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Identification of Land-based Pollution in South Florida Coral Reefs: Host Specific Viruses as Conservative Markers for Human Sewage

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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 Jr 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. 2007) 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

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. 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. 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. 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. 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. 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. 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. 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. 2).

Inlet study. During the outgoing tide, all fecal indicator bacteria tended to be concentrated near the mouth of the inlet (Fig 2) and became more diluted offshore at the surface and at depth (Fig 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 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.

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. 2007), 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. 2007) 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. 2007). 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 conduit 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 1. Primers and probes for detection of enteric viruses. [a] FAM, 6-carboxyfluorescein, fluorescence reporter dye; BHQ, Black Hole Quencher

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)

Table 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

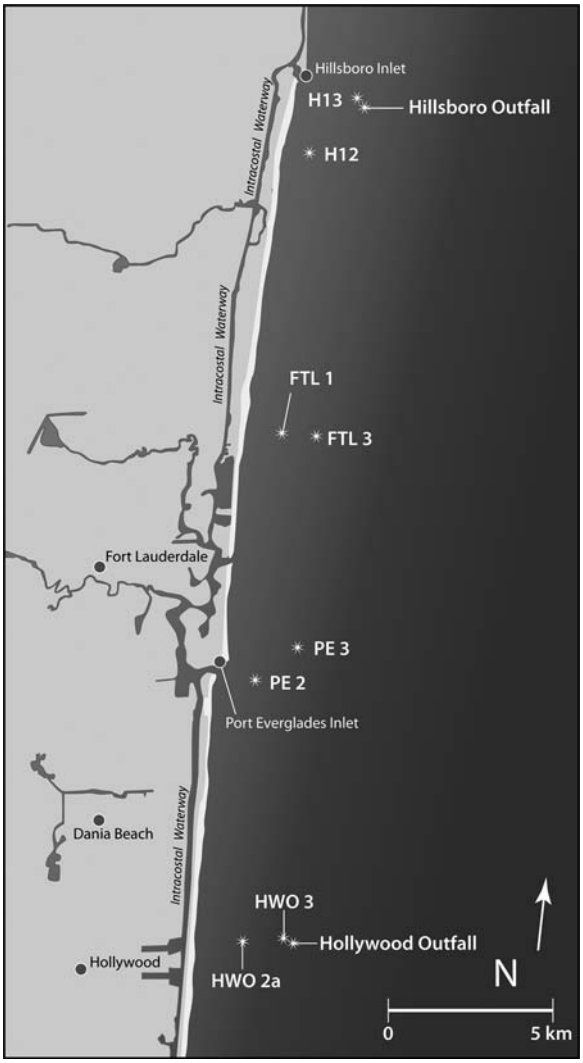
FIGURE LEGENDS

Fig 1A Map of Broward County Offshore Sampling Stations

Fig 1B Map of Port Everglades Inlet Sampling Stations

Fig 2 Fecal Indicator Concentration in Port Everglades Inlet, Broward County

Fig 3 Norovirus prevalence in Port Everglades



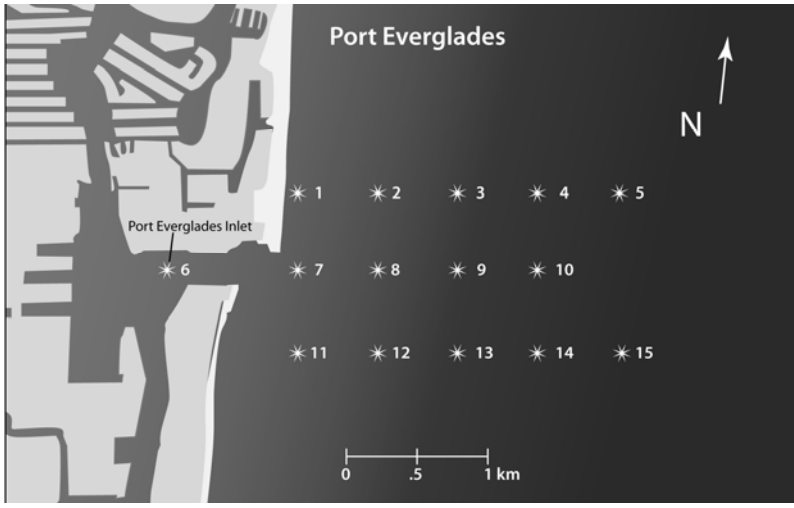


Figure 1 A and B. (A) Map of sampling area. Site HI (stations HI2 and 3) were impacted by the Hillsboro Inlet and the Broward Outfall. Site FTL (Stations FTL 1 and 3) were not immediately influenced by any inlet or outfall. Site PE (stations PE2 and 3) were immediately affected by the outgoing plume from Port Everglades Inlet. Site HWO (stations HWO2 and 3) were impacted primarily by the Hollywood Outfall. (B) Map of sampling grid near the mouth of Port Everglades Inlet

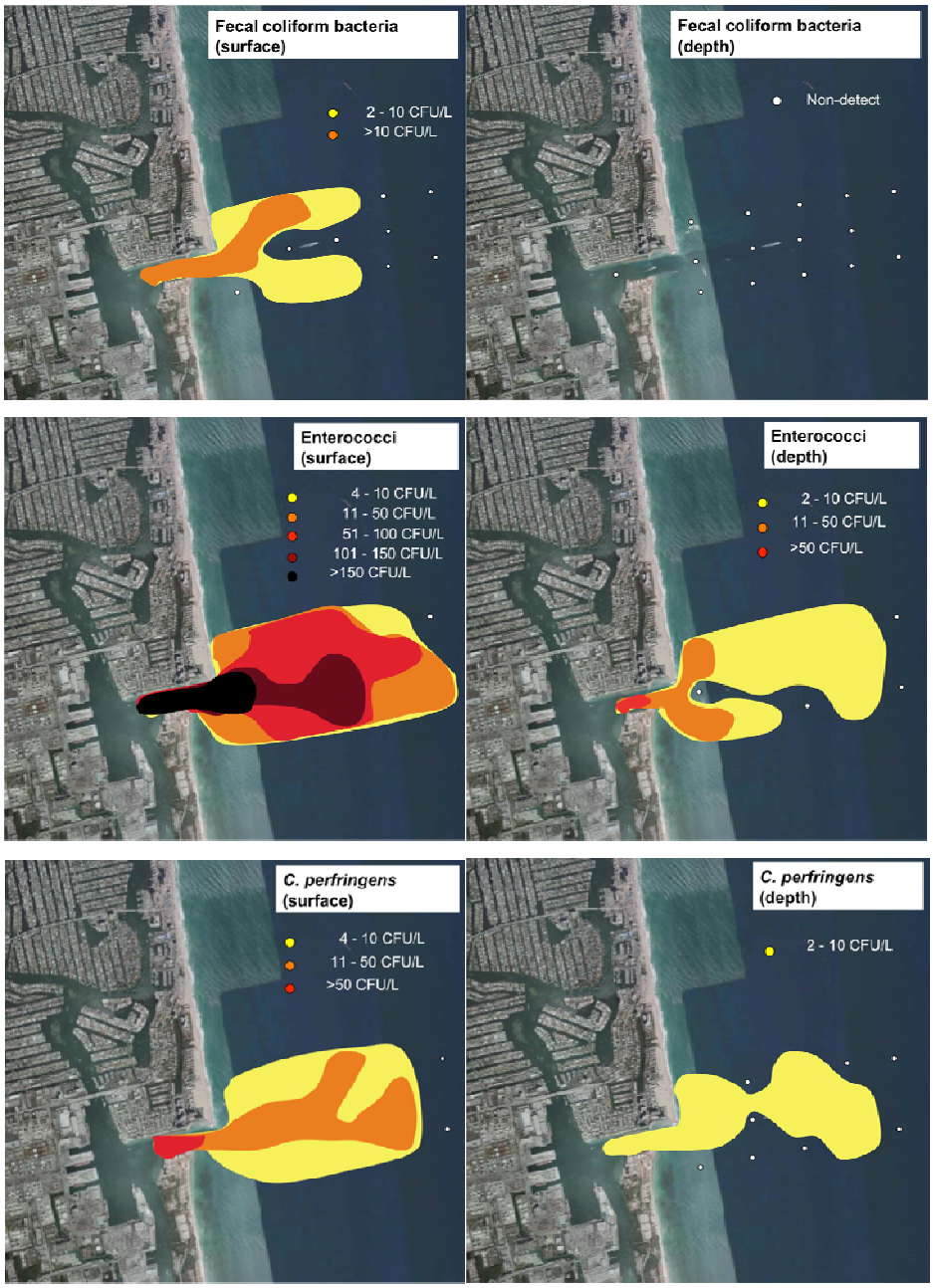


Figure 2. Contour plots of fecal indicator bacteria exiting the Port Everglades Inlet on an outgoing tide. Highest concentrations are noted in black (decreasing through red, orange and yellow). Surface concentrations are shown on the left; concentrations at depth are shown on the right.

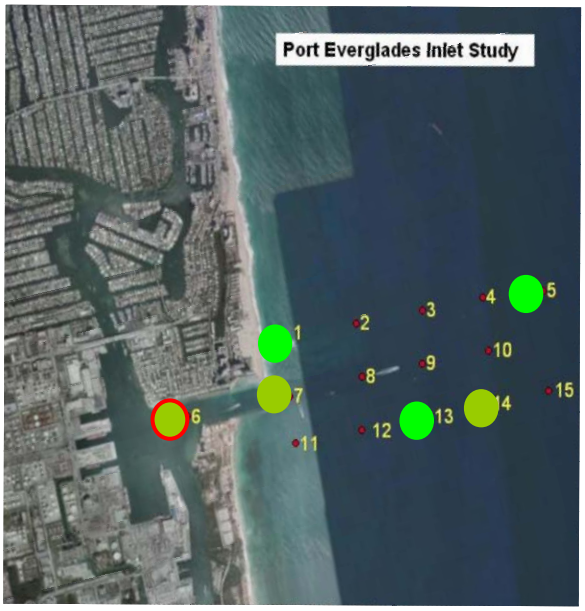


Figure 3. Prevalence of noroviruses (genogroups I or II) on an outgoing tide at Port Everglades Inlet. Bright green shows surface detection; lighter green shows depth. Station 6 in red circle shows simultaneous detection of both genogroups