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Quantitative monitoring of diverse fish communities on a large scale combining eDNA metabarcoding and qPCR

Didier Pont[1] | **Paul Meulenbroek**[1,2] | **Vincenz Bammer**[3] | **Tony Dejean**[4] | **Tibor Erős**[5] | **Pauline Jean**[4] | **Mirjana Lenhardt**[6] | **Christoffer Nagel**[7] | **Ladislav Pekarik**[8] | **Michael Schabuss**[9] | **Bernhard C. Stoeckle**[7] | **Elena Stoica**[10] | **Horst Zornig**[9] | **Alexander Weigand**[11] | **Alice Valentini**[4]

[1]Institute of Hydrobiology and Aquatic Ecosystem Management, University of Natural Resources and Life Sciences, Vienna, Austria
[2]WasserCluster Lunz -Biologische Station GmbH, Lunz am See, Austria
[3]Bundesamt für Wasserwirtschaft, Institut für Gewässerökologie und Fischereiwirtschaft, Abteilung Gewässerökologie, Mondsee, Austria
[4]SPYGEN, Le Bourget du Lac, France
[5]Balaton Limnological Research Institute, Eötvös Lor'and Research Network (ELKH), Tihany, Hungary
[6]Institute for Multidisciplinary Research, Institute for Biological Research “Siniša Stanković,” National Institute of Republic of Serbia, University of Belgrade, Belgrade, Serbia
[7]Technical University of Munich, Chair of Aquatic Systems Biology, Freising-Weihenstephan, Germany
[8]Plant Science and Biodiversity Center, Slovak Academy of Sciences, Bratislava, Slovakia
[9]PRO FISCH OG Ecological Consultants, Vienna, Austria
[10]National Institute for Marine Research and Development “Grigore Antipa,”, Constanţa, Romania
[11]National Museum of Natural History, Luxembourg, UK

Correspondence
Didier Pont, University of Natural Resources and Life Sciences, Vienna, Institute of Hydrobiology and Aquatic Ecosystem Management, Vienna, Austria.
Email: didier.pont@boku.ac.at

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Abstract

Environmental DNA (eDNA) metabarcoding is an effective method for studying fish communities but allows only an estimation of relative species abundance (density/biomass). Here, we combine metabarcoding with an estimation of the total abundance of eDNA amplified by our universal marker (teleo) using a quantitative (q)PCR approach to infer the absolute abundance of fish species. We carried out a 2850-km eDNA survey within the Danube catchment using a spatial integrative sampling protocol coupled with traditional electrofishing for fish biomass and density estimation. Total fish eDNA concentrations and total fish abundance were highly correlated. The correlation between eDNA concentrations per taxon and absolute specific abundance was of comparable strength when all sites were pooled and remained significant when the sites were considered separately. Furthermore, a nonlinear mixed model showed that species richness was underestimated when the amount of teleo-DNA extracted from a sample was below a threshold of 0.65×10^6 copies of eDNA. This result, combined with the decrease in teleo-DNA concentration by several orders of magnitude with river size, highlights the need to increase sampling effort in large rivers. Our results provide a comprehensive description of longitudinal changes in fish communities and underline our combined metabarcoding/qPCR approach for biomonitoring and bioassessment surveys when a rough estimate of absolute species abundance is sufficient.

KEYWORDS
environmental DNA, fish community, metabarcoding, qPCR, quantitative monitoring, rivers

1 | INTRODUCTION

In recent years, the use of extra-organismal DNA (Rodriguez-Ezpeleta et al., 2021) has become a widespread method for monitoring large vertebrate organisms in freshwater, brackish water and marine ecosystems (Harper et al., 2019; Miya, 2022; Sigsgaard et al., 2020; Wang et al., 2021). When targeting only one species (taxon-specific studies), conventional PCR (cPCR) allows the detection of species (Blackman et al., 2020; Jerde et al., 2011), whereas quantitative real-time PCR (qPCR) and droplet digital PCR (ddPCR) are the main environmental DNA (eDNA) methodologies for increasing the species detection sensitivity and quantifying the abundance of DNA sequences (Olsen et al., 2016; Takahara et al., 2012; Thalinger et al., 2019), enabling the indirect estimation of absolute species abundance (Wilcox et al., 2016; Yates, Glaser, et al., 2021). Species assemblages can be identified by metabarcoding after amplification via PCR of one or more genomic regions provided that the appropriate species reference database is available (Miya et al., 2015; Valentini et al., 2016). The number of reads per species is used as a proxy for the relative abundance of species (Di Muri et al., 2020; Goutte et al., 2020; Pont et al., 2018). A Web of Science search (January 2008 to March 2021; key search "TITLE: (environmental DNA) OR TITLE: (eDNA)") considering only studies on aquatic vertebrates (358 selected publications, see Data S1 for methodology) shows that fish are the most targeted group, followed by amphibians, mammals, reptiles and birds (73.3%, 17.4%, 5.1%, 2.3% and 1.8%, respectively). Metabarcoding is less frequently used than taxon-specific studies (117 from a total of 358 publications) and is mainly used for fish (86% of papers) in marine and river ecosystems (36% and 37% of papers, respectively)."

Both taxon-specific and metabarcoding approaches are in general more efficient than traditional sampling methods for detecting species (Czeglédi et al., 2021; Hänfling et al., 2016; McElroy et al., 2020; Pont et al., 2018; Valentini et al., 2016), even if the scale of inference in space and time for an eDNA sample must be better defined (Deiner et al., 2017). Comparisons between taxon-specific and metabarcoding approaches are scarce. The taxon-specific method has been reported to be both more robust and more sensitive than metabarcoding (Bylemans et al., 2018; Wood et al., 2019) or equivalent to metabarcoding (Harper et al., 2018). Depth sequencing, number of technical replicates and occupancy modelling are also key factors that can improve the robustness of metabarcoding (Ficetola et al., 2015; Harper et al., 2019).

The number of eDNA copies in a sample obtained by taxon-specific studies (qPCR) is a significant proxy for both absolute density and biomass (Doi et al., 2015; Takahara et al., 2012; Wilcox et al., 2016) but remains a rough estimate of the abundance of aquatic vertebrates (Ushio et al., 2018). Ninety per cent of a compilation of 63 studies identified significant relationships between eDNA concentrations and the abundance or biomass of target species (Rourke et al., 2021). However, this relationship is generally of medium strength due to the huge numbers of factors affecting the production, degradation, transport, sedimentation and detectability of eDNA particles in relation to ecological/physiological species characteristics, advection/diffusion processes, temperature, pH or bacterial activities (Deiner et al., 2017; Rourke et al., 2021; Yates, Cristescu, & Derry, 2021). A meta-analysis based on 19 studies (Yates et al., 2019) showed that the correlation is stronger in controlled experiments than in the field (82% and 51% of the total variance explained, respectively), partly due to the uncertainties associated with the field estimation of organism abundance by the conventional sampling method (Di Muri et al., 2020).

Metabarcoding provides only a number of reads per taxon that are not related to the amount of corresponding eDNA extracted from the water sample. The relative number of reads is a good proxy for the relative abundance of species when the amplification efficiency is comparable for the different species. Comparison with traditional sampling methods highlights the capacity of eDNA to roughly describe the structure of a vertebrate community (Di Muri et al., 2020; Pont et al., 2018; Sard et al., 2019). Many technical factors can affect the capacity of metabarcoding to deliver "relative" quantitative results (Lamb et al., 2019), but the choice of primers, template competition and the characteristics of the mixture of species are among the most important (Piñol et al., 2019; Wilcox et al., 2020). Some discrepancies are related to the bias of conventional sampling methods, especially in large water bodies (Boivin-Delisle et al., 2021; Pont et al., 2018).

Several technical options have been tested to circumvent the limitation of metabarcoding to deliver absolute quantitative data on the abundance of multiple taxa. Some authors have proposed combining eDNA and animal counts (Chambert et al., 2018). Multiplex real-time PCR enables the simultaneous detection of several fishes (Jo et al., 2020). High-throughput qPCR systems have been tested on fish species and validated by comparison with qPCR (Wilcox et al., 2020). Simultaneous quantification of the eDNA from fish species with qSeq gives results strongly correlated with those obtained with microfluidic ddPCR (Hoshino et al., 2021). Another possibility

(MiqSeq) is the enrichment of the sample with known quantities of DNA fragments from fish species absent from the water sample to estimate the copy number from the number of reads of local species obtained by metabarcoding (Hoshino et al., 2021; Ushio et al., 2018). To date, however, these experiments have only quantified a small number of species simultaneously and have not been tested on species-rich communities.

In this study, we propose a more direct method for inferring the absolute abundance of fish species from multiple sampling locations by combining eDNA metabarcoding with qPCR analysis, which assesses the total abundance of eDNA amplified by the universal marker used for metabarcoding. Fish-specific eDNA concentrations are then calculated from the ratios of fish species-specific read counts over the total read count of a sample (metabarcoding) multiplied by the total eDNA concentration estimated with qPCR (van Bleijswijk et al., 2020).

The effectiveness of this procedure was tested in a fish eDNA metabarcoding survey implemented along the Danube River from source to mouth (2850 km) and its major tributaries (Figure 1). Water samples were collected from shore to shore to provide integrative sampling of the river cross section. Among the 47 sites sampled, 18 were also investigated with a conventional sampling method (traditional electrofishing, TEF) to estimate fish species abundance expressed in density or biomass per ha (Table S1). We performed a previously described eDNA metabarcoding workflow (Pont et al., 2018) using the mitochondrial 12S primer for fish "teleo" (Valentini et al., 2016). Due to the very short length of the marker (<100 bp), the teleo primer is very effective in detecting rare species (Bylemans et al., 2018; Polanco et al., 2021) in large rivers where eDNA is highly diluted (Goutte et al., 2020; Pont et al., 2018). The total abundance of eDNA amplified with "teleo" (teleo-DNA) was estimated by qPCR analysis. Our main objectives were (i) to verify the efficiency of our eDNA sampling strategy to correctly describe the fish communities and the ecological significance of longitudinal taxon profiles, (ii) to evaluate the strength of the correlation between the estimated number of absolute total and specific eDNA copies per litre with the fish abundance obtained by using TEF, and (iii) to model the influence of the total number of eDNA copies per sample on the taxon richness.

2 | MATERIAL AND METHODS

2.1 | Site description and eDNA sampling strategy

From the Black Forest Mountains to the Black Sea, the Danube River is the second largest European river, with a drainage area of 801,093 km^2, a river length of ~2850 km and a mean discharge of 6480 m^3 s^{-1}. The river is divided into three main sections of comparable length, namely the Upper, Middle and Lower Danube (Eros et al., 2017) (Figures 1 and 6). The 18 sampled tributaries, located all along the Danube, have an average flow rate varying from 5 to 1800 m^3 s^{-1} (Table S1) and represent a very diverse range of rivers from torrential, fresh alpine rivers to large warm lowland streams (Kresser & Laszloffy, 1964).

From June 29 to August 6, 2019, 29 and 18 sites were sampled on the Danube River and its tributaries, respectively. During this period, these rivers were close to the average hydrological conditions (Table S1), with a mean daily flow rate of 1716 m^3 s^{-1} at Vienna. The sites located on the main channel of the Danube were distributed regularly from the source to the mouth of the river. The distance between the sites (mean: 99.2 km, *SE*: 26.0 km; range: 38–149 km) was sufficient to avoid the potential influence of eDNA transported downstream from one site to the next (Pont et al., 2018). For the same reason, the sampling sites were not located within several tens of kilometres downstream of the confluence of a major tributary. The tributaries were sampled 1–55 km upstream of their confluence with the Danube. Due to the failure of DNA amplification, the Inn River site was resampled in May 2020. The latter sample is not considered for the longitudinal description of the fish community. At each site, two surface samples were collected and filtered separately either by wading or from a boat moving from shore to shore to provide temporal and spatial integrative sampling of the river cross-section. Each water sample was collected with a peristaltic pump inside a disposable sterile tube and was directly filtered on the boat through a cross-flow filtration capsule (VigiDNA 0.45 µm, SPYGEN), and its volume was measured (3–40 L, mean of 28.73 L, mean filtration time of 22.34 min). At the end of each filtration, the water in the capsule was drained, the capsule was refilled with 80 ml of conservation buffer CL1 (SPYGEN) to prevent eDNA degradation and

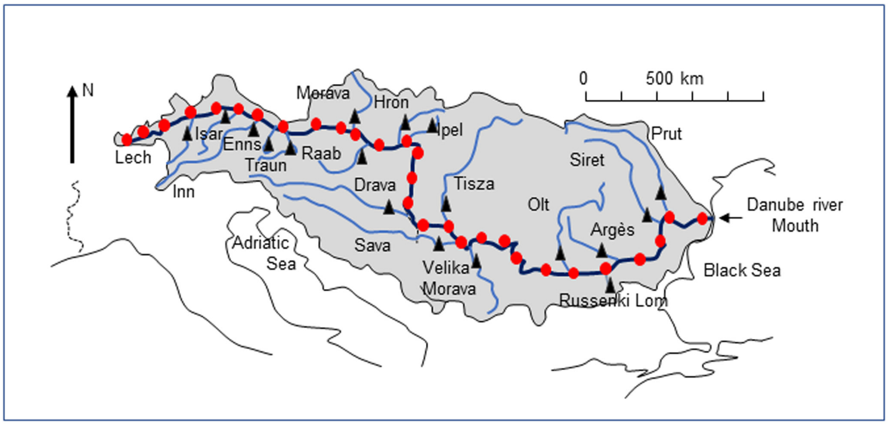


FIGURE 1 Location of sampling sites along the Danube (29 sites, red circles) and tributaries (18 sites, black triangles) near their confluence with the Danube.

kept at room temperature until DNA extraction. A previous study on the influence of the sampling effort on eDNA detection showed that two large samples were sufficient to detect more than 95% of the local species richness (Pont et al., 2018).

2.2 | Conventional fishing

During the same period (July 3 to August 28, 2019), 41 sites were sampled by using TEF along the Danube River and its tributaries (Bammer et al., 2021). Two additional sites were sampled in October 2018 (Ipel River) and January 2020 (Drava River). The sampling procedure followed both the European Standard (CEN, 2003) and recommendations for quantitative sampling in large rivers (Schmutz et al., 2001). Fish were sampled in a single pass along the bank of the main channel and in some places in the connected backwaters. The main mesohabitat types were sampled in their proportional distribution at the site level (length of river site at least 10 times the width of the river) to maximize the representativeness of the fish assemblage. The sampling effort varied between 300 and 28,412 m^2, depending on the diameter of the anode (boom or hand-held) and on the river size. Fish were determined to the species level, measured (±0.5 cm total length) and released alive immediately afterwards. Fish individual biomass was estimated using species-specific length–weight relationships. Data from one site (Rusenski Lom River), known to be a highly polluted tributary (Kirschner et al., 2021), and where only 19 fish were captured, were discarded. Eighteen of the remaining sites were located on the same river stretch as the eDNA sampling sites (distance <20 km), and only the main channel was sampled, allowing comparison between eDNA and TEF sampling methods at these sites (Table S1).

2.3 | eDNA metabarcoding and taxonomic assignment

The eDNA metabarcoding workflow (extraction, amplification using “teleo” primers, high-throughput sequencing and bioinformatic analysis) was performed following a previously described protocol (Pont et al., 2018). After eDNA extraction, 12 PCR replicates were conducted per sample. Twelve libraries were prepared using the Fasteris MetaFast protocol, and 12 independent paired-end sequencing reactions (2 × 125 bp) was carried out on a MiSeq sequencer (Illumina) with the MiSeq Kit version 3 (Illumina) following the manufacturer's instructions at Fasteris facilities. To monitor possible contaminants, 11 negative extraction controls and seven negative PCR controls (ultrapure water) were amplified with 12 replicates and sequenced in parallel with the samples. As sampling large water volumes can increase the potential for PCR inhibition, we applied the recommendations of Sepulveda et al. (2019) and diluted DNA samples to check for inhibition before the amplification with universal primers. Of the 94 samples, only 16 were found inhibited and diluted 5-fold. Sequence reads were analysed using programs implemented in the OBITOOLS package (Boyer et al., 2016). The forward and reverse reads were assembled with the ILLUMINAPAIREDEND program using a minimum score of 40 and retrieving only joined sequences. Then, we assigned the reads to each sample using NGSFILTER software, and a separate data set was created for each sample by splitting the original data set into several files using OBISPLIT. After this step, we analysed each sample individually before merging the taxon list for the final ecological analysis. Strictly identical sequences were clustered together using OBIUNIQ. Sequences shorter than 20 bp, or with fewer than 10 reads or labelled “internal” by the OBICLEAN program were excluded.

To optimize the taxonomic assignment of fish eDNA collected in our water samples, we assembled, in addition to our previous database (Valentini et al., 2016), a complementary “Danubian” reference database (Table S3). Tissue samples for 356 specimens belonging to 73 species were collected at locations situated in the Danube catchment. Total DNA was extracted from 10 mg of muscle tissue following the protocol described in Valentini et al. (2016). The DNA was then amplified using the eDNA metabarcoding protocol with “teleo” primers and each DNA sample was sequenced separately using a MiSeq sequencer at Fasteris facilities (Valentini et al., 2016) with a sequencing depth of 20,000 reads per sample. The sequences obtained were analysed using the OBITOOLS package following the same protocol as the eDNA samples, excluding the taxonomic assignation step. The most abundant sequence was retrieved for reference database construction.

The final taxonomic assignment of molecular operational taxonomic units (MOTUs) was performed using the program ECOTAG, with our two reference databases and the sequences extracted from release 142 (standard sequences) of the ENA database (http://www.ebi.ac.uk/ena). Considering the incorrect assignment of a few sequences to the sample due to tag jumps (Schnell et al., 2015), all the sequences with a frequency of occurrence <0.001 per sequence and per library were discarded. Then, the data were curated for Index-Hopping (MacConaill et al., 2018) with a threshold empirically determined per sequencing batch using experimental blanks (i.e., combinations of tags not present in the libraries) for a given sequencing batch between libraries.

The taxonomic nomenclature used referred to the European Freshwater Fish Fauna (Kottelat & Freyhof, 2007), except for the genera *Cottus* and *Phoxinus* at the species level due to insufficient knowledge of the haplotype diversity within the Danube catchment. The corresponding reference sequences were denominated *Cottus gobio* and *Phoxinus phoxinus* (see Table S2 for species names corresponding to eDNA-detected taxon name abbreviations). When reference sequences from the different reference databases were assigned to the same species, their corresponding number of reads was cumulated. When reference sequences were assigned at the genus level, they were finally denominated at the species level when only one species from the genus was known in the catchment (*Anguilla anguilla*, *Barbatula barbatula*). If not, they were discarded (*Acipenser* spp., *Alburnus* spp., *Barbus* spp., *Rutilus spp.)*, as were sequences assigned to a higher taxonomic level (Cyprinidae, Salmonidae). The molecular markers did not discriminate between two and three detected taxa belonging to the same genus (*Salvelinus*, *Carassius*, *Alosa*, *Acipenser*, *Barbus*, *Lampetra*) and to different genera for five groups (Cyprinids_1, Cyprinids_2, Cyprinids_3, Cyprinids_4, Cyprinids_5). Within all groups, we only considered species-level assignment for taxa known to be present in the Danube catchment (Table S2). Of the two undifferentiated species in the Cyprinids_1 taxonomic group and present in the Danube River catchment (*Chondrostoma nasus*, *Telestes souffia*), only *C. nasus* was captured by using TEF during our survey. Because *T. souffia* is a species well known to occur mainly in upstream fast-flowing river reaches, we considered Cyprinids_1 occurrence to be primarily related to the presence of *C. nasus*. After the final taxonomic sequence identification, three categories of taxa were considered (see Table S2). The first category included all the taxa whose presence in the Danube River was confirmed (Known-taxa) by previous traditional fish sampling surveys (Bammer et al., 2021; Eros et al., 2017) or from the literature (Kottelat & Freyhof, 2007; Meulenbroek et al., 2018; Sommerwerk et al., 2009). The second category (Waste-taxa) included food fish, farmed fish, aquarium fish or fish with any other link to human activity allowing a rejection of extra-organism eDNA in the river (mainly wastewater). The third group included species unknown in the catchment and not known for any human use (Unknown-taxa). *Alosa* spp. were detected in the upper Danube in one sample (KM 843) with only one positive PCR. The presence of this anadromous species in such an upstream location cannot be confirmed by any previous observation and was considered a false positive at this site.

2.4 | Quantification of teleo_eDNA

For the quantification of fish DNA, the samples were amplified in a real-time qPCR setup using the same “teleo” primers as for metabarcoding. qPCR was performed in a final volume of 25 µl, which included 3 µl of DNA, 12.5 µl of SYBR Green Master Mix (BioRad), 8.3 µl of ddH_2O, 0.5 µl of each “teleo” primer (10 mM), 4 µM of human blocking primer (Valentini et al., 2016) and 0.2 µl bovine serum albumin (BSA; Roche Diagnostic). Each sample was analysed in three replicates. To obtain a standard curve, a known concentration of a synthetic gene was diluted from 1.13×10^8 to 1.13×10^5 copies of DNA per reaction. The tubes containing the DNA samples were sealed, and then the qPCR standards were added to the qPCR plate in a room separate from the eDNA extraction room. The qPCR theroprofile and cycling conditions used were as follows: 95°C for 10 min, followed by 55 cycles of 95°C for 30 s and 55°C for 30 s. Melting curves were produced by plotting fluorescence intensity against temperature as the temperature was increased from 65 to 95°C in 0.5°C steps every 5 s. The samples were analysed on a BIO-RAD CFX96 Touch Real-Time PCR Detection System. To test the sensitivity of the primer for quantification, the limit of detection (LOD, i.e., the minimum amount of target DNA sequence that can be detected in the sample) and the limit of quantification (LOQ, i.e., the lowest amount of target DNA that yields an acceptable level of precision and accuracy) were calculated by running a dilution series of a known amount of synthetic gene, ranging from 1 $ng\,\mu l^{-1}$ (1.13×10^9 DNA copies) to 10^{-9} $ng\,L^{-1}$ (1.13 DNA copies) with 12 qPCR replicates per concentration below 10^{-3} $ng\,\mu l^{-1}$.The LOQ (Klymus et al., 2019) was estimated at 10^{-7} $ng\,\mu l^{-1}$, which corresponds to ~500 copies, and the LOD (Klymus et al., 2019) was estimated to be six copies when performing 12 replicates. The quantity of teleo-DNA per sample of Known-taxa (teleo-DNA) was calculated from the ratio of Known-taxa read counts over the total read count, multiplied by the teleo-DNA quantity extracted (van Bleijswijk et al., 2020). A similar computation was applied to each fish taxon, and the final concentration of fish species DNA per litre was computed from the ratio of the quantity of DNA per taxon by the volume sampled for each sample.

2.5 | Statistical treatments

The mean site-specific richness calculated from the eDNA and TEF data was compared using two-tailed Student's *t* test for paired samples (R Core Team, 2020; package MASS, function *t*-test).

Teleo-DNA concentrations and fish biomass/density data were log-transformed to satisfy normality assumptions before modelling the relationship between them using a type II linear regression (R Core Team, 2020; package lmodel2, function lmodel2, “main axis” method). Teleo-DNA concentrations were regressed against the mean annual waterflow values at each site (Kresser & Laszloffy, 1964).

The structures of fish assemblages revealed by eDNA and TEF at the 18 common sites were compared using co-inertia analysis (Doledec & Chessel, 1994; R Core Team, 2020; package ade-4, functions dudi.pca and coinertia). This multivariate method allowed the comparison of the ordinations of two data sets to find the orthogonal co-inertia principal components that maximize the covariance between them. The RV co-inertia criterion (0 to 1) measured the adequacy between the two tables (Dray et al., 2003) and was tested (Monte Carlo test with 10,000 permutations). We only considered common taxa with a similar level of taxonomic resolution (40 species) to test the similarity of the structure of fish assemblages obtained by the eDNA method and TEF abundance expressed in density or biomass.

To test the hypothesis that the number of Known-taxa detected by eDNA was dependent on the quantity of teleo-DNA per sample or on the water volume (*V*) filtered from the 94 samples, we used an asymptotic function to describe our species–sampling effort relationship considering that, at any time, the richness *Y* is finite at a given area (Soberon & Llorente, 1993). The choice of the nonlinear function remains largely empirical (Thompson et al., 2003), and we chose a model (Tjorve, 2003) from the negative exponential family $a * [1 - \exp^{-bX}]$, with an asymptotic value of richness, *b* proportional to the relative rate of *Y* increase while *X* increases, and *X* the sampling effort (teleo-eDNA or *V*). To control for variability in species richness between sites, we used nonlinear mixed-effect (NLME) models (Comets et al., 2017; R Core Team, 2020; package saemix, function saemix.model, 1000 simulations) with sites as a random factor and two alternative fixed effects (teleo-eDNA, *V*). These two models were compared between them and to the model with only the site random effect using the Akaike information criterion (AIC) (Burnham & Anderson, 2002). The significance of the fixed parameters was tested with a Wald chi-square test (Comets et al., 2017), the normality of the residuals with a Shapiro test, and the goodness of fit of the selected model by comparing the observed and predicted values at the individual level.

3 | RESULTS

3.1 | eDNA detected taxon list and comparison with conventional sampling

The total number of sequence reads obtained before and after quality control (metabarcoding bioinformatic process) were 45.999×10^{6} and 36.820×10^{6}, respectively. A total of 53.589×10^{3} reads assigned to Unknown-taxa were discarded. A total of 474.323×10^{3} reads were assigned to Waste-taxa. All the remaining reads were assigned to Known-taxa. The final mean sequencing depth was 34.161 per positive PCR replicate (range 90×10^{3} to 179.209×10^{3}).

Out of a total of 86 taxa detected after the bioinformatic process and reassignment procedure (see Table S1 for taxon abbreviation list), five were classified as Unknown-taxa (*Barbus meridionalis*, *Esox cisalpinus*, *Oncorhynchus clarkii*, *Oncorhincus masou*, *Richardsonius balteatus*) and 21 as Waste-taxa. Among Waste-taxa (Figure 2), the two most abundant (*Oncorhynchus mykiss* and *Salvelinus* spp.) were detected at 15 and seven of the 47 sites, respectively, in the Upper Danube and its tributaries. Most of the other taxa were marine species detected mainly (71% of the total number of occurrences) downstream from Vienna (seven taxa) and on two tributaries, the Arges River (eight taxa) and the Russenki Lom River (five taxa). Of the 60 remaining taxa classified as Known-taxa (Table S1), 48 were identified at the species level, eight at the genus level and four at a higher taxonomic level (taxon groups).

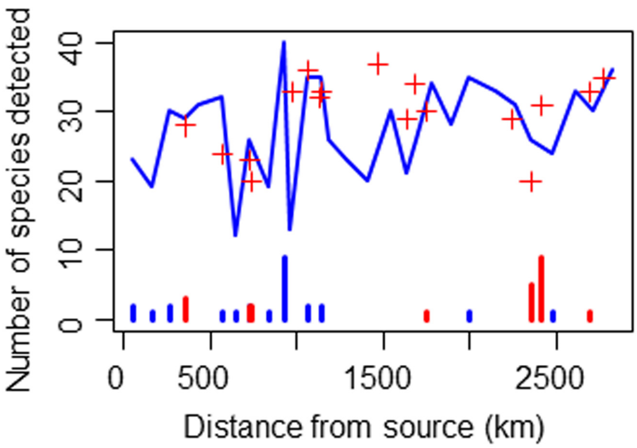


FIGURE 2 Longitudinal change in species richness along the Danube (blue line) and tributaries (red cross). Occurrences of food/aquarium fish at sites along the Danube (blue bars) and tributaries (red bars).

When only considering the 18 sites sampled with both TEF and eDNA, 40 of the 62 species caught by using TEF were also detected at the species level by using eDNA. Eighteen of the remaining species were detected by using eDNA at a higher taxonomic level. Eight species-specific taxa (*Acipenser gueldenstaedtii*, *Acipenser ruthenus*, *Acipenser stellatus*, *Barbus carpathicus*, *Benthophilus* sp., *Pungitius platygaster*, *Romanogobio uranoscopus*, *Umbra krameri*) and one taxon group (*Coregonus* sp.) were detected only by using eDNA. Four species (*Clupeonells cultriventris*, *Eudontomyzon danfordi*, *Eudontomyzon mariae*, *Neogobius eurycephalus*) were caught only by using TEF. Despite the lack of discrimination between certain species by using eDNA, the taxon richness per site obtained by using eDNA was higher than that obtained by using TEF (Figure 3), with mean richness values of 29.7 and 21.6, respectively (Student's *t* test, $t = 5.2147$, $df = 17$, $p < .001$). The difference was slightly greater when species caught by using TEF were grouped together following the taxonomic assignment used for the eDNA taxa (mean TEF species richness of 20.17, $t = 6.1429$, $df = 17$, $p < .001$).

3.2 | Comparison between absolute eDNA copy concentration and TEF abundance

The average amount of teleo-DNA per sample was 4130.634×10^{3} DNA copies (range $50.676–23{,}684\times10^{3}$), corresponding to an average concentration of 1223.819×10^{3} DNA copies per litre (range $7219–9046.465\times10^{3}$). The concentration of teleo-DNA per site decreased along the first 500 km of the Danube and remained stable downstream (Figure 4a). The teleo-DNA concentrations in the tributaries were significantly higher than those in the Danube (Student's *t* test, $t = -5.231$, $df = 44.987$, $p < .001$). For all 47 sites, the teleo-DNA concentrations were negatively correlated with the mean water flow (Figure 4b, Pearson's *R* coefficient = −.740, n = 47, $p < .001$).

The total fish density and biomass estimated by TEF at the 18 common sites were strongly correlated with the teleo-eDNA concentrations (Figure 5a,b): Pearson's *R* coefficients of .821 ($n = 18$, $p = 0.00002$) and .760 ($n = 18$, $p < .001$), respectively. When all the common sites were combined, the correlation between the taxon-specific eDNA concentration per litre and the species-specific abundance/biomass per ha was of comparable intensity: Pearson's *R* coefficients were .763 ($n = 40$, $p < .001$) and .673 ($n = 40$, $p < .001$), respectively (Figure 5c,d). When the sites and species were differentiated, the concentration of taxon-specific eDNA per litre at each site remained significantly correlated with the specific density and biomass per ha estimated from TEF samples but with a lower intensity (Figure 5e,f): Pearson's *R* coefficients of .527 ($n = 224$, $p < .001$) and .397 ($n = 224$, $p < .001$), respectively.

The co-inertia analysis showed a high level of similarity between the structure of the fish assemblages revealed by using eDNA (taxon-specific number of DNA copies per litre) and TEF (specific number of fish caught per ha) at the 18 common sites with RV

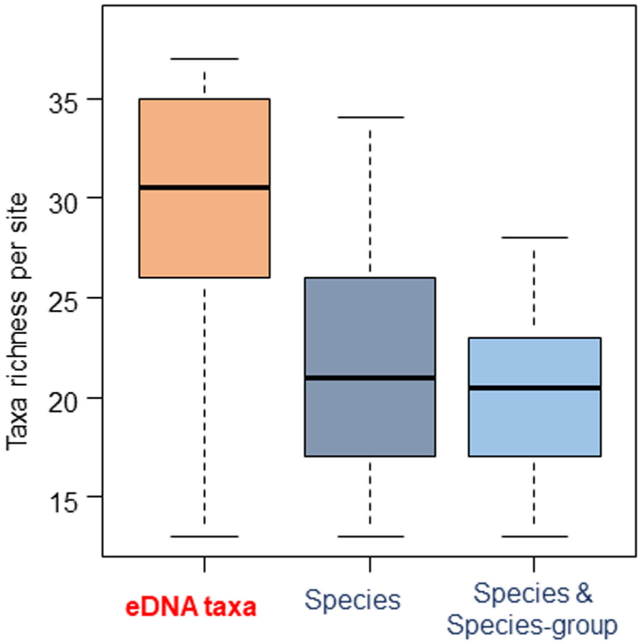


FIGURE 3 Boxplot comparison of the taxon richness obtained from eDNA (red) and (TEF) samples at the 18 common sites. For TEF, richness is expressed at the specific-species level (dark blue) and following the taxonomic assignment used for eDNA taxa (species or species group, light blue).

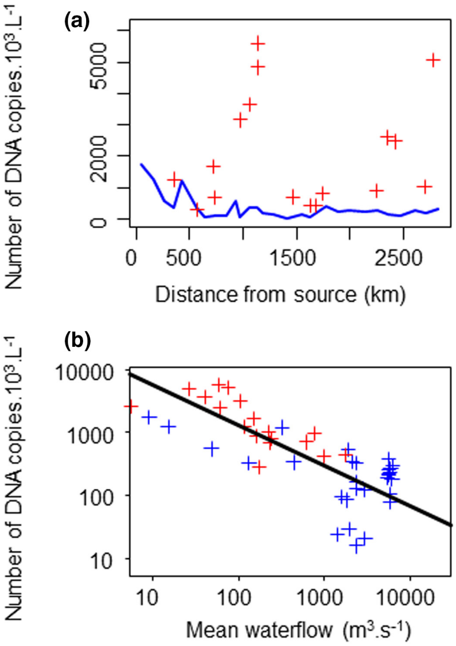


FIGURE 4 (a) Between-site variability of teleo-DNA concentrations in the Danube (blue line) and the tributaries (red cross). (b) Relationship between teleo-DNA concentrations and mean annual waterflow (log scale) at the different sites from the Danube (blue) and tributaries (red).

co-inertia criteria of 0.797 ($p < .001$) and 0.984 ($p < .001$) when the TEF data were expressed in density and biomass, respectively. The coordinates of the eDNA and TEF samples were highly correlated on the first and second co-inertia factors for TEF expressed as density (Pearson's R coefficient = .982, $p < .001$) or as biomass (Pearson's R coefficient = .993, $p < .001$). A direct comparison of the longitudinal distributions of species/taxa obtained with the two methods confirmed their similarity (Figure S1).

The change in the concentrations of the specific taxon DNA copies per litre from the source to the mouth of the Danube River provides evidence of a succession of species (Figure 6). *Barbus barbus*, *C. gob*, *Hucho hucho*, *Lampetra planeri*, *P. phoxinus* and *Thymallus thymallus* were restricted to the upper Danube, while *Ac. ruthenus*, *Neogobius fluviatilis*, *Sabanejewia balcanica* and *Scardinius erythrophthalmus* were detected from Vienna to the mouth of the Danube. *Abramis brama*, *Alburnus alburnus*, *Cyprinus carpio*, *Silurus glanis* and *Zingel streber* were detected along the entire course of the river. *Syngnathus* sp. and *Alosa* spp. were only present in the lower Danube, but *Alosa* spp. were also detected 12 km upstream from Iron Gate I (Figure 6). *Ac. stellatus* and *U. krameri* were limited to the Danube delta.

3.3 | Relationship between the quantity of teleo-DNA extracted and taxonomic richness

The relationship between the number of teleo-DNA copies extracted from a water sample and the number of taxa detected was tested using NLME models with sites as a random factor and two alternative fixed effects: teleo-DNA and water volume (V).

The NLME models with teleo-eDNA as a fixed effect had a lower AIC value than the NLME model with only the random effect (site identity): AIC values of 566.63 and 600.67, respectively. The Wald chi-square test showed a significant effect for the fixed-effect teleo-eDNA (Wald Chi-squared test = 29.973, $df = 1$, $p < .001$). The NLME model with the water volume sampled (V) as a fixed effect had a higher AIC value than the NLME model with only the random effect (835.28 vs. 600.67), and V was not significant (Wald chi-square test = 1.004, $df = 1$, $p > .05$). For the best model including teleo-eDNA (Figure 7), the Pearson's R coefficient between the observed and predicted values at the individual level was .959 ($n = 94$, $p < .001$), and the residuals were normally distributed (Shapiro test, $W = 0.994$, $p > .05$). Asymptotic richness per site and relative growth coefficient estimates and their associated standard errors at the population level were 27.29 (±0.75) and 4.55 (±0.83), respectively, for fixed effects and 17.96 (±5.06) and 4.49 (±0.3.06), respectively, for random effects. At the individual level, asymptotic richness and relative growth coefficients varied from 19.34 to 34.42 and from 0.1793 to 6.2658, respectively, with only one relative growth coefficient value less than 1 (Enns River).

The predicted value of teleo-eDNA needed to detect 95% of the taxon richness was 0.651×10^6 DNA copies when considering the model parameters defined at the population level. At the individual (site) level, this amount varied from 0.252×10^6 to 2.520×10^6 DNA copies after excluding the Enns River site (value of 15.4040×10^6 DNA copies). This high value for the Enns River is due to a very low

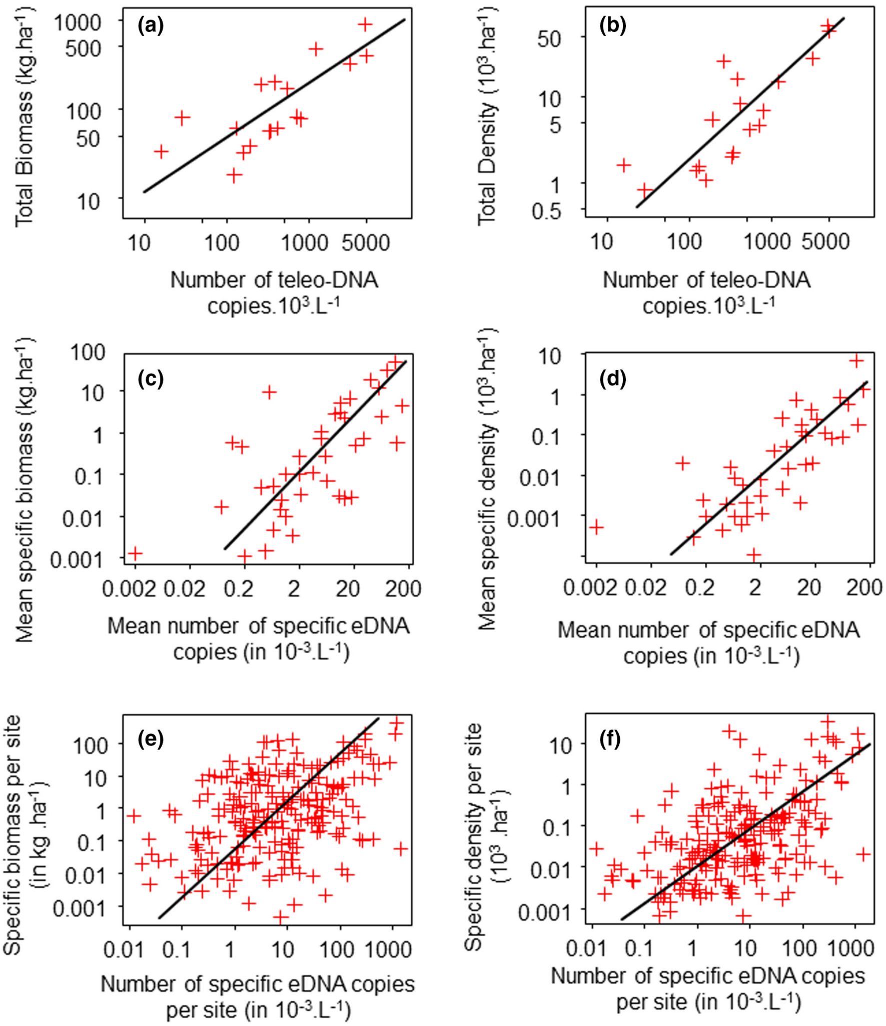


FIGURE 5 Comparison between eDNA and traditional electrofishing methods (TEF) at the 18 common sites sampled by both methods. Regressions (type II) of teleo-eDNA concentration (mitochondrial DNA copies × 10^3 L^{-1}) on total fish biomass (a) and total fish density (b) per site estimated by TEF. Regressions (type II) of mean species-specific eDNA concentration on mean biomasses (c) and mean densities (d) obtained by TEF when all sites are combined. Regressions (type II) of species-specific eDNA concentration per site on species-specific biomasses (e) and species-specific densities estimated by TEF per site(f).

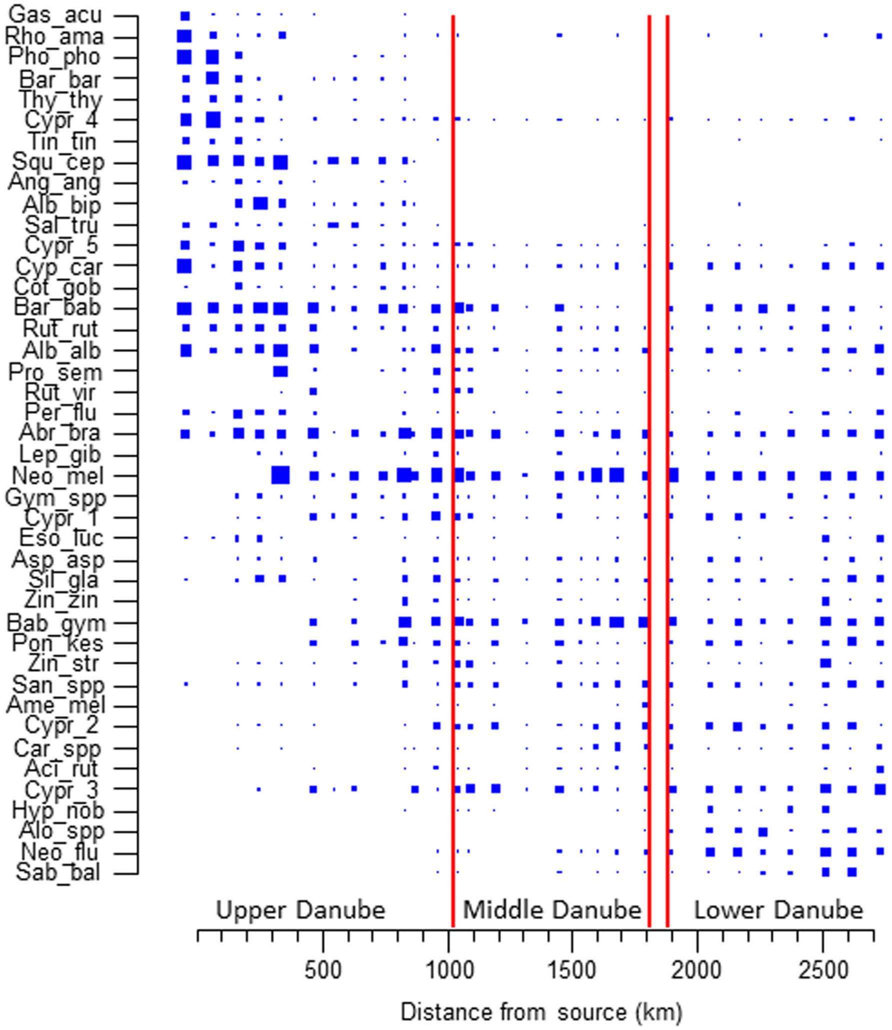
number of species detected in only one of the two samples (nine and 20 species respectively), despite the presence of a significant amount of eDNA.

4 | DISCUSSION

The fish communities of our sampling sites along the Danube and near the mouths of its main tributaries are well known both in terms of the fish species list and the assemblage structure (Eros et al., 2017;Kottelat & Freyhof, 2007; Sommerwerk et al., 2009); thus, these communities are useful for testing the effectiveness of an eDNA metabarcoding strategy. From a total of 86 taxa detected during our study, only five were Unknown-taxa in the Danube catchment (Kottelat & Freyhof, 2007; Sommerwerk et al., 2009). For most of these taxa, the main explanation is probably a misassignment of the detected sequences in relation to insufficient knowledge of their regional haplotype variability. *R. balteatus*, a North American species, *B. meridionalis*, present in rivers draining to the northwestern Mediterranean basin, and *E. cisalpinus* occurring in central and northern Italy (Kottelat & Freyhof, 2007) are species whose teleo-sequences are close to those of *Squalius cephalus*, *B. barbus* and *Esox lucius*, respectively. *O. clarkii* and *O. masou*, two salmonid species inhabiting the northern Pacific Ocean, may also have been confused with 0nc_Myk, but they have also been introduced into European fish farms (Crawford & Muir, 2008), and hybridization with other Salmonid species is conceivable (Chevassus, 1979). The development of a more comprehensive local reference database would reduce this risk of misassignment.

The Waste-taxa category of taxa was composed mainly of food fish according to the eDNA present in urban wastewater. Most of these taxa were detected at only three sites: immediately downstream of the wastewater discharge point of the city of Vienna, on the Argès River and on the Russemski Lom River. The latter two rivers are known to receive insufficiently treated municipal wastewater (Frincu, 2021; Kirschner et al., 2021). eDNA released into the river from wastewater treatment plants can lead to false-positive detection results, and a good knowledge of the regional fauna is needed to identify them. Notably, the detection of marine food fish is a clear sign of local pollution and can be incorporated as a criterion for future bioassessment methods based on eDNA samples (Pont et al., 2021). Two other taxa (*Oncorhynchus mykiss*, *Salvelinus* spp.) are also known as food fish and farmed fish (https://www.helgilibrary.com/indicators/fish-consumption-per-capita/austria/), but they

FIGURE 6 Number of eDNA copies per litre of taxa detected from the source to the mouth of the Danube River (in km). Only species with a relative abundance greater than one per 1000 are represented. The size of the square is a function of the concentration of the corresponding taxon-specific eDNA per litre at a given site (see Table S1 for taxon abbreviation list). The separation of the upper, middle and lower Danube sections (vertical red lines) are based on the locations of the Gabcikovo dam (KM 1029) and the Iron Gate dams I and II (KM 1908 and KM 1987 respectively).



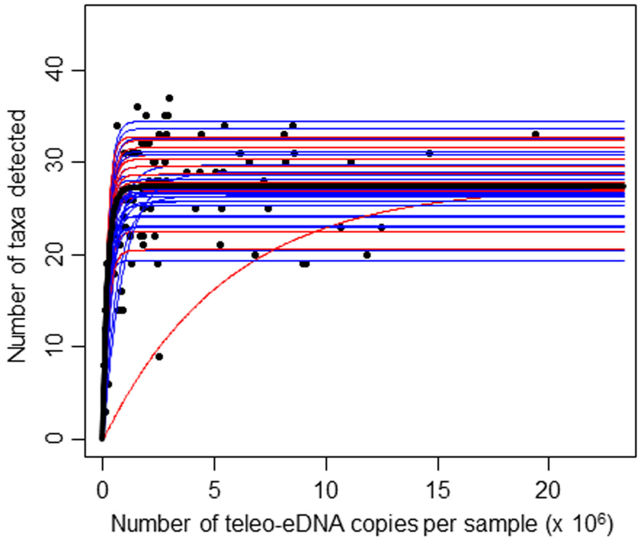


FIGURE 7 Plot of the number of taxa detected by eDNA against the number of teleo-DNA copies per sample for the 47 sites. Fitted curves from parameters estimated from a nonlinear mixed model at the population level (black line) and individual level (red lines: Tributaries, blue lines: Danube River). Longitudinal distribution of species.

are also regularly present in the Upper Danube and its tributaries, mainly due to stocking (Stankovic et al., 2015). Therefore, the presence of their eDNA must be interpreted with caution when detected in a water body that does not correspond to one of their known habitats.

A total of 60 taxa known to occur in the Danube River catchment (Known-taxa) were detected. In addition to the 48 taxa assigned at the species level, the 12 taxa assigned at a higher taxonomic level corresponded to potentially 26 well-known Danubian species, giving a maximum number of 74 species detected. This value was comparable to the total of 71 species caught in the TEF survey conducted in the same period (Bammer et al., 2021). When considering only the 18 sites sampled with both TEF and eDNA, all the species caught by using TEF were detected by using eDNA except four (*C. cultriventris*, *E. danfordi*, *E. mariae*, *N. eurycephalus*), but they were not recorded in our DNA reference database. Six of the eight taxa (*A. gueldenstaedtii*, *A. ruthenus*, *A. stellatus*, *B. carpathicus*, *Benthophilus* sp., *R. uranoscopus*) detected only by using eDNA were benthic species (Kottelat & Freyhof, 2007) mainly inhabiting the Danube itself or its coarse-bottomed tributaries. Similarly, the higher taxonomic richness obtained by using eDNA confirmed the ability of this method to be representative of all fish fauna, especially in deep rivers where a single traditional sampling technique does not allow sampling of the whole river section (Eros et al., 2017). Our results highlight the effectiveness of our integrative sampling strategy in space (the whole section of the river) and time (~30 min) as well as the performance of the teleo primer, even if its discriminating power for some species is limited. For the latter, the analysis of another marker in parallel, such as MiFish, can allow more species to be discriminated (Polanco F et al., 2021).

One of the most original aspects of this study is the strong correlation between teleo-eDNA concentrations and fish abundance estimated by using TEF at 18 common sites. The efficacy of eDNA qPCR data for correctly estimating taxon-specific abundance is well documented (Rourke et al., 2021), but estimation of total fish abundance from total fish eDNA concentration (primer qPCR analysis) has been only tested in mesocosm (Mauvisseau et al., 2021), and once in estuarine environments at three sites only a few kilometres apart (van Bleijswijk et al., 2020). Here, we demonstrate the capability of eDNA metabarcoding to estimate the total absolute abundance of fish at distant sites (i.e., independent of their eDNA contents). The intensity of the correlation between the teleo-eDNA concentration and fish abundance is comparable to results obtained in species-specific qPCR studies in natural environments (Yates et al., 2019). The limited number of sites sampled with both TEF and eDNA is a limitation to our study. However, our sites are located on rivers of different sizes (mean flow from 26 to 5424 $m^3 s^{-1}$) and have distinct fish communities. Moreover, they were sampled by different national TEF teams. All these points increase the robustness of our results despite the limited number of samples. The difference in correlation intensity with fish abundance observed when the eDNA concentration is expressed as density or biomass should be viewed with caution, as no significant effect of the fish abundance metric was found (Yates et al., 2019). The ratios of fish species-specific read counts over the total read count of a sample multiplied by the teleo-eDNA concentration measured with qPCR (van Bleijswijk et al., 2020) were significantly correlated with the fish species abundance obtained by using TEF.

This correlation was higher when all sites were pooled, highlighting the agreement between the two methods for all species and the importance of the associated uncertainties at the site scale. This greater uncertainty at the local scale is probably due to the lack of spatial representativeness of the conventional electrofishing method, which is limited to the bank of large rivers instead of the entire river section for eDNA samples. In addition, eDNA samples describe the fish community at a larger scale than conventional sampling due to the downstream transport of eDNA. It would be interesting to perform a similar methodological comparison in a panel of small, shallow rivers where both conventional and eDNA methods tend to describe the fish community at the same spatial scale."

The very high values of the co-inertia criteria also demonstrate that the descriptions of fish community structures obtained with the TEF (abundance per ha) and eDNA methods (taxon-specific DNA copy numbers per litre) were quite similar. The distribution of species along the entire Danube River obtained by using eDNA was consistent with previous knowledge (Eros et al., 2017) but with a lower between-site variability. For example, *Ac. ruthenus*, a resident sturgeon species, was regularly detected downstream of the first 1000 km of the river by using eDNA, whereas no or few individuals were captured by using traditional methods (Bammer et al., 2021; Eros et al., 2017). The anadromous taxon *Alosa* spp. (*Alosa immaculata*/*Alosa tanaica*) was detected by using eDNA in almost all the sites located downstream of the Iron Gate dams that are known to limit their upstream migration (Sommerwerk et al., 2009). In addition, the detection of *Alosa* spp. 12 km upstream of Iron Gate I dam (KM 1908) is consistent with previous captures of *Al. tanaica* individuals upstream of Iron Gate II (M. Lenhardt, pers. comm.).

Nevertheless, eDNA is only an indirect estimator of organism abundance and is influenced by many physiological processes and environmental conditions, and the uncertainties associated with all factors affecting eDNA concentration in the environment are high (Rourke et al., 2021). eDNA cannot be expected to provide a highly accurate quantification of the fish populations as needed for precise fish stock estimations in fisheries (Boivin-Delisle et al., 2021; Rourke et al., 2021; Yates, Cristescu, & Derry, 2021). For such a purpose, recent technical options could provide a good alternative (Hoshino et al., 2021; Sato et al., 2021; Wilcox et al., 2020; Ushio et al., 2018). However, it must also be considered that most conventional fish sampling methods are associated with many biases and high uncertainties, especially in large water bodies where the spatial representativeness of samples is limited and multiple methods must be used (Eros et al., 2017; Zajiceke & Wolter, 2018). For most biomonitoring purposes, a rough estimation of absolute fish abundance is sufficient, as the main objective is to compare fish assemblages on a large scale or to detect long-term variability in relation to changes in anthropogenic disturbances.

An additional benefit of quantifying total fish eDNA by qPCR is to optimize sampling effort. Our NLME models showed that the species richness was underestimated when the amount of teleo-eDNA extracted from a sample was below a threshold of 0.65×10^6 eDNA copies. Although several authors have recognized the importance of this parameter (Shu et al., 2020; Wang et al., 2021), to our knowledge, no studies have quantified its influence. The value of this threshold should be tested with other observations to better evaluate its possible variability according to the eDNA workflow used and the environmental conditions.

In addition, our results demonstrated the significant influence of river size on the concentration of teleo-eDNA per litre, with values 10–100 times lower in larger rivers. This can be due to different processes (e.g., dilution of eDNA with increasing river depth), as most fish species are confined to the river bottom or shoreline, or the decreased abundance of fish in large rivers compared to small rivers. Further research is needed to better understand the processes that explain such a pattern. As the quantity of teleo-eDNA extracted depends on both its concentration per litre and the water volume sampled, the water volume needed to extract an amount of eDNA over the threshold of 0.65×10^6 eDNA copies is ~40 L for large rivers but only a few litres for smaller rivers. The volume of water to be sampled is the main issue in many studies, with values ranging from <1 L to 68 L (Cantera et al., 2019; Civade et al., 2016; Doi et al., 2017), but no general guidelines have been established (Shu et al., 2020; Wang et al., 2021). This study highlights that river size is one of the main factors that influences the minimum water volume to be sampled. Nevertheless, this result is only valid in the context of our spatial and temporal integrative sampling strategy: the total volume collected must be sufficient to allow the collection of eDNA from the entire river section.

In conclusion, our results show that the combination of qPCR analysis to estimate the total concentration eDNA amplified by the “teleo” primer, an eDNA metabarcoding workflow with a high number of technical replicates, and an integrative sampling strategy allows a correct estimation of species diversity and delivers a good proxy of absolute species abundance (based on taxon-specific DNA copy numbers per litre). Our approach is not appropriate if accurate abundance estimation is required, such as in intensively managed fisheries. However, we consider it sufficient for most biomonitoring and bioassessment purposes, especially given the limited effectiveness of conventional fish sampling methods in most aquatic ecosystems. The efficiency of our procedure needs to be tested in ponds and lakes, estuaries, and marine environments. Our results should inspire a more quantitative approach to aquatic community analysis using eDNA methods.

AUTHOR CONTRIBUTIONS

Didier Pont, Paul Meulenbroek, Michael Schabuss, Horst Zornig and Alexander Weigand designed the study. Michael Schabuss, Horst Zornig, Didier Pont and Paul Meulenbroek collected the eDNA samples in the field, with the participation of Mirjana Lenhardt, Christoffer Nagel, Ladislav Pekarik, Bernhard C. Stoeckle, Elena Stoica and Tibor Erős. Tony Dejean, Pauline Jean and Alice Valentini conducted the laboratory and bioinformatics analyses. Vincenz Bammer analysed the data related to the conventional sampling survey. Didier Pont assisted with data analysis, prepared the figures, and wrote most of the manuscript with significant contributions from the other authors.

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CONFLICT OF INTEREST

‘Teleo’ primers and the use of the amplified fragment for identifying fish species from environmental samples are patented by the CNRS and the Université Grenoble Alpes. This patent only restricts commercial applications and has no implications on the use of this method by academic researchers. SPYGEN owns a licence for this patent. T.D., P.J and A.V. are research scientists at a private company specializing in the use of eDNA for species detection.

DATA AVAILABILITY STATEMENT

Sequences for the reference databases have been uploaded as online supporting information (Table S3) and all Illumina raw sequences data are available on Dryad: https://doi.org/10.5061/dryad.h70rxwdn0. The results of electrofishing sampling at all sites are available from ICPDR (http://www.danubesurvey.org/jds4/)

BENEFIT-SHARING STATEMENT

Benefits Generated: A research collaboration was developed with scientists from the countries providing genetic samples, all collaborators are included as co-authors, and the results of research have been shared with the provider communities and the broader scientific community (see above). More broadly, our group is committed to international scientific partnerships, as well as institutional capacity building (International Commission for the Protection of the Danube River, I.C.P.D.R).

ORCID

Didier Pont https://orcid.org/0000-0001-5187-0135
Alice Valentini https://orcid.org/0000-0001-5829-5479

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