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Development and Employment of Environmental DNA Methods for the Detection of Bull Sharks (*Carcharhinus leucas*) in a Freshwater and Estuarine Interface in the Mobile-Tensaw Delta and Mobile Bay, Alabama

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DEVELOPMENT AND EMPLOYMENT OF ENVIRONMENTAL DNA METHODS FOR THE DETECTION OF BULL SHARKS (*CARCHARHINUS LEUCAS*) IN A FRESHWATER AND ESTUARINE INTERFACE IN THE MOBILE-TENSAW DELTA AND MOBILE BAY, ALABAMA

by

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ABSTRACT

Species conservation and management is influenced by the quality of monitoring methods employed, especially when targeting elusive, but ecologically significant species, like elasmobranchs. Bull Sharks (*Carcharhinus leucas*) are highly mobile predators that rely on estuaries and freshwater rivers for maturation, resources, and refuge; their ability to withstand changing environmental conditions may mean they are linking ecosystems through their habitat usage and movements. Rather than setting nets or attaching acoustic monitoring devices, which can be expensive, time-consuming, and invasive, the analysis of environmental DNA (eDNA) for tracing species presence was used to target *C. leucas* DNA. The present research compared and developed methods to capture and isolate eDNA from northern Gulf of Mexico waters and designed a genetic assay to specifically target *C. leucas* DNA in concentrations as low as 0.6 copies/μL using Droplet Digital™ PCR. The optimal methods determined were employed for samples collected from Mobile Bay, Alabama and the Mobile-Tensaw Delta during two distinct seasons to determine if *C. leucas* was providing linkage between these two regions and if there appeared to be preferential usage of one area over others. Field samples showed strong positive detections for target DNA in the Mobile-Tensaw Delta during the summer season and no strong positive detections during the winter seasons, indicating *C. leucas* is likely using freshwater habitat in the Mobile-Tensaw Delta when temperatures are favorable, but additional sampling is required to make more robust conclusions about the extent to which *C. leucas* is serving as a mobile link between these two habitats.

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BRUVs	baited remote underwater video stations
CN	collection negative
ddPCR	Droplet Digital™ polymerase chain reaction
DO	dissolved oxygen
dUTP	deoxyuridine triphosphate
eDNA	environmental DNA
EN	DNA extraction negative
FN	filter negative
gDNA	genomic DNA
GoM	Gulf of Mexico
IACUC	Institutional Animal Care and Use Committee
IUCN	International Union for Conservation of Nature
LoD	limit of detection
MB	Mobile Bay
MT	manual threshold
MTD	Mobile-Tensaw Delta
mtDNA	mitochondrial DNA
ND2	NADH dehydrogenase 2
PCR	polymerase chain reaction
PCR N	polymerase chain reaction negative
qRT-PCR	quantitative real-time polymerase chain reaction
RED	rare event detection

UV	ultraviolet
UVC	underwater visual census
YOY	young-of-the-year

CHAPTER I – LITERATURE REVIEW

Chapter I of this thesis outlines environmental connectivity and to what capacities mobile link organisms can create connections. It reviews the biology, ecology, and ecological importance of *Carcharhinus leucas* (Bull Shark), as well as habitat use of this species in other areas of the Gulf of Mexico. It describes the molecular approach taken to detect *C. leucas* in the northern Gulf of Mexico.

1.1 Habitat Connectivity

Ecological connectivity encompasses any connections made between habitats within an ecosystem, across ecosystems within the larger seascape, or among food webs in a system (Olds *et al*., 2012). Nutrient flow, transfer of energy, physical organismal movements, gene flow, and predator-prey relationships can all result in physical environmental connections (Lundberg and Moberg, 2003; Rilov and Schiel, 2006; Rosenblatt and Heithaus, 2011; Pittman and Olds, 2015; Olds *et al*., 2017). Mobile species can migrate between distinct habitats to forage, seek refuge from predation, and to reproduce and are thereby serving as 'mobile links' (Lundberg and Moberg, 2003; Polis *et al*., 2004; Darimont *et al*., 2009; Schmitz *et al*., 2010; Rosenblatt and Heithaus, 2011; McCauley *et al*., 2012; Rosenblatt *et al*., 2013), which can influence community and ecosystem stability and function, and trophic dynamics across systems (Rooney *et al*., 2006; Sheaves, 2009; Rosenblatt and Heithaus, 2011; McCauley *et al*., 2012; Rosenblatt *et al*., 2013; Daly *et al*., 2014). One of the best-known cases of mobile linking of habitats is that of anadromous Pacific salmon (*Oncorhynchus* spp.), which link marine, freshwater, and terrestrial habitats (Schindler *et al*., 2003). Semelparous salmonids

migrate from their marine habitats, as adults, to their natal freshwater streams and rivers to spawn (Schindler *et al*., 2003; Schick and Lindley, 2007). The biomass accumulated during their time spent feeding in the marine environment is excreted into the freshwater, stimulating algae and insect larvae production and microbial decomposer growth (Kline *et al*., 1990; Groot and Margolis, 1991; Wipfli *et al*., 1998; Cederholm *et al*., 1999; Holmlund and Hammer, 1999; Gresh *et al*., 2000; Naiman *et al*., 2002). Salmonid eggs provide food for freshwater invertebrates and microorganisms, while adult salmon are preyed or scavenged on by terrestrial mammals and birds (Ben-David *et al*., 1998; Hilderbrand *et al*., 1999; Reimchen, 2000). Due to this complex habitat use and life history of salmonids, declines in their populations have the potential to impact not only marine ecosystems, but also cascade across freshwater and terrestrial environments.

Marine predator species play key roles in influencing the abundance and behavior of other species within communities, both directly and indirectly (Every *et al.* 2017; Engelbrecht *et al*., 2019). Directly, predators contribute to ecosystem health by preying on diseased, weak, or older organisms and sustaining biodiversity (*i.e.*, consumptive predation effects), while indirectly, the presence of predators can influence prey behavior, allowing other non-prey species to flourish (*i.e.*, non-consumptive predation effects) (Creel and Christianson, 2008; Heithaus *et al*., 2008; Orrock *et al*., 2008; Ritchie and Johnson, 2009; Williams *et al*., 2018). Temperate estuarine ecosystems have high levels of biodiversity and support highly mobile predatory species (Kenworthy *et al*., 2018). Red drum (*Sciaenops ocellatus*) are highly mobile predators that use estuarine habitats during the first five years of life and are thought to increase the connectivity between spatially separate marsh habitats within estuaries through wide-spread foraging behaviors

(Scharf and Schlight, 2000; Kenworthy *et al*., 2018). Similarly, low salinity-tolerant American alligators (*Alligator mississippiensis*) are highly mobile predators that have been documented moving from freshwater habitats into brackish estuarine water to forage and are hypothesized to be linking separate freshwater and estuarine food webs (Rosenblatt and Heithaus, 2011). Many elasmobranchs (sharks, skates, and rays) species use different habitats throughout ontogeny, as well as modify their habitat use to respond to changing abiotic and biotic conditions, such as fluctuations in temperature or salinity and the presence of larger predators, respectively (Rosenblatt *et al*., 2013; Schlaff *et al*., 2014). These, in combination with filling predatory roles, shows that elasmobranchs can directly influence the abundance and behavior of prey across environments (Every *et al*., 2017), linking otherwise disparate habitats.

1.2 *Carcharhinus leucas*

1.2.1 Taxonomy, distribution, and physiology

Carcharhinus leucas is an ecologically and commercially important species of requiem shark in the genus *Carcharhinus*, which contains 35 extant shark species worldwide (Ebert *et al*., 2013; White *et al*., 2019). *Carcharhinus leucas* is found in temperate, subtropical, and tropical latitudes globally (Figure 1.1) and are usually encountered in coastal marine waters less than 30 m in depth, rarely in depths greater than 150 m, and it is likely that their range does not extend beyond continental shelves (Compagno, 1984; Ebert *et al*., 2013). The distinctive osmoregulatory abilities and physiological characteristics of *C. leucas* allow individuals to use both low salinity and marine systems for extended periods of time (Thorson, 1962; Thorson, 1971; Oguri, 1964; Thorson *et al*., 1973; Pillans *et al*., 2005). Of the ~1,200 elasmobranch species

described, only about 5% occur in freshwater, including euryhaline *C. leucas* and *Glyphis* spp. (freshwater sharks) in Southeast Asia, New Guinea, and northern Australia (Compagno *et al*., 2008; Pillans *et al*., 2009; Li *et al*., 2015; Lucifora *et al*., 2015; Lyon *et al*., 2017). *Carcharhinus leucas* is well-documented in turbid and warm rivers and lakes throughout their global distribution (Tuma, 1976; Garrick, 1982; Martin, 2005; Ballantyne and Fraser, 2013; Ebert *et al*., 2013) with records thousands of kilometers (km) upstream in the Amazon River, Brazil (Thorson, 1972), Mississippi River, U.S. (Thomerson, 1977), and Tigris River, Turkey (Coad and Papahn, 1988). Tagging and morphometric studies also demonstrated that Lake Izabal, Guatamala, Lake Nicaragua, Nicaragua, and Lake Jamoer, New Guinea contain *C. leucas*, previously thought to be a separate landlocked species (Boeseman, 1964; Thorson *et al.*, 1966; Thorson, 1976).

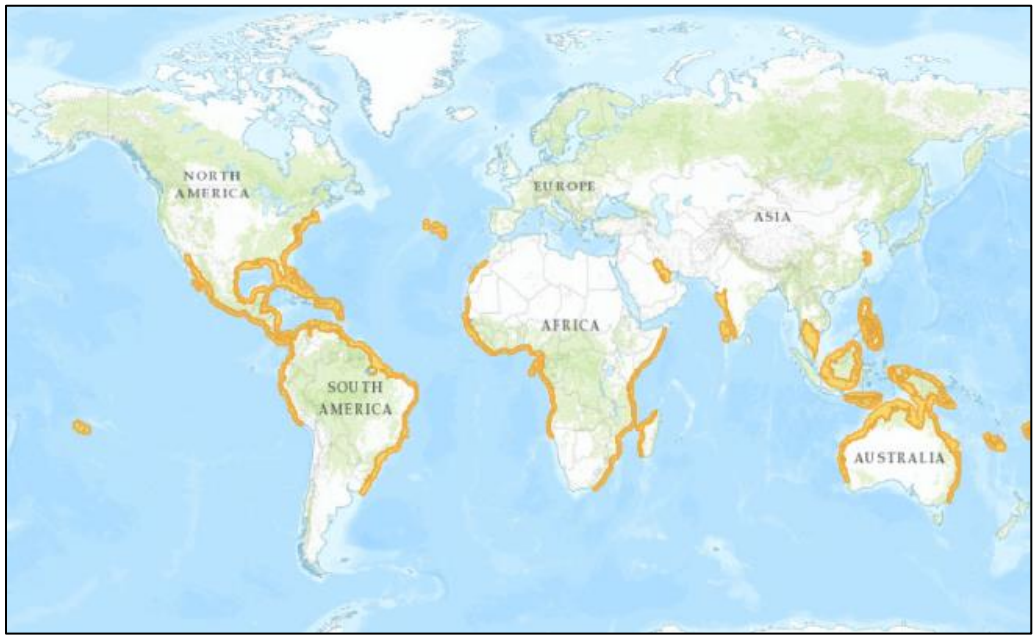

Figure 1.1 *Bull Shark distribution.*

Global distribution of Bull Sharks (*Carcharhinus leucas*) highlighted in orange (from Simpfendorfer and Burgess, 2018).

1.2.2 Life history and ecology

The life cycle of *Carcharhinus leucas* begins via placental viviparity in variable salinity estuaries and near sources of freshwater inflows (Cruz-Martinez *et al*., 2005), where young-of-the-year (YOY) (*i.e.*, individuals <1 year old) and juveniles (individuals >1 year, but not sexually mature) are more common than adults (Caillouet *et al*., 1969; Jenson, 1976; Montoya and Thorson, 1982). YOY and juveniles are often found in freshwater rivers or river mouths, whereas juveniles older than one year are usually further downstream in estuaries (Simpfendorfer *et al*., 2005; Heupel and Simpfendorfer, 2008), though both age groups have been proposed to be using their osmoregulatory ability to remain in low or zero salinity environments in their first years of life to avoid predation by larger sharks (Simpfendorfer *et al*., 2005). The duration spent in these refuge areas by YOY and juvenile *C. leucas* are variable by age group and estuary features (Simpfendorfer *et al*., 2005; Heupel and Simpfendorfer, 2008; Yeiser *et al*., 2008; Heupel *et al*., 2010; Heupel and Simpfendorfer, 2011), although Thorburn and Rowland (2008) estimate the average time to be about four years before moving to coastal ocean habitats in northern Australia. Less is known about *C. leucas* habitat use in the ocean, but it is believed that mature adults (14 – 20 years old, 210 – 220 cm total length for males and >225 cm total length for females) in the northern Gulf of Mexico (GoM) mate offshore (Branstetter and Stiles, 1987; Neer *et al*., 2005; Simpfendorfer *et al*., 2005). Tagging and genetic studies indicate that pregnant females exhibit regional philopatry in the GoM (Chapman *et al*., 2015; Laurrabaquio-A *et al*., 2019) and will return inshore to estuaries and rivers to give birth after a 10 – 11 month gestation period (Last and Stevens, 2009). Average litters are six to 12 young (Last and Stevens, 2009),

each about 56 – 86 cm in total length (Compagno, 1984). Within the first five years of life, *C. leucas* can grow up to 20 cm per year, which gradually slows to 4 – 5 cm per year after sexual maturity is reached, based on vertebral counts (Neer *et al*., 2005). Individuals live about 32 years on average (Compagno *et al*., 2005; Ebert *et al*., 2013) and can reach 350 cm in total length and weigh 230 kg (Castro, 2010).

Carcharhinus leucas fill integral niches in marine and estuarine food webs, functioning as apex predators, mesopredators, and scavengers (Matich *et al*., 2011; Daly *et al*., 2013). Studies conducted on populations around the world show that diet preferences are linked to size, life stage, and available prey in their chosen habitat, with prey size positively correlated to body size of the shark (Bass, 1973; Sadowsky, 1971; Tuma, 1976; Snelson *et al*., 1984; Cliff and Dudley, 1991; Gulak, 2011). Stomach content analyses show that *C. leucas* primarily prey on teleost fish and smaller elasmobranchs although they will opportunistically prey on sea turtles and invertebrates as well as scavenge on deceased marine mammals and birds (Tuma, 1976; Compagno, 1984; Snelson *et al*., 1984; Last and Stevens, 1994). Of nearshore shark species common to the estuarine northern GoM, young *C. leucas* are similar or larger in size (Ebert *et al*., 2013), indicating that they have the potential to outcompete other species, like the Atlantic sharpnose shark (*Rhizoprionodon terraenovae*) (Knip *et al*., 2010; Munroe *et al*., 2016). Matich *et al.* (2011) has suggested that for older juvenile *C. leucas* inhabiting estuaries, feeding preferences may not be predictable across individuals. Recent stable isotope analysis for *C. leucas* shows trophic variation among individuals (*i.e*., freshwater specialists, marine specialists, and trophic generalists); Ecosystem factors such as prey availability, inter- and intraspecific competition, spatial overlap of food webs, and food-

predation risk trade-offs likely influence *C. leucas* diet specialization (Matich *et al*., 2011; Matich and Heithaus, 2015).

1.2.3 Habitat use and selection

Nearshore habitats are commonly used as nursery grounds for YOY and juvenile elasmobranch species, providing abundant prey and reduced predatory interactions that contribute to decreased mortality (Branstetter, 1990; Simpfendorfer and Milward, 1993; Parsons and Hoffmayer, 2007; Nagelkerken *et al*., 2015; Drymon *et al*., 2014). Many species of elasmobranchs cannot tolerate the variable abiotic conditions that arise from seasonality, precipitation, and freshwater outflow present in some nearshore habitats, such as freshwater river mouths and estuaries; however, *C. leucas* are able to withstand broad environmental change, making estuaries and river mouths in tropical, subtropical, and warm temperate climes regular nursery areas (Caillouet *et al*., 1969; Montoya and Thorson, 1982; Heupel *et al*., 2007; Ortega *et al*., 2009). For example, studies from the GoM show that YOY and juveniles are more commonly using estuaries and river mouths compared to adults (except when females enter these areas for parturition), which are often found in fully marine coastal areas (Shipley, 2005; Wiley and Simpfendorfer, 2007; Yeiser *et al*., 2008; Carlson *et al*., 2010; Heupel *et al*., 2010; Curtis *et al*., 2011; Hammerschlag *et al*., 2012; Froeschke *et al*., 2010a, b; Matich *et al*., 2017). Studies suggest that young *C. leucas* preference for freshwater habitats may be influenced by their priority to take refuge from larger predators, rather than prey availability (Heupel and Hueter, 2002; Heithaus, 2004; Heithaus and Dill, 2006; Heithaus, 2007). In such habitats within the GoM, YOY and juvenile *C. leucas* exhibit habitat partitioning; YOY are more frequently caught in shallower, lower-salinity waters, while juveniles are found

in deeper, higher-salinity waters (Simpfendorfer *et al*., 2005; Heupel and Simpfendorfer, 2008). This habitat partitioning is thought to reduce intraspecific predation, decrease competition between size and age classes, and take advantage of abundant shallow water resources (Simpfendorfer *et al*., 2005; Heithaus *et al*., 2007; Heupel and Simpfendorfer, 2008; Matich *et al*., 2017). Since *C. leucas* have a high plasticity to exploit a variety of different prey resources (Pillans and Franklin, 2004; Pillans *et al*., 2005; Matich *et al*., 2017; Plumlee *et al*., 2018), low or zero salinity habitats may provide safe havens for smaller *C. leucas* individuals, which may lead to reduced mortality rates in *C. leucas* relative to similarly-sized species, as observed in Florida (Heupel and Simpfendorfer, 2011).

Catch data from studies in Florida, Alabama, Louisiana, and Texas estuaries and rivers show that salinity, freshwater inflow, temperature, dissolved oxygen, water clarity, and proximity to tidal inlets are determining factors affecting distribution of YOY and juvenile *C. leucas* (Simpfendorfer *et al*., 2005; Froeschke *et al*., 2010b; Plumlee *et al*., 2018). When rivers were available as habitat for young sharks in Florida, individuals were likely to move upriver during periods of low outflow and reside in the river mouths during periods of high outflow (Ortega *et al*., 2009; Heupel *et al*., 2010). Moderate to high salinities (5 – 35‰) were preferentially selected for across areas of the GoM (Shipley, 2005; Simpfendorfer *et al*., 2005; Wiley and Simpfendorfer, 2007; Heupel and Simpfendorfer, 2008; Froeschke *et al*., 2010b; Drymon *et al*., 2014), along with warmer water temperatures (>20°C) (Simpfendorfer *et al*., 2005; Shipley, 2005; Froeschke *et al*., 2010b; Curtis *et al*., 2011; Drymon *et al*., 2014), which is thought to decrease osmoregulatory energy cost and maintain optimal ranges for energetic and physiologic

processes, respectively (Curtis, 2008; Matich and Heithaus, 2012; Schlaff *et al*., 2014). Because *C. leucas* experiences little physiological stress from salinity changes, in conjunction with predation across the freshwater and marine continuum (Matich and Haithaus, 2014; Every *et al*., 2017) and dependence on rivers and estuaries for maturation, refuge, and prey abundance (Cruz-Martinez *et al.*, 2005; Every *et al*., 2017; Every *et al*., 2018), it is reasonable to predict that *C. leucas* is functioning as a mobile link predator between freshwater rivers and estuarine habitats (Rosenblatt and Heithaus, 2011).

1.2.4 The Mobile-Tensaw Delta and Mobile Bay, Alabama

Mobile Bay (MB), Alabama (Figure 1.2) is a highly variable estuarine system suggested to function as the northern-most potential nursery area for *C. leucas* in the GoM (Drymon *et al*., 2014). Compared to estuaries in the eastern and western GoM, those in the northern GoM experience more variable abiotic factors, due to higher latitude, greater freshwater inflows from the Mobile-Tensaw Delta (MTD), and characteristic wet and dry seasons (Marr, 2013; Drymon *et al*., 2014). These variables lead to marked seasonal fluctuations in temperature and freshwater input, and stratified salinity changes (Schroeder and Lysinger, 1979; Schroeder and Wiseman, 1988; Schroeder *et al*., 1990; Kim and Park, 2012; Drymon *et al*., 2014), all of which have shown to impact *C. leucas* habitat usage in other areas of the GoM (Simpfendorfer *et al*., 2005; Shipley, 2005; Froeschke *et al*., 2010b; Heupel *et al*., 2010). Drymon *et al.* (2014) found a large proportion of *C. leucas* acoustic detections in the upper MB and given previous findings concerning *C. leucas* freshwater occurrence (Thorson *et al*., 1966; Thorson, 1971; Thorson, 1972; Thorson, 1976; Jenson, 1976; Montoya and Thorson,

1982; Tan and Lim, 1998; O'Connell *et al*., 2007; Thorburn and Rowland 2008; Huepel *et al*., 2010), it is logical to hypothesize that young *C. leucas* use these freshwater systems within the MTD and MB interface for larger predator avoidance and possibly in search of other resources to decrease intraspecific competition (Pillans and Franklin, 2004; Pillans *et al*., 2005; Gulak, 2011). In order to better understand the role of *C. leucas* as an ecological link between habitats, it is vital to understand how human alterations in a habitat may cascade into other habitats (Heithaus *et al*., 2008; Rosenblatt and Heithaus, 2011; Daly *et al*., 2014).

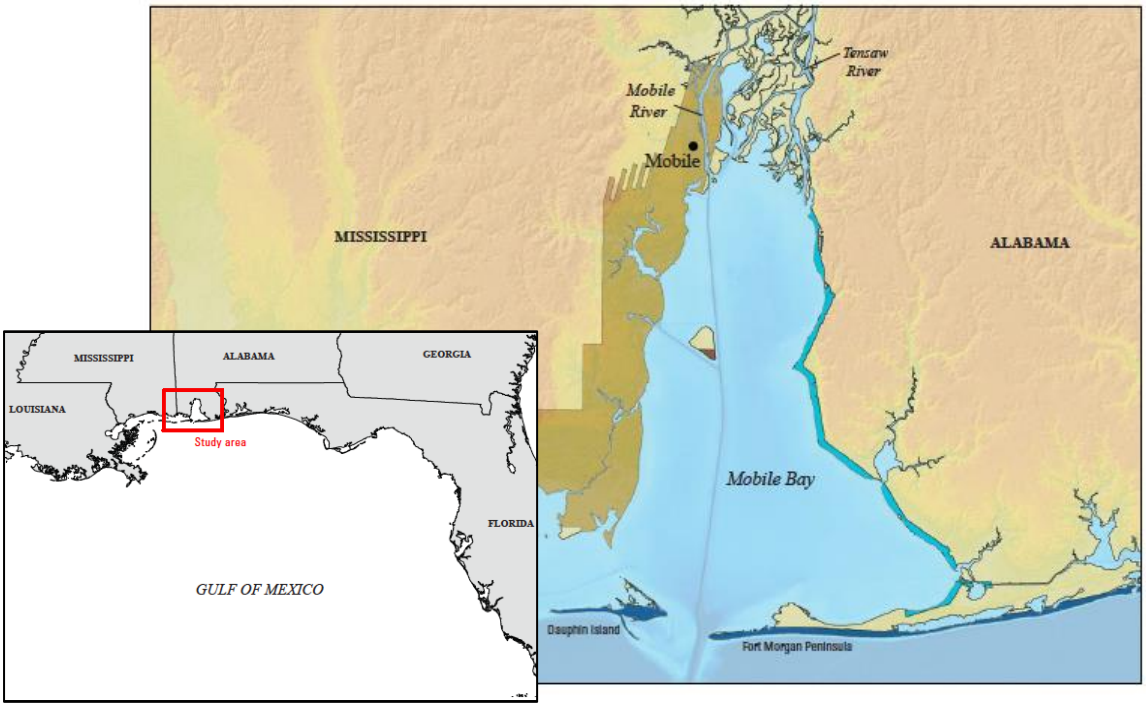

Figure 1.2 *Mobile Bay, Alabama.*

Mobile Bay, Alabama is located in the north central region of the Gulf of Mexico (from Danielson *et al*., 2013).

Globally, 40% of all people live within 100 km of a coast (Sevilla *et al*., 2019) and, as a result, such habitats are at risk from anthropogenic activities, such as fishing

pressure, habitat fragmentation and degradation, coastal development, industrial shipping traffic, environmental pollutants, and effects of climate change (Jackson *et al*., 2001; Sheaves, 2009; Lucifora *et al*., 2015). Mobile Bay and the MTD support one of the largest, intact wetlands in the U.S. (McCreadie, 2002) and has a high diversity of animal and plant species that collectively serves many ecological and economic functions. The western regions of the MTD and MB are substantially more industrialized than the eastern regions, with the Mobile River on the west serving as the southern-most channel of the Tennessee-Tombigbee Waterway (Stine, 1992) and hosting The Port of Mobile, the tenth largest port in the U.S. (U.S. Army Corps of Engineers, 2016). In addition to this port, there are shipbuilding and repair, chemical, and manufacturing facilities present along the Mobile River, as well as Alabama Power's Plant Barry about 30 km upstream from Mobile, which houses an unlined coal ash (material left over after coal is burned) pond just meters from the banks of the Mobile River (Callaway *et al*., 2018). Given that *C. leucas* are so prevalent in nearshore habitats early in life and these same habitats likely provide foraging opportunities for all life stages (Knip *et al*., 2010; Drymon *et al*., 2014), *C. leucas* may be predisposed to impacts of urbanization and industrialization (Curtis *et al*., 2013; Drymon *et al*., 2014). Coastal ecosystem resilience and function are dependent upon healthy populations of predators to aid in combating invasive species, boosting disease resistance, and increasing overall biodiversity (Hoddle, 2004; Heithaus *et al*., 2008; Estes *et al*., 2011; Levi *et al*., 2012; Ritchie *et al*., 2012). A reduction in mobile predators, such as *C. leucas*, or a decrease in suitable habitats may impact established habitat connections that result from direct and indirect predation effects; these have the

potential to upset ecosystem balance that can cascade across freshwater and estuarine environments (Polovina *et al*., 2009; Rosenblatt and Heithaus, 2011; Every *et al*., 2017).

1.3 Molecular Detection of *Carcharhinus leucas* Using Environmental DNA

Carcharhinus leucas habitat use within the GoM has historically been investigated through monitoring programs (longlines, gillnets) and acoustic telemetry (Simpfendorfer *et al*., 2005; Froeschke *et al*., 2010b; Heupel *et al*., 2010; Drymon *et al*., 2014; Plumlee *et al*., 2018). While these traditional field methods are reliable, they are often not time or cost-effective and can be prone to producing false negatives (Pilliod *et al*., 2013; Balasingham *et al*., 2017; Bakker, 2018). A faster, cheaper, and more sensitive molecular alternative to traditional monitoring methods, coined "environmental DNA" (eDNA) (Ficetola *et al*., 2008), has gained momentum in ecological studies. Environmental DNA is genetic material released by all living organisms into their environment, including hair, scales, blood, and feces (Waits *et al*., 2005; Ficetola *et al*., 2008; Valentini *et al*., 2009; Taberlet *et al*., 2012), therefore providing a genetic indicator of recent or current presence (Ficetola *et al*., 2008). Such genetic evidences can be captured in environmental water, soil, or air samples and the DNA isolated, extracted, and analyzed (Waits *et al*., 2005; Ficetola *et al*., 2008; Valentini *et al*., 2009; Taberlet *et al*., 2012). Unlike acoustic monitoring, mark-recapture, or tagging often used in ecology-focused studies of aquatic species, examining ambient water for DNA does not require visualizing or handling the target species (Jerde *et al*., 2011; Wilcox *et al*., 2013).

Environmental DNA has been used to assess biodiversity and threats to biodiversity (Lodge *et al*., 2012; Uchii *et al*., 2016), target invasive, rare, or endemic species (Hunter *et al*., 2015; Simpfendorfer *et al*., 2016; Lafferty *et al*., 2018), estimate

and quantify biomass (Baldigo *et al*., 2017), and determine population characteristics of an Endangered elasmobranch (Pierce and Norman, 2016; Sigsgaard *et al*., 2016). To date, several studies have successfully used eDNA to target molecular signatures from elasmobranch species, including the Critically Endangered largetooth sawfish, *Pristis pristis*, (Simpfendorfer *et al*., 2016), the Endangered whale shark, *Rhincodon typus* (Sigsgaard *et al*., 2016), the Endangered Maugean skate, *Zearaja maugeana* (Weltz *et al*., 2017), the Chilean devil ray, *Mobula tarapacana* (Gargan *et al*., 2017), and the white shark, *Carcharodon carcharias* (Lafferty *et al*., 2018). In addition, studies have used eDNA metabarcoding to illuminate elasmobranch biodiversity in marine areas (Thomsen *et al*., 2016; Bakker *et al*., 2017; Feitosa *et al*., 2018; Boussarie *et al*., 2018; Stat *et al*., 2018). Environmental DNA analysis has proven to be a valuable method when combined with conventional monitoring methods, even outperforming traditional approaches in some cases (Dejean *et al*., 2011; Huver *et al*., 2015; Bakker, 2018). Studies using seines and electrofishing to monitor brook trout (*Salvelinus fontinalis*) and invasive carp (*Hypohthalmichthy*s spp.) populations have confirmed the utility of eDNA to predict presence with about 85% accuracy compared to these traditional methods (Jerde *et al*., 2011; Baldigo *et al*., 2016; Evans *et al*., 2017). When eDNA used for biodiversity detection was compared to trawling in subarctic deep seas, eDNA accurately detected 93% of the species observed from trawl nets, including species that easily avoids trawls, such as the Greenland shark (*Somniosus microcephalus*) (Thomsen *et al*., 2016). Recently, Bakker (2018) compared Underwater Visual Census (UVC) and Baited Remote Underwater Video stations (BRUVs) against eDNA metabarcoding and found that eDNA technology was able to detect 44% more shark species compared to UVC and BRUVs.

The most widely-used method of isolating eDNA from water samples is through vacuum filtration via filter membranes (Goldberg *et al*., 2016). After filtration, used filters are preserved and the DNA is extracted, although multiple methodological variations exist in the literature. Polymerase Chain Reaction (PCR) is performed on the DNA extracts to identify DNA from the target(s), if present. Universal PCR primers are often used for metabarcoding studies to identify biodiversity or calculate biomass and custom-developed species-specific PCR primers are used to detect a target(s) species (Rees *et al*., 2014; Andruszkiewicz *et al*., 2017). The present study targets *C. leucas* within the northern GoM and, therefore, the genetic assay employed must be able to successfully differentiate *C. leucas* DNA from non-target species DNA (Wilcox *et al*., 2013). Once eDNA is released from an organism into the surrounding aqueous environment, it begins to break down immediately due to abiotic and biotic conditions (Thomsen *et al*., 2012a, b), including water quality, mechanical forces, and microbial activity (Lindahl, 1993; Nielsen *et al*., 2007). Rate of eDNA degradation and eDNA transport varies by ecosystem; research indicates that eDNA is detectable for less than one month upon organism removal from controlled freshwater systems in some cases (Dejean *et al*., 2011), but is generally thought to degrade sooner in the natural marine environment (marine eDNA degradation has been suggested to be ~10 – 50 hours) (Dell'Anno and Corinaldesi, 2004; Thomsen *et al*., 2012a; Sassoubre *et al*., 2016; Collins *et al*., 2018), with eDNA showing degradation about 1.6 times faster in coastal systems that have terrestrial influence compared to the offshore environment (Collins *et al*., 2018). Environmental DNA has been observed being transported from its origin horizontally (*i.e*., downstream) (Deiner and Altermatt, 2014), vertically (*i.e*., settling

down to the sediment) (Turner *et al*., 2015), and through repeated tidal action (Kelly *et al*., 2018). Environmental decay of eDNA results in minute concentrations (*e.g*., <200 pg/L) (Takahara *et al*., 2012; Pilliod *et al*., 2013) comprised of short fragments of DNA, meaning that custom-designed species-specific assays should be sensitive and target short fragments ~50 – 250 base pairs in length (Ficetola *et al*., 2008; Minamoto *et al*., 2012; Goldberg *et al*., 2016; Gargan *et al*., 2017). In eukaryotic cells, mitochondria contain thousands of copies of mitochondrial DNA (mtDNA) per cell, whereas each nucleus of a cell contains only two copies of nuclear DNA (Wilcox *et al*., 2013). Because of this, the likelihood of detecting mtDNA is far greater than nuclear DNA, which is essential when targeting minute quantities in vast ecosystems. Environmental DNA species-specific primer design requires primers be developed in a region of the mtDNA that is variable from closely related species; however, for targeted eDNA species detections in elasmobranchs, short target fragments in the mitochondrial genome can be difficult to design because of highly conserved areas and slow mutation rates characteristic of this group compared to other vertebrates (Martin *et al*., 1992; Martin, 1995; Dudgeon *et al*., 2012).

Project Aims

The overall aim of this project is to better understand if *C. leucas* are using the freshwater and estuarine habitats in Alabama waters and the extent to which they are potentially serving as a mobile link between habitats. Furthermore, whether *C. leucas* shows seasonal usage of the freshwater rivers and/or preferential usage of one river over others will be investigated. Specifically, this study involves collecting water samples from different river systems (within the Mobile-Tensaw Delta) in Alabama and Mobile Bay, and will complete the following:

1) Determine the optimal eDNA capture and isolation methods for detecting *C. leucas* in the northern Gulf of Mexico.
2) Develop a genetic assay to specifically detect *C. leucas* eDNA.
3) Obtain *C. leucas* eDNA from an *ex situ* closed system to serve as the positive reference for field samples.
4) Apply the developed methods to water samples collected from Mobile Bay and the Mobile-Tensaw Delta.

Using eDNA analysis to investigate the degree of freshwater habitat usage will aid in the understanding of *C. leucas* serving as a potential predatory mobile link between freshwater rivers in the Mobile-Tensaw Delta and estuarine Mobile Bay, Alabama, which is currently not well explored in the northern Gulf of Mexico. Additionally, if data show *C. leucas* eDNA presence in a more human-influenced area, this can aid in further research focused on anthropogenic impacts on mobile marine predators' habitat usage and preference.

CHAPTER II – DEVELOPMENT OF HIGHLY SENSITIVE ENVIRONMENTAL DNA METHODS FOR THE DETECTION OF BULL SHARKS, *CARCHARHINUS LEUCAS* (MÜLLER AND HENLE, 1839), USING DROPLET DIGITAL™ PCR

Schweiss, K. E.[1], Lehman, R. N.[1], Drymon, J. M.[2], & Phillips, N. M.[1] (In press). 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.* doi:10.1002/edn3.39

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2.1 Abstract

As apex and mesopredators, elasmobranchs play a crucial role in maintaining ecosystem function and balance in marine systems. Elasmobranch populations worldwide are in decline as a result of exploitation via direct and indirect fisheries mortalities and habitat degradation; however, a lack of information on distribution, abundance, and population biology for most species hinders their effective management. Environmental DNA analysis has emerged as a cost-effective and non-invasive technique to fill some of these data gaps, but often requires the development of species-specific methodologies. Here, we establish eDNA methodology appropriate for targeted species detections of Bull Sharks, *Carcharhinus leucas*, in estuarine waters in the northern Gulf of Mexico, with a comparison of different QIAGEN® DNeasy® extraction kit protocols and the development of a species-specific *C. leucas* eDNA assay. We designed species-specific primers and an

internal probe to amplify a 237 base pair portion of the ND2 gene in the mitochondrial genome of *C. leucas* for a Droplet Digital™ PCR (ddPCR) assay, which has the ability to detect target DNA at concentrations in a reaction as low as 0.6 copies/µL. To validate the developed methods, water samples were collected from known *C. leucas* habitat and from an *ex situ* closed environment containing a single *C. leucas* individual. DdPCR reactions performed on water samples from known habitat and 30 minutes after a shark was added to the closed environment contained 1.62 copies/µL and 166.6 copies/µL of target *C. leucas* eDNA, respectively. The effectiveness of the assay in an open environment was then assessed by placing one *C. leucas* into a flow-through mesocosm system and water samples were collected every 30 minutes for three hours. *Carcharhinus leucas* eDNA was detected in this system within 30 minutes, but concentrations remained low and variable throughout the duration of the experiment.

2.2 Introduction

Elasmobranchs (sharks, skates, and rays) play a crucial role in marine ecosystems as apex and mesopredators, influencing prey abundance, behavior, and trophic interactions across multiple trophic levels in marine food webs (Ferretti *et al*., 2010; Ritchie *et al.,* 2012). Healthy elasmobranch populations help to maintain ecosystem function, increase biodiversity, and buffer against invasive species and transmission of diseases (Heithaus *et al*., 2008; Ritchie *et al.*, 2012). However, many elasmobranch populations are in decline as a result of exploitation via direct and indirect fisheries mortalities and habitat degradation (Dulvy *et al.,* 2014). The life history strategies of many elasmobranchs are characterized by late maturity, longevity, and low fecundity, making the recovery of exploited populations a biologically slow process (García *et al*.,

2008; Hoenig and Gruber, 1990). According to the International Union for Conservation of Nature (IUCN) Red List of Threatened Species, one-quarter of elasmobranch species are estimated to be threatened with extinction and almost one-half are categorized as Data Deficient, meaning there is insufficient data to properly assess their conservation status (Dulvy *et al*., 2014). Robust data on species distribution, abundance, biology, and population biology are necessary to enact appropriate conservation strategies for the maintenance of healthy elasmobranch populations; unfortunately, such data are often incomplete or lacking for many species (Dulvy *et al*., 2014).

Analysis of environmental DNA (eDNA) has recently emerged as an alternative, powerful approach to fill data gaps on the distribution, habitat use, abundance, and population biology of aquatic species (Ficetola *et al*., 2008), including elasmobranchs (Sigsgaard *et al*., 2016). All organisms leave traces of DNA in the environment through shedding of cellular debris, skin cells, blood, and biological waste, all of which can be collected in water samples (Rees *et al*., 2014); however, differences in how organisms shed DNA (*i.e*., mucus, scales, feces) suggest that eDNA accumulation may differ across species (Le Port *et al*., 2018), requiring taxon-specific research. In targeted species detections, water samples are typically filtered, DNA extractions are performed on the resulting particulate material, and extracted DNA samples are analyzed using a quantitative real-time Polymerase Chain Reaction (qRT-PCR) platform with species-specific primers, developed to amplify a small DNA fragment in the target species (Foote *et al.*, 2012; Taberlet *et al*., 2012). The collection of water samples is a cost-effective and efficient method of surveying elasmobranch populations when compared to traditional survey methods involving setting nets or lines, which can have high incidence of bycatch

and inflict varying degrees of stress to both target and non-target species (Larson *et al*., 2017; Lewison *et al*., 2004). Post-release recovery and survival tends to vary widely across species, with some species being particularly sensitive to net capture and handling (Stobutzki *et al*., 2002). With a well-designed sampling scheme, eDNA methodologies offer increased sensitivity for detecting the presence of rare species while negating the need to capture, handle, or even observe the target species (Port *et al*., 2016; Rees *et al*., 2014). In elasmobranchs, eDNA methods have been used in targeted species detections for the Critically Endangered Largetooth Sawfish, *Pristis pristis* (Simpfendorfer *et al*., 2016), the Endangered Maugean Skate, *Zearaja maugeana* (Weltz *et al.*, 2017), the Vulnerable Chilean Devil Ray, *Mobula tarapacana* (Gargan *et al*., 2017), and the Vulnerable White Shark, *Carcharodon carcharias* (Lafferty *et al*., 2018). Furthermore, eDNA has been used to assess population characteristics in the Endangered Whale shark, *Rhincodon typus* (Sigsgaard *et al*., 2016) and to estimate shark diversity in tropical habitats using metabarcoding (Bakker *et al*., 2017; Boussarie *et al.*, 2018).

Bull Sharks, *Carcharhinus leucas* (Müller and Henle, 1839), are found in temperate, subtropical, and tropical latitudes globally and are distinctive as one of only a few sharks that can use freshwater for extended periods of time (Thorson, 1962; Thorson, 1971; Thorson *et al*., 1973). As upper trophic level predators, they play a crucial role in maintaining ecosystem health across both marine and freshwater habitats (Every *et al*., 2017; Polovina *et al*., 2009; Ritchie *et al*., 2012). Using acoustic telemetry data to examine the habitat use of *C. leucas* in northern Gulf of Mexico waters, Drymon *et al*. (2014) found *C. leucas* may preferentially select higher-quality, less-urbanized rivers, although a spatially-limited acoustic array hindered a full evaluation of this pattern.

Targeted eDNA surveys of *C. leucas* could provide a cost-effective, sensitive method to examine this pattern more widely, as there could be substantial ecological implications of such habitat preference. Here, we establish an eDNA methodology appropriate for targeted species detections of *C. leucas* in estuarine waters in the northern Gulf of Mexico. Specifically, we compare total eDNA yields for different QIAGEN® DNeasy® DNA extraction kit protocols and develop a species-specific *C. leucas* eDNA assay using a relatively novel, Bio-Rad® Droplet Digital™ PCR (ddPCR), platform to detect low quantities of target DNA. Finally, we apply these methods to investigate the detectability of *C. leucas* eDNA in known habitat in the northern Gulf of Mexico and in *ex situ* closed and flow-through environments containing a single *C. leucas* individual.

2.3 Materials and Methods

2.3.1 Laboratory controls

Strict lab controls were implemented throughout this study to reduce the risk of cross-contamination and contamination by exogenous DNA (see Deiner *et al*., 2015; Goldberg *et al.*, 2016). Water processing, DNA extractions, and PCR amplifications were conducted in physically separated lab spaces to prevent cross-contamination between stages. Negative controls were incorporated into every stage of sample processing and PCR was performed on them to check for potential contamination. Filter negatives contained target-free, autoclaved deionized water, DNA extraction negatives contained no filtered particulate material, and PCR amplification negatives contained no DNA; all negative controls produced negative results, indicating no contamination had occurred. The ddPCR assay conditions used to carry out these negative control tests are described below.

2.3.2 Water sample collection and filtration

Water samples throughout this study were collected just below the surface of the water in 1 L high-density polyethylene Nalgene® bottles pre-cleaned in a 10% bleach solution and sanitized under ultraviolet (UV) light for 20 minutes. New gloves were used to collect each water sample and samples were stored on ice in a cooler until filtration using a vacuum pump could take place, which occurred within 24 hours of collection (see Pilliod *et al*., 2013), except where otherwise noted. Water samples were filtered in a dedicated, pre-cleaned lab space that had never had *C. leucas* tissue or total genomic DNA (gDNA) present. Each 1 L water sample was inverted at least three times to ensure homogenization of particulate matter and was then vacuum-filtered using 47 mm diameter, 0.8 μm nylon filters, which were replaced when clogging occurred every ~350 mL (*e.g.*, three filters per 1 L) and preserved in 95% ethanol at room temperature, unless noted otherwise (see 2.6.1 Supporting Information 1). During all water filtration, filters were handled with designated sterile forceps for each sample and gloves were changed in between samples to avoid cross-contamination.

2.3.3 DNA extraction methods

Due to the wide variety of DNA extraction methods used in eDNA literature (Renshaw *et al*., 2015), we compared eDNA extraction kits to establish an appropriate method for the nylon filters used to filter water samples in this study. The QIAGEN® DNeasy® Blood & Tissue Kit is a frequent choice for DNA extractions from filters in eDNA studies, but with numerous variations (see Rees *et al.* 2014). The performance of this kit using the Goldberg *et al.* (2011) variation incorporating QIAshredder™ spin columns was compared to that of an extraction kit designed specifically for water

samples, the QIAGEN® DNeasy® PowerWater® Kit. The Goldberg *et al.* (2011) protocol incorporating QIAshredder™ spin columns was selected because in preliminary trials it yielded higher relative quantities of DNA compared to some other variations (2.6.2 Supporting Information 2). Additionally, four variations of physical disruption methods to dislodge the particulate matter from the filters prior to digestion were tested with each extraction method: 1) no physical disruption, 2) bead beating, 3) filter scraping, and 4) freezing filters with liquid nitrogen and crushing them using an autoclaved mortar and pestle. The QIAGEN® DNeasy® PowerWater® Kit contained bead beating as part of the standard manufacturer's protocol, so this step was eliminated for the no physical disruption variation to determine if this step was a critical factor in DNA yields. Three × 1 L water sample replicates were used in each extraction/physical disruption treatment, collected from Mobile Bay, Alabama using the water collection and filtration protocols described. To eliminate the filter preservation step, the filters for each 1 L sample were immediately placed into the appropriate lysis buffers (see Hinlo *et al*., 2017). The DNA extracts for each 1 L water sample were combined and the DNA qualities were assessed using 2% agarose gel and the relative quantities were measured using Thermo Fisher Scientific NanoDrop™ spectrophotometer technology, with each extract measured four times.

2.3.4 Development of a species-specific assay

To develop a species-specific assay, primers and an internal probe were manually designed in conserved regions of the mitochondrial (mtDNA) NADH dehydrogenase 2 (ND2) gene within *C. leucas*, but variable regions across 23 genetically similar, exclusion elasmobranch species, using sequences available from GenBank and aligned via

CodonCode Aligner v. 7.0 (see 2.6.3 Supporting Information 3). Forward (BULLND2F6: 5'-TCCGGGTTTATACCCAAATG-3') and reverse (BULLND2R5: 5'-GAAGGAGGATGGATAAGATTG-3') primers were designed first to PCR-amplify a 237 base pair portion of the mtDNA ND2 gene in *C. leucas.* The primers were first tested using gDNA extracted from five *C. leucas* individuals from northern Gulf of Mexico waters using conventional PCR. Each PCR reaction consisted of 10 mM TAQ buffer, 1.5 mM $MgCl_2$, 0.3 μM of each primer, 0.1 mM dNTPs, 1 U of *Taq* polymerase, ~25 ng/μL of each DNA extract, and PCR-grade water for a final reaction volume of 25 μL. PCR cycling conditions began with initial denaturation at 94°C for 5 minutes, followed by 35 cycles of 94°C for 30 seconds, 59°C for 30 seconds, and 72°C for 30 seconds, final extension at 72°C for 7 minutes, and a final hold at 4°C. Primers were also tested against one individual of each of 18 other genetically similar, local exclusion species, collected from the Gulf of Mexico (Table 2.1) to assess specificity. The primers amplified DNA in the target species, *C. leucas,* but also amplified DNA from some of the non-target species tested. To increase the species-specificity of the assay, an internal PrimeTime® double-quenched ZEN®/IOWA Black FQ® probe labeled with 6-FAM (BULL_IBFQ: 5'-CAACACTAACTATAAGTCCTAACCCAATC-3') was designed to amplify the target gene in only *C. leucas*.

DdPCR reaction mixtures and cycling conditions were optimized for *C. leucas* by systematically adjusting the concentrations of primers (300 – 1,000 nM) and internal probe (100 – 250 nM), cycle number (30 – 40 cycles), ramp rate (0.5 – 2.0°C/s), annealing temperature (54 – 66°C), elongation time (1 – 2 minutes), and the amount of gDNA (0.2 – 25.0 ng/μL). The optimized ddPCR reaction mixture contained 1X Bio-

Rad® ddPCR supermix for probes (no deoxyuridine triphosphate (dUTP)), 750 nM of each primer, and 250 nM of probe, and 1.1 µL of extracted DNA, adjusted to a final volume of 22 µL with PCR-grade water. DdPCR droplets were generated for each 22 µL reaction using the Bio-Rad® QX200™ AutoDG™ Droplet Digital™ PCR System (Instrument no. 773BR1456) and thermal cycling conditions were as follows, using a ramp rate of 1°C/s: initial denaturation at 95°C for 10 minutes, followed by 35 cycles of 94°C for 30 seconds and 56°C for 2 minutes, followed by enzyme deactivation at 98°C for 10 minutes, and a final hold at 4°C. To ensure the optimized assay was species-specific for *C. leucas* using the ddPCR platform, the primers and probe were tested using these ddPCR reaction and cycling conditions, in replicates of three, with 0.2 ng/µL of gDNA extracted from five *C. leucas* individuals and one individual of each of 18 other genetically similar, local exclusion species, collected from the Gulf of Mexico (Table 2.1).

Table 2.1

Eighteen genetically similar exclusion elasmobranch species found in the Gulf of Mexico.

Common Name	**Species Name**
Nurse Shark	*Ginglymostoma cirratum*
Shortfin Mako	*Isurus oxyrinchus*
Dusky Smoothhound	*Mustelus canis*
Tiger Shark	*Galeocerdo cuvier*
Great Hammerhead	*Sphyrna mokarran*
Scalloped Hammerhead	*Sphyrna lewini*
Bonnethead	*Sphyrna tiburo*
Atlantic Sharpnose Shark	*Rhizoprionodon terraenovae*
Lemon Shark	*Negaprion brevirostris*
Finetooth Shark	*Carcharhinus isodon*
Blacknose Shark	*Carcharhinus acronotus*
Sandbar Shark	*Carcharhinus plumbeus*
Spinner Shark	*Carcharhinus brevipinna*
Dusky Shark	*Carcharhinus obscurus*
Silky Shark	*Carcharhinus falciformis*
Blacktip Shark	*Carcharhinus limbatus*
Cownose Ray	*Rhinoptera bonasus*
Atlantic Stingray	*Hypanus sabina*

These 18 exclusion species, and the Bull Shark (*Carcharhinus leucas*) were tested for species-specificity of the developed primers and internal probe on the Bio-Rad® QX200™ Droplet Digital™ PCR platform. All tissue samples were collected from the Gulf of Mexico.

All ddPCR data were analyzed with the Bio-Rad® QX200™ Droplet Reader and QuantaSoft™ software using the Rare Event Detection (RED) analysis, a manual detection threshold of 3,000 amplitude (Figure 2.1), and a limit of detection (LoD) of the developed assay. The LoD is considered the lowest concentration of *C. leucas* DNA that can reliably be detected using the optimized assay conditions. The lower LoD was

determined by conducting ddPCR reactions with gDNA from two *C. leucas* individuals using a 6-fold series of 10X dilutions (*e.g.*, 1:10 to 1:1,000,000), from a starting concentration of 25.0 ng/µL. Means and standard errors of detected DNA concentration (copies/µL) were calculated for each individual, across the three ddPCR replicates for each dilution.

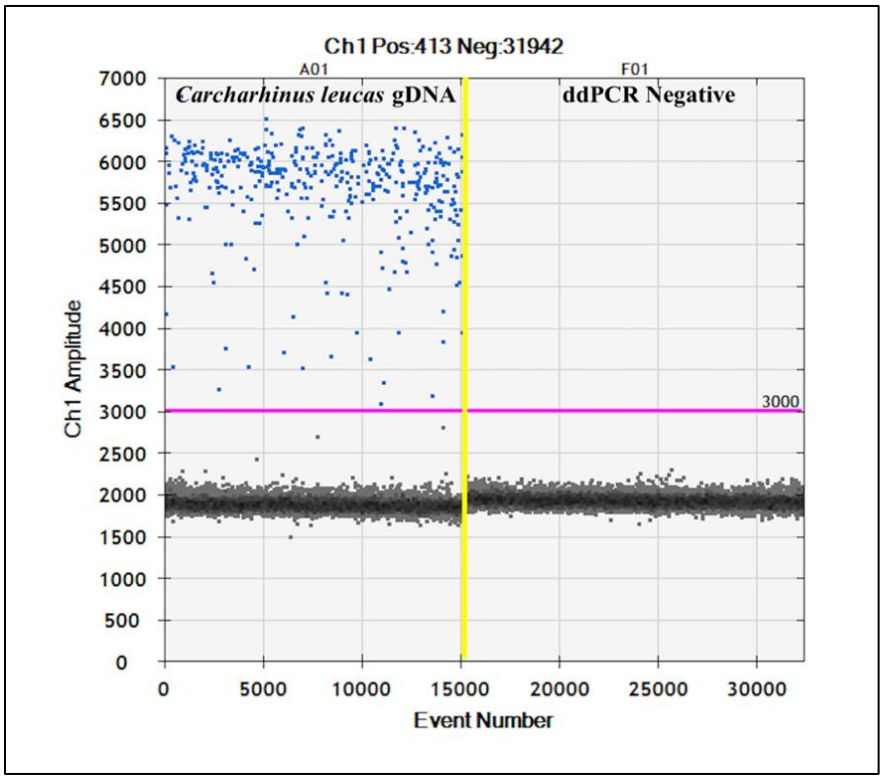

Figure 2.1 *Optimized Droplet Digital™ PCR reaction for Bull Sharks*.

Raw output of the optimized Droplet Digital™ PCR (ddPCR) reaction for the designed Bull Shark (*Carcharhinus leucas*) specific assay showing one ddPCR replicate for one individual (0.2 ng/µL of gDNA) and one replicate for the ddPCR negative from the Bio-Rad® QX200™ Droplet Reader. Each droplet in each well was classified as either positive (blue droplets) or negative (grey droplets) for target DNA, based on a manual detection threshold set to 3,000 amplitude (the horizontal pink line) using the QuantaSoft™ Rare Event Detection analysis. Event Number refers to the number of droplet events generated for a given well or sample; Ch 1 Amplitude measurement refers to the level of fluorescence emitted by a droplet event; and each column is a single well.

2.3.5 Collection of positive water samples

Carcharhinus leucas eDNA samples were obtained via the collection of water samples from known *C. leucas* habitat and *ex situ* experiments. These experiments were conducted in accordance with the laws of the state of Alabama and under the IACUC protocols (IACUC Protocol Number 974304). All measures were taken to reduce the pain or stress the animal underwent during testing; therefore, the water used in the *ex situ* experiments were from natural shark habitat. Water was collected from the coastal waters of Mobile Bay, Alabama, known *C. leucas* habitat, in April 2017 and placed into a pre-cleaned, circular fiberglass, closed-system tank (~120 cm wide and held a volume of ~711 L) and six × 1 L water samples were immediately collected from this tank to determine whether target eDNA was present in the ambient water. A bubbler was added to the tank to keep the system oxygenated and one wild-caught juvenile male *C. leucas*, ~930 mm total length, was added to the tank. To acquire a confirmed positive *C. leucas* eDNA sample, after 30 minutes, six × 1 L water samples were again collected from the tank. These water samples were used in aspects of method development (see 2.6.1 Supporting Information 1) and to validate the developed genetic assay.

To test the effectiveness of the developed *C. leucas* assay in an open system with a single target species present, a flow-through mesocosm (~365 cm wide containing a volume of ~14,500 L) at Dauphin Island Sea Lab, Alabama was maintained in April 2017. The flow rate of the mesocosm was designed to mimic flow in a coastal system at ~30 cm^3/hour, with complete system turnover at approximately two hours. One wild-caught juvenile male *C. leucas*, ~930 mm total length, was introduced to this system and five × 1 L water samples were collected immediately (time 0.0), spanning the diameter

of the mesocosm; this sampling regime was repeated every 0.5 hours for three hours, allowing for complete turnover of the system. Water samples were stored in a -20°C freezer for one month, due to lab equipment constraints, similar to Bakker *et al*. (2017) and Gargan *et al.* (2017), and were thawed at room temperature prior to filtration.

Water samples from these experiments were vacuum-filtered using 47 mm diameter nylon 0.8 μm filters (three per 1 L), which were preserved in 95% ethanol at room temperature (2.6.1 Supporting Information 1) and DNA extractions followed the Goldberg *et al*. (2011) protocol incorporating the QIAshredder™ spin columns (2.6.2 Supporting Information 2). DdPCR amplifications were carried out in replicates of five, using the optimized *C. leucas* assay previously described in this study. All ddPCR reactions were set up using aerosol barrier filter pipette tips and designated pipettes, separate from those used in setting up PCR reactions, were used to add eDNA extracts to the reactions. DdPCR results were analyzed using the Bio-Rad® QX200™ Droplet Reader and QuantaSoft™ RED analysis, a manual detection threshold of 3,000 amplitude, and the LoD.

2.4 Results

2.4.1 Optimal eDNA methods

The Goldberg *et al*. (2011) protocol using the QIAGEN® DNeasy® Blood & Tissue Kit and QIAshredder™ spin columns yielded higher relative quantities of total eDNA from filters compared to the QIAGEN® DNeasy® PowerWater® Kit protocol, across all variations in physical disruption methods (Figure 2.2). The DNA yields from the four physical disruption methods used with the Goldberg *et al.* (2011) protocol were similar: no physical disruption yielded a total DNA average of 61.19 ng/μL (SE = 1.65),

bead beating the filters yielded 56.83 ng/µL (SE = 6.75), filter scraping yielded 56.78 ng/µL (SE = 1.77), and freezing filters with liquid nitrogen and crushing yielded 64.93 ng/µL (SE = 2.36) (Figure 2.2). Since the total DNA yields were similar across these methods and because the addition of a physical disruption step is time-consuming and allows for an additional opportunity for contamination by exogenous DNA, we determined the optimal DNA extraction method for our purposes to be the Goldberg *et al.* (2011) protocol with no physical disruption method.

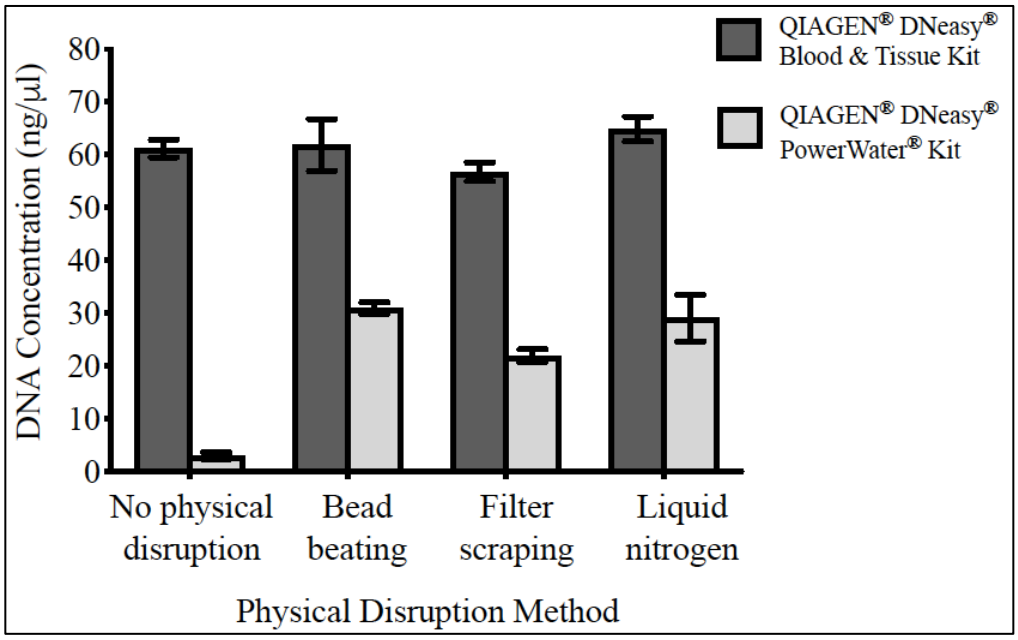

Figure 2.2 *Comparison of QIAGEN® DNeasy® DNA extraction kit protocols.*

Concentrations of DNA extracts from water samples using the QIAGEN® DNeasy® Blood & Tissue Kit with the Goldberg *et al.* (2011) protocol and the QIAGEN® DNeasy® PowerWater® Kit, in combination with additional physical disruption methods. SE bars were used to show the error in mean DNA concentrations between categories, using four Thermo Fisher Scientific NanoDrop™ spectrophotometer readings per sample. Each category contained three × 1 L water sample replicates.

The combination of primers and probe designed in this study were demonstrated to be species-specific for *C. leucas* in our study area by successfully amplifying target

DNA in all ddPCR replicates for the five *C. leucas* individuals and not amplifying DNA in any of the ddPCR replicates of the 18 local exclusion species or PCR negative controls. The LoD, as determined using the Bio-Rad® QX200™ Droplet Reader and QuantaSoft™, was the 1:10,000 dilution, corresponding to 2.5 pg of target DNA in the reaction (Figure 2.3). There were several positive droplets present above the manual threshold in the 1:10,000 dilutions and the standard errors did not include zero or overlap with those of the 1:100,000 dilutions. In contrast, there were no positive droplets detected in the 1:100,000 dilutions, and the standard errors overlapped with zero, indicating no *C. leucas* DNA could reliably be detected (Figure 2.3). Using the number of copies of target DNA/μL in the 1:10,000 dilutions and applying the lower standard error as the relaxed detection threshold for each of the two individuals (see Baker *et al*. 2018), the average LoD threshold was determined to be 0.6 copies/μL.

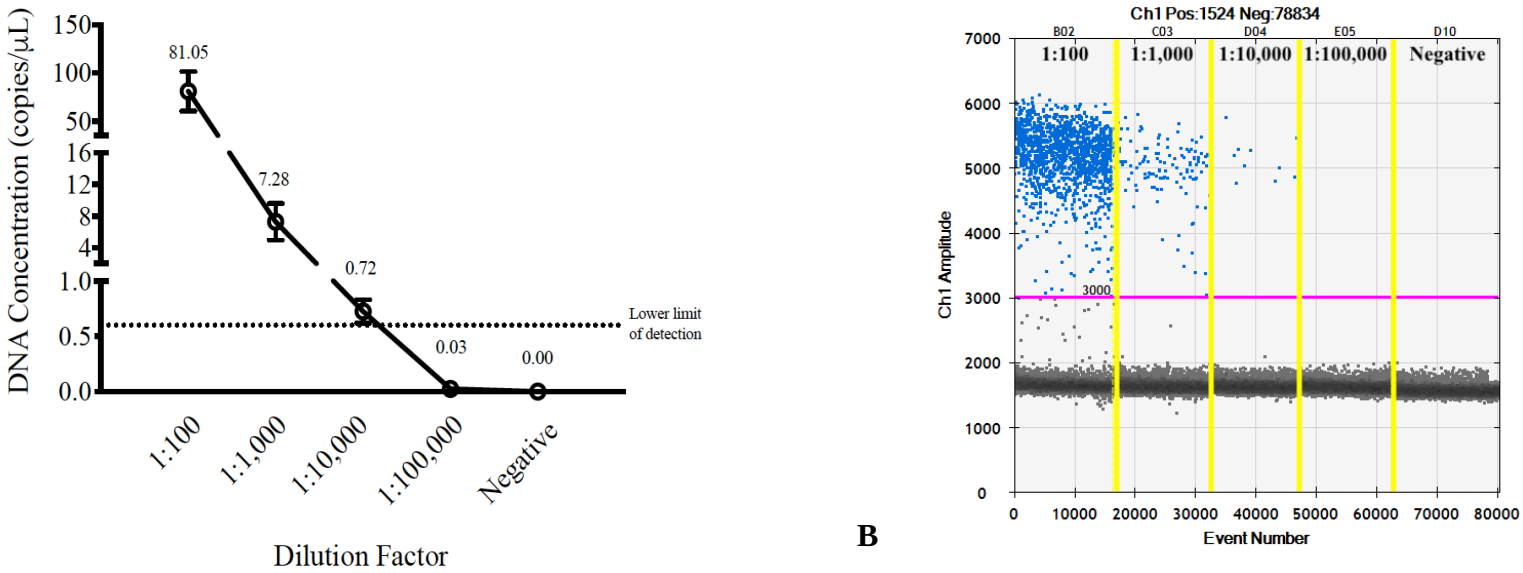

Figure 2.3 *Detection estimates and corresponding droplet outputs for a 10X dilution series.*

Limit of detection (LoD) tests using a 6-fold 10X dilution series (1:10 – 1:100,000) of total genomic DNA from two Bull Shark (*Carcharhinus leucas*) individuals from the northern Gulf of Mexico. (**A**) The mean DNA concentrations (copy number/µL) and standard error bars were calculated from three Droplet Digital™ PCR (ddPCR) replicates for each of two individuals, using a manual detection threshold of 3,000 amplitude and the Rare Event Detection analysis setting on the Bio-Rad® QX200™ Droplet Reader and QuantaSoft™ software. The 1:10 and 1:1,000,000 were not graphed due to oversaturation of the PCR product, and the lack of DNA copies present showing no positive droplet detections, respectively. The LoD (0.6 copies/µL) is represented by a dotted line**.** (**B**) Raw droplet output of ddPCR serial dilution products from one ddPCR replicate of one *C. leucas* individual detected by the Bio-Rad® QX200™ Droplet Reader and QuantaSoft™ software. Each droplet in each well was classified as either positive (blue droplets) or negative (grey droplets) for target DNA. Each well is separated by yellow bars and corresponds to the same dilution concentrations graphed in Figure 2.3A; labeled with each dilution series it represents.

2.4.2 Analysis of positive water samples

Using the developed ddPCR assay and the QuantaSoft™ RED analysis with a manual detection threshold of 3,000 amplitude, an average of 1.62 copies/μL (SE = 0.12) of *C. leucas* DNA was detectable from five 22 μL ddPCR reactions from known habitat, Mobile Bay, without visually confirming the presence of *C. leucas* (Figure 2.4). In the *ex situ* positive eDNA experiment, 30 minutes after a *C. leucas* was added to the closed tank, large amounts of target eDNA were present, with an average concentration of 166.6 copies/μL (SE = 3.01) from five 22 μL reactions (Figure 2.4).

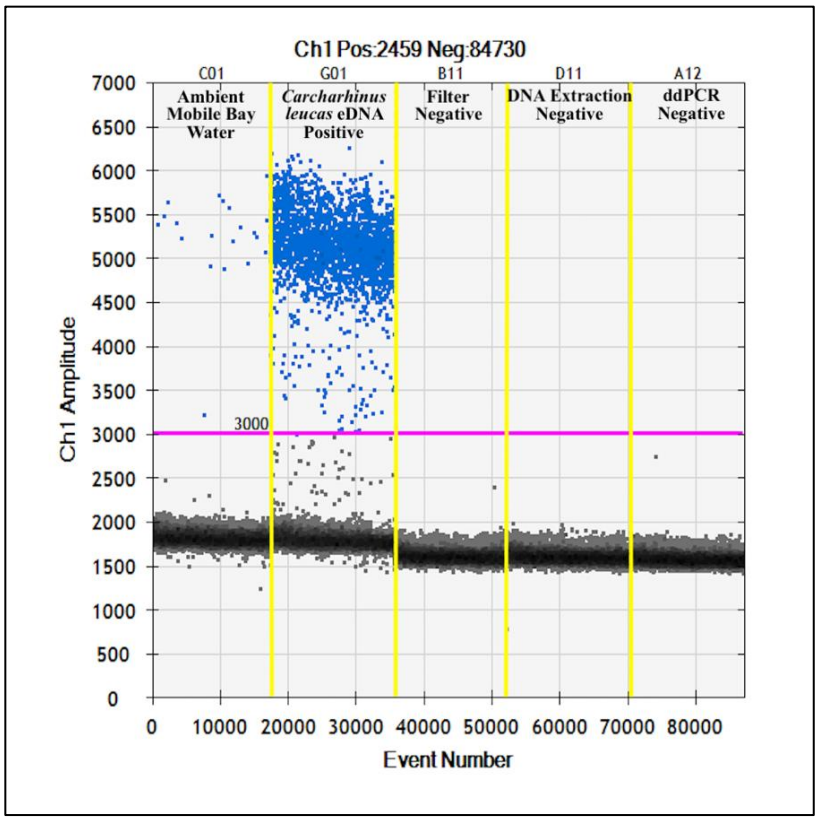

Figure 2.4 *Droplet Digital™ PCR output from positive water sample collection.*

Raw Droplet Digital™ PCR (ddPCR) output from the ambient water sample in Mobile Bay, the Bull Shark (*Carcharhinus leucas*) eDNA positive water sample taken from a closed system 30 minutes after adding the shark, and each negative control from the Bio-Rad® QX200™ Droplet Reader. Each droplet in each well was classified as either positive (blue droplets) or negative (grey droplets) for target DNA based on a manual detection threshold set to 3,000 amplitude (the horizontal pink line) using the QuantaSoft™ Rare Event Detection analysis. Event Number refers to the number of droplet events generated for a given well or sample; Ch 1 Amplitude measurement refers to the level of fluorescence emitted by a droplet event; and each column is a single well. Columns, or wells, are separated by yellow bars; Column C01 corresponds to one ddPCR replicate from the ambient Mobile Bay water sample and G01 corresponds to one ddPCR replicate from the *C. leucas* eDNA positive water sample. Columns B11, D11, and A12 correspond to one ddPCR replicate from each negative control incorporated and shows no contamination occurred during any stage of this experiment.

In the flow-through mesocosm experiment, when applying a lower LoD of 0.6 copies/µL to the data analysis, target *C. leucas* DNA was not detectable in any of the ddPCR replicates at time 0.0 but was detectable in all ddPCR replicates 0.5 hours after

the shark was added (Figure 2.5). Average target eDNA concentration peaked by 1.0 hour, with an average of 5.8 copies/µL (SE = 0.27) across all ddPCR replicates, and then declined over the next hour (Figure 2.5). By 2.0 hours, the average concentration of *C. leucas* eDNA dipped below the LoD, with positive detections in only two of the five ddPCR replicates for this sample (Figure 2.5). There was a second, smaller spike in *C. leucas* eDNA by 2.5 hours, that again decreased, but the average concentration of target. DNA remained detectable at 3.0 hours, although only two of the five ddPCR replicates for this sample had concentrations above the LoD (Figure 2.5).

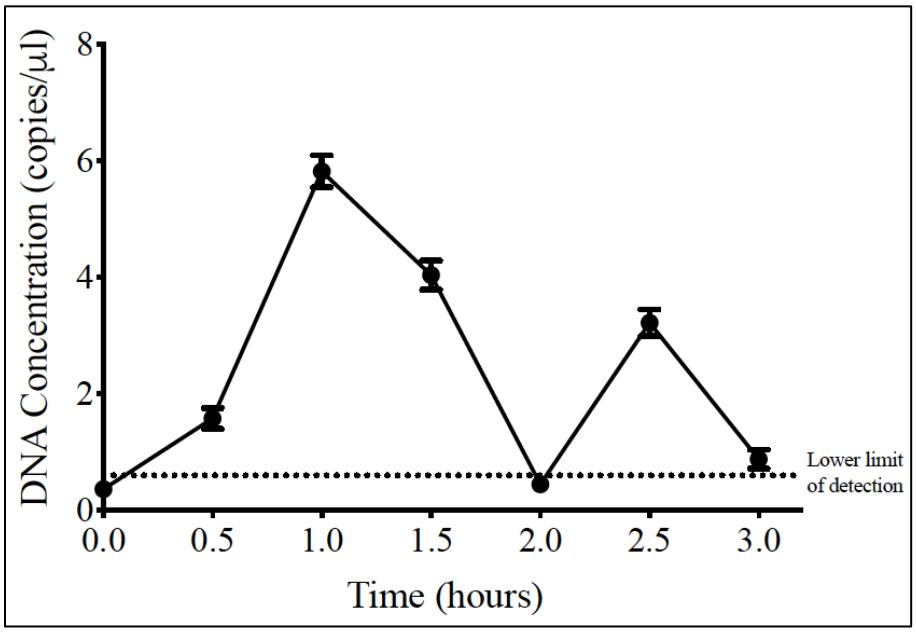

Figure 2.5 *Bull Shark environmental DNA concentration estimates from flow-through mesocosm water samples.*

Bull Shark (*Carcharhinus leucas*) mean eDNA concentrations (unit of measure) in a flow-through mesocosm detected using the Bio-Rad® QX200™ Droplet Reader and QuantaSoft™ using a manual detection threshold of 3,000 amplitude with the Rare Event Detection analysis setting. Each time point sample was run in Droplet Digital™ PCR (ddPCR) replicates of five and standard error bars were used to show the variation in concentration estimates across the five ddPCR replicates for each sample. The lower limit of detection, found to be at least 0.6 copies/µL in this study, is indicated by a dotted line.

2.5 Discussion

The use of eDNA as a tool to study the distribution and ecology of marine species has increased substantially in recent years (Bakker *et al*., 2017; Foote *et al*., 2012; Lafferty *et al.*, 2018; Port *et al*., 2016). However, careful consideration and optimization of the methods employed in such studies are necessary, ultimately allowing for an appropriate interpretation of the results. Here, we found filtering water with nylon 0.8 μm filters, preserving the filters in 95% ethanol (2.6.1 Supporting Information 1), and then performing DNA extractions using the Goldberg *et al.* (2011) protocol with the QIAGEN® DNeasy® Blood & Tissue Kit and QIAshredder™ spin columns to be an appropriate method of isolating total eDNA from water collected from the northern Gulf of Mexico. Although the number of replicates in the experiment was small, the Goldberg *et al.* (2011) protocol was found to outperform the PowerWater® kit across all four physical disruption methods, despite the latter being specifically designed and marketed for eDNA extractions from water samples, and at a higher cost. The total DNA yields used to evaluate the performances of these extraction methods are unlikely to be accurate in an absolute sense due to the inability of NanoDrop™ spectrophotometer technology to decipher DNA from other possible biological macromolecules, but the relative differences between DNA yields were substantial. The combination of primers and internal probe for the mtDNA ND2 gene designed in this study are optimized for *C. leucas* in the estuaries in the northern Gulf of Mexico; however, whether they are appropriate (*e.g.*, species-specific) for use in other geographic regions, such as northern Australia, or in fully marine waters, where there may be additional species of closely related carcharhinids present, requires further testing. The LoD determined in this study

shows the sensitivity and detection capability of the developed assay and was demonstrated to be sufficient for *C. leucas* eDNA detection in Mobile Bay and in *ex situ* positive samples. However, the LoD may require further refinement through additional dilution series between the 1:10,000 and 1:100,000 dilutions before being used in data analysis for large numbers of field samples. Furthermore, due to potential differences across ddPCR machines, we recommend the LoD to be refined independently for each machine, using the LoD here as a starting reference point for this assay.

The ability of ddPCR to detect low concentrations of target DNA, *e.g.*, 2.5 pg of *C. leucas* DNA in this study, means this platform may be less likely to produce false negatives when used alongside an appropriate sampling regime and water processing methods (*e.g.*, spatial and depth coverage, volume collected, filter pore size). False negatives can occur when target DNA is captured in water samples but is not detected due to limitations of the genetic assays employed (Darling and Mahon, 2011; Ficetola *et al.*, 2015; Goldberg *et al.*, 2016; Lahoz-Monfort *et al*., 2016). To date, the majority of studies that use eDNA in targeted species detections have used qRT-PCR, but the detection capabilities of this platform be may limited, when compared to those of ddPCR (Doi *et al.*, 2015a, b). The difference in detection abilities between the two PCR platforms are likely due to fundamental differences in how they quantify target DNA. DdPCR quantifies the starting DNA copy number present in a sample using end-point PCR without reference to a standard (absolute quantification) (Whale *et al.*, 2012), making it a more sensitive and precise assay, ideal for eDNA applications targeting a single target species. Additionally, the RED analysis setting using the Bio-Rad® QuantaSoft™ software is designed to identify low copy numbers of target DNA in a

background largely composed of non-target DNA copies (Bio-Rad® Droplet Digital™ PCR Applications Guide). Given the ability of ddPCR to detect such low quantities of DNA, it may replace qRT-PCR in eDNA research (Doi *et al.*, 2015b; Nathan *et al*., 2014) assessing the distribution, habitat use, and abundance of species found in low abundance and/or are of conservation concern (Baker *et al*., 2018; Hunter *et al.*, 2018; Tréguier *et al.*, 2014), including elasmobranchs (Bohmann *et al.*, 2014; Lafferty *et al.*, 2018). However, we caution that the ability to detect such low quantities of DNA also increases the potential for false positives (Goldberg *et al.*, 2016; Huggett *et al*., 2015). All eDNA studies, but especially those using ddPCR, require strict field and laboratory controls and procedures be in place to reduce the potential for false positives, typically the result of contamination by exogenous DNA or cross-contamination of samples (see Ficetola *et al*., 2016). In addition to the contamination controls described by Goldberg *et al*. (2016), Deiner *et al.* (2015), and Port *et al*. (2016), when using ddPCR, we also suggest: 1) using two cleaning methods for decontamination of all field and water filtration equipment (*e.g.*, a bleach wash, plus autoclaving and/or UV light exposure), 2) that water filtration is conducted in a lab space that has never had tissue or gDNA from the target species present, 3) that gloves and any tools are changed in between samples during water filtration (see Pilliod *et al*., 2013), 4) that negatives be incorporated into field collection, water filtration, DNA extraction, and PCR, with each negative run through to PCR (see Bakker *et al*., 2017; Jerde *et al*., 2011), 5) that a designated pipette, separate from that used to set up reactions, be used to add DNA extracts to ddPCR reactions, and 6) that multiple replicates for each sample are run during ddPCR (see Rees *et al.*, 2014). Strict field and lab controls will ensure the authenticity and reliability of eDNA results, which

is increasingly critical in eDNA research using highly sensitive technologies, such as ddPCR, especially when the results of such studies will be used to inform conservation and management initiatives (Hunter *et al*., 2018).

Fundamental research on the accumulation, persistence, and degradation of elasmobranch eDNA is necessary to improve the interpretation of results in eDNA field research. Here, we have shown that after adding a shark into closed and flow-through systems, target eDNA was detectable within 30 minutes. In the flow-through system, the initial spike in target eDNA that occurred between 0.5 and 1.0 hours could be due to initial stress experienced by the shark after being added to the mesocosm, causing it to expel more DNA (*e.g*., Barnes *et al*., 2014). The overall decrease in target eDNA between 1.0 and 2.0 hours may be the result of the shark acclimating to the environment and releasing less DNA or turn-over of water in the mesocosm if the shark is releasing DNA into the system in pulses rather than continuously, however this has not been explicitly explored in elasmobranchs. The inability to detect *C. leucas* DNA in some of the ddPCR replicates at 2.0 and 3.0 hours, despite the confirmed presence of a shark and the use of a highly sensitive ddPCR assay, suggests there may have been very little *C. leucas* DNA present at those times, which could occur if DNA was shed in pulses, and then flowed out of the mesocosm. However, this pattern could also be indicative of sampling error, where *C. leucas* DNA was present, but not captured; highlighting the need for careful consideration of sampling regime as well as the interpretation of the results of eDNA studies. Because mesocosm water samples were frozen after collection, it cannot be completely ruled out that the eDNA degraded prior to filtration (Hinlo *et al*., 2017; Takahara *et al*., 2015); however, the concentrations of the total eDNA extracts

from these samples were not unusually low compared to the other eDNA extracts analyzed for this study. Furthermore, other eDNA studies have frozen water samples prior to filtration without apparent negative effects (Bakker *et al*., 2017; Gargan *et al*., 2017) making it unlikely to be the sole explanation for the observed patterns of *C. leucas* DNA detected in this experiment. Ideally, these experiments should have been replicated and included a second tank without a shark as a negative control, with water samples filtered immediately after collection; however, due to limited facilities and the constraints of using live animals, these improvements to the study design were not feasible. Regardless, this is the first elasmobranch eDNA study that has placed a single target animal into closed and then open, flow-through systems to quantify target eDNA from a single animal over time, creating a baseline for future *ex situ* research. In comparison, other eDNA studies of elasmobranchs have acquired positive eDNA samples by collecting water samples from aquaria with the target species present (*e.g*., Simpfendorfer *et al.*, 2016) or collecting water samples from known habitats, but without visually confirming the presence of the target species (*e.g.*, Weltz *et al.*, 2017). Future studies should assess DNA accumulation over different timescales than presented here, as well as how altered flow rates, water conditions (pH, temperature), weather conditions (photoperiod, cloud cover), and number and size of target species impact the accumulation and persistence of elasmobranch eDNA in marine systems.

2.6 Supporting Information

2.6.1 Supporting Information 1: Filter pore size and preservation tests

The optimal filter size for water filtration is environment-specific and requires testing prior to starting eDNA field studies (*e.g.*, Simpfendorfer *et al*., 2016; Bakker *et*

al., 2017). To determine the best filter pore size for our study area, the positive *Carcharhinus leucas* eDNA water samples collected (*i.e.*, Mobile Bay ambient water and 30 minutes after a shark was added to the closed tank) were vacuum-filtered using the laboratory protocols described and 47 mm diameter nylon filters of three different pore sizes; 0.45 micron (μm), 0.8 μm, and 1.0 μm (see Rees *et al.*, 2014). For each pore size, 2 L of each positive eDNA water sample was filtered, and to test the most effective method for filter preservation, the filters from 1 L were preserved in 95% ethanol at room temperature and the filters from the other 1 L were stored at -80°C. To assess which pore size captured the most particulate material in the water samples and which preservation method maintained higher qualities and quantities of DNA, DNA extractions were conducted on the filters using the optimal protocol: Goldberg *et al*. 2011 QIAGEN® DNeasy® Blood & Tissue Kit with the QIAshredder™ spin columns. The DNA extracts for each 1 L water sample were combined and the DNA qualities were assessed using 2% agarose gel and quantities measured using Thermo Fisher Scientific NanoDrop™ spectrophotometer technology, with each extract measured four times.

The three pore sizes tested recovered slightly different amounts of eDNA, with the 0.8 μm pore size yielding higher quantities of total eDNA in the positive *C. leucas* eDNA samples when compared to the 0.45 μm and the 1.0 μm pore sizes, but slightly less than the 0.45 μm and about equal to the 1.0 μm in the ambient water from Mobile Bay (Figure 2.6). Notably, the 0.8 μm filter pore size took roughly 20 minutes to filter a 1 L water sample and used three filters, whereas both 0.45 μm and 1.0 μm each took ~45 minutes to filter a 1 L water sample and required four filters. Therefore, we chose 0.8 μm pore size to filter water in this study because there was not a substantial difference in total

eDNA captured by the different pore sizes (Figure 2.6) and using 0.8 μm meant that water filtration was more likely to be completed within 24 hours of collection and decreased the cost of water filtration, via the use of fewer filters. The Mobile Bay system is highly variable and receives the fourth largest river discharge in the United States, primarily from the Alabama and Tombigbee Rivers (Morisawa, 1968); therefore pre-filtering of water samples using a larger pore size (*e.g.*, 5 μm) may be necessary before filtering with a 0.8 μm filter under conditions or seasons where the particulate content is higher. Storing filters in 95% ethanol at room temperature yielded only slightly higher quantities of total eDNA in both the Mobile Bay ambient water and the positive *C. leucas* eDNA samples for all pore sizes after five days of storage (Figure 2.6). Storing filters in 95% ethanol is a sufficient preservation method for filters and will facilitate water filtration in the field, where freezing filters can be logistically challenging, particularly in remote locations.

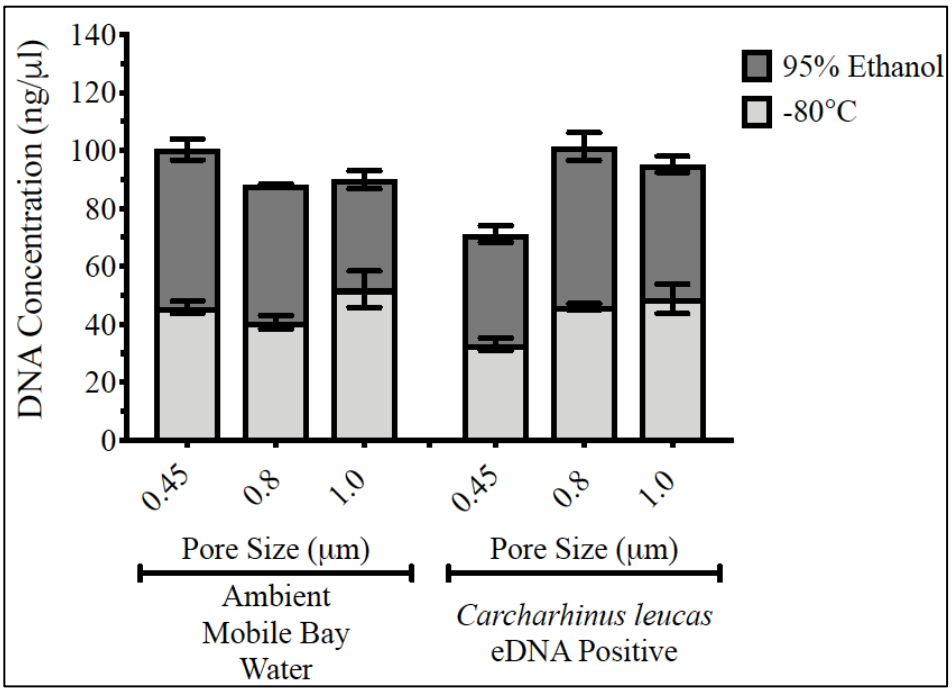

Figure 2.6 *Total environmental DNA concentration yields.*

Mean total environmental DNA concentration yields (ng/µL) for each 1 L replicate of each nylon filter pore size and preservation method used to vacuum-filter ambient Mobile Bay water and positive Bull Shark (*Carcharhinus leucas*) eDNA water samples. DNA concentrations were quantified using a Thermo Fisher Scientific NanoDrop™ spectrophotometer, with each extract measured four times. Standard error bars show the error in mean DNA concentration measurements for 1 L per category.

2.6.2 Supporting Information 2: Comparison of QIAGEN® DNeasy® Blood & Tissue Kit extraction protocols

The QIAGEN® DNeasy® Blood & Tissue Kit is a frequent choice for DNA extractions from filters in eDNA studies, but with numerous variations (Rees *et al*., 2014). We tested three of these variations to determine which yielded higher qualities and quantities of total DNA: 1) the manufacturer's protocol, 2) the Yamamoto *et al.* (2016) protocol, and 3) the Goldberg *et al*. (2011) protocol incorporating QIAshredder™ spin columns. The primary differences between these variations in methods include the types of lysis buffers used during digestion, the duration of digestion, and the incorporation of

QIAshredder™ spin columns into the Goldberg *et al.* (2011) protocol. Three × 1 L water sample replicates were used to test each DNA extraction method variation, which were collected from Lake Byron, Mississippi using the water collection and filtration protocols described and 47 mm diameter, 0.8 μm nylon filters (2.6.1 Supporting Information 1). DNA extractions on the filters were started immediately to eliminate the filter preservation step (see Hinlo *et al*., 2017). The DNA extracts for each 1 L water sample were combined and the DNA qualities were assessed using 2% agarose gel and quantities measured using Thermo Fisher Scientific NanoDrop™ spectrophotometer technology, with each extract measured four times for accuracy.

The Goldberg *et al.* (2011) protocol incorporating QIAshredder™ spin columns yielded higher quality DNA on 2% agarose gels and yielded substantially greater quantities of DNA from filters (mean = 56.79 ng/μL; SE = 2.87) when compared to the manufacturer's protocol (mean = 19.18 ng/μL; SE = 1.11) and the Yamamoto et al. (2016) protocol (mean = 15.58 ng/μL; SE = 0.62) (Figure 2.7).

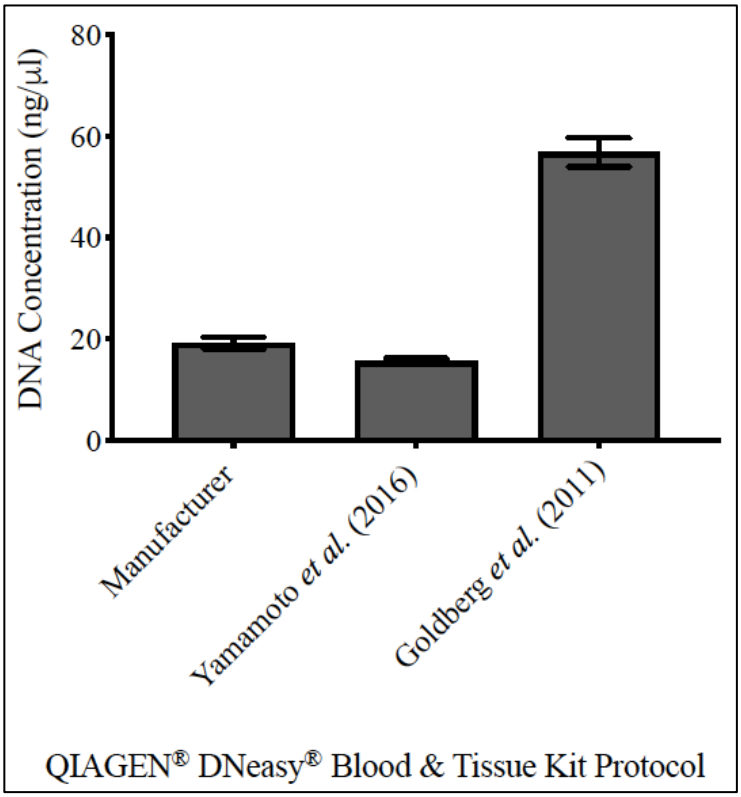

Figure 2.7 *Total environmental DNA concentration yields.*

Mean total environmental DNA yields (ng/µL) for each 1 L replicate of each QIAGEN® DNeasy® Blood & Tissue Kit extraction protocol. DNA concentrations were quantified using a Thermo Fisher Scientific NanoDrop™ spectrophotometer, with each extract measured four times. Standard error bars show the error in mean DNA concentrations across three ×1 L replicates per extraction method.

2.6.3 Supporting Information 3: Bull Shark (*Carcharhinus leucas*) assay design

Table 2.2

Bull Shark and 23 exclusion elasmobranch species used for assay design.

Species	Forward primer nucleotide mismatches	Reverse primer nucleotide mismatches	Probe nucleotide mismatches	GenBank Accession Numbers
Bull Shark, *Carcharhinus leucas* (target)	**0**	**0**	**0**	**KF646785.1**
Blacknose Shark, *Carcharhinus acronotus*	2	4	3	KF728380.1
Bignose Shark, *Carcharhinus altimus*	1	1	4	JQ518603.1
Spinner Shark, *Carcharhinus brevipinna*	2	2	4	KM244770.1
Silky Shark, *Carcharhinus falciformis*	2	1	4	KF801102.1
Finetooth Shark, *Carcharhinus isodon*	2	5	5	JQ518626.1
Blacktip Shark, *Carcharhinus limbatus*	2	4	3	JN082202.1
Oceanic Whitetip Shark, *Carcharhinus longimanus*	3	2	3	KM434158.1
Dusky Shark, *Carcharhinus obscurus*	2	2	2	KC470543.1
Sandbar Shark, *Carcharhinus plumbeus*	1	1	5	KJ740750.1
Smalltail Shark, *Carcharhinus porosus*	1	1	5	JQ519077.1
Night Shark, *Carcharhinus signatus*	2	4	4	JQ518631.1
Spottail Shark, *Carcharhinus sorrah*	2	3	1	KF612341.1
Sand Tiger, *Carcharias taurus*	4	*	5	KF569943.1
Tiger Shark, *Galeocerdo cuvier*	2	4	8	KF111728.1
Atlantic Stingray, *Hypanus sabina*	4	*	13	JQ518787.1
Dusky Smoothhound, *Mustelus canis*	1	2	4	JQ518711.1
Atlantic Sharpnose Shark, *Rhizoprionodon terraenovae*	2	3	4	JQ51865.1
Scalloped Hammerhead, *Sphyrna lewini*	3	4	6	JX827259.1
Great Hammerhead, *Sphyrna mokarran*	3	7	8	DQ422103.1
Bonnethead, *Sphyrna tiburo*	3	6	6	KM453976.1

Table 2.2 *(continued).*

Species	Forward primer nucleotide mismatches	Reverse primer nucleotide mismatches	Probe nucleotide mismatches	GenBank Accession Numbers
Spiny Dogfish, *Squalus acanthias*	1	8	9	Y18134.1
Greeneye Spurdog, *Squalus cholorocculus*	1	8	9	JQ519006.1
North Pacific Spiny Dogfish, *Squalus suckleyi*	1	8	9	JQ518977.1

Target Bull Shark (*Carcharhinus leucas*) and 23 exclusion elasmobranch species (with GenBank accession numbers) aligned to manually design species-specific primers and an internal probe in the mitochondrial NADH dehydrogenase 2 (ND2) gene. * indicates that no data was available for that species at the specific nucleotide location in the ND2 gene from GenBank.

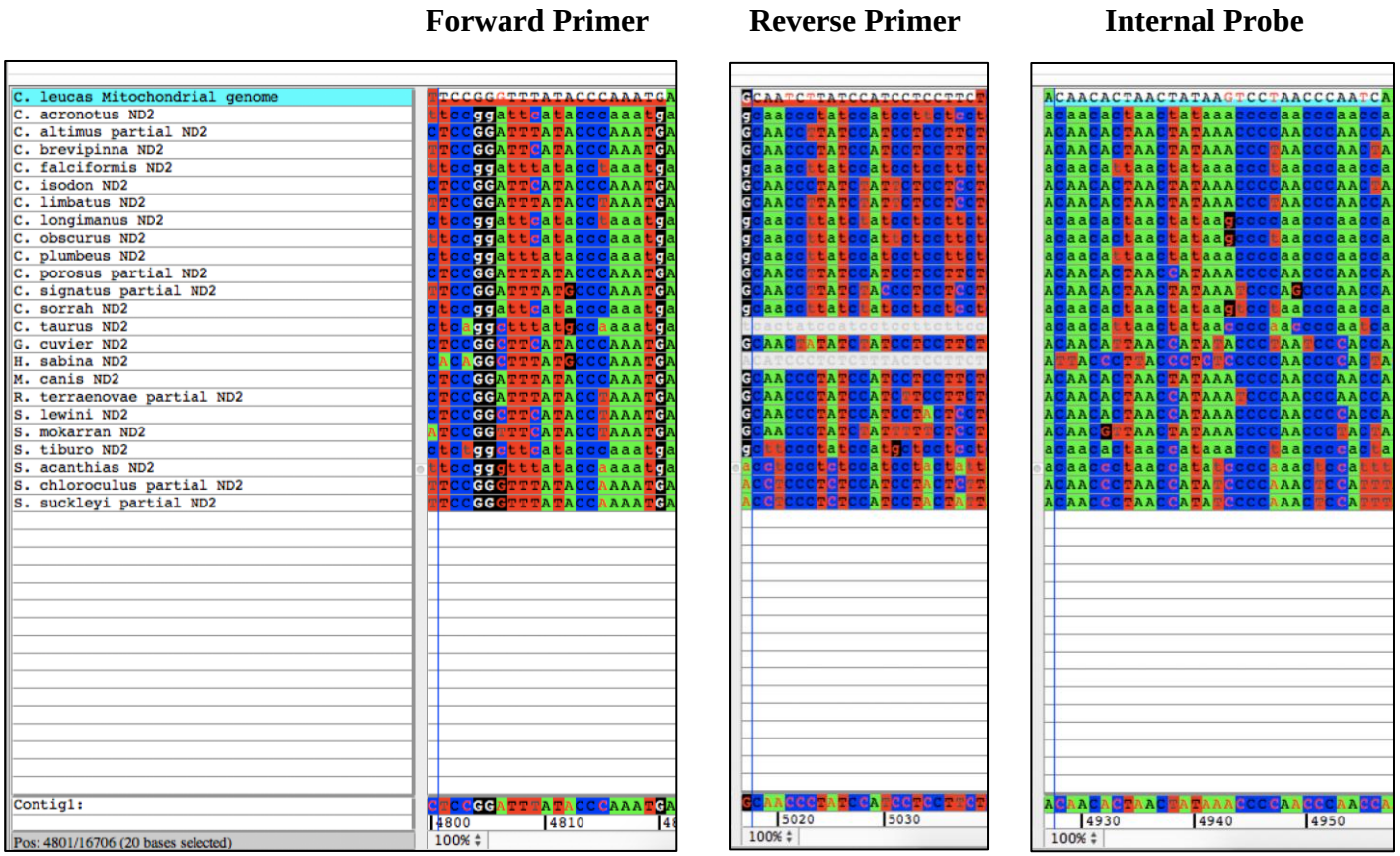

Figure 2.8 *Bull Shark and exclusion species sequence alignments.*

NADH dehydrogenase 2 (ND2) gene of the mitochondrial genome of the Bull Shark (*Carcharhinus leucas*) and 23 exclusion elasmobranch species were aligned using CodonCode Aligner v. 7.0 and used to manually design species-specific primers and an internal probe for *C. leucas*. Each alignment screengrab lists *C. leucas* first, with the primer or probe sequence highlighted in white.

CHAPTER III – ENVIRONMENTAL DNA DETECTION OF BULL SHARKS (*CARCHARHINUS LEUCAS*) IN THE WESTERN AND EASTERN MOBILE-TENSAW DELTA AND MOBILE BAY, ALABAMA DURING TWO DISTINCT SEASONS USING DROPLET DIGITAL™ PCR

3.1 Abstract

Elasmobranchs are ecologically vital; positioned at or near the top of marine trophic systems, they help to maintain ecosystem function and stability by directly influencing the behavior, abundance, and distribution of other species. Bull Sharks (*Carcharhinus leucas*) are highly mobile predators that spend their first years of life maturing in estuarine systems and have also been observed taking advantage of connecting freshwater rivers. *Carcharhinus leucas* can withstand large changes in salinity and forage on a wide variety of prey items, indicating they are serving as a mobile link between freshwater rivers and estuaries. To investigate this potential link in the northern Gulf of Mexico, the developed environmental DNA methods were used to analyze water samples collected from Mobile Bay and the Mobile-Tensaw Delta once during the winter wet season and once during the summer dry season to test for target *C. leucas* DNA. Using a species-specific genetic assay on the highly sensitive Droplet Digital™ PCR platform and three criteria for positive target detection, two adjacent sites in the Alabama River in the Mobile-Tensaw Delta produced strong positive detections during the summer season, while no strong positive detections were produced at any site for the winter season. These results suggest that *C. leucas* does use habitat within the Mobile-Tensaw Delta when environmental conditions are favorable and may show preference for a less-urbanized

habitat over a more-urbanized habitat; however, more frequent water sampling events over many seasons would help to resolve whether *C. leucas* ecologically links the freshwater Mobile-Tensaw Delta and estuarine Mobile Bay through habitat usage and movement.

3.2 Introduction

Elasmobranchs (sharks, skates, and rays) are often highly mobile predators positioned near the middle or top of trophic systems; meaning their presence has the ability to influence the behavior and abundance of prey species in their environments (Every *et al*., 2017; Engelbrecht *et al*., 2019), regulating overall ecosystem health and biodiversity (Miller *et al*., 2001; Ritchie *et al*., 2012; Every *et al*., 2017). Bull Sharks (*Carcharhinus leucas*) are temperate, subtropical, and tropical coastal elasmobranchs that are observed in freshwater and brackish areas globally (Thomerson, 1977; Garrick., 1982; Coad and Papahn, 1988; Martin, 2005; Ebert *et al*., 2013) due to their ability to osmoregulate in a wide range of salinities (Thorson, 1962; Thorson, 1971; Oguri, 1964; Thorson *et al*., 1973; Pillans *et al*., 2009). While maturing in low-salinity areas, *C. leucas* have been observed moving across habitats and altering their usage patterns with fluctuating environmental variables, such as salinity and temperature (Simpfendorfer *et al*., 2005; Froeschke *et al*., 2010b; Curtis *et al*., 2011; Drymon *et al*., 2014). The propensity of *C. leucas* to occupy ranges of different habitats within ecosystems, combined with their diverse diet of teleost fishes, other elasmobranchs, and occasional larger vertebrates like turtles and birds (Tuma, 1976; Compagno, 1984; Snelson *et al*., 1984; Last and Stevens, 1994), demonstrates that *C. leucas* functions as a predatory mobile link between freshwater and estuarine habitats (Tillett *et al*., 2012; Laurrabaqio-A

et al., 2019); therefore, *C. leucas* is able to directly influence prey species abundance across these environments (Rosenblatt and Heithaus, 2011; Every *et al*., 2017; Engelbrecht *et al*., 2019) and contribute to ecosystem stability and function (Rooney *et al*., 2006; Sheaves *et al*., 2009; Rosenblatt *et al*., 2013).

Carcharhinus leucas are present throughout the Gulf of Mexico (GoM), but Mobile Bay (MB), Alabama in the northern GoM is an ideal setting in which to examine the potential connection with adjoining freshwater areas, because it experiences high freshwater inflow from multiple riverways in the Mobile-Tensaw Delta (MTD) (Figure 3.1) forming a variable salinity gradient, as well as more seasonal environmental conditions than other areas where *C. leucas* is commonly observed (Drymon *et al*., 2014). Freshwater influx into MB is the fourth largest river discharge in the United States (Morisawa, 1968) and is characterized by distinct wet and dry seasons; the wet season in late winter and early spring has a mean discharge of 2637 m^3sec^{-1} and the dry season during late summer and early fall has a mean discharge of 802 m^3sec^{-1} (Marr, 2013).

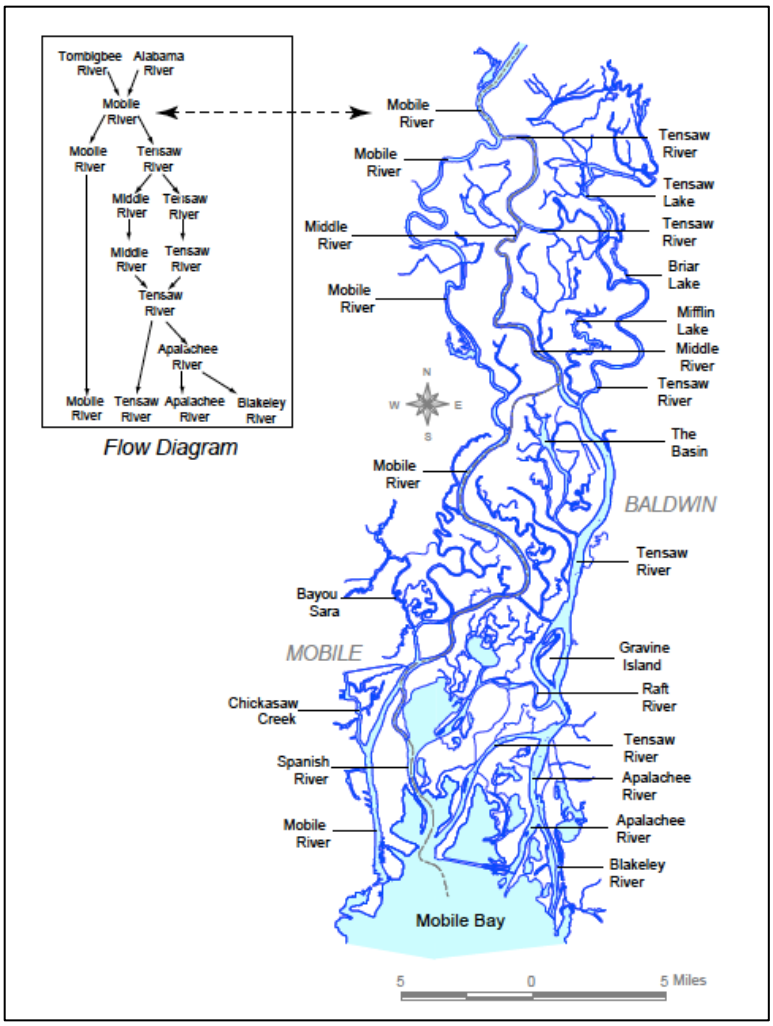

Figure 3.1 *Rivers in the Mobile-Tensaw Delta, Alabama.*

These rivers within the Mobile-Tensaw Delta that discharge into Mobile Bay, Alabama (from Mettee *et al*., 2006).

Although it is well known that *C. leucas* can withstand large changes in salinity, acoustic monitoring of young has shown patterns of movement upriver during periods of low outflow, while residing in river mouths during periods of high outflow (Ortega *et al*., 2009; Heupel *et al*., 2010). The use of environmental DNA (eDNA), genetic material shed by living organisms that is freely present in the environment, for the monitoring of species presence presents an alternative to time-consuming and often intensive traditional

monitoring methods such as setting nets, mark and recapture, or acoustic telemetry. EDNA has been analyzed across a wide variety of taxa, including invertebrates, elasmobranchs, reptiles, amphibians, and mammals to assess ecosystem biodiversity and health, monitor target species presence or absence, and determine population characteristics (Lodge *et al*., 2012; Hunter *et al*., 2015; Sigsgaard *et al*., 2016; Simpfendorfer *et al*., 2016; Uchii *et al*., 2016; Lafferty *et al*., 2018). Despite the rapid deterioration of eDNA once dispelled and its constant transport throughout the environment (Barnes and Turner, 2016), the presence of target eDNA in surface water implies a fairly recent presence of the organism and facilitates in estimating where certain species may occur (Lodge *et al*., 2012). To investigate the potential linkage between estuarine MB and the freshwater MTD provided by predatory *C. leucas* through their habitat use and movement, surface water samples were collected once during the wet season and once during the dry season from MB, Alabama and multiple rivers within the MTD to determine if target *C. leucas* eDNA was detectable in the collected water using the methodology developed in Schweiss *et al*. (In press; Ch. II) and a highly sensitive platform, Droplet Digital™ PCR (ddPCR).

3.3 Materials and Methods

3.3.1 Study region and sampling regime

Water samples were collected at 21 sites in Alabama and across two river systems within the MTD in both summer (dry season; August 2018) and winter (wet season; February 2018). At each site, a YSI™ ProDSS multiparameter water quality meter was used to measure four separate abiotic variables at the surface of the water: water temperature (°C), salinity (ppt), dissolved oxygen (DO; mg/L), and pH. Three collection

sites spanned the length of MB (Sites 1 – 3) and continued into the freshwater river habitats, western and eastern sampling transects (Figure 3.2). The western sampling transect included four sites within the Mobile River and extended north with five sites in the Tombigbee River (Sites 4 – 12), while the eastern sampling transect included five sites within the Alabama River and extended south with four sites in the Tensaw River (Sites 13 – 21) (Figure 3.2). On each transect, samples were collected up to the Coffeeville Lock and Dam and the Claiborne Lock and Dam, respectively (Figure 3. 2 and Figure 3.3A, B).

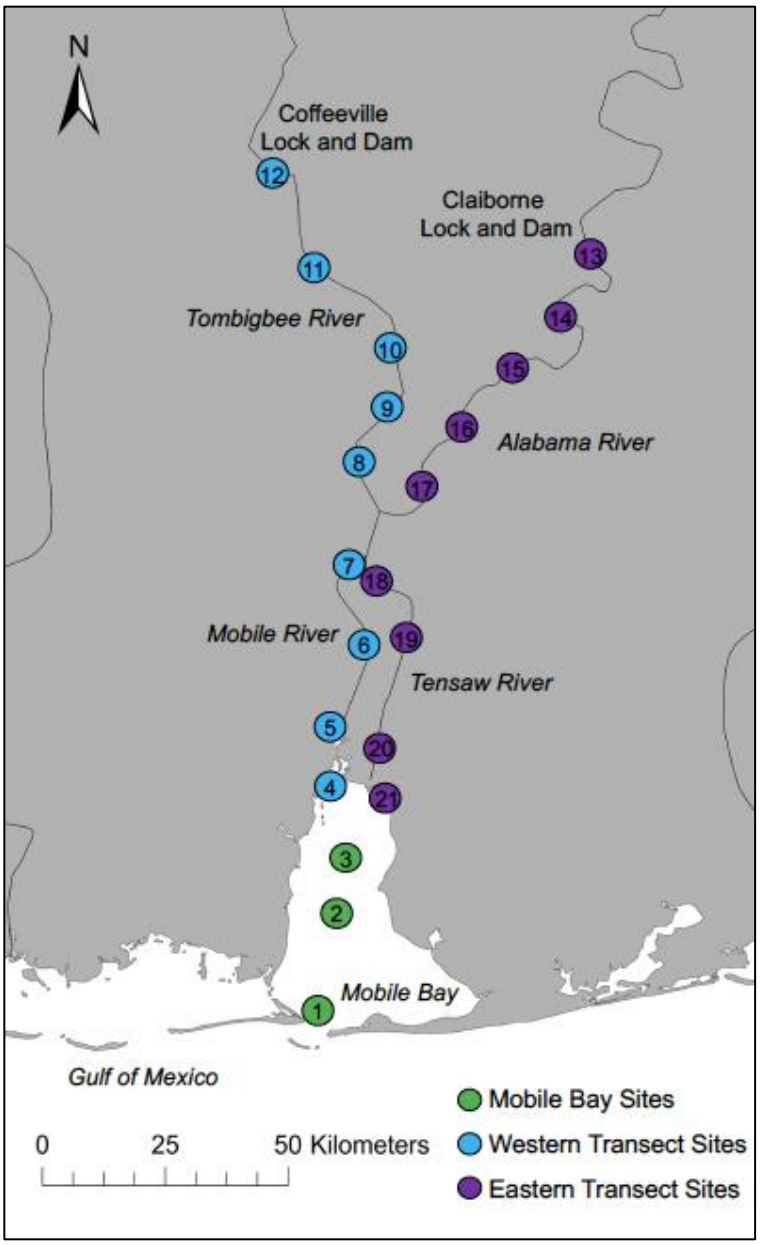

Figure 3.2 *Field sampling sites.*

Mobile Bay and the Mobile-Tensaw Delta with Mobile Bay sites designated by green dots, the western sampling transect sites designated by blue dots, and the eastern sampling transect sites designated by purple dots.

A  **B** 

Figure 3.3 *Coffeeville, Alabama and Claiborne, Alabama Lock and Dam sites.*

(**A**) Coffeeville, Alabama Lock and Dam site spanning the width of the Tombigbee River on the western sampling transect. Photograph credit: U.S. Army Corps of Engineers; (**B**) Claiborne, Alabama Lock and Dam site spanning the width of the Alabama River, on the eastern sampling transect. Photograph credit: U.S. Army Corps of Engineers.

The study region encompassed ~300 km, so the 21 sampling sites were sampled during two consecutive days of each season. Sites 1 – 12 were sampled the first day, and sites 13 – 21 were sampled on the second day (Figure 3.2). Five × 1 L water samples were collected at each of the 21 collection sites. Collection sites 1 and 2 were located at structures in MB: the first at an oilrig (Figure 3.4A) and the second at Middle Bay Lighthouse (Figure 3.4B). The third station was located near the eastern edge of Gaillard Island (Figure 3.4C). Collection sites 1 and 2 were sampled around the perimeter of the structures and collection site 3 was sampled off the eastern shore of Gaillard Island. The remaining sites for each transect were riverine; at each of these sites, five × 1 L samples were collected across the width of the site (Figure 3.5).

A

B

C

Figure 3.4 *Collection sites 1 – 3 in Mobile Bay, Alabama.*

Collection sites in Mobile Bay, Alabama were visited on the first day for both winter and summer sampling events. **(A)** Collection Site 1 at Dauphin Island Rig. Photograph credit: Emily Seubert; (**B**) Collection Site 2 at Middle Bay Lighthouse. Photograph credit: Emily Seubert; (**C**) Gaillard Island with collection Site 3 on the eastern edge circled. Photograph credit: Joey Hunsinger.

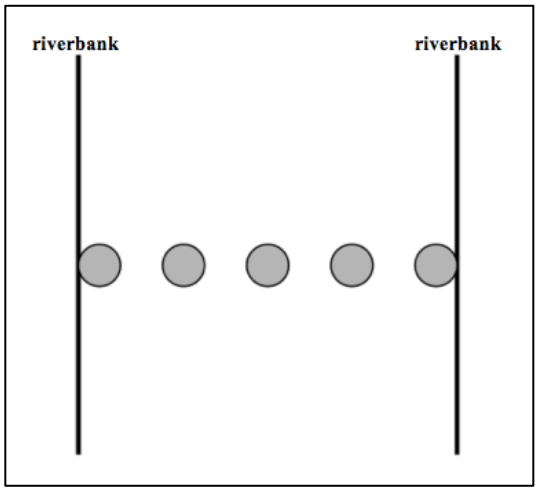

Figure 3.5 *Field sampling regime.*

This sampling regime was used in riverine sites (Sites 4 – 21).

3.3.2 Field and laboratory controls

Field equipment consisted of 1 L Nalgene® high-density polyethylene bottles that were cleaned using a 10% bleach solution and sanitized under 20 minutes of ultraviolet (UV) light prior to collection, and marine coolers for sample bottle storage that were cleaned with a 10% bleach solution prior to field use. Gloves were changed between water collection sites to reduce the risk of cross-contamination. All laboratory controls and procedures followed that of Schweiss *et al*. (In press; Ch. II) with an additional water sample collection negative control. The collection negative control was composed of autoclaved deionized water, taken onto the boat, placed in the cooler on ice for each day of sampling, and remained closed to check for sterility of Nalgene® bottles (Jerde *et al*., 2011; Bakker *et al*., 2017).

3.3.3 Filtration and genetic methods

All water samples were vacuum-filtered using 47 mm 0.8 μm nylon filters and preserved in 95% ethanol at room temperature. DNA extractions from filters followed the Goldberg *et al.* (2011) QIAGEN® DNeasy® Blood & Tissue Kit protocol incorporating the QIAshredder™ spin columns. All Droplet Digital™ PCR (ddPCR) amplifications were carried out in replicates of five, using the optimized *C. leucas* assay determined in Schweiss *et al*. (In press; Ch. II).

3.3.4 Data analysis

All ddPCR data were analyzed with the Bio-Rad® QX200™ Droplet Reader and QuantaSoft™ software using the Rare Event Detection (RED) analysis set with a manual detection threshold of 3,000 amplitude and a limit of detection (LoD) of 0.6 copies/μL (see Schweiss *et al*. In press; Ch. II). Sample replicates were determined to be strongly

positive for the target species, *C. leucas*, if they met three prescribed criteria: 1) droplets above the defined manual threshold (MT) of 3,000 amplitude; 2) the droplets were within the known positive droplet range (see Ch. II, Schweiss *et al.* (In press)); and 3) the number of copies/μL was greater than or equal to the LoD. The known positive droplet range using the developed assay and *C. leucas* eDNA was ~4,000 – 6,000 amplitude (Figure 3.6). Sample replicates were considered to be potential positives if at least one of the three criteria were met. Each of the assay runs for the two sampling seasons contained the respective negative controls for that season and a *C. leucas* positive eDNA reference sample (see Ch. II) to ensure that the reactions were successful (Appendix A).

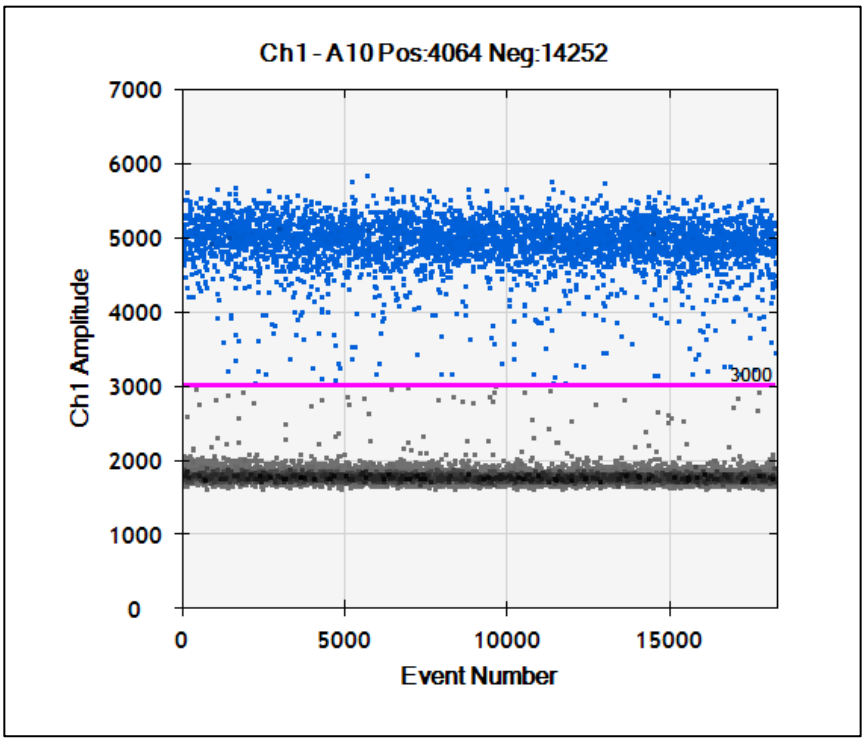

Figure 3.6 *Droplet Digital™ PCR scatter plot with Bull Shark environmental DNA.*

Raw Droplet Digital™ PCR (ddPCR) output from one replicate reaction of the Bull Shark (*Carcharhinus leucas*) eDNA positive water sample in a closed system after 30 minutes from the Bio-Rad® QX200™ Droplet Reader showing the known positive range for the target species. Each droplet in each well was classified as either positive (blue droplets) or negative (grey droplets) for target DNA based on a manual detection threshold set to 3,000 amplitude (the horizontal pink line) using the QuantaSoft™ Rare Event Detection analysis. Event Number refers to the number of droplet events generated for a given well or sample and Ch 1 Amplitude measurement refers to the level of fluorescence emitted by a droplet event.

3.4 Results

3.4.1 Abiotic measurements and analysis of winter field samples

During the winter (wet season) sampling event, the surface water temperatures for all regions combined showed a range of 11.3 – 14.8°C (Table 3.1). The surface salinity in MB was low, at 2.61 parts per thousand (ppt) at Site 1 and steadily decreased further north into MB. Site 5 in the Mobile River was the first to show extremely low salinity, at

0.08 ppt. Surface salinity remained extremely low at 0.06 – 0.07 ppt for each site thereafter in both the western and eastern transects (Table 3.1). Dissolved oxygen (DO) in MB and the western transect were all measured to be ~9 mg/L, while the eastern transect appeared to experience slight flux (~6 – 8 mg/L) (Table 3.1). All sites in MB and each transect showed a consistent pH, remaining within the 6 – 8 range (Table 3.1). While water depth was not measured during this sampling event, excessive flood waters were observed throughout the MTD on each transect.

Table 3.1

Winter field sampling average abiotic measurements.

Site Location (Transect)	**Surface Temperature (°C)**	**Surface Salinity (ppt)**	**Dissolved O_2 (mg/L)**	**pH**
Mobile Bay	13.9	1.4	9.7	7
Mobile River (W)	11.8	0.2	9.2	7.8
Tombigbee River (W)	11.5	0.06	9.3	8
Alabama River (E)	11.9	0.06	10.3	7.8
Tensaw River (E)	13.1	0.07	9.1	7.9

Using the developed ddPCR assay with the QuantaSoft™ RED analysis and a 3,000 amplitude MT, the known positive droplet range of target *C. leucas* DNA, and a lower LoD of 0.6 copies/µL, none of the sample reactions from the winter collection season met all three criteria for a strong positive detection. There were, however, six reactions that adhered to at least one of the criteria and were considered potential positives (Table 3.2). Mobile Bay, the western transect, and the eastern transect each showed two instances of potential positives: Mobile Bay showed a potential positive in both the southern and northern regions, the western transect had two potential positives in

the Tombigbee River and the eastern transect showed one potential positive in both the Alabama and Tensaw Rivers (Figure 3.7). Four of the six reactions met the first two criteria (Sites 1, 3, 8, and 20), each containing one droplet above the MT of 3,000 amplitude and within the known positive droplet range for *C. leucas* (Table 3.2), whereas the remaining two reactions each showed one positive droplet above the MT, but both below the known positive droplet range (Sites 11 and 13) (Table 3.2). No sample reactions from this season met the third criterion of an estimated concentration of target DNA equal to or greater than the LoD of 0.6 copies/μL (Table 3.2).

Table 3.2

Winter field samples Droplet Digital™ PCR reaction results.

Sample Name	Site Number	Site Location (Transect)	Reactions with Droplets Above MT	Reactions with Droplets in Positive Range	Reactions with Droplets Above LoD
3CleueDNA001	1	Mobile Bay	1/5	1/5	0/5
3CleueDNA002	2	Mobile Bay	0/5	0/5	0/5
3CleueDNA003	3	Mobile Bay	1/5	1/5	0/5
3CleueDNA004	4	Mobile River (W)	0/5	0/5	0/5
3CleueDNA005	5	Mobile River (W)	0/5	0/5	0/5
3CleueDNA006	6	Mobile River (W)	0/5	0/5	0/5
3CleueDNA007	7	Mobile River (W)	0/5	0/5	0/5
3CleueDNA008	8	Tombigbee River (W)	1/5	1/5	0/5
3CleueDNA009	9	Tombigbee River (W)	0/5	0/5	0/5
3CleueDNA010	10	Tombigbee River (W)	0/5	0/5	0/5
3CleueDNA011	11	Tombigbee River (W)	1/5	0/5	0/5
3CleueDNA012	12	Tombigbee River (W)	0/5	0/5	0/5
3CleueDNA013	13	Alabama River (E)	1/5	0/5	0/5
3CleueDNA014	14	Alabama River (E)	0/5	0/5	0/5
3CleueDNA015	15	Alabama River (E)	0/5	0/5	0/5

Table 3.2 *(continued).*

Sample Name	Site Number	Site Location (Transect)	Reactions with Droplets Above MT	Reactions with Droplets in Positive Range	Reactions with Droplets Above LoD
3CleueDNA016	16	Alabama River (E)	0/5	0/5	0/5
3CleueDNA017	17	Alabama River (E)	0/5	0/5	0/5
3CleueDNA018	18	Tensaw River (E)	0/5	0/5	0/5
3CleueDNA019	19	Tensaw River (E)	0/5	0/5	0/5
3CleueDNA020	20	Tensaw River (E)	1/5	1/5	0/5
3CleueDNA021	21	Tensaw River (E)	0/5	0/5	0/5

Each sample and negative control for the winter field season is listed with the number of reactions that adhered to three criteria that determine a negative, a potential positive, or a strong positive for the target species, Bull Shark (*Carcharhinus leucas*).

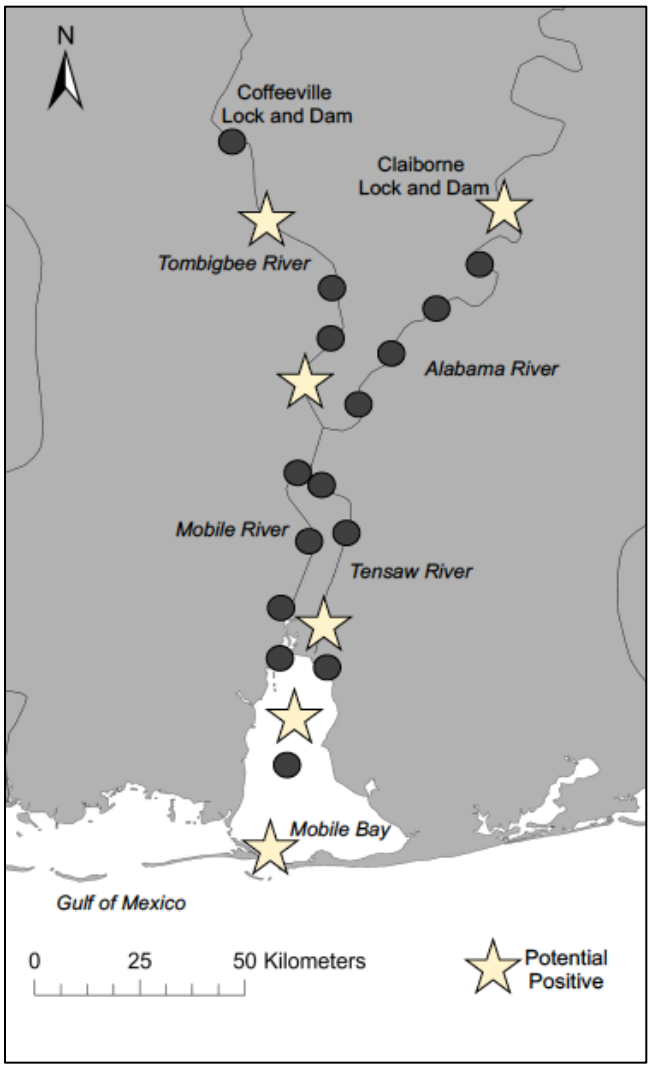

Figure 3.7 *Mobile Bay, Alabama and the Mobile-Tensaw Delta with potential positive Bull Shark detections for the winter field season.*

Mobile Bay and the Mobile-Tensaw Delta showing two instances of potential positive detection in Mobile Bay for the target species, Bull Shark (*Carcharhinus leucas*), two potential positive detections for *C. leucas* in the western transect in the Tombigbee River, and two potential positive detections for *C. leucas* in the eastern transect in each of the Alabama and Tensaw Rivers.

When applying the same three criteria to each filter negative control (FN) reaction for each of the three assay runs performed for the winter season, none of the FN control reactions met all three criteria for a strong positive detection. However, there were three

reactions that adhered to the first criterion (Table 3.3), indicating potential contamination. Potential positive detections were present in two separate FN controls and one of those controls contained two reactions with positive detections, while the other had one reaction with a positive detection (Table 3.3). The filter negative control on 2/19/18 produced two reactions (out of 15 total), each occurring in separate runs, that had positive droplet detections above the MT of 3,000 amplitude, but neither were within the known positive droplet range. The first of these reactions showed the droplet above the known positive droplet range, while the second showed the droplet below the known positive range. The filter negative on 2/20/18 produced one reaction (out of 15 total) that had a positive droplet detection above the MT of 3,000 amplitude but was below the known positive droplet range.

Using the same three detection criteria for each DNA extraction negative control (EN) for each of the three assay runs performed for the winter season, none of the negative control reactions met all three criteria for a strong positive detection (Table 3.3). Three individual EN controls produced droplets above the MT of 3,000 amplitude (Table 3.3), with two of those producing reactions that adhered to the first and second criteria, indicating potential contamination. The DNA extraction negative control on 9/15/18 produced two reactions (out of 15 total), each occurring in separate runs, that had positive droplet detections. The first of these reactions showed the droplet within the known positive droplet range, while the second showed the droplet below the known positive droplet range. The second DNA extraction negative used on 9/20/18 produced one reaction (out of 15 total) that had a positive droplet detection above the MT, but above the known positive droplet range. The third DNA extraction negative on 12/13/18

produced one reaction (out of 15 total) that had a positive droplet detection above the MT of 3,000 amplitude and within the known positive droplet range. The PCR negative controls (PCR N) used for each of the runs performed on the winter samples did not produce any positive droplet detections above the MT.

Table 3.3

Winter field and laboratory negative controls Droplet Digital™ PCR reaction results.

Negative Control	Reactions with Droplets Above MT	Reactions with Droplets in Positive Range	Reactions with Droplets Above LoD
FN 2/19/18	2/15	0/15	0/15
FN 2/20/18	1/15	0/15	0/15
FN 3/15/18	0/15	0/15	0/15
FN 3/23/18	0/15	0/15	0/15
FN 4/9/18	0/15	0/15	0/15
FN 6/22/18	0/15	0/15	0/15
EN 9/15/18	2/15	1/15	0/15
EN 9/20/18	1/15	0/15	0/15
EN 11/12/18	0/15	0/15	0/15
EN 12/13/18	1/15	1/15	0/15
PCR N 2/14/19	0/15	0/15	0/15

Each negative control for the winter season is listed with the number of reactions that adhered to three criteria that determine a negative, a potential positive, or a strong positive detection for the target species.

3.4.2 Abiotic measurements and analysis of summer field samples

During the summer (dry) season sampling event, the surface water temperatures for all regions combined showed a range of 27.6 – 31.1°C (Table 3.4). The salinity in MB was considerably greater than during the winter season. Site 1 surface salinity was

measured to be 23.9 ppt and steadily decreased further north in MB, with an average of 17.2 ppt for Sites 1 – 3 (Table 3.4). Site 6 in the Mobile River was the first to show extremely low salinity at 0.08 ppt. Surface salinity remained low at less than 1 ppt for each site thereafter in both the western and eastern transects. DO in MB and each transect saw a slight flux, showing a range of ~6 – 8.5 mg/L and all sites in MB and each transect showed a consistent pH, remaining within the 7 – 8 range (Table 3.4).

Table 3.4

Summer field sampling average abiotic measurements.

Site Location (Transect)	**Surface Temperature (°C)**	**Surface Salinity (ppt)**	**Dissolved O_2 (mg/L)**	**pH**
Mobile Bay	28	17.2	6.6	7.7
Mobile River (W)	29.7	1.5	6.8	7.8
Tombigbee River (W)	29.9	0.1	7.5	7.5
Alabama River (E)	29.4	0.07	7.6	7
Tensaw River (E)	30.3	0.1	7.3	7.1

Using the developed ddPCR assay with the QuantaSoft™ RED analysis and a 3,000 amplitude MT, the known positive droplet range of target *C. leucas* DNA, and a lower LoD of 0.6 copies/µL, two sites were determined to be potentially positive and two sites were determined to be strongly positive (Table 3.5). Two sites on the eastern transect, adjacent to one another in the Alabama River (Figure 3.8), indicated strong positive detection for target DNA with one or more reactions in each site meeting all three positive detection criteria (Table 3.5). Three reactions in Site 16 adhered to all three positive detection criteria, while two reactions in Site 17 adhered to all three criteria (Table 3.5). Potential positive detections were indicated by two sites on the western

transect (Figure 3.8), with Site 5 in the Mobile River meeting the first two detection criteria by showing a droplet above the 3,000 amplitude MT and within the known positive droplet range and Site 10 in the Tombigbee River meeting the first and third criteria showing a droplet above the MT, but below the known positive droplet range, and an estimated concentration of target DNA greater than the LoD of 0.6 copies/μL (Table 3.5).

Table 3.5

Summer field samples Droplet Digital™ PCR reaction results.

Sample Name	Site Number	Site Location (Transect)	Reactions with Droplets Above MT	Reactions with Droplets in Positive Range	Reactions with Droplets Above LoD
3CleueDNA022	1	Mobile Bay	0/5	0/5	0/5
3CleueDNA023	2	Mobile Bay	0/5	0/5	0/5
3CleueDNA024	3	Mobile Bay	0/5	0/5	0/5
3CleueDNA025	4	Mobile River (W)	0/5	0/5	0/5
3CleueDNA026	5	Mobile River (W)	1/5	1/5	0/5
3CleueDNA027	6	Mobile River (W)	0/5	0/5	0/5
3CleueDNA028	7	Mobile River (W)	0/5	0/5	0/5
3CleueDNA029	8	Tombigbee River (W)	0/5	0/5	0/5
3CleueDNA030	9	Tombigbee River (W)	0/5	0/5	0/5
3CleueDNA031	10	Tombigbee River (W)	1/5	0/5	1/5
3CleueDNA032	11	Tombigbee River (W)	0/5	0/5	0/5
3CleueDNA033	12	Tombigbee River (W)	0/5	0/5	0/5
3CleueDNA034	13	Alabama River (E)	0/5	0/5	0/5
3CleueDNA035	14	Alabama River (E)	0/5	0/5	0/5
3CleueDNA036	15	Alabama River (E)	0/5	0/5	0/5
3CleueDNA037	16	Alabama River (E)	3/5	3/5	3/5

Table 3.5 *(continued).*

Sample Name	Site Number	Site Location (Transect)	Reactions with Droplets Above MT	Reactions with Droplets in Positive Range	Reactions with Droplets Above LoD
3CleueDNA038	17	Alabama River (E)	2/5	2/5	2/5
3CleueDNA039	18	Tensaw River (E)	0/5	0/5	0/5
3CleueDNA040	19	Tensaw River (E)	0/5	0/5	0/5
3CleueDNA041	20	Tensaw River (E)	0/5	0/5	0/5
3CleueDNA042	21	Tensaw River (E)	0/5	0/5	0/5

Each sample and negative control for the summer field season is listed with the number of reactions that adhered to three criteria that determine a negative, a potential positive, or a strong positive for the target species, Bull Shark (*Carcharhinus leucas*).

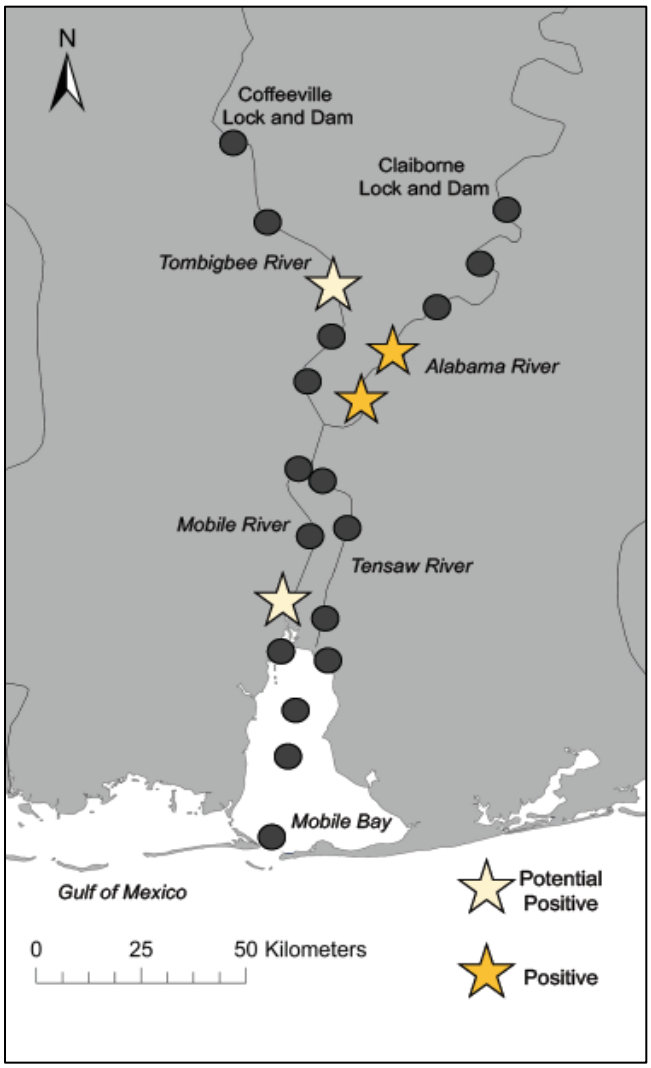

Figure 3.8 *Mobile Bay, Alabama and the Mobile-Tensaw Delta with potential and strong positive Bull Shark detections for the summer field season.*

Mobile Bay and the Mobile-Tensaw Delta showing two instances of potential positive detection for the target species, Bull Shark (*Carcharhinus leucas*), on the western transect in each of the Mobile and Tombigbee Rivers and two strong positive detections for *C. leucas* on the eastern transect in the Alabama River for *C. leucas*.

When applying the three detection criteria to each collection negative control (CN) reaction for each of the five assay runs performed for the summer season, none of the CN control reactions met any of the three criteria for a strong positive detection.

However, when analyzing the FN controls, there were six reactions that adhered to at

least one criterion (Table 3.6), indicating potential contamination. Potential positive detections were present in five separate FN controls and one of those controls contained two reactions with positive detections, while the remaining four FN controls each had one positive detection (Table 3.6). The filter negative control on 8/23/18 produced one reaction (out of 25 total) with a positive droplet detection above the MT and above the known positive droplet range. The filter negative controls on 8/21/18, 9/21/18, and 9/24/18 (each had 25 reactions total) each produced one reaction with a positive droplet detection above the MT of 3,000 amplitude and within the known positive droplet range. The filter negative control on 9/28/18 produced two reactions (out of 25 total), each occurring in separate runs, that had positive droplet detections above the MT of 3,000 amplitude and within the known positive droplet range.

The three detection criteria were applied to each EN control for each of the five assay runs performed for the summer season and none of the negative control reactions met all criteria for a strong positive detection (Table 3.6). One individual EN control contained one reaction (out of 25 total) that produced a positive detection above the MT and within the normal positive droplet range, indicating potential contamination (Table 3.6). While this DNA extraction negative on 12/13/19 did meet the first two detection criteria, it is possible that the detection criteria previously used was not appropriate for this specific run, as the run did not generate the expected quantity of droplets per reaction and the efficiency of the assay was unusually low (see below). The PCR N controls used for each of the five runs performed on the summer samples did not produce any reactions that met all three detection criteria. One PCR N reaction (out of 25 total) did produce a positive detection that met the first two criteria of falling above the MT and within the

normal positive droplet range, but this PCR negative reaction, on 2/13/19, was present on the run that did not generate the expected quantity of droplets per reaction and the assay efficiency was unusually low (see below).

Table 3.6

Summer field and laboratory negative controls Droplet Digital™ PCR reaction results.

Negative Control	Reactions with Droplets Above MT	Reactions with Droplets in Positive Range	Reactions with Droplets Above LoD
CN 8/21/18	0/25	0/25	0/25
CN 8/22/18	0/25	0/25	0/25
FN 8/21/18	1/25	1/25	0/25
FN 8/22/18	0/25	0/25	0/25
FN 8/23/18	1/25	0/25	0/25
FN 9/20/18	0/25	0/25	0/25
FN 9/21/18	1/25	1/25	0/25
FN 9/24/18	1/25	1/25	0/25
FN 9/28/18	2/25	2/25	0/25
EN 1/10/19	0/25	0/25	0/25
EN 1/11/19	0/25	0/25	0/25
EN 1/12/19	1/25	1/25	0/25
PCR N 2/14/19	1/25	1/25	0/25

Each negative control for the summer season is listed with the number of reactions that adhered to three criteria that determine a negative, a potential positive, or a strong positive detection for the target species.

Based on the *C. leucas* eDNA positive reference used for each of the five assay runs to complete this season of samples, one of the ddPCR runs appeared to not perform efficiently (Figure 3.9A) in comparison to other runs (Figure 3.9B). The assay runs that performed at full efficiency contained reactions that each generated ~15,000 droplets and

the positive droplet populations around 5,000 amplitude, whereas the less-efficient run produced a range of ~4,000 – 9,000 droplets per reaction and the positive droplet population lower in amplitude. The subset of samples in the less-efficient assay included Sites 16 – 20 and the five strong positive detection reactions, as well as four negative control potential positive detection reactions (Appendix A, Figure A.7), including two separate FN reactions, one EN reaction, and one PCR N reaction.

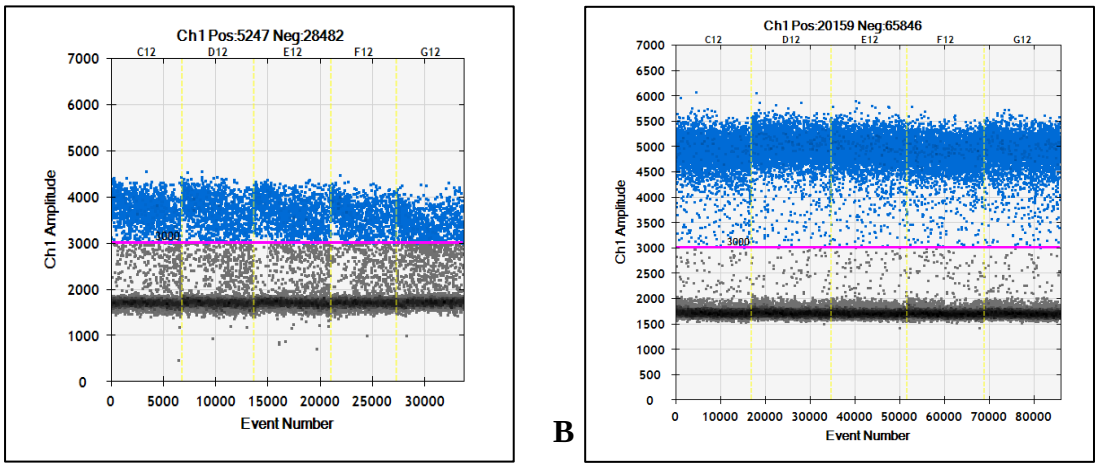

Figure 3.9 *Comparison of two Droplet Digital™ PCR scatter plots of Bull Shark environmental DNA for different assay runs.*

Raw Droplet Digital™ PCR (ddPCR) outputs from one replicate reaction for two different runs of the Bull Shark (*Carcharhinus leucas*) eDNA positive water sample from the Bio-Rad® QX200™ Droplet Reader showing the known positive range for the target species. Each droplet in each well was classified as either positive (blue droplets) or negative (grey droplets) for target DNA based on a manual detection threshold set to 3,000 amplitude (the horizontal pink line) using the QuantaSoft™ Rare Event Detection analysis. Event Number refers to the number of droplet events generated for a given well or sample and Ch 1 Amplitude measurement refers to the level of fluorescence emitted by a droplet event. (**A**) DdPCR assay that shows lesser efficiency, which can be seen by the lower amplitude positive droplet range and the lower number of droplets generated in comparison to B. (**B**) DdPCR assay run that shows full efficiency.

3.5 Discussion

Environmental DNA analysis as an approach to monitor species presence has increased in practice almost exponentially in the last five years, with an ever-growing list of applications (see Goldberg *et al*., 2016). This technique has the potential to fill data gaps that traditional ecological monitoring methods cannot, especially for aquatic species that are elusive or occur in habitats that are logistically difficult to access, as is the case for some elasmobranchs (Sigsgaard *et al*., 2016; Simpfendorfer *et al*., 2016; Bakker *et al*., 2017; Weltz *et al*., 2017). This research used a species-specific ddPCR assay to determine if *C. leucas* was serving as a predatory mobile link between estuarine MB and the freshwater MTD through habitat use and movement. The ability of *C. leucas* to regularly inhabit low salinity estuaries, bays, and freshwater rivers as young-of-the-year and juveniles (Caillouet *et al*., 1969; Montoya and Thorson, 1982; Pillans *et al*., 2005; Heupel *et al*., 2007; Ortega *et al*., 2009), move across habitats with fluctuating abiotic conditions (Simpfendorfer *et al*., 2005; Froeschke *et al*., 2010b; Plumlee *et al*., 2018), and forage on a wide variety of prey (Pillans and Franklin, 2004; Pillans *et al*., 2005; Matich *et al*., 2011; Daly *et al*., 2013; Matich *et al*., 2017; Plumlee *et al*., 2018) means immature *C. leucas* could be serving as predatory mobile links between MB and freshwater rivers in the MTD (Rosenblatt and Heithaus, 2011), similar to other areas where immature *C. leucas* are heavily studied (Simpfendorfer *et al*., 2005; Heupel and Simpfendorfer, 2008; Heupel *et al*., 2010; Tillett *et al*., 2012; Laurrabaqio-A *et al*., 2019). Preliminary results from this research suggest that *C. leucas* demonstrates both temporal and spatial habitat usage patterns in the MTD and MB; strong positive detections of target eDNA were found during the summer field season on the less-

urbanized transect, while no strong positive detections were found during the winter field season in any region sampled.

We have found, when applying the three positive detection criteria to field samples, two adjacent sites in the Alabama River on the eastern transect in the MTD (~70 km north of MB) were strongly positive for the target *C. leucas* during the summer season. The abiotic conditions measured during the summer season coincided with the range reported (pers. comm.) and *C. leucas* presence in other areas of the GoM (Simpfendorfer *et al*., 2005; Shipley, 2005; Heupel and Simpfendorfer, 2008; Froeschke *et al*., 2010a, b; Curtis *et al*., 2011; Matich and Heithaus, 2012; Drymon *et al*., 2014). The lack of strong positive detections in MB and the MTD during the winter season may be indicative of cold-water temperatures forcing *C. leucas* to warmer water (Matich *et al*., 2012). Preferential water temperature for *C. leucas* has often shown to be greater than 20°C to maintain ideal ranges for energetic and physiologic functions (Simpfendorfer *et al*., 2005; Shipley, 2005; Curtis, 2008; Froeschke *et al*., 2010b; Curtis *et al*., 2011; Matich and Heithaus, 2012; Drymon *et al*., 2014; Schlaff *et al*., 2014).

The strong positive detections found during the summer field season were located in the Alabama River on the eastern transect. Drymon *et al.* (2014) found immature *C. leucas* individuals in the northern GoM may show preference for less-urbanized, higher-quality habitat, although this pattern was not fully evaluated due to spatial array limitations (Schweiss *et al*., In press), and similar patterns have been found in Australia (Werry *et al*., 2012). The Alabama and Tensaw Rivers are less-urbanized compared to the Mobile and Tombigbee Rivers, indicating that urbanization and industrialization could be a contributing factor to strong positive detections observed in only the former and no

strong positives observed in the western transect during the summer season. Though with only two replicate sampling events, it is difficult to make statistically-robust conclusions. Nevertheless, investigating this spatial pattern further can assist in identifying critical habitat for *C. leucas* in this region, which will emphasize where potential habitat linkages resulting from this species' movement may be more likely to occur.

Three detection criteria were used to determine negative samples from strongly positive samples. The advantages of using a three-criteria analysis to call a sample strongly positive means a lesser chance of calling false positives. However, the limitation of this approach could also be that these methods are too strict and result in false negatives. If false positives occur and these results were to be communicated to managers, funding bodies, or other researchers, there could be detrimental downstream effects. Not only could funding and resources be illegitimately directed to a certain cause, but the prioritization of research based on weak data can negatively impact other species or environments of conservation concern and result in substantial economic repercussions. On the other hand, if false negatives were to occur and results communicated, management and conservation strategies and specific protections developed for threatened species and/or vital habitat could be prematurely discontinued or the implementation of certain protections and strategies delayed, slowing the overall recovery of species and/or habitat. Additionally, false negatives could lead to inaccurate interpretations of the data, resulting in research questions not being fully explored and potential patterns of certain eDNA presence overlooked. Some samples in this research were classified as potentially positive by meeting only one or two of the detection criteria. Potential positives were detected in each sampling region during the summer and

winter field seasons and could be indicative of *C. leucas* using habitat equally throughout MB and the MTD regardless of season or level of anthropogenic influence; however, discerning a lower LoD than what was used in this study would be necessary to evaluate the validity of these samples categorized as potentially positive, rather than strongly positive for target DNA.

When interpreting positive detections, where "positive" refers to a detection of an organism's DNA, in lotic environments, such as those in Alabama examined during this research, it is imperative that detections are interpreted with the environment in mind. Target eDNA captured in one location within a flowing river does not imply that the target eDNA originated from that location or the target species was ever in that location. EDNA is both transported and further degraded in moving systems (Ficetola *et al*., 2008; Pilliod *et al*., 2013; Strickler *et al.,* 2015); the concentration and distribution of target eDNA when positively identified in a system needs to be taken into consideration when developing an eDNA sampling regime and accounted for in order to accurately and usefully interpret the results. The data obtained in this research is unable to fully answer whether *C. leucas* serves as an ecological mobile link between MB and the MTD due to the limited data set, but it is able to inform how changes can be implemented into the current sampling regime and sample collection in the future and serves as a baseline for others that may be looking to use eDNA detection to answer similar questions.

While conducting ddPCR reactions, the *C. leucas* eDNA positive sample in a closed system obtained in Ch. II was used for each run to ensure the assay proceeded successfully. In molecular biology, it is common practice to include a positive reference sample in a PCR to confirm the reaction proceeds as expected; however, the *C. leucas*

eDNA reference positive contained a high concentration of target DNA (see Ch. II), which may not be ideal to run in conjunction with field samples that are likely to contain low quantities and qualities of target DNA. It is possible that the strongly positive results (in Sites 16 and 17) were products of cross-contamination of the positive reference. Additionally, this same ddPCR run that included strong positive detections did not perform at full efficiency; the normal expected number of droplets per reaction was not generated, the positive droplet population showed a lesser amplitude than expected, and there was an increase in "rain" (*i.e*., droplets that fall between the positive and negative droplet populations). According to the Bio-Rad® ddPCR Applications Manual, there are a number of reasons why these situations could occur including the use of expired droplet generation oil in the Bio-Rad® QX200™ AutoDG™ Droplet Digital™ PCR System, the physical disruption of droplets after PCR and before droplets are analyzed in the Bio-Rad® QX200™ Droplet Reader, particulate matter carried over from sample preparation and left in the samples, or if reagents used for reactions are degraded. The lower efficiency that occurred indicates that the MT and known positive droplet range defined in this study are likely not appropriate for this run; therefore, concentration quantification estimates may be artificially high per reaction and droplets read as positive using the previous 3,000 amplitude MT and the known positive droplet range may be inaccurate. Reperforming this less-efficient ddPCR that produced the strong positive detections while omitting the positive eDNA reference and using new reagents and fresh aliquots could help to clarify if strong positive detections are valid results (Bustin and Mueller, 2005; Kriger *et al*., 2006; Goldberg *et al*., 2016).

Environmental DNA technology has the capability to detect the recent presence of organisms in an area without needing a visual confirmation and offers a vast array of applications, however, special attention to contamination risks should be among the highest of priorities in these studies. Contamination concerns and maintaining clean laboratory practices are vital to eDNA studies that often require repeatable and accurate results in order to make inferences about species detections (Goldberg *et al*, 2016). The results in this study highlight the need to take precautions to avoid contamination by exogenous DNA or cross-contamination between samples and steps of sample processing to avoid generating spurious data. As performed here, water filtering, DNA extractions from filters, and PCR amplifications should be carried out in physically separated lab spaces to prevent cross-contamination between stages and negative controls should be incorporated into each step and PCR-analyzed to check for potential contamination. However, as the results clearly show, contamination can occur even when protocols are followed, demonstrating the sensitivity of targeted genetic assay eDNA detection. Some negative control samples met one or two of the criteria, suggesting there may have been contamination during sample processing. The potential positive detections in each of the winter and summer season FN controls and EN controls may be attributed to handling a large subset of samples at once, where tools could have been reused or mixed up by mistake, gloves mistakenly not replaced between handling sites, or liquid spraying from closing tube caps on extraction tubes kept in close proximity to one another during DNA extractions. The potential positive detection in the PCR N control likely indicates that the ddPCR assay was contaminated with the *C. leucas* positive eDNA reference sample. To reduce the risk of false positives likely resulting from cross-contamination of samples

(Ficetola *et al*., 2016), complete focus and a distraction-free environment is absolutely critical when processing samples. FN control potential positive detections may indicate that water filtration equipment needs to soak in a higher percentage bleach bath (*i.e*., 50%, instead of 10%) (Kemp and Smith, 2005; Champlot *et al*., 2010; Goldberg *et al*., 2016) before being followed by an autoclave cycle or exposure to UV light. To avoid potential positive detections in CN, FN, and EN controls, extraction tubes from different sites and for each negative control should be kept separately from one another, such as a different tube rack for each. Assay runs that show positive detections in any of the negative controls should be interpreted with caution and ideally, rerun without the positive eDNA reference. Following strict field and lab protocols to reduce the likelihood of contamination and cross-contamination will ensure the robustness and reliability of eDNA data obtained. Sound results are absolutely vital when used to inform and create species conservation and management policies (Hunter *et al*., 2017; Hunter *et al*., 2018) regarding issues such as early invasive species detection, assessing community composition and combating biodiversity loss, and estimating species abundance or population characteristics (Barnes and Turner, 2016).

CHAPTER IV – CONCLUSIONS

4.1 Summary

Environmental DNA (eDNA) used for the monitoring of aquatic species is a rapidly evolving field with improved methods for isolation and detection of eDNA presented often. Using a tool as sensitive as eDNA for species detection requires that special attention be given to the effectiveness of methods developed for targeted detection or biodiversity assessments and that precautions are taken to avoid risks of contamination or cross-contamination that could occur in the field and the laboratory. This research aimed to develop methods to isolate eDNA from water samples collected from the northern Gulf of Mexico (GoM), develop a species-specific genetic assay to target Bull Shark, *Carcharhinus leucas,* DNA, and optimize the genetic assay for *C. leucas* detection using the Droplet Digital™ PCR (ddPCR) platform. Using 10X serial dilutions (1:10 – 1:1,000,000) prepared from *C. leucas* genomic DNA (~25 ng/µL), the developed and optimized assay could reliably detect an average of 0.6 copies/µL of target DNA from the 1:10,000 dilutions, indicating that this was the lower limit of detection (LoD) threshold. The developed species-specific assay was then verified for target eDNA by acquiring a live *C. leucas* specimen from Mobile Bay (MB), Alabama, in the northern GoM, and the detectability of target DNA was assessed in both closed and flow-through systems with the target species present using the lower LoD. Water samples from both the closed and flow-through systems suggested that *C. leucas* DNA was detectable within 30 minutes when the target was present in each.

The final aim of this research was to use the developed optimal eDNA methods to assess habitat use of *C. leucas* in MB and the Mobile-Tensaw Delta (MTD); specifically, 1) if there was any use of freshwater rivers in the MTD, 2) if there was seasonal use of these freshwater rivers, and 3) if there was preferential usage of one river over others. When the developed species-specific assay was used to evaluate field samples, there were strong positive detections for target DNA in two separate, but adjacent sites in the Alabama River on the eastern transect during the summer season, while no strong positives were detected at any site during the winter season. These results showing strong positives could be indicative that *C. leucas* individuals are more likely to use less-urbanized, more-pristine habitat in the eastern portion of MB and the MTD, aligning with acoustic monitoring results in Drymon *et al*. (2014). *Carcharhinus leucas* are ecologically vital predators that are dependent upon coastal habitat for maturation, resources, and refuge and are more vulnerable to the impacts of urbanization and industrialization. The lack of strong positives detected during the winter was not unexpected based on unideal water temperatures for *C. leucas* survival (Matich and Heithaus, 2012). Using the three detection criteria, potential positives were detected in both summer and winter seasons, throughout MB and each western and eastern transect. It is possible that these potential positive detections were not considered strong positives because the LoD determined in Schweiss *et al*. (In press; Ch. II) is not the true lowest reliable detection limit and needs further refinement (see below). The results of Ch. III show there is some degree of usage occurring within the MTD, but whether *C. leucas* ecologically links the MTD and MB remains unresolved. The current sampling regime employed one sampling event per season, which may not be enough to understand the

full scope of *C. leucas* prospective habitat usage in MB and the MTD and could result in false negatives. In order to draw more firm conclusions about habitat linkage between the two regions resulting from *C. leucas* habitat use and movement, it would be beneficial for sampling to continue. Ideally, water sampling should occur more than once for each season and for multiple seasons to expand the data set. In addition, using modeling to determine the approximate radius for the presence of organisms by taking into consideration how the degradation and transport of eDNA in lotic systems can impact detectability will be fundamental for accurate interpretation of those results. Nevertheless, using targeted eDNA detections of *C. leucas* in this region can help lay the groundwork regarding the extent of their freshwater usage.

Although methods and precautions used during eDNA studies seem straightforward, contamination control is of the upmost importance when performing eDNA analysis and may occur despite stringent controls and following clean laboratory protocols, as shown in this research. There was potential contamination present in each summer and winter data set in Ch. III, which will require further investigation by running the assays without the *C. leucas* positive eDNA reference sample obtained in Ch. II. The potential contamination observed reiterates the need to employ negative controls at each step of sample collection, processing, and analysis to ensure the validity of results, especially when they are used to advise conservation and management strategies. Applying eDNA technology as a tool for elasmobranch detection offers an alternative to often invasive and involved traditional monitoring techniques. Effective management strategies for elasmobranchs and other elusive species relies on strong monitoring data and while eDNA detection continues to evolve with improved techniques and

applications, the implementation of this technology today can complement traditional methods for species management and conservation.

4.2 Methodological Considerations and Future Directions

While the practice of using eDNA for species detection has been shown to perform equal to, and in some cases better than, traditional monitoring methods (Thomsen *et al*., 2016; Bakker, 2018), the technique is not without concerns. It is probable that the LoD determined in Schweiss *et al*. (In press; Ch. II) is not the true lowest concentration threshold for detection using the developed assay and should be further refined for future analysis. The present LoD was determined by using *C. leucas* genomic DNA 10X serial dilutions and found to be 0.6 copies/μL in the 1:10,000 dilutions with no detection in the following dilutions of 1:100,000. In order to use the three detection criteria as a reliable and useful analysis method for low-quality and low-quantity DNA (Goldberg *et al*., 2016), it is recommended that the assay be tested with an additional serial dilution series, using more than three technical replicates, between these two extremes to determine if a LoD lower than 0.6 copies/μL can reliably be achieved and repeated. While the LoD found in Ch. II is similar to those found in other eDNA studies (Baker *et al*., 2018), if a lower LoD is reliably determined using the *C. leucas* specific assay and an additional dilution series, it should be implemented into the positive detection criteria used in Ch. III and data should be reanalyzed using the updated criteria. For example, it is possible that some samples that were analyzed as potentially positive due to meeting only some of the criteria could actually have target *C. leucas* DNA present. If potential positive detections from samples are actually strong positive detections, it could demonstrate habitat linkage occurring across each region sampled as

each region showed potential positives. It could also indicate that perhaps no preferential habitat selection is occurring among *C. leucas* in MB and the MTD and less-urbanized and more-urbanized habitats are used interchangeably.

The preliminary field data from this research indicates there were some unresolved issues with the analysis criteria to determine a positive detection and potential contamination to be considered; therefore, robust conclusions about linkage across freshwater and estuarine interfaces in the northern GoM resulting from *C. leucas* habitat use and movement were unable to be made. In order to address this and related questions further, reconsidering the effectiveness of the current sampling regime and sample collection and filtration methods may be beneficial for future detectability. Because the sampling region being investigated was a large area with high inflows, future sampling events should take replicate samples at each site, while decreasing the amount per sample to increase the overall volume collected, which will consequently increase the likelihood of detecting minute quantities of target DNA, if present. Additionally, to address the matter of *C. leucas* habitat usage of one location in MB and the MTD compared to other locations, continuous sampling northward to each dam site in both the western and eastern transects may be unnecessary at this stage. To decrease the amount of time spent in the field and the duration of time samples wait to be filtered, future events could begin in the same location in MB, and continue north into the Mobile River on the western transect; however, rather than traveling further north into the Tombigbee River, the brief connection of the transects could be used to loop around and continue south through the Tensaw River in the eastern transect back towards MB. This change in the sampling regime could also allow for more sites to be visited within each river sampled. In terms of

sample collection, there are numerous techniques described in the literature for capture and isolation of total eDNA (see Rees *et al*., 2016 and Goldberg *et al*., 2016). During the sampling events in 2018, collections were made a few centimeters from below the surface of the water due to ease and following suit for other elasmobranch eDNA studies. The literature has shown that eDNA is found in higher concentrations in the benthic sediments than suspended in the water column (Turner *et al*., 2015). Although *C. leucas* is not a benthic elasmobranch, future sampling for targeted detections of *C. leucas* may benefit from collecting samples from the entire depth of the water column, from surface to bottom, as shed eDNA is documented to settle (Turner *et al*., 2015). During method development, three filter pore sizes were tested and the smallest pore size that filtered samples in the timeliest manner was used. However, when field samples were filtered using this previously established size, filtration was slower than what was observed during the development stages, perhaps due to the dynamic nature of river water and/or intense flooding observed during the winter season. To remedy this, larger pore sizes could be tested, in similar fashion to Ch. II, which would allow for larger volumes of water to be filtered, or different brands of filters using the same pore size could be tested to gauge if there are differences in filtration rates between commercially available brands. Lastly, this research also suggested possible contamination was detected and could imply that the controls alone are not enough and should continuously be tested through negatives, continuously be evaluated, and improved upon when necessary for future analyses. Implementing and taking into consideration these suggested changes for future research concerning habitat connectivity of estuarine MB and the freshwater MTD through *C. leucas* movement and usage could aid in increasing reliable positive

detections of the target *C. leucas* when present and may increase our understanding of certain habitat preferences previously observed.

APPENDIX A – FIELD SAMPLE DROPLET DIGITAL™ PCR PLATES

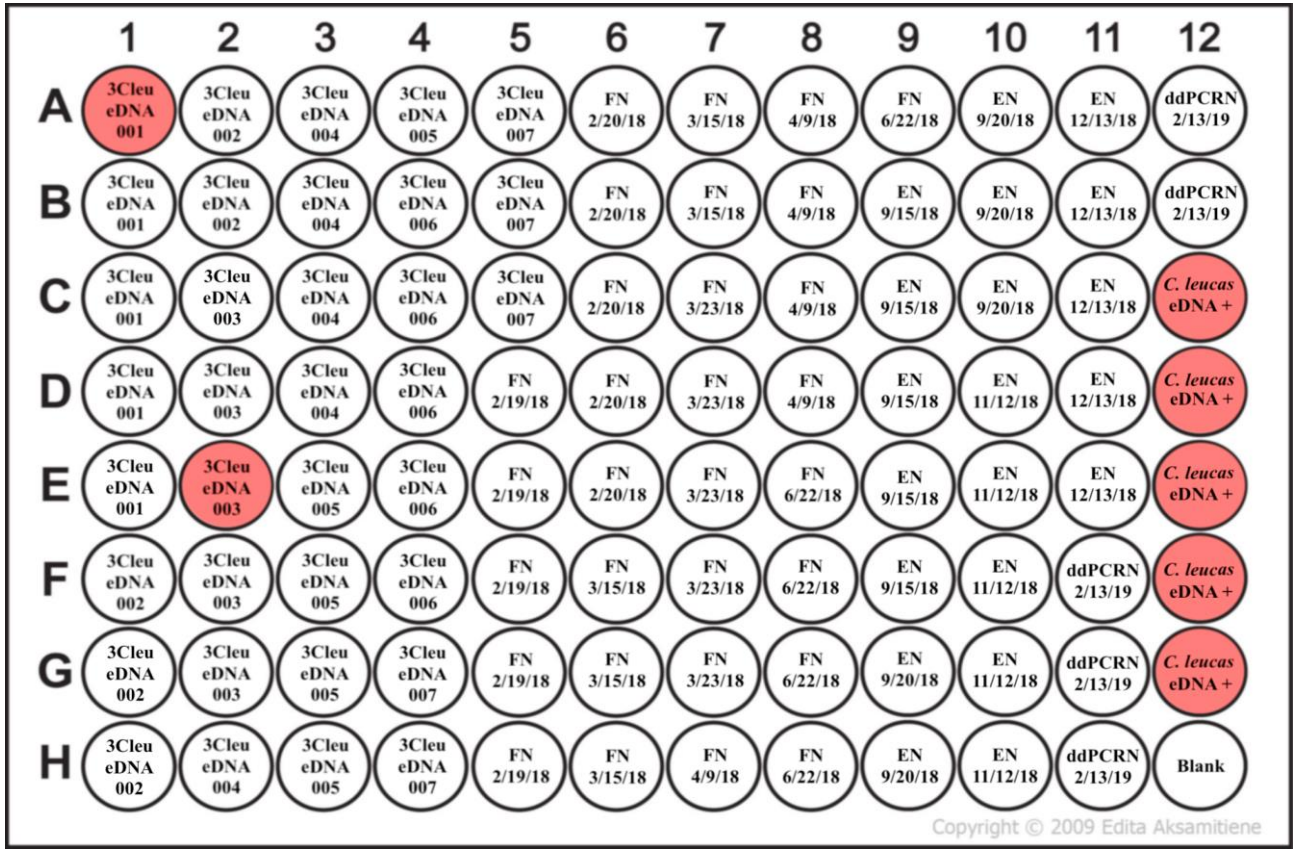

Figure A.1 *Sites 1 – 7 winter season samples and negative controls reaction wells (red wells contained droplet/s above the manual threshold of 3,000 amplitude using the Rare Event Detection analysis).*

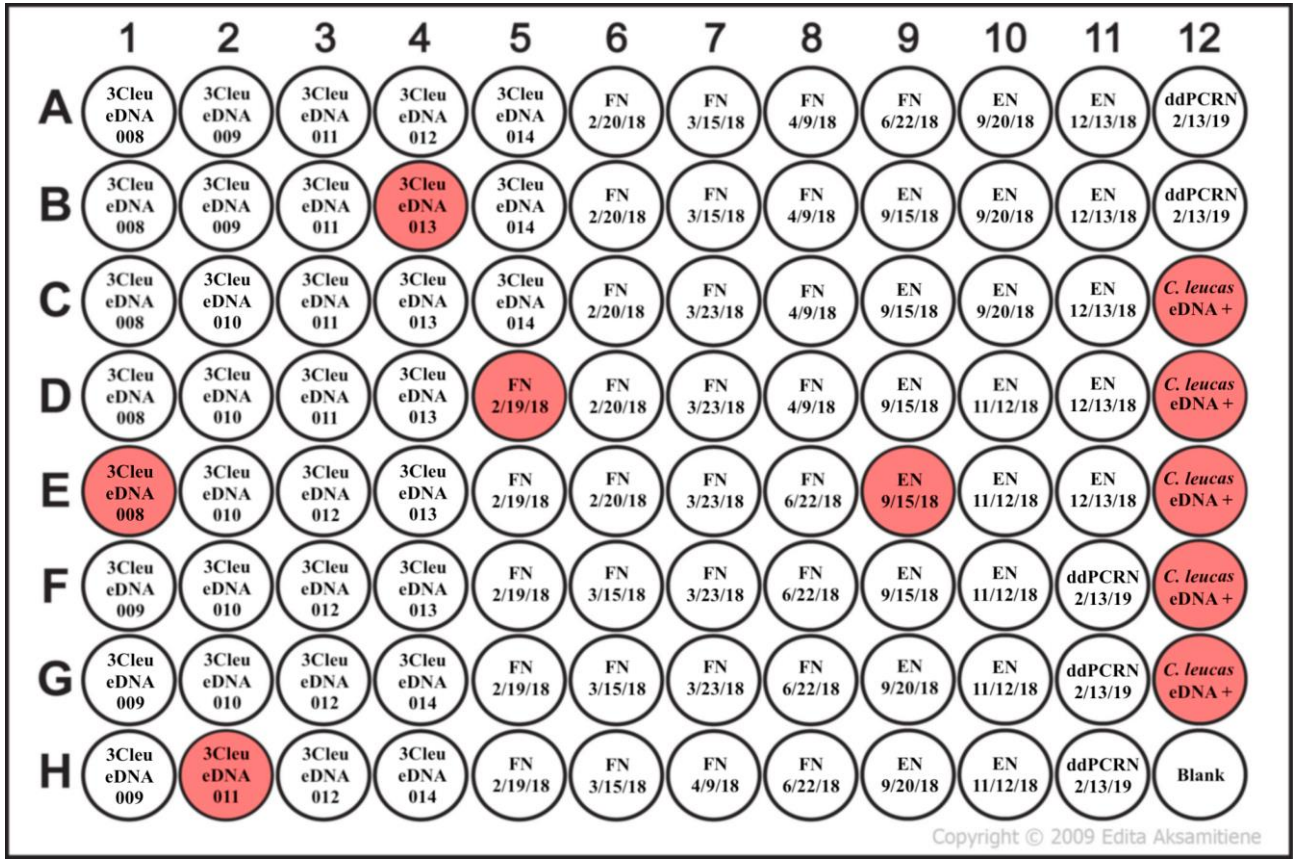

Figure A.2 *Sites 8 – 14 winter season samples and negative controls reaction wells (red wells contained droplet/s above the manual threshold of 3,000 amplitude using the Rare Event Detection analysis).*

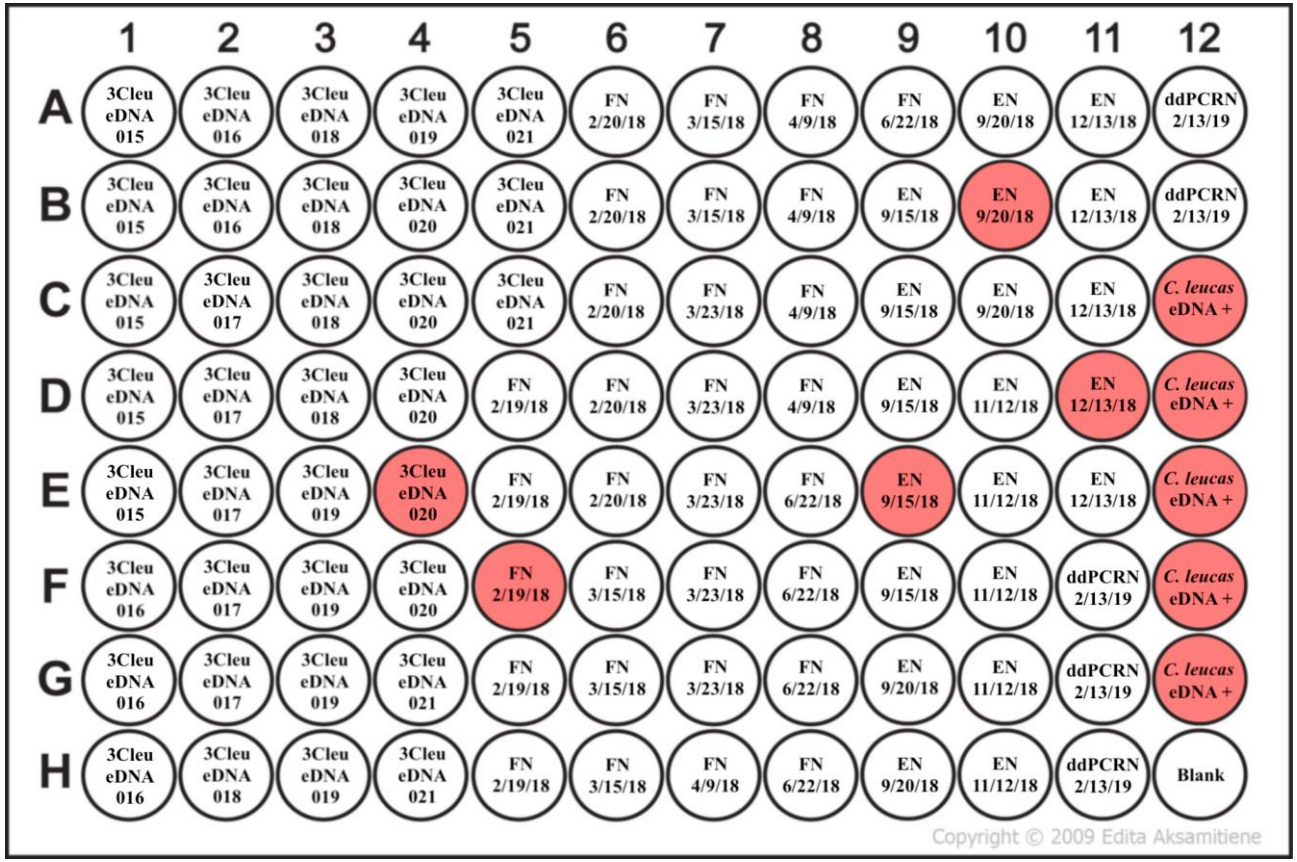

Figure A.3 *Sites 15 – 21 winter season samples and negative controls reaction wells (red wells contained droplet/s above the manual threshold of 3,000 amplitude using the Rare Event Detection analysis).*

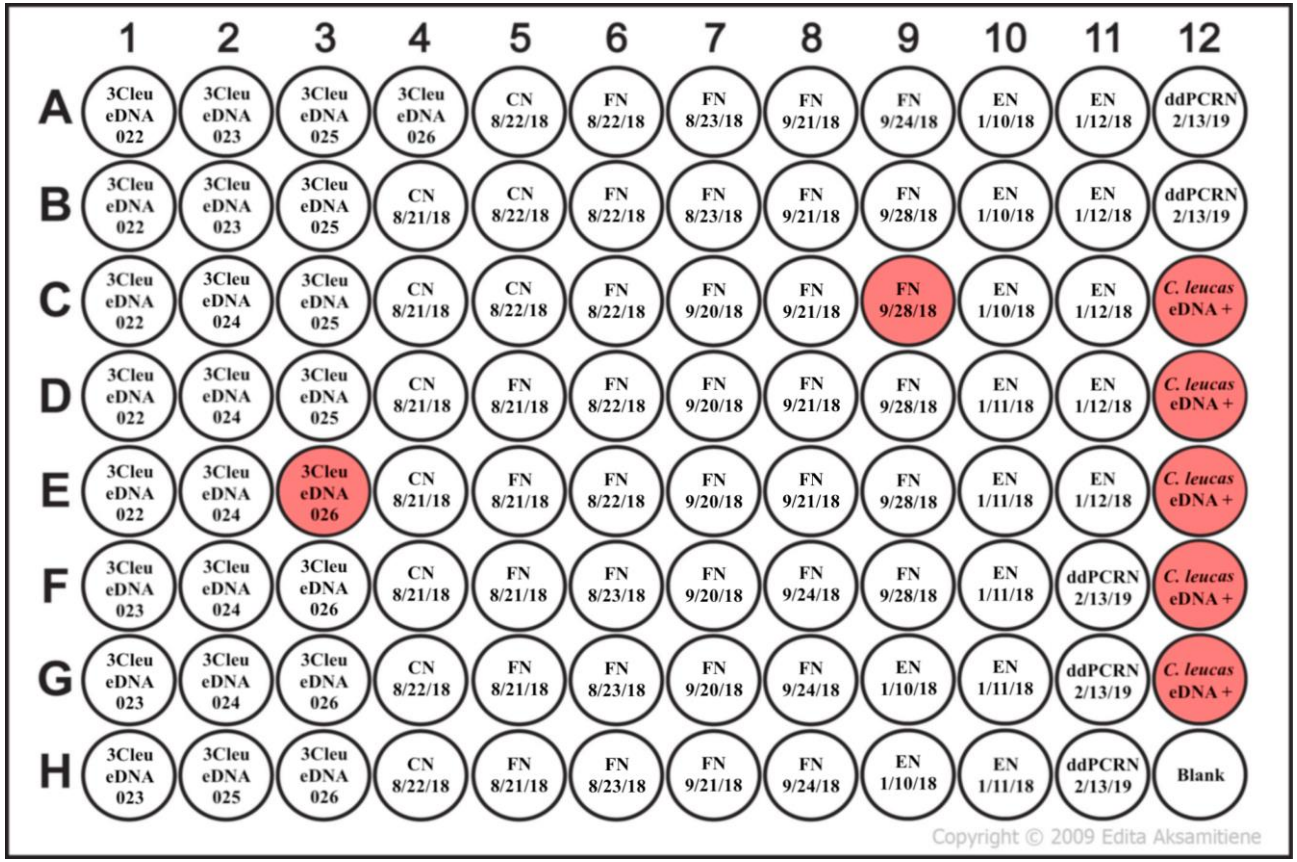

Figure A.4 *Sites 1 – 5 summer season samples and negative controls reaction wells (red wells contained droplet/s above the manual threshold of 3,000 amplitude using the Rare Event Detection analysis).*

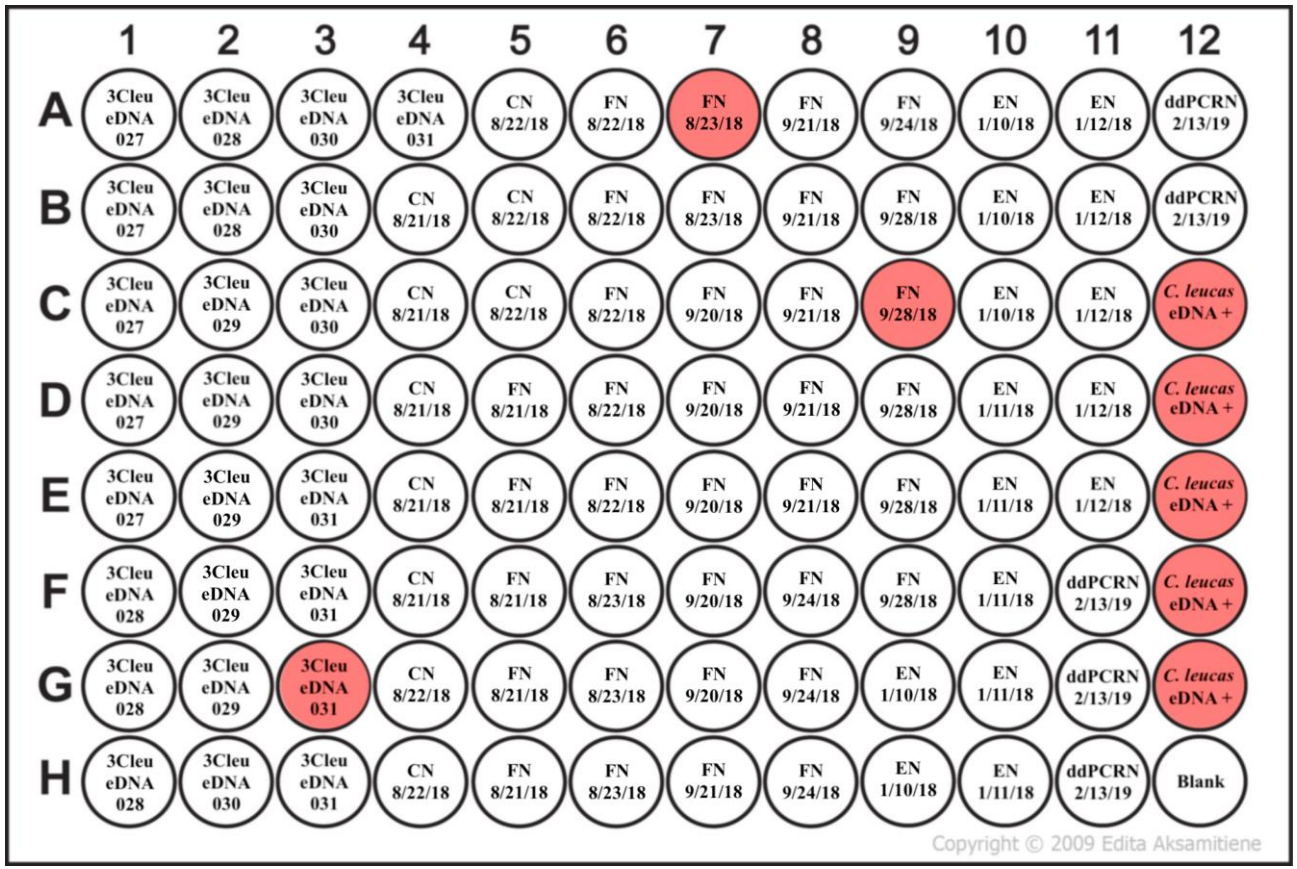

Figure A.5 *Sites 6 – 10 summer season samples and negative controls reaction wells (red wells contained droplet/s above the manual threshold of 3,000 amplitude using the Rare Event Detection analysis).*

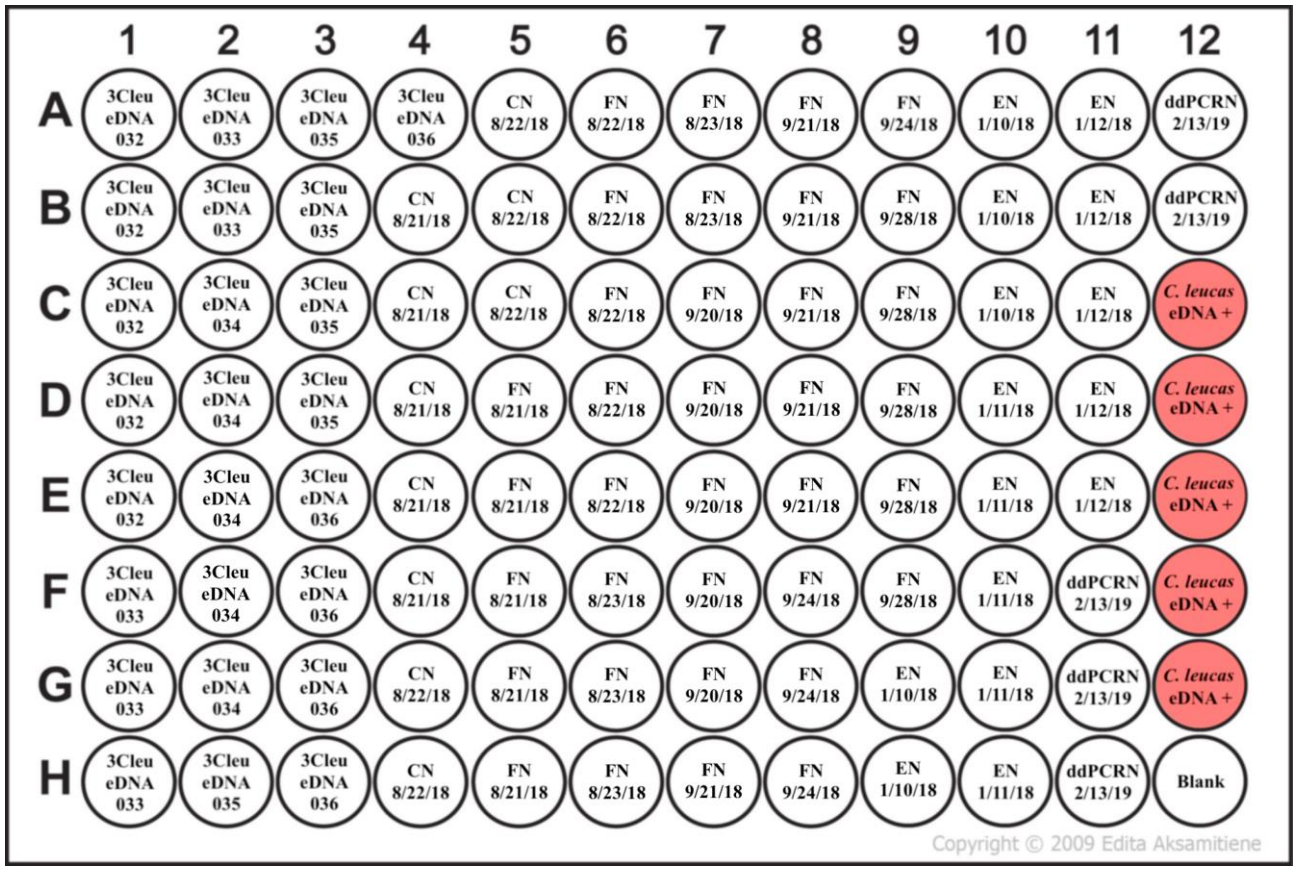

Figure A.6 *Sites 11 – 15 summer season samples and negative controls reaction wells (red wells contained droplet/s above the manual threshold of 3,000 amplitude using the Rare Event Detection analysis).*

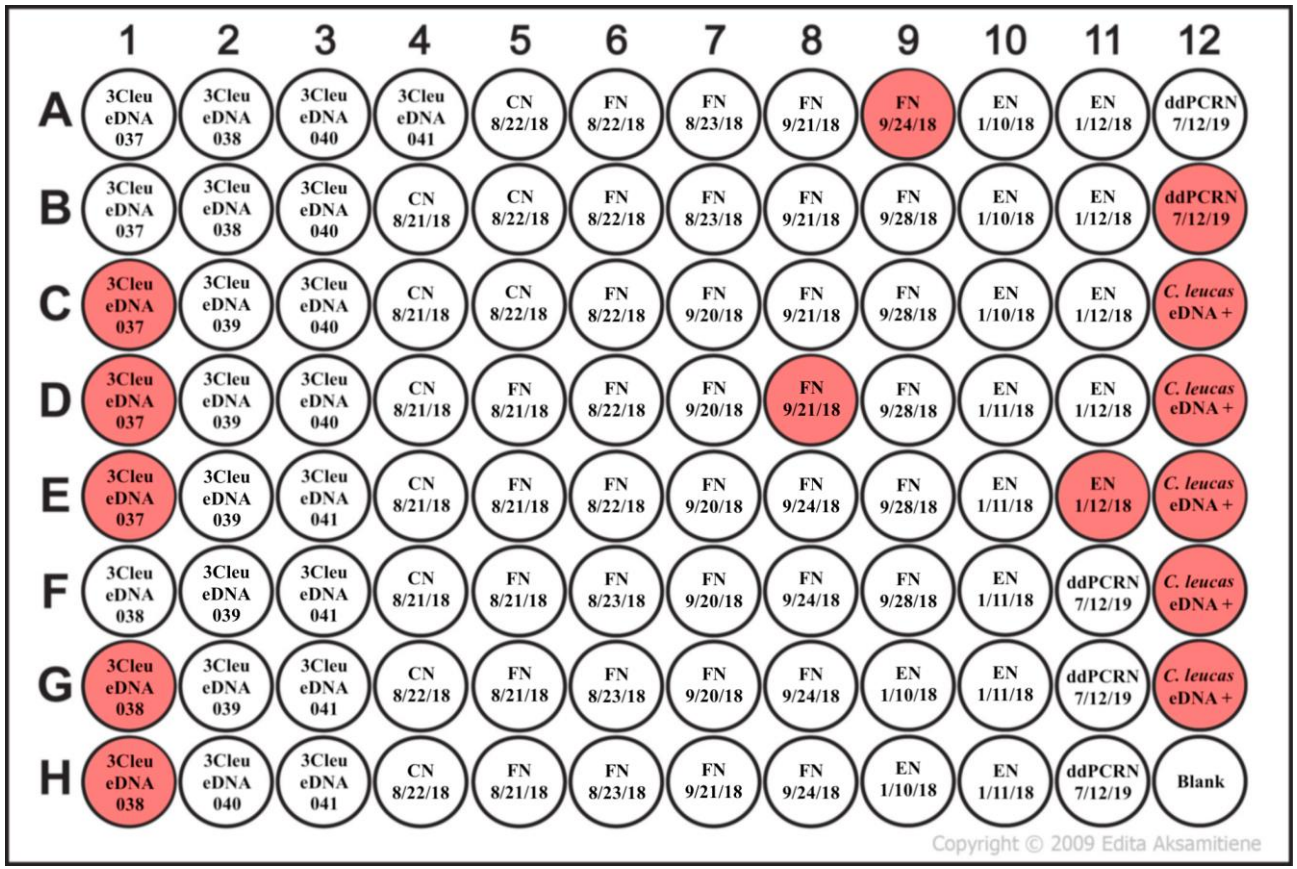

Figure A.7 *Sites 16 – 20 summer season samples and negative controls reaction wells (red wells contained droplet/s above the manual threshold of 3,000 amplitude using the Rare Event Detection analysis).*

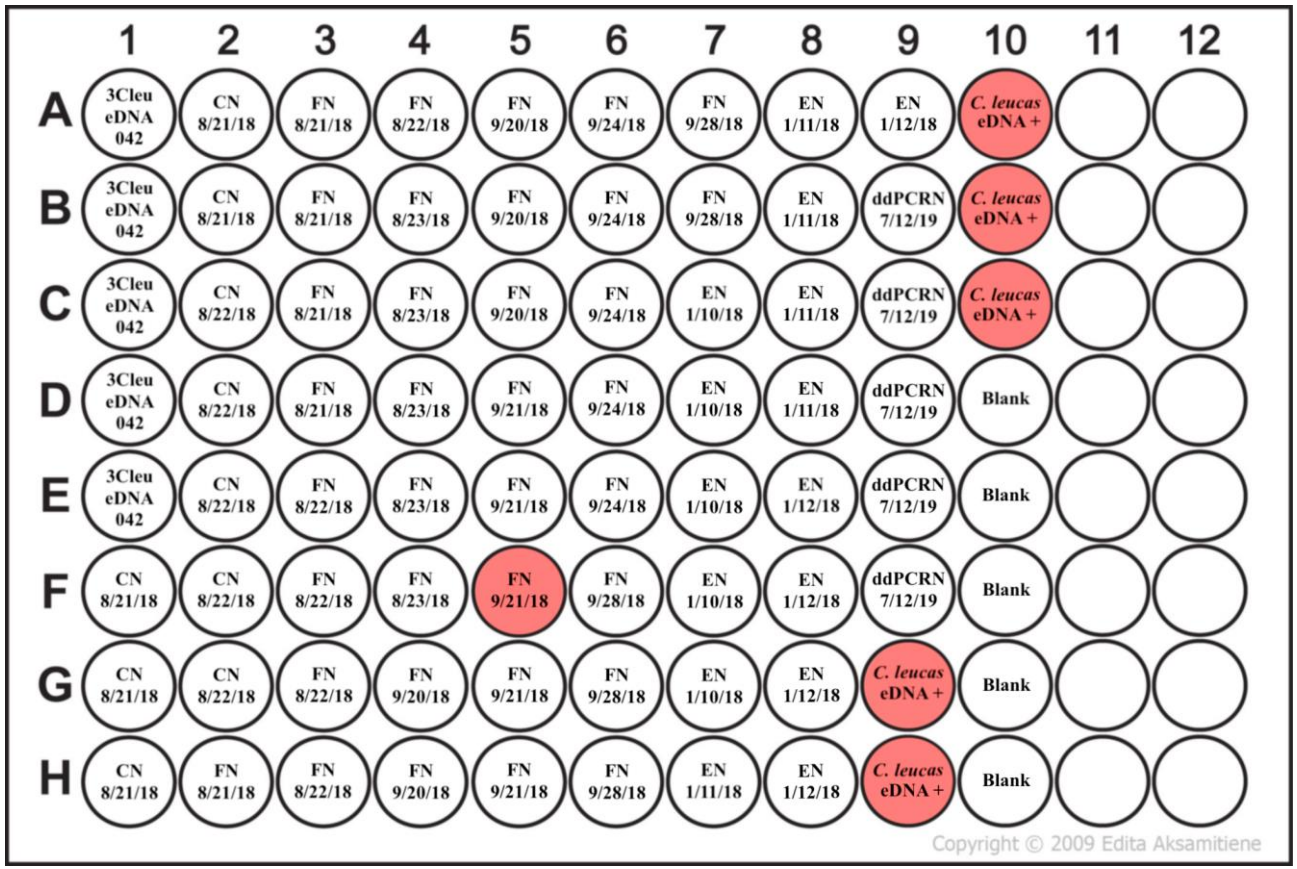

Figure A.8 *Site 21 summer season sample and negative controls reaction wells (red wells contained droplet/s above the manual threshold of 3,000 amplitude using the Rare Event Detection analysis).*

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