridge	Budapest MO-Bridge
Code	JDS4-1	JDS4-6	JDS4-9	JDS4-10	JDS4-11	JDS4-12	JDS4-12	JDS4-14	JDS4-14	JDS4-23	JDS4-24
Total SPM [g]	62	17047	16512	3806	49	109	33	8671	1447	1352	242
% of Total SPM											
> 1000 µm	0,35	0,07	0,23	0,17	5,50	0,47	0,41	0,27	1,86	0,53	1,26
> 500 µm	0,21	0,43	0,28	1,71	3,96	1,78	1,11	0,09	0,06	0,38	1,93
< 500 µm	69,86	88,82	86,82	79,71	15,78	77,92	2,75	89,18	93,98	84,08	16,42
> 100 µm	0,70	0,95	1,67	0,75	2,23	1,16	1,43	0,43	0,34	0,52	4,75
< 100 µm	28,88	9,73	11,00	17,66	72,53	18,66	94,29	10,02	3,75	14,49	75,65
Sum %	100	100	100	100	100	100	100	100	100	100	100

Location	Budapest MO-Bridge	Bezdan	Pancevo	Pancevo	Radujevac/Timok	Giurgiu	Ruse	Vilkove - Chilia/Kilia arm	Brno - WWTP	Tisza	Sava
Code	JDS4-24	JDS4-29	JDS4-37	JDS4-37	JDS4-41	JDS4-47	JDS4-46p	JDS4-51	no JDS 04 sample	no JDS 04 sample	JDS4-36
Total SPM [g]	1209	680	341	139	411	3276	2233	5202	194	2202	285
% of Total SPM											
> 1000 µm	0,07	0,00	0,04	0,10	0,47	0,42	0,04	0,01	0,17	5,73	0,48
> 500 µm	2,93	0,22	0,00	0,13	3,62	0,04	0,08	0,08	0,25	1,85	0,12
< 500 µm	89,06	88,78	68,87	73,66	81,25	93,19	93,43	86,94	86,46	66,17	79,53
> 100 µm	0,22	0,27	0,28	0,21	0,55	3,77	0,17	0,21	3,32	3,09	0,23
< 100 µm	7,72	10,73	30,82	25,90	14,11	2,58	6,28	12,76	9,79	23,17	19,64
Sum %	100	100	100	100	100	100	100	100	100	100	100

Only a few samples were characterized by pyrolysable organic matter content (see Table 3 for the organic content). Most samples were associated with a high minerogenic content. For comparison with other data, the TGA organic content (Thermo Gravimetric Analysis) is converted to the actual determination of LOI (loss on ignition) at 450 °C in an oxygen atmosphere. Routinely, the TGA is operated with an inert nitrogen atmosphere for the pyrolysis.

The TGA-data obtained from the TED-GC/MS were analyzed and compared to the results of the initial TGA measurements to assess the homogeneity within the sample. The organic matter contents differed from 0.9 ± 0.4 % to a maximum of 36.4 ± 0.7 %, the water contents from 0.0 ± 0.9 % to a maximum of 7.2 ± 0.6 % and the non-pyrolyzable mass residue from 56.5 ± 1.5 % to a maximum of 99.0 ± 0.8 %. The results based on the duplicate determination showed great homogeneity (≤ 1% deviation) in the majority of the analyzed parameters organic matter content, water content and mass residue. The maximum total deviation was 2%. Consequently, the microplastic detection results generated by the TED-GC/MS can be interpreted as representative and reproducible.

Table 3: Parameters of the analyzed samples (= values for samples after density separation).*

Country	Sampling location	Faction	TGA organic content 600 °C N_2	TGA converted to LOI 450% O_2 organic content	Residue [%]
Germany	Böfinger Halde	> 100 µm	16,1	18,8	79,6
		< 100 µm	6,6	7,8	90,2
	Jochenstein	> 100 µm	1,7	2,0	98,1
		< 100 µm	1,9	2,2	98,0
		> 100 µm*	19,3	22,5	76,1
		< 100 µm*	30,9	36,1	63,0
Austria	Klosterneuburg	> 100 µm	0,9	1,0	99,0
		< 100 µm	1,5	1,7	98,5
		> 100 µm*	9,2	10,8	90,0
		< 100 µm*	36,4	42,6	56,5
	Hainburg	> 100 µm	2,4	2,8	96,7
		< 100 µm	1,4	1,7	98,2
Czech Republic	Pohansko	> 100 µm	16,2	18,9	78,8
		< 100 µm	9,9	11,6	84,6
	Brno-STP	> 100 µm	11,7	13,6	84,5
		< 100 µm	6,3	7,4	86,5
	Lanzhot	> 100 µm	9,5	11,1	86,5
		< 100 µm	7,6	8,9	87,9
		> 100 µm	9,3	10,9	87,5
		< 100 µm	5,2	6,1	91,8
Slovak Republic	Bratislava	> 100 µm	6,5	7,6	91,7
		< 100 µm	2,7	3,1	96,6
		> 100 µm	3,3	3,9	95,8
		< 100 µm	7,6	8,9	89,1

Country	Sampling location	Faction	TGA organic content 600 °C N_2	TGA converted to LOI 450% O_2 organic content	Residue [%]
Hungary	Budapest MB	> 100 µm	4,6	5,4	93,8
		< 100 µm	4,4	5,2	94,4
	Budapest MO	> 100 µm	5,9	6,9	92,6
		< 100 µm	6,3	7,4	91,5
		> 100 µm	8,8	10,3	88,4
		< 100 µm	8,0	9,4	89,8
Republic of Serbia	Bezdan	> 100 µm	11,3	13,3	85,7
		< 100 µm	8,1	9,5	89,3
	Pancevo	> 100 µm	12,7	14,9	84,4
		< 100 µm	8,7	10,2	88,3
		> 100 µm	15,4	18,0	80,6
		< 100 µm	8,0	9,3	87,8
	Sava	> 100 µm	15,3	17,9	80,2
		< 100 µm	9,0	10,5	87,0
	Radujevac	> 100 µm	8,3	9,7	89,2
		< 100 µm	7,2	8,4	88,9
Romania	Giurgiu	> 100 µm	1,3	1,5	98,4
		< 100 µm	8,8	10,3	87,2
Bulgaria	Ruse	> 100 µm	7,4	8,7	90,5
		< 100 µm	7,3	8,5	89,0
Ukraine	Tisza	> 100 µm	5,2	6,1	93,1
		< 100 µm	5,2	6,1	93,0
	Vilkove - Chilia/Kilia arm	> 100 µm	4,4	5,1	94,7
		< 100 µm	3,2	3,7	95,3

42.3.2 Plastic polymer analyses

Since the samples showed a great diversity in their organic matter contents, the 44 samples were divided into three sample mass categories to ensure an optimal SorbStar load of 1.5 mg. Due to the limited capacity and delays caused by the COVID-19 pandemic, only the samples > 100 µm and < 100 µm were analyzed using TED-GC/MS with sample masses of 10 mg, 20 mg and 50 mg, respectively.

Almost all analyzed polymers were screened for a set of polymers and only 4 were quantified in all the samples (see Table 4). The relative frequency of detection decreases in order: PE > SBR > PS > PP. First marker for NR and PMMA (Poly (methyl methacrylate) were identified and no PET-marker was detected in any of the samples.

Table 4: Content of microplastics [µg/mg SPM] in the analyzed samples (ds = density separated).

Place	Country	Fraction	PE [µg/mg SPM]	PP [µg/mg SPM]	SBR [µg/mg SPM]	PS [µg/mg SPM]
Böfinger Halde	DE	> 100 µm	2,00	0,19	0,47	0,58
Böfinger Halde	DE	< 100 µm	0,73	0,00	0,15	0,03
Jochenstein	DE	> 100 µm	1,64	0,00	0,00	0,00
Jochenstein	DE	< 100 µm	0,28	0,00	0,00	0,00
Klosterneuburg	AT	> 100 µm	0,49	0,00	0,00	0,00
Klosterneuburg	AT	< 100 µm	0,16	0,00	0,00	0,00
Hainburg	AT	> 100 µm	1,32	0,00	0,00	0,00
Hainburg	AT	< 100 µm	0,17	0,00	0,00	0,00
Pohansko	CZ	> 100 µm	10,63	0,09	0,00	0,00
Pohansko	CZ	< 100 µm	1,65	0,00	0,00	0,05
Brno-STP	CZ	> 100 µm	2,52	0,08	0,69	0,47
Brno-STP	CZ	< 100 µm	0,25	0,00	0,00	0,06
Lanzhot	CZ	> 100 µm	2,21	0,00	0,16	0,05
Lanzhot	CZ	< 100 µm	0,96	0,00	0,00	0,06
Lanzhot	CZ	> 100 µm	2,42	0,00	0,13	0,05
Lanzhot	CZ	< 100 µm	0,64	0,00	0,21	0,09
Bratislava	SK	> 100 µm	3,18	0,08	0,00	0,03
Bratislava	SK	< 100 µm	0,36	0,00	0,00	0,00
Bratislava	SR	> 100 µm	1,60	0,00	0,02	0,02
Bratislava	SR	< 100 µm	0,59	0,00	0,05	0,01
Budapest MB	HU	> 100 µm	2,97	0,00	0,00	1,37
Budapest MB	HU	< 100 µm	0,46	0,00	0,00	0,01
Budapest MO	HU	> 100 µm	2,50	0,00	0,00	0,44
Budapest MO	HU	< 100 µm	0,41	0,00	0,00	0,02
Budapest-MO	HU	> 100 µm	3,10	0,00	0,18	0,30
Budapest-MO	HU	< 100 µm	2,33	0,00	0,17	0,06
Bezdan	RS	> 100 µm	2,09	0,00	0,05	0,04
Bezdan	RS	< 100 µm	2,38	0,00	0,10	0,02
Pancevo	RS	> 100 µm	9,14	0,32	0,17	0,22
Pancevo	RS	< 100 µm	0,53	0,00	0,00	0,04
Pancevo	RS	> 100 µm	3,44	0,00	0,00	0,00
Pancevo	RS	< 100 µm	0,79	0,00	0,08	0,01
Sava	RS	> 100 µm	6,01	0,08	1,03	0,48
Sava	RS	< 100 µm	0,52	0,00	0,18	0,00
Radujevac	RS	> 100 µm	1,74	0,00	0,00	0,06
Radujevac	RS	< 100 µm	0,51	0,00	0,00	0,01
Giurgiu	RO	> 100 µm	0,17	0,00	0,00	0,00
Giurgiu	RO	< 100 µm	0,30	0,00	0,00	0,00

Place	Country	Fraction	PE [µg/mg SPM]	PP [µg/mg SPM]	SBR [µg/mg SPM]	PS [µg/mg SPM]
Ruse	BG	> 100 µm	5,36	0,00	0,37	0,19
Ruse	BG	< 100 µm	0,42	0,00	0,00	0,00
Tisza	UA	> 100 µm	1,01	0,00	0,00	0,02
Tisza	UA	< 100 µm	1,41	0,00	0,00	0,00
Vilkove - Chilia/Kilia arm	UA	> 100 µm	2,14	0,07	0,00	3,32
Vilkove - Chilia/Kilia arm	UA	< 100 µm	0,05	0,00	0,00	0,01
Jochenstein (ds)	DE	> 100 µm	12,03	0,18	0,00	0,70
Jochenstein (ds)	DE	< 100 µm	19,09	0,39	0,31	0,28
Klosterneuburg (ds)	AT	> 100 µm	5,25	0,23	0,31	0,23
Klosterneuburg (ds)	AT	< 100 µm	22,24	0,00	0,00	0,00
Min			0,05	0,00	0,00	0,00
Max			22,24	0,39	1,03	3,32

PE was quantified in the highest amount in majority of the samples. The values were particularly high in the density separated samples (ds) from Jochenstein and Klosterneuburg, due to the relative enrichment of the organic fraction through the density separation. Some of the data are preliminary and their validation is underway. In some of the samples a co-elution with unwanted components was registered in mass chromatograms and the data are currently undergoing a further quality check. Nevertheless, the obtained data represent a first set of quantitative microplastic determinations for the entire DRB.

There is no clear trend of increasing or decreasing microplastic contents along the Danube River. In the fraction > 100 µm the content tends to be slightly higher than in the finer fraction < 100 µm.

The samples from the tributaries Morava (CZ), Sava (RS) and Tisza (UA) show similar contents of microplastics as the samples from the Danube River. The contents of individual microplastic polymers (see Table 4) are illustrated in Figure 2. The highest contents were analyzed in the samples after the confluence with the Inn river, in the sample from Pohansko (CZ) and in the downstream sample from Pancevo (RS).

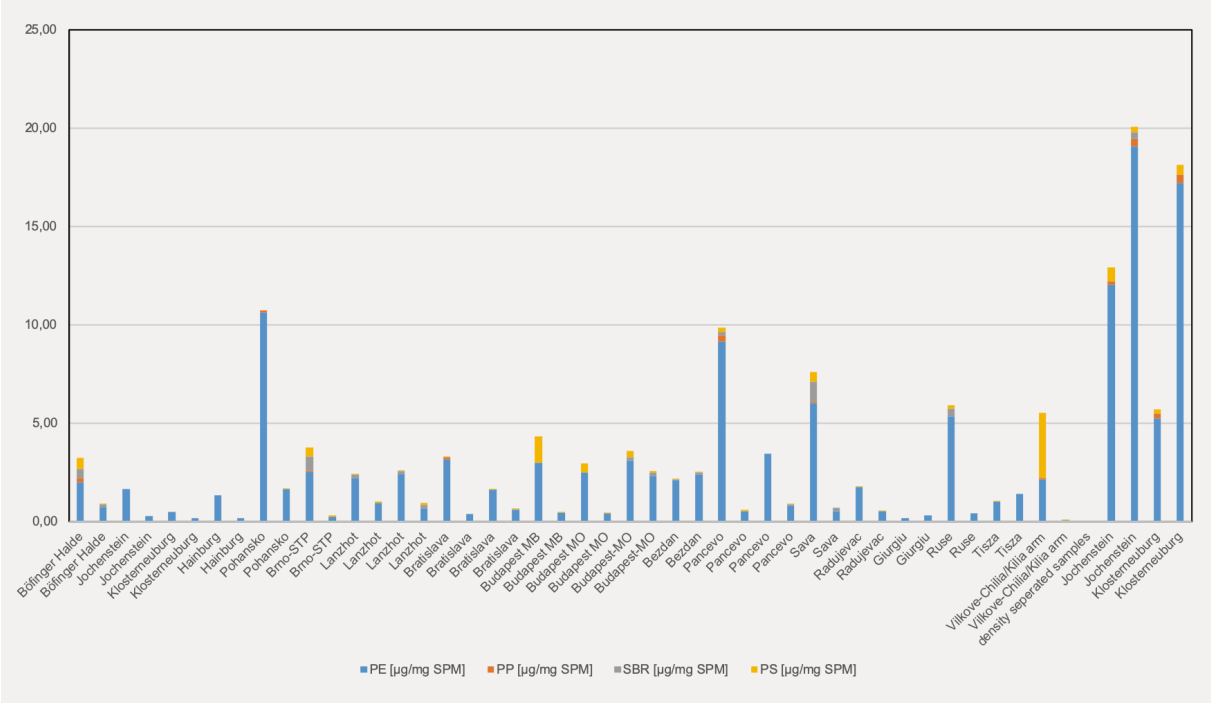

Figure 2: Comparison of the PE, PP, SBR and PS contents (in [µg/mg SPM]) in the samples (> 100 µm – left column and < 100 µm – right column) along the Danube in 2019. The density separated samples are displayed in the far-right position.

42.4 Conclusions

A comprehensive screening of microplastics in the Danube and its tributaries provided a first impression about the degree of its occurrence in the period from June to December 2019. In all samples, plastic polymers were detected, in one case close to their respective limit of detection. The results represent a first set of quantitative data, establishing a baseline of pollution by microplastics in the DRB. For a more comprehensive assessment of the Danube with regard to plastic (nano to macro- plastic), an extensive project was submitted to the EU and unfortunately not funded. Such a project should cover all aspects (sources, transport, sinks) and investigate the effect on biota.

The amount of the studied microplastic polymers was determined at different levels. The relative frequency of detection decreased in order: PE > SBR > PS > PP. Additionally, specific thermal decomposition products were detected, which could give indications about the presence of NR and PMMA. PET was not detectable in any sample. PE was detected as the most abundant component of microplastics in all (but Vilkove – Chilia/Kilia (UA) samples, however the correction of the obtained signals for co-eluting substances is still under progress. The highest contents of PE were observed at Pohansko (CZ) and Pancevo (RS) for the non-density separated samples, whereas the highest PE content in density separated samples were found at Jochenstein (DE) and Klosterneuburg (AT), but they were the only sampling sites with results for density separated samples. The highest content of PS was determined in Vilkove – Chilia/Kilia arm (UA). SBR was analyzed in samples from the DRB for the first time, and its presence in samples indicates an influence of pollution from urban areas and traffic (tyre abrasion). The highest SBR-contents were found in samples from the Sava river (RS) and in the effluent water from WWTP Brno (CZ). The other polymers (PE, PP, PS) are typically assigned as indicators of the sewage treatment effluent efficiency, however, they may also originate from other diffuse sources and non-treated sewage.

A statistical evaluation of the results indicated that the mass content of SPM does not correlate with the content of microplastics. Due to the lack of data on microplastics in SPM in other rivers, a comparison with other studies is difficult. Such comparison is also hampered by the lack of harmonized investigation concepts and methods. There is an obvious need to obtain further data using the same methodology in the DRB, but also in other large European rivers, such as the Rhine and Elbe.

The screening has demonstrated that the chosen investigation approach (sampling, sample preparation, detection) is feasible and provides robust results inter-comparable at the basin scale. However, further developments - also based on the investigations carried out in JDS4 - are already foreseeable. This includes improvement of the sampling scheme, employing active sampling by a pump to control and record the exact sample volume. In the future a sampling with the standing wave should be performed. Applying this approach, a virtual water package is sampled with the flowing wave from the source to the sea.

It is also foreseeable that an exposure of the sedimentation box for one week might be sufficient. The water flow velocity in the river during the sampling should also be more closely monitored in future programs.

Photo 1: Czech national microplastics team installing the box with a barrel in the Dyje.

42.5 Acknowledgements

BMBF-Project: Weiterentwicklung erster fachlicher Ansätze für ein staatenübergreifendes Monitoring für die quantitative und qualitative Erfassung von Plastik in Flusssystemen und zur Vorbereitung eines europäischen Förderantrags (Code 100375834).

BMU-Project: Bilanzierung der Plastikfracht im Rhein. Quantitative Erfassung, Bilanzierung und Bewertung von Mikrokunststoffen in den internationalen Flussgebieten Rhein und Donau; FKZ 3719 22 301 0.

42.6 References

AKDOGAN, Z., GUVEN, B. 2019: Microplastics in the environment: A critical review of current understanding and identification of future research needs. Environmental Pollution, 254, 113011.

BANNICK, C.G., SZEWZYK, R., RICKING, M., SCHNIEGLER, S., OBERMAIER, N., BARTHEL, A.K., ALTMANN, K., EISENTRAUT, P., BRAUN, U. 2019: Development and testing of a fractionated filtration for sampling of microplastics in water. Water Research, 149, 650-658.

DUEMICHEN, E., EISENTRAUT, P., CELINA, M., BRAUN, U. 2019: Automated thermal extraction-desorption gas chromatography mass spectrometry: A multifunctional tool for comprehensive characterization of polymers and their degradation products. Journal of Chromatography A, 1592, 133-142.

EISENTRAUT, P., DÜMICHEN, E., RUHL, A.S., JEKEL, M., ALBRECHT, M., GEHDE, M. AND BRAUN, U. 2018: Two birds with one stone – fast and simultaneous analysis of microplastics: microparticles derived from thermoplastics and tire wear. Environmental Science and Technology Letters, 5, 10, 608–613.

FAIRBROTHER, A., MUIR, D., SOLOMON, K.R. et al. 2019: Towards sustainable environmental quality: Priority research questions for North America. Environmental Toxicology and Chemistry, 38, 1606-1624.

HERZKE, D., ANKER-NILSSEN, T., HAUGDAHL NØST, T., GÖTSCH, A., CHRISTENSEN-DALSGAARD, S., LANGSET, M., FANGEL, K. AND KOELMANS, A.A. 2016: Negligible impact of ingested microplastics on tissues concentrations of persistent organic pollutants in Northern Fulmars off Coastal Norway. Environmental Science and Technology, 50, 1924-1933.

HOHENBLUM, P., FRISCHENSCHLAGER, H., REISINGER, H., KONECNY, R., UHL, M., MÜHLEGGER, S., HABERSACK, H., LIEDERMANN, M., GMEINER, P., WEIDENHILLER, B., FISCHER, N. RINDER, R. 2015: Plastik in der Donau. Untersuchung zum Vorkommen von Kunststoffen in der Donau in Österreich, Reports, REP-0547, Umweltbundesamt Wien.

KAWECKI, D., NOWACK, B. 2019: Polymer-specific modeling of environmental emissions of seven commodity plastics as macro- and microplastics. Environmental Science and Technology, 53, 9664-9676.

LAW, K.L., MOREIT-FERGUSON, S., MAXIMENKO, N.A., PROSKUROWSKI, G., PEACOCK, E.E., HAFNER, J., REDDY, C.M. 2010: Plastic accumulation in the North Atlantic Subtropical Gyre. Science, 329, 1185-1188.

LIEBMANN, B., SEXLINGER, K. 2020: Mikroplastik in der Umwelt – Statusbericht 2019; Report REP-0727, Bundesministerium Landwirtschaft, Regionen und Tourismus,

MANI, T., HAUK, A., WALTER, U., BURKHARDT-HOLM, P. 2015: Microplastics profile along the Rhine River. Scientific Reports, 5-17988.

MAXIMENKO, N., HAFNER, J., AND NIILER, P. 2012: Pathways of marine debris derived from trajectories of Lagrangian drifters. Marine Pollution Bulletin, 65, 51–62.

PAN, Z., LIU, Q., SUN, Y., SUN, X., LIN, H. 2019: Environmental implications of microplastic pollution in the Northwestern Pacific Ocean. Marine Pollution Bulletin, 146, 215-224.

SCHERER, C., WEBER, A., STOCKH, F., VURUSIC, S., EGERCI, H., KOCHLEUS, C., ARENDT, N., FOELDI, C., DIERKES, G., WAGNER, M., BRENNHOLT, N., REIFFERSCHEID, G. 2020: Comparative assessment of microplastics in water and sediment of a large European river. Science of the Total Environment, 738, 139866.

SLOBODNIK, J., ALEXANDROV, B., KOMORIN, V., MIKAELYAN, A., GUCHMANIDZE, A., ARABIDZE, M., KORSHENKO, A. 2020 (eds): National Pilot Monitoring Studies and Joint Open Sea Surveys in Georgia, Russian Federation and Ukraine, 2017, EU/UNDP Project: Improving Environmental Monitoring in the Black Sea – Phase II (EMBLAS-II) ENPI/2013/313-169.http://emblasproject.org/wp-content/uploads/2019/07/EMBLAS-II_NPMS_JOSS_2017_ScReport_FinDraft2.pdf. Accessed on 28 September 2020.

SCHMIDT, C., KRAUTH, T., WAGNER, S. 2017: Export of plastic debris by rivers into the sea. Environmental Science and Technology, 51, 12246-12253.

SCHÖNLAU, C., LARSSON, M., DUBOCQ, F., ROTANDER, A., VAN DER ZANDE, R., ENGWALL, M. AND KÄRMAN, A. 2019: Effect-directed analysis of Ah receptor-mediated potencies in microplastics deployed in a remote tropical marine environment. Frontiers in Environmental Science (7): Article 120.

SCHUBERT, B., HEININGER, P., KELLER, M., RICKING, M., CLAUS, E. 2012: Monitoring of contaminants in suspended particulate matter and sediments – a comparison; Trends in Analytical Chemistry, 36, 58-70.

SIEGFRIED, M., KOELMANS, A.A., BESSELING, E., KROEZE, C. 2017: Export of microplastics from land to sea. A modelling approach. Water Research 127, 249-257.

VAN CAUWENBERGHE, L., VANRESUEL, A., MEES, J., JANSSEN, C.R. 2013: Microplastic pollution in deep-sea sediments. Environmental Pollution, 182, 495-499.

WOODALL, L.C., SANCHEZ-VIDAL, A., CANALS, M., PATERSON, G.L.J., COPPPOCK, R., SLEIGHT, V., CALAFAT, A., ROGERS, A.D., NARAYANASWAMY, B.E., THOMPSON, R.C. 2014: The deep sea is a major sink for microplastic debris. Royal Society Open Science, 1, 140317.

YAO, P., ZHOU, B., LU, Y., YIN, Y., ZONG, Y., CHEN, M., O´DONNELL, Z. 2019: A review of microplastic in sediments: Spatial and temporal occurrences, biological effects, and analytical methods. Quaternary International, 519, 274-281.

43 Nanoparticle inventory in a sediment core from the Iron Gate I reservoir

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Abstract

River sediments are a sink for natural and anthropogenic nanoparticles. Given their risk to harm ecosystems and humans the latter are among contaminants of emerging concern. Here we present multi-element single-particle data of a Danube sediment core, aiming to identify anthropogenic nanoparticles and elucidate their occurrence at different sediment depths. A fly ash dump near Kostolac, Serbia, on the right bank of the Danube River is a likely point source of anthropogenic fly ash particles. Kostolac fly ash particles are enriched in elements such as Cu, Ni, and V. The signatures of these elements within nanoparticles of the sediment core reveal four potential events of fly ash release into the Danube.

43.1 Introduction

The formation of nanoparticles is a natural phenomenon. Physical weathering, volcanism, and sea spray are just some of the sources and the resulting particles have been around since Earth's formation, 4.54 billion years ago (Hochella et al., 2019). Particularly since the industrial revolution, humans are increasingly releasing nanoparticles into the environment with still largely unknown ecosystem effects. While plants and wildlife may be well adapted to deal with naturally occurring nanoparticles, the composition and shape of anthropogenic particles can vastly differ from natural ones and potentially be harmful (Lead et al., 2018). Incidental nanoparticles produced in high-temperature industrial processes, such as coal combustion, may be enriched in harmful heavy metals, posing a risk to human health when inhaled, ingested, or through dermal contact (Kittner at al., 2018). Therefore, anthropogenic nanoparticles are considered contaminants of emerging concern (EPA, 2010; Sauvé and Desrosiers, 2014).

One large source of anthropogenic nanoparticles are coal-fired power plants. During the coal combustion, a range of particles with different sizes, shapes, and compositions is formed. Fly ash, consisting of mostly spherical, micrometre and nanometre sized particles, is of particular interest due to the aforementioned potential for heavy-metal enrichment and the associated health risks. The composition of the ash depends on the coal composition and further alteration by fractionation processes during the combustion (Davison et al., 1974). Highly volatile elements, such as C, Hg, and Se escape into the vapour phase, while less volatile elements condensate onto ash particles as temperature falls. The latter, including Ni, Co, and Cu are thereby enriched in the smallest ash fraction, which provides the largest surface area per particle mass. Elements, such as Al, Ca, and Ti, largely stay in the solid phase, exhibiting no fractionation with particle size (Hower et

al., 2020). The fractionation process therefore assures that the smallest ash particles carry the strongest and thus the most characteristic signature for anthropogenic particles related to high temperature coal combustion.

Identification of anthropogenic nanoparticles in the environment remains a tremendous challenge. In river sediments, natural nanoparticles outnumber the anthropogenic ones on average by several orders of magnitude (Hochella et al., 2019). At these low relative particle numbers, the element signature of anthropogenic particles may remain undetected in bulk sediment analyses. A new generation of mass spectrometers equipped with time-of-flight mass analysers is able to capture multi-element signals for thousands of individual particles per minute. This technique allows us to identify anthropogenic nanoparticles even at very low particle concentrations and thus assess the nanoparticle inventory of river sediments.

43.2 Materials and Methods

43.2.1 Sample material

A core of Danube River sediment was obtained 1077 km upriver from the Black Sea between the Serbian towns of Stara Palanka and Ram, 12.5 km downstream of an uncovered coal ash dump. A polyvinyl chloride liner (60 mm in diameter) was manually pushed into the sediment. The core was kept vertically and was deep-frozen on return to the laboratory. A coal ash sample and a river sediment grab sample, collected during JDS2 in 2007, were analysed as references for the pure anthropogenic source and a coal ash-free sediment, respectively. The coal ash sample was taken from the top of the dump site located on the right riverbank near two coal-fired power plants of Kostolac, Serbia. The sediment grab sample, 1097 km upriver from the Black Sea, originated from the right riverbank 7.5 km upstream of the dump, (Table 1; Figure 1). Given that fluvial transport of fly ash upstream of the dump can be excluded and atmospheric transport is not expected to deposit considerable amounts of fly ash, this sediment was considered to be fly ash-free. As the coal ash produced in the Kostolac coal-fired power plants consists of 95% fly ash (Popovic et al., 2011), it is hereafter referred to as fly ash.

Table 1: Location, type, and identification (ID) of materials used in this study.

Sample ID	Latitude	Longitude	Sample Type	Material
JDS4-RAM-M	44.805793	21.300343	Sediment core (0–32 cm depth)	River sediment
JDS2-KOS-0	44.750764	21.173237	Profile sample (surface)	Fly ash
JDS2-57-R	44.729433	21.127183	Surface grab sample	River sediment

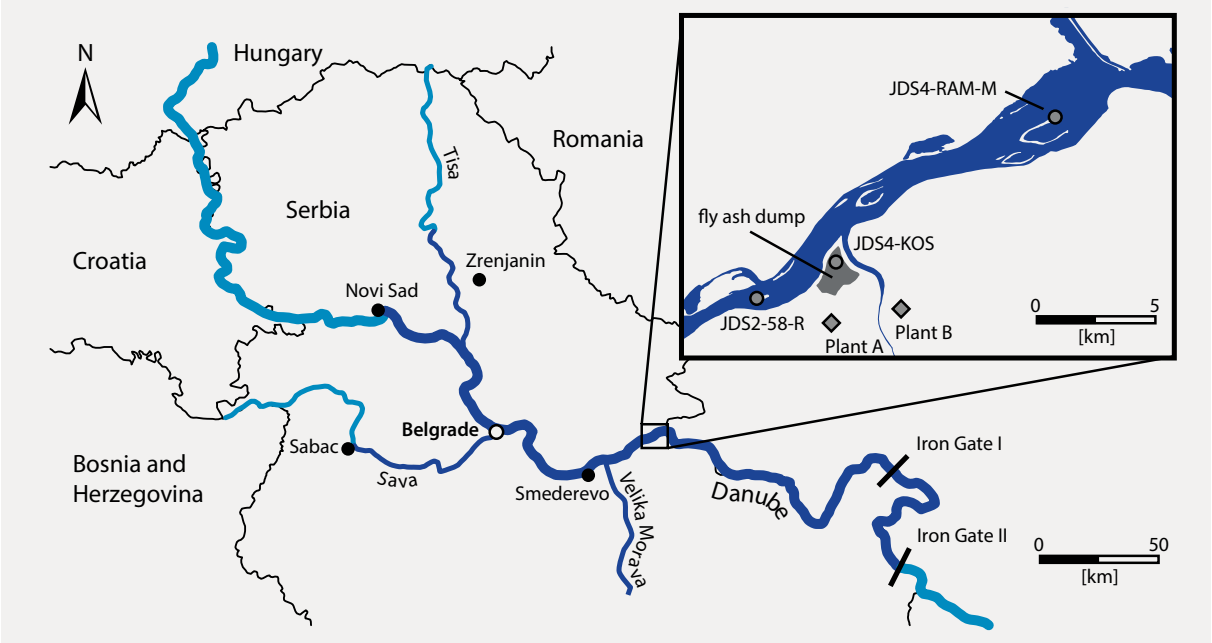

Figure 1: Map of the Danube River through Serbia, including its main tributaries, the dams Iron Gate I and II, and major cities. The river section in dark blue marks the extent of the backwater zone of the Iron Gate I dam (map adapted from Babic Mladenovic et al., 2013). The inset map shows the area surrounding Kostolac, including its two coal-fired power plants A and B, their collective fly ash dump, and all sampling locations detailed in Table 1.

43.2.2 Characterization of the Kostolac fly ash and the fly ash-free sediment reference

43.2.2.1 Mineralogy

The mineralogy of the fly ash and sediment reference samples were investigated by X-ray diffraction (XRD) analysis using a PANalytical X-ray diffractometer (X'pert pro) with Cu Kα radiation (λ = 1.54 Å), operating at 40 kV and 40 mA, together with a linear detector X'Celerator and a secondary flat monochromator. Samples were placed on a zero-background silicon plate and the diffraction pattern recorded within a range from 2 to 70 °. The International Center for Diffraction Data PDF-2 database was used with the X'Pert Highscore Plus software (PANalytical) to identify the mineral phases from the obtained XRD patterns. A selected segment of the sediment core was also included to check for the presence of fly ash-specific minerals.

43.2.2.2 Main and trace element composition

The main and trace element composition of the fly ash and sediment reference samples was determined by inductively coupled plasma optical emission spectrometry (ICP-OES; Optima 5300 DV, PerkinElmer) and inductively coupled plasma mass spectrometry (ICP-MS; 7700x, Agilent), respectively.

A 100 mg sample aliquot and 900 mg of the digestion agent lithium tetraborate (Spectromelt® A10, Merck) were melted in a platinum crucible in an induction heated fusion unit (Lifumat 1.2 Table-Ox, Linn High Therm GmbH) by heating it to a temperature of 1050 °C for 9 minutes. The melt was then dissolved in a mixture of HNO_3 and HF and diluted subsamples were used for subsequent ICP-OES and ICP-MS analyses.

43.2.2.3 Morphology of the Kostolac fly ash

The morphology and particle size of the fly ash was examined by a scanning electron microscope (SEM, Inspect S50; FEI) with an Everhart–Thornley detector, operated at a high vacuum and an acceleration voltage of 10 kV. The grab reference sediment upstream of the dump and a selected segment of the sediment core downstream of the dump were also included to inspect a potential presence of the fly ash particles.

43.2.3 Initial description and bulk elemental composition of the sediment core

Element concentration trends along the sediment core were determined semi-quantitatively by high resolution X-ray fluorescence (XRF) scanning. The analysis was performed on a microXRF core scanner (ITRAX, COX Analytics). The core was defrosted and split lengthwise prior to XRF analysis. The fresh split surfaces were briefly inspected visually, smoothed with a plastic spatula, and covered by a thin translucent protective plastic film. The core was scanned by an RGB camera for high resolution images and the X-ray module, for XRF analyses at 1000 µm resolution. The XRF spectra were analysed with Q-spec 8.6.0 (COX Analytics).

43.2.4 Nanoparticle analysis

The multi-element assessment of single sub-micron sized particles was performed on an inductively coupled plasma time-of-flight mass spectrometer (ICP-TOF-MS; icpTOF 2R, Tofwerk).

43.2.4.1 Preparation of nanoparticle suspensions

Particle suspensions were prepared by dispersing approximately 100 mg sample material in 10 mL of a 0.1% alkaline detergent (FL-70, Fisher Scientific) inside a metal-free conical centrifugation tube, using a Vortex-Genie 2 (Scientific Industries Inc.) for a couple of seconds. The tube was subsequently sonicated in a vial tweeter (UP200St, Hielscher) to an energy target of 1000 Ws/mL. Afterwards, the sample was centrifuged (CR 4 22, Jouan) to a cut-off of 2 µm (after Plathe et al., 2010) and the supernatant pipetted into an acid-washed 10 mL vial. The supernatant, containing the sub-micron sized particles, was diluted with ultrapure water with a factor of 1:1000 for fly ash analysis and with a factor of 1:10,000 for sediment analysis to reduce the particle number concentration and thus avoid both detector saturation as well as particle event coincidence during the measurements.

43.2.4.2 Single-particle measurements

The nanoparticle suspensions were introduced into a desolvating nebulizer (Apex Omega, Elemental Scientific) by a single-loop syringe pump before entering the mass spectrometer. Drying of the aerosol in the membrane desolvation system both increases the sensitivity of the ICP-TOF-MS and decreases formation of oxides. The icpTOF 2R's time-of-flight analyser outputs a continuous spectrum of mass to charge ratios from 7 to 280 in millisecond intervals (continuous acquisition mode) or microsecond intervals (triggered acquisition mode). Consequently, for every particle event lasting ~700 µs, the elemental composition across virtually the entire periodic table is measured quasi-simultaneously. In our measurements, the dwell time was set to 3 ms to obtain the highest temporal resolution in the continuous acquisition mode. Each sample was measured for 60 s.

43.2.4.3 Single-particle data analysis

The raw ICP-TOF-MS spectra were processed using a particle detection and quantification script in Python 2.7 (liq_quant_main_v_0_10_0, Tofwerk). Particles manifest as transient signals in the time-resolved spectra. The script identifies the particle events through iterative outlier detection and removal (Tuoriniemi et al., 2012). The transport efficiency was determined after Pace et al. (2011), using a calibration of gold as a dissolved- (Merck) and particle standard (BBI Solutions; 100 nm diameter). All elements reported were measured as dissolved standards (Merck; Inorganic Ventures) at four points (0, 0.1, 1, and 10 ppb) which in conjunction with the transport efficiency enables a counts-to-particle mass calibration. Any further analyses, including additional post-processing such as merging or exclusion of particle events split across multiple dwell times, element ratio analysis, and plotting, were done using custom scripts in Python 3.7.

43.2.4.4 Selection of chemical elements and element ratios for the single-particle data interpretation

The major and trace element analysis of the bulk fly ash and reference grab sediment samples elucidated elements elevated in the Kostolac fly ash compared to the reference Danube sediment. Additionally, focus was given to elements with intermediate volatility, which are expected to be enriched in the smallest particle size fraction following the fractionation during coal combustion. Selected elements from the bulk analysis as well as the element masses from the single-particle analysis were normalized to Ti, a non-fractionating element during the coal combustion. For the final assessment of the nanoparticle inventory in the Danube sediment core, we used only the element ratios for which there were a sufficient amount of data points, i.e. particle events

43.3 Results and Discussion

43.3.1 Compositional differences between Kostolac fly ash and Danube sediment

The bulk analysis revealed compositional differences between the Kostolac fly ash and the reference Danube sediment, apparent both in their mineralogy as well as in their major- and trace element composition.

The Kostolac fly ash and the reference Danube sediment both contained quartz, feldspars, and muscovite. In the fly ash sample, these common minerals may partially derive from atmospheric deposition of clastic material onto the uncovered dump. Chlorite, Mg-hornblende, and carbonates, the remaining minerals in the Danube River sediments at this location, were absent in the fly ash sample (Figure 2). This was also reflected in the chemical composition, with lower concentrations of K, Na, Ca, and Mg in the fly ash compared to the reference sediment. In contrast, the fly ash contained hematite and mullite, the latter being a rare high-temperature silicate mineral commonly found in fly ash (Gomes and François, 2000). Bulk Al and Fe concentrations were consequently enriched in fly ash (data not shown). These fly ash-specific minerals were not identified in the segment of the downstream sediment core by the XRD but might have been present at amounts below its detection limit of 2wt%.

The bulk materials also differed in their trace element content. Kostolac fly ash exhibited higher concentrations of Sc, V, Cu, Ga, As, Sr, and Mo compared to the reference Danube sediment. These elements were thus considered potential markers for Kostolac fly ash. The remaining trace element concentrations were either lower in fly ash or showed no discernible differences to the river sediment (data not shown).

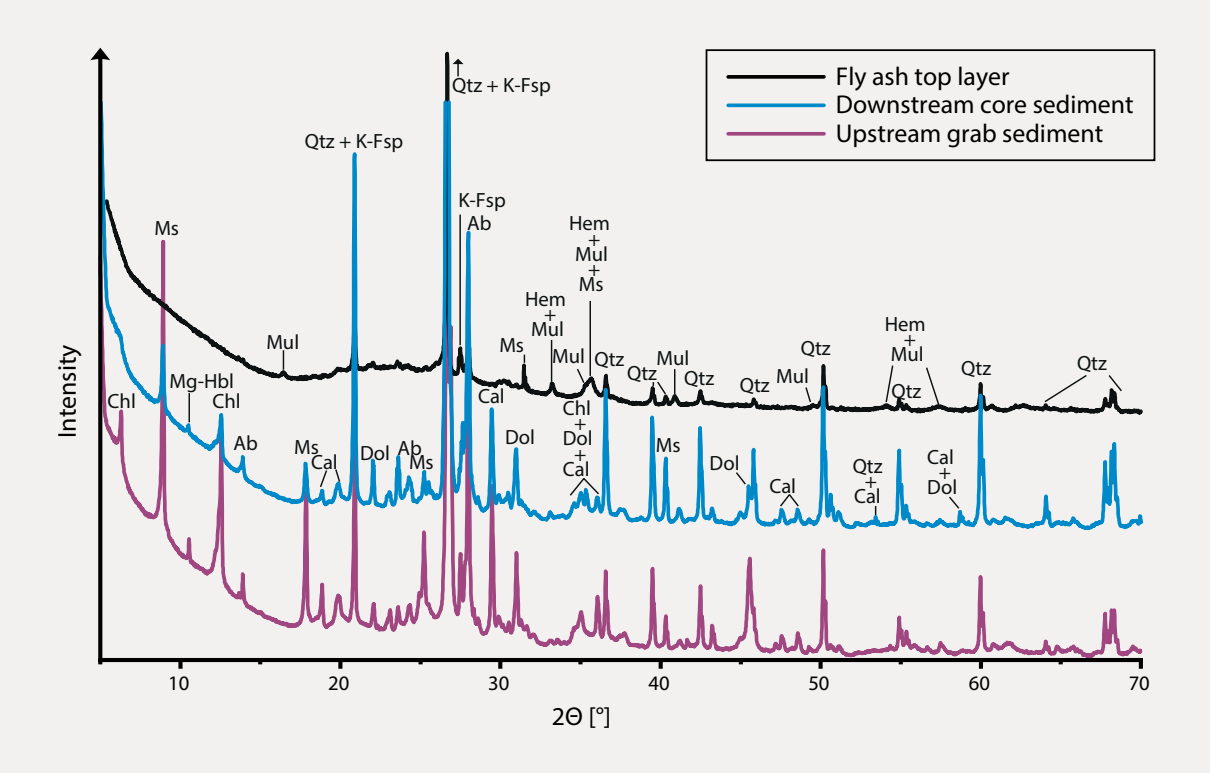

Figure 2: X-ray diffractogram of Kostolac fly ash and the reference Danube sediment, upstream of the fly ash dump. For comparison, a segment of the sediment core, downstream of the dump, is also included. Ab – albite; Cal - calcite; Chl - chlorite; Dol – dolomite; Hem – hematite; K-Fsp –K-feldspar; Mg-Hbl – Mg-hornblende; Mul – mullite; Ms – muscovite; Qtz – quartz.

43.3.2 Visual core description and microXRF core scanning

The sediment core was dark grey to black, consisting of mostly silt and clay, as suggested by visual and tactile inspection (Figure 3, left). Fine sand layers were deposited at 5–7 and 30–32 cm core depth, reflecting higher river discharge events. The brightened RGB images revealed a horizontal layering with thickness from a few mm to several cm. This indicated a generally undisturbed sedimentation mostly under slow flow velocities and no disturbance by burrowing organisms.

MicroXRF core scanning revealed pronounced Ga/Ti and Sr/Ti ratio peaks (potential markers for Kostolac fly ash) in the coarser core layer at around 32 cm depth. The second highest peak in Sr/Ti ratios was located in the coarser layer at 6 cm depth. This peak had no congruent counterpart in the Ga/Ti ratios, which peaked at 4 cm core depth, but was only slightly outside its variability range within the core. As/Ti, V/Ti, and Cu/Ti ratios (additional potential fly ash markers) all peaked at the coarser 6 cm core segment but showed no clear congruent trends along the core (Figure 3 right). These results pointed towards a different or additional source of sediment material at 5–7 and 30–32 cm core depth, most likely related to much higher river discharge events, given their textural differences. However, the trends in As, Cu, Ga, Sr, and V, marker elements in bulk Kostolac fly ash, exhibit partially conflicting trends allowing no clear attribution to a fly ash input into the Danube sediment, by the XRF data.

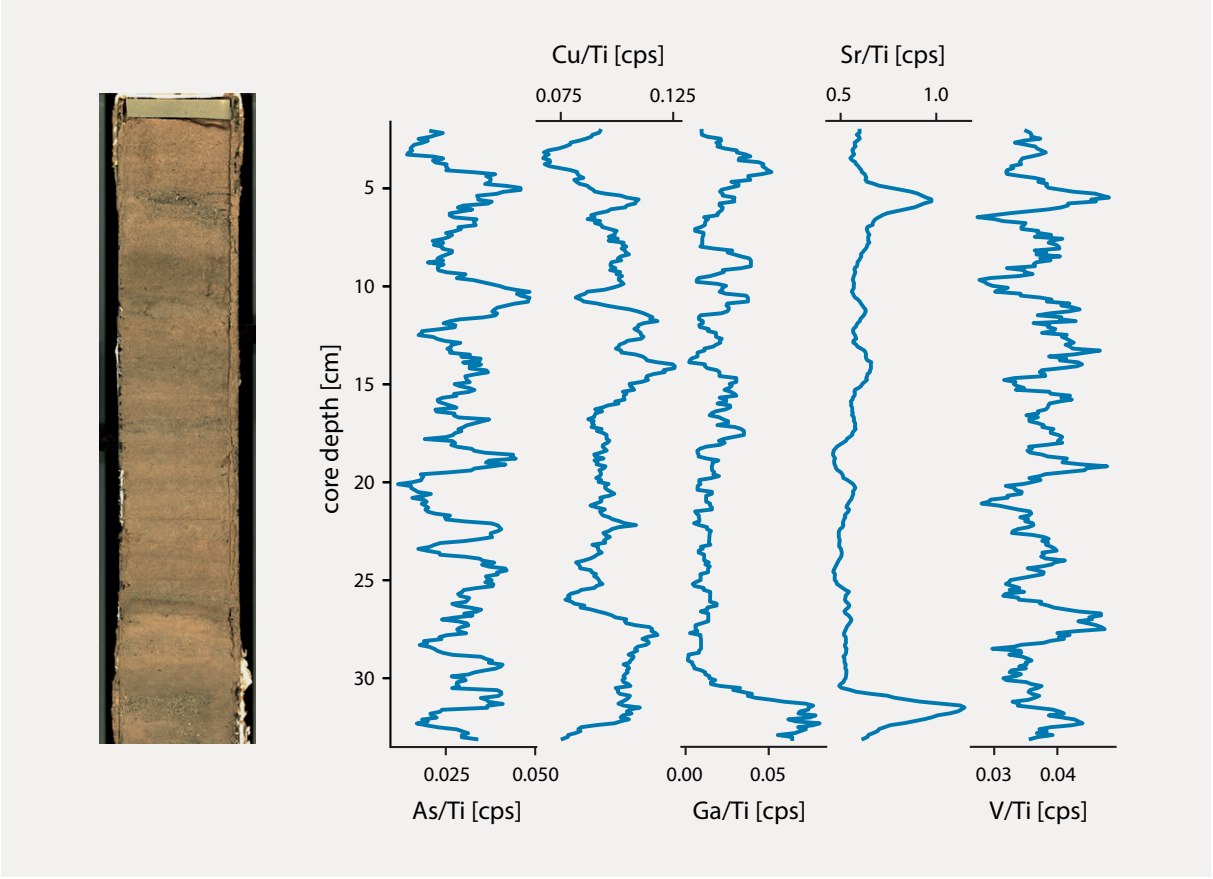

Figure 3: RGB image (left) and selected element ratios (right) for the Danube sediment core downstream of the dump site; cps – counts per second.

43.3.3 Single-particle analysis

The scanning electron micrographs of fly ash particles revealed a high heterogeneity of this material. It covered a range of particle sizes from 500 µm to less than 1 µm (Figure 4). A vesicular texture with numerous pores left by outgassing of the volatiles was common. Particularly towards the smaller particle sizes, spherical shapes were more prevalent. These particles, formed through condensation of the cooling flue gas, are expected to have experienced the strongest fractionation and subsequently to be enriched in elements such as Ni, Cu, and V (Davison et al., 1974; Hower et al., 2020).

The ratios of these elements to Ti in the single particle measurements spanned multiple orders of magnitude. While the mean values were similar between the fly ash and Danube sediments, the fly ash clearly exhibited outliers orders of magnitude above the averages. Such a pattern surely resulted from fractionation during the coal combustion. We therefore focused on those particle events which had elevated element ratios of Co, Cr, Cu, Ni, Pb, and V to Ti, indicative of fly ash-derived nanoparticles. These elements all exhibited strong fractionation and enrichment in the fly ash.

The number of particle events with enriched signature-elements was normalized to the total number of particle events in each measurement to mitigate effects in material heterogeneity. Co, Cr, Cu, Ni, Pb, and V exhibited similar trends within the sediment core downstream of the fly ash dump (Figure 5). Four zones of elevated particle numbers were identified in this core at depths of 5–7, 16, 28, and 31 cm. The first and last zones were congruent to the core depths of elevated Sr and Ga in the bulk analysis and the coarser grain sizes, indicating that the fly ash likely entered the river during major discharge events. For the two other

zones (at 16 and 28 cm), the fly ash likely entered the river during normal river discharge, allowing only the smallest fly ash particles to deposit further downstream, while the coarser grains possibly remained in the vicinity of the dump. The fly ash likely enters the Danube when the fly ash dump is overflowed by strong rain events or increased input of fly ash suspensions from the power plants. These overflowing waters can enter the Danube directly or via the Mlava tributary (Popovic and Djinovic, 2006). Moreover, larger amounts of fly ash may enter the river if the fly ash retaining dike fails, as happened prior to the JDS2 in 2007 (JDS2 diary, http://www.danubesurvey.org/jds2/node/117.html).

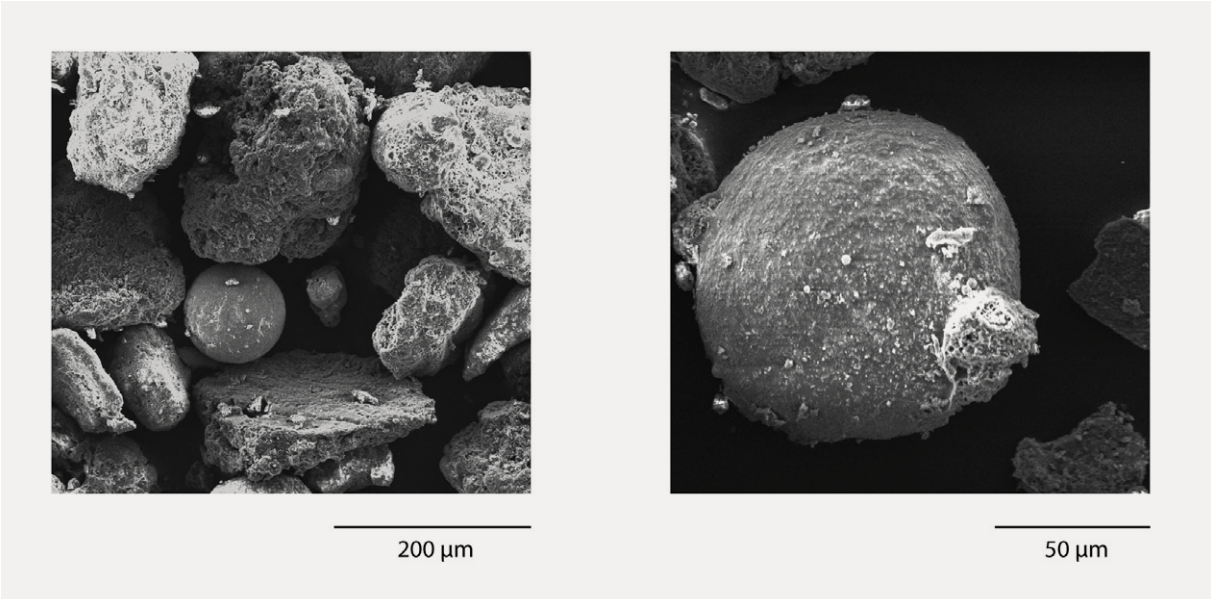

Figure 4: Scanning electron micrographs of Kostolac fly ash at 500x and 1500x magnification exhibiting both irregularly shaped particles with vesicular texture and smooth spherical particles of different sizes.

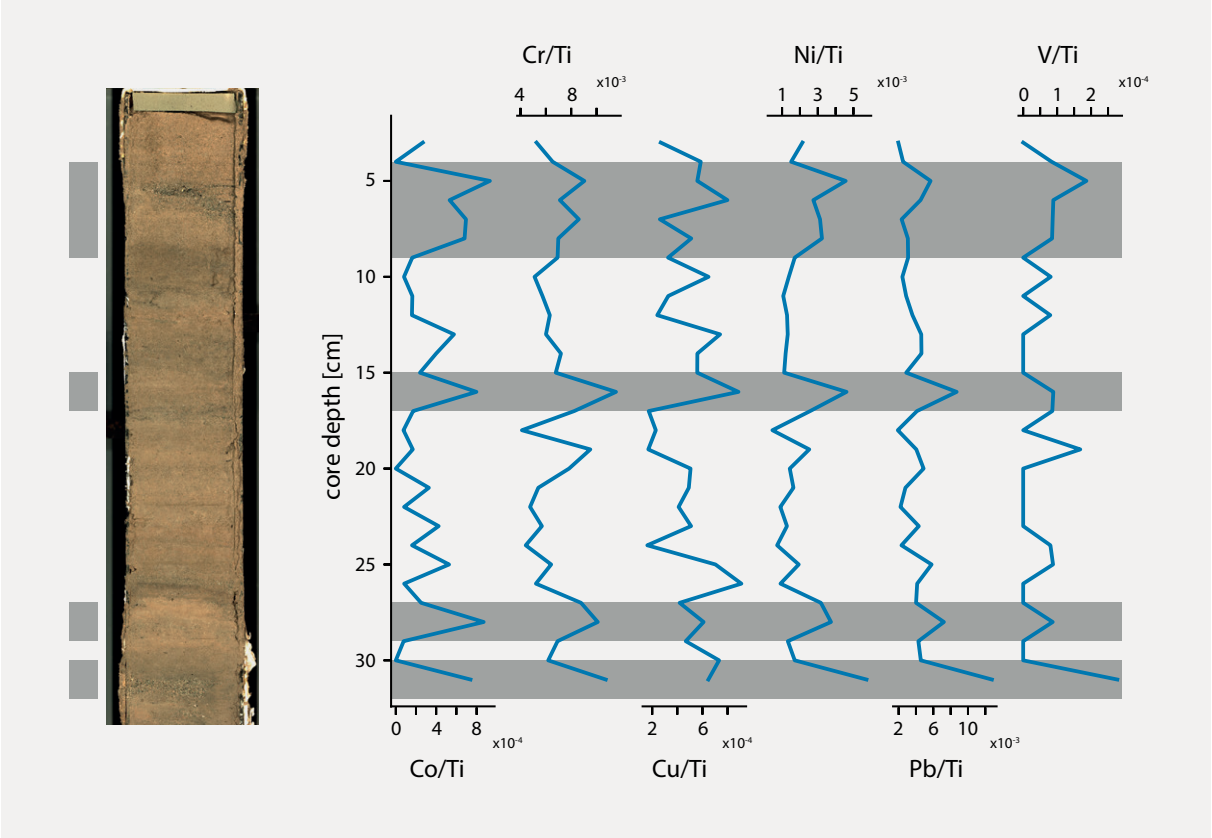

Figure 5: RGB image (left) and number of particle events with elevated ratios for Co, Cr, Cu, Ni, Pb, and V to Ti, normalized to the number of events in each measurement (right) for the Danube sediment core downstream of the fly ash dump site.

43.4 Conclusions

Nanoparticles of a Danube sediment core downstream of the Kostolac fly ash dump were investigated and compared to the dump's fly ash nanoparticles, as well as to a Danube reference sediment upstream of the dump. Several elements that typically fractionate during the coal combustion, such as Cu, Ni, and V, were enriched in a portion of the Kostolac fly ash particles and were orders of magnitude more abundant in fly ash compared to the reference sediment. These were regarded as fly ash-signature elements. While virtually absent in the sediment sample upstream of the fly ash dump, nanoparticles enriched in these fly ash-signature elements were found in the sediment core downstream of the dump. Their number varied with sediment depth, exhibiting four zones with congruently elevated particle counts. This indicated that there is an occasional release of fly ash particles into the Danube from the uncovered fly ash dump located only 50 m from the river. Two of the fly ash release events were associated with major river discharge episodes. The remaining two events apparently occurred during normal river discharge, allowing the transport of only the smallest fly ash particles to the coring site, 12.5 km downstream. The release of fly ash into the Danube through overflow from the dump may be caused either by strong rain events or increased fly ash suspension input.

43.5 References

DAVISON, R. L., NATUSCH, D. F. S., WALLACE, J. R., & EVANS, C. A. (1974). Trace elements in fly ash. Dependence of concentration on particle size. Environmental Science and Technology, 8(13), 1107–1113.

BABIC MLADENOVIC, M., KOLAROV, V., & DAMJANOVIC, V. (2013). Sediment regime of the Danube River in Serbia. International Journal of Sediment Research, 28(4), 470–485.

EPA. (2010). Emerging Contaminants – Nanomaterials At a Glance Fact Sheet. Solid Waste and Emergency Response, EPA 505-F-.

GOMES, S., & FRANÇOIS, M. (2000). Characterization of mullite in silicoaluminous fly ash by XRD, TEM, and 29Si MAS NMR. Cement and Concrete Research, 30(2), 175–181.

HOCHELLA, M. F., MOGK, D. W., RANVILLE, J., ALLEN, I. C., LUTHER, G. W., MARR, L. C., … YANG, Y. (2019). Natural, incidental, and engineered nanomaterials and their impacts on the Earth system. Science, 363(6434), eaau8299.

HOWER, J. C., FU, B., & DAI, S. (2020). Geochemical partitioning from pulverized coal to fly ash and bottom ash. Fuel, 279(May), 118542.

ICPDR. (n.d.). Hotspots and hormones. Retrieved July 29, 2020, from JDS2 Diary website: http://www.danubesurvey.org/jds2/node/117.html

KITTNER, N., FADADU, R. P., BUCKLEY, H. L., SCHWARZMAN, M. R., & KAMMEN, D. M. (2018). Trace Metal Content of Coal Exacerbates Air-Pollution-Related Health Risks: The Case of Lignite Coal in Kosovo. Environmental Science & Technology, 52(4), 2359–2367.

LEAD, J. R., BATLEY, G. E., ALVAREZ, P. J. J., CROTEAU, M.-N., HANDY, R. D., MCLAUGHLIN, M. J., … SCHIRMER, K. (2018). Nanomaterials in the environment: Behavior, fate, bioavailability, and effects-An updated review. Environmental Toxicology and Chemistry, 37(8), 2029–2063.

PACE, H. E., ROGERS, N. J., JAROLIMEK, C., COLEMAN, V. A., HIGGINS, C. P., & RANVILLE, J. F. (2011). Determining transport efficiency for the purpose of counting and sizing nanoparticles via single particle inductively coupled plasma mass spectrometry. Analytical Chemistry, 83(24), 9361–9369.

PLATHE, K. L., VON DER KAMMER, F., HASSELLÖV, M., MOORE, J., MURAYAMA, M., HOFMANN, T., & HOCHELLA, M. F. (2010). Using FlFFF and aTEM to determine trace metal – nanoparticle associations in riverbed sediment. Environmental Chemistry, 7, 82–93.

POPOVIC, A., & DJINOVIC, J. (2006). Influence of coal ash and slag dumping on dump waste waters of the Kostolac power plants (Serbia). Energy Sources, Part A: Recovery, Utilization and Environmental Effects, 28(13), 1189–1201.

POPOVIC, A., DJORDJEVIC, D., RELIC, D., & MIHAJLIDI-ZELIC, A. (2011). Speciation of trace and major elements from coal combustion products of Serbian power plants (I)-"Kostolac A" power plant. Energy Sources, Part A: Recovery, Utilization and Environmental Effects, 33(21), 1960–1968.

SAUVÉ, S., & DESROSIERS, M. (2014). A review of what is an emerging contaminant. Chemistry Central Journal, 8(1), 15.

TUORINIEMI, J., CORNELIS, G., & HASSELLÖV, M. (2012). Size discrimination and detection capabilities of single-particle ICPMS for environmental analysis of silver nanoparticles. Analytical Chemistry, 84(9), 3965–3972.

44 Microplastics in biota – Asian clam case

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Abstract

The study of microplastics was conducted within JDS4 along with other analyses. Freshwater mussel Asian clam Corbicula fluminea (Müller, 1774) was used as the test organism and collected from 23 sites along the Danube River and main tributaries. In total, 216 specimens were used for analysis. After repeated rinsing and separation of tissue, the samples were processed in order to separate particles suspected to consist of different types of plastic materials (KOH digestion and filtration). Isolated particles were photographed, measured, counted and classified to 5 major microplastic categories: fiber, hard plastic, nylon, rubber or miscellaneous. Particles were subcategorized based on the coloration. Microplastic debris ingested by organisms was represented mostly by fragmented hard plastics, within the size range from 0.02 to 4.67 mm and fibers. A total of 1,998 microplastic particles were collected with an average of 9.25 particles per organism or 26.4 particles g-1 wet body weights. JDS4 microplastic study in biota provided comparable information on plastic particles in biota, indicating considerably higher presence of microplastic debris on sites JDS4-4, JDS4-23, JDS4-24, JDS4-40 and JDS4-41 along the Danube, as well as on tributaries - JDS4-20 (Hron), JDS4-35, JDS4-36 (Sava), JDS4-38 (Velika Morava), JDS4-44 (Iskar) and JDS4-45 (Jantra), compared to other sites. The data could indicate important influences of tributaries and some settlements (Belgrade) on the microplastic load in the Danube. Based on microFTIR spectroscopy analysis, particles were characterized as Polycarbonate, Polyethylene Terephthalate (PET), Polypropylene-polyethylene copolymer, Nylon (Polyamide) and Cellophane, with domination of PET, used for production of plastic bottles.

Key words: microplastic debris, aquatic organisms, Corbicula fluminea, Danube River.

44.1 Introduction

Plastic is an organic polymer synthesized by humans from natural derivatives, mainly extracted from oil, natural gas or coal. Particles size range between 1 µm (Gigault et al., 2018) up to 5 mm (Thompson et al., 2009) is considered as microplastics. Plastic degrades through a combination of mechanical erosion, influence of insolation and biological degradation by bacteria or fungi (Andrady, 2011). Further, global mishandling of synthetic organic polymers waste and low recovery rates have led to a significant rise in plastics pollution. Its ubiquitous presence and non-degradable characteristics are directly connected to its persistence in the environment (Andrady, 2011). The following data illustrate the level of pressure to the environment caused by plastic litter. In 2017, the world's production of plastic was estimated to be 348 million metric tons (MT), a year later it was 359 million MT; in Europe, annual production in 2017 and 2018 has been assessed to be 64.4 and 61.8 million MT respectively (Plastics Europe, 2019). A rough estimate predicts that 80% of plastic debris in marine ecosystems is land-based and its pathways are rivers (IOC, 2010). The same source underlines that annual production of plastic has rapidly increased since 1960s.

During the JDS 4 project, we investigated plastic debris in Danube River, from Germany to the Black Sea. The main aim of the study was to categorize and to quantify microplastic particles captured in macroinvertebrate organisms. Freshwater Asian clam *Corbicula fluminea* (Müller, 1774) was used as the test organism. Due to their intensive filter-feeding activities, as well as their benthic way of life, bivalves accumulate considerable quantities of microplastics from the environment and in recent years, they have been extensively used in microplastics studies (Li et al., 2018). The Asian clam, with native range in Asia, can be found nowadays in a wide range of freshwater habitats across the world, including the whole navigable stretch of the Danube River (Paunović et al., 2015). The advantage of using bivalves in microplastic studies is the low mobility, thus indicating the situation on the place of sampling. In addition, in comparison to other aquatic macroinvertebrates, bivalves have a longer life cycle. Having in mind their availability along the Danube in high abundance (Paunović et al., 2007), but also other river systems, the Asian clam was considered the optimal solution for this microplastics study, in order to provide comparability of the data along the river and with other studies.

44.2 Methods

Asian clam *C. fluminea* was used as sentinel organism. Samples were collected by benthic hand nets in summer 2019 by national JDS4 teams. Out of 51 sites, *C. fluminea* was available for analyses at 23.

Ten specimens were randomly selected from each of the 23 sites along 2,040 km of the Danube River. At 3 sampling sites the number of collected specimens was lower (Table 1).

Table 1: Number of Corbicula fluminea specimens per sample.

No.	JDS4 locality codes	No. of specimens	No.	JDS4 locality codes	No. of specimens
1	JDS4-3-L-MC	10	**13**	JDS4-33-L-MC	10
2	JDS4-4-MC	2	**14**	JDS4-35-MC	10
3	JDS4-9-MC	10	**15**	JDS4-36-R-MC	10
4	JDS4-17-L-MC	10	**16**	JDS4-37-L-MC	10
5	JDS4-20-MC	5	**17**	JDS4-38-R-MC	10
6	JDS4-22-MC	10	**18**	JDS4-40-R-MC	10
7	JDS4-23-MC	9	**19**	JDS4-41-R-MC	10
8	JDS4-24-MC	10	**20**	JDS4-44-MC	10
9	JDS4-27-MC	10	**21**	JDS4-45-MC	10
10	JDS4-29-L-MC	10	**22**	JDS4-47-MC	10
11	JDS4-31-L-MC	10	**23**	JDS4-48-MC	10
12	JDS4-32-MC	10			

Stainless steel instruments and glass pots were used during the manipulation of the material. All instruments and pots were rinsed with 70% ethanol and pre-filtered deionised water in order to avoid post-sampling contamination.

Deionized water used for rinsing was filtered through 0.5 µm pore size, 47 mm GF/B glass microfibres (Whatman) in order to eliminate potential presence of microplastic and post-sampling contamination.

The preparation of the material for isolation, as well as further manipulation (except infrared measurements on FTIR) was done in sterile chamber with air circulation through a system of filters used to eliminate particles from the air (original use is for tissue culture).

Before further processing, each specimen was washed with pre-filtered deionised water in order to prevent contamination from the surface of the organisms.

Isolation of microplastics

In total, 216 specimens of *C. fluminea* were measured using analytical scale with the aim to estimate potential microplastic litter per individual and per biomass (g wet body weights).

The shell length and height were measured by the use of a Nonius ruler. The total weight was recorded on analytical scale. The shells were opened with a dissecting knife and the body weight was measured after shell removal. The soft tissue of each specimen was again rinsed with pre-filtered deionized water and placed into glass beakers labelled with JDS4 sample code (Figure 1).

The samples were processed using alkaline protocol (Li et al., 2018) – treatment with 10% potassium hydroxide (KOH) and incubation for 12h at 65 °C in the water bath with a rotation speed of 80 rpm. The solution was filtrated through a 0.5 µm mesh size glass microfibre filters. Each sample was stored in a rinsed sterile glass Petri dish (Figure 1).

Figure 1: Isolation procedure.

The filtrated material was treated with 30% hydrogen peroxide to remove remaining organic matter. One third of each filter paper was examined for microplastic using binocular magnifier and microscope. The minimal size of examined particles was 0.01 mm. The total number of particles was extrapolated to the total filter area. Particles were photographed, measured, counted in the program ImageJ (Ferreira and Rasband, 2012).

In order to assess potential post-sampling contamination in the laboratory with microplastic during the experimental procedure, the filtration process was repeated with distilled water 10 times ("blank") and "blank" filters have been checked for microplastic residues.

Analyses

Microplastic particles of a size range of 0.01 - > 5mm were processed. The particles were photographed, measured, counted and classified to one of 5 categories: fiber, hard plastic, nylon, rubber or miscellaneous and further divided in subcategories based on the coloration (Figure 2).

The classification of particles in 5 categories was done by visual examination and checking of flexibility, fragility and hardness of particles with forceps and entomological needle under the binocular magnifier.

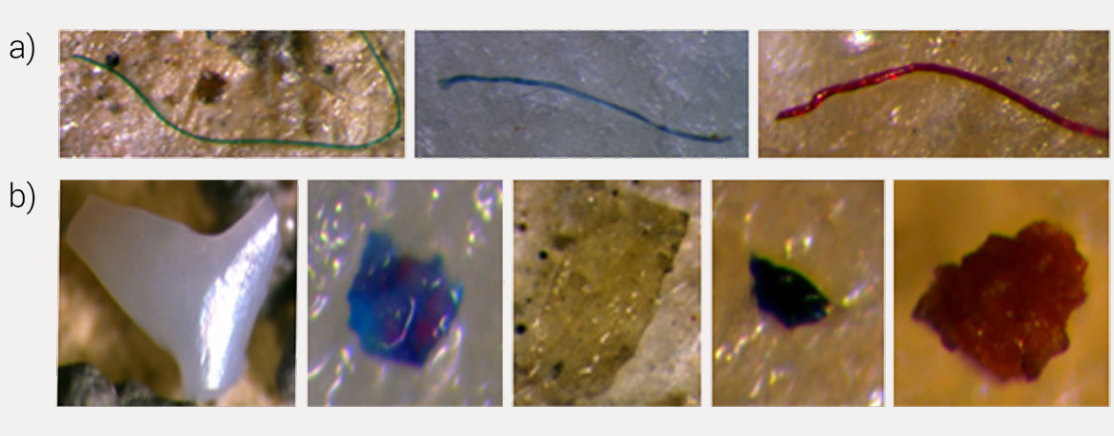


Figure 2: Photographs of microplastic particles isolated from clams tissue: a) Blue, green and red fibres; b) Hard plastic, nylon or rubber particles (white, transparent, blue, red or black particles).

Microplastic chemical identification

Particles identified to be of synthetic origin by their morphology were further processed to be chemically characterized. Infrared measurements were performed using a Nicolet iN10 Fourier transform infrared microscope with micro ATR accessory and cooled MCT detector, using 128 scans at resolution of 4 cm^{-1} (Figure 3).

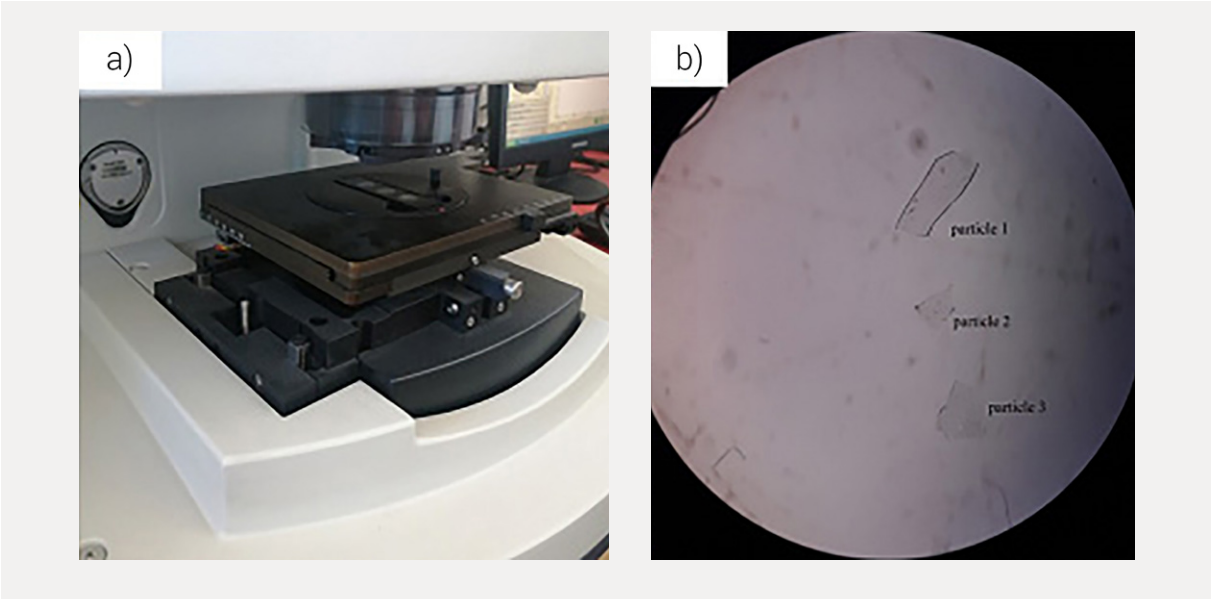


Figure 3: MicroATR spectroscopy (a) and particles prepared for analyses (b).

The Fourier Transforming InfraRed Spectroscopy (FTIR) method for the identification of microplastic particles provides confident data on the chemical composition (Hidalgo-Ruz et al., 2012). Out of a total of 23 localities, 14 were selected for infrared measurements based on the number and diversity of particles in the sample. The position along the longitudinal profile of the Danube was also considered as selection criteria, in order to provide data for the main Danube sections.

At least 3 particles from each selected locality were isolated, according to size, colour and hardness, so that particles from different categories are equally represented in the analysis. A total of 46 particles, from 14 sampling sites were analysed.

The identification of the spectra of the compounds was performed using OMNIC Spectra software (Thermo Scientific™ OMNIC™ Picta Software) through comparison with the spectra of the following spectral libraries: HR Nicolet Sampler Library and Hummel Polymer Sample Library.

44.3 Results and Discussion

The minimum, maximum and average weight of analysed individuals of *C. fluminea* is presented in Table 2.

Table 2: Minimum and maximum values of measured parameters: TW- Total wet weight; BW- Body wet weight.

VALUE	TW [g]	BW [g]
minimum	0.045	0.006
maximum	5.260	1.991
mean	1.8 g ± 0.9	0.3 g ± 0.2

The majority of the analysed individuals were of medium size, with an average length of 14.23 mm (SD 3.78) and height of 15.81 mm (SD 3.89).

Microplastic particles were detected in all 216 individuals of *C. fluminea*. In total 1,998 microplastic particles were detected.

In "blank" filters (filtration process repeated with distillate water) only fibres were identified (1-5 per filter, in average 2 per probe), indicating contamination. Possible sources of background contamination with microfibers could be associated with contamination of ambient air, but also from abrasion from synthetic clothing (Wesch et al., 2017).

Based on our results, an average of 9.25 particles per organism or 26.4 particles/g wet body weights have been recorded.

The number of particles per sampling sites, mean number of particles per individual and per g wet weight are presented in Figure 4.

The data presented show a higher presence of microplastic debris in Asian clams at sites JDS4-4 (Niederalteich – Mühlau), JDS4-23 (Budapest upstream – Megyeri Bridge), JDS4-24 (Budapest downstream – M0 bridge), JDS4-40 (Banatska Palanka/Baziaš) and JDS4-41 (upstream Timok mouth, Radujevac/Gruia) along the Danube, as well as on tributaries – JDS4-20 (Hron), JDS4-35, JDS4-36 (Sava), JDS4-38 (Velika Morava), JDS4-44 (Iskar) and JDS4-45 (Jantra). The data could indicate an important influence of tributaries on microplastic load in the Danube. Furthermore, the rise in the presence of microplastic debris on sites JDS4-37 (downstream Pančevo), JDS4-40 (Banatska Palanka/Baziaš) and JDS4-41 41 (upstream Timok mouth, Radujevac/Gruia), beside influence of tributaries (Sava and Velika Morava) could indicate the influence of Belgrade.

Particles ingested by organisms were represented mostly by fragmented plastic particles within the size range from 0.02 to 4.67 mm (Figure 5), with occasionally presence of nylon and micro-fibres. Blue coloured fibres were dominant among fibres (81 %), while transparent particles were found to be the most abundant among hard ones (42.8 %).

In order to confirm chemical composition of isolated micro-litter, 46 plastic particles from 14 sampling sites were isolated and analysed.

Fibres were excluded from the analyses of chemical composition, due to confirmed contamination with this category of microplastic based on "blank" probe.

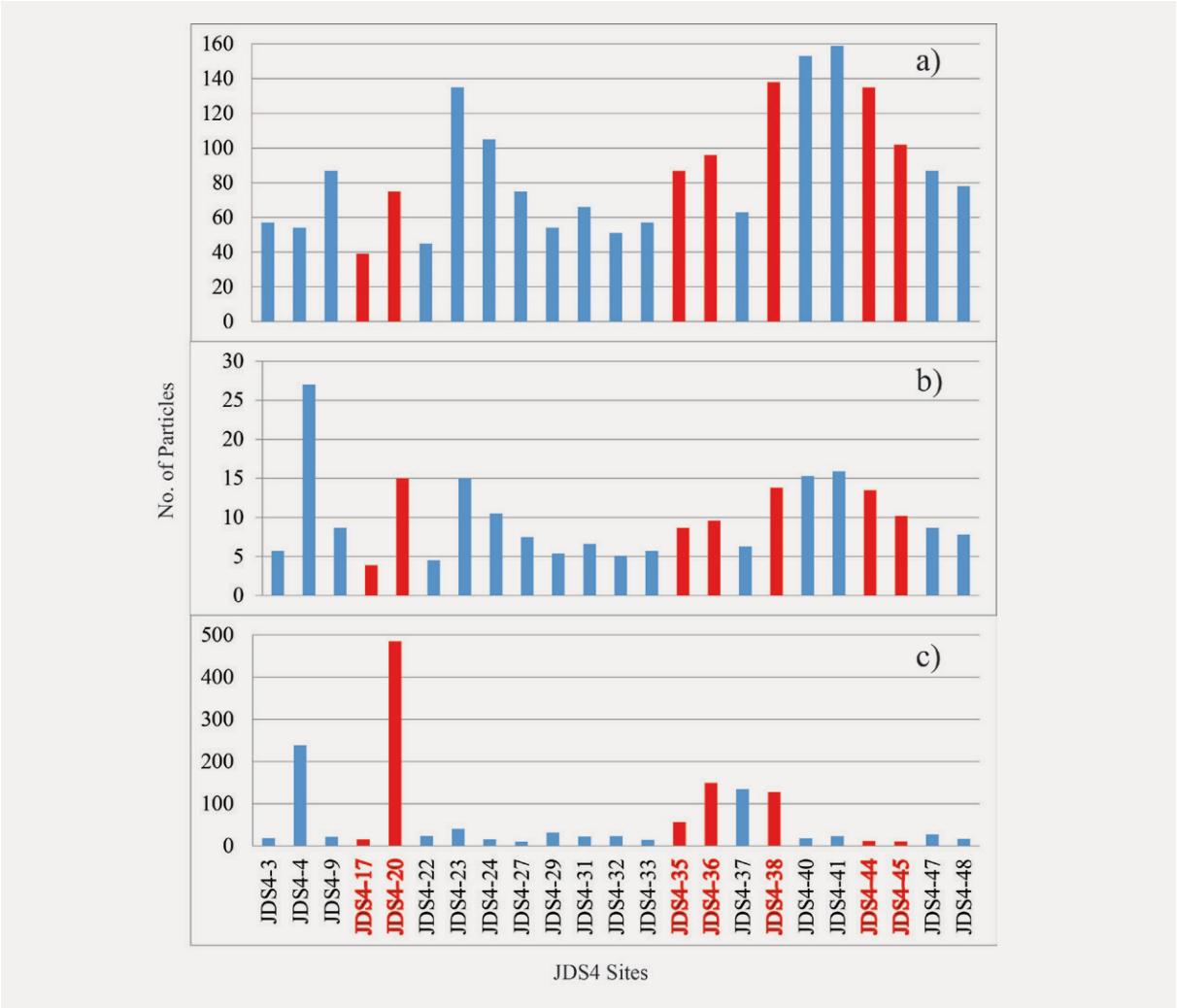


Figure 4: Total number of particles per site, mean No. of particles per individual and mean No. of particles per biomass (g wet weight); sites on tributaries and side arms are presented in red colour.

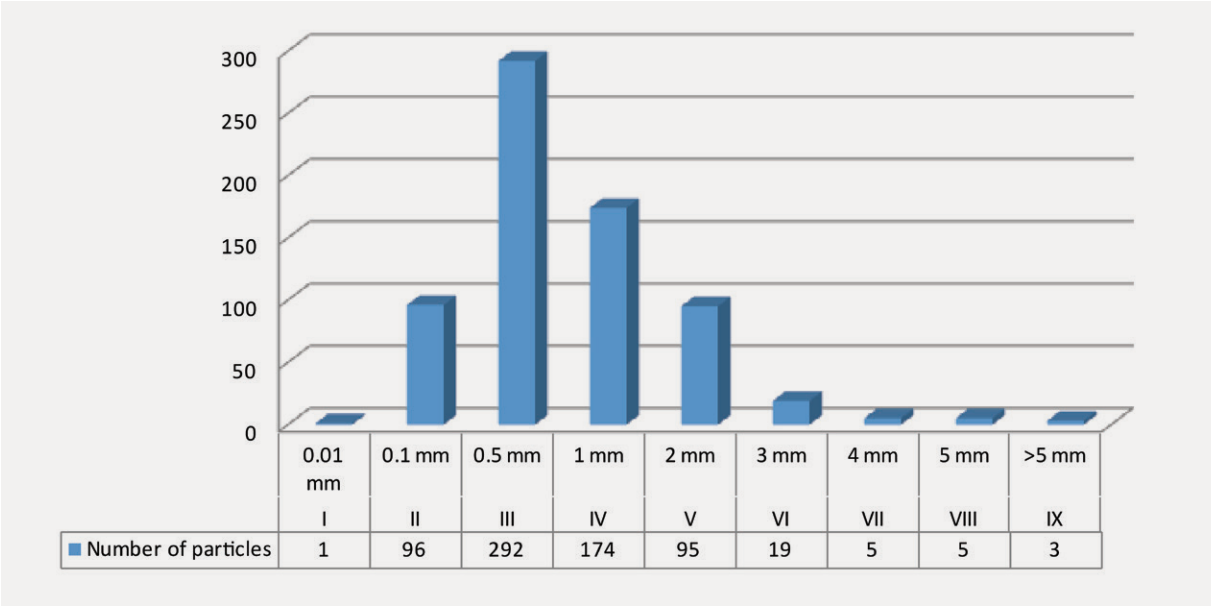


Figure 5: Range of lengths of measured microplastics particles.

Out of 46 particles preselected during microscopic analyses 40 were confirmed as plastic polymers using microFTIR analyses.

Analysed particles were detected as Polycarbonate, Polyethylene Terephthalate (PET), Polypropylene-polyethylene copolymer, Nylon (Polyamide) and Cellophane. (Table 3, Figure 6).

Table 3: Identified microplastics particles per localities.

Locality	Samples	Component identified	Locality	Samples	Component identified
JDS4-3	JDS4-3_1	Cellophane	**JDS4-31**	JDS4-31_1	Polyethylene Terephthalate (PET)
	JDS4-3_2	-		JDS4-31_2	Polyethylene Terephthalate (PET)
	JDS4-3_3	Polycarbonate		JDS4-31_3	5,8,11,14,17-eicosapentaenoic acid
	JDS4-3_4	Polycarbonate	**JDS4-35**	JDS4-35_1	Polyethylene Terephthalate (PET)
JDS4-9	JDS4-9_1	-		JDS4-35_2	Polyethylene Terephthalate (PET)
	JDS4-9_2	-		JDS4-35_3	Polyethylene Terephthalate (PET)
	JDS4-9_3	Polycarbonate	**JDS4-37**	JDS4-37_1	Polyethylene Terephthalate (PET)
JDS4-17	JDS4-17_1	Cellophane		JDS4-37_2	Polyethylene Terephthalate (PET)
	JDS4-17_2	Polypropylene-polyethylene copolymer		JDS4-37_3	Polyethylene Terephthalate (PET)
	JDS4-17_4	Polyethylene Terephthalate (PET)	**JDS4-41**	JDS4-41_1	Polyethylene Terephthalate (PET)
JDS4-20	JDS4-20_1	Polycarbonate		JDS4-41_2	Polyethylene Terephthalate (PET)
	JDS4-20_2	Polycarbonate		JDS4-41_3	Polyethylene Terephthalate (PET)
	JDS4-20_3	Polycarbonate	**JDS4-44**	JDS4-44_1	Polycarbonate
JDS4-24	JDS4-24_1	Polyethylene Terephthalate (PET)		JDS4-44_2	-
	JDS4-24_2	Polyethylene Terephthalate (PET)		JDS4-44_3	Polycarbonate + inorganic
	JDS4-24_3	Polypropylene-polyethylene copolymer	**JDS4-47**	JDS4-47_1	Polyethylene Terephthalate (PET)
	JDS4-24_4	Polyethylene Terephthalate (PET)		JDS4-47_2	Polycarbonate
JDS4-27	JDS4-27_1	-		JDS4-47_3	Polycarbonate
	JDS4-27_2	Polypropylene-polyethylene copolymer	**JDS4-48**	JDS4-48_1	Polyethylene Terephthalate (PET)
	JDS4-27_3	Polyethylene Terephthalate (PET)		JDS4-48_2	Polyethylene Terephthalate (PET)
	JDS4-27_4	Polycarbonate		JDS4-48_3	Polyethylene Terephthalate (PET)
JDS4-29	JDS4-29_1	-			
	JDS4-29_2	Polycarbonate			
	JDS4-29_3	Nylon (Polyamide)			
	JDS4-29_4	Polyethylene Terephthalate (PET)			

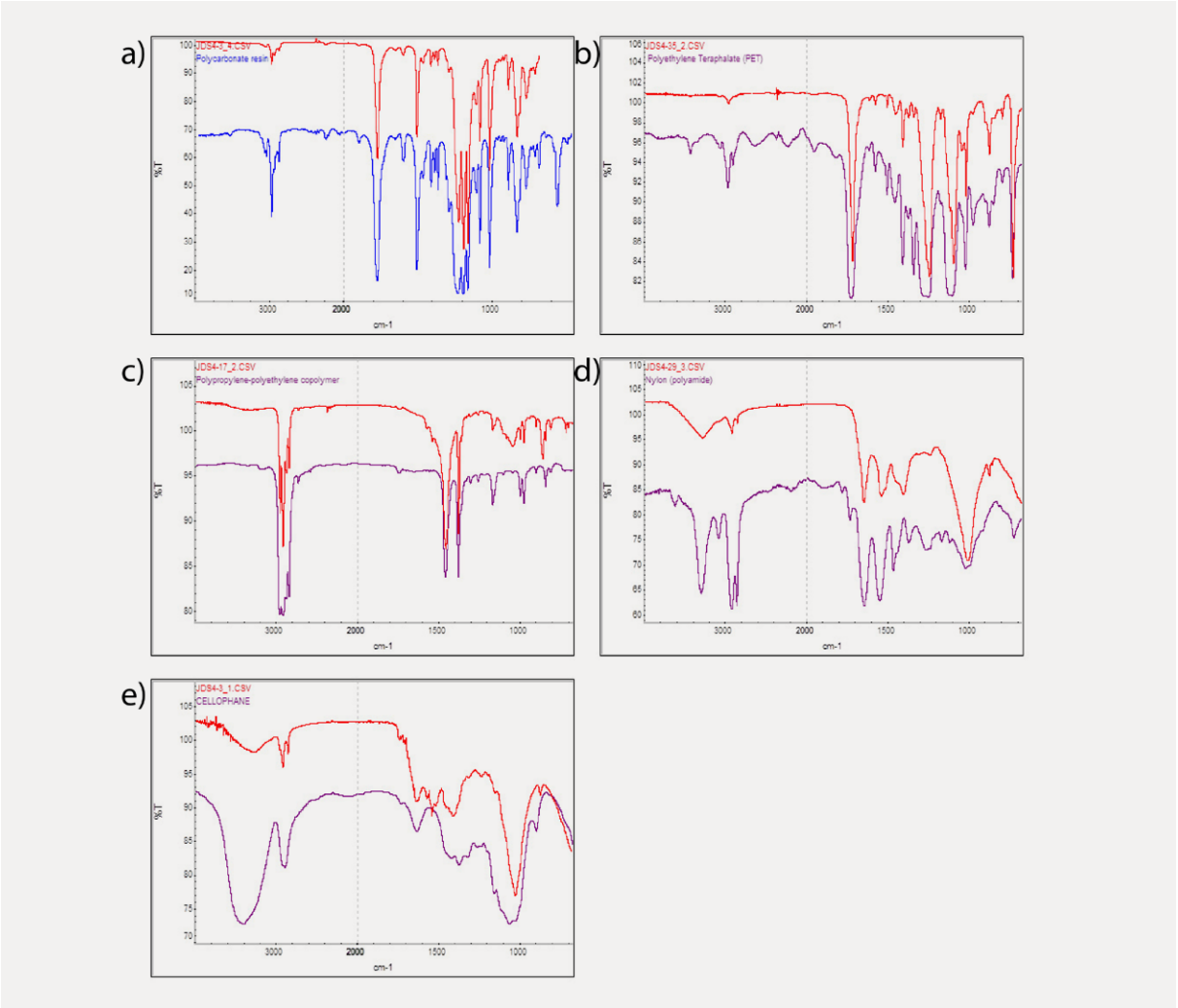


Figure 6: Results of microFTIR analyses (red line) and comparison with data from the chemical substance standards database (blue/purple line): Polycarbonate - JDS4-3_4; Polyethylene Terephthalate (PET) - JDS4-35_2; Polypropylene-polyethylene copolymer - JDS4-17_2; purple line; Nylon (Polyamide) - JDS4-29_3; Cellophane - JDS4-3_1.

The JDS4 microplastic study in biota provided comparable information on the plastic particles in biota along a considerable stretch of the Danube, and thus represents a contribution to the general knowledge on their distribution in biological systems. To the best of our knowledge, beside the study of Su et al., (2018), this is the second study on microplastic in biota in freshwater ecosystems on a large spatial scale.

Detected particles were classified as Polycarbonate, Polyethylene Terephthalate (PET), Polypropylene-polyethylene copolymer, Nylon (Polyamide) and Cellophane, with PET being the most dominant polymer among microplastic particles (58%).

Our results are different in respect to those obtained by the analyses of screening of content of various plastics in suspended particular matter (SPM – see Chapter 42 of this report). According to the results of analyses of SPM samples using TED GC/MS (ThermoExtractionDesorption-GasChromatography-MassSpectrometry), the relative frequency of detection decreases in order: PE > SBR > PS > PP. NR, PMMA, while PET was not detected above the LOD in analysed samples. Our study in Asian clams showed that PET is the most dominant polymer among microplastic particles (58%).

These contrasting findings are rather the result of different target matrixes (SPM versus biota) in studies of concern, than different detection methodology (TED GC/MS versus FTIR spectroscopy). The distribution of different plastic types in respect to particle size is not well known. Our study is focused on larger particles (based on the analyses of particle size frequency, majority of debris is larger than 0.5mm), while study of SPM involves smaller particles, as well (fraction < 500 µm, which was further fractionated into > 100 µm and < 100 µm).

Thus, we are of the opinion that confident monitoring of microplastic should comprise analyses of more than one target sample group. Thus, standardisation of microplastic monitoring is needed in order to avoid misleading results on the distribution of different fractions.

For a more detailed study of peculiarity of distribution of different plastic debris along the watercourse, a denser sampling network is needed. In addition, to achieve higher confidence, a larger share of detected debris needs to be characterized using FTIR spectroscopy. Additional methods of characterisation of polymers may be also applied to provide more accurate results.

A detailed study of Hohenblum et al., (2015) reported a concentration range in water of 0.039–0.205 mg/m^3 and 0.029–0.516 mg/m^3, in the entry and exit points of the Danube Austrian stretch, respectively, with over 50% of the extracted plastic particles consisted of fragments, 4–10 % were pellets and 2.1–2.8% were green lenticular flakes. The annual average range of transport of microplastic particles was calculated from 6 – 66 kg per day in the Austrian Danube River.

Su et al., (2018) provided the results of microplastic particles analyses in the same test organism used in our study in the rivers, lakes and estuarine areas of the Middle and Lower Yangtze River Basin. Their results showed that the Asian clam is a good medium for describing microplastic pollution, especially for sediment. Su et al., (2018) found microplastics in 61 out of 63 samples of Asian clams, with the abundance range from 0.3-4.9 items/g wet body weight and 0.4-5.0 items/individual. Results of microplastic pollution in *C. fluminea* from the Taihu Lake (China) revealed an abundance of microplastic debris in Asian clams, within the range 0.2-12.5 items/g wet weight (Su et al., 2016). Our results showed a higher abundance of microplastic particles in Asian clams investigated in comparison to the mentioned studies in China, indicating an existing pressure caused by plastic pollution in the Danube Basin.

Su et al., (2016) detected Cellophane, Polyethylene Terephthalate, Polyester, Terephthalatic Acid and Polypropylene in Asian clams from the Taihu Lake, with dominance of Cellophane, followed by Polyethylene Terephthalate. Our study showed that particles of Polyethylene Terephthalate (used for production of plastic bottles) are dominant in *C. fluminea* samples from the Danube, while Cellophane particles were found in the Upper and Middle Danube, with lower abundance.

44.4 Conclusions

The JDS4 microplastic study in biota provided comparable information on the plastic particles in mussels (*C. fluminea*) along a considerable stretch of the Danube (along 2,040 river kilometres) and to the best of our knowledge, this is the second study on microplastics in freshwater biota on a large spatial scale.

Analysed parameters (No. of particles per site, mean number of particles per individual per site and mean number of particles per body mass – g/wet weight) indicated a higher microplastic load for tributaries, as well as an important influence of tributaries and settlements on the presence of microplastic debris in the Danube.

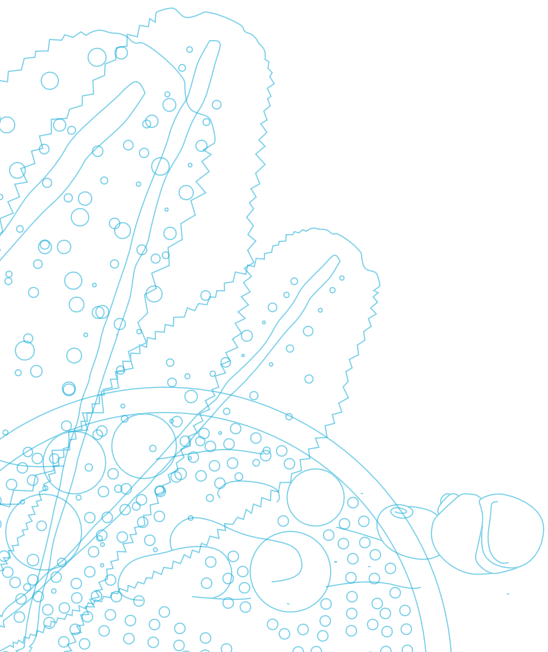
Analyses revealed the presence of the following types of microplastic particles: Polycarbonate, Polyethylene Terephthalate (PET), Polypropylene-polyethylene copolymer, Nylon (Polyamide) and Cellophane, with PET being the most dominant and frequent polymer (58%).

The results of the JDS4 microplastic study confirmed that bivalves are effective test organisms for the assessment of microplastic load in the aquatic environment.

Further standardised studies providing comparable data on microplastic in biota within the Danube River Basin using Asian clams are needed, but also other test organisms, in order to assess the microplastic load and possible consequences more accurately.

The difference of results obtained by the screening of content of various plastics in suspended particular matter (SPM – please, see Chapter 42 of this report) and our study that are the result of different target matrixes (SPM versus tissue) in studies of concern shows that confident monitoring of microplastic requires analyses of more than one target compartment, as well as the standardization of procedures.

For a more detailed study of the peculiarities of the distribution of different plastic debris within the Danube River Basin, a denser sampling network is needed. In addition, to achieve higher confidence, a larger share of the detected debris needs to be characterized using FTIR spectroscopy. Additional methods for the characterization of polymers may be also applied to provide more accurate results.



44.5 References

ANDRADY, A.L., 2011. Microplastics in the marine environment. Mar. Pollut. Bull. https://doi.org/10.1016/j.marpolbul.2011.05.030

FERREIRA, T., RASBAND, W., 2012. ImageJ User Guide User Guide ImageJ. Image J user Guid. https://doi.org/10.1038/nmeth.2019

GIGAULT, J., HALLE, A. TER, BAUDRIMONT, M., PASCAL, P.Y., GAUFFRE, F., PHI, T.L., EL HADRI, H., GRASSL, B., REYNAUD, S., 2018. Current opinion: What is a nanoplastic? Environ. Pollut. https://doi.org/10.1016/j.envpol.2018.01.024

HIDALGO-RUZ, V., GUTOW, L., THOMPSON, R.C., THIEL, M., 2012. Microplastics in the marine environment: A review of the methods used for identification and quantification. Environ. Sci. Technol. https://doi.org/10.1021/es2031505

HOHENBLUM, P., LIEBMANN, B., LIEDERMANN, M., 2015. Plastic and Microplastic in the environment. The Environment Agency Austria, Vienna.

IOC, 2010. The GESAMP International Workshop on micro-plastic particles as a vector in transporting persistent, bio-accumulating and toxic substances in the oceans. … Prot.

LI, J., GREEN, C., REYNOLDS, A., SHI, H., ROTCHELL, J.M., 2018. Microplastics in mussels sampled from coastal waters and supermarkets in the United Kingdom. Environ. Pollut. 241, 35–44. https://doi.org/10.1016/j.envpol.2018.05.038

PAUNOVIĆ, M., CSÁNYI, B., KNEŽEVIĆ, S., SIMIĆ, V., NENADIĆ, D., JAKOVČEV-TODOROVIĆ, D., STOJANOVIĆ, B., CAKIĆ, P., 2007. Distribution of Asian clams Corbicula fluminea (Müller, 1774) and C. fluminalis (Müller, 1774) in Serbia. Aquat. Invasions 2. https://doi.org/10.3391/ai.2007.2.2.3

PAUNOVIĆ, M., CSÁNYI, B., SIMONOVIĆ, P., ZORIĆ, K., 2015. Invasive Alien Species in the Danube, in: The Danube River Basin. pp. 389–409. https://doi.org/10.1007/698_2015_376

PLASTICS EUROPE, 2019. Plastics — the facts 2018. An analysis of European plastics pro- duction, demand and waste data. https://www. plasticseurope. org., 2019. Plásticos – Situación en 2019. Plast. Eur.

SU, L., CAI, H., KOLANDHASAMY, P., WU, C., ROCHMAN, C.M., SHI, H., 2018. Using the Asian clam as an indicator of microplastic pollution in freshwater ecosystems. Environ. Pollut. https://doi.org/10.1016/j.envpol.2017.11.075

SU, L., XUE, Y., LI, L., YANG, D., KOLANDHASAMY, P., LI, D., SHI, H., 2016. Microplastics in Taihu Lake, China. Environ. Pollut. https://doi.org/10.1016/j.envpol.2016.06.036

THOMPSON, R.C., SWAN, S.H., MOORE, C.J., VOM SAAL, F.S., 2009. Our plastic age. Philos. Trans. R. Soc. B Biol. Sci. https://doi.org/10.1098/rstb.2009.0054

WESCH, C., ELERT, A.M., WÖRNER, M., BRAUN, U., KLEIN, R., PAULUS, M., 2017. Assuring quality in microplastic monitoring: About the value of clean-air devices as essentials for verified data. Sci. Rep. 7, 5424. https://doi.org/10.1038/s41598-017-05838-4

45 Radioactivity

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Abstract

The radioactivity content of river sediments is an unerring radio-ecological indicator for the contamination of the environment. The results of the radiometric analysis of the JDS4 sediment samples show that the radio-ecological development of the Danube continues to be promising. The radioactive contamination of the Danube with the long-lived artificial nuclear fission radionuclides ^{137}Cs and ^{90}Sr has decreased by two orders of magnitude since the atmospheric nuclear weapons tests period in the northern hemisphere (1945 - 1963) and the Chernobyl nuclear power plant accident (1986). Furthermore, the activities of the geogenic radionuclides of the natural decay chains and other primordial natural radionuclides (^{40}K, ^{210}Pb, ^{226}Ra and ^{228}Ra) remain at common levels. Thus, there is currently no indication of hazardous man-made radioactive contamination of the Danube ecosystem compartments.

45.1 Introduction

Since the middle of the 20th century the radioactive emissions from atmospheric nuclear weapons testing as well as nuclear reactor accidents - most prominently the Chernobyl accident in Ukraine - have had an impact on the environment. This includes, quite prominently, freshwater resources. Radioecological research on ^{137}Cs and ^{90}Sr in the Danube region began as early as 1967 (Frantz, 1967, Rank, 1976, Tschurlovits, et al., 1980). In subsequent years, naturally occurring radionuclides such as ^{210}Pb, ^{226}Ra, ^{228}Ra, and ^{40}K were also included in the environmental monitoring programs (Maringer, 1996).

Elevated levels of long-lived artificial radionuclide (e.g. ^{137}Cs, ^{90}Sr) concentrations in rivers can lead to increased health risks for populations drinking processed river water or consuming river fish. The use of contaminated river water for irrigation can increase health risks through the consumption of the agricultural products produced in the irrigated areas. Therefore, it is of importance to monitor the radioecological status of the Danube River ecosphere regularly in order to assess the impact on the health of the population living in the Danube Basin. Because of the sustainable enrichment of radionuclides in sediment particles, river sediment samples are the best radioecological indicators for environmental radioactive contamination monitoring in rivers (Maringer, 1994).

The ^{137}Cs activity concentration (half-life of 30.05 ± 0.08 years) in Danube water and riverbed sediments originates primarily from the nuclear power accident in Chernobyl (April-May 1986) and secondarily from

atmospheric nuclear weapons testing during the 1950s and 1960s. ^{90}Sr (half-life of 28.80 ± 0.07 years) originates primarily from atmospheric weapons tests and to a much lesser extent from Chernobyl fallout. In many countries measuring the activity concentration of ^{90}Sr in soil and food (e.g. milk) is part of the monitoring programme for environmental radioactivity. Data are also collected on ^{90}Sr in sediments of lakes, e.g. Lerman and Taniguchi (1972) studied Lake Superior and Lake Ontario in Canada and Mundschenk (1994) studied German inland waters.

Recent radioecological research of the Danube River radioactivity in the framework of the ICPDR Joint Danube Surveys in 2007 and 2013 clearly showed decreasing man-made radioactive contamination of the freshwater compartments (Maringer et al., 2015; 2017).

45.2 Methods

45.2.1 Sample preparation

After collecting two litres of riverbed sediments, the wet samples were dried at 45°C until they reached constant weight. Then the dried samples were homogenized roughly with a wooden roller. After that, a two-step dry sieving procedure followed: (1) stainless steel sieving <125 µm and then (2) homogenization and milling by an agate mill plus final sieving to get the sediment grain size fraction <90 µm.

This procedure was chosen to obtain the highest amount of fine sediment possible from the 2 litres of wet riverbed sediment samples in order to supply enough material for all intended analyses.

By applying this sample preparation, the final sediment samples for analysis were not the 'natural' <63µm fractions of the original sediments but instead the <125 µm fraction of the original sediments which were then additionally milled into a grain size fraction of <60…90 µm.

Therefore, there is a significant difference between the JDS2 and JDS3 (wet on-board sieved <63 µm-fraction) sediment samples compared to the JDS 4 sediment samples (milled <125 µm, <90 µm fraction).

This sample preparation impact must be considered when interpreting / comparing the radioactivity results of the different JDSs, because most radionuclides show higher concentrations within the fine grain fraction of the mineralogical sediment particles (clay, silt / <63µm). The difference in the grain size distribution between the sediment samples of JDS2 / JDS3 and JDS4 must also be considered for chemical pollutants (e.g. heavy metals) when comparing / interpreting the results.

45.2.2 Gamma-ray spectrometry

Generally, the radiometric processing and analysing methods are the same as the ones that were applied in former ICPDR JDS research (Maringer et al., 2017). The dried sediment samples were transferred into a sample container with a well calibrated measurement geometry (a "D-100" container with about 100 g sample mass). Measurements were conducted using a high purity germanium (HPGe) detector for gamma-ray spectrometry. At first a measurement time of 10 000 seconds was chosen. However, since the desired detection limits were unfortunately not reached, the measuring time had to be increased to 80 000 seconds.

All samples that had already been measured for 10 000 seconds were measured for an additional 70 000 seconds in order to reach a total measurement time of 80 000 seconds. During the analysis of the

measurement data, the energy calibration and sample parameters were double-checked. To determine the activity concentration of ^{226}Ra the results of ^{214}Pb and ^{214}Bi were used. ^{228}Ra was determined through ^{228}Ac.

For the evaluation of the gamma-ray spectroscopy, a test spectrum was used to check whether the measurements were significant enough and then the evaluation of the individual radionuclides was started. Figure 1 illustrates the gamma-ray spectrum of sample JDS4-11 and which gamma-ray emitting radionuclides were detected and identified.

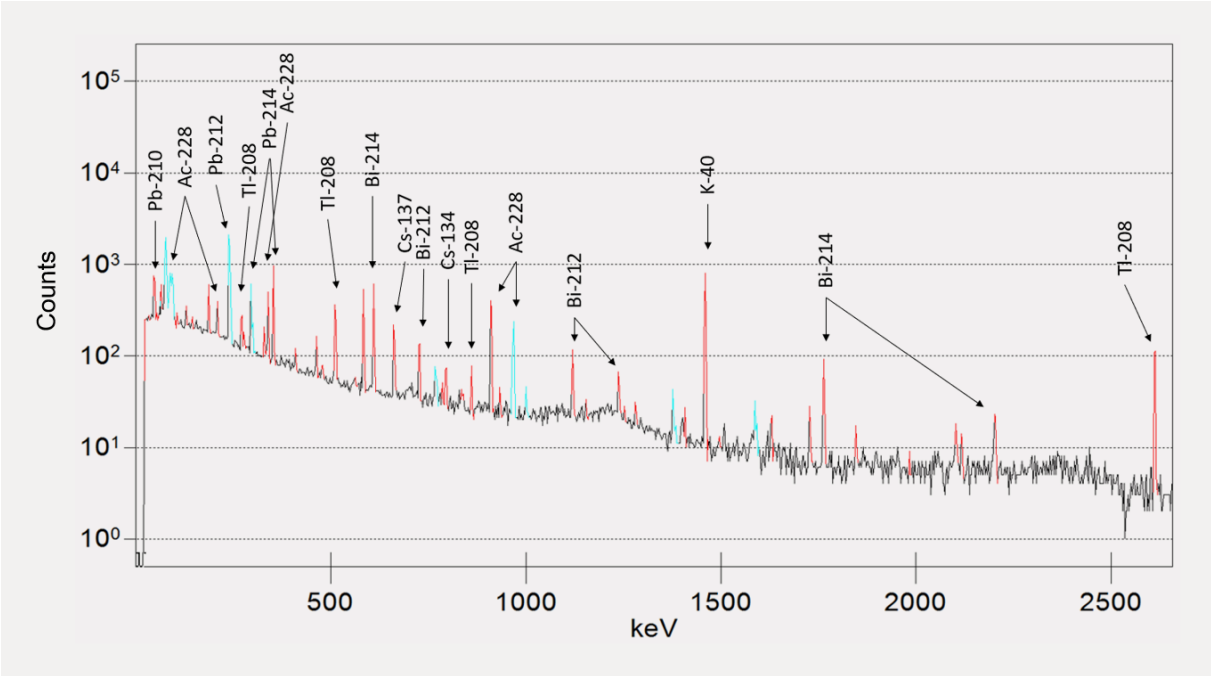


Figure 1: Gamma-ray spectrum of sample JDS4-11 (02.07.2019, Morava, river km 17, Pohansko, Morava mouth at Danube km 1880,3) detected via HPGe-detector system (80 000 seconds).

45.2.3 Radiochemical analysis of ^{90}Sr

After gamma-ray spectrometry, approximately 5 g of the prepared samples were removed for radiochemical analysis. During the subsequent ashing, the samples were incinerated at 700°C for 12 hours. This step removes the organic substances and prevents colour quenching during the ^{90}Sr measurement. In the next step two aliquots of 1 g were taken from each sample and digested in a microwave oven. The first digestion was to determine the original ^{88}Sr and ^{86}Sr content in the sample by ICP-MS. In the second digestion, a Strontium carrier was added beforehand in order to determine the losses during digestion, the subsequent filtration and the separation with the cartridges. For the separation of the Sr-analyte extraction chromatography was applied. Sr-cartridges with SR resin from "TrisKem International" and a vacuum box were used in a newly developed partially automated method for the determination of strontium isotopes and transuranium elements. The vacuum box separation process is mainly applied to samples with small quantities of material like Danube sediment samples.

Before the counting of the sample an aliquot was taken to measure the remaining ^{88}Sr and ^{86}Sr concentration by ICP-MS to calculate the chemical recovery/yield. Finally, the liquid scintillation counting measurements were performed by two Perkin Elmer Quantulus® 1220 LSC instruments, always including a standard and a blank sample prepared with the same method.

45.3 Results and Discussion

45.3.1 Man-made radionuclides

Caesium 137

In the upper part of the Danube (> km 1790), all JDS4 sediment samples (including sediments of tributaries) show ^{137}Cs activity concentrations of below 10 Bq/kg (grain size fraction < 125 µm, dry weight, Figure 2). A similar situation was observed in the Lower Danube (< km 953). In the middle part of the Danube (km 1790 – km 943), the ^{137}Cs activity concentration in the sediments is higher than in the other sections but remains below 30 Bq/kg. The ^{137}Cs activity concentrations in the tributaries' sediments samples are in the same range as the concentrations in their corresponding Danube River samples (Figure 2).

When applying the verified range of the mean equilibrium distribution coefficient $K_D = a/c_A = 10^4 - 10^5$ (Bq/kg)/(Bq/l) of ^{137}Cs in the Danube River (depending primarily on the mineralogical constitution of the sediment; Maringer et al., 1997), the mean ^{137}Cs activity concentration $c_A(^{137}Cs)$ in Danube water ranges between 3×10^{-4} and 3×10^{-3} Bq/l.

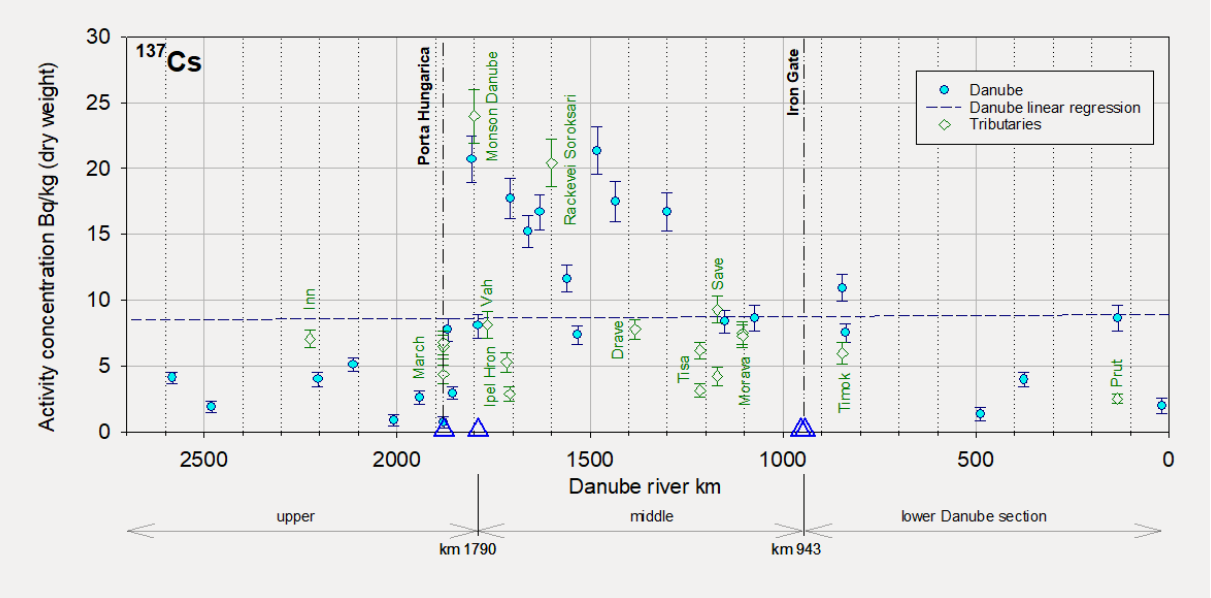


Figure 2: ^{137}Cs activity concentration in JDS 4 sediment samples < 125 µm.

Strontium 90

All ^{90}Sr activity concentrations of the analysed sediment samples were below the detection limit of 4.5 Bq/kg. The individually calculated decision limits for the ^{90}Sr activity concentration in the analysed sediment samples are shown in Figure 3. The decision limits have been calculated based on ISO 11929:2010.

When applying the verified range of the mean ^{90}Sr distribution coefficient $K_D = 3 \times 10^3 - 3 \times 10^4$ (Bq/kg)/(Bq/l) in the Danube River, the mean ^{90}Sr activity concentration $c_A(^{90}Sr)$ in Danube water is below 3×10^{-3} Bq/l.

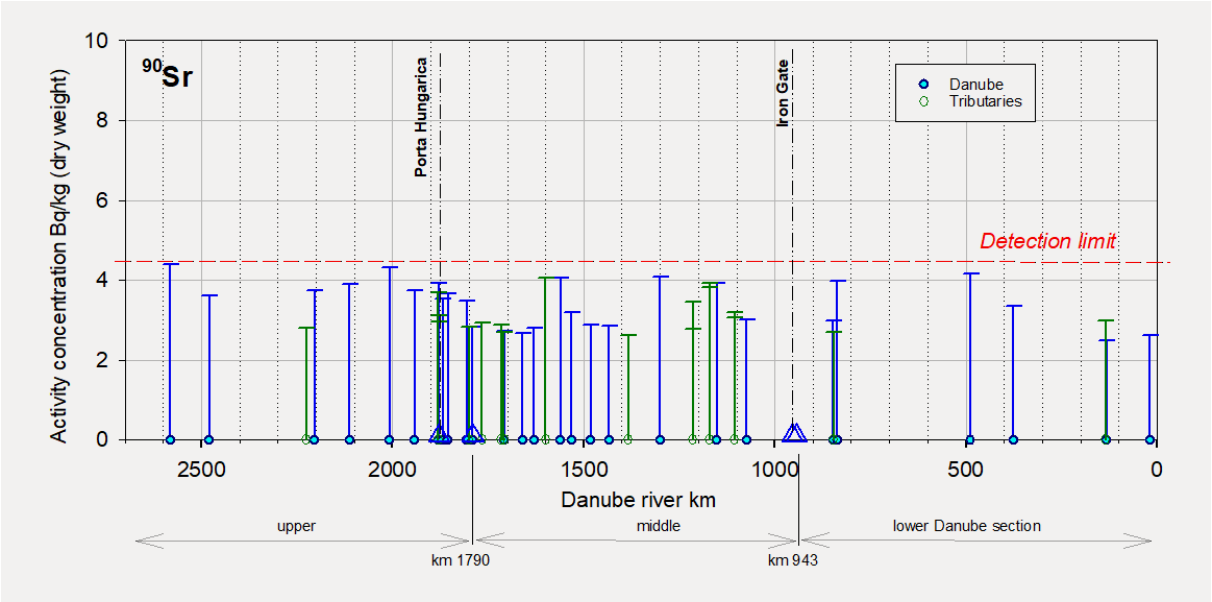


Figure 3: ^{90}Sr activity concentration in JDS 4 sediment samples < 125 µm (individually calculated decision limits).

45.3.2 Natural radionuclides

Potassium 40

The activity concentration of the natural radionuclide ^{40}K in the sediment samples shows an increasing tendency along the course of the river with a mean activity concentration of about 450 Bq/kg (Figure 4). The results are in the typical range of activity concentrations of Danube River sediments for sediment particles with grain size fraction < 125 µm. All ^{40}K activity concentrations of the analysed sediment samples are below 800 Bq/kg.

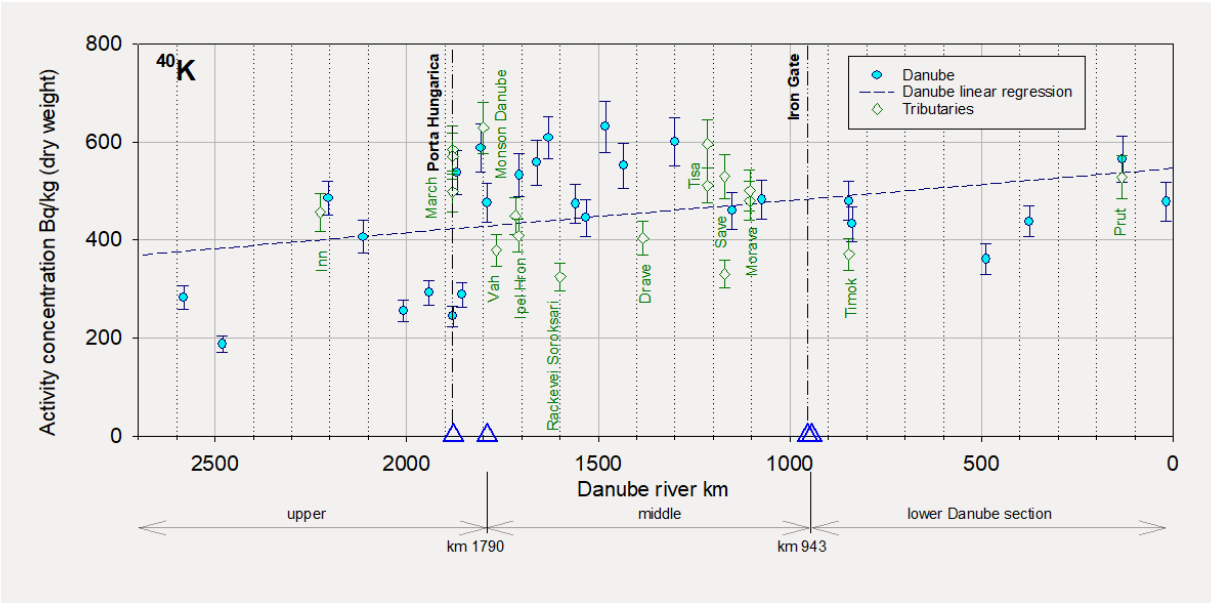


Figure 4: ^{40}K activity concentration in JDS 4 sediment samples < 125 µm.

There is a fixed correlation between the mass concentration of natural potassium c_M and the radioactive isotope ^{40}K: $c_M^{\%} = c_A^{Bq/kg} \times 0.00325^{\%/(Bq/kg)}$. This means, the mass concentrations of all analysed samples are below $c_M = 800 \times 0.00325 = 2.6\%$. In general, potassium in river sediments is an indicator of clay particles and fertilisers.

Radium 226

The most prominent decay product of the natural uranium(^{238}U)-radium decay chain is ^{226}Ra. It is of natural origin and part of soil particles that are washed into the river by soil erosion in the catchment area. The analysis results show typical ^{226}Ra activity concentrations in the JDS4 sediment samples (Figure 5).

The ^{226}Ra activity concentrations are below 60 Bq/kg in all samples except for one sample (Radujevac) and generally they are increasing along the course of the river. Higher activity concentrations were found in the sediments collected in the middle part of the Danube between km 1790 and km 1200. This could be caused by a finer grain size distribution of the bottom sediment samples due to slower flow velocities at these sampling locations. ^{226}Ra and many other radionuclides are enriched in fine grain clay particles (Maringer, 1996).

An uncommonly high ^{226}Ra activity concentration of 231 ± 13 Bq/kg was found in the sediment sample at the location JDS4-41, Radujevac, RS. This high value may be caused locally by industrial processing of materials with elevated levels of natural radioactivity ("NORM industry", e.g. production of mineralogical building materials, phosphate, or gypsum production, etc.). Although this ^{226}Ra value is high, no health risks are associated with it.

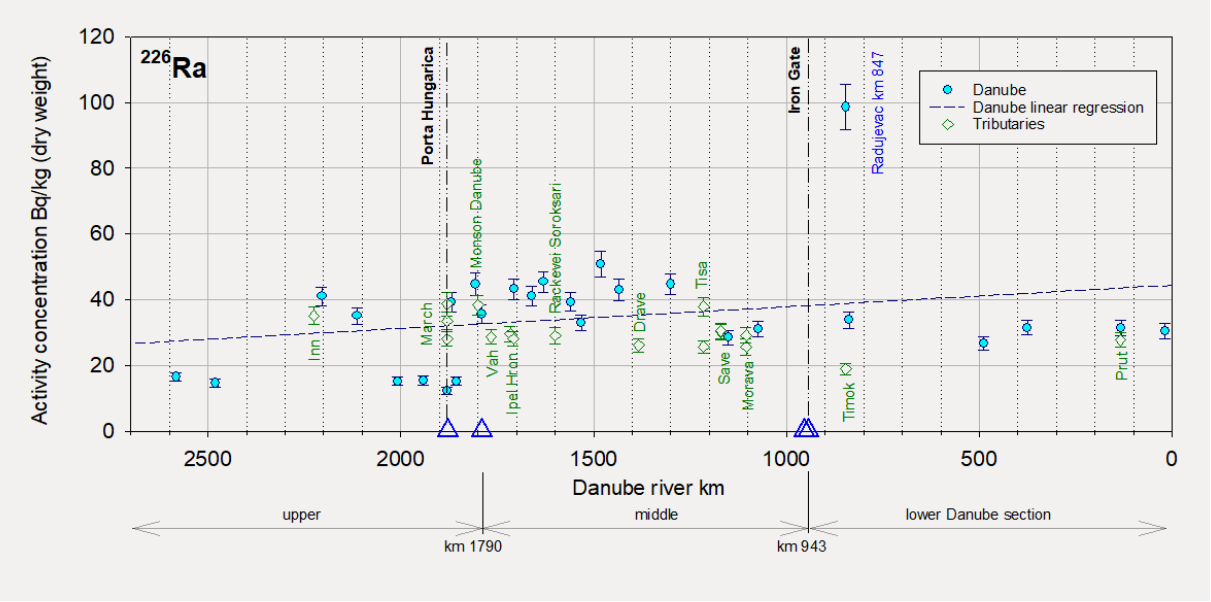


Figure 5: ^{226}Ra (214Pb) activity concentration in JDS 4 sediment samples < 125 µm.

Radium 228

^{228}Ra is part of the natural thorium (^{232}Th) decay chain. The results for this natural radionuclide show commonly found activity concentrations in the analysed sediment samples. All values were below 60 Bq/kg and seem to increase along the course of the Danube (Figure 6).

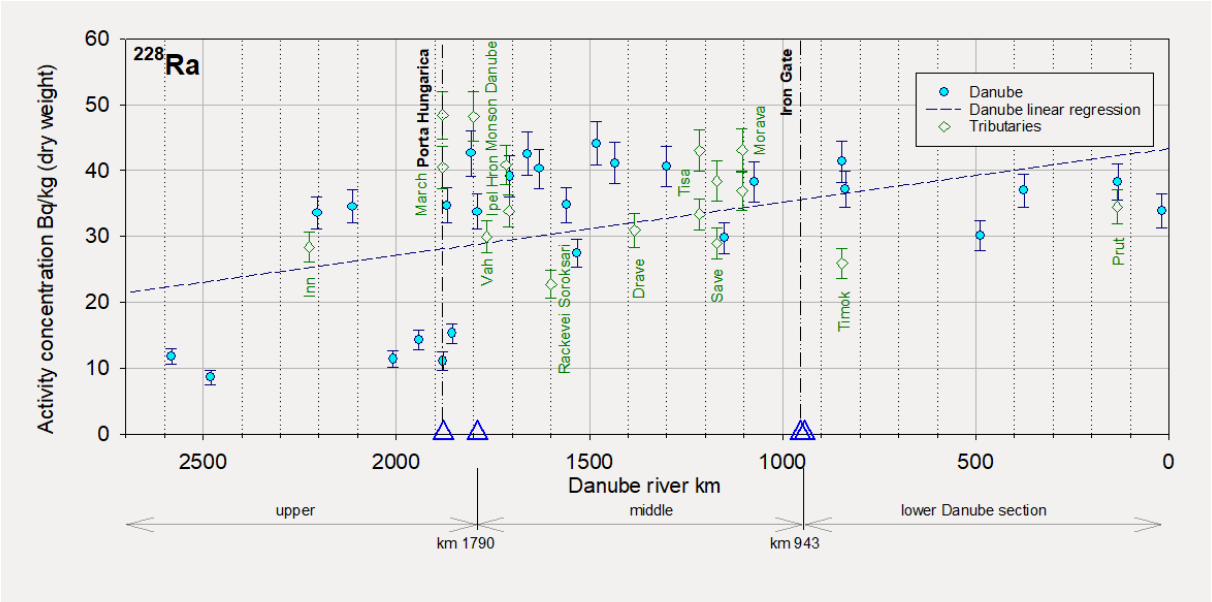


Figure 6: ^{228}Ra (^{228}Ac) activity concentration in JDS 4 sediment samples < 125 µm.

The ratio between the activity concentrations of the two natural radium isotopes $c_A(^{228}Ra)$ / $c_A(^{226}Ra)$ is shown in Figure 7. This relationship is a useful indicator / isotopic "finger-print" of the petrological / mineralogical composition of the (washed-out) soil in the local catchment area. The impact of environmental parameters is equally effective on both radium isotopes. Therefore, only the mineralogical composition of the sediment particles and relation of uranium-radium and thorium decay chain radionuclides in the specific catchment area respectively is relevant to the found ratio.

At location JDS4-41, Radujevac, RS, this isotopic ratio shows a relatively low value of about 0.42 compared to the mean ratio of 0.95. This low ratio means no health effects.

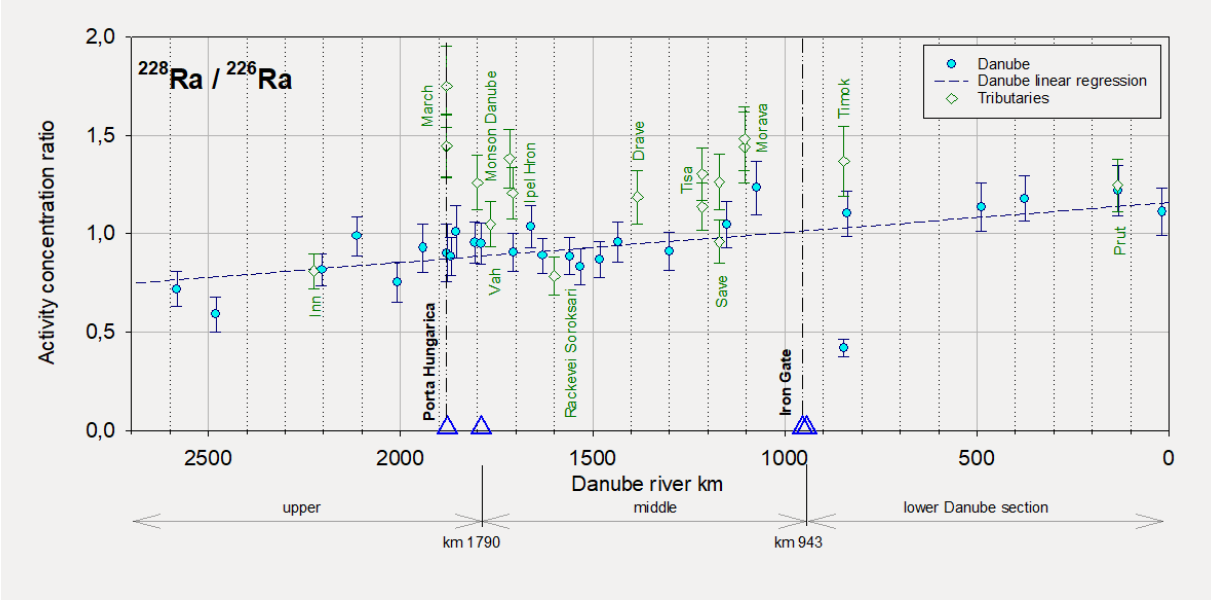


Figure 7: ^{228}Ra (^{228}Ac) / ^{226}Ra (^{214}Pb) activity concentration ratio in JDS 4 sediment samples < 125 µm.

Lead 210

The natural radionuclide ^{210}Pb is the long-lived radioactive progeny of the short-lived ^{222}Rn (half-life about 3.8 days) in the uranium-radium decay chain. ^{210}Pb is generated ("supported") by its preceding radionuclides (^{226}Ra → ^{222}Rn → …) in the sediment particles and additionally brought into the river sediment by wash-out of land soil particles that were atmospherically enriched with ^{210}Pb (Maringer, 1996). Figure 8 shows the total ^{210}Pb activity concentration of the sediment samples whereas in Figure 9 only the unsupported atmospheric "excess" part of the ^{210}Pb activity concentration is shown.

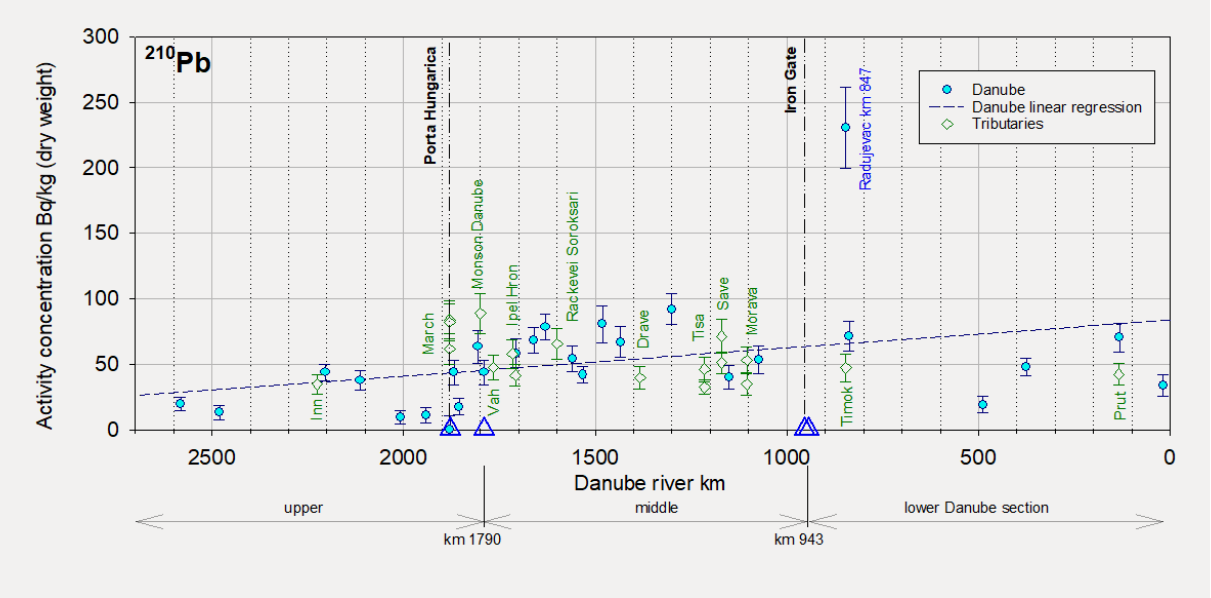


Figure 8: ^{210}Pb activity concentration in JDS 4 sediment samples < 125 µm.

All activity concentrations of ^{210}Pb and ^{210}Pb$_{excess}$ are in the normal range except the value of the sediment sample at location JDS4-41, Radujevac, RS, in line with the uncommonly high ^{226}Ra value found there. Industrial processing of materials with high natural radioactivity (e.g. NORM industry) can also contribute locally to increased excess Pb-210 through atmospheric emissions.

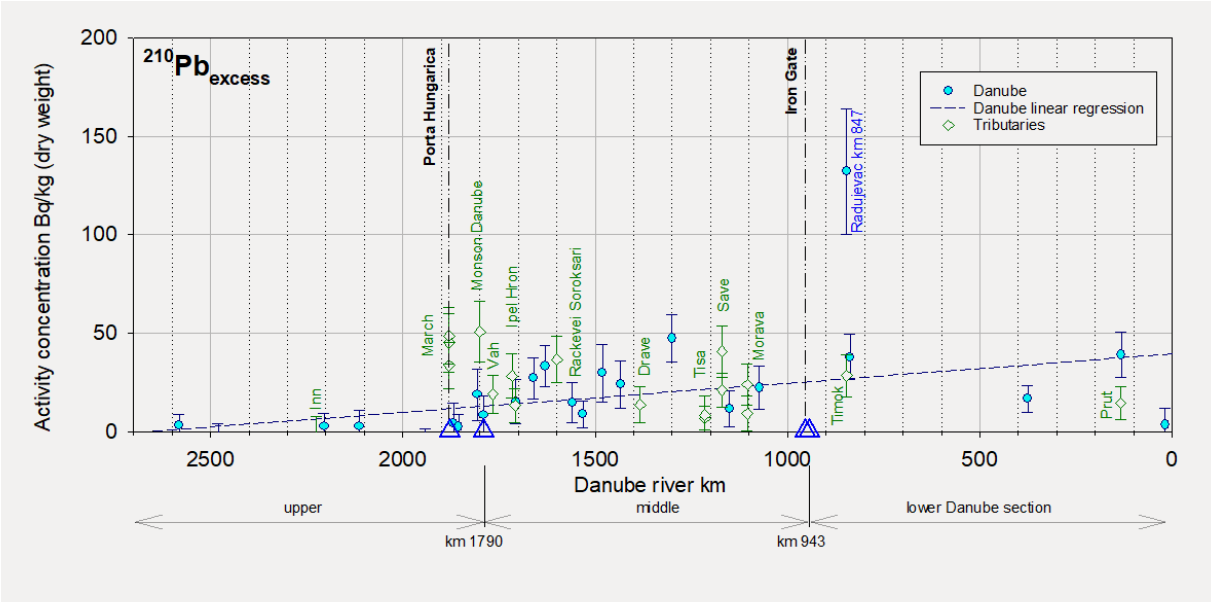


Figure 9: $^{210}Pb_{excess}$ activity concentration (atmospheric origin) in JDS 4 sediment samples < 125 µm.

45.3.3 Chronological sequence of ^{137}Cs

In Figure 10, the distributions of the ^{137}Cs activity concentrations in the sediment samples collected during three different Joint Danube Surveys (JDS2, JDS3 and JDS4) are represented by box plots (10% / 25% / 50%/ 75% / 90% and value dots <10% and >90%). In all three parts of the Danube (upper/middle/lower part), the ^{137}Cs activity concentration in the sediments has decreased since 2007 – with a calculated "radioecological half-life" of about 5 years. This is in accordance with the results of previous radioecological research at the Danube (Maringer, 1994).

The ^{137}Cs values of the JDS4 samples are too low as expected from the JDS2 and JDS3 values because of the different sample preparation applied to the JDS4 sediments. To account for the difference in their grain sizes (<63 µm in JDS2/JDS3 versus <125 µm in JDS4) the ^{137}Cs activity concentrations of the current survey were mathematically adjusted according to Maringer (1994). This calculation is based on a default grain size distribution of the Danube sediments. The adjusted values shown in Figure 11 enable a more realistic comparison of the JDS4 results with those of JDS2 and JDS3.

Like in Figure 10 also in Figure 11 the increase of the ^{137}Cs activity concentrations of the JDS4 samples from the upper part to the middle part of the Danube is visible. This could be an effect of the coarser grain size of the upper part sediment samples and/or (with lower impact / likelihood) a general long-term transport and enrichment of ^{137}Cs with sediment particles down the Danube.

Additionally, continuous monitoring of the Danube in Austria is conducted on behalf of the Federal Ministry for Climate Action, Environment, Energy, Mobility, Innovation and Technology. For this purpose sediment samples from continuous sampling with a filter (MF-Millipore Membrane, mixed cellulose esters, hydrophilic) of 47 mm diameter and 0.45 µm pore size at the power stations Aschach (km 2162,67), Wallsee-Mitterkirchen (km 2094,5), Greifenstein (km 1949,18) and Freudenau (km 1921,05) are analysed.

The JDS4 sediment samples were taken at the beginning of July 2019. During this period (1 - 31 July 2019) the ^{137}Cs activity concentrations found in the sediment samples of the Austrian monitoring programme were between 3.8 ± 0.9 $Bq/kg_{(dry\ weight)}$ and 15.8 ± 2.7 $Bq/kg_{(dry\ weight)}$. In the month before that (June 2019), the ^{137}Cs activity concentration was between 7.4 ± 0.8 $Bq/kg_{(dry\ weight)}$ and 12.0 ± 1.3 $Bq/kg_{(dry\ weight)}$.

Whereas in the following month (August 2019) the values were between 13.7 ± 2.6 Bq/kg$_{(dry\ weight)}$ and 38.6 ± 4.7 Bq/kg$_{(dry\ weight)}$. The mean as well as the median value in the year 2019 was about 19 Bq/kg$_{(dry\ weight)}$. Hence, during the sampling period the ^{137}Cs activity concentration was extremely low. This also seems to be reflected in the JDS4 results for ^{137}Cs in the upper part of the Danube.

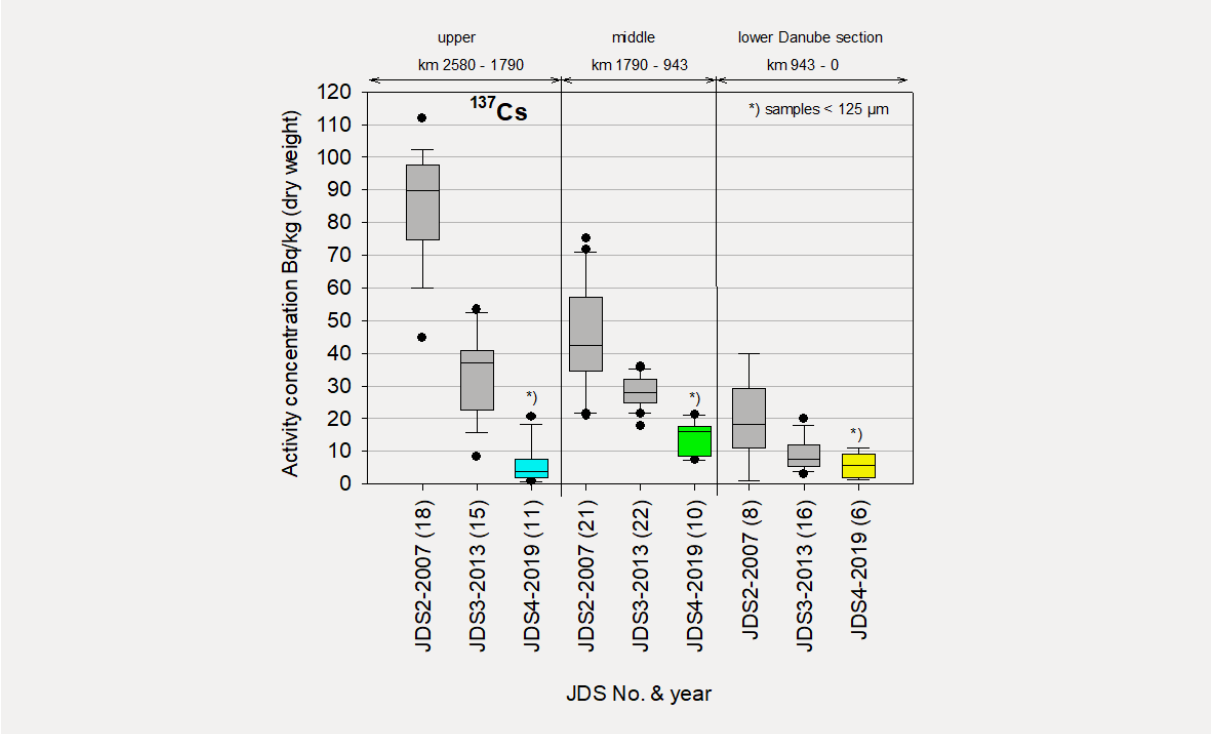


Figure 10: ^{137}Cs activity concentration in Danube sediment samples < 63 µm (grey) and < 125 µm (coloured) box-plot 10% / 25% / 50% / 75% / 90% and value dots <10% and >90%.

45.4 Conclusions

The radioactive contamination of the Danube with the long-lived artificial radionuclides ^{137}Cs and ^{90}Sr after the atmospheric nuclear weapons tests (1963) and the Chernobyl accident (1986) has continuously decreased and is now two orders of magnitude lower than at its maximum in 1986.

The radioecological evaluation of the natural key radionuclides ^{210}Pb, ^{226}Ra, ^{228}Ra, and ^{40}K in the JDS4 sediment samples showed normal activity concentration levels. Only the sediment sample at the location JDS4-41, Radujevac, RS showed uncommonly high ^{226}Ra and ^{210}Pb activity concentrations, which are probably a result of the industrial processing of materials with elevated levels of natural radioactivity.

The results of the radioactivity analysis of the JDS4 sediment samples show undoubtedly that there is no indication of hazardous man-made radioactive contamination of the Danube ecosystem compartments. According to the EU Radiation Protection Directive 2013/59/EURATOM, and with a view towards public radiation protection, it can be stated that the Danube has continued to maintain a healthy radioecological status throughout the year 2019.

45.5 Acknowledgements

The Federal Ministry of Climate Action, Environment, Energy, Mobility, Innovation and Technology subsidised the measurements of the Joint Danube Survey 4 as well as the measurements of the continuous monitoring in Austria. GZ: BMLFUW-U.W.1.1.10/0348-I/7/2017.

The samples for the continuous monitoring in Austria were provided by via donau – Österreichische Wasserstraßen-Gesellschaft mbH.

45.6 References

ISO 11929:2010. Determination of the characteristic limits (decision threshold, detection limit and limits of the confidence interval) for measurements of ionizing radiation – Fundamentals and application.

FRANTZ, A. (1967). The radioactivity of the Danube. Limnology of the Danube, Volume IV, pp. 84–96.

LERMAN, A., TANIGUCHI, H. (1972). Strontium-90 — diffusional transport in sediments of the Great Lakes. Journal of Geophysical Research 77(3), pp. 474-481.

MARINGER F.J. (1994). The behaviour of radionuclides in water, suspended matter, and bottom sediments in the river Danube. (Das Verhalten von Radionukliden im Wasser, Schwebstoff und Sediment der Donau). Dissertation, TU Wien (1994) (in German).

MARINGER F.J. (1996). The Partitioning of Natural Radionuclides in a Large Alpine River. Env. Int. 22, Suppl. 1, 323-331.

MARINGER, F.J., RANK, D., TSCHURLOVITS, M. (1997). Recent radioecological investigations in the Austrian part of the Danube. In: Freshwater and estuarine Radioecology. Ed. G. Desmet. Elsevier Science B.V., pp 249-254.

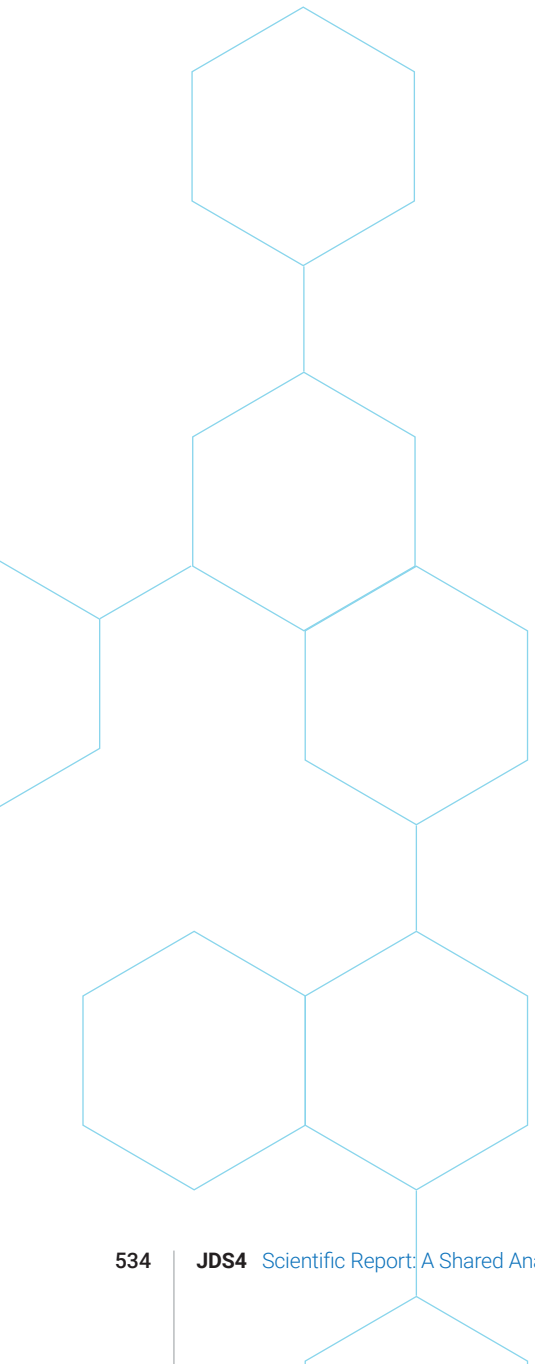
MARINGER, F. J., SEIDL, C., BAUMGARTNER, A., & STIETKA, M. (2015). Radioactivity in the Danube. In I. Liska, The Danube River Basin. Handbook of Environmental Chemistry, 39, pp. 271-284.

MARINGER, F.J., ACKERL, C., BAUMGARTNER, A., BURGER-SCHEIDLIN. C., KOCADAG, M., STERBA, J.H., STIETKA, M., WELCH, J.M. (2017). Joint Danube Survey 3. Summary report on radioactivity. ICPDR - International Commission for the Protection of the Danube River

MUNDSCHENK, H. (1994). Verfahren zur Bestimmung von Strontium-89 und Strontium-90 in Oberflächenwasser im Normalfall, Leitstelle für Oberflächenwasser, Schwebstoff und Sediment in Binnengewässern, ISSN 1865-8725 (in German)

RANK, D. (1976). Measurement of tritium and radio-carbon in the environment. OESRAD-Meeting, Vienna.

TSCHURLOVITS, M., BUCHTELA, K., SAS-HUBICKI, J. & UNFRIED, E. (1980). Determination of Cs-137, Sr-90 and gamma spectroscopy of water samples from the Danube River. IAEA-TECDOC-229, pp. 9–22.



46 Stable isotopes of water and nitrate

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Abstract

In the Danube River Basin agriculture is the main source of nitrate, but its pathways, degradation and dilution processes, as well as the role of other point sources, are not fully understood. However it is important to adopt cost-efficient agri-environmental measures in the Danube River Basin linked to the EU Nitrates Directive.

To assess mixing processes of different water sources and the origin of nitrate in the Danube River Basin water stable isotope and stable isotopes of nitrate were measured during JDS4 and compared to past surveys. The results indicated that snowmelt was more significant in 2019 in comparison to past surveys and that the water fraction of the Inn River is controlling the water chemistry and dilution of pollutants for several hundred kilometers. Nitrate concentrations and its isotopic compositions suggest that nitrate mainly originates from soil nitrate with smaller admixtures of wastewater or manure. The relatively constant nitrate concentrations and similar isotopic results during the JDS2 survey revealed that nitrate originating from diffuse sources is mainly transported via baseflow/groundwater inputs, rather than direct discharges. In-situ processes, like nitrate denitrification, assimilation or nitrification could not be detected from the isotopic compositions of this longitudinal survey.

46.1 Introduction

Stable isotopes have become a standard tool in environmental sciences in order to observe biological, physical, geological and hydrological processes. The stable isotopes of the water molecule (H_2O) ($^{18}O/^{16}O$ and $^{1}H/^{2}H$) are used to, e.g. characterize the origin of water, evaporation, and mixing processes within a watershed. Isotopes of nitrate (NO_3^-) ($^{15}N/^{14}N$ and $^{18}O/^{16}O$) can be used to trace nitrate sources (e.g. mineral fertilizer, manure and wastewater) but also processes such as denitrification, nitrification, and assimilation. In the Danube River Basin agriculture is the main source of nitrate, but its pathways, degradation and dilution processes, as well as the role of other point sources (e.g. NO_3^- discharge from wastewater treatment plants) are not fully understood. The adoption of cost-efficient agri-environmental measures in the Danube River Basin linked to the EU Nitrates Directive is required to meet the objective to reduce nitrogen pollution in surface and groundwaters.

The objective of this study was to examine the water stable isotopes and stable isotopes of nitrate in order to evaluate hydrological processes within the Danube River and its tributaries as well as the origin of nitrate. Water stable isotopes were already analysed during a Danube Survey in 1988 (Rank et al. 1990) and during JSD2 in 2007 (Newman et al. 2008). Also nitrate isotopes were analysed in 2007, which

allows the comparison of data over one and even two decades. As such, large river longitudinal surveys are rare, this study provides a unique insight into the outcomes of long-term-monitoring efforts and the information gained on observing long-term isotopic changes in the nutrient and water cycle of the Danube River Basin.

46.2 Methods

The water samples were taken during the special survey of the JDS4 in July 2019. Water stable isotope samples were filtered through 0.45 µm cellulose syringe filters and stored in 60 mL HDPE bottles without headspace. Nitrate, ammonia, and nitrate isotope samples were collected in 110 mL HDPE bottles, field filtered through 0.45 µm nylon syringe filters, and preserved by adding 1 mL of 2.5 mM sulfanilic acid in 10 % HCl per 100 mL of sample, which would also remove any nitrite if present. The samples were cooled during transport and stored in a fridge at 4 °C until analysis in IAEA laboratories.

Water stable isotopes were analyzed in the IAEA/FAO Soil and Water Management & Crop Nutrition Laboratory, Seibersdorf, Austria. The oxygen and hydrogen isotope compositions were analyzed using Picarro L2130i Wavelength–Scanned Cavity Ring Down Spectroscopy (WS–CRDS) system. The standard deviation was ± 0.06 ‰ for oxygen and ± 0.4 ‰ for hydrogen.

Nitrate and ammonia, as well as stable isotopes of nitrate were analyzed in the IAEA Isotope Hydrology Laboratory, Vienna, Austria. Nitrate concentration analysis followed the ISO/DIS 15923–1 method. Ammonium was measured according the Standard Methods 4500–NH3 G, at 660 nm. Both measurements were performed using a discrete analyser (AQ1, Seal Analytical, Germany). The analytical error for the nitrate and ammonia is 0.5 mg/L and 0.03 mg/L, respectively. The isotopes of nitrates were prepared using the Titanium (III) Chloride method (Altabet et al. 2019) and analyzed on an Isoprime-100™ continuous-flow isotope-ratio mass spectrometer. Precision and accuracy were 0.5 ‰ for $\delta^{15}N–NO_3$ and $\delta^{18}O–NO_3$.

46.3 Results and Discussion

46.3.1 Water stable isotopes

Based on its water stable isotope compositions for the summer of 2019 (see Fig 1) the Danube River can be divided (isotopically) into three sections: (1) *the upper part* of the Danube River, which is drained by lowland and small alpine rivers, which have relatively positive $\delta^{18}O–H_2O$ values; (2) *the middle part,* which starts with a rapid decrease in δ values after the confluence with the Inn River, which is depleted in ^{18}O, and changing towards more positive δ values after progressive longitudinal mixing with lowland rivers (3) and the *lower part,* which starts after the confluence of two relatively large and ^{18}O enriched rivers (Tisa and Sava River) until the river mouth at the Black Sea. Only a few peaks of $\delta^{18}O–H_2O$ toward more positive values revealed areas of incomplete mixing of tributaries within the mainstem. In summary, the results of the stable isotope research showed that the 2019 JDS4 survey occurred during a period of intense snowmelt contribution and that the water fraction of the Inn River is controlling the water chemistry and dilution of pollutants for several hundred kilometers. The dilution factor and water origin may therefore be important when interpreting other parameters obtained during this JDS4 survey.

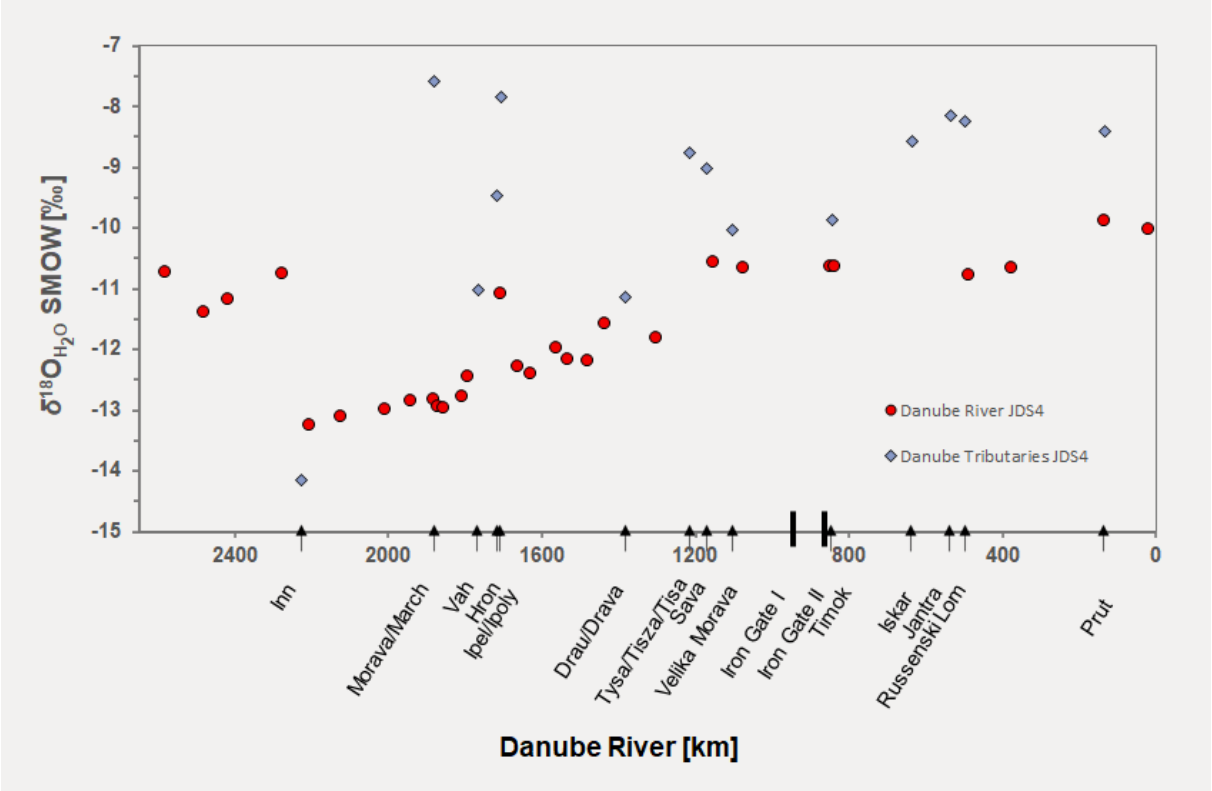

Figure 1: Water stable isotope compositions JDS4.

In comparison to the 1988 survey and JDS2 survey (2007) (See Fig. 2) the water samples taken during JDS4 (2019) were comparatively depleted in ^{18}O, which shows that the snowmelt was more significant in 2019 than in past surveys. This is because JDS4 was implemented in late June/early July, when snow and ice meltwater was coming from higher altitudes, whereas the 1988 survey was conducted in March and the 2007 survey over a 1 1/2-month low discharge period from mid-August to September. Moreover, snow cover in winter 2018/2019 was relatively high in the Inn River catchment.

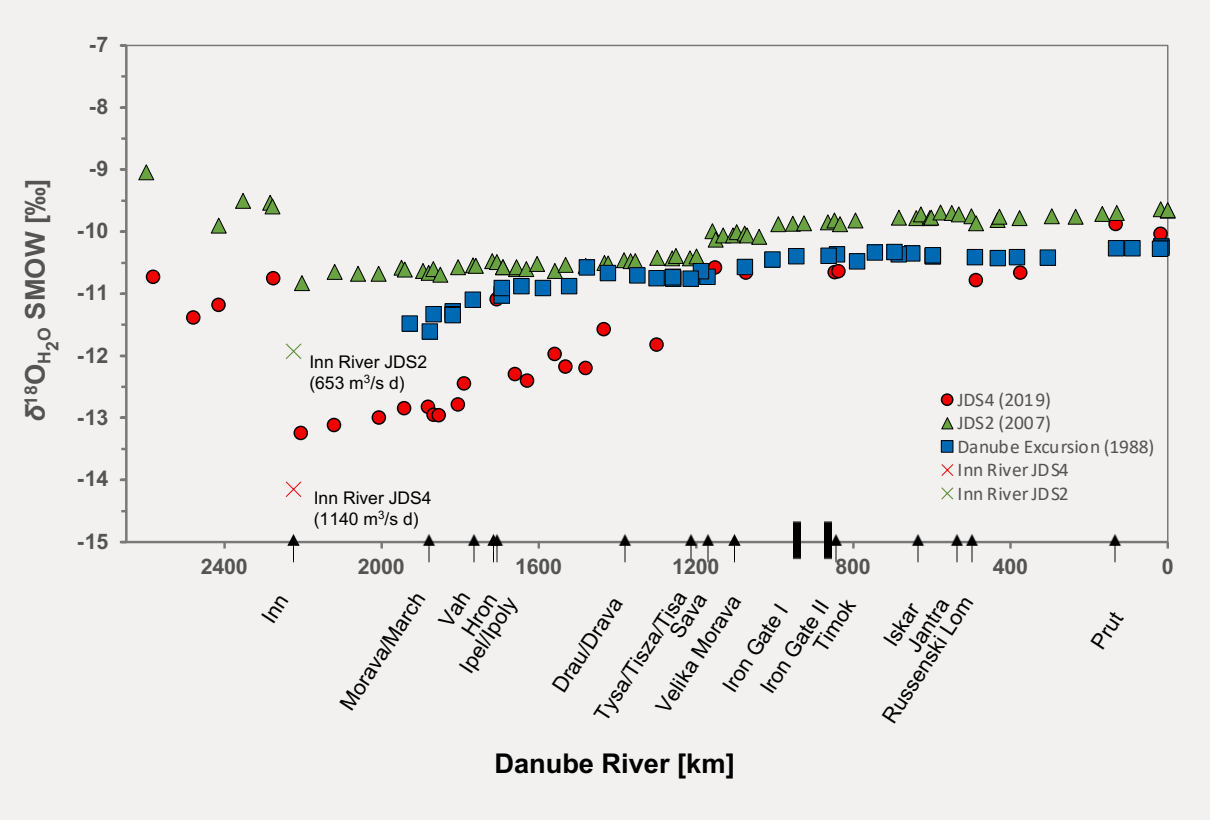

Figure 2: Water stable isotope compositions 2019/2007/1988.

Data from JDS2 are taken from Newmann et al. 2008 and data from the Danube Excursion 1988 from Rank et al. 1990.

46.3.2 Nitrate concentrations and nitrate isotopes

The highest nitrate concentrations were found in the upper part of the Danube and were clearly diluted by alpine rivers like the Lech and Inn River, which both have a low NO_3 load. After the confluence with the Inn River, NO_3 concentrations were relatively constant, although inflowing tributaries had distinctly lower or higher nitrate concentrations. Moreover, no clear NO_3 peaks (or hotspots) from point sources as e.g. wastewater outlets were detected along the longitudinal NO_3 profile. When comparing the 2001, 2007, 2013, and 2019 surveys, it can be seen that the NO_3 concentrations and patterns have stayed within a similar range and pattern over the past decades. The 2019 survey revealed that, on average, the lowest NO_3 concentrations were related to relatively high discharge in 2019 and high dilution with snowmelt, but also to the fact that past surveys were conducted from mid-August to end of September, when NO_3 values tend to be higher under baseflow conditions (ICPDR 2020). Nitrite and ammonia were only detected in tributaries in the lower section of the Danube starting from the Sava River confluence and in most Danube water samples over last 1,000 stream km.

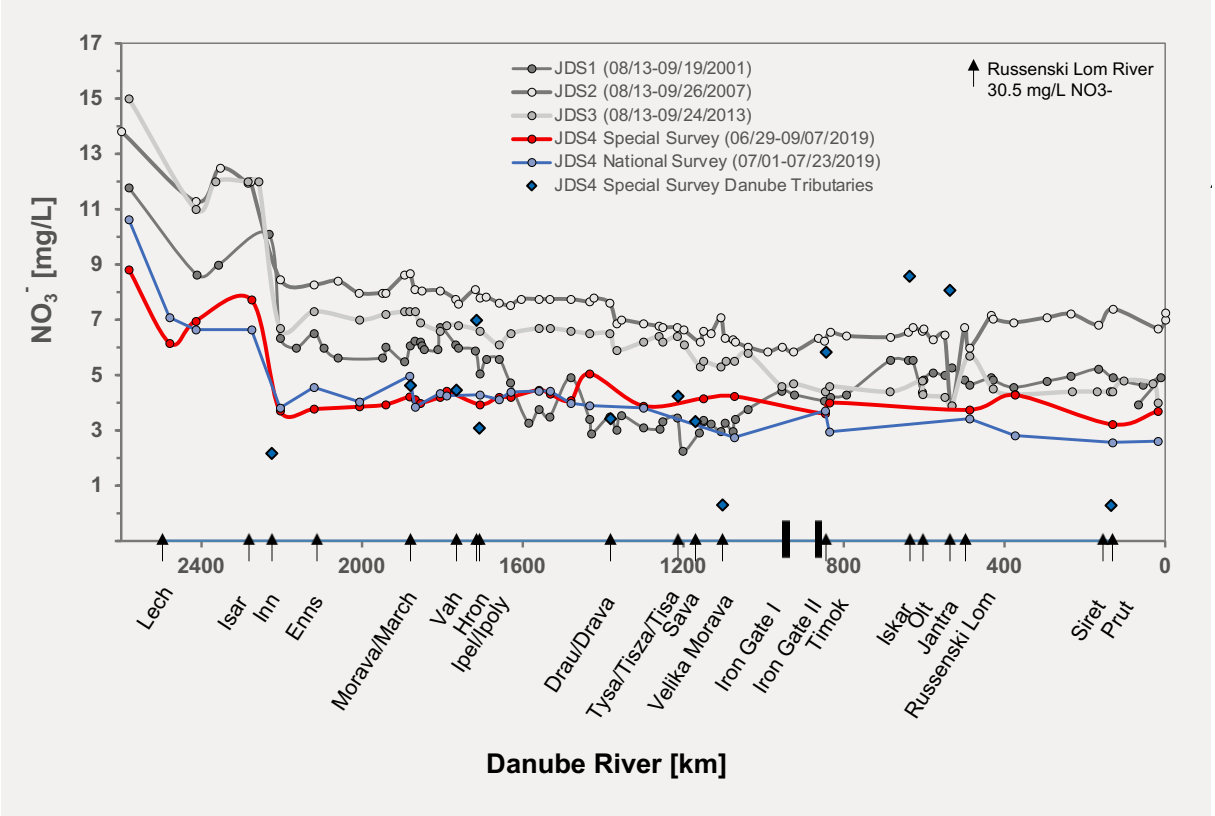

Figure 3: Longitudinal nitrate concentrations.

The isotopic compositions of NO_3–N remained relatively constant over the longitudinal transect, especially over the last 1300 river km (see Fig.4). There were, however two peaks with increasing $\delta^{15}N$–NO_3 values: one after the confluence of the Ipel River and one at 1434 km. In comparison to results from JDS2 (2007) these values were comparable and showed a similar spatial trend. During the 2019 survey, which contained more data points, a decrease in $\delta^{15}N$–NO_3 values was observed after the confluence of the Inn River until mixing with the Morava River. This variation may be related due to the tributary mixing since both tributaries are adding NO_3 with distinctive $\delta^{15}N$–NO_3 values (The Inn River relatively low and the Morava River relatively high values).

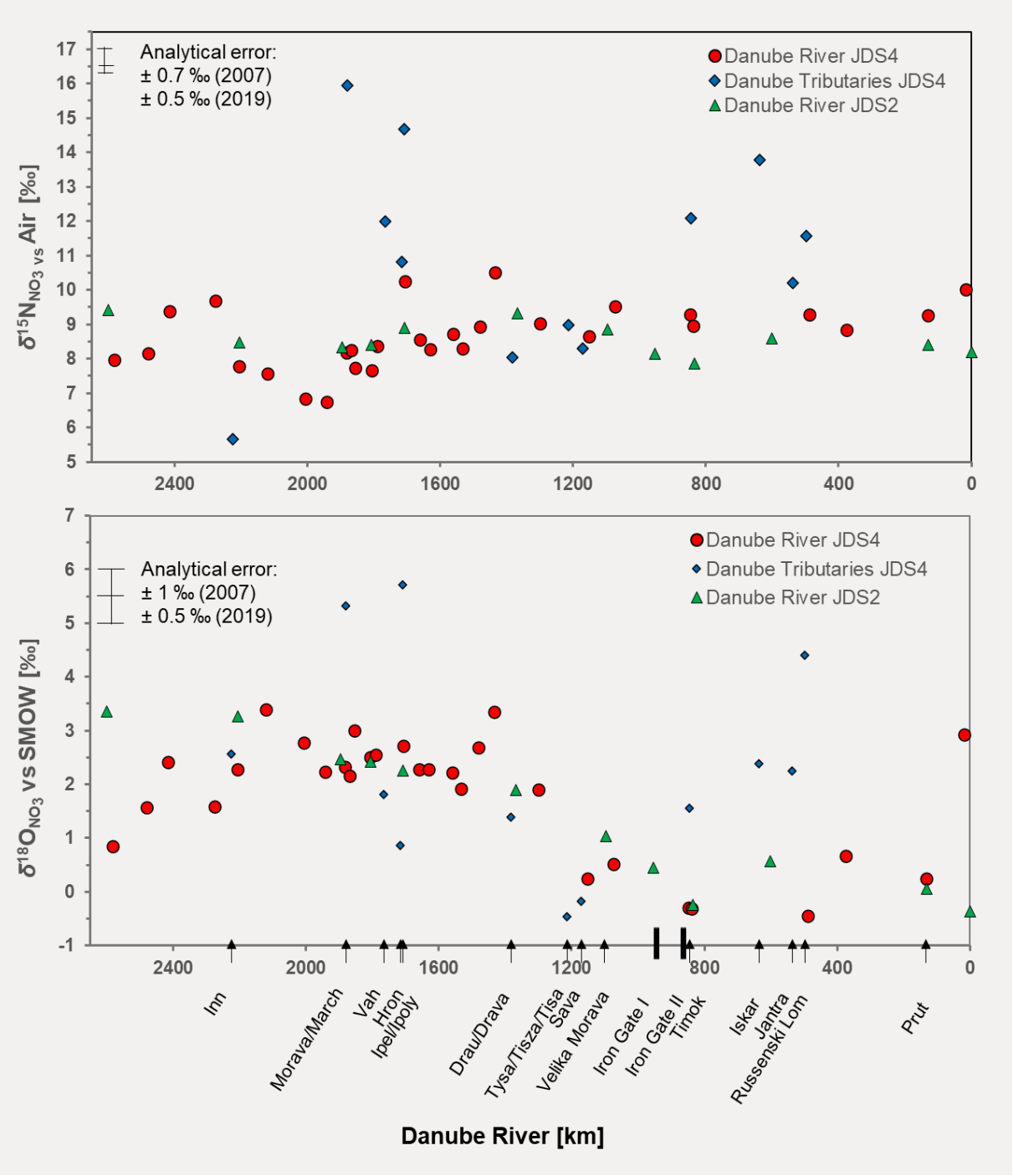

Figure 4: Stable isotopes of nitrate JDS4 and JDS2.
Data from JDS2 are taken from Newmann et al. 2014.

On the longitudinal transect, $\delta^{18}O–NO_3$ values (See Fig. 4) were lower in the Upper Danube headwaters and remained relatively constant until the confluence of the Tisa and Sava River. After the confluence of these rivers and the Iron Gate, $\delta^{18}O–NO_3$ values reach a minimum for about 400 km, despite smaller tributaries adding NO_3 with higher $\delta^{15}N$ values along this transect. A similar longitudinal $\delta^{18}O–NO_3$ profile was observed during the 2007 JDS2 survey.

The fact that nitrate concentrations and its isotopic compositions were relatively constant in the Danube, leads to following conceptualization: nitrate mainly stems from diffuse sources like soil nitrate, agriculture, or septic tanks and is transported via baseflow/groundwater inputs as proposed by daNUbs (2005). The similar isotopic compositions indicated that the nitrate sources, processes and mixing patterns have not changed significantly in a decade (2007-2019). This would not be the case if large amounts of background nitrate were derived from point sources, like surface run-off due to over-fertilization or from wastewater. It suggests rather that groundwater residence time and contribution to baseflow as well as riparian zones buffer average nitrate concentrations infiltrating into the Danube, as suggested also by Malago et al (2017).

Moreover, based on the $\delta^{15}N–NO_3$ values, it is clear that nitrate exported or discharged into the Danube River is mainly from soils with an additional admixture of either wastewater or manure. The contribution of wastewater/manure admixture was slightly higher at 4 sampling points ($\delta^{15}N–NO_3$ > 9.5 ‰): JDS4-4 (Mühlau GER); JDS4-22 (Szob HU/SK); JDS4-29 (Hercegszántó HU); JDS4-51 (Vilkove RO/UA). Tributaries had much higher $\delta^{15}N–NO_3$ values, indicating a relatively higher contribution from wastewater/manure. In total there were 6 tributaries (Morava, Hron, Ipel, Drava, Jantra, and Rusenski Lom river) with $\delta^{15}N–NO_3$ values higher than 10 ‰, along with detected ammonia (except Hron River), indicating an important fraction of wastewater derived nitrate. The Vah, Timok, Iskar River, on the other hand, had $\delta^{15}N–NO_3$ values >10 ‰ but no detection of ammonium. Here the fraction of nitrate could also originate from manure. The Inn River was the only sampling point with a low $\delta^{15}N–NO_3$ value (5.7 ‰) in a range of unpolluted soil-derived nitrate.

The range of $\delta^{18}O–NO_3$ and $\delta^{15}N–NO_3$ values in the Danube indicated that the contribution of mineralized fertilizers or atmospheric deposition was absent or minor. This supports the outcome of a nutrient emission study by Malago et al. (2017) estimating that nitrate input to the Danube by agriculture is substantially reduced by crop uptake, soil denitrification and riparian filter strips. Moreover, this 2019 survey supports the interpretation of the of the JDS2 survey results (Newmann et al. 2014) that nitrate contributions to the river from mineral nitrate fertilizers or from atmospheric N sources are insignificant.

In the Danube River water column there was no evidence for in-situ denitrification as river water exhibited well-oxygenated conditions likely to preserve the nitrate oxyanion (dissolved oxygen levels well above 2 mg/L). Moreover, $\delta^{18}O–NO_3$ and $\delta^{15}N–NO_3$ values would both simultaneously increase if there would be important denitrification processes, which was not the case. There was no decreasing profile in NO_3 concentrations or increasing $\delta^{15}N$-values along the Iron Gates reservoir stretch, indicating denitrification processes in this reservoir. Denitrification within the Danube River sediments or the riparian zone cannot be excluded, but the isotopic signal linked to this process cannot be reliably detected. It is also likely that assimilation and nitrification occur in the water column of the Danube River, however no parallel enrichment in $\delta^{15}N–NO_3$ and $\delta^{18}O–H_2O$ values of residual NO_3 was observed, nor a trend towards a more enriched ^{15}N residual pool.

46.4 Conclusions

In summary, the isotope study revealed that the groundwater/baseflow is an important nitrate source to the Danube River. Groundwater observation networks in the Danube River Basin should therefore ideally be coupled with the river monitoring station network and nitrate concentrations evaluated and compared. The results suggest furthermore, that inputs from smaller diffuse sources, as e.g. septic tanks, should also be considered as contributing to the nitrate load in the Danube River Basin. Wastewater seems to be the most important point source, adding directly nitrate to the Danube River, which highlights the importance of enhancing nitrogen removal efficiency of domestic wastewater in the catchment. The study proposes moreover that riparian protection and buffer zones are an important measure, avoiding significant nitrate spillage from mineral fertilizers and allowing denitrification processes during the transport. As groundwater residence time may be long within the watershed, the observed time frame (2007 and 2019) may also be too short to observe implemented remediation or beneficial land management practices with the objective to decrease nitrate sources in the catchment.

In addition, snowmelt water is an important dilution factor of nitrate concentrations and decreasing snow cover in winter could increase nitrate concentrations in summer the future.

46.5 References

ALTABET, M.A., WASSENAAR, L.I., DOUENCE, C. & Roy. R. (2019). A Ti(III) reduction method for one-step conversion of seawater and freshwater nitrate into N2O for stable isotopic analysis of 15N/14N, 18O/16O and 17O/16O. Rapid Commun Mass Spectrom 33(15):1227-1239.

ICPDR 2020. TNMN – TransNational Monitoring Network https://www.icpdr.org/main/activities-projects/tnmn-transnational-monitoring-network

DaNUbs 2005. Nutrient Management in the Danube Basin and its impact on the Black Sea. Final Report. http://danubs.tuwien.ac.at

MALAGO, A. BOURAOUI, F., VIGIAK, O., GRIZETTI, B & PASTORI, M. (2017). Modelling water and nutrient fluxes in the Danube River Basin with SWAT. Science of The Total Environment 603–604: 196-218.

NEWMAN, B., PAPESCH, W., RANK, D., VITVAR, T., HUDCOVA, H., AGGARWAL, P. & GROENING M (2008). Isotope survey of the Danube. In: International commission for the protection of the Danube River, Joint Danube Survey 2, Final Scientific Report. ICPDR, pp 209–214.

NEWMAN, B., AGGARWAL, P., ARAGUAS-ARAGUAS, L.J., HUDCOVA, H., PAPESCH, W., RANK, D. & VITVAR, T. (2014). Hydrological and Biogeochemical Characterization of the Danube River System Using Isotopes. In: LISKA, I. (ed.), The Danube River Basin, Hdb Env Chem, Springer-Verlag Berlin Heidelberg.

RANK D., PAPESCH, W. & RAJNER, V. (1990). Tritium(3H) und Sauerstoff (18O) Gehalt des Donauwassers zur Zeit der Internationalen Donaubereisung 1988. In: Ergebnisse der Internationalen Donauexpedition (1988) (ed) Internationale Arbeitsgemeinschaft Donauforschung, Wien, pp 307-312.

47 Conclusions

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The first Joint Danube Survey (JDS) in 2001 was an ICPDR initiative tasked with complementing the water quality data received from its regular monitoring program: The Trans-National Monitoring Network (TNMN). It was also designed to help the Danube countries to meet the requirements of the EU Water Framework Directive (WFD). The original primary objective of JDS to produce comparable and reliable information on a wide range of water quality elements for the whole of the length of the Danube River including the major tributaries on a short-term basis has been supplemented over time by other key objectives: to provide an opportunity for harmonization and training in WFD-related monitoring and to fill information gaps ahead of the Danube River Basin Management Plan Update.

Additional ambitions of JDS4 stem from the parallel use of classical monitoring methods in biology and chemistry, with novel approaches such as (e)DNA and target & non-target screening. This parallel application of standard and new monitoring techniques at the large scale of the Danube River offered the opportunity of assessing the potential of these new approaches.

JDS4 was a milestone in the series of Joint Danube Surveys. The three past Surveys were based on a principle that a Core Team of leading experts did all the sampling and, in case of biology, microbiology and hydromorphology, analysis of samples too, while the national experts joined the Core Team only when in their respective country and mostly observed how the work was done (sometimes they provided assistance to the Core Team). JDS4 was organized the other way round: a significant part of the job during the survey (biology, hydromorphology, physico-chemical analyses) was accomplished by the national experts while the Core Team had a coordinating and advisory role to ensure coherence between the approaches used by the national experts. This approach, along with training workshops for each biological quality element organized prior to the survey, provided an excellent opportunity for harmonization and training in WFD-related monitoring giving this way the above-mentioned long-term key objective of JDS a prominent place. The sampling for analysis and screening of chemical pollutants and of environmental DNA was performed by special monitoring teams.

JDS4 was organized on the Danube River including its major tributaries, with a sampling program focused on 51 sites nominated by the ICPDR experts. The sites comprised TNMN sites, JDS3 sites and sites for national surveillance monitoring in 2019. Seven additional groundwater sites and 11 urban wastewater treatment plants (WWTPs) were nominated by the ICPDR to widen the scope of the survey. The ambitious program of JDS4 necessitated the inclusion of additional specific sampling sites for passive sampling, eDNA analysis of fish and microbiological as well as microplastics monitoring. Following the survey's

completion in autumn 2019, the collected samples were analysed in laboratories and scientific institutes across Europe, which produced the data that were used as the basis for preparing this report.

Due to the active engagement of national teams and extremely wide scope JDS4 mobilised the largest amount of actively cooperating experts in the history of the ICPDR. The program of the survey brought together the majority of the ICPDR expert bodies: the Monitoring and Assessment Expert Group as the principal survey organiser, HYMO Task Group focussing on hydromorphological assessment, Groundwater Task Group organising the groundwater monitoring, Information Management and GIS Expert Group dealing with the data management, Public Participation Expert Group taking care of public outreach and communication, the Pressures and Measures Expert Group dealing with the wastewater assessment and the River Basin Management Expert Group utilising the JDS4 results for preparation of the Danube River Basin Management Plan Update 2021.

This report preparation was affected by the pandemic of coronavirus disease in Europe in 2020. The COVID-19 lockdowns adversely impacted the time plan of the analysis of JDS4 samples and consequently some of the results will have to be published later than planned.

Similarly to JDS3, the findings of JDS4 are supportive to the implementation of EU WFD providing an extensive homogeneous dataset acquired by the WFD compliant methods. Even though these data have no ambition to replace the national data used for the assessment of the ecological and chemical status they are an excellent reference database which can be used for WFD assessment methods harmonization throughout the Danube River Basin and for the new derivation and prioritization of the Danube River Basin Specific Pollutants.

Hydromorphology

Based on the results of JDS3 for the continuous overall and WFD 3-digit hydromorphological assessments of 10-rkm sections of the Danube, JDS4 delivered hydromorphological data for changes (improvements/deteriorations) to channel, banks and floodplains. In line with the new approach of JDS4, the data were collected for the first time by the national experts who uploaded harmonized data via the JDS4 web-based portal. The cooperation aspect was strengthened by an intensive preparation phase organised by the ICPDR HYMO Task Group contributing a great harmonisation and training value. The centralised evaluation of changes and finally the reassessment of segments resulted in 73 observed changes (54 improvements and 19 deteriorations) within 55 monitoring segments. The reassessment of JDS3 showed several improvements in the still strongly altered Upper and Middle Danube and slight deteriorations in the Lower Danube. In most cases these changes led only to the reassessment of individual parameters, but not to the shift of overall assessment classes for entire segments. This corresponds to the past assessments which detected numerous alterations along the Upper and Middle Danube, in contrast to the Lower Danube with much less alterations. A general clear trend for the entire Danube cannot be observed for the given period, however the intensified restoration activity on the Upper and Middle Danube and the slight deterioration of the Lower Danube suggest a positive outlook. Regarding the WFD 3-digit assessment, four segments have profited from fish passes in Austria, reconnecting seven segments in total (70 km) for fish migration.

Biology

JDS4 biological monitoring provided a homogeneous internationally coordinated scientific snapshot of the whole Danube at a given time. To strengthen the links to the WFD, an indication of the ecological status was presented for the sites using a harmonised approach regardless of whether or not these sites were located in natural or heavily modified water bodies. The WFD assessment of the ecological status for each water body being a legally mandatory task for the EU Member States is based on a complex methodology requiring monitoring activities over a certain timespan and thus from legal and logistical reasons it could not be carried out during JDS4.

Biological quality elements indicating pressure from nutrients and oxygen depletion by biodegradable substances – phytoplankton, macrophytes, phytobenthos, partly macrozoobenthos – indicated a good status at many sites and pointed at local pressure only. Fish and macrozoobenthos however indicated impacts induced by hydromorphological pressures at most of the sites. In general, an improvement of the indicative ecological status since the previous surveys is not visible along the whole length of the Danube except for some sites. Trends of deteriorating status may also be linked to the use of more effective methodologies and increasing pressure from invasive alien species.

Fish

Fish sampling has been conducted using a standardised procedure at 43 sampling sites (51 sampling sets due to parallel activities by the national teams at bilateral sites). The results showed that still most species of the reference communities could be found at nearly all sites. This is even true for strongly altered hydromorphological stretches in the Upper Danube section. The species compositions at the different sampling sites reflect the wide range of aquatic habitats in this large stream and the combination of rhitral and potamal elements. In total 76,265 specimens of 72 fish and three jawless species were detected during JDS4. This underlines the importance of the Danube as a substantial source of fish biodiversity in Europe. Similar to the previous Joint Danube Surveys, an extraordinary dominance of bleak (*Alburnus alburnus*), a typical swarm fish which prefers the upper water column close to the surface, and of round gobies (*Neogobius melanostomus*) which hide in cavities of the littoral rip-rap structures, was detected. This must be seen in context with the species selectivity of electric fishing, as both species can be collected quite easily with electric fishing in relatively high abundances.

The indication of the ecological status assessment showed that the fish community is threatened along the whole river course. Several indices were used by the experts and all of them show the deficits of the fish community caused by hydromorphological pressures (indication of good status according to FIS: 11% of sites, EFI: 23%, FIA: 25%). Those indices were not developed and are not suitable for the whole length of the Danube, however, the national assessments also show that only 17% of the sites indicated good status. Two thirds of the sites classified as not good show the same indication of status by using the MMI for benthic invertebrates. However, taking into account that the diversity of fish taxa is still high, it is believed that effective restoration measures can help to improve the ecological status in order to meet the WFD goals.

Macroinvertebrates

The tiny stream bed inhabiting animals of the biological quality element macrozoobenthos are indicators for oxygen depletion due to pollution by degradable organic substances (Index: SI, saprobic index) as well as for general habitat degradation (index: MMI, multi-metric index). The results of saprobic index analysis show that organic pollution is a local problem, because 81% of sites show an indication of good or high status. As also known from past surveys and TNMN data the indication of good and high status decreases downstream – 91% of sites in the Upper Danube, 80% in the Middle Danube and 67% in the Lower Danube. The multi-metric index shows a quite different picture: only 37% of sites reach an indication of good status, the situation is better in the Upper Danube (45%) and in the Lower Danube (50%) compared to the Middle Danube (20%). The MMI is an indicator for habitat degradation and these results show hydromorphological deficits of the ecosystem in habitat quality caused by a variety of pressures.

In the majority of cases, only one side of the river was selected for sampling, though at transboundary sites, both sides were usually sampled. In total, 484 taxa were found belonging to 19 higher taxonomical groups, 394 taxa were found in the Danube River and 287 taxa in tributaries.

Phytobenthos

Benthic algae (phytobenthos) are found in nearly all running waters and their assemblages are usually attached to substrate. Their growth and prosperity responds directly and sensitively to physical, chemical and biological variables of the river water.

During JDS4, diatom communities differed between the Danube types and Danube reaches from the Upper to the Lower Danube. Diatom species structure reflected the diversity of environmental conditions, ranged from oligotraphentic to hypereutraphentic and from oligosaprobic to polysaprobic. The environmental variables, which most significantly influenced diatom species composition and diatom metrics (diatom indices, diatom life-forms and partly diatom ecological guilds) were general descriptors (e.g. geographical coordinates), and physico-chemical variables (concentrations of nutrients and of organic pollution). Indicative status of the Danube samples was generally getting worse from the Upper Danube towards the mouth. In general, indicative status of tributaries was found to be better in comparison to the Danube itself.

Phytoplankton

Phytoplankton was analysed at 26 sampling sites in the Danube River and 10 sampling sites in the tributaries. The flexible setup of JDS4 enabled phytoplankton monitoring over the whole year, so the samples were collected monthly from April to September in 2019. A total of 682 taxa were identified. Diatoms were the dominant taxonomic group, mostly represented by planktic taxa like *Stephanodiscus hantzschii, Cyclostephanos dubius, Cyclotella meneghiniana, Skeletonema potamos*, or benthic ones like *Diatoma vulgaris*. The application of functional groups revealed more detailed composition and dynamics. The functional group approach was proven to be an excellent tool for interpretation of the phytoplankton composition, and in the case of the Danube River, it precisely reflects existing hydrological and trophic conditions. The concentration of chlorophyll *a* and total biomass of phytoplankton showed temporal and longitudinal dynamics. The highest chlorophyll *a* (55.7 μgL^{-1}) and biomass (21.4 mgL^{-1}) values were measured in the Middle Reach of the Danube River. Among the tributaries the Morava, Ipeľ and Rackevei-Soroksari Danube Arm had the highest values. The peak of chlorophyll *a* was characteristic in late spring for the Upper Danube, and in mid-summer in the rest of the Danube and its tributaries. Phytoplankton-based ecological status assessment ranged from low to high status, but it was deemed good on most of the sampling sites.

Macrophytes

Macrophytes, or aquatic plants visible to the naked eye, are an important part of the aquatic ecosystems. JDS4's national experts sampled and analysed macrophytes at 38 sampling sites during July 2019. The area of the Danube River was covered by 27 sampling sites, with 11 additional sampling sites assigned along select sections of tributaries. A total of 132 taxa of bryophytes, pteridophytes and angiosperms were identified. Bryophytes were a dominant plant group in the Upper Reach of the Danube River, while angiosperms prevailed in the Middle and Lower Reach, as well as in the tributaries. Hydrophytes were a dominant life form in the Danube River and the tributaries, but helophytes also showed their dominance on a few occasions. Results showed that the most relevant variables influencing water plants in the Danube are water temperature, dissolved oxygen, nitrates, and conductivity. Mosses preferred colder and more oxygenated water, rich in nitrates in the Upper Reach that has shaded banks with hard substrate. Floating or rooted angiosperms and pteridophytes preferred warmer, nutrient, and organically rich water. Beside these relationships, the water plants are well known indicators for hydromorphological alterations. The abundance of floating macrophytes in the Middle and Lower Reach of the Danube River suggests good lateral connectivity to backwaters. Just as was found in three Joint Danube Surveys before, the results demonstrate that there is a natural lack of microhabitats with proper conditions for the successful growth of macrophytes in certain river stretches. This causes almost plant-free river sections with no macrophytes or with insignificant abundance – making the assessment difficult to impossible. Based on the comparison of outcomes of previous Joint Danube Surveys, the composition of macrophytes was found to be stable in terms of richness and diversity across several years.

Invasive alien species

JDS4 results reconfirmed that the Danube River and its main tributaries are under considerable influence from biological invasions. The number of alien species recorded and the values of the pressure indices revealed a better situation in the Lower Danube when compared to Upper and Middle reaches, mainly because the Lower Danube can be considered as a native area of distribution for Ponto-Caspian taxa, which are considered alien to the Middle and Upper Danube.

Compared to results from previous JDSs, an increase in the number of identified alien species has been recorded but the data analysis shows that the pressure caused by biological invasions is relatively stable. The reason is that not all alien species are also invasive therefore the assessment of bioinvasion pressure has to take this into account.

The (e)DNA-based detection of aquatic IAS was effective and it revealed the presence of a non-indigenous snail species that was not detected earlier for the Danube – *Bulinus umbilicatus*. Moreover, this method discovered the presence of four additional non-native aquatic macroinvertebrate species that were not detected by other methods during JDS4.

For the first time, a smartphone application for invasive species detection was used in JDS4. The application was found to be a helpful tool that greatly facilitates access to and update of records on invasive species. It has a very broad usage, not only for public users, but also for researchers. Its broader usage may contribute to IAS awareness-raising in the Danube countries and involve actively the citizens in future surveys.

Zooplankton

The analysis of the zooplankton (rotifera, cladocera, copepoda) samples from the Danube River and the selected tributaries revealed differences in the composition and density of zooplankton assemblages from the Upper to Lower Danube. 157 taxa (118 rotifera, 21 cladocera, 18 copepoda) were identified in 39 JDS4 sites (27 from the Danube and 12 from tributaries). In opposition to previous JDS results, high rotifera species richness was observed in the Upper Danube and there was no longitudinal trend of zooplankton abundance along the Danube. During the four JDSs, the species richness of rotifera gradually increased indicating the importance of cross-sectional sampling and improving ecological conditions of the Danube River. The longitudinal changes of the species richness and abundance of planktonic zooplankton were different in the JDS4 when compared to JDS1-3, the maximum values were observed in the Upper Danube. These results could be explained by the unstable hydrological conditions due to the high water-levels before and during sampling.

(e)DNA-based activities

JDS4 provided an excellent opportunity to evaluate (e)DNA-based approaches in an applied, international and highly integrative setting. The fish community of the Danube, its macrozoobenthos (MZB), phytobenthos and sediment fauna were assessed using group-specific metabarcoding approaches. While a certain degree of methodological variation still exists, the outcomes clearly demonstrate the huge potential of DNA and environmental DNA-based approaches for biodiversity and ecological risk and status class assessments: eDNA water analysis of fish revealed most of the taxa also detected by the traditional fish survey, but was particularly effective in detecting the hard-to-capture benthic taxa (including endangered sturgeon species). The (e)DNA-based taxalists of the MZB likewise covered many of the traditionally assigned species but included a plethora of additional chironomid and oligochaete species. Molecular ecological status class assessments based on presence-absence values of MZB species were also largely congruent to traditional abundance or presence-absence-based outcomes. Although the molecular assessment of the phytobenthos revealed fewer species than traditional light microscopy, many more taxa were detected, which await a species-level taxonomic annotation in the future. Metabarcoding of the sediment community enabled the comprehensive assessment of the meiofaunal community (i.e. an often neglected but ecologically highly sensitive component of the Danube biodiversity) and the molecular inference of fine sediment quality based on local community structures of vulnerable nematode species. Finally, all (eDNA)-based taxalists were compiled to effectively inform invasive alien species detection in the Danube River Basin.

In a pilot comparison exercise, the indicative status for benthic invertebrates based on the Austrian indices SI and MMI and on eDNA were calculated for three sampling sites and the results were found to be astonishingly similar to each other. In another exercise, intercalibration common metrics were used for ecological assessment of sites using data from classical fish survey and from eDNA analysis. For 46% of the sites the same status class was found and for 70% of the sites the final classification of reaching or failing the WFD objective of good status was identical.

The application of (e)DNA-based tools during JDS4 has been found very effective for a comprehensive assessment of the Danube biodiversity (i.e. fish, macrozoobenthos, phytobenthos, sediment community and invasive alien species detection) and showed very promising potential for ecological status class assessments. A complementary approach of traditional assessment techniques and (e)DNA-based tools has a promising potential for WFD ecological status assessments.

Microbiology

The extent and origin of microbial faecal pollution along the Danube and its most important tributaries was determined based on the standard faecal indicator bacterium E. coli and genetic microbial source tracking markers. In total, 72 samples were collected at 36 sites. 56 samples (78%) displayed little or moderate pollution levels as it can be expected for rivers with state-of-the-art wastewater management. 14 samples (19%) showed critical and 2 samples (3%) strong pollution levels. No site with excessive pollution level was observed during JDS4. Hotspots of microbial faecal pollution were identified in the middle and lower section of the Danube and in the tributaries Arges, Rusenski Lom and Drava. A slight yet statistically insignificant trend towards lower values in comparison to JDS3 was observed. However, a classification according to the EU Bathing Water Directive was not directly possible since the bathing water quality assessments comprise at least 16 samples and a percentile evaluation.

Corresponding to earlier investigations, human-associated genetic faecal markers were detected in a high percentage of samples showing that human faecal contamination is the major source of microbial faecal pollution in the Danube River basin. Only at very few sites, were low concentrations of ruminant- and pig-associated source tracking markers found.

Antibiotic resistant bacteria

The occurrence of human induced antibiotic resistant bacteria (ARB) is not only limited to clinical surroundings, they can also be found in the human population, animals and the water environment. In particular the large river systems are of great concern regarding the spreading of ARB. The aim of JDS4 activities was to analyze the *Escherichia coli* population of the Danube for presence of human induced resistances. The obtained data were compared with the occurrence of ARB that were isolated in 2013 from the Danube River during JDS3.

The results show a significant increase in multi-resistance (acquired resistances to antibiotics from three or more tested antibiotic classes) and extended spectrum beta-lactamase (ESBL) phenotype (ESBLs are enzymes produced by a great variety of bacteria and hydrolyze beta-lactam antibiotics such as penicillins, cephalosporins and monobactams). The accumulation of resistance mechanisms in the Danube River *E. coli* population has continued over the last six years. From 797 *E. coli* isolates, 110 (13.8%) were multi-resistant, 198 (24.8%) showed resistances to one or two classes of antibiotics and 489 (61.4%) revealed no acquired resistance to the antibiotics tested. 18 isolates (2.26%) expressed the ESBL phenotype. The most common resistances were those to ampicillin (198 isolates, 24.8%) and tetracycline (192 isolates, 24.1%), respectively. No resistances were detected to imipenem, meropenem, tigecycline, amikacin and colistin.

A separate study investigated changes in the microbial community composition in eight selected sampling points along the Danube River by using a 16S rDNA sequencing approach. In accordance with the data reported during the JDS2, it was observed that Proteobacteria, Actinobacteria and Bacteroidota were the most dominant phyla detected in the river. The antibiotic resistant genes (ARG) against antibiotics belonging to β-lactams (Bla_{TEM}), sulfonamides (*Sul1*) and quinolones (*qnrS1*) were also identified, which are among the main used in human and veterinary medicine. Due to the increasing use of antibiotics, their concentration in waterbodies is increasing and can contribute to the spread of the antimicrobial resistance. The results of the study showed that these ARG were present in at least one sampling point.

Chemistry

Physico-chemical parameters and nutrients

The results obtained during JDS4 reconfirmed the main findings of both the investigative monitoring of the JDS type and the long-term surveillance monitoring carried out in the framework of the TNMN, driven by the ICPDR. A comparison of the nutrients data produced within the four JDSs organised so far and the nitrates data produced by TNMN laboratories showed a high degree of comparability, even though the samples were completely different (different sampling dates and sampling teams).

The results for general physico-chemical parameters measured were typical for the survey time (July) and the geographical area. The spatial patterns previously identified were re-confirmed during JDS4: decrease of the Total Nitrogen and increase of the Total Phosphorous profiles from the Upper to Middle and Lower Danube. Some of the "*hot-spots*" in tributaries identified in previous surveys were confirmed also in JDS4 (*Russenski Lom*) whereas some of them showed an improved situation (*Iskar* and *Jantra*).

Target analysis of organic substances in water

Nineteen priority substances regulated by the EU WFD were analysed. Only for cypermethrin and cybutryne concentrations above the Environmental Quality Standards (EQS) according to the Directive 2013/39/EU were observed at a few sampling sites. All other priority pollutants showed concentrations below the respective EQS.

From the existing list of Danube River Basin Specific Pollutants one pharmaceutical, four pesticides and one metabolite were found in relevant concentrations at a few sampling sites. Ten substances from the EU Watch List were analysed and elevated concentrations could be detected for the pharmaceutical diclofenac, the natural hormone 17-beta-estradiol and the insecticide imidacloprid.

In addition, very low concentrations of 1,4-dioxane and 14 flame retardant substances were found to be present in waters, thus posing no risk to the Danube River Basin.

The overall results of target analysis of organic substances in water show a satisfactory situation. Only for a few substances at a few sampling sites were the effect thresholds exceeded. Often the highest concentrations were found in tributaries, whereas in the Danube itself, dilution led to significantly lower values.

Target analysis of organic substances and metals in biota

Directive 2013/39/EU lists EQS in biota for 11 compounds. 9 of these compounds were analysed in biota during JDS4 (at 44 sites in fish, and at 26 sites in mussels). Hexachlorobenzene and hexachlorobutadiene were not analysed, as the results from JDS2 and JDS3 did not show an exceedance in fish muscle and liver samples for either.

The results of the monitoring show a quite satisfactory picture for most of the parameters. For the parameters dicofol, HCBDD, PFOS and benzo(a)pyrene all sites show concentrations below the EQS. For dioxins and dioxin-like compounds, heptachlor and fluoranthene, concentrations above the biota EQS were found only at single sites.

The results for mercury and BDE are quite opposite with all sites showing concentrations higher than the EQS. Both compounds are considered as ubiquitous persistent, bioaccumulative and toxic substances (uPBTs). Whether the existing mitigation measures for these compounds are effective has to be shown in future monitoring programs.

Groundwater monitoring

Seven groundwater monitoring sites along the Danube River (with expected interconnection with the water from the Danube River through bank-filtration) were sampled and the results were compared to the concentrations detected at the closest Danube sites to identify any kind of interaction. In total 286 pesticide substances, pharmaceuticals, drugs, artificial sweeteners, industrial substances, isotopes, dissolved organic matter and rare earth elements, which are usually not monitored within standard monitoring programs, were detected in either groundwater or in a Danube monitoring site closest to a monitored GW-site.

The analysis showed that in many cases the bank-filtration process contributes to a smaller number of substances and lower concentrations being detected in groundwater than in the Danube River. Nevertheless, this effect cannot be generalised and is compound- and site-specific. For many of the detected substances the situation is opposite and the concentration in groundwater is often higher than in the Danube. Even so, a considerable number of substances (23%) were only detected in a groundwater site and not found in any of the adjacent Danube sites, which indicates that pollution of groundwater is being caused by local or regional polluting activities.

A broad range of chemical substances is widely used in industrial, medical and agricultural activities and thus many of those compounds were also present in the groundwater samples but most of the findings were at a concentration range of few ng/L or even pg/L. Nevertheless, it has to be pointed out that certain substances may have adverse (e.g. endocrine) effects even at such low concentration levels. None of the pesticide substances and metabolites for which European quality standards for groundwater and drinking water exist, have exceeded these standards. For the majority of detected substances, however no quality standards exist, for some of them (PFAS and bisphenol A) drinking water standards are under discussion. For PFAS the discussed standards would not have been compromised, whereas for bisphenol A all the seven detected concentrations in groundwater would have exceeded the discussed drinking water quality standard of 0.01 µg/L by 9- to 16-times.

Screening methods

The anthropogenic pollution of water resources with organic and inorganic chemicals is a major global challenge. More than 350,000 chemicals are already used in commerce and thousands of new chemicals enter the marketplace annually. Many of them are expected to be found in the environment due to their emissions to air, water and soil. The problem of the current chemical water pollution assessment is the focus primarily on Priority Substances (according to the Directive 2013/39/EU) determining the chemical status and on River Basin Specific Pollutants considered for the ecological status assessment. In addition to that few emerging chemicals from the EU Watch List are being investigated. The strategy to overcome the limits of classical target analysis are wide-scope chemical target screening and non-target screening approaches utilising high performance liquid- and gas-chromatography coupled with high resolution mass spectrometers (HRMS). The advantage is that the HRMS technology allows a measurement and also digital archiving of signals of all compounds in a sample, while target analysis only records specific signals for compounds selected prior to the analysis.

The ambition of JDS4 was to apply diverse wide-scope chemical target screening and non-target screening approaches to enable a highly intensified acquisition of data on occurrence of chemical substances in the DRB and to explore the potential of these novel techniques. These approaches were already applied during JDS3 in the form of pilot studies and JDS4 aimed at an intensive application of various screening methods.

MAXX large volume solid phase extraction (MAXX LVSPE) was used during JDS3 for the first time in a larger survey and it was re-applied during JDS4. This method is based on the on-site extraction of larger water volumes without need to bring the water sample to the laboratory, which prevents the danger of the alteration of the sample or a secondary contamination of the samples. The target screening enabled reporting on 150 highest ranked substances ordered by their maximum concentration value. The concentration levels span over 2-3 orders of magnitude. The compound with the maximum concentrations was 2,4-dichlorobenzoic acid, but it was found only at two sites (site JDS4-3 (Kelheim) with 44,000 ng/L and site JDS4-46 (Russenski Lom tributary) with 376 ng/L). Metformin, a type 2 diabetes drug, was detected at 50 sites with a maximum concentration of 25,000 ng/L at JDS4-42 (Timok tributary). Using non-target screening, mainly PEG-based surfactants could be identified as the predominant, ubiquitous compounds in the Danube River Basin, with higher concentration levels at the lower stretches and adjacent tributaries.

Using the wide-scope target and non-target screening of surface water samples by direct injection LC-HRMS different herbicides or their transformation products (TPs) such as azines, bentazone, metolachlor and nicoforone were identified at all sites. A second prominent group of compounds were pharmaceuticals such as the transformation products of the analgesic drug aminopyrine, N-formyl-4-aminoantipyrine and N-acetyl-4-aminoantipyrine, the anticonvulsants carbamazepine, lamotrigine and gabapentin-lactam as well as the angiotensin II receptor (alpha) blockers valsartan, candesartan, losartan and telmisartan, the beta-blocker metoprolol, the antidiabetic metformin and the pain drug tramadol. An important industrial chemical identified was isophorone, a precursor in the production of polymers also used as a solvent. Chemicals of daily use found in the Danube River and its tributaries were the corrosion inhibitors 1H-benzotriazole and 4- as well as 5-methyl-benzotriazole.

To provide a comprehensive picture on the presence of endocrine disrupting compounds, MAXX LVSPE extracts were analysed for 85 compounds (mainly natural and synthetic estrogens, androgens, glucocorticoids, progestagens as well as phenolic xenoestrogens) by LC-MS/MS and LC-HRMS, and tested using the Yeast Estrogen Screen assay combined with high-performance thin-layer chromatography and high-throughput reporter gene assays for estrogen receptor α and glucocorticoid receptor activity. Chemical analysis showed the presence of low levels of estrogens (estrone and estriol, up to 3 ng/L) and androgens (androsterone, epiandrosterone and androstenedione, up to 7.5 ng/L) in most samples, while progestagens (progesterone and different synthetic ones, up to 2 ng/L) and several glucocorticoids (up to 3 ng/L) were present only in a few samples. The concentrations of phenolic xenoestrogens were typically higher, bisphenol A and methylparaben (both ranging from 1 up to several hundred ng/L) showed the highest frequency of occurrence. The YES-HPLTC approach suggested that 17ß-estradiol and estrone were mainly responsible for the observed estrogenic effects, while the high-throughput reporter gene assays for ERα and GR did not detect any effects due to a masking by cytotoxicity of the extracts.

A state-of-the-art wide scope target screening of more than 2,400 chemicals and their transformation products was carried out in samples of influent and effluent wastewater, groundwater, river water, sediments and biota, collected within JDS4 as a collaborative study of three reference laboratories of the NORMAN network. The analysed contaminants of emerging concern were divided into five main categories based on their use: plant protection products, industrial chemicals, pharmaceuticals (including antibiotics), drugs of abuse (including tobacco ingredients) and miscellaneous chemicals. In total, 580 contaminants of emerging concern were detected in the samples. The removal of industrial chemical and plant protection

products by the WWTPs was investigated, their fate in the catchment was reported, and the attention was drawn to nineteen plant protection products and eight industrial chemicals that exceeded their respective ecotoxicological thresholds in various matrices.

Wide-scope target screening of >1,300 illicit drugs, pharmaceuticals, antibiotics and personal care products in wastewater, groundwater, river water, sediments and biota by liquid chromatography coupled with high resolution mass spectrometry detected 287 of these substances in wastewater, 140 were detected in surface water samples, 41 were found in biota and 31 in river sediments. Although more than 300 compounds were detected in the samples, only ca. 5% exceeded their ecotoxicological threshold values. These substances were included among the potential Danube RBSPs. Antibiotics were the most frequently detected class of compounds in water matrices. The detected concentration levels of illicit drugs and their transformation products seem to pose no environmental risk. The antipsychotic drugs sulpiride and temazepam exceeded the respective PNECs (predicted no effect concentration) in biota. Majority of illicit drugs and drugs of abuse that were detected in surface water in JDS3 were determined at significantly lower concentration levels in JDS4 samples. The most prominent from the group of illicit drugs was benzoylecgonine – the main metabolite of cocaine, detected in all 11 tested wastewater samples. In general, concentrations of illicit drugs represented only ca. 1% of the overall load of studied substances and were reduced significantly during the treatment at WWTPs.

In another study, five methods for the analysis of organic micropollutants were tested: one target method based on SPE, LC-MS/MS and GC-HRMS, and four screening methods, of which three were based on SPE and LC-HRMS and one on direct injection LC-HRMS. The different methods were focused either on specific compound classes or aimed at a wide scope screening, and the overlap of all five methods was just 10 compounds. The methods differed considerably in the approaches used for calibration and the number of calibration points. A comparison of concentrations of the 10 compounds analysed by all methods showed in most cases a good agreement within a factor of 3, but in some cases considerable deviations were observed.

Stationary passive samplers were deployed for 100 days at nine sites close to sites where fish was also caught for analysis. Passive samplers provide a time-integrated image of pollution in the aqueous phase over extended time period, providing a representative picture of the surface water quality in summer 2019. The results showed that the spatial variability of investigated hydrophobic priority substances in surface water in the Danube is low. No deterioration of Danube surface water contamination by hydrophobic priority substances was observed in JDS4 in comparison with the results from JDS3. Among investigated organochlorine compounds and PAHs at JDS4-15, a significant concentration decreasing trend was observed for hexachlorobenzene, PCB 28, PCB 52 and p,p'-DDE, whereas no significant temporal trend was found for PCBs with a higher degree of chlorination or for priority PAHs. Passive sampling of hydrophobic substances in surface water provides a worst-case scenario of fish exposure to those substances and should be considered as a viable alternative to biota monitoring. Target analysis of 154 polar contaminants at 9 JDS sites revealed two distinct longitudinal concentration profiles. Whereas concentrations of pharmaceuticals and benzotriazole continuously decreased downstream the Danube, there was an apparent increase of concentrations of currently used pesticides in the Lower Danube downstream from the Iron Gates dam.

Influent and effluent samples from 11 wastewater treatment plants (WWTPs) in 11 countries in the Danube River Basin (DRB) were collected. To assess the performance of the wastewater abatement process in the selected WWTPs, the actual removal rates of the initial list of 11 proposed indicator substances by NORMAN and Water Europe were determined. The removal rates of 12 additional indicator substances used to evaluate the effectiveness of treatment in WWTPs with either ozone or activated carbon (AC), were also calculated. Rather alarmingly, eight out of the 20 indicator substances (the two indicator groups

having 3 common substances) were eliminated with a removal rate below 50%. To address mixture toxicity (combined adverse effect of multiple contaminants), the effluent wastewater samples were also analysed with a battery of seven NORMAN/SOLUTIONS in vitro bioassays covering a wide spectrum of effect endpoints. The results, including those obtained in the previous surveys in the DRB, indicate that the current water treatment technologies used in the studied WWTPs are unable to remove efficiently groups of contaminants of emerging concern (CECs) that cause specific effects such as estrogenicity, PAH activity, xenobiotic metabolism and oxidative stress. The top 17 substances that potentially pose a risk for the Danube and originate from wastewater accompanied with their respective Emission Limit Values (ELVs) were proposed to be considered for inclusion in the monitoring plans of the WWTPs in the DRB.

The analyses of cytotoxic and genotoxic activity of surface water samples extracted by Horizon LV SPE technique providing an enrichment factor of 25,000 were carried out. Initial screening in prokaryotic model was performed by SOS/umuC assay while testing in eukaryotic model comprised integrated zebrafish-based battery of bioassays including testing of cytotoxicity, genotoxicity (comet assay) and cell cycle analyses on zebra fish liver (ZFL) cell line and embryotoxicity (zFET). The results demonstrated that about 46% of the extracts were cytotoxic while about 38% of the extracts were found to have DNA damaging potential to certain extent. Most of the samples active in applied bioassays were collected from the middle Danube section. None of the extract had embryotoxic activity at the highest tested REF 100.

The momentum of JDS4 was used to organise the NORMAN / ICPDR interlaboratory trial for non-target screening and effect-based tools which was combined with a training for Danube laboratories with a view to enhance the knowledge on non-target screening principles and techniques.

The results of the suspect screening of compounds spiked in an extract of a reference natural water sample were quite promising. Many of the most important spiked compounds were identified by the participants of the chemical analytical part. It has been shown that vendors' software is not necessarily better than in-house or open-source software tools to assess mass spectral data.

The interlaboratory trial demonstrated that the existing effect-based methods are powerful tools to discriminate low-toxicity from more toxic samples (WWTP effluents, rivers with high wastewater fraction, agriculturally impacted streams etc) and to quantify their toxic burden, while a quantitative assessment in highly diluted surface waters is currently still not possible.

Prioritisation of Danube RBSPs

The prioritisation exercise was performed on the unique dataset of wide-scope target and suspect screening data obtained within JDS4 with the goal to identify Danube RBSPs in water and biota compartments. Additionally, the study aimed at assessment of the chemical pollution risk for sediment and groundwater matrices.

The samples of the Danube River water, wastewater, groundwater, sediments and biota obtained within JDS4 were screened by different laboratories for several thousands of organic pollutants and their transformation products by wide-scope target (>2,600 substances) and suspect (>65,000 substances) screening techniques. Substances detected in the samples were prioritised in each matrix separately using the NORMAN Prioritisation Framework algorithm and indicators for assessment of the risk, hazard and exposure score of each compound. The used concept divides all detected substances into six 'action categories', where e.g. Category 1 groups substances ready for regulatory monitoring, Category 2 suggests compounds with a need for more monitoring data (Danube Watch List) etc. The risk score, expressing at how many places and how much the ecotoxicity threshold value of a pollutant is exceeded, was used as a primary indicator for ranking substances within each category. Prioritisation of target screening

surface water data has indicated that only three out of 45 (PFOS, cybutryn, cypermethrin) WFD priority substances are of concern in the DRB. In general, attention should be paid to monitoring of 52 candidate RBSPs. Biota results indicated that monitoring of three legacy substances (BDEs, mercury and PFOS) might be justified, while suggesting additional 16 candidate RBSPs. Several substances frequently detected at high concentration levels in wastewater effluents were identified as a clear source of RBSPs in the surface water. Pollutants detected by target screening in seven groundwater sites along the Danube do not seem to pose any significant risk. Suspect (non-target) screening revealed numerous substances in each studied matrix, which might be of concern at the DRB level. A wealth of chemical target analysis and screening data obtained during JDS4 make the DRB arguably the best investigated river basin in Europe and globally. The obtained data stored in a well-organised database system are ready to be used by the EC to support its 'zero-pollution policy' and put eventually a ban on the production, use or import of the chemicals endangering Europe's environment.

Microplastics

Nowadays, the presence of microplastics in the environment is subject of scientific and regulatory discussions. Their inputs from land ultimately end up in the oceans, where they remain for a long time. River systems represent an important path of microplastics entry into the oceans.

A comprehensive screening of microplastics in the Danube and its tributaries was carried out on 12 sites. Sampling was performed by means of deploying sedimentation boxes into the river for 14 days; followed by thermo-analytical detection (TED-GC/MS) for determination of the total content of various plastic polymers in the collected suspended particulate matter samples. In all samples, plastic polymers were detected. The results represent a first set of quantitative data, establishing a baseline of occurrence of microplastics in the DRB.

In almost all samples, all analysed polymers were detected and quantified, whereas there was no clear trend observed of increasing or decreasing microplastics content along the Danube. The content ranged between 0.05 – 22.24, 0.00 – 0.45, 0.00-1.03 and 0.00 – 3.32 [µg/mg SPM] for polyethylene, polypropylene, styrene-butadiene rubber and polystyrene, respectively. Additionally, specific thermal decomposition products were detected, which could give indications about the presence of natural rubber and poly (methyl methacrylate). Polyethylene was detected as the most abundant component of microplastics in almost all samples. Styrene-butadiene rubber was analysed in samples from the DRB for the first time, and its presence in samples indicates an influence of pollution from urban areas and traffic (tyre abrasion). The other polymers (polyethylene, polypropylene, polystyrene) are often found in treated wastewater but also in untreated run-offs, mixed water overflows and so typically assigned as indicators of wastewater, however, they may also originate from other diffuse sources.

The study of microplastic was also conducted in freshwater mussel Asian clam (*Corbicula fluminea*) collected from 23 sites along the Danube River and main tributaries. In total, 216 specimens were used for analysis. Analyses revealed that the following types of microplastic particles were present in mussels: polycarbonate, polyethylene terephthalate (PET), polypropylene-polyethylene copolymer, Nylon (polyamide) and Cellophane; with PET being the most dominant and frequent polymer in the analysed mussels (58%). Microplastic debris ingested by organisms was represented mostly by fragmented hard plastics, within the size range from 0.02 to 4.67 mm and fibers. A total of 1,998 microplastic particles were collected with an average of 9.25 particles per organism or 26.4 particles g-1 wet body weights. The fact that PET was not detected in water and was dominant in biota indicates that a comprehensive monitoring of microplastics in rivers requires analysis of all relevant matrices, as well as standardization of procedures.

Characterization of dissolved organic matter

The structures of the dissolved organic matter (DOM) of samples collected during JDS4 were characterized by easy-to-perform optical methods, i.e. UV-visible spectroscopy and synchronous fluorescence spectroscopy. As provided by the UV-visible spectroscopy, the DOM aromaticity and molecular weight are moderately variable in the Danube River and its investigated tributaries. The contribution of humic substances to the DOM fluorescence is variable with no specific trend up to the confluence with the Timok River. It increases downstream. The protein-like fluorescence is correlated to the chlorophyll-a concentration and to a lesser extent to organic and ammonium nitrogen. It results from a combination of processes such as in-water biological reactions, watershed run-off and poorly treated urban sewage.

Rare Earth Elements

Rare Earth Elements, which include Sc (scandium), Y (yttrium) and the lanthanides, have been monitored for the first time along the Danube River and in some of its tributaries. The concentrations found in the surface water samples have been normalized to a reference rock type (Post Archean Australian Shale) to detect potential anomalies. A negative anomaly was observed for cerium (Ce): it is natural and related to the redox behaviour of this element. A large positive anomaly was observed for gadolinium (Gd): it is due to the use of contrast agents incorporating Gd to perform Magnetic Resonance Imaging exams in health facilities. As the contrast agent is not eliminated in wastewater treatment plants, Gd is ultimately disseminated in the aquatic environment. There are no European quality standards established for Gd, neither for groundwater nor for drinking water.

Nanoparticle inventory in a sediment core

Nanoparticles of a Danube sediment core downstream of the Kostolac fly ash dump were investigated and compared to the dump's fly ash nanoparticles, as well as to a Danube reference sediment upstream of the dump. Several elements that typically fractionate during the coal combustion, such as Copper, Nickel and Vanadium, were enriched in a portion of the Kostolac fly ash particles and were orders of magnitude more abundant in fly ash compared to the reference sediment. While virtually absent in the sediment sample upstream of the fly ash dump, nanoparticles enriched in these fly ash-signature elements were found in the sediment core downstream of the dump. This indicated that there is an occasional release of fly ash particles into the Danube from the uncovered fly ash dump located only 50 m from the river.

Radioactivity

The radioactivity content of river sediments is an unerring radio-ecological indicator for the radioactive contamination of the environment. The results of the radiometric analysis of the JDS4 sediment samples show that the radio-ecological development of the Danube continues to be promising. The radioactive contamination of the Danube with the long-lived artificial nuclear fission radionuclides ^{137}Cs and ^{90}Sr has decreased by two orders of magnitude since the atmospheric nuclear weapons tests period in the northern hemisphere (1945 - 1963) and the Chernobyl nuclear power plant accident (1986). Furthermore, the activities of the geogenic radionuclides of the natural decay chains and other primordial natural radionuclides (^{40}K, ^{210}Pb, ^{226}Ra and ^{228}Ra) remain at common levels. Thus, there is currently no indication of hazardous man-made radioactive contamination of the Danube ecosystem compartments.

Stable isotopes of water and nitrate

To assess mixing processes of different water sources and the origin of nitrate in the Danube River Basin water stable isotope and stable isotopes of nitrate were measured during the JDS4 and compared to past surveys. The results indicated that snowmelt was more significant in 2019 in comparison to past surveys and that the water fraction of the Inn River is controlling the water chemistry and dilution of pollutants for several hundred kilometres. Nitrate concentrations and its isotopic compositions suggest that nitrate mainly originates from soil nitrate with smaller admixtures of wastewater or manure. The relatively constant nitrate concentrations and similar isotopic results during the JDS2 survey revealed that nitrate originating from diffuse sources is mainly transported via baseflow/groundwater inputs, rather than direct discharges. In-situ processes, like nitrate denitrification, assimilation or nitrification could not be detected from the isotopic compositions of this longitudinal survey.

Lessons learned

Hydromorphology

The results obtained serve as a general estimation of trends along the entire Danube and they should encourage further detailed in-situ measurement and assessment work (which has to be carried out according to the WFD at waterbody level). For documenting the changes and as a monitoring tool for the six-year WFD cycle, the approach taken was proved to be feasible and affordable.

It is recommended to take into consideration the future outcomes of the Interreg Danube Transnational Programme (DTP) DanubeFloodplain project for the improvement of floodplain connectivity with the river and the outcomes of the completed Interreg DTP Danube Sediment project for a better understanding of sediment balance and morphological development.

Macroinvertebrates

For ensuring best results, both riverbanks should be sampled during the next survey. The application of different sampling methods always provides better data in several aspects, however from a practical point of view, national teams should focus only on one main sampling technique (e.g. MHS or DWS in the Lower Danube River Reach). Assistance of external experts with most problematic groups, e.g. Oligochaeta and Chironomidae (Diptera), could be recommended for each participating country. This will ensure data comparability (especially for statistical methods) of the most abundant groups.

The Slovak Multi-metric index seems not to be suitable for the tributaries' assessment. Hence, the large tributaries along the Danube River deserve their own particular approach. For the next JDS, assessment methods should be tested on JDS4 data from main channel and tributaries separately.

(e)DNA-based activities

To streamline future monitoring activities and to benefit from the molecular data generated, the following topics were suggested for consideration:

- A higher proportion of species-level annotations can be achieved for all organism groups investigated when gaps in DNA barcode reference libraries are specifically addressed for Danube biota. A gold standard here would be a well curated DNA barcode reference library for Danube biota.
- Besides the focus on classical biological quality elements, (e)DNA-based approaches enable us to integrate additional ecologically sensitive target groups into environmental assessments (e.g. nematodes).
- When generating biodiversity patterns and investigating correlations to environmental parameters, analyses could more explicitly focus on patterns of genetic diversity additional to Linnaean species. As such, the full potential of (e)DNA-based approaches can be released, and the Danube biodiversity more fully accessed (i.e. by integrating cryptic lineages and intraspecific genetic diversity as one pillar of biodiversity).
- Furthermore, a metadata analysis of (e)DNA patterns combined with the outcomes of non-target analytics / effect-based tools holds great promise to understand the ecological drivers of habitat changes and shifts in community composition.
- To reduce the plethora of methodological variation, standardisation work has to be conducted and a limited set of well-performing and praxis-oriented (e)DNA-based approaches selected. A good framework for such standardisation work might be the newly installed CEN working group WG28 "DNA and eDNA methods".
- New or adapted (e)DNA-based biotic indices for ecological status assessment should be more explicitly tested and intercalibration experiments performed.
- The national authorities should be educated and trained in state-of-the-art molecular tools, fostering the development of a strong collaborative international network between all parties involved
- In connection to the analysis of invasive alien species, it would be of great significance to use in future the (e)DNA data for the assessment of bioinvasion pressure based on quantitative approach.

Microbiology

Corresponding to earlier investigations, human-associated genetic faecal markers were detected in a high percentage of samples showing that human faecal contamination is the major source of microbial faecal pollution in the Danube River Basin. Only at very few sites, were low concentrations of ruminant- and pig-associated source tracking markers found. A valuable addition in the future would be the application of genetic faecal markers for bird-associated faecal pollution, but unfortunately up to date there are no such methods available that have been tested in the Central European region.

Screening methods in chemical analysis

A comparison of concentrations of the 10 compounds analysed by different screening methods showed in most cases a good agreement within a factor of 3, but in some cases considerable deviations were observed. Some of these deviations are likely related to the different calibration strategies, as they were highest at low concentration levels, but also occurred for certain compounds and sample sub-sets, pointing at specific interferences for one method. This calls for a strict application of quality assurance and quality

control measures and a system for reporting the reliability for individual compounds, as different methods will not perform equally well on the same compound. Interlinking chemical screening and effect-based monitoring data with results of biological monitoring, and especially eDNA, remain a challenge. This is directly related to a need to account for toxicity of chemical mixtures and improved prioritisation of RBSPs. A capacity building of Danube laboratories responsible for regulatory monitoring is needed to be able to carry out NTS and EBM on a routine basis.

Microplastics

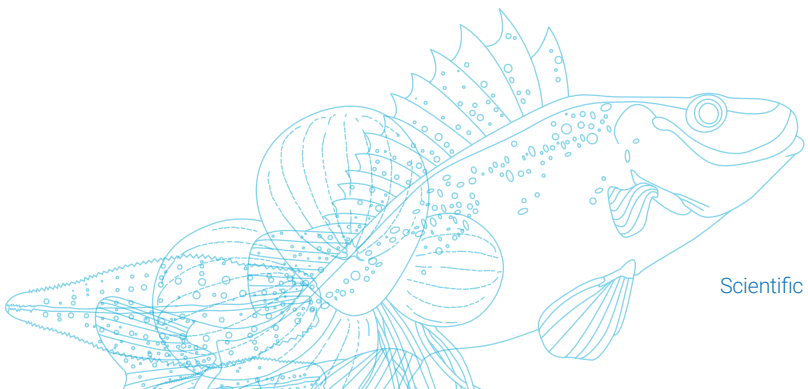
The screening has demonstrated that the chosen investigation approach (sampling, sample preparation, detection) is feasible and provides robust results inter-comparable at the basin scale. However, further developments – also based on the investigations carried out in JDS4 – are already foreseeable. This includes improvement of the sampling scheme, employing active sampling by a pump to control and record the exact sample volume. In the future a sampling with the standing wave should be performed. Applying this approach, a virtual water package is sampled with the flowing wave from the source to the sea.

It is also foreseeable that an exposure of the sedimentation box for one week might be sufficient. The water flow velocity in the river during the sampling should also be more closely monitored in future programs.

The analysis of microplastic in biota has been proved to be important for understanding of the fate of plastic particles in the aquatic ecosystem. JDS4 showed that freshwater mussels are an effective indicator of presence of microplastic particles in living organisms. For reliable monitoring of microplastics in water analyses of all relevant target matrices are required in combination with the standardization of monitoring and assessment procedures.

Stable isotopes of water and nitrate

The isotope study revealed that the groundwater/baseflow is an important source of nitrate in the Danube River. Groundwater observation networks in the Danube River Basin should therefore ideally be coupled with the river monitoring station network and nitrate concentrations evaluated and compared. The results suggest furthermore that inputs from also smaller diffuse sources, as e.g. septic tanks, should be considered contributing to the nitrate load in the Danube River Basin. Wastewater seems to be the most important point source, adding directly nitrate to the Danube River, which highlights the importance of enhancing nitrogen removal efficiency of domestic wastewater in the catchment. The study proposes moreover that riparian protection and buffer zones are an important measure, avoiding significant nitrate spillage from mineral fertilizers and allowing denitrification processes during the transport. As groundwater residence time may be long within the watershed, the observed time frame (2007 and 2019) may also be too short in order to observe implemented remediation or beneficial land management practices with the objective to decrease nitrate sources in the catchment. These results are in agreement with the results from the Moneris model used by the ICPDR in 2015 indicating baseflow as the most important pathway of nitrogen pollution and wastewater and agriculture as the most relevant sources.



Key conclusions

Six years ago, the key conclusions began with the statement that JDS3 provided a unique opportunity to assess the water quality in the whole Danube and provided the largest ever amount of knowledge about Danube water pollution collected within a single scientific exercise. Looking at JDS4, this general conclusion can be repeated because the amount of knowledge collected during JDS4 is again the largest ever – but there is also another substantial added value: the greatest number of actively participating experts in the history of ICPDR activities resulted in a very intense hands-on monitoring exercise, which not only generated another huge amount of valuable data about the water quality in the Danube but also significantly strengthened the cooperation in WFD-related monitoring and assessment between the countries in the Danube River Basin. And we cannot forget the intensive public outreach disseminating JDS4 news to the stakeholders and wider public. The JDS4 motto '*Discover Danube*', was designed as a call to public action towards a healthier and cleaner Danube. The communication to the public included the use of social media and helped to increase the public visibility of JDS4. All results and facts on Joint Danube Surveys are available on www.danubesurvey.org

From the technical point of view, the more active approach which was implemented during JDS4 (sampling methods and strategy were harmonized prior to the survey at workshops and the field and lab work was done by the national teams) could add a bit higher variability to the data (caused by, e.g. slightly varying working routines, taxonomic skills and identification processes, differences in sampling time in relation to discharge) but on the other hand this was exactly what the EC WFD intercalibration process is looking for. Thus, it will be possible to use the high-quality data collected during JDS4 and compare it with the results from other national assessments to figure out the variability of national methods and separate this way the methodological differences from natural variability. This will lead to the elevated precision and accuracy of status assessment results in the Danube River Basin which is instrumental to the proper design of future measures.

The **hydromorphological reassessment** of the situation from six years ago showed intensified restoration on the still strongly altered Upper and Middle Danube and only insignificant deteriorations on the Lower Danube, the long reaches of which are still only slightly to moderately altered.

Biological quality elements indicating pressure from nutrients and oxygen depletion by biodegradable substances – phytoplankton, macrophytes, phytobenthos, partly macrozoobenthos – indicated a good status at many sites and pointed at local pressure only. Fish and macrozoobenthos however indicated impacts induced by hydromorphological pressures at most of the sites. The indication of the ecological status assessment showed that the **fish community** is threatened along the whole river course. There is however a very good chance for improvement as the JDS4 results showed that still most species of the reference fish communities could be found at nearly all sites. This is even true for strongly altered hydromorphological stretches in the Upper Danube section. In total 76,265 specimens of 72 fish and three jawless species were detected. This underlines the importance of the Danube as a substantial source of fish biodiversity in Europe.

JDS4 results reconfirmed that the Danube River and its main tributaries are under considerable influence from **invasive alien species**. Comparing the results from previous JDSs, an increase in the number of identified alien species has been recorded but the data analysis shows that the pressure caused by biological invasions is relatively stable. The reason is that not all alien species are also invasive therefore the assessment of pressures by invasive alien species has to be done using a wider perspective.

The parallel application of traditional biological assessment techniques and modern molecular methods demonstrated a big potential of **DNA and environmental DNA-based approaches** for biodiversity and WFD ecological status class assessments. eDNA water analysis of fish revealed most of the taxa also detected by the traditional fish survey, but was particularly effective in detecting the hard-to-capture benthic taxa (including endangered sturgeon species). The (e)DNA-based taxalists of the MZB likewise covered many of the traditionally assigned species but included a plethora of additional chironomid and oligochaete species. Indication of status by the traditional biological assessment techniques and by modern molecular methods showed a promising correlation for fish and macrozoobenthos.

Assessment of **faecal pollution** showed that 78% of samples displayed little or moderate pollution levels as it can be expected for rivers with state-of-the-art wastewater management. 19% samples showed critical and 3% samples strong pollution levels. No site with an excessive pollution level was observed during JDS4.

The analysis of **antibiotic resistant bacteria** showed a significant increase in multi-resistance (acquired resistances to antibiotics from three or more tested antibiotic classes). The accumulation of resistance mechanisms in the Danube River *E. coli* population has continued over the last six years. The most common resistances were those to ampicillin and tetracycline. No resistances were detected to imipenem, meropenem, tigecycline, amikacin and colistin.

Comparison of the **nutrients** data produced over last 20 years within the four JDS and by ICPDR annual TNMN monitoring showed high degree of comparability, despite the variability in sampling dates and personnel.

Nineteen **priority substances** regulated in the European Water Framework Directive were analysed in water. Only for cypermethrin and cybutryne the concentrations above the Environmental Quality Standards (EQS) were observed at a few sampling sites. All other priority pollutants showed concentrations below the respective EQS.

Ten substances from the **EU Watch List** were analysed in water and elevated concentrations were detected for the pharmaceutical diclofenac, the natural hormone 17-beta-estradiol and the insecticide imidacloprid.

The results for **mercury and brominated diphenylethers in biota** showed concentrations higher than the EQS at all sites. Both compounds are considered as ubiquitous persistent, bioaccumulative and toxic substances. Whether the existing mitigation measures for these compounds are effective has to be shown in future monitoring programs. For dioxins and dioxin-like compounds, heptachlor and fluoranthene the concentrations higher than the biota EQS were found at only a few sites.

The analysis of **groundwater** showed that in many cases the bank-filtration process contributes to a smaller number of substances and lower concentrations being detected in groundwater than in the Danube River. Nevertheless, this effect cannot be generalised and is compound- and site-specific. For many of the detected substances the situation is opposite and the concentration in groundwater is often higher than in the Danube. None of the pesticide substances and metabolites for which European quality standards for groundwater and drinking water exist, have exceeded these standards. However, for bisphenol A, all seven detected concentrations in groundwater would have exceeded the discussed drinking water quality standard of 0.01 µg/L by 9- to 16-times.

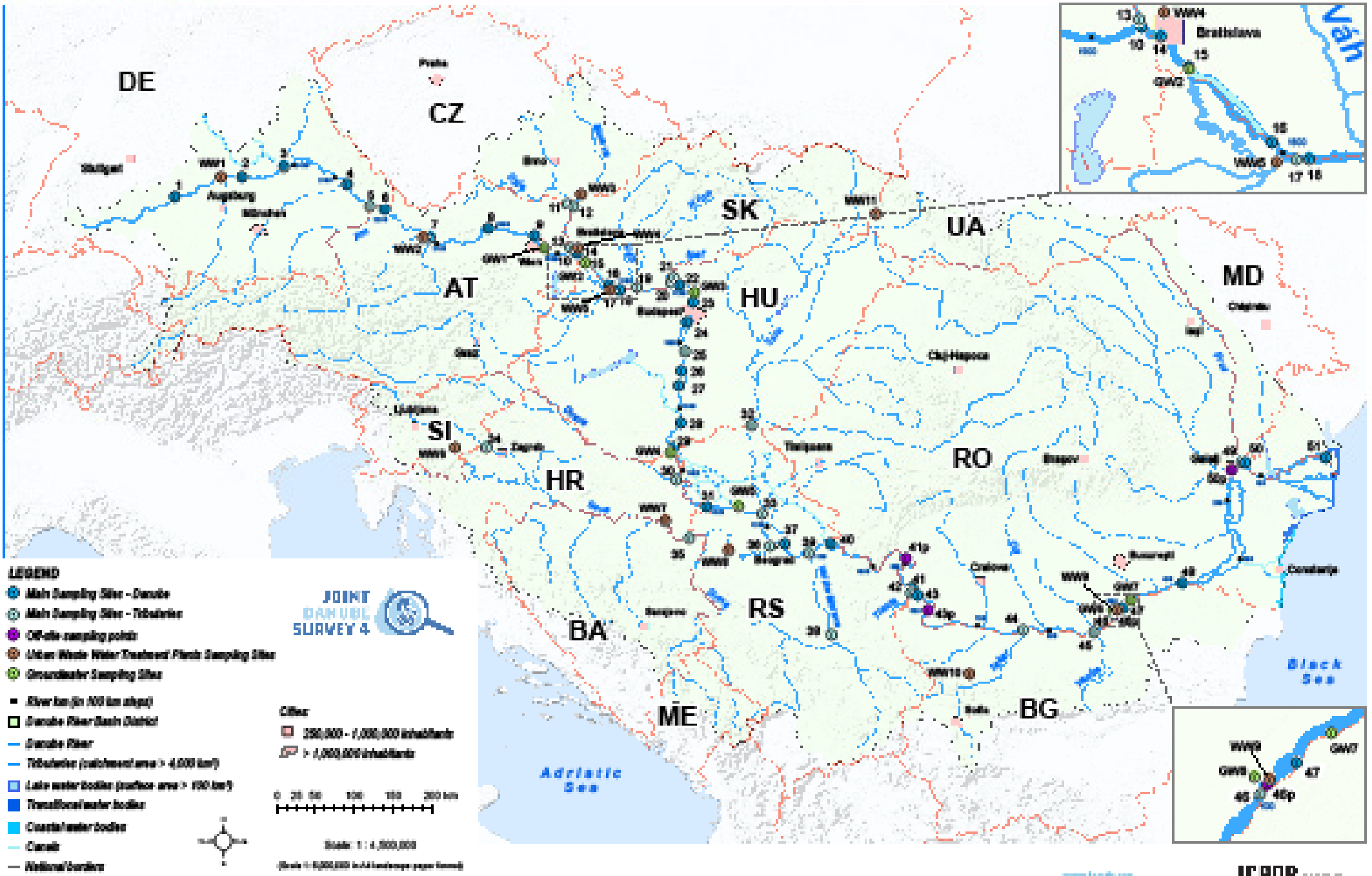
Current chemical river pollution monitoring is focussed on target analysis of Priority Substances and on River Basin Specific Pollutants. In addition to that few emerging chemicals from the EU Watch List are being investigated. The strategy to overcome the limits of classical target analysis includes **wide-scope chemical target screening and non-target screening approaches in combination with effect-based monitoring**

which are on the threshold to become regular tools for WFD-compliant monitoring. A handful of diverse target screening methods were applied during JDS4 focussing on several thousands of compounds. Hundreds of compounds were detected. This comprehensive use of screening techniques enabled their comparison to be made, and interlaboratory trials and training for the Danube laboratories to be completed. Acquiring this huge dataset from screening methods (>2,600 substances from wide-scope target screening, >65,000 substances used for suspect/non-target screening and altogether >300,000 results) made it possible to perform prioritisation of pollutants in water, biota, sediment, wastewater and groundwater leading to specification of tens of substances with the proven most adverse effects to the Danube ecosystem.

The first ever comprehensive screening of **microplastics** along the whole Danube established a baseline of pollution by microplastics in the DRB. In all water samples plastic polymers were detected and polyethylene was detected as the most abundant component of microplastics in almost all water samples. The screening of mussels discovered the presence of microplastics at all sites and revealed polyethylene terephthalate as the dominant plastic pollutant.

The results of the **radiometric analysis** of the JDS4 sediment samples show that the radio-ecological development of the Danube continues to be promising. There is currently no indication of hazardous man-made radioactive contamination of the Danube ecosystem compartments.

JDS4 Sampling Sites



This ICPDR product is based on the Joint Danube Survey (JDS) data. National borders data were provided by the Contracting Parties to the ICPDR, and CIA WDPA data was used for national borders of AL, MK, XK. Shuttle Radar Topography Mission (SRTM) from USGS.
The rest of the background layers displayed in the legend were taken from the ICPDR's GeoNode. Seamless Data Distribution System was used as topographic layer. Data from the European Commission Joint Research Centre was used for the outer border of the EPSG of AL, BY, MK and XK.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ISBN: 978-3-200-07450-7