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# Monitoring of multiple fish species by quantitative environmental DNA metabarcoding surveys over two summer seasons

Running title: Quantitative eDNA metabarcoding of fish

Luhan WU[1*], Tomonori OSUGI[2], Takashi INAGAWA[3], Jiro OKITSU[3], Shogo SAKAMOTO[3], Toshifumi MINAMOTO[1]

[1] Graduate School of Human Development and Environment, Kobe University: 3-11, Tsurukabuto, Nada-ku, Kobe City, Hyogo 657-8501, Japan

[2] Water Resources Environment Center: 2-14-2, Kouji-machi, Chiyoda-ku, Tokyo 102-0083, Japan

[3] OYO Corporation: 275, Nishikata-Ishibatake, Miharu Town, Fukushima 963-7722, Japan

* Corresponding author

Luhan WU

Graduate School of Human Development and Environment, Kobe University: 3-11, Tsurukabuto, Nada-ku, Kobe City, Hyogo 657-8501, Japan

wuluhan12358@outlook.com

+81-78-803-7991 (Tel/Fax)

**Abstract**

Periodic monitoring can provide important information for the protection of endangered fish, sustainable use of fishery resources, and management of alien species. Previous studies have attempted to monitor fish using non-invasive environmental DNA (eDNA) technology, generally employing quantitative PCR (qPCR) to quantify the eDNA concentration. However, the throughput was limited. High-throughput metabarcoding technology can detect the DNA of multiple species simultaneously in a single experiment but does not provide sufficient quantification. In this study, we applied a quantitative metabarcoding approach to simultaneously quantify the eDNA concentration of an entire fish assemblage in a small reservoir over two summer seasons. Traditional surveys were also conducted to investigate the individuals of fish. The eDNA concentrations were quantified using quantitative metabarcoding, and the fish species detected using this approach were highly consistent with the results of traditional fish monitoring. A significant positive relationship was observed between the eDNA concentration and fish species abundance. Seasonal changes in fish community structure were estimated using eDNA concentrations, which may reveal the activity seasons of different fish. The eDNA concentrations of different fish species peaked at different water temperatures, reflecting the differential responses of fish species to this environmental factor. Finally, by detecting outlier eDNA concentrations, the spawning activities of 13 fish species were estimated, 12 of which were roughly consistent with the current knowledge of fish spawning periods. These results indicate that quantitative eDNA metabarcoding with dozens of sampling times is useful for the simultaneous ecological monitoring of multiple fish species.

43 **Key-words**: environmental DNA (eDNA), MiFish, quantitative metabarcoding, spawning,
44 reservoir

## Introduction

Ecological monitoring of changes in fish community structure, fish distribution associated with environmental variables, and the time and location of spawning activities can provide important information for the protection of endangered fish (Chollett et al., 2020), sustainable use of fishery resources (King et al., 2009; Erisman et al., 2017), and management of alien species (Jackson et al., 2004). Traditional methods involved ecological surveys of fish include visual inspection and electrofishing (Copp and Peňáz, 1988). However, visual surveys are time-consuming and generally inefficient (Rowland, 1999), whereas electrofishing can harm fish and may interfere with their natural spawning activities (Snyder, 2003). A non-invasive method that can efficiently monitor fish spawning activities would be a valuable tool for the management of aquatic biodiversity.

Environmental DNA (eDNA) encompasses the DNA of all organisms present in environmental samples, including microbial, meiofaunal, and macrobial taxa (Rodriguez-Ezpeleta et al., 2021). The eDNA technique is a non-invasive method that is widely used in ecological surveys. The technology involved only requires the collection of DNA in water for analysis; therefore, it has the advantage of greatly reducing the cost and labor required for field investigations while avoiding damage to the environment and interference with the natural activities of organisms (Thomsen and Willerslev, 2015) and may be a powerful tool for resource managers for long-term resource monitoring (Mize et al., 2019). The eDNA technique has been commonly used to monitor fish (Jerde et al., 2011; Takahara et al., 2013), amphibians (Ficetola et al., 2008; Pilliod et al., 2013), crustaceans (Tréguier et al., 2014; Wu et al., 2018), reptiles (Hunter et al.,

2015; Davy et al., 2015), birds (Ushio et al., 2018a), mammals (Foote et al., 2012; Ushio et al., 2017), and aquatic plants (Scriver et al., 2015, Fujiwara et al., 2016).

Spawning is the basis for population establishment and development. Monitoring the timing and location of spawning activities can improve our understanding of fish ecology and provide important information for fish conservation and management (Scott et al., 2006). The use of eDNA techniques for non-invasive investigation of fish spawning sites and spawning times is gaining attention (Spear et al., 2015). External fertilization of fish results in the release of a large number of sperm and eggs into the water during spawning, resulting in a sharp rise in eDNA concentration and nuclear eDNA/mitochondrial eDNA ratio in a short period of time (Bylemans et al., 2017; Tsuji & Shibata, 2021; Wu et al., 2022). This allows the eDNA approach to accurately monitor the daily fish spawning activities. A previous study attempted to estimate fish spawning activity by investigating high eDNA concentrations and ratios using quantitative polymerase chain reaction (qPCR; Wu et al., 2023). The use of qPCR to quantify DNA concentrations is common for environmental samples (Doi et al., 2017; Langlois et al., 2021); however, the quantitative performance of this method may be affected by inhibition (Lance & Guan, 2020), and a single qPCR experiment can only quantify a single target species, with repeated experiments required to target multiple species.

Metabarcoding techniques are widely used in multi-species studies to avoid repetition (Miya et al., 2015; Nakagawa et al., 2018). PCR is performed using universal primers combined with high-throughput sequencing to detect multiple target species simultaneously. Metabarcoding provides species information for a single experiment. However, amplification bias can exist

between species, and the uneven distribution of sequencing reads among different samples makes it impossible to quantify the resultant data. Previous studies used metabarcoding to investigate fish species composition based on relative abundance (Bagley et al., 2019; Xie et al., 2021). Another study used the peak relative abundance calculated from metabarcoding reads to estimate fish spawning activity (Di Muri et al., 2022). However, the relative abundance was affected by the combined effects of eDNA released by all detected species, and the validity of using relative abundance to estimate spawning activity requires verification. Therefore, further exploration is needed to develop efficient eDNA methods for simultaneous monitoring of the spawning activities of multiple fish.

Quantification can be achieved by adding internal standard DNAs to the metabarcoding approach and converting the sequencing reads into copy numbers (Ushio et al., 2018b). This method can simultaneously quantify multiple species with a higher efficiency than traditional metabarcoding and species-specific qPCR. Some studies have adopted this method to measure the copy numbers of multiple species (Tsuji et al., 2022a; Nakagawa et al., 2022) with significant positive relationships identified between eDNA concentrations and abundance among species (Tsuji et al., 2022b), but this approach has rarely been applied to long-term field surveys (Ushio et al., 2022). Another study showed a very high variance in the number of reads sequenced from similar amounts of DNA, preventing accurate quantification (Fernández et al., 2018). These contrasting experimental results necessitate further verification of the quantitative effectiveness of this method. eDNA concentration is affected by biomass, and previous studies have used qPCR to determine eDNA concentrations and estimate fish biomass (Lacoursière-

Roussel et al., 2016; Doi et al., 2017). Therefore, the effectiveness of quantitative metabarcoding can be verified by comparing it with data obtained from traditional surveys. Subsequently, the eDNA concentration data obtained using this method can be used to estimate changes in the fish community structure and spawning activity.

In the present study, a quantitative metabarcoding approach was used to simultaneously quantify the eDNA concentrations of multiple fish species in a small freshwater reservoir in Japan over two summer seasons. The data were then combined with a method used in a previous study to estimate the spawning activity of multiple fish species in the reservoir. We try to compare among fish species to look for general patterns in fish abundance, and then looking within fish species to look for patterns in spawning activity. The three main research objectives were: 1) to monitor the changes in eDNA concentrations of multiple fish species using quantitative metabarcoding and compare them with the results of traditional surveys to help validate the method of quantitative metabarcoding; 2) investigate fish species composition and changes in fish-specific eDNA concentrations with environmental variables; and 3) estimate the spawning activities of multiple fish species using quantitative data.

## Materials and Methods

The survey sites were located in the Hebisawagawa front reservoir, which has a total area of approximately 44,000 $m^2$, in the Miharu Reservoir in Fukushima Prefecture, Japan (Figure 1). This front reservoir is connected to the main reservoir by a channel with a width of 5 m and a depth of 5 m and is isolated during the summer season (June 11th to October 10th) when the

water level is low. Traditional and eDNA surveys were conducted to investigate individual counts and eDNA concentrations in fish, respectively. All eDNA samples were collected in a previous study that attempted to establish a method for monitoring fish spawning activity based on eDNA data obtained via species-specific qPCR (Wu et al., 2023); therefore, the details of the sampling sites, measures to prevent cross contamination, and DNA extraction are only briefly described here. In the present study, this method was applied to quantitative metabarcoding data to monitor the spawning activities of multiple fish species simultaneously.

Traditional surveys

The traditional surveys were conducted in the Hebisawagawa front reservoir between 2007 and 2010. A partition net with a mesh size of 10 mm was installed to isolate the front reservoir from the main reservoir during periods of high water levels. The fish were captured using the partition and fixed nets. A partition net was employed using the drawdown operation of the main reservoir when the water level was lowered in preparation for summer floods. The fixed net procedure was performed in two stages. In the first stage, the drawdown operation was used to catch fish in the enclosed area when the water level dropped and the shallows dried. Nets with a mesh size of 10 mm were installed at two locations to surround a 1.5 m water depth range in the shallow area of the reservoir shoreline. The total enclosed area was approximately 7,500 $m^2$. In the second stage, the valve at the bottom of the front reservoir was opened to drain the water by 1.5 m to enable fish to be captured in the fixed net. The captured fish were placed in continuously aerated buckets and released after the survey. The traditional surveys were

permitted by Fukushima Prefecture (Special permission No. 19-5 for 2007, No. 20-3 for 2008, No. 21-9 for 2009 and No. 22-5 for 2010). Owing to the limitations of the research program, traditional surveys were conducted only between 2007 and 2010. Although the survey data were old, the ecology of the target reservoir was relatively stable, and the composition of fish species did not change significantly. In addition to the above surveys, the 2019 traditional survey data were obtained from the River Environmental Database (Ministry of Land, Infrastructure, Transport and Tourism, 2022) and used to complement the traditional survey data. In contrast to the traditional surveys from 2007–2010, the 2019 survey was conducted using cast nets, gill nets, and other methods.

eDNA sampling and extraction

Water samples were collected weekly from three sites (MHS1–MHS3; Figure 1) from March 26 to August 13, 2019, and March 3 to August 25, 2020. A benzalkonium chloride solution (1 mL, 10% mass/volume) was immediately added to water samples to prevent DNA degradation (Yamanaka et al., 2017). A total of 141 samples (47 weeks at two years × three sites) were collected. Water temperature (WT), pH, and electrical conductivity (EC) were recorded during water sample collection. The samples were then filtered until clogging (up to 1 L) using two 0.7 μm pore size 47 mm glass-fiber filters (GF/F; GE Healthcare Japan, Tokyo, Japan). Ultrapure water (1 L) was filtered as a negative control, yielding 47 filtered negative controls. The filters were stored at −25 °C until DNA extraction.

Total DNA was extracted using a DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany)

according to the method recommended by the eDNA Society (Minamoto et al., 2021). Briefly, two filters for each sample were combined in a single Salivette tube (Sarstedt, Nümbrecht, Germany). Buffer AL (400 μL) and Proteinase K (40 μL) were mixed as lysis solutions and added to the filters. The filters were incubated at 56 °C for 30 min and centrifuged at 5,000 × *g* for 3 min to collect eDNA. TE buffer (220 μL) was added to the filters and re-centrifuged at 5,000 × *g* for 1 min to increase the DNA yield. The DNA was purified according to the manufacturer's instructions. Total DNA was eluted in 100 μL AE buffer and stored at −25 °C until paired-end library preparation.

Paired-end library preparation

Five different internal standard DNAs were designed and prepared as described previously (Ushio et al., 2022). The MiFish-U primer set (forward:5′-ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TCT NNN NNN GTC GGT AAA ACT CGT GCC AGC-3′ and reverse:5′-GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG ATC TNN NNN NCA TAG TGG GGT ATC TAA TCC CAG TTT G-3′) was used to amplify the hypervariable region of mitochondrial 12S rRNA gene (Miya et al., 2015). Two PCRs were performed to amplify the DNA of the target region and to add sequencing primers.

In the first-round PCR (1st PCR), each 12 μL PCR mixture contained 6.0 μL 2 × KAPA HiFi HotStart ReadyMix (Roche, Basel, Switzerland), 0.36 μL of each primer with 300 nM final concentration, 1.0 μL template DNA, 1.0 μL internal standard DNA mix containing 40, 20, 10, 5, and 1 copy per reaction and 3.28 μL pure water. The thermal cycling profile was as follows:

an initial 3 min denaturation at 95 °C, 40 cycles of 98 °C for 20 s, 65 °C for 15 s, and 72 °C for 15 s, with a final extension at 72 °C for 5 min. PCR negative controls with internal standard DNAs were used for each 1st PCR run to monitor contamination during the experiments. Because these samples were verified to be free from contamination by qPCR in a previous study (Wu et al. 2023), only seven of the 47 filtering negative controls were selected for experimentation. Four replicates were performed for each sample, and a negative control was used to minimize PCR dropouts. In total, 152 samples (141 samples [47 sampling times × three sites], seven filtering negative controls, and four PCR negative controls) were treated. For each sample, the 1st PCR products of the replicates were pooled and size-selected for 200–400 bp using SPRIselect (Beckman Coulter, Brea, CA, USA) according to the manufacturer's instructions. The concentrations of the size-selected amplicons were quantified using a Qubit fluorometer 3.0 (Thermo Fisher Scientific, Waltham, MA, USA) with a Qubit dsDNA HS assay kit, and then diluted to 0.1 ng/μL with sterilized distilled $H_2O$. All diluted products were frozen at −25 °C until second-round PCR (2nd PCR).

The 2nd PCR was performed by adding Illumina P5/P7 adaptor sequences and 8-bp index sequences to both ends of the amplicon. Each 12 μL PCR mixture contained 6 μL of 2 × KAPA HiFi HotStart ReadyMix, 2 μL of each primer with 300 nM final concentration, 1 μL of diluted 1st PCR product, and 1 μL of pure water. The thermal cycle profile was as follows: an initial 3 min denaturation at 95 °C, 12 cycles of 98 °C for 20 s and 72 °C for 20 s, and a final extension at 72 °C for 5 min. All 2nd PCR products had different indices; therefore, every 38 products were mixed into a single tube as a library for a total of four libraries. The libraries were purified

and size-selected (approximately 370 bp) using E-Gel SizeSelect 2% (Thermo Fisher Scientific). The size distribution of the purified libraries was determined using an Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA). Finally, the four libraries were diluted to 1 nM with sterilized distilled $H_2O$ and sequenced respectively on the iSeq 100 platform (Illumina, San Diego, CA, USA) with iSeq 100 i1 Reagent v2 (Illumina) for 2 × 150 bp pair-ends according to the manufacturer's instructions. On average, approximately 80,000 reads were assigned to each sample to ensure that sufficient reads were assigned. Four iSeq runs were conducted.

Data preprocessing and taxonomic assignment

Raw iSeq data were preprocessed using USEARCH v11.0.667 (Edgar 2010) to generate zero-radius operational taxonomic units (ZOTUs) according to the steps described by Sakata et al. (2020) with modifications. The "fastq_mergepairs" command was used to merge the paired-end reads; "fastx_truncate" to remove the primer sequences; "fastq_filter" to remove low-quality reads with an expected error rate of > 1% (Edgar & Flyvbjerg, 2015) and short reads < 140 bp; "fastx_uniques" to dereplicate the reads and remove singletons; "unoise3" to generate ZOTUs, with chimeras and ZOTUs of less than eight reads removed; "otutab" to generate the ZOTU table under 97% identity threshold, and "usearch_global" to compare the ZOTUs to the local database to determine the internal standards with a sequence identity of > 98.5% (two nucleotide differences allowed) to the reference sequences and a query coverage of ≥ 90%. Finally, the ZOTUs were compared to the NCBI nr/nt database using BLASTN under the same conditions to perform taxonomic assignments.

234 The DNA copy numbers were calculated from the sequence reads according to the method
235 described by Ushio et al. (2018b). Briefly, linear regression with an intercept set to zero was
236 performed to examine the relationship between sequence reads and the copy numbers of the
237 internal standard DNAs for each sample. Sequence reads of non-standard fish DNA were
238 converted to copy numbers by dividing the number of iSeq sequence reads by the sample-
239 specific regression slope. Each PCR negative control corresponded to a portion of the sample,
240 and copy numbers in the PCR negative controls were subtracted from the corresponding
241 samples to remove contaminants. The copy number per filter volume (copies/L filter volume)
242 was calculated as the eDNA concentration based on the filtration volume of each sample. Owing
243 to the absence of research on the limit of quantification in quantitative eDNA metabarcoding,
244 eDNA concentrations of less than one copy/L were denoted as zero copies/L (approximately
245 equal to discarding fewer than four reads of ZOTUs per sample). Fish species detected only
246 once in 141 samples were excluded.
247
248 Data analysis
249 A phylogenetic tree based on the neighbor-joining method was constructed to demonstrate the
250 relationship between different fish species and the potential associations between this
251 relationship and fish species composition. Because eDNA concentrations are affected by fish
252 biomass (Doi et al., 2017), the quantification performance of quantitative metabarcoding can
253 be verified by the correlation between abundance and eDNA concentrations across different
254 fish species. In 2019, the eDNA concentrations of the same fish species from different sites and

255 months were summed and a linear regression model was used to evaluate the relationship
256 between fish eDNA concentration and the number of individual fish obtained from traditional
257 surveys. Data from multi-year eDNA and traditional surveys were also summed and analyzed.
258 Due to the presence of heteroskedasticity, robust standard errors were calculated by
259 heteroskedasticity-consistent covariance matrix estimator (HC2 estimator) for all linear
260 regressions model (MacKinnon & White, 1985; Samii & Aronow, 2012). Permutational
261 multivariate analysis of variance (PERMANOVA) using Bray–Curtis dissimilarity was
262 performed to test for statistical differences in fish eDNA composition between samples from
263 different months, years, and sites, with 9,999 permutations. A permutation test for the
264 homogeneity of multivariate dispersions was performed to check the consistency of the
265 dispersion. The eDNA concentration varied greatly from sample to sample and from fish to fish.
266 To alleviate this variation, concentration data were log-transformed based on natural logarithms
267 before calculating the Bray–Curtis dissimilarity, and all data were +1 to avoid 0 values. Because
268 nonmetric multidimensional scaling (NMDS) contains random processes, Bray–Curtis based
269 principal coordinate analysis (PCoA) was performed to demonstrate differences in fish eDNA
270 composition between samples, and “lingoes” method was chosen as correction for negative
271 eigenvalues (Legendre & Anderson, 1999). Generalized additive models (GAMs) were fitted
272 to the ordination axis scores for the environmental variables (water temperature, pH, and EC),
273 and the smoothed surfaces were plotted over the PCoA using the “ordisurf” command to show
274 the variation of environmental variables between samples (Deveautour et al., 2022).
275 Although different fish species may respond differently to environmental variables, they should

have similar responses to at least some variable gradients, and modeling each fish species separately would lead to loss of this shared information. Therefore, based on the assumption of nonlinearity between fish eDNA and environmental factors, a hierarchical generalized additive model (HGAM) was used to estimate the changes in fish eDNA concentration with environmental variables, and data with 0 copies were not included in the analysis. The model structure was designed as a global smoother plus fish-level smoothers that have the same wiggliness (Pedersen et al., 2019): log(Copy) ~ s(WT,m=2) + s(WT,Fish,bs="fs",m=2) + s(pH,m=2) + s(pH,Fish,bs="fs",m=2) + s(EC,m=2) + s(EC,Fish,bs="fs",m=2) + s(Site,Year,bs="re"). "s()" means a smooth term, "bs" indicating the smoothing basis to use, "fs" means factor smooth interactions, "re" means random effect, "m" means the order of the penalty for this smooth term. Restricted maximum likelihood (REML) was used to select the smoothing parameter (Wood, 2011). This model builds global response curves for eDNA to environmental variables and provides fish-specific response curves for each fish species with an additional smooth term by specifying bs="fs". Random effects between different sites and years were also considered in the model by specifying bs="re".

The spawning activity of the fish species was estimated according to a previously described method (Wu et al., 2023). In brief, the data were grouped by site and year, and the fish were considered to have spawned if the eDNA concentration was greater than the 3rd quartile + 1.5×interquartile range. However, despite meeting the above conditions the target species do not spawn at water temperatures exceeding 30 °C; therefore, no spawning activity was considered in such condition. Based on the spawning/no spawning data obtained by the above

297 method, another HGAM with binomial distributions was generated as follows: spawning ~
298 log(copy) + log(copy)*Fish + s(WT,m=2) + s(WT,Fish,bs='fs',m=2). REML was also used.
299 The model was then used to predict spawning probability based on eDNA sample data. A
300 previous study verified the feasibility of this method by comparing it with the traditional method
301 (Wu et al., 2023). PERMANOVA and PCoA were applied using the *vegan* package (Oksanen
302 et al., 2022), HGAMs using the *mgcv* package (Wood, 2006), robust standard errors using the
303 *estimatr* package (Blair et al., 2022) and the phylogenetic tree and other graphs were created
304 using the *ggtree* (Yu, 2022) and *ggplot2* (Wickham, 2016) packages. All analyses were
305 performed using R (version 4.2.1; R Core Team, 2022).
306
307 **Results**
308 The iSeq paired-end sequencing of the 152 samples (141 samples [47 sampling times × three
309 sites], seven filtering negative controls, and four PCR negative controls) yielded a total of
310 12,630,140 reads, of which 12,288,756 (97.3%) passed the merging processes and 10,771,744
311 (85.3%) passed the quality control processes. Subsequently, 657 ZOTUs were generated after
312 denoising, and 12,055,551 (95.5%) reads were matched to the ZOTUs (>97% identity). Finally,
313 11,558,502 (91.5%) reads of 42 ZOTUs were assigned to standard DNA and fish taxa (>98.5%
314 identity). Of the 11,558,502 reads, 4,342,992 were non-standard fish iSeq reads (37.6%), and
315 4,334,000 (99.8%) of these were from fish DNAs in field samples, while 8,992 (0.2%) were
316 from negative controls. The median adjusted $R^2$ value for the 152 linear regressions generated
317 by the internal standard DNAs was 0.987, and most of the adjusted $R^2$ values were above 0.96.

318 The specific distributions are shown in Figure S1. The detailed results of the linear regression
319 are shown in Table S1. Residual analysis was used to demonstrate the reliability of the linear
320 regression (Figure S2). The copy number of the four PCR-negative controls accounted for
321 approximately 0.03% of that of all samples and filtered negative controls, and that portion of
322 the copy number was removed from the corresponding samples and filtered negative controls.
323 The average copy number ratio of the seven filtered negative controls to the corresponding
324 sample copy number was 0.46%, which could be considered negligible contamination.
325 Therefore, no additional treatment was performed for the copy numbers in the filtered negative
326 controls. There were 779 data points equal to zero copies/L, and 1,336 data points greater than
327 zero copies/L in the final dataset, of which 869 were within the range of the standards, 422 were
328 below the range, and 45 were above the range.
329 Fifteen fish species were detected in 2019 and 2020 using the quantitative eDNA metabarcoding
330 approach (Figure 2). The traditional survey results for 2007, 2008, 2009, 2010, and 2019
331 showed the presence of 19 fish species (6,700 individuals; Figure 2, Table S2). A total of 20
332 species of fish were detected using the two methods, and the dominant species belonged to the
333 Cyprinidae and Centrarchidae families. Traditional and eDNA surveys detected an average of
334 13.4 and 14.5 fish species per year, respectively. *Ctenopharyngodon idella* was only detected
335 using the eDNA approach, and single individuals of *Tridentiger brevispinis*, *Oncorhynchus*
336 *masou*, two individuals of *Rhynchocypris steindachneri*, three individuals of *Anguilla japonica,*
337 and ten individuals of *Rhodeus ocellatus* were detected using the traditional approach. *Anguilla*
338 *japonica* was also detected using the eDNA approach, but was removed from the data because

it was detected only once in 141 eDNA samples. Except for extremely rare fish, all species were detected by the quantitative eDNA metabarcoding approach, including *Rhinogobius* sp., *Gymnogobius urotaenia*, *Micropterus salmoides*, *Lepomis macrochirus*, *Silurus asotus*, *Misgurnus anguillicaudatus*, *Tribolodon hakonensis*, *Zacco platypus*, *Carassius* sp., *Carassius cuvieri*, *Cyprinus carpio*, *Hemibarbus barbus*, *Gnathopogon elongatus*, and *Pseudorasbora parva*. The changes in eDNA concentrations over time for the eight fish species with the highest eDNA concentrations are shown in Figure S3. Linear regression was used to evaluate the relationship between eDNA concentration and the number of individual fish investigated using traditional surveys. The results of the 2019 eDNA and traditional surveys showed a statistically significant positive correlation between the eDNA copy numbers and fish abundance ($p = 0.023$, adjusted $R^2 = 0.202$, Figure 3, Table S3). Multi-year merged data from two years of eDNA surveys and five years of traditional surveys also showed a statistically significant relationship ($p = 0.028$, adjusted $R^2 = 0.3$; Figure S4, Table S3).

PERMANOVA results showed that the fish community structure estimated by eDNA concentration was significantly different between months ($R^2 = 0.104$, $p < 0.001$), and the differences between sampling sites ($R^2 = 0.018$, $p = 0.007$) and years ($R^2 = 0.015$, $p < 0.001$) were minimal but also significant. The results of the pairwise comparisons showed that the difference in fish community structure was smaller between March–April and July–August ($R^2 = 0.053$, adjusted $p = 0.036$; $R^2 = 0.053$, adjusted $p = 0.048$; Table S4), and no significant difference was found between the June–July period ($R^2 = 0.029$, adjusted $p = 0.578$; Table S4). The PCoA results showed a changing trend in fish eDNA composition in various months and

the fish species that induced these changes (Figure 4a). A scree plot of the eigenvalues is shown in Figure S5. The permutation test for the homogeneity of multivariate dispersions showed variance heterogeneity among samples from different months ($p < 0.001$), and paired comparisons showed that the dissimilarity between samples from March and April was larger than that from May to August (Figure S6), which may exaggerate the difference between samples from different months. The dominant fish species were *C. carpio*, *C. cuvieri* and other species of the *Carassius* genus in March and April; *C. idella*, *S. asotus*, *G. urotaenia*, *G. elongatus* and *M. anguillicaudatus* in May; *M. salmoides*, *L. macrochirus*, *P. parva* and *H. barbus* from June to August (Figure 4a). The GAM results for the PCoA axis and environmental variables showed that the change in the eigenvalue of the axis could explain the variation in water temperature (deviance explained = 51.8%, $p < 0.001$), pH (deviance explained = 25.3%, $p < 0.001$), and EC (deviance explained = 14.3%, $p = 0.002$) between samples to some extent. The diagnostic information for this part of the GAMs is shown in Figures S7–S9.

Changes in fish eDNA concentrations with environmental variables were fitted using HGAM. The deviance explained by the HGAM was 47.2%. The modeling outcomes are presented in Table 1, and the results of the basis dimension (k) checking and residual analysis are presented in Table S5 and Figure S10, respectively. Figure 5 shows the relationship between the fish-specific eDNA concentrations and environmental variables. The plots without data points are shown in Figure S11. Water temperature, pH, and EC significantly affected the changes in fish eDNA concentration, with the influence of water temperature and pH showing different trends, whereas the influence of EC exhibited no statistical difference among the fish species (Table 1).

The eDNA concentrations of different fish species peaked at different water temperatures: *T. hakonensis*, *C. cuvieri*, *C. carpio*, *G. elongatus* at 10–20 °C; and *G. urotaenia*, *L. macrochirus*, *Z. platypus*, *H. barbus* and *P. parva* at 20–30 °C (Figure 5). The variation in eDNA concentration with pH differed slightly among the various fish species, and showed a decreasing trend for all fish species at pH values above 8.5 (Figure 5). The eDNA concentrations in all fish species showed a uniform downward trend with increasing EC (Figure 5).

By detecting outliers in the eDNA data, the spawning activities of 13 of the 15 fish species were estimated. Of the 141 samples, *S. asotus* and *C. idella* were detected four and 19 times, respectively, and spawning activities could not be estimated because of the failed identification of outlier values. The outcomes and residual analyses of the HGAM model used to predict spawning probability are shown in Table S6 and Figure S12. The estimated spawning activities of *G. urotaenia* using eDNA concentrations were inconsistent between the two years, with outlier values mainly concentrated in June and July in 2019, whereas those in 2020 were mainly concentrated in March and April (Figure 6). The spawning activities of all fish species except *G. urotaenia* were similar to those of the fish spawning period recorded by the Research Institute of Environment, Agriculture and Fisheries, Osaka Prefecture (http://www.kannousuiken-osaka.or.jp/zukan/) (Figure 6, dashed box). According to the results of traditional surveys, *Rhinogobius* sp. and *Carassius* sp. are mainly composed of *Rhinogobius kurodai* and *Carassius auratus langsdorfii*; therefore, the spawning periods of these two fish species were determined.

## Discussion

An eDNA survey was conducted at three sites in the Hebisawagawa front reservoir over two summer seasons. A quantitative metabarcoding approach was used to quantify the eDNA concentrations of 15 fish species in 141 samples. The results of the survey showed that the fish species detected by the quantitative eDNA metabarcoding approach were highly consistent with the historical results of traditional surveys. The relationship between the eDNA concentrations of different fish species detected by quantitative metabarcoding and the number of individual fish recorded by traditional surveys was statistically significant, demonstrating that the quantitative metabarcoding approach has a certain quantitative performance. Simultaneous measurement of eDNA concentration in multiple fish species allowed the variations in fish eDNA composition within different samples to be revealed using PCoA. The relationships between eDNA concentration and environmental variables in 15 fish species were analyzed using HGAM. The spawning activities of the 13 fish species were estimated by detecting outliers in the eDNA data. Most of the estimated spawning activities were consistent with recognized fish spawning periods, demonstrating the potential of long-term quantitative eDNA metabarcoding to simultaneously monitor the spawning activities of multiple fishes.

### Quantitative metabarcoding

Residual analysis of the linear regression generated from the internal standard DNA revealed heteroscedasticity in the model (Figure S2). However, heteroscedasticity does not affect the expectation of a parameter (MacKinnon & White, 1985). Therefore, it can be considered that

423 the method of converting fish DNA reads to copy numbers using the slope of linear regression
424 is still feasible. It should be noted that, in the process of calculating the fish DNA copy number,
425 approximately 35% of the data points exceeded the regression limit, and we assumed that the
426 data beyond the regression limit remained linear, which could lead to inaccurate results.
427 Although the total number of fish species detected by eDNA and traditional surveys was not
428 identical, the difference was mainly caused by very rare fish species, such as *T. brevispinis*, *O.*
429 *masou*, and *R. steindachneri* (Figure 2). The average number of fish species detected by the two
430 methods in a single survey was similar, confirming that the two survey methods have similar
431 detection capabilities for fish species (Keck et al., 2022). The relative abundance calculated
432 from fish eDNA concentration was not completely consistent with that calculated from the
433 number of individual fish in traditional surveys (Figure 2). *L. macrochirus* had a high relative
434 abundance in the traditional survey but a low value in the eDNA survey, whereas *C. Cuvieri*
435 showed the opposite trend (Figure 2). The rate of eDNA release and number of mitochondria
436 contained in a single cell may vary among fish species (Charitonidou et al., 2022), leading to
437 differences in the relationship between individual numbers and eDNA copy numbers across
438 species. The time interval between eDNA surveys and traditional surveys and the natural
439 changes in fish community structure may also explain this change. Electrofishing is a common
440 practice in the Miharu Reservoir for controlling the number of *M. salmoides* and *L. macrochirus*,
441 and manual intervention may also be an important factor affecting this change. The differences
442 between eDNA and traditional surveys also imply that the two approaches have different
443 species sensitivities. eDNA is not always the best approach (Ulibarri et al., 2017); however, it

444 can be considered that the complementarity of the two approaches can provide enriched
445 information for ecological management (Euclide et al., 2021). There was a significant positive
446 correlation between eDNA concentrations and fish abundance for both the 2019 data (Figure 3)
447 and the multi-year merged data (Figure S4). This indicated the feasibility of using quantitative
448 metabarcoding to simultaneously estimate eDNA concentrations in multiple fish species. For
449 the 2019 data, the adjusted $R^2$ value of the linear regression was lower than that calculated using
450 the multi-year merged data. This may be because the aggregated data from the multiyear
451 surveys moderated the data volatility. Another study showed that different markers produce
452 different amplification biases (Fernández et al., 2018). Although the MiFish primers used in
453 this study were checked to some extent by Ushio et al. (2018b) to ensure interpretability of the
454 quantitative results, further validation is required. Although there was a significant positive
455 correlation between the eDNA concentration and fish abundance, the adjusted $R^2$ value of the
456 model was not high. The residuals of the model may have originated from differences in eDNA
457 release rates among fish species or from PCR bias, making it difficult to accurately estimate
458 fish abundance from eDNA concentrations (Danziger et al., 2022; Lacoursière-Roussel et al.,
459 2016). However, it can still be considered a good complement to traditional surveys, and can
460 provide information for ecological investigations from a molecular perspective. The model will
461 be further improved in the future with increased understanding.
462
463 Fish species composition, eDNA concentration, and environmental variables
464 The reservoir ecosystem is relatively closed compared to flowing waters and sea areas.

Therefore, although the results of PERMANOVA showed differences in fish community structure in different months, this cannot be simply attributed to changes in fish biomass. For eDNA released into the water by different fish, the diffusion and decomposition caused by water flow and other environmental factors may have an impact. However, we believe that the rate of eDNA release by fish is not constant; more active fish may release more eDNA, and fish activity may be an important factor affecting the eDNA composition. Therefore, the differences in fish community structure in different months, calculated by eDNA concentration, may reflect the activities of fish, such as migration and spawning (Thalinger et al., 2019; Bylemans et al., 2017). Because all eDNA samples were collected from shallow water areas, it was not possible to examine the local changes in population structure caused by fish migration between shallow water and deep water; therefore, changes in fish community structure between months can be considered as changes in fish activity in shallow water areas, whereby *C. carpio*, *C. cuvieri,* and other fishes of the *Carassius* genus are mainly active in March and April; *T. hakonensis*, *C. idella*, *S. asotus* in April and May; and *M. salmoides* and *L. macrochirus* in June, July, and August (Figure 4a). The fish community structures in June and July were similar (Figure 4a), indicating that these two months had comparable composition of active fish. When the water temperature was low, most fish were inactive in shallow water areas and the eDNA concentration decreased, resulting in a reduction in detection efficiency. This may explain the large dissimilarity between the samples collected in March and April. Therefore, the eDNA-based fish species composition can be interpreted as changes in fish activity driven by environmental variables (Figure 4b, c, d).

Water temperature affects the distribution of fish (Stefan et al., 1996), and fish eDNA concentrations vary with water temperature (Figure 5), reflecting the differential responses of fish species to water temperature. The eDNA concentration of some fish species, such as *M. salmoides* and *Carassius* sp., increased with higher water temperatures but did not show a downward trend, which may be related to the life history of the fish. In Miharu Reservoir, *M. salmoides* juveniles swarm in large numbers near the lakeshore, which could cause a sharp increase in the local eDNA concentration. The water samples in this study were collected from the shoreline area, and sampling during periods of high water temperature may reflect juvenile swarming. To a certain extent, pH and EC reflect water quality, and the change in the eDNA concentrations of fish species with pH reflects their pH preferences. In this study, the pH fluctuation range was small; therefore, the eDNA concentration of most fish species did not show a significant peak with a change in pH (Figure 5). EC significantly affected fish eDNA concentrations, but the change in this concentration with EC was not statistically different among the fish species (Table 1), which may indicate that some underlying water quality changes affected fish activity. The deviation explained by the HGAM based on environmental variables was only 47.2%, implying that the model was still subject to a large uncertainty and could be further explained by other environmental variables. Although the k checking of the model shows that k may be too low, the effective degrees of freedom (edf) does not increase substantially even when k was increased (Table S5), therefore, the model can be considered to have sufficient k (Wood, 2017).

507 Fish spawning activities

508 The spawning activities of the 13 fish species were simultaneously estimated using quantitative
509 eDNA metabarcoding (Figure 6). Most of the estimated activity dates were within the
510 recognized fish spawning period, suggesting that quantitative eDNA metabarcoding has the
511 potential to provide useful information for characterizing the spatial and temporal nature of fish
512 spawning in reservoirs. The spawning activities of *Carassius* sp., *C. cuvieri*, and *C. carpio*
513 occurred mainly in April and May, and July, forming two spawning periods. This may be
514 because *C. cuvieri* and *C. carpio* can spawn multiple times (Fernández-Delgado, 1990). The
515 estimated spawning activities of some *L. macrochirus*, *M. anguillicaudatus*, *Carassius* sp., and
516 *H. barbus* occurred later than the common spawning period, which may have been caused by
517 climatic differences or swarming of the juveniles after hatching. The estimated spawning
518 activities of *G. urotaenia* varied widely between the two years, and the results for 2019 deviated
519 from the recognized spawning period. Historical records from traditional surveys have shown
520 that the number of *G. urotaenia* individuals in the Hebisawagawa front reservoir is low. The
521 weekly water sampling schedule may have failed to capture the eDNA concentration peaks
522 caused by spawning activity, and the eDNA detected in 2019 may have been derived from *G.*
523 *urotaenia* juveniles rather than from spawning activities. Therefore, for fish species with few
524 individuals, more frequent sampling is required to ensure the accurate monitoring of spawning
525 activity. It is important to note that eDNA concentrations are driven by multiple factors such as
526 abundance, spawning, spreading, and degradation (Barnes & Turner, 2016; Bylemans et al.,
527 2017). The processes and variables affecting eDNA concentrations are complex. Therefore,

528 although high eDNA concentrations have the potential to indicate fish spawning activities, the
529 influence of other confounding factors should be considered when interpreting the results.
530 Previous studies have shown that the large amount of sperm released during spawning leads to
531 a sharp increase in the nuclear DNA/mitochondrial DNA ratio, owing to the low sperm
532 mitochondrial content (Bylemans et al., 2017; Wu et al., 2022). Therefore, it is also possible to
533 estimate fish spawning activity by developing universal primers for fish nuclear DNA and
534 calculating nuclear DNA/mitochondrial DNA ratios. Significant differences in the monitoring
535 results of spawning activities at different sites imply that fish populations are not uniformly
536 distributed in the reservoir and that high concentrations of eDNA released from spawning
537 activities are confined to their release sites. This implies that monitoring fish spawning activity
538 using eDNA requires intensive spatial sampling. It can be assumed that this uneven distribution
539 also affects other eDNA-based surveys such as fish community structure and biomass surveys.
540 In eDNA surveys with small sample sizes, the ecological significance may be misinterpreted
541 because of sampling differences, and reasonable conclusions may not be drawn because of large
542 differences between samples. Therefore, when conducting ecological surveys of water bodies
543 using eDNA, intensive sampling at both the spatial and temporal scales is necessary to obtain
544 more accurate conclusions. Such intensive sampling increases the workload of subsequent
545 molecular analyses and the need for techniques, such as quantitative metabarcoding, to improve
546 efficiency.
547 In conclusion, quantitative metabarcoding was used to simultaneously measure eDNA
548 concentrations in multiple fish species. The results showed that this method can achieve species

549 detection capabilities similar to those of traditional surveys and provides quantitative
550 capabilities compared to general metabarcoding. Based on the eDNA concentration calculated
551 by this method, the fish species composition within samples and the relationship between the
552 fish-specific eDNA concentration and environmental variables were estimated to further our
553 understanding of fish ecology. By using the outliers of the eDNA data proposed in a previous
554 study, the spawning activities of 13 fish species were estimated, with 12 species showing
555 activities roughly consistent with the documented spawning period of fish. These results show
556 that long-term quantitative eDNA metabarcoding has the potential to be applied in simultaneous
557 ecological investigations of multiple fish species. The study of problems with amplification
558 bias and exceeding the limit of quantification can help further improve the reliability of this
559 technique.
560

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## References


Bagley, M., Pilgrim, E., Knapp, M., Yoder, C., Santo Domingo, J., & Banerji, A. (2019). High-throughput environmental DNA analysis informs a biological assessment of an urban stream. Ecological indicators, 104, 378-389.

Barnes, M. A., & Turner, C. R. (2016). The ecology of environmental DNA and implications for conservation genetics. Conservation genetics, 17(1), 1-17.

Blair, G., Cooper, J., Coppock, A., Humphreys, M., Sonnet, L. (2022). estimatr: Fast Estimators for Design-Based Inference. R package version 1.0.0, <https://CRAN.R-project.org/package=estimatr>.

Bylemans, J., Furlan, E. M., Hardy, C. M., McGuffie, P., Lintermans, M., & Gleeson, D. M. (2017). An environmental DNA-based method for monitoring spawning activity: A case study, using the endangered Macquarie perch (Macquaria australasica). Methods in Ecology and Evolution, 8(5), 646-655.

Charitonidou, K., Panteris, E., & Ganias, K. (2022). Ultrastructural changes in mitochondria during oogenesis in two phylogenetically close fish species. Journal of Morphology, 283(4), 502-509.

Chollett, I., Priest, M., Fulton, S., & Heyman, W. D. (2020). Should we protect extirpated fish spawning aggregation sites?. Biological Conservation, 241, 108395.

Copp, G. H., & Peňáz, M. (1988). Ecology of fish spawning and nursery zones in the flood plain, using a new sampling approach. Hydrobiologia, 169(2), 209-224.

Danziger, A. M., Olson, Z. H., & Frederich, M. (2022). Limitations of edna analysis for *carcinus maenas* abundance estimations. BMC Ecology and Evolution, 22(1), 14.

Davy, C. M., Kidd, A. G., & Wilson, C. C. (2015). Development and validation of environmental DNA (eDNA) markers for detection of freshwater turtles. PloS one, 10(7), e0130965.

Deveautour, C., Rojas-Pinzon, P. A., Veloso, M., Rambaud, J., Duff, A. M., Wall, D., ... & Brennan, F. (2022). Biotic and abiotic predictors of potential $N_2O$ emissions from denitrification in Irish grasslands soils: A national-scale field study. Soil Biology and Biochemistry, 168, 108637.

Di Muri, C., Handley, L. L., Bean, C. W., Benucci, M., Harper, L. R., James, B., ... & Hänfling, B. (2022). Spatio-temporal monitoring of lake fish spawning activity using environmental DNA metabarcoding. Environmental DNA. https://doi.org/10.1002/edn3.343

Doi, H., Inui, R., Akamatsu, Y., Kanno, K., Yamanaka, H., Takahara, T., & Minamoto, T. (2017). Environmental DNA analysis for estimating the abundance and biomass of stream fish. Freshwater Biology, 62(1), 30-39.

Edgar, R. C. (2010). Search and clustering orders of magnitude faster than BLAST. Bioinformatics, 26(19), 2460-2461.

Edgar, R. C., & Flyvbjerg, H. (2015). Error filtering, pair assembly and error correction for next-generation sequencing reads. Bioinformatics, 31(21), 3476-3482.

Erisman, B., Heyman, W., Kobara, S., Ezer, T., Pittman, S., Aburto-Oropeza, O., & Nemeth, R. S. (2017). Fish spawning aggregations: where well-placed management actions can yield big benefits for fisheries and conservation. Fish

607 and Fisheries, 18(1), 128-144.
608 Euclide, P. T., Lor, Y., Spear, M. J., Tajjioui, T., Vander Zanden, J., Larson, W. A., &
609 Amberg, J. J. (2021). Environmental DNA metabarcoding as a tool for
610 biodiversity assessment and monitoring: reconstructing established fish
611 communities of north-temperate lakes and rivers. Diversity and Distributions,
612 27(10), 1966-1980.
613 Fernández-Delgado, C. (1990). Life history patterns of the common carp, Cyprinus
614 carpio, in the estuary of the Guadalquivir River in south-west Spain.
615 Hydrobiologia, 206(1), 19-28.
616 Fernández, S., Rodríguez, S., Martínez, J. L., Borrell, Y. J., Ardura, A., & García-
617 Vázquez, E. (2018). Evaluating freshwater macroinvertebrates from eDNA
618 metabarcoding: A river Nalón case study. PloS One, 13(8), e0201741.
619 Ficetola, G. F., Miaud, C., Pompanon, F., & Taberlet, P. (2008). Species detection using
620 environmental DNA from water samples. Biology letters, 4(4), 423-425.
621 Foote, A. D., Thomsen, P. F., Sveegaard, S., Wahlberg, M., Kielgast, J., Kyhn, L. A., ...
622 & Gilbert, M. T. P. (2012). Investigating the potential use of environmental DNA
623 (eDNA) for genetic monitoring of marine mammals.
624 Fujiwara, A., Matsuhashi, S., Doi, H., Yamamoto, S., & Minamoto, T. (2016). Use of
625 environmental DNA to survey the distribution of an invasive submerged plant in
626 ponds. Freshwater Science, 35(2), 748-754.
627 Hunter, M. E., Oyler-McCance, S. J., Dorazio, R. M., Fike, J. A., Smith, B. J., Hunter, C.
628 T., ... Hart, K. M. (2015). Environmental DNA (eDNA) sampling improves
629 Jackson, J. E., Raadik, T. A., Lintermans, M., & Hammer, M. (2004). Alien salmonids in
630 Australia: impediments to effective impact management, and future directions.
631 New Zealand Journal of Marine and Freshwater Research, 38(3), 447-455.
632 Jerde, C. L., Mahon, A. R., Chadderton, W. L., & Lodge, D. M. (2011). “Sight-unseen”
633 detection of rare aquatic species using environmental DNA. Conservation letters,
634 4(2), 150-157.
635 Keck, F., Blackman, R. C., Bossart, R., Brantschen, J., Couton, M., Hürlemann, S., ... &
636 Altermatt, F. (2022). Meta-analysis shows both congruence and complementarity
637 of DNA and eDNA metabarcoding to traditional methods for biological
638 community assessment. Molecular Ecology, 31(6), 1820-1835.
639 King, A. J., Tonkin, Z., & Mahoney, J. (2009). Environmental flow enhances native fish
640 spawning and recruitment in the Murray River, Australia. River Research and
641 Applications, 25(10), 1205-1218.
642 Lacoursière-Roussel, A., Rosabal, M., & Bernatchez, L. (2016). Estimating fish
643 abundance and biomass from eDNA concentrations: variability among capture
644 methods and environmental conditions. Molecular ecology resources, 16(6),
645 1401-1414.
646 Lance, R. F., & Guan, X. (2020). Variation in inhibitor effects on qPCR assays and
647 implications for eDNA surveys. Canadian Journal of Fisheries and Aquatic
648 Sciences, 77(1), 23-33.
649 Langlois, V. S., Allison, M. J., Bergman, L. C., To, T. A., & Helbing, C. C. (2021). The

need for robust qPCR-based eDNA detection assays in environmental monitoring and species inventories. Environmental DNA, 3(3), 519-527.

Legendre, P., & Anderson, M. J. (1999). Distance-based redundancy analysis: testing multispecies responses in multifactorial ecological experiments. Ecological monographs, 69(1), 1-24.

MacKinnon, J. G., & White, H. (1985). Some heteroskedasticity-consistent covariance matrix estimators with improved finite sample properties. Journal of econometrics, 29(3), 305-325.

Minamoto, T., Miya, M., Sado, T., Seino, S., Doi, H., Kondoh, M., ... & Uchii, K. (2021). An illustrated manual for environmental DNA research: Water sampling guidelines and experimental protocols. Environmental DNA, 3(1), 8-13.

Miya, M., Sato, Y., Fukunaga, T., Sado, T., Poulsen, J. Y., Sato, K., ... & Iwasaki, W. (2015). MiFish, a set of universal PCR primers for metabarcoding environmental DNA from fishes: detection of more than 230 subtropical marine species. Royal Society open science, 2(7), 150088.

Ministry of Land, Infrastructure, Transport and Tourism, 2022. River Environmental Database (in Japanese). http://www.nilim.go.jp/lab/fbg/ksnkankyo/dl_82_index.html. (Accessed 25 August 2022).

Mize, E. L., Erickson, R. A., Merkes, C. M., Berndt, N., Bockrath, K., Credico, J., ... & Monroe, E. (2019). Refinement of eDNA as an early monitoring tool at the landscape-level: Study design considerations. Ecological Applications, 29(6), e01951.

Nakagawa, H., Fukushima, K., Sakai, M., Wu, L., & Minamoto, T. (2022). Relationships between the eDNA concentration obtained from metabarcoding and stream fish abundance estimated by the removal method under field conditions. Environmental DNA. https://doi.org/10.1002/edn3.346

Nakagawa, H., Yamamoto, S., Sato, Y., Sado, T., Minamoto, T., & Miya, M. (2018). Comparing local-and regional-scale estimations of the diversity of stream fish using eDNA metabarcoding and conventional observation methods. Freshwater Biology, 63(6), 569-580.

Oksanen, J., Simpson, G., Blanchet, F., & Weedon, J. (2022). vegan: Community Ecology Package. R package version 2.6-2, https://CRAN.R-project.org/package=vegan

Pedersen, E. J., Miller, D. L., Simpson, G. L., & Ross, N. (2019). Hierarchical generalized additive models in ecology: an introduction with mgcv. PeerJ, 7, e6876.

Pilliod, D. S., Goldberg, C. S., Arkle, R. S., & Waits, L. P. (2013). Estimating occupancy and abundance of stream amphibians using environmental DNA from filtered water samples. Canadian Journal of Fisheries and Aquatic Sciences, 70(8), 1123-1130.

R Core Team (2022). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. https://www.R-

project.org/.
Rodriguez-Ezpeleta, N., Morissette, O., Bean, C. W., Manu, S., Banerjee, P., Lacoursière-Roussel, A., ... & Deiner, K. (2021). Trade-offs between reducing complex terminology and producing accurate interpretations from environmental DNA: Comment on "Environmental DNA: What's behind the term?" by Pawlowski et al.,(2020). Molecular Ecology, 30(19), 4601-4605.
Rowland, W. J. (1999). Studying visual cues in fish behavior: a review of ethological techniques. Environmental Biology of Fishes, 56(3), 285-305.
Sakata, M. K., Yamamoto, S., Gotoh, R. O., Miya, M., Yamanaka, H., & Minamoto, T. (2020). Sedimentary eDNA provides different information on timescale and fish species composition compared with aqueous eDNA. Environmental DNA, 2(4), 505-518.
Samii, C., & Aronow, P. M. (2012). On equivalencies between design-based and regression-based variance estimators for randomized experiments. Statistics & Probability Letters, 82(2), 365-370.
Scott, B. E., Marteinsdottir, G., Begg, G. A., Wright, P. J., & Kjesbu, O. S. (2006). Effects of population size/age structure, condition and temporal dynamics of spawning on reproductive output in Atlantic cod (*Gadus morhua*). Ecological Modelling, 191(3-4), 383-415.
Scriver, M., Marinich, A., Wilson, C., & Freeland, J. (2015). Development of species-specific environmental DNA (eDNA) markers for invasive aquatic plants. Aquatic Botany, 122, 27-31.
Snyder, D. E. (2003). Invited overview: conclusions from a review of electrofishing and its harmful effects on fish. Reviews in fish biology and fisheries, 13(4), 445-453.
Spear, S. F., Groves, J. D., Williams, L. A., & Waits, L. P. (2015). Using environmental DNA methods to improve detectability in a hellbender (*Cryptobranchus alleganiensis*) monitoring program. Biological Conservation, 183, 38-45.
Stefan, H. G., Hondzo, M., Fang, X., Eaton, J. G., & McCormick, J. H. (1996). Simulated long term temperature and dissolved oxygen characteristics of lakes in the north-central United States and associated fish habitat limits. Limnology and Oceanography, 41(5), 1124-1135.
Takahara, T., Minamoto, T., & Doi, H. (2013). Using environmental DNA to estimate the distribution of an invasive fish species in ponds. PloS one, 8(2), e56584.
Thalinger, B., Wolf, E., Traugott, M., & Wanzenböck, J. (2019). Monitoring spawning migrations of potamodromous fish species via eDNA. Scientific reports, 9(1), 15388.
Thomsen, P. F., & Willerslev, E. (2015). Environmental DNA–An emerging tool in conservation for monitoring past and present biodiversity. Biological conservation, 183, 4-18.
Tréguier, A., Paillisson, J. M., Dejean, T., Valentini, A., Schlaepfer, M. A., & Roussel, J. M. (2014). Environmental DNA surveillance for invertebrate species: Advantages and technical limitations to detect invasive crayfish P rocambarus clarkii in freshwater ponds. Journal of Applied Ecology, 51(4), 871-879.

736 Tsuji, S., Inui, R., Nakao, R., Miyazono, S., Saito, M., Kono, T., & Akamatsu, Y. (2022b).
737 Quantitative environmental DNA metabarcoding shows high potential as a novel
738 approach to quantitatively assess fish community. Scientific Reports, 12(1).
739 https://doi.org/10.1038/s41598-022-25274-3
740 Tsuji, S., Nakao, R., Saito, M., Minamoto, T., & Akamatsu, Y. (2022a). Pre-
741 centrifugation before DNA extraction mitigates extraction efficiency reduction of
742 environmental DNA caused by the preservative solution (benzalkonium chloride)
743 remaining in the filters. Limnology, 23(1), 9-16.
744 Tsuji, S., & Shibata, N. (2021). Identifying spawning events in fish by observing a spike
745 in environmental DNA concentration after spawning. Environmental DNA, 3(1),
746 190-199.
747 Ulibarri, R. M., Bonar, S. A., Rees, C., Amberg, J., Ladell, B., & Jackson, C. (2017).
748 Comparing efficiency of American Fisheries Society standard snorkeling
749 techniques to environmental DNA sampling techniques. North American Journal
750 of Fisheries Management, 37(3), 644-651.
751 Ushio, M., Fukuda, H., Inoue, T., Makoto, K., Kishida, O., Sato, K., ... & Miya, M. (2017).
752 Environmental DNA enables detection of terrestrial mammals from forest pond
753 water. Molecular Ecology Resources, 17(6), e63-e75.
754 Ushio, M., Furukawa, S., Murakami, H., Masuda, R., & Nagano, A. J. (2022). An
755 efficient early-pooling protocol for environmental DNA metabarcoding.
756 Environmental DNA. https://doi.org/10.1002/edn3.337
757 Ushio, M., Murakami, H., Masuda, R., Sado, T., Miya, M., Sakurai, S., ... & Kondoh, M.
758 (2018b). Quantitative monitoring of multispecies fish environmental DNA using
759 high-throughput sequencing. Metabarcoding and Metagenomics, 2, e23297.
760 Ushio, M., Murata, K., Sado, T., Nishiumi, I., Takeshita, M., Iwasaki, W., & Miya, M.
761 (2018a). Demonstration of the potential of environmental DNA as a tool for the
762 detection of avian species. Scientific reports, 8(1), 1-10.
763 Ushio, M., Sado, T., Fukuchi, T., Sasano, S., Masuda, R., Osada, Y., & Miya, M. (2022).
764 Temperature sensitivity of the interspecific interaction strength of coastal marine
765 fish communities. bioRxiv. https://doi.org/10.1101/2022.06.02.494625
766 Wickham, H. (2016). Data analysis. In ggplot2 (pp. 189-201). Springer, Cham.
767 Wood, S. N. (2006). Generalized additive models: an introduction with R. chapman and
768 hall/CRC.
769 Wood, S. N. (2011). Fast stable restricted maximum likelihood and marginal likelihood
770 estimation of semiparametric generalized linear models. Journal of the Royal
771 Statistical Society: Series B (Statistical Methodology), 73(1), 3-36.
772 Wood, S.N. (2017). Generalized Additive Models: An Introduction with R (2nd edition).
773 CRC/Taylor & Francis.
774 [dataset] Wu, L., Osugi, T., Inagawa, T., Okitsu, J., Sakamoto, S., & Minamoto, T. (2023).
775 Quantitative eDNA metabarcoding survey at the Hebisawagawa front reservoir.
776 DDBJ Sequence Read Archive (https://www.ddbj.nig.ac.jp/dra/index-e.html),
777 PRJDB15018.
778 Wu, L., Yamamoto, Y., Yamaguchi, S., & Minamoto, T. (2022). Spatiotemporal changes

779 in environmental DNA concentrations caused by fish spawning activity.
780 Ecological Indicators, 142, 109213.
781 Wu, L., Wu, Q., Inagawa, T., Okitsu, J., Sakamoto, S., & Minamoto, T. (2023).
782 Estimating the spawning activity of fish species using nuclear and mitochondrial
783 environmental DNA concentrations and their ratios. Freshwater Biology, 68(1),
784 103-114.
785 Wu, Q., Kawano, K., Uehara, Y., Okuda, N., Hongo, M., Tsuji, S., ... & Minamoto, T.
786 (2018). Environmental DNA reveals nonmigratory individuals of Palaemon
787 paucidens overwintering in Lake Biwa shallow waters. Freshwater Science, 37(2),
788 307-314.
789 Xie, R., Zhao, G., Yang, J., Wang, Z., Xu, Y., Zhang, X., & Wang, Z. (2021). eDNA
790 metabarcoding revealed differential structures of aquatic communities in a
791 dynamic freshwater ecosystem shaped by habitat heterogeneity. Environmental
792 research, 201, 111602.
793 Yamanaka, H., Minamoto, T., Matsuura, J., Sakurai, S., Tsuji, S., Motozawa, H., ... &
794 Kondo, A. (2017). A simple method for preserving environmental DNA in water
795 samples at ambient temperature by addition of cationic surfactant. Limnology,
796 18(2), 233-241.
797 Yu, G. (2022). Data Integration, Manipulation and Visualization of Phylogenetic Trees.
798 CRC Press.

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## Data Accessibility Statement

The raw data are available at DDBJ Sequence Read Archive (BioProject: PRJDB15018). Raw iSeq data: DRR426842-DRR426993. Sample information stored using the MIMS water package: SAMD00567991-SAMD00568142. The R script and related files used to analyze the data are accessible at https://github.com/LHWuGH/MHS_quantitative_eDNA_metabarcoding .

## Benefit-Sharing Statement

The benefits of this research accrue from the sharing of our data and results in public databases, as described above.

## Author Contributions

LW and TM conceived of and designed the study. OT performed the traditional surveys. TI and JO collected the eDNA water samples. LW performed the molecular experiments and statistical analyses of the data. LW and TM wrote and edited the manuscript. All the authors discussed the results and contributed to the development of the manuscript.

## Conflict of Interest

Toshifumi Minamoto is an inventor of the patent for the use of Benzalkonium Chloride for eDNA preservation.

## Figure Legends

Figure 1. Map of the sampling sites within the Hebisawagawa front reservoir.

Figure 2. Phylogenetic tree based on the neighbor-joining method and the relative abundance of 20 fish species. Relative abundance represents species composition across a given year and survey type.

Figure 3. Linear regression to test the correlation between the eDNA concentration and individual numbers of fish from the 2019 eDNA survey and the traditional survey. Each point represents a different species of fish. Shading = 95% confidence interval based on robust standard errors calculated by heteroskedasticity-consistent covariance matrix estimator (HC2 estimator).

Figure 4. Principal coordinate analysis (PCoA) with fitted generalized additive model (GAM) surfaces overlaid to illustrate the relationships between fish species composition and environmental variables. Shaded = 80% confidence ellipse; DE = deviance explained. *G. urotaenia* = *Gymnogobius urotaenia*; *M. salmoides* = *Micropterus salmoides*; *L. macrochirus* = *Lepomis macrochirus*; *S. asotus* = *Silurus asotus*; *M. anguillicaudatus* = *Misgurnus anguillicaudatus*; *T. hakonensis* = *Tribolodon hakonensis*; *Z. platypus* = *Zacco platypus*; *C. idella* = *Ctenopharyngodon idella*; *C. cuvieri* = *Carassius cuvieri*; *C. carpio* = *Cyprinus carpio*; *H. barbus* = *Hemibarbus barbus*; *G. elongatus* = *Gnathopogon elongatus*; *P. parva* = *Pseudorasbora parva*.

Figure 5. Relationships between fish-specific eDNA concentration and environmental variables. The x-axis represents environmental variables. The y-axis represents the linear predictor of component smooth functions. Points represent partial residuals. Shading = 95% confidence interval. *G. urotaenia* = *Gymnogobius urotaenia*; *M. salmoides* = *Micropterus salmoides*; *L. macrochirus* = *Lepomis macrochirus*; *S. asotus* = *Silurus asotus*; *M. anguillicaudatus* = *Misgurnus anguillicaudatus*; *T. hakonensis* = *Tribolodon hakonensis*; *Z. platypus* = *Zacco platypus*; *C. idella* = *Ctenopharyngodon idella*; *C. cuvieri* = *Carassius cuvieri*; *C. carpio* = *Cyprinus carpio*; *H. barbus* = *Hemibarbus barbus*; *G. elongatus* = *Gnathopogon elongatus*; *P. parva* = *Pseudorasbora parva*.

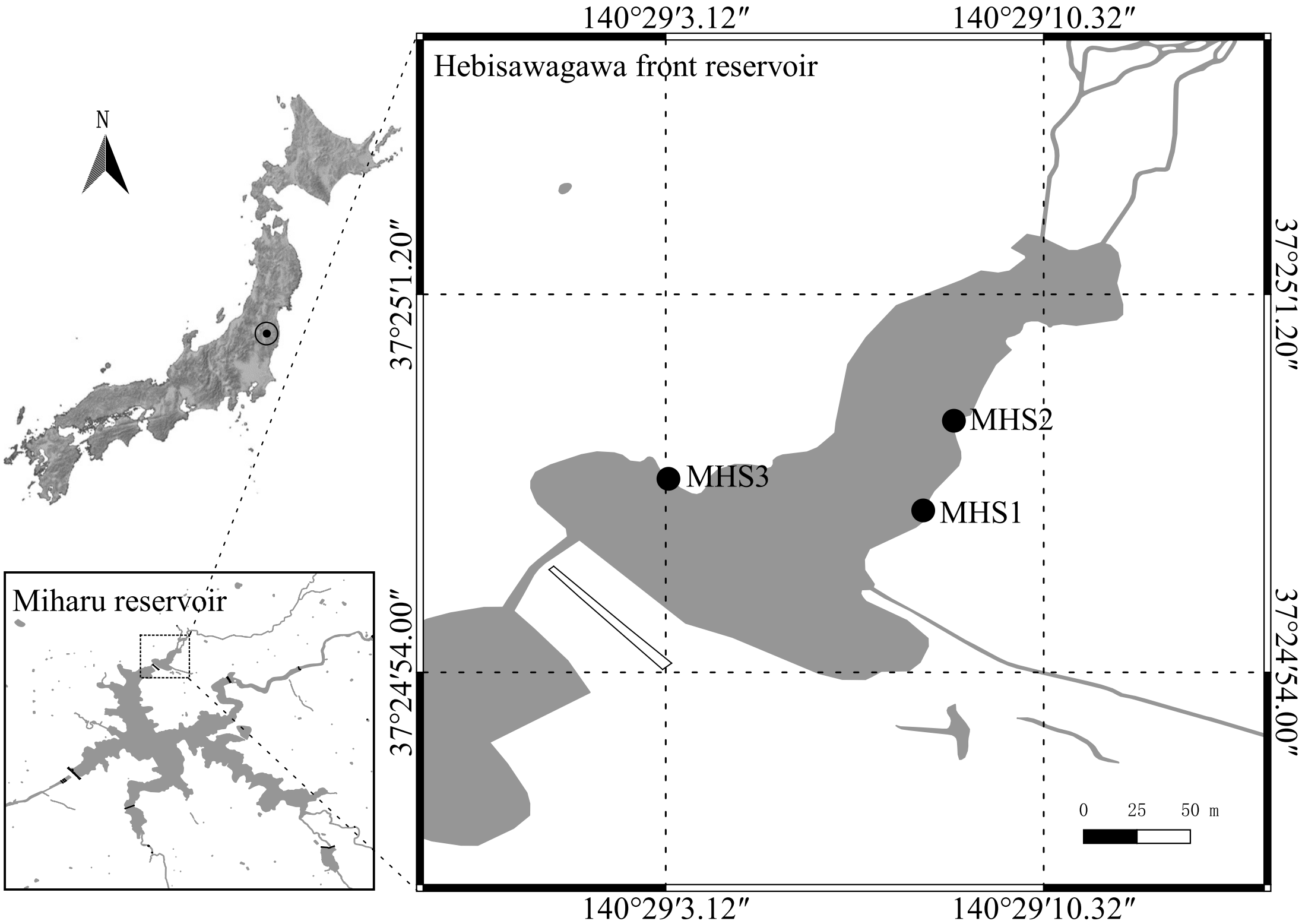
Figure 6. Spawning probabilities estimated by eDNA concentration. The outliers of eDNA concentration were used to establish a hierarchical generalized additive model (HGAM), and then the model was used to predict the spawning probability based on the eDNA sample data. The x-axis represents sampling time. The y-axis represents years and sites. The dashed box refers to the fish spawning period recorded by the Research Institute of Environment, Agriculture and Fisheries, Osaka Prefecture. *G. urotaenia* = *Gymnogobius urotaenia*; *M. salmoides* = *Micropterus salmoides*; *L. macrochirus* = *Lepomis macrochirus*; *M. anguillicaudatus* = *Misgurnus anguillicaudatus*; *T. hakonensis* = *Tribolodon hakonensis*; *Z. platypus* = *Zacco platypus*; *C. cuvieri* = *Carassius cuvieri*; *C. carpio* = *Cyprinus carpio*; *H.*

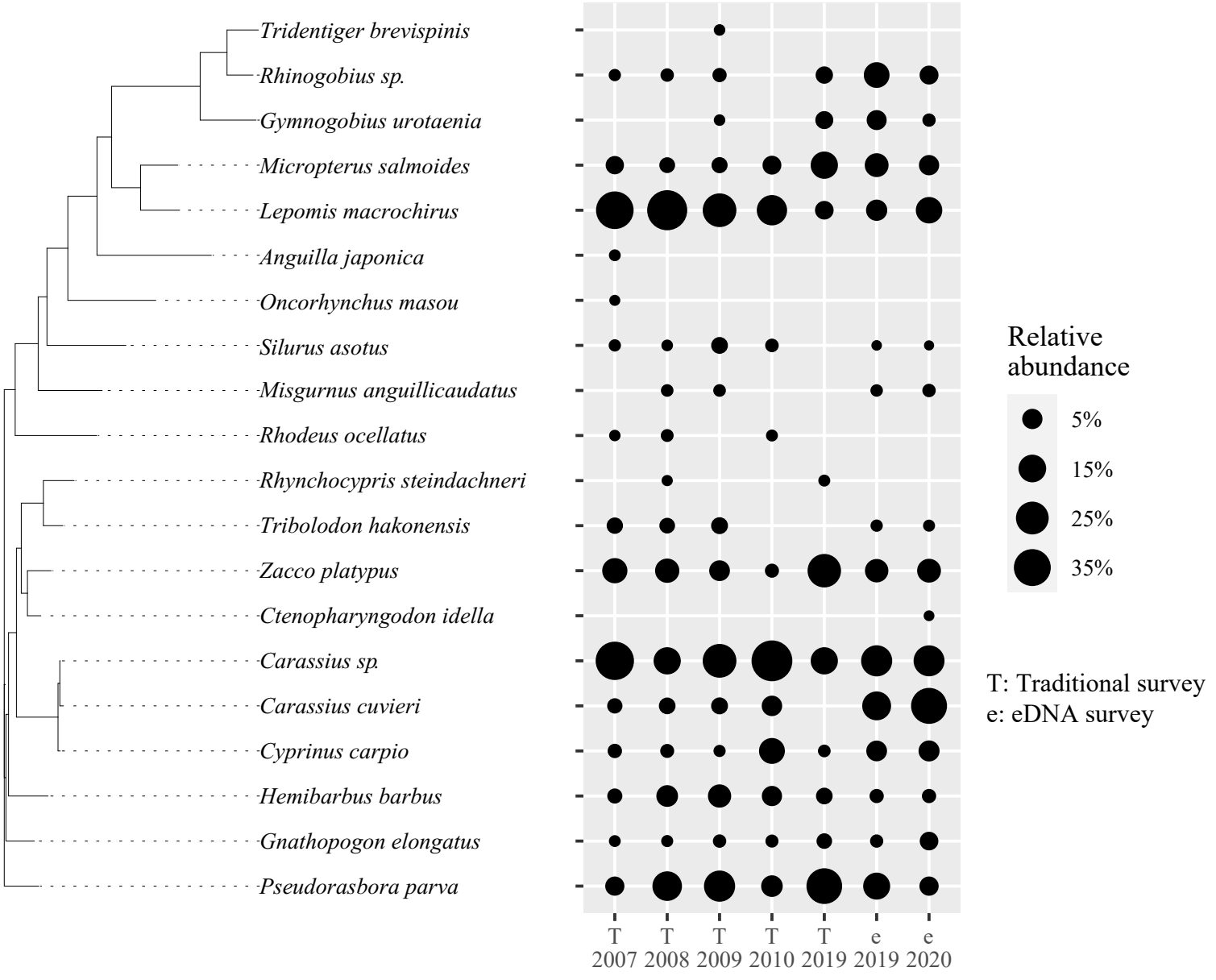
*barbus* = *Hemibarbus barbus*; *G. elongatus* = *Gnathopogon elongatus*; *P. parva* = *Pseudorasbora parva*.

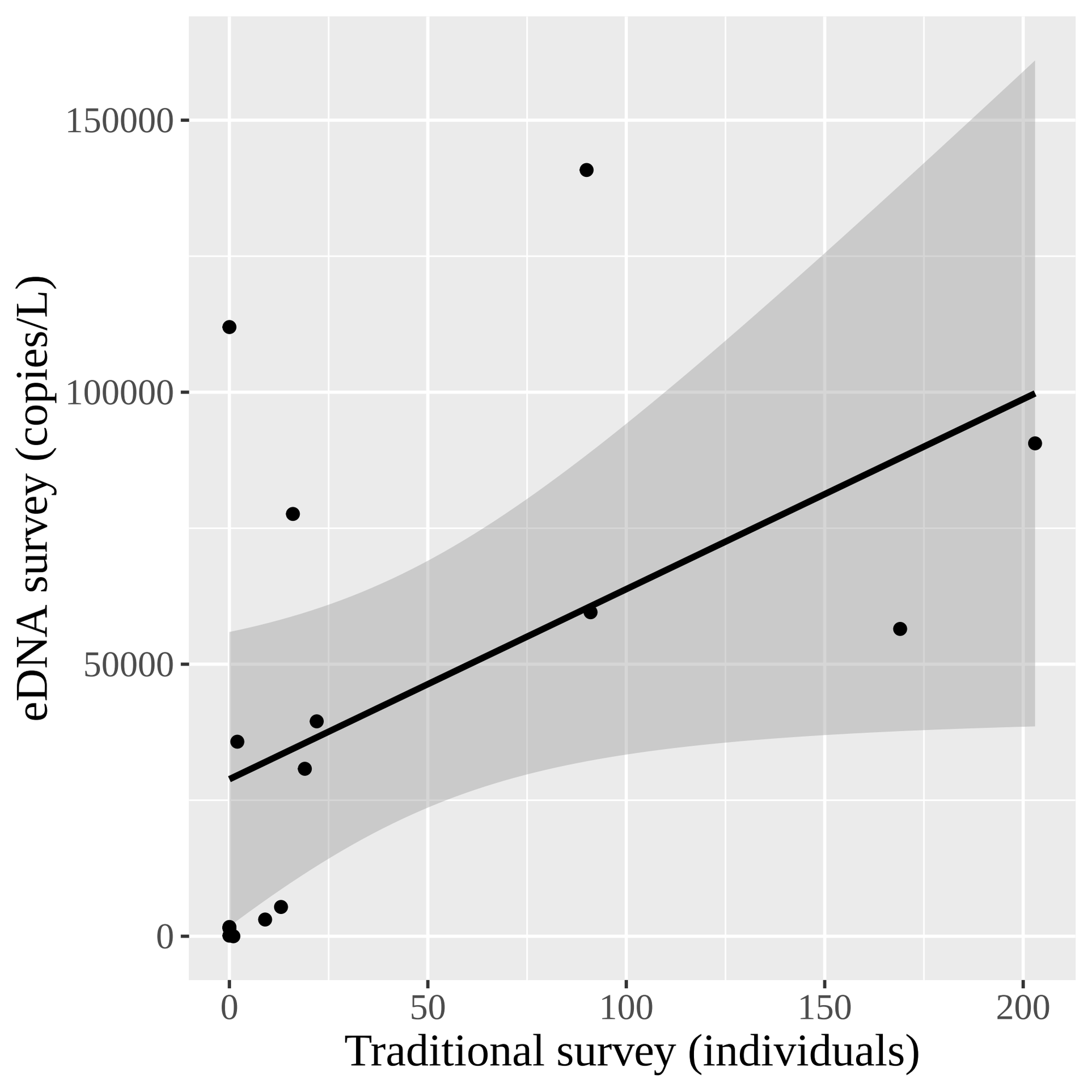
Table 1. Results of the hierarchical generalized additive model used to estimate the changes in fish eDNA concentration with environmental variables

| | edf | p-value |
|---|---|---|
| s(WT) | 2.799 | 0.038 * |
| s(WT,Fish) | 32.581 | <0.001 *** |
| s(pH) | 2.765 | 0.031 * |
| s(pH,Fish) | 14.704 | <0.001 *** |
| s(EC) | 1.001 | <0.001 *** |
| s(EC,Fish) | 0.721 | 0.392 |
| s(Site,Year) | 4.749 | <0.001 *** |

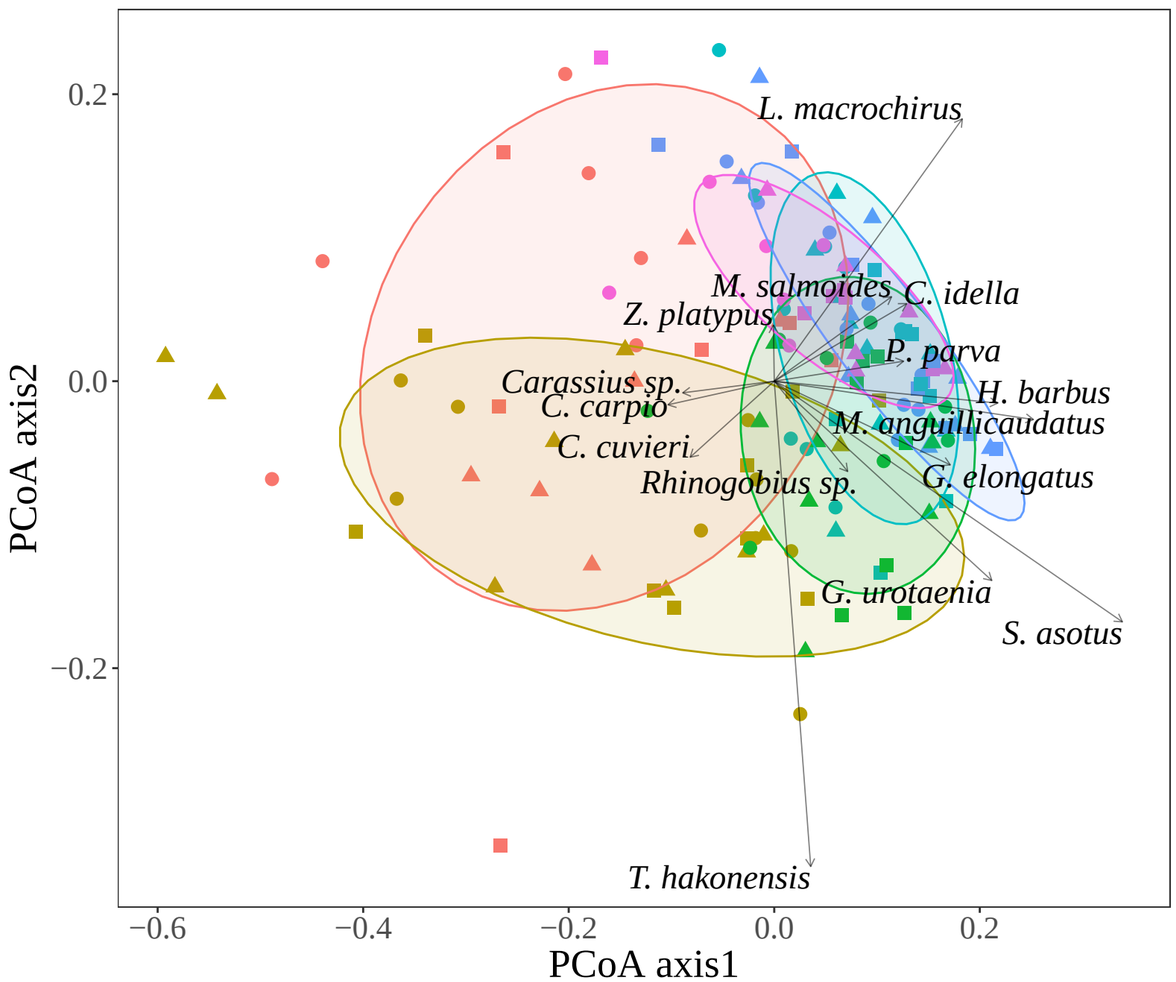
Significance level: *** <0.001; ** < 0.01; * <0.05
WT = water temperature; EC = electrical conductivity;
s() = a smooth term; edf = effective degrees of freedom


140°29′3.12″
140°29′10.32″
Hebisawagawa front reservoir
N
37°25′1.20″
37°24′54.00″
MHS2
MHS3
MHS1
0 25 50 m
Miharu reservoir

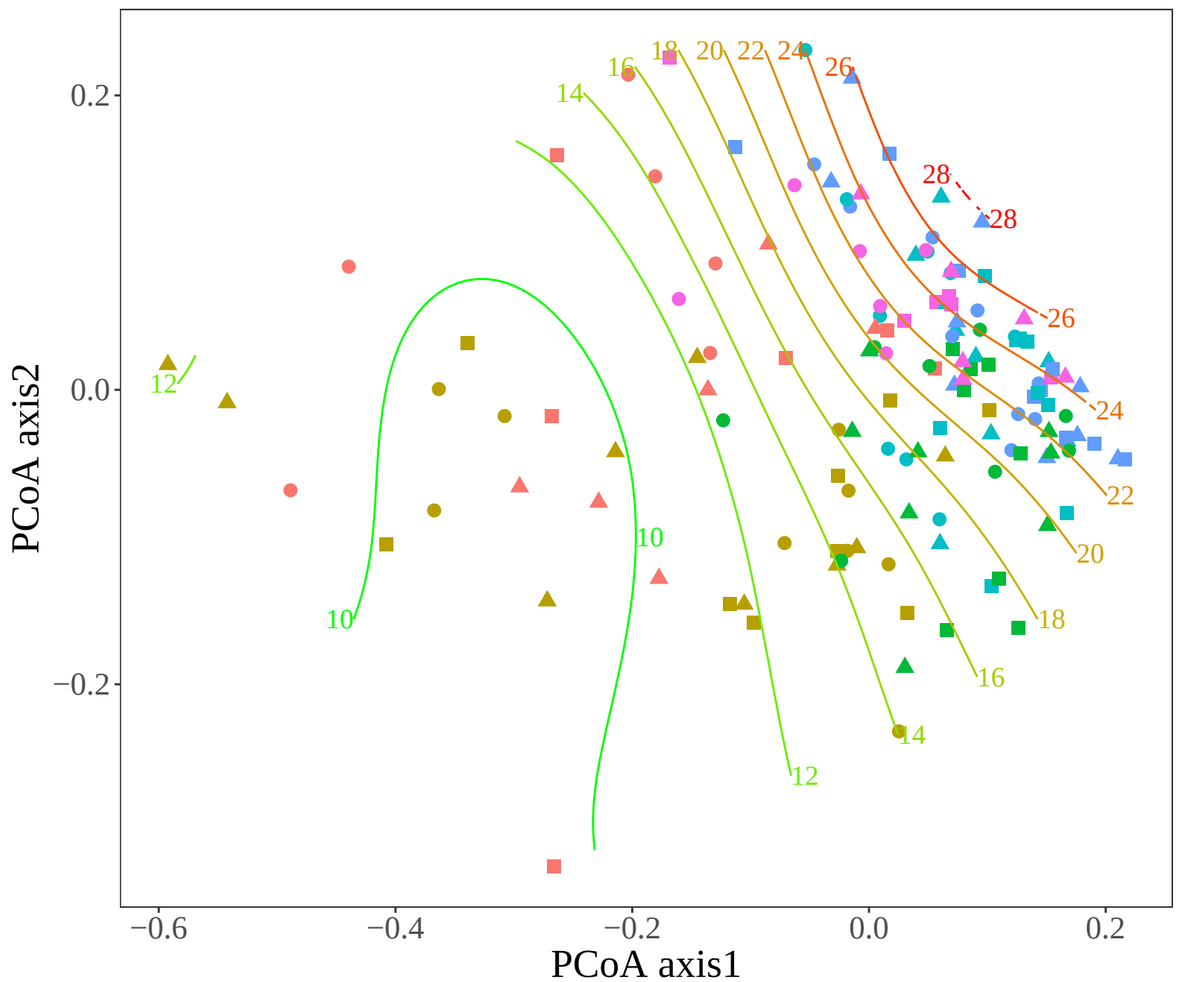

Tridentiger brevispinis
Rhinogobius sp.
Gymnogobius urotaenia
Micropterus salmoides
Lepomis macrochirus
Anguilla japonica
Oncorhynchus masou
Silurus asotus
Misgurnus anguillicaudatus
Rhodeus ocellatus
Rhynchocypris steindachneri
Tribolodon hakonensis
Zacco platypus
Ctenopharyngodon idella
Carassius sp.
Carassius cuvieri
Cyprinus carpio
Hemibarbus barbus
Gnathopogon elongatus
Pseudorasbora parva
T 2007
T 2008
T 2009
T 2010
T 2019
e 2019
e 2020
Relative abundance
5%
15%
25%
35%
T: Traditional survey
e: eDNA survey


150000
100000
50000
0
eDNA survey (copies/L)
0
50
100
150
200
Traditional survey (individuals)

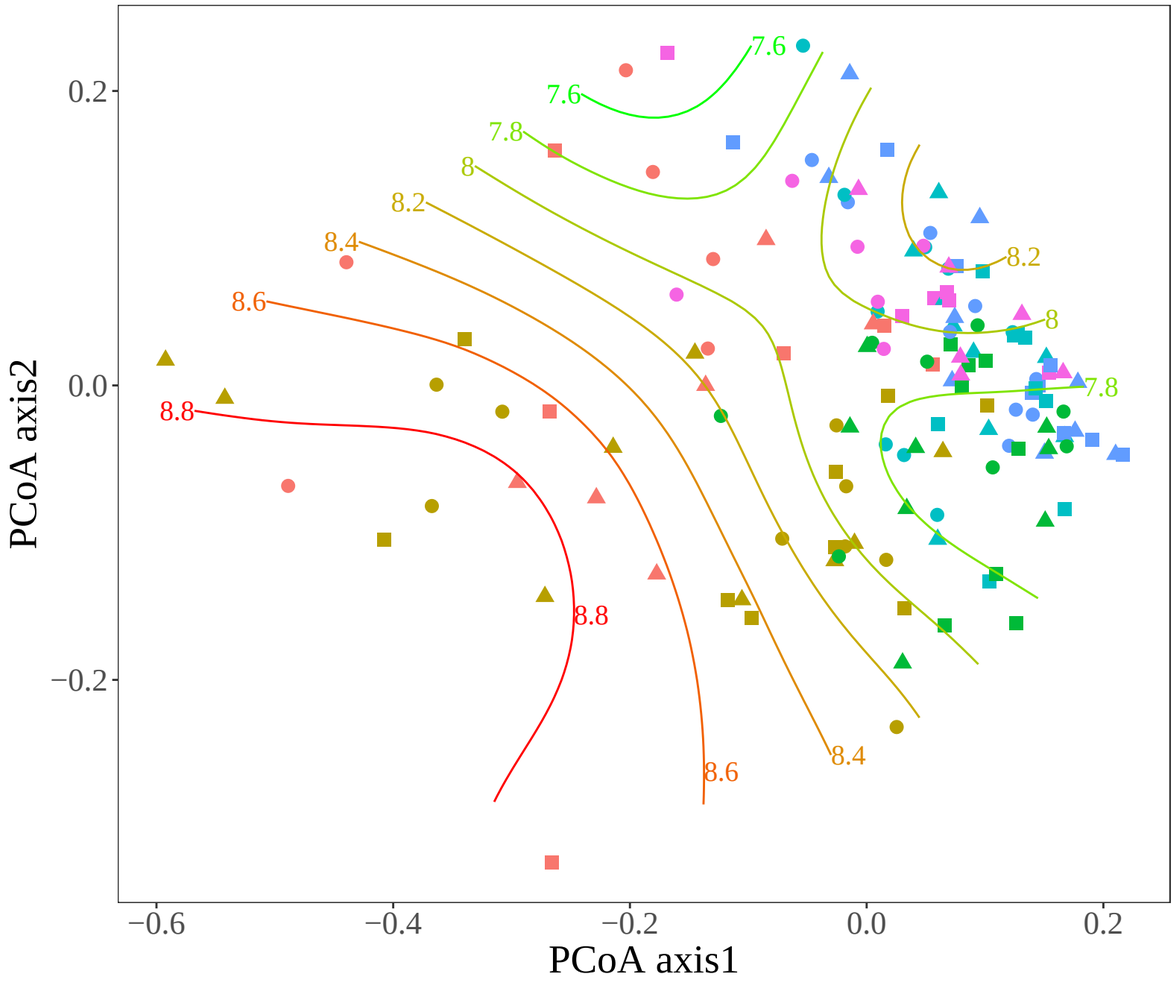
a. PCoA based on Bray−Curtis dissimilarity



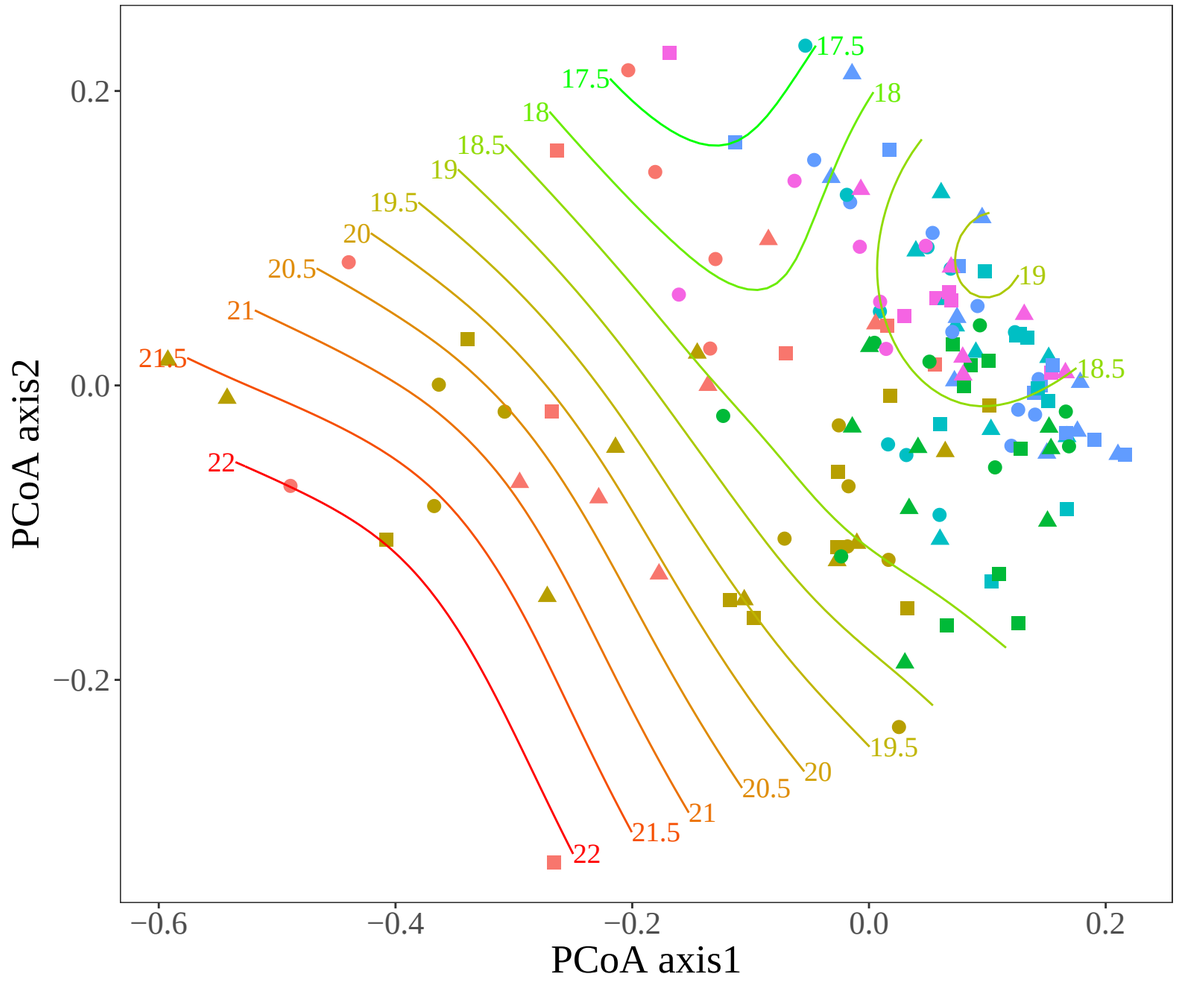

b. Water temperature (°C), $p < 0.001$, DE = 51.8%



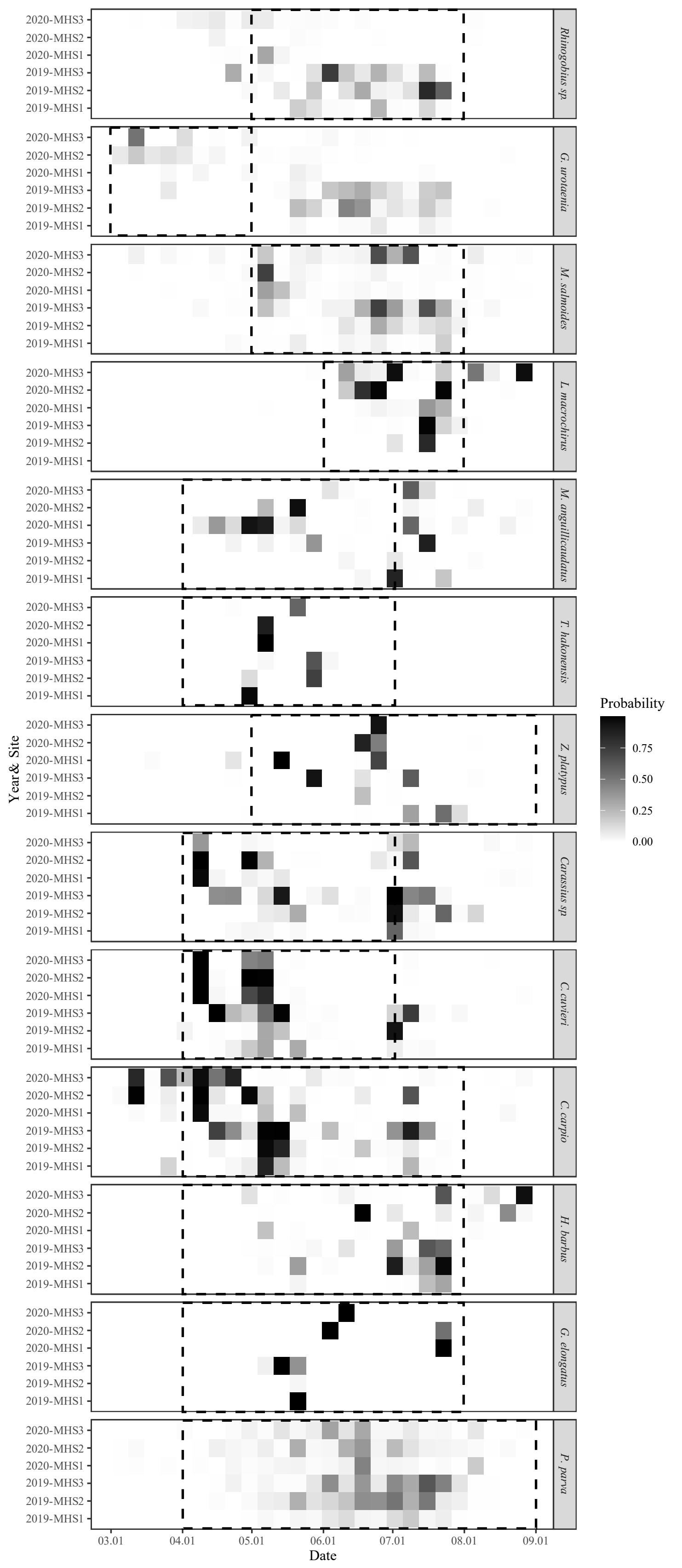

c. pH, $p < 0.001$, DE = 25.3%




d. EC (mS/m), $p = 0.002$, DE = 14.3%



Water temperature (°C)
pH
EC (mS/m)
*Rhinogobius sp.*
*G. urotaenia*
*M. salmoides*
*L. macrochirus*
*S. asotus*
*M. anguillicaudatus*
*T. hakonensis*
*Z. platypus*
*C. idella*
*Carassius sp.*
*C. cuvieri*
*C. carpio*
*H. barbus*
*G. elongatus*
*P. parva*
Component smooth function
Environment variable


Rhinogobius sp.
G. urotaenia
M. salmoides
L. macrochirus
M. anguillicaudatus
T. hakonensis
Z. platypus
Carassius sp
C. cuvieri
C. carpio
H. barbus
G. elongatus
P. parva
2020-MHS3
2020-MHS2
2020-MHS1
2019-MHS3
2019-MHS2
2019-MHS1
Year& Site
03.01
04.01
05.01
06.01
07.01
08.01
09.01
Date
Probability
0.75
0.50
0.25
0.00