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Annual time-series analysis of aqueous eDNA reveals ecologically relevant dynamics of lake ecosystem biodiversity

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1 **Title**

2 **Annual time-series analysis of aqueous eDNA reveals ecologically relevant dynamics of**
3 **lake ecosystem biodiversity.**

4
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24 **Abstract**

25 The use of environmental DNA (eDNA) in biodiversity assessments offers a step-change in
26 sensitivity, throughput and simultaneous measures of ecosystem diversity and function.
27 There remains, however, a need to examine eDNA persistence in the wild through
28 simultaneous temporal measures of eDNA and biota. We used metabarcoding of two
29 markers of different lengths, derived from an annual time-series of aqueous lake eDNA to
30 examine temporal shifts in ecosystem biodiversity and in an ecologically important group of
31 macroinvertebrates (Diptera: Chironomidae). The analyses allow different levels of
32 detection and validation of taxon richness and community composition (β -diversity) through
33 time, with shorter eDNA fragments dominating the eDNA community. Comparisons
34 between eDNA, community DNA, taxonomy and UK species abundance data further show
35 significant relationships between diversity estimates derived across the disparate
36 methodologies. Our results reveal the temporal dynamics of eDNA and validate the utility of
37 eDNA metabarcoding for tracking seasonal diversity at the ecosystem scale.

38 **Introduction**

39 The maintenance of biodiversity underpins the stability of ecosystem processes in
40 constantly changing environments ¹. Consequently, biodiversity loss not only affects
41 ecosystem function and services, but also society as a whole ². One major impediment for
42 elucidating the relationship between biodiversity and ecosystem health is a need for robust
43 and detailed understanding of biodiversity processes and dynamics in time and space ³. To
44 halt or reverse contemporary species loss and habitat degradation, there is a need for
45 increasingly reliable and cost effective methods for biodiversity assessment, since widely
46 employed traditional approaches fall short in many cases ⁴. Currently, species identification
47 of individuals at immature life stages and among closely related species is difficult and
48 requires high-level, labour-intensive taxonomic expertise, thereby rendering large scale
49 ecosystem-wide assessments expensive, time consuming and potentially unrepresentative
50 of the ecosystem sampled ⁵. However, recent advances in molecular detection techniques,
51 most notably the application of environmental DNA (eDNA), offer exciting new
52 opportunities to improve existing biodiversity assessment procedures.

53 Environmental DNA (eDNA) is DNA extracted directly from an environment sample (e.g.,
54 water, soil or air), without prior isolation of the organisms themselves ⁶. Sources of eDNA
55 include sloughed skin cells, urine, faeces, saliva or other bodily secretions ⁷, and consist of
56 both free molecules (extracellular DNA) and free cells ⁸. Furthermore, eDNA collected from
57 water samples has highly sensitive detection capability and is non-invasive to the sampled
58 biota⁹, thereby potentially improving environmental management and assessment of
59 freshwater ecosystems ^{4,10}.

60 Previous work with eDNA of aquatic invertebrates is dominated by PCR-based approaches,
61 which are limited in assessing biodiversity^{11–13}. However, high throughput sequencing (HTS)
62 applications, such as metabarcoding, are already advancing prospects in ecology¹⁴, offering
63 comprehensive and efficient tools for measuring and assessing total biodiversity¹⁵. High
64 throughput sequencing has successfully been used for sequencing whole communities of
65 invertebrates (bulk samples)^{16–18}, though only a few studies have employed metabarcoding
66 of aqueous eDNA^{19,20}, and even fewer for invertebrates³. Most aqueous eDNA studies
67 have focused on macroorganisms, including fish and amphibians^{19–21}, with limited focus on
68 arthropods^{22,23}. Nevertheless, the combination of HTS and eDNA is poised to become a
69 prominent tool for ecosystem assessment^{10,22} by simultaneously assessing a plethora of
70 organisms, including associated organism interactions, with a throughput sufficient for rapid
71 whole community assessment.

72 Regardless of the increasing number of eDNA studies, several factors of eDNA research
73 demand clarification, including persistence of eDNA²⁴. Persistence of eDNA is the time that
74 eDNA remains detectable (e.g., in the water) after removal or loss of the organism from the
75 environment, which influences the timeframe for biodiversity assessment⁶. Investigating
76 the temporal relationship between community DNA²⁵ and eDNA is vital, since accurate
77 (extant) biodiversity assessment requires detection of contemporary, and ecologically
78 relevant, biodiversity. The persistence of eDNA for several different species has been
79 studied mainly in artificial systems, including aquaria and mesocosms^{6,11,22,26}. Notably,
80 persistence of short eDNA fragments, in artificial environments, was found to vary between
81 days to weeks after removal of the study organisms, depending upon biotic and abiotic
82 factors²⁷.

83 Species identity by eDNA is typically undertaken by detection of short DNA fragments ⁷, a
84 practise possibly influenced by ancient DNA work, which utilises highly fragmented DNA ²⁸.
85 For the detection of rare and evasive species, short DNA fragments might indeed increase
86 detection, although with some risk of errors if not properly analysed. Possible biases when
87 using short fragments include inadvertently sampling old eDNA fragments which have
88 demonstrated remarkable persistence ⁸, especially when bound to sediments where the
89 degradation rate is slower, due to protection of DNA molecules and inactivation of
90 extracellular nucleases ²⁷. Conversely, DNA fragments of several hundred base pairs length
91 are less likely to persist after release into the environment due to rapid degradation ²⁹ and
92 may represent a less abundant, but more contemporary, biodiversity signal ³⁰.

93 While the ecological value of collecting temporal data is established, most ecological studies
94 focus on spatial data ³¹. Similarly, many existing eDNA studies have focused on spatial
95 detection, such as early detection of invasive species ^{11,32} and presence of rare, or
96 endangered species ³³. Temporal estimates have been relatively neglected in eDNA studies
97 (but see ³³ for repeated seasonal sampling), and an understanding of temporal relationships
98 between eDNA and community biodiversity remains a key knowledge gap ³. Additionally
99 there are no published studies, to our knowledge, employing temporally collected data that
100 incorporate seasonal variation across an annual cycle from aqueous eDNA for ecosystem-
101 wide biodiversity level analysis.

102 Furthermore, overall ecosystem biodiversity characterisation, using indicator taxonomic
103 groups, can facilitate comparisons between taxonomically identified biodiversity over time
104 (e.g. collection of invertebrate samples) and eDNA detection. One such indicator group is
105 the Chironomidae or non-biting midges (Diptera: Chironomidae), which exhibit specialised

106 responses to ecological stressors and are acknowledged as one of the most informative
107 macroinvertebrate groups for monitoring lake ecosystem health ^{34,35} . Importantly, samples
108 can be collected after adult emergence in the form of shed skins of the pupae (pupal
109 exuviae) that float on the water surface. The exuviae technique allows for integrated
110 sampling of lake ecosystems from all aquatic microhabitats of the lake, and sample
111 identification can yield insights on ecosystem-wide biodiversity ³⁴ .

112 Accordingly, here we (a) investigate whether metabarcoding of lake eDNA is effective for
113 the detection of community diversity and temporal shifts in an ecologically important
114 sentinel group of macroinvertebrates, via comparison to the molecular and morphological
115 analysis of chironomid exuvial bulk samples; (b) investigate the use of eDNA analyses for
116 characterising whole-ecosystem biodiversity patterns; and (c) explore the effects of
117 amplicon length on detection of contemporary diversity. We show that freshwater lake
118 eDNA analyses capture seasonally coherent biodiversity patterns across the tree of life and
119 that shorter fragments of eDNA dominate natural ecosystems. Moreover, species incidence
120 measured by metabarcoding of eDNA and DNA derived from communities overlap
121 substantially with traditional taxonomic assessment. Collectively, we examine the ecological
122 relevance of eDNA by exploring mechanisms underpinning the temporal dynamics of eDNA
123 and the biological community at the ecosystem scale in nature.

124

125 **Results**

126 **Sequencing results.** After stringent filtering and quality control, 13,100,236 reads were
127 obtained for: (1) the full-length COI barcoding region (658bp) (amplicon COIF 6,659,598
128 reads) and (2) a 235bp fragment on the 5' region of the COI barcoding region (amplicon COIS

129 6,440,638 reads), from 32 samples comprising 16 eDNA and 16 invertebrate community
130 DNA samples. Data for these two amplicons were obtained from a larger dataset including
131 additional amplicon libraries, sequenced on two lanes of MiSeq. Overall, the eDNA samples
132 (extracted from filtered water samples) achieved good sequence coverage (mean number of
133 reads per sample (\pm SD): COIF: 269,769 \pm 57,427; COIS: 259,723 \pm 85,437) (for exact number
134 of reads per sample, see Supplementary Table 1). Some of the community DNA samples that
135 contained only small amounts of pupal exuviae resulted in a lower number of reads for both
136 amplicons.

137 **Control samples.** During PCR screening of negative controls, no band (no amplification) was
138 observed on agarose gels. Regardless of no visual proof of amplification, each sample was
139 sequenced and a very low number of reads was returned. After PCR and sequencing of the
140 negative control samples, COIS detected only two OTUs, which were BLAST-identified as
141 bacteria. For COIF, again only two OTUs were detected, identified as Gastropoda and
142 Diptera. The Gastropoda OTU was represented by 240 reads in one of the controls while the
143 Dipteran OTU was only represented by 10 reads in total across all types of negative controls.

144 The positive controls yielded good results for both amplicons, with 547,730 (COIS) and
145 393,341 (COIF) reads after quality control. Detection success was 100% for COIS (all 30
146 species detected) and 87% for COIF (26 species detected). Amongst the species that were
147 not detected was a mayfly species (*E. danica*), which also failed to amplify and sequence
148 during individual barcoding of specimens, using the Folmer primers (Supplementary Table
149 2). BLAST identification and screening of positive control reads resulted in >99.9% of the
150 reads being assigned to the target species known to be present in the positive control. The

151 relative abundance of OTUs found in the positive control which were attributed to non-
152 target taxa was 0.026% for the COIS and 0.007% for the COIF (Supplementary Table 3).

153 **Abundance filtering and rarefaction analysis.** Following investigations of how screening
154 different levels of abundance of rare OTUs affected overall OTU richness (including no
155 filtering, and removal of OTUs that were present at less than 0.01% and 0.02%), a filtering
156 level of 0.01% was set for all ecological analyses. Removal of OTUs present at less than
157 0.01% yielded equivalent levels of OTU genus richness for the community DNA (37 genera)
158 and eDNA (43 genera) according to year 2014 Chironomidae records of Llyn Padarn (31
159 genera) (Fig. 1). Furthermore, filtering of reads below 0.01% was within the limits of a small
160 number of non-target reads detected in the positive control samples. The genus richness
161 comparisons employed COIS data to ensure comparability between eDNA and community
162 DNA for the Chironomidae below. According to the analysis of OTU accumulation curves
163 (Supplementary Figs. 1 and 2) versus sequence coverage, a rarefaction depth of 57,869
164 reads was applied across all water samples (Supplementary Fig. 1a). To subsample Animalia
165 OTUs in our samples a rarefaction depth of 24,914 reads per sample was used
166 (Supplementary Fig. 1b).

167 **Total taxonomic diversity.** OTU clustering of the combined eDNA and community DNA
168 datasets at 97% similarity cut-off (after removal of low abundance OTUs) yielded: 442
169 (eDNA) and 309 (community DNA) OTUs for COIF, and 482 (eDNA) and 394 (community
170 DNA) OTUs for COIS. Taxonomic assignment through BLAST identified the majority of OTUs
171 from Animalia and Protista (Supplementary Fig. 3). From the eDNA samples, COIF identified
172 170 (35.3%) Animalia OTUs, of which 91 comprised Arthropoda (including 42 Insecta), whilst
173 COIS identified 251 Animalia OTUs (56.8%), of which 212 were Arthropoda (including 167

174 Insecta) (Supplementary Fig. 4). For the community DNA samples, COIF detected 219
175 (43.6%) Animalia OTUs, of which 171 were Arthropoda (including 132 Insecta), whilst COIS
176 recovered 227 (73.5%) Animalia OTUs, of which 212 consisted of Arthropoda (including 184
177 Insecta).

178 Although not the focus of the study, metabarcoding of the eDNA samples (COIS used here as
179 an example) also yielded matches to fish (*Phoxinus phoxinus*), amphibian and terrestrial
180 OTUs represented at high read frequencies or distributed across numerous independent
181 samples. Of the terrestrial taxa, spider OTUs from the Segestriidae (3,753 reads) and
182 Thomisidae (1,858 reads) families, a millipede OTU (7,312 reads), orthopteran OTU (14,237
183 reads) and 2,114 reads from domesticated cow (*Bos taurus*) were recovered from multiple
184 samples throughout the year, in addition to a broader diversity of terrestrial groups
185 represented at lower frequencies in the dataset.

186 **Temporal trends of OTU richness from eDNA samples.** Measures of OTU richness were
187 calculated exclusively for eDNA samples and plotted against time to detect possible
188 seasonal variations (Supplementary Fig. 5). All samples were rarefied at an equal depth
189 appropriate for each amplicon (total diversity dataset: 57,869 reads per sample, animal
190 diversity dataset: 24,914 reads per sample, for all water samples).

191 Mean Animalia richness for COIS (\pm SD) was 37.8 (\pm 10.4), and for COIF, 31.4 (\pm 11.4)
192 (Supplementary Fig. 5a). A significant correlation was detected (Spearman's correlation,
193 $p < 0.05$) between the OTU Animalia richness estimates derived from COIF with time and
194 temperature, but not with pH or dissolved oxygen (D.O.). Additionally, mean total richness
195 for COIS (\pm SD) was 73.1 (\pm 21.2), and for COIF, 88.1 (\pm 26.9) (Supplementary Fig. 5b). A
196 significant correlation was detected (Spearman's correlation, $p < 0.05$) between the COIF

197 (total richness), time, temperature and D.O., but not pH. No significant correlation was
198 found for COIS for the Animalia and total richness and any of the above parameters.

199 **Community structure (β -diversity) from eDNA samples.** We used eDNA samples to look
200 into possible changes in community structure over time, for the Animalia identified diversity
201 as well as the total diversity in the dataset. For the eDNA samples, nMDS analysis (Sørensen
202 index) of total diversity for both amplicons (Fig. 2), delimited patterns of seasonal variations
203 driving community composition with qualitatively higher temporal resolution recovered
204 from the smaller amplicon COIS. ANOSIM analyses also supported two main groupings,
205 'winter' (Nov-April) and 'summer' samples (April–Oct) (COIF: ANOSIM sig. level=0.1%, Global
206 R = 0.717, COIS: ANOSIM sig. level = 0.2%, Global R = 0.475, with outlying samples from
207 winter sampling). Additional analysis of the total diversity supports similar findings [two
208 main groupings: 'winter' (Nov-April) and 'summer' samples (April–Oct) (COIF: ANOSIM sig.
209 level=0.1%, Global R = 0.777, COIS: ANOSIM sig. level = 0.1%, Global R = 0.703)]
210 (Supplementary Fig. 6).

211 **Temporal trends in Chironomidae richness.** Analyses of un-trimmed COIF Chironomidae
212 data suggested that temporal richness patterns between eDNA and community DNA
213 samples were comparable (Spearman's correlation $p < 0.01$ between eDNA and community
214 DNA for COIF un-trimmed data) (Supplementary Fig. 7). Nevertheless, the sequencing
215 coverage of Chironomidae from the eDNA samples were approximately an order of
216 magnitude lower for COIF than for COIS (Supplementary Fig. 2). Subsequently, in order to
217 maintain a sufficient sequencing depth across samples, COIF was not retained for further
218 Chironomidae related analyses and rarefied incidence based data were used with 4,000
219 sequencing reads per sample, for COIS only (Supplementary Fig. 2).

220 For the Chironomidae assigned OTUs, COIS identified 103 OTUs from eDNA and 94 OTUs
221 from community DNA samples (138 unique OTUs in total). Using a combination of BLAST ID
222 $\geq 99\%$ and the online Barcode of Life Database (BOLD) species assignment tool³⁶, 73 OTUs
223 (53% out of 138 unique) were assigned species level taxonomic information. Analysis of
224 historical species occurrence data collected by the Environment Agency (EA) (summer
225 surveys 2003 – 2013) in Llyn Padarn (N. Wales, UK) indicated the presence of ≥ 99
226 Chironomidae species from 57 genera. Moreover, Fig.1 illustrates the qualitative overlap
227 between the number of chironomid genera delimited by the current community DNA (65%),
228 eDNA (61%) and taxonomy approaches. Similarly, see Supplementary Fig. 8 for overlap
229 between each method for the four summer time points used for analysis.

230 To visualise the empirically derived annual diversity patterns, OTU and genus richness was
231 assessed against time (Fig. 3) using a polynomial model. Observed OTU richness ranged
232 from 5-27 OTUs for eDNA and 1-27 OTUs for community DNA over time (Fig. 3a).
233 Conversely, genus level richness ranged from 5-19 for eDNA, 1-16 for community DNA. For
234 the data derived from taxonomic identification of invertebrate (exuviae) community
235 samples, genus level richness ranged from 10-18 (green points, restricted to 4 summer
236 sampling times) (Fig. 3b). Please also note that sampling points spanning the winter months
237 (days 36 -190), which did not yield data, represented samples which contained very low
238 physical numbers of exuviae. Consequently, they were not sequenced to an adequate depth
239 in a mixed Illumina sequencing library, and could not be retained for analysis.

240 Significant associations were detected between time and Chironomidae OTU and genera
241 richness derived from community DNA (OTU richness: polynomial regression, $R^2 = 0.890$, p-
242 value = 0.008; Genera richness: $R^2 = 0.849$, p-value = 0.017). However Chironomidae OTU

243 and genera richness derived from eDNA samples did not differ significantly over time (OTU:
244 polynomial regression $R^2 = 0.187$, p-value = 0.460; Genera: $R^2 = 0.128$, p-value = 0.635) (Fig.
245 3). Taxonomic richness (genus level) also did not differ significantly over the limited time
246 points available from seasonal sampling.

247 **Temporal variation of OTU Abundance.** We assessed the annual variation in OTU
248 abundance from metabarcoding sequencing reads between eDNA and community DNA
249 sampling methods using a generalized additive model (GAM). To allow across method
250 comparisons we compared OTU abundances for Chironomidae OTUs occurring in both
251 eDNA and community DNA datasets (45 OTUs). Abundances differed significantly among
252 different OTUs (GAM, $F = 4.688$, p-value <0.001) with a significant effect of the temporal
253 smoothing term (GAM, $F = 2.561$, $p = 0.047$) (Table 1). Additionally, abundances did not
254 differ significantly between methods (GAM, $F = 0.013$, p-value = 0.908), but a significant
255 OTU identity x method interaction (GAM, $F = 1.733$, p-value = 0.003) was found. The
256 abundance of OTU reads was also found to be significantly positively correlated with
257 expected species frequency (ranging from 0.01 to 0.79) across 97 sites in the United
258 Kingdom (UK) (two-way ANOVA, $R^2 = 0.087$, p-value = 0.003) (Table 1), using previously
259 catalogued Chironomidae species frequency data ³⁷ (Fig. 4).

260 **Discussion**

261 We present here one of the first temporal studies of aqueous eDNA and community DNA
262 biodiversity from a lake ecosystem, in addition to targeting a specific group of ecological
263 sentinel macroinvertebrates. In contrast to previous analyses that have used PCR (qPCR) to
264 infer presence/absence of a small number of target species (e.g. macroinvertebrates) from
265 eDNA samples ^{12,13}, we employed HTS of amplicon libraries (metabarcoding) to assess

266 temporal trends in total biodiversity. Such methodology allows for the characterisation of
267 the entire community, which is not possible through targeted individual-species sequencing
268 that employs taxon-specific primers. Simultaneously, we provide among the first accounts of
269 temporally collected biodiversity data from an annual series of eDNA samples compared
270 simultaneously with a series of invertebrate community DNA samples. Our findings yield an
271 informative characterisation of temperate lake ecosystem-wide biodiversity, through
272 detection of multiple groups of organisms from invertebrates to macro-organisms, of
273 primarily freshwater, but also terrestrial origins. Furthermore, the biodiversity of the
274 indicator taxon group used (Chironomidae) was successfully detected throughout the year,
275 from both eDNA and community DNA samples, exhibiting substantial overlap with
276 traditional taxonomic data. In addition, OTU sequence abundances were significantly
277 positively associated with expected chironomid species abundance based on UK taxa
278 occurrence data (Table 1, Fig. 4). Such direct coincidence, despite potential biotic and
279 abiotic variability in the release, transport and persistence of eDNA⁸, demonstrates the
280 value of eDNA metabarcoding for biodiversity characterisation and ecosystem monitoring³⁸.

281 Both metabarcoding amplicons detected large amounts of Animal phylum level diversity
282 from eDNA samples, showing broad representation across the freshwater taxonomic
283 biosphere, including the Arthropoda (Supplementary Fig. 4). Within the Arthropoda, the
284 dominance of Insecta, Maxillopoda and Malacostraca (Crustacea) also demonstrates the
285 utility of eDNA metabarcoding for characterisation of freshwater ecosystem-wide
286 biodiversity. There is increasing exposure of the use of eDNA metabarcoding for the
287 detection of fish and amphibians^{19,20}, as also recorded here. A more novel concept is the
288 ability of freshwater systems to integrate eDNA biodiversity information from terrestrial
289 sources. Terrestrial species found in our dataset, such as spider, millipede and orthopteran

290 species, or the ubiquitous *Bos taurus* (please also note, that no bovine serum products were
291 used in the HTS library preparations), are all commonplace in the surrounding area of the
292 study site and were detected by the analysis of eDNA residing in the lake water samples.
293 The ability of freshwater catchments to contain eDNA from broader habitat biodiversity
294 therefore presents an opportunity for further research regarding the relationship between
295 aqueous eDNA and biodiversity at the landscape scale.

296 Focusing on the Chironomidae richness estimates derived from the analysis of the short COI
297 fragment (Fig. 3), we can see that the COIS amplicon yielded 138 unique OTUs from both
298 sample types throughout the year. The analysis of the COIS amplicon therefore provided
299 valuable comparative qualitative and quantitative data both within the metabarcoding
300 datasets and between the historically collected data for Llyn Padarn and the rest of the UK
301 ³⁷. Other eDNA studies have focused mainly on macro-organisms such as fish or amphibians
302 whereby skin cells and mucus are a likely primary source of eDNA ⁸. While aquatic
303 invertebrates such as chironomids are individually typically much smaller, the accumulated
304 biomass of the community clearly produces sufficiently detectable and persistent amounts
305 of eDNA (from natural shedding, moulting and death) for meaningful biodiversity
306 assessment. Additional quantitative studies are required to determine the effects of
307 invertebrate community biomass on levels of eDNA in environmental samples ²¹.

308 Sequencing of the complete COI region (COIF ~658bp) from eDNA samples was successful in
309 detecting several genera of chironomids and provided biodiversity estimates comparable
310 with community DNA biodiversity patterns (Supplementary Fig. 7). However, it was not
311 possible to retain the COIF locus throughout all analyses after applying strict abundance
312 filtering of OTUs. Low sequence coverage of the COIF for the Chironomidae (primarily in the

313 water eDNA and not the community DNA samples, Supplementary Fig. 2) meant that more
314 robust, ecological comparisons were more effectively achieved using the short eDNA
315 fragment (COIS). Possible reasons for the discrepancies in coverage of the two amplicons
316 could be related to variations in primer specificity, with the COIS primers being more
317 successful than COIF primers in amplifying Chironomidae ³⁹ (please also see the limitations
318 of the Folmer COI barcoding primers for metabarcoding analyses in ⁴⁰). Nevertheless, we did
319 not detect substantial phylogenetic biases in OTUs recovered from the two primer pairs
320 (Supplementary Fig. 9) and coverage of the Chironomidae was only depleted in the water
321 eDNA samples for the COIF. Alternatively, the discrepancy in different amplicon success may
322 be due to the reduced availability of longer sized eDNA fragments in a natural ecosystem ³⁰.

323 After DNA is released into the environment, the degradation process likely begins, breaking
324 down DNA and yielding shorter fragments. It has been shown that ~400bp length fragments
325 remain detectable in water for days to weeks ^{6,11}, with the rate of degradation depending
326 upon various biotic and abiotic factors ²⁷. Overall, smaller fragments degrade slower
327 compared to longer fragments, suggesting an enhanced probability of detection by studies
328 targeting shorter DNA fragments ⁴¹. The present data support the enhanced detection of
329 shorter eDNA fragments, as evidenced by higher sequence coverage of the Chironomidae by
330 the shorter COIS amplicon in the water eDNA samples. Nevertheless, the data additionally
331 show that longer fragments are available at likely lower concentrations in the wild ³⁰
332 (represented by the COIF amplicon) (Supplementary Fig. 2). Using time vs. DNA
333 fragmentation as a working hypothesis for eDNA degradation, longer fragments are
334 predicted to represent more recently living cellular material. It is also therefore noteworthy
335 that among the water eDNA analyses, only the biodiversity delimited by the COIF amplicon
336 yielded significant associations with time/temperature (Spearman's correlation, $p < 0.05$)

337 (Supplementary Fig. 5), most likely representing more rapid breakdown of longer eDNA
338 fragments in the lake environment. Nevertheless, higher sequence coverage, or methods
339 that preferentially amplify longer amplicons, are needed to enhance amplification
340 probability for potentially smaller concentrations of longer eDNA fragments in natural
341 systems. Such solutions include the combination of multiple primer pairs¹⁷, or use of taxon
342 specific/blocking primers. Other suggested strategies for enhancing HTS of eDNA (where
343 concentrations are sufficiently high) involve direct shotgun sequencing or use of capture
344 probes ^{28,42}.

345 Amongst the concerns regarding the utility of eDNA to assess biodiversity, is whether or not
346 species detection represents living or recently living organisms, or communities of 'zombie'
347 DNA (i.e. historically distant DNA from organisms that previously lived in the ecosystem a
348 substantial time ago)³⁸. If eDNA exhibited long persistence times in the wild, temporal
349 patterns of β -diversity would be predicted to be extremely low (i.e., non-existent), especially
350 when derived from smaller fragments. However, here we have clearly shown that temporal
351 turnover (β -diversity) was observed for both the animal level (Fig. 2), and total diversity
352 derived eDNA biodiversity analysis (Supplementary Fig. 6), including temporal patterns of
353 seasonal biodiversity groupings over the year. Similar temporal results were observed for
354 both amplicons, with the short eDNA amplicon providing higher temporal resolution. Some
355 winter samples (Nov 25th and Dec 17th) in the COIS nMDS analysis displayed high levels of
356 β -diversity, since they either contained higher richness (Supplementary Fig. 5, days 57 and
357 79) or additional cohorts of taxa not present in the remaining samples (Supplementary Fig.
358 4). In the absence of technical artefacts, the additional turnover in β -diversity observed
359 could be the consequence of extreme storm events that coincided with the winter 2013-
360 2014 sampling ⁴³, inputting additional allochthonous eDNA from outside the study area. The

361 time points defining the separation of the two main seasonal biodiversity groups were
362 identified over November and late April, times which also correspond to water temperature
363 below 8 °C (winter samples) and above 10 °C (summer samples). Changes in observed
364 community composition (β -diversity) over April and November (Fig. 2, Supplementary Fig. 6)
365 most likely reflect seasonal turnover, possibly attributed to lake inversion effects⁴⁴. It is
366 known that changes in water temperature around these times of the year (Spring and
367 Autumn), can trigger the loss of water column stratification by mixing due to changes in
368 surface water temperature⁴⁴. Collectively, the demonstration of seasonal turnover of lake
369 eDNA β -diversity supports empirical studies using model ecosystems⁴⁴. Previous laboratory
370 and mesocosm studies have demonstrated the short-term temporal decay of eDNA in
371 artificial environments (e.g. 2-6 weeks)^{8,22,26} and the present data show that the eDNA signal
372 in the wild is of a contemporary nature.

373 Metabarcoding sequencing of invertebrate communities directly reveals the
374 presence/absence of living, or recently living communities²⁸. Hence, the insights provided
375 by community DNA samples here offered an essential benchmark to serve as a proxy for the
376 contemporary invertebrate community. The biodiversity estimates derived from
377 metabarcoding of the community DNA (Fig. 3, Supplementary Fig. 7, orange lines) matched
378 literature-based estimations of seasonal variation of Chironomidae for Northern
379 Hemisphere temperate latitudes⁴⁵, with a decrease in species richness over winter (often
380 represented by 'null' samples due to low numbers of collected exuviae) and a summer
381 increase related to rising water temperature (Fig. 3). Since the emergence patterns of
382 Chironomidae through the year are strongly related to changes in temperature and
383 photoperiod⁴⁵ (Supplementary Methods), rapid turnover in emerging communities are
384 apparent and can yield biased estimates of ecological status due to short-term shifts of

385 species emergence⁴⁶. One of the advantages of metabarcoding over traditional analysis is
386 the ability to analyse many samples simultaneously, and so using molecular approaches for
387 biodiversity assessment presents the opportunity to intensify ecological assessment and
388 derive greater precision in ecosystem health assessment³.

389 The companion analysis of the chironomid eDNA did not follow the expected emergence
390 pattern in richness, despite detecting Chironomidae turnover throughout the year from
391 community DNA samples (Fig. 3). The combination of the β -diversity turnover in eDNA
392 composition (Fig. 2), seasonally fluctuating community DNA richness (Fig. 3, orange lines)
393 and a lack of coherent seasonal shifts in eDNA richness (Fig. 3, blue line) thereby provides an
394 annual model of 'community DNA – eDNA' dynamics. The data thereby suggest that there
395 will likely be a standing resource of eDNA for biodiversity detection in lake ecosystems that
396 experience annual species turnover⁴⁴ (Fig. 2). Compositional turnover is thereby expected
397 to result from seasonal variation in species abundances, increasing sources of contemporary
398 eDNA, and environmental degradation decreasing levels of past eDNA accumulation.

399 Using GAM modelling facilitated comparison between read abundances of individual OTUs
400 derived from eDNA and community DNA analyses. Numbers of read abundances differed
401 between OTUs over time and between eDNA and community DNA abundances at the
402 individual OTU level (Table 1). There was also a significant positive association between the
403 abundance of sequencing reads derived from the present study and species frequency at
404 the national scale (Fig. 4). Therefore, lower abundance OTUs from the present study occur
405 at lower frequencies and more abundant OTUs are more common, according to an
406 extensive database of Chironomidae occurrence across the UK³⁷ (Fig. 4).

407 In combination, the analyses provide an overview of chironomid lake eDNA dynamics. Some
408 species will inevitably yield higher levels of eDNA than others, in relation to life history
409 stage, moulting rates/frequency, abundance, biomass, or cellular content/mitochondrial
410 densities^{3,7,8}. In addition, the relationship between eDNA and community DNA is affected by
411 biophysical characteristics and interactions between biotic and abiotic factors (e.g. microbial
412 activity, UV radiation and temperature) that affect persistence and degradation rates
413 throughout the year^{8,27}. Despite such dynamic interactions, numerous broad quantitative
414 associations have been reported for a range of taxa and their eDNA profiles, including data
415 from artificial, semi-natural and natural aquatic ecosystems⁴⁷⁻⁵². Here also, regardless of
416 which methodology was employed, metabarcoding of both eDNA and community DNA
417 reflected general Chironomidae species frequencies across the UK³⁷ (Fig. 4) and overlapped
418 with biodiversity estimates derived from taxonomy analyses (Fig. 1).

419 In summary, we have shown that eDNA from water samples collected consecutively over an
420 annual cycle in a lake ecosystem reveals ecologically representative species and community-
421 level shifts in diversity. Importantly, such patterns were validated both by independent
422 assessments of changes in physical presence in a key indicator group of macroinvertebrates,
423 as well as coinciding with established seasonal trends in indicator species emergence and
424 traditional taxonomy. Collectively, the findings address key outstanding questions related to
425 the ecological relevance and temporal persistence of freshwater eDNA in a natural
426 ecosystem, with significant implications for biomonitoring and the future investigation of
427 biodiversity ecosystem functioning relationships.

428 **Methods**

429 **Field sampling.** Samples (chironomid pupal exuviae and water samples) were collected
430 during Sept 2013 – Sept 2014 from Llyn Padarn, UK (Supplementary Methods), an
431 oligotrophic lake ecosystem located in Snowdonia National Park (53.130051, -4.135567), N.
432 Wales, UK (Supplementary Fig. 10) (Approximate surface area is 97.6 ha, maximum depth
433 27m). The site has been monitored regularly by the UK Environment Agency (EA), and more
434 recently by Natural Resources Wales (NRW) for indicator species of Chironomidae and other
435 invertebrate communities, providing important historical data. Two sites at opposite sides
436 of the lake were selected for sampling: Site 1 (S1: NW: 53.139106, -4.153975) and Site 2 (S2:
437 SW: 53.122414, -4.126761) (Supplementary Fig. 10). Using two locations increases potential
438 for species detection based on both eDNA and invertebrate sampling. Sampling was
439 conducted at approximately three-week intervals for 1 year (16 time points), using
440 standardised sampling methodology, and collecting simultaneously water and Chironomidae
441 samples. The two sites were sampled always in the same sequence (S1, then S2) between
442 8:30am–11:30am, including consecutive collection of water samples, invertebrate samples,
443 followed by water metadata (pH, Dissolved Oxygen (D.O.), conductivity and water
444 temperature), using a calibrated YSI Pro Plus multi-meter. As only water and exuviae (shed
445 skins) were collected and the work was performed in collaboration with the EA and NRW, a
446 permit was not required.

447 **Chironomid exuviae collection and eDNA filtration.** Invertebrate samples in the form of
448 chironomid exuviae (shed pupal skins) were collected using the field collection protocols for
449 the Chironomid Pupal Exuviae Technique (CPET)⁵³, with a 250µm mesh collection net
450 (Supplementary Methods). The floating insect skins were collected on the leeward side
451 (accumulation area) of each sampling site following described methods³⁴ and placed in a
452 sterile container. Upon returning to the lab, the sample was coarsely sorted to remove

453 excessive plant debris, fixed in 100% ethanol and stored at 4°C on the same day of
454 collection, until further processing.

455 For eDNA samples, one litre of surface water was collected using sterile glass Nalgene
456 bottles from each site, which was transferred on ice and placed at 4°C immediately after
457 return to the laboratory. Filtration was completed within 6 hours in a PCR-free separate
458 room. Sterilised, reusable funnel filtration units (Nalgene filter holders with funnel) were
459 used with 0.45µm cellulose nitrate filter membranes and a high-pressure vacuum pump.
460 The filter membranes were stored in sterile 15ml falcon tubes at - 80°C until DNA extraction.

461 **Equipment Sterilization and negative control samples.** All equipment was thoroughly
462 sterilized between sampling visits. The glass Nalgene bottles used for water collection,
463 filtration units and forceps would undergo consecutive cleaning rounds including wash and
464 overnight soak with 10% Trigene (Ammonium chloride & hydrochloride, Medichem Int.),
465 thorough rinse, UV treatment for 5 min and autoclaving. All additional equipment used for
466 invertebrate collection (net, meters, boots) was also thoroughly washed with 10% Trigene.
467 For eDNA extractions, single-use pre-sterilised scissors and forceps were used to handle the
468 filter membranes, and the exterior of storage tubes was wiped with 10% Trigene before
469 handling. During field surveys, to minimise cross contamination from consecutive sampling
470 points, the water samples were collected first, before any other samples or measurements
471 were taken and prior to invertebrate collection. **Negative controls** were collected by
472 filtration of 1 litre of distilled water through the filtration funnels and filter membranes
473 processed. Blank extractions of reagents (reagent controls) and filters (filter controls) were
474 also extracted with the same Phenol Chloroform extraction protocol (PCI) ⁵⁴. The negative
475 control equipment would undergo the same cleaning steps as stated above. All negative

476 controls were amplified with both primer pairs and MiSeq library preparation steps (as
477 below), and sequenced on an Illumina MiSeq.

478 **DNA extractions for eDNA filter membranes and invertebrate samples.** Environmental DNA
479 (eDNA) was extracted from the filter membranes, using a modified Phenol Chloroform
480 protocol (PCI), adapted from Renshaw et al ⁵⁴, with an added digestion step with the
481 addition of 20µl Proteinase K (20mg/µl) (Sigma – Aldrich) and incubation at 60°C for 1 hour.
482 This protocol was selected after rigorous in-house testing of available eDNA capture and
483 extraction protocols (Supplementary Methods). In Renshaw *et al.* ⁵⁴ it was demonstrated
484 that the latter protocol yielded the highest number of DNA copies of targeted eDNA
485 fragments. Furthermore, the combination of filtration and PCI has been shown to optimise
486 DNA yields, performing equally well in eukaryotes and prokaryotes, with enhanced
487 detection of diversity than other methods ⁵⁵. Two individual extractions were performed for
488 each sample, which were subsequently pooled. Extractions were performed in a different
489 building to PCR library construction where no invertebrate DNA had been handled
490 previously. Extracts were stored in a clean room with no post PCR processing.

491 DNA extraction from the bulk pupal exuviae samples (community DNA) was performed
492 using a modified QIAmp Blood Maxi Kit protocol, with an added Proteinase K overnight
493 incubation step. Due to seasonal variation of chironomid emergence ⁴⁵, the mass of the
494 collected invertebrate skin material varied, with some of the winter samples containing
495 smaller amounts of tissue. In order to optimise extraction efficiency, 1g of dry invertebrate
496 material was subsampled from large samples. Conversely, for some low-density winter
497 samples, 1g of exuviae was not available and so in these instances, the whole sample was
498 used for analysis. DNA extraction was performed in standard Qiagen Blood and Tissue kit

499 columns for small winter samples and QIAmp Blood Maxi Kit columns for all other samples
500 with an added 20µl Proteinase K (20mg/µl) overnight incubation step. Both kits are verified
501 by Qiagen to use the same chemistry and differ with respect to the use of columns of
502 different volume capacity to prevent clogging of the membrane. Following separation from
503 the ethanol preservative, the community samples were allowed to air-dry for approximately
504 1 hour and then were homogenised using a sterile mechanical drill and pestle. For detailed
505 information on each extracted sample, see Supplementary Tables 4 and 5.

506 **Primer selection and MiSeq Library preparation.** To fulfil the overarching aims of the study,
507 we required (a) metabarcoding primers that would amplify across a broad range of taxa (in
508 particular, lake occurring taxa), (b) a marker enabling the best annotation power for
509 macroinvertebrates and in particular, the Chironomidae, (c) a combination of two primer
510 pairs providing different length amplicons.

511 Accordingly, two amplicons of different sizes of the mitochondrial Cytochrome Oxidase I
512 gene (COI) were selected for sequencing. The full-length COI barcoding region (658bp),
513 using the universal Folmer primers LCO1490 - HCO2198⁵⁶ (amplicon COIF) and a 235bp
514 fragment (amplicon COIS) using the forward primer LCO1490 and the reverse COIA-R primer
515 (reversed forward COI-A primer by³⁹) (See Supplementary Table 6 for primer sequences).

516 Initially, the forward COI-A primer was designed by³⁹ specifically for amplification of
517 Chironomidae from environmental samples. Two Illumina MiSeq dual indexed amplicon
518 libraries were prepared using a two-step PCR protocol⁵⁷. The first round amplification was
519 performed using template-specific primers with 5' Illumina tails [TruGrade, by IDT,
520 Integrated DNA Technologies (Coralville, USA)], followed by Agencourt AMPure magnetic
521 bead purification. A second round amplification was performed using Illumina adapters with

522 8-nucleotide Nextera indexes (Supplementary Table 6). A 5N sequence was implemented
523 between the forward universal tail and the template specific primer, which is known to
524 improve clustering and cluster detection on MiSeq sequencing platforms⁵⁷. Using primers
525 with identical tails in the first step and indexed primers in the second, is a protocol
526 specifically developed by Illumina to reduce bias caused by variable index sequences in
527 mixed environmental samples^{58,59}.

528 Each sample was amplified in triplicate, the final products were pooled and purified with
529 AMPure beads and quantified using a dsQubit assay. Final library pooling was performed in
530 equimolar quantities for all samples. Sequencing was performed at the Liverpool Centre for
531 Genome Research, distributed across two independent lanes (for the COIS and COIF
532 amplicons) of Paired-end Illumina MiSeq (2x300) sequencing.

533 **PCR protocols for MiSeq Library Preparation.** PCRs were performed in 25µl reaction
534 volumes containing, for Round 1: 12.5µl Q5[®] Hot Start High-Fidelity 2X Master Mix, 10.5µl
535 PCR water, 0.5µl (10nmole/µl) of each forward and reverse primer and 1µl DNA (10ng/µl).
536 For Round 2: 12.5µl Q5[®] Hot Start High-Fidelity 2X Master Mix, 6.5µl PCR water, 0.5µl of
537 each forward and reverse primer and 5µl Purified PCR product from Round 1. The following
538 thermo-cycling parameters were used: Round 1: COIF: Denaturation at 98°C for 30 sec, 20
539 cycles of: 98°C for 10 sec, 46°C for 30 sec, 72°C for 40 sec, followed by a 10min extension at
540 72°C, hold at 4°C. COIS: Denaturation at 98°C for 30 sec, 20 cycles of: 98°C for 10 sec, 45°C
541 for 30 sec, 72°C 30 sec, followed by a 10min extension at 72°C, hold at 4°C. Round 2: both
542 amplicons: Denaturation at 98°C for 30 sec, 15 cycles of: 98°C for 10 sec, 55°C for 30 sec,
543 72°C for 30 sec, followed by a 10min extension at 72°C, cool at 4°C for 10min. Round 1 PCRs
544 were performed using Illumina-tailed primers and Round 2 using Illumina indexes.

545 **Positive control samples.** To account for efficiency of amplification protocols and
546 sequencing, a composite positive control sample comprising 30 invertebrate DNA extracts,
547 including Amphipoda, Coleoptera, Diptera, Ephemeroptera, Gastropoda, Hemiptera,
548 Isopoda and Trichoptera, was also amplified in triplicate with both primer pairs, and
549 sequenced alongside eDNA and community samples on MiSeq (Supplementary Table 2).

550 **Bioinformatics and statistical analysis.** Sequences, including positive and negative controls,
551 were de-multiplexed and Illumina adapters trimmed using Cutadapt⁶⁰ and Sickle⁶¹. A 10%
552 level of mismatch (2 bases) was allowed for primer removal. Filtering and quality control
553 were then performed using USEARCH v7⁶². Sequence quality was visualised using FastQC
554 (www.bioinformatics.babraham.ac.uk) and only sequences with a Phred quality score >25
555 were retained for analysis. Using USEARCH (fastq_maxee = 1) sequences with a maximum
556 expected error (maxee) > 1 were discarded. Maxee is the expected number of errors as sum
557 of the error probabilities (provided by Phred scores). Filtering was performed after merging
558 of R1 and R2 reads (minimum overlap 25bp), which allows recalculation of the error
559 probabilities for the combined sequences and increased accuracy. Sequences shorter than
560 100bp were discarded. The remaining sequences were de-replicated and sorted by cluster
561 size (cluster abundance) and sequences with <2 clusters (singletons) were removed. For the
562 COIF amplicon, the whole barcoding region was amplified and sequenced, but because of
563 the current limitations of MiSeq sequencing read lengths, only the forward reads (R1) were
564 used for analysis. Consequently, the per base quality drop expected in Illumina MiSeq data
565 at the tail of the forward reads was inspected in FastQC and all reads were truncated at
566 250bp and then quality filtered as above. Next, chimeras were removed (uchime_denovo)
567 using a *de novo* delimitation approach. An operational taxonomic unit (OTU) table was
568 created using OTU clustering at 97% similarity (USEARCH). Clustering at 97% similarity level

569 was chosen based on existing knowledge of intraspecific diversity for Chironomidae ³⁹, since
570 previous studies suggest that chironomid intraspecific diversity ranges between 0-4.2% ³⁹ or
571 0-4.9% ⁶³.

572 Taxonomy was then assigned to the OTU table using BLAST+ (megablast) ⁶⁴ against a
573 reference COI database. The reference library was compiled from NCBI GenBank, by
574 downloading all COI sequences, >100bp, excluding environmental sequences (20th June
575 2015, N = 807,388 sequences) and higher taxonomic level information was edited using the
576 GALAXY online software platform ⁶⁵. Taxonomic assignment of the OTU tables and
577 subsequent analysis was performed in QIIME ⁶⁶. All analyses involving USEARCH, QIIME and
578 BLAST+ were performed using the High Performance Computing (HPC) Wales systems.

579 Given the potentially sensitive nature of eDNA metabarcoding, low frequency sequences
580 can either represent less abundant taxa, or possible false positives and low level
581 contaminant OTUs ⁶⁷. In order to reduce the error associated with low frequency sequences,
582 and also focus analyses on predicted levels of richness ⁶⁸, we used two types of analysis.
583 First, we identified the frequency of potential contaminant reads in the positive control.
584 Second, we compared chironomid eDNA richness with variable levels of relative abundance
585 filtering (no filtering, 0.01% and 0.02%), against historical records of richness (genus level
586 only available) for Llyn Padarn (based on summer surveys for Llyn Padarn, 2003 – 2013).
587 Consequently, abundance filtering was performed on the OTU tables at the level that most
588 closely emulated expected chironomid richness and within the limits associated with
589 empirically observed low-level contamination in the sequencing dataset.

590 The validity of the Chironomidae OTUs identified by BLAST and retained after abundance
591 filtering was checked using a phylogenetic approach. The BLAST identified Chironomidae

592 OTUs were aligned with barcodes from 24 Chironomidae and 40 Trichoptera species
593 obtained herein, sequenced from UK samples using universal primers⁵⁶. Alignment, testing
594 for the presence of stop codon and insertions and bootstrapped phylogenetic tree
595 construction were performed in MEGA⁶⁹. Ultimately, only the OTUs that grouped closely
596 with known chironomid sequences on the phylogenetic tree were included in further
597 analysis.

598 For downstream analyses, the appropriate depth of coverage per sample was determined
599 according to OTU accumulation vs. sequence coverage curves generated in QIIME. Samples
600 were subsequently normalised using rarefaction in QIIME at appropriate depth for each
601 amplicon⁷⁰.

602 **Taxonomic identification of invertebrate community samples.** To provide a comparison
603 with community DNA and eDNA sequenced samples, chironomid exuviae community
604 samples from 4 time points (T10: April 30, T11: May 20, T14: July 23, T16: September 04)
605 were taxonomically identified according to standard CPET methodology used by the EA.
606 More specifically, 200 chironomid exuviae were subsampled from the total community
607 sample and identified to the highest possible level (genus or species) by specialised EA staff.
608 The results of the taxonomic identification were used to compare chironomid richness at
609 the genus level with metabarcoding-generated richness (see below).

610 **Calculation of diversity measures.** OTU richness (total diversity and Chironomidae diversity)
611 was calculated in QIIME. Furthermore, for Chironomidae with good taxonomic
612 identification, richness was also calculated at the genus level. To assess variation of richness
613 over time polynomial regression was performed using R version 3.2.4 (2016).

614 The PRIMER-E software ⁷¹ was used to calculate β -diversity based on the Sørensen index for
615 total diversity and Animalia only diversity detected from aqueous eDNA samples and for
616 Chironomidae OTUs for both sample types. Non-metric multi-dimensional scaling (nMDS)
617 and Hierarchical Clustering (HC) analysis were used to represent community similarity
618 between samples. Analysis of similarity (ANOSIM) was used to test for significant effects of
619 time in relation to community composition.

620 **Chironomidae OTU read abundance (eDNA vs community DNA).** In order to explore
621 relationships between the numbers of metabarcoding sequence reads, individual OTUs and
622 methodology (eDNA vs. community DNA), we used a generalized additive model (GAM),
623 with time as a smoothing term, using the R-package mgcv ⁷². In the GAM model, abundance,
624 calculated as total normalised reads per OTU and standardized per method (to allow for
625 across method comparison), was assessed in relation to OTU identity and method (eDNA vs
626 community DNA). Additionally, we assessed the ecological relationship between OTU
627 abundance (log transformed) in Llyn Padarn and species frequency (i.e. abundances derived
628 from ecological assessment) across the UK, by performing a two-way ANOVA, using the lm
629 function in R. UK species frequencies were derived from a Chironomidae inventory of 435
630 species across 220 UK lakes ³⁷. We restricted the species frequency data to 97 sites where
631 species frequency was inventoried at the national level and observed in this study.

632 **Data availability.** Sequencing data reported here have been deposited in GenBank
633 (Submission IDs: 1966226, 1966195) and the European Nucleotide Archive (ENA) (Accession
634 number: PRJEB13009).

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636

637 **References**

638

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835 **Author contributions**

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837 IB, SC, GRC: Designed experiment. IB: Performed lab work, fieldwork, bioinformatics and
838 statistical analysis. MS: Performed statistical analysis and data modelling, DL: Contributed in
839 optimisation of analytical pipelines. MH, MC, KW: Participated in experimental design. IB, SC,
840 GRC: Wrote manuscript. IB, SC, GRC, MS, KW, DL, and MH: Edited manuscript.

841

842 **Additional information**

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844 **Competing financial interests.** The authors declare no competing financial interests.

845

846 **Figures legends**

847 **Figure 1 | Venn diagram showing genera richness detected for all three methods.** Number
848 of Chironomidae Genera per sample type (purple: eDNA, orange: community DNA, green:
849 taxonomic identification) and the number of genera common between sample types (overlap
850 area). (Four time points, 45 Chironomidae genera).

851

852 **Figure 2 | Animal eDNA β -diversity – nMDS (Sørensen index).** a. COIF, b. COIS amplicon
853 (eDNA samples only) (N = 32). Solid green circles: 30% similarity cut-off (corresponding to
854 “winter” –“summer” groups), dashed blue circles: 40% similarity cut-off.

855

856 **Figure 3 | Richness patterns for Chironomidae OTUs and genera.** a: OTU richness. b: Genera
857 richness. Points represent richness values to individual sampling points for eDNA (blue),
858 community DNA (orange) and taxonomic identification of chironomid exuviae (green).
859 Sampling points spanning the winter months (days 36 -190) did not yield data due to very
860 low physical numbers of exuviae. Best fitted, significant lines from polynomial regressions
861 for eDNA samples (blue) and community DNA (orange), plotted against time (x –axis: Sep.
862 2013 – Sep 2014) (a.) eDNA: p-value = 0.46, community DNA: p-value= 0.008, (b.) eDNA: p-
863 value=0.017, community DNA p-value=0.635 (N = 29 datapoints).

864

865 **Figure 4 | Abundance patterns for Chironomidae.** The sequencing identified Chironomidae
866 OTUs plotted against species frequency across the UK, according to historical data.
867 Environmental DNA (eDNA) samples (blue) and community DNA (orange) are shown along
868 with the best fitted, significant, linear regression model (black line) ($R^2 = 0.087$, p-value=
869 0.003).

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874 **Tables**

875 **Table 1 | Sequence reads versus OTU and sampling method.** Generalized additive model
 876 (GAM), explaining OTU sequence abundance relative to OTU taxonomic ID (OTU) and
 877 sampling method (eDNA or community DNA - Method) over time. Model estimates and
 878 significances of the smoothing terms are given for the most parsimonious models. ($R^2 =$
 879 0.18, df: degrees of freedom, edf: estimated degrees of freedom).

880

	Df	F	p-value
OTU	44	4.688	<0.01
Method	1	0.013	0.908
OTU x Method	44	1.733	0.003

Approximate significance of smooth terms:	edf	F	p-value
s(Time)	2.899	2.561	0.047

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