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Environmental DNA metabarcoding

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Molecular Ecology Resources

DOI:

[10.1111/mec.14350](https://doi.org/10.1111/mec.14350)

Published: 01/11/2017

Peer reviewed version

[Cyswllt i'r cyhoeddiad / Link to publication](#)

Dyfyniad o'r fersiwn a gyhoeddwyd / Citation for published version (APA):

Deiner, K., Bik, H. M., Machler, E., Seymour, M., Lacoursiere-Roussel, A., Altermatt, F., Creer, S., Bista, I., & Lodge, D. M. (2017). Environmental DNA metabarcoding: transforming how we survey animal and plant communities. *Molecular Ecology Resources*, 26(21), 5872-5895. <https://doi.org/10.1111/mec.14350>

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1 **Environmental DNA metabarcoding: transforming how we survey animal and plant**
2 **communities**

3

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27 **Keywords:** Macro-organism, eDNA, species richness, bioinformatic pipeline, conservation,
28 ecology, invasive species, biomonitoring, citizen science

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31 **Running title:** Macro-organism eDNA metabarcoding

32 **Abstract**

33 The genomic revolution has fundamentally changed how we survey biodiversity on earth. High-
34 throughput sequencing ('HTS') platforms now enable the rapid sequencing of DNA from diverse
35 kinds of environmental samples (termed 'environmental DNA' or 'eDNA'). Coupling HTS with
36 our ability to associate sequences from eDNA with a taxonomic name is called 'eDNA
37 metabarcoding' and offers a powerful molecular tool capable of non-invasively surveying
38 species richness from many ecosystems. Here, we review the use of eDNA metabarcoding for
39 surveying animal and plant richness, and the challenges in using eDNA approaches to estimate
40 relative abundance. We highlight eDNA applications in freshwater, marine, and terrestrial
41 environments, and in this broad context, we distill what is known about the ability of different
42 eDNA sample types to approximate richness in space and across time. We provide guiding
43 questions for study design and discuss the eDNA metabarcoding workflow with a focus on
44 primers and library preparation methods. We additionally discuss important criteria for
45 consideration of bioinformatic filtering of data sets, with recommendations for increasing
46 transparency. Finally, looking to the future, we discuss emerging applications of eDNA
47 metabarcoding in ecology, conservation, invasion biology, biomonitoring, and how eDNA
48 metabarcoding can empower citizen science and biodiversity education.

49 **Introduction**

50 Anthropogenic influences are causing unprecedented changes to the rate of biodiversity
51 loss and, consequently, ecosystem function (Cardinale *et al.* 2012). Accordingly, we need rapid
52 biodiversity survey tools for measuring fluctuations in species richness to inform conservation
53 and management strategies (Kelly *et al.* 2014). Multi-species detection using DNA derived from
54 environmental samples (termed ‘environmental DNA’ or ‘eDNA’) using high-throughput
55 sequencing (‘HTS’) (Box 1), is a fast and efficient method to survey species richness in natural
56 communities (Creer *et al.* 2016). Bacterial and fungal taxonomic richness (i.e., richness of
57 microorganisms) is routinely surveyed using eDNA metabarcoding and is a powerful
58 complement to conventional culture-based methods (e.g., Caporaso *et al.* 2011; Tedersoo *et al.*
59 2014). Over the last decade, it has been recognized that animal and plant communities can be
60 surveyed in a similar fashion (Taberlet *et al.* 2012b; Valentini *et al.* 2009).

61 Many literature reviews summarize how environmental DNA (eDNA) can be used to
62 detect biodiversity, but they focus on single species detections, richness estimates from
63 community DNA (see Box 1 for definition for how this differs and can be confused with eDNA),
64 or general aspects of using eDNA for detection of biodiversity in a specific field of study (Table
65 S1). To compliment these many recent reviews, here we concentrate on four aspects: a summary
66 of eDNA metabarcoding studies on animals and plants to date, knowns and unknowns
67 surrounding the spatial and temporal scale of eDNA information, guidelines and challenges for
68 eDNA study design (with a specific focus on primers and library preparation), and emerging
69 applications of eDNA metabarcoding in the basic and applied sciences.

70

71 **Surveying species richness and relative abundance with eDNA metabarcoding**

72 Conventional physical, acoustic, and visual-based methods for surveying species richness
73 and relative abundance have been the major ways we observe biodiversity, yet they are not
74 without limitations. For instance, despite highly specialized identification by experts, in some
75 taxonomic groups identification errors are common (Bortolus 2008; Stribling *et al.* 2008).
76 Conventional physical methods can also cause destructive impacts on the environment and to
77 biological communities (Wheeler *et al.* 2004), making them difficult to apply in a conservation
78 context. Furthermore, when a species' behavior or size makes it difficult to survey them (e.g.
79 small bodied or elusive species), conventional methods can require specialized equipment or
80 species-specific observation times, thus making species richness and relative abundance
81 estimates for entire communities intractable (e.g., many amphibians and reptiles, Erb *et al.* 2015;
82 Price *et al.* 2012). These reasons highlight the continued need to develop improved ways to
83 survey global biodiversity, and the unique ways eDNA metabarcoding can complement
84 conventional methods.

85 *Species richness: eDNA metabarcoding compared with conventional methods*

86 Environmental DNA metabarcoding can complement (and overcome the limitations of)
87 conventional methods by targeting different species, sampling greater diversity, and increasing
88 the resolution of taxonomic identifications (Table 1). For example, Valentini *et al.* (2016)
89 demonstrated that, for many different aquatic systems, the number of amphibian species detected
90 using eDNA metabarcoding was equal to or greater than the number detected using conventional
91 methods. When terrestrial hematophagous leeches were used as collectors of eDNA (blood of
92 hosts), endangered and elusive vertebrate species were detected using eDNA metabarcoding and
93 served as a valuable complement to camera trap surveys in a remote geographic region (Schnell
94 *et al.* 2015b). In plants, Kraaijeveld *et al.* (2015) demonstrated that eDNA metabarcoding of

95 filtered air samples allowed pollen to be identified with greater taxonomic resolution relative to
96 visual methods.

97 The ways that eDNA can complement and extend conventional surveys are promising,
98 but the spatial and temporal scale of inference is likely to differ between conventional and
99 molecular methods. For example, in a river Deiner *et al.* (2016) showed on a site by site basis
100 that the eDNA metabarcoding method resulted in higher species detection compared to a
101 conventional physical-capture method (i.e., kicknet sampling) (Table 1). However, eDNA in
102 this case may have detected greater species richness at a site not because the species themselves
103 are present, but rather because their DNA has been transported from another location upstream,
104 creating an inference challenge in space and time for eDNA species detections. Therefore,
105 research is needed to understand the complex spatiotemporal dynamics of the various eDNA
106 sample types (Fig 1), which at present we know very little about. In addition, all sampling
107 methods have inherent biases caused by their detection probabilities. Detection probabilities
108 often vary by species, habitat, and detection method (e.g., the mesh size of a net or a primer's
109 match to a target DNA sequence) and use of bias-corrected species richness estimators will be
110 important to account for these biases when conducting statistical comparisons between the
111 outcomes in measured richness (Gotelli & Colwell 2011; Olds *et al.* 2016).

112 Future methodological comparisons could also benefit from a quantitative ecological
113 approach in the design of sampling by matching sample effort and scope of sampling between
114 eDNA and conventional methods. Multimethod species distribution modeling or site occupancy
115 modeling is one example for how this can be achieved and has been demonstrated in cases
116 comparing qPCR for a single species and conventional methods (Hunter *et al.* 2015; Rees *et al.*
117 2014a; Schmelzle & Kinziger 2016; Schmidt *et al.* 2013), but rarely for eDNA metabarcoding

118 (Ficetola *et al.* 2015). Thus, we expect the robustness of eDNA metabarcoding to reveal species
119 richness estimates for animals and plants will be improved by coupling distribution or occupancy
120 modeling with studies to determine the scale of inference in space and time for an eDNA sample
121 (Fig. 1).

122 *Species relative abundance: eDNA metabarcoding compared with conventional methods*

123 Estimating a species' relative abundance using eDNA metabarcoding is an intriguing
124 possibility. Here we focus on the evidence from animals in aquatic systems. Controlled studies
125 based on detection of a single animal species in small ecosystems, such as in aquaria and
126 mesocosms (e.g., Minamoto *et al.* 2012; Moyer *et al.* 2014; Pilliod *et al.* 2013; Thomsen *et al.*
127 2012a), in natural freshwater systems (e.g., Doi *et al.* 2017; Lacoursière-Roussel *et al.* 2016a)
128 and marine environments (Jo *et al.* 2017; Yamamoto *et al.* 2016) demonstrate that eDNA can be
129 used to measure relative population abundance with a species specific primer set and qPCR.
130 While many more controlled experiments are needed in all ecosystems to determine the
131 relationship of abundance to copy number observed in qPCR, evidence thus far from water
132 samples signifies that eDNA contains information about a species' relative abundance.

133 Overall, ascertaining abundance information using metabarcoding of eDNA for whole
134 communities still lacks substantial evidence, but some studies in aquatic environments have
135 shown positive relationships with between the relative number of reads and relative or rank
136 abundance estimated with conventional methods. Evans *et al.* (2016) showed in a mesocosm
137 setting that relative abundance of individuals and biomass was correlated with relative read
138 abundance in mesocosms containing fishes and an amphibian. In a natural lake, Hänfling *et al.*
139 (2016) found that the rank abundance derived from long-term monitoring was correlated with
140 read abundance for fish species, and positively correlated with gillnet surveys conducted at the

141 same time as eDNA sampling. In deep sea habitats, Thomsen *et al.* (2016) found that when reads
142 for fish were pooled to the taxonomic rank of families, there was a correlation with relative
143 abundance of individuals and biomass captured in trawls. While these examples are promising,
144 not all studies support such findings (e.g., Lim *et al.* 2016).

145 Challenges to accurate abundance estimation through eDNA metabarcoding stem from
146 multiple factors in the field and the lab (Kelly 2016). In the field, the copy number of DNA
147 arising from an individual in an environmental sample is influenced by the characteristics of the
148 ‘ecology of eDNA’ (e.g., its origin, state, fate, and transport) (Barnes & Turner 2016). Because
149 different animal and plant species are likely to have different rates of eDNA production or
150 ‘origin’ (Klymus *et al.* 2015), exhibit different ‘transport’ rates from other locations (Civade *et*
151 *al.* 2016; Deiner & Altermatt 2014), or stability or ‘fate’ of eDNA in time (Bista *et al.* 2017;
152 Yoccoz *et al.* 2012), eDNA in an environmental sample could be inconsistent relative to a
153 species’ true local and current abundance. Therefore, continued research on how the origin, state,
154 fate, and transport of eDNA influences estimates of relative abundance is needed before we can
155 understand the error this may generate in our ability to estimate abundance.

156 In the lab, primer bias driven by mismatches with their target have been shown to skew
157 the relative abundance of amplified DNA from mock communities (Elbrecht & Leese 2015;
158 Piñol *et al.* 2015). Similarly, the same mechanism could alter the relative abundance of a species’
159 DNA amplified from eDNA (Fig. 2). Primer bias results in an increased variance in abundance
160 of reads observed relative to their true abundance in an environmental sample (Fig.2). Another
161 source of error is related to library preparation methods. Analysis of mock communities has
162 shown that amount of subsampling during processing steps can drive the loss of rare reads
163 (Leray & Knowlton 2017) and likely occurs for eDNA samples as well (Shelton *et al.* 2016). The

164 combination of primer bias and library preparation procedures alone could cause a large variance
165 in reads observed for any given species and could prevent rare species detection altogether (Fig
166 2). Technical approaches and potential solutions to alleviate primer bias and alternative library
167 preparation methods are discussed in the “Challenges in the field, in the laboratory, and at the
168 keyboard” section. While in the end, it may be that eDNA metabarcoding is not the most
169 accurate method for simultaneously measuring the relative abundance for multiple species from
170 eDNA, researchers should consider whether the eDNA metabarcoding method is accurate
171 enough for application in a particular study or an applied setting. Other methods such as capture
172 enrichment are being examined and are promising because they avoid PCR and hence the bias
173 this may cause, but they do require extensive knowledge of the biodiversity to design targeted
174 gene capture probes and they come with a greater costs for analysis (Dowle *et al.* 2016). Future
175 studies comparing qPCR, eDNA metabarcoding, and capture enrichment will be beneficial to
176 determine which method yields accurate estimates of relative abundance from eDNA.

177 Before ruling out the plausibility entirely, in the short term, simulations could certainly
178 be used to test the effects of technical laboratory issues and account for the ecology of eDNA to
179 decipher under what conditions reliable estimates for abundance can be achieved from eDNA
180 metabarcoding. Promising steps in this direction have been investigated through simulation to
181 learn the nature of how datasets deliberately “noised” conform to neutral theory parameters in
182 estimation of rank abundance curves (Sommeria-Klein *et al.* 2016). Results from simulations
183 studies such as this could then be used to inform mock community experiments and test
184 hypotheses (e.g., type of error distribution expected) under realistic semi-natural environments.

185

186 **Ecosystems, their sample types and known scales of inference in space and time**

188 Environmental DNA metabarcoding of different sample types has been highly successful
189 in obtaining species richness estimates for animals in aquatic systems (Fig.1, Table 1). In one of
190 the first seminal studies, Thomsen and colleagues (2012a) used surface water from lakes, ponds,
191 and streams in Denmark to demonstrate that eDNA contained information about aquatic
192 vertebrate and invertebrate species known from the region. However, there are a notable lack of
193 eDNA metabarcoding studies assessing living aquatic plant communities, and this remains an
194 open area for further research.

195 Mounting evidence suggests that the spatial and temporal scale of inference for eDNA
196 sampled from surface water differs for rivers and lakes (Fig. 1). Specifically, river waters
197 measure species richness present at a larger spatial scale (Deiner *et al.* 2016) compared to eDNA
198 in lake surface waters (Hänfling *et al.* 2016). Differences between lake and river eDNA signals
199 may be due to the transport of eDNA over larger distances in rivers compared to longer retention
200 times of water in lake systems (Turner *et al.* 2015). However, lakes and ponds with river and
201 surface runoff inputs, combined with lake mixing or stratification, may serve as eDNA sources
202 for catchment level terrestrial and aquatic diversity estimates similar to rivers (Deiner *et al.*
203 2016). No studies to date have estimated the sources of eDNA in surface water from a lake's
204 catchment and related it to the diversity locally occurring in the lake. However, ancient DNA
205 from sediment cores in lakes (sedaDNA) has been used to determine historical plant (e.g., Pansu
206 *et al.* 2015b; Parducci *et al.* 2013) and livestock communities (Giguet-Covex *et al.* 2014), thus
207 indicating that lakes do receive DNA from species in their catchments which can be incorporated
208 into their sediments. For a more extensive review of sedaDNA being used to reconstruct past
209 ecosystems see Pederson *et al.* (2015) and Brown and Blois (2016).

210 Most often, species richness estimates generated from eDNA in surface waters of lakes
211 and rivers reflects recent site biodiversity, while those from eDNA found in surface sediments
212 may reflect a temporally extended accumulation of eDNA. For example, Shaw *et al.* (2016)
213 compared estimates of fish species richness from water and surface sediment samples. Generally
214 they found species were detected in both samples, but estimates of species richness from water
215 samples were in better agreement with the species physically present at the time of sampling.
216 The temporal scale of inference in surface sediments is largely unknown and needs further
217 examination (Fig. 1).

218 In addition to surface freshwater (~1%), groundwater (~30%) and ice (~69%) comprise
219 much of Earth's freshwater (Gleick 1993). While the other freshwater habitats far surpass the
220 amount of surface water, their extant biodiversity is rather poorly described (Danielopol *et al.*
221 2000). Groundwater is known to harbor a wide range of specialist taxa which are difficult to
222 assess using conventional survey methods due to the inaccessibility of these habitats (Danielopol
223 *et al.* 2000). Groundwater micro-organism metabarcoding studies have shown high fungal
224 (Sohlberg *et al.* 2015) and bacterial (Kao *et al.* 2016) diversity, and there are examples of
225 species-specific studies on the cave-dwelling amphibian *Proteus anguinus* (e.g., Gorički *et al.*
226 2017; Vörös *et al.* 2017). However, there is a clear lack of eDNA metabarcoding studies that
227 could shed light on the diversity of a wide range of macro-organisms known to inhabit
228 groundwater; including turbellarians, gastropods, isopods, amphipods, decapods, fishes and
229 salamanders. The spatiotemporal scale of inference of eDNA samples from groundwater is
230 currently unknown. Surveying eDNA in systems with knowledge of the complex hydrology and
231 interactions between surface and ground water will be interesting places to start to reveal the
232 scale of inference for eDNA surveys for these environments.

233 Environmental DNA found in sediment cores and ice core sediments generally reflects a
234 historical biodiversity sample (Fig. 1) and is more commonly used as a source of ancient DNA
235 (Willerslev *et al.* 2007). To date animal and plants surveyed from lake sediment cores suggest
236 that information about terrestrial and aquatic communities can be estimated as far back as 6 to
237 12.6 thousand years before present (Giguet-Covex *et al.* 2014; Pedersen *et al.* 2016), whereas
238 eDNA from sediments in ice cores have successfully been used to reconstruct communities as far
239 back as 2000 years before present (Willerslev *et al.* 1999). The spatial scale of inference for
240 sediment samples types has not been tested, but when samples from multiple locations are
241 combined, large areas can be surveyed for the past presence of species (Anderson-Carpenter *et*
242 *al.* 2011). For modern communities, snow has served as a viable sample type and enabled a local
243 survey of wild canids in France (Valiere & Taberlet 2000). Environmental DNA metabarcoding
244 of water from glacial runoff will also likely be a valuable tool to survey animal and plant
245 richness living in glacial and subglacial habitats, which are undergoing dramatic change because
246 of climate warming (Giersch *et al.* 2017).

247 *Marine ecosystems*

248 The use of eDNA metabarcoding is often described as challenging in marine ecosystems,
249 due to the potential dilution of eDNA in large volumes of water and additional abiotic factors
250 (salinity, tides, currents) that likely impact eDNA transport and degradation (Foote *et al.* 2012;
251 Port *et al.* 2016; Thomsen *et al.* 2012b), not to mention the logistics involved in undertaking
252 such surveys. Nonetheless, eDNA metabarcoding surveys of marine fish from coastal water
253 samples have demonstrated that eDNA can detect a greater taxonomic diversity compared to
254 conventional survey techniques (Table 1), while simultaneously improving detection of rare and
255 vagrant fish species, and revealing cryptic species otherwise overlooked by visual assessments

256 (O'Donnell *et al.* 2017; Port *et al.* 2016; Thomsen *et al.* 2012b; Thomsen *et al.* 2016). Marine
257 mammals have been surveyed with acoustic surveys and eDNA metabarcoding, and here the
258 conventional acoustic methods detected a greater species richness (Foote *et al.* 2012).
259 Nevertheless, this study used low sample volumes compared to other marine studies (15 – 45 mL
260 vs. 1.5 – 3.0 L) and the authors concluded that larger sample volumes would likely lead to
261 greater similarity between eDNA and conventional methods. In Monterey Bay, California, water
262 sampled from depths less than 200 m or greater 200 m were used to detect marine mammals such
263 as seals, dolphins, and whales in addition to many fishes and sharks (Andruszkiewicz *et al.*
264 2017). The taxonomic groups detected were spatially explicit and were found more or less in
265 water associated with their expected habitat.

266 Longitudinal transport of animal and plant eDNA in marine environments is not well
267 studied. But, similar to freshwater sediment cores from lakes, vertical transport into marine
268 sediments is likely to preserve a large proportion of eDNA from particulate organic matter or
269 eDNA that has become directly adsorbed onto sediment particles. This absorption shields
270 nucleotides from degradation (particularly oxidation and hydrolysis) and facilitates long-term
271 preservation of genetic signals over potentially large spatiotemporal scales (Fig. 1). Marine
272 sediment eDNA concentrations have been shown to be three orders of magnitude higher than in
273 seawater eDNA (Torti *et al.* 2015) and eDNA from both ancient and extant communities is
274 typically recovered (Lejzerowicz *et al.* 2013). Similar to lake sediments, marine sediments can
275 accumulate genetic information from both terrestrial and pelagic sources (Torti *et al.* 2015).

276 Marine sediments are difficult to sample because of the logistical effort involved in
277 obtaining samples, which often requires ship time and specialized coring equipment. Even
278 though much work remains to be done to understand the spatiotemporal scale of inference for

279 marine sediment cores, comparisons between eDNA and environmental RNA (eRNA)
280 metabarcoding are hypothesized to allow inference between present and past diversity.
281 Environmental RNA is thought to be only available from live organisms in the community, thus
282 the comparison between eDNA and eRNA has been investigated. In applied settings, eDNA
283 metabarcoding of surface sediments has revealed benthic impacts of aquaculture for Atlantic
284 salmon farming on short spatial scales using both eDNA and eRNA (Pawlowski *et al.* 2014).
285 Guardiola *et al.* (2016) showed through a comparison of eDNA and eRNA that spatial trends in
286 species richness from these two sources were similar, but that eDNA detected higher diversity.
287 Overall, the fate, transport, and decomposition of animal and plant eDNA in marine
288 environments is poorly known compared to other environments, and there is pressing need for
289 further studies.

290 *Terrestrial and aerial ecosystems*

291 Environmental DNA from terrestrial sediment cores is a valuable tool for investigating
292 past environments and reconstructing animal and plant communities (Fig. 1, Haouchar *et al.*
293 2014; Jørgensen *et al.* 2012; Willerslev *et al.* 2003). Animal remains also provide opportunities
294 to reconstruct past trophic relationships. For example, eDNA metabarcoding of pellets in
295 herbivore middens have been used to identify species in ancient animal and plant communities
296 (Fig. 2, Murray *et al.* 2012) and DNA traces from microplant fossils within coprolites were used
297 to reconstruct former feeding relationships in rare and extinct birds (Wood *et al.* 2012). Again
298 here, the recent reviews of Brown & Blois (2016) and Pedersen *et al.* (Pedersen *et al.* 2015)
299 provide a more extensive overview for how ancient DNA is used to uncover past animal and
300 plant communities.

301 In modern environments, eDNA isolated from top soils has been used to characterize
302 biodiversity in earthworms (Bienert *et al.* 2012; Pansu *et al.* 2015a), invertebrates (McGee &
303 Eaton 2015), plants (Taberlet *et al.* 2012c; Yoccoz *et al.* 2012) and vertebrate species (Andersen
304 *et al.* 2012). In what is perhaps the most comprehensive analysis using eDNA metabarcoding for
305 any environment, Drummond *et al.* (2015) simultaneously surveyed all three domains of life in
306 top soil using PCR primers that amplified five different metabarcoding regions, thus
307 demonstrating the power of this method for assessing total richness for an area. However, the
308 spatial scale of inference for many terrestrial eDNA samples is an open question (Fig. 1).
309 Research on the time scale of inference for DNA in top soil suggests that long fragments of DNA
310 break down quickly, but short fragments remain detectable for days to years after the presence of
311 the species (Taberlet *et al.* 2012c; Yoccoz *et al.* 2012). Thus, the fragment length amplified can
312 change the temporal resolution of a soil sample.

313 There are many additional sources for eDNA sampling besides soil in terrestrial
314 ecosystems. For animals, blood meals from leeches (Schnell *et al.* 2012) and carrion flies
315 (Calvignac-Spencer *et al.* 2013) have been used to survey mammal diversity. Saliva on browsed
316 twigs was tested as a source of eDNA to survey ungulates (Nichols *et al.* 2012) and on predated
317 eggs and carcasses of ground-nesting birds to discover predators or scavengers (Hopken *et al.*
318 2016). DNA extracted from spider webs has also been used to detect spiders and their prey (Xu
319 *et al.* 2015). For plants, pollen within honey has revealed honey bee foraging preferences (De
320 Vere *et al.* 2017; Hawkins *et al.* 2015). Craine *et al.* (2017) surveyed dust from indoor and
321 outdoor environments throughout the United States and found that plant DNA from known
322 allergens was almost twice as high outdoor compared with indoor environments. In addition to
323 allergen detection from pollen, there remain many potential applications of dust eDNA to assess

324 animal species richness. Fecal DNA has also been used as a source of eDNA to assess diet
325 composition, but most studies utilizing this source of eDNA are focused on single species
326 detections and population genetic inferences (see review from Rodgers & Janečka 2013) and are
327 not necessarily using eDNA sources from fecal DNA to estimate species richness of terrestrial
328 communities. Boyer *et al.* (2015) proposed that surveys of feces from generalist predators can act
329 as ‘biodiversity capsules’ and analysis of this eDNA source should give rise to biodiversity
330 surveys for prey communities in landscapes. While all of these sources are available, most of
331 these sample types (e.g., leaves from a tree, fecal pellets, spider webs, and dust) do not have a
332 known scale of inference in space and time. A single sample of eDNA from these sources is not
333 likely to confirm species richness for more than a local scale, but combination of multiple sample
334 sources (e.g., leaves, fecal pellets, and spider webs throughout a park) and sampled over time
335 may allow for spatial and temporal estimates of terrestrial species richness.

336 Surveys of airborne eDNA have placed greater emphasis on the detection of bioaerosols
337 that cause infection or allergic responses in animals and plants (West *et al.* 2008). For example,
338 Kraaijeveld (2015) investigated airborne pollen that can cause hay fever and asthma in humans
339 and showed that the source of allergenic plant pollen could be identified more accurately using
340 eDNA from plant pollen filtered from the air compared to microscopic identification. A
341 particularly interesting area for further research is to gain an understanding of the scale of
342 inference for air samples in space and time (Fig. 1). While plant eDNA can be ascertained,
343 surveying other species such as birds and insects from aerial eDNA sources has not been tested
344 to our knowledge.

345

346 **Challenges in the field, in the laboratory, and at the keyboard**

347 Despite the obvious power of the approach, eDNA metabarcoding is affected by a host of
348 precision and accuracy challenges distributed throughout the workflow in the field, in the
349 laboratory, and at the keyboard (Thomsen & Willerslev 2015). Following study design (e.g.,
350 hypothesis/question, targeted taxonomic group, *etc.* Fig. 3), the current eDNA workflow consists
351 of three components: field, laboratory, and bioinformatics. The field component consists of
352 sample collection (e.g., water, sediment, air) that is preserved or frozen prior to DNA extraction.
353 The laboratory component has four basic steps: 1) DNA is concentrated (if not done in the field)
354 and purified, 2) PCR is used to amplify a target gene or region, 3) unique nucleotide sequences
355 called ‘indexes’ (also referred to as ‘barcodes’) are incorporated using PCR or are ligated onto
356 different PCR products, creating a ‘library’ whereby multiple samples can be pooled together, 4)
357 pooled libraries are then sequenced on a high-throughput machine (most often the Illumina
358 HiSeq or MiSeq platform). The final step after laboratory processing of samples is to
359 computationally process the output files from the sequencer using a robust bioinformatics
360 pipeline (Fig. 3, Box 2). Below we emphasize the important and rapidly evolving aspects of the
361 eDNA metabarcoding workflow and give recommendations for ways to reduce error.

362 *In the field*

363 As for any field study, the study design is of paramount importance (Fig. 3, Box 2), since
364 it will impact the downstream statistical power and analytical interpretation of any eDNA
365 metabarcoding dataset. For example, sampling effort and replication (especially biological), are
366 positively correlated with the probability of detecting the target taxa (Furlan *et al.* 2016;
367 Willoughby *et al.* 2016). Despite the extensive evidence of the occurrence of macro-organism
368 DNA in the environment, our fundamental understanding of what ‘eDNA’ is from any
369 environmental sample is still lacking. For an illustration of this challenge, we summarize what is

370 known about eDNA in freshwater environments. The current state-of-the-art relies on the fact
371 that we can access eDNA by precipitating DNA from small volumes of water samples (e.g., 15
372 mL, Ficetola *et al.* 2008), or filter eDNA from the water column using a variety of filter sizes
373 (0.22 μm and upwards) (Rees *et al.* 2014b). Filtration protocols lead to a working hypothesis that
374 aqueous eDNA is either derived from cellular or organellar sources (e.g., mitochondria,
375 Lacoursière-Roussel *et al.* 2016b; Turner *et al.* 2014; Wilcox *et al.* 2015), and precipitation
376 protocols suggest extracellular sources (Torti *et al.* 2015). It is clear that at least some freshwater
377 eDNA comes from intact cellular or organellar sources because it has recently been
378 demonstrated to be available in the genomic state (Deiner *et al.* 2017b). Thus, eDNA in water
379 exists in both un-degraded and degraded forms (Deiner *et al.* 2017b). However, continued
380 research on the origin, state, and fate of eDNA will greatly inform numerous strategies regarding
381 its acquisition (filtering, replication, sample volumes and spatial sampling strategies) (Barnes &
382 Turner 2016). Many methods for solving current challenges of false negatives (e.g., use of
383 biological replicate sampling, improved laboratory methods) and false positives (e.g., use of
384 negative controls) in the field are explored in a recent review (Goldberg *et al.* 2016), we
385 therefore refer readers to this review rather than treat those topics in-depth here.

386

387 *In the laboratory*

388 There are a number of recent studies that focus on the capture, preservation, and
389 extraction of eDNA and the literature reviewed therein summarizes the important considerations
390 and trade-offs that should be tested before a large scale study is conducted (e.g., Deiner *et al.*
391 2015; Renshaw *et al.* 2015; Spens *et al.* 2017). Rather than reiterate those aspects here, we focus
392 on primer choice and library preparation. For animal and plant studies, PCR primers most often

393 target mitochondrial or plastid loci or nuclear ribosomal RNA genes (Table S2). The standard
394 barcoding markers defined by the Consortium for the Barcode of Life (CBOL) are Cytochrome c
395 oxidase subunit I (COI or *cox1*), for taxonomical identification of animals (Hebert *et al.* 2003),
396 and a 2-loci combination of *rbcL* and *matK* as the plant barcode (Hollingsworth *et al.* 2009) with
397 ITS2 also suggested as valid plant barcode marker (Chen *et al.* 2010). However, there are
398 limitations for using the standard barcoding markers in macro-organism eDNA metabarcoding.
399 Specific to COI, other DNA regions are commonly used because not all taxonomic groups can be
400 differentiated to species equally well (Deagle *et al.* 2014) and because it is challenging to design
401 primers in this gene for a length that is suitable for short amplicon analysis, but some regions
402 have been identified (Leray *et al.* 2013). The most common alternative markers used are
403 mitochondrial ribosomal genes such as 12S and 16S or protein coding genes such as Cytochrome
404 B (Table S2). Specific to the plant barcoding loci, the 2-loci primarily used for barcoding plants
405 can be independently generated, but is not always possible to recover which fragment from each
406 gene is associated with each other in an eDNA sample; rendering species identification using the
407 standard plant barcode challenging. Bioinformatic methods can help resolve these situations to
408 some extent, and may work when diversity is low in a sample (Bell *et al.* 2016). Therefore, often
409 one or different markers are used (e.g., P6 loop of the *trnL* intron (Sørenstebø *et al.* 2010; Taberlet
410 *et al.* 2007)) (Table S2).

411 Additionally, some highly-evolving non-coding loci, such as ITS rRNA, are used (Table
412 S2), but these markers do not always allow for the construction of alignments to determine
413 MOTUs during data analysis because they have intragenomic variation that complicates their use
414 in biodiversity studies (plant ITS rRNA may be an exception (Bell *et al.* 2016)). For these loci,
415 an unknown environmental sequence is often discarded unless it has an exact database match

416 reducing a dataset to only known and sequenced biodiversity. Due to these factors, other
417 metabarcoding loci such as 18S rRNA genes may be more appropriate (e.g., in studies of marine
418 invertebrates, Bik *et al.* 2012), especially if phylogenetic analysis is needed to narrow down
419 taxonomic assignments and circumvent database limitations (Box 3).

420 Once the locus or loci are chosen, primers are then designed based on the taxonomic
421 group(s) of interest within a study, and the need for broad (multiple phyla) vs. narrow (single
422 order) coverage to test study-specific hypotheses (Fig. 3). When choosing previously designed
423 primers (Table S2) or when designing new primers it is important to perform rigorous testing, *in*
424 *silico*, *in vitro* and *in situ* to infer their utility for metabarcoding eDNA in a new study system
425 (Elbrecht & Leese 2017; Freeland 2016; Goldberg *et al.* 2016). Amplicon size is also an
426 important consideration because there may be a trade-off in detection with amplicon length (e.g.,
427 short fragments are more likely to amplify). However, short fragments may persist longer in the
428 environment and increase the inference in space or time that can be made from an environmental
429 sample (Bista *et al.* 2017; Deagle *et al.* 2006; Jo *et al.* 2017; Yoccoz *et al.* 2012). Additionally,
430 use of more than one locus for a target group can allow for tests of consistency between loci and
431 increase stringency of detection for any species (Evans *et al.* 2017).

432 Once primers are designed and PCR products are amplified, eDNA metabarcoding relies
433 on multiplexing large numbers of samples on HTS platforms in order to make the tool cost
434 effective. Illumina (MiSeq and HiSeq) sequencing platforms at the moment outperform other
435 models for accuracy (Loman *et al.* 2012) and multiplexing samples is usually achieved by the
436 incorporation of sample-specific nucleotide indices and sequencing adapters during PCR
437 amplification. However, multiplexing creates opportunities for errors and biases. In this facet of
438 the workflow it is important to avoid methods that induce sample specific biases in amplification

439 (O'Donnell *et al.* 2016) and to reduce the potential for index crossover, or “tag jumping” (see
440 Box 2) (Schnell *et al.* 2015a). To address these issues, Illumina has developed a two-step PCR
441 protocol using uniformly tailed primers across samples for the first step and sample specific
442 indexes for the second PCR, which could reduce bias related to index sequence variations (Berry
443 *et al.* 2011; Miya *et al.* 2015; O'Donnell *et al.* 2016). Regardless of the strategy employed
444 extreme care is needed to ensure primer quality control (e.g., both use of small aliquots from
445 stocks as well as proper cleaning of PCR amplified products to remove indexing primers after
446 amplification (Schnell *et al.* 2015a). When a species detection is suspected as highly unlikely in
447 a sample, single-species quantitative PCR (qPCR) can be used to verify its presence from the
448 same eDNA sample because qPCR does not suffer from the same technical sources of error.
449 Additional suggestions for dealing with multiplexing artifacts are suggested in Box 2 under
450 “abundance filtering”.

451 In addition, both positive and negative controls must be used in the lab to ensure sample
452 integrity (Fig. 3). Use of positive control samples (either from pooled DNA extracts derived from
453 tissue at the PCR stage, or used at the extraction stage alongside that of eDNA samples) can help
454 evaluate sequencing efficiency and multiplexing errors in the eDNA metabarcoding workflow
455 (Hänfling *et al.* 2016; Olds *et al.* 2016; Port *et al.* 2016). Careful thought in the construction of
456 the mock community is needed. Typically, species not expected in the study area are used (Olds
457 *et al.* 2016; Thomsen *et al.* 2016) such that if there is contamination during the workflow their
458 reads can be identified, removed and serve as a control for detecting contamination when it
459 occurs.

460 Negative controls should be introduced at each stage of lab work (i.e., filtration - if done
461 in the lab, extraction, PCR, and indexing). We recommend that an equivalent amount of

462 technical replication should be used on negative and positive controls as that carried out on
463 actual samples (Ficetola *et al.* 2015). Furthermore, it is becoming important that negative
464 controls are sequenced regardless of having detectable amounts of DNA because contamination
465 can be below detection limits of quantification and sequences found in these controls can be used
466 to detect de-multiplexing errors or used in statistical modeling to rule out false positive
467 detections (Olds *et al.* 2016).

468 Finally, an important but often neglected consideration for the eDNA metabarcoding
469 workflow, is the identification of technical artifacts that arise independently of true biological
470 variation. For example, recently in a study focused on bacterial biodiversity using the 16S locus
471 it was shown that a run effect can be confounded with a sample effect if it is not accounted for
472 (e.g., by splitting sample groups across multiple Illumina runs, Chase *et al.* 2016); however, it
473 remains to be seen whether such technical artifacts are also prevalent for loci used for
474 metabarcoding plant and animals from eDNA (COI, 18S, ITS, etc.) and more research is needed.
475 Until then, careful thought into how samples are pooled and run on a sequencer seems warranted
476 in order to not confound the hypotheses being tested.

477 *At the keyboard*

478 Bioinformatic processing of high throughput sequence datasets requires the use of UNIX
479 pipelines (or graphical wrappers of such tools, Bik *et al.* 2012). Metabarcoding of animal and
480 plant community DNA is comprehensively outlined in Coissac *et al.* (2012). Below and in Box 3
481 we highlight the common practices to community DNA metabarcoding and deviations for studies
482 focusing on macro-organism eDNA metabarcoding.

483 Bioinformatic pipelines and parameters must be carefully considered (Box 2) and it is
484 important to work with a knowledgeable computational researcher to understand how processing

485 can impact the biological results and conclusions. Before computationally processing an eDNA
486 metabarcoding dataset, perhaps the strongest message from Coissac *et al.* (2012) is to identify
487 the differences between the analysis of data derived from microbial and macro-organismal
488 groups. Since microbial ecologists have been inspired to use sequence-based identification of
489 taxa over the past 40 years (Creer *et al.* 2016), the range of software solutions to analyze
490 microbial metabarcoding datasets is unsurprisingly extensive (Bik *et al.* 2012). Perhaps more
491 importantly, a number of established and maintained databases exist featuring many of the
492 commonly used microbial taxonomic markers for prokaryotes (Cole *et al.* 2009), microbial
493 eukaryotes (Guillou *et al.* 2013; Pruesse *et al.* 2007; Quast *et al.* 2012) and fungi (Abarenkov *et*
494 *al.* 2010), meaning that microbial datasets can be analyzed and taxonomic affiliations established
495 are established in a straight forward way.

496 For macro-organism communities, pre-processing and initial quality control of eDNA
497 metabarcoded data sets is not different from that of microbial datasets and can be acquired using
498 packages developed either for microbial (Caporaso *et al.* 2010), or macro-organism data (Boyer
499 *et al.* 2016), but taxonomic assignment will require a robust dataset of locus-specific reference
500 sequences and the associated taxonomic data from a reference database (Coissac *et al.* 2012)
501 (Box 3). Currently the two most common reference sources for macro-organisms are NCBI's
502 nucleotide database (Benson *et al.* 2013) and the Barcode of Life Database (Ratnasingham &
503 Hebert 2007). The utility and taxonomic breadth of these databases can be enhanced by the
504 creation of custom-made or hybrid databases, with the obvious additional workload and cost
505 depending on the number of focal taxa missing from current data sources. Recently, Machida *et*
506 *al.* (2017) have assembled and proposed metazoan mitochondrial gene sequence datasets that can
507 be used for taxonomic assignment for environmental samples. While these datasets do not

508 account for future growth, their methods could be repeated at the time of any new study to
509 generate a custom reference dataset for taxonomic assignment.

510 Macro-organism eDNA metabarcoding datasets are associated with advantages compared
511 to microbial datasets because the number of taxa in any survey will be comparatively low,
512 reducing the computational time needed for taxonomic annotation. Moreover, the species
513 delimitation concepts and taxonomic markers associated with macro-organisms are well-
514 developed (de Queiroz 2005) and can even be used to analyze population genetic structure
515 (Sigsgaard *et al.* 2016; Thomsen & Willerslev 2015), or delimit species boundaries (Coissac *et*
516 *al.* 2012; Hebert *et al.* 2003; Tang *et al.* 2014). Reliance on the vast knowledge we have for
517 animal and plant taxonomy and biogeography is a distinct advantage for eDNA metabarcoding
518 because of the independent test that it provides to calibrate and test the tool for its precision and
519 accuracy (Deiner *et al.* 2016).

520 *Data archiving for transparency*

521 As eDNA applications continue to develop, all procedures used in the field, lab, and
522 during bioinformatic data processing require a strong commitment to transparency on the part of
523 researchers (Nekrutenko & Taylor 2012). Here, we outline best practices for eDNA
524 metabarcoding studies of macro-organisms, following on from well-established standards in the
525 fields of microbiology and genomics (Yilmaz *et al.* 2011). First, raw FASTQ files from any
526 HTS run need to be submitted to the Sequence Read Archive (SRA) of NCBI or the European
527 Nucleotide Archive (ENA) and other such public national data bases before publication.
528 Archiving raw data in publicly available databases is common practice in virtually all genomics
529 and transcriptomic studies because it allows studies to be re-analyzed with new computational
530 tools and standards. In fact, archiving raw data is becoming increasingly mandatory at many

531 evolutionary and ecology biology journals, inclusive of Molecular Ecology. Second, researchers
532 should adhere to minimum reporting standards defined by the broader genomics community,
533 such as the MIMARKS (Minimum information about a marker gene sequence) and MIxS
534 (minimum information about any “x” sequence) specifications (Yilmaz *et al.* 2011). Goldberg *et*
535 *al.* (2016) have made specific recommendations for upholding these reporting standards specific
536 to eDNA studies (see Table 1 in Goldberg *et al.* 2016).

537 Third, computational processing of data needs to be reproducible (Sandve *et al.* 2013).
538 For eDNA metabarcoding studies, it is increasingly common to deposit a comprehensive sample
539 mapping file (e.g., formatted in the QIIME tab-delimited style, containing the indexes used for
540 creating libraries so that raw data can be de-multiplexed and properly trimmed) along with
541 MOTU clustering or taxonomic binning of results, and documentation of all bioinformatics
542 commands, in a complementary repository such as Dryad (<http://datadryad.org/>), GitHub
543 (<https://github.com/github>), or FigShare (<http://figshare.com>). Sandve *et al.* (2013) provide 10
544 rules that can be followed to ensure such reproducibility, and we strongly encourage researchers
545 using eDNA metabarcoding methods to uphold these practices and take advantage of archiving
546 intermediate steps (Box 2) of their analysis for full transparency.

547

548 **Emerging applications for eDNA metabarcoding**

549 *Applications in ecology*

550 Quantifying the richness and abundance of species in natural communities is and will
551 continue to be a goal in many ecological studies. Information about species richness garnered
552 from eDNA is not necessarily different from conventional approaches (Table 1), but the scale,
553 speed, and comprehensiveness of that information is (Fig. 4). For example, Drummond *et al.*

554 (2015) demonstrated the near-complete analysis of biodiversity (e.g., from bacteria to animals
555 and plants) from top soil is possible. Collection of data on this taxonomic scale opens up new
556 opportunities with respect to measuring community composition and turnover across space and
557 time. In addition to estimating species richness, a major area of research in ecology is
558 determining whether observed community changes surpasses acceptable thresholds for certain
559 desired ecosystem functions (Jackson *et al.* 2016). Biodiversity and ecosystem functioning
560 research requires tracking species in multiple taxonomic groups and trophic levels, along with
561 changes in ecosystem function. Environmental DNA metabarcoding has the potential to facilitate
562 biodiversity and ecosystem function research by improving our knowledge of predator/prey
563 relationships, mutualisms such as plant-pollinator interactions, and food webs in highly diverse
564 systems composed of small cryptic species (e.g., De Vere *et al.* 2017; Hawkins *et al.* 2015; Xu *et*
565 *al.* 2015). Knowledge of species co-occurrences and interactions in these instances will
566 additionally foster the study of meta ecosystems and provide data to guide management
567 decisions at the ecosystem scale (Bohan *et al.* 2017). What will remain challenging is moving
568 beyond richness estimates to also obtaining species abundance data (Fig. 2 & 4).

569

570 *Applications in conservation biology*

571 Given the rapid rate at which biodiversity is declining worldwide (Butchart *et al.* 2010), it
572 is critical that we improve the effectiveness of strategies to halt or reverse this loss (Thomsen &
573 Willerslev 2015; Valentini *et al.* 2016). Accordingly, developing tools that enable rapid, cost-
574 effective and non-invasive biodiversity assessment such as eDNA metabarcoding, especially for
575 rare and cryptic species, is paramount (Fig. 4). Improved estimates of the distribution of
576 vulnerable species, and done so non-invasively, would facilitate policy development and allow

577 for efficient targeting of management efforts across habitats (Kelly *et al.* 2014; Thomsen &
578 Willerslev 2015). For example, documenting the presence of threatened species in a habitat can
579 trigger a suite of actions under laws pertaining to biodiversity conservation (e.g., US Endangered
580 Species Act). Frequently, data relevant to policy are derived from monitoring efforts mandated
581 by environmental laws imparting a significant consequence to the data collected (Kelly *et al.*
582 2014).

583 Environmental DNA-based monitoring is likely to be a tremendous boon to often
584 underfunded public agencies charged with compliance to data-demanding laws. Specifically,
585 eDNA metabarcoding will be useful for monitoring communities when many species are of
586 conservation concern. Vernal pools throughout California are a prime example because they
587 contain 20 US federally listed endangered or threatened species of plants and animals.
588 Monitoring species richness with soil and water samples from a habitat such as this would
589 provide a comprehensive sampling method to ascertain needed community data for their
590 conservation and management (Deiner *et al.* 2017a). However, while eDNA metabarcoding may
591 be important for non-invasively gaining access to the distribution of vulnerable species, it cannot
592 be used to differentiate between alive and dead organisms or estimate many demographic
593 parameters important of population viability analysis (Beissinger & McCullough 2002).

594 Quantifying baselines of animal and plant species richness and departures from those
595 baselines, is central to the assessment of environmental impact and conservation (Taylor &
596 Gemmell 2016). The application of eDNA metabarcoding methods to different samples types,
597 which taken together allow inference across time (e.g., surface water and sediment layers from a
598 core in a lake, Fig. 1) provides a unique tool to document local extinctions and long-term
599 changes in ecosystems. Extinction models often rely on and understanding extinction timelines

600 (reviewed in Thomsen & Willerslev 2015). The efficiency of eDNA metabarcoding to track the
601 timing of extinctions associated with previous glacial events has been demonstrated in mammals
602 (Haile *et al.* 2009) and plants (Willerslev *et al.* 2014). Thus, environmental DNA metabarcoding
603 of different sample types from the same site offers an excellent opportunity to better understand
604 the extinction consequences of perturbations and could inform scenario modeling under climate
605 change.

606

607 *Applications in invasion biology*

608 Because one of the first applications of eDNA to macro-organisms was the detection of
609 North American bullfrogs in French ponds (Ficetola *et al.* 2008), the method immediately came
610 to the attention of researchers interested in invasion biology (e.g., Egan *et al.* 2013; Goldberg *et*
611 *al.* 2013; Jerde *et al.* 2011; Takahara *et al.* 2013; Tréguier *et al.* 2014). These initial studies, as
612 well as much ongoing research, continue to be based on species-specific primers, where positive
613 amplification provides occurrence evidence for a particular invasive species. In invasion biology
614 with eDNA, such a targeted approach is referred to as “active” surveillance (Simmons *et al.*
615 2015).

616 On the contrary, eDNA metabarcoding makes it possible to detect the presence of many
617 species simultaneously, including species not previously suspected of being present. This
618 broader untargeted approach is called “passive” surveillance in management applications (Fig. 4)
619 (Simmons *et al.* 2015). On the down side, due to a trade-off in primer specificity, we expect that
620 eDNA metabarcoding may be less sensitive in detecting some species or that the detection rate of
621 a species can change depending on species richness. Adopting a dual approach of passive and
622 active surveillance could be considered in cases where the risk of a new invasion is high, and

623 where cost effective eradication plans for undesirable species are likely to be successful (Lodge
624 *et al.* 2016).

625 Avoiding future introductions and reducing the spread of exotic species is paramount in
626 natural resource policy (Lodge *et al.* 2016). Environmental DNA metabarcoding relevant to
627 management includes early detection of incipient invasive populations in the environment,
628 surveillance of invasion pathways, e.g., ballast water of ships (Egan *et al.* 2015; Zaiko *et al.*
629 2015), and the live bait trade (Mahon *et al.* 2014). While eDNA metabarcoding is not yet
630 routinely used for biosecurity regulation of invasive species or enforcement in many settings, it
631 has the potential to become valuable monitoring tool for biological invasions. An important
632 challenge for the use of eDNA metabarcoding in invasive species detections are false positives
633 and false negatives since both outcomes can trigger action or inaction when not required, causing
634 a potentially large burden on entities responsible for invasive species mitigation and control (Fig.
635 4). Therefore, continued research to reduce or understand the nature of false positives and false
636 negatives will reduce uncertainty in the tool and facilitate greater adoption.

637

638 *Applications in biomonitoring*

639 Pollution of air, water, and land resources generated from processes such as urbanization,
640 food production, and mining is one of the many emerging global challenges we are facing in the
641 21st century (Vörösmarty *et al.* 2010). Determine the origin, transport, and effects of most
642 pollution is challenging because it accumulates through both point sources (e.g., wastewater
643 effluent) and diffused sources related to land-use types (e.g., agriculture or urbanization). In this
644 context, the presence of tolerant or absence of sensitive organisms has been used to determine
645 the consequences of pollution on ecosystem health throughout the world and is termed biological

646 monitoring or ‘biomonitoring’ (Bonada *et al.* 2006). The extent to which animals and plants have
647 been used in biomonitoring depends on the unique characteristics of the taxonomic group
648 monitored and their relationship to the pollution of interest (Bonada *et al.* 2006; Stankovic *et al.*
649 2014). Most biomonitoring programs take community composition and often abundance of taxa
650 into account and calculate what is known as a biotic index (Friberg *et al.* 2011). Biotic indices
651 take many forms and are typically surrogates for the impacts of pollution (e.g., SPEAR index for
652 toxicant exposure in water, Liess *et al.* 2008).

653 Applying eDNA metabarcoding in the context of biomonitoring is a major avenue of
654 research. Metabarcoding of community DNA samples has shown greater sensitivity for detecting
655 cryptic taxa or life stages and can alleviate the problem of identifying damaged specimens of
656 which render morphological tools ineffective (Gibson *et al.* 2014; Hajibabaei *et al.* 2011). These
657 two issues alone are known to create large variances in biotic index estimation (Pfrender *et al.*
658 2010). Application of eDNA metabarcoding to animals and plants used in biomonitoring requires
659 in-depth testing of conventional survey methods and eDNA-based approaches (Fig. 4), to
660 understand whether species richness estimates derived from the two methods result in a similar
661 measure for the biotic index of interest or whether new biotic indices need to be development
662 that can simultaneously consider both forms of information. Promising steps forward are being
663 made through the DNA AquaNet COST Action (<http://dnaqua.net/>) which is a consortium of
664 over 26 European union countries and four international partners working together to develop
665 genetic tools for bioassessment of aquatic ecosystems in Europe (Leese *et al.* 2016).

666

667 *Applications in citizen science and biodiversity education*

668 The simplicity of the protocol used to collect environmental samples has created an
669 avenue for citizen scientist programs to be built around surveying for biodiversity using eDNA
670 (Biggs *et al.* 2015). With the development of sample kits from commercial companies
671 specifically used for eDNA analysis (e.g., GENIDAQS, ID-GENE, Jonah Ventures,
672 NatureMetrics, Spygen) there now exists a novel opportunity to engage the public in biodiversity
673 science, which could accompany already established biodiversity events, such as BioBlitz
674 (National Geographic Society). Use of eDNA metabarcoding in this context will likely provide
675 an unprecedented tool for education and outreach about biodiversity, and increase awareness
676 about its decline. Challenges that hinder integration of eDNA metabarcoding in citizen science
677 projects and educational opportunities are the time and costs needed to process samples and user
678 friendly data visualization tools to allow exploration of the data once provided. Thus, finding
679 ways to cut costs and speed up data generation (a goal common for any application of the tool),
680 as well as creation of applications for exploration of data on smart phones and desktops alike is
681 needed to propel the use of eDNA applications in citizen science and education.

682

683 **Conclusions**

684 As the tool of eDNA metabarcoding continues to develop, our understanding regarding
685 the analysis of eDNA from macro-organismal communities, including optimal field, laboratory,
686 and bioinformatics workflows will continue to improve in the foreseeable future. Concurrently,
687 we need to gain a better understanding of the spatial and temporal relationship between eDNA
688 and living communities to improve precision, accuracy, and to enhance the ecological and policy
689 relevance of eDNA (Barnes & Turner 2016; Kelly *et al.* 2014). Ultimately, the errors and
690 uncertainties associated with eDNA metabarcoding studies can often be mitigated by thoughtful

691 study design, appropriate primer choice, and robust sampling and replication: as Murray et al.
692 (2015) emphasize, “no amount of high-end bioinformatics can compensate for poorly prepared
693 samples, artefacts or contamination.”

694 Over time, a loop in which improved eDNA metabarcoding methods reduce uncertainty
695 about the meaning of both positive and negative eDNA detections for a species will in turn
696 generate the motivation for continued improvements and use of eDNA metabarcoding methods.
697 Thus, resulting in the adoption of eDNA metabarcoding as a comparable method for estimating
698 species richness. We predict that over the next decade eDNA metabarcoding of animals and
699 plants will become a standard surveying tool that will complement conventional methods and
700 accelerate our understanding of biodiversity across the planet.

701 **Box 1: Community DNA versus environmental DNA metabarcoding of plants and animals**

702

703 **Terms:**

704 **Environmental DNA (eDNA).** DNA captured from an environmental sample without first
705 isolating any target organisms (Taberlet *et al.* 2012a). Traces of DNA can be from feces, mucus,
706 skin cells, organelles, gametes or even extracellular DNA. Environmental DNA can be sampled
707 from modern environments (e.g., seawater, freshwater, soil or air) or ancient environments (e.g.,
708 cores from sediment, ice or permafrost (e.g., cores from sediment, ice or permafrost, see
709 Thomsen & Willerslev 2015).

710 **Community DNA.** DNA is isolated from bulk-extracted mixtures of organisms separated from
711 the environmental sample (e.g., soil or water).

712 **Macro-organism environmental DNA.** Environmental DNA originating from animals and
713 higher plants.

714 **Barcoding.** First defined by Hebert *et al.* (2003), the term refers to taxonomic identification of
715 species based on single specimen sequencing of diagnostic barcoding markers (e.g., COI, *rbcL*).

716 **Metabarcoding.** Taxonomic identification of multiple species extracted from a mixed sample
717 (community DNA or eDNA) which have been PCR amplified and sequenced on a high
718 throughput platform (e.g., Illumina, Ion Torrent).

719 **High Throughput Sequencing (HTS).** Sequencing techniques which allow for simultaneous
720 analysis of millions of sequences compared to the Sanger sequence method of processing one
721 sequence at a time.

722 **Community DNA metabarcoding:** HTS of DNA extracted from specimens or whole organisms
723 collected together, but first separated from the environmental sample (e.g., water or soil).

724 **Molecular Operational Taxonomic Unit (MOTU):** Group identified through use of cluster
725 algorithms and a predefined percent sequence similarity (e.g., 97%) (Blaxter *et al.* 2005).

726

727 Since the inception of High Throughput Sequencing (HTS, Margulies *et al.* 2005), the
728 use of metabarcoding as a biodiversity detection tool has drawn immense interest (e.g., Creer *et al.*
729 *et al.* 2016; Hajibabaei *et al.* 2011). However, there has yet to be clarity regarding what source
730 material is used to conduct metabarcoding analyses (e.g., environmental DNA versus community
731 DNA). Without clarity between these two source materials, differences in sampling, as well as
732 differences in lab procedures, can impact subsequent bioinformatics pipelines used for data
733 processing, and complicate the interpretation of spatial and temporal biodiversity patterns. Here
734 we seek to clearly differentiate among the prevailing source materials used and their effect on
735 downstream analysis and interpretation for environmental DNA metabarcoding of animals and
736 plants compared to that of community DNA metabarcoding.

737

738 With community DNA metabarcoding of animals and plants, the targeted groups are
739 most often collected in bulk (e.g., soil, malaise trap, or net), individuals are removed from other
740 sample debris and pooled together prior to bulk DNA extraction (Creer *et al.* 2016). In contrast,
741 macro-organism eDNA is isolated directly from an environmental material (e.g., soil or water)
742 without prior segregation of individual organisms or plant material from the sample and
743 implicitly assumes that the whole organism is not present in the sample. Of course, community
744 DNA samples may contain DNA from parts of tissues, cells, and organelles of other organisms
745 (e.g., gut contents, cutaneous intracellular or extracellular DNA, *etc.*). Likewise, macro-organism

746 eDNA samples may inadvertently capture whole microscopic non-target organisms (e.g.,
747 protists, bacteria, *etc.*). Thus, the distinction can at least partly breaks down in practice.
748

749 Another important distinction between community DNA and macro-organism eDNA is
750 that sequences generated from community DNA metabarcoding can be taxonomically verified
751 when the specimens are not destroyed in the extraction process. Here sequences can then be
752 generated from voucher specimens using Sanger sequencing. Since the samples for eDNA
753 metabarcoding lack whole organisms, no such *in situ* comparisons can be made. Taxonomic
754 affinities can therefore only be established by directly comparing obtained sequences (or through
755 bioinformatically generated operational taxonomic units (MOTUs)), to sequences that are
756 taxonomically annotated such as NCBI's GenBank nucleotide database (Benson *et al.* 2013),
757 BOLD (Ratnasingham & Hebert 2007), or to self-generated reference databases from Sanger-
758 sequenced DNA (Olds *et al.* 2016; Sønstebo *et al.* 2010; Willerslev *et al.* 2014). Then, to at least
759 partially corroborate the resulting list of taxa, comparisons are made with conventional physical,
760 acoustic, or visual-based survey methods conducted at the same time or compared with historical
761 records from surveys for a location (see Table 1).
762

763 The difference in source material between community DNA and eDNA, therefore, has
764 distinct ramifications for interpreting the scale of inference for time and space about the
765 biodiversity detected. From community DNA it is clear that the individual species were found in
766 that time and place, but for eDNA, the organism which produced the DNA may be upstream
767 from the sampled location (Deiner & Altermatt 2014), or the DNA may have been transported in
768 the feces of a more mobile predatory species (e.g., birds depositing fish eDNA, Merkes *et al.*
769 2014) or was previously present, but no longer active in the community and detection is from
770 DNA that was shed years to decades before (Yoccoz *et al.* 2012). The latter means that the scale
771 of inference both in space and time must be considered carefully when inferring the presence for
772 the species in the community based on eDNA.

773 **Box 2. Basic bioinformatic pipeline for eDNA metabarcoding for plants and animals**

774 Bioinformatic processing of sequence data is one of the most critical aspects of eDNA
775 metabarcoding studies, helping to substantiate research findings, following field and lab work
776 components. Standardization of bioinformatics in a ‘pipeline’ can ensure quality and
777 reproducibility of findings; however, some level of customization is required across studies.
778 Customization is needed to compensate for advances in sequencing technology, software
779 workflows, and the question being addressed. Therefore, taking raw read data and turning it into
780 a list of taxa, requires multiple quality assurance steps – some necessary, others optional.
781 Reaching an absolute consensus for the approaches and software used is not necessary as these
782 will always be in flux, but here we advise careful consideration of the following pre-processing
783 steps *at a minimum* for HTS data before embarking on further analyses (e.g., for biodiversity
784 estimates and statistical significance). We focus primarily on processing Illumina generated data
785 sets and therefore if the technology is different, many of the bioinformatic tools highlighted and
786 advice is transferable to pre-processing of data produced on other platforms, but may be
787 different.

788

789 **Terms:**

790 **Chimeras:** PCR artefacts made of two or more combined sequences during the extension step of
791 PCR amplification.

792 **Phred quality score:** Quality scoring per nucleotide for Illumina sequencing providing the
793 probability that a base call is incorrect.

794 **Sequence merging:** Combining forward (R1) and reverse (R2) reads from paired – end (PE)
795 sequencing, using criteria such as minimum overlap or quality score.

796 **Sequence trimming:** The process of cutting / removing the beginning or end of sequencing
797 reads. Can be performed either by searching for a specific sequence (removal of adaptors,
798 indexes and primers) or based on quality score.

799 **Singletons:** MOTUs that appear only once in the data are likely to be rare taxa, false positives,
800 low level contamination, or unremoved chimeras, and should be treated with appropriate
801 consideration.

802

803 **Primer – adaptor trimming.** Preliminary steps of bioinformatics processing include de-
804 multiplexing of the samples based on the indices used (unique nucleotide tags incorporated into
805 raw sequence data) and trimming (i.e., removal) of the adaptor sequences. The adaptors are
806 specific DNA fragments which are added during library preparation for ligation of the DNA
807 strands to the flow cell during Illumina sequencing. Additionally, the index sequences
808 themselves and the primer sequences should be trimmed (e.g. using software such as Cutadapt,
809 Trimmomatic, QIIME), allowing either zero or a low level of mismatch between the exact
810 sequence of the primer or index and the observed reads.

811

812 **Merging or end trimming.** Sequences from Illumina runs tend to drop in quality towards the 3’
813 end of the reads, as phasing leads to increased noise (and lower signal) in later chemistry cycles.
814 Thus, the quality score of reads should be reviewed to allow informed decisions on the
815 appropriate length of end trimming (single – end runs), merging (paired end runs) and
816 subsequent sequence quality filters. Visualizing the quality scores from raw reads or de-
817 multiplexed sequences (using software like FastQC) will help with the selection of downstream
818 quality cut-off levels.

819 When paired end (PE) sequencing is used for an amplicon of suitable size, the forward
820 (R1) and reverse (R2) reads should be combined (merged) to form the complete amplicon. Using
821 merged sequences improves accuracy since the lower quality bases at the tail ends of individual
822 reads can be corrected based on the combined reads. Here, the minimum overlap for R1 and R2
823 reads should be specified and ‘orphan’ reads with little or no overlap between forward and
824 reverse pairs can be discarded. Inspection of the quality scores, as mentioned above, can provide
825 an estimate of optimal parameters for merging of R1 and R2 reads. Even though a specific
826 consensus does not exist yet, in many cases an overlap of at least > 20bp is selected (Deiner *et al.*
827 2015; Gibson *et al.* 2015).

828
829 **Quality filtering.** For most HTS platforms, a Phred score is calculated and subsequently used to
830 determine the maximum error probabilities (Bokulich *et al.* 2013). Selected strategies include
831 filtering based on a lower Phred score cut-off, usually set at least above 20 or 30 (Bista *et al.*
832 2017; Elbrecht & Leese 2015; Hänfling *et al.* 2016). Quality filtering can also be performed
833 based on maximum error (maxee) probability, which is also derived from Phred scores. The
834 lower the maximum error, the stricter the cut-off. Selection of a maximum error filtering level of
835 1 or 0.5 is common in macro-organism studies (Bista *et al.* 2017; Pawlowski *et al.* 2014; Port *et*
836 *al.* 2016). Additionally, in the case of single-end sequencing, or when long amplicons without
837 sufficient overlap of the forward and reverse reads are used, it is advised that trimming should be
838 performed from the appropriate end. It is often the case that reads are trimmed to a common
839 length, which facilitates alignment downstream and minimizes miscalled bases since a merging
840 step cannot be used.

841
842 **Removing short reads.** Many studies also select to remove short reads from the dataset before
843 clustering since the presence of high length variation could influence the clustering process (see
844 USEARCH manual, Edgar 2010). These sequences could result from sequencing of primer
845 dimers which have not been removed (Pawlowski *et al.* 2014). Different studies select a variety
846 of minimum length reads, from very short 20bp (Valentini *et al.* 2016), to medium 60 – 80 bp
847 (Pawlowski *et al.* 2014; Shaw *et al.* 2016) and up to 100 bp (Bista *et al.* 2017; Gibson *et al.*
848 2015; Hänfling *et al.* 2016; Pawlowski *et al.* 2014). Note that some de-multiplexing or quality
849 filtering workflows may automatically set a minimum sequence length when processing input
850 data and it is advisable to check whether such a parameter is included by default.

851
852 **Removing singletons and chimeras.** Important steps after MOTU clustering involve removal of
853 singletons and chimeras. Chimeras are by-products of the PCR amplification process from two or
854 more parental sequences (chimeric), most commonly produced through an incomplete extension
855 step (Edgar *et al.* 2011). It has been shown that when unique reads, such as chimeras and
856 singletons, are withheld in analysis, the estimation of diversity can be severely inflated (Kunin *et*
857 *al.* 2010). The nature of the chimeric sequences, which can be present as high quality reads, does
858 not enable their removal directly through quality based end-trimming (Coissac *et al.* 2012).
859 Removal of chimeras can be performed either *de novo* or based on a reference database. Most
860 common practice to date is the *de novo* method since a sufficient reference database may not be
861 available. Despite the variation in software used such as UCHIME (Edgar *et al.* 2011), obitools
862 (Boyer *et al.* 2016), or ChimeraSlayer (Haas *et al.* 2011), there is a consensus regarding the
863 importance of removing chimeras and singletons as a minimum quality control for
864 bioinformatics pipeline.

865

866 **Abundance filtering.** In addition to quality filtering based on quality scores and removal of
867 chimeras and singletons, many studies also employ further filtering for removal of low
868 abundance sequences (Murray *et al.* 2015). This step arises from the need to control for
869 laboratory contamination or because of cluster contamination on the flow cell (unique to
870 Illumina platforms) (Olds *et al.* 2016).

871 The process of applying abundance filtering requires setting an MOTU abundance
872 threshold by which MOTUs are only retained in analysis if their relative abundance is higher
873 than the selected threshold (Bokulich *et al.* 2013). Selection of a threshold varies between studies
874 and there is no generally accepted definition of what constitutes an insufficiently abundant read
875 (Murray *et al.* 2015), perhaps with the exception of singletons. Abundance filtering may be
876 applied minimally or avoided entirely, especially if stringent quality trimming parameters are
877 applied to raw reads and detection of “rare” MOTUs is an important aspect of a study (Bokulich
878 *et al.* 2013). Another option that could be used involves selection of a threshold based on
879 availability of empirical data as was done in Valentini *et al.* (2016). An increasing number of
880 studies have employed the sequencing of positive controls to establish a threshold level
881 (Hänfling *et al.* 2016; Port *et al.* 2016; Stoeckle *et al.* 2017). Technical replicates can also be
882 used to assess consistency as was shown to be effective with assessing omnivore diets (De Barba
883 *et al.* 2014).

884 Using a positive control defined error level works by identifying the abundance of
885 sequences in the control sample that belong to non-target taxa and can be the result of errors such
886 as contamination. Furthermore, the distribution of *phiX* reads assigned to target samples has been
887 used to investigate the presence of “tag-jumps” (Schnell *et al.* 2015a) and mis-assigned reads
888 during de-multiplexing (Hänfling *et al.* 2016; Olds *et al.* 2016). The exact mechanisms for mis-
889 assignment of reads remain unknown, but increasingly many studies are reporting this error to be
890 between 0.01 and 0.03 % of reads (Hänfling *et al.* 2016; Olds *et al.* 2016; Stoeckle *et al.* 2017).
891 Adjustments for this include use of a threshold approach based negative and/or positive controls
892 and removes a low number of reads from any given sample. The issue of abundance filtering
893 most significantly causes uncertainty in low abundance MOTUs and will continue to be a
894 problem for detection of rare species. Therefore, to avoid negative impacts to scientific insights
895 or management decisions, careful consideration and transparency regarding how technical
896 artifacts are dealt with during bioinformatic data analysis is needed until these artifacts are well
897 understood.

898

899 **Recording removed data.** For all quality control steps the data removal should be transparent.
900 Often studies report the total number of sequences obtained, but then rarely show how each
901 quality filtering step affects the number of sequences used in testing ecological hypothesis nor do
902 researchers provide the subset of sequences that were retained or omitted. Deleting data without
903 a clear justification does not allow transparency. Therefore, including a supplemental table in
904 eDNA metabarcoding studies showing the number of sequences remaining after each filtration
905 step is advised and archiving the subset of reads retained after each filtering step on a platform
906 such as Dryad (<http://datadryad.org/>) or archiving the exact pipeline with version control
907 information on a platform such as GitHub (<https://github.com/>) will allow for greater
908 transparency and reproducibility of quality filtering.

909 **Box 3: How to transform reads from HTS platforms into measures of richness**

910
911 **MOTU clustering.** While this step is not always necessary and depends on the target set of taxa
912 (Lacoursière-Roussel *et al.* 2016), the amplicon length sequenced (Deiner *et al.* 2016), and
913 completeness of the reference database (Chain *et al.* 2016), clustering of sequencing reads into
914 MOTUs is often performed prior to taxonomic assignment. MOTU clustering is the process
915 whereby multiple reads are grouped according to set criteria of similarity based on an initial seed
916 (Creer *et al.* 2016; Egan *et al.* 2013). Here, a centroid sequence is selected and depending on the
917 set radius or similarity cut-off, closely related sequences are grouped under each centroid
918 sequence (USEARCH, Edgar 2010). The level of similarity selected depends on the study and
919 taxon used, based on the knowledge of intraspecific diversity of the studied taxon. Commonly
920 used cut-offs range from 97% to 99% (Bista *et al.* 2017; Fahner *et al.* 2016; Olds *et al.* 2016).
921 For example, the cut-off selected could depend on known levels of intraspecific diversity of the
922 studied taxon, which could be estimated from an existing reference database. Some commonly
923 used clustering algorithms include USEARCH (Edgar 2010), VSEARCH (Rognes *et al.* 2016),
924 CROP (Bayesian clustering algorithm) (Hao *et al.* 2011), swarm (Mahé *et al.* 2014), and mothur
925 (an alignment-based clustering method, Schloss *et al.* 2009).

926
927 **Taxonomic assignment.** Identification of HTS reads is achieved through a comparison of
928 anonymous MOTU clusters/centroid sequences or direct comparisons of reads remaining after
929 quality filtering against a reference database. Depending on the taxon of study and the marker
930 used, the reference database may consist of publicly available sequences or study-generated
931 reference sequences.

932
933 The challenges of taxonomic assignment have been the subject of a considerable literature so we
934 only briefly discuss this important aspect of the bioinformatics pipeline (e.g., Bazinet &
935 Cummings 2012). A number of different approaches have been suggested including assignment
936 based on sequence similarity via alignment programs like BLAST or similarity searches using
937 Hidden Markov Models such as jMMOTU (Jones *et al.* 2011), MG-RAST (Glass *et al.* 2010),
938 sequence composition and machine learning approaches (e.g., RDP (Wang *et al.* 2007), TACO
939 (Diaz *et al.* 2009)), phylogenetic placement (e.g., pplacer Matsen *et al.* 2010), probabilistic
940 taxonomic placement (e.g., PROTAX (Somervuo *et al.* 2016; Somervuo *et al.* 2017), minimum
941 entropy decomposition (e.g., oligotyping Eren *et al.* 2015), MEGAN (Huson *et al.* 2007), and
942 ecotag (Boyer *et al.* 2016). A number of widely used programs use combinations of these
943 methods, for example, the program SAP (Munch *et al.* 2008) uses BLAST searches of the NCBI
944 database and phylogenetic reconstruction to establish taxonomic identity of query sequences.
945 Most of these methods and various derivatives are nicely discussed and compared by Bazinet and
946 Cummings (2012). Two major determinants of the utility of these different approaches are the
947 specific eDNA markers and the breadth and resolution of reference databases. Some markers
948 have better representation in available databases and greater coverage of relevant species
949 diversity. Taxonomic assignment using the BLAST algorithm (Camacho *et al.* 2009) is
950 commonly used and depending on the study, different selection criteria are specified, such as e-
951 value, maximum ID or length of matching sequence, number of top hits selected, etc. Caution is
952 warranted in strictly relying on this approach, since errors in the curation of sequences in
953 publicly available databases can propagate through the analysis and lead to misidentification of
954 sequences. Ideally, a combination of approaches is used and when feasible the resultant species

955 assignments should be vetted with independent data based on the known distribution and ecology
956 of the species.

957

958 **Diversity analysis.** The goal of most eDNA metabarcoding studies is to accurately characterize
959 the species richness of the community under study. Calculation of diversity indices using
960 appropriate software allows modeling and ecological association of sequencing results.
961 Important considerations when attempting ecological associations include appropriate data
962 standardization to account for variations in sequencing depth and the careful selection of
963 diversity indexes. The most common assessments include alpha-diversity (rarefaction,
964 visualization of taxonomic profiles), and beta-diversity (Principal Components/Coordinates
965 Analysis, NDMS ordination, etc.), prior to hypothesis testing via downstream statistical analysis.

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1477

1478 Table 1: Representative studies comparing richness estimates with traditional sampling or historical data for a geographic location to
 1479 that of eDNA metabarcoding.

Habitat	Macro-organism taxonomic focus	eDNA sample type	Traditional sampling method	eDNA efficacy finding*	Authors	Year
Air	Plants	air pollen trap	morphological identification	Better taxonomic resolution	Kraaijeveld <i>et al.</i>	2015
Freshwater	Fish	flowing water	depletion-based electro fishing	Higher diversity	Olds <i>et al.</i>	2016
Freshwater	Invertebrates	flowing water	kicknet in stream and historical data	Higher diversity	Deiner <i>et al.</i>	2016
Freshwater	Fish	stagnant water	gill-net, trapping, hydroacoustics, analysis of recreational anglers' catches	Complementary	Hänfling <i>et al.</i>	2016
Freshwater	Reptiles, amphibians	stagnant water	species distribution model based on historical data (i.e. distribution range and habitat type)	Increase species distribution knowledge	Lacoursière-Roussel <i>et al.</i>	2016
Freshwater	Amphibians, fish	stagnant water; flowing water	amphibians: visual encounter survey, mesh hand-net; Fish: electrofishing, and/or netting protocols (fyke, seine, gill)	Greater detection probability	Valentini <i>et al.</i>	2016
Freshwater	Amphibians, fish, mammals, invertebrates	stagnant water; flowing water	active dip-netting, fresh tracks or scat, electrofishing with active dip-netting	Complementary	Thomsen <i>et al.</i>	2012
Freshwater	Fish	stagnant water; flowing water; surface sediment	fyke net	Higher diversity	Shaw <i>et al.</i>	2016
Freshwater	Invertebrates	water column; surface sediment	sediment collected using a Van Veen grab	Higher diversity	Gardham <i>et al.</i>	2014

Freshwater	Fish / Diptera	Surface and bottom water column	Long-term data, electro fishing (fish) and emerging traps (Diptera) at time of eDNA sampling	Higher diversity compared to sampling but lower diversity compared to long-term data	Lim <i>et al.</i>	2017
Marine	Fish	Surface and bottom water column	Long term observation	Complementary	Yamamoto <i>et al.</i>	2017
Marine	Fish	Bottom water column	Trawl catch data	Similar Family richness	Thomsen <i>et al.</i>	2016
Marine	Fish	water column	scuba diving	Higher diversity	Port <i>et al.</i>	2015
Terrestrial	Plants	honey	melissopalynology (i.e. pollen grains retrieved from honey are identified morphologically)	Complementary	Hawkins <i>et al.</i>	2015
Terrestrial	Mammals, plants	midden pellets	historical surveys	Higher diversity	Murray <i>et al.</i>	2012
Terrestrial	Mammals	saliva	local knowledge (i.e. physical evidence) and camera data	Complementary	Hopken <i>et al.</i>	2016
Terrestrial	Birds, invertebrates, plants	top soil	invertebrates: leaf litter samples & pitfall traps; reptiles: pitfall traps and under artificial ground covers; birds: distance sampling method; plants: above-ground surveys	Complementary for plants & invertebrates	Drummond <i>et al.</i>	2015
Terrestrial	Earthworms	top soil	irrigated quadrats with 10 L of allyl isothiocyanate solution and hand collected emerging worms	Complementary	Pansu <i>et al.</i>	2015
Terrestrial	Plants	top soil	historical surveys	Complementary	Jørgensen <i>et al.</i>	2012
Terrestrial	Plants	top soil	above-ground surveys	Complementary and better taxonomic resolution	Yoccoz <i>et al.</i>	2012

Terrestrial	Vertebrates	top soil	local knowledge from safari parks, zoological gardens and farms; visual observations; historical surveys	Complementary	Andersen <i>et al.</i>	2012
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1480

1481

* Complementary means the two survey methods detected different diversity, but does not exclude that some of the diversity was

1482

detected by both methods. Higher diversity means the study found more diversity was detected compared to conventional, but does

1483

not exclude that some of the diversity was *not* detected by both methods. Better taxonomic resolution means that sequence based

1484

identifications could be resolved to a lower taxonomic rank compared with the conventional method.

1485 **Figure legends**

1486 **Figure 1: Environmental DNA sample types have different spatial and temporal scopes of**
1487 **inference from different habitats.** Consider each sample type as a single sample from that
1488 environment. Placement of a sample type in a quadrant is not quantitative, but represents a
1489 common scale at which it has been used. Dashed arrows indicate the potential for a sample type
1490 to confer information at multiple scales of inference, but additional research to quantify these
1491 possibilities is needed.

1492

1493 **Figure 2: Challenges for estimating abundance from environmental DNA metabarcoding.**

1494 For simplicity, assume one DNA molecule depicted in the pond is equal to one organism and
1495 colors represent different species. Additionally for this example, assume that sampling is no
1496 biased (i.e., DNA copies are sampled in their true abundance), that boxes surrounding DNA
1497 molecules represent 1 uL and one DNA molecule represents 1 ng of DNA. Thus, values
1498 illustrated show the effect of primer bias, sub-sampling and their combination on the ability to
1499 estimate abundance.

1500

1501 **Figure 3: Important guiding questions for consideration in the design and implementation**
1502 **phases of an environmental DNA metabarcoding study.**

1503

1504 **Figure 4: Opportunities and challenges of using environmental DNA as a tool for assessing**
1505 **community structure in different fields of study.** The tool is reliant on a foundation (blue half
1506 circle) of continued research to improve technological aspects and continued development of
1507 DNA-based reference libraries for the identification of sequences found in the environment.

1508 **Acknowledgements**

1509 We thank Nigel G. Yoccoz Tromsø and three additional anonymous reviewers whose feedback
1510 was valuable in revising our manuscript. We thank Kristina Davis for help in drafting figures.
1511 Manuscript collaboration was facilitated by the National Science Foundation through the Coastal
1512 SEES grant to DML (EF-1427157; KD, DML, MEP), a DoD SERDP grant to DML (W912HQ-
1513 12-C-0073 (RC-2240); KD, DML, MEP); and a NOAA CSCOR grant to DML (KD, DML).
1514 National Science Foundation Research Coordination Network award to HMB (DBI-1262480)
1515 supported a related eDNA-focused Symposium at the 2016 Annual Meeting of the Ecological
1516 Society of America organized by KD, DML and MEP. In addition this article is based upon
1517 work from COST Action DNAqua-Net (CA15219; KD, FA, SC), supported by the COST
1518 (European Cooperation in Science and Technology) program, by the Swiss National Science
1519 Foundation Grant No PP00P3_150698 (to FA) and Eawag (FA and EM); the Natural
1520 Environment Research Council (NERC): NBAF pilot project grant (NBAF824 2013-14),
1521 Standard Grant PollerGEN (NE/N003756/1) and Highlight Topic Grant LOFRESH
1522 (NE/N006216/1) and the Freshwater Biological Association (FBA) (Gilson Le Cren Memorial
1523 Award 2014). IB was funded by a Knowledge Economy Skills Scholarship (KESS) a pan-Wales
1524 higher-level skills initiative led by Bangor University on behalf of the HE sector in Wales. It is
1525 part funded by the Welsh Government's European Social Fund (ESF) convergence programme
1526 for West Wales and the Valleys.

1527

1528 **Author contributions**

1529 K.D. outlined and edited the review. All authors contributed at least one section of primary
1530 writing and contributed to editing of the manuscript. K.D., H.M.B, and E.M. synthesized sections
1531 and drafted figures.

1532

1533 **Data Accessibility**

1534 No data are associated with the manuscript

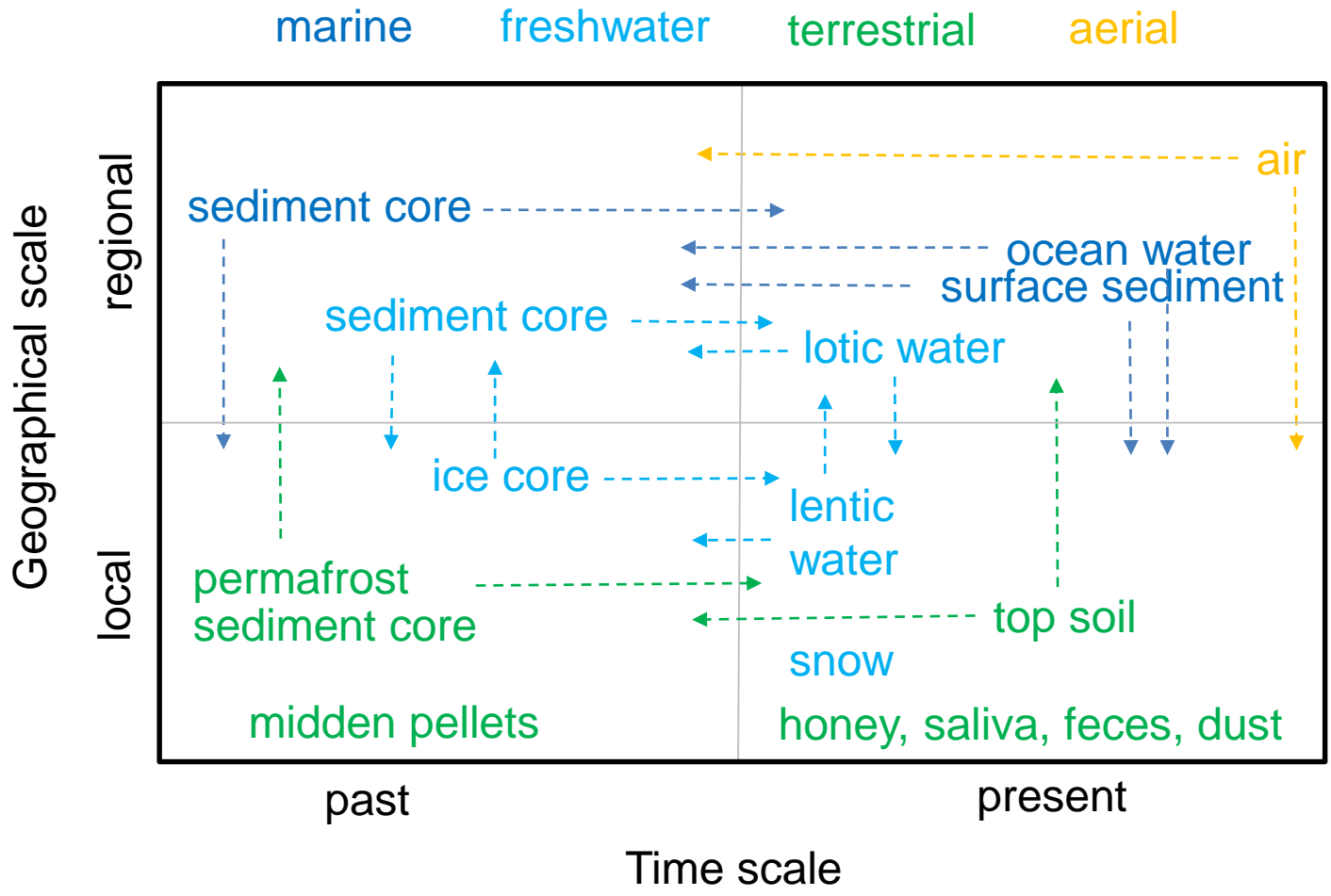
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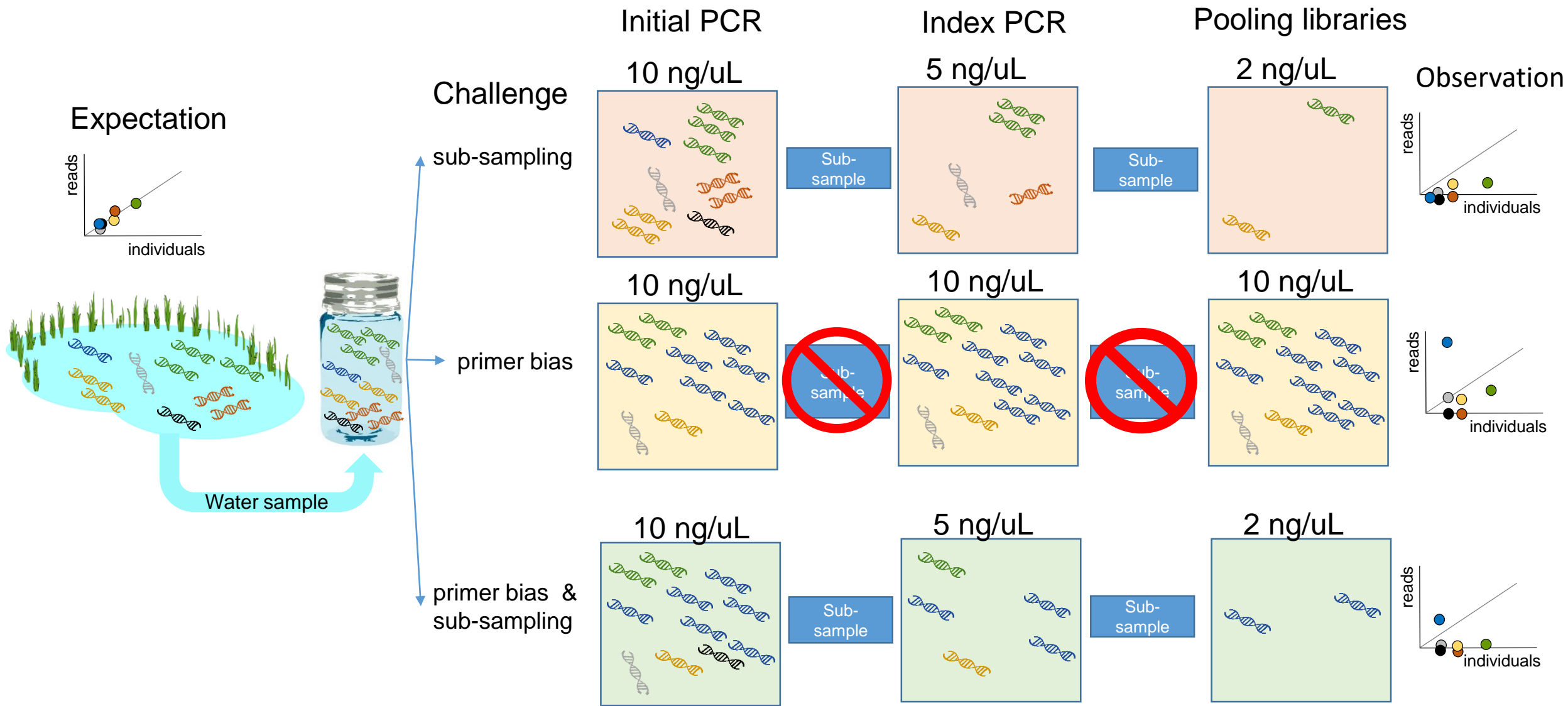
1536 **Supplemental Material**

1537 Table S1: Reviews about use of environmental DNA for species detection

1538

1539 Table S2: Review of primers used in eDNA metabarcoding





Study design



In the field



In the laboratory



At the keyboard



Basic science or applied?
(e.g., environmental biomonitoring)

What is your study goal?

- presence/absence
- diversity assessment
- absolute quantification

What taxa will you target?

Is the scale of inference for your sample type appropriate to your question?

Can you compare complementary data types? (e.g. traditional vs. eDNA)

Does your sampling/replication scheme provide good statistical power?

What type of sample is needed? (water, soil, air)

What metadata should you collect?

How many replicates will you collect?

Does your sampling protocol minimize/control for:

- **contamination**
(e.g., positive and negative controls)
- **any known biases**
(e.g., inhibitors, sample volume)

Sample Handling Phase

What extraction method?
(physical vs. chemical)

How much sample?

What locus and primers?

Do you need to generate reference sequence data?

Are technical replicates needed?

What library preparation method will you use?

How many samples will you index and pool?

What sequence depth is needed per sample?

What read length will you use?

DNA Processing Phase

What sequencing platform will you use?

Do you need paired end sequencing?

Have you included appropriate quality assurances?
(e.g., mock community, qPCR, bioanalyzer traces)

Does your laboratory protocol minimize/control for:

- **contamination**
(e.g., positive and negative controls)
- **any known biases**
(e.g., primer bias, coverage, taxonomic resolution)

How complete is the reference database?

Do you have adequate sequencing coverage across samples?

Are you using appropriate choices for software tools, parameters?

Are your biological conclusions upheld using alternative parameters and workflows?

Are you including appropriate quality filtering of your data?
(see Box 2)

ECOLOGY

Technical issues estimating abundance

Comprehensively estimate biodiversity

Quantify species richness
to measure biodiversity and ecosystem functioning

Measure diversity at different scales
complementary to conventional sampling

CONSERVATION BIOLOGY

Cannot estimate population viability or demography

Rapid, cost effective, non-invasive sampling
to estimate baselines and departures

Standardize sampling from various ecosystems
to compare extinction timelines

INVASION BIOLOGY

False negatives and false positives

Detect species early at low abundance

Employ "passive" surveillance

Detect vectors and pathways of dispersal

BIO-MONITORING

Need in-depth tests comparing biotic index estimated from eDNA vs. conventional sampling

Detect cryptic taxa or life stages with greater sensitivity

Alleviate specimen damage issues

Challenges

Potential impacts

eDNA
of animals and plants

Precision & Accuracy

Sample Scope & Scale

Reference Database