Efficacy of natural marine sponges as a passive environmental DNA sampler for freshwater fish diversity monitoring

Author's Name: **Ryohei Nakao[1], Manami Inaba[1], Seiji Miyazono[1], Minoru Saito[1,2], Keita Maruyama[1,4], Fumiko Imamura[3], Yoshihisa Akamatsu[1]**

Institution:

[1] Graduate School of Science and Technology for Innovation, Yamaguchi University, Yamaguchi University, 2-16-1, Tokiwadai, Ube, Yamaguchi, Japan

[2] Japan International Research Center for Agricultural Sciences, 1-1 Ohwashi, Tsukuba, Ibaraki, Japan

[3] Research and Development Center, Nippon Koei Co., Ltd, 2304, Inarihara, Tsukuba, Ibaraki, Japan

[4] Department of Ocean Sciences, Tokyo University of Marine Science and Technology, 4-5-7 Konan, Minato-ku, Tokyo 108-8477, Japan

Correspond Author: Ryohei Nakao, Graduate School of Science and Technology for Innovation, Yamaguchi University, 2-16-1, Tokiwadai, Ube, Yamaguchi, 755-8611, Japan.

E-mail: lineck92@yahoo.co.jp

Abstract

Environmental DNA (eDNA) analysis is a cost-effective and non-invasive tool for species and biodiversity monitoring in aquatic environments. Passive eDNA sampling method is focused as a novel and alternative to conventional sampling methods, such as water filtration. In this study, we examined the efficacy of the sponge skeleton as an eDNA passive sampler for the freshwater fish monitoring tool. We compared the performance of passive sampling method with standard water filtration in the river environment. A total of five different DNA extraction methods were also conducted in three experiments, and a suitable DNA extraction method from sponge skeleton was estimated. Quantitative fish metabarcoding using MiFish primers revealed no significant difference in species richness between water and passive sampling methods. While both sampling methods showed comparable trends in fish community structures, different clusters were identified for water sampling and passive samplers by the differences in DNA concentration of each fish species. Fish diversity in passive samples was comparable among the four DNA extraction methods using the Direct Capture method, except for the filtration method. Our results demonstrate the efficacy of passive eDNA sampling for monitoring freshwater fish diversity and potential use of sponge skeletons for the absorption material of the eDNA passive sampler.

Keywords: environmental DNA, metabarcoding, passive sampler, river ecosystem, freshwater fish

Introduction

Environmental DNA (eDNA) analysis is increasingly used to monitor the biodiversity in various ecosystems. This method can detect diverse organisms by capturing, extracting DNA from excrements and extracellular DNA from different environmental samples, such as water, soil, and air (Turner et al. 2015, Tsuji et al. 2019, Lynggaard et al. 2022). The eDNA analysis is also a sensitive, non-invasive and broadly applicable tool for species detection. One of eDNA approach, eDNA metabarcoding, which can detect multiple species simultaneously (Miya et al., 2015; Deiner et al., 2017), is useful for performing rapid and large-scale multi-species surveys (Olds et al., 2016; Yamamoto et al., 2017). In particular, eDNA metabarcoding has been used in fish diversity monitoring in a variety of water bodies, ranging in size and type of aquatic ecosystems (Hänfling et al. 2016, Nakagawa et al. 2018, Oka et al. 2021). Many studies generally show that eDNA metabarcoding is a more effective and sensitive method for fish diversity assessment compared with the conventional approaches such as visual census, fyke net capturing and electrofishing (Yamamoto et a. 2017, Shaw et al. 2016).

Water eDNA, an environmental DNA collected from water samples, is most commonly used for fish diversity monitoring in the eDNA approach. Water eDNA of fishes is generally collected and concentrated from water samples by the filtration using artificial membranes (Yamamoto et al. 2017, Ushio 2019, Jeunen et al. 2019). However, fish diversity in water eDNA is often a snapshot information, and insufficient sampling effort could underestimate the fish diversity

58 (Zinger et al. 2019). For example, if the concentration of target DNA is low, such as rare species,
59 small sampling volumes and few replicates can lead to false negatives of these species.
60 Furthermore, since eDNA concentrations reflect the activity levels of target species (e.g., diurnal or
61 nocturnal), the mismatch of timing between the water sampling and fish ecology may increase false
62 negative detections. Large volume of water filtration and sampling replicate should be needed to
63 remove these false negatives and to provide reproducibility in fish diversity. However, the
64 conventional sampling method with water filtration requires a lot of time and cost to achieve such
65 improvements.
66 Passive eDNA collection methods, which directly submerge the sampling material in the
67 water body, facilitate the increase of reproducible biomonitoring as a simpler and more cost-
68 effective sampling method than traditional active filtration methods (Bessey et al., 2021; Kirtane et
69 al., 2020; Zhang et al. 2024). Absorption materials of passive eDNA sampling used in previous
70 studies generally have porous substances, adsorption capabilities enough to detect the target eDNA,
71 and simple or user-friendly form. For example, submersion materials in the passive eDNA sampling
72 method include activated carbon, montmorillonite (Kirtane et al., 2020), living sponge (Mariani et
73 al., 2019; Cai et al., 2022), and biofilm (Rivera et al., 2021). In the case of artificial materials,
74 Verdier et al. (2022) created the passive, 3D-printed and easy-to-use eDNA sampler using
75 hydroxyapatite, a natural mineral with a high DNA adsorption capacity. Yan et al. (2024) deployed
76 the passive sampling using GF filters by angling (short time immersion at site) and trolling (towing

77 in the water) method in large lake environments. These studies showed that passive collection
78 methods detected fish as effectively as conventional active filtration methods and provided similar
79 estimates of total fish biodiversity. In addition, because passive eDNA samplers require minimal or
80 no supporting equipment (e.g., no vacuum or peristaltic pumps), it is suitable deployment to remote
81 environments, and for non-experts.

82 In particular, natural and artificial sponges are expected to be superior eDNA passive
83 samplers due to their ubiquitous distribution, high regeneration ability, and ease of sampling. For
84 example, filter-feeding organisms have been shown to naturally accumulate eDNA in their tissue
85 matrices (Jeunen et al., 2021). Mariani et al. (2019) showed that eDNA extracted from alive marine
86 sponges can detect variety of marine fishes and mammals in the Mediterranean Sea and Antarctic
87 Ocean. In addition, Turon et al. (2020) highlighted the suitability and cost-effectiveness of alive
88 marine sponges as an eDNA passive sampler to describe tropical fish communities from coral reefs
89 in Southeast Asia. Jeunen et al. (2022) suggest that more porous passive filter materials, such as
90 artificial sponges, potentially achieve higher detection rates through an adsorption and retaining
91 effect of less-abundant DNA and DNA bound to particulate matter. Therefore, natural sponge
92 skeletons potentially achieve the effective material of passive eDNA sampler. However, knowledge
93 on the application of sponge skeletons to eDNA studies is insufficient, not only in marine but also
94 in freshwater environments, especially in rivers.

95 In this study, we examined the efficacy of the sponge skeleton as an eDNA passive sampler
96 (sponge sampler) in freshwater river environment. We submerged sponge samplers in the river and
97 estimated the freshwater fish diversity from the sponge eDNA samples using eDNA metabarcoding.
98 In addition, to examine the performance of eDNA accumulation with long-term immersion of
99 sponges, we also collected river water eDNA samples, the major material in eDNA surveys, in
100 parallel with sponge eDNA samples, and compared differences in the number of fish species and
101 fish species composition between passive and water samples. Furthermore, several sampling and
102 DNA purification methods in passive samplers were examined to estimate the optimal passive
103 sampling method using the sponge skeletons.
104

105 **Materials and Methods**

106 *Study field and environmental DNA samplings*

107 In this study, field surveys were conducted in the middle reaches of the Saba River Basin,
108 Yamaguchi Prefecture, Japan in July 30–31, and October 3–4 2021, and May 9–10 2022 (Fig.1A,
109 B). Fish activity times in rivers are different for species (e.g., diurnal or nocturnal), so the species
110 composition detected may not be identical depending on the timing of the water sampling. Sponge
111 passive samplers could potentially overcome these variabilities of species detection through long-
112 term eDNA adsorption on the sponge skeleton in the water. In order to accurately assess the
113 function of eDNA adsorption on the passive sampler, we carried out multi-time water sampling

114 during the immersion of the passive sampler. In water sampling, 3 × 1 L of surface water samples
115 were directly collected in the center of riffle using bleached plastic bottles every 2 hours (0, 2, 4, 6,
116 8, 10, 12, 14, 16, 18, 20, 22, 24 h in 2021) or 3 hours (0, 3, 6, 9, 12, 15, 18, 21, 24 h in 2022) after
117 submerging the equipment (Fig.1C). In this study, we started all experiments at 11.00. Therefore, in
118 Experiments 1 and 2 in this study, we defined 0-6 h (11:00-17:00) and 20-24 h (7:00-11:00) as
119 daytime and 8-18 h (19:00-5:00) as nighttime. In Experiment 3, we also defined 0-6 hours (11:00-
120 17:00) and 21-24 hours (8:00-11:00) as daytime and 9-18 hours (20:00-5:00) as nighttime. Water
121 samplings were also carried out the downstream of the equipment to prevent the contamination due
122 to river-bed disturbance caused by human movement. Water samples were immediately filtered on
123 site using a portable pump (Sentino microbiology pump; Cytiva, Tokyo, Japan) and GF/F glass
124 fiber filter (pore size 0.7μm; Cytiva). After filtration, used equipment was bleached to prevent DNA
125 cross-contamination between water samples. Filters were stored at -16 °C in the ice box and taken
126 back to the laboratory at 12 and 24 hours, respectively. Then, filters were stored at -20°C until DNA
127 extraction.

128 We used a specific passive sampling equipment for submerging the sponge samplers (Fig.
129 1B, C). This equipment consists of the weight and float connected by ropes. A sponge skeleton of
130 bath sponge *Spongia* spp. species was used as the adsorption material for the sponge sampler. To let
131 the sponges float in the middle layer of the water, sponge samplers were attached to the midpoint of
132 the rope tie-wraps before submerging the equipment. The equipment was placed in run near the

133 thalweg of the river for 24 hours for passive eDNA sampling. After 24 hours, the sponge samplers
134 were collected from the equipment and stored according to the specific procedures in each
135 experiment. After collection of the sponge samplers, the equipment was removed from the river. In
136 this study, two different sizes of sponges were used as the passive sampler: medium-sized sponges
137 (M-sponge; ca. 100 mm in diameter) and small-sized sponges (S-sponge; ca. 3–40 mm in diameter).
138 In this study, we compared various methods in each experiment to examine the optimal
139 method to recover eDNA from sponge samplers. In Experiment 1 (July 30–31, 2021), six M-sponge
140 samplers were used and 100 mL of pressed solutions were extracted from each samplers using a
141 handy juicer (Fig. 1C). Then, 100 mL of distilled water was added to the M-sponge to obtain an
142 additional 100 mL of pressed solution. Addition of distilled water and re-extraction were carried out
143 twice and a total of 300 mL of the pressings were obtained. Three of six pressed solutions were
144 additionally separated into 3 × 100 mL using 100 mL polyethylene bottles (M-solution). M-
145 solutions were stored at 4°C in a cooler box and taken back to the laboratory, and then stored at -
146 20°C until DNA extraction. The 300 mL of other three M-sponge solutions in Experiment 1 were
147 filtered with GF/F and filters were stored at -16 °C in the ice box and taken back to the laboratory,
148 and then stored at -20°C until DNA extraction (M-filter). In Experiment 2 (October 3–4, 2021), six
149 M-sponge samplers were used and all pressed solutions were handled as M-solution samples and
150 stored in the same methods as in Experiment 1 (Fig. 1C). In Experiment 3 (May 21–22, 2022), four
151 different passive sampling methods were examined. Three M-sponge samplers and six S-sponge

152 samplers were submerged in the river and collected after 24 hours. In Experiment 3, 100 mL of first
153 pressed solutions were only extracted from each M-sponge sampler and used as M-solution2
154 samples (Fig. 1C). Six S-sponge samplers were collected from the equipment and stored in separate
155 syringes. Three of the six S-sponge samplers were pressed in syringes and separated into syringe
156 samples (S-syringe) and pressed solutions (S-solution). Approximate 50 mL S-solution sample was
157 extracted from each S-sponge sampler and collected using 100 mL polyethylene bottles. The other
158 three S-sponge samplers were stored in syringes without pressing (S-syringe-NP). In Experiment 3,
159 all passive samples were stored at 4°C in a cooler box and taken back to the laboratory, and then
160 stored at -20°C until DNA extraction.
161
162 *DNA extraction from water and sponge samples*
163 Total eDNA of filter sample was extracted and purified following by Tsuji et al. (2022).
164 Briefly, each filter was placed in the upper part of a salivette tube (Sarstedt, Nümbrecht, Germany)
165 and pre-centrifuged at 5000 ×g for 1 min to remove the excess water. Each filter was soaked with
166 440 μL of a mixture composed of 200 μL Buffer AL, 200μL of H_2O and 20 μL of proteinase K, and
167 were incubated at 56°C for 45 min. Afterward, the cartridges were centrifuged at 5000 ×g for 3 min,
168 and the extract solutions of each tube were collected. Total eDNA was purified from extract
169 solution using DNeasy Blood & Tissue Kit (QIAGEN, Hilden, Germany) and was eluted in 100 μL
170 buffer AE from spin column according to the manufacturer's instructions.

171 Total eDNA of solution samples in passive sampling methods (M-solution and S-solution)
172 was extracted and purified using Maxwell® RSC Enviro Total Nucleic Acid Kit (Promega,
173 Madison, WI, USA) according to the manufacturer's instructions (hereafter, Direct Capture (DC)
174 method). Briefly, 12.5 μL of proteinase solution from the DC kit was added per 1 mL of pressed
175 solution, and samples were incubated for 30 min at ambient temperature. The samples were then
176 centrifuged at 3000 xg for 10 min at room temperature to remove the precipitation. The supernatant
177 was divided equally into two 50 mL tube and 6.0 mL of Binding Buffer 1 and 0.5 mL of Binding
178 Buffer 2 were added to the supernatant, respectively. The 24 mL of isopropanol was added to the
179 mixture, gently mixed and passed through a PureYield Midi Binding Column using a VacMan
180 Vacuum Manifold. Nucleic acid captured on the PureYield Midi Binding Column was washed with
181 5 mL of Column Wash 1 followed by 20 mL of Column Wash 2. Nucleic acid was eluted with 750
182 μL of pre-warmed (60°C) nuclease-free water. Then, the eluate was transferred to a Maxwell® RSC
183 Cartridge and the cartridge was held in a Maxwell® deck tray. DNA purification and elution of
184 nuclease-free water were performed on a Maxwell® instrument according to the manufacturer's
185 instructions. After completed procedures, the eluate was collected from the deck tray and 100 μL of
186 eDNA solution was obtained. UV light treatment was then performed on the instrument to avoid the
187 cross-contamination among samples.
188 Total eDNA of syringe samples in passive sampling methods (S-syringe and S-syringe-NP)
189 was extracted and purified using the extraction kit same as sponge solution samples with the slight

190 modification in the incubation step). Briefly, proteinase K powder (approximate 30 units/mg;
191 Nacalai tesque, Kyoto, Japan) was dissolved in TE buffer (pH 8.0) to prepare the proteinase K stock
192 solution (10 units/mL). Then, DNA elution mixture was prepared by adding 2.0 mL of Proteinase K
193 stock solution to 18 mL of TE buffer (pH 8.0). S-syringe sample was submerged in this solution in
194 each syringe, and incubated for 45 min at 56°C. The solution sample was discharged to a 50 mL
195 tube from each syringe and 6.0 mL of Binding Buffer 1 and 500 μL of Binding Buffer 2 were added
196 to the sample, respectively. The samples were then centrifuged at 3000 xg for 10 min at room
197 temperature to remove the precipitation. Following protocols (DNA purification, elution and
198 collection) were same as that of sponge solution samples. All eDNA samples were stored at -20°C
199 until 1st polymerase chain reaction (PCR) step in the eDNA metabarcoding.
200
201 *Quantitative fish environmental DNA metabarcoding*
202 In this study, we used the quantitative eDNA metabarcoding (qMiSeq, Ushio et al. 2019)
203 method to identify fish species composition and to quantify fish eDNA concentration,
204 simultaneously. In general, sequence reads obtained from high-throughput sequencing does not
205 necessarily correspond to the eDNA concentration because of the differences in PCR efficiency
206 (primer bias, PCR inhibition, etc.). In qMiSeq method, sequence reads can be converted to DNA
207 concentration (DNA copies/template) using sample-specific standard regression lines.

208 Amplicon libraries were prepared according to the following protocols (detailed
209 information in Supplementary Information S1). Fish eDNA of each sample was amplified using
210 MiFish primers (MiFish-U, -O, and -L; Miya et al. 2020), and internal standard DNA mix (5, 25, 50
211 copies /template) was added in each PCR reaction mix. The first PCR was repeated eight times for
212 each sample, and the replicated samples were pooled as a single first PCR product for use in the
213 subsequent step. In the first PCR step, the PCR negative controls (eight replicates) were included in
214 each experiment. In second PCR, the 8-bp dual indices were added to the purified first PCR
215 amplicons. The second PCR amplicons were pooled and the target library size (ca. 370 bp) of
216 second PCR was collected using E-Gel SizeSelect agarose gels (Thermo Fisher Scientific). Purified
217 library was sequenced using iSeq i1 Reagent and iSeq 100 platform with 30% PhiX control
218 (Illumina, San Diego, CA, USA). Sequencing dataset output from iSeq platform was subjected to
219 bioinformatic analysis. After bioinformatic analysis, sequence reads of each fish species and
220 standard DNA mix in each sample were obtained and subjected to qMiSeq analysis. To obtain
221 sample-specific regression lines, the regression analysis was performed using sequence reads and
222 DNA concentration of internal standard DNA mix. Slopes and R^2 values of each regression line
223 were shown in Supplementary Table S1–3. The R^2 values of all regression lines were higher than
224 0.98. In this study, no obvious PCR inhibition was identified in all water and passive DNA samples.
225
226 *Statistical analysis*

227 In this study, differences in the number of detected species among the methods (water and
228 each passive sampling) were assessed in each experiment. In each experiment, to examine the
229 difference in the diurnal conditions, the number of species in water samples was pooled together
230 into three categories: water (all), water (day) and water (night). The water (day) contains daytime
231 samples (0–6h and 20–24h), water (night) contains nighttime samples (8h–18h), and water (all)
232 contains all water samples (day and night). Kruskal-Wallis test was conducted to examine
233 differences in the number of species among all survey protocols (three conditions of water samples
234 and passive sampling methods) in each experiment. Then, Steel-Dwass multiple comparison was
235 performed in the experiment with significant differences.
236 Using fish eDNA concentration dataset, non-metric Multidimensional Scaling (nMDS) in
237 1000 separate runs was performed to visualize differences in fish community structure among
238 sampling methods. Stress value was used to measure the indicator of nMDS fitting. Threshold
239 stress values in the nMDS were less than 0.2, and the lowest of result was chosen. In addition,
240 permutational multivariate analysis of variance (PERMANOVA) were used to examine the
241 statistical differences in fish community structure among the sampling methods, and permutational
242 analysis of multivariate dispersions (PERMDISP) were used to assess the variation in species
243 composition among the sampling methods. Bray-Curtis dissimilarity of fish community structure
244 between samples in each experiment was calculated and β-diversity plots of each samples were
245 visualized in each experiment. In each experiment, fish species that affected differences in

246 community structure between sampling methods were estimated using “envfit” function. In this
247 study, the threshold of p-value was <0.01 in order to choose species which have more obvious
248 effects on community structure. All statistical analyses and plotting were performed using R ver.
249 4.02. For nMDS, PERMANOVA and PERMDISP, the “metaMDS”, “adonis” and “betadisper”
250 functions of the vegan package were used.
251

252 **Results**

253 In this study, 8,296,115 reads were obtained from 105 water samples and 41 passive
254 samples, and 6,903,093 reads were remained after pre-processing (Supplementary Table S4). The
255 number of species detected in Experiment 1 (July, 2021) ranged from 15–22 for water samples (n =
256 39) and 7–14 (M-filter, n = 3) and 15–22 (M-solution, n = 8) for passive samples, respectively (Fig.
257 2). Due to the loss of one of the M-solution samples from Experiment 1 (M-solution 3-3) from the
258 equipment, the total number of M-solution samples in Experiment 1 was eight (Supplementary
259 Table S1). The number of species detected in Experiment 2 (October, 2021) ranged from 15–23 for
260 water samples (n = 39) and 19–25 for M-solution samples (n = 18). In Experiment 3 (May, 2022),
261 the number of species ranged from 18–24 for water samples (n = 27) and 21–23 (M-solution, n = 3),
262 21–23 (S-solution, n = 3), 16–19 (S-syringe, n = 3) and 19 (S-syringe-NP, n = 3) for passive
263 samples, respectively (Fig. 2). Steel-Dwass multiple comparison was performed, as the significant
264 differences were confirmed in results of Kruskal-Wallis test in all experiments (Supplementary

265 Table S5). However, significant differences were only confirmed in Experiment 1 in the results of
266 the Steel-Dwass test (Fig. 2). In Experiment 1, significant differences were observed between the
267 number of species detected from M-filter and that of water (all), water (day), water (night) and
268 between water (day) and water (night), respectively ($p < 0.05$). In Experiment 2, the number of
269 species detected from M-solution sample tended to be higher than water (all) and water (day),
270 although the differences were not statistically significant ($p = 0.055$ and 0.072). In Experiment 3,
271 there were no statistically significant differences in the number of species among all sample types
272 (Supplementary Table S5).

273 Cumulative fish diversity was highest for water sampling accumulated between 0 and 24
274 hours (3 samples × 13 timings) than passive sampling methods in all Experiments (Fig. 3).
275 Specifically, cumulative number of fish species in Experiment 1 was 25, 15, 23, 24 and 19 in water,
276 M-filter, M-solution 1, 2, and 3 sample, respectively. Cumulative number of fish species in
277 Experiment 2 was 28 and 31 in water and M-solution sample. In Experiment 3, cumulative number
278 of fish species was 26, 23, 24, 21 and 21 in water, M-solution, S-solution, S-syringe and S-syringe-
279 NP sample, respectively. Passive sampling methods also showed high cumulative fish diversity,
280 similar to water, with the M-solution in Experiment 2 in particular showing the highest diversity
281 (Supplementary Table S1–3).

282 Fish species composition was substantially similar in all experiments (Fig. 4). The number
283 of common species between water (day) or water (night) and passive samples (M-solution, S-

284 solution, S-syringe or S-syringe-NP) was much higher than other combinations in all experiments.
285 However, common fish species in the two water samples (9 species) were not detected in M-filter
286 samples in Experiment 1 (Fig. 4). On the other hand, the comparison with M-solution showed a
287 decrease in the number of species detected only in the two water samples and an increase in the
288 number of species common to the three methods, compared to the M-filter. Cumulative fish species
289 compositions detected by each sampling method in Experiments 1-3 are listed in Supplementary
290 Table S6. In Experiment 1, *Oncorhynchus masou* subsp. was specifically detected from water (day)
291 and *Pseudogobio agathonectris* in passive samples (M-filter and M-solution). In Experiment 2,
292 three fish species (*Acheilognathus rhombeus*, *Hemibarbus* sp. and *Salvelinus leucomaenis* subsp.)
293 were only detected in M-solution samples and the species detected only in two types of water
294 samples were not observed. In Experiment 3, *Tanakia limbata* was only detected in water (day)
295 samples and *Hemibarbus* sp. in S-syringe samples. In Experiment 3, the number of common species
296 between the two types of water sample and four passive sampling methods was much similar. Fish
297 species detected by eDNA metabarcoding were generally known to inhabit the study site in the
298 previous years. (River Environmental Database system, 2018). However, a tropical garfish
299 *Atractosteus tropicus*, listed as alien species in Japan, was newly detected with high DNA
300 concentration from water (night), M-solution, S-solution samples.
301 In this study, nMDS plot was used to assess the differences in community structure
302 considering the eDNA concentration of each fish species (Fig. 5). Fish community structure in

303 Experiment 1 was much different among sample types (PERMANOVA: $p < 0.01$, PERMDISP: $p =$
304 0.441). In particular, M-filter samples showed greater variation among samples than the other
305 sample types. While the number of species in the M-solution samples was similar to the two types
306 of water samples, a few differences were observed in the community structure. Number of fish
307 species affecting the differences in community structure was 8 species, and 2 of these species
308 (*Hemibarbus longirostris* and *Tachysurus nudiceps*) were not detected in M-filter samples. Other
309 species were detected in all sample types, but DNA concentrations of these species were much
310 higher in water samples than in passive samples (Supplementary Table S1). In Experiment 2, the
311 fish community structure of water (night) was included in that of water (day) and the M-solution
312 fish community was plotted outside the two types of water samples. However, the differences in
313 community structure among the three types could not be precisely assessed (PERMANOVA: $p <$
314 0.01, PERMDISP: $p < 0.05$). There could not be also assessed the differences in community
315 structure between water (all) and M-solution samples (PERMANOVA: $p < 0.01$, PERMDISP: $p <$
316 0.05). Number of fish species affecting the differences in community structure was 14 species in
317 Experiment 2, and *Hemibarbus* sp. was only detected in M-solution samples. Other species were
318 detected in all sample types, and trend in DNA concentration of these species was similar to
319 Experiment 1 (Supplementary Stable S2). In Experiment 3, there were slight differences in fish
320 community structure between water (day) and water (night) samples. Also in the passive samples,
321 the community structure was different between the solution types (M-solution and S-solution) and

322 the syringe types (S-syringe and S-syringe-NP). In Experiment 3, Fish community structure was
323 much different among sample types (PERMANOVA: $p < 0.01$, PERMDISP: $p = 0.11$). Number of
324 fish species affecting the differences in community structure was 13 species in Experiment 3. All
325 species were detected in all sample types, except for Misgurnus anguillicaudatus in S-syringe
326 samples. Trend in DNA concentration of these species was also similar to Experiment 1 and 2
327 (Supplementary Table S3).
328

329 **Discussion**

330 In this study, we assessed the effectiveness of eDNA passive sampling methods compared
331 to water sampling in riverine environment. In addition, in the passive sampling method, some DNA
332 extraction methods were examined to estimate the optimal eDNA recovery method from sponges.
333 Our results showed that the passive sampling method using sponge skeletons had similar or higher
334 number of fish species than water samples. Our results in the present study would support previous
335 studies showing similar trends (Jaunen et al. 2024, Zhan et al. 2024, Mlinarec et al. 2025).
336 However, in Experiment 1, the number of species of M-filter sample was significantly lower than
337 other methods (water and other passive samples) and the variation of fish community composition
338 was higher than other methods. However, as no PCR inhibition was observed in all DNA samples,
339 other factors may have affected the reduction in the number of species in the M-filter samples.
340 These suggest that DNA recovery rate of M-filter was less efficient than other methods. Water

341 samples pressed from sponges contain large amount of material besides eDNA (e.g., sand, mud,
342 biofilm, etc.) and DNA in pressed solutions could be captured to these materials. This can easily be
343 assumed from the work of previous studies using soil particles and biofilms as the capturing
344 materials of passive sampling (Kirtane et al., 2020, Rivera et al., 2021). DNA extraction method
345 from GF/F filter used in the M-filter might not have efficiently recovered the eDNA captured on
346 these materials. On the other hand, DC method applied to passive samplers in this study suggests
347 that efficiency of DNA recovery was improved by direct DNA extraction from pressed solutions or
348 by direct immersion of sponge skeletons in the elution mixture. In fact, M-solution in the passive
349 sampling method showed similar fish diversity in all experiments, both in the number of species
350 and the cumulative number of species, as in the water samples. Furthermore, in Experiment 2, fish
351 species detected only in M-solution were observed. The cumulative number of samples in passive
352 sampling methods was larger than for water samples (Fig.4). These results show that passive
353 samples (sponge skeletons) of 24-hour immersion have the same detection performance as 24-hour
354 water samples taken every two hours. Slightly lower number of species were observed in other
355 passive sampling methods (S-solution, S-syringe and S-syringe-NP) compared to water samples,
356 but the statistically significant differences were not found. The high cumulative fish diversity for S-
357 solution and the presence of specific species for S-syringe, respectively, may also indicate the
358 effectiveness of each method (Fig. 3, Fig.4). However, the passive sampling method in Experiment
359 3 could have been difficult to find the difference from other methods because of the lack of

360 replicate samples (triplicate in each sample). Therefore, the effectiveness of these methods may
361 need to be carefully evaluated with further work.
362 In Experiments 1 and 3, the number of detected species at night-time tended to be higher
363 than that of daytime in the water samples (Fig.3). Cumulative number of species in the water
364 samples in Experiments 1 and 2 varied considerably with collection timing (Fig.4). On the other
365 hand, the number of detected and cumulative species in passive samples, except for the M-filter
366 sample, tended to be more homogeneous than in water samples in all experiments. These
367 differences could be due to dissimilarities in the DNA information of freshwater fishes in the water
368 and passive samples. In general, eDNA information on water samples contains the snapshot
369 information reflecting the collection timing (Spear et al. 2015, Bowers et al., 2021; Jensen et al.,
370 2022). Therefore, eDNA shed from rare species or species with coincidental appearance could be
371 rapidly diluted and dispersed, and elude detection via instantaneous water sampling (Kirtane et al.,
372 2020). On the other hand, passive sampling methods could have accumulated more eDNA by
373 filtering the large water volume through long-term immersion in the water. Jeunen et al. (2022)
374 indicated that more porous passive filter materials potentially achieve higher detection rates through
375 an adsorption and retaining effect of low-abundant DNA and DNA bound to particulate matter.
376 Differences in temporal resolution between sampling methods could be reflected in such variations
377 and differences in fish species diversity.

Experiments 1 and 3 showed differences in fish community structure between methods in the nMDS plot and statistical analyses. In all experiments, some fish species also affected differences in community structure among sample types. In Experiment 1, this is reasonable as fish diversity and DNA concentrations of each fish species in the M-filter samples were much lower than other methods. However, in other experiments, the fish diversity and species composition are few different among sampling methods (Fig.3, Fig. 4). Therefore, these variations in community structure among methods could be attributed to differences in the eDNA concentration in each fish species. In fact, there were differences in fish DNA concentrations among sampling methods, and that of common fish species (*Zacco platypus*, *Nipponocypris temminckii* and *Pungtungia herzi*) observed in the survey site showed large differences between water and passive samples in all experiments (Supplementary Table 1–3). As fish DNA concentration of each sample was standardized by adding the standard DNA mix, these differences indicate that eDNA concentration of fishes in passive samples is lower than in water samples. Passive sampling methods store the aquatic eDNA by capturing and accumulating the collection materials. Cananzi et al. (2025) showed that passive samplers contain eDNA of diverse taxa and particle sizes and are more efficient for detecting micro-invertebrates and amphibians compared to water samples by using multi-marker approach. Similarly in our study, large amounts of eDNA from non-fish organisms (e.g., aquatic plants, insects and micro-organisms) may have been captured by sponge skeletons during long-term immersion. As a result, fish eDNA could be relatively reduced in passive samples, causing the

397 differences in proportion of DNA concentration between water samples and passive samples.
398 Differences in eDNA concentrations may also reflect differences in DNA extraction efficiency
399 between sponge skeletons and GF/F filters. While optimization of DNA extraction procedures from
400 GF/F filters has been examined by previous studies (Minamoto et al. 2021), that of sponge
401 skeletons may still be insufficient. Previous studies have also shown that the efficiency of DNA
402 recovery in passive samples varied greatly depending on the sampler material and immersion
403 method (Verdier et al. 2022, Saltonstall et al. 2024, Stevens et al. 2024). Therefore, future
404 improvements in methodology will enhance the efficacy of the passive sampling method using
405 sponge skeletons.
406 In conclusion, we showed that the eDNA passive sampling method using sponge skeletons
407 is one of the most efficient methods for monitoring freshwater fishes in riverine environments.
408 Passive sampling methods enable more cost-effective and efficient eDNA monitoring than
409 conventional filtering methods. Passive sampling methods also requires no need for time-
410 consuming filtration or the preservation of large volumes of water until filtering. Furthermore,
411 passive sampling methods minimize sampling and processing time in the field and accumulate
412 eDNA with long-term immersion, and enable time-efficient biomonitoring. Indeed, our passive
413 sampling method could detect similar or higher fish species diversity than that of water sampling
414 due to long-term and cumulative eDNA adsorption on sponge skeletons. However, there are some
415 challenges in the passive sampling method. One of the challenges of passive sampling method is

416 that the actual amount of water passing through the passive collection device during submersion is
417 difficult to quantify (Zhang et al. 2024). Currently, this method may be unsuitable for estimating
418 eDNA concentration of target species and quantitative assessments of species abundance and
419 biomass. In this study, eDNA concentration of each fish species was estimated by qMiSeq method,
420 but the accuracy of DNA quantification could not be evaluated. This challenge could be solved by
421 measuring flow rates and by estimating the water volume through the sponge skeleton.
422 Furthermore, passive sampling methods could concentrate PCR inhibitors as well as eDNA with
423 long-term immersion. Organic compounds (e.g., humic and fulvic acid) produced by biological
424 processes of phytoplankton and from terrestrial detritus inputs were contained in aquatic systems,
425 and can be the major cause of PCR inhibition (Hellings et al. 1999, Albers et al., 2013, Uchii et al.
426 2019). These substances may be adsorbed and concentrated on the sponge skeleton as well as
427 eDNA, increasing the probability of PCR inhibition. Further improvements of methodology, such as
428 the effect of PCR inhibition, quantitative assessment, and immersion period, should be required so
429 that passive sampling method using sponge skeletons will be an effective tool for the eDNA survey.
430

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Conflicts of interest

We have read the journal's policy and the authors of this manuscript have the following competing interests: F.I. is currently a paid employee of Nippon Koei Co., Ltd. We further declare that the methodology and device of the passive sampling method using sponge skeletons has a patent pending under the application number PCT/JP2023/036721.

Data accessibility

All sequence data will be published in DRA (Accession number:) after accept of the manuscript and detailed information of fish community structure and methodological descriptions of library preparation are available in the Supplementary materials.

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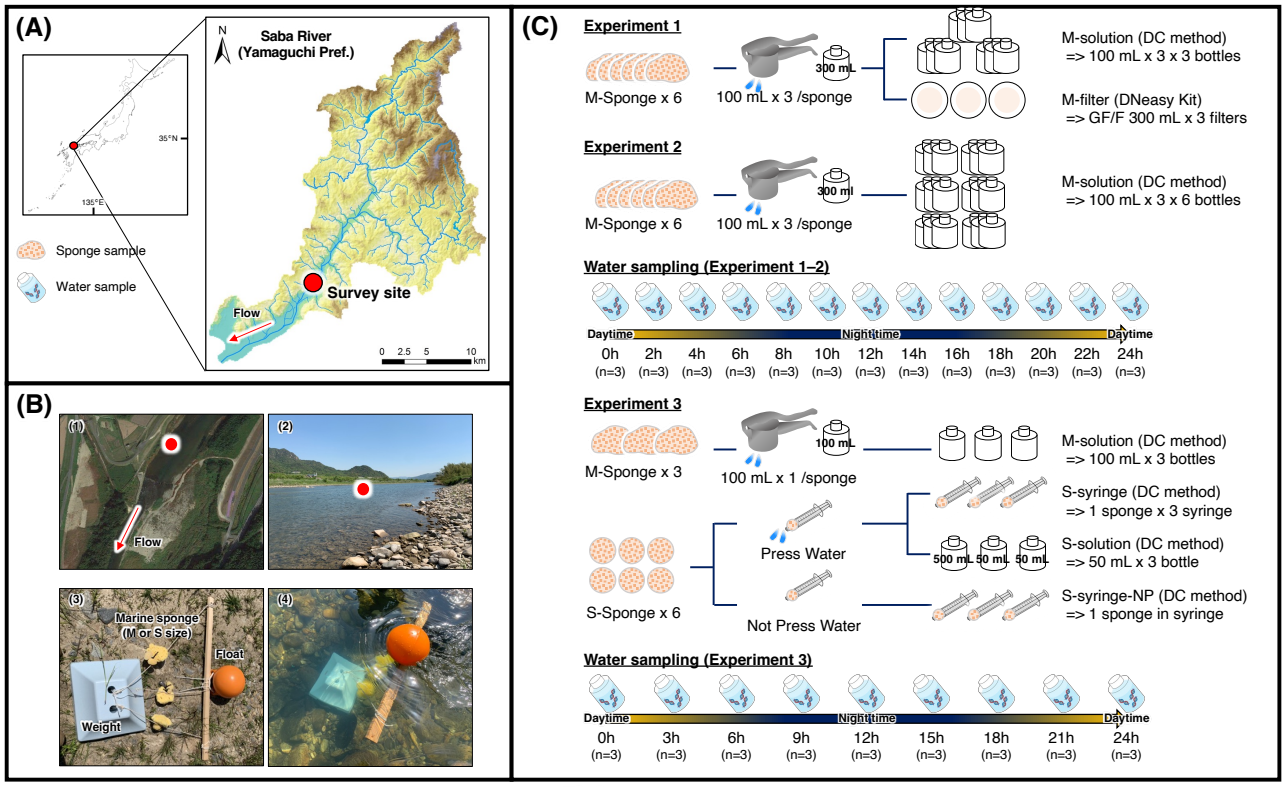
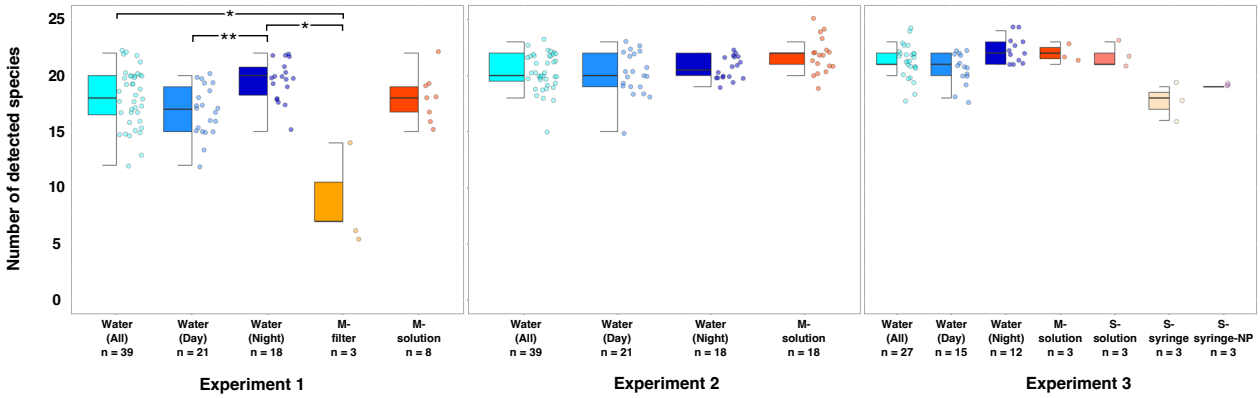


Fig.1

Figure 1

Study site information and descriptions of experimental design in this study. (A) descriptions of survey site, (B) detailed information of survey site and passive sampler, (C) Methodological procedures of water and passive sampling in each Experiment.



588

589 **Figure 2**

590 Boxplots in the number of detected species in each experiment. Asterisks indicate the significant

591 differences among sample categories (**: $p < 0.01$, *: $p < 0.05$).

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593

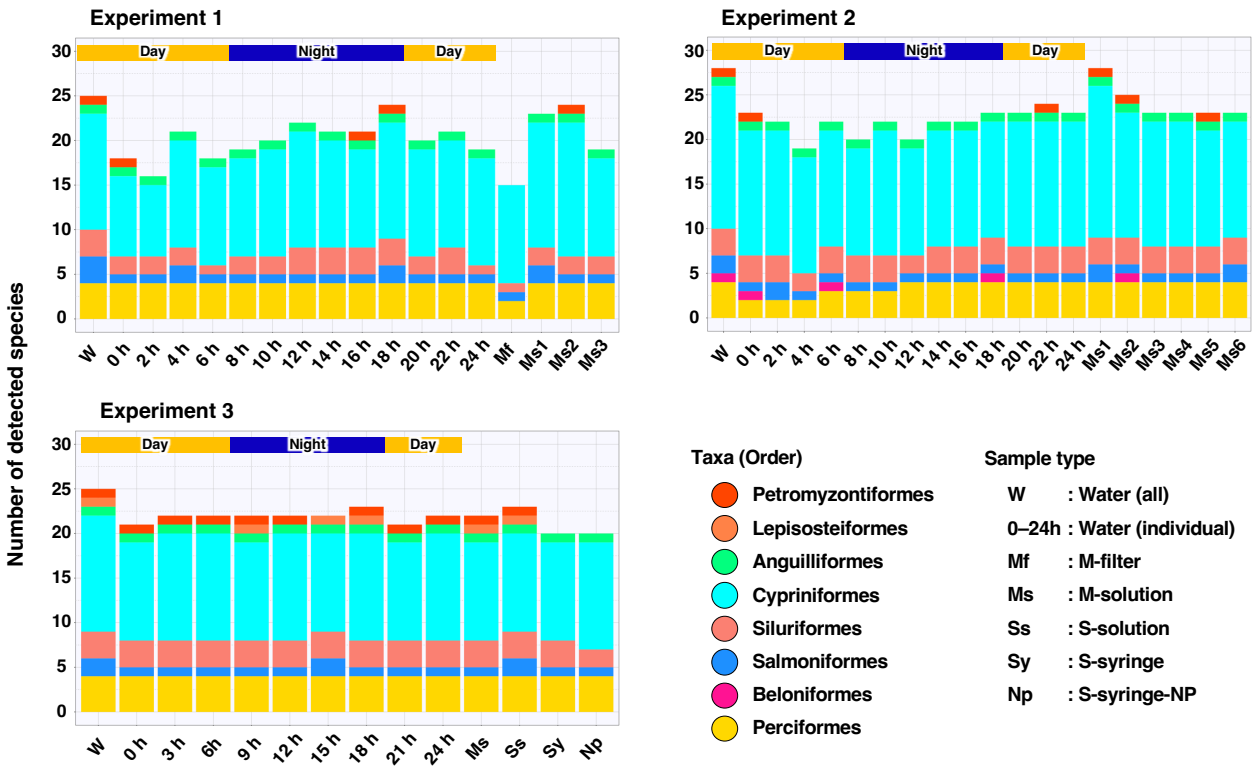

Fig.3 Cumulative number of species detected in each collection timing and DNA extraction method.

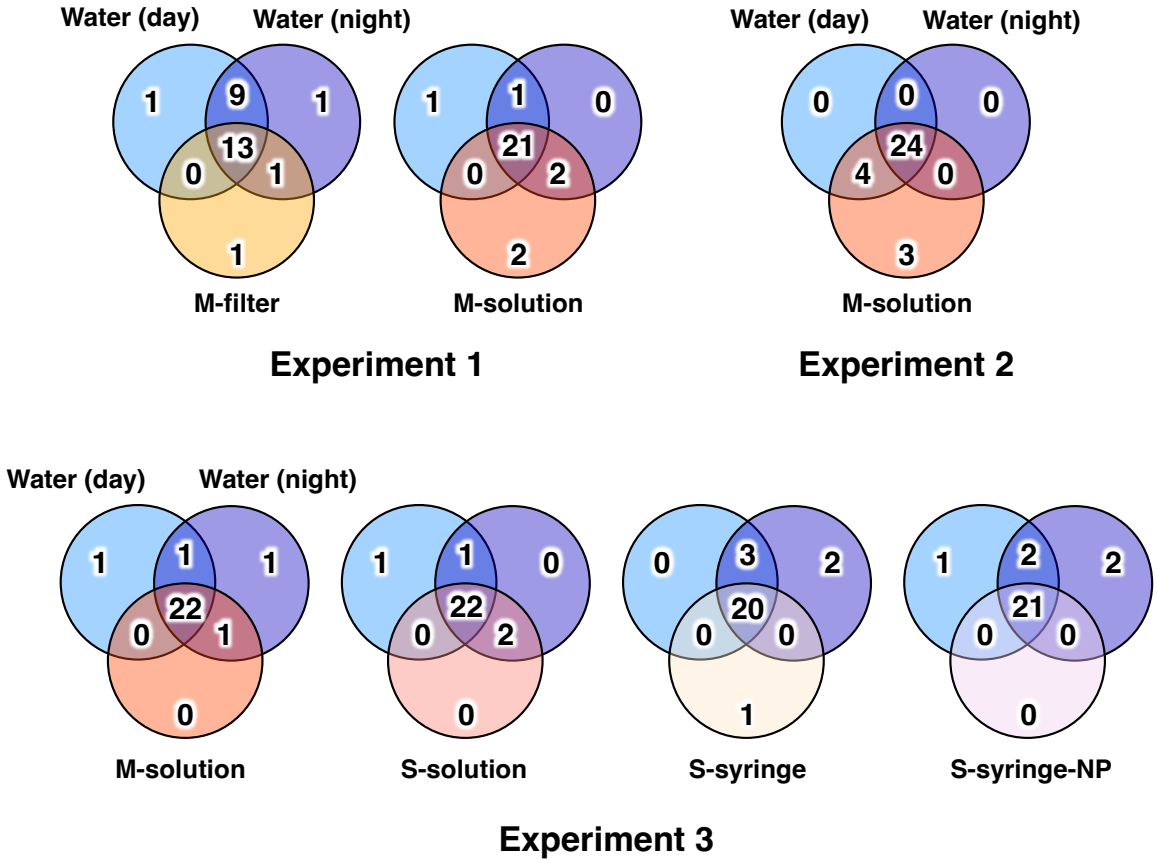

Figure 4

Venn-diagrams showing the number of fish species belonging to each sampling method that were detected by eDNA metabarcoding in each Experiment. Detailed information of fish species compositions in each Experiment was described in Supplementary Table S6.

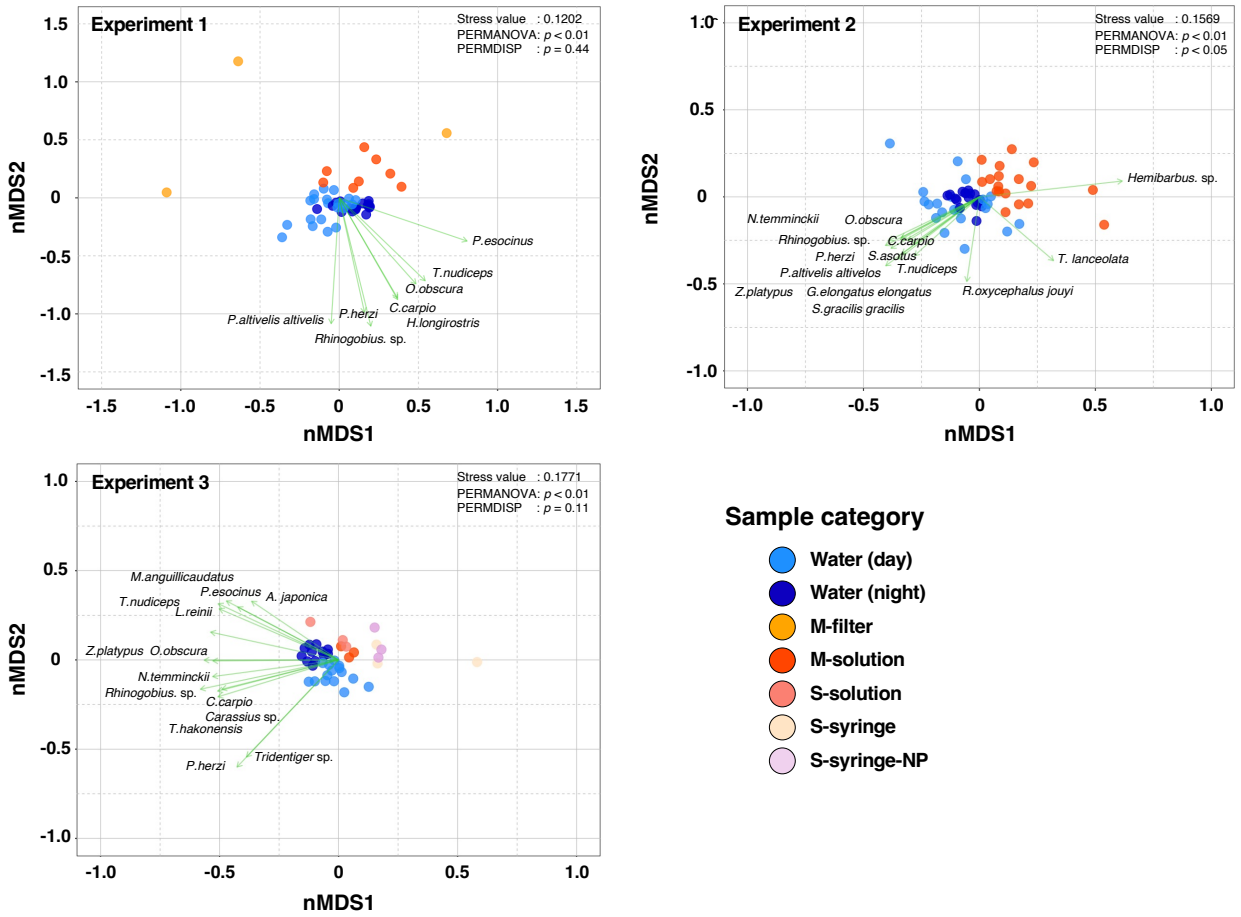

Figure 5 The nMDS plots in each experiment. Green arrows represent the fish responsible for causing the significant difference among sampling methods.