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.

References

Bij de Vaate A, Jazdzewski K, Ketelaars HA, Gollasch S, Van der Velde G (2002): Geographical patterns in range extension of Ponto-Caspian macroinvertebrate species in Europe. Can. J. Fish. Aquat. Sci. 59 1159–1174 https://doi.org/10.1139/f02-098

Borza P, Csányi , Huber T, Leitner P, Paunović M, Remund N, Szekeres J, Graf W (2015): Longitudinal distributional patterns of Peracarida (Crustacea, Malacostraca) in the River Danube. Fundam. Appl. Limnol. 187 113–126 https://doi.org/10.1127/fal/2015/0769

Csányi B, Weipeth A, Zorić K, Brammer V, Borza P, Trichkova T, Weigand MA, Cardoso AC, Očadlík M, Bubikova K, Stanković I, Todorov M, Botev I, Kenderov M, Hubenov Z, Paunović M (2021): Invasive alien species. (in: Liška I, Wagner F, Sengl M, Deutsch K, Jaroslav S, Paunović M (eds), Joint Danube Survey 4 Scientific Report: A Shared Analysis of the Danube River. ICPDR – International Commission for the Protection of the Danube River, Vienna 93–106

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

Liška I, Wagner F, Sengl M, Deutsch K, Jaroslav S, Paunović M, eds. (2021), Joint Danube Survey 4 Scientific Report: A Shared Analysis of the Danube River. ICPDR – International Commission for the Protection of the Danube River, Vienna

Panov VE, Alexandrov B, Arbačiauskas K, Binimelis R, Copp GH, Grabowski M, Lucy F, Leuven RS, Nehring S, Paunović M, Semenchenko V, Son MO (2009): Assessing the risks of aquatic species invasions via european inland waterways: From concepts to environmental indicators. Integr. Environ. Assess. Manag. 5 https://doi.org/10.1897/IEAM_2008-034.1

Paunović M, Csányi B (2018): Guidance document on Invasive Alien Species (IAS) in the Danube River Basin, version March 2018; ICPDR – International Commission for the Protection of the Danube River, pp 61

Todorov M, Kenderov M, Botev I, Hubenov Z, Trichkova T (2020): First records of Pectinatella magnifica (Leidy, 1851) (Bryozoa: Plumatellida: Pectinatellidae) in the Bulgarian shoreline zone of the Danube River. Acta Zool. Bulg. 72, 507–515

Trichkova T, Todorov M, Kenderov M, Hubenov Z, Botev I, Stefanov T, Georgiev D, Jurajda P (2021): Invasive alien species of benthic macroinvertebrates and fish in the Bulgarian sector of the Danube River – results of the Joint Danube Survey 4 (JDS4). Water (Submitted)

Zorić K, Simonović P, Dikanović V, Marković V, Nikolić V, Simić V, Paunović M (2014): Checklist of non-indigenous fish species of the River Danube. Arch. Biol. Sci. 66 https://doi.org/10.2298/ABS1402619Z

Zorić K, Szekeres J, Csányi B, Kolarević S, Marković V, Paunović M (2015): Distribution of the non-native Bryozoan Pectinatella magnifica (Leidy, 1851) in the Danube River. Acta Zool. Bulg. 67 (2) 241-247

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

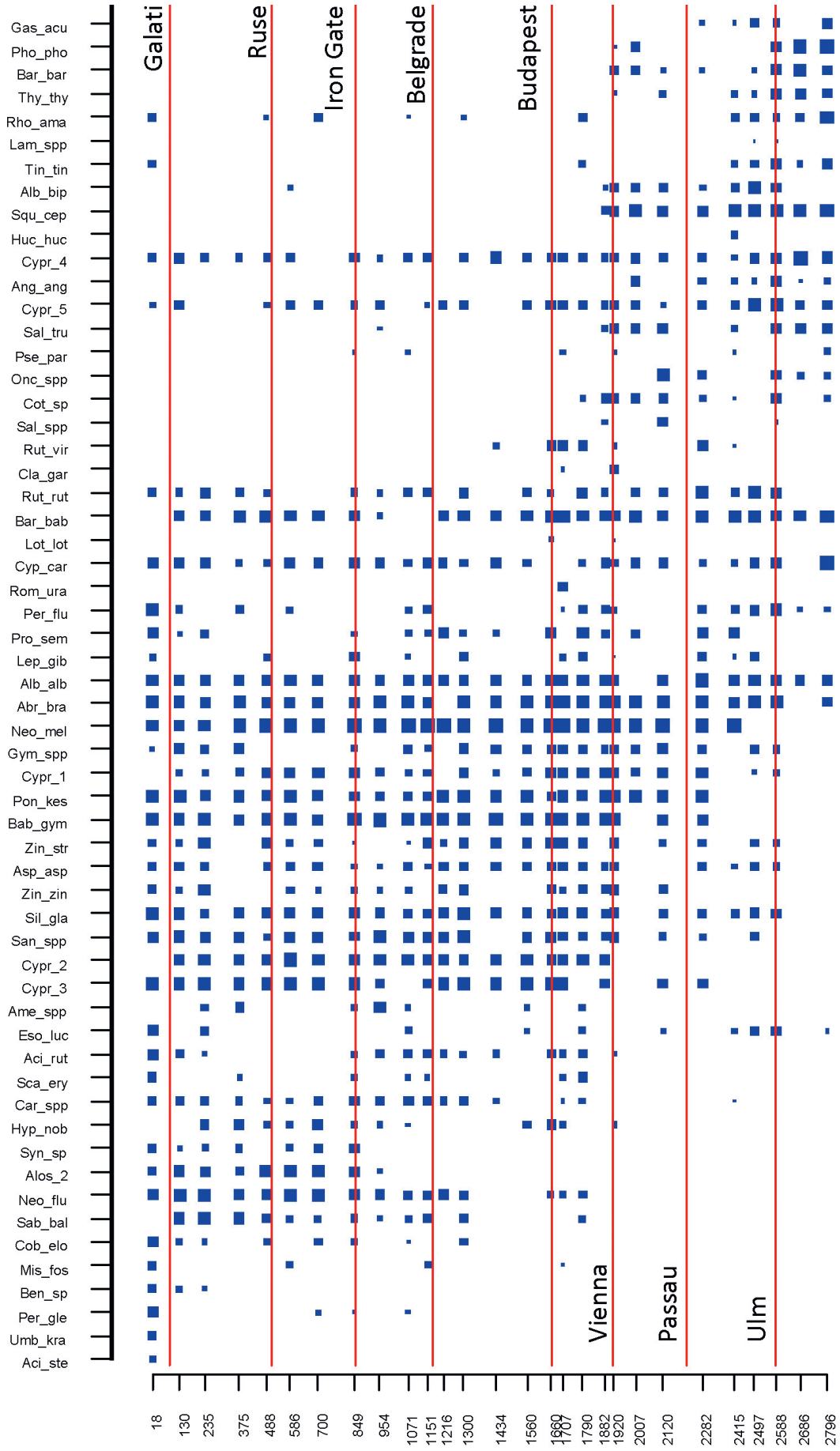

Figure 2: Relative abundance of the 57 taxa detected along the Danube River, from rkm 18 to rkm 2796. The size of the square is a function of the relative abundance of the corresponding taxa in the sample at a given site (see Table 1 for corresponding taxa names). The sites are located at rkm: 2796,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

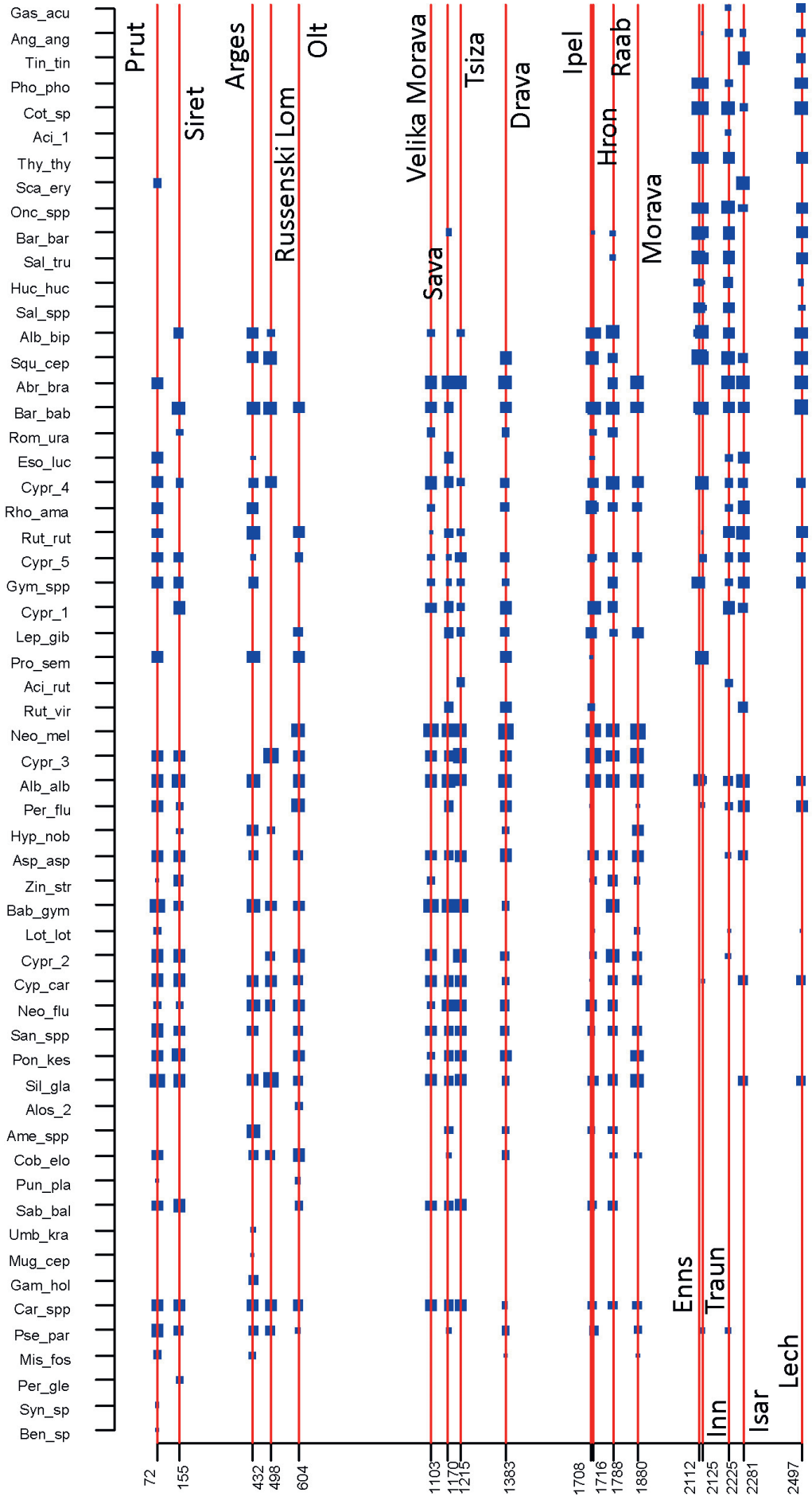

Figure 3: Relative abundance of the 59 taxa detected along the 18 tributaries of the Danube River (rkm 72 to rkm 2497). The size of the square is a function of the relative abundance of the corresponding taxa in the sample (see Table 1 for corresponding taxa names).

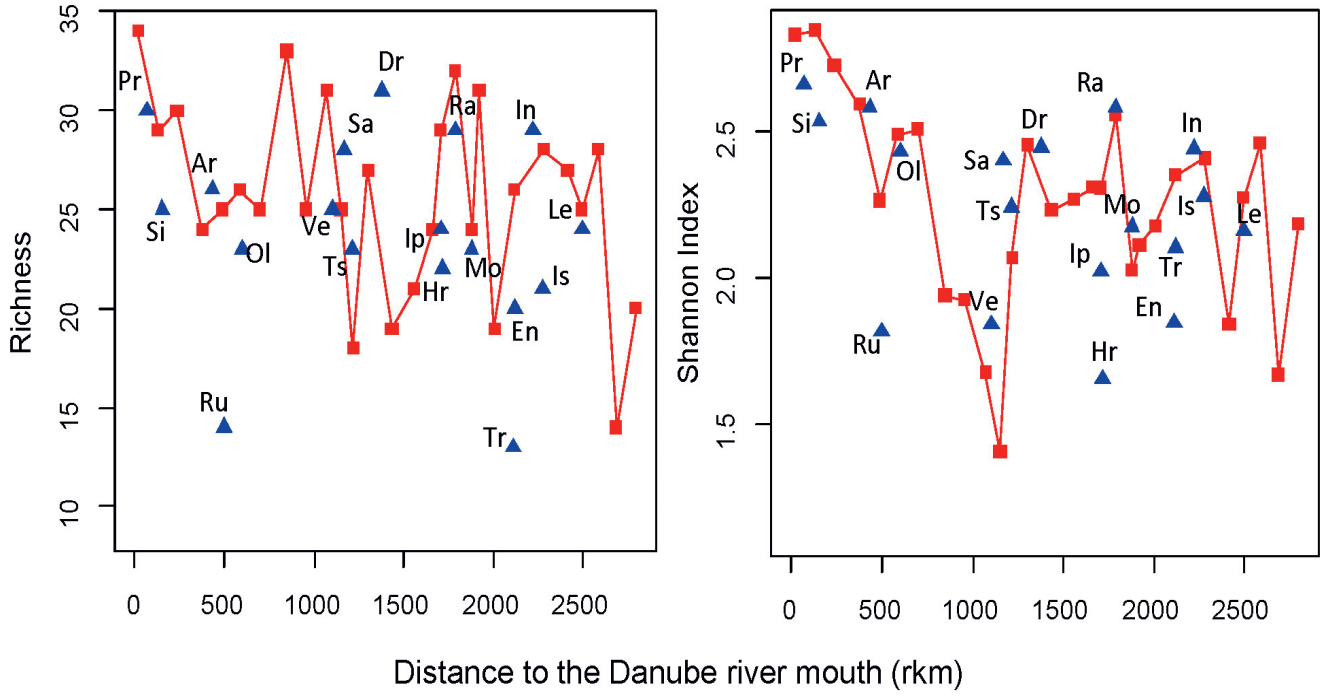

Figure 4: Changes in species richness and diversity (Shannon Index) along the Danube (red) and in major tributaries (blue). Tributary names from upstream to downstream: Lech (Le), Isar (Is), Inn (In), Traun (Tr), Enns (En), Morava (Mo), Raab (Ra), Hron (Hr), Ipel (Ip), Drava (Dr), Tsiza (Ts), Sava (Sa), 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-

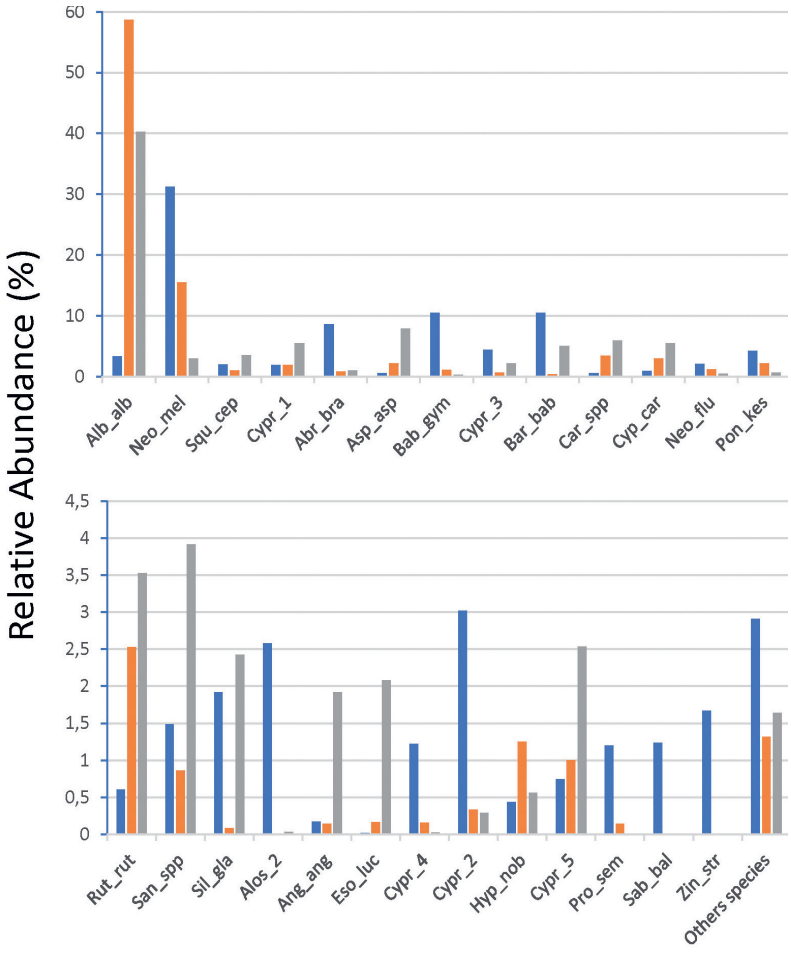

Figure 5: Mean relative abundance of taxa detected by eDNA (blue). Mean relative abundance (orange) and mean relative biomass (grey) of species caught by TFS. Only the 26 most abundant species (> 1%) detected among the 13 common Danube sites are individually represented.

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

References

Bammer V, Apostolou A, Bulat D, Dumitrascu OC, Effenberger M, Erös T, Hortic S, Kováč v, Simononvić P (2021): Fish. In: Liška I, Wagner F, Sengl M, Deutsch K, Slobodník J, Paunović M (eds): Joint Danube survey 4 - Scientific Report: A Shared Analysis of the Danube River. Vienna, Austria, 41-54.

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

Erös T, Bammer V, György AI, Pehlivanov L, Schabuss M, Zornig H, Weiperth A, Szalóky Z (2017): Typology of a Great River Using Fish Assemblages: Implications for the Bioassessment of the Danube River. River Research and Applications 33(1), 37-49.

Kottelat M, Freyhof J (2007): Handbook of European freshwater fishes. Cornol, Switzerland.

Milhau T, Valentini A, Poulet N, Roset N, Jean P, Gaboriaud C, Dejean T (2021): Seasonal dynamics of riverine fish communities using eDNA. Journal of Fish Biology 98, 387–398, DOI: 10.1111/jfb.14190.

Pont D, Keskin E, Meulenbroek P, Ünal EM, Bammer V, Dejean T, Er A, Erős T, Jean P, Lenhardt M, Nagel C, Pekarik L, Schabuss M, Stoeckle B, Stoica E, Valentini A, Zornig H, Weigand A. (2021): Metabarcoding of fish eDNA samples. In: Liška I, Wagner F, Sengl M, Deutsch K, Slobodník J, Paunović M (eds): Joint Danube survey 4 - Scientific Report: A Shared Analysis of the Danube River. Vienna, Austria, 133-144.

Pont D, Rocle M, Valentini A, Civade R, Jean P, Maire A, Roset N, Schabuss M, Zornig H, Dejean T (2018): Environmental DNA reveals quantitative patterns of fish biodiversity in large rivers despite its downstream transportation. Sci Rep 8, 13.

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

Sommerwerk N, Baumgartner C, Bloesch J, Hein T, Ostojic A, Paunovic M, Schneider-Jakoby M, Siber R, Tockner, K. (2009): The Danube River Basin. In: Tockner K, Uehlinger, Robinson CT (eds): Rivers of Europe. Elsevier Academic Press, Amsterdam, 59-112

Valentini A, Taberlet P, Miaud C, Civade R, Herder J, Thomsen PF et al. (2016): Next-generation monitoring of aquatic biodiversity using environmental DNA metabarcoding. Molecular Ecology 25, 929-942.

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