

2017

## Evaluation of Filtration and DNA Extraction Methods for Environmental DNA Biodiversity Assessments across Multiple Trophic Levels

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### Scholar Commons Citation

Djurhuus, Anni; Port, Jesse; Closek, Collin; Yamahara, Kevan; Romero-Maraccini, Ofelia; Walz, Kristine; Goldsmith, Dawn; Michisaki, Reiko; Breitbart, Mya; Boehm, Alexandria; and Chavez, Francisco, "Evaluation of Filtration and DNA Extraction Methods for Environmental DNA Biodiversity Assessments across Multiple Trophic Levels" (2017). *Marine Science Faculty Publications*. 677.  
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# Evaluation of Filtration and DNA Extraction Methods for Environmental DNA Biodiversity Assessments across Multiple Trophic Levels

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## OPEN ACCESS

### Edited by:

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### Specialty section:

This article was submitted to  
Marine Molecular Biology and Ecology,  
a section of the journal  
Frontiers in Marine Science

**Received:** 23 July 2017

**Accepted:** 15 September 2017

**Published:** 13 October 2017

### Citation:

Djurhuus A, Port J, Closek CJ,  
Yamahara KM, Romero-Maraccini O,  
Walz KR, Goldsmith DB, Michisaki R,  
Breitbart M, Boehm AB and  
Chavez FP (2017) Evaluation of  
Filtration and DNA Extraction Methods  
for Environmental DNA Biodiversity  
Assessments across Multiple Trophic  
Levels. *Front. Mar. Sci.* 4:314.  
doi: 10.3389/fmars.2017.00314

Metabarcoding of marine environmental DNA (eDNA), originating from tissue, cells, or extracellular DNA, offers the opportunity to survey the biological composition of communities across multiple trophic levels from a non-invasive seawater sample. Here we compare results of eDNA metabarcoding of multiple trophic levels from individual seawater samples collected from a kelp forest in Monterey Bay, California in order to establish methods for future cross-trophic level eDNA analysis. Triplicate 1 L water samples were filtered using five different 47 mm diameter membrane filters (PVDF, PES, GFF, PCTE, and NC) and DNA was extracted from triplicates of each filter-type using three widely-used extraction methods (the DNeasy Blood and Tissue kit, the MoBio PowerWater DNA Isolation kit, and standard phenol/chloroform methods) resulting in 45 individual eDNA samples prepared with 15 workflow combinations. Each DNA extract was amplified using PCR primers for the 16S rRNA gene (microorganisms; Bacteria and Archaea), 18S rRNA gene (phytoplankton), and the 12S rRNA gene (vertebrates), and PCR products were sequenced on an Illumina MiSeq platform. The richness and community composition of microbial, phytoplankton, and vertebrate OTUs were not significantly different between any of the 0.2  $\mu$ m pore-size filter types extracted with the DNeasy or MoBio kits. However, phenol/chloroform extraction resulted in significantly different community structures. This study provides insight into multiple choices for extraction and filtration methods to use eDNA metabarcoding for biodiversity assessment of multiple trophic levels from a single sample. We recommend any combination of either DNeasy or MoBio with PES, PCTE, PVDF, or NC filters for a cross trophic level comparison.

**Keywords:** environmental DNA, microorganisms, vertebrates, phytoplankton, trophic levels, marine ecosystems, biodiversity

## INTRODUCTION

Marine environmental DNA (eDNA) is the DNA suspended in seawater in the form of tissue, cells, or extracellular DNA. Biodiversity studies using eDNA involve sampling environmental media (e.g., water, soil, air), extracting genomic DNA, and using metabarcoding to determine the diversity of organisms present. The application of metabarcoding (i.e., PCR amplification and sequencing of specific gene targets) to eDNA present in both marine and terrestrial environments has opened new windows into monitoring species diversity (Thomsen and Willerslev, 2015; Kelly et al., 2017). For over three decades, researchers have used metabarcoding to amplify marker genes from complex mixtures of single-celled organisms, often captured on filters from liquid matrices, to describe the diversity and composition of marine microbial and phytoplankton communities (Pace et al., 1985; Pace, 1997; Hugenholtz et al., 1998). Recent research has expanded upon these methods by exploiting the fact that multi-cellular organisms release eDNA into the marine environment. The majority of eDNA investigations have used taxon-specific sampling and processing methods to identify target organisms, with lower trophic levels, microorganisms (Wu et al., 2010) and phytoplankton (Cermeño et al., 2010), receiving the greatest attention. Recently, methods for eDNA metabarcoding to assess taxon-specific biodiversity of higher organisms such as invertebrates (Machida et al., 2009; Yu et al., 2012; Klymus et al., 2015; Aylagas et al., 2016) and vertebrates (Kelly et al., 2016; Port et al., 2016) have been developed and benchmarked against well-established techniques (e.g., dive-surveys and microscopy).

The discovery that eDNA is released into the marine environment by higher trophic levels (e.g., Foote et al., 2012; Thomsen et al., 2012) opens up the possibility of utilizing a single water sample collection (i.e., the same filter and DNA extract) to assess biodiversity from multiple trophic levels (Kelly et al., 2016). The capture, extraction, amplification, and analysis of eDNA originating from organisms from multiple trophic levels offers a practical and comprehensive means for monitoring marine biodiversity over vast spatial domains and over time. In order to perform cross-trophic level analyses of biodiversity from a single water sample and to ensure accurate comparisons across multiple locations and studies, a critical first step is evaluating the results of multiple eDNA filtration and extraction methods. For a one-size-fits-all approach, a balance must be struck between reducing false-negative rates (i.e., maximizing the likelihood of identifying a given species present in a sample) and capturing biodiversity across multiple trophic levels (Kelly et al., 2016). In this study, we assess the influence of filter membrane and DNA extraction method on the detection of eDNA from three genetic loci representing different trophic levels, ranging from microbes (bacteria and archaea) to vertebrates. Although previous studies have compared different genetic markers (Kelly et al., 2017), filters (Pilliod et al., 2013), or extraction methods (Lakay et al., 2006; Roh et al., 2006) on eDNA samples, this study is the first to our knowledge to investigate comprehensive outputs of next generation sequencing of different filter types, extraction types, and different genetic markers simultaneously.

We aim to highlight the response of three genetic markers (16S rRNA, 18S rRNA, and 12S rRNA) to different treatments, with the ultimate goal of reducing the methodological uncertainty in marine eDNA analysis and providing recommendations for suitable methods for the simultaneous analysis of several trophic levels using eDNA to fulfill the needs of emerging programs such as the Marine Biological Observation Networks (MBON).

## METHODS

### Sample Collection and Filtration

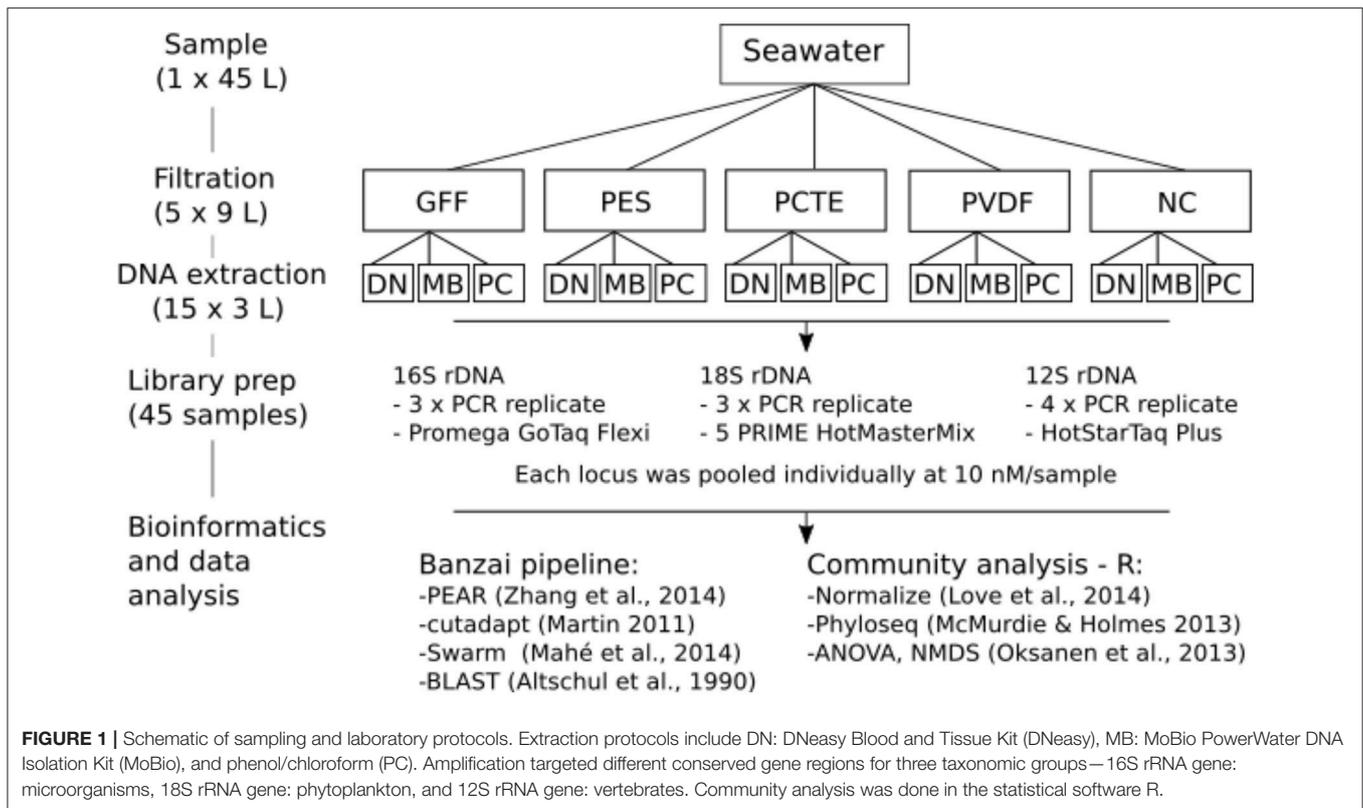
A total of 45 L of seawater were collected in November 2014 within Lovers Point—Julia Platt State Marine Reserve in Monterey Bay, California, using a 10% HCl acid-rinsed, hand-deployed 5 L Niskin bottle at ~10 m depth from a single location adjacent to kelp beds and ~200 m offshore from Hopkins Marine Station (Stanford University). Sampled water was homogenized by mixing and combining into 10% HCl acid-rinsed carboys. The 45 L sample was divided into fifths and each 9 L volume assigned to one of five different 47 mm diameter filter membrane types: Polyvinylidene difluoride (PVDF, 0.2  $\mu\text{m}$ , Fisher Scientific, SVHV 010 RS), Polyethersulfone (PES, 0.2  $\mu\text{m}$ , Sterlitech Corporation, PES0247100), Nitrocellulose (NC, 0.2  $\mu\text{m}$ , Millipore, GSWP4700), Polycarbonate Track Etch (PCTE, 0.2  $\mu\text{m}$ , Sterlitech Corporation, PCT0247100), and Glass microfiber filter (GFF, 0.7  $\mu\text{m}$ , Whatman, 28418314; **Figure 1**). The 1 L aliquots were vacuum-filtered through each filter type ( $n = 9$  filters per filter type) and stored in 5 ml sterile transport tubes (E&K Scientific) at  $-80^{\circ}\text{C}$  until DNA extraction (within 2 months). In parallel with sample filtering, 1 L of MilliQ water was filtered through a single filter of each type to serve as a filtration blank for monitoring contamination during the filtration process.

### DNA Extraction

Each set of 9 filters of a given type was divided into three subsets and DNA was extracted from each subset ( $n = 3$  filters) using the Qiagen DNeasy Blood and Tissue Kit (DNeasy), the MoBio PowerWater DNA Isolation Kit (MoBio), or the phenol/chloroform method (Ph-Ch; **Figure 1**). An extraction blank (i.e., the extraction method run using the methods outlined below but without a membrane filter) and a filtration blank (defined above) were included for each combination of filter type and extraction method. The GFF-MoBio treatment combination yielded no DNA and was omitted from further analyses.

### DNeasy Blood and Tissue Kit

The DNeasy protocol was modified to include an initial bead-beating step whereby 1 g of 0.5 mm and 1 g of 0.1 mm glass beads (BioSpec Products) along with 900  $\mu\text{l}$  ATL Buffer (Qiagen) were added to each filter tube. Before use, glass beads were sterilized by soaking in a 10% bleach solution for 20 min and rinsing at least three times with MilliQ water to remove the bleach. Beads were then dried by incubating overnight at  $65^{\circ}\text{C}$ . Tubes were shaken in a vortexer with a bead-beater adapter at maximum speed for 45 s, followed by incubation at  $56^{\circ}\text{C}$  for 30 min and a second round of bead-beating and incubation. Next, 100  $\mu\text{l}$  of Proteinase K (2 mg/L final concentration) was added to each tube,



vortexed for 10 s, and then incubated at 56°C for 2 h in a shaking incubator. Samples were then vortexed for 15 s and centrifuged for 1 min (4,000 × g). The supernatant from each tube (650–900 μl; volume dependent on the filter type) was transferred into a new 2 ml tube and centrifuged for 1 min at 13,000 × g. Then 650 μl of bead-free supernatant was transferred to a new 2 ml tube. Hereafter the manufacturer's protocol was followed with the following modifications: 650 μl AL Buffer, 650 μl ethanol, and final elution steps of 2 × 50 μl AE Buffer for each sample.

### MoBio PowerWater DNA Isolation Kit

For the MoBio extraction, manufacturer's instructions were followed including a 5 min bead-beating step.

### Phenol/Chloroform

The third extraction method was a Ph-Ch extraction; here 900 μl SDS buffer (10 mM Tris-HCl, 100 mM EDTA, 200 mM NaCl, 1% SDS) and 100 μl of lysozyme (1 mg/ml final conc.) were added to each filter tube and incubated at 37°C for 30 min (Sambrook and Russell, 2001; Corell and Rodríguez-Ezpeleta, 2014). Next, 50 μl proteinase K (~1 mg/ml, final conc.) was added to each tube and incubated overnight at 55°C in a shaking incubator (Boström et al., 2004; Corell and Rodríguez-Ezpeleta, 2014). Then, tubes were vortexed for 15 s and centrifuged at 4,000 × g for 1 min. One milliliter of the supernatant was transferred to a clean 2 ml tube, mixed with 1 ml of phenol/chloroform/isoamyl alcohol 25:24:1, vortexed for 5 s, and centrifuged at 12,000 × g for 10 min. The aqueous phase (800 μl) was transferred to a new 2 ml tube,

mixed with 800 μl (one volume) of chloroform/isoamyl alcohol 24:1, vortexed for 5 s, and centrifuged at 12,000 × g for 10 min. The supernatant containing the DNA (650 μl) was mixed with 65 μl (1/10 volume) of 5 M NaCl and 1.3 ml (2 volumes) of 100% ethanol and incubated at –20°C overnight (Barnes et al., 2014; Deiner and Altermatt, 2014; Renshaw et al., 2015). Next, the DNA was isolated by centrifuging at 20,000 × g for 20 min at 4°C and the ethanol was poured out. Salts were removed by washing the pellet twice with 500 μl of 70% ethanol, centrifuging at 20,000 × g for 20 min at 4°C, and removing the ethanol. Once dried, the pellet was dissolved in 100 μl AE Buffer (Qiagen).

### Quality Control of DNA Extractions

To account for the presence of inhibitory factors in seawater, DNA extracts were subsequently purified using One-step PCR inhibitor removal columns (Zymo Research; McKee et al., 2015). Subsequently, total DNA was quantified using a Qubit 2.0 Fluorometer and the Qubit dsDNA HS assay (Invitrogen, CA, USA). DNA extracts were divided into three aliquots and distributed to three separate research laboratories for marker-specific processing: 16S rRNA gene at the University of South Florida, 18S rRNA gene at the Monterey Bay Aquarium Research Institute, and the 12S rRNA gene at Stanford University. For the 12S rRNA gene analysis, we generated mock vertebrate communities to serve as positive controls for sequencing. The mock community represented a mix of total DNA extracts from tissues of ten species of bony fishes in equal concentrations

(extractions performed with the Qiagen DNeasy Blood and Tissue Kit) as in Port et al. (2016).

## PCR and Library Prep

DNA extracts were amplified with primer sets targeting different trophic levels (**Table 1**). The 16S rRNA gene was amplified from microbes, the 18S rRNA gene was amplified from phytoplankton, and a vertebrate-specific primer set was used to amplify the mitochondrial 12S rRNA gene of birds, fishes, and marine mammals. Sample-specific indexing, library preparation, and PCR profiles varied between the primer sets, utilizing previously optimized protocols for each gene as specified below. No attempts to standardize library preparation conditions were made because this study solely focused on determining the effects of filter-extraction combinations and not on comparing loci. In particular, the goal was to determine which filters and extraction methods would produce DNA of a sufficient quantity and quality to enable individual research groups, who each focused on a specific trophic level, to successfully analyze biodiversity through eDNA metabarcoding. Prior to sequencing, the PCR products for each gene were pooled into an equimolar concentration of 10 nM/sample to produce a similar sequencing depth per sample for accurate comparisons of results obtained from an equal amount of DNA. For samples with a concentration below detection limits (<0.5 ng/ $\mu$ l; i.e., negatives and extraction blanks), 3  $\mu$ l of PCR product was used for the 16S rRNA and 18S rRNA genes, however for the 12S rRNA gene the entire volume was added. All sequencing was performed at the Stanford Functional Genomics Facility on an Illumina MiSeq platform using paired-end sequencing (Miseq Reagent kit v2) and a 20% PhiX174 spike-in control to improve the quality of low-diversity samples for 12S rRNA gene and 18S rRNA gene (Kircher et al., 2009); since the 16S rRNA gene captures diverse communities the addition of PhiX174 was lower (10%). Library construction for Illumina sequencing followed the KAPA low-throughput library prep kit with real-time library amplification protocol (KAPA Biosystems). Library size and concentration were assessed using a Bioanalyzer with the High Sensitivity DNA assay (Agilent Technologies).

## Microorganisms (16S rRNA Gene)

Both forward (515fB) and reverse (806rB) primers for the 16S rRNA gene, targeting microorganisms, were tagged yielding dual-indexed reads (Kozich et al., 2013; Apprill et al., 2015;

Parada et al., 2016; **Table 1**). PCR reactions were carried out in triplicate according to Apprill et al. (2015) with 1:10 dilutions on all extracts. Briefly, 1  $\mu$ l DNA template was added to the master mix consisting of 14.75  $\mu$ l sterile water, 5.0  $\mu$ l 5X buffer AE (Qiagen), 0.5  $\mu$ l dNTPs, 0.25  $\mu$ l Taq polymerase (Promega GoTaq Flexi) and MgCl<sub>2</sub> and 0.5  $\mu$ l of each primer at a final concentration of 5  $\mu$ M). Cycling parameters were 95°C for 2 min followed by 30 cycles of 95°C for 20 s, 55°C for 15 s, and 72°C for 5 min, and lastly 72°C for 10 min. The PCR products were run through a 1.5% agarose gel stained with ethidium bromide to confirm the presence of the targeted band and clean negative controls. All non-template controls (NTCs) and blanks were negative. PCR product concentrations were quantified using a Qubit dsDNA HS Assay Kit with a Qubit 3.0 Fluorometer according to the manufacturer's instructions.

## Phytoplankton (18S rRNA Gene)

PCR reactions were run in triplicate 25  $\mu$ l reactions for each sample using 12-basepair Golay barcoded reverse primers (Amaral-Zettler et al., 2009; **Table 1**). PCR reactions for the 18S rRNA gene were carried out using 1  $\mu$ l DNA extract (no dilution), 10.0  $\mu$ l 5 PRIME HotMasterMix (5 PRIME Inc, USA), 1  $\mu$ l each of forward and reverse primers (final concentration 5  $\mu$ M), 4  $\mu$ l 10  $\mu$ M mammalian blocking primer (Earth Microbiome Project; Vestheim and Jarman, 2008), and 8  $\mu$ l molecular-biology grade water (Sigma-Aldrich). PCR reactions were run in 96-well plates with a NTC run in triplicate for each plate. Cycling parameters were 94°C for 3 min followed by 35 cycles of 94°C for 45 s, 65°C for 15 s, 57°C for 30 s, and a final step of 72°C for 90 s. The pooled PCR products were run through a 1.5% agarose gel stained with Gelgreen (Biotium Inc.) to confirm the presence of target bands and clean NTCs and absence of any non-specific amplification. All NTCs were negative. PCR products were purified and size selected using the Agencourt AMPure XP bead system (Beckman Coulter, USA). A second agarose gel was run to confirm primer removal and retention of target amplicons after purification. Purified products were then quantified using Quant-It Picogreen dsDNA Assay (Life Technologies) on an FMAX Molecular Devices Fluorometer with SoftMaxPro v1.3.1.

## Vertebrates (12S rRNA Gene)

The forward and reverse 12S rRNA gene primers (**Table 1**; Riaz et al., 2011) were modified by the addition of sample-specific nucleotide tags on the 5' ends to allow for the assignment

**TABLE 1** | List of primers used in this study.

Taxon	DNA region	Primer name	Forward/reverse	Primer sequence 5' – 3'	Amplicon length (bp)	References
Microorganisms	16S rRNA gene	515fB	Forward	GTGYCAGCMGCCGCGGTAA	291	Apprill et al., 2015 Parada et al., 2016
		806rB	Reverse	GGACTACNVGGGTWCTAAT		
Phytoplankton	18S rRNA gene	1391F	Forward	GTACACACCGCCGTC	150	Amaral-Zettler et al., 2009
		EukBr	Reverse	TGATCCTTCTGCAGGTTACCTAC		
Vertebrates (Fish and mammals)	12S rRNA gene	12SV5-F	Forward	ACTGGGATTAGATACCC	106	Riaz et al., 2011
		12SV5-R	Reverse	TAGAACAGGCTCCTCTAG		

of sequence reads to the correct sample during bioinformatic processing (Valentini et al., 2009). Tags were designed using the OLIGOTAG program (Coissac, 2012) with a Hamming distance of at least three bases between tags. Tags were preceded by NNN (De Barba et al., 2014) and the forward and reverse primers for a given sample had identical tags.

PCR reactions were carried out using 5 µl DNA template, 12.5 µl HotStarTaq Plus Master Mix (Qiagen), 1 µl of each primer (10 µM) and 5.5 µl molecular-biology grade water (Sigma-Aldrich). Cycling parameters were 95°C for 5 min followed by 40 cycles of 95°C for 15 s, 55°C for 30 s and 72°C for 30 s. Four replicate PCR assays were performed for each sample and then pooled. A negative control was included for each filter by extraction replicate to account for master mixes with different tagged primer sets. The pooled PCR products were run through a 1.5% agarose gel stained with ethidium bromide to confirm the presence of the 12S rRNA gene target band and clean NTCs and absence of any non-specific amplification. All NTCs were negative. PCR products were purified and size selected using the Agencourt AMPure XP bead system (Beckman Coulter) and then quantified using the Qubit 2.0 Fluorometer and the Qubit dsDNA HS Assay Kit (Invitrogen).

Two pools were prepared to allow the same tag to be sequenced more than once and subsequently de-multiplexed during data analysis.

## Bioinformatics

All data from this study can be accessed from GenBank (SUB2901791). Sequence data were processed using a Unix shell script written to analyze Illumina-generated eDNA data (<https://github.com/jimmyodonnell/banzai>). This pipeline includes the following steps: merging of paired reads using PEAR v0.9.2 (Zhang et al., 2014), quality filtering with USEARCH (Edgar, 2010), demultiplexing by primer tag or library barcode with the programming language AWK, primer removal with cutadapt v.1.4.2 (Martin, 2011) allowing for no mismatches in the primer sequence, de-replication, singleton removal, Operational Taxonomic Unit (OTU) clustering using Swarm (Mahé et al., 2014), taxonomic annotation by nucleotide BLAST (BLASTN; Altschul et al., 1990) against a reference database (Table 2), and secondary taxonomic assignment using the lowest common ancestor (LCA) algorithm in MEGAN (Huson et al., 2007). Parameters for these programs were locus-specific (Table 2). To minimize the presence of chimeric sequences and tag jumping, only those reads containing the same tag sequence at both the 5' and 3' ends were retained (Schnell et al., 2015). For all primer sets, reads with homopolymers >7 bases were also omitted.

We further filtered the OTU tables using a suite of R scripts (<https://github.com/marinebon/MBON>). We removed putative contamination (cross-contamination among samples or contamination sourced from the field or lab) by calculating the maximum proportional representation of each OTU across all control samples [i.e., positive (mock community) and negative controls (Kelly et al., 2016)]. These OTU proportions were then subtracted from each OTU in the field samples.

**TABLE 2 |** Bioinformatic parameters used for data processing by primer set.

Step	Program	Parameter	16S rRNA	18S rRNA	12S rRNA
Paired-end merging	PEAR	Min. overlap size	75	50	100
		Max. assembly length	151	300	161
		Min. assembly length	75	75	151
		Quality score threshold	15	15	15
		p-value	0.01	0.01	0.01
Quality filtering	Swarm	Expected no. of errors per read	0.5	0.5	0.5
		Min. sequence length	150	150	154
Primer removal	Cutadapt	No. of mismatches	0.1	0.1	0
OTU clustering	Swarm	Cluster radius	1	1	1
		Percent identity	97	97	98
Taxonomic annotation	BLASTN	E-value	$1 \times 10^{-5}$	$1 \times 10^{-5}$	$1 \times 10^{-5}$
		Word size	20	20	24
		Database	Silva	Silva	NCBI nt
Lowest common ancestor	MEGAN	Min. bit score	160	160	150
		Top percent	2	2	2
		LCA percent	90	90	70

## Statistics

Sequence counts were normalized per locus with the R package DESEQ2 v1.6.2 (Love et al., 2014). This method accounts for differential sample depth (correcting for uneven numbers of reads per tag) and is appropriate for normalizing high-variance data sets from high-throughput sequencing (McMurdie and Holmes, 2014). All other statistical analyses were performed using the VEGAN package v2.2.0 in R (Oksanen et al., 2013). A binary version of the data set was created to determine species richness. Species richness was calculated by summing the number of taxa present per treatment (i.e., filter-type with DNA extraction combination) after data filtering, whereby a taxon was deemed present if its sequence was present in one or more of the three replicates per treatment. Prior to any statistical analyses the data were checked for a normal distribution with a Shapiro–Wilk test of normality. Analyses of variance (ANOVAs) followed by Tukey Honest Significant Difference (Tukey HSD) tests were performed to determine whether DNA concentration before amplification or species richness significantly differed across treatments. Non-metric multidimensional scaling (NMDS) was performed using the Bray-Curtis dissimilarity indices with the function *metaMDS*. The *adonis* function was used to parse the data according to the different treatments [permutational multivariate analysis of variance (PERMANOVA)]. For the heatmap the top 20 ranks were picked based on total abundance from all samples using

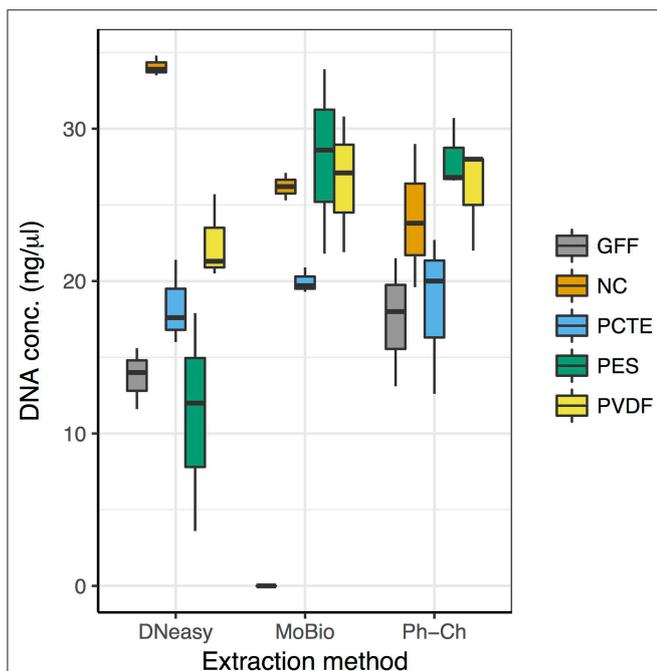
the R package PHYLOSEQ (McMurdie and Holmes, 2013). The heatmap was plotted using the package “superheat” (Barter and Yu, 2015). All analyses were done using the statistical software R (R Development Team, 2009) and can be accessed on <https://github.com/marinebon/MBON>.

## RESULTS

### DNA Yield

DNA yield was highly variable between the three different extraction methods, especially between different filter types extracted with the DNeasy Blood and Tissue Kit (DNeasy; **Figure 2**). The NC-DNeasy combination yielded the highest concentration of DNA of any method ( $34 \pm 0.67$  ng/ $\mu$ l) and GFF-MoBio PowerWater DNA Isolation Kit (MoBio) had the lowest yield ( $<0.5$  ng/ $\mu$ l). Regardless of extraction method, the NC and PVDF filters yielded higher DNA concentrations than PCTE and GFF filters. The PES filter yielded the lowest DNA concentration for DNeasy but highest for MoBio and phenol/chloroform (Ph-Ch) extraction methods (**Figure 2**).

For DNeasy there was a statistically significant difference in DNA yield between NC and all other filter types, with NC-DNeasy having the highest DNA yield of all treatments. For MoBio there was a statistically significant difference between GFF and all other filters (Tukey HSD: adjusted  $p < 0.01$ ) with GFF-MoBio yielding the lowest amount of DNA of all treatments. DNA yield between the filter types extracted with Ph-Ch was fairly uniform with no significant difference between the extractions from Ph-Ch (**Figure 2**).



**FIGURE 2** | Distributions of DNA concentrations (ng/ $\mu$ l) in extractions obtained using the 15 different combinations of filter types and extraction methods.  $n = 3$  for each box.

### OTU Richness and Community Structure Microorganisms (16S rRNA Gene)

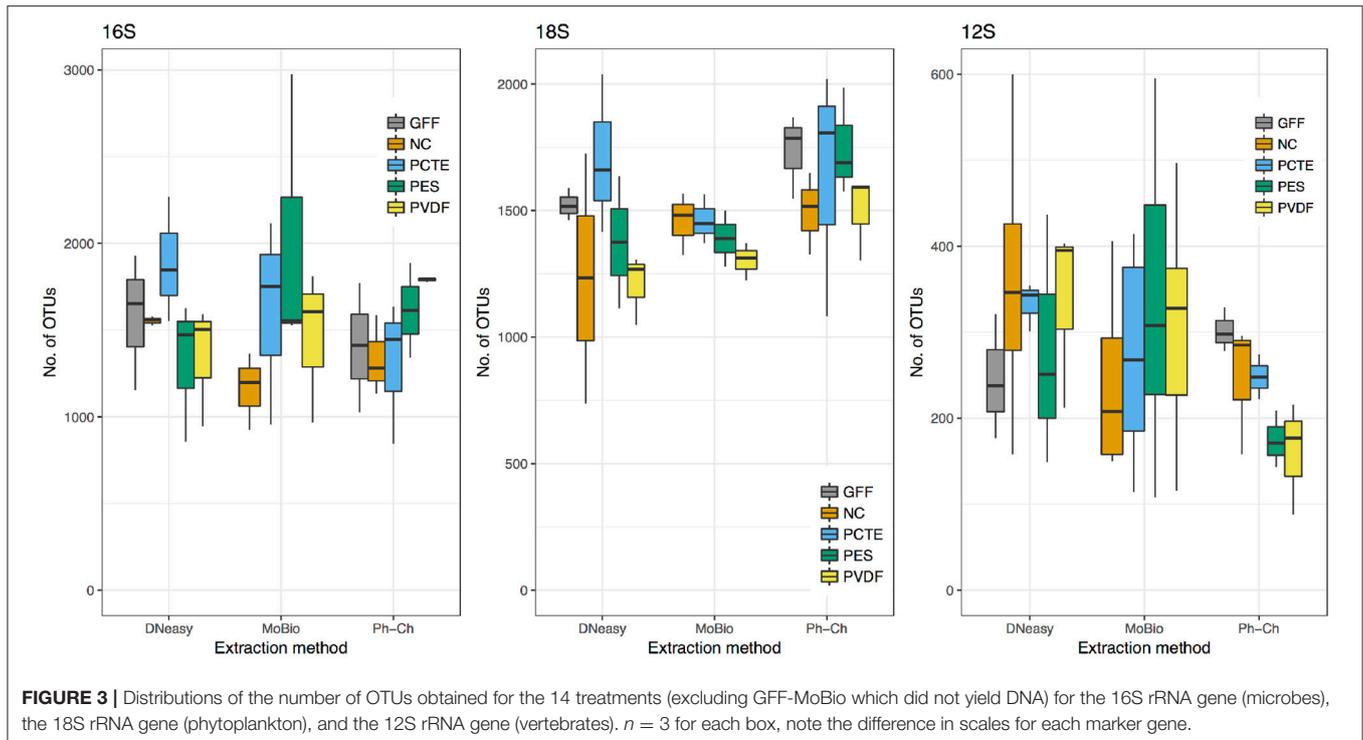
The average richness per sample of microbial communities as measured through 16S rRNA gene sequencing was 1,575 OTUs. The highest average richness was obtained from the PCTE-DNeasy combination and the lowest from the NC-MoBio combination (**Figure 3** and **Table 3**); however, based on the ANOVA there was no significant difference in richness between any of the filter types or extraction methods for 16S rRNA gene (Figures S1, S2). Rank abundances between all extraction methods were similar and the dominant annotated OTU, SAR11, was the same between all filters and extraction methods (**Figure 4**).

### Phytoplankton (18S rRNA Gene)

The average richness of phytoplankton communities as measured through 18S rRNA gene sequencing was 1,458 OTUs. The highest average richness was obtained from the PCTE- Ph-Ch combination (**Figure 3** and **Table 3**) and the lowest from the NC-DNeasy combination. The differences between 18S rRNA gene richness are most notable between the MoBio and Ph-Ch extraction methods, with Ph-Ch yielding a significantly higher average richness than MoBio (Tukey HSD:  $p = 0.034$ ; **Figure 3** and **Figure S1**). There are fewer shifts in the rank abundance of dominant species between the DNeasy and MoBio extractions methods than between MoBio and Ph-Ch (**Figure 4**). This is also reflected in the NMDS analysis, as the phytoplankton community was significantly different between Ph-Ch and the other two extraction methods (PERMANOVA:  $df = 2$ ,  $p = 0.0019$ ). In addition, the filter membrane PCTE had a community structure that was significantly different from the other filter types on an OTU level (PERMANOVA:  $df = 4$ ,  $p = 0.043$ ). The top four highest ranked phytoplankton OTUs were the same between all extraction methods for this marker. The two most abundant OTUs were both annotated as Chloroplastida, and the third and fourth most abundant OTUs were annotated as Syndiniales group II and Syndiniales group I, respectively.

### Vertebrates (12S rRNA Gene)

The average richness of vertebrate communities as measured through 12S rRNA gene sequencing was 277 OTUs distributed among 51 families (**Table 3**). The DNeasy-PVDF combination yielded the highest average richness and the PES-Ph-Ch combination yielded the lowest (**Figure 3** and **Table 3**). The difference in richness was not significant between any pair of extraction method and filter type treatment combinations (Figure S1, S2). However, within Ph-Ch extractions, the filter types PVDF and GFF yielded statistically different numbers of OTUs (Tukey HSD: adjusted  $p = 0.039$ ; **Figure 3**). There are a few variations in rank abundance between DNeasy, MoBio, and Ph-Ch extraction methods (**Figure 4**). The top five overall ranks (families: Phalacrocoracidae (cormorant), Cottidae (sculpins), Phocidae (earless seal), Sebastidae (rockfish), and Clinidae (blennies) (**Figure 4**) shift in order between the three extraction methods but remain the top five ranks for all treatments. There is one notable change between DNeasy and MoBio where the 12th ranked OTU (Paralichthyidae: Flounder) in the



**TABLE 3 |** Number of OTUs per treatment and genetic marker with their respective standard deviations.

Extraction	16S rRNA	18S rRNA	12S rRNA
DNeasy	1,538 ± 356	1,411 ± 327	317 ± 117
MoBio	1,698 ± 605	1,340 ± 246	290 ± 132
Ph-Ch	1,490 ± 314	1,623 ± 256	226 ± 68
FILTER			
GFF	1,740 ± 509	1,487 ± 401	274 ± 57
NC	1,350 ± 234	1,395 ± 294	280 ± 125
PCTE	1,603 ± 476	1,601 ± 315	281 ± 92
PVDF	1,651 ± 570	1,540 ± 284	281 ± 153
PES	1,533 ± 344	1,336 ± 173	288 ± 132

DNeasy extraction was the most abundant OTU in the MoBio extraction. The only significant difference in the total community structure on an OTU level appears between Ph-Ch and the other extraction methods (PERMANOVA,  $df = 2$ ,  $p = 0.0039$ ; Figure 5).

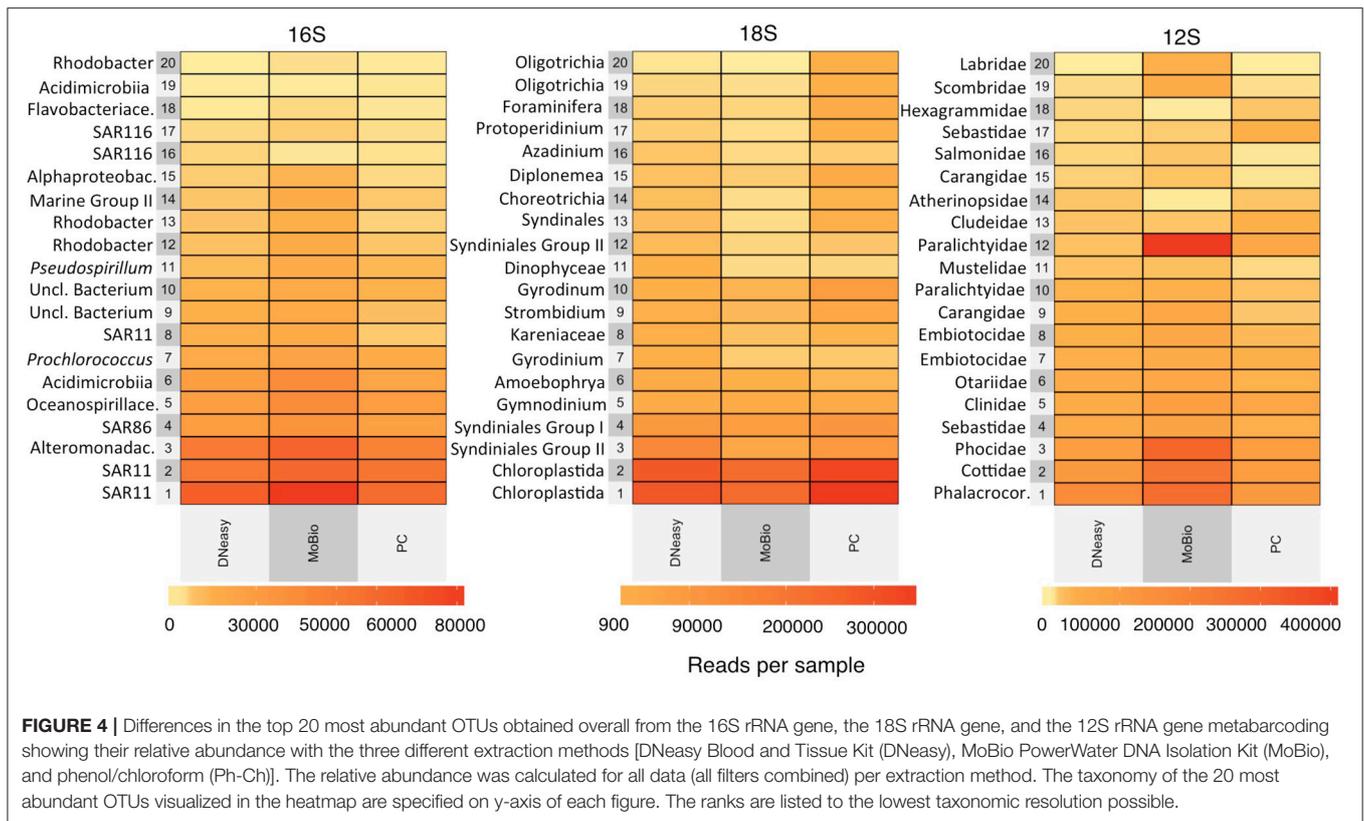
## DISCUSSION

### DNA Yield and Richness Differences between Treatments

In contrast to Pilliod et al. (2013) this study found differences in DNA yield and species richness between some of the different filter type and extraction treatment combinations. Overall, DNA extraction using the three techniques on samples collected on the 0.2  $\mu\text{m}$  pore-size filters tested in this study

(PCTE, NC, PES, PVDF) resulted in similar DNA yields and richness estimates across the three loci. The differences observed among 0.2  $\mu\text{m}$  filters, although not statistically significant, could have resulted from biological heterogeneity. Although the total water-mass for collection was mixed thoroughly prior to filtration, patchiness could still occur (Port et al., 2016).

DNA yield from GFF filters was poor relative to other filters (Figure 2). The very low DNA recovery using the MoBio kit was likely due to the absorbance of lysis buffer by the GFF filters, which resulted in reduced lysate recovery volumes. However, another contributing factor to the lower DNA yield from GFF filters is that the effective pore size of GFF filters is 0.5  $\mu\text{m}$  (Chavez et al., 1995), while the other filters tested were all 0.2  $\mu\text{m}$  pore size and therefore would likely have captured more small particles. Environmental DNA ranges from 1 to 10  $\mu\text{m}$  in size (Turner et al., 2014) and phytoplankton should be retained on all filters tested in this study; however, microorganisms are often smaller than 0.5  $\mu\text{m}$  and would in some cases pass through filters with pore sizes larger than 0.2  $\mu\text{m}$  (Azam and Godson, 1977; Rappé et al., 2002). An important conclusion of this study is that DNeasy or Ph-Ch extraction methods are more effective than MoBio for extracting DNA from GFF filters (Figure 2) and that these filters provide similar taxa richness to the other filters for all loci (Figure 3). These filters can be used for phytoplankton, zooplankton, and vertebrate eDNA but are not optimal for studies involving microorganisms as they may miss free-living small microorganisms or bias the community composition toward those associated with particles (DeLong et al., 1993).



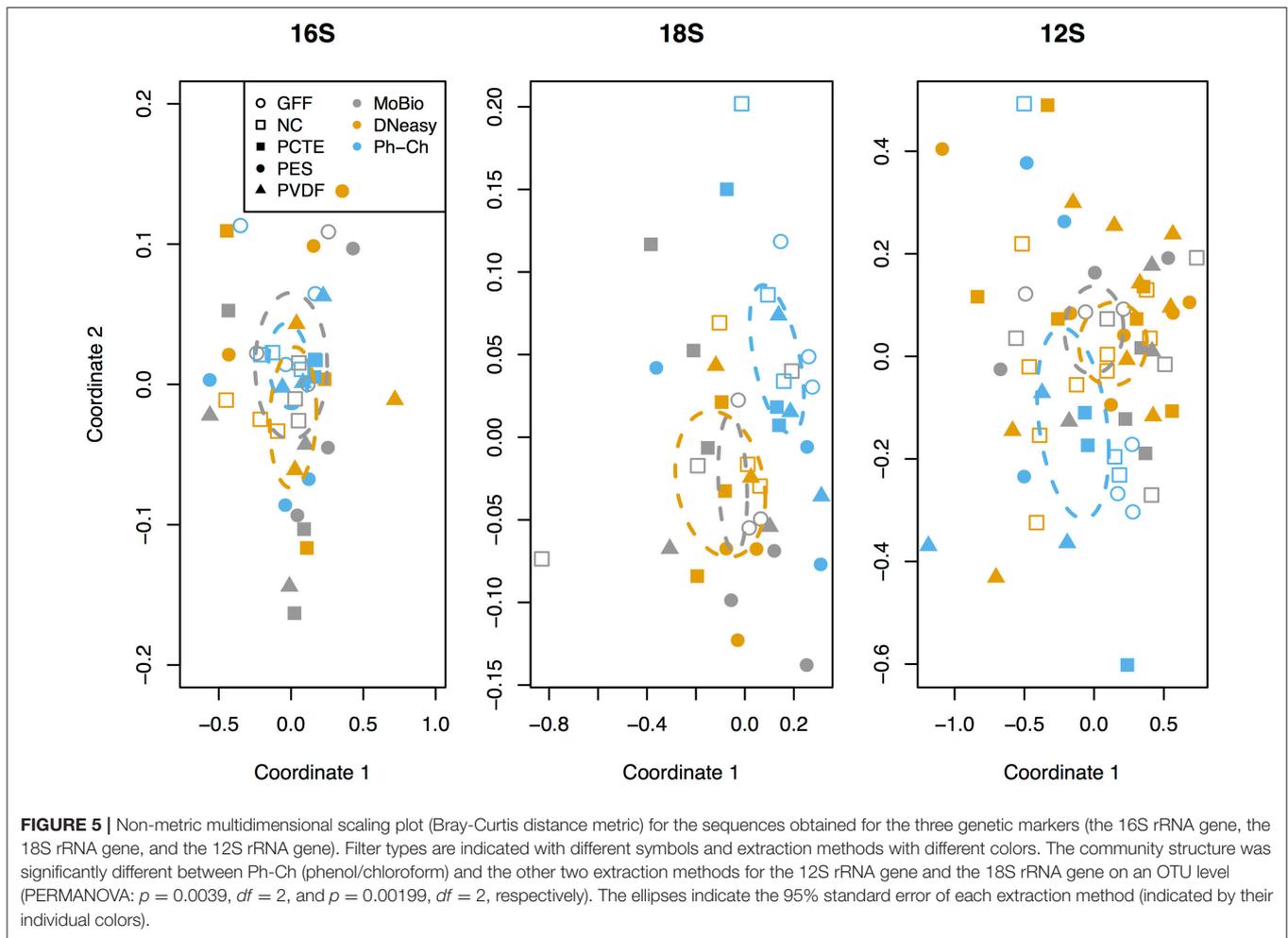
## OTU Community Structure with Treatment

Similar to the results of Kelly et al. (2017) each of our survey methods yielded a distinct set of organisms from the same location. DNeasy and MoBio extraction methods resulted in similar community compositions for both the 12S rRNA gene and the 18S rRNA gene that were different from those obtained with Ph-Ch (Figures 4, 5), suggesting that the Ph-Ch extraction method selects for or against certain organisms. The relationship between sequence abundance and cell abundance is not always straightforward and biases are inevitable in amplification and sequencing (Caporaso et al., 2012). For microorganisms there is a continuous progression of primer redesign to enhance the specificity and inclusiveness of amplification of the desired target region of the 16S rRNA gene and ensure that the amplified community reflects the initial community composition (Caporaso et al., 2011; Apprill et al., 2015; Parada et al., 2016). This effort has most likely improved the relatively conserved rank order of dominant OTUs observed amongst extraction methods in this study. In addition, the microbial cells targeted with the 16S rRNA gene primers are smaller and generally easier to lyse than individual plant cells, the targets of the 18S rRNA gene (Yuan et al., 2015). Some phytoplankton and plant cell walls are composed of cellulose (macroalgae) and silica formed into frustule (diatoms), which require manual lysis (i.e., bead beating) to break down the cell wall to release the cell's nucleic acids. The chemical lysis of the Ph-Ch extraction does not lyse cells as thoroughly as manual lysis (Yuan et al., 2015). For both MoBio and DNeasy we performed bead beating, which we suspect is the

reason that these methods yield a different community structure than the Ph-Ch extraction. Therefore, Ph-Ch extraction is not recommended for cross-trophic level comparisons (Deiner et al., 2015; Yuan et al., 2015).

The MoBio kit is the most common extraction method recommended for large observation networks such as the Earth Microbiome Project (EMP) and Ocean Sampling Day (OSD; Gilbert et al., 2011). Since MoBio achieved good DNA yield for most of the filter types (except GFF) and comparable richness estimates for all genetic markers, this extraction method is appropriate for cross-trophic level analyses. In addition, the MoBio method is less time consuming compared to DNeasy and does not require manual filling of tubes with beads for bead beating. However, our results show that either DNeasy or MoBio with any of the 0.2  $\mu\text{m}$  filter types (PCTE, PES, NC, and PVDF) will produce an OTU richness and community structure that enable cross-study comparisons.

The 12S rRNA gene is the least studied of the genetic markers used in this study (Port et al., 2016). Since there is limited information on the variability in sequencing data from this genetic marker, it is worth noting that the PVDF-DNeasy combination yielded the highest richness. This combination could be considered when designing a study focused on vertebrates or multiple trophic levels. The use of multiple primer sets per sample can be additionally informative as different genetic markers may not be equally applicable for phylogenetic vs. quantitative analyses due to primer and gene copy variation biases (Kelly et al., 2017).



As suggested by Rees et al. (2014), a direct comparison of filtration and extraction methods to monitor the effects of different processing methods on eDNA extraction and detection would be useful. This study lays the groundwork regarding acceptable sample collection, filtration, and DNA extraction methods for cross-trophic level eDNA analyses within emerging observation frameworks (e.g., MBON) to establish baselines and monitor changes in biodiversity over space and time in marine ecosystems.

In conclusion this study analyzed three different extraction methods with five different filter-types to investigate the variability in community structure of microbes, phytoplankton, and vertebrates using the 16S rRNA gene, the 18S rRNA gene, and the 12S rRNA gene metabarcoding data, respectively. Although variation was observed between the filter-extraction combinations, several combinations (DNeasy and MoBio extraction methods and all filter-types except GFF) produced comparably high DNA yield, richness, and community structure. Due to their larger pore size, GFF filters may not capture some small (<0.5  $\mu\text{m}$ ) organisms and are not recommended for cross-trophic level analyses that include microorganisms. In addition, GFF filters should not be used with the MoBio extraction kit

due to low DNA yields. However, GFF filters extracted with the other methods yielded similar species richness results as the 0.2  $\mu\text{m}$  filters, indicating that filters collected and archived for other purposes can provide valid results, especially when extracted with the DNeasy extraction kit.

The Ph-Ch extraction method resulted in a significantly different community structure compared to DNeasy and MoBio for the 18S rRNA gene and the 12S rRNA gene; thus it is not recommended for cross-trophic level analyses due to selection for/against certain organisms, probably because this method does not utilize bead beating. Extraction of DNA using either the DNeasy or MoBio kits from 0.2  $\mu\text{m}$  filters composed of NC, PCTE, PES, and PVDF can be used to obtain statistically comparable results across multiple trophic levels, making them suitable for a one-size-fits-all approach to monitoring marine biodiversity through eDNA.

## DATA ACCESSIBILITY

Data have been deposited to NCBI with the accession number SUB2901791.

## AUTHOR CONTRIBUTIONS

JP, AD, MB, FC, KY, and AB conceived the study. JP, OR, KW, and DG conducted sample collection and lab analyses. Data analysis was done by AD, JP, CC, RM, and KY. AD and JP drafted the manuscript. All authors revised and edited the manuscript.

## ACKNOWLEDGMENTS

The authors acknowledge Kate Hubbard, Enrique Montes-Herrera, and Natalie Sawaya for their constructive feedback on evaluating these methods and discussing the outcome. This

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work was supported by NASA grant NNX14AP62A “National Marine Sanctuaries as Sentinel Sites for a Demonstration Marine Biodiversity Observation Network (MBON)” funded under the National Ocean Partnership Program (NOPP RFP NOAA-NOS-IOOS-2014-2003803 in partnership between NOAA, BOEM, and NASA), and the U.S. Integrated Ocean Observing System (IOOS) Program Office.

## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmars.2017.00314/full#supplementary-material>

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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