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Evaluation of sewage source and fate on southeast Florida coastal reefs

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ABSTRACT

Water, sponge and coral samples were collected from stations impacted by a variety of pollution sources and screened for human enteric viruses as conservative markers for human sewage. While human enteroviruses and adenoviruses were not detected, noroviruses (NoV; human genogroups I and II) were detected in 31% of samples (especially in sponge tissue). Stations near inlets were the only ones to show multiple sample types positive for NoV. Fecal indicator bacteria and enteric viruses were further evaluated at multiple inlet stations on an outgoing tide. Greatest indicator concentrations and highest prevalence of viruses were found at the mouth of the inlet and offshore in the inlet plume. Results suggest that inlets moving large volumes of water into the coastal zone with tides may be an important source of fecal contaminants. Efforts to reduce run-off or unintended release of water into the Intracoastal Waterway may lower contaminants entering sensitive coastal areas.

1. Introduction

The reef tract off the coast of southeast Florida extends from the Florida Keys in the south through coastal Miami-Dade, Broward, and Palm Beach Counties to the north, with hard bottom communities extending through Martin Counties. Although less well known than the reefs of the Florida Keys National Marine Sanctuary, these more northern systems provide extensive reef habitat, including unique, sizeable stands of staghorn coral (*Acropora cervicornis*) (listed as threatened under the US Endangered Species Act; Hogarth, 2006). These reef communities are mainly comprised of sponges, sea fans, and a sparse cover of stony coral and provide vital economic resources, contributing approximately $1.2 billion in income each year (Johns et al., 2003). However, as with many coastal areas, population growth in this already urbanized 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 reef ecosystem integrity as well as public health.

Southeast Florida is highly urbanized, with a population of 1695 people km^{-2} in the coastal zone extending from Miami-Dade to northern Palm Beach County (urbanized area code, UA:56602; census.gov), making it the eighth most densely populated area in the U.S. (census.gov). Furthermore, the population is expected to at least double by 2020 to a total 15 million (Finkl and Charlier, 2003). Broward County, the focus area of this study, is the second largest county in the state and the 15th in the nation, with over 1.7 million people as of 2006. Such concentrated populations place increased burden on existing sewage treatment and disposal infrastructure, contributing to greater point sources of pollution. Increased population density also leads to greater levels of impervious surfaces, which, in turn, facilitate storm water runoff into local waterways.

Water quality along the reef tract of southeast Florida is likely to be affected by multiple sources of pollution, which can contribute opportunistic pathogens against corals (e.g., Sutherland et al., 2010) or viruses and other microbes that may harm humans (e.g., Futch et al., 2010). In the four-county area of Miami-Dade, Broward, Palm Beach and Martin counties, centralized sewers service 57% of the population while 43% rely on in-ground disposal of wastewater, which may or may not be treated (23% through septic system and 20% through injection wells). Secondarily treated wastewater in southeast Florida is discharged directly to the coastal environment through a series of six treated wastewater 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, 2006).

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

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Beach Counties account for ~77% of all injection well wastewater disposal for the entire state.

Storm-water from the region is channeled to regional streams and canals, which ultimately lead to the Intracoastal Waterway. There are approximately 4800 storm sewer outfalls in Broward County alone (Reich et al., 2008). Stormwater run-off 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 during outgoing tides.

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; 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 on reef health and provides information on possible public health risks in this coastal environment.

2. Materials and methods

2.1. Field study sampling

2.1.1. Offshore and reef survey

Surface water, sponge clippings, and coral surface mucopolysaccharide layers (SML) were collected from eight 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 (treated wastewater 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).

Surface water was collected as grab samples 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 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 h 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 °C within 2 h 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).

2.1.2. Inlet study

For inlet sampling, surface water and mid water column-depth (3–16 m) 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 sequentially at sample points along the grid shown in Fig. 1B, ending with station 15. Sampling was completed at all stations within 45 min. 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

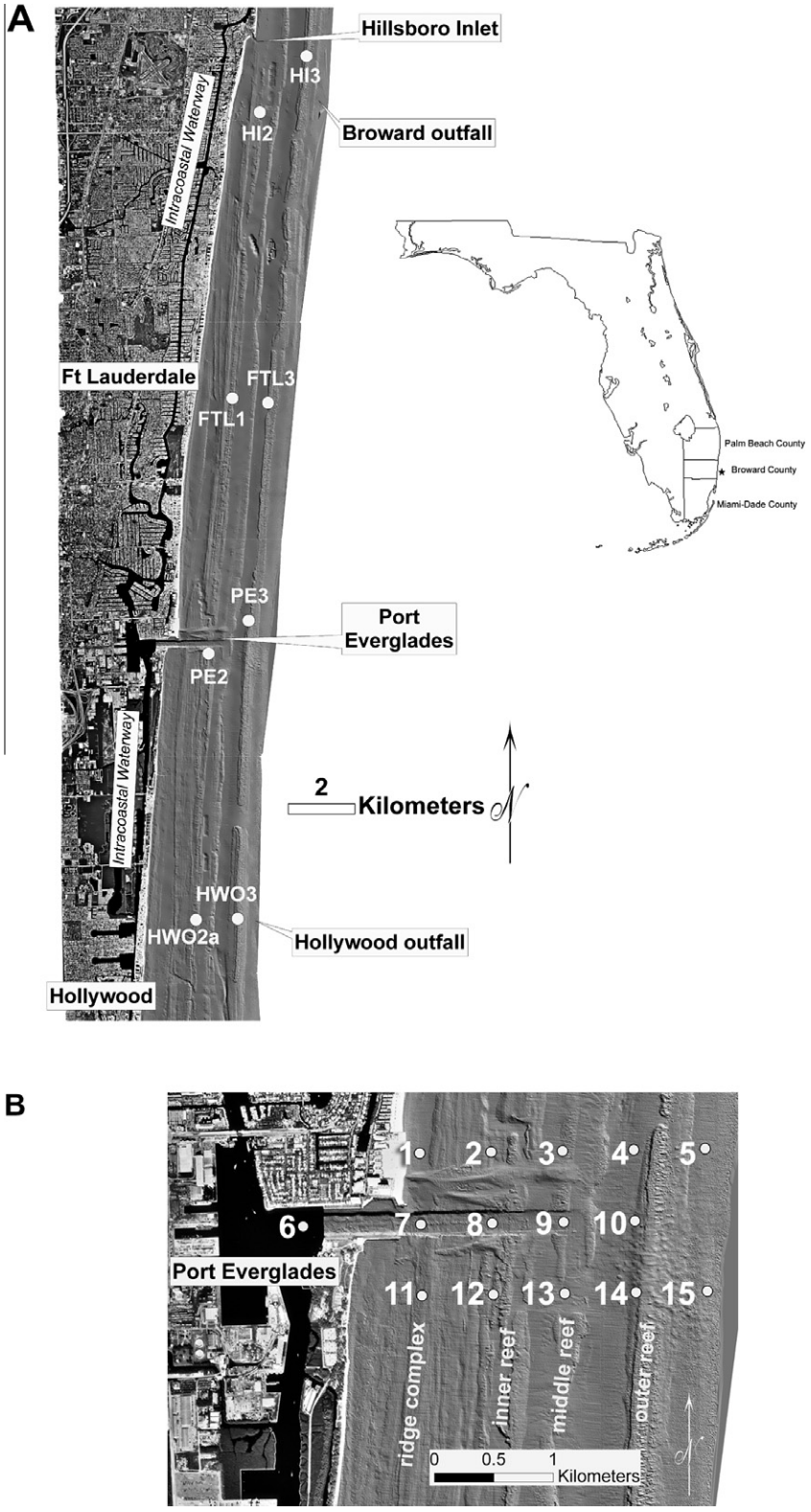

Fig. 1. (A) Broward County offshore sampling stations showing aerial view of coastal development and bathymetry of offshore area; the three parallel reef ridges are visible. Star on the inset map of Florida indicates location of Broward County, situated between Palm Beach County to the north and Miami-Dade County to the south. (B) Port Everglades Inlet sampling stations. Locations of the inner, middle and outer reefs of the reef ridge complex are indicated.

trends associated with land-based sources of pollution (i.e., FIB). To illustrate indicator densities, contour plots were hand drawn in PowerPoint using Google Earth images as a base map.

2.1.3. Environmental parameters

For all water samples, physico-chemical data were collected. Temperature, salinity and pH were measured using a

Table 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, 28, 37, 40, 41, and simian adenovirus 25	He and Jiang (2005)
AD3	GACTCYTCWGTSAGYGGCC		
ADP	**FAM**-AACCAGTCYTTGGTCA TGTTRCATTG-**BHQ**		
Enterovirus			
EV-U	GGCCCCTGAATGCGGCTAAT	192 base pair region of 5′ untranslated region (UTR)	Donaldson et al. (2002)
EV-D	CACCGGATGGCCAATCCAA		
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.

YSI-multi-parameter sonde (Yellow Spring, OH). Weather conditions and tide were also noted.

2.2. Sample processing

2.2.1. Fecal indicator bacteria

Indicator bacteria (fecal coliform bacteria, enterococci, and *C. 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).

2.2.2. 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 min. A sterile 15 ml tube containing a neutralization solution of 0.1 ml 50 mM H_2SO_4 and 0.1 ml of 100× 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. A known strain of human adenovirus type 2 (courteously provided by Dr. C.P. Gerba at the University of Arizona) was used as a 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) (Table 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 set described by Donaldson et al. (2002) targets a 192 base pair region of the 5′ untranscribed region (UTR) of the enteroviral genome. Poliovirus vaccine strain Lsc1 (courteously provided by Dr. C.P. Gerba, University of Arizona) was used as a positive control in all reactions.

RNA from human norovirus (NoV) genogroups I and II 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 120 nM. Two primer and probe sets were used for each genogroup as described by Gentry et al. (2009) (Table 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. RNA transcripts for NoV genogroups I and II (courteously provided by Dr. J. Vinjé (Centers for Disease Control and Prevention) and originally described in Gentry et al. (2009)) were used as positive controls in all reactions. When calculating percent positive for noroviruses in this study, a sample was considered 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.

3. Results

3.1. Offshore and reef study

In all, 80 samples were collected between 2007 and 2008, including 16 water, 48 sponge and 16 pooled coral SML samples from the eight stations. As both sampling events took place in late July, environmental parameters varied little between the two years with temperatures averaging 29.2 (ranging from 28.7 to 29.9 °C), 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 NoV were detected in 25/80 samples (31.0% among all sample types).

NoV genogroup I (GI) was detected in 11/80 samples (13.8%), while genogroup II (GII) was detected in13/80 samples (16.3%). 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 GI and from 15.0% of samples (12/80) for GII. Those described by Kageyama et al. (2003) yielded positive results in only 2.5% (2/80) samples for GI and 1.3% (1/80) of samples for GII.

3.1.1. Water

NoV prevalence in the surface water was 12.5% (2/16 samples). Genogroups I and II were each detected once in separate samples (both in 2007); GI was detected at station HWO2 and GII was detected at station PE3.

3.1.2. 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 genogroups I and II, simultaneously.

3.1.3. Sponges

NoV was detected in 19 of 48 sponges (40.0%). GI was detected in 20.8% of samples (10/48) and GII 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.

3.1.4. Location and putative pollution sources

NoV detection rates varied among the four sampling sites (and putative pollution sources; Table 2). Stations located near the Port Everglades Inlet (PE2 and PE3) were most often positive for NoV (35.0%; 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. GI was detected in 3 samples (20.0%) and GII was detected in five samples (25.0%). Two samples (1 sponge [PE3] and 1 coral SML [PE2]) were concurrently positive for both genogroups (Table 2).

Samples off of Ft. Lauderdale Beach, with no presumed direct impact from outfalls or inlets (FTL1 and 2), were positive for NoV 30.0% of the time (6/20 samples, all types combined); however, noroviruses were only detected in sponges (Table 2). Among all samples, 10.0% (2/20) of samples were positive for GI and 20.0% (4/20) of samples were positive for GII. No samples were concurrently positive for both genogroups.

Stations at the HWO sites (HWO2 and HWO3), presumably influenced by the Hollywood Outfall were positive for NoV 25.0% of the time (5/20) among all samples. NoV were detected from water and sponge, but not from coral samples (Table 2). GI was detected in 15.0% of samples (3/20) and GII was found in 10.0% of samples (2/20). No samples were concurrently positive for both genogroups.

At the Hillsboro Inlet site (stations HI2 and HI3), 20.0% of all samples (4/20) were positive for NoV. Sponges were the only sample type positive for NoV. Three of the 20 samples (15.0%) were positive for GI and 2 of 20 (10.0%) were positive for GII. One sample (HI2 sponge in 2008) was simultaneously positive for both genogroups (Table 2).

3.1.5. 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. *C. 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).

Table 2
Summary of norovirus detection among offshore reef sites including all sample types (for each site $N = 20$). Sites are listed from north to south. HI: Hillsboro Inlet; FTL: Ft. Lauderdale Beach; PE: Port Everglades; and HWO: Hollywood Outfall.

Site	Source[a]	# Positive (%) either genogroup	# Positive (%) genogroup I	# Positive (%) genogroup II	# Positive (%) both genogroups	Sample type positive
HI	Inlet and outfall	4 (20.0%)	3 (15.0%)	2 (10.0%)	1 (5.0%)	Sponge
FTL	Non-point source	6 (30.0%)	2 (10.0%)	4 (20.0%)	0 (0.0%)	Sponge
PE	Inlet	7 (35.0%)	3 (15.0%)	5 (25.0%)	2 (10.0%)	Sponge coral water
HWO	Inlet	5 (25.0%)	3 (15.0%)	2 (10.0%)	0 (0.0%)	Sponge coral

[a] Putative source of fecal pollution.

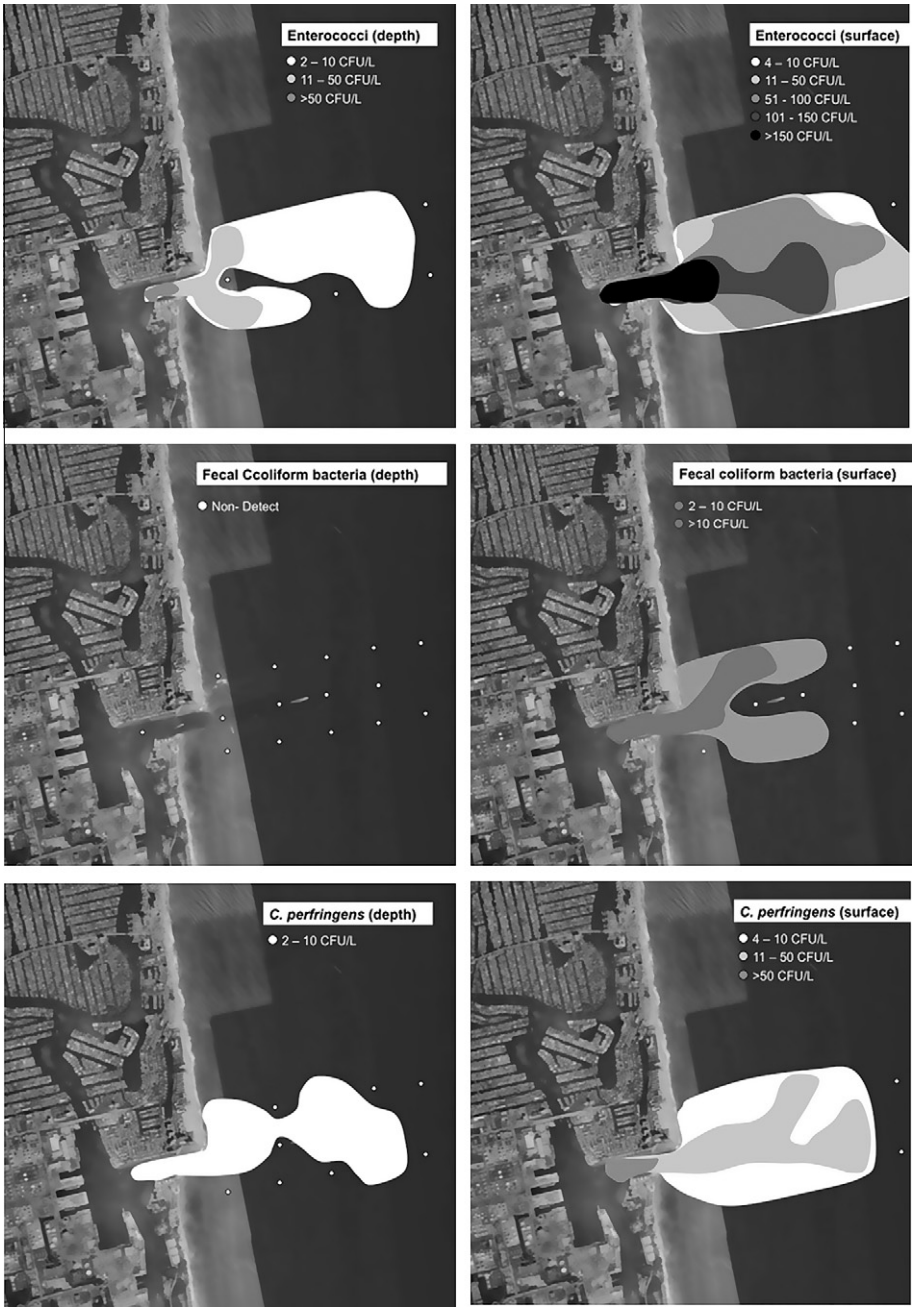

Fig. 2. Contour plots of fecal indicator concentrations in the Port Everglades Inlet, Broward County during an outgoing tide, July 2007. Samples were collected at the surface (left column) and at depth (right column) from a 15 point grid. Concentrations were plotted as contours to show the movement of the plume out of the inlet.

Neither human adenovirus nor human enterovirus were detected during the inlet study (outgoing tide); however, human norovirus was detected in 16.7% of all samples (5/30). Overall, 20.0% 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, GII was more prevalent with

13.3% (4/30) of samples positive (depth stations 6 and 14 and surface stations 1 and 13). Genogroup I was only detected once (3.3%) at surface station 5.

3.2. Treated wastewater 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 NoV GI.

4. 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 noroviruses, 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 (Noble and Fuhrman, 2001; Griffin et al., 2003; Rosario et al., 2009; Futch et al., 2010). Traditional fecal coliform assessments of water quality may be 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., Griffin et al., 1999; Lipp et al., 2001; Futch et al., 2010). In these earlier 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 (RT) PCR approach used here, or may simply reflect slight differences in amplification between gene target region(s). Among the NoV detected in this study, GI and GII were found at similar frequencies with ~14% and 16% of samples from reef stations positive, respectively. However, within the samples collected on the outgoing tide at the Port Everglades Inlet, GII was much more prevalent than GI, which was only detected once. While both genogroups are associated with human disease, GII is more commonly detected in outbreak investigations and is generally considered to cause the greatest burden of disease (Arias et al., 2010). 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.

Noroviruses reached their highest prevalence at reef stations near the Port Everglades Inlet (35.0% samples positive) followed by stations offshore of Ft. Lauderdale beach with no apparent direct point source inputs (30.0% 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, 10.0% of samples were simultaneously positive for both genogroups. The co-occurrence of more than one genogroup provides a more robust indication of human sewage pollution; additionally, given the suspected rates of inactivation between different source materials (Futch, unpublished data), the co-occurrence among all samples types suggests that these enteric viruses were introduced frequently. The only other site in which both genogroups co-occurred was near the Hillsborough Inlet (also near the Broward Outfall); however, only sponges were positive. At the Hollywood Outfall site, no genogroups co-occurred but both sponges 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 (Donaldson et al., 2002); in the case of Ft. Lauderdale Beach, these sponges may also be accumulating viruses from 'downstream' pollution sources, including inlets and ocean outfalls 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 showing a higher potential sewage impact (i.e., higher viral detection rate) at Port Everglades Inlet is similar to a study at nearby Boynton Inlet in 2007 (Carsey et al., 2007), suggesting that the inlets may be a significant source of contamination to the coastal zone even compared to outfalls. Interestingly, Station HI (which presumably is influenced by both the Hillsboro Inlet and Broward Outfall) had a lower virus prevalence than those stations influenced by Port Everglades Inlet, alone and showed a similar prevalence to those stations influenced only by an outfall (Hollywood Outfall). This is likely attributable to the relative sizes and activity between the Hillsboro and Port Everglades Inlets. While discharge is similar between the two ocean outfalls (Koopman et al., 2006), the volume of water passing through the Port Everglades Inlet is significantly greater than that of the Hillsboro Inlet. The Hillsboro Inlet is 473 m long and 141 m wide at the throat with an average depth of only ~2 m (Stauble, 1993; Butler and McAllister, 2000). The Port Everglades Inlet is one of the largest commercial ports on the east coast of Florida and one of the busiest cruise ports worldwide (e.g., NOS, 2010). The inlet entrance measures over 641 m long and 295 m wide at the throat and has an average depth of 13 m (Stauble, 1993; NOS, 2010). Large 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 and discharge large volumes of water twice daily on a semidiurnal tidal cycle. 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 (Carsey et al., 2007) data showing that inlets are a major source of microbial and nutrient inputs. In the 2007 study, over 50.0% of samples were positive for human viruses on an outgoing tide (Carsey et al., 2007).

Non-point pollution sources likely contribute not only to contaminants carried through the inlets along the coast of southeast Florida but may also reach coastal areas directly through surface run off and submarine groundwater discharge. Using an average annual rainfall of 1.46 m $year^{-1}$ in southeast Florida the calculated average runoff rate estimate is $\sim 1.65 \times 10^{12}$ L $year^{-1}$ (Sherwood et al., 1973). 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 7.80×10^{10} L $year^{-1}$ 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 (1.81×10^{10} L $year^{-1}$ which is ~18.9% of the yearly

county-wide sewage flow) it can be estimated that $\sim 4.88 \times 10^8$ L year^{-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 4.54×10^9 L year^{-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), which would also be transported offshore through inlets during ebb tides. This type of non-point source pollution may also help to explain the consistently high levels of norovirus across all sites sampled (20.0–35.0% positive).

Among the six ocean outfalls along the coast of southeast Florida discharging secondarily to advanced treated wastewater, the two located in Broward County were each sampled once during this study. The Broward County outfall discharges on average $\sim 1.38 \times 10^8$ L d^{-1} and is located approximately 2.12 km offshore at a depth of 29 m (Marella, 1999; Koopman et al., 2006). The City of Hollywood outfall discharges on average $\sim 1.50 \times 10^8$ L d^{-1} and is located 3.06 km offshore at a depth of 28.5 m (Marella, 1999; Koopman et al., 2006). These outfalls may contribute to loading of viruses that survive secondary treatment of wastewater, including noroviruses and other viruses found in human feces (Rosario et al., 2009).

Marine sponges were the most common sample type found to harbor human NoV. The high frequency of detection in sponges noted in this study is consistent with reports of Donaldson et al. (2002), suggesting the active filtration and concentration of water may make sponges an excellent *in situ* biosensor or sentinel for sewage contamination in reef habitats. While human viruses do not directly infect these marine organisms, they do provide signal of sewage contamination, which may contain nutrients, toxins or opportunistic pathogens that contribute to coral stress and/or disease (e.g., Sutherland et al., 2011). Work in the Florida Keys, has shown that coral SML is also an effective area for concentration of viruses (Lipp et al., 2002, 2007; Lipp and Griffin, 2004; 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. Given the relatively transient nature of coral mucus secretion and sloughing, viral detection in this matrix may serve as an indicator of a recent sewage exposure while sponges may more provide a longer record of exposure through effective and persistent accumulation of these viruses in their tissues.

While descriptive trends suggest a role for contamination *via* inlets, the overall prevalence of enteric viruses in this study was remarkably similar across all sites and potential sources, suggesting that contamination may be widespread, or that contaminants may be quickly transported and mixed, along the coast of this densely populated area. Additional research is needed to fully account for all pollution sources offshore; however, this research provides an estimate of the potential microbial contaminant introduction from major inlet and outfall sources. These results suggest that large inlets and associated movement of large volumes of water into the coastal zone should be considered an important source of fecal contaminants. Efforts to reduce run-off or other unintended release of water into the Intracoastal Waterway may lower contaminants entering sensitive coastal areas.

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