An investigation of the bacterial profile recovered from the oral cavity of sharks, on the coast of KwaZulu-Natal, South Africa

Nasreen Khan

Submitted in fulfillment of the academic requirements for the degree of Master of Science (MSc) in Environmental Science

November 2015

Preface

This work was carried out at the University of KwaZulu-Natal, College of Agriculture, Engineering and Science, School of Agriculture, Earth and Environmental Sciences, Biomedical Resource Unit: Antibiotic Research Unit, in collaboration with KwaZulu-Natal Sharks Board (KZNSB), under the supervision of Professor Mihai Serban Proches, Dr Linda A. Bester and Dr Sanil Singh.

Submission of dissertation for examination

Approved by:

Supervisor

1. Professor Serban Proches

Co-supervisors

2. Dr. Linda A. Bester
3. Dr. Sanil Singh

Supervisors: 1.	
2.	
3.	

Date: _____

Acknowledgements

I could not have succeeded without the help of many friends, colleagues and family members, who offered support and encouragement during what seemed to be a 'never ending journey'. I sincerely wish to thank and acknowledge the part they have played in helping me reach the goal-post!

Firstly I am indebted to my supervisors who never stopped believing in me and encouraging me. Dr Linda Bester, Dr Sanil Singh, Professor Serban Proches and Dr Helen Watson thank you for being incredible mentors, I could not have asked for any better. Thank you for knowing my needs, for many inspirational discussions and for all the advice and encouragement. This work could not have been done without the funding made available by Dr Watson and Dr Singh.

The KwaZulu-Natal Sharks Board; thank you Geremy Cliff, Sabine Wintner, Kristina Naidoo and Augustina Ganesan. I gained a great deal of knowledge and experience working at Sharks Board, a special thank you to Emmanuel and Phillip for the incredible technical support my knowledge on shark physiology increased abundantly under their supervision. Also thanks to everyone from KZNSB for the support during the Sharks International Conference.

The Biomedical Resource Unit; thank you for the amazing three year journey, for supporting me the way you did and for being a part of my life through all its meanderings. Thank you for the impact you've had in my life and for never giving up on me. Ritta Radebe, Dr Linda Bester, Dr Singh, Vijay, Kristina Naidoo and my lab mates Jacqueline and Christiana.

My Cameroonian sister Julienne Ngo Yebga who encouraged me to start my MSc and then the lovely Marian Bezuidenhout who helped me through the initial teething phase once the MSc was off the ground, thank you ladies.

The UKZN underwater club; Caroline Reid, Heyne van Altena, Bryan Wilson and Alex van Altena stood by me through rough rocky roads, and supported me thoroughly throughout the long journey. The club became my second home where I could work peacefully on my MSc, I honestly would not have survived it without you always being in my corner. Caroline, thank you for the MSc support, for unending laughter and for the incredible dives.

My Naidoo sisters and mum, especially my besty Urisha, I am blessed to have you girls in my life. Thank you for the unwavering support and love.

My supervisors Brent Newman and Steven Weerts at CSIR, a massive thank you for being so understanding and allowing me time away from work to complete my dissertation. To my colleagues at CSIR, you filled 2014 up with incredible memories and supported me through this year. Yes Tarryn Newman, this would have been impossible without you! ☺

The Greenpeace crew, especially Tshepo Peele and Dianne Mc Alpine, thank you for letting me slack on GP duties to finally get my MSc done.

My Buckas and Joshua family who stood by me from the very beginning, my first days at university till the end of my MSc. They are my pillars of strength.

To my incredible friends thank you for being my backbone and for supporting me unconditionally; Nicola Carrasco, Aadila Omarjee, Surashna Huripurshad, Poovashen Pillay (my conscience, always scolding me to stop procrastinating), Christopher Jones, Tyesha Reddy, Natasha Rambaran, Sarisha Perumal (my online study buddy), Tasneem Nakhooda, Kerina Ramparsad (my partner in crime), Ismael Mansoor, Edrich Lubbe, Preven Chetty, Shivanee Pillay, Shaun Booval (who kept checking if the lack of sleep and gallons of Redbull had not yet killed me), Samantha Naidoo and Kirby Waddington (kept me company during late nights at campus), and Timothy Wiggill who understood the agony of the many hidden variables this project had, and the web of data I was entangled in, thank you for the objectivity and the advice.

And then there are those, where words just cannot do any justice, Natasha Govender, Uncle Bienie Garnie, Annika Hagberg (my light), Sumaiya Arabi and Zunaid Raja. Thank you! (for keeping me sane ☺)

This is dedicated to Mummy, Papa, Apa and Yunus papa. A family whose strength, unity and love never ends.

Declaration

I, Nasreen Khan declare that this dissertation contains my own work except where specifically acknowledged.

This research has not been previously accepted for any degree and is not being currently considered for any other degree at any other university.

Signed: _____

Date: _____

Declaration Two: Plagiarism

I, Nasreen Khan declare that:

1. The research reported in this thesis, except where otherwise indicated, is my original research.
2. This thesis has not been submitted for any degree or examination at any other university.
3. This thesis does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.
4. This thesis does not contain other persons' writing, unless specifically acknowledged as being sourced from other researchers.
5. This thesis does not contain text, graphics or tables copied and pasted from the internet, unless specifically acknowledged, and the source being detailed in the thesis and in the references section.

Signed: _____

Date: _____

Supervisors:1.

2.

3.

Date signed:

Summary

Shark attacks are a rare occurrence globally; however quick treatment of a contaminated wound is imperative. Failure to treat infections in a timely manner may result in fatalities as marine bacteria have opportunistic qualities. In addition, limited knowledge is available on antibiotic resistance of bacteria associated with marine top-predators. A cross-sectional study was, therefore, performed to investigate the bacterial profile of a shark's oral cavity. During 2012 to 2013, oral swabs were taken from sharks caught in protective gill-nets along the KwaZulu-Natal coastline in South Africa. Isolates were characterised by Gram-stain morphology and identified using biochemical tests and MALDI-ToF MS (Matrix assisted laser desorption/ionisation time-of-flight mass spectrometer). MICs (minimal inhibitory concentration) were performed using agar dilution against clinically important antibiotics. Data presented includes 205 isolates from 34 sharks. A total of ten species of sharks were caught. Ragged-tooth *Carcharias taurus* was the most frequently caught at 24% (8/34), the least frequent was smooth hammerhead *Sphyrna lewini* and copper *Carcharhinus brachyurus* at 3% (1/34). The highest prevalence of bacterial isolates were found in great white, *Carcharodon carcharias* (20%), scalloped hammerhead *Spyrna lewini* (16%) and mako *Isurus oxyrhincus* (14%) sharks. A Pearson correlation was used to calculate the similarities between sharks based on bacterial assemblages and shark-phylogeny. A trend was seen, however, no statistical significance was found. A plausible connection could be established with a higher sample number. In this study *Micrococcus*, *Staphylococcus*, *Vibrio* and *Pseudomonas* species rank among the four most frequently found bacteria in sharks. MICs revealed bacterial resistance of 50% to cefuroxime, 38% to ampicillin, 18% to nalidixic acid, 14% to tetracycline, 11% to erythromycin, 10% to ceftriaxone and lowest is 2% to ciprofloxacin. No resistance to gentamicin was found, highlighting its value in wound management. This primary data suggests the presence of clinically important bacteria in sharks transferable to humans, requiring specific treatments regimes.

Keywords shark-attacks, marine bacteria, MALDI-ToF, antibiotic resistance

Table of Contents

List of Figures

List of Tables

Chapter 1

1.1 Introduction

1.1.1 Background

The microbiology of a shark's oral cavity and that of shark-bite wounds are poorly understood (Buck et al., 1984, Rtshiladze et al., 2011). Despite the life-threatening infections following non-fatal shark bites, little research has been undertaken in this field (Buck et al., 1984, Interaminense et al., 2010, Rtshiladze et al., 2011). Treatment of shark bites is, therefore, uncertain and broad-spectrum antibiotics are dispensed to wounded patients. This uncertainty persists mainly because culturing of wounds from shark attacks for antibiotic susceptibility tests is not a routine hospital practice (Buck et al., 1984, Interaminense et al., 2010, Rtshiladze et al., 2011). Moreover, the logistics of shark research is challenging with regards to their availability and capture. This could be the reason for lack of research on oral bacterial flora of cartilaginous fish, globally.

Research on marine bacteria is crucial because of the potential risk to human health, for example, certain marine bacteria are considered to be the main cause of seafood toxicity (Howard and Bennett, 1993, Rtshiladze et al., 2011). Moreover, marine bacteria are opportunistic in most cases and quickly infect wounds sustained in marine or estuarine environments (Buck et al., 1984). Edmonds and Thomas (1972) and Halstead (1980) have indicated that some marine bacteria may be virulent and resistant to antibiotics. It is therefore important in this case, with regards to infectious wounds caused by shark attacks, that a scientific survey be undertaken.

Wounds sustained in the water of oceans, estuaries and rivers are exposed to bacteria that are rarely encountered in land-based injuries, and can thus, be potentially pathogenic in some cases (Greer and Noonburg, 2005). Pathogenic sources can be found in the patients' existing skin flora, the environment (seawater/estuarine water) and in the oral cavity of sharks when introduced into the wound by a bite (Interaminense et al., 2010, Rtshiladze et al., 2011).

Bacteria isolated from wounds in previous studies have revealed members of the family Vibrionaceae such as *Vibrio* spp., *Aeromonas hydrophila* and *Plesiomonas* sp. (Matsiota and Nauciel, 1993, Greer and Noonburg, 2005). The above-mentioned species are usually found

in aquatic environments and can be pathogenic in animals and man (Rtshiladze et al., 2011). Interaminense et al. (2010) found an array of bacteria present in the oral cavity of sharks. Eighty-one different bacterial species were isolated from the teeth, majority belonging to the Gram-negative *Enterobacter*, *Proteus*, *Citrobacter*, as well as Gram-positive cocci such as *Staphylococcus* and *Streptococcus* sp.

1.1.2 Aims and objectives

Against this background, this study aimed to identify the bacterial profile of the oral cavity of sharks found off the coast of KwaZulu-Natal, and to investigate the antibiotic susceptibility competence of recovered bacteria. To complete this study the following objectives were fulfilled;

1. To investigate oral cavity bacteria by swabbing the oral cavity of sharks (caught in gill nets) at the KwaZulu-Natal Sharks Board (KZNSB) within 48 hours;
2. To isolate all aerobic bacterial colonies for subculture;
3. To preliminary identify bacterial samples using phenotypic and biochemical testing;
4. To confirm species found, using MALDI-ToF MS;
5. To compare bacterial profiles among sharks species;
6. To analyse antibiotic susceptibility tests on a wide range of clinically relevant antibiotics; and
7. To determine prevalence of antibiotic resistant bacteria in sharks.

The outcomes of this study include: understanding the bacterial profile found in top predators and the microfloral threats that are present in our coastal waters, which can be transferred to humans, should an attack occur. The antibacterial susceptibility outcomes can assist medical personnel better understand antibacterial measures that can be taken and the appropriate antimicrobial prophylaxis that can be used in an event of a shark attack.

Chapter 2

2.1 Literature Review

Chapter two provides background information on several aspects relevant to this study. Bacteria are described in very broad morphological terms, with emphasis on marine bacteria. The culturing of bacteria from the oral cavity of sharks has not been a common area of research and therefore there are only a few studies highlighted.

This chapter provides background information on the KZNSB gill nets; location and extent of the sample area. The historical background, physical description and the shark net capture (of various shark species), of this bather safety device are outlined.

This chapter also provides background information on the distribution and foraging behavior of each shark species. Shark attacks, bacterial infections, anti-microbial treatments and anti-microbial resistance are also covered. The two methods for bacterial identification used in this study are described; the classic biochemical way of identification, and the fairly novel mass spectrometer bacterial identification method.

2.1.1 Bacteria

Bacteria are classified according to their morphological features as rod (bacilli), round (cocci) or spiral-shaped (spirilli) bacteria (Starr et al., 2010). Further classification is based on cell wall characteristics and the reaction to Gram-staining. Further categories are: aerobic forms, bacteria that can function with oxygen, anaerobic bacteria, bacteria that cannot grow in the presence of oxygen. These two groups are subdivided into facultative anaerobes (bacteria that can grow with or without oxygen) and obligate anaerobes (bacteria that are poisoned by oxygen (Starr et al., 2010). Marine bacteria are an integral component in the marine environment, as they are a primary food source and are at the bottom of the food chain (Zubkov and Tarran, 2008). In addition marine bacteria are considered to be 'nature's recyclers'. Heterotrophic nanoflagellates are an example of bacteria being 'recyclers', these are important bacterial grazers, driving key ecosystem processes and biogeochemical cycling in the ocean (Kirchman, 2008). This comes from the role they play in the global carbon cycle when recycling carbon and nutrients by feeding on dissolved organic debris (Starr et al., 2010). Whitman et al. (1998) estimated that the world's oceans contained 10^{29} bacteria, amounting to a biomass far exceeding the combined mass of all zooplankton and fish. Aside

from being important members of marine ecosystems, bacteria can pose a grave threat to an array of would-be hosts, and can become pathogenic in a host body (Blake et al., 1979).

2.1.2 Culturing bacteria from a shark's oral cavity

The first recorded swabbing of a sharks oral cavity was on August 1983 after a sports fisherman from Block Island, United States, harpooned a great white shark (Buck et al., 1984). This provided an opportunity to culture bacteria from a shark's oral cavity for the first time. The bacteria isolated and recovered from this research was found to be normal constituents of the marine environment and included highly infectious flora, like *Vibrio* spp., *Staphylococcus* spp., *Pseudomonas* spp., *Citrobacter* spp. and *Micrococcus* spp. (Buck et al., 1984). In Recife, Brazil, between the years 2006 and 2008 sterile swabs were swabbed from the teeth and under the gums of four captured Zambezi sharks (*Carcharhinus leucas*) and five tiger sharks (*Galeocerdo cuvier*) (Interaminense et al., 2010). Clinically important Gram-positive and Gram-negative bacteria were found, with members of the family Enterobacteriaceae (*Enterobacter* sp., *Citrobacter* sp., *Proteus* sp., *Escherichia coli*) being the most prevalent (Interaminense et al., 2010). In 2013, Dr. Robert Borrego, medical director at St Mary's trauma centre in Florida found the need to begin researching and swabbing shark teeth (as seen in Figure 2-1). It became crucial for Dr. Robert Borrego to have the correct antimicrobial knowledge for post shark-bite-wounds, after having previously treated several shark bite victims, without knowing the best antimicrobial to prescribe to his patients. This research was driven by the paucity in literature on the microflora of a shark's oral cavity (Unger et al., 2014). Live sharks were used in Borrego's research; this differed from the current and previous research on a shark's oral cavity. Other researchers in the field, for example, Blake et al. (1979) and Royle et al. (1997) also differed as they swabbed bite wounds and not the oral cavity of the animal.

Figure 2-1 The oral cavity of a live shark was swabbed by Dr. Robert Borrego and team (Unger et al., 2014), in 2013, for research conducted on the bacterial flora in the oral cavity of sharks

The use of live sharks was not an option in this study, firstly because of the logistical dangers and manpower needed to handle live sharks, secondly because of animal ethics, and not causing harm to the shark whilst retrieving oral-cavity swabs. The sharks used in this study were sharks found dead by the KZNSB in the gill nets off of Durban, the eastern shores of South Africa. [The KZNSB staff have however, mentioned that it is regrettable that gill nets have proven to be the only method, at this stage, capable of providing safe bathing grounds along the KZN coast, preventing highly dangerous species such as the great white, mako, Zambezi and tiger shark from interacting with bathers (Cliff and Wilson, 1994).]

2.1.3 Shark-nets

Along the KwaZulu-Natal (KZN) coastline, an array of sharks and other marine life get caught in gill nets (commonly known as shark nets), installed 400 m from the coast, at several localities, from Port Edward to Richards Bay. The reason for the net installation dates back to 1940 to 1959, when 41 shark attacks occurred along the KZN coastline (Davies, 1963). Of these attacks, 19 were fatal (Davies, 1963), and the increased negative publicity from these attacks proved to be of high economic importance, threatening the multi-million rand tourist industry in Durban (Wallace, 1972, Cliff and Wilson, 1994). Therefore bather safety measures had to be put in place quickly.

The first set of nets was placed along Durban's beachfront in 1952, in order to protect bathing beaches (Wallace, 1972, Cliff and Wilson, 1994). The organisation in charge of maintaining

the gill nets is the KwaZulu-Natal Sharks Board (KZNSB). In 1962, the KZNSB extended the installation of nets to other beaches, resulting in 394 nets set at 46 beaches, totaling approximately 8% of KZN's coastline (Cliff and Wilson, 1994). The gill nets are set behind the breaker zone, due to heavy surf conditions. The nets are not continuous but adjacent to each other, with 20 m overlapping at each end (Wallace, 1972). Each length of net is 305 m in length and 7.6 m deep, altogether catching 800-2200 sharks per annum (Cliff and Wilson, 1994).

2.1.4 Net capture

Some shark species caught in the nets die because they need to keep moving with open mouths in order to get oxygen from the water to breathe (Dapp et al., 2015). This means that when they are caught in the nets, they are unlikely to survive for extended periods before KZNSB are able to release them alive (Cliff and Wilson, 1994). Some species of shark and fish are attracted closer to the shore because they scavenge on debris being flushed down from the rivers into the oceans and fish amassing in this area would also attract sharks (Cliff and Wilson, 1994). Other sharks move closer inshore to use estuaries as nursery sites. Catches increase during what is locally called the 'sardine-run'. This phenomenon occurs when *Sardinops sagax* migrate closer inshore, along the KZN coast in winter (Cliff and Wilson, 1994, Cliff and Dudley, 2011). Flooding indirectly increases catch rate as the turbidity of the surrounding water makes animals disorientated and unable to avoid the nets, resulting in the capture of various shark families (Lamnidae, Carcharhinidae, Odontaspididae and Sphyrnidae) in the gill nets.

2.1.5 Family Lamnidae

The family Lamnidae is represented by the great white shark (*Carcharodon carcharias)* and the shortfin mako (*Isurus oxyrinchus*). Both these sharks are found in offshore or coastal regions, surface or intertidal and in enclosed bays (Bruce et al., 2006). Great whites can reach depths of 1,280 m. These sharks favour cold water but are also found in tropical and subtropical regions such as the KZN coastline. The gill nets record an average by-catch of 20-50 per annum for great white sharks. The great white sharks' behavior has made it notorious for being the most dangerous shark of all, responsible for more attacks on man and boats than any other species (Bruce et al., 2006). Adult great white sharks have heavily serrated, triangular cutting teeth. Adult mako sharks are equipped with long pointed teeth, narrow in profile and double edged without serrations, they are capable of grasping fish-prey and

swallowing it whole. The great white has jaws capable of feeding on large prey, like other sharks, rays, turtles, seabirds, seals, sea lions and porpoises (Cliff and Wilson, 1994, Cliff et al., 1989, Cliff et al., 1990). The diet of a mako shark comprises of small to large bony (ostheichthyes) fish and cephalopods (Cliff and Wilson, 1994, Cliff et al., 1989, Cliff et al., 1990).

2.1.6 Family Carcharhinidae

Requiem sharks from the family Carcharhinidae comprise the tiger shark *Galeocerdo cuvieri*, the Zambezi (bull) shark *Carcharhinus leucas*, the blacktip shark *Carcharhinus limbatus* and the copper shark *Carcharhinus brachyurus*. Tiger sharks favour the KZN coast because of the warmer waters and are very rarely found in the cooler Western Cape shores. The blacktip and Zambezi shark can be found in river mouths, coastal waters off Mozambique and inhabit the warm nearshore waters of KZN with blacktip sharks sometimes being found in the Cape (McCord and Lamberth, 2009). The somewhat similar copper shark is mainly found in the colder inshore waters of the Cape; however, it can venture into KZN waters following the migrating sardines (Smale, 1991). The environmental conditions across these shark distributions are diverse. They can be found far out to sea or close in shore, in turbid coastal waters of KZN coast or clear coral and rocky reefs of Mozambique. These sharks can enter large rivers, estuaries and lakes, using estuaries like those in St. Lucia and Richards Bay as nursery grounds. The scavenger tendencies of a tiger shark allow it to be easily caught up in the nets when scavenging on by-catch already entangled. They are also able to survive longer if caught in the gill nets, as they have the ability to pump water over their gills. Their catch numbers are 30-50 per annum. The Zambezi shark net-capture amounts to 50 per annum. For the blacktip, 100-200 sharks are caught per annum. The copper shark's net capture rate depends on the sardine migration, and is 10-400 per annum depending on the migration and on the prompt removal of nets during this event (Smale, 1991, Wirsing et al., 2006).

The tiger shark is easily stimulated by food, and is considered to be one of the most dangerous sharks in tropical waters (Simpfendorfer et al., 2001). These sharks have massive jaws and heavily serrated cockscomb-shaped teeth. Their teeth are flat, triangular and serrated; there is a notch on the other margin enabling this species to easily cut through hard shells of turtles and able to grip large chunks of marine mammals and other sharks (Simpfendorfer et al., 2001). Juvenile tiger sharks have a liking for sea snakes. Adults are

scavengers, and do not discriminate much in their diet, as various types of items and animals are ingested, from tin cans and plastics to seabirds, bony fish (osteichthyes) or cartilaginous fish (chondrichthyes), cephalopods, marine mammals and turtles (Budker, 1971, Cliff and Wilson, 1994, Wirsing et al., 2006).

Similar behaviour is seen in the Zambezi; this shark is an active predator and scavenger; it feeds on almost anything it encounters, it scavenges near and in rivers and has a variety of food items, feeding on both bony fish (osteichthyes) and cartilaginous fish (chondrichthyes) like skates and sand sharks (Budker, 1971, Cliff and Wilson, 1994, McCord and Lamberth, 2009). It has triangular cutting teeth and a wide jaw. It is considered extremely dangerous and has been implicated in many shallow water attacks.

The blacktip is considered potentially dangerous and has been involved in a few attacks; it is a fast and active hunter, feeding on small condrichthyans, cephalopods and various osteichthyans (Cliff and Wilson, 1994, McCord and Lamberth, 2009).

2.1.7 Family Odontaspididae

The spotted ragged-tooth shark *Carcharias taurus*, from the family Odontaspididae, occurs at the bottom and in shallow inshore waters reaching depths of 191 to 1200 m. These sharks are found in warm temperate and tropical seas. Juvenile sharks of this species are found in the Eastern Cape, mature females have been observed in Zululand. Every year 100-200 sharks are caught in the nets, however, these sharks are not regarded as dangerous unless provoked. They feed on cephalopods, large crustaceans and an array of bony fish (osteichthyes) and cartilaginous fish (condrichthyes), the fish comprise of shoaling fish (tuna), small sharks and small rays (Govender et al., 1991, Smale, 2005).

2.1.8 Family Sphyrnidae

The scalloped and smooth hammerhead shark (*Sphyrna lewini* and *Sphyrna zygaena,* respectively) belong to the family Sphyrnidae. Both are confined to offshore, continental, coastal and insular waters. Sphyrnidae are all found in warm temperate and tropical seas, along the warm waters of the KZN coastline and in the cooler waters of the Cape coast. The gill net capture for the scalloped hammerhead is at 100-200, and for the smooth hammerhead, at approximately 50 per annum. They are not considered dangerous to man unless provoked;

they feed on small bony fish (osteichthyes) and cephalopods. These species have flat triangular teeth with serrated edges, similar to a great white. Both have a wide range in diet. Sphyrnidae are known to feed heavily on crustacean's (crabs and shrimp), chondrichthyans (batoids, other sharks), osteichthyans (bony fish) and cephalopods (Budker, 1971, Cliff and Wilson, 1994, Smale, 1991).

The sharks mentioned above are mostly found along this coastline and are important when considering shark attacks as they swim fairly close to shore. This can possibility lead to interaction with bathers, surfers and divers, as the nets are not solid barriers (Davies, 1963, Cliff and Wilson, 1994). Whilst being near shore, they are also exposed to anthropogenic stresses of our coastal waters and antibiotic-resistance pressures on bacteria. The three main species involved in fatal attacks around the world are the white shark, *Carcharodon carcharias*, the tiger shark, *Galeocerdo cuvier*, and the Zambezi shark, *Carcharhinus leucas* (ISAF, 2014).

2.1.9 Shark attack and infection

Shark attacks occur when sharks are provoked or when the animal is disorientated during times of low visibility at dusk or dawn (Rtshiladze et al., 2011). Activities commonly associated with attacks are surfing, windsurfing or when humans are mistaken for prey (Rtshiladze et al., 2011). In 2008 for example, statistics revealed 59 unprovoked shark attacks worldwide (Rtshiladze et al., 2011) with USA and Australia ranking highest on the list. There were 53 people bitten within a 17 year period in Brazil for example, 20 died as a result of bleeding. Rescue operations following an attack are difficult owing to damaged nerves, blood vessels and bone. The rescue operation is further complicated when a wound is infected (Maslin et al., 2000). In order to avoid wound infections, survivors of shark attacks are generally treated with broad spectrum non-specific antibiotics, because of the uncertainty with regards to the microbiology of shark bites (Rtshiladze et al., 2011). The quick and proper treatment of a contaminated wound makes this study important when selecting an appropriate antimicrobial prophylaxis. The potential sources of pathogens can be found in the oral cavity of sharks when introduced into the wound by a shark bite, from the surrounding seawater and/ or the patient's existing skin flora (Blake et al., 1979, Interaminense et al., 2010, Rtshiladze et al., 2011). In hospitals, infected wounds should be cultured on

appropriate culture mediums, to better understand the state of probable infection (Pavia et al., 1989, Caldicott et al., 2001).

2.1.10 Techniques of identifying bacteria

Gram identification

The traditional method of bacterial identification relied on the classic method of Gram-staining. A Gram-stain is a classification first proposed in 1884 by a Danish physician, Christian Gram. It separates bacteria into two distinctive groups; Gram-negative and Gram-positive, staining them either purple or red (Beveridge, 2001). The difference in colour staining is because in Gram-positive bacteria the peptidoglycan layer is thicker (20-80 nanometers) than in Gram-negative bacteria (7-8 nanometers). The peptidoglycan helps to maintain the structural strength of the cell. It forms 90% of the dry weight of Gram-positive bacteria but only 10% of the dry weight in Gram-negative bacteria (Beveridge, 2001). A futher explanation on this is given in the methods section (Chapter 3).

Biochemical tests

Following the morphological characteristics used by Gram-staining, metabolic and enzymatic characteristics are used in identifying microflora. Bacteria ferment carbohydrates in patterns characteristic to their genus and species. Fermentation products are used in bacterial identification. Catalase, oxidase and phenotypic identifications using biochemical commercial tests kits are used; an example of this kit is the API (analytical profile index) test kit (Murray et al., 2007). These tests take time as the kit needs to be incubated overnight before a reading can be made (Murray et al., 2007, Carbonnelle et al., 2011). These have been known to be imprecise as many environmental variables can affect the condition of the culture and thus the test outcome (Seng et al., 2009).

Molecular-based identification

Molecular methods of identification are used in addition or instead of biochemical tests. Molecular methods involve the examination of DNA in question. The disadvantage of this method, is the requirement for high level expertise and the process can also accrue lofty costs (Couzinet et al., 2005). The need for new, rapid identification was consequently in demand, and ‘new approaches’ led to the developments of using protein profiles from bacterial colonies for identification via the MALDI-ToF MS (Seng et al., 2009).

MALDI-ToF MS

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has proved to be the most effective for bacterial identification (Couzinet et al., 2005, Seng et al., 2009). The technique was proposed and described between the years 1985 and 1988; Karas et al. (1985) initially demonstrated it as the soft ionisation technique with a laser desorption mass spectrometry (LDMS). Today the technology has advanced and has been named MALDI-ToF MS, and in microbiology it is used for the swift identification of bacterial samples (Murray et al., 2007, Carbonnelle et al., 2011).

Laser desorption, stands for a 'soft ionisation technique'. This is ideal for the ionisation of proteins. Soft means that mass spectra are produced with little to no fragmentation, forming ions without breaking any bonds (Seng et al., 2009). 'Matrix assisted' means that a matrix compound (cyano-4-hydroxy-cinnamic acid) is used to assist the soft ionisation technique. MALDI-ToF MS works with two steps, first is the desorption process; the matrix absorbs the UV (ulta-violet) laser light beam leading to removal of ~ 1μm of matrix material. The hot plume resulting from this step is the matrix being deprotonated (this is when a molecule loses a proton and becomes more negatively charged), this ionises the analyte molecules. The matrix molecules are now in the negative-ion mode (Seng et al., 2009). Once the sample has been ionised, ToF (time-of-flight) uses an electric field to accelerate the ions. ToF measures the time it takes the ion to reach the detector. This is governed by mass; therefore the lighter ions will reach faster (Seng et al., 2009).

A mass spectrometer measures the motion of a charged particle in a vacuum and then produces a mass spectrum. A mass spectrum is patterns or spectra representing the distribution of ions by mass. More precisely, it is the intensity verses the mass to charge ratio of an analyte. Mass to charge ratio is represented as m/z, m= molecular or atomic mass number, z = charge of the ion. So the analyte is bombarded by a laser in order to ionize the sample (Moore, 1983).

The bacteria are identified by placing a sample of bacteria onto a steel target plate overlaid with the matrix compound. Once the plate is inside the MALDI-ToF apparatus, the analyte is bombarded with a laser. The laser produces mass spectra, which are described as bacterial

fingerprints as each bacterium has its own unique spectrum. These 'spectral fingerprints' are compared with the MALDI-ToF mass spectral database to determine a species identification. This technique ensures a speedy, proficient and precise identification of an unknown bacterial sample (Seng et al., 2009).

2.1.11 Antimicrobials and resistance

Antimicrobials impede important life processes of a bacterium (Blackburn et al., 2010). The purpose is to either kill or damage a bacterium. The agent that kills is known as bactericidal, the agent that stops the proliferation of a bacterium is bacteriostatic (Wilson, 2008). Antimicrobials have become more refined and sensitive over time. Each new drug is developed from other bacteria or similar drugs, the original being called first (1^{st}) generation; drugs developed after these are second-generation (2^{nd}) drugs then third-generation (3^{rd}) and so on (Woodrow, 2007). The disadvantage with antibiotics is the adverse effects it has on patients, these include allergies, ototoxicity, nephroxicity and hepatotoxicity (Woodrow, 2007).

The early first-generation cephalosporins were highly toxic, especially affecting the kidneys (nephrotoxicity). Cefuroxime (CEF) is a 2^{nd} generation drug which is available orally, has similar activity to ampicillin however it is poorly absorbed. The 3^{rd} generation agent, ceftriaxone is more active than 2^{nd} line cephalosporin, and is active against Gram-negative bacteria, including pseudomonads. Ceftriaxone (CFX) is used in the management of severe infections, for example: bacterial meningitis, septicaemia, and bacterial endocarditis (Greene and Harris, 2008). Resistance called extended spectrum beta lactamase (ESBL) is common for this group, most ESBL producing Ecoli are resistant to cephalosporin.

The most widely used agent is gentamicin (GEN); it is the aminoglycoside of choice in the UK, and is used as a single low dose prophylaxis. Its action is against Gram-negative bacteria, in particular pseudomonads. When gentamicin resistance from pseudomonads occurs, amikacin can be used. It is also useful against staphylococcal infections. It works in synergy with penicillin, against *Enterococcus faecalis* for example. The disadvantages of aminoglycosides are that they are the most toxic among antibiotic classes. Exposure to gentamicin for a period longer than seven days can cause hearing impairments (Greene and Harris, 2008).

For the macrolides, the most important in this group is erythromycin (ERY). This group is useful to people who are allergic to penicillin. The disadvantage is that resistance is now common and therefore there is limited treatment against Gram-negatives. It was also used primarily for infections caused by *Staphylococcus* infection (Greene and Harris, 2008).

Tetracyclines (TET) are these are broad spectrum bacteriostatic agents are called. The disadvantage is that *Pseudomonas* sp. and *Proteus* sp. are intrinsically resistant to tetracyclines (Greene and Harris, 2008). Quinolones such as Nalidixic acid (NA) has low activity and poor tissue concentration, therefore this has resulted in the increased development of resistance of bacteria to antibiotics (Greene and Harris, 2008). Within the fluoroquinolones class, the agent ciprofloxacin (CIP) is active against aerobic Gram-negative infections. This group has wide therapeutic options. The disadvantage is that all quinolones are liable to cause gastrointestinal disorders and central nervous system effects- headaches, dizziness and sleep disturbance (Greene and Harris, 2008). Among the penicillin class, the agent ampicillin is a broad-spectrum penicillin, completely ineffective against *Pseudomonas* spp. because Gram-negative organisms are resistant to ampicillin (Greene and Harris, 2008).

2.1.12 Antimicrobial resistance

Antibiotic resistance is a serious public health problem, often leading to a lack of therapeutic options in clinical settings. Over a period of 60 years, there has been a mounting use and also misuse of antibiotics. Response by bacterial exposure to environmental stresses like antimicrobial treatment has resulted in the selection of resistant forms (Levy and Marshall, 2004, CDC, 2012). Following this, the spread of resistant genes and the propagation of bacterial progeny that is not susceptible to antibiotic treatment occur (CDC, 2012).

Bacterial resistance to antibiotics includes direct/primary and indirect/secondary pathways. Primary pathways are mutations in the gene, which encode for resistance toward mechanisms of particular antibiotics; an example of this is the adaptation of the ribosomal site of *M. tuberculosis* to the antibiotic streptomycin (Bester and Essack, 2010). Secondary pathways include the acquisition of small DNA fragments (which code for resistance) by a recipient bacterium, and this transfer can occur via various genetic means (WHO, 2015). These secondary mechanisms include; transformation and conjugation (Bester and Essack, 2010).

These mechanisms are collectively termed 'horizontal gene transfer' (HGT) and are responsible for the constant evolution of bacterial species (CDC, 2012). HGT was first discovered by Griffith in 1929, which later became known as transformation. Resistance is amplified when organism have all three mechanisms (CDC, 2012).
When cells make contact, they transfer genetic material via mobile genetic elements; utilising this pathway allows the bacteria to transfer or take-up DNA from other bacterial species (WHO, 2015). These mobile genetic elements are: plasmids, phages, transposons and pathogenecity islands. These gene distribution systems help bacteria counteract threats posed to their existence (CDC, 2012).

Transformation is when a dying bacterium releases DNA fragments or plasmids into the environment and it is incorporated into new strains (Bester and Essack, 2010). An example is inter-Gram genetic exchange between Gram-negative and Gram-positive bacteria (CDC, 2012). Plasmids are an extrachromosomal agent moving genes between bacteria of different species. Acquisition of resistance genes occurs through a transformation pathway of the cell wall whereby the recipient bacterium can utilise the material to its own benefit against antimicrobials (Bester and Essack, 2010).

Conjugation is when DNA is transferred during cell to cell contact. This occurs via the conjugation pathway which is a 'hair-like' attachment on the surface of the bacterial cell and is constructed of a protein that acts as a bridge puling two cells together (Bester and Essack, 2010). One cell is the donor of the genetic material and the other is the host (Bester and Essack, 2010).

Transduction is the transfer of genes by bacteriophage particles. Bacteriophages can move or deliver chromosomal-associated resistant genes or plasmid associated resistant genes to a new bacterial host (Bester and Essack, 2010).

Understanding the mechanisms and developments of innate and acquired resistance, including its complexities, is important when attempting to treat bacterial wound infections with antimicrobials.

Chapter 3

3.1 Material and Methods

3.1.1 Ethical clearance

Ethical clearance was submitted and obtained from the animal ethics sub-committee of the University of KwaZulu-Natal. No live animals were used in this study. This study's ethical clearance number is: 065/12/Animal (Appendix A).

3.1.2 Study site

Sharks were collected from protective gill nets off the coast of KZN, South Africa, from Port Edward to Richards Bay. These nets are 400 m offshore, at a depth of 7.6 m along selected beaches. The animals which get entrapped in these protective gill nets are collected at first-light of every day by the KwaZulu-Natal Sharks Board (KZNSB), who are responsible for releasing live animals or, if found dead, taken to the KZNSB wet laboratory for analysis, data capturing and further research.

3.1.3 Study animals

Due to the limited duration of this study and the uncertainty of what species would become available via gill-net stranding, it was decided not to discriminate shark samples, by choosing chiefly dangerous species related to shark attacks. Hence, any species caught was used in this investigation. After meshing of the nets, the sharks were brought to the KZNSB wet lab. The sharks were weighed, measured, and teeth from the lower and upper jaw of the oral cavity were swabbed. A detailed description of each shark was reported in a KZN Sharks Board dissection form (Appendix B) during the analysis. This process was done rigorously, from mid-2012 to mid-2013.

3.1.4 Isolation of bacterial samples

The two swabs used to swab the oral cavity of the shark's oral cavity were carefully transported to the antibiotic research laboratory at the Biomedical Resource Unit (BRU) the same day, where each swab was streaked onto 3 replicates of Nutrient agar (NA[1]).

[1] NA CM0003 (Oxoid LTD, Basingstoke, Hampshire, England)

Presumptive isolates from the Nutrient agar (NA[1]) replicates were recovered from all plates, sub-cultured for purity and maintained on nutrient agar slants. Isolates were additionally stored at -60°C in 1 ml vials of TSB[1] plus 10% glycerol[2].

3.1.5 Identification of bacterial samples

Biochemical tests

Identification of bacterial samples was done using phenotypic and biochemical testing. Gram-staining of bacterial colonies were undertaken to determine Gram-positive or Gram-negative bacteria, including determining the morphology of each bacterial isolate.

Gram-positive and Gram-negative bacteria were confirmed with a string test using 3% potassium hydroxide (3% KOH). As a single colony of bacteria is placed on a droplet of 3% KOH, the cell walls of Gram-negative bacteria (are thinner than the cell wall of Gram-positive bacteria) break down, and form a string-like substance confirming a Gram-negative samp (Murray et al., 2007).

Triple sugar iron (TSI[3]) agar further supported the identification of different bacteria, by placing bacteria into categories of glucose, lactose or sucrose fermenters, as well as identifying if the bacteria were H_2S or gas producers (Phillips, 1993). This determined which API test kit was to be used; 20E[6] or 20NE[7].

Analytical Profile Index (API)

Gram-negative sugar fermenting rods were identified by the API 20E[4] system and Gram-negative non-sugar fermenting rods, by the API 20NE[5] system. The identification of Gram-positive bacteria and Gram-negative cocci was outsourced to Vetdiagnostix Veterinary Pathology Services (Pty) Ltd, Pietermaritzburg[6]. Since all samples that were sent to the

[2] Glycerol AR (Associated Chemical Enterprises (PTY) LTD. Southdale 2135, South Africa)

[3] TSI CM0277 (Oxoid LTD, Basingstoke, Hampshire, England)

[4] API 20 E (bioMerieux sa, Marcy I'Etoile – France)

[5] API 20 NE (bioMerieux sa, Marcy I'Etoile – France)

[6] Veterinary pathology services: www.vetdiagnostix.co.za, KZN division, Pietermaritzburg

Vetdiagnostix laboratory were Gram-positive and Gram-negative cocci, carbohydrate fermentation procedures were used to determine bacterial species as described by Holt (2000).

3.1.5.1 MALDI-ToF MS

Mass spectrometry identification was done to further complement the identification obtained by the Vetdiagnostix laboratory and by the API identification system. In this process a single colony of each isolate was taken directly from the agar plate of fresh bacteria after 18-24 h incubation. The colony was carefully placed on a single target spot of a microtitre 384 polished steel target plate[7], using a sterilised wooden stick and dried at room temperature. Directly after the sample dried, one microlitre of matrix solution (saturated solution of a-Cyano-4-hydroxy-cinnamic acid[8]) was placed onto each target sample spot, and then air dried at room temperature (Wieser et al., 2012).

The target plate with samples were measured by an AutoFlex III Smartbeam MALDI-ToF MS apparatus (Bruker Daltonics, Germany) and the mass spectra of each sample was acquired by an Ultraflex III ToF/ToF mass spectrometer (Bruker Daltonics, Germany) which has a 200-Hz smartbeam 1 laser. The FlexControl 3.0 programme was used to obtain mass spectra; the pre-programmed MBT_FC.par method was selected, other parameters included the voltage of the ion source one, set at 20.08 kV and ion source two, set at 18.57 kV. The instrument was calibrated using a bacterial control standard (BTS[9]) of *Escherichia coli.* This was used for each analysis, to validate the accuracy of mass spectral data generated by the instrument. The smartbeam laser discharged 600 shots to obtain a spectrum for each sample spot, in the positive linear mode.

The MALDI Biotyper 4.0 software (Bruker Daltonics, Germany) was used to analyse the raw spectra. The software generates a list of peaks up to a 100 for each sample, which is then

[7] MTP 384 target plate polished steel TF #209520 (Bruker Daltonics, Bremen, Germany, sales@bdal.de)

[8] HCCA, portioned, package of 10 tubes #255344 (Bruker Daltonics, Bremen, Germany, sales@bdal.de)

[9] BTS, Bruker Bacterial Test Standard, package of 5 tubes #255343 (Bruker Daltonics GmbH, Bremen, Germany, sales@bdal.de)

matched against the software's reference library. Scores obtained are matched to the Bruker Daltonics mass spectral database to identify microorganisms. The Bruker Daltonics MALDI Biotyper database currently holds reference spectra for 3995 microorganisms. An identification with a score of >2.00 is considered correct on the species level, between 1.7 and 1.999 correct at the genus level, and inconclusivewith a score of <1.7 as shown in Table 2.

Table 3-1 Interpretation of MALDI-ToF score values relating to the identification of bacterial samples matched against the Bruker Daltonics mass spectral database

Range	Description	Colour
2.300 to 3.000	Highly probable species identification	Green
2.000 to 2.299	Secure genus and probable species identification	Green
1.700 to 1.999	Probable species identification	Yellow
0.000 to 1.699	No reliable identification	Red

Identification of bacterial samples between the two methods can be seen in appendix P, table 6.18, identification of samples can be seen, these were identified using the MALDI-ToF and biochemical tests. The grey highlighted section is the identity chosen between the two methods, the results for both tests were carefully evaluated, taking in to consideration the score values and the identification percentage value.

3.1.6 Antimicrobials used

Eight antibiotics were chosen for this study, each of which had different clinical significance and had varied modes of action, as each were from a different taxonomic-class of antibiotics, as can be seen in Table 3-2. See Tables 4-6 to 4-16, Chapter 4, for the clinical breakpoints used for each antibiotic (CLSI, 2009).

Table 3-2 Antibiotics chosen for this study, its classification and action

	Antibiotics	Classification	Inhibits
1	Tetracyline	Tetracyclines	Inhibits protein synthesis
2	Erythromycin	Macrolides	
3	Gentamicin	Aminoglycosides	

4	Nalidixic acid	Quinolones	Inhibitors nucleic acid synthesis
5	Ciprofloxacin	Fluoroquinolone	
6	Ampicillin	ß-Lactams (Penicillins): Aminopenicillins (pen A)	Inhibitors cell wall synthesis
7	Cefuroxime	ß-Lactams (Cephems) : 2nd Generation Cephlasporins (C2G)	
8	Ceftriaxone	: 3rd Generation Cephlasporins (C3G)	

3.1.7 Minimal inhibitory concentration (MIC)

Antibiotic tests and appropriate breakpoints for chosen antibiotics were carried out according to the Clinical and Laboratory Standards Institute (CLSI) recommendations (CLSI, 2008). The MIC is the antimicrobial concentration where total bacterial growth is inhibited. MICs were done by adding a serial-dilution of the antimicrobial to Mueller-Hinton agar (MH[19]),the plate was inoculated with 0.5 McFarland bacteria suspension using a Multi-elite automated inoculator[10]. The plates were incubated for 24 h at 37ºC and examined the following day for the presence or absence of a colony followed by further examinations for at least 72 h.

3.1.8 Phylogenetic analysis

Phylogenetic relationships between shark species were considered based on Vélez-Zuazo and Agnarsson (2010). The percentage similarities between shark species based on their phylogenetic relationships were tabulated. Conversely, the percentages of similarity between shark species based on their microflora were also recorded, and the two were plotted against each other, based on a presence/absence table of each shark species and each bacterial genus.

3.1.9 Statistical analysis

Comparative analysis was done between the bacterial profile of shark species and the percentages of similarity based on phylogeny. A Pearson's Correlation Coefficient was used to determine if a relationship existed between closely related shark species and its oral microflora. The statistical software, PASW version 18.0.3 (SPSS Inc. - Statistical Package for Social Sciences), was used to interpret the data statistically.

[10] Multi PointElite SCAN 4000, automated inoculator Mast Group Ltd, Merseyside, UK

Chapter 4

4.1 Results

4.1.1 Shark data

This study concluded with a total of ten species of sharks, with each species varying in catch number, ending with an overall total shark-sample number of n = 34 sharks. Among all shark species, the most frequently found shark caught in the nets were ragged-tooth *Carcharias taurus* (24%), spinner *Carcharhinus brevipinna* (21%) and the tiger shark *Galeocerdo cuvier* (18%). The least caught were, one smooth hammerhead *Sphyrna lewini* and one copper *Carcharhinus brachyurus* (3%) (Figure 4-1). The sex of each species was also established, and the overall number was skewed towards females with a total of 25 females and eight males (Figure 4-1) Appendix C. No males were found in great white *Carcharodon carcharias*, smooth hammerhead *Sphyrna lewini*, blacktip *Carcharhinus limbatus* and ragged-tooth *Carcharias taurus* sharks. Neither was there female copper *Carcharhinus brachyurus* and scalloped hammerhead *Sphyrna zygaena* in the sample (Figure 4-1). The maturity of each shark was determined, and nine juveniles were found and 19 mature sharks dominated the sample population (Table 4-1).

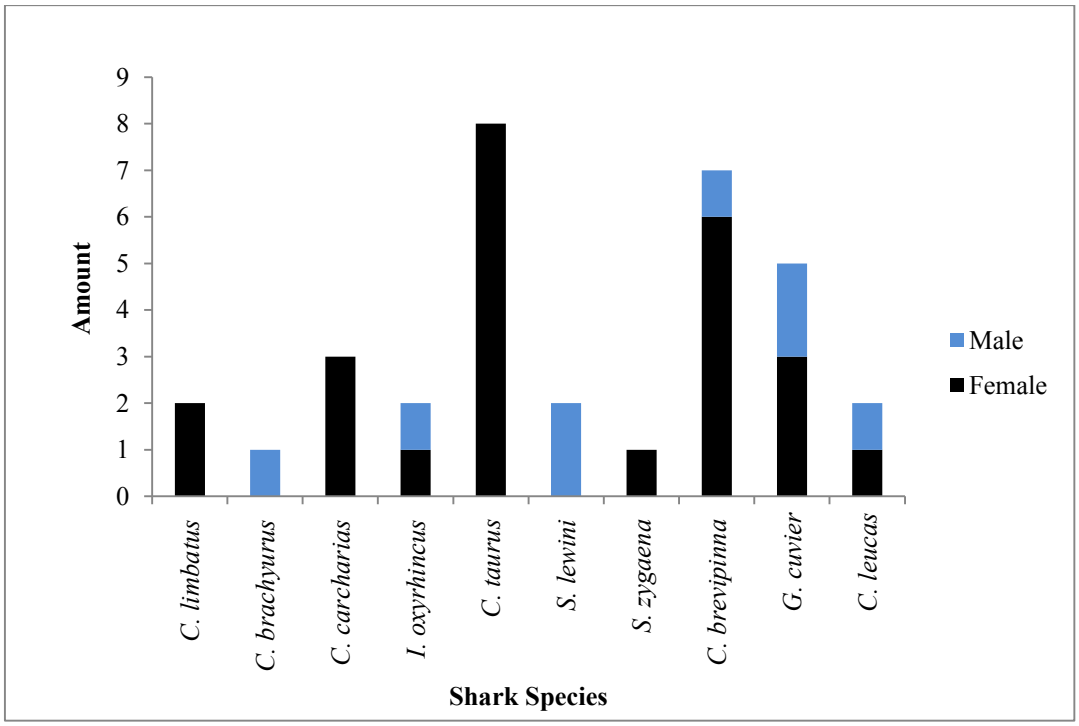

Figure 4-1 Total number of male and female shark species. Total sample number, n = 34 sharks

Table 4-1 The age-class distribution for each shark species represented in an inverse order

Shark species	Shark maturity			Total
	1	**2**	**3**	
Carcharodon carcharias	1	2		3
Carcharhinus brachyurus			1	1
Sphyrna lewini	1			1
Sphyrna zygaena	1		1	2
Carcharhinus brevipinna	1	1	5	7
Galeocerdo cuvier	3		2	5
Carcharhinus limbatus			2	2
Carcharhinus leucas	2			2
Isurus oxyrinchus			2	2
Carcharias taurus		2	6	8
Total	9	5	19	33

Column three represents the mature stage in a shark's life, two is an intermediate stage and one a juvenile stage.

The phylogenetic relationships between sharks were also taken into consideration as seen in Table 4-2. The phylogenetic relationship is described by the number of nodes that separate each shark species from the other on a phylogenetic tree. The table shows that copper *Carcharhinus brachyurus* and spinner sharks *Carcharhinus brevipinna* are sister lineages as they fall in the same clade with one node separating them. The same applies to smooth hammerhead *Sphyrna zygaena*, scalloped hammerhead *Sphyrna lewini*, great white *Carcharodon carcharias* and mako sharks *Isurus oxyrhincus*. The high numbers of six or five nodes indicate that the species are nested in two different sets of clades and are not closely related. Among all shark species, the ragged-tooth shark (*Carcharias taurus*) is the 'outgroup', as it is not closely related to any other species in this study.

Table 4-2 Shared phylogenetic branch length between shark species

Shark species	*Carcharias taurus* (Ragged-tooth)	*Carcharodon carcharias* (G.White)	*Isurus oxyrhincus* (Mako)	*Galeocerdo cuvier* (Tiger)	*Sphyrna zygaena* (Smooth)	*Sphyrna lewini* (Scalloped)	*Carcharhinus limbatus* (Blacktip)	*Carcharhinus brachyurus* (Copper)	*Carcharhinus brevipinna* (Spinner)	*Carcharhinus leucas* (Zambezi)
Carcharias taurus (Ragged-tooth)	0	67	75	75	80	80	75	83	83	80
Carcharodon carcharias (G.White)		0	0	75	80	80	75	83	83	80
Isurus oxyrhincus (Mako)			0	75	80	80	75	83	83	80
Galeocerdo cuvier (Tiger)				0	50	50	67	80	80	75
Sphyrna zygaena (Smooth)					0	0	67	80	80	75
Sphyrna lewini (Scalloped)						0	67	80	80	75
Carcharhinus limbatus (Blacktip)							0	50	50	0
Carcharhinus brachyurus (Copper)								0	0	50
*Carcharhinus brevipinna (*Spinner)									0	50
Carcharhinus leucas (Zambezi)										0

The phylogenetic distance for each pair of shark species as taken from (Vélez-Zuazo and Agnarsson, 2010) which provided the 'most up to date tool for the comparative phylogenetic studies of sharks'.

4.1.2 Microbial data

Interpretation of microbial data was based on bacterial identification from biochemical tests and the MALDI-ToF instrument. Between the two means of bacterial identification the most reliable form of identifaction was utilised. The identification based on the MALDI-ToF instruments contained score values relating to the identification of bacterial samples matched against the Bruker Daltonics mass spectral database. The identifications that had a score of 2.30-3.00; termed, "highly probable species identification" and the identifications that had a score between 2.00 – 2.29; termed, "a secure genus and probable species identification" were used (Table 3-1). This was taken as a reliable identification. The biochemical test kits used in the identification process had produced a percentage indicating the reliability of the interpretation; percentages ranging between 80-99% were taken as a reliable identification in this study.

The dataset consisted of 205 isolates. Of the 205 isolates, 28 could not be identified; and this could be the result of various technical reasons (further information on this can be found in Chapter 5, under the section, '5.1.8 Caveats'). The microbial sample size was therefore 177. A total of 19 bacterial families were identified from the 177 identified isolates. Among the microbial genera *Micrococcus* sp. *Staphylococcus* sp., *Pseudomonas* sp., *Bacillus* sp., *Vibrio* sp. and *Kocuria* sp., account for the top six bacterial genera predominantly found in shark samples (Appendix D). On a species level *Micrococcus luteus, Bacillus cereus* and *Vibrio alginolyticus* rank as the top three bacterial species predominantly found in most sharks (Table 4-3).

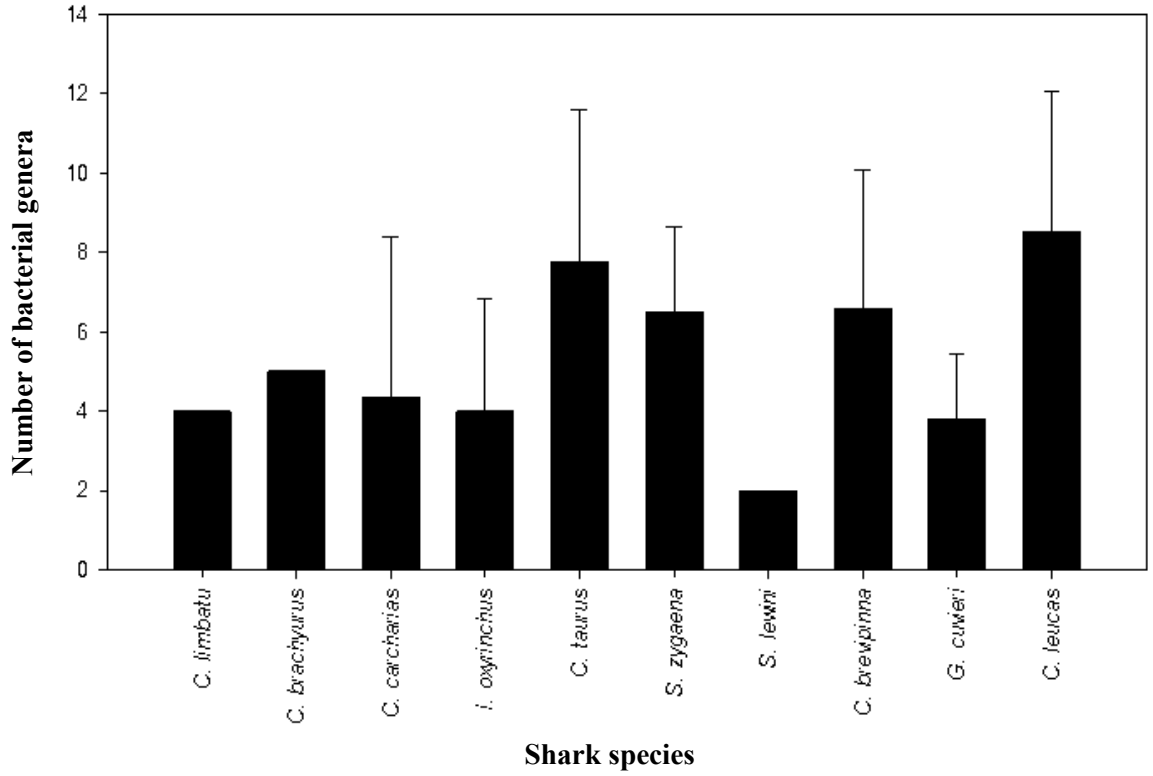

Figure 4-2 Error bar plot of bacterial species-richness between shark species

The data in Figure 4-2 of *C. taurus, C. brevipinna* and *C. leucas* illustrating these species as having the highest species richness of bacterial flora, concurrently the overlapping error bar show a similarity between each of these shark species. The shark species *C. carcharias* and *S. zygaena* shows a similar bacterial species richness and are not significantly different from each other. More detail on Figure 4-2 and actual numbers can be seen in Appendix C, and in depth detail for each sharks bacterial numbers are shown in Appendix D- Appendix N.

A presence/absence table (Table 4-3) was created to gauge the total bacteria present in a specific shark (vertical-total) and the horizontal-total describes the overall bacterial richness in the study. In addition, shark species similarly based on bacterial assemblages was calculated from Table 4-3 and displayed in Table 4-5. Here the similarities between shark species based on its oral flora were revealed; the highest similarity of 91% was between smooth hammerhead *Sphyrna* zygaena and copper sharks *Carcharhinus brachyurus*, smooth hammerhead *Sphyrna* zygaena and blacktip sharks *Carcharhinus limbatus,* and between blacktip *Carcharhinus limbatus* and mako sharks *Isurus oxyrhincus*. The lowest percentage (38%) of similarity was between species of the same genus, i.e., the smooth hammerhead *Sphyrna zygaena* and scalloped hammerhead sharks *Sphyrna lewin*i.

Table 4-3 Presence / Absence table of bacteria in sharks '1' = present and '0' = absent, describing the presence or absence of each bacterial genus in shark species.

Shark species ← **Bacteria genera →**	*Aeromonas*	*Alcaligenes*	*Achromobacter*	*Aerococcus*	*Bacillus*	*Lysinibacillus*	*Brevibacterium*	*Ochrobactrum*	*Brevundimonas*	*Delftia*	*Dermacoccus*	*Kytococcus*	*Brachybacterium*	*Proteus*	*Enterobacter*	*Serratia*	*Enterococcus*	*Micrococcus*	*Kocuria*	*Arthrobacter*	*Microbacterium*	*Acinetobacter*	*Moraxella*	*Pseudomonas*	*Mesophilobacter*	*Staphylococcus*	*Macrococcus*	*Shewanella*	*Sphingobacterium*	*Vibrio*	*Photobacterium*	*Stenotrophomonas*	**Total**
C. Taurus	1	1	0	0	1	1	0	0	0	0	0	1	0	1	1	0	0	1	1	1	1	1	0	1	0	1	1	0	1	1	1	0	**18**
C. carcharias	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	1	0	1	0	1	0	1	1	0	**8**
I. oxyrhincus	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	**4**
G. cuvier	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	1	1	0	0	0	0	0	0	0	0	1	0	1	0	0	**6**
S. zygaena	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	**2**
S. lewini	0	0	0	1	0	1	0	0	0	0	0	1	0	0	0	1	0	1	1	0	0	1	0	1	1	0	0	0	1	1	0	1	**12**
C. limbatus	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	1	0	1	0	0	0	0	0	0	**5**
C. brachyurus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	1	0	0	0	0	0	0	0	0	**3**
C. brevipinna	0	1	1	0	1	0	0	1	1	1	0	0	0	0	0	0	1	1	0	0	1	0	1	1	0	1	1	1	1	1	1	1	**18**
C. leucas	0	0	0	0	0	0	0	0	1	0	1	0	1	0	0	0	0	1	1	0	0	0	0	0	0	1	1	1	0	0	0	0	**8**
Total	**1**	**2**	**1**	**1**	**5**	**2**	**1**	**1**	**2**	**1**	**2**	**2**	**1**	**2**	**1**	**1**	**1**	**9**	**5**	**2**	**3**	**2**	**1**	**7**	**1**	**7**	**3**	**4**	**3**	**5**	**3**	**2**	**84**

Table 4-4 The diversity of 'culturable'bacterial populations found in a shark's oral cavity from various studies

	Proteus mirabilis	*Vibrio alginolyticus*	*Vibrio fluvialis*	*Vibrio parahaemolyticus*	*Vibrio carchariae*	*Pseudomonas* species	*Shewanella putrefaciens*	*Staphylococcus hominis*	*Staphylococcus cohnii*	*Staphylococcus epidermidis*	*Staphylococcus aureus*	*Micrococcus* species	*Aeromonas caviae*	*Aeromonas hydrophila*	*Proteus* species	*Enterococcus* species	*Micrococcus* species	*Moraxella* species	*Enterobacter cloacae*	*Bacillus* species	*Klebsiella oxytoca*	*Klebsiella pneumoniae*	*Clostridium freundii*	*Pasteurella* species	*Streptococcus* species
Buck et al. (1984)		✓	✓	✓		✓																			
Royle et al. (1997)				✓									✓	✓	✓	✓					✓	✓	✓		
Interaminense et al. (2010)	✓														✓	✓									✓
Rtshiladze et al. (2011)																									✓
Unger et al. (2014)		✓				✓	✓	✓	✓	✓	✓					✓	✓	✓	✓	✓		✓		✓	✓
This Study (2015)	✓	✓	✓	✓		✓	✓		✓	✓		✓	✓	✓	✓	✓	✓	✓	✓	✓					

Table 4-5 Similarities between shark species based on bacterial assemblages. The percentage of bacteria shared between shark species, describing the level of similarity between each shark based on bacterial assemblages.

Shark species	*Carcharias taurus* (Ragged-tooth)	*Carcharodon carcharias* (G.White)	*Isurus oxyrhincus* (Mako)	*Galeocerdo cuvier* (Tiger)	*Sphyrna zygaena* (Smooth)	*Sphyrna lewini* (Scalloped)	*Carcharhinus limbatus* (Blacktip)	*Carcharhinus brachyurus* (Copper)	*Carcharhinus brevipinna* (Spinner)	*Carcharhinus leucas* (Zambezi)
Carcharias taurus (Ragged-tooth)	100	63	50	50	50	56	59	53	50	44
Carcharodon carcharias (G.White)		100	81	75	81	56	84	78	69	69
Isurus oxyrhincus (Mako)			100	69	88	56	91	84	50	69
Galeocerdo cuvier (Tiger)				100	81	63	78	78	44	81
Sphyrna zygaena (Smooth)					100	63	91	91	50	81
Sphyrna lewini (Scalloped)						100	66	66	38	50
Carcharhinus limbatus (Blacktip)							100	88	53	78
Carcharhinus brachyurus (Copper)								100	47	72
Carcharhinus brevipinna (Spinner)									100	50
Carcharhinus leucas (Zambezi)										100

A Pearson's correlation test was attempted to determine if a correlation existed between phylogenetically-similar species of shark and similarity of shark based on its oral flora; the data were normally distributed, however, it did not meet the assumption of linearity (even after log transformation). Thereafter, a non-parametric Spearman's rank correlation test was performed. The correlation coefficient was 0.154, with a p-value >0.05. Consequently, the null hypothesis was accepted, revealing that no correlation existed between the data (Table 4-6).

Table 4-6 Spearman's rank correlation analysis of phylogenetic relationships between shark species and its oral microflora. A Spearmans rank correlation analysis was done, correlation coefficient = 0.154 and the p-value = 0.311 >0.05. Thus the H_0: the population correlation coefficient = 0, meaning no linear correlation exists between the data.

Correlation			Bacterial similarities	Phylogenetic similarities
Spearman's rho	Bacterial similarities	Correlation Coefficient	1.000	-.154
		Sig. (2-tailed)	.	.311
		N	45	45
	Phylogenetic similarities	Correlation Coefficient	-.154	1.000
		Sig. (2-tailed)	.311	.
		N	45	45

4.1.3 Morphological characteristics

The Vibrionacea

The morphological characteristics of these bacteria were Gram-negative, appeared to be straight/curved rods with circular, and raised, yellowish-brown, opaque colonies.

The Aeromonadacea

Aeromonas sp. morphological characters appeared white, circular and convex and its colonies appear raised, and translucent.

The Enterobacteriaceae

Morphological characteristics of the colonies appeared greyish-white, smooth, circular, raised or convex. All were rod-shaped and Gram-negative. Species of Proteus tended to swarm on agar, and appeared translucent.

The Pseudomonadaceae

Pseudomonas aeruginosa is member of the gamma proteobacteria class of bacteria. Morphological characters show that bacteria in this family are Gram-negative straight/curved rods. It produces yellowish-green, glistening colonies, and appears florescent under ultraviolet light.

The Micrococcaceae

Micrococcus species are Gram-positive cocci that are 0.5 to 3.5 micrometers in diameter and usually arranged in tetrads or irregular clusters. *M. luteus* had produced yellow colonies.

4.1.4 Antibiotic data

Among all the bacterial species analysed, the highest bacterial resistance was recorded for Cefuroxime (CFX) (Figure 4-3). Of the *Bacillus*, 59% demonstrated resistance to NA and 64% to AMP (Table 4-7). In Table 4-8 and 4-9, 13% of *Micrococcus* and 13% *Kocuria* showed resistance to CIP. Of the *Microbacterium*, 67% demonstrated resistance to CFX (Table 4-10). Further, 75% of *Acinetobacter* demonstrated resistance to AMP and 50% to CTR (Table 4-11). Of the *Staphylococcus*, 33% showed resistance to CFX and 41% to CTR (Table 4-12). High prevalence of resistance, 83%, was observed for *Proteus* against TET and NA. Less *Proteus* resistance, 20% and 17% *was* noted against CTR and GEN respectively (Table 4-13). Of *Shewanella*, no resistance was found for TET, CIP and GEN, with slight resistance of 22% and 33% toward AMP and CTR respectively (Table 4-14). Of the *Vibrio*, 67% showed resistance to CFX and 45% to AMP (Table 4-15). A high 75% of *Photobacterium* and *Pseudomonas* was resistance to CTR (Table 4-16 and Table 4-17 respectively). Among all antibiotics, bacterial species showed the highest % of susceptibility to Gentamicin, and Cefuroxime had the highest bacterial resistance (Figure 4-3).

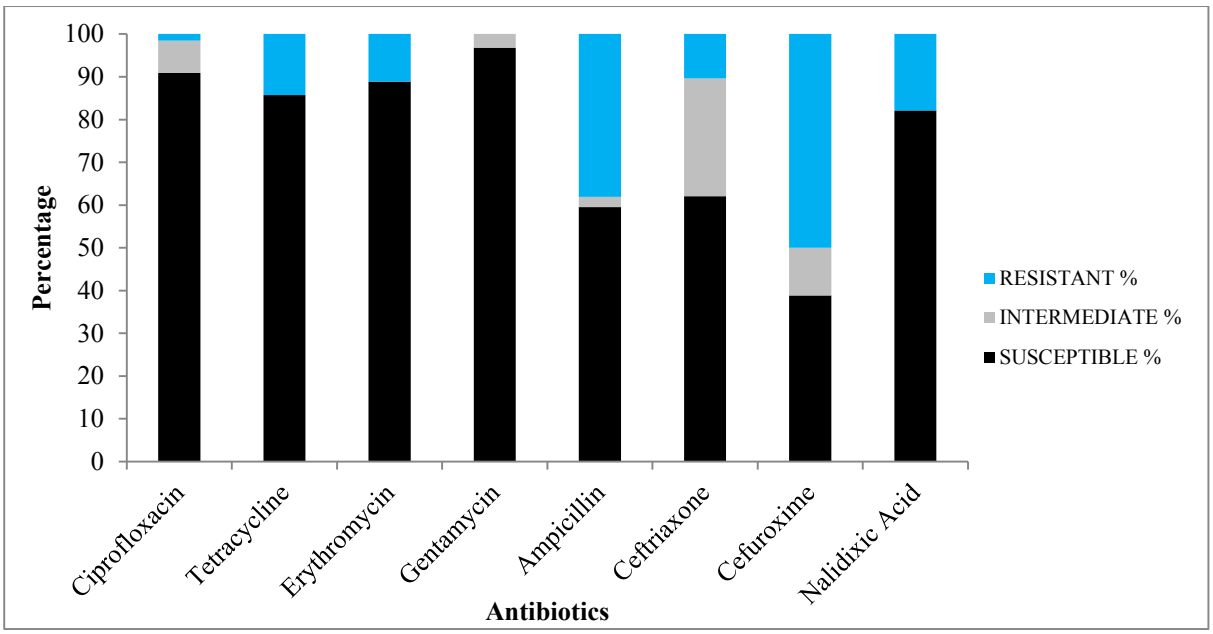

Figure 4-3 Percentage resistance and susceptibility of all bacterial isolates to 8 antibiotics

Table 4-7 MIC distributions and clinical breakpoints for *Bacillus* (n=19)

Bacillus														
ANT	**≤ 0.03**	**0.06**	**0.13**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**≥64**	**ND[c]**	**MIC[b]**
TET		5	2	2	1	1	1		1				5	1[c]
CIP	5	3	4	2		1							3	0.25[e]
ERY	3		6	1	2		1			1		1	3	N/A
GEN	1		1	1	3	6	1	1	1				3	4
AMP	3	1	1				2			3		4	4	0.125[d]
CTR	2					3		3				6	4	8
NA	1			2	1	3	2	2	3	2		1	1	1
CFX[a]		1		1								1		8

[a]CFX not all isolates tested
[b]MIC clinical breakpoints reference – Table 2B-5 Other non-enterobacteriaceae (CLSI, 2009),
[c]not determined,
[d]MIC clinical breakpoints reference – Penicillin MO7 (CLSI, 2009),
[e]MIC clinical breakpoints reference – *Bacillus anthracis*, Potential Bacterial Agents of Bioterrorism MO7 (CLSI, 2009),
N/A- no interpretations available in this family and order, ERY was, however, tested as it was part of a series of tests.

Table 4-7 Percentage resistance (grey shading) of *Bacillus* to the following antibiotics amounted to TET: 2/13 (15%), CIP: 1/15 (7%), GEN: 1/15 (7%), AMP: 9/14 (64%), CTR: 6/14 (43%), NA: 10/ 17 (59%), CFX: 1/ 3 (33%).

Table 4-8 MIC distributions and clinical breakpoints for *Micrococcus* (n=21)

Micrococcus														
ANT	**≤ 0.03**	**0.06**	**0.13**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**≥64**	**ND[c]**	**MIC[b]**
TET	1	4		4	4	2	1				1		3	1[d]
CIP	2	2	2	3		4	2						5	1
ERY	3	2	1					1	2	1	1		9	16[f]
GEN	3	1	1	3	5	1							6	16[e]
AMP	4	2	2			2		1		1			8	N/A
CTR	3	2		1	2	1	2	1		1	1		6	16[g]
NA		1		1	1		4	1	2	1		5	4	N/A
CFX[a]		1		2		2					1	1	13	16[g]

[a]CFX not all isolates tested,
[b]MIC clinical breakpoint reference (CLSI, 2009),
[c]not determined,
[d]MIC clinical breakpoint reference – Doxycycline,
[e]MIC clinical breakpoint reference – Amikacin,
[f]MIC clinical breakpoint reference – Clarithromycin,
[g]MIC clinical breakpoint reference – Cefoxitin
N/A- no interpretations available in this family and order, AMP and NA were however tested as it was part of a series of tests.

Table 4-8 percentage resistance (grey shading) of *Micrococcus* to the following antibiotics amounted to TET: 2/17 (12%), CIP: 2/15 (13%), ERY: 1/ 11 (9%), GEN: 0/14, CTR: 1/14 (7%), CFX: 2/ 7 (29%).

Table 4-9 MIC distributions and clinical breakpoints for *Kocuria* (n=8)

Kocuria														
ANT	**≤ 0.03**	**0.06**	**0.13**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**≥64**	**ND[c]**	**MIC[b]**
TET		1	1		4								2	1[d]
CIP	1	1		1	1	3	1							1
ERY	1	2	1		2			1					1	16[f]
GEN	1		1	1	3	1							1	16[e]
AMP	3		1		1		1						2	N/A
CTR	1	1				1		1		1	2		1	16[g]
NA							1				1	5	1	N/A
CFX[a]							1	1	1	1		1	3	16[g]

[a]CFX not all isolates tested,
[b]MIC clinical breakpoint reference – (CLSI, 2009),
[c]not determined,
[d]MIC clinical breakpoint reference – Doxycycline,
[e]MIC clinical breakpoint reference – Amikacin,
[f]MIC clinical breakpoint reference – Clarithromycin,
[g]MIC clinical breakpoint reference – Cefoxitin
N/A- no interpretations available in this family and order, AMP and NA were however tested as it was part of a series of tests.

Table 4-9 Percentage resistance (grey shading) of *Kocuria* to the following antibiotics amounted to TET: 0/6, CIP: 1/8 (13%), ERY: 0/7, GEN: 0/7, CTR: 2/7 (29%), CFX: 1/5, (20%).

Table 4-10 MIC distributions and clinical breakpoints for *Microbacterium* (n=8)

Microbacterium														
ANT	**≤ 0.03**	**0.06**	**0.13**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**≥64**	**ND[c]**	**MIC[b]**
TET		2		1	4								2	1[d]
CIP	1		2	2	1		1						2	1
ERY		1	1					1					6	16[f]
GEN		1		2		2	2						2	16[e]
AMP	1					2	2						4	N/A
CTR		1			2			1		1	1		3	16[g]
NA							1		2		2	2	2	N/A
CFX[a]									1		2		6	16[g]

[a]CFX not all isolates tested,
[b]MIC reference –Tortoli (2003) (CLSI, 2009),
[c]not determined,
[d]MIC clinical breakpoint reference – Amikacin,
[e]MIC clinical breakpoint reference – Doxycycline,
[f]MIC clinical breakpoint reference – Clarithromycin,
[g]MIC clinical breakpoint reference – Cefoxitin
N/A - no interpretations available in this family and order, NA were however tested as it was part of a series of tests.

Table 4-10 percentage resistance (grey shading) of *Microbacterium* to the following antibiotics amounted to TET: 0/7, CIP: 1/7 (14%), ERY: 0/ 3, GEN: 0/7, CTR: 1/6 (17%), CFX: 2/3 (67%).

Table 4-41 MIC distributions and clinical breakpoints for *Acinetobacter* (n=4)

Acinetobacter														
ANT	**≤ 0.03**	**0.06**	**0.13**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**≥64**	**ND[c]**	**MIC[b]**
TET					1	2				1				4
CIP	1	1		1	1									1
ERY						1			1	1	1			N/A
GEN				2	1	1								4
AMP			1							2		1		8
CTR						1			1			2		8
NA							2			2				N/A
CFX[a]												1	3	8[d]

[a]CFX not all isolates tested,
[b]MIC clinical breakpoint reference – Table 2B-2 *Acinetobacter* spp. M02 and M07 (CLSI, 2009)
[c]not determined,
[d]MIC clinical breakpoint reference – Ceftazidime,
N/A - no interpretations available in this family and order, ERY and NA were however tested as it was part of a series of tests

Table 4-11 percentage resistance of *Acinetobacter* to the following antibiotics amounted to TET: 1/4 (25%), CIP: 0/4, GEN: 0/4, AMP: 3/4 (75%), CTR: 2/4 (50%), CFX: 1/1 (100%).

Table 4-52 MIC distributions and clinical breakpoints for *Staphylococcus* (n=24)

Staphylococcus														
ANT	**≤ 0.03**	**0.06**	**0.13**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**≥64**	**ND[c]**	**MIC[b]**
TET	2	2		3	6	1	2				1		8	4
CIP	4	4	3	7	2								5	1
ERY	4		5	2	1			1	1	1		1	9	0.5
GEN	2	3	2	8	1								9	4
AMP	4	1	4		1	2	2	1					10	0.25
CTR	2				1		1	5	1	5	2		8	8
NA	1					1	3		1	1	1	13	4	N/A
CFX[a]				1		1		1	1		1	1	19	8

[a]CFX not all isolates tested,
[b]MIC clinical breakpoint reference – Table 2C *Staphylococcus* spp. M02 and M07 (CLSI, 2009),
[c]not determined,
N/A - no interpretations available in this family and order, NA was however tested as it was part of a series of tests

Table 4-12 percentage resistance (grey shading) of *Staphylococcus* to the following antibiotics amounted to TET: 1/17 (6%), CIP: 0/20, ERY: 4/16 (25%), GEN: 0/16, AMP: 6/15 (40%), CTR: 7/17 (41%), CFX: 2/6 (33%). No grey shading shown in CIP (indicating resistance), as all data fell below the clinical breakpoint of 1.

Table 4-63 MIC distributions and clinical breakpoints for *Proteus* (n=7)

Proteus														
ANT	**≤ 0.03**	**0.06**	**0.13**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**≥64**	**ND[c]**	**MIC[b]**
TET						1				1	3	1	1	4
CIP	2	1				3							1	1
ERY											1	5	1	N/A
GEN				1	1		2	1	1				1	4
AMP				1		1	1					2	2	8
CTR					2		1	1			1		2	8
NA								1		2	1	2	1	16
CFX[a]							1				1	1	4	8

[a]CFX not all isolates tested,
[b]MIC clinical breakpoint reference – Table 2A-enterobacteriaceae M02 and M07 (CLSI, 2009),
[c]not determined,
N/A- no interpretations available in this family and order, ERY was however tested as it was part of a series of tests.

Table 4-13 percentage resistance (grey shading) of *Proteus* to the following antibiotics amounted to TET: 5/6 (83 %), GEN: 1/6 (17 %), AMP: 2/5 (40%), CTR: 1/5, (20%), NA: 5/6 (83%), CFX: 2/3 (67%).

Table 4-74 MIC distributions and clinical breakpoints for *Shewanella* (n=9)

Shewanella														
ANT	**≤ 0.03**	**0.06**	**0.13**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**≥64**	**ND[c]**	**MIC[b]**
TET					3	5	1							4
CIP	1		1	4	3									1
ERY						1	1	5			2			N/A
GEN				1	3	1	4							4
AMP	3				3	1						2		16[e]
CTR	3			1		2				1		2		8[d]
NA					1	2		2				1	3	N/A
CFX[a]													9	8[d]

[a]CFX not all isolates tested,
[b]MIC clinical breakpoint reference – Table 2B-5 Other non-enterobacteriaceae (CLSI, 2009),
[c]not determined,
[d]MIC clinical breakpoint reference – Ceftazidime,
[e]MIC clinical breakpoint reference – Piperacillin,
N/A- no interpretations available in this family and order, NA and ERY were however tested as it was part of a series of tests.

Table 4-14 percentage resistance (grey shading) of *Shewanella* to the following antibiotics amounted to TET: 0/9, CIP: 0/9, GEN: 0/9, AMP: 2/9 (22%), CTR: 3/9, (33%).

Table 4-15 MIC distributions and clinical breakpoints for *Vibrio* (n=16)

Vibrio														
ANT	**≤ 0.03**	**0.06**	**0.13**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**≥64**	**ND[c]**	**MIC[b]**
TET	1	1		4	2	2	1	1					4	4
CIP	5	1	6	1		1							2	1[e]
ERY	1	2	4	1		2		2	2				2	N/A
GEN		1		3	3	5							4	4[e]
AMP		1					4		1	2		3	5	8
CTR				3	1	1		2		2	1		6	8[e]
NA	1			1	5	1	3	1		1	1		2	N/A
CFX[a]								1			1	1	13	8[d]

[a]CFX not all isolates tested,
[b]MIC clinical breakpoint reference – Table 2I *Vibrio cholerae* M02 and M07 (CLSI, 2009),
[c]not determined,
[d]MIC clinical breakpoint reference – Ceftazidime,
[e]MIC clinical breakpoint reference – Table 2B-5 Other non-enterobacteriaceae (CLSI, 2009),
N/A - no interpretations available in this family and order, ERY and NA were however tested as it was part of a series of tests.

Table 4-15 percentage resistance (grey shading) of V*ibrio* to the following antibiotics amounted to TET: 0/12, CIP: 0/14, GEN: 0/12, AMP: 5/11 (45%), CTR: 3/10, (20%), CFX: 2/3 (67%).

Table 4-16 MIC distributions and clinical breakpoints for *Photobacterium* (n=6)

Photobacterium														
ANT	**≤ 0.03**	**0.06**	**0.13**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**≥64**	**ND[c]**	**MIC[b]**
TET				1	1		1						3	4
CIP	1			1			1						3	1[e]
ERY	1	1						1	1				2	N/A
GEN	1						1		1				3	4[e]
AMP				1	1							1	3	8
CTR	1									2	1		2	8[e]
NA						1				1		1	3	N/A
CFX[a]										1			5	8[d]

[a]CFX not all isolates tested,
[b]MIC reference – Table 2I *Vibrio cholerae* M02 and M07 (CLSI, 2009),
[c]not determined,
[d]MIC reference – Ceftazidime,
[e]MIC reference – Table 2B-5 Other non-enterobacteriaceae (CLSI, 2009),
N/A - no interpretations available in this family and order, NA and ERY were however tested as it was part of a series of tests.

In Table 4-16 the percentage resistance (grey shading) of *Photobacterium* to the following antibiotics amounted to TET: 0/3, CIP: 1/3 (33%), GEN: 1/3 (33%), AMP: 1/3 (33%), CTR: 3/4, (75%), CFX: 1/1 (100%).

Table 4-87 MIC distributions and clinical breakpoints for *Pseudomonas* (n=14)

Pseudomonas														
ANT	**≤ 0.03**	**0.06**	**0.13**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**≥64**	**ND[c]**	**MIC[b]**
TET					1	2	3	2		2	1	1	2	4
CIP		3	1	2	2								6	1[e]
ERY	1								1	1	3	4	4	N/A
GEN	1			2	2		2	1					6	4[e]
AMP	1							1	1	1	2	2	6	16
CTR							2		1	2	2	1	6	8[e]
NA			1					1	2	2		2	6	N/A
CFX[a]							1					2	11	8[d]

[a]CFX not all isolates tested,
[b]MIC clinical breakpoint reference – Table 2B-5 Other non-enterobacteriaceae (CLSI, 2009),
[c]not determined,
[d]MIC clinical breakpoint reference – Ceftazidime,
[e]MIC clinical breakpoint reference – Table 2B- 1 *Pseudomonas aeruginosa* M02 and M07 (CLSI, 2009),
N/A - no interpretations available in this family and order, NA and ERY were however tested as it was part of a series of tests.

In Table 4-17 the percentage resistance (grey shading) of *Pseudomonas* to the following antibiotics amounted to TET: 4/12 (33%), CIP: 0/8, GEN: 0/8, AMP: 4/8 (50%), CTR: 5/8, (62%), CFX: 2/3 (67%).

Chapter 5

5.1 Discussion

5.1.1 Introduction

The bacterial profile of ten shark species was determined and compared. Conventional biochemical methods and mass spectrometry was used to identify the bacteria. Once the bacteria were identified the antibiotic susceptibility competence of each bacterial genus was established on a range of antibiotic classes. Research on the oral bacterial component of each shark species in this study is limited or lacking, globally. Therefore, data obtained can help fill the gaps in this research area by; equiping researchers with a better understanding of the microbial community present in large coastal predators and indirectly the surrounding environment they inhabit and by assisting medical personnel make informed decisions when administering treatment to shark bite victims. Antibiotic resistance in certain bacterial species was also found. Antibiotic resistance data in bacteria found in top predators and thus, along our coastline, will add baseline information toward future studies and management processes, as this poses a health risk to society if not properly monitored.

The aims and objectives of this study; identifying bacterial isolates from the oral cavity of sharks, for antibiotic resistant screening, to obtain antimicrobial therapies for shark bitten victims have been met.

In South Africa, there have been 40 shark attacks since 2008. The highest numbers of attacks have taken place in Port St John's, in the Eastern Cape. This beach has become increasingly notorious for its danger and is dubbed the 'world's deadliest' (Maclean, 2012). The impact and occurrence of human-shark encounters vary with the distribution and behaviour of each shark species, with a notorious handful being extremely dangerous and commonly implicated in attacks; these are the great white, tiger and Zambezi sharks (Caldicott et al., 2001).

A review of 86 attacks in South Africa, found that the great white was involved in 49% of attacks, and Zambezi sharks in 7% (Woolgar et al., 2001), however, true numbers of human-shark encounters for each shark species are largely unknown. Reported cases are not always true representations because of assumptions made by witnesses or victim accounts. Additionally, the tooth morphology of species in the same family is in most instances highly

similar, leading to almost identical wound patterns, thereby adding to confusion when detecting species-specific attacks (Woolgar et al., 2001).

It was, therefore, important that this study conduct research on an array of species for a better and broader understanding. Furthermore, historic data from the International Shark Attack files show that all ten species of shark in this study have had some contact with humans (ISAF, 2014, Burgess, 2015). Therefore even minor injuries with less dangerous sharks that are not commonly documented in shark attack files, can lead to infections, and those infections can be fatal if the correct treatment is not administered. Researchers in the field have stated the impending danger of marine bacteria in human health, because many species have been associated with wound infection toxicity (Kueh et al., 1992, Howard and Bennett, 1993).

5.1.2 Microbial pathology

The sea is not a sterile environment, and it is home to halophillic marine bacteria present like *Vibrio* and *Aeromonas*. These bacteria are capable of establishing rapid and progressive cellulitis or myositis, within hours of exposure (Royle et al., 1997). *Vibrio* has been previously cultured from shark-inflicted wounds (Pavia et al., 1989). The diet of the shark can also lead to the oral cavity housing infectious bacteria (Caldicott et al., 2001).

A broad overview of bacteria that can be plated from a shark's oral cavity can be seen in Table 4.4, in this table, it can be clearly seen that most of the bacteria found in this study, was also found in previous research studies. So perhaps we can infer that most of these bacteria can possibly part of a shark's oral cavity.

The Vibrionaceae

The *Vibrio* family namely; *Vibrio*, *Photobacterium*, and *Aeromonas,* have all been found in this study. The genera *Vibrio* and *Photobacterium* have many common characteristics, these two are closely related, and both are ubiquitous in marine and brackish environments (Thompson et al., 2004). Over the years, many researchers have isolated both *Vibrio* and *Aeromonas* from aquatic animals; today both are considered to be part of the indigenous microflora of marine, fresh water fish, shellfish and the aquatic environments (Colwell and Grimes, 1984, Olafsen, 2001, Uhland, 2011).

The *Vibrio* spp. found in this study is; *V. harveyi*, *V. alginolyticus*, *V. metschnikovii and V. parahaemolyticus*. This study found that five of the 10 shark species sampled, namely; ragged-tooth shark *Carcharias taurus,* tiger shark *Galeocerdo cuvier*, great white *Carcharodon carcharias*, spinner *Carcharhinus brevipinna* and scalloped hammerhead *Sphyrna zygaena* had the above Gram-negative bacteria (*Vibrio*, *Photobacterium*, *Aeromonas*) as part of its oral microflora.

In the environment and in particular, the water column, Simidu (1980) found that *Vibrio* spp. accounted for 80% of surface seawater microflora in the Pacific. The family vibrionaceae is one of the most important bacterial groups in marine environments and members of this family often predominate in the bacterial flora of seawater, plankton, and fish (Kita-Tsukamoto et al., 1993). *Vibrio* spp. that inhabit the surface water layers grow faster because they have access to easily degradable organic matter, and can utilise the carbohydrates and organic acids present. Vibrios in the middle and bottom depths utilised particulate organic matter found in deeper waters (Simidu and Tsukamoto, 1985).

Vibrio spp. such as *V. alginolyticus*, *V. parahaemolyticus, V. anguillarum* (Austin et al., 1995, Hjelm et al., 2004, Radjasa et al., 2007) compete in the environmental with other bacterial populations through antibacterial activities. These antibacterial activities influence microbial populations in the environment (Levy, 2002). This would also then influence the oral cavity's microflora population, with a predisposition for *Vibrio* spp. being among the highest bacteria present. This was true as *Vibrio* spp. was found in 50% of sharks' species and ranked the highest in species richness after *Micrococcus* spp., *Staphylococcus* spp. and *Pseudomona*s spp. respectively.

As early as (1957), Liston found that the *Vibrio* spp. dominated the intestinal flora of fish. *Vibrio* spp. have been previously isolated from organs like the kidneys, or lesions on diseased fish (Uhland, 2011). Five of the 19 *Vibrio* species are notorious for causing disease in fish and shellfish and are important pathogens for both humans and animals and as mentioned above, have been previously isolated from the intestine and surface of freshwater and marine fish.

Clinically, halophilic lactose-fermenting *Vibrio* spp. found in patients who had eaten raw oysters resulted in septicemia, 11 of the 24 patients died as a result. Another set of 15 patients, who, after being exposed to sea water or from the handling of crabs, had wound infections

relating to *Vibrio* spp, of these 15 patients, one patient died. This is because some *Vibrio* spp. are pathogenic and should be considered in the diagnosis of septicemia if patients have been exposed to seawater, sustains an injury at sea or is attacked (Blake et al., 1979).

The genus *Aeromonas* was initially in the family Vibrionaceae, however, in (1984), Colwell and Grimes proposed a newly created family. *Aeromonas* was transferred to the aeromonadaceae family (Colwell and Grimes, 1984). In the environment, *Aeromonas* is globally recognised as an opportunistic fish pathogen. It is part of intestinal microflora of fish and is, therefore, commonly isolated from the intestine, internal organs of diseased fish, including external surfaces or lesions of the fish and the environment, and known to cause furunculosis (abscess formation) (Hanninen et al., 1997).

Clinically, the diseases in humans caused by *Aeromonas* range from wound infections, bacteraemia, meningitis, pulmonary infections and gastroenteritis (Tsai et al., 2007). *Aeromonas* and *Vibrio* have become recognized as pathogens capable of causing severe infections leading to disease in humans. These genera are exposed to various antimicrobials, and therefore, there is a possible development of resistance (Uhland, 2011).

The Enterobacteriaceae

The Enterobacteriaceae family can be isolated from a variety of places, for example water, food, sewage and soil. It has also been commonly found in the intestine of animals and man (Munn, 2004). These genera are thus known as the enterics and are opportunistic pathogens of fish. This family consists of the genera *Escherichia*, *Serratia*, *Enterobacter*, *Proteus and Plesiomonas*.

In the environment, enterobacteria are found in coastal waters polluted by terrestrial sources, and are hence found in the gut of fish and marine mammals. They are not indigenous marine organisms and are indicators of fecal pollution (Munn, 2004). This makes sense because certain sharks were found in Durban, Richards Bay and Uvongo and these areas are subjected to anthropogenic pressures because of the high proximity to terrestrial influences, from rivers, waste water treatment plants (wwtp) and estuaries along the coastline.

Enterobacter cloacae are clinically important bacteria, because infections have the highest mortality rate compared to other *Enterobacter* infections(Rose et al., 2009). *Enterobacter cloacae* is a human clinical pathogen that can cause a range of infections such as bacteremia, lower respiratory tract infection, skin and soft tissue infections, urinary tract infections, endocarditis, intra-abdominal infections, septic arthritis, osteomyelitis, and ophthalmic infections (Rose et al., 2009).

Treatment with cefepime and gentamicin has been reported (Barnes et al., 2003). These bacteria contain beta-lactamase, which is undetectable in vitro, these infections can lead to morbidity and mortality and the infection is hard to manage due to their multiple antibiotic resistances such as third generation cephalosporin.

The results of this study found that three of the 10 shark species sampled, namely two ragged-tooth sharks *Carcharias taurus,* two tiger sharks *Galeocerdo cuvier* and two scalloped hammerhead sharks *Sphyrna zygaena* had enterobacteriaceae species part of its oral microflora. *Proteus vulgaris* was found in the oral cavity of two ragged-tooth sharks *Carcharias taurus* and one tiger shark *Galeocerdo cuvier. Proteus mirabilis* was found in a juvenile tiger shark *Galeocerdo cuvier*, the gut contents of this shark contained; Spotted grunter, longfinned batfish, rat, whale, unidentified shark and octopus. *Serratia marcescens* was found in the oral cavity of a mature scalloped hammerhead shark *Sphyrna zygaena*, in Richards Bay. Terrestrial influence is imminent as can be seen from the ingested rat. Inferences on bacteria obtained, can be drawn from gut content fauna, as diet also influences oral cavity flora.

The Pseudomonadaceae

The genus *Pseudomonas* includes 27 Gram-negative species; with only two species capable of causing diseases in fish. *P. fluorescens* is one of these species, and can be isolated from the internal organs of diseased fish or surfaces of fish (Munn, 2004).

Pseudomonas aeruginosa is an opportunistic pathogen, as it exploits a host when the hosts defenses are compromised, thus initiating infection. *Pseudomonas aeruginosa* is the epitome of an opportunistic pathogen of humans. Several different epidemiological studies have tracked its importance, because antibiotic resistance has increased in clinical isolates. The

bacterium is widespread in the environment, and is ubiquitous in soil and water. P*seudomonas aeruginosa* has an affinity for growth in moist environments.

This study found that seven of the 10 shark species sampled, namely four ragged-tooth sharks *Carcharias taurus,* two spinners sharks *Carcharhinus brevipinna*, one; great white *Carcharodon carcharias*, mako *Isurus oxyrhincus*, scalloped hammerhead *Sphyrna zygaena*, blacktip *Carcharhinus limbatus* and copper shark *Carcharhinus brachyurus* had *Pseudomonas* sp. as part of their oral microflora, namely*; P. stutzeri*, *P. aeruginosa*, *P. mendocina*, *P. fluorescens*, *P. putida* and *P. fulva*.

Only a few antibiotics are effective against *Pseudomonas*, including ciprofloxacin – this is mirrored in the current study, as only 2 % was found to be resistant to CIP (other fluoroquinolones). Other antibiotics that can be used are gentamicin and imipenem; however these antibiotics are not effective against all strains.

The group of Gram-positive cocci, in the family Micrococcaceae

The genera include *Micrococcus*, *Aerococcus* and historically the genera *Staphylococcus* is included in this family however, by molecular and chemical analysis, studies show that *Staphylococcus* sp. which are non-motile and non-spore forming is more closely related to bacillaceae, planococcaceae, and listeriaceae and therefore falls under the order bacillales (Bauman, 2007). *Micrococcus* sp. *M. luteus* and *Kocuria varians* is the most commonly found halotolerant species on human skin. Kocuria fall in the family micrococcaceae and are nonmotile, nonsporing, aerobic Gram-positive cocci (Payne et al., 2003).

The skin colonizers *Micrococcus* sp., *Kocuria* sp. and *Kytococcus* sp. can be easily confused with coagulase negative *staphylococci*, which are different as they grow aerobically and produce coagulase. These are not recognised as major fish pathogens, but have been previously isolated from external surfaces and the intestine of fish. It is occasionally associated with skin lesions (Munn, 2004). The genus *Aerococcus* includes one species *A. viridians,* which causes the disease gaffkemia in lobsters (*Homarus americanus* and *Homarus vulgaris*).

It is easy to overlook *Micrococcus* as the cause of infections as disease caused by this bacterium are rare and is in addition a natural part of the skin's bacterial flora. This bacterium

is widespread in the environment, and is ubiquitous in soil and water. Moreover, micrococcus is generally thought of as a harmless bacterium, but in rare cases it has caused infections in imuno-compromised patients (Payne et al., 2003).

The endospore-forming Gram-positive rods and cocci group

The bacilli and cocci present in this group are significant in the environment and health care settings in which it exists. This group contains genera like *Enterococcus* spp., *Bacillus* spp., and *Staphylococcus* spp. The genus *Bacillus* is capable of producing endospores. During early growth these rods are Gram-negative, but can become Gram-variable after 24 hours of incubation. The genus *Bacillus* is found on the external surfaces and in the digestive tract of fish, it is part of the non-pathogenic microorganisms found in fish (Munn, 2004). The genera *Streptococcus* and *Enterococcus* are Gram-positive cocci that cause wound infections, of leading to pneumonia and scarlet fever (Bauman, 2007).

The genus *Staphylococcus* is of clinical significance. It is known to cause bacteremia endocarditis and urinary tract infections (Bauman, 2007). Half of all species in this genus is found on human skin. The most common *staphylococci* causing disease are *S. epidermidis, S. haemolyticus, S. capitus, S. saprophyticus* which are all coagulase-negative in contrast *S. aureus* and *S. delphini* produces coagulase (Wilson, 2008). This study found that eight of the 10 shark species sampled, namely; six ragged-tooth *Carcharias taurus*, one great white *Carcharodon carcharias*, two mako *Isurus oxyrhincus*, one scalloped and smooth hammerhead *Sphyrna zygaena* and *Sphyrna lewini*, two blacktip *Carcharhinus limbatus* and six spinner sharks *Carcharhinus brevipinna* contained species from this bacterial group. Six of eight ragged-tooth sharks *Carcharias taurus* which were mainly found in Richards Bay contained different species of staphylococci *(S. cohnii*, *S. heamolyticus*, *S. equorum/simulans*, *S. sciuri/lugdenensis, S. delphini*) and *Bacillus* (*B. cereus*, *B. thuringiensis)* in its oral cavity.

5.1.3 Concluding bacterial report

Wound infection is often polymicrobial; having an extensive mix of Gram-negative and Gram-positive bacteria including aerobic and anaerobic forms. According to Abrahamian and Goldstein (2011) the bacteria infecting the wound mirrors the oral flora of the biting animal. It can also be reflective of its geographic location, ontogeny, age and perchance the microbiome of the prey items ingested by the shark (Abrahamian and Goldstein, 2011). These are all

global concerns relating to therapeutic options for the treatment of shark bites, other marine-sustained wounds, and antibiotic resistance.

Bacteria recovered in this study commonly occur in non marine, aquatic environments, including those from terrestrial sources, that reaches the ocean through effluent and runoff; therefore we cannot report that any species was exclusive to the teeth of the shark.

No significant relationship was found between the relatedness of the shark species and the relevant bacterial assemblages (Table 4-6). Even sharks of the same genus, the smooth hammerhead *Sphyrna zygaena* and scalloped hammerhead sharks *Sphyrna lewini*, showed only a low 38 % chance of similarity. This gives us some indication that perhaps physiology and dentition is not a determining or contributing factor when microflora establish in a shark.

The oral flora of sharks from this study, found in diverse locations hundreds of kilometers apart, namely Richard Bay, Port Edward, Amanzimtoti, Durban and Margate, show no clear relationship between location and bacterial content. The analysis of gut contents of these shark species revealed ingestion of a small unidentified shark, the bony fish *Epinephelus andersoni*, an unidentified whale, a tiger shark, a turtle, two large unidentified fish and a bird. No correlations could be drawn from diet and oral cavity bacterial content. However, possible inferences could be made from future studies with a larger shark sample size.

Among shark species, collectively taken into consideration, the most commonly isolated bacteria and its subsequent percentage of species-richness were *Micrococcus* sp. at (90%), *Staphylococcus* sp. (70%), *Pseudomonas* sp. (70%), *Bacillus* sp. (50%), *Vibrio* sp (50%) and *Kocuria* sp. (50%) (Table 4-3) They account for the top six bacteria species predominantly found in shark samples (Table 4-5). In comparison with a very recent study conducted by Dr Borrego and team (Unger et al., 2014), these findings differed slightly, as the bacterial species most commonly found in shark samples were *Vibrio* and *Pasteurella* sp. This shows the commonalities and differences between the two studies.

Certain bacteria, common in the environment where the sharks were found are pathogenic, and known to cause life-threatening infections in humans. Marine vibrios for example, are extremely important to human health and are highly infectious in marine-related wounds. *V.*

parahaemolyticus and *V. alginotyticus* found in the sharks oral cavity is pathogenic, capable of causing septicemia (Blake et al., 1979).

Other bacteria, aside from being pathogenic are enteric organisms, these are non-fermenting Gram-negative bacilli (e.g. *Pseudomonas* sp., *Shewanella putrefacians)*, enterobacteriaceae (e.g. *Enterobacter* sp.) and *S. aureus*, found in this study, are organisms that show exposure to sewage effluents (Unger et al., 2014). This makes sense because; along the length of the shark nets there are various estuaries that have waste-water treatment plants upstream. These treatment plants offload effluent into the estuaries and this ultimately reaches the ocean.

The following halotolerant bacteria found on the teeth of four different shark species: Zambezi, spinner, great white and tiger sharks; was *Shewanella putrefaciens*, *Staphylococcus* sp. and *Micrococcus* sp., these findings are in accordance with previous studies (Pien et al., 1983). This also confirms an early prediction of Hugh and Gilardi (1980) who stated that *Shewanella putrefaciens* might be found on the teeth of sharks and could cause health risks during wound infection (Colwell and Grimes, 1984).

Human pathogens like *Staphylococcus* sp. and *Streptococcus* sp. are commensal on human skin and can easily be driven into the wound by a shark bite, or contamination due to first-aid effort (Rtshiladze et al., 2011). Therefore it is essential that patients suffering from staphylococcal infections are prescribed suitable antimicrobial therapy (Unger et al., 2014).

Considering the large number of potential contaminants, one study (Royle et al., 1997) suggested the use of swabbing bite wounds to help target therapy according to the results, once swabbed, bacteria infecting the wound would be known and targeted therapy can commence. Using MALDI-ToF MS for identification this procedure can be performed within 24 hrs (MALDI-ToF is increasingly been used in commercial diagnostic laboratories (e.g. Lancet Pathology Services, Durban).

5.1.4 Antibiotic resistant concerns

The increasing incidence of bacterial resistance to antibiotics is a concern for public health (Schaefer et al., 2009, Blackburn et al., 2010). The extent of the resistance in oceans and estuaries illustrates ill-exposure of antibiotics to non-target populations and subsequent transfer of resistant strains (Koonin et al., 2001, Schaefer et al., 2009).

Antibiotic contamination of waterways due to anthropogenic effects by discharge of effluent or runoff causes 'selective pressures' in oceans and estuaries, and result in the surfacing of antibiotic resistant bacteria (Blackburn et al., 2010). Antibiotic resistance does, however, occur naturally, but the high levels of exposure to anthropogenic antibiotic-pollutants via use in aquaculture, agriculture and human disease-control have led to antibiotic resistance being found and documented more widely. Moreover, this continued anthropogenic impact on the environment, causes bacterial gene mutation or gene transfer between bacteria (Rose et al., 2009, Blackburn et al., 2010).

Bacteria aquire resistance through DNA transfer, this is a result of bacteria evolving and adapting to environmental stresses. Antibiotics are excreted by animals and individuals and the antibiotic continues to exert its selective pressures. In the environment, antibiotics are in their 'post treatment period' (after having being administered/ prescribed); it is dispersed in diluted amounts into the environment, allowing ample time to select resistant organisms. This allows bacteria to adjust and survive in a stressed environment (Arnold, 2011).

Previous studies on marine animals have found multiple drug resistant organisms in sea-birds, sharks, dolphins, demersal and pelagic fish, and pinnipeds. These animals can potentially act as reservoirs of antibiotic resistant bacteria in the marine environment (Blackburn, 2003, Rose et al., 2009, Schaefer et al., 2009). It becomes a public health concern when resistance builds and spreads and when bacteria become multiple drug resistant. Antibiotic-pollution enters the ocean via effluent or runoff, thus bacteria in the ocean are continually being exposed to this. Marine bacteria are able to colonise a host (fish or invertebrates), and this host could become a prey items for top predators like humans and large predatory animals like sharks. Resistant bacteria are thus able to make their way to the top of the food chain. Sharks have the potential to become vectors for antibiotic resistant bacteria, and if, for example, a non-fatal shark attack should occur, untreated infections could lead to fatalities.

5.1.5 Antibiotic treatment

Globally, the oral flora of cartilaginous fish is largely unknown (Rtshiladze et al., 2011), and the relevant literature reveals only a few authors incorporating antibiotic therapy into the management of shark bites (Buck et al., 1984, Rtshiladze et al., 2011, Woolgar et al., 2001). In

a South African review on shark attacks, 19 cases reported no antibiotic use (Woolgar et al., 2001), while some cases recommended antibiotic prophylactic broad spectrum antibiotics. This can cause problems, as broad spectrum antibiotics result in lowering the function of the immune system (Woodrow, 2007).

Early literature shows therapeutic options supporting a schedule of cefotaxime and metronidazole intravenously, followed by ciprofloxacin orally (Buck et al., 1984). Moreover, these authors felt that the microflora of the tissue involved in the attack (e.g. enteric organisms in the case of abdominal wounds) should also be considered when administering treatment.

For a Zambezi attack on a 32-year old male surfer that took place at Bondi Beach in Sydney, Australia, Royle et al. (1997) reported initial use of intravenous ciprofloxacin twice daily and Tazocin (piperacillin/tazobactam) three times a day. A naval diver, training in the Sydney harbour, who was struck by an inquisitive juvenile great white, was administered with ceftriaxone, metronidazole and gentamicin intra-operatively. Post-operatively, the use of a 5-day course of tazocin (piperacillin/tazobactam) and ciprofloxacin was specified. This was thought to offer a broader cover as a result of the large number of potential contaminants including both Gram-negative and Gram-positive bacteria (Royle et al., 1997), the above treatment regimes makes sense when looking at an overview of the present studies data; as a very small percent of resistance to ciprofloxacin and no ressitance toward gentamicin was seen. More recent work in Florida, by Dr. Borrego and his team, recommends empiric treatment with a fluoroquinolone or a combination of a 3rd generation cephlasporin plus doxycycline for any blacktip shark victims (Unger et al., 2014). Treatment regimen advocates for the use of aminopenicillins and aminoglycosides to expand the therapeutic options for the treatment of shark bites.

In South Africa, 18 shark attack cases used the following antibiotics, second-generation cephlasporin, together with amoxicillin or clauvanic acid or metronidazole (Woolgar et al., 2001). Interaminense et al. (2010) found that levofloxacin was effective against all bacteria tested, and reported its effectiveness as a single agent. Interaminense et al. (2010) also found that *Proteus* sp., Gram-positive cocci and *Staphylococcus* sp. showed susceptibility toward aminoglycosides which is similar to susceptibility results found in this current study. Further treatment that was included was second and third generation cephalosporin, tetracycline and chloramphenicol (Interaminense et al., 2010, Rtshiladze et al., 2011). *Pseudomonas*

aeruginosa is frequently resistant to many commonly used antibiotics. Although many strains are susceptible to gentamicin, tobramycin, colistin, and fluoroquinolones, resistant forms have developed. The combination of gentamicin and carbenicillin have been previously used to treat severe *Pseudomonas* infections (Greene et al., 1973). Antibiotic resistance for the composit *Pseudomonas* spp. in this study had found similar trends, with no resistance shown toward gentamicin and ciprofloxacin, but more than 60% resistance toward cephalosporins. The present study examined antibiotic resistance on a genera level and not species level. This occurred because the data sets for each species needs to be increased and further examined in the future. Antibiotic resistance is best understood when examining species specific data and not on a general genus/genera level. Therefore future studies should expand on this current study, more specifically looking into species specific resistance, and not a broad overview of genus/genera antibiotic resistance. This will result in better understanding biological information conveyed when viewing species specific resistance.

A patient should be carefully evaluated when determining the organism causing the infection; this will help choose the most effective drug treatment for that organism (Edmunds, 2006). The more clinically significant bacteria were targeted in this study however future studies need to accommodate for the growth needs of marine environmental flora and other fastidious organism requiring specific growth regimes. Other improvements in study design are explained fully below.

5.1.6 Caveats of study

In every study there are confounding factors that arise in the sampling process or study design. The best thing to do, is to understand what the confounding factors are, the affects they bring upon the study.

Firstly, the use of dead sharks can be seen as a confounding factor, the possibilities of terrestrial contamination and new microbial growth on dead shark samples need to be considered wen examining data; however it must be noted that the strictest of care was taken, so as not to contaminate the sample. Swabs were taken as far back as possible from the mouth opening, from the lower and upper jaw of the oral cavity. Additionally, the KZNSB staff ensured that each sample was ‘fresh’, meaning that if it had been entangled in the nets and died, it was brought back to the wet laboratory that very day. Information on the length, sex,

gut contents, including some microbial gut analysis was collected for each sample; this study therefore holds more detail for each study animal than previous studies on this topic.

There were certain bacteria in several shark species that yielded no results in terms of bacterial identification. The reason could be, that the unidentified organism is not covered in the API 20 NE, 20 N databases (bioMérieux, Marcy-l'Etoile, France) or in the Bruker Daltonics mass spectral database and therefore identification could not be made.

Moreover, this study only considered aerobic Gram-positive and negative organisms. Certain organisms may not have been harvested during incubation, due to very strict environmental conditions which are required for their growth and survival. If rigid requirements and specific techniques are not followed to accomodate for the organisms needs, they may not flourish, and would go undetected.

In addition, cultures were not plated on marine agar, but on nutrient agar (which does have a percent of sodium chloride in it) however growth requirements for certain marine species could have occurred, this was initially done based on the original objective of investigating clinically important bacteria. Further studies on this can be 'fine-tuned' toward selecting for marine bacteria. The study outcome has a descriptive advantage and allowed for the identification of previously unreported bacteria in South African sharks.

The issue of 'time' was also an aspect; there was a cut-off time of 72 hours, because samples were destroyed by the overgrowth of certain species. Cultures needing longer incubation time were automatically excluded in the study. Culturing the entire microbial community by having all the necessary requirements to accomodate 'blindly' for every would-be species was out of scope for this study, as this would far exceed the time-frame required for an MSc dissertation. Therefore there are vast amounts of aspects untouched and many questions remain unanswered; further research in this field is certainly needed.

The small sample size of each bacterium to each shark is also unfortunate, because in some cases, only one or two bacterial species was obtained, this led to difficulties in the data processing step, in order to overcome this, species of bacteria had to be group into genera, thus increasing the sample size and assisting in easier data analysis. This is unfortunate because, various *Vibrio* groups for example, show vast differences in physiology and biochemical

activities, these activities are directly proportional to their niche in the environment (Simidu and Tsukamoto, 1985). Therefore samples for both shark species and bacterial species needs to increase for future studies.

5.2 **Conclusion**

The observations derived from this study confirm that the teeth from ten different species of shark found in areas along the KwaZulu-Natal coast are a source of infectious bacteria. The bacterial assemblages reported are diverse and depend on the sharks' species and characteristics, although more closely related sharks do not necessarily harbour more similar assemblages.

Although shark attacks are rare, there are places in the world which are regarded as 'hot spots' for shark attacks. The induced trauma from an attack is dramatic but more often than not the bite is not fatal, yet the imminent infection can be. By increasing our awareness regarding potential pathogens in our ocean environment, and associated with the top predators that frequent our coasts will allow for rapid, appropriate and more targeted treatment. The antibiotic susceptibility patterns reported here, including those susceptible to aminoglycosides and aminopenicillins, can add to the therapeutic options for the treatment of shark bites.

The prevalence of a typical wound infection, post shark bite, is extremely high, if the clinician fails to provide rapid and appropriate antibiotic therapy, this will result in increased mortality and morbidity. Increased survival rate will depend upon, improved first responder training and improved presumptive antibiotic therapy (Pavia et al., 1989, Auerbach, 1993, Howard and Bennett, 1993, Burnett, 1998).

Managing of infections is extremely important (Buck et al., 1984) The recommended treatment in this case is empirical antibiotic therapy. Antibiotics prescribed need to cover for *Vibrio* spp. and according to Buck et al, (1984), this should be third generation cephalosporin or ciprofloxacin. Infection by *Aeromonas* spp. should require imipenem or an aminoglycoside. Infections via *Staphylococcal* and *Streptococcal* are also common infections and must also be covered in the treatment process. Abdominal injuries require antibiotics effective against enteric organisms. Infected wounds, if not properly managed, can result in; fulminant infections including myositis and necrotizing fasciitis. *V. parahaemolyticus* is clinically significant and causes 30% clinical bacteraemia (Caldicott et al., 2001).

Another aspect of concern is antimicrobial resistance. The findings of this study confirm antibiotic resistance in the bacterial flora found in the oral cavities of sharks. Bacteria in the ocean are continually being exposed to antibiotic pollution via terrestrial effluent or runoff. In this study, the oral cavity of sharks provided a snapshot into the multi-drug resistant bacteria present in our environment. It becomes a public health concern when resistance builds and spreads, and even more when humans are exposed to these bacteria directly or indirectly.

The coastal marine environment could possibly be a reservoir filled with antimicrobial-resistant bacteria. Therefore, future surveillance of predatory fish should continue. Predatory fish are long-lived, slow-growing and face extended exposure to antimicrobial-resistant bacteria. For these reasons, they can serve as valuable sentries for future antimicrobial resistance studies, where the evolution of resistance in some systems can be monitored and managed.

References

ABRAHAMIAN, F. & GOLDSTEIN, E. 2011. Microbiology of animal bite wound infections. *Clinical Microbiology Reviews,* 24**,** 231–46.

ARNOLD, C. 2011. To Share and Share Alike. Bacteria swap genes with their neighbors more frequently than researchers have realized. *Scientific American,* 304**,** 30-31.

AUERBACH, P. S. 1993. The 'problem' of shark attacks upon humans. *Journal of Wilderness Medicine,* 4**,** 5-10.

AUSTIN, B., STUCKEY, L., ROBERTSON, P., EFFENDI, I. & GRIFFITH, D. 1995. A Probiotic Strain of *Vibrio alginolyticus* Effective in Reducing Diseases Caused by *Aeromonas salmonicida*, *Vibrio anguillarum* and *Vibrio ordalii*. *Fish Disease,* 18**,** 93–96.

BARNES, B., WIEDERHOLD, N., MICEK, S., POLISH, L. & RITCHIE, D. 2003. *Enterobacter cloacae* ventriculitis successfully treated with cefepime and gentamicin: case report and review of the literature. *Pharmacotherapy,* 23**,** 537-42.

BAUMAN, R. 2007. *Innate Immunity,* New York, Pearsons International.

BESTER, L. A. & ESSACK, S. Y. 2010. Antibiotic resistance via the food chain: Fact or fiction? *African Journal of Science,* 106**,** 1-5.

BEVERIDGE, T. 2001. Use of Gram stain in microbiology. *Biotechic & Histochemistry,* 76**,** 111-118.

BLACKBURN, J. K. 2003. *Characterizing Spatially Explicit Patterns of Antibiotic Resistance in the Marine Environment Using Top-level Marine Predators.* MSc, Louisiana State Univ

BLACKBURN, J. K., MITCHELL, M. A., BLACKBURN, M.-C. H., CURTIS, A. & THOMPSON, B. A. 2010. Evidence of Antibiotic Resistance in Free-Swimming, Top-Level Marine Predatory Fishes. *Journal of Zoo and Wildlife Medicine,* 41**,** 7-16.

BLAKE, P., MERSON, M., WEAVER, R., HOLLIS, D. & HEUBLEIN, P. 1979. Disease caused by a marine Vibrio. Clinical characteristics and epidemiology. *New England Journal of Medicine,* 300**,** 1-5.

BRUCE, B., STEVENS, J. & MALCOLM, H. 2006. Movements and swimming behaviour of white sharks (*Carcharodon carcharias*) in Australian waters. *Marine Biology,* 150**,** 11.

BUCK, J., SPOTTE, S. & GADBAW, J. J. 1984. Bacteriology of the teeth from a great white shark: potential medical implications for shark bite victims. *Journal of Clinical Microbiology,* 20**,** 4.

BUDKER, P. 1971. *The life of sharks,* New York, Columbia University Press.

BURGESS, G. 2015. *Reducing the Risk of a Shark Encounter: Advice to Aquatic Recreationists* [Online]. United Statesof America University of Florida. Available: http://www.flmnh.ufl.edu/fish/sharks/Attacks/relariskreduce.htm [Accessed 21 February 2015].

BURNETT, J. 1998. Aquatic adversaries: shark bites. *Cutis,* 61**,** 317.

CALDICOTT, D. G. E., MAHAJANI, R. & KUHN, M. 2001. The anatomy of a shark attack: a case report and review of the literature. *Injury,* 32**,** 445-453.

CARBONNELLE, E., MESQUITA, C., BILLE, E., DAY, N., DAUPHIN, B., BERETTI, J.-L., FERRONI, A., GUTMANN, L. & NASSIF, X. 2011. MALDI-TOF mass spectrometry tools for bacterial identification in clinical microbiology laboratory. *Clinical Biochemistry* 44**,** 104–109.

CDC. 2012. *Antimicrobial resistance* [Online]. USA: Centers for Disease Control and Prevention Available: http://www.cdc.gov/drugresistance/ [Accessed 5 April 2015].

CLIFF, G. & DUDLEY, S. 2011. Reducing the environmental impact of shark-control programs: a case study from KwaZulu-Natal, South Africa. *Marine and Freshwater Research,* 62**,** 700-709.

CLIFF, G., DUDLEY, S. F. J. & B, D. 1989. Sharks caught in the protective gill nets off Natal, South Africa. 2. The great white shark *Carcharodon carcharias* (Linnaeus). *African Journal of Marine Science,* 8**,** 13.

CLIFF, G., DUDLEY, S. F. J. & DAVIS, B. 1990. Sharks caught in the protective gill nets off Natal, South Africa. 3. The shortfin mako shark *Isurus oxyrinchus* (Rafinesque). *African Journal of Marine Science,* 9**,** 11.

CLIFF, G. & WILSON, R. 1994. *Natal Sharks Board field guide to sharks and other marine animals,* Natal Sharks Board, Umhlanga Rocks.

CLSI 2008. Performance Standards for Antimicrobial Susceptibility Testing: Eighteenth Informational Supplement M100-S18. Clinical and Laboratory Standards Institute Wayne, PA.
CLSI 2009. Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically 29.
COLWELL, R. R. & GRIMES, D. J. 1984. Vibrio diseases of marine fish populations. *Helgoland Marine Research,* 37**,** 265-287.
COUZINET, S., JAY, C. & BARRAS, C. 2005. High-density DNA probe arrays for identification of staphylococci to the species level. *Journal of Microbiology Methods,* 61**,** 201-209.
DAPP, D. R., WALKER, T. I., HUVENEERS, C. & REINA, R. D. 2015. Respiratory mode and gear type are important determinants of elasmobranch immediate and post-release mortality. *Fish and Fisheries*.
DAVIES, D. H. 1963. Shark attack and its relationship to temperature, beach patronage and the seasonal abundance of dangerous sharks. *Oceanographic research institute Investigational Report 6***,** 43.
EDMONDS, C. & THOMAS, R. L. 1972. Medical aspects of diving. *Medical Journal of Australia,* 2**,** 1256-1260.
EDMUNDS, M. W. 2006. *Introduction to clinical pharmacology,* Missouri USA, Mosby – Elsevier.
GOVENDER, A., KISTNASAMY, K. & VAN DER ELST, R. 1991. Growth of spotted ragged-tooth sharks *Carcharias taurus* (Rafinesque) in captivity. *African Journal of Marine Science,* 11**,** 15-19.
GREENE, R. J. & HARRIS, N. D. 2008. *Pathology and therapeutics for pharmacists: a basis for clinical pharmacy practice,* Cambridge, UK, Pharmaceutical Press.
GREENE, W. H., MOODY, M., SCHIMPFF, S., YYOUNG, V. M. & WIERNIK, P. H. 1973. Pseudomonas aeruginosa resistant to carbenicillin and gentamicin: epidemiologic and clinical aspects in a cancer center. *Annals of internal medicine,* 79**,** 684-689.
GREER, E. & NOONBURG, M. 2005. Management of Extremity Trauma and Related Infections Occurring in the Aquatic Environment *The American Academy of Orthopaedic Surgeons*.
HALSTEAD, B. W. 1980. Dangerous marine animals. *Cornell Maritime Press, Inc* 2.
HANNINEN, M. L., OIVANEN, P. & HIRVELA-KOSKI, V. 1997. Aeromonas species in fish, fish-eggs, shrimp and freshwater. *International Journal of Food Microbiology,* 34**,** 17-26.
HJELM, M., RIAZA, A., FORMOSO, F., MELCHIORSEN, J. & GRAM, L. 2004. Seasonal Incidence of Autochthonous Antagonistic *Roseobacter* spp. and Vibrionaceae Strains in a Turbot Larva (*Scophthalmus maximus*) Rearing System. *Applied Environmental Microbiology,* 70**,** 7288–7294.
HOLT, J. G. 2000. *Bergey's Manual of Determinative Bacteriology,* Philadelphia USA, Lippincott Williams and Wilkins.
HOWARD, R. J. & BENNETT, N. T. 1993. Infections Caused by Halophilic Marine Vibrio Bacteria. *Annals of Surgery,* 217**,** 525-531.
HUGH, R. & GILARDI, G. 1980. *Pseudomonas, in "Manual of Clinical Microbiology,"* American Society for Microbiology, Washington, DC.
INTERAMINENSE, J., NASCIMENTO, D. & VENTURA, R. 2010. Recovery and screening for antibiotic susceptibility of potential bacterial pathogens from the oral cavity of shark species involved in attacks on humans in Recife, Brazil. *Journal of Medical Microbiology,* 59**,** 7.
ISAF. 2014. *International Shark Attack File. Stats, Trends, Analysis* [Online]. Florida; United States of America.: University of Florida, Florida Museum of Natural History. Available: http://www.flmnh.ufl.edu/fish/Sharks/ISAF/ISAF.htm [Accessed 5 May 2015 2015].
KARAS, M., BACHMANN, D. & HILLENKAMP, F. 1985. Influence of the Wavelength in High-Irradiance Ultraviolet Laser Desorption Mass Spectrometry of Organic Molecules. *Annals of Chemistry,* 57**,** 2935-2939.
KIRCHMAN, D. L. 2008. *Microbial Ecology of the Oceans,* New Jersey Canada, Wiley Blackwell.
KITA-TSUKAMOTO, K., OYAIZU, H., NANBA, K. & SIMIDU, U. 1993. Phylogenetic relationships of marine bacteria, mainly members of the family Vibrionaceae, determined on the basis of 16S rRNA sequences. *International journal of systematic bacteriology,* 43**,** 8-19.
KOONIN, E. V., MAKAROVA, K. S. & ARAVIND, L. 2001. Horizontal gene transfer in prokaryotes: Quantification and Classification. *Annual Review in Microbiology,* 55**,** 709–42.

KUEH, C. S., KUTARSKI, P. & BRUNTON, M. 1992. Contaminated marine wounds – the risk of acquiring acute bacterial infection from marine recreational beaches. *Journal of Applied Bacteriology,* 73**,** 412-420.
LEVY, S. 2002. Factors impacting on the problem of antibiotic resistance. *Antimicrobial Chemotherapy,* 49**,** 25-30.
LEVY, S. B. & MARSHALL, B. 2004. Antibacterial resistance worldwide: causes, challenges and responses. *Nature medicine,* 10**,** S122-S129.
LISTON, J. 1957. The occurrence and distribution of bacterial types on flatfish. *Journal of General Microbiology,* 16**,** 205-216.
MACLEAN, S. 2012. Surfer bitten to death by a shark at South African beach dubbed 'the world's deadliest'. *DailyMail*, 07:49 GMT, 17 January 2012
MASLIN, J., MENARD, G., DROUIN, C. & POLLET, L. 2000. Dangerous sharks in tropical seas. *Medicine Tropical (Mars),* 60**,** 171-1798.
MATSIOTA, B. & NAUCIEL, C. 1993. Vibrio alginolyticus wound infectionafter exposure to sea water in an air crash. *European Journal of Clinical Microbiology and Infectious Diseases,* 12**,** 474–475.
MCCORD, M. & LAMBERTH, S. 2009. Catching and tracking the world's largest Zambezi (bull) shark Carcharhinus leucas in the Breede Estuary, South Africa: the first 43 hours. *African Journal of Marine Science,* 31**,** 107-111.
MOORE, W. J. 1983. *Basic physical chemistry*, Prentice Hall.
MUNN, C. B. 2004. *Marine Microbiology, ecology and applications,* New York USA, Garland Science/BIOS Scientific Publishers.
MURRAY, P., BARON, E., JORGENSEN, J., LANDRY, M. & PFALLER, M. 2007. *Manual of Clinical Microbiology, 9th Edition Edited* Washington DC, ASM.
OLAFSEN, J. A. 2001. Interactions between fish larvae and bacteria in marine aquaculture. *Aquaculture,* 200**,** 223-247.
PAVIA, A. T., BRYAN, J. A., MAHER, K. L., HESTER, J. T. R. & FARMER, I. J. J. 1989. Vibrio carchariae Infection after a Shark Bite. *Annals of Internal Medicine,* 111**,** 85-86.
PAYNE, J. H., WELCH, J. C. & VORA, A. J. 2003. Fatal Pulmonary Hemorrhage Associated With Micrococcal Infection in Two Children With Acute Lymphoblastic Leukemia. *Journal of Pediatric Hematology/Oncology,* 25**,** 969-974.
PHILLIPS, I. 1993. Cowan and Steel's Manual for the Identification of Medical Bacteria. *Journal of Clinical Pathology,* 46**,** 975-975.
PIEN, F. D., ANG, K. S., NAKASHIMA, N. T., EVANS, D. G., GROTE, J. A., L.HEFLEY, M. & KUBOTA, E. A. 1983. Bacterial flora of marine penetrating injuries. *Diagnostic Microbiology Infectious Disease,* 1**,** 229–232.
RADJASA, O., SABDONO, A., ZOCCHI, J. & ZOCCHI, E. 2007. Richness of secondary metabolite-producing marine bacteria associated with the sponge *Haliclona sp*. *International Journal of Pharmacology,* 3**,** 275–279.
ROSE, J. M., GAST, R. J., BOGOMOLNI, A., ELLIS, J. C., LENTELL, B. J., TOUHEY, K. & MOORE, M. 2009. Occurrence and patterns ofantibiotic resistance invertebrates off the North eastern United States coast. *Federation of European Microbiological Societies: Microbiology Ecology* 67 421–431.
ROYLE, J., ISAACS, D. & EAGLES, G. 1997. Infections after shark attacks in Australia. *Pediatric Infectious Disease Journal,* 16**,** 531–2.
RTSHILADZE, M. A., ANDERSEN, S. P., NGUYEN, D. Q. A., GRABS, A. & HO, K. 2011. The 2009 Sydney shark attacks: case series and literature review. *ANZ Journal of Surgery,* 81**,** 345-351.
SCHAEFER, A. M., GOLDSTEIN, J. D., REIF, J. S., FAIR, P. A. & BOSSART, G. D. 2009. Antibiotic-Resistant Organisms Cultured from Atlantic Bottlenose Dolphins (*Tursiops truncatus*) Inhabiting Estuarine Waters of Charleston, SC and Indian River Lagoon, FL. *EcoHealth,* 6**,** 33–41.
SENG, P., DRANCOURT, M., GOURIET, F., LA SCOLA, B., FOURNIER, P., ROLAIN, J. & RAOULT, D. 2009. Ongoing revolution in bacteriology: routine identification of bacteria by

matrix-assisted laser desorption ionization time-of-flight mass spectrometry. *Clinical Infectious Diseases,* 15**,** 43-51.
SIMIDU, U., TAGA, N., COHVELL, R. R. & SCHWARTZ, J. R. 1980. Heterotrophic bacterial flora of the seawater from the Nansei Shoto (Ryukyu Retto) area. *Bull Japan SOC Scientific Fisheries,* 46**,** 505-510.
SIMIDU, U. & TSUKAMOTO, K. 1985. Habitat segregation and biochemical activities of marine members of the family Vibrionaceae. *Applied and environmental microbiology,* 50**,** 781-790.
SIMPFENDORFER, C., GOODREIDC, A. & MCAULEYA, R. 2001. Size, sex and geographic variation in the diet of the tiger shark, Galeocerdo cuvier, from Western Australian waters. *Environmental Biology of Fishes,* 61**,** 37–46.
SMALE, M. 1991. Occurrence and feeding of three shark species, *Carcharhinus brachyurus*, *C. obscurus* and *Sphyrna zygaen*a, on the Eastern Cape coast of South Africa. *African Journal of Marine Science,* 11**,** 31-42.
SMALE, M. J. 2005. The diet of the ragged-tooth shark *Carcharias taurus* Rafinesque 1810 in the Eastern Cape, South Africa. *African Journal of Marine Science,* 27**,** 331-335.
STARR, C., EVERS, C. & STARR, L. 2010. *Biology: Concepts and Applications Without Physiology,* United Kingdom, Cengage Learning.
THOMPSON, F., IIDA, T. & SWINGS, J. 2004. Biodiversity of *Vibrios*. *Microbiology Molecular Biology,* 68**,** 403–431.
TSAI, Y.-H., HSU, R. W.-W., HUANG, T.-J., HSU, W.-H., HUANG, K.-C., LI, Y.-Y. & PENG, K.-T. 2007. Necrotizing Soft-Tissue Infections and Sepsis Caused by *Vibrio vulnificus* Compared with Those Caused by *Aeromonas Species*. *The Journal of Bone & Joint Surgery,* 89**,** 631-636.
UHLAND, F. C. 2011. *Characterization of antimicrobial resistance in Aeromonas and Vibrio isolated in Canada from fish and seafood* MSc, Université de Montréal
UNGER, N., RITTER, E., BORREGO, R. & OSIYEMI, O. 2014. Antibiotic Susceptibilities of Bacteria Isolated within the Oral Flora of Florida Blacktip Sharks: Guidance for Empiric Antibiotic Therapy. *PLOS ONE,* 9.
VÉLEZ-ZUAZO, X. & AGNARSSON, I. 2010. Shark tales: A molecular species level phylogeny of sharks (Selachimorpha, Chondrichthyes). *Molecular Phylogenetics Evolution,* 58**,** 207-217.
WALLACE, L. 1972. *Reactions of the sharks Carcharhinus leucas (Muller & Henle) and Odontaspid taurus (Rafinesque) to gill net barriers under experimental conditions,* South African Association for Marine Biological Research, The Oceanographic Research Intstitute.
WHITMAN, W. B., COLEMAN, D. C. & WIEBE§, W. J. 1998. Prokaryotes: The unseen majority. *Proceedings of National Academy of Sciences,* 95**,** 6578–6583.
WHO. 2015. *Antimicrobial resistance* [Online]. USA: World Health Organisation. Available: http://www.who.int/mediacentre/factsheets/fs194/en/ [Accessed May 2015 2015].
WIESER, A., SCHNEIDER, L., JUNG, J. & SCHUBERT, S. 2012. MALDI-TOF MS in microbiological diagnostics—identification of microorganisms and beyond (mini review). *Applied microbiology and biotechnology,* 93**,** 965-974.
WILSON, M. 2008. *Bacteriology of Humans. An ecological perspective,* London, Blackwell publishing Ltd.
WIRSING, A. J., HEITHAUS, M. R. & DILL, L. M. 2006. Tiger shark (Galeocerdo cuvier) abundance and growth in a subtropical embayment: evidence from 7 years of standardized fishing effort. *Marine Biology,* 149**,** 961-968.
WOODROW, R. 2007. *Essentials of pharmacology for health occupations,* NY USA, Thompson Delmar learning.
WOOLGAR, J., CLIFF, G. & NAIR, R. 2001. Shark attack: review of 86 consecutive cases. *Journal of Trauma Injury Infection and Critical Care,* 50**,** 887–91.
ZUBKOV, M. V. & TARRAN, G. A. 2008. High bacterivory by the smallest phytoplankton in the North Atlantic Ocean. *Nature,* 455**,** 224-226.

Chapter 6 Appendices

6.1 Appendix A- Ethical clearance

6.2 **Appendix B- KZNSB Dissection form**

6.3 Appendix C- Bacterial isolates recovered from each species of shark

No.	Sharks	No. of sharks	No. of isolates	Mean no. of isolates
1	Smooth Hammerhead (*Sphyrna lewini*)	1	2	2
2	Tiger (*Galeocerdo cuvieri*)	6	18	4
3	Blacktip (*Carcharhinus limbatus*)	2	6	4
4	Great white (*Carcharodon carcharias*)	3	13	5
5	Copper (*Carcharhinus brachyurus*)	1	3	5
6	Mako (*Isurus oxyrinchus*)	2	9	6
7	Scalloped Hammerhead (*Sphyrna zygaena*)	2	13	7
8	Spinner (*Carcharhinus brevipinna*)	7	39	7
9	Ragged tooth (*Carcharias taurus*)	8	57	8
10	Zambezi (*Carcharhinus leucas*)	2	17	9
	Total	34	205	56

6.4 Appendix D- Total number of isolates for each family of bacteria listed

The 19 bacterial families found in this study, including the total number of isolates of each family. The highest number of isolates was found in Micrococcaceae, Staphylococcaceae, Vibrionaceae and Bacillaceae, respectively.

	Family	**Number of isolates**
1	Micrococcaceae	32
2	Staphylococcaceae	28
3	Vibrionaceae	22
4	Bacillaceae	21
5	Pseudomonadaceae	15
6	Enterobacteriaceae	11
7	Shewanellaceae	9
8	Microbacteriaceae	8
9	Dermacoccaceae	7
10	Moraxellaceae	5
11	Alcaligenaceae	4
12	Sphingobacteriaceae	3
13	Xanthomonadaceae	3
14	Brucellaceae	2
15	Caulobacteraceae	3
16	Aerococcaceae	1
17	Aeromonadaceae	1
18	Brevibacteriaceae	1
19	Comamonadaceae	1
	Total	177

6.5 Appendix E- Bacterial isolates found in *C. carcharias*

	Family	Genus	Species	Number of isolates
1	Vibrionaceae	*Vibrio*	*metschnikovii*	1
2	Pseudomonadaceae	*Pseudomonas*	*fluorescens/ putida*	2
3	Vibrionaceae	*Vibrio*	*parahaemolyticus*	2
4	Bacillaceae	*Bacillus*	*marisflavi*	1
5	Micrococcaceae	*Micrococcus*	*luteus*	1
6	Microbacteriaceae	*Microbacterium*	*species*	1
7	Staphylococcaceae	*Staphylococcus*	*cohnii*	1
8	Shewanellaceae	*Shewanella*	*putrefaciens*	1
9	Vibrionaceae	*Vibrio*	*alginolyticus*	2
10	Vibrionaceae	*Photobacterium*	*damselae*	1
	Total			13

6.6 Appendix F- Number of bacterial isolates found in *G. cuvieri*

	Family	Genus	Species	Number of isolates
1	Vibrionaceae	*Vibrio*	*alginolyticus*	6
2	Enterobacteriaceae	*Proteus*	*mirabilis*	4
3	Micrococcaceae	*Micrococcus*	*luteus*	3
4	Shewanellaceae	*Shewanella*	*putrefaciens*	2
5	Dermacoccaceae	*Dermacoccus*	*nishinomiyaensis*	1
6	Enterobacteriaceae	*Proteus*	*vulgaris*	1
7	Micrococcaceae	*Kocuria*	*rhizophila*	1
8	Total			18

6.7 Appendix G- Bacterial isolates found in *C. taurus*

	Family	Genus	Species	Number of isolates
1	Micrococcaceae	*Micrococcus*	*luteus*	8
2	Bacillaceae	*Bacillus*	*cereus*	7
3	Vibrionaceae	*Vibrio*	*alginolyticus*	5
4	Vibrionaceae	*Photobacterium*	*damselae*	4
5	Pseudomonadaceae	*Pseudomonas*	*stutzeri*	3
6	Micrococcaceae	*Kocuria*	*marina*	2
7	Dermacoccaceae	*Kytococcus*	*sendentarius*	2
8	Enterobacteriaceae	*Proteus*	*vulgaris*	2
9	Micrococcaceae	*Arthrobacter*	*creatinolyticus*	2
10	Pseudomonadaceae	*Pseudomonas*	*aeruginosa*	2
11	Staphylococcaceae	*Staphylococcus*	*haemolyticus*	2
12	Staphylococcaceae	*Staphylococcus*	*equorum*	2
13	Aeromonadaceae	*Aeromonas*	*hydrophila*	1
14	Alcaligenaceae	*Alcaligenes*	*faecalis*	1
15	Bacillaceae	*Lysinibacillus*	*fusiformis*	1
16	Enterobacteriaceae	*Enterobacter*	*cloacae*	1
17	Micrococcaceae	*Kocuria*	*kristinae*	1
18	Micrococcaceae	*Kocuria*	*palustris*	1
19	Microbacteriaceae	*Microbacterium*	*species*	1
20	Moraxellaceae	*Acinetobacter*	*genomospecies*	1
21	Moraxellaceae	*Acinetobacter*	*baumannii*	1
22	Moraxellaceae	*Acinetobacter*	*species*	1
23	Staphylococcaceae	*Staphylococcus*	*sciuri*	1
24	Staphylococcaceae	*Staphylococcus*	*cohnii*	1
25	Staphylococcaceae	*Macrococcus*	*caseolyticus*	1
26	Sphingobacteriaceae	*Sphingobacterium*	*spiritivorum*	1
27	Xanthomonadaceae	*Stenotrophomonas*	*maltophilia*	1
28	Caulobacteraceae	*Brevundimonas*	*vesicularis*	1
	Total			57

6.8 Appendix H- Bacterial isolates found in *C. limbatus*

	Family	Genus	Species	Number of isolates
1	Bacillaceae	*Bacillus*	*pumilus*	1
2	Micrococcaceae	*Kocuria*	*marina*	1
3	Micrococcaceae	*Micrococcus*	*luteus*	1
4	Pseudomonadaceae	*Pseudomonas*	*stutzeri*	1
5	Staphylococcaceae	*Staphylococcus*	*equorum*	1
6	Staphylococcaceae	*Staphylococcus*	*epidermidis*	1
	Total			6

6.9 Appendix I- Bacterial isolates found in *I. oxyrinchus*

	Family	Genus	Species	Number of isolates
1	Bacillaceae	*Bacillus*	*cereus*	3
2	Bacillaceae	*Bacillus*	*thuringiensis*	1
3	Brevibacteriaceae	*Brevibacterium*	*linens*	1
4	Pseudomonadaceae	*Pseudomonas*	*fulva*	1
5	Staphylococcaceae	*Staphylococcus*	*haemolyticus*	1
6	Staphylococcaceae	*Staphylococcus*	*epidermidis*	1
7	Staphylococcaceae	*Staphylococcus*	*saprophyticus*	1
	Total			9

6.10 Appendix J- Bacterial isolates found in *C. brachyurus*

	Family	Genus	Species	Number of isolates
1	Micrococcaceae	*Micrococcus*	*luteus*	1
2	Micrococcaceae	*Arthrobacter*	*creatinolyticus*	1
3	Pseudomonadaceae	*Pseudomonas*	*fulva*	1
	Total			3

6.11 **Appendix K- Bacterial isolates found in *S. zygaena***

	Family	**Genus**	**Species**	**Number of isolates**
1	Enterobacteriaceae	*Serratia*	*marcescens*	2
2	Aerococcaceae	*Aerococcus*	*viridans*	1
3	Bacillaceae	*Lysinibacillus*	*fusiformis*	1
4	Dermacoccaceae	*Kytococcus*	*sendentarius*	1
5	Micrococcaceae	*Micrococcus*	*luteus*	1
6	Micrococcaceae	*Kocuria*	*palustris*	1
7	Moraxellaceae	*Acinetobacter*	*baumannii*	1
8	Pseudomonadaceae	*Pseudomonas*	*mendocina*	1
9	Pseudomonadaceae	*Mesophilobacter*	*marinus*	1
10	Sphingobacteriaceae	*Sphingobacterium*	*mizutaii*	1
11	Vibrionaceae	*Vibrio*	*harveyi*	1
12	Xanthomonadaceae	*Stenotrophomonas*	*maltophilia*	1
	Total			13

6.12 **Appendix L- Bacterial isolates found in *C.brevipinna***

	Family	**Genus**	**Species**	**Number of isolates**
1	Microbacteriaceae	*Microbacterium*	*species*	6
2	Micrococcaceae	*Micrococcus*	*luteus*	3
3	Alcaligenaceae	*Alcaligenes*	*faecalis*	2
4	Bacillaceae	*Bacillus*	*cereus*	2
5	Pseudomonadaceae	*Pseudomonas*	*aeruginosa*	2
6	Staphylococcaceae	*Staphylococcus*	*delphini*	2
7	Staphylococcaceae	*Staphylococcus*	*warneri*	2
8	Alcaligenaceae	*Achromobacter*	*spanius*	1
9	Bacillaceae	*Bacillus*	*pumilus*	1
10	Bacillaceae	*Bacillus*	*megaterium*	1
11	Brucellaceae	*Ochrobactrum*	*intermedium*	1
12	Brucellaceae	*Ochrobactrum*	*tritici*	1
13	Caulobacteraceae	*Brevundimonas*	*diminuta*	1
14	Comamonadaceae	*Delftia*	*acidovorans*	1
15	Enterococcaceae	*Enterococcus*	*species*	1
16	Moraxellaceae	*Moraxella*	*cuniculi*	1
17	Pseudomonadaceae	*Pseudomonas*	*fluorescens*	1
18	Staphylococcaceae	*Staphylococcus*	*cohnii*	1
19	Staphylococcaceae	*Macrococcus*	*caseolyticus*	1
20	Staphylococcaceae	*Staphylococcus*	*equorum*	1
21	Staphylococcaceae	*Staphylococcus*	*simulans*	1
22	Staphylococcaceae	*Staphylococcus*	*epidermidis*	1
23	Shewanellaceae	*Shewanella*	*putrefaciens*	1
24	Sphingobacteriaceae	*Sphingobacterium*	*multivorum*	1
25	Vibrionaceae	*Photobacterium*	*damselae*	1
26	Vibrionaceae	*Vibrio*	*harveyi*	1
27	Xanthomonadaceae	*Stenotrophomonas*	*maltophilia*	1
	Total			39

6.13 **Appendix M- Bacterial isolates found in *S. lewini***

	Family	Genus	**Species**	**Number of isolates**
1	Micrococcaceae	*Micrococcus*	*luteus*	1
2	Staphylococcaceae	*Staphylococcus*	*saprophyticus*	1
	Total			2

6.14 **Appendix N- Bacterial isolates found in *C. leucas***

	Family	**Genus**	**Species**	**Number of isolates**
1	Shewanellaceae	*Shewanella*	*putrefaciens*	5
2	Staphylococcaceae	*Staphylococcus*	*xylosus*	3
3	Dermabacteraceae	*Brachybacterium*	*faecium*	2
4	Micrococcaceae	*Micrococcus*	*luteus*	2
5	Staphylococcaceae	*Macrococcus*	*caseolyticus*	2
6	Caulobacteraceae	*Brevundimonas*	*diminuta*	1
7	Dermacoccaceae	*Dermacoccus*	*nishinomiyaensis*	1
8	Micrococcaceae	*Kocuria*	*kristinae*	1
	Total			17

6.15 **Appendix O- Total number of males and females of each shark species**

Shark species	Sex		Total
	Female	Male	
Great White (*Carcharodon carcharias*)	3	0	3
Copper (*Carcharhinus brachyurus*)	0	1	1
Smooth Hammerhead (*Sphyrna lewini*)	1	0	1
Scalloped Hammerhead (*Sphyrna zygaena*)	0	2	2
Spinner (*Carcharhinus brevipinna*)	6	1	7
Tiger (*Galeocerdo cuvieri*)	3	2	5
Blacktip (*Carcharhinus limbatus*)	2	0	2
Zambezi (*Carcharhinus leucas*)	1	1	2
Mako (*Isurus oxyrinchus*)	1	1	2
Ragged-tooth (*Carcharias taurus*)	8	0	8
Total	25	8	33

6.16 **Appendix P- Bacterial similarity between two S. *zygaena* sharks**

In table 7.15, all species were unique for each shark, none were similar. Both sharks were male, but differed in maturity and therefore size, shark 1 was found in Richards Bay

Bacteria	1	2	3	4	5	6	7	8	9	10	11	12
Scalloped hammerhead												
1	✓	✓	✓	✓	✓							
2						✓	✓	✓	✓	✓	✓	✓

6.17 **Appendix Q- Table bacterial similarity between S. *zygaena* and S. *lewini***

In above table, although these hammerheads share the same genus, the bacterial assemblages are vastly different, with only *Micrococcus luteus* being similar.

Bacteria	1	2	3	4	5	6	7	8	9	10	11	12	13
Scalloped hammerhead (1) vs Smooth hammerhead (2)													
1	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	
2												✓	✓

Key, Appendix P & Q

1	*Vibrio harveyi*
2	*Acinetobacter baumannii*
3	*Kocuria palustris*
4	*Kytococcus sedentarius*
5	*Aerococcus viridians*
6	*Mesophilobacter marinus*
7	*Lysinibacillus fusiformis*
8	*Stenotrophomonas maltophilia*
9	*Serratia marcescens*
10	*Pseudomonas mendocina*
11	*Sphingobacterium mizutaii*
12	*Micrococcus luteus*
13	*Staphylococcus saprophyticus*

6.18 **Appendix P- Identification with the MALDI-ToF MS instrument and biochemical microbial techniques**

	Ragged-tooth shark	Maldi-ToF MS	Value	Biochemical tests	Value
1	Female, 2670m, maturity 3, Park Rynie, Gut empty	*Proteus vulgaris*	2.35	*Micrococcus luteus*	
		Enterobacter asburiae	1.943	*Enterobacter cloacae*	80.3%
		Micrococcus luteus	2.276	Unacceptable ID	
		-	-	*Kocuria (Micrococcus) kristinae*	
		Staphylococcus haemolyticus	2.076	*Staphylococcus lugdenensis*	
2	Female, 2518m, maturity 3, Willards net 3, Gut empty	*Bacillus cereus*	2.174	-	
		Not reliable ID	-	Non fermenter species	32%
		Kocuria marina	1.766	*Dermacoccus nishinomiyaensis*	
		Micrococcus luteus	1.874	-	
		Not reliable ID	-	*Brevundimonas vesicularis*	91.1% Good
		Kocuria palustris	1.782	-	
3	Female, 2050m, maturity 2, Richards Bay net 2, Gut empty	-	-	*Acinetobacter baumannii*	
		Not reliable ID	-	-	
		Pseudomonas stutzeri	2.144	*Pseudomonas putida*	61.30%
		Not reliable ID	-	-	
		Vibrio alginolyticus	2.046	*Vibrio alginolyticus*	99% Very good
		Bacillus cereus	2.358	-	
		Not reliable ID	-	-	
		Vibrio alginolyticus	2.176	*Weeksella virosa*	57.9% Low discrimination
		-	-	*Stenotrophomonas maltophilia*	
		Photobacterium damselae	2.23	*Pasturella species*	41%
		Staphylococcus cohnii	2.171	*Staphylococcus cohnii*	

		Staphylococcus haemolyticus	2.114	-	
		Kocuria marina	1.979	-	
4	Female, 2532m, maturity 3, Richards Bay, Gut empty	*Pseudomonas aeruginosa*	2.424	*Ochrobacterium anthropi*	33% Low discrimination
		Vibrio alginolyticus	1.921	*Vibrio alginolyticus*	96.8% Doubtful profile
		Pseudomonas aeruginosa	2.303	*Pseudomonas aeruginosa*	98.1% Very good
		Microbacterium species	1.913	*Chryseobacterium*	63%. Low discrimination
		Alcaligenes faecalis	2.038	*Ralstonia piketti*	88.2% Acceptable
5	Female, 2562m, maturity 2, Tweni net 1, Gut empty	*Macrococcus caseolyticus*	1.728		
		Vibrio alginolyticus/ parahaemolyticus	2.139	*Vibrio alginolyticus*	99.20%
		Photobacterium damselae	1.949	*Acinetobacter haemolyticus*	
		Photobacterium damselae	2.13	*Micrococcus luteus*	
		Arthrobacter creatinolyticus	2.005	*Micrococcus luteus*	
		Sphingobacterium spiritivorum		*Sphingobacterium spiritivorum*	
		Proteus vulgaris	2.342	-	
6	Female, 2460m, maturity 3, Richards Bay net 5, Gut: small shark	*Bacillus cereus*	2.257	*Vibrio parahaemolyticus*	
		-	-	*Microbacterium species*	
		Bacillus cereus	2.118	*Bacillus cereus*	N/A Vetdiagnostix
		Aeromonas hydrophila	2.111	*Aeromonas hydrophila/caviae/sabria*	89.8% Excellent ID to the genus
7	Female, 2630m, maturity 3, Richards Bay net 2, Gut empty	*Arthrobacter creatinolyticus*	1.986	*Micrococcus varians*	
		Lysinibacillus fusiformis	2.348	*Acinetobacter haemolyticus*	87.1% Acceptable
		Staphylococcus equorum	2.372	*Staphylococcus simulans*	
		Bacillus cereus	2.183	*Bacillus cereus*	
		Staphylococcus equorum	1.735	*Staphylococcus simulans*	
		Micrococcus luteus	1.995	*Stenotrophomonas maltophilia*	65%
		Micrococcus luteus	2.264	*Micrococcus luteus*	

		Pseudomonas stutzeri	2.017	*Stenotrophomonas maltophilia*	65%
		Kytococcus (Micrococcus) sendentarius	1.764	*Dermacoccus (Micrococcus)nishinomiyaensis*	
		Kytococcus (Micrococcus) sendentarius	1.965	*Kytococcus (Micrococcus) sendentarius*	
		Micrococcus luteus	1.763	*Dermacoccus (Micrococcus) nishinomiyaensis*	
		Bacillus cereus	2.151	*Planococcus species*	
		Micrococcus luteus	1.747	*Achromobacter xylosoxidan*	
		Not reliable ID	-	-	
8	Female, 2744m, maturity 3, Richards Bay net 4, Gut: cuttlefish	*Acinetobacter species*	1.802	*Microbacterium species*	
		Staphylococcus sciuri	2.073	*Staphylococcus lugdenensis*	
		Bacillus cereus	2.249	*Weeksella virosa*	
		Acinetobacter genomospecies	2.132	-	
		Staphylococcus cohnii	2.073	*Staphylococcus cohnii/delphini*	N/A Vetdiagnostix (closely resembles)
		Pseudomonas stutzeri	2.116	-	
	Great white	**Maldi-ToF MS**	**Value**	**Biochemical tests**	**Value**
1	Female, 3144m, maturity 2, Port Edward, Gut: Carcharinidae	*Vibrio alginolyticus*	2.162	*Vibrio alginolyticus/cholerae*	Unacceptable profile
		Micrococcus luteus	1.943	*Micrococcus luteus*	
2	Female, 3080m, maturity 2, Richards Bay net 2, Gut empty	-	-	*Vibrio alginolyticus/ parahaemolyticus*	97.70%
		Pseudomonas mendocina	1.702	*Micrococcus luteus*	
		Bacillus cereus	2.25	*Vibrio metschnikovii*	99.70%
		Bacillus marisflavi	2.046	*Aureobacterium species*	
		Staphylococcus cohnii	1.763	*Staphylococcus cohnii*	
		Shewanella putrefaciens group	2.047	*Shewanella putrefaciens group*	99.90%
		Pseudomonas mendocina	1.82	*Pseudomonas fluorescens/ putida*	94.1 % Good
3	Female, 2536m, maturity 1, Margate net 7, Catface	*Microbacterium species*	1.966	-	

	rockcod (*Epinephelus andersoni*)	*Photobacterium damselae*	2.28	-	
	Mako shark	**Maldi-ToF MS**	**Value**	**Biochemical tests**	**Value**
1	Female, 3500m, maturity 3,Amanzimtoti net 1, Gut: Ray	*Bacillus cereus*	2.072	-	
		Bacillus thuringiensis	1.769	-	
		Brevibacterium linens	1.79	-	
		Staphylococcus epidermidis	1.742	-	
		Staphylococcus haemolyticus	2.076	*Micrococcus lylae*	N/A Vet Diagnostics (closely resembles)
		Pseudomonas fulva	2.213	-	
2	Male, 2390m, maturity 3,Richards Bay net 2, Gut: shark	*Bacillus cereus*	1.779	-	
		Not reliable ID	-	*Staphylococcus saprophyticus*	
	Tiger shark	**Maldi-ToF MS**	**Value**	**Biochemical tests**	**Value**
1	Male, 3272m, maturity 3, Uvongo net D4, Gut: Whale, Tiger shark	*Micrococcus luteus*	1.995	*Non fermenter*	65%
		Proteus vulgaris	2.286	-	
		Vibrio alginolyticus	2.045	*Vibrio alginolyticus*	42.9% Low discrimination
2	Female, 2548m, maturity 1, Amanzimtoti net 9, Gut: unidentified bird	*Vibrio alginolyticus*	2.336	-	
		Micrococcus luteus	2.086	-	
3	Male, 3500m, maturity 3, Richards Bay, Gut: Turtle and 2 large fish	*Micrococcus luteus*	1.907	*Micrococcus luteus*	
		Dermacoccus nishinomiyaensis	1.734	*Kocuria (Micrococcus) kristinae*	
		Vibrio alginolyticus	1.978	*Vibrio alginolyticus*	99%
4	Female, 1624m, maturity 1, Amanzimtoti net 7, Gut: seaweed, fish, shark, cuttlefish	*Kocuria rhizophila*	1.937	-	
		Shewanella putrefaciens group	2.225	-	
		Not reliable ID	-	-	
		No peaks found	-	-	
		Not reliable ID	-	-	

5	Female, 2500m, maturity 1, Durban net 1, Gut: Spotted grunter, longfinned batfish, rat, whale, unidentified shark, octopus	*Proteus mirabilis*	2.373	*Proteus mirabilis*	99%
	Smooth hammerhead	**Maldi-ToF MS**	**Value**	**Biochemical tests**	**Value**
1	Female, 2460m, maturity 1,Durban net 12, Gut: cuttlefish and fish	*Micrococcus luteus*	2.182	-	
		Staphylococcus saprophyticus	1.95	-	
	Scalloped hammerhead	**Maldi-ToF MS**	**Value**	**Biochemical tests**	**Value**
1	Male, 1612m, maturity 1, Durban net 2, Gut: fish	*Vibrio harveyi*	2.17	*Vibrio alginolyticus*	(unacceptable profile)
		Acinetobacter baumannii		*Acinetobacter baumannii*	
		Kocuria palustris	1.861	*Micrococcus luteus*	
		-	-	*Kytococcus sendentarius*	
		Aerococcus viridans	1.798	*Enterococcus species*	N/A Vet Diagnostics (closely resembles)
2	Male,3020m, maturity 3, Richards Bay net 4, Gut empty	-	-	*Mesophilobacter marinus*	
		Lysinibacillus fusiformis	2.012	*Shewanella putrefaciens group*	96.70%
		Stenotrophomonas maltophilia	1.818	*Pseudomonas aeruginosa*	62%
		Serratia marcescens	2.389	-	
		Pseudomonas mendocina	1.903	*Ralstonia piketti*	52.70%
		Sphingobacterium mizutaii	1.886	-	
		Micrococcus luteus	2.022	*Micrococcus luteus*	
	Blacktip shark	**Maldi-ToF MS**	**Value**	**Biochemical tests**	**Value**
1	Female, 2052m, maturity 3, Durban net 1, Gut: shad, sand soldier	*Kocuria marina*	2.097	*Micrococcus luteus*	
		Staphylococcus equorum	1.829	*Dermacoccus (Micrococcus) nishinomiyaensis*	
		Staphylococcus epidermidis	1.776	*Staphylococcus simulans*	

		Micrococcus luteus	1.76	*Micrococcus lylae*	
2	Female, 2166m, maturity 3, Thompsons Bay, Gut: Blenny (Blenidae)	Not reliable ID	-	Unacceptable ID	
		Bacillus pumilus	1.941	-	
		Pseudomonas stutzeri	2.038	-	
		Not reliable ID	-	-	
	Copper shark	**Maldi-ToF MS**	**Value**	**Biochemical tests**	**Value**
1	Male, 2610m, maturity 3, St. Michaels net 1, Gut: fish	Not reliable ID	-	-	
		Micrococcus luteus	2.153	-	
		Arthrobacter creatinolyticus	2.239	Unacceptable ID	
		Pseudomonas fulva	2.039	-	
		Not reliable ID	-	-	
	Spinner shark	**Maldi-ToF MS**	**Value**	**Biochemical tests**	**Value**
1	Female, 1362m, maturity 1, Umhlanga net 5	*Pseudomonas fluorescens*	2.393	*Pseudomonas fluorescens*	88.2 % Acceptable
		Pseudomonas aeruginosa	2.318	*Pseudomonas aeruginosa*	99.9% Very good
		Bacillus cereus	1.992	*Pseudomonas aeruginosa*	
2	Female, 2482m, maturity 3, Umtentweni net 1, Gut empty	*Microbacterium species*	1.723	*Aureobacterium species*	
		Macrococcus caseolyticus	2.062	*Staphylococcus delphini*	
3	Female, 2382m, maturity 3, Umtentweni net 1, Gut empty	*Ochrobactrum intermedium*	2.218	*Sphingomonas paucimobilis*	
		Bacillus cereus	2.139	*Bacillus cereus*	
		Brevundimonas diminuta	1.931	*Pseudomonas stutzeri*	93%
		Macrococcus caseolyticus	1.87	*Moraxella species*	N/A Vet Diagnostics (closely resembles)
		Microbacterium species	1.837	*Aureobacterium species*	
		-	-	*Moraxella cuniculi*	
		Microbacterium species	1.951	*Aureobacterium species*	

		Achromobacter spanius	1.93	-	
		Microbacterium species	1.969	*Aureobacterium species*	
4	Female, 1804m, maturity 2, Park Rynie net 2, Gut empty	*Ochrobactrum tritici*	1.951	*Rhizobium radiobacter*	53.8 % Low discrimination
		Stenotrophomonas maltophilia	2.518	-	
		Staphylococcus epidermidis	1.996	-	
		No peaks found	-	-	
		Delftia acidovorans(was not reliable)	1.953	*Delftia acidovorans*	99.8% Doubtful profile
		Micrococcus luteus	1.758	*Sphingomonas paucimobilis*	N/A Vet Diagnostics
		Entercoccus species	1.879	*Entercoccus species*	N/A Vet Diagnostics
		Micrococcus luteus	1.745	*Chryseobacterium*	49.3%. Doubtful profile
		Not reliable ID	-	*Stenotrophomonas maltophii*	95.40%
		Staphylococcus warneri	2.023	-	
5	Female, 2054m, maturity 3, Park Rynie net 1, Gut empty	*Bacillus megaterium*	2.301	*Bacillus licheniformus*	N/A Vet Diagnostics (closely resembles)
		Staphylococcus equorum	1.91	*Micrococcus lylae*	
		Micrococcus luteus	1.701	*Aeromonas salmonicida*	73.5% not valid
6	Female, 2102m, maturity 3,Scottburgh net 1, Gut empty	*Microbacterium species*	1.771	*Micrococcus luteus*	N/A Vet Diagnostics (closely resembles)
		Staphylococcus delphini	1.999	*Staphylococcus delphini*	N/A Vet Diagnostics (closely resembles)
		Staphylococcus epidermidis	1.733	*Staphylococcus simulans*	N/A Vet Diagnostics (closely resembles)
		Staphylococcus cohnii	1.825	*Staphylococcus cohnii*	N/A Vet Diagnostics (closely resembles)
		Acinetobacter species	1.89	*Alcaligenes faecalis*	N/A Vet Diagnostics (closely resembles)
		Vibrio harveyi	2.06	*Vibrio alginolyticus*	74.4% Doubtful profile.
7	Male, 1740m, maturity	*Shewanella putrefaciens group*	1.871	*Shewanella putrefaciens group*	N/A Vet Diagnostics

	1,Scottburgh net 13, Gut empty	Not reliable ID	-	-	
		Not reliable ID	-	-	
		Not reliable ID	-	-	
		Microbacterium species	2.175	*Microbacterium species*	N/A Vet Diagnostics (closely resembles)
		Not reliable ID	-	-	
		Sphingobacterium multivorum	1.99	-	
		Photobacterium damselae	2.118	-	
	Zambezi shark	**Maldi-Tof MS**	**Value**	**Biochemical tests**	**Value**
1	Male, 1940m, maturity 1, Zinkwazi net 2, Gut: fish	*Macrococcus caseolyticus*	2.077	*Kocuria (Micrococcus) kristinae*	
		Macrococcus caseolyticus	1.876	*Kocuria (Micrococcus) kristinae*	
		Staphylococcus xylosus	2.196	-	
		-	-	*Dermacoccus (Micrococcus) nishinomiyaensis*	N/A Vet Diagnostics
		Staphlococcus warneri	1.927	*Kocuria (Micrococcus) kristinae*	
		Brachybacterium faecium	1.723	-	
		Staphlococcus xylosus	1.708	*Staphlococcus delphini*	
		Brachybacterium faecium	1.828	*Micrococcus luteus*	
		Micrococcus luteus	1.775	*Micrococcus luteus*	
		Staphlococcus xylosus	2.077	*Staphlococcus warneri*	
2	Female, 1872m, maturity 1, Richards Bay net 5, Gut empty	*Shewanella putrefaciens group*	2.035	*Shewanella putrefaciens group*	
		Brevundimonas diminuta	1.978	*Deleya aquamarinus*	
		Micrococcus luteus	1.762	-	
		-	-	*Kocuria (Micrococcus) kristinae*	

In Table 6.18. The grey highlighted section is the identity chosen between the two methods, the results for both tests were carefully evaluated, taking in to consideration the score values and the identification percentage value.