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Microbial Community Richness Distinguishes Shark Species Microbiomes in South Florida

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Microbial Community Richness Distinguishes Shark Species Microbiomes in South Florida

By

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ABSTRACT

The microbiome (microbial community) of individuals is crucial when characterizing and understanding processes that are required for organism function and survival. Microbial organisms, which make up an individual's microbiome, can be linked to disease or function of the host organism. In humans, individuals differ substantially in their microbiome compositions in various areas of the body. The cause of much of the composition diversity is yet unexplained, however, it is speculated that habitat, diet, and early exposure to microbes could be altering the microbiomes of individuals (Human Microbiome Project Consortium, 2012b, 2012a). To date, only one study has reported on microbiome characterization in a shark (Doane et al., 2017; skin microbiome of the common thresher shark). A comparative characterization of microbiomes sampled from different shark species and anatomical locations will allow an understanding of the differences in microbiomes that may be explained by variance in shark habitat and diet. Florida leads as shark bite capitol of the world, with 778 unprovoked bites recorded since 1837, or 4-5 average bites per year. With only a few bites a year, there is not a lot of opportunities to study these bites. What can be studied, however, is how the microbial environment in shark's teeth is composed. To understand overall microbiome composition, and if microbiomes are distinct from the environment, or specific by species or anatomical location (henceforth location), we characterized microbiomes from the teeth, gill, skin, and cloacal microbiomes of 8 shark species in south Florida (nurse, lemon, sandbar, Caribbean reef, Atlantic sharpnose, blacktip, bull, and tiger) using high throughput DNA sequencing of the 16S rRNA gene V4 region. There was a significant difference in microbial community richness among species, sample location, but not the interaction between species and location. Microbial diversity by location was significantly different for both the Shannon index and Inverse Simpson index. Samples examined by species had no significant difference in microbial community diversity overall for both Shannon and Inverse Simpson indexes. Microbial community diversity of samples by location and species combined significantly differed when submitted to an analysis of variance with the Shannon index, but not the Inverse Simpson index. Teeth microbial communities showed the most diversity based on both Shannon and Inverse

Simpson indices. Teeth microbiomes are distinct but also share taxa with the water they inhabit, including potentially pathogenic genera such as *Streptococcus (*8.0% ± 9.0%) and *Haemophilus* (2.9% ± 3.3%) in the Caribbean reef shark. The lemon shark teeth hosted *Vibrio* (10.8% ± 26.0%) and the *Corynebacterium* genus (1.6%±5.1%). The *Vibrio* genus (2.8% ± 6.34%), *Salmonella enterica* (2.6% ± 6.4%), and the genus *Kordia* (3.1% ± 6.0%) are found in the nurse shark teeth microbial community. Strikingly, the *Vibrio* genus was represented in the sandbar shark (54.0% ± 46.0%) and tiger shark (5.8% ±12.3%) teeth microbiomes. One OTU related to traditionally non-pathogenic family *Phyllobacteriaceae* appear to be driving up to 32% of variance in teeth microbiome diversity. We conclude that south Florida sharks host distinct microbiomes from the surrounding environment and vary among species due to differences in microbial community richness. Future work should focus on bacteria found in shark teeth to determine if those present are pathogenic and could provide insights to bite treatment.

Keywords: Elasmobranch, Microbiology, Microbiome, Microbial Community, Ecology, Composition, Diversity, Alpha Diversity, Beta Diversity, Comparative

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CHAPTER 1:

INTRODUCTION

SIGNIFICANCE OF SHARKS

BACKGROUND

The oceans provide home to more than 400 species of shark, many of which have declining populations due to bycatch and overfishing (Gibson and Carter, 2002). Pelagic species of sharks are dwindling the most rapidly, due to fishing malpractice, particularly in longline fisheries (Baum and Myers, 2004; Gallagher *et al.*, 2014; Oliver *et al.*, 2015). Sharks represent apex predators, which often can have a large effect on the structure of marine ecosystems. This includes top-down control on food webs and effects on prey species by behaviorally mediated indirect interactions (BMIIs) which can alter the relationship between predator and prey (Heithaus *et al.*, 2008; Whitney *et al.*, 2016).

Over the 350 million years that sharks have inhabited the Earth, very little has changed about them; yet they are able to inhabit and thrive in every ocean and sea on the planet (Gibson and Carter, 2002).What has changed over these 350 million years, however, is the amount of human interaction with sharks. Interference with sharks and their environment by humans has been shown to increase instances of shark bites, with bites in Recife, Brazil occurring at a higher frequency in months where ships entering the harbor is greater than 30 (Hazin *et al.*, 2008).

Additionally, the increase of shark bites in Recife, Brazil coincided directly with the construction of a nearby commercial port which introduced pollutants into the environment, potentially causing more interactions between sharks and beach-goers (Hazin and Afonso, 2014). Even with extensive human interference in sharks' habitat and lifestyle, these creatures have endured in a variety of ecosystems and conditions. Such durability suggests a unique and superiorly adapted immune system of the apex predator, but it also suggests a constantly evolving microbiome in order to adjust to the changes in their environment (Criscitiello, 2014).

Although sharks are found in virtually all areas of the ocean, they can be difficult to study in the field because of their somewhat elusive nature, in some cases, as well as the sheer size of the organism in other instances. Additionally, sharks do not have as

much of a commercial value as other fishes, with the exception of finning, and so often are not as economically interesting, meaning funding is less available for research on the group compared to bony fish, with a sustainable commercial value (Castro, 2010). Characterizing the microbiome of sharks can provide valuable insight into the lifestyle of sharks, which otherwise may be difficult to understand, and could reveal many interesting details about an individual (gender, habitat, feeding habits, etc.), as well as serve as a unique identifier which could potentially applied to shark bites (Kupferschmidt, 2016).

Shark bites are rare, with 98 unprovoked bites occurring in 2015, with only 6 of these bites being fatal (George H. Burgess, 2016a). From 2006 to 2010, there were only 3 fatal bites out of a total of 179 (Oceana, 2011). Although they are not often fatal, shark bites exhibit a constantly growing risk of bacterial infection with no specific and targeted treatment, as the microbiome of shark teeth and other oral areas have not been characterized (Fleshler, 2013). Because of this lack of knowledge, shark bites are not immediately treated with antibiotics, but instead the main priority is to stop the bleeding and treat shock (Hughes, 2014). In transport to the closest hospital equipped to treat a shark bite, the bacteria could have ample time to colonize and potentially infect the victim.

The ocean itself is home to many bacteria which can cause severe infection within hours of exposure, such as *Vibrio* and *Aeromonas* (Lupkin, 2014). Only once the victim is put under with anesthesiology is the wound cleaned; sometimes with a 'shotgun' method where a general antibiotic is used (Fleshler, 2013), and other times only with sterile water (Lupkin, 2014). In addition, there is a continuous increase of drug resistance in bacteria found in the ocean and its inhabitants. Drug resistant strains of *Staphylococcus* and *E. coli* have been found in shark cloaca off the coast of Massachusetts, further showing that the threat of drug resistance is impending. However, it is unclear whether the cloacal samples harbor a higher incidence of drug resistant virulent strains of *Staphylococcus* and *E. coli* when compared to the surrounding ambient environment (Blackburn *et al.*, 2010) . If potentially harmful and drug resistant bacteria are also characterized in shark mouths, these bacteria could explain high infection rates in shark bites, and offer insight towards a more efficient and effective treatment of bite wounds.

SPECIES IN THIS STUDY

NURSE SHARK

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The Nurse shark (*Ginglymostoma cirratum*) is the only representative of the *Ginglymostoma* genus found in the western Atlantic. These sharks reach a maximum size of around 14 ft., with 7.5 ft. being the average for maturity. Nurse sharks are nocturnal, and are typically bottom feeders that are sluggish in nature (Parsons, 2006). They tend to behave in a defensive manner, having exhibited both "hiding behavior" and "substrate resemblance", in addition to having preference for shelters such as holes and crevices (Garla *et al.*, 2015).

Nurse sharks prefer shallow temperate or tropical waters, and are typically found on hard bottoms where the temperature, dissolved oxygen, salinity, and water clarity are high, but can also be found at depths of up to 230 ft. Nurse sharks are able to remain on the bottom for long periods of time because they utilize buccal pumping to push water over their gills (Gibson and Carter, 2002; Hannan *et al.*, 2012). There are also records of Nurse sharks being found in the Mississippi Sound, which has a high input of fresh river and estuarine water, showing how versatile the species can be (Hendon *et al.*, 2013). As bottom feeders, Nurse sharks feed on a variety of different prey, including but not limited to shrimps, crabs, lobsters, squids, fishes, snails, and octopuses (Parsons, 2006). Nurse sharks are considered crucial in shark research, as they are abundant, hearty, and useful when examining immunological characteristics (Castro, 2000).

LEMON SHARK

Lemon sharks (*Negaprion brevirostris*) are migratory viviparous sharks, which means that they give birth to live offspring which are nourished *in utero* (Beck, 2016).

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Lemon sharks tend to favor inshore and coastal waters, and have adapted to be tolerant of low oxygen environments, and can enter freshwater (Ebert *et al.*, 2015; Stafford-Deitsch, 2000). Additionally, Lemon sharks are known to inhabit depths of 300 ft. or deeper during migration, but tend to move to shallower waters for birthing (Beck, 2016). Lemon sharks have the ability to supplement ram ventilation (having to remain in motion to push water over the gills) with buccal pumping, which is a process which allows the shark to remain still on the ocean bottom (or otherwise) while pumping water over their gills to breathe (Brooks *et al.*, 2011).

The Lemon shark mostly feeds on bony fish, but will also prey upon rays or crayfish and are known to be opportunistic feeders, particularly as juveniles (Beck, 2016; Stafford-Deitsch, 2000). Lemon sharks can reach a length of 12 feet, with maturity between 7.4 and 8 ft. (Parsons, 2006). The lifespan of Lemon sharks was increased from the previously accepted 20 years to 37 years based on recent research (Brooks *et al.*, 2016). These sharks are slow growing, and have a low fecundity with females producing 4-18 pups every other year after maturity. Based on this, the IUCN (International Union for the Conservation of Nature) has listed them as a near-threatened species (Reyier *et al.*, 2014).

SANDBAR SHARK

Sandbar sharks (*Carcharhinus plumbeus*) are a coastal species with a wide range in tropical and temperate regions (McElroy *et al.*, 2006). This species can be found at depths of 900 ft., but are found typically at 300 ft. or less where they forage sea beds for prey (IUCN, 2007). Sandbars tend to feed on teleosts, with occasional cephalopods and crustaceans, with more crustaceans taking up a larger portion of younger sharks' diets (McElroy *et al.*, 2006). This change in diet with age points to a shift in feeding preference with maturity from benthic to pelagic (Harrison, 2015). Total length of Sandbar sharks varies with gender, with females growing up to 8.5 feet and males up to 6 feet (Baremore and Hale, 2012).

https://upload.wikimedia.org/wikipedia/commons/f/f7/Sandbar_shark_newport.jpg

Age ranges for the Sandbar shark are accepted to be from 12-30 years (Romine *et al.*, 2006). Sandbar sharks are a migratory species, with seasonal migrations from north to south on the eastern coast of the United States (Romine *et al.*, 2006). It is known that the Chesapeake Bay serves as a large nursery for Sandbar sharks, where females birth an average of 8 pups, either biennially or triennially (Baremore and Hale, 2012). Due to overfishing and lack of stock assessment, the IUCN has the Sandbar shark listed as vulnerable (IUCN, 2007).

TIGER SHARK

https://upload.wikimedia.org/wikipedia/commons/3/39/Tiger_shark.jpg

Tiger sharks (*Galeocerdo cuvier*) can reach lengths of more than 18 feet, and have been documented as deep as 900 ft. of water. Little is known with certainty about the depth range of tiger sharks, but the species has also been encountered in very shallow water (IUCN, 2005b). Tiger sharks grow relatively rapidly compared to other shark species, and are estimated to live to be around 45-50 years (Meyer *et al.*, 2014).

This species of sharks is known to consume garbage of human origin, including plastics, metals, and scraps. The tiger shark's normal diet is also quite diverse, with known prey including teleosts, rays, other sharks, turtles, birds, dolphins, seals, cephalopods, sea snakes, lobsters, crabs, gastropods, and jellyfish. Tiger sharks will also feed on carrion and is not known to shy away from baited hooks (Randall and Randall, 1992). Smaller and younger tiger sharks appear to be mostly nocturnal and bottom feeders. Larger and more mature tiger sharks feed near the bottom nocturnally, but have also been known to surface feed during the daytime. It appears that tiger sharks are very opportunistic feeders that prey upon what is readily available and accessible (Lowe *et al.*, 1996). Tiger sharks have the ability to switch between buccal pumping and ram ventilation as needed, which allows for change in swimming speed (Dapp *et al.*, 2016).

CARIBBEAN REEF SHARK

The Caribbean reef shark (*Carcharhinus perezi*) is classified as Near Threatened on the IUCN red list, due to the large loss of individuals and populations to bycatch and the high gestation period of about one year, which limits the species ability to bounce back after loss (IUCN, 2006). Caribbean reef sharks range in length up to and including

10 feet and are found to be abundant in the Caribbean and also on coral reefs, in a depth range of 150-900 ft. (Brooks *et al.*, 2012).

https://upload.wikimedia.org/wikipedia/commons/5/59/Caribbean_reef_shark.jpg

The diet of the Caribbean reef shark consists of bony fish and other elasmobranchs, as well as occasional cephalopods. It is unclear if this species moves long distances regularly or not, and at what depth they spend most of their time (IUCN, 2006). However, the Caribbean Reef shark has both a wide vertical and temperature range which allows for them to inhabit both shallow and deeper reef ecosystems (Chapman *et al.*, 2007)

BLACKTIP SHARK

Blacktip sharks (*Carcharhinus limbatus*) can reach a total length of 9 feet and reproduce biennially, with a litter size of 4-7 (Johnson *et al.*, 2017). The blacktip shark mates predominantly in Bulls Bay, South Carolina, in the summer months and give birth in the shallow coast waters of the Carolinas about one year later. Nurseries are in both Georgia and the Carolinas in coastal areas (Castro, 1996). This species is found in tropical and warmer temperate waters, and tends to stay close to shore (Kajiura and Tellman, 2016).

The feeding behaviors of this species include feeding primarily upon teleosts, and also crustaceans, cephalopods, and other elasmobranchs (Kajiura and Tellman, 2016). Blacktip sharks are migratory, and aggregate in Southeast Florida to overwinter in the waters near the shore. Their migratory path is thought to be dominated by water temperature, and tends to coincide with the spawning of bait fish species. This aggregation tends to disperse by late spring (April-May) and peaks again in the following January (Kajiura and Tellman, 2016).

ATLANTIC SHARPNOSE SHARK

As a species of Least Concern on the IUCN red list, it is believed that the Atlantic sharpnose (*Rhizoprionodon terraenovae*) shark is abundant in coastal, warm or temperate waters in the western Atlantic Ocean. This species reproduces annually, with the litter size ranging from 1-7 pups. Nursery sites for atlantic sharpnose sharks include sounds and enclosed bays (IUCN, 2005c). It has been shown that not all populations of atlantic sharpnose sharks are synchronous in reproduction, with ovulatory females present from March to October (Hoffmayer *et al.*, 2013).

The lifespan of the atlantic sharpnose shark is relatively short, and thought to be a maximum of 12 years, with approximately 11 months' gestation period (Borucinska & Adams, 2013). The diet of the atlantic sharpnose has not been extensively researched, but it is thought that it is dominated by teleosts and crustaceans, and can include molluscs (IUCN, 2005c). Recently, it was found that some populations of atlantic sharpnose prey upon juvenile loggerhead turtles (*Caretta caretta*) (Delorenzo *et al.*, 2015).

BULL SHARK

The bull shark (*Carcharhinus leucas*) is the only species of shark which can survive for extended amounts of time in fresh water, and can be found long distances up rivers. This species is considered Near Threatened, as they are closer in proximity to humans because of their ability to thrive in fresh water, and therefore are more susceptible to habitat loss and human impacts. Bull shark populations are found worldwide in tropical and warm temperate waters, and seasonally are found in cool waters (IUCN, 2005a).

Bull sharks primarily thrive in continental shelf waters at a depth of around 450 ft., but can be found in shallow freshwater communities as well. This is mostly a continental species, but it has been shown that populations can exist near islands such as the Philippines and Fiji (IUCN, 2005a). Bull sharks can travel far distances for reproductive purposes, with one pregnant female travelling from Seychelles across the

open ocean to Madagascar and staying for a prolonged period of time at a shallow depth before returning (Lea *et al.*, 2015).

HISTORY OF MARINE MICROBIOLOGY AND MICROBIOLOGY ROLE OF MICROBIOMES

BACKGROUND

Understanding and characterizing microbiomes of an organism can reveal much about the organism's habits and health issues. Microbiomes are an extension of the organism, a separate functioning entity which can affect the organism's health, function, and potentially serve as a unique and specific form of identification. Microbes which thrive in and on humans outnumber the germ and somatic cells which are found in an individual by 10-fold (Turnbaugh *et al.*, 2007). Studies have characterized human microbiomes previously, with the salivary microbiome of humans showing importance in health and disease (Yamashita and Takeshita, 2017)

Microbiome refers to the community of Bacteria and Archaea that inhabit a habitat or organism. Recent research has shown that microbiomes are often crucial to key metabolic processes in higher organisms, and interruptions in the microbiome of an organism can lead to reduced functional abilities and/or disease (Human Microbiome Project Consortium, 2012b, 2012a).For example, *Bacteroides thetaiotaomicron* has been examined for an effect on the gastrointestinal metabolic function of its host (Human Microbiome Project Consortium, 2012b). Changes in microbiome composition have been correlated in humans to frailty in older individuals, risk for type 2 diabetes, metabolic disease, and inflammatory bowel syndrome (Long *et al.*, 2017). The composition of the human microbiome differs substantially across different anatomical locations. Much of the bacterial composition diversity remains to be unexplained, however, it is suggested that habitat, diet, and also early exposure to microbes could be altering the microbiomes of individuals (Human Microbiome Project Consortium, 2012b, 2012a).In contrast to humans, sharks have not been widely characterized in respect to their microbiome. This study begins to remedy this lack of knowledge, in hopes of finding or facilitating further

questions to inspire research to achieve similar results to those found in the human microbiome project.

CURRENT METHODS

A common protocol for determining the taxonomy of members in a microbial community is based on the most variable regions of the small subunit 16S rRNA gene. This gene is used mainly for identifying prokaryotes. By analyzing this gene, the phyla, in most cases, of the bacteria found in the microbial community can be determined. Because of its universal function as part of the protein translation apparatus, ribosomal RNA molecules were widely characterized (Woese *et al.*, 1980) before becoming an accepted comparative tool for bacteria and microbial communities. The gene that encodes for the 16S molecule is made up of 9 variable regions (V1-V9), with the V2 and V4 regions having the lowest error rates (Wang *et al.*, 2007).

The rRNA molecule is ideal as a taxonomic marker, as it can be found in almost all bacteria (Janda and Abbott, 2007). The function of rRNA has remained widely unchanged over time which allows for the comparison of many species on a rather broad level. Typically, microbes can be identified to the genus level using the 16S rRNA marker, but not always to the level of species with certainty due to the fact that only the part of the gene which corresponds to the 16S rRNA marker is being sequenced (Janda and Abbott, 2007). Additionally, most species of marine bacteria are not cultured (Bruns *et al.*, 2002), and so identification with next generation sequencing is more difficult and results in novel taxa.

PREVIOUS RESEARCH

There have been previous studies which have examined cultured bacteria from shark teeth, but to date, none have used next-generation sequencing to investigate the microbiome. Shark species which have been investigated utilizing bacterial culturing techniques include the blacktip shark (*Carcharhinus limbatus*) (Unger et al., 2014), white shark (*Carcharodon carcharias*) (Buck et al., 1984), bull shark (*Carcharhinus leucas*), and tiger shark (*Galeocerdo cuvier*) (Interaminense *et al.*, 2010). Blacktip sharks hosted bacteria with an overall resistance rate of 12.0%. 43.0% were resistant to one antibiotic,

and 4.0% were multidrug resistant (Unger *et al.*, 2014). Bull shark cultures included bacteria species which were 17.0% resistant to antibiotics, and tiger shark bacteria had a 22.0% average overall resistance to antibiotics (Interaminense *et al.*, 2010). White shark cultures contained bacteria which were resistant to penicillin, macrolide, and cephalosporin (Buck et al., 1984).

In addition to these findings from culturing studies for shark teeth, shark gills contain fungi, which may have medicinal applications. *Penicillium* sp., *Aspergillus* sp., *Mucor* sp., and *Chaetomium* sp. were the dominant taxa in *Carcharodon carcharias* (great white shark) gills, were shown to inhibit cancer proliferation in cells (Zhang *et al.*, 2016). A recent report also investigated the skin microbiome of the thresher shark, finding that the skin microbiome was significantly different and distinguished from the water column, but mostly due to enriched taxa which are already found in the water column (Doane *et al.*, 2017). Significant research has not been done into the microbiome of the cloaca, but there has been some research on cultured bacteria. Drug resistant strains of *Staphylococcus* and *E.coli* have been found in shark cloaca off the coast of Massachusetts. However, it is unclear whether the cloacal samples harbor a higher incidence of drug resistant virulent strains of *Staphylococcus* and *E.coli* when compared to the surrounding ambient environment (Blackburn *et al.*, 2010).

Although the culturing techniques used gave some insight as to which bacteria are being hosted by sharks, they are severely limited in what they can successfully detect from the overall microbiome. The majority of all bacteria and archaea are currently unculturable (Vartoukian *et al.*, 2010), with half of the total estimated 61 phyla of bacteria having no members which are culturable (Hugenholtz *et al.*, 2009). For marine bacteria, the efficiency of cultivation can range anywhere from .001% to .10% of all cells for either open ocean or coastal communities (Bruns *et al.*, 2002). In symbiotic communities, such as the human gut microbiome, the cultivation success is between 20.0% and 40.0% (Dave *et al.*, 2012)

Shark microbiome research is still in its infancy, with no standard of data collection or analysis in place for these organisms. Most of the studies that have been done on the microbial communities of sharks do not focus on the overall microbiome of the individual, but only one or two specific areas (namely, the gut). In addition, the most

common current technique is to culture bacteria from the samples, which almost certainly overlooks some community members. This study comprehensively examines the microbiome of sharks including 5 species and four sample locations per individual (skin, teeth, cloaca, and gills) to determine if unique bacteria exist when compared to the surrounding environment. By using next generation sequencing, we will be able to understand the composition of shark microbiomes on a much larger scale, and build upon the current research including isolation by culturing techniques.

HYPOTHESES

This study tests the following hypotheses:

1. Elasmobranch microbiome composition is unique from the surrounding environment.
2. Anatomical locations on individual sharks will have distinct community compositions for all shark species.
3. Microbial communities will differ based on the species of the shark, and each location should be more similar among individuals of the same species than individuals of different species.

METHODS

SHARK SAMPLE COLLECTION

Individuals were caught and released once samples and measurements are taken. The sharks were fished for using a rig that contains a fifty-pound weight and a line with a buoy on the top, labelled with "GHRI" (Guy Harvey Research Institute) and the license number that permits fishing with such gear. Gear was set in groups of 10, with two at each of the following depths: 25ft, 40ft, 60ft, 80ft, and 100ft. Attached to the weight was a 100ft 900lb tested microfilament line with a circle hook and atlantic bonito (*Sarda sarda*) as bait on the end so that sharks could swim and continuously pump water over their gills while the lines and gear were being retrieved. When the line was picked up, it was pulled up by hand and the shark was secured with a tail loop and the line from the

hook so that measurements and samples could be safely taken. After securing the shark, four anatomical locations were sampled using dual tipped sterile swabs

(Henry Schein, Cat. 1228715) which were transported in a cooler to the Microbiology and Genetics lab at the Halmos College of Natural Science and Oceanography. For each individual, four samples were taken: mouth, gills, skin, and cloaca. Samples were collected from eight total species, five of which had sufficient sample size for statistical analysis. These 8 species represent a wide range of habitat preference and diet. Bull, Black tip, and Atlantic Sharpnose sharks were only included in analyses which only examined one factor (species or location), but not when looking at the interaction between the two, as one individual is not sufficient sampling size for in depth and specific statistical analysis.

ENVIRONMENTAL SAMPLE COLLECTION

Additionally, seawater samples were taken for each individual when possible, and filtered after each trip so that any environmental microbes could later be characterized and possibly discounted. One-liter of seawater was sampled from the ocean surface in sterilized plastic Nalgene bottles concurrent with sampling of each shark. These bottles were submerged in surface water and rinsed once before filling with the actual sample. Water samples were transported on ice to the lab and were filtered using a .45 µm filter membrane. Experimental design followed the tenets of (Knight *et al.*, 2012) for the minimum number of samples required, as well as including all possible "metadata" associated with each sample (Sample Code, Associated Shark Species, Month, Latitude, and Longitude).

SAMPLE PREPARATION

Environmental DNA (water samples) was extracted with the DNeasy PowerLyzer PowerSoil kit (Cat# 12855-100), and swabs were extracted using the QIAamp BiOstic Bacteremia DNA kit (Cat# 12240-50) (MoBio Laboratories Inc.). The extracted DNA was amplified using PCR while targeting the V4 region of the 16S rRNA gene. The

primers used for the PCR were R806 and F515, which were developed specifically for the V4 region of the 16S rRNA (Caporaso *et al.*, 2011). Amplicons were sequenced with an Illumina MiSeq sequencing platform equipped with a V2 chemistry 500 cycle cartridge (Caporaso *et al.*, 2012) yielding paired-end 250 bp amplicons. Initial processing of sequence data was performed in MacQIIME (Quantitative Insights into Microbial Ecology) version 1.9.1(Caporaso *et al.*, 2010).

SEQUENCE ANALYSIS

Joining of paired end sequences was done with "join_paired_ends.py" with fastq-join. Mapping files were compared for errors using "validate_mapping_file.py", before demultiplexing and quality filtering with "split_libraries_fastq.py". Raw sequences were quality filtered to remove all chimeric and low quality (quality score < 30) sequences. These sequences were then clustered into 97.0% similar operational taxonomic units (OTUs) using a combination of open and closed reference OTU clustering strategies. OTUs were picked using the "pick_open_reference_otus.py" script. This script prefilters and picks closed reference OTUs, and any that do not have corresponding OTUs in the reference database are filtered out and clustered de novo before being compared to the database once more. Utilizing these OTUs, an OTU map is created that represents the OTUs as matched to the reference database. OTUs were picked based on the Silva (Release 128) database instead of Greengenes, because of the frequency of updates and ease of accessibility (Quast *et al.*, 2013; DeSantis *et al.*, 2006) . These OTUs were then used to identify which bacteria were present at a sampling area on an individual. The composition of each sample was compared to determine significant differences and similarities between individuals.

STATISTICAL ANALYSIS

Analysis was executed with the RStudio software (version 3.2.1), with the added libraries 'picante' and 'vegan' to examine general ecology of the microbiome (Oksanen, 2017a; Kembel *et al.*, 2010). 16S rRNA sequence data was transformed to reflect the

relative abundance to normalize sequencing depth among samples, using the "decostand" tool in vegan. Variation associated with species and location were analyzed using these tools.

Alpha diversity was measured by calculating OTU richness, inverse Simpson's index, and the Shannon index for each sample. The latter two indices consider richness and evenness when examining alpha diversity. Shannon index assumes all species are represented and sampled randomly, can be less effective with rare species. The Inverse Simpson index removes bias by pooling the total diversity so that the average of the pooled communities is greater than or equal to the diversity within communities (Lande, 1996). Differences in alpha diversity among species and locations were assessed using an analysis of variance (ANOVA) and a Tukey's HSD Test was done to as a post hoc test to assess pairwise differences among groups (Tukey, 1949).The Tukey test introduces intervals which are based on a range of sample means, instead of the individual differences which are examined in a normal t-test and is adjusted to account for sample size for unbalanced designs (Bates, 2017).

Beta diversity was measured by calculating Bray-Curtis dissimilarity among samples, which was calculated to understand beta diversity by determining the dissimilarity between groups or clusters (species, location, or environment) while considering the variation found in composition which is categorized by OTUs (Field, 1982). Bray-Curtis values range in value from 0 to 1, with low values indicating that two samples have the very similar compositions, and high values indicating that two samples are highly dissimilar (Bray and Curtis, 1957). Data was represented as distance matrices and significant differences were assessed using a permuted multivariate ANOVA (implemented using the 'adonis' function). Differences in beta diversity among species, locations, and environment (environment vs shark) was assessed using a permuted multivariate ANOVA (PERMANOVA) (Kelly *et al.*, 2015).

Beta dispersion analysis based on the Bray Curtis Distance of the samples by species was done to check if any species are significantly more variable than the others (homogeneity of variances). ANOVA showed that there was a significant difference in the average distance to the spatial median between species. A SIMPER test with 499

permutations show the taxa which are driving pairwise differences across species, location, and environmental microbial communities (Tyler *et al.*, 2014). SIMPER performs comparisons of data in a pairwise fashion which results in the average contributions of each sampling unit to the overall dissimilarity of the samples (Oksanen, 2017a). The SIMPER analysis was used to determine which taxa are driving the differences in species, location, or environmental microbiomes (Rees *et al.*, 2004).

CHAPTER 2:

RESULTS

A total of 12,374,571 MiSeq reads and 26,309 OTUs were generated across all samples in this study (Table 1). From the initial 136 samples collected, 127 were successfully sequenced (31 cloaca, 32 gills, 32 skin, and 32 teeth) with a mean read depth of 97,438. Samples with fewer than 1000 sequences were excluded, due to inadequate sequencing depth. The following results include assessment of all anatomical locations for shark species (n = 127).

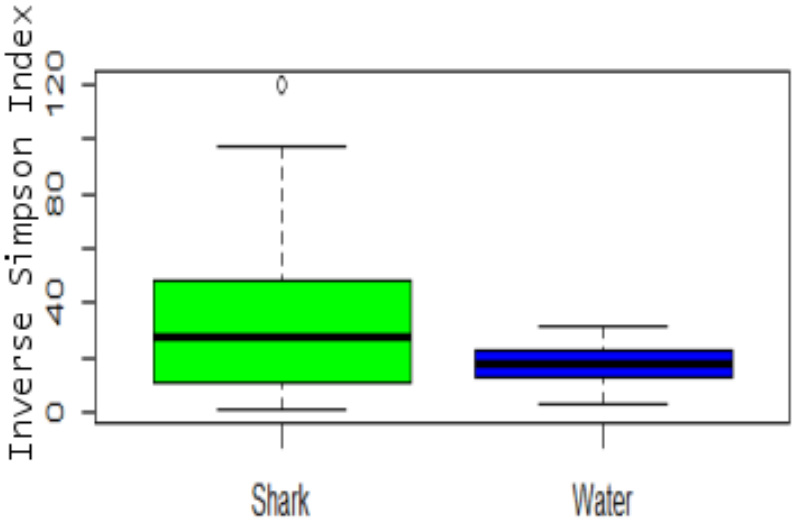

Figure 1. Box plot of mean species diversity comparing water to shark samples based on the Inverse Simpson index. (ANOVA, df=1, F=7.724, p=.006). Seawater microbial communities have significantly less diversity compared to sharks.

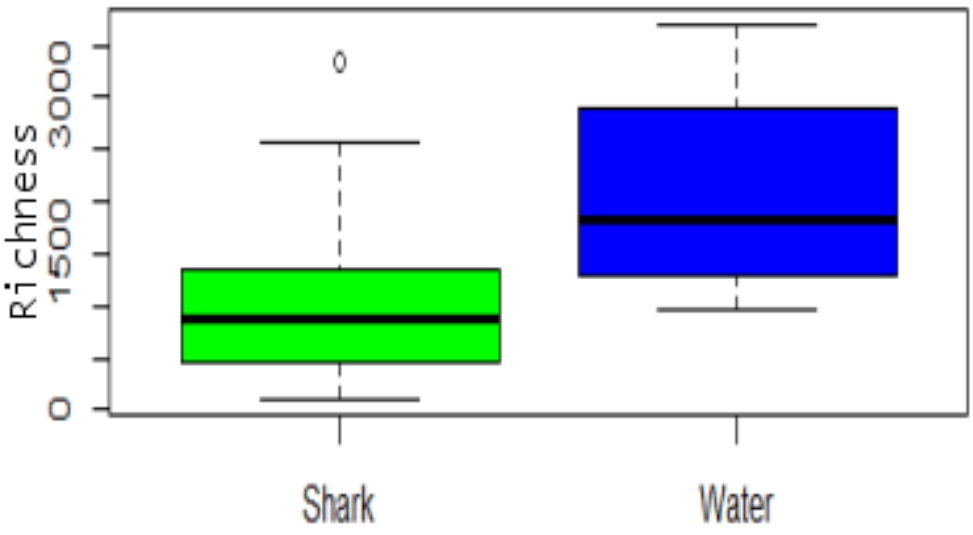

Figure 2. Box plot of mean species richness comparing water to shark samples (ANOVA, df=1, F=42.19, p<.001). The seawater microbial environments hosted more rich communities.

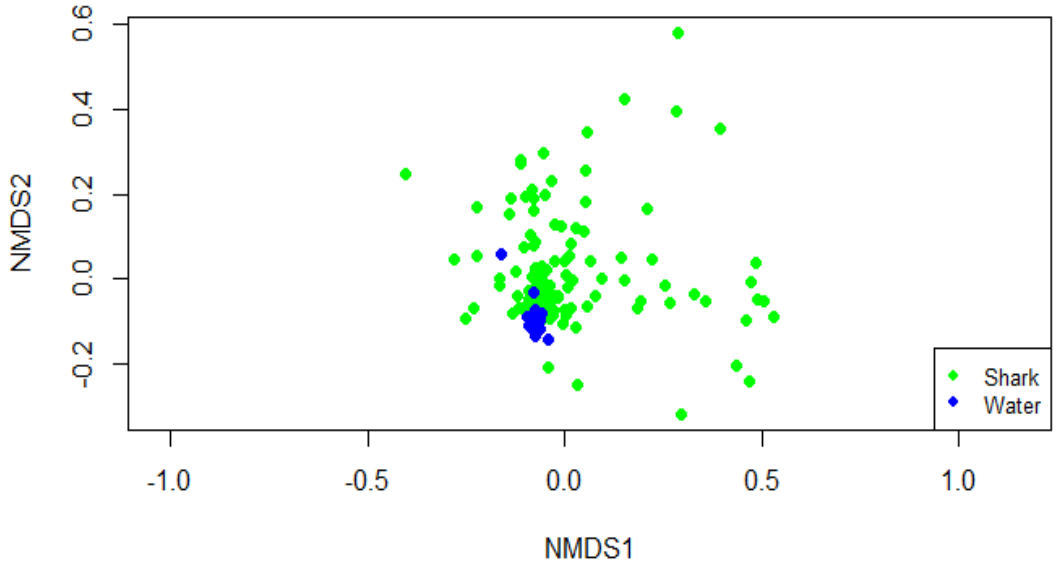

Figure 3. Non-metric dimensional scaling of shark and water samples. (R^2=0.77, stress=0.1833) The water samples are much more clustered than the shark samples, which are quite disperse.

There is a significant difference between the water and shark samples by the inverse Simpson index, and a larger range of diversity in sharks than the water, due to rare taxa (Figure 1). Shark samples had significantly less microbial richness than water. Sharks had more evenness than water, with a higher microbial diversity (Figure 2). There is a 33.0% overlap of OTUs between water and shark samples. Sharks and the water they inhabit are clearly sharking some taxa, but still have distinct microbial environments (Figure 3).

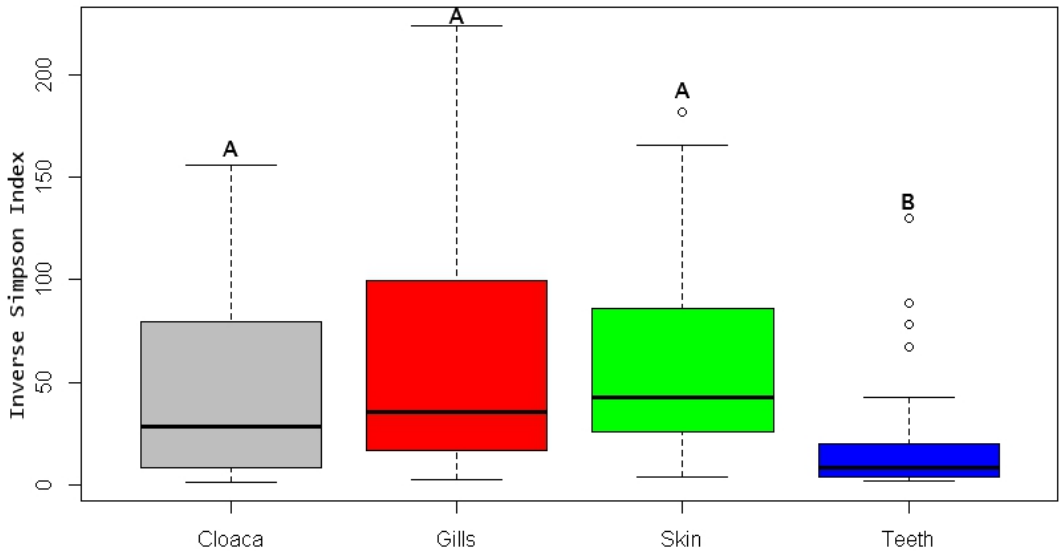

Figure 4. Box plot of mean species diversity of location by the Inverse Simpson index. (ANOVA, df=3, F=4.952, p=.0029). Teeth have a significantly lower diversity than other sampled locations.

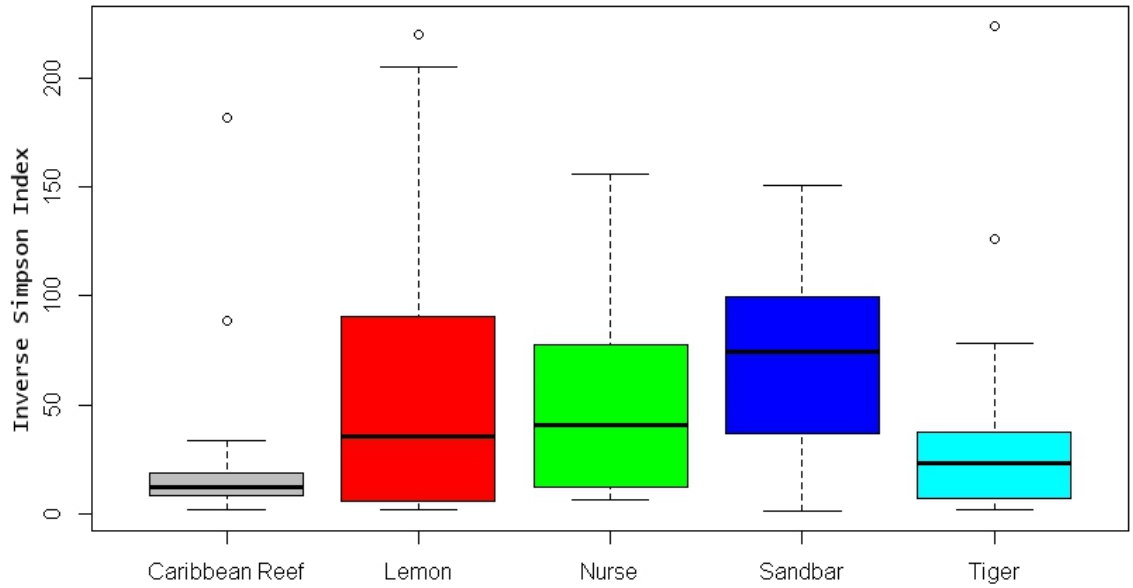

Figure 5. Box plot of mean species diversity of species by the Inverse Simpson index. (ANOVA, df=4, F=1.58, p=.184)

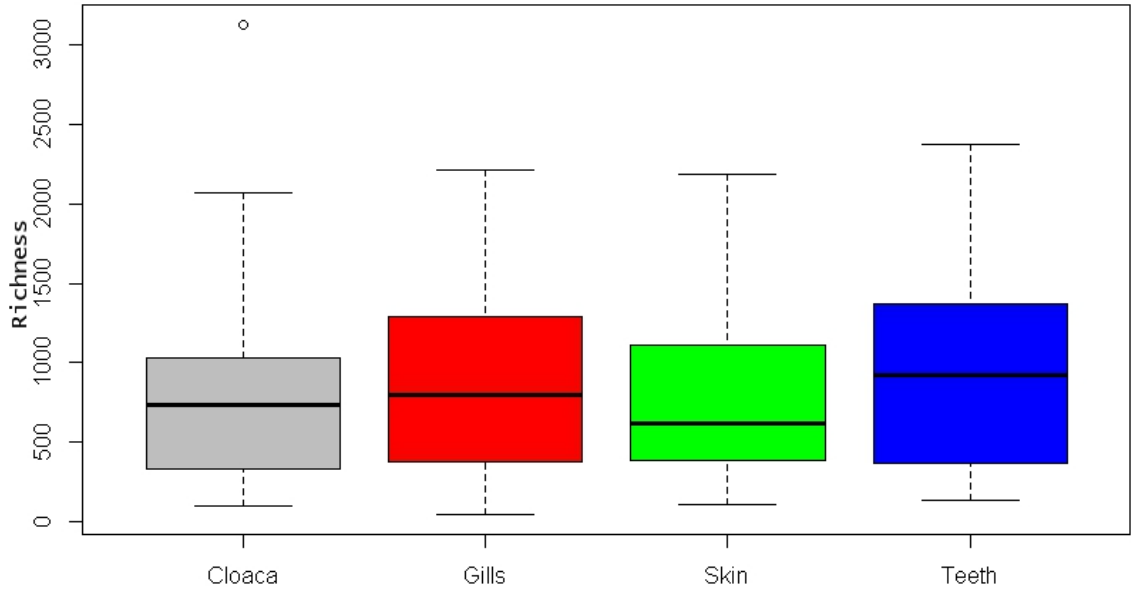

Figure 6. Box plot of mean species richness by location. (ANOVA, df=3, F=.351p=.788) No significant differences are found among locations for richness.

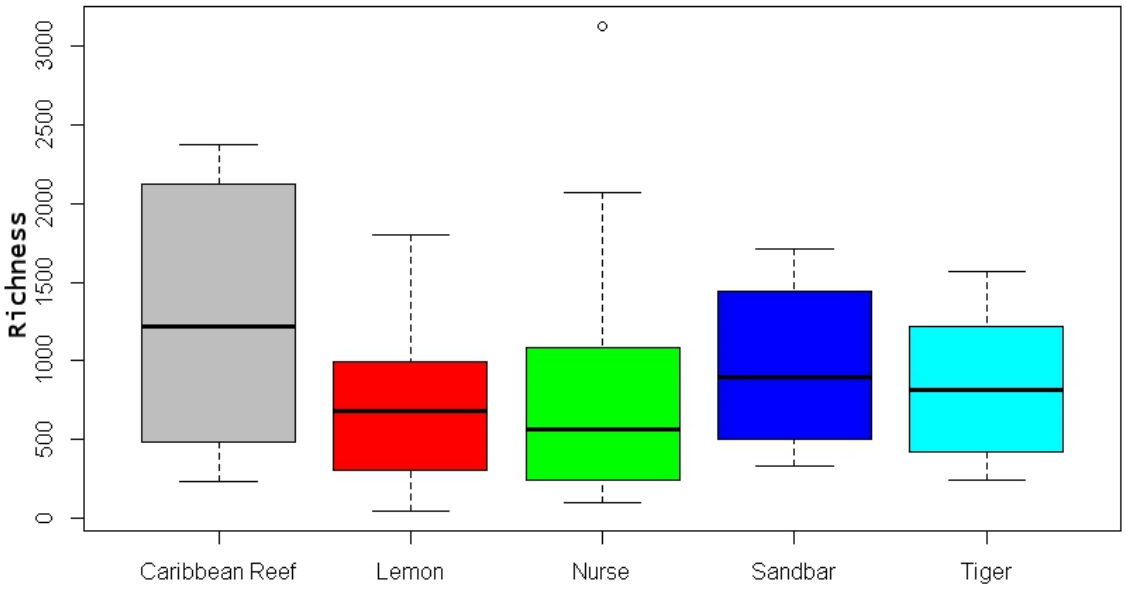

Figure 7. Box plot of mean species richness by species. (ANOVA, df=4, F=2.888, p=.0256). There is a significant difference between groups, but no two species are driving the significant differences.

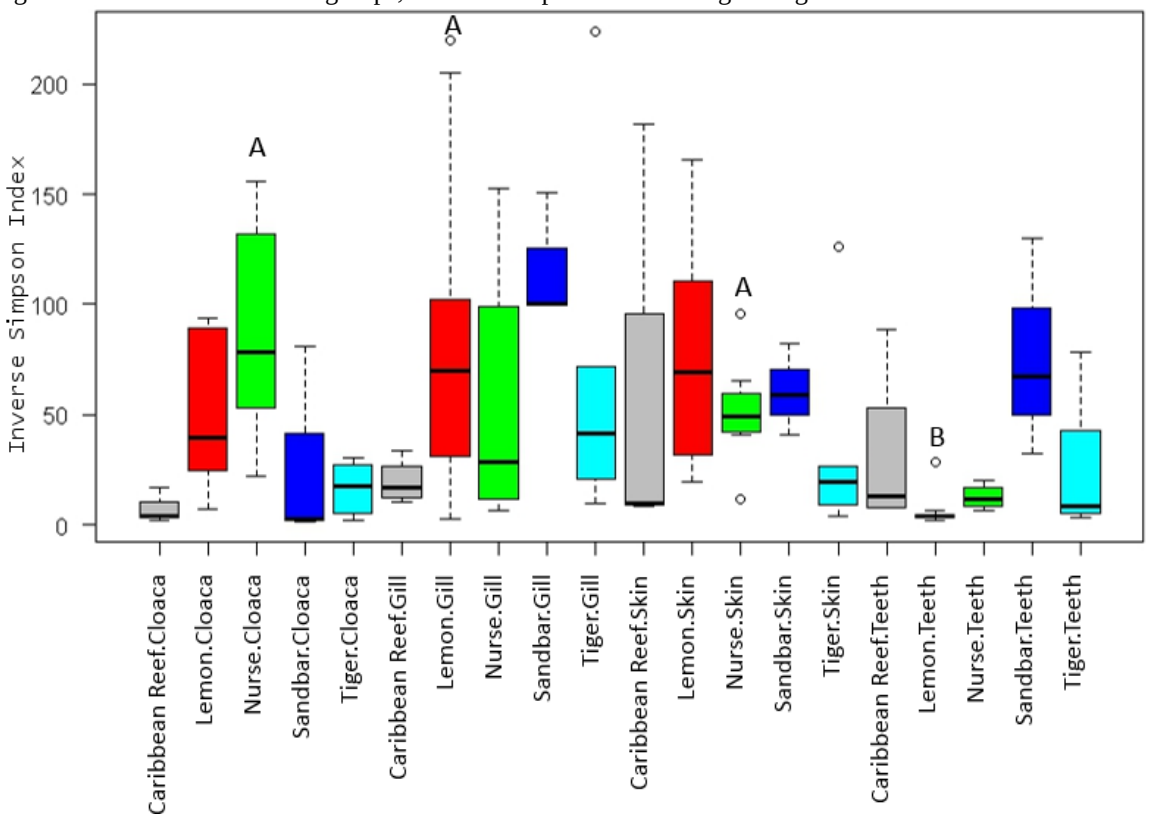

Figure 8. Box plot of mean species diversity of the interaction between species and location by the Inverse Simpson index. (ANOVA, df=12, F=1.764, p=.065) Lemon teeth are significantly different from nurse cloacal and gill samples.

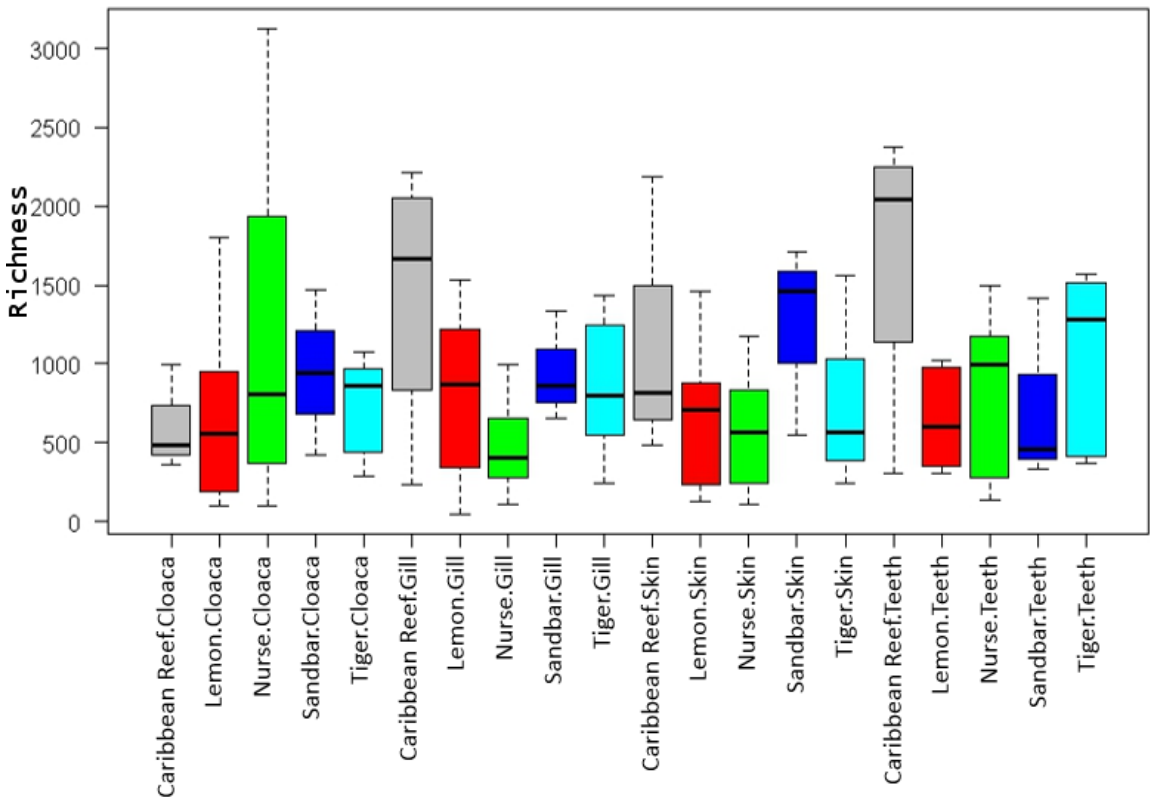

Figure 9. Box plot of mean species richness of the interaction between species and location. (ANOVA, df=12, F=1.323, p=.213) There are no statistically significant differences in richness.

Significant differences were found in microbial community richness among species (ANOVA, df=4, F=2.888, p=.0256) (Figure 7), but not among locations (ANOVA, df=3, F=.351p=.788) (Figure 6), or the interaction of species and location (ANOVA, df=12, F=1.323, p=.217) (Figure 9). The Shannon index showed significant differences among locations (ANOVA, df=3, F=9.832, p<.001) and the interaction between species and location (ANOVA, df=12, F=4.05, p=<.001), but not by species alone (ANOVA, df=4, F=.512, p=.727). Samples collected from teeth were significantly different than those from gills and skin (Tukey's HSD $P < 0.05$). Of the species sampled, the sandbar and lemon shark teeth were driving most of the differences in the interaction of species and location. Diversity as measured by the Inverse Simpson index showed significant differences in community diversity among locations (ANOVA, df=3, F=4.952, p=.0029) (Figure 4), but not by species (ANOVA, df=4, F=1.58, p=.184)

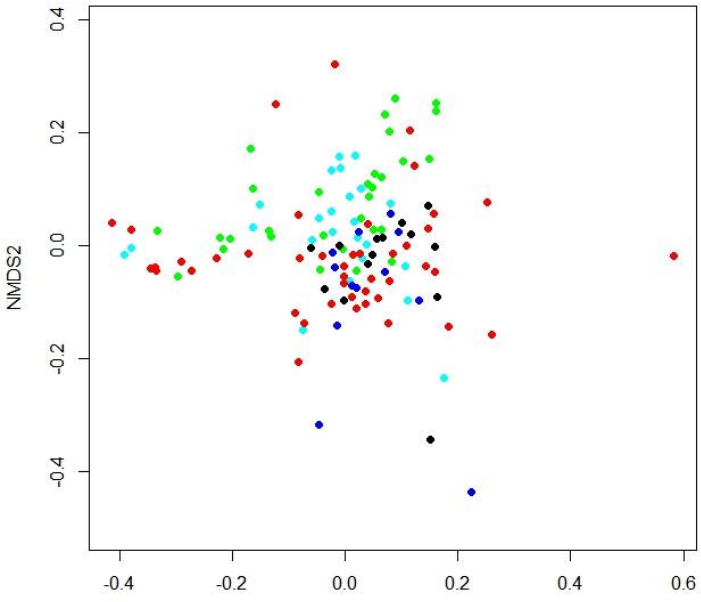

Figure 10. NMDS analysis of all samples by species based on the relative abundance of microbial taxa. All sample locations are included in this plot, as a general microbiome analysis. (Red=lemon, Cyan= tiger, Green=nurse, Blue=sandbar, Black=Caribbean reef). There are clear outliers from the rest of the data points here.

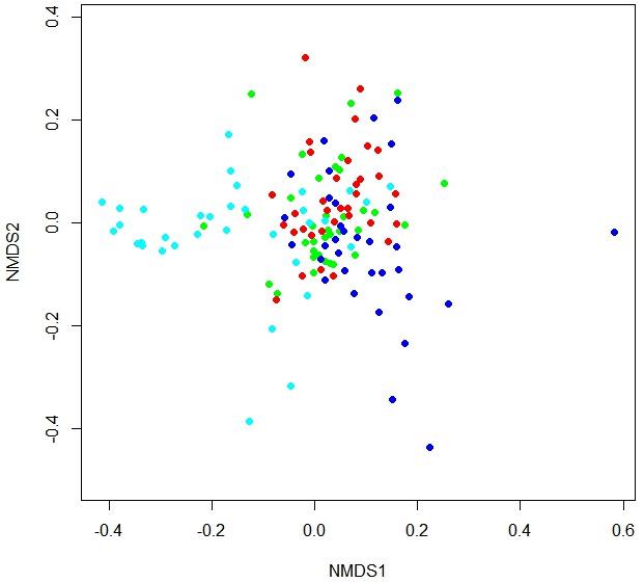

Figure 11. NMDS analysis of all samples by location based on the relative abundance of microbial taxa. All sample locations are included in this plot, as a general microbiome analysis. (Red=Skin, Cyan=Teeth, Green=Gills, Blue=Cloaca) Outliers are mostly teeth and cloacal samples.

(Figure 5) or the interaction of species and location (ANOVA, df=12, F=1.764, p=.065) (Figure 8). Like the Shannon index, significant results were driven by differences in teeth samples compared to gills (Tukey's HSD $P < 0.05$), skin (Tukey's HSD $P < 0.05$), and cloaca (Tukey's HSD $P < 0.05$) samples.

NMDS analysis and visualization of the data by species revealed that lemon, tiger, and nurse sharks had data points which were different from the bulk of the data (NMDS, R^2=.080, p=.001) (Figure 10). Location NMDS visualization revealed that those data points that were most different from the bulk of the data were teeth and cloacal samples (NMDS, R^2=.075, p=.001) (Figure 11). The interaction between species and location show that those data points which are most different were the teeth samples from tiger, lemon, and nurse sharks (NMDS, R^2=.15, p=.004) (Figure 12).

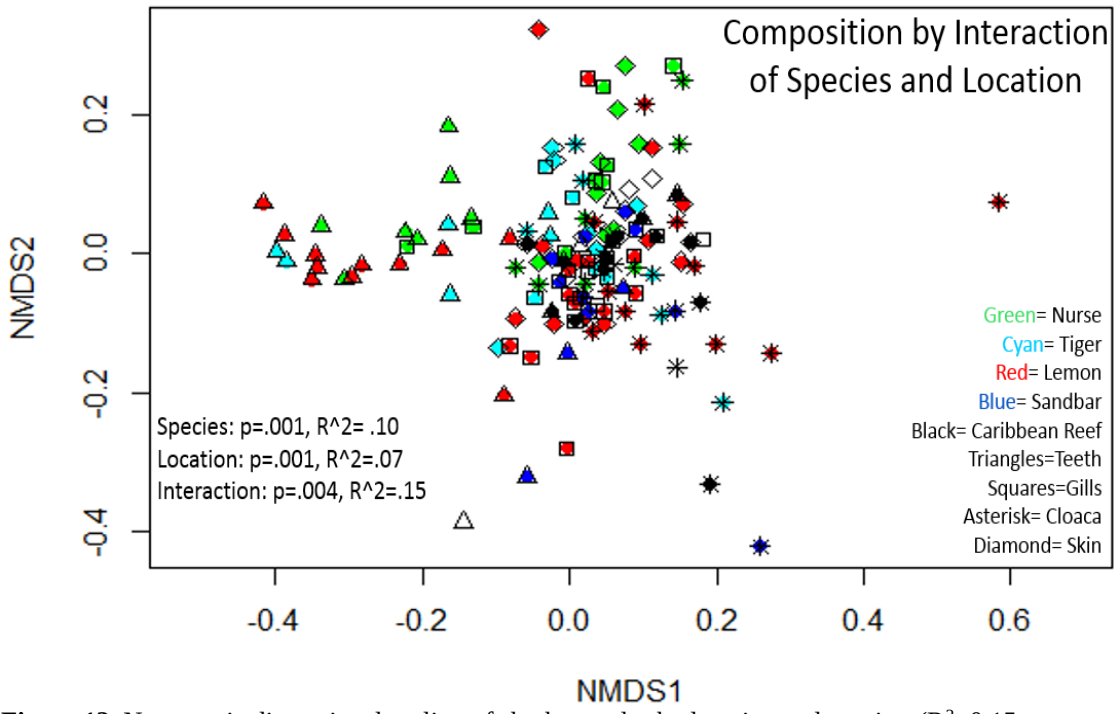

Figure 12. Non-metric dimensional scaling of shark samples by location and species. (R^2=0.15, stress=0.22) Outliers are mostly lemon, nurse, and tiger teeth samples.

Assessment of homogeneity of variances among sample groups indicated significant differences in the average distance to the spatial median among species (ANOVA, df=4, F=3.774, p=.006). Lemon sharks had the highest average distance to the median (0.6342), followed by nurse sharks (0.6221), tiger sharks (0.6096), sandbar sharks (0.5899), and Caribbean reef sharks (0.5832). Lemon and nurse sharks displayed significantly more variation among samples within their respective groups compared to other species (Figure 13). Adonis showed that the interaction between species and location has a higher impact on the differences between groups (PERMANOVA, df=21, F=1.12, R^2=.152, p=.005) than either species (PERMANOVA, df=7, F=2.12, R^2=.104, p=.001) or location (PERMANOVA, df=3, F=3.38, R^2=.069, p=.001) alone.

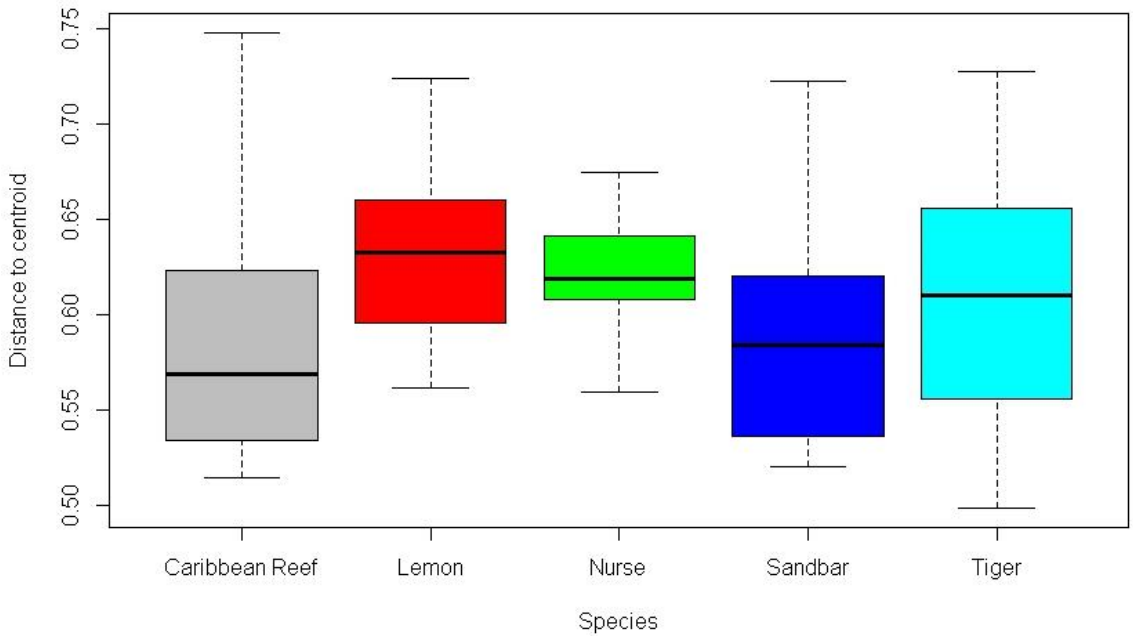

Figure 13. Boxplot of Beta Dispersion analysis based on the Bray Curtis Distance between samples by species, showing the average distance from the spatial median. Average distances were as follows: lemon-.6342, nurse-.6221, tiger-.6096, sandbar-.5899, and Caribbean reef-.5832. (Red=lemon, Cyan= tiger, Green=nurse, Blue=sandbar, Black=Caribbean reef)

BROADER DISCUSSIONS

SHARKS VERSUS ENVIRONMENT

Many OTUs are shared between sharks and the seawater they inhabit, but sharks still host a distinct microbial environment from the seawater. A major difference between shark teeth and seawater is an increased abundance of the genus *Prochlorococcus*. This genus includes species which are among the main primary producers in the ocean, and are very much environmentally associated (Kettler *et al.*, 2007).

Based on these results, sharks in South Florida have specific microbial communities in respect to richness and diversity when compared to the water in which they inhabit. This study does not allow for conclusions on the function or cause of this unique microbiome to be asserted. However, this study was designed based on the human microbiome project, which found that human microbiomes evolve with age, gender, reproductive cycle, and disease. It was found that specific microbial communities were

essential in maintaining health in humans, and can even provide anti-inflammatory compounds (Sokol *et al.*, 2008).

ANATOMICAL LOCATION

When microbial communities of the anatomical locations were compared across all samples, there was a significant difference in the diversity of these communities, but not in the richness (Table 2). This means that these sample locations have similar amounts of microbe species, but vary in the evenness of these species. When an SIMPER test was performed, distinct microbial communities on shark teeth were the primary drivers for compositional differences among anatomical locations. When also examining the location with relation to species, it was narrowed down further to show that the differences were being driven by lemon shark teeth communities.

The OTU driving most of the significant differences in teeth microbial communities is GU118128.1.1417, which is representative of the family *Phyllobacteriaceae*. This family of bacteria includes species which are plant and environmentally associated, and have a heterotrophic, respiratory metabolism that utilizes oxygen as the terminal electron acceptor (Willems, 2014). When examining the SIMPER results for the comparison of teeth to the ambient water, this OTU accounted for only 9.0% of the differences in samples. However, comparing only within all teeth samples across species, this OTU explained between 18.0% and 32.0% of differences.

SIMPER results comparing lemon to Caribbean reef sharks showed OTUs which are driving the differences between these two species' teeth microbiomes, such as one representing the genus *Haemophilus*, which explains 7.0% of the differences of the teeth microbiomes of these species. *Haemophilus* includes some pathogenic species, such as *Haemophilus influenzae*, but also has been associated with the saliva microbiome in humans. Thirdly, the OTU FJ983094.1.1542, representing the genus *Streptococcus*, explains 2.0% of the variance between Caribbean reef and lemon shark teeth microbiomes, being present in the top 10 most abundant OTUs for Caribbean reef, but not lemon shark microbiomes (Figure S2, S4). Taxonomic representations were generated

as Krona plots (Ondov *et al.*, 2011).This genus includes some pathogenic species, but also species which are associated with the human microbiome as important to overall function (Human Microbiome Project Consortium, 2012b).

When examining the SIMPER comparison between nurse and lemon shark teeth, it was shown that 2.0% of differences were explained by the OTU GQ274041.1.1514, which represents the genus *Kordia,* which includes species that have exhibited strong algicidal effects on diatoms. Species of this genus have been isolated in a junction between the ocean and a stream of fresh water (Park *et al.*, 2014). This genus could be relevant in explaining microbial community differences in the lemon and nurse shark teeth samples due to the lemon shark's unique ability to survive in freshwater environments (Ebert *et al.*, 2015).

SPECIES ANALYSIS

When analyzing samples by differences in shark species, there was not a significant difference in overall microbial community diversity (Table 3). There was a significant difference in richness, however, meaning that some shark species have varying amounts of microbe species in their overall microbiome than others, but not a significant difference in the evenness of these species. Richness does not account for how many individuals of each species are present, however, so it is possible that some shark species have a few rare taxa compared to another species. It is clear by looking at the top ten most abundant taxa that some are far more represented then others. The interaction between species and location showed significant differences in diversity, which were explained by significant differences between sandbar shark cloacal samples and lemon and nurse sharks. There were also significant differences by species and location interaction explained by differences in Caribbean reef teeth and lemon and nurse shark teeth

Based on Beta Dispersion analysis, it was shown that lemon sharks had the furthest distance from the spatial median of all other species, with nurse sharks just slightly less, followed by tiger sharks. All three of these species can utilize buccal

pumping- or in the case of the nurse shark, rely solely on it. Buccal pumping allows species to remain still for long periods of time, typically on the sea floor, while pumping water over their gills to breathe (Dapp *et al.*, 2016; Gibson and Carter, 2002; Brooks *et al.*, 2011). This could account for the slight difference in microbial communities that was shown in the Beta Dispersion analysis. Additionally, the nurse and lemon sharks are known to enter fresh water environments on occasion, which could explain why these species have microbial communities which are more similar to each other than other species (Ebert *et al.*, 2015; Stafford-Deitsch, 2000; Hendon *et al.*, 2013).

When examining the taxonomy of the species utilized in this study, the nurse shark is the only shark which is not considered a requiem shark, but instead a carpet shark. Lemon sharks belong to the *Negaprion* genus, which is different from the sandbar, Caribbean reef, and tiger sharks, which belong to the *Carcharinus* genus. The fact that lemon and nurse sharks have different genus' than the other species sampled could explain the microbial community differences seen in the Beta Dispersion analysis (Gibson and Carter, 2002).

SUMMARY AND CONCLUSIONS

This study accomplished the three main hypotheses and goals as laid out in the original proposal. Microbial communities of sharks in South Florida were compared to samples of water in which they were obtained to find. This revealed that sharks have significantly different microbiomes from the environment in which they live. Examination of microbial communities of different anatomical locations revealed that there is a significant difference in microbial diversity between sample locations (gills, teeth, skin, cloaca), and that most of these differences are driven by the microbial diversity of the teeth communities. The comparison of microbial communities across species showed that sharks do not have significantly different microbiomes by diversity, but that there could be rare taxa which are allowing for a significant difference in richness.

APPENDICES

I.TABLES

Test	P-value
Richness	$9.23e^{-06}$
Diversity-Shannon	.469
Diversity-Inverse Simpson	.00154
Adonis	.001

Table 1. p-values of statistics when environmental data was compared to all shark samples.

Test	P-value
Richness	.781
Diversity-Shannon	$8.45e^{-06}$
Diversity-Inverse Simpson	.0029
Adonis	.001

Table 2. p-values of statistics when data was compared by sample location.

Test	P-value
Richness	.0297
Diversity-Shannon	.707
Diversity-Inverse Simpson	.184
Adonis	.001

Table 3. p-values of statistics when data was compared by shark species.

Supplementary Tables:

Sample	Species	Location	Month	Type	Latitude	Longitude	Gender
B051316	Bait	Bait	May	Environment	26.09582	80.04554	
B051816	Bait	Bait	May	Environment	26.14134	80.04847	
B052416	Bait	Bait	May	Environment	26.13789	80.039	
B052616	Bait	Bait	May	Environment	26.21083	80.03233	
B060116	Bait	Bait	June	Environment	26.09485	80.04416	

B060316	Bait	Bait	June	Environment	25.58992	80.05762	
B062216	Bait	Bait	June	Environment	26.03651	80.05088	
B063016	Bait	Bait	June	Environment	26.03651	80.05088	
B091716	Bait	Bait	Sept	Environment	26.12633	80.04755	
B092216	Bait	Bait	Sept	Environment	26.18271	80.04049	
B092316	Bait	Bait	Sept	Environment	26.00659	80.05802	
B111116	Bait	Bait	Nov	Environment	26.02991	80.0556	
C236C	Caribbean Reef	Cloaca	Sept	Shark	26.18271	80.04049	F
C236G	Caribbean Reef	Gills	Sept	Shark	26.18271	80.04049	F
C236S	Caribbean Reef	Skin	Sept	Shark	26.18271	80.04049	F
C236T	Caribbean Reef	Teeth	Sept	Shark	26.18271	80.04049	F
C236W	Water	Water	Sept	Environment	26.18271	80.04049	F
C247C	Caribbean Reef	Cloaca	Nov	Shark	26.00662	80.05114	F
C247G	Caribbean Reef	Gills	Nov	Shark	26.00662	80.05114	F
C247S	Caribbean Reef	Skin	Nov	Shark	26.00662	80.05114	F
C247T	Caribbean Reef	Teeth	Nov	Shark	26.00662	80.05114	F
CR202C	Caribbean Reef	Cloaca	June	Shark	26.09485	80.04416	F
CR202G	Caribbean Reef	Gills	June	Shark	26.09485	80.04416	F
CR202S	Caribbean Reef	Skin	June	Shark	26.09485	80.04416	F
CR202T	Caribbean Reef	Teeth	June	Shark	26.09485	80.04416	F
CR202W	Water	Water	June	Environment	26.09485	80.04416	

L079C	Lemon	Cloaca	May	Shark	26.13549	80.04914	F
L079G	Lemon	Gills	May	Shark	26.13549	80.04914	F
L079S	Lemon	Skin	May	Shark	26.13549	80.04914	F
L079T	Lemon	Teeth	May	Shark	26.13549	80.04914	F
L079W	Water	Water	May	Environment	26.13549	80.04914	
L110C	Lemon	Cloaca	May	Shark	26.09582	80.04554	M
L110G	Lemon	Gills	May	Shark	26.09582	80.04554	M
L110S	Lemon	Skin	May	Shark	26.09582	80.04554	M
L110T	Lemon	Teeth	May	Shark	26.09582	80.04554	M
L110W	Water	Water	May	Environment	26.09582	80.04554	
L164C	Lemon	Cloaca	Sept	Shark	26.00399	80.05626	M
L164G	Lemon	Gills	Sept	Shark	26.00399	80.05626	M
L164S	Lemon	Skin	Sept	Shark	26.00399	80.05626	M
L164T	Lemon	Teeth	Sept	Shark	26.00399	80.05626	M
L164W	Water	Water	Sept	Environment	26.00399	80.05626	
L169C	Lemon	Cloaca	Sept	Shark	26.00429	80.05195	F
L169G	Lemon	Gills	Sept	Shark	26.00429	80.05195	F
L169S	Lemon	Skin	Sept	Shark	26.00429	80.05195	F
L169T	Lemon	Teeth	Sept	Shark	26.00429	80.05195	F
L169W	Water	Water	Sept	Environment	26.00429	80.05195	M
L191C	Lemon	Cloaca	June	Shark	26.03369	80.05599	M
L191G	Lemon	Gills	June	Shark	26.03369	80.05599	M
L191S	Lemon	Skin	June	Shark	26.03369	80.05599	M
L191T	Lemon	Teeth	June	Shark	26.03369	80.05599	M
L191W	Water	Water	June	Environment	26.03369	80.05599	M
L221C	Lemon	Cloaca	May	Shark	26.22286	80.03008	M
L221G	Lemon	Gills	May	Shark	26.22286	80.03008	M
L221S	Lemon	Skin	May	Shark	26.22286	80.03008	M
L221T	Lemon	Teeth	May	Shark	26.22286	80.03008	M
L221W	Water	Water	May	Environment	26.22286	80.03008	
L223.W	Water	Water	June	Environment	26.09695	80.0447	
L223C	Lemon	Cloaca	June	Shark	26.09695	80.0447	F
L223G	Lemon	Gills	June	Shark	26.09695	80.0447	F

L223S	Lemon	Skin	June	Shark	26.09695	80.0447	F
L223T	Lemon	Teeth	June	Shark	26.09695	80.0447	F
L224C	Lemon	Cloaca	June	Shark	25.58992	80.05762	M
L224G	Lemon	Gills	June	Shark	25.58992	80.05762	M
L224S	Lemon	Skin	June	Shark	25.58992	80.05762	M
L224T	Lemon	Teeth	June	Shark	25.58992	80.05762	M
L224W	Water	Water	June	Environment	25.58992	80.05762	
L225C	Lemon	Cloaca	May	Shark	26.13789	80.039	M
L225G	Lemon	Gills	May	Shark	26.13789	80.039	M
L225S	Lemon	Skin	May	Shark	26.13789	80.039	M
L225T	Lemon	Teeth	May	Shark	26.13789	80.039	M
L225W	Water	Water	May	Environment	26.13789	80.039	
L231C	Lemon	Cloaca	Sept	Shark	26.1303	80.04121	F
L231G	Lemon	Gills	Sept	Shark	26.1303	80.04121	F
L231S	Lemon	Skin	Sept	Shark	26.1303	80.04121	F
L231T	Lemon	Teeth	Sept	Shark	26.1303	80.04121	F
L231W	Water	Water	Sept	Environment	26.1303	80.04121	
L238C	Lemon	Cloaca	Sept	Shark	26.00765	80.05163	M
L238G	Lemon	Gills	Sept	Shark	26.00765	80.05163	M
L238S	Lemon	Skin	Sept	Shark	26.00765	80.05163	M
L238T	Lemon	Teeth	Sept	Shark	26.00765	80.05163	M
L238W	Water	Water	Sept	Environment	26.00765	80.05163	
N080C	Nurse	Cloaca	April	Shark	26.13353	80.08902	M
N080G	Nurse	Gills	April	Shark	26.13353	80.08902	M
N080S	Nurse	Skin	April	Shark	26.13353	80.08902	M
N080T	Nurse	Teeth	April	Shark	26.13353	80.08902	M
N082C	Nurse	Cloaca	April	Shark	26.13642	80.08358	F
N082G	Nurse	Gills	April	Shark	26.13642	80.08358	F
N082S	Nurse	Skin	April	Shark	26.13642	80.08358	F
N082T	Nurse	Teeth	April	Shark	26.13642	80.08358	F
N113C	Nurse	Cloaca	April	Shark	26.05427	80.09445	F
N113G	Nurse	Gills	April	Shark	26.05427	80.09445	F
N113S	Nurse	Skin	April	Shark	26.05427	80.09445	F

N113T	Nurse	Teeth	April	Shark	26.05427	80.09445	F
N114G	Nurse	Gills	April	Shark	26.36457	80.06137	F
N114S	Nurse	Skin	April	Shark	26.36457	80.06137	F
N114T	Nurse	Teeth	April	Shark	26.36457	80.06137	F
N157C	Nurse	Cloaca	May	Shark	26.02676	80.06018	F
N157G	Nurse	Gills	May	Shark	26.02676	80.06018	F
N157S	Nurse	Skin	May	Shark	26.02676	80.06018	F
N157T	Nurse	Teeth	May	Shark	26.02676	80.06018	F
N197C	Nurse	Cloaca	June	Shark	26.03651	80.05088	M
N197G	Nurse	Gills	June	Shark	26.03651	80.05088	M
N197S	Nurse	Skin	June	Shark	26.03651	80.05088	M
N197T	Nurse	Teeth	June	Shark	26.03651	80.05088	M
N197W	Water	Water	June	Environment	26.03651	80.05088	
N203C	Nurse	Cloaca	June	Shark	26.03651	80.05088	F
N203G	Nurse	Gills	June	Shark	26.03651	80.05088	F
N203S	Nurse	Skin	June	Shark	26.03651	80.05088	F
N203T	Nurse	Teeth	June	Shark	26.03651	80.05088	F
N203W	Water	Water	June	Environment	26.03651	80.05088	
N235C	Nurse	Cloaca	Sept	Shark	26.13051	80.0434	M
N235G	Nurse	Gills	Sept	Shark	26.13051	80.0434	M
N235S	Nurse	Skin	Sept	Shark	26.13051	80.0434	M
N235T	Nurse	Teeth	Sept	Shark	26.13051	80.0434	M
N235W	Water	Water	Sept	Environment	26.13051	80.0434	
SB159C	Sandbar	Cloaca	May	Shark	26.09699	80.04415	F
SB159G	Sandbar	Gills	May	Shark	26.09699	80.04415	F
SB159S	Sandbar	Skin	May	Shark	26.09699	80.04415	F
SB159T	Sandbar	Teeth	May	Shark	26.09699	80.04415	F
SB159W	Water	Water	May	Environment	26.09699	80.04415	
SB174C	Sandbar	Cloaca	May	Shark	26.07791	80.05269	F
SB174G	Sandbar	Gills	May	Shark	26.07791	80.05269	F
SB174S	Sandbar	Skin	May	Shark	26.07791	80.05269	F
SB174T	Sandbar	Teeth	May	Shark	26.07791	80.05269	F
SB174W	Water	Water	May	Environment	26.07791	80.05269	

SB198C	Sandbar	Cloaca	June	Shark	25.58854	80.05157	F
SB198G	Sandbar	Gills	June	Shark	25.58854	80.05157	F
SB198S	Sandbar	Skin	June	Shark	25.58854	80.05157	F
SB198T	Sandbar	Teeth	June	Shark	25.58854	80.05157	F
SB198W	Water	Water	June	Environment	25.58854	80.05157	
T209C	Tiger	Cloaca	May	Shark	26.20859	80.0344	F
T209G	Tiger	Gills	May	Shark	26.20859	80.0344	F
T209S	Tiger	Skin	May	Shark	26.20859	80.0344	F
T209T	Tiger	Teeth	May	Shark	26.20859	80.0344	F
T209W	Water	Water	May	Environment	26.20859	80.0344	
T228C	Tiger	Cloaca	Sept	Shark	26.00659	80.05802	F
T228G	Tiger	Gills	Sept	Shark	26.00659	80.05802	F
T228S	Tiger	Skin	Sept	Shark	26.00659	80.05802	F
T228T	Tiger	Teeth	Sept	Shark	26.00659	80.05802	F
T228W	Water	Water	Sept	Environment	26.00659	80.05802	
TGH107C	Tiger	Cloaca	May	Shark	26.09456	80.05056	F
TGH107G	Tiger	Gills	May	Shark	26.09456	80.05056	F
TGH107S	Tiger	Skin	May	Shark	26.09456	80.05056	F
TGH107T	Tiger	Teeth	May	Shark	26.09456	80.05056	F
TGH116C	Tiger	Cloaca	Nov	Shark	26.02914	80.05036	F
TGH116G	Tiger	Gills	Nov	Shark	26.02914	80.05036	F
TGH116S	Tiger	Skin	Nov	Shark	26.02914	80.05036	F
TGH116T	Tiger	Teeth	Nov	Shark	26.02914	80.05036	F
TGH117C	Tiger	Cloaca	Nov	Shark	26.08349	80.04573	F
TGH117G	Tiger	Gills	Nov	Shark	26.08349	80.04573	F
TGH117S	Tiger	Skin	Nov	Shark	26.08349	80.04573	F
TGH117T	Tiger	Teeth	Nov	Shark	26.08349	80.04573	F
TGH117W	Water	Water	Nov	Environment	26.08349	80.04573	
TGH240C	Tiger	Cloaca	Nov	Shark	26.08018	80.05205	M
TGH240G	Tiger	Gills	Nov	Shark	26.08018	80.05205	M
TGH240S	Tiger	Skin	Nov	Shark	26.08018	80.05205	M
TGH240T	Tiger	Teeth	Nov	Shark	26.08018	80.05205	M

Table S1. Sample table summarizing all environmental and shark samples

II. SUPPLEMENTAL FIGURES

Figure S1. Graphical representation of the 10 most abundant taxa in all sample locations of the nurse sharks sampled. Percentages are calculated based on overall relative abundance across all nurse sharks.

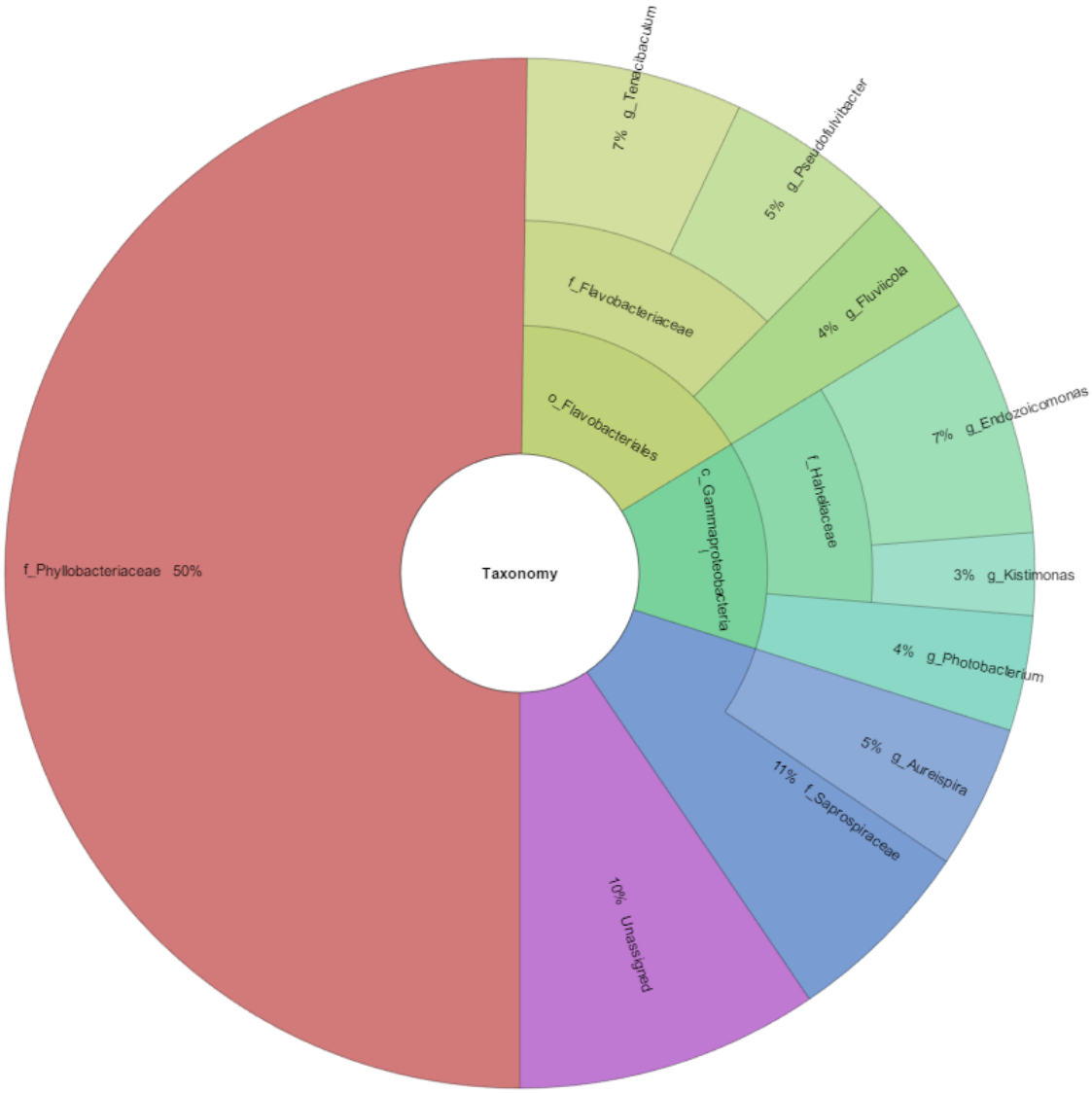

Figure S2. Graphical representation of the 10 most abundant taxa in all sample locations of the lemon sharks sampled. Percentages are calculated based on overall relative abundance across all lemon sharks.

Figure S3. Graphical representation of the 10 most abundant taxa in all sample locations of the tiger sharks sampled. Percentages are calculated based on overall relative abundance across all tiger sharks.

Figure S4. Graphical representation of the 10 most abundant taxa in all sample locations of the Caribbean reef sharks sampled. Percentages are calculated based on overall relative abundance across all Caribbean reef sharks.

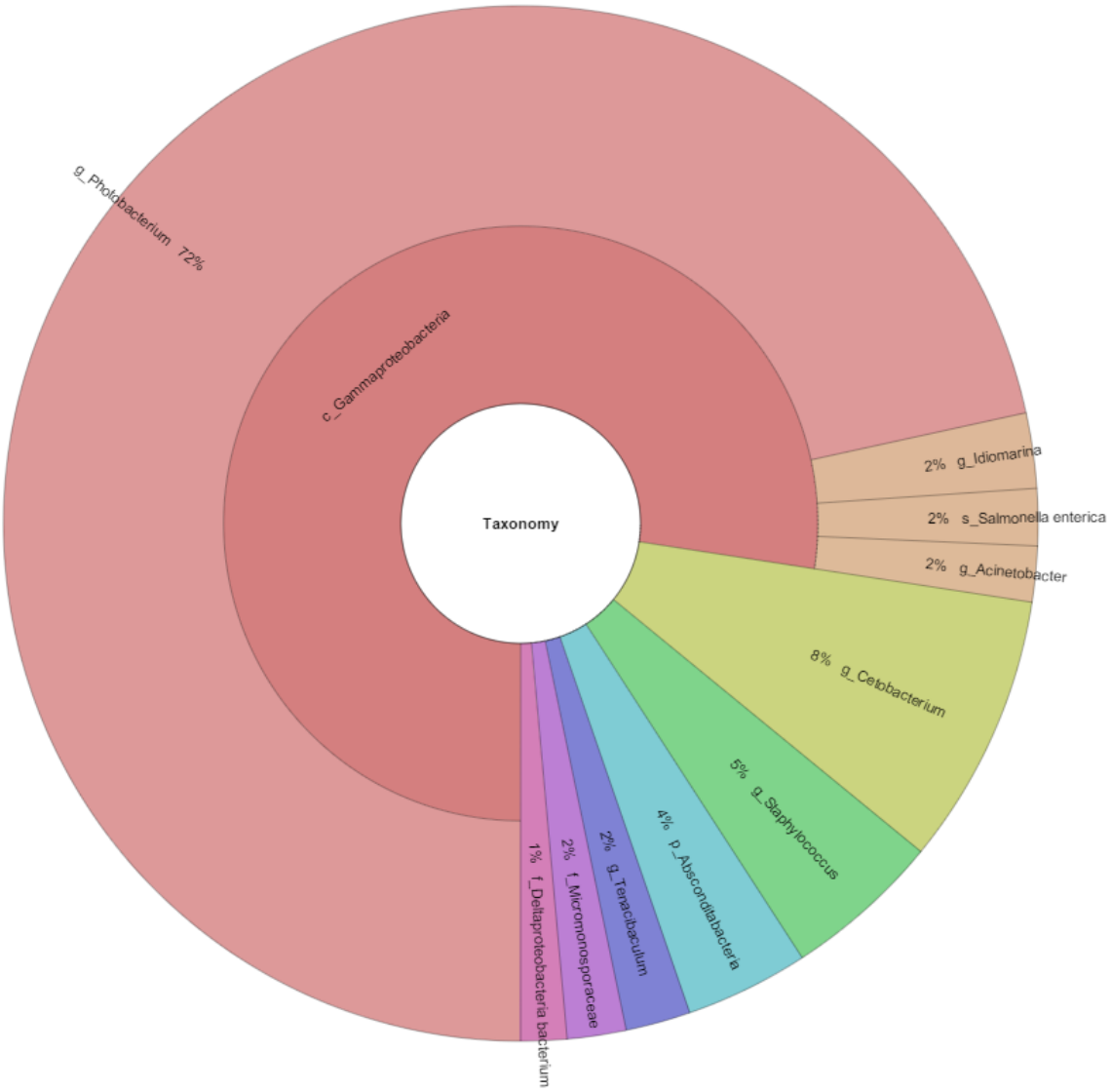

Figure S5. Graphical representation of the 10 most abundant taxa in all sample locations of the sandbar sharks sampled. Percentages are calculated based on overall relative abundance across all sandbar sharks.

CHAPTER 3:

South Florida Shark Teeth Host Uniquely Diverse and Enriched Potentially Infectious Microbial Communities

Abstract

Florida leads as shark bite capitol of the world, with 778 unprovoked bites recorded since 1837. Using high throughput DNA sequencing of the 16S rRNA V4 region, we characterized gill, teeth, skin, and cloacal microbiomes of 5 shark species in south Florida (nurse, lemon, sandbar, Caribbean reef, and tiger). Teeth microbial communities showed the most diversity of all locations based on both Shannon and Inverse Simpson indices. Teeth microbiomes are distinct but share taxa with the seawater, such as *Streptococcus* (8.0% ± 9.0%) and *Haemophilus* (2.9% ± 3.3%) in the Caribbean reef shark. The lemon shark teeth hosted *Vibrio* (10.8% ± 26.0%) and the *Corynebacterium* genus (1.6%±5.1%). The *Vibrio* genus (2.8% ± 6.34%), *Salmonella enterica* (2.6% ± 6.4%), and the genus *Kordia* (3.1% ± 6.0%) are found in the nurse shark teeth microbial community. Strikingly, the *Vibrio* genus was represented in the sandbar (54.0% ± 46.0%) and tiger shark (5.8% ±12.3%) teeth microbiomes. We conclude that south Florida sharks host distinct microbiomes from the surrounding environment and vary among species due to differences in microbial community richness. Future work should focus on bacteria in shark teeth to determine if they are pathogenic, providing insights to bite treatment.

Introduction

The oceans are home to more than 400 species of shark (family *Carcharhinidae*), many of which have declining populations due to bycatch and overfishing. Over the 350 million years that sharks have inhabited the Earth, very little has changed about them. What has changed over these 350 million years, however, is the amount of human interaction with sharks, which has been shown to increase shark bites (Hazin *et al.*, 2008). Even with extensive human interference in sharks' habitat and lifestyle, these creatures have endured in many conditions, suggesting a uniquely adapted immune

system of the apex predator, but it also a constantly evolving microbiome to adjust to changes in their environment (Criscitiello, 2014). These microbiomes include holobionts of the host individual, which are microbiota which coevolved with the host and can change due to environmental stressors (Zilber-Rosenberg and Rosenberg, 2008). Microbiomes of sharks could serve as a unique identifier which could potentially applied to shark bites and treatment.

Shark bites rarely occur, with 84 unprovoked bites worldwide occurring in 2016, four of these being fatal (George H. Burgess, 2016b) . Yet, more than half of the unprovoked bites have occurred in the United States with 778 shark bites for the US. The second most abundant area for unprovoked shark bites is Australia, with only 607 bites since 1580 (George H. Burgess, 2016a). Although not often fatal, shark bites increase the risk of bacterial infection with no specific antibiotic treatment (Fleshler, 2013).Only once the victim is under anesthesia is the wound cleaned with a broad spectrum antibiotic (Fleshler, 2013), or with sterile water (Lupkin, 2014). A thorough characterization of the microbiomes of shark mouths and the taxa which inhabit them could lead to more informed treatment procedures for shark bites.

Current research has shown that the microbiomes of some species can be pivotal and required for the organism to survive. Microbiome importance can be linked to the beneficial functions contributed by symbiont flora, or a shift to a disease state (Caporaso *et al.*, 2011; Sanders *et al.*, 2015; Nelson *et al.*, 2015; Doane *et al.*, 2017; Llewellyn *et al.*, 2014; Colston and Jackson, 2016). In most organisms, individuals have significantly different microbiome compositions in various areas of the body. Much of the compositional diversity remains to be fully unexplained, though likely based on habitat, pH, diet, and varying life stage exposure to microbes (Human Microbiome Project Consortium, 2012a, 2012b).

A common protocol for determining taxonomy of members in a microbial community relies on the most variable regions of the small subunit 16S rRNA gene. By analyzing these gene regions, bacterial taxa in the microbial community, sometimes down to genus can be determined (Woese *et al.*, 1980). Genus was determined for many taxa in this study. The gene that encodes for the 16S molecule is made up of 9 variable

regions (V1-V9) which have different rates of evolution and diversity in their sequence (Wang *et al.*, 2007).

This study examines three main hypotheses concerning five south Florida shark microbiomes. First, we hypothesize that *Elasmobranch* bacterial microbiomes vary in community composition when compared to the surrounding environment. Secondly, individual composition of the microbial communities varies based on the sample area (location) on the organism. Lastly, microbial communities of shark species will have varying compositions. Therefore, each site should be more similar among individuals of the same species than individuals of different species. To test these hypotheses, we have applied routine high throughput DNA sequencing of the 16S V4 amplicon libraries, followed by rigorous statistical analyses of all shark microbiomes.

Materials and Methods

Sample Collection

Individuals were caught and released once samples and measurements are taken. The sharks were caught using a rig that contains a fifty-pound weight and a line with a buoy on the top. Attached to the weight was a 30.49m (meter) 408.22kg (kilogram) tested microfilament line with a circle hook and atlantic bonito as bait (*Sarda sarda*) to attract sharks nonspecifically. Gear was set in groups of 10, with two at each of the following depths: 7.6m, 12.2m, 18.3m, 24.4m, and 30.5m. Samples were collected opportunistically and randomly, resulting in 8 total species, but only 5 species had sufficient sample size. Four samples per individual (gills, teeth, skin, cloaca) were taken using dual sterile swabs (Henry Schein, Melville, NY, Cat. 1228715), which were transported in a cooler to the Microbiology and Genetics lab at the Halmos College of Natural Science and Oceanography (Dania Beach, FL). Experimental design followed the tenets of (Knight *et al.*, 2012) for the minimum number of samples required, as well as including all possible "metadata" associated with each sample.

1 liter samples were taken off the back of the boat when sharks were caught, with sterilized Nalgene bottles. These bottles were submerged in surface water and rinsed once before filling with the actual sample. Water samples were transported in a cooler full of

ice to the lab for filtration. Water samples filtered with a .45 µl filter after each trip so that any environmental microbes could later be characterized. After filtration, environmental DNA (water samples) was extracted with the DNeasy PowerLyzer PowerSoil kit (Cat# 12855-100), and swabs were extracted using the QIAamp BiOstic Bacteremia DNA kit (Cat# 12240-50) (MoBio Laboratories Inc.). Purified DNA was amplified using PCR (Polymerase Chain Reaction) and primers R806 and F515 targeting the V4 region of the 16S rRNA gene.(Caporaso *et al.*, 2011). Amplicons were sequenced with an Illumina MiSeq sequencing platform equipped with a V2 chemistry 500 cycle cartridge (Caporaso *et al.*, 2012). Initial processing of sequence data was performed in MacQIIME (Quantitative Insights into Microbial Ecology) version 1.9.1 (2016). All sequences were submitted to the Sequence Read Archive (SRA) under the project accession number: SRP111970 (Release date: 07-14-2017)

Statistical Analysis

Raw sequences were quality filtered to remove all chimeric and low quality (quality score < 30) sequences. These sequences were then clustered into 97.0% similar Operational taxonomic units (OTUs) using open reference OTU clustering strategies. Data processing was executed using MacQIIME 1.9.1 ("MacQIIME - Werner Lab," 2016). OTUs were picked based on the SILVA database instead of Greengenes, because of the frequency of updates and ease of accessibility. Microbial community differences were examined between sampling areas (gills, teeth, skin, and cloaca), species, individuals, and the environmental communities. OTUs which were associated with only water samples were removed from the other samples. There were no OTUS which were found solely in teeth microbial communities. Analysis was executed with the RStudio software (RStudio version 3.2.1), with the added libraries 'picante' and 'vegan' to examine general ecology of the microbiome (Kembel *et al.*, 2010; RStudio Team, 2015; R Core Team, 2013). Comparisons of OTU content in the context of bacterial diversity and composition between species was analyzed to determine compositional differences in teeth microbiomes (Caporaso *et al.*, 2010).

Significant differences in Shannon and Inverse Simpson diversity measures were assessed using an analysis of variance (ANOVA) (Oksanen, 2017b). A Tukey's post-hoc test was used to examine pairwise significant differences among groups. A permuted multivariate ANOVA (PERMANOVA; adnois in vegan package) was used to assess significant differences in Bray Curtis dissimilarity among sample groups. A SIMPER test (499 permutations) was then used to discriminate which microbial taxa distinguished groups based on the Bray Curtis dissimilarities. SIMPER performs comparisons of data in a pairwise fashion which results in the average contributions of each sampling unit (OTU) to the overall dissimilarity of shark teeth by species (Oksanen, 2017a). Beta dispersion analysis based on the Bray Curtis Distance of the samples by species was done to check if any species are significantly more variable than the others. It was shown by an ANOVA that there was a significant difference in the average distance to the spatial median among species.

Results

Overall Shark Microbiome

A total of 12,374,571 MiSeq reads and 26,309 OTUs were generated across all samples in this study (Table 4). From the initial 136 samples collected, 127 were successfully sequenced (31 cloaca, 32 gills, 32 skin, and 32 teeth) with a mean read depth of 97,438. Samples with less than 1000 sequences were excluded, due to inadequate representation. There is a significant difference between the water and shark samples by the inverse Simpson index, and a larger range of diversity in sharks than the water, due to rare taxa. Shark samples had significantly less microbial richness than water. Sharks had more evenness than water, with a higher microbial diversity. There is a 33.0% overlap of OTUs between water and shark samples. Sharks and the water they inhabit are clearly sharing some taxa, but still have distinct microbial environments (Figure 14).

Significant differences were found in microbial community richness among species (ANOVA, df=4, F=2.888, p=.0256), but not among location (ANOVA, df=3, F=.351p=.788), or the interaction of species and location (ANOVA, df=12, F=1.323,

p=.2168). The Shannon index showed significant differences by location (ANOVA, df=3, F=9.832, p<.001) and the interaction between species and location (ANOVA, df=12, F=4.05, p=<.001), but not by species alone (ANOVA, df=4, F=.512, p=.727). Diversity as measured by the Inverse Simpson index showed significant differences in communities by location (ANOVA, df=3, F=4.952, p=.0029), but not by species (ANOVA, df=4, F=1.58, p=.184) or the interaction of species and location (ANOVA, df=12, F=1.764, p=.065).

Based on the Tukey post-hoc test, significant differences in diversity by location are explained predominantly by differences between teeth samples and all other locations by the Inverse Simpson index (Teeth-Cloaca, p=.023; Teeth-Gills, p<.001; Teeth-Skin, p<.001). The Shannon index had slightly less significant values, but still set teeth apart from other locations (Teeth-Cloaca, p=.235; Teeth-Gills, p=.002; Teeth-Skin, p=.027). We show by ANOVA there is a significant difference in the average distance to the spatial median between species (ANOVA, df=4, F=3.774, p=.006436). Lemon sharks had the highest average distance to the median of .6342, followed by nurse sharks (.6221), tiger sharks (.6096), sandbar sharks (.5899), and Caribbean reef sharks (.5832). Based on these statistics, it shows that lemon and nurse sharks have a more variable microbiome within their respective groups than the other species which were sampled (Figure 15).

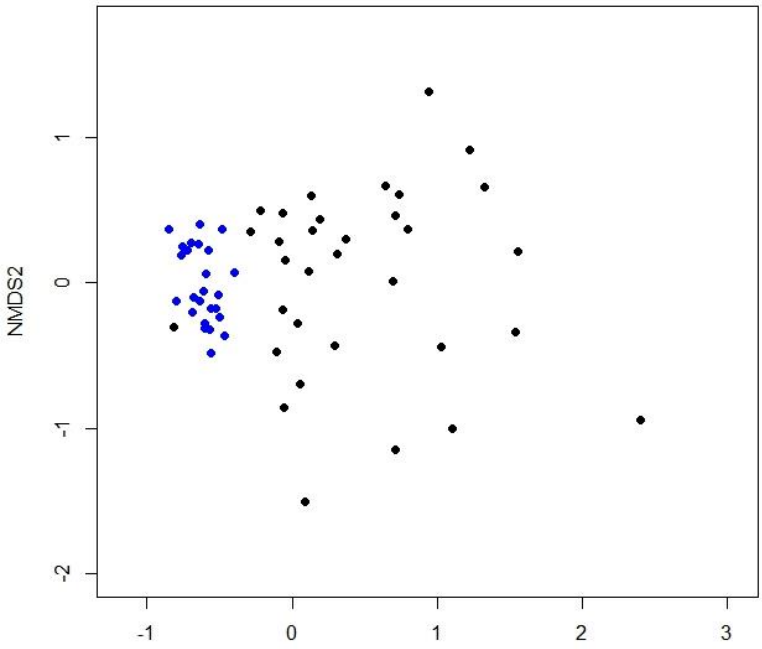

Figure 14. NMDS analysis of teeth samples compared to ambient water samples. This plot shows an overlapping yet distinct microbial community associated with shark teeth. (Green=Teeth, Blue=Water).

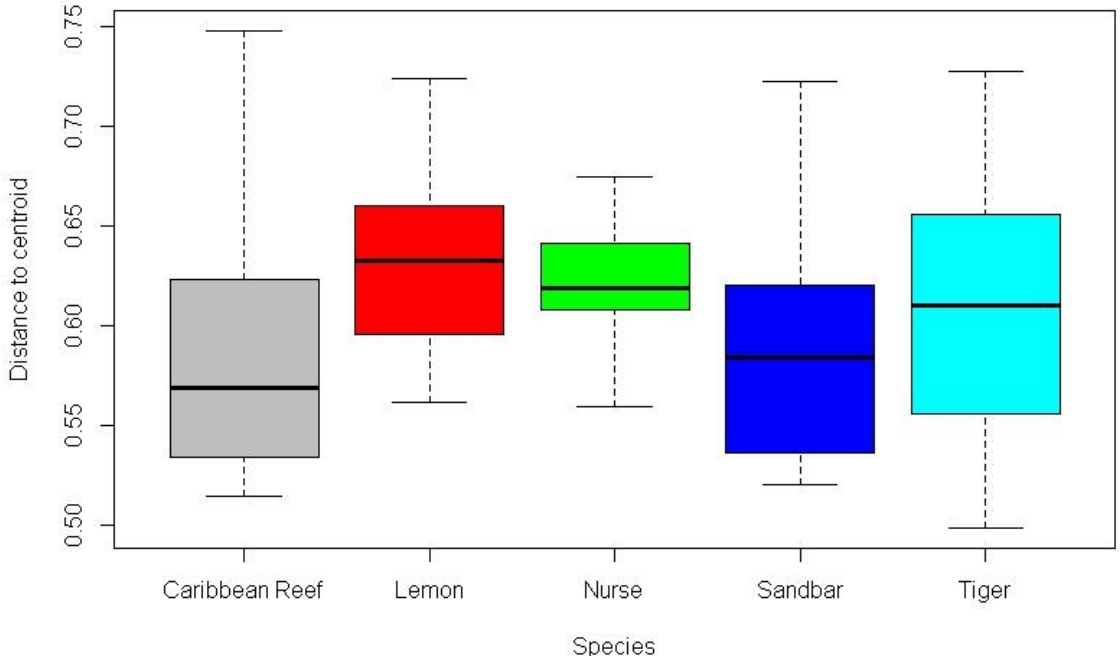

Figure 15. Beta dispersion analysis of teeth samples by species. (ANOVA, df=4, F=3.774, p=.006)

Teeth Microbiome

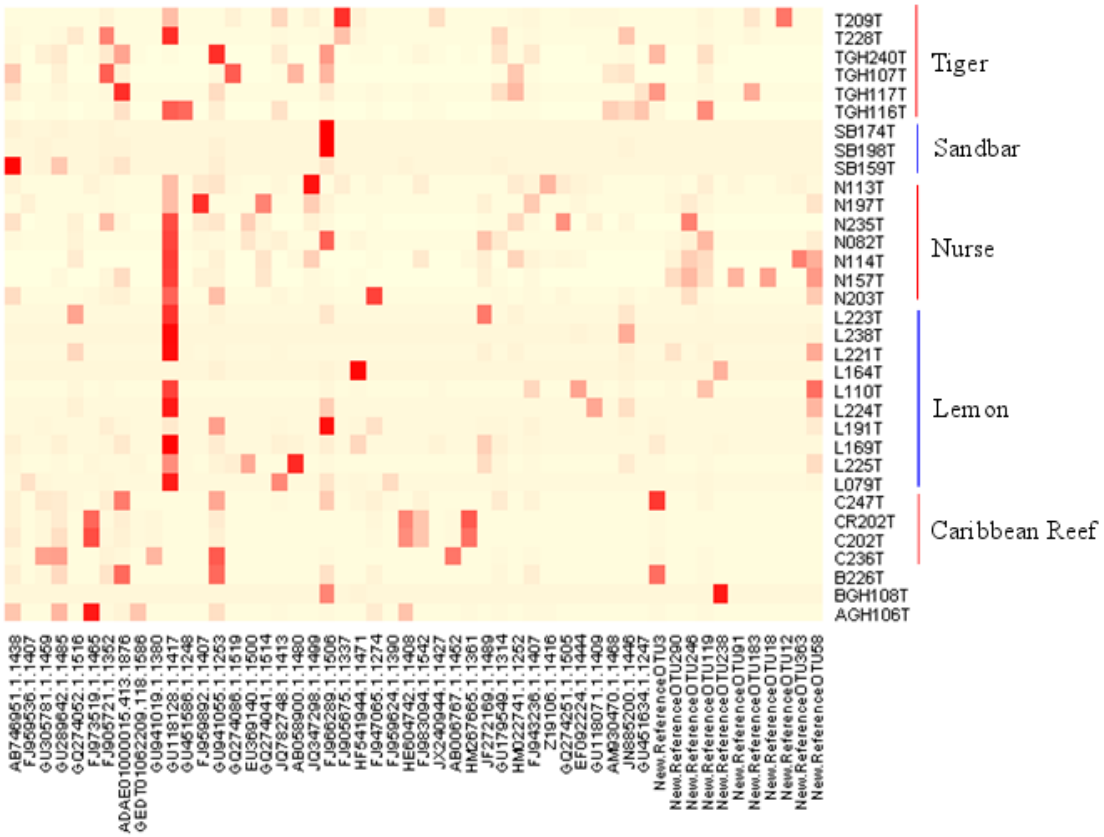

Figure 16. Heatmap showing the most abundant OTUs associated with shark teeth samples. Sample code names are on the y-axis, with OTUs on the x-axis. In the sample codes, T=tiger, SB=sandbar, N=nurse, L=lemon, C or CR= Caribbean reef, B=bull, BGH=blacktip, A=atlantic sharpnose.

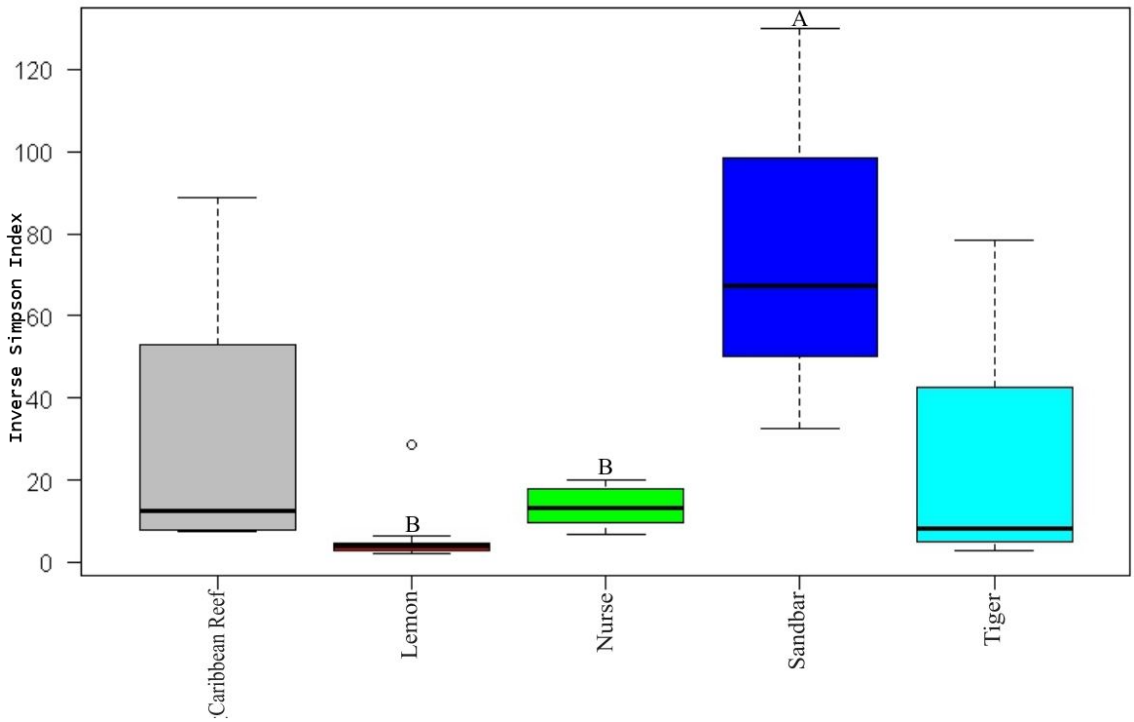

Figure 17. Box plot of mean species diversity across all teeth samples by species based on the Inverse Simpson index. Group B is significantly different from group A. (ANOVA, df=4, F=5.148, p=.0036)

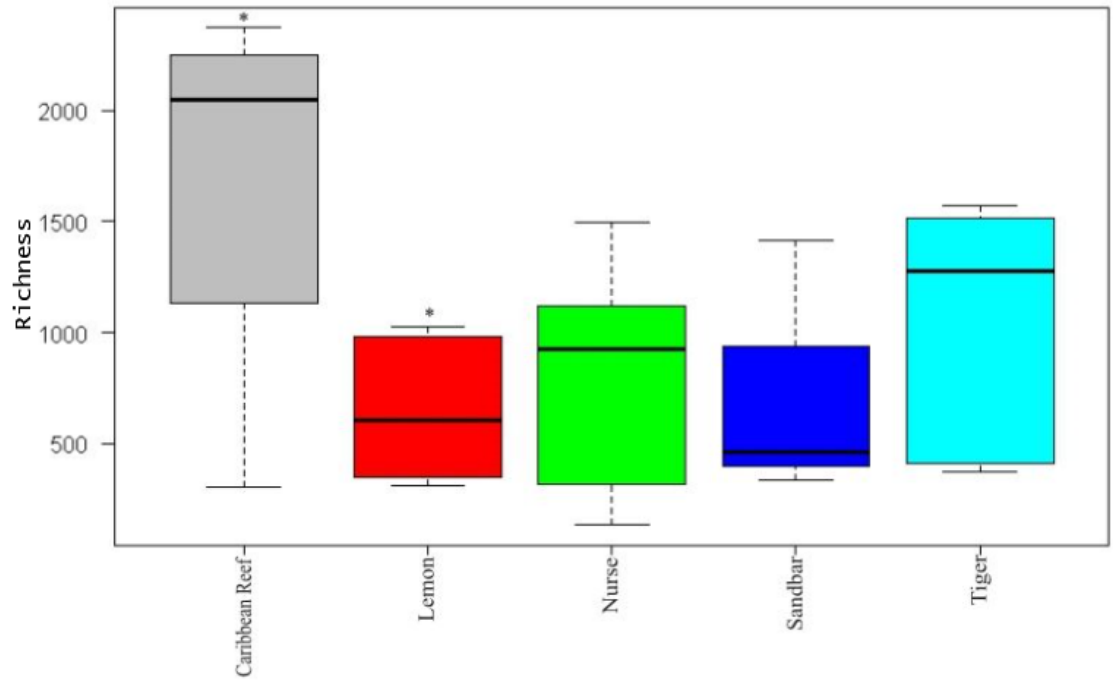

Figure 18. Box plot of mean species richness across all teeth samples by species. "*" represents significant differences. (ANOVA, df=4, F=2.998, p=.0377)

There was a significant difference in the microbial community richness (ANOVA, df=4, F=2.998, p=.0377) (Figure 17), diversity as measured by the Inverse Simpson index (ANOVA, df=4, F=5.148, p=.0036) (Figure 18), and the Shannon index (ANOVA, df=4, F=6.178, p=.00134) among all teeth samples from all species sampled. Significant differences in richness were driven by differences between lemon and Caribbean reef sharks (p=.024), and diversity by differences in lemon and Caribbean reef sharks (p=.006) as well as sandbar and lemon sharks (p=.004).

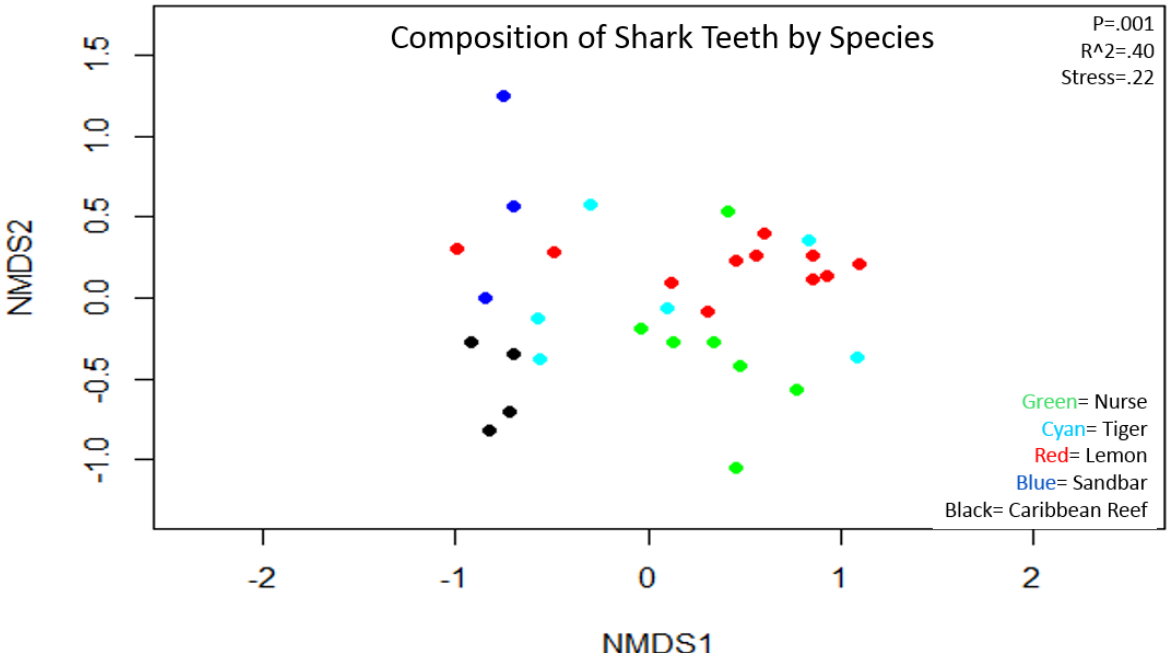

Figure 19. Non-metric dimensional scaling of teeth samples by species. (R2=0.40, stress=0.22, p=.001)

Differences in diversity by the Shannon index were explained by the significant difference between Caribbean reef shark teeth and both sandbar and lemon teeth. The Inverse Simpson index showed that the differences between sandbar and nurse sharks also were significantly driving the overall differences in teeth (p=.008).

NMDS visualization of shark teeth samples among species showed slight clustering by species (NMDS, R^2=0.40, p=.001) (Figure 19). In the context of all locations among species, data points which are most different from the rest of the data are the teeth samples from tiger, lemon, and nurse sharks (NMDS, R^2=.15, p=.004) (Figure 20).

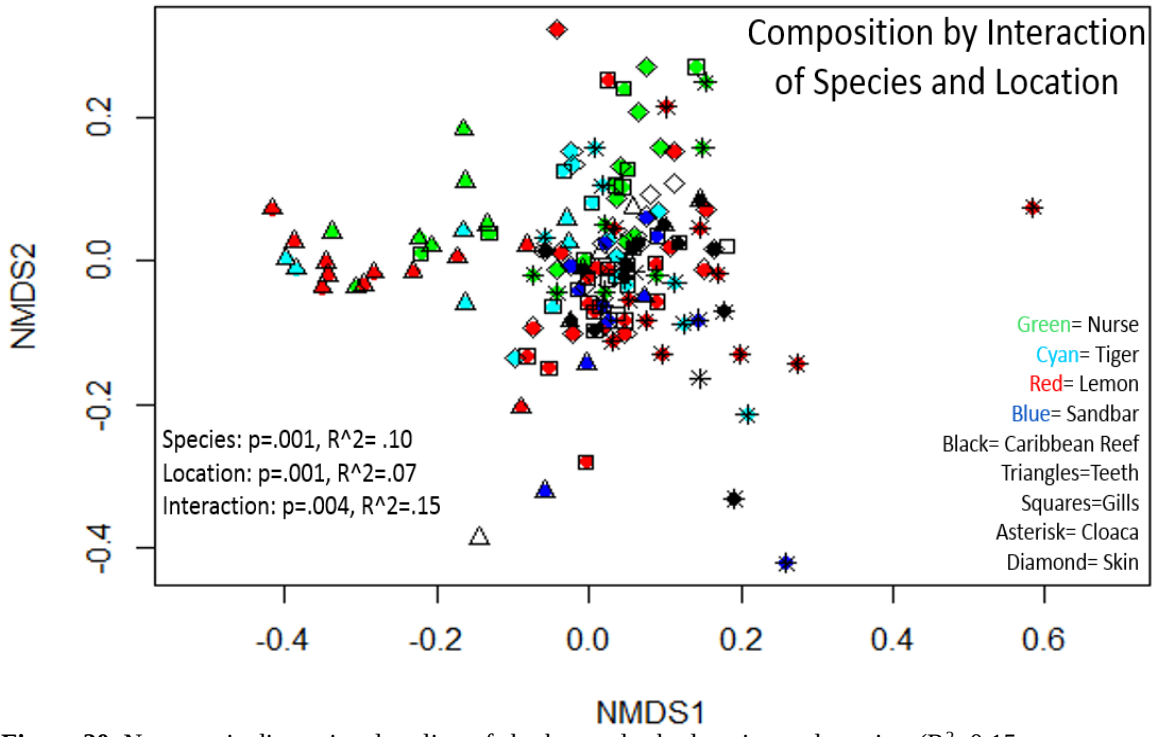

Figure 20. Non-metric dimensional scaling of shark samples by location and species. (R^2=0.15, stress=0.22) Outliers are mostly lemon, nurse, and tiger teeth samples.

A PERMANOVA showed significant differences in teeth communities with (PERMANOVA, df=4, F=2.07, R^2=.249, p=.001) and without (PERMANOVA, df=4, F=2.85, R^2=.313, p=.001) environmental OTUS (Table 5). NMDS (Non-metric multi-dimensional scaling) illustrated further differences in microbial communities between shark teeth and ambient water at the time of the collection (Figure 16). When examining the SIMPER results for the comparison of teeth to the ambient water, *Phyllobacteriaceae* (GU118128.1.1417) was a major driver and explained 18.0%-32.0% of differences among species, and represented a large portion of the teeth microbial community (17.0% ± 20.5%). This family was represented strongly in nurse and lemon sharks (Figure 16).

Potentially Pathogenic Taxa

Vibrio was not found to be in the 20 most prevalent taxa in all teeth samples, and so varies in relative abundance by species. The *Vibrio* genus was represented in the sandbar (54.0% ± 46.0%), tiger (5.8% ±12.3%), nurse (2.8% ± 6.34%), and lemon shark (10.8% ± 26.0%) teeth microbiomes, respectively. There are other taxa or groups which contain pathogenic taxa found in the overall teeth microbiome, however. The

Streptococcus genus (0.8% ± 2.9%) was present in overall teeth microbial composition among species, and Caribbean reef shark (8.0% ± 9.0%) teeth microbial environment. The *Haemophilus* genus is found in the Caribbean reef shark teeth microbiome (2.9% ± 3.3%). In nurse sharks, *Salmonella enterica* is most prevalent among shark species (2.6% ± 6.4%) of the teeth microbial environment.

Discussion

Shark anatomical location analysis

When microbial communities of the anatomical locations were compared across all samples, there was a significant difference in the diversity of these communities, but not in the richness. When an SIMPER test was performed, distinct microbial communities on shark teeth were the primary drivers for compositional differences among anatomical locations. When also examining the location with relation to species, it was narrowed down further to show that the differences were being driven by lemon shark teeth communities.

When examining the SIMPER comparison between nurse and lemon shark teeth, it was shown that 2.0% of differences were explained by the OTU GQ274041.1.1514, which represents the genus *Kordia*, which includes species that have exhibited strong algicidal effects on diatoms. Species of this genus have been isolated in a junction between the ocean and a stream of fresh water (Park *et al.*, 2014). This genus could be relevant in explaining microbial community differences in the lemon and nurse shark teeth samples due to the lemon shark's unique ability to survive in freshwater environments (Ebert *et al.*, 2015).

Shark holobiont behavior

In this study, we provide one of the first comprehensive surveys of shark microbiomes from south Florida using high throughput 16S rRNA analyses. We find that although shark teeth microbiomes vary from the water in which the host inhabits, there is an overlap in the taxa. Additionally, the differences we do find are not explained solely by shark species or location, and there could be other factors influencing microbial

composition. Differences appear partially attributable to shark species' respective ecology and characteristics. For example, blacktip sharks feeding primarily upon teleosts, and also crustaceans, cephalopods, and other *Elasmobranchs* (Kajiura and Tellman, 2016). Blacktip sharks are migratory, and aggregate in southeast Florida to overwinter in the waters near the shore. Their migratory path is thought to be dominated by water temperature, and tends to coincide with the spawning of bait fish species. Several common features of nurse *(Ginglymostoma cirratum*) and lemon (*Negaprion brevirostris*) sharks could affect their similar teeth microbiomes: nurse sharks are nocturnal, sluggish, prefer temperate/tropical waters, and typically bottom feed (Parsons, 2006). Nurse sharks feed on a variety of different prey, including but not limited to shrimps, crabs, lobsters, squids, fishes, snails, and octopuses (Parsons, 2006). Similarly, the lemon shark will prey upon rays or crayfish are known to be opportunistic feeders, particularly as juveniles, though their main prey is bony fish (Beck, 2016; Stafford-Deitsch, 2000). We cannot conclude what other factors are having an effect on distinguishing these microbiomes based on this data, but can infer based on previous studies (Human Microbiome Project Consortium, 2012b, 2012a) that there are likely many factors that are collectively causing varying compositions in microbial environments.

Teeth Microbiome

The OTU driving most significant differences in shark teeth microbial communities is GU118128.1.1417, which is representative of the family *Phyllobacteriaceae*. This family of 13 genera and 72 species are plant and environmentally associated, and have a heterotrophic, respiratory metabolism that utilizes oxygen as the terminal electron acceptor (Willems, 2014). Members of this family are associated with sponges and may undergo adaptive processes, as shown by their occurrence in diverse environments like water and soil, as well as other unicellular organisms (Liu *et al.*, 2012). Lemon and nurse shark teeth are enriched for this OTU compared to other species. SIMPER results comparing teeth to ambient seawater show that this OTU accounted for only 9.0% of differences in samples. However, comparing

only within all teeth samples across species, this OTU explained between 18.0% and 32.0% of differences (Supplementary Figure 7).

SIMPER results comparing lemon to Caribbean reef sharks showed OTUs which are driving the differences between these two species' teeth microbiomes, such as one representing the genus *Haemophilus*, which explains 7.0% of the differences of the teeth microbiomes of these species. *Haemophilus* includes some pathogenic species, such as *Haemophilus influenzae*, but also has been associated with the saliva microbiome in humans. Thirdly, the OTU FJ983094.1.1542, representing the genus *Streptococcus*, explains 2.0% of the variance between Caribbean reef and lemon shark teeth microbiomes. This genus includes some pathogenic species, but also species which are associated with the human microbiome and are important to overall function (Human Microbiome Project Consortium, 2012b). Both taxa are abundant in sandbar shark teeth sampled, and not within the top 10 taxa of the Lemon shark teeth (Figure 3, 4).

When examining the SIMPER comparison between nurse and lemon shark teeth, OTU GQ274041.1.1514 explains about 2.3% of differences. This taxon represents the genus *Kordia*, which includes species that have exhibited strong algicidal effects on diatoms. Species of this genus have been isolated in a junction between the ocean and a stream of fresh water (Park *et al.*, 2014). The *Kordia* genus was found in the nurse shark teeth microbial composition (3.12% ± 6.0%). This genus could be relevant in explaining microbial community differences in the lemon and nurse shark teeth samples due to these species' unique ability to survive in freshwater environments (Ebert *et al.*, 2015).

Because there is an obvious overlap in the taxa found in the microbial community of the water and the teeth, it is important to note some other factors that could be affecting shark teeth microbiomes. Environmental influences could include depth range, migration patterns, and salinity. Lemon sharks (*Negaprion brevirostris*) are migratory and tend to favor inshore and coastal waters, but can enter freshwater (Ebert *et al.*, 2015; Stafford-Deitsch, 2000). Additionally, lemon sharks are known to inhabit depths of 91.44m or deeper during migration, but tend to move to shallower waters for birthing (Beck, 2016). Lemon sharks have the ability to supplement ram ventilation (having to remain in motion to push water over the gills) with buccal pumping, which is a process

which allows the shark to remain still on the ocean bottom (or otherwise) while pumping water over their gills to breathe (Brooks *et al.*, 2011). The lemon shark mostly feeds on bony fish, but will also prey upon rays or crayfish and are known to be opportunistic feeders, particularly as juveniles (Beck, 2016; Stafford-Deitsch, 2000).

Overall Shark Microbiome

When analyzing samples based on differences in shark species, no significant differences in overall microbial community diversity appeared. Significant difference did occur in richness, however, meaning that some shark species have varying amounts of microbe species in their overall microbiome than others, but not a significant difference in the evenness of these species. This suggests that low-abundance populations can be present which are driven by 'rare biospheres' (Sogin *et al.*, 2006). Richness does not account for how many individuals of each species are present, however, so it is possible that some shark species have a few rare taxa compared to another species. It is clear by looking at the top ten most abundant taxa that some are far more represented then others. This explains the significant difference in richness but not diversity. *Photobacterium* include the species *Photobacterium damselae* which is an established pathogen for marine animals such as crustaceans, fish, and molluscs, as well as for humans (Terceti *et al.*, 2016). Additionally, *Photobacterium* belong to the family *Vibrionaceae*, which include bacteria that frequently coexist on a marine animal host, such as potential prey for varying species of shark (Urbanczyk *et al.*, 2011).

Based on Beta Dispersion analysis, lemon sharks had the furthest distance from the spatial median of all other species, with nurse sharks just slightly less, followed by tiger sharks (Figure 15). All three of these species can utilize buccal pumping- or in the case of the nurse shark, rely solely on it. Buccal pumping allows species to remain still for long periods of time, typically on the sea floor, while pumping water over their gills to breathe (Dapp *et al.*, 2016; Gibson and Carter, 2002; Brooks *et al.*, 2011). This could account for the slight difference in microbial communities indicated by the Beta Dispersion analysis. Additionally, the nurse and lemon sharks are known to enter fresh water environments on occasion, which could explain why these species have microbial

communities which are more similar to each other than other species (Ebert *et al.*, 2015; Stafford-Deitsch, 2000; Hendon *et al.*, 2013).

When examining the taxonomy of the species utilized in this study, the nurse shark is the only shark which is not considered a requiem shark, but instead a carpet shark. Lemon sharks belong to the *Negaprion* genus, and sandbar, Caribbean reef, and tiger sharks belong to the *Carcharinus* genus. Lemon and nurse sharks have different genus' than the other species sampled, which could explain the microbial community differences seen in the Beta Dispersion analysis (Gibson and Carter, 2002).

Potentially Pathogenic Taxa

Most often, the bacteria which are isolated from infected bite wounds reflect the oral flora of the organism responsible for the bite. Many taxa which are found in this study are also cause of concern in other animal bite wounds, such as *Streptococcus*, *Staphylococcus*, *Corynebacterium*, *Enterococcus*, and *Haemophilus*. *Vibrio, Salmonella enterica*, *Psychrobacter*, and *Halomonas* all are more specific to be a concern in aquatic organisms and reptiles. *Vibrio* was found to be a concern predominantly in shark bites (Abrahamian and Goldstein, 2011).

All species hosted either a genus or species which are known to be pathogenic to humans. *Vibrio* was not found to be in the 20 most prevalent taxa in all teeth samples, and so varies in relative abundance by species. The *Vibrio* genus is by no means entirely infectious or pathogenic, with 13 species of the total 129 *Vibrio* species (9.9%) causing vibriosis in 2014 in the United States. Vibriosis is most commonly transmitted by water or undercooked seafood (Center for Disease Control, 2015). *Vibrio carchariae* has been previously shown to cause an infection after a shark bite (Pavia *et al.*, 1989). It is recommended that an infection of *Vibrio* is treated immediately to prevent further infection and perhaps mortality (Buck *et al.*, 1984). Although we are not able to determine unequivocally which species of *Vibrio* is present in shark teeth in this study, it is not unreasonable to suggest that because of the high rate of infection and established presence in infections associated with shark bite wounds, it is likely that the *Vibrio*

populations characterized here are pathogenic to humans (Fleshler, 2013) (Cottingham *et al.*, 2003).

Tiger sharks hosted *Vibrio*, and it was the main potentially pathogenic taxa in the teeth microbial community (5.8% ±12.3%) (Figure S3). Tiger sharks have a much more diverse diet than other species sampled, and this could have a confounding effect on the teeth microbiome. This species of sharks is known to consume garbage of human origin, including plastics, metals, and scraps. The normal tiger shark diet is also quite diverse, with known prey including teleosts, rays, other sharks, turtles, birds, dolphins, seals, cephalopods, sea snakes, lobsters, crabs, gastropods, and jellyfish. Tiger sharks will also feed on carrion and are not known to shy away from baited hooks (Randall and Randall, 1992). Tiger sharks have the ability to switch between buccal pumping and ram ventilation as needed, which allows for change in swimming speed (Dapp *et al.*, 2016).

Caribbean reef shark teeth host the most diverse community of potentially pathogenic taxa, compared to other species. Caribbean reef sharks are found to be abundant in the Caribbean and on coral reefs, in a depth range of 45.8-274.3m (Brooks *et al.*, 2012). The diet of the Caribbean reef shark consists of bony fish and other elasmobranchs, as well as occasional cephalopods (IUCN, 2006). The Caribbean reef shark has both a wide vertical and temperature range which allows for them to inhabit both shallow and deeper reef ecosystems (Chapman *et al.*, 2007).

One group which contains pathogenic taxa found in the overall teeth microbiome of Caribbean reef sharks is the genus *Halomonas*. *Halomonas venusta* has been documented to have caused infection after fish bites (von Graevenitz *et al.*, 2000). In this study, we are unable to identify this taxon by species, and only by genus. Another genus isolated from Caribbean reef shark teeth includes the *Haemophilus* genus (2.9% ± 3.3%) , that houses multiple human pathogenic species of bacteria which cause disease and infection at varying success rates (Musher, 1996). Another pathogenic species, *Salmonella enterica* (2.6% ± 6.4%) is found in nurse shark teeth. *Salmonella enterica* is shown to host fish as well as other animals, and cause infections in humans through contact with an infected animal (Government of Canada, 2001). Most human infections

caused by *Salmonella enterica* are associated with undercooked food, and can result in bacteremia, enteric fever, or gastroenteritis (Public Health Agency of Canada, 2010).

Salmonella enterica is typically associated with fresh water instead of saltwater, and so the presence of this species in nurse sharks could be attributable to the unique ability of nurse sharks to enter fresh water, being found in the Mississippi Sound which has a high input of fresh river and estuarine water (Hendon *et al.*, 2013).The nurse shark (*Ginglymostoma cirratum*) is the only representative of the *Ginglymostoma* genus found in the western Atlantic and are sluggish, nocturnal bottom feeders (Parsons, 2006). Nurse sharks prefer shallow temperate or tropical waters, and are typically found on hard bottoms where the temperature, dissolved oxygen, salinity, and water clarity are high, but can also be found at depths of up to 70.10m. Nurse sharks are able to remain on the bottom for long periods of time because they utilize buccal pumping to push water over their gills (Gibson and Carter, 2002; Hannan *et al.*, 2012). As bottom feeders, nurse sharks feed on a variety of different prey, including but not limited to shrimps, crabs, lobsters, squids, fishes, snails, and octopuses (Parsons, 2006).

Even more significant than the Caribbean reef shark teeth is the microbial community on the sandbar shark teeth. Sandbar sharks (*Carcharhinus plumbeus*) are a coastal species with a wide range in tropical and temperate regions (McElroy *et al.*, 2006). This species can be found at depths of 274.3m , but are found typically at 91.44m or less where they forage sea beds for prey (IUCN, 2007). Sandbars tend to feed on teleosts, with occasional cephalopods and crustaceans, with more crustaceans taking up a larger portion of younger sharks' diets (McElroy *et al.*, 2006). Sandbar sharks are a migratory species, with seasonal migrations from north to south on the eastern coast of the United States (Romine *et al.*, 2006). The *Vibrio* genus represents roughly half of the microbial composition of shark teeth (54.0% ± 46.0%). When compared to the microbial community of the gills, teeth, skin, and cloaca combined of the sandbar sharks, *Vibrio* are not found to be in the top ten most abundant OTUs (Supplementary Figure 8).

Conclusions

We conclude that sharks in south Florida host unique microbial communities, with significant differences in composition across all species. Our data show that sharks have teeth microbial communities which are specifically enriched for groups which contain pathogenic taxa when compared to other locations on the individual, as well as to the ambient environment in which they inhabit. Overall, across all sample locations, all shark body microbiomes appear distinct from the surrounding seawater in diversity and richness. We conclude that south Florida sharks host distinct microbiomes from the surrounding environment and vary among species due to differences in microbial community richness. Future work should focus on bacteria found in shark teeth to determine if those present are pathogenic and could provide insights to bite treatment.

Appendices:

I. Tables

Summary Sequencing Statistics	
Total Reads:	12,374,571
Range for Individual Samples:	1,116-545,115
Total OTUs:	26,309

Table 4. Summary sequencing data for all species (nurse, tiger, sandbar, Caribbean reef, and lemon) and water samples. Total reads are a sum of all reads for three separate sequencing runs that the samples were sequenced on. Range for individual species indicates the lowest number of reads among all samples, and the highest among all samples.

Test	**P-value**
Richness	.0377
Diversity-Shannon	.00134
Diversity-Inverse Simpson	.00364
Adonis	.001

Table 5. Summary of p-values of statistics when teeth samples were compared based on shark species, with environmental OTUs removed.

Comparison	Sample Size	Mean Richness	Shannon (ANOVA)	Inverse Simpson
Species	117	765.6± 516.9	df=4, F=.512, p=.727	df=4, F=1.58, p=.184
Location	117	765.6± 516.9	df=3, F=9.832, p<.001	df=3, F=4.952, p=.0029
Species:Location	117	765.6± 516.9	df=12, F=4.05, p=<.001	df=12, F=1.764, p=.065

Table 6. Summary of statistics and sample size for each grouping considered in comparisons of the shark microbiome.

II. Supplemental Figures:

Figure S6. Krona Graphical representation of the 10 most abundant taxa in all sample locations of the nurse sharks sampled. Percentages are calculated based on overall relative abundance across all nurse sharks.

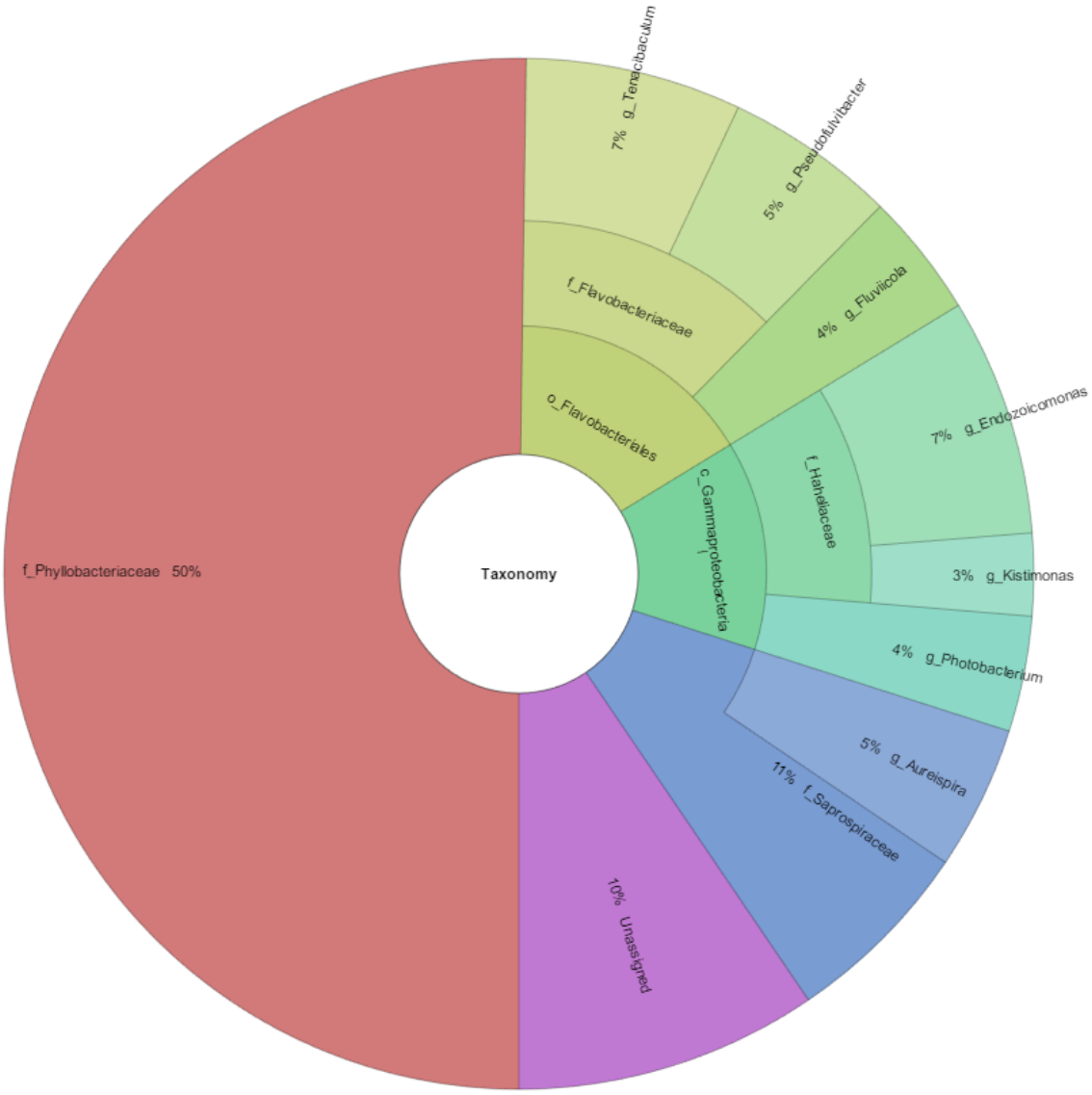

Figure S7. Krona Graphical representation of the 10 most abundant taxa in all sample locations of the lemon sharks sampled. Percentages are calculated based on overall relative abundance across all lemon sharks.

Figure S8. Krona Graphical representation of the 10 most abundant taxa in all sample locations of the tiger sharks sampled. Percentages are calculated based on overall relative abundance across all tiger sharks.

Figure S9. Krona Graphical representation of the 10 most abundant taxa in all sample locations of the Caribbean reef sharks sampled. Percentages are calculated based on overall relative abundance across all Caribbean reef sharks.

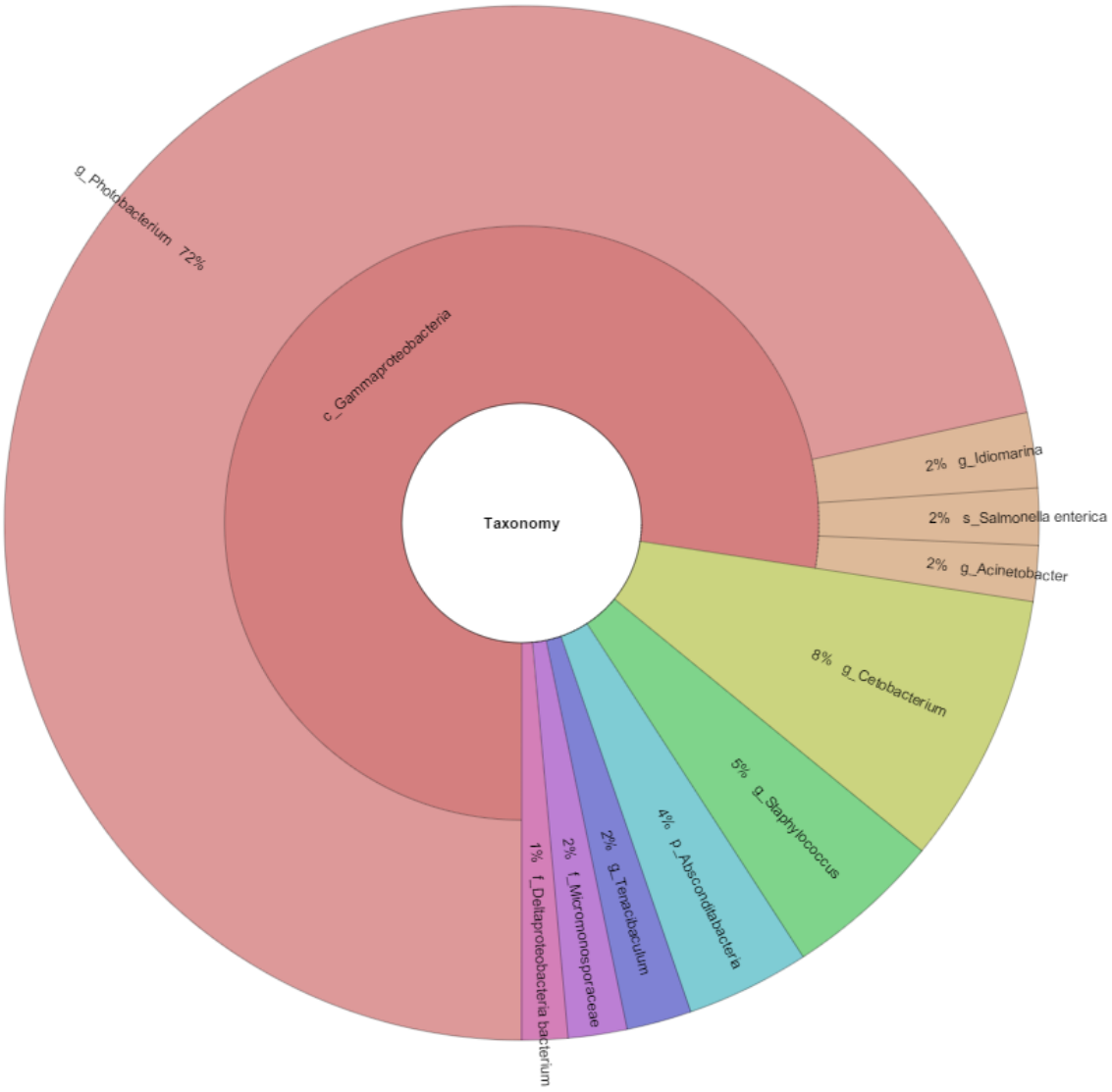

Figure S10. Krona Graphical representation of the 10 most abundant taxa in all sample locations of the sandbar sharks sampled. Percentages are calculated based on overall relative abundance across all sandbar sharks.

Contrast: Teeth_Water

	average	sd	ratio	ava	avb	cumsum	p	
GU941055.1.1253	9.488e-02	4.133e-02	2.2955	1.997e-02	2.091e-01	0.1092	0.002	**
GU118128.1.1417	7.094e-02	8.803e-02	0.8058	1.419e-01	6.965e-06	0.1909	0.030	*
FJ966289.1.1506	4.129e-02	9.923e-02	0.4161	8.312e-02	7.389e-04	0.2384	0.706	
FJ905721.1.1352	2.242e-02	2.652e-02	0.8454	1.791e-02	4.070e-02	0.2642	0.002	**
GU289642.1.1485	1.635e-02	5.344e-02	0.3059	1.022e-02	2.546e-02	0.2830	0.402	
AACY020398456.2130.3630	1.553e-02	5.515e-03	2.8161	3.472e-03	3.453e-02	0.3009	0.002	**
FJ937845.1.1374	1.289e-02	5.701e-03	2.2610	3.231e-03	2.891e-02	0.3157	0.002	**
New.ReferenceOTU58	1.256e-02	2.668e-02	0.4708	2.512e-02	2.407e-06	0.3302	0.752	
New.ReferenceOTU238	1.158e-02	5.291e-02	0.2188	2.315e-02	2.227e-07	0.3435	1.000	
GU940685.1.1378	1.063e-02	6.644e-03	1.6006	1.385e-03	2.256e-02	0.3558	0.002	**
AB746951.1.1438	1.038e-02	3.334e-02	0.3113	2.024e-02	2.683e-03	0.3677	0.820	
HF541944.1.1471	1.006e-02	4.774e-02	0.2108	2.008e-02	5.189e-04	0.3793	0.798	
FJ973519.1.1465	9.758e-03	2.870e-02	0.3401	1.873e-02	1.346e-03	0.3905	0.846	
GU941053.1.1391	9.123e-03	4.666e-03	1.9552	8.612e-03	2.506e-02	0.4010	0.002	**
KC873384.1.1387	8.716e-03	4.268e-03	2.0421	2.777e-03	2.001e-02	0.4111	0.002	**
EU802339.1.1412	8.406e-03	4.593e-03	1.8300	3.250e-03	1.974e-02	0.4207	0.002	**
JN885200.1.1446	7.824e-03	1.975e-02	0.3961	1.565e-02	1.172e-06	0.4297	0.728	
GU940899.1.1230	7.560e-03	8.588e-03	0.8802	3.781e-03	1.646e-02	0.4384	0.002	**
JF272169.1.1489	6.822e-03	2.001e-02	0.3410	1.364e-02	2.451e-06	0.4463	0.846	
FJ905675.1.1337	6.707e-03	2.906e-02	0.2308	1.341e-02	1.557e-06	0.4540	0.886	
FJ960103.1.1365	6.368e-03	9.787e-03	0.6506	1.764e-03	1.277e-02	0.4613	0.002	**
New.ReferenceOTU119	6.225e-03	1.340e-02	0.4644	1.266e-02	2.963e-04	0.4685	0.614	
ADAE01000015.413.1876	6.211e-03	1.026e-02	0.6052	1.231e-02	2.012e-03	0.4757	0.502	
JQ515022.1.1447	5.820e-03	4.819e-03	1.2078	1.595e-03	1.299e-02	0.4824	0.002	**
AB058900.1.1480	5.779e-03	2.479e-02	0.2331	1.156e-02	0.000e+00	0.4890	0.920	
FJ937850.1.1317	5.385e-03	4.547e-03	1.1843	7.673e-04	1.138e-02	0.4952	0.002	**
KF596542.1.1464	5.304e-03	4.025e-03	1.3179	7.621e-04	1.132e-02	0.5013	0.002	**
JQ782748.1.1413	5.196e-03	1.756e-02	0.2959	1.047e-02	1.158e-04	0.5073	0.670	

Figure S11. Simper analysis comparing all teeth to water sample OTUS, up to a cumulative sum of .5 (50.0%). (Significant codes: *= .05 **=.01)

Contrast: Caribbean Reef_Lemon

	average	sd	ratio	ava	avb	cumsum	p	
GU118128.1.1417	2.305e-01	1.391e-01	1.6564	8.984e-05	3.149e-01	0.2380	0.010	**
HM267665.1.1361	6.976e-02	7.698e-02	0.9062	8.128e-02	5.675e-05	0.3100	0.004	**
New.ReferenceOTU58	4.330e-02	5.747e-02	0.7534	0.000e+00	5.975e-02	0.3547	0.182	
JF272169.1.1489	2.623e-02	4.881e-02	0.5375	0.000e+00	3.454e-02	0.3818	0.236	
FJ983094.1.1542	2.550e-02	2.728e-02	0.9350	2.987e-02	2.051e-04	0.4081	0.004	**
JN885200.1.1446	2.427e-02	4.159e-02	0.5835	0.000e+00	3.390e-02	0.4332	0.314	
New.ReferenceOTU238	2.140e-02	6.450e-02	0.3318	0.000e+00	1.627e-02	0.4553	0.536	
AB006767.1.1452	2.130e-02	3.612e-02	0.5895	2.341e-02	2.539e-05	0.4773	0.080	.
AB058900.1.1480	2.126e-02	6.253e-02	0.3400	0.000e+00	2.852e-02	0.4992	0.344	

Figure S12. Simper analysis comparing all Caribbean reef to lemon shark teeth sample OTUS, up to a cumulative sum of .5 (50%). (Significant codes: *= .05 **=.01 '.'=.5)

Contrast: Nurse_Lemon

	average	sd	ratio	ava	avb	cumsum	p	
GU118128.1.1417	1.456e-01	7.568e-02	1.9239	1.291e-01	3.149e-01	0.1793	0.496	
New.ReferenceOTU58	3.916e-02	3.618e-02	1.0821	3.297e-02	5.975e-02	0.2275	0.204	
FJ959892.1.1407	2.941e-02	6.196e-02	0.4747	4.663e-02	0.000e+00	0.2637	0.080	.
JQ347298.1.1499	2.569e-02	3.681e-02	0.6979	4.108e-02	0.000e+00	0.2953	0.012	*
JF272169.1.1489	2.434e-02	3.589e-02	0.6781	1.498e-02	3.454e-02	0.3253	0.288	
New.ReferenceOTU246	2.367e-02	2.245e-02	1.0544	3.534e-02	5.712e-05	0.3544	0.002	**
FJ947065.1.1274	2.024e-02	4.394e-02	0.4606	2.552e-02	2.803e-03	0.3793	0.194	
GQ274041.1.1514	1.969e-02	3.592e-02	0.5483	3.116e-02	7.600e-06	0.4036	0.010	**
JN885200.1.1446	1.907e-02	3.346e-02	0.5699	1.674e-03	3.390e-02	0.4271	0.530	
AB058900.1.1480	1.733e-02	4.964e-02	0.3492	8.260e-04	2.852e-02	0.4484	0.480	
New.ReferenceOTU119	1.603e-02	1.899e-02	0.8441	2.124e-02	1.173e-02	0.4681	0.452	
GQ274052.1.1516	1.526e-02	2.553e-02	0.5979	5.014e-03	2.458e-02	0.4869	0.356	
New.ReferenceOTU238	1.489e-02	4.466e-02	0.3334	0.000e+00	1.627e-02	0.5053	0.764	

Figure S13. Simper analysis comparing all nurse to lemon shark teeth sample OTUS, up to a cumulative sum of .5 (50.0%). (Significant codes: *= .05 **=.01 '.'=.5)

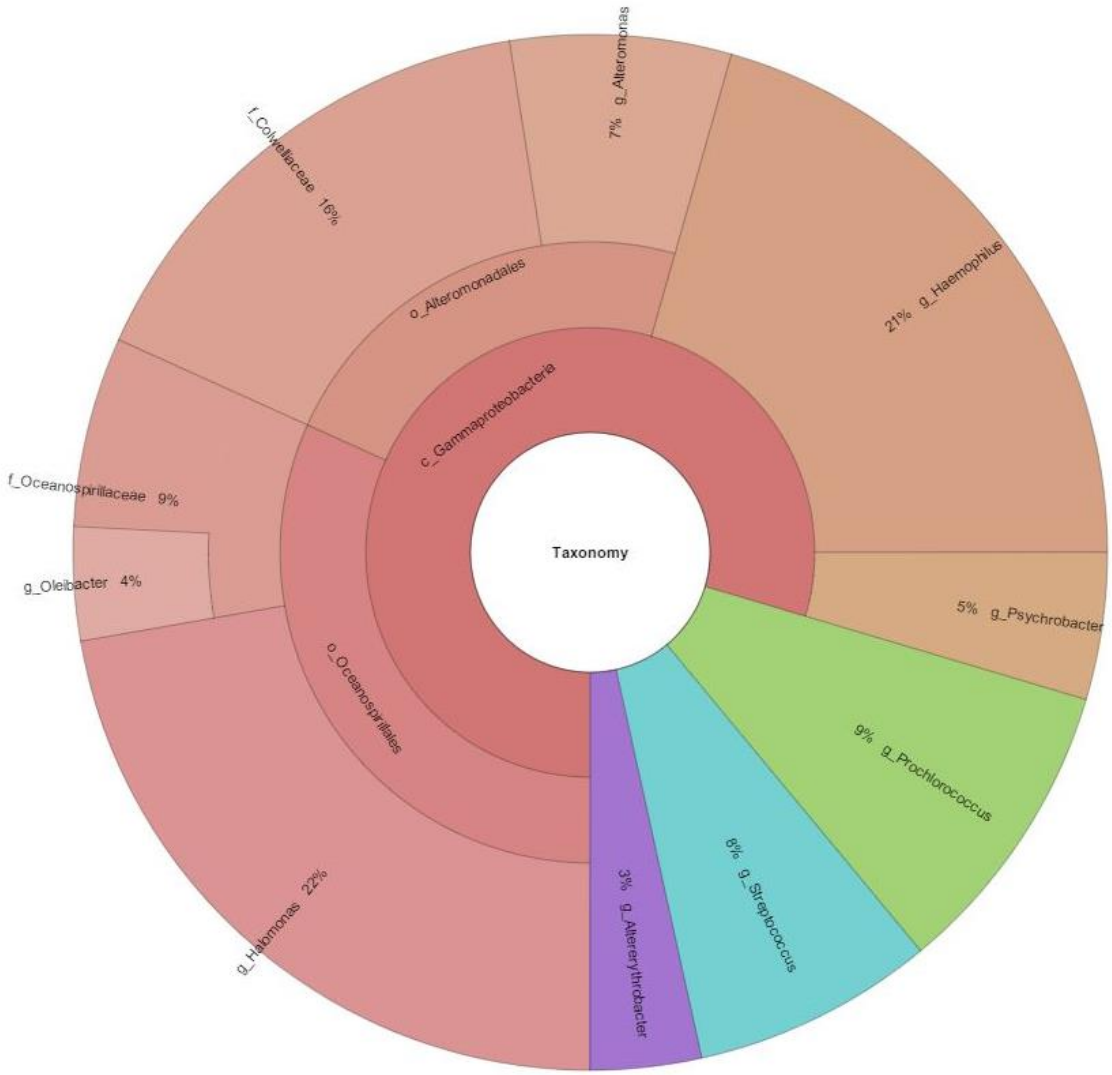

Figure S14. Krona Graphical representation of the 10 most abundant taxa in the microbial community of the teeth of the Caribbean reef sharks sampled. Percentages are calculated based on overall relative abundance across all Caribbean reef shark teeth samples.

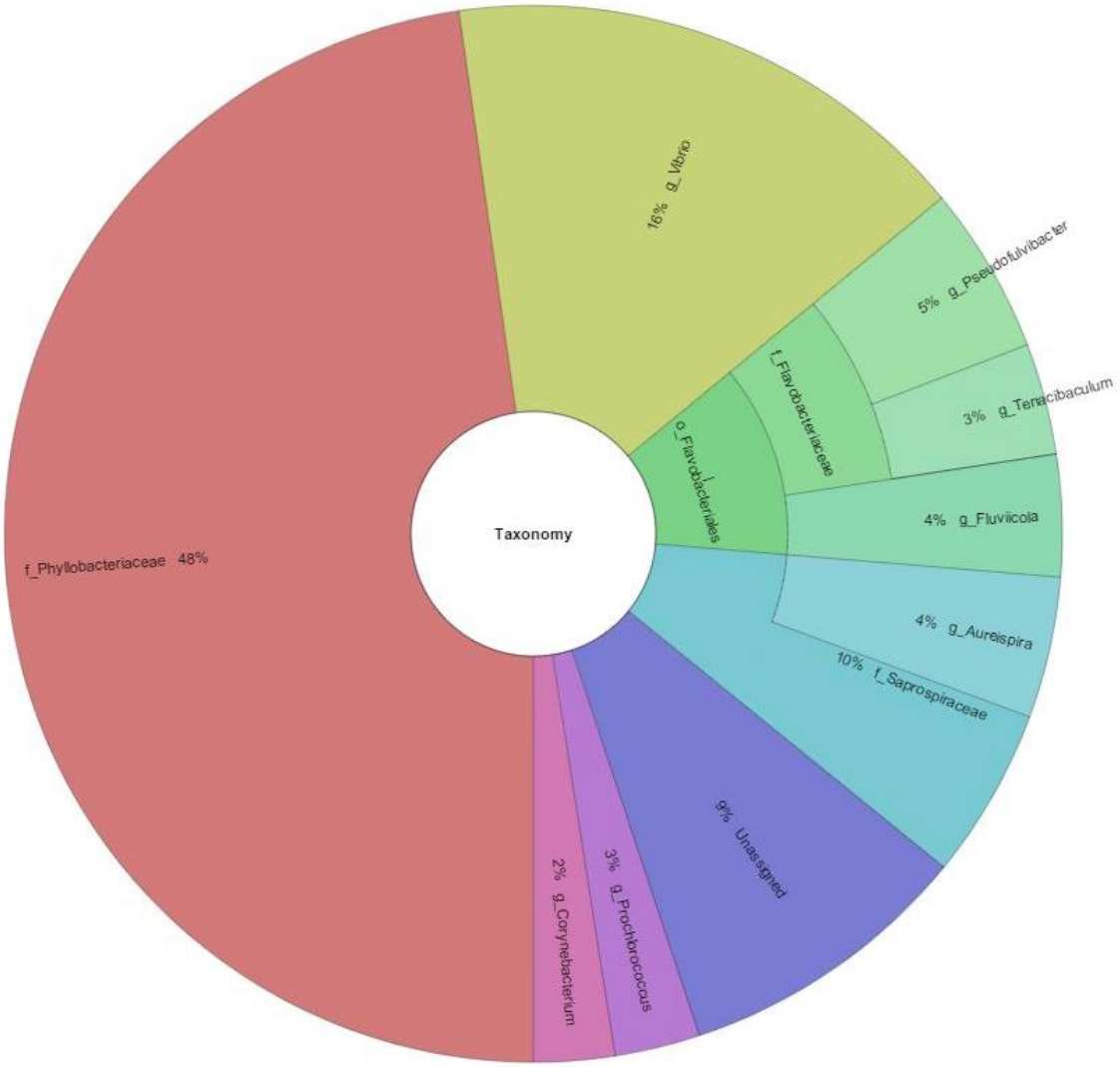

Figure S15. Krona Graphical representation of the 10 most abundant taxa in the microbial community of the teeth of the lemon sharks sampled. Percentages are calculated based on overall relative abundance across all lemon shark teeth samples.

Figure S16. Krona Graphical representation of the 10 most abundant taxa in the microbial community of the teeth of the nurse sharks sampled. Percentages are calculated based on overall relative abundance across all nurse shark teeth samples.

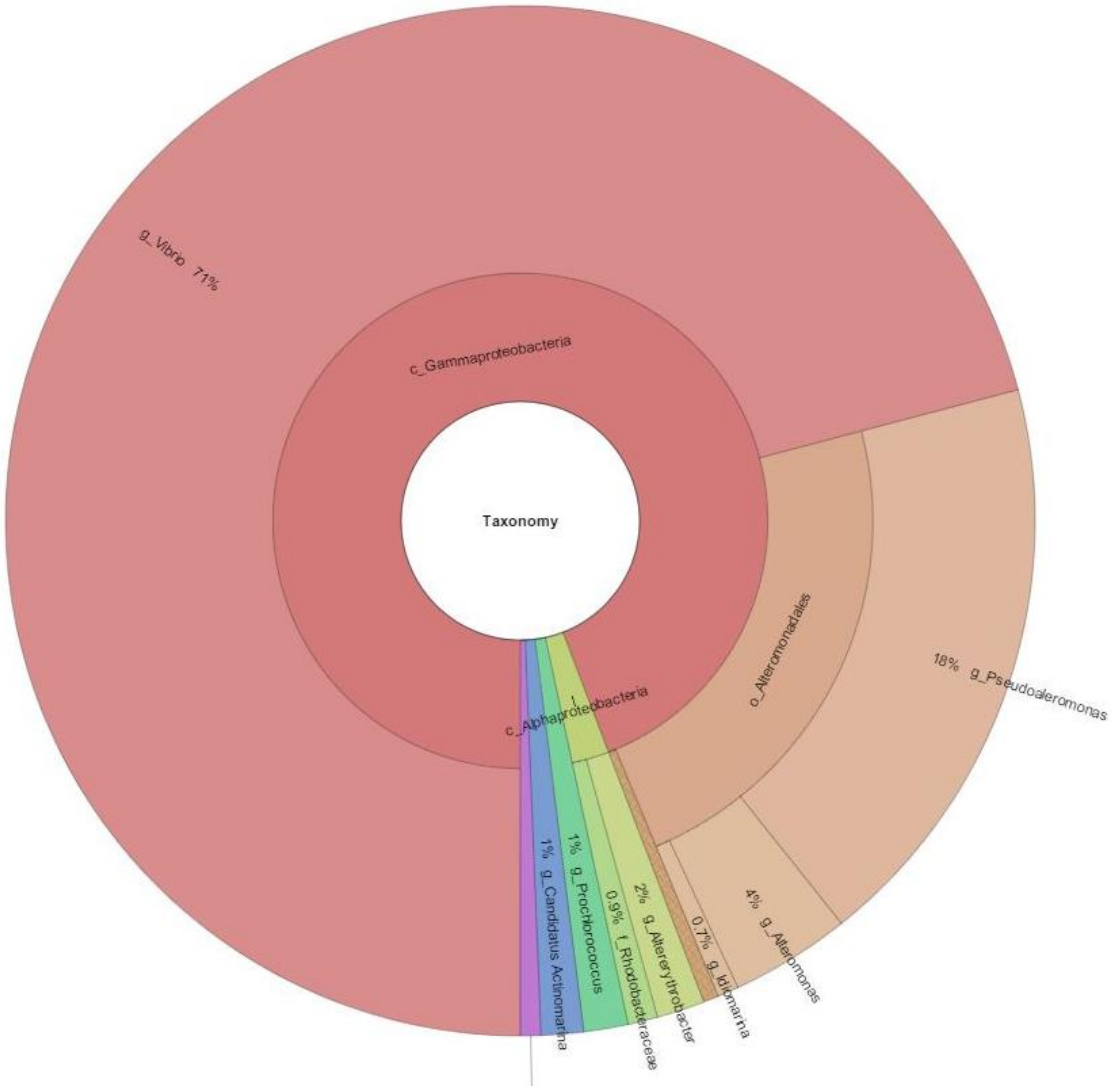

Figure S17. Krona Graphical representation of the 10 most abundant taxa in the microbial community of the teeth of the sandbar sharks sampled. Percentages are calculated based on overall relative abundance across all sandbar shark teeth samples. (Purple=f_NS9 marine group, .6%)

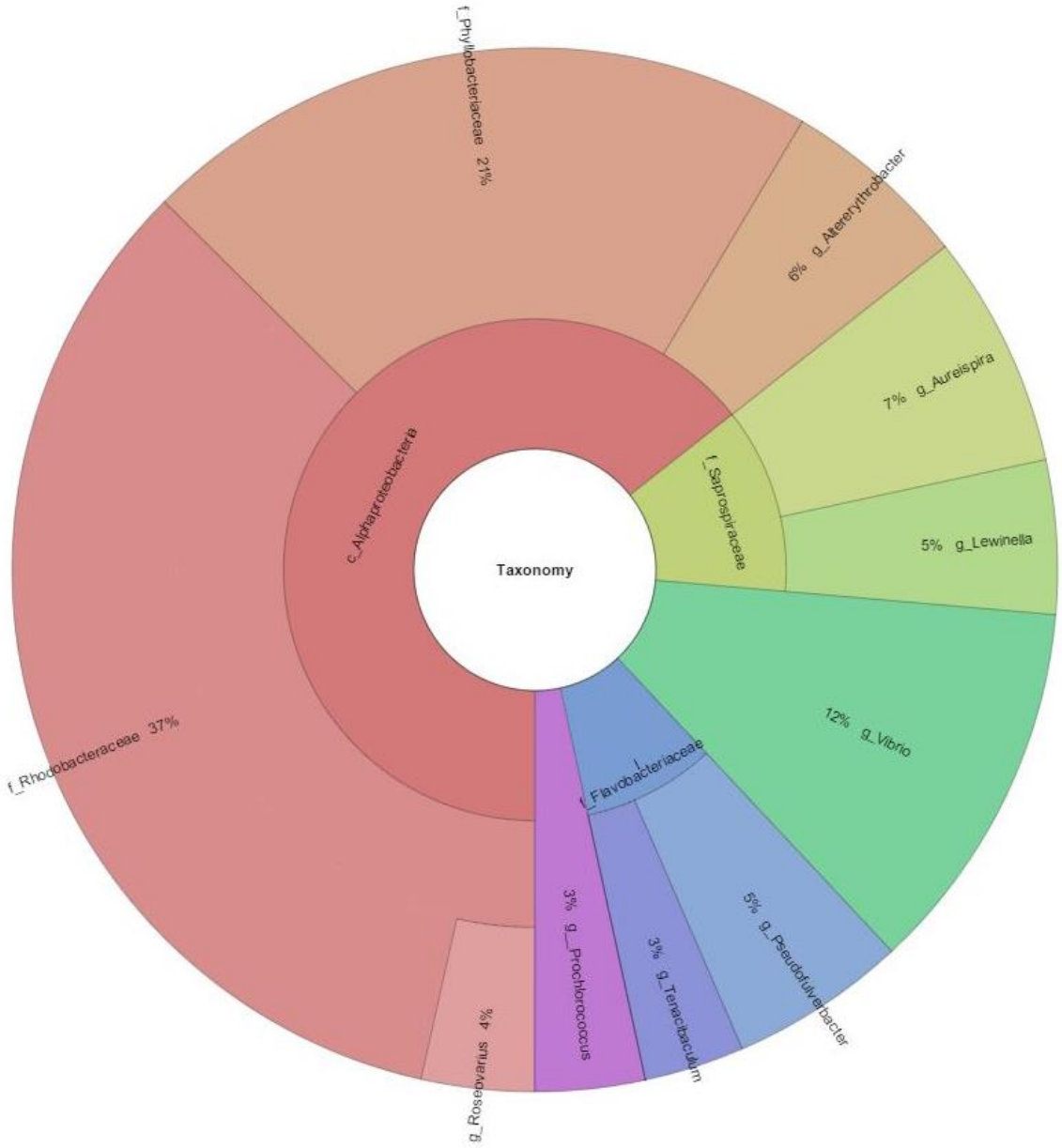

Figure S18. Krona Graphical representation of the 10 most abundant taxa in the microbial community of the teeth of the tiger sharks sampled. Percentages are calculated based on overall relative abundance across all tiger shark teeth samples.

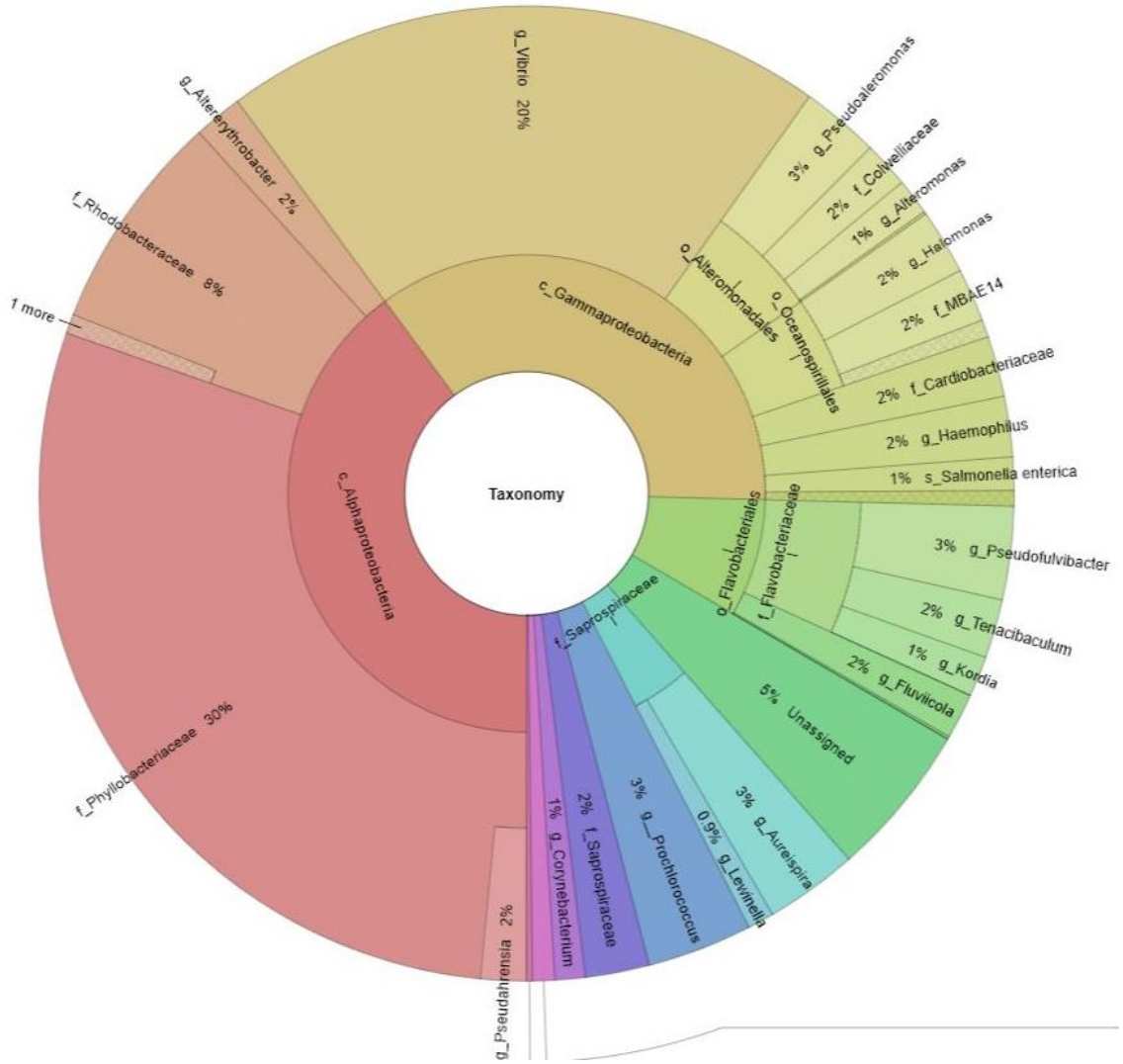

Figure S19. Krona Graphical representation of the 10 most abundant taxa in the microbial community of the teeth of sharks sampled. Percentages are calculated based on overall relative abundance across all sandbar shark teeth samples. (Purple=g_*Streptococcus*, .7%, Pink=G_*Canditatus Actinomarina*, .2%)

IV. R STUDIO CODE

All code associated with this thesis can be found at https://github.com/rckarns/Masters-thesis-code

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