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.