Gene 928 (2024) 148720

Contents lists available at ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

Research paper

Ecological forensic testing: Using multiple primers for eDNA detection of marine vertebrates in an estuarine lagoon subject to anthropogenic influences

Kelcie L. Chiquillo[a,c,1,*], Juliet M. Wong[b,c,1,**], Jose M. Eirin-Lopez[c]

[a] Department of Biology, University of Puerto Rico Río Piedras, P.O. Box 23360, San Juan, PR 00931 USA
[b] Division of Marine Science and Conservation, Nicholas School of the Environment, Duke University Marine Lab, Beaufort, NC, USA
[c] Environmental Epigenetics Laboratory, Institute of Environment, Florida International University, Miami, FL, USA

ARTICLE INFO

Edited by: Carolina Bonin

Keywords:
Metabarcoding
Biscayne Bay
Biodiversity
Urbanization
Environmental Stress
Monitoring
Vertebrates

ABSTRACT

Many critical aquatic habitats are in close proximity to human activity (i.e., adjacent to residences, docks, marinas, etc.), and it is vital to monitor biodiversity in these and similar areas that are subject to ongoing urbanization, pollution, and other environmental disruptions. Environmental DNA (eDNA) metabarcoding is an accessible, non-invasive genetic technique used to detect and monitor species diversity and is a particularly useful approach in areas where traditional biodiversity monitoring methods (e.g., visual surveys or video surveillance) are challenging to conduct. In this study, we implemented an eDNA approach that used a combination of three distinct PCR primer sets to detect marine vertebrates within a canal system of Biscayne Bay, Florida, an ecosystem representative of challenging sampling conditions and a myriad of impacts from urbanization. We detected fish species from aquarium, commercial, and recreational fisheries, as well as invasive, cryptobenthic, and endangered vertebrate species, including charismatic marine mammals such as the protected West Indian manatee, *Trichechus manatus*. Our results support the potential for eDNA analyses to supplement traditional biodiversity monitoring methods and ultimately serve as an important tool for ecosystem management. This approach minimizes stress or disturbance to organisms and removes the intrinsic risk and logical limitations of SCUBA diving, snorkeling, or deploying sensitive equipment in areas that are subject to high vessel traffic and/or low visibility. Overall, this work sets the framework to understand how biodiversity may change over different spatial and temporal scales in an aquatic ecosystem heavily influenced by urbanization and validates the use of eDNA as a complementary approach to traditional ecological monitoring methods.

1. Introduction

Current approaches to monitoring biodiversity include metabarcoding from environmentally derived samples (Deiner et al., 2017, Aylagas et al., 2018). Environmental DNA (eDNA) metabarcoding uses genetic material captured from environmental samples to query species' presence and ecosystem biodiversity (Taberlet et al., 2012). Its application is a valuable biological monitoring tool (Ruppert et al., 2019, Beng and Corlett, 2020, Gold et al., 2021, Rourke et al., 2022) that leverages that organisms constantly shed their DNA through their feces,

Abbreviations: eDNA, Environmental DNA; Niskin, A type of water sampling bottle; L, Liters; km, Kilometers; DNA, Deoxyribonucleic Acid; rRNA, Ribosomal Ribonucleic Acid; PCR, Polymerase Chain Reaction; MiFish, Primer set for metabarcoding analysis for 12S rRNA region; MarVer1, Primer set for metabarcoding analysis 12S rRNA region; MarVer3, Primer set for metabarcoding analysis 16S rRNA region; µM, Micromolar; mM, Millimolar; BSA, Bovine Serum Albumin; DI, Deionized water; UV, Ultraviolet light; HABs, Harmful Algal Blooms; kb, Kilobases; UF ICBR, University of Florida's Interdisciplinary Center for Biotechnology Research; ASV, Amplicon Sequence Variant; MIDORI2, A database of eukaryotic mitochondrial sequences; Chordates, A phylum of animals with a notochord; R, The programming language used for statistical computing and graphics; IUCN, International Union for Conservation of Nature; NCBI, National Center of Biotechnology Information; BLASTN, Basic Local Alignment Search Tool for Nucleotides.

* Corresponding author at: University of Puerto Rico Río Piedras, PO Box 23360, San Juan, PR, USA.
** Corresponding author at: Duke University Marine Lab, 135 Duke Marine Lab Rd, Beaufort, NC 28516, USA.
E-mail addresses: kelcie.chiquillo@upr.edu (K.L. Chiquillo), juliet.wong@duke.edu (J.M. Wong), jeirinlo@fiu.edu (J.M. Eirin-Lopez).
[1] Co-first author.

https://doi.org/10.1016/j.gene.2024.148720
Received 1 March 2024; Received in revised form 12 June 2024; Accepted 20 June 2024
Available online 25 June 2024
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urine, mucus, skin cells, etc., leaving behind a biological signature in their environment. By collecting and analyzing eDNA, it is possible to trace and identify the species present in a given study area (Baldigo et al., 2017, Carvalho et al., 2022, Carvalho and Leal, 2023). Because it is a non-invasive genetic technique, species presence can be determined with minimal impact or disturbance to the ecosystem or the organisms themselves (Ruppert et al., 2019, Beng and Corlett, 2020, Castro-Cubillos et al., 2022).

The number of published papers describing and using eDNA rapidly rose between 2008 and 2020, increasing by ~ 4000 % during that time (Beng and Corlett, 2020). Environmental DNA approaches have been used for a variety of ecology and conservation studies across a wide range of habitats and taxa, from microbes to plants to megafauna (Tsuji et al., 2019). It can provide improved species detection (Senapati et al., 2019), particularly of cryptic (Bessell et al., 2023), rare (Perl et al., 2022), endangered (Qu and Stewart, 2019), invasive (Larson et al., 2020), nocturnal (Shiozuka et al., 2023), or highly mobile taxa (West et al., 2021, Easson et al., 2020). Additionally, it has been demonstrated to be instrumental in monitoring species diversity (Hinlo et al., 2017; Monuki et al., 2021) and distribution (Thomsen et al., 2016; Sanchez et al., 2022). Furthermore, eDNA metabarcoding has been proven effective for monitoring harmful biological agents (Marshall and Stepien, 2019), understanding host-parasite relationships, predator–prey trophic interactions (Moran et al., 2019, Mata et al., 2019), and identifying spawning sites and different life cycle stages (Takeuchi et al., 2019, Bracken et al., 2019).

Environmental DNA metabarcoding has been and continues to be used to detect species within areas that are subject to anthropogenically-derived stressors, often where traditional biodiversity monitoring methods can be challenging to implement (Thomsen et al., 2012; Lacoursière-Roussel et al., 2016). Traditional surveying methods depend on the physical identification and characterization of species by counting individuals in the field (Brock, 1982). In environments in which traditional visual monitoring methods are challenging to conduct due to limited site accessibility (Biggs et al., 2015) or poor water clarity (Kundu and Kumar, 2018; Rourke et al., 2023), an eDNA approach can be used to complement or substitute these methods for the purpose of assessing biodiversity. Its application for marine conservation efforts is appealing, as experiments have shown that eDNA can routinely detect more species than traditional monitoring techniques (Boussarie et al., 2018, Port et al., 2016, Stat et al., 2019, Afzali et al., 2021, Gold et al., 2023). Thus, in ecosystems facing a myriad of impacts such as urbanization, and where traditional surveying methods are challenging to perform, it is critical to implement eDNA-based species detection.

Biscayne Bay in South Florida is a representative model ecosystem in which to assess the potential of eDNA to study biodiversity in highly developed coastal areas. It is a subtropical lagoon that is threatened by anthropogenically derived stressors and is valued for its wildlife and ecosystem services (Serafy et al., 2003). Covering over 400 square miles, Biscayne Bay includes two state aquatic preserves, a critical wildlife area, a national park, and a national marine sanctuary. It is home to critical wildlife that inhabit shoreline, hardbottom, mangrove, and seagrass communities (Gregg, 2013, Lirman et al., 2019). It also provides numerous ecosystem services to people living in and visiting South Florida, serving nearly 2.8 million residents and millions of visitors annually (Alleman, 1995, https://www.miamidade.gov/). Given its close proximity to human activity, Biscayne Bay is subject to increasing levels of urbanization, pollution, and other environmental disruptions (Santos et al., 2016, Singh et al., 2010, Castillo et al., 2023, Meeder and Boyer, 2001, Lapointe et al., 2004, Harlem, 1979). Biscayne Bay has also suffered extensive harmful algal blooms and seagrass die-offs, which have led to recurrent massive fish mortality events of increasing magnitude (Robblee et al., 1991, Lapointe et al., 2004; Tiling and Edward Proffitt, 2017).

Biscayne Bay has been the subject of multiple diversity and conservation studies, such as the identification of nursery habitats for hammerhead sharks (Macdonald et al., 2021). Unfortunately, traditional methods for monitoring biodiversity, such as visual surveys or video surveillance, can face significant challenges because much of the Bay, especially in and around beaches and canals, is subject to high vessel traffic which can be hazardous to snorkelers, divers, or the deployment of sensitive equipment. Additionally, several waterways within Biscayne Bay have poor water visibility due to high turbidity (Caccia and Boyer, 2005). In spite of these challenges, no prior biodiversity studies have incorporated the use of eDNA in Biscayne Bay with the exception of a study that aimed to optimize eDNA extraction methods (Anderson and Thompson, 2022). Furthermore, most eDNA studies conducted throughout other areas of Southeast Florida have been restricted to single species, or communities inhabiting rivers and other waterways (Orzechowski et al., 2019, Hunter et al., 2019, Hunter et al., 2018).

The study presented here contributes to the pioneering efforts of using eDNA approaches to assess the biodiversity of vertebrates within areas of Biscayne Bay that are characterized by high human activity (e.g., boating, fishing, land use/development), low water visibility, and the influence of anthropogenic environmental stressors. The obtained results support the feasibility and benefits of using an eDNA approach in areas that are difficult to access and where traditional biodiversity monitoring methods are challenging to employ. Importantly, this study establishes a framework for monitoring biodiversity in Biscayne Bay by creating a baseline against which future studies can be compared. Overall, this work supports the integration of eDNA approaches into standard biomonitoring schemes to fine-tune biodiversity assessments.

2. Materials and methods

2.1. Field sites

For this study, vertebrate animals were targeted for eDNA analyses because they encompass a wide variety of categories commonly prioritized for ecology and conservation studies, such as endangered species, commercially valuable species, cryptobenthic species, and nonnative species; many of these species fulfill critical roles in aquatic ecosystems (Kelley et al., 2016) and provide a source of protein for humans (Tucker and Rogers, 2014). Sampling sites were selected within two areas of Biscayne Bay that are approximately 30 miles apart from each other (Fig. 1A), based on their diversity of benthic habitat structure (e.g., seagrass habitat, mud/sand, or coral reef) as well as proximity to human activity and sources of environmental stressors (i.e., recreational boating, fishing, and both public and private land use), representing the diversity of habitats found in Biscayne Bay (Supplementary Table 1).

Seven field sites were selected in total: one site was selected within northern Biscayne Bay adjacent to Florida International University's Biscayne Bay Campus (FIU BBC) (Fig. 1B) and is frequented year-round by tourists to Oleta River State Park, which features a hardbottom, rocky substrate with sparse stony corals, amid a historically bulkheaded shoreline with limited mangroves and declining benthic vegetation (Cantillo et al., 2000). This site served as a comparative site to Sites 1–6 at Paradise Point which is primarily a private residential area in Palmetto Bay, Miami-Dade County, FL, bordered to the east by the Deering Estate North Addition Preserve and near the Deering Bay Marina (Fig. 1C), to further test the eDNA approach in areas with low visibility and high boat traffic, usually areas difficult to sample with traditional surveys.

2.2. Sample collection and water filtration

Water sampling was conducted across two consecutive days on April 28 (Site 7) and April 29 (Sites 1—6) in 2022. This minimized the potential effects of seasonal variation in biological, chemical, and environmental conditions across sites. Water collections were performed (n = 3 replicate deployments) at each site using a 5 L Niskin bottle at a

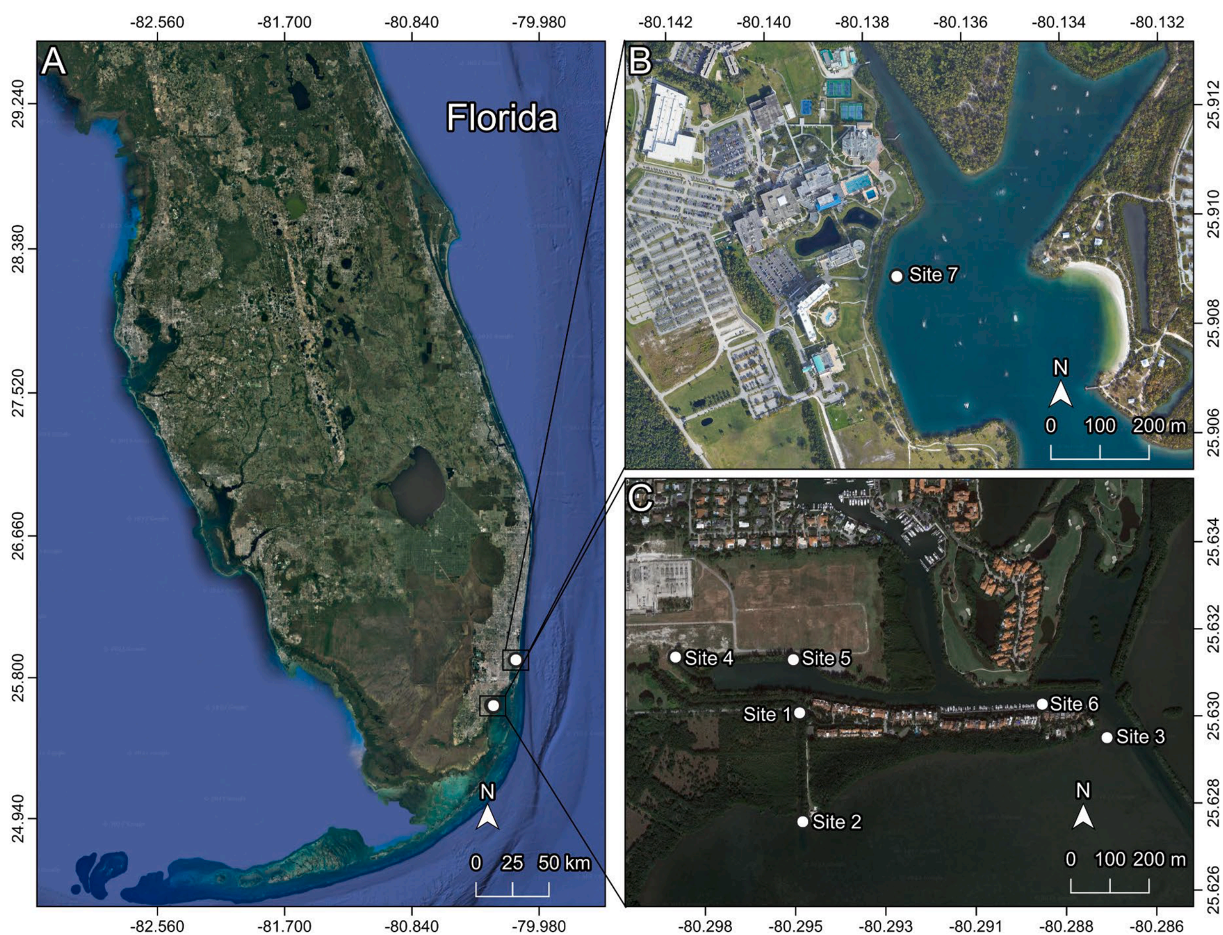

Fig. 1. Sampling locations in Biscayne Bay, Florida. **A.** Water sampling sites in two areas of Biscayne Bay, South Florida. **B.** Site 7 was located near Florida International University's Biscayne Bay Campus. C. Sites 1—6 were located further south in Biscayne Bay, within a canal system at Paradise Point, adjacent to the Deering Estate North Addition Preserve and private residences.

depth of 0–1 m. The water from each Niskin deployment was stored within three 1.5 L water collection bottles that had been previously cleaned via immersion in a 5 % bleach solution, followed by a DI water rinse, a 70 % ethanol rinse, and a final sterilization under ultraviolet light for 15 min. Prior to sampling each site, all collection equipment (5 L Niskin bottle, plastic tubing) was sterilized using 5 % bleach and rinsed with deionized (DI) water. This was followed by the collection of a negative field control sample, in which the Niskin bottle was used to collect 1.5 L of DI water. Negative field controls were used to detect and remove any incidental DNA contamination introduced to the samples during collection and processing. Sampling produced nine 1.5 L field samples and one 1.5 L negative control sample for each site, for a total of n = 70 water samples across all sites. All water samples were immediately transported to facilities at FIU BBC where they were frozen at −20 °C until processing. A vacuum pump (model: Rocker300, 23 L/min) was used to filter each water sample through a 0.22 μM Sterivex filter. All filtration processes were conducted within a clean fume hood to minimize potential contamination. All equipment was sterilized between samples using 5 % bleach, DI water, and 70 % ethanol. All 0.22 μM filters from each of the 70 samples were subsequently stored at −70 °C in FIU's Environmental Epigenetics Lab until DNA extractions were performed.

2.3. Environmental DNA isolation and PCR amplifications

DNA extractions were performed using a Qiagen DNeasy Blood and Tissue kit following the manufacturer's instructions, with minor modifications optimized for isolating eDNA from the Sterivex filters. Specifically, lysis was performed in a greater reagent volume per sample (720 μL of Buffer ATL and 80 μL of proteinase K solution) to ensure the filters were completely immersed in lysis buffer, and samples were incubated overnight at 56 °C with end-over-end rotation. DNA purity, quantity, and quality were verified using a NanoVue Plus, Qubit 2.0, and gel electrophoresis. A total of 70 eDNA samples were isolated, including those extracted from negative field control water samples (i.e., deionized water that was collected and preserved concurrently with field water sampling).

Three primer sets (Table 1) were used to target and amplify gene regions corresponding to teleost fish and broader marine vertebrate taxa: 1) MiFish, developed by Miya et al., 2015 to amplify a section of the 12S rRNA gene region; 2) MarVer1, developed by Valsecchi et al., 2020 to amplify a section of the 12S rRNA gene region; and 3) MarVer3, developed by Valsecchi et al., 2020 to amplify a section of the 16S rRNA gene region. These primer sets were selected to compare their efficiency in detecting marine vertebrate taxa and to maximize the capture of taxa that a single primer set alone may otherwise fail to detect.

PCRs were performed on each water sample in triplicate for each of the three primer sets, resulting in a total of 630 PCR products (70 eDNA samples x 3 primer sets x 3 reactions; Fig. 2). The MiFish PCR thermocycler protocol employed a touchdown profile following Pitz et al., 2020. The MarVer1 and MarVer3 PCR thermocycler protocol employed touch-down profiles following Valsecchi et al., 2020. For every PCR reaction mixture created, a negative control sample was included in which molecular grade water replaced the addition of the eDNA template.

All PCR products were visualized via electrophoresis on 2 % agarose gels to ensure amplification success and correct product size. PCR

Table 1
Primer sets used to target and amplify gene regions corresponding to teleost fish and broader marine vertebrate taxa.

Primer Name	Primer ID	Primer Sequence (5′-3′)	Size	Region	Avg Amplicon Size	Primer Source
MiFish	MiFish-U-F	GTCGGTAAAACTCGTGCCAGC	21 bp	12S	170 bp	Miya et al., 2015
	MiFish-U-R	CATAGTGGGGTATCTAATCCCAGTTTG	27 bp	12S	170 bp	
MarVer1	MarVer1F	CGTGCCAGCCACCGCG	16 bp	12S	202 bp	Valsecchi et al., 2020
	MarVer1R	GGGTATCTAATCCYAGTTTG	20 bp	12S	202 bp	
MarVer3	MarVer3F	AGACGAGAAGACCCTRTG	18 bp	16S	245 bp	Valsecchi et al., 2020
	MarVer3R	GGATTGCGCTGTTATCCC	18 bp	16S	245 bp	

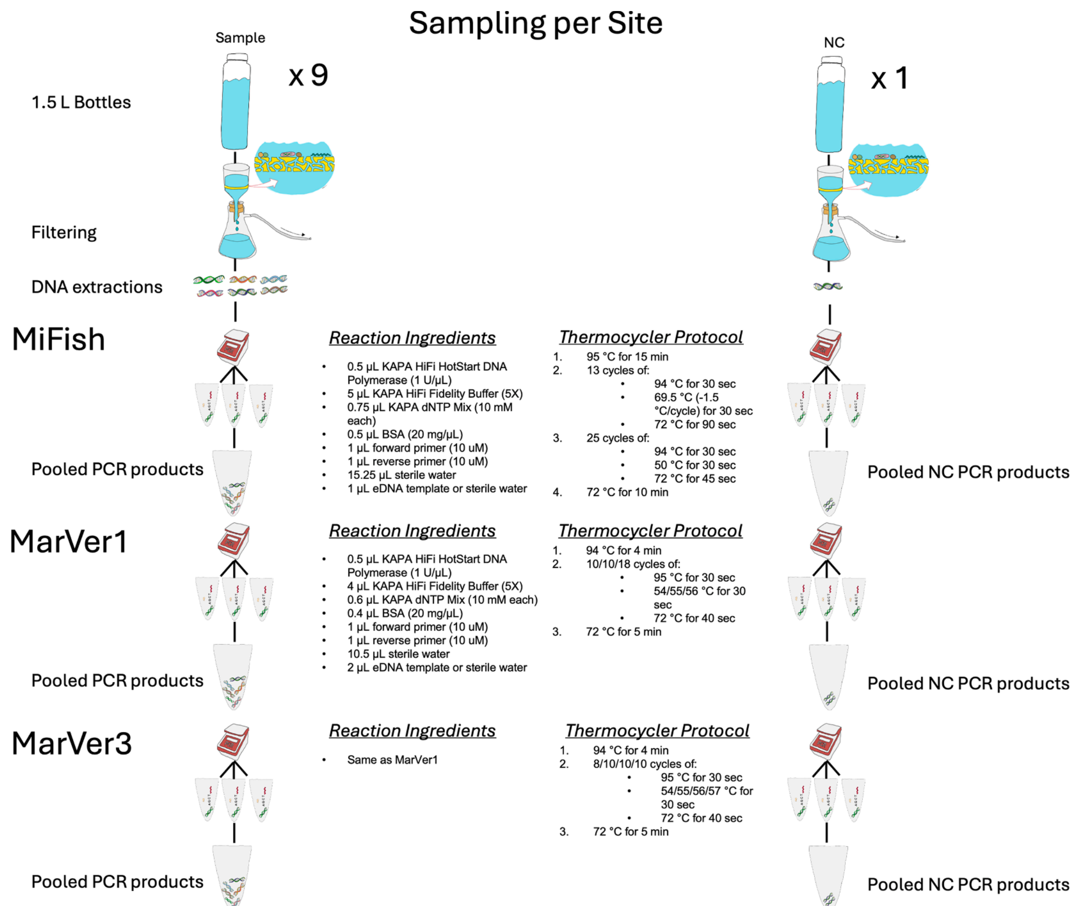

Fig. 2. Sampling and experimental design. Nine 1.5 L sample bottles were collected at each of seven sites (three 1.5-L of water was collected from each of three 5 L Niskin deployments per site). Each water sample was filtered using a Sterivex filter. PCR amplifications were performed in triplicate for each DNA extraction and for each set of primers. PCR replicates were pooled to produce 24 PCR product samples with final concentrations of 100 ng/uL, consisting of one from each of the seven sites for each of the three primer pairs. Negative controls (NC) were pooled together as one field control sample for each of the three primer pairs. PCR products from negative control samples were at lower concentrations than from site samples; the final concentration of each negative control pool was 10 ng/uL.

products were quantified using a Qubit dsDNA HS (High Sensitivity) Assay Kit and Qubit 2.0 fluorometer. PCR products were pooled so that equal amounts of amplified DNA were added to ensure equal representation for every collection site, and so that equal amounts of amplified DNA were used for the library preparation for each site for each primer set. Overall, a total of 24 PCR product pooled samples were produced (one sample from each of the seven sites, for each of the three primer pairs). A field control sample for each of the three primer pairs was also included (Fig. 2).

2.4. Sequencing and bioinformatic analyses

Pooled PCR products were submitted to the NextGen DNA Sequencing core facility at the University of Florida's Interdisciplinary Center for Biotechnology Research (UF ICBR). Samples that contained a short 40–50 bp peak were removed and amplicons were size selected for library peaks below 1000 kb. After quantification of pooled PCR products, libraries were constructed with a limited PCR cycle to ligate overhang adaptor sequences that are compatible with Illumina sequencing adapters and dual indexes. Library preparation was performed for paired end 2x300 bp sequencing on an Illumina MiSeq, and libraries were normalized prior to sequencing.

The bioinformatics workflow for post sequencing data was processed and adapted from the DADA2 pipeline (https://benjjneb.github.io/dada2/tutorial.html). Accordingly, primer sequences were first removed from the beginning and ends of the sequence FastQ files using "cutadapt" (Martin, 2011) in R. FastQ files were then fed into the DADA2 package to model DNA sequencing error on an Illumina run, controlling for read quality and picking Amplicon Sequence Variable (ASV) sequences that represent biological variability. Reads were

subsequently trimmed to remove low-quality regions and filtered by quality score, which was visualized using "plotQualityProfile" to identify sequences that did not meet the quality score requirements and remove them from the dataset using the "filterAndTrim" function, with a low level mismatch between exact sequences of primers and observed reads. After error profiles were characterized, forward and reverse reads were merged using "mergePairs", and chimeras were removed using "removeBimeraDenovo".

Once sequences were trimmed and assessed for quality control, each sequence was given a taxonomic identity using the "assignTaxonomy" algorithm, which matched sequences to a reference database (minimum threshold of 50 %, which were the default settings). Here, the MIDORI2 database of eukaryotic mitochondrial sequences (Leray, Knowlton, and Machida, 2022), which includes reference files for both 12S and 16S rRNA, was used for all three primer sets (i.e., the 12S rRNA reference for MiFish and MarVer1 datasets, and the 16S rRNA reference for the MarVer3 dataset). Sequences were combined with field site metadata using Phyloseq (Mcmurdie & Holmes, 2013). For each primer set, ASVs identified in the negative control were removed from all of the samples (Supplementary Table 2). Any taxa that were unidentified (i.e., did not match to the reference database) at the order level or above were also excluded; taxa identified to the family, genus, or species level remained in the dataset. Data were subset further to exclude all taxa except for chordates and then conglomerated by genus using the "tax_glom" function in phyloseq to simplify downstream presence-absence tables for each site.

Taxonomic identities were assigned to all three primer sets. Primer sets were analyzed to determine the identity and quantity of taxa that were detected by more than one primer set as well as the identity and quantity of taxa that were only identified by one of the three primer sets. In a subsequent analysis, the taxa that were detected by all three primer sets, and therefore have high confidence that their presence was not a false positive, were explored further. After examining how taxa identification varied by primer set, the results from all primers were combined into one dataset. A taxon was included in the dataset as long as it fulfilled the requirement that at least one primer set had detected it in at least one of the seven study sites. Although read counts were collected as part of the dataset, for the present study they were not used to estimate organism abundance (see Discussion). Therefore, only taxa presence or absence per site was assessed.

3. Results

For the eDNA metabarcoding analyses, raw reads were demultiplexed fastq files and each primer pair was processed separately. For the MiFish primer set, an average of 118,461 reads per sample (i.e., seven sites and one negative control) were obtained (Supplementary Table 3). After removing primers, filtering and trimming for quality, merging paired reads, and removing chimeras, 83 % of the raw reads remained for downstream analyses (i.e., an average of 98,084 MiFish reads per sample) (Supplementary Table 3). For the MarVer1 primer set, an average of 42,291 raw reads per sample were obtained, 87 % of which were used for downstream analyses (i.e., an average of 36,590 reads per sample remained after cleaning and merging). For the MarVer3 primer set, an average of 190,954 raw reads per sample were obtained, 80 % of which were used for downstream analyses (i.e., an average of 151,886 MarVer3 reads per sample remained after cleaning and merging).

3.1. Marine vertebrate taxa identified by three primer sets

The combined use of the three primer sets resulted in the identification of a total of 145 unique vertebrate taxa (Fig. 3; Supplementary Table 4). MarVer1 detected the greatest number of taxa (n = 88; Supplementary Table 5), followed by MiFish (n = 79 taxa; Supplementary Table 6), while MarVer3 detected the fewest (n = 47 taxa; Supplementary Table 7). A total of 53 taxa were identified by two or more of the

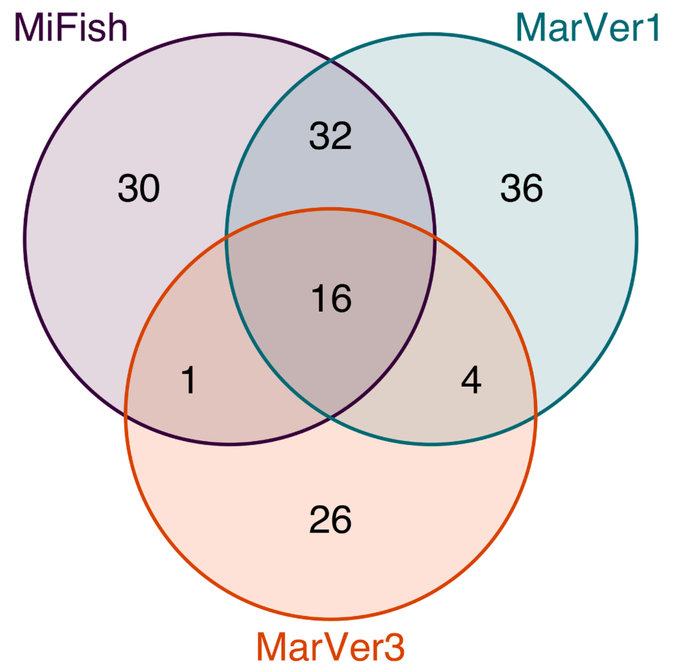

Fig. 3. Venn diagram depicting the number of vertebrate taxa detected across all study sites in Biscayne Bay using three primer sets: MiFish (12S), MarVer1 (12S), and MarVer3 (16S).

primer sets, with the most detection overlap (n = 48 taxa) occurring between the two 12S primer sets (i.e., MiFish and MarVer1). However, 92 taxa were detected by only one of the three primer sets (i.e., were uniquely identified by one primer set, and were not detected using the other two). Thirty taxa were only identified using MiFish (i.e., were not identified by MarVer1 or MarVer3); 29 of these MiFish-only taxa were ray-finned fishes (Class Actinopterygii) and one was a marine mammal, the Clymene dolphin *Stenella clymene*. Thirty-six taxa were only identified using MarVer1, but not MiFish or MarVer3, which included 19 ray-finned fishes, seven cartilaginous fishes (Class Chondrichthyes), six birds (Class Aves), three reptiles (Class Reptilia), and one mammal (Class Mammalia). Twenty-six taxa were only identified using MarVer3, but not MiFish or MarVer1, which included 24 ray-finned fishes, one cartilaginous fish, and one reptile. Each primer set successfully identified taxa at all seven sites, with varying degrees of detection overlap between the three primer sets at each site (Fig. 4).

A total of n = 16 taxa were identified by all three primer sets (Table 2). These included 13 commercially valuable taxa (Table 2). Some of these are collected for the aquarium trade (e.g., *Abudefduf saxatilis* and *Stegastes adustus*), while others are targeted for recreational fishing purposes, both as small bait fish (e.g., *Atherinomorus stipes*, *Jenkinsia lamprotaenia*, and *Mugil cephalus*) as well as larger fish species targeted for sport (e.g., *Centropomus undecimalis*, *Thunnus* sp., and *Tylosurus crocodilus*). All three primer sets also detected cryptobenthic species, *Gobiidae* sp. and *Syngnathus scovelli*. Lastly, two of the species detected by all three primer sets have a vulnerable IUCN status (IUCN, 2022), the West Indian manatee *Trichechus manatus* and the sperm whale *Physeter catodon* (Table 2). Although *Physeter catodon* is listed in the MIDORI2 and other reference databases, the accepted nomenclature for the sperm whale is now *Physeter macrocephalus*.

3.2. Trophic variation across sites

The relative distribution of herbivorous, carnivorous, and omnivorous taxa that composed the vertebrate communities were relatively similar across all sites, with the possible exception of Site 3 (Fig. 5; Supplementary Table 8). At Site 3, which is located above a seagrass

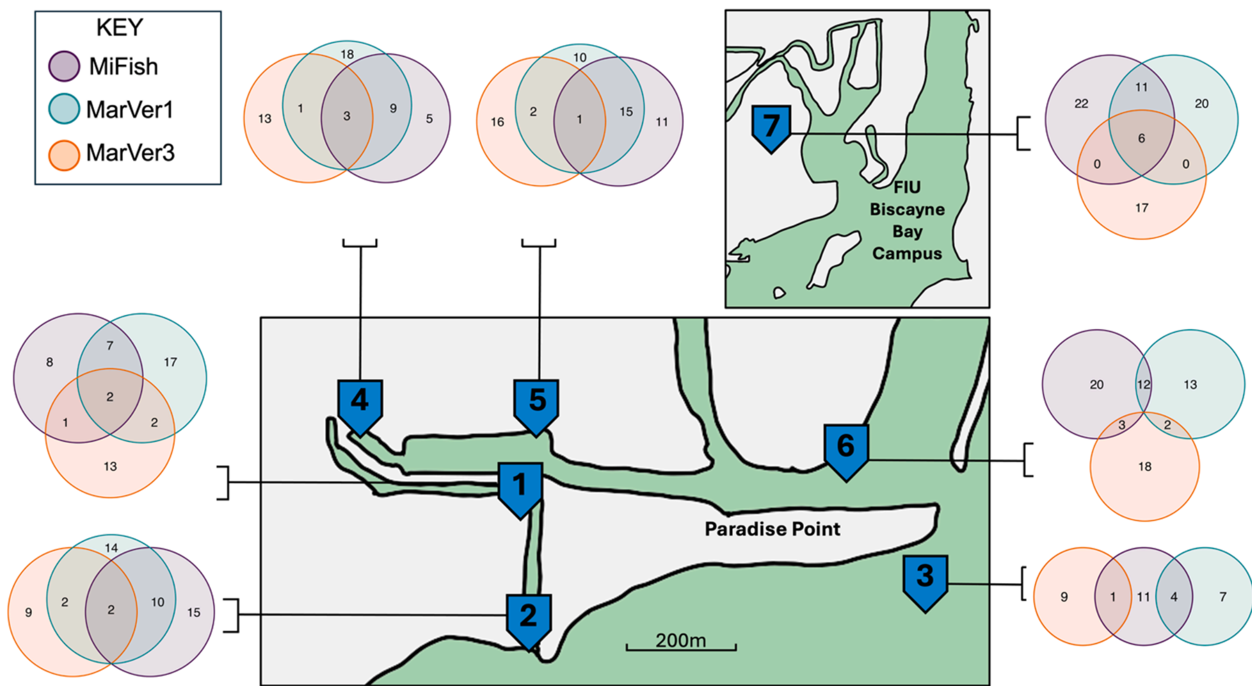

Fig. 4. – Venn diagrams depicting the detection of vertebrate taxa using each of the primer sets– MiFish (12S), MarVer1 (12S), and MarVer3 (16S)– across individual study sites within Biscayne Bay.

Table 2
Taxa identified using the three primer sets combined and associated characteristics.

Taxon	Common name	Endemic Status	Fishery Importance	Cryptobenthic vs Conspicuous	IUCN STATUS	Trophic Category
Abudefduf saxatilis	Sergeant major	Native	minor commercial; aquarium	Conspicuous	Least Concern	Omnivore
Atherinomorus stipes	Hardhead silverside	Native	minor commercial; bait usually	Conspicuous	Least Concern	Omnivore
Centropomus undecimalis	Common snook	Native	commercial; aquaculture: gamefish	Conspicuous	Least Concern	Carnivore
Eucinostomus gula	Jenny mojarra	Native	minor commercial; bait usually	Conspicuous	Least Concern	Carnivore
Gobiidae	Unidentified goby	Native	minor commercial	Cryptobenthic	Least Concern	Omnivore
Harengula jaguana	Scaled herring	Native	minor commercial	Conspicuous	Least Concern	Omnivore
Jenkinsia lamprotaenia	Dwarf herring	Native	minor commercial; bait usually	Conspicuous	Least Concern	Omnivore
Lucania goodei	Bluefin killifish	Native	commercial; aquarium	Cryptobenthic	Least Concern	Omnivore
Mugil cephalus	Flathead grey mullet	Native	highly commercial; aquaculture: gamefish; bait occasionally	Conspicuous	Least Concern	Omnivore
Mugil sp.	Unidentified mullet	Native	highly commercial; aquaculture: gamefish; bait occasionally	Conspicuous	Least Concern	Omnivore
Physeter macrocephalus	Sperm whale	Native	no value	Conspicuous	Endangered	Carnivore
Stegastes adustus	Dusky damselfish	Native	commercial; aquarium	Conspicuous	Least Concern	Herbivore
Syngnathus scovelli	Gulf pipefish	Native	no value	Cryptobenthic	Least Concern	Carnivore
Thunnus sp.	Unidentified tuna	Native	highly commercial; aquaculture: experimental; gamefish	Conspicuous	Least Concern	Carnivore
Trichechus manatus	West Indian manatee	Native	no value	Conspicuous	Vulnerable	Herbivore
Tylosurus crocodilus	Houndfish	Native	commercial; gamefish	Conspicuous	Least Concern	Carnivore

community, only 12.5 % of the taxa were generalist omnivores while 81 % of the taxa were carnivores. All other sites had > 20 % omnivorous taxa, with Site 1 having the highest percentage of omnivores (32 %) of all sites. At every site, the composition of vertebrates was dominated by carnivores, with Site 3 having the greatest percentage (81 %) and Site 1 having the smallest (58 %). In contrast, obligate herbivores made up the smallest percentage of taxa at each site, ranging from only 1.8 % of the taxa at Site 5 to 10 % of the taxa at Site 1. Although Site 7 was the only site located further north within Biscayne Bay and the only site with a hard bottom benthic structure, the relative distribution of herbivores, carnivores, and omnivores detected at Site 7 was highly similar to that found within vertebrate communities at Sites 1, 2, 4, 5, and 6.

3.3. Species richness and taxa characterization

Ray-finned fishes (Class Actinopterygii) composed the majority of the species detected at every study site (Fig. 6A; Supplementary Table 8). These included gobies, jacks, snooks, cichlids, killifish, mullets, mollies, pufferfish, and barracudas. Cartilaginous fishes (class Chondrichthyes) composed the second-most abundant class found at each site. In addition to fishes, other vertebrates including multiple mammal, bird, and reptile taxa were also identified (Fig. 6A; Supplementary Table 8).

Species richness varied by site, ranging from 32 taxa at Site 3 to 75 taxa at Site 7 (Fig. 6A, Supplementary Table 8). At Site 1 (n = 50 taxa), a total of 11 taxa were exclusively found within this site (Fig. 6B,

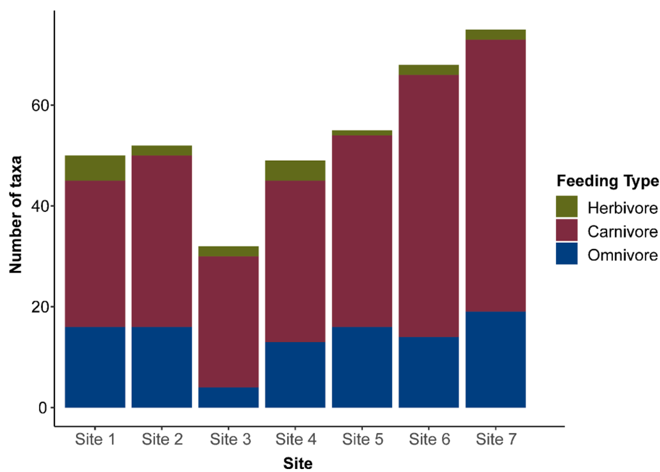

Fig. 5. Number of taxa identified using eDNA metabarcoding by trophic level across study sites in Biscayne Bay.

Supplementary Table 3). These included the striped mojarra *Eugerres plumieri*, the three-barbeled catfish *Rhamdia* cf. *jequitinhonha*, the nurse shark *Ginglymostoma cirratum*, the snakebird *Anhinga anhinga*, and the striated heron *Butorides striata*. Site 1 did, however, share 30 taxa with Site 2, the site to which it is geographically the closest. There were 5 taxa that were only detected at Site 2, including the Atlantic bonefish *Albula goreensis* and the Diamondback terrapin *Malaclemys terrapin.*

Site 3 displayed the fewest number of taxa (n = 32 taxa) (Fig. 6A; Supplementary Table 8), including four ray-finned fishes that were not present at any of the other study sites (Fig. 6B; Supplementary Table 8), such as the Atlantic silverside *Menidia menidia*. In contrast, neither the crested goby *Lophogobius cyprinoides* nor jacks of the family Carangidae were detected at Site 3, although these taxa were detected at all of the other study sites. A few taxa were uniquely identified at Site 4 only, including the grey heron *Ardea cinerea*, the green sea turtle *Chelonia mydas,* and the fantail mullet *Mugil gyrans*. There were no taxa that were detected at all other study sites except for Site 4 (i.e., taxa missing only from Site 4) (Fig. 6B; Supplementary Table 8). Sites 4 and 5, which are geographically closest to one another, shared 36 taxa. The flagfin mojarra *Eucinostomus melanopterus* was the only taxa that was detected at Site 5 but at none of the other study sites.

A total of 68 taxa were detected at Site 6, of which 14 were exclusive (i.e., were not detected at any of the other sites) (Fig. 6; Supplementary Table 8). These included the honeycomb cowfish *Acanthostracion polygonius*, the bar jack *Carangoides ruber*, the Atlantic spadefish *Chaetodipterus faber*, the striped blenny *Chasmodes bosquianus*, the shortfin pipefish *Cosmocampus elucens*, and the double crested cormorant *P. auritus*. In contrast, the checkered puffer *Sphoeroides testudineus* was found at all other study sites except for Site 6.

The greatest number of taxa was found at Site 7 (n = 75), with 23 being unique to this site (Fig. 6; Supplementary Table 8). These included the yellow jack *Carangoides bartholomaei*, the graysby grouper *Cephalopholis cruentata*, the long-spine porcupinefish *Diodon holocanthus*, the tomtate grunt *Haemulon aurolineatum*, the leather jack *Oligoplites saurus*, the king mackerel *Scomberomorus cavalla*, the yellow stingray *Urobatis jamaicensis*, and the brown pelican *Pelecanus occidentalis*. In contrast, there were only three taxa that were present at Sites 1—6 but were absent at Site 7 (Fig. 6B; Supplementary Table 8): the seaboard goby *Gobiosoma ginsburgi*, the code goby *Gobiosoma robustum*, and the West Indian manatee *T. manatus*.

Seven taxa were detected at all seven of the study sites (Fig. 6B; Supplementary Table 7), including 6 ray-finned fishes: the hardhead silverside *Atherinomorus stipes*, the common snook *Centropomus undecimalis*, the slender mojarra *Eugerres plumieri*, the gray snapper *Lutjanus griseus*, the Thoburn's mullet *Mugil thoburni*, and the great barracuda

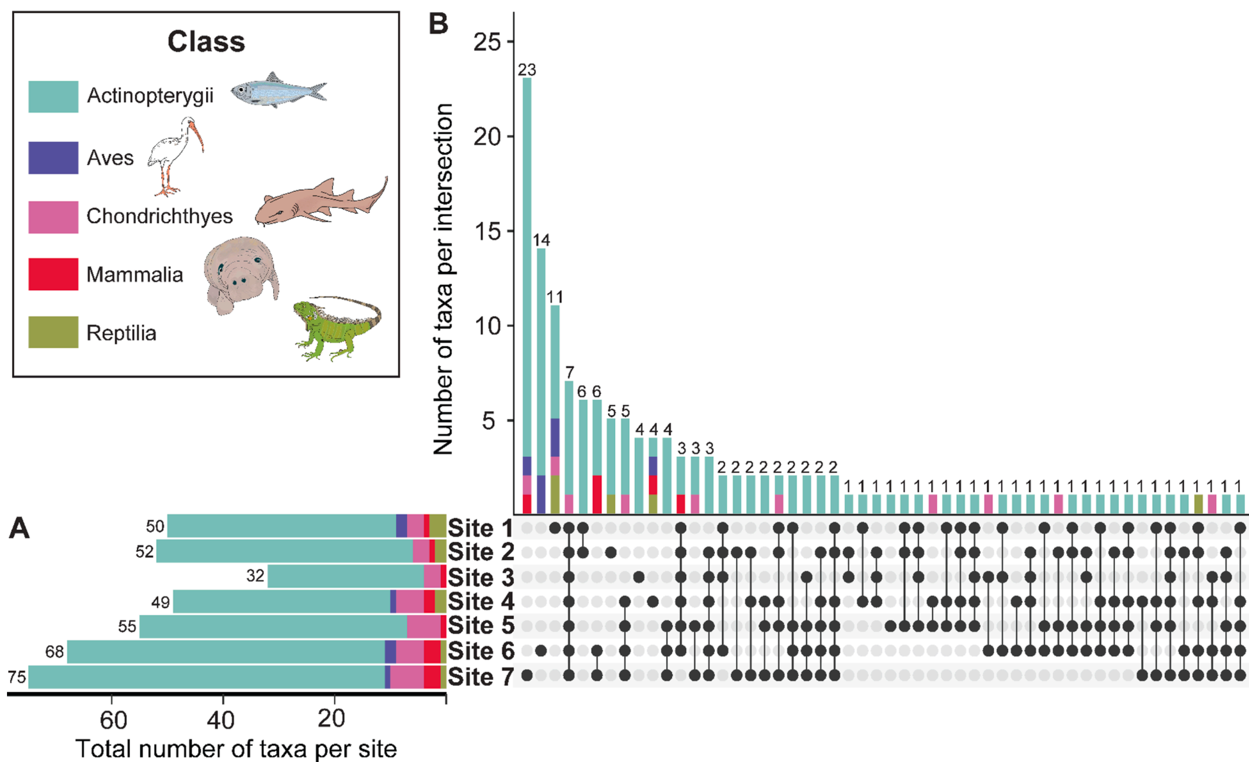

Fig. 6. Shared and distinct taxa across sites. Vertebrate taxa abundance detected at Biscayne Bay study sites 1—–7. **A.** A horizontally stacked bar chart showing the total number of taxa per site. **B.** UpSet plot with a matrix depicting intersecting sets (black dots with vertical connecting lines) between sites, and the number of taxa per intersection is shown in a vertically stacked bar chart. Each bar is labeled with the total number of taxa it represents and is color-coded by taxonomic class according to the key.

Sphyraena barracuda. There was also an unidentified requiem shark (family Carcharhinidae) detected at all seven sites.

4. Discussion

A large number of taxonomically diverse vertebrate taxa (145 total) were identified in the present study using an eDNA approach in locations where traditional biodiversity monitoring can be particularly challenging, due to boating traffic hazards and visibility limitations. The present study supports the feasibility of employing eDNA approaches for the study of the selected sites in Biscayne Bay, which further supports its applicability in other aquatic environments. These data can be used to supplement or validate findings from other biodiversity assessments, compare species composition across sites, and trace particular species of interest (e.g., cryptic, protected, or commercially valuable species).

4.1. Marker selection in assessing vertebrate biodiversity

The MarVer1 primer set (Valsecchi et al., 2020) appeared to be the most optimal of the primers selected, detecting the greatest number of taxa (n = 88) of the three markers. Furthermore, taxa detected by MarVer1 that went undetected by MiFish or MarVer3 (n = 36 taxa uniquely identified by MarVer1) spanned five separate classes of vertebrates. The two 12S primer sets, MiFish and MarVer1, had the greatest overlap in detected taxa (n = 48). Despite this overlap, there were still 71 taxa that differed between the two 12S markers. The 16S primer set, MarVer3, appeared to perform the poorest of the three markers, detecting the fewest number of taxa (n = 47). Nonetheless, MarVer3 detected several taxa that the other two did not (n = 26 taxa uniquely identified by MarVer3).

Previous environmental DNA studies in marine and other aquatic ecosystems have recommended employing a combination of multiple primer sets, particularly those that target different gene regions (Liu and Zhang, 2021, Kumar et al., 2022). The present study also highlights the value of this approach, as the combination of the three primer sets enabled a greater coverage of identifiable vertebrates in Biscayne Bay across a wide diversity of taxonomic classes. Furthermore, the use of more than one marker can reduce the likelihood of false positives and avoid the pitfalls of a single marker for species assignments. For example, there is high confidence that the 16 taxa that were identified by all three of the primer sets were present at the study sites. We therefore recommend combining these three primer sets when investigating broad vertebrate biodiversity or richness in a study area.

Conversely, users may find that selecting a single primer is appropriate for fulfilling their study's outcomes. MiFish was developed for teleost fish (Miya et al., 2015), whereas MarVer1 and MarVer3 were developed more broadly for marine vertebrates, including marine mammals (Valsecchi et al., 2020). Therefore, applying MiFish alone may be more time and cost effective when targeting fishes, and not all vertebrates, as the primary taxa of interest. The present study can be used as a guide to determine which primer set(s) are adequate for detecting a specific species of interest in Biscayne Bay. For example, to detect the presence of invasive iguanas, only MarVer1 should be used as the other two primers were unable to identify this taxon. Our results also support that any, but not necessarily all, of the three primers could be used to detect the presence of sperm whales. Overall, a study's objectives should dictate which and how many primers are selected. Our findings can be used to inform this selection for future eDNA studies that wish to balance the efficient use of resources with the reduction of false positives.

4.2. Vertebrate biodiversity varied across Biscayne Bay study sites

A relatively small overlap of taxa was found between Sites 1 and 2 (n = 30), even though these sites are separated by only about 275 m (Fig. 1). Since water movement appeared minimal at the time of sample collection, the amplified eDNA was likely captured from animals within close proximity of the collection location, rather than being transported passively between these sites due to current and water flow. Such a conclusion is supported by a study conducted within a kelp forest ecosystem (Port et al., 2016), which successfully used eDNA to distinguish vertebrate community assemblages between habitats that were separated by only 60 m. Two bird species, the snakebird *A. anhinga,* and the striated heron *B. striata*, were detected at Site 1. Both species perch and nest in mangroves (Owre, 1967, Mohd-Taib et al., 2020), and likely shed DNA into their environment through fecal matter as well as while feeding on aquatic prey (Mojica et al., 2021). In contrast, no bird taxa were detected at Site 2 where nesting locations are absent, suggesting a lack of passive eDNA transport via water movement between Sites 1 and 2. There were also species currently listed with a vulnerable IUCN status (IUCN, 2022) detected in this canal, including the nurse shark *G. cirratum* (Site 1), and the diamondback terrapin *M. terrapin* (Site 2).

The presence of seagrass meadows at Site 3 (composed of *Thalassia testudinum*, *Syringodium filiforme*, and *Halodule wrightii*) could be indicative of greater species richness, as they tend to act as nursery grounds for a large variety of fish species (Unsworth et al., 2019, Nordlund et al., 2018). However, this site displayed the lowest vertebrate species richness (n = 32) among all sites studied. Disturbance from the high occurrence of motorized boats traveling between the Paradise Point canal system and the larger Cutler Channel via Site 3 (e.g., noise pollution, strikes, displacement by pressure waves) could account for such an observation by altering organism physiology, triggering avoidance behavior, and leading to decreases in species abundance (Whitfield and Becker, 2014, Becker et al., 2013). Interestingly, since Site 3 is adjacent to Cutler Channel where there are increased currents and greater water flow that can transport DNA away from the source organism from which the DNA originates, there may be lower retention rates of eDNA shed by animals in this area (Larson et al., 2022, Harrison et al., 2019). Studies have measured retention and resuspension of eDNA in transport and found unexplained variability, but detection of species followed predictable plume dynamics in rivers and streams (Wood et al., 2021).

The study of Site 4 samples revealed the presence of the West Indian manatee *T. manatus*, a species that was also detected at all other Paradise Point locations (i.e., Sites 1—6). This species has a vulnerable status (IUCN, 2022) protected under the Endangered Species Act and the Marine Mammal Protection Act. The presence of *T. manatus* was visually confirmed at this site, where four individuals were observed resting in the area at the time of water sampling. Manatees were only otherwise visually observed at Site 6, where they were seen swimming towards the canal opening. Manatee responses to boating activity include avoidance behavior (Rycyk et al., 2018, Buckingham et al., 1999). Motorized boats have limited access to Site 4 and there are no residences at the end section of the canal. Therefore, we hypothesize that manatees actively travel throughout this canal system but may use Site 4 as a refuge to rest as there is minimal human activity in this area.

In contrast to Site 4, Sites 5 and 6 are within areas of the northern Paradise Point canal that are more heavily influenced by human activity. These sites are immediately adjacent to residences, many of which have privately-owned boat docks within the canal and are subject to recreational fishing. Many species commonly targeted for fishing were detected in these sites, including the crevalle jack *C. hippos*, the common snook *C. undecimalis*, the barracuda *S. barracuda*, the gray snapper *L. griseus*, and an unidentified tuna species (*Thunnus* sp.). As such, these sites are likely to continue to be popular fishing locations. Based on our results, it is possible to link the used eDNA approach to the monitoring of these species at Sites 5 and 6, and whether their distributions are affected over time by ongoing human influences, including pressures from fishing activities.

Site 7 was the richest in terms of species diversity (n = 75) and had the most exclusive taxa, which was not surprising given it is nearly 30 miles north of the Paradise Point sites (Sites 1—6). Additionally, unlike the canal sites where the benthos is muddy and silty, Site 7 has a rocky

bottom community with a patchy distribution of stony corals. Indeed, eDNA from reef-associated species was detected at Site 7, including the graysby *C. cruentata*, the redlight goby *Coryphopterus urospilus*, and the long-spine porcupinefish *Diodon holocanthus*. These findings align with the expectation of distinct species assemblages found across sites that differ in their habitat structure and characteristics.

4.3. The potential of eDNA to unbiasedly improve management and conservation

In this study, metabarcoding eDNA enabled the assessment of vertebrate biodiversity within areas of Biscayne Bay where visual biodiversity surveys are challenging to conduct. In addition to water clarity, visual surveys can be affected by other local conditions such as currents, light, and structural complexity of the habitat (Valdivia-Carrillo et al., 2021, Bozec et al., 2011). Visual detection can also be affected by the experience, training level, and pre-existing expectations of the observer (Williams et al., 2006, Thompson and Mapstone, 1997). Many surveys are conducted using a pre-established list of taxa; this may bias observers to note species that are already assumed to be present and may decrease the likelihood of recording newly invasive, rare, or unanticipated species.

Analyzing eDNA from water samples can help avoid observer bias because assumptions of which species are present do not influence the dataset. For example, the sperm whale *P. macrocephalus* was detected in water samples at Sites 6 and 7, which was an unexpected result. To verify this finding, the relevant sequences from our reference dataset were compared to the National Center of Biotechnology Information (NCBI) database using BLASTN (Altschul et al., 1990) and were found to match *P. macrocephalus*. This finding is also supported by recent reports of sperm whale strandings in South Florida, including a stranding that occurred in Biscayne Bay in January 2023 (Aguirre, 2023). Lastly, all three primer sets used in this study (i.e., MiFish, MarVer1, and MarVer3) detected *P. macrocephalus* at Site 7, increasing confidence that this is not a false positive result.

Biodiversity surveys in aquatic ecosystems are often performed during the daytime due to logistical constraints. However, this practice likely leads to the underestimation of nocturnal species. In this study, many nocturnal taxa (i.e., sharks and rays) were detected despite water samples being collected during the day. Many “shy” taxa, which tend to exhibit avoidance behavior (Bozec,et al., 2011), were also identified. Furthermore, this study detected multiple cryptic taxa, which are difficult to visually detect even in pristine water conditions. This highlights the value of using eDNA in ecological assessments to capture species that may otherwise be overlooked during traditional visual surveys.

Although the majority of taxa that were detected are obligately aquatic species, bird and reptile eDNA were also found in the water. Several of the identified bird species act as predators to fish and are important contributors to nutrient cycling within Biscayne Bay (Cummings, 1987, Wright et al., 2009, Kushlan and Frohring, 1985). Also identified was the green iguana, which is highly invasive in Florida, and is known to cause landscape damage, erosion due to burrowing activity, and potential transmission of Salmonella (Krysko et al., 2007). Therefore, an eDNA approach can also be useful for detecting taxa that might not always be physically present in the water but are still important contributors or disruptors to the ecosystem.

There remains a growing interest to implement eDNA as a tool for the management and conservation of endangered species (Rees et al., 2014; Gold et al., 2022). Several vulnerable species were identified within our Biscayne Bay study sites, including the protected West Indian manatee, which was detected within the Paradise Point canal system. This study highlights how these species can be detected and monitored using an approach that does not require direct interaction with the organisms to obtain samples and minimizes disturbing their natural behavior. This is particularly useful for highly protected species for which obtaining sample permits can be restricted, time-consuming, or costly.

4.4. Standing challenges for the application of eDNA approaches

Several works have raised questions regarding the sensitivity and accuracy of eDNA approaches (Hansen et al., 2018, Jerde, 2021), as the detection of species diversity can vary by species traits (Thalinger et al., 2021), strength in hydrological flow (Jane et al., 2015), and even elevated temperatures. Environmental DNA analyses are highly reliant upon the completeness and validity of the reference sequence databases used. Some of the taxa identified in the present study, for instance, are unlikely to be present within Biscayne Bay. This poses a significant challenge to distinguish between false positives and novel findings (e.g., of newly invasive species). Environmental DNA analysis has been touted as a useful method for the early detection of invasives (Larson et al., 2020). However, we recommend validating the detection of invasive species with visual confirmation or other follow-up studies to avoid potential errors.

For example, both the green iguana *I. iguana* and the Lesser Antillean iguana *I. delicatissima* were detected in this study. While *I. iguana* is known to be highly invasive in Florida (Krysko et al., 2007), *I. delicatissima* is endemic to the Lesser Antilles and has a critically endangered IUCN status as of 2018 (Knapp et al., 2014; IUCN, 2022). To the best of our knowledge, *I. delicatissima* has never been reported in Florida; rather, it has a much narrower distribution and its populations have faced dramatic declines (Knapp et al., 2014). Misidentification between the two *Iguana* species can occur due to morphological overlap and high variability among individuals (Bochaton et al., 2016). Furthermore, genetic evidence supports that hybridization has occurred between the two species in the Lesser Antilles (Vuillaume et al., 2015, Pounder et al., 2020). We hypothesize that the detection of *I. delicatissima* in the present study is the result of an error in the reference sequences likely due to the misidentification of an *I. iguana* or hybrid specimen.

A major limitation of eDNA metabarcoding is that the technique is optimal for detecting the presence of a species, but not the absence of a species. DNA recovery is dependent on primer-specificity biases (Kelly et al., 2014; Castro et al., 2021), sequencing depth (Sawaya et al., 2019), varying eDNA shedding rates (Wood et al., 2020), decaying rates (Sassoubre et al., 2016), and other abiotic factors including flow rates (Itakura et al., 2020), water temperature, UV-B exposure (Hansen et al., 2018) and other conditions (Pilliod et al., 2013). For example, *Boa constrictor* have been found at Deering Estate at Cutler, Miami-Dade County since the 1970s (Snow et al., 2007), however, we failed to detect the presence of this species in our analyses.

In this study, we opted not to use sequence counts to estimate abundance or biomass. Although eDNA sequence abundance has been positively correlated with population density and population biomass (Rourke et al., 2022), various factors affect eDNA concentrations, such as the intrinsic variability in eDNA production among biological organisms (Klymus et al., 2015), metabolism and size variation that alter shedding rates, the spatial distribution of organisms (Eichmiller et al., 2016, Coulter et al., 2019), various eDNA decay rates (Sassoubre,et al., 2016, Perez et al., 2017), as well as eDNA capture, isolation, and amplification methods (Piggott, 2016). Further, studies that compared relative sequence abundance and field-based methods showed the same quantitative information, however, results changed across sites, and in some cases was not correlated with abundance (Shelton et al., 2019).

Because this study was exploratory and aimed to capture a broad range of taxa, a relatively large volume of water was sampled in triplicate at each site for a total of 13.5 L per site. We were able to isolate a minimum of 3.3 μg of DNA from each 1.5 L of seawater; this was well in excess of the amount of template needed for PCR amplifications. Future eDNA studies in Biscayne Bay may wish to reduce the volume of water sampled and the amount of replication to improve efficiency while maintaining sufficient taxa capture. Decreasing water volume and processing workload will enable the reallocation of time, money, and other resources that should instead be used to sample additional locations or

time points.

5. Conclusions

As eDNA methods continue to rapidly evolve, their application holds enormous potential to facilitate monitoring, conservation, and impact assessments. For instance, in 2018 a “Fish Chat” from a group of scientific experts from multiple organizations that engage in “bioblitzes” discussed the use of eDNA to sample non-native fishes in South Florida (Schofield, 2020). Fortunately, the accuracy and precision of species identification via eDNA analyses are likely to improve as more genetic data is collected and made available.

Paradise Point is next to a housing community and is subject to effects from residential development, disturbance created by recreational activities, and pollution. This study spanned a variety of distinct habitat types and provided baseline biodiversity information for future ecological research within Biscayne Bay, which may compare the performance of eDNA with field-based visual surveys to look at changes in trends and abundance of taxa across multiple sites. Future studies may also examine how this baseline may shift temporally due to seasonal variation or disturbances. This is particularly important as climate change continues; eDNA approaches can be used to monitor how climate change impacts biodiversity in an area over time, such as before and after extreme weather events.

Although there still exist concerns and caveats regarding the detection capacity and accuracy of using eDNA (Beng and Corlett, 2020, Pinfield et al., 2019), this study demonstrates the value of using an eDNA approach to assess biodiversity in an ecosystem that provides a great number of services but is also heavily impacted by human activity. This technique limited organism/ecosystem disturbance and avoided the hazards of deploying personnel or sensitive equipment in areas regularly experiencing high vessel traffic. A broad diversity of vertebrate taxa across different spatial scales was identified, including commercially valuable, protected, and invasive species.

CRediT authorship contribution statement

Kelcie L. Chiquillo: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Juliet M. Wong:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Jose M. Eirin-Lopez:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data, results, and code are available on Dryad (DOI: https://doi.org/10.5061/dryad.h70rxwdrk).

Acknowledgments

This work was funded by grants from the Herbert W. Hoover Foundation and the National Science Foundation (NSF-HRD-2111661, awarded to Florida International University as part of the Centers of Research Excellence in Science and Technology CREST Program). This is contribution #1729 from the Institute of Environment at Florida International University. KLC and JMW were supported by the NSF Postdoctoral Research Fellowships in Biology Program under Grants No. 2109466 and 2010791, respectively, in the Environmental Epigenetics lab within the Institute of Environment at Florida International University. We acknowledge the assistance of colleagues Dr. Piero Gardinali, Dr. Milena Ceccopieri, Dr. Kassidy Troxell, Dr. Stefanie Landeweer, Caiti Waks, Bill Chamberlain, Dr. Paulo Braga Mascarenhas Jr., and Dr. Guilherme Maricato.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gene.2024.148720.

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