Validating environmental DNA metabarcoding for marine fishes in diverse ecosystems using a public aquarium

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Abstract
Environmental DNA metabarcoding has been widely touted as a powerful tool for monitoring biodiversity in marine ecosystems. However, this method still requires thorough validation and standardization before it can be widely applied for ecological monitoring. The potential utility of environmental DNA metabarcoding is greatest in systems with high levels of diversity, yet environmental DNA metabarcoding has typically been validated in closed systems with relatively low levels of diversity. Additionally, the use of a multiple marker approach has been minimally explored under controlled, closed systems in the literature. Using a pilot study, we assess the ability of eDNA metabarcoding to capture biodiversity in a highly diverse closed system at the Ripley’s Aquarium of Canada in Toronto, Ontario. Our pilot study highlights several key knowledge gaps that must be addressed before metabarcoding can be employed for widespread use in ecological monitoring. We found that environmental DNA metabarcoding recovered just over 50% of target species and 80% of target genera within a closed marine system containing 107 species and 44 genera using previously published markers for COI, 12S, and 16S. Additionally, COI and 12S were found to identify fewer target species than 16S, with COI generating the most off-target identifications, but maximum detection success was achievable by combining all three markers. We discuss numerous key limitations which currently present barriers to the application of eDNA metabarcoding for studying highly diverse marine systems. These include marker selection, data validation and confidence, and the complexity of abundant and diverse novel systems. This study highlights important, yet incompletely resolved, challenges of environmental DNA metabarcoding for the detection of marine fishes in a diverse closed system environment using a multiple marker approach.

KEYWORDS
biodiversity, biomonitoring, closed system, DNA metabarcoding, eDNA, marine, marker selection
1 | INTRODUCTION

Monitoring the diversity of complex aquatic ecosystems is a significantly challenging but fundamental component of applied ecology. Traditionally, ecological monitoring efforts have been conducted using methods requiring intensive and expensive field surveying which require physical capture of an organism in the environment. However, the use of environmental DNA (eDNA) for the detection of aquatic species has seen increasing consideration as a complimentary, and sometimes alternative, approach to ecological monitoring (Cristescu and Hebert, 2018). Once released into the environment, DNA constantly degrades and our ability to record the diversity of eDNA in a system fluctuates over time due to this degradation (Eichmiller, Best, and Sorensen, 2016). As a result, the use of eDNA for ecological monitoring can provide snapshots of the current level of biodiversity existing within an aquatic system.

The advancement of high-throughput sequencing technologies has created opportunities for the collection of greater quantities of increasingly complex sequencing data, but guidelines on the selection of appropriate molecular gene markers are lacking. Reliance on short sequence lengths is often required for aquatic metabarcoding studies due to the suspected highly degraded nature of free eDNA in aquatic environments, though the use of multiple primer sets targeting smaller overlapping fragments to construct larger sequences has seen use in the detection of aquatic eDNA (Zhang, Chain, Abbott, and Cristescu, 2018; Deiner, Renshaw, et al. 2017). Commonly cited metabarcoding markers (such as COI, 12S, and 16S) have a variety of fragment lengths and associated primer sets for use with marine fishes in the literature. When developing eDNA metabarcoding studies, researchers have traditionally selected single markers for identification due to factors like cost and availability of high-quality reference sequence databases. Studies using a multiple marker approach, however, are seeing increased prominence due to the increased confidence that comes with detection by multiple markers (Borrel, Miralles, Do Huu, and Mohammed-Geba 2017; Pitz et al. 2017; Kelly et al. 2017). It is critical that we evaluate whether a multiple marker approach to metabarcoding sufficiently increases the detection of species and confidence in positive detections, or whether such approaches are too cost-prohibitive or limited by insufficient reference sequence availability, to determine whether the use of multiple markers is an efficient way of improving metabarcoding study quality. Understanding the benefits and drawbacks of the different selected gene markers is a key next step to widely using eDNA to characterize diversity in marine environments.

The utility of eDNA metabarcoding has been evaluated by controlled studies in closed systems, such as aquariums, and when evaluated has typically focused on low species abundance ecosystems (Kelly, Port, Yamahara, and Crowder, 2014; Evans et al. 2016). Other studies for testing the effectiveness of eDNA protocols have been conducted in various closed and real-world systems (e.g., Thomsen, Kielgast, Iversen, Moller, et al. 2012; Evans et al. 2017). While studies like these have helped improve our understanding of the success of eDNA metabarcoding, it is still unclear how these findings translate in systems with increased taxonomic diversity. This knowledge gap is disconnected with some of the current real-world applications of eDNA metabarcoding, in which researchers are increasingly turning to high-throughput sequencing methods for large-scale biomonitoring in diverse aquatic ecosystems (Taberlet, Coissac, Pompanon, Brochmann, and Willerslev 2012; Andruszkiewicz et al. 2017; Klymus, Marshall, and Stepien, 2017; Yamamoto et al. 2017) where it is cumbersome and problematic to characterize whole communities by individually assessing the presence of each species (Rees, Maddison, Middleditch, Patmore, and Gough, 2014). However, there need to be more validation and standardization conducted under relevant controlled settings before this tool is used in uncontrollable environments to understand technical, practical, and bioinformatic limitations.

In this study, our objective is to evaluate the effectiveness of multiple marker eDNA metabarcoding in a highly diverse aquatic environment using a large public aquarium as a closed study system. The use of controlled aquaria as a closed system is incredibly valuable because these systems allow for many of the environmental conditions known to influence eDNA recovery to be controlled, including (but not limited to) species presence, relative abundance, species biomass, salinity, system flow rate, and temperature (Barnes and Turner, 2016). In collaboration with Ripley’s Aquarium of Canada located in Toronto, Ontario, Canada, water collected from the “Rainbow Reef” tank was used to assess the ability of eDNA metabarcoding to detect species present within the tank using previously published COI, 12S, and 16S markers for identification. The Rainbow Reef is a marine mesocosm containing 107 Indo-Pacific teleost fish species and is an ideal system for exploring eDNA metabarcoding methods related to diversity and marker selection. Due to practical issues surrounding eDNA metabarcoding identified while conducting this pilot study, the dataset collected is used primarily to frame a discussion around currently unresolved or minimally discussed methodological concerns for conducting eDNA metabarcoding, including marker selection, the erroneous detection of species, and detection success in highly diverse aquatic systems. We discuss the findings of our pilot study in the context of important ongoing concerns regarding the current use of eDNA metabarcoding and its applications in marine environments.

2 | METHODS

2.1 | Study location

The Rainbow Reef tank at Ripley’s Aquarium of Canada in Toronto, Ontario, is a 237,080-L tank with a 75,700-L basin and is inhabited by 107 Indo-Pacific species of teleost fishes representing 44 genera and 17 families (Table S1). Seawater is artificially generated on-site and filtered both mechanically by sand filter and biologically using a degas tower before entering the Rainbow Reef tank through a supply line. The Rainbow Reef tank is held at a target temperature of 24.4°C, a salinity ranging from 31 to 34‰, and a pH ranging from
7.9 to 8.3. The tank has two water inflow sources and three outflow skimmers along the walls and numerous outflow sources hidden among the substrate.

2.2 | Sample collection

Water samples were collected in July of 2015 using 500-mL bottles (Nalgene) sterilized with a 10% bleach solution for at least 30 min, then rinsed three times with de-ionized water, allowed to evaporate dry, and then sealed and opened immediately prior to sample collection. One 1 L water sample was collected directly from the saltwater supply line (referred to as the loop line henceforth) prior to the water entering the Rainbow Reef. Three 1 L water samples were then collected from the center of the Rainbow Reef tank: One was collected by hand at the surface of the tank, and the other two were collected by a diver at the middle and bottom of the tank, respectively. Diving suits are shared between all tanks at Ripley’s Aquarium of Canada and are rinsed with tap water before each dive, presenting an additional source of possible aquatic eDNA belonging to organisms found outside the Rainbow Reef tank. Additionally, a 1 L water sample of de-ionized water prepared in laboratory was used to control against cross-contamination of water samples during processing. Water samples were held on ice until filtration.

Feed samples recently used in the Rainbow Reef tank prior to the sampling date were collected to identify the source of any DNA introduced by the feed. Dry feed samples consisted of New Era® Aegis Pellets and New Era® Large Grazing Diet grazing logs (Balance Aquatic Nutrition). Wet feed samples consisted of krill (Euphausia pacifica and E. superba), Atlantic surf clam (Spisula solidissima), capelin (Mallotus villosus), Atlantic herring (Clupea harengus), rainbow smelt (Osmerus mordax), squid (Loligo sp.), and bok choy (Brassica rapa). All feed samples were collected in sterile Whirl-Paks (Nasco), then held on ice for several hours before being stored at −20°C.

2.3 | Sample filtration

All tools and surfaces were sterilized with a 10% bleach solution for at least 30 min, then rinsed three times with sterile de-ionized water, allowed to evaporate dry, and packaged in UV-sterilized Whirl-Paks prior to filtration. Filtration was conducted in a food preparation room on-site at the aquarium after floors, and countertops had been bleach sterilized. One liter water sample was vacuum-filtered using a sink aspirator pump (Ace Glass Incorporated) onto 4.25 cm 1.5 µm pore Whatman® glass microfiber filters (Whatman). Water samples were filtered in order of collection (Pre-tank Loop Line, Top, Middle, Bottom, and de-ionized water control) and were filtered within one hour of being collected. Filters were then placed inside of sterile petri dishes stored inside sterile Whirl-Paks on ice until extracted.

2.4 | DNA extraction

DNA was extracted at the University of Guelph (ON, Canada) from the filters for each of the five samples within seven hours of filtration using a PowerSoil DNA Isolation Kit (MoBio Laboratories). The PowerSoil DNA Isolation Kit was selected for use as it had been shown to be one of the most successful commercially available DNA extraction kits for maximizing detection of fish species from eDNA captured on filter paper at the time sampling was conducted (Eichmiller, Miller, and Sorensen, 2015). Extractions were conducted inside of a fume hood sterilized with 10% bleach solution for 30 min, then rinsed with de-ionized water once. Filters were extracted in the order of collection using a sterile 3-mm diameter biopsy punch. Each filter was separated in half and each half was then extracted separately, for a total of 10 extractions from aquaria and control water samples. Extractions were conducted following the manufacturer’s protocols and DNA was eluted to a final volume of 100 µl and stored at 4°C. The success of extraction was assessed by examining the fragment size distribution for each extraction using a high-sensitivity dsDNA assay chip run on a 2100 Bioanalyzer (Agilent Technologies) at the Advanced Analysis Center at the University of Guelph (University of Guelph).

DNA was extracted from each of the wet and dry food controls separately using a Qiagen DNeasy Blood and Tissue DNA Extraction kit (Qiagen) following manufacturer’s protocols to a final volume of 200 µl, for a total of 10 extracts from wet and dry foods. Extraction success was confirmed using a NanoDrop® ND-8000. DNA extracts for the wet and dry food samples were pooled to form one food control sample for sequencing.

2.5 | PCR amplicon library preparation and next-generation sequencing

Extracted DNA was amplified using previously published next-generation sequencing PCR primers shown to successfully amplify DNA from marine fishes for 12S, 16S, and COI (Table 1). Primers were selected from the literature based on specificity, taxa, fragment size, and reported amplification success for each marker. An additional COI primer set was initially tested (Shokralla, Hellberg, Handy, King, and Hajibabaei, 2015), but due to poor amplification on the extracted eDNA samples, an alternative COI primer set which did provide amplification was used (Leray et al. 2013). Additionally, all species were tested in silico for primer compatibility when reference sequences were available on GenBank using Geneious Prime® v2019.2.3 (Biomatters Ltd.) (see Table S1) due to the importance of validating the presence of true false negatives (Elbrecht and Leese, 2017).

After testing each of the primer sets on the extracted eDNA samples, all DNA samples were amplified in accordance with the cycling protocols and reagent quantities prescribed within their respective studies. Three PCR reactions were run per sample for each marker, and amplicons were pooled for equal volume
TABLE 1 Specification for primers used in amplicon prep for next-generation sequencing

| Primer name       | Primer sequence (5'−3')           | Target marker | Target fragment size | Citation                        |
|-------------------|-----------------------------------|---------------|----------------------|---------------------------------|
| mtCOI intF        | GGWACWGGWTGAACWGTWTAYCCYCC        | COI           | 313−319 bp           | Leray et al. (2013)             |
| jjHCO2198         | TAIACYTCGGRTGGICCRRAAAAYCA        |               |                      |                                 |
| MiFish-U-F        | GTCGGTAAAACCTGTG GCCAGC           | 12S           | 170 bp               | Miya et al. (2015)              |
| MiFish-U-R        | CATAGTGGGCTAATCCAGTTG             |               |                      |                                 |
| Chord_16S_F_TagA  | ATGGGAGAGACCTRTGGAGCT             | 16S           | 116 bp               | Deagle, Kirkwood, & Jarman (2009)|
| Chord_16S_R_Short | CCTGGGTCCGCCCCAAC                 |               |                      |                                 |

The expected fragment length for each marker to their respective maximum lengths (Table 1). All remaining sequences shorter than the maximum expected fragment length for each marker (whether due to quality truncation or small original amplification size) were padded with “N” bases. This was required for the USEARCH dereplication algorithm to properly compare sequences for uniqueness, as all fragments of different lengths are deemed as being unique de facto (Edgar 2013).

Dereplication of sequences was conducted using the USEARCH command “-DEREP_FULLLENGTH” with size annotations appended to each unique sequence representing the number of times that sequence appeared repeated in the sample. Once dereplicated, sequences were clustered into operational taxonomic units (OTUs) using the USEARCH command “-CLUSTER_OTUS” with a 97% clustering threshold.Singletons were included in the OTU clustering analyses for each marker to increase the likelihood of catching rare species present within the tank. Additionally, during the clustering of OTUs USEARCH evaluated dereplicated sequences for the presence of sequence chimeras and removed them from the analysis (Edgar 2013).

Clustered OTUs were taxonomically identified using the BLASTN search function of GenBank for each marker on 2 January 2020 and were visualized and grouped by their NCBI taxonomy using MEGAN6. A threshold of 97% sequence match to a published reference sequence was required in order for an OTU or group of OTUs to be assigned a species-level identification. Any OTUs with greater than 97% sequence match to more than one species were assessed individually and species identifications made matching the list of known species present in the aquarium were considered as possible, but unconfirmable, identifications. Remaining OTUs with greater than 97% sequence match to one or more species not present in the tank or to the genus-level only were assigned a genus-level identification. Categorization of true and false positives, false negatives, and plausible identifications is explained in Table 2.

2.6 | Bioinformatic analysis

Bioinformatic analyses were conducted using USEARCH version 8.1.1861 (Edgar 2013), MEGAN6 (Huson, Auch, Qi, and Schuster, 2007) and cutadapt version 1.9.1 (Martin 2011) on a Linux-based platform. For all bioinformatic protocols, each gene marker was processed as described by their respective studies to compare each primer set under their previously determined optimal conditions (Table 1). When parameter specifications were unavailable, markers were addressed equally. Raw sequence reads underwent paired-end merging using the “-FASTQ_MERGEPAIRS” command in USEARCH. Sequence merging was conducted with a maximum merged sequence length of 218 bp, 155 bp, and 365 bp for 12S, 16S, and COI, respectively, to eliminate all sequences longer than the expected sequence size with forward and reverse primers attached from the raw sequence reads. Additionally, sequences were merged with a minimum sequence overlap of 10 bp and a maximum percentage mismatch within the overlap of 10%. Once merged, primers were removed from sequences using cutadapt allowing for three, zero, or two mismatches in removed primer sequences for 12S, 16S, and COI, respectively, and with the removal of any erroneous insertions or deletions as a result of primer removal for all markers.

Remaining sequences were put through a highly stringent quality filtering protocol using the USEARCH command “-FASTQ_FILTER.” Sequences were filtered if they had greater than 0 ambiguous bases, if they were shorter than 50% of the total length of their expected final fragment size on a per-marker basis, or if they had a maximum cumulative expected error value for all base pairs in the sequence of 1.0. Additionally, all sequences were truncated at the first base pair with a Phred quality score lower than 25. Sequences successfully passing filtration were truncated again using the “-FASTX_TRUNCATE” command to trim any remaining sequences longer than the expected fragment length for each marker to their respective maximum lengths (Table 1). All remaining sequences shorter than the maximum expected fragment length for each marker (whether due to quality truncation or small original amplification size) were padded with “N” bases. This was required for the USEARCH dereplication algorithm to properly compare sequences for uniqueness, as all fragments of different lengths are deemed as being unique de facto (Edgar 2013).

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Clustered OTUs were taxonomically identified using the BLASTN search function of GenBank for each marker on 2 January 2020 and were visualized and grouped by their NCBI taxonomy using MEGAN6. A threshold of 97% sequence match to a published reference sequence was required in order for an OTU or group of OTUs to be assigned a species-level identification. Any OTUs with greater than 97% sequence match to more than one species were assessed individually and species identifications made matching the list of known species present in the aquarium were considered as possible, but unconfirmable, identifications. Remaining OTUs with greater than 97% sequence match to one or more species not present in the tank or to the genus-level only were assigned a genus-level identification. Categorization of true and false positives, false negatives, and plausible identifications is explained in Table 2.

2.7 | Data analysis

Identification success for each marker was determined as the number of positive taxonomic identifications made at the species- and genus-level for all taxa present on the list of species for the tank that had at least one published reference sequence available.
Secondly, the amount of off-target identification noise was determined by comparing the proportion of OTUs which identified to a specific phylum (Chordata, Mollusca, Arthropoda, and Cnidaria) or as belonging to any other phyla which received low OTU clustering (Other). Species identified within both controls and tank samples were not removed before these analyses were conducted, but rather were accounted for later in downstream interpretations of detection success.

Logistic regression was used to determine whether variable detection success of different species was a result of differences in abundance of those species for each of the three markers (using a GLM in R v. 3.6.2). Each logistic regression was compared to a null model to evaluate the significance of abundance in predicting detection success. Species that were undetectable for each marker because of a lack of reference sequences were removed from this analysis (8 species for 12S, 11 species for 16S, and 2 species for COI were removed, respectively, see Table S1).

## Results

### Sequencing results

Sequences were successfully generated from all treatments and controls for all three markers tested. Sequencing efforts generated a total of 28,079,780 raw unpaired sequence reads across all samples, and stringent quality filtering resulted in 2,189,462 high-quality pair-merged sequences for 12S, 3,753,631 high-quality pair-merged sequences for 16S, and 724,525 high-quality pair-merged sequences for COI. Of these, 10,728, 1,012,185, and 101,072 sequences (reflecting 0.490%, 26.97%, and 13.95% of filtered sequences) were generated from the aquarium tank samples for 12S, 16S, and COI, respectively. After dereplication, sequence chimeras were successfully removed and remaining sequences were successfully clustered into OTUs. For a summary of sequence processing, refer to Table 3.

### 3.2 Identification success

In silico primer binding tests found that each marker had differing levels of primer binding site presence in sequences available from GenBank (Table S1). The 16S marker selected bound both primers to at least one reference sequence for 89 species, while 12S matched to 66 species and COI to 27 species. However, the COI marker had 80 species where at least one primer bound and the remaining reference sequence was too short to complete the expected fragment length, while 12S had 34 species and 16S had 6 species which met this criterion. Lastly, the 12S marker could not bind to reference sequences for 31 species while COI could not bind to 7 species and 16S to 6 species.

For all markers, a greater proportion of genus-level identifications were made than species-level identifications when comparing to the list of known species (Figures 1 and 2). The 12S marker produced 13 species-level identifications (4 unique to 12S) and 16 genus-level identifications (0 unique to 12S), missing a possible 51 species-level and 27 genus-level identifications based on available reference sequences. The 16S marker produced 48 species-level identifications (37 unique to 16S) and 35 genus-level identifications (16 unique to 16S), missing a possible 21 species-level and 7 genus-level identifications based on available reference sequences. The COI marker produced 14 species-level identifications (37 unique to COI) and 35 genus-level identifications (16 unique to COI), missing 54 species-level and 28 genus-level identifications based on available reference sequences. When pooling all markers tested, a total of 54 species (50.9%) and 35 genera (79.5%) were successfully identified with 20 species (18.9%) and 9 genera (20.5%) missed.

### 3.3 Off-target identification noise

When compared to 12S and 16S, the selected COI marker generated a much greater proportion of OTUs which identify either as
nontarget taxa or taxa which could not be confirmed to be present in the tank samples, which we refer to as off-target identification noise. When grouping identifications by phyla, 5.11% of COI OTUs identified to be the phylum Chordata (with 3.25% to fish and 1.86% to nonfish chordates) while 94.19% (91.86% to fish and 2.33% to nonfish chordates) and 37.41% (35.12% to fish and 2.29% to non-fish chordates) of 12S and 16S OTUs, respectively, identified as chordates (Figure 3). Of the 5.81% of off-target taxa identified by 12S, 1.74% represent Cnidaria, and 0.58% represent other phyla. Of the remaining OTUs, 1.16% do not identify to phylum level, 1.16% could not be assigned identifications, and 1.16% had no matching hits when compared against GenBank. Of the 62.59% of off-target taxa identified by 16S, 2.77% represent Cnidaria, <0.01% represent Porifera, 0.09% represent Arthropoda, 0.02% represent Mollusca, and 1.17% represent all other phyla. Of the remaining OTUs, 0.16% do not identify to phylum level, 0.04% could not be assigned identifications, and 58.34% had no matching hits when compared against GenBank. Lastly, of the 94.89% of off-target taxa identified by COI,
6.82% represent Cnidaria, 2.30% represent Porifera, 15.19% represent Arthropoda, 1.97% represent Mollusca, and 35.89% represent all other phyla. Of the remaining OTUs, 21.25% do not identify to phylum level, 1.46% could not be assigned identifications, and 10.19% had no matching hits when compared against GenBank. Similar trends were observed when looking at sequences recovered overall from the negative (de-ionized water and loop line) and positive (feed) controls.

4 | DISCUSSION

4.1 | Using a plurality of markers to maximize utility

We found that detection success varied greatly across the genetic markers, but that all three markers had notable difficulties with capturing biodiversity in a highly diverse system. Overall, 20% of all known species with reference sequences (Figure 1) and 20.5% of all known genera with reference sequences (Figure 2) were undetected by any marker tested. Regardless of the level of taxa assessed, 16S was found to outperform both COI and 12S, both of which perform similarly poorly at the species-level (Figure 1) and genus-level (Figure 2). In our study, the 12S marker only certainly identified 13% of species with reference sequences; however, other studies published in the literature conducted in closed systems have found 12S able to detect over 90% of species present across four aquaria (Miya et al. 2015) and 87.5% in the Monterey Bay Aquarium (Kelly et al. 2014). One contributing factor to this low rate of success could
be quality of reference sequence availability. Reference sequences which bound the 12S primer set in silico were found less frequently than the 16S marker tested; however, when aligning small 12S sequences with whole mitochondrial genomes many sequences which could not bind the primers were found to align in between the primers used. Despite this, many of these sequences are small in nature and this may have affected the ability for generated sequences to meet bioinformatic thresholds required for species identification. Additionally, the relatively low identification success and relatively high off-target identification noise (Figure 3) suggest that the COI marker selected performed poorly as an identification marker in this study. The standard COI barcode region is known to have issues with reliable species discrimination when only using partial fragments of the full-length sequence due to fewer conserved sites for primers to bind to inside the COI barcode (Deiner, Renshaw, et al. 2017; Miya et al. 2015; Shokralla et al., 2015; Zhang et al., 2018). Indeed, when testing the COI marker used in this study in silico it was found that the majority of taxa present in the sampled waters had the target fragment span across the 3’ end of the standard COI DNA barcode region and this likely influenced the ability to generate species-specific identifications for this marker. Overall, we found eDNA metabarcoding was less successful at generating species-level or genus-level identifications in our study than would be expected based on previous literature and quality of available reference sequence libraries likely influenced the level of success observed.

We found that the maximum number of species- and genus-level identifications were made when utilizing all three markers together (Figures 1 and 2, “Total”) due to the presence of uniquely identified species detected by each marker. In our study, we also found that each marker differed in the resolution and consistency in which they could identify specific families and genera. Typically, genera belonging to the families Acanthuridae, Chaetodontidae, Ephippidae, Kuhlidae, Labridae, Pomacentridae, and Siganidae were detected across markers, with the genera Acanthurus, Siganus, Kuhlia, and Naso being the most consistently found across markers. Interestingly, there is no readily apparent relationship between intragenus diversity, abundance, and likelihood of detection, with some of the least abundant taxa from diverse genera (e.g., Naso unicornis, Acanthurus mata, or ChatoDON vagabundus) being identified across markers while some of the most abundant taxa from minimally represented genera (e.g., Sphaeramia nematoptera and Zebrasoma velifer) are not. There were notable differences in marker success, however, in the resolution of identifications within diverse genus, with 16S consistently distinguishing between species within the genera Acanthurus, Chaetodon, Naso, and Pomacanthus when COI and 12S could not. As previously described, the quality and coverage of available reference sequences for the COI and 12S marker likely play a major role in the ability to differentiate the more diverse genera present in this study. Despite the relatively low success of individual markers, the use of multiple markers in this study allowed for the overlapping recovery of taxa and increased confidence in identifications in the dataset.

It is also important to note that the interpretation of successful identifications in this study differs based on whether individual or multiple markers are being considered. This is an important distinction as detection success has not been consistently defined in the literature (Evans et al. 2017). In some cases, multiple markers may be evaluated at the start of an experiment based on likelihood of success on a group of taxa, but later reduced to the use of just one marker in practice (e.g., Klymus et al. 2017). Occasionally, multiple markers have been employed to increase the coverage of taxa that can be targeted (Borrell et al. 2017; Menning, Simmons, and Talbot, 2018; Kelly et al. 2017). However, attempts to use multiple markers for detection can suffer from bioinformatic challenges, such as marker-specific parameterization and stringency with error removal (Pitz et al. 2017; Evans and Lamberti, 2018). Research has started to become available exploring solutions to the bioinformatic challenges that come with multiple marker eDNA metabarcoding (Alberdi et al. 2017; Deiner, Bik, et al. 2017) and the field would benefit from continued research in this area. Collaborative efforts to create standards for working with eDNA have occurred in response to a need for increased consistency in field and laboratory protocols (Goldberg,
FIGURE 4 Detection by Species Abundance. Logistic regression of species abundance against binary success in species detection (0 = undetected, 1 = detected). Log-transformed species abundance was found to significantly predict detection success for 12S ($p = .0097$) but did not significantly predict detection success for the COI ($p < .1804$) or 16S ($p = .4481$) markers tested. All markers successfully failed to detect some of the most highly abundant species while also detecting some of the most rare species present in the tank.
and would be similarly beneficial to incorporate bioinformatic frameworks in these efforts as well. Our dataset highlights the importance of using multiple markers for eDNA metabarcoding and draws attention to unresolved experimental and bioinformatic issues which influence our ability to interpret multiple marker study data.

4.2 Validating successful identification by assessing detection errors and contamination

In addition to a greater number of true positive identifications, we found that both the 16S and 12S markers tested generated fewer false positive identifications than did the tested COI marker and were found to generate a much greater proportion of identifications to marine fish taxa than to nonmarine fish taxa (Figure 3). This suggests that both the 16S and 12S markers selected are more highly specific to the recovery and identification of marine fish eDNA than the COI marker tested. The COI marker amplified a greater proportion of nonchordate taxa when compared to both the 16S and 12S markers, which is unsurprising because it was originally designed to broadly target metazoans for marine mammal gut content analyses (Leray et al., 2013). For 12S and 16S, all nonlist species identified were also detected within the loop line and food controls or were likely closely related misidentified species. Examples of taxa that were considered false positive for these markers included squid, clams, and various freshwater salmon species. However, a large proportion of identifications that were made for COI were unique to the tank sample when compared to controls, suggesting presence in the Rainbow Reef, but could not reasonably be assumed to be present within the tank. Examples of taxa that we considered to be false positive identifications for the COI marker include a variety of terrestrial mammals, beetles, flies, moths, and millipedes. Off-target identifications in eDNA metabarcoding studies can be problematic because they may reduce the number of useful sequences detected, and because determining the accuracy of such identifications may not be possible in open ecosystems. Using a multiple marker approach allows us to more confidently parse through the expected and unexpected species identifications recovered during metabarcoding within this study.

We found strong evidence of contamination within our dataset despite many precautions taken to prevent and minimize contamination. Contaminants included the detection of common freshwater fish species studied within our laboratory space, terrestrial species known to be studied within our research facility, and species known to be present within the aquarium in other unsampled and separated tanks (such as sharks or macroinvertebrates) in the water blank controls included. These contaminants were also detected despite the single-use nature of many tools, the rigorous sterilizations of tools and workspaces which were reused, and the protection and preservation of filters and extracts during and after processing. It is difficult to identify the source of all contaminations identified within this study; however, some potential sources could include incomplete sterilization of DNA from diving equipment between aquaria within the facility, or contamination of sterilized tools in transit or sample processing and an increased frequency of decontamination may have helped to resolve some of these issues. The highly sensitive nature of eDNA analysis means that contamination poses a serious and widespread issue for maintaining confidence in research findings. Strategies have been suggested for minimizing the risk of contamination with the use of clean workflows (Goldberg et al., 2016) or for quantifying the level of contamination present in samples (Ficetola et al., 2015; Ballenghien et al., 2017).

Though eDNA metabarcoding studies commonly note that contamination was detected and accounted for, the methodological actions taken to account for contamination are minimally reported or explained within studies. One potential solution involves screening out contaminants based on expected species within the ecosystem, which may be viable when there is an expectation of local species compositions (i.e., freshwater vs. marine and local histories of biodiversity) (Shaw et al., 2016). Other strategies include the use of mock community controls (Klymus et al., 2017; Bylemans et al. 2018) and attempt to use species occupancy modelling for the confirmation of a detection (Pitz et al., 2017). Alternatively, cross-contamination thresholds with control samples have been used for determining an acceptable level of detection required for inclusion (Evans et al., 2017; Serrao et al., 2017). However, when there is no a priori knowledge of local species composition the screening process could bias species recovery with increased false negatives for species truly present which were not predicted beforehand. This is a fundamental shortcoming when looking to apply this tool for the assessment of novel aquatic environments or aquatic environments undergoing rapid change in community composition due to ecological or anthropogenic disturbance—some of the most potentially valuable applications of eDNA metabarcoding (Taberlet, Coissac, Hajibabaei, et al., 2012; Valentini et al., 2016). Before a wider adoption of metabarcoding for biomonitoring takes place, it is important to further explore a standardized approach (in terms of sampling, bioinformatic processing, and analyses) to assess and account for contamination to increase the confidence of recovered and reported data.

4.3 Suitability for highly diverse and novel systems

We found that detection success was only weakly related to the abundance of a species in the aquarium, suggesting that abundance alone does not reliably predict detection success with eDNA metabarcoding (Figure 4). While only 12S showed a significant relationship between detection and organismal abundance, all three markers successfully detected several species in very low abundance and failed to detect several species in high abundance (Figure 4), including abundant species which were confirmed in silico as being compatible with the selected primer sets (See Table S1). Though the sampling efforts in this study were restricted for use as a pilot study to mimic efforts for individual sampling sites originally, additional sequencing replication could increase the
Monitoring and surveying complex aquatic ecosystems with high levels of diversity are a significant challenge due to the amount of expertise, time, effort, and money required. eDNA metabarcoding is a promising solution to a number of these issues and is likely to become more refined with time and further research. eDNA metabarcoding has seen extensive application due to the potential power and promises it can bring to species detection projects; however, before eDNA metabarcoding can be reliably and widely employed for aquatic bio-monitoring, there is still much work to be done to further understand its uses and limitations. This study highlights limitations related to the assessment of eDNA metabarcoding for the detection of marine fishes using a multi-marker approach in a large controlled closed ecosystem, though these limitations likely apply to taxa beyond just marine fishes. Additionally, we discussed key uncertainties related to marker selection and use, validation of findings, and the suitability and use of this tool in highly diverse systems that need to be explored more extensively before eDNA metabarcoding should see wider adoption in bio-monitoring applications outside of the research community. Future research should continue to address the effectiveness of multiple marker eDNA metabarcoding with increased levels of biodiversity and should further address the potential limitations of different markers before a marker receives widespread adoption or rejection. Once the critical limitations of eDNA metabarcoding are addressed, this technique could very well live up to its potential as a powerful biomonitoring tool for marine ecosystems.

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AUTHORS CONTRIBUTIONS

KCM, TJB, and RHH designed the experiment; KCM and TJB conducted field collections and sample processing; KCM conducted the laboratory work; KCM and TJB conducted the data analysis; KCM, TJB, and RHH interpreted the data; KCM, TJB and RHH wrote and revised the manuscript.

DATA AVAILABILITY STATEMENT

Sequence reads generated for this study have been archived in the Sequence Read Archive hosted by the National Center for Biotechnology Information (NCBI), with the accession number PRJNA604594.

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REFERENCES

Andruszkiewicz, E. A., Starks, H. A., Chavez, F. P., Sassoubre, L. M., Block, B. A., & Boehm, A. B. (2017). Biomonitoring of marine vertebrates in Monterey Bay using eDNA metabarcoding. *PLoS One*, 12(4), e0175343. https://doi.org/10.1371/journal.pone.0175343

Alberdi, A., Aizpurua, O., Gilbert, M. T. P., & Bohmann, K. (2017). Scrutinizing key steps for reliable metabarcoding of environmental samples. *Methods in Ecology and Evolution*, 9, 134–147. https://doi.org/10.1111/2041-210X.12849
sequencing with multiple genetic markers for marine observation programs. *Biodiversity Information Science and Standards*, 1, e20548. https://doi.org/10.3897/tdwproceedings.1.20548

Rees, H. C., Maddison, B. C., Middleditch, D. J., Patmore, J. R. M., & Gough, K. C. (2014). The detection of aquatic animal species using environmental DNA – a review of eDNA as a survey tool in ecology. *Journal of Applied Ecology*, 51(5), 1450–1459. https://doi.org/10.1111/1365-2664.12306

Serrao, N. R., Reid, S. M., & Wilson, C. C. (2017). Establishing detection thresholds for environmental DNA using receiver operator characteristic (ROC) curves. *Conservation Genetics Resources*, 10(3), 1–8. https://doi.org/10.1007/s12686-017-0817-y

Shaw, J. L. A., Ckarjem, K. C. M., Wedderburn, S. D., Barnes, T. C., Weyrich, L. S., & Copper, A. (2016). Comparison of environmental DNA metabarcoding and conventional fish survey methods in a river system. *Biological Conservation*, 197, 131-138. https://doi.org/10.1016/j.biocon.2016.03.010

Shokralla, S., Hellberg, R., Handy, S. M., King, I., & Hajibabaei, M. (2015). A DNA mini-barcoding system for authentication of processed fish products. *Scientific Reports*, 5, 15894. https://doi.org/10.1038/srep15894

Taberlet, P., Coissac, E., Pompanon, F., Brochmann, C., & Willerslev, E. (2012). Towards next-generation biodiversity assessment using DNA metabarcoding. *Molecular Ecology*, 21(8), 2045-2050. https://doi.org/10.1111/j.1365-294X.2012.05470.x

Taberlet, P., Coissac, E., Hajibabaei, M., & Rieseberg, L. H. (2012). Environmental DNA. *Molecular Ecology*, 21(8), 1789-1793. https://doi.org/10.1111/j.1365-294X.2012.05542.x

Thomsen, P. F., Kielgast, J., Iversen, L. L., Moller, P. R., Rasmussen, M., & Willerslev, E. (2012). Detection of a diverse marine fish fauna using environmental DNA from seawater samples. *PLoS One*, 7, e41732. https://doi.org/10.1371/journal.pone.0041732

Thomsen, P. F., Kielgast, J., Iversen, L. L., Wiuf, C., Rasmussen, M., Gilbert, M. T. P., ... Willerslev, E. (2012). Monitoring endangered freshwater biodiversity using environmental DNA. *Molecular Ecology*, 21(11), 2565-2573. https://doi.org/10.1111/j.1365-294X.2011.05418.x

Valentini, A., Taberlet, P., Miaud, C., Civade, R., Herder, J., Thomsen, P. R., ... Dejean, T. (2016). Next-generation monitoring of aquatic biodiversity using environmental DNA metabarcoding. *Molecular Ecology*, 25(4), 929–942. https://doi.org/10.1111/mec.13428

Wilcox, T., Mkelvey, K. S., Young, M. K., Jane, S. F., Lower, W. H., Whiteley, A. R., & Schwartz, M. K. (2013). Robust detection of rare species using environmental DNA: The importance of primer specificity. *PLoS One*, 8, e59520. https://doi.org/10.1371/journal.pone.0059520

Yamamoto, S., Masuda, R., Sato, Y., Sado, T., Araki, H., Kondoh, M., ... Miya, M. (2017). Environmental DNA metabarcoding reveals local fish communities in a species-rich coastal sea. *Scientific Reports*, 7, 40368. https://doi.org/10.1038/srep40368

Zhang, G. K., Chain, F. J. J., Abbott, C. L., & Cristescu, M. E. (2018). Metabarcoding using multiplexed markers increases species detection in complex zooplankton communities. *Evolutionary Applications*, 11(10), 1901-1914. https://doi.org/10.1111/eva.12694

Zhou, J., Wu, L., Deng, Y. E., Zhi, X., Jiang, Y.-H., Tu, Q., ... Yang, Y. (2011). Reproducibility and quantitation of amplicon sequencing-based detection. *The ISME Journal*, 5, 1303-1313. https://doi.org/10.1038/isrne.2011.11

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