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SPECIAL ISSUE – Selected results of Joint Danube Survey 4

Editorial

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Dear Reader,

For a period of more than ten years, I had the pleasure to shape work and the profile of the International Association for Danube Research together with my colleagues of the presidency and many other motivated members. Many highlights happened during this time. I am particularly grateful that international cooperation in Danube related research has intensified. This was possible for example within the frame of the European Strategy for the Danube Region (EUSDR), for which we developed transnational and interdisciplinary flagship projects such as Sturgeon 2020 or Danube:Future and IAD people were founding members of the Danube Sturgeon Task Force (DSTF). Also, IAD members coordinated or contributed to several projects within Interreg addressing Danube countries, most specifically to the Danube Transnational Program, which started in 2014. IAD advanced scientific research of the Danube River Basin. It took a leading and coordinating role in sturgeon research as well as in investigating Invasive Alien Species. Many of these projects support achieving healthy and lively rivers in the Danube basin and have an important societal bearing. Another major initiative addresses the river-sea-nexus via the contribution of IAD to the European Research Infrastructure 'International Centre for Advanced Studies on River-Sea Systems' DANUBIUS-RI.

As the EUSDR acknowledges, education is one of the major topics in Danube countries. The network 'Ecology and Management of aquatic ecosystems in Central, East and Southeast Europe' (EcoManAqua) funded by the Central European Exchange Program for University Studies (CEEPUS) supports mobility of students and teachers and has a strong focus on the Danube area. All these activities helped to strengthen our IAD family and will certainly do so in the future. Due to the COVID19-pandemic since 2020, we faced losses and limitations in networking. Nevertheless, IAD remained active and organized the IAD conference, scientific contributions, was observer at ICPDR meetings and continued to issue the Danube News. At this point, I would like to thank all IAD members and especially the current IAD president und the general secretary of IAD for their cooperation, their friendship and support!

Introducing this issue of Danube News will be one of my last activities as member of the IAD-Presidency. The issue is dedicated to Joint Danube Survey 4 to which 13 countries and hundreds of experts from 140 laboratories and institutes contributed. The key objective of this largest and most comprehensive river survey of the world was to provide comparable and reliable information on a multitude of water quality elements. JDS4 followed a new monitoring concept. While before transnational core teams were responsible for sampling over the whole river lengths, national teams took now a leading role in their respective countries. Core teams mainly coordinated and observed the whole activities. This strategy offered a valuable opportunity of exchange among national experts during the training workshops organized for each quality element. The results of JDS 4 helped closing information gaps for the Danube River Basin Management Plan Update 2021.

In this issue of Danube News, we asked experts of selected biological quality elements to share their results with the IAD community. This is of course just a small fraction of the many surveys, which were done during JDS4. If you want to get a comprehensive picture, please visit http://www.danubesurvey.org/jds4/about. We would like to thank the authors for their efforts to contribute to DN 44.

Enjoy reading!

The Most Comprehensive River Survey in the World

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The concept of Joint Danube Surveys (JDS) is driven by two legal instruments: the Danube River Protection Convention (DRPC) and the EU Water Framework Directive (WFD). The DRPC initiated establishing of the TransNational Monitoring Network (TNMN) in the Danube River Basin aiming 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 on a regular basis. The EU WFD requires that countries in the Danube River Basin periodically assess in their territory a quite comprehensive system of water quality elements.

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 monitoring 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 International Commission for the Protection of the Danube River (ICPDR) Ministerial Meeting in 2016) 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 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 JDS4 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 has demonstrated a significant benefit 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.

The key advantages of the new approach used for JDS4 were confirmed by the survey outcomes and these include:

- 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 six 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 prepared for JDS4 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 of 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, 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 (https://youtu.be/iI1Xw58kQ94). 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.

The gratitude of JDS4 organizers 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.

Figure 1. Sampling teams during JSD4

Update of hydromorphological assessment in the framework of ICPDR JDS4

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Abstract

For Joint Danube Survey (JDS) 4 the assessment prepared for the JDS3 in 2013 had to be updated by 2019. This concerns the update of 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).

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. 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 – despite of negative influence of sediment balance due to Iron gate dams – a character of fewer alterations (less stabilized banks and rectification of channel, more bars and islands).

1. Introduction

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 hydromorphological (HYMO) assessment of the predefined 10-rkm-segments with regard to changes (deteriorations, improvements) of channel, banks and floodplain. The data collection and assessment was performed by national experts (deskwork) supported by a consultant and the ICPDR Secretariat providing a specific data upload tool (Schwarz & Höbart 2021).

2. Approach

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. 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. For the detailed method compare JDS3 documentation (Schwarz, Holubova, et.al. 2015) and for JDS4 see Schwarz & Höbart (2021).

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):

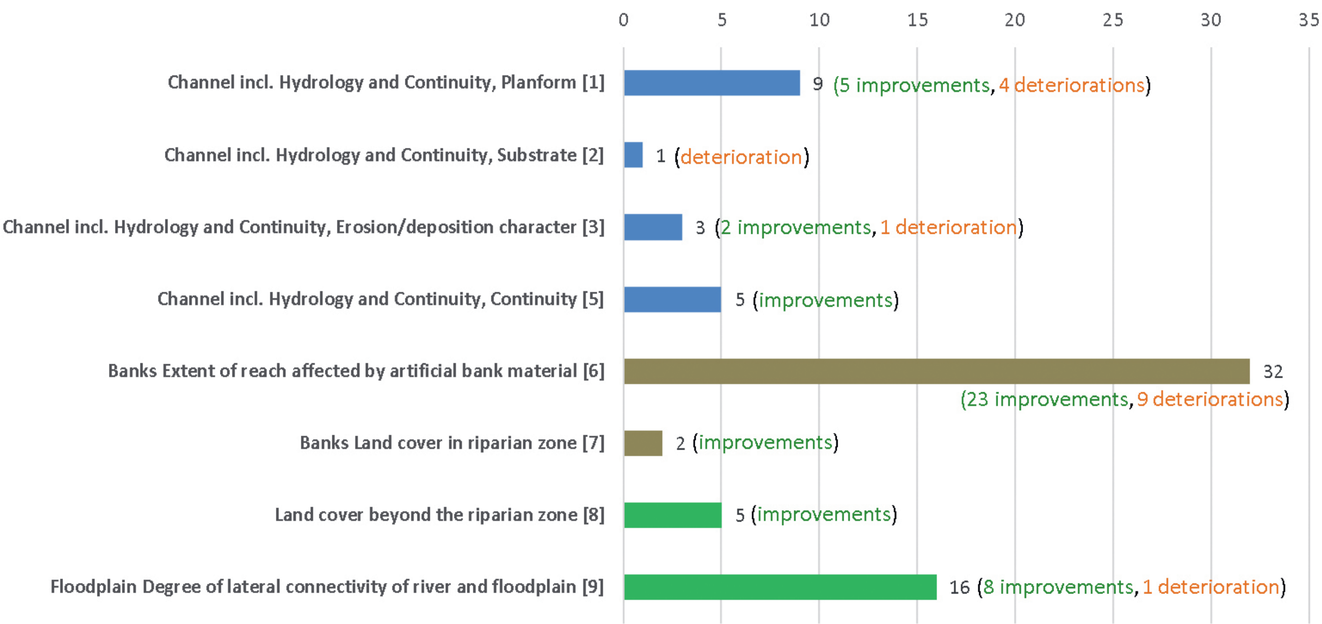


Figure 1: Types of restoration/alteration per all individual changes (blue for "Channel", brown for "banks" and green for "floodplains") and number of improvements/deteriorations per type.

- 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 rip-rap, 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.

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 assesment had to be revised for the reported 10-rkm-segments with changes.

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.

While for the Upper Danube and the Slovak-Hungarian reach of the Middle Danube reported changes are frequent, long reaches on the Lower and Middle Danube segments have no change.

In total, the recorded changes comprise 54 improvements and 19 deteriorations (total number 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,

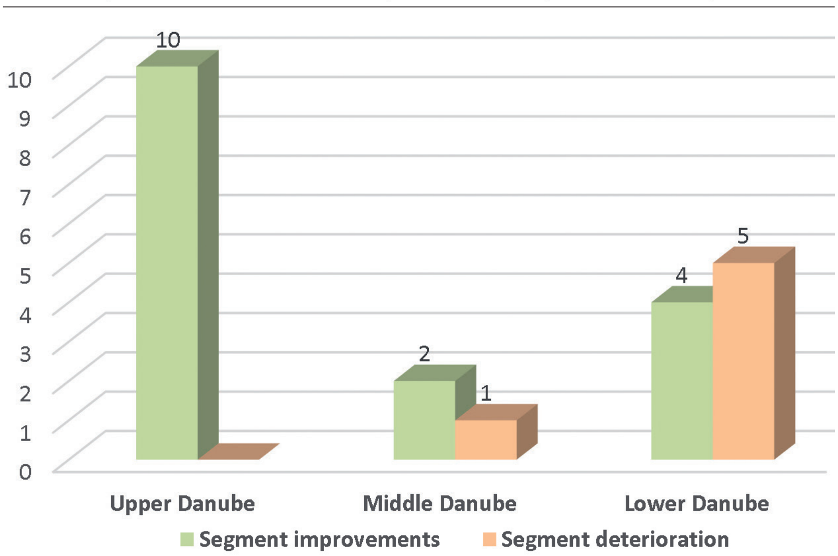


Figure 2: Overview of segments with changes for at least one parameter group (channel, banks, and floodplain) along the three main section of Danube

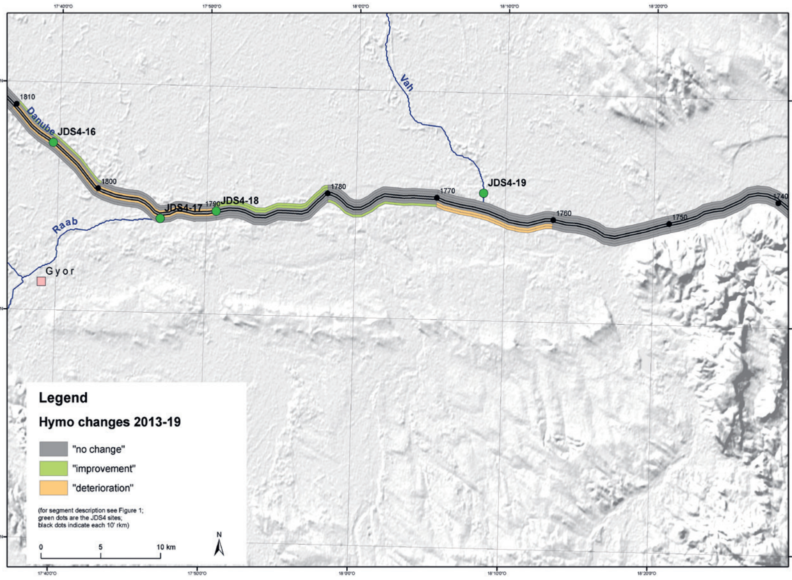


Figure 3: Example for detailed 'change' map: The border stretch SK-HU is characterised by the restoration of two larger side channels in SK and one floodplain improvement in HU. However, the ongoing deepening of the channel downstream of the Gabčikovo dam 'neutralise' from an international viewpoint the development. In Komárom new flood protection reduces the right floodplain area in Hungary.

Figure 4: Example for floodplain restoration near Deggendorf/Germany (Google Earth (2019): Satellite images worldwide. DigitalGlobe 2019. http://www.earth.google.com)

banks right/left and floodplain right/left) 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 find in HU, RS, BG, RO and UA.

The analysis of changes is based on the total number of recorded changes (73) to keep transparently all records sent by the countries (from 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.

The total lengths of all changes (73) 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. 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.

Most of the changes are related to riverbank development [parameters 6 & 7] with in total 34 changes *(fig. 1).* The removal of rip-rap clearly prevails with 23 cases. Side channel connections [9] as main improvements are rather frequent (8 times, out of other non-structural improvements in floodplains) 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.

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 *(fig. 2).*

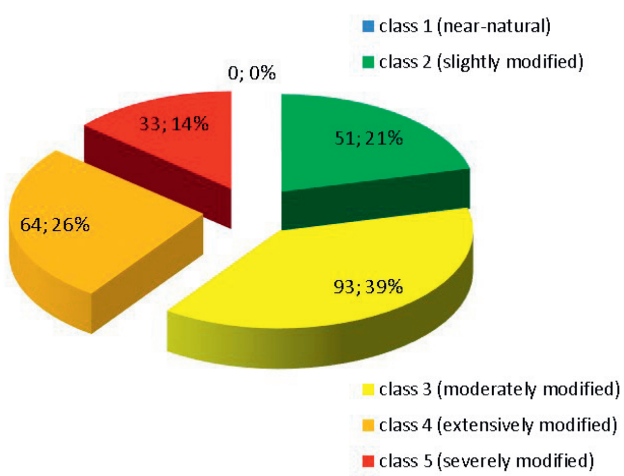
After screening and comparing the changes in detail (starting with major changes > 1 km length and by overlaying changes within one and the same segment), only two segments changed in overall assessment, two in the worse direction, but already having been close to poor assessments before *(fig. 5).* Those are the segments just downstream of Iron Gate II in Serbia (the bank assessment was reduced from three to class 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 fail to shift in a better class due to close boundaries, but being 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 though the barrier is assessed with as '3' for 'partial passable' (for fish but not for sediment).

The WFD 3-digit analysis for the entire Danube *(fig. 6)* indicates the general alteration similar to the overall assessment (prevailing classes 3-5 for the 241 10-km-segment), 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 functioning 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 Austrain 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 dams in Altenwörth and Ybbs-Persenbeug remain, but will be equipped within next years, 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 right 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.



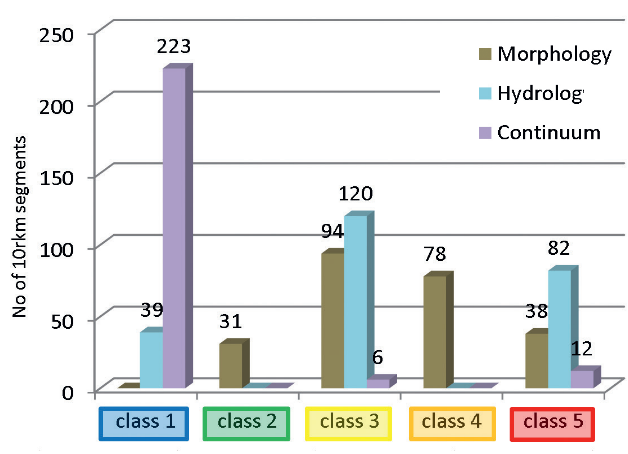


Figure 5 & 6: Above: Overall assessment of JDS4 as based on JDS3 with only slight changes (shift of two segments from class 3 to 4, no change in percentage). Below: WFD 3-digit assessment as based on JDS3, mainly changed for the continuity for fish by the construction of fish passes in AT (hydrology and continuum were assessed only in classes 1, 3 or 5).

In general, the recorded changes imply many improvements in the strongly altered Upper and partial the Middle Danube while on the Lower Danube a few deteriorations prevail, 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 instead two.

4. Conclusions

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 for the period 2013-2019.

Several small deteriorations (and renovation of already existing structures) as well as some improvements fall under the thresholds and cannot be considered for the overall assessment. In addition, the limited dredging data for various purposes (navigation, flood, commercial, and restoration) cannot be clearly addressed to obvious changes (compare evaluation by the DanubeSediment project, Habersack et al. 2019 & 2020). Therefore, 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.

To scope and fulfil the requirements as under the new CEN Standard (CEN 2020) 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 (Habersack et al. 2019 & 2020) delivered already many extremely valuable quantitative hydromorphological data including longitudinal profiles, channel incision stretches, historical comparisons and morphological river types and made first technical proposals how to assess sediment transport, to improve monitoring, both essential parts of the future hydromorphological assessment. Furthermore, it is recommended to take into consideration the Interreg Danube Transnational Programme Danube Floodplain project outcomes and related solutions for the improvement of floodplain connectivity with the river.

The continuation of restoration measures improving the hydromorphological conditions along the entire Danube is of great importance and monitoring and evaluation of previous restoration projects should be used to improve new projects. However fresh bank revetments and reinforcement or additional groynes should be managed to the absolute minimum and must be compensated by extensive restoration measures (banks and side-channels).

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JDS4: Biology and indication of ecological status

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With the data from Joint Danube Survey 4 (JDS4) for biological quality elements values for the indication of the ecological status for the sampling sites were estimated. Those results are not approved ecological status assessments for water bodies on national level as not all required WFD criteria could be met by the JDS design (e.g. not considering aspects regarding representative site selection, choice of sampling time in relation to season and discharge, selection of assessment indices). Additionally many water bodies of the Danube are designated as Heavily Modified Water Bodies – for them, on a binding national level other legal objectives, summarized in the ecological potential, come into place and replace the ecological status.

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, because 81% of sites (67% of samples) show an indication of good or high status. As also known from past surveys and TNMN (Transnational Monitoring Network of the ICPDR) 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 different picture: only 37% of the sites reach an indication of good status, pointing at hydromorphological deficits caused by a variety of pressures.

Fish

Most species of the reference communities can still be found at nearly all sites, even at hydromorphologically strongly altered 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 (Fish Index Slovakia): 11% of sites, EFI (European Fish Index): 23%, FIA (Fish Index Austria): 25%, see contribution of Pont et al. this issue). Those indices were not developed and are not suitable for the whole length of the Danube, however, the national assessments with the same data also show corresponding low 17% of the sites reaching the objective of good status.

Phytobenthos

The indicative status of benthic diatoms (index: Slovakian IPS - Indice de Polluosensibilité Specifique, Specific Pol-

Figure 1: MZB sampling at site with gravel in Upper Danube (© F. Wagner)

Figure 2: MZB sampling in Lower Danube (© M. Paunovic)

lution Sensitivity Index) 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 differ essentially from this indicative assessment, especially for the Lower Danube. Additionally, nutrient levels do not reflect the differences in phytobenthos assessment – diatoms are used particularly as indicators for nutrient pollution.

Macrophytes

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 two sites were classified as indication for moderate status.

Is the ecological status of the Danube improving?

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. Details can be found in the contribution of Schwarz (this issue) and in the final scientific JDS4 report.

Invasive alien species

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

Figure 3: Dikerogammarus villosus in the Upper Danube (© W. Graf)

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.

Regarding macrozoobenthos at some sampling sites invasive alien species reach extremely high abundances. For example, in the upstream reaches of the Danube the genera Dikerogammarus sp. and Echinogammarus sp. accounted for 99% of species diversity and biomass. The invasive crayfish *Faxonius limosus* was present along the entire Danube, with larger abundance in Lower Danube. 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, but was detected only in low densities during JDS4.

Figure 4: Sampling fine sediment for DNA analysis of sediment inhabiting invertebrates (© F. Wagner)

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.

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.

For benthic invertebrates, the sites were compared by using the Austrian SI (saprobic index) and MMI (multi-metric-index). Both indices were calculated with species data originating from classical MHS sampling (multi-habitat-sampling), DNA from bulk samples (like classical samples – all material mixed together) and 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 did not deliver abundance estimates but presence/absence-values). 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 to 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. For the MMI the identical status classes identified by the three different methods is few percent lower but follows the same pattern as described above for the SI.

For the information if the site reaches or fails the quality objective of the WFD – the good ecological status – the accordance between classical sampling and molecular methods is even higher and reaches up to 93%.

For 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. The results are astonishingly close together and when looking at the index values they are even closer.

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 more details see the final scientific JDS4-report at: http://www.danubesurvey.org/jds4/publications/scientific-report

Invasive alien species of macroinvertebrates along the Danube River – JDS4 screening

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Introduction

Several international Danube surveys have proven that invasive alien species (IAS) have a profound influence on native biodiversity of the Danube River Basin (DRB) (Zorić et al. 2014, 2015, Borza et al. 2015, ICPDR 2015, Csányi et al. 2021, Trichkova et al. 2021). The Danube River connects the Black Sea Basin to Western Europe as dominant water route of the 'Southern Invasion Corridor', forming the European Invasion Network (Panov et al. 2009). The spread and expansion of IAS can happen in both directions: upstream and downstream. According to the origin of invasive species, some of them are alien to Europe, others are native to Europe (outside the Ponto-Caspian region), while significant share of these taxa has Ponto-Caspian origin. The latter are in immediate hydrological connection with their native area. Several species of macroinvertebrates (mainly belonging to the crustaceans) and fish (Gobiidae) expanded their range from the Black Sea area and the Lower Danube to the Middle and Upper Danube River during the last decades and appeared in new habitats, even as far as Western European rivers (Bij de Vaate et al. 2002). Considering the importance of IAS in terms of the implementation of the Water Framework Directive, a specific IAS program has been developed and implemented during Joint Danube Survey 4 (JDS4) at regional and national levels (Csányi et al. 2021, Trichkova et al. 2021). The evaluation of the dataset collected during the survey is described here with special attention to the distribution of macroinvertebrate species alien to the Danube River Basin.

Materials and Methods

The survey was conducted between July and October 2019 at JDS4 sites and additional sites in the Danube River, tributaries and adjacent standing water bodies (e.g. 82 sites in Bulgaria). The main data referring to IAS was gathered from the original dataset related to different biological quality elements collected during the JDS4 program. The overall harmonized sampling methodology for macroinvertebrates was based on the Multi-Habitat-Sampling (MHS) procedure (AQEM Consortium 2002) but 'Kick and Sweep' (K&S) sample collection and LiNi crayfish traps were applied as well (Liška et al. 2021). In order to collect detailed, high-quality data for IAS, some additional sampling methods were used at the Bulgarian, Hungarian and Serbian Danube River sections: deep-water dredging and additional sampling effort for mussel collection, and electrofishing, dip nets and detailed hand searching for crayfish collection (Csányi et al. 2021, Trichkova et al. 2021). Data on macroinvertebrate IAS were analyzed according to ICPDR guidance document on IAS relevant to the DRB (Paunović & Csányi 2018). Data from (e)DNA sampling related to macroinvertebrate IAS were also considered in the analysis (Liška et al. 2021).

Results

A total of 35 macroinvertebrate IAS taxa were detected in the Danube River and the studied tributaries and adjacent standing water bodies, using all sampling methods, during JDS4. Compared to previous JDSs, this number has increased almost three times: 12 (JDS1, 2001), 20 (JDS2, 2007) and 34 (JDS3, 2013) (Csányi et al. 2021). Three of these species, the crayfish *Faxonius limosus, Pacifastacus leniusculus* and *Procambarus clarkii,* are IAS of European Union concern, while the rest are IAS of DRB concern. In terms of origin, the species are native to North America (6 species), Asia (4), New Zealand (1), Africa (1), Europe outside Ponto-Caspian region (1), and Ponto-Caspian Region (22). The results show that similar to previous JDSs the invasive species of Ponto-Caspian origin represent the most numerous group and they also dominate in abundance. During JDS4, 393 macroinvertebrate taxa were detected in total in the Danube River by the MHS method. The 17 most abundant taxa provided 80% of the total abundance value. Further, the first seven most abundant species have Ponto-Caspian origin and they represent more than 60% of the total abundance of the overall collected macroinvertebrates. Based on the combined data on macroinvertebrate and fish IAS, 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 by IAS in the Lower Danube River is explained by the fact that Ponto-Caspian species are considered native in this section (Csányi et al. 2021). The integrated biocontamination by type of water bodies for Bulgaria (Lower Danube) ranged from moderate in the shoreline zone of the Danube River, through moderate to high in the canals and lakes adjacent to the Danube River, to severe in the Danube tributaries and studied reservoirs (Trichkova et al. 2021). More detailed information is presented below for some frequently found and abundant macroinvertebrate IAS in the DRB.

Invasive macroinvertebrate species alien to the DRB

Pectinatella magnifica (Bryozoa): The presence of this North American bryozoan species *(fig. 1)* is well known in the Middle Danube since 2011. It was detected later in the backwater section of the Iron Gate I (Zorić et al. 2015). The MHS method did not indicate its presence in the Danube during JDS4. Only K&S and hand search was able to prove its presence in the Hungarian Ráckevei-Soroksári Danube, at JDS4 site at Tass, at the downstream end of this Danube side arm. The species was recorded at two sites in Bulgaria, which are the first records of this species in the Bulgarian shoreline zone of the Danube River (Todorov et al. 2020).

Figure 1: Magnificent bryozoan Pectinatella magnifica, Danube River at VidinTown (Bulgaria), 23.10.2019 (© Teodora Trichkova)

Potamopyrgus antipodarum (Gastropoda): This small snail species coming from New Zealand has been previously abundant along the Upper and the Middle Danube River sectors. During JDS4, it was detected only in the German Danube section in large numbers and one specimen in Hainburg by the MHS method. Present results show that it may be absent from the large part of the Danube River.

Sinanodonta woodiana (Bivalvia): The Chinese pond mussel *(fig. 2)* was detected only at six locations out of the totally sampled 36 sites during JDS4 using the MHS method. One location was at Pancevo, all the others were downstream of the Iron Gate in the Lower Danube. High abundance of the species within one AQEM sample (40 individuals) was detected at Bazias in Romania. In Bulgaria, the species had comparatively low frequency of occurrence (17.86%, out of 38 sampled sites, by dredging) in the Danube River, but much higher in the tributaries (45% out of 28 sampled sites). However, the abundance of the species was not high. In comparison, during JDS3, totally 143 individuals were found at 25 out of 52 sites sampled in the Danube River.

Figure 2: Chinese pond mussel Sinanodonta woodiana (Bulgaria), 15.08.2019 (© Milcho Todorov)

Corbicula fluminea (Bivalvia): Only two JDS4 sites sampled by the MHS method showed high individual numbers of the Asian clam (Kelheim: 200 individuals, and Rudujevac / Gruia – Romanian side: 602 individuals). The total number of individuals collected by the MHS method was 909. An overall decrease in the former abundance of C. *fluminea (fig. 3)* was reported in some Danube River sections during JDS4 compared with previous JDSs when three different sampling methods (K&S, MHS and deep-water dredging) were used. In Bulgaria, the sampling for macroinvertebrates in the Danube River was carried out by dredging at two levels of water depths: at 0-2 m and at 2-4.5 m. At the depths of up to 2 m, although with lower values than the native gastropods, the Asian clam had the highest frequency (53.57%) and relative abundance (4.35%) compared to all other mussels. At depths of 2-4.5 m the species had the highest frequency (90.91%, found at 10 of 11 studied sites) and the highest relative abundance (76.46%) compared to all other species. We observed unusual massive mortality of this species during the survey, especilly in July 2019. Large amounts of soft tissues flowed in the water, while numerous shells and dying individuals were stranded within shallow disconnected pools. This could be owed to abrupt changes in water level in combination with other factors. In the Danube tributaries, the Asian clam occurred most frequently (85%) and showed the highest relative abundance (70.66%) among all macroinvertebrate species.

Figure 3: Asian clam Corbicula fluminea, Voinishka River at Dunavtski Village (Bulgaria), 16.08.2019 (© Milcho Todorov)

Dreissena rostriformis bugensis (Dreissenidae): The quagga mussel *(fig. 4)* was found at only one location by the MHS method (Ilok / Backa Palanka, left – Serbian side). The plausible explanation is the high-water level that made it impossible to approach the stable mussel colonies during the sampling period in July. In the Bulgarian Danube, the species was recorded at the two sampling depths by dredging, with higher frequency of occurrence at the higher depths: 14.29% up to 2 m, and 27.27% at depths of 2-4.5 m. Its relative abundance at the higher depths ranked second after this of the Asian clam, although with a much lower value (14.57%).

Figure 4: Quagga mussel Dreissena rostriformis bugensis, Ogosta Reservoir, Montana Town (Bulgaria), 26.10.2019 (© Teodora Trichkova)

Faxonius limosus (Decapoda): The North American spiny-cheek crayfish *(fig. 5)* was detected only at two sites by the MHS method: Banatska Palanka / Bazias and Novo Selo. However, additional efforts and methods (e.g. LiNi traps, dip nets) showed different results. In the Hungarian Danube, the species was frequently found, e.g., it was detected at all sites (nine sites, 21 individuals) by using the LiNi traps. It also had the highest abundance among all crayfish species (one native and three IAS), using all sampling methods *(fig. 6)*. In the Bulgarian Danube River sector, the American spiny-cheek crayfish was found at only one site, and its relative abundance was close to that of the native *Pontastacus leptodactylus*. However, in the tributaries, the frequency of occurrence and relative abundance of this species was almost two times higher than the native crayfish.

Figure 5: Spiny-cheek crayfish Faxonius limosus, Danube River at Vidin Town (Bulgaria), 24.10.2019 (© Milcho Todorov)

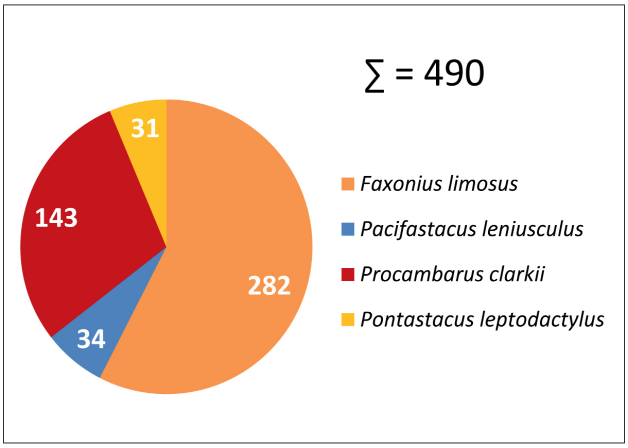

Figure 6: The percentage abundance of three invasive alien crayfish species (Faxonius limosus, Pacifastacus leniusculus and Procambarus clarkii) and one native species (Pontastacus leptodactylus) detected in the Hungarian Danube by different sampling methods.

Pacifastacus leniusculus (Decapoda): The North American signal crayfish was not detected by the MHS method but the LiNi traps proved its presence in the Upper Hungarian Danube at two sites: Mosoni Danube at Vének and Danube at Gönyű. Electrofishing and hand search sampling resulted in 31 specimens at these two sites, including one specimen that was found at Szob, detected by hand search. The latter record indicated the spread of the signal crayfish downstream along the Danube River.

Procambarus clarkii (Decapoda): The North American red swamp crayfish was detected only in the Hungarian section, mainly around Budapest. A total of 143 individuals were collected by all sampling methods. The results show that this species has spread within a hundred km long section in the Danube River, occurring from Dunaföldvár downstream to Paks.

Invasive macroinvertebrate species of Ponto-Caspian origin

Clathrocaspia knipowitschii (Gastropoda): This snail *(fig. 7)* is the newest invader in the Middle Danube River. It was detected for the first time during JDS3 at Vrbica-Simian cross section by deep-water dredging. During JDS4 it was recorded using the same method at a new locality in Hungary at Gönyű, on the Slovakian side. However, the (e)DNA bulk sample showed the presence of this species upstream of this location, at Medve. Detailed search for this species requires long-lasting low water level and deep-water dredging because the changing water level and discharge makes it difficult for this small snail to colonize the littoral zone. The recent data show that it is widespread in the Middle Hungarian Danube, between Gönyű and Budapest (Csányi et al. in press).

Corophiidae (Amphipoda): Three corophiid IAS were recorded during JDS4. *Chelicorophium curvispinum* was the most abundant almost everywhere, except in the Middle Danube, while *C. robustum* and *C. sowinskyi*

Figure 7: Juvenile Clathrocaspia knipowitschii at Gönyű, Hungarian Danube, April 2021 (© Béla Csanyi)

were rarely found (at seven and four sites, respectively). During JDS3, the abundance of these species was much higher.

Gammaridae (Amphipoda): Three main genera were dominant along the Danube River according to the JDS4 results. The genus *Dikerogammarus* was represented by three species, of which *D. villosus* and *D. haemobaphes* were widespread, while *D. bispinosus* was detected mostly in the Middle Danube, being totally absent from Paks. The second genus was represented by *Obesogammarus obesus.* Large proportion of the members of the genus *Echinogammarus* was not determined to species level in the Austrian section, although their individual number represented more than 20% of the total macroinvertebrate abundance. *Echinogammarus ischnus* ranked second in relative abundance (more than 10%). The latter two taxa were distributed only along the sector Jochenstein – Bratislava, reaching around 30% of the total macroinvertebrate abundance. The abundance of the gammarid taxa was much lower compared to JDS3.

Conclusions

JDS4 confirmed the results of previous surveys that the Danube River and its tributaries are under considerable influence of biological invasions. The number of recorded macroinvertebrate IAS has increased three times compared to JDS1 (2001). The level of biocontamination of the Danube River was estimated from moderate to high. Although the biocontamination index was lower in some sectors of the Danube, e.g., from low to moderate in the Lower Danube, the IAS pressure in the Danube tributaries and the adjacent standing water bodies was much higher as some of the IAS find suitable habitats and establish abundant populations in these water bodies.

The JDS4 sampling experience concerning several taxonomic IAS groups (e.g., Decapoda, Gastropoda, Bivalvia) showed that the datasets were not homogenous. For future IAS monitoring programs, the development of training programs is recommended, as well as the adaption and application of additional efforts and methods of sampling, which may be more efficient for IAS early detection related to particular group of species and habitats. The comprehensive assessment of the IAS pressure on aquatic communities will provide valuable information and support for the implementation of the national and EU IAS and water policies in the DRB.

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An eDNA metabarcoding survey of fish communities along the Danube river and its tributaries[1]

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[1]This article is a shortened version of the according chapter in the Scientific report on the Joint-Danube Survey 4 (Pont et al. 2019)

Abstract

Water samples were collected at 29 Danubian River sites and 18 tributaries, and their fish-eDNA contents 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 are identified at the species level. Further, six taxa groups each comprising of two to three species of the same genus were built, as well as five taxa groups each comprising of 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 – in particular sturgeons. Relative abundance of sequence reads per site allowed to describe the longitudinal structure of the fish community efficiently.

Introduction

In complement to the traditional fish survey along the Danube, a fish eDNA metabarcoding-based survey has been implemented along the Danube River at 20 sites within the framework of the JDS4 monitoring programme organised by ICPDR and DNAqua-Net. A collaboration with the INTEREG project MEASURES (DTP2-038-2.3) and support from the Austrian Federal Ministry of Agriculture, Regions and

Figure 1: Location of sampling sites along the Danube (29 sites, red circles) and on tributaries (18 sites, black triangles) near their confluence with the Danube.

Tourism (BMLRT) and the ÖK-IAD (Österreichisches Komitee der Internationalen Arbeitsgemeinschaft Donauforschung) allowed to sample 9 and 17 additional sites respectively on the Danube itself and the main tributaries *(see fig. 1).*

Methods

For the 29 Danube sampling sites, 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 sampling site 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 respectively 22.34 min and 28.73 L (3 to 40 L) 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 (Polymerase Chain Reaction) 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. 2021, JDS4) 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 kilometers) *(see fig. 1).*

All statistical analyses were conducted in R, version 3.3.3 (R Core Team, 2018).

Results and discussion

Species inventory

No DNA amplification could be obtained from the Inn river samples, although additional eDNA testing was re-run to ensure no inhibition existed. Sites downstream of its confluence in Austria (in particular JDS4-6 and JDS4-10) also showed a very low number of detections compared to

Species Names	Abbreviation	SpeciesNames	Abbreviation
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			Aci_1
Hypophthalmichthys molitrix / Ctenopharyngodon idella			Alos_2
Ballerus sapa / Blicca bjoerkna / Vimba vimba			Car_Spp
Gobio gobio / Romanogobio albipinnatus / R. kesslerii / R. vladykovi			Gym_spp
Leuciscus idus / L. leuciscus / Pelecus cultratus			Sal_spp
			Sam_spp

Table 1: List of taxa detected. Species unknown from the Danube catchment (false positive) excluded.

other sites. At its confluence, the Inn has a mean discharge comparable to that of the Danube and probably much more 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 the detection of a number of taxa comparable to the other Danubian sites.

80 taxa were detected from a total of 35,060,453 sequence reads. At nine sites basically located downstream of large cities and wastewater input, 19 taxa (4.7% of the total number of sequence 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 had more than one of these taxa: Arges and Russenski Lom tributaries, Vienna (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. Also, one occurrence of Alosa spp. on the Upper Danube (Oberloiben) was omitted. Of the remaining 61 taxa, 50 taxa are identified at the species level, six taxa correspond to two to three species of the same genus, and five taxa two to three species of different genera *(tab. 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. Hence, the 61 taxa detected correspond to 61 to 79 species (i.e. some taxa comprise of 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.

Longitudinal organisation of fish communities

The longitudinal distribution of fish species *(fig. 2 and 3)* 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 at the furthest downstream site (Danube delta). The species richness tended to increase from upstream to downstream whereas the diversity showed a sharp decrease from downstream

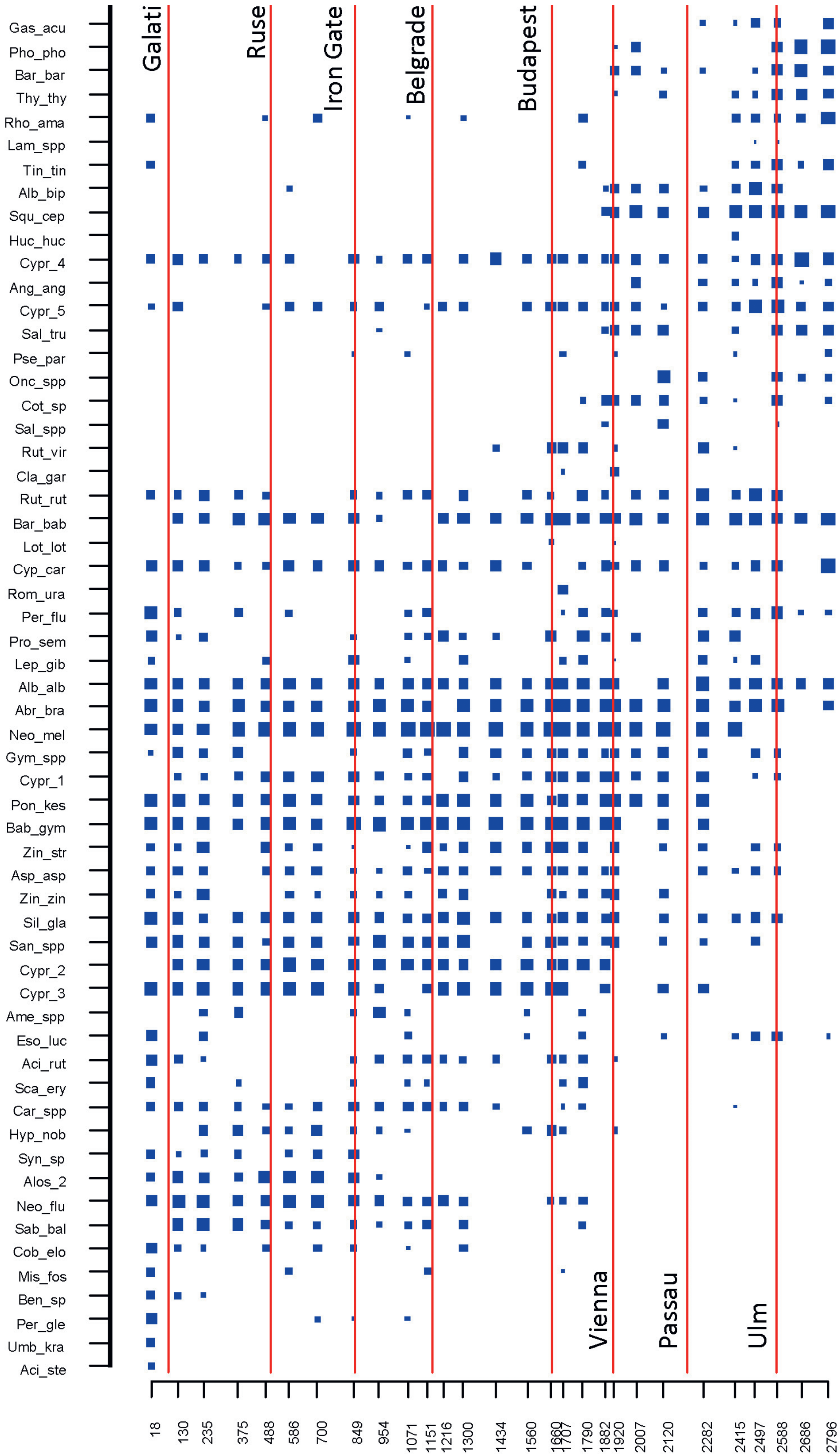

Figure 2: Relative abundance of the 57 taxa detected along the Danube River, from rkm 18 to rkm 2796. The size of the square is a function of the relative abundance of the corresponding taxa in the sample at a given site (see Table 1 for corresponding taxa names). The sites are located at rkm: 2796, 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.*

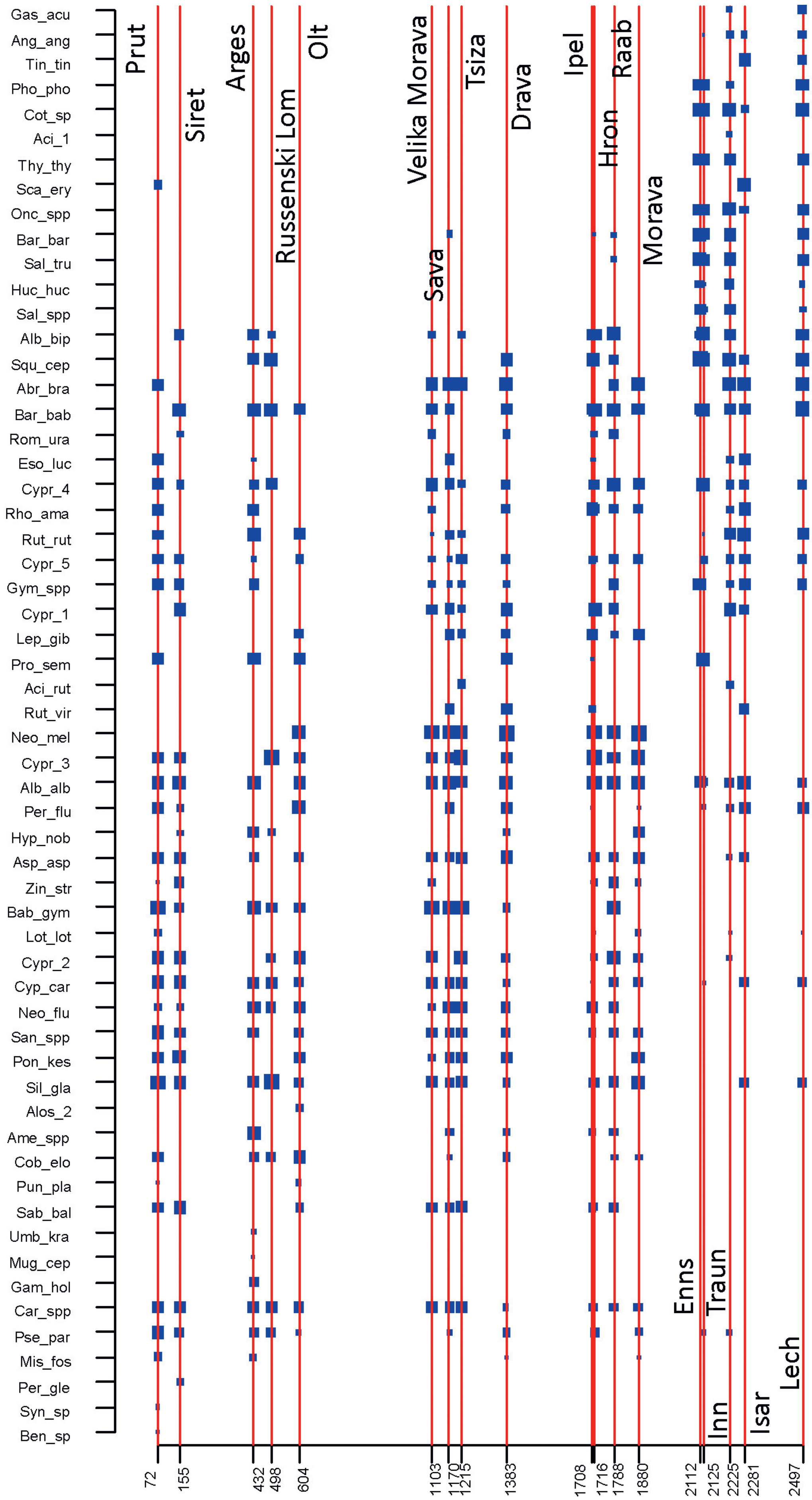

Figure 3: *Relative abundance of the 59 taxa detected along the 18 tributaries of the Danube River (rkm 72 to rkm 2497). The size of the square is a function of the relative abundance of the corresponding taxa in the sample (see Table 1 for corresponding taxa names).*

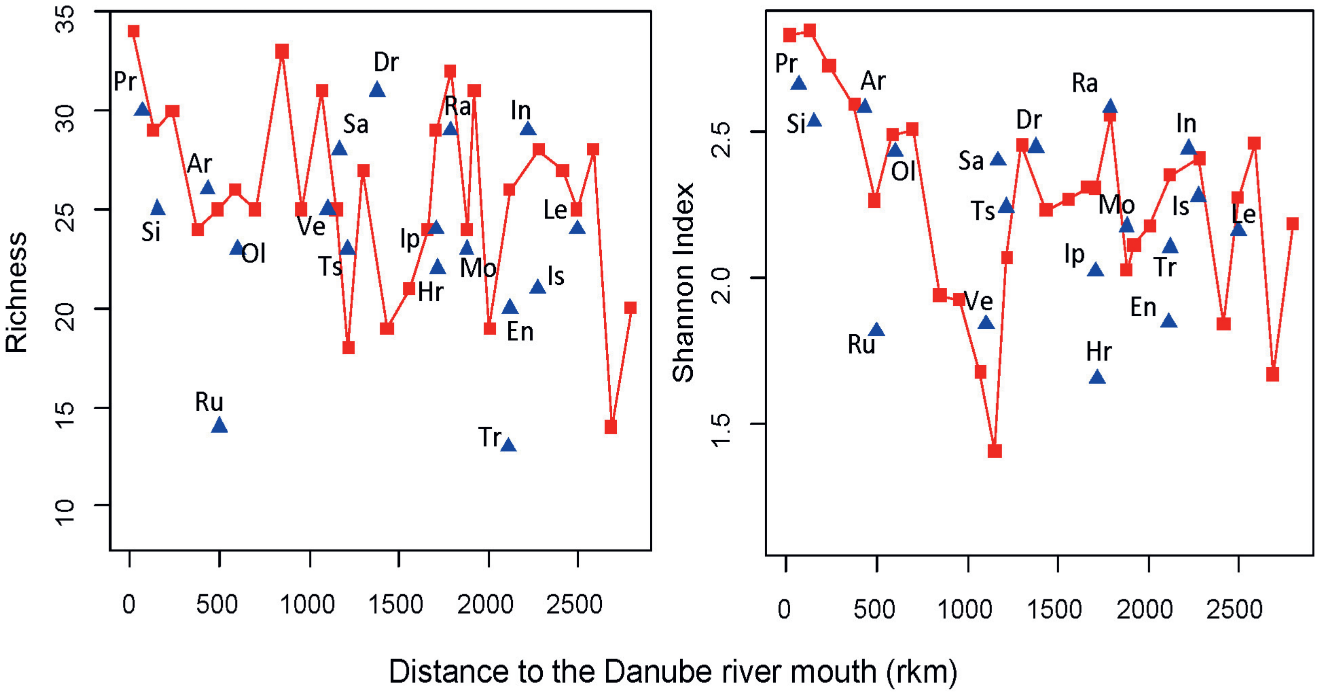

Figure 4: *Changes in species richness and diversity (Shannon Index) along the Danube (red) and in major tributaries (blue). Tributary names from upstream to downstream: Lech (Le), Isar (Is), Inn (In), Traun (Tr), Enns (En), Morava (Mo), Raab (Ra), Hron (Hr), Ipel (Ip), Drava (Dr), Tsiza (Ts), Sava (Sa), Velika_Morava (Ve), Olt (Ol), Russenski_Lom (Ru), Arges (Ar), Siret (Si), Prut (Pr).*

Pancevo (rkm 1151) to upstream_Timok (rkm 849), including the Velika Morava River *(fig. 3).*

Comparison with JDS4 traditional fish survey (TFS)

69 and 57 taxa 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).*

The relative abundance (based on individuals or biomass and sequence reads, respec-

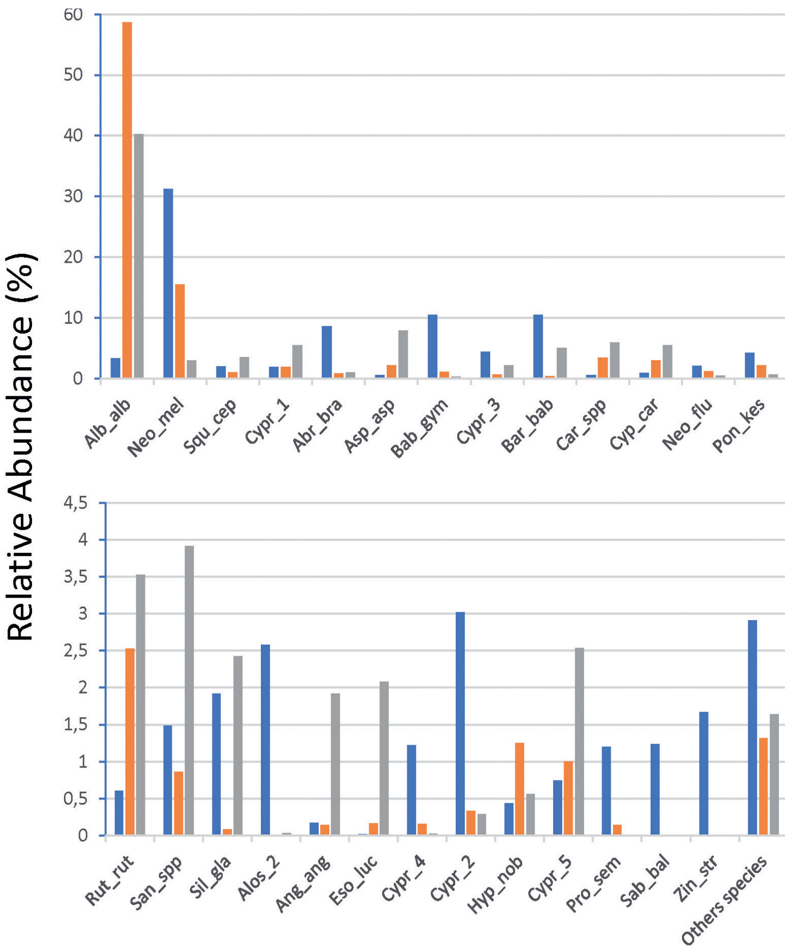

Figure 5: *Mean relative abundance of taxa detected by eDNA (blue). Mean relative abundance (orange) and mean relative biomass (grey) of species caught by TFS. Only the 26 most abundant species (> 1%) detected among the 13 common Danube sites are individually represented.*

tively) 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.

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 similar, 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) hamper successful eDNA metabarcoding application

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Chemical pollution in the Danube River Basin: critical review based on the outcomes of JDS4

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Each Joint Danube Survey (JDS) is bigger than the previous one in terms of number of laboratories involved, parameters measured, data produced and state-of-the-art scientific challenges tackled. Summarising the outcomes, it can be stated with confidence that JDS4 is indisputably the biggest river basin survey ever globally. An attempt has been made here to summarise outcomes of its chemical part.

According to the EU Water Framework Directive (WFD 2000), priority substances (PS; EQSD 2013) 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, waste water and ground water 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 and ca. eight million of suspect analyses has been compiled. In comparison, 719 substances were screened for, and ca. 47,000 data entries were generated in JDS3 in 2013 (Liska et al. 2015). When analysing the data, six questions inadvertently arose.

Why are WFD priority substances and River Basin Specific Substances not 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. The concept of monitoring WFD PS 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. The outcomes of JDS4 showed that only three WFD PS (Perfluorooctanesulfonic acid (PFOS), cybutryn, cypermethrin) were exceeding their Environmental Quality Standard (EQS) values. Elevated concentrations could be detected only for three EU Watch list substances: the pharmaceutical diclofenac, natural hormone 17-beta-estradiol and insecticide imidacloprid. Similarly, in biota, only mercury and flame retardants (brominated diphenyl ethers (BDEs)) were exceeding EQS values in all samples, whereas the rest of legacy substances were not of a basin-wide concern.

The findings of JDS3 (2013) and JDS4 (2019) indicate that WFD-compliant monitoring of all PS 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 EU level.

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. 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.

The high-resolution mass spectrometry (HRMS) techniques typically detect 2,000-5,000 substances and their transformation products (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 Digital Sample Freezing Platform (DSFP; Alygizakis et al. 2019) and are available for retrospective analysis indicating compound's presence/absence and providing semi-quantitative concentration. At the time of reporting results of JDS4, the number of substances which had been searched for in each sample was 65,960 and it is expected that the same samples could be screened for more than 106,000 substances in early 2021; without a need for additional sampling!

The use of these retrospective screening techniques might prevent argumentation of some industries claiming that their products/substances are safe and cannot be found at ecotoxicologically relevant concentration levels in the environment. In such cases, the results can be directly used in support of the EU Chemicals Strategy, REACH regulation (Registration, Evaluation, Authorisation and Restriction of Chemicals) 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, 2020) 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 toxicity threshold 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 the Limit of Quantification (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 toxicity threshold values).

The above approach does not account for mixture (chemical cocktails) toxicity effects. Therefore, a complementary prioritisation of substances was applied in JDS4, using 'toxic units' (TU) - sum of 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. TU assessment for surface water in JDS4 showed the importance of pesticides such as pyrethroids, organophosphate and a 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 drive r for daphnia. For algae, different herbicides such as terbutryne, MCPA, cybutryne, diuron, metolachlor or nicosulforon dominated the ranking of compounds based on TU.

The two above complementary prioritisation approaches often bring to attention the same compounds, however, the outcomes of 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.

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 liquid chromatography-HRMS (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.

There was also a concern that non-target screening (NTS) and effect-based methods (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. The results of the suspect screening of compounds spiked in an extract of a reference natural water sample were quite promising. Regarding EBM, it has been concluded that currently used methods are powerful tools to discriminate low-toxicity from more toxic samples (wastewater treatment plant (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.

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 (https://www.solutions-project.eu/). The in vitro battery was applied on JDS4 wastewater effluent samples. Additionally, a high-throughput high performance thin layer chromatography (HPTLC) methodology with four bioassays has been used by LW Langenau as an example of a rapid EBM screening tool. 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 water pollution over an extended period of time; and it gave a representative picture of the surface water quality in summer 2019. The JDS4 results has shown that the spatial variability of investigated hydrophobic PS in surface water of the Danube is low. No deterioration of Danube surface water contamination by hydrophobic PS was observed in JDS4 in comparison with the results from JDS3. Among investigated organochlorine compounds and polyaromatic hydrocarbons (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 para-para-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 was 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 revealed application of pesticides in agriculture as the main contamination source.

Passive samplers (sometimes nicknamed as ‘plastic fish’) were installed at the same sites from where fish samples were collected for the follow up chemical analyses. It has been demonstrated that 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.

Conclusions and future perspectives

The results of JDS4 have shown that only a handful of WFD PS and surface water Watch List substances were posing a threat to Danube fauna and flora. A potential of wide-scope target and suspect screening techniques, together with EBM, to be used in regulatory monitoring has been demonstrated. Chemical screening data were used for drafting a list of candidate Danube RBSPs in surface water and biota.

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 waste water effluent samples indicated that inefficient treatment in WWTPs across the basin is among the main sources of DRB chemical pollution. EBM tools have been used for measurements of toxicity effects of mixtures of chemicals and effectiveness of their use was demonstrated for waste water and more polluted surface water samples. Waste water 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). JDS4 provided a possibility to test at a large geographical scale how the revised EU UWWTD might work in practice.

Passive sampling results have shown that the spatial variability of investigated hydrophobic priority substances in surface water of the Danube is low and that the technique should be considered in the EU regulatory monitoring framework. Similarly, pollutants in ground water 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.

Interlinking chemical screening and EBM data with results of biological monitoring, and especially eDNA remains 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.

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News and reports from the Expert Groups

Macrophytes

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One of the Expert Groups of IAD is EGM, the group working on 'Aquatic Macrophytes', which are vascular plants, also called 'Higher Plants', in contrast to e.g. mosses or algae. Its scope regards, i.a., the composition of aquatic plants in different aquatic environments.

The main activities were focused on a survey of macrophytes in Lake Neusiedl, known as a 'Shallow Lake' (max depth of 1,5m in some parts, in the past). It is located in the steppe regions of the 'Little Hungarian Plain', shared between Austria and Hungary. EGM-Partners surveyed the macrophytes in three important regions of the lake which, in the end, will be compared with historical data. At present much detail has been worked on, but final presentation will be in the next year. To finalise our work on development of the lake's aquatic plants, surveys had been performed from 1998 to 2020, of course with intervals.

Upcoming activities of EGM will be based on older studies on aquatic plants in different water bodies, which had been studied in the past. That may help to prepare a basis for future investigations. The topic is focused on floodplain water bodies e.g. on the Lainsitz River in its Austrian section at Gmünd, close to the Czech border. Two earlier surveys had shown the dynamics of the oxbow lakes, but dryer climatic conditions had adverse effects, e.g. excessive progress of common reed against the water plants. Work on other floodplain water bodies may follow in the future.

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Hydrological catchment of the River Danube

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