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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.

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Short title: eDNA detection for red-eared slider

Abstract

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 scripta elegans*) 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.

Introduction

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

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

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

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

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

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

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

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

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

Quantitative real-time PCR (qPCR)

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

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

PCR inhibition test

We compared the Ct shift between the samples and controls with the same number of known target DNA copies, based on the method by Doi *et al*. (2017b), to confirm the degree of PCR inhibition. Ct is defined as the number of cycles required for enough amplified PCR product to accumulate that it surpasses a threshold recognized by the real-time PCR instrumentation. The Ct is inversely related to the starting quantity of the target DNA in a reaction and is used to calculate this quantity. The presence of PCR inhibitors will shift (delay) the Ct for a given quantity of the template DNA. To test for inhibition in the DNA samples, 1 μL of the plasmid, including the cytochrome b gene from *Trachurus japonicus* (1.5 × 104 copies), which is a marine fish that does not inhabit the sampled ponds, was added to the PCR tempelate with 1 μL of DNA-free distilled water. We used the primer and probe set that was reported by Yamamoto *et al*. 2016: forward primer: 5′-CAGATATCGCAACCGCCTTT-3′; reverse primer: 5′-CCGATGTGAAGGTAAATGCAAA-3′; and probe: 5′-FAM-TATGCACGCCAACGGCGCCT-TAMRA-3′. The PCR conditions were the same as above. Each real-time PCR assay included three no template controls. We used the average value of the replicates for each Ct value. $\Delta Ct \geq$

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

Water quality analysis

We measured phosphate (PO_4-P), nitrate (NO_3-N), total phosphorus (TP), total nitrogen (TN), dissolved organic matter (DOM), and total organic matter (TOM) from the filtrate, according to the methods of Saijo and Mitamura (1995), using a spectrophotometer (HITACHI U-5100, Hitachi, Tokyo, Japan). The absorbance of DOM was read at 254 nm, using samples not in an autoclave.

SS measurement

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

Chl. a measurement

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

(UNECSO 1969):

Chl. *a* (mg L-1) = {(11.64 × E663 - 2.16 × E645 + 0.1 × E630) × k} / V

where, k: ethanol for extraction (mL); E663, E645, and E630: each absorbance at 663, 645, 630 nm, excluding the absorbance at 750 nm; and V: water samples (L).

Statistical analysis

We used a linear model (LM) to evaluate the relationship between the eDNA concentration of the red-eared slider and the environmental factors: Chl. *a*, SS, PO_4-P, NO_3-N, TP, TN, DOM, and TOM. We used the data of the site detected eDNA, regardless of the visual observations (Table S1). Prior to the LM, we used a variance inflation factor (VIF) to check the collinearity of the factors. The maximum VIF was 59.685, indicating that co-linearity among the factors would influence the results of the LM. Thus, we removed the factors with a VIF > 5, to reduce the collinearity effect on the LM, resulting with Chl. *a* and TP left in the final LM analysis. All statistical analysis and graphics were conducted in R ver. 3.4.1 (R Core Team, 2018) with the "ggplot2" package.

Results

The relationships between eDNA measurements and visual observations

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

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

The relationship between eDNA concentration and water quality

The results of the water quality analysis are shown in Table S1 of the SEM [Chl. *a*: 0.04 ± 0.10 μg L^{-1}, SS: 35.0 ± 44.34 μg L^{-1}, PO_4-P: 0.78 ± 0.87 μmol L^{-1}, NO_3-N: 38.93 ± 18.25 μmol L^{-1}, TP: 1.00 ± 0.99 μmol L^{-1}, TN: 29.53 ± 30.77 μmol L^{-1}, DOM: 0.07 ± 0.04 (abs = 254 nm), and TOM: 0.00 ± 0.01 (abs = 254 nm), mean ± 1 SD]. The VIFs of the LM for each factor were shown in Table S2 in the SEM, of which the VIFs of Chl. *a* (4.254) and TP (1.859) were lower than 5. The LM, without the factors and with a VIF of > 5, showed that Chl. *a* was positively related with the eDNA concentration (LM, $p < 0.0001$), while there was no significant relationship between the eDNA concentration and other factors (Fig. 3, Table S1).

Discussion

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

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

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

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

Our result that only Chl. *a* had a significant affect, might suggest that Chl. *a* influences 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 (Barns *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

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319

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324

325 ## References

326 Barnes MA, Turner CR, Jerde CL, Renshaw MA, Chadderton WL, Lodge DM. 2014.

327 Environmental conditions influence eDNA persistence in aquatic systems. *Environmental*

328 *Science & Technology* 48:1819-1827. http://dx.doi.org/10.1021/es404734p.

329 Barnes MA, Turner CR. 2016. The ecology of environmental DNA and implications for

330 conservation genetics. *Cross Mark* 17:1-17. https://doi.org/10.1007/s10592-015-0775-4.

331 Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl

332 MW, Shipley GL, Vandesompele SJ, Wittwer CT. 2009. The MIQE guidelines: minimum

333 information for publication of quantitative real-time PCR experiments. *Clinical Chemistry*

334 55:611-622. https://doi.org/10.1373/clinchem.2008.112797.

335 Davy CM, Kidd AG, Wilson CC. 2015. Development and validation of environmental DNA
336 (eDNA) markers for detection of freshwater turtles. *PLoS ONE* 10:e0130965.
337 https://doi.org/10.1371/journal.pone.0130965.
338 De Souza LS, Godwin JC, Renshaw RA, Larson E. 2016. Environmental DNA (eDNA)
339 detection probability is influenced by seasonal activity of organisms. *PLoS ONE* 11:1-15.
340 https://doi.org/10.1371/journal.pone.0165273.
341 Deiner K, Bik HM, Mächler E, Seymour M, Lacoursière-Roussel A, Altermatt F, Creer S, Bista
342 I, Lodge DM, Vere N, Pfrender ME, Bernatchez L. 2017. Environmental DNA metabarcoding:
343 Transforming how we survey animal and plant communities. *Molecular Ecology* 26:5872-5895.
344 https://doi.org/10.1111/mec.14350.
345 Doi H, Katano I, Sakata Y, Souma R, Kosuge T, Nagano M, Ikeda K, Yano K, Tojo K. 2017b.
346 Detection of an endangered aquatic heteropteran using environmental DNA in a wetland
347 ecosystem. *Royal Society Open Science* 4:170568. https://doi.org/10.1098/rsos.170568.
348 Eichmiller JJ, Miller LM, Sorensen PW. 2016a. Optimizing techniques to capture and extract
349 environmental DNA for detection and quantification of fish. *Molecular Ecology Resources*
350 16:56-68. https://doi.org/10.1111/1755-0998.12421.
351 Eichmiller JJ, Best SE, Sorensen PW. 2016b. Effects of temperature and trophic state on
352 degradation of environmental DNA in lake water. *Environmental Science & Technology*
353 50:1859-1867. http://doi.org/10.1021/acs.est.5b05672.
354 Ficetola GF, Miaud C, Pompanon F, Taberlet P. 2008. Species detection using environmental
355 DNA from water samples. *Biology Letters* 4:423-425. https://doi.org/10.1098/rsbl.2008.0118.

356 Fujiwara A, Matsuhashi S, Doi H, Yamamoto S, Minamoto T. 2016. Use of environmental DNA
357 to survey the distribution of an invasive submerged plant in ponds. *Freshwater Science* 35:748-
358 754. https://doi.org/10.1086/685882.
359 Fukumoto S, Ushimaru A, Minamoto T. 2015. A basin-scale application of environmental DNA
360 assessment for rare endemic species and closely related exotic species in rivers: a case study of
361 giant salamanders in Japan. *Journal of Applied Ecology* 52:358-365.
362 https://doi.org/10.1111/1365-2664.12392.
363 Goldberg CS, Sepulveda A, Ray A, Baumgardt J, Waits LP. 2013. Environmental DNA as a new
364 method for early detection of New Zealand mudsnails (*Potamopyrgus antipodarum*). *Freshwater*
365 *Science* 32:792-800. https://doi.org/10.1899/13-046.1.
366 Hartman LJ, Coyne SR, Norwood DA. 2005. Development of a novel internal positive control
367 for TaqMan® based assays. *Molecular and Cellular Probes* 19:51-59.
368 https://doi.org/10.1016/j.mcp.2004.07.006.
369 Hashizume H, Sato M, Sato MO, Ikeda S, Yoonuan T, Sanguankiat S, Pongvongsa T, Moji K,
370 Minamoto T. 2017. Application of environmental DNA analysis for the detection of
371 *Opisthorchis viverrini* DNA in water samples. *Acta Tropica* 169:1-7.
372 https://doi.org/10.1016/j.actatropica.2017.01.008.
373 Huver JR, Koprivnikar J, Johnson PTJ, Whyard S. 2015. Development and application of an
374 eDNA method to detect and quantify a pathogenic parasite in aquatic ecosystems. *Ecological*
375 *Applications* 25(4):991-1002. https://doi.org/10.1890/14-1530.1.
376 Katano I, Harada K, Doi H, Souma R, Minamoto T. 2017. Environmental DNA method for

377 estimating salamander distribution in headwater streams, and a comparison of water sampling
378 methods. *PLoS ONE* 12(5):e0176541. https://doi.org/10.1371/journal.pone.0176541.
379 Lacoursière-Roussel A, Dubois Y, Normandeau E, Bernatchez L. 2016. Improving
380 herpetological surveys in eastern North America using the environmental DNA method. *Genome*
381 59(11):991-1007. https://doi.org/10.1139/gen-2015-0218.
382 Lawson G. 2017. Development of eDNA techniques for the detection of *Trachemys scripta* in
383 aquatic samples and detection of prey items (*Danio rerio*) in Trachemys scripta fecal samples.
384 Faculty of California State University, Chico. http://hdl.handle.net/10211.3/200130.
385 Lennon JD. 2007. Diversity and metabolism of marine bacteria cultivated on dissolved DNA.
386 *Applied and Environmental Microbiology* 73:2799-2805. http://doi.org/10.1128/AEM.02674-06
387 Lever C. 2003 Naturalized reptiles and amphibians of the world. Oxford: Oxford University
388 Press. pp.17.
389 Mahon AR, Jerde CL, Galaska M, Bergner JL, Chadderton WL, Lodge DM, Hunter ME, Nico
390 LG. 2013. Validation of eDNA surveillance sensitivity for detection of Asian carps in controlled
391 and field experiments. *PLoS ONE* 8:e58316. https://doi.org/10.1371/journal.pone.0058316.
392 Maruyama A, Nakamura K, Yamanaka H, Kondoh M, Minamoto T. 2014. The release rate of
393 environmental DNA from juvenile and adult fish. *PLoS ONE* 9:e114639.
394 Matheson CD, Gurney C, Esau N, Lehto R. 2010. Assessing PCR Inhibition from Humic
395 Substances. *The Open Enzyme Inhibition Journal* 3:38-45.
396 https://doi.org/10.1371/journal.pone.0114639.
397 Minamoto T, Yamanaka H, Takahara T, Honjo MN, Kawabata Z. 2012. Surveillance of fish

398 species composition using environmental DNA. *Limnology* 13:193-197.
399 https://doi.org/10.1007/s10201-011-0362-4.
400 Opel KL, Chung D, McCord BR. 2009. A study of PCR inhibition mechanisms using real time
401 PCR. *Journal of Forensic Sciences* 55:25-33. https://doi.org/10.1111/j.1556-4029.2009.01245.x.
402 Pilliod DS, Goldberg CS, Arkle RS, Waits LP. 2013. Estimating occupancy and abundance of
403 stream amphibians using environmental DNA from filtered water samples. *Canadian Journal of*
404 *Fisheries and Aquatic Sciences* 70:1123-1130. https://doi.org/10.1139/cjfas-2013-0047.
405 Pyšek P, Richardson DM. 2010. Invasive Species, Environmental Change and Management, and
406 Health. *Annual Review of Environment and Resources* 35:25-55.
407 https://doi.org/10.1146/annurev-environ-033009-095548.
408 Ramsay NF, Ng PKA, O'Riordan RM, Chou LM. 2007. The red-eared slider (*Trachemys scripta*
409 *elegans*) in Asia: a review. In: Gherardi F, ed. *Biological invaders in inland waters*: *Profiles,*
410 *distribution, and threats*. Dordrecht, The Netherlands: Springer Verlag. 161-174.
411 https://doi.org/10.1007/978-1-4020-6029-8_8.
412 R Core Team 2018 R: A language and environment for statistical computing, R Foundation for
413 Statistical Computing, Vienna, Austria, https://www.R-project.org/ (2018/05/15).
414 Rees HC, Maddison BC, Middleditch DJ, Patmore JR, Gough KC. 2014. REVIEW: The
415 detection of aquatic animal species using environmental DNA–a review of eDNA as a survey
416 tool in ecology. *Journal of Applied Ecology* 51:1450-1459. https://doi.org/10.1111/1365-
417 2664.12306.
418 Saijo Y. Mitamura O. 1995. *Shinpen Kosyo tyosaho* method in limnological research (in

419 Japanese). Kodansya, Tokyo.

420 Song JW, Small MJ, Casman EA. 2017. Making sense of the noise: The effects of hydrology on

421 silver carp eDNA detection in the Chicago area waterway system. *Science of the Total*

422 *Environment* 605-606:713-720. https://doi.org/10.1016/j.scitotenv.2017.06.255.

423 Sterrett SC, Smith LL, Schweizer SH, Maerz JC. 2010. An assessment of two methods for

424 sampling river turtle assemblages. *Herpetological Conservation and Biology* 5:490-497.

425 Strickler KM, Fremier AK, Goldberg CS. 2014. Quantifying effects of UV-B, temperature, and

426 pH on eDNA degradation in aquatic microcosms. *Biological Conservation* 183:85-92.

427 https://doi.org/10.1016/j.biocon.2014.11.038.

428 Takahara T, Minamoto T, Yamanaka H, Doi H, Kawabata Z. 2012. Estimation of fish biomass

429 using environmental DNA. *PLoS ONE* 7:e35868. https://doi.org/10.1371/journal.pone.0035868.

430 Takahara T, Minamoto T, Doi H. 2013. Using environmental DNA to estimate the distribution of

431 an invasive fish species in ponds. *PLoS ONE* 8:e56584.

432 https://doi.org/10.1371/journal.pone.0056584.

433 Thomsen PF, Kielgast J, Iversen LL, Wiuf C, Rasmussen M, Gilbert MTP. Orland L, Willerslev

434 E. 2012a. Monitoring endangered freshwater biodiversity using environmental DNA. *Molecular*

435 *Ecology* 21:2565-2573. https://doi.org/10.1111/j.1365-294X.2011.05418.x.

436 Thomsen PF, Kielgast J, Iversen LL, Møller PR, Rasmussen M, Willerslev E. 2012b. Detection

437 of a diverse marine fish fauna using environmental DNA from seawater samples. *PLoS ONE*

438 7:e41732. https://doi.org/10.1371/journal.pone.0041732.

439 Thomsen PF, Willerslev E. 2015. Environmental DNA–an emerging tool in conservation for

monitoring past and present biodiversity. *Biological Conservation* 183:4-18. https://doi.org/10.1016/j.biocon.2014.11.019.

Tréguier A, Paillisson JM, Dejean T, Valentini A, Schlaepfer MA, Roussel JM. 2014. Environmental DNA surveillance for invertebrate species: advantages and technical limitations to detect invasive crayfish *Procambarus clarkii* in freshwater ponds. *Journal of Applied Ecology* 51:871-879. https://doi.org/10.1111/1365-2664.12262.

Uchii K, Doi H, Minamoto T. 2016. A novel environmental DNA approach to quantify the cryptic invasion of non-native genotypes, *Molecular Ecology Resources* 16:415-422. https://doi.org/10.1111/1755-0998.12460.

Yamamoto S, Minami K, Fukaya K, Takahashi K, Sawada H, Murakami H, *et al*. 2016. Environmental DNA as a "Snapshot" of fish distribution: a case study of Japanese Jack Mackerel in Maizuru Bay, Sea of Japan. *PLoS ONE* 11:e0149786. https://doi.org/10.1371/journal.pone.0149786.

Yamanaka H, Minamoto T, Matsuura J, Sakurai S, Tsuji S, Motozawa H, Hongo M, Sogo Y, Kakimi N, Teramura I, Sugita M, BabYa M, Kondo A. 2016. A simple method for preserving environmental DNA in water samples at ambient temperature by addition of cationic surfactant. *Limnology* 18:233-241. https://doi.org/10.1007/s10201-016-0508-5.

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 km^2 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

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.

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

Figure 1

Figure 1 Study sites represented by red points.

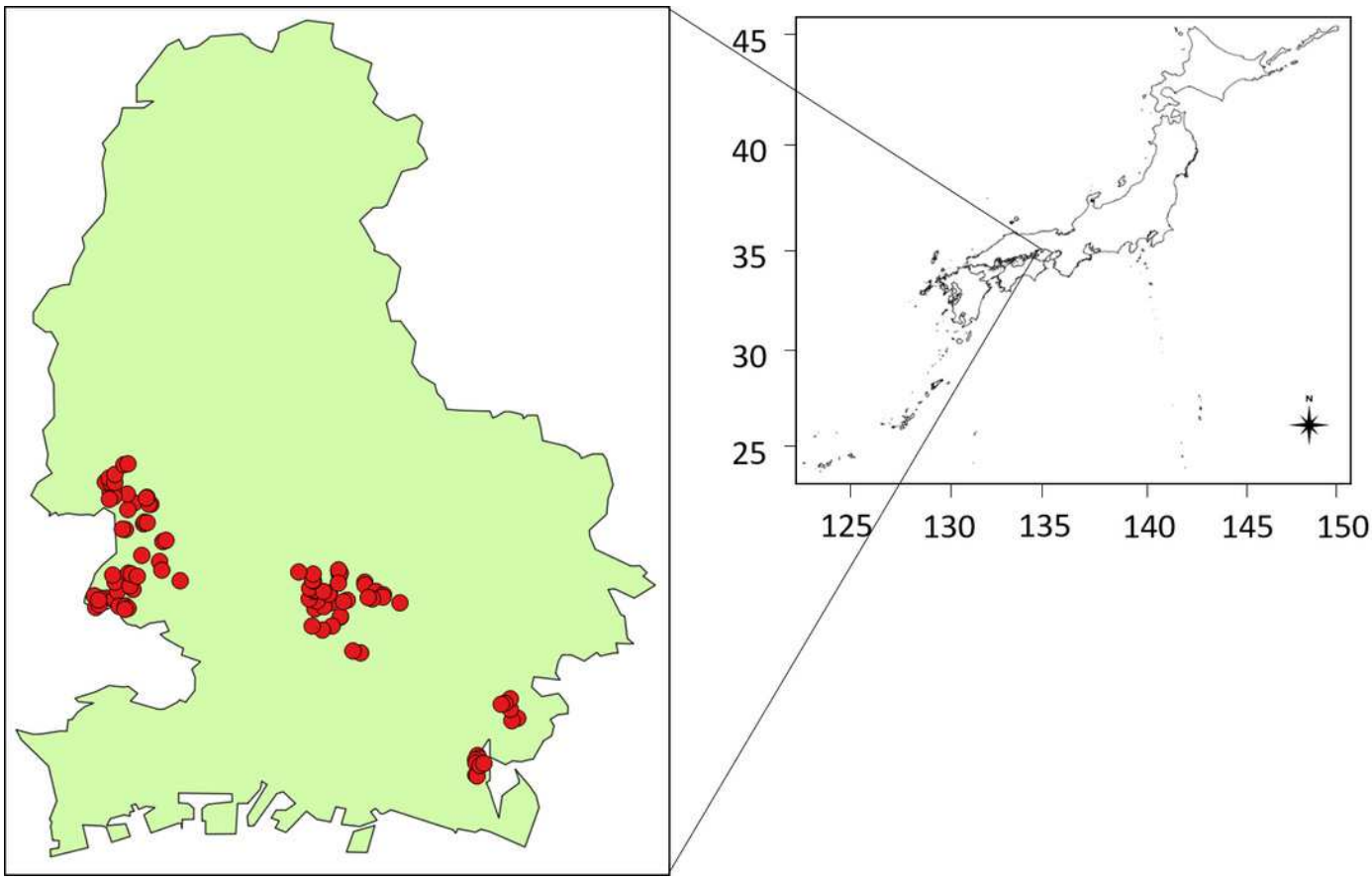

Figure 2

Figure 2 Relationship between the visual observation number of red-eared sliders (*Trachemys scripta elegans*) per km^{2}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.

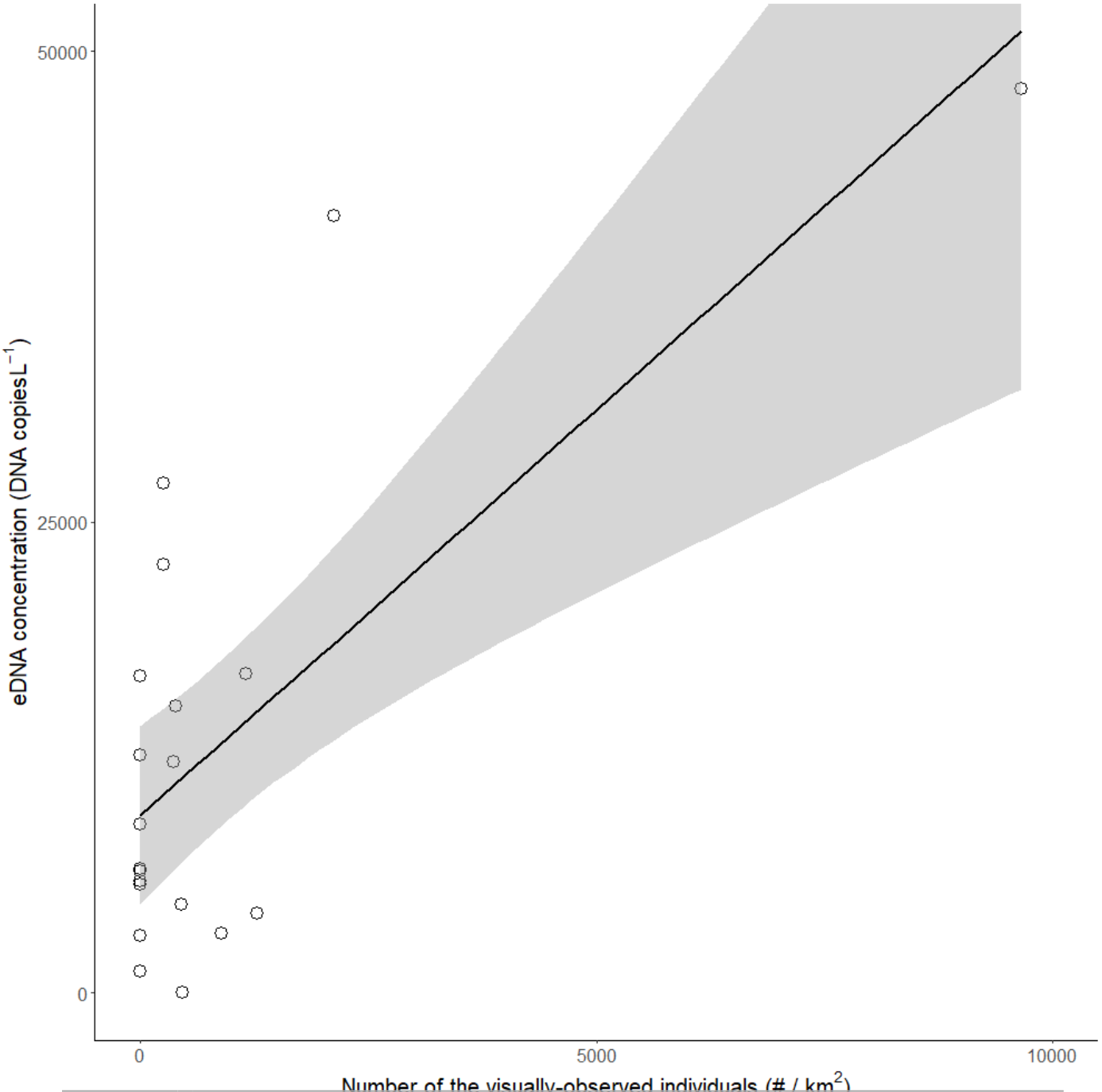

Figure 3

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. *a*was drawn by linear regression with 95% confidence intervals for the slope.

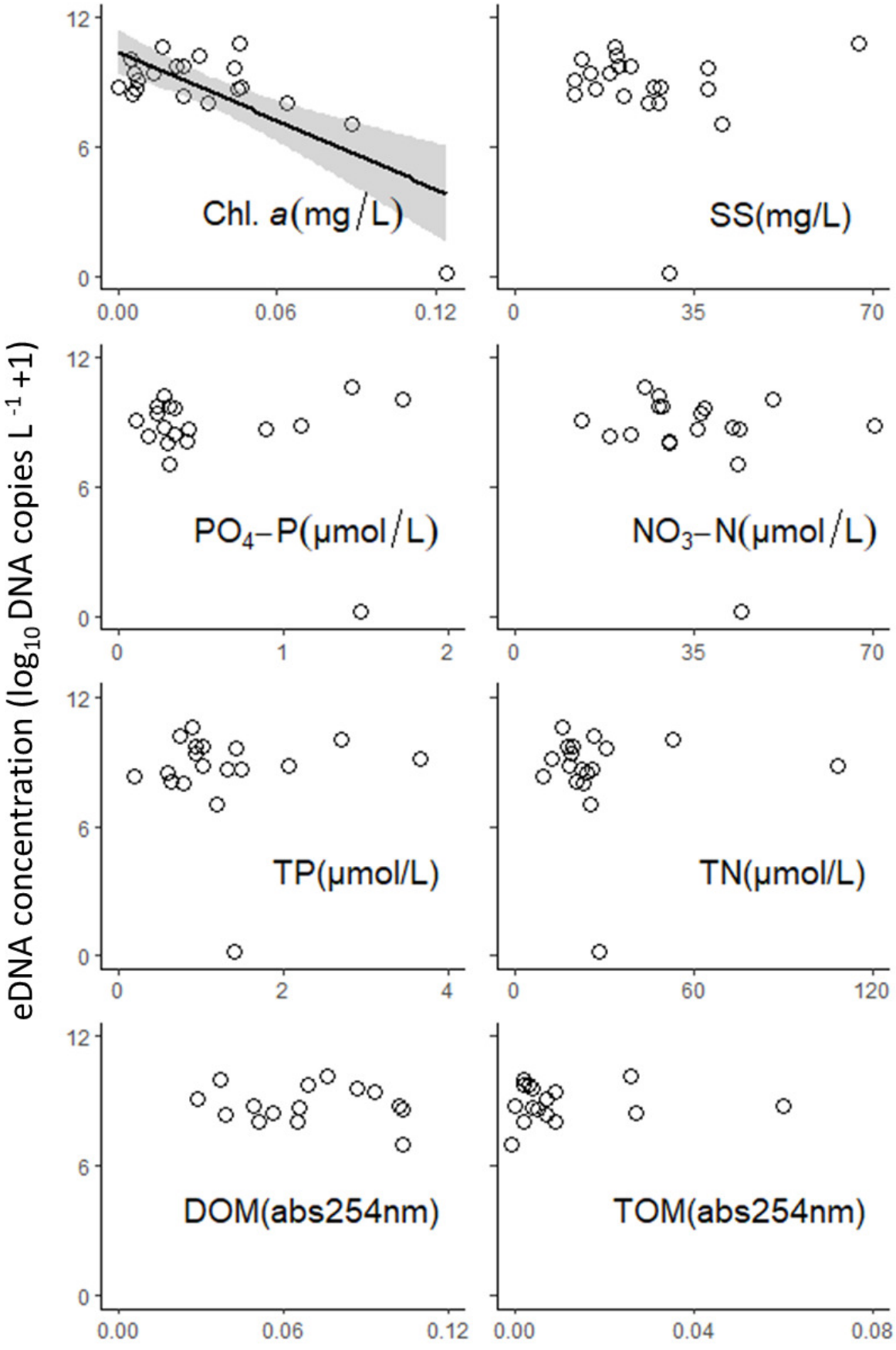

Table 1(on next page)

Table 1 Sampling date, location, the detection ofred-eared slidereDNA,and the number of red-eared slidersvisually 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	Longtitude	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	-	-

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Table 2(on next page)

Table 1 Continued. Sampling date, location, the detection ofred-eared slidereDNA,and the number of red-eared slidersvisually 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	Longtitude	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	-	-

1

Table 3(on next page)

Table 2 a) Linear regression slopes with a ± 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	*t* value	*p* value
Chl. *a*	-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
F value	13.89
p value	0.000
R^2	0.649
Adjusted R^2	0.603

1