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METHOD

Optimizing an enclosed bead beating extraction method for microbial and fish environmental DNA

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Abstract

DNA extraction is a critical step in processing environmental DNA (eDNA), influencing biodiversity estimates and ecosystem monitoring. Sterivex filters have become popular for collecting aquatic eDNA, allowing for on-site filtration and reduced contamination. Yet, extracting eDNA from an enclosed filter remains laborious. Recent methods have improved extraction of microbial DNA from Sterivex by adding lysis buffer and beads directly to the filter; however, it remains unclear how bead size affects eDNA recovery across multiple trophic levels. Further, the method has not been scaled up for 96-well magnetic bead extraction kits and robotic manipulation. We collected surface water from Bear Cut (Biscayne Bay, Florida) and performed enclosed Sterivex DNA extractions with different bead size combinations (0.1, 0.5, 0.1 + 0.5 mm, and no beads) and two magnetic kits (Zymo and NucleoMag). We employed 16S and 12S rRNA gene metabarcoding to assess extraction effects on microbes (bacteria, archaea, and eukaryotes) and bony fishes. We polled local experts to verify fish eDNA, revealing several non-native taxa originating from a nearby aquarium. DNA yield, microbial richness, and diversity were not influenced by beads. Bacterial relative abundance (family to ASV) was conserved across bead treatments, while several hard-to-lyse eukaryotes (diatoms and chlorophytes) were less abundant (or absent) when extracted without beads. Higher fish richness was detected with larger beads; however, relative abundance and composition were not associated with beads, likely reflecting heterogeneity or low concentrations of fish compared to microbial eDNA. We recommend using larger or differently sized beads for Sterivex extractions to detect a range of marine life, with increased water volume (>2 L per filter) or replication as necessary to enhance fish eDNA capture.

KEYWORDS
beads, bony fishes, eDNA extraction, marine life, microbes, Sterivex

1 | INTRODUCTION

Amplicon metabarcoding of environmental DNA (eDNA) has become a widely used approach to characterize marine biodiversity (Deiner et al., 2017), with implications for accurate ecosystem management and conservation strategies (Gilbey et al., 2021; Goodwin et al., 2017; Hajibabaei et al., 2016). Compared to traditional biomonitoring, such as fish trawls or microscopy, eDNA metabarcoding is non-invasive, sensitive to rare or cryptic species, and can distinguish multiple groups of organisms (microbes to mammals) from a single sample (Deiner et al., 2017; Djurhuus et al., 2020; Stat et al., 2017). Marine (and other aquatic) eDNA represents whole microbial cells and genetic material shed from larger organisms, including skin cells, mucus, scales, or other extracellular sources (Thomsen & Willerslev, 2015). Despite its utility, there remains a lack of consensus on best practices for eDNA sampling and processing (Goodwin et al., 2017), as almost every major step in the eDNA workflow (e.g., filtration, primer design, library preparation, and bioinformatics) can vary between lab groups (Eble et al., 2020; Kumar et al., 2020; Ruppert et al., 2019) and differently influence downstream biodiversity estimates. Further testing on how different eDNA methods affect downstream measurements will improve our ability to monitor marine populations, predict ecosystem status (e.g., fisheries information), and guide efforts toward standardized and reproducible eDNA sampling.

Collection and filtration of eDNA from water samples is a critical first step in eDNA processing (Eble et al., 2020; Goldberg et al., 2016). Only a handful of studies have examined different eDNA extraction methods in aquatic systems. These have revealed the effects of water filtration volume, filter type or size, and commercial extraction kit on DNA yield, species richness, and diversity (Bessey et al., 2020; Capo et al., 2020; Deiner et al., 2015, 2018; Djurhuus et al., 2017; Piggott, 2016). Optimal filtration and extraction methods also depend on the sampling environment. For instance, coastal areas with high microbial biomass and particulate matter may limit filtration volumes and increase the risk of PCR inhibition, while offshore areas may have more dilute DNA concentrations and require greater filtration volumes (Kumar et al., 2020, 2021; Li et al., 2018; Tsuji et al., 2019). Sterivex filters have become an increasingly popular method to collect marine eDNA, as they are simple to use, can be employed in the field on research cruises, and can be immediately fixed (or frozen) on site to avoid DNA degradation (Bryant et al., 2016; Ushio, 2019; Wong et al., 2020). Most importantly, the use of Sterivex filters may mitigate contamination during filtration or handling, as the filter is encased in plastic and individually pre-packaged.

Though Sterivex filters may promote sterile sampling conditions, extracting eDNA from an enclosed filter remains challenging. One method is to crack open the filter casing (e.g., with pliers) and process the filter with a commercial DNA extraction kit (Cruaud et al., 2017), which may introduce contamination. A less invasive method involves adding lysis buffer directly to the enclosed filter and vortexing to remove DNA material off the filter. This has been shown to yield similar (or higher) DNA concentrations and species richness compared to filtering and processing with flat filters (Miya et al., 2016; Spens et al., 2017). Recently, work by Ushio (2019) expanded on this method by adding lysis buffer and zirconia beads directly to Sterivex filters, followed by vortex bead beating in the enclosed filter. Compared to only adding lysis buffer or cracking open the filter, enclosed bead beating resulted in higher bacteria richness and bulk DNA concentrations across a range of aquatic habitats (Ushio, 2019). Even so, Sterivex extractions remain untested on other important groups of marine organisms, including photosynthetic eukaryotes and bony fishes. The effects of bead size(s) on Sterivex extractions are also poorly understood, as is the application of magnetic bead-based kits that are compatible with automated instrumentation (Ruppert et al., 2019). Better understanding of these extraction methods, and their ability to be scaled up via automated extraction, will be important in future efforts to streamline biodiversity monitoring across temporal and spatial scales.

Here, we build upon work by Ushio (2019) and test the impact of enclosed bead beating treatments on a wide group of organisms (bacteria, photosynthetic eukaryotes, and fish) from coastal Florida waters (Bear Cut, Biscayne Bay). Using water samples collected on two separate days (in May and August 2020), we tested a range of bead sizes/combinations (0.1 mm, 0.5 mm, 0.1 + 0.5 mm, and no beads) and two common magnetic-based DNA extraction kits (Zymo Research and Takara Bio). We performed metabarcoding of 16S and 12S rRNA gene regions to assess how different Sterivex bead treatments influence the diversity, composition, and relative abundance (class to ASV level) of coastal microbial and fish assemblages. Finally, our study marks a novel use of 12S MiFish primers in coastal Florida, so we polled expert fish biologists and other scientists familiar with the region to assess whether observed sequences would be expected in Bear Cut. Our study provides critical information on extracting eDNA from Sterivex filters, with implications for accurately estimating species distributions and community biodiversity in new and diverse aquatic habitats.

2 | MATERIALS AND METHODS

2.1 | Sample collection and filtration

Surface water (1 m) was collected on two separate days (May 22 and August 27, 2020) from Bear Cut, Biscayne Bay, Florida (latitude, 25°43'53.6"N; longitude, 80°09'47.5"W), accessible by dock at the University of Miami's Rosenstiel School of Marine and Atmospheric Science. Located in the northern section of the bay, Bear Cut is a shallow (~5 m) and well-mixed subtropical lagoon, experiencing strong semidiurnal tides (0.5–0.7 m s^{-1}) entering from the Atlantic Ocean (Biber, 2007; Criales et al., 2000). Sampling occurred within ~1 h of high (May) or low (August) tides; tide predictions were obtained for Virginia Key, Biscayne Bay (NOAA Station ID: 8723214). Seawater (~20 L) was collected via Niskin bottles into pre-cleaned (2% bleach) carboys and transferred (within 30 min) to the nearby NOAA Atlantic Oceanographic and Meteorological Laboratory

(AOML) for filtration. Surface water temperature and salinity (practical salinity unit or psu) were measured on site using a YSI Pro30 probe (YSI Inc.).

Prior to filtration, lab surfaces and pump tubing were sterilized with 2% bleach to avoid contamination, and tubing was rinsed with molecular grade water (Milli-Q). On both days, seawater samples (~1 L) were filtered in triplicate from the same carboy through 0.22-μm Sterivex filters (Millipore; CAT# SVGP01050) via a peristaltic pump (at 100–150 rpm). Filter inlets and outlets were sealed with caps (outlet: STOCKCAP CAT# 300676; inlet: Cole-Parmer CAT# 30800–30), and filters were stored at −80°C until processing (within 3–4 months). Negative sample controls (Milli-Q) were also filtered in triplicate at the time of collection.

2.2 | DNA extractions

Our DNA extraction protocol included several variations using different bead compositions (or no beads) and two different magnetic bead kits, with different combinations of beads and kits tested for May and August samples (Table 1). For May samples, we tested enclosed Sterivex bead beating using the following four bead treatments: small beads (0.1 mm), large beads (0.5 mm), both beads (0.1 + 0.5 mm homemade), and Zymo beads (0.1 + 0.5 mm premade). We also included a treatment without beads (called "no beads"), where only lysis buffer was added to the Sterivex filters (Table 1). Homemade bead mixtures (2 g total per sample) were prepared with 0.1-mm silica (MP Biomedicals; CAT# SKU 116540428) and 0.5-mm yttria-stabilized zirconium beads (MP Biomedicals; CAT# SKU 116540436). Premade Zymo beads (manufactured in "BashingBead" lysis tubes) contained a 2-g mixture of 0.1 + 0.5 mm beads. May samples were all extracted using the ZymoBIOMICS 96 DNA/RNA MagBead kit (Zymo; CAT# D4308). For August samples, we again tested premade Zymo beads vs. no beads, as well as an additional no beads treatment, extracting a set of filters with the NucleoMag DNA/RNA Water kit (Takara; CAT# 744220.1; Table 1). All treatments included three replicate Sterivex filters taken from the same source water (May or August). Milli-Q blanks from May were extracted with Zymo beads and the ZymoBIOMICS Kit, while August blanks were extracted via the NucleoMag kit. A positive bacteria control (via Zymo) was also sequenced.

To prepare for extraction, filters were thawed on ice, inlet caps were removed, and excess water was dried (via Kimwipes) from filter inlets to improve the flow of beads into the Sterivex. Outlet caps were sealed with parafilm to prevent leakage during bead beating (using a Vortex-Genie 2). As in Ushio (2019), beads were added directly to Sterivex filters, which in our case varied depending on bead treatment; however, unlike Ushio (2019), we added beads after water filtration. Lysis buffer was added to the filters for Zymo (1000 μl) and Takara (900 μl) samples and subsequent extraction steps followed manufacturer instructions for each kit. For Zymo samples, Sterivex filters were vortexed for 40 min at maximum speed (~3200 rpm). DNA lysates were transferred to 2-ml LoBind tubes (Eppendorf) via syringe and centrifuged for 1 min at 10,000 *g*. Supernatant (~600 μl per sample) was transferred to a KingFisher 96-well plate and split across three wells (200 μl per well). Zymo MagBinding buffer (600 μl) and beads (25 μl) were added to each well in the sample plate and pipette mixed. Additionally, each run included three wash plates with 500–900 μl per well of MagWash and an elution plate with 50 μl per well of molecular grade water.

Samples (three filters) from August were processed with the NucleoMag kit and followed a slightly different extraction protocol, including a separate KingFisher run. NucleoMag filters were vortexed at maximum speed for 5 min, incubated in a water bath (70°C) for 5 min, and vortexed again for 5 min. DNA lysates were removed via syringe, centrifuged (1 min at 10,000 *g*), and transferred to a KingFisher plate across two wells (450 μl per well). NucleoMag buffer (475 μl) and beads (25 μl) were added to each well and pipette mixed; three wash plates (850 μl per well) and an elution plate (100 μl per well) were included. Both protocols were run on an automated KingFisher Flex system (Thermo Fisher) using custom-made scripts provided by the manufacturer (available at https://github.com/aomlomics/sterivex). Eluted DNA was combined for each unique sample (~150–200 μl). DNA concentrations were measured using a Qubit ds DNA HS kit (Thermo Scientific).

2.3 | PCR and library preparation

Two separate PCRs were run with triplicate reactions (25 μl) per sample, targeting either the 12S (fish mitochondria) or 16S (bacteria, archaea, and photosynthetic eukaryotes) rRNA gene. Both PCRs were carried out using 1 μl of DNA, while volumes of other reagents varied slightly between gene regions. For each 12S PCR reaction, we used 12.5 μl of AmpliTaq Gold (Thermo Fisher; 10 μl for 16S), 9.5 μl of molecular water (12.5 μl for 16S), and 1 μl (10 μM)

TABLE 1 Bead treatments used for Sterivex eDNA extraction testing for each sampling day. Combinations were based on bead size (mm), type (homemade vs. premade), and extraction kit used

Treatment	Day	Bead(s)	Bead type	Kit
Small beads	May	0.1 mm	Homemade	Zymo
Large beads	May	0.5 mm	Homemade	Zymo
Both beads	May	0.1 + 0.5 mm	Homemade	Zymo
Zymo beads	May/Aug	0.1 + 0.5 mm	Premade	Zymo
No beads	May/Aug	NA	NA	Zymo
NucleoMag	Aug	NA	NA	NucleoMag

of each primer (0.75 μl for 16S). We used V4-V5 primers (515F and 926R) to target 16S amplicons (Parada et al., 2016) and MiFish-U primers to target 12S amplicons (Miya et al., 2015; Table 2). A slight modification was applied to the second base pair (T to C) in the forward 12S primer (Sales et al., 2021; Table 2). Primers were constructed with Fluidigm common oligos CS1 forward (CS1-TS-F: 5′-ACACTGACGACATGGTTCTACA-3′) and CS2 reverse (CS2-TS-R: 5′-TACGGTAGCAGAGACTTGGTCT-3′) fused to their 5′ ends, to enable two-step library preparation at the Michigan State University Research Technology Support Facility.

We followed the CALeDNA Touchdown PCR method for 12S rRNA (Pitz et al., 2020), with an initial denaturation step at 95°C for 15 min, 13 cycles of 94°C for 30 s, 69.5°C for 30 s, and 72°C for 90 s (annealing temperature reduced by 1.5°C each cycle), followed by 25 cycles of 94°C for 30 s, 50°C for 30 s, and 74°C for 45 s, and a final extension of 72°C for 10 min. 16S PCR conditions consisted of an initial denaturation at 95°C for 2 min, 25 cycles of 95°C for 45 s, 50°C for 45 s, and 68°C for 90 s, followed by a final elongation step of 68°C for 5 min (Parada et al., 2016). Negative template controls were included for each PCR run. PCR products were pooled (75 μl), run through a 2% agarose gel in Tris-borate-EDTA (TBE) buffer to confirm amplification, and size selected with AMPure XP Beads (Beckman Coulter) at a ratio (beads to sample) of 1.2× and 0.8× for 12S and 16S, respectively. 12S gels distinguished two separate bands, one at ~300 bp (fish) and a faint band at ~400 bp, indicating possible bacteria amplification (Figure S1; Gold et al., 2021). Two sample plates (16S and 12S) were submitted to the Michigan State University Research Technology Support Facility Genomics Core for secondary PCR and sequencing. Secondary PCR used dual-indexed, Illumina-compatible primers, targeting the Fluidigm CS1/CS2 oligomers at the ends of the PCR products. PCR conditions for the secondary run included an initial denaturation step at 95°C for 3 min, 11 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 60 s, followed by elongation at 72°C for 3 min. Two sequencing runs were performed on an Illumina MiSeq (2 × 250 bp).

2.4 | Bioinformatics processing

Amplicon sequence data were analyzed separately (16S and 12S) using either stand-alone QIIME 2 (Bolyen et al., 2019) or Tourmaline (https://github.com/aomlomics/tourmaline), a Snakemake pipeline that wraps QIIME 2, providing reproducible metabarcoding analysis. Primers were removed from demultiplexed FASTQ reads using Cutadapt (Martin, 2011). Sequence quality (per base) cutoffs (0.7 for 16S and 0.75 for 12S) were assessed with MultiQC and FastQC (Ewels et al., 2016). Paired-end DADA2 (Callahan et al., 2016) was used to infer amplicon sequence variants (ASVs). 16S taxonomy was assigned using a Naïve Bayes classifier (Bokulich et al., 2018) trained against the SILVA database (Version 138.1; Pruesse et al., 2007). Chloroplast reads (417 ASVs) were filtered out from the 16S set and mapped against 16S plastid-specific Protist Ribosomal Reference (PR2) files (Version 4.12; Guillou et al., 2013) that had been formatted from the PhytoRef database (Decelle et al., 2015), allowing for taxonomic assignment of photosynthetic eukaryotes. 12S taxonomy was assigned using consensus-vsearch (Bokulich et al., 2018) and QIIME 2 compatible reference files from Mitohelper (March 2021 release; Lim & Thompson, 2021), a metabarcoding tool that provides collated and up-to-date 12S, 16S, and 18S rRNA sequence datasets from SILVA, MitoFish (Iwasaki et al., 2013), NCBI (Sayers et al., 2019), and *Fishes of the World* (Nelson et al., 2016).

2.5 | Statistical analyses

QIIME 2 taxonomy, count, and phylogenetic tree files (.qza files) were imported into R (Version 3.6.3; https://cran.r-project.org) using the read_qza function from the qiime2R package (https://github.com/jbisanz/qiime2R) for downstream analysis. We processed amplicon sequences (12S, 16S, and 16S chloroplasts) separately in R using a variety of packages, including phyloseq (McMurdie & Holmes, 2013), vegan (Oksanen et al., 2018), and tidyverse (Wickham, 2011). Rarefaction curves were generated using the ggrare function in the R package ranacapa (https://github.com/gauravsk/ranacapa). Samples were rarefied to the minimum read count in each respective dataset. Richness (number of ASVs) and Shannon diversity were estimated with the estimate_richness function in phyloseq. DNA concentration and diversity data (for all gene regions) were normally distributed, as verified by Shapiro-Wilk's normality tests. Significant differences in richness, diversity, and initial DNA concentrations were assessed between bead treatments using one-way Analysis of Variance (ANOVA) with Tukey post hoc tests (p-value <0.05).

For the remaining analyses, read counts were transformed to relative abundance and singletons were removed. Community composition was observed via principal coordinates analysis (PCoA)

TABLE 2 Information on forward (Fwd) and reverse (Rev) primers used to target different groups of organisms in this study. Microbes included bacteria, archaea, and some photosynthetic eukaryotes (via chloroplasts)

Group	Region	Primer name	Fwd /Rev	Primer sequence 5′–3′	Length (bp)	Ref.
Microbes	16S rRNA V4–V5	515F	Fwd	GTGYCAGCMGCCGCGGTAA	412	1
		926R	Rev	CCGYCAATTYMTTTRAGTTT		
Bony fishes	12S rRNA	MiFish-U-F	Fwd	GCCGGTAAAACTCGTGCCAGC	163–185	2
		MiFish-U-R	Rev	CATAGTGGGGTATCTAATCCCAGTTTG		3

References: (1) Parada et al. (2016). (2) Miya et al., (2015). (3) Sales et al. (2021).

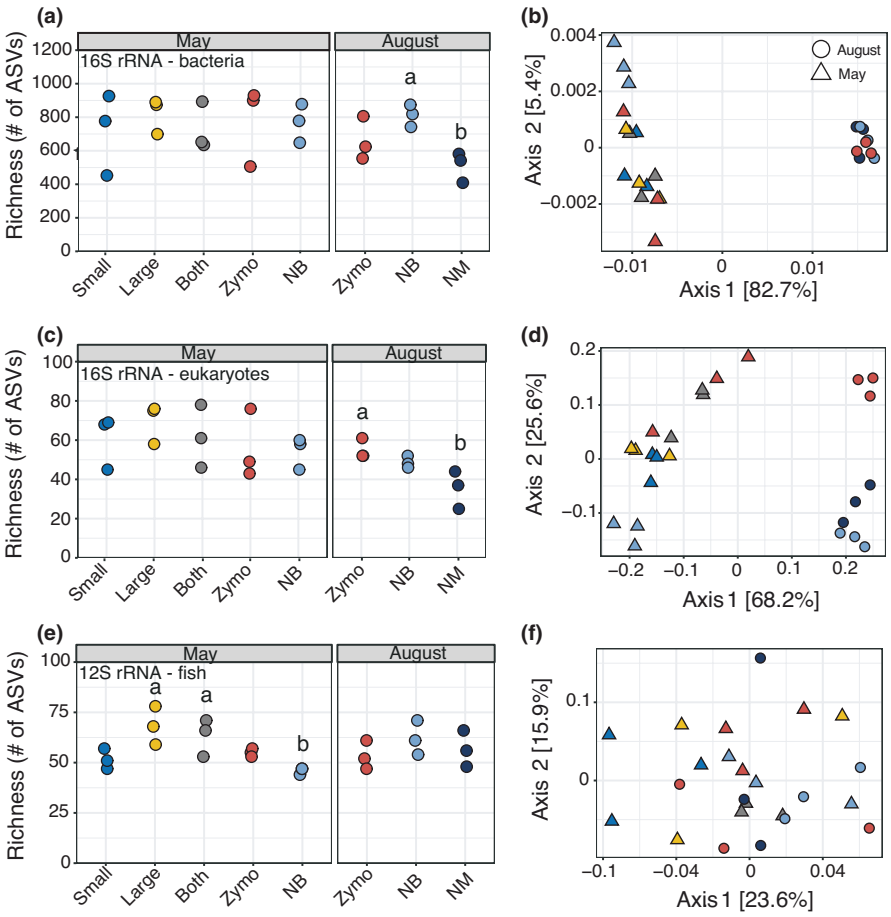


FIGURE 1 Species richness (# of ASVs) and principal coordinates analysis (PCoA) estimated across bead treatments for bacteria (a, b), eukaryotes (c, d), and fishes (e, f). Sampling days are faceted by month (May or August) in richness plots, while days are distinguished by shape (May = triangles; August = circles) in the PCoA plots. Samples are colored based on bead treatment: blue = small beads (Small); yellow = large beads (Large); gray = both beads (Both); red = Zymo premade beads (Zymo); light blue = no beads (NB); dark blue = NucleoMag (NM). Letters indicate significant differences ($p < 0.05$) in mean richness between treatments

using weighted UniFrac distances (ordinate function in phyloseq). Permutational multivariate analysis of variance (PERMANOVA) tests were employed to distinguish significant differences (p-value <0.05) in composition based on bead treatment or sampling day. Relative abundance taxonomy bar plots were observed at the family (12S and 16S) or class level (16S chloroplasts) over the bead treatments for taxonomic groups having relative abundance >5% in at least one sample. Dot plots were used to observe patterns (both presence and magnitude) of the top 20 most relatively abundant ASVs across bead treatments for each marker gene. The ps_venn function in the MicEco package (https://github.com/Russel88/MicEco) was used to estimate the percent of all ASVs from a given dataset that were shared between bead treatments. Percent shared was estimated based on presence/absence or by weighting ASVs by relative abundance.

2.6 | Poll of local fish experts

To verify fish observed in our dataset, we polled 20 fish biologists and other scientists from the University of Miami, Nova Southeastern University, NOAA Southeast Fisheries Science Center, and the NOAA coral listserv, with knowledge of Bear Cut fishes. Participants were asked if they would expect to find our top 40 most abundant fish ASVs (across all samples), with the following possible responses: "yes," "no," "unsure," "yes, though not directly observed." Several fishes were re-classified after the initial poll, and so, there presence/absence status was confirmed via primary literature or estimated from FishBase (Froese & Pauly, 2021). Relative abundance boxplots and poll results (pie charts) were constructed for the top 20 fish taxa. Poll respondents were asked to provide possible sources of introduction for fishes that were considered to be non-native to the region.

3 | RESULTS

3.1 | Sampling conditions and DNA concentrations

We collected surface water from Bear Cut (Biscayne Bay, FL) on two days, encompassing both high (May) and low tide conditions (August). On each day, water depth varied by ~0.6 m between semi-diurnal tides. Water temperature was similar between days (28 and 30°C), although salinity was expectedly higher in May (38 psu at high tide) compared to August (32 psu at low tide) samples. DNA concentrations ranged from 2.4 to 9.95 ng μl^{-1} and were not significantly different (ANOVA, p-values >0.05) between bead treatments on either day (Figure S2).

3.2 | 16S rRNA – bacteria

On average, 16S rRNA sequencing yielded 139,457 sequence reads per sample (range 77,964–185,031 reads) over the two days that were resolved into 10,494 bacterial ASVs (Figure S3). There was no significant difference in 16S richness (number of ASVs) between treatments in May, while in August, richness was significantly higher in the no beads vs. NucleoMag treatment (ANOVA, p-value = 0.02; Figure 1a). Shannon diversity was not significantly different across bead treatments in May but was higher for no beads vs. Zymo or NucleoMag (ANOVA, p-values <0.01) in August (Figure S4). Bacteria communities were significantly separated by bead treatment in May (PERMANOVA $R^2 = 0.38$, p-value = 0.03), not significantly clustered in August (p-value =0.08; Figure 1b), and more strongly separated (PERMANOVA $R^2 = 0.89$, p-value = 0.001) between sampling days (Figure 1b).

Bacteria were highly conserved across bead treatments at multiple taxonomic levels (Figure 2a, b). At the family level, relative abundance (on average) was dominated by common coastal microbes like Cyanobiaceae (22%), Rhodobacteraceae (12%), Flavobacteriaceae (12%), and SAR11 Clade (10%), with little variation between treatments (Figure 2a). Certain groups like SAR11 Clade and Rubritaleaceae were less important (<5%) to community relative abundance in August (Figure 2a). There was remarkable similarity (presence and magnitude) among bead treatments for the top 20 most relatively abundant 16S ASVs observed in May (Figure 2b) and August (Figure S5). Over the entire dataset, >50% of 16S ASVs were detected in all bead treatments on a given day, reaching >98% when weighted by relative abundance (Figure 3b). A higher number of 16S ASVs were shared (>70%) when considering only Zymo vs. no beads (Figure 3a).

3.3 | 16S rRNA – chloroplasts

Photosynthetic eukaryotes identified via 16S chloroplast reads (average 2,963; 603–6,595 reads) were assigned to 417 ASVs over both days (Figure S3). As with bacteria, there was no difference in eukaryotic richness between treatments in May; however, richness was significantly higher in the Zymo beads vs. NucleoMag treatment in August (ANOVA, p-value = 0.02; Figure 1c). Shannon diversity values were not significantly different (p-values > 0.05) between bead treatments on either day (Figure S4). Photosynthetic eukaryote communities clustered by day (PERMANOVA $R^2 = 0.5$, p-value = 0.001), but were also significantly separated by bead treatment in both May and August (PERMANOVA $R^2 = 0.57$ and 0.63, p-values <0.01), with noticeable clustering of treatments without vs. with beads (Figure 1d).

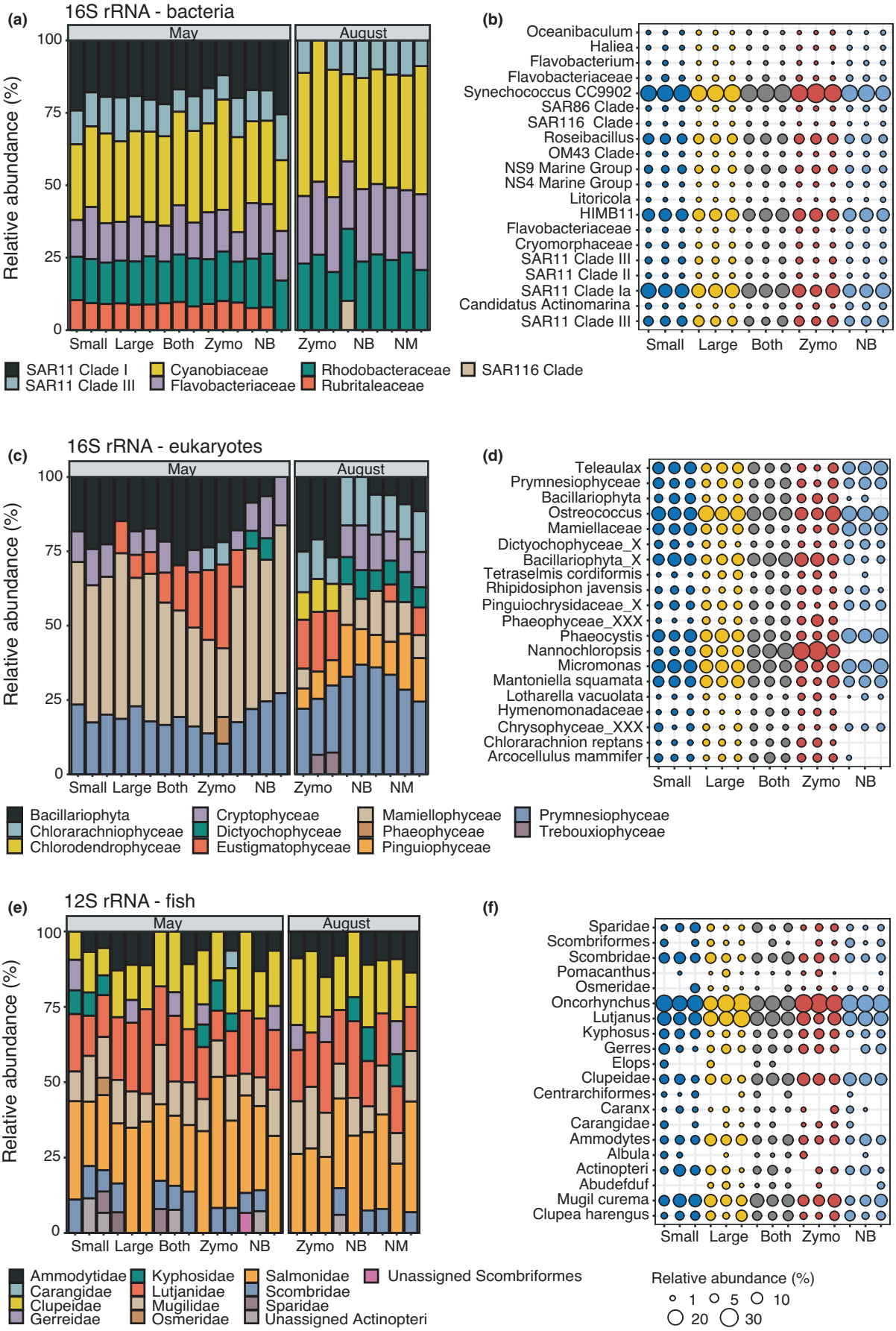
At the class level, eukaryotes were largely assigned (on average) to Mamiellophyceae(25%),Prymnesiophyceae(19%),andBacillariophyta (13%). Interestingly, Bacillariophyta relative abundance was reduced in treatments without beads (Zymo no beads and NucleoMag) compared to treatments with beads (Figure 2c). Relative abundance of other groups, like Eustigmatophyceae, Chlorodendrophyceae, and Trebouxiophyceae, were also reduced in May/August treatments without beads (Figure 2c). Similar trends were observed among the most abundant ASVs in May (Figure 2d) and August (Figure S5), with ASVs from Bacillariophyta (e.g., *Arcocellulus mammifer*) and Chlorodendrophyceae (e.g., *Tetraselmis cordiformis*) being less abundant or entirely absent in treatments without beads. Overall, eukaryotic ASVs were poorly conserved across all bead treatments and between Zymo vs. no beads (20%–49% shared), increasing to 55%–68% shared when weighted by abundance (Figure 3b).

3.4 | 12S rRNA – fish

Overall, 1,134 ASVs were inferred via 12S metabarcoding, of which 679 ASVs were assigned to fish (60%). On average, 79,091 sequence reads across all samples (range 40,838–116,101 reads) were mapped to fish ASVs (Figure S3). In May, 12S richness was significantly higher when extracted with large or both beads compared to no beads (ANOVA, p-values ≤0.05), while in August, there was no significant difference between treatments (Figure 1e). Shannon diversity values for 12S were not significantly different (p-values >0.05) on average across bead treatments for either day (Figure S4). Fish communities were not significantly clustered (p-values >0.05) by bead treatment and only weakly separated (PERMANOVA $R^2 = 0.06$, p-value = 0.05) by day (Figure 1f).

Thirty-nine fish families were observed over both days, with relative abundance dominated (on average) by Salmonidae (24%), Lutjanidae (15%), Clupeidae (14%), and Mugilidae (11%). In general, the same major 12S families were present in all samples; however, group-specific differences in relative abundance were observed between bead treatments and replicates (Figure 2e). For instance, less abundant families, like Gerreidae, Kyphosidae, and Sparidae contributed to >5% of total relative abundance in some treatments (and replicates) but not others, with no clear bead-specific trends (Figure 2e). While the most relatively abundant ASVs (e.g., *Oncorhynchus*, *Lutjanus*, and *Mugil curema*) were detected across bead treatments, other top ASVs varied in magnitude or were entirely absent depending on bead treatment or replicate (Figure 2f;

FIGURE 2 Taxonomic variability across bead treatments, as shown by stacked relative abundance bar plots (family or class level) or dot plots (ASV level) for bacteria (a, b), eukaryotes (c, d), and fishes (e, f). Taxonomy bar plots display taxa which have relative abundance >5% in a sample and are faceted by sampling day. Dot plots display the top 20 most relatively abundant ASVs across all May samples (see Figure S5 for August) and are sized based on relative abundance (scale shown). ASVs are labeled by lowest possible taxonomic identification. Dots are colored by bead treatment: blue = small beads (Small); yellow = large beads (Large); gray = both beads (Both); red = Zymo premade beads (Zymo); light blue = no beads (NB). Replicates are shown


(a)
16S rRNA - bacteria
May
August
Relative abundance (%)
100
75
50
25
0
Small Large Both Zymo NB Zymo NB NM
SAR11 Clade I
SAR11 Clade III
Cyanobiaceae
Flavobacteriaceae
Rhodobacteraceae
Rubritaleaceae
SAR116 Clade
(b)
Oceanibaculum
Haliea
Flavobacterium
Flavobacteriaceae
Synechococcus CC9902
SAR86 Clade
SAR116 Clade
Roseibacillus
OM43 Clade
NS9 Marine Group
NS4 Marine Group
Litoricola
HIMB11
Flavobacteriaceae
Cryomorphaceae
SAR11 Clade III
SAR11 Clade II
SAR11 Clade Ia
Candidatus Actinomarina
SAR11 Clade III
Small Large Both Zymo NB
(c)
16S rRNA - eukaryotes
May
August
Relative abundance (%)
100
75
50
25
0
Small Large Both Zymo NB Zymo NB NM
Bacillariophyta
Chlorarachniophyceae
Chlorodendrophyceae
Cryptophyceae
Dictyochophyceae
Eustigmatophyceae
Mamiellophyceae
Phaeophyceae
Pinguiophyceae
Prymnesiophyceae
Trebouxiophyceae
(d)
Teleaulax
Prymnesiophyceae
Bacillariophyta
Ostreococcus
Mamiellaceae
Dictyochophyceae_X
Bacillariophyta_X
Tetraselmis cordiformis
Rhipidosiphon javensis
Pinguiochrysidaceae_X
Phaeophyceae_XXX
Phaeocystis
Nannochloropsis
Micromonas
Mantoniella squamata
Lotharella vacuolata
Hymenomonadaceae
Chrysophyceae_XXX
Chlorarachnion reptans
Arcocellulus mammifer
Small Large Both Zymo NB
(e)
12S rRNA - fish
May
August
Relative abundance (%)
100
75
50
25
0
Small Large Both Zymo NB Zymo NB NM
Ammodytidae
Carangidae
Clupeidae
Gerreidae
Kyphosidae
Lutjanidae
Mugilidae
Osmeridae
Salmonidae
Scombridae
Sparidae
Unassigned Actinopteri
Unassigned Scombriformes
(f)
Sparidae
Scombriformes
Scombridae
Pomacanthus
Osmeridae
Oncorhynchus
Lutjanus
Kyphosus
Gerres
Elops
Clupeidae
Centrarchiformes
Caranx
Carangidae
Ammodytes
Albula
Actinopteri
Abudefduf
Mugil curema
Clupea harengus
Small Large Both Zymo NB
Relative abundance (%)
1 5 10
20 30

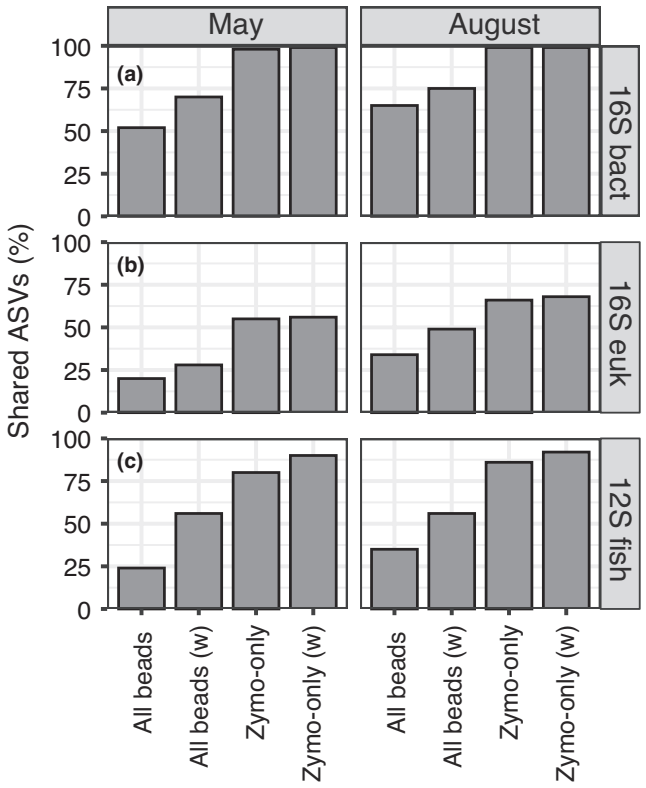


FIGURE 3 Shared ASVs (%) between bead treatments for bacteria (a), eukaryotes (b), and fishes (c). Plots are faceted by sampling day. Shared ASVs were compared between all bead treatments in May ($n = 5$) or August ($n = 3$), as well as between Zymo-only treatments tested on both days (i.e., Zymo vs. no beads). Weighted comparisons (w) account for ASV relative abundance

Figure S5). 12S ASVs were poorly shared between all bead treatments in May (24%) and August (35%), although more ASVs were shared (56%) between Zymo-only treatments and/or with weighted abundance (80%–92%; Figure 3c).

Bacteria ASVs (455) inferred via 12S rRNA metabarcoding were distinct from 16S rRNA communities of the same water samples. At the family level, relative abundance of 12S bacteria were composed mainly of AEGEAN-169 Marine Group and Halieaceae across all samples (Figure S6), both of which were rare (<5% relative abundance) in the 16S rRNA dataset.

3.5 | Bear Cut fishes – poll results

Nearly 69% of the time, a positive fish observation in Bear Cut was directly acknowledged by poll participants ($n = 10$) or through additional resources (Figure 4; Table S1). Many of the top 20 most relatively abundant fish ASVs were observed by all participants (Figure 4), including several genus or species level assignments like *Lutjanus* (snappers), *Mugil curema* (white mullet), *Gerres* (mojarras), *Caranx* (jacks), *Thunnus* (tunas), and *Albula* (bonefishes). *Oncorhynchus* (Pacific salmonids/trouts) was not observed by any participants, while other groups like *Clupea harengus* (Atlantic herring), *Ammodytes* (sand lances), and members of the family Osmeridae (smelts and capelins) garnered mixed consensus from participants (Figure 4). According to poll participants, the most likely cause of non-native taxa in our dataset (e.g., salmon or herring) was input from a nearby aquarium or introduction via fish bait (Table S1).

4 | DISCUSSION

4.1 | Effects of enclosed Sterivex extraction on microbes and fish

In contrast to prior Sterivex extraction testing (Ushio, 2019), total DNA yield and microbial (bacteria and eukaryotes) richness measured in our study was not significantly influenced by the addition of beads. Instead, richness (and bacterial diversity) was enhanced in the Zymo no beads vs. NucleoMag treatments, indicating that extraction kit rather than bead size impacted microbial communities. Previous extraction testing with polycarbonate or glass fiber flat filters has also observed differences in aquatic bacterial richness when using varying commercial kits (Deiner et al., 2015, 2018; Djurhuus et al., 2017), often favoring manual extraction with Qiagen kits, like DNeasy and PowerWater (Kumar et al., 2020; Tsuji et al., 2019). Magnetic bead-based (and automation friendly) kits have been largely overlooked in the context of extraction testing, even if such protocols promote reproducible and scaled-up eDNA workflows that may minimize user contamination. In our case, variability in microbial richness between kits may have been attributed to differences in the protocols, as NucleoMag involved a shorter vortexing step (10 min) relative to Zymo (40 min) and included an additional heating step. Moreover, though using similar reagent chemistry, once on the KingFisher, protocols slightly deviated in terms of reagent volume added to the magnetic bead, wash, and elution plates, which may have affected downstream community dynamics.

Regardless of bead size or extraction kit, relative abundance of bacteria was conserved at the family to ASV level. This was not true for photosynthetic eukaryotes, as several major groups, including Bacillariophyta (diatoms) and Chlorodendrophyceae (chlorophytes), were underestimated in terms of relative abundance and often entirely absent at the ASV level when extracted in Sterivex without beads. Bacteria, dominated in our study by ubiquitous heterotrophs (SAR11 and Flavobacteriaceae) and autotrophs (Cyanobiaceae), are often easier to lyse compared to phytoplankton (Djurhuus et al., 2017; Santos et al., 2017; Yuan et al., 2015). This is especially true for diatoms and chlorophytes that have rigid silica frustules or cellulose thecate exteriors (Becker et al., 1998; Hamm et al., 2003). Such extraction bias among eukaryotes likely contributed to strong separation in community composition based on bead content, while bacteria communities were only weakly clustered by beads. Therefore, while it may be unnecessary for most bacteria, the addition of beads (representing at least one size) to Sterivex filters is needed to effectively extract and release DNA from hard-to-lyse eukaryotes and

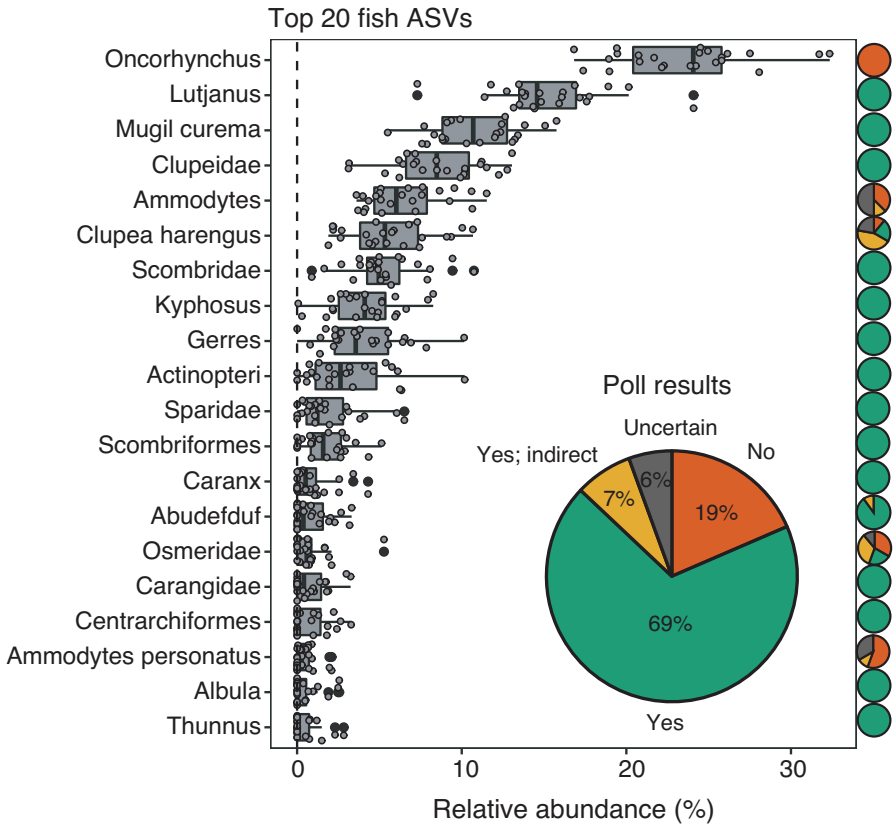


FIGURE 4 Box plots displaying mean relative abundance (individual points show) for the top 20 most abundant 12S ASVs over all treatments and days ($n = 24$ samples). ASVs are labeled by lowest possible taxonomic identification. Dotted line indicates relative abundance of zero. Overall poll results from Bear Cut fish experts (40 fish; $n = 10$ participants) are shown in the large pie chart, indicating a successful fish observation (Yes; green), no observation (No; red), uncertain observation (gray), and an indirect (expected) observation (yellow). Poll results corresponding to each individual ASV are shown to the right as smaller pie charts

should be considered in future Sterivex extractions that target phytoplankton communities.

Bead addition did affect species richness of bony fishes, with significantly higher richness in treatments with larger (or both) beads compared to no beads. Unlike eukaryotes, where ASVs were poorly detected without beads, fish ASV abundances were variable (even between replicates) and were not associated with any single treatment. Fish community composition was also less structured compared to microbes, failing to cluster by sampling day. Such variability may be driven by the heterogeneous nature or low concentration of fish eDNA in the marine environment (Li et al., 2018; Port et al., 2016), especially when compared to whole microbial cells. Fish eDNA originates from complex sources (e.g., mucus, tissue, and scales), is released at varying rates (~10^2–10^3 Pg DNA g^{-1} h^{-1}) depending on species (or size), and is readily transported to new areas by active swimming (Barnes & Turner, 2016; Sassoubre et al., 2016). Additionally, the fate and decay (hours to days) of eDNA in general is influenced by physical, environmental, and biological processes (Andruszkiewicz Allan et al., 2021; Andruszkiewicz et al., 2017; Barnes et al., 2014; Seymour et al., 2018), all determining eDNA detection rates in a water sample.

Fish eDNA sampling may also be more patchy inshore, with DNA degrading faster under warmer and/or more saline conditions (Andruszkiewicz Allan et al., 2021; Collins et al., 2018) and being more freely distributed by tides or runoff. Ultimately, it may be important to adjust sampling strategies to accurately monitor fish and other vertebrate DNA in dynamic coastal habitats (Bessey et al., 2020; Miya et al., 2020). For instance, recent studies have suggested that increasing filtration volumes to >2 L per filter may be necessary to capture fish biodiversity (Bessey et al., 2020; Kumar et al., 2021), although such work has not been systematically tested for Sterivex filters and requires optimization based on sampling environment to avoid filter clogging or PCR inhibition (Kumar et al., 2021). In addition to increased filtration volumes, fish biodiversity monitoring may be improved with higher spatial resolution or by increasing the number of technical replicates (e.g., 4–6 filters).

The use of MiFish primers for 12S rRNA metabarcoding has steadily increased, incorporated globally in eDNA monitoring programs to characterize marine and freshwater fish communities (reviewed in Miya et al., 2020). As with any eDNA protocol, species detection errors can occur during sampling, laboratory manipulation, or bioinformatics analysis (Doi et al., 2019; Ruppert et al., 2019). Particularly, PCR amplification of non-target organisms (Collins et al., 2019) can confound sequencing results and waste resources. In our study, we observed non-target amplification of bacteria (~400 bp), accounting for 40% of total ASVs inferred with MiFish primers. However, bacteria ASVs were only responsible for 14%–35% of total sequencing reads, in line with weaker gels bands compared to fish amplicons. Furthermore, non-target bacterial taxa largely belonged to groups (Halieaceae and AEGEAN Marine Group) that were rare among 16S rRNA sequences from the same samples. Gold et al. (2021) similarly observed non-target amplification of bacteria with MiFish primers, although as in our case, proportion of 16S reads were low (<25%) and mapped to uncultured taxa. Issues with bacterial amplification may be exacerbated in productive coastal marine areas where microbial biomass is high (Gold et al., 2021; Miya et al., 2020). Although

qualitative, we performed a cleanup step (1.2× AMPure beads) that resulted in a weaker bacteria band compared to pre-cleaned amplicons, suggesting the importance of post-PCR cleanup with this primer set. Nevertheless, careful attention should be given to off-target amplification with 12S primers (Gold et al., 2021) and researchers should consider classifying sequences against databases that include both 16S and 12S barcodes (e.g., Mitohelper). Future eDNA metabarcoding studies that overlook 16S reads, classifying them as unassigned fish, may overestimate true fish species richness and diversity.

4.2 | Verifying fish eDNA taxonomy

Accurate interpretation of eDNA metabarcoding results can be challenging, particularly when resident species are absent (false negatives) or non-native taxa are detected (false positives) in the dataset (Goldberg et al., 2016). Even so, eDNA sampling has become an important technique to monitor invasive (or non-native) aquatic organisms (Robson et al., 2016; Ruppert et al., 2019). Although efforts to characterize marine life via eDNA metabarcoding have recently been employed off coastal Florida (Ames et al., 2021; Kumar et al., 2021; Sawaya et al., 2019), 12S MiFish primers have not yet been used to identify fish in this region. To establish a baseline understanding of fish observed in our eDNA dataset, including several taxa that stood out as non-native (salmonids and herrings), we polled 20 fisheries biologists and other scientists affiliated with local universities or NOAA labs. Poll results provided insight into the expected presence or absence of fishes in Biscayne Bay, offering important confirmation of 12S eDNA data.

Many of the top fish ASVs that were positively identified by poll respondents, including coastal and tropical fishes like *Lutjanus* (snappers), *Caranx* (jacks), *Gerres* (mojarras), *Abudefduf* (sergeant majors), *Scarus* (parrotfishes), and *Pomacanthus* (angelfishes), have been observed previously in Biscayne Bay via net tows or visual surveys (Berkeley & Cantillo, 2004; Roessler, 1965; Serafy et al., 2003). Fishes that were not expected to be present (or had mixed consensus), such as *Oncorhynchus* (Pacific salmonid/trout), *Clupea harengus* (Atlantic herring), and members of the family Osmeridae (smelts and capelins), have not been previously reported in Bear Cut or surrounding waters. Although not present in our study, other juvenile bait fishes, including *Opisthonema oglinum* (Atlantic thread herring) and *Harengula jaguana* (scaled sardines), have been observed in the bay (Berkeley & Cantillo, 2004; Houde & Lovdal, 1984). Based on poll responses, input from the nearby aquarium (Miami Seaquarium; ~200 m from the dock) was the most likely source of non-native fishes into the bay. The aquarium confirmed that salmon (*Oncorhynchus* spp.), herrings, and capelins were fed to marine mammals, with remaining fish material being dumped into the bay each night. Given the proximity and strong tidal movement, this offers a compelling explanation for the presence of non-native fish eDNA in our dock samples. In polling local fish experts, we present a simple and alternative means to enhance fish eDNA data, which may be useful when sampling in new areas and when other comparative approaches, like fish trawls or scuba surveys, are not feasible.

5 | CONCLUSIONS

In this study, we evaluated the impact of enclosed Sterivex bead beating (Ushio, 2019), bead sizes, and magnetic kits on diversity estimates of bacteria (16S rRNA), photosynthetic eukaryotes (16S rRNA of chloroplasts), and bony fishes (12S rRNA) from Bear Cut eDNA samples. Bead content did not affect downstream estimates of bacterial richness or diversity, which were instead influenced by the extraction kit used. The Zymo kit resulted in higher bacterial richness and diversity compared to NucleoMag, likely reflecting a longer agitation period with the Zymo protocol (40 vs. 10 min) that more efficiently released microbial DNA off the filter. While not necessary for bacteria, our results demonstrate that vortex bead-beating is essential to properly lyse and extract eDNA from certain phytoplankton groups (diatoms and chlorophytes) and that larger beads (or bead combinations) may improve estimates of fish species richness. Taken together, larger (0.5 mm) or differently sized (0.1 + 0.5 mm) beads, in combination with appropriate agitation (~40 min), are recommended to capture a wide range of marine biodiversity from Sterivex filters, especially if the goal is to barcode across multiple domains of life within a single sample (Stat et al., 2017). Premade bead tubes, as supplied in Zymo MagBead kits, may favor more reproducible and sterile extractions compared to homemade bead preparation. Finally, in line with other studies (Civade et al., 2016; Li et al., 2018), we observed variability in fish eDNA detection between Sterivex treatments and replicates, supporting evidence that fish eDNA signals are heterogeneous (and/or dilute) in seawater. Increasing water filtration volume through the Sterivex (>2 L) or adding technical replicates may improve detection of fish eDNA (Bessey et al., 2020; Kumar et al., 2021; Li et al., 2018). This study establishes baseline effects of enclosed and automated Sterivex extractions across multiple trophic levels, an important step toward accurate, reproducible, and scalable biomonitoring and ecosystem-based management. Further testing is needed to determine best practices in eDNA extraction across different sampling regimes (Deiner et al., 2018) and groups of organisms.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

SRA and LRT designed and performed the research, analyzed the data, and wrote the manuscript.

DATA AVAILABILITY STATEMENT

12S and 16S rRNA sequences were deposited at NCBI SRA under BioProject ID PRJNA728349. Files associated with this project are publicly available on GitHub at https://github.com/aomlomics/sterivex, including R code (via Jupyter notebook), QIIME 2 and Tourmaline output files, sample metadata, and KingFisher extraction scripts. This project has been archived on Zenodo (https://doi.org/10.5281/zenodo.5071380).

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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