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# Optimization of pore size and filter material for better enrichment of environmental DNA

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In the study of environmental DNA (eDNA), the rate of water filtration and the amount of DNA obtained are of general interest to researchers. We compared the PCTE filters with different pore sizes in enriching total DNA and fish eDNA from water samples. We also designed and tested a new filter device with a PET pad (5  $\mu\text{m}$ ) superimposed on a PCTE membrane (3  $\mu\text{m}$ ) and compared it to a pure PCTE filter. A comprehensive evaluation of filtration time, eDNA concentration, fish DNA quantification, and fish species detected showed that the shortest average filtration time was obtained with the 8  $\mu\text{m}$  pore size (2 min 9 s) and the longest average filtration time was obtained with the 0.2  $\mu\text{m}$  pore size (32 min 6 s). The highest average DNA concentration was 3.785 ng/ $\mu\text{L}$  for the 0.2  $\mu\text{m}$  filter and the lowest was 0.577 ng/ $\mu\text{L}$  for the 8  $\mu\text{m}$  filter; meanwhile, the concentrations of fish DNA measured by quantitative PCR were 5.02E+02(8  $\mu\text{m}$ ), 4.79E+02(3  $\mu\text{m}$ ), 1.63E+03(1.2  $\mu\text{m}$ ), 5.95E+03(0.2  $\mu\text{m}$ ) copies of fish DNA per  $\mu\text{L}$  and there was no significant difference in the results between them ( $p > 0.05$ ). The sequencing results showed that 17 fish species were detected in the 0.2  $\mu\text{m}$  filter, 10 species in the 1.2  $\mu\text{m}$  filter, 12 species in the 3  $\mu\text{m}$  filter and 11 species in the 8  $\mu\text{m}$  filter. Comparing to 3  $\mu\text{m}$  and 0.2  $\mu\text{m}$ , the average filtration time in stacked-filter was reduced by 4 and 24 min, respectively, while the average concentration was 2.46 and 1.55 times higher than the former. The qPCR results showed that the number of eDNA copies of the samples enriched with the 3  $\mu\text{m}$  and stacked-filter was 4.79E+02 and 1.53E+04, respectively. 3  $\mu\text{m}$  and stacked-filter showed significant differences ( $p < 0.05$ ), while 0.2  $\mu\text{m}$  and stacked-filter did not show significant differences ( $p > 0.05$ ). Sixteen fish species were detected by the stacked-filter. The number of eDNA obtained and the number of fish species detected were higher than those of the pure PCTE filter, while clogging during filtration was effectively avoided. We recommend using the stacked-filter to enrich eDNA from water samples.

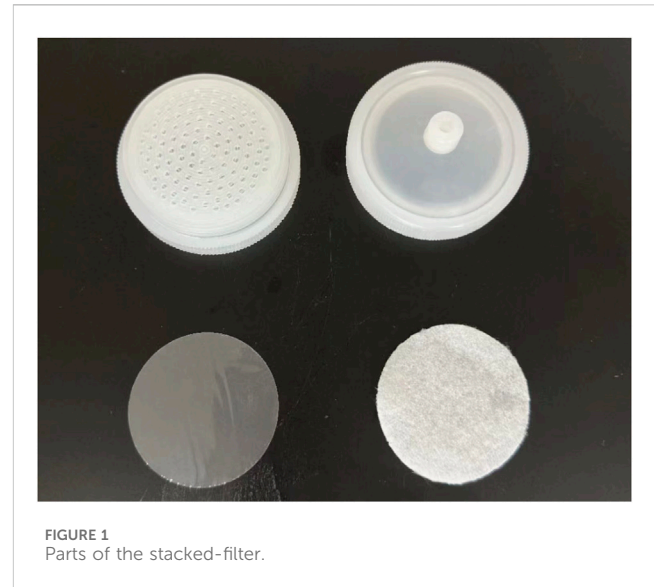
## KEYWORDS

stacked-membrane filter, filter pore size, PCTE, PET, environmental DNA

## 1 Introduction

Environmental DNA (eDNA) can be broadly defined as DNA present in the environment, including DNA released by organisms into the environment as well as DNA of microscopic organisms. Environmental DNA metabarcoding technology is used for qualitative and quantitative analysis of organisms through collection of environmental samples, enrichment, DNA extraction, selection of molecular markers, design of universal primers for their amplification, and high-throughput sequencing. In recent years, eDNA technology has been used in various applications such as species diversity monitoring (Plough et al., 2018; Webster et al., 2020; Zou et al., 2020; Jia et al., 2021; DiBattista et al., 2022; Knudsen et al., 2022), invasive species detection, and biomass assessment (Tillotson et al., 2018; Fraija-Fernandez et al., 2020; Wang et al., 2020; Hanzek et al., 2021). The method has been used to detect target species (Baudry et al., 2021; Matthias et al., 2021; Brandt et al., 2022; Ruppert et al., 2022), for example, in 2021 to detect giant catfish in the Mekong River (Osathanunkul, 2022).

The main methods for eDNA enrichment include alcohol precipitation (Echeverria-Beirute et al., 2021; Persaud et al., 2021), passive eDNA sampling (Kirtane et al., 2020; Verdier et al., 2022), and filtration (Kamoroff and Goldberg, 2018; Jeunen et al., 2019; Muha et al., 2019; Shi et al., 2020; Allison et al., 2021; Bairoliya et al., 2022; Pathiraja et al., 2023; Sahu et al., 2023). Alcohol precipitation involves the addition of anhydrous ethanol to a small amount of aqueous sample, which is centrifuged at high speed to concentrate eDNA (Goldberg et al., 2016). Among these methods, alcohol precipitation is usually used for small sample volumes and the results of alcohol precipitation usually depend on the concentration of target eDNA available in the sample. Ethanol precipitation is only able to enrich sufficient amounts of eDNA when the concentration of target eDNA is very high (Dejean et al., 2012). Passive sampling is the undisturbed collection of eDNA directly from aquatic environments using natural or man-made materials, but it is difficult to expand the scope of passive sampling because it is so dependent on the habits (Zhang et al., 2023). At the same time, a wide variety of equipment and materials are used for passive sampling, and their impact on the sampling results needs to be considered during the sampling process. The filtration method has the advantages of filtering a large volume of water sample, and fast and easy to extract DNA subsequently. However, due to the complex environment of water samples, clogging of filters often occurs (Dunker et al., 2017; Hunter et al., 2019; Kirtane et al., 2020). To mitigate this situation, selection of filters is important. In eDNA applications, membrane pore size ranges from 0.2 to 20  $\mu\text{m}$  (Egan et al., 2013; Jo et al., 2020; Jo and Yamanaka, 2022; Osathanunkul, 2022; Shu et al., 2020; von Ammon et al., 2023). Filtration with large-pore membranes can save time, process large volume of water and presumably reduce DNA degradation (Barnes and Turner, 2015; Zaiko et al., 2022); on the other hand, it may lose fine particles and small DNA fragments, and even possible to increase inhibitory substances (Beng and Corlett, 2020). Nonetheless, using small pore size may increase eDNA yields through retaining small particles, but clogging often occurs, which in turn costs more filtration time and even damages the filter membrane. The effect of pore size on enrichment of eDNA has been studied in different waterbody types (Moushomi et al., 2019; Wu et al., 2019; Fan et al.,



2021). Pre-filtering water samples using membrane with larger pore size or using small-mesh net also was proposed (Takasaki, et al., 2021; Brandt et al., 2021; Zaiko et al., 2022). Although pre-filtering can improve consistency of DNA samples obtained (Takasaki et al., 2021), Majaneva et al. (2018) evaluated the effects of large-aperture prefiltration and found that while pre-filtration in complex environments increased the probability of detecting a single species and yielded higher values of the diversity index and a more consistent community composition, it reduced DNA yield.

Besides, pre-filtration of water samples may increase the risk of sample contamination due to more operation steps, such as changing different filter membranes. In terms of filter membrane material, it can be chosen from glass fiber (GF), mixed cellulose ester (MCE), polycarbonate track etched (PCTE) etc. (Hongo et al., 2021; Wang et al., 2021; Osathanunkul, 2022; Osathanunkul and Madesis, 2022; Uthicke et al., 2022; Xing et al., 2022). Choice of filter material can affect the blockage of filters and subsequent DNA extraction process (Li et al., 2023). For example, with the thin structure and limited number of pores of PCTE membranes, clogging occurs frequently. GF and MCE filters, on the other hand, do not clog as much due to their thickness and almost “three-dimensional” structure, which allows them to hold more particles before they get stuck. However, GF and MCE filters require several steps such as cutting the membrane for incubation during DNA extraction.

CTAB is a cationic detergent with the property of precipitating nucleic acids and acidic polysaccharides from low ionic strength solutions. As such, it is widely used for the extraction of DNA from water samples and the precipitation of environmental DNA (Tsuji et al., 2017; Wang et al., 2021). Meanwhile, Hunter et al. (2019) evaluated Longmire’s storage buffer, Long-CTAB-CI, CTAB-PCI buffer for the removal of PCR inhibitors and concluded that CTAB was the reagent that yielded the highest amount of ambient DNA in short-term storage (5–8 days). Briefly, the filtered membrane can be stored in CTAB solution, then phenol chloroform is added to dissolve the membrane, and after centrifugation at high speed, the supernatant is transferred to a new centrifuge tube for subsequent extraction. No membrane cutting and prolonged

TABLE 1 MiFish primers with inline index (lower case) and the tail of Illumina adapter.

Primer	Sequence (5'to3')	bp
M-F1	ACACTCTTTCCCTACACGACGCTCTTCCGATCTtctgccGTCGGTAAAACTCGTGCCAGC	60
M-R1	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTgaccttCATAGTGGGGTATCTAATCCCAGTTTG	67
M-F2	ACACTCTTTCCCTACACGACGCTCTTCCGATCTgtctctGTCGGTAAAACTCGTGCCAGC	60
M-R2	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTcataaCATAGTGGGGTATCTAATCCCAGTTTG	67
M-F3	ACACTCTTTCCCTACACGACGCTCTTCCGATCTatattgGTCGGTAAAACTCGTGCCAGC	60
M-R3	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTgccgagCATAGTGGGGTATCTAATCCCAGTTTG	67
M-F4	ACACTCTTTCCCTACACGACGCTCTTCCGATCTtggaaGTCGGTAAAACTCGTGCCAGC	60
M-R4	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTcgatcaCATAGTGGGGTATCTAATCCCAGTTTG	67
M-F5	ACACTCTTTCCCTACACGACGCTCTTCCGATCTtctagtGTCGGTAAAACTCGTGCCAGC	60
M-R5	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTttgattCATAGTGGGGTATCTAATCCCAGTTTG	67
M-F6	ACACTCTTTCCCTACACGACGCTCTTCCGATCTagagtaGTCGGTAAAACTCGTGCCAGC	60
M-R6	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTtccgagCATAGTGGGGTATCTAATCCCAGTTTG	67
M-F7	ACACTCTTTCCCTACACGACGCTCTTCCGATCTggccaaGTCGGTAAAACTCGTGCCAGC	60
M-R7	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTcctgaaCATAGTGGGGTATCTAATCCCAGTTTG	67
M-F8	ACACTCTTTCCCTACACGACGCTCTTCCGATCTtatctcGTCGGTAAAACTCGTGCCAGC	60
M-R8	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTattcttCATAGTGGGGTATCTAATCCCAGTTTG	67
M-F9	ACACTCTTTCCCTACACGACGCTCTTCCGATCTttatgcGTCGGTAAAACTCGTGCCAGC	60
M-R9	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTgagactCATAGTGGGGTATCTAATCCCAGTTTG	67
M-F10	ACACTCTTTCCCTACACGACGCTCTTCCGATCTagttggGTCGGTAAAACTCGTGCCAGC	60
M-R10	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTggcttcCATAGTGGGGTATCTAATCCCAGTTTG	67

incubation is required during this process, so it can reduce the risk of cross-contamination (Djurhuus et al., 2017).

Theoretically, the thick PET pad not only helps to trap particles but also reduces the possibility of clogging. Like PCTE membrane, PET pad can be partially dissolved in phenol, so CTAB-phenol-chloroform extraction protocol can be used on the stacked-membrane filter to increase extraction yield and avoid cross-contamination. In this study, we took the advantage of both GF-like and PCTE-like materials, 1) designed a stacked-membrane filter by combing a polyethylene terephthalate pad (5 µm pore size, 2 cm thickness) and a PCTE membrane (3 µm pore size) for eDNA extraction. 2) We also compared the effect of pore size on eDNA enrichment, particularly its effect on DNA of targeted fish species.

## 2 Materials and methods

### 2.1 Sample collection

All sampling tools were bleached and then washed with double distilled water before collecting water samples, such as water sampler (volume 3 L), plastic buckets, etc. Disposable suppliers, such as mask, filter assemblages and forceps were used to avoid cross-contamination. Water samples were collected in mid-December 2021 from a freshwater lake (38°88'72.47"N, 121°89'62.82"E) on campus of the Shanghai Ocean

University, Shanghai, China. The lake hosts both freshwater and some brackish water species, so it is a good sampling place for testing our methods because of its high diversity of fish species. The water sampler and the plastic bucket were rinsed with the water on site for three times. Water samples were taken 10 cm below surface, and then mixed in a plastic bucket. Finally, the mixed water samples were transferred directly to the lab within 30 min. A total of 42 L of water was obtained. The water sample was mixed well before taken for subsequent experiment. Double-distilled water was used as blank control before filtering the samples.

### 2.2 Sample filtration

Polycarbonate track etched filters with a diameter of 47 mm and pore sizes ranging from 8, 3, 1.2 to 0.2 µm were used to filter 1 L of water sample. A vacuum filtration system was used to filter the samples, composed of a 500 mL disposable syringe, a filter assemblage, and a vacuum pump. Six replicates were carried for each pore size, as well as a sample of dd H<sub>2</sub>O used as the blank control. The time required for filtration was recorded. After the filtration was completed, the filter membranes were stored in a 5 mL tube with 2X CTAB buffer added. The samples then were kept in a -20°C freezer before DNA extraction. DNA extraction and subsequent experimental steps are described in the following sections.

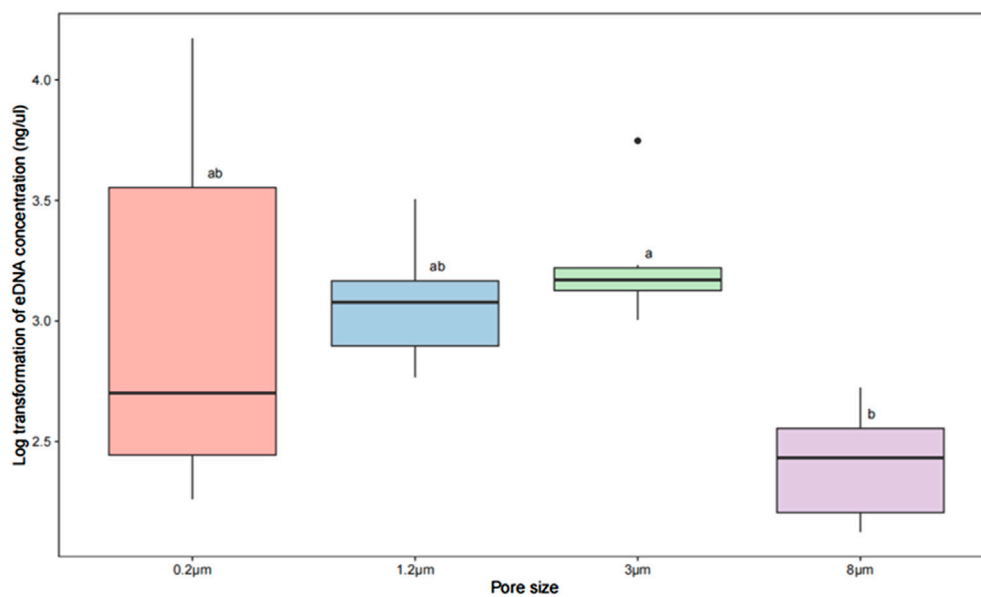


FIGURE 2

Box plot and one-way ANOVA test on log transformed eDNA concentration enriched with filters of different pore size. The results of using log treatment for concentrations extracted from filters with different pore sizes along with differential comparisons using Tukey test. Comparison between the two groups was carried out at different pore sizes. Within the box plots, each group has a label 'a', 'b' 'c' or a combination of letters to show the result of Tukey multiple comparison tests. The results showed no significant difference in total DNA concentrations extracted from the 1.2, 3, 0.2, and 8  $\mu\text{m}$  filters ( $p > 0.05$ ), but those extracted by the 3  $\mu\text{m}$  and 8  $\mu\text{m}$  filters differed significantly ( $p < 0.05$ ).

Water samples tend to filter quickly at the beginning and then become slower and slower until they clog. On this basis, we hypothesized that, rather than increasing the number of particles trapped, prolonging filtration after the filter has clogged would damage the filter and result in loss of the DNA sample. Therefore, we compared the effect of using one membrane to filter 1 L water sample with that of using two membranes, each filtering 0.5 L of water. PCTE with pore sizes of 1.2 and 3  $\mu\text{m}$  were selected for testing. In the two-membrane treatment, each 0.5-L water sample was filtered through one membrane, while in the single-membrane treatment, a 1-L water sample was filtered through one membrane. Each treatment was repeated three times. Record the filtration time. DNA was extracted by placing two membranes in a test tube.

Meanwhile, two different filter combinations were designed: (1) a single membrane filter (PCTE; 3  $\mu\text{m}$ , Safe Lab: #MS-PCTE-47-3.0-10) and (2) a stacked membrane filter combining a PET pad (5  $\mu\text{m}$ , 2 cm thickness) and a PCTE membrane (3  $\mu\text{m}$ ). The top material of the stacked filter is PET with a thickness of 2 cm and pore size of 5  $\mu\text{m}$ . It has a diameter of 47 mm and mainly of three-dimensional structure. Stacked-filter is assembled as follows: place the PCTE membrane on the plastic base, then lay the PET linear flat on the top of the PCTE membrane and tighten the cover (Figure 1). One-liter of water sample was filtered using both filter assemblies. Each treatment was repeated three times. The filtration time was recorded with a timer.

### 2.3 eDNA extraction

The samples were transferred to special 15 mL centrifuge tubes. The CTAB and phenol-chloroform protocols were followed for

DNA extraction with some modifications. Briefly, samples were incubated at 65°C in a water bath for 10 min. Then 1,000  $\mu\text{L}$  of chloroform-isoamyl alcohol mixture (24:1) was added to the sample tube under a fume hood. The tubes were vortexed vertically for 5 min to mix the liquid well, and then the tubes were centrifuged at 15,000 g for 15 min. After that, the supernatant was transferred to a 2 mL Eppendorf tube, and 250  $\mu\text{L}$  of 5 M NaCl solution and 500  $\mu\text{L}$  of cold isopropanol were added to the solution and kept at -20°C for 1 hour.

Samples were removed from the freezer and centrifuged at 15,000 g for 15 min. The supernatant was slowly poured out. Subsequently, 150  $\mu\text{L}$  of 70% ethanol was added to the sample and centrifuged at 15,000 g for 5 min (repeat this step twice). The supernatant was poured out and the sample was dried in a vacuum dryer at 45°C for 10 min. Finally, 100  $\mu\text{L}$  of TE buffer (preheated to 55°C) was added to elute the DNA. The DNA concentration of the samples was determined using a Nanodrop 3,300 fluorescence spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, U.S.A.).

### 2.4 Estimation of fish DNA using quantitative PCR

A real-time fluorescent quantitative PCR system (CFX connect, BIO-RAD, China) was used to quantify fish DNA in the samples using MiFish primers (Miya et al., 2015). The 15  $\mu\text{L}$  reaction system contained 3  $\mu\text{L}$  of template DNA, 1  $\mu\text{L}$  of each of the forward and reverse primers (10  $\mu\text{M}$ ; MiFish-U-F: GTCGGTAAAACCTCGTGCC AGC; MiFish-U-R: CATAGTGGGGTATCTAATCCCAGTTTG), 6  $\mu\text{L}$  of 2X T5 Fast qPCR mix (Tsingke Biotechnology Co., Ltd,

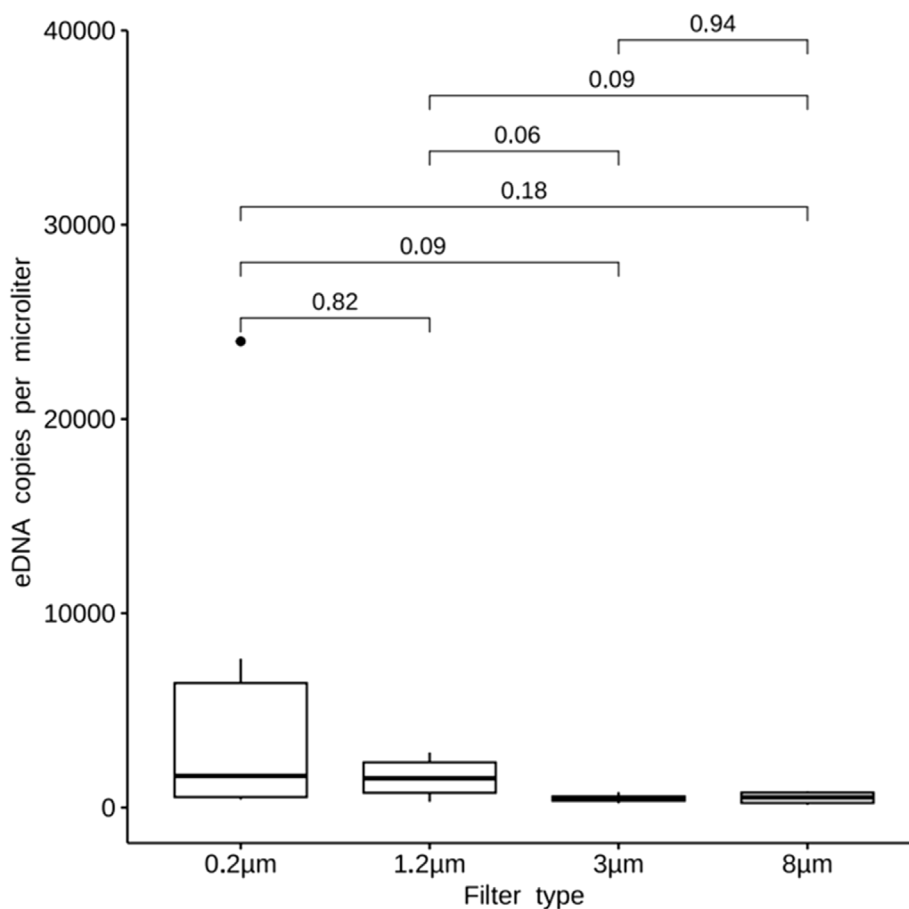


FIGURE 3

Quantity of fish DNA extracted with different filters. Quantification of fish DNA in samples using a real-time fluorescence quantitative PCR system and MiFish primers. For the results obtained the amount of fish DNA was analyzed for variability using one-way ANOVA with SPSS 27. The results of fish DNA quantification were plotted as linear bar graphs. The results shows DNA copy numbers of 8, 3, 1.2, 0.2 μm filters were  $5.02E+02$ ,  $4.97E+02$ ,  $1.63E+03$ ,  $5.95E+03$ , respectively. There was no significant difference between them ( $p > 0.05$ ).

S/N 1FB219010138, China), and 4 μL of dd H<sub>2</sub>O. Amplification products of goldfish samples were used as reference for making standard curves. Double-distilled water was used as negative controls. The cycling conditions for qPCR were as follows: 95°C, 1 min; 40 cycles of 95°C, 10 s, 60°C, 30 s, 72°C, 12 s. Fluorescent signals were collected at 72°C. Concentration of fish DNA in the samples were estimated from three replicates, and the copy number was calculated accordingly.

## 2.5 PCR amplification and sequencing to identify fish species in the eDNA samples

Amplification and sequencing library preparation followed the pipeline of Miya with some modifications. Three replications were performed for each eDNA samples. A pair of inline indices was added to differentiate each PCR replicates and different samples, that is, 6 bp of indices were added between the MiFish primers (Miya et al., 2015) and the Illumina adapter (Wang et al., 2022). The 25 μL reaction system contained 4 μL of template DNA, 1 μL of forward and reverse primers (10 μM), 12.5 μL of PrimeSTAR<sup>®</sup> MAX DNA Polymerase (Takara, CO. NOR045A, BEIJING), and 6.5 μL of PCR

H<sub>2</sub>O. The primer we used is innovative based on MiFish Primer proposed by Maski Miya, and its specific sequence is displayed in Table 1. The cycling conditions for the first round of PCR were as follows: 95°C for 3 min; 35 cycles of 98°C for 10 s, 60°C for 15 s, 72°C for 15 s; 72°C for 5 min and a final hold at 12°C. After the first round of PCR, magnetic beads were used to wash the PCR products. A second round of PCR amplification was performed using the washed products as template and with standard Illumina P5 and P7 primers. The condition for the second PCR was as follows: 98°C for 45 s; 15 cycles of 98°C for 15 s, 60°C for 30 s, 72°C for 45s; 72°C for 1 min and finally held at 12°C. The amplicons were sequenced as PE150 on an Illumina Nova Seq platform (GENEWIZ, Inc., Suzhou, China).

## 2.6 Data analysis

The sequencing data were parsed base on the pair of inline indices. Reads with low quality (Q-score <20) and wrong combination of index were excluded. We uploaded the cleaned sequencing data to the webserver implementing the MiFish pipeline (Sato et al., 2018). MiFish pipeline (<http://mitofish.aori.u-tokyo.ac.jp/mifish/>) server is an all-in-one website that

TABLE 2 Fish species identified in the eDNA samples of different treatments.

Species	Stacked-membrane filter	8 $\mu\text{m}$	3 $\mu\text{m}$	1.2 $\mu\text{m}$	0.2 $\mu\text{m}$
<i>Gobiiformes</i>					
<i>Rhinogobius similis</i>	+	+	+	+	+
<i>Tridentiger bifasciatus</i>					+
<i>Tridentiger barbatus</i>					+
<i>Rhinogobius duospilus</i>	+				
<i>Cypriniformes</i>					
<i>Cyprinus carpio</i>	+	+	+	+	+
<i>Acheilognathus barbatus</i>	+	+	+	+	+
<i>Squaliobarbus curriculus</i>	+				+
<i>Hypophthalmichthys nobilis</i>					+
<i>Pseudobrama simoni</i>	+				+
<i>Chanodichthys erythropterus</i>	+	+		+	
<i>Elopichthys bambusa</i>			+	+	
<i>Ctenopharyngodon idellus</i>	+	+	+		+
<i>Pseudorasbora parva</i>					+
<i>Hemiculter leucisculus</i>	+	+	+		+
<i>Megalobrama amblycephala</i>	+		+	+	+
<i>Saurogobio gracilicaudatus</i>		+	+		
<i>Sarcocheilichthys variegatus</i>				+	
<i>Hemibarbus labeo</i>		+	+		+
<i>Hemibarbus maculatus</i>	+	+	+	+	+
<i>Mugilidae</i>					
<i>Planiliza haematocheilus</i>	+				+
<i>Clupeiformes</i>					
<i>Coilia nasus</i>	+	+			
<i>Siluriformes</i>					
<i>Tachysurus fulvidraco</i>	+		+		
<i>Tachysurus eupogon</i>			+		+
<i>Pseudobagrus albomarginatus</i>	+				
<i>Pempheriformes</i>					
<i>Lateolabrax maculatus</i>				+	
<i>Perciformes</i>					
<i>Siniperca chautsi</i>	+	+		+	+

includes primer removal, sequence assembly, and database sequence comparison. Mitofish (v3.87 version) database was used as the reference for comparison (Iwasaki et al., 2013). The similarity threshold was set to 97%, which means that when the sequence similarity was greater than or equal to 97%, the name of the species corresponding to the eDNA sequence and related information were reported.

The result of the species data was compared using a “Venn” plot implemented in R language, including specific and co-detected species in each DNA sample collected with different pore sizes and different filter materials. Information on all detected species was assembled in a table with the name of the detected species. To evaluate the effect of the stacked-membrane filter and different pore sizes on eDNA enrichment, we compared four parameters: filtration

TABLE 3 Number of species identified in different samples and PCR replicates.

	8 $\mu\text{m}$	3 $\mu\text{m}$	1.2 $\mu\text{m}$	0.2 $\mu\text{m}$	Stacked-filter
Replicate1	4	7	2	—	2
Replicate2	4	4	2	—	2
Replicate3	3	3	2	—	2
Replicate4	2	2	3	2	2
Replicate5	2	2	2	2	3
Replicate6	3	3	2	2	2
Replicate7	3	4	4	4	4
Replicate8	2	2	4	2	2
Replicate9	2	3	3	3	—
Replicate10	3	3	2	4	5
Replicate11	2	3	4	5	7
Replicate12	2	3	2	4	4
Replicate13	3	2	3	3	3
Replicate14	5	5	4	4	3
Replicate15	3	4	3	5	3
Replicate16	3	4	4	4	4
Replicate17	4	2	2	2	9
Replicate18	4	6	3	5	3

time, total eDNA yield, amount of fish DNA, and detection rate of fish species. We used the “gg plot2” in R.4.3.1 to plot the boxplot and performed a log coefficient transformation to match a normal distribution. Comparisons were made for pore size, number of membranes treated and different eDNA concentrations produced by using different filters.

One-way analysis of variance in SPSS27 and t-tests were used to perform significance tests for filtration time, eDNA amount, and fish eDNA for different pore sizes and filters. Fish DNA amounts were presented using a linear bar graph. For species richness in the community, we used accumulation curves for representation. All the R and the package was found in the R Graph Gallery and GitHub.

## 3 Results

### 3.1 Effect of different pore size of filter membrane on eDNA enrichment

The shortest average filtration time for 1 L of water sample was 2 min 9 s using 8  $\mu\text{m}$  membrane, ranged from 1 min 58 s to 3 min 24 s. The next shortest average filtration time was 12 min 8 s for 3  $\mu\text{m}$ , ranged from 11 min 37 s to 14 min 27 s. The average filtration time for 1.2  $\mu\text{m}$  was 14 min 31 s, ranged from 12 min 19 s to 16 min 57 s. The average filtration time for 0.2  $\mu\text{m}$  was 32 min and 6 s, ranged from 28 min to 14 s to 36 min and 12 s. There was no

significant difference between filtration time using membranes with different pore size, except between 0.2 and 8  $\mu\text{m}$  ( $p < 0.05$ ).

The average concentration of total extracted DNA was 0.577 ng/ $\mu\text{L}$  for the 8  $\mu\text{m}$  filter, 2.388 ng/ $\mu\text{L}$  for the 3  $\mu\text{m}$ , 1.404 ng/ $\mu\text{L}$  for the 1.2  $\mu\text{m}$ , and 3.785 ng/ $\mu\text{L}$  for the 0.2  $\mu\text{m}$  (Figure 2). The results showed no significant difference between concentration of extracted total DNA using 1.2, 3, 0.2 and 8  $\mu\text{m}$  filters ( $p > 0.05$ ; Figure 2), excepted between that of 3  $\mu\text{m}$  and 8  $\mu\text{m}$  ( $p < 0.05$ ; Figure 2). There were significantly difference if no identical letters shared between them.

The qPCR result showed that DNA copy numbers of 8, 3, 1.2, 0.2  $\mu\text{m}$  filters were 5.02E+02, 4.97E+02, 1.63E+03, 5.95E+03, respectively. There was no significant difference between them ( $p > 0.05$ , Figure 3).

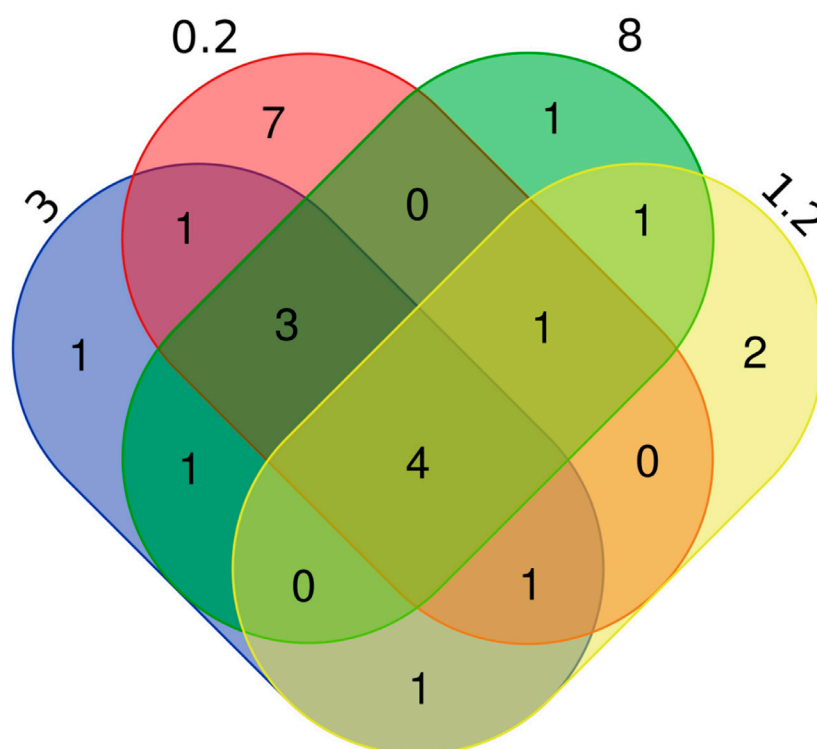
A total of 27 species of fishes were detected in this experiment (Table 2), including four species of the Gobiiformes (14.8%), 15 species of the Cypriniformes (55.6%), three species of the Siluriformes (11.2%), one species of the Perciformes (3.7%), as well as one species each for the Clupeiformes (3.7%), the Mugiliformes (3.7%), the Pempheriformes (3.7%). The fish detected include majorly freshwater fish species, but also some brackish species, which is consistent with that the sampling lake is a freshwater lake having a gated channel connecting to the East China Sea. Seventeen species of fish were enriched in the 0.2  $\mu\text{m}$  treatment, of which seven were specific, *Pseudorasbora parva*, *Planiliza haematocheilus*, *Pseudobrama simoni*, *Hypophthalmichthys nobilis*, *Tridentiger bifasciatus*, *Tridentiger barbatus*, *Squaliobarbus curriculus*. There were ten species of fish obtained in the 1.2  $\mu\text{m}$  treatment, including *Sarcocheilichthys variegatus*, *Lateolabrax maculatus*. There were 11 species of fish enriched in the 8  $\mu\text{m}$  treatment, and the *Coilia nasus* was specifically detected. Twelve fish species found in the eDNA obtained with 3  $\mu\text{m}$  filter, the *Tachysurus fulvidraco* was only found in this filter (Figure 4). Number of species detected in different treatments and PCR replicates can be found in Table 3.

### 3.2 Effect of changing filter membranes on eDNA enrichment

One liter of water sample was filtered with one PCTE membrane or two 0.5 L water samples were filtered separately using one membrane for each. The average concentration of eDNA extracted from 1 L water sample was 1.576 ng/ $\mu\text{L}$  for two membrane trials and 0.4298 ng/ $\mu\text{L}$  for one membrane using the 1.2  $\mu\text{m}$  filter membrane. The average value was 1,337 ng/ $\mu\text{L}$  for a single membrane and 4.056 ng/ $\mu\text{L}$  for two filter membranes in the 3  $\mu\text{m}$  group. The results showed that eDNA concentration was around three times higher using two filter membranes than using one to enrich 1 L of water samples ( $p < 0.05$ ; Figure 5).

### 3.3 Comparison between the stacked-membrane filter and PCTE-only filter

The boxplot showed that mean eDNA concentration from the stacked-membrane filter had a relative wide range than that of the



**FIGURE 4**  
Number of fish species identified in eDNA enriched with different pore sizes. The numbers 0.2, 3, 8, 1.2 represent the different pore sizes (in  $\mu\text{m}$ ) of filtration membranes, showing the number of replicates of species detected between different pore size.

PCTE-only filter. This implies that the results of stacked-filter device were not as consistent as using PCTE-only filters. The PCTE-only filter ( $3\ \mu\text{m}$ ) resulted in an average concentration of  $2.388\ \text{ng}/\mu\text{L}$  of eDNA but stacked-membrane filter generated an average concentration of  $5.875\ \text{ng}/\mu\text{L}$ , which was 2.46 times higher than the former ( $p < 0.05$ ; Figure 6B). The PCTE-only filter ( $0.2\ \mu\text{m}$ ) resulted in an average eDNA concentration of  $3.785\ \text{ng}/\mu\text{L}$ . Again, stacked-filter yielded 1.55 times higher eDNA than using PCTE-only filter ( $p > 0.05$ ; Figure 6A).

The filtration time of the stacked-filter was 8 min 36s in average, which was 4 min shorter than that of the PCTE-only filter ( $p > 0.05$ ). In the  $0.2\ \mu\text{m}$  comparison, we find that the stacked-filter time was reduced by 24 min ( $p > 0.05$ ).

The qPCR results showed that eDNA copy numbers of samples enriched with  $3\ \mu\text{m}$ ,  $0.2\ \mu\text{m}$  and stacked-filters were  $4.79\text{E}+02$ ,  $5.95\text{E}+03$  and  $1.53\text{E}+04$ , respectively. The result showed that there was no significant difference between the  $0.2\ \mu\text{m}$  filters and the stacked filters ( $p > 0.05$ ; Figure 7A) while the difference was a significant between the  $3\ \mu\text{m}$  filters and the stacked filters ( $p < 0.05$ ; Figure 7B).

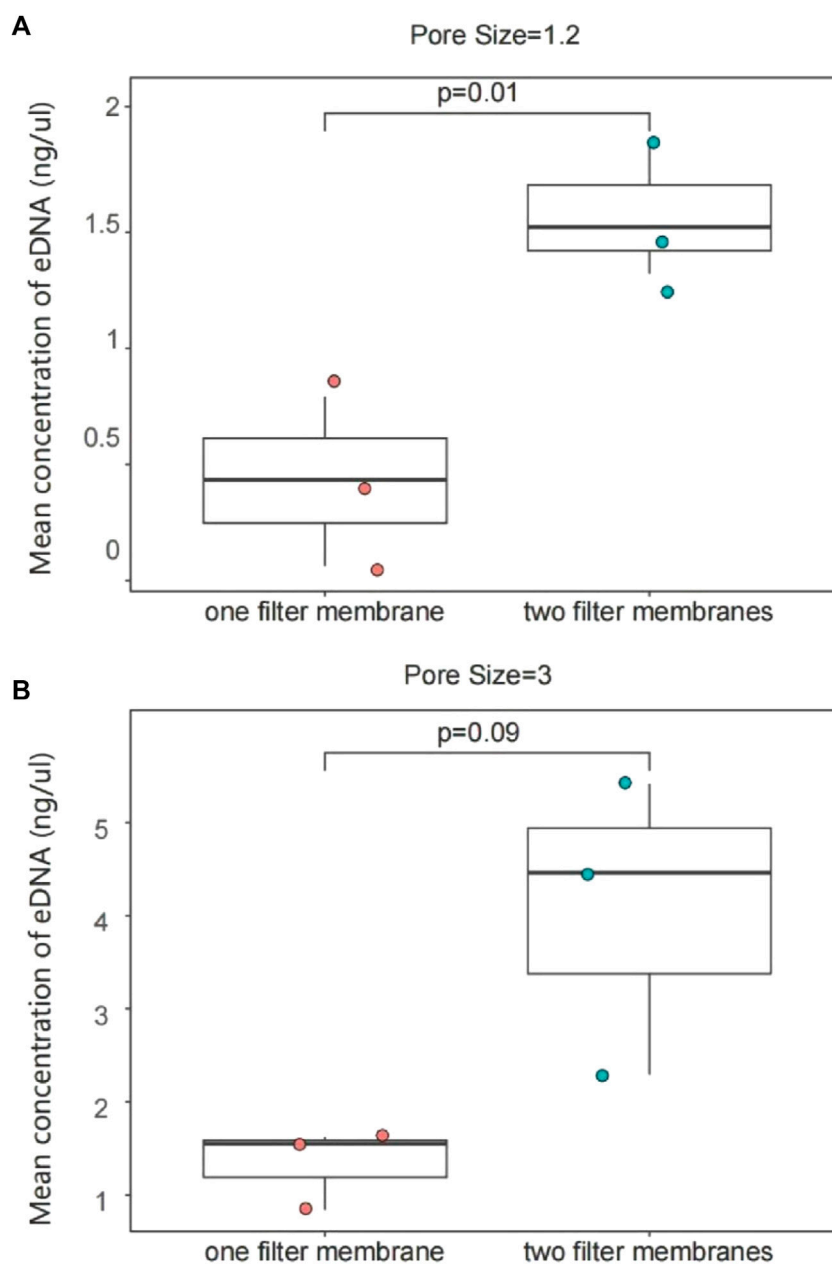
The species detected result shows that sixteen species of fishes were detected in the samples of stacked-membrane filters and twelve species were found in the samples of PCTE-only filters (Table 2). There were eight species shared between the results of stacked-membrane filter and the PCTE-only filter (Figure 8). Species specifically detected by stacked-filter included *Siniperca chuatsi*, *P. haematocheilus*, *P. simoni*, *C. nasus*, *Pseudobagrus albomarginatus*, *Chanodichthys erythropterus*, *Rhinogobius duospilus*, *S. curriculum*. Species only found in the  $3\ \mu\text{m}$  treatment include *Hemibarbus labeo*, *Saurogobio gracilicaudatus*, *Elopichthys bambusa*, *Tachysurus eupogon*.

The result (Figure 9) is based on one sample drawn from fifteen samples, counting 15 draws. Each draw has a corresponding number of species and combining the 25%–75% range of these species is the yellow box. The blue fitted curve behind the yellow box is the fitted curve point confidence interval.

## 4 Discussion

The effect of pore size on the amount of eDNA extracted from the water samples has been studied previously in different ecosystems (Banks et al., 2021; Ma et al., 2022; Turner et al., 2014a; Kumar et al., 2020). It is agreed that the larger pore size of the filter membrane, the shorter filtration time it would cost, but the quantity of eDNA retained may be not optimal. In contrast, small pore size of the membranes can help gathering more eDNA, but the filtration time would be too long (Andruszkiewicz et al., 2017). Our data corroborated this pattern that the  $8\ \mu\text{m}$  membrane used the shortest filtration time comparing to all other pore sizes, but it also obtained the least amount of total eDNA and targeted fish DNA. The amount of eDNA obtained at  $0.2\ \mu\text{m}$  was the highest, but the filtration time was the longest. Although the  $3\ \mu\text{m}$  membrane did not obtain the highest amount of environmental DNA, it was the most consistent performer compared to the  $0.2$  and  $1.2\ \mu\text{m}$  membranes. When detecting concentrations of fish DNA at different pore sizes, the concentration obtained at  $0.2\ \mu\text{m}$  pore size is the highest and the concentration obtained at  $8\ \mu\text{m}$  is the lowest. However, in terms of total DNA extracted and the number of



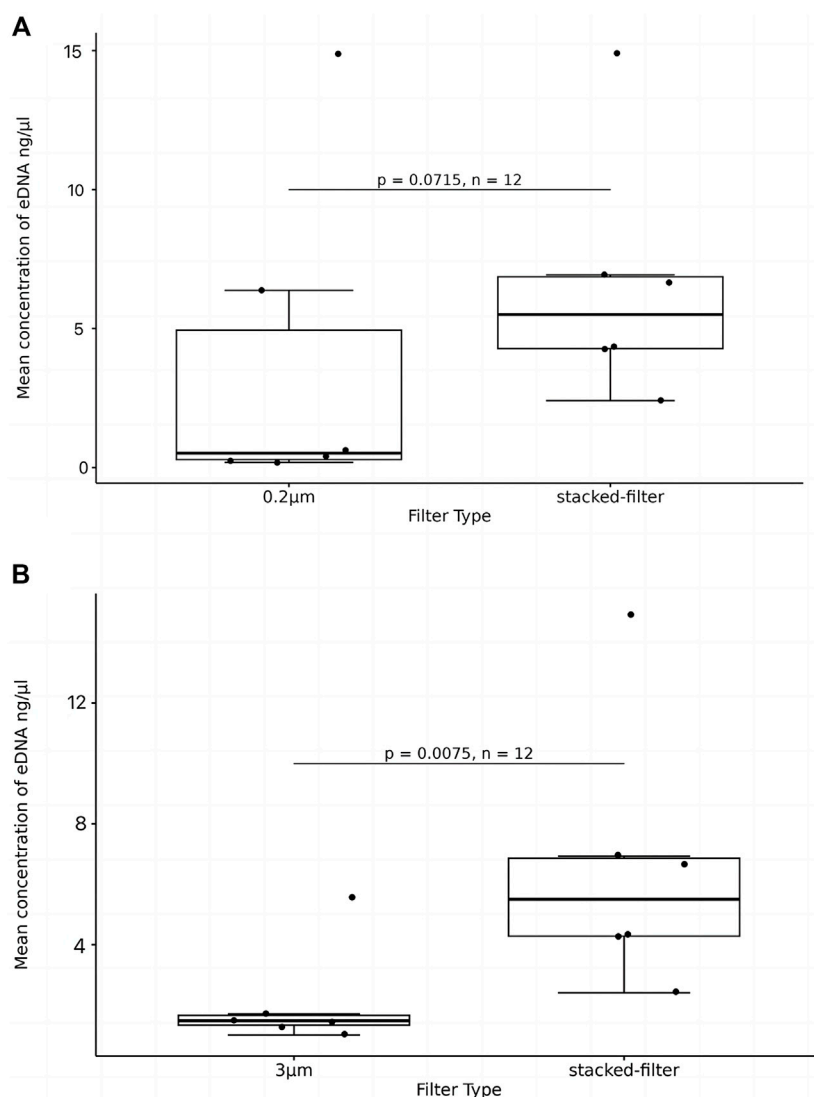


**FIGURE 5** eDNA concentration enriched with one filter membrane or two filter membranes from 1 L of water. 1.2  $\mu\text{m}$  group (A); 3  $\mu\text{m}$  group (B). The results of the eDNA concentration experiments with different numbers of filter membranes between the same pore sizes were interpreted with two sets of plots, AB. (A) The two filter membranes in the 1.2  $\mu\text{m}$  pore size extracted a higher concentration of eDNA; there was also a significant difference; (B) The concentration of eDNA extracted in the 3  $\mu\text{m}$  pore size was also significantly increased. Each sample point in the figure shows the concentration of different replicates.

species detected, 1.2  $\mu\text{m}$  filters performed worse than 3  $\mu\text{m}$  filters. This is contrary to our common perception. We suspected that when the filter membrane was under vacuum pressure for a prolonged period, the integrity of the pore structure of the membrane maybe damaged and small particles maybe lost. The high variance in data collected with 0.2  $\mu\text{m}$  filters also suggests this hypothesis.

Our results showed that when we used two membranes to filter 1 L of water sample sequentially instead of using only one membrane, the amount of eDNA obtained was more than three

times higher. Therefore, when the filtration process is stuck, a long time waiting for the process to complete would not increase the yield of eDNA. This has very significant implications, because the extraction time reported in common practice was often long, e.g., >65 min (Chen et al., 2020), which is bad for eDNA enrichment. Changing membranes during filtration can avoid blocking the pores, but it may also increase the risk of cross-contamination and not easy to operate in the field. Using different filtration devices and materials should help to solve this problem.

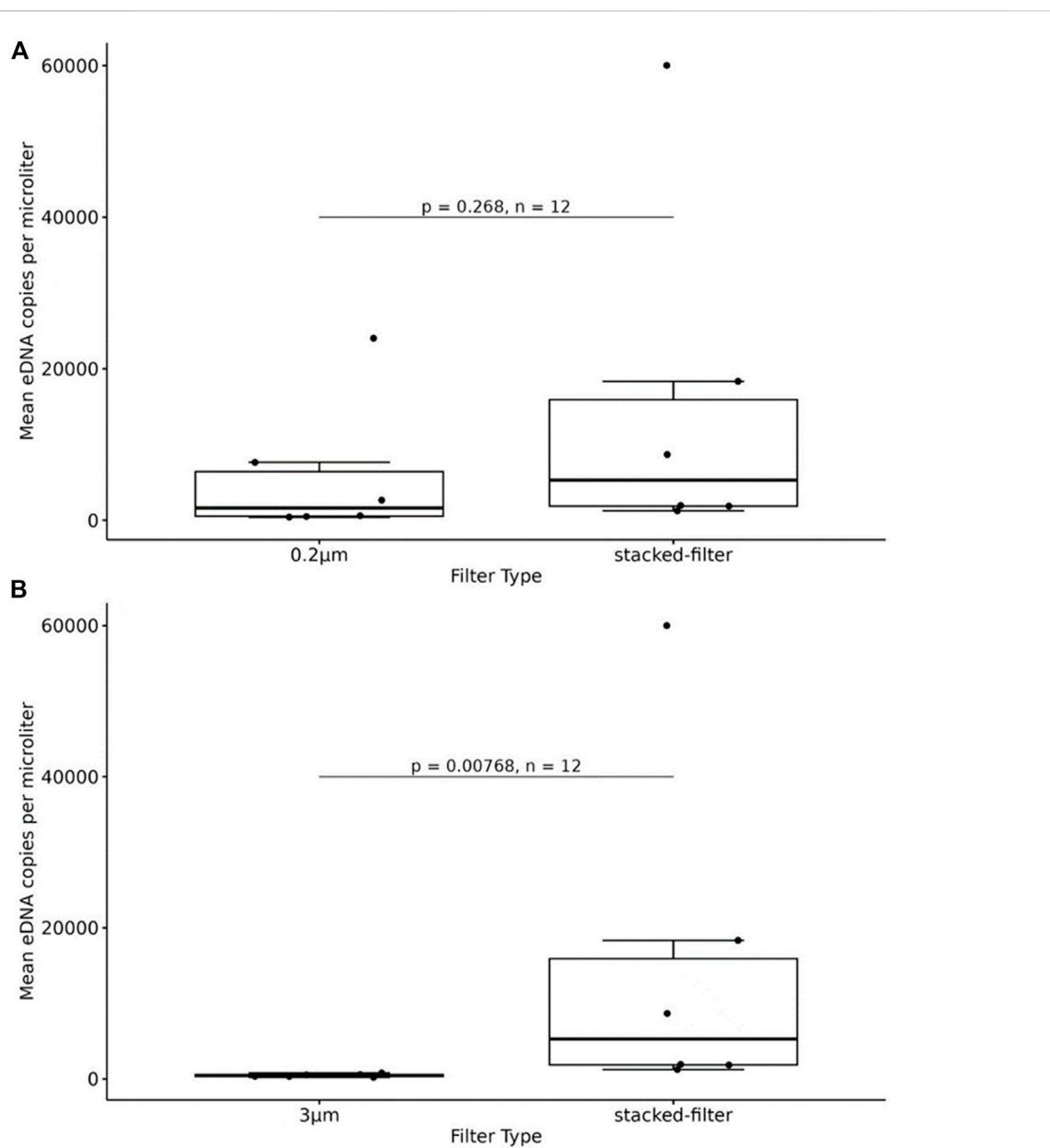


**FIGURE 6**

eDNA concentration enriched with stacked-filters or PCTE-only filters. T-tests were used to compare significant differences and box plots were used to show the results. **(A)** The PCTE-only filter (0.2 μm) resulted in an average eDNA concentration of 3.785 ng/μL. Again, stacked-filter yielded 1.55 times higher eDNA than using PCTE-only filter ( $p > 0.05$ ); **(B)** The PCTE-only filter (3 μm) resulted in an average concentration of 2.388 ng/μL of eDNA but stacked-membrane filter generated an average concentration of 5.875 ng/μL, which was 2.46 times higher than the former ( $p < 0.05$ ).

The quantity of eDNA often is estimated by the concentration of total DNA, not by the DNA of the target taxa, but with some exceptions. For example (Vautier et al., 2023), set an environmental DNA-based qPCR quantification technique to assess the spawning behavior of the target species, European perch and whitefish. Nevertheless, DNA of the target taxa is more relevant, while DNA of other taxa may bias the estimation of eDNA concentration of target taxa. We used qPCR to quantify the eDNA of fish and found that the amount of fish DNA did not differ significantly between samples collected with different pore sizes. In terms of the number of fish species identified, the 3 μm filter resulted in the second highest number of species among the different pore size filters. Combining all evidence, 3 μm would be the best choice of pore size.

Thick filters membranes, such as GF, MCE can hold more particles, but those need to be cut into pieces and incubated with proteinase K for a long time for DNA extraction. The tedious steps of DNA extraction may increase the risk of cross-contamination. It was reported that cross-contamination accounted for 27.01%–83.00% in an eDNA experiment using GF membrane (Wang et al., 2022). The advantage of PCTE membrane is that it is soluble in phenol-chloroform and easy to fit in the DNA extraction protocol without extra manipulation, but the PCTE membrane is easy to be clogged. The stacked-filter we designed not only can filter a large amount of eDNA in a short time, but also can be partially dissolved in phenol chloroform for easy DNA extraction, so to avoid cross-contamination between samples. The average concentration of eDNA enriched with the



**FIGURE 7**

Quantity of fish DNA from samples enriched with the PCTE-only and the stacked-membrane filters, comparing 0.2 μm with stacked-filter. The result showed that there was not a significant difference between the 0.2 μm filters and the stacked filters (A), comparing 3 μm with stacked-filter, the difference was a significant between the 3 μm filters and the stacked filters (B).

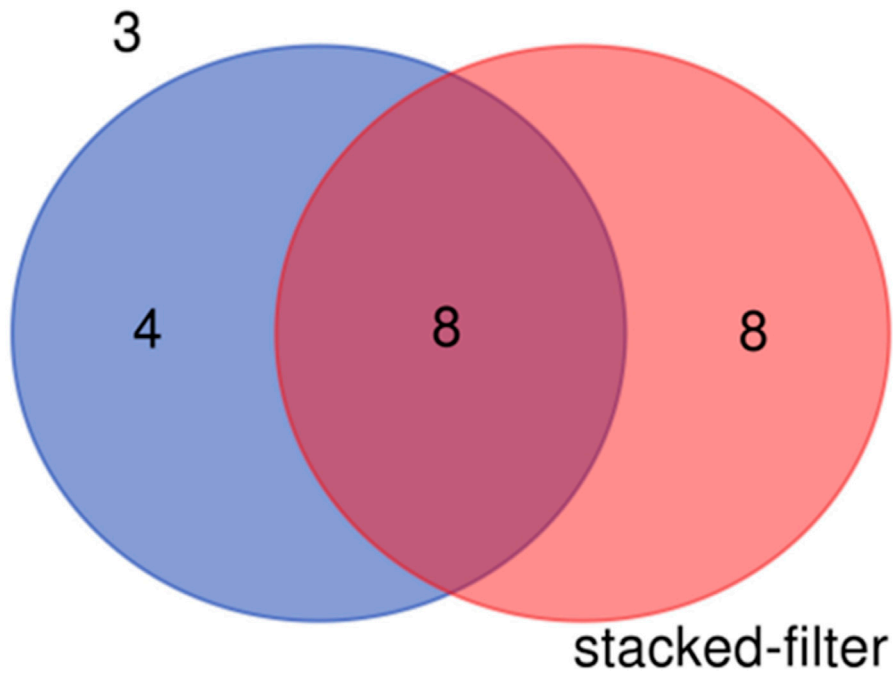
stacked-filter was 1.55 times high than that obtained with PCTE-only filter (0.2 μm) (Figure 8). However, the concentration extracted using stacked-filter had a wider range, between 2 and 14 ng/μL per liter, which may be due to the uneven thickness of the PET pad in the stacked-filter. In the future, different thickness, and materials in addition to PET can be tested to improve the performance of the stacked-filters.

Overall, our results showed that stacked-filter made with phenol-dissolvable material can improve the capture and analysis of environmental DNA. However, cautions should be taken when interpreting the results, since only one type of water body, a campus lake was tested. In future, the filter system should be tested in a range

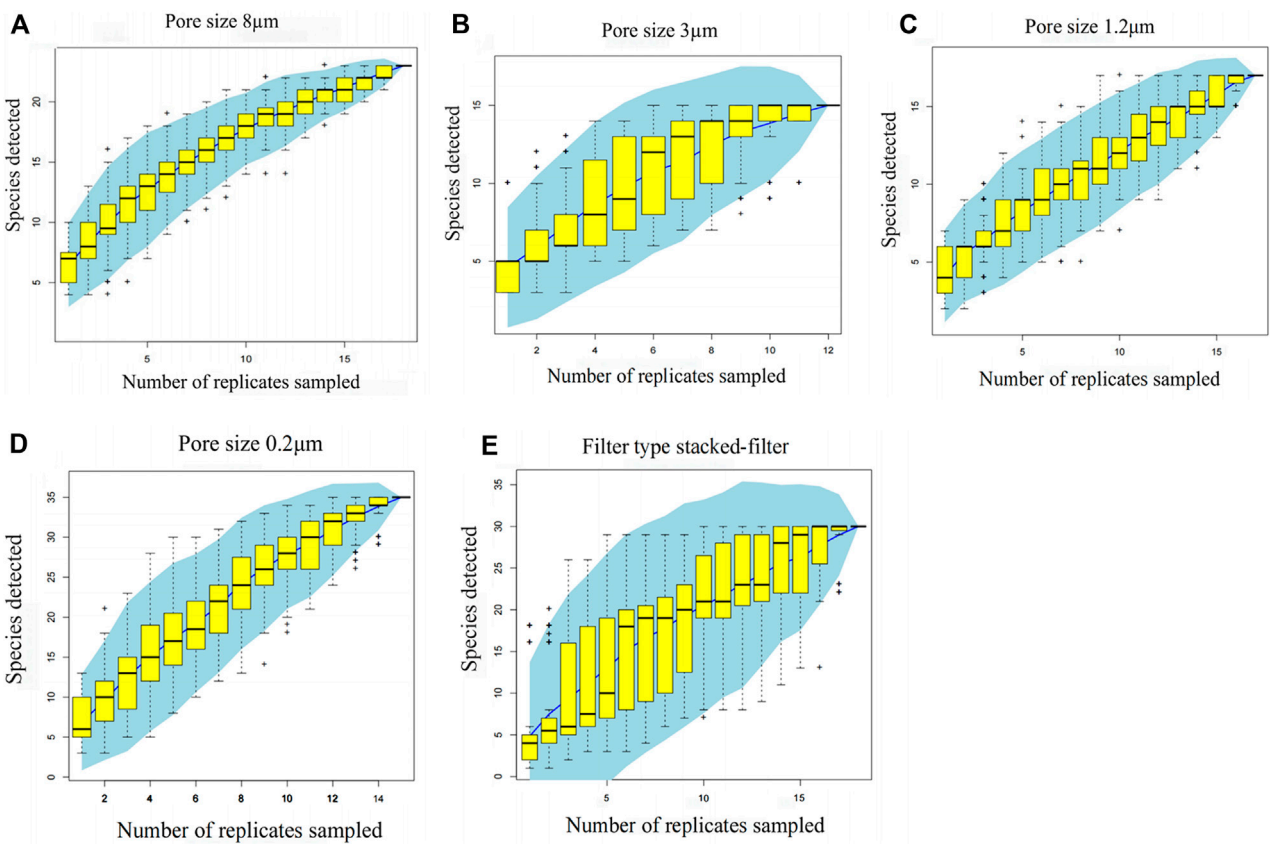
of ecological settings, including both freshwater and marine environments, to better understand its universal applicability.

## 5 Conclusion

Use of 3 μm filter membrane can save time and obtain adequate amounts of eDNA. The stacked-filter designed in this study can trap more particles and hence more eDNA without being stuck. The stacked-filter with a disposable syringe and filter would be useful in the field for fast enrichment of eDNA and for lowering the risk of contamination, which should be evaluated for sampling of eDNA in



**FIGURE 8**  
Fish species detected in the eDNA samples enriched with stacked-membrane filters and PCTE-only filters (3  $\mu\text{m}$ ).



**FIGURE 9**  
Accumulation graphs for different types of filtrations. Pore size 8  $\mu\text{m}$  (A); pore size 3  $\mu\text{m}$  (B); pore size 1.2  $\mu\text{m}$  (C); pore size 0.2  $\mu\text{m}$  (D); filter type stacked-filter (E). The x-axis is the number of samples and the y-axis is the number of species. The result is based on one sample drawn from 15, counting 15 draws. Each draw has a corresponding number of species, and combining the 25%–75% range of these species is the yellow box. The blue fitted curve behind the yellow box is the fitted curve point confidence interval.

different geographical locations, ecological environments, and different turbidity levels of water samples.

## Data availability statement

The original contributions presented in the study are publicly available. This data can be found here: [10.6084/m9.figshare.25838188](https://doi.org/10.6084/m9.figshare.25838188) ([https://figshare.com/articles/dataset/OTU\\_data\\_from\\_filter\\_material\\_of\\_different\\_pore\\_size/25838188](https://figshare.com/articles/dataset/OTU_data_from_filter_material_of_different_pore_size/25838188)).

## Author contributions

QL: Conceptualization, Data curation, Formal Analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing—original draft, Writing—review and editing. JT: Data curation, Formal Analysis, Investigation, Methodology, Writing—review and editing. MW: Data curation, Investigation, Project administration, Supervision, Writing—review and editing. NX: Data curation, Investigation, Methodology, Project administration, Writing—review and editing. RQ: Data curation, Formal Analysis, Writing—review and editing. HW: Conceptualization, Data curation, Formal Analysis, Software, Writing—review and editing.

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## Conflict of interest

CL, QL, HW are authors on a patent applied for by Shanghai Ocean University that covers the stacked-filter designed in this study (202210754981.3).

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