Hydrobiologia
https://doi.org/10.1007/s10750-021-04576-z

PRIMARY RESEARCH PAPER

Metabarcoding meiofauna biodiversity assessment in four beaches of Northern Colombia: effects of sampling protocols and primer choice

Lyda R. Castro · Rachel S. Meyer · Beth Shapiro · Sabrina Shirazi · Samuel Cutler · Ana M. Lagos · Sigmer Y. Quiroga

Received: 31 July 2020 / Revised: 9 March 2021 / Accepted: 18 March 2021
© The Author(s), under exclusive licence to Springer Nature Switzerland AG 2021, corrected publication 2021

Abstract Environmental DNA (eDNA) metabarcoding can enhance understanding of global biodiversity by making it possible to study taxonomic groups that are difficult to sample. However, experimental choices made when generating eDNA data can impact biodiversity surveys and must be carefully considered during study design. Here, we explored the impact of DNA extraction protocol and metabarcode choice on recovery of meiofauna DNA from sand. We extracted DNA from untreated sand and from sand treated with either Ludox or $MgCl_2$ and amplified DNA using the *18S* and *CO1* metabarcodes. We found differences in species composition and richness both between metabarcodes and among sampling strategies, confirming the sensitivity of the experiments to both parameters. Combining data from multiple barcodes and from multiple extraction protocols increased recovered meiofaunal taxonomic diversity. Future metabarcoding studies and meta-analyses should consider the effects of sampling protocols on biodiversity. Our results also highlight the need to continue to improve existing reference databases of morphological and molecular characterization of meiofauna, in particular of the tropics, which are poorly represented in existing databases.

Keywords DNA extraction · Coastal biodiversity · *18S* · *CO1* · Methodology · Environmental DNA metabarcoding · Biomonitoring

Handling editor: Cécile Fauvelot

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10750-021-04576-z.

L. R. Castro (✉)
Grupo de investigación Evolución, Sistemática y Ecología Molecular, Universidad del Magdalena, Carrera 32 # 22-08, Santa Marta DTCH, Colombia
e-mail: lcastro@unimagdalena.edu.co

R. S. Meyer · B. Shapiro · S. Shirazi · S. Cutler
Department of Ecology and Evolutionary Biology, University of California Santa Cruz, Santa Cruz, USA

B. Shapiro
Howard Hughes Medical Institute, University of California Santa Cruz, Santa Cruz, CA 95064, USA

A. M. Lagos · S. Y. Quiroga
Grupo de Investigación en Manejo y Conservación de Fauna, Flora y Ecosistemas Estratégicos Neotropicales MIKU, Universidad del Magdalena, Carrera32 # 22-08, Hangar B, Santa Marta DTCH, Colombia

Published online: 12 April 2021

Introduction

The meiofauna include all benthic animals and protozoa whose sizes range between macro- and microfauna. Meiofauna are defined as organisms that pass through a net with a mesh size of 500–1000 µm and are held back by nets of 20–63 µm mesh size (Fenchel, 1978; Higgins & Thiel, 1988; Giere, 2009; Ptatscheck et al., 2020). Meiofauna are essential members of the marine benthos, as they are basal in the trophic network and contribute to bioturbation through recycling of nutrients and activation of geochemical cycles (Bonaglia et al., 2014). This basal position and biological characteristics including high reproduction rates, short generation times, and the absence of pelagic larval dispersion for some groups, makes these organisms highly sensitive to environmental changes (Bongers & Ferris, 1999; Giere, 2009), and therefore important data sources for environmental impact and monitoring studies (Kennedy & Jacoby, 1999; Moreno et al., 2008; Zeppilli et al., 2015; Martínez et al., 2015).

Many studies have attempted to estimate meiobenthic biodiversity worldwide, of which the vast majority focus on identifying species among the dominant meiofaunal groups using morpho-taxonomy (Appeltans et al., 2012). Despite this work to characterize the marine meiobenthos through traditional taxonomy, several challenges remain, including the lack of specialist taxonomists and the fact that diagnostic morphological features of these organisms are often lost after methodological treatment (Zeppilli et al., 2015). These challenges lead to potentially biased estimates of the richness and distribution of marine benthos species. DNA metabarcoding approaches, in which "barcode" primers are used to amplify all species present in an environmental DNA (eDNA) sample that match the primer sequences, offer a complementary approach to cataloging meiofaunal species of the marine benthos using morphology. eDNA metabarcoding can be more efficient in estimating species richness than the morphotaxonomic approach (Lejzerowicz et al., 2015), and can complement and expand existing approaches to assess overall biodiversity (Fonseca et al., 2017).

While studies of meiofaunal communities using metabarcoding have shown the benefits of the technique for this particular group, many challenges remain. In particular, it would be useful to better understand what biases emerge when cataloging meiofaunal diversity from choice of genetic locus, field collection and DNA extraction protocols, metabarcoding library preparation assays, and bioinformatic approaches, all of which can impact results (Deiner et al., 2015; Clare et al., 2016; Leasi et al., 2018; Fais et al., 2020).

DNA marker choice is a known challenge in metabarcoding studies. The *CO1* gene has been used routinely in both barcoding and metabarcoding studies of animal species (Hebert et al., 2003). Degenerate primers aimed to amplify short fragments have been developed to broaden their taxonomic application and to optimize the amplification of degraded DNA from the environment (Vamos et al., 2017). In particular, primers developed by Leray (2013) target a 313 bp fragment that is commonly used in marine metazoan metabarcoding, as is the combination mlCOIintF + jgHCO2198 primers developed by Geller et al. (2013). Lobo (2013) developed degenerate primers for the same region, and the combination mlCOIintF + LoboR1 was tested and recommended by Haenel et al. (2017) and Fais et al. (2020) for meiofaunal metabarcoding assays. This combination was preferred by Chang et al. (2020) based on costs. The *18S* nuclear gene is also commonly used in metabarcoding studies of marine metazoans, in particular the *18S* V1-V2 region or the *18S* V9 region (Van der Loos & Nijland, 2020), but has been suggested to have less power than *CO1* to discriminate species (Tang et al. 2012, Creer et al., 2010; Leray & Knowlton, 2016). However, Fais et al. (2020) detected similar richness from *CO1* and *18S*, but different meiofauna composition in the assays. Because markers will often amplify different species, combinations of two or more markers are recommended to result in more accurate estimates of species richness and composition (Alberdi et al., 2017).

Experimental design can also impact what taxa are recovered from environmental DNA samples. Many meiofaunal studies undertake a preprocessing step prior to DNA extraction, which is thought to improve DNA recovery of target taxa (Brannock et al., 2015). The most commonly used preprocessing steps are the same as those used prior morphological determination of meiofauna (Montagna et al., 2017; Vand der Loos & Nijland, 2020), and include separation via decantation/flotation with either $MgCl_2$ (Leasi et al., 2018) or Ludox solution (Fonseca et al., 2017; Faria et al.,

2018). Preprocessing is believed to facilitate recovery of largely cellular DNA (Creer et al., 2010). Some studies extract DNA directly from sand or water without preprocessing so as to specifically target extracellular DNA (Guardiola et al., 2015; Pearman et al., 2016), whereas other marine sediment studies focus on total DNA (Lejzerowicz et al., 2015; Nascimento et al., 2018; Fais et al., 2020). Hajibabaei et al. (2019) proposed that direct extraction of total DNA without pre-treatment reduces the probability of identifying many key species, such as benthic bio-indicator taxa. Better understanding of the impact of preprocessing on taxonomic recovery will be useful, in particular as eDNA studies are increasingly used to target large diversity assemblages for ecological analyses (Hermans et al., 2018).

In this study we used eDNA metabarcoding to estimate intertidal meiofaunal biodiversity present in four sandy beaches of Northern Colombia. We performed DNA extraction from samples treated using two common pre-treatment protocols for meiofaunal separation ($MgCl_2$ and Ludox) and from sediment without any pre-treatment (i.e., direct extraction), and amplified DNA using the *18S* and *CO1* markers. We then evaluated the impact of both of these parameters on the inventory of metazoan meiofauna taxa recovered at each beach. We also compared our results with previous meiofauna species reports in the geographic region, with the aim of assessing how eDNA may complement traditional morphological surveys.

Methodology

Sample collection

We collected sand samples from the intertidal in triplicate at four different beaches in Northern Colombia: Santa Marta (11° 14.54′ N, 74° 12.96′ W), Rodadero (11° 12.3′ N, 74° 13.68′ W), Sisiguaca (11° 16′ 15.45″ N, 74° 12′ 2.69″ W) and Monoguaca (11° 16′ 16.77″ N, 74° 12′ 9.74″), between July and August 2019. Sand was extracted with a corer of 10 cm length and an internal diameter of 3.57 cm, which is considered a minimum sampling area of 10 cm^2 (Giere, 2009).

We used two pre-treatment protocols for meiofauna separation from samples collected in each beach. For pre-treatment no. 1, meiofauna was extracted from sand by washing the sample with isotonic $MgCl_2$ solution and decanted by hand through 500 µm and 45 µm sieves before preserving it in a conical tube with absolute ethanol. For pre-treatment no. 2, meiofauna was floated with Ludox TM 50 (specific density 1.15 g/cm^3, De Jonge & Bouwman, 1977), captured on a 45 µm mesh sieve, washed with distilled H_2O, and stored again with absolute ethanol at − 20°C. Additionally, non-treated sand was preserved in absolute ethanol for comparison to the pre-treatments. All samples were centrifuged to eliminate the ethanol and well homogenized using a mortar and a pestle before DNA extraction.

DNA extraction and metabarcoding

We performed DNA extractions using the Qiagen DNeasy PowerSoil Kit (Qiagen, Germantown, Maryland, USA) following the manufacturer's protocol, which has been recommended in other meiofauna and metabarcoding studies using marine sediments (Atherthon & Jondelius, 2020; Pearman et al., 2020; Vand der Loos & Nijland, 2020). We processed one DNA extraction negative control of sterile water only alongside our extracts to control for contamination introduced at the extraction stage. We quantified DNA in each extract with a Qubit fluorometer (dsDNA HS Assay Kit, Invitrogen, Life Technologies, Carlsbad, California, USA) prior to PCR amplification.

We selected the *CO1* and *18S* genes to examine meiofauna composition of our samples. We used the mlCOIintF primer (5′- GGWACWGGWTGAACWGTWTAYCCYCC-3′) introduced by Leray et al. (2013) and the LoboR1 primer (5′- TAAACYTCWGGRTGWCCVRAARAAYCA-3′) by Lobo et al. (2013), which amplify an average 313 base pair (bp) region of the *CO1* gene, and the SSU_FO4 (5′-GCTTGTCTCAAAGATTAAGCC-3′) and SSU_R22 (5′-GCCTGCTGCCTTCCTTGGA-3′) primers which amplify a 360 bp of the V1–V2 regions of the nuclear small subunit *18S* rDNA (Creer et al., 2010; Fonseca et al., 2010). As directed by Murray et al. (2015), to test whether extracted DNA was free of PCR inhibitors and to optimize the number of cycles for amplification of each sample, we performed qPCR reactions on each DNA sample with both primer sets. We varied the input DNA concentration from undiluted to 1:2 and 1:4 dilutions with the expectation that inhibited samples would exhibit better amplification if

diluted. We observed the amplification curve cycle thresholds to determine the sample-specific number of PCR cycles that would avoid overamplification. qPCR reactions were conducted in 25 µl volumes containing 12.5 µl Qiagen Multiplex PCR Mastermix (Qiagen, Germantown, MA, USA); 2 µM of each forward and reverse metabarcoding primer; SYBR green I Dye (1:2000 dilution; Thermo Fisher, Waltham, MS, USA) and 2 µl of DNA extract. qPCR cycling parameters included and initial activation at 95°C for 15 min, followed by 40 cycles of a denaturation step at 94°C for 30 s, an annealing step at 59°C for *CO1* and 58°C for *18S* for 30 s and an extension at 72°C for 1 min.

We prepared metabarcoding libraries using a 'two-step' amplification protocol (described in Nichols et al., 2018) where first a metabarcoding PCR was performed with metabarcoding primers that included TRUSEQ primers, followed by an indexing PCR to incorporate unique dual indexes and Illumina adapters. We performed three technical PCR replicates per sample per primer, and processed PCR negative controls to enable assessment of contamination introduced during library preparation. We conducted each reaction in 25 µl volumes containing 12.5 µl Qiagen Multiplex 2 × Mastermix; 2 µM of each forward and reverse metabarcoding primer and 2 µl of undiluted DNA extract. The reaction conditions for PCR included an initial denaturation step at 95°C for 15 min followed by 20–40 cycles (depending on qPCR for each sample) of denaturation at 94°C for 30 s, annealing at 59°C for *CO1* and 58°C for *18S* for 90 s, and extension at 72°C for 60 s. A final extension at 72°C was performed for 5 min. We cleaned the PCRs using Sera-mag Speed Beads (GE Healthcare Sciences, Marlborough, Mass.) at a 2:1 beads to sample ratio.

In the second amplification step, we conducted reactions in 25 µl volume containing 12.5 µl 2X Kapa HiFi HotStart ReadyMix (Kapa Biosystems, Wilmington, Massachusetts, USA); 1 µl (10 µM) of both unique forward and reverse indices and 5 µl of undiluted cleaned metabarcoding PCR product. PCR conditions were an initial denaturation step at 98°C for 3 min followed by 8 cycles of denaturation at 98°C for 20 s, annealing at 65°C for 30 s, and extension at 72°C for 40 s. A final extension at 72°C was performed for 2 min. We cleaned the indexing PCR products using Sera-mag Speed Beads as described above and quantified each with the Qubit HS Assay Kit (Thermofisher Scientific, 346 Massachusetts, USA). We then pooled samples at an equimolar concentration and sequenced them at the University of California Santa Cruz Paleogenomics Lab on an Illumina NextSeq instrument with a V3 2 × 150 bp kit, targeting a sequencing depth of minimum 20,000 paired reads per sample. Negative controls were sequenced to the same depth.

Bioinformatic and statistical analyses

We performed demultiplexing and FASTQ file generation using the Illumina HiSeq control software. We then filtered the data and assigned reads to taxa using the *Anacapa Toolkit* (Curd et al., 2019) (https://github.com/limey-bean/Anacapa) with default settings, and with custom CRUX reference databases made in October 2019 for *CO1* and *18S V1-V2* loci. These reference databases are available in the Zenodo link. Data QC and filtering followed *Anacapa* default settings in Curd et al. Taxonomy assignment also followed default settings; in brief, we assigned amplicon sequence variants (ASVs) using Bowtie 2 (Langmead & Salzberg, 2012) for alignment and then the minimum Bayesian Least Common Ancestor Bootstrap Confidence Count (BLCA BCC; Gao et al., 2017) of 90 to determine the appropriate taxonomic classification levels. The resulting tables of read counts per taxon were decontaminated by removing taxa that had higher prevalence in the negative controls compared to samples based on a score statistic $P > 0.1$ (Decontam package; Davis et al., 2018), removing taxa with 5 or fewer total reads, and selecting only the taxa with reads in at least two out of the three PCR replicates.

We converted the decontaminated ASV tables into *phyloseq* objects (McMurdie & Holmes, 2013) using *Ranacapa* (Kandlikar et al., 2018) in R 3.3.1. We visually summarized taxonomic diversity with flourish (https://flourish.studio). We generated rarefaction curves in *phyloseq* using 1000 read intervals, and taxon accumulation curves using iNext (Hsieh et al., 2016), and plotted both with ggplot2. We subsequently rarified the taxonomy tables for select analyses where we needed to accommodate differences in sequencing depth across samples that may affect estimates of diversity and community similarity. We chose to rarefy to the number of sequences present in the sample with the lowest number of sequences, because at this level the taxon gain had surpassed the linear growth

phase of both rarefaction and taxon accumulation curves (shown in Results). We used rarefied tables to calculate alpha diversity (Observed, Shannon, Chao1, Simpson) in *phyloseq*. To test whether the differences in diversity were statistically significant, we performed Kruskal–Wallis tests and *post hoc* Dunn tests comparing each group against another. Benjamini Hochberg *P* value adjustment was used with the Dunn tests (Benjamini & Hochberg, 1995). We also performed mixed model ANOVAs to look for interactions between locality and technique using the *aov* function in *R*, which assumed fixed effects.

We tested Beta diversity (Jaccard and Bray–Curtis dissimilarity) across sampling sites and among sampling protocols, and plotted results in Principal Coordinate Analysis (PCoA) ordinations. We tested for statistical significance using ADONIS in *vegan* (Oksanen, 2019) in R.

We computed log twofold differences in taxon abundances between techniques by negative binomial Wald tests using the R package *DESeq2* (Love et al., 2014), with Benjamini–Hochberg False Discovery Rate correction using the R package *MicrobiomeSeq* (Ssekagiri et al., 2018). For all alpha and beta diversity plotting as well as *DESeq2* plotting, we used *ggplot2* (Wickham, 2016). We repeated all statistical analyses with ASV tables including only metazoan meiofaunal groups.

Analysis of species found

We performed a detailed analysis of the species found by searching if they had been reported in databases such as World Register of Marine Species (Worms—marinespecies.org) and if they had been reported in Colombia or nearby countries, using the Global Biodiversity Information Facility database (gbif.org). We also aligned 50 unique amplicon sequence variants (or fewer in cases where 50 sequences were not available) from each of the species identified in our final taxonomy tables using the BLASTn algorithm against the full non-redundant nucleotide database on NCBI.

Results

Raw sequencing results and ASVs generation

We sequenced a total of 2,164,008 raw reads of the *18S* locus and 2,238,690 raw reads of the *CO1* locus. Of these, 773,312 for *18S* and 678,504 for *18S* were assigned to a taxon prior to decontamination, not including controls, and 711,237 for *18S* and 414,053 for *18S* were assigned after decontamination. After decontamination, the average number of reads per sample for *18S* was 20,321 (max 41,769; min 5430), and 11,227 for *CO1* (max 21,212; min 3,735). Raw taxonomy tables had a total of 1849 taxa for *18S* and 685 taxa for *CO1*. After decontamination, this number was reduced to 362 taxa for *18S* and 152 for *CO1*. Metazoan taxa made up 54% of *18S* results and 68% of *CO1* results (Fig. 1, Online Resource 1).

Total observed diversity in *18S* and *CO1*

Metazoan taxa recovered spanned 12 phyla in *18S* and 11 phyla in *CO1* datasets. The taxonomic assignments largely fell into five dominant distinct metazoan groups, the Annelida (19.35% of *18S* metazoan taxa, also 19.35% of *CO1* metazoan taxa), Nematoda (17.42% of *18S* metazoan taxa, 3.22% of *CO1* metazoan taxa), Arthropoda (12.9% of *18S* metazoan taxa, 12.9% of *CO1* metazoan taxa), Mollusca (9.67% of *18S* metazoan taxa, 29% of *CO1* metazoan taxa), and Platyhelminthes (15.48% of *18S* metazoan taxa, 6.45% of *CO1* metazoan taxa) (Fig. 1A, B). Annelida and Nematoda were the most taxon rich phyla identified using *18S*, while Arthropoda and Annelida were the most taxa-rich phyla identified using *CO1* (Fig. 1).

The meiofauna detected include 126 taxa for the *18S* metabarcode and 36 taxa for the *CO1* metabarcode, shown in full taxonomic resolution in Online Resource 1 and summarized within phyla in Table 1. The annelids mainly were composed of Polychaeta, in the order and/or families Capitellida, Protodriliidae, Saccocirridae, Neriliidae, Eunicida, Phyllodocida, Spionida and Terebellida. Within the arthropods, the most abundant taxa were Copepoda, represented by the orders Cyclopoida, Harpacticoida and Siphonostomatoida. Within the arthropods, Trombidiformes (Halacaridae), Ostracoda and Malacostraca were common. Within the Chordata, we exclusively found tunicates (Stolidobranchia). Within the nematodes, the Chromadorea, comprised of the orders Araeolaimida, Chromadorida, Desmodorida and Monhysterida, and the Enoplea, comprised of the Enoplida, were most common. The Platyhelminthes were mainly represented by the Rhabditophora with the orders

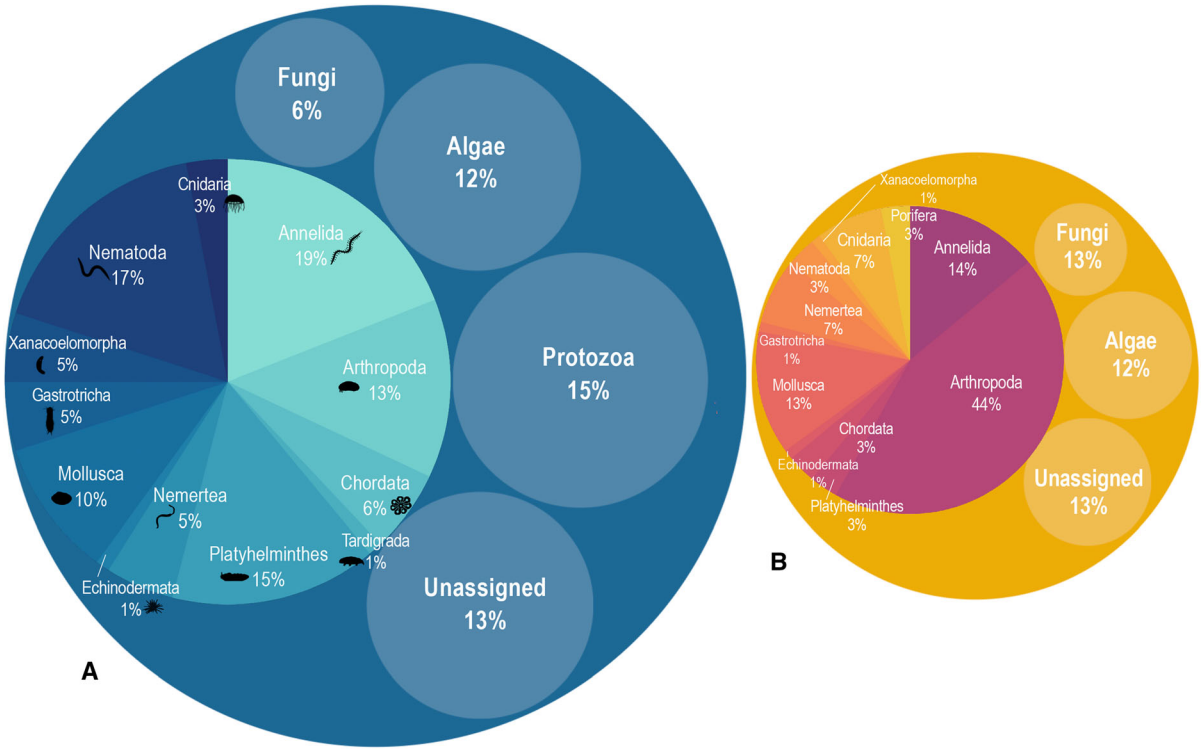

Fig. 1 Combined metabarcoding taxonomic overview of observed taxa across samples from all sites and collection strategy with the *18S* (**A**) and *CO1* (**B**) metabarcodes. Pie charts break down phyla within *Animalia*

Macrostomida, Proseriata, and Rhabdocoela (Table 1; Online Resource 1).

Meiofauna diversity, abundance and composition analyses

We observed differences in taxon abundance between the three pre-extraction treatments (untreated sand, and $MgCl_2$ and Ludox pre-preatments) and per locality (Fig. 2, Online Resources 2 and 3). Rarefaction curves began to asymptote at 3000–5000 reads for *18S* and ~ 2000 reads for *CO1* (Online Resource 4), which were prior to extrapolation points of taxon accumulation curves (Online Resource 5 and 6). We chose to rarefy at a read depth of 5000 reads for *18S* and 3000 reads for *CO1*, which were close to the minimum reads recovered in any sample. Barplots revealed consistency in observed relative abundance of taxa between replicates for each site and sampling technique. Annelida was the most abundant phylum in most of the filtered samples processed either with $MgCl_2$ or Ludox, but it was not well-recovered in the non-treated sand samples. On the other hand, groups like Gastrotrichia were regularly recovered in non-treated sand samples.

In DESeq2 analyses using rarefied data, we detected significant differences in taxa abundances at the genus level between pairwise comparisons of sand, $MgCl_2$ and Ludox techniques. 89 taxa in the *18S* results exhibited treatment-specific abundances, with 47 of those being meiofauna. 22 taxa in *CO1* results exhibited treatment-specific abundances, with 9 of those being meiofauna (Online Resource 7). In these pairwise comparisons of treatment, $MgCl_2$ or Ludox most frequently had overrepresented meiofauna taxa relative to sand, while more genera are underrepresented in $MgCl_2$ relative to Ludox (Fig. 2, Online Resources 7 and 8).

Alpha diversity estimators (Observed, Shannon, Chao1, Simpson) gave different relative diversity among treatment groups, with Observed and Shannon being most often in disagreement. Both the *18S* total metabarcode results (abbreviated as 'All') and *18S* meiofauna-only results showed significant differences

Table 1 Meiofauna community composition at all locations surveyed

Phylum	Class	Order or family	Santa Marta	Rodadero	Monoguaca	Sisguaca
Annelida	Polychaeta	Capitellida	42	309	153	64
		Protodrilidae	2811	5728	1367	4635
		Saccocirridae	7000	12567		
		Neriliidae			103	
		Eunicida			62	265
		Phyllodocida	18230	7004	56033	3008
		Spionida	22	23	103	12
		Terebellida				13
	Clitellata	Enchytraeida				437
		Haplotaxida		34	4069	1969
Arthropoda	Arachnida	Trombidiformes	44		1435	305
	Hexanauplia	Cyclopoida	1532		87	349
		Harpacticoida	155	26	142	86
		Siphonostomatoida	733	10	304	171
	Malacostraca	Decapoda				15
	Ostracoda	Podocopida	1792		38	1161
Chordata	Ascidiacea	Stolidobranchia	25	30	26	30
Platyhelminthes	Hydrozoa	Anthoathecata				
	Rhabditophora		926	237	151	412
		Macrostomida	103	396		97
		Proseriata	5093	3648	16079	5791
		Rhabdocoela	811	2698	706	22203
Echinodermata	Echinoidea					35
Mollusca	Gastropoda			20	240	3877
	Bivalvia	Myoida			87	
		Veneroida				81
Nemertea	Enopla			171	89	17172
		Monostilifera	11	80	34	6904
	Palaeonemertea		3084	2832		1916
	Pilidiophora	Heteronemertea		15		
Nematoda	Chromadorea	Araeolaimida	3283			2417
		Chromadorida	517		189	210
		Desmodorida	3325	257	945	3838
		Monhysterida	62	1902	111	211
		Rhabditida			62	42
	Enoplea	Enoplida	5643	796	11468	7291
Gastrotricha		Chaetonotida	1031	8287	5901	2232
		Macrodasyida	8294	2284		135
Tardigrada	Heterotardigrada	Echniscoidea	55		97	16
Xenacoelomorpha	Acoela		166			27
		Haploposthiidae				726
		Convolutidae	183	127		
		Otocelididae	40			
		Paratomellidae	1334			
		Isodiametridae				108

Table 1 continued

Phylum	Class	Order or family	Santa Marta	Rodadero	Monoguaca	Sisguaca
Cnidaria	Hydrozoa			11		
		Anthoathecata				150

Numbers indicate the total number of reads for the *18S* metabarcode, which was the metabarcode that produced the best results

among choice of sampling protocol [Kruskal–Wallis (Shannon) *18S* All/meiofauna-only, $P = 0.0395/0.0000107$; Kruskal–Wallis (Observed) *18S* All/meiofauna-only, $P = 0.0000047/0.000094$]. *Post hoc* tests revealed significant differences in alpha diversity between $MgCl_2$ and sand and between Ludox and sand, but not between $MgCl_2$ and Ludox (Online Resource 9). With the *CO1* All dataset we found significant differences among sampling treatments; however, we did not with the *CO1* meiofauna dataset [Kruskal–Wallis (Shannon) *CO1* All/meiofauna-only, $P = 0.0281/0.5$; Kruskal–Wallis (Observed) *CO1* All/meiofauna-only, $P = 0.0004/0.96$]. Sand samples frequently had the highest alpha diversity in both *18S* All and *CO1* All results, while $MgCl_2$ and Ludox techniques exceeded sand alpha diversity in the Meiofauna only results (Fig. 3).

Our comparison of the four beaches, which required summation of all rarefied metabarcoding data from all three sample treatments, showed few differences in alpha diversity. Only the Shannon and Simpson tests use *18S* .All data exhibited significant differences among the beaches (Kruskal–Wallis, $P \leq 0.01$). No significant differences in alpha diversity were found among the beach localities when only meiofauna results were analyzed (Fig. 3, Online Resource 9).

Beta diversity, on the other hand, was found to differ by technique as well as by locality (Online Resource 10). We found interaction effects between technique and locality with weak explanatory values ($R^2 = 0.41$ *18S* All, $R^2 = 0.44$ *18S* meiofauna, $R^2 = 0.39$ *CO1* All and $R^2 = 0.35$ *CO1* meiofauna results). PCoA revealed only some samples from the same locality cluster together in the *18S* results when they were prepared with different sampling techniques. In *CO1* results, all samples processed using the sand direct extraction technique clustered together, and samples from the other techniques spread out in the ordination (Fig. 4).

Molecular identifications at the species level

Concerning meiofauna, we could maximally resolve 24 meiofaunal taxa to genus and 34 meiofaunal taxa to species (Table 2). Taxonomic data from the World Register of Marine Species could be retrieved for all of them. Only 5 of these species have been reported in Colombia, while 17 of the species have been reported within the Caribbean or nearby countries (Table 2). BLAST queries of sequences from each of the 34 species-level identifications in Table 2 provide leads of unsequenced taxa (Online Resource 11). At the time of query (May 2020), 14 of the 22 species-level taxa reported in Colombia or nearby countries were strongly supported to be accurately assigned, while 7 additional taxa were not found to have a true match in Genbank suggesting these may be new or unsequenced species. Then, of the 12 species-level taxa that have not been previously reported in this region, only 2 were supported to be accurately assigned, 8 were confirmed by BLAST to be best relative matches but unlikely to be true species matches because sequence similarity was low ($< 97\%$), and 2 could be affirmatively revised to a taxon at the genus level. This suggests many of our taxa may represent undescribed species or unsequenced species related to our tentative assignments at the genus, family, or order levels, as some top sequence similarities were $< 92\%$, which we interpreted to be potential leads for species discovery that would improve reference databases.

Comparisons with previous morphology-based Annelida studies in the area

We made an attempt to compare our metabarcoding results with Lagos et al. (2018) results on the diversity of interstitial annelids in the Caribbean coasts of Colombia, which include Santa Marta and Rodadero beaches. Only Annelida was compared because this is the only study available for the same area. We found

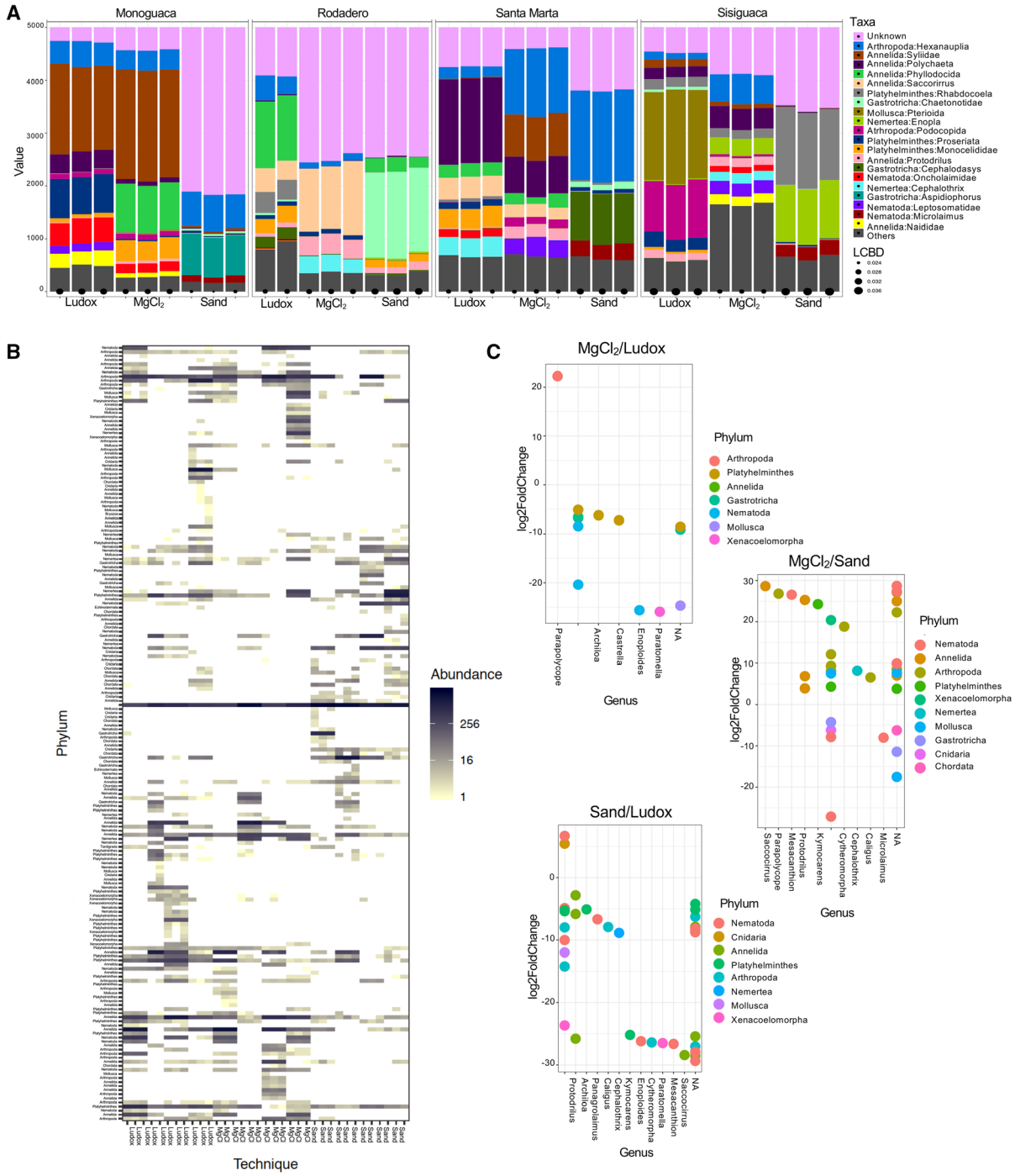

Fig. 2 Select taxonomic profiles represented in **A** Barplots showing relative abundance of the top 21 taxonomic lineages, sorted by the different locations and including local contribution to beta diversity circles sized by the proportion of unique taxa in a sample compared to the rest of the samples in its locality; **B** heatmaps of taxa labeled by their phylum (*y*-axis), with samples plotted on the x-axis and labeled according to their sample processing technique; and **C** DESeq2 plots obtained using the meiofauna datasets with the *18S* and *CO1* metabarcodes. In these, several genera occurring in different phyla are frequently underrepresented in $MgCl_2$ compared to Ludox (top), overrepresented in $MgCl_2$ compared to Sand (middle), and underrepresented in Sand compared to Ludox (bottom). Only genera with significantly different abundance were plotted

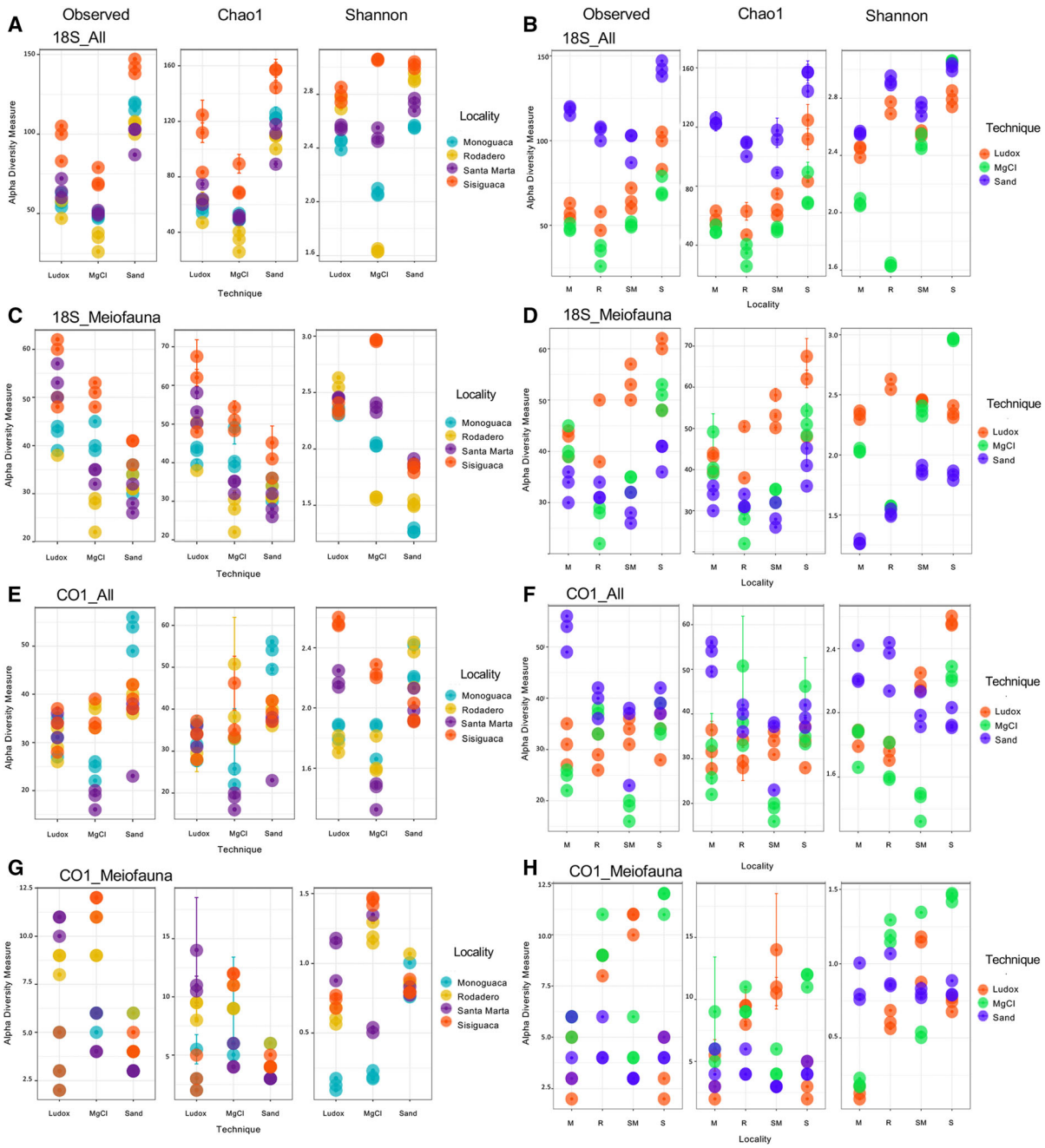

Fig. 3 Shannon, Observed, and Chao1 alpha diversity plots, grouped by treatment (left) and site (right). The technique producing the highest alpha diversity varied with metabarcode and with whether taxa were filtered to only include meiofauna. Likewise, locality also was not consistently ranked in alpha diversity across techniques. **A**, **B** Calculated with all taxa detected with *18S*. **C**, **D** All meiofauna detected with *18S*. **E**, **F**: All taxa detected with *CO1*. **G**, **H** All meiofauna detected with *CO1*

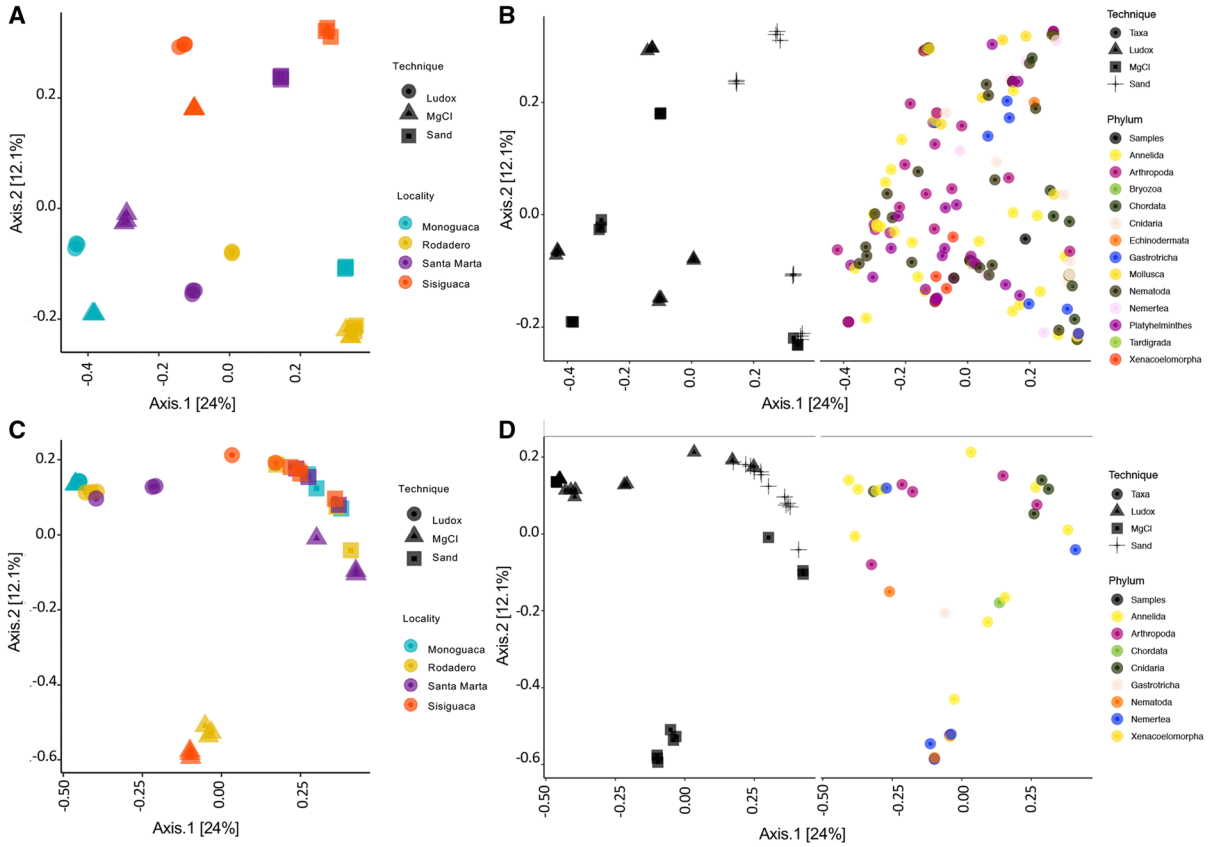

Fig. 4 Principal component analysis (PCoA) plots of samples (left) and present taxa (right) using Jaccard distance ordination, with colors and shapes indicating location and technique. Plots shown are based on the meiofauna tables generated with *18S* (**A** and **B**) and *CO1* (**C** and **D**)

high congruence using the *18S* primers, as all the families and most of the genera were recovered (Table 3). Only one genus reported by Lagos et al. (2018) was missing (*Myrianida*), despite being present in our reference database. On the other hand, the *CO1* only recovered 40% of the families reported by Lagos (2018), even though there were *CO1* sequences from these families in the reference database. Most of the species reported by Lagos et al. (2018) lack *18S* or *CO1* sequences, thus, we were not able to test the reliability of the metabarcoding technique in detecting taxa at the species-level (Table 3).

Discussion

Despite that Colombia has been ranked as the first country in terms of species richness for some taxa (Arbeláez-Cortés, 2013), little is known about the structure and dynamics of meiofauna communities in the country, with few studies available for the Colombian Caribbean (Hummon, 1974; Lorenzen, 1976a, b; Bartsch, 1996; Osorio-Dualiby & Alvarez-Leon, 2011; Lagos et al., 2018). Some of the challenges associated with the study of meiofauna include difficulties in collecting, handling, and identifying species (Giere, 2009; Somerfield & Warwick, 2013; George, 2013; Zeppilli et al., 2015; Kitahashi et al., 2020). Due to these difficulties, metabarcoding approaches could be implemented as an efficient way to complement these other biodiversity inventories.

In this study of Colombian intertidal sediments, Annelida and Nematoda were the most taxonomically rich phyla identified using *18S*, while Arthropoda and Annelida were the most taxonomically rich phyla identified using *CO1*. Other benthic metabarcoding

Table 2 Meiofauna species found in the area

Phylum	Class	Order or Family	Species	SM	R	M	S	gbif.org	GBIF-ID	WoRMS-ID	NCBI-ID
Annelida	Polychaeta	Protodrilidae	*Protodrilus* sp.	✔	✔	✔	✔	✔	5197607	129514	47110
			***Claudrilus corderoi* (du Bois-Reymond Marcus, 1948)**		✔		✔	✔	5197632	998044	1316264
			***Claudrilus ovarium* (Di Domenico, Martínez, Lana & Worsaae, 2013)**	✔	✔	✔	✔	✔	7690222	998038	1317020
			***Claudrilus draco* (Martínez, Di Domenico, Jörger, Norenburg & Worsaae, 2013)**				✔	✔	7882594	998045	1317018
			***Protodrilus smithsoni* Di Domenico, Jörger, Norenburg & Worsaae, 2013**			✔	✔	✔	8327691	744900	1317022
			Protodrilus pythonius Di Domenico, Martínez, Lana & Worsaae, 2013*	✔	✔			✔	-	744904	1462459
		Saccocirridae	***Saccocirrus pussicus* Marcus, 1948**	✔	✔			✔	2320150	331180	1435237
		Neriliidae	*Nerilla antennata* Schmidt, 1848			✔		✔	2318391	130437	47108
		Phyllodocida	*Gyptis* sp.	✔				✔	2308932	129307	378300
			Amphiduros fuscescens (Marenzeller, 1875) (*Gyptis* sp. after BLAST)	✔				✔	2309234	130148	378299
			Syllis sp.	✔				✔	5194522	129680	418360
			Syllis broomensis (Hartmann-Schröder, 1979)	✔				✔	7554671	816639	1761655
			Syllis crassicirrata (Treadwell, 1925)	✔				X	8160669	852359	1761656
			***Pisionidens ixazaluohae* Petersen, Gonzalez, Martínez & Worsaae, 2016**			✔		✔	8465748	881333	1854055
			Eunoe oerstedi Malmgren, 1865*				✔	X	2314982	130746	862927
			*Odontosyllis gibba Claparède, 1863** (Syllidae after BLAST)	✔	✔		✔	X	2309628	131328	199562
		Spionida	***Polydora websteri* Hartman in Loosanoff & Engle, 1943**	✔	✔	✔	✔	✔✔	5197736	153847	1167209
		Orbiniidae	*Naineris laevigata* (Grube, 1855)* (*Naineris* sp. after BLAST)	✔	✔			✔✔	2320012	130515	645996
	Clitellata	Enchytraeida	*Marionina* sp.				✔	X	2308278	137353	253856
Arthropoda	Hexanauplia	Cyclopoida	*Microcyclops* sp.	✔				✔	2116916	-	938114
			Paracyclopina nana Smirnov, 1935 (*Cyclopinella* sp.K102DZMB after BLAST)			✔		✔	4333168	-	565004
	Ostracoda	Podocopida	*Cytheromorpha acupunctata*(Brady, 1880) Hanai, 1962				✔	✔	5862753	461862	182561

Table 2 continued

Phylum	Class	Order or Family	Species	SM	R	M	S	gbif.org	GBIF-ID	WoRMS-ID	NCBI-ID
Platyhelminthes	Rhabditophora	Macrostomida	*Macrostomum* sp.		✔			✔	2499121	142205	39217
			***Macrostomum lineare* Uljanin, 1870**	✔			✔	✔	9051218	-	52057
		Proseriata	*Vannuccia* sp.	✔				✔	2502712	117065	104921
			*Archiloa rivularis*de Beauchamp, 1910	✔	✔	✔		X	2502441	143522	44220
			Monocelis fusca Örsted, 1843		✔			X	2502615	143550	983168
			Otoplana sp.		✔	✔	✔	X	2502400	142494	57455
			Postbursoplana sp.			✔	✔	✔	2502312	142503	859251
		Rhabdocoela	***Adenopharynx mitrabursalis* Ehlers, 1972**	✔				X	4344190	143367	1311954
			Kymocarens sp.			✔	✔	X	4859341	-	1311931
Nemertea	Nemertea incertae sedis	Ototyphlonemertidae	*Ototyphlonemertes* sp.				✔	✔	2507530	122408	66778
			Ototyphlonemertes erneba* Corrêa, 1950		✔			✔✔	2507565	148137	932703
		Monostilifera	***Ototyphlonemertes duplex* Bürger, 1895***				✔	X	2321127	421086	77591
			Ototyphlonemertes lactea* Corrêa, 1954			✔	✔	✔✔	2507546	162318	932705
		Baseodiscidae	*Baseodiscus delineatus* (Delle Chiaje, 1825)* (Rename to phylum after BLAST)	✔				✔✔	2508277	122577	326997
	Palaeonemertea		*Cephalothrix* sp.	✔	✔			✔	2506522	122379	166041
			***Cephalothrix bipunctata* Bürger, 1892**	✔	✔	✔	✔	✔	2508024	122592	1519607
Nematoda	Chromadorea	Araeolaimida	*Axonolaimus paraspinosus* Schuurmans Stekhoven & Adam, 1931	✔				X	4349294	121312	1302885
			Cylindrolaimus sp.	✔				X	2279835	227201	114862
		Chromadorida	*Punctodora ratzeburgensis* (Linstow, 1876) Filipjev, 1929 (*Graphonema* sp. after BLAST)	✔		✔		X	2280927	120717	647165
			Rhips sp.*				✔		2280961	2290	1265449
		Desmodorida	*Microlaimus* sp.	✔	✔	✔	✔	✔	2280387	2366	637441
		Rhabditida	*Panagrolaimus* sp.			✔	✔	✔	2283711	153395	55784
		Monhysterida	*Siphonolaimus* sp.		✔			✔	2280057	2495	2059206
			***Diplolaimelloides meyli* Timm, 1961**			✔		✔	2279701	228785	70221
			Bathylaimus sp.		✔			✔	7020804	2586	320127
		Enoplida	*Enoploides* sp.	✔	✔			✔	2282125	2512	129856
			Cylicolaimus sp.				✔		2282449	2530	1265447
			Mesacanthion sp.			✔	✔	✔	2282096	2517	129890
			Halalaimus sp.	✔				✔	2281918	2548	560183

Table 2 continued

Phylum	Class	Order or Family	Species	SM	R	M	S	gbif.org	GBIF-ID	WoRMS-ID	NCBI-ID
Gastrotrichia		Chaetonotida	*Aspidiophorus* sp.			✓		✓	2254933	114426	233111
		Macrodasyida	*Cephalodasys mahoae*Yamauchi & Kajihara, 2018	✓	✓			X	10161971	1312458	1598081
			Macrodasys sp.				✓	✓	2255269	114451	241703
			***Anandrodasys agadasys* (Hochberg, 2003)**	✓	✓			✓	4351177	378236	1132641
Xenacoelomorpha	Acoela	Convolutidae	***Praeconvoluta tigrina* Hooge & Tyler, 2003**	✓	✓	✓		✓	2498797	158314	188030
		Otocelididae	*Otocelis sandara* Hooge & Tyler, 2003	✓				X	2498886	158303	188044
		Paratomellidae	*Paratomella unichaeta* Dörjes, 1966	✓				X	2499139	142705	188050

One tick indicates the species has been reported within the Caribbean or nearby countries, two ticks indicate the species has been reported in Colombia. A "*" indicates species recovered with the *CO1* metabarcode. Bold denotes this species is well supported by BLAST results (>99% similarity). Underlined denotes this is the best assignment currently possible but the true species is not in Genbank based on low similarity in BLAST results. Renamed species assignments (e.g name, after BLAST) are based on BLAST results. Details in online resource 11. SM (Santa Marta), R (Rodadero), M (Monoguaca), S (Sisiguaca)

studies based on the Operational Taxonomic Unit (OTU) diversity of the *18S* region found the richest phyla varied: top phyla were Nematoda and Platyhelminthes in Fonseca et al. (2010, 2014), Faria et al. 2018; Atherton & Jondelius (2020) and Fais et al. (2020). Annelida and Arthropoda were top in Haenel et al. (2017) and Fonseca et al. (2017). Nematoda and Annelida were top in Bik et al. (2012a, b) and Brannock & Halanych (2015). Nematoda and Arthropoda were top in Lallias et al. (2015), Martínez et al. (2015), Klunder et al. (2019) and Kitahashi et al. (2020). Differences might be related to the type of sediment sampled, as some the cited studies included mud, gravel (Faria et al., 2018) or coarse shell sand (Haenel et al., 2017). Others also sampled at different depths (Bik et al., 2012a, b; Brannock & Halanych, 2015; Fonseca et al., 2017; Kitahashi et al., 2020; Martínez et al., 2015).

On the other hand, Haenel et al. (2017), using *CO1,* also found Annelida, Arthropoda and Mollusca were the most abundant OTUs, and recovered 0% of Nematoda OTUs using *COI*. We only recovered 3% Nematoda of our total metazoan taxa using *CO1* metabarcoding, even though several studies demonstrate that Nematoda is one of the most abundant phyla in sandy beaches (Maria et al., 2016; Fonseca et al., 2017). One reason we may be underestimating Nematoda richness using *CO1* is low diagnostic capacity, as is evident from other studies showing the *CO1* barcode is not adequate for molecular taxonomic identification purposes in nematodes (Blouin et al., 1998; Blouin, 2000), due to the lack of universal *CO1* primer sites within this group (Creer et al., 2010; Weigand & Macher, 2018).

Although we recovered many more meiofaunal taxa using the *18S* metabarcode, the *CO1* metabarcode recovered 5 unique species, corroborating that the integration of multiple barcoding primers is important to ensure better estimates of phylogenetic biodiversity (Zhang et al., 2018). Atherton & Jondelius (2020) also found that fewer meiofauna species DNA signatures were obtained using *CO1* compared to *18S*. The authors found a high proportion of unassigned *CO1* OTUs determined to stem from incompleteness of their *CO1* reference sequences. Although the *CO1* locus remains the most common DNA marker for animals, they also argued there are gaps within Metazoa, especially in understudied taxa, and NCBI Genbank still lacks *CO1* references for several major

Table 3 Comparison of Lagos et al. (2018) results with our metabarcoding results

Family	Genus	Species	*18S* Genbank	*CO1* Genbank	Metabarcoding *18S*	Metabarcoding *CO1*
Hesionidae			✔	✔	✔	X
	Hesionides					
		Hesionides gohari Hartmann-Schröder, 1960	X	X	X	X
	Microphthalmus		X	X	X	X
		Microphthalmus cf. *mahensis* Westheide, 2013				
	Neogyptis		✔	✔	✔	X
		Neogyptis mediterranea (Pleijel, 1993)		✔	X	X
Nerillidae						
	Nerilla		✔	✔	✔	X
		Nerilla cf. m*editerranea* Schlieper, 1925				
Protodrilidae						
	Protodrilus		✔	✔	✔	✔
		Protodrilus cf. *smithsoni* Di Domenico, Jörger, Norenburg & Worsaae, 2013	✔	✔	✔	X
Saccocirridae			✔	✔	✔	X
	Pharyingocirrus					
		Pharyingocirrus cf. g*abriellae* Czerniavsky, 1881				
Syllidae			✔	✔	✔	✔
	Myrianida		✔	✔	X	X
		Myrianida sp.				
	Neopetitia		X	X	X	X
		Neopetitia amphophthalma (Siewing, 1956)				
	Westheidesyllis		X	X	X	X
		Westheidesyllis gesae (Perkins, 1981)				
	Syllis		X	X	X	X
		Syllis beneliahuae(Campoy, 1982)				

marine invertebrate taxa (Curry et al., 2018). This agrees with Van der Loos et al. (2020) literature survey, who showed that when only benthic meiofauna were targeted, *18S* was the marker researchers most frequently chose. When they looked at all the studies using metabarcoding on marine communities, only 28% of all studies used a *CO1* fragment, whereas 59% targeted the nuclear *18S* marker. Although others have suggested that the use of *CO1* for eDNA surveys could provide more accurate estimates of species richness than *18S* (Tang et al., 2012), because of these many mentioned limitations in *CO1* assays, we support that *CO1* be continuously used in conjunction with other metabarcodes.

Our results suggest that general biodiversity meiofauna studies could be biased by the sampling technique chosen. In this study we used two pre-treatment approaches recommended for the recovery of meiofauna DNA from sand, and compared this to a direct extraction approach (no pre-treatment) from

sand sediment. Although $MgCl_2$ and Ludox pre-treatment techniques recovered, as expected, more taxa and meiofauna species than the untreated sand, the untreated approach excelled at recovering some groups, such as gastrotrichs (Fig. 2). The gregarious distribution of many species of gastrotrichs, added to the presence of adhesive organs such as glands and tubes that allow the adhesion of these organisms to sandy sediments (Todaro et al., 2019; Bálsamo et al., 2020), possibly contributed to an adequate and even better recovery of the gastrotrichs in sand samples without further separation treatments. This result corroborates that multiple sample techniques together can broaden surveys.

We found differences in alpha diversity among beach localities using the *18S* All results, and weak differences in beta diversity among localities both with the *18S* and the *CO1* results. A higher number of total taxa and meiofauna taxa were generally recovered in the Sisiguaca samples than in the other samples, followed by Santa Marta, Monoguaca, and Rodadero. Changes in the composition and abundance of meiofauna taxa between beaches may be due to trampling by tourism (Martínez et al., 2020). Two of the beaches included in this study are very touristic beaches (Rodadero and Santa Marta). Monoguaca is less frequented by tourists, and Sisiguaca receives very few visitors of any kind. Negative effects of tourism were suggested by Gheskiere et al., (2005), who demonstrated, by comparing high taxonomic level meiobenthic composition and nematode assemblages between tourist and non-tourist beaches, that tourism-related activities contribute to higher community stress, lower taxonomic range, and lower species diversity assemblages when compared to nearby pristine locations (Gheskiere et al., 2005). Martínez et al. (2015) assessed the impact of tourism on meiofauna including metabarcoding techniques. According to them, even after considering possible confounding effects such as grain size, there was a negative correlation between meiofauna richness and number of tourists. Future studies should also consider other physical and chemical variables such as grain size, temperature, salinity and dissolved oxygen, as well as habitat characteristics, as meiofauna diversity might also be influenced by all these factors (Giere, 2019).

Comparing our results to published morphological reports including the same beaches, we found that only one genus reported by Lagos et al. (2018) was missing in our results (Myrianida), although it was present in our reference database. This could be a result of community turnover among the survey dates as marine coastal communities have been shown to shift in composition over seasons and reproductive cycles (Yin et al., 2017; Srichandan et al., 2019). This also could explain why strict meiofaunal groups such as Gnathostomulida, Kinorhyncha and Loricifera were not recovered. Other reasons for not finding them could, however, be related to their preferred association to other habitats, such as sand enriched with organic detritus for Gnathostomulida (Sterrer, 1998), muddy sediments for Kinorhyncha (Sorensen & Pardos, 2008), or coarse sediments and subtidal habitats for Loricifera (Pardo & Kristensen, 2013). Future studies should consider sampling these beaches in different seasons of the year, and sampling diverse substrates, if trying to characterize the whole diversity associated with the area.

58% of the taxa we recovered through *18S* and *CO1* metabarcoding were resolved only to family or higher levels, while only 20% were assigned to genus and 22% were fully resolved to the species level. This highlights the necessity of further studies combining morphological, barcoding and metabarcoding approaches, as these have become interdependent to fully inventory the phylogenetic breadth of the meiofauna. Metabarcoding largely depends on the taxonomic diversity of sequences deposited in the reference library. Molecular identifications at the species level provided leads for new species occurrence in the area, following the community standard that taxa sharing $< 97\%$ identities with voucher specimen sequences might represent putative different or even novel taxa (López-Escardó et al., 2018). Atherton & Jondelius (2020) also showed evidence of potential new species in their survey in well-studied areas of Sweden, suggesting that the metabarcoding technique could be informative in detecting new meiofaunal biodiversity, even in those areas where these taxa can be considered well documented.

Conclusion

Our study suggests that the *18S* metabarcode recovers more meiofauna taxa than *CO1*. However, some meiofauna species were only recovered with *CO1*,

corroborating that use of multiple markers improves estimates of biodiversity. We also found that using specific pre-treatment separation techniques improved recovery of meiofauna species, but are not sufficient to replace direct extraction of untreated sediment. This result suggests that eDNA-metabarcoding catalogs of meiofauna taxa should employ a variety of treatments. We suggest taking these results into consideration when planning diversity eDNA-metabarcoding studies in general, because the effects of treatment on recovery of different clades remain poorly cataloged.

Comparison of our results to the Lagos et al. (2018) report for Annelids indicate that the *18S* metabarcoding technique is reliable in this particular group. However, it is essential to continue with morphological and barcoding studies in the geographic region, as the availability of the sequences is extremely important to ensure the success of future metabarcoding studies.

Ours is one of the few meiofauna studies of the Colombian Caribbean and the first one to use metabarcoding techniques. Our results not only suggest that this technique is useful to detect novel or yet unreported species, but also that the approach is a fast and useful monitoring tool for future biodiversity and environmental studies.

Acknowledgements Thanks to Maria Victoria Leon, Daniel Giraldo, and Angie Colorado for helping with the collection and processing of samples. This work was supported by a Fulbright visitor research fellowship to Lyda R. Castro and by Fondo Patrimonial de Investigación Fonciencias 2019, Universidad del Magdalena.

Author contributions All authors contributed to the study conception and design. SYQ and AML collected the material; LRC and SC generated the molecular data; LRC, SC, SS, and RSM contributed to the analysis of the data. All the authors contributed to the interpretation of the results. LRC wrote the first draft of the manuscript and all authors contributed to writing the final version. All authors read and approved the final manuscript.

Data availability All raw sequencing data from this study have been deposited in the NCBI Sequence Read Archive (SRA) under BioProject accession number PRJNA694867 (BioSample accessions SAMN17575766– SAMN17575777). All reference databases, taxonomic assignments of Amplicon Sequence Variants, as well as all scripts are available in Zenodo (http://doi.org/10.5281/zenodo.4588923).

Declarations

Conflict of interest The authors declare that there is no competing interest regarding the publication of this paper.

References

Alberdi A, O. Aizpurua, M.T.P Gilbert & K. Bohmann, 2017. Scrutinizing key steps for reliable metabarcoding of environmental samples. Methods in Ecology and Evolution 9: 134–147.

Arbeláez- Cortés, E., 2013. Knowledge of Colombian biodiversity: published and indexed. Biodiversity Conservation, 22: 2875–2906.

Appeltans, W., S.T. Ahyong, G. Anderson, M.V. Angel, T. Artois, N. Bailly, R. Bamber, A. Barber, I. Bartsch, A. Berta, M. Błażewicz-Paszkowycz, P. Bock, G. Boxshall, C.B. Boyko, S.N. Brandão, R.A. Bray, N.L. Bruce, S.D. Cairns, T.-Y. Chan, L. Cheng, A.G. Collins, T. Cribb, M. Curini-Galletti, F. Dahdouh-Guebas, P.J.F. Davie, M.N. Dawson, O. De Clerck, W. Decock, S. De Grave, N.J. de Voogd, D.P. Domning, C.C. Emig, C. Erséus, W. Eschmeyer, K. Fauchald, D.G. Fautin, S.W. Feist, C.H.J.M. Fransen, H. Furuya, O. Garcia-Alvarez, S. Gerken, D. Gibson, A. Gittenberger, S. Gofas, L. Gómez-Daglio, D.P. Gordon, M.D. Guiry, F. Hernandez, B.W. Hoeksema, R.R. Hopcroft, D. Jaume, P. Kirk, N. Koedam, S. Koenemann, J.B. Kolb, R.M. Kristensen, A. Kroh, G. Lambert, D.B. Lazarus, R. Lemaitre, M. Longshaw, J. Lowry, E. Macpherson, L.P. Madin, C. Mah, G. Mapstone, P.A. McLaughlin, J. Mees, K. Meland, C.G. Messing, C.E. Mills, T.N. Molodtsova, R. Mooi, B. Neuhaus, P.K.L. Ng, C. Nielsen, J. Norenburg, D.M. Opresko, M. Osawa, G. Paulay, W. Perrin, J.F. Pilger, G.C.B. Poore, P. Pugh, G.B. Read, J.D. Reimer, M. Rius, R.M. Rocha, J.I. Saiz-Salinas, V. Scarabino, B. Schierwater, A. Schmidt-Rhaesa, K.E. Schnabel, M. Schotte, P. Schuchert, E. Schwabe, H. Segers, C. Self-Sullivan, N. Shenkar, V. Siegel, W. Sterrer, S. Stöhr, B. Swalla, M.L. Tasker, E.V. Thuesen, T. Timm, M.A. Todaro, X. Turon, S. Tyler, P. Uetz, J. van der Land, B. Vanhoorne, L.P. van Ofwegen, R.W.M. van Soest, J. Vanaverbeke, G. Walker-Smith, T.C. Walter, A. Warren, G.C. Williams, S.P. Wilson, M.J. Costello, 2012. The magnitude of global marine species diversity. Current Biology 22: 2189–2202.

Atherton, S. & U. Jondelius, 2020. Biodiversity between sand grains: Meiofauna composition across southern and western Sweden assessed by metabarcoding. Biodiversity Data Journal. 8:e51813.

Balsamo, M., T. Artois, J.P.S. Smith, M.A. Todaro, L. Guidi, B.S. Leander & N.W.L. Van Steenkiste, 2020. The curious and neglected soft-bodied meiofauna: Rouphozoa (Gastrotricha and Platyhelminthes). Hydrobiologia 847: 2613–2644.

Bartsch, I., 1996. New records of Actacarus from the Caribbean area and notes on the subfamily Actacarinae and its species

(Arachnida: Acari: Halacaridae). Senckenbergiana Biologica 75: 229–241.
Benjamini, Y. & Y. Hochberg, 1995. Controlling the false discovery rate: a practical and powerful approach to multiple testing. Journal of the Royal Statistical Society: Series B 57: 289−300.
Bik, H.M., K.M. Halanych, J. Sharma & W.K. Thomas, 2012a. Dramatic Shifts in Benthic Microbial Eukaryote Communities following the Deepwater Horizon Oil Spill. PLoS One 7 e38550.
Bik, H.M., W.A.Y. Sung, P. De Ley, J.G. Baldwin, J. Sharma, A. Rocha-Olivares & W.K. Thomas, 2012b. Metagenetic community analysis of microbial eukaryotes illuminates biogeographic patterns in deep-sea and shallow water sediments. Molecular Ecology 21: 1048−1059.
Blouin, M.S. 2000. Brief communication. Neutrality tests on mtDNA: unusual results from nematodes. Journal of Heredity 91:156–158.
Blouin, M.S., C.A. Yowell, C.H. Courtney & J.B. Dame, 1998. Substitution bias, rapid saturation, and the use of mtDNA for nematode systematics. Molecular Biology and Evolution 15: 1719–1727.
Bonaglia, S., F.J.A. Nascimento, M. Bartoli, I. Klawonn & V. Brüchert, 2014. Meiofauna increases bacterial denitrification in marine sediments. Nature Communications 5: 5133.
Bongers, T. & Ferris H, 1999. Nematode community structure as a bioindicator in environmental monitoring. Trends in Ecology & Evolution 14(6): 224–228
Brannock, P.M. & K.M. Halanych, 2015. Meiofaunal community analysis by high-throughput sequencing: comparison of extraction, quality filtering, and clustering methods. Marine Genomics 23: 67-75.
Clare, E.L., F.J.J. Chain, J.E. Littlefair, M.E. Cristescu & Deiner, K, 2016. The effects of parameter choice on defining molecular operational taxonomic units and resulting ecological analyses of metabarcoding data 1. Genome 59, 981–990.
Chang, J.J.M., Ip, Y.C.A., Bauman, A.G., H. Danwei, 2020. MinION-in-ARMS: Nanopore Sequencing to Expedite Barcoding of Specimen-Rich Macrofaunal Samples From Autonomous Reef Monitoring Structures. Frontiers in Marine Science 7:448.
Creer, S., V.G. Fonseca, D.L. Poraziska, R.M. Giblin-Davis, W. Sung, D.M. Power, M. Packer, G.R. Carvalho, M.L. Blaxter, P.J.D. Lambshead & W.K. Thomas, 2010. Ultrasequencing of the meiofaunal biosphere: practice, pitfalls and promises. Molecular Ecology 19: 4–20.
Curd, E.E., Z. Gold, G.S. Kandlikar, J. Gomer, M. Ogden, T. O'Connell, L. Pipes, T.M. Schweizer, L. Rabichow, M. Lin, B. Shi, P.H. Barber, N. Kraft, R. Wayne & R.S. Meyer, 2019. Anacapa Toolkit: an environmental DNA toolkit for processing multilocus metabarcode datasets. Methods in Ecology and Evolution 10: 1469–1475.
Curry C, J. Gibson, S. Shokralla, M. Hajibabaei & D. Baird, 2018. Identifying North American freshwater invertebrates using DNA barcodes: are existing COI sequence libraries fit for purpose? Freshwater Science 37 (1): 178–189.
Davis, N.M., D.M. Proctor, S.P. Holmes, D.A. Relman & B.J. Callahan, 2018. Simple statistical identification and removal of contaminant sequences in marker-gene and metagenomics data. Microbiome 6 :2-14.
Deiner, K., J.-C. Walser, E. Mächler, F. & Altermatt, 2015. Choice of capture and extraction methods affect detection of freshwater biodiversity from environmental DNA. Biology Conservation 183:53–63.
De Jonge, V. N. & Bouwman, L. A. 1977. A simple density separation technique for quantitative isolation of meiobenthos using the colloidal silica Ludox-TM. Marine Biology 42(2): 143–148.
Estevens, A. & V. Genevois, 2006. Os nematódeos e sua importância nos ecossistemas marinhos. Floresta e Ambiente 13: 113–120.
Faria, L.C. de, M. Di Domenico, S.C.S. Andrade, M.C. dos Santos, G. Fonseca, J. Zanol & A.C.Z. Amaral, 2018. The use of metabarcoding for meiofauna ecological patterns assessment. Marine Environmental Research 140: 160–168.
Fenchel, T. M., 1978. The ecology of micro-and meiobenthos. Annual Review of Ecology and Systematics 9: 99–121.
Fais, M., S. Duarte, P.E. Vieira, R. Sousa, M. Hajibabaei, C.A. Canchaya & F.O. Costa, 2020. Small-scale spatial variation of meiofaunal communities in Lima estuary (NW Portugal) assessed through metabarcoding. Estuarine, Coastal and Shelf Science 238: 106683. https://doi.org/10.1016/j.ecss.2020.106683.
Fonseca, V.G., G.R. Carvalho, W. Sung, H.F. Johnson, D.M. Power, S.P. Neill, M. Packer, M.L. Blaxter, P.J.D. Lambshead, W.K. Thomas & S. Creer, 2010. Second-generation environmental sequencing unmasks marine metazoan biodiversity. Nature Communications 1: 98.
Fonseca, V.G., G.R. Carvalho, B. Nichols, C. Quince, H.F. Johnson, S.P. Neill, J.D. Lambshead, W.K. Thomas, D.M. Power & S. Creer, 2014. Metagenetic analysis of patterns of distribution and diversity of marine meiobenthic eukaryotes. Global Ecology and Biogeography 23: 1293–1302.
Fonseca, V.G., F. Sinniger, J.M. Gaspar, C. Quince, S. Creer, D.M. Power, L.S. Peck & M.S. Clark, 2017. Revealing higher than expected meiofaunal diversity in Antarctic sediments: a metabarcoding approach. Scientific Reports 7: 6094.
Gao, X., H. Lin, K. Revanna, & Q. Dong, 2017. A Bayesian taxonomic classification method for 16S rRNA gene sequences with improved species-level accuracy. BMC Bioinformatics, 18: 247.
Geller, J., C. Meyer, M. Parker, & H. Hawk, 2013. Redesign of PCR primers for mitochondrial cytochrome coxidase subunit I for marine invertebrates and application in all-taxa biotic surveys. Molecular Ecology Resources 13:851–861.
George K.H, 2013. Faunistic research on metazoan meiofauna from seamounts-a review. Meiofauna Marina, 20:1-32.
Gheskiere, T., M. Vincx, J.M. Weslawski, F. Scapini & S. Degraer, 2005. Meiofauna as descriptor of tourism-induced changes at sandy beaches. Marine Environmental Research 60: 245–265.
Giere, O., 2009. Meiobenthology: The microscopic motile fauna of aquatic sediments., Second Edi. ed. Springer-Verlag, Berlin.
Giere, O., 2019. Perspectives in Meiobenthology. Reviews, Reflections and Conclusions. Springer, Berlin.
Guardiola, M., M.J. Uriz, P. Taberlet, E. Coissac, O.S. Wangensteen & X. Turon, 2015. Deep-sea, deep-sequencing:

metabarcoding extracellular DNA from sediments of marine canyons. PLoS ONE 10: e0139633.
Haenel, Q., O. Holovachov, U. Jondelius, P. Sundberg & S.J. Bourlat, 2017. NGS-based biodiversity and community structure analysis of meiofaunal eukaryotes in shell sand from Hållö island, Smögen, and soft mud from Gullmarn Fjord, Sweden. Biodiversity Data Journal e12731–e12731.
Hajibabaei, M., T.M. Porter, C.V. Robinson, D.J. Baird, S. Shokralla & M.T.G. Wright, 2019. Watered-down biodiversity? A comparison of metabarcoding results from DNA extracted from matched water and bulk tissue biomonitoring samples. PLoS One 14:e0225409.
Hebert, P. D. N., A.Cywinska, S. L. Ball & J. R. DeWaard, 2003. Biological identifications through DNA barcodes. Proceedings of the Royal Society B: Biological Sciences, 270: 313–321.
Hermans, S.M., H.L. Buckley & G. Lear, 2018. Optimal extraction methods for the simultaneous analysis of DNA from diverse organisms and sample types. Molecular Ecology Resources 18: 557–569.
Higgins, R. P. & H. Thiel (eds), 1988. Introduction to the study of meiofauna. Smithsonian Institution Press, Washington, DC, London.
Hsieh, T.C., K.H. Ma, K.H. & A. Chao, 2016. iNEXT: an R package for rarefaction and extrapolation of species diversity (Hill numbers). Methods in Ecology and Evolution 7: 1451-1456.
Hummon, W., 1974. Intertidal marine gastrotricha from Colombia. Bulletin of Marine Science. 24: 396-408.
Kandlikar, G.S., Z.J. Gold, M.C. Cowen, R.S. Meyer, A.C. Freise, N.J.B. Kraft, J. Moberg-Parker, J. Sprague, D.J. Kushner & E.E.Curd, 2018. Ranacapa: an R package and Shiny web app to explore environmental DNA data with exploratory statistics and interactive visualizations. F1000 Research 7: 1-19.
Kennedy, A.D. & C.A. Jacoby, 1999. Biological indicators of marine environmental health: meiofauna – a neglected benthic component?. Environmental Monitoring and Assessessment 54: 47–68.
Kitahashi, T., S. Sugime, K. Inomata, M. Nishijima, S. Kato, & H. Yamamoto, 2020. Meiofaunal diversity at a seamount in the Pacific Ocean: a comprehensive study using environmental DNA and RNA. Deep Sea Research Part I: Oceanographic Research Papers. https://doi.org/10.1016/j.dsr.2020.103253
Klunder, L., G.C.A. Duineveld, M.S.S., Lavaleye, H.W., van der Veer, P.J., Palsbøll, & van Bleijswijk, J.D.L. 2019. Diversity of Wadden Sea macrofauna and meiofauna communities highest in DNA from extractions preceded by cell lysis. Journal Sea Research 152:101764.
Lagos, A.M., M.V. Leon, S. Quiroga & A. Martinez, 2018. Interstitial annelids from the Caribbean Coast of Colombia. Revista de Biologia Tropical 66: 658-673.
Lallias, D., J.G. Hiddink, V.G. Fonseca, J.M. Gaspar, W. Sung, S.P. Neill, N. Barnes, T. Ferrero, N. Hall, P.J.D. Lambshead, M. Packer, W.K. Thomas & S. Creer, 2015. Environmental metabarcoding reveals heterogeneous drivers of microbial eukaryote diversity in contrasting estuarine ecosystems. The ISME Journal.9: 208–1221.
Langmead, B. & S. L. Salzberg, 2012. Fast gapped-read alignment with Bowtie 2. Nature Methods 9: 357–359.
Leasi, F., J.L. Sevigny, E.M. Laflamme, T. Artois, M. Curini-Galletti, A. de Jesus Navarrete, M. Di Domenico, F. Goetz, J.A. Hall, R. Hochberg, K.M. Jörger, U. Jondelius, M.A. Todaro, H.H. Wirshing, J.L. Norenburg & W.K. Thomas, 2018. Biodiversity estimates and ecological interpretations of meiofaunal communities are biased by the taxonomic approach. Communications Biology 1: 112.
Lejzerowicz, F., P. Esling, L. Pillet, T.A. Wilding, K.D. Black & J. Pawlowski, 2015. High-throughput sequencing and morphology perform equally well for benthic monitoring of marine ecosystems. Scientific Reports 5: 13932.
Leray, M. & Knowlton, N. 2016. Censusing marine eukaryotic diversity in the twenty-first century. Philosophical Transactions of the Royal Society of London. Series B., Biological Sciences. 371: 20150331. https://doi.org/10.1098/rstb.2015.0331.
Leray, M., J.Y. Yang, C.P. Meyer, S.C. Mills, N. Agudelo, V. Ranwez, J.T. Boehm & R.J. Machida, 2013. A new versatile primer set targeting a short fragment of the mitochondrial COI region for metabarcoding metazoan diversity: application for characterizing coral reef fish gut contents. Frontiers in Zoology 10: 2-14.
Lobo, J., P.M. Costa, M.A.L. Teixeira, M.S.G. Ferreira, M.H. Costa & F.O. Costa, 2013. Enhanced primers for amplification of DNA barcodes from a broad range of marine metazoans. BMC Ecology 13: 34.
López-Escardó, D., J. Paps, C. de Vargas, R. Massana, I. Ruiz-Trillo & J. del Campo, 2018. Metabarcoding analysis on European coastal samples reveals new molecular metazoan diversity. Science Reports 8: 9106.
Lorenzen, S., 1976a. Desmodoridae (nematoden) mit extrem lange spicula aus South America. Mitteilungen aus dem Instituto Colombo-Alemán Investigaciones Científicas Punta Betín 8: 63–78.
Lorenzen, S., 1976b. Calomicrolaimus rugatus n. gen., n.sp. (Desmodoridae, nematodos) in Sandy beaches . Mitteilungen aus dem Instituto Colombo-Alemán Investigaciones Científicas Punta Betín 8: 79–82. https://www.oceandocs.org/bitstream/handle/1834/3283/nematodos.pdf?sequence=1
Love, I.M., W. Huber & S. Anders, 2014. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biology 15:550.
Maria, T.F. J. Vanaverbeke, A., Vanreusel & A.M. Estevens, 2016. Sandy beaches: state of the art of nematode ecology. Anais da Academia Brasileira de Ciências 88 (3): 1635-1653.
Martínez, A., E.M Eckert, T. Artois, G. Careddu, M. Cascu, M. Curini-Galletti, V. Gazale, S. Gobert, V.N., Ivanenko, U. Jondelius, M. Marzano, G. Pesole, A. Zanello, A.M. Todaro & Fontaneto D, 2005. Human access impacts biodiversity of microscopic animals in sandy beaches. Communication Biology 3:175.
Moreno, M., L. Vezzulli, V. Marin, P. Laconi, G. Albertelli & M. Fabiano, 2008. The use of meiofauna diversity as an indicator of pollution in harbours. ICES Journal of Marine Sciences 65: 1428–1435.
Montagna, P.A., J.G. Baguley, C. Hsiang & M.G. Reuscher, 2017. Comparison of sampling methods for deep-sea infauna. Limnology and Oceanography 15 (2): 166–183.

McMurdie, P.J. & S., Holmes, 2013. phyloseq: an R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data. PLoS One 8, e61217.

Murray D.C., M.L. Coghlan & M. Bunce, 2015. From benchtop to desktop: important considerations when designing amplicon sequencing workflows. PLoS ONE 10(4): e0124671.

Nascimento, F.J., D. Lallias, H.M. Bik & S. Creer, 2018. Sample size effects on the assessment of eukaryotic diversity and community structure in aquatic sediments using high-throughput sequencing. Scientific Reports, 8(1): 1–12.

Nichols, R.V., C. Vollmers, L.A. Newsom, Y. Wang, P. D. Heintzman, M. Leighton, R.E. Green & Beth Shapiro, 2018. Minimizing polymerase biases in metabarcoding. Molecular Ecology Resources 8: 927-39.

Oksanen, J., R. Kindt, P. Legendre, R.B. O'Hara, G.L. Simpson, P. Solymos, M.H.H. Stevens & H. Wagner, 2019. Package 'vegan', community ecology package. Version 1.15-1.

Osorio-Dualiby, D. & R. Álvarez-León, 2011. Caracterización del meiobentos en sectores de influencia costera de dos empresas ubicadas en la zona industrial de Mamonal. Arquivos de Ciências do Mar 44: 81–96. http://www.repositorio.ufc.br/handle/riufc/8675

Pardo, F. & R.M. Kristensen, 2013. First record of Loricifera from the Iberian Peninsula, with the description of *Rugiloricus manuelae* sp. nov., (Loricifera, Pliciloricidae). Helgoland Marine Research 67: 623-638.

Pearman, J.K., X. Irigoien & S. Carvalho, 2016. Extracellular DNA amplicon sequencing reveals high levels of benthic eukaryotic diversity in the central Red Sea. Marine Genomics 26: 29–39.

Pearman, J.K., Keeley, N.B., Wood, S.A., Laroche, O., Zaiko, A., Thomson-Laing, G., Biessy, L., Atalah, J., & X. Pochon, 2020. Comparing sediment DNA extraction methods for assessing organic enrichment associated with marine aquaculture. PeerJ 8:e1023.

Ptatscheck, C., S. Gehner, & W. Traunspurger, 2020. Should we redefine meiofaunal organisms? The impact of mesh size on collection of meiofauna with special regard to nematodes. Aquatic Ecology 54:1135–1143.

Somerfield, P. & R. Warwick, 2013. Meiofauna techniques. In: I.A. Eleftheriou (eds), Methods for the Study of Marine Benthos. John Wiley & Sons, Ltd, Chichester: 253–284.

Sørensen, M.V. & F. Pardos, 2008. Kinorhynch systematics and biology – an introduction to the study of kinorhynchs, inclusive identification keys to the genera. In: F. Pfeil (eds), Meiofauna Marina. Peif Verlag, Munich: 21-73.

Srichandan, S., S.K. Baliarsingh, S. Prakash, A.A. Lotliker, C. Parida & K.C. Sahu, 2019. Seasonal dynamics of phytoplankton in response to environmental variables in contrasting coastal ecosystems. Environmental Science Pollution Research 26: 12025–12041.

Ssekagiri, A., W.T. Sloan & U.Z. Ijaz, 2018. MicrobiomeSeq: An R Package for Microbial-Community Analysis in an Environmental Context.

Sterrer, W., 1998. Gnathostomulida from the (Sub)Tropical Northwestern Atlantic. Studies on the Natural History of the Caribbean Region 74: 1-178.

Tang C.Q., F., Leasi, U. Obertegger, A. Kieneke, T.G., Barraclough & D. Fontaneto, 2012. The widely used small subunit 18S rDNA molecule greatly underestimates true diversity in biodiversity surveys of the meiofauna. Proceedings of the National Academy of Sciences of the United States of America, 109:16208–16212.

Todaro, M.A., J.A. Sibaja-Cordero, O.A. Segura-Bermúdez, G. Coto-Delgado, N. Goebel-Otárola, J. D. Barquero, M. Cullell-Delgado & M. Dal Zotto, 2019. An introduction to the study of Gastrotricha, with a taxonomic key to families and genera of the group. Diversity 11:17.

Vamos, E.E., V. Elbrecht & F. Leese, 2017. Short COI markers for freshwater macroinvertebrate metabarcoding. Metabarcoding and Metagenomics 1: e14625.

Van der Loos, L.M. & Nijland, R, 2020. Biases in bulk: DNA metabarcoding of marine communities and the methodology involved. Molecular Ecology. https://doi.org/10.1111/mec.15592.

Weigand, A. & J. Macher, 2018. A DNA metabarcoding protocol for hyporheic freshwater meiofauna: evaluating highly degenerate COI primers and replication strategy. Metabarcoding and Metagenomics 2: 1–15.

Wickham, H., 2016. Ggplot2: Elegant Graphics for Data Analysis. Springer, Berlin.

Yin, S., P. Tan, C. Yuan, J. Hu & X. Liu, 2017. Seasonal dynamics of meiofaunal distribution in the Dagu River Estuary, Jiaozhou Bay, China. Acta Oceanologica Sinica 36: 79–86.

Zhang, G.K., F.J.J. Chain, C.L. Abbott & M.E. Cristescu, 2018. Metabarcoding using multiplexed markers increases species detection in complex zooplankton communities. Evolutionary Applications 11:901-1914.

Zeppilli, D.J. Sarrazin, D. Leduc, P.M. Arbizu, D. Fontaneto, C. Fontanier, A.J. Gooday, R.M. Kristensen, V.N. Ivanenko, M.V. Sørensen, A. Vanreusel, J. Thébault, M. Mea, N. Allio, T. Andro, A. Arvigo, J. Castrec, M. Danielo, V. Foulon, R. Fumeron, L. Hermabessiere, V. Hulot, James, T.R. Langonne-Augen, T. Le Bot, M. Long, D. Mahabror, Q. Morel, M. Pantalos, E. Pouplard, L. Raimondeau, A. Rio-Cabello, S. Seite, G. Traisnel, K. Urvoy, T. Van Der Stegen, M. Weyand & Fernandes, 2015. Is the meiofauna a good indicator for climate change and anthropogenic impacts?. Marine Biodiversity 45:505–535.

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.