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Improved freshwater macroinvertebrate detection from environmental DNA through minimized nontarget amplification

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Abstract

DNA metabarcoding of freshwater communities typically relies on PCR amplification of a fragment of the mitochondrial cytochrome c oxidase I (COI) gene with degenerate primers. The advantage of COI is its taxonomic resolution and the availability of an extensive reference database. However, when universal primers are used on environmental DNA (eDNA) isolated from water, benthic invertebrate read and OTU numbers are typically "watered down," that is, under represented, compared to whole specimen "bulk samples" due to greater co-amplification of abundant nontarget taxa (e.g., fungi, algae, and bacteria). Because benthic stream invertebrate taxa are of prime importance for regulatory biomonitoring, more effective ways to capture their diversity via eDNA isolated from water are important. In this study, we aimed to improve benthic invertebrate assessment from eDNA by minimizing nontarget amplification. Therefore, we generated eDNA data using universal primers BF2/BR2 on samples collected throughout 15 months from a German Long-Term Ecological Research site (Rhine-Main-Observatory, Kinzig River) to identify most abundant nontarget taxa. Based on these data, we designed a new reverse primer (EPTDr2n) with 3'-specificity toward benthic invertebrate taxa and validated its specificity in silico together with universal forward primer fwhF2 using available data from GenBank and BOLD. We then performed in situ tests using 20 Kinzig River eDNA samples. We found that the percentage of target reads was much higher for the new primer combination compared to two universal benthic invertebrate primer pairs, BF2/BR2 and fwhF2/fwhR2n (99.6% versus 25.89% and 39.04%, respectively). Likewise, the number of detected benthic invertebrate species was substantially higher (305 versus 113 and 185) and exceeded the number of 153 species identified by expert taxonomists at nearby sites across two decades of sampling. While few taxa, such as flatworms were not detected, we show that the optimized primer avoids the nontarget amplification bias and thus significantly improves benthic invertebrate detection from eDNA.

Florian Leese and Mandy Sander are shared first authors

KEYWORDS
bioassessment, bioindication, biomonitoring, COI, eDNA, insects, LTER, metabarcoding, primer bias

1 | INTRODUCTION

With environmental DNA (eDNA) extracted from water, aquatic biodiversity can be detected across the tree of life with minimal effort (Bagley et al., 2019; Grey et al., 2018; Li et al., 2018; Taberlet et al., 2018; Zhang et al., 2020). The potential of DNA based methods, in particular eDNA metabarcoding, for aquatic biodiversity and ecological quality assessments is therefore promising (Keck et al., 2017; Pawlowski et al., 2018). Especially for amphibians and fish, eDNA has been reported to capture species diversity in much greater detail than traditional techniques (Li et al., 2019; Pont et al., 2018; Stoeckle et al., 2017; Thomsen et al., 2012; Valentini et al., 2016). In addition, derived ecological status classes or quality ratios (EQRs) based on fish data are consistent between eDNA assessments and traditional techniques (e.g., Hänfling et al., 2016; Pont et al., 2018). Therefore, a roadmap toward the inclusion of eDNA-based methods into regulatory assessment programs is discussed (Hering et al., 2018; Leese et al., 2018; Li et al., 2019; Pont et al., 2019). The big advantage of eDNA-based biodiversity assessments on vertebrate species is that vertebrate-specific primers that target conserved regions of the mitochondrial 12S or 16S genes exist. Therefore, the majority of reads are on-target vertebrate reads. Also owed to this fact, eDNA-based assessments for fish and amphibians are in principle at the level of routine application. For benthic invertebrate species, however, the situation lags behind substantially (Belle et al., 2019; Blackman et al., 2019). One main reason for this is that, in contrast to fish and amphibian eDNA analyses, the metabarcoding fragment typically used is the mitochondrial cytochrome c oxidase I (COI) gene. The advantage of COI is its taxonomic resolution and the availability of extensive reference data (Andújar et al., 2018; Clarke et al., 2017; Ratnasingham & Hebert, 2007; Weigand et al., 2019). However, in contrast to 12S or 16S, COI as a protein-coding gene shows codon degeneracy that limits taxon-specific primer design (Clarke et al., 2014; Deagle et al., 2014; Sharma & Kobayashi, 2014) making it almost impossible so far to design target-specific primers for diverse groups such as "invertebrates" but also fish and amphibians. Elbrecht and Leese (2017a) proposed an approach using degenerate primers for specific taxon groups or taxa of a specific geographical region, depending on the research or applied question. It should be noted, however, that primer degeneracy is no main concern when metabarcoding is used for bulk tissue samples, as this "biodiversity soup" (Yu et al., 2012) mostly contains DNA of the target organisms (Elbrecht et al., 2016, 2017). It becomes a challenge when trying to detect trace amounts of target DNA in pools of nontarget DNA, and this is the case with eDNA extracted from water. Samples are dominated by DNA from living bacteria, fungi, phytoplankton, and other nontarget DNA. When the vast majority of DNA in the sample is nontarget DNA, universal primers for COI will amplify a majority of COI fragments from nontarget organisms even if primers do not match well. This leads to DNA of the target invertebrates becoming "watered down" in the resulting sequenced reads from a sample (Hajibabaei, Porter, Robinson, et al., 2019). This phenomenon has been reported throughout studies using COI for benthic invertebrate assessments from eDNA. For example, Deiner et al. (2016) reported a large diversity of benthic invertebrate families detected from stream eDNA, yet, small nontarget taxa such as Rotifera captured the vast majority of reads. In a study comparing bulk sample and eDNA metabarcoding in New Zealand streams, Macher et al. (2018) detected a vast number of taxa using eDNA, yet only 21% of reads were assigned to Metazoa. When considering species (>97% identity) for the important bioindicator taxa Ephemeroptera, Plecoptera, Trichoptera, and Diptera (EPTD), number of reads only comprised 0.6% compared to about 30% in bulk samples (Macher et al., 2018, Macher pers. comm.). A similar comparison was performed by Gleason et al. (2020) in Canada, also reporting only 12% of the eDNA reads being assigned to benthic invertebrate taxa as opposed to 99% of the reads in bulk samples. Similarly, Beentjes et al. (2019) performed eDNA metabarcoding on water from ponds and recovered a majority of nontarget taxa reads and OTUs (35.7% of reads Metazoa, 14% of the OTUs). As in Deiner et al. (2016) and Macher et al. (2018), most of these were meiofaunal metazoans and only a minor faction (in most of the samples < 5%) were benthic macroinvertebrate taxa (see Beentjes et al., 2019; Figure S3). Thus, especially for indicator taxa, species or OTU diversity was low. In the study by Hajibabaei et al. (2019), which coined the phrase "watered-down biodiversity," the number of reads assigned to benthic invertebrates per eDNA sample was two orders of magnitude lower compared to bulk samples. Likewise, diversity measured as the number of exact sequence variants (ESVs) per sample was one order of magnitude lower. Pereira-da-Conceicoa et al. (2019) showed that water samples from South African streams contained less than 10% reads assigned to targeted benthic invertebrate taxa. As a consequence of ineffective primers for amplification of benthic invertebrates from eDNA, biodiversity assessments for this group still focus on whole organismal samples from mixed communities, that is, sorted bulk samples, to maximize species detection. Several studies demonstrated that DNA metabarcoding data obtained from bulk samples yield robust, powerful, and highly resolved data that can be intercalibrated with current environmental assessment procedures (Aylagas et al., 2014, 2018; Elbrecht et al., 2017; Kuntke et al., 2020). However, collecting and sorting individual benthic invertebrates is a time-consuming (and thus costly) step that greatly hinders the adoption of the methods at a broader scale (see Blackman et al., 2019 for a discussion). As alternative methods that omit the time-consuming step of specimen-picking, three approaches are proposed: (a) using only the preservative liquid (Erdozain et al., 2019; Hajibabaei

et al., 2012; Martins et al., 2019; Zizka et al., 2018), (b) using completely homogenized environmental bulk samples without sorting of benthic invertebrate specimens (Emilson et al., 2017; Hajibabaei 2019; Pereira-da-Conceicoa et al., 2019), or (c) using a water sample as done for fish or amphibian species (Macher et al., 2018; see Blackman et al., 2019 for a review).

As eDNA isolated directly from water is the simplest and most economic approach, it is regarded as the ideal solution when the aim is to maximize data generation for aquatic biomonitoring 2.0 (Baird & Hajibabaei, 2012; Bush et al., 2017). The aim of this study was to minimize the effect of nontarget taxa DNA amplification from eDNA samples by designing and testing (in silico and in situ) a newly designed eDNA primer targeting the COI gene in benthic invertebrates. As a basis, we used data on the most abundant nontarget taxa identified using universal metabarcoding primers. We tested the performance of this primer in situ by comparing the proportion of recovered benthic invertebrate reads and OTUs from the same eDNA samples amplified with three different primer sets. Environmental DNA was sampled at multiple stations of a German Long-Term Ecological Research (LTER) site, the Kinzig River catchment (Mirtl et al., 2018), allowing us to leverage benthic invertebrate biodiversity data, compiled for over two decades, to validate detection results.

2 | MATERIALS AND METHODS

2.1 | Sampling, filtration, and extraction

We collected 102 water samples from one site in the Kinzig River (site 54, see Figure 1) in biweekly intervals within the Long-Term Ecological Research (LTER) site Rhine-Main-Observatory [RMO, https://deims.org/9f9ba137-342d-4813-ae58-a60911c3abc1] from May 2017 to August 2018. LTER sites, such as the RMO, are particularly suited for comparative analyses as they provide a wealth of long-term biodiversity data (Haase et al., 2016, 2018; Kuemmerlen et al., 2016). Three 1-L samples were taken per sampling day: (a) surface water in the middle of the stream, (b) 10 cm above riverbed in the middle of the stream, and (c) at the riverbank. To test the performance of the newly designed primer, a subset of ten of the 102 samples were used. Additionally, ten water samples were collected further upstream in Kinzig River and its tributaries within the RMO in spring 2019 (see Table S1). After sampling 3× 1 L of stream water for the biweekly monitoring (2017, 2018) and 1× 1 L for the Kinzig River network (2019), samples were stored at −20°C until further processing (6 weeks until 12 months, no additives added). Samples were filtered using a vacuum pump and DNA was captured on 0.45-µm Cellulose Nitrate membrane filters (diameter 47 mm; Nalgene). Negative filter controls were included for each filtering day and person that filtered. For the negative filter controls, no water sample was added and only the surrounding air was filtered to check for air contamination. Filtering was processed in a separate room, where no laboratory work was conducted. For filtering, a tube attached to an electric pump was used and for working as sterile as possible, gloves were changed, and the workspace was cleaned with ethanol and bleach (4%–5% sodium hypochlorite, rinsed with distilled water afterward) in between samples. Depending on turbidity, one to three filters were used per sample. Filters were stored in Eppendorf tubes filled with 96% denatured ethanol (Carl Roth). Further eDNA processing steps were carried out in a dedicated eDNA laboratory. Full-body protective equipment was worn, and surfaces were sterilized with UV-C light after each work cycle. For DNA extraction, filters were placed in sterile petri dishes and dried overnight at room temperature. DNA extraction was performed following a salt-precipitation

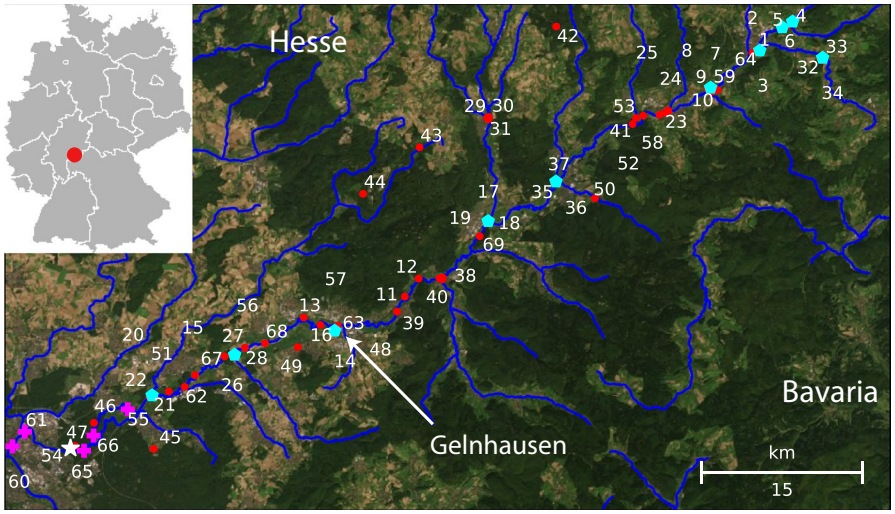

FIGURE 1 Sampling locations within the Long-Term Ecological Research (LTER) site Rhine-Main-Observatory in Hesse, Germany. The white star (site 54) indicates the biweekly sampling site. Purple crosses indicate long-term (morpho-taxonomically surveyed) monitoring sites in close vicinity to site 54 that were used for comparison. Blue pentagons indicate Kinzig River network samples. Red dots represent other (morpho-taxonomically surveyed) monitoring sites. In case of several nearby sampling sites, more than one number is given. The inset figure is Germany. See Table S1 for further details [Colour figure can be viewed at wileyonlinelibrary.com]

protocol (Weiss & Leese, 2016; see Supplementary Information S1). Next, 1.5 µl RNase A (10 mg/ml, Thermo Fisher Scientific) was added to each sample and incubated for 30 min at 37°C and 300 rpm in an Eppendorf ThermoMixer C (Eppendorf AG). Samples were then cleaned up using a Qiagen MinElute Kit to obtain high-quality DNA. Samples were eluted in 30 µl molecular grade H_2O (Carl Roth).

2.2 | DNA metabarcoding with BF2/BR2 for biweekly data set

We applied a two-step PCR approach for the long-term biweekly data set (Zizka et al., 2019). For the first PCR step, universal BF2/BR2 primers (Elbrecht & Leese, 2017a) were used. For the second step, fusion primers with a unique inline shift and Illumina adapters attached to the 5'-end were used (see Figure S1). For the first step, four PCR replicates were processed per extract and for the second step, two of them were pooled together, resulting in two PCR replicates for each sample. All PCR products contained 1× Multiplex PCR Master Mix (Qiagen Multiplex PCR Plus Kit, Qiagen), 0.2 µM of each primer (BF2/BR2: Eurofins Scientific, Luxemburg (1st step); Metabion GmbH (2nd step), fwhF2/fwhR2n: Eurofins Scientific, Luxemburg, EPTDr2n, and 2nd step primers with Illumina index: Metabion GmbH), 0.5 × Q-solution (Qiagen), 1 µl DNA extract/PCR product (concentration not measured) filled up with RNase-free water to a total volume of 50 µl. PCRs were conducted as follows: First-step PCRs consisted of 5 min initial denaturation at 95°C, 30 cycles of 30 s at 95°C, 90 s at 50°C, and 2 min at 72°C, followed by a final elongation for 10 min at 68°C. Second-step PCR was identical to 1st step PCR, only 15 cycles were run using 1 µl of the unpurified PCR product from step 1 with an annealing temperature of 57°C.

The DNA concentration of each PCR product of the second-step PCR was quantified on a Fragment Analyzer™ Automated CE System (Advanced Analytical Technologies GmbH) using the NGS Standard Sensitivity Kit. Equimolar amounts of PCR products were pooled. The pool was purified with a Qiagen MinElute Reaction Cleanup Kit to remove BSA prior to a bead-based 0.76x SPRIselect left-sided selection for BF2/BR2 (Beckman Coulter). Libraries were sequenced on a single flow cell using an Illumina MiSeq system with a 250 bp paired-end read kit v2 with 5% Phi-X spike-in added by the sequencing company CeGaT GmbH. 102 samples were sequenced for the BF2/BR2 primer pairs from site 54 (biweekly sampling).

Reads were demultiplexed and assigned to their original sample using JAMP v0.67 (https://github.com/VascoElbrecht/JAMP; Elbrecht et al., 2018). Default settings in JAMP v0.67 were used to perform subsequent data filtering. Specifically, paired-end reads were first merged using the script module U_merge_PE with fastq_maxdiffs = 99 for the maximum number of mismatches in the alignment and fastq_pctid = 90, for the minimum %id of the alignment. If needed, the reverse complements of the sequences were built (U_revcomp) with usearch v11.0.667 (Edgar, 2010). Primer sequences were removed and sequences of unexpected length were discarded using Cutadapt v2.3 (Martin, 2011) so that only reads with a maximum deviation of 15 bp were used for further analyses (Minmax). To remove the reads with an expected error of > 0.5, the module U_max_ee was used. Singletons were removed before clustering the sequences using Uparse (U_cluster_otus) with ≥97% similarity into OTUs. The dereplicated sequences, including singletons, were then mapped, with a similarity of ≥97%, to the generated OTU dataset to maximize the number of reads retained. OTUs with a minimal read abundance of 0.01% in at least one sample were retained for further analyses, while other OTUs were discarded. Sequences were compared to the BOLD database sequences using BOLDigger 1.1.4 (Buchner & Leese, 2020). For the whole dataset, a similarity threshold of 85% to a reference sequence in BOLD was required to include the OTU into further analysis. This was done because < 85% similarity the hits are often ambiguous and even from different classes or even phyla. For taxonomic assignment of benthic invertebrate organisms, a similarity of > 90% to a reference sequence in BOLD was needed. Every single OTU assigned to an invertebrate taxon was checked manually to test whether conflicting results were found and if assignment was based on specimens identified by taxonomic experts. Still, incorrect taxon assignments may exist sporadically due to incorrect data base entries (Weigand et al., 2019). We excluded terrestrial or planktonic taxa for the comparison of benthic invertebrates.

OTU and read table analyses were performed using R Studio v.3.5.3, and figures were created using the package ggplot2 (Wickham, 2009). Venn diagrams were constructed using http://bioinformatics.psb.ugent.be/webtools/Venn/. The vast majority of reads were assigned to nonarthropod taxa for the 102 samples analyzed from site 54, especially bacteria and unicellular eukaryotes (see Figure S2). This information was used here as one basis to design benthic invertebrate, in particular insect-specific primers.

2.3 | Primer design

To generate reference data for primer design, we used PrimerMiner v0.18 (Elbrecht & Leese, 2017b) to download COI sequences (and full mitogenomes) for 15 important freshwater macroinvertebrate orders from BOLD and NCBI, as well as their mitogenomes if available (taxa listed in Elbrecht and Leese (2017b)) and possible nontarget groups (access date: 8 September 2019, standard settings; downloaded sequences available on request). Sequences were clustered into OTUs with a ≥97% similarity threshold as above. In addition, sequences of nontarget taxa obtained from the 102 eDNA samples were clustered into OTUs as well. These most abundant nontarget OTUs identified from the 102 samples collected over 15 months (i.e., algae, bacteria, fungi with a proportion of at least 1% of total read abundance and a similarity to a sequence from BOLD or NCBI of at least 98%) as well as the sequences with a similarity of at least 98% to these OTUs from NCBI and BOLD were aligned together with OTUs from the 15 benthic invertebrate orders. Alignment was created using MAFFT v7 (Katoh & Standley, 2013) in Geneious v2019.2.1 using the auto setting function. The alignment was then

manually inspected for conserved, diagnostic bases only present in the benthic invertebrate orders but not in sequences of nontarget organisms. These sites were then seen as potential 3' sites for a primer, which were then manually extended neglecting further thermodynamic rules for primer design because of the extreme paucity of diagnostic 3' positions. The positions of primer bases were named relative to a reference sequence of *Drosophila yakuba*, GenBank accession number X03240 (Clary & Wolstenholme, 1985).

2.4 | In silico evaluation

The new EPTDr2n primer was evaluated together with fwhF2 in silico using PrimerMiner v0.21. Again, the 15 most important freshwater benthic invertebrate orders were used to download COI data (see above, scripts and input files available on request). To evaluate the amplification of nontarget organisms, OTU sequences from the 102 BF2/BR2 samples were used (Data S1) and aligned using MAFFT as above . The primer binding regions for fwhF2 and EPTDr2n were extracted from the alignment and in silico evaluated using PrimerMiner on default settings. Penalty scores reflecting primer mismatches (Elbrecht & Leese, 2017b) were grouped on order level (minimum match to reference database 90%) using the information encoded in the sequence IDs. Nontarget organisms were here defined as OTUs that do not match to Arthropoda, Annelida, or Mollusca. R scripts and data used are available on request. OTU tables were extracted as fasta files for each order using an R script and evaluated in silico with default parameters.

2.5 | In situ *primer evaluation*

The newly developed primer was tested using two different test cases: (a) ten samples from the biweekly long-term data series from Kinzig site 54, and (b) ten samples from the Kinzig River network sampling (see Table S1). DNA extraction was similar to the approach outlined above. A two-step PCR was used for the newly developed primer combination fwhF2/EPTDr2n and as a comparison we used the short primer pair fwhF2/fwhR2n (Vamos et al., 2017). For the fwhF2/EPTDr2n and fwhF2/fwhR2n combinations, four length-varying primers were used in the first step with two replicates per sample, with each of them having a universal tail attached (Figure S1). Length variation was due to inline shifts (0–3 Ns) between the universal tail and the primer sequence to maximize diversity (Elbrecht & Leese, 2015). For the second step, primers matching the universal tail with an i5/i7 index and P5/P7 Illumina adapter attached were used. Annealing temperature of the newly developed primer EPTDr2n together with primer fwhR2n was estimated by running a step-down gradient PCR (first 8 × 60°C followed by 32 × 48–58°C) using bulk and eDNA samples. For the first step a step-down gradient PCR with 30 cycles (6 × 60°C for the annealing temperature and 24 × 54°C for fwhF2/fwhR2n and 24 × 48°C for fwhF2/EPTDr2n) was conducted, followed by the second-step PCR with 15 cycles and an annealing temperature of 60°C (see Supplementary information S2 for an updated protocol). Some samples were run with 23 cycles in the second step, due to weak bands on the gel. Purification, left-sided size selection (0.78×), sequencing and sequence analysis were conducted as described above. We performed nonparametric Wilcoxon rank sum tests to assess whether primer sets differed significantly in the proportion of target taxa reads as well as number of target taxa (species, genus, family) resolved.

2.6 | Comparison to morphological data

Classical morphological benthic invertebrate taxa lists were retrieved from LTER monitoring samples of up to 22 sites per year generated over the past 20 years from Kinzig River (RMO). In addition, we used data from the holistic LTER RMO biodiversity database that includes data from other monitoring activities like the Water Framework Directive (WFD) monitoring (https://rmo.senckenberg.de/search/home.php). These lists were compared to taxa lists retrieved from eDNA samples using different primers. Comparisons were done at four different levels: Comparisons with (a) long-term morphological data available for the RMO monitoring site next to study site 54 (W1), (b) five RMO monitoring sites within a radius of 5 km of site 54, (c) all 22 LTER monitoring sites of the RMO, and (d) the entire RMO database covering all monitoring activities in the RMO (e.g., WFD monitoring) (Figure 1). Data from the ten Kinzig River sampling sites were compared similarly. For this comparison, only taxonomic names and not OTUs were considered; that is, OTUs not matching at least at family were discarded. Different OTUs assigned to the same species were merged. This step is similar to Elbrecht et al. (2017) in order to allow for comparison between the two approaches. Taxa with ambiguous assignments (e.g., *Sericostoma personatum/flavicorne*; *Baetis* cf. *scambus*) were reduced to genus level. For statistical comparisons between morphological and eDNA-based approaches at genus level, we also used species level hits but kept only genus information. Likewise, for comparisons at family level we also used species and genus level hits but retained only the information on family.

2.7 | Taxonomic assignment of different primer lengths

The three different COI fragments used in this study are all subfragments of the classical Folmer fragment (Folmer et al., 1994) and differ in their lengths (BR2/BR2: 421 bp, fwhF2/fwhR2n: 205 bp, fwhF2/EPTDr2n: 142 bp). To assess whether the length differences had an impact on the taxonomic assignment and resolution, we performed the following analysis: (a) For all species level hits observed in this study, we downloaded all COI sequences for the respective Folmer region from BOLD. (b) We removed gaps at 5' and 3' ends, sequences < 500 bp as well as sequences containing N's. (c) From the remaining sequences, we excised the 421 bp BF2/BR2 fragment using cutadapt 2.10, and (d) removed identical sequences.

(e) From the remaining sequences, we excised the 205 bp fwhF2/fwhR2n as well as the 142-bp fwhF2/EPTDr2n target fragment. (f) All sequences that did not contain all three primer fragments were rejected so that only target fragments for all three primers were retained. (g) The remaining sequences were reassigned using the BOLD Identification Engine to test how many of the sequences for each of the three primers were correctly assigned as well as how many ambiguous hits were returned using BOLDigger 1.1.10.

An overview of the general workflow followed in this study is given in Figure 2.

3 | RESULTS

3.1 | Primer design and in silico evaluation

For the 102 biweekly samples, we obtained between 27,934 and 282,981 reads per sampling site/time point (Bioproject PRJNA664693). The 8,160,330 reads that passed quality filtering were clustered into 15,812 OTUs and these used for primer design. All analyzed primers are located within the classical Folmer COI fragment (Folmer et al., 1994) (Figure 3a). No fully diagnostic positions were found comparing benthic invertebrate target and nontarget taxa. However, two adjacent positions in the alignment (COI pos. 1979 and 1982, i.e., last and fourth last bases, Figure 3b) differed to a large extent between target and nontarget taxa. These differences were even more obvious when only considering the abundant diatom (Stephanodiscaceae) and bacterial OTUs identified from the 102 biweekly samples analyzed with BF2/BR2 (Figure S2). This region was then chosen to design new primers compatible to existing other universal primers (see Table S2 for list of primers that failed in amplification and were thus rejected). The new EPTDr2n primer can be coupled with one of the universal forward primers (e.g., fwhF2 or BF2, Table 1). In silico analysis supports a good performance, that is, high similarity, of fwhF2 primer across phylogenetically distinct groups with the exception of Actino- and Proteobacteria (Figure 3c). The new reverse primer EPTDr2n had higher penalty scores for both target and nontarget taxa compared to fwhF2. However, penalty scores for abundant nontarget freshwater taxa (e.g., diatoms, bacteria, fungi) were substantially higher and very low for freshwater

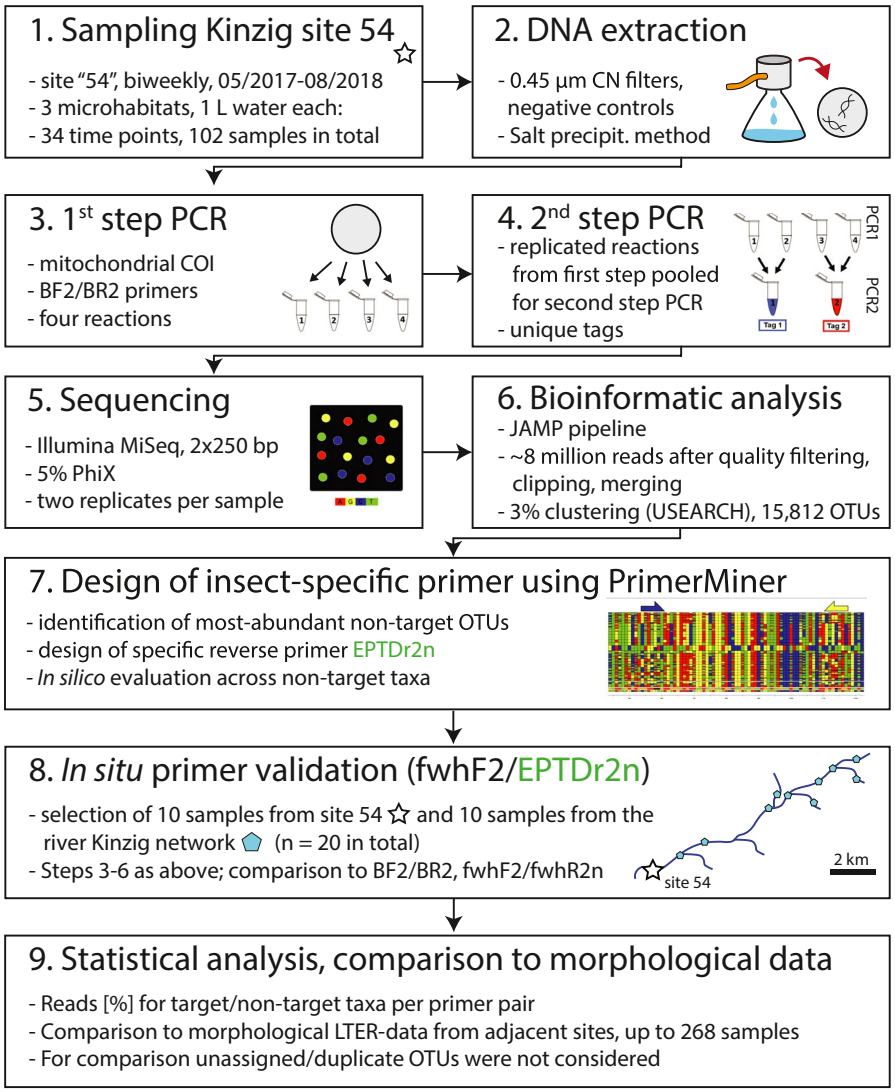

FIGURE 2 Workflow of the present study [Colour figure can be viewed at wileyonlinelibrary.com]

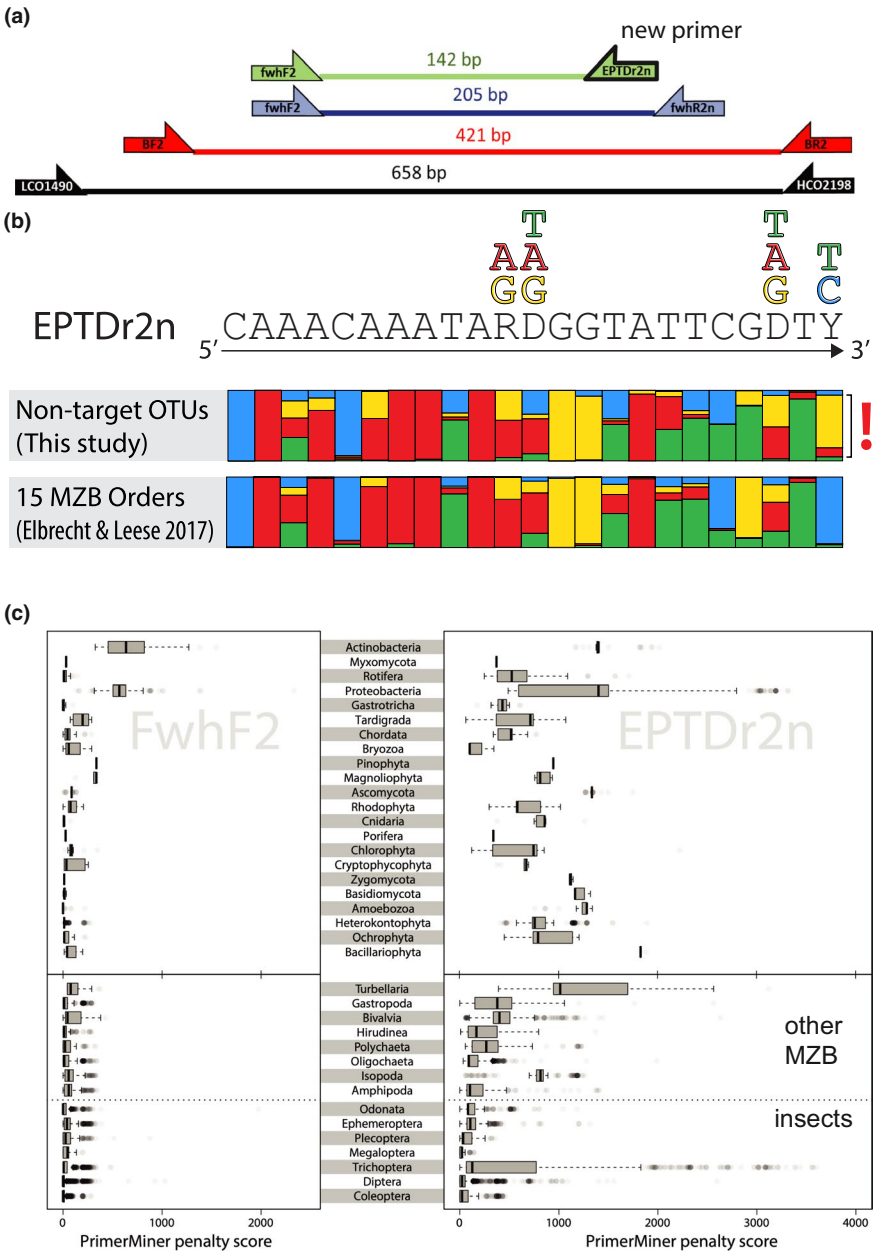

FIGURE 3 In silico primer evaluation. (a) Overview of COI primers used including the newly developed EPTDr2n primer that was applied together with fwhF2. LCO1490 and HCO2198 (Folmer et al., 1994) primers were not used but shown for reference. (b) PrimerMiner plots of the EPTDr2n primer binding site, for 15 macrozoobenthic orders from (Elbrecht & Leese, 2017a) and the nontarget taxa. The quotation mark highlights the proportion of taxa omitted by choosing the last 3' wobble base "Y." (c) Penalty scores for the new primer combination fwhF2 (left) and EPTDr2n (right). Results are listed for macrozoobenthic orders (MZB, bottom) and nontarget groups (top). The higher the penalty score calculated using PrimerMiner (Elbrecht & Leese, 2017a), the worse the primer is expected to perform [Colour figure can be viewed at wileyonlinelibrary.com]

TABLE 1 COI primers used in this study, including the newly developed EPTDr2n primer combined with forward primer fwhF2 in this study. Further primers tested that did not work are listed in Table S2

Primer name	Primer sequence (5´–3´)	Direction	Study
BF2	GCHCCHGAYATRGCHTTYCC	Forward	Elbrecht and Leese (2017a)
BR2	TCDGGRTGNCCRAARAAYCA	Reverse	Elbrecht and Leese (2017a)
fwhF2	GGDACWGGWTGAACWGTWTAYCCHCC	Forward	Vamos et al. (2017)
fwhR2n	GTRATWGCHCCDGCTARWACWGG	Reverse	Vamos et al. (2017)
EPTDr2n	CAAACAAATARDGGTATTCGDTY	Reverse	This study

arthropods with the exception of some trichopteran genera and isopods. Trichoptera target taxa with higher penalty scores belonged to *Hydropsyche*, *Sericostoma*, *Lype*, and *Rhyacophila* and showed the same base as nontarget taxa.

3.2 | In situ evaluation

For the 20 selected samples, sequencing was successfully conducted for BF2/BR2, fwhF2/fwhR2n, and the new fwhF2/EPTDr2n primer combinations. Number of reads for primer pair BF2/BR2 ranged from 29,925 to 147,995, for fwhF2/fwhR2n from 14,256 to 59,333 and for fwhF2/EPTDr2n from 85,849 to 156,992 per sample after excluding OTUs with read numbers < 0.01% per sample (see Figure S6 for rarefaction plots). For BF2/BR2, the vast majority of reads was assigned to diatoms (55.01%), followed by arthropods (23.96%) and then bacteria (7.48%), other algae (7.44%) and fungi (5.79%) (see Figure 4). Total OTU diversity was highest for BF2/BR2 (2,170), lower for fwhF2/fwhR2n (1,334), and lowest for the more specific primer combination fwhF2/EPTDr2n (1,163) (see Data S1).

Across all 20 samples from site 54 and the Kinzig River network, the number of eDNA reads targeting benthic invertebrate taxa was highest for the new primer combination fwhF2/EPTDr2n and lowest for BF2/BR2 (Figure 5a, Figure S7). For the highly degenerate universal primers, BF2/BR2 target read proportion was below 10% for half of the samples and had a maximum of 67.7% of the reads (site 5). For primer pair fwhF2/fwhR2n, the number of reads of target taxa varied tremendously from below 5% at several sites to 99.74% at site 5 (Figure 5a). Target read proportions were significantly higher for fwhF2/EPTDr2n compared to fwhF2/fwhR2n and BF2/BR2 (Wilcoxon rank sum tests: $p = 1.3E^{-5}$ and $2.4E^{-7}$, respectively). No significant difference was found between fwhF2/fwhR2n and BF2/BR2 (Wilcoxon rank sum test: $p = .081$). Highest read numbers were typically assigned to dipterans, especially chironomids, independent of the primer set, but also a few oligochaete taxa (Figure S4, Data S1). While primer combinations BF2/BR2 and fwhF2/fwhR2n had the overall highest OTU numbers, the number of target benthic invertebrate OTUs was highest for the new primer combination fwhF2/EPTDr2n and lowest for BF2/BR2 (Figure 5b). The same was true after taxonomic assignment to species, family, and genus levels (Wilcoxon rank sum tests with $p < .001$ for all pairwise comparisons). Pairwise tests also supported a significantly better performance of fwhF2/fwhR2n compared to BF2/BR2 at family and genus ($p < .01$) as well as species level ($p < .05$).

Interestingly, at site 5 the novel primer combination recovered a larger proportion of OTUs compared to fwhF2/fwhR2n (93.33% versus 66.67%) despite both having similar number of reads assigned to benthic invertebrates. Here, for fwhF2/fwhR2n many reads were assigned to one chironomid OTU (genus *Rheotanytarsus*, OTU 7; 26,021 reads), whereas the new primer combination only recovered this taxon (as well as many more) with a moderate number of reads (OTU 143; 101 reads). A clear outlier site in terms of detected OTU number was site 16 from the Kinzig River network. This site was sampled during the flooding season not in the river but the adjacent flooded riparian vegetation and had many reads assigned to non-target taxa but extremely few OTUs detected with all primer pairs. Almost all reads of the new primer combination were assigned to one chironomid OTU (OTU 9) of the genus *Procladius* (BOLD early release data). All other ten OTUs found in this sample were mostly (semi-)terrestrial taxa with very low read abundances (see Data S1).

Taxon overlap among primer combinations for Ephemeroptera, Plecoptera, Trichoptera, and Diptera (EPTD), which are of special interest for regulatory biomonitoring and bioindication, showed that only a few EPTD taxa were exclusively found by primer combination BF2/BR2 (7; Figure 5c). Primer combination fwhF2/fwhR2n detected a moderate number of exclusive EPTD taxa (25) and shared the majority either with both or at least with the fwhF2/EPTDr2n primer combination. The largest proportion of exclusively detected EPTD taxa was found with the new fwhF2/EPTDr2n primer combination (136 exclusive EPTD taxa). When performing the comparison

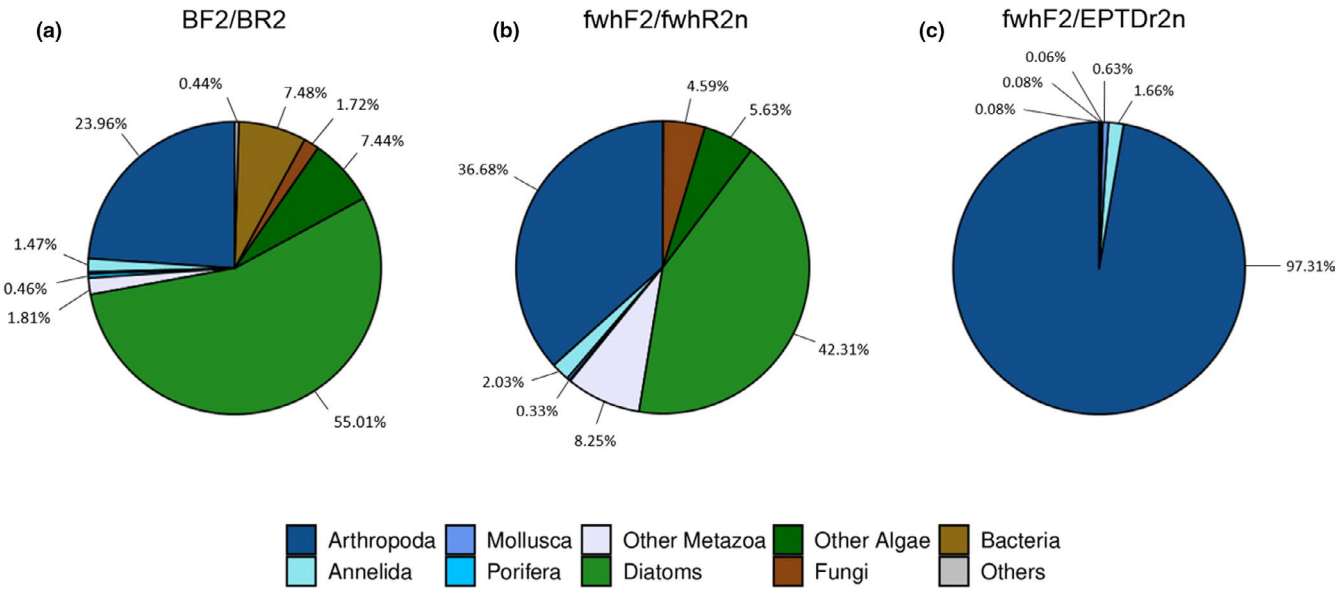

FIGURE 4 In situ primer evaluation using the 20 eDNA samples from the RMO LTER site 54. Pie charts show proportion of reads assigned to phylogenetically distinct groups (see legend) using primer combinations BF2/BR2 (a), fwhF2/FwhR2n (b), and fwhF2/EPTDr2n (c) [Colour figure can be viewed at wileyonlinelibrary.com]

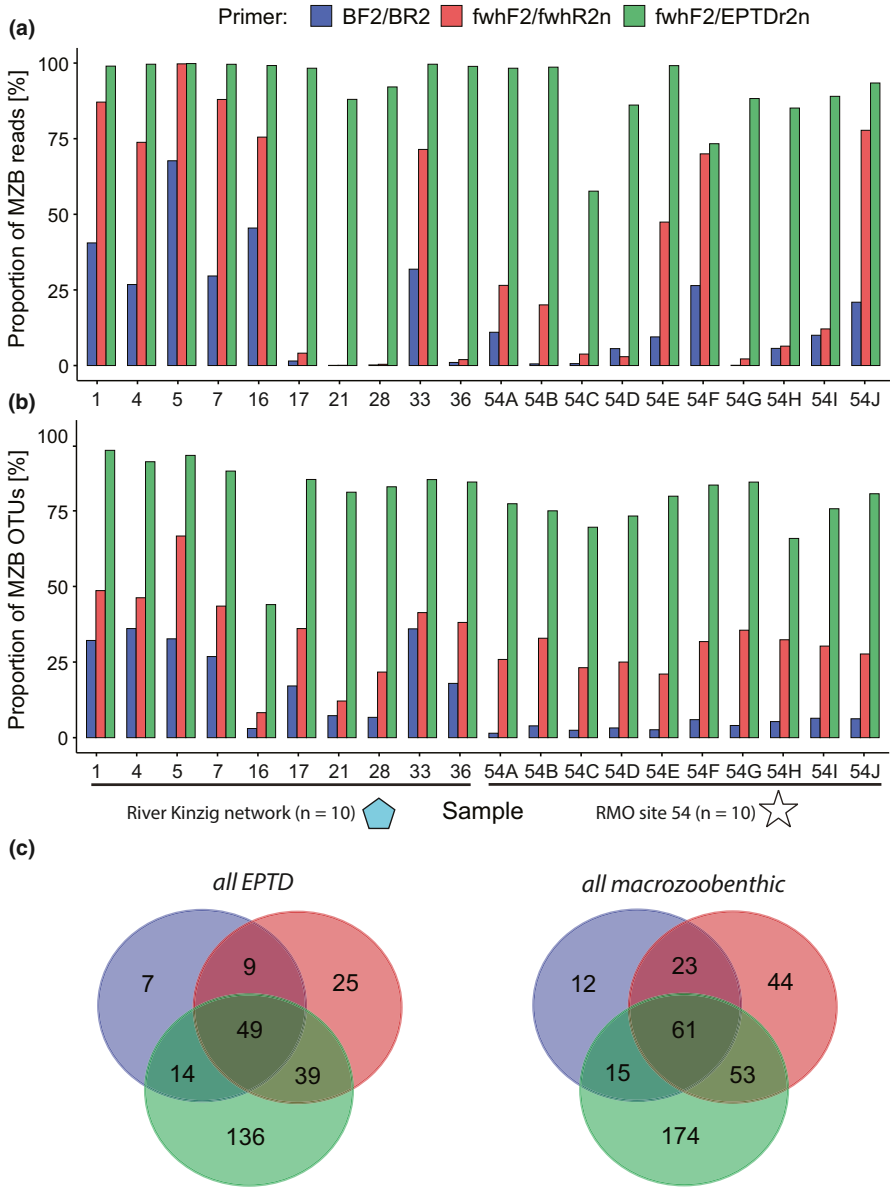

FIGURE 5 Comparison of primer performance for the two in situ analysis test cases. (a), Read proportion per primer pair that target macrozoobenthic taxa (MZB). (b), Proportion of target MZB OTUs per primer pair. Different samples (x-axis) represent different time points (site 54, left) and different sites (Kinzig River network; right). (c), Overlap of identified OTUs for all ephemeropteran, plecopteran, trichopteran, and dipteran (EPTD) taxa as well as all MZB taxa. Note: Site 16 from Kinzig River network was an eDNA sample obtained from a floodplain. See also Figure S7 [Colour figure can be viewed at wileyonlinelibrary.com]

across all benthic invertebrate taxa that are part of the German regulatory operational taxa list, the same trend was found (Figure 5c).

We found no differences in the proportion of correctly assigned taxa between the three different primer pairs of different lengths when using the BOLD database as references (fwhF2/EPTDr2n: 89.7%; fwhF2/fwhR2n: 90.1%; BF2/BR2: 89.5%). Also, when removing ambiguous hits from the returned taxa lists, no difference related to amplicon length was found (see Figure S8 and Data S5).

3.3 | Comparison to morphological data

For the comparison with morphological data, only OTUs with assigned taxonomy (similarity > 90%) were considered. In both in situ comparisons, BF2/BR2 had consistently the poorest performance in terms of number of identified taxa (family, genus, and species levels) as well as in terms of shared taxa with morphotaxonomic lists (Table 2). Primer combination fwhF2/fwhR2n performed better in 20 out of 24 and the new primer combination fwhF2/EPTDr2n best in all cases in terms of total number of taxa, except for the four comparisons on family level for the ten biweekly samples. With 305 species detected with the new primer from only 20 eDNA samples, this number was already higher than the total number of species (153) reported by expert morphological data at all 22 RMO long-term sampling site samples collected for up to two decades (n = 263; Figure 6 top). In four of the 24 comparisons (Table 2), the number of taxa shared between eDNA and morphological datasets was slightly higher for fwhF2/fwhR2n. Here, especially trichopteran species diversity was lower for fwhF2/EPTDr2n.

3.3.1 | Site 54

The ten samples collected at site 54 were compared with the 28 samples from the adjacent RMO long-term monitoring site W1 (taxa

TABLE 2 Comparison of long-term taxa lists generated through morphotaxonomic assessment and ten eDNA samples obtained from site 54 (left) and the ten eDNA samples from Kinzig River network (right)

Morphological data	Site 54 (biweekly), $n = 10$			Kinzig River network, $n = 10$		
Species level	**BF2/BR2**	**FWh2**	**EPTDr2n**	**BF2/BR2**	**FWh2**	**EPTDr2n**
W1	11/23/62	26/99/47	21/218/52	14/87/59	21/105/52	16/204/57
W1, W2, S1, S2, O1	12/22/99	31/94/80	26/213/85	20/81/91	26/100/85	21/199/90
RMO MZB all sites	9/25/144	34/91/119	38/201/115	25/76/128	36/90/117	38/182/115
BioDiv database	11/23/592	52/73/551	70/169/533	32/69/571	46/80/557	63/157/540
Genus level						
W1	10/23/51	29/84/32	28/142/33	18/61/43	27/74/34	26/127/35
W1, W2, S1, S2, O1	11/22/80	36/77/55	36/134/55	24/55/67	33/68/58	32/121/59
RMO MZB all sites	11/22/118	40/73/89	44/126/85	30/49/99	43/58/86	49/104/80
BioDiv database	11/22/287	47/66/251	56/114/242	33/46/265	43/58/255	51/102/247
Family level						
W1	14/7/38	31/25/21	30/24/22	19/9/33	31/14/21	30/25/22
W1, W2, S1, S2, O1	14/7/ 54	35/21/33	35/19/33	20/8/48	34/11/34	34/21/34
RMO MZB all sites	14/7/72	38/18/48	43/11/43	23/5/63	39/6/47	44/11/42
BioDiv database	13/8/94	37/19/70	44/10/63	22/6/85	36/9/71	43/12/64

Note: Rows list different sampling sites/site combinations of morphologically identified specimens. W1 is next to the site analyzed by eDNA (biweekly site 54, Figure 1). Sites W1, W2, S1, S2, and O1 represent all samples ($n = 66$) in a 5 km radius around site 54. "RMO MZB" stands for all macrozoobenthic samples ($n = 263$; from 22 sites) surveyed in the course of LTER monitoring for up to 20 years. The BioDiv database covers all benthic invertebrate records ($n = 926$) within the RMO (including, e.g., data from the Water Framework Directive monitoring of local studies but also terrestrial assessments). The table is split according to three taxonomic assessment levels: species (top), genus (middle), family (bottom). The numbers in the cells, separated by "/" indicate (i) taxa found by both methods/(ii) only DNA metabarcoding/(iii) only morphology for the three primer pairs (columns).

list compiled over two decades of sampling, reported 73 species, 61 genera, and 52 families, Figure 6 bottom). Across all primer pairs, the number of shared species between the two methods was below 40%, and highest for primer pair fwhF2/fwhR2n (26 out of 73 species shared). However, with the exception of BF2/BR2, a much higher number of benthic invertebrate species was reported for primers fwhF2/fwhR2n (125) and fwh2/EPTDr2n (239). This included especially many insect species (Data S1, Data S4). Number of taxa exclusively reported via morphology was larger than the fraction of taxa identified with both approaches. Using the new primer combination, dipterans, but also coleopterans, plecopterans, ephemeropterans, and oligochaetes had greater taxa numbers compared to classical morphotaxonomic long-term data (Data S3, Data S4, Figure S4). The total number of shared taxa between eDNA and morphotaxonomic taxa lists increased when considering taxa lists obtained from sites in a 5 km radius around site 54 ($n = 66$ samplings), all 22 Rhine-Main-Observatory (RMO) long-term monitoring sites ($n = 263$ samplings), or the entire RMO Biodiversity database ($n = 926$ assessments that includes also lentic and few terrestrial taxa; see Table 2).

3.3.2 | Kinzig River network

Patterns of taxon diversity among primers were similar to the patterns reported for the ten "site 54" samples. However, species reported from both eDNA and morphological assessments were mostly lower given that the sites were further upstream (Figure 1). Site 16, which was in the flooding zone, was a clear outlier and had very low benthic invertebrate OTU numbers (Table S1, Data S1).

4 | DISCUSSION

Our study used a global as well as a site-informed in silico approach to design optimized primers for the detection of benthic invertebrates from eDNA samples. The validation of the new primer combination on eDNA samples from a long-term monitoring observatory in Germany convincingly showed that the nontarget amplification bias using eDNA samples can be significantly reduced.

4.1 | In silico analysis

We used the idea of region and ecosystem specific primers to maximize DNA metabarcoding efficiency proposed by Elbrecht and Leese (2017a) to identify and then omit nontarget taxa amplification using eDNA. Environmental DNA metabarcoding data available from the 102 samples obtained at an LTER site (site 54) from Kinzig River allowed us to identify the most abundant nontarget taxa throughout a 15-month period using amplified eDNA with the rather unspecific primers BF2/BR2.

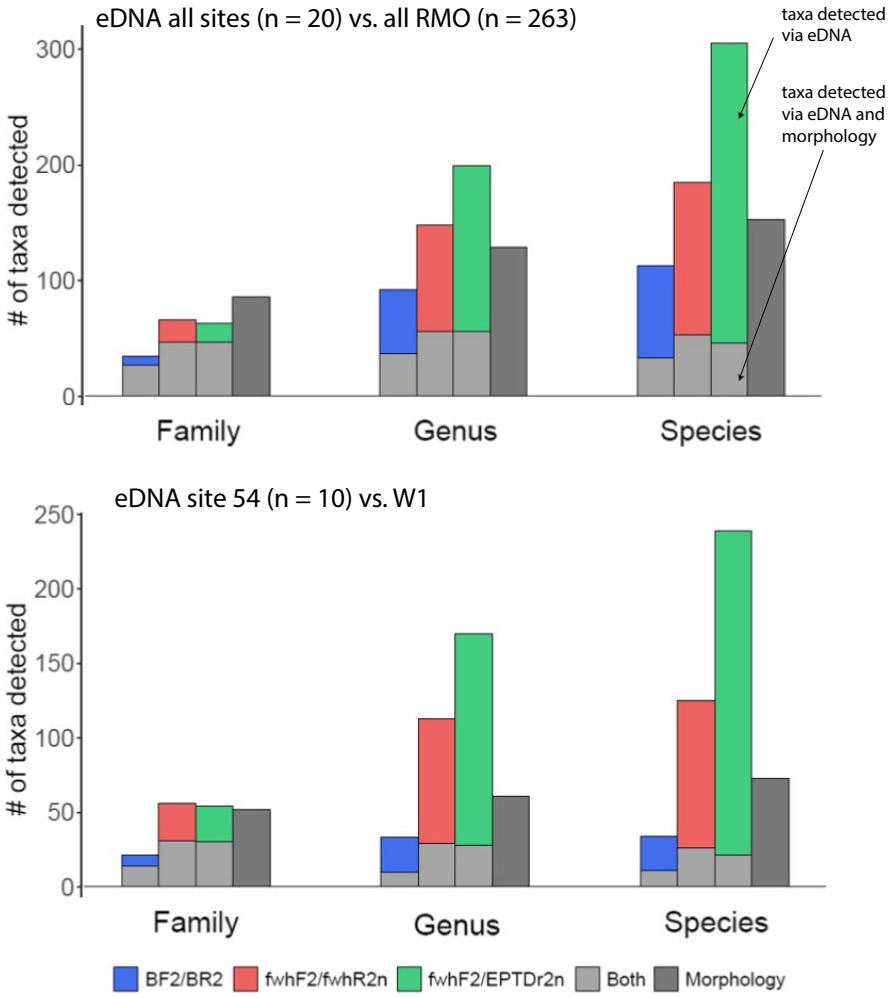

FIGURE 6 Top panel: Comparison of detected taxa between the 20 eDNA and 263 morphological assessments in the RMO. The shaded portions in the columns indicate the number of taxa shared between eDNA and morphology-based assessment. Bottom panel: Comparison of detected taxa between the "site 54" eDNA samples and the long-term morphological assessment data from the adjacent W1 site [Colour figure can be viewed at wileyonlinelibrary.com]

We found that Stephanodiscaceae (diatoms) dominated read abundances. In silico analysis revealed that they differed from most of the target taxa at position 1979 of the alignment by having a "C," whereas target taxa had primarily a "G." Few exceptions were flatworm, crustacean, and bivalve taxa that had an "A" at this position. Flatworms in part showed a "C" like the diatoms. Our decision to include 3' pyrimidine bases (Y, i.e., either C or T) at this position restricted the amplification of the respective flatworm taxa but promised to avoid the abundant diatoms. The second most important position to promote amplification of target taxa was at position 1982. Here, the trade-off was more difficult because an "A" was present in all nontarget taxa but in very few of the target taxa. However, isopods as well as few Trichoptera taxa (*Rhyacophila*, *Hydropsyche*, *Lype*, and *Sericostoma*) were identified to share this "A." As these taxa had a match at the last 3'-base, we accepted this mismatch to avoid higher nontarget taxon binding affinity. Also, some gastropods and hirudinean taxa showed different bases at the second- and third-last position.

The in silico analysis performed without special emphasis on target OTUs detected at site 54 (Figure 3) supported the general specificity of the primer, suggesting that it might not only be applicable to nontarget taxa only occurring in that region. However, the analysis also highlighted the difficulty of finding suitable primer regions that clearly separate distinct target from nontarget groups (Clarke et al., 2014; Sharma & Kobayashi, 2014). The global in silico analysis also confirmed that some taxa of Turbellaria, Mollusca, Trichoptera, and Isopoda had higher penalty scores, suggesting that they might be underrepresented due to primer bias. The newly designed primer EPTDr2n was located more in the center of the "Folmer fragment" compared to fwhR2n (Figure 3). The decision to use it together with fwhF2 and not BF2 was because fwhF2 showed a particularly good match for all target groups and because the amplified region was shorter (142 bp). Environmental DNA shed from target taxa can be degraded quickly (Moushomi et al., 2019; Seymour et al., 2018).

4.2 | In situ analysis

Analysis with universal primer pair BF2/BR2 recovered mostly nontarget taxa, especially diatoms, similar to reported observations from other studies using universal COI primers (Beentjes et al., 2019; Deiner et al., 2016; Gleason et al., 2020; Hajibabaei, Porter, Robinson, et al., 2019; Macher et al., 2018), leaving only a small number of reads and OTUs of target taxa recovered from the extracted eDNA. The amplification of nontarget DNA from extracted eDNA samples was consistently observed across all 20

samples. A reduction of nontarget eDNA was less observed for the shorter universal freshwater invertebrate primer pair fwhF2/fwhR2n, where on average 39% of the reads were assigned to benthic invertebrate target taxa. However, the new primer combination fwhF2/EPTDr2n completely changes this picture by yielding on average >99% of the reads from benthic invertebrate taxa and, importantly, a highly significant higher number of target OTUs. The latter becomes obvious when comparing eDNA results from site 54 to long-term data generated for site W1, for which 117 taxa were identified by classical morphotaxonomy, whereas 329 were detected with eDNA metabarcoding. When restricting the analysis to the years 2017/2018, the difference became even larger (Figure S5, Data S3, Data S4). Especially for trichopterans, plecopterans, and dipterans, the number of taxa identified morphologically decreased considerably and is even lower than the number of taxa identified with the universal fwhF2/fwhR2n primer pair. Furthermore, the majority of taxa detected with eDNA metabarcoding could be assigned to species level in comparison with taxa reported from morphology-based bioassessments at site W1. This is not unexpected given that a higher taxonomic rank for benthic invertebrates is often used to avoid species misidentifications, which are especially known from small specimens using morphology (Haase et al., 2006, 2010). Even when considering the taxon number for all 263 samples obtained from 22 long-term monitoring sites in the past 5–20 years, the number of benthic invertebrate species and genera detected is greater for the eDNA samples of the two case studies compared to the number of morphologically retrieved taxa. It must be noted, however, that family diversity was higher using the long-term morphological data. This is owed to the fact that (a) primer bias omits taxa such as some flatworms and molluscs, and (b) a much smaller temporal sampling is captured with the few eDNA samples. Overall, the dominating taxa in eDNA metabarcoding were insects, especially dipterans (chironomids, simuliids), ephemeropterans, coleopterans, and plecopterans but also oligochaetes (see Figure S4). The high proportion of reads and OTUs assigned to chironomids (Data S1) are expected as nonbiting midges are known to dominate freshwater ecosystems in terms of abundance and diversity (Armitage et al. 1995; Pinder 1986). Applying bulk metabarcoding Beermann et al. (2018) found 183 chironomid OTUs in a small German low-mountain stream using the same 3% distance threshold as applied here, which is similar to the high OTU diversity found here. While the new primer combination comes with a substantial gain in arthropod, in particular insect DNA amplification, the increased specificity also has a down-side because using the new EPTDr2n also excluded some derived arthropod target taxa that have the same nucleotide as the nontarget taxa, for example, Trichoptera genera *Rhyacophila*, *Hydropsyche*, and the isopod *Asellus aquaticus*. These were captured with primer pair fwhF2/fwhR2n (and in part with BF2/BR2). Likewise, flatworms and molluscs were also not reliably detected with the new primer combination. For molluscs and crustaceans, specific primers targeting the 16S gene have been proposed (Klymus et al., 2017; Komai et al., 2019) and should be considered as a complement depending on the goal of a study.

4.3 | Amplicon length and taxonomic assignment

While the power to discriminate species can become lower with short sequences, our COI data here, as well as studies performed earlier (Meusnier et al., 2008; Yeo et al., 2020), showed that the discrimination power with short markers ("mini-barcodes") can still be high. Our analysis found almost identical taxonomic assignment reports for the 142-bp fragment as compared to the 205- and 421-bp fragment. Best hits as well as the fraction of unambiguous hits was similar and not dependent on the amplicon length for the herein tested communities.

4.4 | Considerations for benthic invertebrate bioassessment

The choice of primers is always a difficult quest and depends on the aim. Clearly, no "one-fits-all" solution exists (Clarke et al., 2017; Elbrecht et al., 2019; Elbrecht & Leese, 2017a; Grey et al., 2018; Hajibabaei et al., 2019; Tab erlet et al., 2018). For terrestrial insect bulk samples, good primers exist according to a mock community study using 374 individual and phylogenetically different insect species (Elbrecht et al., 2019). If the aim of a study is to capture the greatest number of benthic invertebrate taxa, in particular insects from eDNA for bioassessment with one primer pair, the newly designed fwhF2/EPTDr2n combination fits that purpose. It clearly outperforms even catchment-wide benthic invertebrate species numbers (Figure 6). However, the overlap between eDNA and morphological assessments is moderate. This may also be due to the very limited number of samples and time points collected via eDNA. If the aim is to maximize the phylogenetic diversity captured in a sample, either a more conservative marker such as 18S can be appropriate (Bagley et al., 2019; Deagle et al., 2014; Li et al., 2018), or—for metazoan taxa—it might be important to consider the use of multiple COI primers (Corse et al., 2019; Hajibabaei, Porter, Wright, et al., 2019) as with the more specific primer combination clearly a few macrozoobenthic taxa within Trichoptera, Mollusca, and Isopoda will be missed due to primer bias. It should be noted that also for broad eukaryotic markers the diversity of benthic invertebrate species can still be underrepresented and read numbers dominated by meiofaunal groups that live in the habitat (Rotifera, Copepoda; see Li et al., 2018) but which are usually not part of the regulatory biomonitoring.

The new primer combination developed here has successfully been tested in silico and used in situ for samples from one German LTER site. Nontarget taxa show much lower binding affinities at the 3'-end compared to most arthropod taxa in the analysis (Figure 3b) and thus we are confident that the same primer will work also in other regions / aquatic habitats and also on sample preservative liquid and completely homogenized environmental samples (Zizka et al., 2018, Beerman et al. unpublished data; Pereira-da-Conceicoa et al., 2019; Blackman et al., 2019). Yet, this remains to be tested. It can possibly be further improved by adding further variability, yet too many degenerate bases also limit amplification success (see

Table S2 for primers not working). The increased resolution for ecologically important taxa such as chironomids and oligochaetes can be of immense relevance for biomonitoring (Beermann et al., 2018; Macher et al., 2016; Milošević et al., 2013; Vivien et al., 2020; Vivien et al., 2015, 2016). They dominated eDNA signals here and were often site-specific. The same has been shown for meiofaunal stream biota recovered from the large Yangtze River in China (Li et al., 2018). Therefore, the increased resolution of such ecologically important indicator taxa is a clear benefit of using our primer combination when it comes to biomonitoring (Pawlowski et al., 2018). Of course, signals derived from eDNA collected from water in rivers will often integrate across a greater section of the river (Deiner et al., 2016; Pont et al., 2018) and thus intercalibration with traditional site-based methods is difficult (Gleason et al., 2020). However, recent studies showed that eDNA signals can pick up local signals despite the flow current regime (Jeunen et al., 2019; Li et al., 2018; Macher et al., 2018). Thus, it is worth considering eDNA metabarcoding as a complementary tool for bioassessment and monitoring of stream invertebrates, including often neglected taxa like chironomids.

In conclusion, our study shows that the detection of benthic invertebrate taxa from eDNA isolated from stream water is greatly increased with a new specific primer combination that avoids non-target taxa amplification. We therefore caution the eDNA field to consider that a nondetection of eDNA from a sample does not mean that a taxon is not there but rather that we are still in the optimization phase of method development. In this respect, the primer and approach proposed here offer a solution to the common problem of "watered-down" benthic invertebrate biodiversity in COI eDNA metabarcoding and may thus improve future biodiversity assessment and monitoring of freshwaters.

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AUTHOR CONTRIBUTION

FL and PH: performed the conception and design of study. MS, DB, and PH: involved in the acquisition of field data. MS, DB, and VMAZ: performed the laboratory analysis. FL, MS, VMAZ, VE, and DB: involved in the primer design and evaluation. VMAZ, MS, DB, and VE: performed the bioinformatic analysis. All authors analyzed and interpreted the data. FL wrote the manuscript with help of all authors.

DATA AVAILABILITY STATEMENT

All read data can be found in the Short-Read Archive, Project number: PRJNA664693.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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