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Characterization of Microbial Communities Across Disease States and Environmental Conditions in Kemp's Ridley (Lepidochelys Kempii) and Green Sea Turtles (Chelonia Mydas)

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CHARACTERIZATION OF MICROBIAL COMMUNITIES ACROSS DISEASE STATES AND ENVIRONMENTAL CONDITIONS IN KEMP'S RIDLEY (*LEPIDOCHELYS KEMPII*) AND GREEN SEA TURTLES (*CHELONIA MYDAS*)

A Dissertation Presented

by

KERRY L. McNALLY

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ABSTRACT

CHARACTERIZATION OF MICROBIAL COMMUNITIES ACROSS DISEASE STATES AND ENVIRONMENTAL CONDITIONS IN KEMP'S RIDLEY (*LEPIDOCHELYS KEMPII*) AND GREEN SEA TURTLES (*CHELONIA MYDAS*)

May 2020

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All species of sea turtles are threatened or endangered, with various diseases and conditions affecting populations around the world. Understanding healthy populations as well as populations beset by disease conditions, such as fibropapillomatosis and cold-stunning, could lead to helpful tools in the conservation management and medical treatment needed to protect these species. Microbial communities, or the microbiome, at different body sites of sea turtles likely play important roles in the health of these animals, from aiding in digestion to immune system regulation. Disruption of these communities, either through

disease and/or environmental factors, may play a role in disease processes and recovery in sea turtle species.

Given the importance of microbial communities in health and disease, my dissertation sought to: 1) characterize the microbiome of two species of sea turtles, Kemp's ridley and green turtles, from the same habitat in the wild, 2) characterize the microbiome of cold-stunned Kemp's ridley turtles through rehabilitation, and 3) investigate the respiratory microbiome of Kemp's ridley turtles in relation to radiographic lung abnormalities and diagnostic tools. To carry out these objectives, I used sequencing of the 16S rRNA gene to identify microbial community composition of various body sites from sea turtles for each experiment. In wild turtles, I identified distinct core microbes from the oral cavity and cloaca of two species of healthy, wild caught sea turtles. In stranded turtles, I characterized the same body sites, oral cavity and cloaca, throughout rehabilitation and found shifts in the microbial community composition throughout hospitalization, including alterations due to antibiotic therapy. I also found that the microbiome did not correlate with disease condition or physiological abnormalities in stranded cold-stunned turtles. Since lung abnormalities are prevalent in cold-stunned turtles, I also examined the respiratory microbiome through tracheal washes and necropsy samples. I found that lungs contained a diverse and variable microbial community and identified limitations of tracheal washes as a diagnostic tool. Taken together, these results contribute to understanding the microbiome of sea turtles across disease states and environmental conditions by identifying the microbial community composition at different body sites, through different methods, and based on different disease conditions.

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GENERAL INTRODUCTION

Introduction to Sea Turtles

Seven species of sea turtles can be found worldwide, and all except the flatback (*Natator depressa*) are found in waters of the United States. All sea turtles have long lifespans, some estimated to be more than 100 years. The age of maturation ranges from approximately 10 years to higher estimates of 30 years (Heppell et al., 2003; Wyneken et al., 2006). The smallest sea turtle species is the Kemp's ridley (*Lepidochelys kempii*) and the largest is the leatherback (*Dermochelys coriacea*), both of which mature at the younger end of the range, at 10-15 years (Heppell et al., 2003; Wyneken et al., 2006). The distribution ranges of sea turtles are wide and include ontogenetic shifts during their life cycles, one of which is a shift from pelagic to benthic areas for feeding (Morreale and Standora, 2005; Heppell et al., 2003). Adult females of all species go through a nesting cycle, depositing 50 to 130 eggs on an ocean facing beach and some species nest up to 3 times in one nesting season (Heppell et al., 2003). Kemp's ridley turtles nest in a mass event called an arribada, in which females emerge at the same time to oviposit, and this repeats in approximately 30 days (Wyneken et al., 2006; Bevan et al., 2016). The long life span, late reproductive maturity,

and low survival rates of nests all contribute to the susceptibility of sea turtles to various threats.

Sea Turtle Threats

All sea turtle species are endangered or threatened in the United States. The International Union for the Conservation of Nature (IUCN) lists the flatback (*Natator depressa*), the olive ridley (*Lepidochelys olivacea*), the loggerhead turtle (*Caretta caretta*), and the leatherback (*Dermochelys coriacea*) as vulnerable. Green turtles (*Chelonia mydas*) are endangered, and the hawksbill (*Eretmochelys imbricata*) and Kemp's ridley (*Lepidochelys kempii*) are critically endangered (http://www.iucnredlist.org). Sea turtle populations declined due to anthropogenic effects of habitat loss and degradation, human interaction (i.e. poaching), interactions with the commercial and recreational fishing industries (i.e. trauma and entanglement), and pollution (Heppell et al., 2003; Wyneken et al., 2006). Kemp's ridley turtles, in particular, are known to have had a drastic decline in population size between the 1940s and the 1980s, where scientists estimate a 99% decline in nests (Marquez et al., 2005; Bevan et al., 2016). The decline for this species is likely due to geographically limited nesting sites, with the primary nesting beach limited only to Rancho Nuevo, Mexico, where there is high natural predation. Exploitation of eggs for human consumption, along with the expansion of the shrimp industry also led to decreased hatching and increased mortalities from incidental captures in the Gulf of Mexico (Wibbels and Bevan, 2016). The decline in Kemp's ridley turtle populations and the need for increased conservation efforts arise from a combination of these threats.

Sea Turtle Conservation

Established conservation efforts reduce threats to sea turtle populations by targeting methods such as outreach, sustainable use, habitat restoration, and fishing gear modifications. Education is crucial in communities where sea turtles are not a protected species, as they are in the United States. Sustainable use practices, although a controversial method, allows reductions in poaching or egg harvesting and establishes guidelines in egg-collecting techniques for regions that are unable to completely end turtle harvesting (Campbell, 2003). While some fishery interactions are still a concern, such as longlines, turtle excluder devices (TEDs) were implemented and successfully reduce incidental take in trawls (Campbell, 2003; Marquez et al., 2005). For Kemp's ridley turtles, the Mexican government provided protection of the nesting beaches, and the United States National Park Service (NPS) established a secondary nesting beach for this species at the Padre Island National Seashore (PAIS) in Texas. A head start program initiated through this plan involved excavation of nests in Mexico, transport of eggs to Texas, raising the hatchlings in captivity for 9 to 10 months and then releasing turtles when they were at a larger size to increase survival (Marquez et al., 2005; Wibbels and Bevan, 2016). Another valuable conservation method is the protection of nesting beaches, which involves fencing off the nests to prevent natural predation, translocation of eggs to areas above the high tide line to prevent wash out, and education of residents to reduce lights from coastal communities that are known to cause disorientation of the hatchlings (Hamann et al., 2003; Wyneken et al., 2006; Wibbels and Bevan, 2016). There are encouraging trends in sea turtle populations through conservation

efforts, but threats remain, and nesting is still lower than historic levels (Bevan et al., 2016; Heppell et al., 2003).

Sea Turtle Health and Disease

Understanding sea turtle health and disease can lead to insights into conservation efforts and population monitoring, as well as expand the ability for proper medical intervention when needed. Common medical problems of sea turtles include trauma, infectious diseases (bacterial, fungal, and viral), and exposure to environmental toxins (Wyneken et al., 2006). Trauma occurs for a variety of reasons, ranging from natural predation such as shark bites to interactions with humans such as boat strikes or dredges. In addition to trauma caused by entanglement in fishing lines, ingested fish hooks may lead to impactions or perforations of the gastrointestinal tract (Wyneken et al., 2006). Bacterial, fungal, and parasitic infections are common in sea turtles, especially those that are already immunocompromised. Coccidian pathogens can cause deaths in both loggerheads and green turtles, and mycotic infections can affect Kemp's ridley turtle lungs and other organs (Manire et al., 2002; Wyneken et al., 2006; Stockman et al., 2013). Many of these infections result from environmental stressors such as drastic temperature changes or diminished water quality.

Fibropapillomatosis

Fibropapillomatosis (FP) is a common disease of sea turtles in tropical waters. It is characterized by proliferative fibroepithelial lesions. Although FP exists in all species of sea turtles, the disease is most prevalent in green turtles (Wyneken et al., 2006; Jones et al., 2016). The lesions can grow on the skin, oral cavity, eyes, and internal organs of turtles.

Severe lesions, based on size and number, can be debilitating to the animal by limiting mobility, ability to feed, and by reducing vision (Jones et al., 2016). Internal lesions indicate a poor prognosis due to the severity and untreatable status of the disease (Wyneken et al., 2006). FP is associated with a herpesvirus infection. Specifically, Chelonid herpesvirus 5 (ChHV5) is currently thought to be the likely etiological agent of FP (Jones et al., 2016). In addition to the virus, several possible co-factors of the disease include poor water quality, pollutants or toxins, water temperature, and algal blooms, all of which cause disruptions to the immune system (Wyneken et al., 2006; Jones et al., 2016).

Cold-Stunning

Several species of sea turtles in the United States are susceptible to cold-stunning, or hypothermia, when exposed to water temperatures below 10˚C, including loggerhead, green, and Kemp's ridley turtles (Still et al., 2005; Wyneken et al., 2006; Roberts et al., 2014; Innis and Staggs, 2017; Shaver et al., 2017). Cold-stunning occurs from a combination of natural conditions, including geography, such as semi-enclosed and/or shallow bays, and meteorological conditions such as cold-front storms, that cause drastic drops in air temperature and an increase in winds (Still et al., 2005, Roberts et al., 2014; Shaver et al., 2017; Griffin et al., 2019). Cold-stunning is classified as acute or chronic, with acute being common for the southern United States. Acute cold stunning typically involves high numbers of turtles that strand from a sudden drop in water temperature of shallow bay waters (Innis and Staggs, 2017; Shaver et al., 2017). The typical mortality rate of acute events is approximately 30% while chronic events can have a mortality rate up to 85% (Wyneken et al., 2006; Innis and Staggs, 2017; Shaver et al., 2017). Chronic cold-stunning occurs in the

northeastern United States where species such as the Kemp's ridley, loggerhead, and green turtle are seasonal inhabitants that forage during warm summer months. Turtles that do not migrate south before water temperatures drop during the autumn are then susceptible to chronic cold-stunning (Morreale and Standora, 2005; Still et al., 2005).

The typical ailments due to chronic cold-stunning include frostbite, dehydration, malnutrition, and immunosuppression, often requiring intensive medical management over several months of hospitalization (Wyneken et al., 2006; Innis et al., 2009). Turtles admitted to rehabilitation are evaluated through physical exams, hematology and plasma biochemical evaluations, and radiography, to prescribe the proper medical treatment and care (Wyneken et al., 2006; Stockman et al., 2013). Pathologic conditions that are common to cold-stunned turtles, especially juvenile Kemp's ridleys, include pneumonia, systemic bacterial and fungal infections, buoyancy disorders, osteomyelitis, and necrotizing enterocolitis (Wyneken et al., 2006; Innis et al., 2009). Pneumonia is one of the major life-threatening complications of cold-stunning, with over 50% of Kemp's ridley turtles in rehabilitation affected by bacterial and/or fungal pneumonia (Innis et al., 2009; Stockman et al., 2013).

Microbial Communities in Health and Disease

Microbes, including bacteria and fungi, are naturally found within and on the surface of all animals. This microbiome influences, and is influenced by, the host and the environment, and aids in host physiology, immune response, and development (Ley et al., 2008; Colston and Jackson, 2016). Growing evidence suggests that gut microbial communities co-evolved with the host, contributing to host nutrition, behavior, and overall health (Ley et al., 2008; Amato, 2013). Next-generation sequencing has allowed the

characterization of the microbiome in various hosts, including humans and other animals. Most microbiome studies have focused on humans, and those studies of non-human mammals primarily focus on captive rather than wild animals. Further, most studies focus solely on mammals, which comprise only ~10% of vertebrate species (Keenan, 2013; Colston and Jackson, 2016). To truly understand the relationships and interactions between animals and their microbiome, more knowledge is needed on diverse species (Amato, 2013; Colston and Jackson, 2016; Apprill, 2017).

Emerging research shows the critical role that the microbiome plays in health and in disease and inflammatory conditions of various species. Humans have been the focus of studies describing associations of the microbiome with healthy and disease states. Research on the human microbiome reveals that dysbiosis, or an imbalance of the microbial communities at particular body sites, contribute to metabolic and immunological disorders (Althani et al., 2015; Moffatt and Cookson, 2017). For example, the lung microbiome is altered in every study of lung disease in humans, where the dysbiosis may either be the primary cause of disease, or a secondary response to the disease (Dickson et al., 2016). The microbiome of one body site can also influence that of another. In humans, the gut microbiome is a strong influence on shaping the systemic immune response, meaning that a disruption in the intestinal microbiota can lead to immune responses at different mucosal sites, including allergic reactions in the lungs and neurodegenerative diseases of the brain (Segal and Blaser, 2014; Althani et al., 2015).

Most non-mammalian studies on the role of microbial communities in organismal health and disease focus on diseases of coral and the skin of amphibians. The coral

microbiome project aims to understand coral-microbe interactions of healthy coral, which provides a stepping stone to better investigate disease processes and management options for the increasing threat to coral reefs (Bourne et al., 2009). Although the microbiome of coral tissue shifts based on disease status, it is unknown whether the change in community structure is a cause or a result of the disease (Bourne et al., 2009; Roder et al., 2014). For example, the microbial communities of coral infected with White Plague Disease were distinct from healthy coral, and this relationship could provide a community profile for a particular disease (Roder et al., 2014). The majority of amphibian skin microbiome research focuses on the emerging infectious disease Chytridiomycosis, caused by the pathogen *Batrachochytrium dendrobatidis* (Bd) that is resulting in mass extinctions of amphibians around the globe (Jani and Briggs, 2014; Colston and Jackson, 2016). Not only does Bd induce an alteration in the natural microbial communities of amphibian skin, but also the healthy skin microbiome provides a protection or resistance to Bd that may be useful in conservation efforts (Jani and Briggs, 2014; Woodhams et al., 2016).

Monitoring health through animal microbiome research provides information on disease states, which is extremely important in disease management. Studies of healthy humpback whale respiratory vapor demonstrate a core set of microbes unique to all samples collected. Deviations from this core microbiome could indicate a disease related shift in animal health (Apprill et al., 2017). The American alligator gastrointestinal (GI) microbiota is not only affected by diet but is different at sites along the GI tract. This information could help establish the functional roles of dominant bacteria present at the particular body sites, which could be used to evaluate disease processes (Keenan and Elsey, 2015). For example, a

change in the proportion of the dominant gut bacteria of Firmicutes and Bacteroidetes in both humans and alligators is linked to changes in immune function and overall health, including obesity (Ley et al., 2008; Keenan and Elsey, 2015). The knowledge gained from understanding the microbiome in healthy organisms and in various disease states can lead to insight into potential disease biomarkers, improved diagnostic methods, and improved medical therapies (Apprill et al., 2017; Moffatt and Cookson, 2017). As this information is gathered across a range of host taxa, including sea turtles, individual medical care and conservation medicine can improve.

Dissertation Objectives

The overall objective of my dissertation is to characterize the microbiome of sea turtles and investigate host-microbe relationships based on health, disease, and the environment, providing insights into the biology of an important and critically threatened reptilian species. To understand the role of the microbiome in sea turtles, it is important to establish a baseline from healthy wild turtles. The goal of my first chapter is to assess wild caught turtle microbiomes through sample collection (oral and cloaca swabs) of wild Kemp's ridley and green sea turtles. Comparing two species of wild turtles from the same habitat can help us to understanding how environmental factors, such as habitat and diet, influence the microbiome of turtles. The main objective of this work is to determine whether a core microbiome exists among each turtle species or among sea turtles in general that can provide a background for monitoring the health of wild turtles in the future. Diverse disease states also exist in the wild; for example, green sea turtles have varying severity of fibropapillomatosis in tropical waters. Thus, in my dissertation I also examine the

microbiome associated with FP to better understand the role microbes may play in this disease.

Monitoring the health of an animal through shifts in microbial community assemblages is an important step that can result in improved medical care or husbandry. The second chapter of my dissertation focuses on understanding the clinical and environmental effects of rehabilitation on Kemp's ridley sea turtles through characterization of the microbiome at different body sites (oral and cloaca) throughout the time course of rehabilitation. The first objective for this research is to identify which bacteria compose the microbial communities of the oral cavity and cloaca of cold-stunned sea turtles. The next objective is to determine whether antibiotics alter the microbiome over time, and if the communities return to a healthy composition prior to the release of turtles into the wild. Lastly, I explore whether microbial community analysis can provide diagnostic information on clinical status through correlations with disease condition or hematologic relationships. These objectives provide insight into the role the microbiome plays in the health and disease of sea turtles in rehabilitation.

The respiratory microbiome of Kemp's ridley turtles is the primary focus of my third chapter, with the goal of understanding pneumonia as a disease process and evaluating tracheal washes as a standard diagnostic tool. Samples from cold-stunned Kemp's ridley turtles from the New England Aquarium (NEAq) were collected to understand radiographic lung abnormalities that are commonly diagnosed during rehabilitation. The first objective of this chapter is to determine whether the degree of radiographic lung abnormalities in turtles alter the microbial community. Tracheal washes are considered a standard in characterizing

pneumonia, so my next objective is to investigate whether this method truly captures the microbes inhabiting the lungs and whether the microbiome varies at different sites along the respiratory tract (i.e. glottis, trachea, lung). The fluid acquired from tracheal washes is typically sent to a diagnostic lab for culture, but it is unknown whether the results truly represent the causative agents of pneumonia in sea turtles. Investigating these diagnostic techniques and culture-dependent methods can lead to insight into the approaches currently used to diagnose pneumonia in sea turtles and how they may improve both diagnostic abilities and treatment options.

Overall, the research objectives serve to advance our understanding of microorganisms in sea turtle health and disease. Advancing knowledge on the biology of sea turtles, which includes microbial communities, contributes to conservation management and improvement in medical treatment. Establishing what constitutes a healthy microbiome, identifying normal differences between species, evaluating effects of common conditions such as cold-stunning and antibiotic therapy, and assessing diagnostic tools through culture-independent methods provides important information for an endangered species.

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CHAPTER 1

THE MICROBIOME OF WILD CAPTURED KEMP'S RIDLEY (*LEPIDOCHELYS KEMPII*) AND GREEN SEA TURTLES (*CHELONIA MYDAS*) IN CRYSTAL RIVER, FLORIDA

Abstract

Conservation efforts for endangered sea turtle species, such as Kemp's ridley (*Lepidochelys kempii*) and green turtles (*Chelonia mydas*), may benefit from information on the microbial communities that contribute to host health. Here, I characterized the microbiome of the oral cavity and cloaca from wild captured Kemp's ridley and green turtles off the west coast of Florida, U.S.A. by using Illumina sequencing to analyze the 16S rRNA gene. Microbial communities were distinct between body sites (oral cavity and cloaca) as well as between turtle species, suggesting that the turtle species is more important than the local environment in determining the microbiome of sea turtles. I identified the core microbiome for each species at each body site and determined that there were very few bacteria shared among the oral samples of both species, and no taxa co-occurred in the cloaca samples among both species. The core microbiome of the green turtle cloaca was primarily

from the order Clostridiales, which play an important role in digestion for herbivorous species such as the green turtle. Due to high prevalence of fibropapillomatosis in the green turtles (90%), I also investigated the correlation between the microbiome and the severity of fibropapillomatosis, and I identified shifts in microbial community composition associated with tumor scores. This study provides the first glimpse of the microbiome in two co-located species of sea turtle and sheds an important species-specific light on the microbiome of these critically endangered animals.

Introduction

Sea turtles are found worldwide, and all except one species are found in waters of the United States. All sea turtles have long lifespans, some estimated to be more than 100 years. They also have complex life histories involving a diversity of habitats, including nesting on beaches, initial development in the open ocean, and foraging in coastal waters (Bolten, 2003). Diets vary between sea turtle species, with species such as the loggerhead turtle (*Caretta caretta*) eating hard shelled invertebrates and the leatherback turtle (*Dermochelys coriacea*) exclusively feeding on jelly organisms (Bjorndal and Jackson, 2003). The shallow coastal areas along the northwest coast of Florida, U.S.A. are critical developmental and foraging habitat for multiple species of sea turtle, where juvenile green turtles (*Chelonia mydas*) transition from being omnivores to herbivores in the shallow seagrass beds, and Kemp's ridley turtles (*Lepidochelys kempii*), which are carnivores, primarily feed on crustaceans in the coastal habitat (Bjorndal, 1997). Sea turtles are susceptible to environmental pressures, including anthropogenic disturbances such as pollution and habitat destruction. All sea turtle species are endangered or threatened in the United States. For

example, The International Union for the Conservation of Nature (IUCN) lists the loggerhead turtle as Vulnerable (Casale and Tucker, 2017), the green turtle as Endangered (Seminoff, 2004) and the Kemp's ridley turtle as Critically Endangered (Wibbels and Bevan, 2019).

Like other marine vertebrates, sea turtles are considered sentinels of ecosystem health due to their wide distribution, long lifespans, and occurrence in multiple ecosystems. Fibropapillomatosis (FP) is one prominent indicator of sea turtle health, with its distribution and prevalence increasing over the last several decades (Jones et al., 2016). This infectious disease is found in all species of sea turtles, although it has reached epizootic proportions in green turtles (Aguirre and Lutz, 2004; Page-Karjian et al., 2019). FP is characterized by proliferative fibroepithelial lesions, and the likely etiological agent is the herpesvirus, Chelonid herpesvirus 5 (ChHV5) (Wyneken et al., 2006; Jones et al., 2016). There are multiple factors influencing the expression of the virus by causing disruptions in the immune system, including environmental co-factors such as water quality, temperature, pollutants or toxins, and algal blooms (Wyneken et al., 2006; Jones et al., 2016; Page-Karjian and Herbst, 2017). The interactions between FP and the turtle microbiome remains unclear, however, documenting these interactions is a critical step in understanding the consequences of FP for host health.

Microbes are considered a fundamental part of the life history of animals (McFall-Ngai et al., 2013; Colston and Jackson, 2016), including sea turtles. In recognition of the central role of microbes, animals and their microbiomes are now considered holobionts, which recognizes that ecological and evolutionary forces act on both the host and host-associated microbial communities (Bourne et al., 2009; Bordenstein and Theis, 2015).

Microbial communities play a role in host development and function, including nutrition, metabolism, immune response, behavior, and sociality (Ley et al., 2008b; Amato, 2013; McFall-Ngai et al., 2013; Bordenstein and Theis, 2015; Colston and Jackson, 2016). Further, the holobiont is nested within an ecosystem, resulting in important influences from the local environment (i.e. habitat, temperature), the host (i.e. age, diet), and interactions with other microbes (Keenan and Elsey, 2015; Colston and Jackson, 2016). The role that these microbes play in the health of sea turtles remains unclear. Thus, it is important to examine wild turtle microbiomes to begin to understand their microbial community structure and the role that it plays in turtle health.

Thus far, studies of sea turtle microbial communities focused on the gut of loggerhead or green turtles (Abdelrhman et al., 2016; Ahasan et al., 2017a; Ahasan et al., 2017b; Price et al., 2017; Biagi et al., 2018; Campos et al., 2018; Bloodgood et al., 2020). Differences in the cloacal microbiome of green turtles were evident between different habitats (pelagic vs. neritic), which suggests that environmental and dietary factors contribute to microbial community composition in that turtle species (Price et al., 2017; Campos et al., 2018). A core microbial community from feces in wild-captured green turtles and stranded green turtles showed that *Bacteroides* dominated both groups, indicating the likely importance of this taxa to the host (Ahasan et al., 2017b). The fecal microbiota of loggerhead turtles studied during rehabilitation highlighted turtle resilience to a captive environment and showed distinct differences compared to the green turtle (Biagi et al., 2018). Additional studies on wild turtles and on a variety of turtle species, including the critically endangered Kemp's ridley turtle, are needed to assess whether turtles have a microbial community structure of

important bacterial taxa that are found in the majority of individuals, or whether the microbiome varies at different life stages, habitats, or in different environments.

Determining the nature of the core microbiota of an organism is important to understanding the healthy and stable microbiome of hosts, including sea turtles. A core microbiome is defined as the microbes that are common or consistent across microbial communities of similar habitats (Turnbaugh et al., 2007; Shade and Handelsman, 2012). Core microbiome analysis is useful as a first step in identifying the healthy microbes of a host, thus allowing for monitoring organism health or predicting potential perturbations and/or the effects of dysbiosis (Shade and Handelsman, 2012; Apprill et al., 2014). For example, in healthy humpback whale respiratory vapor and skin, a core microbiome was identified for each body site, which is important for identifying and assessing the importance of atypical microbes or the absence of members of the typical core communities (Apprill et al., 2014; Apprill et al., 2017). Characterizing an animal's specific core microbial community can also be used to develop a screening tool that could identify host health, immunity, and disease. Establishing the core microbiome of endangered species of sea turtles could lead to improvements in conservation and rehabilitation management through monitoring this indicator of overall host health.

Evidence from other animals indicates that dysbiosis of the microbial communities can be both a cause and a consequence of metabolic and immunological disorders (Althani et al., 2015; Moffatt and Cookson, 2017). Understanding the microbiome of marine hosts such as sea turtles is important for developing insight into the changing marine environment and the environmental factors that may contribute to dysbiosis and may promote

immunocompromising disorders or disease (Egan and Gardiner, 2016; Apprill, 2017) such as FP. Understanding dysbiosis and its cause is also critical to endangered species conservation in order to manage habitat restructuring and captive rearing or rehabilitative care (West et al., 2019). The consequences of dysbiosis in sea turtle microbial communities is unknown, compromising our ability to protect these endangered animals.

In this study, I investigated the microbiome of two endangered sea turtle species from the same environment. My objective was to characterize the oral and cloacal microbiome of wild Kemp's ridley and green turtles from the same habitat. Sampling from the same habitat allows me to control for local environmental variation that may influence the microbiome. I hypothesized that the turtle species would have distinct microbial communities from each other but that there would be a core microbiome that exists for each body site. Additionally, I investigated microbial community differences based on severity of fibropapillomatosis. I hypothesized that there would be a relationship between the microbial community composition and severity of disease due to the likely links between immune system function and the microbiome.

Methods

Sample Collection

I collected green and Kemp's ridley sea turtles by hand or net capture in the St. Martins Marsh Aquatic Preserve of Crystal River, Florida from June 12 to June 17, 2017 in collaboration with the Inwater Research Group (IRG). For each turtle, we collected blood when the turtle was initially brought aboard the boat followed by performing physical exams. I obtained body temperature using a non-contact digital infrared thermometer (Lasergrip 774;

Etekcity Corp, Anaheim, CA) in the pectoral or femoral regions upon initial boarding of the boat and heart rates using a fetal doppler (Pocket-Dop3; Nicolet Vascular, Madison, WI) prior to release. As part of the physical exam, we examined all turtles for fibropapillomatosis by evaluating the total number of tumors (total tumor score) and assigning a Balazs tumor score which considers the size as well as the number of tumors (Balazs, 1991; Table 1.1). Once the exam was completed and all samples were collected, we released the turtle from the side of the boat.

During the exams, I took an oral swab by gently swabbing the glottis of the turtle with a sterile cotton tipped applicator. I then took a cloaca swab by inserting a cotton tipped applicator gently into the cloaca approximately 2.5 cm and swabbing the interior mucosa. I placed swabs in individual cryovials that were immediately set on dry ice after collection. Upon return from the field, I transferred the samples to a cryogenic dewar of liquid nitrogen for storage until the field work was complete and samples could be transported to the laboratory. After arrival at the laboratory, ranging from 5 to 10 days after initial collection, I moved all samples to an ultra-low freezer (-80˚C) for storage until DNA extraction and sequencing.

To characterize the microbial community of the marine system, I also collected one liter of water at the site of the last turtle release on each day. Within one hour after return from the field (approximately one to two hours after collection), I filtered the water through a 0.22 µM Sterivex^{TM} filter and placed the filter in a labeled whirl-pak bag to store in the liquid nitrogen dewar. Additionally, immediately upon return from the field each day, I collected swabs of the boat deck where the turtles were being held for exams using a sterile cotton

tipped applicator. I placed the swab in a cryovial and stored it in the liquid nitrogen dewar until the samples were transferred to an ultra-low freezer for long-term storage. I collected the boat deck samples to assess it as a source of influence on the turtle samples.

Animal sampling was approved by the New England Aquarium (NEAq) Institutional Animal Care and Use Committee (Protocol #2017-07), and samples were collected under NMFS permit #16598-03 and Florida Fish and Wildlife Conservation Commission permit # MTP-17-125A.

DNA Extraction

I extracted DNA from swabs using a phenol:chloroform:isoamyl extraction protocol adapted from Mettel et al. (2010). I first suspended the swabs in PBL lysis buffer (water saturated phenol, disodium EDTA, sodium dodecyl sulfate, tris HCL, pH 5.7) by vortexing and centrifuging. I removed the supernatant and placed it in a clean tube. After removal of the supernatant, I added TPM buffer (50 mM Tris, pH 7.0, polyvinyl pyrrolidone, and $MgCl_2$) to the original tube with the swab; after vortexing and centrifuging, I then added the supernatant to the tube with the first supernatant. I supplemented the combined supernatant with 800 µL of a phenol:chloroform:isoamyl alcohol solution (pH 6.7+, 25:24:1) and centrifuged. I transferred the upper aqueous layer to a sterile tube and added 0.7 volumes of 100% isopropanol and 0.1 volumes of 3 M sodium acetate. After centrifugation, the supernatant was decanted, I washed the pellet with 70% ethanol, and allowed it to air dry. I then resuspended the dried pellet in 50 µL nuclease-free water and stored it at -80°C until amplification. I verified all DNA extracts by gel electrophoresis, including negative controls

of unused sterile swabs to ensure there was no contamination from supplies and solutions used in the extraction.

I extracted DNA from water samples using an adaptation from manufacturer's guidelines of the MoBio PowerWater® Sterivex™ DNA Isolation Kit. I followed manufacturer's instructions to generate the lysate (up through step 12 in the manufacturer's protocol). I removed the lysate with a 3 mL syringe and added it to clean 2 mL sterile tubes. To these tubes, I added 800 µL of phenol:chloroform:isoamyl alcohol solution (pH 6.7+, 25:24:1) and centrifuged. I transferred the upper aqueous layer to a sterile tube and added 0.7 volumes of 100% isopropanol and 0.1 volumes of 3 M sodium acetate. After centrifugation, the supernatant was decanted, I washed the pellet with 70% ethanol, and allowed it to air dry. I then resuspended the dried pellet in 50 µL nuclease-free water and stored it at -80°C until amplification.

After verification, I amplified DNA extracts in triplicate using bacterial specific (515F and 806R), uniquely barcoded, 16S rRNA primers containing adaptors for Illumina sequencing (Caporaso et al., 2012). Each 25 µL PCR reaction contained 12.5 µL Phusion Master Mix (ThermoFisher), 0.5 µL primers, 11 µL diethylpyrocarbonate (DEPC) water, and 1 µL of DNA. I verified the PCR product via gel electrophoresis, excised the target bands, and purified them using the QIAquick PCR Purification Kit (QIAGEN, Valencia, CA, USA) following the manufacturer's protocols. I then quantified the purified product using a Qubit 2.0 Fluorometer (ThermoFisher, Waltham, MA, USA) and pooled it in equimolar concentrations. Sequencing was performed on the Illumina MiSeq platform with a paired-end V2 300 cycle kit.

Data Analysis

Paired-end reads were demultiplexed using Illumina-utils version 2.0.2 (Eren et al., 2013). I performed quality filtering, merging of paired reads, and amplicon sequence variant (ASV) clustering using DADA2 version 1.12.1 (Callahan et al., 2016) in R version 3.6.1 (R Core Team, 2019). I assigned taxonomy using IDTAXA from the DECPHER package version 2.12.0 (Murali et al., 2018) with the Silva Small Subunit (SSU) 132 training set for classification. I used the phyloseq package version 1.28.0 in R to perform visualizations and statistical tests (McMurdie and Holmes, 2013). I used Bray-Curtis distance metrics to evaluate the differences between each body site (oral cavity and cloaca) for each species. I used principal coordinates analysis (PCoA) to visualize variations in the microbial communities and I tested for significant differences using permutational multivariate analysis of variance (PERMANOVA). I calculated alpha diversity metrics and tested for significance using pairwise Wilcoxon tests, and tested for the significance of FP total tumor scores and Balazs score on the microbial communities using PERMANOVA for Bray-Curtis distance metrics and pairwise Wilcoxon test for alpha diversity metrics.

I identified important taxa between the microbial communities of each species using the DESeq2 package version 1.24.0 (Love et al., 2014), which identifies features that are differentially abundant across samples. I defined the core microbiome as ASVs present in a minimum of 90% of the turtle samples specific to each body site and each species, which I identified using the microbiome package version 1.6.0 (Lahti et al., 2017).

Results

Sample Data

I successfully examined a total of 30 Kemp's ridley turtles and 20 green turtles in Crystal River, Florida (Figure 1.1). One Kemp's ridley turtle had a straight standard carapace length (SSCL) of 24.6 cm and weight of 2.2 kg. The other 29 Kemp's ridley turtles were more similar in size with a mean SSCL of 46.7 cm and a mean weight of 15.4 kg (Table 1.2). We captured all Kemp's ridley turtles by hand except for the smallest animal, which was captured with a dip net. All 30 Kemp's ridley turtles appeared clinically healthy with no visible injuries. We captured five green turtles by hand and the remaining 15 were captured with a dip net. The mean SSCL of the green turtles was 38.0 cm (Table 1.2) and the mean weight was 7.0 kg (Table 1.2). Two green turtles had no external evidence of FP, with the remaining 90% of captured green turtles having visible tumors (total tumor score mean 17.2, Balazs score mean 1.6, Table 1.2). No Kemp's ridley turtles had visible tumors consistent with FP. Water temperature at the site of collection and animal body temperature were approximately equivalent between the green and Kemp's ridley turtles while heart rates of green turtles were slightly higher than Kemp's ridley turtles (Table 1.2).

The oral and cloacal microbiome

Across all samples, sequencing of the 16S rRNA gene resulted in 2,727,027 reads after joining paired-ends and quality filtering, which included the removal of chimeras, singletons, chloroplasts, mitochondrial DNA, and archaea. Out of 107 samples, one sample (a cloaca sample from a green turtle) did not yield enough sequences to be included in analysis and was removed from downstream analyses. The mean sequence counts per sample

was 25,727 (median 23,173) and range was 7,306 to 88,976 counts per sample. These sequences were assigned to 1335 unique amplicon sequence variants (ASVs; a measure of sequence similarity that can be used to differentiate taxa) across 181 different families.

Kemp's ridley turtles had significantly higher Shannon diversity at each body site compared to green turtles for oral samples and cloaca samples (Figure 1.2). Oral samples had higher Shannon diversity than cloaca samples in Kemp's ridley turtles, but the Shannon diversity was similar between oral and cloaca samples of green turtles (Figure 1.2). The oral samples of Kemp's ridley turtles also had higher Shannon diversity than the water samples which had a mean of 2.8 and standard deviation of 0.5 (Wilcoxon, $p = 0.00054$). The water samples were similar in diversity to the Kemp's ridley turtle cloaca samples, while higher in diversity compared to green turtle oral and cloaca samples (Wilcoxon, $p = 0.029$). The boat deck samples had the highest Shannon diversity of all other samples (mean 3.7, standard deviation 0.1).

The oral and cloacal microbial communities were distinct from each other within each species and the structure of the microbial communities was significantly different between each body site and species based on Bray-Curtis dissimilarity (Figure 1.3). The oral microbiome of Kemp's ridley turtles was dominated by bacteria in the family Flavobacteriaceae, with a mean abundance of 34.8%, followed by Arcobacteraceae (11.6%) and Rhodobacteraceae (8.7%), while the green turtle oral cavity was dominated by Pasteurellaceae (44.8%), followed by Arcobacteraceae (15.6%), Campylobacteraceae (9.9%), and Desulfobulbaceae (9.2%). The cloaca samples of both turtle species had high proportions of Neisseriaceae, though they were relatively more abundant in the green turtles (29.2%) than

in the Kemp's ridley turtles (10.4%). Green turtles also had a high percentage of the family Arcobacteraceae (14.7%) and Desulfobulbaceae (11.4%). In addition to the Neisseriaceae, Kemp's ridley cloaca samples had a large proportion of Cardiobacteriaceae (16.5%) and Flavobacteriaceae (15.5%) (Figure 1.4).

Water and deck samples were distinct from all the turtle samples (Figure 1.4C). Water samples were dominated by bacteria in the family Rhodobacteraceae (33.9%), Flavobacteriaceae (19.4%), Thioglobaceae (15.5%), and Litoricolaceae (9.2%). The boat deck, which was in contact with the turtle skin, sea water, and humans, had highest proportions of Idiomarinaceae (24.0%), Marinobacteraceae (19.1%), Halomonadaceae (12.1%), and Alteromonadaceae (9.8%). Since I did not see discrete signatures of these taxa in the turtle microbiomes, I focused on turtles only for the remaining analyses.

I found 204 ASVs with significant differences in abundance between the green turtle and Kemp's ridley turtle oral samples, and the cloaca samples had 108 significantly different ASVs between the two species. The oral cavity ASVs that had highest relative abundance in the Kemp's ridley turtles compared to green turtles largely consisted of bacteria from the families Arcobacteraceae and Flavobacteriaceae (Table 1.3). ASVs that were more abundant in green turtles were similarly from the family Arcobacteraceae, but also included bacteria in the families Desulfobulbaceae and Campylobacteraceae (Table 1.3, Figure 1.5A). ASVs with the largest difference in cloaca samples between species include the families Arcobacteraceae, Desulfobulbaceae, Leptotrichiaceae, and Campylobacteraceae, which were more abundant in green turtles; whereas Rhodocyclaceae, Cardiobacteriaceae,

Campylobacteraceae, Endozoicomonadaceae, Tannerellaceae, and Flavobacteriaceae were significantly more abundant in Kemp's ridley turtles (Table 1.3, Figure 1.5B).

Core microbiome analysis

I found only four ASVs shared in 90% of oral samples across both turtle species and no ASVs were shared across both species in the cloaca samples (Figure 1.6). Among the individual body sites within each species, however, there were many ASVs that had greater than 90% prevalence (Table 1.4, Figure 1.6). The oral samples of green turtles had 11 ASVs present in at least 90% of the samples and the green turtle cloacal microbial community shared 11 ASVs that were in at least 90% of all samples. The Kemp's ridley oral samples had 23 ASVs; by contrast, the cloaca samples only had two ASVs common to at least 90% of the samples.

Fibropapillomatosis and the turtle microbiome

FP tumors were present in 90% of the green turtles captured and none of the Kemp's ridley turtles. Green turtles had a total tumor score mean of 17.2 (range 0 to 38) and a Balazs score mean of 1.6 (range 0 to 3). Bray-Curtis dissimilarity was significantly different for green turtle cloaca samples based on total tumor score, and not significantly different in oral samples (Figure 1.7). There was no significant difference in Shannon diversity among green turtle oral microbial communities based on Balazs score. Cloacal microbial communities also did not differ in Shannon diversity based on Balazs score. I also examined the change in community as a function of Balazs score (Figure 1.8). The turtle with the highest Balazs score (most severe level of infection) had higher relative abundances of Fusobacteriaceae and

Acidaminococcaceae, and lower abundances of Arcobacteraceae compared to oral samples from the other Balazs scores.

Discussion

Variation in microbial communities within a species can be caused by the organism's local environment, life stage, and diet, among other things. In this study, I characterized the microbiome of juvenile Kemp's ridley and green turtles from coastal western Florida. Several green sea turtle studies from various regions of the world examined microbial communities of either cloaca or fecal samples (Ahasan et al., 2017; Price et al., 2017; Campos et al., 2018). Price et al. (2017) characterized the juvenile green turtle cloacal microbiome from different regions of Florida and from two habitats (pelagic and neritic). The turtles from the coastal habitat were most similar to the turtles in this study which were also collected from coastal systems. The families of Neisseriaceae, Arcobacteraceae, Campylobacteraceae, and Desulfobulbaceae were relatively abundant in the green turtles of both studies. By contrast, fecal samples collected from wild green turtles from Australia and Brazil had microbiomes dominated by the families Bacteroidiaceae, Lachnospiraceae, Clostridiaceae, and Porphyromonadaceae (Ahasan et al., 2017; Campos et al., 2018). Although present in low abundances in the turtles from Florida, they were not dominant in the cloaca samples of juvenile green turtles in our study. This could be due to differences in location, life stage, or the section of the gastrointestinal tract that was sampled. For example, in alligators, fecal samples were significantly different from other parts of the gastrointestinal tract (Keenan et al., 2013; Keenan and Elsey, 2015). Alligators had fecal samples primarily composed of the phylum Fusobacteria and intestinal samples with higher proportions of

Firmicutes, which shifted to predominantly Proteobacteria during the winter months (Keenan et al., 2015). Thus, it is reasonable that the cloaca and fecal samples are different in sea turtles.

This is the first study to report the Kemp's ridley turtle microbiomes. The only comparable studies from a carnivorous sea turtle are from loggerhead turtles stranded along the Mediterranean coast, which primarily focused on fecal microbial communities (Abdelrhman et al., 2016; Biagi et al., 2018; Arizza et al., 2019). Abdelrhman et al. (2016) examined six samples from the cloaca or intestine (deceased animals) from stranded loggerheads and found that the classes Clostridia and Bacilli were most abundant. Although the Kemp's ridley cloaca had a small proportion of Clostridia in the microbiome, the dominant classes were Gammaproteobacteria and Bacteroidia. The differences between the loggerhead turtle and Kemp's ridley turtle microbial communities are not limited to species differences; other variables including health condition (the loggerheads were stranded), geography, and diet could also explain these differences.

Thus far, studies of microbial communities in sea turtles focused on fecal or cloacal microbiomes. This study is unique in that I also characterized the microbiome of the oral cavity. The importance of the oral microbiome is unknown in many animals. The oral microbes of other species of reptiles has been evaluated for the alligator and Komodo dragon. In the alligator, the oral samples had higher alpha diversity than the lower GI tract (Keenan et al., 2013), consistent with what I observed with the Kemp's ridley turtles (Figure 1.4). The salivary microbiome of Komodo dragons has higher Shannon diversity than fecal samples (Hyde et al., 2016). A similar pattern was seen in marine mammals, with oral specimens

having higher alpha diversity than rectal samples (Bik et al., 2015). This is thought to be due to greater interaction with transient microbes from the environment entering the oral cavity. However, I did not observe this pattern in green turtles. In fact, green turtles had lower alpha diversity at both body sites compared to the surrounding water, indicating again that the water column microbes had little influence on the turtle microbiome. Kemp's ridley turtles also had higher Shannon diversity than the green turtles at each body site. This result was unexpected because other studies, particularly in mammals, have indicated that herbivores have higher alpha diversity compared to carnivores possibly due to the need for more diverse bacteria to effectively ferment the plant cell wall polysaccharides (Ley et al., 2008a). Two species of herbivorous iguanas, however, had different levels of alpha diversity, which may be due to the complexity of the specific vegetation being consumed (Hong et al., 2011). It is possible that despite green turtles being herbivores, Kemp's ridley turtles still require a diverse microbial community to digest their hard shelled food items.

I sampled Kemp's ridley and green turtles from the same environment, ruling out location-specific environmental variables as the cause of species differences in microbial communities. Although there are no previous studies of Kemp's ridley turtles for comparison, they are clearly different from the co-located green turtles. In the oral and cloaca samples, *Campylobacter* sp. and *Arcobacter* sp., both in the order Campylobacterales, comprised the biggest differences between the turtle species, with both genera having multiple ASVs that were differentially abundant in one or the other turtle species (Table 1.3). For example, Kemp's ridley turtles had 3 ASVs from the *Arcobacter* genus that were more abundant than green turtle oral or cloaca samples, but different ASVs of this genus were

more abundant in green turtles. This could be due to the diverse bacteria from this genus found in the environment, including food items, of the sea turtles. *Arcobacter* is common in sea water, oysters, and even sewage, and it has also been found in intestine samples and feces of farm animals (Collado and Figueras, 2011). Although it is associated with disease (particularly causing abortion) in some farm animals, it is more commonly found in healthy animals (Collado and Figueras, 2011).

Multiple ASVs of the family Flavobacteriaceae were more abundant in Kemp's ridley oral or cloaca samples compared to those of green turtles. Flavobacteriaceae is common in marine environments, particularly in shellfish (Jooste and Hugo, 1999). Flavobacteriaceae is in many marine mammal microbiomes, including oral and gastric samples from sea lions (Bik et al., 2015) and humpback whale respiratory vapor and skin (Apprill et al., 2014; Apprill et al., 2017). Although common to the marine environment, I found this family of bacteria only in the Kemp's ridley turtles, not green turtles. Thus, they may be essential to Kemp's ridley turtles, potentially playing a role in digestion or they could be transiently carried to the Kemp's ridley turtles from a particular food item. In humpback whales, the Flavobacteriaceae on the skin is thought to provide a protective function by predating on other types of bacteria (Apprill et al., 2014), providing another possible role for this family of bacteria in Kemp's ridley turtles. It appears unlikely that Flavobacteriaceae found in Kemp's ridley turtles is pathogenic, as it was highly prevalent and all turtles in this study appeared healthy.

Cardiobacteriaceae is another family of bacteria that I found in high abundance in Kemp's ridley turtle cloaca samples that was not present in green turtles. This family is also

found in dolphin and whale respiratory vapor (Lima et al., 2012; Apprill et al., 2017) though its function in those environments is unknown. Cardiobacteriaceae may be responsible for human illnesses such as endocarditis and wound infections (Das et al., 1997), but pathogenicity in Kemp's ridley turtles is extremely unlikely due to its common presence and high abundance in seemingly healthy animals.

Several of these ASVs are part of the core microbiome (shared among 90% of samples) of the green turtle or Kemp's ridley turtle cloaca (Table 1.4). Additional members of the core microbiome include Lachnospiraceae, a family in the order Clostridiales, which was in 100% of the green turtle cloaca samples. This bacterial family consists of anaerobes with the ability to degrade polysaccharides, which is essential in herbivores such as marine iguanas and green turtles (Hong et al., 2011; Campos et al., 2018). Families in the order Clostridiales play a role in herbivore digestion by breaking down cellulose (Yuan et al., 2015), which is likely the reason multiple ASVs from this order constitute the cloacal microbiome of the herbivorous green turtle. I found *Snodgrassella* sp. (family Neisseriaceae) to be highly abundant in 100% of green turtle cloaca samples as well. Neisseriaceae is a common and diverse bacterial family inhabiting mucosal surfaces of humans and many other animals such as dogs, cats, dolphins, and iguanas (Lie et al., 2015).

There were no ASVs shared between the Kemp's ridley and green turtle cloaca samples, indicating their distinct microbiomes at this body site, which is likely due to their different diet requirements (carnivore vs. herbivore) and subsequent gut morphology (Ley et al., 2008a; Hong et al., 2011; Yuan et al., 2015, Campos et al., 2018). There were four ASVs from different families found in 90% of the oral samples from both turtle species. Two of

which, Pasteurellaceae and Moraxellaceae, were also in high abundance across the oral samples. Pasteurellaceae may be more common than originally expected, as it has been found in human oral microbiome studies (Contreras et al., 2010), European bats (Mühldorfer et al., 2014) and the oral cavity of sea lions and walruses (Hansen et al., 2012). Specifically, *Phocoenobacter* sp., a genus within Pasteurellaceae that was found in the oral samples, was first described in a harbor porpoise uterus (Foster et al., 2000). Moraxellaceae is commonly found in the marine environment, but also includes species that colonize mucosal membranes or the skin of humans and animals (Teixeira and Merquior, 2014), including the oral cavity of dolphins (Bik et al., 2015) as well as the cloaca of green turtles (Price et al., 2017) and feces of loggerhead turtles (Arizza et al., 2019).

I found a high prevalence of FP in the green turtles I sampled, which may be due to the shallow/inshore habitat, higher water temperatures in the summer months, biotoxin exposure, or unidentified water quality disturbances from this region (Jones et al., 2016; Page-Karjian and Herbst, 2017). There is weak clustering, based on Bray-Curtis dissimilarity of cloacal microbial communities, by total number of tumors (tumor score) in the green turtles (Figure 1.7), but the small sample size makes this significance difficult to interpret. There was only one turtle with a Balazs score of 3, the most severe, and this individual had a drastically different oral microbiome compared to other oral samples. In particular, Acidaminococcaceae was highly prevalent in this turtle due to one ASV, an *Acidaminococcus* sp. This bacterial genus is not well understood in animals, and although it increases in abundance in infants with chronic malnutrition (Gough et al., 2015), this turtle appeared to have good body condition. *Fusobacterium* sp. was also dominant in the turtle

with a Balazs score of 3, but it was found in low abundance in several other turtles with lower tumor scores. It was also identified in loggerhead fecal samples although not consistently or in much lower abundance in herbivores (Biagi et al., 2018). Clustering by total tumor score for cloaca samples may be driven by lower abundance of Vibrionaceae and Arcobacteraceae in the turtles with more tumors. *Vibrio* sp. were cultured in most turtles with increased severity of FP in Hawaii (Work et al., 2003), but I found this genus to be in low abundance and not associated with FP severity. More turtles with severe cases of FP would need to be examined to determine whether the patterns observed here are, in fact, a direct result of FP. Location of the tumors on a turtle may also have a direct influence on the microbial communities at different body sites, either through physical contact with the tumors or by influencing exposure to transient bacteria from the local environment. The Balazs score, which is the most widely used scoring system in the field, only evaluates the number and size of the tumors, so future studies should examine a more clinically based scoring system (taking into account location on the turtle and morphology of the tumors) to further evaluate severity of the disease and effect on microbial communities (Page-Karjian et al., 2014; Page-Karjian and Herbst, 2017; Page-Karjian et al., 2019). Since I did find a significant relationship between FP and microbial community structure, despite the small sample size, additional investigations are essential to expand our identification of microbial correlations with disease and immune system function for this multifactorial disease in endangered sea turtles.

Conclusions

I provided the first characterization of the oral and cloacal microbiome of two wild caught sea turtle species, green and Kemp's ridley turtles, from the same environment, allowing us to identify differences in microbial community composition between species. I added microbiome data of green turtle cloaca samples to a growing field of studies and provide a first glimpse into the green turtle oral microbiome. I also provided valuable new information to understand the microbial composition of healthy Kemp's ridley turtles for both the oral cavity and cloaca from this endangered species. I identified a core microbiome for each species at each body site, allowing me to understand the potential importance of these microbes to the health of the turtle, including potential contributions to digestion based on diet. I also provided data on the correlation between the severity of FP in green turtles, and I identified the need for increased sample sizes and a higher resolution scoring system as important to further understand the role of turtle microbiomes in health and disease. Understanding the microbiome from wild populations provides a foundational baseline for comparison that will allow for enhanced monitoring of sea turtle health in future studies.

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Table 1.1. The tumor score calculator. An index used to assess the degree of severity of fibropapillomatosis based on the number and size of external lesions (Balazs, 1991).

Balazs Tumor Score Calculator				
Tumor Size	**0**	**1 (Light)**	**2 (Moderate)**	**3 (Heavy)**
< 1 cm	0	1 - 5	> 5	> 5
1-4 cm	0	1 - 5	> 5	> 5
> 4 - 10 cm	0	0	1 - 3	> 4
> 10 cm	0	0	0	> 1

Table 1.2. Health assessment information and morphometric data of sampled sea turtles. Data is separated by species. Mean ± Standard Deviation (Range). SSCL = Straight standard carapace length. HR = Heart rate (beats per minute).

Variable	**Kemp's ridley (n=30)**	**Green (n=20)**
Water Temp (°C)	28.5 ± 1.4 (26.6-30.8)	28.6 ± 1.0 (26.7-30.8)
Body Temp (°C)	28.6 ± 1 (26.9-30.8)	28.8 ± 1.4 (26.9-31.8)
HR (bpm)	49 ± 8 (32-66)	56 ± 7 (44-66)
Weight (kg)	15 ± 4.8 (2.2-23.5)	7.0 ± 2.2 (4.1-13.1)
SSCL (cm)	45.9 ± 6.2 (24.6-53.6)	38 ± 4 (31-47)
Tumor score	0	17.2 ± 14 (0-38)
Balazs Score	0	1.6 ± 1 (0-3)

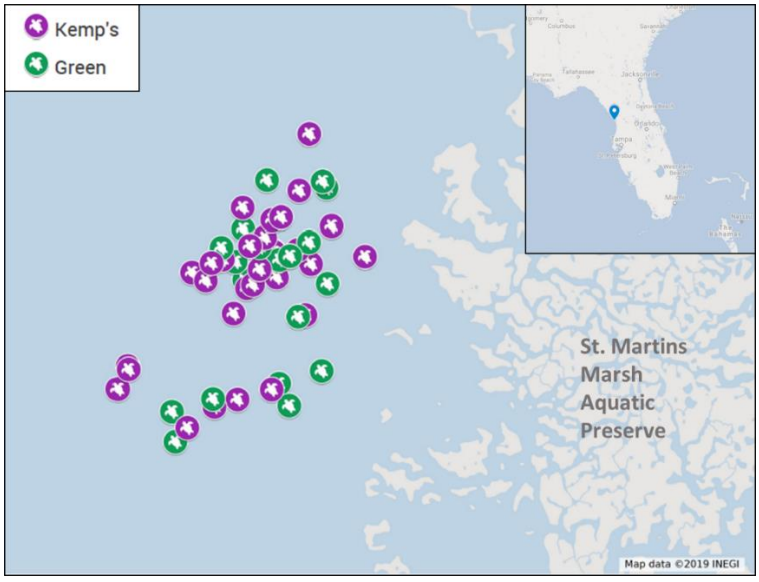


Figure 1.1. Map of locations where turtles were collected in Crystal River, FL. Color indicates species of turtle (Purple = Kemp's ridley, Green = green turtle).

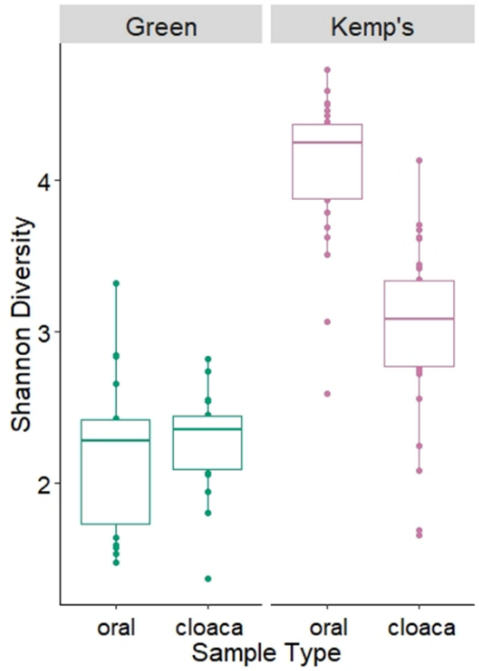


Figure 1.2. Shannon diversity for each body site. Color indicates turtle species. Wilcoxon comparisons between Kemp's oral and green oral (p = 8.10e-13), Kemp's cloaca and green cloaca (p = 1.80e-06), Kemp's oral and Kemp's cloaca (p = 8.803e-10).

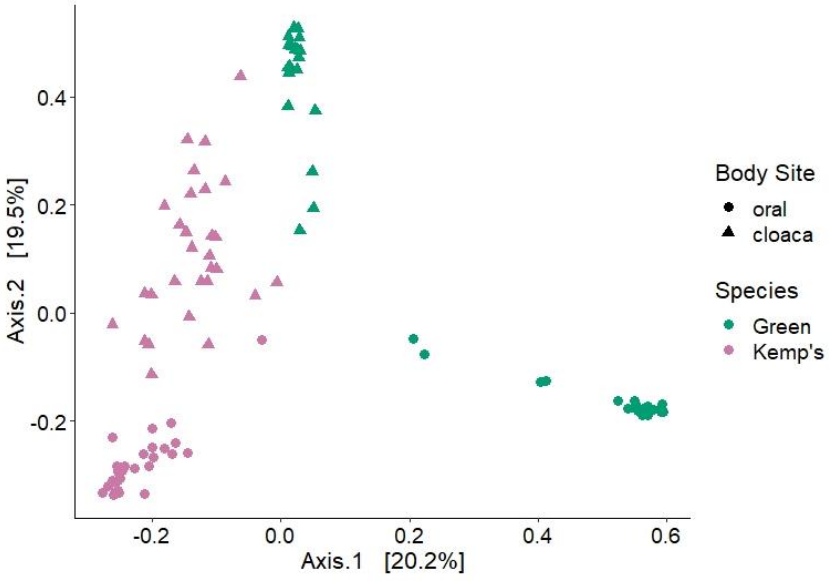
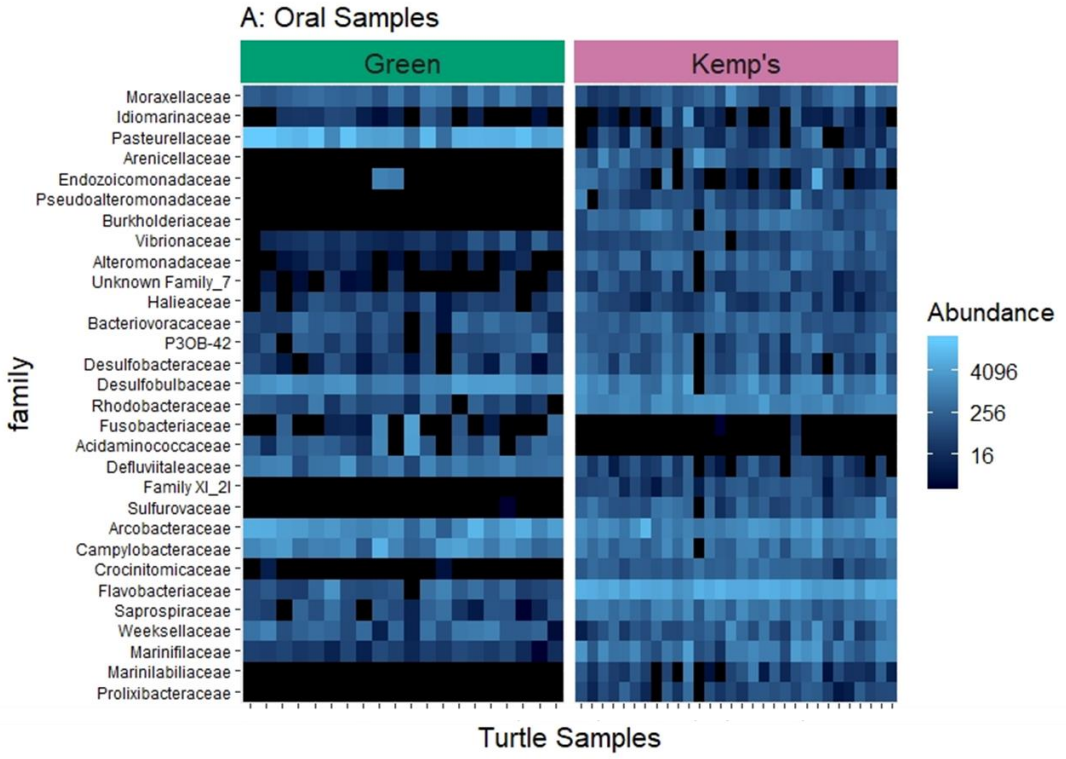
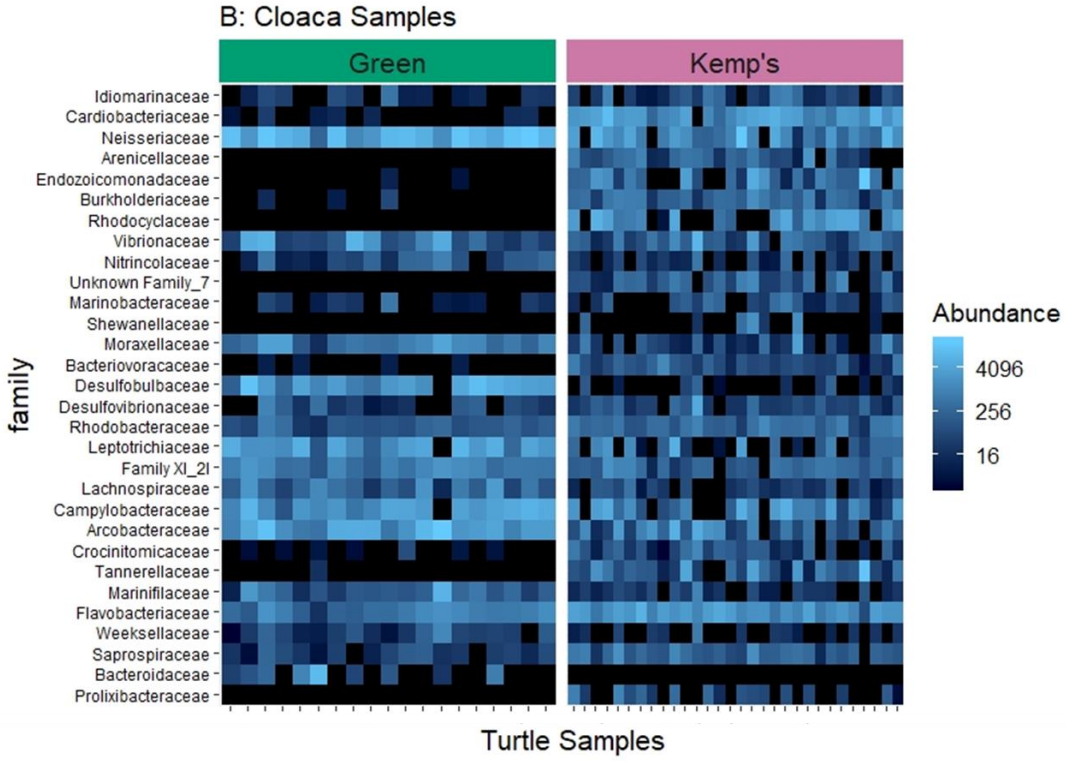


Figure 1.3. PCoA plots of samples based on Bray-Curtis dissimilarity. Color indicates turtle species and shape indicates body site (PERMANOVA, p = 0.001).


A: Oral Samples
Green
Kemp's
Moraxellaceae
Idiomarinaceae
Pasteurellaceae
Arenicellaceae
Endozoicomonadaceae
Pseudoalteromonadaceae
Burkholderiaceae
Vibrionaceae
Alteromonadaceae
Unknown Family_7
Halieaceae
Bacteriovoracaceae
P3OB-42
Desulfobacteraceae
Desulfobulbaceae
Rhodobacteraceae
Fusobacteriaceae
Acidaminococcaceae
Defluviitaleaceae
Family XI_2I
Sulfurovaceae
Arcobacteraceae
Campylobacteraceae
Crocinitomicaceae
Flavobacteriaceae
Saprospiraceae
Weeksellaceae
Marinifilaceae
Marinilabiliaceae
Prolixibacteraceae
family
Abundance
4096
256
16
Turtle Samples


B: Cloaca Samples
Green
Kemp's
Idiomarinaceae
Cardiobacteriaceae
Neisseriaceae
Arenicellaceae
Endozoicomonadaceae
Burkholderiaceae
Rhodocyclaceae
Vibrionaceae
Nitrincolaceae
Unknown Family_7
Marinobacteraceae
Shewanellaceae
Moraxellaceae
Bacteriovoracaceae
Desulfobulbaceae
Desulfovibrionaceae
Rhodobacteraceae
Leptotrichiaceae
Family XI_2I
Lachnospiraceae
Campylobacteraceae
Arcobacteraceae
Crocinitomicaceae
Tannerellaceae
Marinifilaceae
Flavobacteriaceae
Weeksellaceae
Saprospiraceae
Bacteroidaceae
Prolixibacteraceae
family
Abundance
4096
256
16
Turtle Samples

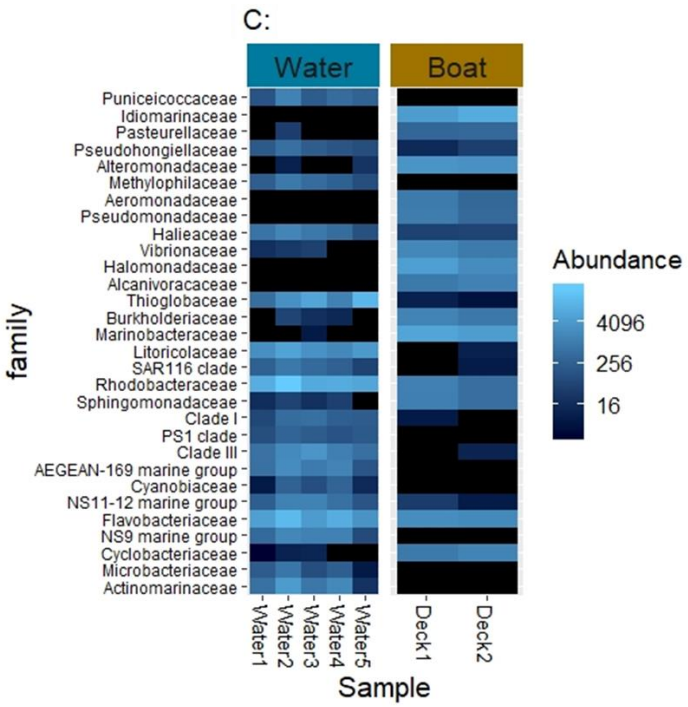


Figure 1.4. Heatmap of top 30 families composing the bacterial communities of each turtle at each body site. Body sites are oral cavity (A) and cloaca (B). Water and boat deck samples are displayed in C. Heatmaps were generated using the plot_heatmap function in phyloseq package.

Table 1.3. The top 20 ASVs that displayed the largest difference between sea turtle species. Relative abundance (%) and standard deviation (SD, %) are displayed for green and Kemp's ridley turtles in both oral (top chart) and cloaca (bottom chart) samples.

ORAL	**Taxonomy**			**Green**		**Kemp's**	
ASV	**Order**	**Family**	**Genus**	**Relative Abundance %**	**SD%**	**Relative Abundance %**	**SD%**
ASV1016	Campylobacterales	Campylobacteraceae	*Campylobacter*	2.25	3.45	0.05	0.10
ASV627	Clostridiales	Defluviitaleaceae	UCG-011	1.94	1.72	0.00	0.00
ASV709	Desulfobacterales	Desulfobulbaceae	NA	2.44	3.62	0.00	0.01
ASV272	Campylobacterales	Arcobacteraceae	*Arcobacter*	9.31	8.76	0.00	0.00
ASV197	Campylobacterales	Campylobacteraceae	*Campylobacter*	6.59	9.79	0.00	0.00
ASV492	Campylobacterales	Arcobacteraceae	*Arcobacter*	2.75	2.10	0.00	0.00
ASV452	Desulfobacterales	Desulfobulbaceae	*Desulforhopalus*	2.32	2.03	0.00	0.00
ASV560	Desulfobacterales	Desulfobulbaceae	NA	1.72	1.55	0.00	0.00
ASV545	Bacteroidales	Marinifilaceae	*Marinifilum*	0.00	0.00	1.48	1.85
ASV279	Flavobacteriales	Flavobacteriaceae	NA	0.00	0.00	1.17	1.49
ASV924	Flavobacteriales	Flavobacteriaceae	NA	0.01	0.02	1.75	1.43
ASV1291	Flavobacteriales	Flavobacteriaceae	NA	0.00	0.00	1.55	1.68
ASV377	Campylobacterales	Arcobacteraceae	*Arcobacter*	0.00	0.00	3.00	8.98
ASV43	Flavobacteriales	Flavobacteriaceae	NA	0.00	0.00	2.55	2.45
ASV467	Bacteroidales	Marinifilaceae	*Marinifilum*	0.00	0.00	1.65	1.51
ASV917	Flavobacteriales	Flavobacteriaceae	NA	0.00	0.00	3.55	4.27
ASV619	Betaproteobacteriales	Burkholderiaceae	NA	0.00	0.00	1.96	2.01
ASV1111	Flavobacteriales	Flavobacteriaceae	NA	0.00	0.00	5.23	5.15
ASV1104	Campylobacterales	Arcobacteraceae	*Arcobacter*	0.00	0.00	2.95	2.45
ASV508	Campylobacterales	Arcobacteraceae	*Arcobacter*	0.00	0.00	2.73	2.06

CLOACA	Taxonomy			Green		Kemp's	
ASV	**Order**	**Family**	**Genus**	**Relative Abundance %**	**SD%**	**Relative Abundance %**	**SD%**
ASV166	Campylobacterales	Arcobacteraceae	*Arcobacter*	6.99	8.28	0.15	0.70
ASV1302	Campylobacterales	Arcobacteraceae	*Arcobacter*	3.38	4.95	0.21	1.12
ASV411	Campylobacterales	Arcobacteraceae	*Arcobacter*	3.78	4.06	0.01	0.05
ASV1068	Desulfobacterales	Desulfobulbaceae	NA	7.53	8.04	0.03	0.18
ASV721	Desulfobacterales	Desulfobulbaceae	NA	3.53	3.97	0.10	0.24
ASV313	Fusobacteriales	Leptotrichiaceae	NA	9.47	8.58	2.16	4.27
ASV517	Campylobacterales	Campylobacteraceae	*Campylobacter*	9.64	6.46	0.83	1.23
ASV726	Cardiobacteriales	Cardiobacteriaceae	NA	0.00	0.00	2.29	3.76
ASV222	Clostridiales	Family XI_2I	*Fusibacter*	0.00	0.00	1.09	1.43
ASV514	Betaproteobacteriales	Rhodocyclaceae	NA	0.00	0.00	7.89	9.04
ASV1245	Campylobacterales	Campylobacteraceae	*Campylobacter*	0.03	0.10	4.70	6.53
ASV935	Flavobacteriales	Flavobacteriaceae	NA	0.00	0.00	1.15	1.82
ASV1104	Campylobacterales	Arcobacteraceae	*Arcobacter*	0.00	0.00	1.64	3.45
ASV1164	Cardiobacteriales	Cardiobacteriaceae	*Cardiobacterium*	0.00	0.01	12.39	11.49
ASV798	Flavobacteriales	Flavobacteriaceae	NA	0.00	0.00	1.78	2.33
ASV1046	Oceanospirillales	Endozoicomonadaceae	*Endozoicomonas*	0.00	0.01	4.97	9.56
ASV1310	Bacteroidales	Tannerellaceae	*Macellibacteroides*	0.01	0.02	3.43	6.69
ASV882	Flavobacteriales	Flavobacteriaceae	NA	0.00	0.00	2.75	3.56
ASV1072	Arenicellales	Arenicellaceae	*Arenicella*	0.00	0.00	1.52	2.69
ASV619	Betaproteobacteriales	Burkholderiaceae	NA	0.00	0.00	1.19	1.36

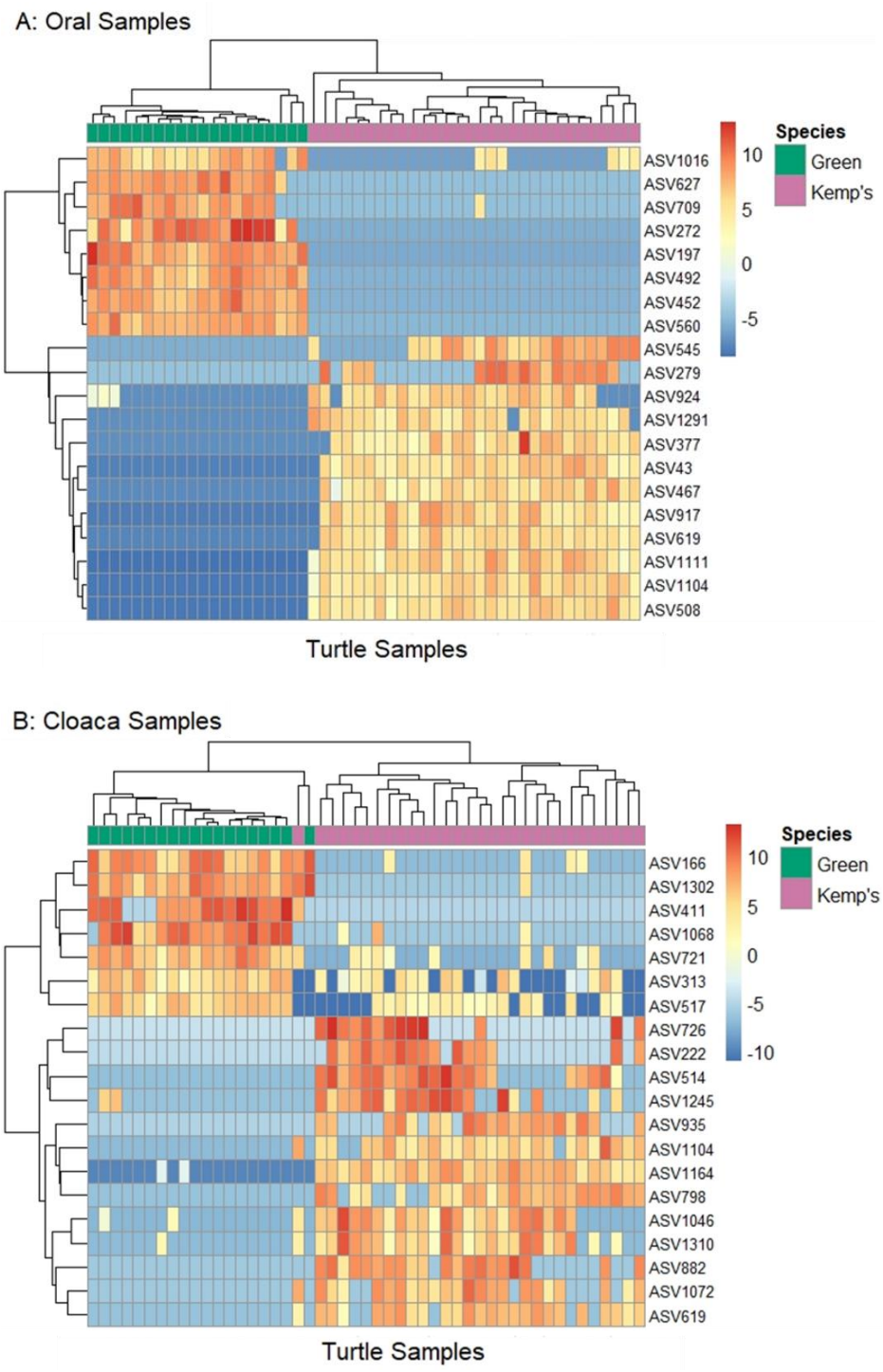


Figure 1.5. Heatmap of the top 20 ASVs that displayed the largest difference between sea turtle species. Sample color (top bar) indicates the turtle species, Kemp's ridley turtles and green turtles, for oral samples (A) and cloaca samples (B). Counts were normalized using variance stabilizing transformation. Taxonomy of the ASVs is shown in Table 1.3.

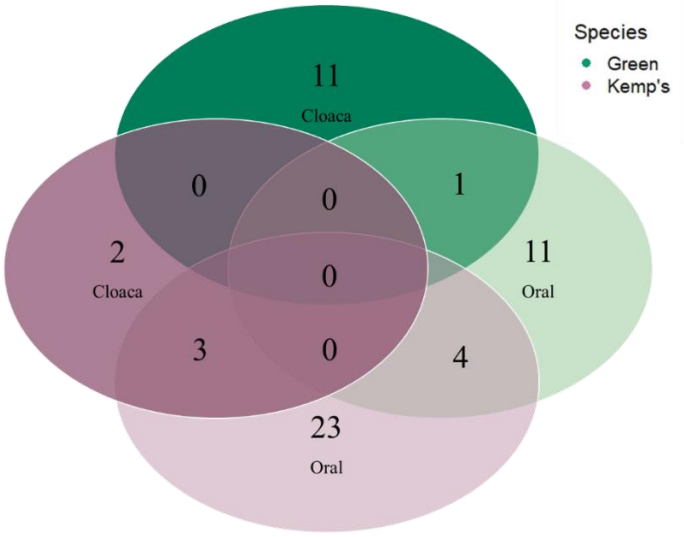


Figure 1.6. Venn diagram of the number of ASVs shared in 90% of samples. Color indicates species and shade indicates body site; darker shades = cloacal samples; lighter shades = oral samples.

Table 1.4. Core microbiome. The ASVs that are shared among species and body sites with taxonomy and prevalence (proportion of samples in which the ASV is found) across the indicated sample type. Core was defined as minimum prevalence of 0.90 (90%).

Turtle Species Body Site	ASV	Order, Family, Genus	Prevalence
Green Cloaca	ASV19	Clostridiales, Lachnospiraceae, NA	1.00
	ASV40	Pseudomonadales, Moraxellaceae, *Moraxella*	0.95
	ASV166	Campylobacterales, Arcobacteraceae, *Arcobacter*	1.00
	ASV313	Fusobacteriales, Leptotrichiaceae, NA	0.95
	ASV381	Flavobacteriales, Flavobacteriaceae, NA	1.00
	ASV517	Campylobacterales, Campylobacteraceae, *Campylobacter*	0.95
	ASV721	Desulfobacterales, Desulfobulbaceae, NA	0.95
	ASV761	Clostridiales, Family XI_2I, *Fusibacter*	0.95
	ASV978	Clostridiales, Family XI_2I, *Fusibacter*	1.00
	ASV1130	Betaproteobacteriales, Neisseriaceae, *Snodgrassella*	1.00
	ASV1302	Campylobacterales, Arcobacteraceae, *Arcobacter*	1.00
Kemp's Cloaca	ASV917	Flavobacteriales, Flavobacteriaceae, NA	0.93
	ASV1164	Cardiobacteriales, Cardiobacteriaceae, *Cardiobacterium*	0.97
Green Oral	ASV197	Campylobacterales, Campylobacteraceae, *Campylobacter*	1.00
	ASV272	Campylobacterales, Arcobacteraceae, *Arcobacter*	0.95
	ASV439	Vibrionales, Vibrionaceae, NA	0.95
	ASV452	Desulfobacterales, Desulfobulbaceae, *Desulforhopalus*	1.00
	ASV492	Campylobacterales, Arcobacteraceae, *Arcobacter*	1.00
	ASV560	Desulfobacterales, Desulfobulbaceae, NA	1.00
	ASV584	Clostridiales, Defluviitaleaceae, NA	1.00
	ASV669	Campylobacterales, Arcobacteraceae, *Arcobacter*	0.95
	ASV1016	Campylobacterales, Campylobacteraceae, *Campylobacter*	0.95
	ASV1161	Pasteurellales, Pasteurellaceae, *Phocoenobacter*	1.00
	ASV1281	Pseudomonadales, Moraxellaceae, NA	1.00

Table 1.4 (continued). Core microbiome.

Turtle Species Body Site	ASV	Order, Family, Genus	Prevalence
Kemp's Oral	ASV41	Campylobacterales, Campylobacteraceae, *Campylobacter*	0.97
	ASV43	Flavobacteriales, Flavobacteriaceae, NA	0.97
	ASV187	Campylobacterales, Campylobacteraceae, *Campylobacter*	0.93
	ASV377	Campylobacterales, Arcobacteraceae, *Arcobacter*	0.93
	ASV434	Desulfobacterales, Desulfobulbaceae, *Desulforhopalus*	0.97
	ASV467	Bacteroidales, Marinifilaceae, *Marinifilum*	0.97
	ASV499	Campylobacterales, Sulfurovaceae, *Sulfurovum*	0.97
	ASV508	Campylobacterales, Arcobacteraceae, *Arcobacter*	1.00
	ASV546	Flavobacteriales, Flavobacteriaceae, NA	0.93
	ASV575	Flavobacteriales, Flavobacteriaceae, *Maritimimonas*	1.00
	ASV619	Betaproteobacteriales, Burkholderiaceae, NA	0.97
	ASV687	Flavobacteriales, Weeksellaceae, NA	0.93
	ASV798	Flavobacteriales, Flavobacteriaceae, NA	0.93
	ASV834	Chitinophagales, Saprospiraceae, NA	0.97
	ASV843	Myxococcales, P3OB-42, NA	0.97
	ASV917	Flavobacteriales, Flavobacteriaceae, NA	0.97
	ASV935	Flavobacteriales, Flavobacteriaceae, NA	0.93
	ASV1104	Campylobacterales, Arcobacteraceae, *Arcobacter*	1.00
	ASV1111	Flavobacteriales, Flavobacteriaceae, NA	1.00
	ASV1112	Bdellovibrionales, Bacteriovoracaceae, *Peredibacter*	0.97
	ASV1281	Pseudomonadales, Moraxellaceae, NA	0.97
	ASV1291	Flavobacteriales, Flavobacteriaceae, NA	0.93
	ASV1305	Flavobacteriales, Flavobacteriaceae, *Maritimimonas*	0.97
Green & Kemp's Oral	ASV687	Flavobacteriales, Weeksellaceae, NA	0.90
	ASV843	Myxococcales, P3OB-42, NA	0.92
	ASV1161	Pasteurellales, Pasteurellaceae, *Phocoenobacter*	0.90
	ASV1281	Pseudomonadales, Moraxellaceae, NA	0.98
Green, Oral & Cloaca	ASV439	Vibrionales, Vibrionaceae, NA	0.92
Kemp's Oral & Cloaca	ASV917	Flavobacteriales, Flavobacteriaceae, NA	0.95
	ASV1104	Campylobacterales, Arcobacteraceae, *Arcobacter*	0.95
	ASV1164	Cardiobacteriales, Cardiobacteriaceae, *Cardiobacterium*	0.92

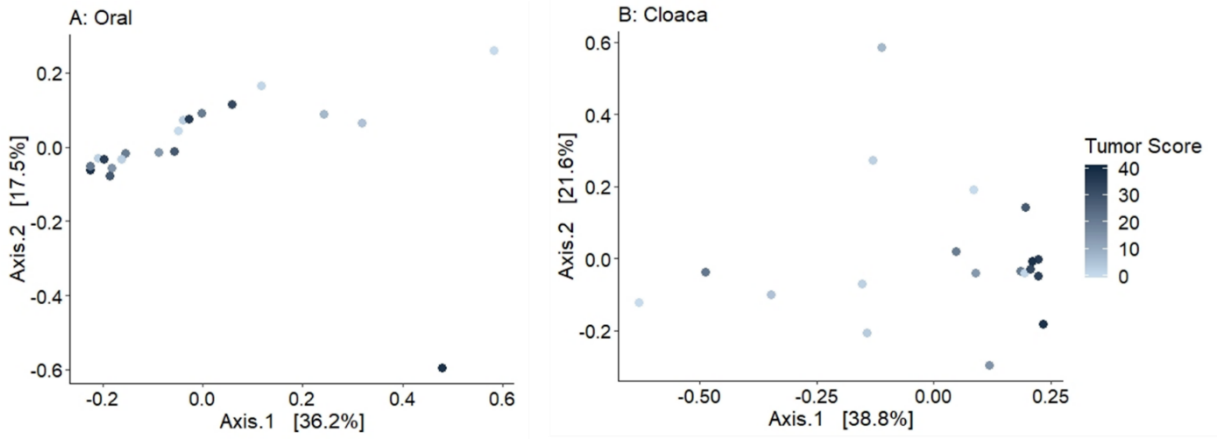


Figure 1.7. PCoA plots of green turtle microbial communities. Oral (A; PERMANOVA, p = 0.175) and cloacal (B; PERMANOVA, p = 0.008) microbial communities are based on Bray-Curtis dissimilarity and colored by total tumor score (number of tumors).

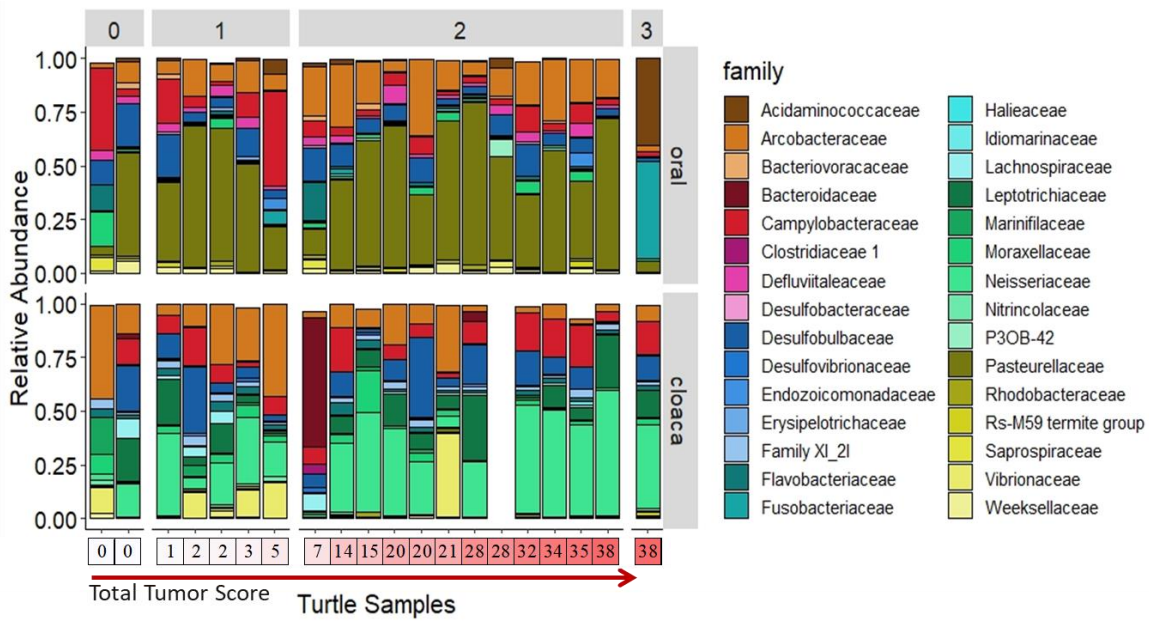


Figure 1.8. Taxa plots of green turtle microbial communities. Plots represent the relative abundance of the top 30 bacterial families. Plots are separated by Balazs score (0 – 3) and each body site (oral or cloaca). The turtle samples are ordered by total tumor score (number of tumors) with 0 tumors on the left to the most tumors (38) on the right.

CHAPTER 2

CHARACTERIZATION OF ORAL AND CLOACAL MICROBIAL COMMUNITIES IN COLD-STUNNED KEMP'S RIDLEY SEA TURTLES (*LEPIDOCHELYS KEMPII*) DURING THE TIME COURSE OF REHABILITATION

Abstract

Microbial communities of animals play a role in health and disease, including immunocompromising conditions. In the northeastern United States, cold-stunning events often cause endangered Kemp's ridley turtles (*Lepidochelys kempii*) to become stranded on beaches in autumn. These sea turtles are admitted to rehabilitation facilities when rescued alive and are presumed immunocompromised secondary to hypothermia. To better understand the role that microbes play in the health of cold-stunned sea turtles, I characterized the oral and cloacal microbiome from Kemp's ridley turtles at multiple timepoints during rehabilitation, from intake to convalescence, by using Illumina sequencing to analyze the 16S rRNA gene. Microbial communities were distinct between body sites and among turtles that survived and those that died. I found that stranding location influenced the cloacal microbiome, but clinical parameters such as presence of pneumonia or values for

various blood analytes did not correlate with oral or cloacal microbial community composition. I also investigated the effect of antibiotics on the microbiome during rehabilitation and prior to release and found that the type of antibiotic altered the microbial community composition, yet overall taxonomic diversity remained the same. The microbiome of cold-stunned Kemp's ridley turtles gradually changed through the course of rehabilitation with environment, antibiotics, and disease status all playing a role in those changes and ultimately the release status of the turtles.

Introduction

Kemp's ridley turtles (*Lepidochelys kempii*) are listed as Critically Endangered by the International Union for the Conservation of Nature (Wibbels and Bevan, 2019). The species faces global challenges due to fisheries interactions, legal and illegal harvest, habitat loss, pollution, vessel strike, and climate change (Heppell et al., 2003; Wyneken et al., 2006; Wallace et al., 2011). In addition to anthropogenic causes of population decline, sea turtles are also susceptible to several diseases and presumed immunocompromising conditions that require rehabilitation and medical intervention (Wyneken et al., 2006; Innis and Staggs, 2017). One example of this is cold-stunning, or hypothermia. Cold-stunning occurs when turtles are exposed to water temperatures below 10°C (Witherington and Ehrhart, 1989; Still et al., 2005; Wyneken et al., 2006; Innis and Staggs, 2017). Large cold-stunning events involving juvenile Kemp's ridley, green (*Chelonia mydas*), and loggerhead turtles (*Caretta caretta*) occur annually in Massachusetts when turtles do not migrate south before water temperatures drop during autumn (Morreale et al., 2005; Still et al., 2005). Cold stunned turtles cease swimming and may become stranded on beaches when forced ashore by tidal

activity and wind. Warming sea water temperatures are predicted to cause a continued trend of increasing numbers of stranded Kemp's ridley turtles by increasing the distribution of turtles to the northeastern United States and creating a bridge from the Gulf Stream to nearshore waters (Griffin et al., 2019). Common sequelae resulting from chronic cold-stunning include cardiorespiratory depression, dehydration, reduced renal function, pneumonia, sepsis, osteomyelitis, and death (Innis et al., 2007, 2009a, 2009b, 2014, 2016; Solano et al., 2008; Keller et al., 2012; Kennedy et al., 2012). Kemp's ridley turtles comprise the majority of turtles that strand each year in the northeastern U.S., and when found alive, they are transported to wildlife hospitals, such as the New England Aquarium (NEAq), for medical care. NEAq is the primary rehabilitation center for sea turtles stranded in Massachusetts, where turtles are triaged and rehabilitated over several months until release or transportation to secondary facilities for continued care until release. Affected turtles often require intensive medical management over several months of hospitalization, during which they are serially evaluated by physical examinations, hematology and plasma biochemical evaluations, radiography, and other methods needed to guide their recovery (Wyneken et al., 2006; Innis and Staggs, 2017). It is not known whether cold-stunned turtles' microbial communities are affected during rehabilitation, but it is possible that medical management and the captive environment could lead to a dysbiosis, or shift in the microbiome of the affected turtles.

Chronic disease conditions or environmental stressors can cause dysbiosis in humans and other animals, but there is limited information on sea turtle microbiomes (Dickson et al., 2014; Bourne et al., 2016; Duvallet et al., 2017; Zaneveld et al., 2017). Research reveals that

diseases of many species may not be caused by a single agent, but rather by dysbiosis of the microbial communities that play a role in health and immunity (McFall-Ngai et al., 2013; Egan and Gardiner, 2016; Zaneveld et al., 2017). Perturbation of natural microbial communities drives many chronic diseases in humans, including gut, oral, skin, and lung disorders; thus, understanding dysbiosis may improve diagnostic and therapeutic management (Egan and Gardiner, 2016). Further, dysbiosis of one body site may affect other sites because of microbial communities' effect on the host immune system. For example, the gut microbiome plays a role in host immune system function, directly influencing diseases of the gut, but also affecting other sites such as the nervous system or respiratory tract (McFall-Ngai et al., 2013).

Dysbiosis may present differently depending on the disease. In some human diseases such as colorectal cancer (CRC), the microbial community shifts to an increased proportion of pathogenic bacteria. Other diseases, such as in inflammatory bowel disease (IBD) may reveal a decrease in health-associated bacteria (Duvallet et al., 2017). A complete restructuring of the microbial community may also characterize dysbiosis. For irritable bowel syndrome (IBS), inflammation is associated with alterations in the proportions of microbes, including an increase in the amount of *Ruminococcus* and *Clostridium*, and reduction in *Bifidobacterium* and *Faecalibacterium* (Althani et al., 2015). Complicating the situation, dysbiosis may be a consequence of a disease, rather than the cause. In lung diseases of humans, a dysbiosis-inflammation cycle suggests that the relationship is bidirectional between host response and lung microbiome alterations (Dickson et al., 2016). Disentangling

whether dysbiosis in microbial communities leads to or is a result of disease is essential for understanding the mechanism of disease transmission.

Zaneveld et al. (2017) conceptualize dysbiosis in microbiomes based on the opening sentence of Tolstoy's novel *Anna Karenina*, which states that "All happy families are all alike; each unhappy family is unhappy in its own way". In other words, healthy microbiomes are similar, but a dysbiotic or unhealthy microbiome is each altered in a unique way (Dickson et al., 2016; Zaneveld et al., 2017). Healthy microbiomes have little variability, while stressed or diseased microbiomes have a wider range of altered compositions compared to the healthy microbiomes (Zaneveld et al., 2017). For example, frogs infected with the fungus, *Batrachochytrium dendrobatidis* (Bd) showed more variability in their skin microbiome than frogs that were not infected (Jani and Briggs, 2014). In addition to recognizing deterministic changes in microbial community structure, it is also important to document the stochastic changes in microbiomes due to stressors and/or pathogens (Zaneveld et al., 2017). Diseases of corals, such as black band disease and white-plague disease, are associated with shifts in microbial communities, including increases in opportunistic pathogens, polymicrobial infection, reduction in commensal bacteria, or enrichment of bacteria with pathogenic potential (Egan and Gardner, 2016; Apprill, 2017). Many variables may lead to dysbiosis in sea turtles during rehabilitation, including the initial cold-stunned event, stress of rehabilitation, and captive diet.

Thus far, insights into the microbiome of sea turtles focused on the gut microbiome (cloaca or feces) of loggerhead and green turtles (Abdelrhman et al., 2016; Ahasan et al., 2017a; Ahasan et al., 2017b; Price et al., 2017, Biagi et al., 2018, Arizza et al., 2019). The

fecal microbiome was distinct between wild-captured green turtles and stranded green turtles; stranded animals had a higher proportion of Proteobacteria, specifically Gammaproteobacteria, compared to wild turtles that had feces dominated by Firmicutes (Ahasan et al., 2017b). Rehabilitation also appears to affect the microbiome. The cloacal microbial communities differed before and after rehabilitation of green turtles, with post rehabilitation turtles having more similar microbiomes due to both environmental effects and controlled diet during hospitalization (Ahasan et al., 2017a). Rehabilitating green turtles also have shifts in their microbiome attributed to receiving a high protein diet during recovery (Bloodgood et al., 2020).

During rehabilitation for cold-stunning, Kemp's ridley turtles are treated with antibiotics to prevent or treat secondary infections (Stamper et al., 1999; Innis et al., 2011; Innis et al., 2017). Although antibiotics are important to treating infectious diseases, these medications interact with entire microbial communities, which can affect immune homeostasis of the host and potentially lead to dysbiosis (Willing et al, 2011; Blaser, 2016; Ferrer et al., 2017). In humans, even a short term course of antibiotic treatment can have a long lasting impact. For example, the microbiome of the throat and gut became altered after just one week of treatment with clarithromycin and metronidazole, and the microbiome remained perturbed, in some cases, for up to four years after treatment (Jakobsson et al., 2010). Different antibiotics have different effects on the shift in microbial community assemblage, but all generally result in a decrease in diversity of the microbial community, coupled with varying timeframes until the community returns to the pretreatment state (Willing et al., 2011). Exposure to antibiotics can also lead to antibiotic resistance due to

increases in antibiotic resistance genes in the microbial community (Jakobsson et al., 2010; Zaura et al., 2015). Pigs that received in-feed antibiotics had an increase in antibiotic resistance genes and larger *Escherichia coli* populations compared to animals that were not provided prophylactic antibiotic treatment (Looft et al., 2012; Looft et al., 2014). Understanding the affect that antibiotic treatment, and the rehabilitation process more broadly, has on the microbiome of Kemp's ridley turtles is important for optimizing their chances for success once they are reintroduced to the wild.

In this study, I investigated the microbial communities of cold-stunned Kemp's ridley turtles through the time course of rehabilitation at NEAq. My first objective was to characterize the oral and cloacal microbiome of the cold-stunned turtles. I hypothesized that there was a distinct and diverse microbial community at each body site. Second, I identified bacteria that were associated with mortality versus survival and I evaluated the microbiome at intake to determine if there were correlations with clinical variables such as hematologic parameters (i.e. complete blood counts) or disease status (i.e. pneumonia). I also evaluated temporal effects of rehabilitation to determine alterations to the turtle microbiome from the time they were admitted to the hospital through the end of their hospitalization. I hypothesized that the microbiome of turtles at intake (i.e. directly from the wild) would shift when cold-stunned turtles were hospitalized, brought to appropriate body temperatures, and medically managed. I also hypothesized that antibiotics would alter microbiomes compared to turtles that were not administered antibiotics and that the microbial community assemblages would converge toward that of the turtles that did not receive antibiotics once

they were considered clinically healthy (i.e. after discontinuing antibiotics and prior to release).

Methods

Sample Collection

I collected oral and cloaca samples from Kemp's ridley turtles admitted to NEAq during the 2015 cold-stun event (November and December 2015). I chose turtles at random to be enrolled in the study and to have radiographs taken prior to their intake exam to assess the degree of lung abnormalities. The attending veterinarian categorized the turtle as pneumonia or non-pneumonia based on their interpretation of the radiographs. Clinicians collected blood during physical exams from the dorsal cervical sinus and analyzed it immediately using a blood gas and biochemical analyzer pHOx Ultra (NOVA Biomedical, Waltham, MA). At day three of rehabilitation, additional blood samples were collected and transported to a commercial veterinary diagnostic laboratory (IDEXX Laboratories, North Grafton, MA) where a complete blood count and chemistry panel was performed.

Prior to the intake physical exam on day 0, I collected samples for microbiome analysis from the oral cavity and cloaca of each animal. I took an oral swab by gently swabbing the glottis of the turtle with a sterile cotton tipped applicator. I then took a cloaca swab by inserting a cotton tipped applicator gently into the cloaca approximately 2.5 cm and swabbing the mucosa. I placed swabs into individual cryovials and immediately placed them on dry ice after collection, then moved them to an ultra-low freezer (-80°C) within 15 minutes for later DNA extraction and sequencing.

NEAq veterinarians prescribed antibiotics (ceftazidime 22 mg/kg intramuscular or oxytetracycline 42 mg/kg subcutaneous) for the turtles, as necessary, based on radiograph findings and blood analysis. In some cases, additional antibiotics or a change in antibiotic was prescribed later in rehabilitation based on clinical needs. I sampled the surviving turtles throughout rehabilitation at timepoints dependent on clinical status. I collected oral and cloaca swabs at a second timepoint, four weeks after the turtles were admitted. Timepoint 3 was slated to be at eight weeks after admittance but, in some cases, was conducted as early as six weeks to ensure that the sample was collected prior to the discontinuance of antibiotics. Timepoint 4a was collected when the turtle was classified as convalescent, or clinically healthy, which was defined as 30 days after antibiotics were discontinued. If a turtle was not on antibiotics, convalescence was determined based on when the animal was ready for release, which depended on appetite, physical exam, and transport preparation. I collected oral and cloaca swabs at an additional timepoint, timepoint 4b, prior to release if the turtle remained at NEAq more than 4 weeks after timepoint 4a was collected. Overall, turtles in the longitudinal study received 2 to 5 oral and cloaca swabs during their time in rehabilitation, except those that died after intake sampling (Figure 2.1). During rehabilitation, turtles were maintained in tanks of filtered saltwater at approximately 24°C and they were offered food items of herring and squid once to twice daily.

This study was approved by the NEAq Institutional Animal Care and Use Committee (Protocol #2015-16) and conducted under the US Department of the Interior Fish and Wildlife Service Permit# TE-697823.

DNA Extraction

I extracted DNA from the swabs using a phenol:chloroform:isoamyl extraction protocol adapted from Mettel et al., (2010). I first suspended the swabs in PBL lysis buffer (water saturated phenol, disodium EDTA, sodium dodecyl sulfate, tris HCL, pH 5.7) by vortexing and centrifuging. I removed the supernatant and placed it in a clean tube. After removal of the supernatant, I added TPM buffer (50 mM Tris, pH 7.0, polyvinyl pyrrolidone, and $MgCl_2$) to the swab; after vortexing and centrifuging, I then added the second supernatant to the tube with the first supernatant. I supplemented the combined supernatant with 800 µL of a phenol:chloroform:isoamyl alcohol solution (pH 6.7+, 25:24:1) and centrifuged. I transferred the upper aqueous layer to a sterile tube and added 0.7 volumes of 100% isopropanol and 0.1 volumes of 3 M sodium acetate. After centrifugation, the supernatant was decanted, I washed the pellet with 70% ethanol, and allowed it to air dry. I then resuspended the dried pellet in 50 µL nuclease-free water and stored it at -80˚C until amplification. I verified all DNA extracts by gel electrophoresis, including negative controls of unused sterile swabs, to ensure there was no contamination from supplies and solutions used in the extraction.

After verification, I amplified DNA extracts in triplicate using bacterial specific (515F and 806R), uniquely barcoded, 16S rRNA gene primers containing adaptors for Illumina sequencing (Caporaso et al., 2012). Each 25 µL PCR reaction contained 12.5 µL Phusion Master Mix (ThermoFisher), 0.5 µL primers, 11 µL diethylpyrocarbonate (DEPC) water, and 1 µL of DNA. The PCR product was verified via gel electrophoresis, and I excised the target bands and purified them using the QIAquick PCR Purification Kit (QIAGEN,

Valencia, CA, USA) following the manufacturer's protocols. I then quantified the purified product using a Qubit 2.0 Fluorometer (ThermoFisher, Waltham, MA, USA) and pooled it in equimolar concentrations. Sequencing was performed on the Illumina MiSeq platform with a paired-end V2 300 cycle kit.

Any samples that had poor sequencing read quality or low sequencing depth were reamplified in triplicate as described above. However, for these samples I purified the resulting PCR product using AMPure XP (Beckman Coulter, Inc. Indianapolis, IN, USA) following manufacturers guidelines using the 0.8:1.0 ratio of bead:sample to target 300 bp and above. After purification, I quantified the DNA using the Agilent D1000 ScreenTape System (Agilent Technologies, Inc, Waldbronn, Germany) following manufacturers guidelines for more precise quantification and band size visualization. I pooled the purified PCR product to equimolar concentration based on the concentration of the desired band size range. I used a BluePippin™ (Sage Science Inc., Beverly, MA, USA), following manufacturer's instructions, to size select the target bands. I also sequenced these samples on an Illumina MiSeq platform and the sequences were used for downstream analyses.

Data Analysis

Paired-end reads were demultiplexed using Illumina-utils version 2.0.2 (Eren et al., 2013). I performed quality filtering, merging of paired reads, and amplicon sequence variant (ASV) clustering using DADA2 version 1.12.1 (Callahan et al., 2016) in R version 3.6.1 (R Core Team, 2019). I assigned taxonomy using IDTAXA from the DECPHER package version 2.12.0 (Murali et al., 2018) with the Silva Small Subunit (SSU) 132 training set for

classification. I used the phyloseq package version 1.28.0 in R to perform diversity metric visualizations and statistical tests (McMurdie and Holmes, 2013).

The differences between each body site (oral cavity and cloaca) for each species were evaluated using Bray-Curtis distance metrics. I tested for significant differences of Bray-Curtis distance metrics using permutational multivariate analysis of variance (PERMANOVA) for variables including survival, stranding location, disease condition (pneumonia vs. non-pneumonia), and arrival day at NEAq (day of stranding or next day) for each body site. I performed hierarchical clustering using the simple average method to evaluate the differences between intake samples and convalescent samples (timepoint 4a). Random forest models were used to determine which ASVs were associated with survival (survived vs. died) using the randomForest package version 4.6-14 (Liaw and Wiener, 2002). I used the betadisper function to analyze the variance among groups and tested for significance with analysis of variance (anova) in R.

Correlation between microbial communities at each body site and clinical parameters were determined using the envfit function with the vegan package version 2.5-6 in R (Oksanen et al., 2019). This function fits vectors representing environmental factors, in this case hematologic values, to ordination plots and tests for statistical significance with 999 random permutation tests. The blood gas, biochemical, and hematologic analytes included pH, partial pressure of carbon dioxide (pCO_2), partial pressure of oxygen (pO_2), bicarbonate (HCO_3), sodium (Na), potassium (K), chloride (Cl), ionized calcium, glucose, blood urea nitrogen (BUN), uric acid, lactate, hematocrit (Hct), white blood cell count, relative heterophil count, and relative lymphocyte count.

Within a given body site (oral cavity or cloaca), I used principal coordinates analysis (PCoA) to visualize variations in the microbial communities across timepoints and days in rehabilitation, and I tested for significant differences using PERMANOVA. Shannon diversity index was calculated for each body site at each timepoint, and significance was tested by pairwise Wilcoxon tests.

To test the hypothesis that antibiotics affected the microbial communities of turtles and that the microbiome became similar at convalescence to turtles that were not on antibiotics, I calculated Bray-Curtis dissimilarity for the communities at each timepoint. I used PERMANOVA to determine significant differences based on antibiotic exposures or antibiotic type. To test whether alpha diversity changed based on antibiotic exposure or drug type, I calculated Shannon diversity, and performed significance testing by pairwise Wilcoxon tests. If there was a significant difference between antibiotic types at a timepoint, I performed a similarity percentages breakdown (SIMPER) analysis (Clarke, 1993), to identify abundant ASVs that contribute most to the Bray-Curtis dissimilarity between antibiotic groups.

Results

Sample Data

At intake, I collected oral and cloaca swabs from a total of 35 Kemp's ridley turtles (Figure 2.1). Seven turtles died shortly after being admitted to the hospital, and two turtles died later in rehabilitation (after timepoint 2 samples were collected). Overall, 26 turtles had serial samples from intake to convalescence, varying from two to five timepoints depending on clinical status (Figure 2.1). Of the 35 turtles, veterinarians categorized 15 turtles as non-

pneumonia and 20 as pneumonia based on initial radiographs. Veterinarians did not prescribe antibiotics to 11 turtles (four of these died) and prescribed antibiotics to 24 turtles (five of these died). The antibiotic was either oxytetracycline (turtles with ID numbers that were even, n = 8 surviving turtles) or ceftazidime (turtles with ID numbers that were odd, n = 11 surviving turtles). Veterinarians prescribed additional antibiotics to five of the turtles that initially received ceftazidime because their disease condition was not improving (typically after one or two months of rehabilitation). These additional antibiotics varied depending on diagnostics, so they are reported as 'other' for the purpose of this study. Seven turtles that survived did not receive any systemic antibiotics.

Out of 230 oral and cloaca samples, sequencing of the 16S rRNA gene resulted in 2,309,082 reads after joining paired-end reads and quality filtering, which included the removal of chimeras, singletons, chloroplasts, mitochondrial DNA, and archaea. The mean sequence counts per sample was 10,039 (median 7,429) and range was 777 to 80,792 counts per sample. These sequences were assigned to 1,528 unique ASVs across 218 different families.

The Oral and Cloacal Microbiome at Intake

Oral samples had significantly higher Shannon diversity than cloaca samples at intake (Wilcoxon, $p = 0.000025$; oral mean 3.45 ± 0.47; cloaca mean 2.90 ± 0.52) and the oral and cloacal microbiomes were significantly different from each other at intake based on Bray-Curtis dissimilarity (PERMANOVA, $p = 0.001$), which I visualized via hierarchical clustering of the Bray-Curtis dissimilarity values (Figure 2.2). The oral microbial communities at intake were dominated by bacteria in the family Flavobacteriaceae, with a

mean abundance of 30.0%, followed by Rhodobacteraceae (13.7%), Vibrionaceae (9.0%), and Porticoccaceae (6.0%). The microbiome of cloaca samples at intake were dominated by Vibrionaceae (23.1%), Arcobacteraceae (11.8%), Shewanellaceae (7.7%), and Rhodobacteraceae (6.7%). There was significant clustering within the oral (PERMANOVA, $p = 0.035$) and cloacal (PERMANOVA, $p = 0.047$) microbiomes by survival, based on Bray-Curtis dissimilarity (Figure 2.2). Other variables that did not contribute to clustering include disease condition (pneumonia vs. non-pneumonia) and arrival day (same day as stranding or next day). The stranding location (town) had a significant influence on the cloacal microbial communities (PERMANOVA, $p = 0.008$), but not on the oral samples. There were no significant correlations between blood analytes and microbial communities for either body site at intake.

Random forest modelling predicted ASVs that differ between the turtles that survived and those that died for both oral samples (Figure 2.3A) and cloaca samples (Figure 2.3B). These analyses had an out-of-bag error rate of 22.9% and 25.7% error rates, respectively. The model correctly predicted turtle survival based on the microbiome from 26 oral samples (100%) and correctly predicted turtle mortality in only one oral sample (12%). For cloaca samples, the model correctly predicted turtle survival of 25 cloaca samples (96%) and correctly predicted turtle mortality of one cloaca sample (12%). Thus, although good at predicting survival, the model struggled to find key indicators that could be used to predict turtle mortality. The top 10 ASVs most important in distinguishing those turtles that survived included taxa from the families Flavobacteriaceae and Rhodobacteraceae for oral samples (Table 2.1). The oral samples from turtles that died were more variable in the abundance of

the important ASVs, but primarily had lower abundance of ASV1513, *Thalassobius* sp., from the family Rhodobacteraceae. For cloaca samples, the top 10 ASVs that differed between those turtles that survived and those that did not included diverse taxa from 9 different families (Table 2.1). There was high variability in the composition of these ASVs in the turtles that died, with some of the turtles having high proportions of Rhodobacteraceae, Fusobacteriaceae, and Ruminococcaceae, and lower abundance of Burkholderiaceae compared to turtles that survived. Overall, the oral microbiome was more variable in turtles that died compared to those that survived (anova, $p = 0.041$). The cloacal microbiome of turtles that died were not more variable statistically (anova, $p = 0.074$).

Temporal Shifts in Microbial Communities During Rehabilitation

There was a slight trend of increasing Shannon diversity from intake to rehabilitation samples for both the oral and cloacal microbiome (Figure 2.4). I found significant differences between TP4b (pre-release) and intake for oral samples (Wilcoxon, $p = 0.0076$), and between TP3 (in rehab) and intake for cloaca samples (Wilcoxon, $p = 0.028$). The PCoA of each body site for all samples showed significant differences between timepoints based on Bray-Curtis dissimilarity (PERMANOVA, $p = 0.001$, Figure 2.5). The largest separation along the principal axis resulted from the difference between intake samples and the remaining samples (Figure 2.5). After intake, the turtles were in a shared environment at a consistent temperature, so I re-evaluated shifts in microbial communities during rehabilitation by excluding the intake samples (Figure 2.6). The microbial communities of both oral and cloaca samples continued to shift based on the number of days in rehabilitation (PERMANOVA, $p = 0.001$, Figure 2.6). The shift appeared to stabilize after approximately

100 days, with less variability in the microbiome the longer the turtles were in rehabilitation (Figure 2.6). It is important to note that these shifts during rehabilitation were consistent among turtles that received antibiotics and those that did not.

Antibiotic Effects During Rehabilitation

I observed changes in turtle microbiomes due to antibiotic exposure at specific timepoints during rehabilitation. At timepoint 2 (approximately four weeks after the start of antibiotic treatment, for those turtles that received antibiotics), there was a significant difference by antibiotic type based on Bray-Curtis distance for cloaca samples, but not for oral samples (Figure 2.7). Turtles not receiving antibiotics, as well as those on specific antibiotic types (ceftazidime or oxytetracycline), had distinct cloacal microbial communities (PERMANOVA, $p = 0.001$). SIMPER analysis indicated that the families Bacteroidaceae, Enterobacteriaceae, and Pseudomonadaceae were more abundant in cloaca samples of turtles receiving oxytetracycline (Figure 2.8). Vibrionaceae was more prevalent in turtles not receiving antibiotics, while Flavobacteriaceae was more abundant in the turtles receiving ceftazidime (+/- other). Shewanellaceae was consistently present in turtles that received oxytetracycline and in turtles that received no antibiotic.

Bray-Curtis distances at convalescence, which was 30 days after the antibiotic was discontinued, were significantly affected by antibiotic type among both oral and cloaca samples (PERMANOVA, oral $p = 0.013$, cloaca $p = 0.004$, Figure 2.9). SIMPER analysis indicated that turtles receiving any type of antibiotics had oral samples with higher abundance of an ASV in the family Microscillaceae, *Microscilla* sp. Oral samples of turtles that never received antibiotics had higher prevalence of Saprospiraceae (*Aureispira* sp.), and

turtles that received antibiotics in addition to ceftazidime (ceftaz, other) had higher abundance of ASVs specific to the families Flavobacteriaceae (*Maritimimonas* sp.), Rubritaleaceae (*Rubritalea* sp.), and Kangiellaceae (*Aliikangiella* sp.), all of which are marine bacteria. The cloaca samples from turtles on antibiotics had an increased presence of the Vibrionaceae *Photobacterium* sp. (more consistent with turtles that never received antibiotics), but the turtles that had other antibiotics in addition to ceftazidime also had some samples with higher abundance of Fusobacteraceae (*Fusobacterium* sp.). All of the ASVs in these families, except for Vibrionaceae, made up a low relative abundance compared to other ASVs in the same families.

Oral and Cloacal Microbiome at Convalescence

At convalescence, I saw that Bray-Curtis distance remained significantly different between oral and cloaca samples (PERMANOVA, $p = 0.001$, Figure 2.9). Since convalescent samples were different from intake samples, I also wanted to characterize the differences between body sites prior to release (Figure 2.9). The oral microbial communities at convalescence were dominated by bacteria in the family Flavobacteriaceae (22.5%) and Rhodobacteraceae (20.6%) followed by an unassigned Gammaproteobacteria family (12.1%) and Saprospiraceae (10.8%). The microbiome of cloaca samples at convalescence were dominated by Flavobacteriaceae (17.0%), Vibrionaceae (13.6%), Arcobacteraceae (10.3%), and Rhodobaccteraceae (9.1%). The Shannon diversity of oral microbial communities remained higher compared to the cloaca samples at convalescence (Wilcoxon, $p = 0.00065$; oral mean 3.64 ± 0.39; cloaca mean 3.19 ± 0.44).

Discussion

I characterized the oral and cloacal microbiomes of cold-stunned Kemp's ridley turtles through the course of rehabilitation, from stranding to release. This is the first investigation of the microbiome of this species, and the first investigation of the microbiome during rehabilitation after cold stunning for any turtle species. In Kemp's ridley turtles, the oral and cloacal microbial communities were distinct from each other in composition and taxonomic diversity when they arrived at the rehabilitation center and through the course of treatment. The lower Shannon diversity of cloaca samples compared to oral samples is not unique to Kemp's ridley sea turtles and is thought to be due to greater interaction with transient microbes from the environment that enter the turtle via the oral cavity (Bik et al., 2015; Hyde et al. 2016).

Compared to wild Kemp's ridley turtles, the oral microbiome of cold-stunned turtles at intake share similar predominant bacterial families, including Flavobacteraceae and Rhodobacteraceae (McNally et al., in preparation). The cloaca samples, by contrast, were different between the healthy wild Kemp's ridley turtles and the cold-stunned stranded turtles with no shared dominant families between the two groups (McNally et al., in preparation). Cardiobacteraceae, Flavobacteraceae, and Neisseriaceae were most prevalent in the wild turtles, while Vibrionaceae, Arcobacteraceae, Shewanellaceae, and Rhodobacteraceae were prevalent from the intake cloaca samples of cold-stunned turtles. This difference in cloaca samples could be due to diet influences between the regions, last meal for the cold-stunned turtles (which tend to be malnourished due to stranding), or the more integrated relationship of immune system with gastrointestinal microbes.

Cold-stunned turtles strand with a variety of clinical derangements. Plasma biochemical and hematologic analyses are important for evaluating the health and monitoring the recovery of these animals (Innis and Staggs, 2017). I did not find a correlation of blood analytes with the microbiome of either the cloaca or oral cavity of cold-stunned turtles. This was unexpected since blood parameters are useful in diagnosing diseases, metabolic disorders, and immunological disorders that have been linked to the microbiome (Althani et al., 2015; Dickson et al., 2016; Moffatt and Cookson, 2017). Some blood analytes of turtles had great variability at intake, which could be one reason for lack of correlation with microbial communities. For example, glucose at admission was highly variable, with hypoglycemia likely indicating exhaustion, anorexia, or sepsis, and hyperglycemia indicating a stress response, liver disease, or pancreatic disease (Innis et al., 2007; Innis et al., 2009b; Stacy and Innis, 2017). White blood cell counts also can be indicative of several conditions including inflammation, immune response, or systemic pathologic conditions (Innis et al., 2009b), but were variable in these turtles at stranding resulting in no clear association with the microbiome. Blood pH, pCO_2, pO_2, and potassium concentrations in particular are good predictors of mortality in cold-stunned Kemp's ridley turtles (Stacy et al., 2013), yet they were also not associated with microbial communities. Future studies focusing on specific immune assays of Kemp's ridley sea turtles in addition to these traditional blood analytes might provide further insight into the relationship between the microbiome and the immune system for this species.

Although the clinical parameters and disease conditions did not strongly predict the microbiomes of cold-stunned turtles at intake, I was able to identify ASVs that were different

between turtles that survived and those that died. For example, ASV1044, from the genus *Phaebacter*, was found in higher abundance in initial cloaca samples of turtles that died (Figure 2.3). This genus is an antibiotic producing bacterium that is found in sea water and on marine surfaces (Breider et al., 2017). *Phaebacter* sp. strongly shaped the microbiome of microalga by changing the proportions of other metabolite producing bacteria such as *Vibrio* sp. (Dittman et al., 2019), so this genus could play an important role in altering the sea turtle microbial communities. Cloaca samples of turtles that died also had higher abundance of ASVs in families that are common to marine environments such as Shewanellaceae and Rhodobacteraceae. The oral microbiome of turtles that died were also more variable in composition compared to turtles that survived due to their more severe debilitated state. This may be an indication of dysbiosis in which more diseased animals show greater variability in microbial composition (Dickson et al., 2016; Zaneveld et al., 2017). Increased beta diversity has also been associated with coral mortality, in which above average temperatures caused increases in various opportunistic microbes leading to stochastic changes (Zaneveld et al., 2017). Although all stranded cold-stunned turtles are not considered healthy, the turtles that died still had more variability than the those that survived. Thus, mortality may be further associated with stochastic changes rather than specific pathogens.

The turtles that survived had microbiomes that shifted through rehabilitation as they stabilized in a controlled environment and recovered from cold-stunning. The local environment shapes distinct microbial communities as seen in other reptiles and aquatic animals (Kueneman et al., 2014; Bik et al., 2015; Yuan et al., 2015; Hyde et al., 2016). Shannon diversity was also lower in the intake samples compared to later timepoints in

rehabilitation, further suggesting a dysbiosis resulting from cold-stunning (Figure 2.4). Initially, turtles may remain inappetent for days to weeks after stranding, which could cause differences in the microbiome until they are eating consistently (Costello et al., 2010; Keenan et al., 2013). Other reptiles had lower alpha diversity during periods of fasting compared to during the feeding season (Costello et al., 2010). Thus, disease recovery and feeding status may be variables that further shift the oral and cloacal microbiome during rehabilitation. There are also changes in the number of turtles in the tanks during the first few months of rehabilitation, which influences the bioload of the system in turn adding additional variables that could influence microbial community composition. During the first few months of rehabilitation, cold stunned turtles are in various states of disease and drug exposure. It may take several months before turtles recover from cold-stunning, and this appears to apply to their microbiome as well.

In addition to microbiome changes over time, I found changes due to antibiotic exposure, but not as many changes as might be expected based on what is known about the effect of antibiotics in other animals. For example, I did not see the typical reduction in Shannon diversity of turtles on antibiotics versus those not on antibiotics (Jakobsson et al., 2010; Willing et al., 2011), but I did find differences in beta diversity (Figure 2.7). The ASVs that were more abundant in cloaca samples of turtles receiving oxytetracycline were from the families Pseudomonadaceae, Vibrionaceae, and Enterobacteriaceae. Although these are all bacterial families targeted by the drug, antibiotic resistance and transient microbes continuously passing through the gastrointestinal tract cause them to remain present in the microbiome. I may be capturing transient microbes that are constantly being introduced by

the local environment and food, thus antibiotics would not affect their presence in the samples collected. Ceftazidime and oxytetracycline are so commonly used in sea turtles that antibiotic resistance is a concern at rehabilitation facilities (Innis et al., 2017). Although antibiotic resistance was outside the scope of what I investigated, bacteria in the families Pseudomonadaceae and Enterobacteriaceae have shown high resistance to tetracycline classes of antibiotics, such as oxytetracycline, in sea turtles (Ahasan et al., 2017c; Pace et al., 2019). There were also some strains that were resistant to ceftazidime, though the majority were susceptible in loggerhead turtle cultures (Pace et al., 2019). In addition to some bacterial families that were present despite being targets of the antibiotics, several ASVs were reduced in the cloaca samples of turtles that received antibiotics compared to those that did not, revealing that the antibiotics were having an influence despite transient microbes and potential antibiotic resistance. Vibrionaceae, specifically the ASV matching to *Photobacterium damselae* which is a marine bacterium capable of causing infection in animals (Lee et al., 2018), was more prevalent in turtles that did not receive antibiotics. *Shewanellae algae* was identified as the ASV in the Shewanellaceae family that had lower abundance in the cloaca of turtles that received ceftazidime, showing the effect these antibiotics have on a variety of bacteria.

During rehabilitation, antibiotics did not affect the oral microbiome as much as they affected the cloacal microbiome. Different body sites may be affected by antibiotics in different ways. The salivary microbiome of humans was not affected by antibiotics although fecal microbial communities were highly affected (Zaura et al., 2015). Route of administration (injectable or oral) may also play a role, especially depending on drug

excretion routes (Zhang et al., 2013). Drugs such as ceftazidime and oxytetracycline are excreted through the kidneys (Innis et al., 2017), therefore there may be minimal drug exposure of the oral cavity and gut if delivered by injection (Zhang et al., 2013).

At convalescence, the oral and cloacal microbiomes were both different based on antibiotic type (Figure 2.9). Although there are no analogous studies in turtles, in humans, throat and gut microbiomes were also both perturbed by antibiotics, but each subject responded uniquely (Jakobsson et al., 2010). The time of recovery to a pre-treatment state varied as well, ranging from weeks to several years in humans (Jakobsson et al., 2010). There were several differences in composition between convalescent microbial communities and intake samples for each body site of turtles. Flavobacteriaceae became the most abundant bacterial family of the cloaca at convalescence and remained the most abundant in oral samples. Oral samples also had higher proportions of the marine environmental bacteria Saprospiraceae and an unknown family of Gammaproteobacteria at convalescence. Differences from intake cloaca samples to convalescence were seen in green turtles as well, and the presence of *Salmonella* in convalescent samples specifically indicated introduction from the hospital tanks (Ahansan et al., 2017a). I did not see *Salmonella* in our samples, although I did have the closely related *Citrobacter* sp., which is also a coliform bacterium from the family Enterobacteraceae, but this was present in low abundance in both the intake and convalescent samples indicating the hospital environment was not the primary source for this family of bacteria. In addition to the local environment, captivity also plays a role in altering microbial communities due to diet, so mimicking the wild is important to maintaining proper functioning upon release (Gibson et al., 2019; Sun et al., 2019). During

rehabilitation, Kemp's ridley turtles are fed high calorie herring and squid compared to the crustaceans they eat in the wild. Shifting the diet to crustaceans during rehabilitation may aid in restoring normal cloacal microbial communities, as was suggested for green turtles transitioning to an herbivorous diet as soon as possible in rehabilitation (Bloodgood et al., 2020). Further evaluating diet as well as the functional microbiome might be a useful method of future studies to determine differences between wild healthy turtles and captive turtles prior to release.

Conclusions

I characterized the oral and cloacal microbiome of cold-stunned Kemp's ridley turtles throughout rehabilitation, allowing me to investigate differences in microbial communities based on survival and disease condition. I sampled at multiple timepoints in rehabilitation, from admission to the hospital, during rehabilitation, and at convalescence, providing first glimpses into the changes that occur during recovery from cold-stunning. I identified ASVs that are important to predicting survival or mortality of turtles after stranding. An important contributing variable to microbial communities is exposure to antibiotics, which I investigated as well and found that antibiotics did lead to an altered state. My findings indicate that the microbiome of cold-stunned Kemp's ridley turtles is affected by disease status, the local environment, and antibiotics, all of which ultimately play a role in the recovery and release status of the turtles.

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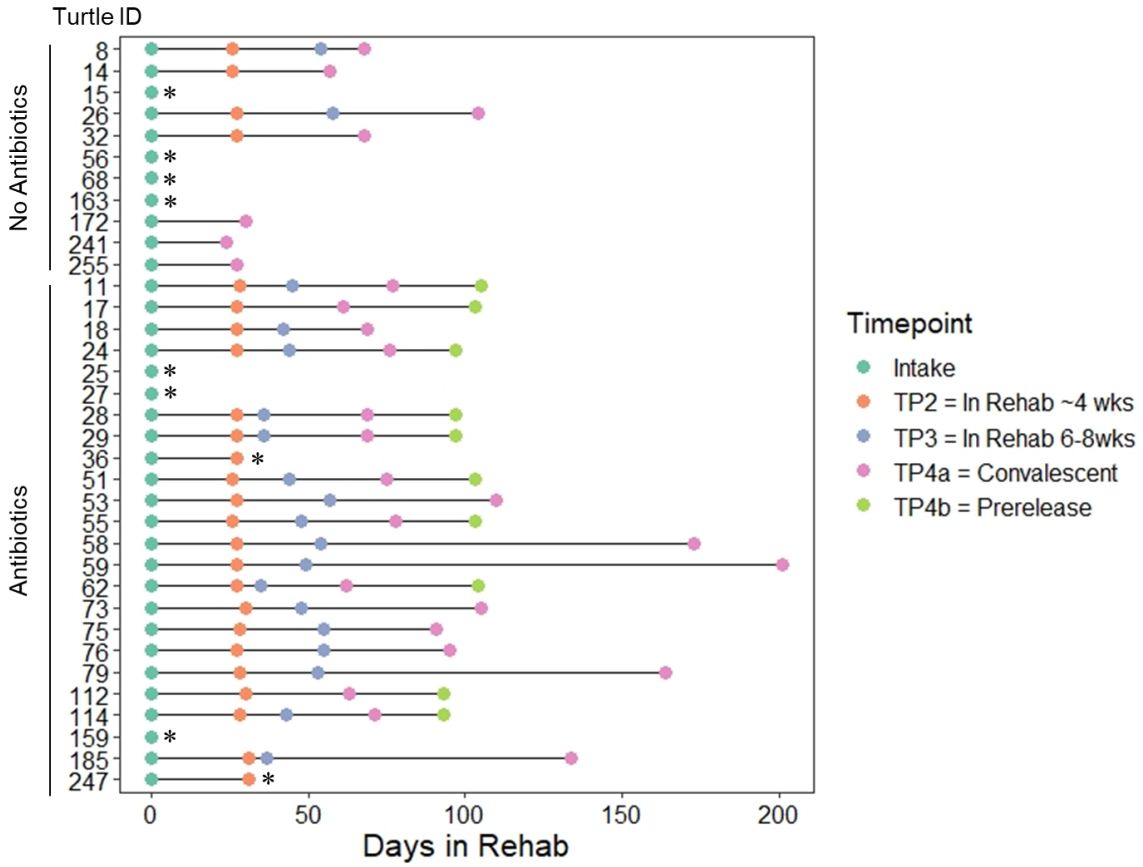

Figure 2.1. Oral and cloaca samples collected for each individual turtle during rehabilitation. Turtle identification numbers are listed on the y-axis. Each color denotes the timepoint in rehab. Convalescence (TP4a) is when the turtle is considered clinically healthy. Prerelease (TP4b) was only collected on turtles that remained in the hospital >30 days after TP4a was collected. Whether antibiotics were received or not is indicated on the y axis. * indicates that the turtle died after that sample was collected.

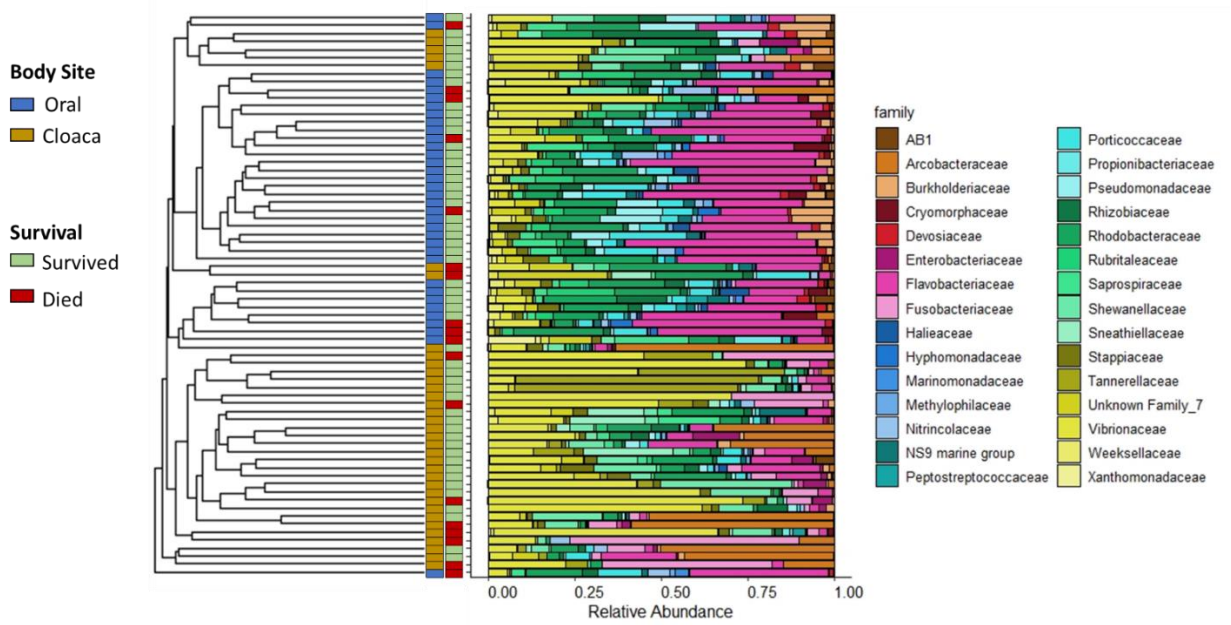

Figure 2.2. Hierarchical clustering of intake samples with corresponding stacked bar plots. Taxa plots represent the relative abundance of community composition at the family level (top 30 bacterial families). Colored bars at the center specify body site and survival.

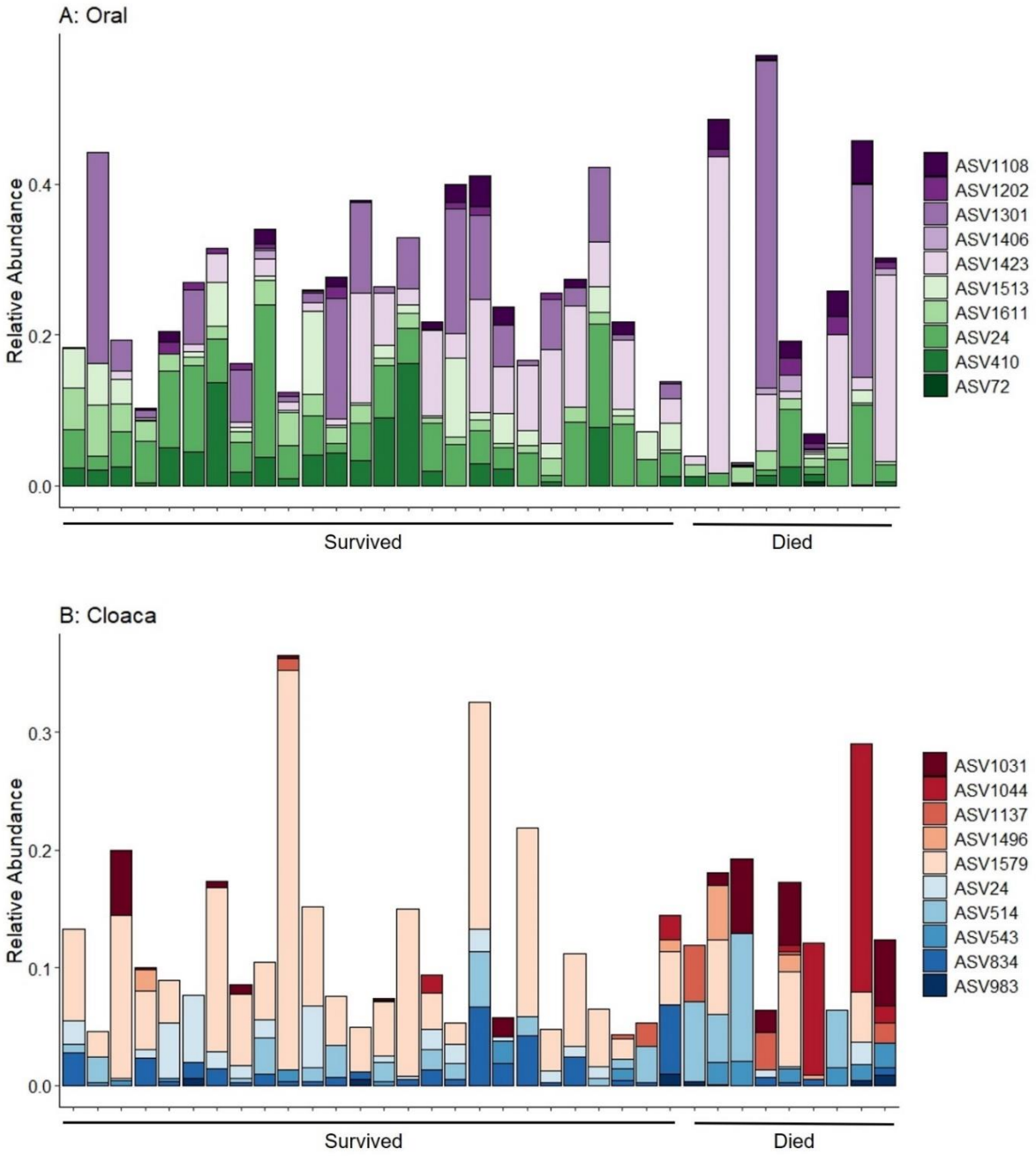

Figure 2.3. Random forest model predictions. The top 10 most significant ASVs at intake that differ between turtles that survived and those that died for oral samples (A: top) and cloacal samples (B: bottom). Taxonomy information for these ASVs are provided in Table 2.1.

Table 2.1. Taxonomy of the top 10 ASVs predicting survival by random forest modelling. Table is separated by oral (top) and cloaca (bottom) samples at intake.

ORAL	**Taxonomy**			
ASV	**Class**	**Order**	**Family**	**Genus**
ASV24	Gammaproteobacteria	Cellvibrionales	Porticoccaceae	*Porticoccus*
ASV72	Gammaproteobacteria	Arenicellales	Arenicellaceae	HTCC5015
ASV410	Bacteroidia	Flavobacteriales	Flavobacteriaceae	*Aquimarina*
ASV1108	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	NA
ASV1202	Bacteroidia	Flavobacteriales	Flavobacteriaceae	NA
ASV1301	Bacteroidia	Flavobacteriales	Flavobacteriaceae	*Kordia*
ASV1406	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	*Pseudophaeobacter*
ASV1423	Gammaproteobacteria	Vibrionales	Vibrionaceae	NA
ASV1513	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	NA
ASV1611	Alphaproteobacteria	Rhizobiales	Stappiaceae	NA

CLOACA	**Taxonomy**			
ASV	**Class**	**Order**	**Family**	**Genus**
ASV24	Gammaproteobacteria	Cellvibrionales	Porticoccaceae	*Porticoccus*
ASV514	Gammaproteobacteria	Vibrionales	Vibrionaceae	*Photobacterium*
ASV543	Gammaproteobacteria	Alteromonadales	Shewanellaceae	*Shewanella*
ASV834	Gammaproteobacteria	Betaproteobacteriales	Burkholderiaceae	NA
ASV983	Alphaproteobacteria	Rhodospirillales	Rhodospirillaceae	*Candidatus Riegeria*
ASV1031	Fusobacteriia	Fusobacteriales	Fusobacteriaceae	*Cetobacterium*
ASV1044	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	NA
ASV1137	Clostridia	Clostridiales	Ruminococcaceae	NA
ASV1496	Campylobacteria	Campylobacterales	Arcobacteraceae	*Arcobacter*
ASV1579	Gammaproteobacteria	Alteromonadales	Shewanellaceae	*Shewanella*

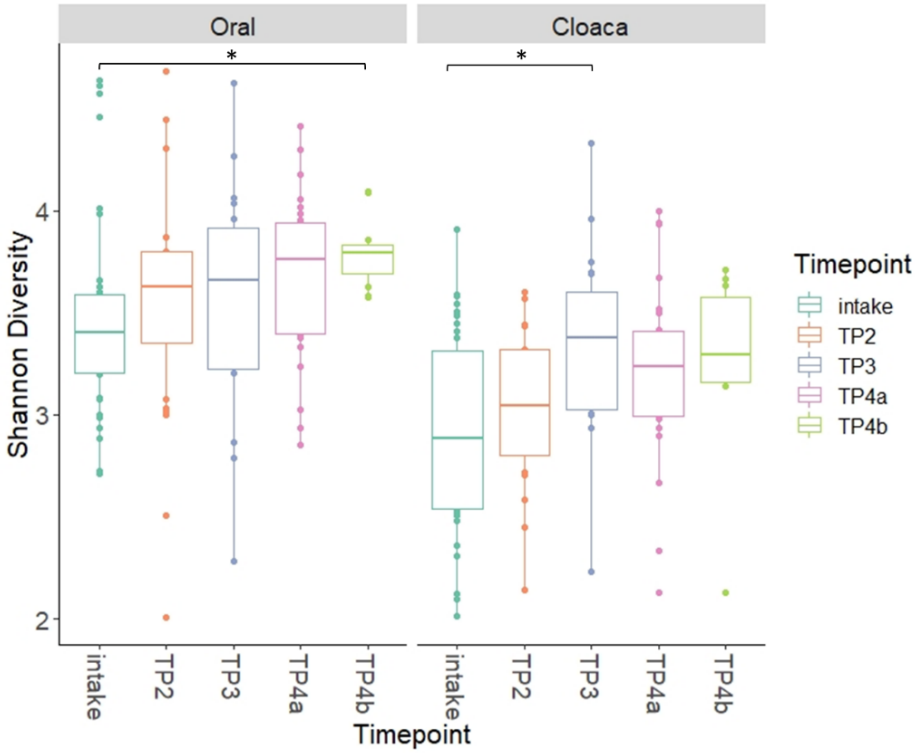

Figure 2.4. Shannon diversity index. Oral samples (left) and cloaca samples (right) are shown at each timepoint during rehabilitation. * indicates $p < 0.05$ (Wilcoxon test)

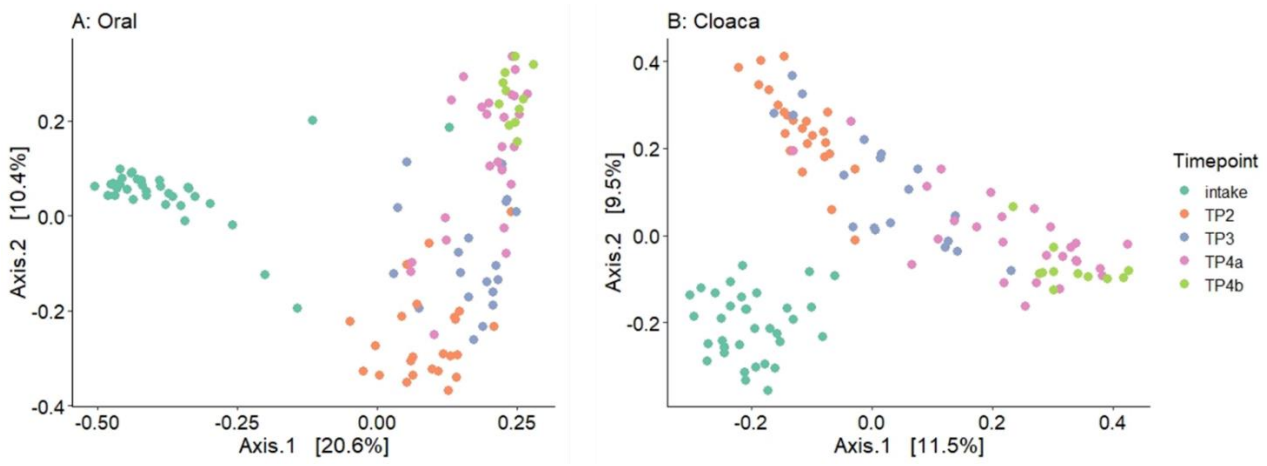

Figure 2.5. PCoA plots across all timepoints. Oral samples (A) and cloaca samples (B) are based on Bray-Curtis distance. Color indicates timepoint in rehabilitation from intake to convalescence (TP4a)/pre-release (TP4b).

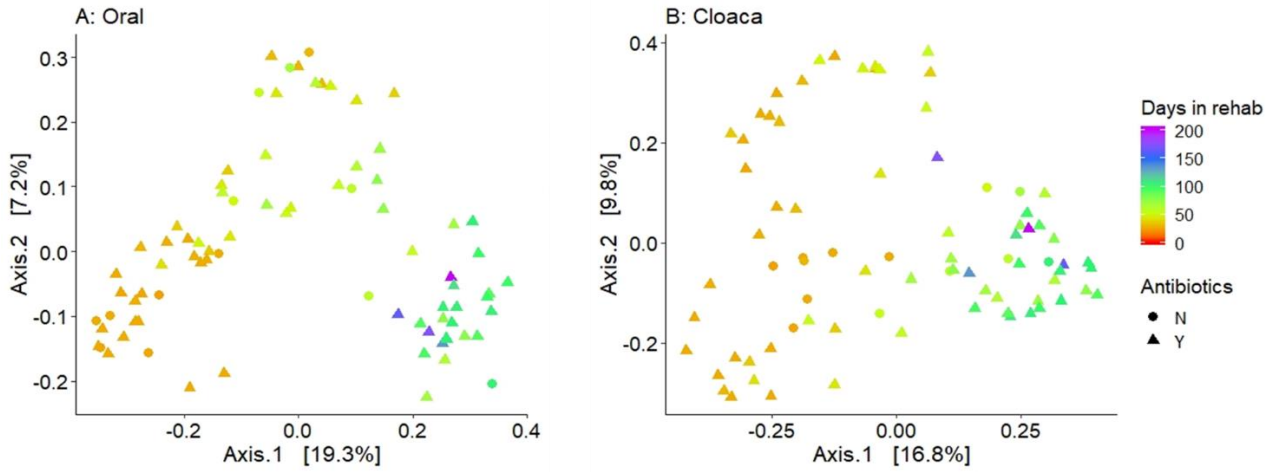

Figure 2.6. PCoA plots during rehabilitation. Oral samples (A) and cloaca samples (B) are based on Bray-Curtis distance. Color indicates number of days in rehabilitation. Shape indicates whether a turtle was not on antibiotics (circle, N) or on antibiotics (triangle, Y) during rehabilitation.

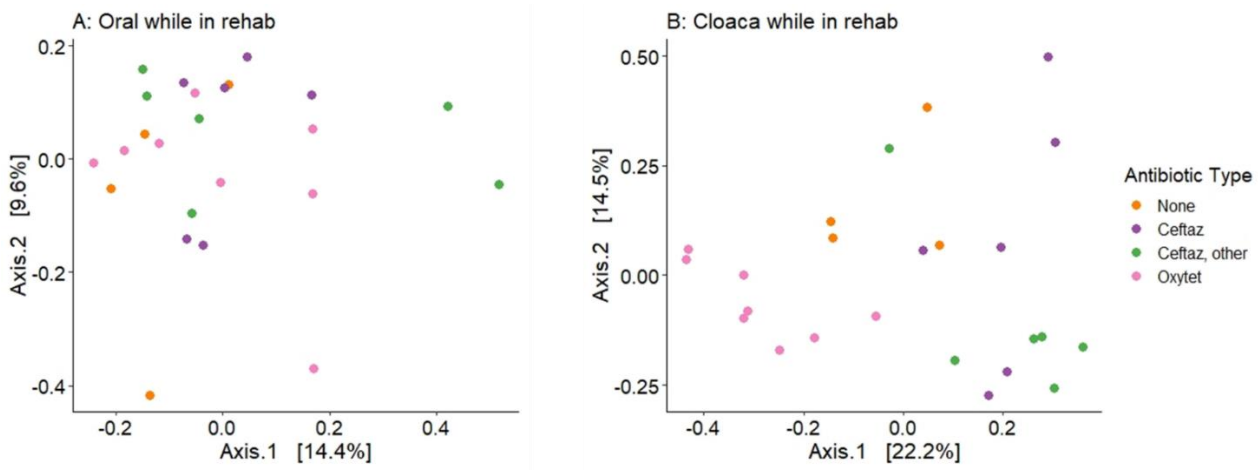

Figure 2.7. PCoA plots for timepoint 2 (in rehabilitation). Oral (A) and cloaca (B) samples are based on Bray-Curtis distance. Colors specify the type of antibiotic the turtle was on during hospitalization.

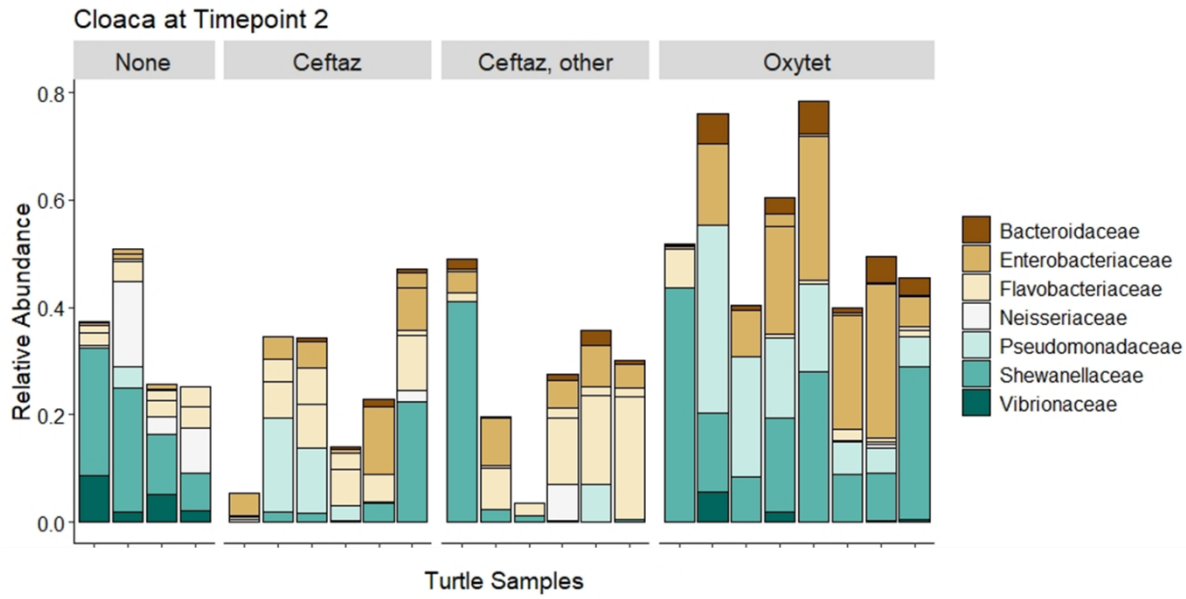

Figure 2.8. Relative abundance of ASVs that significantly contribute to the differences between antibiotic type of cloaca samples while in rehabilitation. Taxonomy is colored by family.

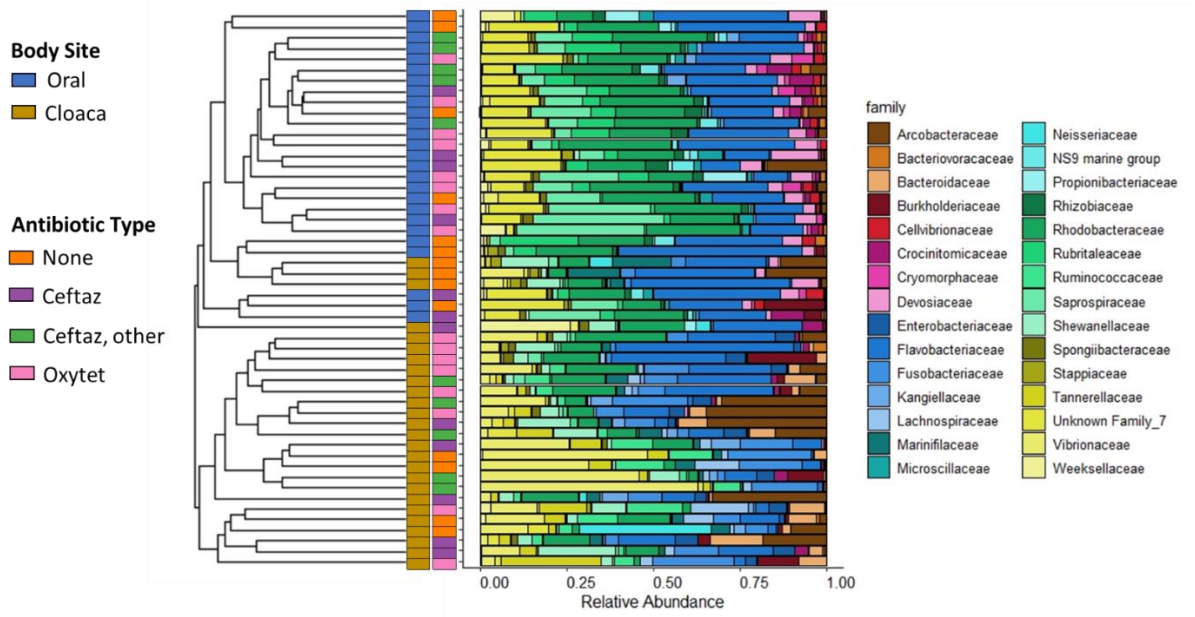

Figure 2.9. Hierarchical clustering of convalescent samples (timepoint 4a) with corresponding stacked bar plots. Taxa plots represent the relative abundance of community composition at the family level (top 30 bacterial families). Colored bars at the center specify body site (1st bar) and antibiotic type (2nd bar).

CHAPTER 3

EVALUATION OF THE RESPIRATORY MICROBIOME TO ASSESS TRACHEAL WASHES AS A DIAGNOSTIC TOOL IN COLD-STUNNED KEMP'S RIDLEY SEA TURTLES (*LEPIDOCHELYS KEMPII*)

Abstract

Over half of the cold-stunned Kemp's ridley turtles (*Lepidochelys kempii*) that strand annually in the northeastern United States have radiographic lung abnormalities and are presumed to have pneumonia. Although culture-dependent methods are typically used to characterize microbes associated with pneumonia and to determine treatment, culture-independent methods could provide a deeper understanding of pathogenesis and lead to more accurate diagnosis and enhanced treatment outcomes. In this study, I characterized the tracheal wash microbiome from cold-stunned Kemp's ridley turtles at three timepoints during rehabilitation (intake, rehab, convalescence) by analyzing the 16S rRNA gene collected from tracheal washes. A radiographic scoring system was developed to grade the severity of lung abnormalities in these turtles and I found no differences in diversity or composition of microbial communities based on score of radiographic lung abnormalities. I

also found that the culture isolates from tracheal washes of turtles with pneumonia and other previously reported sea turtle pathogens were present in variable abundance across all sequenced samples and were entirely absent in some samples. In addition to tracheal washes, I characterized microbial communities from other segments of the respiratory tract (glottis, trachea, anterior lung, posterior lung) from deceased turtles, and I found a high degree of variability within turtles and a high degree of dissimilarity between the different segments of the respiratory tract and the tracheal wash collected from the same turtle. In summary, I found that pneumonia pathogenesis in cold-stunned sea turtles is complex and does not correlate with the microbial community of the tracheal washes, underscoring the limitations of using tracheal washes as a diagnostic tool in this disease.

Introduction

Juvenile Kemp's ridley turtles, a critically endangered species, strand annually in Cape Cod Bay, Massachusetts, USA from cold-stunning, or hypothermia, when water temperatures drop during autumn (Morreale et al., 2005; Still et al., 2005; Griffin et al., 2019; Wibbels and Bevan, 2019). Wildlife rehabilitation hospitals such as the New England Aquarium (NEAq) admit live cold-stunned turtles for triage and rehabilitation with the goal of returning healthy animals back to the wild. Cold-stunned Kemp's ridley turtles present with a wide range of pathologic findings including cardiorespiratory depression, dehydration, sepsis, reduced renal function, and death (Innis et al., 2007, 2009a, 2009b, 2014, 2016; Keller et al., 2012; Kennedy et al., 2012). Pneumonia is extremely common, with an average of half of the Kemp's ridley turtles per cold-stun event presenting with respiratory abnormalities during rehabilitation at NEAq (Innis et al., 2009a; Stockman et al., 2013).

Since respiratory abnormalities have a high prevalence in cold-stunned Kemp's ridley turtles in rehabilitation, veterinary staff use a variety of diagnostic methods to appropriately characterize and identify the causative microbes (bacteria and/or fungi) to determine the appropriate course of medical intervention. Initial diagnosis of pneumonia typically relies on radiographic evidence of lung abnormalities by identifying patterns of radiopacities, which may increase with inflammation, edema, and fibrosis (Stockman et al., 2013; Boylan et al., 2017). The radiographic views most useful in evaluating the lungs are whole body dorsoventral view (vertical beam), craniocaudal view (horizontal beam), and lateral view (horizontal beam). Dorsoventral projections are useful in identifying radiopacities, but lateral and craniocaudal views limit superimposition of visceral organs allowing further characterization of distribution, such as changes that are diffuse or ventral in the lungs (Boylan et al., 2017; Pease et al., 2017). Abnormalities found on radiographs that typically indicate pneumonia can include focal, multifocal, and generalized interstitial patterns to a reticular (honeycomb) pattern (Stockman et al., 2013), all of which help veterinarians identify the severity and location of infection.

Tracheal lavage, or "tracheal wash", is a diagnostic tool that is commonly used in animals, including sea turtles, for characterizing pneumonia via cytology and culture (Murray, 2006; Boylan et al., 2017). The tracheal wash process involves infusing sterile saline into the trachea or lungs (typically 0.5 to 1.0% of the body weight, although less is common and adequate), which then comes into contact with the biofilm and epithelial cells of the respiratory tract so that when it is gently suctioned out, the sample can be cultured to identify potential causative microbes (Boylan et al., 2017). Culture results from tracheal

washes guide treatment based on the bacteria and/or fungi that are isolated and the associated antibiotic susceptibility testing. Although diagnosis via this method can result in successful rehabilitation, it is not known whether the tracheal washes are truly representative of the microbes responsible for causing pneumonia. Characterizing the microbial communities of the respiratory system may help us to better understand the causes of respiratory disease, as well as the reliability of common diagnostic methods such as tracheal washes in sea turtles.

Research on the human respiratory microbiome over the past decade recognizes the importance of microbial communities in health and chronic respiratory diseases, including pneumonia (Dickson et al., 2013; Dickson et al., 2014a). Microbial communities that inhabit healthy individuals become altered in taxonomic identity, diversity and richness as healthy lungs transition to various disease states (Dickson et al., 2016). Several factors influence the lung microbiome, including microbes immigrating to the lungs through aspiration or inhalation, microbial emigration through host immune defenses, and growth conditions for community members, such as temperature (Dickson and Huffnagle, 2015; Martin et al., 2015; Dickson et al., 2016; Ho Man et al., 2017). Respiratory disease alters the composition of the lung based on these factors, and diseased lungs vary from patient to patient more so than healthy individuals (Dickson et al., 2016; Zaneveld et al., 2017). Lung diseases of humans that are associated with microbial dysbiosis include asthma, chronic obstructive pulmonary disease (COPD), and cystic fibrosis (CF). Research shows that environmental factors play a role in asthma, such that exposure to a rich and diverse environmental microbial community early in life can protect against the onset of the disease (Martin et al., 2015; Dickson et al., 2016; Moffatt and Cookson, 2017). Alterations of the microbiota in

asthma patients include higher proportions of *Haemophilus* and *Neisseria* spp., which are potential pathogens, and lower numbers of commensal species such as *Prevotella* and *Veillonella* spp. (Moffatt and Cookson, 2017). Similar to asthma patients, COPD patients' symptoms may be exacerbated by the presence of *Haemophilus influenzae* (Martin et al., 2015; Moffatt and Cookson, 2017). Changes in respiratory microbial communities, and the interaction of microbes along the respiratory tract can influence the complex pathogenesis of diseases such as pneumonia (Dickson et al., 2014a). Identifying potential pathogens and important commensal species in these complex systems can lead to a better understanding of diseases and treatment options.

Microbes also play a role in respiratory health and disease in non-human vertebrates, including sea turtles (Vital et al., 2015; Lima et al., 2016; Klima et al., 2019; Vientós-Plotts et al., 2019). Mice are a commonly used model organism for studying human diseases, so the murine respiratory microbiome is the focus of most respiratory microbiome research, including the effects of alterations to the gut microbiome on allergic airway disease and understanding the lung microbiome in relation to other body sites such as the intestine (Barford et al., 2013; Vital et al., 2015). Respiratory microbiome research focusing on non-human diseases is also important. For example, bovine respiratory diseases can be identified by analysis of bacterial pathogens present in the upper respiratory tract or lower respiratory tract through bronchoalveolar lavage (BAL; Lima et al., 2016; Klima et al., 2019). The causes of pneumonia in dogs are better understood based on sequencing results versus standard culture, with BAL microbial communities demonstrating distinct signatures based on pneumonia type (i.e. overgrowth of a single microbe in community-acquired pneumonia).

Taxa identified via cultured isolates from canine BAL often differed from the dominant taxa in sequencing data (Vientós-Plotts et al., 2019).

Studying the healthy lung is important to understanding the composition, diversity, and variation of the microbial communities of the respiratory system. In sheep, there is spatial variation among the lung microbial communities, with microbiome differences based on depth in the respiratory tract, or distance from the glottis (Glendinning et al., 2016). The marine mammal respiratory microbiome has been evaluated extensively by sampling blow (respiratory vapor ejected during an exhale through the blowhole), and some studies evaluated a swab of the blowhole (Lima et al., 2012; Nelson et al., 2015; Bik et al., 2016; Apprill et al., 2017; Raverty et al., 2017). These results indicate a core, or shared, microbiome within a specific whale species as well as a core across multiple whale species, suggesting some microbes are specialized to marine mammals as a group, which can provide useful information for monitoring their health (Nelson et al., 2015; Apprill et al., 2017).

Different diagnostic tools will provide different results for both culture-dependent and culture-independent methods. In cattle, tracheal washes had drastic differences in cytological findings compared to BAL due to the location of the respiratory tract sampled (trachea vs bronchioles), and tracheal washes had higher nasopharyngeal contamination (Abutarbush et al., 2019). Further, cultures of deep oral swabs are not appropriate surrogates to tracheal washes in dogs, as each resulted in different isolates (Sumner et al., 2011).

There may be some limitations in the use of culture in diagnosing pneumonia as well (Dickson et al., 2014b). Less than 1% of bacteria in nature can be cultured or grown in media (Ritz, 2007; Stewart, 2012) due to a lack of knowledge of the specific growth requirements

of most microbes, as well as to difficulty in replicating environmental conditions in the laboratory (Stewart, 2012). This is true for host associated microbes as well. Standard medical culture media only identifies 70% of bacteria in the human body, leading to difficulty in diagnosing potential pathogens (Dickson et al., 2013). For example, 75% of humans diagnosed with pneumonia have no specific pathogen identified, creating difficulty in treating the infection when drug sensitivity cannot be ascertained (Dickson et al., 2013).

In this study, I investigated the respiratory microbiome of cold-stunned Kemp's ridley turtles with and without lung abnormalities throughout the duration of their rehabilitation. My first objective was to characterize the turtles' tracheal wash microbiome. I hypothesized that turtles with lung abnormalities (i.e. pneumonia) would have a distinct microbial community in tracheal wash samples from turtles with no lung abnormalities. The conventional view in veterinary medicine is that an overgrowth of a pathogenic bacteria causes infection, so I hypothesized that the microbial community of turtles that present with signs of pneumonia have a lower overall diversity due to a higher abundance of one or a few specific bacteria associated with the disease. Additionally, I compared culture-dependent and culture-independent methods to determine whether different conclusions about animal health can be drawn from diagnosis based on tracheal wash cultures compared to high throughput sequencing of the microbial community. I hypothesized that the culture results of tracheal washes were not truly capturing potential causative agents for pneumonia in turtles. I also characterized microbial communities in other locations along the respiratory tract of deceased cold-stunned turtles and compared them to the tracheal wash fluid from the same turtles. I hypothesized that the tracheal wash fluid was similar to the trachea microbiome, but

distinct from the lungs, therefore tracheal washes may not be the most valuable diagnostic tool in characterizing pneumonia in cold-stunned sea turtles. Finally, I identified the prevalence of previously identified sea turtle pathogens in the tracheal wash microbiome. Evaluating the whole microbial community in tracheal wash samples could demonstrate the utility of the method in diagnosing pneumonia in sea turtles when it is placed in the context of disease severity, corresponding culture results, and when compared to other locations along the respiratory tract.

Methods

This study was approved by the NEAq Institutional Animal Care and Use Committee (Protocol #2015-16) and conducted under the US Department of the Interior Fish and Wildlife Service Permit # TE-697823.

Sample Collection

Kemp's ridley sea turtles were admitted to NEAq during the 2015 cold-stun event (November and December 2015). I chose turtles at random to have radiographs taken on the day of admission to assess for lung abnormalities. Radiographic results dictated whether the turtle was enrolled in the study, with the goal of having an approximately equal number of turtles with radiographically normal lungs and those with evidence of pneumonia. One of three attending veterinarians categorized the turtle as having pneumonia or not having pneumonia (hereafter referred to as "non-pneumonia") based on their interpretation of the radiographs.

I conducted tracheal washes on the day of admission ("Intake") without sedation, as turtles were hypothermic and minimally reactive. Tracheal washes were performed using the

following process. First, I intubated the trachea of the turtle with a Cole-style endotracheal tube (Jorgensen Labs, Loveland, CO) that was selected based on the size of the turtle. Then I inserted a sterile 5 French (1.7 mm) diameter red rubber catheter as far as possible into the endotracheal tube. I infused five mL of sterile saline through the catheter while the turtle was gently rocked side to side to promote fluid contact with the lung tissue. I gently suctioned back the saline with a syringe, recovering a range of two to four mL. If an animal had been classified as a pneumonia turtle, I aliquoted a portion of the fluid and debris into three empty sterile vials. If an animal had been classified as a non-pneumonia turtle, I saved two vials. For pneumonia turtles, one vial was submitted for aerobic, anaerobic, fungal, and mycobacterial cultures to a commercial veterinary diagnostic laboratory (IDEXX Laboratories, North Grafton, MA). I immediately placed the remaining vials of tracheal wash material on dry ice after collection and moved them to an ultra-low freezer (-80˚C) within 15 minutes of collection for DNA extraction and sequencing at a later date.

NEAq veterinarians prescribed antibiotics for the turtles as necessary based on radiographic findings and blood analysis. I sampled surviving turtles at two additional timepoints during the rehabilitation process. The “Rehab” sample was collected approximately eight weeks after admission but, in some cases, was conducted as early as six weeks after admission to ensure the sample was collected prior to the discontinuance of antibiotics. The “Convalescent” sample was collected when the turtle was classified by the attending veterinarian as clinically healthy (based on appetite, physical exam, serial blood data, radiographs, etc.), approximately 30 days after antibiotics were discontinued (if antibiotics had been used). If a turtle was not on antibiotics, convalescence was determined

based on veterinarian evaluation of clinical status and ability to be released (dependent on appetite, physical exam, and transport preparation). At each of these timepoints I repeated tracheal washes, sedating the turtles to ensure safe restraint. For sedation, veterinary staff administered 0.1 mg/kg dexmedetomidine intravenously (IV) and allowed it to take effect for approximately 10 minutes before the tracheal wash was performed. Heart rate and palpebral reflex were monitored throughout the process. Once the tracheal wash was completed, the sedative was reversed by administering 1.0 mg/kg atipamezole intramuscularly (IM).

I performed necropsies on eight turtles, four of which were already enrolled in the study and received trachea washes while alive, and four of which had not been previously enrolled in the study but were utilized opportunistically. Necropsies were performed within 12 hours of death. During the time of necropsy, I examined all organ systems. I performed a post-mortem tracheal wash if it had not already been done within the previous two days. I used sterile cotton tipped applicators to swab other portions of the respiratory tract which included the glottis, the trachea (cranial to the bifurcation), the anterior right lung, and the posterior right lung. I placed swabs and tracheal wash fluid in labelled cryovials and immediately stored them on dry ice until moving them to a -80°C freezer within 15 minutes of collection for DNA extraction and sequencing at a later time. I collected a swab of grossly visible respiratory lesions using the Fisherfinest® Transport Swab with Amies gel (Fisher HealthCare, Pittsburg, PA) for culture submission to IDEXX Laboratories. I also collected a set of tissues in 10% neutral buffered formalin that I submitted for histopathology to National Marine Fisheries Service Office of Protected Resources Pathology Consultation at University of Florida, Gainesville, Florida.

Radiographic Score

In addition to initial radiographs, veterinary staff obtained radiographs of the turtles throughout rehabilitation using a standardized method, including a dorsoventral and craniocaudal horizontal beam view via a veterinary radiographic system (MinXray HF100+, MinXray Inc, Northbrook, Illinois, USA) with digital x-ray cassettes (Kodak DirectView CR cassette, Carestream Health Inc., Rochester, New York USA) at a focal distance of 1 m. Typical exposure factors for both projections were 75 kVp and 7.5 mAs. A single, board-certified veterinary radiologist, blinded to the clinical status of the turtles, retrospectively reviewed the radiographs obtained closest to the time of each tracheal wash and scored the pulmonary changes according to the grading scale as detailed in Table 3.1. Additionally, the radiologist further categorized radiographic lung abnormalities according to distribution (left, right, bilateral, diffuse, or ventral).

DNA Extraction and sequencing

I extracted DNA from necropsy swabs and tracheal wash fluid using a phenol:chloroform:isoamyl extraction protocol adapted from Mettel et al., (2010). I first suspended the swabs or tracheal wash fluid in PBL lysis buffer (water saturated phenol, disodium EDTA, sodium dodecyl sulfate, tris HCL, pH 5.7) by vortexing and centrifuging. I removed the supernatant and placed it in a clean tube. After removal of the supernatant, I added TPM buffer (50 mM Tris, pH 7.0, polyvinyl pyrrolidone, and $MgCl_2$) to the original tube; after vortexing and centrifuging, I then added the supernatant to the tube with the first supernatant. I supplemented the combined supernatant with 800 µL of a phenol:chloroform:isoamyl alcohol solution (pH 6.7+, 25:24:1) and centrifuged. I transferred

the upper aqueous layer to a sterile tube and added 0.7 volumes of 100% isopropanol and 0.1 volumes of 3 M sodium acetate. After centrifugation, the supernatant was decanted, I washed the pellet with 70% ethanol, and allowed it to air dry. I then resuspended the dried pellet in 50 µL nuclease-free water. I proceeded with a clean-up protocol to concentrate the DNA by adding 0.1 volume 3 M sodium acetate and 2 volumes of 100% isopropanol, vortexing briefly, and then freezing the sample for 15 minutes. I then centrifuged the sample, removed the supernatant and washed the pellet with 70% ethanol, decanted, and centrifuged again. After allowing the pellet to air dry, I then resuspended the dried pellet in 12 µL nuclease-free water and stored it at -80˚C until amplification. I verified all DNA extracts by gel electrophoresis, including negative controls of sterile saline and unused sterile swabs to ensure there was no contamination from supplies and solutions used in the extraction.

After verification, I amplified DNA extracts in triplicate using bacterial specific (515F and 806R), uniquely barcoded, 16S rRNA primers containing adaptors for Illumina sequencing (Caporaso et al., 2012). Each 25 µL PCR reaction contained 12.5 µL Phusion Master Mix (ThermoFisher), 0.5 µL primers, 10 µL diethylpyrocarbonate (DEPC) water, and 2 µL of DNA. After I verified the PCR product via gel electrophoresis, I purified the PCR product using AMPure XP beads (Beckman Coulter, Inc. Indianapolis, IN, USA) following manufacturers guidelines and using the 0.8:1.0 ratio of bead:sample to target 300 bp and above. After purification, I quantified the DNA using the Agilent D1000 ScreenTape System (Agilent Technologies, Inc, Waldbronn, Germany) following manufacturers guidelines. I pooled the purified PCR product to equimolar concentrations. To ensure proper DNA size selection from the pool, I size selected the product using a BluePippin™ (Sage Science Inc.,

Beverly, MA, USA) following manufacturer's instructions. Sequencing was performed on the Illumina MiSeq platform with a paired-end V2 500 cycle kit.

Data Analysis

Paired-end reads were demultiplexed using Illumina-utils version 2.0.2 (Eren et al., 2013). I performed quality filtering, merging of paired reads, and amplicon sequence variant (ASV) clustering using DADA2 version 1.12.1 (Callahan et al., 2016) in R version 3.6.1 (R Core Team, 2019). I assigned taxonomy using IDTAXA from the DECPHER package version 2.12.0 (Murali et al., 2018) with the Silva Small Subunit (SSU) 132 training set for classification. I used the phyloseq package version 1.28.0 in R to further process the sequences, evaluate taxonomy, and perform diversity metric visualizations and statistical tests (McMurdie and Holmes, 2013).

I used Bray-Curtis distance metrics to analyze microbial community differences in tracheal wash samples and used principal coordinates analysis (PCoA) to visualize these differences. I tested for significant differences using permutational multivariate analysis of variance (PERMANOVA) for variables including disease condition (non-pneumonia or pneumonia), radiographic score, survival, and timepoint. I calculated Shannon diversity and tested for significance using pairwise Wilcoxon tests.

I compiled the culture reports of tracheal washes from IDEXX Laboratories and identified the isolates grown in culture at the different sampling times and the percentage of antibiotic sensitivity for those isolates. I then identified ASVs in the corresponding sequence data that had the same genus as the isolates, and performed a BLASTN (Zhang et al., 2000) search to determine the closest bacterial species. The ASVs were then tracked through the

time course of the animal's rehabilitation. To further characterize the respiratory tract, I visualized microbial community composition of necropsy samples using taxa plots for each sample to identify the individual turtle's variability. I used Bray-Curtis distance metrics to determine the similarity of the tracheal wash microbial community to other sites along the respiratory tract (oral/glottis, trachea, anterior lung, and posterior lung). I also reviewed and summarized the histopathology reports for the turtles that had tissues submitted. I screened the tracheal wash microbiome sequences from admission for the presence of potential pathogens common to sea turtles at the genus and family level based on pathogens identified by Innis and Frasca (2017).

Results

Sample Data

I collected tracheal wash samples for sequencing from 20 turtles on the day of admission, 10 of which were classified as non-pneumonia and 10 as pneumonia. Six of these turtles died during rehabilitation (prior to "Rehab" sample collection), and of these, I necropsied four. Necropsies were not performed on the other two mortalities due to use of the carcasses for further diagnostic analysis. I collected additional tracheal wash samples from 11 turtles in rehabilitation (mean 48 days in rehab, range 30 – 58 days) and from 14 of the turtles at convalescence (mean 100 days in rehab, range 24 – 201 days).

Radiographic scores were assigned to 22 turtles at admission (Table 3.2). Fifteen turtles had bilateral lung abnormalities at admission, four of which were more severe on the right lung and one that was more severe on the left lung (Table 3.2). Only five turtles had completely unilateral abnormalities, four on the right lung only and one on the left lung only.

Approximately half of the turtles with lung abnormalities had diffuse patterns. The radiologist noted a reticular pattern in seven turtles, primarily associated with a radiographic score of 5, and four of these turtles died in rehabilitation. Examples of score 0 (normal lungs), score 2 (mild abnormalities), and score 5 (severe abnormalities) are provided in Figure 3.1. No turtles had a score of 5 at convalescence, although one turtle still had a radiographic score of 4 based on remaining lung abnormalities despite being clinically healthy. Table 3.2 provides detailed information on radiographic scores assigned to each turtle as well as the location and patterns described.

Out of 44 tracheal wash samples, sequencing of the 16S rRNA gene resulted in 718,312 reads after joining paired-end reads and quality filtering, which included the removal of chimeras, singletons, chloroplasts, mitochondrial DNA, and archaea. The mean sequence counts per sample was 16,325 (median 13,752) and range was 267 to 42,742 counts per sample. These sequences were assigned to 1,331 unique ASVs across 218 different families. Negative control samples had no reads remaining after quality filtering and were thus not included in any sequence analysis.

I collected 38 samples at necropsy from eight turtles, which included four body sites (glottis/oral, trachea, anterior right lung, and posterior right lung) and tracheal washes for all except two turtles who had tracheal washes performed within the previous 48 hours (Turtle ID 56 and 68). From these 38 necropsy samples, sequencing of the 16S rRNA gene resulted in 980,793 reads after joining paired-end reads and quality filtering as described above. One sample (Turtle ID 27 anterior lung) had low sequence depth and was removed during quality

filtering. The mean sequence count per sample was 27,244 (median 12,215) and range was 463 to 183,855 counts per sample. These sequences were assigned to 1,179 unique ASVs.

The Tracheal Wash Microbiome

The tracheal wash of cold-stunned Kemp's ridley turtles at admission was significantly different from other timepoints during rehabilitation based on Bray-Curtis distance metrics (Figure 3.2). The rehab timepoint and convalescent tracheal washes, however, were not significantly different from each other. There was no difference in Shannon diversity among all timepoints. At admission, Shannon diversity of tracheal wash samples was not significantly different as a function of survival of the turtles (those that survived vs. those that died), disease condition (non-pneumonia vs. pneumonia), radiographic scores, location of radiographic abnormalities, or radiographic abnormality patterns. Bray-Curtis distance was also not significantly different between these variables.

Bacterial families prevalent in 100% of the tracheal washes at admission include Vibrionaceae, with a mean of 21.1% relative abundance, Rhodobacteraceae (9.3%), and Flavobacteriaceae (8.8%). Vibrionaceae, although present in all the samples, had high variability with a range of 0.2% to 70.0% relative abundance across samples. Shewanellaceae was also highly variable with a mean of 3.7% and range of 0.03% to 32.0%. Pseudomonadaceae was present in 85% of the tracheal wash samples with a mean of 5.2% and range of 0 to 40% abundance (Figure 3.3).

In comparison to samples collected upon admission, rehab and convalescent tracheal wash microbial community composition had different proportions of the most abundant bacterial families (Figure 3.3). They had a lower abundance of Vibrionaceae than found in

intake samples, with a mean of 3.8% (range 0 – 11.4%) in rehab samples and 4.8% (range 1.6 – 12.0%) in convalescent samples. Both timepoints were dominated by Flavobacteriaceae (rehab 15.8%, convalescent 12.2%) and Rhodobacteraceae (rehab 9.4%, convalescent 11.7%). Pseudomonadaceae remained highly variable in abundance at each of these timepoints (rehab mean 13.3%, range 0.5% - 47.5%; convalescent mean 4.2%, range 0% - 27.2%).

Culture

I submitted seven tracheal wash samples to IDEXX Laboratories for culture at each timepoint (total 21 samples). The laboratory reported positive cultures as presence of growth and, where possible, they reported the identification of the isolate. Anaerobic and fungal cultures all had no growth. Aerobic cultures resulted in more isolates from the intake tracheal washes and rehab samples compared to convalescent samples (Table 3.3). *Vibrio* sp. were cultured at all three timepoints from admission to convalescence, and not always from the same turtle. Most turtles typically had one to two isolates that were cultured per sample, with no growth on two turtles at intake, two during rehab, and four at convalescence. Several cultures also grew non-enteric gram negative bacteria that could not be speciated. Specific species that were only isolated at admission were *Raoultella planticola* and *Shewanella putrefaciens*. *Pseudomonas* sp. and *Enterococcus* sp. were both only isolated from tracheal washes of turtles in rehabilitation.

Drug sensitivities were also reported for each of the isolates, except for one *Enterococcus* sp., which did not receive a complete panel (Table 3.3). All isolates were sensitive to several antibiotics, including amikacin, ciprofloxacin, enrofloxacin, gentamycin,

tetracycline, and tobramycin. Isolates had the highest percentage of resistance to amoxicillin and cephalexin. All isolates except *Vibrio* sp. were completely sensitive to ceftazidime, but some *Vibrio* isolates had intermediate resistance.

Culture isolates that matched at the genus level in the sequence data include *Vibrio* (3 ASVs), *Pseudomonas* (9 ASVs), and *Shewanella* (3 ASVs). The BLAST matches of these ASVs resulted in 1 to 3 possible species based on 100% identity across the length of the 16S rRNA fragment (Table 3.4). The *Vibrio* ASVs had highest abundance in the intake samples and lower abundance in samples in rehab and at convalescence (Figure 3.4A). *Pseudomonas* had variable abundance throughout rehabilitation, with ASV1394 (*Pseudomonas flourescens*) having greater than 40% relative abundance in some samples (Figure 3.4B). *Shewanella woodyi* (ASV53) had high abundance in some intake tracheal washes, while *Shewanella algae* (ASV1267) was prevalent in most tracheal washes throughout rehabilitation (Figure 3.4C). None of the genera were specific to higher radiographic scores as they were variable across samples with pneumonia (Figure 3.4).

Necropsy Samples

From 8 necropsies, I found high variability in the abundance of taxa at each body site, with some turtles having high abundance (greater than 40%) of Flavobacteriaceae, Enterobacteriaceae, Marinomonadaceae, Burkholderiaceae, Bacteroidaceae, Pseudomonadaceae, Shewanellaceae, or Vibrionaceae in the trachea and/or lung samples (Figure 3.5). Due to the variability between turtles, I only compared Bray-Curtis similarity among the body sites of each turtle to the corresponding tracheal wash of that turtle (that is, I only performed intra-turtle comparisons, not comparisons across individuals) and found that

most samples were distinctly different from the tracheal wash microbial community (Figure 3.6). I also found that necropsy samples were more similar among body sites within a turtle rather than in the same body site across all turtles (PERMANOVA, $p = 0.001$).

Histology reports for turtles with gross lung lesions confirmed pneumonia (Table 3.5). Three out of the four necropsied turtles had granulomatous pneumonia with intralesional fungi +/- bacteria. The fourth turtle also had intralesional bacteria. The tracheal wash cultured isolates did not correspond to the cultured isolates from necropsy samples of the lungs (Table 3.5). Fungi were cultured from two out of three necropsy lung samples, despite no positive fungal cultures from tracheal washes at admission.

Sea Turtle Pathogens in Sequence Data

I found several previously reported sea turtle pathogens in low abundance at the genus level in the tracheal wash microbial communities including *Corynebacterium*, *Flavobacterium*, *Acinetobacter*, and *Mycobacterium*, meanwhile the pathogens *Pseudomonas* sp., *Shewanella* sp., and *Vibrio* sp. were in higher abundance at the genus level (Table 3.6). Since taxonomic identification did not reach the genus level for the majority of the ASVs, I found that at the family level, there was high abundance of Flavobacteriaceae (8.82%) and Vibrionaceae (21.14%) across intake tracheal washes with 48 ASVs and 12 ASVs respectively (Table 3.6). Pseudomonadaceae and Shewanellaceae had similar prevalence as the genus level but with eight ASVs and four ASVs respectively.

Discussion

Respiratory diseases such as pneumonia are typically associated with a higher bacterial biomass, decreased bacterial community diversity, and a shift in composition to a

higher abundance of single pathogens (Dickson et al., 2014a, 2014b; Dickson and Huffnagle, 2015; Dickson et al., 2016; Faner et al., 2017; Vientós-Plotts et al., 2019). In the present study, characterization of tracheal wash microbial communities did not identify any of these typical signatures of pneumonia. I identified pneumonia based on radiographic scores of lung abnormalities and found no difference in bacterial diversity nor composition in comparison to the severity of radiographic lung abnormalities at the time of admission.

The respiratory tract is a heterogenous ecosystem with a continuous mucosal surface and a continuously varying microbial topography (Dickson et al., 2013). Even in healthy lungs of humans, the microbial communities are not consistent across samples (Charlson et al., 2011). The lung microbiome of mice is also highly variable and clusters by different habitats in the respiratory system (Dickson et al., 2017). Similarly, the upper respiratory tract of tortoises does not have a strong core nasal microbiome within a tortoise species but has shared microbiomes of various sizes based on habitat (Weitzman et al., 2018). Similar to mammals, the local environment likely influences the respiratory microbiome of sea turtles through microbial immigration, such as microaspiration, inhalation of microbes, and dispersal along mucosal surfaces; and elimination, including exhalation and removal via host immune defenses (Dickson et al., 2013, 2014a, 2014b). Cold-stunned sea turtles in this study all stranded from the waters of Cape Cod Bay, but the exact stranding location along the coast of Cape Cod was variable, allowing for potential geographic variation in microbial exposure. Also, turtles stranded in various states of debilitation, with potential variation in the amount of sea water aspirated, respiratory rates, immunologic status, and other physical and physiologic conditions that are not necessarily associated with radiographic lung

abnormalities. It is possible that cold-stunned sea turtles further complexify pneumonia pathogenesis since pneumonia leads to a disruption in the homeostasis of the immigration and elimination of microbes that can occur from changing conditions such as the environment of the lung (i.e. systemic vasoconstriction from hypothermia, hypoxia), host inflammatory response (i.e. immunocompromised state from cold-stunning), and interactions with other bacteria that may change from aspiration/introduction of new microbes or environmental conditions (Dickson et al., 2014a).

Changing conditions during rehabilitation likely caused the shift in tracheal wash microbial communities after admission since the water temperature became consistent, turtles were medically treated for cold-stun clinical signs (dehydration, metabolic derangements, etc.), and their local environment (i.e. tank water) became constant. Concurrent with this shift in environment once in rehabilitation, the microbial community composition also stabilized. Temporal stability in bottlenose dolphin blowhole microbial communities also showed host specificity, where the samples of individual dolphins were more similar over time than to other dolphins (Lima et al., 2012). Even though overall communities were not different between rehab and convalescent samples, variability remained within the individual turtles, which was different than the pattern observed in dolphins (Lima et al., 2012).

The radiographic scoring system was useful in identifying the most critically ill turtles. Further categorizing the scores into unilateral or bilateral, and ventral or diffuse provided some insight into potential causes of pneumonia in cold-stunned sea turtles. Potential causes include colonization of opportunistic microbes and/or aspiration of sea water (Innis et al., 2009a; Stockman et al., 2013). Although I primarily saw bilateral lung

abnormalities, the right lung tended to be more severely affected radiographically than the left as described in a previous study (Stockman et al., 2013). If aspiration of sea water contributes to pneumonia in these cases, it seems reasonable that the right lung would be more severely affected since the entrance to the right bronchus is ventral to the entrance of the left bronchus anatomically, allowing water and debris to gravitate in that direction (Stockman et al., 2013). Radiographic score did not correlate with survival, as reported in other studies (Stockman et al., 2013), but I did see that the reticular pattern was associated with the highest radiographic scores. This reticular pattern corresponds to the structure of the airways and is possibly representative of edicular wall thickening. I found that most turtle radiographic lung abnormalities resolved or improved by convalescence, but there were still some that had moderate abnormalities. These turtles were still considered clinically stable and ultimately released. Since it is difficult to radiographically distinguish chronic pneumonia (infection still present) from fibrotic changes (infection resolved, but residual scarring present) clinicians often rely on the total body of clinical information to determine convalescence and suitability for release to the wild.

Comparison of culture-dependent and culture-independent methods highlights the limitations of culture in diagnosing diseases such as pneumonia. I found some cultured isolates were not present in the sequences or possibly not detectable due to extremely low abundance (*Raoultella planticola*, *Enterococcus* sp.). Also, several samples had isolates that could not be speciated (reported as non-enteric gram negative rods) so I could not examine them in the sequence dataset. Of the ones that were identified, *Vibrio* sp., *Pseudomonas* sp., and *Shewanella* sp. were all isolated at various timepoints, and they were also found in

various abundances in the 16S rRNA sequences (Figure 3.4). *Vibrio* sp. are gram negative rods associated with the marine environment, yet some species have been found in septic sea turtles (Wellehan and Divers, 2019). The tracheal washes at admission had higher abundances of *Vibrio* overall than rehab and convalescent samples. This could indicate influence of environment or immune status during the rehabilitation process. One *Vibrio* (ASV1445) appeared to be more prevalent in some turtles with higher radiographic scores. The BLAST match of this ASV was *Vibrio anguillarum*, which is a known pathogen of marine fish, bivalves, and crustaceans, so may have pathogenicity in sea turtles as well (Frans et al., 2011). *Pseudomonas* sp. were also found in a varying abundance across tracheal wash samples. *Pseudomonas* are found in the marine environment, have extensive resistance profiles, and are opportunists (Wellehan and Divers, 2019). The ASV with relatively high abundance in several samples was *Pseudomonas flourescens,* which produces antimicrobial metabolites that may be useful in defense roles (Gross and Loper, 2009). Lastly, *Shewanella* sp., in particular *Shewanella algae* was abundant in most tracheal washes at each timepoint. This marine bacterium can tolerate a wide range of temperatures and salinity (Tseng et al., 2018). Although it has been shown to be a pathogenic agent (Tseng et al., 2018), it was present in convalescent turtles, so was unlikely always pathogenic in this context.

The mismatch between cultures and sequence data could result from several possibilities. Culture relies on morphological and biochemical identification which can lead to decreased specificity (Stewart, 2012). I saw this in the culture-based identification of "non-enteric gram negative rods", and several isolates that were only identified to the genus level. Growth can also be limited by conditions of the media environment (temperature, pH,

nutrients) or inhibition by other microbes (Stewart, 2012; Innis and Frasca, 2017; Wellehan and Divers, 2019). A negative culture may reflect the presence of culture inhibitors such as *Pseudomonas flourescens*, which actively impedes in vitro growth of other microbes by producing antimicrobial metabolites (Gross and Loper, 2009; Dickson et al., 2014b). Concurrent or recent antibiotic use could also inhibit bacterial viability in cultures. Lastly, some of the pathogens that may be causing pneumonia may be uncultured microbes not previously identified (Vientós-Plotts et al., 2019). Although cultures have limitations and 16S rRNA data provide a more comprehensive profile of bacterial taxa in samples, antibiotic susceptibility testing from cultured isolates does seem to provide clinical benefit in identifying sensitivity and resistance for drug selection (Innis and Frasca, 2017). Unfortunately, it often remains unclear whether the cultured isolate is truly the causative agent for pneumonia.

Necropsy tissue microbial community analysis not only revealed high variability between individual turtles, but also between sites along the respiratory tract (Figure 3.5). Lung brush samples in sheep found similar patterns in that multiple samples along the respiratory tract were more similar in the same sheep than between samples of different sheep, yet spatial variability was still present (Glendinning et al., 2016). The variability along the respiratory tract may be due to regional changes of physiological parameters, such as gas concentrations, osmolarity, temperature, pH, and blood flow (West, 1978; Zeltner et al., 1990; Dickson et al., 2014a; Glendinning et al., 2016). These differences demonstrate that certain parts of the respiratory tract cannot be considered to be surrogates for other parts. For example, oral microbial communities were found to provide different culture results than

tracheal washes in dogs with pneumonia, and thus could not be considered a replacement diagnostic tool (Sumner et al., 2011). I also found differences within the same lung of individual turtles, so dissimilarity of the tracheal wash microbial communities to other parts of the respiratory tract was somewhat expected.

Since tracheal washes were not similar to other sites of the respiratory tract (Figure 3.6), the site of infection may not be sampled effectively by tracheal wash, further limiting the value of culture results. Tracheal washes are a blind diagnostic technique, in that the clinician does not know how deep and into which lung the saline is being infused. Since lung abnormalities develop in different locations (i.e. unilateral vs. bilateral) and in different patterns within the lung (ventral vs. diffuse), failure to sample the infected region of the lung could result in false negatives, or misleading results. Endoscopically-guided lung washes allow visualization of the lung, may provide guidance to the site of infection, and are recommended for cases of chronic or resistant pneumonia if resources are available (Boylan et al., 2017). Although performed as sterilely as possible, the oral cavity may still be a cause of contamination in tracheal washes. For example, cattle had more evidence of oral microbial contamination in tracheal washes compared to BAL (Abutarbush et al., 2019). I ruled out contamination of the saline during the DNA extraction and amplification process with negative controls, but influence of the oral cavity environment cannot be completely ruled out for tracheal washes. In humans, BAL were more similar to the mouth than lung brushings, which reflects the difference in sampled surface areas (Dickson et al., 2017). Brushings, which can be done by bronchoscopy, may provide more thorough and representative samples by improved contact with the lung biofilm and epithelial cells since

tracheal washes potentially have a dilution effect from the saline and a lack of clarity around what surface area was actually lavaged (Dickson et al., 2014b). Histology reports also highlight the limitations of tracheal washes in that bacteria and/or fungi were typically intralesional. Tracheal washes would not penetrate well encapsulated granulomas, and since granulomatous pneumonia was common in the histological diagnosis of turtles with lung lesions (Table 3.5), biopsy may be a more valuable approach. Overall, I found that the use of tracheal washes as a diagnostic tool was limited due to the wide differences in the microbial communities between the tracheal wash microbial communities and those found in the necropsy samples.

Despite the limitations in tracheal washes as a diagnostic tool, I screened the sequences for previously reported pathogens of sea turtles and found low abundance of several pathogens at the genus level and high abundance in some bacteria at the family level (Table 3.6). Although the known pathogen *Flavobacterium* sp. was in low abundance, sequence data showed 48 ASVs in the corresponding family, Flavobacteriaceae. Flavobacteriaceae is common in marine environments (Jooste and Hugo, 1999), which likely explains the high number of ASVs contributing to the family. *Pseudomonas, Shewanella,* and *Vibrio* were identified in low abundance at the genus level, but *Vibrio* at the family level (Vibrionaceae) was found in high abundance likely due to its association with the marine environment and not necessarily due to its pathogenicity in tracheal washes. It is important to note that many pathogens appear in low abundance even in healthy animals, but they could lead to secondary invasion in immunocompromised states (Dickson et al., 2014a; Weitzman et al., 2018; Vientós-Plotts et al., 2019). Since detection of sea turtle pathogens has primarily

relied on culture-dependent methods, potential pathogens are not limited to those in Table 3.6 and could be captured with future microbial community analysis.

Conclusions

I established that the lungs of Kemp's ridley sea turtles are not sterile, but rather contain a diverse assortment of microbial taxa. This data demonstrate that the microbial communities recovered from tracheal washes are diverse and variable between cold-stunned sea turtles at stranding, rehabilitation, and convalescence. The radiographic scoring system identified severity of lung abnormalities, however, tracheal wash microbial communities did not cluster by radiographic score, likely because pneumonia pathogenesis in cold-stunned sea turtles is complicated and variable. Bacteria isolated from culture-dependent methods, which in many cases could not be assigned taxonomy at the species level, had variable abundance in culture-independent methods. I found some cultured isolates in the sequence data across many samples, even at convalescence, and they were not necessarily dependent on radiographic severity. I also found that tracheal washes were not representative of other segments of the respiratory tract in sea turtles, which is likely due to a combination of regionality of the lungs, granulomatous lesions/focal sites of infection, and limitations of the technique itself (access to lungs, contamination, visualization, surface area sampled). I documented several previously identified bacterial pathogens in varying abundances in the tracheal wash microbial communities of both pneumonia and non-pneumonia turtles, suggesting that these organisms may be opportunistic pathogens that can be present in turtles that do not have pneumonia. My findings suggest that pneumonia in cold-stunned sea turtles

has a complex pathogenesis, and tracheal washes may not be the most appropriate diagnostic tool for determining causative agents for treatment selection.

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Table 3.1. Radiographic scoring system established to identify degree of lung abnormalities for turtles. Radiographs were evaluated and assigned a score by a veterinary radiologist blinded to the clinical history of each turtle in this study.

Score	Description	Radiographic features
0	Normal	No radiographic abnormalities of the pulmonary parenchyma
1	Minimal	Interstitial, increased opacity with ill-defined pulmonary vasculature
2	Mild	Diffuse interstitial
3	Mild-Moderate	Mixed interstitial and alveolar pattern, increased opacity with obscured pulmonary vasculature
4	Moderate	Alveolar focal or multifocal, mild reticular
5	Marked	Diffuse bilateral alveolar or marked reticular pattern

Figure 3.1. Examples of radiographs from turtles with different radiographic scores. A: dorsoventral view of a normal turtle (Score 0); B: craniocaudal view of a normal turtle (Score 0); C: dorsoventral view and D: craniocaudal view of Score 2 with abnormalities in the ventral left lung; E: dorsoventral view and F: cranioventral view of Score 5, most severe with diffuse reticular (honeycomb) pattern in both lungs, worse in right lung. Details of scores are found in Table 3.1 and Table 3.2.

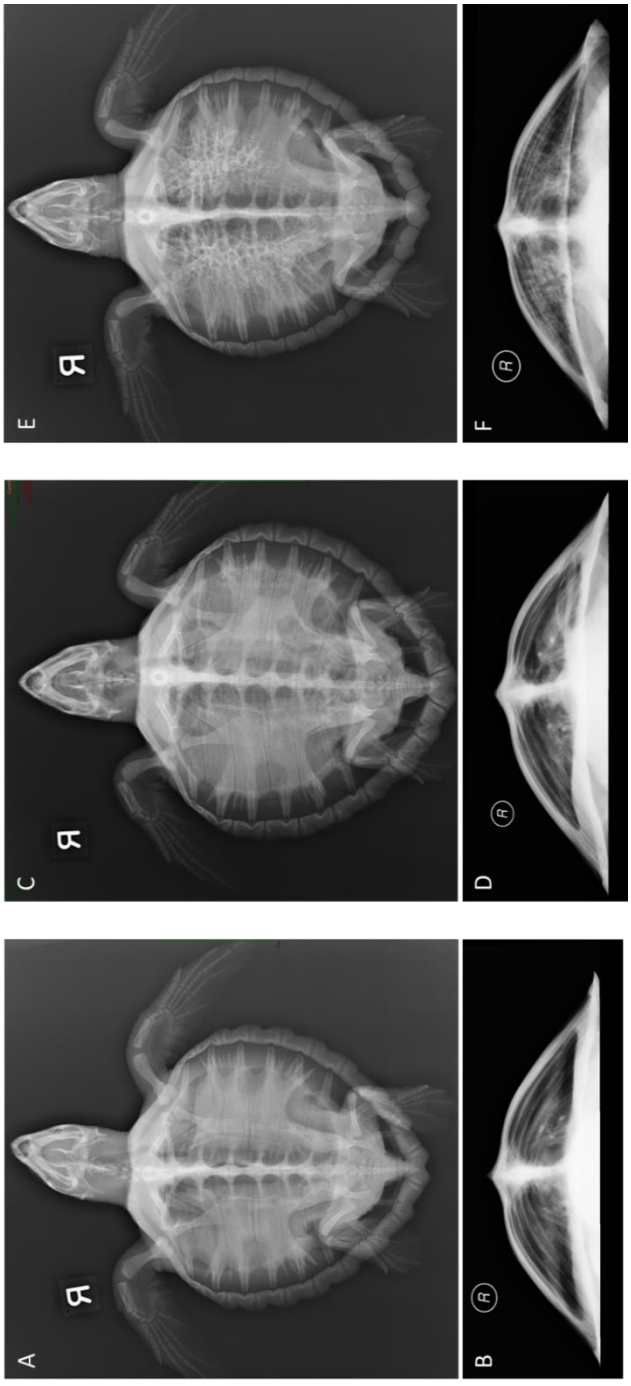


Table 3.2. Radiographic scores assigned to study turtles. Disease Condition (Dis Cond) refers to whether the turtle was diagnosed as non-pneumonia (N) or pneumonia (P) by clinical veterinarians (unrelated to the score assigned). Location (Loc) refers to whether the abnormalities were unilateral or bilateral. r = right lung; l = left lung; b = bilateral lungs; r,b = bilateral but worse in right lung; l,b = bilateral but worse in left lung; vent-med = ventromedial; ^ indicates reticular pattern; * indicates the turtle died during rehabilitation; N superscript = turtle was necropsied only and did not have a tracheal wash while alive; na = not applicable

Turtle ID	Dis Cond	Intake			Rehab			Convalescent		
		Score	Loc	Pattern	Score	Loc	Pattern	Score	Loc	Pattern
112	N	0	na	normal	0	na	normal	0	na	normal
114	N	0	na	normal	0	na	normal	0	na	normal
241	N	0	na	normal	na	na	na	0	na	normal
8	N	1	b	diffuse	0	na	normal	0	na	normal
14	N	1	b	ventral	na	na	na	1	b	diffuse
56 *	N	1	b	ventral	na	na	na	na	na	na
159 *	N	1	b	diffuse	na	na	na	na	na	na
15 *	N	2	b	diffuse	na	na	na	na	na	na
32	N	2	l	ventral	na	na	na	1	b	ventral
26	N	3	b	diffuse	2	b	diffuse	3	b	diffuse
58	P	1	r	ventral	1	b	ventral	1	b	ventral
185	P	3	r	ventral	2	b	diffuse	3	b	diffuse
247 * N	P	3	b	diffuse ^	na	na	na	na	na	na
18	P	4	r,b	ventral	1	b	vent-med	2	l,b	diffuse
53	P	4	l,b	diffuse	4	b	diffuse	1	b	ventral
68 *	P	4	r	vent-med	na	na	na	na	na	na
73	P	4	r	ventral ^	3	b	ventral	2	b	ventral
79	P	4	r,b	vent-med ^	4	b	ventral	3	b	ventral
25 *	P	5	b	diffuse ^	na	na	na	na	na	na
27 * N	P	5	b	diffuse ^	na	na	na	na	na	na
36 *	P	5	r,b	diffuse ^	na	na	na	na	na	na
59	P	5	r,b	diffuse ^	4	b	diffuse	4	b	vent-med

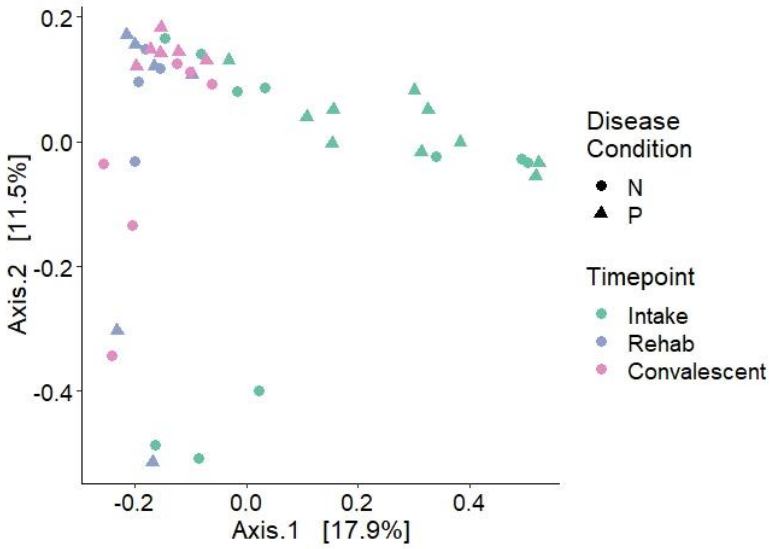


Figure 3.2. PCoA plot of Bray-Curtis distance for tracheal wash samples. Color indicates the timepoint during hospitalization. Shape indicates whether the turtle was categorized as a non-pneumonia (N) or pneumonia (P) patient (PERMANOVA, p = 0.001).

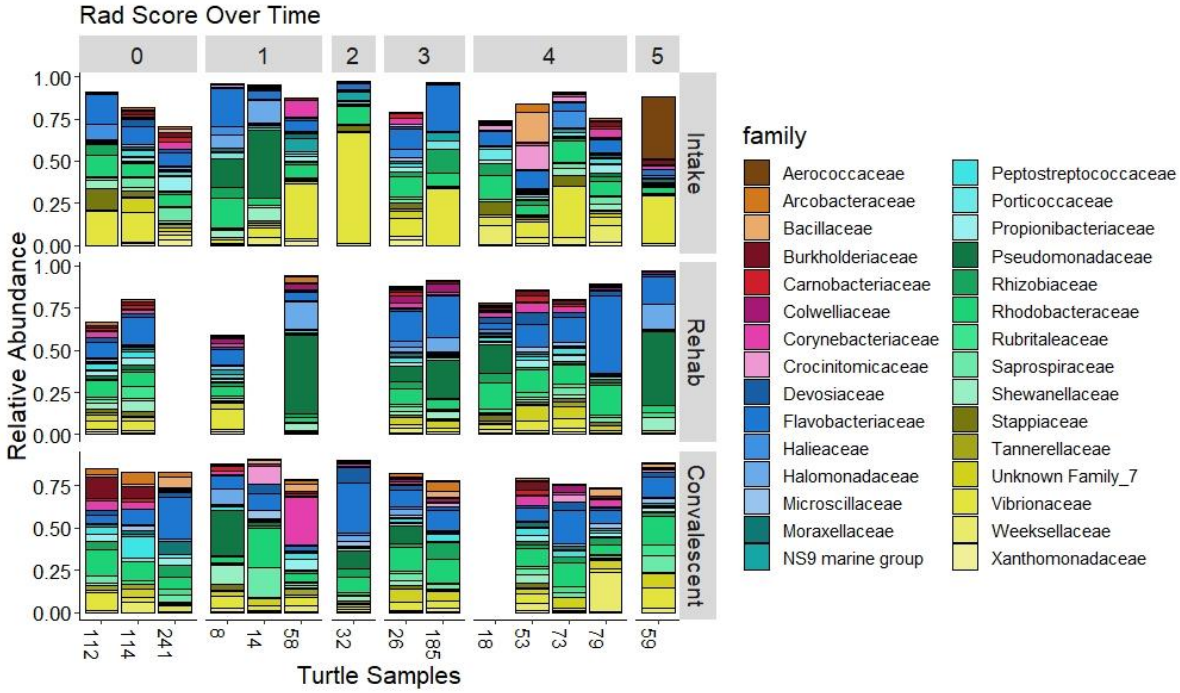


Figure 3.3. Taxa plots of tracheal washes. Plots represent the relative abundance of the top 30 bacterial families across samples of turtles that survived. Plots are separated by radiograph (rad) score at time of intake (0 through 5) and timepoint during hospitalization. A turtle has no sample during Rehab if it was considered convalescent before a sample could be collected. Any other samples that are missing are due to low quality sequencing results and were removed during quality filtering.

Table 3.3. Identity and percentage of antibiotic sensitivity for bacteria cultured from the tracheal wash samples. Samples are from turtles classified as Pneumonia. *n* = number of samples from which the bacteria were isolated; Conv = Convalescent; AM = Amoxicillin; AMK = Amikacin; CEPH = Cephalexin; CFR = Ceftiofur; CFZ = Ceftazidime; CIPR = Ciprofloxacin; ENRO = Enrofloxacin; GENT = Gentamicin; TET = Tetracycline; TOB = Tobramycin; NE = Not Examined

	Intake	In Rehab	Conv	Antibiotic								
	n	*n*	*n*	AM	AMK	CEPH	CFR	CFZ	CIPR	ENRO	GENT	TET	TOB
Vibrio alginolyticus	1	1	0	0	100	0	100	100	100	100	100	100	100
Pseudomonas sp.	0	2	0	0	100	0	0	100	100	100	100	100	100
Raoultella planticola	1	0	0	0	100	100	100	100	100	100	100	100	100
Vibrio sp.	2	3	3	25	100	25	87.5	87.5	100	100	100	100	100
Shewanella putrefaciens	1	0	0	100	100	0	100	100	100	100	100	100	100
Enterococcus sp.	0	1	0	100	NE	NE	NE	NE	100	100	NE	100	NE
Non-enteric gram neg rod	3	2	1	100	100	80	100	100	100	100	100	100	100
No growth	2	2	4										

Table 3.4. Taxonomy of ASVs matching at the genus-level to culture results. Sequences of each ASV were input into the NCBI BLASTN database. The top species level results are included which match the ASV at 100% sequence identity across the length of the 16S rRNA gene fragment.

ASV	Genus	BLAST Matches
ASV784	*Vibrio*	*chagasii, cuclitrophicus, splendidus*
ASV1445	*Vibrio*	*anguillarum, cortegadensis*
ASV1456	*Vibrio*	*tapetis*
ASV1225	*Pseudomonas*	*graminis, viridiflava, donghuensis*
ASV125	*Pseudomonas*	*marginalis, grimontii, rhodesiae*
ASV1394	*Pseudomonas*	*flourescens, veronii*
ASV1443	*Pseudomonas*	*koreensis*
ASV1450	*Pseudomonas*	*pachastrellae, aestusnigri*
ASV215	*Pseudomonas*	*oleovorans, stutzeri, taeanensis*
ASV250	*Pseudomonas*	*arsenicoxydans, yamanorum, proteolytica*
ASV669	*Pseudomonas*	*paralactis, tolaasii, gessardii*
ASV808	*Pseudomonas*	*entomophila, mosselii, guariconensis*
ASV1096	*Shewanella*	*marisflavi, fidelis, schlegeliana*
ASV1267	*Shewanella*	*algae*
ASV53	*Shewanella*	*woodyi*

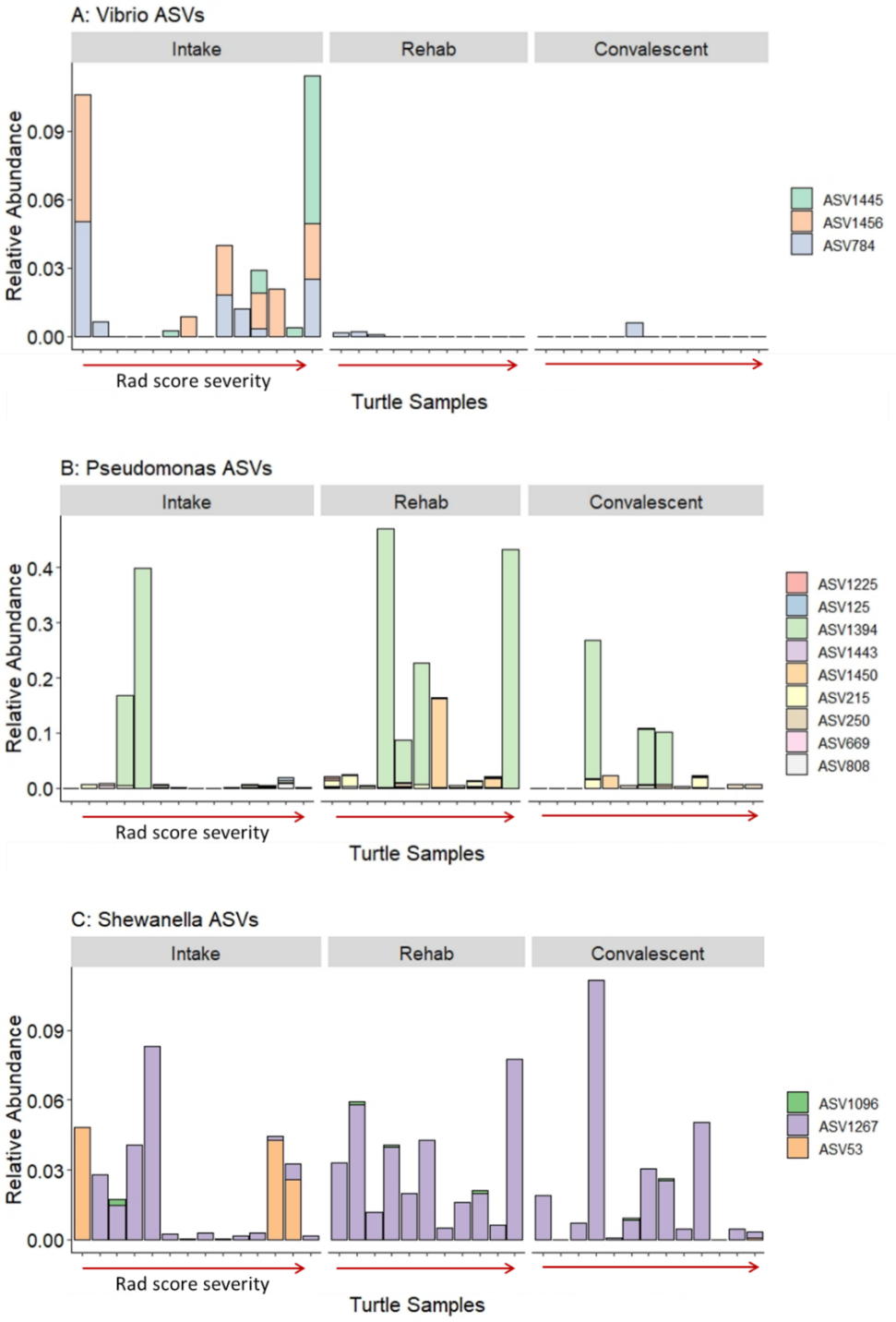
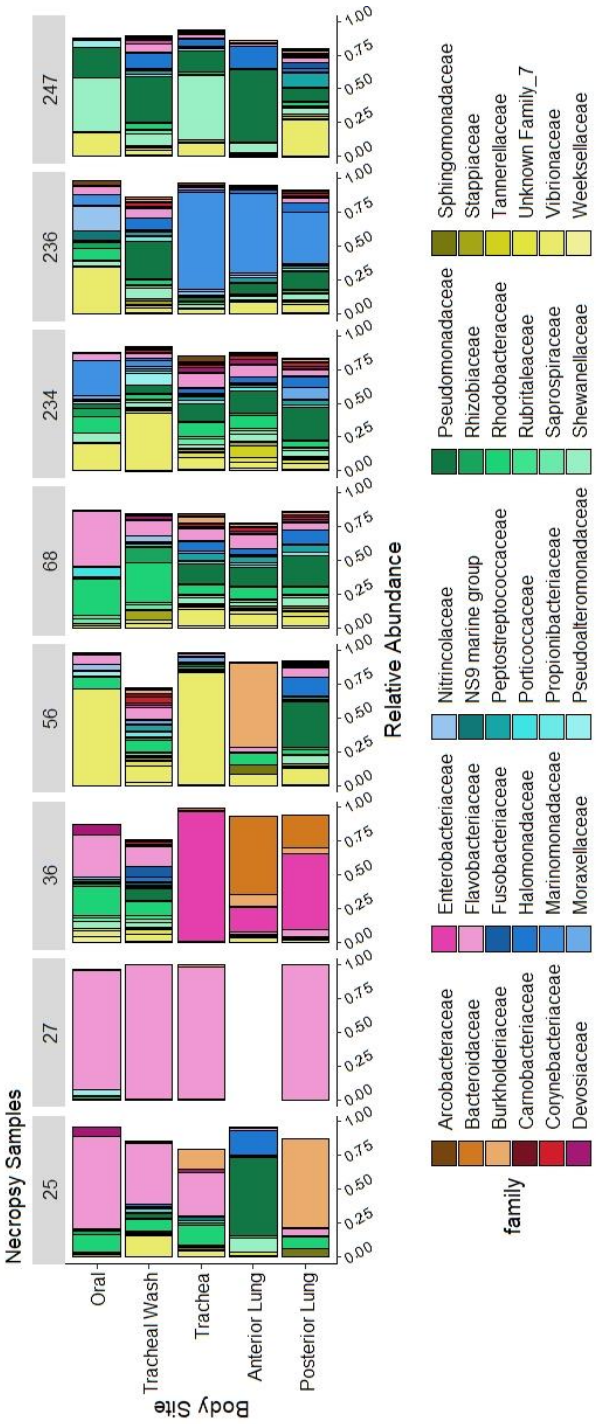


Figure 3.4. Bar plots of the ASVs that match the genus level of culture results. Only 3 cultured bacteria were identified at the genus level in the sequence dataset including Vibrio (A), Pseudomonas (B), and Shewanella (C). The turtle samples are ordered by their intake radiographic score from 0 (normal) on the left to 5 (severe) on the right at each timepoint during hospitalization.

Figure 3.5. Taxa plots of necropsy samples. Plots represent the relative abundance of the top 30 bacterial families at each site of the respiratory system, including a tracheal wash. Plots are separated by individual turtles (Turtle ID numbers are listed at the top).



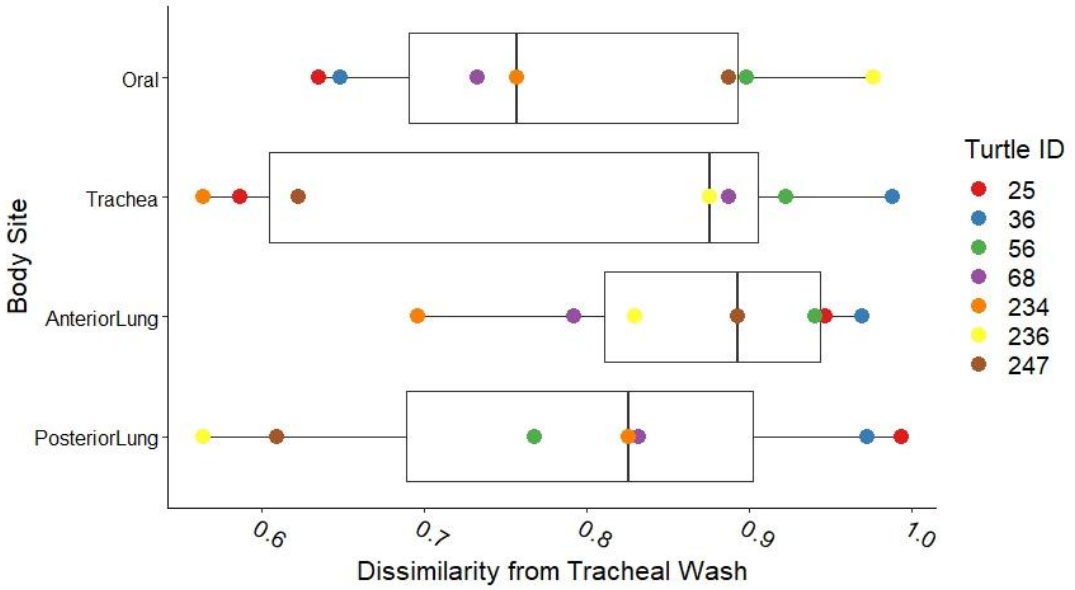


Figure 3.6. Boxplots of Bray-Curtis dissimilarity of each necropsied turtle's samples. Samples are from along the respiratory tract and directly compared to that individual's tracheal wash. Colored points represent the Turtle ID number.

Table 3.5. Summary of histology results from necropsy samples that were submitted to pathologists. The corresponding lung culture results and tracheal wash culture results from intake are also displayed. NE = Not examined; * indicates that the culture of the lung was taken while the turtle was still alive (via a biopsy) prior to mortality.

Turtle ID	Date of Admit	Date of Necropsy	Histologic Diagnoses (Lung)	Aerobic Culture Isolates from Tracheal Wash at Admit	Aerobic Culture Isolates at Necropsy
25	11/23/2015	12/14/15	Severe necrotizing and granulomatous pneumonia with intralesional fungal hyphae (with vascular invasion)	*Serratia liquefaciens* *Shewanella* sp. *Vibrio* sp. Non-enteric gram neg rod	*Hafnia (Enterobacter) alvei*
27	11/23/2015	12/02/15	Heterophilic bronchopneumonia with intralesional bacteria	NE	NE
36	11/24/2015	12/26/15	Severe necrotizing, heterophilic, and granulomatous pneumonia with numerous intralesional fungi and bacteria	*Vibrio* sp.	*Serratia marcesens* *Citrobacter braakii* *Providencia rettgeri*
247	12/20/2015	02/14/16	Severe, diffuse, chronic granulomatous pneumonia with intralesional fungal hyphae	NE	gram neg rods (rare) *

Table 3.6. Previously reported bacteria associated with infection in sea turtles. The prevalence (%, relative abundance) in tracheal wash samples from study turtles at intake are displayed. Prevalence is listed both at the genus level and family level with the number of ASVs composing each. SD = Standard Deviation.

Bacteria	Genus prevalence %	Genus SD %	Number of ASVs in Genus	Family	Family prevalence %	Family SD %	Number of ASVs in Family
Aeromonas hydrophila	0	0	0	Aeromonadaceae	0	0	0
Bacillus sp.	0	0	0	Bacillaceae	1.3	3.5	7
Corynebacterium sp.	1.7	2.1	8	Corynebacteriaceae	2.1	2.4	13
Hafnia alvei	0	0	0	Enterobacteriaceae			
Citrobacter braakii	0	0	0	Enterobacteriaceae	0.35	0.43	2
Citrobacter freundii	0	0	0	Enterobacteriaceae			
Escherichia coli	0	0	0	Enterobacteriaceae			
Enterococcus spp.	0	0	0	Enterococcaceae	0.09	0.16	1
Flavobacterium sp.	0.07	0.12	1	Flavobacteriaceae	8.82	6.45	48
Acinetobacter calcoaceticus	0.66	0.7	5	Moraxellaceae	1	0.9	10
Chromobacterium violaceum	0	0	0	Neisseriaceae	0.17	0.33	4
Pseudomonas sp.	5.10	12.3	7	Pseudomonadaceae	5.15	12.2	8
Shewanella algae	3.60	7.2	3	Shewanellaceae	3.66	7.14	4
Vibrio alginolyticus				Vibrionaceae			
Vibrio fluvialis	2.10	3.0	4	Vibrionaceae	21.14	20.13	12
Vibrio cholerae				Vibrionaceae			
Mycobacterium spp.	0.03	0.1	1	Mycobacteriaceae	0.03	0.1	1

GENERAL CONCLUSIONS

My dissertation provides insights into the microbial communities of endangered sea turtles. The microbial communities of Kemp's ridley turtles had not been evaluated in any context before this research. Further, this work adds data for green turtle microbiomes to a growing field and provides a first glimpse into the oral microbes for any species of sea turtles. In addition to characterizing the Kemp's ridley turtle microbiome for the first time, I investigated how the microbial communities are affected by the local environment, disease, and medical treatment. This information is useful in understanding effects of disease and rehabilitation, and for assessing diagnostic methods.

By characterizing the microbiome of healthy wild-caught green and Kemps ridley sea turtles from the same environment, I identified distinct differences due to species, ruling out location-specific environmental variables as the primary source of microbes for these turtle species. Evaluating microbiomes of healthy wild animals provides a background for monitoring health and disease states in future studies. I identified the core microbes for each species, creating a baseline against which we can make future comparisons to assess changes in the health of this population. I also evaluated correlation between the microbiome of green

turtles with the incidence of fibropapillomatosis, showing a potential relationship worth exploring in future studies of this infectious disease.

I also examined cold-stunned Kemp's ridley turtles throughout rehabilitation and found a lack of correlation with clinical status and the microbial communities at each body site. I did find differences between turtles that survived and those that died from cold-stunning and identified ASVs important to predicting survival of the turtles. The microbiome shifts throughout rehabilitation as the turtles recover and are exposed to antibiotic therapy. These results show that microbial communities of sea turtles shift based on local environmental conditions, disease states, and antibiotic exposure, ultimately playing a role in their recovery and release status.

Since pneumonia is a highly prevalent complication of cold-stunned Kemp's ridley turtles, I examined how this disease is correlated with the respiratory microbiome. I found no differences in microbial communities based on radiographic lung abnormalities but identified a high degree of variability among individual turtles. By comparing culture results to sequence data and analyzing multiple sites along the respiratory tract, I also concluded that tracheal washes are not truly representative of the lungs and site of infection. Understanding microbial communities in disease and through diagnostic methods not only improves understanding of pathogenesis, but also allows us to understand ways to improve diagnostic tools in characterizing diseases.

In summary, these three chapters contribute to understanding microbial communities in health and disease of sea turtles. I found core microbial communities in two species of wild sea turtles, identifying important species differences, and providing the first

characterization of microbial communities of Kemp's ridley turtles. I also investigated the many effects that cold-stunning and rehabilitation have on stranded sea turtles in the northeastern United States based on environmental influences, survival, clinical condition, and antibiotic exposure. Further, I highlighted the complex pathogenesis of pneumonia in cold-stunned turtles and identified limitations of common diagnostic tools in characterizing pneumonia. As the environment changes, disease conditions occur, and rehabilitation efforts are utilized, microbial communities of sea turtles are playing a role in health and disease. It is therefore important to understand how the sea turtle microbial communities correlate with health and disease of these endangered species.

BIOGRAPHICAL SKETCH OF AUTHOR

Kerry McNally graduated with a Bachelor of Arts with a major in Biology from Occidental College in May 2005. During the following years, she worked in domestic animal veterinary practices until beginning work as a Biologist at the New England Aquarium (NEAq) in Boston, MA in November 2008. Her position most recently is a Senior Biologist in the Animal Health Department at NEAq. She started graduate school in January 2015 at University of Massachusetts Boston in the Environmental Sciences program, focusing her research on microbial communities of sea turtles, integrating her studies with her work at NEAq.