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# Development of Three Novel eDNA Assays for Detecting Coastal Sharks

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# Development of Three Novel eDNA Assays for Detecting Coastal Sharks

By

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## Abstract

Environmental DNA (eDNA) analysis is an emerging, non-invasive community monitoring tool. This study aimed to determine if eDNA methods can be reliably used in a large brackish, partially mixed estuary by developing and testing three novel eDNA primers, for Sandbar Sharks (*Carcharhinus plumbeus*), Blacknose Sharks (*Carcharhinus acronotus*), and Bonnetheads (*Sphyrna tiburo*). These primers were designed to target 109, 156, and 120 base pair (bp) fragments, respectively, of the highly conserved NAD2 gene in the mitochondrial genome of each species. Primer function was validated through testing against 102 known genomic source samples and 25 filtered water samples from aquaria in which the species were exhibited. A total of 198 water samples were collected alongside active longlines in Winyah Bay, South Carolina, and extracted for eDNA analysis. We created three species-specific eDNA primers for the target species and validated them against 8+ target genomic samples and 14 other local elasmobranch species as negative controls. Detection was successful when applied to aquarium samples gathered from five separate institutions. Large, naturally occurring organic compounds in Winyah Bay consistently inhibited PCR detection from the raw water samples, thereby limiting the utility of eDNA in the study system. This study provided evidence that species-specific primers of closely related carcharhinid species can be developed and utilized while also showcasing the challenges of eDNA detection in a highly productive marine environment. Further study in waters of lower organic content, or with advanced techniques is needed to demonstrate the full functionality of the primer.

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## Introduction

Many elasmobranch communities are heavily impacted by overexploitation and habitat destruction (Worm *et al.*, 2013, Dulvy *et al.*, 2014Dulvy *et al*., 2021, Flowers *et al*., 2022). As a result of these threats, the International Union for Conservation of Nature (IUCN) currently lists 218 species of shark as "Near Threatened" or worse. Unfortunately, the life history strategies of many elasmobranch species, characterized by late maturity and low fecundity, make species recovery a slow biological process (Abel & Grubbs, 2020). Assisting in the recovery of these communities requires effective management of their ecosystems due to the numerous trophic roles sharks and rays fill and their impacts within food webs (Navia *et al*., 2017; Burkholder *et al*., 2013). Success of species recovery and management is heavily influenced by the quality of the monitoring methods used during a population or community assessment (Lodge *et al*., 2012; Bylemans *et al*., 2016; Schweiss *et al*., 2019). More detailed monitoring methods are particularly important for ecologically significant, yet transient species such as elasmobranchs (Jerde et al., 2011, Dulvy *et al*., 2014). Current community monitoring methods include fishing by longlining or gillnetting, acoustic and satellite tagging and tracking, baited remote underwater video (BRUV) observation, underwater visual census, and ecological and fisheries-dependent surveys (Bakker, 2018). These survey methods have biases and challenges, leaving room for improvement in the conservation monitoring field. While traditional survey methods have proven useful, they can be time-

consuming, logistically difficult to carry out in isolated areas, and may fail to detect animals that are present in an area (Simpfendorfer *et al*., 2016). As there is no systematic global survey to monitor shark and ray populations, much of the data used for managing populations comes from national fisheries landing records (Dulvy *et al*., 2014). While fisheries monitoring is improving, only one-third of reportings reach a species level (Fischer *et al*. 2013). In order to gather the data necessary for a complete understanding of elasmobranch habitat partitioning and successful species management, a cost-effective and scalable alternative is needed (Postaire *et al*., 2020).

Environmental DNA (eDNA) assessment is a promising, emerging tool for species-level community monitoring. Previous studies have focused on answering questions about rare or invasive species in a wide variety of taxa and habitats, including freshwater and marine environments (e.g., Goldberg *et al.,* 2011; Thomsen *et al*., 2012; Bylemans *et al*., 2016; Simpfendorfer *et al*., 2016; Lafferty *et al*., 2018; Matthias *et al*., 2021). All organisms leave behind fragments of genetic material in their environments via skin cells, defecation, or gamete emission; these left-behind fragments are collectively called eDNA (Foote *et al*., 2012). The number of fragments left behind by an individual varies greatly based on its condition and the environment. In an aquatic environment DNA is subject to degradation in the form of UV-B rays and flow velocity, due to this degradation the average length of these fragments are only a couple hundred base pairs (Strickler *et al*., 2015; Scriver *et al*., 2023). Although these fragments are short, advances in genetic monitoring technologies have allowed researchers to take advantage of these materials in a cost-effective, non-invasive method that can gather data about multiple species' presence and absence as well as an index of population size from just one sample

(Thomsen *et al*., 2015; Bakker *et al*., 2017; Boussaire *et al*., 2018; Johri *et al*., 2019). Genetic material left behind in an aquatic environment can be as unique an indicator for a species as a track print is for terrestrial species, thus allowing for a presence and absence analysis (Cristecu & Hebert 2018). Moreover, since eDNA analysis is a non-invasive detection method, there is no need for a species of interest to be caught to establish a known range of presence in the field. Without the need for a visual sighting, eDNA analysis makes positive identification of like species and detection of rare species easier (Dejean *et al*., 2011, Huver *et al*., 2015).

There are two main approaches to studying eDNA of aquatic species. The first method is to develop species-specific primers from the mitochondrial DNA of the target species. Primers are then applied to filtered and extracted water samples to detect target species through visualization on gel electrophoresis or quantitative Polymerase Chain Reaction (qPCR) (Goldberg *et al*., 2011, Thomsen *et al*., 2012, Postaire *et al*., 2020). This process is limited in that only one species can be detected at a time. The second method is known as metabarcoding, in which universal primers allow for simultaneous multiple-species identification from a single environmental sample. These primers are designed to amplify a fragment of DNA shared by multiple species, but retains the ability to distinguish closely related species from one another (Deiner *et al*., 2017, Takeuchi *et al*., 2019; Liu *et al*., 2022).

While eDNA assays often provide improved detection assessments over current survey methods, they are not a panacea for conservation monitoring. eDNA detection poses its own challenges, including non-standardized protocols, PCR inhibition, and

environmental influences on DNA sample quality (Olson *et al*., 2012, Spangler *et al*., 2018).

Presently, freshwater environments are where most eDNA studies take place. This precedent can be attributed to the methodological challenges of higher salinities and increased dilution effects associated with larger water bodies (Foote *et al*., 2012). Additionally, in aquatic studies, PCR inhibition based on environmental factors is also of particular concern. Inhibition is caused by particles in the water containing inhibitory compounds that can interfere with PCR amplification, completely masking the detection of target DNA (Cao *et al*., 2015, Griener-Ferris, 2020). A study done in the Florida Panhandle confirmed that higher levels of inhibition were correlated with tannins in the water produced by vegetation in the surrounding habitat (Hunter *et al*., 2019). However, another study at Southern Mississippi was able to successfully amplify target DNA in a similarly productive environment (Schweiss, 2019).

*Uses and efficacy of eDNA with rare and invasive species*

Some of the most cited studies utilizing eDNA analysis focus on establishing a more accurate habitat range of rare or invasive species. For instance, Olson *et al*. (2012) developed a species-specific primer for the Eastern Hellbender (*Cryptobranchus a. alleganiensis*). Eastern Hellbenders are large, nocturnal salamanders often found concealed beneath rocks (Matasich *et al*., 2003). This behavior, in combination with their IUCN status of "Near Threatened" (IUCN 2022), made the species a prime candidate for an early application of eDNA in conservation. As one of the pioneer aquatic-based eDNA studies, the methodology of the project is important to understanding more recent project design as well. This study used sites with previously identified population density

estimates to validate their primer assays. Samples confirmed the previous classification levels of density, with number of positive detections increasing as known density level increased. However, this study showed that prior population estimates for the Eastern Hellbender were too high; the species was approaching its lowest reported natural density (Olson *et al*., 2012). The conclusions of this study demonstrate that eDNA surveys can be used to establish broad-scale population patterns of density for species that commonly evade other traditional survey methods.

As technology and trust in the methodology improves, researchers can make informed management decisions based on eDNA studies. A 2016 study in Australia was able to use species-specific qPCR results to recommend a location for wildlife management organizations to erect an exclusion barrier for invasive Redfin Perch (*Perca fluviatilis*) (Bylemans *et al*., 2016). This study is one of the first examples of eDNA surveys being used to inform species management and improve the success of containment actions. As a tool for containing invasive species, the amount of time from sample collection to visualization of amplification is often an obstacle. Until recently, samples had to be returned to the lab for qPCR, taking days or weeks for results. A study published in 2019 presents a new set of machinery intending to produce in-field qPCR results. In their field experiments, 38% of samples produced a positive detection, while traditional laboratory qPCR methods produced a positive detection 55% of the time (Thomas *et al*., 2020). While the study that is the focus of this thesis will not utilize the newest methodology, the results of Thomas *et al*., (2020) show that the in-field qPCR is not yet up to the same standard, they also demonstrate an improvement in eDNA technology and the investment in improving the methodology.

*eDNA and elasmobranchs*

Currently, only a handful of species-specific primers exist for eDNA application for elasmobranch species. Existing primers include sets for Blacktip Shark (*Carcharhinus acronotus*) (Postaire *et al.*, 2020), Bull Shark (*Carcharhinus leucas*) (Schweiss *et al*., 2019), Largetooth Sawfish (*Prisitis pristis*) (Simpfendorfer *et al*., 2016), Whale Shark (*Rhincodon typus*) (Sigsgarrd *et al*., 2017), White Shark (*Carcharodon carcharias*) (Lafferty *et al*., 2018) and Devil Rays (*Mobula mobular*) (Gargan *et al*., 2017). The first study to apply eDNA methodologies to elasmobranch ecological research was conducted by Simpfendorfer *et al*. (2016). This study aimed to isolate a portion of the Largetooth Sawfishes COI gene (GenBank accession NC_039438) to serve as a primer assay to confirm species identity. In this study, every water sample taken from controlled aquaria environments produced a positive detection, and when the primer assay was applied to environmental water samples, seven of the eight locations produced a positive detection in PCR trials. Simpfendorfer et al. (2016) showed that applying eDNA methodologies outside of an aquarium setting for wild presence of an elasmobranch species was possible. Many of the identified habitats were in hard-to-reach locations, making traditional survey methods difficult to complete. Success with eDNA analysis allowed for the possibility of a broad-scale survey of other hypothesized habitats to gain a more complete picture of species distribution. Since the work done by Simpfendorfer *et al*. (2016), sawfishes have become a key group of species in advancing eDNA methodology for elasmobranch studies. These studies (Bonfil *et al*., 2021; Sani *et al*., 2021) focus on identifying the presence of *P. pristis* in its historical home ranges where detection of the species via visual observation has declined. More recently, Bonfil

et al. (2021) in Mexico used the Largetooth Sawfish primer assay with short gillnet sets to confirm the presence of Largetooth Sawfish. Results showed positive species detection in all three sampling locations, while gillnetting resulted in zero catches (Bonfil *et al*., 2021). The results of this study further support the need and practicality of using eDNA analysis to detect rare species rapidly. Sani *et al*., (2021) used the methods established by Simpfendorfer *et al*. (2016) for primer design to create a primer for Knifetooth Sawfish (*Anoxypristis cuspidata*), another member of the Pristidae family (Sani *et al*., 2021). Positive eDNA detections occurred in 6 locations along Indonesian reefs, not all of which were known to be reefs the species was still present on, suggesting that the current distribution maps of the species are inaccurate.

The number of studies utilizing eDNA to detect elasmobranch species is increasing, including work done on Devil Rays (*Mobula mobular*) (Gargan *et al*., 2017), Blacktip Sharks (Postaire *et al*., 2020), and Whale Sharks (Sigsgarrd *et al*., 2017). Most recently, eDNA metabarcoding methods have been used to evaluate a more extensive range of species presence and diversity, although these studies have had mixed results in differentiating between closely related species in the same water body (Bakker *et al*., 2017; Liu *et al*., 2022).

Mixed results can be attributed to the inability to differentiate between closely related members of the same family. At this time, a universal metabarcoding primer that can reliably differentiate between closely related carcharhinids does not yet exist (Bakker *et al*., 2017). A few elasmobranch metabarcoding primers have been created, but they have been unable to distinguish between closely related carcharhinid species. For example, primers to a 127-base pair fragment of the mitochondrial oxidase subunit 1

(COI) gene found ambiguity in the distinction of species within the genera *Carcharhinus*, *Rhizoprionodon*, and *Negaprion* when the primer was applied to filtered water samples from the Caribbean (Bakker *et al.,*2017). This ambiguity in carcharhinid results may be attributed to the paraphyletic structure of most families within the order, suggesting current issues with the taxonomy (Vélez-Zuazo & Agnarsson, 2010). If a more definitive metabarcoding primer were produced, its application has the potential to reveal hundreds of taxa and their abundances from a single environmental sample (Bakker 2018). For this reason, species-specific primer studies are still preferred in environments in which carcharhinids are the dominant taxa.

*Elasmobranchs of Winyah Bay*

The Coastal Carolina University (CCU) Shark Lab and the South Carolina Department of Natural Resources (SCDNR) have been extensively studying the elasmobranch community of Winyah Bay since 2001. Winyah Bay is a 65 $km^2$ partially mixed estuary located in South Carolina between Myrtle Beach and Charleston. Previous studies have identified a shark assemblage in Winyah Bay that is more diverse than other estuaries in the southeastern United States (Abel *et al*., 2007; Gary, 2009; Peterson *et al*., 2017). According to the most recent Cooperative Atlantic States Shark Pupping and Nursery Program (COASTSPAN) survey (NOAA, 2021), Winyah Bay was the most diverse estuary sampled within the program with a total of 23 species of sharks and rays collected, of which 10 were carcharhinids.

Long-term longline studies show that Sandbar Sharks (*Carcharhinus plumbeus*) dominate this system (Abel *et al*., 2007; Gary, 2009). During summer months, neonates, young-of-year (YOY), juveniles, and adults are routinely caught in Winyah Bay, along

with other carcharhinids including Bull, Blacktip, Finetooth, (*Carcharhinus isodon*), Lemon (*Negaprion brevirostris*), Blacknose (*Carcharhinus acronotus*), and Spinner (*Carcharhinus brevipinna*) sharks, plus Bonnethead, (*Sphyrna tiburo*), and Scalloped Hammerhead (*Sphyrna lewini*) sharks (Abel *et al*., 2007, Gary 2009). In order to more completely assess the elasmobranch community assemblage of an environment researchers need more species-specific primer pairs. The following thesis study will aim to develop primer sets for Sandbar, Blacknose, and Bonnethead Sharks. These species represent both the most commonly caught species (*C. plumbeus*) as well as species that have been caught in past CCU studies but are not commonly caught during the longline surveys (*C. acronotus* & *S.tiburo*)

Sandbar Shark is the dominant elasmobranch species in Winyah Bay (Abel *et al*., 2007; Gary, 2009; Fryman, 2013). During a two-year study performed by Abel et al., (2007) 49.8 % of elasmobranchs caught within the bay were Sandbar sharks, ranging in life history stage from young-of-year to adults. This same study found that Sandbar shark presence decreased as sampling progressed north into the bay. This trend was credited to the influence of freshwater in the system, resulting in a division of the bay into three sub-sections based entirely on the salinity gradient. The study also acknowledged that CPUE rates changed in each sub-section based on the region's rainfall, suggesting that this species' usage of the bay varies significantly from season to season. Considering the species' ecological importance and prevalence in the environment an additional aspect of this thesis study is to analyze their usage of the bay through eDNA application.

*Driving questions*

Given the mobility of elasmobranch species and the discussed challenges of traditional sampling methods, as noted by Dulvy *et al*. (2014) and Postaire *et al*. (2020), the following study proposes to utilize eDNA analysis to answer the following questions about the elasmobranch community assemblage in Winyah Bay:

1. Can eDNA methods be reliably used in the brackish environment of Winyah Bay that is fed by multiple blackwater river sources?
2. Can species-specific primer pairs be developed for *C. plumbeus, S. tiburo*, and *C. acronotus*?
3. How do positive eDNA detections compare to documented catches in completed longline surveys? Do longlines and eDNA analysis reflect a similar community structure, or are more species identified using a small set of validated eDNA assays?
4. Do eDNA samples reflect the same distribution patterns of *C. plumbeus* in Winyah Bay that historic longline surveys demonstrate?

## Materials and Methods

*Study area*

Winyah Bay is a 65 $km^2$ coastal estuary located in northeast South Carolina (Figure 1, adjacent to the city of Georgetown, SC. The bay is fed by the confluence of four blackwater rivers: Black, Pee Dee, Waccamaw, and Sampit (Goñi, Teixeira, & Perkey 2003). During periods of low river flow, Winyah Bay is a partially mixed estuary. However, under high flow conditions, the upper and middle thirds of the bay function as a salt wedge estuary (Bloomer 1973). The saltwater inflow during a normal tidal cycle reaches just north of the US-17 highway bridge. Winyah Bay's tidal flow is semi-diurnal with a mean amplitude of 1.4 m with salinities along the bay's axis ranging from 0 to 34 parts per thousand (ppt), with greater values at the mouth (Goñi, Teixeira, & Perkey 2003). The deepest portion of the bay is also located at the mouth, with depths reaching greater than 10 m. Average depth around the bay is 4 m (Abel *et al*., 2007). Water temperatures vary seasonally from means of 9°C in February to 30°C in July.

Along much of the bay margin, the dominant plant is *Spartina alterniflora*. Sediments of Winyah Bay consist of mud, sand, silt, and clay, with river-deposited sediments dominating the upper estuary (Patchineelam & Kjerfve, 2004). Although the main shipping channel had been maintained for decades, the town of Georgetown stopped dredging in 2008 and is currently exploring alternative options to maintain the bottom structure of the bay (T. Hannebuth, pers. comm 10 August 2021).

Based on a previous study by Abel *et al.* (2007), Winyah Bay can be divided into three subsections based on a salinity gradient. The subsections are referred to as upper, middle, and lower bay, with each region corresponding to the ecosystem's average salinity of fresh, brackish, and marine, respectively.

*Longlines and eDNA water sample collection*

Water samples were collected for eDNA analysis from June through November 2021 and April through July 2022. Water samples collected alongside active longlines were meant to enable a comparison between eDNA detection methods and the species composition that was observed via a more traditional survey method, while a second set of water samples collected throughout the bay was intended to establish habitat usage patterns of the most frequently caught species, Sandbar sharks. All samples were collected from the subsurface of the water column from 2.7–3.3 meters of depth via a Model 1010 Niskin Water Sampler (1.2 L). Day, time, and GPS location were recorded at each sample location, along with the ambient environmental conditions; water temperature (°C), salinity (ppt), dissolved oxygen (mg/L), Secchi depth (cm), wind speed (km/hr), and tidal stage defined as time passed from predicted slack high tide and the recorded set time of the line.

Bottom longline sampling was conducted from June–November 2021 and April–July 2022 aboard the Coastal Carolina University vessel, RV *Coastal Research*. At each sampling location, five separate 150 m lines were set. Each 150 m longline consisted of 25, 1 m gangions with 0.5 m steel braided leader and 0.5 m mono-filament line attached to 16/0 circle hooks. Individual gangions were set approximately 4.5 m apart and baited with Boston Mackerel (*Scomber scrombus).* All longlines were deployed during or

slightly before high slack tide and were soaked for 45–60 minutes. Soak time was measured as the time from the first hook in the water until the last hook was out of the water. Tide predictions were based on the National Oceanographic and Atmospheric Association (NOAA) tide charts for Mosquito Creek (ACT6536).

A 1 L water sample was collected alongside each longline, creating five replicates for each survey date. All water samples were collected before each baited longline was set according to recommendations from the eDNA Society (2019). Upon completion of soak, longlines were retrieved, and any sharks smaller than 1.5 m total length (TL) were brought on board to be identified, measured, and tagged. Once onboard, individuals were identified to species and classified by sex. Measurements of a full workup included precaudal length (PCL), fork length (FL), and stretched tail length (TL), all recorded in centimeters. All captured sharks were processed and tagged unless physical stress indicators were observed, such as a delayed nictitating membrane reflex or blotchy coloration. To minimize stress while being handled, sharks were placed in a tub with ambient bay water; however, sharks > 1.5 m were secured alongside the boat for workup and tagging. Shark tags were provided by the NOAA and National Marine Fisheries Service (NMFS) Cooperative Shark Tagging Program. Sharks that did not attempt to swim away upon release were revived beside the boat to aid in post-capture survivability (Gary, 2009).

Samples intended to monitor *C. plumbeus* usage of Winyah Bay were collected monthly from July–November 2021 aboard the CCU vessel RV *Brooks McIntyre*. Due to their distribution throughout the entirety of the bay these samples necessitated a separate trip from those collected alongside the longlines. Five sampling locations per each

subsection of the bay were identified for water collection (figure 2). Locations were selected based on previous location data from Abel et al., (2007) and depth. Depth was considered in an effort to standardize the depths of the water sample with those at longline sampling locations. Again, using the Model 1010 Niskin Water Sampler (1.2 L), 1-liter of water was collected from each of the 15 locations for analysis.

eDNA sample collection procedures to minimize cross-contamination due to handling were followed for all field collections (Ma *et al*., 2016; Greiner-Ferris, 2020), bottles were handled only when wearing powder-free sterile gloves. Water sample collection bottles and coolers were cleaned with a 20% bleach solution in order to remove any traces of elasmobranch DNA prior to each sampling event (Ma *et al.*, 2016). Additionally, an extra sterilized collection bottle filled with DI water was kept in the cooler to monitor for contamination between water samples during sampling events. Upon collection, samples were placed directly in the cooler with ice to be transported back to the laboratory on CCU's main campus, where filtration took place. Filtration of the water sample allowed genetic material to be separated from the environmental medium. Samples were filtered within 24 hours through a 47 mm mixed cellulose ester (MCE) filter paper with a 0.45 μm pore size (Ma *et al.*, 2016; Postaire *et al.*, 2020). The filtration unit was rinsed with a 20% bleach solution between each sample. To monitor for cross-contamination via lab practices, 1 L of DI water was filtered through the filtration system after each sterilization. Those filters were stored separately and tested later for lab contamination. All control and experimental filters were removed and folded into a 2 mL loBind tube and stored at -20℃ until eDNA extraction.

*Genomic controls*

For future considerations of validating the primer assay, tissue samples in the form of fin clips were collected to serve as known genomic source DNA. The final collection included fin clips from 15 local elasmobranch species commonly caught using traditional survey methods (*Carcharhinus plumbeus, C. limbatus, C. leucas, C. acronotus, C. isodon, Negaprion brevirostris, Rhizoprionodon terraenovae, Sphyrna tiburo, S. lewini, Squalus acanthias, Hypanus americanus, Hypanus sabina, Gymnura micrura, Myliobatis freminvillii, Rhinoptera bonasus*). During the 2022 season, a fin clip from the dorsal fin of each individual shark caught during the longline surveys was collected and stored at -20°C in a 2 mL loBind tube. Additional fin clips were from stored tissues from CCU Shark Lab projects, tissues donated by the University of North Carolina Institute of Marine Science (UNC-IMS Plumlee), and Bimini Biological Field Station (BBFS). From these tissues, a genomic DNA (gDNA) collection of 15 species was extracted (Table 1). Concentrations of gDNA (µg/µL) were measured using a Thermofisher Nanodrop™ spectrophotometer measuring nucleic acid concentration (260/280 ratio) and double-strand DNA (dsDNA) concentration (µg/µL).

*Aquaria controls*

To assess the function of the primer assays' ability to detect small fragments of DNA in a controlled environment water was collected from aquarium environments. Five institutions known to house the study's species of interest participated in the study. Water samples were obtained in triplicate from systems that were known to house *C. plumbeus* and *S. tiburo* from Pine Knoll Shores Aquarium, Fort Fisher Aquarium, Ripley's Aquarium of Myrtle Beach, Ripley's Aquarium of the Smokies, and the

Mississippi Aquarium (Table 2). The Mississippi Aquarium was the only participating institution with a system that housed *C. acronotus*. Water samples were obtained in triplicate from this system to confirm functionality of the *C. acronotus* primer. For each system sampled, 3–4 L of water were collected along with target species housed, number of individuals in residence, other elasmobranchs in residence, the volume of the system, and details about the most recent water change. Samples were shipped overnight to CCU and, within 24–48 hours from collection, filtered through a 47 mm mixed cellulose ester (MCE) filter paper with a 0.45 μm pore size (Ma *et al*., 2016; Postaire *et al.*, 2020). All aquaria control filters were removed and folded into 2 mL loBind tubes and stored at -20 °C until eDNA extraction.

*eDNA extraction*

DNA extraction protocols for the previously collected fin clips, and filter material followed the same protocol. Extraction was performed using the DNeasy Blood and Tissue kit from Qiagen following the extraction protocol per Goldberg *et al.* (2011). Due to cost limitations, the protocol was slightly modified; instead of using Qiashredder spin columns, all filters will be manually finely shredded using sterilized tools. Filters were pulled from the freezer in one-sample-day increments. To begin extraction, the workbench and tools needed for shredding were sterilized with a 10% bleach solution. Working with one filter at a time, the filters were scored down the middle of the grid; one half was placed back into the loBind tube for storage while the other was shredded into roughly 1 square millimeter-sized pieces and transferred to a 1.5 mL centrifuge tube. Initially, 20 μL of proteinase K and 180 μL of ATL buffer solution were added to the centrifuge tube; if the filter material was not fully immersed, up to 50 μL of ATL buffer

was added as needed and notated. Centrifuge tubes were capped with parafilm and incubated at 59℃ for 24 hours in a water incubator. Samples were vortexed periodically over the 24 h period. The workbench and tools were sterilized with a 10% bleach solution between every sample. After incubating at 59 ℃ for 24 hours, samples were vortexed and spun down to separate excess debris. All liquid was then transferred to a DNeasy Spin Column, and the manufacturer protocol was followed until the elution step. For the final product, elution buffer AE was warmed to 70 ℃, then 100 µL was added to the membrane and left to sit at room temperature for ten minutes before the final collection of the eluate.

*PCR assay development*

Five primer sets and PCR assays for detection of species within Winyah Bay were used. Two previously published assays (Postaire *et al*., 2020; Schweiss *et al*., 2019), and three novel (*C. plumbeus, C. acronotus, S. tiburo*). The previously published assays were designed to detect Blacktip Sharks (588 F-limbatus-NADH2: 5′-TGCCCCCAATCTCACCTTAC-3′ and 776 R-limbatus-NADH2: 5′-CCGGAAAGTGGGGGTAATCC-3′) (Postaire *et al.*, 2020) and Bull Sharks ((BULLND2F6: 5’-TCCGGGTTTATACCCAAATG-3' and BULLND2R5: 5'-GAAGGAGGATGGATAAGATT-3') with probe (BULL_IBFQ: 5'-CAACACTAACTATAAGTCCTAACCCAATC-3') (Schweiss *et al.*, 2019).

Three sets of oligonucleotide PCR primers were designed to target the conserved regions of mitochondrial DNA (mtDNA) nicotine adenine dinucleotide dehydrogenase subunit 2 (NADH2) gene for Sandbar Sharks (NCBI accession number KY909609.1, Vella *et al.*, 2017), Blacknose Sharks (National Center for Biotechnology Information

(NCBI) accession number DQ422101.1 Lopez *et al.*) and Bonnetheads (NCBI accession number OM165197.1, Villate M.). Mitochondrial DNA sequences of the target species and 14 other local non-target elasmobranch species were aligned using GeniousPrime (v. 2022.2.1) and the Muscle 3.8.425 plugin for the software. All genetic sequences were downloaded from GenBank and the MiFish databases (Table 3). Primer pairs were designed to amplify 109–150 base pair fragments (Simpfendorfer *et al.*., 2016, Postaire *et al.*, 2020) exclusively within the target gene region. This was achieved by maximizing the number of mismatches; primer pair parameters required 2–3 mismatches with local non-target species, 50-60% GC content, and melting temperatures ($T_m$) between 59–64 ℃. A BLAST search for each primer pair compared primer sequences to all available sequence data in the NCBI genetic database to confirm that the primer pairs did not match any already known non-target sequences.

Each PCR reaction for all five tested assays consisted of 12.5 µL DreamTaq MasterMix 2× (Thermofisher Scientific), 5.8 µL of PCR grade water (Thermofisher Scientific), 3.4 µL of extracted genomic DNA (10µg/µL), and 1.7 µL of each primer (10 µM) for a total reaction volume of 25 µL.

For Sandbar Sharks, forward (C.plu-NADH2-312-F: 5’-AAAAATTGGCCTCGCACCAC-3') and reverse (C.plu-NADH2-402-R: 5'-TGGCGAATGGGGCTAGTTTT-3') primer sequences were designed to PCR amplify a 109 base pair region of the mtDNA NADH2 gene in *C. plumbeus*. The primer pair was tested using gDNA extracted from 12 *C. plumbeus* individuals from Winyah Bay, South Carolina, using conventional PCR. PCR cycling conditions began with initial denaturation of 94℃ for 2 minutes followed by 35 cycles of 95℃ for 30 seconds, 64℃

for 30 seconds, 72℃ for 30 seconds, final extension was at 72℃ for 5 minutes. Primer pairs were also tested against one individual of 14 other genetically similar, local exclusion species.

For Blacknose Sharks, forward (C.acr-NADH2-757-F: 5'-GGACTTCCTCCACTTTCCGG-3') and reverse (C.acr-NADH2-913-R: 5'-ATGTTGGTTGGGTTGGGGTT-3') primer sequences were designed to amplify a 175 base pair region of the mtDNA NADH2 gene in *C. acronotus*. The primer pair was first tested using gDNA of eight *C. acronotus* individuals collected by Bimini Biological Field Station in 2019 from waters surrounding the Florida Keys. PCR cycling conditions began with initial denaturation of 95 ℃ for 2 minutes followed by 35 cycles of 95 ℃ for 30 seconds, 63 ℃ for 30 seconds, 72 ℃ for 30 seconds, final extension was at 72 ℃ for 5 minutes. Primer pairs were also tested against one individual of 14 other genetically similar, local exclusion species.

For Bonnetheads, forward (S.tib-NADH2-848-F: 5'-CCCTCATAGCCCTCCTCAGT-3') and reverse (S.tib-NADH2-968-F: 5'-TGAGGTTAGGAGGGTGAGGG-3') primer sequences were designed to amplify a 139 base pair region of the mtDNA NADH2 gene in *S. tiburo.* The primer pair was first tested using gDNA of six *S. tiburo* individuals collected from coastal South Carolina waters. PCR cycling conditions began with initial denaturation of 94 ℃ for 2 minutes followed by 35 cycles of 94 ℃ for 30 seconds, 59 ℃ for 30 seconds, 72 ℃ for 30 seconds, final extension was at 72 ℃ for 5 minutes. The primer pair was also tested against one individual of 14 other genetically similar, local exclusion species.

Published primer assays for *C. leucas* (Schweiss *et al.*, 2019) and *C. limbatus* (Postaire *et al.*, 2020) were also tested to verify species-specificity in this study. Genomic DNA for *C. leucas* was obtained from eight individuals collected by Bimini Biological Field Station in 2019 from waters surrounding the Florida Keys, while gDNA for *C. limbatus* was obtained from nine individuals from Winyah Bay, SC. PCR assays and primers followed according publication details for Bull Sharks (Schweiss *et al*., 2019) and Blacktips (ref). These primer assays were also tested against one individual of 14 other genetically similar, local exclusion species.

To determine the functionality of the novel primer pairs in eDNA assays, each primer pair was applied to three types of extracted filter samples: known aquarium sources, environmental control samples, and water samples collected from Winyah Bay. Environmental control samples were 1 L water samples taken from the holding container aboard the RV *Coastal Research* after a species of interest had been placed into it. Unfortunately, we did not catch a single Blacknose Shark during either sampling season, and so an environmental control does not exist for this species. All three newly designed primer sets, as well as the published Bull Shark primer set (Schweiss *et al*., 2019), were applied to all aquarium samples, whether the system held the target species or not. Finally, all three primer pairs were also applied to water samples taken from Winyah Bay. When a primer pair was applied to a filtered sample, the reagent reaction and PCR cycle specifications were tailored to the species-specific primer pair that was used.

The quality of all amplifications was assessed by electrophoresis. Product volume electrophoresed was 7 µL across all wells in 1.5% agarose gels stained with SYBR Safe Gel Stain in 0.5× TBE (Thermofisher Scientific). Each gel contained a positive control of

target species gDNA, a negative template control (NTC), and a 50 bp ladder (Thermofisher Scientific) to aid in visualization. Amplification was considered successful when a single band was observed at the expected fragment size (Beauclerc *et al.*, 2018). Any run that showed evidence of contamination was noted, and the PCR was rerun with new components to produce a clean run.

## Results

*Specificity and validation of previously published eDNA assays*

The previously published *C. leucas* (Bull Shark) primer set and probe from Schweiss *et al.* (2019) were validated for specificity using eight gDNA samples of *C. leucas* as positive controls and 14 other genetically similar, local elasmobranch species as negative controls (Table 4). The primer assay successfully amplified seven of the eight gDNA samples and did not amplify any of the 14 other local species (Figure 3). No aquarium facility holding this species participated in the study.

The previously published *C. limbatus* primer assay from Postaire *et al.* (2020) was tested for specificity using nine gDNA samples of *C. limbatus* (Blacktip Shark) and 14 other genetically similar, local elasmobranch species. The primer assay amplified all nine gDNA samples, positive detections were observed for 8 of the 14 species used as negative controls (figure 4). Those species with positive detections using the primer assay were *R. terraenovae, C. isodon, S. lewini, G. micrura, H. sabina, M. freminvillii, C. acronotus,* and *S. acanthias*. This result was double-checked using a second gDNA sample from all 14 other species; the same result was obtained upon electrophoresis completion. Due to the non-specificity of the primer assay, it was not used for any further analysis.

*Specificity and validation of novel eDNA assays*

Three primer assays were developed for elasmobranch species local to Winyah Bay. For Sandbar Sharks, forward (C.plu-NADH2-312-F: 5'-AAAAATTGGCCTCGCACCAC-3') and reverse (C.plu-NADH2-402-R: 5'-TGGCGAATGGGGCTAGTTTT-3') primer sequences were designed, and successfully amplified 12 gDNA samples taken from Winyah Bay.

For Blacknose Sharks, forward (C.acr-NADH2-757-F: 5'-GGACTTCCTCCACTTTCCGG-3') and reverse (C.acr-NADH2-913-R: 5'-ATGTTGGTTGGGTTGGGGTT-3') primer sequences were designed and successfully amplified eight gDNA samples taken by Bimini Biological Field Station from individuals in the Florida Keys.

For Bonnetheads forward (S.tib-NADH2-848-F: 5'-(CCCTCATAGCCCTCCTCAGT-3') and reverse (S.tib-NADH2-968-R: 5'-TGAGGTTAGGAGGGTGAGGG-3') primer sequences were designed, and successfully amplified six gDNA samples taken from coastal South Carolina waters.

Gel electrophoresis after PCR confirmed species-specificity for all three primer assays as none of the primer pairs amplified any of the 14 other local species (Figure 5--7).

The functionality of the primer assays' ability to detect small amounts of genomic material in an environment was confirmed via aquarium samples. When the primer pairs were applied to filtered samples that housed their particular species of interest a positive detection was always obtained (figures 8-10). To ensure specificity in an environmental setting, the primer pairs were applied to samples from systems that were known not to

host their particular species of interest. False positive detections were not obtained from these reactions (Table 5)

*Application of eDNA assays in Winyah Bay, South Carolina*

The four validated species-specific assays were applied to filtered water samples (n=275) from Winyah Bay, South Carolina. No positive detections (0 of 275) were obtained from any eDNA samples extracted from waters of Winyah Bay for any of the species-specific PCR assays. DNA purification was attempted through ethanal precipitation of nucleic acids following the Eppendorf Protocol (OpenWetWare, 2015). It should be noted that the average Nanodrop™ spectrophotometer concentration of the samples extracted from Winyah Bay was significantly higher ($p<0.001$) than the concentrations recorded from the gDNA samples (Figure 11). Further concentrating the DNA precipitate did not yield a positive detection in subsequent PCR reactions. No further analysis was possible with the given results. Therefore, we could not compare community assemblages as surveyed via longline vs eDNA analysis or how eDNA analysis assessed Sandbar shark habitat distribution of the bay.

## Discussion

Novel oligonucleotide primer pairs for Sandbar Sharks, Blacknose Sharks, and Bonnetheads were designed and validated to detect eDNA from captive-housed sharks. We were able to provide support for their functionality via aquarium water samples, as each primer was able to detect the species it was designed for. Unfortunately, we were unable to demonstrate the full functionality of the assays in an environmental setting with the water samples obtained from Winyah Bay.

*Species-specificity*

When designing primers, the length of the primer's target fragment length is of important consideration. Targeting short fragments of DNA increases the likelihood of amplifying eDNA, especially in environments with highly fragmented genomic material (Strickler *et al.*, 2015, Axtner *et al.*, 2018). However, targeting longer fragments of DNA can allow for higher taxonomic resolution or the ability to make a distinction among closely related species (Bylemans *et al.*, 2018). Current studies suggest that primer pair sensitivity in estuarine and coastal environments decreases with increasing target fragment length (Axtner *et al.*, 2018, Bakker *et al.*, 2018). Environmental DNA, species-specific primer design requires primers be developed in a region of the mtDNA that is diagnostically variable from closely related species; however, short target fragments in the mitochondrial genome of elasmobranchs can be difficult to design. This is due to the highly conserved areas of the genome and slow mutation rates characteristic of this group

compared to other vertebrates (Martin, 1995; Dudgeon *et al.*, 2012). Previously published elasmobranch eDNA primer sets have an amplicon range from ~50-250 bp (e.g., Simpfendorfer *et al.*, 2016, Lafferty *et al.*, 2018, Schweiss *et al.*, 2019). The products of this study also stayed within that target range. Previous studies have focused on the COI gene and struggled to put forth primer sets that could differentiate between carcharhinid species (Bakker *et al.*, 2017, Boussaire *et al.*, 2018, Liu *et al.*, 2022). This result is often attributed to the non-monophyly of many of the families within the order (Vélez-Zuazo & Agnarsson, 2010). This study, in combination with Schweiss *et al.* (2019), provides support that differentiation between carcharhinids can be determined by utilizing the NADH2 gene to create primer sets.

*PCR inhibition*

There are many ways by which PCR reactions may be inhibited (Balasingham *et al.*, 2017); one that may have contributed to the inhibition in this project was high organic material concentrations. Inhibition is caused by co-extracted substances that interfere with PCR amplification and can limit or completely mask the detection of target DNA (Mckee *et al.*, 2015). In lentic systems, the majority of these compounds are humic substances (Albers *et al.*, 2013). Estuaries such as Winyah Bay receive input of organic material from multiple allochthonous sources, including groundwater, rivers, and tidal movements, and autochthonous sources such as the native vegetation (Patchineelam & Kjerfve, 2004). Goñi *et al.* (2003) documented higher ratios of organic matter from surrounding vegetation from May - November in Winyah Bay. This time frame of elevated organic matter correlates directly with the sampling season of this project. Similarly, a study from the Florida panhandle that was conducted in coastal, brackish

waters confirmed high levels of inhibition in connection to the tannins produced by the vegetation surrounding the body of water (Hunter *et al.*, 2018). Additionally, tidal diffusion is known to be a dominant factor in flushing PCR inhibitors out of a tidal salt marsh system (Kjerfve *et al.*, 1991). Sampling on the incoming high tide may not have allowed for the full diffusion of PCR inhibitors out of the system. Performance quality of the eDNA extractions was evaluated via DNA yields with Nanodrop™ spectrophotometer technology. However, this evaluation is likely to be inaccurate due to the inability of the Nanodrop™ to discriminate DNA molecules from all other possible biological macromolecules. This may offer an explanation as to why the Nanodrop™ readings were significantly higher in concentration than the gDNA samples. It is important to note that water samples were not analyzed for organic content at any point so this explanation can not be supported with any degree of confidence.

Potential solutions for decreasing the impact of inhibitors are dependent on the molecules present. There are three methods commonly used to try to reduce the impact of inhibitors in a sample they are 10-fold dilution in water, the use of Bovine Serum Albumin (BSA), and spin column purification (Mckee *et al.*, 2015). Based on the results of a 2015 study (Mckee *et al.*,) spin column purification was the best option for this study. Despite efforts to increase DNA concentration through purification, PCR reactions were still inhibited in the Winyah Bay samples, as evidenced by the lack of positive detection. 10-fold dilutions in water were also shown decrease inhibition in the 2015 study's coastal samples; however, diluting samples can decrease the sensitivity of the assay, especially when target DNA is present in low concentrations (Goldberg *et al.*, 2011). All methods to reduce PCR inhibitors from environmental samples can increase

measurement error and the risk of sample contamination (Cao *et al.*, 2015). However, if inhibitors are not removed, interference with PCR amplification can profoundly affect detectability and lead to underestimation of the target species' presence (Cao *et al.*, 2015).

*Practical applications of eDNA*

The use of eDNA as a survey tool has grown exponentially in recent years, but appreciation of the tool and considerations of its limitations have not grown in time with its use (Beng & Corlett, 2020). As supported by the results of this study, eDNA analysis does not always work, or curated results do not always provide the information the project hoped to gain. However, utilizing an assay in more advantageous conditions can make the detection and monitoring of a species more efficient when compared to traditional survey methods that can be labor intensive and time-consuming (Zhang *et al.*, 2020). Additionally, recent studies that aimed to analyze the cost efficiency of eDNA analysis found that the survey method is 2-10 times cheaper when compared to traditional visual surveys (Davy *et al.*, 2015, Sigsgaard *et al.*, 2015). When designing an eDNA study, it is important to consider whether or not it is the appropriate technique to answer the questions of the study. Environmental DNA studies cannot yet determine life history stages, sex ratio, body condition, or reliably quantify abundance of target species, although that is an avenue of active research (Griener-Ferris, 2020, Doi *et al.*, 2021, Doi & Nakamura, 2022). Thus, at this stage in technological development, most studies should use eDNA analysis as a preliminary tool for ecological surveys or concurrently with an additional survey method in order to gather the most robust data (Beng & Corlett, 2020). For example, in line with the original goals for this study, eDNA analysis can be used concurrently with longline sampling for a community structure survey. The longline

analysis would provide important ecological information about the individuals caught, while the eDNA analysis could provide information about the unseen portion of the community. Furthermore, eDNA may be the preferred method to reliably detect rare or elusive species or to quickly and efficiently gather data about the distribution of an invasive species (Beng & Corlett, 2020, Doi & Nakamura, 2023). As technology continues to improve and the limitations of the survey method are better understood, eDNA analysis can be improved upon and more efficiently applied in the field of ecological research.

## Conclusion

This study provides evidence that species-specific eDNA primers can be made for closely related carcharhinid species by using the NADH2 gene for specificity. Optimized primer pairs successfully detected 109, 175, and 139 base pair fragments of Sandbar Sharks, Blacknose Shark, and Bonnetheads, respectively, in aquarium systems known to house the individual species. While successful in design and controlled aquarium environments, eDNA analysis did not detect DNA from common species of sharks in Winyah Bay, even from waters collected in the presence of captured individuals of those species. The results of this study suggest that further use of the methodology be suspended in this waterbody until there are improvements in the technology or its limitations in an environment containing high amounts of dissolved organic material are better understood.

The results of this study contribute to the general knowledge and design of eDNA primers for elasmobranch species while also providing three new species-specific primers for future analysis. This study highlights the limitations of eDNA analysis and the challenges of utilizing developing technology in ecological research. Future studies should consider the abiotic factors of the survey medium and attempt to apply the newly developed primers to an environment of lower organic content. Continued contributions of eDNA studies will further improve the understanding of the technique and will

assuredly lead to a reliable, cost-effective, non-invasive monitoring technique for the marine environment.

## Tables

**Table 1:** Genomic DNA collection includes the total number of tissue samples collected per species, year(s) of collection, and institutions that collected them. Participating institutions include Coastal Carolina University Shark Project (CCU), Bimini Biological Field Station (BBFS), and University of North Carolina- Institute of Marine Science (UNC-IMS)

| Species | Number of Samples | Year Collected | Collecting Institution |
|---|---|---|---|
| Sandbar | 14 | 2022 | CCU |
| Blacknose | 10 | 2019 | BBFS (8)/ UNC-IMS (2) |
| Bonnethead | 6 | 2022 | CCU |
| Bull | 8 | 2019 | BBFS |
| Blacktip | 9 | 2019/2022 | UNC-IMS (2)/ CCU (7) |
| Finetooth | 8 | 2019/2022 | UNC-IMS (3)/ CCU (5) |
| Lemon | 8 | 2019 | BBFS |
| Atlantic Sharpnose | 10 | 2022 | CCU |
| Scalloped Hammerhead | 10 | 2019/2022 | UNC-IMS (4)/ CCU (6) |
| Spiny Dogfish | 2 | 2019 | UNC-IMS |
| Southern Ray | 6 | 2022 | CCU |
| Butterfly Ray | 1 | 2022 | CCU |
| Atlantic Ray | 5 | 2022 | CCU |
| Cownose Ray | 2 | 2022 | CCU |
| Bullnose Ray | 1 | 2022 | CCU |

**Table 2:** Details of aquarium samples, including the institution they came from, the volume of water in the system, the target species present in the system and their abundance, and any non-target elasmobranch(s) in the same system. Non-target elasmobranchs surveyed include; Sand Tiger Sharks (ST), Nurse Sharks (NS), Southern Ray (SR), Largetooth Sawfish (LTS), Blacktip Sharks (BS), Whitetip Reef Sharks (WRS), Leopard Sharks (LS), Cownose Ray (CR), Spotted Eagle Ray (SER), White Spotted Bamboo Shark (WSBS), Brown Banded Bamboo Shark (BBBS), Epaulette Shark (ES), Yellow Ray (YR), Blue Spot Ray (BSR), Shovelnose Guitarfish (SG), Zebra Shark (ZS) and the Atlantic Ray (AR)

| System Name | Institution | Volume (gallons) | Target Species | #of Target Species | Non-target Species |
|---|---|---|---|---|---|
| Living Shipwreck | North Carolina Aquariums-Pine Knoll Shores | 306,000 | Sandbar Shark | 3 | ST, NS |
| Cape Fear Shoals | North Carolina Aquariums-Fort Fisher | 235,000 | Bonnethead | 1 | SR, ST |
| Dangerous Reef | Ripley's Aquarium of Myrtle Beach | 550,000 | Sandbar Shark | 5 | SR, ST, NS, LTS |
| Quarantine 4 | Ripley's Aquarium of Myrtle Beach | 12,800 | Bonnethead | 1 | N/A |
| Shark Lagoon | Ripley’s Aquarium of the Smokies | 750,000 | Sandbar Sharks | 3 | SR, BS, NS, ST, WRS, LS, LTS |
| Ray Bay | Ripley’s Aquarium of the Smokies | 84,000 | Bonnethead | 8 | SR, CR, SER, BS, WSBS, ES |
| Aquatic Wonders | Mississippi Aquarium | 3-5,000 | Sandbar Shark/Bonnethead | 1/ 4 | SR, CR, YR, BSR, AR, SG, ES, BBS, WSBS, ZS |
| Quarantine 3 | Mississippi Aquarium | 16,000 | Blacknose | 2 | N/A |

**Table 3:** Genbank Accession Numbers for mtDNA sequences of target and non-target local elasmobranch species

| Species | Genbank Accession Number | Author |
|---|---|---|
| **Sandbar Shark, *Carcharhinus plumbeus* (target)** | KY909609.1 | Vella *et al.*, 2017 |
| **Blacknose Shark, *Carcharhinus acronotus* (target)** | DQ422101.1 | Lopez *et al.* |
| **Bonnethead, *Sphyrna tiburo* (target)** | OM165197.1 | Villate, M. |
| **Bull Shark, *Carcharhinus leucas*** | OM165108.1 | Villate, M. |
| **Blacktip Shark, *Carcharhinus limbatus*** | JN082204.1 | Moore *et al.* |
| **Finetooth Shark, *Carcharhinus isodon*** | KU255142.1 | Portnoy, D. |
| **Lemon Shark, *Negaprion brevirostris*** | L08039.1 | Martin at al., 1993 |
| **Atlantic Sharpnose, *Rhizoprionodon terraenovae*** | HM991199.1 | Mendonca *et al.*, 2011 |
| **Scalloped Hammerhead, *Sphyrna lewini*** | MT881538.1 | Budd *et al.*, 2021 |
| **Spiny Dogfish, *Squalus acanthias*** | MT263531.1 | Gracan *et al.*, 2020 |
| **Southern Ray, *Hypanus americanus*** | MT319684.1 | Petean *et al.*, 2020 |
| **Butterfly Ray, *Gymnura micrura*** | JN184295.1 | Aschliman *et al.*, 2011 |
| **Atlantic Ray, *Hypanus sabina*** | JQ518787.1 | Naylor *et al*., 2012 |
| **Cownose Ray, *Rhinoptera bonasus*** | KX151652.1 | White *et al*., 2018 |
| **Bullnose Ray, *Myliobatis freminvillii*** | JN184302.1 | Aschliman *et al*., 2011 |

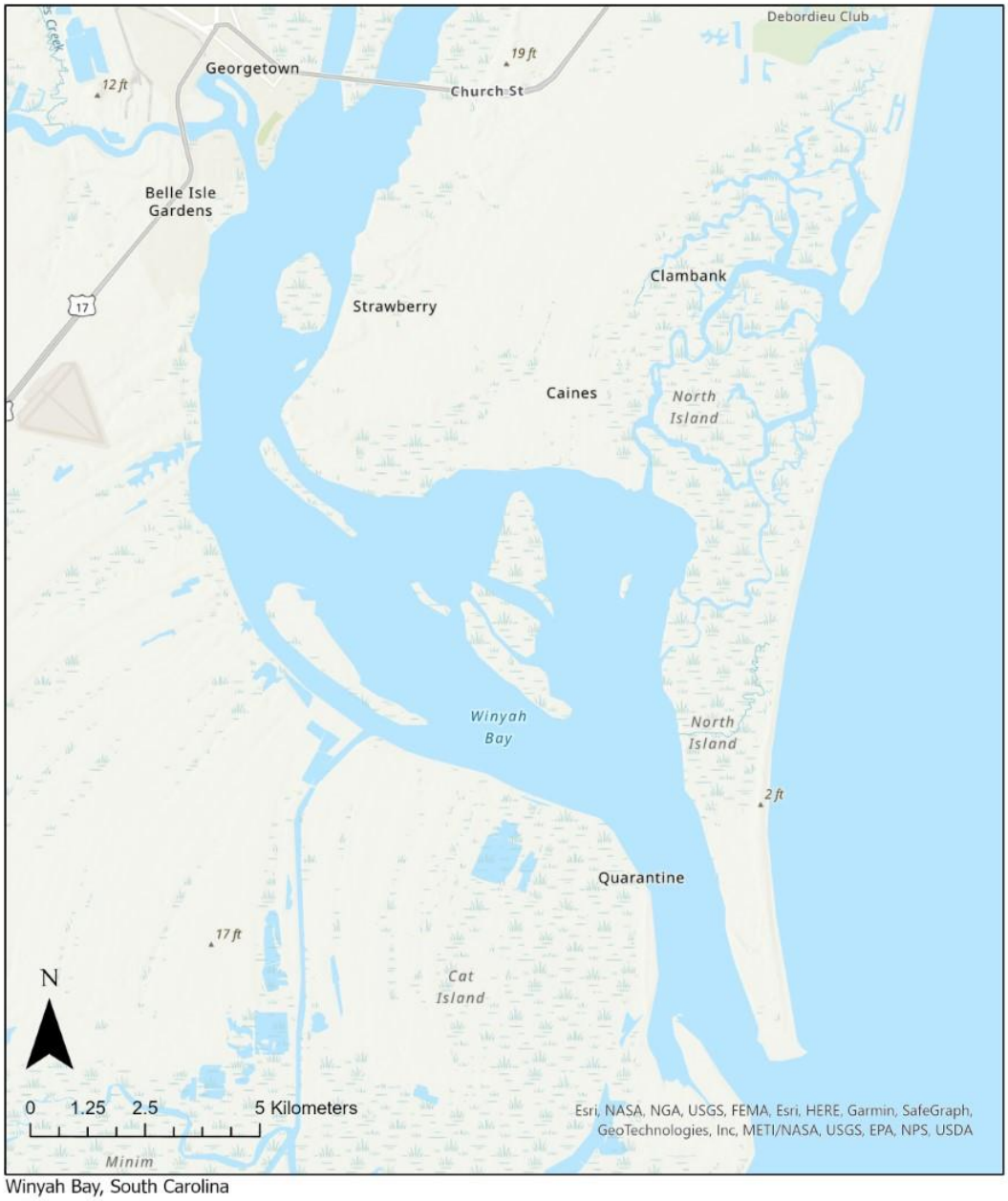
**Table 4:** Primers used for amplification of Blacktip Sharks, Bull Sharks, Sandbar Sharks, Blacknose Sharks, and Bonnetheads. Details include annealing temperature, guanine, and cytosine content (GC%) and total product length (bp).

| Primer Name | Author | Primer Sequence (5'-3') | Tm | GC | Product Length |
|---|---|---|---|---|---|
| 588 F-limbatus-NADH2 | Postaire | TGCCCCCAATCTCACCTTAC | 63 | N/A | 149 |
| 776 R-limbatus-NADH2 | Postaire | CCGGAAAGTGGGGGTAATCC | 63 | N/A | |
| BULLND2F6 | Schweiss | TCCGGGTTTATACCCAAATG | 59 | N/A | 237 |
| BULLND2R5 | Schweiss | GAAGGAGGATGGATAAGATT | 59 | N/A | |
| BULL_IBFQ | Schweiss | CAACACTAACTATAAGTCCTA ACCCAATC | 56 | | |
| C.plu-NADH2-312-F | Flanigan | AAAAATTGGCCTCGCACCAC | 63.7 | 50 | 109 |
| C.pluNADH2-421-R | Flanigan | TGGCGAATGGGGCTAGTTTT | 64.2 | 50 | |
| C.acr-NADH2-757-F | Flanigan | ATGTTGGTTGGGTTGGGGTT | 62.8 | 50 | |
| C.acr-NADH2-932-R | Flanigan | GGACTTCCTCCACTTTCCGG | 63.2 | 60 | 175 |
| S.tib-NADH2-848-F | Flanigan | CCCTCATAGCCCTCCTCAGT | 57.7 | 60 | |
| S.tib-NADH2-987-R | Flanigan | TGAGGTTAGGAGGGTGAGG G | 56.8 | 60 | 139 |

**Table 5: PCR** detection results of novel primers to aquarium samples

| System | Institution | Target Species Housed | Primer Set Applied | Positive Detection Observed |
|---|---|---|---|---|
| Living Shipwreck | North Carolina Aquariums-Pine Knoll Shores | Sandbar Shark | *C. plumbeus* | Yes |
| | | | *C. acronotus* | No |
| | | | *S. tiburo* | No |
| Cape Fear Shoals | North Carolina Aquariums-Fort Fisher | Bonnethead | *C. plumbeus* | No |
| | | | *C. acronotus* | No |
| | | | *S. tiburo* | Yes |
| Dangerous Reef | Ripley's Aquarium of Myrtle Beach | Sandbar Shark | *C. plumbeus* | Yes |
| | | | *C. acronotus* | No |
| | | | *S. tiburo* | No |
| Quarantine 4 | Ripley's Aquarium of Myrtle Beach | Bonnethead | *C. plumbeus* | No |
| | | | *C. acronotus* | No |
| | | | *S. tiburo* | Yes |
| Shark Lagoon | Ripley's Aquarium of the Smokies | Sandbar Shark | *C. plumbeus* | Yes |
| | | | *C. acronotus* | No |
| | | | *S. tiburo* | No |
| Ray Bay | Ripley's Aquarium of the Smokies | Bonnethead | *C. plumbeus* | No |
| | | | *C. acronotus* | No |
| | | | *S. tiburo* | Yes |
| Aquatic Wonders | Mississippi Aquarium | Sandbar Shark/Bonnethead | *C. plumbeus* | Yes |
| | | | *C. acronotus* | No |
| | | | *S. tiburo* | Yes |
| Quarantine 3 | Mississippi Aquarium | Blacknose | *C. plumbeus* | No |
| | | | *C. acronotus* | Yes |
| | | | *S. tiburo* | No |

# Figures



Winyah Bay, South Carolina
Upper Left: 79°19'45"W 33°22'43"N

**Figure 1:** Map of the study site

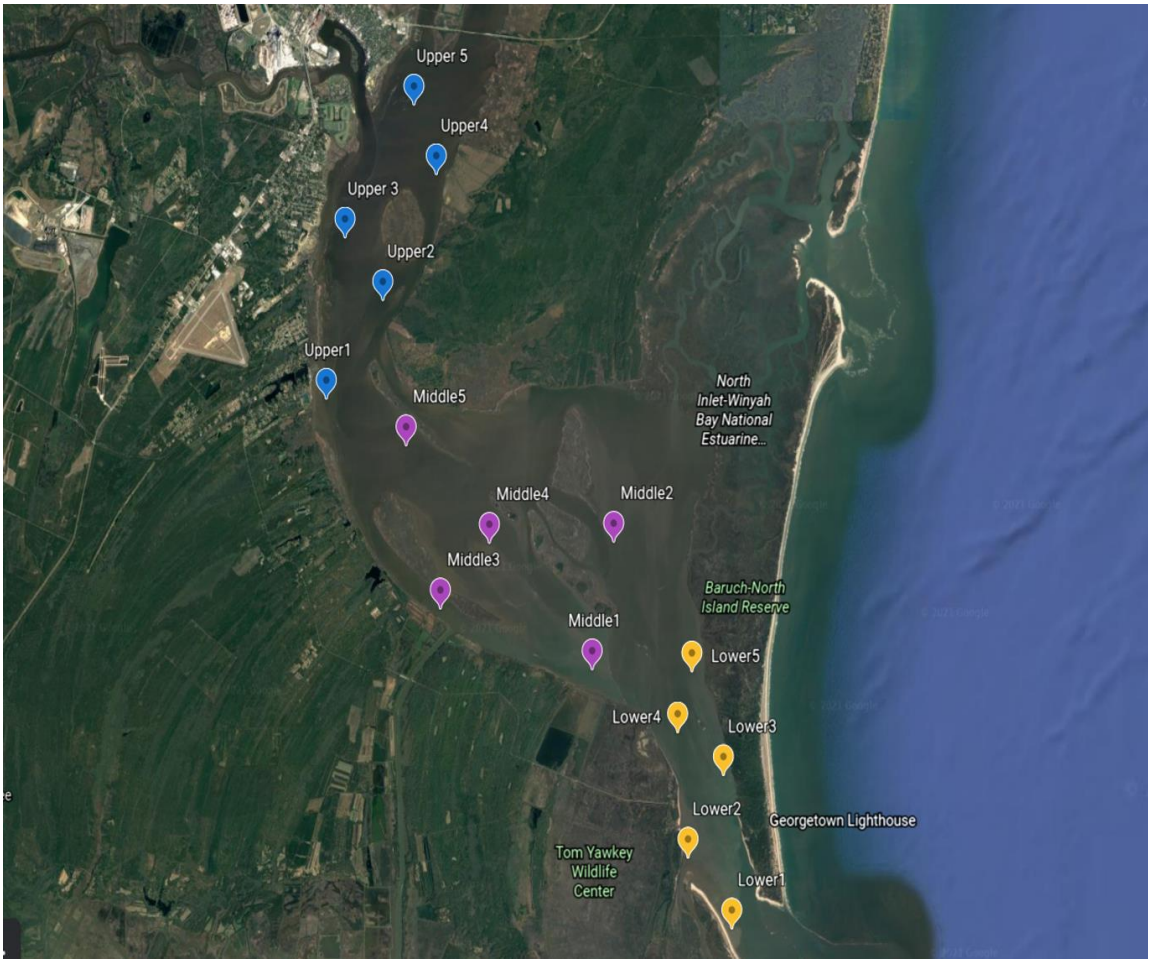



**Figure 2:** Map of habitat distribution sampling sites. The color differences refer to the changes of the subsection of the bay as determined by the salinity gradient. Blue=upper bay, purple =middle bay, yellow= lower bay

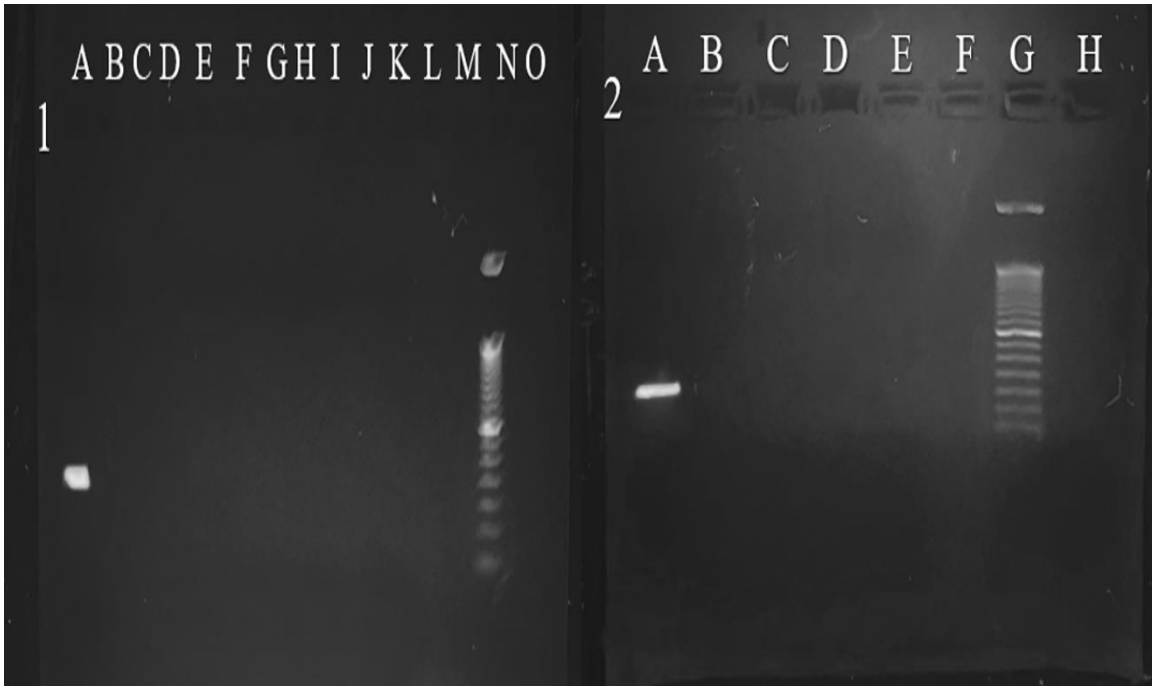



**Figure 3:** 237 bp region of Bull Shark ND2; (1A) Carcharhinus leucas (1B) C. limbatus, (1C) Rhizoprionodon terraenovae, (1D) C. isodon, (1E) Sphyrna lewini, (1F) Hypanus americanus (1G) Gymnura micrura, (1H) S. tiburo, (1I) Hypanus sabina, (1J) Myliobatis freminvillii, (1K) C. plumbeus, (1L) empty, (1M) empty, (1N) 50 bp ladder, (1O) No template control, (2A) C. leucas, (2B) C. acronotus, (2C) Squalas acanthias, (2D) Rhinoptera bonasus, (2E) Negaprion breviriostris, (2F) empty, (2G) 50 bp ladder, (2H) No template control

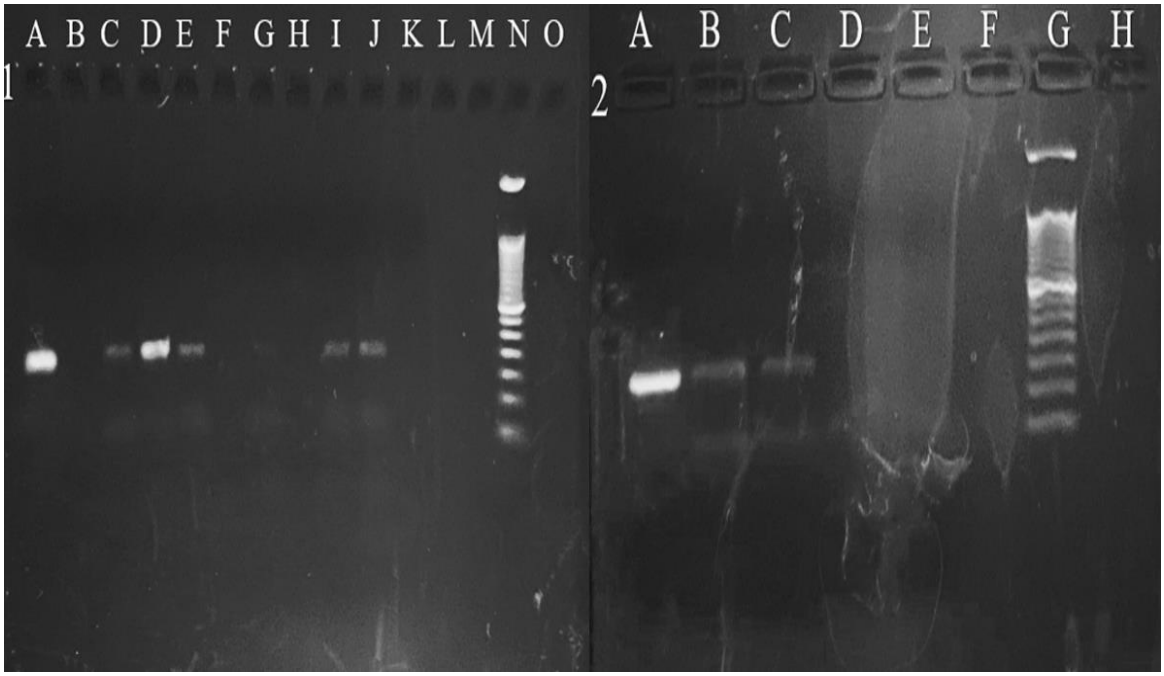



**Figure 4:** 149 bp region of Blacktip NADH2; (1A) Carcharhinus limbatus (1B) C. plumbeus, (1C) Rhizoprionodon terraenovae, (1D) C. isodon, (1E) Sphyrna lewini, (1F) Hypanus americanus (1G) Gymnura micrura, (1H) S. tiburo, (1I) Hypanus sabina, (1J) Myliobatis freminvillii, (1K) C. leucas, (1L) empty, (1M) empty, (1N) 50 bp ladder, (1O) No template control, (2A) C. limbatus, (2B) C. acronotus, (2C) Squalas acanthias, (2D) Rhinoptera bonasus, (2E) Negaprion breviriostris, (2F) empty, (2G) 50 bp ladder, (2H) No template control

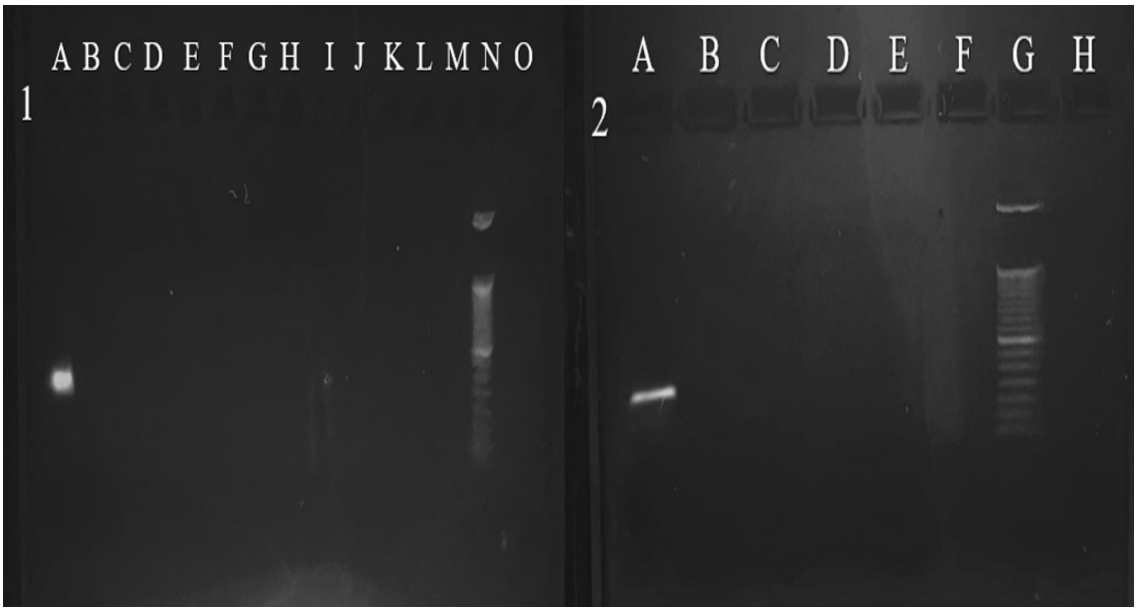



**Figure 5:** 109 bp region of Sandbar Shark NADH2; (1A) Carcharhinus plumbeus (1B) C. limbatus, (1C) Rhizoprionodon terraenovae, (1D) C. isodon, (1E) Sphyrna lewini, (1F) Hypanus americanus (1G) Gymnura micrura, (1H) S. tiburo, (1I) Hypanus sabina, (1J) Myliobatis freminvillii, (1K) C. leucas, (1L) empty, (1M) empty, (1N) 50 bp ladder, (1O) No template control, (2A) C. plumbeus, (2B) C. acronotus, (2C) Squalas acanthias, (2D) Rhinoptera bonasus, (2E) Negaprion breviriostris, (2F) empty, (2G) 50 bp ladder, (2H) No template control

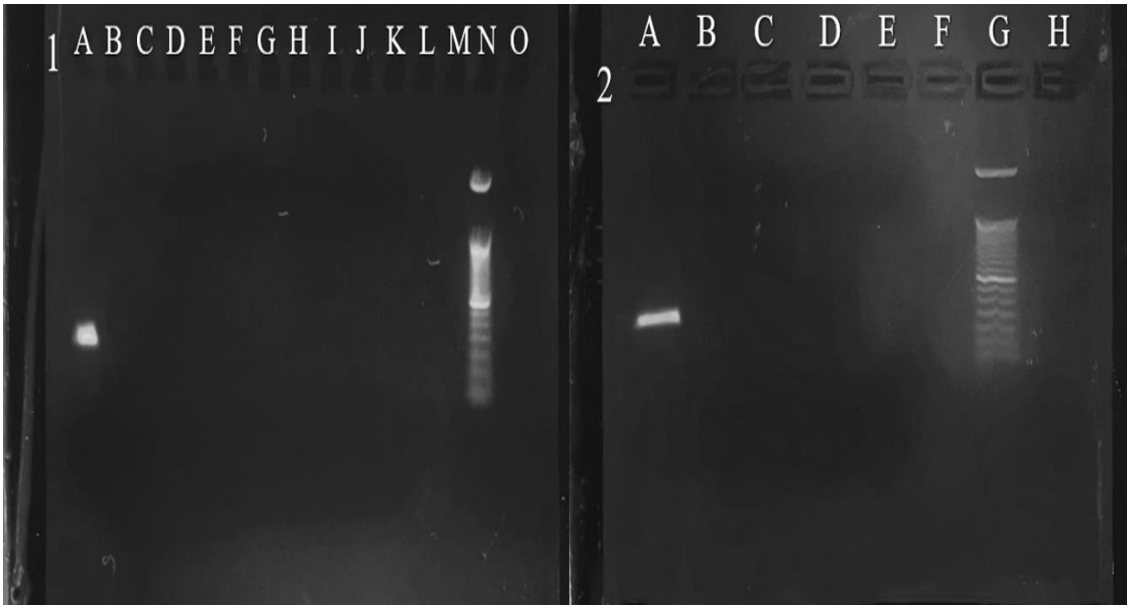



**Figure 6**: 175 bp region of Blacknose Shark NADH2; (1A) Carcharhinus acronotus (1B) C. limbatus, (1C) Rhizoprionodon terraenovae, (1D) C. isodon, (1E) Sphyrna lewini, (1F) Hypanus americanus (1G) Gymnura micrura, (1H) S. tiburo, (1I) Hypanus sabina, (1J) Myliobatis freminvillii, (1K) C. leucas, (1L) empty, (1M) empty, (1N) 50 bp ladder, (1O) No template control, (2A) C. acronotus, (2B) C. plumbeus, (2C) Squalas acanthias, (2D) Rhinoptera bonasus, (2E) Negaprion brevirostris, (2F) empty, (2G) 50 bp ladder, (2H) No template control

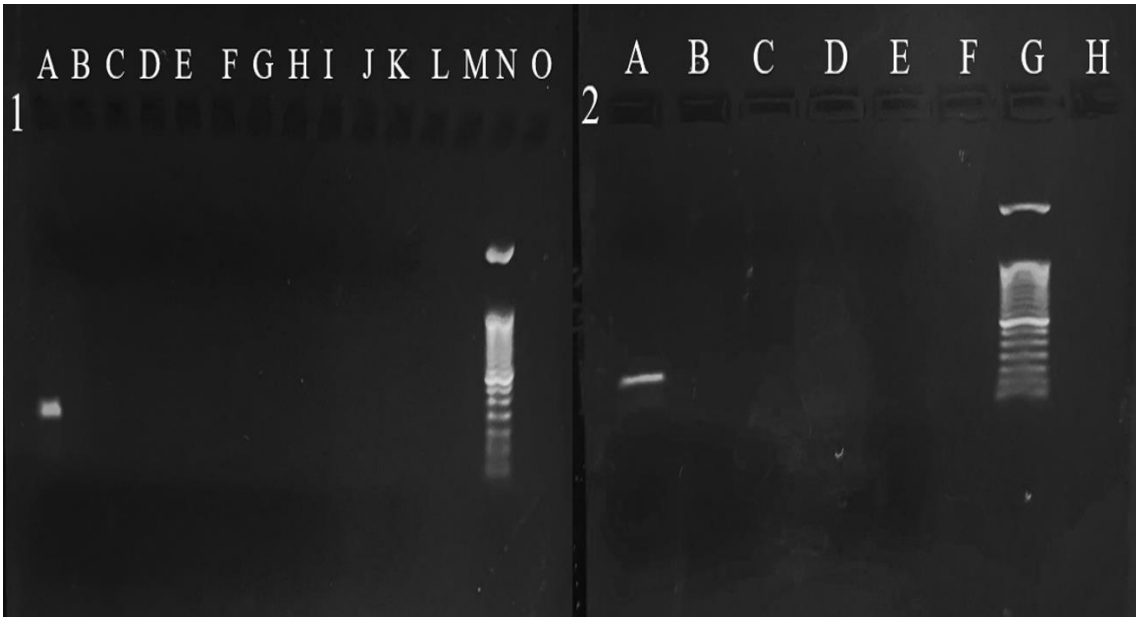



**Figure 7:** 149 bp region of Bonnethead NADH2; (1A) Sphyrna tiburo (1B) C. limbatus, (1C) Rhizoprionodon terraenovae, (1D) C. isodon, (1E) S. lewini, (1F) Hypanus americanus (1G) Gymnura micrura, (1H) C. plumbeus, (1I) Dasyatis sabina, (1J) Myliobatis freminvillii, (1K) C. leucas, (1L) empty, (1M) empty, (1N) 50 bp ladder, (1O) No template control, (2A) S. tiburo, (2B) C. plumbeus, (2C) Squalas acanthias, (2D) Rhinoptera bonasus, (2E) Negaprion brevirostris, (2F) empty, (2G) 50 bp ladder, (2H) No template control

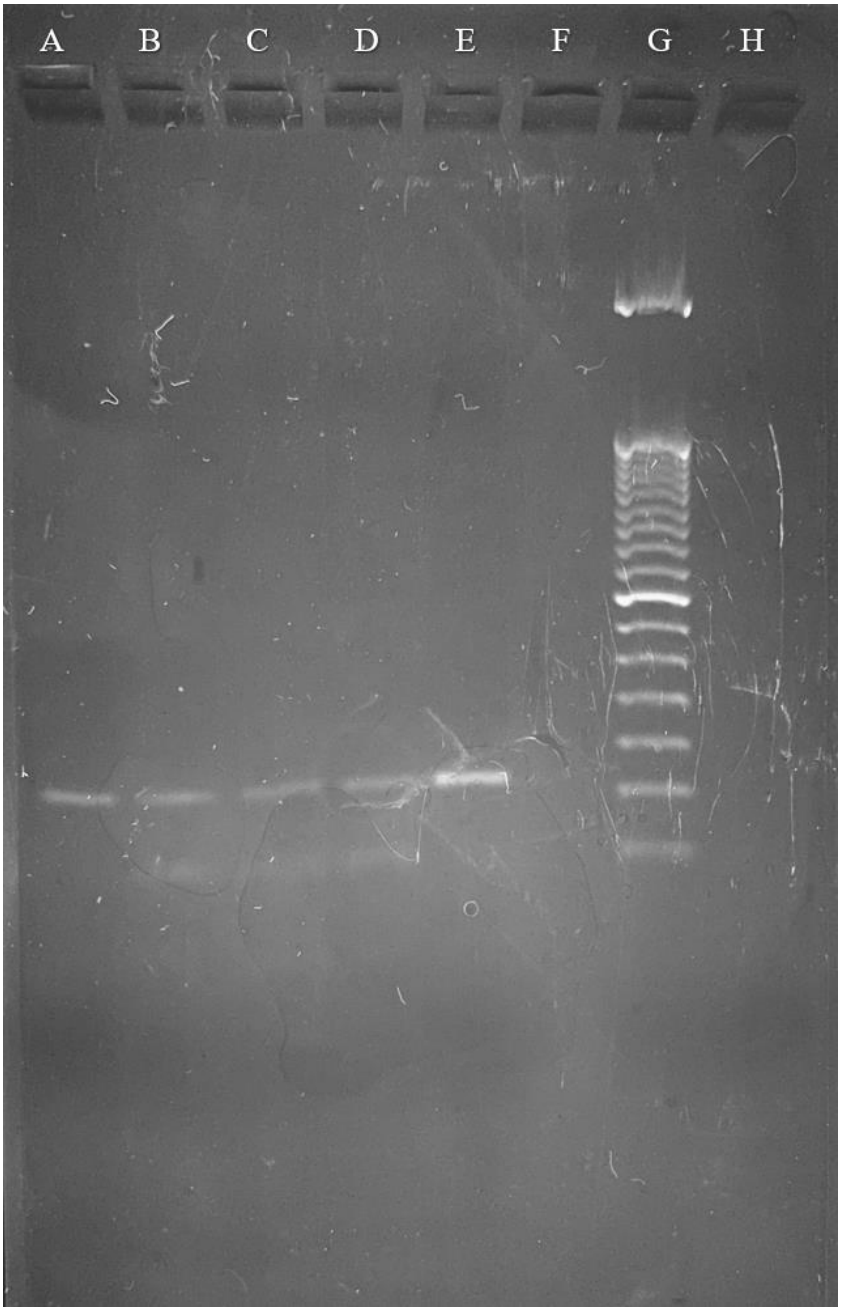



**Figure 8:** Sandbar Aquarium Samples: 109 bp region of Sandbar Shark NADH2; (A) Ripley's Aquarium of the Smokies-Shark Lagoon (B) Mississippi Aquarium- Aquatic Wonders, (C) Pine Knoll Shores Aquarium-Living Shipwreck, (D) Ripley's Aquarium of Myrtle Beach-Dangerous Reef, (E) Carcharhinus plumbeus (F) empty (G) 50 bp ladder (H) No template control

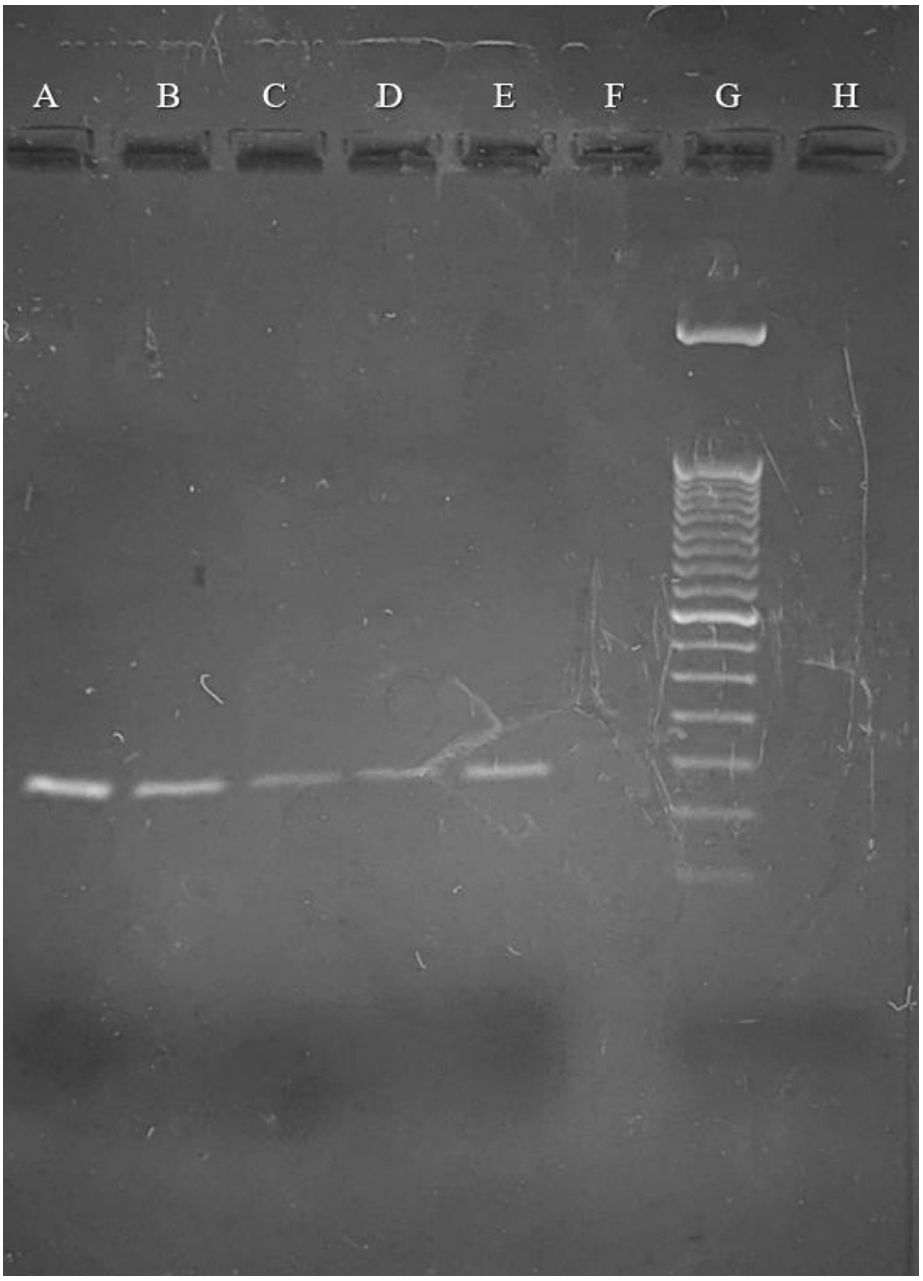



**Figure 9:** Bonnethead Aquarium Samples: 1 bp region of Bonnethead NADH2; (A) Sphyrna tiburo (B) Ripley's Aquarium of the Smokies-Ray Bay (C) Ripley's Aquarium of Myrtle Beach-Quarantine 4, (D) Fort Fisher Aquarium- Cape Fear Shoals, (E) Mississippi Aquarium- Aquatic Wonders (F) empty (G) 50 bp ladder (H) No template control

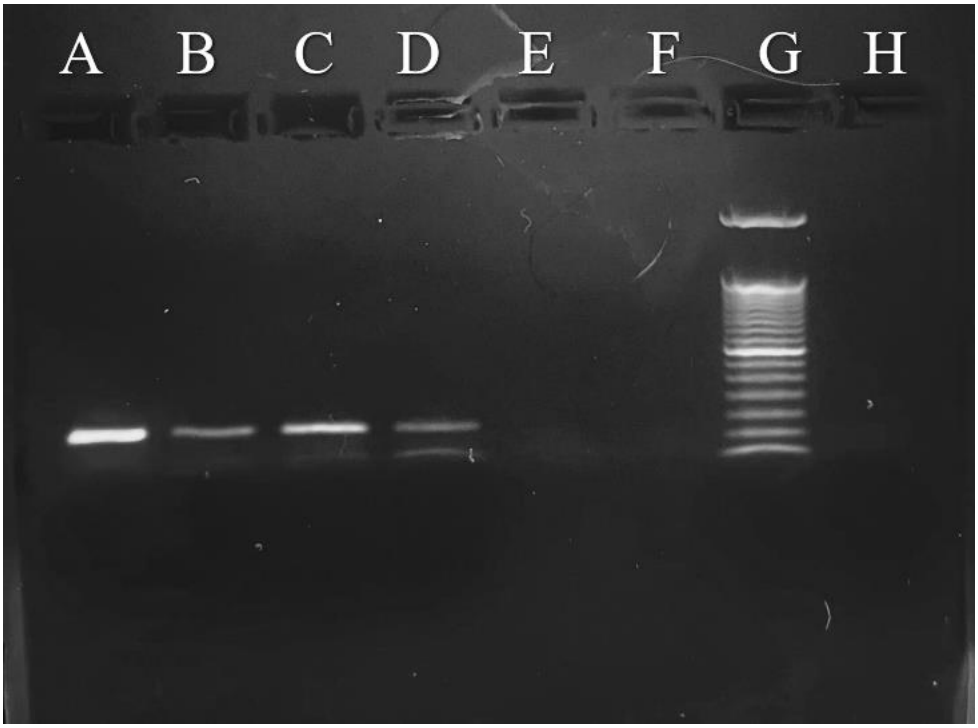



**Figure 10:** Blacknose Shark Aquarium Samples: (A) Carcharhinus acronotus (B) Mississippi Aquarium- Quarantine 3 S1 (C) Mississippi Aquarium- Quarantine 3 S2, (D) Mississippi Aquarium- Quarantine 3 S3, (E) empty (F) empty (G) 50 bp ladder (H) No template control

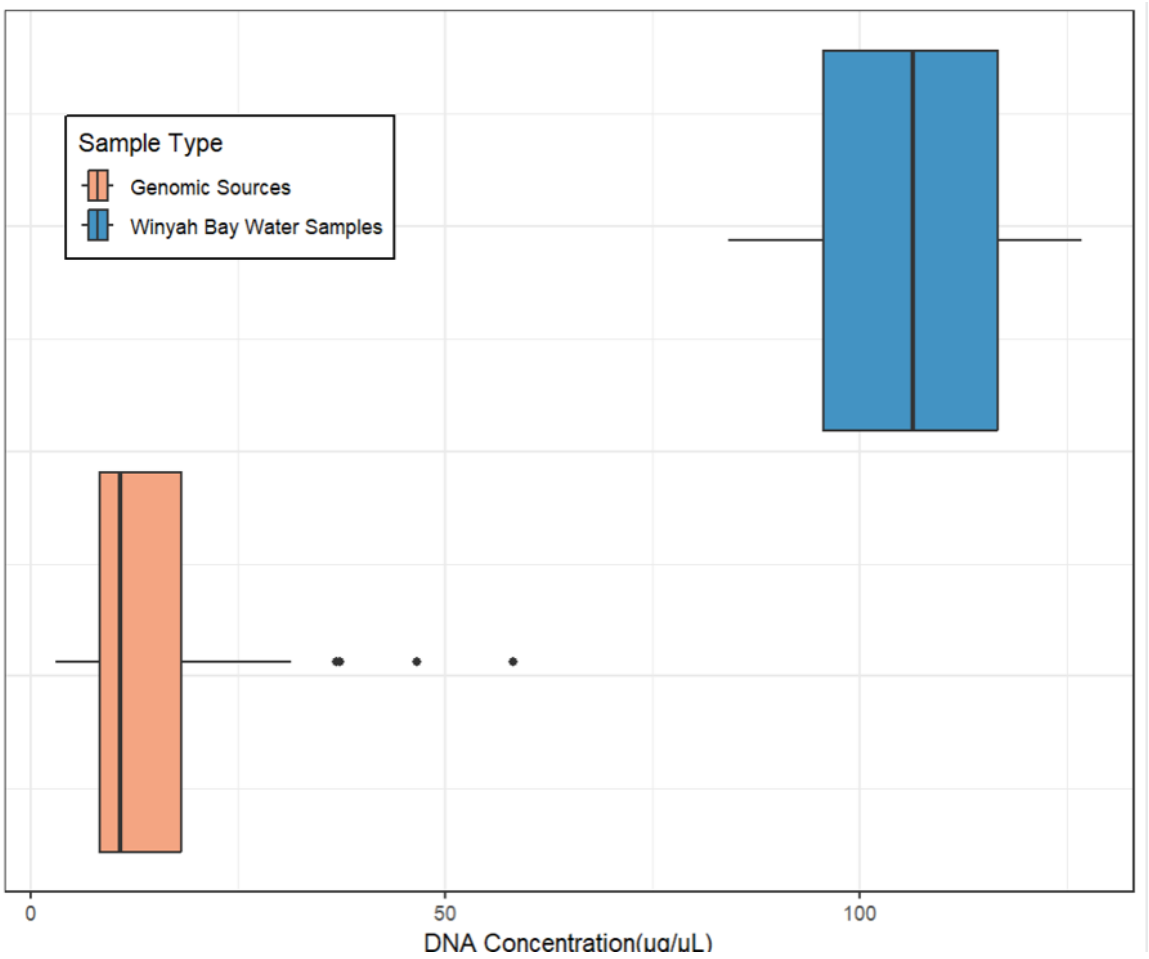



**Figure 11:** Boxplot of DNA concentrations recorded by Nanodrop™ spectrophotometer. Mean concentrations of DNA (µg/µL) differ significantly ($p<0.001$) between genomic sources ($\bar{x}=12.7$) and Winyah Bay Water Samples ($\bar{x}=109.3$)

## References:


Abel, D. C., Young, R. F., Garwood, J. A., Travaline, M. J., & Yednock, B. K. (2007). Survey of the Shark Fauna in Two South Carolina Estuaries and the Impact of Salinity Structure. American Fisheries Society Symposium,

Abel, D. C., & Grubbs, D. (2020). *Shark Biology and Conservation: essentials for educators, students, and enthusiasts*. Johns Hopkins University Press.

Albers, C. N., Jensen, A., Bælum, J., & Jacobsen, C. S. (2013). Inhibition of DNA Polymerases Used in Q-PCR by Structurally Different Soil-Derived Humic Substances. *Geomicrobiology Journal*, *30*(8), 675-681. https://doi.org/10.1080/01490451.2012.758193

Axtner, J., Crampton-Platt, A., Horig, L. A., Mohamed, A., Xu, C. C. Y., Yu, D. W., & Wilting, A. (2019). An efficient and robust laboratory workflow and tetrapod database for larger scale environmental DNA studies. *Gigascience*, *8*(4). https://doi.org/10.1093/gigascience/giz029

Bakker, J., Wangensteen, O. S., Chapman, D. D., Boussarie, G., Buddo, D., Guttridge, T. L., Hertler, H., Mouillot, D., Vigliola, L., & Mariani, S. (2017). Environmental DNA reveals tropical shark diversity in contrasting levels of anthropogenic impact. *Sci Rep*, *7*(1), 16886. https://doi.org/10.1038/s41598-017-17150-2

Bakker, J. (2018). *e(lasmo)DNA: The role of environmental DNA (eDNA) analysis in marine fish biodiversity assessment with special focus on elasmobranchs* [Thesis (PhD), University of Salford]. University of Salford E-theses.

Balasingham, K. D., Walter, R. P., & Heath, D. D. (2017). Residual eDNA detection sensitivity assessed by quantitative real-time PCR in a river ecosystem. *Mol Ecol Resour*, *17*(3), 523-532. https://doi.org/10.1111/1755-0998.12598

Beauclerc, K., Wozney, K., Smith, C., & Wilson, C. (2018). Development of quantitative PCR primers and probes for environmental DNA detection of amphibians in Ontario. *Conservation Genetics Resources*, *11*(1), 43-46. https://doi.org/10.1007/s12686-017-0962-3

Beng, K. C., & Corlett, R. T. (2020). Applications of environmental DNA (eDNA) in ecology and conservation: opportunities, challenges and prospects. *Biodiversity and Conservation*, *29*(7), 2089-2121. https://doi.org/10.1007/s10531-020-01980-0

Bloomer, D. R. (1973). *A hydrographic investigation of Winyah Bay, South Carolina and the adjacent coastal waters* [Masters of Science, Georgia Institute of Technology]. Georgia Tech Theses and Dissertations. http://hdl.handle.net/1853/25984

Bonfil, R., Palacios-Barreto, P., Vargas, O. U. M., Ricaño-Soriano, M., & Díaz-Jaimes, P. (2021). Detection of critically endangered marine species with dwindling

populations in the wild using eDNA gives hope for sawfishes. *Marine Biology*, *168*(5). https://doi.org/10.1007/s00227-021-03862-7

Boussarie, G., Bakker, J., Wangensteen, O. S., Mariani, S., Bonnin, L., Juhel, J.-B., Kiszka, J. J., Kulbicki, M., Manel, S., Robbins, W. D., Vigliola, L., & Mouillot, D. (2018). Environmental DNA illuminates the dark diversity of sharks. *Science Advances*, *4*(5), eaap9661. https://doi.org/doi:10.1126/sciadv.aap9661

Burkholder, D. A., Heithaus, M. R., Fourqurean, J. W., Wirsing, A., & Dill, L. M. (2013). Patterns of top-down control in a seagrass ecosystem: could a roving apex predator induce a behaviour-mediated trophic cascade? *J Anim Ecol*, *82*(6), 1192-1202. https://doi.org/10.1111/1365-2656.12097

Bylemans, J., Furlan, E. M., Pearce, L., Daly, T., & Gleeson, D. M. (2016). Improving the containment of a freshwater invader using environmental DNA (eDNA) based monitoring. *Biological Invasions*, *18*(10), 3081-3089. https://doi.org/10.1007/s10530-016-1203-5

Bylemans, J., Furlan, E. M., Gleeson, D. M., Hardy, C. M., & Duncan, R. P. (2018). Does Size Matter? An Experimental Evaluation of the Relative Abundance and Decay Rates of Aquatic Environmental DNA. *Environ Sci Technol*, *52*(11), 6408-6416. https://doi.org/10.1021/acs.est.8b01071

Cao, Y., Griffith, J. F., Dorevitch, S., & Weisberg, S. B. (2012). Effectiveness of qPCR permutations, internal controls and dilution as means for minimizing the impact of inhibition while measuring Enterococcus in environmental waters. *J Appl Microbiol*, *113*(1), 66-75. https://doi.org/10.1111/j.1365-2672.2012.05305.x

Cristescu, M. E., & Hebert, P. D. N. (2018). Uses and Misuses of Environmental DNA in Biodiversity Science and Conservation. *Annual Review of Ecology, Evolution, and Systematics*, *49*(1), 209-230. https://doi.org/10.1146/annurev-ecolsys-110617-062306

Davy, C. M., Kidd, A. G., & Wilson, C. C. (2015). Development and Validation of Environmental DNA (eDNA) Markers for Detection of Freshwater Turtles. *PLoS One*, *10*(7), e0130965. https://doi.org/10.1371/journal.pone.0130965

Deiner, K., Bik, H. M., Machler, E., Seymour, M., Lacoursiere-Roussel, A., Altermatt, F., Creer, S., Bista, I., Lodge, D. M., de Vere, N., Pfrender, M. E., & Bernatchez, L. (2017). Environmental DNA metabarcoding: Transforming how we survey animal and plant communities. *Mol Ecol*, *26*(21), 5872-5895. https://doi.org/10.1111/mec.14350

Dejean, T., Valentini, A., Duparc, A., Pellier-Cuit, S., Pompanon, F., Taberlet, P., & Miaud, C. (2011). Persistence of environmental DNA in freshwater ecosystems. *PLoS One*, *6*(8), e23398. https://doi.org/10.1371/journal.pone.0023398

Doi, H., Minamoto, T., Takahara, T., Tsuji, S., Uchii, K., Yamamoto, S., Katano, I., & Yamanaka, H. (2021). Compilation of real-time PCR conditions toward the standardization of environmental DNA methods. *Ecological Research*, *36*(3), 379-388. https://doi.org/10.1111/1440-1703.12217

Doi, H., & Nakamura, K. (2022). Special issue: Environmental DNA as a practical tool for aquatic conservation and restoration. *Landscape and Ecological Engineering*, *19*(1), 1-2. https://doi.org/10.1007/s11355-022-00534-6

Dudgeon, C. L., Blower, D. C., Broderick, D., Giles, J. L., Holmes, B. J., Kashiwagi, T., Kruck, N. C., Morgan, J. A., Tillett, B. J., & Ovenden, J. R. (2012). A review of the application of molecular genetics for fisheries management and conservation of sharks and rays. *J Fish Biol*, *80*(5), 1789-1843. https://doi.org/10.1111/j.1095-8649.2012.03265.x

Dulvy, N. K., Fowler, S. L., Musick, J. A., Cavanagh, R. D., Kyne, P. M., Harrison, L. R., Carlson, J. K., Davidson, L. N., Fordham, S. V., Francis, M. P., Pollock, C. M., Simpfendorfer, C. A., Burgess, G. H., Carpenter, K. E., Compagno, L. J., Ebert, D. A., Gibson, C., Heupel, M. R., Livingstone, S. R., . . . White, W. T. (2014). Extinction risk and conservation of the world's sharks and rays. *Elife*, *3*, e00590. https://doi.org/10.7554/eLife.00590

Dulvy, N. K., Fowler, S. L., Musick, J. A., Cavanagh, R. D., Kyne, P. M., Harrison, L. R., Carlson, J. K., Davidson, L. N., Fordham, S. V., Francis, M. P., Pollock, C. M., Simpfendorfer, C. A., Burgess, G. H., Carpenter, K. E., Compagno, L. J., Ebert, D. A., Gibson, C., Heupel, M. R., Livingstone, S. R., . . . White, W. T. (2014). Extinction risk and conservation of the world's sharks and rays. *Elife*, *3*, e00590. https://doi.org/10.7554/eLife.00590

Fischer, J., Erikstein, K., D'Offay, B., Guggisberg, S., & Barone, M. (2012). Review of the implementation of the internal plan of action for the conservation and management of sharks. *FAO Fisheries and Aquaculture Circular; Rome*(C1076), 121.

Flowers, K. I., Babcock, E. A., Papastamatiou, Y. P., Bond, M. E., Lamb, N., Miranda, A., Nuñez, R., Valentin-Albanese, J., Clementi, G. M., Kelley, M. C., & Chapman, D. D. (2022). Varying reef shark abundance trends inside a marine reserve: evidence of a Caribbean reef shark decline. *Marine Ecology Progress Series*, *683*, 97-107. https://doi.org/10.3354/meps13954

Foote, A. D., Thomsen, P. F., Sveegaard, S., Wahlberg, M., Kielgast, J., Kyhn, L. A., Salling, A. B., Galatius, A., Orlando, L., & Gilbert, M. T. (2012). Investigating the potential use of environmental DNA (eDNA) for genetic monitoring of marine mammals. *PLoS One*, *7*(8), e41781. https://doi.org/10.1371/journal.pone.0041781

Fryman, K. (2013). *Shark Community Structure in Winyah Bay, SC*. 27. [Honors Thesis, Coastal Carolina University]. CCU Digital Commons.

Gargan, L. M., Morato, T., Pham, C. K., Finarelli, J. A., Carlsson, J. E. L., & Carlsson, J. (2017). Development of a sensitive detection method to survey pelagic biodiversity using eDNA and quantitative PCR: a case study of devil ray at seamounts. *Marine Biology*, *164*(5). https://doi.org/10.1007/s00227-017-3141-x

Gary, S. (2009). *Shark Population and Structure and Partitioning in Winyah Bay, SC* (Publication Number 77) [Master of Science, Coastal Carolina University]. CCU Digital Commons.

Goldberg, C. S., Pilliod, D. S., Arkle, R. S., & Waits, L. P. (2011). Molecular detection of vertebrates in stream water: a demonstration using Rocky Mountain tailed frogs and Idaho giant salamanders. *PLoS One*, *6*(7), e22746. https://doi.org/10.1371/journal.pone.0022746

Goñi, M. A., Teixeira, M. J., & Perkey, D. W. (2003). Sources and distribution of organic matter in a river-dominated estuary (Winyah Bay, SC, USA). *Estuarine, Coastal and Shelf Science*, *57*(5-6), 1023-1048. https://doi.org/10.1016/s0272-7714(03)00008-8

Greiner-Ferris, K. (2020). *Testing the Potential of Environmental DNA: Genetic Monitoring of Estuarine and Coastal Bottlenose Dolphin (Tursiops truncatus) Stocks in South Carolina* (Publication Number 118) [Master of Science, Coastal Carolina University]. CCU Digital Commons.

Hunter, M. E., Meigs-Friend, G., Ferrante, J. A., Takoukam Kamla, A., Dorazio, R. M., Keith-Diagne, L., Luna, F., Lanyon, J. M., & Reid, J. P. (2018). Surveys of environmental DNA (eDNA): a new approach to estimate occurrence in Vulnerable manatee populations. *Endangered Species Research*, *35*, 101-111. https://doi.org/10.3354/esr00880

Huver, J. R., Koprinvinikar, J., Johnson, P. T. J., & Whyard, S. (2015). Development and application of an eDNA method to detect and quantify a pathogenic parasite in aquatic ecosystems. *Ecological Society of America*, *25*(4), 991-1002. https://doi.org/10.1890/14-1530.1

IUCN (2022). The IUCN Red List of Threatened Species. Version 2022-2. https://www.iucnredlist.org. Accessed on 12/20/2022.

Jerde, C. L., Mahon, A. R., Chadderton, W. L., & Lodge, D. M. (2011). "Sight-unseen" detection of rare aquatic species using environmental DNA. *Conservation Letters*, *4*(2), 150-157. https://doi.org/10.1111/j.1755-263X.2010.00158.x

Johri, S., Doane, M., Allen, L., & Dinsdale, E. (2019). Taking Advantage of the Genomics Revolution for Monitoring and Conservation of Chondrichthyan Populations. *Diversity*, *11*(4). https://doi.org/10.3390/d11040049

Kjerfve, B., Miranda, L. B., & Wolanski, E. (1991). Modelling water circulation in an estuary and intertidal salt marsh system. *Netherlands Journal of Sea Research*, *28*(3), 141-147. https://doi.org/10.1016/0077-7579(91)90013-Q

Lafferty, K. D., Benesh, K. C., Mahon, A. R., Jerde, C. L., & Lowe, C. G. (2018). Detecting Southern California's White Sharks With Environmental DNA. *Frontiers in Marine Science*, *5*. https://doi.org/10.3389/fmars.2018.00355

Liu, Z., Collins, R. A., Baillie, C., Rainbird, S., Brittain, R., Griffiths, A. M., Sims, D. W., Mariani, S., & Genner, M. J. (2022). Environmental DNA captures elasmobranch diversity in a temperate marine ecosystem. *Environmental DNA*, *4*(5), 1024-1038. https://doi.org/10.1002/edn3.294

Lodge, D. M., Turner, C. R., Jerde, C. L., Barnes, M. A., Chadderton, L., Egan, S. P., Feder, J. L., Mahon, A. R., & Pfrender, M. E. (2012). Conservation in a cup of water: estimating biodiversity and population abundance from environmental DNA. *Mol Ecol*, *21*(11), 2555-2558. https://doi.org/10.1111/j.1365-294X.2012.05600.x

Ma, H., Stewart, K., Lougheed, S., Zheng, J., Wang, Y., & Zhao, J. (2016). Characterization, optimization, and validation of environmental DNA (eDNA) markers to detect an endangered aquatic mammal. *Conservation Genetics Resources*, *8*(4), 561-568. https://doi.org/10.1007/s12686-016-0597-9

Martin, A. P. (1995). Mitochondrial DNA sequence evolution in sharks: rates, patterns, and phylogenetic inferences. *Mol Biol Evol*, *12*(6), 1114-1123. https://doi.org/10.1093/oxfordjournals.molbev.a040285

Matthias, L., Allison, M. J., Maslovat, C. Y., Hobbs, J., & Helbing, C. C. (2021). Improving ecological surveys for the detection of cryptic, fossorial snakes using eDNA on and under artificial cover objects. *Ecological Indicators*, *131*. https://doi.org/10.1016/j.ecolind.2021.108187

McKee, A. M., Spear, S. F., & Pierson, T. W. (2015). The effect of dilution and the use of a post-extraction nucleic acid purification column on the accuracy, precision, and inhibition of environmental DNA samples. *Biological Conservation*, *183*, 70-76. https://doi.org/10.1016/j.biocon.2014.11.031

Navia, A., Mejía-Falla, P., Lopez, J., Giraldo, A., & Cruz-Escalona, V. (2017). How many trophic roles can elasmobranchs play in a marine tropical network? *Marine and Freshwater Research*, *68*(7), 12. https://doi.org/10.1071/MF16161

National Oceanic Atmospheric Administration (NOAA)., (2021). COASTSPAN Survey Available at: The Search for Atlantic Shark Nurseries (arcgis.com) Accessed on 12/20/2022

Olson, Z. H., Briggler, J.T., Williams, R.N. (2012). An eDNA approach to detect eastern hellbenders (Cryptobranchus a. alleganiensis) using samples of water. *Wildlife Research*, *39*(7), 629-636. https://doi.org/10.1071/WR12114

OpenWetWare contributors., (2012) Ethanol precipitation of nucleic acids *OpenWetWare* Available at: http://openwetware.org/index.php?title=Ethanol_precipitation_of_nucleic_acids&oldid=611420 Acessed on 11/11/2022

Pacoureau, N., Rigby, C. L., Kyne, P. M., Sherley, R. B., Winker, H., Carlson, J. K., Fordham, S. V., Barreto, R., Fernando, D., Francis, M. P., Jabado, R. W., Herman, K. B., Liu, K. M., Marshall, A. D., Pollom, R. A., Romanov, E. V., Simpfendorfer, C. A., Yin, J. S., Kindsvater, H. K., & Dulvy, N. K. (2021). Half a century of global decline in oceanic sharks and rays. *Nature*, *589*(7843), 567-571. https://doi.org/10.1038/s41586-020-03173-9

Patchineelam, S. M., & Kjerfve, B. (2004). Suspended sediment variability on seasonal and tidal time scales in the Winyah Bay estuary, South Carolina, USA. *Estuarine, Coastal and Shelf Science*, *59*(2), 307-318. https://doi.org/10.1016/j.ecss.2003.09.011

Peterson, C. D., Belcher, C. N., Bethea, D. M., Driggers, W. B., Frazier, B. S., & Latour, R. J. (2017). Preliminary recovery of coastal sharks in the south-east United States. *Fish and Fisheries*, *18*(5), 845-859. https://doi.org/10.1111/faf.12210

Plumlee, J. D., Dance, K. M., Matich, P., Mohan, J. A., Richards, T. M., TinHan, T. C., Fisher, M. R., & Wells, R. J. D. (2018). Community structure of elasmobranchs in estuaries along the northwest Gulf of Mexico. *Estuarine, Coastal and Shelf Science*, *204*, 103-113. https://doi.org/10.1016/j.ecss.2018.02.023

Postaire, B. D., Bakker, J., Gardiner, J., Wiley, T. R., & Chapman, D. D. (2020). Environmental DNA detection tracks established seasonal occurrence of blacktip sharks (Carcharhinus limbatus) in a semi-enclosed subtropical bay. *Sci Rep*, *10*(1), 11847. https://doi.org/10.1038/s41598-020-68843-0

Sani, L. M. I., Husna, A. K., Subhan, B., & Madduppa, H. (2021). Environmental DNA (eDNA) reveals endangered narrow sawfish across Indonesian Reefs. *IOP Conference Series. Earth and Environmental Science*, *1*(1), 9. https://doi.org/10.1088/1755-1315/944/1/012020

Schweiss, K. (2019). *Development and Employment of Environmental DNA Mathods for the Detection of Bull Sharks (Carcharhinus leucas) in a Freshwater and Estuarine Interface in the Mobile-Tensaw Delta and Mobile Bay, Alabama* [Master of Science, University of Southern Mississippi]. Marine Biology Commons.

Schweiss, K. E., Lehman, R. N., Drymon, J. M., & Phillips, N. M. (2019). Development of highly sensitive environmental DNA methods for the detection of Bull Sharks,Carcharhinus leucas (Müller and Henle, 1839), using Droplet Digital™ PCR. *Environmental DNA*, *2*(1), 3-12. https://doi.org/10.1002/edn3.39

Scriver, M., Zaiko, A., Pochon, X., & von Ammon, U. (2023). Harnessing decay rates for coastal marine biosecurity applications: A review of environmental DNA and RNA fate. *Environmental DNA*. https://doi.org/10.1002/edn3.405

Sigsgaard, E. E., Carl, H., Møller, P. R., & Thomsen, P. F. (2015). Monitoring the near-extinct European weather loach in Denmark based on environmental DNA from water samples. *Biological Conservation*, *183*, 46-52. https://doi.org/10.1016/j.biocon.2014.11.023

Sigsgaard, E. E., Nielsen, I. B., Bach, S. S., Lorenzen, E. D., Robinson, D. P., Knudsen, S. W., Pedersen, M. W., Jaidah, M. A., Orlando, L., Willersleve, E., Moller, P. R., & Thomsen, P. F. (2017). Population characteristcs of a large whale shark aggregation inferred from seawater environmental DNA. *Nature Ecology and Revolution*, *1*. https://doi.org/10.1038/s41559-016-0004

Simpfendorfer, C. A., Garin, F. G., Wiley, T. R., & Heupel, M. R. (2005). Distribution and Habitat Partitioning of Immature Bull Sharks (Carcharhinus leucas) in a Southwest Florida Estuary. *Estuaries*, *28*(1), 8. https://doi.org/ 10.1007/BF02732755

Simpfendorfer, C. A., Kyne, P. M., Noble, T. H., Goldsbury, J., Basiita, R. K., Lindsay, R., Shields, A., Perry, C., & Jerry, D. R. (2016). Environmental DNA detects Critically Endangered largetooth sawfish in the wild. *Endangered Species Research*, *30*, 109-116. https://doi.org/10.3354/esr00731

Sorenson, L., Santini, F., & Alfaro, M. E. (2014). The effect of habitat on modern shark diversification. *J Evol Biol*, *27*(8), 1536-1548. https://doi.org/10.1111/jeb.12405

Spangler, M. A., Huettmann, F., Herriott, I. C., & López, J. A. (2017). Development, validation, and evaluation of an assay for the detection of wood frogs (Rana sylvatica) in environmental DNA. *Conservation Genetics Resources*, *10*(4), 631-633. https://doi.org/10.1007/s12686-017-0881-3

Strickler, K. M., Fremier, A. K., & Goldberg, C. S. (2015). Quantifying effects of UV-B, temperature, and pH on eDNA degradation in aquatic microcosms. *Biological Conservation*, *183*, 85-92. https://doi.org/10.1016/j.biocon.2014.11.038

Takeuchi, A., Sado, T., Gotoh, R. O., Watanabe, S., Tsukamoto, K., & Miya, M. (2019). New PCR primers for metabarcoding environmental DNA from freshwater eels, genus Anguilla. *Sci Rep*, *9*(1), 7977. https://doi.org/10.1038/s41598-019-44402-0

Thomas, A. C., Tank, S., Nguyen, P. L., Ponce, J., Sinnesael, M., & Goldberg, C. S. (2019). A system for rapid eDNA detection of aquatic invasive species. *Environmental DNA*, *2*(3), 261-270. https://doi.org/10.1002/edn3.25

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

Thomsen, P. F., & Willerslev, E. (2015). Environmental DNA – An emerging tool in conservation for monitoring past and present biodiversity. *Biological Conservation*, *183*, 4-18. https://doi.org/10.1016/j.biocon.2014.11.019

Velez-Zuazo, X., & Agnarsson, I. (2011). Shark tales: a molecular species-level phylogeny of sharks (Selachimorpha, Chondrichthyes). *Mol Phylogenet Evol*, *58*(2), 207-217. https://doi.org/10.1016/j.ympev.2010.11.018

Worm, B., Davis, B., Kettemer, L., Ward-Paige, C. A., Chapman, D., Heithaus, M. R., Kessel, S. T., & Gruber, S. H. (2013). Global catches, exploitation rates, and rebuilding options for sharks. *Marine Policy*, *40*, 194-204. https://doi.org/10.1016/j.marpol.2012.12.034

Zhang, Y., Pavlovska, M., Stoica, E., Prekrasna, I., Yang, J., Slobodnik, J., Zhang, X., & Dykyi, E. (2020). Holistic pelagic biodiversity monitoring of the Black Sea via eDNA metabarcoding approach: From bacteria to marine mammals. *Environ Int*, *135*, 105307. https://doi.org/10.1016/j.envint.2019.105307