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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

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

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

42 Introduction

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

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

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

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

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

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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

A photopolymerizable organic resin (3D Mix, 3DCeram Company, HAP, Bonnac-la-108 Côte, France) containing 40-60% (w/v) of hydroxyapatite powder ($Ca_{10}(PO_4)_6(OH)_2$, 109 stoichiometric hydroxyapatite), a synthetic calcium phosphate with Ca/P atomic ratio 110 of 1.67, was the raw material used to fabricate the samplers. The samplers were built 111 112 from this hydroxyapatite-enriched resin using a 3D stereolithographic printer (CERAMAKER C900, 3DCeram Company, with 55 mW laser power and 100 µm 113 layer thickness). Two prototypes of HAp samplers were produced: a first test 114 prototype (P1) corresponding to 10 pieces cut from a 3D-printed mesh with (Fig. 1A) 115 to test the concept and the material, and a second prototype (P2) printed in 25 copies 116 (Fig. 1B) with a higher ratio porosity/surface and an optimised design for laboratory 117 and field manipulations. P1 prototypes have an exposed surface of 240 mm² and a 118 macroporosity of 500 μ m in diameter. P2 has a total surface of 480 mm² and a 119 macroporosity of 400 µm in diameter. 120

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

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

135 1.2 Expected DNA recovery from HAp samplers

We used the term "DNA recovery" to define the quantity of DNA adsorbed and 136 desorbed from the HAp samplers. A first estimation of the maximum DNA recovery 137 (DNA_{max}) can be obtained by hypothesising that a single layer of DNA molecules 138 would bind on the HAp surface of the samplers. According to equation 1, the number 139 of DNA molecules that can adsorb to the surface is obtained by dividing the exposed 140 surface (Se) of a sampler (P1 = 240 mm², P2 = 480 mm²) by the surface of a DNA 141 base pair (Sd = 6.46^{-10} mm²). The surface of a DNA base pair was calculated 142 according to Mandelkern et al (1981) (diameter = 2 nm, length = 3.4 nm). The 143 number of DNA molecules per sampler is then divided by Avogadro's constant (NA = 144

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

148 DNAmax = (St/Sd)/NAxW Equation 1

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

154 1.3 Protocol of DNA adsorption and desorption

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

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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 of168 various sizes

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

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185 1.4.2 Experiment 2: repeatability

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

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

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

238 1.4.4 Experiment 4: eDNA sampling experiment in situ

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

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

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

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

1.6 DNA quantification and analysis

284 1.6.1 Quantification of DNA size marker

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

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

301 1.6.2 Quantitative PCR assay for A. aquaticus eDNA

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

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Laboratories, Inc., Hercules, CA) in duplicate for experiment 3, as we had a limited 313 amount of DNA material, and in triplicate for experiment 4. gPCR cycle started with 314 an incubation at 95 °C for 10 min followed by 45 cycles of denaturation at 95 °C for 315 10 sec and an annealing/extension step at 64 °C for 20 sec before a final melt curve 316 317 from 65-95 °C (0.5 °C increments). For the microcosm experiments (experiment 3), the gPCR plates included a single seven-fold dilution series of the purified 16S A. 318 aquaticus amplicons between 2.5 x 10^{10} and 2.5 x 10^{3} copies/µL as guantified by a 319 OuBit 3.0 assay. As we expected very low levels of A. aquaticus eDNA in the pond 320 (experiment 4), gPCR plates were run with a single seven-fold dilution series of 321 purified 16S amplicons with a copy number ranging from 2.5 x 10^7 and 2.5 x 10^1 322 copies/ μ L. In both gPCR analysis, the R² values and PCR efficiency (%) of the 323 calibration curves were 0.99 and 101.5 \pm 0.1 (mean \pm SD). 324

325 1.6.3 Limit of detection and quantification

The limit of detection (LOD) was determined as the lowest DNA concentration at which there is 95% of detection across replicates and the limit of quantification (LOQ) as the lowest concentration at which the coefficient of variation is below 35% (Klymus et al., 2019). LOD and LOQ were determined via a 4-fold serial dilution of a 16S *A*. *aquaticus* amplicon from 2.5 x 10³ to 2.5 DNA copies/µL. Each dilution was run in 18 qPCR replicates. The LOQ was 2.5 x 10³ and the LOD 2.5 x 10² copies/µL.

332 1.7 Statistical analysis

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

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

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

370 2.2 Experiment 2: Repeatability of HAp samplers over time

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

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

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

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

404 2.3 Experiment 3: eDNA sampling in microcosms

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

precipitation. Using concentrated genomic DNA as a positive control, the samplers recovered up to 2.4×10^{10} DNA copies/sampler, one magnitude more than with precipitation. In the microcosms that contained isopods, the amount of 16S eDNA molecules was above the LOB in seven HAp samplers and in four precipitated water samples out of the eight replicates. The number of 16S eDNA molecules recovered was similar between precipitated water samples and HAp samplers (Wilcox-test, p = 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 conditions by immersing them for 24 hours in a freshwater pond that is inhabited by a 424 population of *A. aquaticus*. We compared the amount of DNA recovered from the 425 samplers with that obtained by the filtration of nine 1 L samples of water. Despite the 426 very low levels of eDNA amplified (i.e. below the limit of detection; 2.5x10² 427 copies/µL), A. aquaticus was detected by seven HAp samplers replicates and in 428 three filtered water samples out of the nine samples (Fig. 8). On average over the 429 nine replicates, HAp samplers recovered a higher amount of DNA than filtration 430 431 (samplers = 6 DNA copies, filtration = 15 DNA copies). Detections of target DNA at lower copy numbers than the LOD are common in eDNA studies due to the low DNA 432 concentrations of the target species in natural environments (i.e. < 100 target copies/ 433 reaction; Ellison et al., 2006). Here, we assume that the detections are true positives 434 because 1) all samples amplified below 40 cycles (Ct), 2) the melting curve was 435

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

439 3 Discussion

3.1 HAp samplers recover DNA fragments of various sizes

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

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.

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

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

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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

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

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

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

3.3 Repeatability issues

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

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

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

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

554 Conclusion

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

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776 Data Accessibility

The fluorescence data, DNA concentrations and DNA copy numbers are available on

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
- characterised by HR, ST and LG. Laboratory experiments were conducted by HV
- 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.

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- Table 1. Process parameters for debinding HAp samplers.
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 HAp samplers (three replicates per marker solution) measured by QuBit ® .
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- Figure 4: (A) experiment 1 layout, (B) electropherograms of the long and short DNA fragments desorbed from the HAp samplers. The most concentrated DNA replicates were selected to get a clearer description of the fragment distribution (P1-B, P1-E, P2-A, P2-D, Table 2). As standards, the curves 1 and 4 represent the profile of the initial DNA markers. The horizontal axis represents the size of DNA fragments in bp,

and the vertical axis represents fluorescence. The left-most (1 bp) and right-most (20 000 bp) peaks are internal markers.

Figure 5: Percentage of DNA recovered by two prototypes (P1 and P2) of HAp samplers in three consecutive experiments (A, B and C). Five P1 and 25 P2 samplers are sorted according to their DNA recovery variance. Samplers that recovered no DNA are in the red box.

Figure 6: Relationship between DNA desorbed from HAp samplers and residual DNA in supernatants (%) in three consecutive experiments (A, B and C). The lines connect the same sampler used in experiments B and C.

Figure 7: Number of copies (in log scale) of A. aquaticus 16S rDNA recovered by the 811 two HAp prototypes (P1 = triangle, P2 = circle) and in precipitated water samples 812 (blue triangle) after 24 hours of incubation in microcosm containing A. aquaticus 813 genomic DNA used as a positive control (gDNA) or five individuals of A. aquaticus 814 (eDNA). Two types of negative controls were used: control microcosms without DNA, 815 and control HAp samplers without microcosm incubation. Red lines correspond to the 816 limits of blanks (LOB) obtained with HAp samplers (LOB HAp samplers) and with 817 precipitation (LOB precipitation). The LOB corresponds to the highest DNA 818 concentration measured in microcosms without DNA. 819

Figure 8: Number of copies of *A. aquaticus* eDNA recovered from HAp samplers P2 (orange triangles) and in 1 L filtered water samples (blue circles). Sampling locations in the pond are ranging from S1 to S9. Each point represents the average number of DNA copies over three qPCR measurements per sample, the vertical bar represents the lowest and highest estimates.

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833 Tables and figures

Table 1. Process parameters for debinding HAp samplers

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	-

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

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

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



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





Figure 4: (A) experiment 1 layout, (B) electropherograms of the long and short DNA fragments desorbed from the HAp samplers. The most concentrated DNA replicates were selected to get a clearer description of the fragment distribution (P1-B, P1-E, P2-A, P2-D, Table 2). As standards, the curves 1 and 4 represent the profile of the initial DNA markers. The horizontal axis represents the size of DNA fragments in bp, and the vertical axis represents fluorescence. The left-most (1 bp) and right-most (20 000 bp) peaks are internal markers.



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Figure 8: Number of copies of *A. aquaticus* eDNA recovered from HAp samplers P2 (orange triangles) and in 1 L filtered water samples (blue circles). Sampling locations in the pond are ranging from S1 to S9. Each point represents the average number of DNA copies over three qPCR measurements per sample, the vertical bar represents the lowest and highest estimates.



