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Effects of sampling strategies and DNA extraction methods on eDNA metabarcoding: A case study of estuarine fish diversity monitoring

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ABSTRACT

Environmental DNA (eDNA) integrated with metabarcoding is a promising and powerful tool for species composition and biodiversity assessment in aquatic ecosystems and is increasingly applied to evaluate fish diversity. To date, however, no standardized eDNA-based protocol has been established to monitor fish diversity. In this study, we investigated and compared two filtration methods and three DNA extraction methods using three filtration water volumes to determine a suitable approach for eDNA-based fish diversity monitoring in the Pearl River Estuary (PRE), a highly anthropogenically disturbed estuarine ecosystem. Compared to filtration-based precipitation, direct filtration was a more suitable method for eDNA metabarcoding in the PRE. The combined use of DNeasy Blood and Tissue Kit (BT) and traditional phenol/chloroform (PC) extraction produced higher DNA yields, amplicon sequence variants (ASVs), and Shannon diversity indices, and generated more homogeneous and consistent community composition among replicates. Compared to the other combined protocols, the PC and BT methods obtained better species detection, higher fish diversity, and greater consistency for the filtration water volumes of 1 000 and 2 000 mL, respectively. All eDNA metabarcoding protocols were more sensitive than bottom trawling in the PRE fish surveys and combining two techniques yielded greater taxonomic diversity. Furthermore, combining traditional methods with eDNA analysis enhanced accuracy. These results indicate that methodological

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decisions related to eDNA metabarcoding should be made with caution for fish community monitoring in estuarine ecosystems.

Keywords: eDNA metabarcoding; Fish diversity; Sampling strategies; DNA extraction; Estuarine ecosystem

INTRODUCTION

Accurately describing species composition is not only necessary for evaluating the characteristics of ecosystems but is also the cornerstone for establishing biodiversity protection systems (Blowes et al., 2019; Dixon et al., 2019). As one of the most productive ecosystems worldwide, nutrient-rich estuaries play important ecological functions and provide critical animal breeding and nursery habitats (Bhakta et al., 2019). Overfishing, pollutant discharge, and offshore construction have led to a significant deterioration in estuarine ecosystem functions, including rapid decreases in fish stock and biodiversity (Irfan & Alatawi, 2019; Yan et al., 2021). Fish assemblages are particularly sensitive to environmental stresses and are therefore considered good indicators of aquatic ecosystem health, even in highly modified and threatened estuaries (Santos et al., 2017; Zhou et al., 2019). Fish diversity is traditionally monitored through netting, trapping, and electrofishing. However, these methods require the physical capture of animals and are therefore labor intensive, potentially dangerous, and require high taxonomic expertise (Bessey et al., 2020). Furthermore, the limitations of capture-based methods hinder the management and protection of fishery resources in estuarine ecosystems, necessitating the development of more efficient and beneficial techniques for fish-diversity monitoring.

Environmental DNA (eDNA) metabarcoding is an emerging non-invasive and cost-effective detection method and has been successfully applied to predict fish species assemblages by extracting DNA from the environment (Bessey et al., 2020; Coutant et al., 2021; Stat et al., 2019; Valdivia-Carrillo et al., 2021). eDNA metabarcoding is not dependent on taxonomic experts for species identification, as eDNA sequences can be assigned to taxa or operational taxonomic units (OTUs) by matching sequences to those in the reference database of fish DNA (Taberlet et al., 2018).

However, fish in different ecosystems and habitats are likely to generate eDNA with spatial and temporal heterogeneity, leading to potential inconsistencies in analysis (Barnes & Turner, 2016; Beng & Corlett, 2020; Troth et al., 2021). Variation among eDNA protocols can also influence species detection and results, leading to imprecision ecological inferences and biodiversity assessments (Bessey et al., 2020; Jeunen et al., 2019; Majaneva et al., 2018). At present, laboratory protocols involving eDNA metabarcoding methods remain incongruent and non-standardized (Shu et al., 2020). As there is no one-size-fits-all eDNA metabarcoding process for aquatic ecosystems, investigators must first test the efficacy of a variety of methods to determine the optimal tool to achieve their goals in the target ecosystem (Jeunen et al., 2019). Consequently, comparing methodologies is critical for understanding the potential influences of different protocols and for obtaining reliable biodiversity estimates.

Molecular workflows applied to analyze eDNA include DNA collection from environmental samples, eDNA extraction and amplification, high-throughput sequencing (HTS), and bioinformatics analysis (Deiner et al., 2017; Mirimin et al., 2021; Wang et al., 2021). Each step can affect biodiversity assessment, and various strategies have been applied in aquatic eDNA-based studies. For instance, direct filtration, ethanol precipitation, and centrifugation are three major first-step filtration methods used for concentrating eDNA from aquatic environments (Tsuji et al., 2019). DNA collection and extraction are crucial for capturing eDNA from water, and their success is dependent on the selected filtration and extraction methods, as well as filtration water volumes (Sakata et al., 2021). Notably, different extraction techniques can result in differences in DNA quantity and quality, which can subsequently impact biodiversity assessment (Coutant et al., 2021; Deiner et al., 2015; Jeunen et al., 2019; Piggott, 2016; Wittwer et al., 2018). At present, studies show considerable variation in the protocols and steps involved in aquatic eDNA metabarcoding, which can impact the probability of detecting species as the protocols are not specific for fish and quantitative standards for estuarine ecosystems are lacking (Kumar et al., 2020; Lear et al., 2018; Stauffer et al., 2021). Thus, understanding the differences in DNA yields generated by different protocol combinations is essential for the successful application of eDNA metabarcoding in estuarine ecosystems.

The Pearl River Estuary (PRE) is an important estuary linking material exchange between the Pearl River Basin and the South China Sea. The PRE is characterized by high fishery production and rich biodiversity (Zou et al., 2020), and provides a favorable habitat for many important marine, estuarine, and freshwater species (Li et al., 2018; Sun & Chen, 2013; Zheng, 1989). However, due to the recent rapid economic growth of the surrounding cities and the increasing impact of human activities, biodiversity and fishery resources have declined, with deterioration of the bioecological integrity of the estuarine ecosystem (Zhang et al., 2015). At present, most investigations on fishery resources in the PRE are based on traditional survey methods, which can be destructive for both species and habitat (Hiddink et al., 2019; Liu et al., 2018). As an effective and non-invasive technology, eDNA metabarcoding is a promising alternative approach for investigating fish diversity in aquatic ecosystems such as the PRE (Boulanger et al., 2021; Gold et al., 2021; Prié et al., 2021; Sales et al., 2021). Because determining differences in DNA detection capabilities among protocols requires a realistic eDNA metabarcoding approach, we explored sampling, filtration, and extraction method combinations to reliably monitor fish diversity in the PRE. We compared the eDNA yields, detection probability, and consistency of two filtration- (filtration-based precipitation and direct filtration) and three extraction-based methods (DNeasy Blood & Tissue Kit (BT), traditional phenol/chloroform extraction (PC), and DNeasy PowerWater Kit (PW) extraction) using three filtration water volumes (500, 1 000, and 2 000 mL). We evaluated the performance of different sampling and extraction combinations

to assess PRE fish diversity. Finally, we compared the accuracy of fish diversity assessment between bottom trawling and eDNA metabarcoding.

MATERIALS AND METHODS

Sampling sites and fish species collection

Both eDNA and fish samples were collected from the PRE in January 2018 (Figure 1). Prior to sampling, the hydrophore sampler and sterile plastic bottles were cleaned with sterile deionized water and repeatedly rinsed with local water at least three times. The experimental design for comparative analysis is shown in Figure 1 and Supplementary Figure S1. Estuarine water was sampled using two filtration methods to collect DNA in water, i.e., direct filtration and filtration-based precipitation. For direct filtration, we collected 32 L of water in sterile plastic bottles using a hydrophore. For filtration-based precipitation, the samples were a mixture of natural PRE water, absolute ethanol, and 3 mol/L sodium acetate at a ratio of 32:16:1. Triplicate samples were collected for each method, with each duplicate sampled at different spots in the sampling area. Each eDNA sample was a mixture of surface and bottom water. Blanks (triplicate samples) were established using 6 L of distilled and deionized water to check for contamination during field collection. All samples were kept on ice in the field, then transported to the laboratory in less than 4 h and filtered within 6 h. Fish specimens were captured using traditional bottom trawling on the same day as water sampling and transported to the laboratory for species identification. Morphological examination was carefully performed by ichthyologists at the South China Agricultural University and South China Sea Fisheries Research Institute, and fish were identified to species level after each campaign (Li et al., 2018; Sun & Chen, 2013; Zheng, 1989). All animal procedures performed in this study were in accordance with the ethical standards of the Animal Care and Use Committee of South China Agricultural University (SCAU) and all animal research was conducted under the guidance of the SCAU Institutional Animal Care and Use Committee.

Filtration water volume for eDNA concentration

Before filtering and subsequent experiments, all equipment was soaked with bleach containing 500 mg/L available chlorine to reduce DNA contamination, then thrice rinsed with distilled water. Among the filters frequently used in eDNA studies, nitrocellulose membranes achieve higher DNA yields than polyethene sulfone, polyvinylidene fluoride, and polycarbonate filters (Liang & Keeley, 2013; Majaneva et al., 2018). Therefore, in the laboratory, water samples were filtered through nitrocellulose membranes (0.45 μm pore) for analysis of fish diversity in the PRE, as used previously (Jeunen et al., 2019; Zou et al., 2020). Water samples at three filtration volumes were established (500, 1 000, and 2 000 mL (three replicates each) and filtered using single filter membranes. Negative controls were established using filtered sterilized double-distilled water (500, 1 000, and 2 000 mL) to monitor contamination during filtering and subsequent DNA extraction. The filtering processes were implemented in a clean specialized room. All filters were fixed with absolute ethanol and stored at −80 °C until eDNA extraction.

eDNA extraction protocols

Three DNA extraction protocols were compared, i.e., DNeasy PowerWater Kit (PW) (Qiagen, Santiago, USA), DNeasy Blood & Tissue Kit (BT) (Qiagen, Hilden, Germany), and traditional phenol/chloroform extraction (PC) (Figure 1), with negative controls also included. The PW and BT eDNA extraction procedures were conducted following the manufacturers' instructions. The PC extraction protocols were as follows. We mixed 800 μL of buffer solution, consisting of 1.4 mol/L sodium chloride (NaCl), 0.1 mol/L tris (hydroxymethyl) aminomethane hydrochloride (Tris, pH=8), and 0.02 mol/L ethylene diamine tetraacetic acid (EDTA) (referred to as "STE"), and 160 μL of 10% sodium dodecyl sulfate (SDS) to sterilized centrifuge tubes, and added filters (cut up using clean and sterile scissors) to each tube, followed by incubation at 65 °C for 20 min. The tubes were vortexed for 15 s and centrifuged for 15 min at 14 000 *g* at room temperature. The resulting supernatant was transferred to a clean 2 mL tube and an equal volume of phenol:chloroform:isoamyl alcohol (PCI) (25:24:1) was added. The tube was then mixed upside down and centrifuged at 13 000 *g* for 10 min at room temperature. The aqueous phase was transferred to a new 2 mL tube, mixed with an equal

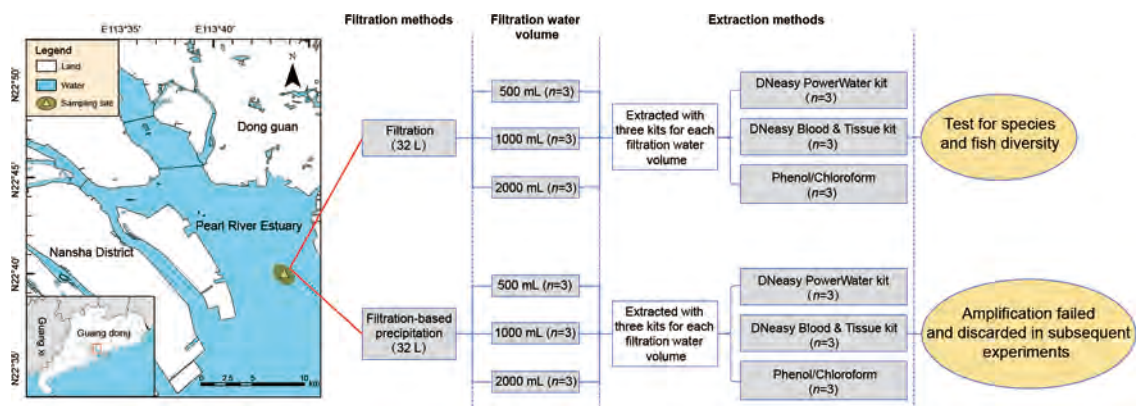

Figure 1 Experimental design used to evaluate influences of different methods on eDNA metabarcoding

Sampling strategies included two filtration methods, three filtration water volumes, and three eDNA extraction methods.

volume of chloroform:isoamyl alcohol (24:1), and centrifuged for 10 min at 13 000 *g* at room temperature. The supernatant containing DNA was mixed with cold isopropanol (DNA: isopropanol solution (10:7)) and incubated at –20 °C overnight to precipitate DNA. After centrifugation for 10 min at 13 000 *g* at room temperature, the supernatant was discarded. The remaining pellet (i.e., extracted DNA) was washed twice with 500 μL of 75% ethanol and removed through 5 min centrifugation at 13 000 *g* at room temperature. The pellet was dried at room temperature for 5 min and finally dissolved in 100 μL of AE Buffer (Qiagen, Hilden, Germany). Intact DNA was detected by 1% agarose gel electrophoresis, and DNA quality and yield were measured using a NanoDrop 2000 spectrophotometer (ThermoFisher Scientific, Waltham, USA) and Qubit™ 4 Fluorometer (ThermoFisher Scientific, Woodlands, Singapore). Negative controls were established during each extraction. All extraction processes were conducted in a clean specialized laboratory (dedicated to eDNA metabarcoding) at South China Agricultural University. Subsequently, polymerase chain reaction (PCR) amplification was performed using the eDNA obtained from different combinations of protocols as a template.

DNA amplification

The teleo_R_H1913 (5'-CTTCCGGTACACTTACCATG-3') and teleo_F_L1848 primers (5'-ACACCGCCCGTCACTCT-3'), which target the 12S rDNA region of the mitochondrial gene in teleosts, were used to amplify 110 bp fragments of the extracted eDNA samples (Valentini et al., 2016). Three PCR replicates were performed for each sample. Each set of replicates contained eDNA samples, blanks, and negative controls. DNA amplifications consisted of a two-step PCR protocol. The first PCR step was performed in a 20 μL reaction containing 10 μL of PCR Mixture (Novogene, Tianjin, China), 0.4 μL of the teleo_R_H1913 and teleo_F_L1848 primers (each 10 μmol/L), 2 μL of template (eDNA samples (30 ng/μL), blanks, and negative controls), and molecular grade water added to 20 μL. Thermal conditions for the first PCR step were: initial denaturation at 95 °C for 5 min, followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at 56 °C for 20 s, elongation at 72 °C for 30 s, and final elongation at 72 °C for 10 min. The first-step PCR products were diluted 10 times with molecular biological-grade water and used as the template for the second PCR step. The second-step PCR system included 25 μL of PCR Mixture (Novogene, Tianjin, China), 1 μL of 10 μmol/L forward and reverse primers (6 bp barcode sequences) (Novogene, Beijing, China), 3 μL of template, and deionized double-steamed water added to 50 μL. The second-step PCR thermal conditions were similar to the first, except the number of cycles was 20. The PCR products were mixed in equal-density ratios, followed by 2% agarose gel electrophoresis for detection and gel cutting for PCR product purification. The PCR products were purified using a TIANgel Purification Kit (Tiangen Biotech, Beijing, China). All DNA amplification and PCR product purification procedures were conducted in the same clean specialized laboratory as used for eDNA extraction.

Library preparation and sequencing

A TruSeq® DNA PCR-Free Sample Preparation Kit (Illumina, USA) was used for DNA sequencing library construction according to the manufacturer's recommendations, with index codes added. Library quality was evaluated using a Qubit 2.0 fluorometer (ThermoFisher Scientific, Waltham, USA) and Agilent Bioanalyzer 2100 (Agilent Technologies Inc., California, USA). The library was then sequenced on the Illumina NovaSeq platform (Novogene, Beijing, China) and 120 bp paired-end reads were generated.

Bioinformatics

Paired-end reads were assigned to samples based on their unique barcodes and truncated by cutting off the barcode and primer sequences. To utilize Illumina error profiling in the dada2 denoising step, the forward and reverse sequences were first isolated and processed independently using Cutadapt (v2.10). This was achieved by filtering the R1 fastq files for reads with the forward PCR primer and then with the reverse PCR primer. The reads were then demultiplexed by tag, followed by primer and barcode trimming using Cutadapt (v2.10) (Martin, 2011). Paired-end reads were merged using FLASH (v1.2.7; http://ccb.jhu.edu/software/FLASH/) (Magoč & Salzberg, 2011). The successfully spliced sequences were named as raw reads, with a total of 2 256 325 reads attained. Amplicon sequencing bioinformatics was performed under specific filtering conditions to obtain high-quality clean reads (Bokulich et al., 2013) using the QIIME (v1.9.1, http://qiime.org/scripts/split_libraries_fastq.html) quality-control processes (Caporaso et al., 2010). Clean read sequences were compared with the reference Silva database (https://www.arb-silva.de/) using the UCHIME algorithm (http://www.drive5.com/usearch/manual/uchime_algo.html) (Edgar et al., 2011) to detect and eliminate chimeric sequences and obtain effective reads. An average of 47 173 valid reads per sample were obtained after quality control, resulting in an effective rate of 78.68%.

The remaining bioinformatics steps were carried out using dada2 (v1.10.1) (Callahan et al., 2016). Quality trimming was carried out using default settings, except for tag truncation length "truncLen", which was determined to provide an approximate 30 bp overlap between forward and reverse reads. The reads were then denoised, dereplicated, merged, cleaned of chimeras, and re-orientated using the dada2 workflow. A homology filter was then implemented by aligning the amplicon sequence variants (ASVs) against a hidden Markov model of an expected fragment using HMMER hmmsearch, and non-homologous reads were discarded. The sequences were clustered into 130 observed ASVs.

Taxonomic assignment of the ASVs produced by dada2 was carried out using a multi-step procedure, incorporating the Kimura two-parameter (K2P) nucleotide evolution model and neighbor-joining (NJ) method (Kimura, 1980; Saitou & Nei, 1987). First, each ASV was locally blasted against the Nucleotide Sequence database (NT) of the National Center for Biotechnology Information (NCBI, https://www.ncbi.nlm.nih.gov/accessed; 23 March 2020) using *blastn* (v2.9.0) ("-task blastn -evalue 1000 -word size 11 -max target seqs 500"). The results were filtered to obtain a rough taxonomic classification based on the best-scoring blast hit. The ASVs of non-fish (i.e., humans, microbes, and other vertebrates) were

removed, including 20 ASVs (87 483 reads) not annotated to a species and three ASVs (5 813 reads) annotated to terrestrial animals (*Anas platyrhynchos*, *Anser answer*, and *Gallus gallus*). Next, a more stringent procedure was carried out, with putative fish sequences extracted from the initial blast result subjected to a second search. If the coverage between the full-length amplicon and NCBI sequence was ≥95% and had more than 96% sequence identity, the sequence was considered as the fish species. ASVs that did not meet the above principles or failed to match any fish species were not considered in downstream analysis. Fourteen ASVs (946 671 reads) were annotated to the family level (cyprinid) at this step. To ensure the correctness of the diversity results, the ASVs were artificially blasted in the NCBI database one-by-one to annotate taxonomic information. If the annotated fish did not match published records for the Pearl River and its estuary (Li et al., 2018; Sun & Chen, 2013; Zheng, 1989), the ASV was discarded. According to the local checklist of fish species in the PRE, the sequences of more than 90% of species are found in the NCBI database. Two ASVs (12 498 reads) were annotated to fish species (*Sprattus sprattus* and *Serranochromis robustus*) never previously recorded in the PRE. Finally, 71 ASVs (424 559 reads) were retained in the following analyses.

Statistical analysis

ASV abundance was normalized using a standard sequence number corresponding to the sample with the least sequences. Alpha diversity (Shannon index) was performed based on the normalized output data and calculated with PRIMER v5. The Jaccard index was calculated as: $J(A, B)=|A \cap B|/|A \cup B|$, where A and B represent two different sets of data, respectively. All data were analyzed using SPSS v17.0 based on one-way analysis of variance (ANOVA) and least significant difference (LSD). Significant and very significant differences were recognized at $P<0.05$ and $P<0.01$, respectively. Graphical visualizations of the DNA concentrations, A260/A280 nm ratio, A260/A230 nm ratio, relative abundance, and species detection were performed using GraphPad Prism v8.01, and graphical visualizations of OTU number, Shannon index, and Jaccard similarity index were displayed using R (v3.6.3). A Venn diagram showing overlapping species between eDNA metabarcoding and bottom trawling was constructed using InteractiVenn (http://www.interactivenn.net/) (Heberle et al., 2015).

RESULTS

DNA yields of different filtration and extraction methods

The DNA yields produced by the three filtration water volumes and three extraction methods are shown in Figure 2A, B and Supplementary Figure S2. Generally, the concentration of eDNA increased with increasing estuarine water volume when sampled using the two filtration methods. The eDNA concentration determined using the Qubit fluorometer was much lower than that determined using the NanoDrop spectrophotometer. For direct filtration, the highest concentration of eDNA was obtained using the BT extraction method with 2 000 mL of filtration water (NanoDrop: 72.59±10.13 ng/μL; Qubit: 46.93±9.82 ng/μL), which was significantly different from the other protocols ($P<0.05$, Figure 2A, B). For filtration-based precipitation, the eDNA concentrations ranged from 6.24±1.88 ng/μL to 45.08±3.89 ng/μL when using the NanoDrop spectrophotometer and from 0.20±0.11 ng/μL to 13.47±2.91 ng/μL when using the Qubit fluorometer (Supplementary Figure S2).

Except for PW extraction with 500 mL of water, no significant differences were identified for direct filtration of the 500 and 1 000 mL water volumes (Figure 2A, B). However, significant differences were found between PW and the other extraction methods and between NanoDrop and Qubit. In addition, highly significant differences ($P<0.01$) in eDNA concentrations were detected between the PC extraction method and other protocols when water was sampled by filtration-based precipitation (Supplementary Figure S2). For filtration-based precipitation, PC extraction with 2 000 mL of filtration water resulted in the highest eDNA concentration (NanoDrop: 45.08±3.89 ng/μL; Qubit: 13.47±2.91 ng/μL).

eDNA quality under different strategies

The DNA quality is shown in Figure 2C, D and Supplementary Figure S3. The eDNA A260/A280 results ranged from 0.97 to 3.65 (direct filtration) and 1.14 to 5.01 (filtration-based precipitation), with most not reaching optimal genomic DNA quality (1.8–2.0) (Figure 2C; Supplementary Figure S3). The eDNA A260/A230 results ranged from 0.80 to 1.62 (direct filtration) and 0.56 to 1.87 (filtration-based precipitation), with most not reaching 2.0 (Figure 2D; Supplementary Figure S3). No significant differences were found among the different protocols.

Results showed that the amplified products attained the target band when eDNA was obtained from direct filtration. However, the target band was not detected under multiple amplifications when eDNA was captured by filtration-based precipitation. Thus, filtration-based precipitation was discarded in the following experiments.

Diversity detected by different protocol combinations

For the PW and PC extraction methods, the greatest number of available ASVs was obtained when using 1 000 mL of filtering water. The BT method captured higher ASV richness than the other two extraction methods, with the highest richness obtained with the 2 000 mL filtration water volume (Figure 3A). Based on ANOVA, significant differences were found in available ASV number between BT and the two other protocols (PW: 0.002, PC: 0.037). The Shannon index is a common indicator used to assess community diversity. In the current study, based on ASVs, the highest Shannon index was obtained with the PC method, while BT and PW showed similar diversity levels (Figure 3B). The Shannon index results followed the order PW-0.5<PW-2<PW-1 (where 0.5, 1, and 2 indicate 500, 1 000, and 2 000 mL filtration water volumes, respectively), BT-1<BT-0.5<BT-2, and PC-0.5<PC-2<PC-1, respectively, with no significant differences detected among them ($P>0.05$).

Influences on species detection and reproducibility

Based on the eDNA metabarcoding results, BT-0.5, BT-1, and BT-2 detected 39, 35, and 42 fish species, PW-0.5, PW-1, and

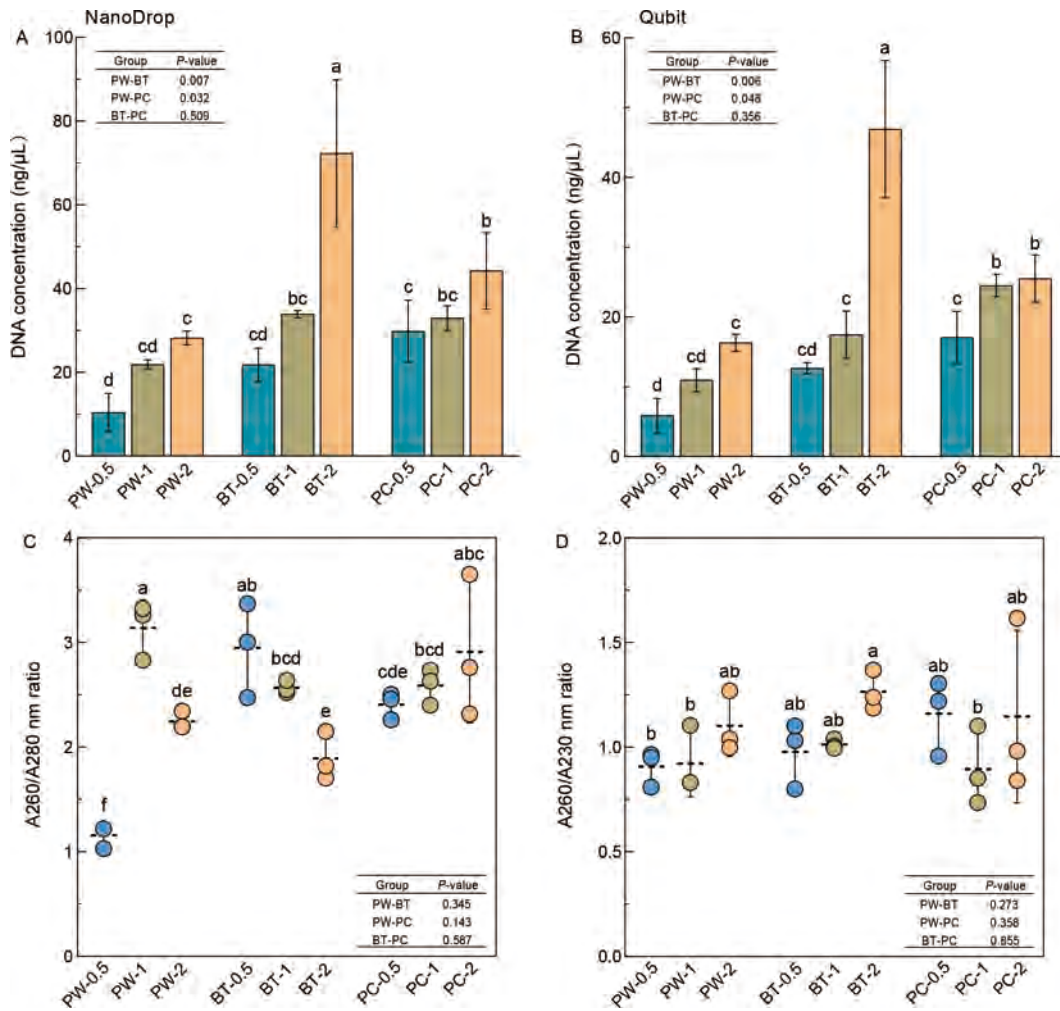

Figure 2 DNA concentrations (ng/μL) and quality captured by different filtration water volumes and extraction methods with direct filtration in Pearl River Estuary (PRE)

A: DNA concentrations measured by NanoDrop (spectrometry); B: DNA concentrations measured by Qubit (fluorometry); C: A260/A230 ratio; D: A260/A230 ratio. Bar frame shows means of each protocol; error bars show standard deviation. Letters a, b, c, and d indicate significant differences among means based on least significant difference (LSD, α=0.05). Dotted line indicates means of each group; error bars show standard deviation. PW, BT, and PC represent DNeasy PowerWater Kit, DNeasy Blood and Tissue Kit, and phenol/chloroform extraction, respectively. Numbers 0.5, 1, and 2 represent 500, 1 000, and 2 000 mL filtration water volumes, respectively.

PW-2 detected 28, 38, and 39 species, and PC-0.5, PC-1, and PC-2 detected 35, 40, and 31 species, respectively (Figure 4A). The BT method with 2 000 mL of filtration water generated the greatest number of species, and significant differences were found between BT-2 and the other protocols (except for BT-0.5 and PC-1, Figure 4B). Significant differences were also found between the BT and PW methods (P=0.013, <0.05), but no significant differences were found between the BT and PC (P=0.059, >0.05) and PC and PW methods (P=0.481, >0.05; Figure 5B). Additionally, only 11 species were detected by bottom trawling, including seven Perciformes, three Clupeiformes, and one Mugiliformes species, whereas four species (*Planiliza affinis*, *Odontamblyopus lacepedii*, *Johnius belangerii*, and *J. carutta*) captured by bottom trawling were not identified by the eDNA protocols (Figure 4A). These results indicate that eDNA metabarcoding can more comprehensively monitor fish species than bottom trawling.

Among the 49 species captured by eDNA metabarcoding, 17 exhibited high reproducibility and were captured by all protocol combinations (Figure 5). *Cirrhinus molitorella*, *Parasinilabeo assimilis*, and *Plectropomus leopardus* showed the lowest reproducibility and were only detected by BT-0.5, PW-2, and PW-2, respectively. *Inegocia japonica* (detected by BT-1 and PW-1), *Macrognathus aculeatus* (detected by BT-0.5 and BT-2), and *Mylopharyngodon piceus* (detected by PW-1 and PC-1) showed the second lowest reproducibility (Figure 5). The species detection rates (ratio of species detected by each method to all species detected by eDNA metabarcoding) of the nine protocol combinations ranged from 0.58 (PW-0.5) to 0.86 (BT-2) (Figure 5). The species detection rate of BT (including BT-0.5, BT-1, and BT-2) ranged from 0.71 to 0.86, and a higher species detection rate was achieved compared to PC and PW.

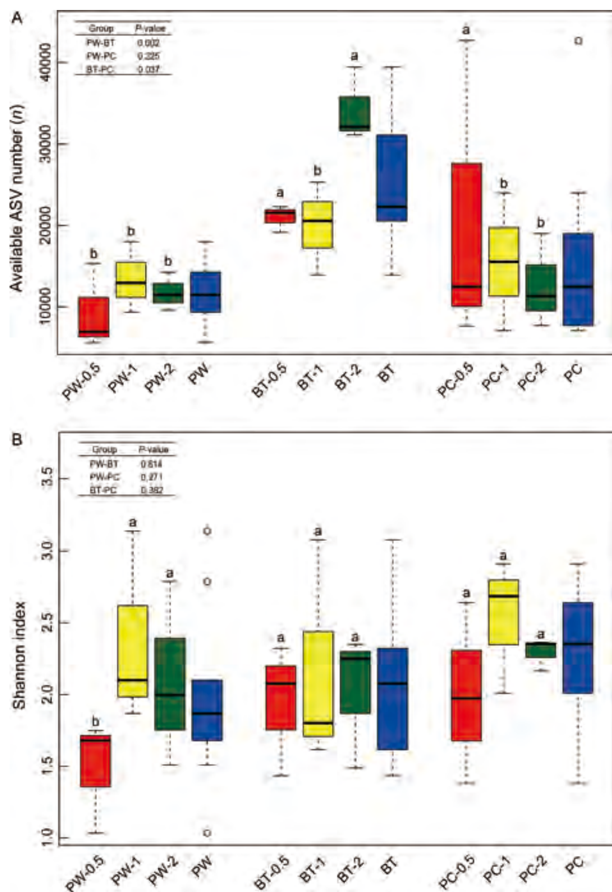

Figure 3 Available amplicon sequence variant (ASV) number (A) and Shannon index (B) obtained by each combined protocol.

Clupanodon thrissa, *Coilia grayii*, *C. mystus*, *Dendrophysa russelii*, *Glossogobius giuris*, *Nuchequula nuchalis*, and *Siganus fuscescens* were detected by all eDNA metabarcoding combinations and bottom trawling (Figure 4A).

Compared to bottom trawling, BT extraction showed the greatest congruence, with seven (BT-0.5), six (BT-1), and seven (BT-2) shared species, respectively (Figure 6). The PW-0.5, PW-1, and PW-2 protocols shared four, seven, and six species with bottom trawling, while PC-0.5, PC-1, and PC-2 shared six, six, and six species, respectively (Figure 6). These results indicate that the fish community obtained using BT more closely resembled the fish community obtained by bottom trawling. The BT-2 combination detected the largest number of species (42), followed by PC-1 (40), with PW-0.5 detecting the lowest number of species (28) (Figure 6).

Consistency of each protocol

To evaluate replicate consistency for each protocol, the Jaccard index of species was calculated. In terms of extraction methods, PC (0.74) had a higher mean Jaccard similarity coefficient compared to BT (0.71) and PW (0.64), indicating the most similar community composition (Figure 7A). No significant differences in the similarity coefficients were found among the three extraction methods. In addition, the mean Jaccard similarity coefficients for the 500, 1 000, and 2 000 mL filtration water volumes were 0.68, 0.67, and 0.71, respectively (Figure 7B), which were not significantly different (P>0.05).

DISCUSSION

As a promising method for the assessment of aquatic species diversity (Ahn et al., 2020; Polanco et al., 2021; Tsuji et al., 2019), eDNA metabarcoding is strongly dependent on a molecular workflow, which involves eDNA capture, extraction, and bioinformatics analyses. Comparative studies have tested the efficiency of eDNA capture using different filtration and extraction methods as well as filtration water volumes in various settings (Cantera et al., 2019; Deiner et al., 2015; Djurhuus et al., 2017; Shu et al., 2020). However, the high complexity of estuarine and marine ecosystems requires consistent comparison of eDNA metabarcoding procedures. Therefore, we evaluated the efficiency of a variety of filtration

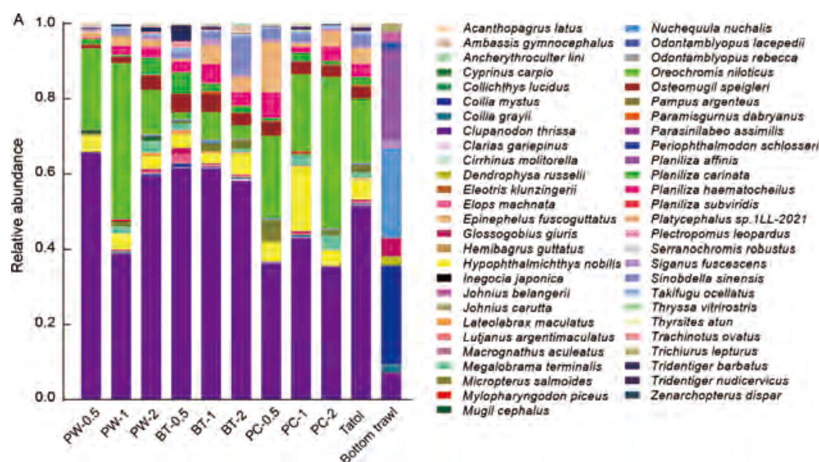

Figure 4 Relative abundances detected by different protocols and bottom trawling and differentiation of species number for each protocol

A: Relative abundances of fish species for different combined protocols and bottom trawling. Total represents total relative abundance of species detected by all eDNA metabarcoding protocols; B: Statistical comparisons based on LSD test (α=0.05) of species number between different protocols.

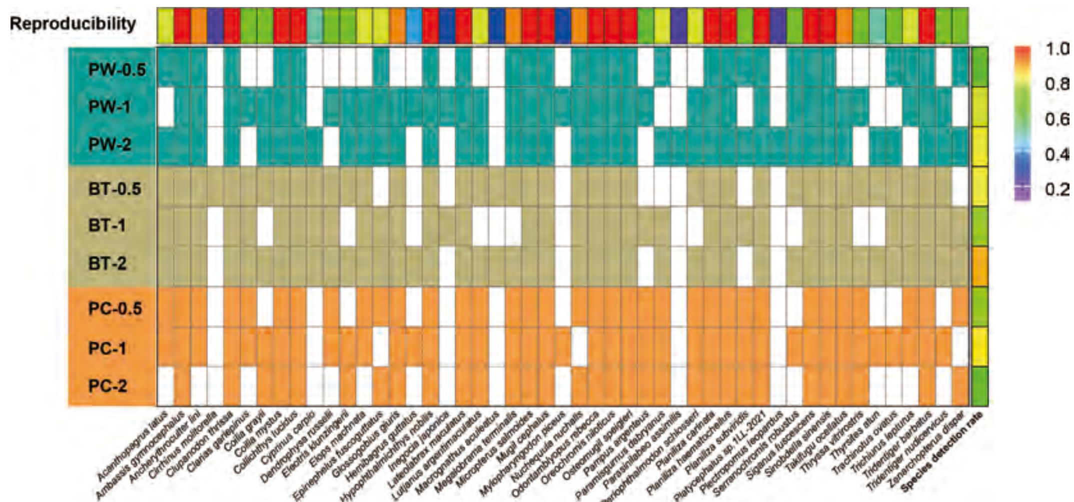

Figure 5 Distribution of detected species using different combined protocols

Colorized rectangle indicated that the protocol detected species; blank rectangle represents that the protocol failed to detect species. Rainbow-colored rectangles at top and right represent recurrence and detection rates, respectively. Ratio level is expressed with different colors.

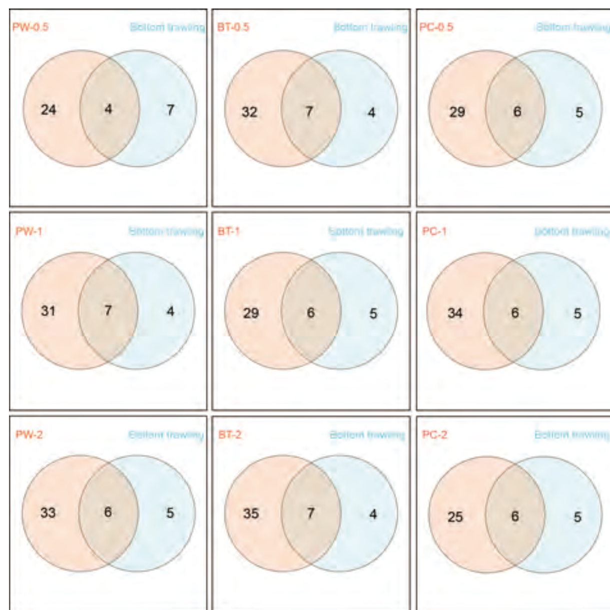

Figure 6 Overlap between fish species found by different eDNA metabarcoding protocols (pink) and bottom trawling (blue)

Numbers represent number of species in each set.

methods, filtration water volumes, and eDNA extraction methods at a single site to determine suitable eDNA metabarcoding protocols for fish-diversity assessment in the PRE. Different combinations of protocols had different impacts on eDNA concentrations and detection sensitivity. Based on the effective eDNA yield, diversity, reproducibility, and consistency of the tested methods, direct filtration of a 2 000 mL water sample combined with BT extraction showed the best results for fish diversity monitoring by eDNA metabarcoding in the PRE ecosystem. The BT and PC extraction methods were both appropriate for extracting eDNA from PRE water and for eDNA metabarcoding to monitor fish diversity.

Evaluation of sample treatments for eDNA collection

Ethanol precipitation and direct filtration are two common strategies used to concentrate eDNA from water samples (Goldberg et al., 2016; Wittwer et al., 2018). Results showed that significantly less eDNA was extracted from samples through filtration-based precipitation than through direct filtration (Figure 2A, B; Supplementary Figure S2). There are two potential explanations. For ethanol precipitation, more than twice the volume of ethanol is typically added to the water sample, which restricts the maximum sample volume and detection power of the eDNA techniques. First, less water is obtained under the same sampling volume when using the filtration-based precipitation. Less natural water is available for precipitation than for direct filtration under the same volume due to the addition of absolute ethanol and sodium acetate, leading to a disproportionate concentration of eDNA between samples (Foote et al., 2012). Second, the recovery of eDNA from water samples may be low with filtration-based precipitation of natural water from the PRE. Minamoto et al. (2016) did not recommend precipitation-based methods for fish detection in rivers, and estuarine water samples are even more challenging for eDNA analyses due to the larger body of source water, strong tide and current action, and higher turbidity and salinity.

We next amplified the captured eDNA for a specific gene target (i.e., metabarcode analysis) and categorized it into biodiversity units. The purity and effective concentration of eDNA can affect subsequent PCR amplification. In this study, the purity and concentration of the extracted eDNA was tested using both the NanoDrop and Qubit fluorimeter systems. The Qubit system detected much lower eDNA concentrations than the NanoDrop system (Supplementary Figure S2; Figure 2A, B), indicating very low purity and low effective concentration of the captured eDNA in the PRE. The A260/A280 and A260/A230 results provided further evidence of low eDNA purity (Supplementary Figure S3; Figure 2C, D). Furthermore, eDNA captured through filtration-based precipitation was unable to obtain the effective target band during the PCR

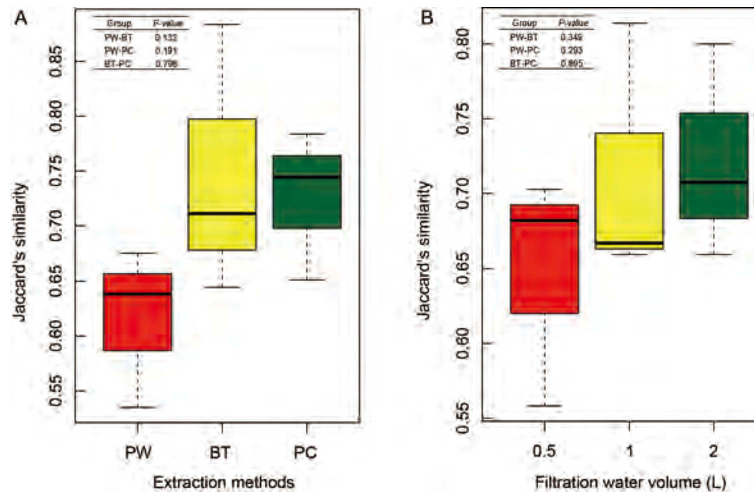

Figure 7 Pairwise Jaccard similarity values for replicates

A: Jaccard similarity values for extraction methods; B: Jaccard similarity values for filtration water volumes. Boxplots summarize species similarity values (n=9 per method) among extraction methods and filtration water volumes (n=9 per volume), respectively.

process. This may be due to several possible reasons. First, the target barcode could not be amplified due to insufficient effective eDNA during precipitation (Supplementary Figure S2; Figure 2A, B). Similarly, previous studies have demonstrated that direct filtration captures more effective eDNA than precipitation (Minamoto et al., 2016; Turner et al., 2014). Direct filtration also shows greater detection rates than precipitation in assessment of eukaryotic diversity through eDNA metabarcoding (Deiner et al., 2015). Second, the PCR procedure used in this study may be unsuitable for amplifying eDNA extracted via precipitation. The addition of absolute ethanol and sodium acetate in filtration-based precipitation may inhibit eDNA amplification by PCR. Therefore, further studies are needed to increase the filtration water volume and optimize the PCR procedure for filtration-based precipitation methods.

Performance of eDNA extraction methods and filtration water volumes

Various commercial kits and internal formulations are currently available for eDNA metabarcoding. However, interactions between extraction methods and filtering materials can impact eDNA yield. For example, PC extraction with nitrocellulose membranes shows better performance than with glass fiber filter membranes (Deiner et al., 2018; Djurhuus et al., 2017). In this study, we filtered all estuarine water samples using nitrocellulose membranes, and then extracted eDNA via three different methods. Our results are consistent with previous aquatic studies reporting that the BT and PC extraction methods with direct filtration through nitrocellulose membranes show better eDNA extraction (Jeunen et al., 2019), with significant differences found between the PW and other two extraction methods (Figure 2). The BT method also detects fish more consistently than other extraction methods (Hermans et al., 2018). Our results showed greater fish species and higher Jaccard similarity for the BT and PC replicates (Figures 6, 7), implying higher fish community composition consistency under the BT and PC DNA extraction methods for water samples from the PRE.

An ideal eDNA extraction method should obtain a high DNA yield and high PCR inhibitor removal rate. However, a high DNA yield does not necessarily indicate high biodiversity (Deiner et al., 2015; Djurhuus et al., 2017). Accordingly, extracted DNA concentrations may not directly reflect the diversity of eDNA in a water sample. Therefore, it is necessary to consider available ASV number and fish diversity results obtained through eDNA metabarcoding using different extraction methods. In this study, BT and PC extraction led to significantly more available ASVs and higher fish diversity (Shannon index) than obtained via PW extraction (Figure 3). This can be explained as follows. The PW method employs a bead-beating approach to break open cells, which increases the degree of DNA breakage and can negatively affect subsequent PCR amplification (Callahan, 2009; Rajendhran & Gunasekaran, 2008; Tringe & Rubin, 2005). Second, eDNA samples collected from estuarine ecosystems are complex and heterogeneous, and the ability to remove inhibitory substances varies among eDNA extraction methods, leading to variation in the resulting biodiversity information. Inhibitory removers are added to the purification columns in the BT kits for higher DNA concentrations in water extracts and more accurate PCR results (Jerde et al., 2013; Lear et al., 2018). Third, cell lysis and biochemical methods are considered more suitable for extracting DNA from animal tissues, and more conducive to PCR amplification (Wintzingerode et al., 1997). The BT method uses proteinase K to split cells, whereas the PC method uses phenol and chloroform chemical reagents. Consequently, the BT and PC extraction methods performed better during eDNA metabarcoding of the PRE water in terms of available ASV number and fish diversity.

Theoretically, larger water volumes should result in higher eDNA yields for target taxa. In this study, DNA yields and Jaccard similarity increased with filtration water volumes using the different filtration strategies (Figures 2, 7B). However, the available ASV number, Shannon index, and fish species number results differed using the PC extraction method, with the highest fish diversity obtained with the combination of PC

extraction and 1 000 mL filtration water volume (Figures 3, 6). Natural water usually contains microbes and chlorophyll, and differences in their abundance and concentration can impact eDNA persistence (Barnes et al., 2014). Notably, increasing the filtration water volume can lead to greater microbial and chlorophyll residues on membranes, resulting in the introduction of more PCR inhibitors into the eDNA experiments (Koziol et al., 2019; Shaw et al., 2016). Due to a lack of inhibitory removers during the whole extraction process, impurities increased with the water sample volume during PC extraction, which may have restricted subsequent eDNA amplification by PCR. Hence, for eDNA metabarcoding, PC extraction using a 1 000 mL filtration water volume resulted in higher fish diversity than that obtained using the 500 or 2 000 mL filtration water volumes in the PRE ecosystem.

Taxonomic identification compared to traditional bottom trawling

In this study, a total of 49 fish species were detected by the nine eDNA-based protocol combinations (i.e., three direct filtration strategies×three extraction methods), including 17 species with 100% reproducibility (Figures 4, 5). The 17 species of fish with 100% reproducibility detected in this study are common in the PRE and have been reported in previous studies (Li et al., 2018; Sun & Chen, 2013; Zheng, 1989; Zou et al., 2020), indicating that these species exist in the PRE even though they were not detected by traditional bottom trawling (e.g., *Oreochromis niloticus*, *Hypophthalmichthys nobilis*, *Planiliza haematocheilus*, and *Osteomugil speigleri*) (Figures 4, 5). In some detected species, the similarity between technical duplicates did not reach 100%, and higher similarity was found between duplicates using the BT and PC extraction methods (Figure 7A). These results demonstrate that BT and PC extraction can be replicated more easily than PW extraction.

Accurate detection of all species present in an environment is important for fish diversity surveys. Similar to Hermans et al. (2018), we obtained high similarity in fish species using the BT and PC extraction methods, indicating that species detected by these methods more closely resembled the true community of the study area (Figures 6, 7). In addition, eDNA metabarcoding revealed some species overlooked by visual assessment and identified greater taxonomic diversity compared to conventional methods (Dubart et al., 2019; Zou et al., 2020). In this study, eDNA metabarcoding produced much higher fish species diversity than obtained by bottom trawling, regardless of the protocol combination (Figures 4, 6). Traditional survey techniques are primarily performed via hand and/or net, making it difficult to catch some fish species, including endangered, nocturnal, and cave-dwelling species such as *Hemibagrus guttatus*, *Macrognathus aculeatus*, and *Paramisgurnus dabryanus*, which were only identified by eDNA metabarcoding in this study. In contrast, species with localized habitat requirements or small population size may not be detected by eDNA metabarcoding due to lower concentrations of released eDNA and short transport distances (Nukazawa et al., 2018). In the present study, *J. belangerii*, *J. carutta*, *Planiliza affinis*, and *Odontamblyopus lacepedii* were only caught by bottom trawling as these species tend to inhabit the bottom of natural water bodies with low abundance (Figure 4). Species caught by bottom trawling are indisputably present in the survey area. Bottom trawling can obtain more species information, e.g., length, weight, and age, and performs well for catching demersal species in fish diversity surveys (Murphy & Jenkins, 2010). The catching efficiency of bottom trawling is less affected by population size, but can be influenced by fish escape behavior, equipment properties, and fishing operations (Costello et al., 2017). In summary, despite sampling at the same site under similar conditions, eDNA metabarcoding and traditional methods produced different fish abundance and assemblage composition results. Therefore, although eDNA metabarcoding is a promising, feasible, and sensitive approach for fish diversity assessment (Sales et al., 2021; Valdivia-Carrillo et al., 2021), combining it with traditional methods may be more effective for enhancing fish diversity surveys.

Additional considerations

Several factors must be considered when incorporating sampling, filtration, and extraction protocols into a workflow for fish diversity assessment using eDNA metabarcoding. Substantial heterogeneity in eDNA detection probabilities across sites can lead to inference bias in the biodiversity results when site replication used for eDNA-based analysis is low (Buxton et al., 2021). In the current study, only one site was used to compare the performance of different protocols for capturing eDNA, which may have resulted in less biometric information than would have been obtained at multiple sites. However, the probability of capturing species based on eDNA metabarcoding can be increased by collecting multiple water samples at a single site (Lugg et al., 2018; Mauvisseau et al., 2019). Nine sampling replicates for each extraction method were performed at the sampling site, which may have offset any bias in the biodiversity. Fish diversity at the study site was estimated using 27 replicates, achieving saturation of biodiversity accumulation statistics (Supplementary Figure S4). Undoubtedly, a single study site could result in undetected species (e.g., rare or transient species, species with localized habitats), and multiple sites would be more integrative. Adequate water-sampling procedures for aquatic ecosystems are important for eDNA studies. Previous research has demonstrated that a large volume of filtration water is needed to represent real fauna in habitats (Cantera et al., 2019; Polanco et al., 2021; Pont et al., 2018; Stauffer et al., 2021; Valentini et al., 2016). The three filtration water volumes tested in this study are commonly used in eDNA metabarcoding studies, with high success rates for species detection (Sales et al., 2021; Valdivia-Carrillo et al., 2021). Typically, 1 000 or 2 000 mL of water is collected in the field, and standard volumes of 1 000 or 2 000 mL of water are commonly utilized for filtration and purification of DNA in water samples (Shu et al., 2020). Monitoring fish diversity through eDNA metabarcoding in a region requires filtration water from many sites. Filtering large water volumes from each one site will greatly increase transportation costs and technical requirements. Due to financial and technical limitations, the filtration water volumes tested in this study were convenient

and cost-effective for eDNA inventories. Finally, attention should be paid to the limitations of the barcode and reference database using eDNA metabarcoding for regional fish diversity monitoring. The barcode is not particularly resolutive for cyprinids (Taberlet et al., 2018), and 14 discarded ASVs were annotated to the cyprinid level in this study. Furthermore, the NCBI database does not contain a complete reference of PRE fish species, with the sequences of ~10% of local fish species not currently found in the database. Thus, it is possible to underestimate fish diversity in the PRE due to these limitations. Multi-marker approaches and local reference databases should be considered in future research.

CONCLUSIONS

As a potential tool for fish diversity monitoring, eDNA metabarcoding shows great promise. To date, however, the methodologies and protocols for eDNA metabarcoding remain unstandardized. Therefore, it is imperative to evaluate the performance of different methods of eDNA metabarcoding before fully integrating them into standard biomonitoring approaches. Environmental sample collection and eDNA extraction protocols are crucial to the eDNA capture process and are heavily impacted by environmental and biological variables in ecosystems. In this study, we conducted comparative assessments of DNA yields, fish detection ability, fish diversity, reproducibility, and consistency among different filtration methods, filtration water volumes, and extraction methods in the PRE. Based on our results, direct filtration can be used to conduct eDNA metabarcoding procedures for fish diversity analysis in the PRE. In addition, the BT and PC extraction methods performed much better than PW extraction in terms of eDNA yield, ASV number, fish diversity, species detection, reproducibility, and consistency. Overall, our results indicated that direct filtration of 1 000 mL of water with PC extraction or 2 000 mL of water with BT extraction is a good strategy for revealing fish community composition in the turbid, eutrophic, and environmentally complex estuarine ecosystem of the PRE.

DATA AVAILABILITY

The datasets analyzed during the current study are available in the NCBI Sequence Read Archive repository under BioProjectID PRJNA736834.

SUPPLEMENTARY DATA

Supplementary data to this article can be found online.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

H.T.R., K.S.Z., and M.L. designed the study, performed the experiment, and wrote the original manuscript. R.L.W. and H.T.L. organized sampling, conducted fieldwork, and analyzed data. L.L. and T.X.K. analyzed data. K.S.Z. and M.L. reviewed and edited the manuscript. All authors read and approved the final version of the manuscript.

REFERENCES

Ahn H, Kume M, Terashima Y, Ye F, Kameyama S, Miya M, et al. 2020. Evaluation of fish biodiversity in estuaries using environmental DNA metabarcoding. *PLoS One*, **15**(10): e0231127.

Barnes MA, Turner CR. 2016. The ecology of environmental DNA and implications for conservation genetics. *Conservation Genetics*, **17**(1): 1−17.

Barnes MA, Turner CR, Jerde CL, Renshaw MA, Chadderton WL, Lodge DM. 2014. Environmental conditions influence eDNA persistence in aquatic systems. *Environmental Science & Technology*, **48**(3): 1819−1827.

Beng KC, Corlett RT. 2020. Applications of environmental DNA (eDNA) in ecology and conservation: opportunities, challenges and prospects. *Biodiversity and Conservation*, **29**(7): 2089−2121.

Bessey C, Jarman SN, Berry O, Olsen YS, Bunce M, Simpson T, et al. 2020. Maximizing fish detection with eDNA metabarcoding. *Environmental DNA*, **2**(4): 493−504.

Bhakta D, Meetei WA, Vaisakh G, Kamble S, Das SK, Das BK. 2019. Finfish diversity of narmada estuary in Gujarat of India. *Proceedings of the Zoological Society*, **72**(3): 257−262.

Blowes SA, Supp SR, Antão LH, Bates A, Bruelheide H, Chase JM, et al. 2019. The geography of biodiversity change in marine and terrestrial assemblages. *Science*, **366**(6463): 339−345.

Bokulich NA, Subramanian S, Faith JJ, Gevers D, Gordon JI, Knight R, et al. 2013. Quality-filtering vastly improves diversity estimates from Illumina amplicon sequencing. *Nature Methods*, **10**(1): 57−59.

Boulanger E, Loiseau N, Valentini A, Arnal V, Boissery P, Dejean T, et al. 2021. Environmental DNA metabarcoding reveals and unpacks a biodiversity conservation paradox in Mediterranean marine reserves. *Proceedings of the Royal Society B:Biological Sciences*, **288**(1949): 20210112.

Buxton A, Matechou E, Griffin J, Diana A, Griffiths RA. 2021. Optimising sampling and analysis protocols in environmental DNA studies. *Scientific Reports*, **11**(1): 11637.

Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJA, Holmes SP. 2016. DADA2: high-resolution sample inference from Illumina amplicon data. *Nature Methods*, **13**(7): 581−583.

Callahan H. 2009. Inhibitor-free DNA purification from water samples. *BioTechniques*, **46**(6): 473.

Cantera I, Cilleros K, Valentini A, Cerdan A, Dejean T, Iribar A, et al. 2019. Optimizing environmental DNA sampling effort for fish inventories in tropical streams and rivers. *Scientific Reports*, **9**(1): 3085.

Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, et al. 2010. QIIME allows analysis of high-throughput community sequencing data. *Nature Methods*, **7**(5): 335−336.

Costello MJ, Basher Z, McLeod L, Asaad I, Claus S, Vandepitte L, et al. 2017. Methods for the study of marine biodiversity. *In*: Walters M, Scholes RJ. The GEO Handbook on Biodiversity Observation Networks. Cham: Springer, 129–163.

Coutant O, Cantera I, Cilleros K, Dejean T, Valentini A, Murienne J, et al. 2021. Detecting fish assemblages with environmental DNA: does protocol matter? Testing eDNA metabarcoding method robustness. *Environmental DNA*, **3**(3): 619−630.

Deiner K, Bik HM, Mächler E, Seymour M, Lacoursière-Roussel A, Altermatt F, et al. 2017. Environmental DNA metabarcoding: transforming how we survey animal and plant communities. *Molecular Ecology*, **26**(21):

5872−5895.

Deiner K, Lopez J, Bourne S, Holman L, Seymour M, Grey EK, et al. 2018. Optimising the detection of marine taxonomic richness using environmental DNA metabarcoding: the effects of filter material, pore size and extraction method. *Metabarcoding and Metagenomics*, **2**: e28963.

Deiner K, Walser JC, Mächler E, Altermatt F. 2015. Choice of capture and extraction methods affect detection of freshwater biodiversity from environmental DNA. *Biological Conservation*, **183**: 53−63.

Dixon KM, Cary GJ, Worboys GL, Banks SC, Gibbons P. 2019. Features associated with effective biodiversity monitoring and evaluation. *Biological Conservation*, **238**: 108221.

Djurhuus A, Port J, Closek CJ, Yamahara KM, Romero-Maraccini O, Walz KR, et al. 2017. Evaluation of filtration and DNA extraction methods for environmental DNA biodiversity assessments across multiple trophic levels. *Frontiers in Marine Science*, **4**: 314.

Dubart M, Pantel JH, Pointier JP, Jarne P, David P. 2019. Modeling competition, niche, and coexistence between an invasive and a native species in a two-species metapopulation. *Ecology*, **100**(6): e02700.

Edgar RC, Haas BJ, Clemente JC, Quince C, Knight R. 2011. UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics*, **27**(16): 2194−2200.

Foote AD, Thomsen PF, Sveegaard S, Wahlberg M, Kielgast J, Kyhn LA, et al. 2012. Investigating the potential use of environmental DNA (eDNA) for genetic monitoring of marine mammals. *PLoS One*, **7**(8): e41781.

Gold Z, Sprague J, Kushner DJ, Marin EZ, Barber PH. 2021. eDNA metabarcoding as a biomonitoring tool for marine protected areas. *PLoS One*, **16**(2): e0238557.

Goldberg CS, Turner CR, Deiner K, Klymus KE, Thomsen PF, Murphy MA, et al. 2016. Critical considerations for the application of environmental DNA methods to detect aquatic species. *Methods in Ecology and Evolution*, **7**(11): 1299−1307.

Heberle H, Vaz Meirelles G, da Silva FR, Telles GP, Minghim R. 2015. InteractiVenn: a web-based tool for the analysis of sets through Venn diagrams. *BMC Bioinformatics*, **16**(1): 169.

Hermans SM, Buckley HL, Lear G. 2018. Optimal extraction methods for the simultaneous analysis of DNA from diverse organisms and sample types. *Molecular Ecology Resources*, **18**(3): 557−569.

Hiddink JG, Jennings S, Sciberras M, Bolam SG, Cambiè G, McConnaughey RA, et al. 2019. Assessing bottom trawling impacts based on the longevity of benthic invertebrates. *Journal of Applied Ecology*, **56**(5): 1075−1084.

Irfan S, Alatawi AMM. 2019. Aquatic ecosystem and biodiversity: a review. *Open Journal of Ecology*, **9**(1): 1−13.

Jerde CL, Chadderton WL, Mahon AR, Renshaw MA, Corush J, Budny ML, et al. 2013. Detection of Asian carp DNA as part of a Great Lakes basin-wide surveillance program. *Canadian Journal of Fisheries and Aquatic Sciences*, **70**(4): 522−526.

Jeunen GJ, Knapp M, Spencer HG, Taylor HR, Lamare MD, Stat M, et al. 2019. Species-level biodiversity assessment using marine environmental DNA metabarcoding requires protocol optimization and standardization. *Ecology and Evolution*, **9**(3): 1323−1335.

Kimura M. 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution*, **16**(2): 111−120.

Koziol A, Stat M, Simpson T, Jarman S, Dibattista JD, Harvey ES, et al. 2019. Environmental DNA metabarcoding studies are critically affected by substrate selection. *Molecular Ecology Resources*, **19**(2): 366−376.

Kumar G, Eble JE, Gaither MR. 2020. A practical guide to sample preservation and pre-PCR processing of aquatic environmental DNA. *Molecular Ecology Resources*, **20**(1): 29−39.

Lear G, Dickie I, Banks J, Boyer S, Buckley HL, Buckley TR, et al. 2018. Methods for the extraction, storage, amplification and sequencing of DNA from environmental samples. *New Zealand Journal of Ecology*, **42**(1): 10−50A.

Li GF, Zhou L, Ye SH, Zeng L, Weng SP, Liu L, et al. 2018. Conservations of Fish Diversity and Resource in the Pearl River Estuary. Beijing: China Agriculture Press. (in Chinese)

Liang ZB, Keeley A. 2013. Filtration recovery of extracellular DNA from environmental water samples. *Environmental Science & Technology*, **47**(16): 9324−9331.

Liu D, Cai L, Yang YO, Hu JW. 2018. Length‐weight relationships of two fish species caught from the estuary of the Pearl River. *Journal of Applied Ichthyology*, **34**(4): 1071−1072.

Lugg WH, Griffiths J, van Rooyen AR, Weeks AR, Tingley R. 2018. Optimal survey designs for environmental DNA sampling. *Methods in Ecology and Evolution*, **9**(4): 1049−1059.

Magoč T, Salzberg SL. 2011. FLASH: fast length adjustment of short reads to improve genome assemblies. *Bioinformatics*, **27**(21): 2957−2963.

Majaneva M, Diserud OH, Eagle SHC, Boström E, Hajibabaei M, Ekrem T. 2018. Environmental DNA filtration techniques affect recovered biodiversity. *Scientific Reports*, **8**(1): 4682.

Martin M. 2011. Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet Journal*, **17**(1): 10−12.

Mauvisseau Q, Burian A, Gibson C, Brys R, Ramsey A, Sweet M. 2019. Influence of accuracy, repeatability and detection probability in the reliability of species-specific eDNA based approaches. *Scientific Reports*, **9**(1): 580.

Minamoto T, Naka T, Moji K, Maruyama A. 2016. Techniques for the practical collection of environmental DNA: filter selection, preservation, and extraction. *Limnology*, **17**(1): 23−32.

Mirimin L, Desmet S, Romero DL, Fernandez SF, Miller DL, Mynott S, et al. 2021. Don't catch me if you can – Using cabled observatories as multidisciplinary platforms for marine fish community monitoring: an *in situ* case study combining Underwater Video and environmental DNA data. *Science of the Total Environment*, **773**: 145351.

Murphy HM, Jenkins GP. 2010. Observational methods used in marine spatial monitoring of fishes and associated habitats: a review. *Marine and Freshwater Research*, **61**(2): 236−252.

Nukazawa K, Hamasuna Y, Suzuki Y. 2018. Simulating the advection and degradation of the environmental DNA of common carp along a river. *Environmental Science & Technology*, **52**(18): 10562−10570.

Piggott MP. 2016. Evaluating the effects of laboratory protocols on eDNA detection probability for an endangered freshwater fish. *Ecology and Evolution*, **6**(9): 2739−2750.

Polanco FA, Martinezguerra MM, Marques V, Villa‐Navarro F, Pérez GHB, Cheutin MC, et al. 2021. Detecting aquatic and terrestrial biodiversity in a tropical estuary using environmental DNA. *Biotropica*, **53**(6): 1606−1619.

Pont D, Rocle M, Valentini A, Civade R, Jean P, Maire A, et al. 2018. Environmental DNA reveals quantitative patterns of fish biodiversity in large rivers despite its downstream transportation. *Scientific Reports*, **8**(1): 10361.

Prié V, Valentini A, Lopes-Lima M, Froufe E, Rocle M, Poulet N, et al. 2021. Environmental DNA metabarcoding for freshwater bivalves biodiversity assessment: methods and results for the Western Palearctic (European

sub-region). *Hydrobiologia*, **848**(12): 2931−2950.

Rajendhran J, Gunasekaran P. 2008. Strategies for accessing soil metagenome for desired applications. *Biotechnology Advances*, **26**(6): 576−590.

Saitou N, Nei M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology Evolution*, **4**(4): 406−425.

Sakata MK, Watanabe T, Maki N, Ikeda K, Kosuge T, Okada H, et al. 2021. Determining an effective sampling method for eDNA metabarcoding: a case study for fish biodiversity monitoring in a small, natural river. *Limnology*, **22**(2): 221−235.

Sales NG, Wangensteen OS, Carvalho DC, Deiner K, Præbel K, Coscia I, et al. 2021. Space-time dynamics in monitoring neotropical fish communities using eDNA metabarcoding. *Science of the Total Environment*, **754**: 142096.

Santos RVS, Ramos S, Bonecker ACT. 2017. Can we assess the ecological status of estuaries based on larval fish assemblages?. *Marine Pollution Bulletin*, **124**(1): 367−375.

Shaw JLA, Clarke LJ, Wedderburn SD, Barnes TC, Weyrich LS, Cooper A. 2016. Comparison of environmental DNA metabarcoding and conventional fish survey methods in a river system. *Biological Conservation*, **197**: 131−138.

Shu L, Ludwig A, Peng ZG. 2020. Standards for methods utilizing environmental DNA for detection of fish species. *Genes*, **11**(3): 296.

Stat M, John J, Dibattista JD, Newman SJ, Bunce M, Harvey ES. 2019. Combined use of eDNA metabarcoding and video surveillance for the assessment of fish biodiversity. *Conservation Biology*, **33**(1): 196−205.

Stauffer S, Jucker M, Keggin T, Marques V, Andrello M, Bessudo S, et al. 2021. How many replicates to accurately estimate fish biodiversity using environmental DNA on coral reefs?. *Ecology and Evolution*, **11**(21): 14630−14643.

Sun DR, Chen Z. 2013. Retrieval of Fish in South China Sea. Beijing: Ocean Press. (in Chinese)

Taberlet P, Bonin A, Zinger L, Coissac E. 2018. Environmental DNA: for Biodiversity Research and Monitoring. Oxford: Oxford University Press.

Tringe SG, Rubin EM. 2005. Metagenomics: DNA sequencing of environmental samples. *Nature Reviews Genetics*, **6**(11): 805−814.

Troth CR, Sweet MJ, Nightingale J, Burian A. 2021. Seasonality, DNA degradation and spatial heterogeneity as drivers of eDNA detection dynamics. *Science of the Total Environment*, **768**: 144466.

Tsuji S, Takahara T, Doi H, Shibata N, Yamanaka H. 2019. The detection of aquatic macroorganisms using environmental DNA analysis—A review of methods for collection, extraction, and detection. *Environmental DNA*, **1**(2): 99−108.

Turner CR, Barnes MA, Xu CCY, Jones SE, Jerde CL, Lodge DM. 2014. Particle size distribution and optimal capture of aqueous macrobial eDNA. *Methods in Ecology and Evolution*, **5**(7): 676−684.

Valdivia-Carrillo T, Rocha-Olivares A, Reyes-Bonilla H, Domínguez-Contreras JF, Munguia-Vega A. 2021. Integrating eDNA metabarcoding and simultaneous underwater visual surveys to describe complex fish communities in a marine biodiversity hotspot. *Molecular Ecology Resources*, **21**(5): 1558−1574.

Valentini A, Taberlet P, Miaud C, Civade R, Herder J, Thomsen PF, et al. 2016. Next-generation monitoring of aquatic biodiversity using environmental DNA metabarcoding. *Molecular Ecology*, **25**(4): 929−942.

Wang SP, Yan ZG, Hänfling B, Zheng X, Wang PY, Fan JT, et al. 2021. Methodology of fish eDNA and its applications in ecology and environment. *Science of the Total Environment*, **755**: 142622.

Wintzingerode FV, Göbel UB, Stackebrandt E. 1997. Determination of microbial diversity in environmental samples: pitfalls of PCR-based rRNA analysis. *FEMS Microbiology Reviews*, **21**(3): 213−229.

Wittwer C, Nowak C, Strand DA, Vrålstad T, Thines M, Stoll S. 2018. Comparison of two water sampling approaches for eDNA-based crayfish plague detection. *Limnologica*, **70**: 1−9.

Yan HF, Kyne PM, Jabado RW, Leeney RH, Davidson LNK, Derrick DH, et al. 2021. Overfishing and habitat loss drive range contraction of iconic marine fishes to near extinction. *Science Advances*, **7**(7): eabb6026.

Zhang BJ, Mo JH, Mai JB, Li M, Huang LB, Mai GQ, et al. 2015. The fish population composition and the variations of the fishery resources in Humen Harbor Waters. *Freshwater Fisheries*, **45**(5): 50−58. (in Chinese)

Zheng CY. 1989. Pearl River Fishes. Beijing: Science Press. (in Chinese)

Zhou L, Wang GP, Kuang TX, Guo DL, Li GF. 2019. Fish assemblage in the Pearl River Estuary: spatial‐seasonal variation, environmental influence and trends over the past three decades. *Journal of Applied Ichthyology*, **35**(4): 884−895.

Zou KS, Chen JW, Ruan HT, Li ZH, Guo WJ, Li M, et al. 2020. eDNA metabarcoding as a promising conservation tool for monitoring fish diversity in a coastal wetland of the Pearl River Estuary compared to bottom trawling. *Science of the Total Environment*, **702**: 134704.