Extracellular DNA in environmental samples: Occurrence, extraction, quantification, and impact on microbial biodiversity assessment

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Abstract

Environmental DNA, i.e., DNA directly extracted from environmental samples, has been applied to understand microbial communities in the environments and to monitor contemporary biodiversity in the conservation context. Environmental DNA often contains both intracellular DNA (iDNA) and extracellular DNA (eDNA). eDNA can persist in the environment and complicate environmental DNA sequencing-based analyses of microbial communities and biodiversity. Although several studies acknowledged the impact of eDNA on DNA-based profiling of environmental communities, eDNA is still being neglected or ignored in most studies dealing with environmental samples. In this article, we summarize key findings on eDNA in environmental samples and discuss the methods used to extract and quantify eDNA as well as the importance of eDNA on the interpretation of experimental results. We then suggest several factors to consider when designing experiments and analyzing data to negate or determine the contribution of eDNA to environmental DNA-based community analyses. This field of research will be driven forward by: (i) carefully designing environmental DNA extraction pipelines by taking into consideration technical details in methods for eDNA extraction/removal and membrane-based filtration and concentration; (ii) quantifying eDNA in extracted environmental DNA using multiple methods including qPCR and fluorescent DNA binding dyes; (iii) carefully interpretating effect of eDNA on DNA-based community analyses at different taxonomic levels; and (iv) when possible, removing eDNA from environmental samples for DNA-based community analyses.

Keywords: extracellular DNA, environmental DNA, eDNA, microbial biodiversity

36 **1. Introduction**

37 In recent years, analysis of environmental DNA, *i.e.*, DNA directly extracted from environmental
38 samples, has been applied to understand microbial communities in the environments and to
39 monitor contemporary biodiversity in the conservation context. DNA from an environmental
40 sample often contains both intracellular DNA (iDNA) representing DNA contained in intact cells
41 and extracellular DNA (eDNA) representing DNA outside organisms (1-4). DNA can be
42 released into the environment from organisms through cell lysis mediated by predation or phage
43 infection and release of membrane vesicles (5, 6). Studies have shown that eDNA can persist and
44 accumulate in various environments such as soil, e.g., relic DNA and adsorbed DNA (ads_DNA)
45 (2, 7), and aquatic systems, e.g., dissolved DNA (dis_DNA) (8), although the abundance of
46 eDNA might vary in different environmental systems (7).

47 eDNA can serve as a source of nutrient supporting microbial growth, free genetic material
48 helping in iDNA repair and the acquisition of favourable functions, and a structural component
49 in biofilms (5). In environmental DNA-based biodiversity monitoring through DNA sequencing,
50 eDNA may complicate the determination of the structure and potential function of the
51 community inhabiting the sampled environment. As eDNA in environmental samples is a
52 mixture of genomic DNA derived from cell lysis along with plasmids etc., general
53 bioinformatics pipelines cannot distinguish between eDNA and iDNA effectively, highlighting
54 the need to mitigate the effect of eDNA in the design of experiments. Although several studies
55 acknowledged the impact of eDNA on DNA-based profiling of environmental communities,
56 eDNA is still being neglected or ignored in most studies dealing with environmental samples. In
57 this article, we summarize key findings on eDNA in environmental samples and discuss the
58 methods used to extract and quantify eDNA as well as the importance of eDNA on the
59 interpretation of experimental results. We then suggest several factors to consider when
60 designing experiments and analyzing data to negate/determine the contribution of eDNA to
61 environmental DNA-based community analyses.

62 **2. Occurrence of eDNA in environmental samples**

63 eDNA is ubiquitous in nature. It has been found in natural systems such as soil, sediments, water,
64 and air, as well as engineered systems such as wastewater treatment plants, drinking water and
65 anaerobic digesters (Figure 1). In soil samples, eDNA concentration ranges from 0.3 - 200 µg/g

of soil (9) and varies with the soil type and depth from the surface. For instance, podzol was found to contain 2 µg eDNA/g of soil, luvisol contained 0.08 µg eDNA/g of soil (10), fine-loamy soil contained 60 µg eDNA/g of soil (11), and Cambic Umbrisol contained 6.07 µg eDNA/g of soil (12) in the topsoil layer. eDNA concentration is seen to decrease with an increase in depth of soil. In the subsoil layer, 0.4 ng eDNA/g of soil was found in luvisol, 5.2 ng eDNA/g of soil in podzol, and 10.8 µg eDNA/g of soil in fine-loamy soil. Sediment samples exhibit a similar trend. A study on sediments from lake Towuti in Indonesia showed that the highest concentration of eDNA was found in the top 5 cm layer of sediments at both shallow and deep sites (0.5 - 0.6 µg eDNA/g wet-sediment) and decreased to below detection limits at a depth of 30-35 cm (13). Varying amounts of eDNA has been successfully extracted from the sediments, which have been summarized recently by Torti et al (3). Studies on sediments from the Baltic sea, Barents Sea, South pacific Gyre (14), Adriatic sea, Mediterranean sea and South Pacific Ocean (15) have reported high amounts of eDNA which are 6-68 folds higher than the amount of iDNA extracted from the same samples. Mao et al. (16) also reported higher eDNA than iDNA extracted from river sediments. A recent study on sediments from Aarhus Bay showed that only 40% of the total DNA was extracellular in nature (17). Globally, the top 10 cm of deep sea sediments is the largest reserve of eDNA, accounting for ~90% of the total DNA (18).

In the marine environment, eDNA in water columns can range between 0.2 – 44 µg eDNA/ L of water depending on the location of sampling (estuarine vs coastal vs offshore), while freshwater ecosystems can harbor 0.5 – 25.6 µg eDNA/ L of water(3). More recently, 10.3 µg eDNA/ L of water was found in the hypersaline lake environment (20). Monochloraminated drinking water system was also shown to have a low quantity (33 – 386 ng/L) but a significant proportion (~50%) of eDNA in total DNA (24). Another study detected 0.12 – 2.5 µg eDNA/ L in Tama river water (21). Zhang et al. (22) showed the presence of extracellular antibiotic resistance genes (eARGs) in wastewater treatment plants and the eARGs proportion increased along the treatment plant. eARGs were also seen to decay slowly in the treated water indicating their persistence. Calderon et al. (23) estimated influent and effluent wastewater to contain 12.5 and 8.6 µg eDNA/ L respectively. 12.3 µg eDNA/ L of activated sludge was also found (23). Though lesser amounts of eDNA are found in the water column as compared to the soil and sediment, eDNA still forms a major proportion of the total DNA extracted from water samples.

96 The presence of eDNA in the discussed system has been known for 3 decades with the earliest
97 reports arising in the mid-1980s. A new study showed that apart from these conventionally
98 studied systems, air can also contain eDNA. The study focuses on the presence of eARGs
99 adsorbed to the PM2.5 particles and shows that the ARGs in the iDNA fraction are distinct from
100 the eDNA fraction (19). Overall, eDNA contributes significantly to total DNA extracted from
101 environmental samples and further studies are needed to understand its implication, fate, and
102 biological significance in different environments.

103 **3. Persistence of eDNA in environmental samples**

104 The interaction of eDNA with extracellular nucleases is one of the most important factors
105 influencing its persistence in the environments. Environmental temperature affects nuclease
106 activity and hence, eDNA persistence. DNA molecules are known to adsorb onto particles such
107 as sands, clays and minerals (25), which can protect them from nucleases (26). Length of eDNA
108 inversely correlates to its adsorption potential and hence, smaller eDNA fragments are more
109 persistent than longer fragments (27). Other factors such as low temperature and anoxic
110 conditions help in the preservation of eDNA (28, 29). Another major factor influencing eDNA
111 persistence is its metabolism and uptake by microorganisms. Certain groups of bacteria (such as
112 *Lutibacter*, *Shewanella*, '*Candidatus* Izemoplasma' and *Fusibacteraceae*) in deep sea sediments
113 have been shown to be capable of utilizing eDNA as a carbon source. In soil samples, bacteria
114 belonging to the genera *Arthrobacter* and *Nocardioides* may also play a role in the degradation
115 of eDNA (30, 31).

116 Extracellular DNA degradation in various environments have been previously summarized by
117 Nielsen et al. (1) and Pietramellara et al. (9) and can vary between different environmental
118 matrices (Figure 2). More recently, Sirois et al. (32) reported that the persistence of eDNA in the
119 soil is positively correlated with organic matter content in the soil while higher moisture and
120 temperature enhanced eDNA degradation. Although the eDNA standard (469 bp *htrA* gene
121 fragment) was observed to decline rapidly upon its introduction into soil microcosms (~99%
122 reduction in 7 days), the eDNA standard could be detected up to 80 days. A similar phenomenon
123 was reported by Kunadiya et al. (33), where degradation of DNA from *Phytophthora cinnamomi*
124 was accelerated in moist soils as compared to dry soils. eDNA in dry soils could be easily
125 detected for up to 90 days using qPCR. Gordon et al. (34) also reported the successful detection

126 of *Phaeocollybia* eDNA up to 60 days in soil samples. Plasmids incubated in soil microcosms
127 are also known to persist for at least 28 days (35). eDNA introduced as cell lysate into soils was
128 seen to persist for 24 weeks (36).

129 DNA is effectively preserved in the marine sediments evidenced by retrieval of eDNA as old as
130 217000 years (44, 45) and the successful amplification of 16S rRNA genes from eDNA extracted
131 from sediment samples that were 10000 years old (46). Using microcosms, Mao et al. (16)
132 reported that eDNA degraded faster in Haihe river water (~100% within 7 days) than in the
133 Haihe river sediments (detectable after 12 weeks). Deere et al. (41), also observed eDNA to
134 persist in the water column for 10 days, whereas it could still be detected in sediments for up to
135 13 weeks. Dell'Anno et al. (37) estimated the turnover time for DNA in sediments to be longer
136 than water (29-93 days in sediments vs 10 h in seawater). In forest pond sediment-water
137 microcosms England et al. (38) reported that viral eDNA from *Baculovirus* could be detected for
138 24 h, whereas in laboratory microcosms, the eDNA could persist for days. Saito et al. (40)
139 reported that eDNA was not degraded in purified water over the experimental period of 7 days,
140 whereas it persisted in pond water for 5 days and could be detected in sea water after 7 days.
141 Similar to Mao et al. (16), Saito et al. (40) also observed rapid initial decay of eDNA in
142 environmental water samples, which were attributed to the microbial activity and action of
143 extracellular enzymes. However, a study by Bukh et al. (42) reported eDNA to persist for at least
144 28 days in hot tap water. The rapid decay of eDNA in environmental waters as compared to
145 engineered water systems could also be attributed to dissolved organic matter, e.g., humic
146 substances, organic acids, and carbohydrates. These organic matter can be photosensitized by
147 sunlight, resulting in formation of reactive oxygen species (ROS) and hydroxyl radicals which
148 facilitate the decay of DNA (47, 48). Overall, DNA seems to persist longer in sediments and
149 soils as compared to water samples. This might be attributed to faster eDNA decay in water
150 because of reduced protection from extracellular nucleases, increased microbial activity (40, 49),
151 high chance of ROS-induced DNA damage and higher temperature (50, 51) or reduced detection
152 efficiency due to lower DNA concentrations in water environments and DNA binding to organic
153 matter (52).

154 Due to its persistence, eDNA serves as a possible reservoir of antibiotic resistance genes (ARGs)
155 for horizontal gene transfer via transformation. Uptake of eDNA by competent bacterial cells can

156 result in dissemination of these genes on a large scale. Mao et al. (16) demonstrated the uptake of
157 kanamycin resistance gene by an indigenous sediment bacterium under selective pressure.
158 Recent research has suggested that disinfection by-products from chlorine and chloramine can
159 lead to an increase in uptake of exogenous DNA (53). Disinfection using chlorine, chloramine
160 (54) and solar irradiation (55) have also been seen to enhance the transformation process through
161 upregulation in DNA uptake and repair proteins caused by the ROS stress response. Biofilms in
162 estuarine systems have been shown to accumulate ARGs in eDNA (56) facilitating the horizontal
163 gene transfer process (57). However, most studies are performed under ideal laboratory
164 conditions using model organisms and plasmids. Further studies are required to determine the
165 real contribution of eDNA in the environment as a pool for horizontal gene transfer.

166 4. Extraction and quantification of eDNA in environmental samples

167 Table 1 summarizes previous studies on eDNA in environmental samples. The most common
168 method to extract eDNA from solid phases such as soil samples is to desorb the ads_eDNA using
169 an alkaline sodium phosphate buffer (NaP) (58) (Figure 3). The excess phosphate in NaP
170 competes with eDNA for binding sites on clay particles, enabling the desorption and recovery of
171 eDNA from the samples. This method has been widely used with various modifications over the
172 last 3 decades (11, 13-15, 59). A remarkable improvement in the yield of eDNA (~4 to 10-fold)
173 was observed when the samples were pre-treated with proteinase K (21). Another method for
174 eDNA extraction from solid phases is to dissolve eDNA in Tris-EDTA (TE) buffer from the
175 environmental samples (10, 17, 60) (Figure 1). Treatment with NaP/TE or proteinase K does not
176 cause cell lysis, thereby preventing contamination of eDNA with iDNA. The obtained crude
177 extracts of eDNA can be purified using conventional DNA extraction techniques such as cetyl
178 trimethylammonium bromide (CTAB) method, ethanol precipitation, or chromatography. These
179 methods have mainly been applied to soil, sediments, and sludge samples due to practical
180 feasibility as the particles can be resuspended and washed in the extraction buffer.

181 To extract eDNA from liquid environmental samples, we usually need to concentrate it first
182 (Figure 3). For example, eDNA in water samples can be concentrated through adsorption by the
183 Nucleic Acid Adsorption Particles (NAAPs - silica coated with aluminum hydroxide) followed
184 by elution and precipitation (61). This method has mainly been used to extract extracellular
185 antibiotic resistant genes (eARG) from different water systems as the NAAPs favor adsorption of

186 short-length linear DNA (61). Another method uses hollow fiber membrane (MWCO of 30 kDa
187 and a surface area of 25 m^2) and silica adsorption to concentrate and extract eDNA, from Tama
188 river water (21). Compared with the NAAPs method, this method gives a higher recovery by
189 allowing concentration of all DNA above 50 bp. Calderon et al. (23) used anion exchange
190 chromatography for eDNA extraction from wastewater samples and found that plasmids and
191 transposable elements were highly enriched in the extracted eDNA. Yuan et al. (62) reported the
192 use of a prefiltration technique combined with magnetic beads to isolate eDNA from water and
193 sludge samples. Using the optimized method, large quantities of free and adsorbed eDNA from
194 small volumes of wastewater and activated sludge samples could be extracted.

195 Different filter membranes have been used to concentrate eDNA from water samples. Glass fiber
196 (GF) and cellulose nitrate (CN) membranes exhibit the highest affinity to eDNA (~30-35% of
197 eDNA retained) (63), followed by polyvinylidene difluoride (PVDF), mixed cellulose esters
198 (MCE), polyether sulfonate (PES) and polycarbonate (PC) membranes (64). Hence, GF or CN
199 filter membranes are recommended for extracting eDNA from the filter retentate, while PC and
200 PES membranes are preferable for separating eDNA from cells and recovering eDNA from the
201 filtrate (Figure 3). In addition to the adsorption by membrane filters, the retention of eDNA on
202 filters may also attribute to other components in the samples. For example, using PC membrane
203 for the filtration of storm water, Liang et al. (64) found that 7-13% of their qPCR signals from
204 the filter retentate were derived from the spiked plasmid DNA (eDNA proxy) even though they
205 observed only ~2% eDNA retention in optimization experiments with PC membranes. The
206 increased detection of the spiked plasmid DNA in the retentate can be attributed to the
207 adsorption of eDNA on sand and clay particles retained on the filter membranes (64).

208 Extracted eDNA is usually quantified by using fluorometric DNA dyes such as Picogreen (10,
209 60, 65), Hoechest (8, 11), SYBR Green (15), Qubit dsDNA assay kits (2, 21, 24) or DAPI (63).
210 DNA quantification using fluorometric dyes is very specific and accurate as compared to
211 absorption spectroscopy methods which are non-specific. For instance, freshwater ecosystems
212 usually harbor 0.5 – 25.6 µg eDNA/ L of water (3). A study conducted in Haihe river quantified
213 eDNA using absorption spectroscopy and reported eDNA concentrations of ~2000 µg eDNA/ L
214 in the water samples (16). This concentration is likely overestimated due to the choice of the
215 non-specific DNA quantification method.

216 **Table 1**. Summary of previous studies dealing with eDNA in environmental samples. Cellulose acetate (CA), Polycarbonate (PC), Glass
217 fibre (GF), Polyether sulfonate (PES), Phenol-Chloroform-Isoamyl alcohol (PCIA); Sodium Phosphate Buffer (NaP); Propidium
218 Monoazide (PMA); Ethidium Monoazide (EMA); Cetyl Trimethylammonium Bromide (CTAB); Nucleic Acid Adsorption Particles
219 (NAAP); Diethylaminoethyl cellulose (DEAE); Denaturing Gradient Gel Electrophoresis (DGGE).

Sample	Filter	Starting material	Method of extraction/treatment	Quantification	eDNA yield	Recovery /removal efficiency	Impact on 16S/18S rDNA detection	Ref
Soil	CA	2 g	TE Buffer dissolution	Picogreen	Podzol: 1949.5 ng/g; Luvisol: 80.8 ng/g (top layer).	-	-	(10)
	-	10 g	NaP wash	Hoechst 33258	~18.9 µg/g (top layer), 10-60% eDNA over all horizons	-	Yes - DGGE	(11)
	-	0.5 g	NaP wash	Hoechst 33258	Total eDNA: ~8.2 µg/g soil Free eDNA: ~1.4 µg/g soil Bound eDNA: ~6.9 µg/g soil (Top layer)	-	Yes - DDGE	(59)
	-	0.25 g	PMA/EMA	Picogreen/Nanodrop	Total DNA reduced by 14.9% (PMA, 0.56 µg/g soil) and 16.4% (EMA, 0.74 µg/g soil)	-	No - DGGE	(65)
	-	0.03 g	PMA	qPCR	~41 % of amplifiable soil DNA pool	100% recovery of spiked eDNA	Yes	(2)

	-	0.03 g	PMA	qPCR	30-97 % of amplifiable soil DNA pool	-	Yes	(66)
	-	0.03 g	PMA	Qubit ds DNA	Not reported. Calculated: ~1.4 µg/g soil (top layer) in control samples.	-	No	(67)
Sediment	-	100 g	NaP wash	Spectroscopy	1 µg/g sediment	-	-	(58)
	PC	Buffer/Sediment ratio ~2.5 (v/wt)	TE Buffer dissolution - Enzymatic treatment	Diaminobenzoic acid	7.8 - 22 µg/g sediment (top layer)	Higher than alkaline method	-	(68)
	Anopo-re	2.5 g	NaP wash	SYBR green I	6.7 - 24.3 µg/g sediment	34-60% using internal DNA standard	No	(15)
	CA	Buffer/Sediment ratio ~2 (v/wt), 2-2.5 g	NaP wash	Qubit ds DNA	699 - 889.6 ng/g sediment	13-91%	Yes - RFLP	(14)
	-	0.2 g	Carbonate dissolution followed by alkaline treatment and TE buffer dissolution	Picogreen, qPCR	10–83% of total DNA based on qPCR, 49-61% based on fluorescence spectroscopy	-	-	(60)
	-	1 g	Carbonate dissolution - alkaline treatment - TE buffer dissolution	Qubit ds DNA	0.6 to 0.5 µg/g sediment (top layer)	-	Yes	(13)
	-	0.1 cm^3 of fresh wet sediment	PMA	qPCR	Pacific 36–50%; Arctic 28%	PMA-treatment removed	Minimal difference	(69)

						73–98% of eDNA		
	-	0.2 g	Carbonate dissolution - alkaline treatment - TE buffer dissolution	Picogreen, qPCR	0.03-4.45 µg/cm^3, 28-58% (Picogreen), 20-57% and 33-69% (qPCR)	-	Yes	(17)
Water	GF/D, PC	100-1000 mL	0.2 µm prefiltration – filtrate precipitated with ethanol	Hoechst 33258	Dissolved DNA constituted 11-226% of the particulate DNA. Marine environment 0.41-14.52 µg/L, Freshwater 1.7-7.8 µg/L	85-95%	-	(8)
	PC	100-1000 mL	0.2 µm prefiltration - Filtrate precipitated by CTAB-NaCl	DAPI	2.5-72 µg/L	80-90%	-	(63)
	-	1000 mL	PMA treatment	-	-	-	No	(70)
	PC	100 mL	0.2 µm filtration - eDNA extraction from filter residue	qPCR	7-13% of spiked plasmid recovered from storm water		-	(64)
	NAAP	10 L	NAAP adsorption	TaqMan qPCR	-	95%	-	(61)
	GF/F	15 L	DNase treatment	Qubit dsDNA assay	Location 1: 85-386 ng/L, Location 2: 33-58 ng/L	~99% eDNA removal	Yes	(24)
	PES	20 L	Proteinase K + NaP wash	Qubit dsDNA assay, qPCR	iDNA: 0.15–7.40 µg/L, Ads_eDNA: 0.06–0.45 µg/L,	38-63%	-	(21)

					Dis_eDNA: 0.06–2.37 μg/L			
Multiple	PVDF	Sediment: 1 g, Water: 200 mL	NaP wash	Spectrophotometer	Haihe River sediment: 96.8 ± 19.8 μg/g, Haihe River water: 2.2 ± 0.8 μg/mL	37-81%	-	(16)
	PES	250 mL, 0.25 g	DNase treatment	qPCR	Soil: ~22%, Sediment: ~30%, Gut: ~40%, Water: ~48%, range 0 – 83%	98% eDNA removal	Minimal difference	(7)
	PES	1000 mL	0.2 μm Prefiltration - DEAE cellulose chromatographic column	Qubit dsDNA assay	Influent: ~12.5 μg/L, Activated sludge: ~12.3 μg/L, Effluent: ~8.6 μg/L	-	No	(23)

220

221 qPCR can also be used to quantify eDNA using specific target genes such as the 16S rRNA or
222 18S rRNA gene. Interestingly, results from fluorometric assays and qPCR are not always
223 congruent. For example, studies using the alkaline method for eDNA extraction reported a very
224 high fraction of eDNA in total DNA with the eDNA/iDNA ratio of 6-68 (14, 15) in sediment
225 samples whereas qPCR suggested only ~10-80% (average ~ 40%) of the 16S rRNA genes
226 originated from eDNA (7, 60). The discrepancy could be because the use of qPCR for target
227 genes such as the 16S rRNA gene often substantially underestimate eDNA content in
228 environmental samples. For example, although eDNA was abundant in marine sediments, qPCR
229 failed to amplify the 1500 bp 16S rRNA gene from the eDNA fraction (15). Low integrity of
230 eDNA, the presence of PCR inhibitors, the copy number of target marker gene, and target
231 amplicon size are factors contributing to the high variability in eDNA quantification by qPCR.
232 For example, in Aarhus Bay sediment samples, qPCR for bacteria and archaea suggested that
233 eDNA as 42-51% and 29-71% of total DNA, respectively, while fluorescence spectroscopic
234 measurement showed the eDNA fraction to be 49-61% (60).

235 **5. Effect of eDNA on DNA-based community analyses and its mitigation**

236 The effect of eDNA on metagenomic studies may vary for different environmental samples.
237 Lennon et al. (7) showed, using a statistical model, that if the pool of eDNA is similar to iDNA,
238 the presence of eDNA would not affect DNA-based biodiversity analyses. Otherwise, eDNA
239 could cause over- or under-estimation of the biodiversity. Hence, the effect of eDNA on DNA-
240 based community analyses depends on factors causing dissimilarity between eDNA and iDNA,
241 for example, decay of eDNA in the given environmental conditions. Lennon et al. (7) reported
242 that although eDNA accounted for a high fraction of total DNA in sediment, soil, gut and surface
243 water samples, no significant effect of eDNA on the richness and evenness of the detected
244 microbial communities was observed. Gustavo et al. (69) observed significant differences in 16S
245 rRNA gene copy numbers in 3 out of 12 samples. After removing eDNA signal from the 3
246 samples, no significant change was observed for community composition. However, 3 out of the
247 top 16 detected OTUs showed eDNA-based fluctuations across different depths in the sediment
248 samples. Gustave et al. (67) reported minimal influence of eDNA on community composition in
249 paddy soil microbial fuel cells, but also showed significant changes in relative abundance of
250 certain taxa at genus level after removing eDNA signal.

251 Carini et al. (2) reported a significant difference of eDNA (~40% of total DNA) removal on the
252 soil community detection. The presence of eDNA in soil samples resulted in over- or under-
253 estimation of some bacterial and fungal taxa via amplicon analysis. In addition, the spatial (71)
254 and temporal (66) shift in community structure were found more apparent in analyses excluding
255 eDNA in soil samples and anaerobic digesters (72). Torti et al. (17) also reported OTUs unique
256 to eDNA in sediment samples from the Aarhus bay. In our recent study, we reported a significant
257 effect of eDNA removal on community analysis at the genus level in drinking water (24).

258 The effect of eDNA on community characterization is more discernible at the ASV/OTU or
259 genus level. Abundance of a higher taxonomic level (e.g., phylum or class level) is a summation
260 of abundances of lower taxonomic level (i.e., abundance of a class is a summation of abundances
261 of all orders under it. The abundance of each order is a summation of abundances of family
262 under it and so on). Thus, increase or decrease in abundance of a genera at a higher taxonomic
263 level (e.g., phylum or class level), may not be evident or accurately determined; at lower
264 taxonomic levels, upon eDNA removal, some taxa are under-detected while some are over-
265 detected. At higher taxonomic ranks, such changes would be confounded and become
266 undetectable.

267 To mitigate the effect of eDNA on DNA-based community analyses, we can either remove
268 eDNA from the total DNA or inhibit the amplification of eDNA in PCR using propidium
269 monoazide (PMA) for DNA metabarcoding (Figure 4). eDNA can be extracted using the
270 discussed method (Figure 3) and the remaining biomass can be used to extract iDNA. Nucleases
271 can also be used to degrade eDNA, while iDNA may remain intact when the treatment
272 conditions are carefully optimized (7, 24, 73). Lennon et al. (7) reported an eDNA removal
273 efficiency of 97-99% using DNase on soil samples, while for drinking water samples we
274 reported ~99% eDNA degradation efficiency (24). DNA-intercalating dyes such as PMA can be
275 used to inhibit PCR amplification of eDNA (2, 65, 66, 69, 74). Treatment of samples with
276 optimized protocols for PMA treatment minimized contamination of eDNA with iDNA-based
277 community analyses. Nocker et al. (75) evaluated PMA for cell lysis of various Gram-positive
278 and Gram-negative bacteria and concluded that it did not lyse intact cells. Similarly, Carini et al.
279 (2) reported that PMA treatment did not cause lysis of exponentially growing cells of *E. coli* and
280 *S. cerevisiae*. However, the efficiency of these treatments might vary from sample to sample. In

281 a comparative study, Villarreal et al. (73) reported comparable performance of DNase and PMA
282 treatments in differentiating live/dead cells in drinking water biofilms and suggested DNase
283 treatment as a "more practical alternative" to the PMA-qPCR method. Using PMA, Gustavo et
284 al. (69) reported an eDNA PCR signal removal efficiency of 73–98% in sediment samples, while
285 Carini et al. (2) reported that PMA could not remove PCR signals of the spiked DNA in one
286 sample type. Wagner et al. (65) reported no difference in DGGE patterns observed between total
287 and PMA-treated soil samples (~15% reduction in DNA yield after PMA treatment), whereas
288 Agnelli et al. (11) have observed significant differences in DGGE banding of total and
289 extracellular DNA (10-60% of total DNA) extracted using the alkaline method. These studies
290 suggest that the removal efficiency of PCR signals from eDNA using the PMA treatment may
291 vary substantially for different environmental samples, depending on the physicochemical
292 properties of the sample matrices and other factors influencing PCR efficiency. Although DNase
293 treatment has been demonstrated effective in mitigating the influence of eDNA on metagenomic
294 studies, it is not applicable to environmental samples containing DNase inhibitors that may
295 substantially reduce eDNA removal efficiency.

296 The choice of approach depends largely on the research objectives and practical considerations,
297 for example, sample types, number of samples and inhibitors. Extraction of eDNA and iDNA
298 separately can be used if the research questions require characterization of eDNA. This approach
299 differentiates the two fractions of total DNA, allowing determination of the true community
300 structure and metabolic potential by analyzing the iDNA fraction. eDNA analysis can be used to
301 determine its composition and if it harbors important genes such as antibiotic resistance genes
302 and mobile genetic elements which can be disseminated to naturally competent bacterial cells via
303 transformation. A major limitation of this method is the recovery efficiency of eDNA from
304 environmental samples. Due to intrinsic variability in environmental samples, most studies report
305 a large variation in extraction efficiencies ranging from 10-90%, which may cause discrepancy in
306 data interpretation (Table 1). In contrast, DNA removal strategies (DNase/PMA treatment) show
307 a higher efficiency of eDNA removal across studies (73-99%). However, these treatments
308 complicate downstream computational analyses. Optimization is needed as the effectiveness of
309 DNase/PMA treatment varies between different sample types. Using these approaches, the
310 contribution of eDNA may be indirectly inferred by examining the difference in total DNA- and
311 iDNA-based community analyses.

6. Concluding Remarks

DNA-metabarcoding and metagenomics have revolutionised the way we understand natural and engineered ecosystems. While the methods are evolving, there are some fundamental issues that need to be addressed. Specifically, there is an immediate need for researchers to acknowledge the persistence of eDNA in environmental samples and mitigate its effect on DNA-based community analyses. This field of research will be driven forward by: (i) carefully designing environmental DNA extraction pipelines by taking into consideration technical details in methods for eDNA extraction/removal and membrane-based filtration and concentration; (ii) quantifying eDNA in extracted environmental DNA using multiple methods including qPCR and fluorescent DNA binding dyes; (iii) carefully interpreting effect of eDNA on DNA-based community analyses at different taxonomic levels; and (iv) when possible, removing eDNA from environmental samples for DNA-based community analyses.

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Notes

The authors declare no competing financial interest.

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537 **Figure Legends**

538

539 **Figure 1.** eDNA is ubiquitous in nature and can be found in natural (sediments (1, 3, 13-18), soil
540 (1, 9-12), air (19), freshwater and seawater (1, 3, 20, 21)) and engineered ecosystems (waste
541 water treatment plant (22, 23) and drinking water distribution systems (24)).

542

543 **Figure 2.** Persistence of eDNA in water (△), soil (□) and sediments (○). eDNA can persist in
544 water for 10 h to 28 days(16, 37-42) while eDNA in soil(1, 32-36, 43) and sediments(16, 37, 41,
545 44-46) can persist for a minimum of 24-28 days up to thousands of years. Numbers next to the
546 symbols are reference numbers for the sources of literature. The reported results are observations
547 from different studies, considering all factors, including environmental matrices, that may
548 influence eDNA persistence in respective experimental systems.

549

550 **Figure 3.** Isolation of eDNA from solid and liquid environmental samples. NaP: Sodium
551 phosphate buffer, TE: Tris-EDTA buffer, iDNA: Intracellular DNA, eDNA: Extracellular DNA,
552 Dis_eDNA: Dissolved eDNA, Ads_eDNA, NAAP: Nucleic acid adsorption particles, GF: Glass
553 fiber membrane, MC: Mixed Cellulose ester membrane, NC: Nitrocellulose membrane, PES:
554 Polyether sulfonate membrane, PC: Polycarbonate membrane, AEX: Anion Exchange Column,
555 MB: Magnetic Beads, Pk: Proteinase K.

556

557 **Figure 4.** A schematic illustration showing the effects of eDNA on metagenomic studies and
558 ways to mitigate them. In a typical metagenomic study, microbial communities obtained from
559 the environments (**1**) are subjected to DNA extraction (**2**) to obtain total DNA containing both
560 iDNA and eDNA. The DNA can be analysed for the presence of marker genes by amplifying
561 them using PCR followed by sequencing the amplicons (**3**). The DNA can also be directly
562 sequenced using shotgun sequencing (**4**) and genomes of sampled organisms (MAGs,
563 metagenome-assembled genomes) can be reconstructed. Effects of eDNA can be mitigated using
564 PMA treatment (**T1**) or DNase treatment (**T2**). PMA treatment inhibits PCR amplification of
565 marker genes from eDNA, while DNase treatment removes eDNA from the environmental
566 samples before DNA extraction.

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