Universal performance of benzalkonium chloride for the preservation of environmental DNA in seawater samples

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Abstract

Environmental DNA (eDNA) analysis allows non-invasive and cost-effective monitoring of species distribution and composition in aquatic ecosystems. Benzalkonium chloride (BAC) treatment is an inexpensive and simple method for preserving macrobial eDNA in water samples, which is suitable for maximizing both the number of sampling replicates and water volume. However, its preservation performance has been evaluated in a limited manner by species-specific assays, targeting short fragments of mitochondrial DNA in freshwater and brackish ecosystems. Here, we examined the performance of BAC in preserving eDNA in seawater samples, targeting different fragment lengths of mitochondrial and nuclear eDNA, and community information inferred by eDNA metabarcoding. First, we quantified the time-series changes of Japanese jack mackerel (*Trachurus japonicus*) eDNA concentrations in experimental tanks and inshore seawater to compare the yields and decay rates of eDNA between BAC treatments. As a result, BAC addition increased the eDNA yields at the start of the experiment and substantially suppressed the initial phase of rapid degradation but not the subsequent phase of slower degradation. In addition, we performed eDNA metabarcoding targeting fish community, showing that BAC addition suppressed the decrease in species richness, where the number of fish species hardly varied throughout the day. Findings of the present and previous studies indicate high versatility of BAC in preserving qualitative (species richness) and quantitative (copy number) information on aqueous eDNA under various environmental conditions. BAC should therefore be used to minimize the false-negative detection of eDNA, regardless of target genetic regions, fragment sizes, environmental conditions, and detection strategies.

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eDNA preservation using BAC in marine systems

Abstract

Environmental DNA (eDNA) analysis allows non-invasive and cost-effective monitoring of species distribution and composition in aquatic ecosystems. Benzalkonium chloride (BAC) treatment is an inexpensive and simple method for preserving macrobial eDNA in water samples, which is suitable for maximizing both the number of sampling replicates and water volume. However, its preservation performance has been evaluated in a limited manner by species-specific assays, targeting short fragments of mitochondrial DNA in freshwater and brackish ecosystems. Here, we examined the performance of BAC in preserving eDNA in seawater samples, targeting different fragment lengths of mitochondrial and nuclear eDNA, and community information inferred by eDNA metabarcoding. First, we quantified the time-series changes of Japanese jack mackerel (*Trachurus japonicus*) eDNA concentrations in experimental tanks and inshore seawater to compare the yields and decay rates of eDNA between BAC treatments. As a result, BAC addition increased the eDNA yields at the start of the experiment and substantially suppressed the initial phase of rapid degradation but not the subsequent phase of slower degradation. In addition, we performed eDNA metabarcoding targeting fish community, showing that BAC addition suppressed the decrease in species richness, where the number of fish species hardly varied throughout the day. Findings of the present and previous studies indicate high versatility of BAC in preserving qualitative (species richness) and quantitative (copy number) information on aqueous eDNA under various environmental conditions. BAC should therefore be used to minimize the false-negative detection of eDNA, regardless of target genetic regions, fragment sizes, environmental conditions, and detection strategies.

Keywords:

benzalkonium chloride (BAC); DNA fragment size; environmental DNA (eDNA); marine; metabarcoding; nuclear DNA

Introduction

Effective monitoring of species distribution and abundance is the first step in the conservation of biodiversity and ecosystems (Margules & Pressey, 2000), as well as the proper management of fishery resources (Jackson et al., 2001). However, traditional methods that rely on capturing and morphological identification of species require substantial effort and cost, resulting in insufficient and biased monitoring and damage to individuals and their habitats (Thomsen & Willerslev, 2015). To overcome these limitations, analysis of environmental DNA (eDNA), which is defined as the total pool of DNA isolated from environmental samples (Pawlowski et al., 2020), has been developed (Ficetola et al., 2008; Minamoto et al., 2012; Deiner et al., 2017a). Macro-organisms such as fish are reported to produce eDNA from mucus, scale, feces, and gametes (Barnes & Turner, 2016). The PCR-based detection of eDNA in water samples enables non-invasive and cost-effective surveillance of species distribution and composition in aquatic ecosystems (Takahara et al., 2013; Yamamoto et al., 2017; Lawson Handley et al., 2019); thus, eDNA analysis is a promising tool for biological conservation and fishery resource management.

To achieve high accuracy and reliability of eDNA detection and quantification, eDNA must be preserved as soon as possible after water sampling because of its rapid degradation. There are a variety of preservation

strategies for aqueous eDNA (Table 1), which primarily depend on whether water filtration is performed in the field (on-site) or in the laboratory (in-lab). On-site filtration allows the immediate storage of filter samples *via* the addition of a buffer (Renshaw et al., 2015; Spens et al., 2017) or desiccation (Thomas et al., 2019), whereas in-lab filtration is generally feasible to maximize the number of sampling sites per survey, as water filtration in the field is not required. In case of in-lab processing, to suppress eDNA degradation during transportation to the laboratory, water samples have been chilled and frozen (Takahara et al., 2015; Jo et al., 2020a), precipitated using organic solvents (Doi et al., 2017; Ladell et al., 2019), and directly added with Longmire's buffer after collection (Williams et al., 2016).

Recently, benzalkonium chloride (BAC) has been used as an inexpensive and simple preservative for macrobial eDNA in water samples (Yamanaka et al., 2017). BAC is a cationic surfactant that inhibits bacterial function by adsorbing onto their cell surfaces (Ziani et al., 2011). The preservation strategy does not necessarily require elaborate work (e.g., the use of a pipette) and an equipment to chill the sample (e.g., cooler box and refrigerator). Yamanaka et al. (2017) reported that the addition of BAC at a final concentration of only 0.01% preserved 92% of bluegill sunfish (*Lepomis macrochirus*) eDNA in water samples after 8 hours at ambient temperature (˜ 25 °C) compared to only 14% in untreated water samples. Moreover, BAC addition allowed the retention of 50% of target eDNA in water samples after 10 days compared to non-detection in untreated water. To the best of our knowledge, BAC treatment is among the most suitable eDNA preservation strategies to maximize both the number of sampling sites and sampling volume (hundreds to thousands of milliliters). It allows intensive monitoring of species distribution and abundance *via* eDNA analysis over a short period of time.

Nevertheless, the performance of BAC in eDNA preservation has not necessarily been evaluated fully because most eDNA studies using BAC targeted short fragments (up to 200 bp) of mitochondrial DNA (mtDNA) in freshwater ecosystems (e.g., Sakata et al., 2017; Yamanaka et al., 2017; Hayami et al., 2020). Therefore, the present study investigated the performance of BAC in preserving eDNA in water samples from three aspects: (i) genetic region, (ii) DNA fragment size (i.e., the length of PCR amplicon), and (iii) marine ecosystems. First, given the possibility and prospect of using nuclear DNA (nuDNA) and longer DNA fragments in eDNA analyses for population-level inferences, such as population status and genetic diversity (Deiner et al., 2017b; Sigsgaard et al., 2020), it is important to verify whether BAC can be effective in preserving them from degradation. Moreover, BAC has only been applied to brackish water in a single experiment by Takahara et al. (2020); however, no study has examined its performance in eDNA preservation targeting seawater samples. Some water chemistry parameters, such as pH, salinity, and ionic content, are generally higher in marine systems than in freshwater systems (Okabe & Shimazu, 2007; Collins et al., 2018), which may affect the performance of BAC in eDNA preservation. Using Japanese jack mackerel (*Trachurus japonicus*), an economically important marine fish in East Asia, including Japan, we examined the preservative performance of BAC targeting different fragment sizes of nuDNA and mtDNA in seawater samples. Furthermore, Yamanaka et al. (2017) anticipated that BAC should enable the preservation of community information inferred by eDNA metabarcoding; however, this has not been verified yet. Thus, we performed eDNA metabarcoding using MiFish primers (Miya et al., 2015) and examined whether BAC could be effective in preserving genetic information of fish communities.

Materials and methods

Experimental design and water sampling

We conducted tank experiments and field sampling at the Maizuru Fisheries Research Station (MFRS) of Kyoto University, Japan, which is located in front of Maizuru Bay, in October 2020 (Fig. 1; Table S1). Two 60-L aliquots of rearing water were simultaneously transferred to two other tanks (sampling tanks) from a 200-L tank (stock tank), in which five Japanese jack mackerel individuals were kept (total length: approximately 20 cm). We then added 60 mL of BAC solution (Osvan S, Nihon Pharmaceutical Co., Ltd, Japan; 0.01% final concentration of BAC in the sampling tank) to one of the sampling tanks and thoroughly mixed the water. Subsequently, we collected four replicates of 1000 mL water samples using plastic bottles from both sampling tanks (defined as time 0). Subsequently, water sampling was performed 6, 12, 24, 48, 72, and 96 hours after

time 0 (i.e., at time 6, 12, 24, 48, 72, and 96, respectively), during which the water temperature was also measured in the sampling tanks (Table S1). The sampling tanks were aerated by a pump and placed in a water bath to minimize fluctuations in water temperature throughout the experiment. After water collection, water samples were immediately filtered with a 47-mm-diameter GF/F glass microfiber filter (nominal pore size, 0.7 μm; GE Healthcare Life Science, UK). At each time point, 1000 mL distilled water was filtered as a negative filtration control. All filtered samples were kept at -20 °C until eDNA extraction.

In addition, we collected 18 1-L seawater samples using plastic bottles from a floating pier in the MFRS. Surface seawater temperature and salinity were 20.9 °C and 30.9solution to nine of the collected seawater samples and thoroughly mixed them. Subsequently, we randomly collected three seawater samples with and without BAC addition and filtered them in the same manner as described above (defined as time 0). The remaining seawater samples were placed in a water bath at a constant temperature (18 +- 1degC). Further, after 6 and 24 hours (i.e., at time 6 and 24), we randomly sampled three seawater samples with and without BAC addition, respectively, and filtered them in the same manner as above. For each time point, 1000 mL distilled water was filtered as a negative filtration control. Throughout the experiments, we wore disposable gloves to collect and filter water samples and bleached the filtering devices (i.e., filter funnels [Magnetic Filter Funnel, 500 mL capacity; Pall Corporation, USA], 1 L beakers, tweezers, and sampling bottles) before every use in 0.1% sodium hypochlorite solution for at least 5 min (Yamanaka et al., 2017).

DNA extraction and quantitative real-time PCR

Total eDNA on the filter was extracted using the DNeasy Blood and Tissue Kit (Qiagen, Germany), according to the method described by Jo et al. (2017). We estimated eDNA concentration in water samples by quantifying the copy number of CytB genes and ITS1 regions using the StepOnePlus Real-Time PCR system (Thermo Fisher Scientific, USA). In this study, we targeted four types of Japanese jack mackerel eDNA, short and long fragments of the mitochondrial cytochrome b (CytB) gene and nuclear internal transcribed spacer-1 (ITS1) region in the ribosomal RNA (rRNA) gene (mtS, mtL, nuS, and nuL, respectively) for the eDNA quantification. We cited primers/probe sets amplifying mtS, mtL, and nuS of Japanese jack mackerel eDNA from previous literature (Table 2), and newly developed the primers/probe sets that amplify 603 bp fragments of the ITS1 region in Japanese jack mackerel (Table S2; Appendix S1). Each 13.3 μL of TaqMan reaction contained 2 μL template DNA, a final 900 nM concentration of both the forward and reverse primers, and 125 nM of TaqMan probe in 1 × TaqPath^{TM} qPCR Master Mix, CG. We simultaneously analyzed 2 μL of pure water as a negative PCR control. We performed qPCR using a dilution series of standards containing 3×10^1–3×10^4 copies of a linearized plasmid containing synthesized artificial DNA fragments from the CytB gene (1141 bp) or ITS1 region (666 bp) of target species. All eDNA samples, standards, and negative controls were performed in triplicates. The thermal conditions for qPCR were as follows: 2 min at 50 °C, 10 min at 95 °C, 55 cycles of 15 s at 95 °C and 1.5 min at 60 °C (2-step PCR) for mtS and nuS and 2 min at 50 °C, 10 min at 95 °C, 55 cycles of 15 s at 95 °C, 30 s at 60 °C, and 1 min at 72 °C (3-step PCR) for mtL and nuL. We calculated eDNA concentrations by averaging the triplicate, and each PCR-negative replicate (indicating non-detection) was regarded as containing zero copies (Ellison et al., 2006).

Library preparation, iSeq sequencing, and bioinformatics

We performed eDNA metabarcoding using seawater samples to assess the differences in marine fish communities inferred by eDNA between BAC treatments. Each 12 μL of first-round PCR contained 1 μL template DNA, a final 300 nM concentration of MiFish-U primers, which amplify approximately 170 bp fragments of mitochondrial 12S rRNA regions from teleost fish (Miya et al., 2015), in 2 × KAPA HiFi HotStart ReadyMix (KAPA Biosystems, Wilmington, USA). The thermal conditions of the first PCR were as follows: 3 min at 95 °C, 40 cycles of 20 s at 98 °C, 15 s at 65 °C, and 15 s at 72 °C, followed by 5 min at 72 °C. PCR for eDNA samples and negative controls (1 μL of pure water instead of template DNA) was performed in eight replicates. After the first PCR, eight replicates from each sample were pooled and purified using the SPRIselect Reagent Kit (Beckman Coulter, Brea, CA, USA) according to the manufacturer's instructions. We then quantified the total DNA concentrations of the purified PCR products using a Qubit dsDNA HS assay kit and a Qubit fluorometer 3.0 (Thermo Fisher Scientific) and diluted them to 0.1 ng/μL.

Each 12 μL of second-round PCR contained adapter and 8-bp index sequences for high-throughput sequencing added to the first PCR products, as well as 1 μL template DNA and a final concentration of 300 nM for each forward and reverse primer in 2 × KAPA HiFi HotStart ReadyMix. The thermal conditions of the second PCR were as follows: 3 min at 95 °C, 12 cycles of 20 s at 98 °C, and 20 s at 72 °C, followed by 5 min at 72 °C. After pooling all second PCR products, we selected the product size (approximately 370 bp) of the library sample by electrophoresis using E-Gel SizeSelect 2% (Thermo Fisher Scientific) with the E-Gel Precast Agarose Electrophoresis System (Thermo Fisher Scientific), which was confirmed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). The library sample was then sequenced using an Illumina iSeq with 2 × 150 bp paired-end kits (Illumina, San Diego, USA). We performed data preprocessing and analyses of iSeq raw reads using USEARCH v10.0.240 (Edgar, 2010) according to the method described by Sakata et al. (2020a). We discarded all reads from seawater samples corresponding to (i) freshwater fish, regarding it as contamination from the rivers flowing into Maizuru Bay, and (ii) some bony fish, which were regarded as contamination of domestic wastewater (detailed information can be seen in Appendix S2).

Statistical analyses

All statistical analyses were performed using R version 4.0.4 (R Core Team, 2021). The decay rates of Japanese jack mackerel eDNA were estimated using the time-series changes in their eDNA concentrations from each sampling tank. Previous studies estimated eDNA decay rates by fitting a monophasic exponential decay model (Strickler et al., 2015; Jo et al., 2020b) as follows:

$$C_t = C_0 e^{-kt}$$

where C_t is the eDNA concentration (copies) at time t[hour], C_0 is the eDNA concentration at time 0, and k is the decay rate constant (/hour). We used a linear model to compare the decay rates of each type of eDNA between BAC treatments, where log-transformed eDNA concentration was included as the dependent variable and sampling time point (hour), BAC treatment, and their interaction were included as explanatory variables.

Alternatively, following Eichmiller et al. (2016), we used a biphasic exponential decay model if the fitness of a monophasic decay model was poor and there was an obvious breakpoint between two distinct phases of eDNA degradation as follows:

$$C_t = C_0 e^{-k_1 t'} e^{-k_2 (t - t')}$$

where k_1 and k_2 are the eDNA decay rate constants at the initial rapid and following slower phases, respectively, and t' is the time of breakpoint between different degradation phases (hour). We estimated eDNA decay rates with 95% confidence intervals (CIs) and breakpoints using the package 'segmented' (Muggeo, 2017). We compared the fitness of monophasic and biphasic decay models between BAC treatments by calculating Akaike's information criterion (AIC). All eDNA samples with concentrations below one copy per reaction were excluded.

Furthermore, we compared time-series changes in fish species composition inferred by eDNA metabarcoding between BAC treatments. For each time point, the number of fish species detected by eDNA metabarcoding was compared between BAC treatments using the exact McNemer test in the package 'exact2×2' (Fay, 2010). We then visualized the community compositions based on Jaccard dissimilarities using a two-dimensional non-metric multidimensional scaling (nMDS) with 10000 permutations by *vegdist* and *metaMDS* functions in the package 'vegan' (Oksanen et al., 2019). In addition, we performed a permutational multivariate analysis of variance (PERMANOVA) with 10000 permutations using *adonis* function to examine whether the community compositions were different among BAC treatments and/or time points.

Results

In the tank experiment, regardless of BAC treatment, we observed biphasic exponential degradation of all types of Japanese jack mackerel eDNA (Fig. 2; Table 3). All types of eDNA concentrations were higher in the treatment with BAC addition than in those without BAC at time 0, which lasted throughout the sampling period. The decay rates at the initial phase (k_1) were substantially lower in the treatment with BAC addition (31.0 to 53.0% relative to the treatment without BAC), while those at the following slower phase (k_2) were not significantly different between BAC treatments (Fig. 3). In contrast, in field sampling, we observed monophasic exponential degradation of shorter fragments of eDNA (Fig. 4). Linear models showed a significant interaction between sampling time points and BAC treatments for nuS, indicating that eDNA decay rates were significantly lower in the treatment with BAC than in those without BAC ($P < 0.05$; Table S3). Although we did not confirm a significant interaction for mtS, target eDNA was detected for 24 hours from the seawater samples with BAC addition, whereas it was hardly detected in the samples without BAC addition (Fig. 4). We did not evaluate the effect of BAC addition on the degradation of longer eDNA fragments (mtL and nuL) because of their poor detection relative to that of shorter eDNA fragments. The overall PCR efficiencies and R^2 values of the standard curves are shown in Table S4. A few filtration negative controls in the tank experiment showed PCR amplification, whose concentrations were less than one copy per PCR and much less than those of the sampling tank at the corresponding time points. No amplification was observed in any of the PCR-negative controls throughout the study.

Moreover, the number of fish species detected by eDNA metabarcoding was higher in the treatment with BAC over time (Fig. 5a). In total, 65 marine and brackish fish were detected in 18 of 1-L seawater samples, wherein 58 and 45 species were detected in the samples with and without BAC addition, respectively; 36, 40, and 38 species were detected in samples with BAC, whereas 36, 28, and 27 species were detected in samples without BAC at time 0, 6, and 24, respectively, when sampling triplicates were pooled (Table 4; the number of detected species per sample is shown in Table S5). Exact McNemer tests showed significant differences in the number of fish species between BAC treatments at time 6 and 24 (both $P < 0.05$), while no statistical difference was observed at time 0 ($P = 1.00$). In addition, PERMANOVA tests showed a significant difference in community composition between BAC treatments ($P < 0.05$) but not between time points ($P = 0.79$) (Fig. 5b). We additionally confirmed that the variances of the compositions were not statistically different among treatments (PERMDISP; both $P > 0.1$). After preprocessing the iSeq raw reads and removing potential contaminations, none of the eDNA reads were detected from all filtration and PCR negative controls (Table S6). All the rarefaction curves, generated by *rarecurve* function in the package 'vegan', showed that the number of species detected from each sample was saturated and the library sample was satisfactorily sequenced (Fig. S1).

Discussion

Although BAC is an effective tool for suppressing eDNA degradation in water samples, its preservative performance has only been confirmed by species-specific detection targeting shorter fragments of mitochondrial genes. In the present study, targeting different fragment sizes of mtDNA and nuDNA, we demonstrated that BAC suppressed the degradation of various types of eDNA in seawater samples and increased eDNA yields. Moreover, BAC addition suppressed the time-series changes in species richness inferred by eDNA metabarcoding. Taking previous findings of BAC performance in freshwater and brackish environments into account (Yamanaka et al., 2017; Takahara et al., 2020), our findings indicated a high versatility of BAC in preserving aqueous eDNA regardless of genetic regions, DNA fragment sizes, and environmental conditions.

The tank and field experiments showed that BAC addition increased the yield of Japanese jack mackerel eDNA at time 0 and suppressed the degradation of eDNA. Similar tendencies were reported by Takahara et al. (2020); even at the start of water collection, target eDNA concentrations were higher in the treatment with BAC addition, regardless of species. These results imply that both the suppression of eDNA degradation and the increase in initial eDNA concentrations could substantially contribute to the preservation of eDNA in water samples *via* BAC. Adding a surfactant such as BAC to water samples might agglutinate a variety of suspended particles, including eDNA, which may allow eDNA to be captured by a filter more frequently. The apparent particle size distribution of eDNA in water samples might shift in the larger size fraction by

adding BAC. On the other hand, depending on water quality, it is also possible for BAC to agglutinate PCR inhibitory substances such as humic, fulvic, and tannic acids. Sales et al. (2019) reported that the number of fish species detected by eDNA metabarcoding (MOTUs) was slightly lower in samples stored at ambient temperature with BAC addition than in those stored in a cooler box with ice. Such tropical freshwater ecosystems are typically characterized by turbidity due to high sediment loads and algae, and the result might thus have included the effect of PCR inhibition by BAC.

We observed biphasic degradation of the target eDNA in the tank experiment. Some previous studies estimating eDNA decay rates reported similar processes of eDNA degradation and implied that a part of eDNA degraded rapidly, and subsequently, the rest degraded slowly (Eichmiller et al., 2016; Bylemans et al., 2018; Shogren et al., 2018). In particular, Bylemans et al. (2018) reported that the initial rapid degradation of eDNA might be caused by intra-cellular nuclease activities and/or microbial digestion, and slower degradation might reflect other degradation factors such as hydrolytic and oxidative decomposition of DNA molecules. Considering that BAC inactivates bacterial functions by adsorbing to their cell surfaces (Ziani et al., 2011), this hypothesis is consistent with our findings that BAC substantially suppressed the initial rapid degradation of eDNA but had little effect on subsequent slower degradation in the tank experiment. Moreover, Jo et al. (2019) reported that the inflow of degraded eDNA from larger (e.g., intra-cellular DNA) to smaller size fractions (e.g., extra-cellular DNA) could prolong the apparent persistence of smaller-sized eDNA compared to larger-sized ones. Altogether, BAC mainly preserves intra-cellular eDNA, such as cell and tissue fragments, by weakening microbial activities in water.

Conversely, in field experiments, we observed the monophasic degradation of eDNA. This could simply be explained by a lower concentration of target eDNA, a shorter experimental period, and fewer sampling time points relative to those in the tank experiment. Alternatively, an aerobic environment of seawater samples, where sampling tanks were continuously aerated, might have inflated the decay rates and influenced the degradation processes of aqueous eDNA in the tank experiment (Weltz et al., 2017). In any case, the finding that eDNA in seawater samples collected from the field scarcely degraded throughout the day by BAC addition would indicate a high suitability of BAC for preserving eDNA in marine ecosystems. Unfortunately, longer fragments of eDNA were rarely detected in field samples, which could be improved by collecting water samples in the warmer season because Japanese jack mackerels are abundant in Maizuru Bay from July to August (Masuda, 2008). Jo et al. (2017) actually detected 719 bp fragments of its mitochondrial eDNA collected in the summer season here.

In addition to the species-specific analyses using quantitative real-time PCR described above, we revealed that the richness of fish communities inferred by eDNA metabarcoding did not vary among sampling time points by BAC addition, although species richness decreased with time without BAC addition. Surprisingly, the number of species detected from seawater samples was not different throughout the day by BAC addition. Smaller eDNA decay rates by BAC addition would allow the detection of more fish species with low eDNA concentrations in seawater samples. Although PERMANOVA tests showed the differences in community compositions between BAC treatments but not time points, considering the nMDS plot, it is likely that compositions between BAC treatments were relatively similar just after seawater sampling (i.e., time 0), followed by larger differences in compositions between BAC treatments over time (i.e., time 6 and 24). Our study is the first to show that BAC is effective in preserving qualitative eDNA information, such as species richness, as well as quantitative information such as copy number. These findings would partly support the reasonability of using BAC to preserve community information inferred by eDNA metabarcoding from water samples, including previous studies (e.g., Hayami et al., 2020; Sakata et al., 2020b).

Conclusions

Targeting various fragment sizes of mitochondrial and nuclear genes of Japanese jack mackerels, we showed that the addition of BAC suppressed eDNA degradation and increased its initial concentration. In addition, BAC enabled to maintain the number of marine fish species detected by eDNA metabarcoding in seawater samples. Our study suggests the use of BAC for the preservation of various types of eDNA from water samples under various environmental conditions regardless of eDNA detection strategies (i.e., species-specific

or metabarcoding assay); accordingly, we could increase the yield of target eDNA and the number of detected species, which may prevent the underestimation of species abundance and richness *via* eDNA analysis.

Some issues remain to be addressed in the future. First, all eDNA studies using BAC have targeted fish and amphibians, and it is unknown whether BAC effectively preserves eDNA released from other vertebrates and invertebrates. Different production sources of eDNA among taxa might lead to different performances of BAC in preserving aqueous eDNA. In addition, understanding the interactions between BAC and environmental factors is necessary. Takahara et al. (2020) reported a statistically marginal interaction between BAC treatment and storage temperature on eDNA yields. Further studies are required to determine the effects of water chemistry and environmental conditions on the eDNA preservation performance of BAC. Information on the performance of BAC, including that revealed in this study, would simplify the application of eDNA analysis in natural environments and enable the effective and precise monitoring of biodiversity conservation and resource management.

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Data Accessibility

The raw data of qPCR and iSeq raw reads are to be uploaded to the Dryad Digital Repository upon acceptance.

Authors' contributions

T.J., R.M., and T.M. conceived the experiments. T.J. and H.M. performed tank experiments and field sampling. T.J. and M.K.S. performed the molecular analyses and bioinformatic analyses. T.J. analyzed the data and wrote the first draft of the manuscript. All authors have edited and provided feedback on the manuscript.

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Figures

Figure 1. Overall flowchart of the experiments in this study. We transferred 60-L aliquots of rearing water from a stock tank, where Japanese jack mackerels were kept, into two sampling tanks and added BAC solution to one of them (a). In addition, we collected 18 seawater samples from a floating pier in the MFRS and added BAC solution to nine of them (b). Time-series water sampling and filtration was performed for 96 hours (a) or 24 hours (b) followed by DNA extraction, quantitative real-time PCR, and high-throughput sequencing.

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Figure 2. Decay curves of Japanese jack mackerel eDNA for (a) mtS (164 bp fragment of CytB gene), (b) mtL (682 bp fragment of CytB gene), (c) nuS (164 bp fragment of ITS1 region), and (d) nuL (603 bp fragment of ITS1 region) observed in the tank experiment. Concentrations of target eDNA (log-transformed) in sampling tanks with and without BAC addition are shown as circles and triangles respectively. Breakpoints between different phases of degradations are shown as dotted lines, which were estimated by the package 'segmented' in R.

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Figure 3. Comparison of eDNA decay rates among eDNA types (mtS, mtL, nuS, and nuL) and BAC treatments. Decay rate constants estimated from sampling tanks with and without BAC addition are shown as circles and triangles, and those estimated from initial rapid (k_1) and following slower (k_2) phases are shown as closed and open plots. Error bars indicate the 95 % CIs of decay rate constants of each target eDNA between BAC treatments (Y: with BAC addition; N: without BAC addition).

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Figure 4. Comparisons of time-series changes of Japanese jack mackerel eDNA concentrations for (a) nuS and (b) nuL observed in field sampling. Circles and triangles in the plots show the time-series changes of target eDNA concentrations (original concentrations + 0.1 followed by log-transformed) with and without BAC addition, where regression lines are indicated in black and gray lines and the corresponding 95 % CIs are indicated in solid and dotted lines, respectively. In (a), we note that the lower CI of the regression line in the treatment without BAC addition is not visualized.

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Figure 5. (a) Comparisons of species richness inferred by eDNA metabarcoding between BAC treatments for each sampling time point. Each Venn diagram shows the differences of the number of fish species detected by eDNA metabarcoding between BAC treatments. Each sampling triplicate is pooled here. (b) A two-dimensional nMDS plot based on Jaccard dissimilarity among BAC treatments and time points. Each symbol represents BAC treatments (Y: with BAC addition; N: without BAC addition) and time points (0, 6, or 24). Plots based on each triplicate in the same treatment are encircled by triangles.

Tables

Table 1. Overview of major strategies for preserving macrobial eDNA in water.

Literature	Preserved sample	Method	Water volume per sample	Major findings
Takahara et al. (2015)	Water	Freezing (-30 °C)	1000 mL	Detection rate of common carp (*Cyprinus carpio*) eDNA was lower in frozen samples than in non-frozen samples, while eDNA concentrations were not different between treatments.
Williams et al. (2016)	Water	Longmire's solution[1] (1: 3 of preservative to water) freezing (-80 °C)	15 mL	Detectability of wild pig (*Sus scrofa*) eDNA did not decrease in both treatments for 58 days.
Doi et al. (2017)	Water	Isopropanol precipitation (1: 1 of preservative to water) Ethanol precipitation (1: 2 of preservative to water)	27 mL (Isopropanol) 15 mL (Ethanol)	Yields of common carp eDNA were not different between treatments.
Yamanaka et al. (2017)	Water	BAC[2] solution (0.01 % of preservative to water)	500 mL	92 and 50 % of bluegill sunfish (*Lepomis macrochirus*) eDNA retained after 8 hours and 10 days at ambient temperature.
Ladell et al. (2019)	Water	Ethanol precipitation (1: 2 of preservative to water) cooling (4 °C)	15 mL	Silver carp (*Hypophthalmichthys molitrix*) eDNA was amplified from the samples preserved by ethanol precipitation after up to 7 days even at room temperature.

Literature	Preserved sample	Method	Water volume per sample	Major findings
Sales et al. (2019)	Water	BAC[2] solution (0.01 % of preservative to water) cooling	1000 mL	MOTUs were higher in the samples stored in ice than those preserved in BAC at ambient temperature.
Takahara et al. (2020)	Water	BAC[2] solution (0.01 % of preservative to water) cooling (4 °C) freezing (-25 °C)	500 mL	Preservative effect of BAC on eDNA degradation was similar among species but not among storage temperature.
Renshaw et al. (2015)	Disk filter	Longmire's buffer[1] CTAB buffer[3]	250 mL	Bluegill sunfish eDNA concentrations did not decrease in both treatments for 2 weeks and was higher in the filter in Longmire's buffer.
Spens et al. (2017)	Enclosed filter	Freezing (-20 °C) Ethanol Longmire's buffer[1] RNAlater	15 mL	European perch (*Perca fluviatilis*) eDNA yields were higher in the filter in ethanol or Longmire's buffer.

[1]100 mM Tris, 100 mM EDTA, 10 mM NaCl, and 0.5 % (w/v) SDS; [2]abbreviation of 'benzalkonium chloride';[3]1.4 M NaCl, 2 % (w/v) cetyltrimethyl ammonium bromide, 100 mM Tris, 20 mM EDTA, and 0.25 mM polyvinylpyrrolidone.

Table 2. Primers/probe sets used in this study.

ID	Target species	Target genetic region	Sequence (5'-3')	Tm [°C]	Reference
Tja_CytB_F	Japanese jack mackerel (*Trachurus japonicus*)	Mitochondrial cytochrome b (CytB)	CAG ATA TCG CAA CCG CCT TT	58.7	Jo et al. (2020b)
Tja_CytB_-R164			TTC TTT GTA GAG GTA CGA GCC G	59.8	

ID	Target species	Target genetic region	Sequence (5'-3')	Tm [°C]	Reference
Tja_CytB_-R682			ATT GAT CGG AGA ATG GCG TAT	57.3	Jo et al. (2017)
Tja_CytB_P			[FAM]- TAT GCA CGC CAA CGG CGC CT -[TAMRA]	67.9	Jo et al. (2020b)
Tja_ITS1_F164		Nuclear rRNA internal transcribed spacer-1 (ITS1)	GCG GGT ACC CAA CTC TCT TC	60.1	Jo et al. (2020b)
Tja_ITS1_F603			TCT TTG GCT TTA ACT TGC CCG	59.4	This study
Tja_ITS1_R			CCT GAG CGG CAC ATG AGA G	63.2	Jo et al. (2020b)
Tja_ITS1_P			[FAM]- CTC TCG CTT CTC CGA CCC CGG TCG -[TAMRA]	70.8	

Note: We changed the reverse primer for the CytB gene and the forward primer in the ITS1 region to alter the length of the PCR amplicon.

Table 3. Results of model fitting to eDNA decay curves observed in the tank experiment.

Target eDNA	BAC	AIC	AIC	Intercept	Intercept	Intercept	k_1 [per hour, ×-1]	k_1 [per hour, ×-1]	k_1 [per hour, ×-1]
		Monophasic	Biphasic	Estimate	SE	P value	Estimate	SE	Lower (2.5 %)
mtS	Y	-13.1	-73.9	4.183	0.023	***	0.029	0.002	0.026
	N	19.6	-3.6	3.493	0.103	***	0.094	0.024	0.044
mtL	Y	9.0	-33.0	3.701	0.047	***	0.045	0.003	0.038
	N	32.6	15.9	3.284	0.136	***	0.085	0.018	0.048
nuS	Y	-12.9	-27.3	4.804	0.052	***	0.045	0.004	0.053
	N	37.4	-18.1	3.777	0.072	***	0.111	0.009	0.130
nuL	Y	11.1	10.4	4.582	0.121	***	0.057	0.016	0.089
	N	44.3	21.3	3.903	0.165	***	0.166	0.039	0.247

Target eDNA	BAC	k_2 [per hour, ×-1]	k_2 [per hour, ×-1]	k_2 [per hour, ×-1]	k_2 [per hour, ×-1]	k_2/k_1	Breakpoint	Breakpo
		Estimate	SE	Lower (2.5 %)	Upper (97.5 %)		[hour] Estimate	[hour] SE
mtS	Y	0.007	0.001	0.005	0.009	23.6 %	43.9	2.9
	N	0.010	0.001	0.007	0.014	11.1 %	10.1	2.4
mtL	Y	0.007	0.002	0.003	0.010	15.0 %	27.7	2.9
	N	0.012	0.003	0.005	0.018	13.8 %	16.2	3.4
nuS	Y	0.027	0.002	0.031	0.023	59.8 %	37.4	7.3
	N	0.010	0.001	0.013	0.007	9.3 %	15.2	1.2
nuL	Y	0.032	0.003	0.037	0.026	55.9 %	16.3	8.7
	N	0.020	0.003	0.026	0.013	11.9 %	9.8	2.2

Note: Abbreviations 'Y and 'N' mean the treatments with and without BAC addition, respectively. Asterisks indicate a significant intercept in the linear regression (*** $P < 0.001$).

Table 4. Fish species detected from seawater samples by eDNA metabarcoding.

BAC addition	Yes	Yes	Yes	No	No	No
Species name / Time point [hour]	0	6	24	0	6	24
Ablennes hians	41	0	0	0	0	0
Acanthogobius flavimanus	0	36	0	68	5	0
Acanthopagrus schlegelii	2606	2234	3792	2987	1740	1779
Acentrogobius pflaumii	0	0	0	9	0	0
Anguilla japonica	177	0	0	0	0	0
Chaenogobius gulosus	0	5	122	122	0	0
Decapterus maruadsi	365	514	488	281	527	1805
Dictyosoma burgeri	54	88	44	573	251	226
Ditrema spp.	0	112	0	0	17	0
Engraulis japonicus	537	180	596	125	256	408
Epinephelus akaara	0	6	0	0	0	0
Epinephelus awoara	0	0	0	0	0	176
Equulites rivulatus	62	6	347	157	84	82
Eviota abax	0	0	0	48	0	0
Girella punctata	4	259	48	97	0	0
Halichoeres poecilopterus	6	0	0	0	0	9
Halichoeres tenuispinis	0	0	0	5	0	0
Hexagrammos agrammus	37	0	5	35	120	307
Hypoatherina valenciennei	0	17	0	0	157	0
Hyporhamphus sajori	2120	1938	2682	1700	2218	582
Istigobius campbelli	6	0	7	0	0	0
Jaydia lineata	0	0	0	0	35	0
Kaiwarinus equula	23	0	0	0	0	0
Konosirus punctatus	535	574	584	424	492	391
Lateolabrax japonicus	360	321	285	231	1552	599
Leucopsarion petersii	0	0	277	22	0	0
Luciogobius pallidus	4	0	0	0	0	0
Mugil cephalus	83	0	96	118	16	14
Muraenesox cinereus	98	0	0	0	0	0
Nuchequula nuchalis	146	50	257	4	213	0

BAC addition	Yes	Yes	Yes	No	No	No
Omobranchus elegans	0	38	0	63	0	84
Omobranchus punctatus	4	0	0	67	0	0
Oncorhynchus keta	0	24	0	0	0	0
Ostorhinchus semilineatus	0	12	0	53	0	0
Pagrus major	0	17	117	27	114	0
Parablennius yatabei	269	567	1017	329	498	712
Paralichthys olivaceus	44	13	68	0	0	0
Parapercis snyderi	0	0	0	10	0	0
Parapristipoma trilineatum	0	0	6	0	95	0
Petroscirtes breviceps	0	0	176	0	0	0
Pholis nebulosa	4	0	88	0	0	0
Plotosus japonicus	0	0	5	7	0	4
Psenopsis anomala	0	0	23	0	0	0
Pseudolabrus sieboldi	12221	12948	25	43	34	12
Repomucenus spp.	0	62	0	0	0	0
Rhynchopelates oxyrhynchus	0	10	0	0	0	0
Rudarius ercodes	6	9	0	0	0	0
Saurida wanieso	0	12	0	0	0	0
Scomber japonicus	419	1053	1376	374	715	2407
Scomberomorus niphonius	1921	546	900	520	1129	999
Sebastes spp.	0	11	84	0	0	129
Sebastiscus marmoratus	0	69	9	0	0	0
Seriola dumerili	107	43	101	0	125	575
Seriola lalandi	23	0	0	0	0	0
Seriola quinqueradiata	1314	1084	1098	497	901	1403
Siganus fuscescens	153	243	159	486	1127	335
Sillago japonica	0	0	0	0	0	40
Sphyraena pinguis	232	272	506	239	434	432
Stephanolepis cirrhifer	67	51	28	12	0	0
Strongylura anastomella	0	121	0	0	0	0
Takifugu spp.	1071	77	453	1461	692	1319
Thamnaconus modestus	0	58	315	82	0	0
Trachurus japonicus	267	492	1699	364	260	529
Trichiurus japonicus	0	0	5	0	0	0
Tridentiger trigonocephalus	19	25	14	69	300	9
Total number of fish species	36	40	38	36	28	27

Note: Numerals indicate the sum of the number of eDNA reads among sampling triplicates after preprocessing of iSeq raw data, except for the total number of fish species. Some fish were assigned to the genus level because of poor taxonomical discrimination.

Supporting Information

Appendix S1. Information on primers/probe development.

Appendix S2. Information on data preprocessing and analyses of iSeq raw reads.

Figure S1. Rarefaction curves in eDNA metabarcoding using seawater samples.

Table S1. Detailed information on water sampling.

Table S2. Details of sequence information from the National Center for Biotechnology Information (NCBI) for the development of primers and probe.

Table S3. Results of linear models for field samplings.

Table S4. R^2 values, slopes, and Y intercepts of the calibration curves, and the PCR efficiencies (mean ± 1 SD) for each type of eDNA.

Table S5. Detailed list of fish species detected from seawater samples by eDNA metabarcoding.

Table S6. Detailed information on fish eDNA metabarcoding using seawater samples.