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JOINT DANUBE SURVEY 4

JOINT DANUBE SURVEY 4 SCIENTIFIC REPORT:

A SHARED ANALYSIS OF THE DANUBE RIVER

ICPDR IKSD
International Commission for the Protection of the Danube River
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14 Metabarcoding of fish eDNA samples

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Abstract

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

14.1 Introduction

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

14.2 Methods

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

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

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

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

14.3 Results and discussion

14.3.1 Species inventory

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

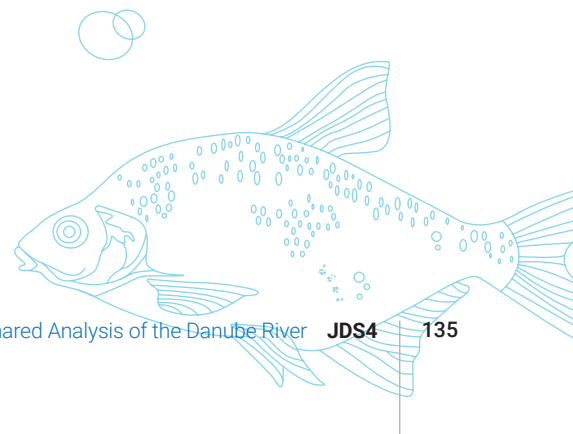

*Table 1: List of taxa detected. *: Species absent from the Danube catchment are excluded.*

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

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

14.3.2 Longitudinal organisation of fish communities

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

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

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

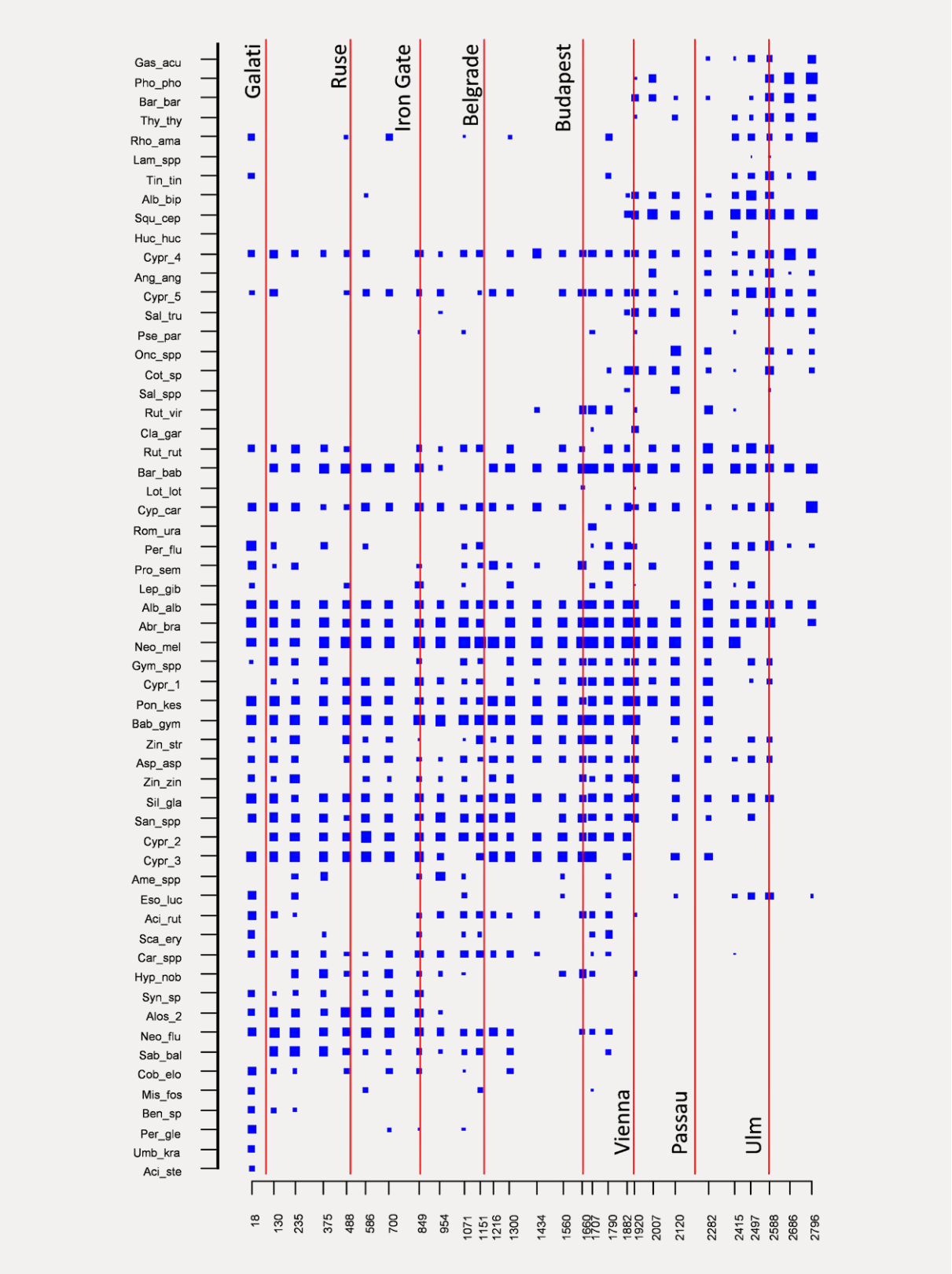

Figure 1: Relative abundance of the 57 taxa detected along the Danube River, from rkm 18 to rkm 2796. The size of the square is a function of the relative abundance of the corresponding taxa in the sample at a given site (see Table 1 for corresponding taxa names). The sites are located at rkm: 2796,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

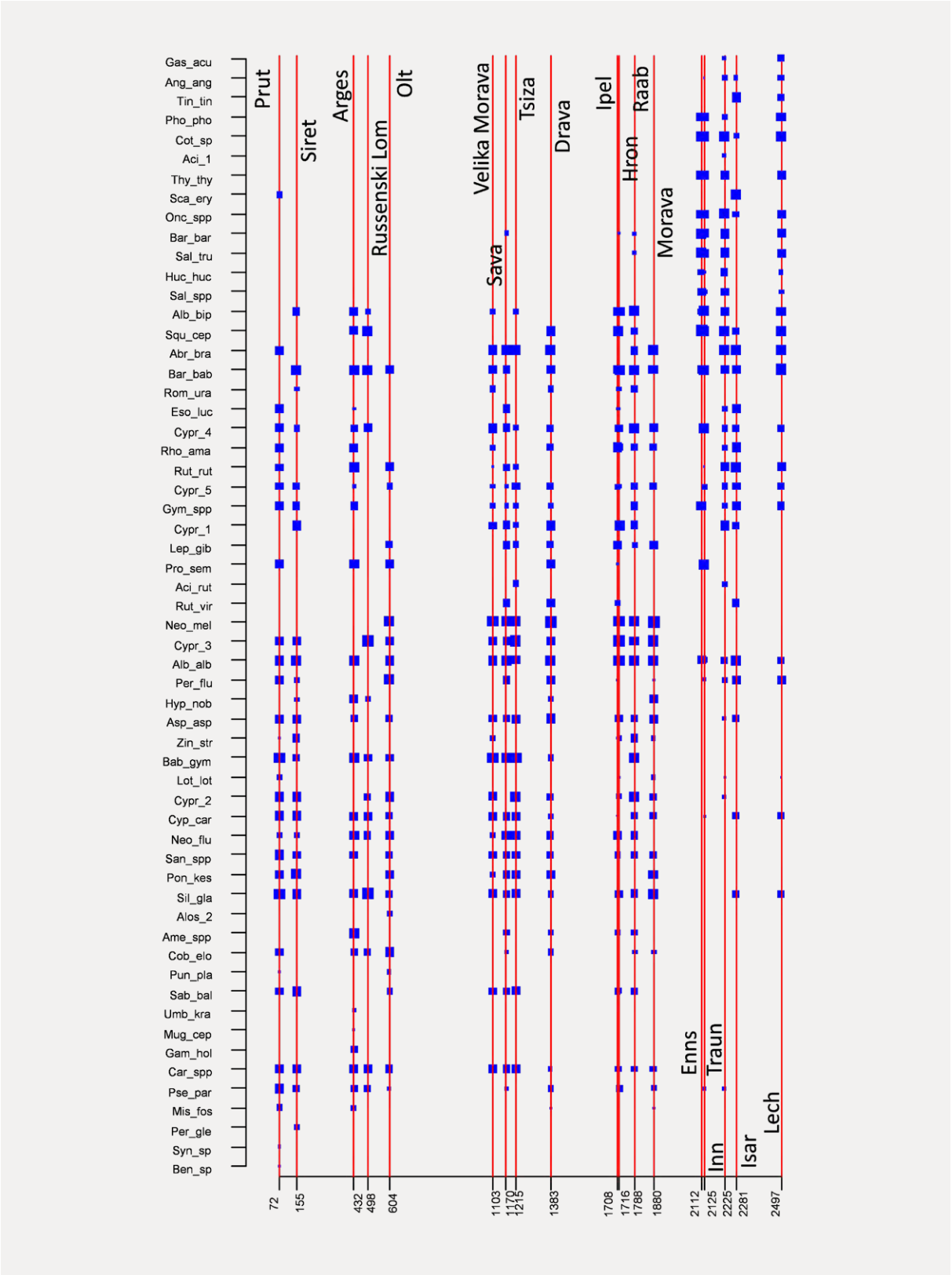

Figure 2: Relative abundance of the 59 taxa detected along the 18 tributaries of the Danube River (rkm 72 to rkm 2497). The size of the square is a function of the relative abundance of the corresponding taxa in the sample (see Table 1 for corresponding taxa names).

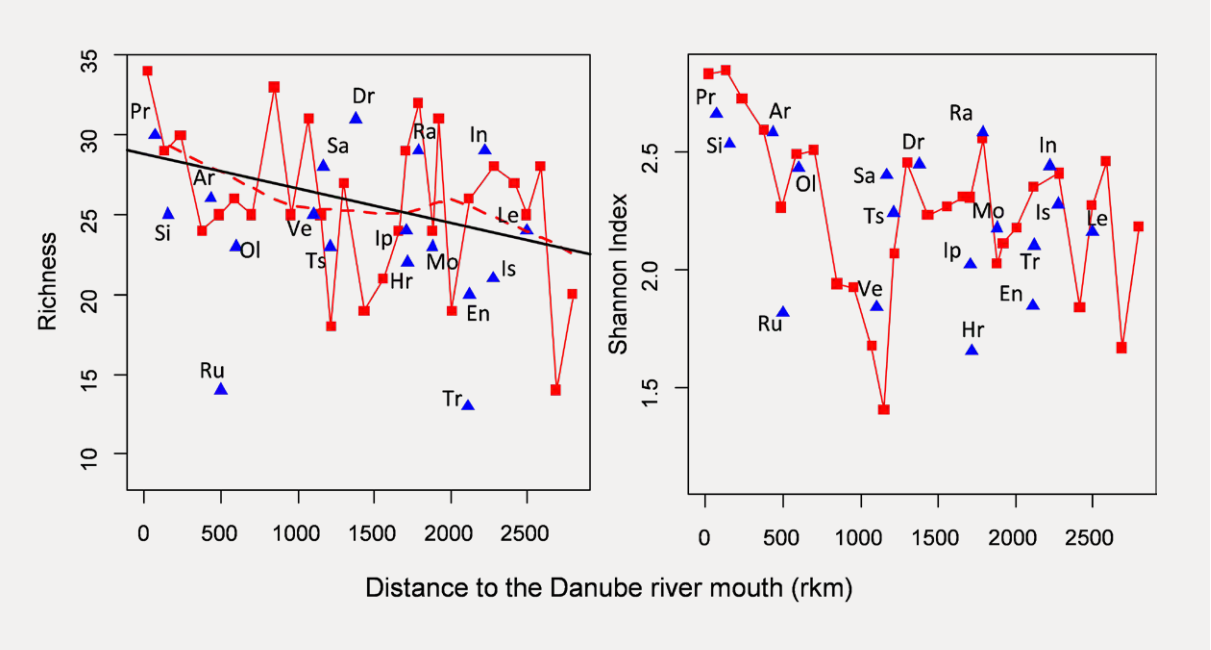

Figure 3: Changes in species richness and diversity (Shannon Index) along the Danube (red) and in major tributaries (blue). Tributary names from upstream to downstream: Lech (Le), Isar (Is), Inn (In), Traun (Tr), Enns (En), Morava (Mo), Raab (Ra), Hron (Hr), Ipel (Ip), Drava (Dr), Tsiza (Ts), Sava (Sa),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

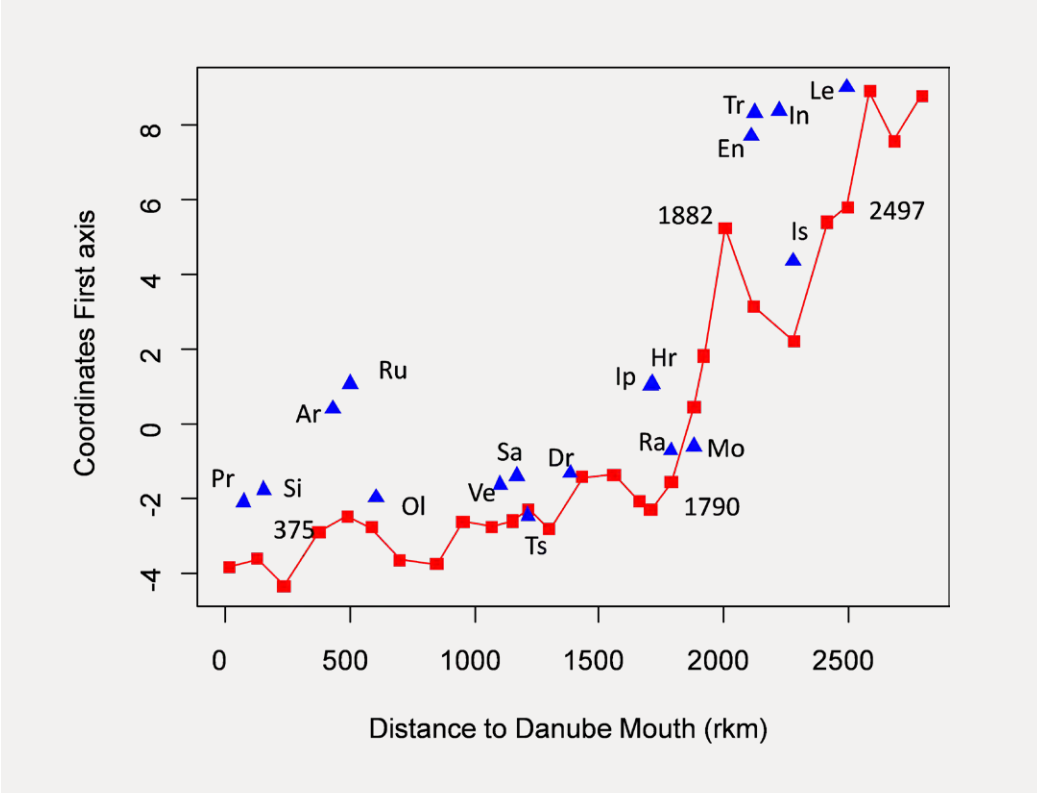

Figure 4: Longitudinal changes in site coordinates on the first axis of a principal component analysis (log-transformed standardized number of reads per taxa).

14.3.3 Comparison with JDS4 traditional fish survey (TFS)

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

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

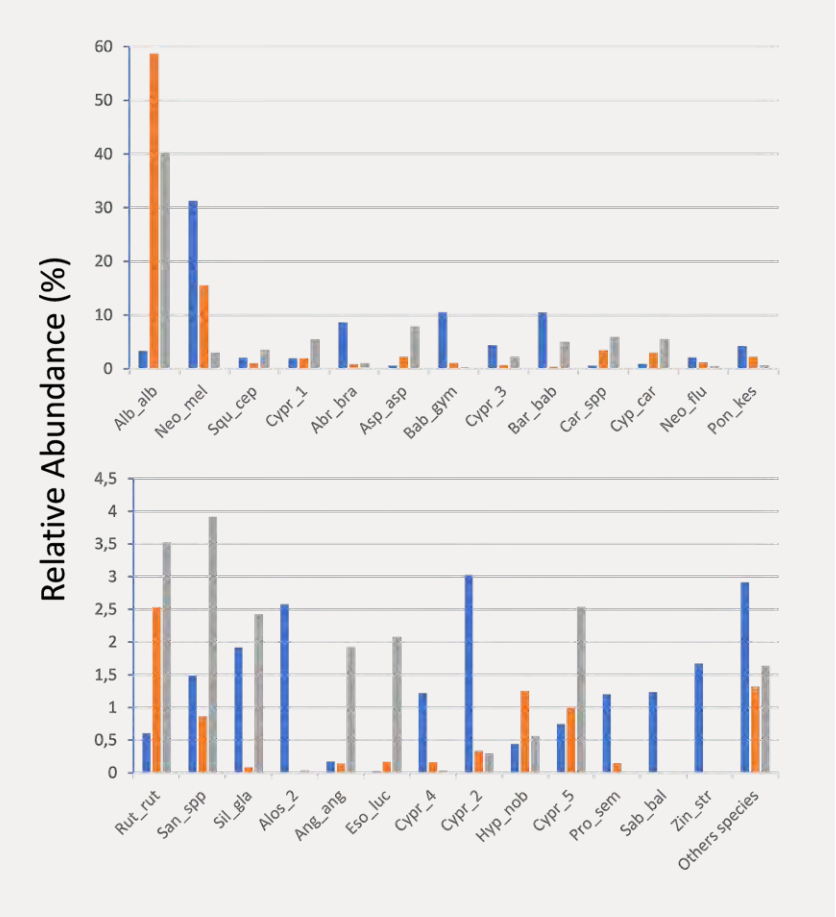

Figure 5: Mean relative abundance of taxa detected by eDNA (blue).
Mean relative abundance (orange) and mean relative biomass (grey) of species caught by TFM.
Only the 26 most abundant species (> 1%) detected among the 13 common Danube sites are individually represented.

14.3.4 Fish-based assessment using eDNA data

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

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

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

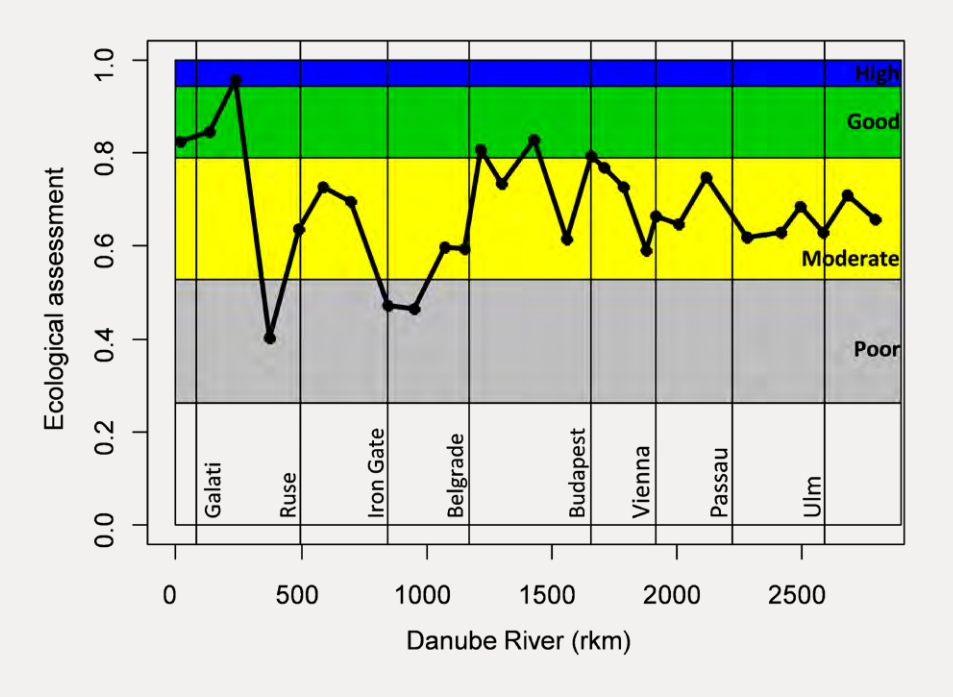

Figure 6: eDNA fish-based ecological assessment of the Danube River.

14.3.5 Comparison of eDNA markers and reference libraries for fish

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

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

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

14.4 Conclusions

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

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