MICROBIAL METHODS FOR ASSESSING HUMAN SEWAGE SOURCES AND FATE IN COASTAL WATERS AND HABITATS OF THE SOUTHEAST UNITED STATES

by

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(Under the Direction of Erin Lipp)

ABSTRACT

Little is known about how water quality in offshore recreational environments may be impacted by microbes associated with human waste. Point and non-point sources contribute to loading of human enteric viruses to offshore marine waters. In the Florida Keys, the ability of contaminated ground water to reach the offshore reef tract was assessed by sampling surface water, groundwater, and coral mucus along an onshore to offshore transect for fecal indicator bacteria and human enteric viruses. Coral mucus harbored both fecal coliform bacteria and enterococci at levels 10-fold greater than in the corresponding water samples. Results showed that human enteric viruses reached the outer reef tract via contaminated groundwater arising from extensive use of septic systems in the Florida Keys. In densely populated southeast Florida, ocean outfalls and inlets are additional conduits of sewage contamination to the offshore environment. Water, sponge and coral samples were assayed at stations near sewage outfalls, the Port Everglades Inlet, and beaches for presence of human enteric viruses. Noroviruses were detected at high rates in sponges throughout the area but detection in coral and water samples were more prevalent near the Port Everglades Inlet. The high rate of norovirus detection indicates a potential human health threat. Given the common detection of enteric virus nucleic

acid in coastal waters, marine mammals (dolphins, *Tursiops truncatus*) were screened for carriage of similar viruses; dolphin feces were tested for a suite of human-specific and zoonotic pathogens to assess their role as a sentinel species of human health in contaminated waters. Results show that these mammals are exposed to human enteric viruses but the low detection frequency does not indicate primer cross reactivity. The zoonotic bacterium, *Campylobacter*, was common among surveyed dolphins and indicates a potential threat for both humans and marine mammals. Human enteric pathogens may be ubiquitous in the marine environment, concentrating in sessile organisms (i.e., sponges and corals). Risk assessment studies are needed to determine the weight of these findings for human and ecosystem health. Water column samples alone may underestimate exposure risk.

INDEX WORDS: Enteric Viruses, Fecal Indicators, Marine Water Quality

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DEDICATION

This work is dedicated to my family for surviving six years of stress and life changes. Most importantly, I want to dedicate this to my grandfather who was so proud of my progress and achievements.

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TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER 1

INTRODUCTION

PURPOSE OF STUDY

Coastal development poses a serious threat to coastal water quality throughout the world, including the subtropical marine waters of the southeastern United States. As more development occurs, more sewage waste and storm runoff enter the ocean. The natural buffer between the ocean and human population has been diminishing. Toxic chemicals and pathogens impact the coastal ecosystem by contaminating ocean food sources, recreational waters, destroying coral reef habitats, and promoting new marine mammal diseases. This is a global problem, where populations have become increasingly dense near coastal regions.

Many different monitoring approaches have been proposed, ranging from documenting wastewater pollution to determining risks to humans and the environment. Strategies range from studying habitats as sentinel areas to marine mammals and sponges as sentinel organisms of human sewage impact, or adding a suite of indicator organisms to more correctly identify pollution and potential health risk to humans (Bossart, 2006; Griffin et al., 2001a; Stewart et al., 2008).

Risks from wastewater contamination in marine waters have been known to impact human health via exposure to pathogenic microbes (Cabelli et al., 1983; Colford Jr et al., 2007; Donovan et al., 2008; Fleisher et al., 1998; Haas et al., 1999; Wade et al., 2003); however, increasingly threats from human sewage are affecting more than humans in coastal and marine water. Approximately 50% of the world's populations live within a few kilometers within marine

waters (Shuval, 2003). Urbanization in coastal regions is increasing; two thirds of all cities worldwide (with populations greater than 2.5 million) are on the coast (GESAMP 2001).

In the Caribbean approximately 97% of diseased coral are found in areas impacted, either highly or moderately, by human activity (Burke and Maidens, 2004; Green and Bruckner, 2000a). Sewage-derived strains of at least one enteric bacterium (*Serratia marcescens*) can cause disease and mortality in the threatened elkhorn coral (*Acropora palmata*) (Sutherland et al. 2010). Additionally, marine mammals can incur a variety of diseases and ailments from swimming in polluted marine waters. These include, but are not limited to, toxoplasmosis in sea otters, urogenital cancer (caused by a novel herpes virus) in sea lions, and papillomas in bottlenose dolphins (herpes virus) (Bossart 2006); there is growing evidence for carriage of zoonotic agents in marine mammals that are often associated with human disease.

Human sewage can introduce a variety of contaminants in the form of pathogens, chemicals, and nutrients. Research is needed to fully understand the anthropogenic impact from sewage contamination and run-off resulting from increasing coastal development. The results of this research may provide baseline knowledge to link human activity and coral disease, address management needs for reef environments, and provide support for changes to the current indicator standards utilized to determine recreational water quality in marine environments.

The four goals of this dissertation are 1) to evaluate the use of human specific microbial markers as sewage indicators in coral reef environments, 2) to assess the major sources of wastewater pollutants, 3) to determine use of coral (mucus) and sponges as indicators (or sentinels) of sewage impact in offshore environments, and 4) to evaluate the carriage of human enteric pathogens in marine mammals (Atlantic bottlenose dolphins).

A comprehensive review of the literature concerning marine pollution, human and ecosystem impacts from pathogens in human sewage and the role for sentinels in better measuring risk is presented in Chapter 2. In Chapter 3, evidence is presented on the role of contaminated groundwater (from septic systems) in transporting and delivering human enteric bacteria and viruses to offshore waters of the Florida Keys; additionally, data suggests that coral mucus layers accumulate these microbial contaminants in an otherwise dilute environment, acting as a "sentinel" habitat. In Chapter 4, the role of ocean outfalls and inlets are examined for their contribution to the prevalence and distribution of human enteric viruses in reefs of southeast Florida and further support the notion that sponges may act as effective sentinel habitats. In Chapter 5, the focus shifts to the role of marine mammals as reservoirs or sentinels of sewage contamination in the southeastern U.S. Dolphin fecal samples were obtained from an existing study that focused on dolphin health near the waters of Charleston, S.C and the Indian River Lagoon of Florida. This chapter examines the possibilities of marine mammals as carriers of sewage indicators and the potential cross-reactivity of dolphin enteric viruses with primers of known human enteric viruses. The last chapter, Chapter 6, provides concluding remarks on these studies and their significance to the research as a whole.

CHAPTER 2

LITERATURE REVIEW

INTRODUCTION

Coastal development and the increasing population pressures introduce sewage waste and storm water runoff to nearby marine waters. This threat to coastal waters occurs world-wide, including the subtropical marine water of the southeastern United States. Coastal urbanization diminishes the natural buffer between the ocean and human population by creating impermeable surfaces that act as a conduit for polluted waters to enter the ocean. Toxic chemicals and pathogens contaminate not only ocean food sources but recreational waters, contributing to the destruction of coral reef habitats and intensifying marine mammal diseases. Contamination impacts local and global populations where population densities have increased near coastal regions.

ECONOMIC IMPACTS OF DETERIORATION IN COASTAL WATER QUALITY

Deteriorating water quality in coastal waters impacts the economy of a region by affecting fisheries, economic loss from tourism dollars when beaches are closed, and the direct cost of medical treatment when people become ill due to exposure to contaminated seafood or water. Coastal populations are most likely to be impacted by waste contaminated water, which causes a range of illness (dermal, gastrointestinal to respiratory infections) and economic impacts. Shuval (2003) calculated the clinical cases per year that are associated with gastroenteritis globally to be 120,312,000. These cases combined with respiratory diseases (acute febrile respiratory infections estimated to be 48,125,000 cases per year and lower

respiratory tract infections estimated to be 1,636,250 cases per year) create great economic strains to the amount of $700 million/year. This figure only reflects the impact of bathing and swimming in contaminated recreational marine waters, and does not include fisheries loss or habitat destruction.

Contaminated seafood can cause severe illness and even death. Filter feeders, such as shellfish, accumulate both naturally occurring (i.e., *Vibrio*) and enteric (sewage-associated) pathogens, and cause serious illness upon consumption. One of the more notable pathogens is the hepatitis A virus (HAV) which causes acute liver disease. The death rate of a hepatitis A and E infection is 1:100, and Shuval (2003) estimates these viruses cause about 4×10^6 cases per year due to contaminated shellfish. A study by Rose and Sobsey (1993) of shellfish taken from marine waters meeting the United States' bacteriological standards for shellfish growing and harvesting, showed a mean virus concentration of 10 PFUs (plaque-forming units) per 100 grams of shellfish meat. Loss of money from contaminated seafood alone has been estimated at $11.6 billion/yr worldwide (Shuval, 2003).

COASTAL ECOSYSTEM SERVICES & IMPACTS FROM ANTHROPOGENIC POLLUTION

Healthy marine ecosystems provide many services, from food to medicine, and support marine life in various ways, from viable habitats to species survival (Barbier, 2000; Barbier et al., 2002; Hoeller et al., 2001; Nagai et al., 2003). Both sessile organisms (hard and soft corals, sponges and coralline algae) and larger vertebrates (including marine mammals) can suffer from poor water quality. Harmful algal blooms, toxins, and pathogens all contribute to the degradation of coastal environments.

Coral reefs are in decline worldwide due to many factors including, climate change, over fishing, disease and pollution (Pandolfi et al., 2005). In the Caribbean approximately 97% of diseased coral are found in areas impacted, either highly or moderately, by human activity (Green & Bruckner, 2000). In particular, human sewage can introduce a variety of contaminants in the form of pathogens, chemicals, and nutrients. Research is needed to fully understand the anthropogenic impact from sewage contamination and run off resulting from increasing coastal development. This would include baseline studies linking human activity and ecosystem impairment (including coral disease), to address management needs for coastal environments (especially coral reefs), and to provide support for changes to the current indicator standards utilized to determine recreational water quality in marine environments.

Sewage Pollution in Tropical Marine Water and Coral Reefs.

Sewage and associated nutrients can threaten the oligotrophic conditions necessary for coral reef development. Yet the range of impacts on reefs from sewage, including loading of heterotrophic bacteria and potential pathogens remains unclear. Kleypas and Eakin (2007) surveyed participants of the 10th International Coral Reef Symposium in Okinawa, Japan to obtain an idea of perceived threats on coral reefs from experts in the scientific community. They found that overfishing, coastal development, unnatural sedimentation, nutrient enrichment, and habitat destruction were among the top five threats. All of the top five threats are anthropogenic. Lirman and Fong (2007) tested the hypothesis that proximity to the onshore land-based source of stressors influenced the conditions of corals. However, the results of the study were not conclusive. While land-based pressure does exert stress upon corals, the relationship involves many factors before coral disease is the outcome. There are a complex multitude of factors that

influence the condition of coral reef habitats, such as temperature, stress, water quality, and physical damage.

South Florida and the Florida Keys offer an ideal area to study this impact. The bedrock of the Florida Keys is comprised of a porous limestone (karst), which quickly carries groundwater (including untreated human waste from septic systems and injection wells) reefward (Lipp et al., 2002; Paul et al., 2000; Paul et al., 1995a; Paul et al., 1995b; Paul et al., 1997). The Florida Keys depend upon their coral reefs for tourism and fishing. Porter et al. (2001) and Dustan et al. (1987) suggest that reef decline in the Florida Keys is due to multiple stressors including local, regional, and global influences. Throughout the Florida Keys and South Florida, in general, local land-based sources of pollution, primarily associated with human waste, continue to be a critical source of stress. In the Keys, the widespread use of in-ground wastewater disposal (~30,000 septic tanks and cess pits) (Shinn et al. 1994) has been shown to contribute to nearshore contamination, with both nutrients and enteric microbes (Paul et al. 1995a; Paul et al. 1995b; Griffin et al. 1999; Lipp et al. 2002; LaPointe et al. 1990). In the densely populated area of southeast Florida extending from Dade County in the south to Martin County in the north, there are multiple sources of potentially human-derived pollution including several ocean outfalls which discharge secondarily treated sewage, septic systems and a series of inlets carrying large volumes of water offshore with each tidal cycle. Broward County alone claimed a population of over 1.7 million people in 2009 (U.S. Census 2009, www.census.gov) discharging an average of 2.78×10^8 L d^{-1} from the Broward County outfall, alone, located 2.12 km offshore (in all there are six outfalls in southeast Florida; two in Broward County). Additionally, submarine groundwater discharge is estimated to contribute 4.54×10^9 L yr^{-1} of effluent, combining septic tank and injection well estimates. The large numbers of navigational

inlets throughout the coastal areas of southeast Florida and the Intracoastal Waterway also serve as a conduit of contamination (FACE 2007). Despite these estimates of effluent reaching the ocean via many pathways, little is known of the impact to offshore waters and reefs.

It is known, however, that sewage-derived or other introduced inorganic nutrients lead to a shift in the marine benthic community away from corals and towards algae (LaPointe 1992). Sewage constituents may cause or work in conjunction with existing opportunistic pathogens to exacerbate or introduce coral disease (Kline et al., 2006). Bruno et al. (2003) demonstrated that increased nutrient input in the presence of aspergillosis (disease agent is the fungus *A. sydowii*) in the sea fan and yellow band disease in *Montastraea annularis* and *M. franskii* (disease agent is *Vibrio* sp.) caused significant enhancement of disease, with higher mortality and more rapid disease progression compared to non-nutrient amended controls (Bruno et al., 2003). In 2002, Patterson et al. documented that white pox disease of elkhorn corals in the Florida Keys is caused by an enteric bacterium with potential links to human sewage (Sutherland et al., 2010). Looney et al. (2010) showed that the white pox pathogen (*Serratia marcescens*) persisted longer in seawater with elevated nutrients (especially phosphorous and dissolved organic carbon). However, Lesser et al. (2007) suggests that coral mortality is likely due to a wide mixture of variables. He argues that physiological stress put upon corals, such as increases in seawater temperature, may increase the corals' susceptibility to opportunistic pathogens and cites work by Martinez and Baquero (2002) suggesting that many bacteria express the virulence genes at elevated temperatures. This combination of factors promotes progression of disease.

Sewage contamination can both directly and indirectly have adverse affects on both ecosystem health and human health. The importance of sewage is suspected, but it is difficult to detect dilute concentrations of it, making the full impact unknown. In order to understand the

impact sewage contamination, good indicators of sewage presence are needed to identify acute or chronic impact from human enteric pathogens (Griffin et al. 2001).

Methods for Assessing Sewage Contamination in Tropical Marine Waters and Coral Reefs

Ideal characteristics for fecal indicators include the following: 1) they occur when pathogens do, 2) are found in higher numbers than pathogens, 3) can be easily isolated and counted, 4) are only found in sewage, 5) cannot re-grow in the environment, and 6) have densities that relate to the degree of contamination, and therefore the health hazard for this type of pollution. To date, no single indicator meets these criteria.

Briefly, the search for water quality indicators began with total coliforms and fecal coliforms. With case studies and research, the bacterial indicator has been updated from total coliforms to fecal coliforms to *Clostridium perfringens* and enterococci, which the US EPA currently recommends for monitoring marine microbial water quality. However, these indicators remain far from ideal. They need an incubation time of 12-18 hrs before obtaining results on media. They also do not detect the viable but-nonculturable portion of the pathogen community. Therefore, a suite of new indicators of sewage contamination have been proposed from coliphages to different bacteria to enteric viruses, chemicals, and proposals for a combination of indicators (Savichtcheva and Okabe, 2006).

Fecal Indicator Bacteria and the Indicator System for Water Quality

Fecal indicator bacteria (FIB), fecal coliform bacteria, *enterococci*, and *Clostridium perfringens*, are widely accepted as markers of human sewage pollution. However, this indicator system is not ideal. FIB tend to lose culturability in marine waters (Vasconcelos and Swartz, 1976; Fujioka et al., 1981; Perez-Rosas and Hazen, 1988; Fujioka and Yoneyama, 2002). They

may also be derived from non-human sources (Griffin et al. 2003). Some species of enterococci and fecal coliform bacteria also grow in some tropical marine environments (Fujioka et al., 1988b; Hardina and Fujioka, 1991). Ideal indicators must not grow in the environments, must be indicative of a public health risk, must be specific to sewage, must be found in higher concentrations than the pathogen of concern, and occur where pathogens do (Cabelli, 1977). Recent research has shown that bacterial fecal indicators are not always present, even when viral indicators are (Griffin et al., 1999; Lipp et al., 2002).

Human Enteric Viruses

Enteric generally refers to the intestines, and enteric pathogens live and are excreted from the gut. Pathogens, such as human enterovirus (hEV), human adenovirus (hAdV), and human norovirus (hNoV), are frequently detected in contaminated marine waters. Given their human host specificity, their presence suggests not only a health risk to humans, but a clear indication of human sewage contamination. Human enteroviruses are single stranded RNA viruses, which include rhinovirus (agent of the common cold), coxsackievirus, echovirus, enterovirus, and poliovirus. The most common symptoms of enteroviruses range from acute respiratory to gastrointestinal infections with more severe species causing poliomyelitis, among other severe conditions (Zaoutis and Klein, 1998). Human adenoviruses are double stranded DNA viruses that are non-enveloped. They tend to cause respiratory disease, conjunctivitis, and gastroenteritis in humans (Rotbart, 1995; Zaoutis and Klein, 1998). Noroviruses are RNA viruses that cause around 90% of the outbreaks (non-bacterial) of gastroenteritis worldwide (Lindesmith et al., 2003) and are believed to be the most common cause of diarrhea and vomiting among adults. This virus is commonly referred to as the cruise-ship virus because of its recent notoriety due to continued outbreaks on multiple cruise ships. In addition to being waterborne

and foodborne, norovirus can be easily transmitted person to person and disease will pass quickly within tight quarters. There are five genogroups of noroviruses, genogroups 1 and 2 are the most commonly associated diarrhea outbreaks (Atmar and Estes, 2006; Gentry et al., 2009; Vinjé et al., 2004).

Problems Using Traditional Water Quality Measures

From a public health standpoint, methods for assessing sewage contamination in water are generally designed for assessing risk to humans from enteric pathogens (using an indicator or proxy) in nearshore coastal waters (i.e., beaches) and are based on only a handful of beach studies, mostly in temperate waters, that demonstrated an association between fecal indicator levels and health risk (Seyfried et al., 1985). Despite distinct differences in conditions in subtropical and tropical waters, compared to beaches in New York [one of the key health effects study sites (Cabelli et al., 1982)] and the fact that in areas such as the Florida Keys more recreational swimming takes place offshore rather than on the limited number of beaches, no methods are in place to assess sewage contamination and human risk in offshore tropical marine waters. Furthermore, the traditional indicator bacteria (e.g. *E. coli*) appear to decay quickly in highly transparent and warm marine water. Therefore, even if monitoring took place, the results may be ineffective at predicting health exposure to other pathogens, such as viruses. Hanes and Fragala (1967) experimented with the impact of seawater concentrations on indicator bacteria. Enterococci survived almost 2 days longer on average than *E. coli*, which degraded much more rapidly with increased sunlight intensity. Despite enterococci being a hardier bacterial indicator, it does grow naturally in some marine environments (Anderson et al., 2005; Fujioka et al., 1988a; Hardina and Fujioka, 1991) and may, therefore, not correlate with presence of human

viruses or other non-bacterial pathogens (Jiang et al., 2001b; Noble and Fuhrman, 2001; Schvoerer et al., 2001).

While not associated specifically with human health risk, analysis of nutrient input is also commonly used to determine pollution loads affecting marine ecosystems. Use of stable nitrogen isotopes have been used extensively to trace the origin of nitrogen to specific environments (Heikoop et al., 2000; Lapointe et al., 2004; Lapointe et al., 2005; Ward-Paige et al., 2005). Although nitrogen enrichment is a key concern in reef health contributing to increased disease severity (as described above), in the Florida Keys, some studies suggest that offshore N-enrichment may be due to regional upwelling, rather than anthropogenic sources (Leichter et al., 2003; Swart et al., 2005; Szmant, 2002). Yet others find results to suggest a clear influence of human sewage in the isotope data (Ward-Paige et al. 2005).

Clearly a definitive (unambiguous) and conservative marker of human sewage would be a valuable tool in determining contamination in reef environments. Areas showing the presence of sewage markers could then be targeted for more in-depth studies on possible nutrient enrichment, disease, and human risk from recreational exposure.

Enteric viruses, which are strictly host-specific, can be used as indicators for human sewage and may provide a conservative marker for the presence of sewage contamination (Fong and Lipp, 2005). Enteric viruses are excreted in the feces of infected individuals, which may contain 10^5 to 10^{11} viral particles per gram of stool (Farthing and Kline, 1989). Given the low infective dose of viruses (Haas et al., 1993), contaminated water pose a real health risk, especially to immune suppressed or immune compromised persons (Gerba et al., 1996). Enteric viruses tend to be more persistent in the marine environment than bacterial indicators (Wetz et al., 2004) and can remain infective for long periods of time. Previous studies have shown

survival for up to 120 days in freshwater and sewage, and 130 days in seawater (Jiang et al., 2001b). Unlike bacteria, viruses need a host for replication; they cannot grow in the environment. Finally, previous work has shown that viruses may accumulate in the secreted surface mucopolysaccharide layer (SML) of coral, which provides a concentrated source of material relative to the overlying water column (Lipp et al., 2001a; Lipp and Griffin, 2004). Not only do the presence of viruses within the coral mucus indicate sewage impact and potential threat to coral health but also increased risk to the many snorkelers and divers that frequent the coral reefs (Schijven and de Roda Husman, 2006).

RESERVOIRS AND SENTINEL HABITATS OF OCEAN AND HUMAN HEALTH

Marine Ecosystems as Sentinel Habitats

While assessment of coastal waters using fecal indictors, whether microbes or nutrients, is frequently used, the presence or absence of these sewage proxies do not indicate the actual risk to ecosystems, or for that matter to human populations. Sentinel organisms have been proposed to better indicate health risk. These are organisms that provide an early warning for the contaminant of interest and are available for use as biomonitors. The idea of a sentinel suggests that key habitats or organisms are susceptible to low levels of contaminants, where their presence may be more easily measured, they may concentrate contaminants or an assessment of the health of the sentinel can provide information about the actual impact of the contaminant. Tropical, subtropical marine waters, including offshore waters and coral reefs, are prime candidates for assessing the utility of sentinels given the sensitivity of these ecosystems to environmental change and the poor utility of traditional indicators.

Role of Coral Mucus as a Sentinel Microbial Habitat

Mucus acts in defense, feeding, and locomotion, but also serves to protect against desiccation, aids in shedding sediments, provides sunscreen against UV-radiation, and serves in reproduction and settlement behavior (Brown and Bythell, 2005). Mucus also plays an important role in coral disease (Porter et al., 2001). It functions as a protective barrier (Hayes and Goreau, 1998; Peters, 1997; Santavy and Peters, 1997; Sutherland et al., 2004), a growth medium for bacteria (Banin et al., 2001; Lipp et al., 2002; Toren et al., 1998), and may accumulate microbes from the surrounding water column (Lipp and Griffin, 2004).

Coral mucus has been described in various terms, such as the coral surface microlayer (CSM), the surface mucopolysaccharide layer (SML), and the mucopolysaccharide layer (MPSL) by researchers working on microbial communities associated with corals (Brown and Bythell, 2005). For the purpose of this dissertation, coral mucus is defined as the polysaccharide-protein complex on the surface of the corals. This includes the associated aggregates of bacteria, zooxanthellae, filamentous algae, plankton, and sediments and the water in the immediate area of the surface layer. This natural mucus-associated microbiota may serve in a symbiotic relationship with the coral (Reshef et al., 2006b). Corals obtain some of their nutritional needs from particulate organic matter that is captured in the mucus and catabolized by resident bacteria (Goreau et al., 1971; Reshef et al., 2006). The bacteria are able to break down particulates, such as chitin and algae, which corals are not able to process. The coral shares photosynthetic products produced by the symbiotic algae (zooxanthellae) as well. Excess photosynthate contributes to mucus production. Coral mucus and its resident bacterial community may also be involved in host defense from infection. Similarly, studies have shown that some sponges and

soft corals have bacteria that produce antibiotics, which protect their host from planktonic bacteria (Castillo et al., 2001; Kelman et al., 2006; Thakur et al., 2005).

The Role of Marine Sponges As Microbial Reservoirs and Sentinels

Marine sponges are sessile organisms that filter feed from their surrounding environment (Reiswig, H.M. 1971). They filter and circulate water through tiny pores on their surface. Some sponges can filter large amounts of water, from 0.002 to 0.84 cm^3/s for each cm^3 of sponge tissue (Osinga et al., 2003; Reiswig and Rützler, 1990). This interaction with the surrounding water column may make them excellent indicator species of human sewage impact. The unique filtration system may capture sewage constituents from the surrounding current. Sponges frequently inhabit reef environments and some species appear to survive poor water quality better than many stony corals. However, it is known that these organisms have innate immune defense from viruses, protozoa, and bacteria. They produce secondary metabolites to defend themselves against harmful microorganisms (Becerro et al., 1994; Blunt et al., 2007; Wiens et al., 2005). It is also known that sponges carry their own host-specific bacteria (Taylor et al., 2007) that might outcompete bacterial sewage constituents for food and space resources; they may also ingest captured microbes. Further research on human sewage indicator interaction with natural sponge defenses is needed to determine whether sponges are a good indicator organism and whether they may concentrate viruses from the water column.

The Role of Marine Mammals As Pathogen Reservoirs or Sentinel Species

Marine mammals can develop diseases from contact with contaminants originating from anthropogenic sources. Due to the carriage and impact of possible zoonotic pathogens and the effects of anthropogenic toxins, many researchers have suggested that these mammals could serve as sentinel species for the health of the ocean (Bossart 2006). Atlantic bottlenose dolphins,

West Indian manatees, California sea lions, and sea otters are known examples where these animals are directly impacted by marine contamination from anthropogenic sources (Bossart, 2006) resulting in acute and chronic diseases and mortality. A clear zoonotic disease that occurs from water contamination is toxoplasmosis, which can develop in sea otters along coastal southern California (Miller et al., 2002). Toxoplasmosis is caused by *Toxoplasma gondii*, a parasitic protozoa whose definitive host is the cat, but it can also infect a range of secondary hosts including humans and other mammals (Frenkel and Dubey, 1972). The parasite forms long-lived cysts in muscle tissue, the nervous system, and other vital tissues and can be fatal to fetuses of pregnant women. Toxoplasmosis is unique in that it is one of the few marine zoonotic diseases with a distinct link to both land-based sources of pollution and human pathogens.

Brucella sp. is a pathogen of increasing concern in marine mammals. *Brucella* is a Gram-negative bacterium that can cause respiratory infections in humans. It also infects terrestrial wildlife, as well as marine mammals such as seals, porpoises, and common dolphins (where it was first discovered in marine mammals). Recently, the discovery of *Brucella* has expanded to other populations of marine mammals (Denner et al., 2003; Ewalt et al., 1994; Foster et al., 1996; Gonzalez et al., 2002; Groussaud et al., 2007; Jahans et al., 1997; McDonald et al., 2006; Miller et al., 1999; Whatmore et al., 2008).

Viruses are also being introduced to marine mammals. Vesiviruses, members of the genus Caliciviridae, were found in walrus fecal matter (Ganova-Raeva et al., 2004). Caliciviruses (which include, but are not limited to, the norovirus genus) have a wide host range and some are known to infect multiple host species (Lamarque et al., 1997). This poses a threat to humans and marine mammals alike. Sewage contamination may not only release viruses into the ocean but may also introduce novel marine viruses and diseases.

Persistent pollutants may cause neoplastic disease and contribute to the emergence or resurgence of infectious diseases (Bossart et al., 1997; Bossart et al., 2002; Bossart et al., 2005; Rehtanz et al., 2006). Urogenital cancer in sea lions is associated with chemical contaminants (such as PCBs and DDTs) as well as a novel herpes virus.

Bottlenose dolphins make a good sentinel species on the southeast coast due to their high site fidelity and general appeal as a charismatic megafauna. These mammals carry zoonotic diseases and infective bacteria, such as *Brucella*, *Leptospira*, and *Mycobacterium*, which can infect humans (Thompson et al. 1993 and Brew et al. 1999). They also develop lesions and health problems from chemical contaminants. These marine mammals, therefore, make an excellent indicator for anthropogenic pollution. More research is needed on marine viruses to compare to known human sewage viruses. This will help determine the full impact of human enteric viruses on marine populations as well as produce information about the path of these viral indicators in marine habitats. However, it is unknown if many human enteric pathogens can infect, or merely pass through, the digestive systems of these mammals.

SUMMARY

The search for an ideal human fecal indicator is a complex and ongoing process. This search involves many factors from different habitat types (strictly marine, estuary influence, temperate, subtropical, and tropical) to the most appropriate marine organisms (dolphins, reef, sponges) to the impact of the human population (direct recreational exposure to consumption of contaminated marine organisms). These problems extend well beyond environmental and public health concerns. They impact economies on the coast and inward by impacting fishing, acquisition of natural antimicrobial products (sponges), recreational options, and tourism income

of coastal cities. The importance of protecting marine recreational waters from land-based (mostly human influenced) contamination is undeniable.

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CHAPTER 3

PREVALENCE AND DISTRIBUTION OF ENTERIC MICROBIOTA IN CORAL REEFS AND UNDERLYING GROUNDWATER IN THE FLORIDA KEYS[1]

[1]J. Carrie Futch, Dale W. Griffin, Erin K. Lipp. 2010. *Environmental Microbiology*. 12: 964 – 974. Reprinted with permission of publisher

SUMMARY

To address the issue of human sewage reaching corals along the main reef of the Florida Keys, samples were collected from surface water, ground water and coral (surface mucopolysaccharide layers [SML]) along a 10 -km transect near Key Largo, FL. Samples were collected semi-annually between July 2003 and September 2005 and processed for fecal indicator bacteria (fecal coliform bacteria, enterococci and *Clostridium perfringens*) and human-specific enteric viruses (enterovirus RNA and adenovirus DNA) by (RT)-nested PCR. Fecal indicator bacteria concentrations were generally higher nearshore and in the coral SML. Enteric viruses were evenly distributed across the transect stations. Adenoviruses were detected in 37 of 75 samples collected (49.3%) whereas enteroviruses were only found in 8 of 75 samples (10.7%). Both viruses were detected twice as frequently in coral compared to surface or groundwater. Offshore, viruses were most likely to be found in groundwater, especially during the wet summer season. These data suggest a possible geological conduit from polluted onshore and nearshore waters to the outer reef environment in the Florida Keys.

INTRODUCTION

In the Caribbean approximately 97% of diseased coral are found in areas impacted, either highly or moderately, by human activity (Green and Bruckner, 2000b). Researchers have reported a decrease in coral diversity and an increase in coral disease throughout the Florida Keys National Marine Sanctuary since at least 1996 (Porter et al., 2001). The Florida Keys, which is already near the northern extent of typical coral range has a resident population of ~80,000 and is visited by another 3 million people per year.

Porter et al. (2001) and Dustan and Halas (1987) suggest that reef decline in the Florida Keys is due to multiple stressors including local, regional, and global influences. A critical local

and regional source of stress is the widespread use of in-ground wastewater disposal (~30,000 septic tanks and cess pits) (Shinn et al., 1994), which contributes to nearshore contamination by enteric microbes, nutrients and other components (Griffin et al., 1999; Lapointe et al., 1990; Lipp et al., 2002; Paul et al., 1995b). Sewage-derived or other introduced nutrients lead to a shift in the benthic community from corals towards more algae and sponges (Lapointe, 1997). Enteric pathogens found in human waste contaminate nearshore waters, beaches, and canals of the Keys (Griffin et al., 1999; Lipp and Griffin, 2004; Lipp et al., 2001b), and have led to beach closures and swimming-associated disease in recent years impacting the local economy and public health (Nobles et al., 2000). Furthermore in 2002, Patterson et al. documented that white pox disease, which affects the threatened elkhorn coral *(Acropora palmata*), in the Florida Keys is caused by an enteric bacterium with potential links to human sewage (Patterson et al., 2002b).

While nearshore impacts of human sewage are well documented (Griffin et al., 1999; Jiang et al., 2001; Lapointe et al., 1990; Lipp et al., 2002), little is known about the migration and impact of this sewage to offshore reef environments. Several researchers have shown that nutrients (especially nitrogen) and microbial indicators of fecal pollution migrate from septic tanks into the groundwater and nearshore surface waters of the Keys (Lapointe et al., 1990; Paul et al., 2000; Paul et al., 1995a; Paul et al., 1997). In a series of seeded tracer studies, migration of dyes and bacteriophage consistently showed reef-ward transport through the subsurface (Dillon et al., 1999; Paul et al., 2000; Paul et al., 1995a; Paul et al., 1997). Viral tracers traveled at rates ranging from 35 m hr^{-1} in Key Largo to 141 m hr^{-1} in the lower Keys, and migrated at least 2,500 m (Paul et al., 2000; Paul et al., 1997). Despite the indication from these studies that sewage constituents are moving rapidly reef-ward in the subsurface, tools to date have not been able to adequately document if human sewage or wastewater is reaching the outer reef tract.

Issues in detecting a sewage signal offshore include rapid die-off of traditional fecal indicator bacteria in marine water (Fujioka et al., 1988a; Garcia-Lara et al., 1991; Griffin et al., 2001b; Lipp and Griffin, 2004), adsorption of nutrients in the subsurface or uptake of nutrients by biota in nearshore surface waters (Corbett et al., 1999; Lapointe et al., 1990), or dilution effects. Furthermore, while seepage of groundwater in coral reefs where porosity in the subsurface tends to be high is well documented (Simmons Jr, 1992), there has been no systematic evaluation of contamination in groundwater underlying the outer reef tract.

Given corals' adaptation to oligotrophic environments, traditional approaches to reef water quality have relied heavily on analyses for nutrient levels; however, while increasing nutrient concentrations are an important contributor to changes in reef habitat (i.e., bottom-up controls on benthic communities leading to preferential growth of sponges and algae over corals due to high nutrient levels (Lapointe, 1997)]), it can be difficult to assign excess nutrients specifically to human sources (Leichter et al., 2003). Although stable isotope assays (nitrogen) are used as effective indirect markers for sewage, especially in nearshore environments (Ward-Paige et al., 2005), there has been some controversy regarding its use and application (Swart et al., 2005).

Microbial fecal indicators have long been used in assessing quality of nearshore coastal waters but their application to coral reef water quality, especially at distances exceeding 10 km from shore, has not been frequently reported in the literature (i.e., Bonkosky et al. 2009, Lipp et al. 2007). Fecal indicator bacteria, traditionally used by regulators for evaluating water quality, tend to rapidly lose culturabilty in marine water (Fujioka and Yoneyama, 2002; Fujioka et al., 1981) and may be derived from sources other than human (Griffin et al., 2003). Additionally, some fecal indicators, including species within enterococci and fecal coliform groups, may grow

in certain tropical environments (Davies et al., 1995). Enteric viruses, which are strictly host-specific, can be used as alternative indicators for human sewage and may provide a conservative marker for the presence of sewage contamination (Fong et al., 2005). Enteric viruses are excreted in the feces of infected individuals, which may contain 10^5 to 10^{11} viral particles per gram of stool (Farthing et al., 1989). Enteric viruses tend to be more persistent in the marine environment than bacterial indicators (Jiang et al., 2001b; Wetz et al., 2004). Also, unlike bacteria, viruses need a host for replication; they cannot re-grow in the environment. Finally, previous work has shown that viruses may accumulate in the secreted surface mucopolysaccharide layer (SML; or mucus) of coral, which provides a concentrated source of material relative to the overlying water column (Lipp and Griffin, 2004; Lipp et al., 2002).

Here, the extent of human sewage contamination was evaluated along a near-shore to offshore transect in the Upper Keys using traditional fecal indicator bacteria and human enteric virus nucleic acids as sewage markers. Distribution was compared between surface water, groundwater, and coral surface mucopolysaccharide layer (SML) to determine potential sources and fates of these sewage constituents. The overall objectives of this study were two-fold: 1) to determine if waters and corals of the main reef tract in the Florida Keys (>10 km from shore) were exposed to human sewage, and 2) to determine if groundwater contamination is a plausible source of enteric microbes to the offshore environment.

RESULTS

Physical Chemical Characteristics

A total of 25 samples each from coral SML (pooled), surface water and groundwater were collected between July 2003 and September 2005. Surface water temperatures ranged from a mean of 25.4 ˚C in the spring (April 2004 and 2005) to a mean of 29.9 ˚C in the summer (July

2003 and 2004 and September 2005). Relative to surface waters, groundwater temperatures were slightly higher in the spring (26.2 ˚C) and lower in the summer (28.6 ˚C). The pH ranged from 7.0 to 8.4 for all sample types but tended to be highest in the surface water in spring (mean of 8.1) and lowest in the summer groundwater samples (mean of 7.4). On average, salinity in the groundwater was similar to the surface water (36.9 and 36.5, respectively); however, summer samples were generally less saline (35.9 and 35.7 for groundwater and surface water, respectively) than those collected in the spring (38 and 37.3 for groundwater and surface water, respectively). Precipitation ranged from a monthly mean of 3.7 cm in the spring (5.13 cm and 2.28 cm for April 2004 and 2005, respectively) to 20.5 cm in the summer (N = 3; 5.2 cm, 22.9 cm and 33.4 cm for July 2003, July 2004 and September 2005, respectively).

Bacterial Fecal Indicators

Fecal coliform counts ranged from non-detectable to 105 CFU 100 ml^{-1} for SML (geometric mean of 0.5 CFU 100 ml^{-1}), non-detectable to 5.5 CFU 100 ml^{-1} for surface water (geometric mean of 0.3 CFU 100 ml^{-1}), and non-detectable to 3.5 CFU 100 ml^{-1} for groundwater (geometric mean of 0.1 CFU 100 ml^{-1}). Enterococci counts ranged from non-detectable to 700 CFU 100 ml^{-1} in SML (geometric mean of 0.8 CFU 100 ml^{-1}). The counts for surface water and groundwater ranged from non-detectable to 10 CFU 100 ml^{-1} (geometric mean of 0.2 CFU 100 ml^{-1}) and non-detectable to 41 CFU 100 ml^{-1} (geometric mean of 0.3 CFU 100 ml^{-1}), respectively. *C. perfringens* counts ranged from non-detectable to 10 CFU 100 ml^{-1} for SML (geometric mean of 0.1 CFU 100 ml^{-1}), non-detectable to 5 CFU 100 ml^{-1} for surface water (geometric mean of 0.04 CFU 100 ml^{-1}), and were not detected in groundwater. For all indicators, the limit of detection was 5 CFU 100 ml^{-1} coral SML and 2 CFU 100 ml^{-1} for surface and groundwater. While there was no significant difference in any of the indicator levels

between the different sample types ($P < 0.05$), all were higher in SML relative to both types of water (Fig. 1). Additionally, all indicator levels were within recreational standards set under the Florida Healthy Beaches Program (classified as "Good") (Chapter 62-302.530, Florida Administrative Code); <199 CFU fecal coliform bacteria 100 ml^{-1} and <35 CFU enterococci 100 ml^{-1}, for a single sample.

Although indicator levels were low at all stations, when all sample types were combined (groundwater, surface water and coral), they showed a declining trend moving offshore (Fig. 2); however, correlations with distance from shore were only significant for enterococci ($r = -0.93$; $p = 0.023$). Likewise, enterococci concentrations were significantly higher ($p \leq 0.05$) at KL1 than all other stations, except for KL2 (the other nearshore station). Statistically, there were no significant differences between mean levels for fecal coliform bacteria or *C. perfringens* between stations.

All indicators were more frequently detected in summer months, with all three bacteria occurring in all sample types. While enterococci were detected from all sample types during both summer and spring, fecal coliform bacteria and *C. perfringens* were detected only in surface water in the spring. There were no significant differences in mean concentrations between the seasons for any indicator.

Enteric Viruses

Adenovirus DNA was found at a significantly greater frequency from all sample types when compared to enterovirus RNA ($p < 0.05$). Adenoviruses were detected in 37 of 75 samples collected (49.3%) whereas enteroviruses were only found in 8 of 75 samples (10.7%). When compared by sample type, both viruses were detected more often in coral SML than in either water type. For both adenovirus and enterovirus, the frequency of coral-positive stations was

approximately double that of groundwater and surface water (Tbl. 1); however, the difference was only statistically significant for adenovirus ($p < 0.05$).

Unlike bacterial indicators, the enteric virus detection frequency varied little between stations across the nearshore to offshore transect. Adenovirus detection ranged between 46.7% (7/15) to 53.3% (8/15) of samples positive per station (Fig. 4). While frequency of detection was lower overall for enteroviruses, the pattern was similar with detection frequencies ranging from 6.7% (1/15) to 13.3% (2/15) for each of the five stations (Fig. 3). For adenoviruses, positive results were obtained from all sample types at all stations, except for KL2 which was always negative for groundwater (Fig. 3). Conversely, enteroviruses showed distinct partitioning between the sample types. At KL1, enteroviruses were found equally between the coral SML and surface water. At KL2 and KL3, only coral SML was positive whereas offshore, at KL4 and KL5, coral SML samples were negative but groundwater was positive at both stations (Fig. 3). For both virus types, frequency of groundwater positives was greater at these offshore stations. Enteroviruses were found only in the SML in the spring (2 of 30 samples; 6.7%); surface water and ground water were always negative in the spring samples. In the summer months, the overall detection of enteroviruses was greater (6 of 45 samples; 13.3%) and was evenly distributed between sample types (Fig. 4). Human adenoviruses were detected in ~50% of all samples in both seasons. Similar to enteroviruses, adenoviruses were dominant in coral SML in spring samples (9 of the 10 spring coral SML samples [90%] were positive). Likewise, adenovirus prevalence in the surface water was similar between the seasons, but detection in groundwater accounted for 31.5 % (7/22) of the positives in the summer and only 6.7 % (1/15) positives in the spring (Fig. 4).

DISCUSSION

Several tracer studies have revealed that viruses in septic tanks and injection wells in the Florida Keys can reach distances up to and exceeding 3,922 m within 27 hours (Paul et al., 2000; Paul et al., 1995a; Paul et al., 1997), which suggests that wastewater contamination may be impacting more than just the nearshore environment (Lipp et al. 2007). To date, little is known about how such contamination affects offshore recreational environments or the associated risks to both humans and the environment. Beach closures and loss of coral due to disease and habitat changes have major economic impacts on areas such as the Florida Keys. Tourism revenues associated with the Florida Keys reef amount to $1.6 billion annually (Bryant et al., 1998). The economic impact of reef degradation could greatly affect the surrounding communities. Effective methods to determine sewage impacts offshore are needed for improved resource management. A conservative marker of human sewage would be a valuable tool in determining contamination in reef environments; areas showing the presence of sewage markers could then be targeted for more in-depth studies on possible nutrient enrichment, disease, and human risk from recreational exposure. This study shows that both bacterial and human viral fecal indicators can be detected in surface water, groundwater and corals of the Upper Florida Keys and associated reef communities and shows that constituents of human sewage can reach the outer reef tract.

Fecal Indicator Bacteria

Fecal indicator bacteria followed a declining trend in concentration and frequency of detection from nearshore to offshore; however, all were generally found at low levels. All three indicators were found at the nearshore stations KL1 and KL2 but in offshore stations (KL4 and KL5) only enterococci were detected (in coral SML and groundwater). Fecal indicator bacteria

can be derived from a variety of sources in addition to humans (Griffin et al., 2001b) and this trend may reflect the multiple land based sources of pollution to this area. Entercocci, which was recommended in 1986 as a superior indicator of human health risk in marine waters compared to fecal coliform bacteria (USEPA 1986; USEPA 1997), may be more predictive of human wastewater sources. In general, bacterial indicators may decline offshore due to a high degradation rate in tropical marine waters, high temperatures and damage from UV irradiation (Bordalo et al., 2002; Fujioka and Yoneyama, 2002).

Enteric Viruses

Unlike the fecal indicator bacteria, human enteric viruses were detected at similar frequencies at all stations over the entire transect. Enteric viruses commonly do not correlate well with fecal indicator bacteria (Griffin et al., 2001b; Lipp et al., 2001b) due to different susceptibilities to biotic and abiotic degradation factors (Gerba et al., 1979), differences in host ranges and excretion patterns. Enteroviruses were found at KL1 13.3% of the time and at KL5 6.7% of the time; however, they tended to accumulate in the coral SML in the near to mid-shore portion of the stations (KL1 – KL3) and were only detected in the groundwater offshore (KL4 and KL5). Adenoviruses ranged between 46.7% and 53.3% positive from nearshore to offshore but did not show a similar trend in partitioning as the enteroviruses except that detection in groundwater also increased at the offshore stations.

The higher overall detection rate of adenoviruses may be related to increased loading (Irving and Smith, 1981), extended period of shedding and prolonged persistence relative to the RNA-based enteroviruses (Enriquez et al., 1995). Adenoviruses were also detected at higher rates in all sources relative to enteroviruses; similar detection patterns have also been noted throughout the Florida Keys (Lipp et al., 2007). These DNA-based viruses (or their genomes)

may persist longer in marine environments than the RNA-based enteroviruses. Enriquez et al. (1995) demonstrated that the time required for loss of 99% of adenovirus was up to 85 days in seawater at 15^{o} C, which is 4.7 times longer than the 99% loss observed for poliovirus (enterovirus) within 18 days. Of particular interest is the ability of adenoviruses to withstand degradation (including heat and UV irradiation). Additionally, studies showing enterovirus infectivity and PCR signal loss (using poliovirus as the model) suggest that RNA-based enteroviruses are relatively short-lived in natural marine conditions (Wetz et al., 2004). Furthermore, loss of poliovirus genetic material (i.e., PCR signal) and infectivity occur at similar rates (Enriquez et al., 1993; Wetz et al., 2004). Based on these studies and other field results, Lipp et al. (2007) suggested that enteroviruses might better indicate recent contamination, which is also consistent with results from this study.

Seasonal Patterns

Fecal indicator bacteria and enterovirus detection rates were higher in summer sampling months. The mean enterovirus detection rate in summer was double that observed in spring, consistent with clinical infection rates and patterns of excretion, which both peak in summer months (Khetsuriani et al., 2006; Sedmak et al., 2003). While adenovirus detection was similar for both seasons, also consistent with clinical infection and excretion patterns (Pina et al., 1998), both virus types were detected more frequently groundwater in the summer months. This trend may be explained by seasonal weather patterns; summer is the wet season in south Florida and total monthly rainfall for the sampled summer months was over 5-fold higher than the sampled spring months. Groundwater also tended to be fresher in summer months compared to spring samples. Increased contamination of coastal waters during storm events is common (Lipp et al., 2001b). In particular, Paul et al. (Paul et al., 1995a; Paul et al., 1997) noted increased migration

rates of viral tracers in groundwater of the Florida Keys during wet weather. Population effects may also explain some of the observed trends; all summer samples were collected during the "Special Two Day Sport Season" for lobster (the 'lobster mini-season') (July 2003 and 2004) or over Labor Day (September 2005; during regular lobster season) which attracts a large number of tourists to the Keys (Sharp et al., 2005). The combined factors of increased population, increased excretion (enteroviruses) and wet weather-facilitated transport may explain the seasonal differences observed here; however, data collected over additional seasons and over a greater time period are needed to confirm this trend.

Spatial Patterns

Fecal indicator bacteria and viral data both suggest that nearshore waters are impacted by human sewage and other land based sources of contamination. Offshore, monitoring wells were frequently found to contain genetic material from enteroviruses, adenoviruses, or both suggesting that certain components of human sewage, most likely originating from the widespread use of in-ground waste disposal, are reaching distances exceeding 10 km offshore. Shinn et al. (Shinn et al., 1994) previously reported that groundwater from KL5 was enriched in ammonium, but found that nearshore wells were lower in concentration, and based on available chemical data suggested that the offshore ammonium source was probably not sewage; however, using the same wells, Paul et al. (Paul et al., 1995b) detected fecal coliform bacteria in the groundwater offshore at KL5 and nearshore at KL1 and KL2 but not at the mid-transect stations (i.e., KL3 and 4). The results of these studies in combination with our findings of human enteric viruses in the same offshore wells indicate that human sewage has contaminated the groundwater underlying the Florida reef tract, but does not address the rate of movement. Although our sampling scheme followed a linear pattern offshore, it is not known if the transect actually matched real

groundwater flow patterns; therefore, lack of detection of high levels of indicator bacteria or enteric viruses in groundwater nearshore might be due to non-linear flows in the subsurface in this highly heterogeneous substrate (Personné et al. 1998). Likewise, the frequent appearance of enteric viruses in groundwater at offshore stations may also be explained by heterogeneity of the limestone bedrock and numerous cracks and fissures, which would facilitate preferential flow of groundwater through these large pores and result in accumulation of sewage constituents in offshore or distant areas (Paul et al., 1995b). Additionally, for much of the study area a layer of Holocene mud acts as an effective confining layer against vertical migration of ground and surface waters but may tend to force groundwater to move laterally (Shinn et al., 1994). This lateral movement has been shown to be most often toward the reef tract because of hydraulic conductivity (i.e., Florida Bay tends to have a higher sea level than the Atlantic) (Paul et al., 2000; Paul et al., 1995a; Paul et al., 1997).

The significance of contaminated groundwater reaching the outer reef tract is important because groundwater discharge is more probable in reef environments due to increased vertical porosity relative to mud and sediment (Shinn et al., 1994). Submarine groundwater discharges have been documented along the reef margin in the Florida Keys with discharge values up to 8.9 L m^{-2} d^{-1} (Simmons Jr, 1992). The groundwater underlying these offshore reefs is likely composed of both land derived sources, as evidenced by sewage markers such as those reported here and elsewhere (Paul et al., 1995b; Shinn et al., 1994) and mixing with the offshore water column (Simmons Jr, 1992). Therefore submarine discharge of groundwater along the Florida Reef Tract may provide a mechanism to move sewage derived constituents into the water column and reef environment. Our results indicate that enteric microbes accumulate along coral surfaces (SML) relative to the surrounding water, which is consistent with previous reports (Lipp and

Griffin, 2004; Lipp et al., 2002). This has potential implications both for both human health and the health of the coral community.

While previous work in the Florida Keys has suggested that the offshore environment and associated reefs are likely impacted by waste disposal practices on land (e.g., Paul et al. 1995a; Paul et al. 1995b, LaPointe et al. 1991; Lipp et al. 2007), this is the first study to systematically evaluate this trend using human-specific sewage markers. This works builds upon the important work of Shinn et al. (1994) and Paul et al. (1995a), which first used this Key Largo transect to document fecal indicator bacteria in the groundwater. Furthermore, in 2007, Lipp et al. showed a high frequency of human enteric viruses at reefs offshore of the main population centers in the Florida Keys, including Key Largo. The work presented here bridges these studies and provides evidence that groundwater beneath the Florida Keys contains genetic material of human viruses found in human sewage at distances greater than 10 km offshore. The seepage of groundwater at porous reefs could provide a mechanism for sewage contaminants, including human and potential coral pathogens (Patterson et al., 2002b), to travel while shielded from potential UV damage, increasing survival time, to the offshore water column (Lipp et al. 2007). While the full effects of the multiple constituents of human sewage (i.e., pharmaceuticals, hormones, etc.) on coral reefs is unknown, this study provides a definitive link between wastewater disposal practices on land and contamination of off-shore and suggests that additional research is needed to determine specific risks to corals and other benthic organisms, as well as public health.

EXPERIMENTAL PROCEDURES

Sampling

Surface water, groundwater and coral surface mucopolysaccharide layers (SML) were collected from each of five stations along a 10-km transect originating at Port Largo Canal in

Key Largo, Florida and extending out to the Florida Keys barrier reef tract near Molasses Reef (Fig. 5). There were two near shore stations; station KL1, located at the mouth of the Port Largo Canal, and station KL2, immediately south, which was the origin of the offshore transect (KL2 through KL5). Samples were collected twice yearly beginning in 2003, in the spring (April 2004 and 2005) and summer (July 2003 and 2004 and September 2005).

Surface water was collected as grab samples at each site in sterile polypropylene bottles (3 L)(Lipp et al., 2002). Groundwater was pumped from pre-drilled monitoring wells, as originally described by Shinn et al. (Shinn et al., 1994). Briefly, SCUBA divers attached tubing to the PVC well head (2.54 cm diameter) and the well was purged for 5 minutes at a flow rate of 11.4 L min^{-1} (approximately 5 well volumes). After purging, samples were collected in sterile polypropylene bottles (3 L). Screened well depths ranged from 13 to 20 m (Tbl. 2; (Shinn et al., 1994). Tubing was disinfected with a 10% bleach solution for 15 min followed by a rinse with sodium thiosulfate to de-chlorinate in between each sample collection. Additionally, three individual coral colonies were selected in an arbitrary fashion near the well head at each station. When possible, sampling was constrained to *Siderastrea* spp. (*radians* or *siderea*) or *Porites astroides* because these were present at all stations. One hundred and fifty ml of the coral SML was aspirated from each colony using sterile syringes without needles. Material was transferred to sterile 50 ml conical tubes at the surface. All samples were held on ice until processing (<6 h).

At each station, time of collection, temperature, salinity (measured by refractometry), and pH of both water types were noted. Daily precipitation estimates were obtained from the NOAA National Climate Data Center Summary of the Day for station 084095-1 at Homestead, Florida; the nearest station to the study area with a complete data set for the study period.

Fecal Indicator Bacteria

Indicator bacteria (fecal coliform bacteria, enterococci, and *Clostridium perfringens*) were concentrated from water and SML by membrane filtration and enumerated using standard methods. Up to 50 ml of surface and ground water and 10 ml of well-vortexed coral SML were analyzed in duplicate for each indicator bacterium. Samples were filtered through sterile 47-mm, 0.45-µm pore size mixed cellulose ester membranes. The membranes were then placed on selective agar media: mFC, mEI, and mCP, for fecal coliform bacteria, enterococci, and *C. perfringens,* respectively. mFC plates were incubated at 44.5 ˚C for ≥ 18 h; blue colonies were counted as fecal coliform bacteria. mEI plates were incubated at 41˚C for ≥ 18 h and all colonies with a blue halo were recorded as enterococci (USEPA 1997). mCP plates were incubated at 45 ˚C for ≥ 18 h; yellow colonies that turned pink colonies upon exposure to ammonium hydroxide fumes (30 s) were counted as *C. perfringens* (Bisson and Cabelli, 1979). For all indicators, the limit of detection was 5 CFU 100 ml^{-1} coral SML and 2 CFU 100 ml^{-1} for surface and groundwater.

Enteric Viruses

Viruses were concentrated using an adsorption-elution technique modified from Katayama et al. (2002) and described by Fong et al. (2005). Briefly, the pH of each 2-l water sample or 50 ml coral SML sample was adjusted to ~4 with 10% acetic acid and passed through a type HA, negatively charged membrane (Millipore, Billerica, MA) with a 47-mm or 90-mm diameter and a pore size of 0.45 µm. Final volumes filtered were recorded; these varied between samples depending upon turbidity. After adsorption, 100 ml of 0.5 mM H_2SO_4 was passed through the membrane. Viruses were then eluted with 10 ml of 1 mM NaOH, which was exposed to the membrane for ~1 minute. The eluate was then added to a sterile 15 ml tube

containing a neutralization solution of 0.1 ml 50 mM H_2SO_4 and 0.1 ml of 100X TE buffer. Centriprep YM-50 concentrator columns (Millipore, Billerica, MA) were used to concentrate and desalt the eluates to a final volume of ~2 ml, which were split and stored at -80 ˚C.

Commercially available kits (DNeasy Tissue kits and RNeasy Mini Spin kits [Qiagen, Valencia, CA]) were used to extract and purify DNA for detection of adenoviruses and RNA for detection of enteroviruses. For both virus types, extracts were obtained from 200 µl of concentrated eluate. According to the manufacturer's protocol, DNA was eluted and re-suspended in 50 µl Buffer AE, provided by kit and RNA was eluted and re-suspended in 30 µl RNase free water. Samples were stored at -20 ˚C, if not processed immediately. RNA was held less than 24 h before processing while DNA was stored for up to 3 days.

To minimize contamination from post-PCR carry over, nucleic acid extraction took place in a separate room from any post-PCR analysis. This room was equipped with an individual entry, separate air handling and dedicated equipment. Additionally, separate PCR workstations fitted with germicidal lamps were used with one dedicated for preparation of master mix (no nucleic acids) and one for DNA and RNA extraction.

Enterovirus RT PCR

Human enterovirus RNA reverse transcribed and cDNA amplified using nested-polymerase chain reaction (PCR) as described by Fong et al. (2005) using a commercially available RNA-PCR core kit (Applied Biosystems; Foster City, CA). Concentrated and purified RNA (2.5 µl) was added to the RT reaction mixture (7.5 µl), resulting in a final concentration of 5.0 mM $MgCl_2$, 1X PCR Buffer II (Applied Biosystems), 0.75 mM dNTPs, 2.5 µM Random Hexamer, 2.5 U μl^{-1} Reverse Transcriptase, 1.25 U μl^{-1} RNase Inhibitor. RNA was reverse transcribed at 22˚C for 10 min, 42˚C for 15 min, 99˚C for 5 min, and finally cooled to 4˚C. All

reactions took place in a DNA Engine® PTC-0200 thermal cycler (MJ Research, Inc. Waltham, MA). For PCR round 1, a reaction mixture of 20 µl was added directly to the RT product. The PCR 1 reaction provided a final concentration of 2.9 mM $MgCl_2$, 0.2 µM each primer (ENT-up-1 [5'- GTAGATCAGGTCGATGAGTC-3'] and ENT-down-1 [5'- ACYGGRTGGCCAATC – 3']), 0.4 mM dNTPs, 1X PCR buffer II (Applied Biosystems), 1X TaqMaster (Eppendorf), and 0.625 U μl^{-1} *Taq* polymerase. PCR round 1 was performed with 40 cycles of denaturing at 95 ˚C for 30 sec, annealing at 57.7 ˚C for 30 sec, and extension at 72 ˚C for 45 sec and a final extension at 72 ˚C for 5 min. The resulting amplicon for this cycle was 333 base pairs. 1 µl of PCR round 1 product was transferred into a 49 µl of reaction mixture for PCR round 2 with a final concentration of 2.0 mM $MgCl_2$ (including $MgCl_2$ provided by PCR Buffer), 0.2 µM each primer (ENT-up-2 [5'-CCTCCGGCCCCTGAATG-3'] and ENT-down-2 [5'-ATTGTCACCATAAGCAGCC-3']), 1X PCR Buffer (Eppendorf), 0.2 mM dNTPs, 1X TaqMaster (Eppendorf), and 1.25 U *Taq* polymerase enzyme. PCR round 2 consisted of 40 cycles denaturing at 95 ˚C for 30 sec, annealing at 56.5 ˚C for 30 sec, and extension at 72 ˚C for 30 sec and a final extension at 72 ˚C for 5 min. The resulting amplicon was 154 base pairs. A positive control, consisting of extracted RNA from poliovirus Lsc-1 (courteously provided by Dr. C.P. Gerba at the University of Arizona), and a negative (no-template) control were included in every PCR run.

Adenovirus PCR

Using the protocol described by Pina et al. (Pina et al., 1998), human adenovirus DNA was amplified using nested PCR. 1.5 µl of purified DNA was added to a PCR round 1 reaction mixture at final concentrations of 0.2 mM dNTPs, 0.8 µM each of primers AVA1 (5'-GCCGCAGTGGTCTTACATGCACATC-3') and AVA2 (5'-

CAGCACGCCGCGGATGTCAAAGT-3'), 1.5 mM $MgCl_2$, 1X PCR Buffer (Eppendorf), 1X TaqMaster (Eppendorf), and 0.25 U μl^{-1} *Taq* polymerase, for a final volume of 25 µl. PCR round 1 consisted of initial denaturing at 94 ˚C for 4 min and 40 cycles denaturing at 94 ˚C for 30 sec, annealing at 60 ˚C for 30 sec, and extension at 72 ˚C for 1 min and a final extension at 72 ˚C for 5 min. 3 µl of product from PCR round 1 was added to 47 µl of reaction mixture for PCR round 2. Concentrations were identical to PCR round 1 except that primers AVB1 (5'-GCCACCGAGACGTACTTCAGCCTG - 3') and AVB2 (5'-TTGTACGAGTACGCGGTATCCTCGCGGTC-3'). PCR round 2 was subjected to the same cycling conditions as PCR 1. The resulting amplicon of PCR 1 was 330 base pairs and 142 base pairs in PCR 2. A positive control, consisting of extracted DNA from adenovirus type 2 (courteously provided by Dr. C.P. Gerba at the University of Arizona), and a negative (no-template) control were included in every PCR run.

PCR Product Visualization and Confirmation

PCR products were separated by gel electrophoresis using a 2.2% Omnipur® agarose gel (EM Science, Darmstadt, Germany) and viewed under UV transillumination after staining in ethidium bromide. To confirm positive results and increase limits of detection (Fong et al., 2005; Griffin et al., 1999), all PCR products and controls were hybridized against a biotin-labeled (at the 5' end) internal probe (5'- ACGCACGACGTAACCACAGAC-3') specific to each amplified region using a dot-blot platform (Bio-Dot, BioRad). All blots were hybridized overnight at 37 ˚C, with a stringency wash at 47 ˚C for enteroviruses and 65 ˚C for adenoviruses (Fong et al., 2005). Labeled and hybridized probes were detected by chemiluminescence using the Southern-Light protocol (Tropix) as described by Fong et al. (2005).

All post-PCR analyses were performed in a physically separated lab with controlled access to prevent carry-over to other samples.

Statistical Analysis

Bacterial indicator counts (colony forming units [CFU]) were log transformed for all statistical analyses. Means were reported as geometric mean. Analysis of variance (ANOVA) and Tukey post hoc tests were used to evaluate differences in indicator levels between station, sample type, and date or season of collection (GraphPad Prism version 5.01; La Jolla, CA). The association between indicator levels and distance from shore was determined by Pearson correlation analysis (GraphPad Prism version 5.01; La Jolla, CA). Proportion of samples positives for each of the virus types was compared using Tukey's honest significant difference test (MULTPROP.MAC macro in Minitab v.14). Individual stations were considered positive for the virus in question if at least one sample from that station was positive (coral, groundwater or surface water). The three coral SML collected per station and sample point were pooled and treated as one sample. For both bacteria and viruses, non-detects were considered as zeros in all statistical analyses. In all cases, significance was declared at $p \leq 0.05$.

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TABLES

Table 3.1. Enteric virus detection between sample types

Sample Type	Enterovirus detection frequency	Adenovirus detection frequency
Coral SML[a]	16% (4/25)	72% (18/25)[b]
Water Column	8% (2/25)	44% (11/25)
Ground Water	8% (2/25)	32% (8/25)

a. Detection of adenovirus in coral SML was significantly greater than in other fractions ($P < 0.05$).
SML, surface mucopolysaccharide layer.

Table 3.2. Description and geological setting of sampling stations and monitoring wells

Station	Distance from shore (km)	Water Depth (m)	Well Depth (m)	Substrate
KL1	0.06	1.1	13.7	Grainstone, Packstone
KL2	0.34	1.8	13.7	Grainstone, Packstone
KL3	5.2	4.3	19.8	Holocene Sediment
KL4	8.0	4.6	15.9	Carbonate Sand
KL5	10.3	4.9	18.3	Coral Reef, Grainstone

FIGURE LEGEND

Figure 3.1. Mean concentration of fecal indicator bacteria among the three sample types (coral surface mucopolysaccharide layer [SML], surface water and groundwater) for all stations combined.

Figure 3.2. Mean concentration of fecal indicator bacteria from all sources combined (coral surface mucopolysaccharide layer [SML], surface water and groundwater) at each station (nearshore [KL1] to offshore [KL5]).

Figure 3.3. Frequency of detection of enteric viruses at sampled stations and among sample types. Bar height represents the total frequency of positive samples at each station (N=15). Each bar is subdivided to represent the relative contribution of positives from coral surface mucopolysaccharide layers [SML] (N=5), surface water (N=5) and groundwater (N=5) to this total.

Figure 3.4. Seasonal prevalence of enteric viruses and distribution between sample types. Bar height represents the total frequency of positive samples in summer (N=45) and spring (N=30). Each bar is subdivided to represent the relative contribution of positives in surface mucopolysaccharide layers [SML], surface water and groundwater (for each, summer N=15 and spring N=10).

Figure 3.5. Map of Key Largo transect and sampling stations.

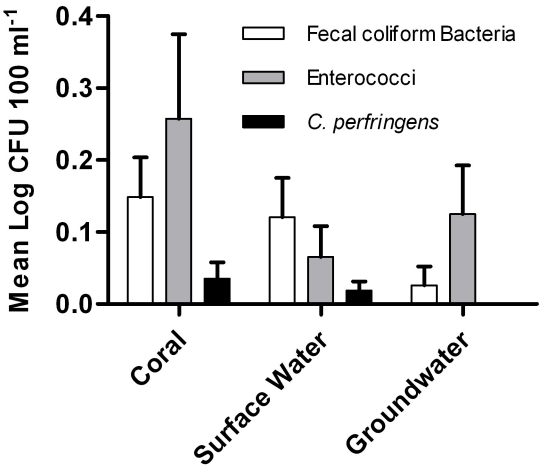
Mean Log CFU 100 ml-1
0.4
0.3
0.2
0.1
0.0
Fecal coliform Bacteria
Enterococci
C. perfringens
Coral
Surface Water
Groundwater

Figure 3.1.

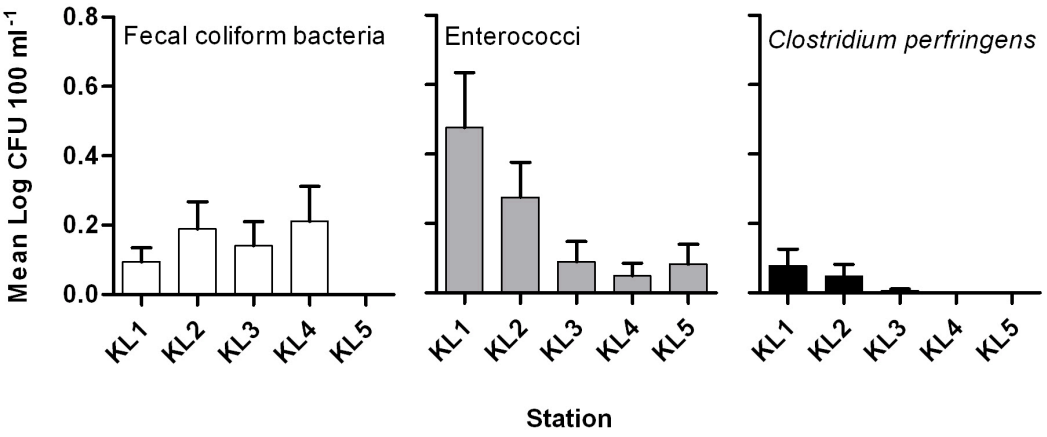
Mean Log CFU 100 ml-1
0.8
0.6
0.4
0.2
0.0
Fecal coliform bacteria
Enterococci
Clostridium perfringens
KL1
KL2
KL3
KL4
KL5
Station

Figure 3.2.

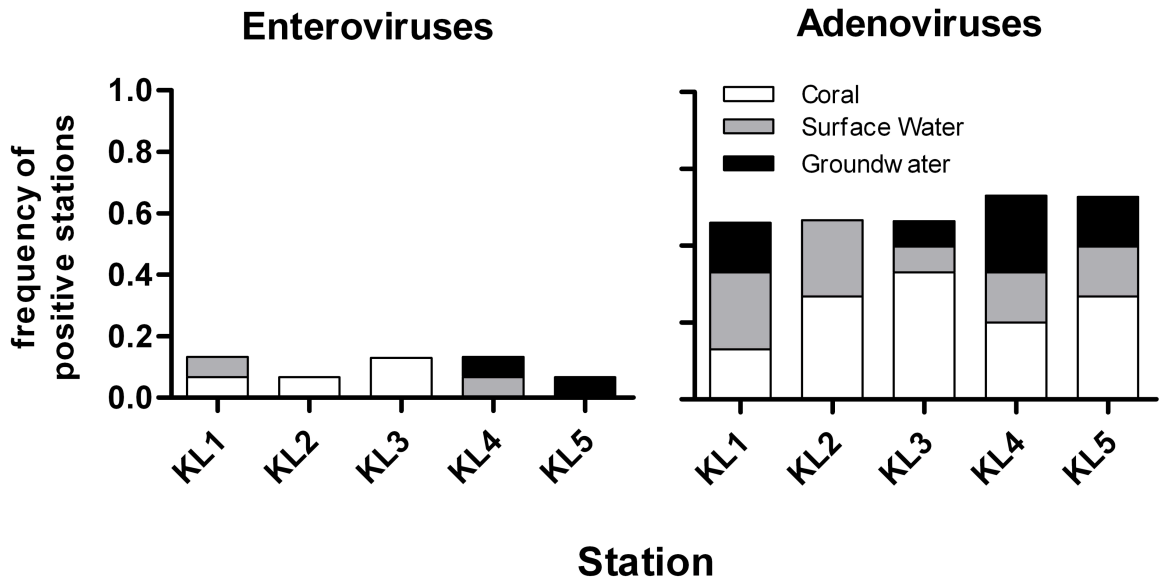
Enteroviruses
Adenoviruses
frequency of positive stations
1.0
0.8
0.6
0.4
0.2
0.0
Coral
Surface Water
Groundw ater
KL1
KL2
KL3
KL4
KL5
Station

Figure 3.3.

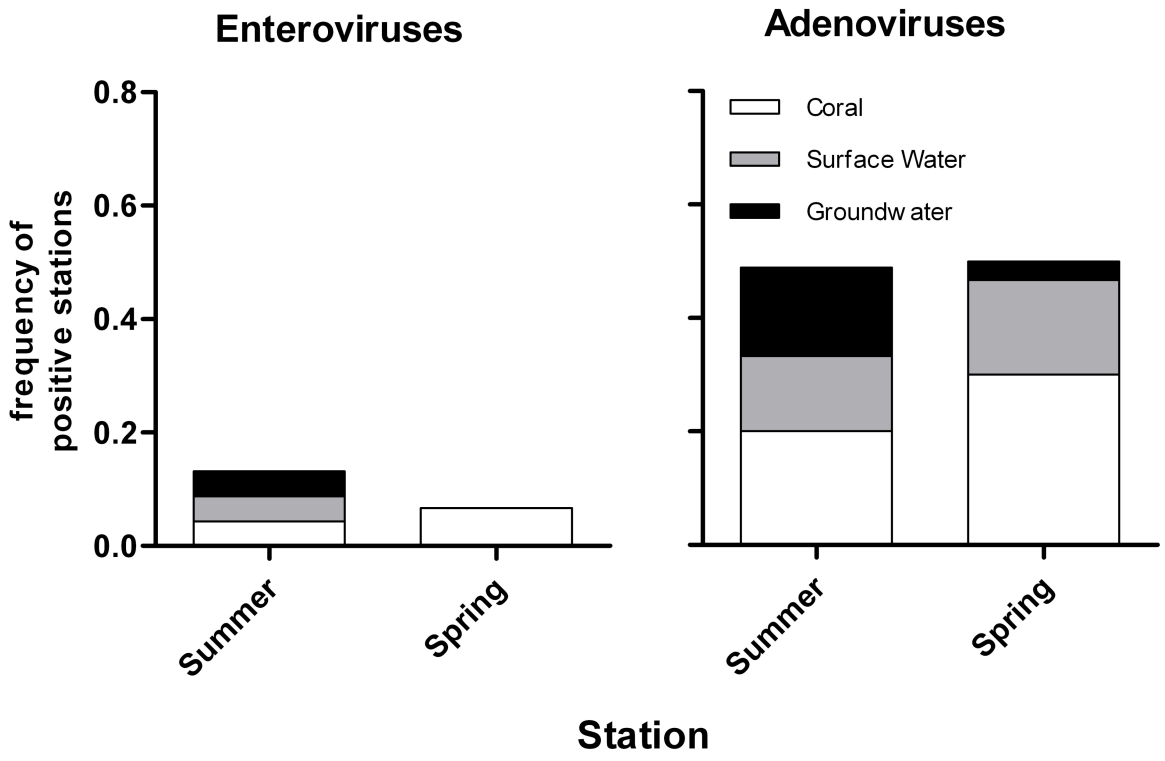
Enteroviruses
Adenoviruses
frequency of positive stations
0.8
0.6
0.4
0.2
0.0
Coral
Surface Water
Groundw ater
Summer
Spring
Summer
Spring
Station

Figure 3.4.

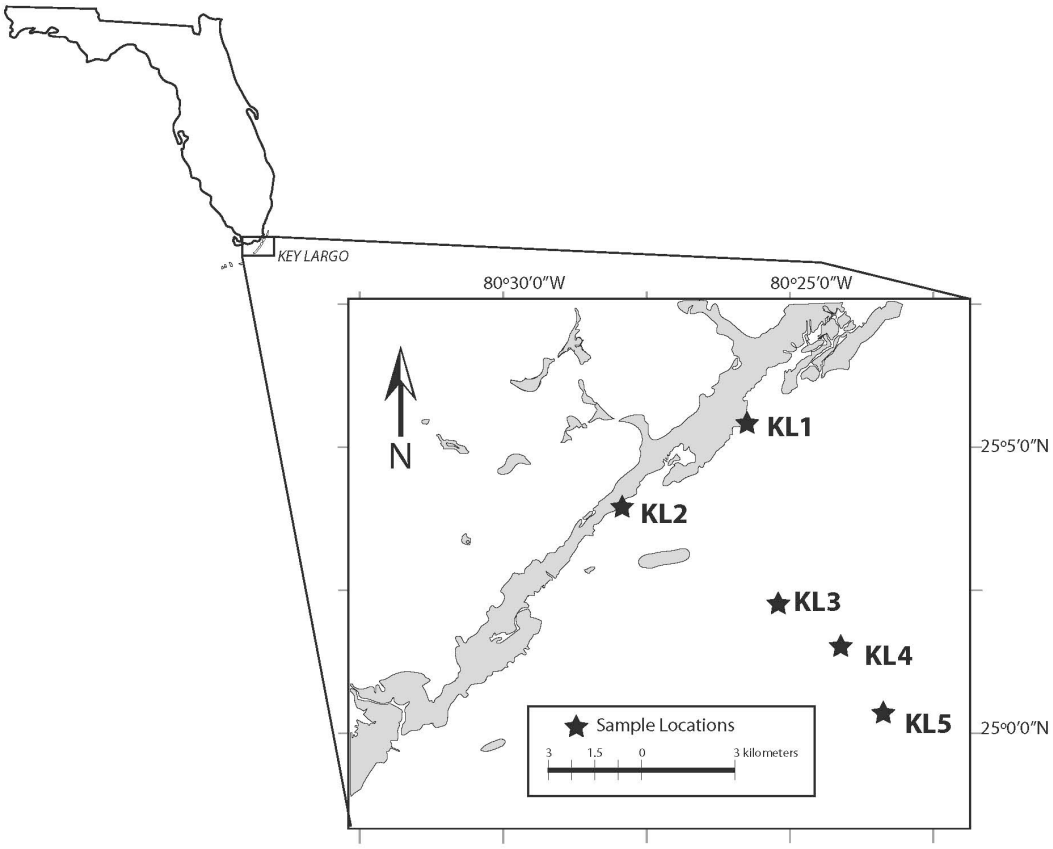
KEY LARGO
80°30'0"W
80°25'0"W
25°5'0"N
25°0'0"N
N
KL1
KL2
KL3
KL4
KL5
Sample Locations
3
1.5
0
3 kilometers

Figure 3.5.

CHAPTER 4

IDENTIFYING OCEAN OUTFALL IMPACTS ON SOUTHEAST FLORIDA COASTAL REEFS

INTRODUCTION

The reef tract off the coast of southeast Florida extends from the Florida Keys and Dry Tortugas to the south through the lower eastern counties that include Miami-Dade, Broward, Palm Beach and Martin Counties to the north. Although often less well known than the reefs of the Florida Keys National Marine Sanctuary, these more northern systems along the coast of southeast Florida provide extensive reef habitat, including a unique and sizeable stand of staghorn coral (*Acropora cervicornis*) (listed as threatened under the US Endangered Species Act; Hogarth 2006). These reefs are mainly comprised of sponges, sea fans, and a low percent of stony coral. The reef tract between Miami-Dade and Martin counties is a vital element of the economy, contributing approximately 1.8 billion dollars in income each year (Johns et al. 2003). As with many coastal areas, population growth in this region poses a serious threat to local coastal water quality (Colford et al., 2007; DiDonato et al., 2009; Fong and Lipp, 2005; Futch et al., 2010; Jiang et al., 2001; Lipp et al., 2007; McQuaig et al., 2006), which in turn may adversely affect the reef ecosystem integrity as well as public health.

Southeast Florida is densely populated, with a human population of 4,000 people mi^{-2} (www.censusscope.org) that is expected to at least double by 2020 to a total 15 million (Finkl and Charlier, 2003). In terms of population, Broward County is the second largest county in the state and the 15^{th} in the nation, with over 1.7 million people as of 2006. Such concentrated populations place increased burden on existing infrastructure in dealing with sewage treatment

and disposal. The increasing population densities cause increases in the amount of impervious surfaces, which facilitate storm water runoff into local waterways. Both point and non-point source pollution have a significant impact on coastal water quality.

Water quality along the reef tract of southeast Florida is impacted by multiple point and non-point sources of pollution. Among this four-county area of Miami-Dade, Broward, Palm Beach and Martin, centralized sewers service 57% of the population while 43% rely on in-ground disposal of wastewater (23% through septic system and 20% through injection wells). Treated wastewater in southeast Florida is discharged directly to the coastal environment through a series of six ocean outfalls (Carsey et al. 2008) offshore of Miami-Dade, Broward and Palm Beach Counties. Broward County utilizes two ocean outfalls for approximately 42.9% of its treated wastewater disposal (USEPA 2006a).

Injection wells are an important source of submarine groundwater discharge (SGD). Within Broward County alone there are 10 Class-I injection wells, 6 of which are used for wastewater disposal and 4 of which are used for reverse osmosis concentrate disposal (Maliva et al., 2007). Collectively, Miami-Dade, Broward and Palm Beach Counties account for ~77% of all injection well wastewater disposal for the entire State.

In the coastal counties of southeast Florida, storm-water is channeled to regional streams and canals; there are approximately 4,800 storm sewer outfalls in Broward County alone (Reich et al. 2008). Stormwater run off water can result in a significant quantity of surface pollutants (e.g., fertilizers, pesticides, automotive road deposits, animal feces) being mobilized and transported to local and distant marine environments. A series of interconnected networks of canals serve as a stormwater drainage system and periodically drain water from Lake Okeechobee as part of "controlled" releases (Finkl et al., 2005). Additionally, navigational

canals provide access to waterfront homes throughout this region. These constructed and natural channels and canals all flow into the Intracoastal Waterway, and many carry contaminants derived from both storm water and groundwater discharge. This water is ultimately transported to the Atlantic Ocean through a series of ocean inlets.

Collectively these point and non-point sources of contamination introduce a variety of pollutants, including chemicals, nutrients, and microorganisms, that may affect both public and ecosystem health. Nutrient-rich waters emanating from anthropogenic sources have already been implicated in recent blooms of macroalgae off the southeast Florida coast (Lapointe et al. 2005).

Microbial contamination from sewage affects public heath by increasing the risk for exposure to sewage-associated pathogens in the marine recreational environment (Cabelli et al., 1983; Griffin et al., 2003; Yau et al., 2009). Swimming, snorkeling, SCUBA diving and other recreational activities expose people to pathogens within the water column. Studies have documented the impact of contaminated marine waters on swimmers (Cabelli et al., 1983; Yau et al., 2009) citing symptoms ranging from ear, eye, and nose infections to gastrointestinal symptoms (Nobles et al. 2000). Sewage contamination may also impact reef health. Introduction of coral pathogens directly (i.e., *Serratia marcesscens*; Patterson et al. 2002, Sutherland et al. 2010), opportunistic enteric heterotrophic bacteria (Frias-Lopez et al. 2002; Lipp et al. 2002) or nutrients and other potentially toxic compounds, may cause or even exacerbate certain coral diseases (Bruno et al. 2003; Looney et al. 2010).

Currently, there are little data available to enable accurate assessments of risk to the reef habitat or associated recreational waters in southeast Florida that is due to anthropogenic pollutants. Fong and Lipp (2005) proposed that enteric viruses are a promising host-specific tool to assess water quality and improve public health. Enteric viruses have been widely used as a

biomarker for the presence of human sewage in many aquatic environments including lakes, rivers, estuaries, and marine beaches (e.g., Jiang et al. 2001, Katayama et al. 2002, Noble et al. 2003, and Wetz et al. 2004). More recently coral mucus has been found to naturally concentrate these viruses. Their detection is an effective marker for sewage contamination in coral reef systems (Lipp et al. 2007; Futch et al. 2010). Using molecular techniques to track the presence of human enteric viruses in the environment, this study aimed to evaluate the relative levels of sewage exposure among reefs impacted by ocean outfalls, inlets and non-point sources along the southeast Florida coast. This work provides a benchmark for understanding the influence of sewage pollution in reef health and provides information on possible public health risks in this coastal environment.

MATERIALS AND METHODS

Field Study Sampling

Offshore and reef survey. Surface water, sponge clippings, and coral surface mucopolysaccharide layers (SML) were collected from 8 stations, representing a range of potential pollution sources, located offshore of Broward County, Florida in July 2007 and 2008 (Fig. 1a). The eight sample stations were divided into four sites representing probable pollution source types (outfall, inlet, outfall plus inlet and no direct point source). The reef system of southeast Florida exists as a series of three reefs that run parallel to the shoreline. Samples from each site were collected from two of these parallel reefs, designated by number (1 was the most shoreward reef, 2 the mid reef and 3 the most offshore reef). From the north, site HI (stations HI2 and HI3) were proximal to both the Hillsboro Inlet and the Broward outfall. Site PE (stations PE2 and PE3) was close to the Port Everglades Inlet. Site FTL (stations FTL1 and FTL3) was expected to be primarily affected by non-point sources and was located offshore of Ft.

Lauderdale Beach. Finally, site HWO (stations HWO2 and HWO3) was located near the Hollywood Outfall at the southern end of Broward County. Surface water samples were also collected immediately above both Broward and Hollywood ocean outfalls (Hollywood was sampled in both years while Broward was sampled only in 2007) (Fig. 4.1a).

All surface water samples were grab samples collected in sterile polypropylene bottles (3 l) from a small boat; SCUBA divers collected coral and sponge samples. From each offshore site, three individual coral colonies (*Porites astreoides*) were selected in an arbitrary fashion at each station for collection of coral surface mucopolysaccharide layer (SML). Approximately 150 ml of the coral SML was aspirated from each colony using three 60-ml sterile syringes without needles. Material was transferred to sterile 50 ml conical tubes at the surface. The three coral samples per site were pooled for analysis (~150 ml) in a sterile 250 ml polypropylene bottle in the lab within 2 hours of collection. Three sponges were also selected in a haphazard fashion from each offshore sampling site. Species collected varied by station and were selected based on convenience and close proximity to sampled corals. Tissue was excised from the outer regions of each sponge using fresh razor blades. The size of clippings ranged from 3 to 4 cm in length with varying diameters. Clippings were placed in sterile conical tubes (50 ml) immediately upon collection (tissue was suspended in water at the collection point); sponge samples were frozen to -20^{o} C within 2 hours of collection. All samples were held on ice until processing (or freezing in the case of sponges) (<6 h). At each station, time of collection, temperature, salinity (measured by refractometry), and pH were noted.

All offshore samples (including water over the two ocean outfalls) were processed and analyzed for the presence of human enteric viruses (adenoviruses, enteroviruses, and human

noroviruses), which were used as conservative marker of human sewage (Fong and Lipp, 2005; Futch et al. 2010). Analysis is described below.

Inlet study. For inlet sampling, surface water and mid-depth (3-16 m) water samples (3 L each) were collected from 15 stations originating just inside the mouth of the Port Everglades Inlet in on July 31, 2007 (Fig. 4.1b). All samples were taken on an outgoing tide beginning at 11:25 AM. The first sample was taken in the mouth of the inlet (station 6) and subsequent samples were taken as the boat moved offshore following the transect, ending with station 15. Sampling was completed at all stations within 45 minutes. Surface water samples were collected by hand in sterile polypropylene bottles. Depth samples were collected using Niskin bottles; water was transferred to sterile polypropylene bottles on the deck of the boat. Niskin samplers were decontaminated between each sample using a 10% bleach solution followed with a sodium thiosulphate rinse to neutralize the chlorine. Samples were held on ice (<6 h) until processing.

Inlet samples were processed for both human enteric viruses as well as fecal indicator bacteria (FIB: enterococci, fecal coliform bacteria, and *Clostridium perfringens*) to assess both conservative markers of human waste (i.e., enteric viruses) as well as general trends associated with land-based sources of pollution (i.e., FIB).

Sample processing

Fecal indicator bacteria. Indicator bacteria (fecal coliform bacteria, enterococci, and *Clostridium perfringens*) were concentrated from inlet water samples using membrane filtration and grown on standard selective and differential media. Samples (up to 250 ml) were filtered in duplicate through sterile 47-mm, 0.45-µm pore size mixed cellulose ester membranes for each of the three FIB targets. The membranes were then placed on selective agar media: mFC, mEI, and mCP, for fecal coliform bacteria, enterococci, and *C. perfringens,* respectively. mFC plates were

incubated at 44.5 ˚C for ≥ 18 h; blue colonies were counted as fecal coliform bacteria (APHA 1995). mEI plates were incubated at 41˚C for ≥ 18 h and all colonies with a blue halo were recorded as enterococci (USEPA 1997). mCP plates were incubated at 45 ˚C for ≥ 18 h; yellow colonies that turned pink upon exposure to ammonium hydroxide fumes (30 s) were counted as *C. perfringens* (Bisson and Cabelli 1979).

Human enteric viruses. Viruses from all water samples and coral SML were concentrated based on the adsorption-elution technique originally described by Katayama et al. (2002), and modified for use in reef samples as described by Futch et al. (2010) and Lipp et al. (2007). Briefly, using a 10% solution of glacial acetic acid, the pH of each water sample (~2 L each) or 150 ml pooled coral SML sample was adjusted to ~4 and then passed through a type HA, negatively charged membrane (Millipore, Billerica, MA) with 90-mm diameter and a pore size of 0.45 μm. Sample volumes were recorded, as they varied between samples depending upon turbidity, and final collected volume was recorded. Membranes were rinsed with 100 ml of 0.5 mM H_2SO_4. To elute the viruses, the vacuum seal to the manifold was broken and membranes were exposed to 10 ml of 1 mM NaOH for ~1 minute. A sterile 15 ml tube containing a neutralization solution of 0.1 ml 50 mM H_2SO_4 and 0.1 ml of 100X TE buffer was used to catch the eluate. Tubes were stored at -20 ˚C until further processing. To concentrate and desalt the marine eluates, Centriprep YM-50 concentrator columns (Millipore, Billerica, MA) were used to obtain a final concentrated volume of ~2 ml, which was split and stored at -80 ˚C.

Sponge tissues were thawed and divided into equivalently sized triplicate pieces using sterile techniques. Each tissue replicate plus ~1.5 ml of associated interstitial sponge water was then placed into individual 2 ml cryovials. One aliquot was used immediately for extraction of

RNA or DNA while the others were stored at -80 ˚C. Each sponge aliquot was vigorously vortexed for approximately 2 min. Liquid was then carefully squeezed from the sponge tissue using sterile forceps as described by Donaldson et al. (2002). Tissue was discarded and the sponge slurry water was then used for RNA and DNA extraction.

From all concentrated samples (water and coral SML) and sponge water, 200 µl aliquots were used for extracting DNA or RNA using commercially available kits [DNeasy Tissue kits and RNeasy Mini Spin kits (Qiagen, Valencia, CA)]. DNA was eluted and re-suspended in 50 µl Buffer AE, provided by kit and RNA was eluted and re-suspended in 30 µl RNase free water, both according to the manufacturer's protocol. Samples were then stored at -20 ˚C, if not processed immediately. The RNA, prone to quicker degradation, was held less than 24 h before processing while DNA was stored for up to 3 days.

Human adenovirus (hAdV) DNA was amplified by real-time PCR using a commercially available TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA). Concentrated and purified DNA (2.5 µl) was added to the PCR reaction mixture (22.5 µl), with a primer concentration of 300 nM and probe concentration of 200 nM, as described by He and Jiang (2005). The reaction was carried out on an Applied Biosystems StepOne™ Real-Time PCR System under the following conditions: 95 ˚C for 15 s, 56 ˚C for 15 s, and 62 ˚C for 30 s for 45 cycles. Primer and probe sequences are listed in Table 1. The assay targets serotypes 1-5, 9, 16, 17, 19, 21, 28, 37, 40, 41, and simian adenovirus 25. Standard curves were based upon the concentration (PFU ml^{-1}) of a known strain of human adenovirus type 2 (courteously provided by DR. C.P. Gerba at the University of Arizona). This strain was also used as positive control in all reactions.

Human enterovirus (hEV) RNA was amplified by real-time reverse transcription (RT)-PCR using a commercially available AgPath-ID™ One-Step RT-PCR Kit [Applied Biosystems (Foster City, CA)]. Concentrated and purified RNA (2 µl) was added to the PCR reaction mixture (23 µl), with a primer concentration of 600 nM and probe concentration of 250 nM, as described in Donaldson et al. (2002) (Tbl. 4.1). The reaction was carried out on an Applied Biosystems StepOne™ Real-Time PCR System under the following conditions: RT for 10 min at 45 ˚C, 10 min at 95 ˚C, and 45 cycles of 10 s at 95 ˚C, 30 s at 55 ˚C, and a final extension of 15 s at 72 ˚C. The primer/probe sets described by Donaldson et al. (2002) target a 192 base pair region of the 5' untranscribed region (UTR) of the enteroviral genome. Standard curves were based upon an extraction of a known concentration (PFU ml^{-1}) of poliovirus vaccine strain Lsc1 (courteously provided by Dr. C. P. Gerba, University of Arizona). RNA from this strain was also used as a positive control in all reactions.

RNA from human norovirus (NoV) Genogroups 1 and 2 were also amplified for real-time RT-PCR using a commercially available AgPath-ID™ One-Step RT-PCR Kit [(Applied Biosystems (Foster City, CA)]. For each genogroup, concentrated and purified RNA (2 µl) was added to two PCR reaction mixtures (23 µl ea), with primer concentrations of 400 nM and probe concentrations of 120nM. Two primer and probe sets were used for each genogroup as described by Gentry et al. (2009) (Tb. 4.1). Reactions were carried out on an Applied Biosystems StepOne™ Real-Time PCR System under the following conditions: RT for 10 min at 45 ˚C, 10 min at 95 ˚C, and 45 cycles of 10 s at 95 ˚C, 30 s at 55 ˚C, and a final extension of 15 s at 72 ˚C. Standard curves were based on RNA transcripts for NoV genogroups 1.4 and 2.4 courteously provided by Dr. J. Vinjé (Centers for Disease Control and Prevention) and originally described in Gentry et al. (2009). These transcripts were also used as positive controls in all reactions. When

calculating percent positive for NoV in this study, a sample was considered NoV-positive based on detection with any primer set (i.e., only one primer set needed to be positive to consider the sample positive).

To maintain quality control standards, PCR master mix was prepared inside a designated hood in a separate room from any amplified product. Extractions were also carried out in a second designated hood and in a separate room from any amplified products. No-template negative controls were included in all reactions. Finally, no equipment or re-entrance was allowed inside the master mix and extraction room following any exposure to amplified product.

RESULTS

Offshore and Reef Study

In all, 80 samples were collected including 16 water, 48 sponge and 16 pooled coral SML samples from the eight stations between 2007 and 2008. As both sampling events took place in late July, environmental parameters varied little between the two years with temperatures averaging 29.2 ºC, salinity averaging 31.8 (ranging from a low of 30 at stations HI2 and PE2 to a high of 35 at station HI3) and pH averaging 8.06 (ranging from a low of 8.01 at station HWO2 to a high of 8.10 at station HI3).

Human adenovirus DNA and human enterovirus RNA were never detected during this study period. Pooling data from 2007 and 2008, human noroviruses (NoV) were detected in 25/80 samples (31% among all sample types).

NoV Genogroup 1 (G1) was detected in 12/80 samples (15%), while Genogroup 2 (G2) was detected in13/80 samples (16%). Among the two primer/probes sets utilized, those described by Jothikumar et al. (2005) yielded positive results from 12.5% of samples (10/80) for G1 and from

15% of samples (12/80) for G2. Those described by Kageyama et al. (2003) yielded positive results in only 2.5% (2/80) samples for G1 and 1.25% (1/80) of samples for G2.

Water. Norovirus prevalence in the surface water was 12.5% (2/16 samples). Genogroups 1 and 2 were each detected once in separate samples (both in 2007); G1 was detected at station HWO2 and G2 was detected at station PE3.

Coral SML. Among coral SML samples, 1/16 samples were positive for NoV (6.3%); however, this single sample (station PE2, collected in 2007) was positive for both Genogroup 1 and 2, simultaneously.

Sponges. NoV was detected in 19 of 48 sponges (40%). G1 was detected in 20.8% of samples (10/48) and G2 was detected in 22.9% (11/48). Two sponge samples (4.2%) were concurrently positive for both genogroups including station PE3 in 2007 and HI2 in 2008.

Location and putative pollution sources. NoV detection rates varied among the 4 sampling sites (and putative pollution sources). Samples located near the Port Everglades Inlet (PE2 and PE3) were most often positive for NoV (35%; 7/20 among all sample types combined). All sample types collected (water, coral and sponge) were positive at least once during the course of this study; this was the only site in which all sample types were found to be positive for NoV. G1 was detected in 3 samples (20%) and G2 was detected in 5 samples (25%). Two samples (1 sponge [PE3] and 1 coral SML [PE2]) were concurrently positive for both genogroups (Tbl. 4.2).

Samples off of Ft. Lauderdale Beach, with no direct impact from outfalls or inlets (FTL1 and 2), were positive for NoV 30% of the time (6/20 samples, all types combined); however, noroviruses were only detected in sponges (Tbl. 4.2). Among all samples, 10% (2/20) of samples were positive for G1 and 20% (4/20) of samples were positive for G2. No samples were concurrently positive for both genogroups.

Stations at the HWO site (HWO2 and HWO3), influenced by the Hollywood Outfall, were positive for NoV 25% of the time (5/20) among all samples. NoV were detected from water and sponge, but not from coral samples (Tbl. 4.2). G1 was detected in 15% of samples (3/20) and G2 was found in 10% of samples (2/20). No samples were concurrently positive for both genogroups.

At the Hollywood Inlet site (stations HI2 and HI3), 20% of all samples (4/20) were positive for NoV. Sponges were the only sample type positive for NoV. Three of the 20 samples (15%) were positive for G1 and 2 of 20 (10%) were positive for G2. One sample (HI2 sponge in 2008) was simultaneously positive for both genogroups (Tbl. 4.2).

Inlet study. During the outgoing tide, all fecal indicator bacteria tended to be concentrated near the mouth of the inlet (Fig 4.2) and became more diluted offshore at the surface and at depth (Fig 4.2); however, concentrations were always below actionable levels for recreational water (USEPA 1986). Fecal coliform bacteria averaged 9.2 CFU L^{-1} among all surface samples (N=15) and <2 CFU L^{-1} at depth (N = 15). The highest concentrations were noted in the surface at stations 2 (48 CFU L^{-1}), 7 (42 CFU L^{-1}) and 6 (34 CFU L^{-1}). Enterococci averaged 78 CFU L^{-1} among surface samples (N=15) and 8.5 CFU L^{-1} at depth. The highest concentrations were noted in the surface samples at stations 6 (310 CFU L^{-1}), 7 (154 CFU L^{-1}) and 9 (124 CFU L^{-1}). The highest concentration at depth, 64 CFU L^{-1}, was also found at station 6. *Clostridium perfringens* followed a similar trend, averaging 9.2 CFU L^{-1} among all surface samples (N=15) and 2 CFU L^{-1} at depth. The highest concentrations were noted at 34 CFU L^{-1} in the surface and at 6 CFU L^{-1} at depth, both at station 6 (Fig 4.2).

Neither human adenovirus nor human enterovirus were detected during the inlet study (outgoing tide); however, human norovirus were detected in 16.7 % of all samples (5/30).

Overall, 20% of surface water samples (3/15; stations 1, 5 and 13) and 13.3% of samples at depth (2/15; stations 6 and 14) were positive for NoV. Among the genogroups, G2 was more prevalent with 13.3% (4/30) of samples positive (depth stations 6 and 14 and surface stations 1 and 13). Genogroup 1 was only detected once (3.3%) at surface station 5.

Outfalls

Outfall samples were taken in 2007 from both the Broward and Hollywood Outfalls. At the Broward Outfall, fecal coliform bacteria were found at a concentration of 236 CFU L^{-1}, enterococci at 66 CFU L^{-1}, and *C. perfringens* at 56 CFU L^{-1}. FIB analysis was not performed for samples from the Hollywood Outfall. Enteric viruses, adenoviruses and enteroviruses were not detected; however, both outfalls were positive for norovirus G1.

DISCUSSION

In densely populated coastal areas, such as southeast Florida, land based sources of pollution to marine environments are becoming increasingly significant for their potential negative impacts to coastal marine ecosystems. Pollution causes harmful algal blooms and creates human health risk. The full impact of sewage in offshore reef environments and recreational waters is yet to be monitored or fully investigated, including where the most significant source of contaminants may arise (e.g., inlets, outfalls, submarine groundwater discharge, among others).

Data from this study show that NoV, a primary source of adult and childhood gastroenteritis (Atmar and Estes, 2006) and prevalent in human sewage, are widespread along the coast of Broward County, despite the relatively low levels of fecal indicator bacteria at inlets and outfalls. The lack of correlation between enteric virus detection and fecal indicator bacteria

is consistent with previous studies and is a common finding in coastal waters. Traditional fecal coliform assessments of water safety are inadequate in making a public health risk assessment.

The lack of any detection of enteroviruses and adenoviruses was surprising given the relatively high rates of detection in previous studies in coastal Florida (e.g., Futch et al. 2010, Lipp et al. 2001). In these previous studies, nested (RT-) PCR in combination with dot blot hybridization was used to detect enteric viruses, which may have been more sensitive and perhaps less selective than the real time PCR approach used here, or may simply reflect slight differences in amplification between gene target region(s). Among the NoV detected in this study, G1 and G2 were found at similar frequencies with 15% and 16% of samples from reef stations positive, respectively. However, within the samples collected on the outgoing tide at the Port Everglades Inlet G2 was much more prevalence than G1, which was only detected once. While both genogroups are associated with human disease, G2 is more commonly detected in outbreak investigations and is generally considered to cause the greatest burden of disease. G1 has been speculated to lead to more sporadic cases (which are often underreported) and may be more environmentally stable (Gentry et al., 2009). While two primer and probe assays were used in this study, those described by Jothikumar et al. (2005) detected NoV more frequently than those described by Kageyama et al. (2003), which was also noted in other environmental surveys and suggests that this assay may be more effective in detecting human noroviruses from marine samples (Gentry et al. 2009) or warrant further investigation for cross reactivity with other targets (Futch et al. Chapter 5).

In this study, the most detected enteric virus was norovirus, which reached its highest frequency of detection at reef stations near the Port Everglades Inlet (35% samples positive) followed by stations offshore of Ft. Lauderdale beach with no direct point source inputs (30%

positive) compared to outfall impacted sites (Hillsboro and Hollywood). Furthermore, stations near the Port Everglades inlet were the only reefs in which all three samples types were positive (water, sponge and coral); and, 10% of samples were simultaneously positive for both genogroups. The only other site in which both genogroups co-occurred was at the Hillsborough Inlet (also near the Broward Outfall); however, only sponges were positive. At the Hollywood Outfall site no genogroups co-occurred but both sponge and water were positive for NoV. Finally, while the non-point source impact site off Ft. Lauderdale Beach had the second highest prevalence rate, no samples contained both genogroups and positives were only found in sponges. Sponges, being natural filters of the surrounding water, seem to concentrate these enteric viruses or their nucleic acids. The dilution effect in the water column, as well as UV degradation may play a role in the lack of detection within the surface water.

The trend with the Port Everglades inlet having a higher sewage influence is consistent with a study at nearby Boynton Inlet in 2007 (Carsey et al. 2008), suggesting that the inlets may be a greater source of contamination compared to the outfalls. Inlets may act as point sources or conduits to the nearshore marine environment because they contain a high concentration of contaminants from a wide variety of sources. Bacterial indicator data from the surveyed outgoing tide event also suggest that the Port Everglades inlet may be a point source for pollution offshore. This is consistent with National Oceanic and Atmospheric Administration (NOAA) Florida Area Coastal Environment (FACE report; Carsey et al. 2008) data showing that inlets are a major source of microbial and nutrient inputs. In a 2007 study, over 50% of samples were positive for human adenovirus on an outgoing tide (Carsey et al. 2008). However, prevalence of enteric viruses was remarkably similar across all stations, suggesting that contamination is

widespread along the coast of the densely populated area. Therefore, inlets may be an important condiut of contamination, but there is a high degree of mixing among the sampled sites.

Non-point pollution sources likely contribute not only to contaminants carried throughout the inlets along the coast of southeast Florida but may also reach coastal areas through surface run off and submarine groundwater discharge. Using an average annual rainfall of 1.46 m yr^{-1} in southeast Florida the calculated average runoff rate estimate is ~1.65 x 10^{12} L yr^{-1}. This 1 trillion plus liters of water can result in a significant quantity of surface pollutants to coastal waters. Additionally, using submarine ground water discharge (SGD) estimates and current annual rainfall data, approximately 7.8 x 10^{10} L yr^{-1} of rainwater migrates to and is discharged on the Broward County offshore shelf (Sherwood et al. 1973). Using the same SGD rates and the current volume of septic tank effluent being discharged in Broward County (1.81 x10^{10} L yr^{-1} which is ~18.9% of the yearly county-wide sewage flow) it can be estimated that ~4.88 x 10^{8} L yr^{-1} exit the offshore shelf via SGD. If the same estimate were applied to injection well effluent that may be escaping to the offshore shelf via fissure/cracks or gaps in the confining layers, then approximately 4.54 x 10^{9} L yr^{-1} would be discharged into the marine environment off Broward County. This estimate may be conservative in nature given that day-to-day pumping may result in pressure-enhanced transport. Injection wells and septic systems may be a primary source of contaminants to the marine environment, especially those systems located in coastal settings or more inland in the vicinity of marine access canal systems (i.e., Paul et al. 2000, Futch et al. 2010). This type of non-point source pollution may also help to explain the consistently high levels of norovirus across all site sampled (20% - 35% positive).

Among the six ocean outfalls along the coast of southeast Florida discharging secondarily to advanced treated wastewater, two are located in Broward County and were each sampled once

during this study. The Broward County outfall discharges on average ~2.78 x 10^8 L d^{-1} (range is 2.94 to 3.03 x 10^8 L d^{-1}) and is located approximately 2.12 km offshore at a depth of 29 m. The City of Hollywood outfall discharges on average ~1.59 x 10^8L day^{-1} and is located 3.06 km offshore at a depth of 28.5 m.

Among sample types, marine sponges were the most common sample type found to harbor human NoV. Previous work by Donaldson et al. (2002) suggested that sponges may be a good bio indicator organism and concentrator for microorganisms. The high concentration noted in this study is also consistent with reports of Donaldson et al. (2002), suggesting the active filtration and concentration of water may make sponges an excellent natural bioindicator or sentinel for sewage contamination in reef habitats. Work in the Florida Keys, has shown that coral SML are also effective areas for concentration of viruses (Lipp et al. 2002, 2004, 2007; Futch et al. 2010); however, detection in this study was limited to only those sites impacted by the Port Everglades Inlet while detection in sponges was noted throughout the study sites.

While clearly more research is needed to fully account for all pollution sources offshore, this research provides an estimate of the potential microbial contaminant introduction from major inlet and outfall sources. Norovirus data shows that these viruses, indicative of sewage pollution, are widespread in this area despite relatively low levels of fecal bacterial indicators (inlet data). In an attempt to determine the greatest source input of sewage contamination, we concentrated on the impact of inlets, ocean outfalls, and areas with no direct inlet or outfall link (based upon proximity). The inlet stations (esp. Port Everglades, a highly trafficked and large inlet) had the highest percent of samples present and included multiple detects from the same station. The study reveals that not only are sewage constituents reaching the coral reef, but also the presence of human enteric viruses may pose a significant health risk to recreational

swimmers. These viruses are not regularly monitored for and are found in high levels and without the presence of fecal bacterial indicators in some areas (Futch et al. 2010). This research is especially significant to the Broward County area due to the proximity of the inlet to public beaches, and its potential impact to recreational swimmers, as well as the proximity to significant thickets of a threatened coral species.

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TABLES

Table 4.1. Primers and probes for detection of enteric viruses.

Primer/Probe	Sequence (5' to 3')[a]	Target/Location	Reference
Adenovirus			
AD2	CCCTGGTAKCCRATRTTGTA	Serotypes 1–5, 9, 16, 17, 19, 21,	He & Jiang 2004
AD3	GACTCYTCWGTSAGYGGCC	28, 37, 40, 41, and simian adenovirus 25	
ADP	**FAM**-AACCAGTCYTTGGTCA TGTTRCATTG-**BHQ**		
Enterovirus			
EV-U	GGCCCCTGAATGCGGCTAAT	192 base pair region of	Donaldson et al. 2002
EV-D	CACCGGATGGCCAATCCAA	5' untranslated region (UTR)	
EV-Pr	**FAM**-CGGACACCCAAAGTAGTCGGTTCCG-**BHQ**		
Norovirus Genogroup I			
COGIF	CGYTGGATGCGNTTYCATGA	5291–5310	Kageyama et al. (2003)
COGIR	CTTAGACGCCATCATCATTYAC	5375–5358	Kageyama et al. (2003)
Ring1a	**FAMc**-AGATYGCGATCYCCTGTC CA-**BHQ**	5340–5359	Kageyama et al. (2003)
Ring1b	**FAM**-AGATCGCGGTCTCCTGTCCA-**BHQ**	5340–5321	Kageyama et al. (2003)
JJVIF	GCCATGTTCCGITGGATG	5282–5299	Jothikumar et al. (2005)
JJVIR	TCCTTAGACGCCATCATCAT	5377–5358	Jothikumar et al. (2005)
JJVIP	**FAM**-TGTGGACAGGAGATCGCAATCTC-**BHQ**	5319–5341	Jothikumar et al. (2005)
Ring1b	**FAM**-AGATCGCGGTCTCCTGTCCA-**BHQ**	5340–5321	Kageyama et al. (2003)
Norovirus Genogroup II			
COG2F	CARGARBCNATGTTYAGRTGGATGAG	5003–5023	Kageyama et al. (2003)
COG2R	TCGACGCCATCTTCATTCACA	5100–5080	Kageyama et al. (2003)
Ring2	**FAM**-TGGGAGGGCGATCGCAATCT-**BHQ**	5048–5067	Kageyama et al. (2003)
JJV2F	CAAGAGTCAATGTTTAGGTGGATGAG	5003–5028	Jothikumar et al. (2005)
COG2R	TCGACGCCATCTTCATTCACA	5100–5080	Kageyama et al. (2003)
Ring2	**FAM**-TGGGAGGGCGATCGCAATCT-**BHQ**	5048–5067	Kageyama et al. (2003)

[a] FAM, 6-carboxyfluorescein, fluorescence reporter dye; BHQ, Black Hole Quencher

Table 4.2. Summary of norovirus detection among offshore reef sites including al sample types (for all N =20). Sites are listed from north to south.

Site	Putative Source	# positive (%) Either genogroup	# positive (%) Genogroup 1	# positive (%) Genogroup 2	# positive (%) Both genogroups	Sample Types Positive
HI	Inlet and Outfall	4 (20%)	3 (15%)	2 (10%)	1 (5%)	Sponge
FTL	Non-point source	6 (30%)	2 (10%)	4 (20%)	0 (0%)	Sponge
PE	Inlet	7 (35%)	3 (15%)	5 (25%)	2 (10%)	Sponge Coral Water
HWO	Outfall	5 (25%)	3 (15%)	2 (10%)	0 (0%)	Sponge Coral

FIGURE LEGENDS

Figure 4.1A Map of Broward County Offshore Sampling Stations

Figure 4.1B Map of Port Everglades Inlet Sampling Stations

Figure 4.2 Fecal Indicator Concentration in Port Everglades Inlet, Broward County


Hillsboro Inlet
H13
Hillsboro Outfall
H12
Intracostal Waterway
Intracostal Waterway
FTL 1
FTL 3
Fort Lauderdale
PE 3
PE 2
Port Everglades Inlet
Dania Beach
Intracostal Waterway
HWO 3
Hollywood Outfall
Hollywood
HWO 2a
N
0
5 km

Figure 4.1A.

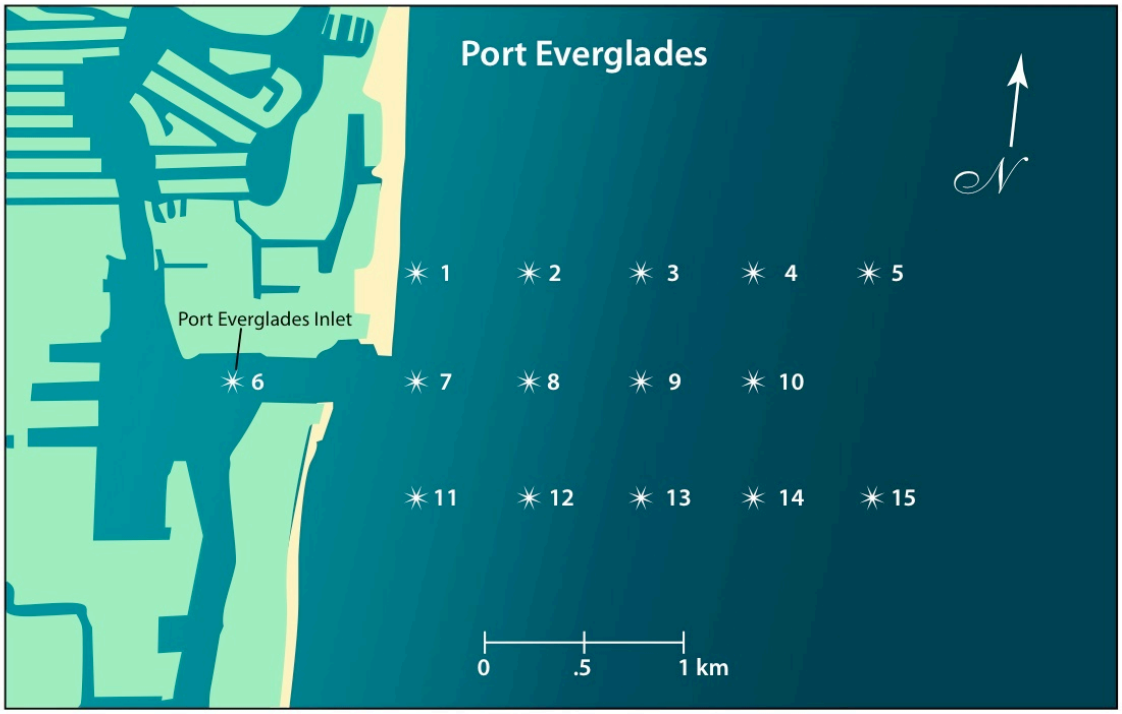
Port Everglades
N
1
2
3
4
5
Port Everglades Inlet
6
7
8
9
10
11
12
13
14
15
0
.5
1 km

Figure 4.1B.

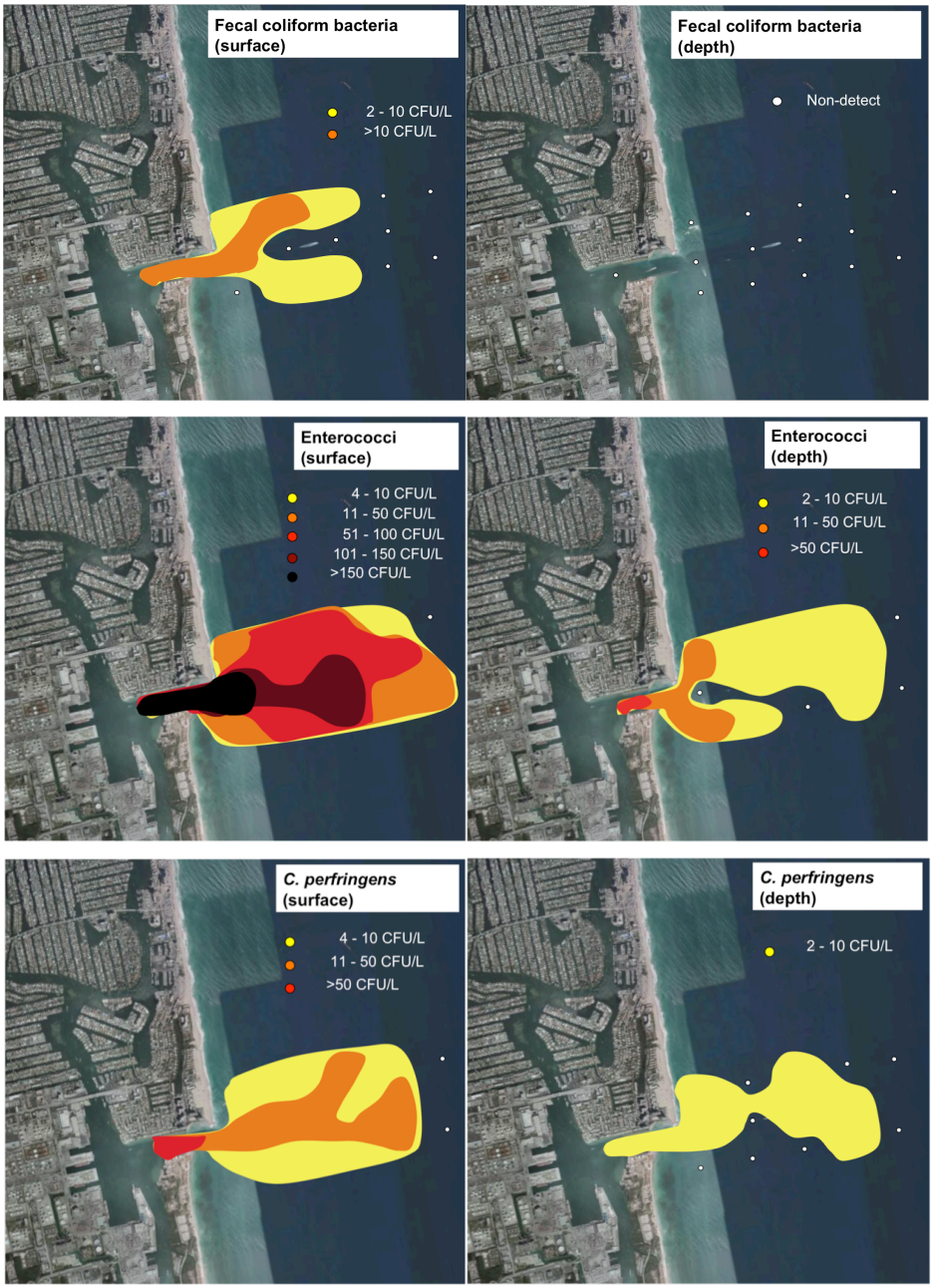
Fecal coliform bacteria (surface)
2 - 10 CFU/L
>10 CFU/L
Fecal coliform bacteria (depth)
Non-detect
Enterococci (surface)
4 - 10 CFU/L
11 - 50 CFU/L
51 - 100 CFU/L
101 - 150 CFU/L
>150 CFU/L
Enterococci (depth)
2 - 10 CFU/L
11 - 50 CFU/L
>50 CFU/L
C. perfringens (surface)
4 - 10 CFU/L
11 - 50 CFU/L
>50 CFU/L
C. perfringens (depth)
2 - 10 CFU/L

Figure 4.2.

CHAPTER 5

DOLPHINS AS SENTINEL ANIMALS OF OCEANS AND HUMAN HEALTH: SURVEYING CARRIAGE OF HUMAN ENTERIC PATHOGENS USING MOLECULAR TECHNIQUES

INTRODUCTION

Recent reports have highlighted a surge in marine mammal illnesses (Baskin, 2006), many linked to anthropogenic sources, which has prompted much interest in the role of marine mammals as sentinels of human health associated with marine exposure (Bossart 2006). In addition to diseases from exposure to chemical contaminants associated with human activity, increasingly zoonotic diseases that occur in humans are observed in populations of marine mammals. Sea lion populations in California are affected by toxoplasmosis as the *Toxoplamsa gondii* parasite typically found in felids, enters coastal waters via storm water run off (Miller et al. 2002). Marine mammals have also been documented as a reservoir for *Brucella* sp., causing brucellosis in humans (Godfroid et al., 2005; McDonald et al., 2006).

Concomitant with this recognition of human pathogens affecting marine mammals, research over wide geographic areas have documented a high rate of detection of human enteric viruses in marine waters and coastal environments (Donaldson et al., 2002; Griffin et al., 2003; Jiang et al., 2001; Lipp and Griffin, 2004), where microbial contaminants affect public health as well as the health of sensitive coastal ecosystems, such as coral reefs (Patterson et al., 2002a; Sutherland et al., 2010). Leaky septic systems, insufficient wastewater treatment, as well as storm water run-off coupled with the increasingly dense population of coastal cities create

conditions that threaten both the public health and the marine environment. This study looks at the role of human enteric pathogens and their possible impact upon marine mammals, and considers whether they may be a reservoir of possible human enteric pathogens or a sentinel for exposure to such pathogens in contaminated coastal water.

In this study, we evaluated the overall detection by standard molecular methods of selected human bacterial and viral pathogens among feces and rectal swab samples of Atlantic bottlenose dolphins in two anthropogenically impacted coastal areas. Our goal was to develop a baseline of data for the prevalence of these pathogens and to begin to distinguish the role of marine mammals as possible reservoirs of these microbes or true sentinels for exposure to human waste.

SAMPLE PROCESSING

Source of Specimens

Dolphin fecal samples and rectal swabs were collected as part of a dolphin capture-release health assessment study (Bottlenose Dolphin Health and Risk Assessment [HERA]), (Reif et al., 2008). This study took place in Charleston Harbor (South Carolina) and the Indian River Lagoon (Florida) (Fig. 1) during the summers of 2005 and 2007. Fecal samples were stored immediately in cryovials at -80^{o} C in aliquots of 60 – 80 mg; rectal swabs were stored in 500 µl of 1 x PBS (phosphate buffered saline) in 15 ml conical tubes at -80^{o} C. Additionally, a small number of fresh fecal samples were collected in May of 2009 from stranded dolphins in coastal South Carolina and were used within 24 h of collection for analysis of culturable bacteria.

The Charleston Harbor site is located near the estuarine waters of Charleston, SC. This site includes the Charleston Harbor and the Stono Estuary, as well as the channels and creeks of the Ashley, Cooper, and Wando Rivers. It is the third largest estuarine drainage location in

South Carolina and is impacted by both urban and residential development. As of 2009, the population of Charleston, SC was estimated to be 107,845 (US Census Bureau; www.census.gov).

The Indian River Lagoon is located on the Atlantic Coast of Florida; this area encompasses a population of greater than 325,008 (including population estimates from 2006 for Titusville, Palm Bay, Ft. Pierce, and Port St. Lucie) (www.census.org). The capture sites included Mosquito Lagoon as the northernmost site and St. Lucie Inlet as the southernmost site. This lagoon may be more susceptible to pollution concentration due to a limited flushing capability and a long residence time (Phlips et al., 2010; Steward and Lowe, 2010; Zingone et al., 2010). A highly dense human population and intensive agriculture also impact the Indian River Lagoon (Lu et al., 2010; Phlips et al., 2010). Dolphins in these areas were previously shown to have a strong site fidelity (Zolman 2002; Mazzoil et al. 2005, and Speakman et al. 2006).

Culturable Enteric Bacteria

Aliquots from fresh fecal samples were evaluated for the presence of fecal indicator bacteria (fecal coliform bacteria and enterococci), *Vibrio* spp. and *Salmonella* spp. Samples (10 – 100 µl) were spread, in duplicate, onto selective mFC and mEI agar for the detection of fecal coliform bacteria and enterococci, respectively; plates were incubated at 44.5˚C and 41˚C for 18 – 24 h for fecal coliform bacteria and enterococci, respectively. Blue colonies on mFC were counted as fecal coliform bacteria (APHA 1995). All colonies on mEI with a blue halo were recorded as enterococci (USEPA 1997). Vibrios were detected by spread-plating up to 100 µl in duplicate onto TCBS (thiosulphate citrate bile salts sucrose; Oxoid) agar and incubating overnight at 35˚C. All green and yellow colonies were counted as presumptive *Vibrio* spp.

Additionally, to increase detection of vibrios, enrichment was prepared with 1 ml alkaline peptone water (APW; 1% peptone 1% NaCl at pH 8.6) inoculated with 100 µl of fresh fecal matter. This enrichment was incubated overnight at 37˚C, and then 10 µl was spread onto TCBS agar and incubated at 35˚C overnight. A random selection of yellow and green colonies from both direct plating and enrichment plating were picked, streaked three times for isolation and saved on agar slants for identification by carbon substrate utilization pattern analysis using the BioLog system (Microlog, Hayward, CA).

To detect culturable *Salmonella* spp., a two-step enrichment process was utilized (Haley et al., 2009). Fresh fecal sample (100 µl) was added to 10 ml 1% peptone and incubated overnight at 35˚C for pre-enrichment. Then 100 µl of the overnight culture was transferred to 10 ml Rappaport-Vassiliadis (RV) broth and incubated overnight at 43˚C for selective enrichment. A loopful of overnight growth from RV broth was then spread onto selective xylose-lysine-deoxycholate (XLD) agar and incubated overnight (up to 48 hrs) at 37˚C. All black colonies or yellow colonies with black halos were counted as presumptive *Salmonella* sp.; colonies were picked, streaked three times for isolation and saved for final biochemical confirmation of genus using the Enterotube II system (Becton Dickinson, Franklin Lakes, NJ). Additionally, isolates were screened by PCR for the *Salmonella* genus as described below.

Molecular Detection of Zoonotic Enteric Bacteria

Fecal solids and rectal swabs were screened directly for *Salmonella* and *Campylobacter* using conventional PCR (Tbl 5.1a). Any presumptive *Salmonella* isolates from XLD agar plates were confirmed using the same PCR assay. DNA was extracted from 100 µl of thawed fecal/swab samples using the DNeasy Tissue Kit (Qiagen); DNA was eluted in 50 µl of buffer AE provided by the kit. DNA was extracted directly from overnight cultures of presumptive

Salmonella isolates by boiling 100 µl of culture for 10 min. The boiled cells were spun down to pellet out debris (10,000 x g for 5 min) and the supernatant saved and diluted before storing at -20˚C.

Extracted DNA was subjected to conventional PCR using a commercially available PCR Master Mix kit (Eppendorf, Hauppauge, NY) and targeted the genus-specific *SipB/C* gene for *Salmonella* (Carlson et al. 1999) or the thermophilic *Campylobacter* spp. using the 23s rRNA gene (Eyers et al. 1993) (Tbl 5.1a). For both targets, extracted DNA (1 µl) was added to the PCR reaction mixture (24 µl), with a final forward and reverse primer concentration of 800 nM, a final dNTP concentration of 200 µM, 1X PCR Buffer (final concentration), 1X PCR Enhancer (final concentration), 2.5 mM MgCl and 0.625 U μl^{-1}.

The *Salmonella* reaction was carried out under the following conditions: initial denaturing at 95 ˚C for 10 m, with 40 cycles of 94 ˚C for 20 s, 60 ˚C for 30 s, and 72 ˚C for 30 s, and a final extension at 72 ˚C for 5 m. A *Salmonella Sp*. strain was used for a positive control. The thermophilic *Campylobacter* reaction was carried out under the following conditions: initial denaturing at 94 ˚C for 3 m, with 45 cycles of 94 ˚C for 1 m, 54 ˚C for 1 m, and 72 ˚C for 1 m, and a final extension at 72 ˚C for 8 m. *Campylobacter jejuni* was used for a positive control. No template negative controls using PCR grade water were included in all reactions. Assays were performed in a DNA Engine® PTC-0200 thermal cycler (Bio Rad, Hercules, CA).

Molecular Detection of Human Enteric Viruses

Enteric viruses were assayed using standard protocols for the molecular detection in environmental water quality studies. For enteroviruses and adeonoviruses in addition to real time (RT) PCR, which is increasingly the detection method of choice given its high specificity and ability to provide quantitative information, conventional (RT) PCR was used for detection in this

study given the long history of using these genetic targets in studies of coastal water quality (Bosch, 1998; Fong et al., 2005; Fong and Lipp, 2005; Griffin et al., 2003; Griffin et al., 1999; Lipp et al., 2007; Lipp et al., 2001c; Wetz et al., 2004). For noroviruses, only real time RT-PCR was evaluated as previous research has demonstrated that the short amplicons of the real time RT-PCR are high specific for differentiating the genogroups of norovirus (Gentry et al., 2009; Jothikumar et al., 2005; Kageyama et al., 2003) and are the primary assay used by regulatory agencies (Chan et al., 2006; Nordgren et al.).

For the detection of enteric viruses, nucleic acids were extracted from a loopful of solid or 100 µl of liquid feces (the condition of the fecal samples ranged from solid to liquid with varying degrees in between), using commercially available DNeasy Tissue kits (Qiagen, Valencia, CA) for human adenoviruses and RNeasy Mini Spin kits (Qiagen, Valencia, CA) for human noroviruses and enteroviruses. For samples that were very thick or partially dehydrated, a Qiashredder was utilized as part of the extraction process to aid the break down of the samples and reduce viscosity prior to extraction of nucleic acids (Qiagen, Valencia, CA). Tubes containing rectal swabs stored in 1 x PBS were vortexed vigorously for ~1 min. Excess water from swabs was removed by gently pressing against side of the tube. From the remaning solution, 100 µl was collected for DNA and RNA extraction. 50 µl of Buffer AE, provided by kit, was used to elute and re-suspend DNA while RNA was eluted and re-suspended in 30 µl RNase free water, provided by the kit and per manufacturer protocol. DNA samples were stored at -20º C for up to 3 d if not processed immediately. RNA was held at 4º C for less than 24 h if not processed immediately.

Adenoviruses

Human adenoviruses (hAdV) were amplified by real time PCR using a commercially available TaqMan Universal PCR Master Mix from Applied Biosystems (Carlsbad, CA) on a Bio-Rad iQ5 Real-Time PCR Detection System (Hercules, CA). The eluted DNA (2.5 µl) was added to a PCR reaction mixture (22.5 µl), with a primer concentrations of 300 nM (final), probe concentration of 200 nM and 1 X TaqMan Universal Master Mix (which includes AmpliTaq Gold DNA Polymerase, dNTPs, passive reference, and optimized buffer components, as indicated in the manufacturer's product literature). Cycling conditions followed those described by He and Jiang (2005) (Tbl 5.1b). Probe and primer sequences for all viral targets are listed in Tbl 5.1b. This assay targets human adenovirus serotypes 1-5, 9, 16, 17, 19, 21, 28, 37, 40, 41, and simian adenovirus 25. Standard curves were based upon a known strain of human adenovirus (Type 2) courteously provided by Dr C.P. Gerba at the University of Arizona.

Human adenoviruses were also assayed using conventional nested PCR based on the protocol originally described by Allard and colleagues (1990; Tbl 5.1c). Purified DNA (1.5 µl) was added to the first-round PCR reaction mixture at final concentrations of 0.2 mM dNTPs, 0.8 mM each of the outer forward and reverse primers (Tbl. 5.1c), 1.5 mM $MgCl_2$, 1 x PCR Buffer (Eppendorf), 1X TaqMaster (Eppendorf) and 0.25 U ml^{-1} *Taq* polymerase, for a final volume of 25 ml. PCR round 1 consisted of initial denaturing at 94°C for 4 min and 40 cycles denaturing at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 1 min and a final extension at 72°C for 5 min. Three microlitres of product from PCR round 1 was added master mix for round 2 PCR to obtain a final volume of 50 µl. Concentrations and reagents were identical to first round PCR except that internal forward and reverse primers were used (Tbl. 5.1c). PCR

round 2 was subjected to the same cycling conditions as PCR 1. The resulting amplicon of PCR 1 was 330 base pairs and 142 base pairs in PCR 2. A positive control (human adenovirus type 2, as described previously) and a negative (no-template) control were included in every PCR run. PCR products were separated on a 2.2% Omnipur® agarose gel (EM Science, Darmstadt, Germany) by gel electrophoresis, stained in ethidium bromide and viewed under UV transluminator. All PCR products (regardless of the presence of a band on electrophoesis gels) were subjected to final confirmation against a biotin labeled probe internal to the amplified region (Tbl. 5.1c) using dot blot hybridization and chemiluminescent detection with the Tropix CSPD substrate (Applied Biosystem) as previously described by Griffin *et al.* (1999) and Fong *et al.* (2005).

Enteroviruses

Using the real time RT-PCR protocol described by Donaldson et al. (2002), extracted RNA (2 µl) was added to a 23 µl PCR reaction mixture (23 µl) using components of the Ag-Path-ID™ One-Step RT-PCR Kit (Applied Biosystems, Foster City, CA)]. Final concentrations were 400 nM for primers and 120 nM for the probe (Tbl. 5.1b). Reactions were carried out on a Bio-Rad iQ5 Real-Time PCR Detection System (Hercules, CA). Standard curves were based upon an extraction from a known concentration of poliovirus (vaccine strain Lsc1, courteously provided by Dr. C. P. Gerba, University of Arizona).

Human enterovirus RNA was also assayed using conventional nested RT-PCR. Using the RNA-PCR core kit (Applied Biosystems; Foster City, CA), RNA was reverse transcribed and cDNA was amplified under the conditions described by Fong and colleagues (2005), and based on the primers originally described by DeLeon et al. (1990). A positive control (poliovirus Lsc 1 as described above) and a negative (no-template) control were included in every PCR run. PCR

visualization took place as described with the human adenovirus technique, including internal probe hybridization (Tbl.5.1c) for increased sensitivity in detection and further confirmation (Fong et al. 2005).

Noroviruses

Human Norovirus Genogroups 1 and 2 were amplified in a Bio-Rad iQ5 Real-Time PCR Detection System (Hercules, CA) for real time RT-PCR using an AgPath-ID™ One-Step RT-PCR Kit by Applied Biosystems (Foster City, CA). Extracted RNA (2 µl) was added to each of two PCR reaction mixtures (23 µl ea) for both genogroup 1 and 2, with primer/probe concentrations and reaction conditions as first described by Jothikumar et al. (2005) and evaluated by Gentry et al. (2009) (Tbl 5.1b). Standard curves were based on RNA run-off transcripts for Norovirus types1.4 and 2.4 which were courteously provided by Dr. J. Vinjé (Centers for Disease Control and Prevention) and described in Gentry et al. 2009. These transcripts were also used as positive controls in all reactions. When calculating prevalence for noroviruses in this study, samples were considered to be positive if either of the primer sets resulted in detection.

To maintain quality control, extractions were performed and preparation of PCR master mix was prepared inside individual dedicated hoods in a separate room from any amplified product. No equipment or re-entrance was allowed inside the mastermix and extraction room following any exposure to amplified product.

RESULTS

In all, feces or rectal swabs representing 56 individual dolphins were screened in this study. In total, 81 samples were processed, which included 45 fecal solids and 36 rectal swabs; all but 5 samples were frozen. The remaining 5 samples were collected fresh and processed

immediately. Concurrently collected rectal swabs and fecal solids were available for 22 of the 56 sampled (39.3%) dolphins For 12 dolphins (24.1%) only rectal swabs were obtained and only fecal solids were available for 9 animals (16.1%). Thirteen of the 45 solid fecal samples were from stranded (i.e., dead) dolphins; all other dolphins were alive and health status was recorded as healthy, concerned or diseased by trained professionals (Reif et al., 2008).

Enteric Viruses

Overall, 5 of 56 dolphins (8.9%) were positive for at least one of the human enteric viruses screened in this study; however, none of the three virus targets were found concurrently in the same sample. There were matched fecal solids and rectal swabs for three of the positive animals and in only one case were positive results repeated for the matched samples (norovirus detected in a female captured in Charleston Harbor, described below); however, in all five cases, positive results were obtained from the fecal solids. The only case in which a positive result was obtained for a rectal swab, the matched fecal solid was also positive. Of the five animals positive for an enteric virus, four were captured (or collected) from Charleston Harbor or the surrounding area and one was captured in the Indian River Lagoon.

By specific virus target, 1 dolphin out of 56 (1.8%) was positive for human enterovirus RNA (only conventional RT-PCR resulted in detection); the detection was from a 5-yr old diseased male from Charleston Harbor (Tbl. 5.2). Two dolphins (3.57%) were positive for human adenovirus (only conventional PCR resulted in detection); the animals included a diseased male of unknown age from the Indian River Lagoon captured and a stranded male of unknown age collected near the Charleston area (this was a fresh fecal sample) (Tbl. 5.2). Finally, 2/56 (3.57%) dolphins were positive for human norovirus genogroup I (none were positive for genogroup 2). Both of the animals in which norovirus RNA was detected were 23-yr

old females captured in Charleston Harbor one listed as concerned and the other with a normal health status (Tbl 5.2). Norovirus genogroup 1 was detected from both fecal solids and rectal swabs in the healthy female (captured on 10 August 2005).

Enteric and Zoonotic Bacteria

Only one fecal sample was collected and delivered to the lab at a short enough interval to warrant assaying for culturable bacteria. The sample was obtained from a male dolphin stranded on 22 May 2009 in coastal South Carolina. The sample was positive for enterococci and *Vibrio* spp., both at levels too numerous to count, but *Salmonella* and fecal coliform bacteria were not detected. In all, 3 presumptive *Vibrio* isolates and 8 presumptive enterococcoi isolates were subjected to carbon substrate utilization pattern analysis (Biolog) to assign a putative identification. The vibrios were identified as *Vibrio carcariae* (87% probability), *V. parahaemolyticus* (85% probability) and *Photobacterium damselae* (96% probability). Of the 8 presumptive enterococcus islolates, only two could be identified at a probability level above 75%; both were identified as *Enterococcus casseliflavus* (100% probability for both isolates).

Frozen samples from all 56 animals were assayed for *Salmonella* and thermophilic *Campylobacter* using conventional PCR. None of the samples were positive for *Salmonella* but thermophilic *Campylobacter* spp. were detected in 28.6% of the dolphins (16/56). Detection rate was similar between the two sites with 9/31 animals positive in or near Charleston Harbor (2 of which included stranded dolphins and the remaining 7 were from captured and released dolphins) and 7/25 positive from Indian River Lagoon. *Campylobacter* detection was also evenly distributed between males and females with 9/27 males positive (33.3%) and 5/16 females positive (31.3%). Health status also appeared to have little relationship with *Campylobacter* detection rates: 7 of 11 healthy animals were positive (63.6%); 2 of 10 listed as concerned were

positive (20%); 5 of 21 diseased animals were positive (23.8%); and, 2 of the 13 stranded (dead) dolphins were positive (15.4%). Interestingly, there was an inverse trend between dolphin age (and weight and height) and prevalence of *Campylobacter*, with the highest detection rates in the youngest and smallest animals (63.6% positive for the youngest quartile [less than 6 years of age] and 9% positive for the oldest quartile [14 – 25 years old]) Fig. 5.2). Only data from the capture and release were examined for these trends as the specific age as well as weight or length at time of death could not be accurately measured for the stranded dolphins.

DISCUSSION

Traditional indicators of fecal pollution, including fecal coliform bacteria and enterococci, do not fully reflect potential public health hazards or the impact on marine life that may occur from other sewage pathogens, such as enteric viruses. Traditional fecal indicators poorly correlate with enteric viruses, such as enteroviruses, noroviruses, among others, which are often found in the absence of bacterial indicators (Gerba et al., 1979; Griffin et al., 1999; Noble and Fuhrman, 2001). An ideal indicator for human fecal contamination is the goal of many published papers, which cite bacterial indicators as inadequate to protect public health (Anderson et al., 2005; Fujioka et al., 1981; Garcia-Lara et al., 1991; Gerba et al., 1979; Griffin et al., 2001b; Henrickson et al., 2001; Sinton et al., 1999). Additionally, the EPA is considering human specific targets to replace indicators in their new water quality criteria (EPA 823-R-07-006 2007). Among these alternative indicators, human enteroviruses and human adenoviruses are being studied as indicators of sewage impact and associated public health risks. However, the application of viral indicators is currently hindered by lack of studies establishing viral standards as well as limited information about the potential carriage of human enteric viruses by marine

mammals or potential cross reactivity of viruses from marine mammals with current human viral targets.

Given the interest in using viruses as an assessment tool, several lines of additional research are needed including (but not limited to) epidemiological studies to determine acceptable threshold levels and a more complete understanding of methodological limitations in detection from marine environments. Specifically, given that rapid detection using PCR based methods is the tool of choice (Donaldson et al., 2002; Gentry et al., 2009; He and Jiang, 2005; Mackay et al., 2002;,Gubbins, 2002) it is imperative to rule out any viruses shed by marine animals that might be cross reactive with the currently utilized primers. Although BLAST-searches in GenBank show that the primers and probes for human enteric viruses such as those utilized in this study are homologous only to viruses of human origin, there has been no systematic study of similar viruses in fecal samples of marine organisms, and therefore it is unknown if currently used PCR assays are compromised by cross reactivity with non-human viruses.

On the other hand, if dolphins or other marine mammals are indeed carrying human strains of enteric viruses, these animals could serve as a sentinel species for assessing risk in marine waters, especially given the high site fidelity of many marine mammals (including Atlantic bottlenose dolphins (Gubbins, 2002; Hanson and Defran, 1993; Rossbach and Herzing, 1999; Wells, 1991) and the fact that these animals, by their nature of constant immersion, are exposed to potentially contaminated water and much higher levels than humans. There have been many studies in the past looking at dolphins to evaluate exposure to anthropogenic pollution, especially chemical contaminants. While many studies to date have focused predominantly on toxicology and chemical exposure (Fair et al., 2010) rather than pathogens,

there is an established framework for evaluating dolphins as sentinel marine mammals (Bossart, 2006; Murdoch et al., 2008; Schaefer et al., 2009). Given recent reports on zoonoses in marine mammals (Austin, 2010; Fayer, 2004; Godfroid et al., 2005; Higgins, 2000; McDonald et al., 2006) and their potential high levels of exposure to human enteric bacteria and viruses in polluted nearshore waters, it is logical to further evaluate dolphins and other marine mammals for exposure to human fecal pathogens. Therefore, these marine mammals may not only serve as sentinels for water quality but also potential human health risk.

Dolphins as sentinels mammals of marine water pollution may be an expensive indicator to measure. The capture release study conducted by Reif et al. (2006) provided a unique opportunity to survey both toxicological effects and infectious disease in the bottlenose dolphin populations of Charleston Harbor and the Indian River Lagoon. Frozen material available from this study provided a platform for the analysis of human enteric microbes presented here; however, our results show that fecal solids may be required for proper assessment of microbial exposure given that only one rectal swab provided positive results. Captured wild dolphins were evaluated by a suite of tests performed by marine veterinarians and technicians, which included general physical exams, diagnostic ultrasounds, hematology, serum analyses, microbial tests, among others. While the results of this study predominantly focused on toxicological data (Fair et al. 2006), it is clear that dolphins are indeed exposed to anthropogenic sources of pollution, which is also confirmed by the presence of human enteric bacteria and viruses.

In this study, evidence of human enteric viruses was found in only 5 animals and included human adenoviruses, human enteroviruses and human norovirus genogroup 1. While the overall prevalence of these viruses was low, these are considered to be human-specific viruses and therefore conservative markers for human sewage. However, it remains unclear

whether the dolphins were capable of being infected by these pathogens or were simply carriers of them. The hEV and hAdV primer sets, especially the conventional PCR primers, are 'broadly reactive' and are designed to target multiple viral types within each viral group. In this respect, it would seem plausible to have a higher rate of detection or cross-reactivity of these assays with possible dolphin viruses [i.e., newly identified dolphin enterovirus (Nollens et al. 2009)]. Yet, only one or two of each virus type were found among the 56 sampled individuals. On the other hand, human NoV primer and probe sets for real time RT PCR are highly specific to individual genogroups and therefore may be expected to result in a lower risk of cross reactivity. Human NoV genogroup 1 was detected in two animals (including matched fecal and rectal swab samples). Given the relatively low prevalence and incidence of detection of the enteric viruses, it suggests that if cross reactivity with non-specific targets exists, it is rare in these populations of dolphins. Therefore, the primers utilized in this study are likely still effective at differentiating human waste.

Attempts to determine the exact virus types detected in these dolphins by sequence analysis were unsuccessful due to low recovery of amplified product in conventional PCR (samples were only positive by dot blot hybridization for hEV and hAdV; bands were not detected using gel electrophoresis) and inability to obtain sufficient DNA for cloning. For NoV, the product length from the real time RT PCR (amplified region <100 bp) was insufficient to determine the exact virus type. Lastly, the amount of fecal material available for analysis was depleted before other attempts at sequencing could be completed. Despite the lack of sequences, the specificity of this primer set and the low prevalence of detection suggests that dolphins might be exposed to human enteric viruses in these coastal waters. Additional research is needed to

determine whether dolphins merely carry these viruses (passing through their system) or are indeed infected with them.

Results for enteric bacteria from this study are consistent with previous findings showing that *Vibrio* spp. are commonly found as enteric flora in dolphins and marine mammals (Buck and McCarthy, 2008; Buck et al., 2006; Schroeder et al., 1985; Tangredi and Medway, 1980). Among the fecal indicator bacteria, enterococci were detected at high levels but fecal coliform bacteria were not detected. It is difficult to extrapolate these results given that only one sample was analyzed for culturable bacteria. The zoonotic bacterium *Salmonella enterica*, which is among the most common bacterial agents of enteric diseases among humans, was never detected by either culture or conventional PCR. However, another epidemiologically important zoonotic bacterium, the thermophilic *Campylobacter* spp. (which include *C. jejuni, C. coli, C. lari* and *C. upsaliensis* and are the species most often associated with human illness) were frequently detected by traditional PCR.

Thermophilic *Campylobacter* spp. was the most common enteric pathogen detected in this study. Interestingly, prevalence was highest among the young (small) dolphins, which is consistent with human epidemiology of campylobacteriosis, where it is most prevalent among young children. Given the high rate of detection, it is reasonable to suspect that this pathogen may be actively infecting these animals. Whether this infection results in disease is yet unknown and further research is warranted to understand the relationship between *Campylobacter* and a possible dolphin host.

The goal of this research was to begin to address the question of whether marine mammals (dolphins) that reside in contaminated coastal waters may serve as a sentinel for human health risk or if they are reservoirs (carriers) of these enteric agents. Data provided here

provides some of the first assessment of multiple human enteric pathogens, including human specific viruses, in a marine mammal population. While more research is needed to finally determine if dolphins are sentinels or reservoirs, the results of this study suggest that dolphin populations can carry human pathogens and therefore may be first in line for exposure to human sewage pollution in coastal waters, which ultimately may affect dolphin health as well as providing information about human health risk. Given the technical difficulties in obtaining fecal specimens from live animals, additional research should focus on human pathogen detection from more easily accessible tissue types to truly support the use of dolphins and marine mammals as sentinels for enteric disease.

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Table 5.1A. Primers and probes for detection of zoonotic bacteria by traditional PCR.

Primer/Probe	Sequence (5' to 3')[a]	Target/Location	Reference
Salmonella			
SipB/C (forward)	ACAGCAAAATGCGGATGCTT	*SipB/C* gene; n.t. position 2305-2324	Carlson et al. 1999
SipB/C (reverse)	GCGCGCTCAGTGTAGGACTC	*SipB/C* gene; n.t. position 2555-2536	
Campylobacter			
Therm 1	TATTCCAATACCAACATTAGT	23S rRNA	Eyers et al. 1993
Therm 2	GATTACAACGGGCATGGC		

Table 5.1B. Primers and probes for detection of human enteric viruses by real time (RT) PCR.

Primer/Probe	Sequence (5' to 3')[a]	Target/Location	Reference
Adenovirus			
AD2	CCCTGGTAKCCRATRTTGTA	Serotypes 1–5, 9, 16, 17, 19, 21,	He & Jiang 2004
AD3	GACTCYTCWGTSAGYGGCC	28, 37, 40, 41, and simian adenovirus 25	
ADP	**FAM**-AACCAGTCYTTGGTCATGTTRCATTG-**BHQ**		
Enterovirus			
EV-U	GGCCCCTGAATGCGGCTAAT	192 base pair region of	Donaldson et al. 2002
EV-D	CACCGGATGGCCAATCCAA	5' untranslated region (UTR)	
EV-Pr	**FAM**-CGGACACCCAAAGTAGTCGGTTCCG-**BHQ**		
Norovirus Genogroup I			
JJVIF	GCCATGTTCCGITGGATG	5282–5299	Jothikumar et al. (2005)
JJVIR	TCCTTAGACGCCATCATCAT	5377–5358	Jothikumar et al. (2005)
JJVIP	**FAM**-TGTGGACAGGAGATCGCAATCTC-**BHQ**	5319–5341	Jothikumar et al. (2005)
Ring1b	**FAM**-AGATCGCGGTCTCCTGTCCA-**BHQ**	5340–5321	Kageyama et al. (2003)
Norovirus Genogroup II			
JJV2F	CAAGAGTCAATGTTTAGGTGGATGAG	5003–5028	Jothikumar et al. (2005)
COG2R	TCGACGCCATCTTCATTCACA	5100–5080	Kageyama et al. (2003)
Ring2	**FAM**-TGGGAGGGCGATCGCAATCT-**BHQ**	5048–5067	Kageyama et al. (2003)

[a] FAM, 6-carboxyfluorescein, fluorescence reporter dye; BHQ, Black Hole Quencher

TABLE 5.1C. Primers and probes for detection of human enteric viruses by conventional (RT) PCR

Primer/Probe	Sequence (5' to 3')[a]	Target/Location	Reference
Adenovirus			
AV-A1	GCCGCAGTGGTCTTACATGCACATC	Hexon gene	Fong et al. 2005
AV-A2	CAGCACGCCGCGGATGTCAAAGT		
AV-B1	GCCACCGAGACGTACTTCAGCCTG		
AV-B2	TTGTACGAGTACGCGGTATCCTCGCGGTC	Hexon gene	
AV-probe	ACGCACGACGTAACCACAGAC		
Enterovirus			
ENT-up-1	GTAGATCAGGTCGATGAGTC	5'UTR	Fong et al. 2005
ENT-down-1	ACYGGRTGGCCAATC	5'UTR	
ENT-up-2b	CCTCCGGCCCCTGAATG		
ENT-down-2b	ATTGTCACCATAAGCAGCC		
EV-probe	TACTTTGGGTGTCCGTGTTTCc 12		

Table 5.2. Description of those samples positive for human enteric viral nucleic acids.

Site	Virus	Date Collected or Captured	Source
Charleston Harbor (South Carolina)	Human Enterovirus	9 Aug 2005	Fecal solids (frozen)
	Human Adenovirus	1 May 2009	Fecal solids (fresh)
	Human Norovirus Genogroup 1	2 Aug 2005	Fecal solids (frozen)
	Human Norovirus Genogroup 1	10 Aug 2005	Fecal solids (frozen) Rectal swab (frozen)
Indian River Lagoon (Florida)	Human Adenovirus	28 June 2007	Fecal solids (frozen)

FIGURE LEGEND

Figure 5.1. Map of Charleston Harbor and Indian Lagoon Sampling Area for the HERA Study (Reshef et al., 2006a).

Figure 5.2. Frequency of detection of thermophilic *Campylobacter* by Age, Weight and Length quartiles.

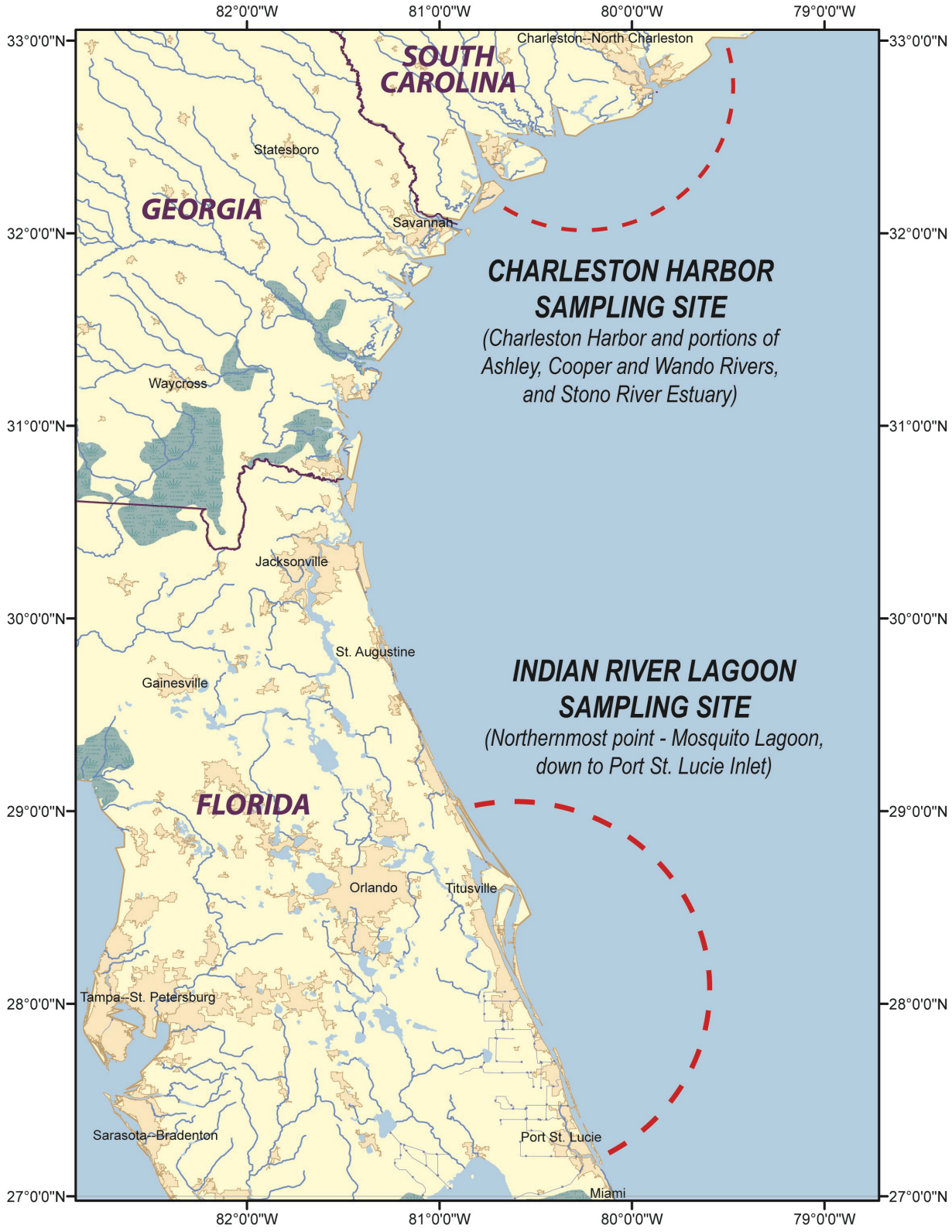
82°0'0"W
81°0'0"W
80°0'0"W
79°0'0"W
33°0'0"N
32°0'0"N
31°0'0"N
30°0'0"N
29°0'0"N
28°0'0"N
27°0'0"N
SOUTH CAROLINA
GEORGIA
FLORIDA
Charleston--North Charleston
Statesboro
Savannah
Waycross
Jacksonville
St. Augustine
Gainesville
Orlando
Titusville
Tampa--St. Petersburg
Sarasota--Bradenton
Port St. Lucie
Miami
CHARLESTON HARBOR SAMPLING SITE
(Charleston Harbor and portions of Ashley, Cooper and Wando Rivers, and Stono River Estuary)
INDIAN RIVER LAGOON SAMPLING SITE
(Northernmost point - Mosquito Lagoon, down to Port St. Lucie Inlet)

Figure 5.1.

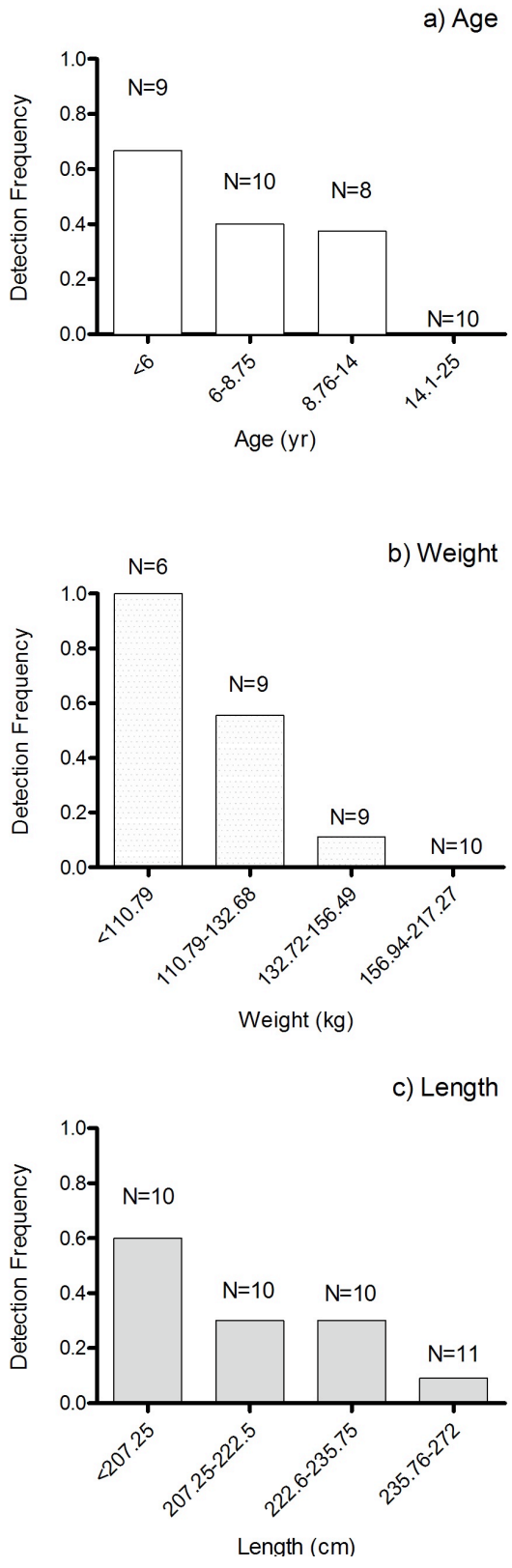
a) Age
Detection Frequency
1.0
0.8
0.6
0.4
0.2
0.0
N=9
N=10
N=8
N=10
<6
6-8.75
8.76-14
14.1-25
Age (yr)
b) Weight
N=6
N=9
N=9
N=10
<110.79
110.79-132.68
132.72-156.49
156.94-217.27
Weight (kg)
c) Length
N=10
N=10
N=10
N=11
<207.25
207.25-222.5
222.6-235.75
235.76-272
Length (cm)

Figure 5.2.

CHAPTER 6

CONCLUSION

There is an ongoing search for human specific microbial markers to monitor offshore marine recreational waters. An ideal microbial indicator of sewage contamination currently does not exist. Fecal bacterial indicators grow differently in temperate, subtropical, and tropical environments, reducing their general applicability. There are also differences in land use that influence which microbial inputs reach the ocean and which to monitor, e.g. industrial runoff and input may require an additional toxicological indicator, unlike agricultural runoff which introduces nutrients from fertilizers and if livestock is present, general fecal bacterial indicators. Human populations and high use recreational waters need better monitoring to identify and quantify human health risk. Given all these variables, it is imperative to design fecal indicators based upon numerous factors.

Beach monitoring for human health risk is a state-level process.. It is up to each state to set the standards that meet the US EPA's acceptable risk threshold. This creates a patchwork of regulation in marine beach water quality. What is alarming is that there are no guidelines, state or federal, mandating microbial water quality offshore. Recreational activities around coral reefs are a major contribution to the economy of Florida and other tropical areas. Potential sewage contamination can not only increase the price of health care for those exposed, but can tarnish the reputation of this highly visited tourist locations. People are not the only ones that would

benefit for better monitoring standards. Coral reefs are subject to human sewage impacts, with nutrient and pathogen inputs creating or increasing coral disease.

In addition to monitoring microbial water quality for coral reef and human health risk, the source of pollution needs to be identified. In order to maintain water quality and remediate polluted water, microbial fecal indicators need to also point to a source. Currently, techniques for fecal bacterial indicators are not specific enough to identify host. Therefore, host specific techniques, such as polymerase chain reaction (PCR), are designed to better identify the host source. More research is needed however to definitively separate marine mammal primers from those that are human specific. Also, it is important to acknowledge that identifying the pollution source will impact a wide variety of policy changes, such as discontinuing use of ocean outfalls, creating stricter no dumping zones for marine vessels, and monitoring inlets as potential sources of contamination. These societal regulations should be informed and influenced by factual accounts and relevant monitoring. Currently, the link between science and policy is weak in the area of sewage contamination of nearshore marine waters.

Microbial indicators monitored in the water column are not sufficient for predicting the total impact to coral reefs. Adding marine sentinel organisms, such as dolphins, corals, and marine sponges gives researchers a better idea of the impact of pollution and where it may be accumulating. Coral mucus and marine sponges accumulate particles from the water and may serve as a signal for acute and chronic exposure to microbial contaminants. The discovery of RNA-based human enteric viruses in these organisms identify acute exposures to viruses due to the more rapid degradation of RNA in the marine environment, while the presence of DNA based viruses could be a signal of chronic exposure. More research is needed to address how the natural defense mechanisms of these organisms interact with the microbes utilized for fecal

indicators. Dolphins are a potential reservoir for contamination and monitoring their health via fecal processing and other techniques can not only detect land-based contamination but may also contribute to identifying and perhaps addressing zoonotic pathogens before serious problems develop in marine organisms. Brucellosis has become an increasing widespread pathogen.

The current monitoring process of marine recreational water is insufficient. Ideally, increasing research of indicators, including microbial, organism, or habitat indicators, will alleviate the monitoring gaps and bring about changes in policy that consider the impact to offshore environments (habitats, marine organisms, and public health risk). Given my research findings, it is apparent that human sewage constituents are reaching the coral reefs of the Florida Keys and are found in coral mucus in both the Florida Keys and Broward County. Marine sponges also contain enteric viruses indicative of human sewage contamination. Here we find that the inlet is a major source of sewage contamination, and we also find human norovirus in the water (both in the inlet and offshore). Human norovirus is also found in fecal matter of dolphins off the Charleston harbor, but it is yet unknown how these viruses interact with these marine mammals (infection or merely carriage). These results indicate a widespread impact of human sewage in various habitats, organisms, and from various sources. My research also identifies the enteric viruses, enteroviruses, adenoviruses, and noroviruses, as strong candidates for fecal contamination detection in many sample types.