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MINIREVIEW

Extracellular DNA in Environmental Samples: Occurrence, Extraction, Quantification, and Impact on Microbial Biodiversity Assessment

Sakcham Bairoliya,[a,b] Jonas Koh Zhi Xiang,[a] Bin Cao[a,b]

[a]Singapore Centre for Environmental Life Sciences Engineering, Interdisciplinary Graduate Program, Nanyang Technological University, Singapore
[b]School of Civil and Environmental Engineering, Nanyang Technological University, Singapore

ABSTRACT Environmental DNA, i.e., DNA extracted directly from environmental samples, has been used to understand microbial communities in the environment 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 suggested 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 interpreting the 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

INTRODUCTION

In recent years, analysis of 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. DNA from an environmental sample often contains both intracellular DNA (iDNA), which represents DNA contained within intact cells, and extracellular DNA (eDNA), which represents DNA outside an organism (1–4). DNA can be released into the environment from organisms through cell lysis and is mediated by predation or phage infection and the release of membrane vesicles (5, 6). Studies have shown that eDNA can persist and accumulate in various environments, such as soil (e.g., relic DNA and adsorbed DNA [ads_DNA] (2, 7)), and aquatic systems (e.g., dissolved DNA [dis_DNA] (8)), although the abundance of eDNA might vary in different environmental systems (7).

eDNA can serve as a source of nutrients to support microbial growth, as a source of free genetic material for iDNA repair and the acquisition of favorable functions, and as a structural component in biofilms (5). In environmental DNA-based biodiversity monitoring through DNA sequencing, eDNA may complicate the determination of the

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Address correspondence to Bin Cao, bincao@ntu.edu.sg.
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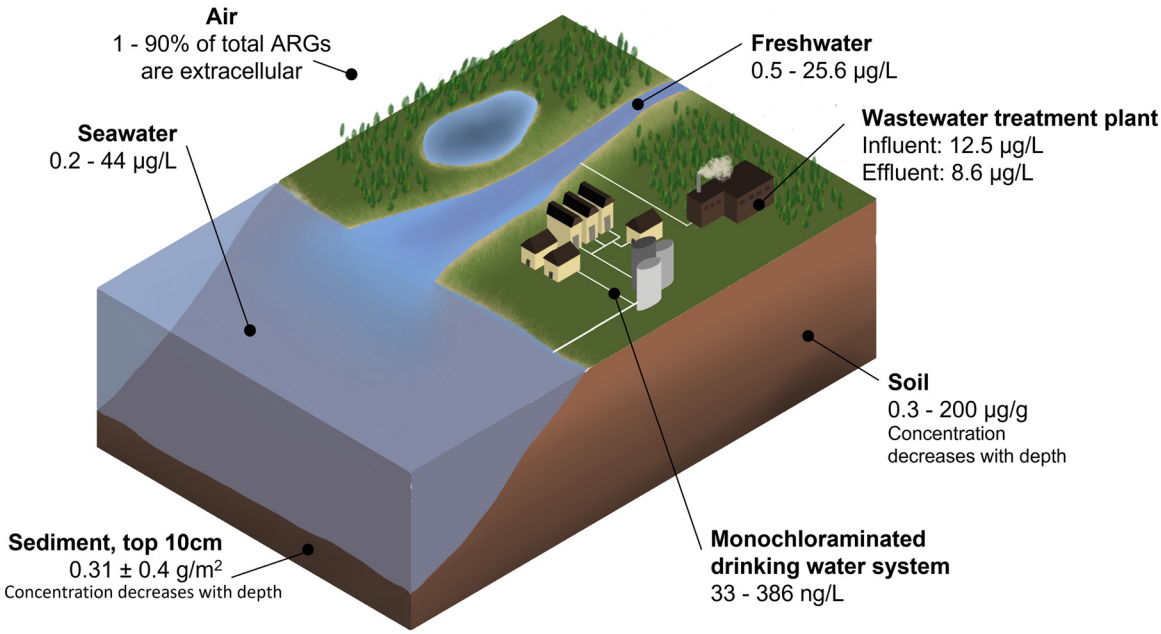

FIG 1 eDNA is ubiquitous and can be found in natural ecosystems (sediments [1, 3, 13 to 18], soil [1, 9–12], air [24], freshwater, and seawater [1, 3, 19, 21]) and engineered ecosystems (wastewater treatment plant [22, 23] and drinking water distribution systems [20]).

structure and potential function of the community inhabiting the sampled environment. As eDNA in environmental samples is a mixture of genomic DNA derived from cell lysis along with plasmids etc., general bioinformatics pipelines cannot effectively distinguish between eDNA and iDNA, highlighting the need to mitigate the effect of eDNA in the design of experiments. 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. Here, we summarized key findings on eDNA in environmental samples and discussed the methods used to extract and quantify eDNA as well as the importance of eDNA on the interpretation of experimental results. We then suggested several factors to consider when designing experiments and analyzing data to negate/determine the contribution of eDNA to environmental DNA-based community analyses.

OCCURRENCE OF eDNA IN ENVIRONMENTAL SAMPLES

eDNA is ubiquitous. It has been found in natural systems, such as soil, sediments, water, and air as well as in engineered systems, such as wastewater treatment plants, drinking water, and anaerobic digesters (Fig. 1). In soil samples, eDNA concentrations range from 0.3 to 200 μg/g of soil (9) and vary 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 concentrations have been observed to decrease with an increase in the 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 sediment at both shallow and deep sites (0.5 to 0.6 μg eDNA/g wet sediment) and decreased to below detection limits at a depth of 30 to 35 cm (13). Various amounts of eDNA have 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 large amounts of eDNA, which are 6- to 68-fold 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 (17). Globally, the top 10 cm of deep-sea sediment 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 to 44 μg eDNA/L of water depending on the location of sampling (estuarine versus coastal versus offshore) while freshwater ecosystems can harbor 0.5 to 25.6 μg eDNA/L of water (3). More recently, 10.3 μg eDNA/L of water was found in the hypersaline lake environment (19). Monochloraminated drinking water systems were also shown to have a low quantity (33 to 386 ng/L) but a significant proportion (~50%) of eDNA in total DNA (20). Another study detected 0.12 to 2.5 μg eDNA/L in the 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 with 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 μg and 8.6 μg eDNA/L, respectively. Activated sludge was also found to contain substantial amount of eDNA (12.3 μg/L) (23). Although reduced amounts of eDNA were found in the water column compared to the soil and sediment, eDNA still formed a major proportion of the total DNA extracted from water samples.

The presence of eDNA in the discussed system has been known for 3 decades with the earliest reports arising in the mid-1980s. A new study showed that, apart from these conventionally studied systems, air can also contain eDNA. The study focused on the presence of eARGs adsorbed to the PM2.5 particles and showed that the ARGs in the iDNA fraction were distinct from the eDNA fraction (24). Overall, eDNA contributes significantly to total DNA extracted from environmental samples, and further studies are needed to understand its implication, fate, and biological significance in different environments.

PERSISTENCE OF eDNA IN ENVIRONMENTAL SAMPLES

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

Degradation of eDNA in various environments has been previously summarized by Nielsen et al. (1) and Pietramellara et al. (9) and can vary between different environmental matrices (Fig. 2). More recently, Sirois et al. (32) reported that the persistence of eDNA in soil is positively correlated with its organic matter content while higher moisture and temperature enhanced eDNA degradation. Although the eDNA standard (469 bp *htrA* gene fragment) was observed to decline rapidly upon its introduction into soil microcosms (~99% reduction in 7 days), the eDNA standard could be detected for up to 80 days. A similar phenomenon was reported by Kunadiya et al. (33), where degradation of DNA from *Phytophthora cinnamomi* was accelerated in moist soils compared to dry soils. eDNA in dry soils could be easily detected for up to 90 days using qPCR. Gordon et al. (34) also reported the successful detection of *Phaeocollybia* eDNA up to 60 days in soil samples. Plasmids incubated in soil microcosms are also known to persist for at least

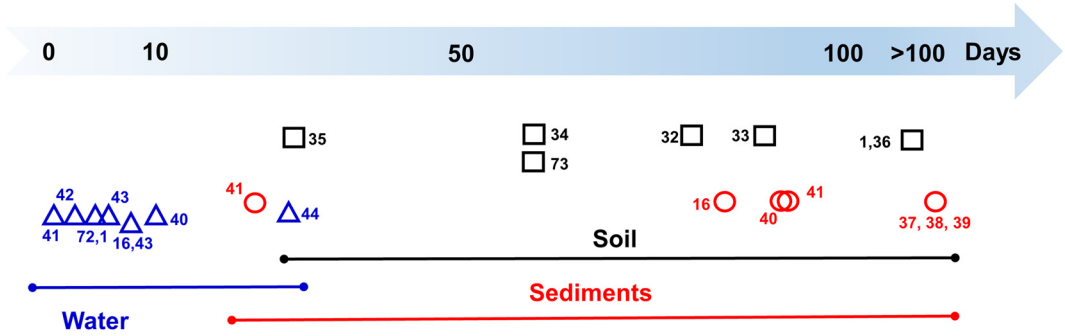

FIG 2 Persistence of eDNA in water (△), soil (□) and sediments (○). eDNA can persist in water for 10 h to 28 days (16, 40–44, 72), while eDNA in soil (1, 32–36, 73) and sediments (16, 37–41) can persist for a minimum of 24 to 28 days up to thousands of years. Numbers next to the symbols are reference numbers for the sources of literature. The reported results are observations from different studies, considering all factors, including environmental matrices, that may influence eDNA persistence in respective experimental systems.

28 days (35). eDNA introduced as cell lysate into soils was seen to persist for 24 weeks (36).

DNA is effectively preserved in the marine sediments as evidenced by retrieval of eDNA as old as 217,000 years (37, 38) and the successful amplification of 16S rRNA genes from eDNA extracted from sediment samples that were 10,000 years old (39). Using microcosms, Mao et al. (16) reported that eDNA degraded faster in Haihe river water (~100% within 7 days) than in the Haihe river sediments (detectable after 12 weeks). Deere et al. (40), also observed eDNA to persist in the water column for 10 days, whereas it could still be detected in sediments for up to 13 weeks. Dell'Anno et al. (41) estimated the turnover time for DNA in sediments to be longer than water (29 to 93 days in sediments versus 10 h in seawater). In forest pond sediment-water microcosms, England et al. (42) reported that viral eDNA from *Baculovirus* could be detected for 24 h, whereas the eDNA could persist for days in laboratory microcosms. Saito et al. (43) reported that eDNA was not degraded in purified water over the experimental period of 7 days, whereas it persisted in pond water for 5 days and could be detected in seawater after 7 days. Similar to Mao et al. (16), Saito et al. (43) also observed rapid initial decay of eDNA in environmental water samples, which were attributed to the microbial activity and action of extracellular enzymes. However, a study by Bukh et al. (44) reported eDNA to persist for at least 28 days in hot tap water. The rapid decay of eDNA in environmental waters compared to engineered water systems could also be attributed to dissolved organic matter, e.g., humic substances, organic acids, and carbohydrates. This organic matter can be photosensitized by sunlight, resulting in the formation of reactive oxygen species (ROS) and hydroxyl radicals which facilitate the decay of DNA (45, 46). Overall, DNA seems to persist longer in sediments and soils compared to water samples. This might be attributed to faster eDNA decay in water because of reduced protection from extracellular nucleases, increased microbial activity (43, 47), a high chance of ROS-induced DNA damage and higher temperatures (48, 49), or a reduced detection efficiency due to lower DNA concentrations in water environments and DNA binding to organic matter (50).

Due to its persistence, eDNA serves as a possible reservoir of antibiotic resistance genes (ARGs) for horizontal gene transfer via transformation. Uptake of eDNA by competent bacterial cells can result in the dissemination of these genes on a large scale. Mao et al. (16) demonstrated the uptake of the kanamycin resistance gene by an indigenous sediment bacterium under selective pressure. Recent research has suggested that disinfection by-products from chlorine and chloramine can lead to an increase in uptake of exogenous DNA (51). Disinfection using chlorine, chloramine (52), and solar irradiation (53) have also been seen to enhance the transformation process through upregulation in DNA uptake and repair proteins caused by the ROS stress response.

Biofilms in estuarine systems have been shown to accumulate ARGs in eDNA (54) facilitating the horizontal gene transfer process (55). However, most studies are performed under ideal laboratory conditions using model organisms and plasmids. Further studies are required to determine the real contribution of eDNA in the environment as a pool for horizontal gene transfer.

EXTRACTION AND QUANTIFICATION OF eDNA IN ENVIRONMENTAL SAMPLES

Table 1 summarizes previous studies on eDNA in environmental samples. The most common method to extract eDNA from solid phases, such as soil samples is to desorb the ads_eDNA using an alkaline sodium phosphate buffer (NaP) (56) (Fig. 3). The excess phosphate in NaP competes with eDNA for binding sites on clay particles, enabling the desorption and recovery of eDNA from the samples. This method has been widely used with various modifications over the last 3 decades (11, 13–15, 57). A remarkable improvement in the yield of eDNA (~4 to 10-fold) was observed when the samples were pretreated with proteinase K (21). Another method for eDNA extraction from solid phases is to dissolve eDNA in Tris EDTA (TE) buffer from the environmental samples (10, 17, 58) (Fig. 3). Treatment with NaP/TE or proteinase K does not cause cell lysis, thereby preventing contamination of eDNA with iDNA. The obtained crude extracts of eDNA can be purified using conventional DNA extraction techniques such as the cetyltrimethylammonium bromide (CTAB) method, ethanol precipitation, or chromatography. These methods have mainly been applied to soil, sediments, and sludge samples due to practical feasibility as the particles can be resuspended and washed in the extraction buffer.

To extract eDNA from liquid environmental samples, we usually need to concentrate it first (Fig. 3). For example, eDNA in water samples can be concentrated through adsorption by the nucleic acid adsorption particles (NAAPs; silica coated with aluminum hydroxide) followed by elution and precipitation (59). This method has mainly been used to extract extracellular antibiotic-resistant genes (eARG) from different water systems as the NAAPs favor adsorption of short-length linear DNA (59). Another method used a hollow fiber membrane (MWCO of 30 kDa and a surface area of 25 m^2) and silica adsorption to concentrate and extract eDNA, from Tama River water (21). Compared with the NAAPs method, this method gives a higher recovery by allowing the concentration of all DNA to be above 50 bp. Calderon et al. (23) used anion exchange chromatography for eDNA extraction from wastewater samples and found that plasmids and transposable elements were highly enriched in the extracted eDNA. Yuan et al. (60) reported the use of a prefiltration technique combined with magnetic beads to isolate eDNA from water and sludge samples. Using the optimized method, large quantities of free and adsorbed eDNA from small volumes of wastewater and activated sludge samples could be extracted.

Different filter membranes have been used to concentrate eDNA from water samples. Glass fiber (GF) and cellulose nitrate (CN) membranes exhibit the highest affinity to eDNA (~30 to 35% of eDNA retained) (61), followed by polyvinylidene difluoride (PVDF), mixed cellulose esters (MCE), polyether sulfonate (PES) and polycarbonate (PC) membranes (62). Hence, GF or CN filter membranes are recommended for extracting eDNA from the filter retentate, while PC and PES membranes are preferable for separating eDNA from cells and recovering eDNA from the filtrate (Fig. 3). In addition to the adsorption by membrane filters, the retention of eDNA on filters may also be attributed to other components in the samples. For example, using PC membrane for the filtration of stormwater, Liang et al. (62) found that 7 to 13% of their qPCR signals from the filter retentate were derived from the spiked plasmid DNA (eDNA proxy) even though they observed only ~2% eDNA retention in optimization experiments with PC membranes. The increased detection of the spiked plasmid DNA in the retentate can be attributed to the adsorption of eDNA on sand and clay particles retained on the filter membranes (62).

Extracted eDNA is usually quantified by using fluorometric DNA dyes, such as Picogreen (10, 58, 63), Hoechst (8, 11), SYBR green (15), Qubit dsDNA assay kits (2, 20, 21), or DAPI (61). DNA quantification using fluorometric dyes is more specific and

TABLE 1 Summary of previous studies dealing with eDNA in environmental samples[a]

Sample	Filter	Starting material	Method of extraction/ treatment	Quantification	eDNA yield	Recovery/ removal efficiency	Impact on 16S/ 18S rRNA genes 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	(57)
	-	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	(63)
	-	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	(67)
	-	0.03 g	PMA	Qubit ds DNA	Not reported. Calculated: ~1.4 μg/g soil (top layer) in control samples.	-	No	(65)
Sediment	-	100 g	NaP wash	Spectroscopy	1 μg/g sediment	-	-	(56)
	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	-	(74)
	Anopore	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	-	-	(58)
	-	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 73–98% of eDNA	Minimal difference	(64)
	-	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)

(Continued on next page)

TABLE 1 (Continued)

Sample	Filter	Starting material	Method of extraction/ treatment	Quantification	eDNA yield	Recovery/ removal efficiency	Impact on 16S/ 18S rRNA genes detection	Ref
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%	-	(61)
	-	1000 mL	PMA treatment	-	-	-	No	(75)
	PC	100 mL	0.2 μm filtration - eDNA extraction from filter residue	qPCR	7-13% of spiked plasmid recovered from storm water		-	(62)
	NAAP	10 L	NAAP adsorption	TaqMan qPCR	-	95%	-	(59)
	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	(20)
	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, Dis_eDNA: 0.06–2.37 μg/L	38-63%	-	(21)
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)

[a]CA, cellulose acetate; PC, polycarbonate; GF, glass fiber; PES, polyether sulfonate; PICA, phenol-chloroform-isoamyl alcohol; NaP, sodium phosphate buffer; PMA, propidium monoazide; EMA, ethidium monoazide; CTAB, cetyltrimethylammonium bromide; NAAP, nucleic acid adsorption particles; DEAE, diethylaminoethyl cellulose; DGGE, denaturing gradient gel electrophoresis.

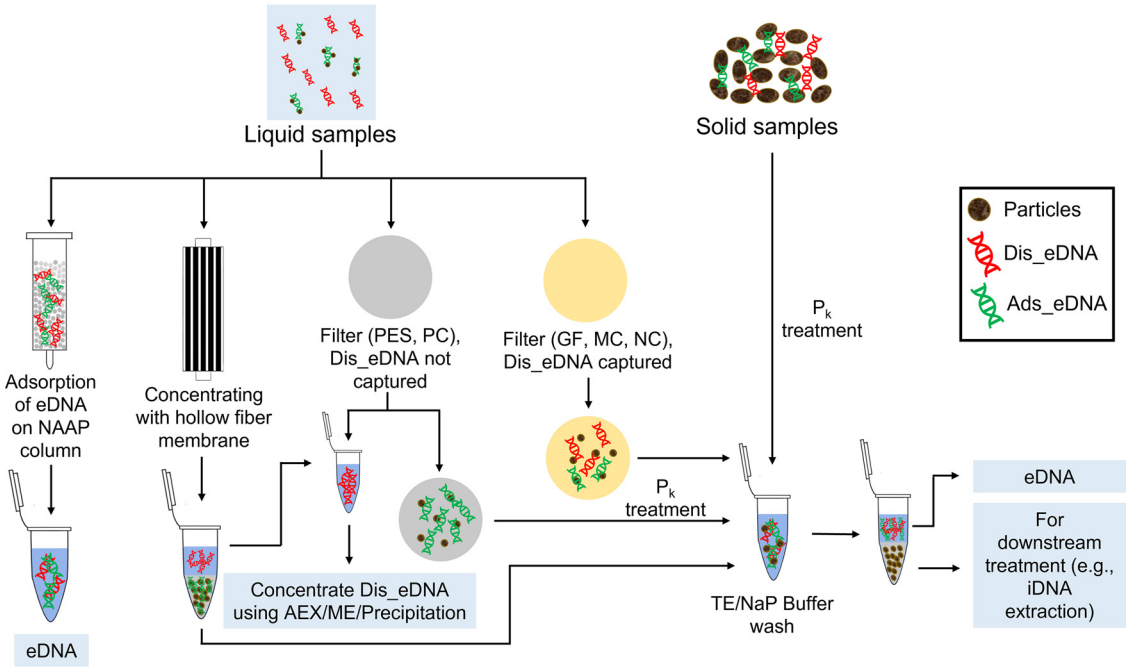

FIG 3 Isolation of eDNA from solid and liquid environmental samples. NaP: sodium phosphate buffer; TE: Tris EDTA buffer; iDNA: intracellular DNA; eDNA: extracellular DNA; Dis_eDNA: dissolved eDNA; Ads_eDNA: adsorbed eDNA; NAAP: nucleic acid adsorption particles; GF: glass fiber membrane; MC: mixed cellulose ester membrane; NC: nitrocellulose membrane; PES: polyether sulfonate membrane; PC: polycarbonate membrane; AEX: anion exchange column; ME: magnetic beads; Pk: proteinase K.

accurate compared to absorption spectroscopy methods which are nonspecific. For instance, freshwater ecosystems usually harbor 0.5 – 25.6 μg eDNA/L of water (3). A study conducted in the Haihe River quantified eDNA using absorption spectroscopy and reported eDNA concentrations of ~2,000 μg eDNA/L in the water samples (16). This concentration is likely overestimated because of the low specificity of the spectrometric DNA quantification method.

qPCR can also be used to quantify eDNA using specific target genes such as the 16S rRNA or 18S rRNA gene. Interestingly, results from fluorometric assays and qPCR are not always congruent. For example, studies using the alkaline method for eDNA extraction reported a very high fraction of eDNA in total DNA with the eDNA/iDNA ratio of 6 to 68 (14, 15) in sediment samples whereas qPCR suggested only ~10 to 80% (average ~40%) of the 16S rRNA genes originated from eDNA (7, 58). The discrepancy could be because the use of qPCR for target genes such as the 16S rRNA gene often substantially underestimates eDNA content in environmental samples. For example, although eDNA was abundant in marine sediments, qPCR failed to amplify the 1,500 bp 16S rRNA gene from the eDNA fraction (15). Low integrity of eDNA, the presence of PCR inhibitors, the copy number of the target marker gene, and target amplicon size are factors contributing to the high variability in eDNA quantification by qPCR. For example, in Aarhus Bay sediment samples, qPCR for bacteria and archaea suggested that eDNA as 42 to 51% and 29 to 71% of total DNA, respectively, while fluorescence spectroscopic measurement showed the eDNA fraction to be 49 to 61% (58).

EFFECT OF eDNA ON DNA-BASED COMMUNITY ANALYSES AND ITS MITIGATION

The effect of eDNA on metagenomic studies may vary for different environmental samples. Lennon et al. (7) showed, using a statistical model, that if the pool of eDNA is similar to iDNA, the presence of eDNA would not affect DNA-based biodiversity analyses. Otherwise, eDNA could cause overestimation or underestimation of biodiversity. Hence, the effect of eDNA on DNA-based community analyses depends on factors causing dissimilarity between eDNA and iDNA, for example, decay of eDNA in the

given environmental conditions. Lennon et al. (7) reported that although eDNA accounted for a high fraction of total DNA in sediment, soil, gut, and surface water samples, no significant effect of eDNA on the richness and evenness of the detected microbial communities was observed. Gustavo et al. (64) observed significant differences in 16S rRNA gene copy numbers in 3 out of 12 samples. After removing the eDNA signal from the 3 samples, no significant change was observed for community composition. However, 3 out of the top 16 detected OTUs showed eDNA-based fluctuations across different depths in the sediment samples. Gustave et al. (65) reported a minimal influence of eDNA on community composition in paddy soil microbial fuel cells but also showed significant changes in the relative abundance of certain taxa at the genus level after removing the eDNA signal.

Carini et al. (2) reported a significant difference of eDNA (~40% of total DNA) removal on the soil community detection. The presence of eDNA in soil samples resulted in the overestimation or underestimation of some bacterial and fungal taxa via amplicon analysis. In addition, the spatial (66) and temporal (67) shifts in community structure were found more apparent in analyses excluding eDNA in soil samples and anaerobic digesters (68). Torti et al. (17) also reported OTUs unique to eDNA in sediment samples from the Aarhus Bay. In our recent study, we reported a significant effect of eDNA removal on community analysis at the genus level in drinking water (20).

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

To mitigate the effect of eDNA on DNA-based community analyses, we can either remove eDNA from the total DNA or inhibit the amplification of eDNA in PCR using propidium monoazide (PMA) for DNA metabarcoding (Fig. 4). eDNA can be extracted using the discussed method (Fig. 3) and the remaining biomass can be used to extract iDNA. Nucleases can also be used to degrade eDNA, while iDNA may remain intact when the treatment conditions are carefully optimized (7, 20, 69). Lennon et al. (7) reported an eDNA removal efficiency of 97 to 99% using DNase on soil samples, while for drinking water samples we reported ~99% eDNA degradation efficiency (20). DNA-intercalating dyes such as PMA can be used to inhibit the PCR amplification of eDNA (2, 63, 64, 67, 70). Treatment of samples with optimized protocols for PMA treatment minimized contamination of eDNA with iDNA-based community analyses. Nocker et al. (71) evaluated PMA for cell lysis of various Gram-positive and Gram-negative bacteria and concluded that it did not lyse intact cells. Similarly, Carini et al. (2) reported that PMA treatment did not cause lysis of exponentially growing cells of *Escherichia coli* and *Saccharomyces cerevisiae*. However, the efficiency of these treatments might vary from sample to sample. In a comparative study, Villarreal et al. (69) reported comparable performance of DNase and PMA treatments in differentiating live/dead cells in drinking water biofilms and suggested DNase treatment as a "more practical alternative" to the PMA-qPCR method. Using PMA, Gustavo et al. (64) reported an eDNA PCR signal removal efficiency of 73 to 98% in sediment samples, while Carini et al. (2) reported that PMA could not remove PCR signals of the spiked DNA in one sample type. Wagner et al. (63) reported no difference in DGGE patterns observed between total and PMA-treated soil samples (~15% reduction in DNA yield after PMA treatment), whereas Agnelli et al. (11) have observed significant differences in DGGE banding of total and extracellular DNA (10 to 60% of total DNA) extracted using the alkaline method. These studies suggest that the removal efficiency of PCR signals from eDNA using the PMA treatment may vary substantially for different environmental samples, depending on the

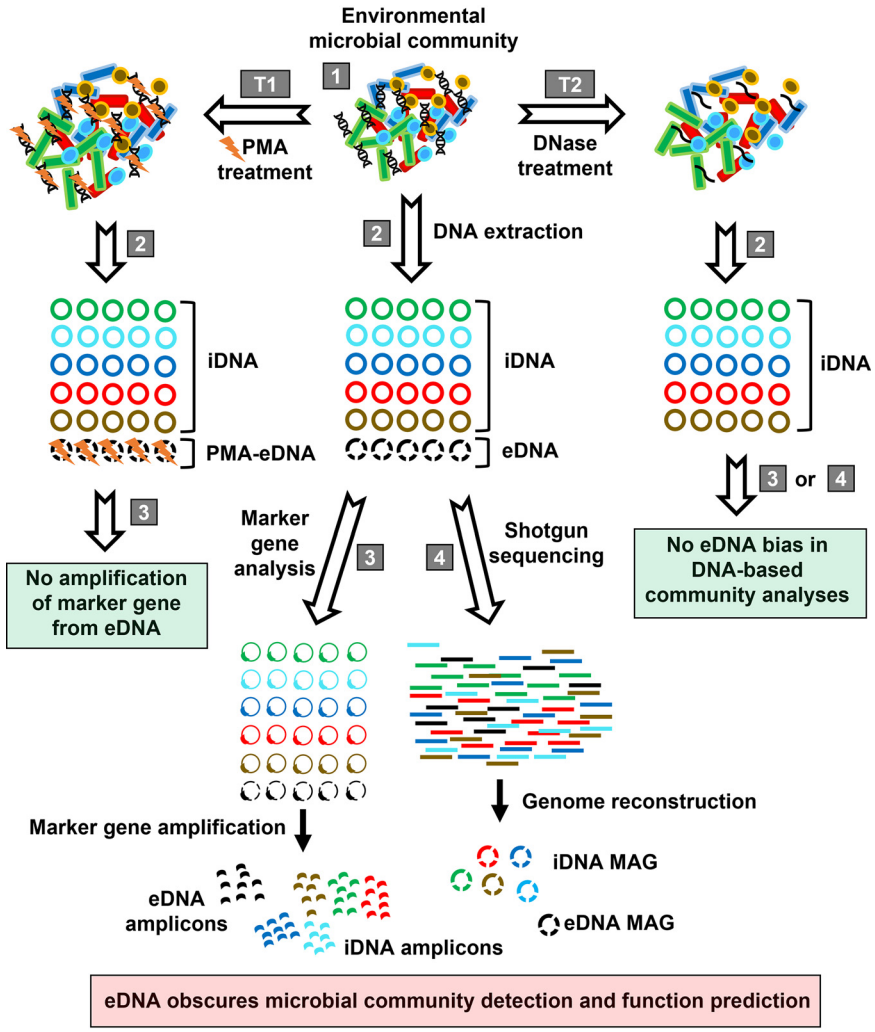

FIG 4 A schematic showing the effects of eDNA on metagenomic studies and ways to mitigate them. In a typical metagenomic study, microbial communities obtained from the environments (1) are subjected to DNA extraction (2) to obtain total DNA containing both iDNA and eDNA. The DNA can be analyzed for the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

physicochemical properties of the sample matrices and other factors influencing PCR efficiency. Although DNase treatment has been demonstrated effective in mitigating the influence of eDNA on metagenomic studies, it does not apply to environmental samples containing DNase inhibitors that may substantially reduce eDNA removal efficiency.

The choice of approach depends largely on the research objectives and practical considerations, for example, sample types, number of samples, and inhibitors. Extraction of eDNA and iDNA separately can be used if the research questions require the characterization of eDNA. This approach differentiates the two fractions of total DNA, allowing the determination of the true community structure and metabolic potential by analyzing the iDNA fraction. eDNA analysis can be used to determine its composition and if it harbors important genes such as antibiotic resistance genes and mobile genetic elements which can be disseminated to naturally competent bacterial cells via transformation. A major limitation of this method is the recovery efficiency of eDNA from environmental samples.

Due to intrinsic variability in environmental samples, most studies report a large variation in extraction efficiencies ranging from 10 to 90%, which may cause a discrepancy in data interpretation (Table 1). In contrast, DNA removal strategies (DNase/PMA treatment) show a higher efficiency of eDNA removal across studies (73 to 99%). However, these treatments complicate downstream computational analyses. Optimization is needed as the effectiveness of DNase/PMA treatment varies between different sample types. Using these approaches, the contribution of eDNA may be indirectly inferred by examining the difference in total DNA- and iDNA-based community analyses.

CONCLUDING REMARKS

DNA-metabarcoding and metagenomics have revolutionized the way we understand natural and engineered ecosystems. While the methods are evolving, some fundamental issues need to be addressed. Specifically, there is an immediate need for researchers to acknowledge the presence and 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 the 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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REFERENCES

1. Nielsen KM, Johnsen PJ, Bensasson D, Daffonchio D. 2007. Release and persistence of extracellular DNA in the environment. Environ Biosafety Res 6:37–53. https://doi.org/10.1051/ebr:2007031.
2. Carini P, Marsden PJ, Leff JW, Morgan EE, Strickland MS, Fierer N. 2016. Relic DNA is abundant in soil and obscures estimates of soil microbial diversity. Nat Microbiol 2:16242. https://doi.org/10.1038/nmicrobiol.2016.242.
3. Torti A, Lever MA, Jørgensen BB. 2015. Origin, dynamics, and implications of extracellular DNA pools in marine sediments. Marine Genomics 24:185–196. https://doi.org/10.1016/j.margen.2015.08.007.
4. Nagler M, Insam H, Pietramellara G, Ascher-Jenull J. 2018. Extracellular DNA in natural environments: features, relevance and applications. Appl Microbiol Biotechnol 102:6343–6356. https://doi.org/10.1007/s00253-018-9120-4.
5. Ibáñez de Aldecoa AL, Zafra O, González-Pastor JE. 2017. Mechanisms and regulation of extracellular DNA release and its biological roles in microbial communities. Front Microbiol 8. https://doi.org/10.3389/fmicb.2017.01390.
6. Biller SJ, McDaniel LD, Breitbart M, Rogers E, Paul JH, Chisholm SW. 2017. Membrane vesicles in sea water: heterogeneous DNA content and implications for viral abundance estimates. ISME J 11:394–404. https://doi.org/10.1038/ismej.2016.134.
7. Lennon JT, Muscarella ME, Placella SA, Lehmkuhl BK. 2018. How, when, and where relic DNA affects microbial diversity. mBio 9:e00637-18.
8. Deflaun MF, Paul JH, Davis D. 1986. Simplified method for dissolved DNA determination in aquatic environments. Appl Environ Microbiol 52:654–659. https://doi.org/10.1128/aem.52.4.654-659.1986.
9. Pietramellara G, Ascher J, Borgogni F, Ceccherini MT, Guerri G, Nannipieri P. 2009. Extracellular DNA in soil and sediment: fate and ecological relevance. Biol Fertil Soils 45:219–235. https://doi.org/10.1007/s00374-008-0345-8.
10. Niemeyer J, Gessler F. 2002. Determination of free DNA in soils. J Plant Nutr Soil Sci 165:121–124. https://doi.org/10.1002/1522-2624(200204)165:2<121::AID-JPLN1111121>3.0.CO;2-X.
11. Agnelli A, Ascher J, Corti G, Ceccherini MT, Nannipieri P, Pietramellara G. 2004. Distribution of microbial communities in a forest soil profile investigated by microbial biomass, soil respiration and DGGE of total and extracellular DNA. Soil Biol Biochem 36:859–868. https://doi.org/10.1016/j.soilbio.2004.02.004.
12. Ascher J, Ceccherini MT, Pantani OL, Agnelli A, Borgogni F, Guerri G, Nannipieri P, Pietramellara G. 2009. Sequential extraction and genetic fingerprinting of a forest soil metagenome. Appl Soil Ecol 42:176–181. https://doi.org/10.1016/j.apsoil.2009.03.005.
13. Vuillemin A, Horn F, Alawi M, Henny C, Wagner D, Crowe SA, Kallmeyer J. 2017. Preservation and significance of extracellular DNA in ferruginous sediments from Lake Towuti, Indonesia. Front Microbiol 8:1440. https://doi.org/10.3389/fmicb.2017.01440.
14. Alawi M, Schneider B, Kallmeyer J. 2014. A procedure for separate recovery of extra- and intracellular DNA from a single marine sediment sample. J Microbiol Methods 104:36–42. https://doi.org/10.1016/j.mimet.2014.06.009.
15. Corinaldesi C, Danovaro R, Dell'Anno A. 2005. Simultaneous Recovery of extracellular and intracellular DNA suitable for molecular studies from marine sediments. Appl Environ Microbiol 71:46–50. https://doi.org/10.1128/AEM.71.1.46-50.2005.
16. Mao D, Luo Y, Mathieu J, Wang Q, Feng L, Mu Q, Feng C, Alvarez PJJ. 2014. Persistence of extracellular DNA in river sediment facilitates antibiotic resistance gene propagation. Environ Sci Technol 48:71–78. https://doi.org/10.1021/es404280v.

17. Torti A, Jørgensen BB, Lever MA. 2018. Preservation of microbial DNA in marine sediments: insights from extracellular DNA pools. Environ Microbiol 20:4526–4542. https://doi.org/10.1111/1462-2920.14401.
18. Dell'Anno A, Danovaro R. 2005. Extracellular DNA plays a key role in deep-sea ecosystem functioning. Science 309:2179. https://doi.org/10.1126/science.1117475.
19. Aldeguer-Riquelme B, Ramos-Barbero MD, Santos F, Antón J. 2021. Environmental dissolved DNA harbours meaningful biological information on microbial community structure. Environ Microbiol 23:2669–2682. https://doi.org/10.1111/1462-2920.15510.
20. Sakcham B, Kumar A, Cao B. 2019. Extracellular DNA in monochloraminated drinking water and its influence on DNA-based profiling of a microbial community. Environ Sci Technol Lett 6:306–312. https://doi.org/10.1021/acs.estlett.9b00185.
21. Liu M, Hata A, Katayama H, Kasuga I. 2020. Consecutive ultrafiltration and silica adsorption for recovery of extracellular antibiotic resistance genes from an urban river. Environ Pollut 260:114062. https://doi.org/10.1016/j.envpol.2020.114062.
22. Zhang Y, Li A, Dai T, Li F, Xie H, Chen L, Wen D. 2018. Cell-free DNA: a neglected source for antibiotic resistance genes spreading from WWTPs. Environ Sci Technol 52:248–257. https://doi.org/10.1021/acs.est.7b04283.
23. Calderón-Franco D, van Loosdrecht MCM, Abeel T, Weissbrodt DG. 2021. Free-floating extracellular DNA: systematic profiling of mobile genetic elements and antibiotic resistance from wastewater. Water Res 189:116592. https://doi.org/10.1016/j.watres.2020.116592.
24. He T, Jin L, Xie J, Yue S, Fu P, Li X. 2021. Intracellular and extracellular antibiotic resistance genes in airborne PM2.5 for respiratory exposure in urban areas. Environ Sci Technol Lett 8:128–134. https://doi.org/10.1021/acs.estlett.0c00974.
25. Gardner CM, Gunsch CK. 2017. Adsorption capacity of multiple DNA sources to clay minerals and environmental soil matrices less than previously estimated. Chemosphere 175:45–51. https://doi.org/10.1016/j.chemosphere.2017.02.030.
26. Demanèche S, Jocteur-Monrozier L, Quiquampoix H, Simonet P. 2001. Evaluation of biological and physical protection against nuclease degradation of clay-bound plasmid DNA. Appl Environ Microbiol 67:293–299. https://doi.org/10.1128/AEM.67.1.293-299.2001.
27. Ogram AV, Mathot ML, Harsh JB, Boyle J, Pettigrew CA. 1994. Effects of DNA polymer length on its adsorption to soils. Appl Environ Microbiol 60:393–396. https://doi.org/10.1128/aem.60.2.393-396.1994.
28. Parducci L, Bennett KD, Ficetola GF, Alsos IG, Suyama Y, Wood JR, Pedersen MW. 2017. Ancient plant DNA in lake sediments. New Phytol 214:924–942. https://doi.org/10.1111/nph.14470.
29. Corinaldesi C, Barucca M, Luna GM, Dell'Anno A. 2011. Preservation, origin and genetic imprint of extracellular DNA in permanently anoxic deep-sea sediments. Mol Ecol 20:642–654. https://doi.org/10.1111/j.1365-294X.2010.04958.x.
30. Morrissey EM, McHugh TA, Preteska L, Hayer M, Dijkstra P, Hungate BA, Schwartz E. 2015. Dynamics of extracellular DNA decomposition and bacterial community composition in soil. Soil Biol Biochem 86:42–49. https://doi.org/10.1016/j.soilbio.2015.03.020.
31. Wasmund K, Pelikan C, Schintlmeister A, Wagner M, Watzka M, Richter A, Bhatnagar S, Noel A, Hubert CRJ, Rattei T, Hofmann T, Hausmann B, Herbold CW, Loy A. 2021. Genomic insights into diverse bacterial taxa that degrade extracellular DNA in marine sediments. Nat Microbiol 6:885–898. https://doi.org/10.1038/s41564-021-00917-9.
32. Sirois SH, Buckley DH. 2019. Factors governing extracellular DNA degradation dynamics in soil. Environ Microbiol Rep 11:173–184. https://doi.org/10.1111/1758-2229.12725.
33. Kunadiya MB, Burgess TI, Dunstan W, White D, StJ Hardy GE. 2021. Persistence and degradation of Phytophthora cinnamomi DNA and RNA in different soil types. Environmental DNA 3:92–104. https://doi.org/10.1002/edn3.127.
34. Gordon M, Van Norman K. 2021. Mycelial DNA persistence in a forest soil. Environmental DNA 3:1208–1213. https://doi.org/10.1002/edn3.242.
35. Wang F, Che R, Xu Z, Wang Y, Cui X. 2019. Assessing soil extracellular DNA decomposition dynamics through plasmid amendment coupled with real-time PCR. J Soils Sed 19:91–96. https://doi.org/10.1007/s11368-018-2176-z.
36. England LS, Lee H, Trevors JT. 1997. Persistence of Pseudomonas aureofaciens strains and DNA in soil. Soil Biol Biochem 29:1521–1527. https://doi.org/10.1016/S0038-0717(97)00013-8.
37. Coolen MJL, Overmann J. 2007. 217000-year-old DNA sequences of green sulfur bacteria in Mediterranean sapropels and their implications for the reconstruction of the paleoenvironment. Environ Microbiol 9:238–249. https://doi.org/10.1111/j.1462-2920.2006.01134.x.
38. Ellegaard M, Clokie MRJ, Czypionka T, Frisch D, Godhe A, Kremp A, Letarov A, McGenity TJ, Ribeiro S, John Anderson N. 2020. Dead or alive: sediment DNA archives as tools for tracking aquatic evolution and adaptation. Commun Biol 3:169. https://doi.org/10.1038/s42003-020-0899-z.
39. Corinaldesi C, Beolchini F, Dell'Anno A. 2008. Damage and degradation rates of extracellular DNA in marine sediments: implications for the preservation of gene sequences. Mol Ecol 17:3939–3951. https://doi.org/10.1111/j.1365-294X.2008.03880.x.
40. Deere D, Porter J, Pickup RW, Edwards C. 1996. Survival of cells and DNA of Aeromonas salmonicida released into aquatic microcosms. J Appl Bacteriol 81:309–318. https://doi.org/10.1111/j.1365-2672.1996.tb04333.x.
41. Dell'Anno A, Corinaldesi C. 2004. Degradation and turnover of extracellular DNA in marine sediments: ecological and methodological considerations. Appl Environ Microbiol 70:4384–4386. https://doi.org/10.1128/AEM.70.7.4384-4386.2004.
42. England LS, Pollok J, Vincent M, Kreutzweiser D, Fick W, Trevors JT, Holmes SB. 2005. Persistence of extracellular baculoviral DNA in aquatic microcosms: extraction, purification, and amplification by the polymerase chain reaction (PCR). Mol Cell Probes 19:75–80. https://doi.org/10.1016/j.mcp.2004.09.004.
43. Saito T, Doi H. 2021. Degradation modeling of water environmental DNA: Experiments on multiple DNA sources in pond and seawater. Environmental DNA 3:850–860. https://doi.org/10.1002/edn3.192.
44. Bukh AS, Roslev P. 2014. Mycobacterium avium complex in day care hot water systems, and persistence of live cells and DNA in hot water pipes. Curr Microbiol 68:428–439. https://doi.org/10.1007/s00284-013-0493-4.
45. Zhang X, Li J, Fan W-Y, Yao M-C, Yuan L, Sheng G-P. 2019. enhanced photodegradation of extracellular antibiotic resistance genes by dissolved organic matter photosensitization. Environ Sci Technol 53:10732–10740. https://doi.org/10.1021/acs.est.9b03096.
46. Zhang X, Li J, Yao M-C, Fan W-Y, Yang C-W, Yuan L, Sheng G-P. 2020. Unrecognized contributions of dissolved organic matter inducing photodamages to the decay of extracellular DNA in waters. Environ Sci Technol 54:1614–1622. https://doi.org/10.1021/acs.est.9b06029.
47. Alvarez AJ, Yumet GM, Santiago CL, Toranzos GA. 1996. Stability of manipulated plasmid DNA in aquatic environments. Environ Toxicol Water Qual 11:129–135. https://doi.org/10.1002/(SICI)1098-2256(1996)11:2<129::AID-TOX8>3.0.CO;2-B.
48. Dupray E, Caprais MP, Derrien A, Fach P. 1997. Salmonella DNA persistence in natural seawaters using PCR analysis. J Appl Microbiol 82:507–510. https://doi.org/10.1046/j.1365-2672.1997.00143.x.
49. Tsuji S, Ushio M, Sakurai S, Minamoto T, Yamanaka H. 2017. Water temperature-dependent degradation of environmental DNA and its relation to bacterial abundance. PLoS One 12:e0176608. https://doi.org/10.1371/journal.pone.0176608.
50. van Bochove K, Bakker FT, Beentjes KK, Hemerik L, Vos RA, Gravendeel B. 2020. Organic matter reduces the amount of detectable environmental DNA in freshwater. Ecol Evol 10:3647–3654. https://doi.org/10.1002/ece3.6123.
51. Mantilla-Calderon D, Plewa MJ, Michoud G, Fodelianakis S, Daffonchio D, Hong P-Y. 2019. Water disinfection byproducts increase natural transformation rates of environmental DNA in Acinetobacter baylyi ADP1. Environ Sci Technol 53:6520–6528. https://doi.org/10.1021/acs.est.9b00692.
52. Zhang S, Wang Y, Lu J, Yu Z, Song H, Bond PL, Guo J. 2021. Chlorine disinfection facilitates natural transformation through ROS-mediated oxidative stress. ISME J 15:2969–2985. https://doi.org/10.1038/s41396-021-00980-4.
53. Augsburger N, Mantilla-Calderon D, Daffonchio D, Hong P-Y. 2019. Acquisition of extracellular DNA by Acinetobacter baylyi ADP1 in response to solar and UV-C254nm disinfection. Environ Sci Technol 53:10312–10319. https://doi.org/10.1021/acs.est.9b01206.
54. Guo X-p, Yang Y, Lu D-p, Niu Z-s, Feng J-n, Chen Y-r, Tou F-y, Garner E, Xu J, Liu M, Hochella MF. 2018. Biofilms as a sink for antibiotic resistance genes (ARGs) in the Yangtze Estuary. Water Res 129:277–286. https://doi.org/10.1016/j.watres.2017.11.029.
55. Abe K, Nomura N, Suzuki S. 2020. Biofilms: hot spots of horizontal gene transfer (HGT) in aquatic environments, with a focus on a new HGT mechanism. FEMS Microbiol Ecol 96. https://doi.org/10.1093/femsec/fiaa031.
56. Ogram A, Sayler GS, Barkay T. 1987. The extraction and purification of microbial DNA from sediments. J Microbiol Methods 7:57–66. https://doi.org/10.1016/0167-7012(87)90025-X.

57. Ceccherini MT, Ascher J, Agnelli A, Borgogni F, Pantani OL, Pietramellara G. 2009. Experimental discrimination and molecular characterization of the extracellular soil DNA fraction. Antonie Van Leeuwenhoek 96:653–657. https://doi.org/10.1007/s10482-009-9354-3.
58. Lever MA, Torti A, Eickenbusch P, Michaud AB, Šantl-Temkiv T, Jørgensen BB. 2015. A modular method for the extraction of DNA and RNA, and the separation of DNA pools from diverse environmental sample types. Front Microbiol 6. https://doi.org/10.3389/fmicb.2015.00476.
59. Wang D-N, Liu L, Qiu Z-G, Shen Z-Q, Guo X, Yang D, Li J, Liu W-l, Jin M, Li J-W. 2016. A new adsorption-elution technique for the concentration of aquatic extracellular antibiotic resistance genes from large volumes of water. Water Res 92:188–198. https://doi.org/10.1016/j.watres.2016.01.035.
60. Yuan Q-B, Huang Y-M, Wu W-B, Zuo P, Hu N, Zhou Y-Z, Alvarez PJJ. 2019. Redistribution of intracellular and extracellular free & adsorbed antibiotic resistance genes through a wastewater treatment plant by an enhanced extracellular DNA extraction method with magnetic beads. Environ Int 131:104986. https://doi.org/10.1016/j.envint.2019.104986.
61. Siuda W, Güde H. 1996. Determination of dissolved deoxyribonucleic acid concentration in lake water. Aquat Microb Ecol 11:193–202. https://doi.org/10.3354/ame011193.
62. Liang Z, Keeley A. 2013. Filtration recovery of extracellular DNA from environmental water samples. Environ Sci Technol 47:9324–9331. https://doi.org/10.1021/es401342b.
63. Wagner AO, Praeg N, Reitschuler C, Illmer P. 2015. Effect of DNA extraction procedure, repeated extraction and ethidium monoazide (EMA)/propidium monoazide (PMA) treatment on overall DNA yield and impact on microbial fingerprints for bacteria, fungi and archaea in a reference soil. Appl Soil Ecol 93:56–64. https://doi.org/10.1016/j.apsoil.2015.04.005.
64. Ramírez GA, Jørgensen SL, Zhao R, D'Hondt S. 2018. Minimal influence of extracellular DNA on molecular surveys of marine sedimentary communities. Front Microbiol 9. https://doi.org/10.3389/fmicb.2018.02969.
65. Gustave W, Yuan Z-F, Sekar R, Toppin V, Liu J-Y, Ren Y-X, Zhang J, Chen Z. 2019. Relic DNA does not obscure the microbial community of paddy soil microbial fuel cells. Res Microbiol 170:97–104. https://doi.org/10.1016/j.resmic.2018.11.002.
66. Pathan SI, Arfaioli P, Taskin E, Ceccherini MT, Puglisi E, Pietramellara G. 2021. The extracellular DNA can baffle the assessment of soil bacterial community, but the effect varies with microscale spatial distribution. FEMS Microbiol Lett 368. https://doi.org/10.1093/femsle/fnab074.
67. Carini P, Delgado-Baquerizo M, Hinckley E-LS, Holland-Moritz H, Brewer TE, Rue G, Vanderburgh C, McKnight D, Fierer N. 2020. Effects of spatial variability and relic DNA removal on the detection of temporal dynamics in soil microbial communities. mBio 11:e02776-19.
68. Nagler M, Podmirseg SM, Mayr M, Ascher-Jenull J, Insam H. 2021. The masking effect of extracellular DNA and robustness of intracellular DNA in anaerobic digester NGS studies: A discriminatory study of the total DNA pool. Mol Ecol 30:438–450. https://doi.org/10.1111/mec.15740.
69. Villarreal JV, Jungfer C, Obst U, Schwartz T. 2013. DNase I and Proteinase K eliminate DNA from injured or dead bacteria but not from living bacteria in microbial reference systems and natural drinking water biofilms for subsequent molecular biology analyses. J Microbiol Methods 94:161–169. https://doi.org/10.1016/j.mimet.2013.06.009.
70. Chiao T-H, Clancy TM, Pinto A, Xi C, Raskin L. 2014. Differential resistance of drinking water bacterial populations to monochloramine disinfection. Environ Sci Technol 48:4038–4047. https://doi.org/10.1021/es4055725.
71. Nocker A, Cheung C-Y, Camper AK. 2006. Comparison of propidium monoazide with ethidium monoazide for differentiation of live vs. dead bacteria by selective removal of DNA from dead cells. J Microbiol Methods 67:310–320. https://doi.org/10.1016/j.mimet.2006.04.015.
72. Zhu B. 2006. Degradation of plasmid and plant DNA in water microcosms monitored by natural transformation and real-time polymerase chain reaction (PCR). Water Res 40:3231–3238. https://doi.org/10.1016/j.watres.2006.06.040.
73. Romanowski G, Lorenz MG, Wackernagel W. 1993. Plasmid DNA in a groundwater aquifer microcosm–adsorption, DNAase resistance and natural genetic transformation of Bacillus subtilis. Mol Ecol 2:171–181. https://doi.org/10.1111/j.1365-294X.1993.tb00106.x.
74. Dell'Anno A, Stefano B, Danovaro R. 2002. Quantification, base composition, and fate of extracellular DNA in marine sediments. Limnol Oceanogr 47:899–905.
75. Nocker A, Richter-Heitmann T, Montijn R, Schuren F, Kort R. 2010. Discrimination between live and dead cells in bacterial communities from environmental water samples analyzed by 454 pyrosequencing. Int Microbiol 13:59–65. https://doi.org/10.2436/20.1501.01.111.

Sakcham Bairoliya is a postdoctoral research fellow in the School of Civil and Environmental Engineering and Singapore Centre for Environmental Life Sciences Engineering, Nanyang Technological University (NTU), Singapore. He received his Ph.D. from NTU on the topic of extracellular nucleic acids in drinking water distribution systems. He is currently working on a project aiming to understand biofilm-plastic interactions in the Southeast Asian seas.

Jonas Koh Zhi Xiang is a Ph.D. student in Singapore Centre for Environmental Life Sciences Engineering under the Interdisciplinary Graduate Program, Graduate College, Nanyang Technological University (NTU), Singapore. He received his BEng from the School of Chemical and Biomedical Engineering, NTU. He is currently working on a project aiming to understand biofilm-plastic interactions in the Southeast Asian seas.

Bin Cao is an Associate Professor in the School of Civil and Environmental Engineering and a Principal Investigator at the Singapore Centre for Environmental Life Sciences Engineering, Nanyang Technological University, Singapore. He received his Ph.D. from the National University of Singapore and later worked as a postdoctoral research associate at Washington State University and Pacific Northwest National Laboratory. In 2011, he joined NTU as an Assistant Professor and was promoted to Associate Professor in 2017. His research interest centers on biofilm-mediated microbial processes, with the goals of understanding environmental biofilm processes and applying the knowledge and insights to harness the power of beneficial biofilms and combat detrimental biofilms.