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Application of environmental DNA metabarcoding in a lake with extensive algal blooms

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Abstract

Recently, environmental DNA (eDNA) metabarcoding techniques have been applied to biodiversity investigations in aquatic ecosystems. However, no study has yet tested whether this technique is effective for water bodies in which extensive algal blooms break out. In this study, fish DNA metabarcoding was carried out in Lake Taihu, which experiences extensive algal blooms, to confirm whether the technique is also effective for fish diversity research in ecosystems with frequent and extensive blooms. In December 2016, three samples were collected, including one collected in the presence of algal blooms and two collected in the absence of algal blooms. In August 2017, six samples were collected, including three collected in the presence of algal blooms and three in the absence of algal blooms. Equal amount of water samples (1 L) were collected from each site; however, the actual amount of filtrate varied with the site. Twenty-seven freshwater fish species were detected from the water samples collected in Lake Taihu. The results showed that the composition of the detected species did not differ whether or not blooms were present. However, the amount of filtration could influence the number of species detected. The results suggest that future eDNA

metabarcoding studies under similar water environments should increase the amount of filtration to maximize number of species detected.

Keywords: algal blooms, environmental metabarcoding analysis, fish species composition, Lake Taihu

Introduction

Global biodiversity is decreasing at an unprecedented rate, far beyond people's imagination (Dirzo and Raven 2003). Human activity is one of the main causes of biodiversity loss, which directly threatens natural ecosystems (Butchart et al. 2010). Freshwater vertebrate species populations are declining at twice the rate of decline of land or ocean vertebrates (Grooten and Almond 2018). Among the 29,500 freshwater dependent species so far assessed in the IUCN Red List, 27% are currently at risk of extinction (Tickner et al. 2020). Among aquatic organisms, fish species play an important role in ecosystem linkages as well as commercial fisheries, yet many fish species are in danger of extinction. In fact, in China, approximately 15% of freshwater fish species are listed as endangered or vulnerable (199 out of the 1323 species registered in freshwater systems) (Xing et al. 2016). Therefore, accurate assessment of fish communities is key to maintaining freshwater fish diversity and freshwater ecosystem protection, and it provides essential information for biological protection (Kwak and Peterson 2007).

53 In recent years, environmental DNA (eDNA) analysis and its usage have received
54 widespread attention. Environmental DNA represents all types of DNA found in the
55 environment, including DNA from organisms in the form of metabolic waste, damaged
56 tissue, or sloughed skin cells (Kelly et al. 2014; Barnes and Turner 2016). A method
57 that retrieves DNA from environmental samples has been used to explore aquatic
58 organisms in conservation and ecological studies (Pompanon et al. 2012; Bohmann et
59 al. 2014; Thomsen and Willerslev 2015; Valentini et al. 20016). Two different
60 approaches can be applied. One approach is eDNA analysis, which can detect a single
61 species by using species-specific primers (Sean et al. 2019; Sakata et al. 2017; Wu et al.
62 2018, 2019; Fujiwara et al. 2016), while the other approach promotes diversity
63 assessments of organisms using universal primers, called eDNA metabarcoding (Miya
64 et al. 2015; Komai et al. 2019). The experimental procedure for eDNA metabarcoding
65 includes collecting water samples and PCR amplification of the eDNA from fish species
66 using universal primers (Miya et al. 2015; Deiner et al. 2016). The species composition
67 was estimated by sequencing the PCR amplicons through next-generation sequencing
68 (NGS). This technique is rapid and non-invasive method that is also cost-effective

(Deiner et al. 2017; Yamamoto et al. 2017). In freshwater fish species, eDNA metabarcoding reveals comparable or higher number of fish species, compared to that from traditional surveys (Hänfling et al. 2016; Shaw et al. 2016; Nakagawa et al. 2018). In recent years, eDNA metabarcoding is used increasingly for characterizing the species composition of ecological communities (Evans et al. 2016; Sato et al. 2017). Therefore, we believe it would be suitable for studying fish biodiversity and composition in lakes with serious eutrophication.

Eutrophication in reservoirs, estuaries, rivers, and lakes is widespread all over the world, and the severity is increasing, especially in developing countries such as China (Yang et al. 2008). When the nitrogen and phosphorus concentrations in a water body reach certain levels, algal blooms can easily occur (Carpenter 2008; Vadeboncoeur, Lodge and Carpenter 2001). Algal blooms can clog the gills of aquatic organisms (Smith and Schindler 2009), and aquatic organisms such as fish and bottom-dwelling animals cannot survive in oxygen-deprived aquatic environments as those in algal blooms (Ansari, Gill and Khan 2010). Researchers studying such aquatic environments have largely performed biodiversity surveys using traditional methods, and the

85 application of eDNA technology has emerged in recent years (Cai et al. 2017; Zhang et
86 al. 2018). In the wake of increased environmental degradation and biodiversity loss,
87 more biodiversity surveys will be required in eutrophic aquatic ecosystems with algal
88 blooms to assess aquatic ecosystem health and the impact of the blooms on the
89 ecosystems. Environmental DNA technology provides information on the presence or
90 absence of organisms based on the genetic information obtained from environmental
91 water samples. Most studies that apply metabarcoding techniques for eDNA analysis
92 have been conducted in oligotrophic or mesotrophic waters. To date, only Handley et al.
93 (2019) has applied the metabarcoding technique to investigate fish composition in
94 eutrophic water. It has been reported that species that were sensitive to the trophic
95 conditions of their habitat were distributed in areas with corresponding trophic
96 conditions, while those that were not were widely distributed regardless of the trophic
97 conditions (Handley et al. 2019). Eutrophic aquatic environments are vulnerable to
98 massive algal blooms, and algae clog filter papers, reducing filtration capacity and
99 organism detection rates in eDNA surveys. Nevertheless, very few studies have been
100 conducted to evaluate the potential application of eDNA technology in aquatic

environments with extensive algal blooms on the surfaces. The present study was conducted in Lake Taihu, a representative highly eutrophic lake with extensive algal blooms, and the results of the study could reveal the feasibility of the survey approach in such environments.

This study aimed to determine whether the eDNA metabarcoding analysis method could be applied to a highly eutrophicated lake by analyzing the effect of algal blooms. As a test area, we selected Lake Taihu, China, a highly eutrophicated lake with frequent extensive algal blooms, where fish are an important aquatic resource. We compared the number of detected species and the differences in species composition with the presence or absence of algal blooms. Finally, species detection using eDNA metabarcoding was compared with the traditional methods and the use of the illustrated book for the fish of Lake Taihu.

Materials and Methods

Study site

116 The study area of Lake Taihu is located in the Yangtze River Delta in eastern China,
117 with a surface area of 2,338 km^2 and an average depth of 1.89 m (Sun and Huang 1993).
118 In the 1980s, there were between 72 and 106 different species of fish in the lake (Zhu et
119 al. 2007). During the 2000s, massive blue-green algal blooms broke out in Lake Taihu
120 (Wang and Shi 2008; Zhu 2008). These led to massive mortalities of fish and shrimp
121 and triggered serious drinking water crises (Huang et al. 2014). In addition, due to the
122 multiple impacts of overfishing and degradation of aquatic vegetation, significant
123 changes took place in the fish community structure of the lake, so that now there are
124 only 40 to 50 fish species in Lake Taihu (Zhu 2004).
125 Water samples were collected during summer and winter in the north of Lake
126 Taihu. We surveyed three sites on December 21, 2016 (Fig. 1, blue circle), and six sites
127 on August 27–28, 2017 (Fig. 1, red circle). The different sampling sites were selected as
128 there were relatively few sites in winter with algal blooms present. Consequently,
129 sampling was carried out across four sites with algal blooms and five sites with no algal
130 blooms. We observed that algal blooms appeared in three sampling sites in summer and
131 one site in winter (Table 1). At site T7, we conducted a survey in both summer (referred

as T7-S) and winter (T7-W). Two bottles of 500 mL water were collected at each site, and 500 µL of 10% (mass/volume) benzalkonium chloride solution was added to each sample to prevent DNA degradation (Yamanaka et al. 2017). We transported the water samples at ambient temperature and filtered them in the laboratory at Shanghai Jiao Tong University.

eDNA filtration and extraction

The water samples were filtered using 47-mm glass-fiber filters (GF/F; GE Healthcare Japan, Tokyo, Japan; nominal pore size = 0.7 µm; cf. Minamoto et al. (2016) for selection of filter type for eDNA sampling). In the present study, we unified the filtration labor, and filtration would continue until it could not be further carried out. The amount of sample filtration varied for each water sample because of filter clogging (Table 1). After filtering the samples, we filtered 1 L of ultrapure water to check for unintended cross-contamination in the laboratory (i.e., filtration blank). To prevent contamination, the equipment was cleaned with a bleach solution (diluted household bleach containing 0.1% sodium hypochlorite) for >5 min before reuse to remove

148 residual DNA. After filtration, the filters were stored at – 20 °C until DNA extraction.

149 Total eDNA was extracted from the filter using a DNeasy Blood and Tissue Kit

150 (Qiagen, Hilden, Germany) following Minamoto et al. (2019). After DNA extraction,

151 DNA was eluted from the DNeasy spin column with 100 μL of Buffer AE (Qiagen) and

152 stored at – 20 °C until metabarcoding analysis.

153

154 **Amplicon library preparation and MiSeq sequencing**

155 Amplicon libraries for fish eDNA metabarcoding were prepared using MiFish-U

156 primers (Miya et al. 2015). The first-round polymerase chain reaction (1st PCR) was

157 performed in a total reaction volume of 12 μL, containing 6.0 μL 2 × KAPA HiFi

158 HotStart ReadyMix (KAPA Biosystems, Wilmington, MA, USA), 3.6 pmol each of

159 MiFish-U primers, 1 μL eDNA template, and ultrapure water. The thermal cycle profile

160 was 95 °C for 3 min; 40 cycles of 98 °C for 20 s, 65 °C for 15 s, and 72 °C for 15 s; and

161 72 °C for 5 min. A polymerase chain reaction was performed with four repetitions for

162 each DNA sample, to reduce potential bias arising through a stochastic variation during

163 the PCR step. Ultrapure water was used instead of eDNA in four reaction mixtures

(non-template negative controls). After four technical replicates of 1st PCR products were pooled into a single tube, unreacted reagents and primer dimers were removed from the 1st PCR products with the SPRI select Reagent Kit (Beckman Coulter, Brea, CA, USA) according to the manufacturer's instructions. The purified 1st PCR product concentration of each sample was measured by Qubit fluorometer 3.0 (Thermo Fisher Scientific, Waltham, MA, USA) using Qubit dsDNA HS assay kit. Then, all samples were diluted to 0.1 ng/μL, and all negative controls were diluted using an average dilution ratio.

The second-round PCR (2nd PCR) was performed to add MiSeq adapter sequences and 8-bp index sequences to both amplicon ends (Hamady et al. 2008). The total reaction volume of the 2nd PCR was also 12 μL, containing 6.0 μL of 2 × KAPA HiFi HotStart ReadyMix (KAPA Biosystems), 3.6 pmol each of forward and reverse primers, 1 μL template, and ultrapure water. The thermal cycle profile for the 2nd PCR was 95 °C for 3 min; 12 cycles of 98 °C for 20 s, 72 °C for 20 s, and 72 °C for 5 min. The 2nd PCR products were pooled in one tube (i.e., one pooled 2nd PCR products that included all samples). The library sample of target size amplicons was obtained by

electrophoresis using E-Gel SizeSelect 2% (ThermoFisher Scientific) with the E-Gel Precast Agarose Electrophoresis System (ThermoFisher Scientific). Subsequently, it was confirmed whether only DNA of the target length (approximately 370 bp) was isolated by using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). The concentration of the DNA library was adjusted to 4 nM. Finally, the library was sequenced using an Illumina MiSeq v2 Reagent kit for 2 × 150 bp PE (Illumina, San Diego, CA, USA).

Sequence data analysis

The row reads obtained from Miseq sequencing were preprocessed and analyzed using USEARCH v10.0.240 (Edgar 2010). First, R1 and R2 reads were assembled using the command "-fastq_mergepairs". Second, the primer sequence was removed from both edges of the assembled reads by using commands "-fastx_truncate", "-stripleft" and "-stripright". Third, low quality reads with an expected error rate (Edgar and Flyvbjerg 2015) of >1% and too short reads of <140 bp were removed by quality filtering (using command "-fastq_filter"). Fourth, the preprocessed reads were dereplicated using the

196 “fastx_uniques” command. Fifth, the dereplicated reads were denoised using the
197 “unoise3” command to generate amplicon sequence variants (ASVs) that remove all
198 putatively chimeric, erroneous sequences (Edgar 2016) and partial ASVs with fewer
199 than 10 reads. Finally, ASVs were subjected to taxonomic assignments to species names
200 using the “BLAST” search program to nucleotide database using a nucleotide query
201 (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSear
202 ch&LINK_LOC=blasthome). In the next step, the search was performed with default
203 settings, and ASVs of sequence identity >98.5% (two nucleotide differences allowed)
204 were judged as that species or was judged a species of that genus in the case of no
205 distinction at the species level. When ASVs of sequence identity of <98.5% were
206 assigned to top hit species, and when the sequence identity was >90% to reference
207 sequences, ASVs were classified as belonging to the genus or family level. In addition,
208 the detected reads in the negative control had possibly been contaminated, and the
209 number of *Carassius spp*. reads detected in the filtration blank of 2016 was removed
210 from reads of all corresponding environmental samples from 2016 (Port et al. 2016;
211 Hayami et al. 2020).

212

213 **Statistical analysis**

214 All analyses were performed using R ver. 3.6.0 (R Development Core Team 2019), the
215 vegan package version 2.5-6 (Oksanen et al. 2019), and the lme4 package version 1.1-
216 21 (Bates et al. 2015). The result of the species accumulation curve generated by
217 "rarecurve" function in the vegan package shows that the number of detected species in
218 each sample is saturated, except for the sample T2. In all analyses, the reads data were
219 converted to presence/absence (Jeunen et al. 2019; Fig. S1).

220 A rarefaction analysis was performed to account for the variations in the number
221 of total reads across samples (Fig S2). The following two analyses were performed
222 individually for un-rarefied and rarefied data, which was obtained by unifying the
223 number of total reads to the fewest reads through rarefying, using the R package
224 "vegan" version 2.5-6. To estimate whether algal blooms (presence/absence) affect the
225 number of fish species detected by eDNA metabarcoding, we used a generalized linear
226 mixed model (GLMM) with a poisson distribution. In the model, we assigned the
227 absence of algal blooms = 0 and the presence of algal blooms = 1 and set the

228 presence/absence of algal blooms as explanatory variables, the number of fish species
229 detected by eDNA metabarcoding as a response variable, filtration volume as an offset
230 term, and site IDs as random effects. Regarding the sampling sites in the present
231 analysis, some studies have reported that the fish community in Lake Taihu is evenly
232 distributed throughout the entire lake since being affected by severe environmental
233 change and overfishing (Tao et al. 2010; Mao et al. 2011). Therefore, we assumed that
234 there was no bias in potential fish communities based on sampling location or season,
235 and the site IDs was randomized in the analysis. Non-metric multidimensional scaling
236 (NMDS) was then performed with "Jaccard methods" and 10,000 permutations to
237 visualize the dissimilarity of fish composition by the presence/absence of algal blooms.
238 In addition, to compare fish composition between the presence and absence of algal
239 blooms, we performed PERMANOVA by function "adonis" in the vegan package. In
240 this analysis, all the data were used to conduct community analysis, because similar
241 result was obtained even at site T2, where only one ASV was detected, was removed
242 (Fig. S3). Two mammals (*Homo sapiens* and *Sus scrofa*) were detected in the eDNA

243 metabarcoding analysis. Mammals were excluded from the statistical analysis. Parts of
244 graphs were drawn using the ggplot2 package version 3.1.1 (Wickham 2019).
245 Finally, the fish composition obtained from eDNA and the latest traditional
246 survey were compared (eDNA survey: this study, traditional survey and the illustrated
247 handbook for the fish of Lake Taihu: Mao et al. 2011; Li and Shimatani 2016).
248
249 **Results**
250 In total, 1,207,279 MiSeq reads were obtained. After bioinformatic filtering (see
251 Materials and Methods), 465,347 reads were retained, corresponding to 38.55% of the
252 total reads. From the filtration blank in 2016, 55 reads of *Carassius spp.* were detected,
253 while no fish DNA was detected in the filtration blank in 2017. In total, 28 freshwater
254 fish species were detected (Table S1).
255 The results for the un-rarefied data showed that the number of detected fish
256 species was significantly lower in the presence of algal blooms (GLMM, $p = 0.016$; Fig.
257 2). In addition, there was no significant difference in the composition of detected
258 species between the presence/absence of algal blooms ($p = 0.969$; Fig. 3). The results

259 for rarefied data indicated the significantly lower number of detected species in the
260 presence of algal blooms (GLMM, p= 0.014; Fig. S4) and non-significant difference in
261 the composition of species (p=0.514; Fig. S5). Therefore, rarefaction did not alter the
262 results. Additionally, among the 27 freshwater fish species detected by eDNA
263 metabarcoding analysis, 10 species appeared in the traditional survey, and 13 species
264 appeared in the illustrated handbook for the fish of Lake Taihu (Li and Shimatani 2016)
265 (Fig. 4). According to the species accumulation curve, the number of detected species
266 did not reach a saturation by metabarcoding, after sampling nine sites (Fig. S6).
267
268 **Discussion**
269 This study attempted to assess the fish diversity in a severely eutrophicated lake with
270 extensive algal blooms using eDNA metabarcoding. Our results showed that fish
271 species could be detected even in eutrophicated areas where extensive algal blooms
272 break out; however, in such an environment, besides the existence of algae blooms,
273 other factors (e.g., filtration volume and PCR inhibitors) could affect the number of

274 species detected. In future research, how the aforementioned factors influence the
275 number of fish species detected should be explored.
276 Although the composition of detected species did not differ by the presence or
277 absence of algal blooms, the number of detected species showed significant differences
278 (Fig. 2, 3). There could be two reasons why the composition of the detected species was
279 not affected. First, since the late 1980s, serious eutrophication has been occurring in
280 Taihu Lake, and fish species sensitive to oxygen-poor environments have been
281 significantly reduced, while those adapted to oxygen-poor environments have survived
282 (Zhu, 2004; Mao et al. 2011). It has also been reported that some fish, such as *Coilia*
283 and *Carassius*, can survive by changing their diet (e.g., consume cyanobacteria) (Liu,
284 2009). As a result, only fish species with strong adaptability to oxygen-poor conditions
285 may remain in such an unfavorable environment. Therefore, the presence or absence of
286 algal blooms may not influence species composition in the lake. In the present study,
287 the filtration volume of the water samples at sites without algal blooms was
288 significantly higher than at sites with algal blooms (Table 1; Fig. S7). When
289 investigating fish diversity in water bodies with extensive and frequent algal blooms

290 using eDNA, algae could clog filters, and, in turn, directly influence filtration yield and
291 indirectly influence DNA recovery. Consequently, the number of species detected could
292 be relatively low, and saturation points may not be achieved in study sites. The obtained
293 reads, classified as fish sequences after filtering, were compared; lower reads were
294 obtained for the sites with algal blooms (Table S1). This might be attributed to the
295 limitations in the PCR amplification, such as PCR inhibition in the samples from sites
296 with algal blooms. Although previous studies have shown that sample dilution could
297 circumvent the effects of inhibition (Biggs et al. 2015; Cilleros et al. 2019), no dilution
298 was performed in the present study because the amount of water that could be filtered
299 was very small and fish DNA concentrations in the samples were assumed to be very
300 low. Previous studies have reported positive relationships between the number of
301 detected species and filtered water volume (Mächler et al. 2016; Sakata et al. 2020).
302 Consequently, increasing the amount of filtration is key for improving species detection
303 rates. This is the first investigation in the water environment rich in algae, and the
304 filtration has been performed according to the standard experimental methods stipulated
305 before. In future studies, multiple filter papers, filter with a larger pore size, or pre-

306 filtration to treat water containing more particles are required. Alternatively, the
307 subsurface water under algal blooms could also be collected as opposed to the surface
308 water covered by algal blooms; this could improve the efficiency of eDNA
309 metabarcoding in eutrophic environments. Meanwhile, some studies have demonstrated
310 DNA in sediment has higher concentrations and can be conserved for longer periods
311 than DNA in water (Corinaldesi et al. 2011; Sakata et al. 2021). Therefore, collecting
312 both water samples and sediment samples could enhance detection capacity.

313 MiFish marker is generally useful for identifying fish species in Lake Taihu. A
314 total of 104 fish species were found in Lake Taihu using traditional methods and an
315 illustrated fish handbook in Taihu Lake (Mao et al. 2011; Li and Shimatani 2016).
316 Excluding 17 species that had no reference data, the rest of the 87 species had reference
317 sequences; however, some species were indistinguishable because the sequences in the
318 marker regions were identical among species, so that some species were not easily
319 identifiable in our data (Table S1). Some DNA sequences were not successfully
320 identified because of high similarity within the genus, resulting in the genus-level
321 classification (i.e., *Carassius*, *Hypophthalmichthys*). The similarity of DNA sequences

322 of cyprinid fish is high within and between genus, which leads to the subfamily level
323 classification (i.e., *Acanthohodeus* and *Cultrinae*). We cannot rule out the possibility
324 that the deposited sequence on NCBI may be wrong or caused by incorrect species
325 identification. Therefore, in future research, it will be essential to establish a perfectly
326 correct database. At the same time, we also found that the sequences of many fishes
327 living in Lake Taihu are very close and cannot be fully identified by using 12S rRNA
328 fragments. It is also suggested that a longer marker for metabarcoding or specific
329 primers for a certain genus is required to solve this identification problem.
330 Environmental DNA metabarcoding analysis has been applied to biomonitoring
331 and has been used to investigate the effect of external factors such as water quality. Our
332 results indicate that the presence of algal blooms did not affect the community structure
333 detected by eDNA metabarcoding, but the number of fish species. A body of essential
334 information of this nature will assist in designing eDNA metabarcoding biomonitoring
335 programs and their application in complex environments, contributing to aquatic
336 biodiversity conservation.
337

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Table 1. Filtration volume of each water sample. + means that we observed the presence of algal blooms at the site and – means that we did not.

Season	Date	Site	Filtration volume	Algal blooms
Winter	Dec-21, 2016	T5	400ml	-
	Dec-21, 2016	T7-W	400ml	-
	Dec-21, 2016	T8	100mL	+
Summer	Aug-27, 2017	T1	350mL	+
	Aug-27, 2017	T2	155mL	+
	Aug-28, 2017	T3	1000mL	-
	Aug-28, 2017	T4	22.5mL	+
	Aug-28, 2017	T6	1000mL	-
	Aug-28, 2017	T7-S	560mL	-

538 **Figure legends**

539 **Figure 1.**

540 Map of the study sites. Green squares mean that we observed the presence of algal blooms at the
541 site. Red and blue circles show the sampling sites in summer and winter, respectively. A red-edged
542 white circle means no fish ASVs have been detected. Circle size indicates the number of detected
543 species. With regard to T7, we conducted surveys in both summer (T7-S) and winter (T7-W).

544

545 **Figure 2.**

546 Number of fish species detected in the presence/absence of algal blooms by environmental DNA
547 metabarcoding. The GLMM result shows that number of species is significant higher when algal
548 blooms are absent than when they are present.

549

550 **Figure 3.**

551 Composition of detected fish species in the presence/absence of algal blooms. Circles and grey solid
552 triangle represent the samples with algal blooms (N = 4), crosses and white dotted triangle represent
553 the samples without algal blooms (N = 5). No fish species was detected from the sample of T7-S
554 without algal blooms, and therefore, only four points for the latter were plotted.

555

556 **Figure 4.**

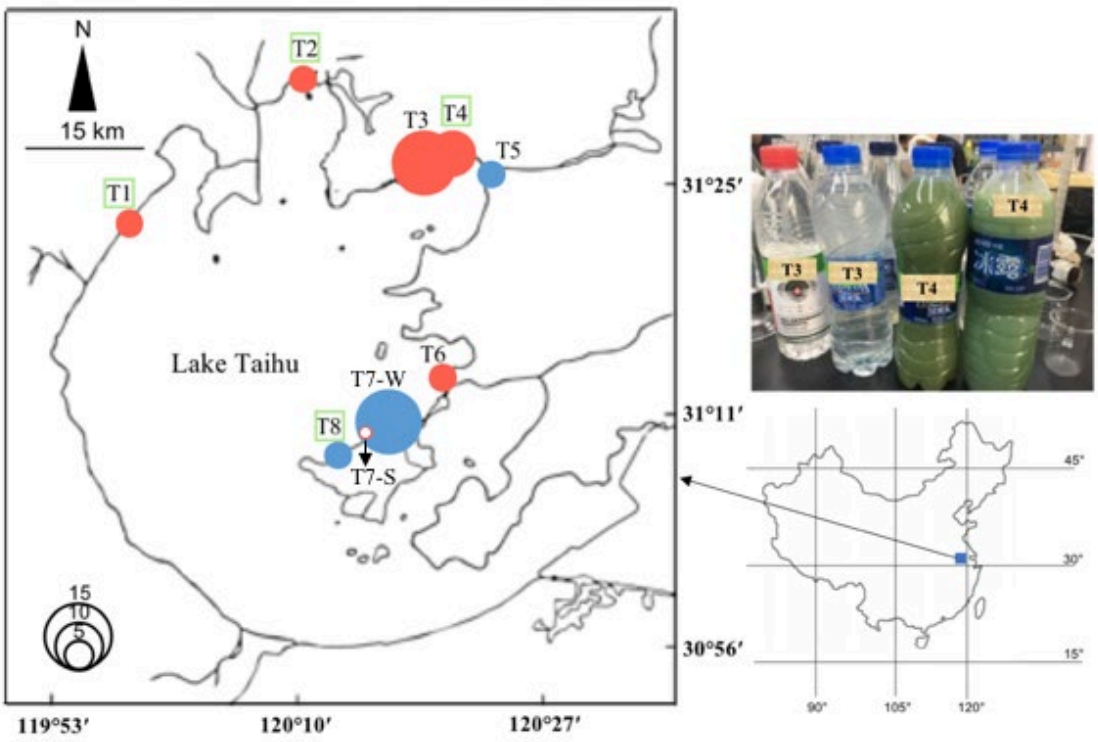
557 Comparison of the results of detected fish in Lake Taihu confirmed by traditional methods and by
558 the environmental DNA (eDNA) method. The upper-left circle represents the result from eDNA,
559 upper-right represents the fish caught by trawl net (Mao et al. 2011), and the bottom shows the fish
560 species in the illustrated handbook of Lake Taihu (Li and Shimatani 2016).

561

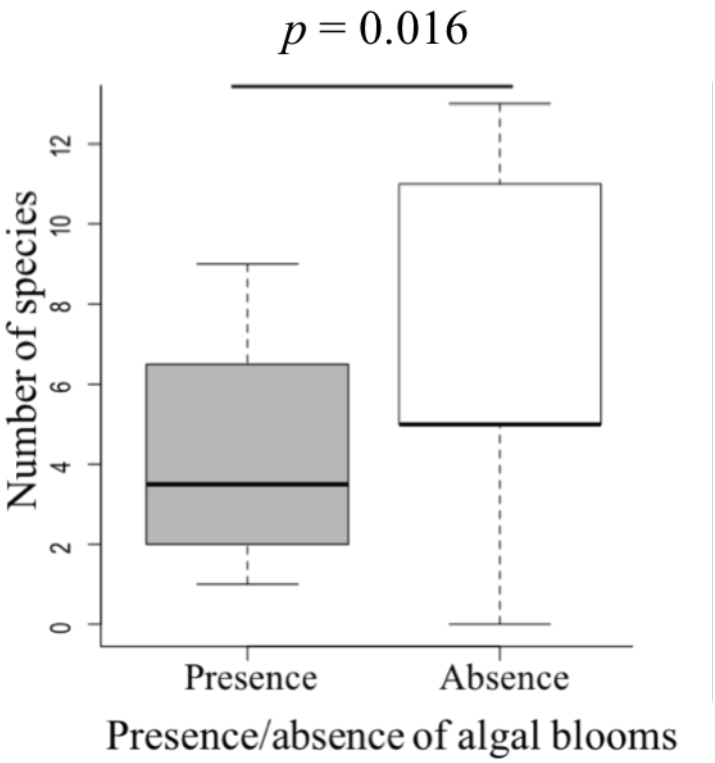
562

Figures

Figure 1.



566 **Figure 2.**



567

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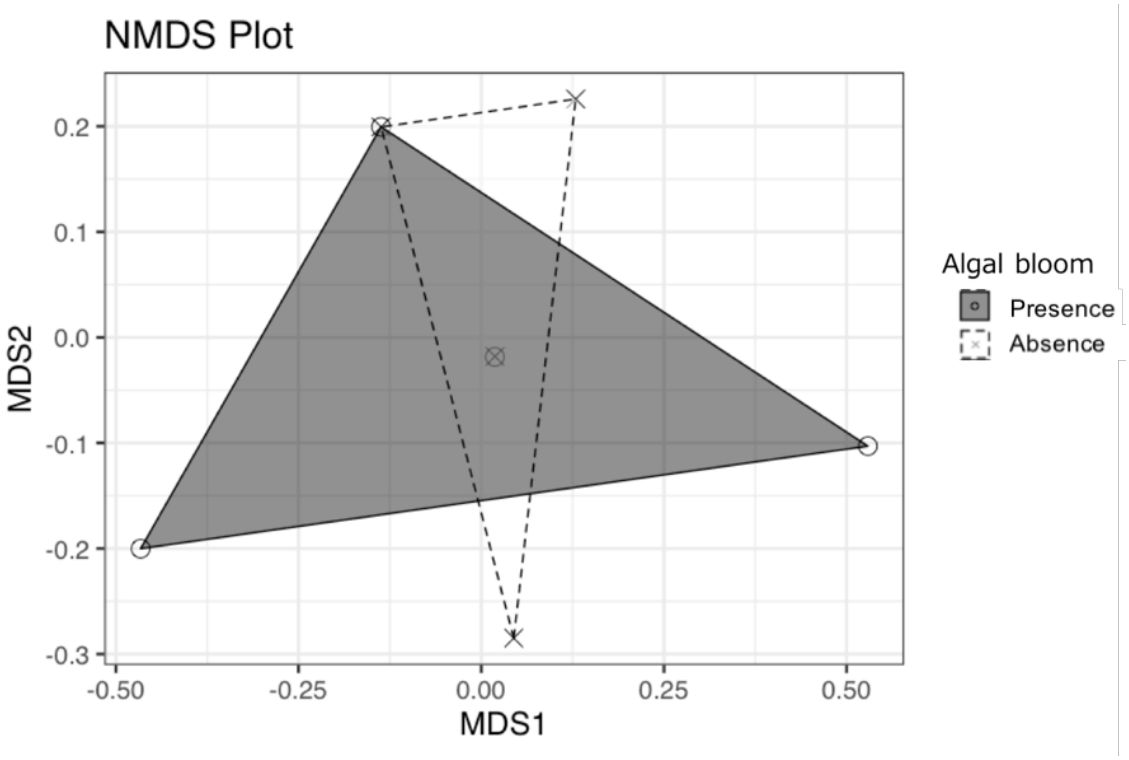
569 **Figure 3.**



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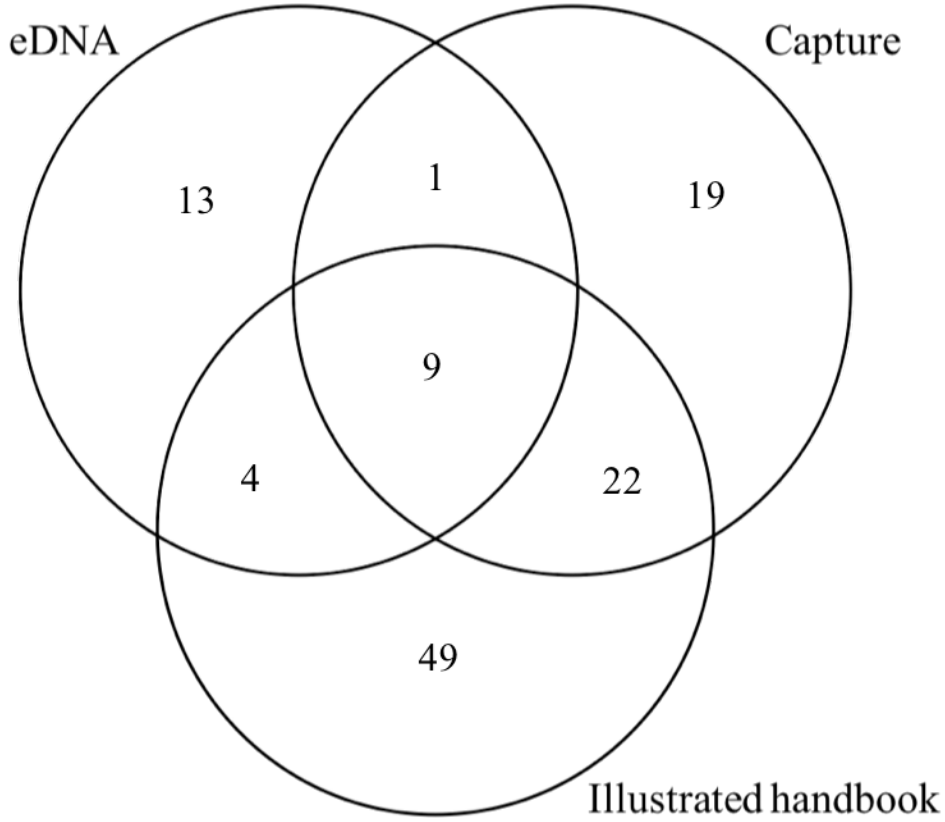
571 **Figure 4.**



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