

A peer-reviewed version of this preprint was published in PeerJ on 6 December 2019.

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Kakuda A, Doi H, Souma R, Nagano M, Minamoto T, Katano I. 2019. Environmental DNA detection and quantification of invasive red-eared sliders, *Trachemy scripta elegans*, in ponds and the influence of water quality. PeerJ 7:e8155 <https://doi.org/10.7717/peerj.8155>

Detection of environmental DNA of the invasive red-eared slider in ponds for evaluating their distribution with comparison to water quality

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Environmental DNA (eDNA) is a powerful tool for monitoring the distribution of aquatic macro-organisms. However, environmental factors, including the water temperature and water quality, can affect the inhibition and/or degradation of eDNA, which prevents accurate estimations of eDNA concentrations and the detection of the presence/absence of species in natural habitats. Also, very few eDNA studies have been conducted for reptiles, especially with respect to estimating their biomass and/or abundances. Here we examined the relationship between the visually-observed number of red-eared sliders (*Trachemys scriptaelegans*) and the eDNA concentrations. Additionally, we evaluated the effect of water quality on the eDNA concentration in 100 ponds. We found that there was a significant positive correlation between the observed number of red-eared sliders and the eDNA concentration in the ponds. On comparing various water quality indicators, including dissolved nitrogen, dissolved phosphorous, organic matter, and chlorophyll a (Chl. a), we found that only Chl. a had a negative correlation with the red-eared slider eDNA concentration, while we did not find any inhibitions in the quantitative PCR. We conclude that concentrations of eDNA can potentially be used for estimating the abundance of the red-eared slider. Additionally, Chl. a might indirectly influence the degradation of eDNA through the microorganisms bonded to the phytoplankton in the ponds, which microbial activity thought to be decreasing eDNA.

1 For PeerJ,

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3 **slider in ponds for evaluating their distribution with**
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19

20 Short title: eDNA detection for red-eared slider

21

22

23 **Abstract**

24 Environmental DNA (eDNA) is a powerful tool for monitoring the distribution of aquatic macro-
25 organisms. However, environmental factors, including the water temperature and water quality,
26 can affect the inhibition and/or degradation of eDNA, which prevents accurate estimations of
27 eDNA concentrations and the detection of the presence/absence of species in natural habitats.
28 Also, very few eDNA studies have been conducted for reptiles, especially with respect to
29 estimating their biomass and/or abundances. Here we examined the relationship between the
30 visually-observed number of red-eared sliders (*Trachemys scripta elegans*) and the eDNA
31 concentrations. Additionally, we evaluated the effect of water quality on the eDNA concentration
32 in 100 ponds. We found that there was a significant positive correlation between the observed
33 number of red-eared sliders and the eDNA concentration in the ponds. On comparing various
34 water quality indicators, including dissolved nitrogen, dissolved phosphorous, organic matter,
35 and chlorophyll a (Chl. *a*), we found that only Chl. *a* had a negative correlation with the red-
36 eared slider eDNA concentration, while we did not find any inhibitions in the quantitative PCR.
37 We conclude that concentrations of eDNA can potentially be used for estimating the abundance
38 of the red-eared slider. Additionally, Chl. *a* might indirectly influence the degradation of eDNA
39 through the microorganisms bonded to the phytoplankton in the ponds, which microbial activity
40 thought to be decreasing eDNA.

42 Introduction

43

44 Environmental DNA (eDNA) methods for monitoring the distribution of aquatic species
45 have recently been developed (Ficetola *et al.*, 2008; Ree *et al.*, 2014; Goldberg and Strickler,
46 2015). The eDNA are DNA fragments released through the mucus, urine, gametes, or feces of
47 species in the environment. We can analyze DNA fragments of target species from a few liters of
48 water (Ficetola *et al.*, 2008; Ree *et al.*, 2014; Goldberg and Strickler, 2015; Denier *et al.*, 2017).
49 As we just sample the water in the field, eDNA methods are non-invasive, take a short time, and
50 cost less compared with traditional monitoring methods (Thomsen & Willerslev, 2015).

51 The eDNA analysis has been applied to various aquatic taxa, for example, fish
52 (Minamoto *et al.*, 2012; Thomsen *et al.*, 2012a; Takahara *et al.*, 2012, 2013; Eichmiller *et al.*,
53 2016a), amphibians (Ficetola *et al.*, 2008; Pilliod *et al.*, 2013; Fukumoto *et al.*, 2015; Katano *et*
54 *al.*, 2017), mollusks (Goldberg *et al.*, 2013), crustaceans (Tréguier *et al.*, 2014), insects
55 (Thomsen *et al.*, 2012b; Doi *et al.*, 2017b), trematode (Huver *et al.*, 2015; Hashizume *et al.*,
56 2017), and aquatic plants (Fujiwara *et al.*, 2016). We can detect the eDNA of various taxa, from
57 which we can estimate their biomass and abundance. However, there are few studies for reptiles,
58 especially on the eDNA quantification of turtles (e.g., Davy *et al.*, 2015; De Souza *et al.*, 2016;
59 Lacoursière-Roussel *et al.*, 2016).

60 Here, we conducted research to detect the distribution and abundance of a turtle
61 species, the red-eared slider (*Trachemys scripta elegans*). The red-eared slider is listed in the top
62 100 of the world's worst invasive species by the World Conservation Union IUCN (Global

63 Invasive Species Database, <http://www.issg.org/database>). In Japan, red-eared sliders were imported
64 from USA as a pet in the 1960s, and since then it has been released into the local natural habitats.
65 This species is an omnivore and it has gained some attention as they affect indigenous animals
66 (Lever, 2003). In 2013, the Nature Conservation Society of Japan conducted a visual survey in
67 the 41 prefectures and found 4,146 red-eared sliders in 39 prefectures, which was 64% of all
68 turtle abundance. Despite the concern that red-eared sliders disrupt the local ecology, there are
69 few studies about the species or its regional distribution in Japan. For all invasive species,
70 including the red-eared sliders, it is important to rapidly and correctly understand its distribution
71 to conserve the ecosystems in which it resides (Pyšek & Richardson, 2010). However, the
72 effective sampling methods for turtles depends on the target species. Sterrett *et al.* (2010),
73 suggested that we might underestimate the abundance of red-eared sliders by using visual
74 observations. Thus, eDNA methods would be a useful tool to replace visual observations for
75 evaluating the turtles distribution.

76 Despite the merits of eDNA methods for turtle surveying, the previous eDNA studies in
77 freshwater systems suggest that a number of environmental factors affect the probability of
78 eDNA detection; for example, the water temperature and water quality, including the pH,
79 suspended solid (SS), total phosphorous (TP), total nitrogen (TN), biological oxygen demand
80 (BOD), and chlorophyll a (Chl. *a*) (e.g., Barnes *et al.*, 2014; Strickler *et al.*, 2014; Eichmiller *et*
81 *al.*, 2016b; Song *et al.*, 2017). These factors can lead to false negative detections, which prevents
82 the accurate evaluation of eDNA concentrations and detection of the distribution/quantification
83 of a species. To improve the eDNA evaluation of distribution and biomass/abundance in natural

84 habitats, we should understand the relationship between eDNA detection rate/concentration and
85 these environmental factors.

86 Two main environmental factors that negatively influence eDNA detection rate are
87 ‘inhibition’ and ‘degradation.’ In fact, for the inhibition of eDNA detection, humic acids inhibit
88 DNA polymerase used in PCR (Matheson *et al.*, 2010). The degradation rate of eDNA decreased
89 with low water temperatures, low UV-B levels, and alkaline (high-pH) conditions in a mesocosm
90 experiment (Strickler *et al.*, 2014). The degradation rate of eDNA decreased along with the
91 increase of BOD, Chl. *a*, and total eDNA concentration in a mesocosm experiment (Barnes *et al.*,
92 2014). Although these studies are based on laboratory experiments, in rivers, eDNA detection
93 rate has been known to decrease with increasing Chl. *a*, but increase with increasing water
94 temperature and pH (Song *et al.*, 2017). Chl. *a* resulted in different outcomes from mesocosm
95 experiments and field observations, suggesting that more evidence is needed to conclude on the
96 effects of water quality on eDNA degradation. It is important to understand how environmental
97 factors, and which ones, affect eDNA degradation and PCR inhibition, however, there are few
98 studies on this conducted in the field.

99 In this study, our aim was to compare the eDNA concentrations of the target species, by
100 using real-time quantitative PCR (qPCR), with visual observations. Additionally, we aimed to
101 evaluate the species abundance with the measurements of eDNA concentrations in 100 study
102 ponds. From the eDNA concentration and water quality data, we examined the relationships
103 between the water quality and eDNA concentrations of the red-eared slider, to consider the water
104 quality effects that influence eDNA degradation and PCR inhibition in the ponds.

105

106 **Materials & Methods**

107

108 *Study site*

109 We conducted the field survey in 100 ponds that were located in Himeji, Japan (34°47' - 34°54'
110 N, 134°35' - 134°45' E, Fig. 1) between July 21 and November 16, 2016. The ponds were
111 located in city, rural, and mountain areas (see Table 1, Figure S1 in Supplemental Electronic
112 Materials (SEM)). There are few ponds in the southern (city area) and northern areas (mountain)
113 because the distribution of the ponds is biased. The field survey and pond sampling was
114 permitted by the land owners, if needed.

115

116 *Field survey and sampling*

117 We recorded the presence/absence and number of red-eared sliders, based on visual observations
118 from the shore line for three minutes by an expert (A. Kakuda). From a point within each pond,
119 500 mL of surface water was collected for eDNA and SS analysis, and a further 100 mL of
120 surface water was collected for Chl. *a* and water quality analysis. We directly sampled the eDNA
121 using a bleached bottle and added 0.5 mL of benzalkonium chloride (BAC) to avoid a reduction
122 of the eDNA concentration in the samples (Yamanaka *et al.*, 2016). Samples were stored in a
123 cooler box with a 'cooler blank.' The 'cooler blank' contained 500 mL of DNA-free water,
124 which we brought to the field, and it was treated identically to the other water samples, except
125 that it was not opened at the field sites.

126

127 *Water preparation*

128 Within six hours after water sampling, the samples were filtered onto a GF/F glass filter (47 mm
129 diameter and 0.7 μm pore size, GE Healthcare Japan, Tokyo, Japan). We used separate filters for
130 the eDNA, SS (from 500 mL water), and Chl. *a* (from 100 mL water) analysis. The filter was
131 then wrapped in commercial aluminum foil and stored at $-20\text{ }^{\circ}\text{C}$ until eDNA extraction, or SS/
132 Chl. *a* measurement. For eDNA samples, the ‘cooler blank’ and ‘filter blank’, DNA-free distilled
133 water, were also filtered in the same way as the samples. To avoid contamination, each piece of
134 equipment that was used in the water sampling or filtration was soaked in a 10% commercial
135 bleach solution (approximately 0.6% sodium hypochlorite) and rinsed using DNA-free distilled
136 water prior to reuse. The 80 mL of the filtrated samples were stored at $-20\text{ }^{\circ}\text{C}$ until further water
137 quality analyses. In total, we measured the water quality of 94 samples (in the 100 samples
138 without six data, see Table S1 in SEM).

139

140 *DNA extraction from the filters*

141 The eDNA was extracted from each filter using a DNeasy Blood & Tissue Kit (Qiagen, Hilden,
142 Germany) based on the method described by Uchii *et al.* 2016. Each filtrate was soaked in 400
143 μL of buffer AL and 40 μL of protease K in a Salivette tube (Sarstedt, Nümbrecht, Germany)
144 and incubated at $56\text{ }^{\circ}\text{C}$ for 30 min. After centrifugation at 5000 *g* for 5 min, 220 μL of TE buffer
145 (pH 8.0) was added to each filter, and the tubes were centrifuged again in the same way after
146 being kept for a minute. The 200 μL of buffer AL and 600 μL of 100% EtOH were then added to

147 each filtrate and mixed by pipetting. The mixture was applied to a DNeasy Mini spin column and
148 prepared according to the manufacture's manual. The sample solution (100 μ L of buffer AE) was
149 stored in a 1.5 mL microtube at -20°C until qPCR analysis.

150

151 *Quantitative real-time PCR (qPCR)*

152 The eDNA was measured with four PCR replicates using a PikoReal Real-Time PCR System
153 (Thermo Scientific, Waltham, MA, USA). To detect and quantify the DNA of the red-eared
154 slider using qPCR, the mitochondrial cytochrome b gene fragments were amplified and
155 quantified with the following primers and probe: Tse-Kako-A-F (5'-
156 CCTCCAACATCTCTGCTTGA -3'), Tse-Kako-A-R (5'- ATTGTACGTCTCGGGTGATG -3'),
157 and Tse-Kako-A-MGB-P (5'-FAM- CGGAATTTTCTTGGCTATAC -MGB-3'). The specificity
158 of the probe and primers was confirmed by Primer-BLAST and testing on Japanese turtles
159 (*Mauremys japonica*, *Mauremys reevesii*, and *Pelodiscus sinensis*). Each TaqMan reaction
160 contained 900 nM of each primer, 125 nM of TaqMan probe, 5 μ L qPCR master mix (TaqMan
161 Environmental Master Mix 2.0, Thermo Scientific, Waltham, MA, USA), 0.2 μ L AmpErase®
162 Uracil N-Glycosylase (UNG, Thermo Scientific, Waltham, MA, USA), and 2 μ L of the DNA
163 solution. The total volume of each reaction mixture was 10 μ L and we performed four replicates
164 for PCR. The PCR conditions were as follows: 2 min at 50°C , 10 min at 95°C , and 55 cycles of
165 15 s at 95°C and 60 s at 60°C . The qPCR results were analyzed using PikoReal software ver.
166 2.2.248.601 (Thermo Fisher Scientific, Waltham, MA, USA). The R² values of the standard
167 curves ranged from 0.960 to 0.989, and PCR efficiency ranged from 73.09 to 118.56%. Each

168 real-time PCR assay included four no template controls (NTCs) and we also measured the cooler
169 and filter blanks with four replicates. We used the average value of the four replicates for each
170 eDNA concentration. All of the above qPCR procedures were based on the MIQE checklist for
171 qPCR (Bustin *et al.*, 2009). We performed the PCR set up and real-time PCR in two separate
172 rooms to avoid contamination.

173

174 *PCR inhibition test*

175 We compared the Ct shift between the samples and controls with the same number of known
176 target DNA copies, based on the method by Doi *et al.* (2017b), to confirm the degree of PCR
177 inhibition. Ct is defined as the number of cycles required for enough amplified PCR product to
178 accumulate that it surpasses a threshold recognized by the real-time PCR instrumentation. The Ct
179 is inversely related to the starting quantity of the target DNA in a reaction and is used to
180 calculate this quantity. The presence of PCR inhibitors will shift (delay) the Ct for a given
181 quantity of the template DNA. To test for inhibition in the DNA samples, 1 μ L of the plasmid,
182 including the cytochrome b gene from *Trachurus japonicus* (1.5×10^4 copies), which is a marine
183 fish that does not inhabit the sampled ponds, was added to the PCR template with 1 μ L of
184 DNA-free distilled water. We used the primer and probe set that was reported by Yamamoto *et*
185 *al.* 2016: forward primer: 5'-CAGATATCGCAACCGCCTTT-3'; reverse primer: 5'-
186 CCGATGTGAAGGTAAATGCAAA-3'; and probe: 5'-FAM-TATGCACGCCAACGGCGCCT-
187 TAMRA-3'. The PCR conditions were the same as above. Each real-time PCR assay included
188 three no template controls. We used the average value of the replicates for each Ct value. $\Delta Ct \geq$

189 3 cycles were considered to be evidence of inhibition (Hartman *et al.*, 2005).

190

191 *Water quality analysis*

192 We measured phosphate (PO₄-P), nitrate (NO₃-N), total phosphorus (TP), total nitrogen (TN),
193 dissolved organic matter (DOM), and total organic matter (TOM) from the filtrate, according to
194 the methods of Saijo and Mitamura (1995), using a spectrophotometer (HITACHI U-5100,
195 Hitachi, Tokyo, Japan). The absorbance of DOM was read at 254 nm, using samples not in an
196 autoclave.

197

198 *SS measurement*

199 We used the GF/F glass filter, which had been burned and dried before the weight was measured
200 by electric balance (Satorius CPA2252), prior to the SS analysis. The filtrate was dried in the 60
201 °C automatic oven (Yamato DX402, Yamato, Japan) over 12 h before the weight was measured.
202 After that, we burned the dried filter at 450 °C for 2 h using an electric muffle furnace (Yamato
203 FO410), then measured the weight again in the same way. The SS content was calculated as
204 follows; (450 °C burned weight) – (60 °C dried weight).

205

206 *Chl. a measurement*

207 We extracted the Chl. *a* of the filter by immersing it in 99.5% ethanol over 12 h. The extracts
208 were measured at 630, 645, 663, and 750 nm absorbances by the spectrophotometer (HITACHI
209 U-5100). The Chl. *a* concentration was determined according to the following equation

210 (UNECSO 1969):

211

212 $\text{Chl. } a \text{ (mg L}^{-1}\text{)} = \{(11.64 \times E663 - 2.16 \times E645 + 0.1 \times E630) \times k\} / V$

213

214 where, k: ethanol for extraction (mL); E663, E645, and E630: each absorbance at 663, 645, 630

215 nm, excluding the absorbance at 750 nm; and V: water samples (L).

216

217 *Statistical analysis*

218 We used a linear model (LM) to evaluate the relationship between the eDNA concentration of

219 the red-eared slider and the environmental factors: Chl. *a*, SS, PO₄-P, NO₃-N, TP, TN, DOM,

220 and TOM. We used the data of the site detected eDNA, regardless of the visual observations

221 (Table S1). Prior to the LM, we used a variance inflation factor (VIF) to check the collinearity of

222 the factors. The maximum VIF was 59.685, indicating that co-linearity among the factors would

223 influence the results of the LM. Thus, we removed the factors with a VIF > 5, to reduce the

224 collinearity effect on the LM, resulting with Chl. *a* and TP left in the final LM analysis. All

225 statistical analysis and graphics were conducted in R ver. 3.4.1 (R Core Team, 2018) with the

226 “ggplot2” package.

227

228 **Results**

229

230 *The relationships between eDNA measurements and visual observations*

231 We detected the red-eared slider by both the eDNA and visual observation methods in 11 ponds
232 (Table 1). Of the 11 ponds, 10 ponds were detected with visual observation, and 9 ponds were
233 detected with eDNA (Table 1). There was a significant positive correlation between the eDNA
234 concentration and number of red-eared sliders identified by visual observations (LM, $R^2 = 0.48$,
235 $p < 0.001$, $N = 20$, Figure 2).

236 The ΔC_t values in 99 of the 100 ponds, except pond No. 33, were lower than 3 (0.27 ± 0.22 ,
237 mean \pm SD), which means that they were lower than the inhibition criteria (Hartman *et al.*,
238 2005). Thus, PCR inhibition was not significant for all samples, but pond No. 33 showed no
239 amplification in the PCR inhibition test. We did not detect any amplifications in the negative
240 controls and equipment blanks, including the cooler and filter blanks.

241

242 *The relationship between eDNA concentration and water quality*

243 The results of the water quality analysis are shown in Table S1 of the SEM [Chl. *a*: 0.04 ± 0.10
244 $\mu\text{g L}^{-1}$, SS: $35.0 \pm 44.34 \mu\text{g L}^{-1}$, $\text{PO}_4\text{-P}$: $0.78 \pm 0.87 \mu\text{mol L}^{-1}$, $\text{NO}_3\text{-N}$: $38.93 \pm 18.25 \mu\text{mol L}^{-1}$,
245 TP: $1.00 \pm 0.99 \mu\text{mol L}^{-1}$, TN: $29.53 \pm 30.77 \mu\text{mol L}^{-1}$, DOM: 0.07 ± 0.04 (abs = 254 nm), and
246 TOM: 0.00 ± 0.01 (abs = 254 nm), mean \pm 1 SD]. The VIFs of the LM for each factor were
247 shown in Table S2 in the SEM, of which the VIFs of Chl. *a* (4.254) and TP (1.859) were lower
248 than 5. The LM, without the factors and with a VIF of > 5 , showed that Chl. *a* was positively
249 related with the eDNA concentration (LM, $p < 0.0001$), while there was no significant
250 relationship between the eDNA concentration and other factors (Fig. 3, Table S1).

251

252 Discussion

253 We detected the eDNA of the red-eared slider in the surface water samples of the ponds. When
254 comparing with the visual observations, we detected the red-eared slider by both the eDNA and
255 visual surveys in 11 ponds. In the nine ponds detected by eDNA, we might fail to detect them by
256 visual observation, due to there being few turtles in the pond. EDNA methods can detect even
257 rare or cryptic species (Barnes & Turner, 2016). Our results support this phenomenon as the
258 turtles were detected not by visual observation, but by eDNA. While, in the ten ponds detected
259 by visual observation, we sampled the eDNA at one point per pond as per the survey design.
260 This result may suggest that it was necessary to sample at several points at each study site, to
261 decrease the false-negative eDNA detections (e.g., Tréguier *et al.*, 2014; Thomsen *et al.*, 2012a).
262 In this study, we designed the red-eared slider-specific primers and probe and showed that we
263 can use them to detect the red-eared sliders in field samples. In summary, we can detect the red-
264 eared sliders by eDNA for almost half of the total detections, by sampling only 500 mL of water
265 at a point of the pond, suggesting that the eDNA method is a useful tool for detecting the turtles'
266 distribution.

267 We also found a significant positive correlation between the eDNA concentration and
268 number of red-eared sliders detected by visual observation. In Takahara *et al.* 2012, there was a
269 highly positive correlation between the eDNA concentration and carp biomass in both the
270 aquaria and outdoor experiments. The field tests for the relationship between eDNA
271 concentration and biomass of amphibians and fish also supported the positive correlation (e.g.,
272 Pilliod *et al.*, 2013; Doi *et al.*, 2017). Although few studies for reptiles estimating the biomass or

273 number of individuals have been reported, in Lacoursière-Roussel *et al.* 2016, the eDNA
274 detection rate was highly correlated with the relative abundance of wood turtles. In our study,
275 likewise, a positive relationship between the eDNA concentration and the abundance of visual
276 detections was observed. Thus, we can possibly use the eDNA concentration for estimating the
277 number of red-eared sliders, especially for the ponds with a high species abundance.

278 From the relationships between water quality and eDNA concentration, Chl. *a* seemed
279 to influence the degradation of eDNA concentration. One of our hypotheses was that water
280 quality would influence/inhibit qPCR for eDNA detection, however, we could not find any
281 inhibition of PCR by inhibition tests, except for a single pond (No. 33). Only pond No. 33 could
282 be inhibited, because it showed no amplification of DNA. The humic acid from the
283 decomposition of leaves potentially inhibited the PCR for pond No. 33, because it was
284 surrounded by forest and its water color was black (Figure S2 in SEM). Thus, Chl. *a* might not
285 be directly related to PCR inhibition; however, it might have an influence with respect to
286 decreasing the eDNA concentration in the water through DNA degradation. For the other water
287 quality characteristics in our study, Eichmiller *et al.* (2016b) measured the effects of Chl. *a*, TN,
288 TP, and SS on the decay rate of carp eDNA in laboratory experiments, however, these variables
289 were not significantly correlated with the eDNA decay rate (Eichmiller *et al.*, 2016b). This result
290 of non-correlations with TN, TP, and SS to eDNA degradation was the same as our results,
291 however, we showed the negative relationship with Chl. *a* and eDNA.

292 Our result that only Chl. *a* had a significant affect, might suggest that Chl. *a* influences
293 the degradation of eDNA in the surface water of ponds. However, this phenomenon seems to be

294 in debate. For example, the eDNA decay rate has a negative relationship with Chl. *a* in a
295 mesocosm experiment for goldfish (Barnes *et al.*, 2014), i.e., the eDNA degradation was less in
296 the higher Chl. *a*. On the other hand, the eDNA detection rate has a negative relationship with
297 Chl. *a* in a field survey for silver carp (Song *et al.*, 2017). In our study, the eDNA concentrations
298 have a negative relationship with Chl. *a*, which supports the results of Song *et al.* 2017. As
299 abiotic environmental factors indirectly influence the increase of microbial activities, eDNA may
300 be decomposed by microorganisms (Barnes *et al.*, 2014). Thus, the eDNA degradation by
301 microorganisms bonded to phytoplankton, for example, indirectly increases microbial activities
302 by providing basal resources (Lennon, 2007), although we did not directly evaluate the microbial
303 activity. Further discussion on the “Chl. *a* hypothesis” on eDNA degradation is required for
304 understanding the mechanisms of eDNA degradation and for developing eDNA methods,
305 especially for eDNA surveys in highly-productive water bodies. In this study, we can provide the
306 hypothesis from the field data, but further field and laboratory experiments controlling the DNA
307 concentration and water conditions, including the water quality and planktonic community, are
308 required for understanding the mechanisms.

309 In conclusion, we could detect the eDNA of red-eared sliders at a similar performance
310 as visual observations and evaluate the abundance by the eDNA concentration. We also provide
311 the “Chl. *a* hypothesis” for eDNA degradation for comparing the water quality of the ponds. For
312 eDNA surveys, we should pay attention to the potential for false-negative detections, probably
313 because of the state of primary production with reference to the Chl. *a* concentration.
314 Understanding the mechanisms in eDNA degradation would provide us with the tools for easy

315 and accurate eDNA methods to evaluate the distribution of aquatic organisms.

316

317 **Acknowledgements**

318 We thank A. Sumi and D. Togaki for their helps on our sampling and experiments.

319

320 **Funding Statement**

321 This study was supported by the Environment Research and Technology Development Fund (4-

322 1602) of the Ministry of the Environment, Japan and JST-CREST (JPMJCR13A2) and JSPS

323 KAKENHI (15K07233) for TM.

324

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459 Figure legends

460

461 Figure 1 Study sites represented by red points.

462

463 Figure 2 Relationship between the visual observation number of red-eared sliders (*Trachemys*

464 *scripta elegans*) per km² and their eDNA concentrations in the ponds. The grey area represents

465 the limits of the 95% confidence interval for the slope of the linear regression.

466

467 Figure 3 Relationships between each water-quality factor and the eDNA concentration of the

468 red-eared slider in the ponds. The regression curve of Chl. *a* was drawn by linear regression with

469 95% confidence intervals for the slope.

470

471 Table 1 Sampling date, location, the detection of red-eared slider eDNA, and the number of red-
472 eared sliders visually observed in the study ponds. Observed by both eDNA and visual
473 observation is ⊙. Observed by only eDNA and by only visual observation are ○ and ●,
474 respectively.

475

476 Table 2 a) Linear regression slopes with a \pm 95% confidence interval, SE, t values, and p values
477 for the relationships between Chl. *a*, TP, and eDNA concentrations in the ponds. Factors with a
478 VIF > 5 were removed. b) The table represents the n, F value, p value of the F value, R², and
479 adjusted R² for the linear regression.

480

Figure 1

Figure 1 Study sites represented by red points.

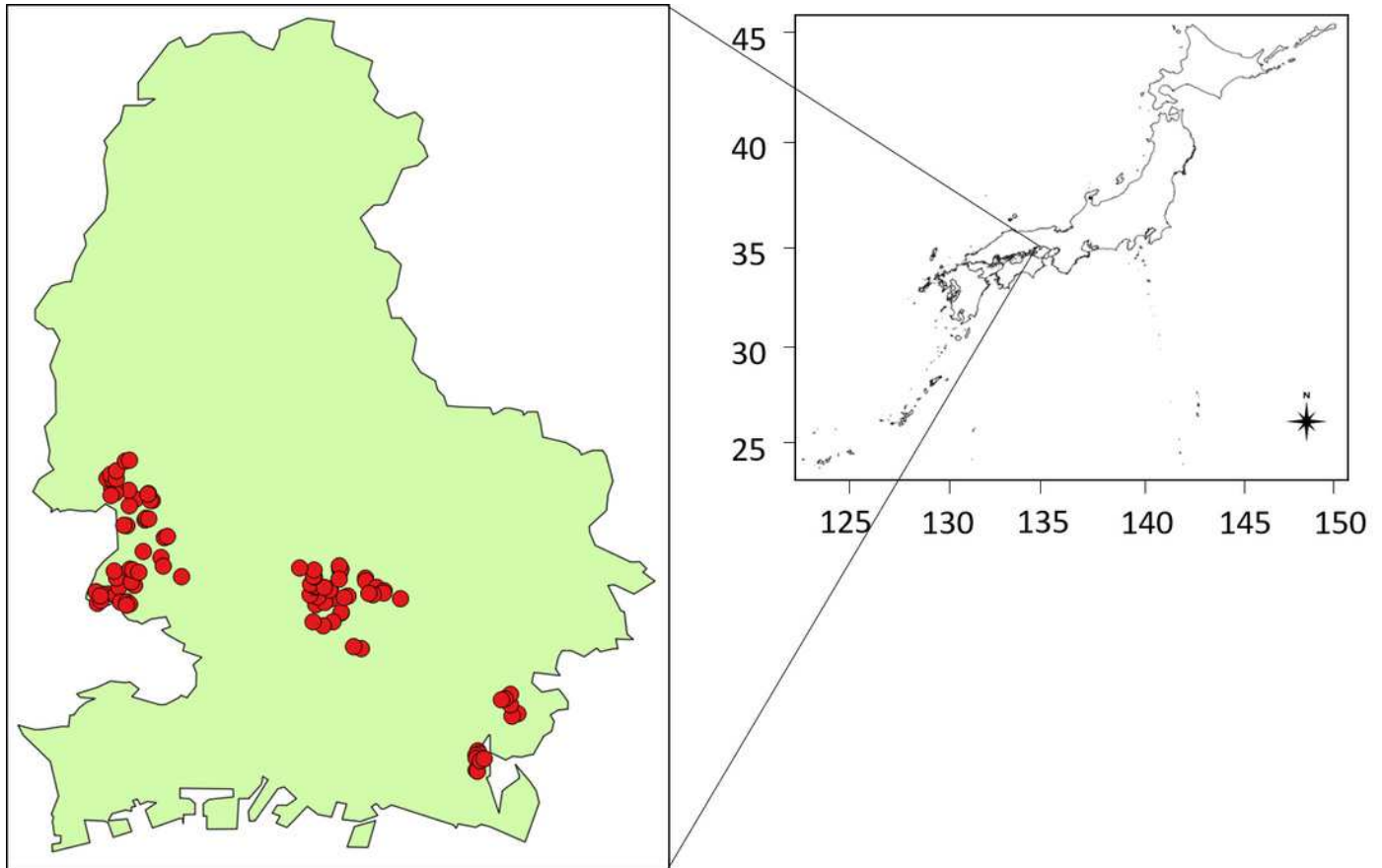


Figure 2

Figure 2 Relationship between the visual observation number of red-eared sliders (*Trachemys scripta elegans*) per km² and their eDNA concentrations in the ponds.

The grey area represents the limits of the 95% confidence interval for the slope of the linear regression.

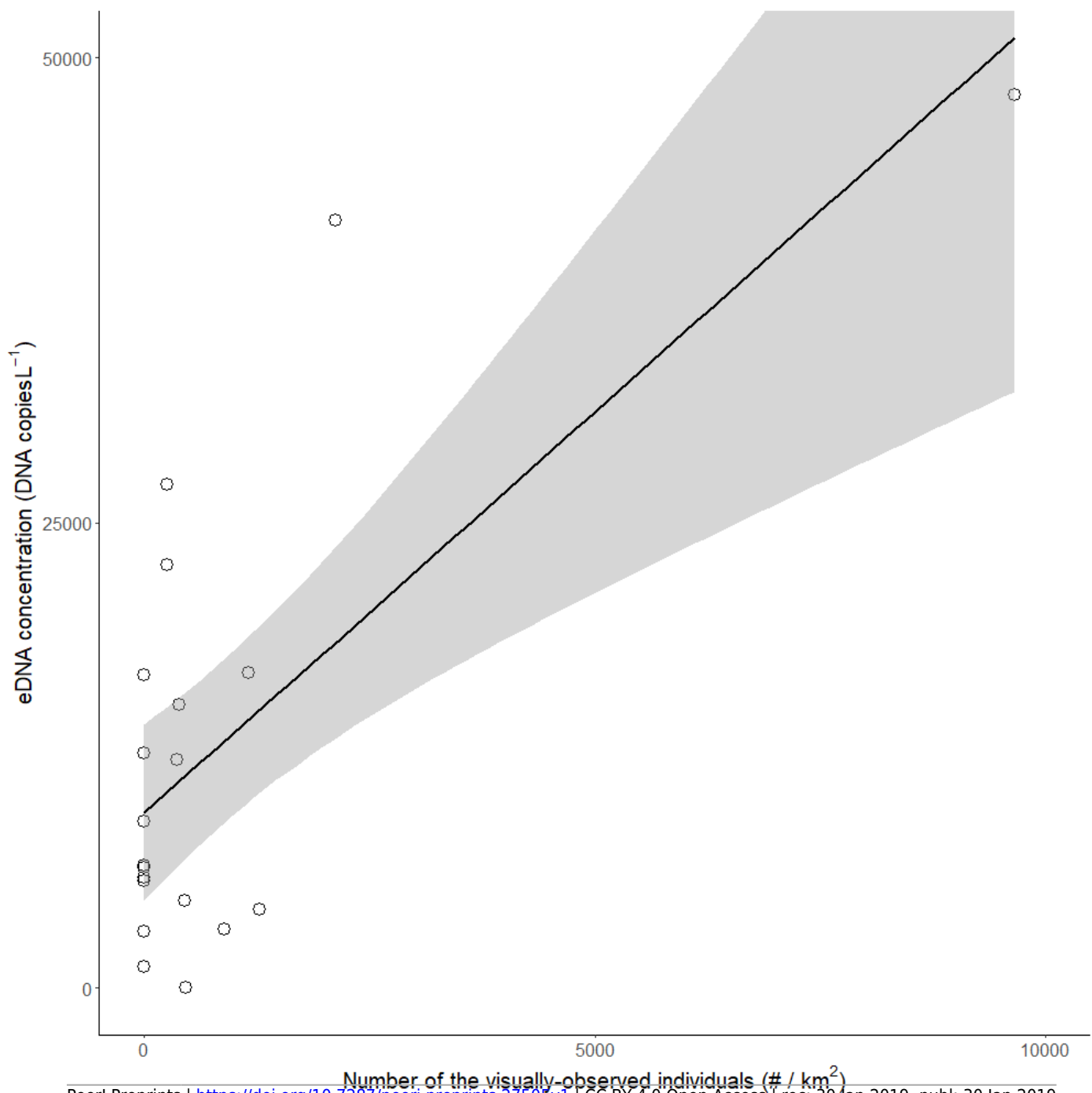


Figure 3

Figure 3 Relationships between each water-quality factor and the eDNA concentration of the red-eared slider in the ponds.

The regression curve of *Chl. awas* was drawn by linear regression with 95% confidence intervals for the slope.

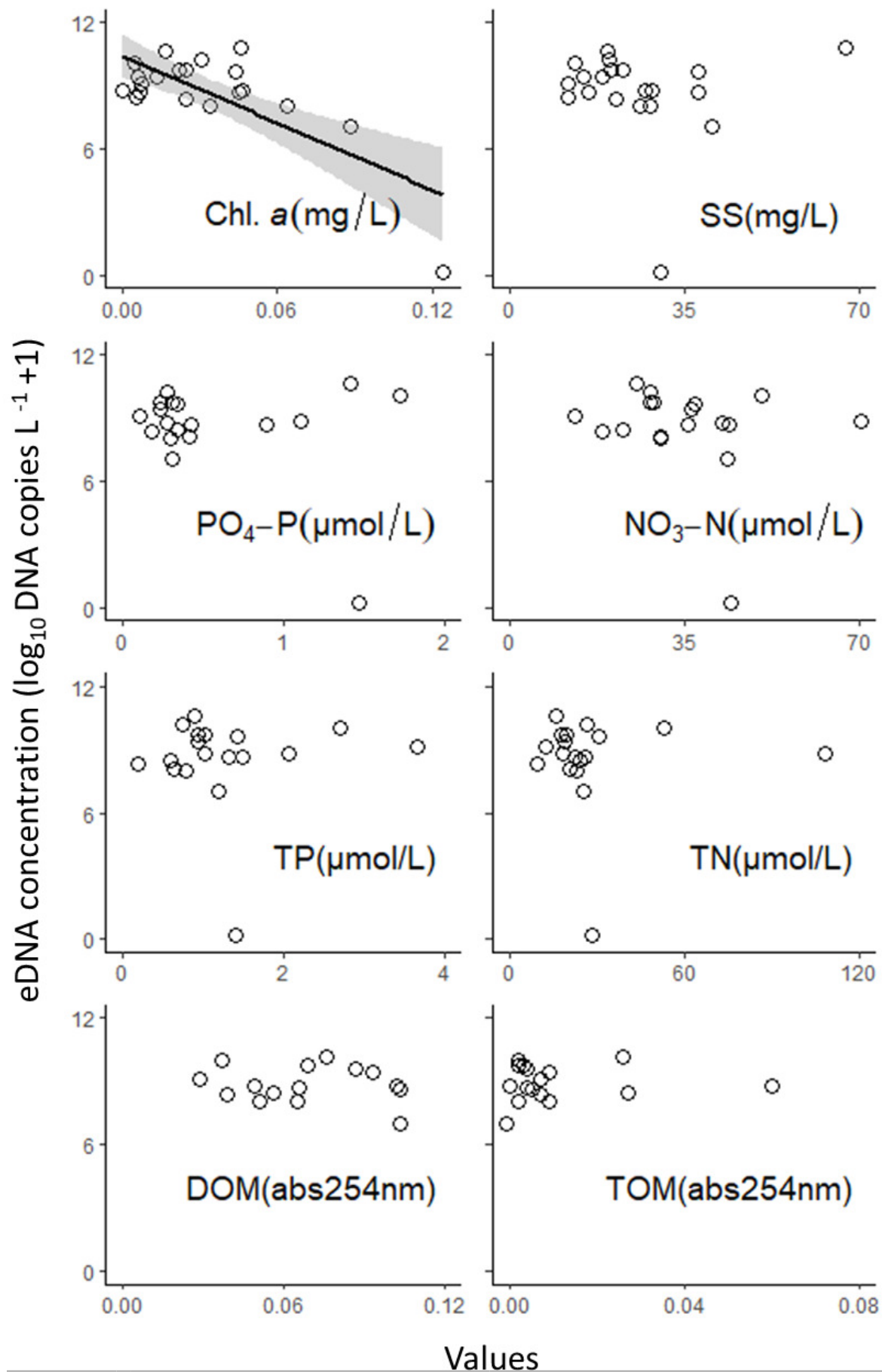


Table 1 (on next page)

Table 1 Sampling date, location, the detection of red-eared slider eDNA, and the number of red-eared sliders visually observed in the study ponds.

Observed by both eDNA and visual observation is ⊙. Observed by only eDNA and by only visual observation are ○ and ●, respectively.

Pond No.	Date	Latitude	Longitude	eDNA detected	Visual
1	2016/7/21	34°51'27"71	134°40'36"11	☉	10
2	2016/7/29	34°51'15"84	134°41'12"84	●	2
3	2016/7/29	34°51'16"92	134°41'12"84	○	-
4	2016/8/4	34°51'02"88	134°41'00"96	-	-
5	2016/8/4	34°50'57"12	134°40'46"92	☉	1
6	2016/8/8	34°51'36"36	134°40'57"57	☉	2
7	2016/8/8	34°51'30"96	134°40'49"08	○	-
8	2016/8/10	34°51'02"88	134°40'32"16	-	-
9	2016/8/10	34°51'50"27	134°40'36"26	-	-
10	2016/8/16	34°51'38"88	134°40'39"39	☉	10
11	2016/8/16	34°51'42"12	134°40'27"84	●	1
12	2016/8/19	34°50'24"24	134°41'42"42	-	-
13	2016/8/19	34°50'26"88	134°41'30"84	☉	3
14	2016/8/26	34°51'52"92	134°40'35"04	☉	1
15	2016/10/7	34°52'15"97	134°41'11"45	-	-
16	2016/10/7	34°52'21"91	134°41'10"54	☉	1
17	2016/10/7	34°52'19"39	134°41'12"48	-	-
18	2016/10/7	34°52'24"05	134°41'10"23	-	-
19	2016/10/7	34°52'06"21	134°41'48"00	●	1
20	2016/10/7	34°52'02"02	134°41'48"27	○	-
21	2016/10/14	34°51'36"40	134°42'38"65	-	-
22	2016/10/14	34°51'52"94	134°42'04"27	-	-
23	2016/10/14	34°51'47"99	134°42'14"86	○	-
24	2016/10/14	34°51'44"67	134°42'14"22	-	-
25	2016/10/14	34°51'42"18	134°41'59"00	-	-
26	2016/10/14	34°51'44"11	134°41'52"91	-	-
27	2016/10/14	34°51'40"18	134°41'22"60	-	-
28	2016/10/14	34°51'38"16	134°41'17"16	●	1
29	2016/10/14	34°51'50"62	134°40'57"02	-	-
30	2016/10/14	34°51'48"96	134°40'56"23	●	3
31	2016/10/17	34°51'54"45	134°40'37"75	-	-
32	2016/10/17	34°51'57"51	134°40'28"63	-	-
33	2016/10/17	34°51'52"84	134°40'48"37	-	-
34	2016/10/19	34°51'43"21	134°35'35"21	○	-
35	2016/10/19	34°51'43"34	134°35'26"03	●	1
36	2016/10/19	34°51'46"64	134°35'17"99	-	-
37	2016/10/19	34°51'41"91	134°35'46"99	-	-
38	2016/10/19	34°51'53"15	134°35'51"31	-	-
39	2016/10/19	34°52'06"27	134°35'47"93	-	-
40	2016/10/19	34°52'16"58	134°35'44"54	-	-
41	2016/10/19	34°51'31"47	134°35'53"56	☉	4
42	2016/10/19	34°51'32"04	134°36'02"88	☉	1
43	2016/10/19	34°51'28"49	134°36'06"63	●	5
44	2016/10/19	34°51'27"27	134°36'02"16	-	-
45	2016/10/19	34°51'29"60	134°35'20"10	-	-
46	2016/10/19	34°51'33"48	134°35'25"09	○	-
47	2016/10/19	34°51'40"47	134°35'24"34	-	-
48	2016/10/21	34°51'55"82	134°36'13"86	-	-
49	2016/10/21	34°52'00"86	134°36'09"34	-	-
50	2016/10/21	34°52'19"20	134°36'07"22	-	-

Table 2 (on next page)

Table 1 Continued. Sampling date, location, the detection of red-eared slider eDNA, and the number of red-eared sliders visually observed in the study ponds.

Observed by both eDNA and visual observation is ⊙. Observed by only eDNA and by only visual observation are ○ and ●, respectively.

Pond No.	Date	Latitude	Longitude	eDNA detected	Visual
51	2016/10/21	34°52'18"12	134°36'11"16	●	3
52	2016/10/21	34°52'14"80	134°36'20"07	-	-
53	2016/10/21	34°52'36"15	134°36'52"11	-	-
54	2016/10/21	34°52'23"71	134°36'55"38	-	-
55	2016/10/21	34°52'08"35	134°37'21"82	-	-
56	2016/10/26	34°48'49"62	134°45'28"46	-	-
57	2016/10/26	34°48'45"99	134°45'20"11	-	-
58	2016/10/26	34°49'02"53	134°45'17"81	-	-
59	2016/10/26	34°49'17"61	134°45'17"76	-	-
60	2016/10/26	34°49'11"13	134°45'09"65	○	-
61	2016/10/26	34°49'09"75	134°45'04"08	○	-
62	2016/10/26	34°47'55"70	134°44'30"60	-	-
63	2016/10/26	34°47'51"66	134°44'32"38	-	-
64	2016/10/26	34°47'49"32	134°44'27"66	-	-
65	2016/10/26	34°47'44"73	134°44'28"25	-	-
66	2016/10/26	34°47'27"68	134°44'28"04	-	-
67	2016/10/26	34°47'26"17	134°44'30"00	-	-
68	2016/10/26	34°47'41"37	134°44'34"11	-	-
69	2016/10/26	34°47'44"19	134°44'39"70	-	-
70	2016/11/2	34°52'04"71	134°41'09"82	○	-
71	2016/11/2	34°52'08"04	134°40'33"96	◎	40
72	2016/11/2	34°52'08"77	134°40'32"74	◎	10
73	2016/11/2	34°52'17"98	134°40'33"59	●	1
74	2016/11/2	34°52'20"97	134°40'12"81	-	-
75	2016/11/10	34°52'44"93	134°36'26"46	-	-
76	2016/11/10	34°53'04"92	134°36'56"88	-	-
77	2016/11/10	34°53'06"74	134°37'01"27	-	-
78	2016/11/10	34°53'22"42	134°36'02"79	-	-
79	2016/11/10	34°53'22"97	134°35'58"44	-	-
80	2016/11/10	34°53'30"58	134°36'29"18	-	-
81	2016/11/10	34°53'33"12	134°36'30"51	-	-
82	2016/11/10	34°53'32"29	134°36'34"27	-	-
83	2016/11/10	34°54'00"96	134°36'15"60	-	-
84	2016/11/10	34°53'51"23	134°36'06"41	-	-
85	2016/11/16	34°53'58"77	134°36'39"56	-	-
86	2016/11/16	34°53'59"25	134°36'36"64	-	-
87	2016/11/16	34°54'09"90	134°36'33"84	-	-
88	2016/11/16	34°54'07"92	134°36'33"12	-	-
89	2016/11/16	34°54'13"64	134°36'05"48	-	-
90	2016/11/16	34°54'19"24	134°35'45"65	-	-
91	2016/11/16	34°54'17"99	134°35'40"68	-	-
92	2016/11/16	34°54'09"87	134°35'46"19	-	-
93	2016/11/16	34°54'06"24	134°35'39"84	-	-
94	2016/11/16	34°54'30"99	134°35'33"98	-	-
95	2016/11/16	34°54'30"37	134°35'41"57	-	-
96	2016/11/16	34°54'36"81	134°35'39"32	-	-
97	2016/11/16	34°54'30"37	134°35'47"51	-	-
98	2016/11/16	34°54'41"97	134°35'47"75	●	1
99	2016/11/16	34°54'56"00	134°36'00"70	-	-
100	2016/11/16	34°54'57"30	134°36'06"47	-	-

Table 3 (on next page)

Table 2 a) Linear regression slopes with a \pm 95% confidence interval, SE, t values, and p values for the relationships between Chl. a, TP, and eDNA concentrations in the ponds.

Factors with a VIF > 5 were removed.

factors	slope	SE	<i>t</i> value	<i>p</i> value
Chl. <i>a</i>	-57.388	10.896	-5.267	0.000
TP	-0.390	0.432	-0.903	0.381
Intercept	10.922	0.794	13.751	0.000

1

Table 4 (on next page)

Table 2 b) The table represents the n, F value, p value of the F value, R^2 , and adjusted R^2 for the linear regression.

N	18
<i>F</i> value	13.89
<i>p</i> value	0.000
<i>R</i> ²	0.649
Adjusted <i>R</i> ²	0.603

1