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Research Article

Beyond fish eDNA metabarcoding: Field replicates disproportionately improve the detection of stream associated vertebrate species

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Abstract

Fast, reliable, and comprehensive biodiversity monitoring data are needed for environmental decision making and management. Recent work on fish environmental DNA (eDNA) metabarcoding shows that aquatic diversity can be captured fast, reliably, and non-invasively at moderate costs. Because water in a catchment flows to the lowest point in the landscape, often a stream, it can collect traces of terrestrial species via surface or subsurface runoff along its way or when specimens come into direct contact with water (e.g., when drinking). Thus, fish eDNA metabarcoding data can provide information on fish but also on other vertebrate species that live in riparian habitats. This additional data may offer a much more comprehensive approach for assessing vertebrate diversity at no additional costs. Studies on how the sampling strategy affects species detection especially of stream-associated communities, however, are scarce. We therefore performed an analysis on the effects of biological replication on both fish as well as (semi-)terrestrial species detection. Along a 2 km stretch of the river Mulde (Germany), we collected 18 1-L water samples and analyzed the relation of detected species richness and quantity of biological replicates taken. We detected 58 vertebrate species, of which 25 were fish and lamprey, 18 mammals, and 15 birds, which account for 50%, 22.2%, and 7.4% of all native species to the German federal state of Saxony-Anhalt. However, while increasing the number of biological replicates resulted in only 24.8% more detected fish and lamprey species, mammal, and bird species richness increased disproportionately by 68.9% and 77.3%, respectively. Contrary, PCR replicates showed little stochasticity. We thus emphasize to increase the number of biological replicates when the aim is to improve general species detections. This holds especially true when the focus is on rare aquatic taxa or on (semi-)terrestrial species, the so-called 'bycatch'. As a clear advantage, this information can be obtained without any additional sampling or laboratory effort when the sampling strategy is chosen carefully. With the increased use of eDNA metabarcoding as part of national fish bioassessment and monitoring programs, the complimentary information provided on bycatch can be used for biodiversity monitoring and conservation on a much broader scale.

Key Words

biomonitoring, birds, bycatch, conservation, environmental DNA, mammals

Introduction

Environmental DNA (eDNA) metabarcoding is a powerful and nowadays frequently applied method to assess and monitor fish biodiversity in streams (Cantera et al. 2019), lakes (Muri et al. 2020), and the sea (Andruszkiewicz et al. 2017). Contrary to conventional methods, such as net trapping or electrofishing, eDNA metabarcoding from water samples is non-invasive, safe and simple, and derived taxonomic richness estimates are generally more complete than classical assessments (Hänfling et al. 2016; Pont et al. 2018; Boivin-Delisle et al. 2021). In view of the maturity of the method, the uptake of fish eDNA metabarcoding into regulatory monitoring programs, such as the European Water Framework Directive (2000/60/EC, WFD), is discussed (Hering et al. 2018; Pont et al. 2021).

In view of global biodiversity loss and the demand for highly resolved spatio-temporal data, eDNA metabarcoding has an additional, so far less explored potential: While fish species are primary targets, eDNA monitoring data can also provide reliable information on many other taxa either living in or in the vicinity of water bodies such as mammals (Andruszkiewicz et al. 2017; Closek et al. 2019), amphibians (Lacoursière-Roussel et al. 2016; Bálint et al. 2018; Harper et al. 2018), and birds (Ushio et al. 2018a; Day et al. 2019; Schütz et al. 2020). While traditional monitoring of birds is usually conducted by many professional and hobby ornithologists, the monitoring of mammals relies on far more advanced, non-invasive, observational methods such as camera traps or identification of field traces (e.g., hair or feces). Nevertheless, semi-aquatic, terrestrial, and aerial species emit genetic material to their environment, which allows their identification with eDNA-based approaches. These bycatches from one monitoring approach, as e.g., fish eDNA metabarcoding from water samples, can become important sources for other regulatory frameworks: While birds and mammals are not considered in the WFD, they are subject to the EU Birds Directive (Directive 2009/147/EC, 2009), the "EU Regulation 1143/2014 on Invasive Alien Species", and the EU Habitats Directive (Council Directive 92/43/EEC, 1992). Monitoring data on birds and mammals are furthermore of major interest under the convention on biological diversity (see https://www.cbd.int/) and may become increasingly the basis of inventory estimates for regional, national, and international red lists (e.g., IUCN). The definition of bycatch and target, is artificially defined by the respective national or international regulations and directives. This differentiation of bycatch and target is irrelevant on the molecular level of eDNA, since eDNA from all different groups can be found in a single water sample. Thus, eDNA metabarcoding allows insights into the whole stream associated vertebrate community (Deiner et al. 2017; Ushio et al. 2017; Mariani et al. 2021), detecting not only aquatic but also semi-aquatic and terrestrial mammals and birds (Figure 1). The collection of eDNA samples during monitoring studies thus can provide highly valuable information on a much broader scale without any additional costs or sampling effort, if the same metabarcoding primers are used. Often universal, i.e., degenerate primers (Riaz et al. 2011; Miya et al. 2015; Taberlet et al. 2018), have the potential to efficiently target fish and lamprey, and moreover also to amplify DNA of species of birds and mammals as a bycatch, without reducing the fish detection rate.

While the perception of water bodies as 'sinks' or 'conveyor belts' (sensu Deiner et al. 2016) is appealing in view of holistic biodiversity monitoring, several issues are obvious. Typically, the non-target (semi-) terrestrial bycatch is difficult to detect. Especially when eDNA is not homogeneously distributed (Furlan et al. 2016; Cantera et al. 2019; Jeunen et al. 2019). Previous studies reported that the number of sampling sites and biological replicates can strongly influence the detected species richness (Civade et al. 2016; Hänfling et al. 2016; Valentini et al. 2016; Evans et al. 2017; Bálint et al. 2018; Doble et al. 2020). This holds true in particular for standing water bodies with strong stratification (Jeunen et al. 2019). For stream ecosystems, however, eDNA distribution can be assumed to be more homogeneous given turbulent flow, yet, only a few studies tested this. For example, in a study by Cantera et al. (2019) tropical fish richness estimates showed that the filtration of 34 to 68 liters was sufficient to inventory the local fish fauna, while the filtration of larger volumes only slightly increased the detected species richness. However, this study focused on total fish diversity and did not consider other taxa. In addition, an important aspect from the practical standpoint of routine biomonitoring are trade-offs between sample number or water volume filtered and the actual increase in species detection with more samples or higher volumes. Given limited resources and time, the best compromise between the number of samples analysed and detection probability is needed.

Therefore, we performed an eDNA metabarcoding survey using universal fish primers on water samples collected from the German river Mulde to assess the fish and stream associated vertebrate (bycatch) community. Our aims were i) to test the effect of biological sample replication on the detected fish species richness, and ii) to investigate the detection rate of usually discarded bycatch vertebrate species.

Methods

Sampling site

The sampling site was located at the Mulde weir in Dessau (Germany, 51°49'56.2"N, 12°15'05.1"E). The river Mulde is a tributary of the Elbe system with an average effluent at the sampling site of 62.7 m^3/s in April (2012–2018; FIS FGG Elbe). From the complete stream system up to 34 fish species are reported (Geisler 2001, MULE fish report 2014), which is close to the total number of 50 fish species reported for the German federal state of Saxony-Anhalt (Kammerad et al. 2020). Amongst these are endangered and strictly protected fish as well as diadromous and invasive species. In accordance with the WFD, a fish ladder was built in 2017 in the stream to surpass the 2.4 m weir and to allow for unimpeded migration of organisms, in particular fish.

eDNA sampling

We collected 18 water samples on a single day in April 2019 over a stretch of 2 km: 4 samples each were collected 1 km upstream of the weir (location S1), directly upstream (S2) and directly downstream of the fish ladder (S3), and 1 km downstream of the weir (S4). Additionally, two samples were taken directly in the

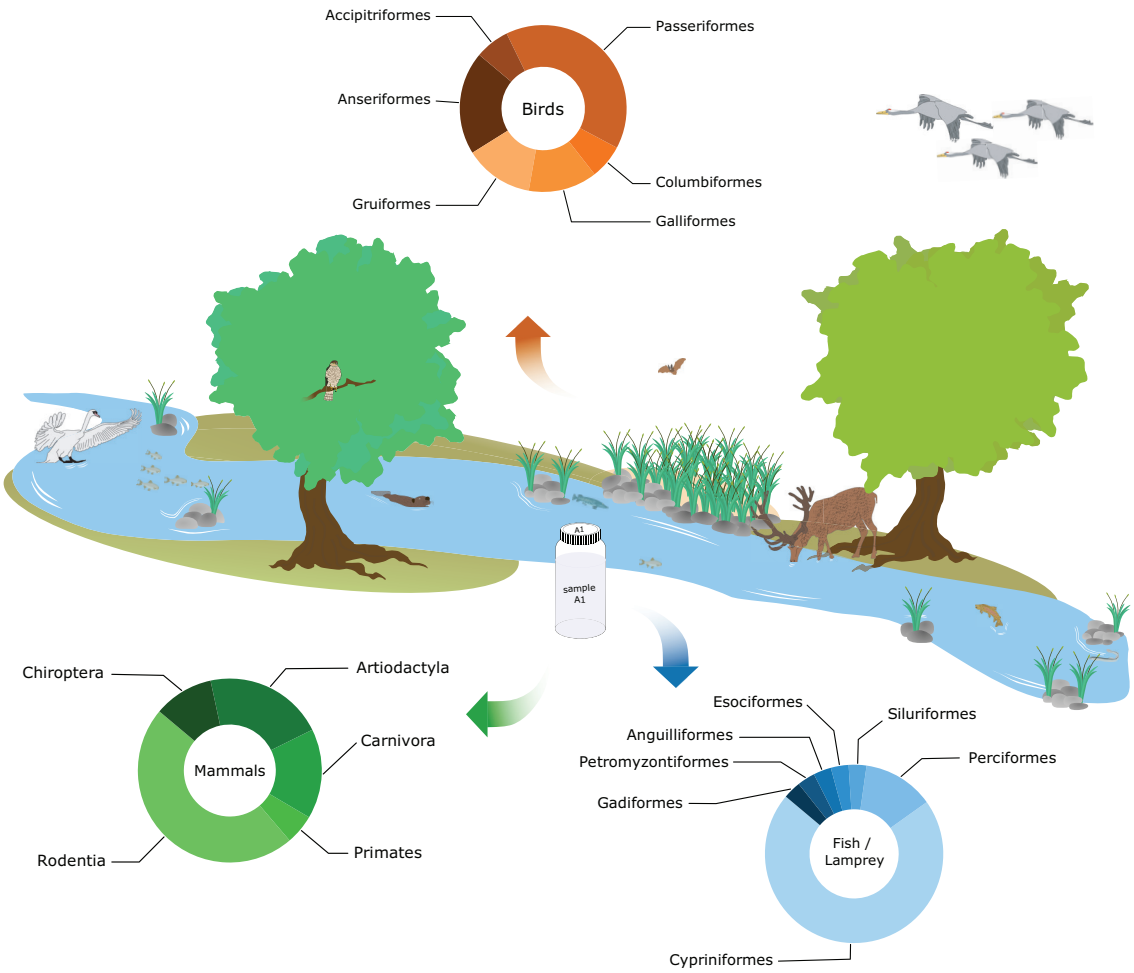

Figure 1. Overview of a freshwater associated vertebrate community including some of the detected species. The OTU richness among the classes of birds, mammals and fish/lamprey found in this study are illustrated in pie charts.

fish ladder itself (S4). For each sample, 1 L of water was collected from the surface in a sterile plastic bottle. To prevent cross-contamination, sterile laboratory gloves were changed between samples. All water samples were filtered on site to avoid contamination and ease the transportation. Open MCE (mixed cellulose ester membrane) filters with a 0.45 µm pore size (diameter 47 mm, Nalgene) were used for the filtration. The filters were handled with sterile forceps (previously bleached with 4%–5% sodium hypochlorite, rinsed with distilled water afterward) and gloves. Both forceps and gloves were changed between each sample. An electric vacuum pump, a disposable funnel filter, and a filter flask were installed for filtering the water. Both the funnel filter and filter flask were used for all samples, since they do not come in contact with the filter membrane. As field blanks, a total of two blank filters were placed on the filter flask, the pump was switched on and the filters were exposed to air for 20 seconds. The filters were transferred to 1.5 mL Eppendorf tubes filled with 96% ethanol, kept at 4 °C during transport and overnight, and then stored at -20 °C until DNA extraction.

DNA extraction

All laboratory steps were conducted under sterile conditions in a dedicated sterile laboratory (UV light, sterile benches, overalls, gloves, and face masks). The filters were dried separately in sterile petri dishes and covered with aluminum foil overnight. Afterwards the filters were torn into pieces using sterile forceps and transferred into new 1.5 mL Eppendorf tubes. Subsequently, filters were eluted in 600 µL TNES-Buffer with 10 µL Proteinase K (300 U/mL, 7BioScience, Neuenburg am Rhein, Germany) and incubated at 55 °C and 1000 rpm for three hours on an Eppendorf ThermoMixer C (Eppendorf AG, Hamburg, Germany). DNA was extracted from the filters following an adapted salt precipitation protocol (Weiss and Leese 2016), eluted in 50 µL PCR-grade water, and stored overnight at 4 °C. Next, 0.5 µL RNase A (10 mg/mL) was added to each sample and incubated for 30 minutes at 37 °C on an Eppendorf ThermoMixer C. Subsequently, samples were purified using the Qiagen MinElute DNeasy Blood & Tissue Kit (Hilden, Germany), following the manufacturer's protocol. Samples were eluted in 30 µL PCR-grade water.

DNA amplification and sequencing

A two-step PCR approach was applied for amplifying the extracted DNA. In the first PCR, the vertebrate tele02 primers (Taberlet et al. 2018) were used that are optimized for European freshwater fish and amplify a 129–209 bp long 12S gene fragment. In total, 100 first step PCR reactions were run, including 5 replicates per sample as well as 8 negative PCR controls and 2 field blanks. The PCR reaction volume was 50 µL and consisted of 21 µL PCR-grade water, 25 µL Multiplex Mastermix (Multiplex PCR Plus Kit, Qiagen, Hilden, Germany), 1 µL of each the tele02 forward and reverse primer (10 µM), and 2 µL DNA template. The first PCR step was carried out at 95 °C for 5 minutes followed by 35 cycles with 94 °C for 30 seconds, 52 °C for 90 seconds, and 72 °C for 90 seconds. The final elongation was carried out at 68 °C for 10 minutes. After the first PCR, all five replicates per sample were pooled together. For the second PCR step, a universal tagging primer set was used (Buchner et al. under review). A total of 52 second-step PCR reactions were run using two PCR replicates per sample, 4 first-step negative controls, 4 second-step negative controls, and 2 field blanks. The PCR mix per sample contained 19 µL of PCR-grade water, 25 µL of Multiplex Mix, 2 µL combined primer (10 µM), and 4 µL PCR product from the first PCR. PCR conditions were 95 °C for 5 minutes followed by 10 cycles at 94 °C for 30 seconds, 62 °C for 90 seconds, and 72 °C for 90 seconds. The final elongation was carried out at 68 °C for 10 minutes. Following the second-step PCR, the PCR products were visualized on a 1% agarose gel to evaluate the amplification success. The samples were subsequently normalized to 25 ng per sample, using a SequalPrep Normalization Plate (Applied Biosystems, Foster City, CA, USA) following the manufacturer's protocol. Subsequently, the normalized samples were pooled into one library. After library-pooling, the samples were concentrated using a NucleoSpin Gel and PCR Clean-up kit (Machery Nagel, Düren, Germany) following the manufacturer's protocol. The final elution volume of the library was 22 µL. The samples were then analyzed using a Fragment Analyzer (High Sensitivity NGS Fragment Analysis Kit; Advanced Analytical, Ankeny, USA) to check for potential primer dimers and co-amplification and quantify the DNA concentration of the library. Primer dimers were removed by extracting PCR products using two lanes (10 µL each) of an E-Gel Power Snap Electrophoresis Device (ThermoFisher Scientific, Germany). This resulting library was sequenced on a MiSeq using the v2 250 bp PE Illumina kit at CeGaT (Tübingen, Germany).

Bioinformatics

Raw reads for both libraries were received as demultiplexed fastq files. The quality of the raw reads was checked using FastQC (Andrews 2010). The sequencing data was processed using a pre-release version of the graphical-user interface pipeline MetaProcessor (available at https://github.com/TillMacher/MetaProcessor). Paired-end reads were merged using VSEARCH version 2.11.1 (Rognes et al. 2016), allowing for up to 25% differences between merged pairs and a minimum overlap of 5 bp. Afterwards, primers were trimmed with cutadapt version 2.8 (Martin 2011), using the linked adapter option without anchoring. Reads were then filtered by length (±10 bp threshold for the amplified 129–209 bp tele02 target fragment) and by maximum expected error (maxee = 1), using VSEARCH. The filtered reads were dereplicated and singletons and chimeras were removed with VSEARCH. All reads were then pooled using a custom python script and again dereplicated (MetaProcessor: v_derep_singletons_uchime.py). Operational taxonomic units (OTUs) were obtained by a 97% similarity clustering and the seeding sequences were extracted as representative OTU sequences. The OTUs were remapped (usearch_global function, 97% similarity) to the individual sample files to create the read table. The read table was filtered by column (read abundance threshold: >0.01% of reads to keep the OTU) and then by row (OTU must be present in at least one of the samples). BLAST searches (web BLAST, blastn suite, nt database, blastn program) were performed against the National Center for Biotechnology Information (NCBI) database (access date: 17.10.2020). The results were downloaded in xml2 format and processed using a custom python script (MetaProcessor: xml2_converter.py). Here, the taxon ID and BLAST similarity columns were fetched from the xml2 file (Suppl. material 1: Table S1 sheet "Raw hits") and the respective taxonomy was downloaded from the NCBI server (Suppl. material 1: Table S1 "Taxonomy added''). The BLAST results were subsequently filtered in three steps. First, only the hit with the highest similarity was kept and duplicate hits were removed. When two or more different taxon names were found, all of them were kept. Subsequently, the hit table was filtered according to the thresholds described in JAMP (https://github.com/Vasco-Elbrecht/JAMP), with a >= 98% cutoff threshold to retain species level, >= 95% for genus, >= 90% for family, >= 85% for order and below 85% for class level (Suppl. material 1: Table S1 "JAMP filtering"). Subsequently, all remaining hits of one OTU were trimmed to their first shared taxonomic rank. Remaining duplicates (i.e., hits of one OTU that share the same taxonomy after the filtering) were dereplicated. Thus, each OTU was assigned to one taxonomic hit in the final taxonomy table (Suppl. material 1: Table S1 "JAMP hit"). Finally, OTUs were matched with the read table and OTUs without assigned taxonomy after BLAST searches were discarded.

Both, the taxonomy and read table file were converted to the TaXon table format (Suppl. material 2: Table S2) for downstream analyses in TaxonTableTools v1.2.4 (Macher et al. 2021). The separately sequenced PCR and field replicates were analyzed using the replicates analysis tools (correlation analyses and shared OTUs) and were subsequently merged. Furthermore, per OTU the sum of reads present in negative controls were subtracted from each sample and only OTUs assigned to the phylum Chordata

were kept for the downstream analyses (Suppl. material 3: Table S3). Spearman rank correlation analysis was performed for i) read numbers and ii) OTU ranks between replicates to test for stochastic deviations. The read proportions, number of OTUs and number of unique species for each class were calculated. A Jaccard distance-based non-metric multidimensional scaling analysis (NMDS) was conducted to test if site effects between the five sampling locations were present or if all samples can be treated as individual field replicates (NMDS settings: 8000 iterations and 400 different initializations). Three incidence data-based rarefaction and extrapolation analyses were performed to calculate the effect of field replicates on the number of obtained species using the iNEXT online calculation tool (https://chao.shinyapps.io/iNEXTOnline/; Hsieh et al. 2016) with 1000 bootstrap repetitions. Here, random sub-samples were drawn from all 18 field replicates and the number of observed fish/lamprey, bird, and mammal species counts were assessed separately. Additionally, an occupancy plot was calculated in TaxonTableTools to investigate the relative occurrence of each species across all replicates. The plot was subsequently adjusted in Inkscape to add an order-specific color code.

Results

The raw data were deposited at the European Nucleotide Archive (https://www.ebi.ac.uk/ena/browser/home) under the accession number PRJEB45400. We obtained 9,906,197 raw reads with 1,193,233 reads assigned to negative controls. After final quality filtering 7,520,725 reads remained (1,646 reads in negative controls), which were clustered into 474 OTUs (97% similarity). The sum of the reads in negative controls after clustering and remapping was 1,376. After the > 0.01% threshold filtering of the read table, 99.7% of reads and 153 OTUs remained of which we could assign 147 taxonomically. In five cases where the marker resolution was too low to distinguish between species, taxonomic annotation was manually edited to retain both possible species names. Therefore, we conservatively counted those cases as one entry in the species list since at least one was present (i.e., *Pipistrellus pipistrellus* / *P. pygmaeus*, *Blicca bjoerkna* / *Vimba vimba*, *Carassius auratus* / *C. carassius*, *Leuciscus aspius* / *Alburnus alburnus*). OTU 17 was automatically assigned only to genus level due to two 100% similarity database sequences representing two different species, the European eel (*Anguilla anguilla*), and the American eel (*Anguilla rostrata*). Since the European eel is the only representative of its genus in Europe, we assigned the OTU manually to *Anguilla anguilla*. Furthermore, we assigned OTU 10 to the mallard (*Anas platyrhynchos*) after manually investigating the taxonomic assignment results. Due to various reference sequences of mallard breeds and one common shelduck breed (*Tadorna tadorna*), the automatic assignment was unable to find a consensus and thus reduced the taxonomic resolution to Anatidae level.

Three OTUs were assigned to Proteobacteria, Verrucomicrobia, and Bacteroidetes and removed for downstream analyses. The majority of reads in negative controls (1371) were found in one field negative control and were mostly assigned to *Sus scrofa*. Thus, the *Sus scrofa* OTU was excluded from the dataset. After merging replicates (OTUs that were not present in both replicates were discarded) and subtraction of reads in negative controls, 137 vertebrate OTUs remained, 64 of which could be assigned to species level (Suppl. material 3: Table S3). Reads were mainly assigned to fish (Actinopterygii, 92% of all reads), while Hyperoartia (only recent representatives are lampreys) accounted for 0.1% of the reads. Mammals were represented by 6% of all reads and birds (Aves) by 2% (Figure 2B). Overall, 74 OTUs were assigned to fish, including 24 different species, while one OTU on species level was assigned to Hyperoartia. Furthermore, 17 OTUs were assigned to 15 bird species and 44 OTUs to 18 different mammal species (Figure 2B). The 25 fish and lamprey species (in the following summarized as fish/lamprey if not stated otherwise) belonged to the orders Cypriniformes, Perciformes, Siluriformes, Esociformes, Anguilliformes, Petromyzontiformes, and Gadiformes (Table 1). They account for 25 of 50 reported fish species from the German federal state Saxony-Anhalt (red list of Saxony-Anhalt, LAU 01/20). The overall 18 mammal species belonged to the orders of Rodentia, Primates, Carnivora, Artiodactyla, and Chiroptera (Table 2), and the 15 bird species to Accipitriformes, Anseriformes, Gruiformes, Galliformes, Columbiformes, and Passeriformes (Table 3). They account for 18 of 81 mammal species (22.2%) and 15 of 202 breeding bird species (7.4%) that are native to Saxony-Anhalt. In terms of read abundance the common dace (*Leuciscus leuciscus*) was the most abundant chordate species with 58% of all reads. Three further fish species showed read proportions of more than 2%, i.e., *Gymnocephalus cernua* (4%), *Abramis brama* (4%), and *Rutilus rutilus* (3%). The only Hyperoartia species we detected was

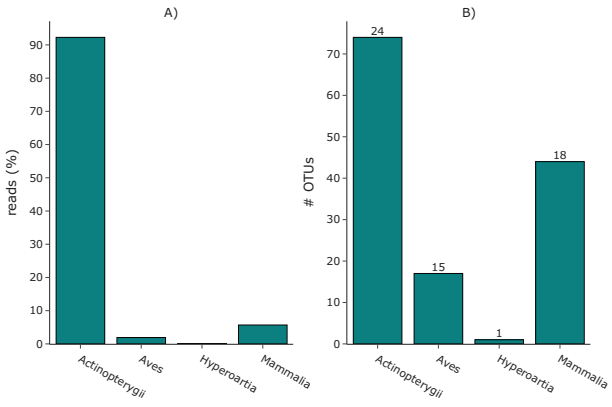

Figure 2. A) Percentage of reads assigned to the classes of Actinopterygii (ray-finned fish), Aves (birds), Hyperoartia (lampreys), and Mammalia (mammals). B) Number of OTUs assigned to the four classes. The number of assigned species is shown above the respective plot.

Table 1. List of detected fish/lamprey species. The IUCN status (LC = least concern, NT = near threatened, VU = vulnerable, EN = endangered, CR = critically endangered, DD = data deficient) and protection status of Saxony-Anhalt (S-A) are presented. Non-native species are marked with an asterisk.

Species name	Common name	IUCN	S-A
Abramis brama	Common bream	LC	
Anguilla anguilla	European eel	CR	NT
Barbatula barbatula	Stone loach	LC	
Barbus barbus	Common barbel	LC	EN
Blicca bjoerkna/Vimba vimba	White bream/bream	LC	/CR
Carassius auratus/carassius	Goldfish/Crucian carp	LC	*/VU
Cobitis taenia	Spined loach	LC	
Ctenopharyngodon idella	Gras carp		*
Cyprinus carpio	Common carp	VU	
Esox lucius	Northern pike	LC	
Gasterosteus aculeatus	Three-spined stickleback	LC	
Gobio gobio	Gudgeon	LC	
Gymnocephalus cernua	Eurasian ruffe	LC	
Hypophthalamichthys nobilis/molitrix	Bighead carp/silver carp		*/*
Lampetra fluviatilis	European river lamprey	LC	VU
Leuciscus aspius/Alburnus alburnus	Asp/Common bleak	LC	
Leuciscus leuciscus	Common dace	LC	
Lota lota	Burbot	LC	VU
Perca fluviatilis	Common perch	LC	
Rhodeus sericeus	European bitterling	LC	
Rutilus rutilus	Roach	LC	
Sander lucioperca	Pikeperch	LC	
Silurus glanis	Wels catfish	LC	
Tinca tinca	Tench	LC	
Vimba melanops	Macedonian vimba	DD	

Table 2. List of detected mammal species. The IUCN status (LC = least concern, NT = near threatened, VU = vulnerable, EN = endangered, CR = critically endangered, DD = data deficient) and protection status of Saxony-Anhalt (S-A) are presented. Non-native species are marked with an asterisk.

Species name	Common name	IUCN	S-A
Apodemus agrarius	Striped field mouse	LC	NT
Apodemus flavicollis	Yellow-necked mouse	LC	NT
Arvicola amphibius	European water vole	LC	
Bos taurus	Cattle		
Canis lupus	Wolf/domestic dog	LC	CR/
Capreolus capreolus	European roe deer	LC	
Castor fiber	Eurasian beaver	LC	VU
Cervus elaphus	Red deer	LC	
Homo sapiens	Human		
Martes foina	Beech marten	LC	
Micromys minutus	Harvest mouse	LC	EN
Microtus agrestis	Field vole	LC	
Myodes glareolus	Bank vole	LC	
Myotis daubentonii	Daubenton's bat	LC	VU
Ondatra zibethicus	Musk rat	LC	*
Procyon lotor	Raccoon	LC	*
Rattus norvegicus	Brown rat	LC	

the European river lamprey (*Lampetra fluviatilis*) with 0.1% of all reads. The mammal species with the highest read abundance was the European beaver (*Castor fiber*) with 4%, while the mallard (*Anas platyrhynchos*) with 1% and the graylag goose (*Anser anser*) with 0.5% were the birds with highest read abundances. A total of 17% of the reads was not assigned to species level. We found positive correlations ($p < 0.05$) for both read counts (rho = 0.843) and number of OTUs (rho = 0.924) between

Table 3. List of detected bird species. The IUCN status and protection status (LC = least concern, NT = near threatened, VU = vulnerable, EN = endangered, CR = critically endangered, DD = data deficient) of Saxony-Anhalt (S-A) are presented. Non-native species are marked with an asterisk.

Species name	Common name	IUCN	S-A
Accipiter nisus	Eurasian sparrowhawk	LC	
Anas platyrhynchos	Mallard	LC	
Anser anser	Grey goose	LC	
Coccothraustes coccothraustes	Hawfinch	LC	
Columba palumbus	Common wood pidgeon	LC	
Cygnus olor	Mute swan	LC	
Emberiza leucocephalos	Pine bunting	LC	*
Emberiza siemsseni	Slaty bunting	LC	*
Gallinula chloropus	Common moorhen	LC	
Gallus gallus	Domestic chicken		
Garrulus glandarius	Eurasian jay	LC	
Grus grus	Common crane	LC	
Phasianus colchicus	Common pheasant	LC	*
Prunella modularis	Dunnock	LC	
Sylvia atricapilla	Eurasian blackcap	LC	

PCR replicate pairs. (Suppl. material 4: Figure S1). Furthermore, PCR replicates showed high similarity values of shared OTUs across all samples, ranging from 85.5% to 97.1% (Suppl. material 5: Figure S2).

No consistent differences in the community composition between the field replicates along the 2 km stretch were found based on the NMDS results (dimensions=3; stress=0.75). Thus, we treated all samples as individual field replicates. To evaluate the effect of sampling effort on the detected species richness, we separately ran rarefaction and extrapolation analyses for fish/lamprey, mammals and birds (Figure 3). Our results showed a substantial increase in detected species richness with increased sampling effort for all three groups. However, we observed a strong disproportionate increase between fish/lamprey species richness and mammal and bird species. Here, the fish/lamprey species showed the lowest increase from an average of 18.8 (±0.7 bootstrap lower and upper confidence limits for the diversity) species in one sample to a maximum of 25 detected species in all 18 samples and 26.2 species in an extrapolated sample size of 36 samples. The detected species richness of both mammals and birds increased substantially more. Here, we observed 5.6 (±0.6) mammal species and 3.4 (±0.5) bird species on average in one sample to a maximum of 18 and 15 species in all 18 samples, and 25 and 24 species in an extrapolated sample size of 36 samples. This accounts for an overall growth in detected species richness of 24.8% for fish/lamprey, 68.9% for mammals, and 77.3% for birds when including all 18 samples. The rarefaction curve for the fish/lamprey species showed its strongest increase in the first 8 replicates, accounting for 80% of the total increase, and then nearing an asymptote towards the maximum number of 18 samples. The rarefaction curves of the mammal and bird species did not reach a plateau but showed a consistent linear increase even beyond the 18

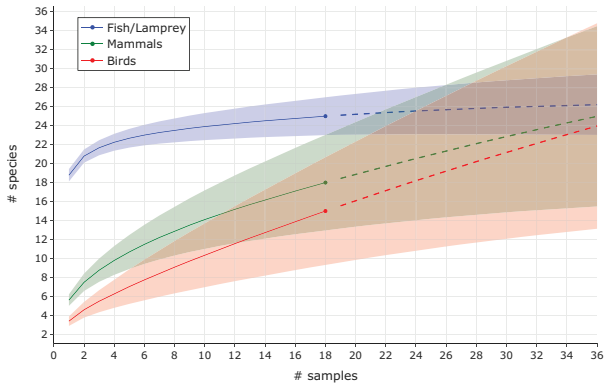

Figure 3. Rarefaction (solid lines) and extrapolation (dashed) curves of the detected species richness of fish/lamprey (blue), mammals (green) and birds (red). The number of observed species is indicated by a dot. The respective bootstrap lower and upper confidence limits for the diversity are represented by shaded areas. Samples were randomly drawn 1000 times for each group to account for stochasticity.

samples. Overall, the majority of fish species (19 of 25) were detected in at least 50% of the samples (Figure 4). Only two fish species were solely detected in one sample (*Lota lota* and *Cobitis taenia*). As for the other vertebrates, the majority of species was detected in less than 50% of the samples, accounting for 13 of 15 bird species and 12 of 17 mammal species.

Discussion

Detected fish biodiversity

Using eDNA metabarcoding, we successfully detected 25 fish species known to occur in the river Mulde and, further, even 50% of all fish species native to Saxony-Anhalt. Most fish species belonged to the order of Cypriniformes (66% of all species), which was expected since they are the dominant group in Central European rivers (Freyhof and Brooks 2011). The species that stood out in terms of read abundance (57.7% of all reads) was the common dace (*Leuciscus leuciscus*), followed by the Eurasian ruffe (*Gymnocephalus cernua*, 4%), and the common bream (*Abramis brama*, 4%). Quantitative interpretations of read counts and biomass or specimens abundance have been reported for fish (Hänfling et al. 2016; Ushio et al. 2018b; Salter et al. 2019; Muri et al. 2020) but can be prone to several sources of bias. In our study, the sampling event took place during the spawning time of various fish species in spring. This can lead to a potential inflation in the number of eDNA molecules of certain species that for example release their eggs and sperm into the open water, such as the common dace (Mills 1981). Furthermore, we cannot rule out a primer-specific bias that in- or deflates read counts of certain species. Thus, we here omitted correlations of read counts to specimen abundance or biomass and merely focused on species occurrence.

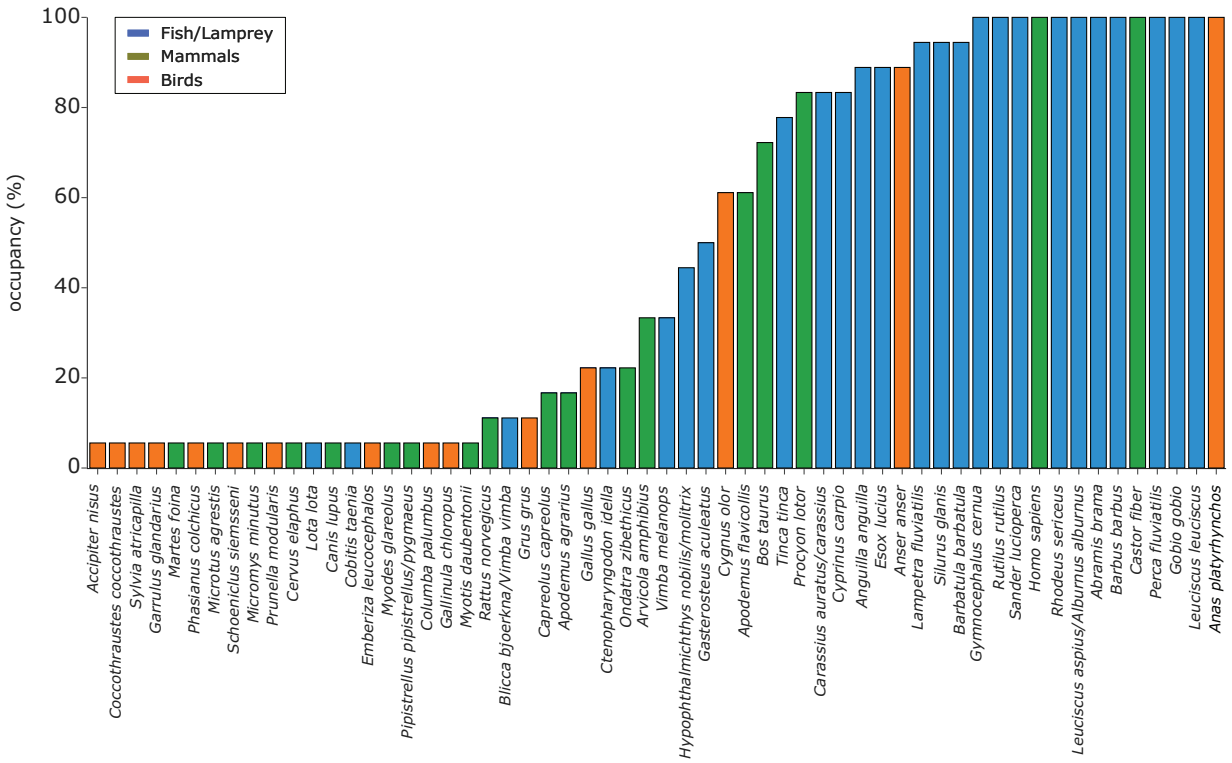

Figure 4. Occupancy of fish/lamprey, bird and mammal species across all samples.

However, not all OTUs were successfully assigned to species level. We found multiple taxa where the 12S marker resolution was too low to distinguish between species and instead two species with identical similarity score were assigned. We manually checked these cases and found several OTUs for which both potential species were reported from the Mulde. For these ambiguous taxonomies we chose a strict approach and counted those cases as one entry. For example, we found the crucian carp and goldfish (*Carassius carassius* and *C. auratus*), where the crucian carp is the ancestry species of the domestic goldfish (Chen et al. 2020). Other closely related species we found are the white bream (*Blicca bjoerkna*) and vimba bream (*Vimba vimba*), the asp (*Leuciscus aspius*) and common bleak (*Alburnus alburnus*), and the invasive bighead and silver carp (*Hypophthalmichthys nobilis* and *H. molitrix*). Furthermore, the record of the Macedonian vimba (*Vimba melanops*) was puzzling, since it does not occur in Germany. We suggest that this hit resembles most likely a vimba bream (*Vimba vimba*), as we found this native species in our dataset and both are closely related (Hänfling et al. 2009), which may impact the taxonomic assignment. These findings confirmed that the fish-specific tele02 marker is not suitable to distinguish all Central European fish at species level.

Beyond fish eDNA metabarcoding: investigating bycatch detection

While most studies discard all non-target sequences (e.g., Evans et al. 2017; Li et al. 2018; Harper et al. 2019; Sales et al. 2020), we explicitly explored the legitimacy of the detected species and discuss whether they can inform other biomonitoring or species conservation activities. We here used the tele02 primer pair that is known to amplify DNA of vertebrate species other than fish (Mariani et al. 2021). First, we could show that many vertebrate species besides fish were found as bycatch in our samples. We were able to detect a notable 22.2% and 7.4% of the whole native mammal and breeding bird fauna reported from Saxony-Anhalt, respectively. While only a minority of the detected species are water-bound or semi-aquatic, the majority inhabit agricultural, forest, and urban habitats, which accompany large parts of the upstream areas of the river Mulde. All organisms depend on water as a drinking source, which makes streams a sink for eDNA signals, transporting them downstream. The most represented group of the vertebrate species bycatch in terms of read proportions and species richness were mammals. The most represented order within the mammals was rodents (Rodentia). Here, high read counts were assigned to the semi-aquatic Eurasian beaver (*Castor fiber*), which is reported to inhabit the river Mulde (German national FFH report, 2019). Furthermore, several terrestrial rodents were found, which often inhabit agricultural and urban environments, such as the striped field mouse (*Apodermus agrarius*) or the Eurasian harvest mouse (*Micromys minutus*). Four species of even-toed ungulates (Artiodactyla) were detected: Cattle (*Bos taurus*) are livestock and graze on fields near the river. Roe deer (*Capreolus capreolus*) and red deer (*Cervus elaphus*) are known to be good swimmers and can easily cross rivers to reach new feeding grounds and thus release traces into the water. Three carnivora species were detected with eDNA. The putative detection of *Canis lupus* is most likely explained by the detection of domestic dogs (*Canis lupus familiaris*), which cannot be distinguished from one another based in the analyzed 12S region. However, since wolf populations have significantly increased over the last decades in central Europe (Chapron et al. 2014) and wolves have been reported from the area of the sampling site (LAU Saxony-Anhalt wolf observation report 2020; J. Arle pers. obs.), a detection of a wild wolf cannot be ruled out. The two other detected carnivore species are the beech marten (*Martes foina*), a generalist and adaptable species inhabiting open areas and forests, and the invasive raccoon (*Procyon lotor*), which inhabits forests or urban areas and is a good swimmer that prefers freshwater associated habitats. Furthermore, three bat species were found, i.e., the Daubenton's bat (*Myotis daubentonii*) and a pipistrelle species (either *Pipistrellus pipistrellus* or *P. pygmaeus*). The Daubenton's bat relies on clean streams or lakes and hunts insects directly over the water surface (Vesterinen et al. 2013, 2016), which makes it very likely to introduce DNA traces into the water by dropping hair, skin, saliva, urine, and feces. The two detected pipistrelle species are closely related and were not distinguishable with the 12S marker. Birds, however, were generally less represented in both read proportions and species richness compared to mammals. An initial observation was that the detected birds are rather common species that occur in high numbers in the area, compared to the detected mammals. Several aquatic and marsh birds were detected, such as the mallard (*Anas platyrhynchos*), the graylag goose (*Anser anser*), the mute swan (*Cygnus olor*), and the common crane (*Grus grus*). While the first three species are common waterfowl in Germany all year round, the common crane is a migratory bird. Its detection falls directly in the spring migration, when large flocks of common cranes travel northwards, which makes a detection with eDNA very likely. Besides the waterfowl, most detected species belonged to passerine birds. Two puzzling species were detected that are not present in Germany: the pine bunting (*Emberiza leucocephalos*) and the slaty bunting (*Schoeniclus siemsseni*). Here, the most likely explanation is that the 12S marker is not suitable to identify them at species level and distinguish them from other bunting species that are inhabiting Germany and are abundant in the area, such as the common reed bunting (*Emberiza schoeniclus*) or the yellowhammer (*Emberiza citrinella*). Furthermore, no amphibians or reptiles were found in the dataset. Particularly the absence of amphibians was notable since at least frog species of the genus *Rana* and toad species of the genus *Bufo* are commonly occurring in streams and ponds in Central Europe. Since the detection of amphibians is possible with the here used tele02 primer (Mariani

et al. 2021) the river Mulde is most likely not a suitable habitat for amphibians, especially during the reproductive season, which falls into the time of the sampling event.

Streams as eDNA conveyor belts

We found no effect of sampling distance on fish species detection. Thus, although samples were collected at five distinct locations of the river Mulde, the 18 collected samples can be considered as individual field replicates rather than 2–4 specific replicates of 5 sites. The lack of a spatial signal is, on the one hand, not unexpected considering that sampling sites were max. 2 km apart, which is well in the range of reported transport distances of eDNA (Deiner and Altermatt 2014; Shogren et al. 2017; Nukazawa et al. 2018). On the other hand, source, state, transport, and fate of eDNA is anything but simple (Barnes and Turner 2016). While eDNA molecules are transported downstream in general, they are influenced by shedding, retention, and resuspension processes along the way (Shogren et al. 2017). Also, location and density of populations thus can greatly influence the detectability (Carraro, Stauffer, and Altermatt 2021). Community signal inferred via eDNA can thus be very site-specific (Cantera et al. 2019). Besides the spatial aspects, sampling time may be even more important in streams, as eDNA concentration can be increased for several taxa due to e.g., seasonal events such as spawning (Wacker et al. 2019) and migration (Thalinger et al. 2019). Also, water discharge drastically differs among seasons thus leading to different baseline concentrations, suggesting the use of hydrological models in eDNA assessments to increase reliability (Carraro et al. 2018).

Disproportionate increase of fish and bycatch detection

Generally, the probability of detecting target DNA when present, i.e., the sensitivity of a method, depends on the concentration and dispersion of target DNA molecules at a site, the sampling design, and the laboratory workflow (Furlan et al. 2016). In previous studies both increased water volume filtered and implementation of field and PCR replicates were found to enhance the sensitivity of eDNA monitoring approaches (Civade et al. 2016; Hänfling et al. 2016; Evans et al. 2017; Beentjes et al. 2019). For example, a previous study on tropical stream fish species suggested that a saturation of tropical stream fish species detection can be reached when sampling 34 to 68 liters per site (Cantera et al. 2019). Our results based on 18 1-L water samples showed that the detection probability of eDNA for non-fish vertebrate species differed substantially among samples. Comparing field samples, we found that fish species richness increased only by 24.8% when considering one versus all 18 samples. A sample volume of 8–10 L can be considered a good tradeoff (sampling effort versus species detection) to monitor the fish fauna of a medium-size river (like the Mulde), since no substantial richness increase was observed by collecting additional 10 L of water. This was different for the detection probability of the non-fish bycatch vertebrate species. For mammals and birds, it increased by 68.9% and 77.3%, respectively, when including 18 field samples. Here, the extrapolation analysis suggested an even further increase of species richness through the collection of additional water samples. While in aquatic organisms such as fish release all their DNA into the surrounding water, only traces of the predominantly terrestrial or aerial bycatch species enter the water leading to a much lower concentration. This expectation is also met when comparing eDNA detection of semi-aquatic bird and mammal species, which were detected in more than 50% of the samples (e.g., mute swan, graylag goose, and Eurasian beaver). Similarly high detection rates, however, were also found for domestic animals living in high abundances in the riparian area of the river (e.g., cattle). Harper et al. (2019) identified similar patterns that terrestrial mammal eDNA signals are weaker and can be detected less frequently than signals from semi-aquatic mammals, using a vertebrate specific primer (Riaz et al. 2011). We also found a high number of human reads in the samples, which are expected in an eDNA assessment in urban environments from various potential sources. Importantly, however, our negative controls did not show many human reads (in our case, a maximum of 4 reads in processed libraries) rendering lab contamination as unlikely. The human traces are likely derived from the original water sample. Nevertheless, low read counts are commonly observed in PCR negative controls and might also originate from laboratory contamination.

eDNA bycatch: unexplored potential for conservation management

While often left aside in studies that focus only on fish biomonitoring, the relevance of detected non-fish bycatch species can be high. This holds true in particular for endangered or protected species that are often difficult to monitor and rely on sighting reports or intensive survey campaigns. Additionally, early reports of invasive species occurrence can also trigger timely management options. For the target taxa, i.e., fish, six of the 25 detected fish/lamprey species are listed as near threatened (European eel), vulnerable (crucian carp, European river lamprey, and burbot), endangered (common barbel), and critically endangered (vimba bream) in the German federal state of Saxony-Anhalt. In the bycatch eDNA data, however, our results detected several mammal species that are classified as protected in Saxony-Anhalt, such as the striped field mouse and yellow-necked mouse (both near threatened), the European beaver and the Daubenton's bat (both vulnerable), the Eurasian harvest mouse (endangered), and possibly the wolf (critically endangered). Although we were able to detect these endangered species, our findings only provide small insights into the whole vertebrate community, since this study was limited in terms of time coverage (one sampling event) and spatial coverage (2 km stretch of one river). The rarefaction analysis

results predicted the detection of more mammal and bird species if more samples were collected. However, it is expected that advances in the standardization and operation of fish eDNA metabarcoding will lead to a higher rate of application in research and regulatory monitoring campaigns in the future. This goes in hand with an increasing amount of available bycatch data that can be analyzed and utilized. With hundreds or thousands of eDNA water samples that are potentially collected each year in countries that apply a nationwide routine monitoring, the coverage of water bodies and different habitats will automatically increase. This opens access to obtain highly resolved spatial and temporal data not only on fish distributions, but also detection patterns of bycatch species. The obtained data could be directly collected in online biodiversity databases and used for more comprehensive insights into vertebrate species occurrence and distribution. The additionally acquired data would then also be available for conservation planning and management and could help to increase the extent and accuracy of regional red lists and lead to a better intercalibration with the international red list. This accounts particularly for conservation monitoring under the EU Birds Directive (Directive 2009/147/EC, 2009), the "EU Regulation 1143/2014 on Invasive Alien Species" or the EU Habitats Directive (Council Directive 92/43/EEC, 1992), where data is generally hard to obtain and striking deficits in the monitoring coverage are known. For example, data on distribution and population sizes of the bird fauna is available in great detail, but observations are often conducted on a non-standardized, voluntary basis. For mammals, however, routine monitoring campaigns are even more scarce, since they are costly and time consuming. Here, the fish eDNA metabarcoding data could provide a notable increase of data points that can be sampled and analyzed under standardized conditions and can be evaluated by experts. The potential of obtaining new, additional information on terrestrial species, in particular elusive, rare or protected species without additional costs is immense and may also stimulate major international conservation initiatives currently developed in the context of the post-2020 CBD-framework.

Nevertheless, the reports of non-target species from fish eDNA metabarcoding have to be interpreted with particular caution. Environmental DNA metabarcoding comes with several challenges that can lead to both false negative and false positive identifications (Barnes and Turner 2016). This accounts particularly for terrestrial and aerial species, which are only temporarily interacting with the water and leave only marginal traces.

We also detected species that are generally unlikely to inhabit the catchment and thus likely represent a false-positive result. Here, potential sources are that the marker resolution is too low to distinguish species, the detected eDNA was already degraded, or the reference sequences are incorrectly labeled. But also, introduction of eDNA via effluents from sewage plants or other influxes can falsify the picture of the species distribution (Yamamoto et al. 2016). Particularly false positive signals must be flagged to avoid biased distribution patterns, when they can be identified as such. It also has to be considered that commonly used fish primers, such as the MiFish (Miya et al. 2015) and tele02 primers, are optimized for fish and discriminate the amplification of other taxa, which will most-likely lead to a lower detection rate compared to fish species. To compensate for the primer bias, universal vertebrate primers (e.g., Riaz et al. 2011) could be used, which would allow to monitor fish, mammal, bird, reptile, and amphibian species at once without additional sampling or laboratory efforts. However, if the main target of the routine biomonitoring remains to detect fish, specific primers might perform better. If the goal is to target groups other than fish, the additional usage of specific primer sets for mammals (MiMammal, Ushio et al. 2017) or birds (MiBird, Ushio et al. 2018) on the same DNA extract is possible, but would come with additional laboratory work and costs. These, however, are small and analysis can be automated (Buchner et al. under review), thus the added value for other specific conservation and management programs can be immense.

Outlook

Our results show that not only target fish but also bycatch species (i.e., birds, mammals) can be assessed reliably using fish eDNA metabarcoding. While the analysis of only few 1-L samples already delivered consistent estimates on fish species richness, the detected richness of non-target bycatch species steadily increased with the number of samples analyzed due to the lower concentration of eDNA molecules of these in the water. In total, we detected a notable 50% of fish species, 22.2% of mammal species, and 7.4% of breeding bird species native to Saxony-Anhalt by sampling a single site at a single day only. In typical fish eDNA metabarcoding assessments, these bycatch data are typically left aside, yet, from a viewpoint of biodiversity monitoring they hold immense potential to inform about the presence of also (semi-)terrestrial species in the catchment. Unlocking these data from the increasingly available fish eDNA metabarcoding information enables synergies among terrestrial and aquatic biomonitoring programs, adding further important information on species diversity in space and time. We thus encourage to exploit fish eDNA metabarcoding biomonitoring data to inform other conservation programs. For that purpose, however, it is essential that eDNA data is jointly stored and accessible for different biomonitoring and biodiversity assessment campaigns, either at state, federal, or international level.

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Supplementary material 1
Table S1. BLAST taxonomy table
Author: Till-Hendrik Macher
Data type: BLAST taxonomy table

Link: https://doi.org/10.3897/mbmg.5.66557.suppl1

Supplementary material 2
Table S2. Raw taXon table as created with TaxonTableTools
Author: Till-Hendrik Macher
Data type: taXon table (raw)

Link: https://doi.org/10.3897/mbmg.5.66557.suppl2

Supplementary material 3
Table S3. Filtered taXon table
Author: Till-Hendrik Macher
Data type: taXon table (filtered)

Link: https://doi.org/10.3897/mbmg.5.66557.suppl3

Supplementary material 4
Figure S1
Author: Till-Hendrik Macher
Data type: image
Explanation note: Spearman correlation analyses between 2nd-step PCR replicates for reads (A) and OTUs (B). Significant correlations ($p \leq 0.05$) are marked with an asterisk.

Link: https://doi.org/10.3897/mbmg.5.66557.suppl4

Supplementary material 5
Figure S2
Author: Till-Hendrik Macher
Data type: image
Explanation note: Proportion of shared OTUs between the two independent 2nd-step PCR replicates of each sample.

Link: https://doi.org/10.3897/mbmg.5.66557.suppl15