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SPECIAL ISSUE ARTICLE

# One size does not fit all: Tuning eDNA protocols for high- and low-turbidity water sampling

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## Abstract

Findings from eDNA metabarcoding are strongly influenced by experimental approach, yet the effect of pre-PCR sample processing on taxon detection and estimates of biodiversity across different water types is still poorly resolved. To fill this data gap, we investigated the impact of sampling effort, extraction method, and filter pore size on DNA yield, PCR inhibition, and 16S rDNA metabarcoding results for fishes in water samples collected from inshore turbid- and offshore clear-water environments. The turbid-water samples had high concentrations of suspended organic and/or inorganic material and yielded ~3.2× more DNA and exhibited high levels of PCR inhibition compared with the low-turbidity, clear-water samples. Importantly, there were no striking differences in the results of our metabarcoding experiments based on extraction method or filter pore size. While a small number of unique species of relatively low read count were detected in all turbid-water treatments, most species were consistently detected across samples. Results for the clear-water samples were strikingly different, with low DNA yield, high levels of variation across replicates, and a high number of non-overlapping species across treatments. These findings indicate a patchy distribution of eDNA in offshore environments, which means higher volumes of water (≥ 2 L per replicate) must be filtered in habitats where target DNA is likely to be sparse. In semi-closed systems such as estuaries, higher concentrations of target DNA are expected, and we found that either a 1.0 or 3.0 µm filter pore size was sufficient to capture standing diversity, while decreasing the risk of clogging. For economical DNA extraction and inhibitor removal, we recommend a combination of Omega Bio-tek E.Z.N.A Tissue DNA kit followed by a PCR inhibitor removal step using the Zymo Kit. Finally, we emphasize that pilot studies should be undertaken whenever sampling in a new environment to identify which protocol is most appropriate.

**KEYWORDS**
biodiversity, DNA extraction, estuarine, filter pore size, fisheries, metabarcoding, PCR inhibition

## 1 | INTRODUCTION

Reliable biodiversity assessments are necessary for addressing essential questions in ecology and conservation biology (Vermeulen & Koziell, 2002). Traditionally, biodiversity surveys in aquatic systems have relied on direct capture or visual observation to determine species distributions and community status. However, these approaches are often limited in scope because they are labor-intensive and commonly require on-site taxonomic expertise. Over the past decade, the utility of environmental DNA (eDNA) as a tool for conducting biodiversity surveys has come into focus (Berry et al., 2019; Collins et al., 2018; DiBattista et al., 2019; Djurhuus et al., 2017; Koziol et al., 2019; Stat et al., 2017; Yoccoz, 2012). While eDNA approaches present unique challenges, including sometimes lengthy protocol development and unique biases that must be considered (Fonseca, 2018; Thomsen & Willerslev, 2015), they have proven to be effective across an array of taxa and in some cases may be more efficient in terms of time and cost than traditional techniques (Cristescu, 2014; Kelly et al., 2014; Sutherland et al., 2019). Because of their demonstrated utility as a biomonitoring tool, eDNA methods are becoming increasingly popular (Eble et al., 2020). However, standardized protocols and best practices have yet to be established, undermining the reliability of comparisons of eDNA findings within and among studies (Kumar et al., 2020).

Estimates of species occurrence and community biodiversity derived from eDNA studies are strongly influenced by the choice of DNA capture and isolation protocols (Deiner et al., 2015; Djurhuus et al., 2017; Vishnivetskaya et al., 2014; Wesolowska-Andersen et al., 2014; Yuan et al., 2012), yet only a handful of replicated experiments have directly compared and evaluated alternative approaches (Djurhuus et al., 2017; Majaneva et al., 2018). Most studies capture eDNA from water samples using filtration (Tsuji et al., 2019), and while filter material has been shown to impact DNA capture and detection (Djurhuus et al., 2017; Miya et al., 2016), cellulose-based filters have been shown to perform consistently in terms of both DNA yield and metabarcoding outcomes (Djurhuus et al., 2017; Hinlo et al., 2017). The influence of filter pore size on metabarcoding outcomes has received less attention, which complicates any comparison of findings within and between studies when different pore sizes are used either due to different laboratory preferences or in response to environmental conditions.

Protocol efficacy can vary significantly in response to local environmental conditions (Piggott, 2016). For example, eDNA in water that has high concentrations of suspended particulates is often bound to particles and more resistant to degradation (Alvarez et al., 1998), resulting in higher eDNA yields (Diaz et al., 2020). However, these same environments often contain high levels of compounds such as humic acid and tannins that inhibit PCR (Albers et al., 2013; McKee et al., 2015). Therefore, what represents best practices in a low-turbidity, oligotrophic systems may not apply to systems with high concentrations of suspended organic and/or inorganic material. Here, we begin to address these data gaps by evaluating the impact of sampling effort, choice of DNA extraction method, and filter pore size on DNA concentration, PCR inhibition, and estimates of fish biodiversity generated using 16S rDNA metabarcoding primers. We ran our experiments in both high-turbidity estuarine and low-turbidity clear-water habitats to assess the impact of water quality on protocol efficacy.

## 2 | MATERIALS AND METHODS

### 2.1 | Experimental design and sample collection

On March 14th, 2019, we collected replicate water samples from a high-turbidity inshore (estuarine) and a low-turbidity offshore site in eastern Florida (Figure 1). High-turbidity samples were collected at Geiger Point near the entrance to Crane Creek in the Indian River Lagoon (IRL), a diverse estuarine ecosystem that runs for ~250 km along the east coast of Florida, USA (Figure 1). This site, like many in the IRL, is notable for low water clarity and high concentrations of suspended organic and inorganic material (Sigua et al., 2000). The Secchi disk depth measurement at this site was 73 cm. The following day, low-turbidity samples were collected in the coastal ocean, approximately a half mile offshore of Sebastian Inlet (Figure 1). Samples were collected in clear water outside the obvious plume formed by outflowing, highly turbid waters from the IRL. The Secchi disk depth measurement at this site was 230 cm. Hereafter, we refer to these sites as our turbid- and clear-water sites, respectively (Figure 1).

From each site, we collected 50 replicate 500 ml samples of surface water using sterile Nalgene bottles. Each collection event included a negative field control consisting of a sterile 500 ml Nalgene bottles filled with store-bought reverse osmosis (RO) water. Samples were immediately stored on ice and filtered back in the laboratory within 6 h of collection. All collection and filtration equipment including Nalgene bottles, filter funnels, plastic containers, and forceps were sterilized in 20% bleach for at least 20 min and rinsed with RO water prior to use. Water samples were filtered using 500 ml filter funnels, a custom-made 6-station PVC manifold, and a standard laboratory vacuum pump. All treatments used mixed cellulose ester (MCE) filters which have been shown to perform well in eDNA experiments (Hinlo et al., 2017). Filters were stored in Longmire's buffer (Longmire, 1997), which has been shown to prevent DNA degradation even at room temperature (Renshaw et al., 2015). Filters in Longmire's were placed in 3 ml tubes and stored at −20°C until DNA extraction. Negative laboratory controls, consisting of 500 ml of RO water filtered using the same protocol as our field samples, were also collected and stored in Longmire's buffer. All extractions were carried out in a dedicated PCR-free room at the University of Central Florida Marine Molecular Ecology and Evolution Laboratory (MMEEL) in a laminar flow cabinet that had been sterilized with 10% bleach followed by 70% ethanol and irradiated with UV for 20 min before and after each round of extractions. Following extraction, DNA was stored at −20°C.

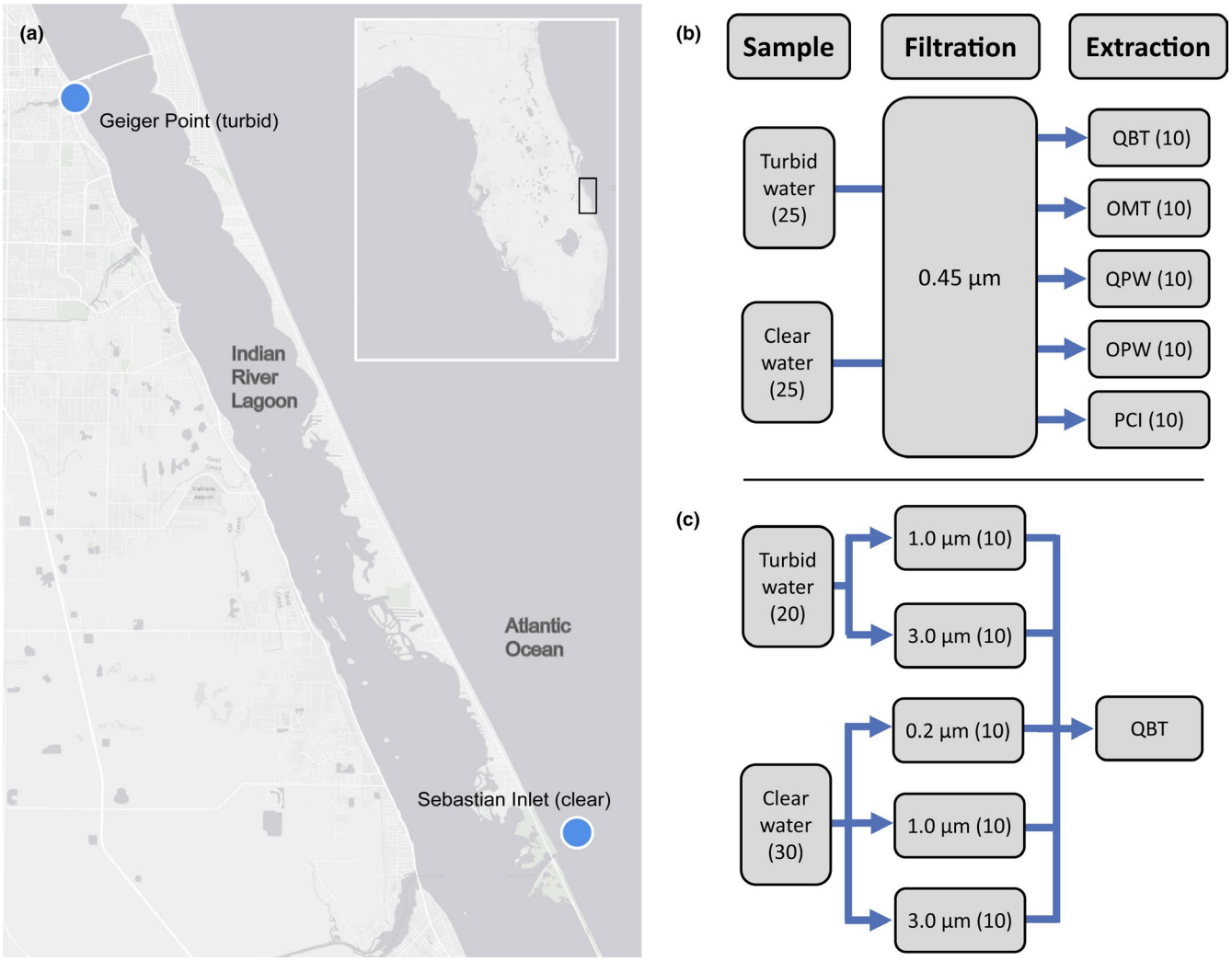


FIGURE 1 Map of study area and experimental design. Map of the central Indian River Lagoon, Florida (a) including our two sampling locations. (b) In Experiment 1, we compared five different extraction methods: Qiagen's DNeasy Blood & Tissue Kit (QBT); Omega Bio-Tek's E.Z.N.A. Tissue DNA Kit (OBT); Qiagen's DNeasy PowerWater Kit (QPW); Omega Bio-Tek's E.Z.N.A. Water DNA Kit (OPW); and Phenol–chloroform–isoamyl alcohol (PCI), while (c) in Experiment 2, we compared four filter pore sizes (0.2, 0.45, 1.0, 3.0 µm) and extracted all of these samples using the QBT kit. Number of technical replicates, each of 500 ml of surface water are in parentheses

## 2.2 | Experiment 1: eDNA extraction method and PCR inhibition

For Experiment 1, 25 samples from each of the two study sites were filtered using 0.45 µm MCE filters (Figure 1). Filters were cut in half and each half was haphazardly assigned to the five extraction protocols (Table 1), which were selected because they are either widely employed (QBT and QPW; Tsuji et al., 2019) or are less expensive alternatives (OMT, OPW, and PCI). For the commercial kits, DNA was extracted following the manufacturer's protocol and the DNA was eluted in two steps, each using 50 µl of elution buffer (100 µl combined volume). Phenol–chloroform–isoamyl alcohol (PCI) extractions were carried out following Djurhuus et al., (2017), excluding the incubation step with lysozyme.

To determine levels of PCR inhibition across extraction methods due to residual compounds either left behind or introduced during DNA extraction, we performed five replicate qPCR assays on DNA serially diluted as follows 1:1, 1:5, and 1:10 (parts DNA: parts water), and evaluated the resulting qPCR-$C_T$ values. The qPCR-$C_T$ value is defined as the number of amplification cycles required for the fluorescent signal to cross a specific threshold. In a perfectly efficient PCR with no inhibition, $C_T$ values will decline or increase in proportion to DNA concentration (e.g., a 5× dilution will result in a 2.3× increase in $C_T$ value). On the other hand, if PCR inhibitors are present, we expect the qPCR-$C_T$ value to initially decline with increasing DNA dilution (reflecting a reduction in inhibition and more efficient PCR) and then increase as inhibition is overcome and DNA template concentration becomes the limiting factor.

Because inhibitor removal kits offer the promise of rapid and relatively affordable inhibitor management, we evaluated the efficacy of the commonly used OneStep PCR Inhibitor Removal Kit (Zymo Research; Zymo Kit) with an assessment of qPCR results from

**TABLE 1** DNA yield across treatments

| Extraction method | Abbreviation | DNA concentration | |
|---|---|---|---|
| | | Turbid-water | Clear-water |
| Qiagen DNeasy Blood and Tissue | QBT | 48.89 ± 2.74[a] | 2.69 ± 1.30[a,c] |
| Omega Bio-tek E.Z.N.A Tissue DNA | OMT | 49.81 ± 4.28[a] | 1.91 ± 1.22[a,c] |
| Qiagen DNeasy PowerWater | QPW | 71.98 ± 19.38[b] | 1.35 ± 1.57[a] |
| Omega Bio-tek E.Z.N.A Water DNA | OPW | 30.11 ± 11.43[c] | 0[b] |
| Phenol-chloroform-isoamyl alcohol | PCI | 107.18 ± 8.15[d] | 3.04 ± 0.70[c] |
| | **Pore size** | | |
| Qiagen DNeasy Blood and Tissue | 0.2 μm | NA | 3.10 ± 1.85 |
| | 1.0 μm | 9.89 ± 1.74 | 2.76 ± 1.82 |
| | 3.0 μm | 9.35 ± 1.74 | 3.08 ± 1.59 |

Note: Extraction method, filter pore sizes, and average DNA concentration (ng/μl) obtained across ten replicates are listed. DNA concentration is reported in ng/μl ± standard deviation. Each value is derived from 500 ml water samples filtered through a mixed cellulose ester (MCE) filter membrane. One-half of each filter was extracted as outlined in the methods section above and quantified using a Qubit 4 fluorometer. In our experiments, the OPW kit failed to yield detectable DNA from the clear-water samples. Those comparisons that were significant using an ANOVA show subscripts with different letters indicating statistical significance at $p < 0.05$ using the post hoc Tukey–Kramer Honest Significant Difference test.

cleaned extractions. This experiment was conducted on only QBT, OMT, and PCI extractions from turbid-water samples (Table 1; $N = 5$ for each method), as these exhibited the highest levels of inhibition.

## 2.3 | Experiment 2: filter pore size

DNA capture rates can vary with filter pore size (i.e., smaller pore size filters capture more DNA than larger pore size filters; Barnes et al., 2020); however, this is not always the case (Eichmiller et al., 2016; Lacoursiere-Roussel et al., 2016). To assess the impact of filter pore size on DNA concentration and metabarcoding results, we filtered samples using 0.2, 0.45, 1.0, and 3.0 μm MCE filters (Figure 1). We included results from Experiment 1 for samples that were filtered using 0.45 μm filter membranes and included an additional 20 and 30 samples collected on May 15th, 2019 from the turbid- and clear-water sites, respectively. Turbid-water samples were filtered using 1.0 and 3.0 μm filter membranes and clear-water samples were filtered using 0.2, 1.0, and 3.0 μm filter membranes ($N = 10$ per filter treatment). We omitted the 0.2 μm filter membranes from the turbid-water site due to filter clogging. All samples were extracted using the commonly employed QBT kit (Table 1) following the manufacturer's protocol and treatment with the OneStep PCR Inhibitor Removal Kit.

## 2.4 | Illumina library preparation and sequencing

Based on evidence of PCR inhibition from Experiment 1, the DNA extractions were either cleaned using the Zymo Kit (QBT, OMT, and PCI) or diluted in a 1:1 ratio (QPW and OPW) prior to beginning library preparation. Amplicon libraries were prepared for Illumina sequencing using a modified two-step PCR protocol (Figure S1). PCR 1 (qPCR) targeted 178–228 bp of the mitochondrial 16S gene in fishes using the Fish16sF/D and 16s2R primers of Berry et al., (2017) with Illumina adapter overhangs (Figure S1; Table S1). Amplifications were carried out in 25 μl reactions that included 2× SsoAdvance Universal SYBR Green Supermix (Bio-Rad), 0.5 μl forward primer (10 μM), 0.5 μl reverse primer (10 μM), 2.0 μl of template DNA, and 9.5 μl of ultrapure water (ThermoFisher Scientific). Each qPCR run included extraction and no-template negative controls to monitor contamination. qPCRs were performed on a CFX96 Touch Real-Time PCR System (Bio-Rad) using the following conditions: 95°C for 3 min followed by 30 cycles at 95°C for 30 s, 54°C for 30 s, 72°C for 30 s, and a final extension at 72°C for 5 min. In samples where DNA concentration was low (0.3–0.9 ng/μl), 11.5 μl of the template DNA was added and the qPCR was carried out for 35 cycles. To minimize cross-contamination between samples, all the PCR reactions were conducted in eight-tube PCR stripes with individually attached snap caps. To compensate for PCR bias, qPCRs were performed in duplicate, pooled, and then purified using E.Z.N.A. Cycle Pure Kits (Omega Bio-tek) following the manufacturer's protocol. DNA concentration was determined using a Qubit 4.0 (Invitrogen).

For PCR 2, amplifications were conducted using forward and reverse primers containing linker sequences complementary to the Illumina adapter, a unique 8-bp Nextera index (different for the forward and reverse primers), and the Illumina MiSeq adapters i5 and i7 (Figure S1; Table S1). Amplifications were conducted in a 25 μl volume containing 12.5 μl IBI Taq 2× Master Mix (IBI Scientific), 0.5 μl forward primer (10 μM), 0.5 μl reverse primer (10 μM), 2.0 μl of purified DNA from PCR 1, and 9.5 μl of ultrapure water. PCRs were performed on an Veriti Thermal Cycler (Applied Biosystem) using the following conditions: 95°C for 3 min; followed by 15 cycles at 95°C for 30 s, 55°C for 30 s, 72°C for 30 s; and a final extension at 72°C for 5 min. Final PCR products were cleaned using E.Z.N.A. Cycle Pure Kits, quantified on a Qubit 4.0, and pooled in

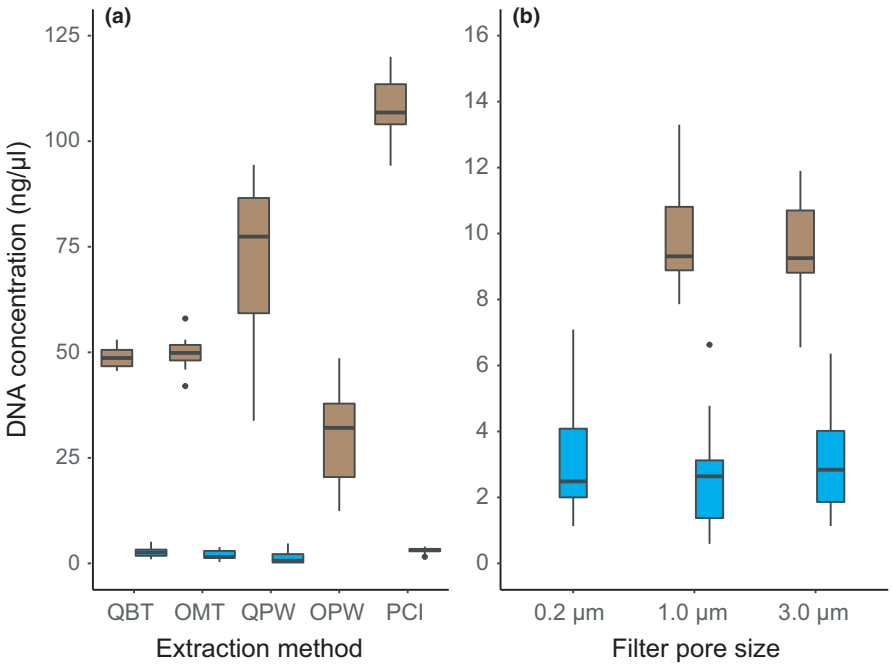


**FIGURE 2** Box plots comparing DNA yield across treatments. (a) DNA yield for each of the five extraction methods and (b) pore sizes tested here in turbid-water (brown bars) and clear-water (blue bars) samples. The black line in each box represents the median while the upper and lower whiskers (lines) show the range of the highest and lowest 25% of the values. Note that the OPW kit failed to yield detectable DNA from the clear-water samples. See Table 1 for abbreviations

equimolar concentrations. The pooled library was then size-selected (300–450 bp) using a PippinHT (Sage Science) and a 2% agarose gel cassette and quantified with a NEBNext Library Quantification Kit for Illumina (New England Biolabs) and a Qubit 4.0. We used the average of these values to adjust the library concentration to 4 nM and denatured it following Illumina protocols. The denatured library was combined with 10% PhiX control to improve sequencing results and sequenced on an Illumina MiSeq (V3, 2× 300 cycles) at the Genomics and Bioinformatics Cluster core lab at the University of Central Florida.

## 2.5 | Bioinformatic analyses and taxonomic assignments

Sequencing data were demultiplexed using the Illumina MiSeq software and downloaded onto an in-house server maintained by the Genomics and Bioinformatics Cluster at the University of Central Florida. Individual FASTQ files were then filtered following a series of quality control steps using USEARCH v10 (Edgar, 2010) and VSEARCH v2.14 (Rognes et al., 2016). Forward and reverse reads were merged in USEARCH using the fastq_mergpairs command with a minimum length of 202 bp, minimum overlap of 100 bp, and a maximum of 3 mismatches. VSEARCH was used to remove primers, de-replicate sequences, and discard sequences with expected errors of >0.5. Unique sequences were then denoised using the UNOISE3 (Edgar, 2016) option implemented in USEARCH. UNOISE3 generates zero radius operational taxonomic units (ZOTUs) by correcting point errors and filtering chimeric sequences (Edgar, 2016). A minimum of five reads were used to identify amplicon sequence variants (ASVs).

Amplicon sequence variants were blasted against the National Center for Biotechnology Information's (NCBI) GenBank nucleotide database using BLASTn with the default parameters. To retrieve the full taxonomic identity, we queried each of the "taxids" from the BLAST results against the NCBI database using the "taxonkit lineage" command of taxonkit (Shen & Xiong, 2019). To reduce uncertainty in taxonomic assignment, we discarded ASVs with a bitscore below 250 and/or query coverage below 100%. Each ASV was then assigned to the lowest taxonomic level based on the percent similarity to NCBI alignments. We used the following threshold criteria for taxonomic assignment: ≥99% similarity for species; 97% for genus; 95% for family; 90% for order; 85% for class; and 80% for phylum (following West et al., 2020). Taxonomic assignments were collapsed to the next highest classification if the assigned taxa were not known to occur in the IRL or central-west Atlantic or if more than one match exceeded the assignment threshold (e.g., 99% species-level assignment). If an ASV fell between the genus and species cutoff (97%–99%) and was the only species of its genus known to occur in the study region, the ASV was assigned to the species level.

## 2.6 | Statistical analyses

Unless otherwise stated, statistical analyses were conducted in JMP Pro 12 (SAS Institute). All datasets were tested for normality using a Shapiro–Wilk test (Shapiro & Wilk, 1965). One-way analysis of variance (ANOVA) was used to determine whether there were significant differences in DNA concentration, number of reads, and ASV and species counts across experimental treatments. When a significant interaction was detected, we performed a post hoc Tukey–Kramer Honest Significant Difference (HSD) test to determine which specific group means were significantly different. To determine the effect of the PCR inhibitor removal step on DNA concentration, we used a paired *t* test. Read copy number per species was visualized using the

R package superheat (Barter & Yu, 2018). To visualize the number of overlapping and unique taxa detected across treatments, Venn diagrams were constructed in R using the VennDiagram package v1.6.2 (Chen & Boutros, 2011). The Bray–Curtis dissimilarity statistic was used to compare community composition across treatments in Vegan and visualized using non-metric multidimensional scaling (NMDS) in RStudio using ggplot2 (Wickham et al., 2016) with two ordination axes. An analysis of similarity (ANOSIM; Clarke, 1993) was used to test for significance. Lastly, species accumulation curves were generated in the R package Vegan (Oksanen et al., 2015) with random sampling and 10,000 permutations.

## 3 | RESULTS

Regardless of the DNA extraction method, we recovered significantly more DNA from the turbid-water samples compared to the clear-water samples (Figure 2; Table 1) with the latter exhibiting notably higher levels of PCR inhibition (Figure 3). The two more expensive power water kits (QPW and OPW) performed well with the turbid-water and were able to alleviate most of the PCR inhibition. However, the OPW kit failed to yield detectable DNA with the clear-water samples indicating its unsuitability for water samples with low DNA concentration. The MiSeq paired-end sequencing (2 × 300 bp) generated a total of 11.86 million reads across all samples, including 4.73 million and 7.13 million from the turbid-water and clear-water samples, respectively (Table 2). Read quality was high with 82.1% of the reads having Phred scores of ≥30. Following quality filtering, merging, primer trimming, and chimera removal, 6.6 million reads remained (56.1%). A total of 36 species were detected at the turbid-water site while 37 species were detected at the clear-water site (Figure 4) with high levels of variation across replicates in the latter.

### 3.1 | Experiment 1—High variability in DNA yield and PCR inhibition

DNA yields differed significantly across extraction methods for the turbid-water samples (ANOVA $p < 0.001$; Table 1, Figure 2) with the PCI method consistently yielding the most DNA. The OBT and OMT extraction kits yielded less but roughly equal amounts of DNA (Table 1). DNA yield also differed significantly across extraction methods in the clear-water samples (ANOVA $p < 0.001$). In this case, the OPW kit, which resulted in the lowest DNA yields in the turbid-water samples, failed to yield detectable DNA in the clear-water samples (Table 1; Table S2; Figure 2). The PCI method resulted in similar DNA yields when compared to the QBT and OMT kits, but significantly more DNA compared to the QPW kit (Tukey-Kramer HSD $p = 0.019$). The QBT, OMT, and QPW kits yielded similar amounts of DNA.

PCR inhibition was common across our experimental treatments but was most pronounced in the turbid-water samples. DNA extractions conducted using the PCI method exhibited the most inhibition (increased $C_T$ value), which was alleviated

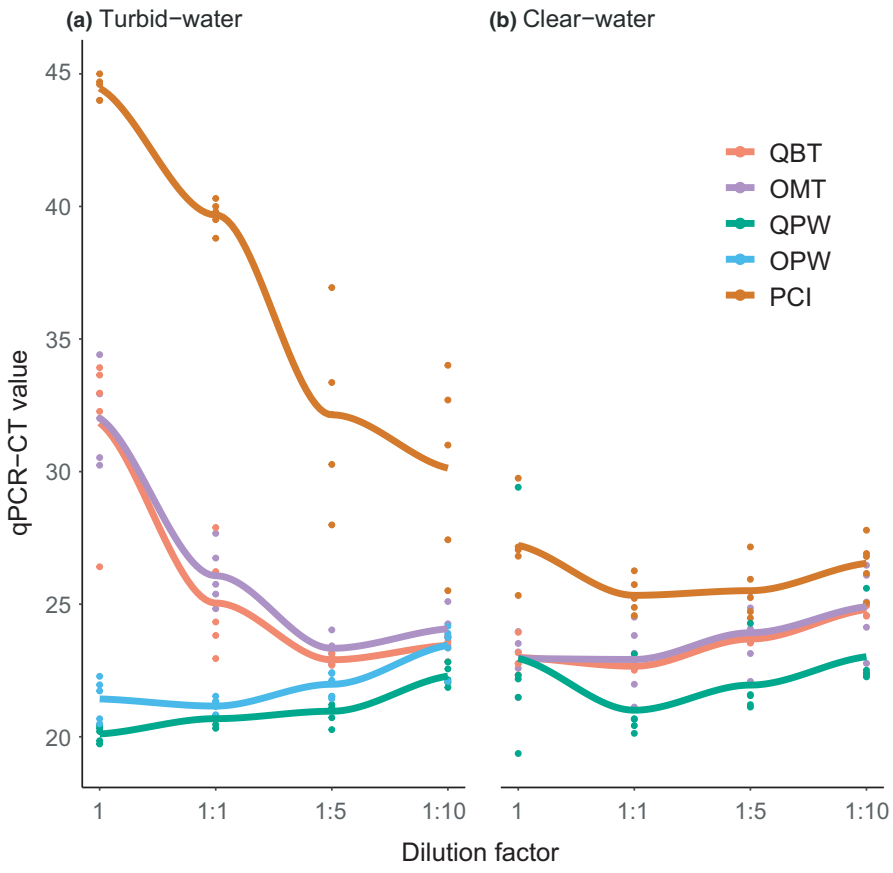


FIGURE 3 Results of tests for PCR inhibition across extraction methods (Experiment 1) in (a) turbid- and (b) clear-water samples. Samples were tested for PCR inhibition at full concentration of the DNA extraction (1) and at 1:1 (1 part extract: 1 part water), 1:5 (1 part extract: 5 parts water), and 1:10 (1 part extract: 10 parts water) dilutions. When PCR inhibitors are present, we expect the qPCR-$C_T$ value to initially decline with increasing DNA dilution (reflecting a reduction in inhibition and more efficient PCR). The solid lines are color coded by extraction method and represent the mean $C_T$ values

**TABLE 2** Number of sequence reads, amplicon sequence variants (ASVs), and species detected across treatments in turbid- and clear-water samples

| | Turbid-water | | | Clear-water | | |
|---|---|---|---|---|---|---|
| | # Reads | # ASVs | # Species | # Reads | # ASVs | # Species |
| Extraction methods (Experiment 1) | | | | | | |
| QBT | 44,531 ± 9,052 | 24.20 ± 2.53 | 17.60 ± 2.01 | 69,204 ± 76,704 | 14.00 ± 3.13$^{a,b}$ | 5.90 ± 0.99$^{a}$ |
| OMT | 45,530 ± 8,048 | 22.90 ± 3.98 | 15.50 ± 1.43 | 53,648 ± 63,887 | 19.50 ± 2.55$^{a}$ | 10.40 ± 1.57$^{b}$ |
| QPW | 46,429 ± 14,485 | 26.70 ± 4.27 | 16.70 ± 2.58 | 34,801 ± 53,230 | 17.50 ± 7.66$^{a,b}$ | 6.70 ± 2.26$^{a}$ |
| OPW | 39,598 ± 17,225 | 24.70 ± 4.14 | 17.30 ± 2.26 | NA | NA | NA |
| PCI | 50,652 ± 14,548 | 25.80 ± 4.24 | 17.10 ± 2.51 | 50,979 ± 77,311 | 13.20 ± 4.78$^{b}$ | 7.90 ± 2.28$^{a}$ |
| Pore size (Experiment 2) | | | | | | |
| 0.2 µm | NA | NA | NA | 49,695 ± 39,013 | 12.60 ± 4.77 | 7.40 ± 2.37$^{a,b}$ |
| 0.45 µm* | 44,585 ± 9,045$^{a}$ | 24.20 ± 2.53$^{a}$ | 17.60 ± 2.01$^{a}$ | 69,204 ± 76,704 | 14.00 ± 3.13 | 5.90 ± 0.99$^{b}$ |
| 1.0 µm | 60,237 ± 16,980$^{b}$ | 18.90 ± 1.60$^{b}$ | 14.20 ± 1.32$^{b}$ | 47,837 ± 58,719 | 13.70 ± 4.95 | 5.50 ± 1.58$^{b}$ |
| 3.0 µm | 44,515 ± 13,066$^{a}$ | 17.00 ± 3.86$^{b}$ | 13.70 ± 2.21$^{b}$ | 26,982 ± 35,085 | 12.00 ± 3.13 | 8.00 ± 1.49$^{a}$ |

Note: In our experiments, the OPW kit failed to yield detectable DNA from the clear-water samples. Those comparisons that were significant using an ANOVA show superscript with different letters indicating statistical difference at $p < 0.05$ using the post hoc Tukey–Kramer Honest Significant Difference test.

*Data from Experiment #1.

in the clear-water samples after a 1:1 dilution while a 1:10 dilution was required to alleviate inhibition in the turbid-water samples (Figure 3). The two power water kits (QPW and OPW) were largely able to prevent PCR inhibition in the turbid-water samples (Figure 3(a)), but this was not the case for the QBT and OMT kits which suffered considerable PCR inhibition. Only modest levels of PCR inhibition were observed in the clear-water samples extracted using the PCI and QPW methods, which was alleviated after a 1:1 dilution (Figure 3(b)). The treatment of extractions with the Zymo Kit (only QBT, OMT, and PCI extractions tested) resulted in a slight but significant decrease (−5.24 ± 2.02 ng/µl; *t*-test $p = 0.001$; Figure S2) in DNA concentration. After Zymo treatment, PCR inhibition was alleviated in the QBT and OMT extractions but not in the PCI samples, which required a 1:5 dilution to eliminate inhibition (Figure S3).

## 3.2 | Experiment 1—The effect of DNA extraction method on metabarcoding results

There was no indication of contamination (no amplification in the qPCR reaction) in any of the extraction or PCR-negative controls. We did detect positive qPCR amplification in the seven of the nine negative field controls. However, after library preparation and sequencing we detected only five reads across three of the seven controls (no reads were detected in four of the controls). Of the five reads, three were detected in a single turbid-water field control (all assigned to the Atlantic croaker *Micropogonias undulates*) and one read was detected in each of two clear-water negative controls (assigned to the Atlantic thread herring *Opisthonema oglinum* and the Atlantic menhaden *Brevoortia tyrannus*, respectively).

In turbid-water, the number of ASVs ranged from 22.90 (±3.98) to 26.70 (±4.27) and the number of species was similarly consistent and ranged from 15.50 (±1.43) to 17.60 (±2.01). A total of 33 species were detected in the turbid-water samples across all extraction methods, with the Bay anchovy *Anchoa mitchilli* being the most dominant species (Figure 4(a)). Of the species detected in turbid-water, 17 were common across methods while 0–2 unique species were identified by a single extraction method (Figure 5(a)) and represented by low read number. In the turbid-water samples, there were no significant differences in species composition (ANOSIM $R = 0.019$, $p = 0.717$) across extraction methods and the NMDS plots showed no separation across treatments (Figure S4) as most of the species were represented by high read number. ASV accumulation curves show overlapping curves with fairly narrow confidence intervals (Figure S5).

Because the OPW kit failed to yield detectable DNA from our clear-water samples, it is not considered here. The average number of reads recovered from the clear-water samples was highly variable across replicates but did not vary significantly across treatments, ranging from 34,801 (±53,230) to 69,204 (±76,704) (Table 2). The number of ASVs detected ranged from 13.20 (±4.78) for the PCI method to 19.50 (±2.55) for the OMT kit and only ASV counts from these two extraction methods were significantly different. The number of species detected ranged from 5.90 (±0.99) for the QBT kit to 10.40 (±1.57) for the OMT kit with the latter being significantly higher than the other methods which did not differ from each other (Table 2). A total of 31 species were detected at the clear-water site in this experiment with the Atlantic thread herring (*O. oglinum*) having the highest read counts across most methods (Figure 4(b)). Only five species were common to all extraction methods and between 3 and 7 unique species were identified by a single method (Figure 5(b)). As

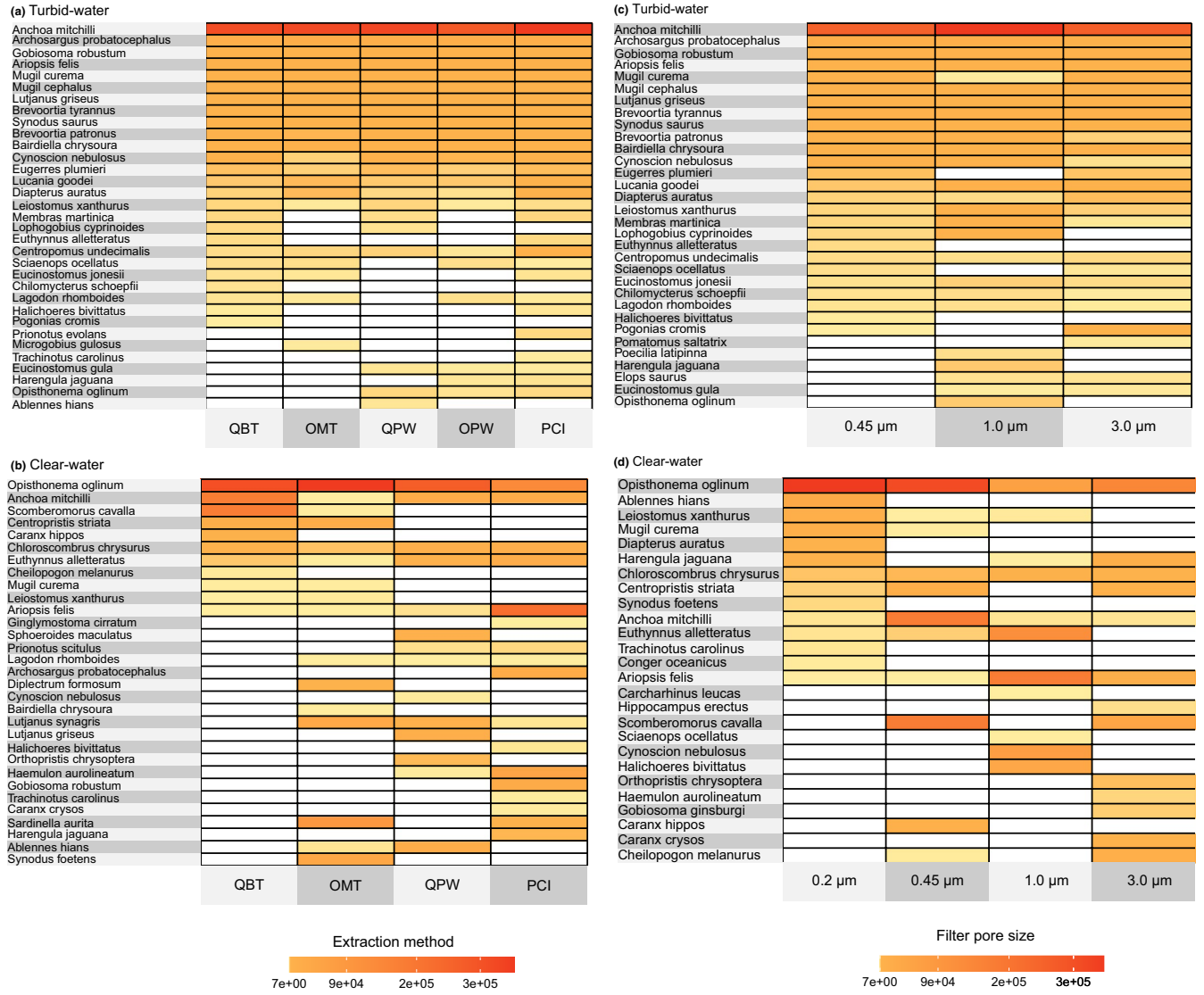


**FIGURE 4** Relative abundance of read counts for fish species detected using five different extraction methods from (a) turbid- and (b) clear-water samples. Data are based on extractions from 0.45 µm pore size filters. Also shown are data for species detected using four different filter pore sizes (0.2, 0.45, 1.0, 3.0 µm) from (c) turbid- and (d) clear-water samples. Data from (c) and (d) are based on filters extracted using the Qiagen's DNeasy Blood & Tissue Kit

above, there were no significant differences in species composition (ANOSIM $R$ = 0.029, $p$ = 0.185) across extraction methods and the NMDS plots showed no separation across treatments (Figure S4). However, the NMDS plots did show significantly more spread across replicates and the ASV accumulation curves showed wide confidence intervals compared with the turbid-water samples (Figure S5).

### 3.3 | Experiment 2: The effect of filter pore size on DNA yield and metabarcoding results

All samples for this experiment were extracted using the QBT kit with the samples for the 0.45 µm filter pore size taken from Experiment 1. We did not statistically compare DNA yield for the 0.45 µm filter membranes (Experiment 1) with yield from the 0.2, 1.0, and 3.0 µm filters because the extraction of 0.45 µm filters was done ~18 months prior to the others. The earlier extractions of the 0.45 µm filters resulted in dramatically (~5-fold) higher DNA yield compared with the filters extracted ~18 months later (Table 1).

As found in Experiment 1, turbid-water samples yielded significantly more DNA than the clear-water samples (9.62 ± 1.71 ng/µl versus 2.98 ± 1.70 ng/µl, respectively; $p$ < 0.001; Table 1). However, there were no significant differences in DNA yield across filter pore sizes within either habitat. The average number of reads recovered from the turbid-water samples was significantly higher for the 1.0 µm filters with 60,237 (±16,980) reads versus 44,515 ± 13,066 and 44,585 ± 9045 for the 3.0 and 0.45 µm filters, respectively (Table 2). Similarly, the number of ASVs detected was significantly higher for the 0.45 µm filters, with 24.20 (±2.53) ASVs compared with 18.90 (±1.60) and 17.00 (±3.86) ASVs

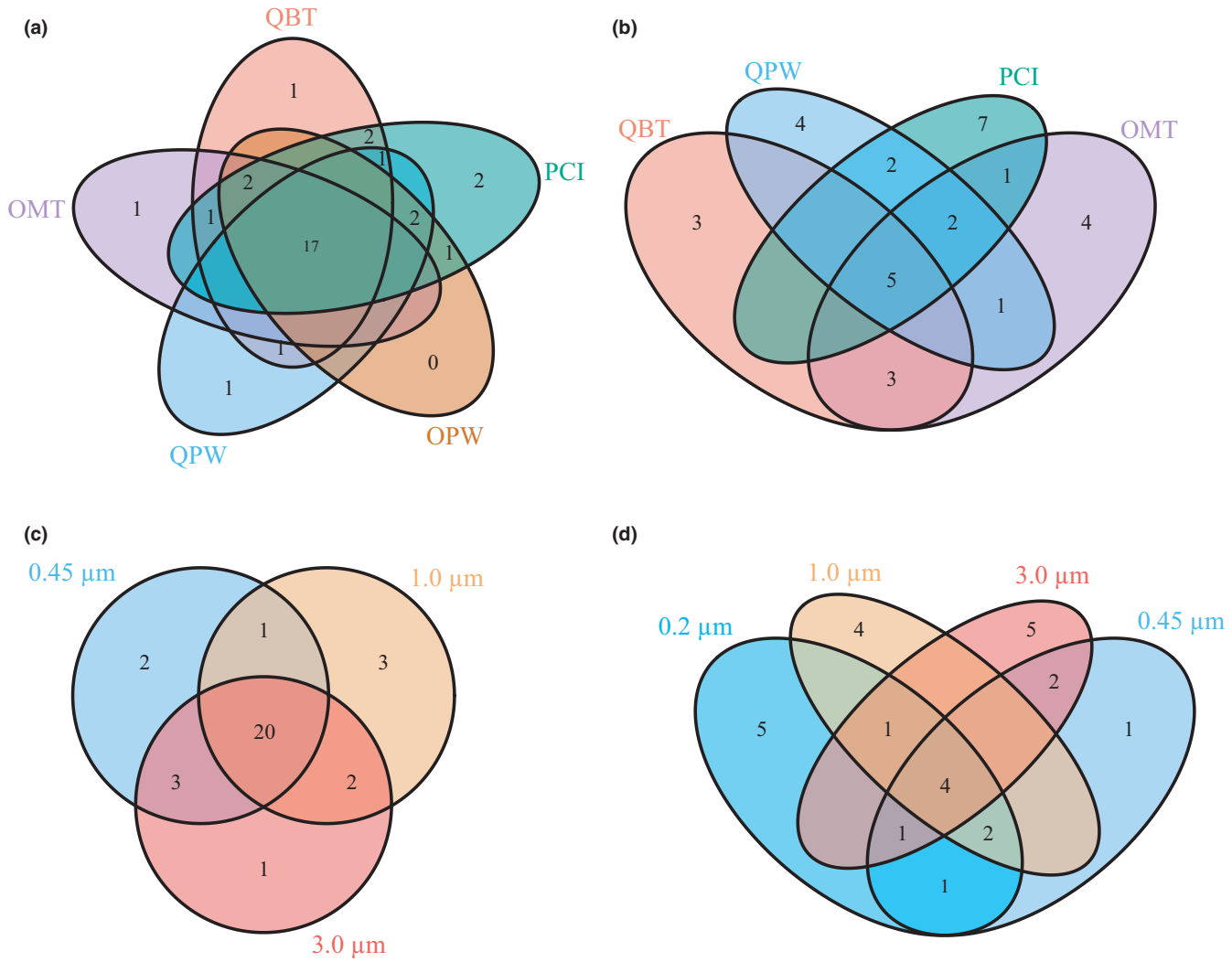


**FIGURE 5** Venn diagrams representing the number of fish species detected in the metabarcoding experiments across five different extraction methods in the (a) turbid- and (b) clear-water samples. Also shown are results based on filter pore size in the (c) turbid- and (d) clear-water samples. The numbers shown in overlap areas reflect shared species. Zeros in areas of overlap are omitted for figure clarity. See Table 1 for abbreviations

for the 1.0 and 3.0 µm filters, respectively. The number of species detected showed a pattern that correlated with the number of ASVs, with significantly more species detected in the 0.45 µm filters at 17.60 (± 2.01) species compared with 14.20 (±1.32) and 13.70 (±2.21) species for the 1.0 and 3.0 µm filters, respectively. A total of 32 species were detected in the turbid-water samples across all treatments. Of the species detected in turbid-water, 20 were common across all pore sizes while 1–3 species were identified by a single treatment and represented by relatively low read number (Figures 4 and 5(c)). Similar to the results above, the Bay anchovy (*A. mitchilli*) was the dominant species detected across all pore sizes (Figure 4(c)). The ANOSIM test revealed significant differences in species composition across treatments ($R$ = 0.759, $p$ < 0.001) which is supported by the NMDS plots that show slight separation among the communities found across all pore sizes (Figure S4). As above, species accumulation curves were broadly overlapping across treatments (Figure S5).

As in Experiment 1, the average number of reads recovered from the clear-water samples was highly variable across replicates but did not vary significantly across treatments ranging from 26,982 (±35,085) for the 3.0 µm filters to 69,204 (±76,704) for the 0.45 µm filters (Table 2). The number of ASVs detected was consistent across treatments and ranged from 12.00 (±3.13) to 14.00 (±3.13). We did detect significantly more species in the 0.2 µm and 3.0 µm filters at 7.40 (±2.37) and 8.00 (±1.49), respectively, compared with 5.90 (±0.99) and 5.50 (±1.58) for the 0.45 and 1.0 µm filters, respectively. In total, we detected 26 species in the clear-water samples in this experiment with only four species detected in all pore sizes (Figures 4 and 5(d)) and 1–5 unique species identified by a single treatment (Figure 4(d)). Similar to Experiment 1, herring (*O. oglinum*) had the highest read counts for the 0.2, 0.45, and 3.0 µm filters whereas the hardhead catfish (*Ariopsis felis*) had the highest read count in the 1.0 µm pore size filters most of which came from just two of the ten replicates (Figure 4(d)). We detected

a small but significant difference in species composition (ANOSIM $R$ = 0.070, $p$ = 0.041) across pore sizes that was not supported by the NMDS plot (Figure S4) which showed widespread across replicates that were broadly overlapping. As in Experiment 1, the ASV accumulation curves for the clear-water samples showed wide confidence intervals (Figure S5).

## 4 | DISCUSSION

There is a growing excitement over the potential of eDNA as a tool for community assessments and ecosystem monitoring (Eble et al., 2020). However, a major hurdle when adopting these new methods is the lack of accepted best practices: a problem that can only be solved with careful experimental design and protocol development (Kumar et al., 2020). Here, we test the effect of DNA extraction method and filter pore size on DNA yield, PCR inhibition, and, ultimately, the results of Illumina metabarcoding experiments using a 16S rDNA primer set for fishes. We ran our experiments using samples from both a high-turbidity estuarine environment as well as a relatively low-turbidity nearshore environment. Our results indicate that the most influential factor was water type (turbid vs clear). We found that DNA yield and PCR inhibition varied significantly across our two experimental sites and extraction methods, but that inhibition could largely be alleviated using commercially available PCR inhibitor removal kits. We found that the commonly used power water kits (QPW and OPW) do relieve most PCR inhibition, but the high cost of these kits must be weighed against the extra step involved for PCR inhibitor removal required for the other methods. We highlight that the less expensive commercial blood and tissue kit (OMT) did as well as the more expensive and commonly used kit tested here (QBT). Most importantly, we found that the volume of water needed to conduct biodiversity assessments will vary greatly by habitat, and in environments where eDNA concentrations are expected to be low (open ocean or oligotrophic habitats) more extensive sampling may be required. Here, we show that 500 ml water volume returned consistently high numbers of species detections across replicates sampled in turbid-water (Table 2) but was insufficient for replicates sampled in (offshore) clear-water environments.

### 4.1 | Effect of extraction methods on DNA yield and PCR inhibition

Consistent with previous studies, we found that the PCI method yielded the most DNA (Djurhuus et al., 2017; Geerts et al., 2018). Because eDNA in aquatic environments is often bound to suspended particulate matter (Turner et al., 2014), our extractions from the turbid-water environment, with high concentrations of suspended material, yielded upwards of 30× more DNA than the samples from our clear-water site (Figure 1). The most consistent DNA yields were obtained from the two blood and tissue kits (QBT and OMT, Table 1, Figure 2) while the highest variation across replicates was obtained with the QPW kit. While other studies have shown lower DNA yield with power water kits (Deiner et al., 2015; Djurhuus et al., 2017), this was not always the case in our study. The QPW kit resulted in high (but variable) DNA yields while the OPW kit resulted in the lowest yields of any method and in fact failed to yield detectible DNA in our clear-water samples (Table 1, Figure 2). Because the commercial kits used here employ similar column-based DNA binding steps, differences in DNA yield may be due to the addition of a bead beating step (employed in the QPW and OPW kits) and/or the formulation of lysis buffers which result in differential breakup of whole cells that impacts DNA yield (Eichmiller et al., 2016; Kumar et al., 2020).

PCR inhibition occurred in samples taken from both experimental sites but was most profound in the turbid-water samples and in the samples extracted using the PCI method (Figure 3(a)). Estuarine habitats are known to have elevated levels of PCR inhibitors, particularly humic acid, which binds to DNA molecules (Matheson et al., 2010) and slows or even prevents PCR. The power water kits successfully removed PCR inhibitors from the turbid-water samples that the blood and tissue kits (QBT and OMT) left behind. While inhibition was not nearly as striking in the latter two kits as for the PCI method, it still required a 1:5 dilution to relieve. While DNA dilution can be effective to reduce PCR inhibition (Figure 3), it is not recommended as it will likely result in false negatives especially when target DNA is present in low concentration. Alternately, the column-based Zymo Kits, which cost ~$2 USD per sample, were able to successfully remove PCR inhibitors and resulted in only a ~3% loss of DNA. Furthermore, (Williams et al., 2017) showed that the Zymo Kits actually performed better than sample dilution and improved sensitivity particularly when samples contained very high concentrations of inhibitors, as was the case here with our turbid-water samples. Even though the Zymo Kits were able to remove most of the residual inhibitors from the QBT and OMT extractions, there was still some inhibition in the PCI extracted samples even after treatment (Figure S3), suggesting that care must be taken when using a PCI method to extract samples collected from sites with high PCR inhibitor loads. Lastly, while the QPW kit did well to remove PCR inhibitors without additional steps, it is also expensive at ~$10 USD per sample (Kumar et al., 2020). A more affordable option that does not compromise eDNA detection might be the less expensive OMT kit (~$1.8 USD per sample) followed by Zymo cleanup.

### 4.2 | Effect of filter pore size on DNA yield

Intuitively, it is expected that smaller pore size filters will retain more eDNA than larger pore size filters. However, this is not supported here or in earlier studies (Eichmiller et al., 2016; Lacoursiere-Roussel et al., 2016). We did not find a significant difference in DNA yield across filter pore sizes which may be because eDNA in aquatic systems is often associated with or bound to suspended particulate matter and not truly free floating (Turner et al., 2014). For instance, size fractionation experiments have shown that mitochondrial eDNA is most abundant in particle size fractions ranging from 1.0

to 10 μm (Turner et al., 2014). Here, we tested filter pore sizes that ranged from 0.2 to 3.0 μm, so perhaps, the bulk of the eDNA in our samples was bound to larger particles or even still encapsulated in whole mitochondria or cells.

### 4.3 | Lysis buffers and effect of filter storage time on DNA yield

Longmire's solution is a lysis buffer that facilitates the release of intracellular DNA by lysing external cellular structures (Longmire, 1997). The samples used in these experiments were collected at roughly the same time but processed in two batches ~18 months apart. As a result, we obtained significantly lower DNA yields for our turbid-water samples (~5× lower; Table 1) stored at −20°C for ~20–21 months (0.2, 1.0, and 3.0 μm filters), compared with the 0.45 μm filters which were processed after just ~2–3 months storage at −20°C. Thus, there was a ~18-month lag between batch processing which we believe contributed to differences in DNA yield. It is interesting to note that we did not observe differences in DNA yield in the clear-water samples processed ~18 months apart. We speculate that increased organic material in the turbid-water samples included whole algal cells and that the increased time in storage resulted in the lysis of those cells and the liberation of their DNA into the buffer, which was not recovered following our methodology (we extracted ½ the filter per replicate and discarded the buffer). Based on this finding, we suggest that eDNA extractions from filters stored in Longmire's be performed soon after collection to avoid the loss of DNA from the filter itself, or extractions should include the entire filter as well as the buffer. Because we do not target algal DNA, we do not believe this had an adverse effect on the results presented here (Figures 4 and 5(c,d); Figure S4), but certainly, this issue is an important consideration when designing future experiments.

### 4.4 | Species diversity and community composition

While DNA yield and PCR inhibition are important factors to consider when designing protocols, of ultimate importance is the impact of the protocol on the outcome of sequencing. A handful of studies have examined aspects of eDNA protocols that we test here (Djurhuus et al., 2017; Hinlo et al., 2017; Majaneva et al., 2018; Sellers et al., 2018); however, few of these studies have taken their samples through to sequencing and thus the impact of their protocols on metabarcoding results is rarely evaluated (Djurhuus et al., 2017; Majaneva et al., 2018). There was no indication that extraction method impacted our biodiversity assessment (Table 2) with the NMDS plots showing complete overlap in community composition estimates for the turbid-water samples and a haphazard pattern in the clear-water samples. However, there was some indication of separation across pore sizes in the turbid-water samples (Figure S4) that does not correlate with any other metric such as read count or number of species detected (Table 2, Figure 5(a)). Therefore, our data suggest that filter pore size up to the 3.0 μm does not significantly impact metabarcoding fish detections.

While the extraction methods and filter pore sizes tested here did not have a major impact on our assessments of fish communities, this was not the case for water quality. There are many biological questions that would require sampling across estuarine and open water environments, yet no published studies have evaluated the impact of water quality (turbid vs. clear) on metabarcoding results. At the highest level, we show that eDNA sampling was able to resolve the unique fish communities at our estuarine (turbid-water) and nearshore (clear-water) sites (Figure 4, Figure S4). This was not surprising as the turbid-water site is located inside the Indian River Lagoon (IRL), one of the richest estuarine communities in North America (Gilmore, 1995; 397 species of fish across 137 families) hosting a distinct fish community from that found outside the lagoon (Gilmore et al., 1977). What was surprising was the high level of variation we detected among replicates taken at the clear-water site. All water samples were taken on the same day and within 15 min, yet the species detected among replicates were highly variable (Figure S4); a trend that is reflected in the shotgun-like spread of the data points on the NMDS plots (Figure S4), wide confidence intervals in the ASV accumulation curves (Figure S5), and fewer overlapping species across experimental treatments (Figure 5). These findings correspond to low concentrations of eDNA in this open water environment that is likely driven by a lower fish biomass to water volume ratio and the processes of advection and mixing (Andruszkiewicz et al., 2019). Taken together, these data highlight the need for sampling relatively large volumes of water in clear-water oligotrophic environments to ensure reliable detection of target species. For instance, working in a coral reef habitat off northwest Australia, Bessey et al. (2020) found high levels of variation across replicate samples and an increasing number of overlapping taxa when the volume of water sampled increased (volume ranged from 25 ml to 2 L). They found that even when 2 L was filtered, they captured only 43% of taxa ultimately detected in the area. Correspondingly, McClenaghan et al., (2020), using samples taken between 500–3000 m, found consistently higher eDNA yields and less variable numbers of sequence variants across replicates using 1.5 L water samples versus 250 ml. In our turbid estuarine habitat, 500 ml was the maximum volume that could be filtered due to clogging. To ensure that samples from our two sites were comparable, we used this same sample volume at our offshore clear-water site. While our data indicate that a 500 ml sample volume resulted in consistently high numbers of species across replicates in turbid-water (Table 2), this was not the case in the offshore environment where replicates samples of at least 2 L would be recommended.

## 5 | CONCLUSIONS

Our study indicates that water quality can strongly influence the performance of eDNA protocols. Like many nearshore environments, our estuarine study site was highly turbid due to high concentrations

of suspended organic and inorganic material. Processing of these samples resulted in high DNA yields as well as high levels of PCR inhibition. Both power water kits (QPW and OPW) performed well and successfully prevented PCR inhibition in these turbid samples, but they are expensive and at up to $10 USD per sample would be cost-prohibitive for many laboratories. The two blood and tissue kits we tested here (QBT and OMT) also worked well but left behind PCR inhibitors that had to be removed with an additional kit (Zymo Kit). While this step added to the cost, it was still a less expensive option than the power water kits alone. The PCI method, which is the least expensive of the extraction methods, is time-consuming and suffered from levels of PCR inhibition that were not fully alleviated even with the added PCR inhibitor removal step, and thus must be used with care. In contrast, sampling in the low-turbidity coastal ocean environment (clear-water site) resulted in little PCR inhibition but also yielded far less DNA. In the environment, we found high variability among our replicates indicating that much larger volumes of water than the 500 ml (per replicate) we sampled here would be needed to adequately characterize this community.

Based on these results, we would recommend a combination of the less expensive Omega Bio-tek E.Z.N.A Tissue DNA kit followed by a PCR inhibitor removal step using the Zymo Kit or equivalent. Furthermore, we would consider using either the 1.0 or 3.0 µm filter membrane filters in the turbid estuarine system to avoid unnecessary filter clogging. In environments where eDNA concentration is expected to be low, the Omega Bio-tek E.Z.N.A Water DNA should be used with caution as it failed to yield DNA from our clear-water samples. In oligotrophic habitats where eDNA is expected to be in low concentration, we recommend the filtration of at least 2 L per replicate, and while we found no discernable differences based on filter pore sizes, we would still recommend the use of small pore size filters (0.2 or 0.45 µm), unless clogging is an issue.

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## CONFLICT OF INTERESTS

Authors declare no conflict of interest.

## AUTHOR CONTRIBUTIONS

The conception and design of the study were done by M.R.G., G.K., and J.A.E. Samples were collected by J.A.E. and M.R.G. Laboratory experiments were performed by G.K., and data were analyzed and interpreted by G.K., M.R.G., E.F., and A.S. Initial draft of the manuscript was written by G.K., and all the authors edited and improved the initial draft. The project was supervised by M.R.G.

## DATA AVAILABILITY STATEMENT

Raw demultiplexed fastq files have been uploaded to GenBank's Sequence Read Archive (SRA; BioProjectID: PRJNA742717) and the associated metadata can be found at the Genomic Observatories MetaDatabase (GEOME; https://geome-db.org/; Deck et al., 2017). ASV files with read counts and corresponding sequences can be found on Data Dryad (https://doi.org/10.5061/dryad.ghx3ffbp6).

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## SUPPORTING INFORMATION

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

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SPECIAL ISSUE ARTICLE

# One size does not fit all: Tuning eDNA protocols for high- and low-turbidity water sampling

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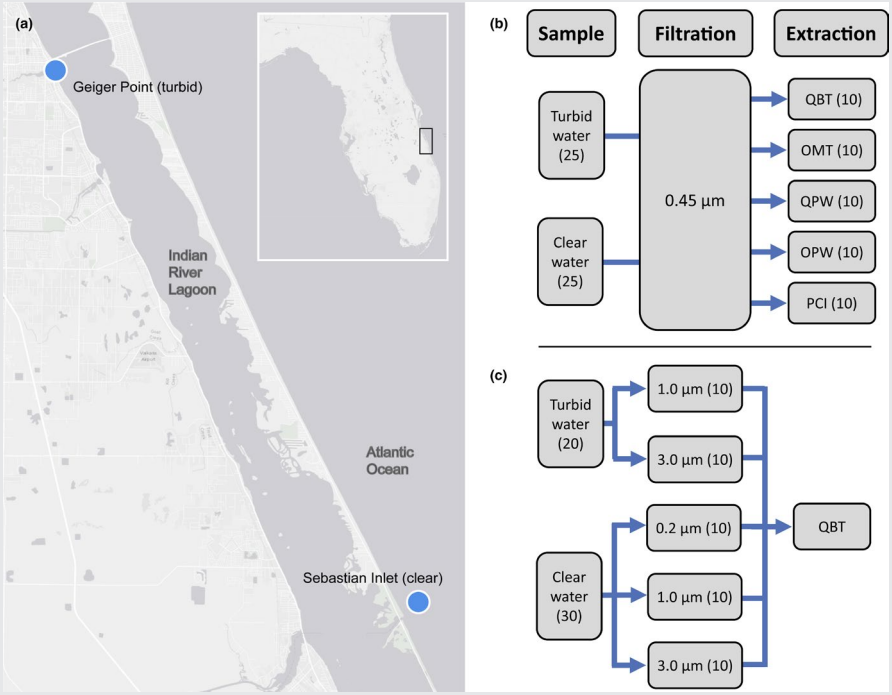
## Graphical Abstract

The contents of this page will be used as part of the graphical abstract of html only. It will not be published as part of main article.




We investigated impact of five different extraction methods and four different filter pore sizes on metabarcoding results of fishes on turbid- and clear-water samples. Our results indicate that the most influential factor on metabarcoding results was water source.