Kobe University Repository : Kernel

PDF issue: 2025-12-05

Particle Size Distribution of Environmental DNA from the Nuclei of Marine Fish

Jo, Toshiaki
Arimoto, Mio
Murakami, Hiroaki
Masuda, Reiji
Minamoto, Toshifumi

(Citation)
Environmental Science & Technology,53(16):9947-9956

(Issue Date)
2019-07-22

(Resource Type)
journal article

(Version)
Accepted Manuscript

(Rights)
© 2019 American Chemical Society. This document is the Accepted Manuscript version of a Published Work that appeared in final form in Environmental Science & Technology, copyright © American Chemical Society after peer review and technical editing by the publisher. To access the final edited and published work see…

(URL)
https://hdl.handle.net/20.500.14094/90008040

1 **Title:**

2 Particle size distribution of environmental DNA from the nuclei of marine fish

3

4 **Authors:**

5 *Toshiaki Jo[1, 2], Mio Arimoto[3], Hiroaki Murakami[4], Reiji Masuda[4], Toshifumi Minamoto[1]

6

7 **Affiliations:**

8 [1]Graduate School of Human Development and Environment, Kobe University: 3-11,

9 Tsurukabuto, Nada-ku, Kobe City, Hyogo 657-8501, Japan

10 [2]Research Fellow of Japan Society for the Promotion of Science, 5-3-1 Kojimachi, Chiyoda-

11 ku, Tokyo 102-0083, Japan

12 [3]Faculty of Human Development, Kobe University: 3-11, Tsurukabuto, Nada-ku, Kobe City,

13 Hyogo 657-8501, Japan

14 [4]Maizuru Fisheries Research Station, Field Science Education and Research Center, Kyoto

15 University, Maizuru, Kyoto 625-0086, Japan

16

17 **Corresponding author:**

18 Toshiaki Jo

19 Graduate School of Human Development and Environment, Kobe University: 3-11,

20 Tsurukabuto, Nada-ku, Kobe City, Hyogo 657-8501, Japan

21 Tel: +81-78-078-803-7743

22 Email: 160d405d@stu.kobe-u.ac.jp

23

24 **Running head:**

25 Estimating nuclear eDNA particle size distribution

26

27 **Keywords:**

28 cytochrome b (CytB), environmental DNA, internal transcribed spacer-1 (ITS1), Japanese

29 Jack Mackerel (*Trachurus japonicus*), mitochondrial DNA, nuclear DNA, particle size

30 distribution

31

32 **Abstract**

33 Environmental DNA (eDNA) analyses have enabled more efficient surveillance of species

34 distribution and composition than conventional methods. However, the characteristics and

dynamics of eDNA (e.g., origin, state, transport, and fate) remain unknown. This is especially limited for the eDNA derived from nuclei (nu-eDNA), which has recently been used in eDNA analyses. Here, we compared the particle size distribution (PSD) of nu-eDNA from Japanese Jack Mackerel (*Trachurus japonicus*) with that of mt-eDNA (eDNA derived from mitochondria) reported in previous studies. We repeatedly sampled rearing water from the tanks with multiple temperature and fish biomass levels, and quantified the copy numbers of size-fractioned nu-eDNA. We found that the concentration of nu-eDNA was higher than that of mt-eDNA at 3-10 μm size fraction. Moreover, at the 0.8-3 μm and 0.4-0.8 μm size fractions, eDNA concentrations of both types increased with higher temperature and their degradation tended to be suppressed. These results imply that the production of eDNA from large to small size fractions could buffer the degradation of small-sized eDNA, which could improve its persistence in water. Our findings will contribute to refine the difference between nu- and mt-eDNA properties, and assist eDNA analyses as an efficient tool for the conservation of aquatic species.

50 **Introduction**

51 Environmental DNA (eDNA) analyses have been developed for improving the conservation
52 and management of aquatic ecosystems in this decade[1-3]. Macro-organisms shed their DNA
53 into the environment as feces, mucus, scales, and gametes[4-7], which is termed eDNA. The
54 presence of target species can be estimated by detecting their eDNA from environmental
55 media such as water and sediment, allowing more efficient and non-invasive surveillance of
56 species distribution and composition than traditional methods[8-12].

57 Most eDNA analyses for macro-organisms have targeted mitochondrial DNA
58 (mtDNA) as a genetic marker due to its abundance in a cell[2, 13-15]. However, recent studies
59 have suggested the applicability of nuclear DNA (nuDNA) marker for eDNA analysis, which
60 targets multiple copies of ribosomal RNA gene such as internal transcribed spacer (ITS)
61 regions[16-18]. The genetic regions have high inter-specific variations and, unlike mtDNA, can
62 provide high resolutions to discriminate closely related targets[19-21]. It is likely that nuDNA
63 markers will become an alternative eDNA tool, whereas the knowledge on the characteristics
64 and dynamics of eDNA derived from nuclei (nu-eDNA) is scarce.

65 Researchers have been interested in how eDNA can be produced and exist in the
66 environment, and therefore have emphasized the necessity to collect such fundamental

67 information on eDNA[22-26]. For example, although there is still much to be verified, several
68 studies have reported the effects of various biotic/abiotic factors on eDNA detectability and
69 persistence[27-33] and the horizontal/vertical transport of eDNA in various aquatic
70 environments[34-38]. However, the information on the physiological origin and state of eDNA
71 (e.g., living/dead cell, intra-/extra-membrane, dissolved/free) is relatively limited, which is
72 rather fundamental for understanding the characteristics and dynamics of eDNA[22, 25]. These
73 eDNA aspects can influence the transport and fate of eDNA[22], where larger and heavier
74 eDNA particles in water can be expected to disperse less and settle more rapidly[39]. In
75 addition, DNA molecules within cell membrane (i.e., intra-membrane DNA) should be
76 attacked less efficiently by microbes and extra-cellular enzymes in environment than extra-
77 membrane free DNA[40-42]. Studying how eDNA can be produced and exist in aquatic
78 environment would substantially contribute to the understanding of eDNA characteristics and
79 dynamics. However, almost all eDNA studies have targeted only mitochondrial eDNA (eDNA
80 derived from mitochondria, hereafter, mt-eDNA).

81 Therefore, we focused on the characteristics and dynamics of nu-eDNA, especially
82 its particle size distribution (PSD) and temporal changes. Previous studies estimated the PSD
83 of eDNA in natural environments using mtDNA marker and found that the largest proportion

84 of fish mt-eDNA was found in the 1-10 um size fraction[43-44]. In addition, Jo et al. (2019)

85 reported that mt-eDNA PSD from Japanese Jack Mackerel (*Trachurus japonicus*) could vary

86 depending on water temperature and time passages after fish removal. These results included

87 various eDNA production and degradation processes, and it remains unknown how each

88 process could contribute to the PSD of eDNA. The state of eDNA (e.g., intra- to extra-

89 membrane) may vary over time until the material is no longer detectable, and such a process

90 would influence the persistence of eDNA. In addition, these processes may differ between nu-

91 and mt-eDNA. In eukaryotic cells, the nuclei have chromatin structures that are 5-10 µm in

92 diameter[45-46], while mitochondria have simple cyclic structures that are generally smaller[47-48].

93 If the PSDs differ between nu-eDNA and mt-eDNA, the selective capture of target eDNA

94 might be possible based on their size. The PSDs of eDNA based on multiple DNA regions or

95 loci would help our understanding of the state and fate of eDNA.

96 This study investigated the PSD of nu-eDNA and its temporal changes through a

97 tank experiment. We used Japanese Jack Mackerel (*Trachurus japonicus*) as a model species

98 due to its previous use in our eDNA studies[30, 49-50] and its economic importance in East Asia

99 including Japan[51]. In addition, focusing on water temperature and fish biomass density, we

100 examined how these biotic/abiotic factors influenced nu-eDNA PSD. Furthermore, we

101 compared these results with those of mt-eDNA PSD from previous studies[30].

102

103 **Materials and methods**

104 *Experimental protocol*

105 We conducted tank experiments at the Maizuru Fisheries Research Station, Kyoto University,

106 Japan, from June 2016 to July 2017 (Figure 1). All the eDNA samples were from Jo et al.

107 (2019) (detailed information about the experimental design, water sampling, and DNA

108 extraction is described in Appendix S1). Briefly, we collected the rearing water from

109 experimental tanks with different temperature (13, 18, 23, and 28 °C) and fish biomass levels

110 (Small, Medium, and Large) with four tank replicates per treatment. Fish biomass levels were

111 based on the difference of total fish biomass in the tank (g/200 L). We performed sequential

112 filtrations using a series of filters with different pore sizes (10, 3, 0.8, and 0.4 or 0.2 μm),

113 extracted total DNA on the filter with DNeasy Blood and Tissue Kit (Qiagen, Hilden,

114 Germany), and quantified Japanese Jack Mackerel's eDNA concentrations at each size

115 fraction. We estimated the concentration of Japanese Jack Mackerel's nu-eDNA in water

116 samples by quantifying the copy number of nuclear internal transcribed spacer-1 (ITS1)

117 regions using the StepOnePlus Real-Time PCR system (Thermo Fisher Scientific, Foster City,

118 CA, US). We used the primers/probe set that specifically amplified the Japanese Jack

119 Mackerel's DNA fragment from the ITS1 region (Jo et al., submitted; Table 1; Appendix S1).

120 ITS1 is a part of ribosomal RNA genes (rDNA), and multiple copies of ITS1 are present in

121 the nuclear genome. We confirmed that our ITS1 primer set amplified only our target species

122 and locus using *in silico* specificity check. Each 20 μL TaqMan reaction contained a 2 μL

123 template DNA, a final 900 nM concentration of forward and reverse primers, and 125 nM of

124 TaqMan probe in 1 × TaqMan Gene Expression PCR Master Mix (Thermo Fisher Scientific).

125 We simultaneously analyzed a 2 μL pure water as a PCR negative control. We performed

126 qPCR using a dilution series of standards containing 3×10^1 - 3×10^4 copies of a linearized

127 plasmid containing synthesized artificial DNA fragments from a partial ITS1 region sequence

128 (237 bp) of a target species. We performed all qPCRs for eDNA extracts, standards, and

129 negative controls in triplicate. Thermal conditions of quantitative real-time PCR were as

130 follows: 2 min at 50 °C, 10 min at 95 °C, 55 cycles of 15 s at 95 °C, and 1 min at 60 °C.

131 Quantification of eDNA copy number for the mitochondrial CytB gene was performed as per

132 the method in Jo et al. (2019). We calculated eDNA concentrations by averaging the triplicate,

133 and each replicate showing non-detection (PCR-negative) was classified as containing a zero

134 copy[52]. The limit of quantification (LOQ) of the qPCR was one copy per reaction with

135 triplicates following previous studies[53, 54] and we classified any eDNA concentration below

136 LOQ as a zero copy.

137

138 *Statistical analysis*

139 We used R version 3.2.4 for all the statistical analyses[55]. Before the analyses, we log-

140 transformed all the eDNA concentrations after adding one to meet the assumption of

141 normality. Using all samples that had passed through sequential filters with 10, 3, 0.8, and 0.4

142 µm pore sizes at time bfr (i.e., before fish removal; Appendix S1), we investigated how the

143 PSD of eDNA related to water temperature, fish biomass, and DNA markers. We performed

144 Multivariate Analysis of Variance (MANOVA) and post-hoc ANOVAs for the eDNA

145 concentrations at each size fraction, where water temperature level, fish biomass, and type of

146 DNA markers (ITS1 or CytB) were included as factors. There were four tank replicates per

147 treatment level (except for 28 °C - Large biomass levels, where three tank replicates were

148 prepared due to fish mortality; Appendix S1). MANOVA can simultaneously evaluate the

149 effects of each factor on multiple response variables, which can reduce the likelihood of Type

150 I errors and increase the statistical powers[56, 57].

151 In addition, we investigated how the PSD of eDNA changed with fish removal
152 using the samples that passed through the sequential filters with 10, 3, 0.8, and 0.2 µm pore
153 sizes at time bfr and 0 (hour). We performed an ANOVA for eDNA concentrations, and
154 included filter pore size, sampling time point (time bfr or 0), type of DNA markers, and all the
155 interactions between them as factors. Furthermore, we investigated temporal changes of nu-
156 and mt-eDNA PSDs after fish removal using the same samples at time 0, 6, 12, and 18 (hour).
157 We performed LMM (linear mixed model) with the function *lmer* of the R package
158 *lmerTest*[58], and included filter pore size, sampling time point, temperature level, fish biomass
159 level, and type of DNA marker as explanatory variables. We considered water temperature as
160 quantitative values and set each temperature level as the increment from the lowest
161 temperature level (13 °C). We also included the interactions between sampling time points
162 and each of the other factors, where we assumed that the temporal degradation of eDNA may
163 vary among size fractions, treatment levels, and DNA markers, and tank replicates as random
164 effects.
165
166 **Results and Discussion**

167 In all qPCR analyses for nu-eDNA including filtration negative controls, the R^2 values, slope,

168 Y-intercept, and PCR efficiency (%) of the calibration curves were 0.984 ± 0.017, −3.586 ±

169 0.208, 44.940 ± 1.567, and 90.615 ± 7.690, respectively (mean ± 1 SD). PCR amplifications

170 were confirmed in some inlet water samples which was pumped from 6 m depth at the station,

171 where Japanese Jack Mackerel is abundant, and filtration negative controls: nu-eDNA

172 concentrations in inlet water samples were 22.3 ± 84.2 copies/reaction. This corresponded to

173 5.2 ± 15.9 % of eDNA concentrations relative to those with sum of sequential filters in water

174 samples at time bfr (mean ± 1 SD, respectively). Besides, nu-eDNA concentrations in

175 filtration negative controls were 11.3 ± 54.5 copies/reaction, which corresponded to 1.2 ±

176 9.2 % of eDNA concentrations relative to those in overall water samples (mean ± 1 SD,

177 respectively). We considered that the Japanese Jack Mackerel eDNA in inlet water and cross-

178 contamination among samples is not likely to have affected our results. We confirmed no PCR

179 amplification from any PCR negative controls (Table S1).

180

181 ***The relationships of eDNA PSD with temperature, fish biomass, and DNA markers***

182 Water temperature, fish biomass, and DNA markers significantly affected the eDNA

183 concentrations at each size fraction (MANOVA, all $P < 0.05$; Figs. 2 and S1; Table 2). Post-

184 hoc ANOVAs showed that the type of DNA marker was a significant factor for 3-10 μm size

185 fraction (P < 0.05), water temperature was for 0.8-3 and 0.4-0.8 μm size fractions (both P <

186 0.01), and fish biomass was for all size fractions (all P < 0.0001). First, the concentration of

187 nu-eDNA was larger than mt-eDNA for the 3-10 μm size fraction (Figs. 2 and S1; Table 2).

188 Although both nu- and mt-eDNA could also be detected at the size of cell or tissue fragments

189 (mainly the >10 μm size fraction in the study), the results may partly reflect the size

190 differences between nuclei and mitochondria; nuclei (around 5-10 μm in diameter) is

191 generally larger than mitochondria (around 0.5-2 μm) in eukaryotic cells[46, 59]. Our study is the

192 first report to estimate of the PSD of nu-eDNA, as well as to find the differences of PSD

193 between nu- and mt-eDNA. Meanwhile, the fact that much of nu- and mt-eDNA was detected

194 at > 3 μm size fractions would also be meaningful, because nu-eDNA could be captured as

195 much as, or more than, mt-eDNA using the filter with same pore sizes. Further study is

196 needed to examine whether these similarities and differences are common among taxa.

197 Second, the concentration of nu- and mt-eDNA generally increased at higher water

198 temperatures for the 0.8-3 μm and 0.4-0.8 μm size fractions (Figs. 2 and S1; Table 2),

199 whereas temperature was not a significant factor for the > 3 μm size fraction. The degradation

200 of eDNA would be likely promoted with higher water temperatures in all size fractions[29, 32],

201 and the warmer temperature could influence fish behavior and increase eDNA shedding[30]. On
202 the other hand, it is also likely that the outer cell membrane of large-sized eDNA (such as
203 cells and tissues) broke down, and the part of them was turned into small-sized eDNA (such
204 as nuclei, mitochondria, and their extra-cellular DNA). The DNA release from prokaryotic
205 cells occurs following viral attacks or enzymatic activity[41, 60-61]. Besides, the activity of
206 microbes and extra-cellular enzymes can be stimulated by moderately higher temperatures (<
207 50 °C)[40, 62]. Thus, it is possible that, through the enzymatic activity, higher temperature
208 facilitates the release of such small-sized eDNA out of the cell membrane. The decrease of
209 eDNA due to degradation at smaller size fractions might be buffered by an increase of eDNA
210 production from larger to smaller size fractions.

211 Third, the concentration of eDNA was much larger in Large biomass level than
212 other biomass levels for all size fractions (Figs. 2 and S1; Table 2). Interestingly, there was
213 almost no difference of eDNA concentrations between Small and Medium biomass levels.
214 The growth model for Japanese Jack Mackerel[63] estimated the ages of both Small- and
215 Medium-sized fishes to be 0+ year, while those of Large-sized fish to be almost 1+ year. The
216 release of eDNA might be similar within the same age group. Further investigation would be
217 needed for the relationship between eDNA release and the age/developmental stage of

218 organisms[64]. Besides, it might be accounted by the effect of fish biomass density in

219 experimental tanks. For example, Sassoubre et al. (2016) reported that eDNA shedding rates

220 per individual of Pacific Sardine (*Sardinops sagax*) and Pacific Chub Mackerel (*Scomber*

221 *japonicus*) increased with larger fish biomass density in the tanks. In our experiment, Large-

222 sized fish might have touched each other more often.

223

224 ***Temporal changes of eDNA PSD***

225 We also studied temporal changes of eDNA PSD (Figs. 3 and S2). At first, immediately after

226 fish removal, the concentration of eDNA increased for all size fractions, which could be due

227 to the handling stress at fish removal. The eDNA concentrations significantly depended on

228 sampling time and filter pore size (ANOVA, all $P < 0.001$; Table 3). The interaction between

229 sampling time and filter pore size was also significant ($P < 0.01$); eDNA increases were not

230 similar among size fractions but were emphasized in >10 µm size fraction. Previous studies

231 have suggested that physical and environmental stresses on organisms could stimulate eDNA

232 release, which could originate from scales and mucus[7, 30, 65-66]. The type of DNA marker and

233 other interactions were not significant (all $P > 0.1$), suggesting that, due to fish removal, there

234 was no difference of eDNA release between nu- and mt-eDNA. Most of the eDNA just after

235 released from aquatic organisms might be intra-cellular DNA such as cells and tissues rather

236 than extra-cellular DNA. Further study is needed to verify the physical forms of eDNA

237 released into natural environments.

238 Following fish removal, the concentration of eDNA decreased over time for all size

239 fractions, while eDNA degradation was suppressed in the smaller size fractions (Figs. 3 and

240 S2). The eDNA concentrations were significantly affected by filter pore size and temperature

241 positively, and time point and fish biomass negatively (LMM, all $P < 0.0001$; Table 3) but did

242 not significantly change with DNA marker ($P = 0.8175$). Besides, all interactions in the

243 analyses were significant ($P < 0.05$). Thus, the significance of main effects of each variable

244 might be restrained. The significant interactions between filter pore size and time point could

245 reflect the reduction of large-sized eDNA toward smaller size fractions as above; some of

246 eDNA at larger size fractions broke down, changed their physical forms, and turned into

247 small-sized eDNA. Especially at the 0.2-0.8 µm size fraction, there were some treatment

248 levels where the concentration of eDNA seemed to rather increase over time (Fig. S2). These

249 results imply that, depending on the size fraction, the production of eDNA from larger to

250 smaller size fractions might sometimes surpass the reduction of eDNA (i.e., non-detection by

251 PCR). If we had continued the experiment another a few days, the shift of eDNA PSD toward

252 smaller size fractions might have been more obvious. Due to the differences of physical
253 forms, small-sized eDNA such as organelles and extra-membrane DNA would likely be more
254 sensitive to enzymatic activity in environment than large-sized eDNA such as cells and
255 tissues. Our study, however, suggested that the production of eDNA from larger to smaller
256 size fractions could occur, which could buffer the degradation of small-sized eDNA and
257 prolong its 'apparent' persistence in water. Our findings imply that the size, and the state, of
258 eDNA could vary over time, which would contribute to the elucidation on the state and fate of
259 eDNA in aquatic environments. On the other hand, there might be some difference of eDNA
260 PSDs between experimental tanks and natural environment. We suggest future study of eDNA
261 PSDs with various environmental conditions (e.g., pH, trophic state, and fish density) like
262 natural conditions and temporal changes of eDNA PSDs in environmental water samples.
263 This might help link our results with actual eDNA dynamics in aquatic environment.
264 Other significant interactions in the LMM analysis offer interesting interpretations
265 (Table 3). The negative interaction between time point and temperature indicates that eDNA
266 degradation was accelerated with higher temperatures, which has been found in previous
267 studies[29, 32]. The positive interaction between time point and fish biomass shows that eDNA
268 degradation was suppressed for Small contrary to Large biomass level. This might be due to

269 an increase of microbial density with fish biomass density in the experimental tanks[27, 30].

270 Moreover, the negative interaction between time point and DNA marker means that nu-eDNA

271 degradation was accelerated compared to mt-eDNA, which may be due to the amplicon sizes

272 of the primer pairs (Table 1). The increase of PCR amplification length results in a decrease of

273 detected DNA copy number[67] and a higher eDNA decay rate[49]. Further studies are needed to

274 show how the relationships between eDNA persistence and various biotic/abiotic factors

275 depend on the size and state of eDNA.

276

277 ***Implications and perspectives***

278 Through the study, by estimating the PSD of nu-eDNA, we obtained important implications

279 on the state and fate of eDNA derived from macro-organisms in aquatic environment. Based

280 upon the present and previous studies, we summarized on the state and fate of eDNA from

281 fish in water (Fig. 4). First, much of eDNA would be released from organisms as relatively

282 large-sized particles (> 10 µm in diameter), originating as intra-cellular DNA such as cell and

283 tissue fragments (Fig. 3). These eDNA could be released into the environment with mucus

284 and scales[7], which may increase the average eDNA size. It is less likely that organisms would

285 directly shed their nuclei, mitochondria, and their intra-membrane DNA; rather, the part of

286 eDNA especially at larger size fractions could break down (e.g., the lysis and fragmentation

287 of cell membrane through the activity of microbes and exonucleases[40, 68]), which might

288 change their physical state and structure, and thus turned them into smaller-sized eDNA. In

289 our study, the degradation of both nu- and mt-eDNA was suppressed in the smaller size

290 fractions, which is likely due to the breakdown of large-sized eDNA (Fig. 3). This tendency

291 might be facilitated by an increase of water temperature and species biomass density since

292 these factors can promote microbial activity (Fig. 2; Table 3). Moreover, because of the size

293 differences between nuclei and mitochondria, nu-eDNA was more detected than mt-eDNA,

294 especially at > 3 μm size fractions (Figs. 2 and S1), which might contribute to the difference

295 of eDNA PSDs between DNA markers.

296 We clarified some aspects of particle size characteristics of fish eDNA, though there

297 are still knowledge gaps that must be verified before this tool can be used in environmental

298 applications. Regardless of the increase of eDNA applications with various taxa[13, 31, 54], the

299 PSD of eDNA has not been reported for taxa other than fish. It could be possible that eDNA

300 PSDs are different among taxa. In addition, PCR efficiencies tended to be slightly lower for

301 nu-eDNA (90.615 ± 7.690) than mt-eDNA (93.789 ± 3.794; mean ± 1 SD). This might partly

302 be due to the difference of amplification length between primers/probe sets (ITS1: 164 bp;

303 CytB: 127 bp). When comparing the results of eDNA detection between different DNA
304 regions or fragment sizes, equalizing PCR efficiencies would be ideal. Furthermore, it will be
305 necessary to understand the physiological and cytological characteristics of eDNA other than
306 its PSD. For example, chromatin structure in nuclei[45] and the fission and fusion of
307 mitochondria for the maintenance of its integrity[69] might influence the detectability and
308 persistence of eDNA. A greater understanding of such fundamental information on eDNA
309 would improve the efficiency of eDNA analyses, and contribute to the validation of its use in
310 natural environments. Our study can be the basis for future eDNA studies, and may help
311 facilitate the use of eDNA analyses as an efficient tool for improving the conservation and
312 management of aquatic ecosystems.
313

314 **Acknowledgements**
315 We thank Dr. Satoshi Yamamoto, Takaya Yoden, Mizuki Ogata, Sachia Sasano, Misaki
316 Shiomi (Kyoto University), Qianqian Wu, Masayuki K. Sakata, Sei Tomita, Mone Kawata,
317 and Saki Ikeda (Kobe University) for supporting the tank experiments. We thank Dr.
318 Masayoshi K. Hiraiwa (National Agriculture and Food Research Organization) for helpful
319 comment on the statistical analysis. We thank three anonymous reviewers and an editor who
320 provided advice to greatly improve the manuscript. This work was supported by JST CREST,
321 Grant Number JPMJCR13A2, Japan, and Grant-in-Aid for JSPS Research Fellow, Grant
322 Number JP18J20979, Japan.
323

324 **Data accessibility**

325 The raw data for the qPCR experiments is included in Supplemental Information.
326
327 **Author contribution**
328 T.J., R.M., and T.M. designed the experiments. T.J. and H.M. performed the tank experiments.
329 T.J. and M.A. performed the molecular analyses, analyzed the data, and wrote the first draft of
330 the manuscript. All authors edited and provided feedback for the manuscript.
331
332 **Supporting Information**
333 Appendix S1. Extended methodological details (Jo et al., 2019; Jo et al., submitted).
334
335 Table S1. Raw values of eDNA concentrations (copies per 2 µL template DNA) in tank
336 samples with nuclear DNA markers.
337
338 Figure S1. Results of the particle size distributions of Japanese Jack Mackerel mt-eDNA at
339 time bfr (data from Jo et al., 2019).
340
341 Figure S2. Results of the temporal changes of Japanese Jack Mackerel eDNA particle size
342 distributions for each treatment level (Dataset of mt-eDNA is from Jo et al., 2019).
343
344 **References**
345 (1) Bohmann, K.; Evans, A.; Gilbert, M. T. P.; Carvalho, G. R.; Creer, S.; Knapp, M.; Yu, D.
346 W.; De Bruyn, M. Environmental DNA for wildlife biology and biodiversity monitoring.
347 *Trends. Ecol. Evol*. **2014**, *29* (6), 358-367.
348
349 (2) Ficetola, G. F.; Miaud, C.; Pompanon, F.; Taberlet, P. Species detection using
350 environmental DNA from water samples. *Biol. Lett*. **2008**, *4* (4), 423-425.
351
352 (3) Minamoto, T.; Yamanaka, H.; Takahara, T.; Honjo, M. N.; Kawabata, Z. I. Surveillance of
353 fish species composition using environmental DNA. *Limnology*. **2012**, *13* (2), 193-197.
354
355 (4) Bylemans, J.; Furlan, E. M.; Hardy, C. M.; McGuffie, P.; Lintermans, M.; Gleeson, D. M.
356 An environmental DNA-based method for monitoring spawning activity: a case study, using

357 the endangered Macquarie perch (*Macquaria australasica*). *Methods Ecol. Evol*. **2017**, *8* (5),
358 646-655.
359
360 (5) Martellini, A.; Payment, P.; Villemur, R. Use of eukaryotic mitochondrial DNA to
361 differentiate human, bovine, porcine and ovine sources in fecally contaminated surface water.
362 *Water Res*. **2005**, *39* (4), 541-548.
363
364 (6) Merkes, C. M.; McCalla, S. G.; Jensen, N. R.; Gaikowski, M. P.; Amberg, J. J. Persistence
365 of DNA in carcasses, slime and avian feces may affect interpretation of environmental DNA
366 data. *PLoS One*. **2014**, *9* (11), e113346.
367
368 (7) Sassoubre, L. M.; Yamahara, K. M.; Gardner, L. D.; Block, B. A.; Boehm, A. B.
369 Quantification of environmental DNA (eDNA) shedding and decay rates for three marine fish.
370 *Environ. Sci. Technol*. **2016**, *50* (19), 10456-10464.
371
372 (8) Biggs, J.; Ewald, N.; Valentini, A.; Gaboriaud, C.; Dejean, T.; Griffiths, R. A.; Foster, J.;
373 Wilkinson, J. W.; Arnell, A.; Brotherton, P.; et al. Using eDNA to develop a national citizen
374 science-based monitoring programme for the great crested newt (*Triturus cristatus*). *Biol.*
375 *Conserv.* **2015**, *183*, 19-28.
376
377 (9) Boussarie, G.; Bakker, J.; Wangensteen, O. S.; Mariani, S.; Bonnin, L.; Juhel, J. B.;
378 Kiszka, J. J.; Kulbicki, M.; Manel, S.; Robbins, W. D.; et al. Environmental DNA illuminates
379 the dark diversity of sharks. *Sci. Adv.* **2018**, *4* (5), eaap9661.
380
381 (10) Fukumoto, S.; Ushimaru, A.; Minamoto, T. A basin-scale application of environmental
382 DNA assessment for rare endemic species and closely related exotic species in rivers: a case
383 study of giant salamanders in Japan. *J. Appl. Ecol.* **2015**, *52* (2), 358-365.
384
385 (11) Li, Y.; Evans, N. T.; Renshaw, M. A.; Jerde, C. L.; Olds, B. P.; Shogren, A. J.; Deiner, K.;
386 Lodge, D. M.; Lamberti, G. A; Pfrender, M. E. Estimating fish alpha- and beta-diversity along
387 a small stream with environmental DNA metabarcoding. *Metabarcoding Metagenomics*.
388 **2018**, *2*, e24262.
389

390 (12) Yamamoto, S.; Masuda, R.; Sato, Y.; Sado, T.; Araki, H.; Kondoh, M.; Minamoto, T.;
391 Miya, M. Environmental DNA metabarcoding reveals local fish communities in a species-rich
392 coastal sea. *Sci. Rep.* **2017**, *7*, 40368.
393
394 (13) Carraro, L.; Hartikainen, H.; Jokela, J.; Bertuzzo, E.; Rinaldo, A. Estimating species
395 distribution and abundance in river networks using environmental DNA. *Proc. Natl. Acad.*
396 *Sci.* **2018**, *115* (46), 11724-11729.
397
398 (14) Deiner, K.; Fronhofer, E. A.; Mächler, E.; Walser, J. C.; Altermatt, F. Environmental
399 DNA reveals that rivers are conveyer belts of biodiversity information. *Nat. Commun.* **2016**,
400 *7*, 12544.
401
402 (15) Takahara, T.; Minamoto, T.; Doi, H. Using environmental DNA to estimate the
403 distribution of an invasive fish species in ponds. *PLoS One.* **2013**, *8* (2), e56584.
404
405 (16) Dysthe, J. C.; Franklin, T. W.; McKelvey, K. S.; Young, M. K.; Schwartz, M. K. An
406 improved environmental DNA assay for bull trout (*Salvelinus confluentus*) based on the
407 ribosomal internal transcribed spacer I. *PLoS One.* **2018**, *13* (11), e0206851.
408
409 (17) Gantz, C. A.; Renshaw, M. A.; Erickson, D.; Lodge, D. M.; Egan, S. P. Environmental
410 DNA detection of aquatic invasive plants in lab mesocosm and natural field conditions. *Biol.*
411 *Invasions.* **2018**, *20* (9), 2535-2552.
412
413 (18) Minamoto, T.; Uchii, K.; Takahara, T.; Kitayoshi, T.; Tsuji, S.; Yamanaka, H.; Doi, H.
414 Nuclear internal transcribed spacer-1 as a sensitive genetic marker for environmental DNA
415 studies in common carp *Cyprinus carpio*. *Mol. Ecol. Resour.* **2017**, *17* (2), 324-333.
416
417 (19) Booton, G. C.; Kaufman, L.; Chandler, M.; Oguto-Ohwayo, R.; Duan, W.; Fuerst, P. A.
418 Evolution of the ribosomal RNA internal transcribed spacer one (ITS-1) in cichlid fishes of
419 the Lake Victoria region. *Mol. Phylogenetics Evol.* **1999**, *11* (2), 273-282.
420

421 (20) Bylemans, J.; Gleeson, D. M.; Hardy, C. M.; Furlan, E. Toward an ecoregion scale
422 evaluation of eDNA metabarcoding primers: A case study for the freshwater fish biodiversity
423 of the Murray-Darling Basin (Australia). *Ecol. Evol.* **2018b**, *8* (17), 8697-8712.
424
425 (21) Hillis, D. M.; Dixon, M. T. Ribosomal DNA: molecular evolution and phylogenetic
426 inference. *Q. Rev. Biol.* **1991**, *66* (4), 411-453.
427
428 (22) Barnes, M. A.; Turner, C. R. The ecology of environmental DNA and implications for
429 conservation genetics. *Conserv. Genet.* **2016**, *17* (1), 1-17.
430
431 (23) Díaz-Ferguson, E. E.; Moyer, G. R. History, applications, methodological issues and
432 perspectives for the use environmental DNA (eDNA) in marine and freshwater environments.
433 *Rev. Biol. Trop.* **2014**, *62* (4), 1273-1284.
434
435 (24) Goldberg, C. S.; Strickler, K. M.; Pilliod, D. S. Moving environmental DNA methods
436 from concept to practice for monitoring aquatic macroorganisms. *Biol. Conserv.* **2015**, *183*, 1-
437 3.
438
439 (25) Hansen, B. K.; Bekkevold, D.; Clausen, L. W.; Nielsen, E. E. The sceptical optimist:
440 challenges and perspectives for the application of environmental DNA in marine fisheries.
441 *Fish Fish.* **2018**, *19* (5), 751-768.
442
443 (26) Stewart, K. A. Understanding the effects of biotic and abiotic factors on sources of
444 aquatic environmental DNA. *Biodivers. Conserv.* **2019**, *28* (5), 983-1001.
445
446 (27) Barnes, M. A.; Turner, C. R.; Jerde, C. L.; Renshaw, M. A.; Chadderton, W. L.; Lodge,
447 D. M. Environmental conditions influence eDNA persistence in aquatic systems. *Environ. Sci.*
448 *Technol*. **2014**, *48* (3), 1819-1827.
449
450 (28) Collins, R. A.; Wangensteen, O. S.; O'Gorman, E. J.; Mariani, S.; Sims, D. W.; Genner,
451 M. J. Persistence of environmental DNA in marine systems. *Commun. Biol.* **2018**, *1* (1), 185.
452

453 (29) Eichmiller, J. J.; Best, S. E.; Sorensen, P. W. Effects of temperature and trophic state on
454 degradation of environmental DNA in lake water. *Environ. Sci. Technol*. **2016**, *50* (4), 1859-
455 1867.
456
457 (30) Jo, T.; Murakami, H.; Yamamoto, S.; Masuda, R.; Minamoto, T. Effect of water
458 temperature and fish biomass on environmental DNA shedding, degradation, and size
459 distribution. *Ecol. Evol.* **2019**, *9* (3), 1135-1146.
460
461 (31) Seymour, M.; Durance, I.; Cosby, B. J.; Ransom-Jones, E.; Deiner, K.; Ormerod, S. J.;
462 Colbourne, J. K.; Wilgar, G.; Carvalho, G. R.; de Bruyn, M.; et al. Acidity promotes
463 degradation of multi-species environmental DNA in lotic mesocosms. *Commun. Biol.* **2018**, *1*
464 (1), 4.
465
466 (32) Strickler, K. M.; Fremier, A. K.; Goldberg, C. S. Quantifying effects of UV-B,
467 temperature, and pH on eDNA degradation in aquatic microcosms. *Biol. Conserv.* **2015**, *183*,
468 85-92.
469
470 (33) Turner, C. R.; Uy, K. L.; Everhart, R. C. Fish environmental DNA is more concentrated
471 in aquatic sediments than surface water. *Biol. Conserv.* **2015**, *183*, 93-102.
472
473 (34) Deiner, K.; Altermatt, F. Transport distance of invertebrate environmental DNA in a
474 natural river. *PLoS One*. **2014**, *9* (2), e88786.
475
476 (35) Jane, S. F.; Wilcox, T. M.; McKelvey, K. S.; Young, M. K.; Schwartz, M. K.; Lowe, W.
477 H.; Benjamin, L. H.; Whiteley, A. R. Distance, flow and PCR inhibition: eDNA dynamics in
478 two headwater streams. *Mol. Ecol. Resour.* **2015**, *15* (1), 216-227.
479
480 (36) Murakami, H.; Yoon, S.; Kasai, A.; Minamoto, T.; Yamamoto, S.; Sakata, M. K.;
481 Horiuchi, T.; Sawada, H.; Kondoh, M.; Yamashita, Y.; et al. Dispersion and degradation of
482 environmental DNA from caged fish in a marine environment. *Fish. Sci.* **2019**, *85* (2), 327-
483 337.
484

485 (37) Nukazawa, K.; Hamasuna, Y.; Suzuki, Y. Simulating the advection and degradation of the
486 environmental DNA of common carp along a river. *Environ. Sci. Technol*. **2018**, *52* (18),
487 10562-10570.
488
489 (38) Shogren, A. J.; Tank, J. L.; Andruszkiewicz, E.; Olds, B.; Mahon, A. R.; Jerde, C. L.;
490 Bolster, D. Controls on eDNA movement in streams: Transport, retention, and resuspension.
491 *Sci. Rep*. **2017**, *7* (1), 5065.
492
493 (39) Wotton, R. S.; Malmqvist, B. Feces in aquatic ecosystems. *BioScience*. **2001**, *51* (7), 537-
494 544.
495
496 (40) Ahrenholtz, I.; Lorenz, M. G.; Wackernagel, W. The extracellular nuclease of *Serratia*
497 *marcescens*: studies on the activity in vitro and effect on transforming DNA in a groundwater
498 aquifer microcosm. *Arch. Microbiol.* **1994**, *161* (2), 176-183.
499
500 (41) Levy-Booth, D. J.; Campbell, R. G.; Gulden, R. H.; Hart, M. M.; Powell, J. R.;
501 Klironomos, J. N.; Pauls, P.; Swanton, C. J.; Trevors, J. T.; Dunfield, K. E. Cycling of
502 extracellular DNA in the soil environment. *Soil Biol. Biochem.* **2007**, *39* (12), 2977-2991.
503
504 (42) Matsui, K.; Honjo, M.; Kawabata, Z. Estimation of the fate of dissolved DNA in
505 thermally stratified lake water from the stability of exogenous plasmid DNA. *Aquat. Microb.*
506 *Ecol*. **2001**, *26*, 95-102.
507
508 (43) Turner, C. R.; Barnes, M. A.; Xu, C. C.; Jones, S. E.; Jerde, C. L.; Lodge, D. M. Particle
509 size distribution and optimal capture of aqueous macrobial eDNA. *Methods Ecol. Evol.*
510 **2014a**, *5* (7), 676-684.
511
512 (44) Wilcox, T. M.; McKelvey, K. S.; Young, M. K.; Lowe, W. H.; Schwartz, M. K.
513 Environmental DNA particle size distribution from Brook Trout (*Salvelinus fontinalis*).
514 *Conserv. Genet. Resour.* **2015**, *7* (3), 639-641.
515
516 (45) Kornberg, R. D. Chromatin structure: a repeating unit of histones and DNA. *Science*.
517 **1974**, *184* (4139), 868-871.

518
519 (46) Lloyd, H. M.; Jacobi, J. M.; Cooke, R. A. Nuclear diameter in parathyroid adenomas. *J.*
520 *Clin. Pathol*. **1979**, *32* (12), 1278-1281.
521
522 (47) Ernster, L.; Schatz, G. Mitochondria: a historical review. *J. Cell Biol.* **1981**, *91* (3), 227s-
523 255s.
524
525 (48) Shadel, G. S.; Clayton, D. A. Mitochondrial DNA maintenance in vertebrates. *Annu. Rev.*
526 *Biochem*. **1997**, *66* (1), 409-435.
527
528 (49) Jo, T.; Murakami, H.; Masuda, R.; Sakata, M. K.; Yamamoto, S.; Minamoto, T. Rapid
529 degradation of longer DNA fragments enables the improved estimation of distribution and
530 biomass using environmental DNA. *Mol. Ecol. Resour.* **2017**, *17* (6), e25-e33.
531
532 (50) Yamamoto, S.; Minami, K.; Fukaya, K.; Takahashi, K.; Sawada, H.; Murakami, H.;
533 Tsuji, S.; Hashizume, H.; Kubonaga, S.; Horiuchi, T.; et al. Environmental DNA as a
534 'snapshot'of fish distribution: A case study of Japanese jack mackerel in Maizuru Bay, Sea of
535 Japan. *PLoS One*. **2016**, *11* (3), e0149786.
536
537 (51) Zhang, C. I.; Lee, J. B. Stock assessment and management implications of horse
538 mackerel (*Trachurus japonicus*) in Korean waters, based on the relationships between
539 recruitment and the ocean environment. *Prog. Oceanogr.* **2001**, *49* (1-4), 513-537.
540
541 (52) Ellison, S. L.; English, C. A.; Burns, M. J.; Keer, J. T. Routes to improving the reliability
542 of low level DNA analysis using real-time PCR. *BMC Biotechnol.* **2006**, *6* (1), 33.
543
544 (53) Doi, H.; Inui, R.; Akamatsu, Y.; Kanno, K.; Yamanaka, H.; Takahara, T.; Minamoto, T.
545 Environmental DNA analysis for estimating the abundance and biomass of stream fish.
546 *Freshwater Biol.* **2017**, *62* (1), 30-39.
547
548 (54) Katano, I.; Harada, K.; Doi, H.; Souma, R.; Minamoto, T. Environmental DNA method
549 for estimating salamander distribution in headwater streams, and a comparison of water
550 sampling methods. *PLoS One.* **2017**, *12* (5), e0176541.

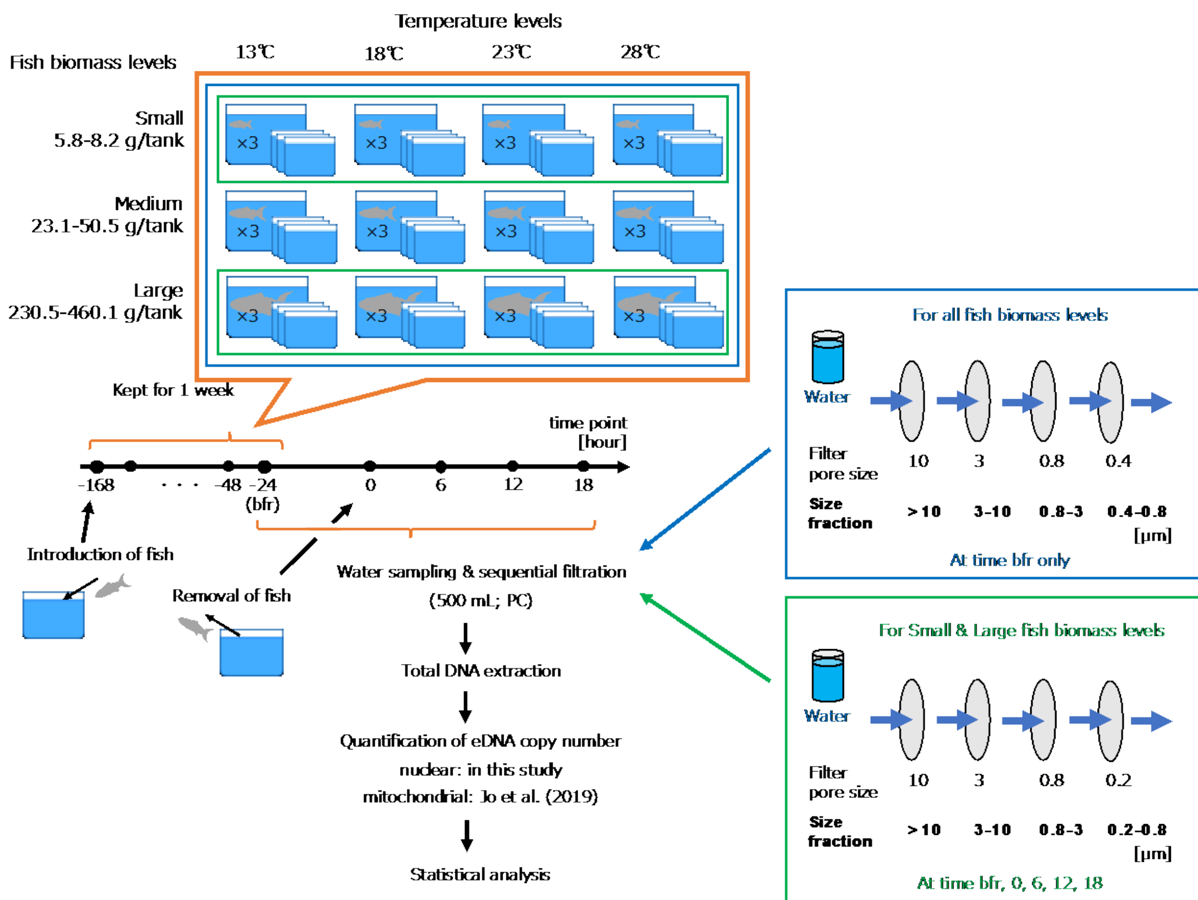
551
552 (55) R Core Team. R: A language and environment for statistical computing. Vienna, Austria:
553 R Foundation for Statistical Computing, **2006**.
554
555 (56) Fish, L. J. Why multivariate methods are usually vital. *Meas. Eval. Couns. Dev.* **1988**, *21*,
556 130-137.
557
558 (57) Warne, R. T. A primer on Multivariate Analysis of Variance (MANOVA) for behavioral
559 scientists. *Pract. Assess., Res. Eval.* **2014**, *19* (17), 1-10.
560
561 (58) Kuznetsova, A.; Brockhoff, P. B.; Christensen, R. H. B. lmerTest package: tests in linear
562 mixed effects models. *J. Stat. Softw.* **2017**, *82* (13), 1-26.
563
564 (59) Wrigglesworth, J. M.; Packer, L.; Branton, D. Organization of mitochondrial structure as
565 revealed by freeze-etching. *Biochim. Biophys. Acta.* **1970**, *205* (2), 125-135.
566
567 (60) Arnosti, C. Patterns of microbially driven carbon cycling in the ocean: links between
568 extracellular enzymes and microbial communities. *Adv. Oceanogr.* **2014**, *2014*, 1-12.
569
570 (61) Torti, A.; Lever, M. A.; Jørgensen, B. B. Origin, dynamics, and implications of
571 extracellular DNA pools in marine sediments. *Mar. Genom.* **2015**, *24*, 185-196.
572
573 (62) Price, P. B.; Sowers, T. Temperature dependence of metabolic rates for microbial growth,
574 maintenance, and survival. *Proc. Natl. Acad. Sci.* **2004**, *101* (13), 4631-4636.
575
576 (63) Mitani, F.; Ida, E. A study on the growth and age of the jack mackerel in the East China
577 Sea. *Bull. Jap. Soc. Sci. Fish.* **1964**, *30* (12), 968-977. (in Japanese)
578
579 (64) Maruyama, A.; Nakamura, K.; Yamanaka, H.; Kondoh, M.; Minamoto, T. The Release
580 Rate of Environmental DNA from Juvenile and Adult Fish. *PLoS One.* **2014**, *9* (12), e114639.
581

582 (65) Bylemans, J.; Furlan, E. M.; Gleeson, D. M.; Hardy, C. M.; Duncan, R. P. Does size
583 matter? An experimental evaluation of the relative abundance and decay rates of aquatic
584 eDNA. *Environ. Sci. Technol*. **2018a**, *52* (11), 6408-6416.
585
586 (66) Pilliod, D. S.; Goldberg, C. S.; Arkle, R. S.; Waits, L. P. Factors influencing detection of
587 eDNA from a stream-dwelling amphibian. *Mol. Ecol. Resour.* **2014**, *14* (1), 109-116.
588
589 (67) Deagle, B. E.; Eveson, J. P.; Jarman, S. N. Quantification of damage in DNA recovered
590 from highly degraded samples–a case study on DNA in faeces. *Front Zool.* **2006**, *3* (1), 11.
591
592 (68) Nielsen, K. M.; Johnsen, P. J.; Bensasson, D.; Daffonchio, D. Release and persistence of
593 extracellular DNA in the environment. *Environ. Biosafety. Res.* **2007**, *6* (1-2), 37-53.
594
595 (69) Suen, D. F.; Norris, K. L.; Youle, R. J. Mitochondrial dynamics and apoptosis. *Genes*
596 *Dev.* **2008**, *22* (12), 1577-1590.
597

598 **Figures**

599 Figure 1. The overall flowchart for the tank experiments. Three Japanese Jack Mackerels were
600 kept in 200 L tanks with four temperature and three biomass levels. After one week, the fish
601 were removed from each tank. Water sampling and sequential filtration were conducted the
602 day before and after fish removal. For all fish biomass levels, water samples were filtered
603 only at time bfr using polycarbonate (PC) filters with 10, 3, 0.8, and 0.4 µm pore sizes. For
604 Small and Large fish biomass levels, water samples were filtered at times bfr, 0, 6, 12, and 18
605 using PC filters with 10, 3, 0.8, and 0.2 µm pore sizes.

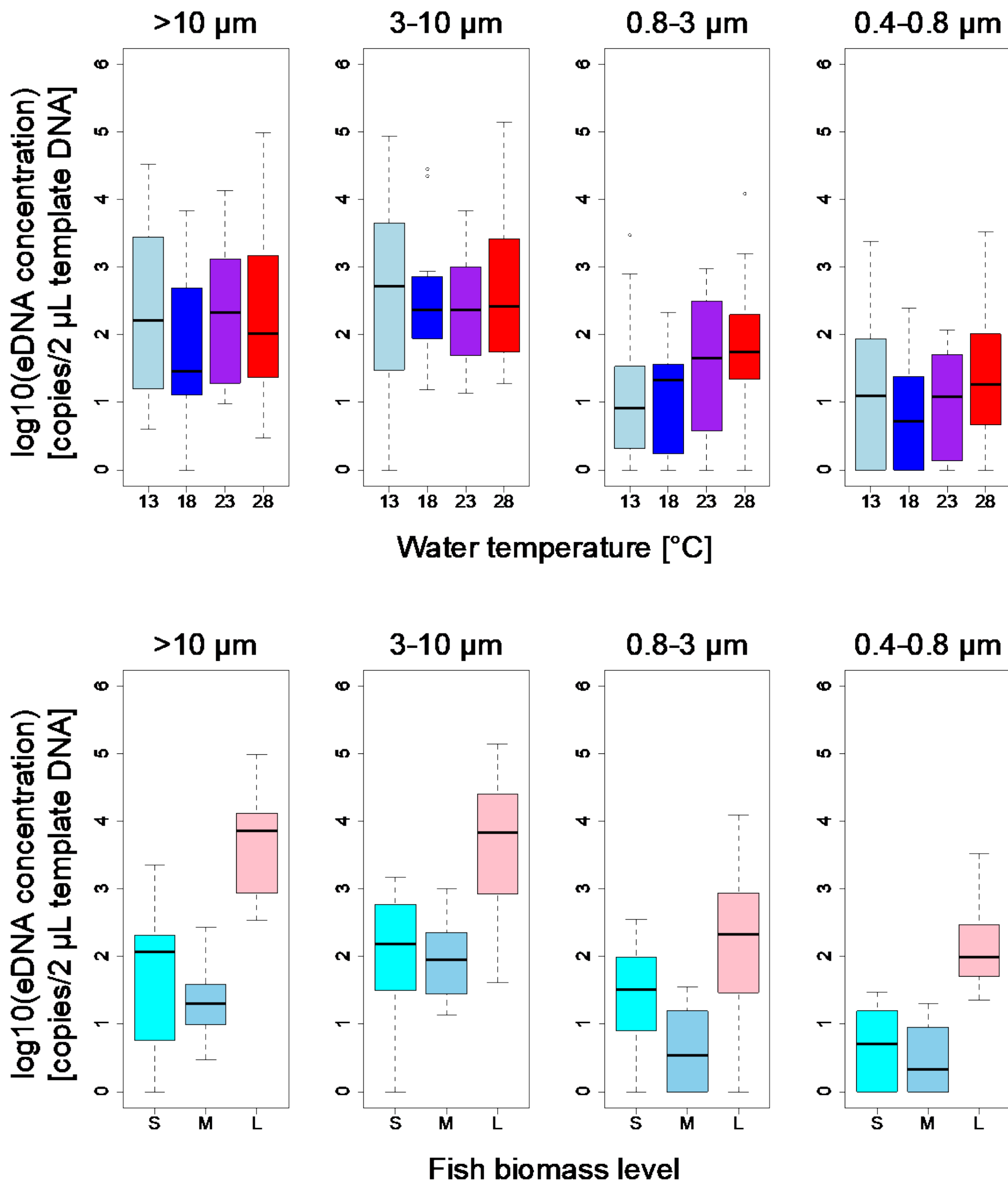
606



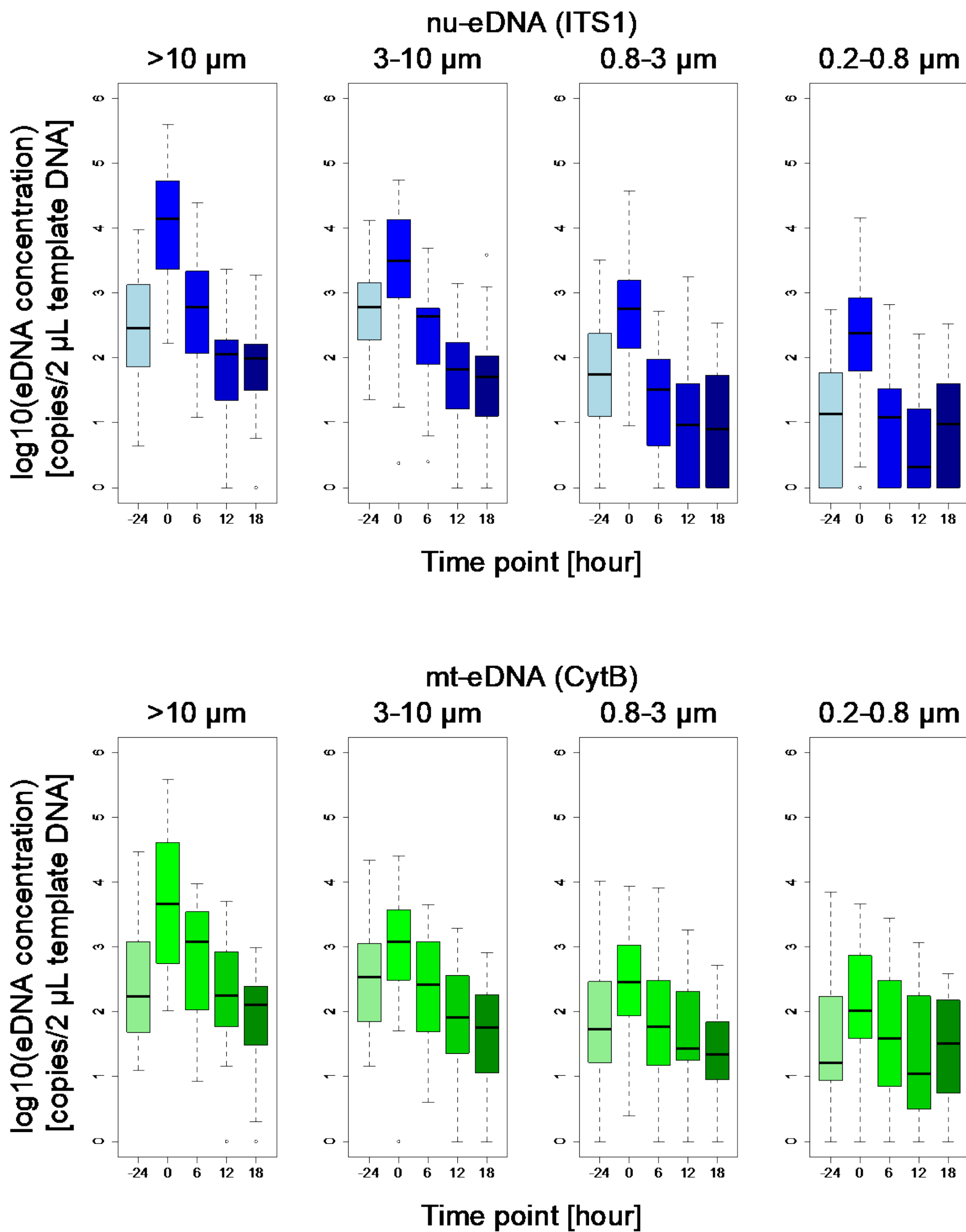
607

608

609 Figure 2. Results of the PSDs of Japanese Jack Mackerel nu-eDNA at time bfr. Upper
610 boxplots show the eDNA PSD at each temperature level (lightblue: 13 °C, blue: 18 °C,
611 purple: 23 °C, and red: 28 °C), where fish biomass levels are pooled. The lower boxplots
612 show the eDNA PSD at each fish biomass level (cyan: Small, skyblue: Medium, and pink:
613 Large), where water temperature levels are pooled.
614

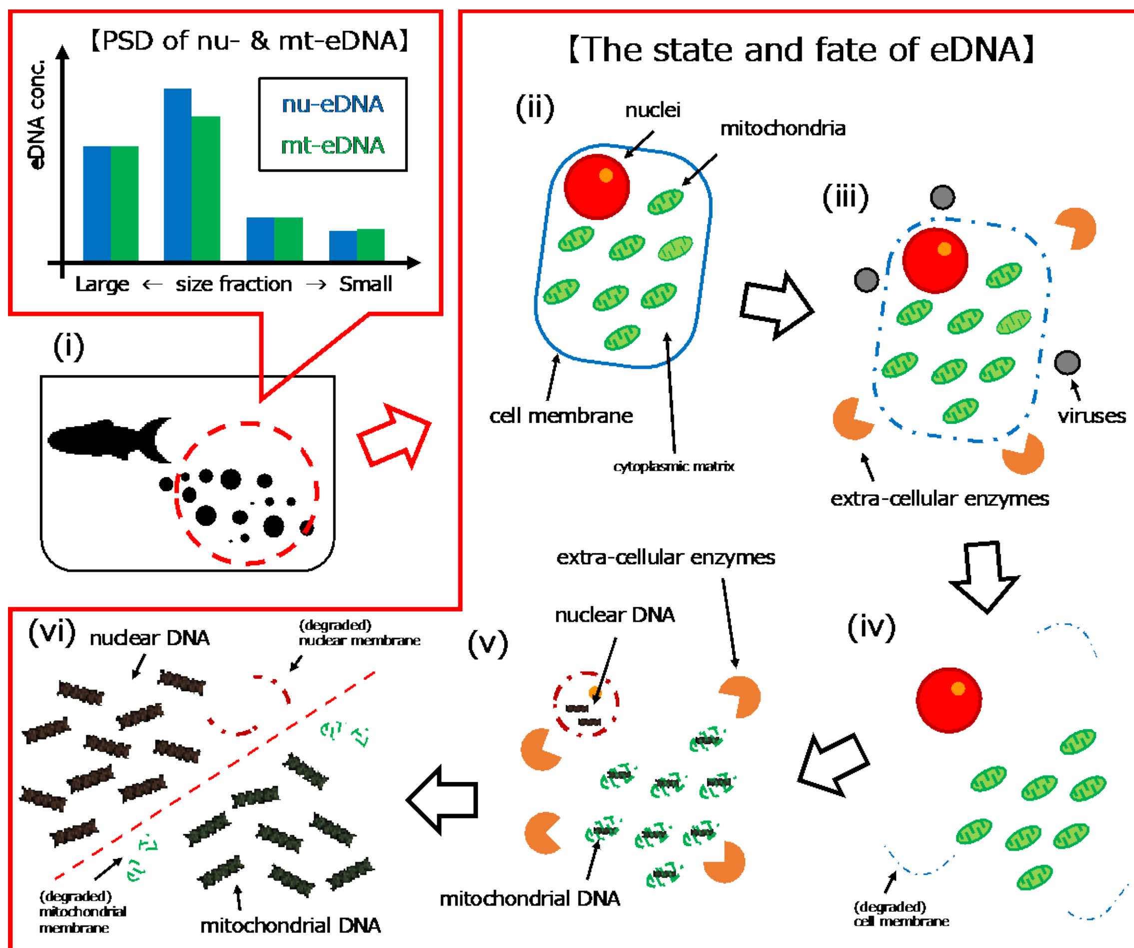


615 Figure 3. Results of the temporal changes of Japanese Jack Mackerel nu-eDNA (Upper) and
616 mt-eDNA (Lower) PSDs. All temperature and fish biomass levels are pooled for both
617 boxplots (Results for each treatment level are shown in Figure S2). Note that the smallest size
618 fraction here is 0.2-0.8 μm.
619



620

621 Figure 4. Schematic depiction of the state and fate of eDNA in water. Macro-organism eDNA
622 can exist in aquatic environments in various sizes and states, most being 1-10 µm in diameter
623 (i). At 3-10 µm size fractions, nu-eDNA can be more detected than mt-eDNA. Just after being
624 released into the water, most eDNA could be intra-cellular DNA within cells and tissues (ii).
625 After the eDNA is released into the water, it could break down by various degradation
626 processes, such as hydrolysis and take-up by extra-cellular enzymes and viral attack (iii),
627 which would result in the shedding of nuclei and other organelles out of degraded cell
628 membrane (iv). Likewise, the outer nuclei membranes of nuclei and mitochondria could also
629 break down by environmental factors (v), and their DNA molecules would be released (vi).
630 These extra-cellular DNA could also be degraded and eventually become undetectable.
631



632

633 **Tables**

634 Table 1. The primers/probe set used in this study.

635

Primer or Probe ID	Target region	Sequences (5’→3’)	Length (bp)	Tm (℃)	Reference
TjaITS1_F	nuclear internal transcribed spacer-1 (ITS1)	GCG-GGT-ACC-CAA-CTC-TCT-TC	164	60.1	Jo et al. (submitted)
TjaITS1_R		CCT-GAG-CGG-CAC-ATG-AGA-G		63.2	
TjaITS1_P		[FAM]-CTC-TCG-CTT-CTC-CGA-CCC-CGG-TCG-[TAMRA]		70.8	
Tja_CytB_F2	mitochondrial cytochrome b (CytB)	CAG-ATA-TCG-CAA-CCG-CCT-TT	127	58.7	Yamamoto et al. (2016)
Tja_CytB_R2		CCG-ATG-TGA-AGG-TAA-ATG-CAA-A		57.6	
Tja_CytB_P2		[FAM]-TAT-GCA-CGC-CAA-CGG-CGC-CT-[TAMRA]		67.9	

636

637

638 Table 2. Results of MANOVA (Upper) and post-hoc ANOVAs (Lower) for the relationships
639 between eDNA concentrations at each size fraction and each factor. Asterisks show the
640 corresponding factors that are statistically significant (* $P < 0.05$; ** $P < 0.01$; *** $P <$
641 0.001). All eDNA concentrations were log-transformed.
642

Response	Factor	P value	
eDNA conc. (for all size fractions)	Temperature	0.0000	***
	Fish biomass	0.0000	***
	DNA marker	0.0353	*

643

Response	Factor	P value	
eDNA conc. (> 10 μm size fraction)	Temperature	0.8906	
	Fish biomass	0.0000	***
	DNA marker	0.2059	
eDNA conc. (3-10 μm size fraction)	Temperature	0.7147	
	Fish biomass	0.0000	***
	DNA marker	0.0254	*
eDNA conc. (0.8-3 μm size fraction)	Temperature	0.0012	**
	Fish biomass	0.0000	***
	DNA marker	0.9596	
eDNA conc. (0.4-0.8 μm size fraction)	Temperature	0.0040	**
	Fish biomass	0.0000	***
	DNA marker	0.7663	

644

645 Table 3. Results of the statistical analyses for temporal change of eDNA PSDs. The upper
646 table shows the results of ANOVA for the differences in eDNA concentrations between time
647 bfr and 0, where bold values represent the statistical significances of these factors ($P < 0.05$).
648 The lower table shows the results of LMM for the relationships between eDNA
649 concentrations and each factor, where asterisks represent the statistical significances of the
650 parameters (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$). In the LMM, variables 'Fish biomass
651 (S)' and 'DNA marker (ITS1)' represent the fixed effects of Small against Large biomass
652 levels, and the markers for nuclear DNA against mitochondrial DNA, respectively. All the
653 eDNA concentrations were log-transformed.
654

Response	Factor	P value
eDNA conc.	Time point	**0.0000**
	Pore size	**0.0000**
	DNA marker	0.4063
	Time point: Pore size	**0.0028**
	Time point: DNA marker	0.1003
	Pore size: DNA marker	0.1903
	Time point: Pore size: DNA marker	0.5425

655

Response	Explanatory	Estimate	SE	P value
eDNA conc.	Intercept	2.3436	0.1302	***
	Time point	-0.0419	0.0095	***
	Pore size	0.1594	0.0116	***
	Temperature	0.0342	0.0082	***
	Fish biomass (S)	-0.8641	0.0908	***
	DNA marker (ITS1)	0.0209	0.0906	
	Time point: Pore size	-0.0050	0.0010	***
	Time point: Temperature	-0.0026	0.0007	***
	Time point: Fish biomass (S)	0.0162	0.0081	*
	Time point: DNA marker (ITS1)	-0.0258	0.0081	**

656
657