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Optimization of environmental DNA analysis using pumped deep-sea water for the monitoring of fish biodiversity

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Deep-sea ecosystems present difficulties in surveying and continuous monitoring of the biodiversity of deep-sea ecosystems because of the logistical constraints, high cost, and limited opportunities for sampling. Environmental DNA (eDNA) metabarcoding analysis provides a useful method for estimating the biodiversity in aquatic ecosystems but has rarely been applied to the study of deep-sea fish communities. In this study, we utilized pumped deep-sea water for the continuous monitoring of deep-sea fish communities by eDNA metabarcoding. In order to develop an optimum method for continuous monitoring of deep-sea fish biodiversity by eDNA metabarcoding, we determined the appropriate amount of pumped deep-sea water to be filtered and the practical number of filtered sample replicates required for biodiversity monitoring of deep-sea fish communities. Pumped deep-sea water samples were filtered in various volumes (5–53 L) at two sites (Akazawa: pumping depth 800 m, and Yaizu: pumping depth 400 m, Shizuoka, Japan) of deep-sea water pumping facilities. Based on the result of evaluations of filtration time, efficiency of PCR amplification, and number of detected fish reads, the filtration of 20 L of pumped deep-sea water from Akazawa and filtration of 10 L from Yaizu were demonstrated to be suitable filtration volumes for the present study. Fish biodiversity obtained by the eDNA metabarcoding analyses showed a clear difference between the Akazawa and Yaizu samples. We also evaluated the effect of the number of filter replicates on the species richness detected by eDNA metabarcoding from the pumped deep-sea water. At both sites, more than 10 sample replicates were required for the detection of commonly occurring fish species. Our optimized method using pumped deep-sea water and eDNA metabarcoding can be applied to eDNA-based continuous biodiversity monitoring of deep-sea fish to better understand the effects of climate change on deep-sea ecosystems.

KEYWORDS

eDNA, biodiversity, deep-sea water, fish monitoring methods, metabarcoding

1 Introduction

The deep sea, which comprises water depths below 200 m, is the largest ecosystem on Earth and has unique characteristics which support one of the highest levels of biodiversity in aquatic environments while harboring an ecosystem distinct from other marine and land-based ecosystems (Ramirez-Llodra et al., 2010; Costello and Chaudhary, 2017). With its large volume, the deep sea remains poorly understood, as there are several difficulties in tackling it: the access to and investigation of deeper oceanic waters are still limited (e.g., high cost, logistic difficulties, and limited opportunities for sampling). Thus, biodiversity in the deep sea has still not been fully elucidated (Ramirez-Llodra et al., 2010; Webb et al., 2010; Mora et al., 2011; Costello and Chaudhary, 2017; Fujiwara et al., 2021). Therefore, there is an urgent need to assess the biodiversity of deep sea ecosystems including biodiversity loss by climate change and anthropogenic activities through continuous monitoring.

Environmental DNA (eDNA) analysis for metabarcoding of macroorganisms using next-generation sequencing has been utilized for monitoring of biodiversity within aquatic ecosystems (Taberlet et al., 2012; Miya et al., 2015; Valentini et al., 2016; Vences et al., 2016). eDNA metabarcoding for simultaneous and highly sensitive detection of multiple species aimed at understanding species presence/absence, distribution of species, biomass estimates, and detection of introduced species, have been examined in various aquatic environments including oceans (Thomsen et al., 2012; Yamamoto et al., 2016; Weltz et al., 2017; Kelly et al., 2018; Ushio et al., 2018; Knudsen et al., 2019). As fish are the most diverse vertebrates, and include large predators with higher trophic levels, they are one of the key components of aquatic ecosystem functioning through the food web (Albouy et al., 2019). They are also sensitive to climate change and human activities (e.g., commercial fishing) (Gallo et al., 2020; Huang et al., 2021; Baag and Mandal, 2022).

Unfortunately, investigation of the deep sea is not easy to conduct and expensive to carry out, thus the opportunities for continuous biodiversity monitoring are limited. As a means of solving this issue, we focused on pumped deep-sea water as an eDNA source. More than ten deep-sea water pumping facilities for commercial and research purposes are located in Japan. Pumped deep-sea water is more easily accessible than the deep-sea water collected using water sampling devices (e.g., CTD with Niskin bottles) operated from a research vessel and is available at all times throughout the year on land. The most important advantage of pumped deep-sea water is that the continuous collection of a large amount of deep-sea water as required is possible. Thus, we utilized it as a source of eDNA for elucidating and continuous monitoring of the biodiversity in deep-sea fish in this study.

In the deep sea, eDNA analysis has focused on the sediments to survey the benthic communities (e.g., Guardiola et al., 2016; Sinniger et al., 2016; Laroche et al., 2021). On the other hand, a few surveys of aquatic communities in the deep sea have been conducted (Frajía-Fernández et al., 2020; McClenaghan et al., 2020; Merten et al., 2021). Recently, a combination of eDNA with other methodologies (e.g., imaging data) is shown to be a more comprehensive method of monitoring deep-sea biodiversity (Fujiwara et al., 2022; Stefanni et al., 2022). However, it is estimated that in the deep sea, where biomass is low, the amount of eDNA sources is lower than in coastal and surface seawater (McClenaghan et al., 2020). Moreover, several biological

factors of deep-sea fish (e.g., slower metabolism) (Drazen and Seibel, 2007) may also affect the amount of eDNA sources released into seawater. Due to lower eDNA concentrations in deep-sea water, large filtration volumes of seawater are needed for biodiversity monitoring to detect a large number of taxa using the eDNA metabarcoding analysis (Bessey et al., 2020; Stauffer et al., 2021). Indeed, a larger number of species could be detected with increasing filtration volume (Miya et al., 2016; Bessey et al., 2020). Filtration volume for fish metabarcoding studies at river, stream, and reef varied from 0.15 – 6 L (Wilcox et al., 2016; Hinlo et al., 2017; Schumer et al., 2019), although 1 – 2 L of water is frequently filtered (reviewed in Bessey et al., 2020). Recent studies indicated that filtration of over 20 L of water from each site is required for detecting species until accumulation curves approach an asymptote (Cantera et al., 2019; Koziol et al., 2019). However, the greater the volume of water that is filtered, the greater the presence of PCR inhibitory substances and non-targeted eDNA, which may result in PCR inhibition as described previously (Deiner et al., 2015; Lear et al., 2017; Harper et al., 2019). Thus, we should also pay attention to the occurrence of such undesirable materials. Another important point of monitoring fish biodiversity using pumped deep-sea water is the number of sample replicates for metabarcoding analysis. Fish eDNA in seawater can be sporadically distributed, thus detection of fish using eDNA metabarcoding is strongly affected by its distribution, as previously reported (Bessey et al., 2020). An adequate number of sample replicates is effective for estimating biodiversity (Bessey et al., 2020).

For continuous monitoring of the biodiversity of deep-sea fish using pumped deep-sea water, seasonal sampling, and the sampling of water after natural disasters (such as earthquakes, extreme floods, and extreme storms), are planned using the deep-sea water from two pumping facilities on the Pacific coast of central Japan. One is in Akazawa (Shizuoka Prefecture, pumping depth: 800 m) facing Sagami Bay, and the other is in Yaizu (Shizuoka Prefecture, pumping depth: 400 m) facing Suruga Bay. In this study, we developed an eDNA metabarcoding analysis using pumped deep-sea water for understanding the biodiversity of deep-sea fish. To evaluate the effect of filtration volume of water on extracted eDNA yields, PCR amplification efficiency, and detected fish diversity in the metabarcoding analysis, various volumes of pumped deep-sea water were filtered. Moreover, we examined the effect of the number of sample replicates on the detection of deep-sea fish. For these purposes, we collected the deep-sea water at Akazawa on September 14–15, 2020, and at Yaizu on September 16–17, 2020.

2 Materials and methods

2.1 Deep-sea water sampling and filtration

Deep-sea water samples were collected from two deep-sea water pumping facilities belonging to DHC corporation, located in Akazawa, Shizuoka, Japan (34°50.331'N, 139°08.192'E, pumping depth: 800 m) on September 14–15, 2020, and Shizuoka prefecture at Yaizu, Shizuoka, Japan (34°51.196'N, 138°21.439'E, pumping depth: 400 m) on September 16–17, 2020. These facilities are located on the Pacific coast of central Japan, facing Sagami Bay and Suruga Bay, respectively. These facilities are located near our laboratory and can be rapidly accessed after natural disasters. Moreover, extensive surveys

on marine organisms have been carried out in these areas and also have been continuously investigated. Environmental data including temperature (°C), salinity (‰), electrical conductivity (mS/cm), σ_T , chlorophyll a (Chl-a in $\mu\text{g/L}$), turbidity (FTU), and dissolved oxygen (DO; mg/L) of collected deep-sea water was measured using Rinko profiler sensors (JFE Advantech Co., Ltd., Nishinomiya, Japan). Collected pumped deep-sea water was immediately filtered on-site using a Sterivex filter cartridge (0.45 μm pore size, PVDF membrane, gamma-irradiated, sterile, SVHVL10RC, Merck KGaA, Darmstadt, Germany). The deep-sea water at Yaizu was filtered using peristaltic pumps (Masterflex L/S peristaltic pump with Masterflex L/S multichannel cartridge pump head and L/S 17G tubing, Masterflex, Radnor, PA, USA), and that at Akazawa was directly filtered by a filtration device driven by pressure due to the height differential (Supplementary Figure 1). At each site, 18 Sterivex filters at Akazawa and 8 at Yaizu were simultaneously used for filtration as one cycle, and at least 2.5 or at least 3 cycles were carried out per day. All filtration equipment used in this study was sterilized by immersion in a 1% or 6% chlorine-based commercial bleach solution before use. After filtration, the outlet of the Sterivex cartridge was capped by a sterilized protective plug (B. Braun, Melsungen, Germany), and approximately 2.0 mL of RNAlater (Thermo Fisher Scientific, Waltham, MA, USA) was immediately filled into the cartridge through the inlet to prevent the degradation of eDNA, and the inlet was capped similarly. Sterivex cartridges were refrigerated at 4°C for transport back to the laboratory and were stored at -30°C in the lab until eDNA extraction. To evaluate the effect of filtration volume of water on extracted eDNA yields, PCR amplification efficiency, and detected fish diversity in metabarcoding analysis, three or four replicates of pumped deep-sea water for each volume (10, 20, 30, 40, 48, 50, and 53 L at Akazawa; and 5, 10, 15, 20, 25, and 30 L at Yaizu) was filtered except for 48 L and 53 L at Akazawa, and 25 L and 30 L at Yaizu. Based on filtration time, filtration volumes were determined. In addition, to evaluate the relationship between the number of sample replicates and the detected fish diversity, we prepared 33 replicates of 20-L filtration from Akazawa, and 27 replicates of 10-L filtration from Yaizu, sharing a portion of filters with the water-volume experiments shown above. Based on the filtration time, the quantity of PCR products, and the ratio of total fish reads, filtration volumes (20-L filtration from Akazawa and 10-L filtration from Yaizu) were determined. Details of deep-sea water samples and environmental data were summarized in Tables 1 and Supplementary Table S1. Additionally, 20 L or 10 L of ultra-purified water (Milli-Q), filled in a single-use sterilized plastic bag (Sekisui Seikei Co., Ltd., Osaka, Japan) or a plastic tank sterilized with 6% chlorine-based commercial bleach solution, and filtered using the Sterivex filter cartridge by the peristaltic pump at each collection site and RNAlater was also filled into the cartridge as the same manner to serve as negative controls (Filtration blank; FB) during the filtration and subsequent experiments. An infiltrated Sterivex filled with RNAlater was also used as a negative control (eDNA elution blank; EB) at each collection site.

2.2 DNA extraction, library preparation, and next-generation sequencing

eDNA collected on a filtration membrane in a Sterivex filter cartridge was extracted using a DNeasy Blood and Tissue Kit

(QIAGEN, Valencia, CA, USA) as described previously (Kawato et al., 2021). In this method, the filter was removed from the Sterivex filter cartridge after opening the cartridge and was cut into small pieces for efficient lysis of eDNA sources (Kawato et al., 2021). eDNA was eluted into 200 μl of the elution buffer. Extracted eDNA concentrations were quantified by a Qubit dsDNA HS Assay Kit with a Qubit fluorometer (Life Technologies, Carlsbad, CA, USA). To evaluate the purity of extracted eDNA, the absorbance at 230 nm (A230), 260 nm (A260), and 280 nm (A280) and the ratio of A260/A230 were measured by a JASCO V-560 ultraviolet-visible spectrophotometer (JASCO, Tokyo, Japan).

Library preparation including two-step PCR for metabarcoding of fish in eDNA from pumped deep-sea water was performed according to a recently updated method (Kawato et al., 2021) which was based on a previously reported method by Miya et al., 2015. In the first round of PCR, multiplex PCR targeting the mitochondrial 12S rRNA gene sequence using two universal primer sets (MiFish-U-Forward, MiFish-E-Forward, MiFish-U-Reverse, and MiFish-E-Reverse; Supplementary Table S3) was performed. These primer sets amplify approximately 170-bp fragments in a hypervariable region of the mitochondrial 12S rRNA gene and append second-round primer binding adapters for the next-generation sequencing at both ends of amplicons (total length: ca. 300bp) (Miya et al., 2015). PCR amplification was carried out as previously described (Kawato et al., 2021) using Platinum SuperFi II DNA polymerase (Thermo Fisher Scientific, Waltham, MA, USA) and 1 X SuperFi II buffer containing 200 μM of each dNTP, 0.6 μM of each primer, and a minimum of 40ng eDNA template. Eight technical replicates for each eDNA template, as described in the previous studies (Miya et al., 2015), were prepared. PCR thermal cycling conditions were as follows; 98°C for 30 s for initial denaturation, followed by 38 cycles of denaturation at 98°C for 10 s, annealing at 60°C for 10 s, and extension at 72°C for 30 s; with a final extension at 72°C for 5 min. After PCR amplification, amplicons were analyzed by an E-gel Precast Agarose Electrophoresis System using an E-gel SizeSelect II Agarose Gels 2% (Thermo Fisher Scientific, Waltham, MA, USA) and an Agilent 2100 Bioanalyzer using an Agilent High Sensitivity DNA Kit (Agilent Technologies, Santa Clara, CA, USA). To evaluate the PCR amplification efficiency, the amount of PCR products represented by fluorescence intensity (i.e., peak area) of corresponding products obtained using the Agilent 2100 Bioanalyzer was calculated.

After the first round of PCR, the second round of PCR and next-generation sequencing was performed by Bioengineering Lab. Co., Ltd (Sagamihara, Japan) as described previously (Kawato et al., 2021). First-round PCR products were purified with AMPure XP (Beckman Coulter, Brea, CA, USA). Second-round PCR was performed to add unique index sequences and Illumina sequencing adapters (2ndF, and 2ndR; Supplementary Table S3) using Ex Taq polymerase (Takara Bio, Shiga, Japan). Adapter-attached PCR fragments were purified using AMPure XP, and then these paired-end sequence libraries were sequenced using Illumina MiSeq (Illumina, San Diego, CA, USA) under 2×300 bp conditions.

To assess potential contamination of exogenous DNA during the experimental procedure including water filtration, eDNA extraction, and PCR amplification, first-round PCR amplifications using eDNA extraction solutions from FB and EB were conducted. All apparatuses and benches used in this procedure were cleaned with a 1% chlorine-

TABLE 1 Summary of filtered samples with filtration volume, recovered eDNA, read number of data processing, and taxon assignment.

Filter No.	Site	Sampling date (JST)	Water sampling time (JST)	Beginning of filtration time (JST)	End of filtration time (JST)	Filtration period (min)	Filtration vol. (L)	DNA conc. (µg/ml)	No. of raw reads	No. of merged reads	No. of quality filtered reads	No. of denoised reads	No. of fish reads
AK6-46	Akazawa	2020.915	11:01-11:22	11:01	11:22	21	10	0.849	118021	109946 (93.16%)	109930 (93.14%)	84409 (71.52%)	84409 (71.52%)
AK6-47	Akazawa	2020.915	11:01-11:22	11:01	11:22	21	10	1.91	122335	115402 (94.33%)	115383 (94.32%)	87279 (71.34%)	87279 (71.34%)
AK6-48	Akazawa	2020.915	11:01-11:23	11:01	11:23	22	10	1.76	121621	114786 (94.38%)	114760 (94.36%)	83818 (68.92%)	83818 (68.92%)
AK6-49	Akazawa	2020.915	11:01-11:45	11:01	11:45	44	20	2.44	132639	125220 (94.41%)	125185 (94.38%)	89887 (67.77%)	89887 (67.77%)
AK6-50	Akazawa	2020.915	11:01-11:43	11:01	11:43	42	20	1.09	129467	122352 (94.50%)	122331 (94.49%)	93210 (72.00%)	93210 (72.00%)
AK6-51	Akazawa	2020.915	11:01-11:43	11:01	11:43	42	20	4.91	134088	127313 (94.95%)	127297 (94.94%)	91217 (68.03%)	91217 (68.03%)
AK6-52	Akazawa	2020.915	11:02-12:12	11:02	12:12	70	30	2.97	143342	135575 (94.58%)	135555 (94.57%)	94163 (65.69%)	94163 (65.69%)
AK6-53	Akazawa	2020.915	11:02-12:11	11:02	12:11	69	30	7.54	147769	139860 (94.65%)	139840 (94.63%)	92760 (62.77%)	92760 (62.77%)
AK6-54	Akazawa	2020.915	11:02-12:00	11:02	12:00	58	30	4.79	139823	131695 (94.19%)	131676 (94.17%)	86802 (62.08%)	86802 (62.08%)
AK6-55	Akazawa	2020.915	11:02-12:50	11:02	12:50	108	40	8.42	166285	156894 (94.35%)	156865 (94.34%)	111659 (67.15%)	111659 (67.15%)
AK6-56	Akazawa	2020.915	11:02-13:03	11:02	13:03	121	40	12.3	149670	140785 (94.06%)	140761 (94.05%)	98611 (65.89%)	98611 (65.89%)
AK6-57	Akazawa	2020.915	11:02-13:01	11:02	13:01	119	40	12.1	135647	127552 (94.03%)	127525 (94.01%)	78942 (58.20%)	78942 (58.2%)
AK6-58	Akazawa	2020.915	11:02-14:12	11:02	14:12	190	50	17.6	140768	132603 (94.20%)	132573 (94.18%)	85513 (60.75%)	85513 (60.75%)
AK6-59	Akazawa	2020.915	11:02-14:08	11:02	14:08	186	50	18.7	139684	132613 (94.94%)	132596 (94.93%)	85783 (61.41%)	85783 (61.41%)
AK6-60	Akazawa	2020.915	11:02-14:09	11:02	14:09	187	50	19.6	154114	145180 (94.20%)	145151 (94.18%)	89106 (57.82%)	89106 (57.82%)
AK6-61	Akazawa	2020.915	11:03-14:54	11:03	14:54	231	53	18	122941	115482 (93.93%)	115470 (93.92%)	71944 (58.52%)	71944 (58.52%)
AK6-62	Akazawa	2020.915	11:03-14:54	11:03	14:54	231	48	17.4	124302	116391 (93.64%)	116378 (93.63%)	70637 (56.83%)	70637 (56.83%)

(Continued)

TABLE 1 Continued

Filter No.	Site	Sampling date (JST)	Water sampling time (JST)	Beginning of filtration time (JST)	End of filtration time (JST)	Filtration period (min)	Filtration vol. (L)	DNA conc. ($\mu\text{g/ml}$)	No. of raw reads	No. of merged reads	No. of quality filtered reads	No. of denoised reads	No. of fish reads
AK6-63	Akazawa	2020.9.15	11:03-14:54	11:03	14:54	231	53	15.9	130289	122456 (93.99%)	122437 (93.97%)	77451 (59.45%)	77451 (59.45%)
AK6-10	Akazawa	2020.9.14	12:27-13:14	12:27	13:14	47	20	4.65	127208	120992 (95.11%)	120974 (95.10%)	85829 (67.47%)	85829 (67.47%)
AK6-11	Akazawa	2020.9.14	12:27-13:20	12:27	13:20	53	20	4.69	138602	131849 (95.13%)	131826 (95.11%)	87087 (62.83%)	87087 (62.83%)
AK6-12	Akazawa	2020.9.14	12:27-13:21	12:27	13:21	54	20	3.85	136870	130437 (95.3%)	130413 (95.28%)	92281 (67.42%)	92281 (67.42%)
AK6-13	Akazawa	2020.9.14	12:27-13:19	12:27	13:19	52	20	5	136762	130258 (95.24%)	130231 (95.22%)	80627 (58.95%)	80627 (58.95%)
AK6-14	Akazawa	2020.9.14	12:27-13:18	12:27	13:18	51	20	4.59	131468	124833 (94.95%)	124815 (94.94%)	81834 (62.25%)	81834 (62.25%)
AK6-15	Akazawa	2020.9.14	12:27-13:18	12:27	13:18	51	20	4.68	140326	133968 (95.47%)	133940 (95.45%)	91254 (65.03%)	91254 (65.03%)
AK6-16	Akazawa	2020.9.14	12:27-13:15	12:27	13:15	48	20	4.12	121219	114770 (94.68%)	114752 (94.67%)	77436 (63.88%)	77436 (63.88%)
AK6-17	Akazawa	2020.9.14	12:27-13:18	12:27	13:18	51	20	4.21	129344	121899 (94.24%)	121873 (94.22%)	84638 (65.44%)	84638 (65.44%)
AK6-18	Akazawa	2020.9.14	12:27-13:22	12:27	13:22	55	20	5.17	141544	135660 (95.84%)	135640 (95.83%)	97109 (68.61%)	97109 (68.61%)
AK6-19	Akazawa	2020.9.14	13:34-14:22	13:34	14:22	48	20	3.9	161840	154583 (95.52%)	154558 (95.5%)	108454 (67.01%)	108454 (67.01%)
AK6-20	Akazawa	2020.9.14	13:34-14:24	13:34	14:24	50	20	4.73	131617	126519 (96.13%)	126495 (96.11%)	85565 (65.01%)	85565 (65.01%)
AK6-21	Akazawa	2020.9.14	13:35-14:27	13:35	14:27	52	20	4.21	143972	137769 (95.69%)	137742 (95.67%)	93194 (64.73%)	93194 (64.73%)
AK6-22	Akazawa	2020.9.14	13:35-14:30	13:35	14:30	55	20	4.54	165128	157774 (95.55%)	157746 (95.53%)	106496 (64.49%)	106496 (64.49%)
AK6-23	Akazawa	2020.9.14	13:35-14:30	13:35	14:30	55	20	4.42	149413	143464 (96.02%)	143448 (96.01%)	103712 (69.41%)	103712 (69.41%)
AK6-24	Akazawa	2020.9.14	13:35-14:39	13:35	14:39	64	20	4.36	131757	125595 (95.32%)	125575 (95.31%)	85738 (65.07%)	85738 (65.07%)
AK6-25	Akazawa	2020.9.14	13:35-14:33	13:35	14:33	58	20	4.32	124206	118597 (95.48%)	118571 (95.46%)	76114 (61.28%)	76114 (61.28%)

(Continued)

TABLE 1 Continued

Filter No.	Site	Sampling date (JST)	Water sampling time (JST)	Beginning of filtration time (JST)	End of filtration time (JST)	Filtration period (min)	Filtration vol. (L)	DNA conc. (µg/ml)	No. of raw reads	No. of merged reads	No. of quality filtered reads	No. of denoised reads	No. of fish reads
AK6-26	Akazawa	2020.9.14	13:35-14:33	13:35	14:33	58	20	4.63	127105	120753 (95.00%)	120738 (94.99%)	79608 (62.63%)	79608 (62.63%)
AK6-27	Akazawa	2020.9.14	13:36-14:24	13:36	14:24	48	20	4.12	151136	142909 (94.56%)	142882 (94.54%)	98306 (65.04%)	98306 (65.04%)
AK6-37	Akazawa	2020.9.14	14:42-15:19	14:42	15:19	37	20	5.25	141351	133818 (94.67%)	133800 (94.66%)	89569 (63.37%)	89569 (63.37%)
AK6-38	Akazawa	2020.9.14	14:42-15:23	14:42	15:23	41	20	5.23	125270	119049 (95.03%)	119032 (95.02%)	84360 (67.34%)	84360 (67.34%)
AK6-39	Akazawa	2020.9.14	14:42-15:20	14:42	15:20	38	20	5.4	140244	132070 (94.17%)	131932 (94.07%)	89846 (64.06%)	89846 (64.06%)
AK6-40	Akazawa	2020.9.14	14:42-15:22	14:42	15:22	40	20	5.37	142320	135029 (94.88%)	134998 (94.86%)	87404 (61.41%)	87404 (61.41%)
AK6-41	Akazawa	2020.9.14	14:42-15:19	14:42	15:19	37	20	4.96	130712	123226 (94.27%)	123201 (94.25%)	87062 (66.61%)	87062 (66.61%)
AK6-42	Akazawa	2020.9.14	14:43-15:22	14:43	15:22	39	20	5.36	127003	120305 (94.73%)	120282 (94.71%)	82330 (64.83%)	82330 (64.83%)
AK6-43	Akazawa	2020.9.14	14:43-15:20	14:43	15:20	37	20	4.95	134512	127581 (94.85%)	127555 (94.83%)	86959 (64.65%)	86959 (64.65%)
AK6-44	Akazawa	2020.9.14	14:43-15:18	14:43	15:18	35	20	4.18	136703	130260 (95.29%)	130238 (95.27%)	88221 (64.53%)	88221 (64.53%)
AK6-45	Akazawa	2020.9.14	14:43-15:20	14:43	15:20	37	20	4.9	160501	152542 (95.04%)	152513 (95.02%)	104989 (65.41%)	104989 (65.41%)
AK6-109	Akazawa	2020.9.15	10:35	10:45	12:17	92	20	3.38	139069	132428 (95.22%)	132408 (95.21%)	94740 (68.12%)	94740 (68.12%)
AK6-110	Akazawa	2020.9.15	10:38	10:43	12:24	101	20	5.57	148530	141266 (95.11%)	141242 (95.09%)	98794 (66.51%)	98794 (66.51%)
AK6-111	Akazawa	2020.9.15	10:40	10:43	12:03	80	20	5.07	154540	147310 (95.32%)	147285 (95.31%)	99754 (64.55%)	99754 (64.55%)
YA6-25	Yaizu	2020.9.17	9:22	9:31	9:48	80	5	1.17	178433	171423 (96.07%)	171396 (96.06%)	142242 (79.72%)	142242 (79.72%)
YA6-26	Yaizu	2020.9.17	9:22	9:31	9:45	14	5	0.783	167928	161749 (96.32%)	161723 (96.30%)	136045 (81.01%)	136045 (81.01%)
YA6-27	Yaizu	2020.9.17	9:22	9:32	9:49	17	5	1.19	141602	135327 (95.57%)	135310 (95.56%)	112367 (79.35%)	112367 (79.35%)

(Continued)

TABLE 1 Continued

Filter No.	Site	Sampling date (JST)	Water sampling time (JST)	Beginning of filtration time (JST)	End of filtration time (JST)	Filtration period (min)	Filtration vol. (L)	DNA conc. (µg/ml)	No. of raw reads	No. of merged reads	No. of quality filtered reads	No. of denoised reads	No. of fish reads
YA6-28	Yaizu	2020.9.17	9:22	9:32	10:05	33	10	2.94	182863	175016 (95.71%)	174987 (95.69%)	128652 (70.35%)	128652 (70.35%)
YA6-29	Yaizu	2020.9.17	9:22	9:37	10:23	46	10	3.18	140933	134120 (95.17%)	134096 (95.15%)	100286 (71.16%)	100286 (71.16%)
YA6-30	Yaizu	2020.9.17	9:22	9:36	10:11	35	10	2.41	184547	176104 (95.43%)	176069 (95.41%)	131821 (71.43%)	131821 (71.43%)
YA6-42	Yaizu	2020.9.17	12:52	12:55	13:53	58	10	2.41	157033	147842 (94.15%)	147816 (94.13%)	121037 (77.08%)	121037 (77.08%)
YA6-41	Yaizu	2020.9.17	12:52	12:56	13:57	61	15	5.6	141071	133090 (94.34%)	133065 (94.32%)	107510 (76.21%)	107510 (76.21%)
YA6-43	Yaizu	2020.9.17	12:52	12:55	14:03	68	15	5.87	136424	130491 (95.65%)	130469 (95.63%)	101130 (74.13%)	101130 (74.13%)
YA6-44	Yaizu	2020.9.17	12:53	12:55	14:01	66	15	5.13	131415	125638 (95.60%)	125618 (95.59%)	91197 (69.40%)	91197 (69.40%)
YA6-34	Yaizu	2020.9.17	9:53	10:04	12:47	163	15	8.47	141987	135454 (95.40%)	135429 (95.38%)	93042 (65.53%)	93042 (65.53%)
YA6-36	Yaizu	2020.9.17	10:27	10:32	12:44	132	20	6.79	147538	138875 (94.13%)	138852 (94.11%)	111034 (75.26%)	111034 (75.26%)
YA6-37	Yaizu	2020.9.17	10:27	10:36	12:14	98	20	7.19	140579	132188 (94.03%)	132162 (94.01%)	105156 (74.80%)	103579 (73.68%)
YA6-38	Yaizu	2020.9.17	10:28	10:33	12:08	95	20	8.7	125715	119103 (94.74%)	119086 (94.73%)	93320 (74.23%)	93320 (74.23%)
YA6-45	Yaizu	2020.9.17	12:20	12:28	14:55	147	20	8.33	132472	126233 (95.29%)	126224 (95.28%)	97124 (73.32%)	97124 (73.32%)
YA6-46	Yaizu	2020.9.17	12:20	12:25	15:39	194	25	10.6	138959	132428 (95.30%)	132409 (95.29%)	86245 (62.07%)	86245 (62.07%)
YA6-47	Yaizu	2020.9.17	12:23	12:27	14:42	135	25	9.09	146941	140473 (95.60%)	140451 (95.58%)	89672 (61.03%)	89672 (61.03%)
YA6-40	Yaizu	2020.9.17	10:48	10:55	15:22	267	30	14.2	98448	93333 (94.80%)	93315 (94.79%)	68381 (69.46%)	68381 (69.46%)
YA6-1	Yaizu	2020.9.16	10:20	10:34	11:15	41	10	2.39	152388	144624 (94.91%)	144591 (94.88%)	111419 (73.12%)	111419 (73.12%)
YA6-2	Yaizu	2020.9.16	10:20	10:32	11:17	45	10	1.87	138567	130731 (94.34%)	130703 (94.32%)	105464 (76.11%)	104810 (75.64%)

(Continued)

TABLE 1 Continued

Filter No.	Site	Sampling date (JST)	Water sampling time (JST)	Beginning of filtration time (JST)	End of filtration time (JST)	Filtration period (min)	Filtration vol. (L)	DNA conc. ($\mu\text{g/ml}$)	No. of raw reads	No. of merged reads	No. of quality filtered reads	No. of denoised reads	No. of fish reads
YA6-3	Yaizu	2020.9.16	10:20	10:34	11:16	42	10	1.84	127072	120658 (94.95%)	120175 (94.57%)	97786 (76.95%)	97786 (76.95%)
YA6-4	Yaizu	2020.9.16	10:20	10:35	11:26	51	10	2.78	139875	132775 (94.92%)	132745 (94.90%)	102396 (73.21%)	101888 (72.84%)
YA6-5	Yaizu	2020.9.16	10:20	10:34	11:25	51	10	2.21	146625	138073 (94.17%)	138042 (94.15%)	109833 (74.91%)	107993 (73.65%)
YA6-6	Yaizu	2020.9.16	10:20	10:32	11:26	54	10	2.68	123572	117165 (94.82%)	117131 (94.79%)	97904 (79.23%)	97904 (79.23%)
YA6-7	Yaizu	2020.9.16	10:20	10:32	11:42	70	10	3.06	148353	139538 (94.06%)	139515 (94.04%)	108537 (73.16%)	108537 (73.16%)
YA6-8	Yaizu	2020.9.16	10:23	10:33	11:31	58	10	3.12	150264	142090 (94.56%)	142059 (94.54%)	118905 (79.13%)	118905 (79.13%)
YA6-9	Yaizu	2020.9.16	11:45	11:55	12:38	43	10	2.48	121823	115596 (94.89%)	115565 (94.86%)	91088 (74.77%)	90219 (74.06%)
YA6-10	Yaizu	2020.9.16	11:45	11:53	12:41	48	10	2.05	125987	119294 (94.69%)	119264 (94.66%)	92356 (73.31%)	89356 (70.92%)
YA6-11	Yaizu	2020.9.16	11:45	11:54	12:41	47	10	1.23	118083	111967 (94.82%)	111947 (94.80%)	87647 (74.22%)	81860 (69.32%)
YA6-12	Yaizu	2020.9.16	11:45	11:54	12:32	38	10	2.67	120160	114326 (95.14%)	114298 (95.12%)	89961 (74.87%)	89961 (74.87%)
YA6-13	Yaizu	2020.9.16	11:45	11:52	12:39	47	10	2.67	139855	130626 (93.40%)	130599 (93.38%)	104876 (74.99%)	104876 (74.99%)
YA6-14	Yaizu	2020.9.16	11:45	11:53	12:29	36	10	2.48	125282	119392 (95.30%)	119366 (95.28%)	87754 (70.05%)	87689 (69.99%)
YA6-15	Yaizu	2020.9.16	11:45	11:52	12:31	39	10	2.01	109925	98854 (89.93%)	98832 (89.91%)	77068 (70.11%)	75949 (69.09%)
YA6-16	Yaizu	2020.9.16	11:48	11:52	12:30	38	10	2.69	128740	121656 (94.50%)	121634 (94.48%)	98287 (76.35%)	98041 (76.15%)
YA6-17	Yaizu	2020.9.16	12:49	12:57	13:47	50	10	3.36	115672	109241 (94.44%)	109218 (94.42%)	73952 (63.93%)	73725 (63.74%)
YA6-18	Yaizu	2020.9.16	12:49	12:57	13:48	51	10	4.05	147192	138716 (94.24%)	138683 (94.22%)	100130 (68.03%)	100130 (68.03%)
YA6-19	Yaizu	2020.9.16	12:49	12:57	13:44	47	10	3.28	109784	104204 (94.92%)	104181 (94.9%)	78144 (71.18%)	78144 (71.18%)

(Continued)

TABLE 1 Continued

Filter No.	Site	Sampling date (JST)	Water sampling time (JST)	Beginning of filtration time (JST)	End of filtration time (JST)	Filtration period (min)	Filtration vol. (L)	DNA conc. ($\mu\text{g/ml}$)	No. of raw reads	No. of merged reads	No. of quality filtered reads	No. of denoised reads	No. of fish reads
YA6-20	Yaizu	2020.9.16	12:49	12:56	14:00	64	10	4.5	121452	112708 (92.80%)	112680 (92.78%)	84624 (69.68%)	82893 (68.25%)
YA6-21	Yaizu	2020.9.16	12:49	12:56	13:33	37	10	3.47	135728	127124 (93.66%)	127105 (93.65%)	90394 (66.60%)	89567 (65.99%)
YA6-22	Yaizu	2020.9.16	12:49	12:56	13:50	54	10	3.96	130636	122533 (93.80%)	122510 (93.78%)	86897 (66.52%)	83376 (63.82%)
YA6-23	Yaizu	2020.9.16	12:49	12:56	13:44	48	10	3.73	120859	113868 (94.22%)	113844 (94.20%)	83515 (69.10%)	80669 (66.75%)
YA6-24	Yaizu	2020.9.16	12:53	12:57	13:37	40	10	3.19	139277	131525 (94.43%)	131497 (94.41%)	97771 (70.20%)	97771 (70.20%)

The numbers in parentheses denote percentages of the raw read numbers.

based commercial bleach before use. Tubes and pipetting tips were also new and sterilized using a UV sterilizer light bulb. Detailed operating procedures employed to avoid any contamination risks were performed according to the previously described methods (Miya et al., 2015; Minamoto et al., 2021) and were performed by staff who were familiar with these procedures.

2.3 Bioinformatics

Amplicon raw sequence data were processed by a previously described method (Oka et al., 2021) using USEARCH ver11.0.667 (Edgar, 2010). Raw paired-end reads were merged, and low-quality (Q score < 2) and short reads (< 100 bp) were filtered out. Merged reads with differences over 5 bp in aligned regions were discarded. After trimming primer sequences, low-quality reads with an expected error rate of over 1% and short reads (<120 bp) were removed. Duplicated sequences from resulting sequences were found and unique sequences without singletons, doubletons, and tripletons which were removed to avoid false positives (Edgar, 2016), were obtained. Obtained sequences were processed to generate amplicon sequence variants (ASVs) and chimeric sequences were removed.

Assignment of operational taxonomic units (OTUs) from ASVs which was based on a previously described report (Oka et al., 2021), was performed by using MiFish database ver. 41. Instead of a faunal survey based on traditional methods, we conducted the literature surveys (e.g., habitat, habitat depth range, and distribution at Akazawa and Yaizu) of the resulting taxonomic hit species using FishBase www.fishbase.org (Froese and Pauly, 2022) and an atlas of Japanese fishes (Nakabo, 2013) which comprehensively catalogs the fish species recorded in Japanese waters. To assess the taxonomic assignment of ASVs, a family-level neighbor-joining (NJ) phylogenetic tree including ASVs was built using MEGAX ver. 10210527 (Kumar et al., 2018) after alignment with MUSCLE (Edgar, 2004) which is a built-in program in MEGAX. ASVs with sequence identity of more than 98.5% (two nucleotide difference allowed) and a clade consisting of the same species were assigned to the species name of the reference sequence, while those with sequence identities of 80–98.5% and a clade consisting same species were named “U98.5” plus corresponding species names with highest identities. ASVs with sequence identities of lower than 80% were removed from taxonomic assignment. Each OTU in a monophyletic clade of species belonging to a particular genus was assigned the genus name followed by “sp.” with a sequential number (e.g., *Lepidotrigla* sp. 1, sp. 2). Each OTU in a clade of a particular family but not in a particular genus was named that family followed by “sp.” with a sequential number (e.g., Synphobranchidae sp. 1, sp. 2 or U98.5_Myctophidae sp. 1, U98.5_Myctophidae sp. 2). Hereafter, the terms, “OTU” and “species” are used interchangeably.

Alpha diversity (Chao1) for OTUs was analyzed using vegan v2.5.7 (Dixon, 2003) packaged in R 4.1.2 (R Core Team, 2021) set in RStudio Version 2021.09.1 + 372 (RStudio Team, 2020). The nonmetric multidimensional scaling (NMDS) based on the presence/absence data of the fish species in individual samples from Akazawa and Yaizu was performed using the Jaccard distance with the metaMDS function in the vegan package in R. Species accumulation curves based on the presence/absence data of the fish

species detected by eDNA were analyzed using iNEXT ver. 2.0.20 packaged-in R 4.1.2 set in RStudio Version 2021.09.1 + 372. Graphics were constructed using the R package ggplot2 ver. 3.3.5 (Wickham, 2016).

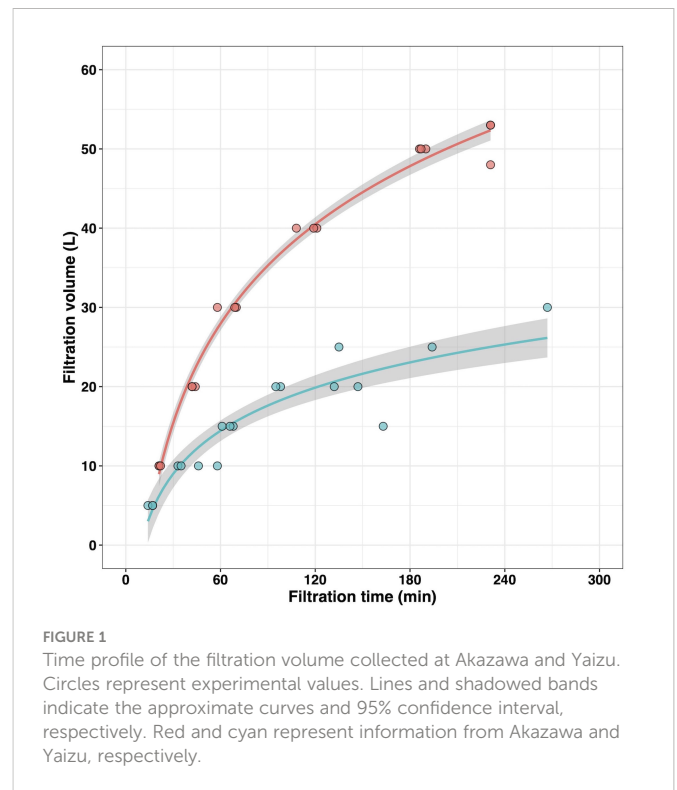
2.4 Statistical analysis

The correlation between fish reads and filtration volumes was investigated by calculating Pearson's product-moment correlation coefficient in R 4.1.2 set in RStudio Version 2021.09.1 + 372. The difference in the number of OTUs between Akazawa and Yaizu which obtained various filtration volumes was performed by using Dunnett's test in multcomp ver. 1.4-17 packaged in R 4.1.2 set in RStudio Version 2021.09.1 + 372. We also compared the difference in the number of OTU from 33 replicates of 20-L filtration from Akazawa, and 27 replicates of 10-L filtration from Yaizu by using Mann-Whitney's U test in exactRankTests ver 0.8-34 in R 4.1. set in RStudio Version 2021.09.1 + 372. A difference at $p < 0.05$ was considered significant in all tests.

3 Results

3.1 Comparison of filtration volume and extracted eDNA yields from pumped deep-sea water

The relationships between filtration water volume, filtration time, yields of recovered eDNA, and efficiency of PCR amplification were evaluated using filtered samples of pumped deep-sea water in varying quantities. At the Akazawa sampling site, we developed a filtration device that utilized the pressure due to the height difference (Supplementary Figure 1). This filtration device has 18 filters that can filter the larger volume of the pumped deep-sea water without an electric power supply. The filtration time across all the filtration volumes ranged from approximately 20 to 230 min for water samples collected at Akazawa and from approximately 15 to 260 min at Yaizu (Figure 1). Concentration and purity (ratio of A230/A260) of extracted eDNA were shown in Supplementary Figures 2, 3. eDNA was recovered from all the filtered samples at both sampling sites, regardless of the volume of filtered deep-sea water. Filtration water volumes had a positive effect on the amount of recovered total DNA, with higher eDNA concentrations recovered from larger filtration volume samples compared to those from smaller ones. DNA concentrations estimated by measuring the A260 absorbance indicated a higher value than that measured using fluorescent reagents, and the difference between them at Yaizu increased with increasing filtration volume (Supplementary Figure 3). For the Yaizu samples, PCR products by adding more template DNA were not detected in the larger samples of filtered water (Supplementary Figure 4). It was noted that the amount of PCR products obtained from 10-L filtered samples were the highest among the Yaizu samples only when the minimum amount of the template DNA solution was added (Supplementary Figures 4, 6). For the Akazawa samples, PCR products decreased only when the maximum amount of the template



DNA solution was added (Supplementary Figure 5). In the negative controls which are filtration blanks including ultra-purified water filtered blanks and eDNA elution ones, no detectable DNA concentration was obtained and any PCR amplifications were not recognized.

3.2 Sequencing summary of eDNA metabarcoding derived from pumped deep-sea water

A total of 90 fish metabarcoding libraries of filtered samples from various filtration-volume series (18 libraries at both Akazawa and Yaizu) and filtered sample replicates from fixed filtration-volume series (30 libraries at Akazawa and 24 libraries at Yaizu) were sequenced by MiSeq (detailed data were shown in Table 1). The number of raw reads (a total of 12,395,136 reads) for each library ranged from 98,448 to 184,547 with an average of 137,724 (Table 1). After sequence processing, a total of 8,450,653 reads were assigned as fish sequences which ranged from 68,381 to 142,242 per sample with an average of 93,896 (Table 1). Negative correlations between filtration volumes and the percentage of assembled fish reads were found at both sampling sites (Akazawa: Pearson's $r = -0.87$, $df = 16$, $P = 2.868e-06$; Yaizu: Pearson's $r = -0.65$, $df = 16$, $P = 0.035$; Table 1; Supplementary Figure 7). A total of 146 OTUs representing fish including 92 identified at the species level, 13 identified at the genus level, and 24 identified at the family level and 17 sequences with identities of 80-98.5% were detected at both sampling sites (Supplementary Table S2). These OTUs consisted of a total of 68 fish families and 88 genera (Supplementary Figure 8A) and most detected OTUs at both sampling sites were benthopelagic, bathydemersal, and bathypelagic species

(Supplementary Table S2, Supplementary Figure 9). A total of 43 and 123 OTUs were detected at Akazawa and Yaizu, respectively (Supplementary Figure 8A). Of these OTUs, 29 OTUs (67.4% of detected total OTUs) at Akazawa and 89 OTUs (72.4% of detected total OTUs) at Yaizu were in good agreement with the known distribution records, i.e., distribution to Sagami Bay and Suruga Bay, respectively (Supplementary Table S2) (Nakabo, 2013). Moreover, the habitat depth range of 27 OTUs (62.8% of detected total OTUs) at Akazawa and 63 OTUs (51.2% of detected total OTUs) at Yaizu corresponded to the depth of each intake of the pumped deep-sea water (Akazawa: 800 m, Yaizu: 400 m, Supplementary Table S2; Figure 2). The species representing incongruent habitat depth range with the depth of each water intake were 9 OTUs (20.9% of detected total OTUs) at Akazawa and 25 OTUs (20.3% of detected total OTUs) at Yaizu (Supplementary Table S2; Figure 2), and 7 OTUs (16.3% of detected total OTUs) at Akazawa and 35 OTUs (28.5% of detected total OTUs) at Yaizu exhibited no records of habitat depth range from FishBase

3.3 Detection of fish OTUs from each filtration volume of pumped deep-sea water

The number of OTUs detected at each filtration volume sample was lower than that of the estimated species richness (Chao1 index) and total detected OTUs (Figure 3). Based on statistical analysis, the number of OTUs detected from the larger samples of filtered water was not significantly higher than in the smaller samples of filtered water at the Akazawa site (Figure 3A). However, a significant increase in the number of detected OTUs was observed in 15-, 20-, and 25-L filtered samples at the Yaizu site (Figure 3B). The total number of OTUs from the samples of various amounts of filtered water was significantly increased at Yaizu (Figure 3). A similar trend was seen in the numbers of shared OTUs between replicates from samples of various amounts of filtered water at Yaizu.

3.4 Relationship between the number of sample replicates and detected fish biodiversity at two sites

In this experiment, each filtration volume was fixed among replicates (20 L at Akazawa, 10 L at Yaizu) based on the result of the evaluation of the filtration time, efficiency of PCR amplification, and number of detected fish reads. The numbers of OTUs detected from replicates were different between the sampling sites (Figure 4). The numbers of detected OTUs ranged from 2 to 10 (means = 6.36) at Akazawa and from 10 to 22 (means = 15.36) at Yaizu, significantly higher at Yaizu than at Akazawa (Mann-Whitney's U test; $p < 0.05$). The variation of detected OTUs among replicates was higher at Yaizu than at Akazawa. The numbers of total OTUs and chao1 index of samples collected from Yaizu (total OTUs: 98; chao1 index: 217.5) were also higher than those from Akazawa (total OTUs, 35; chao1 index, 43.8). A total of 118 OTUs representing fish species including 76 identified genera and 61 identified families were detected (Supplementary Figure 8B). Of these 118 OTUs, the number of

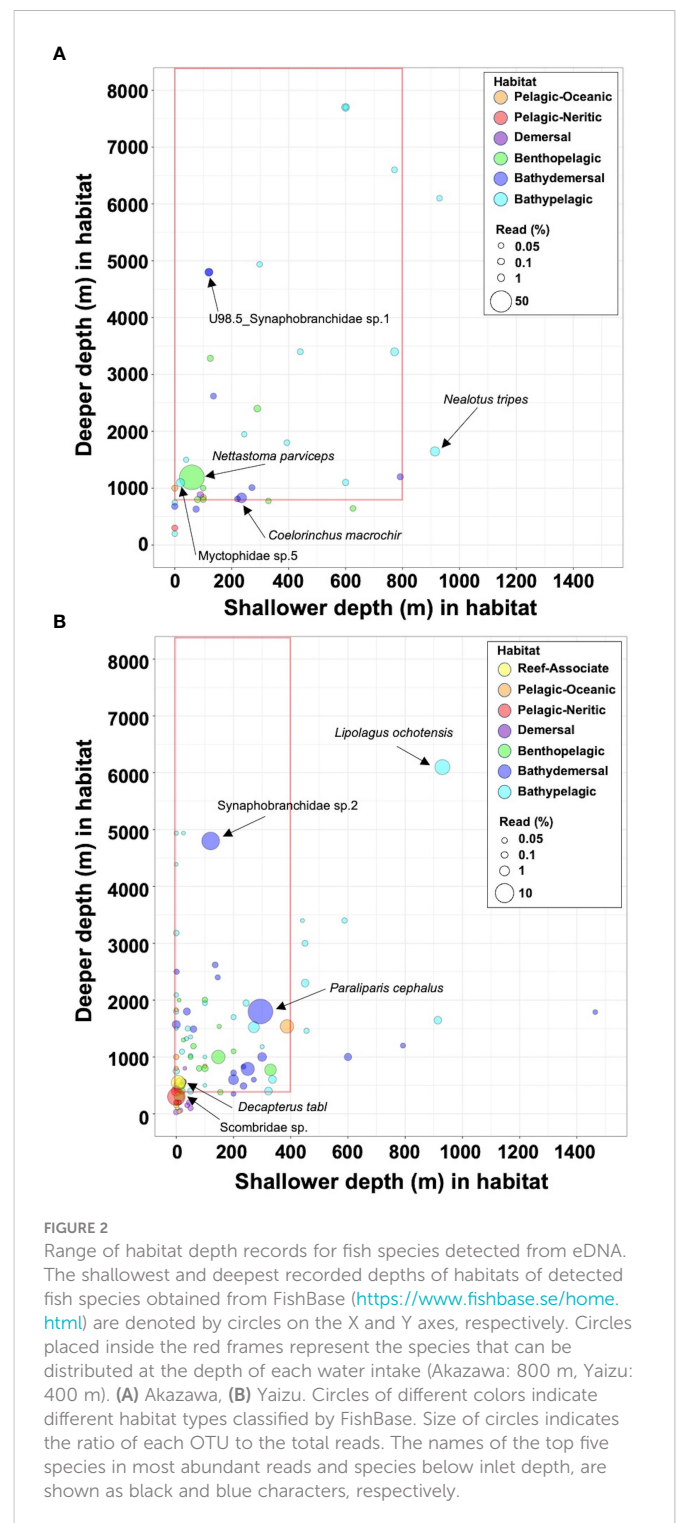


FIGURE 2
Range of habitat depth records for fish species detected from eDNA. The shallowest and deepest recorded depths of habitats of detected fish species obtained from FishBase (<https://www.fishbase.se/home.html>) are denoted by circles on the X and Y axes, respectively. Circles placed inside the red frames represent the species that can be distributed at the depth of each water intake (Akazawa: 800 m, Yaizu: 400 m). (A) Akazawa, (B) Yaizu. Circles of different colors indicate different habitat types classified by FishBase. Size of circles indicates the ratio of each OTU to the total reads. The names of the top five species in most abundant reads and species below inlet depth, are shown as black and blue characters, respectively.

shared OTUs between Akazawa and Yaizu was 15. There was distinct dissimilarity in the composition of fish taxa between the two sampling sites (Figure 5; NMDS stress = 0.844778). The frequency of occurrence among sample replicates (the occurrence ratio of fish OTU among filtered sample replicates) showed that approximately half the number of OTUs from Yaizu and one-third of the OTUs from Akazawa showed a less than 5% in occurrence ratio (Supplementary Figure 10). The cumulative number of OTUs from Akazawa almost converged to 40 in 50 replicates (Figure 6A). In addition, accumulation curves of OTUs

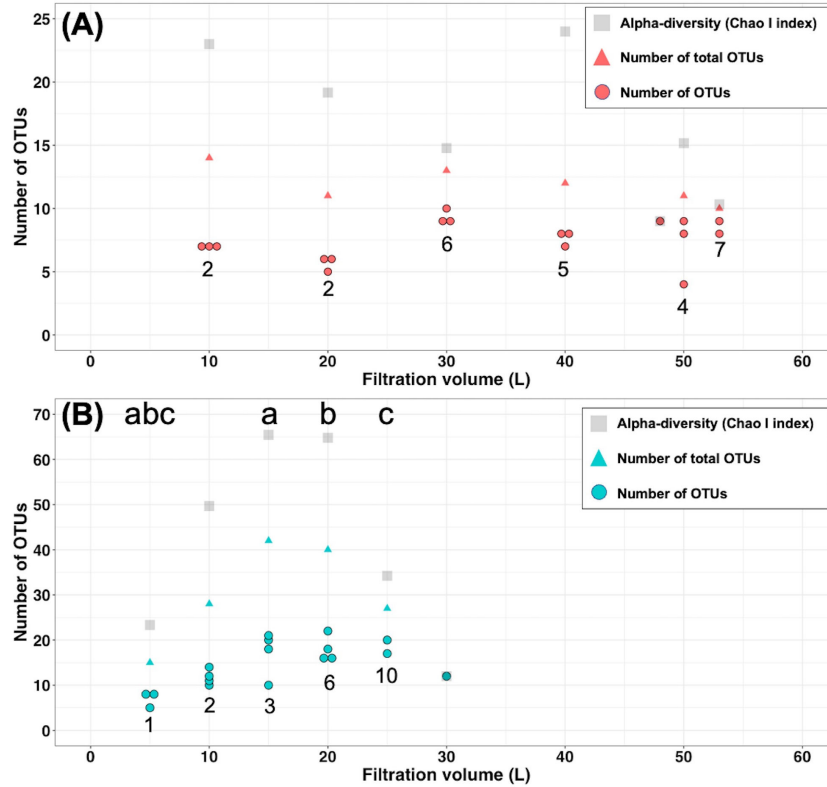


FIGURE 3 Number of fish OTUs identified from different filtration-volume samples in (A) Akazawa, and (B) Yaizu. Circles, triangles, and squares indicate the number of OTUs, number of total OTUs, and alpha-diversity (Chao I index), respectively. The numbers below circles indicate the number of shared OTUs between all replicates. There is a significant difference in the number of OTUs between samples given the same alphabet based on Dunnett’s test ($p < 0.05$).

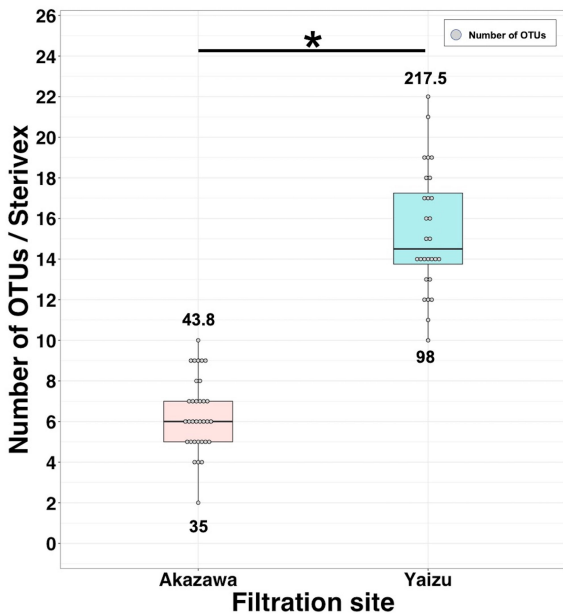


FIGURE 4 Number of fish OTUs obtained from pumped deep-sea water sampled in Akazawa and Yaizu. Filtration volume of samples from Akazawa and Yaizu was 20 L and 10 L, respectively. Boxplots with circles indicate the number of OTUs. Upper and lower values indicate the alpha diversity (Chao I index) and total number of OTUs, respectively. Asterisk indicates significant difference in number of OTUs between Akazawa and Yaizu samples based on Mann–Whitney’s test ($p < 0.05$).

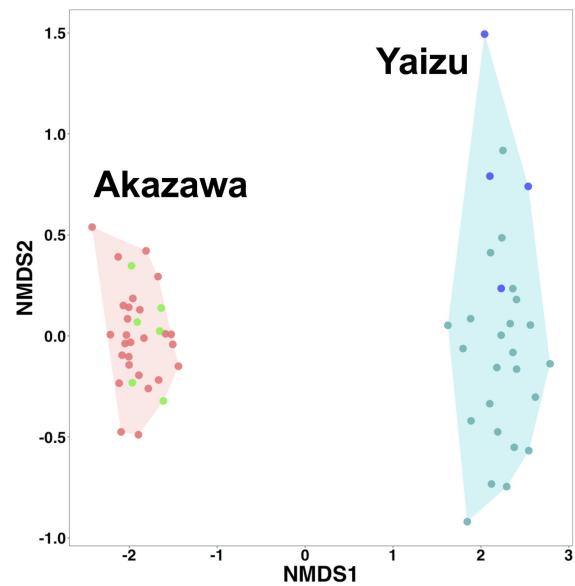


FIGURE 5 Nonmetric multidimensional scaling (NMDS) of fish OTU occurrence obtained from Akazawa and Yaizu. Analysis was performed using OTU read presence and Jaccard distance. Red and green circles indicate the filtered samples collected at Akazawa on September 14 and 15, 2020, respectively. Dark green and blue circles indicate the filtered samples collected at Yaizu on September 16 and 17, 2020, respectively. Convex hulls cluster samples according to collected sampling sites. NMDS stress value was 0.091377.

representing a frequency of more than 5% were saturated within 15 replicates at Akazawa. In contrast to Akazawa, the accumulation curve of OTUs at Yaizu using all OTU data indicated species richness was not fully saturated even in 50-replicate sampling (Figure 6B). Similarly to Akazawa, the OTUs representing a frequency of more than 5%, accumulation curves were also saturated within 15 replicates at Yaizu. Accumulation curves of OTUs using OTU data with more than 5% frequency among replicates indicated that more than 10 sample replicates at both sites were needed for the detection of frequently occurring fish species (Figure 6).

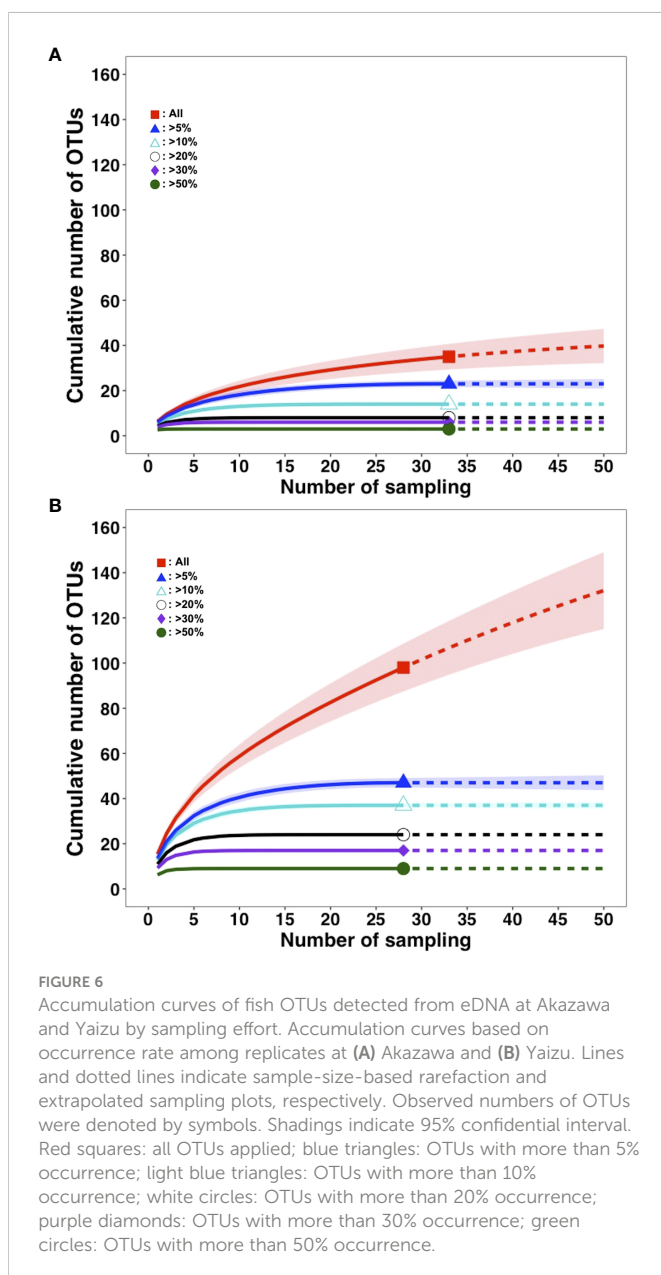
4 Discussion

In this study, we demonstrated the successful application of monitoring of fish diversity using eDNA from pumped deep-sea water and developed a novel filtration device using the siphon effect

which simultaneously filtrates 18 replicates without the need for expensive equipment or power. Our results indicated that large filtration volumes (> 20-L) of pumped deep-sea water presented several technical difficulties and risks. Furthermore, the filtration of 20 L of pumped deep-sea water from Akazawa and filtration of 10 L of pumped deep-sea water from Yaizu were demonstrated to be suitable filtration volumes for monitoring deep-sea fish biodiversity in the present study. Also, our data suggested that sufficient replicate samples are needed to detect comprehensive eDNA of rarely occurring fish in pumped deep-sea water (approximately 10 or more replicates for covering major communities, and much more if it includes minor communities, depending on the sites). Moreover, this study suggested that optimum filtration water volume and appropriate sample replicates vary depending on the filtration system and biodiversity of the survey points, thus, it is important to consider the optimum method for each study site and purpose.

Our results indicated no significant differences in the number of detected OTUs among different filtration volumes of samples at Akazawa. The amount of PCR products was less amplified even though the eDNA extracted from a larger volume of the pumped deep-sea water was utilized. Moreover, a negative correlation between the filtration volume and the total number of assembled fish reads was found. A possible reason for these negative effects with the increase of filtration volume was that the accumulation of PCR inhibitory materials and sources of non-target DNA such as bacterial particles on the filter exceeded the accumulation of fish eDNA sources when the filter began clogging as the amount of filtration increased. It took about 30–45 min for the 10-L filtration at Yaizu and the 20-L filtration at Akazawa, while much larger filtration needed excessive time, and it took more than one hour for 10-L and 20-L filtrations or larger. Large filtration volume (more than 20-L) of pumped deep-sea water presents several technical difficulties and risks, such as the increase of the contamination risk, the degradation of eDNA, and the accumulation of PCR inhibitory materials during longer filtration time. Hence, the appropriate pumped deep-sea water volume of filtration samples should be determined considering those difficulties and risks: the 20-L filtration of pumped deep-sea water from Akazawa and 10-L filtration from Yaizu have been demonstrated to be suitable in the present study, for the following reasons: the quantity of PCR products in more than 10-L filtrations was lower at Yaizu (Supplementary Figures 4, 6) and the ratio of total fish reads in more than 20-L filtration was decreased at Akazawa (Supplementary Figure 7).

Along with filtration volume, the number of replicates of filtered samples is also an important factor for estimating biodiversity using eDNA metabarcoding analysis. The source of eDNA in seawater exhibits sporadic distribution, and the estimation of biodiversity using eDNA is strongly influenced by the sampling effort (Bessey et al., 2020; Stauffer et al., 2021; Ahn et al., 2022). The number of detected species was relatively congruent among replicates, with a variation of up to 8 species between replicates for the Akazawa samples and up to 12 species between replicates for the Yaizu samples. These variations were slightly higher than that of a previous study conducted in tropical streams and rivers (Cantera et al., 2019), but were significantly lower than that of a previous deep-sea study (McClenaghan et al., 2020). The number of detected species at Yaizu among replicates was higher than that of Akazawa, and the



total number at Akazawa and Yaizu was 35 and 98, respectively. Of these OTUs, 25 OTUs (71.4% of the total detected OTUs) at Akazawa and 70 OTUs (71.4% of the total detected OTUs) at Yaizu were in good agreement with previously known distribution records. A previous study indicated that eDNA derived from low-abundant taxa is very localized (Di Muri et al., 2020), and our results indicated that approximately half of the OTUs at Yaizu and one-third of the OTUs at Akazawa were only detected from one replicate (approximately less than 5% occurrence ratio among replicates; Supplementary Figure 10). Except for OTUs with less than a 5% occurrence ratio, accumulation curves of detected OTUs reached the plateau in approximately 10 replicates or more, while that of all detected OTUs at Yaizu was not saturated even in 50 replicates. These data suggested that eDNA from fish in low density shows a sporadic distribution and is rarely pumped. Our results were consistent with previous suggestions that sufficient replicate samples are needed to improve the detection probabilities and to obtain reliable estimates of fish biodiversity (Bessey et al., 2020; Stauffer et al., 2021).

The fish eDNA in the open ocean is thought to be detected at the depth where it was released or deeper, but rarely shallower (Canals et al., 2021). Our results showed that the habitat depth range of detected species corresponded to the depth of each intake of pumped deep-sea water (Akazawa: 800 m, Yaizu: 400 m, respectively). Moreover, approximately 70% of the total detected OTUs at both sampling sites were in agreement with the previous distribution records. Of the remaining OTUs, approximately 14% OTUs at both sampling sites did not find in distribution records from previous reports and other OTUs showed no record data. Thus, deep-sea water pumped at both sampling sites is useful and available for estimating the biodiversity of deep-sea fish. Further imaging studies are needed to directly observe live fish using remotely operated vehicles and/or automated baited cameras to increase the reliability of eDNA metabarcoding data.

A recent study suggests that the predicted changes in temperature and oxygen by climate change are expected to reduce biodiversity in deep-sea demersal fish communities in the Gulf of California (Gallo et al., 2020). Fishes are key organisms in ocean ecosystem functioning through the food web (Albouy et al., 2019). eDNA analysis is a powerful tool for monitoring fish biodiversity comparable to conventional survey methods, such as capture-based sampling (e.g., Yamamoto et al., 2017; Ahn et al., 2020; Bessey et al., 2020; Sigsgaard et al., 2020; Oka et al., 2021) and its utilization to various studies are becoming more popular recently. However, deep-sea field surveys using research vessels equipped with water-collecting devices (e.g., CTD with Niskin bottles) are difficult to conduct, time-consuming, and costly. Recently, new sampling approaches using a novel device attached to trawl nets or the utilization of drip water from trawl nets for simpler and cheaper detection of eDNA have been conducted on bottom trawl fishing vessels (Russo et al., 2021; Maiello et al., 2022). These methods, which are potentially applied to commercial fishing vessels, are useful for the continuous monitoring of biodiversity. Similarly, pumped deep-sea water as an eDNA source supplied by a pumping facility is also cost-effective, foregoing the need for vessels and acts as a window into the near-shore deep-sea environments, which are more affected by human activities and climate change, making it highly effective as a method for the monitoring of deep-sea fish biodiversity. Understanding the

impacts of climate change on the biodiversity of fish is an important issue for predicting the future of marine ecosystems. Our optimized method may provide accurate qualitative information on fish biodiversity in deep-sea water and eDNA metabarcoding analysis using pumped deep-sea water is a cost-effective and useful approach for continuous biodiversity monitoring.

Data availability statement

Raw DNA sequence data from MiSeq are deposited in the DNA Data Bank of JAPAN (DDBJ) Sequenced Read Archive (DRA; <https://www.ddbj.nig.ac.jp/dra/index-e.html>) under accession number DRA013780. The datasets presented in this study can be found in the article/Supplementary Material.

Ethics statement

The animal study was reviewed and approved by All animal experiments were conducted in accordance with the Guidelines for Proper Conduct of Animal Experiments (Science Council of Japan).

Author contributions

TY and MK designed the research. TY, MK, YF, YN, ST, and AY collected and filtrated the deep-sea water. MK performed the experiments. TY and MK annotated the OTUs. TY analyzed all data and wrote the manuscript. All authors approved the final version of the manuscript and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmars.2022.965800/full#supplementary-material>

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