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ORIGINAL ARTICLE

The impact of DNA extract homogenization and replication on marine sediment metabarcoding diversity and heterogeneity

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Abstract

Metabarcoding of environmental DNA (eDNA) is an attractive complement to morphological methods for surveys and routine monitoring of marine sediment benthic communities. However, metabarcoding and other genetic techniques are heavily affected by choices made during sampling, processing, and analysis. Here, we investigated the effect of different eDNA extraction protocols on observed alpha- and beta diversity of replicates from the same grab. Specifically, we compared (A) homogenization intensity during sediment DNA extraction, (B) extraction replicates *vs* larger sediment extraction volume, and (C) pre- and post-PCR extract pooling. Using the 18S V1-V2 region marker, we show that a Precellys homogenizer protocol during DNA extraction can significantly improve sediment metabarcoding results in terms of captured diversity and inter-replicate homogeneity compared to vortexing only. This effect superseded that of increased sediment extract volume. Pre-PCR pooling of DNA extraction replicates increased observed rarefied richness compared to data from single extracts only, but not to the extent of sample extract replicates amplified individually before pooling. We argue that this discrepancy was due to a reduction both in recovered sample diversity, but also the number of PCR artifacts and PCR drift. Our results demonstrate that extraction replicates of smaller sediment volumes, in combination with moderate Precellys homogenization and pre-PCR pooling, are a cost-effective way to increase the amount of organism diversity that is recovered from sediment eDNA metabarcoding samples.

KEYWORDS
Benthos, community ecology, DNA extraction, environmental DNA, metabarcoding

1 | INTRODUCTION

Marine soft bottom sediments form a large part of benthic ecosystems worldwide, are home to diverse metazoan and microbial communities, and provide important ecosystem services at local and global scales (Ramirez-Llodra et al., 2010; Snelgrove, 1997). Traditionally, most studies of the diversity and connectivity of marine benthic communities have focused on morphological identification of macroinvertebrate fauna. Thus, only a small part of the total biological community is investigated. Further, it is time-consuming and dependent on highly specialized taxonomic expertise (Schander & Willassen, 2005).

For these reasons, metabarcoding of environmental or community DNA (here collectively "eDNA") is an attractive alternative or complement to existing morphological methods to investigate benthic community diversity (Baird & Hajibabaei, 2012; Chariton et al., 2010; Cordier et al., 2020; Mohrbeck et al., 2015). Metabarcoding enables faster and more affordable analyses, is less dependent on specialized taxonomic expertise, and may help to enable automated sampling in future. Importantly, eDNA data also allow the study of meio- and microfauna, yielding a more complete picture of benthic communities and functional relationships compared to studies limited to the macrofauna (Bik et al., 2012; Bourlat et al., 2013; Faria et al., 2018; Lanzén et al., 2016).

While the potential advantages of eDNA are well established, many obstacles remain for metabarcoding to become an established technology in routine monitoring of marine benthic biodiversity. To a greater degree than that of morphological taxonomy, choices made during sampling, laboratory work and analysis are decisive factors for obtained results (Deiner et al., 2015; Jeunen et al., 2019; Leray & Knowlton, 2016; Zinger et al., 2019). Numerous recent benthic metabarcoding studies have been published as sequencing has become more affordable and environmental genomics expertise and data sequence analysis tools more accessible. Yet, current metabarcoding studies of benthic habitats still differ in methods and sampling design, and are often one-off events using few technical or biological replicates (Cordier et al., 2020; Makiola et al., 2020). While an increasing number of studies have investigated technical aspects of sediment eDNA metabarcoding, many factors that influence or bias results are still not well understood. Thus, it remains challenging to understand to which degree sediment metabarcoding results are reproducible and representative for any given locality (e.gBrannock & Halanych, 2015; Klunder et al., 2019; Lanzén et al., 2017; Lekang et al., 2015; Nascimento et al., 2018).

The amount of sediment source material used for eDNA extraction is magnitudes smaller than what is necessary for morphological characterization of macrofauna. While the distribution of total community eDNA (including extracellular DNA) does not correspond directly to that of individual organisms, heterogeneous distribution is still a critical concern that has proven challenging to address (Nascimento et al., 2018). Increased sequencing depth (i.e., number of sequence reads per sample) can improve coverage of targeted organisms, estimates of alpha diversity, and the ability to separate samples with different characteristics (Smith & Peay, 2014). Increasing the volume of source material for DNA extraction has an even larger positive effect on coverage, independently of sequencing depth (Nascimento et al., 2018; Penton et al., 2016), but a similar outcome has also been achieved by using several replicates of smaller extraction volumes (Lanzén et al., 2017).

Furthermore, the DNA extraction protocol also has a large impact on results (Lekang et al., 2015). An important step in many sediment DNA extraction protocols is homogenization, where beads are added to the sediment sample and subjected to rapid movement in order to facilitate release of DNA. Smaller volume extracts allow the use of homogenizer instruments that enable higher intensity homogenization than standard vortexing, and are easier to include in automated and semi-automated laboratory processing. Thus, there are several approaches that can be used to increase sediment sampling completeness, and it is not clear which approach yields the most consistent community structure estimates for the least additional cost and effort.

In order to help establish sampling and processing recommendations for benthic eDNA metabarcoding, we investigated the relative effects of homogenization treatment intensity, sediment source volume and number of DNA extract replicates from five van Veen grab samples at an offshore North Sea monitoring station targeting the V1-V2 region of the 18S rRNA gene. This widely used marker targets both a broad range of microeukaryotes and most metazoan groups (see, e.g., Mauffrey et al., 2020). As we anticipated that distribution patterns of metazoans might be different from single-celled eukaryotes, the metazoan fraction was examined separately in several of the analyses.

We hypothesized that the type and intensity of sediment homogenization treatment during DNA extraction has a significant impact on perceived community structure (diversity and composition) and the extent of random sampling effect, that is, perceived inter-replicate heterogeneity. To test this, we investigated the relative effect of low- to high-intensity homogenization treatments of extraction replicates from the same sediment sample on both total eukaryotic and metazoan community DNA coverage.

Moreover, we hypothesized that technical replication of smaller sediment volumes can act as a time- and cost-efficient alternative to increased sediment volume. To this end, we compared the relative effect of increased sediment volume and number of extraction replicates, taking into account that it is easier to homogenize smaller sediment volumes more rigorously.

Finally, we hypothesized that the observed increase in alpha diversity associated with increasing the number of extraction replicates is due to a more complete sampling of the source sediment, rather than technical artifacts caused by the extra PCR replication, and can thus be fully or partially recreated by pooling extraction replicates prior to PCR. To verify this, we compared the effect of pooling DNA extracts before (pre) and after (post) PCR on resultant alpha diversity, using DNA replicates from five grab parallels, and alpha diversity estimates of pre-PCR and in silico pools for each homogenization treatment.

2 | MATERIALS AND METHODS

2.1 | Sediment sampling

The sediment samples used in this study were collected as part of the 2018 environmental monitoring of the oil and gas installations in monitoring Region III, part of the Norwegian shelf in the North Sea. Samples were taken from five van Veen grab parallels (500 g from grab 4 and 50 g each from grabs 1–3 and 5) at station GK-DA-01

(58.5757N, 1.6973E, 116 m depth). Each grab was subsampled so that an equal weight of sediment was taken from three separate surface areas (0–2 cm) and pooled into one sample (Table 1). Sediment samples were immediately frozen and kept at −20°C until DNA extraction. The sediment at the station, examined as part of the regular monitoring, was characterized as fine sand (φ = 3.03, silt and clay = 11.14%, sand = 83.13%, gravel = 5.73%, TOC = 0.48%) (Hatlen et al., 2019).

2.2 | Homogenization and DNA extraction

To test the impact of homogenization on observed diversity and composition, four homogenization treatment groups were defined to obtain individually indexed extraction replicates as well as pre-PCR pools thereof, prepared from the grab 4 sediment sample: (1) *Precellys 1* homogenization of 0.5 g sediment, (2) *Precellys 2* homogenization of 0.5 g sediment, (3) vortexing of 0.5 g sediment, and (4) vortexing of 5 g sediment. To test the relative effect of pre- and post-PCR pooling, five 0.5 g extraction replicates were also made from each of the other four grabs (Figure 1; Table 1).

Whole sediment samples from each grab were thawed in a refrigerator and pre-mixed by hand with a spatula for 1 min before subsamples were taken for DNA extraction. For the 5 g extracts, we used the standard Qiagen DNeasy PowerMax Soil kit following the manufacturer's instructions and a protocol with 20-min vortexing at 2000 rpm. For the 0.5 g extracts, we used Qiagen PowerBead tubes and C1 solution for initial steps and homogenization (analogous to the Qiagen PowerSoil kit), then after centrifugation (10K rpm for 1 min) the Qiagen QIAsymphony SP robot (DSP DNA kit, Tissue LC protocol) for remaining extraction steps. The 0.5 g samples were homogenized using vortexing (2000 rpm for 20 min) or the Precellys 24 homogenizer (Bertin Instruments) with either a high-intensity program of three rounds of 6000 rpm for 40 s with 120 s intervals (*Precellys 1*) or a medium intensity program of a single round of 6000 rpm for 40 s (*Precellys 2*). Extraction negative controls were included for each extraction event. DNA concentrations for all extracts were measured with a Qubit 3.0 fluorometer (Thermo Fisher Scientific), and lack of DNA fragmentation for more rigorous homogenization treatment confirmed using an Agilent 2100 Bioanalyzer.

2.3 | PCR amplification and sequencing

The 18S V1-V2 region (~350–400 bp) was amplified using PCR with the SSU_F04mod/SSU_R22mod primer pair. The SSU_F04mod (5'-GCTTGWCTCAAAGATTAAGCC-3') (Cordier pers. comm.) and SSU_R22mod (5'-CCTGCTGCCTTCCTTRGA-3') (Sinniger et al., 2016) primers are slightly modified versions of the SSU_F04 and SSU_R22 primers (Blaxter et al., 1998). For pre-PCR pooling of extracts, average percent deviations of extract concentrations among individual extracts within each of the four treatment pools were measured as 2.98%–4.95%. Given the small variation in extract concentration, equal volumes of each extract were included directly in extract pools. PCR amplification was performed using the KAPA3G Plant PCR kit (Kapa Biosystems) with primers containing 12 random bases to aid sequencing. The protocol included an initial 3 min step at 95°C, 30 cycles of 30 s each at 95, 57 and 72°C, and a final 10 m step at 72°C. Libraries were prepared with equimolar concentration of PCR product using the TruSeq dual index Illumina i5/i7 barcode set to distinguish individual samples. Extraction and PCR negative controls were used to check sample contamination. Sequencing was performed on an Illumina MiSeq instrument using v3 300 bp chemistry at the Norwegian Sequencing Centre (University of Oslo, Norway).

We sequenced (A) ten replicates for each of the 0.5 g homogenization treatments Vortex, Precellys 1 and Precellys 2, and four additional 5 g Vortex replicates, (B) ten PCR replicates from a single extract, (C) pre-PCR samples pooled from all (0.5 g = 10; 5 g = 4) treatment extracts for each of the four homogenization treatments, and (D) four data replicates from each of the five grabs using either three or five extraction replicates pooled before or after initial PCR amplification. In all, this comprised 68 indexed data replicates (hereafter "samples") from 55 distinct DNA extraction replicates, in addition to negative controls (Table S1).

TABLE 1 Overview of study sediment samples and extracts

Grab sample	Homogenization treatment	Extraction replicates	Extraction subsample (g)	Purpose
1	Precellys 2	5	0.5	P
2	Precellys 2	5	0.5	P
3	Precellys 2	5	0.5	P
4	Vortex	4	5	H, T
4	Vortex	10	0.5	H, T
4	Precellys 1	10	0.5	H, T
4	Precellys 2	10	0.5	H, T, (P n=5)
5	Precellys 2	5	0.5	P

Note: The heading "Purpose" describes the relevant experiment: investigating the relative effect of (H) homogenization, (T) technical replication, and (P) pre- and post-PCR pooling.

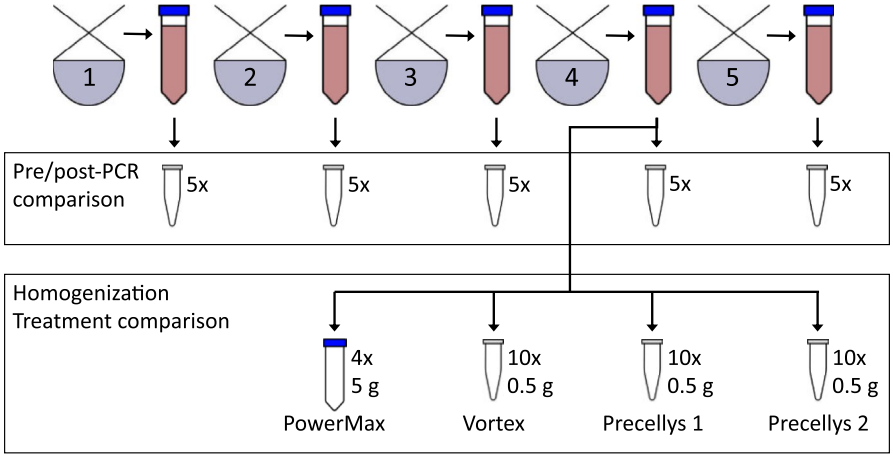

FIGURE 1 Graphical overview of study sampling design. Five 0.5 g DNA extract replicates were sampled from each of five separate grabs to compare the effect of pre- and post-PCR pooling. For tests of homogenization intensity and sediment volume, a total of 4 5 g and 30 0.5 g DNA extracts were sampled using sediment from grab 4. The "PowerMax" and "Vortex" replicates were homogenized using vortexing, while "Precellys 1 " and "Precellys 2" replicates were subjected to two different Precellys homogenizer programs, respectively

2.4 | Data processing

Sequence quality was manually assessed using FastQC v0.11.8 (Andrews, 2010). Quality filtering, clustering into SWARM sequence variants (hereafter "operational taxonomic units," OTUs) and post-clustering correction was carried out using a minimally modified protocol from Lanzén et al., (2020). Briefly, read pair merging, filtering, singleton removal and chimera detection was performed using VSEARCH v2.11.1 (Rognes et al., 2016). A maximum of 20 mismatches were accepted for read pairing, after which primers were trimmed using Cutadapt v1.18 (Martin, 2011) and reads lacking the complete forward and reverse primers discarded. All amplicons (merged, trimmed read pairs) shorter than 330 or longer than 450 bp were also discarded. Clustering was done using SWARM v2.2.1 with default settings, that is, a maximum linkage difference (-d) of 1 and 3 for the fastidious step (-b) (Mahé et al., 2015). Post-clustering correction was carried out using LULU with 97% and 95% minimum similarity and co-occurrence, respectively (Frøslev et al., 2017). Taxonomy was assigned using CREST release 3.2.2 (Lanzén et al., 2012) with the SilvaMod v128 as reference database (https://github.com/lanzen/CREST).

Reads were further filtered using R. OTU cross-contamination among samples was reduced by removing sample occurrences of an OTU when present at very low abundance compared to its average abundance across samples (<1%), similar to the UNCROSS algorithm (Edgar, 2016). All OTUs below the minimum classification threshold or classified to clear non-target, putative contaminating taxa were also removed (Insecta, Mammalia, Arachnida, Actinopterygii). The relative abundance ratio in negative controls *vs*. the average in real samples was used to manually identify and remove any remaining potential contaminant OTUs, resulting in the removal of a single OTU. Datasets were divided into Metazoa and non-Metazoa for certain analyses. Finally, in silico pooling was carried out to compare extraction replicates to total datasets for each homogenization treatment.

2.5 | Statistical analysis

Rarefaction, sub-sampling, and Shannon diversity estimates were calculated using the R package *vegan* v3.2.1 (Oksanen et al., 2019). To facilitate direct comparisons among technical replicates, we calculated expected OTU richness for the read depth corresponding to the smallest sample, using the function *rarefy* (hereafter "rarefied richness"). Shannon diversity (H') was calculated after random subsampling to 40,000 reads.

Bray-Curtis pairwise dissimilarity values were calculated based on Hellinger transformed sample abundance data, filtered using a relative abundance cutoff in order to compensate for differences in sequence depth and random sampling effects. Specifically, we removed all OTUs with an average relative abundance across samples that corresponded to fewer than 3 reads in the least sequenced sample (46,826 reads, i.e., 0.0064% relative read abundance), leaving 770 OTUs in the dataset (8% of total). Non-metric dimensional scaling as implemented in *vegan* (function *metaMDS*) was used to transform and visualize the dissimilarity matrix to a non-linear approximation in 2D space.

To investigate the effect of the number of pooled extraction replicates for, we used random subsampling (100 iterations) as previously described in Lanzén et al., (2017). Briefly, for each iteration we drew a random subset of m_i replicates, for each value of m_i ranging from 1 to n where n was the total number of replicates available for each compared group (n = 4 for Vortex 5 g and n = 10 for other treatments). The function *rrarefy* was then applied to each picked replicate, to randomly subsample $ri = D/m_i$ where D was chosen close to the sequencing depth of the least sequenced sample,

specifically 40,000. In other words, a total simulated sequencing depth of 40,000 reads was kept, regardless of the number of simulated pooled extraction replicates. Standard errors for each treatment group across iterations were calculated for each value of m_i.

Pairwise comparisons among groups were carried out using one-sided Wilcoxon signed-rank tests, since differences (of richness, diversity or dissimilarity values) could not be assumed to be normally distributed.

3 | RESULTS

3.1 | Sequencing data and taxonomical assignment

The total number of raw read pairs was 8.1 million, with 56–163 thousand reads for individual samples. After taxonomy and cross-contamination filtering, 6.8 million total reads, with 47–139 thousand reads per sample and 9,190 total OTUs, were retained (Table 2). The 4 ×5 g vortexed samples yielded a total of 311,337 reads for, and the three distinct treatments based on 10 ×0.5 g sediment yielded 811,299–1,094,423 reads each (Table S1).

The most dominant taxon, in terms of relative read abundance, was unclassified Stramenopiles (most of which are phytoplankton), followed by Enoplida (Nematoda), *Ebria* (Rhizaria) and the cercazoan clade CCW10 (Fig. S1A). One extraction replicate in particular (Precellys 1–1) appeared as an outlier with a strong dominance of reads assigned to the polychaete order Phyllodocida. The 10 PCR replicates derived from the same extraction replicate differed very little from each other. The metazoan composition was more heterogeneous among extraction replicates than the total eukaryote dataset. The three dominating metazoan phyla according were Nematoda, Nemertea, and Annelida (Fig. S1B).

The relative metazoan read abundance for individual samples across all homogenization treatment groups was 26% (ranging from 11%–40% except the Precellys 1 outlier at 72%). This abundance was highest for the 0.5 g Precellys 1 treatment, but differences were not significant. The four pre-PCR pooled extract samples had roughly similar relative metazoan read abundance as individual extracts from the same treatment type (ignoring the Precellys 1 outlier). In contrast, the increased sediment volume of the 5 g vortexed samples resulted in significantly higher OTU richness for individual samples (on average 155) compared to the 0.5 g treatments (91–104), but this trend was not consistent with the pre-PCR pooled samples, where OTU richness was higher for both Precellys treatment pools despite being composed of a smaller amount of total sediment (5 g *vs.* 20 g) (Table 3, Table S1).

3.2 | Effect of homogenization treatment on diversity estimates and community composition

The distributions of rarefied richness and Shannon diversity (40,000 reads) for the different homogenization treatments are shown in Figure 2. Precellys 2 treatment yielded significantly higher median rarefied richness ($p < 0.001$), and both Precellys treatments yielded higher median rarefied richness than both 0.5 and 5 g vortexed samples. The same pattern was repeated for both pre-PCR and *in silico* pooled samples. Shannon diversity (H') was also higher for both Precellys treatments, although the difference here was not significant, and Precellys 1 distribution was strongly impacted by a Phyllodocida-dominated Precellys 1 outlier (Figure 2, Table S1).

Bray–Curtis dissimilarities (based on Hellinger transformed OTU abundances) among individual extraction replicate samples are represented as box and NMDS plots in Figure 3 grouped by homogenization treatment and including PCR replicates (from a single Precellys 2 extract). For the total 18S dataset, excluding the Precellys 1 outlier, median dissimilarities were similar for both Precellys treatments and the 5 g vortexed samples (0.23–0.24), while it was significantly higher for vortexed 0.5 g replicates (0.30; p = 5E-7), and significantly lower for PCR replicates of a single sample (0.075; p = 1E-7) (Figure 3A). The corresponding NMDS plot revealed that treatment groups formed separate clusters, clearly indicating higher intra-treatment than inter-treatment similarity (Figure 3C). Dissimilarities for the 18S metazoan fraction were higher than that of the total eukaryote dataset, especially for all three 0.5 g treatments, but somewhat lower for the 5 g vortexed samples (p < 1E-15) (Figure 3B). Finally, only the Precellys 2 treatment was clearly separate from the rest of the clusters (Figure 3D).

3.3 | Effect of number of technical replicates and replicate pools on alpha diversity estimates

To estimate the effect of additional extract replicates on observed richness, we ran simulations using repeated subsampling at a read depth of 40,000 distributed evenly over each randomly chosen replicate in pools of 1–10 replicates, for each treatment (Figure 4).

These results show that expected rarefied richness increases in an approximately log-linear fashion with the number of extraction replicates, regardless of homogenization treatment. Consistent with median richness of individual samples as well as sequenced pooled extracts (Figure 4A), Precellys 2 treatment provided the highest expected rarefied richness, followed by the Precellys 1, 5 g Vortex,

TABLE 2 Quality control and diversity statistics

Dataset	Reads (% of previous step)	OTUs (% of previous)	Metazoan reads (% abundance)	Metazoan OTUs (% richness)
Raw data	8,068,067 pairs	–	NA	–
After overlap, filtering and SWARM	6,815,525 (84%)	9,349	1,517,554 (22%)	1,344 (14%)
After taxonomic filtering	6,805,250 (99.8%)	9,190 (98%)	1,516,951 (22%)	1,322 (14%)

TABLE 3 Metazoan relative abundance and richness across treatments for 18S. For groups of replicates, averages ± 2x estimated standard deviation is given

Individual averages	Precellys 1 (0.5 g)	Precellys 2 (0.5 g)	Vortex (0.5 g)	Vortex (5 g)
Relative Metazoan abundance	28% (23%) ± 38% (10%)	19% ± 10%	18% ± 4%	18% ± 6%
Metazoan OTU richness	104 (106) ± 24 (21)	98 ± 22	91 ± 23	155 ± 18
Pre-PCR pooled extract samples	**Precellys 1 (5 g total) (×10)**	**Precellys 2 (5 g total) (×10)**	**Vortex (5 g total) (×10)**	**Vortex (20 g total) (×4)**
Metazoan read percentage	40%	21%	17%	18%
Metazoan OTU richness	241	232	165	208

Note: Precellys 1 values without the 72% outlier removed are given in parentheses.

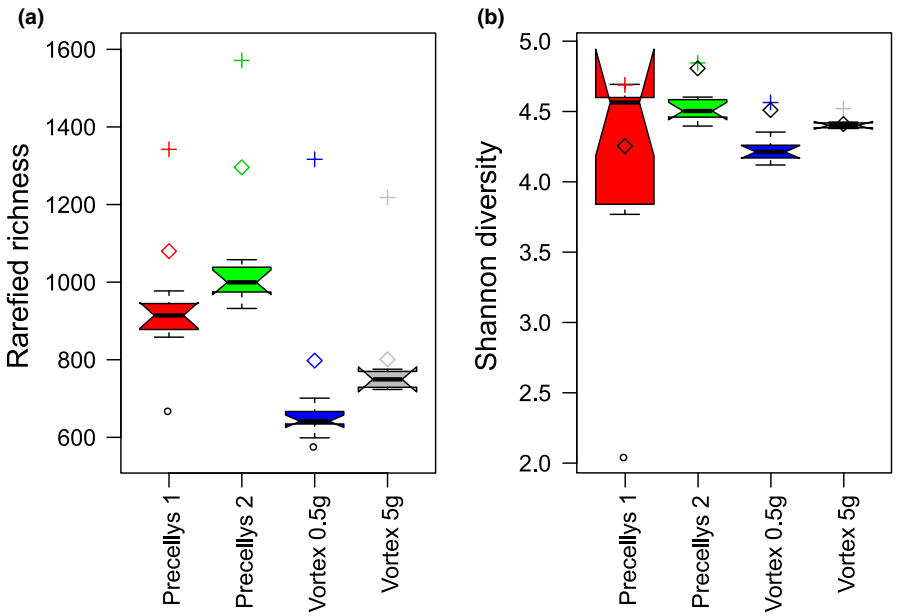

FIGURE 2 Homogenization treatment alpha diversity. Box plots representing (a) rarefied richness and (b) Shannon diversity (40K reads), comparing diversity of extraction by treatment (kit and homogenization method). The slanted notches around medians represent ± 1.58 IQR/sqrt(n), indicating strong evidence that medians differ (Chambers et al., 1983). Crosses represent diversity estimates calculated from *in silico* pooled replicates and diamonds from pre-PCR pools

and 0.5 g Vortex treatments. The rate of absolute change per added replicate was roughly similar for these treatments: Two to three replicates were needed to reach the same expected richness as could be reached with a single Precellys 2 treatment replicate. Richness also increased with the number of pooled PCR replicates, but not to the same extent as the increase observed for extraction replicates, and flattening off after five PCR replicates.

In contrast to the total eukaryote dataset, the 5 g Vortex treatment yielded significantly more OTU-rich data for the metazoan subset in particular, while other treatments performed similarly to each other (Figure 4B; Table S1). Approximately three replicates of either 0.5 g treatment were needed to obtain the same metazoan rarefied richness as one 5 g treatment replicate. For all treatment groups, the increase in metazoan richness with each added replicate remained high, while virtually no richness increase could be detected from adding PCR replicates.

As a separate experiment, we compared the relative effect of pre- and post-PCR pooling on observed alpha diversity by investigating PCR replicates run independently for each extraction before pooling (post-PCR) to first pooling and then running a single PCR amplification. This was carried out with either three or five pooled extracts for all five separate grabs from the same station. There was considerable variance in observed rarefied richness among grabs, but for samples from the same grab, rarefied richness was consistently and significantly higher for three-extract post-PCR pools compared to three-extract pre-PCR pools (p = 0.03, mean relative increase in richness 19%).

4 | DISCUSSION

Based on the comparative homogenization results, using a homogenizer to increase homogenization intensity beyond standard vortexing had a clear and significant positive impact on observed sample diversity and reduced inter-sample dissimilarities, demonstrating more complete recovery of DNA from the source sediment. Interestingly, the choice of homogenization protocol did not significantly change the read abundance ratio of metazoans to non-metazoans, meaning that no particular pro- or anti-metazoan bias was detected due to homogenization intensity.

The positive effect on community recovery from increased homogenization was found to be valid only up to a certain point,

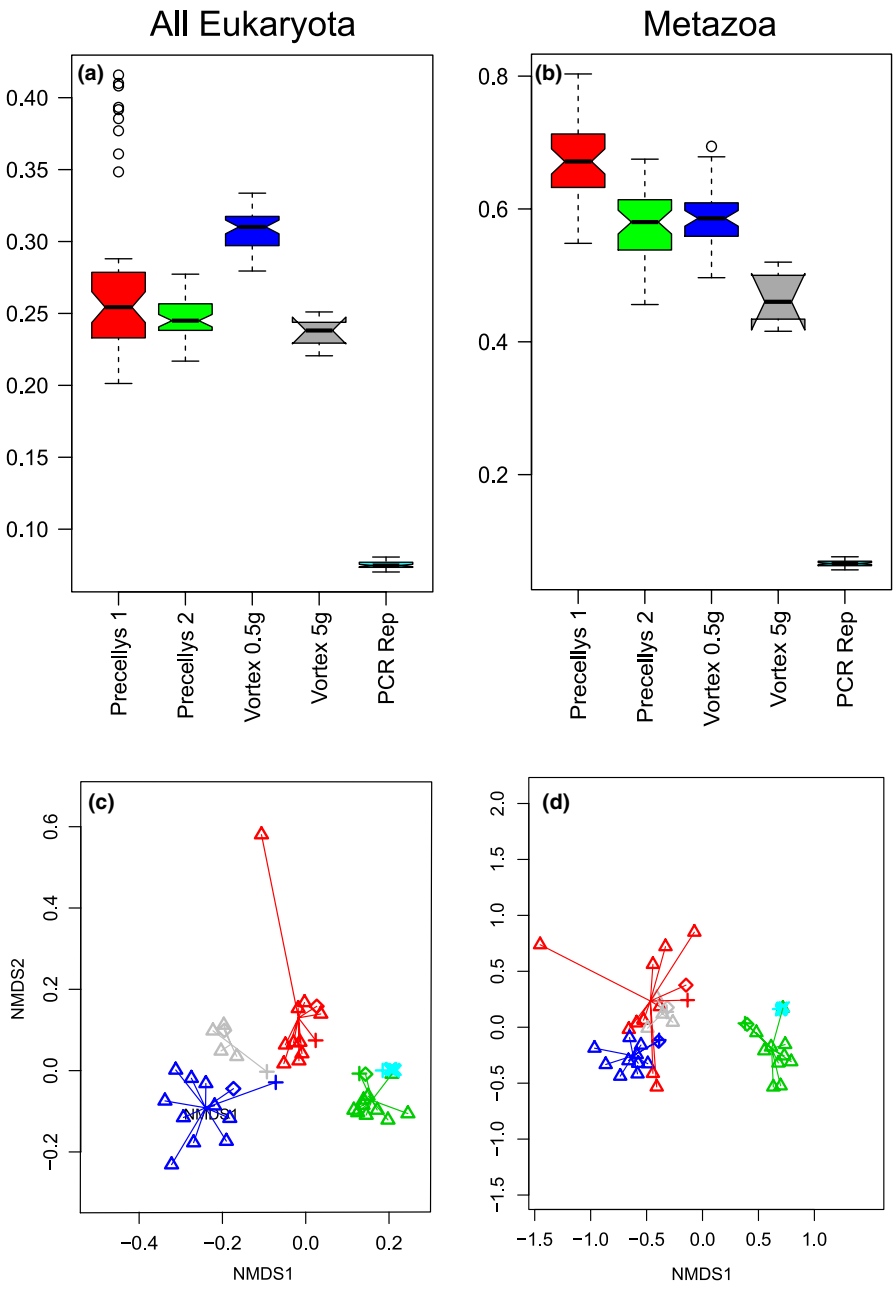

FIGURE 3 Homogenization treatment beta diversity. Bray-Curtis dissimilarities of samples grouped by homogenization treatment and the ten extraction replicates shown as box plots for (a) the total eukaryote dataset and (b) metazoans only, and non-metric dimensional scaling (NMDS) plots for (c) all eukaryotes and (d) metazoans only. Box plot slanted notches around medians represent ± 1.58 IQR/sqrt(n), indicating strong evidence that medians differ (Chambers et al., 1983). NMDS plots are color coded according to treatment using corresponding colors as the box plots

however: The highest diversity and lowest inter-sample dissimilarities were observed for the Precellys 2, rather than the higher intensity Precellys 1 homogenization protocol. This held true even when discounting the Precellys 1 outlier sample. It is particularly interesting since it suggests a homogenization "sweet spot" where maximum diversity can be obtained, but it must be stressed that these results are derived from a single station: DNA adsorbs differently to different types of sediments, especially with regards to grain size, that is, percentage of silt and clay (Levy-Booth et al., 2007). The relative performance of homogenization treatments can thus be expected to vary with sediment composition, and ideally needs be tested under a variety of conditions.

Increased sediment volume did have a small but significant effect on total eukaryote richness. Though still lower than both 0.5 g Precellys homogenizer treatments, 5 g vortexed samples were more OTU-rich than 0.5 g vortexed sediment samples. For metazoans in particular, however, the 5 g sourced samples did provide the best coverage, a result that could be attributed to a more heterogeneous sediment metazoan distribution relative to single-celled microorganisms, though our simulation of replication effects showed that the higher 5 g metazoan coverage could be replicated by using approximately three extraction replicates sourced from a smaller 0.5 g sediment volume (Figure 4). Given the higher cost and time usage per unit for larger volume extraction kits, especially when accounting for the use of an extraction robot protocol for smaller volume extracts as was done here, smaller sediment volumes could thus still be a more cost- and time-efficient sampling design for metazoans as well, even when a higher total number of replicates are needed. Thus, we did not find that sediment volume had the same decisive impact as reported in Nascimento et al., (2018), where larger sample volume

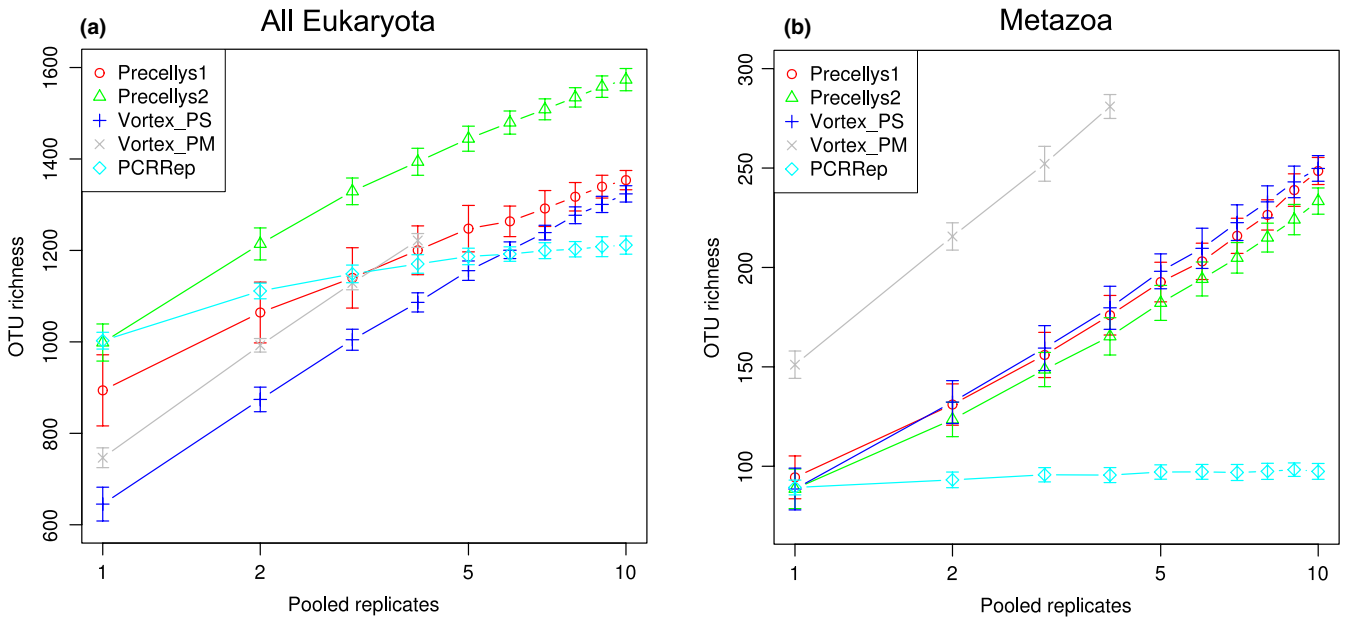

FIGURE 4 Cumulative richness increase. Expected (rarefied) OTU richness as a function of number of extract replicates (keeping the sum of sequence reads across replicates constant), for (a) 18S OTUs, and (b) the subset of 18S OTUs classified as Metazoa. Error bars represent standard error based on bootstrapping and taxonomic subsets

strongly and positively impacted both metazoan and non-metazoan alpha and beta diversity. The discrepancy between this study and Nascimento et al., (2018) could be explained by the inclusion here (and in Lanzén et al., 2017) of a manual pre-mixing step of the whole 50 g sediment subsample, which served to partially homogenize the sediment prior to extraction. With this caveat, our findings strongly support the hypothesis that replication of smaller volume extracts can act as a time- and cost-efficient alternative to the increased sediment volume of larger extraction kits. While the effect of single taxa dominating extract abundance is difficult to avoid completely, this effect can also be diluted by replicate extracts, reducing the total sediment volume necessary.

The results in this study clearly confirm the results of Lanzén et al., (2017) showing that pooled replicates significantly outperform individual extraction replicates at the same sequencing depth in terms of alpha diversity and intra-site *vs* inter-site homogeneity. The present study also compared data from samples pooled as extracts prior to PCR with the same extracts subjected to individual PCR before pooling, and found that pre-PCR pooling of extracts can be used to increase alpha diversity well beyond single extracts, but not to the extent of post-PCR or in silico pools. A failure of mixing individual extracts in an equimolar manner could also have caused this, but considering that pooled replicates had similar DNA concentrations (<± 5% deviation), this is an unlikely explanation. Our interpretation is that, while pre- rather than post-PCR extract pooling reduces recovered sample diversity to some extent, it also reduces the number of unique PCR artifacts in the data, estimated to be a significant fraction of singletons, doubletons, and tripletons (Zhan & MacIsaac, 2015). This is also consistent with our rarefaction analysis predicting the OTU richness captured depending on the number of PCR replicates (Figure 4) as well as the direct comparison of pre- and post-PCR replicates. Whereas about 20% of OTUs appeared to be PCR artifacts at a sequencing depth of 40,000 reads according to this analysis, the relative increase in richness from extraction replicates was at least twice as large for all treatments. This likely explains a significant part of the difference between post and pre-PCR pooling, and suggests that pre-PCR pooling should be considered a feasible and cost-effective alternative to PCR replication for sediment metabarcoding studies.

5 | CONCLUSIONS

Our findings show that choice of DNA homogenization protocol during extraction and use of extraction replicates are critical considerations in the design of sediment metabarcoding studies. For the 18S V1-V2 marker employed here, homogenizer treatment had a significant positive impact on total eukaryote alpha and beta diversity results compared to vortexing only. However, the less rigorous of the homogenizer programs provided the highest diversity, suggesting a "sweet spot" in terms of homogenization intensity that can be expected to vary with sediment composition. Homogenization treatment superseded the effect of increased sediment source volume except for the metazoan data fraction in particular. Still, even in the case of metazoans, three lower volume replicates were able to replicate the metazoan diversity of the larger volume samples.

The effort involved per extract is lower for smaller volume samples, especially considering the ability to partially automate the extraction workflow. Such extracts can also more readily be subjected to homogenizer treatment, and pre-PCR pooling should be considered a viable strategy to save time and reduce artifacts due to an increased number of PCR replicates. Finally, extract pooling lessens

the effect of "large" metazoan specimens randomly dominating read abundances in universal eukaryote data. Thus we argue that smaller volume DNA extraction replicates, combined with pre-PCR pooling, represents the most cost-effective way of maximizing sample coverage and reducing heterogeneity for large scale sediment metabarcoding studies.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

TGD and AL general conceptualized and funded the document. AL and JTH designed the study. JTH and KS performed the laboratory work. AL and JTH analyzed the data. JTH wrote the manuscript; JTH, AL, TGD, and KS edited the manuscript.

DATA AVAILABILITY STATEMENT

Unprocessed sequence read data are available through the NCBI Sequence Read Archive (SRA) as BioProject PRJNA718787 (Hestetun et al., 2021). All bash scripts used for sequence data processing (directory "script") and R code for analysis (directory "R/Paper1_18S") are available at the GitHub repository https://github.com/lanzen/Metamon1/.

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SUPPORTING INFORMATION

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