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Author(s):
Brantschen, Jeanine; Pellissier, Loïc; Walser, Jean-Claude; Altermatt, Florian

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ORIGINAL ARTICLE

Evaluation of primer pairs for eDNA-based assessment of Ephemeroptera, Plecoptera, and Trichoptera across a biogeographically diverse region

Jeanine Brantschen[1,2] | Loïc Pellissier[3,4] | Jean-Claude Walser[3] | Florian Altermatt[1,2,5]

[1]Department of Aquatic Ecology, Eawag, Swiss Federal Institute of Aquatic Science and Technology, Duebendorf, Switzerland

[2]Faculty of Science, Department of Evolutionary Biology and Environmental Studies, University of Zurich, Zurich, Switzerland

[3]Department of Environmental Systems Science, Federal Institute of Technology, Zurich, Switzerland

[4]Department of Landscape Dynamics & Ecology, WSL, Swiss Federal Institute for Forest, Snow and Landscape Research, Birmensdorf, Switzerland

[5]Department of Environmental Systems Science, Genetic Diversity Center, Swiss Federal Institute of Technology, Zurich, Switzerland

Correspondence
Jeanine Brantschen and Florian Altermatt, Department of Aquatic Ecology, Eawag, Swiss Federal Institute of Aquatic Science and Technology, Duebendorf, Switzerland.
Email: jeanine.brantschen@eawag.ch (JB) and florian.altermatt@ieu.uzh.ch (FA)

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Abstract

Macroinvertebrates serve as key indicators in ecological assessments of aquatic ecosystems, where the composition and richness of their communities are indicative of environmental and anthropogenic change. Established monitoring of macroinvertebrates is expensive and time-consuming, and relies on expert taxonomic knowledge. In contrast, biomonitoring based on molecular tools can support faster characterization of aquatic communities but needs validation for the target taxonomic groups and the study region. Here, we used data from a biomonitoring program covering a large biogeographic gradient to compare the routine kick-net method with eDNA metabarcoding. We used two primer pairs targeting COI, one targeting a broad metazoan spectrum (mlCOIintF/jgHCO2198) and another more recently developed primer pair optimized for the detection of freshwater invertebrates (fwhF2/EPTDr2n). We used the data of the macroinvertebrate monitoring with a focus on the orders of Ephemeroptera, Plecoptera, and Trichoptera across 92 rivers in Switzerland, covering four continental drainage basins and an elevational range from 198 to 1650 m a.s.l. Across all sample sites, the kick-net detected more distinct taxa than either of the metabarcoding approaches. At a site level, however, both primer pairs detected on average more species. Comparing both primer pairs, the fwhF2/EPTDr2n primer pair captured more species assigned to the indicator groups Ephemeroptera, Plecoptera, and Trichoptera, and showed a significantly larger overlap with the kick-net method. However, the community composition still varied significantly among the different metabarcoding approaches. Fewer Trichoptera species were recovered by eDNA, whereas the fwhF2/EPTDr2n primer pair detected more Plecopterans than the other two approaches. This study highlights the importance of the optimization and validation of novel molecular approaches under consideration of the target organismal group and the study area.

KEYWORDS
aquatic, biomonitoring, macroinvertebrates, primer choice

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1 | INTRODUCTION

Freshwater biodiversity is sharply declining, and ecosystems are heavily affected by anthropogenic pressures (Dudgeon et al., 2020; Reid et al., 2019). While abiotic physicochemical conditions of aquatic ecosystems can be assessed at a high temporal and spatial resolution, it remains challenging to evaluate the impact of habitat conditions on living organisms and biodiversity comprehensively. Biomonitoring across space and time is central to understanding of ecosystem state and degradation, and to subsequently informing policies that mitigate biodiversity loss (IBPES, 2019). Biological communities are characterizing the ecological integrity of a habitat based on the organisms' ecological requirements and responses to environmental stressors. In routine biodiversity assessments, generally only a subset of the biodiversity is assessed and used as bioindicators. These groups of taxa inspected are often widely distributed across the phylogenetic tree as they are not chosen by taxonomic uniformity but based on practical considerations (size, identifiability, and sensitivity to ecological drivers). Yet, many other taxa that might be equally suited from an indicator perspective are often ignored. Studies on eDNA may allow to also use information on these taxa, and the fast detection of bioindicators with molecular approaches can be considered a complementary tool for established methods in the assessment of ecosystem integrity.

Freshwater invertebrates are frequently used in established freshwater monitoring programs, and especially, insects serve as indicators for water quality and ecological integrity of running waters (Andújar et al., 2018; Barbour et al., 1999; Benetti et al., 2012). Their ecological preferences are well known, as is their response to organic and chemical pollution (Buss et al., 2015). In particular, the orders of Ephemeroptera, Plecoptera, and Trichoptera (short: EPT) are widespread and their habitat requirements and sensitivity to chemicals have been studied extensively, which makes them key indicators for habitat pollution and degradation. However, the sorting and morphological identification of species assemblages is costly in time, reliant on taxonomic expertise 1 and on intact specimens and well-developed late-instar stages. These conditions limit morphological methods to potentially provide biodiversity information at high spatio-temporal resolution (Jones et al., 2008).

Environmental DNA has become a game changer for biodiversity assessments in freshwater systems (Baird et al., 2012; Deiner et al., 2017; Pawlowski et al., 2018; Taberlet et al., 2012). Reliant only on DNA traces extracted from an environmental sample, such as water, macro-organisms are not assessed invasively and are not only detectable through intact larvae specimens (Pawlowski et al., 2020). Routine biomonitoring programs aim to sample communities across large environmental gradients and spatial scales, and therefore, they require sampling methodologies that perform well in various habitats and under changing environmental conditions to capture meaningful baselines of ecological states beyond individual sampling campaigns. With molecular tools, the opportunity arises to upscale routine biomonitoring programs through a non-invasive, fast, and standardized way to sample more sites across larger regions (Carraro et al., 2020; Deiner et al., 2021; Pawlowski et al., 2020; Valentini et al., 2016). These novel tools, like eDNA metabarcoding, require careful assessment and validation in comparison with established methods before they can deliver robust information for management and policy making (Blackman et al., 2019; Makiola et al., 2020; Seymour et al., 2020).

Macroinvertebrates, especially indicator species that belong to the EPTs (Valente-Neto et al., 2018; Wallace et al., 1996) are a prominent target group for DNA-based monitoring (Hering et al., 2018), but approaches are still under development and validation (e.g., Blackman et al., 2019; Brantschen et al., 2021; Fernández et al., 2018; Leese et al., 2021; Macher et al., 2018). As highlighted by a recent meta-analysis (Keck et al., 2022), eDNA metabarcoding of macroinvertebrates gives mixed results compared with other organisms, such as fish, that are more comparable across methods (e.g., Blabolil et al., 2021; Pont et al., 2018; Sard et al., 2019). The assessment of macroinvertebrates for biomonitoring with eDNA metabarcoding compared with established methods remains challenging (Carraro et al., 2020; Elbrecht et al., 2017; Fernández et al., 2019; Mächler et al., 2019). The detection of study organisms relies heavily on methodological choices, such as the barcoding region chosen, or primer types used (e.g., degenerate vs specific) within one barcode (Elbrecht & Leese, 2017; Hajibabaei et al., 2019; Leese et al., 2021). With established methods, EPTs, and macroinvertebrates more generally, are detectable within one sample (such as kick-net) because they share a physical habitat, but this by no means implies that they are also genetically similar for a given barcode. In particular, the Cytochrome Oxidase subunit I (COI) marker region shows a high degeneracy for targeted macroinvertebrates (Hajibabaei et al., 2019; Leray et al., 2019). This high genetic variation complicates their widespread implementation in metabarcoding surveys. Consequently, biomonitoring programs are biased toward a subset of organisms (i.e., established indicators) but constrained by the efficiency of primer design across a wide group. Various primer pairs targeting the COI barcode have been developed and not all of them cover this subset of target organisms equally well. One primer pair that is commonly used is the degenerate primer pair mlCOIintF/jgHCO2198 which targets a broad range of metazoan organisms and was initially developed for invertebrates in gut content (Geller et al., 2013; Leray et al., 2013). While such universal primers cover a broad range of macroinvertebrates, the width of taxonomic coverage comes at the cost of unspecific amplification of non-target DNA (Elbrecht & Leese, 2017; Hajibabaei et al., 2019). Therefore, more specific primer pairs have been developed, among others, the less degenerate primer pair fwhF2/EPTDr2n (Leese et al., 2021; Vamos et al., 2017), showing improved detection of benthic macroinvertebrates in comparison with other previously established primers (Leese et al., 2021). The latter primer pair was developed and tested in a river catchment in Central Germany, covering a relatively narrow biogeographic area and taxonomic breadth of communities.

In this study, we extended the application of the fwhF2/EPTDr2n primer pair to a broader range of communities than hitherto studied. We used samples from a monitoring of more than 90 river sites in

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Switzerland that covers a broad biogeographic and elevational gradient. From this established monitoring, we compared kick-net-based data of EPT distributions and two metabarcoding approaches, for which we used the degenerate primer pair mlCOIintF/jgHCO2198 (targeting a broad array of invertebrates in gut content analysis) and the more specific primer pair fwhF2/EPTDr2n (targeted specifically toward aquatic invertebrates) amplifying fragments within the COI barcode. We evaluated the detection of EPT indicator species by all three approaches, looking at two different primer pairs varying in their specificity toward the target group and the established kick-net method, to test the effectiveness of these approaches for the monitoring of these bioindicators. In particular, we investigated the following:

- We tested the differences in the taxonomic resolution and the diversity of EPT captured by two primer pairs (mlCOIintF/jgHCO2198 and fwhF2/EPTDr2n) that differ in their specificity. Furthermore, we compared the spectrum of species overlapping with established kick-net data.
- We tested whether the species richness (alpha diversity) based on the invertebrate-specific primer pair (fwhF2/EPTDr2n) is higher when restricting the data to EPT species. Furthermore, we hypothesized the richness based on eDNA is equal or higher than with the kick-net method.
- We assessed whether and how the three different approaches capture beta diversity pattern among meta-communities, reflected in a decreasing community similarity with increasing distance between sampling sites.

2 | METHODS

2.1 | Study area and field sampling

In spring 2019, more than 90 river sites were surveyed in a federal biomonitoring program. The sampling sites covered all biogeographic regions of Switzerland ranging from lowland to alpine sites (Figure 1) and intermediate to large river systems under some anthropogenic, agricultural, or industrial impact. In the federal water quality assessment (NAWA: Nationale Beobachtung Oberflächengewässerqualität; FOEN, 2016), rivers are routinely surveyed since 2012 by kick-net, and in 2019, this sampling was complemented by eDNA sampling (paired samples $n = 92$) in addition to the standard kick-net sampling (Stucki, 2010). The sampling of eDNA collected four eDNA filter replicates at each site right before the kick-netting. For each sampling site, two filter replicates were taken per riverbank (total n per site = 4, 2 L of water collected in total) in order to integrate DNA from different microhabitats similar to the river stretch sampled by kick-net. For an eDNA sample, water was filtered in the field using Sterivex filters with a 0.22 µm pore size (Merck Millipore, Merck KgaA). In total, 0.5 L were filtered per replicate, resulting in two liters per sampling site. Sterivex filters were then sealed with Luer caps (Merck Millipore, Merck KgaA) and placed in a cool box

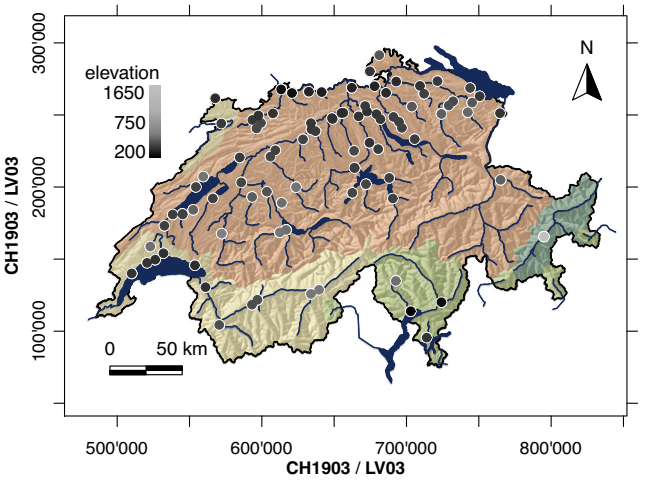

FIGURE 1 Map showing the spatial distribution of the sampled sites across Switzerland. The sampling covered an altitudinal gradient from lowland (198 m a.s.l.) to sub-alpine (1650 m a.s.l.) river systems, indicated by the gradient of gray of the points. The four major river basins (Rhine, Rhone, Ticino, and Inn) are shaded in different background colors.

for short-term storage. The sealed filters were stored in the freezer (−20°C) until further processing. For the negative controls, two replicates of DNA-free water (0.5 L each) were filtered directly in the field at the beginning and the end of every field campaign by a given sampler (total $n = 40$). Those filters were extracted individually, the first PCR performed per filter, and the replicates from one sampling campaign were then pooled for the second PCR step.

The kick-net samples were collected directly after the eDNA sampling. The kick-net sampling was performed using standardized methods for river systems (Stucki, 2010). At each site, the benthic fauna of microhabitats were sampled: 8 individual kick-net subsamples were done at each stream site, and subsequently, all organisms were pooled for quantification and identification according to Stucki, 2010. The macroinvertebrates were preserved with 95%-ETOH on site, and all specimens of EPTs were identified by experts to species level (few were collated in species-complexes, which we excluded for comparability with the eDNA approaches).

2.2 | DNA extraction

The eDNA samples and negative field controls were extracted in a laboratory dedicated to eDNA use (cleanroom environment). The DNA was extracted with the Qiagen PowerWater Sterivex Extraction Kit (Qiagen) according to the protocol of the manufacturer. All samples were extracted in batches of 12 samples at a time. Field filter controls served also as negative extraction controls and were extracted randomly among the samples. The extracted DNA was eluted in 100 µl elution buffer and stored at −20°C until further processing. The extraction kit includes an inhibitor removal step before final elution, and a subset of the samples was tested showing no signs of inhibition.

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2.3 | PCR amplification

Samples were amplified using a fragment of the COI barcoding region (Figure S1). We here used the PCR protocols of the original publications of the respective primer pairs, adopted, and optimized for our samples. Thus, some of the observed differences between the primer pairs may also be due to some of the methodological settings used in the PCR, which, however, are inherently linked to the primers used. The first primer pair used was mICOIintF and jgHCO2198 (Geller et al., 2013; Leray et al., 2013) targeting a 313 bp fragment for a broad array of metazoans. The more specific primer pair was fwhF2 combined with EPTDr2n (Leese et al., 2021; Vamos et al., 2017), targeting a 142 bp fragment. All primers were modified to include the Nextera® transposase sequences and frameshifts bases. All samples as well as negative and positive controls were randomly assigned to four 96-well PCR plates. For the positive control, we extracted DNA from a specimen of a terrestrial beetle (*Abax parallelepipedus*). For each PCR plate ($n = 4$), we added the DNA extract in one of the 96 wells and treated it like a sample in the downstream laboratory processing. The first PCR was carried out in a total volume of 25 µl containing polymerase AmpliTaq Gold 360° (1.25 U/µl), 0.5 µM each of each primer, 1x Buffer I (Thermo Fisher Scientific), BSA (0.1 mg/µl), dNTP (0.2 mM), $MgCl_2$ (1 mM), SigmaFree water, and 2 µl of DNA template was added per reaction. The PCRs were performed in a thermal cycler (Biometra T1 Thermocycler; Analytik Jena GMBH, Ge). The cycler performed the following PCR protocol: initial denaturation at 95°C for 10 min, cycling started with denaturation at 95°C for 15 s, and annealing at 62°C for 30 s, followed by extension at 72°C for 30 s for 25 cycles. After this, the cycler performed 16 cycles where the annealing temperature was reduced by one degree in each cycle, performing the last cycle at a temperature of 45°. The final extension was performed at 72°C for 5 min before the plates were cooled down to 10°C.

The reactions containing the fwhF2/EPTDr2n primer pair were performed according to a touchdown protocol as follows: initial denaturation at 95°C for 10 min, the denaturation temperature of each cycle at 95°C for 15 s, and the annealing step of the first cycle was 60°C for 30 s, and subsequently reduced by 1°C for 9 more cycles. Then, 30 cycles were performed with an annealing step at 50°C. The extension for each cycle was at 72°C for 30 s. The final extension was performed at 72°C for 5 min before the plates were cooled down to 10°C.

The fragment length and amplicon quality of the two amplicons were checked on a Screening Cartridge (AM320) using the QiAxcel electronic gel (Qiagen). First-step PCR products were cleaned with a size-selective bead clean-up to remove primer dimers. For this, the 4× PCR replicates for each sample were pooled to a total volume of 100 µl. We then cleaned the samples with magnetic SPRI beads in a ratio to 0.8× 100 µl following the manufacturer's protocol and eluted the cleaned product in 75 µl. As a second PCR step, the amplicons were indexed using the Illumina Nextera XT Index Kits A and D following the manufacturer's protocol (Illumina, Inc.). One reaction consisted of 25 µl 2x KAPA HIFI HotStart ReadyMix (Kapa Biosystems, Inc., USA), 5 µl of each Nextera XT Index adaptor, and 15 µl of the clean amplicon as a template. The second PCR performed for the attachment of the Illumina adaptors was the same for both libraries (miCOIintF/jgHCO2198 primer pair and fwhF2/EPTDr2n primer pair) and followed the same PCR protocol: initial enzymatic activation at 95°C for 10 min, 8× thermal cycling with a denaturation at 95°C for 30 s; annealing at 55°C for 30 s; and extension 72°C for 30 s. After the last cycle, a final extension was performed at 72°C for 5 min, and the reactions were cooled to 10°C and stored at 4°C for downstream application. Second PCR products were cleaned using the Thermo MG Magjet bead clean-up kit and a customized program for the KingFisher Flex Purification System (Thermo Fisher Scientific Inc.). The clean product was then eluted in 50 µl SigmaWater in a fresh PCR plate and stored at −20°C.

2.4 | DNA quantification and normalization

Both libraries (mICOIintF/jgHCO2198 and fwhF2/EPTDr2n primer pairs) were processed individually as followed: The concentration of each PCR product was quantified in replicates using the Qubit BR DNA Assay Kit (Life Technologies). The DNA concentration of samples and a standard dilution series were measured using a Spark Multimode Microplate Reader (Tecan, US Inc.). Subsequently, the samples were normalized by concentration into equimolar pools according to the calculated concentration using the BRAND Liquid Handling Station (BRAND GMBH + CO KG). Negative controls were pooled equally to samples. The final library was cleaned twice using SPRI beads (0.8× for mICOIintF/jgHCO2198 and 0.75× for fwhF2/EPTDr2n). The concentration of the library was quantified by the Qubit HS Assay Kit, and the amplicon size was confirmed on the Agilent 4200 TapeStation (Agilent Technologies, Inc.). The Nextera XT library prep Kit (Illumina, Inc.) was used to prepare the library for loading onto a flow cell with a target concentration of 10% PhiX. The mICOIintF/jgHCO2198 primer pair library was pair-end sequenced using v3 chemistry with a target concentration of 16 pM, and the fwhF2/EPTDr2n library was pair-end sequenced using v2 chemistry with a target concentration of 14 pM, both on an Illumina MiSeq (Illumina, Inc.) at the Genetic Diversity Center (ETH, Zurich).

2.5 | Bioinformatic sequence analysis and quality filtering

The raw sequencing data for both primer pairs were processed bioinformatically according to this workflow: A Multi FastQC file informed about the data quality for the demultiplexed reads (Andrews, 2012). Please note that the maximum number of reads expected differs for the two primer pairs due to differences in the sequencing kit recommended for the two barcode lengths considered, respectively. First, the raw reads were end-trimmed and merged, and full-length primer sites were removed using usearch (v11.0.667_i86linux64) (Edgar, 2010). Then, reads were quality-filtered using prinseq-lite (v0.20.4).

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The UNOISE3 (usearch v10.0.240) (Edgar, 2016) method with an additional clustering at 99% identity was applied to obtain error-corrected and chimera-filtered sequence variants (zero-distance OTUs) (Edgar et al., 2011). The sequences were checked for stop codons using the invertebrate mitochondrial code; retained were OTUS with open reading frames. These OTUs were then mapped against a customized COI reference (see Brantschen et al., 2021) to assign them taxonomically build from several sources (BOLD, MIDORI and NCBI). The output files of the bioinformatic workflow (Material S7) used in the downstream analysis were an OTU table, a phylogenetic tree, and a reference sequence file. The data set generated with the mlCOIintF/jgHCO2198 primer pair had been already partially analyzed in a previous study (Brantschen et al., 2021). However, the focus of that previous study was on a taxonomy-free approach and aimed at calculation of biotic indices at the level of macroinvertebrate families in general.

2.6 | Comparison of biodiversity across the three approaches

The data were imported into R using the phyloseq package (v1.28.0) (McMurdie & Holmes, 2013). The data were quality-filtered: We used the negative and positive controls to define a sample- and OTU-specific threshold by calculating a ratio between the number of reads of an OTU in the controls and the total number of reads of this OTU. Subsequently, we applied this ratio for every OTU to each sample and removed reads that were below this detection threshold as possible contamination. A first overview of the number of reads and OTUs was done using the phyloseq package. We assessed the differences in detections on a site level across the phylogeny of Ephemeroptera, Plecoptera, and Trichoptera. For this, we extracted a phylogenetic tree for all EPT species from the NCBI taxonomy database using the phylip-tree format and the webtool of NCBI (Figure S2). The tree is scale-free (branches do not reflect evolutionary time). The branching relies on the NCBI taxonomy on the current knowledge of phylogenetic placements of species based on literature. We then tested for significance in the species detection between the detection matrices with Kruskal–Wallis tests with correction for multiple comparisons in vegan (Oksanen et al., 2020).

Furthermore, we looked at the influence of the number of individual counts and site detections on the detectability with the different approaches. Therefore, we fitted a linear model for the specimen counts over the site detections and tested the relationship of each of the two with the detectability for each of the three approaches in vegan.

We decomposed diversity into alpha, beta, and gamma components, which we compared among the approaches. For alpha diversity, we tested for the effect of the sampling method on species richness with a one-way ANOVA following a Tukey HSD test to identify significant differences. We tested the relationship between the number of individuals per site and the number of sites at which it was detected with a linear regression model. For beta diversity, we calculated pairwise dissimilarities between sites within each method based on presence-absence data of EPT species using the betapart (Baselga et al, 2021) package. We then tested the relationship between pairwise dissimilarity and Euclidian or elevational distance using Mantel's test with the vegan package. All analyses were performed in R (v3.6.0) (R Core Team, 2021).

3 | RESULTS

3.1 | Number and taxonomic assignment of OTUs for the mlCOIintF/jgHCO2198 and fwhF2/EPTDr2n primer pairs

Comparing the raw sequencing data recovered with the two primer pairs resulted in 26.4 M raw reads for the mlCOIintF/jgHCO2198 primer pair and 14.76 M raw reads for the fwhF2/EPTDr2n primer pair, respectively. The more degenerate primer pair, that is, the mlCOIintF/jgHCO2198 primer pair, resulted in a higher number of OTUs (n = 7231) than the fwhF2/EPTDr2n primer pair (n = 2648) (Figure 2a,b). However, more OTUs were assigned to a lower taxonomic rank (i.e., to species level) with the fwhF2/EPTDr2n primer pair (see also Table S1). The OTUs assigned to the targeted class of insects made up for 67% of the number of reads with the fwhF2/EPTDr2n primers compared with only 6.2% with the mlCOIintF/jgHCO2198 primer pair. In comparison, the kick-net monitoring identified fewer than 175 taxa based on mixed taxonomic levels where only EPTs were assigned to species and other groups were summarized on a family or higher taxonomic level (Figure 2c). The orders of EPTs make up the highest proportion of taxa in the kick-net data given they are assigned to species level. When looking at the molecular approaches, the fwhF2/EPTDr2n primer pair recovered a higher proportion of reads assigned to EPTs species (Table S2). The mlCOIintF/jgHCO2198 primer pair not only detected more species belonging to other commonly surveyed macroinvertebrate groups, such as Bivalvia, Gastropoda, and Amphipoda (Table S1), but also showed a high proportion of OTUs that yielded in no taxonomic assignment.

3.2 | Site occupancy of EPT species based on kick-net, the mlCOIintF/jgHCO2198 and the fwhF2/EPTDr2n primer pairs

The abundance of EPT species, sorted according to their taxonomy, was visualized for each site and each of the three approaches (Figure 3). Overall, the patterns established by the two molecular approaches showed partial consistency in the detected species, especially for species with high occupancy. Overall, both eDNA approaches detected fewer distinct species than the kick-net method; however, many of the latter being rare in general. The most common species was the Ephemeroptera species *Baetis rhodani*. It was detected at 93% of the sites with the kick-net approach and at 96%

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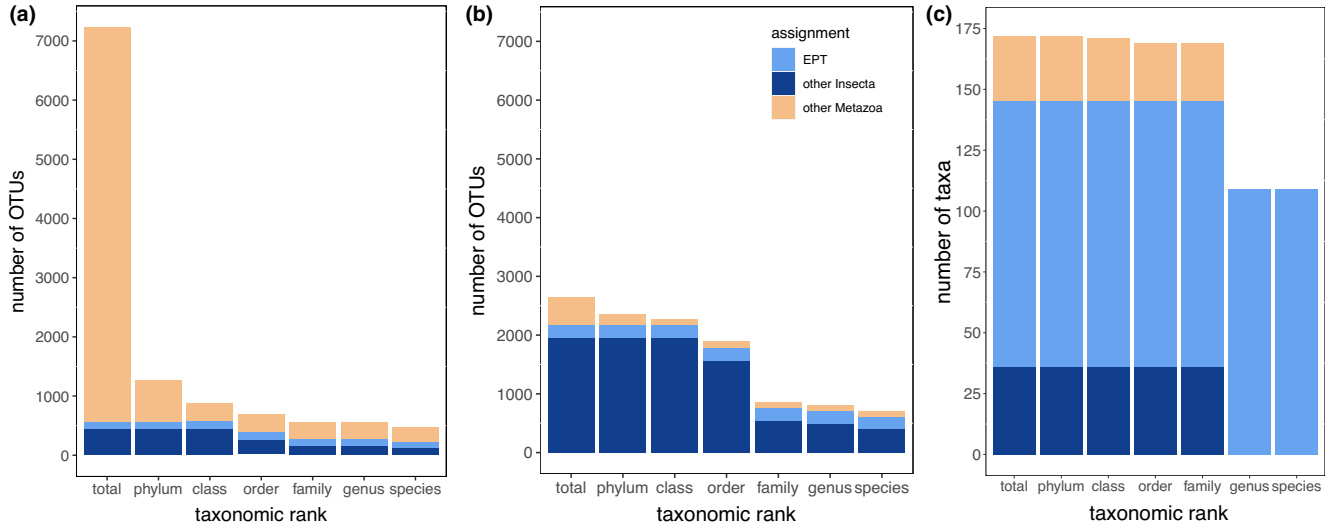

FIGURE 2 Total number of OTUs and assigned to a given taxonomic rank (from phylum to species) using (a) the degenerate mlCOIintF/jgHCO2198 primer pair (b) the fwhF2/EPTDr2n primer pair and (c) the total number of taxa from the kick-net approach morphologically assigned to a given rank (please note the different axis-extend in this panel compared with the two previous panels). Overall, the mlCOIintF/jgHCO2198 primer pair detected more OTUs ($n = 7231$) than the fwhF2/EPTDr2n primers ($n = 2648$). However, the fwhF2/EPTDr2n primer pair yielded a higher proportion of assigned OTUs at lower-level taxonomic assignment than the mlCOIintF/jgHCO2198 primer pair. The kick-net monitoring overall fewer macroinvertebrate taxa ($n = 172$) than either of the two molecular approaches. The color of the bar segments shows the proportion of OTUs assigned to selected target groups used in aquatic biomonitoring (EPT: Ephemeroptera, Trichoptera, Plecoptera; Other Insecta: Class of Insecta without EPTs; Other Metazoa: all Metazoans without Insecta).

of the sites with the eDNA approaches (Figure 3), thus on the one hand common species show very similar site occupancy patterns across the methods. On the other hand, there were some distinct differences in the detectability of species within the three orders. Generally, the molecular approaches detected fewer species belonging to Trichoptera (35 and 33, fwhF2/EPTDr2n and mlCOIintF/jgHCO2198 primers respectively) as compared to the kick-net method (42 species). Although detecting a similar number of species, the two primer pairs detected a different subset of species, for example, the mlCOIintF/jgHCO2198 primer pair recovered several species from the *Hydropsyche* and *Lype* genera, whereas the fwhF2/EPTDr2n did not detect these genera at all (Figure 3, Table S3). The two eDNA approaches did not just cover different subsets of species in one order but also detected different numbers of species within an order. When focusing on Plecoptera only, the fwhF2/EPTDr2n primer pair detected more than double the number of species compared to the mlCOIintF/jgHCO2198 (75 and 35, respectively), and also more than the kick-net approach.

3.3 | Alpha diversity of EPT species compared between kick-net and eDNA approaches

Both eDNA-based approaches detected a higher number of EPT species at the site level (mlCOIintF/jgHCO2198 vs kick-net, $F = 1.7$ $p < 0.08$; fwhF2/EPTDr2n vs kick-net $F = 6$, $p < 0.05$. 1, Figure 4) compared with the number detected with kick-net only. Most of the species were detected by only one method at a site level (mean ± SD for kick-net = 7.4 ±4 taxa, mean for mlCOIintF/jgHCO2198 = 10.3 ±6.2, mean for fwhF2/EPTDr2n = 13.7 ±6.1). The combination of the mlCOIintF/jgHCO2198 and the fwhF2/EPTDr2n primer pairs shared slightly more overlap in detected species than any of the two primer pairs shared with the kick-net. Only a small number of species was consistently detected by all three approaches at a site level.

3.4 | Beta diversity as pairwise dissimilarity between sites based on Jaccard's index

Over the spatial scale of the biogeographic regions, we captured the change in community composition by looking at pairwise comparisons of EPT across all sites. Overall, the beta diversity values did not significantly differ between the two eDNA approaches and the kick-net approach (Figure S3). As expected, the dissimilarity was related to increasing spatial distance between sampled communities. For each method individually, the dissimilarities between communities were plotted relative to the pairwise Euclidean distance between sampling sites (Figure 5). A significant decay of community similarity was detected by a Mantel test for the kick-net method and the fwhF2/EPTDr2n primer pair (Figure 5a,b, respectively). The mlCOIintF/jgHCO2198 primer pair did not capture the increase in dissimilarity in communities that were geographically further apart (i.e., with an increasing Euclidean distance) (Figure 5c). Mantel tests confirmed that distance significantly explained the dissimilarity in EPT communities for the kick-net and the fwhF2/EPTDr2n primer pair ($r = 0.21$ and $r = 0.24$, respectively, $p < 0.001$). For the mlCOIintF/jgHCO2198 primer pair, the

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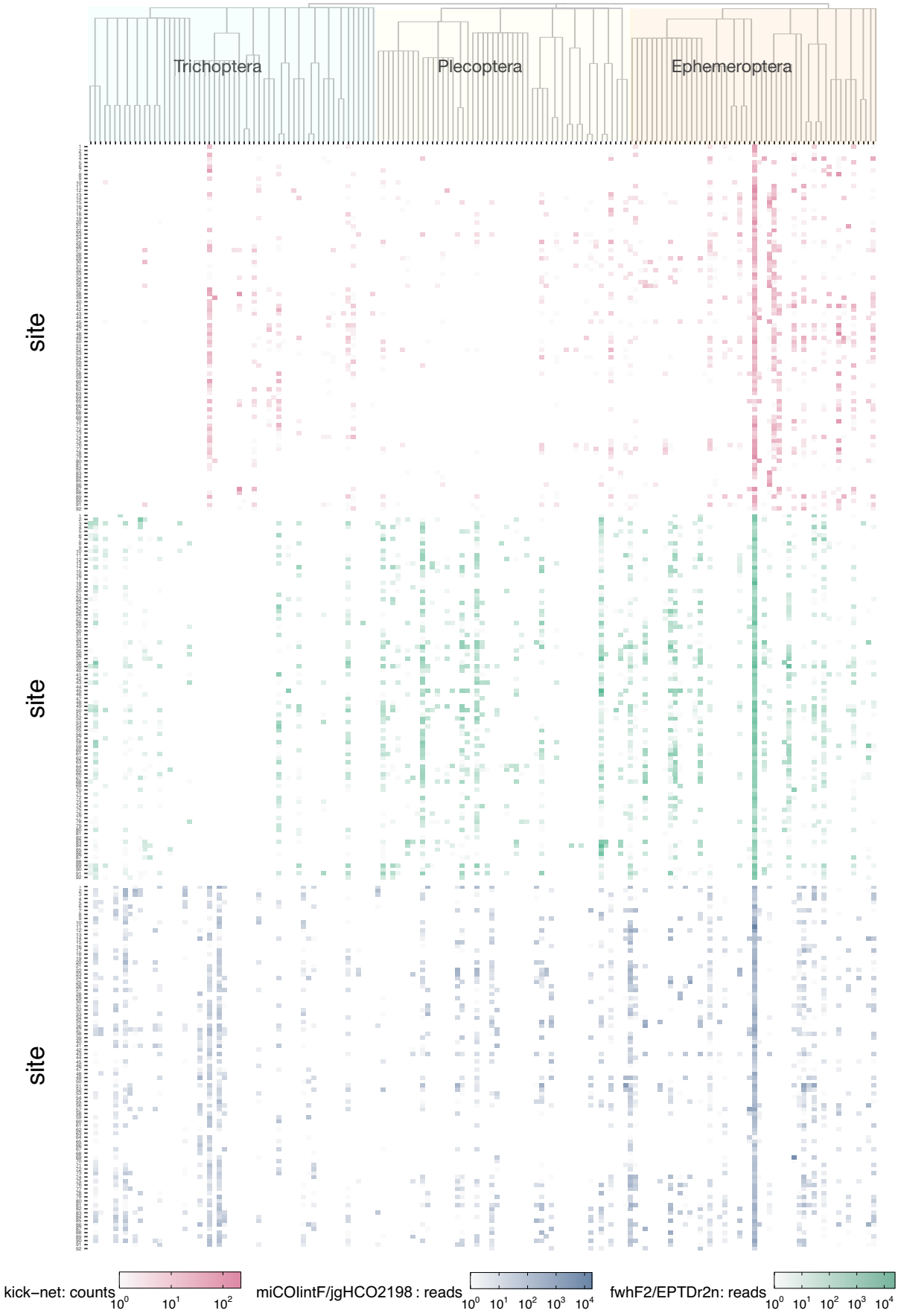

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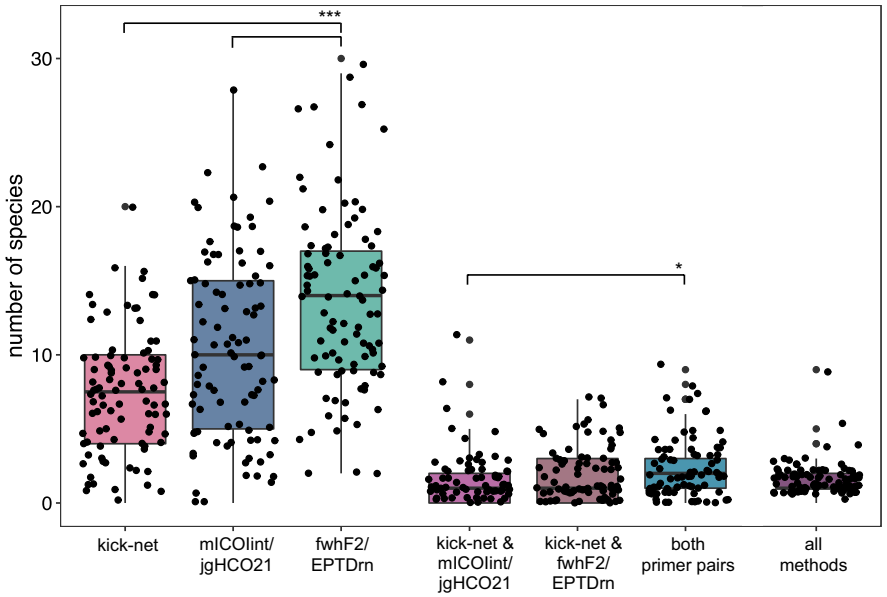
FIGURE 3 Heatmap (site-abundance matrix) of the EPT species detected by the three methods. The species are sorted phylogenetically and cladogram dividing Trichoptera, Plecoptera, and Ephemeroptera and is indicated on top. For each species (column), the abundance of one EPT species (rows) is given as counts for kick-net or read numbers for the eDNA approaches. The three approaches are indicated by the three panels displayed in different colors (red: kick-net, blue: mlCOIintF/jgHCO2198, and green: fwhF2/EPTDr2n).

FIGURE 4 Number of EPT species detected per site, separately given for species found by only one, only two, or by all three approaches. Most species were detected by only one sampling method. At the site level, both eDNA approaches detected significantly more species than the kick-net approach. The fwhF2/EPTDr2n primer pair detected the most species, and the eDNA approaches detected the highest number of species detected with two approaches.



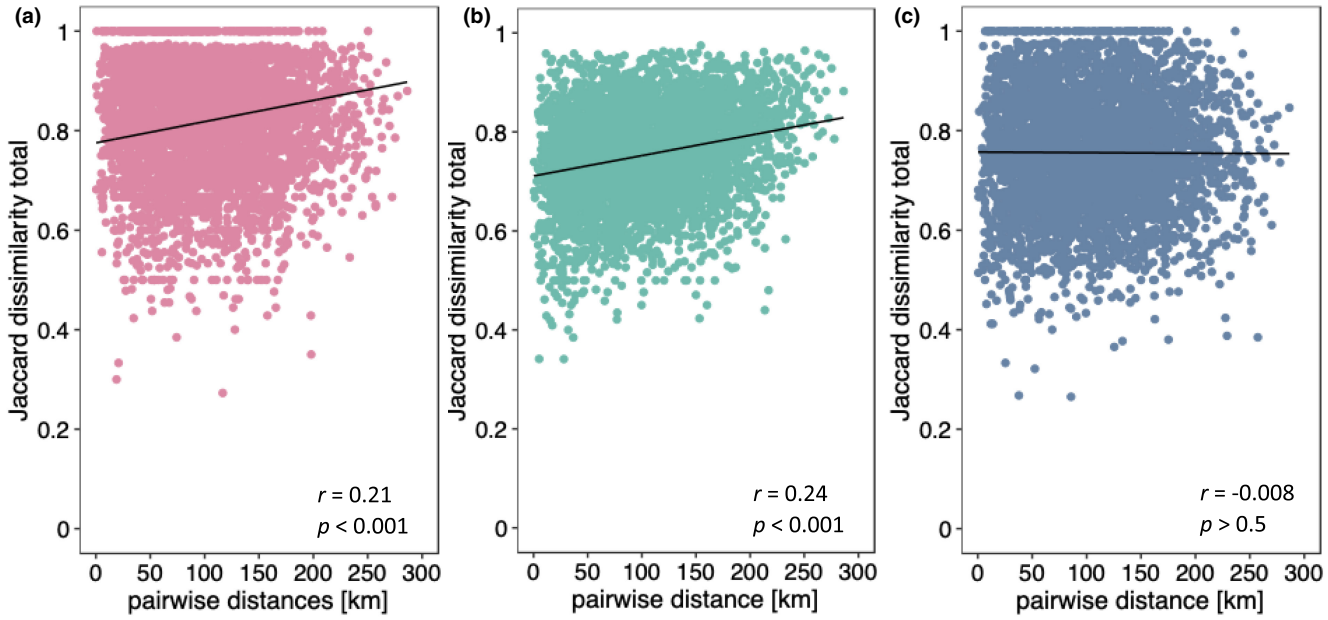


FIGURE 5 Pairwise ß-diversity based on Jaccard's dissimilarity over pairwise distances between sites. A dissimilarity of 0 indicates two identical EPT communities; a dissimilarity of 1 is a complete turnover of community composition. The three panels indicate the turnover component of ß-diversity for EPT taxa for three different approaches: (a) kick-net, (b) fwhF2/EPTDr2n, and (c) mlCOIintF/jgHCO2198. A Mantel test indicates a significant correlation between the change in the community and the pairwise distance between sites for kick-net (Mantel's $r = 0.21$) and the fwhF2/EPTDr2n primer pair (Mantel's $r = 0.24$), but not for the mlCOIintF/jgHCO2198 primer pair (Mantel's $r = -0.008$).

Mantel test showed that distance did not explain the dissimilarity between communities but that the detected EPT species communities were equally dissimilar disregarding their geographical distance ($r = 0.008$, $p > 0.5$). These findings were congruent between total beta diversity and the turnover component, which indicates that beta diversity is mainly driven by a replacement of species, whereas nestedness had no significant effect for any of the methods (Figure S4). Community dissimilarity was also linked to altitude, showing increasing turnover with an increasing elevational gradient (Figure S5).

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3.5 | Overall comparison of species detection as gamma diversity

A comparison of the approaches at the gamma diversity level showed that kick-net sampling detected the highest number of distinct EPT species (higher than any of the two approaches based on eDNA sampling; Figure 6a). A total of 49 EPT species were detected only by kick-net, whereas both molecular approaches together detected 56 species that were not recovered by the kick-net approach. The number of species detected only by the fwhF2/EPTDr2n primer pair was higher than for the mICOIintF/jgHCO2198 primer pair, as was its overlap with the kick-net method. The individual orders of Ephemeroptera, Plecoptera, and Trichoptera showed different detectability, the highest overlap was shown for Ephemeroptera, the lowest for Trichoptera (Figure S6).

Species that were detected at only one or few sites with the kick-net very mainly picked up only by the kick-net method, or the kick-net and one of the other approaches (Figure 6b). The number of individuals detected per species correlated significantly with how widespread a species was over all the sampling sites ($F = 16.4$, $p < 0.01$) as indicated by the linear regression. Species that were detected at more sites were most likely to be picked up by all three approaches or by kick-net and the mICOIintF/jgHCO2198 primer pair (indicated by the shift in the ellipses). Contrastingly, species that were detected at a few sites only were more likely to be detected by only the kick-net method or the kick-net and the fwhF2/EPTDr2n primer pair ($F = 24.6$, $p < 0.01$). The more individuals of a species were found in a kick-net, the higher the chance that the species was detected with multiple approaches. Species for which the kick-net detected only one individual per site were not detected by eDNA-based approaches at all.

4 | DISCUSSION

Novel eDNA methods for the assessment of bioindicators, such as the detection of EPT species in applied ecological monitoring, should be validated in multiple environmental and biogeographic conditions. Here, we used a large-scale monitoring of EPT species across 92 river sites to assess the performance of eDNA-based approaches with two primer pairs (one more degenerate; Geller et al., 2013; Leray et al., 2013) and a recent one more specific to aquatic invertebrates (Leese et al., 2021; Vamos et al., 2017), and compared them with kick-net data from a routine biomonitoring. We combine data from three sources (the kick-net and the two metabarcoding approaches) to evaluate the strengths but also limitations of each approach for the assessment of EPT communities. The EPTDr2n primer, combined with the forward primer fwhF2 (Vamos et al., 2017), has recently been designed for and validated in aquatic insect communities in a relatively narrow biogeographical region in a German river catchment (Leese et al., 2021). Here, the validation of the primer was expanded to meta-communities in a large-scale ecological assessment across all major Swiss catchments, spanning lowland to alpine rivers (range: 198–1650 m a.s.l.) and showed a significant effect of the sampling method (kick-net vs eDNA) but also between the two different eDNA approaches (mICOIintF/jgHCO2198 vs fwhF2/EPTDr2n primer pairs).

The characterization of communities based on eDNA has highlighted that the choice of primers is essential for a successful implementation of molecular approaches (Hajibabaei et al., 2016, 2019; Leese et al., 2021), yet is notoriously challenging with macroinvertebrates (Fernández et al., 2019; Gleason et al., 2021; Mächler et al., 2019). Testing different primer pairs varying in their specificity toward certain macroinvertebrate groups helps to refine eDNA approaches in terms of taxonomic resolution and coverage of target indicator species. Further down the line, these refinements of eDNA approaches help to increase robustness of the application of molecular methods in routine biomonitoring (Pawlowski et al., 2020) and the comparison with established methods. The fwhF2/EPTDr2n primer pair showed a higher overlap of detected EPT species with the kick-net method, detected more species on a site level (alpha diversity), and therefore characterized the communities more comprehensively. This is particularly supported by the finding of a decay in community similarity (given as pairwise Jaccard's dissimilarity over the Euclidean distance between sites) across the large-scale assessment, which was highly congruent with the pattern observed with the kick-net data. It highlights that eDNA monitoring (based on the fwhF2/EPTDr2n primer pair) captures essential turnover in aquatic communities at relevant scales that generally match biogeographic patterns despite the difference in local richness estimates. Our results suggest that the choice of eDNA primers is essential to get robust detections of indicator groups, therefore helping to connect eDNA-based monitoring to previously established methods (Blackman et al., 2019; Leese et al., 2021). Interestingly, the overlap of EPT species between kick-net and the fwhF2/EPTDr2n primer pair was higher than between the two molecular approaches, but kick-net remains the method that detected the most unique species, and each approach recovers a unique set of species that has not been captured by any of the other two methods. Hence, DNA-based and specimen-based samplings capture different aspects of aquatic diversity, introducing an inherent challenge for a one-to-one comparison (Altermatt et al., 2020; Bush et al., 2019; Keck et al., 2022; Seymour et al., 2020).

The mismatches between methods are of particular interest because they can show current limitations of the eDNA approaches such as missing records and thus opportunities to further improve the methods. Generally, species that were rare (few sites and few individuals) in the kick-net observations were also the ones most-overlooked by the eDNA approaches. The non-detection in the eDNA sampling is thus relevant for organisms at low abundance, possibly they leave fewer genetic traces in the water, and we may reach the detection limits of both metabarcoding approaches for the sampled systems. Consequently, we find a higher stochasticity in the species detection. The detection limit depends not just on the amplification bias of the DNA template but also on the sampling effort, that is, the sequencing depth for a given sample, and thus, gathering

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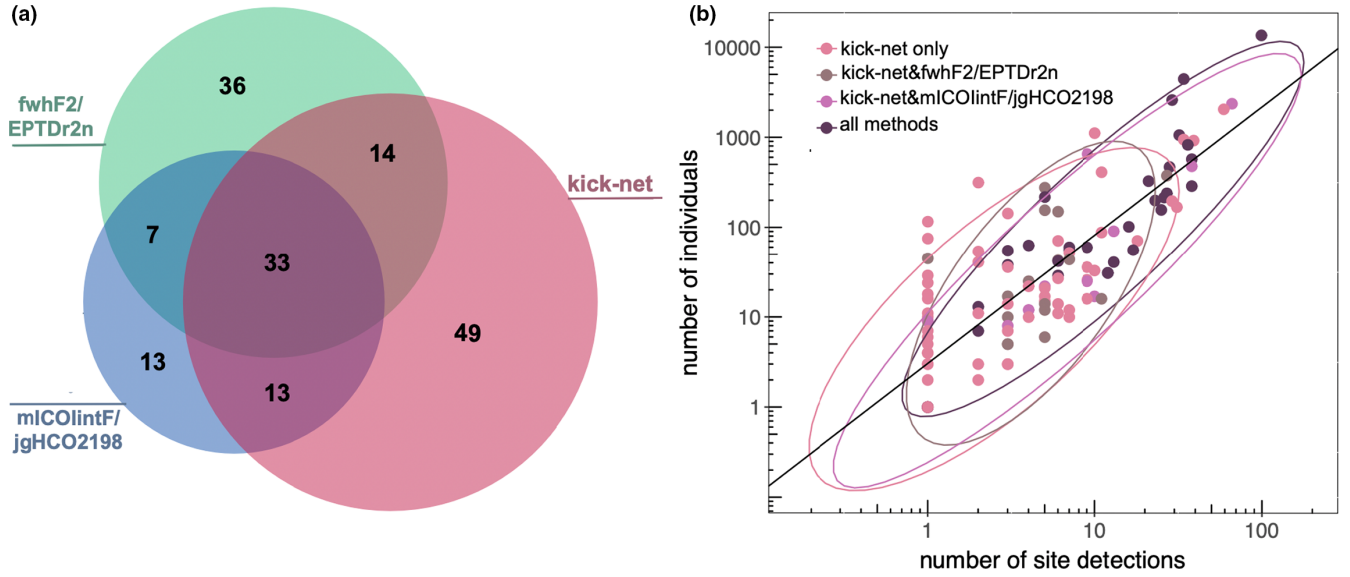

FIGURE 6 Overlap of Ephemeroptera, Trichoptera, and Plecoptera species detected by kick-net, and environmental DNA samples using either the mICOIintF/jgHCO2198 or the fwhF2/EPTDr2n primer pairs. (a) Venn diagram showing the proportion of species detected by each method, the numbers indicate the numbers of species detected by only one, two, or all three approaches. (b) Rank-abundance for all the EPT species detected by the kick-net or kick-net and at least one eDNA approach. For each species detected by kick-net, we compared the total number of individuals with the number of sites at which this species was detected. The number of individuals detected per species was significantly associated with how widespread a species was over all the sampling sites as indicated by the linear regression. Species that were detected in higher numbers were also present at more sites. The ellipses indicate the 95% intervals of rank abundances detected by the different approaches.

more reads could improve the consistency of the detection for low-concentration targets. Here, we have a higher sequencing depth for the primer pair mICOIintF/jgHCO2198 than for the fwhF2/EPTDr2n pair, due do different amplicon lengths and subsequent differences in the sequencing kit. Importantly, however, we have higher richness estimates and a larger overlap with the kick-net approach with the primer pair that generated fewer reads. Thus, our conclusion on the superiority of this primer pair is conservative, and it may even be better with a higher sequencing coverage/depth. Furthermore, the species most abundant in kick-net samples were also recovered by the two metabarcoding approaches, both detecting indicators across the biogeographic and elevational gradient. However, the fwhF2/EPTDr2n primer pair had a higher detection probability toward EPT species that were rare in the kick-net, showing a higher affinity of the primer pair toward the templates of insects as suggested by the in-silico evaluation (see Leese et al., 2021). This is also reflected in the higher local richness estimates when using fwhF2/EPTDr2n primer pair compared with the mICOIintF/jgHCO2198 and kick-net approach, as eDNA integrates genetic traces of organisms upstream (Deiner & Altermatt, 2014; Carraro et al., 2020; Deiner et al., 2017).

Although benthic invertebrates physically share a habitat and might therefore be commonly picked up together in a kick-net sample, they show a high variability in the targeted barcoding region, therefore imposing constraints on a comprehensive metabarcoding approach for indicator species (Elbrecht & Leese, 2017; Hajibabaei et al., 2019). Here, we show that both primer pairs applied in the monitoring of EPT species detect relatively fewer species in the order of Trichoptera, although Trichopterans are commonly found with the kick-net method. This can be explained by a high degeneracy in the primer-binding sites of the COI barcode region, as suggested by an in-silico approach (see Leese et al., 2021), impairing the amplification efficiency of certain species.

The mismatch of community composition inferred from kick-net and eDNA-based approaches is driven by multiple factors, of which DNA transport with water flow is only on possible explanation. The organisms' biology heavily determines the traceability of DNA. First, different organisms have various DNA shedding rates (Allan et al., 2021; Carraro et al., 2020), meaning the amount of DNA in the water does not necessarily scale with their abundance. Larvae of aquatic insects for example can either have an exoskeleton which is sclerotized to different levels, or some, such as Trichopterans, build a housing from abiotic material and thus leave less DNA in their environment. Second, habitat preferences not only impact the detection in kick-net samples but also heavily affect the suspension of DNA in the water column at the riverbank. Taxa that burry themselves or crawl into interstitial places which is characteristic for some Plecoptera might be harder to detect with molecular surveys than when physically disturbing these habitats. Third, the efficiency of detection can depend on the life cycle stage of an organisms, as for example, the order of Ephemeroptera show strong temporal differences in their phenology. All those biological factors impact the production of aquatic eDNA, the concentration of DNA in the water column, and thus lastly the detection of the taxa in eDNA samples.

The discrepancy between the two metabarcoding approaches acknowledges the effect of methodological choices on the

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characterization of communities and on the comparability to established monitoring methods (Blackman et al., 2019). Refined methodological choices and the increasing understanding of the mismatches between molecular and established approaches (Keck et al., 2022) can position eDNA as a monitoring method across large scales and multiple organismal groups.

AUTHOR CONTRIBUTIONS

J.B. and F.A. conceived the study. J.B. performed the laboratory work and J-C.W. did the bioinformatics. J.B. and F.A. did the analysis, generated the figures, and wrote the manuscript. All authors contributed to interpreting the data and commented on the manuscript.

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CONFLICT OF INTEREST

The authors of this study have no conflicts of interest to disclose.

DATA AVAILABILITY STATEMENT

All raw sequencing data produced for this study are archived online and are publicly available on DRYAD under the link: https://doi.org/10.5061/dryad.jsxksn0cq. Further resources or information are available upon request to the corresponding authors.

ORCID

Jeanine Brantschen https://orcid.org/0000-0002-2945-3607
Loïc Pellissier https://orcid.org/0000-0002-2289-8259
Jean-Claude Walser https://orcid.org/0000-0003-1513-0783
Florian Altermatt https://orcid.org/0000-0002-4831-6958

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