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RESOURCE ARTICLE

Molecular diet analysis in mussels and other metazoan filter feeders and an assessment of their utility as natural eDNA samplers

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Abstract

Molecular gut content analysis is a popular tool to study food web interactions and has recently been suggested as an alternative source for DNA-based biomonitoring. However, the overabundant consumer's DNA often outcompetes that of its diet during PCR. Lineage-specific primers are an efficient means to reduce consumer amplification while retaining broad specificity for dietary taxa. Here, we designed an amplicon sequencing assay to monitor the eukaryotic diet of mussels and other metazoan filter feeders and explore the utility of mussels as natural eDNA samplers to monitor planktonic communities. We designed several lineage-specific rDNA primers with broad taxonomic suitability for eukaryotes. The primers were tested using DNA extracts of different limnic and marine mussel species and the results compared to eDNA water samples collected next to the mussel colonies. In addition, we analysed several 25-year time series samples of mussels from German rivers. Our primer sets efficiently prevent the amplification of mussels and other metazoans. The recovered DNA reflects a broad dietary preference across the eukaryotic tree of life and considerable taxonomic overlap with filtered water samples. We also show the utility of a reversed version of our primers, which prevents amplification of nonmetazoan taxa from complex eukaryote community samples, by enriching fauna associated with the marine brown algae *Fucus vesiculosus*. Our protocol will enable large-scale dietary analysis in metazoan filter feeders, facilitate aquatic food web analysis and allow surveying of aquacultures for pathogens. Moreover, we show that mussels and other aquatic filter feeders can serve as complementary DNA source for biomonitoring.

KEYWORDS
environmental DNA, food web, gut content analysis, invasive species, lineage-specific primer, natural eDNA sampler

1 | INTRODUCTION

The immense task of documenting human imprints on ecosystems has been greatly simplified by eDNA metabarcoding (Taberlet et al., 2012). The filtering of water and sequencing of the filtrate is a commonly used approach in aquatic eDNA analysis (Barnes et al., 2014) but various other substrates can serve as eDNA sources. Examples include sediments, marine cobbles or the gut content of detritus feeding animals (Koziol et al., 2019; Shum et al., 2019; Siegenthaler et al., 2019). Another promising source of aquatic eDNA are sponges (Mariani et al., 2019), which behave like a biological eDNA filter, filtering and retaining eDNA particles from their surrounding community.

Besides sponges, a particularly well-suited organism to target as a natural eDNA sampler are mussels. Bivalves are ubiquitous inhabitants of aquatic environments, important components of most limnic and marine food webs (Newell, 2004; Vaughn et al., 2008) and of great economic importance in aquaculture (Shumway et al., 2003). Considering their ecological and economic importance, the efficient characterization of the natural diet of mussels is also of critical importance for aquatic food web analysis, to understand their impact on ecosystems and to optimize and survey aquaculture.

Mussels filter the water column for plankton and detritus (Lavrentyev et al., 1995; MacIsaac et al., 1995; Wai & Levinton, 2004). The filtering mechanism is highly efficient: invasive mussels, which are known to build up high densities within a short time, rapidly alter plankton composition, leading to state shifts in entire ecosystems (Maguire & Grey, 2006; Miller & Watzin, 2007). While mussels can show certain diet selectivity (Baker & Levinton, 2003), their filtering mechanism retains most particles of a certain size range (Sprung & Rose, 1988). The size of particles retained by mussels is well within that of common eDNA water filters (0.2–10 μm; Barnes et al., 2014; Wilcox et al., 2015).

So far, diet analysis in mussels is mostly based on laboratory feeding assays or chemical screens (Fernández et al., 2015; Kreeger, 1993; Pettersen et al., 2010). Metabarcoding approaches now offer a powerful alternative. To characterize a mussel's diet, consumed DNA could simply be amplified from DNA extracts of the mussel's gill and intestinal tissue. Universal PCR primers could be used to enable the recovery of a broad range of dietary taxa. However, with such universal primers, the highly overabundant consumer DNA may outcompete dietary taxa during PCR (Krehenwinkel et al., 2017). A pragmatic solution to this problem is the use of very high sequencing coverage. Consumer sequences are removed from the data before analysis (Piñol et al., 2015). However, the DNA of the dominant taxa is often so overabundant that nearly no desired sequences remain (Krehenwinkel et al., 2017). An alternative solution is the use of blocking primers, which prevent the consumer's DNA from being amplified (Vestheim & Jarman, 2008). Yet another option is the use of diagnostic SNPs at the PCR primer's 3′-end, which are highly efficient at preventing amplification of target lineages (Krehenwinkel et al., 2019; Stadhouders et al., 2010). This allows the recovery of even minute amounts of dietary DNA. Suitable PCR primers can be designed in conserved sequences, assuring a broad taxonomic specificity.

Here, we aimed to: (1) develop a metabarcoding assay that allows the reconstruction of dietary composition of mussels and other metazoan filter feeders, while at the same time excluding the metazoan consumer from amplification by 3′-prime mismatches. Furthermore, we aimed to (2) determine whether this assay provides a suitable alternative or complement to aquatic eDNA analysis of eukaryotic communities from filtered water samples.

For this purpose, we designed nine primers targeting the nuclear 18S ribosomal DNA. These primers were located in highly conserved sequences across the eukaryote tree of life to guarantee a broad taxonomic coverage. At the same time, they encompassed variable loop sequences, which allowed to discriminate closely related taxa. One set of primers efficiently suppressed amplification of *Dreissena* and mussels of 34 other genera in 16 families, due to two lineage diagnostic SNPs. Another set of primers was designed to reduce amplification of all metazoans and recover nonmetazoan dietary taxa from any animal. Finally, we designed a primer set, which particularly targets metazoa and suppresses nonmetazoan taxa.

We tested our primers in various samples. We particularly focused on two species of the genus *Dreissena*; the zebra mussel *D. polymorpha* and the quagga mussel *D. rostriformis*. Both species' native range is in far Eastern Europe, but they are now widespread invasives in Western Europe and America (Garton et al., 2013; Kinzelbach, 1992; Paulus et al., 2014; Son, 2007) where they cause great ecological and economic damage (Maguire & Grey, 2006; Miller & Watzin, 2007). Considering their great impact, a protocol for the detailed assessment of dietary preferences of *Dreissena* spp. is particularly relevant. In parallel, we collected eDNA water samples in close proximity to the sampled *Dreissena* mussel colonies, which were directly compared to the mussel samples. To show a direct application of our protocol, we analysed several standardized time series samples of *D. polymorpha*. The samples were collected in three German rivers over the past 25 years and allow us to explore temporal biodiversity changes in the past decades. To test the broad utility of our assay, we also included samples of the marine blue mussel *Mytilus edulis*. Finally, we tested the option to enrich metazoa from complex eukaryote community sample using samples of the bladder wrack *Fucus vesiculosus*, a marine brown alga.

2 | MATERIALS AND METHODS

2.1 | Design of lineage-specific primers to recover the diet of mussels and other metazoan filter feeders

Mitochondrial COI is a widely used metabarcoding marker in metazoans (Elbrecht & Leese, 2017). Mussels' diet, however, consists of taxonomically diverse eukaryotic plankton, including different plant and algal groups, various protozoans and metazoans. COI is not a well-established barcode marker for many of these groups. A more suitable universal target locus for these taxa is found in the nuclear

ribosomal RNA genes (Capra et al., 2016; Giebner et al., 2020; Seymour et al., 2020). Here, we focused on the 18S rDNA as a potential marker to exclude *Dreissena* and other metazoan filter feeders from amplification, while retaining a broad specificity for various planktonic taxa. We generated an alignment of 158 near-complete sequences of the 18S gene for different eukaryotic taxa (Genbank, assessed September 2020, see Appendix S1) and screened this region for possible priming sites that would suppress the amplification of *Dreissena* mussels, while at the same time retaining a broad specificity for the remaining eukaryotes. A particularly suitable region for our purpose was found in the variable V8 and V9 regions of the gene, which are already widely used to generate barcoding markers for eukaryotes (Choi & Park, 2020; Machida & Knowlton, 2012). A highly conserved fragment at the 5′-end of the gene was used to design reverse primers (See Figure 1 for an overview of primer design). In comparison to other eukaryotes, *Dreissena* spp. show two diagnostic substitutions from AA to TC at this position. This results in an A-A and an A-G mismatch at the last two positions of the primer, leading to a pronounced drop in amplification efficiency of *Dreissena* (Stadhouders et al., 2010). To explore the generality of the observed patterns of mismatches, we downloaded the exact mismatch sequence of more than 10,000 genera across the eukaryotic tree of life (see Appendix S1). Only one random member per genus was included in the analysis, to avoid biases by species-rich genera, or groups where many sequences of single species were deposited. Out of all analysed eukaryotic genera, only 47 (0.42%) showed the *Dreissena* specific sequence (3'-TC; Figure 1b). A total of 34 of these genera were mussels (in 16 families), eight belonged to other metazoans (5 annelids, 1 arthropod and one gastrotrich), and the remaining were 4 fungi and one oomycete.

In addition to reducing *Dreissena* amplification, the fourth, fifth and sixth positions of the priming site contained substitutions discriminating different eukaryotic groups (Figure 1c). Nearly all metazoa (93%, including 98% of mussels), fungi (95%) and cercozoans (80%) showed a TAA, while nearly all remaining eukaryotic groups were distinguished by GAG or GTG at the according position. We thus designed three lineage-specific primers (DreissDiet_TAA_R, DreissDiet_GAG_R, DreissDiet_GTG_R), which were distinguished at the fourth to sixth nucleotide position and should discriminate between these different eukaryotic groups.

However, these primers will not generally suppress amplification of metazoans. To assess the diet of a broader spectrum of filter-feeding animals, we used the three discriminating nucleotide positions at the fourth to sixth position of the *Dreissena* primer. We

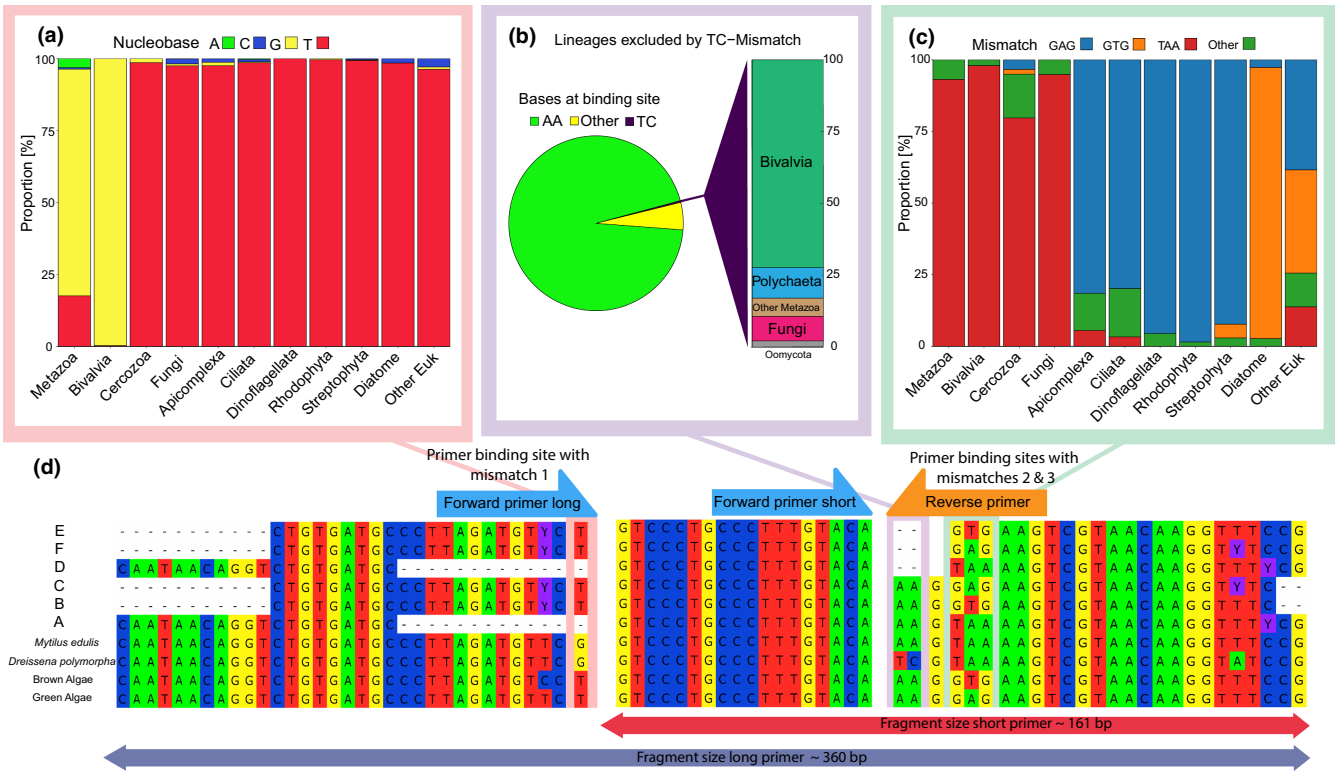

FIGURE 1 Overview of the designed primer combinations and their mismatches with different eukaryotic groups. The lower alignment (d) shows subsections of the amplified fragment, highlighting the amplified fragment lengths and primer binding sites for a *Mytilus* (1) and a *Dreissena* (2), followed by (3) a brown alga and (4) a green alga. The upper six sequences show the different primer combinations we designed (E,F,D,C,B,A). The taxon-specific mismatches are highlighted by coloured bars surrounding the nucleobases. The insets show the specificity of each mismatch across a total of ~10,000 different genera from various eukaryotic lineages. (a) Shows the distribution of a diagnostic 3′-T or G across different eukaryotes. (b) Shows the distribution of 3′-AA versus TC across eukaryotes. Only 0.42% of all tested eukaryote genera show the *Dreissena* sequence TC here as seen in the barplot magnified from the pie chart; and the majority of those genera are bivalves. (c) Shows the distribution of a diagnostic 3′-TAA, GAG or GTG across different eukaryotes.

designed three additional primers, which started at these positions. One of these primers (Metazoa_TAA_R: 3′-TAA) should efficiently amplify metazoans, fungi and cercozoans, while suppressing all other taxa due to up to three 3′-mismatches with other eukaryotes (A-G, T-A and T-G). This primer could be used to specifically enrich metazoan DNA from complex mixture of eukaryotes, for example, an eDNA sample rich in phytoplankton. The other two primers (NonMetazoaDiet_GAG_R: 3′-GAG or NonMetazoaDiet_GTG_R: 3′-GTG) will amplify the majority of other eukaryotes, while reducing the amplification of metazoans, fungi and cercozoans due to up to three 3′-mismatches (T-C, A-A and A-C). These latter two primers should be well suited to enrich nonmetazoan eukaryotic taxa from extracts of filter feeding metazoans. In comparison to the *Dreissena* primers, however, the reduction of consumer amplification should be less efficient, as they only show 2–3 3′-mismatches.

Corresponding forward primers were designed at the 3′-end of the V8 region (Figure 1a). The first forward primer (Euk_F1) was universal for eukaryotes and was designed as a complementary primer to the metazoan primers (DreissDiet_TAA_R, Metazoa_TAA_R). The second primer (NonMetazoa_F1) discriminated bivalves and other metazoans from other eukaryotes by its first base (Figure 1a). While all bivalves (100%) and the majority of metazoans (78.9%) showed a G at that position, nearly all other eukaryotes showed a T (99%–100% depending on the group), resulting in a T-C mismatch with bivalves. This primer was used as complement to the nonmetazoan reverse primers (DreissDiet_GAG_R, DreissDiet_GTG_R, NonMetazoaDiet_GAG_R and NonMetazoaDiet_GTG_R). The resulting PCR fragments of these primer combinations covered the complete V8 and V9 region and reached a length of 360 bp on average. DNA in the guts of predators and eDNA is often degraded and present in small fragment sizes (Krehenwinkel et al., 2017). The relatively long fragment length amplified by our primer combinations may therefore make them unsuitable for some of this highly degraded DNA. To enable the recovery of degraded DNA fractions, we designed an additional eukaryote-specific forward primer (Euk_F2) in the conserved DNA stretch between V8 and V9. This primer can be combined with all other reverse primers and amplifies a fragment length of 161 bp on average. In total, we designed 12 possible primer combinations. Throughout the following text, we use letters to indicate the different primer combinations, according to the annotation in Table 1 and Figure 1. For an overview of all designed primer combinations and their efficiency in reducing consumer amplification see Table 1.

2.2 | Samples and sampling sites

To test our protocol, we collected specimens of the limnic zebra mussel *Dreissena polymorpha* and quagga mussel *D. rostriformis*, widespread invasive species in German lakes and rivers. Zebra and quagga mussels were collected from two sites at the Danube River, Kelheim and Jochenstein. Additional zebra mussels were collected in the Northern German Lake Stechlin (Figure S1). The mussels were collected by hand from hard substrate close to the shore and subsequently stored in the gas phase over liquid nitrogen and transported to Trier University. Here, they were briefly thawed, opened with sterile tweezers and the entire soft tissue carefully removed. Tweezers were flame sterilized between separate samples. For an initial test of our primer sets, DNA was extracted from seven separate zebra mussels from Jochenstein in the Danube using the Qiagen Puregene DNA extraction kit according to the manufacturer's protocols (Qiagen). An additional purification step was added to the DNA extraction to remove possible PCR inhibitors, according to the manufacturer's protocol. To explore the recovery of eDNA and dietary taxa between the two *Dreissena* species and across a broader geographic context, we included samples from the additional sampling sites at Lake Stechlin and Kelheim. To maximize taxon recovery, we combined the tissue of 32 mussels of each species for the two collection sites at the Danube. Due to limited available mussels, we included an extraction of only four pooled mussels from the lake Stechlin. The mussels were dissected as described above, then finely ground in a mortar while adding liquid nitrogen. To avoid cross contamination, the mortar was thoroughly cleaned and treated with bleach after each processed sample. DNA was then extracted from 200 mg of the total mussel homogenate as described above.

Before the collection of the mussels, two water samples of 1 L each were collected at the same locations. These samples served as a direct comparison of the eDNA present in the water column and the recovered eDNA from mussels. The water was taken right next to the mussel colonies, by dipping the closed collection bottle down to the colony and then opening it. The water sample was stored at 4°C during transport back to the laboratory and then immediately filtered using a nitrocellulose filter of 0.45 µm pore size (Thermo Fisher Scientific Inc.). DNA was then extracted from the filter using the Qiagen DNeasy PowerWater kit according to the manufacturer's protocol.

In addition to the hand-collected mussel samples, we included several mussel homogenates of the German Environmental Specimen Bank (hereafter ESB), a Germany-wide long-term biomonitoring programme (Figure S1). These samples served to show a direct application of our protocol to the analysis of time series and to document contemporary biodiversity change. We used homogenates of the zebra mussel from three German rivers, the Saar, the Rhine and the Elbe, which had been collected between 1994 and 2016. DNA extracts from 50 mg of tissue homogenate from a thesis at Trier University (Richter, 2020) were used. ESB samples are collected and processed according to highly standardized protocols (Teubner et al., 2018; Paulus et al., 2014). The mussels are dissected as described above and homogenate samples prepared from pools of several hundred individuals, by grinding them to a powder of 200 µm diameter in a cryomill. The resulting homogenate samples are then permanently stored at ultra-deep temperature below −150°C.

We further included each three marine ESB samples of the blue mussel *Mytilus edulis* and the brown algae *Fucus vesiculosus*, the bladder wrack, which were collected at the same site on the Island Sylt (Figure S1). Both species are widespread along the German coast and collected by the ESB as indicators for marine pollution. While zebra mussel samples are collected only once annually, blue mussel and bladder wrack homogenates comprise pools of six subsamples collected every second month throughout

TABLE 1 18S primer combinations used in this study (see Figure 1 for details on amplification suppression and enrichment of the different primer combinations)

Name	Blocks	Possible targets	Length of amplicon in *D. polymorpha* (bp)	Name (Forward)	Forward (5′- 3′)	Fw primer length (bp)	Name (reverse)	Reverse (5′-3′)	Rv primer length (bp)	*Dreissena* sequence	Recovery of target reads (%)
AL	Dreissena and various other bivalves	Metazoa	363	EukF1	CAATAACAGGTCTGTGATGC	20	DreissDiet_TAA_R	**AAGTAA**AAGTCGTAACAAGGTTTYCG	26	TC/TAA	70.51 ± 5.3
BL	Dreissena and various other bivalves	Brown Algae	352	NonMetazoaF1	CTGTGATGCCCTTAGATGTYC**T**	22	DreissDiet_GTG_R	**AAGGTG**AAGTCGTAACAAGGTTTC	24	G/TC/TAA	99.9 ± 0.009
CL	Dreissena and various other bivalves	Green Algae	352	NonMetazoaF1	CTGTGATGCCCTTAGATGTYC**T**	22	DreissDiet_GAG_R	**AAGGAG**AAGTCGTAACAAGGTYTC	24	G/TC/TAA	99.64 ± 0.50
DL	Phytoplankton	Metazoa	363	EukF1	CAATAACAGGTCTGTGATGC	20	Metazoa_TAA_R	**TAA**AAGTCGTAACAAGGTTTYCG	23	–	99.89 ± 0.23
EL	All metazoa	Brown Algae	352	NonMetazoaF1	CTGTGATGCCCTTAGATGTYC**T**	22	NonMetazoaDiet_GTG_R1	**GTG**AAGTCGTAACAAGGTTTCCG	23	G/TAA	*
FL	All metazoa	Green Algae	363	NonMetazoaF1	CTGTGATGCCCTTAGATGTYC**T**	22	NonMetazoaDiet_GAG_R1	**GAG**AAGTCGTAACAAGGTYTCCG	23	G/TAA	48.11 ± 13.63
AS	Dreissena and various other bivalves	Metazoa	166	EukF2	GTCCCTGCCCTTTGTACA	18	DreissDiet_TAA_R	**AAGTAA**AAGTCGTAACAAGGTTTYCG	26	TC/TAA	63.17 ± 5.7
BS	Dreissena and various other bivalves	Brown Algae	164	EukF2	GTCCCTGCCCTTTGTACA	18	DreissDiet_GTG_R	**AAGGTG**AAGTCGTAACAAGGTTTC	24	TC/TAA	99.93 ± 0.10
CS	Dreissena and various other bivalves	Green Algae	164	EukF2	GTCCCTGCCCTTTGTACA	18	DreissDiet_GAG_R	**AAGGAG**AAGTCGTAACAAGGTYTC	24	TC/TAA	99.46 ± 0.18
DS	Phytoplankton	Metazoa	164	EukF2	GTCCCTGCCCTTTGTACA	18	Metazoa_TAA_R	**TAA**AAGTCGTAACAAGGTTTYCG	23	–	*
ES	All metazoa	Brown Algae	164	EukF2	GTCCCTGCCCTTTGTACA	18	NonMetazoaDiet_GTG_R1	**GTG**AAGTCGTAACAAGGTTTCCG	23	TAA	*
FS	All metazoa	Green Algae	166	EukF2	GTCCCTGCCCTTTGTACA	18	NonMetazoaDiet_GAG_R1	**GAG**AAGTCGTAACAAGGTYTCCG	23	TAA	*

The column "Name" shows the abbreviation for each primer combination used in the main text. The following column shows which taxonomic group is excluded from amplification. The taxon specific sequences, used to suppress mussel amplification are highlighted in bold within the primer sequence. The corresponding sequences to the lineage specific nucleotides in *Dreissena* are shown in the "*Dreissena* sequence" column. The "Recovery of target reads" column represents the proportion of enriched target reads (*only tested in silico)

the whole year. They will thus cover the entire annual phenology of the marine habitat. The blue mussel and bladder wrack samples were extracted from 200 mg of homogenate using a CTAB extraction buffer (OPS Diagnostics) according to the manufacturer's protocols. The blue mussels served as additional species to test the broad applicability of our newly designed metazoan suppressing primer pairs (E and F), while the bladder wrack served to show the utility of the metazoan specific pair (D) in enriching metazoans from samples dominated by algae. This primer pair should enrich the macroalgae-associated fauna.

2.3 | Test of the designed PCR primers

All primer pairs were initially tested with different eukaryotic taxa (several arthropods, one annelid, two mussels, four plants, and a brown alga), in gradient PCRs at annealing temperatures from 50–60°C, to estimate the optimal annealing temperature. PCRs were run in 10 µl volumes with 1 µl of 10 µM primer and 35 cycles using the Qiagen multiplex kit according to the manufacturer's protocols (Qiagen). PCR success was checked on a 1.5% agarose gel.

We then tested the efficiency of eight primer combinations (AL, AS, BL, BS, CL, CS, EL, FL) using the seven separate specimens of the zebra mussel and a water eDNA sample from Jochenstein at the Danube. As a control for the efficient reduction of mussel amplification of the above primers, we also amplified four mussels for the metazoan-specific primer combination DL. The samples were each amplified using the Qiagen Multiplex PCR kit as described above at an optimal annealing temperature of 55°C. To test for the effect of stringent PCR conditions on consumer amplification, we also included samples for which the annealing temperature was increased to 60°C. Negative control PCRs were run alongside both, the water and mussel samples. The negative controls were generally clean and showed only minor carryover of sequences of very abundant taxa, possibly as a result of carryover during indexing.

For a more detailed analysis of spatial diversity patterns recovered by mussel derived eDNA and dietary differences between mussel species and water eDNA samples, we used the extracted mussel pools and water samples from all three sampling sites, including the two species *D. polymorpha* and *D. rostriformis*. PCR amplification was performed as described above. A total of five mussel samples and six water samples were amplified for the three long amplicons (AL, BL, CL). Water samples and mussel samples were amplified separately, to avoid cross-contamination.

To show the broad applicability of our assay, we also included the marine blue mussel and the bladder wrack in our analysis. The three marine samples of the blue mussel were amplified with primer pair EL and FL in a multiplex reaction with equal amounts of the two primers, while the three bladder wrack samples were amplified with the primer combination DL.

To show a direct application of our protocol, we also analysed the 22 *Dreissena* time series samples of the ESB. All *Dreissena* samples were amplified for two *Dreissena* primer pairs (AL and BL).

Our first round PCR primers contained a 5′-tail, on which a second indexing PCR of five cycles was added, as described in Krehenwinkel et al. (2018) and using the same reaction conditions as in the first round PCR. Briefly, 8 bp dual indexes and Illumina Truseq adapters were introduced to each sample, based on tailed primers. The second round PCR products were visualized on a 1.5% agarose gel, pooled in approximately equal concentrations based on gel band intensity and then cleaned of leftover primer with 1x AMPure Beads XP (Beckman & Coulter).

2.4 | Sequencing and sequence analysis

We generated a total of 128 libraries for the different marker combinations and experiments. All libraries were quantified using a Qubit Fluorometer (Thermo Fisher Scientific Inc.), pooled in equimolar proportions and then sequenced on an Illumina MiSeq using V3 chemistry and 600 cycles (Illumina Inc.). The samples were demultiplexed using CASAVA version 1.8.2 (Illumina Inc.) with no mismatches allowed. Demultiplexed fastq files were merged using PEAR (Zhang et al., 2014) with a minimum overlap of 50 and a minimum quality of 20. The merged reads were additionally filtered for a minimum quality of Q30 over >90% of the sequence and then transformed to fasta files using the FASTX-Toolkit (Gordon & Hannon, 2010). PCR primer sequences were then trimmed off from the merged reads using grep and sed in UNIX scripts allowing degenerate bases to vary in the search patterns. The processed reads were dereplicated and clustered into zero radius OTUs (hereafter OTUs) using USEARCH (Edgar, 2010). A minimum cluster size of 8 was used. A de novo chimera removal is included in the clustering pipeline. As the 18S gene is rather conserved, we refrained from clustering into 3% radius OTUs, as this would have probably deflated true taxonomic diversity. Zero radius OTUs essentially correspond to unique sequence variants, with down to a single nucleotide difference distinguishing different zOTUs. The recovered OTU richness for 18S rDNA zero radius OTUs corresponds relatively well with that found with 3% radius OTUs used in mitochondrial COI for metazoans (de Kerdrel et al., 2020). All resulting OTUs were searched against the complete NCBI nucleotide database (downloaded 02/2020) using BLASTn with a maximum of 10 target sequences (Altschul et al., 1990). Taxonomy was then assigned to the resulting BLAST output using a custom python script (de Kerdrel et al., 2020), with a minimum of 90% similarity to a reference being used to classify a sequence. An OTU table was then constructed for all samples using USEARCH.

We first tested the efficiency of our primers for suppressing mussel amplification. For this purpose, we calculated the proportion of recovered non-mussel sequences for the seven separate mussel samples based on the previous taxonomic assignments. The OTU tables were then rarefied to an equal coverage and used to calculate alpha and beta diversity within and between samples using VEGAN (Oksanen et al., 2020) in R version 4.1.1 (R Core Team, 2021) and Rstudio version 1.4.1717 (RStudio Team, 2021; Figure S2). Factors influencing beta diversity were estimated using a PERMANOVA

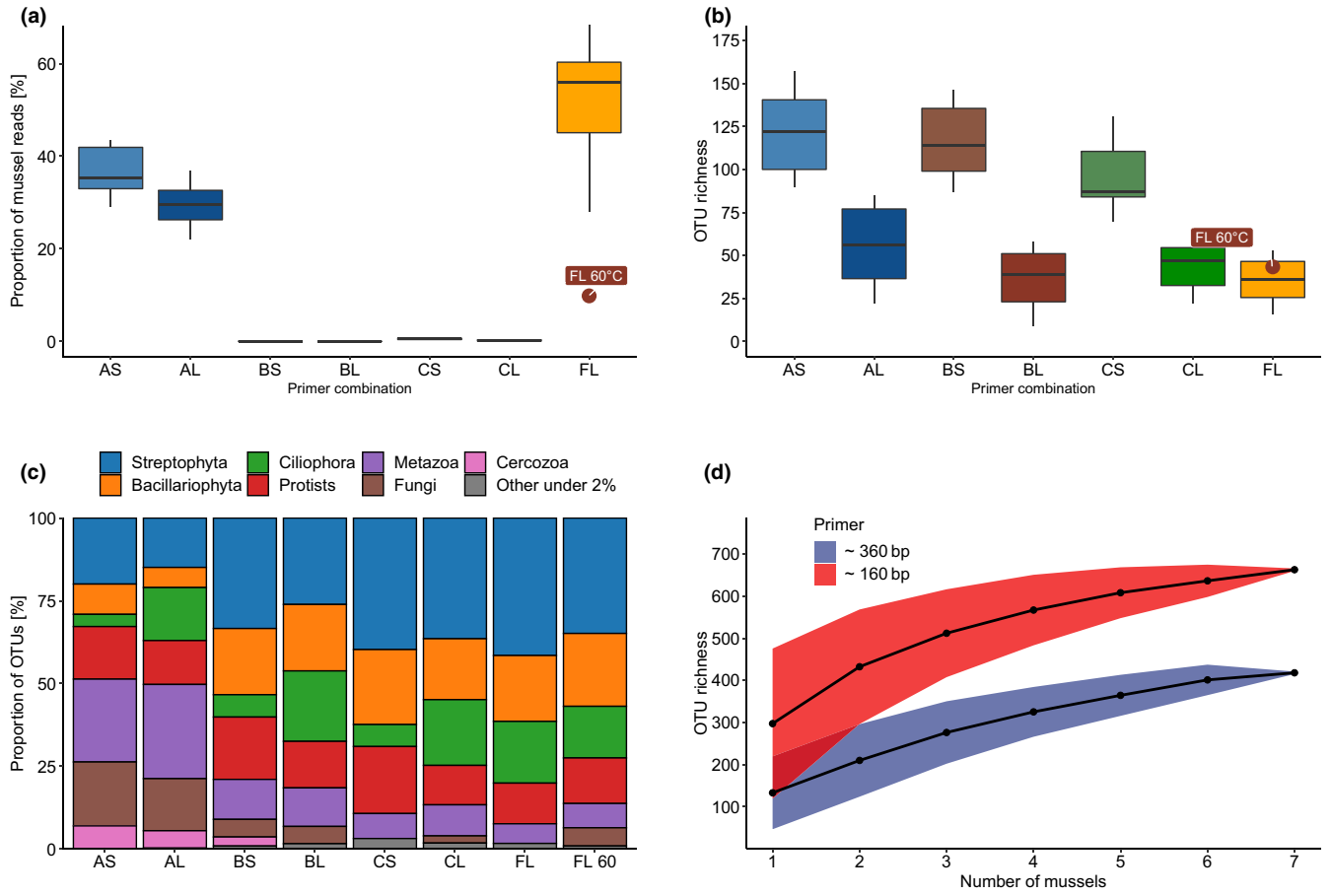

FIGURE 2 (a) Recovered proportion of *Dreissena* reads for different primer combinations. The brown dot indicates the mean read proportion of the FL primer combination at 60°C instead of 55°C annealing temperature. (b) Recovered OTU richness per primer. (c) Higher taxonomic composition of the OTUs recovered by the seven primer pairs. (d) Saturation curve of OTU richness for the aggregated primer paris (a,b,c) by length. The smaller fragment shows a higher OTU richness but a similar saturation rate as the long one. The coloured ribbon shows the standard deviation.

using the adonis function in vegan. Community similarity was visualized using NMDS plots based on the metaMDS function in vegan (distance = "jaccard", binary = T, k = 3, trymax = 999). All plots in this study were created with the tidyverse and ggplot2 packages (Wickham, 2016; Wickham et al., 2019). We also compared the recovered taxonomic composition and diversity in the seven separate mussels as well as the five mussel pools and six filtered water eDNA samples and between the different primer pairs used in our experiments. Using the 22 time series samples, we analysed changes in the mussels' diet composition and their surrounding planktonic communities over time and between rivers.

3 | RESULTS

3.1 | Performance of the designed PCR primers

Using our data set of seven separate mussels, we explored the utility of our lineage-specific primers. The primers performed quite differently in the recovery of nonmussel taxa. Without the diagnostic suppressing nucleotides, the recovered read population consisted almost entirely of mussel DNA (1.76% of nonmussel reads for the primer pair DL). In contrast, the six *Dreissena* primer pairs were all highly efficient at minimizing amplification of the DNA sequences of the mussel (nonmussel sequences primer pair AL = 70.5±5.31, AS = 63.17±5.99, BL = 99.93±0.09, BS = 99.93±0.10, CL = 99.64±0.50, CS = 99.46±0.18; Figure 2a). For the primer pairs that did not contain the additional 3′-TC sequence (EL and FL), an average of 51.9% of the total reads were identified as nonmussel (Figure 2a). However, an increase of the PCR annealing temperature from 55 to 60°C led to a considerable increase of the nonmussel sequences recovered for that primer pair to 90.16%.

A significantly increased richness was recovered for the short amplicons (Richness primer pair AL = 67±49.0, AS = 143±77.2, BL = 45±37.3, BS = 145±89.3, CL = 49±26.1, CS = 123±85.3, FL = 41±25.6, ANOVA, p<.05). Interestingly, the higher richness recovered for the shorter primers in mussels was not found for a water sample (Richness in water sample primer pair AL = 568±35.4, AS = 569±10.6, BL = 450±8.4, BS = 421±4.2, CL = 459±21.2, CS = 402±33.9).

The recovered taxonomic composition of OTUs was comparable between the primers B and C, E and F. A clear difference in

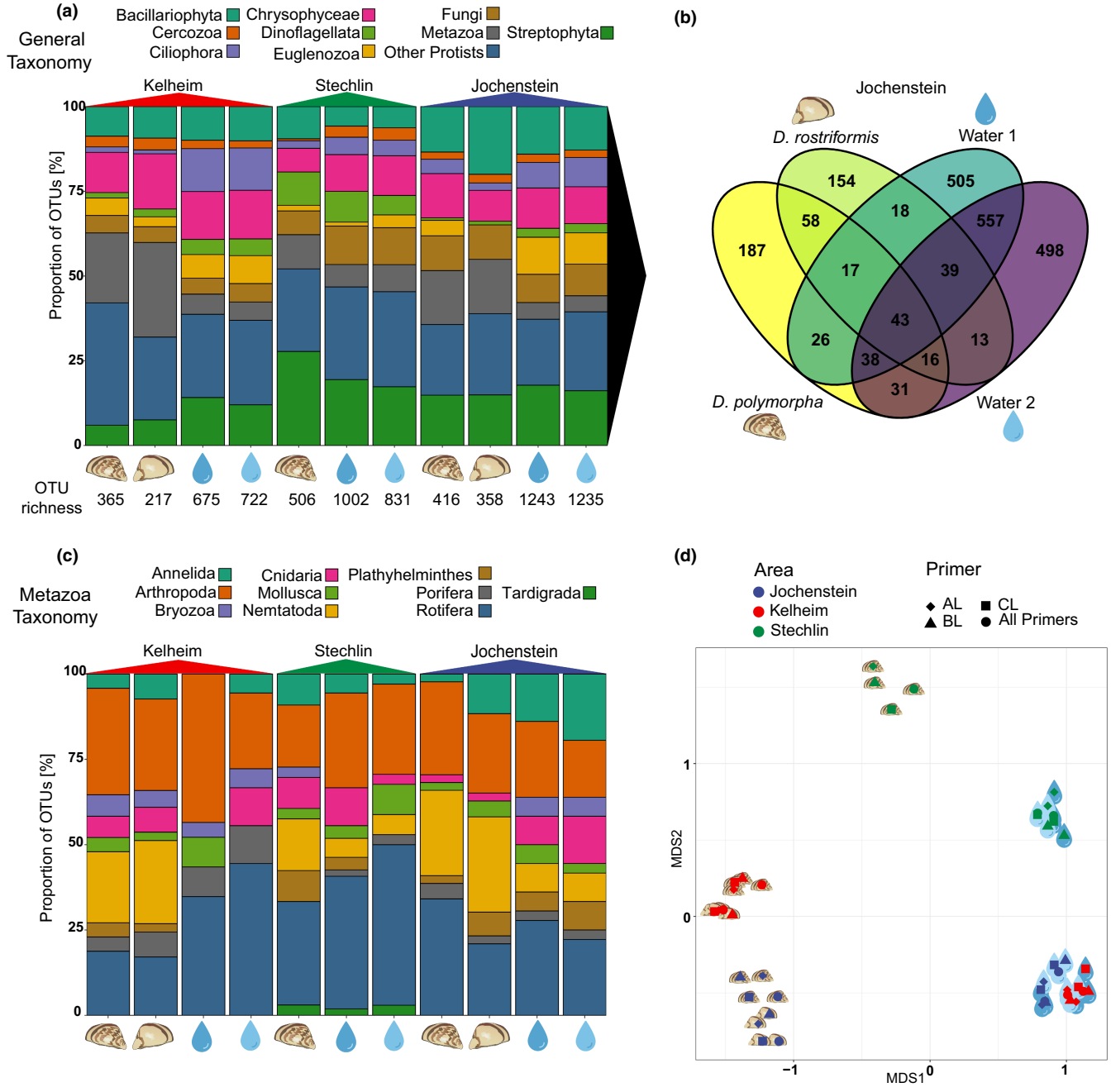

FIGURE 3 (a) Higher taxonomic composition of the recovered OTUs comparing water eDNA samples, *Dreissena polymorpha* (DP) and *D. rostriformis* (DR) DNA extracts from the different sites Kelheim, Stechlin and Jochenstein). The plots show the total number of recovered OTUs in each sample at the bottom, and proportion of different taxa among these OTUs. (b) Venn diagrams showing the recovered OTU numbers and their overlap between *D. polymorpha*, *D. rostriformis* and the two eDNA water samples for Jochenstein. (c) More detailed taxonomic composition of the recovered OTUs on metazoan-level based on the same samples as in. (d) NMDS plots (stress = 0.089) based on Jaccard dissimilarity showing community differentiation of eDNA water samples and *Dreissena* mussel samples for the two sites at the Danube (Kelheim and Jochenstein) and Lake Stechlin. The first axis separates water (right) and mussels (left). The shapes distinguish the different primer combinations used, as well as a combination of all three into a merged data set (Aggregate).

the taxonomic composition of recovered OTUs was found for the metazoan-specific primer pair A. While the primer pairs B, C, E and F recovered significantly more plants (mostly green algae) and Bacillariophyta, primer pair A showed more Metazoa and Cercozoa OTUs (Chi square test, $p < .05$; Figure 2c). The recovered taxa were well expected from the sampling sites. They included various green and brown algae and protozoans, which are all probably diet of the mussels as well as different freshwater animals. We also found 367 OTUs of likely commensals and parasites of the mussels in the data, for example the ciliate *Conchophthirus* sp. and the trematode *Aspidogaster conchicola*. The relative proportion of parasite OTUs was significantly lower for the short markers than for the long ones (ANOVA, $p < .05$).

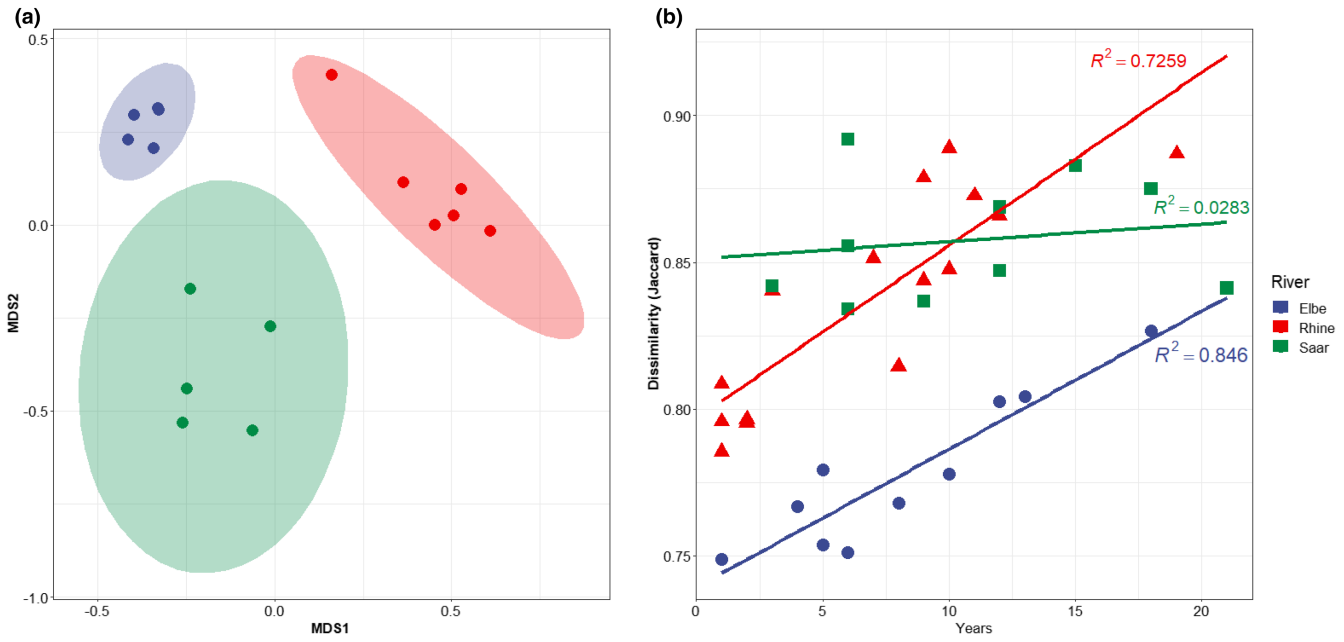

FIGURE 4 (a) NMDS (stress = 0.080) plot based on Jaccard dissimilarity showing differentiation of *D. polymorpha* associated eukaryotic communities between ESB homogenates collected from three different rivers. Ellipses represent the 95% confidence interval. (b) Correlation of Jaccard dissimilarity between the *D. polymorpha* associated communities and the number of years between sampling events.

The higher recovered richness of the short amplicon is also shown in accumulation curves of the combined markers A, B and C. Initially, the inclusion of more mussels in the sample considerably increased richness. But at seven mussels, the amount of recovered OTUs was already approaching saturation. 664 OTUs were recovered for a combined data set of seven mussels for the short and 416 for the long amplicon data set (Figure 2d).

3.2 | Comparison of recovered communities between mussel species, and water samples across space

We used pools of mussels and water eDNA samples from three sites for this analysis. The three *Dreissena* diet primer pairs (AL, BL, CL) were merged into a single data set for each sample. Mussel and water samples recovered similar compositions of higher taxa in our analysis (Figure 3a; Chi square tests, $p > .05$). The similar taxonomic composition recovered by water and mussel samples held true when lower taxonomic ranks were compared, for example phyla within metazoa (Figure 3c). Despite the comparable higher-level taxonomic compositions, water samples and mussels recovered a considerable proportion of unique OTUs (Figure S3). On average, only 7% of the OTUs recovered from the mussels were shared with the water samples, While water samples shared approximately 30% of the recovered OTUs. Also, water samples recovered a significantly higher OTU richness than mussel samples (372.4 vs. 951.3 on average, *t* test, $p < .05$).

The two mussel species *D. polymorpha* and *D. rostriformis* recovered relatively similar richness (288 vs. 391 on average). Comparing the two sites Kelheim and Jochenstein, mussel and water samples showed similar trends of recovered richness, with both showing higher richness in Kelheim than in Jochenstein. The number of mussels in a sample did not appear to contribute to the number of recovered species. The samples of *D. polymorpha* from Lake Stechlin (only 4 mussels) recovered an even higher OTU richness than the other two samples of 32 mussels (506 vs. 391).

The relatively small overlap of recovered OTUs between water and mussel samples (see Figure 3b, Figure S3 and S4) was reflected in an NMDS plot (Figure 3d), where the two sample types were well separated on the first axis. However, the general pattern of differentiation between sites was comparable between water samples and mussels. Samples from Lake Stechlin formed a separate cluster, distant from the two Danube samples. The two Danube sampling sites were also separated by water and mussel eDNA. But their separation was less clear for the mussels (Figure 3d). However, when mussel parasite OTUs were included in the data set, the pattern reversed, with the two sites from the Danube being more distinct in mussels (Figure S5). The mussel eDNA samples also showed a differentiation between the recovered communities of *D. rostriformis* and *D. polymorpha*. The recovered differentiation in the NMDS plot was also supported by a PERMANOVA, suggesting significant ($p < .001$) effects of sample type ($R^2 = 0.193$), sampling site ($R^2 = 0.199$) and mussel species ($R^2 = 0.119$) on the recovered beta diversity pattern. No significant difference was found for the communities recovered by the three different primer sets ($R^2 = 0.053$, $p > .05$).

3.3 | eDNA time series analysis using ESB samples

The ESB zebra mussel homogenate samples recovered a total of 1716 OTUs for the merged data sets of the primer pair B and C. We did not recover significant differences in richness between the

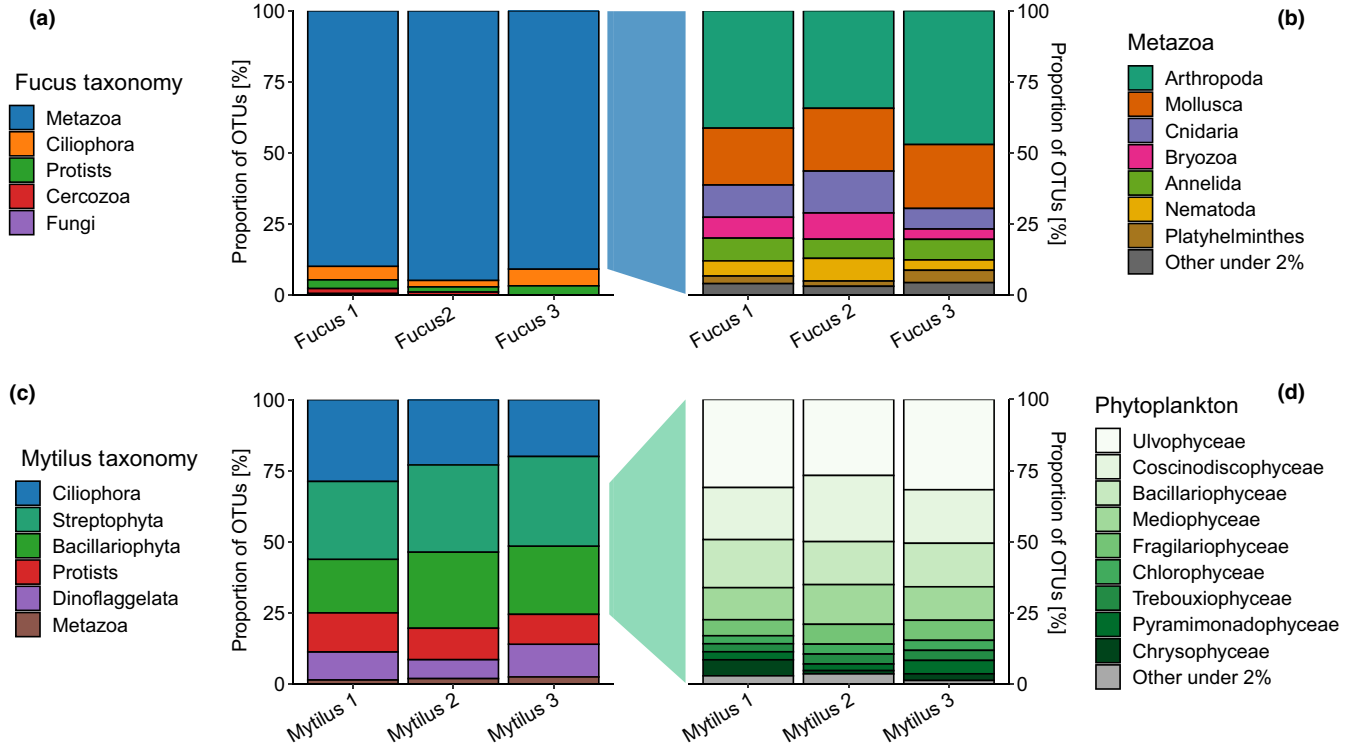

FIGURE 5 (a) Higher taxonomic composition of the ESB bladder wrack samples based on the recovered OTUs on kingdom-level obtained by amplification with the primer combination D. (b) More detailed view of the metazoan community from the bladder wrack samples on phylum level. (c) Higher taxonomic composition of the ESB Mytilus samples based on the recovered OTUs on kingdom-level obtained by amplification with the primer combinations E and F. (d) Detailed view of the phytoplankton composition on class-level.

three sampled rivers (ANOVA, $p > .05$). On average, we found 121 OTUs in the samples from the Rhine, 138 in those from the Saar and 107 in those from the Elbe. For Rhine and Saar, we also did not find significant changes of richness between different sampling periods. For the Elbe, however, we found a significant decline of richness with time from 140 OTUs in 1998 to only 63 in 2016 (Linear model, $R^2 = 0.89$, $p < .05$). Interestingly, we found some simultaneous and significant changes (positive and negative) for the Rhine and Saar rivers for the same OTUs (Table S1). The different rivers were well separated by an NMDS plot (Figure 4a). A PERMANOVA suggests that sampling sites primarily contribute to the observed beta diversity ($R^2 = 0.22$, $p < .001$). However, we also found a strong positive correlation between beta diversity between samples and the temporal distance between sampling events (Linear model, $p < .05$, $R^2_{\text{Rhine}} = 0.73$, $R^2_{\text{Elbe}} = 0.85$) for the river Rhine and Elbe (Figure 4b). The community composition in these rivers strongly turned over in the past 20 years (from 1995 to 2016).

3.4 | Blue mussel and bladder wrack samples

The blue mussel samples recovered 205 OTUs on average for the primer E and F, the bladder wrack samples 106 OTUs for marker D. Our primers proved highly efficient in suppressing the mussel (85.3% nonmussel reads on average) and the bladder wrack (99.89% of nonbladder wrack reads on average). The recovered communities between bladder wrack and blue mussels showed entirely different taxonomic compositions (Figure 5a and c).

The OTUs recovered from blue mussels mostly belonged to different groups of green and brown algae and protists, particularly Ciliates. Many of these taxa are probably elements of the mussel's diet. The majority of OTUs recovered from bladder wrack was metazoan (Figure 5b and d). Visual inspection of actual bladder wrack samples during collection confirmed the actual presence of numerous of the bladder wrack associated animals, which were identified among our OTUs. Examples include different Balanidae, Copepoda and Amphipoda, as well as *Littorina* snails, different mussels, Bryozoans and Polychaeta.

4 | DISCUSSION

4.1 | High throughput diet analysis in mussels and other aquatic metazoans

In the past years, metabarcoding of gut content has developed into a popular tool for food web analysis (Kennedy et al., 2020). However, this method often comes with a tradeoff between maximizing the recovered dietary diversity and minimizing the amplification of consumer DNA (Krehenwinkel et al., 2017). This is clearly shown by our amplification of mussel extracts with metazoan-specific primers, which yield nearly 100% mussel reads. Here, we

introduce primer sets for mussels and other filter-feeding metazoans, which overcome this issue by 3′ mismatches with the consumer (Krehenwinkel et al., 2019; Stadhouders et al., 2010). Our primers suppress consumer amplification efficiently, while recovering a broad spectrum of eukaryote dietary taxa. Based on group-specific mismatches, different primers in our assay target taxonomically different subsets of the eukaryotic diet. As they amplify nearly identical DNA fragments, they can also be used in multiplex reactions (Krehenwinkel et al., 2019).

Our first primer sets are mussel-specific (A, B, C). They are highly efficient in the genus *Dreissena* and 33 other bivalve genera and recover all possible eukaryotic dietary taxa. This marker set will be of great value for studying the impact of invasive *Dreissena* mussels on aquatic ecosystems (Maguire & Grey, 2006; Miller & Watzin, 2007). We here reconstructed a diverse diet of zoo- and phytoplankton taxa from *Dreissena* mussels and highlight a case of possible selective feeding between the two sympatric species *D. polymorpha* and *D. rostriformis*. It has long been assumed that the quagga mussel is replacing the zebra mussel in its current invasive range (Matthews et al., 2014). However, a niche partitioning by particle selectivity and following different dietary resource use (Baker & Levinton, 2003) could allow a long-term coexistence of the two species.

Our second primer set (E and F) is of much broader taxonomic utility, as shown in the analysis of blue mussels. This primer pair will efficiently prevent any metazoan's DNA from amplification. Hence, it opens up the possibility to explore a wide range of nonmetazoan diets of every animal, including other aquatic filter feeder like annelids, bryozoans, sponges or cnidarian, but also terrestrial herbivores. Its reduced efficiency in consumer amplification compared to the *Dreissena* primers can be considerably improved by increasing the PCR stringency with a higher annealing temperature of 60°C. This opens up new possibilities to study aquatic food webs, in which filter-feeding metazoans often play a critical role (Newell, 2004; Vaughn et al., 2008).

Interestingly, our shorter amplicon (~160 bp) recovered significantly more OTUs from mussels than the longer one (~360 bp), while this effect was not found for water samples. Dietary DNA from the mussel's digestive tract will be considerably more degraded than that of freshly ingested prey. Long amplicons may thus only recover very recently ingested dietary items, while the short one will recover a more complete diet. On the other hand, a longer amplicon will contain more genetic variation and probably provide a better taxonomic resolution. A shorter amplicon may also be more prone to detect instances of secondary predation (Sheppard et al., 2005), for example, DNA from phytoplankton ingested by the mussel's zooplankton prey. It is noteworthy that not all taxa recovered from mussels are likely to be their actual diet. Mussels filter the water column for most particles in a certain size range (Sprung & Rose, 1988), but will eject some as pseudofaeces. Such noningested particles could be removed, if only the digestive tract of the mussel is used for DNA isolation.

Besides dietary taxa, our primers also pick up DNA of parasitic and commensal organisms (Molloy et al., 1997). For an accurate dietary analysis, these parasites should be removed from the data set. However, the task is not trivial, as not all parasites will be known. We have only removed taxa, which were clearly known as mussel parasites, but may have omitted others. To accurately distinguish parasites and commensals from diet, mussels should best be collected and then starved until no dietary DNA can be detected anymore. The use of longer amplicons will also help to enrich parasites, as DNA of parasites living in the mussels' tissues will be of high quality. Similarly, RNA extracts could be used for metabarcoding, which will mostly contain the active community in the mussel's tissue. The possibility to detect parasites with our method offers an interesting application for monitoring health in natural mussel populations or in mussel aquaculture, which is of great economic importance in some areas (Shumway et al., 2003). In our analysis, these parasites and commensals were barely detected in water eDNA samples, collected in close proximity to the mussels, hence actual tissue needs to be analysed for accurate pathogen monitoring.

Finally, it should be noted that for small mussels like *Dreissena* spp., bacteria may also be an important part of the diet (Dionisio Pires et al., 2005). We did not analyse this here as we were particularly interested in eukaryotic taxa. Bacterial composition in mussels could be easily scored using 16SrDNA primers and corresponding molecular protocols (Kozich et al., 2013).

4.2 | The utility of natural eDNA samplers

The filter feeding mechanism of many aquatic metazoans is quite similar to the commonly used filtering of water for eDNA analysis (Barnes et al., 2014; Jo et al., 2019; Wilcox et al., 2015). This has led to the suggestion that aquatic filter feeders could be used as natural eDNA samplers (Mariani et al., 2019; Siegenthaler et al., 2019; Turon et al., 2020). Our primer sets are ideally suited to serve as selective markers to monitor different components of eukaryotic plankton. Interestingly, we can not only prevent amplification of the DNA of metazoan filter feeders. Our analysis of bladder wrack samples also shows that primer set D is well suited to enrich metazoan DNA from substrates rich in DNA of other eukaryotes. This is a common issue in filtered eDNA samples, where phytoplankton DNA usually greatly exceeds that of animals (Zou & Smith, 2020).

Mussels should be well suited as eDNA samplers. Their efficient filtering of plankton (Fanslow et al., 1995) is well illustrated by the strong cascading effect of the invasion of *Dreissena* mussels in American lakes on limnic ecosystems (Lavrentyev et al., 1995; MacIsaac et al., 1995; Maguire & Grey, 2006). Their efficient particle removal has even led to the suggestion to use them as biofilters for water clarification (Elliott et al., 2008). Mussels recovered similar taxonomic compositions like water eDNA samples, same held true for trends of α- and β-diversity. However, the recovered richness was considerably higher in the water samples, suggesting a certain selectivity of the mussel's filtering apparatus (Baker & Levinton, 2003). Recent work shows that eDNA particles are present in diverse size ranges, spanning from <0.2 to several 100 μm, but

are particularly abundant at the lower end of the spectrum (Turner et al., 2014). Different studies recommend different filter size from 10 μm down to 0.2 μm (Barnes et al., 2014; Jo et al., 2019; Wilcox et al., 2015). Here, we used a filter pore size of 0.45 μm. *Dreissena* mussels show an imperfect retention of particles larger than 0.7 μm and a total retention only at particle sizes larger than 5 μm (Sprung & Rose, 1988). The retention efficiency shifts to larger particles in larger mussels (Strohmeier et al., 2012). Our mussel-based analysis may thus have omitted very small eDNA particles. As the retention of mussels correlates with their size (MacIsaac et al., 1995), a broader spectrum of taxa could be recovered by choosing mussels of various sizes for the eDNA analysis. Unfortunately, the *Dreissena* mussels used in our experiments where all from the same age cohorts and hence of similar size. However, selecting mussels of different size in the future could be easily done, as different age classes are readily available in mussel colonies.

Interestingly, a noteworthy proportion of taxa, which we detected in mussels, was not found in water samples. Some of these may live in close association with the mussels, for example, parasites and commensals (Molloy et al., 1997), others may be species that simply do not release much eDNA into the water column (Koziol et al., 2019). It is also likely that mussels recover taxa over a broader timescale, as dietary particles will remain in the gut for several hours (Hawkins et al., 1990), while a water eDNA sample is just a snapshot at a certain site. Hence, DNA extracts from filter feeders add a complementary perspective and may even recover taxa, which are not detectable using water samples.

As shown in our saturation analysis, a relatively small number of mussels is sufficient to serve as an eDNA sample. This may also explain the relatively high number of OTUs found in a pool of only four mussels from Stechlin. Also, metazoan filter feeders are often highly abundant and easy to collect. However, it is critical to carefully optimize molecular protocols for different natural samplers. One important consideration is the DNA isolation method. Mussels contain significant amounts of mucus, which is hard to remove by most common DNA extraction protocols and can interfere with PCR efficiency. We initially repurified our DNA extracts, but found that a CTAB method is the most efficient way to achieve high quality extracts. Also, when several mussels are pooled for an eDNA sample, they need to be very thoroughly homogenized. From a well homogenized sample, only a small subset needs to be extracted. However, if homogenization is incomplete, the recovered diversity may be biased between samples. Alternatively, separate mussels would have to be extracted, which, however, significantly increases processing cost and workload.

An ideal eDNA sample should be taxonomically exhaustive, while at the same time minimizing processing effort. A water sample represents an ideal compromise between these two demands. Considering this background, natural samplers are unlikely to replace water samples. Yet, a particularly important future application of our protocol is the analysis of time series samples. Global ecosystems are changing at an unprecedented rate, and the task of biomonitoring is of outmost importance (Gavrilescu et al., 2015; Krehenwinkel, Pomerantz, & Prost, 2019; Pawlowski et al., 2020). However, accurate time series samples for temporal biomonitoring are largely lacking (García-Barón et al., 2021). Using alternative sources of eDNA, like dietary information from archived filter feeders, offers novel opportunities to generate this urgently needed data. Our time series samples suggest a pronounced temporal turnover of the recovered dietary community. In the past decades, decreasing eutrophication and increasing warming have probably led to state shifts, which strongly affect the plankton communities (Abonyi et al., 2018). Such state shifts do not necessarily cause losses of alpha diversity, but are more evident by turnover of the community (Magurran & Henderson, 2010). This is also supported by our data, which show a relatively stable richness in most sites. We are currently processing entire mussel time series of the ESB across Germany to explore temporal changes of planktonic communities in more detail. Yet, the ESB is not the only alternative source of eDNA for retrospective biomonitoring. Mussels are collected across the globe as part of pollution monitoring projects since many decades. Further historical filter feeder samples will probably be available in natural history museums. Such samples offer exiting new perspectives to explore and understand anthropogenic effects on global aquatic biodiversity.

AUTHOR CONTRIBUTIONS

Henrik Krehenwinkel devised the study, Diana Teubner, Manuel Wörner and Lukas Brink performed the fieldwork. Sven Weber, Isabelle Junk and Lukas Brink performed laboratory work. Henrik Krehenwinkel and Sven Weber analysed the data and wrote the manuscript. All authors have read and approved the final version of the manuscript.

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CONFLICT OF INTEREST

The authors declare no conflcts of interest.

DATA AVAILABILITY STATEMENT

All alignments of 18SrDNA sequences for primer design and of the priming sites for different eukaryotic taxa, all raw reads and OTU tables will be made available on Dryad Digital. Repository for review: https://datadryad.org/stash/share/A7XHr1_zraiKuqViCexMBUxuplJSt3gQg8-7UDRpTKk

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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