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

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Abstract

Environmental DNA (eDNA) analysis is a cost-effective and noninvasive tool for species and biodiversity monitoring in aquatic environments. Passive eDNA sampling is a novel alternative to conventional sampling methods such as water filtration. In this study, we examined the efficacy of the sponge skeleton as a passive eDNA sampler for monitoring freshwater fish. The performance of the passive sampling method was compared with that of standard water filtration in a river environment. Five DNA extraction methods were used in three experiments, and a suitable method for DNA extraction from sponge skeleton was identified. Quantitative fish metabarcoding using MiFish primers revealed no significant differences in species richness between the aqueous and passive sampling methods. Although both the sampling methods showed comparable trends in fish community structure, different clusters were identified for water sampling and passive samplers based on the differences in the DNA concentration of each fish species. The fish diversity in the passive samples was comparable among four DNA extraction methods (except for the filtration method) using the direct capture method. Our results demonstrate the efficacy of passive eDNA sampling for monitoring freshwater fish diversity and the potential use of sponge skeletons as absorption materials for passive eDNA samplers.

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

Introduction

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

Water eDNA is an environmental DNA collected from water samples. It is most commonly used to monitor fish diversity. Water eDNA from fish is generally collected and concentrated from water samples by filtration through artificial membranes (Yamamoto et al. 2017, Ushio 2019, Jeunen et al. 2019). However, the fish diversity in water eDNA is generally snapshot information. Moreover, insufficient sampling efforts could result in an underestimation of the fish diversity (Zinger et al. 2019). For example, if the concentration of the target DNA is low (such as for rare species), small sampling volumes or few replicates can yield false negatives. Furthermore, because eDNA concentrations reflect the activity levels of the target species (e.g., diurnal or nocturnal), the mismatch of timing between water sampling and fish ecology may increase false-negative detections. Large volumes of water filtration and sampling replicates are required to remove false negatives and provide reproducibility of fish diversity. However, the conventional sampling method using water filtration requires considerable time and cost to achieve such improvements.

Passive eDNA collection methods directly submerge the sampling material in a water body. These facilitate reproducible biomonitoring as a simpler and more cost-effective sampling method than conventional active filtration methods (Bessey et al., 2021; Kirtane et al., 2020; Zhang et al. 2024). The absorption materials for passive eDNA sampling used in previous studies generally have porous substances, sufficient adsorption capabilities to detect the target eDNA, and simple or user-friendly forms. The submersion materials used in passive eDNA sampling methods include activated carbon, montmorillonite (Kirtane et al., 2020), living sponges (Mariani et al., 2019; Cai et al., 2022), and biofilms (Rivera et al., 2021). In the case of artificial materials, Verdier et al. (2022) created the passive, 3D-printed, and convenient-to-use eDNA sampler using hydroxyapatite (a natural mineral with a high DNA adsorption capacity). Yan et al. (2024) deployed passive sampling using GF filters by the angling (short-term immersion at the site) and trolling (towing in the water) methods in large lake environments. These studies showed that the effectiveness of passive collection methods in detecting fish is similar to that of conventional active filtration methods. Furthermore, these studies provided similar estimates of total fish biodiversity. In addition, because passive eDNA samplers require minimal or no supporting equipment (e.g., no vacuum or peristaltic pumps), these are suitable for deployment in remote environments and by non-experts.

Natural and artificial sponges are likely to be superior passive eDNA samplers owing to their universal distribution, high regeneration capability, and convenience of sampling. For example, filter-feeding organisms have been shown to naturally accumulate eDNA in their tissue matrices (Jeunen et al., 2021). Mariani et al. (2019) showed that eDNA extracted from live marine sponges could detect various marine fishes and mammals in the Mediterranean Sea and Antarctic Ocean. Turon et al. (2020) highlighted the suitability and cost-effectiveness of live marine sponges as passive eDNA samplers for describing tropical fish communities from coral reefs in Southeast Asia. Jeunen et al. (2022) indicated that more porous passive filter materials such as artificial sponges could potentially achieve higher detection rates through the adsorption and retention of less abundant DNA and DNA bound to particulate matter. Therefore, natural sponge skeletons could

potentially be used as effective materials for passive eDNA samplers. However, the knowledge of the application of sponge skeletons to eDNA studies is insufficient for both marine environments and freshwater environments, particularly in rivers.

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

Materials and Methods

Study field and environmental DNA samplings

In this study, field surveys were conducted in the middle reaches of the Saba River Basin, Yamaguchi Prefecture, Japan, on July 30–31, October 3–4, 2021, and May 9–10, 2022 (Fig. 1A, B). The fish activity times in rivers differ across species (e.g., diurnal or nocturnal). Therefore, the species composition detected may vary depending on the timing of water sampling. Sponge-passive samplers can potentially overcome these variabilities in species detection through long-term eDNA adsorption onto the sponge skeleton in water. To accurately assess the function of eDNA adsorption on the passive sampler, we conducted water sampling multiple times during the immersion of the passive sampler. For water sampling, 3 × 1 L of surface water was collected directly from the center of the riffle using bleached plastic bottles every 2 h (0, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, and 24 h in 2021) or 3 h (0, 3, 6, 9, 12, 15, 18, 21, and 24 h in 2022) after submerging the equipment (Fig. 1C). In this study, all the experiments were started at 11.00. Therefore, in Experiments 1 and 2, we

117 defined 0–6 h (11:00–17:00) and 20–24 h (7:00–11:00) as daytime, and 8–18 h (19:00–5:00) as
118 nighttime. In Experiment 3, we also defined 0–6 h (11:00–17:00) and 21–24 h (8:00–11:00) as
119 daytime and 9–18 h (20:00–5:00) as nighttime. Water sampling was also performed downstream of
120 the equipment to prevent contamination from riverbed disturbances caused by human movement.
121 Water samples were filtered immediately on site using a portable pump (Sentino microbiology
122 pump; Cytiva, Tokyo, Japan) and GF/F glass fiber filter (pore size 0.7 μm; Cytiva). After filtration,
123 the equipment was bleached to prevent DNA cross-contamination between water samples. The
124 filters were stored at -16°C in the ice box and taken back to the laboratory at 12 and 24 h,
125 respectively. The filters were stored at -20°C until DNA extraction.

126 Specific passive sampling equipment was used to submerge the sponge samplers (Fig. 1B,
127 C). This equipment consists of a weight and float connected by ropes. The sponge skeleton of the
128 *Spongia* spp. species was used as the adsorption material for the sponge sampler. Sponge samplers
129 were attached to the midpoint of the rope-tip wraps before submerging the equipment to enable the
130 sponges to float in the middle layer of the water. The equipment was placed near the river thalweg
131 for 24 h for passive eDNA sampling. After 24 h, the sponge samplers were collected from the
132 equipment and stored according to specific procedures in each experiment. After the sponge
133 samplers were collected, the equipment was removed from the river. In this study, two sponge sizes
134 were used for passive samplers: medium (M-sponge; approximately 100 mm in diameter) and small
135 (S-sponge; approximately 3–40 mm in diameter).

136 In this study, we compared various methods to determine the optimal method for
137 recovering eDNA from sponge samples. In Experiment 1 (July 30–31, 2021), six M-sponge
138 samplers were used, and 100 mL of the pressed solutions were extracted from each sampler using a
139 handheld juicer (Fig. 1C). Next, 100 mL of distilled water was added to the M-sponge to obtain 100
140 mL of pressed solution. Distilled water was added, and re-extraction was performed two times. A
141 total of 300 mL of the pressing solution was obtained. Three of the six pressed solutions were
142 additionally separated into 3 × 100 mL aliquots using 100 mL polyethylene bottles (M-solution).

143 M-solutions were stored at 4°C in a cooler box and transferred back to the laboratory. Then these
144 were stored at -20°C until DNA extraction. The 300 mL of the other three M-sponge solutions in
145 Experiment 1 were filtered with GF/F. The filters were stored at -16°C in the ice box and
146 transferred back to the laboratory. Then, these were stored at -20°C until DNA extraction (M-filter).
147 Six M-sponge samplers were used in Experiment 2 (October 3–4, 2021). All the pressed solutions
148 were handled as M-solution samples and stored using the methods in Experiment 1 (Fig. 1C). In
149 Experiment 3 (May 21–22, 2022), four passive-sampling methods were used. Three M-sponge and
150 six S-sponge samplers were submerged in the river and collected after 24 h. In Experiment 3, 100
151 mL of the first pressed solution was extracted from each M-sponge sampler and used as the M-
152 solution 2 samples (Fig. 1C). Six S-sponge samples were collected from the equipment and stored
153 in separate syringes. Three of the six S-sponge samplers were pressed into syringes and separated
154 into syringe samples (S-syringes) and pressed solutions (S-solutions). Approximately 50 mL of the
155 S-solution sample was extracted from each S-sponge sampler and collected in 100 mL polyethylene
156 bottles. The remaining three S-sponge samplers were stored in unpressed syringes (S-syringe NP).
157 In Experiment 3, all the passive samples were stored at 4°C in a cooler box and transferred back to
158 the laboratory. Then, these were stored at -20°C until DNA extraction.
159
160 *DNA extraction from water and sponge samples*
161 The total eDNA of the filter samples was extracted and purified as described by Tsuji et al.
162 (2022). Briefly, each filter was placed in the upper part of a Salivette tube (Sarstedt, Nümbrecht,
163 Germany) and pre-centrifuged at 5000 × g for 1 min to remove excess water. Each filter was soaked
164 with 440 μL of a mixture composed of 200 μL of Buffer AL, 200 μL of H_2O, and 20 μL of
165 proteinase K, and incubated at 56°C for 45 min. Subsequently, the cartridges were centrifuged at
166 5000 × g for 3 min, and the extract solutions of each tube were collected. The total eDNA was
167 purified from extract solution using DNeasy Blood & Tissue Kit (QIAGEN, Hilden, Germany) and
168 eluted in 100 μL of Buffer AE from a spin column according to the manufacturer's instructions.

169 The total eDNA of the solution samples in the passive sampling methods (M-solution and
170 S-solution) was extracted and purified using Maxwell® RSC Enviro Total Nucleic Acid Kit
171 (Promega, Madison, WI, USA) according to the manufacturer's instructions (hereafter, the direct
172 capture (DC) method). Briefly, 12.5 μL of proteinase solution from the DC kit was added per 1 mL
173 of pressed solution. The samples were incubated for 30 min at ambient temperature. The samples
174 were then centrifuged at 3000 × g for 10 min at room temperature to remove the precipitate. The
175 supernatant was divided equally into two 50 mL tubes. Furthermore, 6.0 mL of Binding Buffer 1
176 and 0.5 mL of Binding Buffer 2 were added to the supernatant. Then, 24 mL of isopropanol was
177 added to the mixture, mixed gently, and passed through a PureYield Midi Binding Column using a
178 VacMan Vacuum Manifold. Nucleic acids captured on the PureYield Midi Binding Column were
179 washed with 5 mL of Column Wash 1, followed by 20 mL of Column Wash 2. Nucleic acid was
180 eluted with 750 μL of pre-warmed (60°C) nuclease-free water. Then, the eluate was transferred to a
181 Maxwell® RSC Cartridge, and the cartridge was held in a Maxwell® deck tray. DNA purification
182 and elution of nuclease-free water were performed on a Maxwell® instrument according to the
183 manufacturer's instructions. After the procedures were completed, the eluate was collected from the
184 deck tray, and 100 μL of eDNA solution was obtained. The instrument was then subjected to UV
185 light treatment to prevent cross-contamination.
186 The total eDNA of syringe samples in the passive sampling methods (S-syringe and S-
187 syringe-NP) was extracted and purified using the extraction kit for the sponge solution samples with
188 a marginal modification in the incubation step. Briefly, proteinase K powder (approximately 30
189 U/mg; Nacalai Tesque, Kyoto, Japan) was dissolved in TE buffer (pH 8.0) to prepare a proteinase K
190 stock solution (10 U/mL). The DNA elution mixture was prepared by adding 2.0 mL Proteinase K
191 stock solution to 18 mL TE buffer (pH 8.0). An S-syringe sample was submerged in this solution in
192 each syringe and incubated for 45 min at 56°C. The solution sample was discharged to a 50 mL
193 tube from each syringe. Then, 6.0 mL of Binding Buffer 1 and 500 μL of Binding Buffer 2 were
194 added to the sample, respectively. The samples were then centrifuged at 3000 × g for 10 min at

195 room temperature to remove the precipitate. Following protocols (DNA purification, elution, and
196 collection) were identical to those for the sponge solution samples. All the eDNA samples were
197 stored at -20°C until the first polymerase chain reaction (PCR) step in the eDNA metabarcoding.
198
199 *Quantitative fish environmental DNA metabarcoding*
200 In this study, we used the quantitative eDNA metabarcoding (qMiSeq, Ushio et al. 2019)
201 method to identify the fish species composition and quantify the fish eDNA concentrations
202 simultaneously. In general, sequence reads obtained from high-throughput sequencing do not
203 necessarily correspond to eDNA concentrations owing to the differences in PCR efficiency (primer
204 bias, PCR inhibition, etc.). Applying the qMiSeq method, sequence reads can be converted into
205 DNA concentrations (DNA copies/template) using sample-specific standard regression lines.
206 Amplicon libraries were prepared according to the following protocol (detailed information
207 is provided in Supplementary Information S1). Fish eDNA from each sample was amplified using
208 MiFish primers (MiFish-U, -O, and -L; Miya et al. 2020). An internal standard DNA mix (5, 25,
209 and 50 copies/template) was added to each PCR mix. The first PCR was repeated eight times for
210 each sample. The replicated samples were pooled as a single first PCR product for use in
211 subsequent steps. In the first PCR step, PCR-negative controls (eight replicates) were included. In
212 the second PCR, 8 bp dual indices were added to the purified first PCR amplicons. The second PCR
213 amplicons were pooled, and the target library size (approximately 370 bp) of the second PCR was
214 collected using E-Gel SizeSelect agarose gels (Thermo Fisher Scientific). The purified library was
215 sequenced using the iSeq i1 Reagent and the iSeq 100 platform with 30% PhiX control (Illumina,
216 San Diego, CA, USA). The sequencing dataset output from the iSeq platform was subjected to a
217 bioinformatic analysis. After the analysis, sequence reads of each fish species and the standard
218 DNA mix in each sample were obtained and subjected to a qMiSeq analysis. To obtain sample-
219 specific regression lines, a regression analysis was performed using sequence reads and the DNA
220 concentration of the internal standard DNA mix. The slopes and R^2 values for each regression line

221 are listed in Supplementary Tables S1–3. The R^2 values of all the regression lines are higher than
222 0.98. In this study, PCR inhibition was not apparent in any of the water or passive DNA samples.
223
224 *Statistical analysis*
225 In this study, the differences in the number of detected species between the two methods
226 (water and passive sampling) were assessed in each experiment. In each experiment, to examine the
227 differences in diurnal conditions, the number of species in water samples was separated into three
228 categories: water (all), water (day), and water (night). Water (day) contained daytime samples (0–6
229 h and 20–24 h), water (night) contained nighttime samples (8 h–18 h), and water (all) contained all
230 the water samples (day and night). The Kruskal–Wallis test was conducted to examine the
231 differences in the number of species among all the survey protocols (three conditions of water
232 samples and passive sampling methods) in each experiment. Steel–Dwass multiple comparisons
233 were then performed in the experiments with significant differences.
234 Using the fish eDNA concentration dataset, non-metric multidimensional scaling (nMDS)
235 in 1000 separate runs was performed to visualize the differences in fish community structure among
236 the sampling methods. The stress value was used as an indicator of the nMDS fitting. The threshold
237 stress values in the nMDS were less than 0.2, and the lowest value was selected. In addition,
238 permutational multivariate analysis of variance (PERMANOVA) was used to examine the statistical
239 differences in fish community structure among the sampling methods, and permutational analysis of
240 multivariate dispersions (PERMDISP) was used to assess the variation in species composition
241 among the sampling methods. The Bray–Curtis dissimilarity of fish community structure between
242 samples in each experiment was calculated. The β-diversity plots of each sample were visualized in
243 each experiment. In each experiment, fish species that affected the differences in community
244 structure between sampling methods were estimated using the "envfit" function. In this study, the
245 threshold p-value was < 0.01 for selecting species that had higher effects on the community
246 structure. All the statistical analyses were performed using R ver. 4.02. For nMDS, PERMANOVA,

247 and PERMDISP, the “metaMDS”, “adonis”, and “betadisper” functions of the vegan package were
248 used.
249
250 **Results**
251 In this study, 8,296,115 reads were obtained from 105 water samples and 41 passive
252 samples, and 6,903,093 reads remained after preprocessing (Supplementary Table S4). The number
253 of species detected in Experiment 1 (July 2021) ranged from 15 to 22 for the water samples (n = 39)
254 and 7–14 (M-filter, n = 3) and 15–22 (M-solution, n = 8) for the passive samples (Fig. 2). Because
255 of the loss of one of the M-solution samples in Experiment 1 (M-solution 3-3) from the equipment,
256 the total number of M-solution samples in Experiment 1 was eight (Supplementary Table S1). The
257 number of species detected in Experiment 2 (October 2021) ranged from 15 to 23 for the water
258 samples (n = 39) and from 19 to 25 for the M-solution samples (n = 18). In Experiment 3 (May
259 2022), the number of species ranged from 18 to 24 for the water samples (n = 27), and 21–23 (M-
260 solution, n = 3), 21–23 (S-solution, n = 3), 16–19 (S-syringe, n = 3), and 19 (S-syringe-NP, n = 3)
261 for the passive samples (Fig. 2). Steel–Dwass multiple comparisons were performed because
262 significant differences were verified by the results of the Kruskal–Wallis test in all the experiments
263 (Supplementary Table S5). However, the results of the Steel–Dwass test verified significant
264 differences only in Experiment 1 (Fig. 2). In that experiment, significant differences were observed
265 between the number of species detected from M-filter and that of water (all), water (day), water
266 (night) and between water (day) and water (night), respectively ($p < 0.05$). In Experiment 2, the

267 number of species detected in the M-solution samples tended to be larger than that in water (all) and
268 water (day), although the differences were not statistically significant (p = 0.055 and 0.072,
269 respectively). In Experiment 3, there were no statistically significant differences in number of
270 species among the sample types (Supplementary Table S5).

271 The cumulative fish diversity was higher for water sampling accumulated between 0 and
272 24 h (3 samples × 13 times) than for passive sampling methods in all the experiments (Fig. 3).
273 Specifically, the cumulative numbers of fish species in Experiment 1 were 25, 15, 23, 24, and 19 in
274 the water; M-filter; and M-solution 1, 2, and 3 samples, respectively. The cumulative number of
275 fish species in Experiment 2 was 28 and 31 for the water and M-solution samples, respectively. In
276 Experiment 3, the cumulative numbers of fish species in the water, M-solution, S-solution, S-
277 syringe, and S-syringe-NP samples were 26, 23, 24, 21, and 21, respectively. Passive sampling
278 methods also showed a high cumulative fish diversity similar to that for water. Herein, the M-
279 solution in Experiment 2 showed the highest diversity (Supplementary Tables S1–3).

280 The fish species composition was substantially similar in all the experiments (Fig. 4). The
281 number of species common between the water (day) or water (night) samples and the passive
282 samples (M-solution, S-solution, S-syringe, or S-syringe-NP) was significantly larger than that for
283 the other combinations in all the experiments. However, the common fish species in the two water
284 samples (nine species) were not detected in the M-filter samples in Experiment 1 (Fig. 4).
285 Meanwhile, the comparison with the M-solution showed a decrease in the number of species
286 detected only in the two water samples and an increase in the number of species common to the
287 three methods compared with the M-filter. The cumulative fish species composition detected by
288 each sampling method in Experiments 1–3 is listed in Supplementary Table S6. In Experiment 1,
289 *Oncorhynchus masou* subsp. was specifically detected in water (day), and *Pseudogobio*
290 *agathonectris* was detected in the passive samples (M-filter and M-solution). In Experiment 2, three

291 fish species (*Acheilognathus rhombeus*, *Hemibarbus* sp., and *Salvelinus leucomaenis* subsp.) were
292 detected only in the M-solution samples. The species detected only in the two types of water
293 samples were not observed. In Experiment 3, *Tanakia limbata* was detected only in the water (day)
294 samples, and *Hemibarbus* sp. was detected only in the S-syringe samples. In Experiment 3, the
295 number of common species between the two types of water samples and the four passive sampling
296 methods were similar. The fish species detected by eDNA metabarcoding have generally been
297 known to inhabit the study sites in previous capturing surveys (River Environment Database
298 System, 2018). However, the tropical garfish *Atractosteus tropicus* (listed as an alien species in
299 Japan) was newly detected with high DNA concentrations in the water (night), M-solution, and S-
300 solution samples.

301 In this study, an nMDS plot was used to assess the differences in community structure
302 considering the eDNA concentration of each fish species (Fig. 5). The fish community structure in
303 Experiment 1 differed significantly among the sample types (PERMANOVA: $p < 0.01$;
304 PERMDISP: $p = 0.441$). In particular, the M-filter samples showed a higher variation than the other
305 sample types. Although the number of species in the M-solution samples was similar to that in the
306 two water samples, a few differences were observed in the community structure. Eight fish species
307 affected the differences in community structure. Two of these species (*Hemibarbus longirostris* and
308 *Tachysurus nudiceps*) were not detected in the M-filter samples. Other species were detected in all
309 the sample types. However, their DNA concentrations were significantly higher in the water
310 samples than in the passive samples (Supplementary Table S1). In Experiment 2, the fish
311 community structure in water (night) was included in that of water (day), and the M-solution fish
312 community was plotted outside the two types of water samples. However, differences in community
313 structure among the three types could not be assessed precisely (PERMANOVA, $p < 0.01$;
314 PERMDISP, $p < 0.05$). Differences in community structure between the water (all) and M-solution
315 samples could not be assessed (PERMANOVA, $p < 0.01$; PERMDISP, $p < 0.05$). Fourteen fish
316 species affected the differences in community structure in Experiment 2. *Hemibarbus* sp. was

317 detected only in the M-solution samples. Other species were detected in all the sample types. The
318 trend of the DNA concentration of these species was similar to that observed in Experiment 1
319 (Supplementary Table S2). In Experiment 3, the fish community structure differed marginally
320 between the water (day) and nighttime water (night) samples. In the passive samples, the
321 community structure differed between solution types (M-solution and S-solution) and syringe types
322 (S-syringe and S-syringe-NP). In Experiment 3, the fish community structure differed significantly
323 among the sample types (PERMANOVA: $p < 0.01$, PERMDISP: $p = 0.11$). Thirteen fish species
324 affected the community structure in Experiment 3. All the species were detected in all the sample
325 types, except for Misgurnus anguillicaudatus in the S-syringe samples. The trend of the DNA
326 concentration of these species was similar to that observed in Experiments 1 and 2 (Supplementary
327 Table S3).
328
329 **Discussion**
330 In this study, we assessed the effectiveness of passive eDNA sampling methods compared
331 with water sampling in a riverine environment. In addition, in the passive sampling method, several
332 DNA extraction methods were examined to determine the optimal method for eDNA recovery from
333 sponges. Our results showed that the passive sampling method using sponge skeletons had a similar
334 or larger number of fish species than water samples. The results of the present study support
335 previous 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 in the M-filter sample was significantly less than
337 that for the other methods (water and other passive samples), and the variation in fish community
338 composition was larger than that in the other methods. Meanwhile, because no PCR inhibition was
339 observed in any of the DNA samples, other factors may have affected the reduction in the number
340 of species in the M-filter samples. These results indicate that the DNA recovery rate of M-filter was
341 less than that of the other methods. Water samples pressed from sponges contained large amounts of
342 material in addition to eDNA (e.g., sand, mud, and biofilm). The DNA in pressed solutions could be

343 captured by these materials. This could be assumed straightforwardly from previous studies using
344 soil particles and biofilms as capturing materials for passive sampling (Kirtane et al., 2020, Rivera
345 et al., 2021). The method of DNA extraction from the GF/F filter used in the M-filter may not have
346 efficiently recovered the eDNA captured from these materials. In contrast, the DC method applied
347 to the passive samplers in this study indicated that the efficiency of DNA recovery was improved by
348 direct DNA extraction from pressed solutions or by direct immersion of sponge skeletons in the
349 elution mixture. The M-solution in the passive sampling method showed similar fish diversity in all
350 the experiments (in terms of both number of species and cumulative number of species) similarly as
351 in the water samples. Furthermore, in Experiment 2, fish species detected only in M-solution were
352 observed. The cumulative number of samples in the passive sampling methods was larger than that
353 for the water samples (Fig. 4). These results show that passive samples (sponge skeletons) with 24 h
354 immersion displayed detection performance equal to that for 24 h water samples extracted every 2
355 h. The smaller number of species observed for the other passive sampling methods (S-solution, S-
356 syringe, and S-syringe-NP) was marginally less than that in the water samples. However,
357 statistically significant differences were not observed. The high cumulative fish diversity for the S-
358 solution and the presence of specific species in the S-syringe may also indicate the effectiveness of
359 each method (Figs. 3 and 4). However, it could have been difficult to distinguish the passive
360 sampling method in Experiment 3 from the other methods because of the absence of replicate
361 samples (triplicates in each sample). Therefore, the effectiveness of these methods should be
362 evaluated meticulously in future studies.

363 In Experiments 1 and 3, the number of species detected in the water samples at nighttime
364 tended to be larger than that for the day time (Fig. 3). The cumulative number of species in the
365 water samples in Experiments 1 and 2 varied considerably with collection timing (Fig. 4). In
366 contrast, the number of detected and cumulative species in the passive samples, except for the M-
367 filter sample, tended to be more homogeneous than those in the water samples in all the
368 experiments. These differences could be owing to dissimilarities in the DNA information of

369 freshwater fish between the water and passive samples. In general, the eDNA information from
370 water samples contains snapshot information that reflects the collection timing (Spear et al. 2015,
371 Bowers et al., 2021; Jensen et al., 2022). Therefore, eDNA removed from rare species or species
372 with an incidental appearance can be rapidly diluted and dispersed and could elude detection via
373 instantaneous water sampling (Kirtane et al., 2020). In contrast, passive sampling methods could
374 accumulate more eDNA by filtering a large water volume through long-term immersion in water.
375 Jeunen et al. (2022) indicated that more porous passive filter materials could achieve higher
376 detection rates through the adsorption and retention of low-abundance DNA and DNA bound to
377 particulate matter. The differences in temporal resolution between sampling methods could be
378 reflected in these variations and differences in fish species diversity.
379 Experiments 1 and 3 showed differences in fish community structure between the methods
380 in the nMDS plot and statistical analyses. In all the experiments, certain fish species affected the
381 differences in community structure among the sample types. In Experiment 1, this was reasonable
382 because the fish diversity and DNA concentrations of each fish species in the M-filter samples were
383 significantly lower than those in the other methods. However, in the other experiments, the fish
384 diversity and species composition differed among the sampling methods (Figs. 3 and 4). Therefore,
385 the variations in community structure among the different methods could be attributed to the
386 differences in the eDNA concentration of each fish species. There were differences in fish DNA
387 concentrations among the sampling methods. Those of the common fish species (*Zacco platypus*,
388 *Nipponocypris temminckii*, and *Pungtungia herzi*) observed at the survey site showed large
389 differences between the water and passive samples in all the experiments (Supplementary Tables 1–
390 3). The fish DNA concentration of each sample was standardized by adding the standard DNA mix.
391 Therefore, these differences indicated that the eDNA concentration of fish in the passive samples
392 was lower than that in the water samples. Passive sampling methods store aquatic eDNA by
393 capturing and accumulating collection material. Cananzi et al. (2025) showed that passive samplers
394 contain eDNA of diverse taxa and particle sizes and are more efficient for detecting micro-

395 invertebrates and amphibians than water samples using a multi-marker approach. Similarly, in our
396 study, large amounts of eDNA from non-fish organisms (e.g., aquatic plants, insects, and
397 microorganisms) may have been captured by sponge skeletons during the long-term immersion. As
398 a result, fish eDNA was relatively reduced in the passive samples. This generated differences in the
399 proportion of DNA concentration between the water samples and passive samples. The differences
400 in eDNA concentrations may also reflect the differences in DNA extraction efficiency between the
401 sponge skeletons and GF/F filters. Although the optimization of DNA extraction procedures using
402 GF/F filters has been examined in previous studies (Minamoto et al. 2021), the optimization of
403 sponge skeletons may still be insufficient. Previous studies have also shown that the efficiency of
404 DNA recovery from passive samples varies significantly depending on the sampler material and the
405 immersion method (Verdier et al. 2022, Saltonstall et al. 2024, Stevens et al. 2024). Therefore,
406 future improvements in the methodology would enhance the efficacy of the passive sampling
407 method using sponge skeletons.

408 To conclude, we showed that passive eDNA sampling using sponge skeletons is one of the
409 most efficient methods for monitoring freshwater fish in riverine environments. Passive sampling
410 methods enable more cost-effective and efficient eDNA monitoring than the conventional filtering
411 methods. Passive sampling methods also do not require time-consuming filtration or the
412 preservation of large volumes of water. These methods minimize sampling and processing times in
413 the field, accumulate eDNA through long-term immersion, and enable time-efficient biomonitoring.
414 Our passive sampling method could detect similar or higher fish species diversity than water
415 sampling because of the long-term and cumulative eDNA adsorption on sponge skeletons.
416 However, there are challenges associated with passive sampling methods. One of these is that the
417 actual amount of water passing through the passive collection device during submersion is difficult
418 to quantify (Zhang et al. 2024). Currently, this method may be unsuitable for estimating the eDNA
419 concentration of target species or for quantitative assessments of species abundance and biomass. In
420 this study, the eDNA concentration of each fish species was estimated using the qMiSeq method.

However, the accuracy of DNA quantification could not be evaluated. This problem can be resolved by measuring the flow rate and estimating the water volume through the sponge skeleton. Furthermore, passive sampling methods can concentrate PCR inhibitors and eDNA through long-term immersion. Organic compounds (e.g., humic and fulvic acid) produced by the biological processes of phytoplankton and terrestrial detritus inputs are present in aquatic systems and can be the major cause of PCR inhibition (Hellings et al. 1999, Albers et al., 2013, Uchii et al. 2019). These substances may be adsorbed and concentrated on the sponge skeleton, as well as on eDNA. This would increase the probability of PCR inhibition. Further improvements in methodology, such as the effect of PCR inhibition, quantitative assessment, and immersion period, are required so that passive sampling methods using sponge skeletons can be an effective tool for eDNA surveys.

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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 application number PCT/JP2023/036721.

Data accessibility

The entire sequence data would be published in DRA (Accession number:) after the manuscript is accepted. Detailed information on the fish community structure and methodological descriptions of library preparation are available in the Supplementary Materials.

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572 **Figure Captions**

573 **Fig 1** Study site information and descriptions of experimental design in this study. (A) Descriptions
574 of survey site, (B) detailed information of survey site and passive sampler, (C) methodological
575 procedures of water and passive sampling in each experiment

576

577 **Fig 2** Boxplots in the number of detected species in each experiment. Asterisks indicate the
578 significant differences among sample categories (**: $p < 0.01$, *: $p < 0.05$)

579

580 **Fig 3** Cumulative number of species detected for each collection timing and DNA extraction
581 method

582

583 **Fig 4** Venn diagrams showing the number of fish species belonging to each sampling method that
584 were detected by eDNA metabarcoding in each experiment. Detailed information on the fish species
585 compositions in each experiment is presented in Supplementary Table S6

586

587 **Fig 5** nMDS plots for each experiment. The green arrows represent the fish responsible for causing
588 a significant difference among sampling methods

589

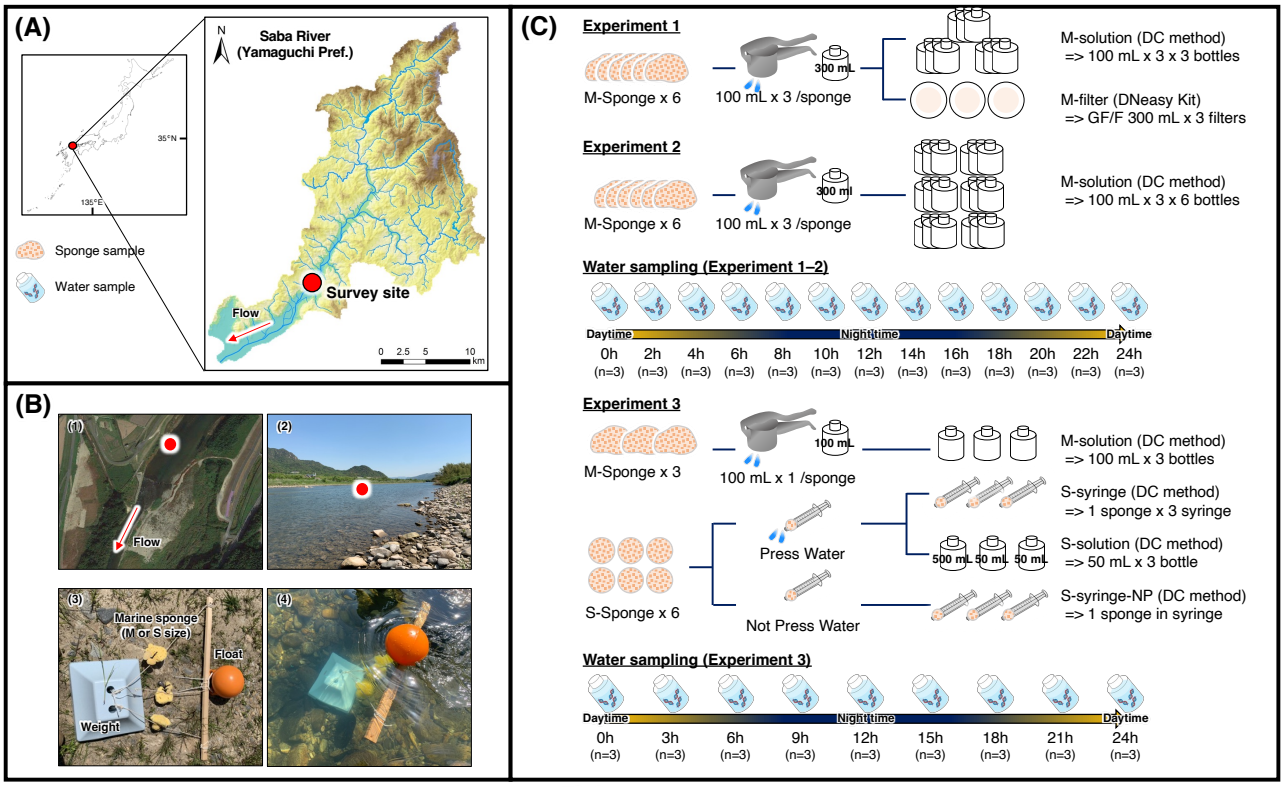
(A)
Saba River
(Yamaguchi Pref.)
N
35°N
135°E
Sponge sample
Water sample
Survey site
Flow
0 2.5 5 10 km
(B)
(1)
Flow
(2)
(3)
Marine sponge
(M or S size)
Float
Weight
(4)
(C)
Experiment 1
M-Sponge x 6
300 mL
100 mL x 3 /sponge
M-solution (DC method)
=> 100 mL x 3 x 3 bottles
M-filter (DNeasy Kit)
=> GF/F 300 mL x 3 filters
Experiment 2
M-Sponge x 6
300 mL
100 mL x 3 /sponge
M-solution (DC method)
=> 100 mL x 3 x 6 bottles
Water sampling (Experiment 1–2)
Daytime
Nighttime
Daytime
0h (n=3)
2h (n=3)
4h (n=3)
6h (n=3)
8h (n=3)
10h (n=3)
12h (n=3)
14h (n=3)
16h (n=3)
18h (n=3)
20h (n=3)
22h (n=3)
24h (n=3)
Experiment 3
M-Sponge x 3
100 mL
100 mL x 1 /sponge
M-solution (DC method)
=> 100 mL x 3 bottles
S-syringe (DC method)
=> 1 sponge x 3 syringe
Press Water
500 mL 50 mL 50 mL
S-solution (DC method)
=> 50 mL x 3 bottle
S-Sponge x 6
Not Press Water
S-syringe-NP (DC method)
=> 1 sponge in syringe
Water sampling (Experiment 3)
Daytime
Nighttime
Daytime
0h (n=3)
3h (n=3)
6h (n=3)
9h (n=3)
12h (n=3)
15h (n=3)
18h (n=3)
21h (n=3)
24h (n=3)

Fig.1

Fig 1

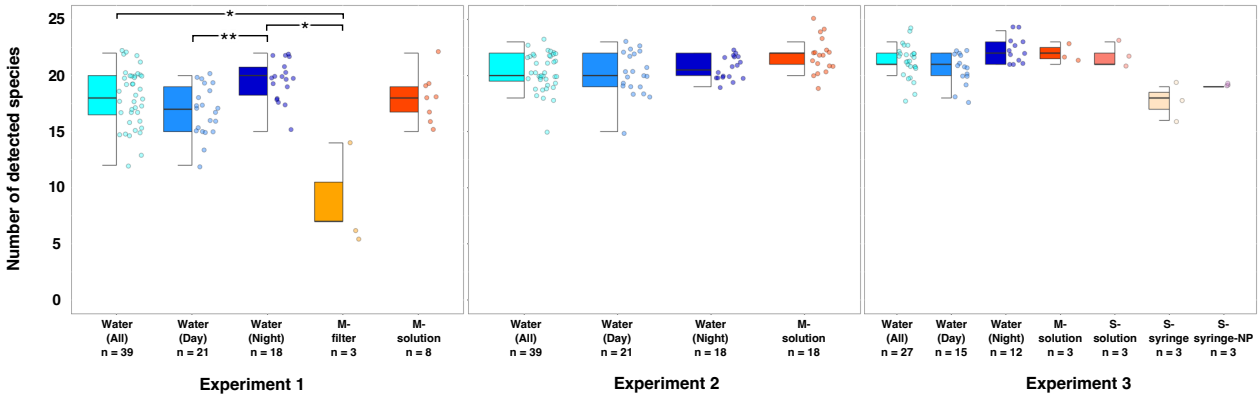
Number of detected species
25
20
15
10
5
0
*
**
*
Water (All) n = 39
Water (Day) n = 21
Water (Night) n = 18
M-filter n = 3
M-solution n = 8
Experiment 1
Water (All) n = 39
Water (Day) n = 21
Water (Night) n = 18
M-solution n = 18
Experiment 2
Water (All) n = 27
Water (Day) n = 15
Water (Night) n = 12
M-solution n = 3
S-solution n = 3
S-syringe n = 3
S-syringe-NP n = 3
Experiment 3

Fig 2

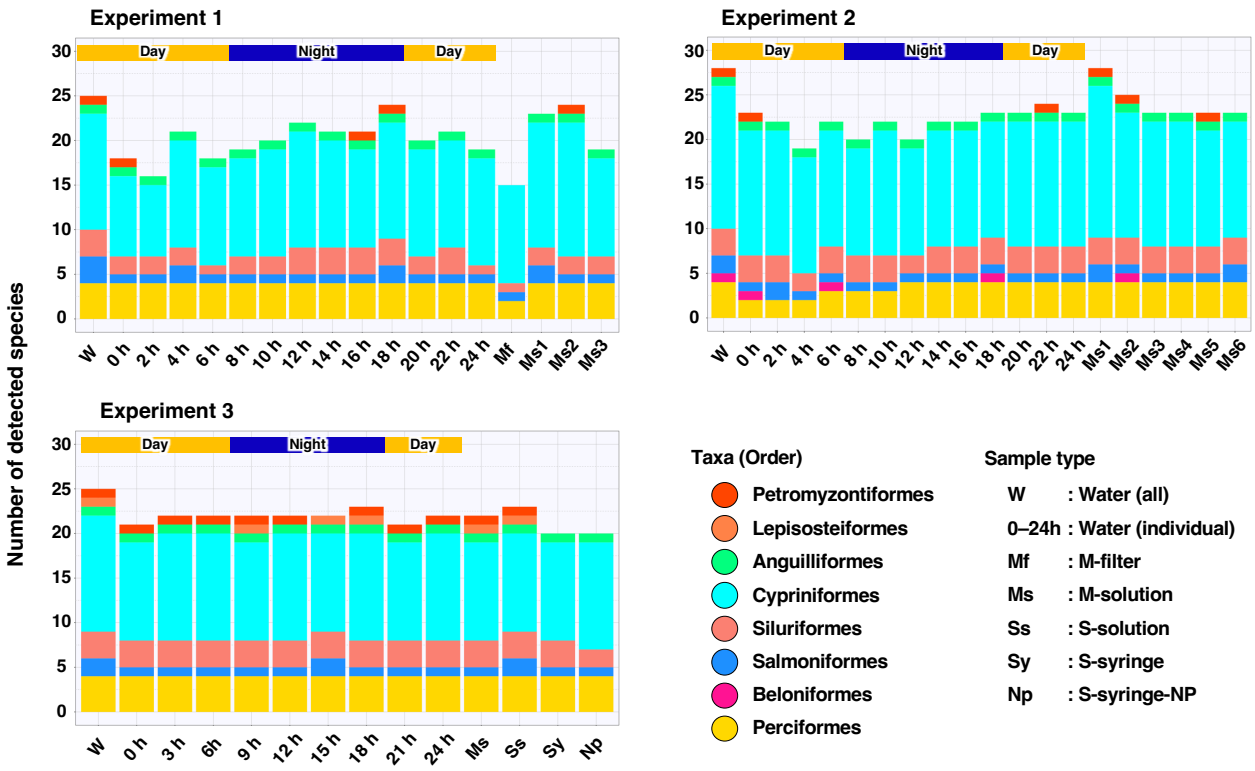
Experiment 1
Experiment 2
Experiment 3
Number of detected species
Day
Night
Day
W
0 h
2 h
4 h
6 h
8 h
10 h
12 h
14 h
16 h
18 h
20 h
22 h
24 h
Mf
Ms1
Ms2
Ms3
Ms4
Ms5
Ms6
3 h
6h
9 h
15 h
21 h
Ms
Ss
Sy
Np
Taxa (Order)
Petromyzontiformes
Lepisosteiformes
Anguilliformes
Cypriniformes
Siluriformes
Salmoniformes
Beloniformes
Perciformes
Sample type
W : Water (all)
0–24h : Water (individual)
Mf : M-filter
Ms : M-solution
Ss : S-solution
Sy : S-syringe
Np : S-syringe-NP

Fig 3

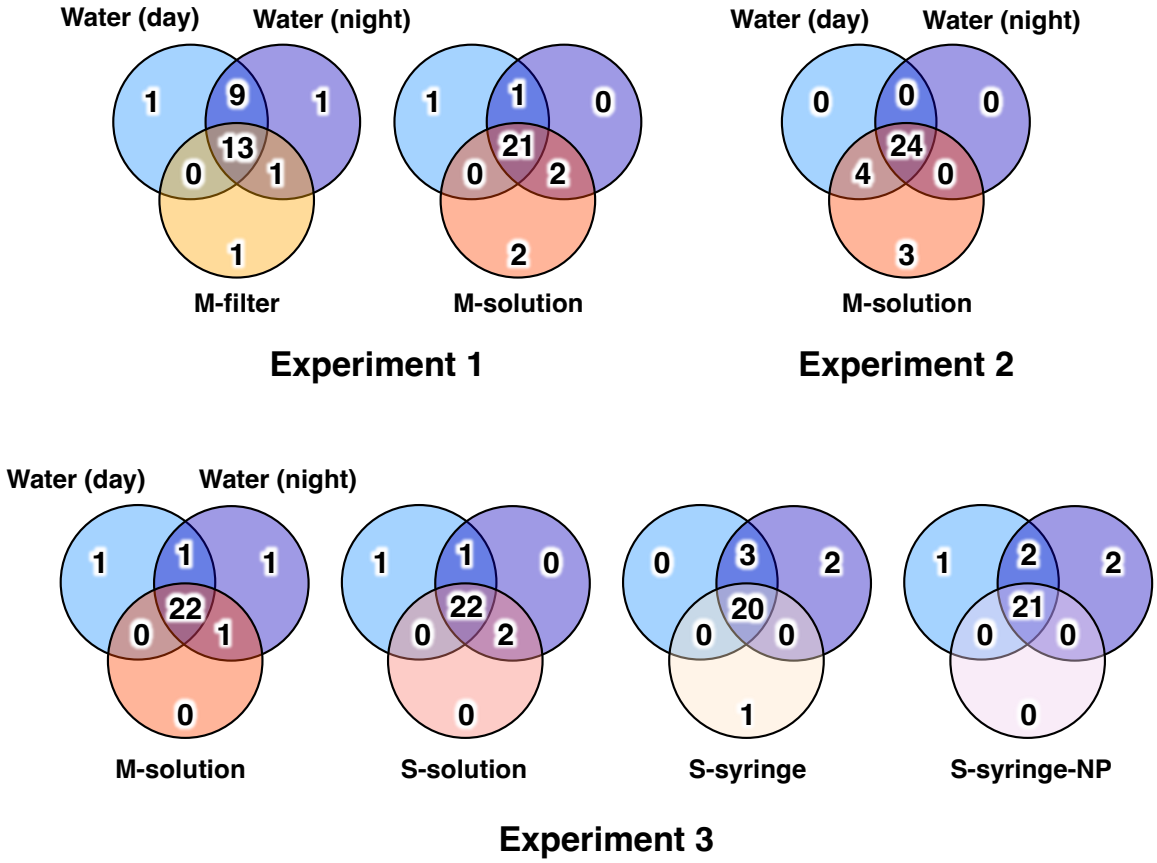
Water (day)
Water (night)
1
9
1
13
0
1
1
M-filter
1
1
0
21
0
2
2
M-solution
Experiment 1
Water (day)
Water (night)
0
0
0
24
4
0
3
M-solution
Experiment 2
Water (day)
Water (night)
1
1
1
22
0
1
0
M-solution
1
1
0
22
0
2
0
S-solution
0
3
2
20
0
0
1
S-syringe
1
2
2
21
0
0
0
S-syringe-NP
Experiment 3

Fig 4

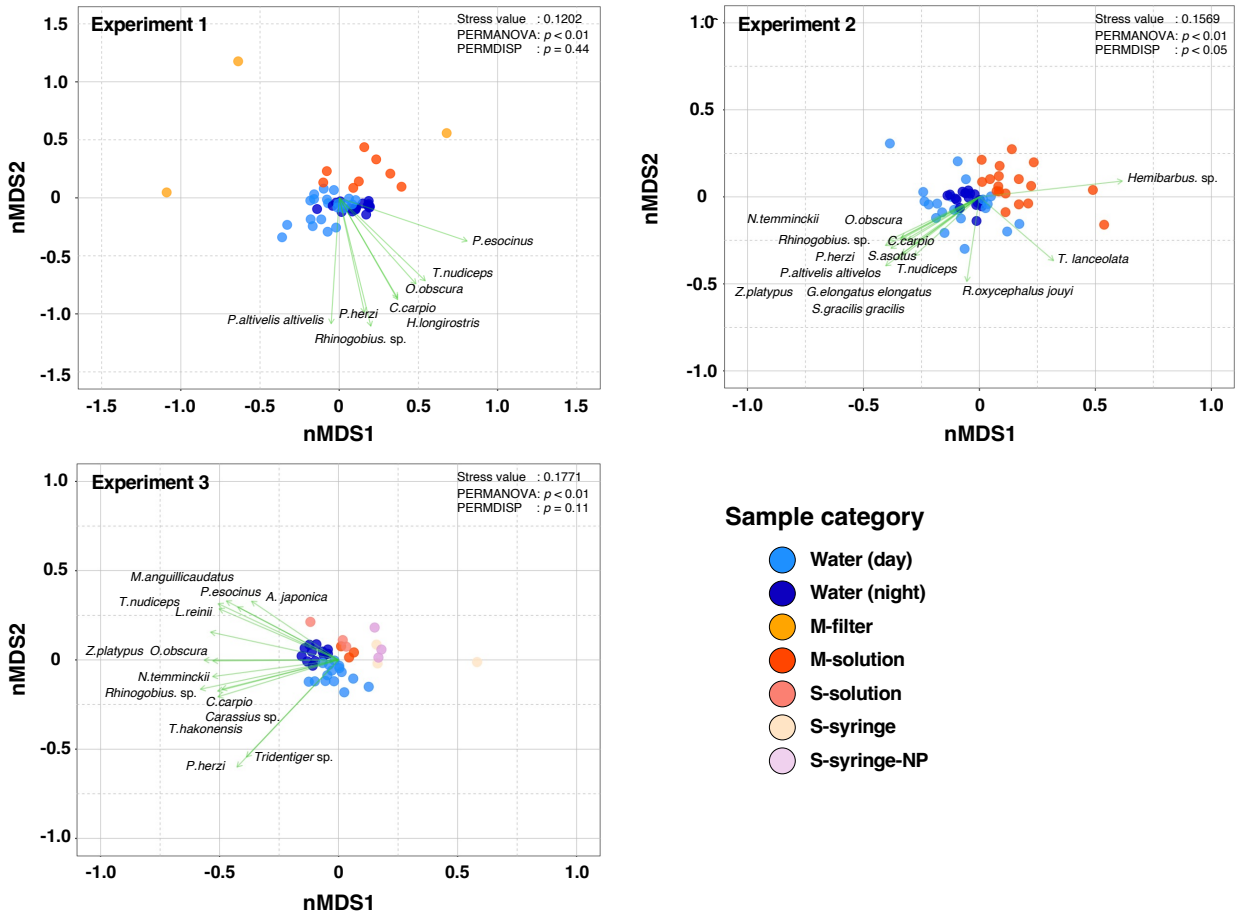
Experiment 1
Stress value : 0.1202
PERMANOVA: p < 0.01
PERMDISP : p = 0.44
P.esocinus
T.nudiceps
O.obscura
C.carpio
H.longirostris
P.herzi
P.altivelis altivelis
Rhinogobius. sp.
Experiment 2
Stress value : 0.1569
PERMANOVA: p < 0.01
PERMDISP : p < 0.05
Hemibarbus. sp.
N.temminckii
O.obscura
Rhinogobius. sp.
C.carpio
P.herzi
S.asotus
P.altivelis altivelos
T.nudiceps
T. lanceolata
Z.platypus
G.elongatus elongatus
S.gracilis gracilis
R.oxycephalus jouyi
Experiment 3
Stress value : 0.1771
PERMANOVA: p < 0.01
PERMDISP : p = 0.11
M.anguillicaudatus
P.esocinus
A. japonica
T.nudiceps
L.reinii
Z.platypus
O.obscura
N.temminckii
Rhinogobius. sp.
C.carpio
Carassius sp.
T.hakonensis
P.herzi
Tridentiger sp.
nMDS1
nMDS2
Sample category
Water (day)
Water (night)
M-filter
M-solution
S-solution
S-syringe
S-syringe-NP

Fig 5