Full title: Effect of salinity and water dilution on environmental DNA degradation in freshwater environments

Short title: Salinity and water dilution affect eDNA degradation in freshwater environments

Tatsuya Saito[1,*] and Hideyuki Doi[2]

[1]Graduate School of Simulation Studies, University of Hyogo, 7-1-28 Minatojima-minamimachi, Chuo-ku, Kobe, 650-0047, Japan

[2]Graduate School of Information Science, University of Hyogo, 7-1-28 Minatojima-minamimachi, Chuo-ku, Kobe, 650-0047, Japan

*Corresponding author

email: tatwoeight630@icloud.com

ABSTRACT

Environmental DNA (eDNA) analysis methods have been developed to detect the distribution and abundance/biomass of organisms in various environments. eDNA generally degrades quickly, thus the study of eDNA degradation is critical for eDNA evaluation. However, there have only been a few studies of eDNA degradation experiments in which the salt concentration and water dilution were controlled. In this study, the effects of degradation were experimentally evaluated by controlling the salinity and water dilution of pond water. An experiment was conducted to evaluate the effects of salinity and dilution on eDNA detection with fragmental eDNA and free cell-derived eDNA using pond water, diluted pond water, and saline pond water. We quantified the eDNA copies of free cells, fragmental DNA, and the eDNA from *Cyprinus carpio*. In both the diluted and saline pond water, we found that the degradation rate of eDNA was much slower than that in pond water. Furthermore, the DNA concentration did not exponentially decrease in both the saline purified water and purified water samples. For the lower degradation rate in salt water, we interpreted that salts may affect DNA degradation factors such as microbe compositions and activities. The effect of salinity and dilution on eDNA detection provides fundamental information

about the degradation process of eDNA, which is essential to understand the behavior of eDNA in natural environments.

KEYWORDS: eDNA, degradation rate, pond, quantitative real-time PCR

INTRODUCTION

Environmental DNA (eDNA) evaluation methods have been developed to monitor macroorganism communities and manage aquatic ecosystems [1–4]. eDNA is the DNA released by organisms into an environment, such as water or soil, and derives from the feces [5], skin cells [1], mucus [6], and secretions [7] of the organisms. In addition, this eDNA can be collected in aquatic systems [1,8]. The DNA sources are mainly fractions of cells or organelles but can also be free DNA fragments suspended in the water [9,10].

An understanding of eDNA degradation, which is a critical eDNA characteristic, is important for eDNA evaluation for both species distribution and abundance/biomass [11–13]. To reveal the states of eDNA, especially its degradation rate, many experiments have been conducted under various conditions [14,15], such as varied temperature [16,17], pH [15,18], and salinity [11].

eDNA is measured over an experimental period to evaluate eDNA release and degradation [14,15,19]. The degradation curves of the eDNA in most experiments have been observed to have exponentially declined [12] and eDNA concentrations can decay below the limit of detection in less than a week [13–15].

In previous meta-analyses of eDNA [12,20](, it was similarly shown that no significant difference could be observed in the eDNA degradation rates between freshwater and seawater. However, the results of degradation experiments using sea and pond water have shown that the eDNA degradation rate would be slower in the sea [13]. Collins et al. [11] found that there is a slower eDNA degradation rate in marine areas with higher salt concentrations. There have been only a few studies of eDNA degradation in which the salt concentration was controlled, despite its importance for eDNA evaluation in the field. Therefore, we evaluated the effect of salinity on eDNA degradation by conducting degradation experiments. Furthermore, water dilution can potentially reduce the factors of eDNA degradation, such as the enzymes and microbes that degrade DNA. Thus, we also tested the effect of water dilution on eDNA degradation in the same manner.

The aim of this study was to observe and compare the effects of salinity and water dilution on the eDNA degradation rate in freshwater environments. To understand the degradation in each DNA source, such as individual-derived, cell-derived, and fragmental DNA [13], we evaluated the effects of salinity and dilution on eDNA detection while considering the fragmental eDNA, free cell-derived eDNA, and eDNA derived from the resident species of the pond.

68

69 **MATERIALS AND METHODS**

70 *Experimental design*

71 We collected pond water, diluted pond water, and salined pond water and divided each
72 water sample into bottles (Wide-Mouth Bottle, 500mL; AS ONE, Osaka, Japan) (Figure
73 1). We collected the pond water from an artificial pond in Kobe (the same pond used in
74 Saito & Doi [13]). A solution of isolated cells (from *Oncorhynchus kisutch*) and
75 fragmental DNA (from an internal positive control [IPC, 207-bp, 1.5×10^5 copies;
76 Nippon Gene, Tokyo, Japan]) was added to each bottle (Figure 1). The pond water
77 contained the eDNA of the resident common carp (*Cyprinus carpio*). We used *O.*
78 *kisutch* tissue for the isolation of cells because this species is not distributed in the pond.
79 We conducted the experiment for seven days. Water samples (500 mL) from each bottle
80 were filtered and collected using a Sterivex filter (0.45 μm pore size; Merck Millipore,
81 Burlington, MA, USA; Figure 1). After extracting eDNA from the Sterivex filter, the
82 copy number of each type of DNA contained in the Sterivex samples and filtrate was
83 estimated by quantitative real-time PCR (qPCR, Figure 1).

84

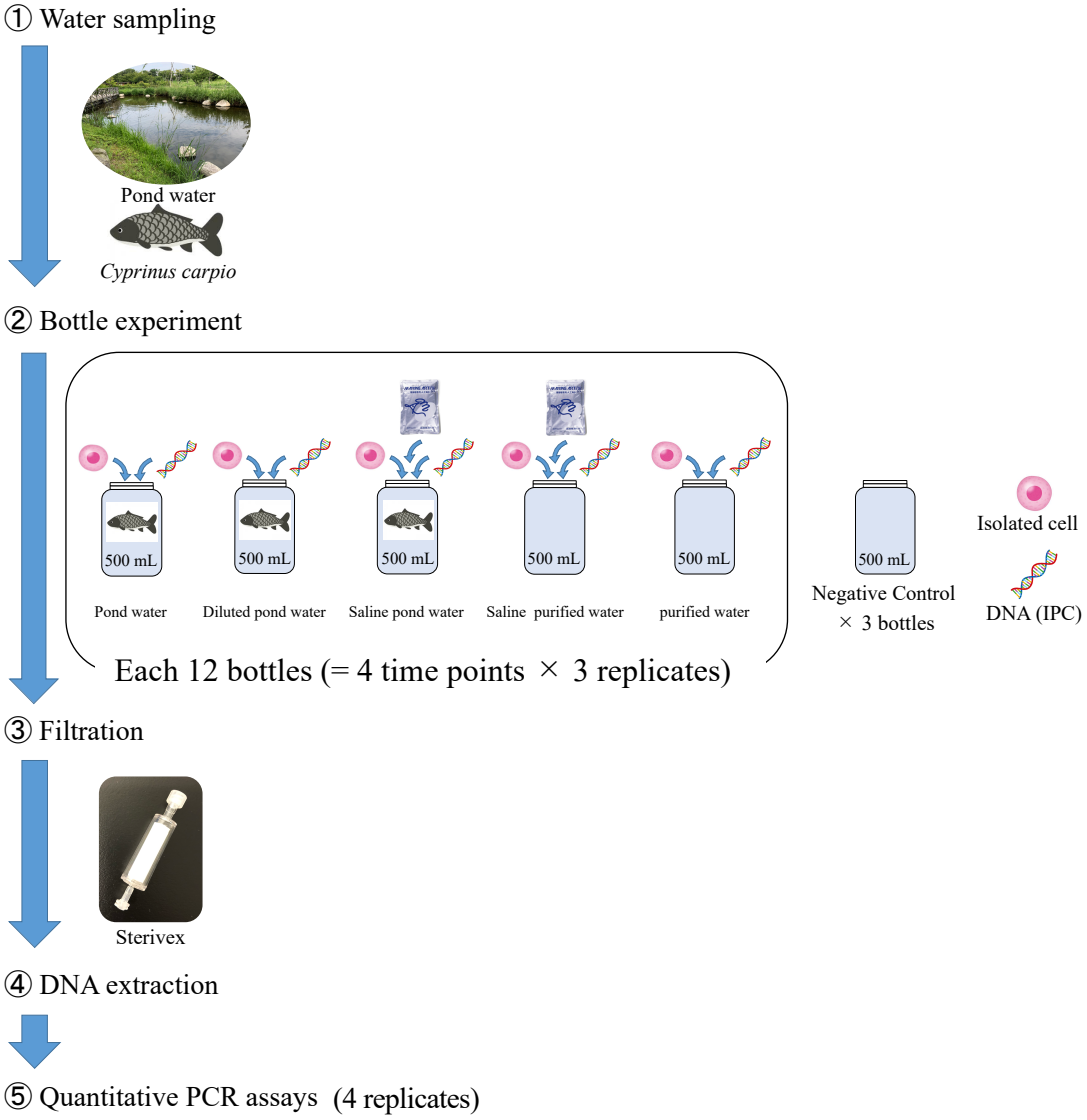


Fig 1. Experimental overview of the bottle experiments. We collected pond water, purified water, diluted pond water, saline pond water, and saline purified water and divided each type of water into 12 bottles. A solution of isolated cells (from *Oncorhynchus kisutch*) and fragmental DNA (IPC) was added to each bottle. The pond water was expected to contain the environmental DNA (eDNA) of *Cyprinus carpio*. We used *O. kisutch* tissue for the isolation of cells. We conducted the experiment for seven

92 days. A Sterivex filter was used to filter 500-mL samples of water from each bottle.
93 After extracting eDNA, the copy number of each type of DNA was estimated by
94 quantitative real-time PCR.

95

96 *Bottle experiment*

97 We collected the pond water from an artificial pond in Kobe, Japan (34°39' 40" N, 135°
98 13' 02" E) on July 16, 2020, using bleached tanks. We measured the salt concentration
99 (salinity) and temperature of the collected water using a salinity meter (CD-4307SD;
100 Mother Tool, Nagano, Japan) and a thermometer (ProODO; YSI, Tokyo, Japan),
101 respectively. The salt concentration (salinity) and water temperature at the time of the
102 water collection were 0.04 and 26.5 °C, respectively.

103 For the saline water, artificial seawater powder (Marine Art BR; Osaka
104 Yakken, Osaka, Japan) was added to the pond water and purified water to increase the
105 salinity to 3.3, the mean seawater salinity around Japan. For the diluted pond water, the
106 pond water and purified water (A300; AS ONE) were mixed at a ratio of 1:9. The pond
107 water, purified water, diluted pond water, saline pond water, and saline purified water
108 were each divided into 12 bottles (500 mL each). The bottles and equipment were

sterilized with 10% commercial bleach (ca. 0.6% hypochlorous acid), (KAO, Tokyo, Japan) and washed with DNA-free distilled water to avoid DNA contamination.

Each bottle received 100 µL of a solution of isolated cells [1.0×10^5 copies] and DNA (IPC). The bottles were incubated in the laboratory at about 25 ºC for a week. We collected and filtered 500 mL of the water from each bottle using 0.45-µm Sterivex filters (Merck Millipore) at 0, 3, 12, and 168 (day 7) h after the introduction of the cells and DNA. After filtration, approximately 2 mL of RNAlater (Thermo Fisher Scientific, Waltham, MA, USA) was injected into the Sterivex. As a filtration blank, the 500 mL of DNA-free water was filtered in the same manner after filtration of the samples to monitor cross-contamination. The Sterivex filters were immediately stored at –20 ºC until further analysis.

DNA extraction

DNA was extracted from the Sterivex filter using a DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) following Miya et al. [21] and Minamoto et al. [22]. The RNAlater was removed using a 50-mL syringe, and 440 µL of the mixture (220 µL of phosphate-buffered saline, 200 µL of Buffer AL, and 20 µL of proteinase K [Qiagen])

was added to the Sterivex filter. We incubated the filters on a rotary shaker (AS ONE) at 20 rpm for 20 min in a 56 ºC dry oven. We transferred the incubated mixture into a new 1.5 mL tube by centrifugation at 5000 g for 5 min. We then purified the mixture using a DNeasy Blood and Tissue Kit, and finally eluted the DNA in 100 µL of buffer AE from the kit. The extracted DNA from both methods was stored at –20 °C until qPCR analysis.

Quantitative PCR assays

We performed the qPCR analysis for *C. carpio* [2], *O. kisutch*, and the IPC [13]. We quantified the DNA concentrations by qPCR using a PikoReal™ qPCR system (Thermo Fisher Scientific). Each TaqMan reaction contained 900 nM of forward and reverse primers and 125 nM of a TaqMan probe in the 1× TaqPath™ qPCR master mix (Thermo Fisher Scientific). To this, 2 µL of the sample template was added to reach a final volume of 10 µL. A four step dilution series containing 1.5×10^1 to 1.5×10^4 copies was prepared and used as quantification standards. For the standard curves, we used target DNA cloned into a plasmid.

142 A quantitative PCR was performed with the following conditions: 2 min at 50
143 °C, 10 min at 95 °C, and 55 cycles of 15 s at 95 °C and 1 min at 60 °C. Four replicates
144 were performed for each sample, and four replicate negative non-template controls
145 (NTC) containing DNA-free water instead of template DNA were included in all PCR
146 plates. We performed the qPCR procedures according to the MIQE checklist [23]. The
147 PCR and qPCR were set up in two separate rooms to avoid DNA contamination.

148 The qPCR results were analyzed using PikoReal software ver. 2.2.248.601
149 (Thermo Fisher Scientific). The R^2 values of the standard curves ranged from 0.985–
150 0.998 (Supplementary Information) and the PCR varied from 91.07–101.68%. The
151 concentration of DNA in the water collected (DNA copies mL^{-1}) was calculated based
152 on the volume of filtered water. DNA copy numbers were evaluated including negative
153 amplifications set as zero values. In our previous study [13], we have already performed
154 a limit of detection (LOD) test for the PCR assay, which resulted in one copy for the
155 LOD.

156

157 *Statistical analysis*

158 Statistical analysis and data plotting were performed using R software version 3.6.0
159 [24]. We used the Single First-Order rate model (SFO) as the degradation model
160 because the SFO was the most effective model of degradation in Saito and Doi [13].
161 The SFO establishes a simple procedure for determining a first-order rate constant from
162 the degradation. The model equation is as follows:

163 $$C = C_0 e^{kt} \text{ (model 1, SFO)} \quad (1),$$

164 where C is the eDNA concentration at time t, C_0 is the eDNA concentration at time 0
165 (i.e., the initial eDNA concentration), and k is the degradation rate constant per hour.
166 We performed modeling using the "mkin" package version 0.9.49.8 in the R software.
167 We evaluated the fit of the models using the chi-squared error level [25](Boesten et
168 al.,2005). Significant differences in the model coefficients were evaluated by
169 overlapping the 95% confidential intervals (CIs) of the coefficients (i.e., $\alpha = 0.05$).

170

171 **RESULTS**

172 *Degradation of eDNA in saline pond water*

We detected all the targeted DNA of *C. carpio*, the *O. kisutch* cells, and the IPC using qPCR in saline pond water (Figure 2). The degradation rates in the saline pond water were significantly lower than those in the regular pond water for all three DNA sources (Table 1, Figure 3). We could not detect the eDNA of *C. carpio,* the *O. kisutch* cells, or the IPC on day seven in the pond water. However, we detected the eDNA of *C. carpio* and the IPC in the saline pond water up to day seven. There were no amplifications from the filter, extraction blanks, and NTCs in this experiment or in the following experiments.

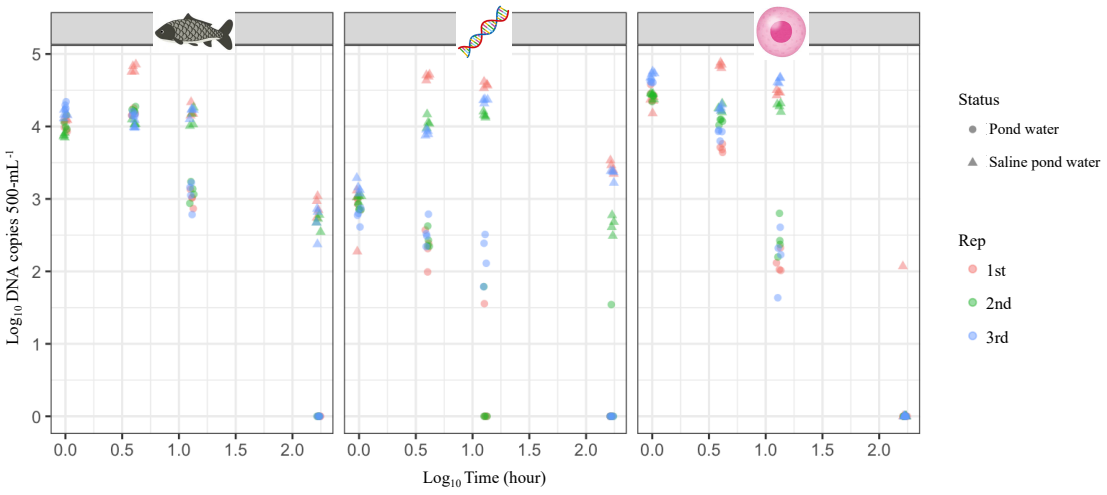


Fig 2. Relationship between the environmental DNA (eDNA) concentrations of the water source (pond water and saline pond water). The dots indicate the eDNA concentrations of the targets at each time point under two water conditions: pond water,

circles; saline pond water, triangles (N = 12 for each time point: 1st replication, red; 2nd replication, green; 3rd replication, blue).

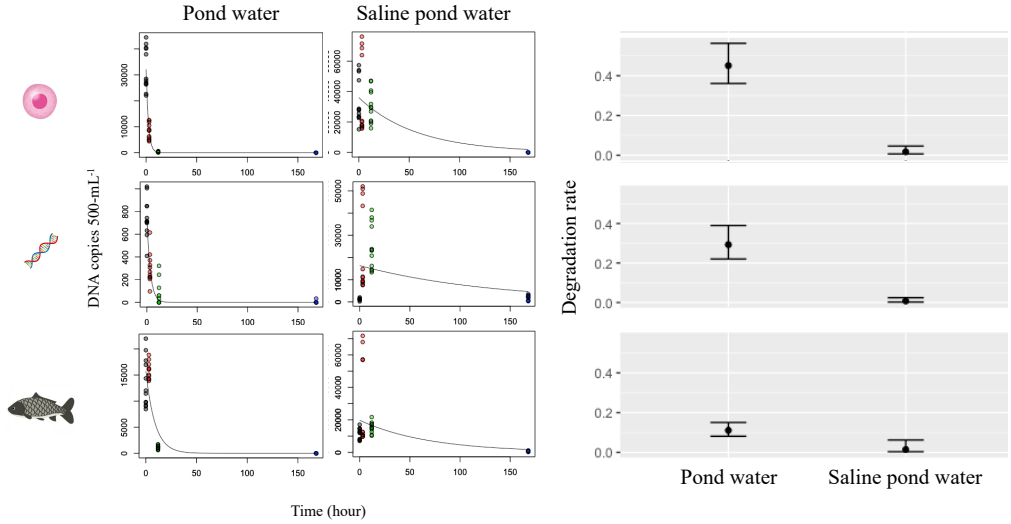


Figure 3. Degradation curves of the Single-First Order (SFO) model and the rate constant for the bottle experiments in the saline pond water and pond water. The dots indicate the environmental DNA concentrations of the targets (*Cyprinus carpio*, the internal positive control [IPC], and *Oncorhynchus kisutch* cells) at each time point with different colors (N = 12 for each time point). The left degradation curves show each target (*O. kisutch* cells, the IPC, and *C. carpio*) in the pond samples. The right decay curves show each target (*O. kisutch* cells, the IPC, and *C. carpio*) in the saline pond samples. In the right-hand plots, the slopes (k) of each target (*O. kisutch* cells, the IPC, and *C. carpio*) are shown with 95% confidential intervals.

198 Table 1. Degradation rate constant of Single-First Order models. The values are slope k

199 and the values in parentheses are the lower and upper confidential intervals of slope k.

Water	IPC	Okis	Cyca
Pond	0.293 (0.2202, 0.3899)	0.4510 (0.3612, 0.5631)	0.1107 (0.0813, 0.1506)
Saline pond	0.00757 (0.002361, 0.024270)	0.01746 (0.00659, 0.04626)	0.01465 (0.00344, 0.06244)
Diluted pond	0.009872 (0.004441, 0.021950)	0.03306 (0.02026, 0.05394)	0.02519 (0.01592, 0.03986)

200 Cyca, *Cyprinus carpio*; IPC, internal positive control; Okis, *Oncorhynchus kisutch* cells.

201

202 The degradation rate constant (k) of the cells, IPC, and *C. carpio* were

203 significantly different between the saline pond and pond samples when comparing the

204 95% CIs (Figures 2 and 3). The degradation rates of the saline pond were significantly

205 lower than those of the pond water for all three DNA sources.

206

207 *Degradation of eDNA in saline purified water*

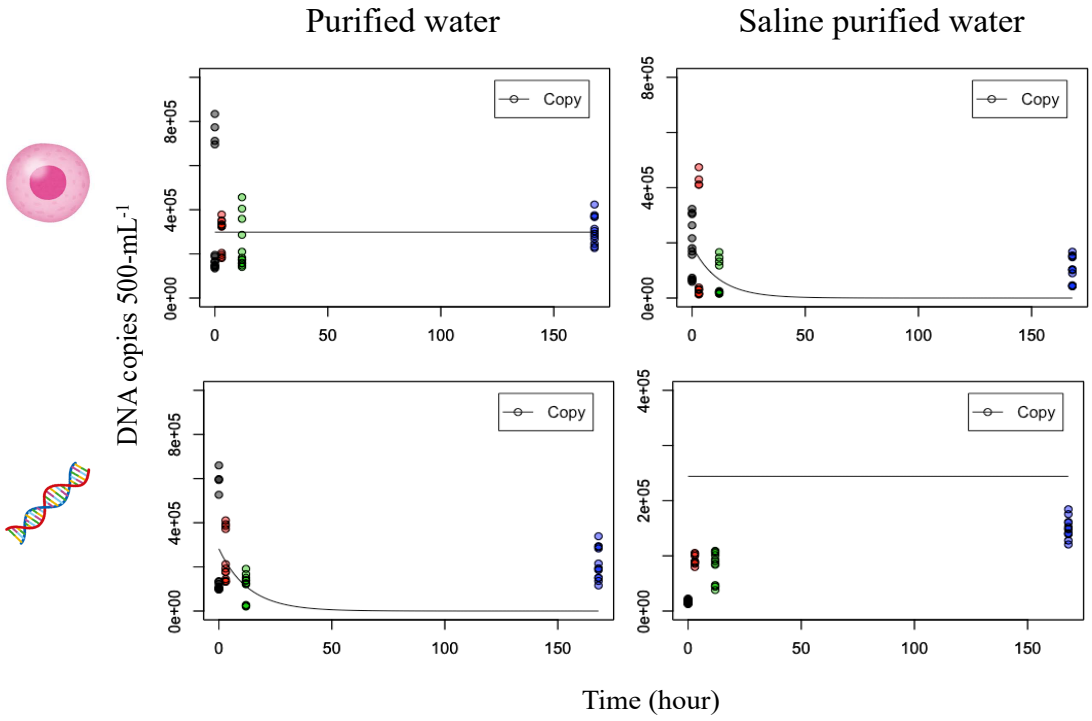
208 We detected all the targeted DNA of the *O. kisutch* cells and the IPC using qPCR in the

209 saline purified water (Figure 4). We detected the *O. kisutch* cells and the IPC DNA in

210 the purified water and saline purified water, respectively, up to 168 h. The DNA

211 concentrations of the cells and IPC DNA did not decrease exponentially after they were

212 added (0 h).



213

214 Fig 4. Degradation curves of the Single-First Order model (SFO) for the bottle

215 experiments in the saline purified water and purified water. The dots indicate the

216 environmental DNA concentrations of the targets (the internal positive control [IPC]

217 and *Oncorhynchus kisutch* cells) at each time point with different colors (N = 12 for

218 each time point). The left degradation curves show each target (*O. kisutch* cells and the

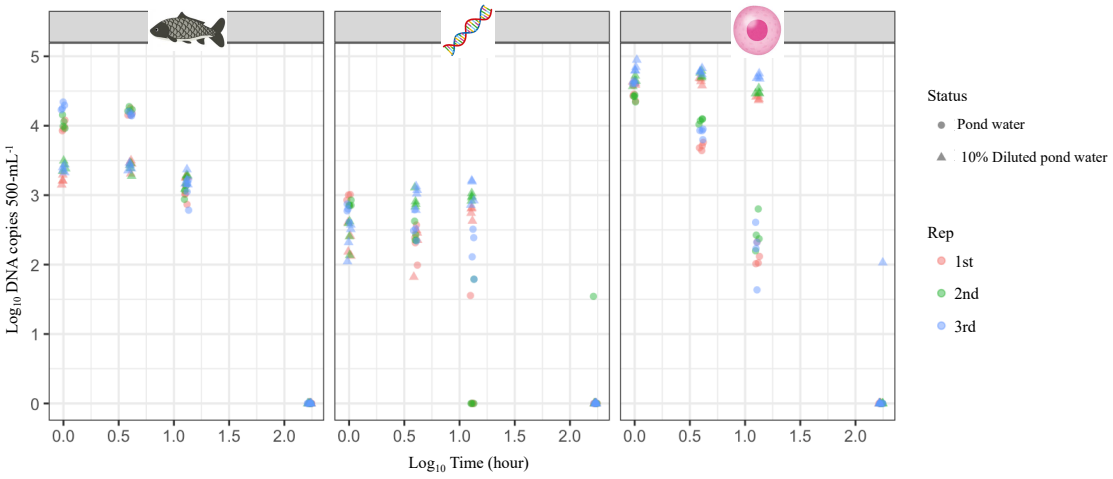
219 IPC) in the purified water samples. The right decay curves show each target (*O. kisutch*

220 cells and the IPC) in the saline purified water samples.

221

222 *Degradation of eDNA in diluted pond water*

223 We detected three of the targeted DNA types, *C. carpio*, *O. kisutch* DNA from the cells,
224 and the IPC, using qPCR in diluted pond water (Figure 5). The degradation rates of the
225 diluted pond were significantly lower than those of the pond water for all three DNA
226 sources (Table 1, Figure 6). We could not detect the eDNA of *C. carpio,* the *O. kisutch*
227 cells, and the IPC at 168 h in the pond water. However, we detected the eDNA of *C.*
228 *carpio* and the IPC in the diluted pond water up to 168 h.

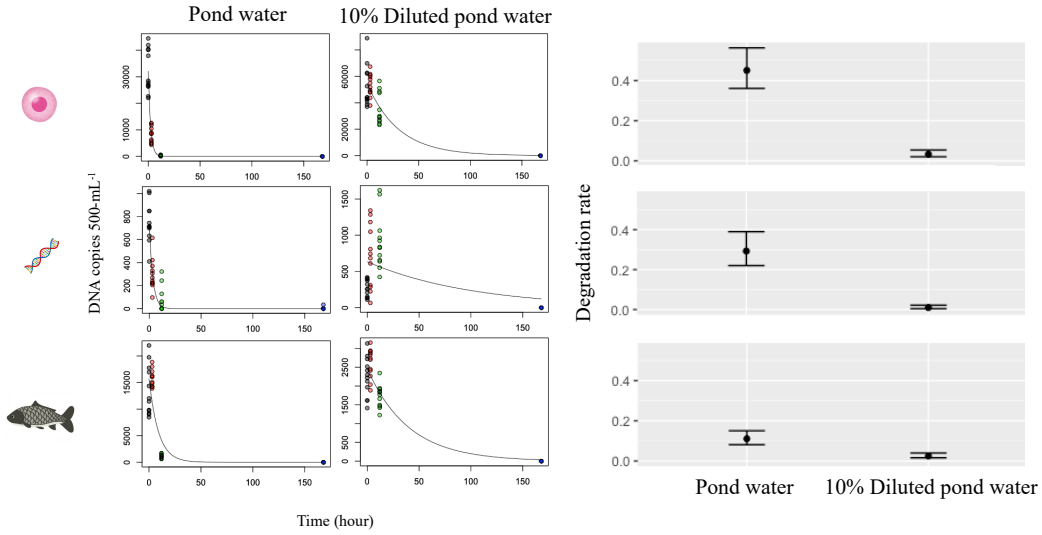


229

230 Fig 5. Relationship between the environmental DNA (eDNA) concentrations of the
231 water source (pond water and 10% diluted pond water). The dots indicate the eDNA
232 concentrations of the targets at each time point under two water conditions: pond water,

233 circles; diluted pond water, triangles (N = 12 for each time point: 1st replication, red;

234 2nd replication, green; 3rd replication, blue).



235

236 Fig 6. Degradation curves of the Single-First Order model (SFO) and the rate constant

237 for the bottle experiments in diluted pond water and pond water. The dots indicate the

238 environmental DNA concentrations of the targets (*Cyprinus carpio*, the internal positive

239 control [IPC], and *Oncorhynchus kisutch* cells) at each time point with different colors

240 (N = 12 for each time point). The left degradation curves show each target (*O. kisutch*

241 cells, the IPC, and *C. carpio*) in the pond samples. The right decay curves show each

242 target (*O. kisutch* cells, the IPC, and *C. carpio*) in the diluted pond samples. In the right-

243 hand plots, the slopes (k) of each target (*O. kisutch* cells, the IPC, and *C. carpio*) are

244 shown with 95% confidential intervals.

The degradation rate constant (k) of the cells, IPC, and *C. carpio* were significantly different between the diluted pond and pond samples when comparing the 95% CIs (Figure 6). The degradation rates in the diluted pond water were significantly lower than those in the pond water for all three DNA sources.

DISCUSSION

We found that the DNA concentrations of the *C. carpio, O. kisutch* cells, and the IPC did not decline exponentially in both the saline purified water and purified water samples. Our present results supported our previous study [13]. Furthermore, we detected the DNA in the saline purified water. This result showed that the increased salinity in the saline sample did not have any effect on DNA detection.

The degradation rates in the saline pond samples were significantly lower than those in the pond water for all three DNA sources. This result might suggest that salinity suppresses the degradation of eDNA. However, the results of a meta-analysis of eDNA degradation showed that the eDNA degradation rates between freshwater and

seawater are not significantly different [12,20]. A previous study [11] found salinity to be a better predictor of eDNA decay than pH, with salinity varying more between locations. They also showed that salinity itself may not be entirely responsible for the difference in degradation rate, but rather that it is associated with abundances or communities of microbes. In fact, the characteristics of microorganisms involved in DNA degradation may vary depending on the environment. For example, microorganisms living in freshwater are susceptible to salinity and cannot adapt to the rapid environmental change caused by salt addition, which is thought to reduce their activity and suppress DNA degradation. Therefore, the results of our study suggest that salts would be unlikely to protect DNA and may affect DNA degradation factors, including microbe composition changes and the activity of DNA enzymes in the water. Further evaluation of microbe compositions and DNA enzymes in salt water is needed to gain a deeper understanding of the degradation of eDNA.

The degradation rates in the diluted pond samples were significantly lower than those in the pond water for all three DNA sources. In the pond water diluted 10 times, the initial DNA concentration was reduced to one-tenth, but the degradation rate was slower than that in the pond water. This is thought to be due to the dilution of the degradation factors as well as the eDNA. Takasaki et al. [26] showed that pre-filters that

279 remove humic substances such as humic acid and fulvic acid are effective in detecting
280 eDNA. Our results showed that simply diluting eDNA without removing its degraders
281 and inhibitors was effective. However, the DNA was not detected after seven days.
282 Therefore, it was found that even if the concentration of the degradation factor is low,
283 the degradation progresses over time.

284 Our experiments provide new findings on eDNA degradation; however, there
285 were some limitations owing to the experimental design. First, we performed the
286 experiment using only one site for collecting the pond samples. Therefore, it is unclear
287 whether similar DNA degradation rates exist seasonally and in the other aquatic habitats
288 such as river and wetlands. Experiments using a selection of site replicates from various
289 habitats need to be performed to achieve a more generalized understanding of eDNA
290 degradation. The evaluation of eDNA degradation while comparing different
291 environmental conditions (e.g., salinity, water temperature, pH, chlorophyll, and
292 microorganism population) may reveal what is affecting eDNA degradation in general.

293 In conclusion, we found that the DNA concentrations of the *C. carpio, O.*
294 *kisutch* cells, and the IPC did not decline exponentially in both the saline purified water
295 and purified water samples. The degradation rates of the saline pond and pond samples

296 were significantly different. The degradation rates of the diluted pond and pond samples
297 were significantly different. A greater understanding of and the accumulation of basic
298 information about eDNA would improve eDNA analysis methods and enable
299 researchers to maximize the potential of future eDNA methods.

300

301 ACKNOWLEDGMENTS

302 This study was supported by the Environment Research and Technology Development
303 Fund (JPMEERF20164002 and JPMEERF20204004) of the Environmental Restoration
304 and Conservation Agency, Japan.

305

306 DATA ACCESSIBILITY

307 All data, including the raw values for the qPCR experiments, are included in the
308 supporting information.

309

310 REFERENCES

311

312 1. Ficetola GF, Miaud C, Pompanon F, Taberlet P. Species detection using
313 environmental DNA from water samples. Biol Lett. 2008; 4(4): 423–425.

314 2. Takahara T, Minamoto T, Yamanaka H, Doi H, Kawabata ZI. Estimation of fish
315 biomass using environmental DNA. PLoS One. 2012; **7**(4): e56584.

316 3. Minamoto T, Yamanaka H, Takahara T, Honjo MN, Kawabata ZI. Surveillance of
317 fish species composition using environmental DNA. Limnology. 2012; 13(2): 193–197.

318 4. Tsuji S, Takahara T, Doi H, Shibata N, Yamanaka H. The detection of aquatic
319 macroorganisms using environmental DNA analysis—A review of methods for
320 collection, extraction, and detection. Environ DNA. 2019; 1(2): 99–108.

321 5. Martellini A, Payment P, Villemur R. Use of eukaryotic mitochondrial DNA to
322 differentiate human, bovine, porcine and ovine sources in fecally contaminated surface
323 water. Water Res. 2005; 39(4): 541–548.

324 6. Merkes CM, McCalla SG, Jensen NR, Gaikowski MP, Amberg JJ. Persistence of
325 DNA in carcasses, slime and avian feces may affect interpretation of environmental
326 DNA data. PLoS One. 2014; 9(11): e113346.

7. Bylemans J, Furlan EM, Hardy CM, McGuffie P, Lintermans M, Gleeson DM. An environmental DNA-based method for monitoring spawning activity: A case study, using the endangered Macquarie perch (*Macquaria australasica*). Methods Ecol Evol. 2017; 8(5): 646–655.

8. Turner CR, Uy KL, Everhart RC. Fish environmental DNA is more concentrated in aquatic sediments than surface water. Biol Conserv. 2015; 183: 93–102.

9. Turner CR, Miller DJ, Coyne KJ, Corush J. Improved methods for capture, extraction, and quantitative assay of environmental DNA from Asian bigheaded carp (*Hypophthalmichthys* spp.). PLoS One. 2014; 9(12): e114329.

10. Minamoto T, Naka T, Moji K, Maruyama A. Techniques for the practical collection of environmental DNA: filter selection, preservation, and extraction. Limnology. 2016; 17(1): 23–32.

11. Collins RA, Wangensteen OS, O'Gorman EJ, Mariani S, Sims DW, Genner MJ. Persistence of environmental DNA in marine systems. Commun Biol. 2018; 1(1): 1–11.

12. Saito T, Doi H. A Model and Simulation of the Influence of Temperature and Amplicon Length on Environmental DNA Degradation Rates: A Meta-Analysis Approach. Front Ecol Evol. 2021a; 9: 139.

13. Saito T, Doi H. Degradation modeling of water environmental DNA: Experiments on multiple DNA sources in pond and seawater. Environ DNA. 2021b; https://doi.org/10.1002/edn3.192.

14. Thomsen PF, Kielgast J, Iversen LL, Møller PR, Rasmussen M, Willerslev E. Detection of a diverse marine fish fauna using environmental DNA from seawater samples. PLoS One. 2012; **7**(8): e41732.

15. Barnes MA, Turner CR, Jerde CL, Renshaw MA, Chadderton WL, Lodge DM. Environmental conditions influence eDNA persistence in aquatic systems. Environ Sci Technol. 2014; 48(3): 1819–1827.

16. Tsuji S, Ushio M, Sakurai S, Minamoto T, Yamanaka H. Water temperature-dependent degradation of environmental DNA and its relation to bacterial abundance. PLoS One. 2017; 12(4): e0176608.

356 17. Jo T, Murakami H, Yamamoto S, Masuda R, Minamoto T. Effect of water
357 temperature and fish biomass on environmental DNA shedding, degradation, and size
358 distribution. Ecol Evol. 2019; 9(3): 1135–1146.

359 18. Tsuji S, Yamanaka H, Minamoto T. Effects of water pH and proteinase K
360 treatment on the yield of environmental DNA from water samples. Limnology. 2017;
361 18(1): 1–7.

362 19. Maruyama A, Nakamura K, Yamanaka H, Kondoh M, Minamoto T. The release
363 rate of environmental DNA from juvenile and adult fish. PLoS One. 2014; 9(12):
364 e114639.

365 20. Jo T, and Minamoto T. Complex interactions between environmental DNA
366 (eDNA) state and water chemistries on eDNA persistence suggested by meta-analyses.
367 Mol Eco Res. 2020; https://doi.org/10.1111/1755-0998.13354.

368 21. Miya M, Minamoto T, Yamanaka H, Oka SI, Sato K, Yamamoto S, et al. Use of a
369 filter cartridge for filtration of water samples and extraction of environmental DNA. J
370 Vis Exp. 2016; Nov (117): e54741.

371 22. Minamoto T, Miya M, Sado T, Seino S, Doi H, Kondoh M, et al. An illustrated
372 manual for environmental DNA research: Water sampling guidelines and experimental
373 protocols. Environ DNA. 2021; 3(1): 8-13.

374 23. Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, et al. The
375 MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time
376 PCR Experiments. Clinical Chemistry. 2009; 55(4): 611– 622.

377 24. R Core Team. R: A Language and Environment for Statistical Computing.
378 Vienna: R Foundation for Statistical Computing. 2020

379 25. Boesten JJTI, Aden K, Beigel C, Beulke S, Dust M, Dyson JS, et al. Guidance
380 document on estimating persistence and degradation kinetics from environmental fate
381 studies on pesticides in EU registration. Report of the FOCUS Work Group on
382 Degradation Kinetics. EC Doc. Ref. 2005; Sanco/10058/2005, version 1.

383 26. Takasaki K, Aihara H, Imanaka T, Matsudaira T, Tsukahara K, Usui A, et al.
384 Water pre-filtration methods to improve environmental DNA detection by real-time
385 PCR and metabarcoding. PLoS One. 2021; 16(5): e0250162.

386

387