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Title

Acidity promotes degradation of multi-species environmental DNA in lotic mesocosms

Authors

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Abstract

Accurately quantifying biodiversity is fundamental to understanding ecosystem function and for environmental assessment. Molecular methods using environmental DNA (eDNA) offer a non-invasive, rapid and cost-effective alternative to traditional biodiversity assessments, which require high levels of expertise. While eDNA analyses are increasingly being utilized, there remains considerable uncertainty regarding the dynamics of multispecies eDNA, especially in variable systems such as rivers. Here, we utilize four sets of upland stream mesocosms, across an acid-base gradient, to assess the temporal and environmental degradation of multispecies eDNA. Sampling included water column and biofilm sampling over time with eDNA quantified using qPCR. Our findings show that the persistence of lotic multispecies eDNA, sampled from water and biofilm, decays to non-detectable levels within two days and that acidic environments accelerate the degradation process. Collectively, the results provide the basis for a predictive framework for the relationship between lotic eDNA degradation dynamics in spatio-temporally dynamic river ecosystems.

MAIN TEXT

Introduction

Accurate biodiversity assessment involves reliable species detection and quantification, and is essential for furthering understanding of the natural world and for implementing effective management practices. Traditional biodiversity assessment methods are increasingly being supplemented, or even replaced, with more rapid and more accurate molecular environmental DNA (eDNA) based approaches. Environmental DNA is obtained by sampling and directly extracting DNA from natural systems, such as river water, without directly isolating the target organism(s); eDNA is thus freely distributed and originates from sources such as decaying tissue, feces, shed exoskeletons, skin, as well as other bodily excretions¹. The successful application of eDNA-based approaches in ecology is relatively recent, but several key eDNA studies have already had major impacts on the management of invasive and endangered species^{2,3}, and in biodiversity and environmental assessments⁴⁻⁷. However, despite the burgeoning applications of eDNA, there still is limited understanding of the temporal, physical and chemical factors that influence eDNA persistence dynamics, including eDNA degradation and transport.

Understanding eDNA persistence dynamics is particularly key to ensure the accuracy and reliability of eDNA biodiversity assessments. Here we define persistence dynamics as the relationship between physical, abiotic or biotic factors and the degradation and localized detection of eDNA in natural ecosystems. Environmental DNA studies to-date, have primarily assessed spatially static or semi-static lentic (e.g. pond and lake) or marine environments⁶⁻⁹. Particularly, physical hydrological processes, including flow, dilution and sediment uptake have been shown to influence eDNA detection¹⁰⁻¹². While lentic eDNA studies have shown reliable analytical species detection in diverse communities^{6,7}, as well as efficient monitoring of rare and low abundance species⁹, the effects of environmental variability among sampling points in relation to findings is largely ignored. Yet, the persistence of eDNA is directly influenced by the physical and abiotic environment¹. Well known to forensic science, tissue and genetic material can persist for extended periods of time in conditions where oxygen and microbial action are reduced or absent, such as DNA extracted from museum specimens or sediment and ice cores¹³. However, DNA can degrade rapidly (e.g. minutes) in aquatic environments due to hydrolysis, oxidation and microbial activity^{14,15}. The perceived low persistence of DNA in aquatic environments makes the application of aquatic eDNA approaches to biodiversity assessments and environmental management quite attractive, as the short persistence time allows for near real-time monitoring.

81 Direct tests of eDNA persistence have been limited to single species exclusion
82 experiments in lentic mesocosms^{4,12,16–20} or stream cages²¹. While microcosm experiments have
83 shown that increased temperature and pH promote eDNA degradation of single species eDNA
84 under control settings^{17,20}, we currently lack an assessment of natural environmental variation on
85 eDNA persistence in the water column across multiple distantly related species. Biotic factors are
86 also expected to influence eDNA persistence in the water column of lotic systems, whereby once
87 eDNA is released, it is expected to settle and accumulate into substrates or biofilms. While higher
88 eDNA concentrations have been found in sediments versus water samples¹⁰, the temporal
89 accumulation of eDNA into lotic or lentic substrate has yet to be empirically tested. Overall,
90 understanding how and where detection rates are influenced by environmental factors is
91 paramount for utilizing eDNA methods effectively across systems in order to assimilate
92 knowledge of biodiversity trends.

93 Despite their ecological and socio-economic importance, lotic systems (i.e. rapidly
94 moving freshwater bodies such as rivers and streams) have rarely been the focus of eDNA
95 investigations. Moreover, the focus of lotic eDNA studies has been on assessing the spatial signal
96 of transporting eDNA, with disparate results suggesting that the eDNA transit distances ranges
97 from meters to kilometers^{11,22–25}. Disparities in these findings likely relates to several physical
98 factors. The transport of a genetic signal will depend on the hydrological dynamics of flow,
99 diffusion/dilution, sinking of the material into the substrate and subsequent resuspension until the
100 eDNA source becomes degraded beyond the level of capture^{10–12}. The range of factors relating to
101 the transit of eDNA will strongly affect our ability to detect biodiversity signals and to date, there
102 have been no studies that assess how environmental factors affect the persistence of lotic eDNA.
103 Consequently, there is a clear need to experimentally assess temporal eDNA dynamics occurring
104 in natural lotic systems.

105 Here we assess the persistence dynamics of lotic eDNA using a replicated set of semi-
106 natural field experimental streams (i.e. mesocosms) to understand the effects of time and abiotic
107 environmental variation on multispecies eDNA detection. Specifically, we test the effects of a
108 wide range of environmental variables routinely measured for environmental quality, UV and
109 temperature and address three key knowledge gaps: 1) How does the temporal degradation of
110 eDNA vary across a range of taxonomically disparate species? 2) Which environmental factors
111 can be attributed to static and temporal variation in the eDNA signal? Finally, 3) does eDNA
112 accumulate in natural stream substrata? Our findings show multi-species lotic eDNA, derived
113 from water and biofilm, degrades rapidly over time following a negative binomial distribution.
114 Additionally, acidic environments accelerate the rate of lotic eDNA degradation.

115 **Results**

116 *Environmental variation*

118 The experimental sites utilized an established set of mesocosms that were designed
119 specifically to allow experimental lotic comparisons across an environmental gradient
120 present across the Welsh upland and more generally, representing land uses across the
121 United Kingdom. The site consisted of four circulating experimental mesocosms, with
122 three channels per mesocosms and with water originating from neighboring streams (Fig.
123 1). More specifically, mean pH for each mesocosm were typical of the Llyn Brianne
124 catchments²⁶ at 6.73 (± 0.01) for Carpenter, 6.82 (± 0.04) for Davies (both circumneutral
125 moorland), 5.90 (± 0.07) for Hanwell and 5.35 (± 0.05) for Sidaway (both conifer forest).
126 Temperature means were 15.29 °C (± 1.80) for Carpenter, 14.72 °C (± 1.52) for Davies,
127 14.47 °C (± 1.87) for Hanwell and 16.16 °C (± 2.57) for Sidaway. Mean total dissolved
128 nitrogen (TDN) was 0.146 mg/L (± 0.03) for Carpenter, 0.14 mg/L (± 0.03) for Davies,

129 0.17 mg/L (± 0.03) for Hanwell and 0.49 mg/L (± 0.20) for Sidaway (Fig. 2). Additional
130 water chemistry data, measured but not included in the final analyses, are included in the
131 methods and supplementary material (Supplementary Table 1). For the source eDNA
132 material, we chose ecologically relevant and taxonomically diverse taxa that could either
133 be cultured, or collected to serve as eDNA source material. Thus, *Daphnia magna*,
134 *Ephemera danica* and *Anguilla anguilla* were selected, thereby facilitating comparisons
135 of eDNA persistence from diverse sources of macroinvertebrates and vertebrates. *Daphnia*
136 *magna* is a small planktonic crustacean, found commonly in lentic environments across
137 the Northern hemisphere and is routinely utilized in ecological and evolutionary studies.
138 *Ephemera danica* is a species of mayfly commonly found in lakes and rivers across
139 Europe. *Anguilla anguilla* is a critically endangered eel species found in marine and inland
140 waters across Europe and Northern Africa.
141

142 *Quantitative PCR*

143 Successful amplification of eDNA from water samples for *D. magna*, *E. danica* and *A.*
144 *anguilla* occurred across time points 0, 1, 3, 7, 19, 29 and 43h, whereas no amplification
145 was observed for all samples at time point -1 (the control sample), where the streams were
146 sampled prior to adding eDNA to the experiment. Additionally, no amplification was
147 evident in the negative PCR controls. Generally, across all species, amplification,
148 calculated as copy numbers, as described in the methods, was greatest at time point 0
149 across all sites (*D. magna*: $\bar{x} = 18.55$ copy numbers ± 34.673 , *E. danica*: $\bar{x} = 56.872$ copy
150 numbers ± 95.991 , *A. anguilla* = 2.97 copy numbers ± 3.405) and degraded over time to
151 near 0 copy numbers or null amplification at hour 43 (Fig. 3). While the added sucrose
152 signal decayed over time indicating uptake by the microbial community, the effects of
153 sucrose on DNA quantification was non-significant. Using a mixed effect generalized
154 linear model with a negative binomial error distribution, the variance among groups was
155 approximately zero after testing the relation between quantification and time. Therefore,
156 sucrose was not retained as a factor in subsequent analyses. Biofilm eDNA quantification
157 was successful for *E. danica*, but failed for *D. magna* and *A. anguilla*, with lower copy
158 numbers at time point 0 ($\bar{x} = 2.003$ copy numbers ± 3.548), compared to the water derived
159 eDNA signal, and degrading to near 0 copy numbers at time 43. We assessed whether the
160 lower detectability associated with the biofilm extracts could be due to PCR inhibition by
161 randomly selecting 7 samples from time point 0 across the mesocosms and using
162 OneStep™ PCR Inhibitor Removal Kit (Zymo Research Corp.) prior to rerunning the
163 qPCR with clean and uncleaned samples. Amplification of the cleaned samples did not
164 differ between the cleaned and uncleaned extractions.

165 We found significant negative effects of time ($P < 0.001$, SE = 0.663, slope = -0.100), a
166 significant positive effect of pH ($P < 0.001$, SE = 0.187, slope = 0.926), and a significant
167 negative effect of time x pH ($P < 0.001$, SE = 0.020, slope = -0.092) on water derived
168 eDNA signal (Table 1, Fig. 3, Fig. 4). Random effects of time and species had non-zero
169 standard deviations of 0.554 and 1.048 respectively, indicating their importance to the
170 model. Temperature and TDN, including their interactions with time, were not
171 significantly related to eDNA quantification and were dropped from the final model.
172 Environmental DNA quantification was typically 1 to 2 orders of magnitude greater in
173 higher pH (>6) sites compared to lower (<6) pH sites (Fig. 4) shortly after the start of the
174 experiment. Decay rates (proportional loss per hour) derived from the model showed rapid
175 eDNA decay calculated at hour 1 and 3 of the experiment, particularly for the acid sites

176 Sidaway (0.982 ± 0.001 ; 0.329 ± 0.001) and Hanwell (0.946 ± 0.005 ; 0.322 ± 0.001)
177 compared to the circumneutral sites of Carpenter (0.674 ± 0.009 ; 0.273 ± 0.001) and
178 Davies (0.602 ± 0.030 ; 0.261 ± 0.005). Biofilm derived eDNA was not detected in the
179 most acidic mesocosms, with quantification levels roughly ten times less than those found
180 in the water derived eDNA. Overall, biofilm derived eDNA was found to decline
181 significantly over time ($P < 0.001$, SE 0.008) and was significantly greater at higher pH
182 ($P < 0.001$, SE 0.184) (Table 2). Decay rates for the biofilm derived eDNA at the onset of
183 the experiment were much slower in the circumneutral mesocosm, Davies (0.085 ± 0.014 ;
184 0.049 ± 0.014) compared to the acidic mesocosm, Hanwell (0.719 ± 0.023 ; 0.246 ± 0.023).

185 186 Discussion

187 Environmental DNA is predicted to be a powerful source of information for
188 assessing species and community dynamics as it allows higher spatial and temporal
189 sampling resolution at increased accuracy compared to traditional methods^{2,27-29}.
190 However, for meaningful inferences from natural systems we need to have a fundamental
191 understanding of the processes that govern the persistence and detection of the eDNA
192 signal when exposed to representative environmental variation. Here we present the first
193 experimental assessment, to our knowledge, of eDNA persistence in lotic environments
194 across multiple species under different pH conditions. We found clear indication that
195 environmental conditions interact with temporal dynamics to influence eDNA persistence.
196 Additionally, we show that short-lived eDNA persistence dynamics are similar across
197 species, indicating a general eDNA persistence model, with a negative binomial
198 distribution, that is particularly relevant for large scale community studies.

199 Localized eDNA persistence dynamics are largely unknown, but are suspected to
200 be influenced by environmental conditions with laboratory assessments of eDNA decay
201 suggesting pH and high temperatures as key explanatory variables^{17,19}. Conversely, a
202 recent field experiment found temperature had no effect on seawater derived *Scomber*
203 *japonicas* (chub mackerel) eDNA degradation³⁰. Here we show that abiotic variation,
204 specifically acidity, decreases eDNA persistence locally and over time. There were no
205 observed effects of nutrient load (e.g. total dissolved nitrogen) or temperature on eDNA
206 degradation rates, but this may be due to the low nutrient levels and relatively
207 homogeneous cooler temperatures, indicative of temperate upland headwater ecosystems.
208 While there has been no assessment of the effects of the abiotic environment on eDNA
209 derived from natural systems, there are some basic laboratory based understandings with
210 regards to DNA degradation that support our empirical observations. The structure of
211 DNA is very stable under dry, anoxic conditions; with an estimated half-life of ~500 years
212 under ideal conditions³¹, but will decay rapidly (minutes) in oxygenated environments,
213 due to effects such as hydrolysis and oxidation¹⁵. Degradation of DNA is particularly
214 likely when positively charged enzymes, indicative of acidic conditions (i.e. low pH), are
215 present¹⁴. The finding of decreased eDNA persistence with decreasing pH and temporal
216 degradation are further supported by a single species eDNA based laboratory study¹⁷,
217 whereby proportional detection of *Lithobates catesbeianus* eDNA was shown to be lower
218 at pH 4 compared to pH 7, however degradation comparisons between pH 7 and pH 10
219 were non-significant. Moreover, DNA is traditionally preserved in alkaline buffers (e.g.
220 Tris, EDTA buffer, pH 9), and will degrade if left in water due to acid hydrolysis,
221 particularly below pH 7.5¹⁵.

222 Temporal persistence of eDNA has thus far been experimentally assessed for
223 individual or closely related species^{11,17,18,32}, with reported persistence times ranging from

224 hours to months. In the Llyn Brianne mesocosms, we observed lotic eDNA persistence
225 over 43 hours for three taxonomically distant species, which validates previous findings.
226 However, a majority (>90%) of the eDNA signal, across all mesocosms, was lost within
227 the first 3 hours of the experiment and within the first hour for the more acidic
228 environments. Nevertheless, the novel observation here was that the prevailing
229 environmental conditions affected the decay dynamics of the disparate forms of multi-
230 species lotic eDNA in a concerted fashion. Although intuitive, harmonized degradation of
231 disparate forms of eDNA suggest that aquatic eDNA is likely derived from the same
232 biological material (e.g. cellular matter)³³. Regarding the variance between different times
233 of recorded eDNA persistence, differences in overall temporal persistence between this
234 study and previous studies are likely attributed to source eDNA concentrations or
235 differences in experimental design such as local environmental or mesocosm
236 environmental factors. For example, Jerde *et al.* (2016) assessed eDNA localized
237 persistence in shallow stream beds and found that eDNA was transported out of the
238 system in minutes by flowing water²³. Likewise, Wilcox *et al.* (2016) determined that
239 50% of *Salvelinus fontinalis* produced eDNA was lost within 100 m of the source (i.e.
240 minutes)²¹. Conversely, Strickler *et al.* (2015) showed that lentic eDNA persisted up to 60
241 days in experimental mesocosms that harbored roughly similar eDNA concentrations as
242 our experiment¹⁷. Additionally, studies assessing eDNA detection dynamics in natural
243 environments suggest that detection is limited to less than one month in static lentic
244 systems⁴, and at least 24 hours across lotic systems²⁴. Overall, the short time persistence
245 found in this study, particularly the rate of decay in the acidic environments, is similar to
246 previous findings looking at lotic eDNA persistence in relation to hydrological dynamics
247 ^{12,21}.

248 Lotic eDNA studies are generally rare, despite the fact that lotic systems are a
249 substantial source of biodiversity information and harbor a disproportionately high amount
250 of Earth's biodiversity (>6%) compared to their low surface coverage (0.8%)³⁴.
251 Additionally, the dendritic interconnected network structure of lotic systems allows for a
252 single river network to encompass a large geographical area, environmental habitats and
253 diverse species groups^{35,36}. According to our empirical data here, eDNA from sites across
254 a river network will be transported downstream, potentially allowing ecologists and
255 managers to utilize eDNA assessed from downstream confluence sites to infer biodiversity
256 and community dynamics across a large geographical range and set of environmental
257 conditions²⁴. Here we demonstrate qPCR detectable eDNA persistence of 43 hours, which
258 corresponds to roughly 35 km in rivers with a flow rate of ~2 m/s, which constitutes an
259 average flowrate in natural rivers. However, other studies show that the eDNA signal will
260 be undetectable downstream from the eDNA source due to dilution by large tributaries at
261 the point of the confluence¹¹. However, if effects of dilution by tributaries are limited
262 within a river network, the eDNA can be traceable for over 12 km from the eDNA source
263 ^{24,37}. Here, we did not include the effects of dilution, as headwater streams are
264 characteristically not influenced by dilution from neighboring streams, although there may
265 be some effect of groundwater flows. Nevertheless, it is essential to consider all factors
266 associated with the transport of eDNA as it moves through different environments as
267 environmental heterogeneity will directly impact the ability to capture the eDNA signal¹.
268 A potential caveat of the persistence of eDNA is the large spatial heterogeneity possibly
269 associated with sampling eDNA, particularly in riverine environments. While some
270 applications may benefit from catchment wide assessments, efforts to characterize
271 localized diversity will require alternative methodologies³⁸. One potential alternative
272 would be to utilize primers targeting longer sequence fragments, which have been shown
273 to degrade faster compared to shorter fragments, thereby likely of more local origin^{9,39}.

274 The fate of eDNA is largely unknown, but is closely linked with persistence. Aside
275 from chemical decomposition of free-floating DNA molecules and liberation of eDNA
276 from the cell matrix, it is suspected that eDNA will settle at the bottom of river beds and
277 become trapped by the biofilm, which in turn will allow microbial organisms to utilize the
278 accumulating eDNA as a food source¹. Here, we found little support for eDNA
279 accumulation in the biofilm as quantification failed for two of the three experimental
280 species and the quantification of the *E. danica* biofilm eDNA was a magnitude lower
281 compared to the water derived *E. danica* quantification. Additionally, the sampled area to
282 total flume area were the same order of magnitude for the water (0.13% of the total
283 volume) and biofilm (0.14% of the total volume) samples. This might suggest that the
284 turbidity of the flowing lotic system does not allow measurable eDNA accumulation. No
285 study has previously assessed eDNA accumulation in biofilm, although previous work by
286 Barnes *et al.*¹⁸ showed that *Cyprinus carpio* eDNA degradation increased under lower
287 aerobic activity and chlorophyll levels, which suggest biological activity is either
288 counterintuitively assisting eDNA preservation, or that the effect of biological utilization
289 of eDNA may be less fundamental than expected. Another recent study also showed that
290 the localized retention and resuspension of eDNA in lotic systems is influenced by the
291 substrate type of the river channel, whereby finer substrate beds allow for greater *C.*
292 *carpio* eDNA substrate uptake¹². The lower accumulation found in our experiment may
293 therefore be due to the coarse substrate hindering absorptions due to negatively charged
294 surface areas or from the utilization of eDNA as a food source by microorganisms in the
295 substrata¹⁵. While the findings presented here suggest limited to no additional effect of
296 biological activity on eDNA persistence, further assessment should be made in higher
297 nutrient (e.g. available nitrogen or phosphorous) sites.

298 This study is the first, to our knowledge, to assess the effect of abiotic factors on
299 eDNA detection and degradation across a suite of ecologically relevant, yet taxonomically
300 divergent taxa in near natural, replicated experimental streams. Overall, the results of this
301 study indicate more rapid eDNA degradation in lotic systems, compared to previous lentic
302 studies, likely attributed to variation in the abiotic environment and physical
303 characteristics of flowing water systems. Additionally, we show that eDNA persistence
304 dynamics are consistent across broad taxonomic groups, further cementing eDNA based
305 approaches as an efficient, robust method for assessing community dynamics. The
306 findings from this study have clear implications for eDNA approaches to measuring
307 biodiversity in flowing waters, highlighting the need to consider environmental variation
308 among sites and spatial-temporal dynamics, which are paramount for robust ecological
309 and environmental assessments of biodiversity. Spatio-temporal patterns of species
310 detection are likely to be predictable across different species and strongly influenced by
311 environmental variation across different river catchments.

314 **Materials and Methods**

315 *Experimental setup*

316 We utilized four, unique experimental stream mesocosms located upstream of the Llyn
317 Brianne Reservoir (UK; 52.132614, -3.752174) in upland Wales
318 (<http://www.cardiff.ac.uk/llyn-brienne-observatory>). Each of the experimental streams,
319 described in detail in Durance *et al.*⁴⁰ (Fig. 1), consisted of 3 circulating channels (20m x
320 20 cm x 20 cm), utilizing cobble (D₅₀ = 5 cm) for substrate, with an average flow rate of
321 ~2m/sec with water sourced directly from adjacent headwater upland streams. The
322 experimental channels, with corresponding site names in parentheses, included two

323 channels feeding from moorland catchments with circumneutral waters at pH ranging
324 from 6.8 to 7.2 (L6-Carpenter, L7-Davies), and two from conifer forest catchments with
325 acidic waters at pH ranging from 5.3-5.8 (L3-Hanwell, and recently logged L8-Sidaway).
326 The mesocosms at the Llyn Brienne observatory are fed directly from natural streams, and
327 so chemical conditions represent the prevailing acid-base gradient in the upper Tywi
328 catchment⁴¹. Moreover, the environmental variation represented in the experiment is
329 representative of wider conditions across the whole of upland Wales and large areas of
330 upland Britain more generally^{42,43}.

331 *Environmental DNA sources and addition*

332 Environmental DNA was sourced from a wide range of taxa including *Daphnia*
333 *magna*, *Ephemera danica* and *Anguilla anguilla*. Species were selected with the aim to
334 acquire broad phylogenetic diversity, and based on locally available non-invasive species
335 that were naturally occurring in the Llyn Brienne catchment. *D. magna* were cultivated in
336 mesocosms (~200 Individuals/L) at Bangor University, which originated from a single
337 clone provided by Birmingham University. *Ephemera danica* were collected near
338 Galsbury, UK and kept in mesocosms (~100 Indv/L) at Bangor University two weeks
339 prior to the experiment. Environmental DNA rich water from the *D. magna* and *E. danica*
340 cultures were collected by sieving individuals from the water using a 250 micron sieve
341 into sterilized plastic containers. *Anguilla anguilla* was sourced from the Cynrig Fish
342 Culture unit (Brecon, UK) where *A. anguilla* juveniles (250 Indv/L) were kept in 4L tanks.
343 Prior to collection, the water from the Cynrig Fish Culture Unit was subjected to
344 ultraviolet light due to water treatment protocols.

345 At each experimental mesocosm, we added 2L of eDNA rich water that had held *D.*
346 *magna*, and *A. anguilla* and 1L of eDNA for *E. danica*. The reduced volume for *E. danica*
347 was due to higher eDNA concentration in the holding tanks. We quantified eDNA
348 concentrations prior to addition thereof to the experimental systems using a Qubit (2.0)
349 fluorometer (Life Technologies, Carlsbad, USA) for each species resulting in 5.45 ng/ μ l
350 (5.45E6 ng/L) for *D. magna*, 7.33 ng/ μ l (7.33E6 ng/L) for *E. danica* and 1.75 ng/ μ l
351 (1.75E6 ng/L) for *A. anguilla*. DNA concentrations were then diluted upon addition to the
352 mesocosms by 1:400 for the *D. magna* (18600 ng/L) and *A. anguilla* (4375 ng/L) and
353 1:800 for the *E. danica* (9162.5 ng/L), which were over five orders of magnitude higher
354 than concentrations found in natural river systems^{44,45}. Starting eDNA concentrations
355 were also quantified using qPCR as described below.

356 Furthermore, to test the effect of increased microbial activity on eDNA persistence, a
357 synthetic form of dissolved organic carbon (DOC) sucrose (>99.0% Sucrose, Sigma-
358 Aldrich, Dorset, UK) was added to one of the three channels in each of the experiment
359 streams to simulate high productivity sites.

360 *Sampling*

361 Water samples were collected, from the water column, over the course of 44 hours,
362 including one hour prior (time point -1) to adding eDNA to the systems (negative control),
363 10 min after adding eDNA to the system (time point 0) and 1, 3, 7, 19, 29 and 43 hours
364 from initializing the experiment. In addition to the T -1 negative control sampling, we
365 took one negative control sample for each time point that consisted of previously
366 autoclaved water kept in the same sampling containers as the samples, and kept among the
367 sampled material during the experiment. For each sampling time, 1L water samples were
368 collected, without replacement, using sterilized Nalgene bottles, in triplicate, from each
369 experimental stream channel, resulting in 36 samples per time point (total 252 samples for
370 the experiment). Compared to the total volume of the mesocosm (800L) each filtered

371 sample constituted 0.13% of the total mesocosm volume. Water samples were filtered on-
372 site using 0.22µm Sterivex filter units with male and female luer ends (Millipore Corp,
373 Bilerica, USA) and a Geotech peristaltic pump (series II Geotech, Denver, USA). The
374 eDNA was preserved by expelling all water from the filter units, capping the male luer
375 end with a luer screw cap, filling the sterivex unit with Longmires solution ⁴⁶ and capping
376 the female luer end. Samples were then transported to Bangor University, kept at 4 °C and
377 DNA extracted within two weeks.

378 To investigate whether eDNA was settling and accumulating on the bed of the
379 channels we took standardized biofilm samples from three of the experimental channels,
380 covering the full environmental variability. Terracotta tiles (15 cm x 15 cm x 5 cm) were
381 added to 1m interval sections of the flumes two weeks prior to the experiment to allow
382 biofilm growth. During each water sampling event, a tile was removed at random, from
383 each of the flumes in the experimental stream and scraped clean into a 50ml tube, using
384 standard biofilm sampling protocols ⁴⁷. Biofilm samples were then stored at -20°C and
385 shipped to Bangor frozen for subsequent analyses. Compared to the total surface area of
386 the mesocosm (160 800 cm²), each biofilm sample (750 cm²) constituted 0.47% of the
387 total sampling surface area.

388 *Water chemistry*

389 Water chemistry measurements were collected daily for aluminium (Al), boron
390 (B), calcium (Ca), iron (Fe), potassium (K), magnesium (Mg), manganese (Mn), sodium
391 (Na), sulfur (S), silicon (Si), total suspended solids (TSS), bromide (Br), chloride (Cl),
392 fluorine (F), Ammonium (NH₄-N), Nitrite nitrogen (NO₂-N), Nitrate nitrogen (NO₃-N),
393 phosphate (PO₄-P), total organic nitrogen (TON), non-purgeable organic carbon (NPOC),
394 total dissolved nitrogen (TDN), pH, alkalinity (GranAlk), and conductivity (Cond.).
395 Additionally, temperature and light data loggers (model 650MDS, YSI Inc, USA) were
396 placed in each experimental channel with measurements taken every 15 minutes during
397 the experiment with daily averages used in subsequent analyses (Supplementary Table 1).

398 *DNA extraction and qPCR analyses*

399 All extractions and qPCR setups were performed in a designated eDNA laboratory
400 at Bangor University, in rooms free of PCR products (i.e. no PCR machines and no prior
401 PCR amplification occurring in the rooms) with positive air flow. The eDNA was
402 extracted from the filters using a modified Qiagen Blood and Tissue DNAeasy (Qiagen,
403 Hilden, Germany) extraction method ^{48,49}. In short, the Longmire's solution was first
404 removed by passing the Longmires through the filter membrane. Lysis buffer and
405 proteinase K were then added to the filter, and the filter placed in a hybridization oven to
406 rotate and incubate at 56 °C overnight. Subsequent extraction steps followed the standard
407 Qiagen DNAeasy extraction protocol. We extracted DNA from biofilm samples using
408 PowerMax Soil DNA isolation kit (MoBio) according to the manufacturer's instructions,
409 following a 20 minute centrifuge spinning of the samples at high speed to pellet the
410 sample.

411 Quantification of extracted eDNA from all water and biofilm samples was
412 performed in triplicate via species-specific targeted qPCR assays (Table 3) developed by
413 Primer Design Ltd (Southampton, UK). Each 20ul reaction contained 1ul primer/probe
414 mix (300nM), 10 µl (2X) PrecisionPLUS Mastermix (Primer Design Ltd.), 2 µl DNA, and
415 7 µl DNase free water. Reactions were run on a QuantStudio™ Flex 6 Real-Time PCR
416 System (Applied Biosystems, USA) with the following protocol: 2 min at 95 °C, followed
417 by 40 cycles of 10s at 95 °C and 60s at 60 °C. Each qPCR plate included a five-fold
418 dilution series of the relevant control DNA (*D. magna* 6500 copies/reaction to 0.65

419 copies/reaction, *E. danica* 4000 copies/reaction to 0.40 copies/reaction, *A. anguilla* 1500
420 copies/reaction to 0.15 copies/reaction) and no template control in triplicate. For each
421 primer set, mean Ct values generated from the control DNA dilution series were plotted
422 against log gene copy number to generate a standard curve and a linear line of best fit to
423 assess amplification efficiency, y-intercept and R² value.

424 *Statistical analyses*

425 All statistical analyses and graphics employed R, version 3.3.1⁵⁰. To assess the
426 relationship between eDNA quantification in relation to time and environmental variation,
427 we fitted a mixed effect generalized linear model with a negative binomial error
428 distribution using quantified eDNA copy numbers as the response variable. Initial
429 explanatory variables included time, pH, TDN, temperature and all two-way interactions
430 between pH, TDN, temperature and time. Water chemistry explanatory variables were
431 selected based on individual variable distributions, particularly avoiding variables with an
432 overabundance of zero values as they likely result from lower detection limitation and
433 may result in type I errors due to zero-inflation⁵¹. Additionally, highly correlated
434 variables were reduced using pairwise comparisons to avoid violation of independence
435 among explanatory variables. Explanatory variables were centered, such that their mean =
436 0, prior to model fitting to avoid unrealistic intercept parameterization. Time and species
437 were included as random effects to account for covariance structure among time points
438 and among species (i.e. starting eDNA concentrations). Models were reduced using
439 backward model selection with Akaike information criterion (AIC) comparisons, such that
440 the final model resulted in time, pH and time:pH as explanatory factors. The relationship
441 between biofilm derived eDNA in relation to time and environmental variation was
442 assessed in a similar fashion as the water-derived eDNA, except a simpler generalized
443 linear model with a negative binomial error distribution was fitted, as it was determined
444 that including random effects did not improve the model fits.

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572
573
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590
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592
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595

596 **Figure legends**

597
598 **Figure 1. Schematic overview of the study design.** The study design includes the sampling
599 workflow for the water and biofilm eDNA sampling. Mesocosms are depicted with their
600 associated names above. The dotted lines represent 1m channel sections (20m in total for
601 each channel) in which terracotta tiles (small brown boxes) were placed for biofilm
602 accumulation. Background colors (blue, green, orange, red) correspond to the natural
603 acidic gradient of the mesocosms.

604 **Figure 2. Environmental variation of the experimental flumes.** Boxplots showing
605 environmental variation across sites (x-axis) for pH (top panel), temperature (middle
606 panel) and total dissolved nitrogen (TDN) (bottom panel). Data shown include daily
607 averages across three days with three samples taken per sampling site (one per channel).
608 The upper and lower whiskers show the standard deviation.

609 **Figure 3. Temporal eDNA dynamics.** Results of the qPCR analysis. Quantity (x-axis) as
610 normalized copy numbers relative to time (y-axis) in hours with each point showing mean
611 quantity values (n=3) for each time point at the respective experimental stream (separate
612 panels). The experiment consisted of 864 data points evenly distributed across three
613 species, four sites, and eight time points with nine samples taken per site per time point (3
614 per channel). Whisker bars show the standard deviation. Lines are the fitted values from a
615 generalized linear mixed effects model. Lines and point data were normalized after fitting
616 the statistical model. Colours represent unique species (*D. magna*, *E. danica*, *A. anguilla*)
617 for each stream replicate (3 per stream).

618 **Figure 4. Acidic effects on eDNA detection.** Barplot showing eDNA quantification (log copy
619 numbers: y-axis) versus pH (x-axis). Each bar depicts the mean quantification value (with
620 accompanying standard deviation) across all samples for a given site/channel, which
621 correspond to a mean pH value for the given sampling location. The experiment consisted
622 of 864 data points evenly distributed across three species, four sites, and eight time points
623 with nine samples taken per site per time point (3 per channel). The different colour bars
624 depict different time points including -1 (control), 0, 1, 3, 7, 19, 29, and 43 hours from the
625 start of the experiment.
626

627 **Tables**

628

629 **Table 1. eDNA Mixed effects model results.** Results of the generalized linear mixed effects
 630 model with negative binomial error distribution describing the relationship between quantified
 631 copy numbers as the response variable, time, pH and time x pH as the explanatory variables
 632 (Fixed-effects) and time and species as the random effects. Provided are the values for the
 633 estimate, z-value, standard error and p-values for the corresponding fixed effects of the model as
 634 well as the variance and standard deviation for the random effect of the model.
 635

Parameter	Estimate	z-value	Standard Error	P-value
Fixed effects:				
intercept	-2.389	0.663	-3.602	
Time	-0.099	0.020	-4.863	<0.001
pH	0.926	0.261	3.549	<0.001
Time:pH	-0.092	0.020	-4.503	<0.001
Random Effects				
	Variance	Standard Deviation		
Time	0.307	0.554		
Species	1.097	1.048		

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638 **Table 2. Biofilm generalized linear model results.** Results of the generalized linear model (glm)
639 with negative binomial error distribution describing the relationship between quantified copy
640 numbers derived from biofilm as the response variable, time and pH as the explanatory variables.
641 Provided are the values for the estimate, z-value, standard error and p-values for the
642 corresponding parameters of the model
643

Parameter	Estimate	Standard Error	z-value	P-value
(Intercept)	-0.444	0.115	-3.855	<0.001
Time	-0.036	0.008	-4.574	<0.001
pH	1.318	0.184	7.162	<0.001

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Table 3. qPCR primer/probe information. Quantitative PCR Sense and AntiSense primer and probe sequences for each target species used for this study.

Target Species	Primer/Probe	SensePrimer
<i>Daphnia magna</i>	Sense	TCGGAATGATCTCTCATATTATCAGTC
	AntiSense	ACCTAAGACACCAATAGCTAATATAGC
	Probe	TCCCAAAGGCTTCCTTCTCCCTCTTTTCG
<i>Ephemera danica</i>	Sense	CTTCCTCCTGCTTTAACACTTCTT
	AntiSense	GGGCGATTCTGCTGCTAA
	Probe	ACAGTTCAACCTGTTCTGCTCCTCTTTCT
<i>Anguila anguila</i>	Sense	GCAGGTATTTTCATCAATTCTAGGG
	AntiSense	GAGTAGTAAAACGGCGGTTACTAA
	Probe	ACCGCCTGCAATTACACAGTACCA

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