An Optional Low-Cost Method of Extracting Environmental DNA of Macro-Organisms from Filter Membranes in Large Scale eDNA Surveys

Zhi Chen[1], Toshifumi Minamoto[2], Longshan Lin[3] and Tianxiang Gao[4],*

[1]*College of Fisheries and Life Science, Hainan Tropical Ocean University, Sanya 572022, China*

[2]*Graduate School of Human Development and Environment, Kobe University, Kobe 657-8501, Japan*

[3]*Marine Life and Ecology Laboratory, Third Institute of Oceanography, State Oceanic Administration, Xiamen 361005, China*

[4]*Fisheries College, Zhejiang Ocean University, Zhoushan 316022, China*

ABSTRACT

Environmental DNA (eDNA) analysis is a powerful tool within ecology for the distribution or abundance study of aquatic species. DNA extracting was an indispensable process to employ this method. In eDNA extracting of macro-organisms, DNeasy Blood and Tissue Kit was commonly used, but its price was about 80 RMB/sample, which was too expensive. Cheaper eDNA extraction kit was a potential aspect for improvement to expand further application of eDNA analysis. In order to examine some cheaper kits' effectiveness of extracting eDNA of macro-organisms and select an optional low-cost kit, a test among three kits was held. 12S rRNA bands of marine fishes on the agarose gel preliminarily revealed that all three kits could successfully extract eDNA from field water samples. Digital PCR results further revealed that the average eDNA concentration of *Sepiella japonica* extracted by three kits were $(4.62 \pm 0.72) \times 10^5$, $(3.83 \pm 0.56) \times 10^5$, and $(2.35 \pm 0.27) \times 10^5$ copies/300 ml, respectively. The eDNA concentrations by AxyPrep DNA Gel Extraction Kit were about 83% of that extracted by DNeasy Blood and Tissue Kit. However, extracting cost of an eDNA sample using AxyPrep DNA Gel Extraction Kit was about 3.75% of that using DNeasy Blood and Tissue Kit. Up to now, many eDNA surveys have collected eDNA samples with large scale, which sometimes required hundreds of filter membranes and a mass of extraction kits. Combined the price and effectiveness, using AxyPrep DNA Gel Extraction Kit as a substitute for DNeasy Blood and Tissue Kit could remarkably cut the eDNA extracting cost. AxyPrep DNA Gel Extraction Kit might be an optional low-cost method of extracting environmental DNA of macro-organisms from filter membranes in large scale eDNA surveys.

INTRODUCTION

Environmental DNA (eDNA) represented all types of DNA present in the environment, DNA from organisms themselves and extracellular DNA (Ficetola *et al*., 2008; Wilcox *et al*., 2013). eDNA approaches for mapping the distribution of organisms were first used for micro-organisms (Lydolph *et al*., 2005). Recently, they have been applied to macro-organisms (Ficetola *et al*., 2008; Jerde *et al*., 2011; Minamoto *et al*., 2012). It was estimated that macro-organisms released their DNA via egestion, excretion, secretion, exfoliation, reproduction and decomposition (Pilliod *et al*., 2013; Minamoto *et al*., 2017), and that the main form of the eDNA would be derived from organelles (Santas *et al*., 2013;

Turner *et al*., 2014). The presence or absence of the eDNA for a specific organism generally indicated the presence or absence of that organism (Turner *et al*., 2014; Biggs *et al*., 2015), and the eDNA concentration was considered to reflect the biomass or abundance of the target organisms (Takahara *et al*., 2012; Yamamoto *et al*., 2016; Doi *et al*., 2017). To date, eDNA surveys of macro-organisms have been conducted in different aquatic systems (Thomsen and Willerslev, 2015; Doi *et al*., 2015b), such as wetlands (Piaggio *et al*., 2014), tanks (Kelly *et al*., 2014; Minamoto *et al*., 2017), streams (Goldberg *et al*., 2011; Jane *et al*., 2014; Sigsgaard *et al*., 2015), rivers (Goldberg *et al*., 2013; Deiner and Altermatt 2014), ponds (Dejean *et al*., 2012; Treguier *et al*., 2014; Takahara *et al*., 2015), lakes (Egan *et al*., 2013; Jerde *et al*., 2013; Eichmiller *et al*., 2014) and oceans (Foote *et al*., 2012; Pilliod *et al*., 2014; Valentini *et al*., 2016). Although this new method was reported to outperform the traditional survey methods including direct catch by angling and netting, direct observation by diving,

and indirect observation by filming and echo sounding (Olson *et al*., 2013; Thomsen and Willerslev, 2015; Minamoto *et al*., 2017), there were still many problems that needed improvement.

For example, to get more reliable data about macro-organisms, researchers sometimes collected eDNA samples in triplicate at each site (Margaret *et al*., 2015; Matthew *et al*., 2015; Merkes *et al*., 2015), which would no doubt increase the cost of eDNA collecting. Besides, DNeasy Blood and Tissue Kit (Qiagen) were universally used to extract eDNA from filter membranes (Takahara *et al*., 2013; Thomsen and Willerslev, 2015; Ikeda *et al*., 2016; Minamoto *et al*., 2017). This kit provided fast and easy silica-based DNA purification in convenient spin-column and 96-well-plate formats. Most tissue and blood samples can be normally extracted according to operating instruction. However, double or more dosage of buffer AL was used because of the obvious water-absorbing quality of filter membranes (Takahara *et al*., 2012; Doi *et al*., 2015a, b; Fukumoto *et al*., 2015; Thomsen and Willerslev, 2015). The price of DNeasy Blood and Tissue Kit (50 times) was usually 2000 RMB, double dosage and triplicate meant approximate 80 RMB/sample and only 8 or 9 sampling sites that such a kit could cover. A large eDNA surveys usually consisted of several temporal times (Dejean *et al*., 2011; Turner *et al*., 2014; Bista *et al*., 2017), and dozens even hundreds of spatial sampling sites (Takahara *et al*., 2012; Yamamoto *et al*., 2016), and sometimes each site was sampled at two different section (surface and bottom) (Yamamoto *et al*., 2016). As a result, a lot of money was spent in eDNA extraction part. Cheaper and efficient method of extracting eDNA of macro-organisms was therefore a significative step in eDNA surveys.

Here, we examined certain cheaper kits, AxyPrep DNA Gel Extraction Kit (Axygen) and Tissue DNA Kit (Omega bio-tek) as the potential DNeasy Blood and Tissue Kit replacer. We used these kits to extract eDNA from filter membranes after filtration. To better reveal the effectiveness of three kits, digital PCR (dPCR) was used by figure out the number of *Sepiella japonica* DNA copies. The reason why we chose *Sepiella japonica* as our target species was that although this species has important economic value and was suffering severe resource declining, there was no eDNA study of *Sepiella japonica*. More sensitive and convenient eDNA resource surveys of *Sepiella japonica* are necessary to be carried out in the future. In present study, both field and indoors water samples were tentatively collected. Because field water was complicated and there was a lot of uncertainty of eDNA distribution (Roussel *et al*., 2015), sometimes specific-target digital PCR (dPCR) of field water might can't be put into effect. If dPCR results revealed that there

was no eDNA copy of *Sepiella japonica*, we would use nonspecific-target agarose electrophoresis to measure the total eDNA of field water samples. Sediment concentration had a severe influence on eDNA yield and varied significantly between water samples (Ficetola *et al*., 2008; Thomsen and Willerslev, 2015). So, we simultaneously measured the sediment concentration of water samples to determine our experiment. We hope this kits' comparison result would cut down the extracting cost and contribute to the subsequent eDNA surveys in the future.

MATERIALS AND METHODS

Species-specific primers and probe design

Cytochrome c oxidase I (COI) sequences of *Sepiella japonica* as well as all Cephalopoda species existing in the coastal waters of Zhoushan were downloaded from Genbank. To give consideration to both the efficiency and species-specificity of primers and probe, we constantly adjusted the parameters (position, length, Tm and so on) of Primer Express 3.0.1 software (Thermo Fisher Scientific, Waltham, MA). Ultimately, the sequences of the designed specific primers and probe were listed as follows: F-2317: 5'-CACCAGACATAGCCTTCC-3'; R-2471: 5'-GCCAGCATGAGATAGATTAC-3'; and Pro-2432: 5'-HEX-TGTTCATCCAGTTCCAGCACCT-TAMRA-3'. To check the specificity of the primers and probe, we performed digital PCR separately by using the DNA of *Octopus variabilis*, *Octopus ocellatus*, *Sepia esculenta*, *Sepiella japonica*, *Loligo kobiensis*, *Sepiola birostrata* and *Loligo beka* (the common species in Zhoushan coastal waters). The digital PCR conditions were described in section 2.5. The digital PCR results confirmed their species-specification.

Water sampling and filtration

Water sampling was conducted in Zhoushan City, Zhejiang Province, because its close distance to laboratory could benefit eDNA transportation, preservation and subsequently filtration. In consideration of difference between field and indoor water environment, both field and indoors studies were carried out. Exactly, four nonspecific-target water sampling sites (not sure whether there was *Sepiella japonica* or not) were set throughout the field river (sample 1 to sample 2) and sea (sample 3 to sample 4) and one water sampling sites (sample 5) specially targeting at *Sepiella japonica* was set at a breeding base (located in Xixuan island, a professional *Sepiella japonica* farm) (Fig. 1). The water sampling started at April 17, 2017 and ended at the same day. Briefly, water samples were collected from the water surface using a bucket and then parallelly divided into 12 plastic cups (three kits

group × triplicate at each site, and the rest three were used to measure sediment concentration of every sample (in section 2.3); volume of all plastic cup was 1.5 L). To minimize cross-contamination, the bucket was bleached using 0.1% sodium hypochlorite for about ten minutes before sampling and washed twice or more with surface water at each sampling site and was then placed in water for several minutes before each water collection (Yamamoto *et al*., 2017). All water samples were simply added the chemicals benzalkonium chloride, at a final concentration of 0.01% to suppress the degradation of eDNA and immediately transported to our laboratory (Yamanaka *et al*., 2017). All water samples were filtered through WCN filters (Whatman Cellulose Nitrate Membrane Filters, 7184-004) of 0.45 µm average pore size. Two sets of filtration equipment (Combisart 3-branch stainless steel manifold, GM-0.33A pump) were used to accelerate the process of experiment. A total of 300 ml of every sample was uniformly filtered because later blocking made some samples cannot be filtered more. The filter funnels and measuring cups used for filtration were also bleached after filtration using 0.1% sodium hypochlorite. In addition, to verify the effectiveness of the bleaching, we filtered artificial seawater with every filter funnel and measuring cup at every site (equipment negative control) (Yamamoto *et al*., 2016).

Measurements of sediment concentration

Sediment concentration had a severe influence on eDNA yield (Turner *et al*., 2014), and in whole coastal of Zhoushan, sediment concentration was high everywhere. Thus it was necessary to consider the influence of sediment concentration. Before extraction, dry weight of each filter membrane was determined by a SECURA225D-1CN electronic balance (Sartorius Mechatronics, weighing range: 0-120g; degree of accuracy: 0.01mg). Afterwards, the water samples targeting at sediment concentration measurements were filtrated as those described in section

2.2. Then, these filter membranes were dried completely in an electro-thermostatic blast oven (Shanghai Hasuc Instrument Manufacture Co., Ltd. DGH-9055A) at 56°C. The above electro-thermostatic blast oven measured these filter membranes again to determine the sediment concentration of water samples.

DNA extraction

In this step, filter samples were subjected to DNA extraction following the method referring to Miya *et al*. (2015), the specification of AxyPrep DNA Gel Extraction Kit and Tissue DNA Kit, respectively. Briefly, each filter was rolled into a cylindrical shape using sterile forceps and placed into a spin column (EZ-10 Spin Column & Collection Tube; Bio Basic Inc., Ontario, Canada). The silica membranes of the columns were removed before use. The columns were centrifuged at 6000g for 1 min and excess water on the filter recovered as the filtrate was discarded. As for DNeasy Blood and Tissue Kit, AxyPrep DNA Gel Extraction Kit, and Tissue DNA Kit, the reagent names and dosage of each step were listed, respectively as follows (three brackets in following steps corresponded with three kits):

1: (200µl ultrapure water, 200 µl buffer AL and 10 µl proteinase K) or (400 µl buffer DE-A and 10 µl proteinase K) or (200 µl ultrapure water, 200 µl buffer TL and 10 µl proteinase K) were dispensed onto the filter, and the spin columns were then incubated at 56°C for 30 min.

2: The spin columns were centrifuged for 1 min at 6000g to elute the filtrate, and this elution was moved to new 1.5-ml micro-tubes.

3: Then, 400 µl of TE buffer was added to each filter and incubated for 1 min at room temperature. Spin columns were centrifuged for 1 min at 6000g to recover any DNA remaining on the filters. The first elutions were then returned to the 2-ml collection tubes containing the second elutions.

4: Then, 200 µl (buffer AL) or (buffer DE-B) or (buffer

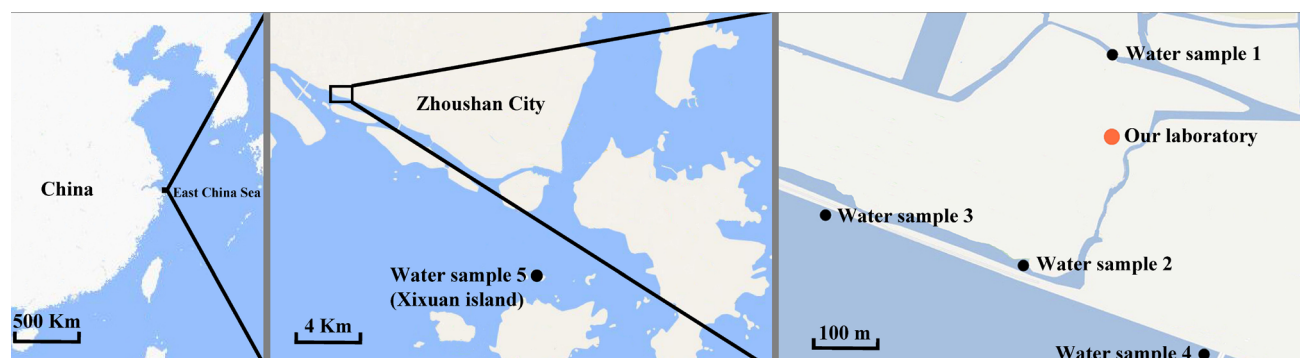
Fig. 1. Water sampling sites of this study. From left to right, the map was gradually enlarged.

BL) and 610 µl ethanol were added to each collection tube and mixed well by pipetting. eDNA in each solution was collected and purified using the three different kits by centrifuging the solution by using the provided respective spin columns in three stages because of the large volume of each filtrate.

5: The silica-gel membrane was washed two times using washing buffers (AW1 and AW2) or (W1 and W2) or (HBC and DNA Wash buffer), according to the manufacturer's instructions.

6: DNA was eluted from the spin columns with 100 µl of the provided (AE) or (Eluent) or (Elution) buffer.

PCR and digital PCR (dPCR)

We performed dPCR by using the *Sepiella japonica* primers and probe. The detail of dPCR was followed the method referring to Doi *et al*. (2015a, b). Each dPCR reaction mixture (20 µl) contained 2 µl of DNA template, 900 nM of each primer, and 125 nM TaqMan probe in a 1 × Bio-Rad Supermix (Bio-Rad, Hercules, CA, USA), which was mixed with Bio-Rad droplet generator oil and partitioned into 15,000–20,000 droplets by using the Bio-Rad QX-100 droplet generator (Bio-Rad). The droplets of individual samples were separately applied to each well of a 96-well PCR reaction plate. PCR was performed in the 96-well plate sealed with pierceable sealing foil by using the Gene Amp 9700 thermocycler (Life Technologies). For dPCR analysis, triplicated PCR negative controls were used in which 1 µl ultrapure water was added to each reaction instead of the eDNA template. PCR conditions were 10 min at 95°C, 40 cycles of denaturation for 30 s at 95°C and extension for 60 s at 60°C with ramp rate of 2.5°C/s, followed by 10 min at 95°C and a hold at 4°C. After PCR amplification, the plate was transferred to the Bio-Rad QX-100 droplet reader (Bio-Rad). Each droplet in the well was checked for TaqMan fluorescence to count the number of droplets that yielded positive/negative results. We used Bio-Rad's Quanta Soft software version 1.3.2.0 to quantify the copies of target DNA. Threshold for a positive signal was determined according to the Quanta Soft instructions. Any droplet beyond the fluorescence threshold was counted as a positive event. We used the mean and standard deviation values for statistical analyses.

At the beginning of our study, we intended to uniformly use eDNA copies of *Sepiella japonica* in both field and indoors water samples to evaluate the extraction efficiency of three kits. However, in whole coastal of Zhoushan, sediment concentration was high everywhere and resulted in rare marine-life survival (Jiang and Guo, 2016). Some previous experimental results also revealed no *Sepiella japonica* inhabiting in these field waters. Thus if dPCR results revealed that there was no eDNA copy

of *Sepiella japonica*, we would use nonspecific-target agarose electrophoresis to measure the total eDNA of field water samples (nonspecific-target meant much more total eDNA could be amplified and would show a more manifesting result). A professional set of universal PCR primers (12S rRNA) for metabarcoding environmental DNA from fishes was used to analyze these 4 field water samples by seeing their agarose gel. The sequence of this universal PCR primers used in this study were: MiFish-U-F (5'-GTCGGTAAAACTCGTGCCAGC-3') and MiFish-U-R (5'-CATAGTGGGGTATCTAATCCCAGTTTG-3'). These two primers were professionally designed for eDNA of teleost fishes (Miya *et al*., 2015). PCR was carried out in a 25 µl reaction mix containing DNA template (1 µl, 50 ng/µl), forward primer (MiFish-U-F, 1 µl, 10 µM/L), reverse primer (MiFish-U-R, 1 µl, 10 µM/L), dNTPs (2 µl, 2.5 mM/L each), EasyTaq DNA Polymerase (0.15 µl, 5 U/µl) and 10 × PCR buffer (2.5 µl, 25 µM/L). A Biometra thermal cycler (Gottingen, Germany) with the following given procedure: one initial denaturation (95°C, 3 min), 35 cycles consisting of denaturation (94°C, 20 s), annealing (50°C, 20 s) and extension (72°C, 20 s), and one final extension (72°C, 3 min), was employed to put PCR amplification into effect. All 12 DNA template samples extracted from 4 field water samples were run in duplicate. Triplicated PCR negative controls were also used in which 1 µl ultrapure water was added to each reaction instead of the eDNA template. 1% agarose gel was employed to check the existence of target fragments. DNA bands were visualized by staining with ethidium bromide.

Statistical analysis

All data on sediment concentration and DNA yield were calculated as milligram (mg) and DNA copies per sample water volume, respectively, and used for statistical analyses. The average concentrations of sediment in five water samples and eDNA of *Sepiella japonica* in the three kits groups were compared by analysis of variance (ANOVA) followed by the Tukey–Kramer post hoc test using R ver. 3.0.2 (R Core Team 2013). The minimum level of significance was set at $p \leq 0.05$.

RESULTS

Sediment concentration

The measured sediment concentrations of five water samples (1 to 5) were: 16.33 ± 2.57, 9.3 ± 1.88, 50.23 ± 3.27, 50.5 ± 1.97, and 27.63 ± 7.06 mg/300 ml water (mean ± standard deviation), respectively (Fig. 2). ANOVA followed by a post hoc test revealed no difference between sample 3 and sample 4 ($p = 0.959$), but did reveal a significant difference in all rest combinations ($p < 0.001$).
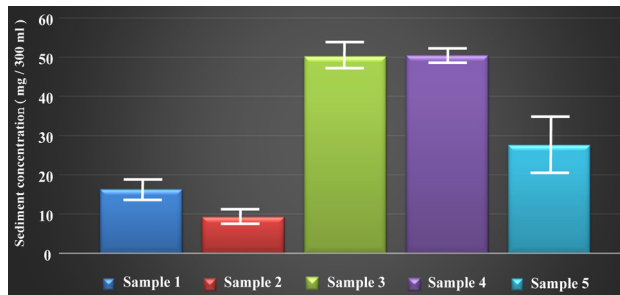
Fig. 2. Sediment concentration of all water samples.

DNA copies of water samples

Blank samples showed negative results for DNA copies. Results of dPCR revealed that there was no *S. japonica* DNA copy in the all four field water samples. The average *Sepiella japonica* DNA concentration (mean ± standard deviation) of indoors water samples (water simple 5) extracted by DNeasy Blood and Tissue Kit, AxyPrep DNA Gel Extraction Kit and Tissue DNA Kit were $(4.62 ± 0.72) × 10^5$, $(3.83 ± 0.56) × 10^5$, and $(2.35 ± 0.27) × 10^5$ copies/300 ml aquaculture water, respectively (Fig. 3). ANOVA analysis revealed no difference between the DNeasy Blood and Tissue Kit and AxyPrep DNA Gel Extraction Kit groups ($p = 0.061$), but did reveal a difference between the DNeasy Blood and Tissue Kit and Tissue DNA Kit groups ($p < 0.001$), and between AxyPrep DNA Gel Extraction Kit and Tissue DNA Kit groups ($p = 0.032$). In comparison with the DNeasy Blood and Tissue

Kit filtration group, the eDNA concentrations of AxyPrep DNA Gel Extraction Kit and Tissue DNA Kit groups were reduced to 83% and 51%, respectively.
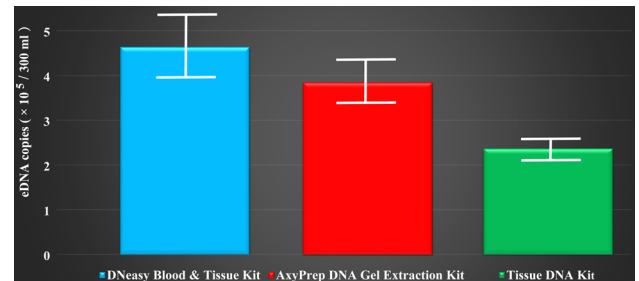

Fig. 3. The DNA yield (copies/300 ml) of *Sepiella japonica* extracted from aquaculture water (indoors water sample). There was no difference in eDNA yield between the DNeasy Blood and Tissue Kit and AxyPrep DNA Gel Extraction Kit groups ($p=0.061$).

Agarose gel electrophoresis

One amplification of water sample 1 extracted by AxyPrep DNA Gel Extraction Kit failed. Others of water sample 1 and sample 2 had a clear and bright major band. Length of PCR products was about 220 bp, which were consistent with the description of this universally PCR primers designed by Miya *et al.* (2015). Water sample 3 and 4 did not amplify at all in all three kits group. Gels in Tissue DNA Kit group showed not only a major band but also some obvious smear bands in the same lane.
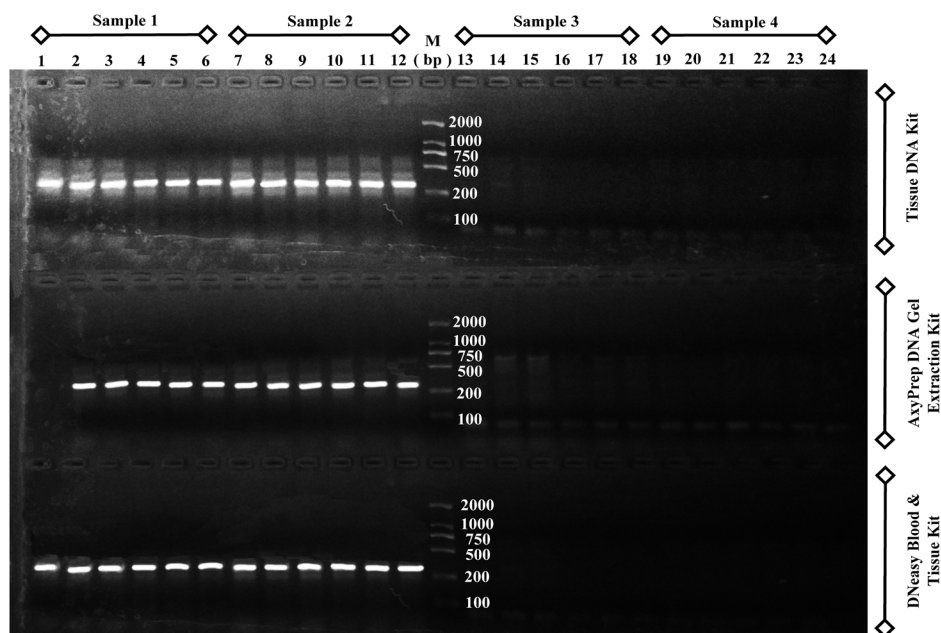

Fig. 4. Agarose gel electrophoresis results of 4 field water samples. M, DNA marker.

DISCUSSION

From the agarose gel image (Fig. 4), all three kits obtained similarly bands. Apart one well of water sample 1 that was not amplified due to certain unknown reason, when DNeasy Blood and Tissue Kit group showed clear and bright major bands in water sample 1 and sample 2, AxyPrep DNA Gel Extraction Kit and Tissue DNA Kit synchronously showed; when AxyPrep DNA Gel Extraction Kit and Tissue DNA Kit could not extract eDNA from water sample 3 and sample 4, DNeasy Blood and Tissue Kit also failed in the same lane position. This fact roughly but clearly suggested that AxyPrep DNA Gel Extraction Kit and Tissue DNA Kit might be able to replace DNeasy Blood and Tissue Kit as the method of extracting environmental DNA from filter membranes. More specifically, lane 1 to lane 12 of Tissue DNA Kit group mingled with smear bands, but those of AxyPrep DNA Gel Extraction Kit and DNeasy Blood and Tissue Kit group didn't, which indicated using Tissue DNA Kit to extract eDNA might produce more impurities.

Sediment concentration had a severe influence on eDNA yield, which was also been explored by Turner *et al.* (Turner *et al.*, 2014). Sampling site 3 and 4 were directly located in the coast of open sea. A sturdy dam was built along this coast and thus resulted in the sediment reduction of sample 1 and 2. Sediment concentration of sample 3 and sample 4 had no difference ($p = 0.959$), but was 3.08 and 5.41 times higher than sample 1 and sample 2 ($p < 0.001$) (Fig. 2), respectively. High sediment concentration made against fish survival (Watts *et al.*, 2003), caused much physical damage to DNA molecule and brought about more extraction impurities. Such highly turbid water also usually contained many PCR inhibition substances. Some fisherman also caught fish by gill nets and cage nets in this area. It was confirmed that fish species existed in the coast of this open sea. The 12S primers were extremely universal as well, which were applicable for 70 families and 152 genera marine fishes at least (Miya *et al.*, 2015). Thus, although we did not further determine what substances they are, it was reasonably inferred that inhibition substances and extraction impurities cannot be completely removed by three kits. As the final reflection of low eDNA yield and high PCR inhibition, in agarose gel, no matter what kits were used to extract water sample 3 and 4, no bands appeared in the lane position of these samples. Although sample 1, 2 and 5 also had a heave weight, the component of them might be organic substance rather than sediment. Because after filtration, color of filter membrane was sandy brown of sample 3 and 4, but slight greenish-yellow of sample 1 and 2 and light black of sample 5.

The digital PCR study suggested that water samples for eDNA analyses could be optionally extracted from filter membranes by using AxyPrep DNA Gel Extraction Kit. On average, DNeasy Blood and Tissue Kit appeared to be the most effective and Tissue DNA Kit was the worst. In digital PCR experiment of *Sepiella japonica*, which focused on DNA copies, reductions in DNA concentration were both observed in AxyPrep DNA Gel Extraction Kit and Tissue DNA Kit group comparing to DNeasy Blood and Tissue Kit. This revealed that the chemical components and physic material might exist some difference in these three kits, and such difference caused the extraction effectiveness variation. However, all three kits were effective in extracting DNA of water samples, as confirmed by the results of PCR and digital PCR. Although the yield of eDNA extracted by DNeasy Blood and Tissue Kit was 1.21 times higher than that extracted by AxyPrep DNA Gel Extraction Kit, no difference between these two groups ($p = 0.061$) was revealed.

Different from the popular and thoroughly developed use of DNeasy Blood and Tissue Kit (Thomsen *et al.*, 2012; Takahara *et al.*, 2013; Miya *et al.*, 2015; Doi *et al.*, 2017; Minamoto *et al.*, 2017), as far as we know, no people before us used AxyPrep DNA Gel Extraction Kit to carry out the eDNA extraction of macro-organisms. The operation procedure of AxyPrep DNA Gel Extraction Kit could promisingly improve in the future. As some studies had revealed, the length of eDNA dissolved in waters was pretty incomplete and short (Deiner *et al.*, 2015; Jo *et al.*, 2017; Tsuji *et al.*, 2017). AxyPrep DNA Gel Extraction Kit also specifically noted that if the DNA fragment was < 400 bp, isopropanol should also be added when extracting. In our present studies, we did not use isopropanol, which might pull down the performance of AxyPrep DNA Gel Extraction Kit. Besides, the incubation object of AxyPrep DNA Gel Extraction Kit was mainly agarose (saccharides), whereas DNA molecule was most wrapped in biological membrane of cells, organelles or tissue fragments consisting of lipid, protein and so on (Deiner *et al.*, 2015; Tsuji *et al.*, 2017). Unified 10 ul proteinase K in AxyPrep DNA Gel Extraction Kit might was not enough to incubate biological membrane. Because as the name suggested, DNeasy Blood and Tissue Kit itself had tissue incubation ability but AxyPrep DNA Gel Extraction Kit didn't. The extraction efficiency of DNA from filter samples might be improved by adding more proteinase K.

Based on the efficiency and promising improvement in the future, the price of kits was a significant factor affecting kit selection in eDNA studies. A large scale eDNA surveys usually consisted of several temporal times (Dejean *et al.*, 2011; Turner *et al.*, 2014; Bista *et al.*, 2017), dozens even hundreds of spatial sampling sites (Takahara *et al.*, 2012; Yamamoto *et al.*, 2016), and each site was

also sampled at two different section (surface and bottom waters) (Yamamoto *et al.*, 2016). Sometimes, triplicate was also done at every sample site (Yamamoto *et al.*, 2016). Thus, such a survey need hundreds to thousands filter membranes to filtrate. The price of DNeasy Blood and Tissue Kit (50 times), AxyPrep DNA Gel Extraction Kit (250 times) and Tissue DNA Kit (50 times) was respectively 2000 RMB, 750 RMB and 550 RMB. Attention should also be paid to DNeasy Blood and Tissue Kit, because double dosage of buffer AL was used when extracting (see section 2.5, or Takahara *et al.*, 2012; Miya *et al.*, 2015; Yamamoto *et al.*, 2016; Minamoto *et al.*, 2017 and so on). Thus the average price of one filter membrane extraction was respectively 80 RMB, 3 RMB and 11 RMB. Tissue DNA Kit had the worst eDNA extraction efficiency, its price was also disadvantaged. DNeasy Blood and Tissue Kit was the most effective kit, but it was too expensive. In small-scale eDNA studies, DNeasy Blood and Tissue Kit might be best; but in large scale eDNA surveys, combined the extraction efficiency, improvement prospect and use cost, we thought AxyPrep DNA Gel Extraction Kit was also a good choice.

CONCLUSION

AxyPrep DNA Gel Extraction Kit as an substitute for DNeasy Blood and Tissue Kit could remarkably cut the extracting cost in eDNA surveys. AxyPrep DNA Gel Extraction Kit might be an optional low-cost method of extracting environmental DNA of macro-organisms from filter membranes in large scale eDNA surveys.

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Statement of conflict of interest
The authors have declared no conflict of interest.

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