VIRAL PATHOGENS AND MICROBIAL INDICATORS IN THE CANALS OF THE FLORIDA KEYS

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ABSTRACT

In order to access the microbial water quality in canal waters throughout the Florida Keys, a survey was conducted to determine the concentration of microbial fecal indicators and presence of human pathogenic microorganisms. Nineteen sites, including 17 canal sites and two nearshore water sites, were assayed for total coliforms, fecal coliforms, *Escherichia coli, Clostridium perfringens*, enterococci, coliphages, F^+ specific RNA coliphages, *Giardia lamblia*, *Cryptosporidium parvum*, and human enteric viruses (Polio, Coxsackie A, Coxsackie B, echoviruses, Hepatitis A viruses, Norwalk viruses and Small Round-Structured Viruses). Coliforms ranged from <1 to 1410, *E.coli* <1 to 130, *Clostridium spp.* <1 to 520 and enterococci from <1 to 800 colony forming units/100ml of sample. Two sites were positive for coliphage but no F+ specific phage were identified. The sites were ranked according to microbial water quality and compared to various water quality standards and guidelines. Seventy-nine percent of the sites were positive for the presence of enteroviruses by reverse transcriptase-polymerase chain reaction (Polio, Coxsackie A, Coxsackie B and echoviruses). Sixty-three percent of the sites were positive for the presence of Hepatitis A viruses. Ten percent of the sites were positive for the presence of Norwalk viruses. One hundred percent of the sites were positive for at least one of the virus groups. These results indicate the canals and nearshore waters throughout the Florida Keys are being impacted by human fecal material carrying human enteric viruses through current wastewater treatment strategies such as septic tanks. Exposure to canal waters through recreation and work may be contributing to human health risks.

INTRODUCTION

The islands that form the Florida Keys are adjacent to the only living coral reef system within the territorial borders of the Continental United States. The island chain extending from Key Largo to Key West supports a significant population of human residents and vacationers and is well known as a premier recreational site. With the exception of a few of the smaller islands within this region, development of residential communities and vacation resorts has been continuous over the last twenty years. The community on Key West is the only population served by a full-scale sewage treatment plant and outfall disposal. Two other communities, Key Colony Beach on Marathon and Ocean Reef Resort on Key Largo are served by full-scale treatment plants and injection well disposal. All other island communities rely on septic tanks, cesspools and package treatment plants combined with injection wells for disposal of sewage. Currently there are approximately 30,000 septic tanks and 600 injection wells utilized in the Keys (1,2). The depths of the injection wells varies from ~1 to 30 meters with current regulations requiring a drill depth of 27.4 meters with casing to 18.3 meters (2). Due to the porous nature of limestone (which makes up the strata of the islands) coupled with natural physical dynamics of the region such as flushing from precipitation and tidal pumping (2), these types of sewage disposal practices may be inadequate for protection of water quality from microbial pollutants. Several studies conducted in the Florida Keys have demonstrated movement of contaminants from septic tanks and injection wells to the surrounding marine environment. One study conducted in the lower Keys demonstrated elevated nutrients in areas where septic tanks were being utilized in comparison to levels in areas outside the influence of septic tanks (1). Studies in the upper Keys demonstrated the presence of fecal indicator bacteria in surface waters and the subsurface aquifer, with levels of microbes decreasing with distance from areas under the influence of septic tanks (3). In the same area viral tracers moved within 11 hours from a seeded septic tank out into the surrounding environment (4). Similar tracer studies conducted in the middle Keys demonstrated an 8 hour migration of viral tracers from a seeded injection well into the surrounding groundwater and a 53 hour migration to surface waters located on the opposite side of the island (5).

One of the concerns of recreational water use is the risk of illness resulting from exposure to waters contaminated by human sewage. Risk to human health associated with contaminated marine water has been well documented. In a study conducted along beaches in Hong Kong, swimmers were at higher risk of illness than non-swimmers, and there was a greater risk at beaches effected by pollution than beaches considered

unpolluted (6). Children swimming in contaminated seawater are more likely to develop symptoms illnesses than those who do not(7). A dose response relationship between the level of indicator organisms in recreational marine waters and risk of illness has been reported (8).

The United States Environmental Protection Agency (USEPA) has suggested an ambient water quality goal for *E.coli* of 126 colony forming units (CFU)/100ml for freshwater environments. The USEPA guidance level for marine waters is a geometric mean of 35 enterococci CFU/100ml for five samples spaced over a 30-day period. (9)). The single sample guidance level of 107 enterococci CFU/100ml of sample has been suggested. Fujioka et al. (10) has suggested that *Clostridium spp.* (< 50 CFU/100ml) is a better indicator for tropical waters. The State of Florida Department of Health (DOH) ambient water quality standards for total coliforms are a geometric mean of 1000 CFU/100ml, a maximum single sample level of 2400 CFU/100ml, and < 1000 CFU/100ml for 80% of samples. The DOH ambient water quality standards for fecal coliforms are a geometric mean of 200 CFU/100ml, a maximum single sample level of 800 CFU/100ml, and < 400 CFU/100ml for 90% of samples.

Total and fecal coliform bacterial indicators often do not indicate the persistence of pathogens, especially viruses in surface waters. Human viral and protozoa pathogens are more persistent in the waters than coliform bacteria, and are not removed as efficiently by treatment processes such as chlorination (12). In addition total and fecal coliforms can readily be isolated in tropical waters from areas far removed from human activity, and thus are not adequate indicators of fecal contamination and human health risks (13). Simultaneous monitoring of water samples for alternate indicators, F^+ specific RNA coliphage, *enterococci, Clostridium perfringens* as well as direct pathogen monitoring for enteroviruses and enteric protozoa (*Cryptosporidium* and *Giardia* spp.) enables better assessment of fecal contamination and public health risks. Protocols are now available for detection of F^+ specific RNA coliphage (14) and enteroviruses, enabling the determination if the fecal source is human or animal. A number of researchers have used enteroviruses to access water quality(15,16,17,18,19,20).

In order to make improvements in the wastewater management in the Florida Keys, a better understanding was needed of the sources of microbial contaminants (human versus animal), their transport, prevalence and fate into the marine environments and the resulting public health risks. This study was

specifically designed to assess microbial water quality in the canal systems of the residential communities of the Florida Keys.

Material and Methods

Sites

Nineteen sites from the northern portion of Key Largo (Ocean Reef Resort) to the Southern Most Point on Key West (Table 1, Figure 1) were sampled. Seventeen of the sites were residential canals identified by the USEPA as sites of suspected poor water quality. Two of the sites were nearshore water sites. Sites 1 through 8 were sampled between September 29, 1997 and October 4, 1997 and sites 9 through 19 were sampled between August 16, 1998 and August 22, 1998. Table 1 lists each sample site and its location.

Detection Protocols

Grab samples were collected using sterile 2L bottles, or concentrated by cartridge filtration (for large volume analysis of protozoa and viruses) and vortex flow filtration (collected with sterile 20L carboys) concentrates (21).

Total Coliforms. Volumes of 50.0ml, 5.0ml (grab samples) and 1.0ml (membrex retentate) of each water sample were filtered through membrane filters (0.45um, 47mm, Gelman Sciences). Each volume was assayed in duplicate. The filters were placed on mENDO medium and incubated for 24 hours at 37°C. The colonies that produced a metallic sheen were enumerated as total coliforms (Standard Methods for the Examination of Water and Wastewater 1998. (22)).

Fecal Coliforms and *Escherichia coli.* Water samples were filtered as described above. The filters were placed on M-FC medium and sealed in plastic bags within 30 min after filtration. The plates were incubated for 24 hours in a water bath at 44.5°C. The bacterial colonies with various shades of blue were counted as fecal coliform bacteria (22). The filters with blue colonies were then transferred to EC-MUG media and incubated at 35.5^{0}C for 24 hours. At 24 hours the filters were exposed to UV-light and fluorescing colonies (*E.coli*) were enumerated.

Enterococci. Water samples were filtered as described above. The filters were placed on MEI media and incubated at 41.0°C. After 24 hours incubation, enterococci showed pink or red colonies on the membrane filters. The colonies, which develop a black or reddish-brown precipitate on the underside of filter, were counted as enterococci (23).

Clostridium perfringens. Water samples were filtered as described above. The filters were placed on the M-CP plates and sealed with anaerobic gas paks (BBL GasPak, Becton Dickinson). After 24 hours incubation at 45.0°C, the yellow colonies were exposed to ammonium hydroxide fumes and the colonies that turned red or dark pink were enumerated as *C. perfringens* (24).

Protozoan Analysis (*Cryptosporidium spp.* and *Giardia spp.*). Samples were processed and assayed for enteric protozoa using filtration and immunofluorescence microscopy techniques (25). Between 50.0 and 220.0 L were collected from each site by filtration through filter cartridges. Volumes were monitored by attached flow meters. After collection, the filters were placed on ice for transport to the University of South Florida where they were processed by cutting the filter and washing the collected material from the filter to recover protozoan cysts and oocysts. The eluent was centrifuged to a concentrated pellet representing the initial volume of water collected. An aliquot of concentrated pellet was then clarified using percoll/sucrose gradient centrifugation. The final concentrates were examined using an indirect antibody FITC (fluoro-iso-thio-cynate)/epifluorescence assay. Equivalent concentrations of cysts and oocysts per 100 L were then calculated.

PCR Detection of Enteroviruses. Approximately 110 L of water was filtered at each site using Filterite filters (DFN 0.45-10UN. Filterite/MEMTEC A. Corp., Timonium, MD) as outlined in Standard Methods for the Examination of Water and Wastewater 1998 (22). Viruses were eluted with beef extract (pH 9.5) and concentrated using organic flocculation. The concentrates were stored at -20°C until analysis. One hundred ul of each sample was purified and concentrated to 60.0ul using spun-column chromatography (Rneasy Mini Kit, QIAGEN, Santa Clarita, CA). Ten ul of this sample was then utilized for RT-PCR for each group of viruses assayed. The primer sets and biotinylated-oligonucleotide probes (Table 2) used for viral detection, included a set for the detection of 25 different enteroviruses, a set for detection of Hepatitis A viruses, a set for the detection of Norwalk viruses (26) and a set for the detection of Small Round-Structured viruses (27). RT-PCR profiles and master mixes were used as published per respective primer groups/sets (separate profiles and master mixes for the Schwab et al. assay (26) versus the Ando et al. assay (27)). Detection of PCR product included gel electrophoresis (gels are stained with 0.5ug/ml ethidium bromide and visualized using UV-light) and chemiluminescent dot blot (Southern-Star, Chemiluminescent Detection System for Biotin-Labeled Probes, Version A.2, Tropix inc., Bedford, MA).

Nonspecific coliphage Assay. Aliquots (1.0ml) of grab samples and sample concentrates were assayed with a lawn of *E. coli* ATCC 15597 using standard overlay technique. The plates were allowed to solidify and then incubated at 35.0°C for 24 hours. Plaques were then enumerated as total coliphage.

Genotyping F^+ RNA Coliphages using Nucleic Probes (modified assay from Hsu et al. (14)). Aliquots (1.0ml) of grab samples and sample concentrates were assayed with a lawn of *E. coli Famp* using standard overlay technique. After overnight incubation at 35.0°C plaques were picked with a Pasteur pipette and suspended in 1.0ml of 0.5M TRIS, pH~8.0. Plaques were then verified by spotting 10.0ul of the TRIS/plaque suspension onto a fresh lawn of *E.coli Famp* using standard media and 10.0ul onto a lawn of *E.coli Famp* using media containing RNase (to control for DNA coliphage). Isolates that resulted in plaques on the standard media plate and didn't produce plaques on the plates containing RNase were picked again using a Pasteur pipette. A total of ten agar plugs were picked from the cleared zone and suspended in a buffer containing 537.2ul of 20X SSC (1 L of 20X SSC = 175.3g NaCL, 88.2g NaCitrate, pH 7.0), 495.6ul of 37% W/W Formaldehyde (Fisher Scientific F79-500) and 400.0ul of filter sterilized water which had been irradiated with UV-light. These suspensions were then incubated at 65.0°C for 30.0minutes. Aliquots of 335.0ul (X4, one for each virus probe group) were then applied to 0.45um nylon filter paper using a dot blot apparatus. Viral RNA was fixed to the filters using UV-light and hybridized with the appropriate probe. The probes (Table 3) for detection of group II and group III F^+ RNA coliphages (found predominately in human feces) and for the detection of group I and group IV F^+ RNA coliphages (found predominately in animal feces) were employed for hybridization and detection. As with the enterovirus protocol, chemiluminescence was used for target/probe detection.

Results

Indicator Water Quality. Six indicators (bacteria and phage) of fecal pollution were assessed at each site (Table 4). Total coliform levels averaged 211.9 CFU/100ml (geometric mean value was 53.8 CFU/100ml). None of the sites were in violation of the State of Florida ambient water quality standard. Fecal coliform levels averaged 124.4 CFU/100ml (geometric mean 24.1 CFU/100ml) and no single site was in violation of the State of Florida ambient water quality standard. *E.coli* concentrations averaged 45.7 CFU/100ml and the geometric mean was 13.0 CFU/100ml. *Clostridium perfringen* levels averaged 31.3 CFU/100ml and the geometric mean value was 2.9 CFU/100ml. A *Clostridium perfringen* level of 520 CFU/100ml was detected at site 19 and was the only site above the Hawaiian guidance level of <50 CFU/100ml.

Enterococci levels averaged 119.8 CFU/100ml and the geometric mean value was 23 CFU/100ml. Three sites (site 3 - 800 CFU/100ml, site 11 - 240 CFU/100ml, and site 19 - 680 CFU/100ml) had levels above a single sample guidance level of 107 CFU/100ml. Nonspecific coliphage were detected in low levels at sites 1 and 4 (10 pfu/100ml in both cases). F^+ specific RNA coliphage were not detected at any of the other sites.

Table 5 lists the sites in order from the North end of Key Largo to the Southern Most Point on Key West. The indicator (bacteria and phage) concentrations were given rankings of 1 (the lowest levels of bacteria and coliphage within each group) to 19 (the highest levels of bacteria and coliphage within each group) in order to compare the sites to each other. After each category was ranked a total score was calculated and an overall ranking assigned per site. Figure 2 illustrates the site locations and their respective site rankings (represented by columns). Site 1, which was located within the Ocean Reef Resort (Key Largo) had the lowest ranking (best microbial water quality). Site 16, which was located on Lower Matecumbe had the highest ranking (worst microbial water quality). Site 19, which was located at the Southern Most Point on Key West, had the second highest rating (rank 18).

Protozoa. No protozoa were detected in any of the samples. The assays detection limits ranged from <2 cysts or oocysts/100L to <23 cysts or oocysts/100L.

Human enteric viruses. Table 6 lists the sites from north to south and includes the prokaryote and coliphage site rankings in addition to the RT-PCR human virus data. The RT-PCR virus data was reported as presence absence. All viral data was confirmed by dot blot. Figure 3 is a photograph of the panenteroviral dot blot. Seventy-nine percent of the sites were positive when assayed with the panenterovirus primer set. Sixty-three percent of the sites were positive for Hepatitis A viruses. Ten percent of the sites were positive for Norwalk viruses. No site was positive for Small Round-structured viruses.

Discussion

Previous research has demonstrated that coliforms are not good indicators in tropical waters (13, 28,29). We have identified numerous *E.coli* isolates in the Marquesas Islands, which is a chain/ring of islands approximately 25 miles due west of Key West (where there are no human residences and minimal human activity). It is believed that these isolates are the result of animal/bird presence and that once deposited in these types of warm shallow marine environments are able to multiply. Research conducted in our laboratory has suggested that Florida waters should be considered tropical waters and coliforms are not adequate predictors of

fecal contamination and public health risks. Others have suggested that alternate indicators are more reflective of pollution for example, the USEPA has promoted enterococci in marine waters as a better indicator of health risks. Cabelli (30) showed that levels above 35 CFU/100ml were associated with increased risk of illness. Average (23 CFU/100ml) in this study was just below that level. Three sites (site 3, site 11 and site 19) with the highest single sample levels were within the highest ranked sites for fecal pollution.

Table 7 lists the sample site/canal descriptions (ages and number of homes on the each canal screened. Sites 1 through 17). Sites 18 and 19, which were both nearshore sites located on Key West were not included in Table 7. Canal type (flow through, multi-canal network, etc.), and age and number of homes on canals within each type, appeared to influence prokaryote/indicator prevalence. The general trend was canals that could flush easily such as the flow through canals and the short single dead-end canals had the lower rankings. Exceptions were generally related to the age of homes. Canals that were part of a multi-canal network generally had the higher rankings. There were four types of canals; 1) flow through canals, 2) long and short single dead-end canals, 3) feeder/side canals in a multi-canal network, and 4) main canals in a multi-canal network. The following sites are described in order from north (Key Largo) to southwest (Key West). The cleanest site (site 16, rank 1) was taken from a flow through canal within the Ocean Reef Resort on the north end of Key Largo. This site differs from the other canal sites in that the homes are connected to sewage treatment lines and thus do not utilize septic tanks for sewage disposal. Sites 6 and 5 were located on Key Largo and both were located in single dead-end canals (ranks of 17 and 8 respectively). The sites had 17 and 22 homes per canal (sites 6 and 5, respectively) with an average age of 23 years. The difference between these sites is that site 5 was a short canal (greater tidal flushing). Site 7 was located in Rock Harbor, in a long single dead-end canal (rank 12). This canal had 15 homes on it with an average age of 30 years. Site 8 was located in Buttonwood Bay, in a flow through canal (rank 6.5). This site had 260 condo units around it. Site 4 was located on Plantation Key, in a long single dead-end canal (rank 5). The canal had 24 homes on it with an average age of 18 years. Site 3 was located on Lower Matecumbe, in the main canal of a multi-canal network and had the highest ranking of all sites (rank 19). The sample site was located at the end of the main canal, which had 27 homes on it with an average age of 15 years. Site 2 was located on Long Key, in a canal (feeder canal) which was attached to a main canal (rank 9). The canal had 7 homes on it with an average age of 17 years. Site 1 was located on Conch Key, in a short single dead-end canal. This site had the highest ranking (rank 14) of all short dead-end canals and was unique in that it

had the homes (15 homes) with the oldest average age (46years). Sites 9, 10, 11, and 15 were located on Big Pine Key (rank 11, 10, 16, and 13 respectively). Site 9 was located in a long single dead-end canal and had 8 homes on it with an average age of 26 years. Site 10 was located in a feeder canal and had 15 homes on it with an average age of 11 years. Site 11 was located in the main canal of a multi-canal network and had 16 homes on it with an average age of 14 years. Site 15 was located in a feeder canal and had 3 homes on it with an average age of 6 years. Site 13 was located on Cudjoe Key, in the main canal of a multi-canal network (rank 3). This site had the lowest ranking of all the multi-canal network sites and was unique in that it had the homes (10 homes) with the lowest average age (5 years), of all sites. Site 17 was located on Sugarloaf Key in a short single dead-end canal (rank 2). This canal had 9 homes on it with an average age of 16 years. Site 14 was located on Saddlebunch Key, in a flow through canal (rank 6.5). This site had 58 homes on it with an average age of 26 years. Site 12 was located on Boca Chica Key, in a short feeder canal (rank 4). This site had 27 homes on it with an average age of 22 years.

The second highest ranked site (rank 18) was located off of the Southern Most Point on Key West. The island of Key West utilizes full-scale sewage treatment. However, sewer lines in this section of the city are in need of replacement as the city experiences a salt-water intrusion level of 65% at this site (65% of the wastewater coming from this section of the city is marine in origin). Site 18, which was taken near Houseboat Row on Key West had a rank of 15. Numerous live-aboard houseboats were (many were destroyed in a Hurricane since this site was sampled) located at this site and the houseboats were hooked up to the City of Key West's sewer system.

The low numbers of coliphage isolated in this study may indicate rapid die-off of phage, which may have been a result of salinity and the high water temperatures noted during the sampling dates. Salinity values ranged from 22 - 37 ppt (average 27ppt) during the first sampling dates and 32 – 36ppt (average 34ppt) during the second sampling dates. Water temperature averaged 29.0^{0}C and 33.0^{0}C respectively. The only phage isolation occurred during the first sample date when both the salinity and water temperature were lower.

The numbers of sites positive for enteroviruses (79%. panenterovirus primer set), Hepatitis A viruses (63% of sites positive) and Norwalk viruses (10% positive) suggest that wastewater is impacting the canals and nearshore waters of the Florida Keys. It should be noted that there was a marked difference in RT-PCR positive control signal (amplicon) between the four primer sets used. The panenterovirus and the HAV primer sets

produced positive control signal, which could be detected by gel electrophoresis The Norwalk and SRSV primer sets required dot blot analysis with overnight x-ray film exposure to detect positive control signal. Published sensitivity of the primer sets used in this study demonstrated that the detection limits vary. The panenterovirus and HAV primer sets sensitivity varied from 10^3 to 0.01 poliovirus/HAV PFU (using various virus recovery techniques. (31, 32)). The Norwalk primer required at least 10^5 amplifiable units (~ number of virons) before amplicon was detected (17). The inefficiency of the SRSV primer sets targeting short regions of polymerase gene has also been noted (33). A new RT-PCR protocol which was published after the start of this study has demonstrated a detection limit of <21.0 SRSV's using nested RT-PCR (34). The authors of that study demonstrated detection of SRSV's in samples (contaminated shellfish) previously determined to be negative by single round RT-PCR. It is also interesting that preliminary recovery assays conducted in our laboratory resulted in enhanced RT-PCR detection of Norwalk and SRSV seeded samples when utilizing magnetic poly-T capture of viral RNA, in comparison to the assay utilized in this study (data not shown).

The panenterovirus RT-PCR data in this study mirrors the results obtained from a similiar study conducted in Sarasota County Florida. The water quality study conducted in Sarasota county (11) on the impact of septic tank effluents on microbial water quality reported 12 of 15 samples in violation of Florida State Standards for Safe Swimming (200 fecal coliforms/100mL) with averages of 152 to 2,780 fecal coliforms/100mL. *Enteroccoci, Clostridium* spp. and coliphage were also found in concentrations indicating significant fecal contamination. Enteroviruses were detected by cell culture in 88% of the samples and by reverse transcriptase-polymerase chain reaction (RT-PCR) in 91% of the samples. In the current study an additional three more viral RT-PCR primer sets/assays were utilized and 100% of the sites were positive for at least one of the groups of viruses. Site 3, which was ranked highest for indicator prevalence, was positive for enteroviruses, HAV and Norwalk viruses. RT-PCR detection of these virus groups does not address the question of viability. Salinity and water temperature as the phage data suggest may significantly impact viral viability. Research has demonstrated that both HAV and Polio I viruses can survive for a period of time in marine/estuarine environments (a 3 log reduction of seeded viruses over 5 days as determined by cell culture. (35)). In contrast to the Sarasota County study (which had lower salinity and water temperatures for both fresh and tidally influenced samples), where a high percent of the samples positive by

RT-PCR were also positive by cell culture, many of the RT-PCR positives in this study may represent inactivated viruses.

The waste disposal studies conducted in the Florida Keys to date have directly demonstrated microbial and nutrient loading in the nearshore water-column throughout the region. Future studies will be conducted on water quality in the Florida Keys. These will include viability assays (cell culture) for those viruses where cell lines exist. Given the high prevalence of these viruses and the high numbers of alternate indicators such as enterococci and *Clostridium* spp. detected in a number of the sites assayed, the data indicates that these waters may present a risk to human heath in regard to recreational water use.

Acknowledgements - This work was funded by a grant from the United States Environmental Protection Agency, Region 4 (#984295-97-0). We would like to thank the following individuals for their assistance on this project: 1) Gus Rios of the Florida Department of Environmental Protection, Marathon, FL., for providing assistance in sample site selection. 2) David Coleman of Lindahl Browning Ferrari and Hellstrom, Inc., Consulting Engineers. Palm City, Florida, for the GIS data. 3) Robert C. Hudson and Doug Wilder of the Florida Department of Environmental Protection, St. Petersburg, FL., for assistance in analyzing the GIS data.

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Table 1. Sample Sites

Sample identification	Site location	Mile marker
EPA-Keys 1	Conch Key, Seaview Av., canal, Gulf side @ bridge.	63
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EPA-Keys 4	Plantation Key, Venetian Shores, Palo-De Oro Dr., canal @ empty lot.	86
EPA-Keys 5	Key Largo, Tropical Ln., mobile home park off a boat ramp, inner most part of the canal	103
EPA-Keys 6	Key Largo, Sexton Cove Estates, corner of Sexton Cove Rd. and Grassy Rd., off end of canal/mobile home park	106
EPA-Keys 7	Rock Harbor, Jolly Roger Dr., Buccaneer point, private boat ramp	99
EPA-Keys 8	Buttonwood Bay, bayside boat ramp just past mm97	97
EPA-Keys 9	Big Pine Key, Whispering Pines subdivision, at the very end of the canal off of Gordon Dr.	30
EPA-Keys 10	Big Pine Key, Doctors Arm subdivision, first canal off of W. Ortega Ln., corner lot.	30
EPA-Keys 11	Big Pine Key, Eden Pines Colony, canal off of W. Shore Dr., corner lot.	30
EPA-Keys 12	Boca Chica Key, Boca Chica Ocean Shores, at the end of the canal off of Scopio Ln.	10
EPA-Keys 13	Cudjoe Key, Cudjoe Gardens, canal behind the Sheriffs Station	21
EPA-Keys 14	Saddlebunch Keys, Bay Point Subdivision, E. Circle Dr. first canal on the right.	15
EPA- Keys 15	Big Pine Key, Port Pine Heights, the canal at Kyle Blvd. and Driftwood St.	30
EPA-Keys 16	Key Largo, Ocean Reef Resort, the canal behind the Chapel, near the bridge.	N/A
EPA-Keys 17	Sugarloaf Key, Sugarloaf Shores, the canal at W. Bonita Ln. and Sugarloaf Blvd.	17
EPA-Keys 18	Key West, Houseboat Row	0
EPA-Keys 19	Key West, near the Southern Most Point, at the seawall located on the end of Simonton St.	0

Table 2. Human virus primer set(s) and probe sequences

Virus	*Primer and probe sequences*	*Amplicon and target*
Panenterovirus	Upstream = 5'-CCTCCGGCCCTGAATG-3' Downstream = 5'-ACCGGATGGCCAATC-3' Probe = 5'-TACTTTGGGTGTCCGTGTTTC-3'	197bp-highly conserved 5' untranslated region
HAV	Upstream = 5'-CAGCACATCAGAAAGGTGAG-3' Downstream = 5'-CTCCAGAATCATCTCCAAC-3' Probe = 5'-TGCTCCTCTTTATCATGCTATG-3'	192bp-VP1 and VP2 capsid protein-interphase
Norwalk	Upstream = 5'-CAAATTATGACAGAATCCTTC-3' Downstream = 5'-GAGAAATATGACATGGATTGC-3' Probe = 5'-ATGTCATCAGGGTCAAAGAGG-3'	260bp- Viral polymerase
SRSV(Ando)	Upstream = 5'-TGTCACGATCTCATCATCACC-3' Downstream = 5'-TGGAATTCCATCGCCCACTGG-3' Probes = 5'-ATGTCAGGGGACAGGTTTGT-3' 5'-ATGTCGGGGCCTAGTCCTGT-3' 5'-ACATCGGGTGATAGGCCTGT-3'	123bp-RNA polymerase region

Table 3. F+ RNA Coliphage probe data

Virus	*Group*	*Probe Sequence*	*Target region*
MS2	I	5'-CTAAGGTATGGACCATCGAGAAAGGA-3'	Maturation protein
GA	IIa	5'-CATGTTATCCCCCAAGTGCTGGCTAT-3'	Maturation protein
	IIb	5'-GTTTTCCTTATGTTTTGCTTTCAGACCCA-3'	
QB	III	5'-ATACTCAGTGAA(A/G)TACTGCTGTGT-3'	5' nontranslated region
SP/FI	IV	5'-GGCATAGATTCTCCTCTGTAGTGCG-3'	5' nontranslated region

Table 4. EPA-Keys Microbial Grab Sample Data (# CFU or Coliphage/100ml)

Site	Total Coliforms	Fecal Coliforms	*E.coli*	Clostridium spp.	Enterococci spp.	Nonspecific Coliphage
1	327.5	56	15	5	78	10
2	15	8.5	8	5	11.5	0
3	480 w/OG	770	120	26	800	0
4	1 w/OG	5.5	5.5	0	0	10
5	36	19	5	1	1	0
6	390	294	180	6	79.5	0
7	30	26	20.5	21.5	29.5	0
8	10.5	9.5	8	0.5	18	0
9	40	20	13	4	51	0
10	60	44	40	0	27	0
11	330	220	170	1	240	0
12	14	1	0	0	80	0
13	20	5	2	0	0	0
14	11	6	1	3	23	0
15	168	150	70	0	94	0
16	0	0	0	0	0	0
17	30	0	0	0	0	0
18	710	130	130	2	63	0
19	1410	600	80	520	680	0
Average	211.9	124.4	45.7	31.3	119.8	1
Geometric mean	53.8	24.1	13.0	2.9	23.0	1.3

OG = overgrowth which prevented accurate enumeration

The coliphage data had a detection limit of <10/100ml as only 10ml of sample was assayed at each site (see the definition for detection limit in section 2.a.).

Table 5. Site rankings using the prokaryote data (sites are listed in order from north to south)

Location	Site	Total Coliforms	Fecal Coliforms	*E.coli*	Clostridium spp.	Enterococci spp.	Coliphage	Total	Rank
Ocean Reef Resort	16	1	1.5	2	3.5	2	8.5	18.5	1
Key Largo	6	16	17	19	16	14	8.5	90.5	17
Key Largo	5	10	9	6	9.5	5	8.5	48	8
Rock Harbor	7	8.5	11	12	17	10	8.5	67	12
Buttonwood Bay	8	3	8	8	8	7	8.5	42.5	6.5
Plantation Key	4	2	5	8	3.5	2	18.5	39	5
Lower Matecumbe	3	18	19	16	18	19*	8.5	98.5	19*
Long Key	2	6	7	8	14.5	6	8.5	50	9
Conch Key	1	14	13	11	14.5	13	18.2	74	14
Big Pine	9	11	10	10	13	11	8.5	63.5	11
Big Pine	10	12	12	13	3.5	9	8.5	58	10
Big Pine	11	15	16	18	9.5	17*	8.5	84	16*
Big Pine	15	13	15	14	3.5	16	8.5	70	13
Cudjoe	13	7	4	5	3.5	2	8.5	30	3
Sugarloaf	17	8.5	1.5	2	3.5	2	8.5	26	2
Saddlebunch	14	4	6	4	12	8	8.5	42.5	6.5
Boca Chica	12	5	3	2	3.5	15	8.5	37	4
Key West, Houseboat Row	18	17	14	17	11	12	8.5	79.5	15
Key West, Southern Most Point	19	19	18	15	19	18*	8.5	97.5	18*

* = exceeds USEPA guidance levels for single sampling (enterococci).

The sites are ranked according to the number of organisms found at each site. A ranking of 1 equals the highest number of pathogens. After each category was ranked (total coliforms, fecal coliforms etc.) the rankings were totaled and an overall ranking was assigned to each site.

Table 6. RT-PCR virus results by site (sites are listed in order from north to south)

Location	Sample Site	Prokaryote and coliphage Ranking	Panentero - Viruses	Hepatitis A Viruses	Norwalk Viruses	Small Round-Structured Viruses
Ocean Reef Resort	16	1	+	-	-	-
Key Largo	6	17	+	-	+	-
Key Largo	5	8	+	+	-	-
Rock Harbor	7	12	+	+	-	-
Buttonwood Bay	8	6.5	+	+	-	-
Plantation Key	4	5	-	+	-	-
Lower Matecumbe	3	19*	+	+	+	-
Long Key	2	9	+	+	-	-
Conch Key	1	14	-	+	-	-
Big Pine	9	11	-	+	-	-
Big Pine	10	10	+	-	-	-
Big Pine	11	16*	+	+	-	-
Big Pine	15	13	+	-	-	-
Cudjoe	13	3	+	-	-	-
Sugarloaf	17	2	-	-	-	-
Saddlebunch	14	6.5	+	+	-	-
Boca Chica	12	7	+	+	-	-
Key West, Houseboat	18	15	+	+	-	-
Key West, Southern Most Point	19	18*	+	-	-	-
Percent Positive for viruses (not including site 17)			79	63	10	0

* = exceeds USEPA guidance levels for single sampling (enterococci).
NC = not completed
- = none detected
+ = detected

Table 7. Site Descriptions and Prokaryote Ranks

Site	Prokaryote Rank	# Homes on Canal/ # Lots on Canal	# Condo/ Apt. Units on Canal	Number of Homes Built by Year (1930's to Current, by Decade)							Avg. Age of Homes	Canal Type
				30	40	50	60	70	80	90		
16	1	66/75	40				28	21	7	10	23	FT
6	17	17/24					4	9	3	1	23	SDE
5	8	22/23					1	18	3	0	23	*SDE
7	12	15/25				2	4	9			30	SDE
8	6.5		260									FT
4	5	24/32					1	9	11	3	18	SDE
3	19	27/33						5	17	5	15	MCN
2	9	7/17						2	4	1	17	FCN
1	14	15/19		2	2	6	3	1			46	*SDE
9	11	8/16					4	2	1	1	26	SDE
10	10	15/45							12	3	11	FCN
11	16	16/24					2	2	7	5	14	MCN
15	13	3/16							1	2	6	FCN
13	3	10/34							2	8	5	MCN
17	2	9/16					1	1	6	1	16	*SDE
14	6.5	58/85				12	14	15	13	4	26	FT
12	7	27/27					3	18	5	1	22	*FCN

FCN = feeder canal in a multi-canal network.
FT = flow through canal.
MCN = main canal in a multi-canal network.
SDE = single dead end canal.
* = canal/multi-canal network ≤ ~100m long.

Figure 1. Florida Keys site map

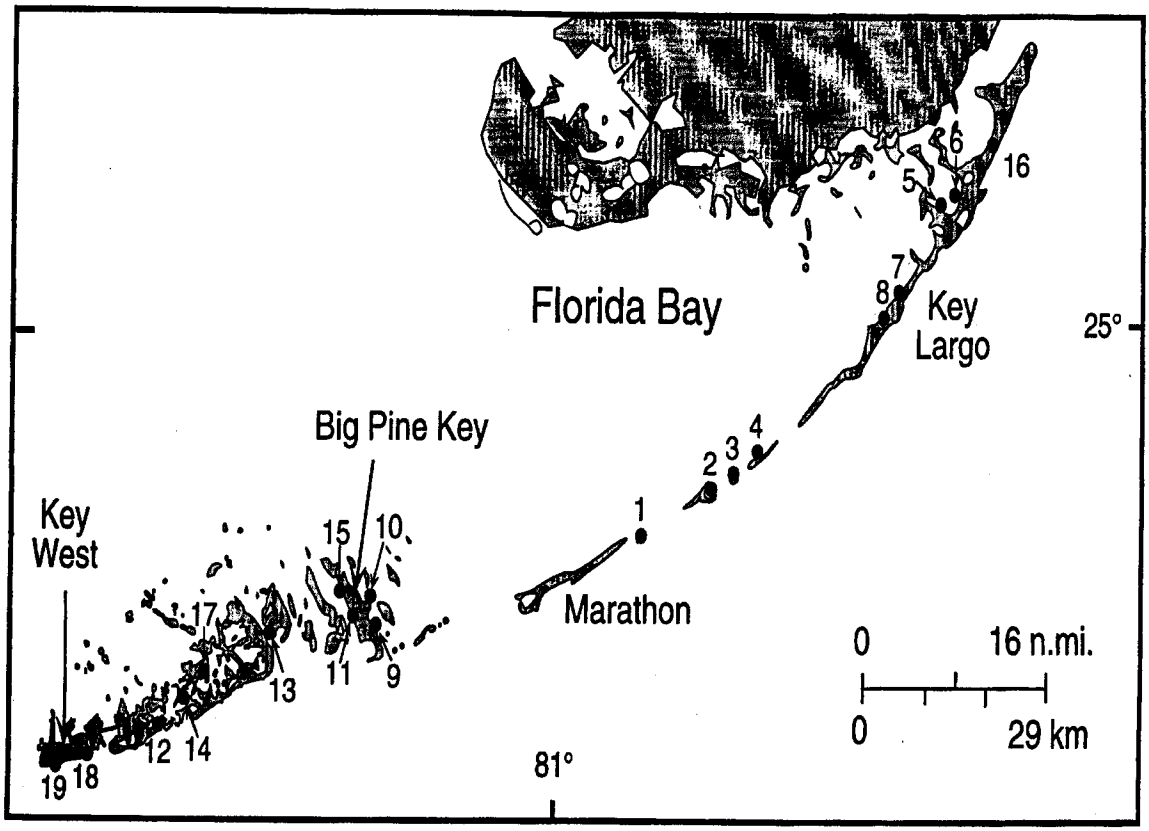
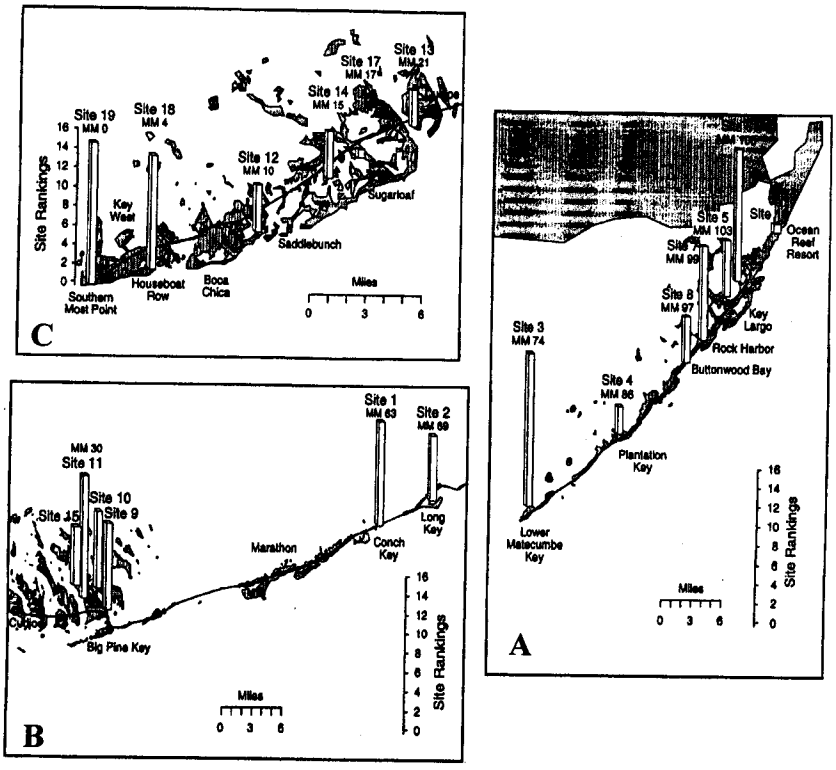
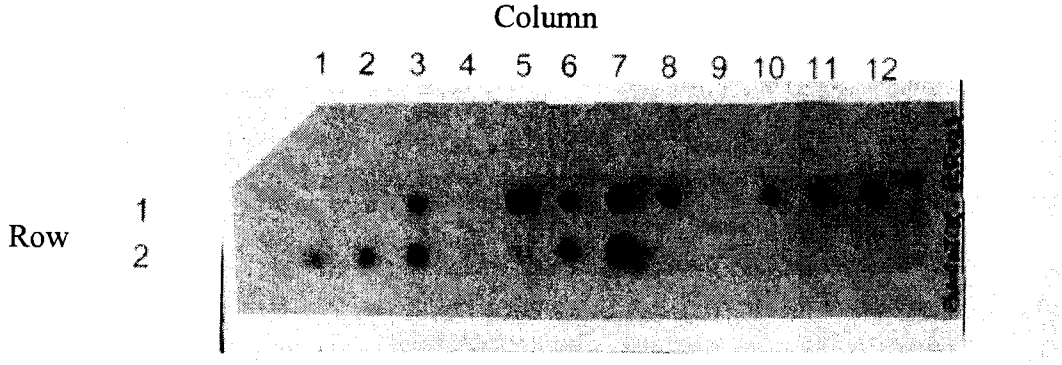


Figure 2a. Site Rankings



A = Upper Keys
B = Middle Keys
C = Lower Keys
See Table 5 for individual site rank values

Figure 3. Panenteroviral dot blot



Row 1, Column 1-12 = sites 1-12 respectively
Row 2, Column 1-4 = sites 13-16 respectively
Row 2, Column 5-6 = sites 18-19 respectively
Row 2, Column 7 = positive control
Row 2, Column 8 = negative control
Site 17 was completed at a later date

VIRAL PATHOGENS AND MICROBIAL INDICATORS IN THE CANALS OF THE FLORIDA KEYS

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Acknowledgements - This work was funded by a grant from the United States Environmental Protection Agency, Region 4 (#984295-97-0).

We would like to thank the following individuals for their assistance on this project:

1) Gus Rios of the Florida Department of Environmental Protection, Marathon, and FL., for providing assistance in sample site selection.

2) David Coleman of Lindahl Browning Ferrari and Hellstrom, Inc., Consulting Engineers. Palm City, Florida, for the GIS data.

3) Robert C. Hudson and Doug Wilder of the Florida Department of Environmental Protection, St. Petersburg, FL., for assistance in analyzing the GIS data.

OBJECTIVES

Examine the microbial water quality at 19 sites in the Florida Keys

1) **17 Canal sites**
   - **16 canals with septic tank waste disposal (ranging from Key Largo to Boca Chica Key)**
   - **1 canal with sewered residences (Ocean Reef Resort, Key Largo)**
2) **2 Nearshore water sites**
   - **Houseboat Row, Key West**
   - **Southern-most Point, Key West**

METHODS

1) **For bacteria – membrane filtration and enumeration on appropriate media**
   - **Total coliforms, fecal coliforms and *E.coli* (standard indicators)**
   - ***Clostridium perfringens* and enterococci (alternate indicators)**

2) **For coliphage – standard overlay technique**
   - **Viruses which attack the coliform *E.coli***

3) **For protozoa – immuno-fluorescent assay (IFA) and epifluorescent microscopy**
   - ***Giardia spp.***
   - ***Cryptosporidium spp.***

4) **For Human Viruses – reverse transcriptase-polymerase chain reaction (RT-PCR) and genetic probes**
   - **Panenterovirus group (Polio, Coxsackie A and B, Echoviruses)**
   - **Hepatitis A Viruses**
   - **Norwalk Viruses**
   - **Small Round Structured Viruses**

*****RT-PCR does not address the question of viability**

Sample Sites

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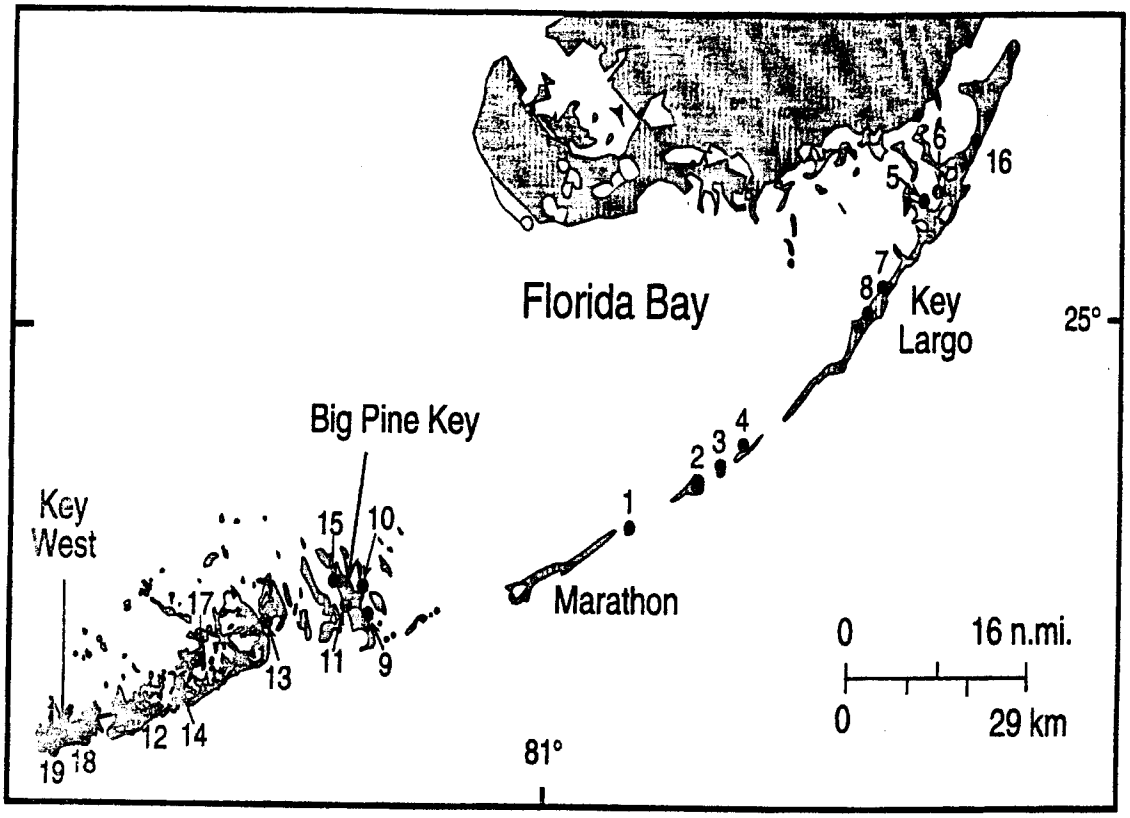

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10	60	44	40	0	27	0
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14	11	6	1	3	23	0
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Big Pine	9	11	-	+	-	-
Big Pine	10	10	+	-	-	-
Big Pine	11	16*	+	+	-	-
Big Pine	15	13	+	-	-	-
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Saddlebunch	14	6.5	+	+	-	-
Boca Chica	12	7	+	+	-	-
Key West, Houseboat	18	15	+	+	-	-
Key West, Southern Most Point	19	18*	+	-	-	-
Percent Positive for viruses (not including site 17)			79	63	10	0

* = exceeds USEPA guidance levels for single sampling (enterococci).
NC = not completed
- = none detected
+ = detected

Summary

1) 79% of the sample sites were positive for the panenterovirus group (Polio, Coxsackie A and B, Echoviruses).
2) 63% of the sample sites were positive for HAV.
3) 10% of the sample sites were positive for Norwalk Viruses.
4) No *Giardia* or *Cryptosporidium spp.* were found in the water column.****
5) Coliphage isolation was low.
6) No site was in violation of the State coliform single sample maximums.
7) Three of the sites were in above the USEPA enterococci guidance level.
8) One of the sites was above the Hawaiian *Clostridium perfringens* guidance level.
9) Pathogen hotspots were distributed throughout the Keys.
10) The cleanest site was within Ocean Reef Resort, Key Largo.

Conclusions

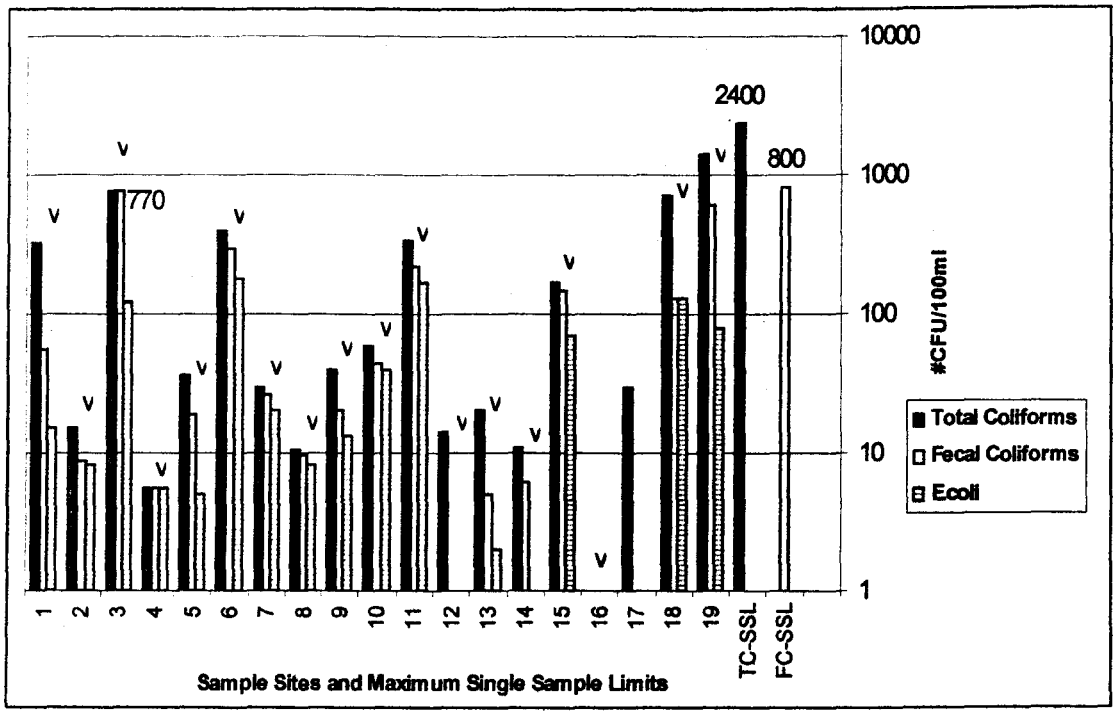
1) Viral data suggests that the canals and nearshore waters throughout the Florida Keys are being impacted by human fecal material.

2) Septic tank waste disposal in this environment is grossly inadequate.

3) Future studies are needed to address viability.

4) The young, aged and immuno-compromised should not swim in the canals of the Florida Keys.

Site Descriptions and Prokaryote Ranks



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16	1	66/75	40				28	21	7	10	23	FT
6	17	17/24					4	9	3	1	23	SDE
5	8	22/23					1	18	3	0	23	*SDE
7	12	15/25				2	4	9			30	SDE
8	6.5		260									FT
4	5	24/32					1	9	11	3	18	SDE
3	19	27/33						5	17	5	15	MCN
2	9	7/17						2	4	1	17	FCN
1	14	15/19		2	2	6	3	1			46	*SDE
9	11	8/16					4	2	1	1	26	SDE
10	10	15/45							12	3	11	FCN
11	16	16/24					2	2	7	5	14	MCN
15	13	3/16							1	2	6	FCN
13	3	10/34							2	8	5	MCN
17	2	9/16					1	1	6	1	16	*SDE
14	6.5	58/85				12	14	15	13	4	26	FT
12	7	27/27					3	18	5	1	22	*FCN