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Comparison of an extracellular *v.* total DNA extraction approach for environmental DNA-based monitoring of sediment biota

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Abstract. Monitoring sediment biota is an essential step for the quality assessment of aquatic ecosystems. Environmental DNA-based approaches for biomonitoring are increasing in popularity; yet, commercial kits and protocols for extracting total DNA from sediments remain expensive and time-consuming. Furthermore, they can accommodate only small amounts of sediments, potentially preventing an adequate representation of local biodiversity, especially for macroorganisms. Here, we assessed the reliability of a cost- and time-effective extracellular DNA extraction approach able to account for large volumes of starting material, for characterising bacterial, eukaryote and metazoan communities in three sedimentary environments. DNA concentrations extracted with the extracellular approach were at least similar to those obtained with the commercial kit. Local diversity estimates were not biased towards any particular extraction method, although specific responses were observed depending of the sediment type. Community composition and β-diversity patterns were moderately affected by the extraction approach and the initial amount of starting material; differences being more important for macro- than microorganisms. Thus, the extracellular DNA approach appears as robust and efficient as those based on the commercially available kit for biomonitoring sedimentary communities. Its low cost and fast processing time make it a promising alternative for large-scale ecological assessments of aquatic environments.

Keywords: benthic communities, biomonitoring, ecological assessment, eDNA protocols, metabarcoding, sample size.

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Introduction

Sedimentary environments are a highly biodiverse component of aquatic ecosystems (Snelgrove 1997; Wang *et al.* 2012). They also play a key role in biogeochemical processes and are intrinsically coupled with the pelagic system (Alkhatib *et al.* 2012; Huettel *et al.* 2014). However, sedimentary environments are highly susceptible to anthropogenic activities because they ultimately act as a repository for many contaminants, resulting in contaminant concentrations often several orders of magnitude higher than that of the overlying water (Simpson and Batley 2016).

As a means of tempering the increasing influence of anthropogenic activities on aquatic systems, many jurisdictions have adopted regulations and guidelines for assessing, monitoring and restoring the ecological integrity of benthic environments (Lyons *et al.* 2010; Carere *et al.* 2012; Simpson *et al.* 2013). Although approaches vary, they generally involve the collection of chemical and physical data, along with ecological information on benthic communities residing in or interacting with sediments. These communities are essential for marine and freshwater ecosystems because they are involved in many ecosystem functions, including trophic transfer and biogeochemical processes (Freckman *et al.* 1997; Snelgrove 1997; Simčič 2005). Information about benthic community composition can further inform ecological risk assessments (Graham *et al.* 2019) and predictions of ecosystem vulnerability to biodiversity loss (Heilpern *et al.* 2018).

The most widespread approach for obtaining such ecological information is via the sorting, identification and enumeration of macrobenthic invertebrate communities (Canfield *et al.* 1994; Canfield *et al.* 1996; Dauer *et al.* 2000; Chariton *et al.* 2010), although other communities, for example diatoms, are also less

commonly examined (Cattaneo *et al.* 2011; Desianti *et al.* 2017). Yet, these essential taxa represent only a minute fraction of benthic communities. As such, constraining ecological monitoring to macrobenthos not only provides a narrow view of biodiversity, but also excludes the crucial roles of meio- and micro-biota, with a growing body of evidence showing that they may be better indicators of disturbance and ecosystem health (Kennedy and Jacoby 1999; Gyedu-Ababio and Baird 2006; Chariton *et al.* 2014). Other major limitations of traditional macrobenthic surveys include the reliance of suitable taxonomic expertise, the high costs of sample processing, and the latency in producing the data (Chariton *et al.* 2010).

Environmental DNA (eDNA, i.e. the DNA of all organisms present in environmental samples; Pawlowski *et al.* 2020) has been increasingly seen as a viable alternative to traditional 'capture and count' methods for biomonitoring and environmental quality assessment (Chariton *et al.* 2010, 2015; Pochon *et al.* 2015; Pawlowski *et al.* 2018; Taberlet *et al.* 2018; Cordier *et al.* 2020; DiBattista *et al.* 2020). Environmental-DNA metabarcoding seeks to identify multiple taxa from DNA retrieved from an environmental matrix such as samples of sediment or water. From a single sample, numerous DNA (meta)barcodes, each characterising a particular taxon, can be amplified and sequenced simultaneously, virtually capturing all life forms. Thousands of samples can be processed in a single analysis, making the approach both time and cost efficient (Taberlet *et al.* 2012*a*). In addition, this approach can also be efficiently implemented over large spatial scales (Bohmann *et al.* 2014; Thomsen and Willerslev 2015), and is now considered as an ecological line of evidence for sediment and water-quality guidelines in an increasing number of jurisdictions (Simpson and Batley 2016; Hering *et al.* 2018).

Protocols for using eDNA-based approaches for environmental monitoring are continually being developed (Taberlet *et al.* 2018), with several studies also comparing intra-laboratory differences in protocols (e.g. Birer *et al.* 2017; Djurhuus *et al.* 2017; Majaneva *et al.* 2018). Consequently, there has been far greater conformity in approaches in recent years. This is critical, given that biomonitoring and assessment is founded on reproducibility. Environmental-DNA extraction is widely recognised as a critical and sensitive step of the process (Natarajan *et al.* 2016; Zinger *et al.* 2016, 2019; Ramírez *et al.* 2018*a*) and, in the case of sediment analysis, the method of eDNA extraction and the initial volume of the sample analysed are important considerations. Standard approaches using commercial kits result in the extraction of total DNA containing both intracellular (resulting from the lysis of living, dormant and dead cells during the extraction process) and extracellular DNA (previously released by lysis of damaged or dead cells or actively excreted into the surrounding environment; Torti *et al.* 2015; Ramírez *et al.* 2018*b*). Although widely used, these kits are expensive (Majaneva *et al.* 2018) and require time and suitable facilities, which make them often unsuitable for regular biodiversity monitoring (Zinger *et al.* 2016). Most importantly, commercial kits allow processing of only small amounts (≤10 g) of sample material, potentially constraining their ability to fully assess local biodiversity (Taberlet *et al.* 2012*b*). This is particularly relevant if macrobenthic invertebrates are being targeted.

More recently, protocols targeting only extracellular DNA have been developed as a cost-effective alternative to rapidly process larger (and potentially more representative) sample volumes of soil, with little to no facilities being required (Taberlet *et al.* 2012*b*; Zinger *et al.* 2016). Once adsorbed onto a mineral matrix (i.e. sediment particles), extracellular DNA is partially protected from degradation, enabling it to persist in the environment (Nielsen *et al.* 2007; Nagler *et al.* 2018). It is, therefore, expected to constitute a substantial fraction of the total eDNA retained within an aquatic ecosystem (Ceccherini *et al.* 2009; Pietramellara *et al.* 2009; Vuillemin *et al.* 2017). Desorption of extracellular DNA from the sedimentary matrix (of any amount) can be easily achieved using a saturated phosphate buffer (Taberlet *et al.* 2012*b*); the cost and processing time for its extraction have been estimated to be five and four times lower respectively than that required for extracting total DNA using a commercially available kit (Zinger *et al.* 2016). However, as the extracellular DNA fraction includes DNA derived from ancient and recent terrestrial and aquatic origins, the biodiversity signal inferred from extracellular DNA can differ from those obtained by total DNA, with some authors arguing that the extracellular DNA-based approaches inflate diversity (Alawi *et al.* 2014; Carini *et al.* 2017). A comparison of both approaches performed on tropical soil samples showed that, while local (α-) diversity was underestimated using extracellular DNA, β-diversity patterns were globally similar, with strong correlations in the relative abundances of clades observed between the two methods (Zinger *et al.* 2016). Despite its use in a few sediment eDNA metabarcoding studies (e.g. Pansu *et al.* 2015*a*; Guardiola *et al.* 2016*a*, 2016*b*), the efficiency of the extracellular DNA approach (hereafter called 'extDNA') has never been properly compared with the total DNA approach (hereafter called 'totDNA') for the purpose of sediment biomonitoring.

In this study, we evaluated the reliability of the extDNA approach for characterising sediment biota via eDNA metabarcoding. For this, we targeted taxonomic groups routinely used in sediment-quality assessments (prokaryote, eukaryote and metazoans) in three different sedimentary environments (freshwater pond, estuary and near-shore marine sands), and assessed the influence of two extraction methods (a bespoke method targeting only extDNA *v.* commercially available kit-based method for extracting totDNA) and the original volume of sampling material on α- and β-diversity patterns of those communities. We hypothesised that (1) the DNA concentration should be higher after totDNA extraction than extDNA extraction, and that (2) totDNA should provide higher estimates of local richness (α-diversity) than does extDNA. However, (3) assemblages based on totDNA and extDNA approaches should not differ significantly in terms of β-diversity patterns and the relative abundance of phyla, although (4) we expect an influence from the original amount of sampling material.

Material and methods

Study sites and sample collection

Sediments were sampled from the following three biomes located in eastern New South Wales, Australia: a freshwater pond at Macquarie University, Sydney (–33°46′9″, 151°6′52″); the Lane Cove River estuary at Cunninghams Reach, Lane Cove (–33°49′42″, 151°8′44″); and a sandy seashore at Umina Beach

(–33°31′42″, 151°18′55″). Sampling occurred between 7 August and 12 September 2018. At each site, five individual samples were collected (total $n = 15$), each of them containing ~500 mL of the top ~10 cm of surficial sediment, the most biologically active part of the sediment (Simpson and Batley 2016). Samples were collected in sterile plastic containers that were rinsed five times in the water at each sample site before collection. Sediment samples were placed immediately on ice and transported to the laboratory where they were stored at –30°C before eDNA extractions.

eDNA extraction

The sediment samples were thawed for 24 h at 4°C and homogenised for 30 min using a roller-mixer before eDNA extractions. To simultaneously assess the influence of the extraction approach (i.e. extDNA *v.* totDNA) and of the initial volume of sediment material on the diversity patterns, we subsampled 1, 10 and 200 g from each sample for the extracellular DNA extraction (Taberlet *et al.* 2012*b*), and 1 and 10 g for the total DNA extraction. In total, each of the 15 samples was subjected to three protocol variants (with changing volume of starting material) for the extDNA extraction method and two for the totDNA method, leading to a total of 75 extractions (Fig. S1 of the Supplementary material).

Total DNA extractions, for both 1- and 10-g subsamples, were performed using the DNeasy PowerMax Soil Kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. This kit is one of the most widely used in eDNA research, and is designed to allow totDNA extraction from up to 10 g of sediment. Extracellular DNA was extracted following the bespoke protocol described in Zinger *et al.* (2016), a modified version of the protocol proposed by Taberlet *et al.* (2012*b*). Briefly, equivalent amounts of phosphate buffer (Na_2HPO_4, 0.12 M, pH ~8) were added to each subsample and mixed for 15 min to extract extracellular DNA. For each extraction, a 2-mL aliquot was then centrifuged at 10 000*g* for 10 min at room temperature; the supernatant was then used in subsequent extraction steps, which were performed using the NucleoSpin Soil Kit (Macherey-Nagel, Düren, Germany), following manufacturer's instructions and skipping the cell-lysis step (Taberlet *et al.* 2012b). For each site, at least two extraction controls were performed, one using phosphate buffer for extracellular DNA extraction and one using solution C2 for the total DNA extraction. In total, 45 extracellular DNA extractions (15 per site) plus four negative controls were conducted, as well as 30 total DNA extractions (10 per site) plus three negative controls. DNA extracts and negative controls ($n = 82$) were stored at –20°C before polymerase chain reaction (PCR) amplification.

PCR amplification and sequencing

Standard PCR methods were used to amplify specific gene regions targeting prokaryotes (bacteria and archaea; *16S* rRNA; Caporaso *et al.* 2011), eukaryotes (*18S* rRNA; Hardy *et al.* 2010) and metazoans (*COI* mtDNA; Leray *et al.* 2013). So as to multiplex PCR products and sequence them together in a single high-throughput analysis, primers were tagged on their 5′ ends with a combination of eight-nucleotide labels differing by at least five nucleotides (Binladen *et al.* 2007; Valentini *et al.* 2009). In addition to samples and extraction controls, two blank PCR controls (using nuclease-free water) and positive controls (using saltwater crocodile, *Crocodylus porosus*, and foolish mussel, *Mytilus trossulus*, DNA for *18S* and *COI*, and a synthetic microbial sequence for *16S*) were included.

Each PCR reaction was performed in a total volume of 20 μL and contained 2 μL of DNA extract and 10 μL of AmpliTaq Gold 360 PCR Master Mix (Applied Biosystems, Foster City, CA, USA). Primer concentration was 0.2 μM for prokaryote primers (515F: 5′-GTGYCAGCMGCCGCGGTAA-3′, Parada *et al.* 2016; 806R: 5′-GGACTACNVGGGTWTCTAAT-3′; Apprill *et al.* 2015), 0.4 μM for eukaryote primers (All18SF: 5′-GGTGCATGG-CCGTTCTTAGT-3′; All18SR: 5′-CATCTAAGGGCATCACA-GACC-3′; Hardy *et al.* 2010) and 0.5 μM for metazoan primers (mlCOIintF: 5′-GGWACWGGWTGAACWGTWTAYCCYCC-3′; jgHCO2198: 5′-TAIACYTCIGGRTGICCRAARAAYCA-3′; Leray *et al.* 2013). UltraPure RNase/DNase-free distilled water (Invitrogen, Carlsbad, CA, USA) was added to the mix to reach a final volume of 20 μL. Thermocycling conditions included an initial denaturing period of 10 min at 95°C, followed by 35 cycles of variable duration and temperature depending of the primer pair, as follows: denaturing at 94°C for 45 s, annealing at 50°C for 1 min and elongation at 72°C for 1.5 min for prokaryote (Caporaso *et al.* 2011); denaturing at 94°C for 1 min, annealing at 50°C for 1 min and elongation at 72°C for 1.5 min for eukaryote (Hardy *et al.* 2010); denaturing at 95°C for 30 s, annealing at 46°C for 30 s and elongation at 72°C for 45 s for metazoan (Deagle *et al.* 2018); and a final elongation at 72°C step for 10 min (prokaryote and eukaryote) or 5 min (metazoan).

Pre- and post-PCR DNA concentrations were determined using Quant-iT PicoGreen dsDNA Reagent assays (Invitrogen) on a PHERAstar FSX plate reader. Amplicon concentration was measured twice and averaged. To avoid overbiased sequencing depth among samples, PCR products were pooled in equimolarity (Harris *et al.* 2010; Leray *et al.* 2016; Lim *et al.* 2018), and PCR products with a low concentration (<2.5 ng μL^{-1}) were excluded. Pooled PCR samples were purified using AMPure XP (Agencourt Bioscience, Beverly, MA, USA). Sequencing libraries were prepared using a TruSeq DNA PCR-free protocol and sequenced on an Illumina MiSeq 2500 platform (Illumina Australia and New Zealand, Melbourne, Vic., Australia; 2 × 250-bp paired-end reads) at the Ramaciotti Centre for Genomics (University of New South Wales, Sydney, NSW, Australia).

Data processing and filtering

Metabarcoding data were processed using the GHAP amplicon clustering and classification pipeline (see https://doi.org/10.4225/08/59f98560eba25). This pipeline combines tools from USearch (Edgar 2010) and the Ribosomal Database Project (RDP) classifier (Cole *et al.* 2007) with locally written scripts to conduct the following steps: (1) demultiplexing to assign reads to their original samples on the basis of the tag information attached to the primers, (2) reads trimming to remove poor-quality tail regions (with an Illumina base calls quality score of <25) by using a windowed quality-score based technique, (3) paired reads merging using the *fastq_mergepairs* command implemented in USearch, non-paired reads being discarded, (4) de-replication of reads with the *fastx_ uniques* command, (5) trimming of sequences outside the expected length range of the marker (245–265 bp for *16S*, 100–220 bp for *18S*, 295–355 bp

for *COI*), (6) operational taxonomic unit (OTU) clustering at 97% similarity using the *cluster_otus* command, which also performs chimera checking, (7) filtering out of non-target gene sequences, and (8) taxonomic assignment. For this latter step, representative sequences from each *18S* and *COI* OTUs were taxonomically assigned against a respective curated reference set derived from the SILVA non-bacterial sequence (V128) reference set (Quast *et al.* 2013) and a custom-made mitochondrial *COI* reference dataset derived from GenBank (with >410 000 reference sequences and >127 000 species being represented), using the *ublast* command implemented in USearch (with the following parameters: e-value = 1E–5, query coverage = 0.8). For taxonomic assignment at the phylum, class, order and family levels, BLAST similarity cut-off values implemented by default in GHAP were used (0.77, 0.8, 0.85 and 0.9 respectively; see https://doi.org/10.4225/08/59f98560eba25); below these thresholds, the OTU was considered as unassigned. *16S* OTUs were classified in two ways to improve confidence in the taxonomic assignment. Classification was undertaken against a set of curated *16S* reference sequences, derived from the RDP *16S* training set and supplemented by sequences from the RefSeq *16S* set, using (1) the RDP Naïve Bayesian Classifier (RDP minimum confidence threshold for classification = 0.5) to assign a taxonomy, possibly down to species level, and (2) the *usearch_global* command to find the closest match to each OTU in the reference set. Finally, the accurate number of reads of each OTU in each sample was finally calculated by mapping all the merged reads back onto the final set of classified OTUs, using the *usearch_global* command, and an OTU × sample table was generated.

The following steps were conducted in R (ver. 3.5.3, R Foundation for Statistical Computing, Vienna, Austria, see https://www.R-project.org/) to further filter and de-noise the datasets. Operational taxonomic units (OTUs) that could not be assigned at the Domain level in *18S* and *16S* datasets were removed (Sutcliffe *et al.* 2017), as well as those that were not assigned to the 'Metazoa' Kingdom in the *COI* one. In the *16S* dataset, we also filtered out OTUs that matched with known chloroplast sequences (Sutcliffe *et al.* 2019). In addition, to remove putative contaminant sequences, OTUs that had their maximal relative abundance in controls were discarded (Pansu *et al.* 2019). Then, only common OTUs representing >0.1% of sequences in at least one sample were retained to limit the inclusion of potentially artefact sequences resulting from PCR or sequencing errors (Deagle *et al.* 2018). Finally, in an effort to reduce the impact of low-abundance false positives resulting from 'tag-jumps' (Carlsen *et al.* 2012; Schnell *et al.* 2015), we considered an OTU as genuinely present in a sample if its abundance in this sample represented at least 0.5% of its total abundance across samples (Zinger *et al.* 2016). In the *COI* dataset, one cross-contaminated sample and two others with a low number of reads (<1000 reads) were discarded. Statistics about the metabarcoding data filter process are provided in Table S1 of the Supplementary material.

Statistical analysis

We first tested the effect of the extraction protocol variants on the DNA yield after extraction and after PCR amplification. Prior to statistical analysis of sequencing results, OTU data were rarefied on the basis of the minimum number of reads observed to 45 000, 7000 and 3000 reads per sample for eukaryotes, bacteria and metazoans respectively. We then investigated the α-diversity of bacterial, eukaryote and metazoan OTUs in relation to the extraction protocol variant used. Three different α-diversity indices (OTU richness, Shannon index and evenness) were estimated on the basis of the average values from 100 rarefactions (Sutcliffe *et al.* 2017). Differences in DNA concentration and OTU richness or diversity were assessed using Kruskal–Wallis tests and *post hoc* Dunn's multiple-comparison tests (with Benjamini–Hochberg correction method) at a significance level (α) of 0.05.

To assess the influence of the extraction protocol variants on β-diversity patterns, rarefied communities were first analysed using correspondence analysis (Borcard *et al.* 2011); initially with all samples, followed by a second separate analysis for each sediment type. We further partitioned the total inertia of each correspondence analysis by sediment type to determine the portion due to the variation between extractions from a same sample and that due to the variation among samples (see the 'Code for the inertia decomposition analysis' section in the Supplementary material). Second, pairwise Bray–Curtis distances among samples were calculated, and permutational analyses of variance (PERMANOVA) were conducted on each full dataset by using the R package 'vegan' (J. Oksanen, F. G. Blanchet, M. Friendly, R. Kindt, P. Legendre, D. McGlinn, P. R. Minchin, R. B. O'Hara, G. L. Simpson, P. Solymos, M. H. H. Stevens, E. Szoecs, and H. Wagner, see https://CRAN.R-project.org/package=vegan) to assess the direct effects of the extraction protocol variants on these dissimilarity values (Anderson 2001). Similar analyses were performed for each sediment type to test for the effect of the DNA extraction approach (extDNA and totDNA). Bray–Curtis distances between samples were also compared between each pair of extraction protocol variants by using a Spearman rank-correlation test and a Mantel procedure with 999 permutations. Finally, for each sediment type, we tested whether the multivariate homogeneity of dispersion values differed between extraction protocol variants using the 'betadisper' function in '*vegan*' with a bias correction for small sample size (Anderson 2006). We specifically expected the different samples from the same biome (sediment type) to be more homogeneous when extracted with larger amounts of sampling material.

To investigate the taxonomic composition of samples, the relative abundance of each phyla was averaged per sediment type for each extraction protocol variant. The fold-change ratio was then determined to quantify differences between pairs of protocol with similar amounts of starting material (Zinger *et al.* 2016). Operational taxonomic units and phylum relative abundance were then averaged per extraction approach (extDNA and totDNA), and we used Spearman rank-correlation tests to assess the congruence between OTUs and phylum relative abundances obtained from both approaches.

Results

DNA concentration

Many marine samples did not amplify with *COI* primers (9 of 25). This was particularly the case with samples extracted with the total DNA (totDNA) approach (6 of 10). Consequently, all marine *COI* amplicons were excluded from subsequent

analyses (Fig. S2), resulting in a total of 46 *COI* samples being analysed for the pond and estuary environments. Samples that did not amplify for *18S* ($n = 1$ of 75) and *16S* ($n = 4$ of 75) were extracted using different extraction protocol variants and came from different sediment types; therefore, no particular bias related to the sediment type or extraction protocol variant was identified for these primers.

The DNA yield obtained from 1 g of sediment extracted with the totDNA approach was significantly lower than that from our other extraction protocol variants (i.e. all those based on the extDNA method and the totDNA one with 10 g of sediments), especially for pond and estuarine sediments (Dunn's multiple-comparison test $P < 0.05$; Fig. 1, Table S2). The mean totDNA yield produced from 10 g of sediment did not differ significantly ($P > 0.05$) from the extDNA yields obtained from 1-, 10- or 200-g samples (Fig. 1). The initial amount of starting material did not have a significant effect on the quantity of extracellular DNA extracted ($P > 0.6$ for all pairwise comparisons; Table S2).

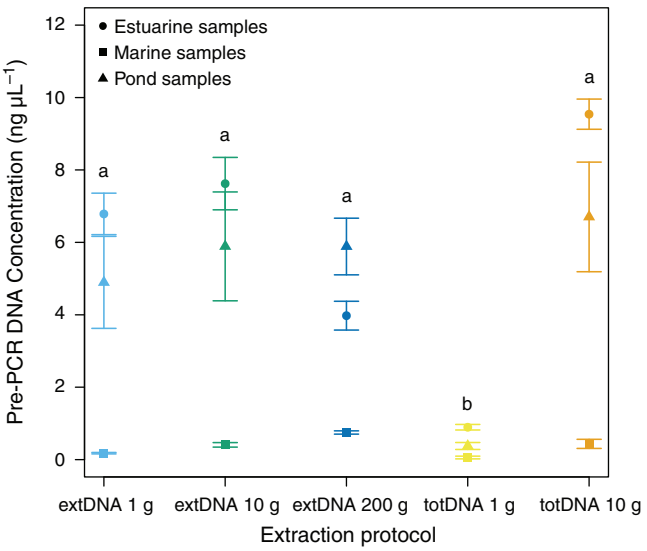

Fig. 1. Efficiency of extraction protocol variants. Each point represents the mean (± s.e.m.) DNA concentration after extraction for each sediment type according to the employed protocol variant. Different letters reflect significant differences between protocol variants.

The sediment type (i.e. pond, estuary and marine) affected the total yield of DNA (Fig. 1). The DNA yield (mean ± s.e.m) was consistently lower in marine sediments (0.37 ± 0.06 ng μL^{-1}) than in both estuarine (5.76 ± 0.65 ng μL^{-1}) and pond (4.75 ± 0.66 ng μL^{-1}) sediments, regardless of the extraction protocol variant used (all marine *v.* all estuary DNA extracts: $Z = 6.01$, $P < 0.001$; all marine *v.* all pond DNA extracts: $Z = -4.70$, $P < 0.001$; all pond *v.* all estuary DNA extracts: $Z = 1.31$, $P = 0.19$).

Alpha-diversity patterns

We found no consistent evidence that totDNA provides higher local richness estimates than does extDNA. There was no significant difference in OTU richness for prokaryotes between extraction protocol variants (Kruskall–Wallis $\chi^2 = 2.04$, d.f. = 4, $P = 0.73$; Table 1). The same pattern was observed for metazoan assemblages, although OTU richness was marginally greater with the totDNA protocol using 10 g of sediment ($\chi^2 = 6.17$, d.f. = 4, $P = 0.19$). By contrast, eukaryote richness using the totDNA extraction protocol with 1 g of sediment had a lower OTU richness than did protocols with larger amounts of starting material, regardless of the extraction approach (totDNA and extDNA alike, $\chi^2 = 11.00$, d.f. = 4, $P = 0.027$), although pairwise comparisons were marginally non-significant (Fig. S3, Table S3). However, there were sediment-specific responses; for example, OTU richness obtained from the pond samples using totDNA was overall higher than those obtained using extDNA, regardless of the primers pair used (Fig. S3). For eukaryotes and prokaryotes, no differences were observed in Shannon diversity ($\chi^2 = 0.40$, d.f. = 4, $P = 0.98$ and $\chi^2 = 2.28$, d.f. = 4, $P = 0.69$, for *18S* and *16S* respectively) and Pielou evenness ($\chi^2 = 1.28$, d.f. = 4, $P = 0.87$ and $\chi^2 = 2.71$, d.f. = 4, $P = 0.61$), indicating that potential differences observed in OTU richness are likely to be due to rare OTUs (Table 1). By contrast, we observed differences in Shannon and Pielou indices for metazoans ($\chi^2 = 16.41$, d.f. = 4, $P < 0.003$ and $\chi^2 = 20.91$, d.f. = 4, $P < 0.001$ respectively). In this case, the observed OTU richness was significantly higher in the totDNA protocol with 10 g of starting material than in all extDNA protocols.

Beta-diversity in community composition

Operational taxonomic unit composition samples clustered primarily by sediment type for all taxonomic groups (Fig. 2*a–c*).

Table 1. Alpha-diversity metrics measured for each taxonomic group according to the employed extraction protocol variant

Values (mean ± s.e.m.) for three different metrics (operational taxonomic unit (OTU) richness, Shannon index and Pielou evenness) are presented. Pairwise differences in OTU richness among extraction protocol variants are reported in Table S3

Extraction protocol	Bacteria (*16S*)			Eukaryote (*18S*)			Metazoan (*COI*)		
	OTU richness	Shannon index	Pielou evenness	OTU richness	Shannon index	Pielou evenness	OTU richness	Shannon index	Pielou evenness
extDNA 1 g	449 ± 16	5.27 ± 0.05	0.864 ± 0.004	254 ± 18	3.36 ± 0.18	0.609 ± 0.028	119 ± 13	2.41 ± 0.19	0.507 ± 0.035
extDNA 10 g	460 ± 11	5.28 ± 0.04	0.862 ± 0.004	290 ± 26	3.48 ± 0.17	0.617 ± 0.028	130 ± 14	2.59 ± 0.15	0.535 ± 0.021
ext DNA 200 g	438 ± 13	5.19 ± 0.05	0.854 ± 0.004	303 ± 23	3.37 ± 0.23	0.59 ± 0.037	125 ± 8	2.54 ± 0.11	0.529 ± 0.021
totDNA 1 g	468 ± 16	5.29 ± 0.05	0.861 ± 0.004	224 ± 14	3.48 ± 0.09	0.647 ± 0.017	131 ± 13	3.31 ± 0.15	0.684 ± 0.020
totDNA 10 g	447 ± 22	5.26 ± 0.05	0.864 ± 0.005	275 ± 18	3.53 ± 0.13	0.634 ± 0.024	163 ± 15	3.09 ± 0.17	0.609 ± 0.025

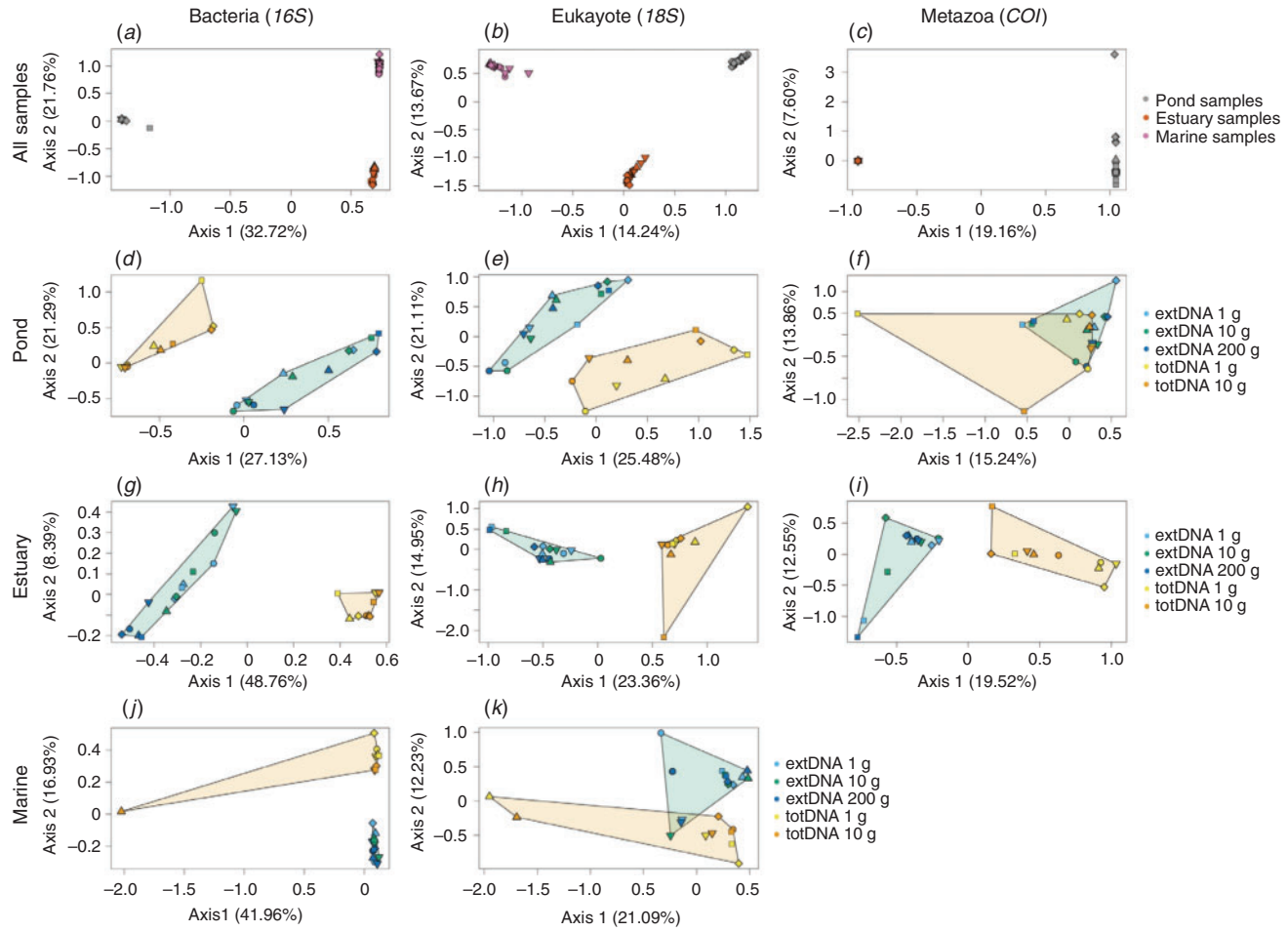

Fig. 2. Correspondence analysis ordinations illustrating the similarities in operational taxonomic unit (OTU) composition of polymerase chain reaction (PCR) products for bacteria (left panels), eukaryote (central panels) and metazoan (right panels) markers. (*a–c*) Ordinations of all samples; colours reflect the sediment type. (*d–k*) Ordinations per sediment type; colours reflect extraction protocol variants and envelops encompass PCR products derived from a similar extraction type (extDNA in green, totDNA in yellow). The shape of points corresponds to the sample in all panels.

This suggests that the extraction protocol only moderately affects the overall β-diversity patterns, with discrimination being, here, predominately due to sediment type. When restricted by sediment type, the two first axes of the correspondence analyses discriminated samples according to the DNA extraction approach (extDNA *v.* totDNA), regardless of the taxonomic group and the sediment type (Fig. 2*d–k*). The inertia decomposition analysis performed per sediment type showed that, for all taxonomic groups, the inertia owing to the variation between extraction protocol variants was far greater than that owing to the variation among samples. For marine and estuarine sediments, 76.8–84.9 and 78.5–87.1% of the inertia respectively was due to differences in extraction protocol variants. The inertia was more evenly distributed between sample and extraction protocol variants for pond samples (58.5–65.0%). Although it was not possible to distinguish the respective effects of the extraction approach and the initial amount of sediment, results suggest that most of the variation was associated with the extraction approach. This was confirmed by the PERMANOVA analyses for each sediment type, which showed that Bray–Curtis distance was influenced by the DNA extraction approach (extDNA *v.* totDNA; Table S4). This was most evident in the estuarine sediments (r^2 = 0.33–0.61, $P < 0.001$); in all other cases, r^2 ranged between 0.16 and 0.36 ($P < 0.002$).

Overall, Bray–Curtis dissimilarities were unaffected by the DNA extraction protocol for prokaryotes (perMANOVA: F_{pseudo} = 0.65, r^2 = 0.038, P = 0.79), eukaryotes (perMANOVA: F_{pseudo} = 1.02, r^2 = 0.056, P = 0.42) or metazoans (perMANOVA: F_{pseudo} = 0.78, r^2 = 0.071, P = 0.65). Beta-diversity patterns among samples were consistent among extraction protocol variants, especially for prokaryotes (Spearman's ρ = 0.89–0.96, P = 0.001, for all pairwise comparisons in the prokaryote dataset; Spearman's ρ = 0.78–0.93, P = 0.001 for eukaryotes; Spearman's ρ = 0.81–0.93, $P < 0.01$ for metazoans; Fig. 3). Eukaryote and metazoan Bray–Curtis distances derived from the totDNA extraction approach using 1 g of sediment were overall more dissimilar to those derived from other protocols (Fig. 3*a–d*), except for prokaryotes.

The effect of the initial amount of sediment on β-diversity patterns was moderate. Bray–Curtis distances obtained using the same extraction approach with varying amount of sampling

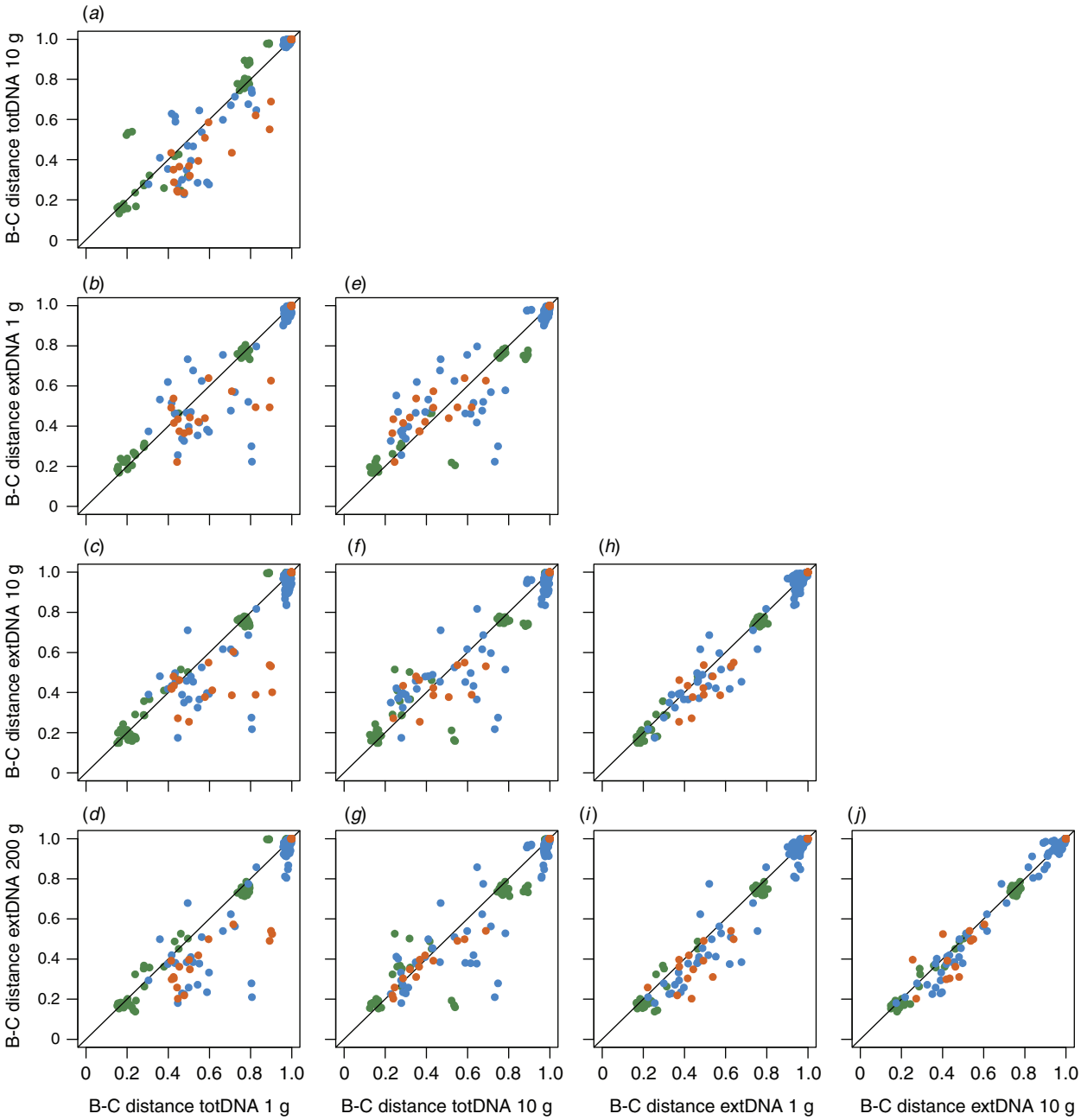

Fig. 3. Comparison of β-diversity patterns (defined as Bray–Curtis (B-C) dissimilarities between samples) among the different extraction protocol variants. Bacteria (green dots); eukaryotes (blue dots); metazoans (orange dots). The solid line indicates a 1 : 1 relationship.

material were highly similar (Fig. 3*a* for totDNA, Spearman's $\rho = 0.94$; and Fig. 3*h–j* for extDNA, Spearman's $\rho = 0.97$–0.98). Eukaryote and metazoan communities from a same sedimentary environment tended to be more homogenous when a larger amount of sampling material was used (multivariate dispersion analysis; Fig. S4), but significant differences among extraction protocol variants were detected only for metazoan communities (ANOVA, $P < 0.05$). No consistent pattern emerged for prokaryote communities, although significant differences were observed (Fig. S4).

Taxonomic composition at the phylum level was very similar among extraction protocols, especially for prokaryotes (Fig. 4*a*). Relative abundances of prokaryote phyla were strongly correlated between extraction approaches (extDNA *v.* totDNA, Spearman's $\rho = 0.97$, $P < 0.001$; Fig. S5). Although slightly weaker, this correlation was also high for eukaryotes and metazoans (Spearman's $\rho = 0.90$ and 0.89 respectively; Fig. 4*b*, *c*, S5). To a lesser extent, this pattern was also observed at the OTU level (Spearman's $\rho = 0.55$, 0.40 and 0.36 for prokaryotes, eukaryotes and metazoans respectively, $P < 0.001$). For a given amount of

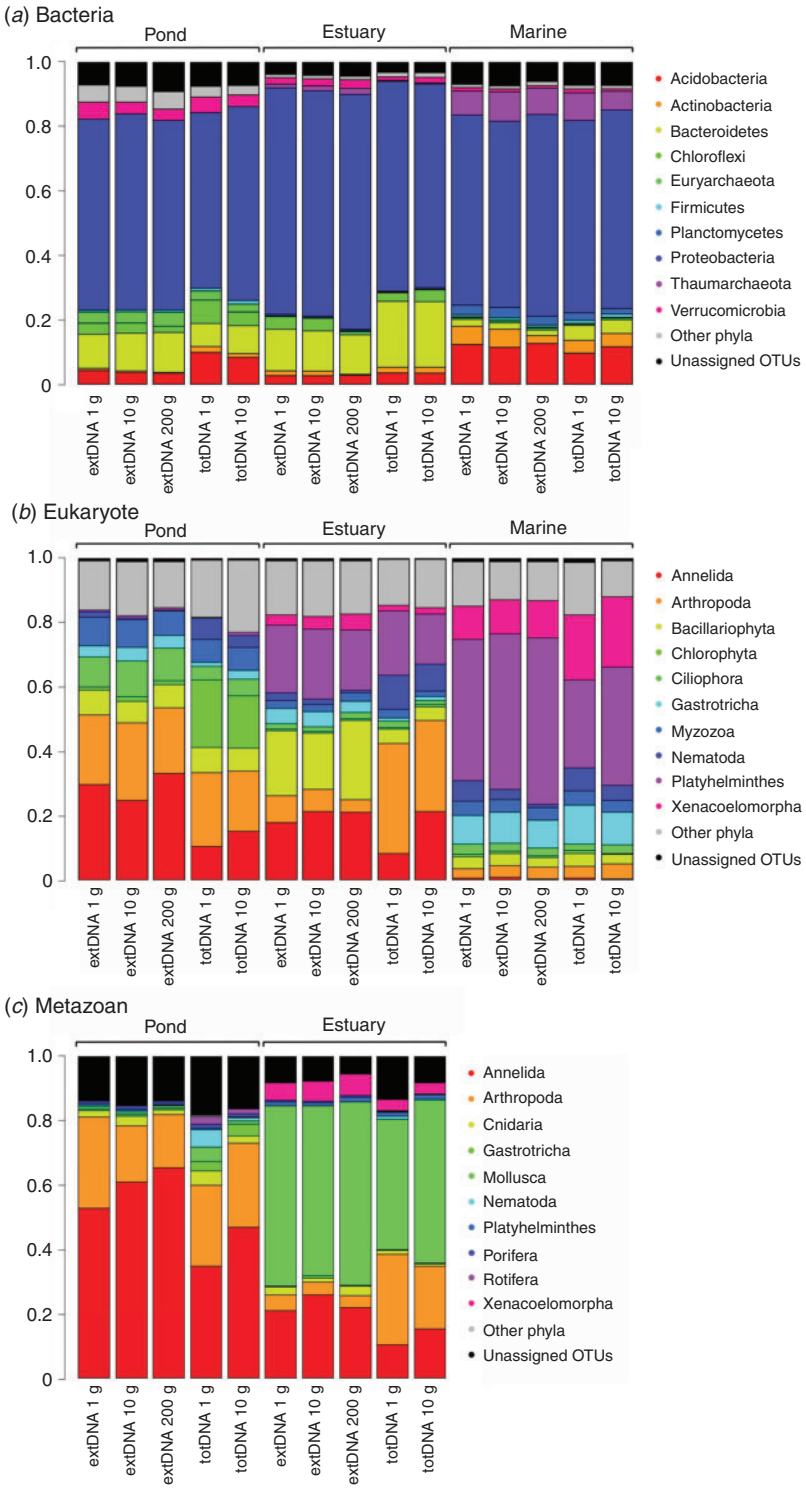

Fig. 4. Average relative abundance of (*a*) bacterial, (*b*) eukaryote and (*c*) metazoan phyla for each sediment type according to the employed extraction protocol variant.

starting material, the average fold-change (AFC) ratio was always <2 for the 10 most abundant bacterial clades, suggesting that there were no marked differences in the relative abundance of bacterial phyla between the extraction methods (extDNA *v.* totDNA; Table S5). For eukaryotes and metazoan primers, marked changes were observed for arthropods and nematodes (both being overestimated with totDNA compared with extDNA), whereas annelids exhibited an opposite trend (Fig. 4, Table S5). Plant (Chlorophyta

phylum) relative abundances determined with the *18S* eukaryotic marker were substantially higher in the totDNA (AFC > 7) than extDNA, whereas diatoms (Bacillariophyta phylum) tended to be overestimated with extDNA.

Discussion

DNA concentration and amplification success

In this study, we examined the potential suitability of extracellular DNA (extDNA) for eDNA-based biomonitoring of sedimentary communities, by comparing this approach with the traditional total DNA (totDNA) extraction approach. The fundamental difference between the two methods is in the eDNA release technique; extDNA is extracted by desorption from sediment matrix by using saturated phosphate buffer, whereas totDNA relies on the mechanical and chemical lysis of cells contained in the matrix, allowing the extraction of both intra- and extracellular DNA (Zinger *et al.* 2016). However, variations in techniques and buffers between extraction protocols are known to lead to slightly different outputs (Ramírez *et al.* 2018*a*; Dopheide *et al.* 2019), and we cannot rule out that factors other than the eDNA fraction targeted (e.g. buffers used) also played a role in differences observed between protocols.

We first compared the yields obtained from the different protocols (Hypothesis 1). Among the three sediment types examined, DNA concentration obtained from extDNA extractions was at least as high as (or even higher than) those obtained with the totDNA protocols (Fig. 1). Specifically, the totDNA concentration from 1 g of sediment was much lower than those obtained with any other protocol. One possible explanation for this result is technical; we used a PowerMax Soil DNA extraction kit (following manufacturer's protocol) to extract totDNA, which is designed for a starting material mass between 5 and 10 g. As such, the protocol may have failed to deal with a smaller amount of starting material because the DNA would be more diluted in the buffer. Nevertheless, even with 10 g of sediment, we found no evidence that totDNA provided a higher DNA yield than did extDNA. This contrasts with Zinger *et al.* (2016) who found extDNA yields from tropical soils to be lower than those obtained using totDNA. Matrix composition and local environmental parameters are known to influence both the persistence and concentration of extDNA in soils and sediments (Pietramellara *et al.* 2009; Nagler *et al.* 2018). Given that aquatic systems have been shown to contain significant amounts of DNA derived from the surrounding catchments (Deiner and Altermatt 2014; Chariton *et al.* 2015), it is likely that extDNA constitutes the dominant fraction of environmental DNA in aquatic ecosystems (Dell'Anno and Danovaro 2005; Ceccherini *et al.* 2009; Vuillemin *et al.* 2017). This can explain the absence of significant differences in the yield obtained from extDNA and totDNA protocols.

In the case of both the *16S* rDNA bacterial and *18S* rDNA eukaryote primers, we found that the proportion of samples that successfully amplified by PCR was similar regardless of the extraction protocol variant or sediment matrix. By contrast, amplification success using the *COI* primers was considerably lower, especially for the marine sediments extracted with the totDNA extraction method where a majority of the samples failed to amplify (Fig. S2). One explanation is the higher sand content of the marine sediments that effectively operates as an abiotic stressor, limiting burrowing capacity of many taxa and, ultimately, leading to reduction in diversity and abundance of organisms (Gray 1974; Snelgrove and Butman 1994). This interpretation is supported by the overall lower DNA yield observed in marine samples (Fig. 1). However, results from the *18S* dataset showed that metazoan DNA was present in marine samples and that metazoan phyla constituted a significant proportion of the eukaryote community (Fig. 4). Consequently, DNA yield is not the only factor contributing to the discrepancy between the *COI* and *18S* datasets; PCR amplification biases are also likely to play a role. This is a known pitfall with the *COI* marker (Clarke *et al.* 2014; Deagle *et al.* 2014). The size range of amplicons can partly explain this pattern; the average length of *COI* amplicons is approximately twice that of *18S* amplicons. Yet, DNA in environmental samples is degraded and shorter fragments are more common, making them more easily amplifiable than are longer fragments (Yoccoz *et al.* 2012).

Alpha-diversity patterns

Contrary to our expectation (Hypothesis 2), we found no evidence, overall, that estimates of local richness were higher with totDNA (containing both extDNA and intracellular DNA) than with extDNA (Tables 1, S3). This result is in line with the supposition that extDNA constitutes most of eDNA found in aquatic sediment (Dell'Anno and Danovaro 2005; Ceccherini *et al.* 2009). In aquatic environments, part of the sedimentary extDNA is of allogenous origin (i.e. coming from the surrounding environment, including terrestrial ones; Torti *et al.* 2015), or from cells that are no longer alive ('relic DNA'; Carini *et al.* 2017). Our results suggest that the totDNA extraction protocol effectively captures this extDNA fraction, and that, in turn, the extDNA extraction protocol effectively integrates the diversity associated with the active communities that continuously release extDNA through biomass turnover. However, sediment- (and taxonomic-) specific responses to the extraction protocol variants in term of richness were observed (Fig. S3), which may reflect the strong influence of the matrix composition on both biological community richness (McLachlan 1996; Seiderer and Newell 1999) and DNA preservation (Ogram *et al.* 1987; Pietramellara *et al.* 2009). Furthermore, community-diversity metrics accounting for both richness and relative abundance of OTUs (i.e. Shannon index) exhibited moderate differences between protocols (excepted for metazoans), suggesting that potential differences observed in OTU richness concern mostly rare OTUs. In the context of biomonitoring, this is unlikely to be of concern, because the primary goal is to obtain representative and reproducible samples of the community, rather than a comprehensive inventory, as in the case of biodiversity studies (Chariton *et al.* 2016).

Beta-diversity patterns

The two extraction approaches used here were tailored to capture different components of sediment eDNA. Although the totDNA undoubtedly includes extracellular DNA (Chariton *et al.* 2015), the approach is designed to maximise the extraction of DNA residing within cells, be it active, dead or dormant. By contrast, the extDNA approach is unlikely to capture recalcitrant DNA within whole cell structures, but rather provides a temporally integrated biodiversity signal, including the presence of 'relic' DNA. Although this can lead to differences in the identity of OTUs retrieved between extraction methods (Taberlet *et al.* 2012*b*; Wagner *et al.* 2015;

Zinger et al. 2016; Nagler et al. 2018), our findings support our hypothesis that the extraction approach has only a moderate effect on β-diversity patterns (Hypothesis 3; Fig. 2a–c, 3). These results are in line with those by Ramírez et al. (2018b), who found only minimum differences between total and intercellular bacterial communities extracted from marine sediments.

Overall, compositional profiles based on the relative abundances of phyla were highly congruent among extraction protocol variants (Fig. 4), and strong correlations in the relative abundances of most phyla were observed between the two extraction approaches (Fig. S5). This was particularly the case for prokaryotes, whereas some eukaryote and metazoan phyla tended to be biased towards a particular extraction approach (Fig. 4, S5, Table S5). Two main distinctions in the methods can explain these differences; in contrast to the totDNA extraction protocol, the extDNA protocol does not include any cell lysis step; active communities with recalcitrant DNA could, thus, be better extracted by the totDNA protocol. However, extDNA is expected to be less biased by the presence of macroremains in the original samples (e.g. rootlets; Pansu et al. 2015a), which can release massive quantity of DNA when those cells are lysed. In that sense, estimates of macro-organism biodiversity made from totDNA can be more sensitive to 'subsampling' effects than are those based on extDNA.

In general, the mass of the extracted sediment had only a moderate effect on community composition (Hypothesis 4), and played a much less important role than the extraction approach. However, the initial starting volume of sediment had a more pronounced effect in the samples extracted using totDNA (Fig. 3a, h–j, 4), with eukaryote and metazoan Bray–Curtis distances derived from the totDNA extraction of 1 g of sediment being more dissimilar to those derived from other extraction protocol variants (Fig. 3a–d). Interestingly, this was not observed for prokaryotes, which is likely to be a reflection of their size and density within a sample, and, thus, the capacity to capture a representative sample using a small amount of sediment. Because of their porosity and water movements, the genetic signal in sediments can be more homogenous than in soils. Here, community composition patterns from samples extracted using the extDNA protocols with 10 and 200 g of sediments (and, to a lower extent, 1 g) were very similar. These results indicated that even small amounts of sediment (<10 g of an original sample of 500 g) extracted with the extDNA protocol can efficiently represent local biodiversity, assuming that the original sample was well mixed. However, even though results were not necessarily significant, replicate samples tended to be more homogenous when a larger amount of sampling material was used for eukaryote and metazoan primers (Fig. S4), suggesting that a larger sample size is necessary to get relevant and consistent patterns of larger organisms (Ranjard et al. 2003; Taberlet et al. 2012b). Given the large body of evidence on how macrofauna (e.g. insects, crustacea, annelids) respond to natural and anthropogenic stressors, they are likely to remain the focus of biomonitoring studies for the foreseeable future. It is imperative, therefore, that biomonitoring approaches using eDNA consider the importance of the starting sample volume.

Conclusions

Extracellular DNA extraction from aquatic sediment provides reliable estimates of community diversity and composition, qualitatively comparable to those obtained with totDNA. Although both methods may provide marginally different 'views' of composition, other parts of the metabarcoding pipeline, including bioinformatics, primer choice and filtering, can also profoundly shape compositional profiles (Clarke et al. 2014; Alberdi et al. 2018; Taberlet et al. 2018; Pauvert et al. 2019). Hence, each extraction method has its own trade-off, and the choice of the most appropriate approach ultimately depends of the study objectives, taxa of interest along with financial and technical constraints. In the context of routine biomonitoring programs, which aims at providing a comprehensive view of the local diversity in a reproducible way, the extracellular DNA approach offers many benefits; namely, it is easy to implement, and cheaper and faster than commercial kits dedicated to total DNA (Taberlet et al. 2012b; Pansu et al. 2015b; Zinger et al. 2016). It allows the processing of a larger number of samples in a reduced amount of time, and, thus, potentially increased sampling intensity or the extent of the study area for a limited financial and quality cost. In doing so, targeting extDNA provides opportunities for biomonitoring in situations where it may have been excluded because of cost, lack of taxonomic expertise and latency in producing data. In addition, extracellular DNA can be extracted from a greater mass of sediment, which can promote the sampling replicability of larger taxa, such as polychaetes, whose responses to a range of environmental stressors have been well documented. We argue that the advantages and the relevance of this approach will contribute to the increased use of sediment eDNA-based biomonitoring for ecological assessment of aquatic environments.

Authors' contribution

A. A. Chariton and G. C. Hose designed the study. A. Chariton and M. B. Chapman collected samples. M. B. Chapman and J. Pansu conducted DNA analyses. J. Pansu processed the data and analysed the results. J. Pansu and A. A. Chariton drafted the manuscript. All authors contributed to manuscript revisions and approved the submitted version.

Data accessibility

Datasets have been deposited in the Dryad depository (https://doi.org/10.5061/dryad.kwh70rz34) and the CSIRO data access portal (see https://data.csiro.au).

Conflicts of interest

J. Pansu and A. A. Chariton are Guest Editors of the Environmental DNA special issue. Despite this relationship, they took no part in the review and acceptance of this or any other manuscript in this issue that they authored. The authors declare that they have no further conflicts of interest.

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