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USING COMPARATIVE METAGENOMICS TO DETERMINE THE ROLE OF ATLANTIC BOTTLENOSE DOLPHINS (TURSIOPS TRUNCATUS) AS SENTINELS FOR HUMAN RESPIRATORY HEALTH

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USING COMPARATIVE METAGENOMICS TO DETERMINE THE ROLE OF ATLANTIC BOTTLENOSE DOLPHINS (*Tursiops truncatus*) AS SENTINELS FOR HUMAN RESPIRATORY HEALTH

by

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DEDICATION

For God, my strength and my refuge, who pulled me out of the mud and the mire and set my feet on solid ground, whose everlasting arms are beneath me, and who freed me from the chains of my dark affliction. For Mick, the love of my life and my very best friend, who has loved me for everything that I am from the very beginning, who keeps me laughing, and whose smile is a light in my heart. You are in every part of me; I’m yours and that’s it forever. For Mutt and Dad, who never gave up. You have given everything; spared no expense and unconditionally loved and supported me through unimaginable circumstances. There is no way to thank you for what you have done; I can only live in such a way that every tear, sleepless night, and moment of helplessness was worth it. For Rach, whose love has ensured that I never wallow in self pity, who has never let me feel sorry for myself or fish for compliments, and who has no patience for weakness. You have kept me strong and taught me the art of both sarcasm and yoga. For Rebie, my baby sister who believed in me through it all. You never wavered, never questioned whether or not I would pull through, and never lost faith. At my lowest, I lived for you. We start with our bunk bed bond and reach across heartsore valleys and fairytale peaks. For Josh, Ash, Dan, Ashlee, Stephen, and Aunt Toni, who really didn’t have to keep loving me or praying for me, but chose to. For Nanallama, Moe, and Matt, my curly-haired towheads, whose laughter is light. Even when you’re grown, you’ll always be my sweet, little babies.
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ABSTRACT

Cetaceans are proposed as sentinel species for assessing the health of marine ecosystems and human coastal populations. This relationship has been based on similarities in the bioaccumulation of pollutants by cetaceans and humans, as well as their susceptibility to infectious disease. Respiratory disease represents a major cause of mortality in humans and cetaceans, and culture-dependent studies and 16S rDNA gene sequencing have identified known human respiratory pathogens from immunocompromised and healthy dolphins.

To broaden our understanding of the microbial community associated with dolphin upper respiratory tracts (URT), we characterized metagenomes generated from the URT of Atlantic bottlenose dolphins (*Tursiops truncatus*) from coastal Georgia. Using MG-RAST, we compared dolphin URT metagenomes to metagenomes from healthy human anterior nares, cystic fibrosis (CF) afflicted human lungs, and healthy human lungs. Human and dolphin respiratory metagenomes were dominated by four bacterial phyla (Actinobacteria, Proteobacteria, Bacteroidetes, and Firmicutes). Ascomycota and Basidiomycota were the only fungal phyla identified in the dolphin URT and human lung, whereas the human nares displayed greater fungal diversity. With mostly unclassified viruses in dolphins, order-level viral comparisons demonstrate similarities between dolphin URT and healthy human lung. However, Genus-level distributions showed differences, with the phage *Chlamydiamicovirus* dominating the
classified dolphin sequences and absent in the human respiratory metagenomes.

Known cetacean respiratory pathogens were detected in the dolphin and human metagenomes, including bacterial pathogens *Staphylococcus aureus*, *Streptococcus* spp., *Nocardia* spp., and *Aeromonas* spp.; fungal pathogens *Aspergillus* spp. and *Cryptococcus* spp.; and the protozoan *Toxoplasma gondii*. The bacterial pathogen *Erysipelothrix rhusiopathiae* and the fungal pathogens *Candida glabrata* and *Ajellomyces dermatitidis* were detected exclusively in the dolphin URT. Human adenoviruses A-G were detected in the dolphin URT and adenovirus C in the nares. Additionally, dolphin URT metagenomes were compared with healthy cetartiodactyls (big horn sheep, okapis, pig, giraffe, and cow) and healthy human fecal metagenomes. Bacteroidetes, Actinobacteria, Firmicutes, and Proteobacteria also dominated the fecal metagenomes, although the percentage of Bacteroidetes in human feces double that observed in the dolphin URT and cetartiodactyl fecal metagenomes. Fecal samples had higher fungal diversity and fewer unclassified viruses than the dolphin URT metagenomes.

Comparison between the dolphin, human and cetartiodactyl metagenomes reveal similarities at higher taxonomic levels. However, dolphin URT microbial community specificity is discernible across genera/species. These results broaden our understanding of the dolphin respiratory microbiome, and represent the first comparison with human respiratory metagenomes. Our findings help to establish a baseline respiratory microflora that can be used to validate the role of cetaceans as sentinels for ecosystem health and indicators of emerging marine mammal and human respiratory pathogens.
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CHAPTER 1: RESPIRATORY DISEASE IN DOLPHINS AND OTHER MARINE MAMMALS

1.1 INTRODUCTION

Over the past 40 years, a 39% increase in the coastal population of the United States has placed over 123 million Americans in close proximity to the marine environment (US Census Bureau 2011). With this surge in coastal populations, there has also been an increase in the number of marine (Harvell et al., 2004) and marine mammal (Dunn et al., 2001; Bossart 2007) diseases documented, suggesting that the relationship between human health and health of the marine environment is an increasingly important one. Our knowledge regarding the potential for the marine environment to serve as a reservoir for human pathogens and the possible threat these and other uncharacterized microorganisms pose to human and marine organism health is limited. Diarrheal pathogens (and proxies for these pathogens) have been systematically monitored in both marine and freshwater environments and the characterization of enteric diseases has been the focus of numerous studies (Fong and Lipp 2005; Hipsey et al., 2008). However the relationship between respiratory microorganisms of human clinical significance and marine organism health in coastal environments has not been clearly elucidated.

Marine mammals, such as cetaceans, are ideal sentinels for ocean health as they often reside in coastal habitats and are exposed to a wide variety of infectious biological
agents and chemical pollutants (Reddy et al., 2001; Wells et al., 2004; Conrad et al., 2005; Bossart, 2007). The bottlenose dolphin (*Tursiops truncatus*) commonly experiences respiratory illness (Lipscomb et al., 1996; Kennedy, 1998; Turnbull and Cowan, 1999; Tsang et al., 2002; Bossart, 2007). Establishing a baseline of microorganisms associated with bottlenose dolphin respiratory tracts will not only assist in determining health risks to wild dolphin populations but also help to identify both known and potentially emerging pathogens associated with bottlenose dolphins in a broader ecosystem context (Marine Mammal Commission, 2004). These baseline findings will shed light on bottlenose dolphins as sentinel species for the health of the coastal zone in ecological risk assessment (Ross 2000), and whether they can act as ecological reservoirs for known and emerging human and marine mammal pathogens.

Cetaceans and humans have physiologically similar respiratory systems (Reidenberg and Laitman 2008; Piscitelli et al., 2013) and are susceptible to a number of respiratory diseases. The respiratory system is a major interface between the body and the external environment; therefore, the normal flora that colonizes the upper respiratory tracts (URT) of both cetaceans and humans may function to protect the host organism from infection (Cangemi de Gutierrez et al., 1999). However, opportunistic microorganisms may become pathogenic when the indigenous microflora of the respiratory tract becomes suppressed (Lidbeck et al., 1987). For cetaceans, such as Atlantic bottlenose dolphins (*Tursiops truncatus*), respiratory illnesses have been proposed as the leading cause of mortality in both captive and wild animals (Johnson et al., 2009). Interestingly, upper and lower respiratory tract perturbations were the leading cause of human death in 2012 worldwide (World Health Organization 2012) and many of
the potential dolphin respiratory pathogens have been isolated from the respiratory system of healthy and diseased humans.

This chapter will review what is currently known about respiratory illness in bottlenose dolphins and other marine mammals. First, the anatomy of the cetacean respiratory system will be reviewed with the inclusion of comments about the specialization of the dolphin respiratory organs for an aquatic existence. Second, comparisons between clinical isolation (culture-dependent) and molecular (culture-independent) approaches to describe respiratory microbial communities will be made. Finally, the known etiologic agents, including bacterial, fungal, and viral agents, of respiratory infections in marine mammals, specifically odontocete cetaceans, will be discussed. The description of each pathogen will include individual case study examples, which will combine details about the infected animals, as well as clinical symptoms and necropsy and histopathological findings. In summary, this chapter will review what is found in the published literature regarding respiratory disease in odontocete cetaceans.

1.2 DOLPHIN LUNG ANATOMY AND PHYSIOLOGY

The anatomical organization of the dolphin’s respiratory tract is well-adapted for the air-breathing, ocean-dwelling lifestyle of a marine mammal. The dolphin “nostril” is in the form of a blowhole dorsally located in a position posterior to the melon, a lipid and connective tissue structure used in echolocation (Berta and Sumich 1999; McKenna et al., 2012) (Figure 1.1). The location of the blowhole allows the dolphin to access air without exerting the energy necessary to lift its head from the water (Reidenberg and Laitman
The blowhole is actively opened during an inhalation and, at all other times, passively sealed by fibrous, fatty connective tissue (Berta and Sumich 1999) further saving energy.

Inspired air travels past the blowhole opening and through the smooth-walled, bony nasal cavity. The nasal cavity is lined by a number of air sacs, diverticula, which transport air back and forth for use in whistle creation and as a means of increasing lung oxygenation (Reidenberg and Laitman 2008). The nasal cavity delivers the air to a highly specialized larynx, which is elongated into a goosebeak-shaped formation (Reidenberg and Laitman 1987) and held upright by sphincter muscles (Lawrence and Schevill 1965). By separating into channels that pass around the laryngeal spout, the oral cavity effectively circumnavigates the air passage, fusing into the esophagus posterior to the larynx. This morphological separation of the digestive tract and the respiratory tract protects the dolphin from drowning during feeding and vocalization (Reidenberg and Laitman 1987). From the larynx, the inspired air travels through the short, wide trachea, the entire length of which is spiraled by flexible cartilaginous rings that serve to reinforce and protect the non-collapsing air passageway (Yablokov 1972; Berta and Sumich 1999; Moore et al., 2014). The trachea bifurcates into bronchi which subdivide into branched bronchioles, each ending in alveoli (Coffey 1977), where gas exchange occurs. The delivery of air to these alveoli is the primary function of the respiratory system in marine mammals.

Because dolphins are unique air-breathing mammals living in an aquatic environment, the evolutionary adaptations seen in the respiratory system are evidenced by a number of unusual morphological features (Bagnoli et al., 2011). In addition to the
unique anatomical organization of the respiratory system, specializations for rapid surfacing and deep diving support the conundrum of an air-dependent animal living in the ocean. In most terrestrial mammals, locomotion and the respiratory system are mechanically coupled, meaning that stamina is dependent upon aeration of the muscles. In dolphins, these systems are decoupled since the dolphin lives and functions on extended breath holds separated by quick, forceful surface respirations (75-90% of total lung volume in 0.3 seconds) (Ridgway et al., 1969). In order to accommodate the rapid and forceful expirations of these mammals, the trachea and bronchi have cartilaginous reinforcements that extend to the entrance of the alveoli. Such reinforcements may also serve to accommodate air from the alveoli during dives (Scholander 1940). It has been theorized that alveolar compression and collapse pushes air into the upper airways during breath-holds, decreasing gas exchange and effectively limiting the dissolution of gasses into the bloodstream that could cause aeroembolism upon ascent (Bostrom et al., 2008).

Because the respiratory system represents a major interface between the marine mammal body and the external environment, another major function of this system is the protection of the respiratory tissues from pathological damage (Suzuki et al., 2008). Due to a mucus layer in the lungs that traps inhaled particles, propelling them upward with cilia to be expectorated or swallowed, the lower respiratory tract of mammals was previously believed to be sterile (Hart and Winstanley, 2002) while the upper respiratory tract is known to be colonized by an indigenous microbiota (Sansonetti 2011; Pettigrew et al., 2012). However, recent evidence suggests that both the upper and lower respiratory tracts contain resident microbes in healthy mammals (Charlson et al., 2011; Beck et al., 2012; Charlson et al., 2012; Blainey et al., 2012).
The anatomy and physiology of the dolphin is specialized for life in an aquatic environment. Unfortunately, the respiratory specializations of these ocean-dwelling mammals are the cause of certain vulnerabilities, as well. While the rapid exchange of air during a surfacing event is a marked adaptation of the dolphin respiratory system, the lung volume utilization of up to 90% puts the animal at increased risk of respiratory infections (Olsen et al., 1969; Ridgway 1972). By using so much of their lung volume (comparatively, humans use only 20%), air is drawn into the dolphin’s deep lung which, combined with the absence of an upper airway filtration system, puts the animal at greater risk for respiratory tract infections (Venn-Watson et al., 2012).

1.3 DOLPHIN RESPIRATORY MICROBIOME

Mammalian respiratory health is greatly influenced by the URT microbiome, a term that refers to the community of commensal, symbiotic, and pathogenic microorganisms sharing the body space of an individual organism (Lederberg and McCray 2001). While little is known about the microorganisms normally associated with the URT of dolphins, respiratory pathogens have been isolated from the URT of healthy animals in a number of studies (Buck et al., 2006; Johnson et al., 2009; Morris et al., 2011). Evidence suggests that pathogens that normally act as benign residents of the microbiome may illicit respiratory disease in immunocompromised animals; therefore, characterizing the dolphin respiratory microbiome will not only allow us to better understand the cause and progression of respiratory diseases in these animals, it will also
help us differentiate a healthy, normal respiratory community from a compromised one. Also, knowledge of the ecology and composition of these resident microorganisms will give insight into the dolphin’s role as a sentinel species for ecosystem health (Bossart 2010; Morris et al., 2011).

Although it is well known that most microorganisms are not culturable using traditional isolation approaches (Amann et al., 1995; Rappe and Giovannoni 2003; Pedrós-Alió, 2006, Dethlefsen et al., 2007), many of the studies on the indigenous microorganisms of the dolphin URT have relied on culture-dependent methods. Gaining a deeper understanding of the dolphin URT normal flora will require techniques that characterize the unculturable portion of the microbial consortium. Combined, the clinical and molecular approaches to assessing the diversity of the dolphin microbiota will help us better determine the suitability of the dolphin as a sentinel species, and generate interesting hypotheses related to the co-evolution of an important marine mammal and its microbiota.

1.3.1 Culture-dependent Studies

Culture-dependent studies offer a means of growing microorganisms in vitro for use in genomic and biochemical studies. From a clinical perspective, cultivation is an important first step for identifying potentially significant microorganisms (Buck et al., 2006). The clinical isolation of microorganisms is essential for phenotypic characterization and sequencing of whole genomes, which has led to key insights into the pathogenicity of infectious diseases (Bryant et al., 2004). Many disease studies in humans
and other animals have relied on culture-dependent approaches to identify etiologic agents, significantly skewing our current realm of knowledge in favor of culturable organisms. Culture-dependent analyses offer an incomplete picture of the microbes present in a given community due to the biases that result from cultivation based on the perception and successful duplication of an ecological niche (Ward et al., 1990). The selectivity of culture media limits which members of a community have the nutrient requirements for growth and, therefore, this technique favors the 1-10% of culturable microbes present in the environment (Torsvik et al., 1990). Furthermore, in the following studies, cultures were dependent upon media that is typically used to identify known human pathogens, meaning that human pathogens were the most abundant in the identified microorganisms.

In 2006, Buck et al. used culture-dependent methods to analyze the blowhole and anal/fecal samples of free-ranging bottlenose dolphins captured and released in Sarasota Bay, FL, Matagorda Bay, TX, and Beaufort, NC. Vibrios were identified in 97% of the blowhole samples, with *V. alginolyticus* and *V. damsela* being recovered most often. *Acinetobacter/Pasteurella/Pseudomonas* spp., *Candida* spp., *Enterobacter* spp., and coryneforms were also identified in 10-20% of the blowhole samples. A 2011 culture-dependent study by Morris et al. analyzed blowhole, gastric, and fecal samples from 180 free-ranging bottlenose dolphins in Charleston Harbor, SC and Indian River Lagoon, FL. The gram-negative bacteria *Aeromonas hydrophila* and *Plesiomonas shigelloides* were the most frequently cultured isolate from the blowhole samples. *Pseudomonas fluorescens* and *Vibrio alginolyticus* were also cultured from 11.7% and 10.2% of blowhole swabs, respectively.
Although we cannot make any definitive assertions as to the role of these organisms as causative agents of respiratory disease, these culture-dependent studies demonstrate that we are able to isolate organisms that are both known human pathogens and known respiratory pathogens from blowhole samples in free ranging dolphins. Because many of the known human pathogens have been isolated from a number of diverse environments, comparing the pathogenicity of these microorganisms between humans and dolphins will require a broader look at the microflora of the dolphin URT.

1.3.2 Culture-independent Studies

A large percentage of microorganisms observable in nature cannot be cultivated by standard laboratory techniques (Hugenholtz et al., 1998); therefore culture-independent studies have emerged as a means of bypassing cultivation. Culture-independent studies allow us to characterize an organism phylogenetically, an assessment that is based on gene sequences that can be taken directly from a sample containing DNA (Hugenholtz and Pace 1996). While culture-independent techniques are greatly advantageous, by helping to sharpen the picture of microbial communities that was previously clouded by culture-dependent limitations, there are still a number of limitations and disadvantages associated with culture-independent studies. For example, dormancy of certain microorganisms may limit the extent to which microbial activity is understood in a sample (Rincon-Florez et al., 2013).
Johnson et al. (2009) used 16S rDNA sequencing to analyze the bacterial communities of the URT of bottlenose dolphins using a live capture and release method. URT samples were obtained non-invasively by gently rubbing a swab along the interior walls of the blowhole. The authors found a novel bacterial community associated with the blowhole of two healthy free-ranging dolphins from Charleston Harbor, SC and two from Indian River Lagoon, FL. The sequences from the swab samples were dominated (96%) by three bacterial phyla in all four dolphins: *Proteobacteria*, *Bacteroidetes*, and *Firmicutes*. These findings indicate that diversity is highest at the species/strain level and that microbial communities were fairly similar at higher taxonomic levels meaning that, among animals in similar states of health, habitat is likely the greatest influence in microbial diversity. A study by Lima et al. (2012) involved the pyrosequencing of the bacterial 16S rRNA gene variable regions with results similar to those described by Johnson et al. (2009). Using non-invasive blow capture methods, the authors sampled Atlantic bottlenose dolphins (*Tursiops truncatus*), Indo-Pacific bottlenose dolphins (*Tursiops aduncus*), and four hybrid *T. truncatus* and *T. aduncus* species that were held in captivity in Queensland, Australia. The sequences were dominated (98%) by six bacterial phyla: *Proteobacteria*, *Firmicutes*, *Fusobacteria*, *Actinobacteria*, *Bacteroidetes*, and unclassified bacteria. Interestingly, *Gammaproteobacteria* accounted for an average of 75% of all blow sample sequences. These findings are similar to those of Johnson et al. (2009), which showed *Gammaproteobacteria* accounting for an average of 55% of all sequences. A comparison by Lima et al. (2012) between the results from these two studies found that 38.8% of the captive sequences showed ≥ 97% similarity across the sequence length to those from free-ranging dolphins. This may indicate that the
microflora of the dolphin URT is not significantly altered when these animals are kept in captivity. Lima et al. (2012) also found that both the captive and the free-ranging (Johnson et al., 2009) community abundances of the *Gammaproteobacteria* were dominated by the family *Cardiobacteriaceae*. Because this particular family of bacteria is responsible for a number of illnesses in humans, such as endocarditis and pneumonia (Das et al., 1997), the phylogenetically distinct clades that were found in the dolphin URT may display the same opportunistic pathogenesis seen in other clades within the *Cardiobacteriaceae* family.

1.4 RESPIRATORY DISEASES IN DOLPHINS (AND OTHER CETACEANS)

In spite of the number of reports detailing respiratory diseases in dolphins, a lack of data has hindered the advancement of our understanding of these diseases. For example, most studies focus on illness in captive or stranded dolphins. There is limited data regarding the prevalence and progression of respiratory diseases in free-ranging dolphin populations. Furthermore, respiratory infection in nearly every study is revealed through necropsy. Even when presented with the opportunity to study a stranded animal, a number of factors may skew the data, leading to inconclusive results. Contamination resulting from the difficulties of sampling in a non-sterile environment may identify a misrepresentative number of primary, opportunistic, commensal, or even environmental microorganisms (Venn-Watson et al., 2008). Another challenge to understanding respiratory infections in dolphins is that disease is rarely detected until a large area of the lung has been damaged (Dunn et al., 2001). This means that respiratory illnesses are
seldom diagnosed until the disease has progressed substantially. Even in captive animals
documenting the progression and cause of a respiratory disease is a challenge because the
primary pathogens may be buried in a mass of secondary and opportunistic
microorganisms. Furthermore, many of the studies on respiratory disease in dolphins
have utilized different methods to identify the responsible pathogens. Some authors
attempt to identify the microorganisms using the most advanced laboratory techniques
while others may simply use cultured human pathogens to identify organisms, limiting
the range of possibilities to only human pathogens (Higgins 2000; Gulland et al., 2007).
Regardless of the varied approaches, there are certain respiratory pathogens that have
been clearly shown to cause disease in cetaceans. Necropsy of the respiratory tract of
diseased captive and free-ranging cetaceans in multiple studies have consistently revealed
the same viral, bacterial, fungal, and protozoal pathogens. Some or all of the respiratory
diseases discussed in this review may be more complex than we currently realize due to
the interactions that are maintained within communities of microorganisms. Additionally,
the small number of individuals in most case studies is insufficient for deducing how
these diseases may impact cetacean populations. The known odontocete respiratory
pathogens, along with a number of case studies detailing the results of primarily necropsy
analyses, are discussed below. The pathogens include viruses, protozoans, fungi, and
bacteria, while the infected animals include stranded, captive, and occasionally free-
ranging animals.
1.4.1 Characterized Respiratory Diseases in Dolphins and Other Cetaceans

**Viral Diseases**

As obligate intracellular parasites, viruses are generally considered nonliving microscopic infectious agents. The replication of viruses, which consist of genetic material encased by a protein, lipid, or glycoprotein shell, depends upon the successful attachment of a parasite to a host cell, which is followed by the injection of genetic material into the cell and subsequent generation of virally encoded enzymes and nucleic acids via the alteration of biosynthetic operations within the host (Madsen 2011). Upon cell death, the newly created parasites continue the cycle of host attachment and infection, resulting in the exponential production of viruses. A number of viruses are known respiratory pathogens, such as respiratory syncytial virus, parainfluenza and influenza virus, and human metapneumovirus (Geretti 2003).

In terms of infectious diseases, viral pathogens are responsible for more marine mammal mass mortality events than any other etiologic organism (Gulland et al., 2007) (Table 1.1). Host specificity varies among marine mammal viruses in that some viral agents are order specific, such as morbillivirus, and others may affect mammals across classes or even phyla (Van Bressem et al., 1999). The importance of viral pathogens in marine mammals has been historically ignored as evidenced by the lack of viral pursuance during histopathological evaluations (Anthony et al., 2013). However, as technology and cetacean virology advances, it is likely that most, if not all, future investigations into cetacean respiratory perturbations will include pathological studies for viral diseases (Van Bressem et al., 1999; Anthony et al., 2013).
Cetacean Morbillivirus (CeMV)

Morbillivirus is perhaps the most frequently documented respiratory disease in cetaceans. Outbreaks of morbillivirus are responsible for numerous mass mortality events in wild marine mammals, specifically bottlenose dolphins (*Tursiops truncatus*) and striped dolphins (*Stenella coeruleoalba*) (Shimizu et al., 2013). The close antigenic and genetic relationships of the four morbilliviruses, including canine distemper virus (CDV), phocine distemper virus (PDV), dolphin morbillivirus (DMV), and porpoise morbillivirus (PMV), coupled with the possibility of multiple morbilliviruses causing infection and the difficulty of laboratory cultivation, greatly complicates the identification of the primary viral agent. Dolphin morbillivirus (DMV) and porpoise morbillivirus (PMV) are so closely related that they are generally consider one species, cetacean morbillivirus (CeMV) (Saliki et al., 2002), which was initially isolated from the lungs of necropsied harbor porpoises (*Phocoena phocoena*) off the coast of Northern Ireland in 1988 (McCullough et al., 1991). Since the initial identification of CeMV, morbillivirus epidemics of striped dolphins in the Mediterranean Sea in 1990-1992 (Domingo et al., 1992; Duignan et al., 1992; Barrett et al., 1995), Atlantic bottlenose dolphins along the eastern coast of the United States in 1987-1988 and in the Gulf of Mexico in 1993-1994 (Lipscomb et al., 1994; 1996), as well as a 2013-2014 unusual mortality event in Atlantic bottlenose dolphins along the eastern coast of the United States have resulted in the mass die-off of thousands of cetaceans (Shimizu et al., 2013). There have been comparatively few reported cases of CeMV in the southern hemisphere and no mass mortality events. Stephens et al. (2014) found a novel strain of CeMV in three Indo-Pacific bottlenose dolphins that died in 2009 off the Swan River in Western Australia, while Stone et al.
(2011) identified morbillivirus in the lung tissue of a stranded Atlantic bottlenose dolphin near Fraser Island, Queensland, Australia. Additionally, Groch et al. (2014) isolated a novel strain of CeMV from the lungs of a Guiana dolphin (*Sotalia guianensis*) stranded off the coast of Brazil.

Though little is known about CeMV pathogenesis or the clinical signs involved with infection, in post-mortem analyses pneumonia is the most common pathological evidence of disease in cetaceans infected with CeMV. Necropsy primarily reveals congested, emphysemic lungs while histopathological findings often consist of necrosis of bronchial and bronchiolar epithelium and serofibronous exudates, leucocytes, and macrophages in the bronchial, bronchiolar, and alveolar lumina (Di Guardo et al., 2005). The defining histopathological finding of CeMV, however, is the presence of Warthin-Finkeldey type syncytia, the giant multinucleated cells characteristic of measles (Nozawa et al., 1994) and HIV-infection (Orenstein 1998), in the bronchial, bronchiolar, and alveolar lumina (Di Guardo et al., 2005).

**Influenza A**

While wildfowl and shorebirds are believed to be the natural reservoirs for the influenza A virus strain, the evolution and ecology of this virus has broadened the pool of potential hosts subsequently facilitating the transmission of influenza A between animal species (Olsen et al., 2006). The rapid evolution observable in the antigenic drift and genetic shift in influenza A viruses in humans and other mammals is of concern due to the many human epidemics that this disease has caused (Webster et al., 1992). Two
subtypes of influenza A, distinguishable from influenza B and C based on the identity of the nucleoproteins and matrix proteins, were identified in a sickly, free-ranging pilot whale (*Globicephala melaena*) near Portland, ME (Hinshaw et al., 1986). Necropsy revealed an enlarged hilar node and hemorrhagic lungs. Influenza A subtype H13N2 was isolated from the hilar node and lung while H13N9 was isolated from the hilar node and both subtypes are believed to have originated from gulls.

**Parainfluenza Virus**

In a study by Nollens et al (2008), a novel form of parainfluenza virus (PIV) closely related to the human and bovine forms was cultured from antemortem samples of fine need aspirates of the lung and postmortem samples of lung tissue of an Atlantic bottlenose dolphin (*Tursiops truncatus*). Parainfluenza viruses are enveloped RNA viruses of the family *Paramyxoviridae*. These nonsegmented, negative-strand viruses commonly cause respiratory infections in humans and other terrestrial mammals (Hall 2001; Dochow et al., 2012). *Tursiops truncatus* parainfluenza virus type 1 (TtPIV-1) was isolated from a dolphin with severe respiratory disease, including bronchointerstitial pyogranulomatous pneumonia, tracheitis, and laryngitis (Nollens et al., 2008). The 19-year old, male animal had raspy breathing patterns, coupled with odorous expirations, as well as cream-colored exudate from the blowhole. Further symptoms included a decrease in appetite and lethargy. Necropsy revealed enlarged, darkened thoracic lymph nodes, fluid in the trachea, severe congestion in the lungs, and purulent discharge from smaller airways. Histopathological studies identified *Escherichia coli* in the pleural fluid and
Candida glabrata, E. coli, Proteus mirabilis, Proteus vulgaris, and Vibrio alginolyticus in the lung tissue. The cause of death was attributed to respiratory disease of undetermined origin due to the complex pathological findings in the lungs, larynx, and trachea. TtPIV-1 was isolated from fine needle aspirates of the lung, lung tissue, pleural fluid, and the pulmonary lymph node. Phylogenetic analysis revealed a close clustering with paramyxoviruses in the subfamily Paramyxovirinae, genus Respirovirus. The authors concluded that TtPIV-1 is most closely related to bovine parainfluenza virus type 3 (BPIV-3) and human parainfluenza virus type 3 (HPIV-3). Categorizing this virus as a novel respirovirus species is further substantiated by the similarities of the clinical manifestations between the infected dolphin and parainfluenza infection in other mammals.

Polyomavirus

A novel cetacean polyomavirus (PyV), described by Anthony et al. (2013), was isolated from the lungs and trachea of a short-beaked common dolphin (Delphinus delphis) stranded in San Diego, CA. PyV infection has been identified in human, non-human mammalian, and avian hosts. In mammals, PyV appears to be benign in the immunocompetent, only presenting with symptoms of disease upon suppression of the immune system (Krumbholz et al., 2009). In the study by Anthony et al. (2013), necropsy revealed multifocal ulcerative lesions in the trachea and bronchi, accompanied by epithelial loss and karyomegaly in the mucosa of the larynx. PyV, which appeared solely in the respiratory organs, was three orders of magnitude higher in the trachea than the
lungs, with no observable pathologic changes in lung histology, suggesting that infection was exclusive to the upper airways.

Protozoan Diseases

Protozoa, unicellular eukaryotes, exist as parasitic heterotrophs or free-living autotrophs. Protozoal respiratory infections in humans may occur through direct damage to the parenchyma, dissemination via the bloodstream, or by direct contact with an adjacent lesion (Martinez-Giron et al., 2008). Protozoal respiratory infections often result from specific instances of immunosuppression, such as cancer or AIDS (Lednicky and Rayner 2006). Currently, the only known protozoan respiratory disease in cetaceans is the cat-associated parasite *Toxoplasmosis gondii* (Table 1.2). This well-documented pathogen, which affects humans and marine and terrestrial animals, has been identified in numerous cases of marine mammal pneumonia.

*Toxoplasmosis gondii*

Infection by the protozoan parasite *Toxoplasma gondii* in marine mammals may indicate oocyst contamination of the ocean environment (Dubey et al., 2009) via runoff of wild and domestic cat excrement (Miller et al., 2002; Conrad et al., 2005). Cats are not only natural reservoirs of *T. gondii*, a parasitic member of the phylum Apicomplexa, they are also the only definitive hosts (Dubey et al., 1970; Dubey 2004). While the sexual cycle and oocyst formation of *T. gondii* occur only in domestic and wild felines, many
warm-blooded animals have been identified as intermediate hosts, including mice, pigs, and birds, as well as humans (Frenkel and Dubey 1972; Dubey 2004; Dubey and Jones 2008). *T. gondii* may infect a host via the ingestion of tissue of an infected animal or consumption of food or drink contaminated with sporulated oocysts (Dubey 2004). Additionally, the possibility of congenital acquisition may lead to fetal death or blindness or mental retardation either at birth or later in childhood (McAuley et al., 1994; Guerina et al., 1994). The first report of toxoplasmosis in cetaceans came from a Guiana dolphin in Brazil (Bandoli and de Oliveira, 1977). Since then, histological examination of deceased animals has revealed *T. gondii* infection in various cetaceans, including the Atlantic bottlenose dolphin (Inskeep et al., 1990). Inskeep et al. (1990) documented the stranding of a female Atlantic bottlenose dolphin and her calf in Tampa Bay, FL. Necropsy revealed severe cases of pneumonia in both animals, as well as lymphadenopathy. The mother had alveolar edema and signs of fibrinous exudation while the calf displayed mild lymphoid atrophy. Necropsy reports from infected cetaceans since Inskeep et al. (1990), including striped dolphins (Dubey et al., 2007; Di Guardo et al., 2009), an Indo-Pacific bottlenose dolphin (*Tursiops aduncus*) (Jardine and Dubey 2002), a spinner dolphin (*Stenella longirostris*) (Migaki et al., 1990), beluga whales (*Delphinapterus leucas*) (Mikaelian et al., 2000), a Guiana dolphin (Gonzales-Viera et al., 2013), and many Atlantic bottlenose dolphins (Dubey et al., 2009(a); 2009(b)) have revealed lymphadenitis, necrotizing adrenal adenitis, myocarditis, acute interstitial pneumonia, non-suppurative encephalitis and systemic disease as the primary manifestations of *T. gondii* infection. As previously mentioned, oocyst ingestion from contaminated food or water and the ingestion of *T. gondii*-infected tissues are the two
main sources of postnatal *T. gondii* infection (Dubey and Jones 2008); curiously, neither source can be easily applied to toxoplasmosis in cetaceans. In addition to feeding solely on cold-blooded animals, cetaceans drink little or no water, meaning that another source of *T. gondii* infection must exist but has not yet been identified (Dubey et al., 2009(a)).

Fungal Diseases

Fungi are eukaryotic, multicellular microorganisms with two basic morphological forms: Yeasts, the unicellular form that reproduces asexually via blastoconidia, and molds, the multicellular form with hyphae capable of asexual or sexual reproduction (McGinnis and Tyring 1996). In human medicine, dimorphic organisms are fungi that may exist in two different phenotypic states, typically as a mold *in vitro* (or at room temperature) and as a yeast *in vivo* (or at human body temperature) (McGinnis and Tyring 1996). The frequency of human fungal infections has significantly increased in recent decades, particularly in immunosuppressed individuals (Karkowska-Kuleta et al., 2009). A number of aerially transmitted fungi have been identified as respiratory pathogens in humans, as well as opportunistic fungi associated with the indigenous flora of the human respiratory tract, including *Aspergillus fumigatus* and *Candida* spp. (Aliouat-Denis et al., 2013). Similarly, primary fungal infections in the cetacean respiratory system occur through the inhalation of fungal pathogens (Reidarson et al., 1999), while some fungal agents exist as part of the normal microflora of the cetacean respiratory tract (Morris et al., 2011). Due to the ease with which these air-breathing marine mammals may contract airborne spores, fungal infection frequently causes
respiratory disease, as evidenced in the study by Reidarson et al. (1999) which found 19 fungal species in 143 infected marine mammals with fungal agents isolated from the lungs of 49% of the animals (Table 1.2).

Aspergillus spp.

Aspergillus spp. infections are the most commonly reported of the systemic cetacean mycoses (Reidarson et al., 1999). Due to the abundance of these fungi in nature, aspergillosis, most often caused by *Aspergillus fumigatus* and less often by *A. niger* and *A. terreus*, occurs worldwide. *A. fumigatus*, a saprophytic fungus, enters the lungs via direct inhalation by the host. After settling within the host lungs, rapid sporulation spearheads the aggressive overtaking of the bronchial tissue. Fungal infection by *Aspergillus* spp. may occur in a chronic and debilitating form, such as allergic aspergillosis, chronic necrotizing aspergillosis or aspergillomas, or it may take the fulminating form, invasive aspergillosis, which spreads throughout the entire internal organ system, leading to a rapid death (Reidarson et al., 1999). In 1998, Reidarson et al. reported the development of a harsh, dry cough in a male Atlantic bottlenose dolphin in captivity at Sea World California. In addition to a cough, the animal was slightly underweight and had uneven inhalations and exhalations. Bronchoscopy was performed, and biopsy of a yellow endobronchial lesion just beyond the carina of the trachea revealed *A. fumigatus*. This animal was successfully treated for *Aspergillus* spp. infection. Abdo et al. (2012) detailed the case of a female orca whale (*Orcinus orca*) in captivity at the Port of Nagoya Public Aquarium, Japan. Lethargy and anorexia were
initially observed and necropsy of the lungs revealed foamy exudate from the bronchi, multifocal consolidation in the caudal lobes, and a multitude of 1-3 cm abscesses. Additionally, the thoracic and visceral lymph nodes showed lymphadenitis with congestion and edema. Lung cultures revealed the presence of *Aspergillus* spp. *Aspergillus* spp. infections have also been found in a narwhal (*Monodon monoceros*), a pygmy sperm whale (*Kogia breviceps*), and California sea lions (*Zalophus californianus*) (Sweeney et al., 1976). Finally, Morris et al. (2011) isolated *Aspergillus fumigatus* and *Aspergillus niger* from the blowhole samples of visually healthy Atlantic bottlenose dolphins, indicating that *Aspergillus* spp. may be part of the normal microflora of the dolphin’s URT. In a report by Young et al. (1999), *Aspergillus fumigatus* was isolated from the air samples of four captive beluga whales. As none of the animals were ill at the time of isolation, nor did any of the animals become diseased afterward, the authors concluded that the fungi were environmental contaminants, rather than pathogens.

*Blastomyces dermatitidis*

*Blastomyces dermatitidis*, the causative agent of blastomycosis, is a dimorphic fungus that has been reported to infect the lungs of humans and several species of animal, including marine mammals (Williamson et al., 1959; Sweeney et al., 1976; Cates et al., 1986; Zwick et al., 2000; Bradsher et al., 2003). *B. dermatitidis* conidia proliferate as saprophytes in nature and infection results from the inhalation of these spores into the alveoli (Migaki and Jones 1983; Bradsher et al., 2003), where the warm environment of the lungs elicits the conversion of *B. dermatitidis* from the mycelial form to the yeast.
form (Bradsher et al., 2003; Smith and Kauffman 2012). In marine mammals, this leads to the development of a primary lesion on the lungs with subsequent metastases to other organs (Migaki and Jones 1983). The clinical manifestation of *B. dermatitidis* in marine mammals is variable, depending on the severity of the lesion and the affected organs (Migaki and Jones 1983). Cates et al., (1986) detailed a case of blastomycosis in a female Atlantic bottlenose dolphin caught in the Mississippi Sound and held in an open ocean pen in Kaneohe Bay, HI. A year after her capture, she developed cranial swelling, along with lethargy and a greatly decreased appetite, and, despite treatment, died four weeks later. Upon necropsy, pulmonary lesions were characterized by hemorrhage and edema and spherical *B. dermatitidis* fungal cells (8 to 12 μm in diameter) were identified on the lungs, liver, kidneys, spleen, and lymph nodes. Severe necrosis was evident in the thoracic lymph nodes, in addition to pyrogranulomatous inflammation. The only other reports of *B. dermatitidis* infection in marine mammals describe cases of blastomycosis in captive sea lions (Williamson et al., 1959; Zwick et al., 2000).

*Coccidioides immitis*

Humans and animals, including marine mammals, may become infected by the fungus *Coccidioides immitis* via the inhalation and subsequent lodging of spores within the lungs (Laniado-Laborin 2007). Although much of what we know is based on studies of *Coccidioides immitis* as a soil mold, the formation of arthroconidia within the hyphae, which are dispersed by the wind and respired by humans and animals, have been shown to cause pneumonic symptoms in the infected individuals (Laniado-Laborin
2007). In 1998, the sole reported case of coccidioidomycosis in cetaceans was described by Reidarson et al. An adult, female bottlenose dolphin rescued from a beach in La Jolla, CA displayed progressively worsening inspiratory dyspnea (shortness of breath) and was underweight. Her illness advanced quickly, resulting in unidirectional listing while swimming, in addition to buoyancy problems and complete cessation of food uptake. At necropsy, large, caseous nodules were found on both the lungs and the perihilar lymph nodes (Reidarson et al., 1998).

*Cryptococcus* spp.

Like *C. immitis*, primary infection by *Cryptococcus neoformans* is the result of inhalation of spores by humans and animals, including marine mammals (Migaki et al., 1978(a); Gales et al., 1985; Levitz 1991; Chayakulkeeree and Perfect 2008). Of the fungi classified in the genus *Cryptococcus*, *Cryptococcus neoformans* and *Cryptococcus gattii* are the main pathogens in humans (Kwon-Chung et al., 2002; La Hoz and Pappas 2013). *C. neoformans* has been closely linked to avian habitats (Emmons 1955; Chayakulkeeree and Perfect 2008) and has been isolated from a wide range of geographical locations (Levitz 1991). *C. gattii*, on the other hand, has proven much more elusive and difficult to isolate. Correlations have been made between *C. gattii* and the eucalyptus tree *Eucalyptus camaldulensis*, with an 8-month study by Ellis and Pfeiffer (1990) finding that *C. gattii* was specifically associated with the wood, bark, and leaves of *E. camaldulensis*, as well as the debris beneath the canopy cover of the tree. Furthermore, the appearance of *C. gattii* was observed to coincide with the flowering of *E. camaldulensis* (Ellis and Pfeiffer...
The first case of *C. neoformans* to be described in a dolphin involved a captive 7-year old, male bottlenose dolphin. A white-colored, subpleural nodule found on the animal’s left lung was found to be caused by *C. neoformans* (Migaki et al., 1978(a)). While a number of seafaring birds were observed to roost near the infected animal’s compound, no definitive source could be established as no studies were performed on the avian waste or nesting materials (Migaki et al., 1978(a)). The first documented case of *C. neoformans* var. *gattii* was in a captive 19-year old, male bottlenose dolphin (Gales et al., 1985). The dolphin displayed signs of illness such as tachypnea, dyspnea and tachycardia. The antibiotic and antifungal treatments that were used proved ineffective. Lesions throughout the lungs and enlarged pulmonary lymph nodes were observed at necropsy, and microscopy revealed many *C. neoformans* yeast cells in the lung tissue.

*Sporothrix schenckii*

*Sporothrix schenckii*, a dimorphic fungus, causes sporotrichosis in humans and various species of animals worldwide (Higgins 2000). Because *S. schenckii* grows in soil or vegetation, human infections are frequent in farmers, gardeners, and miners. Fungal contamination of skin lacerations is the most common cause of *S. schenckii* in humans and some animals (Migaki et al., 1978(b)), although pulmonary infection may occur following the inhalation of fungal spores (Migaki and Jones 1983). The only report of sporotrichosis in a cetacean is from Migaki et al. (1978(b)), describing a Pacific white-sided dolphin (*Lagenorhynchus obliquidens*) that was captured and held in captivity for three years at Sea World, Orlando, FL. While the clinical symptoms were limited to
erratic feeding, necropsy revealed a number of significant findings associated with the respiratory system. Visceral lesions were found on the mediastinal and bronchial lymph nodes, along with abscesses and hemorrhages in the lungs. Also, large amounts of serofibrinous exudate resulted in thickened pleura. Migaki et al. (1978(b)) concluded that the extent of the infection may be indicative of a suppressed immune system. While the origin of the infection in this report was undetermined, skin lacerations caused by the animal rubbing itself against the walls of the holding tank may have provided an entry point for *S. schenckii*.

**Zygomycosis**

A number of different pathogens cause the disease referred to zygomycosis in marine mammals. The term zygomycosis is used to describe any mycosis attributable to the species of the order Mucorales, with the genera *Absidia, Rhizopus, Mucor, Saksenaea, Rhizomucor, Cunninghamamella*, and *Apophysomyces*, and the order Entomophthorales, with the genera *Basidiobolus* and *Conidiobolus* (Ajello et al., 1976; Kwon-Chung 2012). In marine mammals, the clinical signs of zygomycosis are dependent upon the intensity of the infection, along with the specific organs and tissue structures that are affected (Migaki and Jones 1983). Cutaneous tissues and muscular tissues appear to be the primary sites of zygomycotic infection in marine mammals (Robeck and Dalton 2002) but the accessibility, and susceptibility, of the upper respiratory tract provides an alternate entry point for the pathogenic spores (Migaki and Jones 1983). Robeck and Dalton (2002) described four cases of fatal zygomycotic
infections, observable in the lungs, in marine mammals housed at SeaWorld Texas from 1991-2001. The first case, a 14-year old orca whale whose pregnancy was 13 months through a 17 month gestation, died during early labor after exhibiting signs of lethargy and decreased appetite, in addition to an increased white blood cell count. Necropsy of the lungs showed white froth in the bronchi and darkened lungs filled with fluid and white froth. *Saksenaea vasiformis* was identified as the causative fungal pathogen.

Another case documented in the report by Robeck and Dalton (2002) described a 17-year old Pacific white-sided dolphin that initially presented with an increased respiratory rate and a temporary listing to the right side. Upon necropsy, hemorrhaging was present throughout the trachea and large bronchi and the right lung contained a 6-cm diameter hollow cavity. Histopathology revealed hemorrhagic necrotizing pneumonitis in the lungs. The fungal cultures present were found to be *Apophysomyces elegans*. Robeck and Dalton (2002) also detailed zygomycotic in a newborn Atlantic bottlenose dolphin calf. At necropsy, the bronchi were filled with caseous material, the lungs were dark and meaty in texture, and the zygomycotic fungus *Saksenaea vasiformis* was found in the lungs. The final case in the Robeck and Dalton paper (2002) described a 20-month old captive born bottlenose dolphin. Although in good health, biopsies of an infected laceration and coincident lesions revealed cultures of *Apophysomyces elegans*. The same fungus was identified in the small, white nodules seen throughout the lungs during necropsy. Abdo et al. (2012) reported a case of pulmonary zygomycosis in a 28-year old orca whale in captivity at the Taiji Whale Museum in Taiji, Japan. After months of appetite irregularities, and culture samples revealing various fungal infections, the animal succumbed to disease. Necropsy revealed fluid in the thoracic cavity, enlarged bronchial
lymph nodes, and white froth in the trachea and bronchi. Nodules were found on the lungs, which were consolidated, along with a number of 3-5 cm cavities. Purulent bronchopneumonia was identified in the lungs, in addition to a large number of fungal hyphae, which were characterized as *Cunninghamella bertholletiae*. Morris et al. (2011) isolated *Cunninghamella bertholletiae* from the URT of a seemingly healthy free-ranging Atlantic bottlenose dolphin via a blowhole sample. This leads us to believe that certain zygomycotic pathogens may be associated with the normal microflora of species of marine mammals, perhaps as an opportunistic pathogen.

**Bacterial Diseases**

From soil and water to Arctic ice and hot springs, bacteria have been found in every testable ecosystem on Earth. As prokaryotes, bacteria have no nucleus or membrane-bound organelles except ribosomes, as well as pili for genetic material transfer to other cells and flagella for mobility (Nordqvist 2013). Believed to have been the first life forms to inhabit our planet, bacteria may exhibit heterotrophy or autotrophy using nitrogen, sulfur, phosphorous, etc. for synthesizing food. Although the normal flora of the human respiratory tract contains massive quantities of diverse bacteria that change over time, bacterial colonization in the URT may increase during respiratory illness (Faden et al., 1997). Many bacterial pathogens cause respiratory disease in humans, including *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Staphylococcus aureus*. A number of bacterial species have been identified as primary and secondary pathogens in marine mammal respiratory diseases (Dunn et al., 2001). Some microorganisms have
been determined as the cause of death in multiple studies of cetacean respiratory infection, while others have been reported only once or twice (Table 1.3). The greatest limitation in the identification of primary bacterial pathogens in deceased cetaceans, particularly stranded animals, may well be the possibility of a misdiagnosis of secondary infection attributable to malnutrition, parasitic disease, or neoplasia (Howard et al., 1983).

Actinobacillus spp.

Isolates of *Actinobacillus delphinicola* were first recovered post-mortem from stranded cetaceans in the northeast Atlantic by Foster et al. (1996). A 16S rRNA gene sequence analysis revealed that this gram-negative, rod-shaped bacterium represented a previously unknown line of descent in the family *Pasteurellaceae*, leading the authors to classify it as a new species of bacteria. In the report, *A. delphinicola* was isolated from the stomach and intestinal content of harbor porpoises, the lungs, gastric and mandibular lymph nodes, and intestinal content of a striped dolphin, and the lungs of a Sowerby’s beaked whale (*Mesoplodon bidens*) (Foster et al., 1996). Despite bacteriological investigations into a large number of seals, including common seals (*Phoco vitulina*), grey seals (*Halichoerus grypus*), hooded seals (*Cystophara cristata*), and harp seals (*Phoca groenlandica*), Foster et al. (1996) failed to recover *A. delphinicola* isolates. The authors suggest that this novel group of bacteria may be specifically adapted to cetaceans. The authors also mention that these findings reveal no clear pathological significance of *A. delphinicola*. 
In 1998, Foster et al. identified a new *Actinobacillus* spp., *Actinobacillus scotiae*, in three stranded harbor porpoises at three different locations along the Scottish coast. The first animal died from septicemia, which appeared to involve *A. scotiae*. Bacterial isolates were recovered from the brain, lungs, spleen, liver, lymph nodes, blood, and small intestine of the first porpoise. *A. scotiae* was isolated from the lungs, spleen, liver, kidneys, lymph nodes, blood, and small intestines of the second and third animals, although no specific *A. scotiae*-related cause of death could be identified. Similar to the report by Foster et al. (1996) on *A. delphinicola*, the authors draw no clear conclusions as to the pathogenicity of *A. scotiae* (Foster et al., 1998).

*Aeromonas* spp.

Prior to a report by Cusick and Bullock (1973) of *Aeromonas hydrophila* infection in the lungs of an Atlantic bottlenose dolphin, *A. hydrophila* had been isolated from the lungs of a non-human animal only once before in the case of an alligator with pneumonia (Shotts et al., 1972). *Aeromonas* spp. comprises a group of Gram-negative microorganisms that inhabit numerous aquatic environments, including fresh and coastal waters, sewage, and drinking water (Monfort and Baleux, 1990). In addition to their role as primary pathogens in cold-blooded animals (Vivekanandhan et al., 2005), aeromonads are most commonly associated with gastrointestinal disease in humans (Janda and Abbott 1998). A study by Cusick and Bullock (1973) described a case of bronchopneumonia, dermatitis, and septicemia caused by *Aeromonas hydrophila* infection in an Atlantic bottlenose dolphin that had been recently captured to be trained as a spectacle for
entertainment. Upon its death, necropsy revealed mottled dull red and gray lungs and pus-filled lesions throughout the pulmonary tissue. *A. hydrophila* were isolated from the skin, liver, lung, spleen, kidneys, and heart blood. The authors concluded that the suppurative bronchopneumonia observed in the lungs was bacterial, as *Aeromonas* spp. and *Pseudomonas putrefaciens* were both isolated from the lung tissue. Since Cusick and Bullock (1973), many more reports of *Aeromonas* spp. isolations from the lungs of pneumonic and septicemic cetaceans have been reported. Migaki et al. (1990) found *A. hydrophila* in the lungs of a stranded spinner dolphin, while Howard et al. (1983) identified *Aeromonas* spp. in a septicemic Pacific white-sided dolphin. *Aeromonas* spp. was also identified in blowhole cultures from a free-ranging killer whale (Young et al., 1997). *Aeromonas* spp. has been isolated from the respiratory tract of wild and captive visually healthy cetaceans, suggesting that these microorganisms may be part of the normal microbial community associated with the cetacean respiratory system. *A. hydrophila* and *A. salmonicida* were isolated from the respiratory tract of perceivably healthy beluga whales in a study detailing the microbiota changes seen between initial capture and extended captivity (Buck et al., 1989). Both Buck et al. (2006) and Morris et al. (2011) isolated *A. hydrophila* from blowhole samples from visually healthy wild Atlantic bottlenose dolphins.

*Erysipelothrix rhusiopathiae*

The zoonotic bacterium *Erysipelothrix rhusiopathiae* exists pathogenically or commensally in humans and various species of non-human animals including birds and
pigs (Wang et al., 2010), and has been reported in cetaceans since the early 1950’s (Seibold and Neal, 1956). *E. rhusiopathiae* is a non-spore-forming, Gram-positive, facultative anaerobe that can persist for long periods of time in a variety of aquatic environments (Wang et al., 2010). It has been isolated from the exterior mucoid slime of fish (Wood 1975); therefore, consumption of fish prey carrying *E. rhusiopathiae* is believed to be the means through which odontocetes acquire this pathogen (Suer et al., 1988). In marine mammals, *E. rhusiopathiae* infection may take one of two forms: acute septicemia or a self-limiting, sub-acute dermatological disease (Dunn et al., 2001). The progression of the often-fatal septicemic disease may be very rapid, transitioning from asymptomatic to death in less than a day (Waltzek et al., 2012). In 1956, Siebold and Neal reported cases of *E. rhusiopathiae* septicemia in three bottlenose dolphins and one spotted dolphin (*Stenella plagiodon*) that died in captivity at Marine Studios, Inc., Marineland, FL. Symptoms of illness among the three bottlenose dolphins varied in that one died within 20 minutes of showing signs of sickness, another died within 36 hours, and one died after having no observable clinical symptoms at all. Necropsy and histopathological examination revealed thoracic lymph node hemorrhaging in one animal, lung tissue congestion and lymph node hemorrhaging in another, and a lung abscess, as well as congestion and edema of the lung tissue in the third bottlenose dolphin. Bacteria isolated from the tissue surrounding a presumably parasitic lung lesion was believed to be *E. rhusiopathiae* based on morphological similarities and histological similarities to *E. rhusiopathiae* infection in swine. Another early report of erysipelas in cetaceans was from Geraci et al. (1966), which detailed four cases of *E. rhusiopathiae* infection in captive bottlenose dolphins. All four animals had similar respiratory findings at necropsy:
congestion in the lungs and lymphadenitis of the lymph nodes. A 1997 report by Kinsel et al. documented a case of fatal *E. rhusiopathiae* in a captive Pacific white-sided dolphin. The animal showed signs of lethargy and depression over a 6-10 hour period, ending in death. Necropsy revealed enlarged mesenteric and pleural lymph nodes and bacteriology of the lungs, liver, spleen, and kidneys produced heavy growths of *E. rhusiopathiae*. Based on the lack of clinical signs and the rapid progression of disease, the authors believed that this dolphin succumbed to *E. rhusiopathiae* septicemia. Interestingly, although this animal was kept in an enclosure with beluga whales, harbor seals (*Phoca vitulina*), and other dolphins, in addition to being fed the same fish, none of the other animals contracted erysipelas. A 2011 study by Melero et al. reported a case of *E. rhusiopathiae* infection in a stranded Atlantic bottlenose dolphin on the Mediterranean coast of Spain. Necropsy revealed white froth in the trachea, pulmonary congestion, thickened pleura, and edematous and congestive lymph nodes. The gross and microscopic findings led the authors to conclude that the cause of death was *E. rhusiopathiae* septicemia. The septicemic form of *E. rhusiopathiae* has also been reported in a long-finned pilot whale (*Globicephala melas*) (Howard et al., 1983), a captive killer whale (Young et al., 1997), and stranded harbor porpoises (Boseret et al., 2002; Siebert et al., 2009). While erysipelas has been reported in pinnipeds (Suer and Vedros, 1988), the disease is not an important clinical problem for these marine mammals (Sweeney 1974).

*Nocardia* spp.

Because the aerobic, filamentous *Nocardia* bacteria are found in a range of environments, including dust, marine and fresh water, marine sediment, terrestrial soil,
decaying vegetation, and decaying animal excrement, marine mammals have a number of potential routes of infection (St. Leger et al., 2009). The two most frequently observed forms of *Nocardia* spp. infections in mammals are the systemic form, which involves two or more body sites, and the pulmonary form (Beaman and Beaman, 1994; St. Leger et al., 2009). In cetaceans, the systemic form appears often in current literature, while pulmonary nocardiosis is seldom reported (St. Leger et al., 2009). Infection may result from direct ingestion, bloodstream injection, or inhalation (Beaman and Beaman, 1994), although many cetacean reports lack definitive answers into the sources of infection. A 1970 report by Pier et al. described *Nocardia* spp. infection in three cetaceans that resided in a sea life park in Hawaii. The first case, a newly captured pilot whale, had no observable eating or breathing abnormalities immediately prior to death. Necropsy revealed severe suppurative pneumonia and *Nocardia asteroides* was recovered from the lungs. The second case, a newly captured Pacific bottlenose dolphin (*Tursiops gilli*), also maintained normal habits prior to death. Necropsy revealed multiple abscesses in the lungs and lymph nodes and samples from both infected tissues revealed the presence of *Nocardia brasilienis*. The third case, a Pacific bottlenose dolphin that had been in captivity for four years, had loss of appetite and lethargy, followed by irregular respirations. Necropsy showed abscesses and nodules on the lungs, along with pleuritis. *Nocardia caviae* was identified in the lung tissue and determined as the causative agent. Macneill et al. (1978) documented the case of a captive beluga whale that developed weak swimming and listing, as well as loss of appetite. *Nocardia* spp. infection was diagnosed based on postmortem lesions, including thoracic abscesses. Martineau et al. (1988) detailed the necropsy findings of 13 stranded St. Lawrence beluga whales between
1983 and 1986. Pathological findings of the respiratory system include pulmonary abscesses and mineralization of bronchial and bronchiolar cartilages. *Nocardia* spp. was isolated from the lesions, including the lung lesions, of one animal diagnosed with systemic norcardiosis. In 2009, St. Leger et al. facilitated a post-mortem study to evaluate cases of nocardiosis in pinnipeds and cetaceans from American facilities between 1974 and 2007. The cetaceans included two captive and two wild Atlantic bottlenose dolphins, two captive killer whales, and four captive beluga whales. Moderate to severe *Nocardia* spp. infections were found most prominently in the lung and lymph nodes. All three species of cetaceans presented with pulmonary pyogranulomas with thoracic lymph node abscesses. The authors found two different *Nocardia* spp. causing fatal infections among the dolphins, *Nocardia levis* and *Nocardia asteroides*, along with two unidentified species of *Nocardia*. *Nocardia asteroides* and *Nocardia farcinica* were identified as the causative agents in the two killer whales and *Nocardia farcinica*, *Nocardia brasiliensis*, *Nocardia cyriacigeorgica*, and one unidentified species of *Nocardia* were identified as the primary pathogens in the four beluga whales.

*Pseudomonas* spp.

*Pseudomonas* spp., specifically *P. aeruginosa*, has frequently been isolated from cetaceans with respiratory tract infections (Howard et al., 1983; Dunn et al., 2001). *Pseudomonas* spp. has been found to cause bronchopneumonia, dermatitis, osteomyelitis, and septicemia in dolphins (Howard et al., 1983; Avalos-Tellez et al., 2010), although it may be part of the normal microbiota of a healthy animal (see below). *Pseudomonas*
*P. aeruginosa* is a Gram-negative bacterium that can grow in soil, water, and plant and animal tissue (Hardalo and Edberg 1997), as well as in seemingly inhospitable environments such as jet fuel and soap (Botzenhart and Doring 1993). Over the past century, *P. aeruginosa* has emerged as a major opportunistic pathogen in humans, perhaps because of its resistance to antibiotics and disinfectants (Bodey et al., 1983; Stover et al., 2000). One of the first reports of fatal bronchopneumonia caused by *P. aeruginosa* in a dolphin was by Diamond et al. (1979). An Atlantic bottlenose dolphin in ill health caught near Vero Beach, FL died after suffering 70 days of progressive necrosis, the development of skin nodules, and eventual dyspnea and complete loss of appetite. Necropsy revealed a thoracic cyst, a mass in the lung, and fluid in the bronchiolar spaces. *P. aeruginosa* was isolated from the lungs and from skin nodules containing crater-like depressions. The authors concluded that the dolphin’s immunosuppressed state provided an ideal environment for *P. aeruginosa* infection during captivity. A report by Eo and Kwon (2011) detailed the progression of respiratory disease in a Pacific bottlenose dolphin at the Seoul Zoo, Korea. Initially, signs of illness included low activity levels, loss of appetite, and slight listing in the water, along with thick, yellow mucous in the blowhole. Necropsy revealed fibrous pleuropneumonia and pleural effusion in the lungs and thorax. Although exhaled air samples taken at the onset of illness revealed *Staphylococcus aureus* and *Proteus mirabilis*, *P. aeruginosa* was isolated from the blowhole mucus after significant disease progression. Late stage infection may be indicative of the role of *P. aeruginosa* as an opportunistic pathogen; this is especially likely given the evidence that *P. aeruginosa* is present in the normal microbiota of the URT of marine mammals (see below). The post-mortem isolation of *P.
*P. putrefaciens* from the lungs of 12 stranded beluga whales with verminous bronchopneumonia (De Guise et al., 1995). *Pseudomonas* spp. has also been isolated from septicemic California sea lions, a northern elephant seal (*Mirounga leonina*) and a Pacific white-sided dolphin and from the pneumonic lungs of California sea lions, a northern elephant seal, harbor seals, a killer whale, a pilot whale, Pacific white-sided dolphins, and common dolphins (Howard et al., 1983). Furthermore, in a study by Siebert et al. (2009) looking at the regional differences in the bacterial flora of harbor porpoises, the authors identified *P. aeruginosa* as the causative agent of bronchopneumonia in harbor porpoises from the North Sea. Evidence indicates that *Pseudomonas aeruginosa* may be part of the normal microbiota associated with the upper respiratory tract of various cetaceans. In a study on visually healthy animals, Johnston and Fung (1969) found *P. aeruginosa* in wild porpoises and in animals held in captivity for 4-6 weeks and Asper and Odell (1990) found *P. aeruginosa* in blowhole samples from 26 wild Atlantic bottlenose dolphins from the east coast of Florida. Buck et al. (1989) identified *Pseudomonas* spp. in blowhole samples from a number of visually healthy beluga whales captured in the Churchill River in Manitoba, Canada. Chan et al. (2001) monitored 15 captive bottlenose dolphins over a 7-year period and identified *P. aeruginosa* as one of the most common isolated organisms. Finally, Morris et al. (2011) isolated *Pseudomonas* spp. from the blowholes of visibly healthy wild Atlantic bottlenose dolphins from the Southeastern US.
Salmonella spp.

Salmonella spp. has been isolated from orca whales (Howard et al., 1983; Colegrove et al., 2010), bottlenose dolphins (Howard et al., 1983), and harbor porpoises (Foster et al., 1999; Jepson et al., 2000), as well as various seals, sea lions, and sea otters (Howard et al., 1983; Thornton et al., 1998; Smith et al., 2002; Iveson et al., 2009; Stoddard et al., 2008). Although necrotizing enteritis and/or septicemia are the common manifestations of Salmonella spp. infection in marine mammals, lung infection by a monophasic group B Salmonella in harbor porpoises has been described in a number of reports (Foster et al., 1999; Valderrama Vasquez et al., 2008). Foster et al. (1999) reported on the isolation of monophasic group B Salmonella from stranded harbor porpoises around the Scottish coast. Post-mortem evaluation resulted in the isolation of monophasic group B Salmonella from lung tissue in 33 out of 36 of the animals that tested positive for Salmonella. Although Salmonella was found in a variety of tissues in the sampled animals, recovery of monophasic group B Salmonella in the lungs far exceeded the frequency of any other tissue isolations. This type of bacteria has frequently been found in porpoises, including both pneumonic and non-pneumonic porpoises, as well as in healthy animals whose deaths were non-pathological (Foster et al., 1999). This led the authors to conclude that monophasic group B Salmonella may be an opportunistic bacterial pathogen that takes advantage of immunocompromised animals, such as those inflicted with a heavy burden of lungworms (Foster et al., 1999) (See Other Respiratory Perturbations section). While monophasic group B Salmonella was not recovered from any animals analyzed in past studies performed by the authors in Foster et al. (1999), they did find other salmonellae in cetaceans, seals and otters. Salmonella typhimurium DT12
was isolated from a porpoise, *Salmonella typhimurium* DT104 was isolated from a grey seal, *Salmonella tennessee* was isolated from a common seal, and *Salmonella bovismorbificans* was isolated from two grey seals, an otter (*Lutra lutra*), and an undetermined species of seal (Foster et al., 1999), although the body isolation sites were not specified. While the findings of these *Salmonella* spp. are attributable to a common food source, monophasic type B *Salmonella* has been found largely in the lung tissue of porpoises, rather than the gastro-intestinal tract, and could not be recovered from the marine mammals sharing their food source. This implies that monophasic type B *Salmonella* is transferred through some mode other than feeding (Foster et al., 1999). A 2008 study by Valderrama-Vasquez et al. examined 511 stranded or by-caught cetaceans around England and Wales between September 1990 and December 2002. Of the 279 harbor porpoises analyzed, monophasic type B *Salmonella* was isolated from the various tissues of 60 animals, including the lungs, lymph nodes, and pleural fluids. Monophasic type B *Salmonella* was also isolated from the lungs of one of the 58 common dolphins examined. Davison et al. (2010(a)) reported the prevalence of group B *Salmonella enterica* in 28 of 80 harbor porpoise carcasses from along the Devon and Cornwall coastline in England, UK. The authors reported that lung tissue was the most common site of isolation of group B *Salmonella enterica*, with the lung tissue of all 28 porpoises testing positive for this organism.
*Staphylococcus aureus*

Although *Staphylococcus aureus* isolates from marine mammals have not yet been characterized, the pathogenicity of *S. aureus* has been well documented in humans, domestic animals, and other wildlife. This pathogen has been observed to co-evolve with its host species, leading to host species-specific strains of *S. aureus* (Sung et al., 2008). Cross contamination of these host species-specific strains to other species is also possible (Cefai et al., 1994; Simoons-Smit et al., 2000). A 2012 study by van Elk et al. sought to determine whether or not marine mammals carry their own strains of host species-specific *S. aureus*. *S. aureus* isolates were obtained at necropsy from multiple tissue sources, including the lungs and lymph nodes of five stranded and captive harbor porpoises, the brain and nose of three captive harbor seals, the mouth of a stranded grey seal and the mouth of a trapped southern elephant seal. The authors found that four of the *S. aureus* strains had novel sequence types, while the other six had been found previously in terrestrial animals, including humans. Analyzing the clustering of the strains in a clonal complex, the authors further determined that three of the strains may have been host species-specific. In 1976, Streitfeld and Chapman used bacteriologic culture technique to examine staphylococcal isolates from captive dolphins and the humans with whom the animals had contact. The authors intended to determine the extent of cross infection occurring between dolphins and oceanarium personnel, and to determine if pathogenic staphylococci are part of the normal respiratory flora of dolphins. Blowhole and pharynx samples were obtained from 31 healthy dolphins and one dolphin diagnosed with a respiratory infection, along with 31 healthy humans and one human afflicted with illness from the Wometco Miami Seaquarium (Miami, FL) and the Flipper Sea School
(Grassy Key, FL). The results led the authors to conclude that *S. aureus* may be commonly isolated from dolphins in captivity and that healthy dolphins most commonly carry *S. aureus* in their blowhole, as only a few of the pharyngeal cultures tested positive. In 2002, Siebert et al. detailed a case of *S. aureus* septicemia affecting the respiratory system of a harbor porpoise found near Denmark and Germany. The animal, which was deceased upon discovery, was found to have a 2 cm abscess in the retropharyngeal lymph node and bacteriological examination revealed *S. aureus* in the lymph nodes, lungs, and thoracic and pleural cavities. *S. aureus* was also isolated from the lung during postmortem examination of a pneumonic killer whale (Power and Murphy 2002).

In addition to *S. aureus*, methicillin-resistant *Staphylococcus aureus* (MRSA) has been isolated from marine mammals. Morris et al. (2011) isolated MRSA from blowhole swabs of free-ranging dolphins off the coast of Charleston, SC. In 2009, Faires et al. reported the isolation of MRSA from a captive male bottlenose dolphin that died of pneumonia. The death of this animal led to a deeper investigation into the extent of MRSA contamination at the marine park where he had been kept. In total, after monitoring the situation for a year and a half, MRSA was found in five dolphin blowhole samples and three walrus nasal samples. While the possibility of human-to-animal transmission of MRSA in the captive populations is obvious, Schaefer et al. (2009) offer two possible explanations for the introduction of MRSA into the wild populations: antibiotic-resistant organisms from humans or terrestrial animals infect marine mammals after being washed into the coastal waters or pharmaceutical products that are discharged into the waterways lead to the evolution of resistant bacteria.
Streptococcus spp.

In 1980, Higgins et al. documented a case of bronchopneumonia in a stranded pilot whale caused by *Streptococcus equi*. Prior to this report, *S. equi* had rarely been isolated from any animal besides horses, and never from a marine mammal (Higgins et al., 1980; Higgins 2000). *Streptococcus equi* causes strangles, one of the most prominent diseases in horses (Harrington et al., 2002), which is characterized clinically by abrupt fever followed by upper respiratory tract inflammation (Sweeney et al., 2005). Additionally, beta-hemolytic streptococci have been recognized as major pathogens causing septicemia, bronchopneumonia, and abscesses in harbor porpoises (Siebert et al., 1996; Swenshon et al., 2008). A number of reports have documented alpha- and beta-hemolytic streptococci infection in cetaceans. Necropsy by Higgins et al. (1980) on a young North Atlantic pilot whale (*Globicephala melaena*) stranded on Metis Beach, Quebec, Canada, revealed bronchopneumonia in the left lung and bloody exudate in the thoracic cavity. *S. equi* was isolated from the lungs and the pharynx, as well as the pericardial fluid. Swenshon et al. (2008) identified 35 beta-hemolytic streptococci isolated from stranded harbor porpoises or porpoises caught in fishing nets in the North and Baltic Seas. All 35 isolates were classified biochemically and serologically into Lancefield’s serological group L and were identified as *Streptococcus dysgalactiae* subsp. *dysgalactiae*. The authors noted that the deceased porpoises had obvious signs of parasitic respiratory infection and that beta-hemolytic streptococci were frequently isolated from the lungs. A study of 41 stranded harbor porpoises off the coast of the United Kingdom (Baker and Martin 1992) found group L streptococcus in a number of animals that died from bacterial pneumonia. Alpha- and beta-hemolytic streptococci were
also recovered from various stranded, by-caught, or hunted porpoises from North Atlantic waters (Siebert et al., 2009). Streptococci were found to be pathologically associated with bronchopneumonia, enteritis, hepatitis, nephritis, lymphadenitis, and septicemia. Also, Howard et al. (1983) isolated beta-hemolytic streptococci from the respiratory tracts of a killer whale and a Pacific white-sided dolphin. Finally, the alpha-hemolytic, beta-hemolytic, and non-hemolytic forms of Streptococcus spp. have all been isolated from seals and sea lions with pneumonia or septicemia (Howard et al., 1983), including S. zooepidemicus (Baker 1980), S. bovis (Baker and Baker, 1988), S. canis (Baker 1989), and S. phocae (Skaar et al., 1994). S. zooepidemicus has been shown to cause severe respiratory disease in humans (Barnham et al., 1983), while S. bovis and S. canis are associated with meningitis and septicemia in humans (Jacobs et al., 1993; Grant et al., 2000; White et al., 2002).

*Other Respiratory Perturbations*

Although this review has focused primarily on microorganisms as agents of respiratory disease, we think it is important to summarize the published literature for both toxic marine algae and the eukaryotic, macroparasitic lungworms, which are commonly detected in odontocetes with respiratory infections.
**Brevetoxins**

Marine algae may bloom in response to favorable environmental conditions, resulting in dense concentrations of algal cells in the water. While most of the over 5000 species of phytoplankton are harmless, 2% produce toxins that may be released into the air or water (Landsberg 2002). In low concentrations, toxic algae are relatively benign; however, during bloom conditions, biotoxin production increases may greatly affect aquatic organisms, such as marine mammals, as well as humans (Van Dolah 2000). The dinoflagellate *Karenia brevis* produces harmful algal blooms (HABs) referred to as Florida red tide (Pierce and Henry 2008), and releases brevetoxins, neurotoxins which cause neurological and gastrointestinal illness when ingested and severe respiratory illness inhaled (Watkins et al., 2008). A report by Twiner et al. (2011) explored the degree of brevetoxin exposure in Atlantic bottlenose dolphins in Sarasota Bay, FL and found brevetoxins in over a third of the animals studied during a nine-year period during both *K. brevis* bloom and non-bloom conditions. Twiner et al. (2012) examined the role of brevetoxins in three Atlantic bottlenose dolphin mortality events in Florida’s panhandle and found that the presence of *K. brevis* in the water during two of the three mortality events may have caused over 200 bottlenose dolphin stranding deaths. Additionally, McHugh et al. (2011) observed changes in juvenile Atlantic bottlenose dolphins (2-13 years of age) during red tide events, including decreased foraging and increased milling, increased sociality and group size and concentration. Although these changes were likely indirect effects of brevetoxin production in the ecosystem, behavioral changes may create stress and subsequent immunosuppression in dolphins, increasing vulnerability to pathogenic diseases, including respiratory infections.
Lungworms

The highly diverse phylum Nematoda comprises an estimated 100,000 to 1 million extant species (Parkinson et al., 2004) that inhabit a variety of environments, including highly specific habitats such as the placenta of a sperm whale (Goater et al., 2013). These tiny, threadlike animals have a simple body plan including a pseudocoelom body cavity and an anteriorly located mouth. Within the Nematoda phylum, two families, Pseudaliidae and Crassicaudidae, and four genera, *Halocerus*, *Pharurus*, *Pseudalius*, and *Sternurus* (Daily 2001), commonly cause of respiratory infection in odontocetes (toothed whales which form a suborder of the cetaceans). While nematodes that infect terrestrial mammals may have direct life cycles or use vegetation-dwelling snails or slugs as intermediate hosts to infect the mostly herbivorous terrestrial hosts, little is known about the life cycle of these parasites in odontocetes (Gibson et al., 1998). Dailey et al. (1991) described a case of prenatal infection in four wild Atlantic bottlenose dolphin calves from the Atlantic and Gulf coasts of Florida which may be indicative of transplacental infection. In a study by Fauquier et al. (2009) including 22 stranded and 44 live Atlantic bottlenose dolphins from southwestern Florida, the authors found that severity and prevalence of *Halocerus lagenorhynchii* and *Skrjabinalius cryptocephalus* in the lungs were higher in neonates than in adults which may be indicative of transmission through mother’s milk. Woodard et al. (1969) found that lung infection by the nematode species *Halocerus lagenorhynchia* in both wild (stranded) and captive (spontaneous death) Atlantic bottlenose dolphins resulted in pneumonia and mucopurulent bronchiol infection, with pneumonia limited to areas contiguous to the parasite-infected airways
and bronchiolitis exacerbated by hypertrophy of the sphincter muscles of the terminal bronchioles. Host age may play a role in susceptibility as pre- and neonatal parasitic lung infections by *Halocerus* spp. are more prevalent and severe than in older hosts (Dailey et al., 1991; Measures 2001; Fauquier et al., 2009), while prevalence of lung infection by pseudaliids increases with age (Measures 2001).

Lungworms have been detected in individual stranding events and mortality events (Daily et al., 1991; Moser and Rhinehart 1993; Parsons and Jefferson 2000; Bennett et al., 2001; Parsons et al., 2001; Marigo et al., 2002; Siebert et al., 2006; Oliviera et al., 2011; Lehnert et al., 2014) from various odontocete species around the world. A 1998 study by Gibson et al. analyzed parasite samples from over 300 odontocetes stranded along the coast of England and Wales from 1990-1994. The authors identified 22 different helminth species from various body sites and with varying degrees of both host specificity and pathogenic effects. The report by Gibson et al. (1998) reveals that without systematic investigations into the severity, transmission, and etiologic variability of parasitic diseases (Fauquier et al., 2009), the significance of these organisms in cetaceans remains elusive. For example, Fauquier et al. (2009) necropsied 22 stranded Atlantic bottlenose dolphins off the southwestern coast of FL and found a 77% prevalence of lungworm infection with no deaths attributable to the parasites. In the same study, Fauquier et al. (2009) analyzed blowhole swab samples of 44 free-ranging Atlantic bottlenose dolphins and found no lungworm larvae in any of the samples. Also, Baker and Martin (1992) reported on the causes of death of 41 by-caught and stranded harbor porpoises along coastal UK and found that 17% succumbed to parasitic bronchopneumonia, while 10% died of bacterial pneumonias. Comparing and contrasting
these two studies raises questions pertaining to location and environmental factors, host species and age, and parasite species and abundance, which may have combined or separate implications in parasitic infections (Raga et al., 2002) and cannot be answered with the limited data currently available on macroparasitic diseases in cetaceans. In addition to their role in respiratory disease, lungworms may also function as hosts for bacterial pathogens. Foster et al. (1999) suggested that lungworms may operate as vectors for the transmission of *Salmonella* in harbor porpoises. Perrett et al. (2004) isolated a species of *Brucella* from *Pseudoalium inflexus* found in the lungs of a stranded harbor porpoise that likely died of asphyxiation due to congested lungs while Davison et al. (2010b), much like Foster et al. (1999), described numerous instances of the co-occurrence of *Salmonella* in the lungs and lungworms in harbor porpoises. While the significance of lungworms in odontocetes is not fully understood, the prevalence of these parasites in the airways of sick animals likely indicates that these parasites play a role in certain odontocete respiratory diseases. Further research into the host-species relationship between odontocetes and lungworms, in addition to the host-species relationship between lungworms and pathogenic bacteria, will provide answers to current questions about the role that macroparasites play in cetacean respiratory disease.

1.5 CONCLUSION

The majority of published research on respiratory illness in cetaceans has relied primarily on clinical, culture-dependent isolation approaches, which has limited the ability to more broadly identify potential agents of respiratory disease. In addition,
necropsies of stranded animals have provided a large portion of what is described in the published literature about respiratory disease in wild cetacean populations. As a result of the clinically asymptomatic progression of most pathogenic respiratory diseases in cetaceans, there have been few opportunities for antemortem investigations into respiratory diseases in captive animals (Reidarson et al., 1999; Young et al., 1999; Miller et al., 2002). Due to this lack of sufficient data pertaining to pathogenic respiratory infections in cetaceans, diagnoses of disease have been largely based on what is known in humans. While it may appear that nearly all cetacean respiratory diseases have a corresponding human manifestation, this may, in fact, be a result of using known human respiratory pathogens as a guideline for the identification of etiologic agents isolated from cetaceans. Moving forward with cetacean respiratory disease research will benefit from the characterization of the respiratory microbiome of healthy and diseased animals (from captive, wild, and managed populations) in order to establish a baseline of the associated microorganisms present under the varying conditions.

With the established Human Microbiome Project supported by the National Institutes of Health well underway (Aagaard et al., 2013), there are an increasing number of human metagenomes available, including those for the respiratory system. By comparing recently sequenced dolphin respiratory metagenomes with available human respiratory metagenomes, we will be able to better understand the similarities and differences between their microbial communities and the proposed role of human-centric diagnoses that have dominated cetacean respiratory research. In Chapter 2, we will describe seven Atlantic bottlenose dolphin upper respiratory tract (URT, blowhole) metagenomes from the southeastern US. Using metagenomes of the surrounding
seawater as a control, we will compare the dolphin URT metagenomes with healthy and Cystic Fibrosis human lung metagenomes, as well as metagenomes from the anterior nares of healthy humans. Additionally, we will compare dolphin URT metagenomes to fecal metagenomes of various cetartiodactyls (e.g., bighorn sheep, okapis, giraffes, pigs, and cows) and humans to gain insight into the relationship between evolutionarily similar and dissimilar organisms on microbiome composition. Lastly, in Chapter 3, we will discuss the significance of our findings to the potential for bottlenose dolphins to serve as sentinel species for human and ecosystem health in the near coastal environment.
Figure 1.1 Illustration of the dolphin blowhole position and anatomy. Modified and adapted from Cranford et al., 1996.
Table 1.1 Viral respiratory pathogens identified in odontocetes and other marine mammals.

<table>
<thead>
<tr>
<th>Pathogen</th>
<th>Domain</th>
<th>Marine Mammal</th>
<th>Free-ranging, captive, stranded</th>
<th>Reference</th>
</tr>
</thead>
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<td>Influenza A Virus</td>
<td>Virus</td>
<td>Long-finned pilot whale</td>
<td>Free-ranging</td>
<td>Hinshaw et al., 1986</td>
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<td>Morbillivirus</td>
<td>Virus</td>
<td>Atlantic bottlenose dolphin</td>
<td>Stranded</td>
<td>Lipscomb et al., 1994</td>
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<tr>
<td>Parainfluenza virus</td>
<td>Virus</td>
<td>Atlantic bottlenose dolphin</td>
<td>Captive (open ocean enclosure)</td>
<td>Nollens et al., 2008</td>
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<tr>
<td>Polyomavirus</td>
<td>Virus</td>
<td>Short-beaked common dolphin</td>
<td>Stranded</td>
<td>Anthony et al., 2013</td>
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<td>Striped dolphin</td>
<td>Stranded</td>
<td>Domingo et al., 1992</td>
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<td></td>
<td>Harbor porpoise</td>
<td>Stranded</td>
<td>Barrett et al., 1995</td>
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51
Table 1.2 Eukaryotic respiratory pathogens identified in odontocetes and other marine mammals.

<table>
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<th>Free-ranging, captive, stranded</th>
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<td>Reidarson et al., 1998</td>
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<td>Free-ranging</td>
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<td>Beluga whale</td>
<td>Captive</td>
<td>Young et al., 1999</td>
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<td><em>Cunninghamella bertholletiae</em></td>
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Table 1.3 Bacterial respiratory pathogens identified in odontocetes and other marine mammals.

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<td>Sowerby's beaked whale</td>
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<td><em>Aeromonas hydrophila</em></td>
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<td>Cusick and Bullock 1973</td>
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<td></td>
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<td>Buck et al., 2006</td>
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<td>Stranded</td>
<td>Migaki et al., 1990</td>
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<td>Beluga whale</td>
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### Table 1.3 Continued

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<td>St. Leger et al., 2009</td>
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CHAPTER 2: METAGENOMIC CHARACTERIZATION OF THE DOLPHIN URT MICROBIOME AND COMPARATIVE METAGENOMIC ANALYSIS WITH HUMAN RESPIRATORY AND CETARTIODACTYL FECAL MICROBIOMES

2.1 INTRODUCTION

Odontocete cetaceans have long been considered sentinels for human health in relation to the marine environment (Holden 1972) because of their long lifespans in the coastal ecosystems, high trophic feeding level, and propensity for bioaccumulating toxic chemicals in their blubber (Ross 2000; Reddy et al., 2001; Wells et al., 2004). The fact that these animals reside in coastal waters that may be contaminated with anthropogenic pollutants as a result of runoff and increasing coastal populations, or saturated with biotoxins from toxic algal blooms make them ideal indicators of the health and status of the marine ecosystem. Also, the physiological similarities between dolphins and humans suggest that these animals may be used to assess the human health effect of both anthropogenic and biological toxins, as well as the etiologic effects of pathogenic organisms. Enteric pathogens in the coastal ecosystems have long been an area of focus; however, infectious respiratory diseases were the leading cause of human mortality in 2012 (World Health Organization 2012), thus a shift in focus to new and emerging respiratory pathogens is necessary. Interestingly, respiratory illnesses are believed to be the leading cause of death in captive, and possibly free-ranging, dolphins (Johnson et al.,
2009); therefore, it may be possible to link the respiratory health of dolphins to marine ecosystem health and, subsequently, respiratory risk to humans.

In spite of the generally accepted role of dolphins as sentinel species, no definitive link has been established to support this idea. In this study, we examined the relationship between the microbiomes of the bottlenose dolphin upper respiratory tract (URT) and the human respiratory system (including the lungs and anterior nares) in order to assess the credibility of the proposed sentinel role of odontocetes. Our intent was to use comparative metagenomics to investigate the similarities and differences between the microorganisms, particularly the pathogenic species, present in the normal microflora of the respiratory tracts of dolphins and humans.

2.2 MATERIALS AND METHODS

Prior to my participation in this project, in August 2009, blowhole swabs were collected from free ranging Atlantic bottlenose dolphins (*Tursiops truncatus*) captured in the Sapelo Island and Brunswick field sites in Georgia as part of the National Oceanic and Atmospheric Association’s (NOAA) Coastal Georgia Dolphin Health Assessment (Drs. Lori Schwacke and Randy Wells, Project Leaders). While two males and two females from each location, totaling eight dolphins, were selected for this study, only seven metagenomes were analyzed as a result of low recovery of DNA from one of the samples. The following sections will summarize the collection of the bottlenose dolphin (URT) samples, as well as the nucleic acid extraction and sequencing methodology for metagenomic library construction (N. Kimes, W. Johnson, and P.J. Morris, personal
communication). My contribution to the project was comparison of the dolphin URT metagenomes and the comparative metagenomics.

***Site Description***

NOAA’s Coastal Georgia Dolphin Health Assessment area included the north-south stretch of estuarine waters from Sapelo Sound to St. Simons Sound. Further, the assessment area was divided into two field sites: The Brunswick field site and the Sapelo field site. The Brunswick field site included the Turtle/Brunswick River Estuary (TBRE) and all estuarine waters of St. Simons and Altamaha Sounds. PCB contamination of water, sediments, and biota in the TBRE, due to an EPA National Priority List site (Superfund Site), has been well-documented (Winger et al., 1993; Kannan et al., 1997a; Kannan et al., 1997b; Kannan et al., 1998a; Kannan et al., 1998b; Kannan et al., 1999; Sajwan et al., 2009). The Sapelo field site included all estuarine waters north of Altamaha Sound, including Sapelo Sound. In contrast to the Brunswick site, the counties adjacent to the Sapelo site supported a smaller human population of at time of sampling and do not include a historic point-source for chlorinated contaminants.

***Dolphin Capture and Upper Respiratory Tract (Blowhole) Microbiome Sample Collection***

Dolphins were encircled with a seine net and brought aboard a specially designed veterinary examination and sampling vessel. In order to sample the dolphin upper respiratory tract, sterile Dacron swabs were inserted into the dolphin’s blowhole by trained marine mammal veterinarians during an exhalation, swabbed along the wall of the
distal sinus, and removed during the animal’s next breath. Swabs were placed on dry ice and stored at -80°C until they were processed in the Morris laboratory.

**DNA Isolation, Amplification, and Sequencing of Upper Respiratory Tract (Blowhole) Samples**

The metagenomic DNA was isolated from the blowhole samples by modifying the MoBio Power soil nucleic acid extraction kit protocol. The swab tips were cut from their shafts using sterile shears and transferred to MoBio bead beating tubes containing 2.0 mL of TE (10 mM Tris-HCl, pH 9; 1.0 mM EDTA). The tubes were homogenized in a bead-beater at 5000 rpm for two minutes, and then centrifuged at 10,000 x g for five minutes. The 1000 mL of supernatant was transferred to a clean microcentrifuge tube. Potassium acetate was added to a final concentration of 100 mM, and the extracts were mixed by inversion and incubated at 4°C for five minutes. The extracts were once again centrifuged at 10,000 x g for five minutes, the supernatant was transferred to a clean tube, and membrane binding solution was added according to the MoBio kit protocol. The samples were loaded into MoBio glass filters and washed with MoBio wash buffer as described in the manufacturer’s instructions. DNA was eluted from the filters using 50 uL of sterile, nuclease-free water. The resulting metagenomic DNA was amplified using the Repli-G Mini Whole Genome Amplification kit (Qiagen) according to the manufacturer's instructions. The quality of the resulting DNA was visually evaluated by running the amplified samples on a 1% agarose gel and concentrations were determined with a fluorometer using the Quant-iTDNA HS system. The DNA was sequenced at EnGenCore in the University of South Carolina on a Roche 454 FLX pyrosequencing platform.
Metagenomic Sequence Analysis

Sequences were uploaded to the MG-RAST server (Meyer et al. 2008) as a single .sff formatted file for each metagenome. Each upload underwent quality control (QC), which included quality filtering to remove sequences containing five or more ambiguous base pairs, length filtering to remove sequences with lengths greater than or equal to 2 standard deviations from the mean sequence length, and dereplication to remove replicate sequences that are artifacts of shotgun pyrosequencing. All sequences were assigned taxonomic classifications by comparison to the M5NR sequence database with a maximum e-value cutoff of $1.0 \times 10^5$, a minimum percent identity cutoff of 50%, and a minimum alignment length cutoff of 10 bases.

Metagenomic Comparisons

The dolphin upper respiratory tract metagenomes were compared to metagenomes from the lungs of healthy and cystic fibrosis afflicted human patients, from the anterior nares of healthy humans, and from the feces of humans and various cetartiodactyls, along with filtered Sapelo Island tidal creek water publicly available on the MG-RAST server (Table 2.2; Figure 2.1). Metagenomes for comparison were primarily selected on the basis of availability, with the random selection of 10 human anterior nares metagenomes and 5 human fecal metagenomes available via the Human Microbiome Project (HMP). In addition to a metagenome-wide analysis at each taxonomic level, taxonomic lineages within each domain were analyzed. Analyses used the full scope of available metagenomic data, including unassigned, unclassified, and other unknown sequences.
Abundance values from the table feature on MG-RAST were used to determine the percentage of taxa within each metagenome by calculating the sum of abundance values as a total of sequences per metagenome and per domain. For each respective comparison (the dolphin URT metagenomes; the dolphin URT vs seawater; the dolphin URT vs the human nares and lungs; the dolphin URT vs mammalian feces), the abundance values were used to determine the 25 most abundant taxa. The Principal Component Analysis (PCA) tool available in MG-RAST was also used in the metagenomic comparisons. PCA offers a means of reducing large datasets down to the size of only a few variables. In MG-RAST, these variables, or principle components, are ordered so that the smallest numbered component (PC01) represents the direction of highest variation among the metagenomic data and the largest (PC0n) is assumed to represent random fluctuations in the data (Yeung and Ruzzo 2001). For comparisons of known human respiratory pathogens, we compiled a list of 17 common human respiratory pathogens (bacterial, fungal, and viral) from various sources (Dasaraju and Liu 1996; LaRocque and Ryan 2013), including specific respiratory disease fact pages accessible via the Centers for Disease Control and Prevention website (www.cdc.gov). For the comparisons of potential odontocete respiratory pathogens, we used the respiratory pathogens to-date in the published literature (Table 1.1; see Chapter 1 for a review). Presence/absence and percentages of both human and odontocete pathogens were found by examining the species data available via the MG-RAST table tool combined with the aforementioned percentage calculation method.
2.3 RESULTS

We examined the microbial communities associated with the blowholes of seven adult dolphins (D1-D7) representing male (N=4) and female (N=3) animals from Sapelo (N=4) and Brunswick, GA (N=3), each with a different concentration of PCBs in their blubber, ranging from 40 to 769 ppm (Table 2.1; Figure 2.1). Pyrosequencing of blowhole metagenomes resulted in 89.47 Mbp of sequences, with an average of 46,839 reads per dolphin at an average length of 272 bp per read. Taxonomic classification of these sequences using the non-redundant M5NR database within MG-RAST revealed that the dolphin URT metagenomes were dominated by bacterial sequences (68%), followed by eukaryotic (5.3%) and viral (1.3%) sequences (Figure 2.2).

Dolphin URT-associated Bacteria

All seven dolphin URT metagenomes are dominated by the same four bacterial phyla (Figure 2.3a): Bacteroidetes, Proteobacteria, Actinobacteria, and Firmicutes. Each individual exhibited a unique distribution pattern with Bacteriodes dominating D1 (53%), D3 (39%), and D6 (49%), Proteobacteria dominating D2 (55%), D4 (45%), and D5 (45%), and Actinobacteria dominating D7 (63%). Firmicutes, although not dominant in any single individual, were consistently identified in all seven dolphins, representing 5-16% of the bacterial community from each individual.

At the class-level, D1 and D3-D6 exhibit similar class-level distribution patterns, while D2 and D7 present unique patterns. Gammaproteobacteria and Flavobacteria are the two most abundant classes in D1 (16.1% and 39.3%, respectively), D3 (22% and 26.7%, respectively), D4 (29% and 27.4%, respectively), D5 (28.4% and 21%,
respectively), and D6 (28.1% and 34.4%, respectively) (Figure 2.3b). In D2, Gammaproteobacteria (49.3%) and Actinobacteria (22.3%) are the most abundant bacterial classes, with less sequences of Flavobacteriia (3.5%). D7 is dominated by Actinobacteria (62.8%) and contains fewer sequences of Gammaproteobacteria (9%) and Flavobacteriia (1.1%).

All of the dolphin URT metagenomes, with the exception of D2, contain over 500 identified bacterial genera. D1, D3, D4, and D6 are dominated by the same two genera, *Flavobacterium* and *Bacteroides*, in similar proportions (D1: 7.9% and 6.2%, respectively; D3: 5.5% and 5.5%, respectively; D4: 5.3% and 4.7%, respectively; D6: 6.9% and 5.2%, respectively), while D5 is dominated by the same two bacterial genera with a similar proportion of *Flavobacterium* (5.7%) but a higher abundance of *Bacteroides* sequences (12%) (Figure 2.4a). The most abundant genera of D2 and D7 are largely different from the other dolphin URT metagenomes. D2 is dominated by *Psychrobacter* (13%) and *Acinetobacter* (10.6%) with a lower abundance of *Flavobacterium* (0.9%) and *Bacteroides* (1.2%), while D7 is dominated by *Actinomyces* (7.2%) and *Corynebacterium* (5.1%), also with a lower abundance of *Flavobacterium* (0.22%) and *Bacteroides* (1%). While D1 and D3-D6 have similar distributions of the top 15 bacterial genera, including *Gramella*, *Capnocytophaga*, and *Polaribacter* (D1: 5%, 2.6%, and 3.9%, respectively; D3: 2.7%, 2.4%, and 2%, respectively; D4: 3.2%, 2.5%, and 2.3%, respectively; D5: 2.1%, 1.5%, and 2.1%, respectively; D6: 4.2%, 3.3%, and 3%, respectively), the most abundant bacterial genera of D2 and D7 include very few of the most abundant genera of the other dolphin URT metagenomes. The genus *Escherichia* is present in every metagenome (D1: 2.7%; D2: 8.7%; D3: 2.7%; D4: 2.7%;
D5: 5.5%; D6: 1%; D7: 1.8%) but is notably higher in D2 than the other dolphin URT metagenomes. Also, *Streptococcus* makes up a larger percentage of the D2 bacterial genera (2.5%) than any of the other dolphin URT metagenomes (D1: 0.8%; D3: 0.6%; D4: 0.5%; D5: 0.5%; D6: 0.3%; D7: 1.7%). Overall, of the 25 most abundant genera of the dolphin URT metagenomes are similar with D2 containing bacterial sequences for six genera that do not fall within the top 25 of the other metagenomes and D7 containing 13 genera that do not occur in the top 25 of the other dolphin metagenomes.

The distribution of the most abundant bacterial species in the dolphin URT metagenomes are similar for D1, D3, D4, and D6 with *Gramella forsetti* (5%, 2.7%, 3.3%, and 4.3%, respectively), *Flavobacterium johnsoniae* (4.1%, 2.5%, 2.6%, and 3.5%, respectively), and *Flavobacterium psychrophilum* (3.7%, 2.3%, 2.4%, and 3.1%, respectively) falling within the top five bacterial species (Figure 2.4). *Escherichia coli* falls within the five most abundant species of D1 (2.7%), D3 (2.6%), D4 (2.6%), and D7 (1.7%) and it accounts for 0.86% of D6. *E. coli* is the most abundant bacterial species identified in D2 (8.3%) and D5 (4.5%), followed by two *Psychrobacter* spp. in D2 (7.5% and 5.3%, respectively) and *Pasteurella multocida* and *Staphylococcus aureus* in D5 (3% and 2.1%, respectively). There are no *Psychrobacter* spp. within the top 25 bacterial species of any other individual dolphin URT metagenome except for D6 (1%), while *P. multocida* does not fall within the top 25 species of any dolphin URT metagenome except D2 (1.9%) and D6 (1.2%). *S. aureus*, though not among the 25 most abundant bacterial species in the other dolphin metagenomes, has similar percentages in D1 (0.4%), D2 (0.5%), D3 (0.5%), D4 (0.3%), D6 (0.5%), and D7 (0.1%).
Dolphin URT-associated Eukaryota

Ascomycota and Basidiomycota were the only two fungal phyla classified in the dolphin metagenomes, with all seven dolphins having higher abundances of Ascomycota than Basidiomycota and D3 containing no Basidiomycota sequences (data not shown). The phylum Nematoda accounts for 38% of the eukaryotic sequences in D1, 32.5% of the eukaryotic sequences in D6, and 32% of the eukaryotic sequences in D2. In D3 and D5, Nematoda is slightly less abundant (19% and 15.3%, respectively), while making up 6.7% of D7 and 3.4% of D4. Also, the phylum Apicomplexa accounts for 9.8% of the eukaryotic sequences in D6, with smaller percentages in D1-D5 (3.6%, 2.3%, 1.5%, 1%, and 1.2%, respectively) and the lowest abundance in D7 (0.34%).

Dolphin URT-associated Viruses

The dolphin blowhole metagenomes contain a wide range of viral sequences with D1 and D2 exhibiting the highest percentages of total sequence abundance (4.5% and 5.3%, respectively), while D5 and D6 present the lowest percentage of viral sequences (0.07% and 0.11%, respectively) (Table 2.3; Table 2.4). In total, 16 viral families were observed with an average of seven viral families associated with each dolphin (Figure 2.5). For D1-D4 and D7, the greatest percentages of viral sequences were from the families Microviridae and Adenoviridae (D1: 75.5% and 11.9%, respectively; D2: 53.7% and 31.6%, respectively; D3: 60.2% and 23.5%, respectively; D4: 13.2% and 21.5%, respectively; D7: 4.3% and 84%, respectively), while Microviridae and Inoviridae were the most abundant viral families in D5 (73.3% and 16.7%, respectively) and Adenoviridae and Siphoviridae were the most abundant viral families in D6 (36.2% and
26.6%, respectively). Most of the viral families were detected in more than one dolphin URT metagenome with Inoviridae, Siphoviridae, and Herpesviridae, along with Microviridae and Adenoviridae, occurring in at least four of the seven dolphin URT metagenomes.

*Chlamydiamicovirus* is the most abundant viral genus in D1 (47.2% of the viral sequences), D3 (30.5% of the viral sequences), and D5 (43.3% of the viral sequences) (Figure 2.6). Although *Chlamydiamicovirus* is abundant in D2 (27.3%) and present in lower abundance in D4 (7.4%) and D7 (1.8%), it is absent in D6. The genus *Mastadenovirus* exhibits the highest abundance in D2 (31.6%), D6 (36.2%), and D7 (84%), has lower abundance in D1 (11.9%), D3 (23.5%), and D4 (21.5%), and is absent in D5. *Inovirus* and *Bdellovirus* were detected in D1 (4.6% and 3.9%, respectively), D3 (0.36% and 9.5%, respectively), and D4 (4.1% and 3.3%, respectively), while only *Inovirus* was detected in D5 (16.7%) and D6 (7.4%) and only *Bdellovirus* was detected in D2 (7.6%) and D7 (1.1%). The 19 classified viral genera for D1 include eight papillomaviruses and *Varicellovirus*, none of which occur in the other dolphin URT metagenomes. The six classified viral genera of D2 include *Roseolovirus* and *Cyprinivirus*, whereas the 12 classified viral genera of D3 also include *Roseolovirus*, along with *Begomovirus*, *Chlorovirus*, and *Rhadinovirus*. The 12 classified viral genera of D6 include *Rhadinovirus*, as well, but *Mimivirus* and *Lymphocystisvirus* are also present in D6, and additionally present in D4. While there are only two classified viral genera in D5, *Chlamydiamicovirus* and *Inovirus*, D7 has seven classified viral genera including *Iotatorquevirus*. With the exception of D5, all of the dolphin URT metagenomes include phages.
Comparison of Sapelo Island Seawater and Dolphin Upper Respiratory Tract (Blowhole) Metagenomes

We compared the seven free-ranging dolphin URT (D) metagenomes from the southeastern coast of the US (Sapelo Island and Brunswick, GA) with the metagenomes of eight seawater (SW) samples taken from Sapelo Island, GA (Figure 2.1). The SW metagenomic data serves as a control for establishing dolphin URT microbiome specificity from the surrounding seawater. While the dolphin URT consists of 68% bacterial sequences, 5.3% viral sequences, and 1.3% eukaryotic sequences, the SW metagenomes have 83.3% bacterial sequences, 0.85% viral sequences, and 0.74% eukaryotic sequences (Figure 2.2).

The dolphin URT is dominated by the bacterial phyla Bacteroidetes (24.8%), Proteobacteria (23.4%), Actinobacteria (10.8%), and Firmicutes (6%) as was previously discussed, while the SW metagenomes contain the same top three phyla, (Proteobacteria (73.8%), Actinobacteria (3.4%), and Bacteroidetes (2.4%)), as well as Cyanobacteria (1.04%) and Firmicutes (1.03%) (Figure 2.7a). The most abundant classes in the dolphin URT metagenomes and the SW metagenomes are similar but in different distributions (Figure 2.7b). For example, Gamma- (44.3%), Alpha- (26.4%), and Betaproteobacteria (16.2%) make up a large portion of the SW metagenomes, while only accounting for a combined total of 31.5% of the dolphin URT, and the classes Actinobacteria (15.8%), Bacteroidia (8.5%), Bacilli (4.4%), and Clostridia (4%) each make up larger proportions of the dolphin URT than the SW bacterial sequences (4.1%, 0.31%, 0.64%, and 0.55%, respectively). Also, unclassified derivatives of the phylum Cyanobacteria account for 1.2% of the bacterial sequences of the SW classes and slightly less of the bacterial
sequences of the dolphin URT classes (0.77%). While the phylum/class-level comparisons demonstrated similarities between the dolphin URT and SW, the genera/species are more dissimilar (Figure 2.8a,b). The four most abundant bacterial genera of the SW metagenomes are *Pseudoalteromonas* (6.3%), *Pseudomonas* (4.9%), *Vibrio* (3.2%), and *Shewanella* (3.1%), which account for only 0.3%, 1.1%, 1.2%, and 0.89%, respectively, of the dolphin URT bacterial sequences. Interestingly, a handful of marine-associated genera such as *Polaribacter*, *Psychrobacter*, and *Croceibacter* are more abundant in the dolphin URT (2.3%, 2%, and 1.1%, respectively) than the SW metagenomes (0.12%, 0.36%, and 0.12%, respectively). Finally, although the top 25 bacterial species of the dolphin URT contain no *Pseudoalteromonas* spp., 6.2% of the top 25 bacterial species of the SW metagenomes are *Pseudoalteromonas* spp.. Additionally, while no *Pseudomonas* spp. are present in the top 25 bacterial species of the dolphin URT, these species account for 2.5% of the 25 most abundant bacterial species sequences in the SW metagenomes.

The only classified fungal phyla in both the dolphin URT and SW are Ascomycota (75.5% and 92.6% of the fungal sequences, respectively) and Basidiomycota (24.3% and 6.7% of the fungal sequences, respectively) (data not shown). The same fungal classes are present in both the dolphin URT and SW metagenomes with the exception of Pneumocystidomycetes in the SW (0.67% of the fungal sequences). Saccharomycetes, Eurotiomycetes, and Sodariomycetes are both abundant in the dolphin URT (32.5%, 13.3%, and 12.5% of the fungal sequences, respectively) and SW (22.8%, 28.9%, and 26.8% of the fungal sequences, respectively). The three most abundant fungal genera of the SW metagenomes are *Aspergillus* (14.8%), *Magnaporthe* (8.1%), and
Saccharomyces (6.7%) which make up 7%, 0.75%, and 9.5% of the dolphin URT, respectively, while the most abundant fungal species in the SW metagenomes (Aspergillus fumigatus 8.7%) accounts for 3.3% of the dolphin URT fungal sequences and the second-most abundant SW fungal species (Magnaporthe oryzae 8.1%), accounts for 0.75% of the dolphin URT (data not shown).

In the dolphin URT, 96% of the viral sequences at the order-level are unclassified; however, in the SW metagenomes, 76.1% of the viral sequences have the closest homology to Caudovirales, which account for 3.7% of the dolphin URT (data not shown). The two most abundant families in the dolphin URT (Microviridae 60% and Adenoviridae 23.5%) were not detected in the SW samples, whereas the two most abundant SW viral families (Myoviridae 56.4% and Podoviridae 15.7%) make up 0.5% and 0.24% of the dolphin URT viral sequences, respectively (Figure 2.5). Finally, T4-like viruses (31.4% of the viral sequences) and unclassified derivatives of various viral families (53.1% of the viral sequences) dominate the SW metagenomes while T4-like viruses make up 0.1% of the viral sequences in the dolphin URT. However, the dolphin URT and SW metagenomes have similar proportions of phages (37.8% and 34.5% of the viral sequences, respectively).

Comparison of Human Lung, Nares, and Dolphin Upper Respiratory Tract (Blowhole) Metagenomes

We compared the metagenomes of seven free-ranging dolphin upper respiratory tracts (D) from the southeastern coast of the US, 10 healthy human anterior nares (HN), three cystic fibrosis infected human lungs (CF), and two healthy human lungs (H) (Table
While bacterial sequences dominate the dolphin (68%) and human CF and healthy lung metagenomes (65% and 60%, respectively) in similar proportions, bacteria account for nearly all of the human anterior nares metagenome sequences (93%). Eukaryotic sequences have similar representations in both the CF (30%) and healthy (36%) human lung but are comparatively less prevalent in the dolphin URT (5%) and human anterior nares (0.3%). Viral sequences are lowest in the healthy human lung (0.07%) and slightly higher in the CF lung (0.2%), dolphin URT (1.3%), and human anterior nares (2.1%) (Figure 2.2).

The 25 most abundant phyla of the dolphin URT, human nares and lungs, and Sapelo Island seawater metagenomes reveal that Actinobacteria and Firmicutes are more prevalent in the human anterior nares (51.8% and 33.5%, respectively) than in the dolphin URT (10.8% and 6%, respectively) (Figure 2.9). Actinobacteria is less prevalent in the human CF (0.56%) and healthy (1.3%) lung than in the dolphin URT but Firmicutes is more prevalent (21.7% and 22.6%, respectively). Bacteroidetes accounts for similar proportions of the dolphin URT (24.8%) and human CF (30.5%) and healthy (27.9%) lung with much lower representation in the human anterior nares (2.1%). The eukaryotic phylum Nematoda falls within the top 10 phyla of the pooled dolphin metagenomes (1.7%), while Nematoda abundance is low in the human anterior nares (0.009%) and human CF lung (0.01%) and completely absent in the healthy human lung.

The top 25 classes of the pooled dolphin URT, human nares and lungs, and seawater metagenomes reveal that the class Actinobacteria makes up 51.8% of the human anterior nares, 10.8% of the dolphin URT, 0.6% of the healthy and 1.27% of the CF human lung (Figure 2.10). Class Bacilli accounts for 31.9% of the human anterior nares,
17.7% of the CF and 14.8% of the healthy human lung, but only 3% of the dolphin URT, while the class Gammaproteobacteria is more prevalent in the dolphin URT (16.1%) and CF (9.1%) human lung than the human anterior nares (4.5%) and healthy (1.8%) human lung. The CF and healthy human lung sequences have a high abundance of the class Bacteroidia (30.2% and 27.6%, respectively) but the dolphin URT and human anterior nares have lower abundances (5.8% and 2.1%, respectively). At 16.8%, the class Flavobacteria has the highest abundance of all of the dolphin URT classes; interestingly, Flavobacteria is relatively low in the human metagenomes (0.2% in the nares and both CF and healthy lungs).

Comparison of Bacterial Sequences in Human Lung, Nares, and Dolphin Upper Respiratory Tract (blowhole) Metagenomes

The dolphin URT, human anterior nares, and CF human lung are dominated by the same four bacterial phyla: Actinobacteria, Proteobacteria, Bacteroidetes, and Firmicutes (Figure 2.9). The healthy human lung, on the other hand, is dominated by Bacteroidetes (46.6%), Firmicutes (37.8%), Proteobacteria (6.1%), and Fusobacteria (3.5%). The two most abundant phyla of the bacterial sequences of the dolphin URT, Bacteroidetes and Proteobacteria, have similar percentages (36.4% and 34.3%, respectively) followed by Actinobacteria (15.8%) and Firmicutes (8.8%); however, the abundance distribution of the human anterior nares is less proportional than the dolphin URT with the phylum Actinobacteria making up more than half of the entire metagenome (55.7%), followed by Firmicutes (36%), Proteobacteria (5.9%), and Bacteroidetes (2.2%). The three CF and two healthy human lung metagenomes are similar to one another as both have comparable abundances of the top three phyla, Bacteroidetes (46.7% and
46.6%, respectively), Firmicutes (33.2% and 37.8%, respectively), and Proteobacteria (15.8% and 6.1%, respectively), but the healthy human lung differs from the other metagenomes in that Fusobacteria (3.5%) ranks higher in abundance than Actinobacteria (2.1%).

The bacterial class Flavobacteriia (Figure 2.10) is more prominent in the dolphin URT (24.7%) than the other metagenomes (0.02% in the human anterior nares and 0.3% in both the CF and healthy lungs), although D2 and D7 have fewer sequence features with a hit (referred to hereafter as a hit) than the other five dolphin metagenomes. Additionally, the classes Cytophagia and Planctomycetia are more abundant in the dolphin metagenomes than in the human metagenomes and the classes Solibacteres, Thermomicrobia, Ktedonobacteria, Elusimicrobia, Zetaproteobacteria, Gemmatimonadetes, Chrysiogenetes, and Spartobacteria have hits exclusively in the dolphin metagenomes. While no bacterial classes have hits exclusively in the human metagenomes, Fibrobacteria, Negativicutes, and Mollicutes are more prominent in the human anterior nares and lungs than in the dolphin URT.

With nearly double the number of classifications, the bacterial genera associated with the seven dolphin URT metagenomes have more variation than the genera of the human anterior nares and CF and healthy human lung (Figure 2.11a). The genera Bacteroides and Flavobacterium have the highest abundances in the dolphin metagenomes (5.5% and 5.2%, respectively), whereas the highest abundances in the 10 human anterior nares metagenomes are Corynebacterium and Propionibacterium, which together make up a significant percentage of the nares (30.3% and 25%, respectively), and the highest abundances in the three CF human lung metagenomes are Prevotella
(38.8%) and *Streptococcus* (22.1%). The two healthy human lung metagenomes have smaller top percentages, *Prevotella* (22.7%) and *Bacteroides* (22%), and more bacterial diversity than the other human metagenomes. While *Corynebacterium* and *Propionibacterium* make up a large portion of the human anterior nares metagenomes, these two genera have smaller representations in the dolphin URT (1.3% and 0.6%, respectively) and the CF (0.01% and 0.04%, respectively) and healthy (0.09% and 0.08%, respectively) human lung. Additionally, the genus *Prevotella*, while abundant in the CF and healthy human lung, accounts for only 1.1% of the dolphin URT and 0.3% of the human anterior nares. As the higher diversity in the dolphin URT would indicate, there are a number of genera in the dolphin metagenomes that are in low abundance or completely absent in the human metagenomes. The scope of abundance appears to indicate that the dolphin URT is more similar to the human lung than the anterior nares as many more genera have hits in the dolphin URT and human lung than in the dolphin URT and human anterior nares. The genus *Gramella* has 5650 hits in the dolphin URT, 13 hits in the CF and healthy human lung combined, and no hits in the human anterior nares; a similar trend is followed by *Polaribacter* with 4354 hits in the dolphin URT, 4 hits in the CF and healthy human lung combined, and no hits in the human anterior nares. Additionally, the genus *Paludibacter* has 984 hits in the dolphin metagenomes and 31 hits in the CF and healthy human lungs combined and the genus *Candidatus Azobacteroides* has 658 hits in the dolphin URT and 210 hits in H2 but no hits in the human anterior nares, CF human lungs, or H1. Two genera associated with plant pathogenesis, *Leifsonia* and *Clavibacter*, have 732 and 710 hits, respectively, in the dolphin URT, with only *Clavibacter* having hits in the nares (2 hits) and CF and healthy
A number of human pathogen-associated genera, such as *Kingella*, *Arcanobacterium*, and *Weeksella*, have 670, 627, and 464 dolphin URT hits, as opposed to only 11 combined human lung hits, 5 combined human nares and lung hits, and 0 human hits, respectively. Additionally, the bacterial genera *Actinobacillus*, which includes a number of species associated with the pig respiratory tract, has 1329 hits in the dolphin URT, 889 hits in the human anterior nares, and 12 hits in the CF and 75 hits in the healthy human lungs.

In the dolphin URT, no single bacterial species accounts for more than 3% of the metagenomes. *Gramella forsetii* (2.9%), *Flavobacterium johnsoniae* (2.4%), *Escherichia coli* (2.4%), and *Flavobacterium psychrophilus* (2.2%) are the only bacterial species that exceed 2% of the metagenome in the dolphin URT (Figure 2.11b). In the 10 human anterior nares metagenomes, the bacterial species *Propionibacterium acnes* makes up 24.8% of the bacterial sequences, followed by *Corynebacterium accolens* (11.2%) and *Lactobacillus iners* (10.3%). The three CF and two healthy human lung metagenomes are quite different in terms of bacterial species distribution with *Prevotella melaninogenica* (20.7%) having the highest abundance in the CF lung, followed by *Streptococcus pneumoniae* (5.9%) and *Pseudomonas aeruginosa* (5.8%), and *Bacteroides* spp., such as *B. fragilis* and *B. intestinalis*, accounting for 13.7% of the healthy human lung metagenomes. The most abundant bacterial species in the dolphin URT are either very low or completely absent in the human metagenomes, such as *Gramella forsetii* which makes up 0.02% of both the CF and healthy human lungs and has no hits in the human anterior nares. *Escherichia coli* is higher in the human anterior nares (0.4%), CF (0.04%), and healthy (0.02%) human lung than either of the top *Flavobacterium* spp. of the
dolphin URT. *Riemerella anatipestifer*, a common pathogen in water-dwelling birds, has 2667 hits in the dolphin URT and only 68 hits in the human anterior nares, 5 hits in the CF human lung, and 4 hits in the healthy human lung. Similarly, a known respiratory pathogen in poultry, *Ornithobacterium rhinotracheale*, was detected in the dolphin URT (268 hits) and the human anterior nares (2 hits) but not in either the CF or healthy human lungs. The large abundance and high diversity of *Corynebacterium* spp. in the anterior human nares (54476 hits and at least 60 species) is more similar to the dolphin URT (2469 hits and over 20 species) than either the CF (4 hits and three species) or healthy (23 hits and seven species) human lung.

*Comparison of Fungal Sequences in Human Lung, Nares, and Dolphin Upper Respiratory Tract (Blowhole) Metagenomes*

Ascomycota and Basidiomycota are the only two identified fungal phyla (data not shown) in the seven dolphin URT (75.5% and 24.3%, respectively) and the three CF (7.5% and 92.5%, respectively) and two healthy (4.4% and 95.6%, respectively) human lungs metagenomes. The 10 human anterior nares metagenomes have a larger diversity of fungal phyla. In addition to Ascomycota (32.4%) and Basidiomycota (58.5%), the human anterior nares also contain the phyla Chytridiomycota, Glomeromycota, and Blastocladiomycota.

The most abundant fungal classes of the dolphin URT include Saccharomycetes (32.5%), Ustilaginomycetes (19.3%), and Eurotiomycetes (13.3%) (data not shown). While Saccharomycetes rank high in the human anterior nares (20.5%), this class is less prevalent in the CF (6%) and healthy (1.5%) human lungs. Exobasidiomycetes, the most
prevalent fungal class in the human anterior nares (35.3%) and CF (92.5%) and healthy (95.6%) human lungs only makes up 0.3% of the fungal sequences in the dolphin URT. Eurotiomycetes, which does not appear in the CF human lung metagenomes, accounts for 1.5% of the healthy human lung, 9.2% of the human anterior nares, and 13.3% of the dolphin URT.

While there are nearly 200 fungal genera in the human anterior nares and over 40 fungal genera in the dolphin URT metagenomes, both the CF and healthy human lungs had comparatively lower abundances of fungal genera (Figure 2.12). The genus *Malassezia* is the most abundant in the human anterior nares (17%) and the CF (92.5%) and healthy (95.6%) human lungs but it only accounts for 0.3% of the dolphin URT. Conversely, the most abundant genus of the dolphin URT, *Ustilago* (19.3%), makes up 2.3% of the human anterior nares metagenomes and does not appear in either the CF or healthy human lungs metagenomes. While *Candida* was detected in the human anterior nares (9.6%), the CF human lung (4.5%), and the dolphin URT (2%), *Aspergillus* was detected in the human anterior nares (2.4%), the healthy human lung (1.5%), and the dolphin URT (7%). Interestingly, the second and third most abundant genera in the dolphin URT, *Debaryomyces* (10.3%) and *Leptosphaeria* (10%), each make up 1.5% of the CF human lung, while only *Leptosphaeria* has 1 hit in the human anterior nares and neither genus appears in the healthy human lung.

The species *Malassezia globosa* is the most abundant fungal species in the human anterior nares (14.6%), the CF (92.5%), and healthy (95.6%) human lung but has only a single hit in D7 of the dolphin URT metagenomes (data not shown). Of the top fungal species of the dolphin URT, *Debaryomyces hansenii* (10.3% in the dolphin URT) and
Leptosphaeria maculans (10% in the dolphin URT) are found only in the CF human lung (1.5% for both species). Although there are only five fungal species in the CF human lung, two different Candida spp., C. albicans and C. dubliniensis, have hits. Both C. albicans and C. dubliniensis have hits in the human anterior nares and the dolphin URT, along with other various Candida spp. such as C. tropicalis and C. glabrata in the dolphin URT and C. tropicalis, C. castelli, C. metapsilosis, and other Candida spp. in the human anterior nares. No Candida spp. were detected in the healthy human lung. The healthy human lung has six fungal species, including two Aspergillus spp., including A. fumigatus and A. terreus. Both Aspergillus spp. have hits in the human anterior nares and dolphin URT, while no Aspergillus spp. occur in the CF human lung. The seven dolphin URT metagenomes also contain A. niger and A. oryzae while the 10 human anterior nares metagenomes contain A. tubengensis, A. awamori, A. parasiticus, A. niger, A. flavus, and A. kawachii. Similar to the unique occurrence of D. hansenii and L. maculans in the CF human lung and dolphin URT, the healthy human lung contains two fungal species, Neurospora crassa (1.5%) and Yarrowia lipolytica (0.7%), that have hits solely in the dolphin URT (4.5% and 3.3%, respectively).

Comparison of Viral Sequences in Human Lung, Nares, and Dolphin Upper Respiratory Tract (blowhole) Metagenomes

At the order level, 96% of the viruses in the dolphin URT are unclassified while the remaining 4% are divided into the orders Caudovirales and Herpesvirales with 137 and 14 hits, respectively. In the human anterior nares, 99.2% of the viral sequences are classified under the order Caudovirales, with only 0.01% and 0.8% of the viral sequences categorized as Herpesvirales and unclassified, respectively (data not shown). The CF
human lung had the least amount of viral sequences classified as Caudovirales (10.3%) and similar proportions of Herpesvirales (50%) and unclassified sequences (39.7%), whereas the healthy human lung, similar to the dolphin URT, had mostly unclassified sequences (81.3%) distantly followed by Caudovirales (15.6%) and Herpesvirales (2.9%).

The top ranking viral family in the dolphin URT (Microviridae with 60% of the viral sequences) is absent in the human anterior nares, CF, and healthy human metagenomes, while the second most abundant family (Adenoviridae with 23.5% of the viral sequences) has hits only in the human anterior nares (3 hits accounting for 0.01% of the viral sequences) and the third most abundant family (Inoviridae with 3.04% of the viral sequences) has no hits in any of the human metagenomes (data not shown).

Although 97% of the viral sequences of the human anterior nares are categorized as Siphoviridae, this family accounts for 10.3% of the CF and 12.5% of the healthy human lung and only 2.9% of the dolphin URT. Herpesviridae, which has the highest percentage of hits in the CF human lung (49.4%), accounts for 3.1% of the healthy human lung, 0.3% of the dolphin URT, and 0.01% of the human anterior nares while Retroviridae, which has only 1 hit in the human anterior nares and is not detected in the dolphin URT, makes up 34.6% of the CF and 65.6% of the healthy human lung. Finally, the family Myoviridae occurs only in the human anterior nares (2.3%) and the dolphin URT (0.5%).

The viral genera (Figure 2.13) of the dolphin URT, human anterior nares, and human lung are quite variable, with the most abundant dolphin URT genus, *Chlamydiamicovirus* (a Chlamydia phage accounting for 35.2% of the viral sequences), detected only in the dolphin metagenomes. Subsequently, of the other top ranking genera
in the dolphin URT, *Mastadenovirus* (23.5%), *Bdellomicrovirus* (5.03%), and *Inovirus* (2.9%), only *Mastadenovirus* has hits in the human metagenomes (3 hits in the human anterior nares). A large portion of the viral sequences in the human anterior nares are categorized as unclassified derivatives of the family Siphoviridae (94.3%). This category accounts for 0.7% of the viral sequences in the dolphin URT and 10.3% and 12.5% of the CF and healthy human lung, respectively. The next two highest classifications of viral genera in the human anterior nares are bacteriophages (lambda-like (2.5%) and T-4-like (2%)) which account for 2.2% and 0.1% of the dolphin URT, respectively. While 33.3% of the viral sequences in the CF human lung and 50% of the viral genera of the healthy human lung are unclassified derivatives of the family Retroviridae, 1.3% of the CF human lung consists of the genus *Gammaretrovirus* and 18.8% of the healthy human lung is made up of retroviruses including *Alpha-, Beta-,* and *Gammaretrovirus*. The dolphin URT metagenomes have no retroviruses but the human anterior nares metagenomes have 1 *Gammaretrovirus* hit.

**Comparison of Potential Odontocete and Known Human Respiratory Pathogens in the Human Lung, Nares, and Dolphin Upper Respiratory Tract (Blowhole) Metagenomes**

**Comparison of Potential Odontocete Respiratory Pathogens**

Many of the potential odontocete respiratory pathogens appear in the seven dolphin URT metagenomes, as well as the 10 human anterior nares and three CF and two healthy human lung metagenomes (Table 2.3). Interestingly, the CF human lungs have more odontocete respiratory pathogen hits but with less variation among species than the healthy human lungs and the odontocete respiratory pathogens found in both lung
metagenomes are less varied than either the dolphin URT or human anterior nares. *Staphylococcus aureus* is the most prevalent odontocete respiratory pathogen in both the dolphin URT (0.7% of the bacterial sequences) and the human anterior nares (2.5% of the bacterial sequences) but is less present in the CF (0.2% of the bacterial sequences) and healthy (0.03% of the bacterial sequences) human lungs. Unsurprisingly, *Pseudomonas aeruginosa* is the most prevalent odontocete respiratory pathogen in the CF human lung (5.9% of the bacterial sequences) and the least prevalent odontocete respiratory pathogen in the healthy human lungs (with 1 hit), while accounting for 0.2% of the dolphin URT bacterial sequences and 0.1% of the human anterior nares bacterial sequences. The dolphin URT and CF and healthy human lungs have the same two *Streptococcus* spp., *S. equi* (0.04%, 0.08%, and 0.2% of the bacterial sequences, respectively) and *S. dysgalactiae* (0.01%, 0.5%, and 0.14% of the bacterial sequences, respectively) but the human anterior nares have *S. canis* (1 hit) along with *S. equi* (0.02% of the bacterial sequences) and *S. dysgalactiae* (0.003% of the bacterial sequences). The bacterial pathogen *Proteus mirabilis* makes up 0.18% of the dolphin URT, has 3 hits in both the CF human lungs and human anterior nares bacterial sequences, and no hits in the healthy human lungs. *Nocardia farcinica* is the only *Nocardia* spp. odontocete respiratory pathogen with hits in the dolphin URT (0.1% of the bacterial sequences), CF (0.01% of the bacterial sequences), and healthy (0.01% of the bacterial sequences) human lungs but *N. asteroides* (23 hits) and *N. cyriacigeorgica* (1 hit), in addition to *N. farcinica* (4 hits) occur in the human anterior nares metagenomes. *Proteus mirabilis* and *Proteus vulgaris* both occur in the dolphin URT (0.18% and 0.05% of the bacterial sequences, respectively) and the human anterior nares (3 hits and 4 hits, respectively) but only *P.*
*mirabilis* occurs in the CF human lung (0.01% of the bacterial sequences) and neither *Proteus* spp. was detected in the healthy human lung. *Vibrio alginolyticus* occurs in the dolphin URT, healthy human lungs, and human anterior nares but *Erysipelothrix rhusiopathiae* occurs only in the dolphin URT. Also, *Aeromonas hydrophila* and *Aeromonas salmoncida* are found in the dolphin URT, the CF and healthy human lungs, and the human anterior nares.

Potential odontocete cetacean fungal respiratory pathogens make up 8.8% of the fungal sequences of the dolphin URT, 1.5% of the healthy human lungs, 12.6% of the human anterior nares, and none of the potential odontocete fungal respiratory pathogens occur in the CF human lungs (Table 2.3). Only *Aspergillus fumigatus* and *Aspergillus terreus* have hits in the healthy human lungs, while both *Aspergillus* spp. along with a third species, *Aspergillus niger*, was detected in the dolphin URT and human anterior nares. Both the dolphin URT and the human anterior nares contain *Cryptococcus neoformans* but the human anterior nares also contain *Cryptococcus gattii*. Additionally, the zygomycosis-associated species make up 6.1% of the human anterior nares metagenomes while none appear in the dolphin URT. Finally, both *Candida glabrata* and *Ajellomyces dermatitidis* are exclusive to the dolphin URT.

The only identified protozoal odontocete respiratory pathogen, *Toxoplasma gondii*, occurs in both the CF and healthy human lungs and dolphin URT but not the human anterior nares (Table 2.3). It is the most abundant odontocete respiratory pathogen in the healthy human lung with a combined 261 hits in both metagenomes and the third most abundant in the CF human lung with a combined 92 hits in the three CF metagenomes, while only having 4 combined hits in the dolphin URT metagenomes.
Comparison of Known Human Respiratory Pathogens

We compiled a list of 17 common human respiratory pathogens (bacterial, fungal, and viral) from various sources (Dasaraju and Liu 1996; LaRocque and Ryan 2013), including specific respiratory disease fact pages accessible via the Centers for Disease Control and Prevention website (www.cdc.gov) (Table 2.4). Many of these known human respiratory pathogens were detected in the dolphin URT, human anterior nares, and CF and healthy human lungs. *Haemophilus influenzae* makes up 0.38% of the dolphin URT bacterial sequences, 0.27% of the human anterior nares bacterial sequences, 0.17% of the CF, and 0.48% of the healthy human lungs bacterial sequences. *Streptococcus pneumoniae* and *Streptococcus pyogenes* each account for 0.12% of the bacterial sequences in the dolphin URT; 1.4% and 0.16%, respectively, of the bacterial sequences in the human anterior nares; 5.9% and 0.32%, respectively, of the bacterial sequences of the CF; and 3.1% and 0.6%, respectively, of the healthy human lungs. Additionally, the respiratory pathogen *Klebsiella pneumoniae* occurs in all of the metagenomes, making up 0.16% of the dolphin URT, 0.11% of the human anterior nares, 0.04% of the CF, and 0.02% of the healthy human lung bacterial sequences, as does *Legionella pneumophila*, accounting for 0.13% of the dolphin URT bacterial sequences and 11, 1, and 5 hits in the human anterior nares, CF, and healthy human lungs, respectively. Finally, *Mycobacterium tuberculosis* and *Neisseria gonorrhoeae* are both found in all of the metagenomes (0.1% and 0.2% of the dolphin URT bacterial sequences, respectively; 0.01% and 0.08% of the human anterior nares bacterial sequences, respectively; 1 hit and 0.15% of the bacterial sequences of the CF human lung, respectively; 1 hit and 0.53% of the bacterial sequences of the healthy human lung, respectively). Two human respiratory
pathogens from our list were not detected in the healthy human lung, including *Moraxella catarrhalis* (0.15% of the dolphin URT and 1.4% of the human anterior nares bacterial sequences, and 1 hit in the CF human lung) and *Coxiella burnetti* (0.07% of the dolphin URT bacterial sequences and 0.001% of the human anterior nares, and 1 hit in the CF human lung). On the other hand, *Corynebacterium diphtheriae* occurs in the dolphin URT (0.14% of the bacterial sequences), the human anterior nares (2.23% of the bacterial sequences), and the healthy human lungs (0.01% of the bacterial sequences) metagenomes. Both the dolphin URT and the human anterior nares have *Mycoplasma pneumoniae* (0.01% in both metagenomes) and *Mycoplasma hominis* (8 hits and 14 hits, respectively), along with *Chlamydia pneumoniae* (44 hits and 6 hits, respectively). The bacterium *Paracoccidioides brasiliensis* was only detected in the human anterior nares (3 hits).

Many of the fungal human respiratory pathogens were discussed in the previous section (*Comparison of Potential Odontocete Respiratory Pathogens*); however, a few fungal pathogens that cause respiratory infection in humans have not been identified as respiratory pathogens in current literature, only one of which was detected in the dolphin URT and human anterior nares. Although absent in the CF and healthy human lungs, *Aspergillus flavus* had 8 hits in the dolphin URT and 4 hits in the human anterior nares metagenomes. Additionally, the human adenoviruses A, B, C, D, E, F, and G are all found in the dolphin URT (23% of the viral sequences), while only human adenovirus C is found in the human anterior nares (3 hits) and no human adenoviruses are found in the CF or healthy human lungs. The other human respiratory viruses were not detected in the dolphin URT, human anterior nares, or CF or healthy human lung metagenomes.
Comparison of Mammalian Fecal and Dolphin Upper Respiratory Tract (Blowhole) Metagenomes

We compared the upper respiratory tract metagenomes of seven free-ranging Atlantic bottlenose dolphins from the southeastern coast of the US with seven cetartiodactyl and five healthy human fecal metagenomes (Table 2.2; Figure 2.1). Cetartiodactyla is the clade containing the Orders Cetacea and Artiodactyla; therefore, mammals within this group are evolutionarily related to dolphins. The seven cetartiodactyl metagenomes chosen for this project were used in a mammal-gut microbiota co-evolution study by Muegge et al. (2011). Selecting metagenomes of various cetartiodactyl species within the same study minimized disparity in sample retrieval methods and techniques per metagenome. Along this same line of reasoning, the human fecal metagenomes came from the same Human Microbiome Project that provided the human anterior nares samples. Bacteria account for nearly all of the sequences in the cetartiodactyl fecal metagenomes (90%) and human fecal metagenomes (96%), while only accounting for 68% of the dolphin URT metagenomes. As such, the dolphin URT has higher proportions of eukaryota and viruses (5.3% and 1.3%, respectively) than either the cetartiodactyl feces (0.61% and 0.17%, respectively) or the human feces (0.11% and 0.02%, respectively) (Figure 2.2).

The four most abundant phyla of the cetartiodactyl and human fecal metagenomes (data not shown) are the bacterial phyla Bacteroidetes (38.5% and 82.1%, respectively), Firmicutes (36.8% and 10%, respectively), Proteobacteria (6.3% and 1.8%, respectively), and Actinobacteria (4.9% and 0.82%, respectively) (Figure 2.9). While the same four phyla dominate the dolphin URT, the order is slightly different with Bacteroidetes as the
most abundant (24.8%), followed closely by Proteobacteria (23.4%), and Actinobacteria (10.8%) and Firmicutes (6%). Fusobacteria make up similar percentages of the dolphin URT (0.6%) and the cetartiodactyl feces (0.55%), but less of the human feces (0.15%). Similarly, the phylum Cyanobacteria accounts for 0.53% of the dolphin seven URT metagenomes and 0.55% of the seven cetartiodactyl fecal metagenomes but only 0.1% of the five human fecal metagenomes. The eukaryotic phyla Nematoda and Apicomplexa each rank within the top 10 most abundant identified phyla in the dolphin URT (1.7% and 0.3%, respectively) but are less abundant in the cetartiodactyl feces (0.01% for each) and the human feces (0.002% and 0.0007%, respectively).

The seven cetartiodactyl and five human fecal metagenomes are dominated by the same four classes (Figure 2.10): Bacteroidia (36.7% and 80.4%, respectively), Clostridia (27.9% and 8.3%, respectively), Bacilli (6.7% and 1.1%, respectively), and Actinobacteria (4.9% and 0.82%, respectively). While these four classes fall within the top 10 classes of the dolphin URT (5.8%, 2.7%, 3%, and 10.8%, respectively), the dolphin URT metagenomes are dominated by Flavobacteriia (16.8%) and Gammaproteobacteria (16.1%). Flavobacteriia accounts for 1% of the cetartiodactyl fecal metagenomes and 0.76% of the human fecal metagenomes while Gammaproteobacteria accounts for 3.3% of the cetartiodactyl feces and 0.65% of the human feces.

Comparison of Bacterial Sequences in Mammalian Fecal and Dolphin Upper Respiratory Tract (Blowhole) Metagenomes

Like the dolphin URT and human CF and healthy lung and anterior nares metagenomes, the mammalian fecal metagenomes are dominated by the bacterial phyla
Bacteroidetes, Proteobacteria, Actinobacteria, and Firmicutes (Figure 2.9). While the phylum Bacteroidetes has similar percentages in the dolphin URT (36.4%) and cetartiodactyl fecal (42.8%) metagenomes, Bacteroidetes accounts for 85.8% of the human fecal metagenomes. Also, Proteobacteria is the second most abundant phylum in the dolphin URT (23.4%) but is less prevalent in the cetartiodactyl fecal (7.01%) and human fecal (1.8%) metagenomes. Firmicutes is the second-most abundant phylum in both the cetartiodactyl (40.8%) and human fecal (10.5%) metagenomes, while making up 8.8% of the dolphin URT and Actinobacteria accounts for 15.8% of the dolphin URT, 5.4% of cetartiodactyl feces, and only 0.9% of the human feces. After the top four ranking phyla, Fusobacteria takes the fifth spot in all three groups with 0.9% in the dolphin URT and 0.6% in the cetartiodactyl and 0.02% in the human fecal metagenomes.

Flavobacteria, the most prevalent class (Figure 2.14a) in the dolphin URT (24.7%), accounts for 1.1% of the cetartiodactyl and 0.8% of the human fecal metagenomes. Similarly, Gammaproteobacteria makes up 23.7% of the dolphin URT but 3.6% and 0.7% of the cetartiodactyl and human fecal metagenomes, respectively. The most prevalent classes in both the cetartiodactyl and human fecal metagenomes are Bacteroidia (40.7% and 84%, respectively), Clostridia (30.9% and 8.6%, respectively), and Bacilli (7.4% and 1.2%, respectively). Combined, these classes account for nearly 80% of the cetartiodactyl fecal metagenomes and more than 90% of the human fecal metagenomes but less than 20% in the dolphin URT metagenomes.

The cetartiodactyl and human fecal metagenomes show diversity patterns similar to those of the dolphin URT. However, the five most abundant genera of the cetartiodactyl fecal metagenomes account for more than 50% of the bacterial sequences.
and the five most abundant genera of the human fecal metagenomes account for more than 80% of the bacterial sequences while the five most abundant genera of the dolphin URT metagenomes account for less than 20% of the bacterial sequences (Figure 2.14b). Bacteroides is the most prevalent genus in the dolphin URT and cetartiodactyl and human feces (5.5%, 22%, and 74.3%, respectively). While Flavobacterium (5.2%) and Gramella (2.9%) follow Bacteroides in abundances in the dolphin URT, Prevotella (15.3%) and Clostridium (8.9%) have the next highest abundances in the cetartiodactyl feces while Parabacteroides (4.1%) and Alistipes (3%) round out the three genera of greatest abundance in the human feces. A number of genera associated with human and animal intestinal tracts found in the cetartiodactyl and human fecal metagenomes also have hits in the dolphin URT. Faecalibacterium accounts for 2.5% of the cetartiodactyl and 0.7% of the human fecal metagenomes and 0.1% of the dolphin URT metagenomes, while Alistipes makes up 0.8% of the cetartiodactyl feces, 3% of the human feces, and 0.1% of the dolphin URT. Ruminococcus make up 2.4% of the cetartiodactyl fecal metagenomes, 0.7% of the human fecal metagenomes, and 0.2% of the dolphin URT metagenomes while Butyrivibrio account for 0.9% of the cetartiodactyl feces, 0.2% of the human feces, and 0.1% of the dolphin URT.

With the exception of Parabacteroides distasonis (2.1% of the bacterial sequences), the top 30 bacterial species of the five human fecal metagenomes are of the genera Bacteroides and Alistipes (data not shown). Unidentified Bacteroides spp. (12.9%) dominate the bacterial species in the human feces while B. fragilis (6.6%), B. thetaotaomicron (6.2%), B. vulgatus (6%), and B. ovatus (3.5%) account for the four most abundant classified species in the human fecal metagenomes. The top 30 species of
the seven cetartiodactyl fecal metagenomes are from a greater variety of genera than the human fecal metagenomes. Similar to the human feces, the cetartiodactyl feces are dominated by unidentified *Bacteroides* spp. (3.3%), but, unlike the human feces, *Prevotella melaninogenica* (2.8%), *Bacteroides vulgatus* (2.6%), *Faecalibacterium prausnitzii* (2.5%), and *Prevotella ruminicola* (2.4%) make up the four most abundant classified bacterial species. Unidentified *Bacteroides* spp. account for 0.83% of the dolphin URT; also, the four most abundant bacterial species of both the cetartiodactyl and human fecal metagenomes have hits in the dolphin URT. The top bacterial species of the dolphin URT, *Gramella forsetii* (2.9%) and *Flavobacterium johnsoniae* (2.4%), are found in the cetartiodactyl feces (0.1% and 0.2%, respectively) and the human feces (0.07% and 0.14%, respectively). *Escherichia coli*, while prevalent in the dolphin URT (2.4% of the bacterial sequences), are less abundant in the cetartiodactyl (0.67%) and the human (0.03%) fecal metagenomes. While many bacterial species have unique occurrences in the cetartiodactyl and human fecal metagenomes, the few bacterial species exclusive to the dolphin URT metagenomes are largely pathogens. For example, the human respiratory pathogen, *Hafnia alvei*, accounts for 0.27% of the dolphin URT and the human pathogen *Propionibacterium granulosum* accounts for 0.02% of the dolphin URT. Additionally, *Chlamydia suis*, associated with pneumonia in swine, makes up 0.03% of the dolphin URT, while *Bibersteinia trehalosi*, associated with pneumonia in bighorn sheep, makes up 0.01% of the dolphin URT. Finally, the plant pathogen, *Pseudomonas viridiflava*, accounts for 0.01% of the dolphin URT.
Comparison of Fungal Sequences in Mammalian Fecal and Dolphin Upper Respiratory Tract (Blowhole) Metagenomes

Two fungal phyla in the dolphin URT metagenomes, Ascomycota (75.5%) and Basidiomycota (24.3%), rank as the two most abundant phyla in both the cetartiodactyl (86% and 13.3%, respectively) and human (85% and 14.3%, respectively) fecal metagenomes (data not shown). The phylum Glomeromycota accounts for 0.4% of both the cetartiodactyl and human fecal metagenomes while the cetartiodactyl fecal metagenomes additionally contain the phylum Blastocladiomycota (0.1%).

Saccharomycetes ranks as the most abundant fungal class in both the dolphin URT (32.5%) and the human feces (30.8%) and, while this class makes up a similar percentage of the cetartiodactyl feces (21.3%), Eurotiomycetes and Sodariomycetes rank higher in abundance than Saccharomycetes in the cetartiodactyl fecal metagenomes (31.8% and 22.9%, respectively) (data not shown). Eurotiomycetes and Sodariomycetes account for 29.4% and 18.2%, respectively, of the human fecal metagenomes and 13.3% and 12.5%, respectively, of the dolphin URT metagenomes. Additionally, Ustilaginomycetes, which makes up 19.3% of the dolphin URT, accounts for 3.2% of the cetartiodactyl fecal metagenomes and 4.1% of the human fecal metagenomes.

The proportions and distributions of fungal genera across the dolphin URT, cetartiodactyl, and human fecal metagenomes are similar, although the genera of highest abundance differ between the dolphin URT and both fecal metagenomes (Figure 2.15). The most abundant genera in both fecal datasets are Aspergillus (15% of the cetartiodactyl and 17.8% of the human fecal metagenomes) and Gibberella (6% of the
cetartiodactyl and 10.2% of the human fecal metagenomes), which are present in the
dolphin URT but in lower proportions (7% and 0.3%, respectively). The highest genera
of the dolphin URT, *Ustilago* (19.3%), *Debaryomyces* (10.3%), and *Leptosphaeria*
(10%), all appear in the five human fecal metagenomes (4.1%, 3.6%, and 0.4%,
respectively) while only *Ustilago* and *Debaryomyces* appear in the seven cetartiodactyl
fecal metagenomes (3.2% and 0.7%, respectively).

Five fungal species (data not shown) of highest abundance in the cetartiodactyl
and human fecal metagenomes are *Gibberella zeae* (21.9% and 10.1%, respectively),
*Aspergillus fumigatus* (15.3% and 5.9%, respectively), *Saccharomyces cerevisiae* (13.9%
and 5.9%, respectively), *Emericella nidulans* (11.5% and 5.3%, respectively), and
*Neurospora crassa* (12.6% and 4.5%, respectively). All five species appear in the dolphin
URT (3.3%, 3.3%, 9.5%, 1%, and 4.5%, respectively) and, with the exception of
*Cochliobolus heterostrophus, Lyophyllum shimeji, Metarhizium acridum,* and
*Phycomyces blakesleeanus,* every fungal species identified in the dolphin URT has hits in
the cetartiodactyl fecal metagenomes

*Comparison of Viral Sequences in Mammalian Fecal and Dolphin Upper Respiratory
Tract (Blowhole) Metagenomes*

While 96% of the viral sequences in the dolphin URT are unclassified (derived
from viruses) at the order-level (data not shown), 86.4% and 76% of the viral sequences
in the mammalian feces and human feces metagenomes, respectively, are of the order
Caudovirales. Unclassified sequences account for 13.4% and 23.6% of the mammalian
and human fecal datasets while the order Herpesvirales is the lowest across both fecal metagenome groups and the dolphin URT.

The ranking of viral families between the mammalian and human feces are similar in that Siphoviridae (73.2% and 54.3%, respectively) and Myoviridae (4.5% and 17.9%, respectively) account for the top classified viral families in both groups of fecal data and only 2.9% and 0.5%, respectively, of the dolphin URT (Figure 2.16). Microviridae, which ranks first in the dolphin URT (60% of the viral sequences), makes up 0.1% of the mammalian feces and 0.5% of the human feces; however, the presence of microviridae in the fecal metagenomes contrasts with the complete absence of this family in the human anterior nares and human lungs. The second-most abundant family in the dolphin URT, Adenoviridae (23.5%), has no hits in the fecal data.

The most abundant viral genera (data not shown) in both the mammalian and human fecal metagenomes are a mix of unclassified genera derived from the order Caudovirales (4.5% and 1.2%, respectively) and two viral families (Myoviridae (2.4% and 6.4%, respectively) and Podoviridae (0.8% and 0.5%, respectively)) and bacteriophages. The unclassified derivatives of Myoviridae and Podoviridae rank much lower in terms of abundance in the dolphin URT (0.3% and 0.1%, respectively), as do bacteriophages as no single genus makes up more than 0.1% of the dolphin URT. The most abundant viral genera in the dolphin URT, Chlamydiamicovirus (a Chlamydia phage representing 35.2% of the viral sequences), which has no hits in the human anterior nares or lungs, accounts for 0.08% of the mammalian and 0.1% of the human fecal metagenomes.
Comparison of Potential Odontocete and Known Human Respiratory Pathogens in Mammalian Fecal and the Dolphin Upper Respiratory Tract (blowhole) Metagenomes

Comparison of Potential Odontocete Respiratory Pathogens

*Staphylococcus aureus* is the most abundant potential odontocete respiratory pathogen identified in the dolphin URT (0.7% of the bacterial sequences), cetartiodactyl (0.06% of the bacterial sequences), and human (0.01% of the bacterial sequences) fecal metagenomes (Table 2.3). *Pseudomonas aeruginosa* is the second-most abundant odontocete respiratory pathogen in both the dolphin URT (0.2% of the bacterial sequences) and the human feces (0.01% of the bacterial sequences) but *Streptococcus equi* ranks slightly higher in abundance (0.04% of the bacterial sequences) in the cetartiodactyl feces than *P. aeruginosa* (0.03% of the bacterial sequences). *S. equi* makes up 0.04% of the dolphin URT metagenomes and 0.01% of the human fecal metagenomes and *S. dysgalactiae* occurs in the dolphin URT and both mammalian fecal groups but has fewer hits than *S. equi* in all three metagenomes. Both *Proteus mirabilis* and *Proteus vulgaris* appear in the dolphin URT and cetartiodactyl and human fecal metagenomes, along with *Aeromonas hydrophila* and *Aeromonas salmoncida*, *Vibrio alginolyticus*, *Nocardia farcinica*, and *Erysipelothrix rhusiopathiae*. Interestingly, all of the potential odontocete bacterial respiratory pathogens that have hits in the dolphin URT metagenomes occur in both the cetartiodactyl and human fecal metagenomes.

The potential fungal respiratory pathogens include a number of *Aspergillus* spp.. The dolphin URT metagenomes and cetartiodactyl, and human fecal metagenomes contain *A. niger*, *A. terreus*, and *A. fumigatus*. All three groups of metagenomes also
contain *Candida glabrata* and *Cryptococcus neoformans*, while both the dolphin URT and the cetartiodactyl feces contain *Ajellomyces dermatitidis*. The cetartiodactyl and human fecal metagenomes each contain zygomycosis-associated species and *Cryptococcus gatti* occurs exclusively in the cetartiodactyl fecal metagenomes. Finally, the protozoal odontocete respiratory pathogen *Toxoplasma gondii* has 4 hits in the dolphin URT, 1 hit in the cetartiodactyl feces, and 69 hits in the human feces.

**Comparison of Known Human Respiratory Pathogens**

Nearly all of the bacterial human respiratory pathogens from our list occur in the dolphin URT, cetartiodactyl fecal, and human fecal metagenomes (Table 2.4). The human respiratory pathogens *Streptococcus pyogenes* and *Streptococcus pneumoniae* make up 0.04% and 0.03%, respectively, of the bacterial sequences of the human fecal metagenomes, while each account for 0.15% of the bacterial sequences of the cetartiodactyl fecal metagenomes and 0.12% of the bacterial sequences of the dolphin URT metagenomes. Additionally, *Haemophilus influenzae* accounts for 0.38% of the dolphin URT fungal sequences, 0.05% of the cetartiodactyl fecal fungal sequences, and 0.01% of the human fecal fungal sequences. *Chlamydia pneumoniae, Corynebacterium diphtheria, Coxiella burnetti, Klebsiella pneumoniae, Legionella pneumophila, Moraxella catharralis, Mycobacterium tuberculosis, Mycoplasma hominis, Mycoplasma pneumoniae, and Neisseria gonorrhoeae* all have hits in the dolphin URT, the cetartiodactyl fecal, and human fecal metagenomes, while *Paracoccidioides brasiliensis* was found only in the fecal metagenomes and *Chlamydia psittaci* was detected solely in the cetartiodactyl fecal sequences.
While many of the known fungal human respiratory pathogens from our list were discussed above (Comparison of Potential Odontocete Respiratory Pathogens) and many others do not occur in any of the metagenomes, *Aspergillus flavus* makes up 2.3% of the cetartiodactyl fecal and 2.5% of the human fecal metagenomes. Also, *Pneumocystis carinii* has 1 hit in the cetartiodactyl fecal metagenomes. No human adenoviruses had hits either of the fecal datasets, although human adenovirus A, B, C, D, E, F, and G occur in the dolphin URT. None of the other common human respiratory viruses from our list were detected in the dolphin URT or fecal metagenomes.

### 2.4 DISCUSSION

A sentinel animal may be used to detect environmental hazards and warn humans of potential dangers in a particular environment (Rabinowitz et al., 2009). Studies have suggested that dolphins may be sentinels for the health of the aquatic environment (Wells et al., 2004) due to their long life span and long-term residence in the coastal environment, high trophic feeding level, and high blubber content which bioaccumulates PCBs (Reddy et al., 2001). The utilization of dolphins as sentinels is two-fold: they offer insight into the impact of contaminants, marine biotoxins, and pathogens on coastal ecosystems and, because of the similarities between dolphins and humans (including mammalian characteristics, food sources, etc.) they may be used to examine the potential human health risks in the marine environment (Schwacke et al., 2013). We explored the role of dolphins as sentinels for existing or novel respiratory diseases originating from coastal ecosystems. The purpose of our study was to generate foundational data that may contribute to the discussion of marine mammals (particularly dolphins) as sentinels for human respiratory health in relation to the aquatic ecosystem. The similar structure and
function of the dolphin and human respiratory systems offers a unique opportunity to correlate an aquatic organism with humans in order to assess the similarities and differences in the respiratory microbiota of these two species, especially in relation to marine-associated respiratory pathogens. The current state of our knowledge on respiratory diseases in cetaceans is largely based on clinical studies using human pathogens as the primary reference organisms for identifying agents of infection (Chapter 1); therefore, this study represents the first characterization of the dolphin respiratory system using a metagenomic approach, where we compared the respiratory microbiomes of bottlenose dolphins and humans (lungs and anterior nares). Our results reveal broad similarities between the microbial taxonomy of the dolphin URT and the human respiratory system, a finding which may serve as the framework for establishing the respiratory tract as a link between dolphin and human health in relation to the marine environment.

*Ecology of the Dolphin URT*

The dolphin respiratory tract environment is warm and humid (Coulombe et al., 1965) with a complex mix of lipids and proteins (Foot et al., 2006). In an investigation of respiratory water exchange in two dolphin species, Atlantic bottlenose dolphins and Pacific white-sided dolphins, Coulombe et al. (1965) observed that the dolphin URT temperature was maintained 9°C cooler than the mean internal body temperature. Also, while expired air was 4.5°C cooler than air in the URT, it was between 3.5-4.3°C warmer than inhaled air. In the bottlenose dolphin URT, Coulombe et al. (1965) further determined that air was inspired and expired with a 75% mean relative humidity but air remaining in the URT had a mean relative humidity of 95-100%. This warm, moist
setting makes the dolphin URT hospitable for colonization by microorganisms. The microbiota of the dolphin URT is influenced by the unique lifestyle of cetaceans, including pressure and oxygen changes experienced while diving and forceful expirations through the blowhole resulting in mucus expulsion (Ridgway et al., 1969; Johnson et al., 2009). Therefore, the ecology of the dolphin URT makes it commodious for microbial colonization but the pressure/oxygen fluctuations and mucosal expulsions experienced by the host animal inhibits the growth of some microorganisms, effectively creating a dolphin-specific URT microbiome.

Dolphin URT Comparisons

The seven dolphin URT metagenomes demonstrated similarities across the higher level taxonomic lineages. All seven metagenomes were dominated by Bacteroidetes, Proteobacteria, Actinobacteria, and Firmicutes, and the seven metagenomes, with the exception of D2 and D7, had similar proportions of the classes Flavobacteriia and Gammaproteobacteria. In addition to congruous abundance values and distributions in broader taxonomic lineages of the dolphin URT microbiome, the genera/species of closest homology were comparable among the dolphin metagenomes. A number of the most abundant species matches in our dolphin metagenomes contained known human, other mammalian, bird, fish, and plant pathogens, a finding similar to that of Johnson et al. (2009). For example, the fish pathogen Flavobacterium psychrophilum (Starliper 2011) accounts for 1.5% of the total dolphin sequences; Riemerella anatipesifer, which causes septicemia in domestic and wild birds, particularly ducks (Segers et al., 1993; Hinz et al., 1996), accounts for 1% of the total species; and the human pathogen Capnocytophaga ochracea (Hawkey 1984) makes up 0.8% of the total dolphin
sequences. Although the average homology for these pathogens fall between 70-75% in the dolphin URT, which is far below the 97% threshold for verifying species (Schloss and Handelsman 2005), the fact that the genera and species of the dolphin metagenomes were most closely matched with characterized pathogens may indicate the presence of novel dolphin-specific pathogens.

Most of the order-level viral sequences were unclassified in the dolphin metagenomes meaning that numerous novel cetacean viruses could be present in the dolphin microflora. In terms of highest similarity to closest database matches, Enterobacteria phages M13 and lambda (which infect *Escherichia coli* (van Wezenbeek et al., 1980)) and human adenovirus C (a human respiratory pathogen (Table 2.4)) are 99% similar to their closest matches. Of the eight papillomaviruses detected in D1, the human papillomaviruses had 65% average similarity, *Phocoena spinipinnis* papillomavirus (which causes genital warts in Burmeister’s porpoises (Van Bressem et al., 2007)) had 74% average similarity, and *Tursiops truncatus* papillomavirus 1 (which causes papillomatous lesions in bottlenose dolphins (Rector et al., 2008)) had 69% average similarity to the database matches. These weak homologies could imply novel dolphin papillomaviruses in the dolphin URT metagenomes. Although viral metagenomics has exploded in recent years, including studies on viromes of the ocean environments (Angly et al., 2006), viral communities of corals (Marhaver et al., 2008), and viromes of sea lion feces (Li et al., 2011), many viruses are still uncharacterized thus there is currently little available data pertaining to the viral component of the dolphin respiratory microbiome.
The comparisons between the Sapelo Island, GA seawater metagenomes and the dolphin URT metagenomes reveal two distinct profiles (Figure 2.2). The SW phyla is dominated by Proteobacteria (73.8% of the total sequences), followed by Actinobacteria, Bacteroidetes, and Cyanobacteria, while the dolphin URT microbiome has a more even distribution among the four most abundant phyla, Bacteroidetes, Proteobacteria, Actinobacteria, and Firmicutes. The SW classes are dominated by Gamma-, Alpha-, and Betaproteobacteria (36.9%, 22%, and 13.5%, respectively). This finding is similar to that of Mou et al. (2008), which used metagenomics to determine the metabolic function of bacteria in Sapelo Island seawater. Neither Alpha- nor Betaproteobacteria account for large proportions of the dolphin URT metagenomes (2.4% and 2.9%, respectively). At the genus level, the dolphin URT and SW share only two of the 25 most abundant genera with database matches, *Pseudomonas* (which has an average of 70.9% and 83.2% homology in the dolphin URT and SW datasets, respectively) and *Vibrio* (which has an average of 71.9% and 83.5% homology in the dolphin URT and SW dataset, respectively). The differences between the dolphin URT and SW microbiomes suggest that the dolphin’s blowhole system is an efficient mechanism for breathing air in an aquatic environment. The active forceful expirations and passively tight seal of the blowhole (Berta and Sumich 1999) effectively keep seawater out of the dolphin’s airways; therefore, the microbial community of the dolphin URT does not mirror the surrounding seawater but, instead, has a distinctly individual consortium of microbes. Furthermore, these differences may be an indication of the co-evolution of the dolphin and its normal microflora. Cetaceans evolved as terrestrial mammals about 55 Ma and developed into fully aquatic organisms in the middle Eocene (about 40 Ma) (Thewissen
et al., 2007). During the transition from the land to the sea, the co-evolution of the dolphin’s microbiota may have resulted in a complex community structure reflective of the dolphin’s unique evolutionary path with little influence from the animal’s marine habitat.

*Comparison of Human Lung, Nares, and Dolphin Upper Respiratory Tract (Blowhole) Metagenomes*

Prior to the Human Microbiome Project (HMP), the human lung was generally perceived to be sterile while the upper respiratory airways were known to be inhabited by a core consortium of microorganisms (Willner et al., 2009). Advancements in sequencing approaches have revealed that the lungs are indeed populated by microorganisms; however, the efficiency of the lung filtration system may help maintain a smaller community of microbes than that of the upper airways (Hilty et al., 2010). Respiratory infections are pervasive in both humans and dolphins. In 2012, the World Health Organization ranked respiratory diseases as the leading cause of infectious disease mortalities in humans. Also, respiratory illnesses have been proposed as the leading cause of mortality in both captive and wild dolphins (Johnson et al., 2009). We compared the microbiome of the dolphin URT to the microbiomes of the human anterior nares, healthy, and diseased (cystic fibrosis) human lungs. In the United States, cystic fibrosis is the most common genetic disease in Caucasians (Gibson et al., 2003). CF affects the airways and submucosal glands and initial infection leads to a substantial increase in microbial inhabitants of the airways, including opportunistic pathogens that assume etiologic roles as the immunosuppressed CF individual advances in age. An increase in the biofilm of the lungs, particularly with the bacterial species *Staphylococcus aureus* and
*Pseudomonas aeruginosa*, leads to heavy lung congestion resulting in eventual respiratory failure (Gibson et al., 2003; Klepac-Ceraj et al., 2010). The dolphin URT and CF human lungs were dominated by same four bacterial phyla, Bacteroidetes, Firmicutes, Actinobacteria, and Proteobacteria, while the healthy human lungs metagenome had highest abundance of Bacteroidetes, Firmicutes, Proteobacteria, and Fusobacteria.

Because of the protective systems in place that maintain a reduced lung consortium, combined with the function of the blowhole essentially as the dolphin nostril (Berta and Sumich 1999), we hypothesized that the dolphin URT exhibits closer similarity to the human anterior nares than the human lungs. However, although the dolphin URT and human anterior nares have the same four most abundant bacterial phyla (Bacteroidetes, Firmicutes, Actinobacteria, and Proteobacteria), the most abundant phylum of the nares (Actinobacteria), which accounts for over 50% of the sequences, makes up a comparably smaller portion of the dolphin URT metagenomes (10.8%).

Although the phyla Bacteroidetes, Firmicutes, Proteobacteria, and Actinobacteria are large and diverse, each taxon has unique characteristics associated with its respective lineages. For example, members of the phylum Actinobacteria are Gram-positive and have a high DNA G+C content (Ventura et al., 2007). As one of the largest phyla of the domain Bacteria, Actinobacteria exhibit a range of lifestyles, including pathogenic (*Corynebacterium* spp. and *Propionibacterium* spp.) and commensal (*Leifsonia* spp. and *Bifidobacterium* spp.) organisms (Ventura et al., 2007). Firmicutes, on the other hand, exhibit varied Gram-staining characteristics (Sneath et al., 1986) and have low G+C contents in their DNA (Lightfield et al., 2011). With the largest number of characterized species (Dworkin and Falkow 2006), Firmicutes are considered both useful and
troublesome in the food and beverage industries (Caldwell et al., 2013), and may play important roles both beneficially (*Lactobacillus* spp.) and pathogenically (*Erysipelothrix* spp.) in the health of humans and animals (Carr et al., 2002; Wang et al., 2010). The phylum Proteobacteria, the most diverse of the bacterial phyla (Dworkin and Falkow 2006), includes largely Gram-negative organisms (Gupta 2000) and contains many well-known species of bacteria, including pathogens such as *Escherichia coli*, and an abundance of aquatic organisms (Schaechter 2011). The phylum Bacteroidetes is also commonly associated with aquatic environments, including marine (Yoon and Kasai 2014) and freshwater (Lee et al., 2013), as well as hyperthermal (Albuquerque et al., 2011) and hypersaline (Vaisman and Oren 2009) environments. Compared to Proteobacteria, Bacteroidetes has been underrepresented in culture (O’Sullivan et al., 2006); however, the characterizations of novel genera have recently surged, especially within the family Flavobacteriaceae (Hameed et al., 2012; Park et al., 2012; Chen et al., 2013). Finally, Fusobacteria is the fourth-most and fifth-most abundant bacterial phyla in the healthy human lungs and dolphin URT, respectively. Fusobacteria contains many commensals of the human oral microbiome (Eckburg et al., 2005). A number of genera within the phylum Fusobacteria are pathogenic, including *Fusobacterium*, *Leptotrichia*, and *Streptobacillus*. *Fusobacterium* spp., which cause pharyngitis, lung abscesses, and intestinal infections in humans and necrotic infections in many artiodactyl species (Citron 2002; Huggan and Murdoch 2008), account for larger percentages of the total sequences in the dolphin URT and both lungs than the human nares. A similar trend is observable in *Leptotrichia* spp., which causes periodontal diseases and gingivitis (Eribe and Olsen 2008). Also, the Fusobacteria species *Streptobacillus moniliformis*, the agent of rat bite
fever (though rare, human fatalities are often associated with endocarditis, bronchopneumonia, and lymph node hyperplasia (Elliott 2007)), had a greater number of matches in the dolphin URT microbiome than the human microbiomes. Although the average percent identity across *Fusobacterium, Leptotrichia*, and *Streptobacillus* ranges between 70-73% in the dolphin URT, the low homology might indicate the existence of novel Fusobacteria-related pathogens. Also, the CF human lung had lower average percent identities (70-75%) than either the healthy lung or human nares (75-85%) across the Fusobacteria genera. The similar homology of the dolphin URT and CF human lung among these genera may imply that these two microbiomes share novel Fusobacteria-related pathogens.

With the exception of Flavobacteriia in the dolphin URT, some of the most abundant bacterial classes of the dolphin URT and CF and healthy human lung are similar (including Gammaproteobacteria, Bacteroidia, and Bacilli) indicating that these microbial communities are comparable at both the phylum- and class-level. The class Gammaproteobacteria, which accounts for a greater percentage of the nares and CF lungs than the healthy lungs, contains numerous agents of human respiratory infection including *Coxiella burnetii, Haemophilus influenzae, Klebsiella pneumonieae, Legionella pneumophila*, and *Moraxella catarrhalis* (Williams et al., 2010), all of which were detected in the dolphin URT (with average homologies between 70-75%), human nares (with average homologies between 80-90%), and CF lung (with average homologies between 70-90%) metagenomes. Furthermore, Gammaproteobacteria contains a number of pathogens that potentially cause respiratory disease in odontocetes, such as *Actinobacillus* spp., *Aeromonas* spp., *Proteus* spp., *Pseudomonas aeruginosa, Salmonella*
spp., and *Vibrio alginolyticus* (Williams et al., 2010; Chapter 1). *Aeromonas hydrophila*, *A. salmoncida*, and *Pseudomonas aeruginosa* were detected in the dolphin URT (with average homologies between 70-75%), human nares (with average homologies between 85-90%), CF lung (with *Aeromonas* spp. average homology of 79% and *Pseudomonas aeruginosa* average homology of 98%), and healthy lung (with *Aeromonas* spp. average homology of 75% and *Pseudomonas aeruginosa* average homology of 97%). The prevalence of this class has been previously established in the core microflora of the dolphin URT (Buck et al., 2006; Johnson et al., 2009; Morris et al., 2011). A culture-dependent analysis by Buck et al. (2006) found the blowhole of free-ranging dolphins dominated by the bacterial class Gammaproteobacteria while a similar study by Morris et al. (2011) also identified Gammaproteobacteria as the dominant bacterial class in visually healthy bottlenose dolphin blowholes. Additionally, a 16S rDNA study by Johnson et al. (2009) determined that the class Gammaproteobacteria had the highest abundance across visually healthy bottlenose dolphin URT clone libraries. The predominance of Gammaproteobacteria in the dolphin URT, human nares, and CF human lung may indicate a large presence of pathogenic species in these microbiomes. The class-level designations between the dolphin URT and the human nares show more similarity than between the dolphin URT and either human lung, with the same seven most abundant bacterial classes (with the exception of Flavobacteriia in the dolphin URT) occurring in both the dolphin URT and human nares metagenomes, including Gammaproteobacteria, Actinobacteria, Bacteroidia, Bacilli, Clostridia, Alpha- and Beta- proteobacteria. The class Actinobacteria contains not only a number of human respiratory pathogens (such as *Corynebacterium diphtheria* and *Mycobacterium tuberculosis*) (Ventura et al., 2007), but
also a number of potential odontocete pathogens (*Nocardia* spp.) (Chapter 1). All of these pathogens were detected in the dolphin URT and human nares. In the human nares, stronger homology to the human pathogens (90-95%) than the odontocete pathogens (70-80%) was observed, while homologies were equally weak between the human and potential odontocete pathogens in the dolphin URT (70-75%). The class Flavobacteriia, which, along with Gammaproteobacteria, dominates the dolphin URT, makes up a small percentage of the human nares, CF, and healthy human lung. Similar to our findings, Flavobacteriia was identified as the second-most abundant class in blowhole analyses of free-ranging bottlenose dolphins in the study by Johnson et al. (2009). Interestingly, no odontocete pathogens thusly reported falls under the class Flavobacteriia.

The fungal phyla detected in the dolphin URT metagenomes were more similar to the CF and healthy human lungs than the human anterior nares. The fungi within the nares fell under five different phyla (Basidiomycota, Ascomycota, Chytridiomycota, Glomeromycota, and Blastocladiomycota) while the dolphin URT and both lungs were clustered in only two phyla (Basidiomycota and Ascomycota). The specificity of the dolphin microbiome is observable in the 75/25% split of Ascomycota and Basidiomycota, respectively, which is reversed in the CF and healthy lung metagenomes with Basidiomycota accounting for over 90% of the fungal sequences. There were 10 identified taxa in the dolphin URT, while only three and four fungal classes were detected in the CF and healthy human lungs. The class Saccharomycetes, present in all of the dolphin and human respiratory metagenomes and accounting for large portions of both the dolphin URT and human nares, is a diverse class of fungi containing the genera *Aspergillus* and *Candida*. Interestingly, both *Aspergillus* and *Candida* accounted for
approximately 10% of the fungal sequences of the dolphin URT and the human nares, whereas *Aspergillus* was not detected in the CF lung and *Candida* was not detected in the healthy lung. While only a handful of *Candida* spp. are pathogenic to humans (Douglas 2003), *Candida glabrata*, which was detected only in the dolphin URT, has been found in post-mortem analyses of odontocetes with respiratory infection (Nollens et al., 2008). *Aspergillus* spp., on the other hand, are associated with both human (Table 2.4) and odontocete respiratory diseases (Chapter 1). Although *Candida* spp. and *Aspergillus* spp. have weak homologies (between 68-72%) in the dolphin URT, close database matches to pathogenic genera may be indicative of phylogenetically-connected, dolphin-specific genera in the metagenomes.

While the dolphin and human metagenomes all had matches to the protist phylum Apicomplexa, a phylum consisting solely of animal parasites, including the etiologic agents of toxoplasmosis, malaria, and cryptosporidiosis (Morrissette and Sibley 2002), this taxonomic group accounts for 0.3% of the total sequences of the dolphin URT, 0.5% of the total sequences of the CF lung, and 0.9% of the total sequences of the healthy lung, but only 0.001% of the total nares sequences. The malaria pathogens, *Plasmodium* spp., are the most abundant apicomplexans in the dolphin URT and human lungs and nares, accounting for higher percentages in the dolphin URT and CF human lung. Also, the protozoan *Toxoplasma gondii* was detected in the dolphin URT and both the CF and healthy human lungs. This zoonotic parasite, identified in numerous studies as a potential odontocete respiratory pathogen (Chapter 1), accounted for a higher percentage of the eukaryotic sequences in the healthy human lung (1.8%). While the homologies were low for genera and species of the phylum Apicomplexa (averaging between 70-83% in the
dolphin URT, human nares, CF, and healthy human lung), the fact that the closest database matches were apicomplexans may indicate that novel organisms with phylogenetic similarities to these parasites are present in the dolphin URT and human nares and lungs metagenomes.

The largest percentage of viral orders in the dolphin URT was unclassified viral derivatives (96% of the viral sequences) with Caudovirales and Herpesvirales making up the remaining 4%. A similar trend was detected in the healthy human lung with over 80% of the viral orders categorized as unclassified and the remaining orders divided into Caudovirales and Herpesvirales. While the two identified taxa and unclassified category was observable in both the nares and CF lung, the unclassified derivatives made up less than 40% of the CF lung and less than 1% of the human nares. Microviridae, the family of viruses that dominates the dolphin URT, contains bacteriophages that infect pathogens such as *Escherichia coli* and *Chlamydia psittaci* (Pavesi 2006; Roux et al., 2012). *C. psittaci* was not detected in the dolphin URT or human nares or lungs but *E. coli* was detected in all of the dolphin and human metagenomes with stronger homology in the nares (92%) than the dolphin URT and CF and healthy lungs metagenomes (average homology of 75%). While the family Microviridae (and corresponding genera) was not detected in the human respiratory metagenomes, a genus of Microviridae, *Chlamydiamicovirus*, is the most abundant genus identified in the dolphin URT (with 71% average homology). *Chlamydiamicovirus* infects species of *Chlamydia* and *Chlamydophila*, including the human respiratory pathogen *Chlamydia pneumoniae* (King et al., 2012). The specific *Chlamydiamicovirus* phage that infects *C. pneumoniae*
(Chlamydia phage CPAR39) (Hoestgaard-Jensen et al., 2011) was the second-most abundant viral species matched in the dolphin URT (with 73% average homology).

A viral metagenomics study by Willner et al. (2009) on sputum samples from five CF and five non-CF (including one asthmatic and one CF spouse) individuals found distinct differences between the respiratory tract associated phages in the CF (and non-CF asthmatic and non-CF spouse) and the non-CF viromes. Our investigations revealed Staphylococcus phages in the dolphin URT, human nares, and CF lung but not in the healthy human lung. These findings are similar to the results of Willner et al. (2009), which showed a higher abundance of Staphylococcus phages in the CF inflicted lungs than the healthy lungs. Staphylococcus aureus, a potential respiratory pathogen in odontocetes (Chapter 1) and a known respiratory pathogen in humans (Levin et al., 1990), was detected in the dolphin URT, the human nares, and the CF and healthy lungs, with stronger homologies in the human metagenomes (between 80-90%) than the dolphin URT metagenomes (67%). S. aureus was also isolated from the blowhole of visually healthy dolphins in the culture-dependent study by Morris et al. (2011) from Charleston Harbor, SC and Indian River Lagoon, FL. Therefore, the presence of Staphylococcus phages in the diseased human lung, nares, and dolphin URT may indicate that these bacteriophage communities function to control the populations of bacteria in the respiratory human and dolphin microbiomes. Another important correlation between our study and Willner et al. (2009) was that while no Pseudomonas phages were found in any of the metagenomes, Pseudomonas aeruginosa was detected in the dolphin URT, human nares, and CF and healthy human lungs. Willner et al. (2009) suggest that this
discrepancy may be due to an abundance of novel *P. aeruginosa* phages that have not yet been classified.

The family Adenoviridae, the second-most abundant viral family in the dolphin URT, was detected in the human nares but not in the CF-infected or healthy human lungs. The seven species of adenoviruses (A-G) cause a wide range of human illnesses, including respiratory disease (Bhat et al., 1984; Murtagh et al., 1993; Robinson et al., 2011; Cai et al., 2014). While all seven species of human adenoviruses (A-G) were detected in the dolphin URT, accounting for 15% of the viral sequences with an average homology of 86%, the human nares exhibited closest homology to only human adenovirus C (with an average similarity of 95%). The only potential odontocete virus detected in the dolphin and human metagenomes was a polyomavirus in the human nares (with an average homology of 100%). However, the large percentage of unclassified viruses in the dolphin URT evidences the limited data currently available on odontocete viral pathogens; therefore, numerous species specific viruses are likely present in the dolphin URT microbiome but currently uncharacterized.

In terms of bacterial and fungal phyla/classes and viral orders/families, the dolphin URT and human nares and lungs are remarkably similar. We hypothesized that the dolphin URT exhibits closer similarity to the human anterior nares than the human lungs, and, after evaluating the data, we have disproved this hypothesis at the broader taxonomic levels. Our data shows a closer similarity between the dolphin URT and the human lungs than the dolphin URT and the human nares but the broad taxonomic similarities observable between the dolphin URT and the human nares are still substantial. This is especially true considering that dolphins are marine mammals.
inhabiting an aquatic environment while humans reside on land. The similarities that we have shown in the bacterial, fungal, and viral members of the dolphin URT and human nares and lungs microbiomes will be useful for establishing dolphins as sentinels for the relationship between marine ecosystem change and human respiratory health.

Comparison of Mammalian Fecal and Dolphin Upper Respiratory Tract (Blowhole) Metagenomes

In order to examine the similarities and dissimilarities of evolutionarily related microbiomes, we wanted to compare the dolphin metagenomes to other cetartiodactyls (species of the Order Artiodactyla) metagenomes. The Orders Cetacea and Artiodactyla are the only two orders in the Cetartiodactyla clade, placing artiodactyls as the closest evolutionary relatives of cetaceans (Price et al., 2005). As respiratory tract metagenomes for artiodactyls were unavailable (and this study represents the first metagenomic analysis specific to the dolphin URT), artiodactyl fecal metagenomes were used to broadly compare the microbiomes of these evolutionarily related organisms. Overall, the dolphin URT was more similar to the cetartiodactyl fecal metagenomes than the human fecal metagenomes. Both the dolphin URT and cetartiodactyl microbiomes have nearly equal percentages of the two most abundant bacterial phyla (about 35% for Bacteroidetes and Proteobacteria for the dolphin URT and about 41% for Bacteroidetes and Firmicutes in the cetartiodactyl feces) while the human fecal datasets are less evenly distributed with Bacteroidetes accounting for more than 85% and Firmicutes accounting for 10.5% of the sequences. A study by Arumugam et al. (2011), which used the human fecal metagenomes provided via the HMP combined with newly sequenced fecal metagenomes from four countries, also found a predominance of Bacteroidetes and Firmicutes in the
human gut microbiome. The rank of bacterial classes is similar between the cetartiodactyl and human fecal metagenomes (Bacteroidia, Clostridia, Bacilli, Actinobacteria), although the distributions differ. Both Flavobacteriia and Gammaproteobacteria were detected in comparably lower percentages in the fecal metagenomes than the dolphin URT but the percentages were slightly higher in the cetartiodactyls than the humans. The bacterial genus *Bacteroides*, the most abundant in all three datasets, accounts for 75% of the human feces with 86% average database similarity, 22% of the cetartiodactyl feces with 70-80% average database similarity, and 5.5% of the dolphin URT with 70% average database similarity. The cetartiodactyl fecal metagenomes used in this study were originally sequenced by Muegge et al. (2011) in a study on the influence of diet on the gut microbiome of various mammals, including humans. Similar to our findings, Muegge et al. (2011) demonstrated an abundance of the genus *Bacteroides* in the feces of both humans and other mammalian species. *Bacteroides* spp. are well-recognized commensals of the human gut (Wexler 2007) and the high percentage of matches for this genus in the human fecal metagenomes is not surprising; however, the weak homology observed in the dolphin URT and cetartiodactyl feces may be attributable to novel organisms with phylogenetic similarities to *Bacteroides* spp. All of the bacterial potential odontocete respiratory pathogens (Table 2.3) detected in the dolphin URT were detected in both the cetartiodactyl and human feces, although the overall percentages in the dolphin URT were higher than those of either fecal metagenome.

The fungal phyla of closest homology in the dolphin URT were in similar proportions in both the cetartiodactyl and human fecal datasets while the class
Saccharomycetes accounts for between 20-30% of each respective microbiome. Interestingly, the common fungal human respiratory pathogens, including *Aspergillus* spp., *Cryptococcus* spp., *Paracoccidioides brasiliensis*, and *Pneumocystis carinii* (Table 2.4) account for about 16% of each of the fecal metagenomes (with 60-80% average homology) and 9% of the dolphin URT (with 65-70% average homology), while potential fungal odontocete respiratory pathogens make up 18% and 16% of the human (with 60-75% average homology) and cetartiodactyla (with 60-90% average homology) fungal sequences, respectively, and about 9% of the dolphin URT fungal sequences (with 60-70% average homology) (Table 2.3).

The viral family of highest abundance in both fecal metagenomes was Siphoviridae (more than 50% of viral sequences in each metagenome) which contains bacteriophages that infect commensals of the human gut. The dominance of the family Siphoviridae has been demonstrated previously in human feces in unrelated studies by Breitbart et al. (2003) and Reyes et al. (2012). Comparatively, Siphoviridae makes up only 2.9% of the dolphin URT. Also, Microviridae, the family of highest abundance in the dolphin URT, was detected in both fecal datasets (with a higher percentage in the human feces). Microviridae has also been previously associated with the human gut in an unrelated study (Reyes et al., 2012). None of the common human respiratory viruses or potential odontocete respiratory viruses from Tables 2.3 and 2.4 were detected in the fecal metagenomes.

Our results show that the dolphin URT metagenomes were more similar to the cetartiodactyl fecal metagenomes than the human fecal metagenomes. This finding gives insight into the influence of evolutionary relationships on microbiome composition. The
Orders Cetacea and Artiodactyla are the only two Orders in the Cetartiodactyla clade (Rubes et al., 2012) and the fecal metagenomes came from a variety of artiodactyls, including, okapis, sheep, cows, pigs, and giraffes. By determining that the core microbiomes of the evolutionarily similar dolphins and artiodactyls are more comparable than the core microbiomes of dolphins and humans, we demonstrate that evolution may play a role in determining the members of the indigenous microflora.

2.5 CONCLUSION

Dolphins have been proposed as sentinels for the health of marine ecosystems because of their long life spans, coastal residence, elevated trophic level, and unique blubber stores which bioaccumulate toxic contaminants (Reddy et al., 2001). These odontocete-specific characteristics may be useful for assessing the impact of pollutant run off from the coast and the effect of increasing coastal populations through the bioaccumulation of contaminants in the dolphin’s blubber (Reddy et al., 2001). Also, dolphins may be used as sentinels for determining the risk of infection to humans due to fecal contamination in coastal waterways. However, in spite of the fact that infectious respiratory disease is the leading cause of human death (World Health Organization 2012) and respiratory illnesses are believed to be the primary cause of mortality in captive populations of dolphins (Johnson et al., 2009), very little attention has been given to the potential role of dolphins as sentinels for respiratory pathogens in the marine environment.

The purpose of our study was to broaden our understanding of the microbial communities associated with the healthy dolphin URT, as well as determine the
similarities between the dolphin and human respiratory microbiomes. Correlating the dolphin and human respiratory microbiomes will provide the foundational data necessary to establish dolphins as the link for relating marine ecosystem health with the health of human coastal populations. Despite the contrast between the dolphin’s aquatic existence and the human’s terrestrial lifestyle, these two organisms have remarkably similar respiratory anatomies; in fact, a number of studies on the etiologic agents of dolphin respiratory diseases have shown that known human respiratory pathogens may also cause respiratory illness in dolphins (Chapter 1). Most of what is known about respiratory disease in dolphins is based on culture-dependent studies (as outlined in Chapter 1). For example, the most frequently isolated dolphin respiratory agents, *Pseudomonas aeruginosa*, *Erysipelothrix rhusiopathiae*, and *Aspergillus* spp., are well-recognized human pathogens (Bodey et al., 1983; Wang et al., 2010; Arabatzis and Velegraki 2013). Therefore, we ultimately need to determine whether there are novel odontocete-specific respiratory pathogens that are unique to these animals or if the odontocete and human respiratory microbiomes truly are so closely related that they share multiple pathogens. In order to begin answering these questions, we utilized a metagenomic approach to obtain foundational data for microbiomes associated with the URT of seven wild dolphins captured in a NOAA study focused on health assessments in wild dolphins. We found that the seven dolphin URT metagenomes were comparable across the higher-level taxonomic lineages, including sharing the same four most abundant phyla (Bacteroidetes, Actinobacteria, Firmicutes, and Proteobacteria) and the same two most abundant classes (Gammaproteobacteria and Flavobacteriia). We further used metagenomic comparisons to determine the similarity between the dolphin URT microbiome and the human nares.
and lungs microbiomes. Our study revealed broad taxonomic similarities at the phylum- and class-level between the dolphin URT and the human lung and nares microbiomes, including similar phyla (Bacteroidetes, Actinobacteria, Firmicutes, Proteobacteria, and Fusobacteria) and classes (Gammaproteobacteria, Bacteroidia, and Bacilli) of top abundance.

The results of our study give weight to the argument of dolphins as sentinels for respiratory pathogens in the marine environment. The broad taxonomic similarities detected in the core microbial communities the dolphin URT and human nares and lungs offer insight into the comparable nature of the respiratory microbiomes of these two organisms. This means that, with more studies, we will be able to clearly define the sentinel role of dolphins. For example, studies with more samples, studies with samples from a wider variety of locations and different species of dolphins, and studies analyzing the respiratory microbiomes of captive or managed populations of dolphins with diagnosed respiratory diseases will help us better understand the influence of disease on the core respiratory microbiome of these animals. The data presented in this study serves as the foundation for more in-depth research that will lead to definitive conclusions as to the role that dolphins play in assessing the health of marine ecosystems and human coastal population.
Table 2.1 Dolphin identifications and metadata.

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<th>D2</th>
<th>D3</th>
<th>D4</th>
<th>D5</th>
<th>D6</th>
<th>D7</th>
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<td>4466345</td>
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<td>4466347</td>
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<td>M</td>
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<td>M</td>
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<td>H (377)</td>
<td>L (40)</td>
<td>L (70)</td>
<td>H (330)</td>
<td>H (674)</td>
<td>L (207)</td>
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Table 2.3 Sum of abundance values of each metagenomic group showing potential odontocete respiratory pathogens.

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<th>ΣHN</th>
<th>ΣCF</th>
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Table 2.4 Sum of abundance values from each metagenomic group showing common human bacterial, fungal, and viral respiratory pathogens. List compiled from various sources including: Dasaraju and Liu 1996; the 2014 Centers for Disease Control and Prevention fact sheets (http://www.cdc.gov); LaRocque and Ryan 2013. D = dolphin URT Σ(D1-D7); HN = human anterior nares Σ(HN1-HN10); CF = cystic fibrosis affected human lungs Σ(CF1, CF2, CF3); H = healthy human lungs Σ(H1, H2); M = healthy mammalian (cetartiodactyl) feces Σ(O1, O2, BH1, BH2, G1, VW1, C1); HF = healthy human feces Σ(HF1-HF5); SW = Sapelo Island seawater Σ(SW1-SW8).

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Figure 2.1 Principal Component Analysis (PCA) of dolphin URT, CF and healthy human lung, human anterior nares, human fecal, cetartiodactyl fecal, and Sapelo Island seawater metagenomes showing organism classifications.
Figure 2.2 Domain distributions based on the percentage of total sequences within each summed group of metagenomes.
Figure 2.3 Distribution of the 25 most abundant bacterial phyla (a) and classes (b) in the dolphin URT (D1-D7) metagenomes
Figure 2.4 Distribution of the 25 most abundant bacterial genera (a) and species (b) in the dolphin URT (D1-D7) metagenomes.
Figure 2.5 Distribution of viral families detected in the dolphin URT (D1 – D7) and seawater (SW1-SW8) metagenomes.
Figure 2.6 Distribution of the 25 most abundant viral genera of the dolphin URT (D1-D7) metagenomes.
Figure 2.7 Distributions of the 25 most abundant bacterial phyla (a) and classes (b) of the dolphin URT (D1-D7) and Sapelo Island seawater (SW1-SW8) metagenomes.
Figure 2.8 Distribution of the 25 most abundant bacterial genera (a) and species (b) of the dolphin (D1-D7) and seawater (SW1-SW8) metagenomes.
Figure 2.9 Distribution of 25 most abundant phyla in the dolphin (D1-D7), human anterior nares (HN1-HN10), CF human lung (CF1-CF3), healthy human lung (H1, H2), cetartiodactyl fecal (O1, O2, VW1, BH1, BH2, C1, G1), human fecal (HF1-HF5), and seawater (SW1-SW8) metagenomes.
Figure 2.10 Distribution of the 25 most abundant classes in the pooled dolphin URT (D), human anterior nares (HN), CF human lung (CF), healthy human lung (H), cetartiodactyl feces (M), human feces (HF), and seawater (SW) metagenomes.
Figure 2.11 Distribution of the 25 most abundant bacterial genera (a) and species (b) in the dolphin (D1-D7), human anterior nares (HN1-HN10), CF human lung (CF1-CF3), and healthy human lung (H1, H2) metagenomes.
Figure 2.12 Distribution of the 25 most abundant fungal genera of the dolphin (D1-D7), human anterior nares (HN1-HN10), CF human lung (CF1-CF3), and healthy human lung (H1, H2) metagenomes.
Figure 2.13 Distributions of the 25 most abundant viral genera of the dolphin (D1-D7), human anterior nares (HN1-HN10), CF human lung (CF1-CF3), and healthy human lung (H1, H2) metagenomes.
Figure 2.14 Distribution of the 25 most abundant bacterial classes (a) and genera (b) of the dolphin URT (D1-D7), cetartiodactyl fecal (O1,O2, VW1, BH1, BH2, C1, G1), and human fecal (HF1-HF5) metagenomes.
Figure 2.15 Distribution of the 25 most abundant fungal genera of the dolphin URT (D1-D7), cetartiodactyl fecal (O1, O2, VW1, BH1, BH2, C1, G1), and human fecal (HF1-HF5) metagenomes.
Figure 2.16 Distribution of the 25 most abundant viral families of the dolphin URT (D1-D7), cetartiodactyl fecal (O1, O2, VW1, BH1, BH2, C1, G1), and human fecal (HF1-HF5) metagenomes.
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inhabiting estuarine waters of Charleston, SC and Indian River Lagoon, 

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