APPLIED AND ENVIRONMENTAL MICROBIOLOGY, June 1995, p. 2230–2234 Vol. 61, No. 6
0099-2240/95/$04.00+0

Viral Tracer Studies Indicate Contamination of Marine Waters by Sewage Disposal Practices in Key Largo, Florida

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Received 20 December 1994/Accepted 4 April 1995

Domestic wastewater disposal practices in the Florida Keys are primarily limited to on-site disposal systems such as septic tanks, injection wells, and illegal cesspits. Poorly treated sewage is thus released into the highly porous subsurface Key Largo limestone matrix. To investigate the fate and transport of sewage in the subsurface environment and the potential for contamination of marine surface waters, we employed bacteriophages as tracers in a domestic septic system and a simulated injection well in Key Largo, Florida. Transport of bacteriophage ΦHSIC-1 from the septic tank to adjacent surface canal waters and outstanding marine waters occurred in as little as 11 and 23 h, respectively. Transport of the *Salmonella* phage PRD1 from the simulated injection well to a canal adjacent to the injection site occurred in 11.2 h. Estimated rates of migration of viral tracers ranged from 0.57 to 24.2 m/h, over 500-fold greater than flow rates measured previously by subsurface flow meters in similar environments. These results suggest that current on-site disposal practices can lead to contamination of the subsurface and surface marine waters in the Keys.

The Florida Keys is a rapidly growing coastal area that is adjacent to a unique marine environment, the only coral reefs in the continental United States. Public concern for protection of the reef environment has resulted in the creation of the Florida Keys National Marine Sanctuary by the United States federal government. A series of reports on coral mortality, bleaching, and benthic algal proliferation (1, 2, 12) have prompted investigation of the causes of such events. A consensus of reef scientists was that increased nutrification linked to accelerating urbanization in South Florida and specifically the Keys was a leading threat and potential cause of the problems noted in the coral reef environment (3).

A source of nutrification is the sewage disposal practices in the Keys. With the exception of Key West, these are limited to on-site disposal practices, which include injection wells (an estimated 600 to 700), 25,000 septic tanks, and 5,000 illegal cesspools (3, 13). The fate of sewage disposed of by these methods remains largely unknown. Lapointe et al. (6) correlated nutrification of canals in the Keys with the presence of septic tanks. We have detected microbial indicators of sewage pollution (fecal coliforms, enterococci, and *Clostridium perfringens*) in the shallow subsurface aquifer both onshore and offshore and in the surface waters in canals and offshore waters of Key Largo, Florida (9). Shinn et al. (13) have also detected fecal coliforms and fecal streptococci at certain locations in the subsurface aquifer on shore and in shallow coastal waters around the Florida Keys. However, the presence of such indicators of sewage cannot be definitely attributed to any one source. For example, the presence of such microbial indicators has been attributed to contamination of the subsurface aquifer by exchange with surface waters that have been contaminated by boats, birds, or other animal populations or by on-site disposal practices.

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To conclusively determine the effects (if any) of injection well and septic practices on the surface and subsurface waters of Key Largo, a well-designed tracer study was required. Tracer studies have been effectively used to investigate the migration of wastewater and efficiency of sewage treatment (5). Using this approach, we have studied the migration of simulated wastes in the subsurface by seeding a septic tank and a simulated injection well with different bacteriophages and following their fate as a function of time. Our results suggest that sewage materials move rapidly through the subsurface environment in Key Largo and into surface marine waters.

MATERIALS AND METHODS

Phages and strains. The marine bacterium HSIC was isolated from a sample taken from Ke'ehi Lagoon, Honolulu, Hawaii, in July 1993 on an artificial seawater medium (designated ASWJP) at 28°C (7). The microbial population from this site was concentrated by vortex flow filtration (VFF) as previously described (8). The bacteriophage ΦHSIC-1 was isolated, by using host HCIC, from the same retentate by top agar overlay. The phage makes distinctive, polymorphic plaques, in that a certain proportion are haloed and some are clear. The *Salmonella* phage PRD1 was obtained from Charles Gerba, University of Arizona, Tucson, and was maintained on the host *Salmonella typhimurium* (ATCC 19585), which was grown on Trypticase soy broth medium.

Seeding of septic tanks and injection wells. ΦHSIC-1 was grown to a high titer (2×10^{11} PFU/ml) by plaque agar overlay and eluted from plates with 5 ml of 0.5 M Tris-HCl, pH 8.0, per plate. The lysate of over 200 such plates was screened through 0.2-μm-pore-size filters to produce 950 ml of lysate and 1.9×10^{14} PFU of phage. For PRD1, a higher titer than that for ΦHSIC-1 was obtained by plaque agar overlay (10^{12} PFU/ml) and 70 to 100 ml of lysate was prepared. For seeding of a septic tank, 200 ml of ΦHSIC-1 lysate was flushed down the toilet of the National Undersea Research Center on the Port Largo Canal (Fig. 1) every hour for 5 h, starting at 1700 on 6 June 1994. Also, fluorescent spheres (0.7- to 1-μm-diameter Fluoresbrite spheres; Polysciences) were flushed at the same time, at a volume of 10 ml per flushing, for a total of 40 ml and 6×10^{12} spheres. During one flushing, 20 g of fluorescein (Sigma) in 1 liter of deionized water was also flushed.

For seeding of a simulated injection well, the monitoring well KLA (Fig. 1), a 1-in. (2.54-cm)-internal-diameter polyvinyl chloride pipe in a well drilled to a depth of 13.7 m and with a screen interval of 10.7 to 11.9 m, was used. The well was first pumped for 45 min to remove 60 to 80 liters of water prior to seeding with a Shaklee diaphragm pump. The bacteriophage was added by diluting the phage in 10 liters of well water and pumping from 12:50 to 17:50 on 7 June 1994.

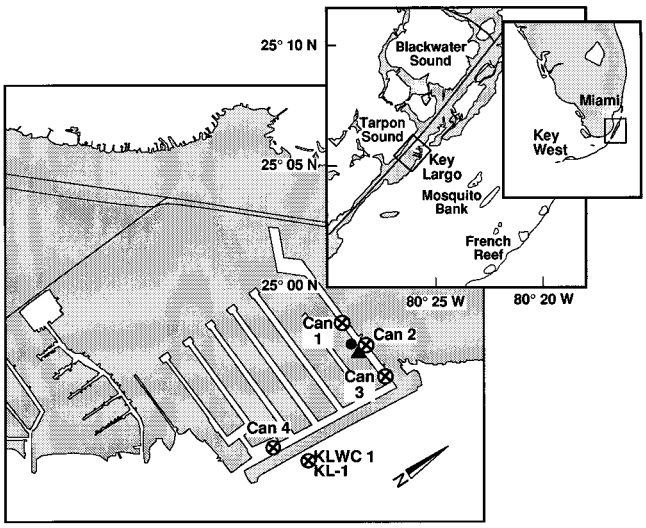

FIG. 1. Location of the study area for bacteriophage seeding experiments described in this paper and in reference to southern Florida (small inset) and the Florida Keys (large inset). The location of both the simulated injection well (KLA) and the shallow monitoring well KLB (filled black circle), the location of the septic tank drain field (filled triangle), and the location of surface water sampling sites (crossed circles) are indicated. The crossed circle labelled KLWC 1 was also the location of offshore deep monitoring well KL1.

Sampling sites. The sites chosen for sampling appear in Fig. 1. Site Can 1 was located on the Port Largo Canal approximately 137 m northwest of the seed site, while Can 2 was located directly adjacent to the seed site and Can 3 and Can 4 were located 167 and 793 m, respectively, down the canal from the seed site. KLWC 1 was located approximately 50 m from the Key Largo shore in the marine environment outside the canals, termed "outstanding marine waters." The monitoring wells sampled included KLB, a 1-in. (2.54-cm)-internal-diameter polyvinyl chloride pipe in the same hole as KLA with a screen interval (effective depth of sampling) of 3.7 m, and KL1, a well drilled to a depth of 12.2 m with a screen interval of 11 to 12.2 m at the same site as KLWC 1 (Fig. 1).

Sampling procedures. Surface water samples were collected by dip bucket and placed into sterile 50-ml centrifuge tubes (stations Can 1 to 3) or into 20-liter carboys that had been sterilized by sodium hypochlorite treatment (stations Can 4 and KLWC 1). The KLA well was sampled with the diaphragm pump, whereas KL1 was sampled with an impeller pump system previously described (9). The sampling interval for Can 1, 2, and 3 and KLB was initially every 4 h for 24 h after the seed, then every 8 h for 48 h, and then twice a day. Can 2 was also sampled at noon each day, and the sample was concentrated by VFF. All KLWC 1 and KL1 (20-liter) samples were concentrated by VFF to 40 to 60 ml as previously described (8).

Phage detection. Volumes of 1 ml and 0.1 ml of each sample in duplicate were assayed for the phage presence on the appropriate host. Plaques were enumerated after 16 to 24 h.

Fluorescent sphere detection. A 20-ml sample was fixed with 1 ml of 37% filtered formalin. The entire sample was filtered onto 0.2-μm-pore-size filters that had been previously counterstained with Irgalan black (4). Spheres were counted by epifluorescence microscopy at a magnification of ×100 with an Olympus BH-2 epifluorescence microscope equipped with blue light excitation. Thirty to sixty fields per sample were counted.

RESULTS

Septic tank seed study. Figures 2 and 3 show the results of the ΦHSIC-1 septic tank seed study. ΦHSIC-1 appeared first and in the largest numbers ($>10^6$ PFU/liter) in shallow monitoring well KLB within 7 h of the beginning of the seed period (Fig. 2C). This well was approximately 20 m from the septic tank drain field. The phage appeared next at the Can 3 site at 11 h after the initiation of the seed period (Fig. 2D). This result was surprising in that the distance from the drain field to Can

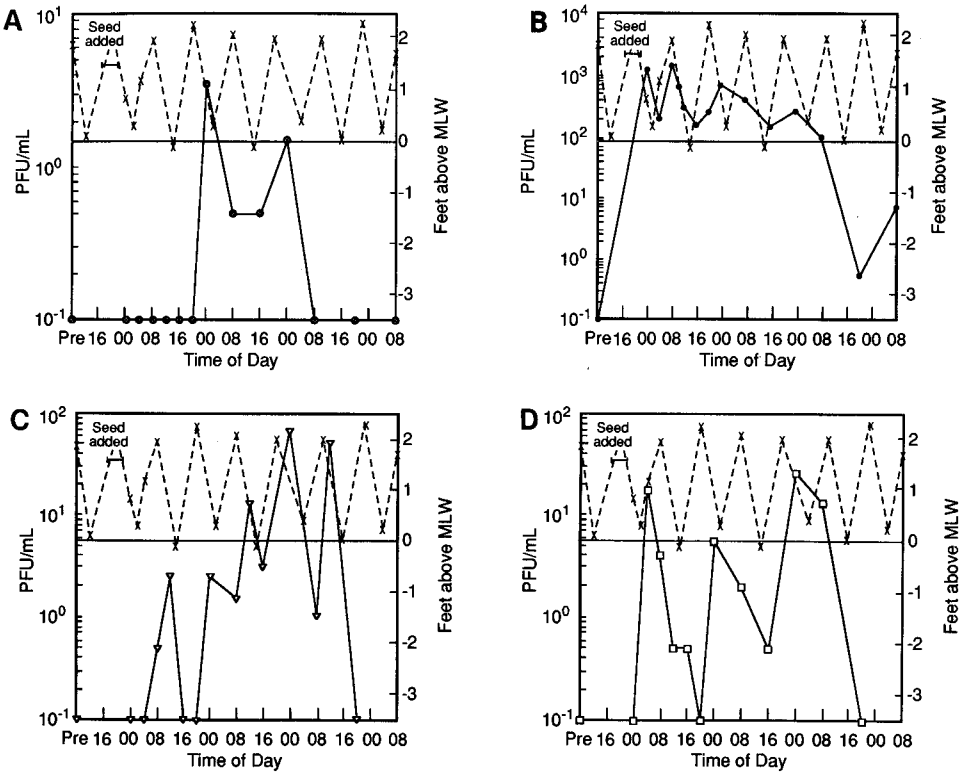

FIG. 2. Appearance of septic tank bacteriophage seed, ΦHSIC-1, as a function of time, in marine surface waters (A, C, and D) and in the shallow onshore monitoring well KLB (B). The marine sampling sites include station Can 1 (A), Can 2 (B), and Can 3 (D). The tidal height above mean low water (MLW) (1 ft = 30.48 cm) as a function of time (dashed line) is plotted. The period of seed addition is indicated by a short horizontal line. Pre, sample taken prior to seeding; ×, tidal levels.

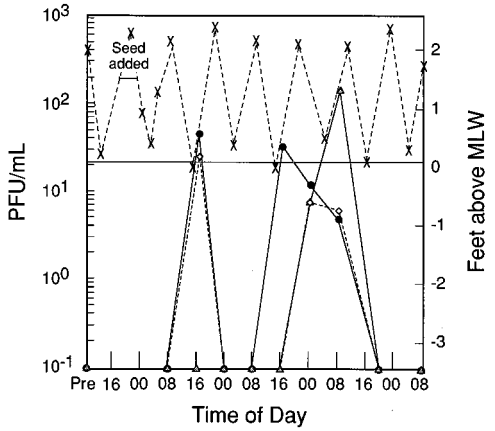

FIG. 3. Appearance of septic tank-seeded ΦHSIC-1 in surface waters at Can 4 (filled circles) and KLWC 1 (diamonds) and in offshore monitoring well KL1 (triangles) as a function of time. The tidal height above mean low water (MLW) (1 ft = 30.48 cm) (dashed line) and the period of seed addition (short horizontal line) are indicated. Pre, sample taken prior to seeding; ×, tides.

3 was approximately 167 m. The phage was next detected (15 h after initiation of the seed period) at Can 2, which was the canal site closest to the septic tank. The phage was next detected at Can 4 and KLWC 1, the stations farthest from the septic drain field, at 23 h after seeding. The seed was detected at Can 1, which was the only shoreward station sampled, at 31 h after seeding and in the offshore monitoring well KL1 after 55 h.

The abundance of ΦHSIC-1 found as a function of time was not uniform but rather showed peaks in concentration. Also plotted in Fig. 2 to 4 are tidal data during the sampling period. Thirteen of fourteen peaks in concentration of viruses in Fig. 2 corresponded with a period of falling tide. Peaks in concentration in the farthest stations (Can 4, KLWC 1, and KL1) appeared immediately following mean low water (within 2 h). These results suggest a relationship between phage movement and tidal action.

Figure 4 shows the results of fluorescent sphere counts. A peak in the fluorescent sphere count was noted in the shallow well at 7 h after seeding. Spheres were also noted at high

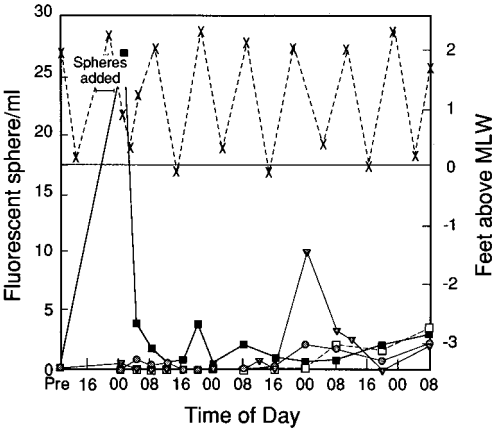

FIG. 4. Appearance of fluorescent spheres added to the septic tank in marine surface waters and shallow well KLB. The tidal height above mean low water (MLW) (1 ft = 30.48 cm) as a function of time (dashed line) and the period of sphere addition (short horizontal line) are indicated. Pre, sample taken prior to seeding. Symbols: ◍, Can 1; ▽, Can 2; □, Can 3; ■, KLB; ×, tides.

concentrations 55 h into the study. The sphere-counting method was not deemed to be as sensitive as the phage tracer method because of the smaller initial seeding and the higher limit of detection (about 5 spheres per ml) and uncertainty in identifying 0.7- to 1-μm spheres at ×100 magnification.

Water samples (unconcentrated and VFF retentates) were assayed for fluorescein fluorescence by fluorometry. The light scatter by particulates in the VFF retentates confounded measurements. However, a fluorescent green "cloud" was observed in the water at Can 2 at noon on 8 June, at approximately 33 h after seeding, indicative that the fluorescein tracer had made its way from the septic tank into the canal.

Injection well seed study. Figure 5 shows the results of the simulated injection well seed study. Unlike with the ΦHSIC-1 tracer, one positive sample (or plaque) was detected prior to the seed interval at Can 3. This finding suggests the potential for a low level of indigenous *Salmonella* phage. Unlike in the septic tank study, the seed was first detected at Can 1, again on a falling tide (Fig. 5A), at approximately 11.2 h after seeding. Again, a pulse-like distribution in concentration was observed, with a second peak 16 h after the first.

The seed was next detected in shallow monitoring well KLB and at Can 3 (Fig. 5B and D). Lateral transport rates were greater than vertical transport rates, because the KLB well sampled the subsurface environment which was directly above the KLA well, whereas Can 3 was 167 m away from the KLA seeding site.

The PRD1 phage was detected last (35 h after the seed) at Can 2, which was the canal site closest to the KLA simulated injection well. This observation argues for rates of deep lateral movement greater than those of vertical movement.

On the basis of the distances travelled and appearance of phage tracers, rates of movement of viruses through the combined subsurface aquifer and surface waters were calculated. These rates are probably conservative because of the limitation imposed by our sampling schedule (i.e., lack of temporal resolution because of infrequent sampling). The near-surface rates (i.e., those obtained from the septic tank seed) ranged from 1.3 m/h obtained at Can 2 to 24.2 m/h for Can 4, with an average rate for all data of 13.5 m/h. These rates are illustrated in the vector arrays displayed in Fig. 6.

The rates for the injection well study ranged from 0.57 m/h at Can 2 to 12.4 m/h at Can 1, for an average of 6.7 m/h. Unlike in the septic tank study, the greatest rate occurred upstream, but this initial rate was only for a very small number of viruses. Again, a vector diagram of flow movement for the injection study appears in Fig. 6.

DISCUSSION

The experiments performed in this study clearly show a connection between a septic tank drain field, well discharge areas, and surface marine waters. Connections between canal surface waters and septic tanks from houses adjacent to canals have been inferred by work with nutrient measurements by LaPointe et al. (6). These investigators correlated the appearance of elevated nutrient levels in canals with septic tanks and showed evidence of tidal action in the transport of this material. However, our estimated migration rates are considerably greater than those previously published. Previous estimates based on measurements with subsurface flow meters ranged from 0 to 1.25 m/day (6), with higher rates in the Key Largo limestone (predominantly in the upper Florida Keys) and lower rates in the Miami oolite (common in the lower Keys). Hydraulic conductivities between surface waters and internal coral waters in Hawaii have been estimated at ~50 m/day (14).

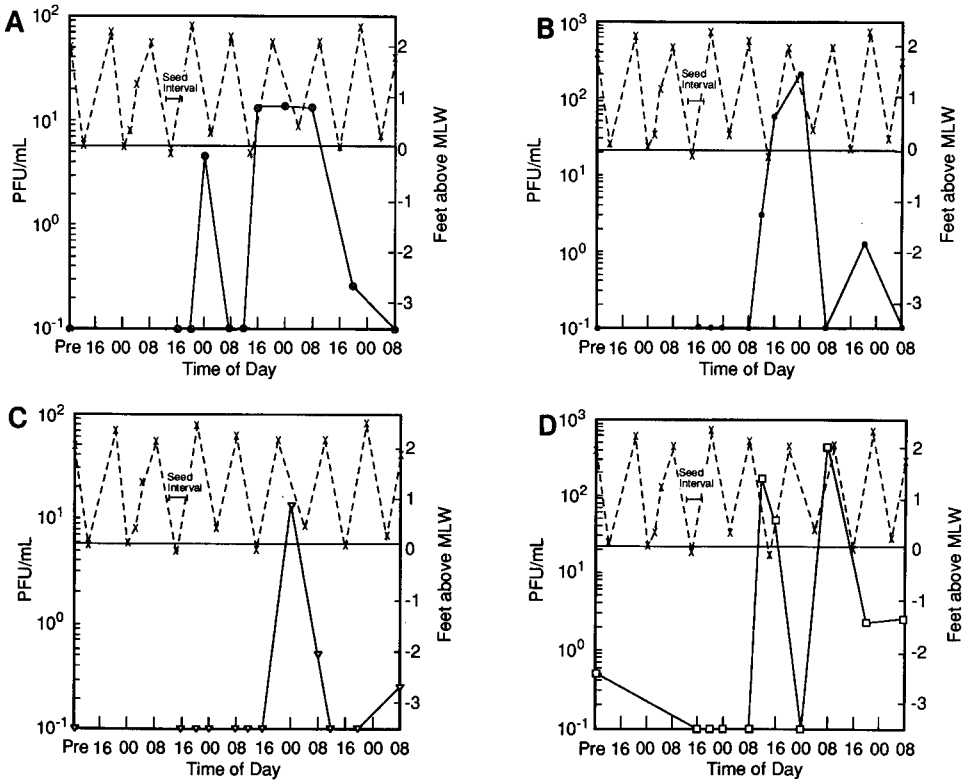

FIG. 5. Appearance of *Salmonella* phage PRD1 at injection well seed sampling sites Can 1 (A), shallow well KLB (B), Can 2 (C), and Can 3 (D) as a function of time. The tidal height above mean low water (MLW) (1 ft = 30.48 cm) as a function of time (dashed line) and the period of seed addition (short horizontal line) are indicated. Pre, sample taken prior to seeding.

Our slowest estimated rate of migration was 0.57 m/h (13.7 m/day), and our fastest estimated rate was 24.2 m/h (581 m/day). Although these rates seem high compared with previous estimates, several factors should be considered. First, the flows we measured may not reflect uniform diffusion through a homogeneous matrix, but rather "channeling" through conduit-like passages in the Key Largo limestone (15). A second consideration is that viruses travel like colloids through subsurface environments, in effect, faster than the bulk water flow rates (10, 11). However, these observations were made for saturated and unsaturated soils and there is no information on microbial movement in the saturated subsurface carbonate environment of Key Largo. Finally, record rainfalls preceded our study and may have been responsible for unusually rapid flow rates in the subsurface aquifer.

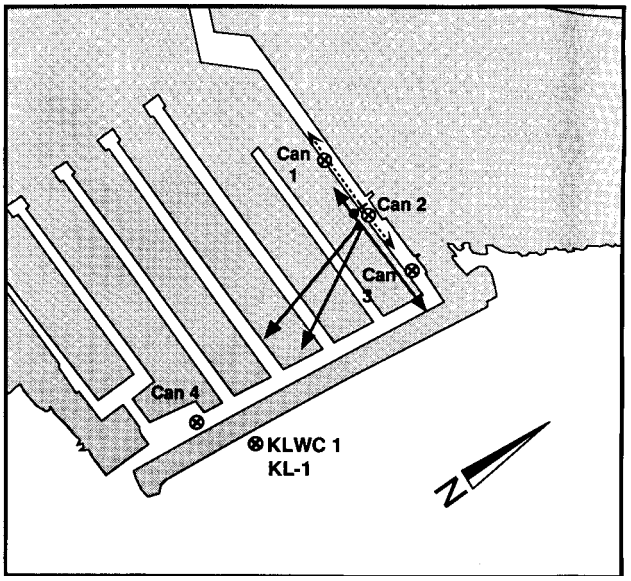

FIG. 6. Vector diagram showing estimated rate and direction of movement of the ΦHSIC-1 (solid arrows) and PRD1 phages (dashed arrows) from the septic tank (filled triangle) and the simulated injection well (filled circle), respectively. Sampling sites are indicated by crossed circles.

The variation in concentrations of viral tracers used in this study showed a correlation with falling tides, suggesting that tidal pumping played a significant role in viral movement. Previous work by Shinn and coworkers (13) has shown that tidal pumping is particularly active in Key Largo, particularly near the shore. Hydraulic heads as great as 7 cm above sea level have been detected in nearshore wells during falling tides. Such a head could transport viruses and other wastes in septic tank effluents and injection well materials through the porous Key Largo limestone matrix.

We and others have previously detected the presence of indicator bacteria in the shallow onshore aquifer and in the offshore subsurface aquifer, as well as in canals (9, 13). Evidence for a rapid exchange between the KLB shallow monitoring well and the marine environment was found when high levels of chlorophyll *a* and indigenous marine bacteriophages (Φ16-like vibriophages) were found in that well (9). The high-level salinity of the KLB well also suggested a rapid exchange with canal waters (9).

Our work suggests that the on-site disposal practices employed in Key Largo lead to contamination of the marine surface waters. These practices could pose a health risk to the human population, when contact (swimming or diving) occurs, particularly in the canals, or from the consumption of seafood harvested from these canals.

Our work also is the first to link microbial contamination of the outstanding marine waters (station KLWC 1) with on-site disposal practices. These data argue for changes in current waste disposal practices in the Florida Keys.

ACKNOWLEDGMENTS

This work was supported by grants from the National Science Foundation and the National Oceanographic and Atmospheric Association's National Undersea Research Center (administered by the University of North Carolina at Wilmington).

Technical assistance was provided by Sara M. Jacob.

REFERENCES

1. **Causey, B.** 1988. Zooxanthellae expulsions in Florida reefs during 1987. Proc. Assoc. Island Mar. Lab. Caribb. **21:**51.
2. **Dustan, P.** 1985. Community structure of reef-building corals in the Florida Keys: Carysfort Reef, Key Largo, and Long Reef, Dry Tortugas. Atoll Res. Bull. **288:**1–27.
3. **Environmental Protection Agency.** 1992. Water quality protection program for the Florida Keys National Marine Sanctuary, phase II. Continental Shelf Associates Inc. and Battelle Ocean Sciences, contract 68-C8-0105. Environmental Protection Agency, Washington, D.C.
4. **Hobbie, J. E., R. J. Daley, and S. Jasper.** 1977. Use of Nuclepore filters for counting bacteria by fluorescence microscopy. Appl. Environ. Microbiol. **33:**1225–1228.
5. **Ijzerman, M. M., C. Hagedorn, and R. B. Renau.** 1993. Microbial tracers to evaluate an on-site shallow-placed low pressure distribution system. Water Res. **27:**343–347.
6. **Lapointe, B. E., J. D. O'Connell, and G. S. Garrett.** 1990. Nutrient couplings between on-site waste disposal systems, groundwaters, and nearshore surface waters of the Florida Keys. Biogeochemistry **10:**289–307.
7. **Paul, J. H.** 1982. The use of Hoechst dyes 33258 and 33342 for the enumeration of attached and pelagic bacteria. Appl. Environ. Microbiol. **43:**939–949.
8. **Paul, J. H., S. C. Jiang, and J. B. Rose.** 1991. Concentration of viruses and dissolved DNA from aquatic environments by vortex flow filtration. Appl. Environ. Microbiol. **57:**2197–2204.
9. **Paul, J. H., J. B. Rose, S. Jiang, C. Kellogg, and E. S. Shinn.** 1995. Occurrence of fecal indicator bacteria in surface waters and the subsurface aquifer in Key Largo, Florida. Appl. Environ. Microbiol. **61:**2235–2241.
10. **Powelson, D. K., and C. P. Gerba.** 1994. Virus removal from sewage effluents during saturated and unsaturated flow through soil columns. Water Res. **28:**2175–2181.
11. **Powelson, D. K., J. R. Simpson, and C. P. Gerba.** 1990. Virus transport and survival in saturated and unsaturated flow through soil columns. J. Environ. Qual. **19:**396–401.
12. **Rutzler, K., and D. L. Santavy.** 1983. The black band disease of Atlantic reef corals. I. Description of the cyanophyte pathogen. P.S.Z.N.I. Mar. Ecol. **4:**301–319.
13. **Shinn, E. A., R. S. Reese, and C. D. Reich.** 1994. Fate and pathways of injection-well effluent in the Florida Keys. Department of the Interior, U.S. Geological Survey open-file report 94-276. U.S. Geological Survey, Washington, D.C.
14. **Tribble, G. W., F. J. Sansone, R. W. Buddemeir, and Y.-H. Ii.** 1992. Hydraulic exchange between a coral reef and surface sea water. Geol. Soc. Am. Bull. **104:**1280–1291.
15. **Vascher, H. L. (University of South Florida, Department of Geology).** 1994. Personal communication.