Received: 14 November 2020 | Revised: 21 February 2021 | Accepted: 23 February 2021

DOI: 10.1111/ddi.13284

BIODIVERSITY RESEARCH

Diversity and Distributions WILEY

Environmental DNA of preservative ethanol performed better than water samples in detecting macroinvertebrate diversity using metabarcoding

Yu Wang[1] | Kai Chen[1] | Jin Gao[1] | Meng Wang[1] | Jie Dong[1] | Yuwei Xie[2] | John P. Giesy[2,3,4] | Xiaowei Jin[5] | Beixin Wang[1]

[1]Department of Entomology, Nanjing Agricultural University, Nanjing, China

[2]Toxicology Centre, University of Saskatchewan, Saskatoon, Saskatchewan, Canada

[3]Department of Veterinary Biomedical Sciences, University of Saskatchewan, Saskatoon, Saskatchewan, Canada

[4]Department of Environmental Sciences, Baylor University, Waco, Texas, USA

[5]China National Environmental Monitoring Centre, Beijing, China

Correspondence
Xiaowei Jin, China National Environmental Monitoring Centre, Beijing 100012, China.
Email: jinxw@cnemc.cn

Beixin Wang, Department of Entomology, Nanjing Agricultural University, Nanjing 210095, China.
Email: wangbeixin@njau.edu.cn

Funding information
National Natural Science Foundation of China, Grant/Award Number: 41771052 and 41977364

Editor: Jun Yang

Abstract

Aim: High-throughput pipelines supported by eDNA metabarcoding have been applied in various freshwater ecosystems. Both eDNA in ethanol (EtOH) samples (ES-eDNA) and in water samples (WS-eDNA) can provide comprehensive classification lists with good taxonomic resolution and coverage for determining freshwater biodiversity and biomonitoring. But, the advantages of ES-eDNA metabarcoding over WS-eDNA metabarcoding remain unclear for routine assessments of diversity of benthic macroinvertebrates in streams.

Location: Qiantang River Basin, China.

Methods: Here, we compared ES-eDNA and WS-eDNA metabarcoding to evaluate the performance of two eDNA workflows in determining biodiversity and recovery of damaged macroinvertebrate communities. All eDNA samples from the environment and bulk specimen of macroinvertebrates were processed into available molecular operational taxonomic units (MOTUs) and identified to the level of genus.

Results: WS-eDNA detected more exact sequence variants (ESVs) (formerly referred to as operational taxonomic units; OTUs), than did ES-eDNA (2,866 vs. 2,406), but fewer macroinvertebrate ESVs (381 vs. 481). Among sampling sites, the two eDNA workflows exhibited relatively large dissimilarity on inferred community composition ($p < .001$). Furthermore, ES-eDNA metabarcoding exhibited more consistent with morphological identification approaches than did WS-eDNA metabarcoding (24.24% vs. 17.63%, $p = .002$), especially for species identified by traditional morphology (morphotaxa).

Main conclusions: Based on the attributes of ES-eDNA and WS-eDNA, it is suggested that ES-eDNA metabarcoding performs better than does WS-eDNA metabarcoding in detecting local biodiversity and was consistent with morphological results, while WS-eDNA was more suitable for exploring biodiversity patterns on a broad scale, as it is the easiest and most convenient way to collect samples. Results of this study suggest ES-eDNA metabarcoding could be an option in building molecular

measurement biomonitoring programme based on EtOH sample used for preserving biological samples.

KEYWORDS
benthic invertebrates, COI, eDNA metabarcoding, freshwater biomonitoring, preservative ethanol, stream

1 | INTRODUCTION

Robust species data from field experiments and investigations are essential for testing ecological theory, estimating the true biodiversity and assessing biological quality of ecosystems (Hooper et al., 2005). Macroinvertebrates are widely used to monitor water quality, assess river health and reflect the impact of human disturbances (Li et al., 2010; Resh & Unzicker, 1975). Accurate macroinvertebrate species data are essential to detect taxon-specific responses to stressors and precisely assign water quality classes as a function of space and time, which is important to water environmental management. However, because they are the most diverse group in freshwater ecosystems and significant proportion of individuals collected are immatures with vague morphological features for identification, it is difficult to accurately classify each macroinvertebrate to the level of species, based on morphological approaches, which could lead to omission of species and underestimating diversity (Jackson et al., 2014; Sweeney et al., 2011). In addition, identification is not only time-consuming, but also expensive, and results of a survey might not be available for months or in some cases even years (Jones, 2008). This means that the information was not timely and thus less useful for managers to conduct quick response to emergency environmental disasters.

Metabarcoding is able to rapidly and efficiently provide lists of species from bulk or environmental samples and could become a robust method for programmes that rely on the identification of species in communities to provide data on status and trends as a function of disturbances (Bush et al., 2019). Metabarcoding can assign sequences to ESVs, providing consistent and good taxonomic resolution, relative to classifications based on morphology (Porter & Hajibabaei, 2018). It allows exploring patterns of diversity at broad spatial scales, detecting responses of specific taxa to stressors and developing reliable metrics for routine environmental monitoring (Beermann et al., 2018; Cilleros et al., 2019; Elbrecht et al., 2017).

Recently, environmental DNA (eDNA, directly extracted from environmental samples) and tissue DNA (extracted from bulk samples) have been widely utilized for metabarcoding of macroinvertebrates (Hajibabaei et al., 2019; Macher et al., 2018). eDNA, which is intracellular and extracellular trace DNA of organisms released into surrounding environments, including air, soil and water, offers a non-destructive scheme that can be replicated to describe diversity of ecosystems (Cilleros et al., 2019; Frøslev et al., 2019; Kraaijeveld et al., 2015). For example, by the application of metabarcoding, eDNA collected from water (WS-eDNA) can be used to detect presence–absence of invasive or endangered species (Bylemans et al., 2016; Keskin et al., 2016; Sigsgaard et al., 2015), assess biodiversity (Stat et al., 2019), track temporal dynamics of populations and biodiversity (Bista et al., 2017) and assess ecological conditions (Yang & Zhang, 2020). However, WS-eDNA in running waters has been reported to be a poor tool to quantify local diversity of macroinvertebrate in comparison with the DNA extracted from bulk samples due to eDNA diffusion and transportation by high water flow and rapid degradation (Hajibabaei et al., 2019).

Preserving specimens in ethanol (EtOH) can assemble abundant DNA shed from stored individuals and have been shown to be an adequate source of eDNA (Erdozain et al., 2019; Hajibabaei et al., 2012). Compared to tissue DNA extracted from bulk samples, eDNA extracted with ethanol (ES-eDNA) allowed non-destructive sampling to effectively obtain even small DNA fragments for evaluating species present and monitoring biodiversity (Shokralla et al., 2010; Zizka et al., 2019). Most ecological studies and some biomonitoring programmes prefer to preserve unsorted macroinvertebrates along with detritus such as organic debris, including branches, leaves and gravel, with large volumes of EtOH in the field and then sort them in the laboratory for more accurate data and reducing duration of sampling time (Hering et al., 2018). ES-eDNA from unsorted sample provides an alternative source of eDNA for monitoring macroinvertebrate diversity (Martins et al., 2019; Zizka et al., 2019) and is comparable to morphological identification based on visual sorted individuals. This unique method to collect DNA also makes preservative EtOH different from water or soil and is not limited to a single ecological environment (Linard et al., 2016) and would be a promising alternative method and provides a diversified parallel detection approach for freshwater biodiversity and biomonitoring. However, comprehensive validation of ES-eDNA is still rarely reported.

In this study, to validate the performance of ES-eDNA, applicability of ES-eDNA metabarcoding for routine biomonitoring programme and ecological research was assessed by comparing the results to those obtained by the use of WS-eDNA metabarcoding, as well as morphological data. Specifically, the completeness of identification of species was compared. Specific objectives were to examine (a) does the ES-eDNA method performed better than WS-eDNA in detecting macroinvertebrate diversity and recovery of morphologically identified taxa and (b) provide an overview on attributes of ES-eDNA, WS-eDNA and tissue-DNA metabarcoding for understanding their advantages and limitations for describing aquatic biodiversity and for routine use in biomonitoring programmes based on the results of this study and previous studies.

2 | METHODS

2.1 | Collection and processing of samples

The study was conducted in the Qiantang River Basin, China. Samples of water and benthic invertebrates were collected at 14 s-order through third-order streams during March 2018 (Figure S1), and EtOH samples were processed from unsorted bulk samples in laboratory (Figure 1).

2.1.1 | Water samples and WS-eDNA

Before collecting macroinvertebrates, 500 ml water was collected from the left, middle, and right portions of the longest transect within a 100 m stream reach. Samples were collected in sterile plastic bottles and then combined into a 1.5 L composite. To capture WS-eDNA, the composite was filtered in the field, through glass microfiber filters (Whatman, 0.7 μm pore size, GF/F), applying reusable filtration units connected to a hand-held vacuum pump. Filters containing WS-eDNA were transferred to a sterile centrifuge tube containing 99% ethanol. The reusable units were immersed in 200 ppm sodium hypochlorite for 30 min at least before next filtration, and then, units were rinsed with fresh water of sampling stream to flush away the remaining sodium hypochlorite and minimize contamination (Nakagawa et al., 2018).

2.1.2 | Macroinvertebrate samples

In the same reach as where eDNA samples were collected, following a routine biomonitoring programme (Barbour et al., 1999), a total of 20 D-fame nets (0.5 mm mesh size, sampling area 0.09 m^2 for each net) were collected to pool a composite macroinvertebrate sample for each site. The 20 nets were assigned to multiple habitats proportionally to frequencies of occurrence of various substrata including

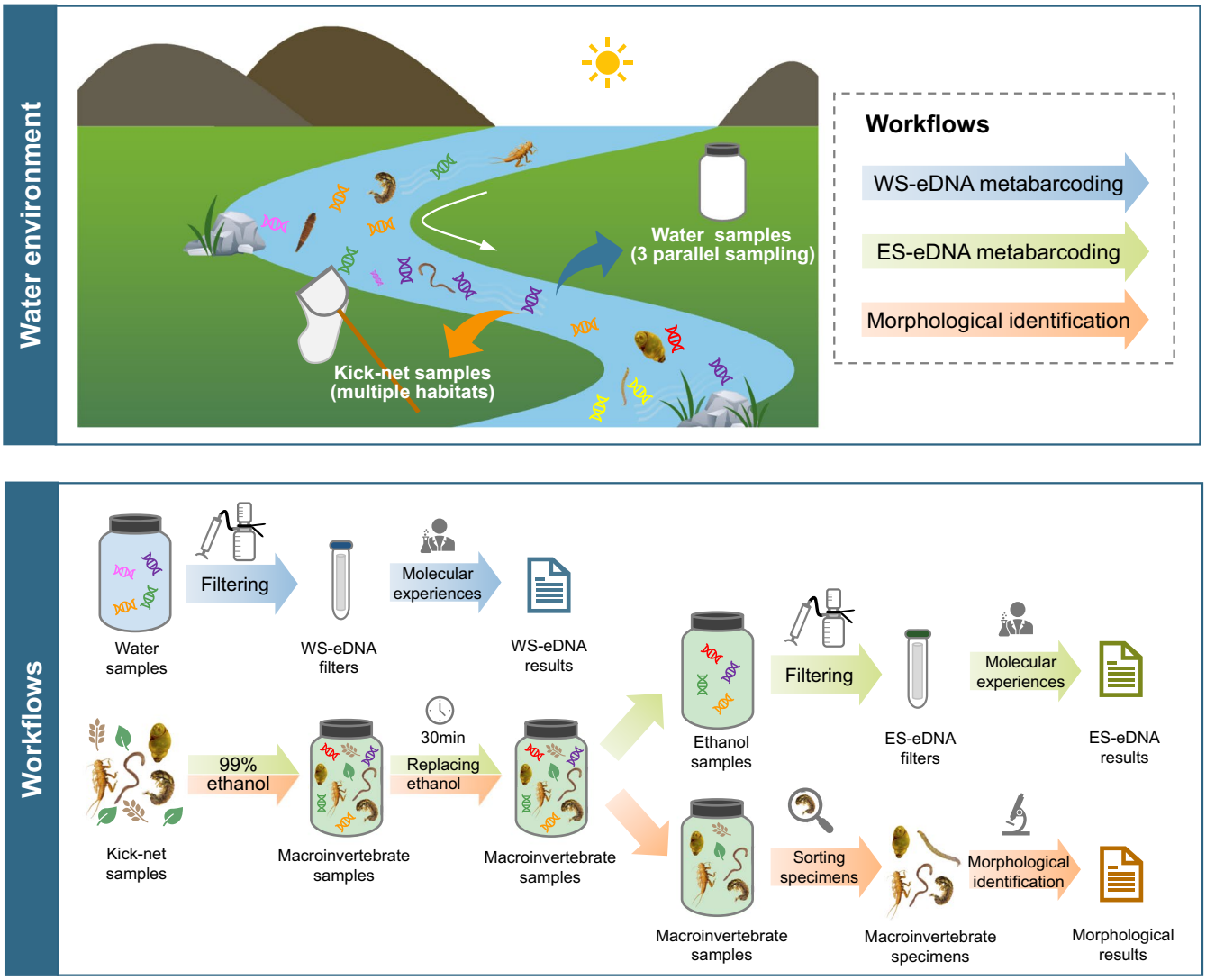

FIGURE 1 Workflows for sample collection of macroinvertebrates, ES-eDNA and WS-eDNA, and morphological identification. Modified from Hering et al. (2018)

silts, sands, pebbles, cobbles and leaf litter packs in the reach, different depths and different current velocities, including runs, riffles and pools as well as patches of aquatic plants. Composite samples were rinsed to remove larger leaves, detritus and stones, and then, remnants were placed into 500 ml of 99% molecular grade EtOH. Half an hour later, the initial solution was replaced with the same amount of new EtOH to preserve macroinvertebrates. Preservative macroinvertebrate samples and WS-eDNA filters were stored inside a portable refrigerator at 4℃ before being transferred to a −20℃ freezer in the laboratory.

2.1.3 | EtOH samples and ES-eDNA

Prior to processing ES-eDNA, containers and instruments were sterilized. We firstly collected EtOH samples by the use of 0.5 mm mesh size, 5 × 5 cm screen on a clean workbench, to remove small plant debris and sand. Then, EtOH samples were filtered through GF/F filters to capture ES-eDNA. An electric vacuum pump was used to accelerate filtration. These filters were kept in 99% EtOH and preserved at a −20°C freezer. eDNA extraction was conducted within one month after filtration.

2.1.4 | Negative controls

Negative controls were separately set for ES-eDNA and WS-eDNA metabarcoding, which were 500 ml EtOH and 1.5 L ultrapure water. The two negative controls were filtered and preserved with process same as the WS-eDNA and ES-eDNA.

2.1.5 | Morphological identification

Individual macroinvertebrates were visually sorted from raw materials and under 10 times microscope soon after collection of EtOH samples and identified to genus, based on morphological characteristics, except for Turbellaria, Oligochaeta, the dipteran family Canacidae and Pteroptera family Taeniopterygidae, which were enumerated without further classification (Morse et al., 1994). Those taxa without confirmation of specific genera were not included in the later analysis.

2.2 | eDNA extraction, PCR amplification and high-throughput sequencing

Each filter was placed on a sterile filter paper for 15 min to volatilize residual EtOH and then cut into 3 × 3 mm square pieces using sterile scissors. DNA was extracted from the square pieces by the use of Ezup Column Animal Genomic DNA Purification Kits (Sangon Biotech). Due to the large volume of filter fragments, extraction was modified so that a larger amount of Buffer ACL and Proteinase K was used and duration of digestion was 6 hr, and 10 μl RNase A (DNase and Protease free; Sangon Biotech) was added prior to purification of DNA. DNA was quantified by the use of Qubit™ dsDNA HS (High Sensitivity) Assay Kits (Thermo Fisher Scientific) on Qubit 3.0 Fluorometer (Life Technologies/Thermo Fisher Scientific) and adjusted to 5 ng/μl with the exception of negative controls.

A pair of universal primers (mlCOIintF: GGWACWGGWTGAACWGTWTAYCCYCC; jgHCO219: TANACYTCNGGRTGNCCRAARAAYCA, I in the original primer was replaced by N) was chosen to amplify a 313 bp fragment of the cytochrome *c* oxidase I (COI) region (Geller et al., 2013; Leray et al., 2013). To distinguish among samples, eight nucleotides were added as tags to the 5'-end of all forward and reverse primers. Three PCR replicates were conducted for each sample of DNA in 50 μl reactions, including 44 μl mix (Tsingke, GoldenStar T6 Super PCR Mix), 2 μl of each primer (10 μM) and 2 μl of template DNA. Amplicons were done by the use of the following temperature programme: initial denaturation at 94°C for 5 min, 16 cycles of 95°C for 10 s, 62°C for 30 s (−1°C per cycle) and 72°C for 60 s, 25 cycles of 95°C for 10 s, 46°C for 30 s and 72°C for 30 s and extension at 65°C for 5 min. Agarose gel with a concentration of 2% was used to examine each PCR amplicon. Three PCR replicates were combined and fully oscillated, and then, concentrations were measured by the use of a Qubit Fluorometer. Mixed PCR products were purified by the use of a SanPrep Column PCR Product Purification Kit (Sangon Biotech, Shanghai, China) and then pooled with 300 ng DNA. Finally, two amplicon pool libraries were individually built with NEBNext® Ultra™ II DNA Library Prep Kit for Illumina® (New England Biolabs®) and sequenced on a HiSeq 2500 platform (2 × 250 bp reads) at Novogene (Beijing, China).

2.3 | Bioinformatics and statistical analysis

Raw sequencing data were processed by the use of the UPARSE pipeline in the USEARCH (Edgar, 2013). First, USEARCH v11.0.667 was applied to join paired-end data with the following settings: -fastq_mergepairs with -fastq_pctid 90, -fastq_maxdiffs 99 and -fastq_trunctail 0. Second, all merged sequences were demultiplexed, based on the tags, stripped of primers from their ends and trimmed to 308–318 bp reads with CUTADAPT version 1.13 (Martin, 2011). Third, high-quality sequences were retained and distinguished based on sample names (-fastq_filter with -fastq_maxee 1.0 and -sample ES/WS_SAMPLE$_i$). After filtering, sequences in two eDNA treatments were combined, dereplicated and singletons discarded, using fastx_uniques with minuniquesize = 2. Finally, cluster_otus was performed to remove chimeras and generate ESVs at similarity of 97% (Edgar, 2013), and a MOTU table was made by the use of the otutab command.

ESVs with sequence abundances greater than 0.005% in each sample (Elbrecht & Leese, 2015; Macher et al., 2018) were retained. ESVs observed in negative controls were deleted. The number of WS-eDNA samples in the following analysis was 12 because no valid

reads were produced in samples of S09 or S11. Taxonomic assignments to available ESVs were performed in a local downloaded COI database (NCBI, 12-06-2019) with sequence lengths ranging from 300 bp to 2,000 bp, by the use of the blastn program in SEED 2 (Seed2.1_64bit, Větrovský et al., 2018). The best hit with a sequence identity of ≥80%, coverage of ≥50% and an E-value threshold of $<10^{-6}$ was retained to achieve representative species of Metazoan. The NCBI-assigned ESVs of Annelida, Arthropoda (Insecta and Crustacea), Mollusca and Platyhelminthes were further identified through the online database (Barcode of Life Data System, BOLD). To facilitate the analysis, orders of some taxa in Mollusca were supplemented according to Bouchet and Rocroi (2005) and Bouchet et al. (2017). Non-target ESVs with blasting parameters of similarity <85%, such as terrestrial animals, plankton, coarse or inaccurate taxonomic resolution according to taxonomy annotation, were discarded.

The Mann–Whitney *U* tests were performed to examine the differences among richness, and PERMANOVA (permutational analysis of variance) with the Bray–Curtis dissimilarity was used to compare composition dissimilarity of macroinvertebrate between ES-eDNA and WS-eDNA metabarcoding in R (v.3.5.3, R Development Core Team, 2019) package vegan (Oksanen et al., 2019). These analyses were applied for all comparisons of ESVs, after deleting low abundance ESVs, Metazoa, macroinvertebrates and typical metrics between two eDNA treatments, which included sensitive indicators of EPT (Ephemeroptera, Trichoptera and Plecoptera), pollution indicators of Oligochaeta that were unable to be stored long-term in EtOH and Chironomidae, a highly diversified insect family and very difficult to morphologically identify each specimen to genus (Buss et al., 2015; Resh & Unzicker, 1975).

For comparison with morphological datasets, only ESVs from NCBI or BOLD databases with similarity greater than 97% and detailed genus information were retained. When conflicting assignments occurred, annotation results of ESVs were returned to the same taxonomic level in both databases. Proportions of genera shared by morphology and eDNA treatments in each site were calculated. Then, a rank abundance curve was drawn to define the rarity in morphological dataset (Siqueira et al., 2012). The inflection point of the rank abundance curve was used to separate common and rare taxa. Detectability of rare (R) and common (C) morphotaxa was evaluated for WS-eDNA and ES-eDNA metabarcoding. Heat maps were drawn with HemI (Heatmap Illustrator, v.1.0, Deng et al., 2014), and a boxplot was generated with R to show proportions of abundances of C and R taxa among all samples.

3 | RESULTS

3.1 | High-throughput sequencing statistics

A total of 2,962,734 (ES-eDNA) and 2,719,130 (WS-eDNA) read pairs were obtained from ES-eDNA and WS-eDNA metabarcoding, respectively. All raw data have been uploaded to the Sequence Read Archive (SRA, accession number SRR12599027 and SRR12599028). After filtering and clustering, 67.6% of total sequences in ES-eDNA samples and 29.7% in WS-eDNA were retained for subsequent analyses. In terms of the negative control of PCR, no obvious amplification bands on agarose gels and few data sequences were returned. Only 17 ESVs were detected in negative controls, including 2 ESVs (2 reads) found in ES-eDNA samples and 15 ESVs (18 reads) found in WS-eDNA. Unexpected sequences with abundances less than 0.005% were deleted, which eliminated a total of 3,997 available taxonomic units, including 2,406 ESVs of EtOH samples and 2,866 ESVs of water samples (Table S1). Metazoan ESVs of EtOH sample were 1866 similar to 1846 of water samples.

3.2 | Richness and inferred macroinvertebrate community composition of eDNA metabarcoding

There assigned to macroinvertebrate ESVs were 481 (19.99%) for ES-eDNA and 381 (13.29%) for WS-eDNA and the proportions of sequences were 42.74% and 29.98%, respectively (Figure S2 and Table S2). Only Annelida richness detected in ES-eDNA was significantly greater than that in WS-eDNA (p = .004, Table 1). However, inferred compositions of macroinvertebrate between

TABLE 1 Sum and mean ESVs (standard deviation) of different taxonomic categories generated by ES-eDNA and WS-eDNA metabarcoding

	ES-eDNA		WS-eDNA		Species richness	Community composition
Category	Sum	Mean (*SD*)	Sum	Mean (*SD*)	*p*-Value	*p*-Value (R^2)
Annelida	63	15.21 (6.15)	29	8.83 (3.90)	**.004**	**<.001 (.22)**
Arthropoda	403	85.14 (25.35)	340	76.50 (34.05)	.347	**<.001 (.12)**
Mollusca	14	7.57 (2.79)	12	6.58 (2.84)	.118	**<.001 (.11)**
Platyhelminthes	1	0.14 (0.36)	/	/	/	/
Macroinvertebrates	481	95.79 (23.79)	381	80.50 (36.25)	.090	**<.001 (.14)**

Note: The difference in MOTU richness between two methods was tested by the Man–Whitney *U* test, while dissimilarity in community composition was tested by PERMANOVAs, except Platyhelminthes because of too much absence in data matrix. Significant differences between two approaches were marked with bold.

ES-eDNA and WS-eDNA metabarcoding were remarkably different (PERMANOVA, $p < .001$), as well as the Annelida, Arthropoda and Mollusca (Table 1).

Four of 20 orders observed in ES-eDNA were not detected by WS-eDNA, including Littorinimorpha, Allogastropoda, Isopoda and Arhynchobdellida (Figure 2b). However, DNA of Unionida was not captured by ES-eDNA metabarcoding. The proportion of Diptera was greatest (ES-eDNA: 61.54%, WS-eDNA: 68.50%), followed by Haplotaxida (ES-eDNA: 12.89%, WS-eDNA: 7.61%) and Ephemeroptera (ES-eDNA: 6.03%, WS-eDNA: 6.04%; Figure 2b and Table S3). Chironomidae accounted for the most richness of ESVs in both dipteran families and macroinvertebrate community (ES-eDNA: 155 ESVs, WS-eDNA: 147 ESVs). In particular, over 80% of sequences in water samples were classified as Diptera, while only 42.22% in EtOH samples (Table S3).

Both EPT and Chironomidae exhibited more shared ESVs of 41 (accounting for 55.41%) and 105 (53.30%), respectively, than exclusive ESVs in either EtOH or water samples (Figure 3a,b). The number of exclusive ESVs from EPT and Chironomidae in ES-eDNA was larger than that in WS-eDNA. However, the 24 shared ESVs (35.82%) of Oligochaeta were fewer than the exclusive ESVs in ES-eDNA (38 ESVs), but greater than that in WS-eDNA (5 ESVs, Figure 3c).

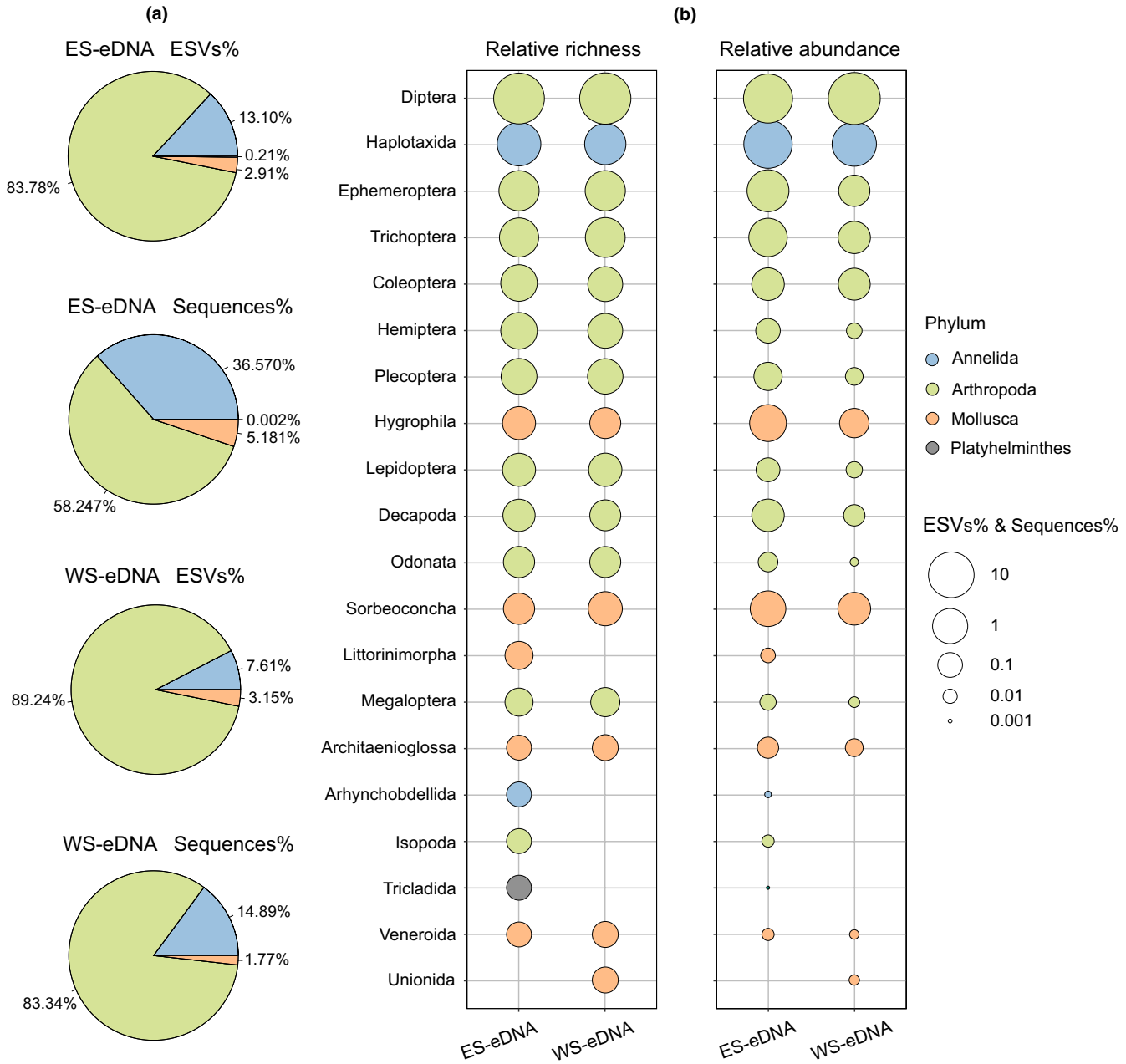

FIGURE 2 Relative ESVs and sequences of macroinvertebrates at taxonomic levels of (a) phylum and (b) order generated by ES-eDNA and WS-eDNA metabarcoding

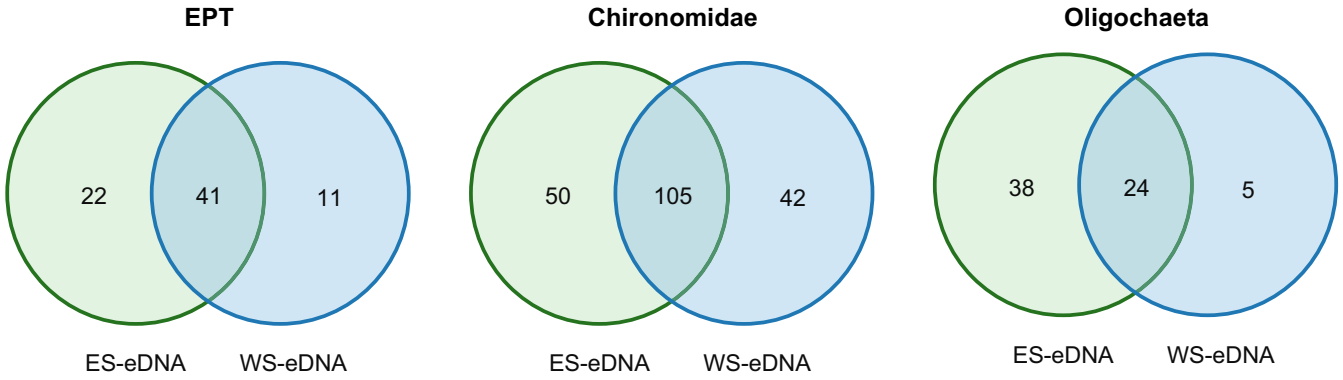

FIGURE 3 Venn diagram showing ESVs shared in EPT, Chironomidae and Oligochaeta between ES-eDNA metabarcoding and WS-eDNA metabarcoding

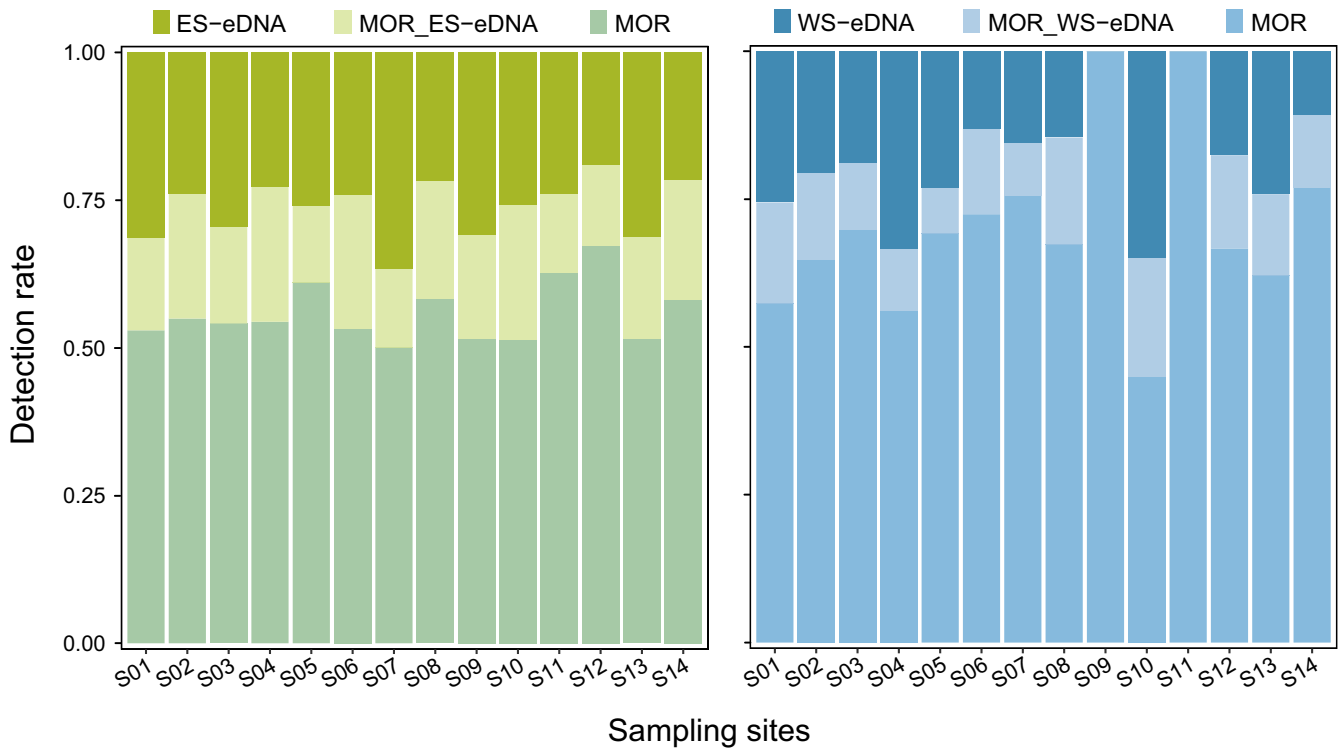

FIGURE 4 Proportions of genera detected by different methods to the total genera observed by all methods at each site. MOR: proportion of morphologically identified genera, MOR_ES-eDNA/MOR_WS-eDNA: proportion of genera detected by both morphology and eDNA metabarcoding, ES-eDNA/WS-eDNA: proportion of genera detected by ES-eDNA or WS-eDNA only

3.3 | Detectability of eDNA metabarcoding on morphologically identified taxa

A total of 155 morphotaxa of macroinvertebrates were identified at 14 sites with a mean taxa richness of 51.57 (*SD* = 7.74, ranging from 36 to 64), which were far less than 202 macroinvertebrate ESVs detected by eDNA metabarcoding (Figure S3), including 170 ESVs in EtOH samples and 140 in water samples.

After assigning ESVs at the level of genus, morphological protocols obtained 129 genera (Table S4), followed by ES-eDNA (82) and WS-eDNA (68). A total of 32 genera identified by morphological identification approaches are detected by both two eDNA metabarcoding. Apart from 32 shared genera, seven genera morphologically identified were detected by ES-eDNA merely and two genera only detected by WS-eDNA (Table S5).

ES-eDNA metabarcoding resulted in a greater rate of detection for taxa identified by the use of morphology to genus, with a mean rate of 24.24% than 17.63% of WS-eDNA (Figure 4). However, a total of 50 assigned genera detected by eDNA metabarcoding, which were mainly Chironomidae and Naididae,

TABLE 2 A summary of assigned ESVs detected by eDNA metabarcoding but not available in morphologically identified data

Phylum	Order	Family	ES-eDNA	WS-eDNA	Total
Annelida	Haplotaxida	Naididae	10	5	11
	Coleoptera	Psephenidae	1	0	1
		Ptilodactylidae	0	1	1
Arthropoda	Diptera	Chironomidae	19	18	21
		Culicidae	1	0	1
		Psychodidae	1	0	1
	Ephemeroptera	Baetidae	2	2	2
		Heptageniidae	1	1	1
	Megaloptera	Corydalidae	1	1	1
	Odonata	Gomphidae	2	1	2
	Plecoptera	Styloperlidae	1	0	1
	Trichoptera	Hydrobiosidae	1	0	1
		Hydroptilidae	1	1	1
		Leptoceridae	1	1	1
Mollusca	Basommatophora	Lymnaeidae	0	1	1
	Littorinimorpha	Assimineidae	1	0	1
	Sorbeoconcha	Pachychilidae	0	1	1
	Unionida	Unionidae	0	1	1
Sum			43	34	50

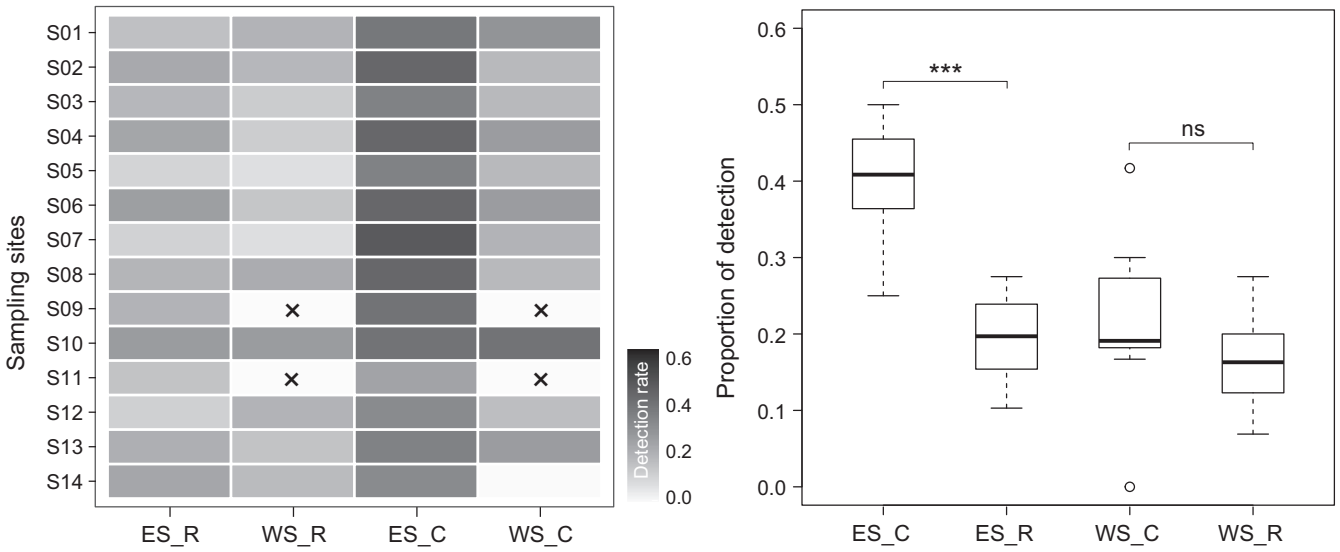

FIGURE 5 Proportions of rare (R) and common (C) morphotaxa detected by ES-eDNA and WS-eDNA metabarcoding. Cross represents no detection. *$p < .05$, **$p < .01$, ***$p < .001$ and ns, $p \geq .05$ by the Mann–Whitney *U* tests

were not included in the lists identified based on morphology (Table 2). In the list to genus level based on morphology, 12 rare genera and 117 common genera were defined based on the inflection point of the rank abundance curve (Figure S4 and Table S4). More than two-thirds (71.43%–90.70%) of morphotaxa were classified as rare taxa across morphological samples (Table S4). Overall, common taxa were detected more frequently than rare taxa by eDNA metabarcoding and the detection rate of common morphotaxa was greater by ES-DNA than by WS-DNA ($p < .001$, Figure 5b).

4 | DISCUSSION

4.1 | Application scenario based on comparison between ES-eDNA and WS-eDNA

eDNA metabarcoding brought convenience and benefits in sample collection and species detection to freshwater biodiversity and biomonitoring (Nakagawa et al., 2018; Stat et al., 2019). In particular, WS-eDNA metabarcoding has been used previously as an auxiliary tool for determining diversity used in developing strategies for the

management of diverse, freshwater habitats (Apothéloz-Perret-Gentil et al., 2017; Shaw et al., 2016; Yang & Zhang, 2020). Recently, ES-eDNA metabarcoding of macroinvertebrates exhibited advantages for non-destructive sampling and a useful alternative to bulk-sample metabarcoding for routine biomonitoring programmes using macroinvertebrates and biodiversity detection (Martins et al., 2019; Zizka et al., 2019).

Samples of water were able to capture signals of cellular and extracellular DNA from various aquatic organisms (Rees et al., 2014) and even terrestrial insects, parasitic animals, etc. However, with the instability and mobility of aqueous aquatic environments (Leff et al., 1992), eDNA released by organisms tended to disperse and transfer rapidly, which decreased the possibility of observing diluted biological signals. Particularly, lesser density of some benthic macroinvertebrates or the lesser amounts of DNA released into surrounding environment water could result in lesser potentials to detect. For example, when abundances of crayfish (*Procambarus clarkii*) were small, due to free DNA in the environment through conventional capture workflow, detection efficiency by the use of WS-eDNA would be relatively poor (Tréguier et al., 2014). When salamanders were introduced into previously unoccupied streams, their DNA could be detected after 6 hr, but only for samples collected close to salamanders (within 5 m) with a relatively high density (Pilliod et al., 2014). Therefore, when individuals were remote from sampling location, false-negative results were observed.

ES-eDNA metabarcoding detected more macroinvertebrate taxa than did WS-eDNA (Table 1). Most benthic fauna prefer to live under the solid substratum and even burrow into soft sediments. Thus, the dual effect of special habitats and large eDNA particles sinking might result in free DNA being more easily detected from sediment than from surface water (Turner et al., 2014, 2015). Collecting surface water samples might miss eDNA signals conveyed from burrowing benthos. This conclusion was also supported by the typical burrowing Oligochaeta taxa, which were of more unique ESVs from ES-eDNA than WS-eDNA samples in our study (Table 1). In contrast, EtOH when used as a preservative could directly capture DNA released from macroinvertebrates living in habitats collected by kick net (Blocksom et al., 2008). The enrichment of target organisms eventually resulted in greater concentrations of ES-eDNA, which might greatly increase probabilities of observation, especially of rater taxa. Alternatively, greater concentrations (e.g. ≥95%) of EtOH denatured proteins that might degrade DNA and protected released DNA from further damage (Nagy, 2010; Stein et al., 2013). Thus, more taxa observed during visual inspections were also detected in concentrated samples of EtOH (Figure 4), which also resulted in a greater rate of detection and proportion of rare and common morphotaxa (Figure 5b).

Compared with WS-eDNA, ES-eDNA was more stable and preserved in EtOH without the influence of multiple environmental factors, such as current, temperature, UV, acidity, alkalinity, biological enzymes and microorganisms. In contrast, WS-eDNA in natural waters might degrade into smaller fragments or even completely disappear (Barnes et al., 2014; Seymour et al., 2018; Strickler et al., 2015). Humic substances and suspended particles could also affect the efficiency of filtration and inhibit the detection of eDNA (Stoeckle et al., 2017). The superposition effect of these adverse conditions might impair efficiencies of eDNA to detect organisms living in water, and even make some water samples unable to be amplified successfully. In addition, the complexity of DNA templates in water samples often brings more unpredictability to PCR amplification and sequencing, including tag jumping and chimera formation. We found that a large number of sequences with poor quality or too long or too short were excluded from WS-eDNA files during quality filtering, which might partly explain why fewer reads were observed in WS-eDNA than ES-eDNA (Table S1).

Results of this study indicated that community composition of macroinvertebrates was significantly dissimilar between ES-eDNA metabarcoding and WS-eDNA metabarcoding (Table 1). Preservation and extraction with EtOH resulted in lists of detected benthic invertebrates that were similar to those based on morphological identification methods (Erdozain et al., 2019; Hajibabaei et al., 2012). However, WS-eDNA was likely biased towards the detection of upstream assemblages (Nakagawa et al., 2018), and inevitably, due to downstream transport of eDNA, result in different assemblages than those derived from traditional investigation tools at local scales (Deiner & Altermatt, 2014), especially in fast-running rivers and streams. Upstream eDNA can affect consistency and accuracy of downstream observations (Pilliod et al., 2014). For example, ESVs of Unionida were not identified from EtOH samples because no mussels were actually collected in the field, but they were captured by WS-eDNA metabarcoding (Figure 2). Also, eDNA from benthic invertebrates in a sampling site might be carried downstream and thus missed. Therefore, water samples covering part of biological data in the upstream and the collection location should be more strongly related to the whole catchments and more reliable in reflecting biodiversity for the whole landscapes (Deiner et al., 2016).

4.2 | Comparison of macroinvertebrates between eDNA metabarcoding and morphological identification protocols

Comparability among methods is of concern for biodiversity and routine biomonitoring programmes. Specifically, can results based on eDNA metabarcoding be compared to those based on conventional morphological approaches (Linard et al., 2016; Shaw et al., 2016)? Generally, molecular methods for rapid identification are expected to generate more taxa in water or EtOH samples by obviating the need to have detailed knowledge to allow for classification specimens including immature or damaged individuals (Bush et al., 2019).

The results of this study yielded larger numbers of ESVs (>3,900) by the use of a combination of ES-eDNA and WS-eDNA metabarcoding, which was attributed, at least in part to the application of degenerate, universal primers, thought to expand the scope for detection of non-target taxa (Horton et al., 2017; Macher et al., 2018). A total of 593 ESVs were assigned to macroinvertebrates, which was

larger than the 155 morphologically identified taxa. Larger number of ESVs indicated that the actual diversity of the macroinvertebrate community might be richer than that determined by routine biomonitoring programme. In particular, environmental samples were advantageous for Chironomidae and Naididae (Table 2), which were difficult to accurately identify to lower taxonomic levels, by the use of current morphological protocols, especially for larval stages (Elbrecht et al., 2017; Jones, 2008).

Ecologists are hesitant to employ eDNA metabarcoding if it cannot associate the molecular units with the binomial taxonomic names (Bush et al., 2019). This is due to the fact that there is a large amount of functional information embodied in natural history information that has been catalogued over time based on traditional identification and classification. Nevertheless, original ESVs list lacks this complete species information, and not all ESVs will obtain accurate and effective taxonomic annotation. Coarse taxonomic resolution is insufficient to reveal specific responses of benthic macroinvertebrates to stressors (Macher et al., 2016); thus, higher taxonomic resolution is indispensable for the assessments of status and trends in assemblages of benthic invertebrates (Jones, 2008), such as life history and functional diversity. In this study, after bioinformatic processing and purposefully screening, assigned ESVs were less than expected. Obviously, at genus level, almost 70% of ESVs were unable to be assigned reliably to traditional named species-based morphological taxonomy, resulting in 88 of 129 morphologically identified genera not reliably detected by two eDNA treatments, especially rare morphotaxa (Figure 5). Therefore, even though the high-throughput molecular scheme provides a promising method to characterize biological communities, it is still unable to match all organic individuals perfectly (Erdozain et al., 2019). This was due to relatively strict quality control (ESVs less than 0.005% read abundances were deleted), a lack of coverage in the COI database (Curry et al., 2018), accuracy of reference sequence (Bridge et al., 2003), primer bias (Deiner et al., 2017; Piñol et al., 2015) and morphological misidentification (Haase et al., 2006; Sweeney et al., 2011). Alternatively, with increasing interest in taxonomy-free methods and more exploration, researchers would find molecular data were getting better and better in assessing ecological status (Yang & Zhang, 2020). Promoting professional and automatic high-throughput genomic workflows are conducive to scientific decision-making associated with biodiversity. However, the supplement and improvement of aquatic biota databases are still a major task to be completed for the use of DNA barcoding or eDNA metabarcoding.

4.3 | Pros and cons of three metabarcoding methods

Here, we summarized the pros and cons of three metabarcoding: WS-eDNA, tissue DNA and ES-eDNA based on the universal applicability, sample processing, persistence and coincidence with morphological data and specimen integrity (Table 3).

Water samples have constantly been used for eDNA surveys in freshwater ecosystems (Fernández et al., 2018; Olds et al., 2016; Walsh et al., 2019), and WS-eDNA has been proposed as a powerful observational tool to improve the monitoring of status in aquatic communities and master the biodiversity at catchment level (Macher et al., 2018). As a repeatable, non-destructive means of sampling, it greatly expands efficiencies of monitoring areas for rare or elusive species, such as some amphibians (Takahara et al., 2020), rare fishes (Brys et al., 2020) and aquatic mammals (Ma et al., 2016). Moreover, differing with in EtOH or bulk samples relying on classical kick-net methods, water samples greatly simplify workflows for field surveys and obviates the need for collections of organisms. But, sampling of water alone might introduce biases due to upstream taxa to the species pool of downstream, especially for energetic streams and rivers that could also result in poor repeatability and consistency with morphological data investigated at specific locations (Carraro et al., 2018; Li et al., 2018). Also, there could be biases due to failure to amplify eDNA for matrix effects in some waters or DNA degradation and even unequal DNA persistence of different taxa (Goldberg et al., 2013; Nevers et al., 2018).

In general, survey results based on bulk sample metabarcoding were similar to those based on traditional non-PCR-based surveys for local biodiversity and, in some cases, were superior to morphological identification of macroinvertebrates (Elbrecht et al., 2017; Rivera et al., 2018). Bulk samples can replace eDNA to become a priority for traditional monitoring and assessment, particularly when water in study areas is quite turbid. However, complicated preprocess of bulk samples requires mixing and homogenizing all biological specimens (Dowle et al., 2016; Elbrecht & Steinke, 2018), or sorting

TABLE 3 Overview of characteristics of three metabarcoding (WS-eDNA, ES-eDNA and tissue DNA) for macroinvertebrates

Characteristics	WS-eDNA	ES-eDNA	Tissue DNA
Universal applicability	Widely	Widely	Widely
Optimal spatial scale	Catchment to river network	Local	Local
Sample processing efforts (field and laboratory)	Cost-effective	Less cost-effective	Medium cost-effective
DNA persistence	Degraded easily	Retained for a relatively long time	Retained for a long time?
Consistency with morphological data	Low	Medium	High
Specimen integrity	Not collected	Non-destructive	Dissected

a piece of tissue or part from each specimen (e.g., leg, abdomen) for DNA extraction (Braukmann et al., 2019). While this method can be employed to construct a library that includes a range of key species from various stages of development, it will involve irreversible destruction of specimens and inevitable sorting of samples, increasing the time–cost invisibly.

The use of samples preserved with EtOH seems to be an appropriate balance between screening of water and bulk samples in improving consistency with morphological data, making effort in sample process and keeping specimen integrity. This eDNA treatment can keep specimens intact without dissecting or homogenizing individuals (Marquina et al., 2019; Zenker et al., 2020), which allows subsequent morphological examination, while preventing DNA from rapid degradation during management of the sample (Moreau et al., 2013; Stein et al., 2013). Moreover, rapid molecular identification schemes based on EtOH samples are not only applicable to newly collected specimens (Barbato et al., 2019; Martins et al., 2019), but also applicable to specimens retained for longer periods of time (Shokralla et al., 2010). Thus, preserved samples of museum historical specimens collected over the past few years can be concentrated on the construction of library. In addition, preservative EtOH is performed by direct contact with objects, regardless of the type of ecosystem. Consequently, it can highly recover the diversity of freshwater macroinvertebrates (Erdozain et al., 2019; Hajibabaei et al., 2012), as well as intestinal microorganisms, mixed terrestrial arthropods and variety of rare biological specimens (Barbato et al., 2019; Marquina et al., 2019).

5 | CONCLUSIONS

In summary, results reported that eDNA metabarcoding detected more species of benthic invertebrates than morphological identification approaches. Of which, ES-eDNA detected more macroinvertebrate ESVs than WS-eDNA and 3 typical taxa (EPT, Chironomidae and Oligochaeta) showed better detectability in ES-eDNA. Due to the difference in eDNA, sources and concentration, inferred community compositions determined by the use of the two methods were significantly dissimilar. Moreover, ES-eDNA metabarcoding recovered more morphotaxa in kick-net samples than did WS-eDNA metabarcoding, especially for common taxa. The use of preservative EtOH from unsorted macroinvertebrate samples was found to be rich sources of eDNA from bulk samples. On balance, the better performance of ES-eDNA over WS-eDNA in local biodiversity detection of macroinvertebrates implied ES-eDNA metabarcoding was a prominent option for establishing DNA-based biomonitoring and biodiversity programme.

ACKNOWLEDGEMENTS

This research was funded by the National Natural Science Foundation of China (NSFC, No. 41771052 and No. 41977364). The authors thank Yanli Hu, Cong Wu, Evance Omondi Mbao, Chaozhong Tan, Huiyu Wei and other colleagues from the Laboratory of Insect Taxonomy and Aquatic Insects, Nanjing Agricultural University, for their help in specimen collection and statistical analysis. Dr. Xie was supported by "Next generation solutions to ensure healthy water resources for future generations" funded by the Global Water Futures Program, Canada First Research Excellence Fund (#419205). Dr. Giesy was supported by the Canada Research Chairs Program of the Natural Sciences and Engineering Research Council of Canada (NSERC) and Distinguished Faculty Chair in the Department of Environmental Sciences at Baylor University, Waco, Texas, USA. We sincerely thank the editor and two anonymous reviewers for their valuable comments, which have greatly improved the manuscript.

PEER REVIEW

The peer review history for this article is available at https://publons.com/publon/10.1111/ddi.13284.

DATA AVAILABILITY STATEMENT

Two sets of sequencing data were uploaded to NCBI SRA, BioProject Number: PRJNA629637.

ORCID

Beixin Wang https://orcid.org/0000-0002-5253-8799

REFERENCES

Apothéloz-Perret-Gentil, L., Cordonier, A., Straub, F., Iseli, J., Esling, P., & Pawlowski, J. (2017). Taxonomy-free molecular diatom index for high-throughput eDNA biomonitoring. *Molecular Ecology Resources*, *17*(6), 1231–1242. https://doi.org/10.1111/1755-0998.12668

Barbato, M., Kovacs, T., Coleman, M. A., Broadhurst, M. K., & de Bruyn, M. (2019). Metabarcoding for stomach-content analyses of Pygmy devil ray (*Mobula kuhlii cf. eregoodootenkee*): Comparing tissue and ethanol preservative-derived DNA. *Ecology and Evolution*, *9*, 2678–2687. https://doi.org/10.1002/ece3.4934

Barbour, M. T., Gerritsen, J., Snyder, B. D., & Stribling, J. B. (1999). *Rapid bioassessment protocols for use in wadeable streams and rivers: periphyton, benthic macroinvertebrates, and fish*, 2nd ed. US Environmental Protection Agency, Office of Water (EPA/841-B-99-002).

Barnes, M. A., Turner, C. R., Jerde, C. L., Renshaw, M. A., Chadderton, W. L., & Lodge, D. M. (2014). Environmental conditions influence eDNA persistence in aquatic systems. *Environmental Science and Technology*, *48*(3), 1819–1827. https://doi.org/10.1021/es404734p

Beermann, A. J., Zizka, V. M. A., Elbrecht, V., Baranov, V., & Leese, F. (2018). DNA metabarcoding reveals the complex and hidden responses of chironomids to multiple stressors. *Environmental Sciences Europe*, *30*(1), 26. https://doi.org/10.1186/s12302-018-0157-x

Bista, I., Carvalho, G. R., Walsh, K., Seymour, M., Hajibabaei, M., Lallias, D., Christmas, M., & Creer, S. (2017). Annual time-series analysis of aqueous eDNA reveals ecologically relevant dynamics of lake ecosystem biodiversity. *Nature Communications*, *8*(1), 14087. https://doi.org/10.1038/ncomms14087

Blocksom, K. A., Autrey, B. C., Passmore, M., & Reynolds, L. (2008). A comparison of single and multiple habitat protocols for collecting macroinvertebrates in Wadeable Streams. *Journal of the American Water Resources Association*, *44*(3), 577–593. https://doi.org/10.1111/j.1752-1688.2008.00183.x

Bouchet, P., & Rocroi, J. P. (2005). *Classification and nomenclator of gastropod families*. ConchBooks.

Bouchet, P., Rocroi, J. P., Hausdorf, B., Kaim, A., Kano, Y., Nützel, A., Parkhaev, P., Schrödl, M., & Strong, E. E. (2017). Revised

classification, nomenclator and typification of Gastropod and Monoplacophoran families. *Malacologia*, *61*(1–2), 1–526. https://doi.org/10.4002/040.061.0201

Braukmann, T. W. A., Ivanova, N. V., Prosser, S. W. J., Elbrecht, V., Steinke, D., Ratnasingham, S., de Waard, J. R., Sones, J. E., Zakharov, E. V., & Hebert, P. D. N. (2019). Metabarcoding a diverse arthropod mock community. *Molecular Ecology Resources*, *19*(3), 711–727. https://doi.org/10.1111/1755-0998.13008

Bridge, P. D., Roberts, P. J., Spooner, B. M., & Panchal, G. (2003). On the unreliability of published DNA sequences. *New Phytologist*, *160*(1), 43–48. https://doi.org/10.1046/j.1469-8137.2003.00861.x

Brys, R., Halfmaerten, D., Neyrinck, S., Mauvisseau, Q., Auwerx, J., Sweet, M., & Mergeay, J. (2020). Reliable eDNA detection and quantification of the European weather loach (*Misgurnus fossilis*). *Journal of Fish Biology*, *98*, 399–414. https://doi.org/10.1111/jfb.14315

Bush, A., Compson, Z. G., Monk, W. A., Porter, T. M., Steeves, R., Emilson, E., Gagne, N., Hajibabaei, M., Roy, M., & Baird, D. J. (2019). Studying ecosystems with DNA metabarcoding: Lessons from biomonitoring of aquatic macroinvertebrates. *Frontiers in Ecology and Evolution*, *7*, 434. https://doi.org/10.3389/fevo.2019.00434

Buss, D. F., Carlisle, D. M., Chon, T.-S., Culp, J., Harding, J. S., Keizer-Vlek, H. E., Robinson, W. A., Strachan, S., Thirion, C., & Hughes, R. M. (2015). Stream biomonitoring using macroinvertebrates around the globe: A comparison of large-scale programs. *Environmental Monitoring and Assessment*, *187*(1), 4132. https://doi.org/10.1007/s10661-014-4132-8

Bylemans, J., Furlan, E. M., Pearce, L., Daly, T., & Gleeson, D. M. (2016). Improving the containment of a freshwater invader using environmental DNA (eDNA) based monitoring. *Biological Invasions*, *18*(10), 3081–3089. https://doi.org/10.1007/s10530-016-1203-5

Carraro, L., Hartikainen, H., Jokela, J., Bertuzzo, E., & Rinaldo, A. (2018). Estimating species distribution and abundance in river networks using environmental DNA. *Proceedings of the National Academy of Sciences of the United States of America*, *115*(46), 11724–11729. https://doi.org/10.1073/pnas.1813843115

Cilleros, K., Valentini, A., Allard, L., Dejean, T., Etienne, R., Grenouillet, G., Iribar, A., Taberlet, P., Vigouroux, R., & Brosse, S. (2019). Unlocking biodiversity and conservation studies in high-diversity environments using environmental DNA (eDNA): A test with Guianese freshwater fishes. *Molecular Ecology Resources*, *19*(1), 27–46. https://doi.org/10.1111/1755-0998.12900

Curry, C. J., Gibson, J. F., Shokralla, S., Hajibabaei, M., & Baird, D. J. (2018). Identifying North American freshwater invertebrates using DNA barcodes: Are existing COI sequence libraries fit for purpose? *Freshwater Science*, *37*(1), 178–189. https://doi.org/10.1086/696613

Deiner, K., & Altermatt, F. (2014). Transport distance of invertebrate environmental DNA in a Natural River. *PLoS One*, *9*(2), e88786. https://doi.org/10.1371/journal.pone.0088786

Deiner, K., Bik, H. M., Mächler, E., Seymour, M., Lacoursière-Roussel, A., Altermatt, F., Creer, S., Bista, I., Lodge, D. M., Vere, N., Pfrender, M. E., & Bernatchez, L. (2017). Environmental DNA metabarcoding: Transforming how we survey animal and plant communities. *Molecular Ecology*, *26*(21), 5872–5895. https://doi.org/10.1111/mec.14350

Deiner, K., Fronhofer, E. A., Mächler, E., Walser, J.-C., & Altermatt, F. (2016). Environmental DNA reveals that rivers are conveyer belts of biodiversity information. *Nature Communications*, *7*(1), 12544. https://doi.org/10.1038/ncomms12544

Deng, W., Wang, Y., Liu, Z., Cheng, H., & Xue, Y. (2014). HemI: A Toolkit for Illustrating Heatmaps. *PLoS One*, *9*(11), e111988. https://doi.org/10.1371/journal.pone.0111988

Dowle, E. J., Pochon, X., C. Banks, J., Shearer, K., & Wood, S. A. (2016). Targeted gene enrichment and high-throughput sequencing for environmental biomonitoring: A case study using freshwater macroinvertebrates. *Molecular Ecology Resources*, *16*(5), 1240–1254. https://doi.org/10.1111/1755-0998.12488

Edgar, R. C. (2013). UPARSE: Highly accurate OTU sequences from microbial amplicon reads. *Nature Methods*, *10*(10), 996–998. https://doi.org/10.1038/nmeth.2604

Elbrecht, V., & Leese, F. (2015). Can DNA-Based Ecosystem Assessments Quantify Species Abundance? Testing Primer Bias and Biomass—Sequence relationships with an innovative metabarcoding protocol. *PLoS One*, *10*(7), e0130324. https://doi.org/10.1371/journal.pone.0130324

Elbrecht, V., & Steinke, D. (2018). Scaling up DNA metabarcoding for freshwater macrozoobenthos monitoring. *Freshwater Biology*, *64*, 380–387. https://doi.org/10.1111/fwb.13220

Elbrecht, V., Vamos, E. E., Meissner, K., Aroviita, J., & Leese, F. (2017). Assessing strengths and weaknesses of DNA metabarcoding-based macroinvertebrate identification for routine stream monitoring. *Methods in Ecology and Evolution*, *8*(10), 1265–1275. https://doi.org/10.1111/2041-210X.12789

Erdozain, M., Thompson, D. G., Porter, T. M., Kidd, K. A., Kreutzweiser, D. P., Sibley, P. K., Swystun, T., Chartrand, D., & Hajibabaei, M. (2019). Metabarcoding of storage ethanol vs. Conventional morphometric identification in relation to the use of stream macroinvertebrates as ecological indicators in forest management. *Ecological Indicators*, *101*, 173–184. https://doi.org/10.1016/j.ecolind.2019.01.014

Fernández, S., Rodríguez, S., Martínez, J. L., Borrell, Y. J., Ardura, A., & García-Vázquez, E. (2018). Evaluating freshwater macroinvertebrates from eDNA metabarcoding: A river Nalón case study. *PLoS One*, *13*(8), e0201741. https://doi.org/10.1371/journal.pone.0201741

Frøslev, T. G., Kjøller, R., Bruun, H. H., Ejrnæs, R., Hansen, A. J., Læssøe, T., & Heilmann-Clausen, J. (2019). Man against machine: Do fungal fruitbodies and eDNA give similar biodiversity assessments across broad environmental gradients? *Biological Conservation*, *233*, 201–212. https://doi.org/10.1016/j.biocon.2019.02.038

Geller, J., Meyer, C., Parker, M., & Hawk, H. (2013). Redesign of PCR primers for mitochondrial cytochrome *c* oxidase subunit I for marine invertebrates and application in all-taxa biotic surveys. *Molecular Ecology Resources*, *13*(5), 851–861. https://doi.org/10.1111/1755-0998.12138

Goldberg, C. S., Sepulveda, A., Ray, A., Baumgardt, J., & Waits, L. P. (2013). Environmental DNA as a new method for early detection of New Zealand mudsnails (*Potamopyrgus antipodarum*). *Freshwater Science*, *32*(3), 792–800. https://doi.org/10.1899/13-046.1

Haase, P., Murray-Bligh, J., Lohse, S., Pauls, S., Sundermann, A., Gunn, R., & Clarke, R. (2006). Assessing the impact of errors in sorting and identifying macroinvertebrate samples. *Hydrobiologia*, *556*, 505–521. https://doi.org/10.1007/978-1-4020-5493-8_34

Hajibabaei, M., Porter, T. M., Robinson, C. V., Baird, D. J., Shokralla, S., & Wright, M. T. G. (2019). Watered-down biodiversity? A comparison of metabarcoding results from DNA extracted from matched water and bulk tissue biomonitoring samples. *PLoS One*, *14*(12), e0225409. https://doi.org/10.1371/journal.pone.0225409

Hajibabaei, M., Spall, J. L., Shokralla, S., & van Konynenburg, S. (2012). Assessing biodiversity of a freshwater benthic macroinvertebrate community through non-destructive environmental barcoding of DNA from preservative ethanol. *BMC Ecology*, *12*(1), 28. https://doi.org/10.1186/1472-6785-12-28

Hering, D., Borja, A., Jones, J. I., Pont, D., Boets, P., Bouchez, A., Bruce, K., Drakare, S., Hänfling, B., Kahlert, M., Leese, F., Meissner, K., Mergen, P., Reyjol, Y., Segurado, P., Vogler, A., & Kelly, M. (2018). Implementation options for DNA-based identification into ecological status assessment under the European Water Framework Directive. *Water Research*, *138*, 192–205. https://doi.org/10.1016/j.watres.2018.03.003

Hooper, D. U., Chapin, F. S., Ewel, J. J., Hector, A., Inchausti, P., Lavorel, S., Lawton, J. H., Lodge, D. M., Loreau, M., Naeem, S., Schmid, B.,

Setälä, H., Symstad, A. J., Vandermeer, J., & Wardle, D. A. (2005). Effects of biodiversity on ecosystem functioning: A consensus of current knowledge. *Ecological Monographs*, *75*(1), 3–35. https://doi.org/10.1890/04-0922

Horton, D. J., Kershner, M. W., & Blackwood, C. B. (2017). Suitability of PCR primers for characterizing invertebrate communities from soil and leaf litter targeting metazoan 18S ribosomal or cytochrome oxidase I (COI) genes. *European Journal of Soil Biology*, *80*, 43–48. https://doi.org/10.1016/j.ejsobi.2017.04.003

Jackson, J. K., Battle, J. M., White, B. P., Pilgrim, E. M., Stein, E. D., Miller, P. E., & Sweeney, B. W. (2014). Cryptic biodiversity in streams: A comparison of macroinvertebrate communities based on morphological and DNA barcode identifications. *Freshwater Science*, *33*(1), 312–324. https://doi.org/10.1086/675225

Jones, F. C. (2008). Taxonomic sufficiency: The influence of taxonomic resolution on freshwater bioassessments using benthic macroinvertebrates. *Environmental Reviews*, *16*, 45–69. https://doi.org/10.1139/A07-010

Keskin, E., Unal, E. M., & Atar, H. H. (2016). Detection of rare and invasive freshwater fish species using eDNA pyrosequencing: Lake Iznik ichthyofauna revised. *Biochemical Systematics and Ecology*, *67*, 29–36. https://doi.org/10.1016/j.bse.2016.05.020

Kraaijeveld, K., de Weger, L. A., Ventayol García, M., Buermans, H., Frank, J., Hiemstra, P. S., & den Dunnen, J. T. (2015). Efficient and sensitive identification and quantification of airborne pollen using next-generation DNA sequencing. *Molecular Ecology Resources*, *15*(1), 8–16. https://doi.org/10.1111/1755-0998.12288

Leff, L. G., Vaun McArthur, J., & Shimkets, L. J. (1992). Information spiraling: Movement of bacteria and their genes in streams. *Microbial Ecology*, *24*(1), 11–24. https://doi.org/10.1007/BF00171967

Leray, M., Yang, J. Y., Meyer, C. P., Mills, S. C., Agudelo, N., Ranwez, V., Boehm, J. T., & Machida, R. J. (2013). A new versatile primer set targeting a short fragment of the mitochondrial COI region for metabarcoding metazoan diversity: Application for characterizing coral reef fish gut contents. *Frontiers in Zoology*, *10*(1), 34. https://doi.org/10.1186/1742-9994-10-34

Li, L., Zheng, B., & Liu, L. (2010). Biomonitoring and bioindicators used for river ecosystems: Definitions, approaches and trends. *Procedia Environmental Sciences*, *2*, 1510–1524. https://doi.org/10.1016/j.proenv.2010.10.164

Li, Y., Evans, N. T., Renshaw, M. A., Jerde, C. L., Olds, B. P., Shogren, A. J., Deiner, K., Lodge, D. M., Lamberti, G. A., & Pfrender, M. E. (2018). Estimating fish alpha- and beta-diversity along a small stream with environmental DNA metabarcoding. *Metabarcoding and Metagenomics*, *2*, e24262. https://doi.org/10.3897/mbmg.2.24262

Linard, B., Arribas, P., Andújar, C., Crampton-Platt, A., & Vogler, A. P. (2016). Lessons from genome skimming of arthropod-preserving ethanol. *Molecular Ecology Resources*, *16*(6), 1365–1377. https://doi.org/10.1111/1755-0998.12539

Ma, H., Stewart, K., Lougheed, S., Zheng, J., Wang, Y., & Zhao, J. (2016). Characterization, optimization, and validation of environmental DNA (eDNA) markers to detect an endangered aquatic mammal. *Conservation Genetics Resources*, *8*(4), 561–568. https://doi.org/10.1007/s12686-016-0597-9

Macher, J. N., Salis, R. K., Blakemore, K. S., Tollrian, R., Matthaei, C. D., & Leese, F. (2016). Multiple-stressor effects on stream invertebrates: DNA barcoding reveals contrasting responses of cryptic mayfly species. *Ecological Indicators*, *61*, 159–169. https://doi.org/10.1016/j.ecolind.2015.08.024

Macher, J. N., Vivancos, A., Piggott, J. J., Centeno, F. C., Matthaei, C. D., & Leese, F. (2018). Comparison of environmental DNA and bulk-sample metabarcoding using highly degenerate cytochrome *c* oxidase I primers. *Molecular Ecology Resources*, *18*(6), 1456–1468. https://doi.org/10.1111/1755-0998.12940

Marquina, D., Esparza-Salas, R., Roslin, T., & Ronquist, F. (2019). Establishing arthropod community composition using metabarcoding: Surprising inconsistencies between soil samples and preservative ethanol and homogenate from Malaise trap catches. *Molecular Ecology Resources*, *19*(6), 1516–1530. https://doi.org/10.1111/1755-0998.13071

Martin, M. (2011). Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet.journal*, *17*, 10–12. https://doi.org/10.14806/ej.17.1.200

Martins, F. M. S., Galhardo, M., Filipe, A. F., Teixeira, A., Pinheiro, P., Paupério, J., Alves, P. C., & Beja, P. (2019). Have the cake and eat it: Optimizing nondestructive DNA metabarcoding of macroinvertebrate samples for freshwater biomonitoring. *Molecular Ecology Resources*, *19*(4), 863–876. https://doi.org/10.1111/1755-0998.13012

Moreau, C. S., Wray, B. D., Czekanski-Moir, J. E., & Rubin, B. E. (2013). DNA preservation: A test of commonly used preservatives for insects. *Invertebrate Systematics*, *27*(1), 81–86. https://doi.org/10.1071/is12067

Morse, J. C., Yang, L. F., & Tian, L. X. (1994). *Aquatic insects of China useful for monitoring water quality*. Hohai University Press.

Nagy, Z. T. (2010). A hands-on overview of tissue preservation methods for molecular genetic analyses. *Organisms Diversity & Evolution*, *10*(1), 91–105. https://doi.org/10.1007/s13127-010-0012-4

Nakagawa, H., Yamamoto, S., Sato, Y., Sado, T., Minamoto, T., & Miya, M. (2018). Comparing local- and regional-scale estimations of the diversity of stream fish using eDNA metabarcoding and conventional observation methods. *Freshwater Biology*, *63*(6), 569–580. https://doi.org/10.1111/fwb.13094

Nevers, M. B., Byappanahalli, M. N., Morris, C. C., Shively, D., Przybyla-Kelly, K., Spoljaric, A. M., Dickey, J., & Roseman, E. F. (2018). Environmental DNA (eDNA): A tool for quantifying the abundant but elusive round goby (*Neogobius melanostomus*). *PLoS One*, *13*(1), e0191720. https://doi.org/10.1371/journal.pone.0191720

Oksanen, J., Blanchet, F. G., Friendly, M., Kindt, R., Legendre, P., McGlinn, D., Minchin, P. R., O'Hara, R. B., Simpson, G. L., Solymos, P., Stevens, M. H. H., Szoecs, E., & Wagner, H. (2019). *vegan: Community Ecology Package*. R package version 2.5-6. https://CRAN.R-project.org/package=vegan

Olds, B. P., Jerde, C. L., Renshaw, M. A., Li, Y., Evans, N. T., Turner, C. R., Deiner, K., Mahon, A. R., Brueseke, M. A., Shirey, P. D., Pfrender, M. E., Lodge, D. M., & Lamberti, G. A. (2016). Estimating species richness using environmental DNA. *Ecology and Evolution*, *6*(12), 4214–4226. https://doi.org/10.1002/ece3.2186

Pilliod, D. S., Goldberg, C. S., Arkle, R. S., & Waits, L. P. (2014). Factors influencing detection of eDNA from a stream-dwelling amphibian. *Molecular Ecology Resources*, *14*(1), 109–116. https://doi.org/10.1111/1755-0998.12159

Piñol, J., Mir, G., Gomez-Polo, P., & Agustí, N. (2015). Universal and blocking primer mismatches limit the use of high-throughput DNA sequencing for the quantitative metabarcoding of arthropods. *Molecular Ecology Resources*, *15*(4), 819–830. https://doi.org/10.1111/1755-0998.12355

Porter, T. M., & Hajibabaei, M. (2018). Scaling up: A guide to high-throughput genomic approaches for biodiversity analysis. *Molecular Ecology*, *27*(2), 313–338. https://doi.org/10.1111/mec.14478

R Core Team (2019). *R: A language and environment for statistical computing*. R Foundation for Statistical Computing.

Rees, H. C., Maddison, B. C., Middleditch, D. J., Patmore, J. R. M., & Gough, K. C. (2014). Review: The detection of aquatic animal species using environmental DNA - a review of eDNA as a survey tool in ecology. *Journal of Applied Ecology*, *51*(5), 1450–1459. https://doi.org/10.1111/1365-2664.12306

Resh, V. H., & Unzicker, J. D. (1975). Water quality monitoring and aquatic organisms: The importance of species identification. *Journal (Water Pollution Control Federation)*, *47*(1), 9–19.

Rivera, S. F., Vasselon, V., Jacquet, S., Bouchez, A., Ariztegui, D., & Rimet, F. (2018). Metabarcoding of lake benthic diatoms: From structure

assemblages to ecological assessment. *Hydrobiologia*, *807*(1), 37–51. https://doi.org/10.1007/s10750-017-3381-2

Seymour, M., Durance, I., Cosby, B. J., Ransom-Jones, E., Deiner, K., Ormerod, S. J., Colbourne, J. K., Wilgar, G., Carvalho, G. R., de Bruyn, M., Edwards, F., Emmett, B. A., Bik, H. M., & Creer, S. (2018). Acidity promotes degradation of multi-species environmental DNA in lotic mesocosms. *Communications Biology*, *1*(1), 4. https://doi.org/10.1038/s42003-017-0005-3

Shaw, J. L. A., Clarke, L. J., Wedderburn, S. D., Barnes, T. C., Weyrich, L. S., & Cooper, A. (2016). Comparison of environmental DNA metabarcoding and conventional fish survey methods in a river system. *Biological Conservation*, *197*, 131–138. https://doi.org/10.1016/j.biocon.2016.03.010

Shokralla, S., Singer, G., & Hajibabaei, M. (2010). Direct PCR amplification and sequencing of specimens' DNA from preservative ethanol. *BioTechniques*, *48*(3), 233–234. https://doi.org/10.2144/000113362

Sigsgaard, E. E., Carl, H., Møller, P. R., & Thomsen, P. F. (2015). Monitoring the near-extinct European weather loach in Denmark based on environmental DNA from water samples. *Biological Conservation*, *183*, 46–52. https://doi.org/10.1016/j.biocon.2014.11.023

Siqueira, T., Bini, L. M., Roque, F. O., Marques Couceiro, S. R., Trivinho-Strixino, S., & Cottenie, K. (2012). Common and rare species respond to similar niche processes in macroinvertebrate metacommunities. *Ecography*, *35*(2), 183–192. https://doi.org/10.1111/j.1600-0587.2011.06875.x

Stat, M., John, J., DiBattista, J. D., Newman, S. J., Bunce, M., & Harvey, E. S. (2019). Combined use of eDNA metabarcoding and video surveillance for the assessment of fish biodiversity. *Conservation Biology*, *33*(1), 196–205. https://doi.org/10.1111/cobi.13183

Stein, E. D., White, B. P., Mazor, R. D., Miller, P. E., & Pilgrim, E. M. (2013). Evaluating ethanol-based sample preservation to facilitate use of DNA barcoding in routine freshwater biomonitoring programs using benthic macroinvertebrates. *PLoS One*, *8*(1), e51273. https://doi.org/10.1371/journal.pone.0051273

Stoeckle, B. C., Beggel, S., Cerwenka, A. F., Motivans, E., Kuehn, R., & Geist, J. (2017). A systematic approach to evaluate the influence of environmental conditions on eDNA detection success in aquatic ecosystems. *PLoS One*, *12*(12), e0189119. https://doi.org/10.1371/journal.pone.0189119

Strickler, K. M., Fremier, A. K., & Goldberg, C. S. (2015). Quantifying effects of UV-B, temperature, and pH on eDNA degradation in aquatic microcosms. *Biological Conservation*, *183*, 85–92. https://doi.org/10.1016/j.biocon.2014.11.038

Sweeney, B. W., Battle, J. M., Jackson, J. K., & Dapkey, T. (2011). Can DNA barcodes of stream macroinvertebrates improve descriptions of community structure and water quality? *Journal of the North American Benthological Society*, *30*(1), 195–216. https://doi.org/10.1899/10-016.1

Takahara, T., Iwai, N., Yasumiba, K., & Igawa, T. (2020). Comparison of the detection of 3 endangered frog species by eDNA and acoustic surveys across 3 seasons. *Freshwater Science*, *39*(1), 18–27. https://doi.org/10.1086/707365

Tréguier, A., Paillisson, J.-M., Dejean, T., Valentini, A., Schlaepfer, M. A., & Roussel, J.-M. (2014). Environmental DNA surveillance for invertebrate species: Advantages and technical limitations to detect invasive crayfish *Procambarus clarkii* in freshwater ponds. *Journal of Applied Ecology*, *51*(4), 871–879. https://doi.org/10.1111/1365-2664.12262

Turner, C. R., Barnes, M. A., Xu, C. C. Y., Jones, S. E., Jerde, C. L., & Lodge, D. M. (2014). Particle size distribution and optimal capture of aqueous macrobial eDNA. *Methods in Ecology and Evolution*, *5*(7), 676–684. https://doi.org/10.1111/2041-210X.12206

Turner, C. R., Uy, K. L., & Everhart, R. C. (2015). Fish environmental DNA is more concentrated in aquatic sediments than surface water. *Biological Conservation*, *183*, 93–102. https://doi.org/10.1016/j.biocon.2014.11.017

Větrovský, T., Baldrian, P., & Morais, D. (2018). SEED 2: A user-friendly platform for amplicon high-throughput sequencing data analyses. *Bioinformatics*, *34*(13), 2292–2294. https://doi.org/10.1093/bioinformatics/bty071

Walsh, J. R., Spear, M. J., Shannon, T. P., Krysan, P. J., & Vander Zanden, M. J. (2019). Using eDNA, sediment subfossils, and zooplankton nets to detect invasive spiny water flea (Bythotrephes longimanus). *Biological Invasions*, *21*(2), 377–389. https://doi.org/10.1007/s10530-018-1862-5

Yang, J., & Zhang, X. (2020). EDNA metabarcoding in zooplankton improves the ecological status assessment of aquatic ecosystems. *Environment International*, *134*, 105230. https://doi.org/10.1016/j.envint.2019.105230

Zenker, M. M., Specht, A., & Fonseca, V. G. (2020). Assessing insect biodiversity with automatic light traps in Brazil: Pearls and pitfalls of metabarcoding samples in preservative ethanol. *Ecology and Evolution*, *10*(5), 2352–2366. https://doi.org/10.1002/ece3.6042

Zizka, V. M. A., Leese, F., Peinert, B., & Geiger, M. F. (2019). DNA metabarcoding from sample fixative as a quick and voucher-preserving biodiversity assessment method. *Genome*, *62*(3), 122–136. https://doi.org/10.1139/gen-2018-0048

BIOSKETCH

The research team is interested in how eDNA metabarcoding technique could be used for routine water quality assessment and biomonitoring programme, including sampling method refine, bioinformatic analysis, establishment of metrics derived from eDNA data and barcode data reference of China macroinvertebrate.

Author contributions: Y.W. designed the research, field collection, data analysis and wrote manuscript draft; J.G. conducted fieldwork and provided morphological data; K.C., M.W., Y.X. and J.D. wrote part of the manuscript; B.W. conceived and supervised the study and refined the MS; and J.P.G. and X.J. refined the MS.

SUPPORTING INFORMATION

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

How to cite this article: Wang Y, Chen K, Gao J, et al. Environmental DNA of preservative ethanol performed better than water samples in detecting macroinvertebrate diversity using metabarcoding. *Divers Distrib*. 2021;27:1989–2002. https://doi.org/10.1111/ddi.13284