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)*

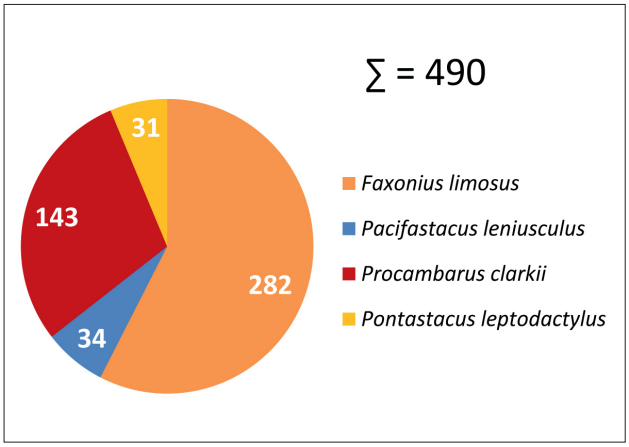

Figure 6: The percentage abundance of three invasive alien crayfish species (Faxonius limosus, Pacifastacus leniusculus and Procambarus clarkii) and one native species (Pontastacus leptodactylus) detected in the Hungarian Danube by different sampling methods.

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