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

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Abstract

1. The study of environmental DNA released by aquatic organisms in their habitat offers a fast, non-invasive and sensitive approach to monitor their presence. Common eDNA sampling methods such as filtration and precipitation are time consuming, require human intervention and are not applicable to a wide range of habitats such as turbid waters and poorly-accessible environments. To circumvent these limitations, we propose to use the binding properties of minerals to create a passive eDNA sampler.

2. We have designed 3D-printed samplers made of hydroxyapatite (HAp samplers), a mineral known for its high binding affinity with DNA. The shape and the geometry of the samplers have been designed to facilitate their handling in laboratory and field. Here we describe and test the ability of HAp samplers to recover artificial DNA and eDNA.

3. We show that HAp samplers efficiently recover DNA and are effective even on small amounts of eDNA (<1 ng). However, we also observed large variations in the amount of DNA recovered even under controlled conditions.

4. By better understanding the physico-chemical interactions between DNA and the HAp sampler surface, one could improve the repeatability of the sampling process and provide an easy-to-use eDNA sampling tool for aquatic environments.

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Key-words : DNA Binding, Environmental DNA, Hydroxyapatite, Passive sampling, 3D-printing

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1 | INTRODUCTION

56 At a time of unprecedented threats on freshwater biodiversity, it is crucial to develop rapid,

57 accurate and minimally invasive tools to monitor aquatic ecosystems. About a decade ago,

58 methods based on the sampling of environmental DNA (eDNA) were proposed as a

59 revolutionary way to survey aquatic macro-organisms (Deiner et al., 2017). Macro-organisms

60 release DNA in their environment through different processes (e.g. faeces, excretion,

61 shedding cells, gametes) and this eDNA can take different forms (tissues, cells, organites,

62 nucleo-proteic complexes, …). The direct sampling of eDNA coupled with molecular analysis

63 methods such as NGS (Shokralla et al., 2012) or quantitative polymerase chain reaction

64 (qPCR) (Langlois et al., 2020) allow the detection and identification of aquatic species while

65 overcoming organism capture. Although eDNA offers many promising applications, several

66 methodological challenges remain.

67

68 One of the most challenging aspects of eDNA-based approaches is the sampling method.

69 eDNA is present in very small quantities and is heterogeneously distributed in aquatic

70 environments (Goldberg et al., 2016). To maximise its recovery, sampling methods must be

71 able to concentrate eDNA (Hinlo et al., 2017). Active filtration of a large volume of water is the

72 most commonly-used method to recover eDNA in aquatic systems. However, filtration has

73 significant methodological limitations. Firstly, it is a long and tedious process requiring human

74 intervention, sometimes difficult to carry out in poorly-accessible habitats. Secondly, the

75 clogging of the filters is a recurrent problem which reduces the volume of water that can be

76 sampled (Williams, Huyvaert and Piaggio, 2017).

77

78   To limit clogging, filtration membranes with large porosity (greater than 0.45 µm) are often

79   used. However, eDNA particles can be present in various forms (intra or extracellular), states

80   (free or complexed with other particles) and sizes (from > 180 to < 0.2µm but most abundant

81   between 0.2 and 10 µm) (Turner et al., 2014; Moushomi et al., 2019 ; Wilcox et al., 2015). As

82   filtration is based on particle size sorting, the use of membranes with large porosity will

83   overlook smaller DNA particles, even though they may be an important source of eDNA

84   (Moushomi et al., 2019). Finally, given the complex dynamic of eDNA in aquatic environments

85   (i.e. pulsed emission , transport, retention, degradation), one filtration sample will provide an

86   instantaneous snapshot which is likely to be poorly integrative of the overall eDNA signals

87   (Pilliod et al., 2013; Spear et al., 2015).

88

89   Passive eDNA sampling using natural substrates is a promising solution to overcome filtration

90   challenges. Organisms such as marine sponges (Mariani et al., 2019), molluscs (Der

91   Sarkissian et al., 2020) and biofilms (Rivera et al., 2021) can trap and accumulate eDNA

92   particles in water. Minerals can also accumulate and protect DNA from enzymatic degradation

93   (Alvarez et al., 1998; Levy-Booth et al., 2007). Indeed, a sample of sediment can contain

94   more eDNA than a water sample (Turner, Uy and Everhart, 2015). Recently, Kirtane and

95   colleagues (Kirtane, Atkinson and Sassoubre, 2020) have shown that montmorillonite and

96   coal-based mineral powders can be used as passive eDNA samplers in aquatic

97   environments. Thanks to good DNA capture and preservation rates (up to 200 µg genomic

98   DNA / g) (Gardner and Gunsch, 2017), sediments and commercial mineral powders may very

99   well be more integrative eDNA substrates than filtration methods. Yet, these substrates are

100   difficult to handle and deploy in the environment, particularly in aquatic systems.

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102 In this study, as an alternative to filtration, we developed proof of concept 3D-printed passive

103 eDNA samplers. 3D printing allows control of the shape and composition of an object. The

104 shape of the samplers were designed with optimised surface/volume -ratio and a shape

105 easing handling in the field and in the lab. The samplers were made of pure hydroxyapatite

106 (HAp), a calcium phosphate mineral naturally present in bones and known for its high binding

107 affinity toward DNA (Okazaki et al., 2001; Brundin et al., 2013). Here we describe the

108 development of hydroxyapatite samplers (HAp samplers) and test their ability to sample

109 eDNA in fresh waters. Two prototypes of samplers will be presented: a first test-version, with

110 which the concept and material will be tested, and a second version which shape and design

111 have been optimised for eDNA sampling. Using controlled laboratory experiments, our

112 objectives are to (i) quantify the HAp samplers DNA binding and release capacity, (ii) assess

113 the range of DNA fragment size sampled, (iii) quantify the repeatability of DNA sampling

114 across several cycles of use of the HAp samplers, and (iv) evaluate the samplers capacity to

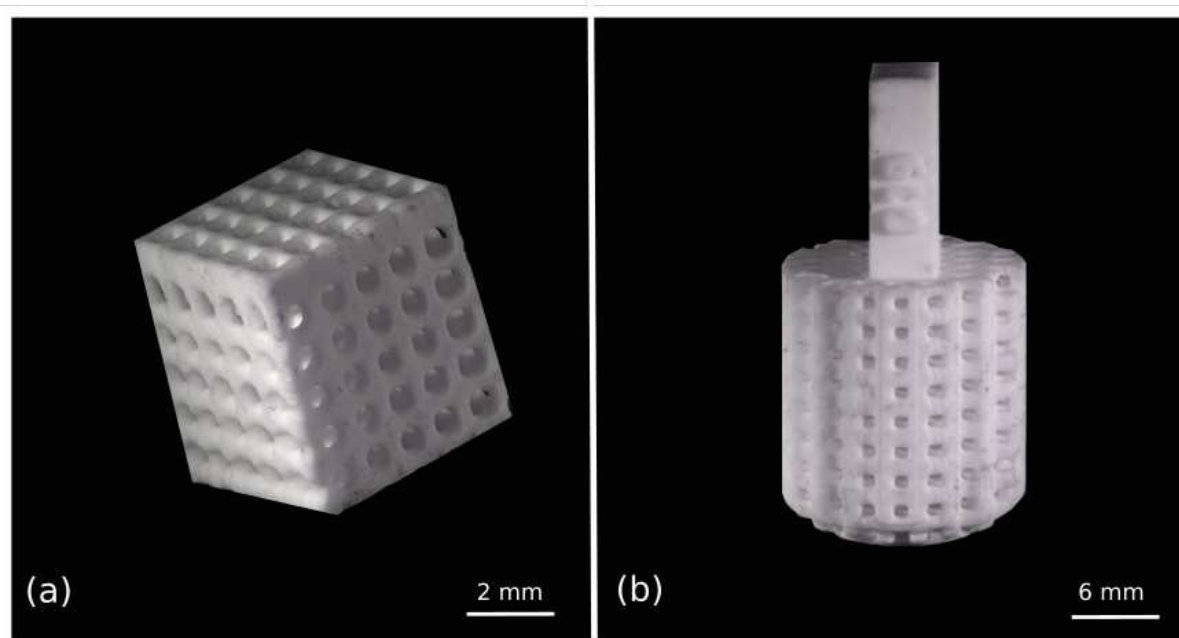115 sample eDNA released by organisms in microcosm.

116

117 **2 | MATERIALS AND METHODS**

118 2.1 | 3D-printed HAp samplers design

119 2.1.1 | Raw material and printing setup

120 A photopolymerizable organic resin (3D Mix, 3DCeram Company, HAP, Bonnac-la-Côte,

121 France) containing 40-60 % (w/v) of hydroxyapatite powder ($Ca_{10}(PO_4)_6(OH)_2$, stoichiometric

122 hydroxyapatite), a synthetic calcium phosphate with Ca/P atomic ratio of 1.67, was the raw

123 material used to fabricate the samplers. The samplers were built from this hydroxyapatite-

124   enriched resin using a 3D stereolithographic printer (CERAMAKER C900, 3DCeram

125   Company, with 55mW laser power and 100µm layer thickness).

126   Two types of prototypes of HAp samplers were produced. The first prototype (P1) is a test

127   version corresponding to 10 pieces cut out of a 3D-printed mesh (Fig. 1a). P1 prototypes

128   have an exposed surface of 240 mm^2 and a macroporosity of 500 µm in diameter. A second

129   more elaborate prototype (P2) was then produced with optimized geometry and porosity, and

130   printed in 25 copies (Fig. 1b). P2 has a total surface of 480 mm^2 and a macroporosity of 400

131   µm in diameter.

132

133   **FIGURE 1** : Images of 3D-printed hydroxyapatite samplers prototype P1 (a) and P2 (b)

134   obtained with a confocal microscope (objective x0.5, LEICA Z16 APO, camera LEICA

135   DMC5400).

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137   2.1.2 | Debinding and sintering steps

138 Once printed, cleaned with a specific solvent (Ceracleaner, 3DCeram Company, Bonnac-la-

139 Côte, France) and dried, the HAp samplers underwent debinding and sintering steps.

140 Debinding aims at removing all organic components (in particular the organic resin) and was

141 conducted in a conventional oven following the thermal cycle described in Table 1.

142 **TABLE 1** : Process parameters for debinding HAp samplers

143

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	-

144

145 Sintering aims at consolidating the samplers by densifying them (creation of necks and

146 reduction of the porosity between the individual ceramic particles) (Rahaman, 2017), and is

147 achieved by a thermal treatment at higher temperature (1°C/min up to 1150°C, 60 min. at

148 1150°C, followed by a second step at 3°C/min up to 1250°C, 60 min at 1250°C, finally cooling

149 to room temperature at 3°C/min). After sintering, no additional processing (i.e. finishing or

150 polishing) was performed. The presence of pure HAp was confirmed by X-ray diffraction

151 (XRD) performed on as-sintered samples.
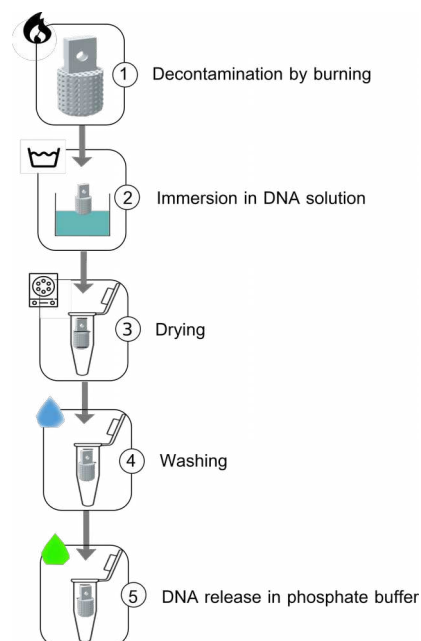
152

153 2.2 | Expected DNA recovery from HAp samplers

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154 We used the term "DNA recovery" to define the quantity of DNA that binds and is released

155 from the HAp samplers. We estimated the theoretical maximum DNA recovery (DNA$_{max}$)

156 based on the hypothesis that a single layer of DNA molecules would bind on the HAp surface

157 of the samplers. According to equation 1, the number of DNA molecules that can bind to the

158 surface is obtained by dividing the exposed surface (Se) of a sampler (P1 = 240 mm^2 , P2 =

159 480 mm^2) by the surface of a DNA base pair (Sd = 6.46E-10 mm^2). The surface of a DNA

160 base pair was calculated according to Mandelkern et al (1981) (diameter = 2 nm, length = 3.4

161 nm). The number of DNA molecules per sampler is then divided by Avogadro's constant (NA =

162 6.02214076 × 1023 mol - 1) to give the number of DNA moles per sampler. The number of

163 moles of DNA is then divided by the molar mass of a DNA base pair (W = 650 daltons) to

164 obtain the total mass of DNA that can bind to a sampler. Being smaller, P1 has a maximum

165 theoretical recovery capacity of 400 ng of DNA per sampler, while P2 has a capacity of 800

166 ng.

167 $$DNAmax = (St/Sd)/N\,A\,x\,W$$ **Equation 1**

168

169 2.3 | Protocol of DNA binding and release

170 The HAp sampler DNA binding and release protocol is composed of 5 steps. First, HAp

171 samplers are decontaminated before each experiment by a thermal treatment in air at 550 ° C

172 for 3 hours (Thermolyne model 30400 furnace), a procedure typically used to decontaminate

173 glassware. Second, DNA is bound to the HAp samplers by immersing them in an aqueous

174 solution (varying composition upon the present study) containing DNA. Third, samplers are

175 transferred to Eppendorf tubes and centrifuged for 1 minute at 3000 rpm to dry them. Fourth,

176 samplers are washed with 1 mL of sterile ultrapure water. Finally, DNA is released from the

8

177   samplers by immersing them in 1 mL of 0.1 M phosphate buffer pH 8, vortexed for 30

178   seconds and incubated at room temperature for 1 hour.

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① Decontamination by burning

② Immersion in DNA solution

③ Drying

④ Washing

⑤ DNA release in phosphate buffer

180   **FIGURE 2** : DNA binding and release protocol

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182   2.4 | DNA sampling experiments by HAp samplers

183   2.4.1 | Experiment 1: DNA binding and release

184   A DNA sampling experiment with concentrated DNA fragments of various sizes (i.e. a DNA

185   size marker) was conducted to validate the binding and release protocol and to assess

186   whether DNA fragments of different sizes have different binding efficiencies. After

187   decontamination, one batch of HAp samplers (P1 and P2) was incubated in tubes (1 sampler/

188   tube) containing 2 mL of a solution of large DNA fragments at 5000 ng / mL (λ DNA / BstEII

189   Digest, 117-8450 bp). A second batch of HAp samplers was incubated in tubes containing 2

190   mL of a solution of short DNA fragments at 2000 ng / mL (PCR 20 bp Low Ladder, 20-2000

191   bp). Both batches were incubated for 17 hours on a rotary shaker (IKA Roller 6 Digital, 40

192   rpm). Controls were tubes with 2 mL of solution and devoided of samplers. Residual DNA in

193   the supernatants was quantified by taking 60 µl aliquots of the supernatant after 45 min and

194   17 h of incubation. After incubation, HAp samplers were removed from the DNA solutions

195   using sterile clamps and DNA was released according to the protocol in section 2.3. All

196   supernatants aliquots and released DNA solutions were stored at -20°C prior to analysis.

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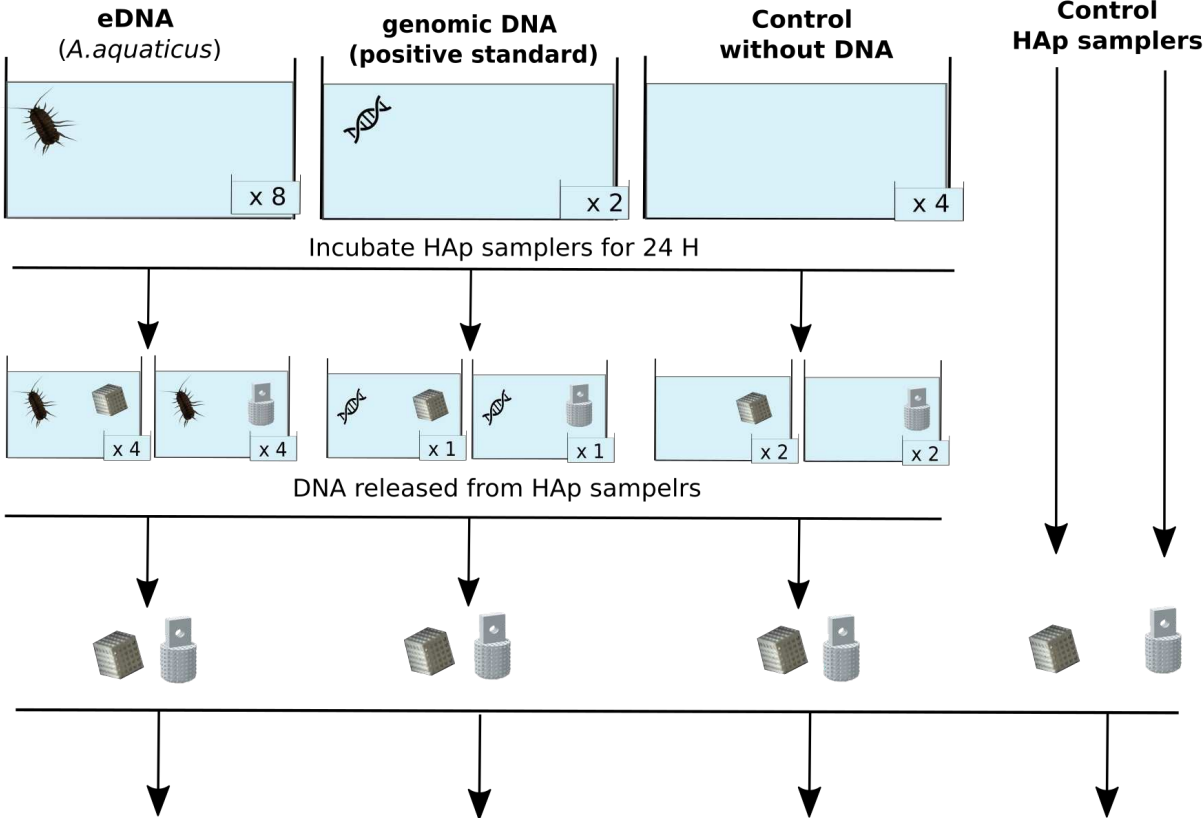198   2.4.2 | Experiment 2: repeatability

199   A quantification of repeatability was conducted to test whether HAp samplers can be reused

200   after several cycles of use. A cycle of use is defined here as a thermic treatment phase

201   followed by a DNA binding and release phase. For this purpose, 5 prototypes 1 and 25

202   prototypes 2 HAp samplers were incubated in a concentrated solution of DNA size marker

203   (λDNA/BstEII Digest 117-8450 pb) at a concentration of 2.8 µg/mL on a rotary shaker (Roller

204   10 Digital IKA) for 17 H. This experiment was carried out three times in a row (hereafter called

205   experiments A, B and C) under strictly identical conditions, at room temperature (24°C $\pm$ 2°C)

206   with decontamination through thermic treatment between each use. After incubation, HAp

207   samplers were removed from the DNA solution with sterile clamp, washed and DNA was

208   released with 1 mL of 0.1 M phosphate buffer pH 8 according to the protocol section 2.3 DNA

209   samples were stored at -20°C prior to analysis.

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211   2.4.3 | Experiment 3: microcosm experiment

212   *Asellus aquaticus,* a small freshwater isopod*,* was used as a target organism to test the

213   capacity of the HAp samplers to collect eDNA. 40 organisms of *A.aquaticus* sampled from a

214   natural pond (Lyon, France) in april 2019 were divided into 8 glass microcosms (5 individuals /

215   microcosm) containing 500 mL of synthetic water (Peltier and Weber, 1985). Positive controls

216   correspond to microcosms where we injected genomic DNA (final microcosm at 1 ng/mL)

217   extracted from a pool of 10 *A.aquaticus*. Negative controls were of two types: control

218   microcosms containing water without DNA and a sampler, and control samplers from which

219   the DNA was released just after the decontamination step (i.e. without incubation in DNA

220   solution). After 24 hours of *A. aquaticus* acclimatization, the two prototypes of HAp samplers

221   were incubated in microcosms (1 sampler / microcosm) for 24 hours. All microcosms were

222   placed in a cold room at 18°C, spaced 0.5 m apart and covered to limit the risk of

223   contamination. The organisms were not fed during the experiment to reduce the amount of

224   allochthonous DNA. After incubation, the HAp samplers were collected with sterile clamps

225   and the DNA was released according to section 2.3 of the protocol. Released DNA was

226   purified (Macherey-Nagel ™ NucleoSpin ™ gel and PCR cleaning kit) to avoid potential

227   inhibition of the downstream qPCR by the phosphate buffer (see next section), following the

228   manufacturer's recommendations. Purified eDNA was stored at -20 ° C prior to analysis.

FIGURE 3 : Experimental design testing HAp samplers efficacy to recover eDNA from *Asellus aquaticus* in microcosms.

2.5 | DNA quantification and analysis

2.5.1 | Quantification of DNA size marker

In the first experiment, DNA binding and release by HAp samplers were evaluated by following the DNA concentration and fragment sizes in three compartments: (1) in the supernatant (i.e. residual DNA), (2) in the washing solution, and (3) in the releasing solution (Fig. 4). DNA was quantified using a QuBit ® 3.0 fluorometer (Invitrogen) with the dsDNA BR kit (broad range, 2 to 1000 ng/µL) according to the manufacturer's protocol. The binding of

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241 large DNA fragments (117-8450 pb) was evaluated using gel electrophoresis (1.3% agarose),

242 and the binding of small fragments (35-2000 bp) using a 2100 Bioanalyzer with an Agilent

243 high-sensitivity DNA chip (Agilent Technologies).

244

245 For the second experiment (repeatability), the amount of DNA released from the HAp

246 samplers was measured by fluorescence (excitation at 480 nm and emission at 520 nm)

247 using an Infinite M200 Pro microplate fluorometer (TECAN, Switzerland). A QuantiFluor®

248 dsDNA kit was used according to the manufacturer's protocol, with a DNA sample volume of

249 10 µL and 190 µL of working solution. A five-fold dilution series (1500-0 ng/µL) of standard

250 DNA (Lambda DNA Standard, 100µg/mL) was used to build the standard curve and calculate

251 the sample DNA concentration in µg/µL. The results are reported in percentage of recovered

252 DNA (i.e. DNA bound and released).

253 *DNAr* is the measured concentration of released DNA (µg/mL) per HAp sampler and DNAtot

254 is the initial DNA concentration added in each tube (DNAtot = 2.8 µg/mL).

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256 2.5.2 | Quantitative PCR assay for eDNA from *A.aquaticus*

257 For the third experiment (microcosm experiment), quantitative PCR (qPCR) was used to

258 quantify the amount of *A.aquaticus* eDNA recovered by the samplers. We designed a pair of

259 primers to specifically amplify a 110 bp fragment of the mitochondrial 16S gene of *A.*

260 *aquaticus (*5' GGTTTAAATGGCTGCAGTATCC 3', 5' CTTGTGTAATAAAAAGCCTACCTC 3').

261 The amplification specificity of the primers was tested *in silico* using primer-BLAST (NCBI)

262 and assessed experimentally through PCR and electrophoresis gel analysis. The qPCR

263 reaction volume was 20 µL consisting of 1X SsoAdvanced Universal SYBR Green Supermix

264 (Bio-Rad Laboratories Inc., Hercules, CA), 0.5 µM of primers and 2 µL of DNA released from

13

265 samplers. All qPCRs assays were run in duplicate in 96 wells plate on a CFX96 Touch™ Real-

266 Time PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, CA). qPCR cycle started

267 with an incubation at 95 °C for 10 min followed by 45 cycles of denaturation at 95 °C for 10

268 sec and an annealing/extension step at 60 °C for 20 sec before a final melt curve from 65-95

269 °C (0.5 °C increments). Each qPCR plate included a five-fold dilution series of the genomic

270 DNA at a concentration between 0 and 2.5 ng/µL quantified by a QuBit 3.0 assay.

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277 2.6 | Statistical analysis

278 Linear mixed-effect models (LMMs) were used to test the influence of the prototype version

279 and of the experiment timing (experiment 2). These models were fitted with the restricted

280 maximum likelihood method using the package nlme. We tested significance of experiment

281 timing and prototype version using likelihood ratio tests (LRT) between the models with and

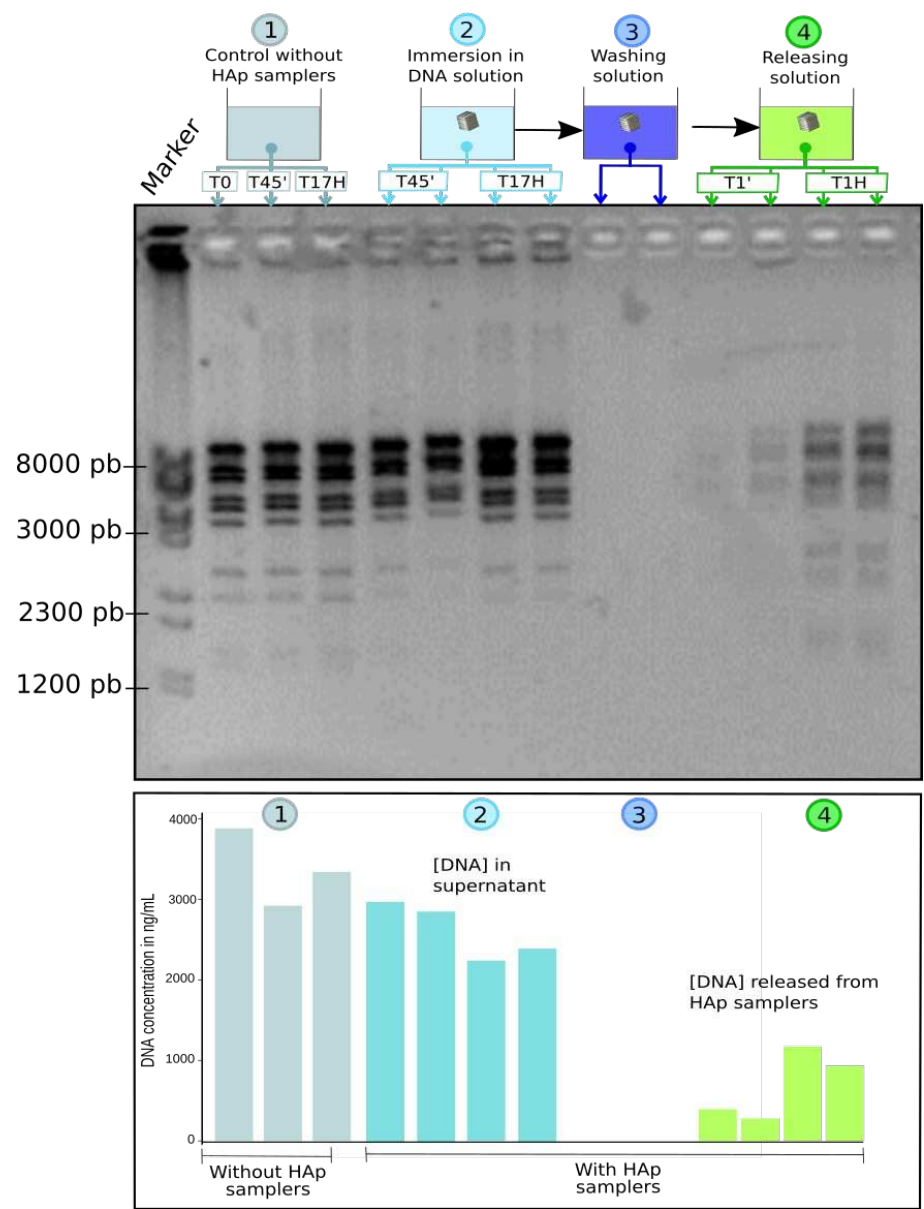282 without the tested variable. All analyses were conducted using R (v 4.0.3).

283

284 **3 | Results**

285 3.1 | Experiment 1: DNA binding and release

286 We tested the hypothesis that 3D-printed HAp samplers can bind DNA of different fragment

287 sizes by exposing them to two solutions of DNA size markers containing either long (117 to

288 8450 bp) or short DNA fragments (35 bp to 2000 bp). In the solution containing long DNA

14

289   fragments, quantification of DNA concentration (Fig. 4, bottom panel) shows a clear reduction

290   in DNA concentration in the supernatant after 17 hours of exposure to the HAp samplers for

291   both sampler replicates. Once immersed in the releasing solution, and after only 1 minute, the

292   HAp samplers started to release DNA. The amount of released DNA then tripled after 1 hour

293   of incubation. By examining the DNA band profiles in the supernatants and in the releasing

294   solution, we found that P1 bound all DNA fragment sizes from 2000 to 8450 bp. Fragments

295   below 2000 bp were not visible on the electrophoresis gel (Fig. 4, top panel). The same

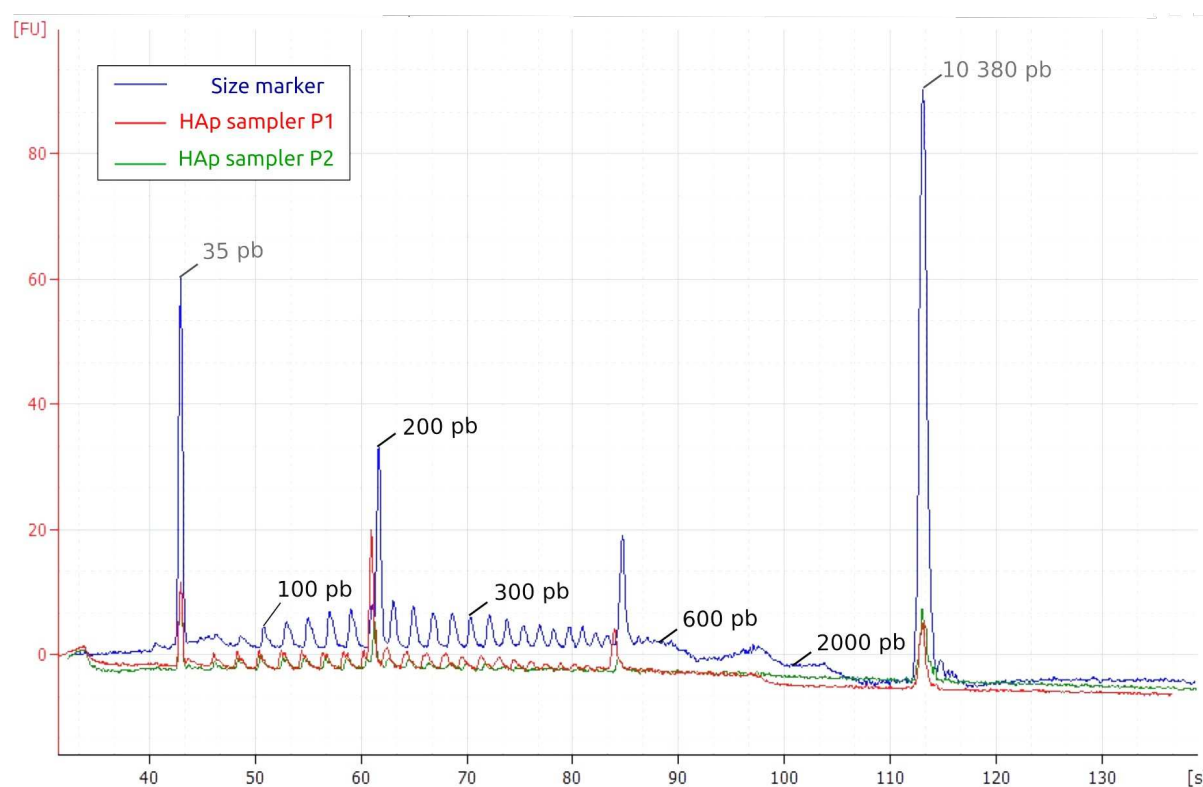296   observations were made on P2 (see supporting information).

FIGURE 4 : DNA binding and release by two replicates of the HAp samplers prototype 1. DNA fragment size (agarose electrophoresis gel, top panel) and concentration (bottom panel) are shown in the following order (left to right): 1) in the control solution without HAp samplers after 0 minute, 45 minutes and 17 hours, 2) in the DNA solution 45 min and 17 H after addition of HAp samplers, 3) in the washing solution and 4) in the releasing solution 1 min and 1 H after immersion of the HAp samplers.

304

305   Repeating the same experiment but using this time short DNA fragments (35-2000 pb) and a

306   microfluidics-based automated electrophoresis system does not show an effect of fragment

307   size on DNA binding (Fig. 5): both samplers prototypes bound and released DNA fragments

308   ranging from 35 to 2000 bp although the resolution of the marker for fragments above 600 bp

309   was not optimal.

310

311

312   **FIGURE 5** : Electropherograms of the DNA fragments bound and released by the prototype 1

313   (red curve) and prototype 2 (green curve). As a standard, the blue curve represents the profile

314   of the initial DNA marker. The horizontal axis represents the migration time of DNA fragments

315   in seconds, and the vertical axis represents fluorescence. The left-most (35 bp) and right-

316   most (10380 bp) peaks are internal markers.

17

317 3.2 | Experiment 2: Reusing HAp samplers over time

318 A repeatability experiment was conducted to test the hypothesis that HAp samplers can be

319 reused and that their binding efficacy is stable after several cycles of use. We performed three

320 consecutive cycles of use (experiment A, B and C), each composed of a decontamination,

321 DNA binding and release steps. The percentage of DNA recovered by the samplers was lower

322 in experiment A compared to experiments B and C, with an average of 8%, 17% and 15%,

323 respectively (Fig. 6). In the meantime, experiment A showed a disproportion of samplers (18

324 out of 30, against 0 for experiment B and C) which failed to recover any DNA compared to the

325 other experiments (Fisher exact test, p < 1E-10). After removing the samplers which failed to

326 capture any DNA, we tested the influence of the experiment and prototype on the percentage

327 of DNA recovered using a linear mixed-effect models with experiments (A, B and C) and

328 sampler prototypes (P1 or P2) as the fixed effects, and samplers as random effect on the

329 intercept. The experiment and the sampler prototype had no significant effect on the

330 percentage of DNA recovered (LRT, sampler prototype : $\chi^2_{df=1}=4.96, p=0.08$, experiment :

331 $\chi^2_{df=2}=1.16, p=0.28$). Nonetheless, while not associated with any experiment in particular, the

332 percentage of DNA recovered was highly variable. The coefficient of variation of DNA

333 recovered was on average 65% considering all the samplers and 34% when excluding the

334 samplers which failed to recover any DNA. Altogether, while we found that the samplers can

335 still recover DNA after several cycles of use, we also discovered that the capacity of HAp

336 samplers to recover DNA is variable and unpredictable: at some times it may not work at all,

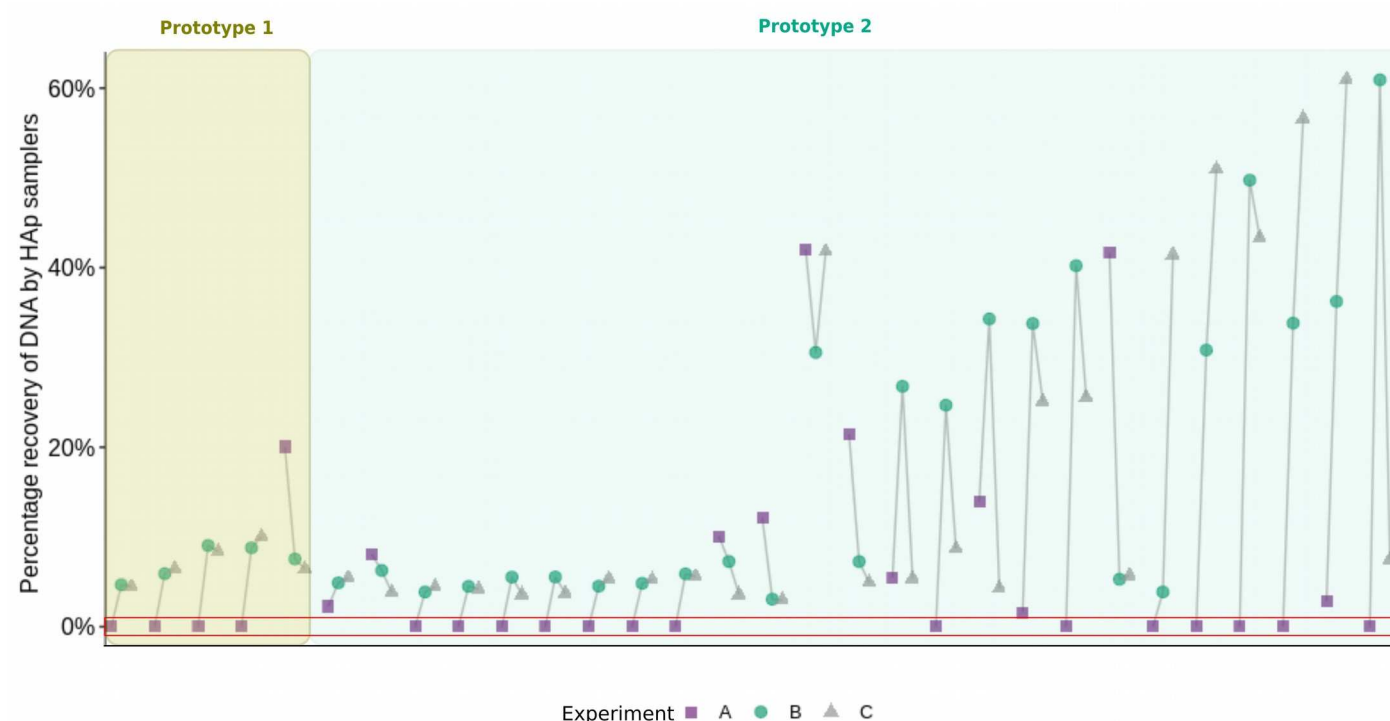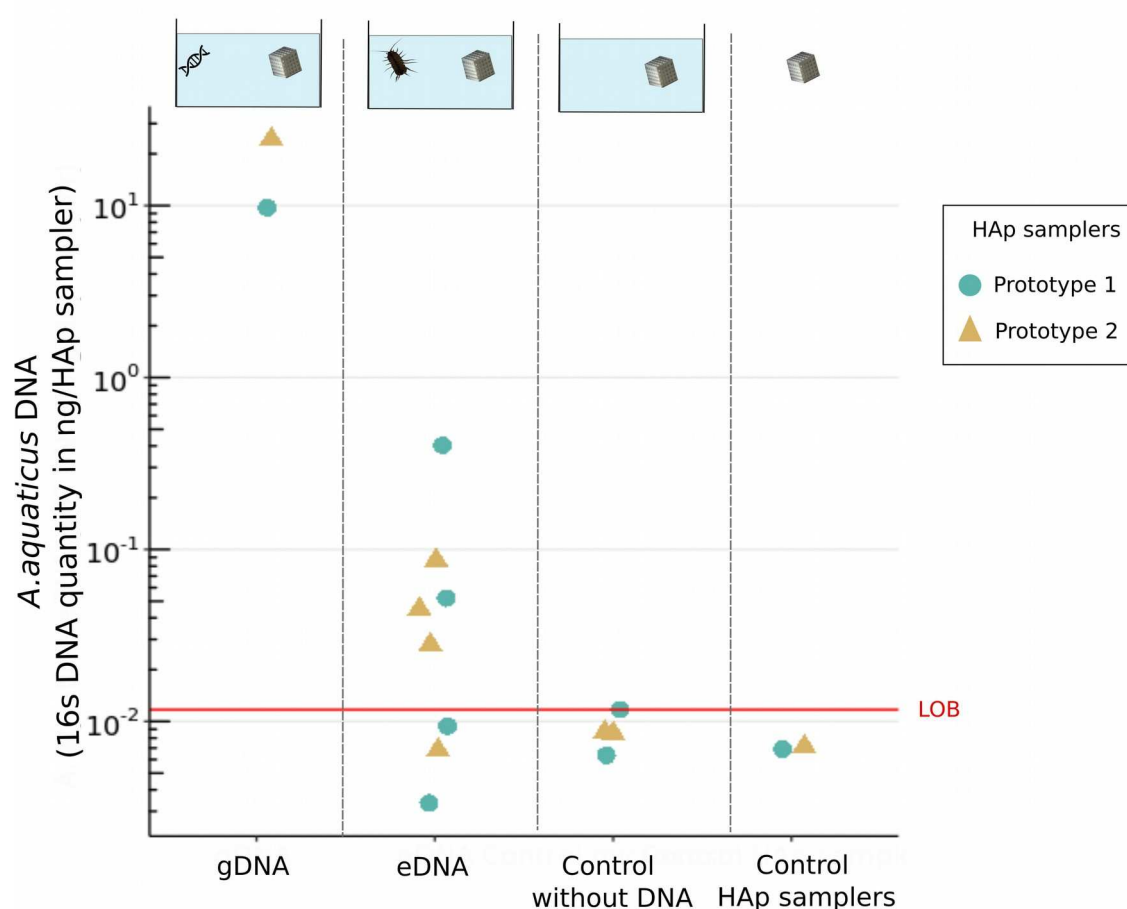337 while at others it may recover a large amount of DNA.

18

339 **FIGURE 6** : Percentage of DNA recovered by two prototypes (P1 and P2) of HAp samplers in

340 three consecutive experiments (A, B and C). 5 P1 and 25 P2 samplers are sorted according

341 to their variance of recovered DNA. Samplers in the red box did not recover any DNA.

342

343 3.3 | Experiment 3: Environmental DNA sampling

344 We deployed the HAp samplers in a microcosm containing isopods (*Asellus aquaticus*) to test

345 their ability to recover eDNA and used qPCR to quantify the amount of *A. aquaticus* 16S gene

346 recovered by the samplers. In a microcosm with no organisms, we observed low levels of

347 DNA that were similar or slightly above the amount of DNA observed in control samplers that

348 were not immersed in a microcosm (Fig. 7). This is indicative of a slight level of cross-

349 contamination between microcosms, and allowed us to determine an amount of 16S DNA

350 below which we cannot differentiate between a contamination and a positive result (limit of

19

351   blank, LOB). As expected, using concentrated genomic DNA as a positive control, the

352   samplers recovered large amounts of 16S DNA molecules (Fig. 7). In the microcosm that

353   contained isopods, the amount of 16S DNA molecules was about 3 orders of magnitude

354   lower, with 3 samplers out of 8 below the limit of blank. Overall, the two HAp prototypes

355   recovered *A. aquaticus* eDNA with the same efficiency (Wilcox-test, p = 0.89).

357

358   **FIGURE 7** : Quantity (in log scale) of *A.aquaticus* 16S gene recovered by the two prototypes

359   of HAp samplers (P1 = circle, P2 = triangle) after 24h of incubation in microcosm containing

360   genomic DNA used as a positive control (gDNA) or five individuals of *A. Aquaticus* (eDNA).

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361 Two types of negative controls were used: one control microcosm without DNA (control

362 without DNA), and HAp samplers without microcosm incubation (control HAp samplers). Red

363 line corresponds to the limit of blanks (LOB).

364

365 **4 | Discussion**

366 4.1 | HAp samplers recover artificial DNA and eDNA

367 Our results demonstrated that HAp samplers can passively recover artificial DNA and eDNA.

368 Using artificial DNA, DNA recovery was fast and optimal. In 17 hours, HAp samplers

369 recovered up to 4 times more DNA (i.e. 1.75 µg) than the theoretical quantity we had

370 estimated using a projection of a DNA monolayer on the surface of the samplers (see

371 methods). These results confirm the high binding affinity between DNA and hydroxyapatite

372 reported in literature (Okazaki et al., 2001 ; Del Valle et al., 2014) and suggest that more than

373 one layer of DNA molecules can bind to the HAp surface. To our knowledge, this is the first

374 time that these binding properties are tested and validated on 3D-printed objects. eDNA

375 experiments showed that HAp samplers recovered eDNA from living macro-organisms (*A.*

376 *aquaticus*). 5 out of 8 HAp samplers allowed a positive detection of *A. aquaticus* after only 24

377 hours of incubation in microcosm. Given the low densities of these small isopods which,

378 unlike large organisms commonly used in eDNA microcosm experiments (e.g. fish,

379 amphibians; Maruyama et al., 2014; Jo et al., 2020), are likely to release very small amounts

380 of eDNA, and given the short experiment duration, this overall high rate of detection

381 demonstrates the high sensitivity of HAp samplers to detect organisms.

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385 4.2 | Binding of different DNA fragment size

386 While it was hypothesized that DNA fragment size would influence DNA binding (Ogram et al.,

387 1994), we did not find any evidence that certain fragment sizes bind preferentially to the HAp

388 samplers. The samplers recovered DNA fragments of various sizes (i.e. 35-8450 bp),

389 although bands below 2000 bp were not visible on the electrophoresis gel, possibly due to a

390 higher concentration of the larger fragments in the marker solution. However, the sensitive

391 microfluidics-based automated electrophoresis analysis showed that smaller fragments

392 (<2000 bp) were bound and released by HAp samplers. eDNA is a complex mixture of genetic

393 material ranging from cells to more or less degraded free DNA fragments (Wilcox et al.,

394 2015). A sampling method that is not biased toward a given range of fragment sizes is a real

395 advantage for eDNA sampling, in particular in environments where eDNA could be rapidly

396 degraded into small free DNA fragments (Seymour et al., 2018). While free DNA binds to the

397 HAp samplers, it remains to be tested whether other forms of eDNA such as proteo-nucleic

398 complexes or even larger particles can also be collected.

399

400 4.3 | Repeatability issues

401 Although HAp samplers show a great potential for DNA sampling, repeatability appears to be

402 a concerning issue. Many HAp samplers showed extreme variability in DNA recovery among

403 experiments carried out under strictly identical conditions (section 3.2). Given the high

404 number of samplers that did not recover any DNA during the first but not the later experiments

405 (Fig. 6), one might have expected that DNA recovery would improve with cycle of use.

406 However, no effect of cycle of use or sampler prototype was found. In some cases, the DNA

407 recovery remained stable over time, in some it increased, and in other it decreased.

408 Surprisingly, while highly variable, there was not a set of samplers or one prototype in

409 particular which was less effective than the others to recover DNA. This unexplained

410 variability highlights   the complexity of the binding mechanism between DNA and

411 hydroxyapatite and the factor that controls it, and reinforces the necessity to better

412 understand the evolution of the HAp surface after several DNA cycles of use. According to

413 Okazaki et al. (2001), the binding affinity is based on an electrostatic interaction between the

414 negative charges of the phosphate groups of DNA to the calcium ions at the surface of the

415 hydroxyapatite. This ionic interaction strongly depends on the physico-chemical properties of

416 the sampler surface and the solution in which the binding reaction takes place (Gallo et al.,

417 2018). Among the surface properties, porosity, specific surface area, crystallinity and

418 stoichiometry of the HAp phase (calcium groups can be substituted by other ions) could play

419 a major role in DNA binding. The different manufacturing steps, such as the HAp densification

420 (i.e. sintering), can greatly influence most of these surface properties. In particular, ionic

421 substitution (e.g. carbonatation) and partial dehydration are known to occur frequently in HAp

422 during thermal treatment (Wang, Dorner-Reisel and Müller, 2004; Lafon, 2004) such as the

423 ones used here to decontaminate the samplers before and between experiments, and might

424 be the source of the observed variability. Surface analyse needs to be carried out to identify

425 the physical (e.g. porosity, crystalline phases) and chemical (e.g. surface ionic groups)

426 parameters involved in DNA binding on the HAp surface and the extent to which these

427 parameters are influenced by the manufacturing and use of the sampler (e.g. sintering,

428 debinding, immersion in DNA solution).

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5 | 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 an alternative sampling solution for eDNA-based biomonitoring. It also opens up an interdisciplinary field at the interface between engineering, surface science and molecular ecology.

AUTHORS' CONTRIBUTIONS

TL and CM conceived the ideas and designed HAp samplers. Experimental design was conceived by TL, LK and HV. HAp samples were thermal treated and characterized 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 manuscript. All authors contributed to the manuscript.

REFERENCES

Alvarez, A. J. *et al.* (1998) 'Amplification of DNA bound on clay minerals', *Molecular Ecology*, 7(6), pp. 775–778. doi: 10.1046/j.1365-294x.1998.00339.x.

Brundin, M. *et al.* (2013) 'DNA Binding to Hydroxyapatite: A Potential Mechanism for Preservation of Microbial DNA', *Journal of Endodontics*, 39(2), pp. 211–216. doi: 10.1016/j.joen.2012.09.013.

Deiner, K. *et al.* (2017) 'Environmental DNA metabarcoding: Transforming how we survey animal and plant communities', *Molecular Ecology*, 26(21), pp. 5872–5895. doi: 10.1111/mec.14350.

Del Valle, L. J. *et al.* (2014) 'DNA adsorbed on hydroxyapatite surfaces', *J. Mater.Chem. B*, 2(40), pp. 6953–6966. doi: 10.1039/C4TB01184H.

Der Sarkissian, C. *et al.* (2020) 'Unveiling the Ecological Applications of Ancient DNA From Mollusk Shells', *Frontiers in Ecology and Evolution*, 8, p. 37. doi: 10.3389/fevo.2020.00037.

Gallo, M. *et al.* (2018) 'Resorption of calcium phosphate materials: Considerations on the in vitro evaluation', *Journal of the European Ceramic Society*, 38(3), pp. 899–914. doi: 10.1016/j.jeurceramsoc.2017.07.004.

Gardner, C. M. and Gunsch, C. K. (2017) 'Adsorption capacity of multiple DNA   sources to clay minerals and environmental soil matrices less than previously estimated', *Chemosphere*, 175, pp. 45–51. doi: 10.1016/j.chemosphere.2017.02.030.

Goldberg, C. S. *et al.* (2016) 'Critical considerations for the application of environmental DNA methods to detect aquatic species', *Methods in Ecology and Evolution*. Edited by M. Gilbert, 7(11), pp. 1299–1307. doi: 10.1111/2041- 210X.12595.

Hinlo, R. *et al.* (2017) 'Methods to maximise recovery of environmental DNA from water samples', *PLOS ONE*. Edited by H. Doi, 12(6), p. e0179251. doi: 10.1371/journal.pone.0179251.

Jo, T. *et al.* (2020) 'Estimating shedding and decay rates of environmental nuclear DNA with relation to water temperature and biomass', *Environmental DNA*, 2(2), pp. 140–151. doi: 10.1002/edn3.51.

Kirtane, A., Atkinson, J. D. and Sassoubre, L. (2020) 'Design and Validation of Passive Environmental DNA Samplers Using Granular Activated Carbon and Montmorillonite Clay', *Environmental Science & Technology*, 54(19), pp. 11961–11970. doi: 10.1021/acs.est.0c01863.

Lafon, J.-P. (2004) Synthèse, stabilité thermique et frittage d'hydroxyapatites carbonatées (pp. 218).

Langlois, V. S. *et al.* (2020) 'The need for robust qPCR☐based eDNA detection assays in environmental monitoring and species inventories', *Environmental DNA*, p. edn3.164. doi: 10.1002/edn3.164.

Levy-Booth, D. J. *et al.* (2007) 'Cycling of extracellular DNA in the soil environment', *Soil Biology and Biochemistry*, 39(12), pp. 2977–2991. doi: 10.1016/j.soilbio.2007.06.020.

Mandelkern, M., Elias, J. G., Eden, D., & Crothers, D. M. (1981). The dimensions of DNA in solution. *Journal of molecular biology*, *152*(1), 153-161. doi: 10.1016/0022-2836(81)90099-1.

Mariani, S. *et al.* (2019) 'Sponges as natural environmental DNA samplers', *Current Biology*, 29(11), pp. R401–R402. doi: 10.1016/j.cub.2019.04.031.

Maruyama, A. *et al.* (2014) 'The Release Rate of Environmental DNA from Juvenile and Adult

503 Fish', *PLoS ONE*. Edited by M. Stöck, 9(12), p. e114639. Doi:
504   10.1371/journal.pone.0114639.
505 Moushomi, R. *et al.* (2019) 'Environmental DNA size sorting and degradation experiment
506   indicates the state of Daphnia magna mitochondrial and nuclear eDNA is subcellular',
507   *Scientific Reports*, 9(1), p. 12500. doi: 10.1038/s41598-019-48984-7.
508 Ogram, A. V. *et al.* (1994) 'Effects of DNA Polymer Length on Its Adsorption to Soils',
509   *Applied and Environmental Microbiology*, 60(2), pp. 393–396. doi:
510   10.1128/AEM.60.2.393-396.1994.
511 Okazaki, M. *et al.* (2001) 'Affinity binding phenomena of DNA onto apatite crystals',
512   Biomaterials, vol. 22, no 18, (pp. 2459-2464). doi : 10.1016/S0142-
513   9612(00)00433-6
514 Pilliod, D. S. *et al.* (2013) 'Estimating occupancy and abundance of stream amphibians
515   using environmental DNA from filtered water samples', *Canadian   Journal of Fisheries
516   and Aquatic Sciences*. Edited by J. Richardson, 70(8), pp.   1123–1130. doi:
517   10.1139/cjfas-2013-0047.
518 Rahaman, M. N. (2017). *Ceramic processing and sintering* (Vol. 1). CRC press.
519 Rivera, S. F. *et al.* (2021) 'Exploring the capacity of aquatic biofilms to act as
520   environmental DNA samplers: Test on macroinvertebrate communities in rivers',
521   *Science of The Total Environment*, 763, p. 144208. doi:
522   10.1016/j.scitotenv.2020.144208.
523 Seymour, M. *et al.* (2018) 'Acidity promotes degradation of multi-species environmental DNA
524   in lotic mesocosms', *Communications Biology*, 1(1), p. 4. doi: 10.1038/s42003-017
525   0005-3.
526 Shokralla, S. *et al.* (2012) 'Next-generation sequencing technologies for   environmental DNA
527   research: NEXT-GENERATION SEQUENCING FOR ENVIRONMENTAL DNA'
528   *Molecular Ecology*, 21(8), pp. 1794–1805. doi: 10.1111/j.1365-294X.2012.05538.x.
529 Spear, S. F. *et al.* (2015) 'Using environmental DNA methods to improve detectability in a
530   hellbender (Cryptobranchus alleganiensis) monitoring program', *Biological
531   Conservation*, 183, pp. 38–45. doi: 10.1016/j.biocon.2014.11.016.
532 Turner, C. R. *et al.* (2014) 'Particle size distribution and optimal capture of aqueous
533   macrobial eDNA', *Methods in Ecology and Evolution*. Edited by M. Gilbert, 5(7), pp.
534   676–684. doi: 10.1111/2041-210X.12206.
535 Turner, C. R., Uy, K. L. and Everhart, R. C. (2015) 'Fish environmental DNA is more
536   concentrated in aquatic sediments than surface water', *Biological   Conservation*, 183,
537   pp. 93–102. doi: 10.1016/j.biocon.2014.11.017.
538 Wang, T., Dorner-Reisel, A. and Müller, E. (2004) 'Thermogravimetric and thermokinetic
539   investigation of the dehydroxylation of a hydroxyapatite powder', *Journal of the
540   European Ceramic Society*, 24(4), pp. 693–698. doi: 10.1016/S0955-2
541   219(03)00248-6.
542 Wilcox, T. M. *et al.* (2015) 'Environmental DNA particle size distribution from Brook   Trout
543   (Salvelinus fontinalis)', *Conservation Genetics Resources*, 7(3), pp. 639–641. doi:
544   10.1007/s12686-015-0465-z.
545 Williams, K. E., Huyvaert, K. P. and Piaggio, A. J. (2017) 'Clearing muddied waters:
546   Capture of environmental DNA from turbid waters', *PLOS ONE*. Edited by H. Doi,
547   12(7), p. e0179282. doi: 10.1371/journal.pone.0179282.