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1 Passive sampling of environmental DNA in aquatic
2 environments using 3D-printed hydroxyapatite samplers

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22 **Abstract**

23 The study of environmental DNA released by aquatic organisms in their habitat offers
24 a fast, non-invasive and sensitive approach to monitor their presence. Common
25 eDNA sampling methods such as water filtration and DNA precipitation are time
26 consuming, require difficult-to-handle equipment and partially integrate eDNA
27 signals. To overcome these limitations, we created the first proof of concept of a
28 passive, 3D-printed and easy-to-use eDNA sampler. We designed the samplers from
29 hydroxyapatite (HAp samplers), a natural mineral with a high DNA adsorption
30 capacity. The porous structure and shape of the samplers were designed to optimise
31 DNA adsorption and facilitate their handling in the laboratory and in the field. Here we
32 show that HAp samplers can efficiently collect genomic DNA in controlled set-ups,
33 but can also collect animal eDNA under controlled and natural conditions with yields
34 similar to conventional methods. However, we also observed large variations in the
35 amount of DNA collected even under controlled conditions. A better understanding
36 of the DNA-hydroxyapatite interactions on the surface of the samplers is now
37 necessary to optimise the eDNA adsorption and to allow the development of a
38 reliable, easy-to-use and reusable eDNA sampling tool.

39 **Key-words:** DNA adsorption, Environmental DNA, Hydroxyapatite, Passive
40 sampling, 3D-printing

41

42 **Introduction**

43 At a time of unprecedented threats on freshwater biodiversity (Dudgeon et al., 2006;
44 Reid et al., 2019), it is crucial to develop rapid, accurate and minimally invasive tools
45 to monitor aquatic ecosystems. About a decade ago, methods based on the sampling
46 of environmental DNA (eDNA) were proposed as a revolutionary way to survey
47 aquatic macro-organisms (Deiner et al., 2017). Macro-organisms release DNA in
48 their environment through different processes (e.g. faeces, excretion, shedding cells,
49 gametes) and this extra-organismal eDNA can take different forms (tissues, cells,
50 organites, nucleo-proteic complexes, ...). The direct sampling of eDNA coupled with
51 molecular analysis methods such as next generation sequencing (Shokralla et al.,
52 2012) or targeted approaches such as quantitative polymerase chain reaction
53 (qPCR) (Langlois et al., 2020) allow the detection and identification of aquatic
54 species while overcoming organism capture. Although eDNA offers many promising
55 applications, several methodological challenges remain (Beng & Corlett, 2020).

56 eDNA sampling is one of the most challenging steps in eDNA-based approaches.
57 Several families of methods exist to sample eDNA from water: precipitation,
58 centrifugation or active filtration, the latter being the most commonly used (Tsuji et
59 al., 2019). Within each method family, there are a multitude of possible strategies
60 (e.g. volume of the water sample, field or laboratory processing, choice of equipment
61 and DNA extraction kits) that vary according to the ecosystem studied, the target
62 species and the downstream analysis (Deiner et al., 2015).

63 However, all these sampling methods are time-consuming, require human
64 intervention and specialised equipment. For example, the filtration of a large volume
65 of water requires pumping systems and filtration funnels which are expensive and
66 difficult to handle. In addition, given the complex dynamics of eDNA in aquatic
67 environments (i.e. pulsed emission, transport, retention, degradation), a single
68 filtered or precipitated water sample will provide a snapshot that is likely to be poorly
69 integrative of the overall eDNA signals (Spear et al., 2015).

70 Passive eDNA sampling appears as a promising and simple solution to overcome
71 challenges associated with conventional methods. Passive eDNA sampling can be
72 defined as the use of natural or artificial substrates that can collect eDNA passively,
73 without human intervention. Substrates such as marine sponges (Mariani et al.,
74 2019), biofilms (Rivera et al., 2021) and filters immersed directly in water (Bessey et
75 al., 2021) have been used successfully to collect eDNA from aquatic systems.
76 Recently, Kirtane and colleagues (Kirtane et al., 2020) have shown that
77 montmorillonite and coal-based mineral powders can be used as passive eDNA
78 samplers in aquatic environments. As a result of good DNA capture and preservation
79 capacity (up to 200 µg genomic DNA per g) (Gardner & Gunsch, 2017), sediments
80 and commercial mineral powders may very well be more integrative eDNA substrates
81 than methods based on a single water sample. Yet, these substrates are difficult to
82 deploy in the environment, particularly in aquatic systems.

83

84 In this study, we developed 3D-printed passive eDNA samplers made of pure
85 hydroxyapatite (HAp), a calcium phosphate mineral naturally present in bones. Due
86 to its biocompatibility in bone contact (Yetgin, 2013), synthetic HAp is widely used in
87 3D-printing for the manufacture of prosthetic implants (Kattimani et al., 2016). In
88 addition to its clinical benefits, HAp has been used since the 1990s in
89 chromatography for its ability to adsorb biomolecules such as DNA (del Valle et al.,
90 2014; Okazaki et al., 2001). The mechanism involved is thought to be a binding
91 between the phosphate groups of the extracellular DNA and the positively charged
92 calcium groups of the hydroxyapatite surface (Brundin et al., 2013). Coupling the use
93 of 3D-printing and DNA adsorption properties of HAp allows the creation of an object
94 whose shape and composition are optimised for eDNA sampling. In this paper, we
95 described the development of hydroxyapatite samplers (HAp samplers) and tested
96 their ability to sample eDNA in fresh waters. Our objectives were to (i) quantify the
97 HAp samplers DNA adsorption and desorption capacity, (ii) assess the range of DNA
98 fragment size sampled, (iii) quantify the repeatability of DNA sampling across several
99 cycles of use of the HAp samplers, and (iv) evaluate the samplers capacity to recover
100 eDNA in microcosms and under natural conditions.

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105 1. Materials and methods

106 1.1. 3D-printed HAp samplers design

107 1.1.1. Raw material and printing setup

108 A photopolymerizable organic resin (3D Mix, 3DCeram Company, HAP, Bonnac-la-
109 Côte, France) containing 40-60% (w/v) of hydroxyapatite powder ($\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$,
110 stoichiometric hydroxyapatite), a synthetic calcium phosphate with Ca/P atomic ratio
111 of 1.67, was the raw material used to fabricate the samplers. The samplers were built
112 from this hydroxyapatite-enriched resin using a 3D stereolithographic printer
113 (CERAMAKER C900, 3DCeram Company, with 55 mW laser power and 100 μm
114 layer thickness). Two prototypes of HAp samplers were produced: a first test
115 prototype (P1) corresponding to 10 pieces cut from a 3D-printed mesh with (Fig. 1A)
116 to test the concept and the material, and a second prototype (P2) printed in 25 copies
117 (Fig. 1B) with a higher ratio porosity/surface and an optimised design for laboratory
118 and field manipulations. P1 prototypes have an exposed surface of 240 mm^2 and a
119 macroporosity of 500 μm in diameter. P2 has a total surface of 480 mm^2 and a
120 macroporosity of 400 μm in diameter.

121 1.1.2. Debinding and sintering steps

122 Once printed, cleaned with a solvent (Ceracleaner, 3DCeram Company, Bonnac-la-
123 Côte, France) and dried, the HAp samplers underwent debinding and then sintering

124 steps. Debinding aims at removing all organic components (in particular the organic
125 resin) and was conducted in a conventional oven following the thermal cycle
126 described in Table 1. Sintering aims at consolidating the samplers by densifying them
127 (creation of necks and reduction of the porosity between the individual ceramic
128 particles) (Rahaman, 2017), and is achieved by a thermal treatment at higher
129 temperature (1 °C/min up to 1150 °C, 60 min. at 1150 °C, followed by a second step
130 at 3 °C/min up to 1250 °C, 60 min at 1250 °C, finally cooling to room temperature at 3
131 °C/min). After these steps, no organic components remain and the samplers are
132 made of pure HAp as confirmed by X-ray diffraction (XRD). However, sintering is
133 accompanied by a ~15% linear shrinkage corresponding to a ~30% decrease of the
134 surfaces of HAp samplers after printing.

135 1.2 Expected DNA recovery from HAp samplers

136 We used the term “DNA recovery” to define the quantity of DNA adsorbed and
137 desorbed from the HAp samplers. A first estimation of the maximum DNA recovery
138 (DNA_{max}) can be obtained by hypothesising that a single layer of DNA molecules
139 would bind on the HAp surface of the samplers. According to equation 1, the number
140 of DNA molecules that can adsorb to the surface is obtained by dividing the exposed
141 surface (S_e) of a sampler ($P1 = 240 \text{ mm}^2$, $P2 = 480 \text{ mm}^2$) by the surface of a DNA
142 base pair ($S_d = 6.46 \cdot 10^{-10} \text{ mm}^2$). The surface of a DNA base pair was calculated
143 according to Mandelkern *et al* (1981) (diameter = 2 nm, length = 3.4 nm). The
144 number of DNA molecules per sampler is then divided by Avogadro's constant ($NA =$

145 6.02214076 × 10²³ mol⁻¹) to give the number of DNA moles per sampler. The
146 number of moles of DNA is then divided by the molar mass of a DNA base pair (W =
147 650 daltons) to obtain the total mass of DNA that can bind to a sampler.

$$148 \quad DNAMax = (St/Sd) / N A \times W$$

Equation 1

149 According to Equation 1, DNA recovery should be correlated with the surface area of
150 the sampler. Being smaller, P1 has a theoretical recovery capacity of 400 ng of DNA
151 per sampler, while P2 has a capacity of 800 ng. Because they are based on a
152 simplistic interaction model, these estimates are likely to be biased, but they will
153 serve as a first estimation to calibrate several experiments of this study.

154 1.3 Protocol of DNA adsorption and desorption

155 The HAp sampler DNA adsorption and desorption protocol was composed of 5 steps
156 (Fig. 2). First, HAp samplers were decontaminated before each experiment by a
157 thermal treatment in air at 550 °C for 3 hours (Thermolyne model 30400 furnace), a
158 procedure typically used to decontaminate glassware. Second, DNA is adsorbed to
159 the HAp samplers by immersing them in an aqueous solution (varying composition
160 upon the present study) containing DNA. Third, samplers are transferred to
161 Eppendorf tubes and centrifuged for 1 minute at 3000 rpm to dry them. Fourth,
162 samplers are washed with 1 mL of sterile ultrapure water. Finally, DNA is desorbed
163 from the samplers by immersing them in 1 mL of 0.1 M phosphate buffer pH 8
164 (Grunenwald et al., 2014), vortexed for 30 seconds and incubated at room

165 temperature for 1 hour.

166 1.4 DNA sampling experiments by HAp samplers

167 1.4.1 Experiment 1: capacity of DNA adsorption and desorption of fragments of
168 various sizes

169 We hypothesised that HAp samplers adsorb free DNA fragments and that longer
170 fragments, having more adsorption sites, were preferentially adsorbed. To test this,
171 we performed a DNA sampling experiment with concentrated DNA fragments of
172 various sizes (i.e. using a DNA size marker). After decontamination, a first batch of
173 six HAp samplers (three P1 and three P2) were incubated in tubes (one
174 sampler/tube) containing 2 mL of a solution of long DNA fragments at 1 ng/ μ L (λ DNA
175 / BstEII Digest, 1260-8450 bp). A second batch of six HAp samplers was incubated in
176 tubes containing 2 mL of a solution of shorter DNA fragments at 1 ng/ μ L (PCR 20 bp
177 Low Ladder, 20-2000 bp). Initial DNA concentration in both solutions was verified by
178 QuBit[®] (High Sensitive assay kit, range: 0.001 to 100 ng/ μ L) quantification before
179 the experiment (1 ng/ μ L \pm 0.2 ng/ μ L). All samplers were incubated for 17 hours on a
180 rotary shaker (IKA Roller 6 Digital, 40 rpm). After 17 hours of incubation, 10 μ L of
181 supernatants in each tube was taken to quantify residual DNA and HAp samplers
182 were removed from the DNA solutions using sterile forceps. The DNA was desorbed
183 from the samplers according to the protocol in section 1.3. The supernatant aliquots
184 and desorbed DNA solutions were stored at -20 °C prior to analysis.

185 1.4.2 Experiment 2: repeatability

186 A quantification of repeatability was conducted to test whether HAp samplers can be
187 reused after several cycles of use. A cycle of use is defined here as a thermic
188 treatment phase followed by a DNA adsorption and desorption phase. For this
189 purpose, five P1 prototypes and 25 P2 prototypes of HAp samplers were incubated in
190 5 mL of a concentrated solution of DNA size marker (λ DNA/BstEII Digest 1260-8450
191 bp) at a concentration of 2.88 ± 0.5 ng/ μ L on a rotary shaker (Roller 10 Digital IKA)
192 for 17 hours. This experiment was carried out three times in a row (hereafter called
193 experiments A, B and C) under strictly identical conditions, at room temperature (24
194 $^{\circ}\text{C} \pm 2$ $^{\circ}\text{C}$) with decontamination through thermic treatment between each use. After
195 incubation, HAp samplers were removed from the DNA solution with sterile clamp,
196 washed and DNA was released with 1 mL of 0.1 M phosphate buffer pH 8 according
197 to the protocol section 1.3. 20 μ L of supernatants was taken to quantify residual
198 DNA. We added a DNA degradation control corresponding to three tubes containing
199 the DNA marker solutions with no HAp sampler. We quantified DNA in the
200 supernatant at the beginning and end of each experiment to calculate a DNA
201 percentage loss (e.g. due to degradation, adsorption to plastic tubes) (Gaillard &
202 Strauss, 1998) and estimate the exact amount of DNA adsorbed by the samplers.
203 DNA samples (in supernatants and desorbed from samplers) were stored at -20 $^{\circ}\text{C}$
204 prior to analysis.

205 1.4.3 Experiment 3: eDNA sampling experiment in microcosm

206 *Asellus aquaticus*, a small freshwater isopod, was used as a target organism to test
207 the capacity of the HAp samplers to collect eDNA in microcosm. *A. aquaticus* is a
208 relevant model because it is easy to rear under laboratory conditions and can survive
209 for several days without feeding, which is an advantage for avoiding exogenous
210 contamination in eDNA experiments. In addition, macroinvertebrates received less
211 attention than fish and amphibians in eDNA-based studies, and the demonstration
212 that eDNA-based tools work on these organisms is essential (Mächler et al., 2014).
213 Last, we have access to genomic resources for this species and related species
214 allowing us to design species-specific primers. Forty individuals of *A. aquaticus*
215 sampled from a natural pond (Lyon, France) in April 2019 were divided into eight
216 glass microcosms (five individuals / microcosm) containing 500 mL of synthetic water
217 (Peltier & Weber, 1985) (Fig. 3). Positive controls correspond to microcosms where
218 we injected genomic DNA (final microcosm at 1 ng/mL) extracted from a pool of 10
219 *A. aquaticus*. After 24 hours of *A. aquaticus* acclimatisation, the two prototypes of
220 HAp samplers were incubated in microcosms (1 sampler / microcosm) for 24 hours.
221 All microcosms were placed in a cold room at 18 °C, spaced 0.5 m apart and covered
222 to limit the risk of contamination. The organisms were not fed during the experiment
223 to reduce the amount of allochthonous DNA. After incubation, the HAp samplers
224 were removed from the microcosms using sterile forceps. At the same time, a 14 mL
225 water sample was taken from each microcosm to compare the amount of *A.*
226 *aquaticus* eDNA obtained using a conventional method (i.e. eDNA precipitation) with

227 the amount of DNA collected by HAp samplers. Directly after the experiment, DNA
228 from HAp samplers was desorbed according to section 1.3. of the protocol and
229 purified (Macherey-Nagel [™] NucleoSpin [™] gel and PCR cleaning kit) to avoid
230 potential inhibition of the downstream qPCR by the phosphate buffer (see next
231 section), following the manufacturer's recommendations. DNA from the water
232 samples was precipitated by adding 35 mL of 96% ethanol, 1.4 mL of 3 M sodium
233 acetate and 10 µL of glycogen in 50 mL falcon tubes. After three days of incubation
234 at -80 °C, the tubes were centrifuged at 1200 rpm for 35 minutes at 4 °C. The pellets
235 obtained were resuspended in 50 µL of 1 X TE buffer and extracted with a
236 commercial kit (Qiagen [™] DNA Blood and Tissue kit). Precipitated DNA and DNA
237 desorbed from HAp samplers were stored at -20 °C prior to analysis.

238 1.4.4 Experiment 4: eDNA sampling experiment *in situ*

239 To test the ability of HAp samplers to sample eDNA in natural conditions, an
240 experiment was conducted on August 27, 2021 in a freshwater pond located in an
241 urban area where a population of *A. aquaticus* is present (Lyon, France).
242 Environmental DNA sampling was carried out at nine locations in the pond. At each
243 location, a P2 HAp sampler was placed at an average depth of 20 cm from the water
244 column, attached with a fishing line perpendicular to the surface and incubated for 24
245 hours. Upon collection of the samplers, a 1 L sample of water was collected using
246 sterile bottles from all nine locations to compare the amount of eDNA obtained using
247 a conventional sampling method, here filtration. Immediately after collection, water
248 samples were filtered in a laminar flow hood (Noroit, H-BOX, France) at the

249 University of Lyon using a vacuum filtration manifold (ThermoScientific [™], Nalgene
250 ®) and 47-mm nitrocellulose filters (0.45 µm pore size, MCE Membrane, Merck
251 Millipore, Germany) placed in disposable single-use filter funnels (Thermo Fisher
252 Nalgene [™]). Instead of precipitation, filtration was chosen to increase the volume of
253 sampled water hence increasing the probability of detection of the targeted species
254 (Hinlo et al., 2017; Piggott, 2016; Spens et al., 2017). After filtration, all filters were
255 placed into 5 mL LoBind ® eppendorf tubes and stored at -20 °C until extraction.
256 DNA from filters was extracted using a modified protocol of the DNeasy Blood and
257 Tissue kit (Qiagen [™] DNA Blood and Tissue kit). Briefly, 500 µL of ATL buffer and 40
258 µL of proteinase K were added into 5 mL tubes containing filter membranes and
259 incubated at 56 °C with shaking for 24 hours. After incubation, 400 µL of AL buffer
260 and 400 µL of ethanol 96% were added in tubes. Next steps were done following the
261 manufacturer's recommendations. After 24 hours of exposure, the HAp samplers
262 were removed from the pond and DNA was desorbed following the protocol detailed
263 in section 1.3 and purified (Macherey-Nagel [™] NucleoSpin [™] gel and PCR cleaning
264 kit) following the manufacturer's protocol. Desorbed DNA from HAp samplers and
265 filtered DNA from water samples were stored at -20 °C until quantification.

266 1.5 Negative controls and contamination tracing

267 Concentrated DNA (i.e. DNA marker and genomic DNA) and eDNA samples were
268 processed separately in dedicated laminar flow hoods and with specific equipment.
269 All equipment such as pipettes, filtration material and consumables were

270 decontaminated before each use with a decontamination solution (DNA AWAY ®,
271 ThermoScientific™, France) followed by a 15-min ultraviolet light (UV) treatment.
272 Forceps, glass bottles and glass microcosms were immersed for 1 hour in a 5%
273 bleach solution, rinsed with sterilised water and were autoclaved before use. To trace
274 potential contamination, we have set up four types of contamination controls
275 throughout each experiment: 1) initial controls (control HAp samplers) corresponding
276 to DNA directly desorbed from HAp samplers without incubation in DNA solution, 2)
277 experimental controls corresponding to microcosms or tubes containing water without
278 target DNA in which the samplers are incubated and the water is precipitated or
279 filtered, 3) extraction controls corresponding to filters extracted with a commercial kit
280 (Blood and Tissue kit, Qiagen) without having been in contact with water, 4)
281 quantification controls corresponding to wells without DNA in qPCR and fluorescence
282 plates.

283 1.6 DNA quantification and analysis

284 1.6.1 Quantification of DNA size marker

285 In the first experiment testing DNA recovery of various DNA fragments, we quantified
286 total DNA desorbed from HAp samplers and residual DNA in supernatants by
287 fluorescence using a QuBit® 3.0 fluorometer (Invitrogen). We used the dsDNA BR
288 kit (broad range, range: 0.2 to 1000 ng/μL) according to the manufacturer's protocol.
289 The results are reported in ng/μL. DNA bands profiles were visualised using a
290 Fragment Analyzer (Agilent Technologies). For the second experiment (repeatability),

291 desorbed DNA from the HAp samplers and residual DNA in supernatants were also
292 measured by fluorescence (excitation at 480 nm and emission at 520 nm) but using
293 an Infinite M200 Pro microplate fluorometer (TECAN, Switzerland) to allow the
294 parallel analyses of many samples (96 samples per reading). A QuantiFluor® dsDNA
295 kit was used according to the manufacturer's protocol, with a DNA sample volume of
296 10 µL and 190 µL of working solution. A five-fold dilution series (1500-0 ng/µL) of
297 standard DNA (Lambda DNA Standard, 100 ng/µL) was used to build the standard
298 curve and calculate the sample DNA concentration in ng/µL. The results are reported
299 in percentage of recovered DNA (i.e. DNA adsorbed and desorbed). All DNA
300 samples and controls were quantified in duplicates.

301 1.6.2 Quantitative PCR assay for *A. aquaticus* eDNA

302 For experiments testing eDNA sampling by HAp samplers in microcosms and *in situ*,
303 quantitative PCR (qPCR) was used to quantify the amount of *A. aquaticus* eDNA. We
304 designed a pair of primers to specifically amplify a 110 bp fragment of the
305 mitochondrial 16S gene of *A. aquaticus* (5' GGTTTAAATGGCTGCAGTATCC 3', 5'
306 CTTGTGTAATAAAAAGCCTACCTC 3'). The amplification specificity of the primers
307 was tested *in silico* using primer-BLAST function (NCBI) and assessed
308 experimentally through PCR and electrophoresis gel analysis on a closely related
309 species (*Proasellus meridianus*). The qPCR reaction volume was 10 µL consisting of
310 1X SsoAdvanced Universal SYBR Green Supermix (Bio-Rad Laboratories Inc.,
311 Hercules, CA), 0.5 µM of primers and 2 µL of DNA. The qPCRs assays were run in
312 96 well plates on a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad

313 Laboratories, Inc., Hercules, CA) in duplicate for experiment 3, as we had a limited
314 amount of DNA material, and in triplicate for experiment 4. qPCR cycle started with
315 an incubation at 95 °C for 10 min followed by 45 cycles of denaturation at 95 °C for
316 10 sec and an annealing/extension step at 64 °C for 20 sec before a final melt curve
317 from 65-95 °C (0.5 °C increments). For the microcosm experiments (experiment 3),
318 the qPCR plates included a single seven-fold dilution series of the purified 16S *A.*
319 *aquaticus* amplicons between 2.5×10^{10} and 2.5×10^3 copies/ μ L as quantified by a
320 QuBit 3.0 assay. As we expected very low levels of *A. aquaticus* eDNA in the pond
321 (experiment 4), qPCR plates were run with a single seven-fold dilution series of
322 purified 16S amplicons with a copy number ranging from 2.5×10^7 and 2.5×10^1
323 copies/ μ L. In both qPCR analysis, the R^2 values and PCR efficiency (%) of the
324 calibration curves were 0.99 and 101.5 ± 0.1 (mean \pm SD).

325 1.6.3 Limit of detection and quantification

326 The limit of detection (LOD) was determined as the lowest DNA concentration at
327 which there is 95% of detection across replicates and the limit of quantification (LOQ)
328 as the lowest concentration at which the coefficient of variation is below 35% (Klymus
329 et al., 2019). LOD and LOQ were determined via a 4-fold serial dilution of a 16S *A.*
330 *aquaticus* amplicon from 2.5×10^3 to 2.5 DNA copies/ μ L. Each dilution was run in 18
331 qPCR replicates. The LOQ was 2.5×10^3 and the LOD 2.5×10^2 copies/ μ L.

332 1.7 Statistical analysis

333 Wilcoxon tests were performed in DNA sampling experiments to test three null
334 hypotheses: (1) HAp samplers recover a similar amount of DNA whether incubated in
335 a solution of short or long DNA fragments, (2) both prototypes of HAp samplers
336 recover a similar amount of DNA, (3) the amount of eDNA recovered by HAp
337 samplers is similar to that of two conventional eDNA sampling methods (i.e.
338 precipitation, filtration). In the repeatability experiment, a Fisher exact test was
339 performed to test whether the number of failed recovery was homogeneous between
340 experiments A, B and C. Linear mixed-effect models (LMMs) were used to test the
341 influence of the prototype version and of the experiment timing (experiment 2). These
342 models were fitted with the restricted maximum likelihood method using the package
343 nlme (Pinheiro et al., 2021). We tested significance of experiment timing and
344 prototype version using likelihood ratio tests (LRT) between the models with and
345 without the tested variable. Plots were made with the ggplot2 package (Wickham et
346 al., 2016) and all analyses were conducted using R (v 4.0.3).

347 **2 Results**

348 2.1 Experiment 1: DNA adsorption and desorption of various 349 fragment sizes

350 We first tested the capacity of the HAp samplers to collect DNA using concentrated

351 DNA solution. To also test whether DNA fragment size influences DNA adsorption on
352 HAp samplers, we exposed them to two DNA size marker solutions at the same
353 concentration containing a pool of either medium to long (1260-8450 bp) or short to
354 medium DNA fragments (20-2000 bp). After 17 hours of exposure to the samplers,
355 DNA concentrations in the supernatants were significantly reduced or too low to be
356 quantified for both solutions and prototypes (Table 2). The concentrations of DNA
357 recovered by the HAp samplers were similar whether incubated in the solution
358 containing the longer or the shorter fragments (Wilcox test, $p = 0.31$). Regarding
359 prototypes, P2 samplers recovered significantly more DNA than P1 samplers
360 regardless of the solution in which they were incubated (Wilcox test, $p = 0.04$).
361 Examination of the DNA band profiles (Fig. 4, bottom panel), despite a slight size
362 shift between the control profile and the DNA desorbed from the HAp samplers,
363 showed that the P1 and P2 HAp samplers recovered all the DNA fragments from
364 both markers. The relative intensity among fragments was preserved except for the
365 DNA fragments above 5000 bp which were less concentrated after the desorption.
366 Following the manufacturer (Agilent, personal communication), the observed size
367 shift is likely to be due to the buffer solution being of different composition and
368 concentration between the control ladder solution (TE buffer) and the HAp samplers
369 DNA desorption solution (phosphate buffer).

370 2.2 Experiment 2: Repeatability of HAp samplers over time

371 A repeatability experiment was conducted to test the hypothesis that HAp samplers

372 can be reused and that their recovery efficacy is stable after several cycles of use.
373 We performed three consecutive cycles of use (experiment A, B and C), each
374 composed of a decontamination, DNA adsorption and desorption steps. The
375 percentage of DNA recovered (adsorbed and desorbed) by the samplers was lower
376 in experiment A compared to experiments B and C, with an average of 8%, 17% and
377 15%, respectively (Fig. 5). Experiment A showed a disproportion of samplers (18 out
378 of 30, against 0 for experiment B and C) which failed to recover any DNA compared
379 to the other experiments (Fisher exact test, $p < 1E-10$). Nonetheless, while not
380 associated with any experiment in particular, the percentage of DNA recovered was
381 highly variable. The coefficient of variation of the proportion of recovered DNA was
382 on average 65% considering all the samplers and 34% when excluding the samplers
383 which failed to recover any DNA. After removing the samplers which failed to recover
384 any DNA, we tested the influence of the experiment and prototype on the percentage
385 of DNA recovered using a linear mixed-effect model with experiments (A, B and C)
386 and sampler prototypes (P1 or P2) as the fixed effects, and samplers as random
387 effect on the intercept. The experiment had no significant effect on the percentage of
388 DNA recovered (LRT, experiment : $\chi^2_{df=2} = 1.16, p = 0.28$). While P2 samplers prototype
389 recover more DNA than P1, the effect is not significant (LRT, sampler prototype:
390 $\chi^2_{df=1} = 4.96, p = 0.08$).

391 If we exclude the results of the first experiment, when looking at the relationship
392 between residual DNA in the supernatant and DNA recovered by the HAp samplers
393 (Fig. 6), we can delimit three types of sampler behaviour: (1) a group of 13 samplers

394 with low adsorption capacities as demonstrated by a low DNA recovery (< 20%) and
395 a high proportion of DNA remaining in the supernatant (>30%); (2) a group of 7
396 samplers with high adsorption and desorption capacities (recovery > 20%,
397 supernatant < 30%); (3) and a group of 5 samplers which adsorbed most of the DNA
398 but did not desorb it (supernatant < 30%, recovery < 20%). For this last group, an
399 alternative explanation could be that in these tubes DNA degraded or was adsorbed
400 on the plastic tubes instead of the samplers. By quantifying residual supernatant
401 DNA at the beginning and at the end of each experiment in three tubes without
402 samplers, we estimated the average percentage of DNA loss at 25% (\pm 4.5%) which
403 is too low to explain the behaviour of this third group of sampler.

404 2.3 Experiment 3: eDNA sampling in microcosms

405 We deployed the HAp samplers in microcosms containing isopods (*Asellus*
406 *aquaticus*) to test their ability to recover eDNA in comparison to a conventional
407 sampling method (here precipitation). In a microcosm with no organisms, we
408 observed low levels of DNA that were similar or slightly above the amount of DNA
409 observed in control samplers that were not immersed in a microcosm (Fig. 7). This is
410 indicative of a slight level of cross-contamination between microcosms. We therefore
411 determined, for each sampling method, a 16S rDNA copy number below which we
412 cannot distinguish between a contamination and a positive result (blank limit, LOB),
413 which is more appropriate in this context than the previously calculated LOD and
414 LOQ. The LOB was 7×10^7 copies for HAp samplers and 10×10^7 copies for

415 precipitation. Using concentrated genomic DNA as a positive control, the samplers
416 recovered up to 2.4×10^{10} DNA copies/sampler, one magnitude more than with
417 precipitation. In the microcosms that contained isopods, the amount of 16S eDNA
418 molecules was above the LOB in seven HAp samplers and in four precipitated water
419 samples out of the eight replicates. The number of 16S eDNA molecules recovered
420 was similar between precipitated water samples and HAp samplers (Wilcox-test, $p =$
421 0.35).

422 2.4 Experiment 4: *in situ* eDNA sampling

423 We tested the ability of nine HAp samplers to recover isopod eDNA under natural
424 conditions by immersing them for 24 hours in a freshwater pond that is inhabited by a
425 population of *A. aquaticus*. We compared the amount of DNA recovered from the
426 samplers with that obtained by the filtration of nine 1 L samples of water. Despite the
427 very low levels of eDNA amplified (i.e. below the limit of detection; 2.5×10^2
428 copies/ μ L), *A. aquaticus* was detected by seven HAp samplers replicates and in
429 three filtered water samples out of the nine samples (Fig. 8). On average over the
430 nine replicates, HAp samplers recovered a higher amount of DNA than filtration
431 (samplers = 6 DNA copies, filtration = 15 DNA copies). Detections of target DNA at
432 lower copy numbers than the LOD are common in eDNA studies due to the low DNA
433 concentrations of the target species in natural environments (i.e. < 100 target copies/
434 reaction; Ellison et al., 2006). Here, we assume that the detections are true positives
435 because 1) all samples amplified below 40 cycles (Ct), 2) the melting curve was

436 uniform (without additional peaks) and 3) no amplifications of the targeted species
437 occurred in any of the negative controls including filtration and extractions control
438 (Klymus et al., 2020).

439 3 Discussion

440 3.1 HAp samplers recover DNA fragments of various sizes

441 Using genomic DNA, we validated the concept of passive HAp samplers and their
442 capacity to recover free DNA fragments of various sizes. In only 17 hours, HAp
443 samplers recovered up to 890 ng in experiment 1 and 1750 ng in experiment 2,
444 which is well above the theoretical maximum quantity we estimated (400 ng for P1
445 and 800 ng for P2 samplers) using a projection of a DNA monolayer on the surface of
446 the samplers (section 1.2). As we predicted, P2 samplers recovered more DNA than
447 P1 samplers during our experiments. These results confirm the high binding affinity
448 between DNA and hydroxyapatite (del Valle et al., 2014; Okazaki et al., 2001), which
449 increase with the HAp mass (Brundin et al., 2013) and suggest that more than one
450 layer of DNA molecules can bind to the HAp surface. Depending on physical
451 parameters such as the microstructure of the surface and the number and size of
452 porosities, DNA molecules can be deposited in different ways on the surface of
453 minerals (Paget et al., 1992). For example, Khanna et al. (1998) found that DNA
454 molecules predominantly adsorb to the edges of clays, and that one end of a DNA
455 fragment could bind when the other end is unbound and extends outwards. So far,

456 DNA adsorption mechanisms have been described on mineral powders or soil
457 particles but remain to be studied on 3D-printed hydroxyapatite surfaces (James
458 Cleaves et al., 2011; Yu et al., 2013). Further experiments are needed to accurately
459 quantify the maximum adsorption capacity of a HAp sampler and to characterise the
460 surface parameters (e.g. microstructure, porosities) that influence DNA adsorption.

461 While we hypothesised that longer DNA fragments would be preferentially adsorbed
462 due to a greater number of binding sites, we found no clear evidence that longer
463 fragments were preferentially adsorbed on the HAp samplers. On the contrary, the
464 examination of DNA band profiles (Fig. 4) suggests that DNA fragments above 5000
465 bp are less well adsorbed or desorbed of the HAp sampler surface. This is consistent
466 with what is observed on soil particles where smaller DNA fragments (< 3 Kb) or
467 those with lower molecular weights are adsorbed more easily than longer fragments
468 (> 7 Kb, Franchi et al., 1999; Ogram et al., 1994). This is explained by an exclusion
469 phenomenon of long DNA fragments in the interstices of the mineral, as well as a
470 difference in the diffusion coefficient between small and long DNA fragments
471 (Ogwada & Sparks, 1986; Yu et al., 2013). As the geometry and microstructure of our
472 HAp samplers differ widely from soil particles, the adsorption processes are certainly
473 very different, explaining why we did not find a strong effect of DNA fragment size.

474 A sampling method that is not strongly biased toward a given range of fragment
475 sizes, or even favours short DNA fragments, is a real advantage for eDNA sampling.
476 eDNA is a complex mixture of genetic material ranging from cells to more or less
477 degraded free DNA fragments (Wilcox et al., 2015). Free DNA fragments may

478 predominate in certain types of environments (e.g. acidic) (Seymour et al., 2018) and
479 may be of different sizes depending on their level of degradation. While free DNA
480 adsorbs to the HAp samplers, it remains to be tested whether other forms of eDNA
481 such as proteo-nucleic complexes or even larger particles can also be sampled.

482 3.2 Environmental DNA sampling

483 Our experiments demonstrated that HAp samplers can sample animal eDNA
484 passively in fresh waters. In controlled and natural conditions, at least 80% of the
485 sampler replicates successfully detected *A. aquaticus* eDNA, after only 24 hours of
486 immersion. Given the low biomass of these small isopods which, unlike large
487 organisms commonly used in eDNA-based studies (e.g. fish, amphibians; Jo et al.,
488 2020; Maruyama et al., 2014), are likely to release very small amounts of eDNA, and
489 given the short experiment duration, this overall high rate of detection demonstrates
490 the high sensitivity of HAp samplers to detect isopods. Although HAp samplers are a
491 promising and simple option for target organism detection, additional field
492 experiments are needed to validate HAp samplers efficiency in other ecosystems
493 (e.g. rivers) to describe whole communities using metabarcoding.

494 The efficiency of HAp samplers was comparable or higher to that obtained with
495 conventional methods used in our study (precipitation and filtration). While the eDNA
496 recovery efficiency of the HAp samplers was similar to DNA precipitation in
497 microcosms, it was higher than filtration in the pond experiment. As filtration is based
498 on sampling a volume of water at a specific time window, it could miss pulse inputs of

499 eDNA (e.g. molts, gametes). Another explanation could be that since eDNA
500 degrades rapidly in lentic systems, as has been shown with fish eDNA (Li et al.,
501 2019), the eDNA could be mainly in extracellular and degraded form and pass
502 through the filters. As the HAp samplers remain in the water longer, they accumulate
503 extracellular DNA over time, and are more likely to catch a weak and temporary
504 eDNA signal. In addition, one of the characteristics of minerals and in particular
505 hydroxyapatite, is to accumulate DNA and protect it from degradation (Pietramellara
506 et al., 2009). DNA adsorbed on mineral surfaces can remain in place for several days
507 or more (Brundin et al., 2013; Cai et al., 2006). Further investigations need to be
508 carried out to assess the residence time of eDNA on the sampler.

509 3.3 Repeatability issues

510 Although HAp samplers show a great potential for DNA sampling, repeatability
511 appears to be a concerning issue. HAp samplers showed extreme variability in DNA
512 recovery among strictly identical conditions (experiment 2). Given the numerous
513 samplers that did not recover any DNA during the first but did recover DNA at the
514 later experiments (Fig. 5), one might have expected that DNA recovery could
515 improve with cycle of use. Nonetheless, no significant difference between the two
516 later experiments was found. Furthermore, by simultaneously monitoring the residual
517 DNA and the desorbed DNA, we were able to determine that this unexplained
518 variability is the result of two variable mechanisms which may act independently: a
519 variable DNA adsorption rate and a variable desorption rate. Ignoring the first

520 experiment (Fig. 6, Exp2.A), 7 samplers adsorbed and desorbed DNA, 13 did not
521 adsorb DNA and 5 adsorbed DNA but partially desorbed it during the two remaining
522 experiments (Fig. 6, Exp2.B,C). To our knowledge, there is no literature reporting an
523 uncoupling between adsorption and desorption rates. However, Pietramellara et al.
524 (2009) found that DNA can be partially desorbed from clay surfaces because the
525 bonds between the DNA molecules and the surface are of different nature, which
526 results in different degrees of strength. Depending on the strength of the bond, some
527 DNA molecules might be hard to desorb. Although electrostatic interactions between
528 the negative charges of the DNA and the surface is the main mechanism of DNA
529 adsorption on hydroxyapatite (Okazaki et al., 2001; S. Yetgin, 2013), other
530 mechanisms are possible such as hydrophobic interaction and OH-bonding
531 (Douarche et al., 2008).

532 The DNA/surface interactions strongly depend on the physico-chemical properties of
533 the sampler surface and the solution in which the binding reaction takes place (Gallo
534 et al., 2018). Among the surface properties, porosity, specific surface area,
535 crystallinity and stoichiometry of the HAp phase (calcium groups can be substituted
536 by other ions) could play a major role in DNA adsorption. The different manufacturing
537 steps, such as the HAp densification (i.e. sintering), can greatly influence most of
538 these surface properties. In particular, ionic substitution (e.g. carbonatation) and
539 partial dehydration are known to occur frequently, and heterogeneously, in HAp
540 during thermal treatment (J-P.Lafon, 2004; Wang, Dorner-Reisel and Müller, 2004)
541 such as the ones used here to decontaminate the samplers before and between

542 experiments, and might be the source of the observed variability. In addition, the
543 chemical properties of the aqueous solution such as the pH (Alvarez et al., 1998;
544 Cortez & Schnitzer, 1981; Khanna & Stotzky, 1992), ionic strength and the
545 concentration of divalent cations (James Cleaves et al., 2011; Saeki et al., 2010)
546 significantly impacts DNA adsorption and desorption. For example Wu et al. (2011)
547 showed that by lowering the pH of the solution from 8 to 5 during the experiment, the
548 binding between the DNA and the surface (here graphene oxide) increased from
549 30% to 100%. Surface analysis needs to be carried out to identify the physical (e.g.
550 porosity, crystalline phases) and chemical (e.g. surface ionic groups) parameters
551 involved in DNA binding on the HAp surface and the extent to which these
552 parameters are influenced by the manufacturing and use of the sampler (e.g.
553 sintering, debinding, immersion in DNA solution).

554 Conclusion

555 In view of the democratisation of the use of eDNA, tools are needed to easily and
556 cost-effectively sample eDNA. We demonstrate that 3D passive hydroxyapatite
557 samplers can be designed and used to collect eDNA, albeit some repeatability
558 issues. Provided we can get a better understanding and control of the interaction
559 between eDNA and HAp, this approach offers a simple alternative solution for eDNA-
560 based biomonitoring. It also opens up an interdisciplinary field at the interface
561 between engineering, surface science and molecular ecology.

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776 **Data Accessibility**

777 The fluorescence data, DNA concentrations and DNA copy numbers are available on
778 Zenodo (Zenodo, <https://doi.org/10.5281/zenodo.5997707>).

779 **Author's contributions**

780 TL and CM conceived the ideas and designed HAp samplers. Experimental design
781 was conceived by TL, LK and HV. HAp samplers were thermal treated and
782 characterised by HR, ST and LG. Laboratory experiments were conducted by HV
783 and LK. Data analysis was conducted by HV and TL. HV and TL led the writing of the
784 manuscript. All authors contributed to the manuscript.

785 **Tables and figure captions**

786 Table 1. Process parameters for debinding HAp samplers.

787

788 Table 2. DNA concentration in supernatants and recovered from six P1 and six P2
789 HAp samplers (three replicates per marker solution) measured by QuBit® .

790 Figure 1: Images of 3D-printed hydroxyapatite samplers prototype P1 (a) and P2 (b)
791 obtained with a confocal microscope (objective x0.5, LEICA Z16 APO, camera LEICA
792 DMC5400).

793 Figure 2: DNA adsorption and desorption protocol

794 Figure 3: Experimental design (experiment 3) testing the efficiency of HAp samplers
795 to recover eDNA from *Asellus aquaticus* in microcosms compared to a conventional
796 sampling method (precipitation).

797 Figure 4: (A) experiment 1 layout, (B) electropherograms of the long and short DNA
798 fragments desorbed from the HAp samplers. The most concentrated DNA replicates
799 were selected to get a clearer description of the fragment distribution (P1-B, P1-E,
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801 initial DNA markers. The horizontal axis represents the size of DNA fragments in bp,

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804 Figure 5: Percentage of DNA recovered by two prototypes (P1 and P2) of HAp
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811 Figure 7: Number of copies (in log scale) of *A. aquaticus* 16S rDNA recovered by the
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821 (orange triangles) and in 1 L filtered water samples (blue circles). Sampling locations
822 in the pond are ranging from S1 to S9. Each point represents the average number of
823 DNA copies over three qPCR measurements per sample, the vertical bar represents
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833 **Tables and figures**

834 Table 1. Process parameters for debinding HAp samplers

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Step	Temperature (°C)	Heating rate (°C/min)	Dwell (min)
1	20-200	0.2	120
2	200-300	0.1	120
3	300-380	0,1	120
4	380-550	0.1	120
5	550-950	1	0
6	950-20	2	-

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837 Table 2. DNA concentration in supernatants and recovered from six P1 and six P2
838 HAp samplers (three replicates per marker solution) measured by QuBit® .

839

HAp-samplers	DNA marker	Residual DNA in supernatants after 17h (ng/μL)	DNA desorbed from HAp samplers (ng/μL)
P1-A	short	0.105	0.2
P1-B	short	ND	0.215
P1-C	short	0.183	0.153
P2-A	short	ND	0.677
P2-B	short	ND	0.567
P2-C	short	ND	0.551
P1-D	long	0.062	0.52
P1-E	long	ND	0.88
P1-F	long	ND	0.087
P2-D	long	ND	0.89
P2-E	long	0.053	0.774
P2-F	long	0.061	0.554

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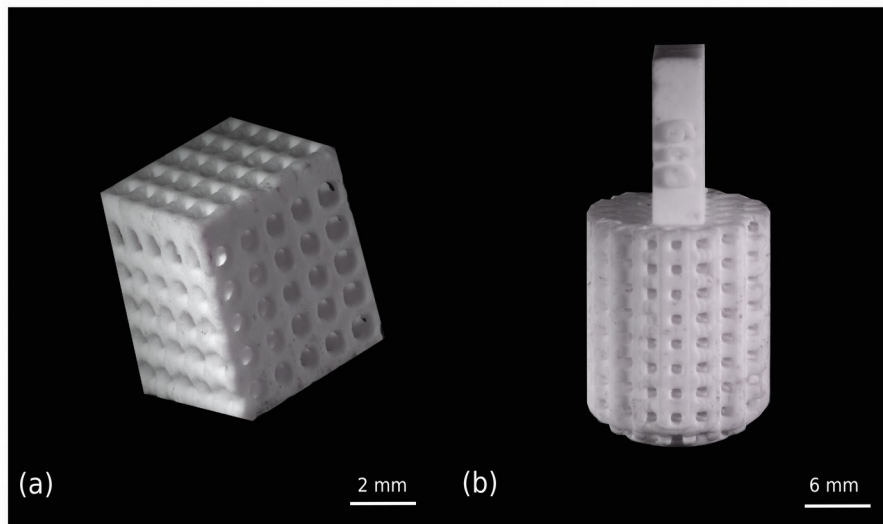
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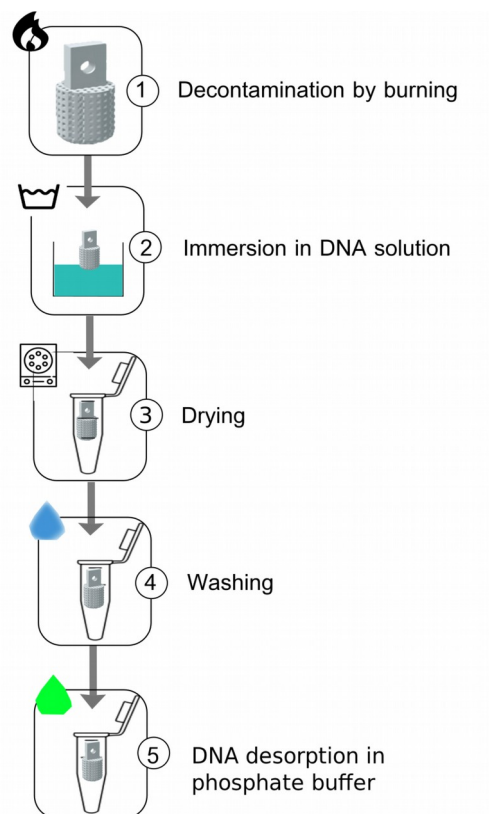
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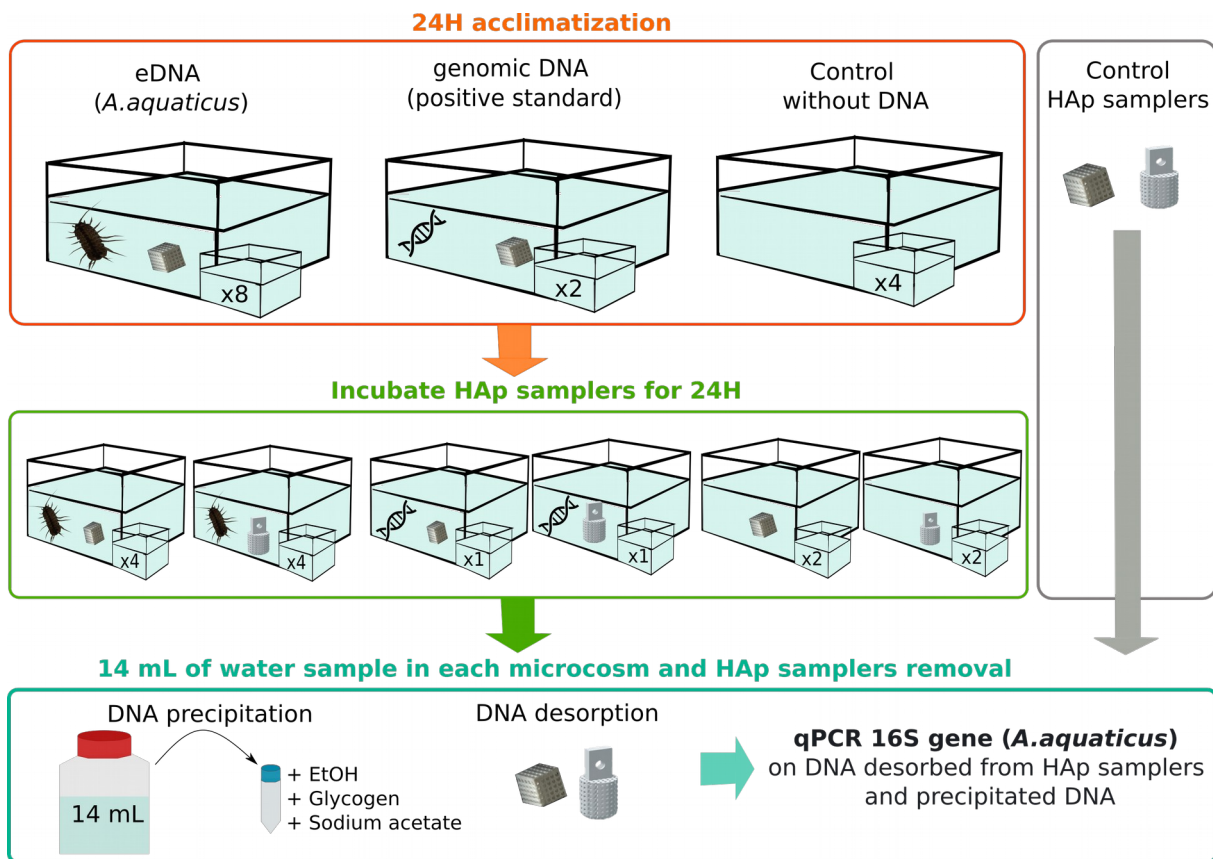
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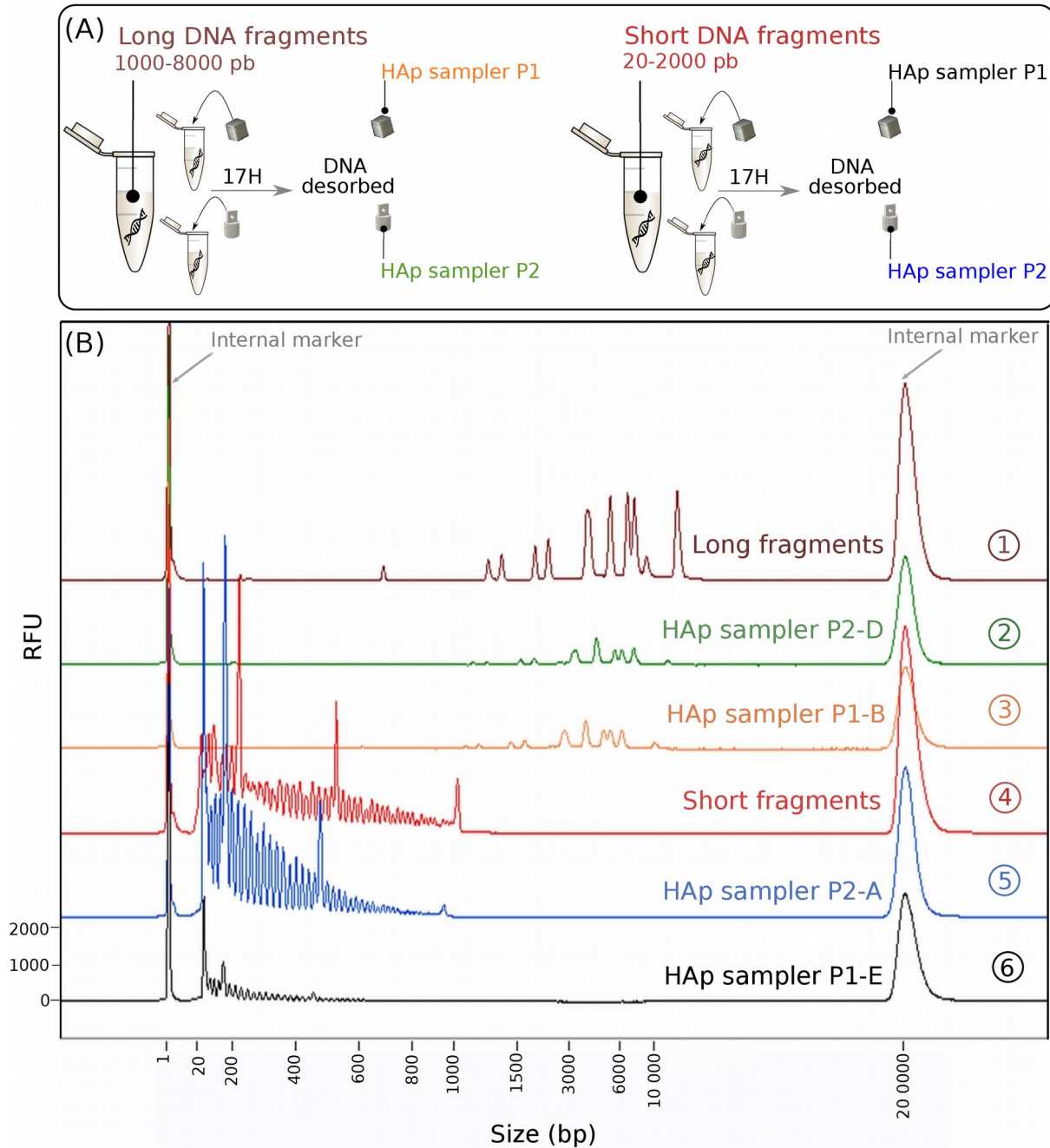
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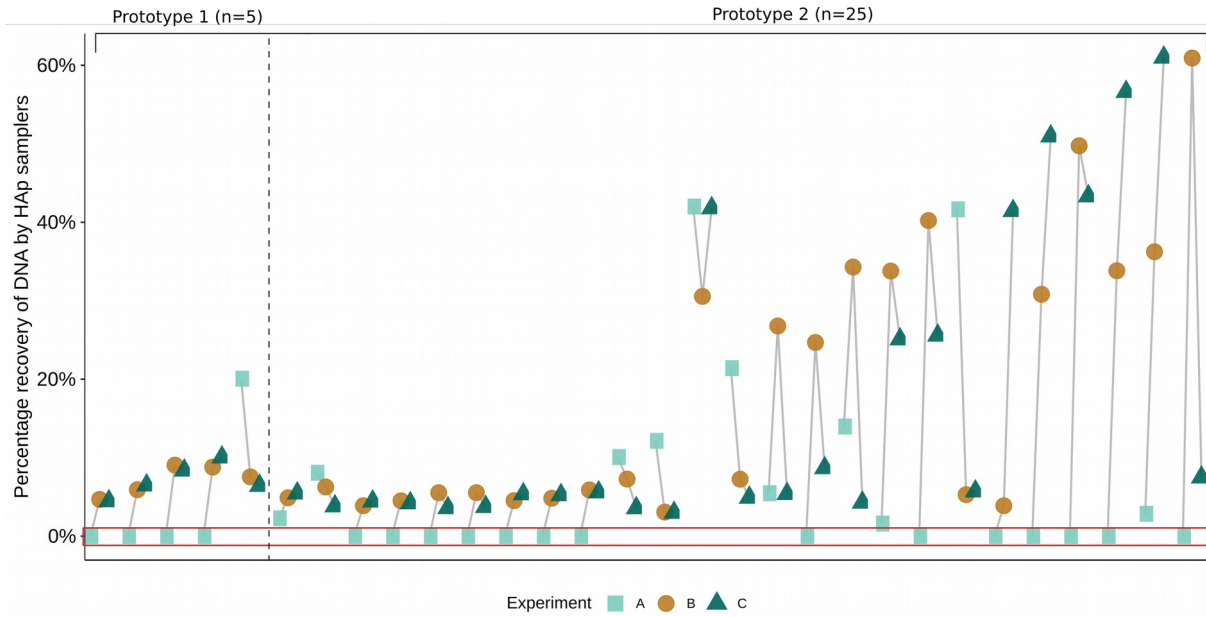
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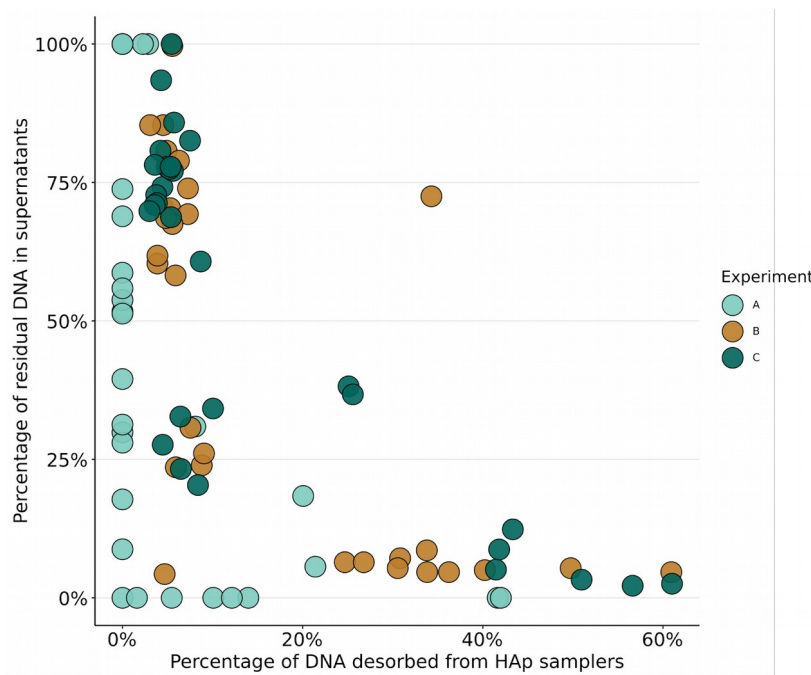
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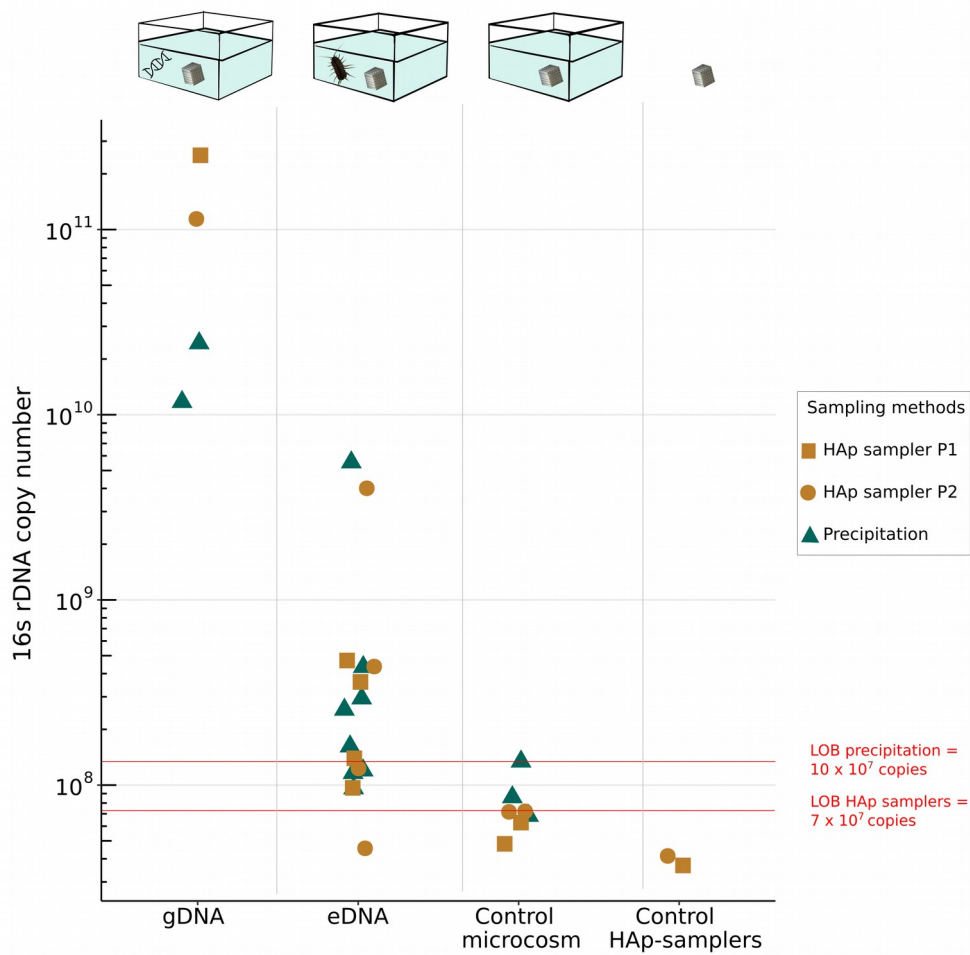


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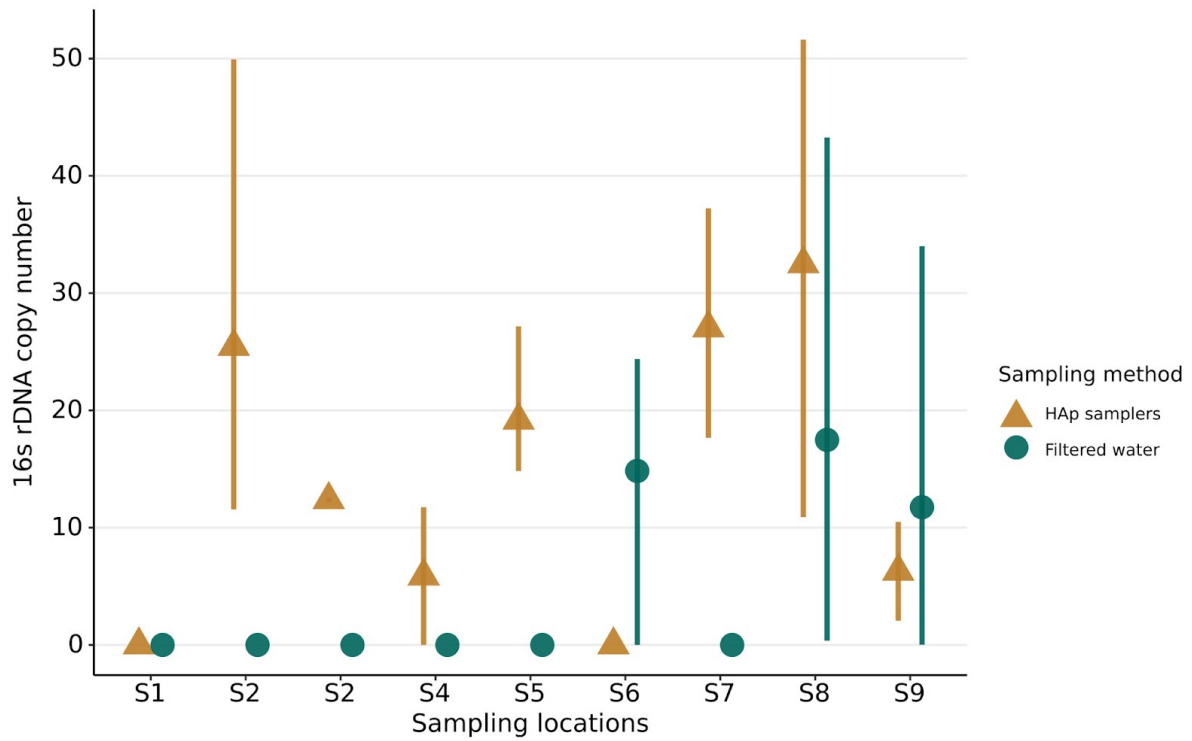
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