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Running head:

eDNA preservation using BAC in marine systems

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Abstract

Environmental DNA (eDNA) analysis allows non-invasive and cost-effective monitoring of macroorganisms' distribution and composition in aquatic ecosystems. Benzalkonium chloride (BAC) is an inexpensive and simple preservative for eDNA in water samples, and has been

used in many eDNA studies for preventing its degradation during transportation. However, its preservation performance has limitedly been evaluated 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. 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. For both tank and field samples, BAC treatment substantially suppressed the degradation of all types of target eDNA and increased the eDNA yields at the start of the experiment. In addition, we performed eDNA metabarcoding targeting fish community to compare the species richness and composition in seawater samples between BAC treatments, showing that the number of fish species in field samples hardly varied throughout a day by BAC treatment. Our findings indicate high versatility of BAC in preserving both the quantitative (copy number) and qualitative (species richness) information on various types of aqueous eDNA in 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.

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; Rodriguez-Ezpeleta et al., 2021), 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. It is considered that eDNA degradation is caused by microbial activities and various abiotic factors such as high temperature and acidic conditions (Strickler et al., 2015; Jo & Minamoto, 2021). There are a variety of preservation strategies for aqueous eDNA, 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; Mauvisseau et al., 2021) 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; Mauvisseau et al., 2021), 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; Sales et al., 2019). BAC is a cationic surfactant that inhibits bacterial function by adsorbing onto their

87 cell surfaces (Ziani et al., 2011). The preservation strategy does not necessarily require
88 elaborate work (e.g., the use of a pipette) and an equipment to chill the sample (e.g., cooler
89 box and refrigerator). Yamanaka et al. (2017) reported that the addition of BAC at a final
90 concentration of only 0.01% preserved 92% of bluegill sunfish (*Lepomis macrochirus*) eDNA
91 in water samples after 8 hours at ambient temperature (~ 25 °C) compared to only 14% in
92 untreated water samples. Moreover, BAC addition allowed the retention of 50% of target
93 eDNA in water samples after 10 days compared to non-detection in untreated water. To the
94 best of our knowledge, BAC treatment is among the most suitable eDNA preservation
95 strategies to maximize both the number of sampling sites and sampling volume (hundreds to
96 thousands of milliliters). It allows intensive monitoring of species distribution and abundance
97 *via* eDNA analysis over a short period of time.
98 Nevertheless, the performance of BAC in eDNA preservation has not necessarily
99 been evaluated fully because most eDNA studies using BAC targeted short fragments (up to
100 200 bp) of mitochondrial DNA (mtDNA) in freshwater ecosystems (e.g., Sakata et al., 2017;
101 Yamanaka et al., 2017; Sales et al., 2019; Hayami et al., 2020). Therefore, the present study
102 investigated the performance of BAC in preserving eDNA in water samples from three
103 aspects: (i) genetic region, (ii) DNA fragment size (i.e., the length of PCR amplicon), and (iii)

104 marine ecosystems. First, given the possibility and prospect of using nuclear DNA (nuDNA)
105 and longer DNA fragments in eDNA analyses for population-level inferences, such as
106 population status and genetic diversity (Deiner et al., 2017b; Sigsgaard et al., 2020), it is
107 important to verify whether BAC can be effective in preserving them from degradation.
108 Moreover, BAC has only been applied to brackish water in a single experiment by Takahara et
109 al. (2020); however, no study has examined its performance in eDNA preservation targeting
110 seawater samples. Some water chemistry parameters, such as pH, salinity, and ionic content,
111 are generally higher in marine systems than in freshwater systems (Okabe & Shimazu, 2007;
112 Collins et al., 2018), which may affect the performance of BAC in eDNA preservation. Using
113 Japanese jack mackerel (*Trachurus japonicus*), an economically important marine fish in East
114 Asia, including Japan, we examined the preservative performance of BAC targeting different
115 fragment sizes of nuDNA and mtDNA in seawater samples. Furthermore, Yamanaka et al.
116 (2017) anticipated that BAC should enable the preservation of community information
117 inferred by eDNA metabarcoding; however, this has not been verified yet. Recently, Holman
118 et al. (2021) reported that time-series eDNA degradation also decreases the number of eDNA
119 reads and ASVs (amplicon sequence variants) based on metabarcoding, and 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 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 30.9, respectively. We added 1 mL BAC solution 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 ± 1°C). 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,

154 and sampling bottles) before every use in 0.1% sodium hypochlorite solution for at least 5
155 min (Yamanaka et al., 2017).
156
157 *DNA extraction and quantitative real-time PCR*
158 Total eDNA on the filter was extracted using the DNeasy Blood and Tissue Kit (Qiagen,
159 Germany), according to the method described by Jo et al. (2017). We estimated eDNA
160 concentration in water samples by quantifying the copy number of CytB genes and ITS1
161 regions using the StepOnePlus Real-Time PCR system (Thermo Fisher Scientific, USA). In
162 this study, we targeted four types of Japanese jack mackerel eDNA, short and long fragments
163 of the mitochondrial cytochrome b (CytB) gene (mtS and mtL respectively) and nuclear
164 internal transcribed spacer-1 (ITS1) region in the ribosomal RNA (rRNA) gene (nuS and nuL
165 respectively) for the eDNA quantification. We cited primers/probe sets amplifying mtS, mtL,
166 and nuS of Japanese jack mackerel eDNA from previous literature (Table 1), and newly
167 developed the primers/probe sets that amplify 603 bp fragments of the ITS1 region in
168 Japanese jack mackerel (Table S2; Appendix S1). Each 13.3 μL of TaqMan reaction contained
169 2 μL template DNA, a final 900 nM concentration of both the forward and reverse primers,
170 and 125 nM of TaqMan probe in 1 × TaqPath^{TM} qPCR Master Mix, CG. We simultaneously

171 analyzed 2 μL of pure water as a negative PCR control. We performed qPCR using a dilution
172 series of standards containing 3×10^1–3×10^4 copies of a linearized plasmid containing
173 synthesized artificial DNA fragments from the CytB gene (1141 bp) or ITS1 region (666 bp)
174 of target species. All eDNA samples, standards, and negative controls were performed in
175 triplicates. The thermal conditions for qPCR were as follows: 2 min at 50 °C, 10 min at 95 °C,
176 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
177 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
178 PCR) for mtL and nuL. We calculated eDNA concentrations by averaging the triplicate, and
179 each PCR-negative replicate (indicating non-detection) was regarded as containing zero
180 copies (Ellison et al., 2006).
181
182 *Library preparation, iSeq sequencing, and bioinformatics*
183 We performed eDNA metabarcoding using seawater samples to assess the differences in
184 marine fish communities inferred by eDNA between BAC treatments. Each 12 μL of first-
185 round PCR contained 1 μL template DNA, a final 300 nM concentration of MiFish-U
186 primers, which amplify approximately 170 bp fragments of mitochondrial 12S rRNA regions
187 from teleost fish (Miya et al., 2015), in 2 × KAPA HiFi HotStart ReadyMix (KAPA

188 Biosystems, Wilmington, USA). The thermal conditions of the first PCR were as follows: 3
189 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
190 72 °C. PCR for eDNA samples and negative controls (1 μL of pure water instead of template
191 DNA) was performed in eight replicates. After the first PCR, eight replicates from each
192 sample were pooled and purified using the SPRIselect Reagent Kit (Beckman Coulter, Brea,
193 CA, USA) according to the manufacturer's instructions. We then quantified the total DNA
194 concentrations of the purified PCR products using a Qubit dsDNA HS assay kit and a Qubit
195 fluorometer 3.0 (Thermo Fisher Scientific) and diluted them to 0.1 ng/μL.
196 Each 12 μL of second-round PCR contained adapter and 8-bp index sequences for
197 high-throughput sequencing added to the first PCR products, as well as 1 μL template DNA
198 and a final concentration of 300 nM for each forward and reverse primer in 2 × KAPA HiFi
199 HotStart ReadyMix. The thermal conditions of the second PCR were as follows: 3 min at
200 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
201 all second PCR products, we selected the product size (approximately 370 bp) of the library
202 sample by electrophoresis using E-Gel SizeSelect 2% (Thermo Fisher Scientific) with the E-
203 Gel Precast Agarose Electrophoresis System (Thermo Fisher Scientific), which was
204 confirmed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA).

205 The library sample was then sequenced using an Illumina iSeq with 2 × 150 bp paired-end
206 kits (Illumina, San Diego, USA). We performed data preprocessing and analyses of iSeq raw
207 reads using USEARCH v10.0.240 (Edgar, 2010) according to the method described by Sakata
208 et al. (2020a). We discarded all reads from seawater samples corresponding to (i) freshwater
209 fish, regarding it as contamination from the rivers flowing into Maizuru Bay, and (ii) some
210 bony fish, which were regarded as contamination of domestic wastewater (detailed
211 information can be seen in Appendix S2).
212
213 *Statistical analyses*
214 All statistical analyses were performed using R version 4.0.4 (R Core Team, 2021). The decay
215 rates of Japanese jack mackerel eDNA were estimated using the time-series changes in their
216 eDNA concentrations from each sampling tank. Previous studies estimated eDNA decay rates
217 by fitting a monophasic exponential decay model (Strickler et al., 2015; Jo et al., 2020b) as
218 follows:
219 $$C_t = C_0 e^{-kt}$$
220 where C_t is the eDNA concentration (copies) at time t [hour], C_0 is the eDNA
221 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

239 community compositions based on Jaccard dissimilarities using a two-dimensional non-metric
240 multidimensional scaling (nMDS) with 10000 permutations by *vegdist* and *metaMDS*
241 functions in the package 'vegan' (Oksanen et al., 2019). In addition, we performed a
242 permutational multivariate analysis of variance (PERMANOVA) with 10000 permutations
243 using *adonis* function to examine whether the community compositions were different among
244 BAC treatments and/or time points.
245
246 **Results**
247 In the tank experiment, regardless of BAC treatment, we observed biphasic exponential
248 degradation of all types of Japanese jack mackerel eDNA (Fig. 2; Table S3). All types of
249 eDNA concentrations were higher in the treatment with BAC addition than in those without
250 BAC at time 0, which lasted throughout the sampling period. The decay rates at the initial
251 phase (k_1) were substantially lower in the treatment with BAC addition (31.0 to 53.0%
252 relative to the treatment without BAC), while those at the following slower phase (k_2) were
253 not significantly different between BAC treatments (Fig. 3). In contrast, in field sampling, we
254 observed monophasic exponential degradation of shorter fragments of eDNA (Fig. 4). Linear
255 models showed a significant interaction between sampling time points and BAC treatments

256 for nuS, indicating that eDNA decay rates were significantly lower in the treatment with BAC
257 than in those without BAC ($P < 0.05$; Table S4). Although we did not confirm a significant
258 interaction for mtS, target eDNA was detected for 24 hours from the seawater samples with
259 BAC addition, whereas it was hardly detected in the samples without BAC addition (Fig. 4).
260 We did not evaluate the effect of BAC addition on the degradation of longer eDNA fragments
261 (mtL and nuL) because of their poor detection relative to that of shorter eDNA fragments. The
262 overall PCR efficiencies and R^2 values of the standard curves are shown in Table S5. A few
263 filtration negative controls in the tank experiment showed PCR amplification, whose
264 calculated concentrations were less than 0.01 copy per PCR reaction and the potential
265 contamination during water filtration was negligible. No amplification was observed in any of
266 the PCR-negative controls throughout the study.
267 Moreover, the number of fish species detected by eDNA metabarcoding was higher
268 in the treatment with BAC over time (Fig. 5a). In total, 65 marine and brackish fish were
269 detected in 18 of 1-L seawater samples, wherein 58 and 45 species were detected in the
270 samples with and without BAC addition, respectively; 36, 40, and 38 species were detected in
271 samples with BAC, whereas 36, 28, and 27 species were detected in samples without BAC at
272 time 0, 6, and 24, respectively, when sampling triplicates were pooled (Table S6). 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 S7). 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 mostly 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, the increase in eDNA yields may depend on filter pore size and material considering the diverse particle size and state of eDNA (Jo et al., 2019). In addition, depending on water quality, it is also possible for

307 BAC to agglutinate PCR inhibitory substances such as humic, fulvic, and tannic acids. Sales
308 et al. (2019) reported that the number of fish species detected by eDNA metabarcoding
309 (MOTUs) was slightly lower in samples stored at ambient temperature with BAC addition
310 than in those stored in a cooler box with ice. Such tropical freshwater ecosystems are typically
311 characterized by turbidity due to high sediment loads and algae, and the result might thus
312 have included the effect of PCR inhibition by BAC.

313 We observed biphasic degradation of the target eDNA in the tank experiment. Some
314 previous studies estimating eDNA decay rates reported similar processes of eDNA
315 degradation and implied that a part of eDNA degraded rapidly, and subsequently, the rest
316 degraded slowly (Eichmiller et al., 2016; Bylemans et al., 2018; Shogren et al., 2018). In
317 particular, Bylemans et al. (2018) reported that the initial rapid degradation of eDNA might be
318 caused by intra-cellular nuclease activities and/or microbial digestion, and slower degradation
319 might reflect other degradation factors such as hydrolytic and oxidative decomposition of
320 DNA molecules. Considering that BAC inactivates bacterial functions by adsorbing to their
321 cell surfaces (Ziani et al., 2011), this hypothesis is consistent with our findings that BAC
322 substantially suppressed the initial rapid degradation of eDNA but had little effect on
323 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.

In field experiments, we observed the monophasic degradation of eDNA. This could simply be explained by fewer sampling time points relative to those in the tank experiment, which would not be enough to determine a monophasic or biphasic degradation pattern. Nevertheless, 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 due to the stochasticity of retrieving such long fragment in natural systems. This point could be improved by collecting water samples with larger volume and/or 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 strongly 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. Moreover, this study did not compare the effectiveness of BAC treatment against other preservation strategies. Although the comparison of eDNA preservation performance among the strategies was reported a bit (e.g., Spens et al., 2017; Mauvisseau et al., 2021), researchers have only reached a little consensus about it. Further studies would be required to compare the effectiveness of eDNA preservation (e.g., eDNA persistence time and survey cost-performance) among the strategies including BAC. Nonetheless, 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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Table

Table 1. Primers/probe sets used in this study.

ID	Target species	Target genetic region	Sequence (5'→3')	Amplicon size with the other side of primer [bp]	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	164	59.8	
Tja_CytB_R682			ATT GAT CGG AGA ATG GCG TAT	682	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	164	60.1	Jo et al. (2020b)
Tja_ITS1_F603			TCT TTG GCT TTA ACT TGC CCG	603	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.

Figure legends

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.

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.

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).

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.

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

661 the number of fish species detected by eDNA metabarcoding between BAC treatments. Each
662 sampling triplicate is pooled here. (b) A two-dimensional nMDS plot based on Jaccard
663 dissimilarity among BAC treatments and time points. Each symbol represents BAC
664 treatments (Y: with BAC addition; N: without BAC addition) and time points (0, 6, or 24).
665 Plots based on each triplicate in the same treatment are encircled by triangles.
666

(a) Storage temperature: 15.9 to 19.2 °C

Stock tank

BAC

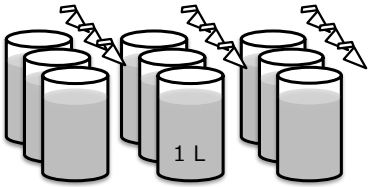
60 L
BAC presence

60 L
BAC absence

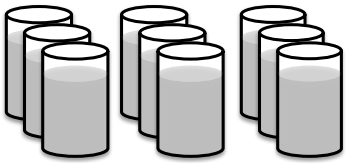
Sampling tank

(b)

Storage temperature: 18 ± 1 °C



BAC presence

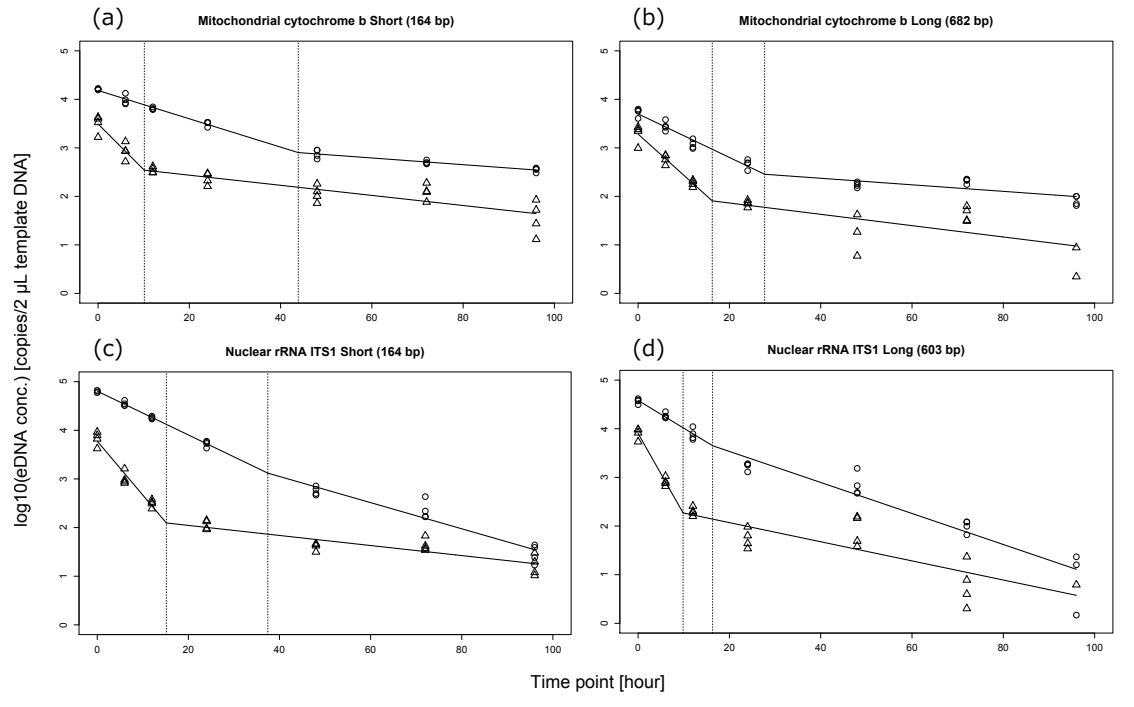


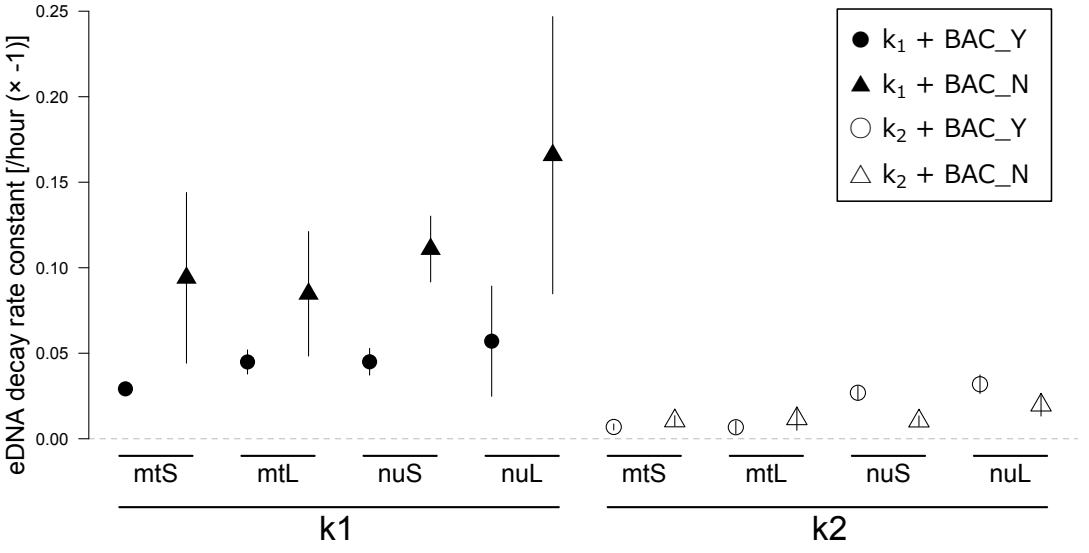
BAC absence

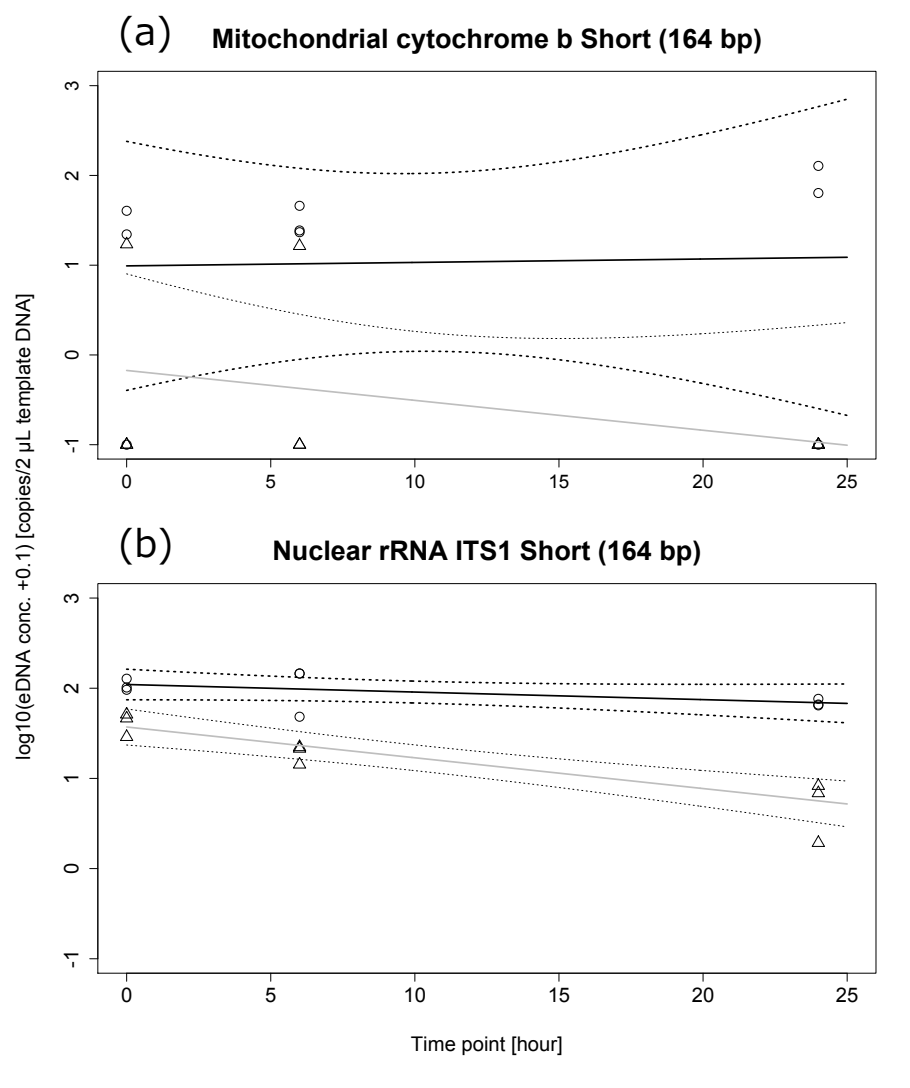
Water sampling & filtration

(a)	(b)
0	0
6	6
12	
24	24
48	
72	
96	

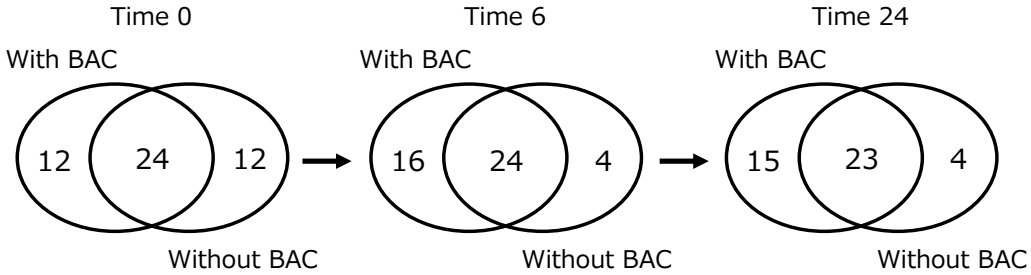
[hour]


(a)
Mitochondrial cytochrome b Short (164 bp)
(b)
Mitochondrial cytochrome b Long (682 bp)
(c)
Nuclear rRNA ITS1 Short (164 bp)
(d)
Nuclear rRNA ITS1 Long (603 bp)
log10(eDNA conc.) [copies/2 µL template DNA]
Time point [hour]

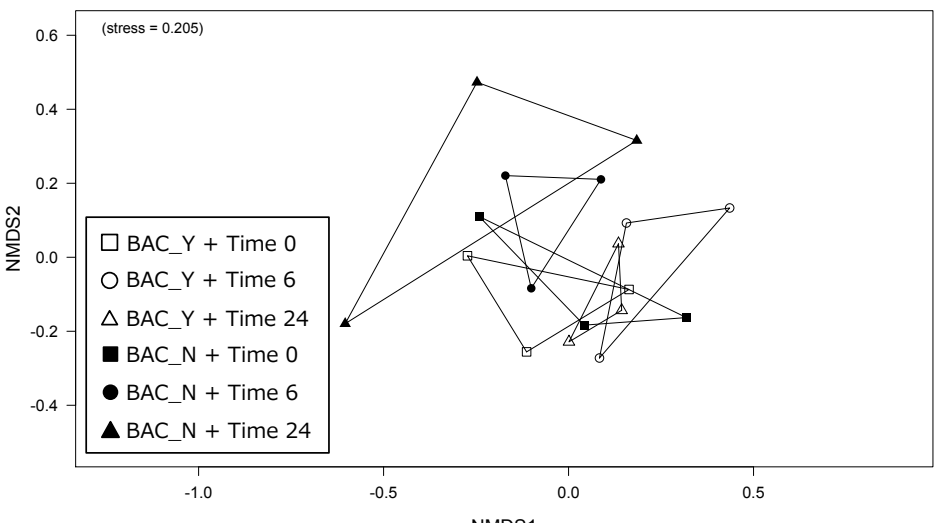

eDNA decay rate constant [/hour (× -1)]
0.25
0.20
0.15
0.10
0.05
0.00
k1 + BAC_Y
k1 + BAC_N
k2 + BAC_Y
k2 + BAC_N
mtS
mtL
nuS
nuL
mtS
mtL
nuS
nuL
k1
k2


(a)
Mitochondrial cytochrome b Short (164 bp)
(b)
Nuclear rRNA ITS1 Short (164 bp)
log10(eDNA conc. +0.1) [copies/2 µL template DNA]
Time point [hour]
3
2
1
0
-1
0
5
10
15
20
25

(a)


Time 0
With BAC
12
24
12
Without BAC
Time 6
With BAC
16
24
4
Without BAC
Time 24
With BAC
15
23
4
Without BAC

(b)


(stress = 0.205)
NMDS2
NMDS1
0.6
0.4
0.2
0.0
-0.2
-0.4
-1.0
-0.5
0.0
0.5
BAC_Y + Time 0
BAC_Y + Time 6
BAC_Y + Time 24
BAC_N + Time 0
BAC_N + Time 6
BAC_N + Time 24