Estuaries and Coasts Vol. 29, No. 2, p. 246–256 April 2006

Application of *Bacteroides fragilis* Phage as an Alternative Indicator of Sewage Pollution in Tampa Bay, Florida

MOLLY R. MCLAUGHLIN[1,†] and JOAN B. ROSE[2,*]

[1] *University of South Florida, College of Marine Science, 140 7th Avenue South, St. Petersburg, Florida 33701*

[2] *Homer Nowlin Chair in Water Research, Department of Fisheries and Wildlife, 13 Natural Resources Building, Michigan State University, East Lansing, Michigan 48824*

ABSTRACT: Traditional fecal coliform bacterial indicators have been found to be severely limited in determining the significance and sources of fecal contamination in ambient waters of tropical and subtropical regions. The bacteriophages that infect *Bacteroides fragilis* have been suggested as better fecal indicators and at least one type may be human specific. In this study, the phages that infect *B. fragilis* host RYC2056 (RYC), including phage B56-3, and host ATCC 51477-HSP40 (HSP), including the human specific phage B40-8, were evaluated in the drainage basins of Tampa Bay, 7 samples (n = 62), or 11%, tested positive for the presence of phages infecting the host HSP, whereas 28 samples, or 45%, tested positive using the host RYC. A survival study was also done to compare the persistence of phages B56-3 and B40-8 to MS2 coliphage in seawater at various temperatures. The decay rates for MS2 were 0.239 $\log_{10}$ d^{-1} at 10°C, but increased to 0.896 at 20°C and 2.62 $\log_{10}$ d^{-1} at 30°C. The two *B. fragilis* phages persisted much longer in the seawater compared to the coliphage and showed little variation between the temperatures. All sewage influents sampled from area wastewater treatment plants contained phages that infected the two *B. fragilis* hosts at levels from 1.2×10^4 to 1.11×10^5 pfu 100 ml^{-1} for host RYC and 67 to 350 pfu 100 ml^{-1} for host HSP. Of the 7 chlorinated effluent samples tested, 3 were positive for the presence of the phage using the host RYC and the phage enrichment method, with levels estimated to be <10 pfu 100 ml^{-1}. No phages were detected using the host HSP in the treated sewage effluent. Coliphages were found in 3 of the 7 effluent samples at a range of 30 to 1.2×10^3 pfu 100 ml^{-1}.

Introduction

Contamination of marine recreational waters can occur through sewage disposal into estuaries, contaminated river flow into coastal areas, and marine sewage outfalls (Yates and Scott 1988; Griffin et al. 2001). Over 18,000 d of beach closures and advisories were reported in 2003 (for both marine and freshwater) in the United States, with 88% of those due to bacteria levels exceeding the health and safety standards for that area (NRDC 2004).

There are several inherent disadvantages to using fecal coliforms as indicators of wastewater contamination of ambient waters in tropical and subtropical regions. Both total and fecal coliforms are found in the intestinal flora of mammals other than humans and may replicate in tropical environments (Gallagher and Spino 1968; Yates et al. 1988; Kreader 1995; Edberg et al. 1997; Byappanahalli and Fujioka 1998). In addition to questions about source, fecal coliforms are also less resistant to environmental stress, water treatment, and disinfection than some pathogens, such as viruses and parasites (Yates and Scott 1988; Fleisher et al. 1996; Griffin et al. 2001).

In Europe, the *Bacteroides fragilis* bacteriophages have been proposed as a possible alternative indicator of sewage pollution (Jofre et al. 1986; Tartera and Jofre 1987; Tartera et al. 1988; Puig et al. 1999). Most phages of *B. fragilis* belong to the taxonomic group Siphoviridae and contain double-stranded DNA. These somatic phages have icosahedral heads, exhibit flexible tails, and are often observed in star-shaped clusters when viewed under an electron microscope (Ackermann and DuBow 1987; Lasobras et al. 1997). According to Lasobras et al. (1997), bacteriophages with flexible tails are often the most resistant to environmental stress. *B. fragilis* phages showed no replication in the environment and demonstrated decay rates similar to human enteric viruses, coliphages, and poliovirus (Jofre et al. 1986; Tartera and Jofre 1987), and their persistence in seawater was similar to Hepatitis A virus (Yates and Scott 1988; Chung and Sobsey 1993). The phage was more resistant to chlorination than *E. faecalis*, *Escherichia coli*, and some enteroviruses (Gallagher and Spino 1968; Bosch 1989; Sinton et al. 1998); they displayed a positive

* Corresponding author; tele: 517/432-4412; fax: 517/432-1699; e-mail: rosejo@msu.edu

† Current address: U.S. Geological Survey, Center for Coastal and Watershed Studies, 600 4th Street-South, St. Petersburg, Florida 33701.

correlation with the levels of enteroviruses and rotaviruses (Jofre et al. 1989). Grabow et al. 1995 found that the phage B40-8 (host ATCC 51477-HSP40 [HSP]) was found in the feces of 13% of humans tested, but not in animals or birds. This phage was highly specific to human sewage, but natural variations exist in the gastrointestinal flora due to diet, stress, and environment (Holdeman et al. 1976; Havelaar et al. 1986; Grabow et al. 1995; Puig et al. 1997). The phage B56-3 (host RYC2056 [RYC]) has been isolated from feces of 28% of humans, 31% of pigs, and 29% of poultry tested. It has not been isolated from cattle, sheep, or horse fecal samples (Puig et al. 1997, 1999).

No major studies have been published on the use of this indicator group in U.S. waters. In order to evaluate the potential for the *B. fragilis* phage assay to be used as an indicator of fecal contamination in subtropical waters in the U.S., this assessment focused on three main areas: a survey of ambient waters in the Tampa Bay area including 22 sites and 62 total samples for the presence of the *B. fragilis* phages that infect the hosts RYC and HSP compared to traditional and other alternative indicators (fecal coliforms, enterococci, *Clostridium perfringens*, and coliphages); the persistence of *B. fragilis* phage B40-8 (host HSP) and B56-3 (host RYC) and MS2 coliphage in seawater at various temperatures; and a preliminary domestic wastewater survey to determine background levels in the local population and a possible environmental source of the *B. fragilis* phages and comparing background levels to standard indicators.

Materials and Methods

Site Selection

Study sites (22) were chosen as part of the Tampa Bay Healthy Beaches Project and a total of 104 samples were analyzed for the environmental survey of the *B. fragilis* phage prevalence and concentration. The sites were chosen to represent the major drainage basins leading into Tampa Bay and to provide several different water types, including marine, fresh, urban, and rural, as well as low, intermediate, and high pollution areas (Fig. 1).

Sites TB1 through TB11 were in a predominately rural area with several cattle ranches and small communities with septic tank disposal systems. Sites TB3, 4, 6, 7, and 8 were in various locations along Bullfrog Creek in South Hillsborough county, which has a history of high levels of agricultural pollution. Sites TB12 through TB21 were in a highly populated section of Pinellas county. Sites TB13, 16, 19, and 20 were beach sites. Site TB22 was a control site from the middle of Tampa Bay.

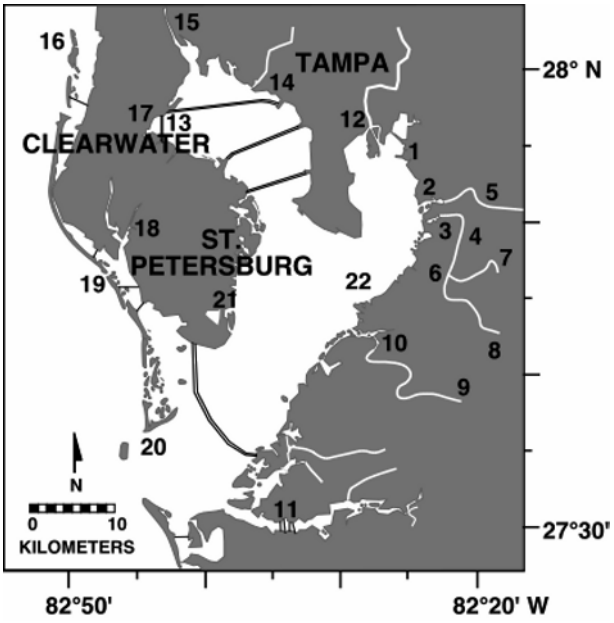

Fig. 1. Map of Tampa Bay, Florida, showing sampling sites.

Bacteria and Phage Strains

The *B. fragilis* bacteriophages B56-3 (host RYC) and B40-8 (host HSP) and their corresponding host bacteria were kindly provided by Dr. Juan Jofre, Dr. Francisco Lucena, and Dr. Javier Mendez at the University of Barcelona, Spain.

Quality Control Organisms

Enterococcus faecalis (ATCC 19433) was used as a positive control for the enterococci indicator assay. *C. perfringens* (ATCC 13124) was used as a positive control for the *Clostridium* indicator assay. *E. coli* (ATCC 15597) was used as both a positive control for *E. coli* and as the host bacterium for phage MS2, which should host both somatic and F-specific type phages.

Sample Collection

Grab samples were collected in sterile 1-l nalgene bottles and placed on ice for transportation to the lab. Samples were processed within 8 h of collection.

Fecal Indicators

Samples were assayed using membrane filtration techniques (0.45-μm cellulose filters from Osmonics, Westborough, Massachusetts, with a 47-mm Gelman filter funnel). Assay volumes were determined by the fecal contamination level present at each site. Fecal coliforms were enumerated according to Standard Methods for Examination of Water and Wastewater (APHA 1998), using mFC agar (Difco, Becton Dickinson, Maryland). Dark blue colonies that

developed after 18 to 24 h incubation at 44.5°C in a waterbath were counted as fecal coliforms. *E. coli* were enumerated by taking those plates that were positive for fecal coliforms, transferring the membrane filter to EC with MUG agar (Difco) and incubating for an additional 24 h at 37°C. Colonies that fluoresced under ultraviolet light (MUG positive) were counted as *E. coli* (APHA 1998). Enterococci were enumerated according to U.S. Environmental Protection Agency (USEPA) method 1600 (USEPA 1986) using mEI agar (Difco). Colonies exhibiting a blue halo after 18 to 24 h at 41°C were counted as enterococci. *C. perfringens* were enumerated using mCP agar (Acumedia Manufacturers, Inc., Baltimore, Maryland) and incubated anaerobically in GasPak jars (BD-BBL GasPak, Becton Dickinson Co., Maryland) for 18 to 24 h at 45°C (Bisson and Cabelli 1979). Yellow colonies that turned pink or red when exposed to ammonium hydroxide fumes were counted as *C. perfringens*. Coliphages (somatic and F-specific) were enumerated according to Standard Methods for Examination of Water and Wastewater (APHA 1998), using the standard overlay assay and ATCC 15597 *E. coli* host. Each sample was assayed using 10 replicate plates, with 1 ml of water sample each. All indicators were calculated in colony forming units (cfu) or plaque forming units (pfu) per 100 ml of water.

B. fragilis Overlay Protocol

The overlay protocol and media used in this study were as stated in the International Standards Organization protocol CD 10705-4 (2001). The *B. fragilis* hosts (RYC and HSP) were grown to log phase in *Bacteroides* Phage Recovery Medium (BPRM) broth (equivalent to 10^8 host cells per ml of broth) using 10 ml screw-top sterile glass culture tubes filled to the top with broth to provide an anaerobic environment and incubated at 37°C. The time required for the cells to reach log phase was 4 to 5 h.

Log-phase host cells (1 ml) and room temperature water samples (1 ml) were added to a tube containing 2.5 ml of melted BPRM soft agar. The sample was poured onto the surface of a BPRM agar plate and the agar was allowed to solidify. The plate was inverted and incubated for 18 to 24 h at 37°C anaerobically in a GasPak jar. Plaques appearing on the bacterial lawn were considered to be phages infecting the hosts RYC and HSP. In some cases, replicates of 10 were used for each sample, increasing the limit of detection for this assay to 10 pfu 100 ml^{-1}.

B. fragilis Bacteriophage Enrichment Assay

Prefiltered seawater (100 ml through a 0.45-µm Millipore polyvinylidene fluoride (PVDF) filter; Tartera et al. 1992; Araujo et al. 1993; Lucena et al. 1995) was added to 100 ml of double-strength BPRM broth in a sterile 250-ml glass flask with a screw-top lid. Both were prewarmed to at least room temperature. A log-phase culture of either *B. fragilis* hosts RYC or HSP (30 ml) was added, and the flask was filled to the top with regular strength BPRM broth to provide an anaerobic environment. Positive and negative control flasks were used for each host. All flasks were incubated at 37°C for 48 h. A 1-ml aliquot was taken from each flask and placed in a 1.5-ml sterile microcentrifuge tube with 0.4 ml of chloroform and vortexed. The tubes were spun in the microcentrifuge at 3,000 g for 5 min. Log-phase host culture (1 ml) was added to a tube containing 2.5 ml of melted BPRM soft agar and overlayed on BPRM agar plates. For each sample, 1 µl of the chloroform-treated aliquot was dotted in triplicate on the surface of the appropriate host lawn without disturbing the surface of the agar. The plates were allowed to solidify and were inverted and incubated at 37°C for 24 h in a GasPak anaerobic jar. Samples were considered positive for the presence of the bacteriophage if clearing was noted on the lawn. All samples positive for the enrichment assay were repeated using 10 ml of unfiltered sample and the standard overlay procedure.

Statistical Analysis for Environmental Samples

All indicator data was log transformed to calculate monthly geometric means. Correlations, means, and survival statistics were computed using Microsoft Excel (Microsoft Corp, Redmond, Washington). Binary logistic regressions were determined using MINTAB (Minitab, Inc., State College, Pennsylvania).

Transmission Electron Microscopy

Samples of a phage dilution (1 µl) were dotted onto Formvar-coated copper electron microscopy (EM) grids, negatively stained with 2% uranyl sulfate, and viewed using a Hitachi H7100 transmission electron microscope.

Phage Survival Study

In order to determine the effects of temperature and salinity on the persistence of the *B. fragilis* phage B56-3 (host RYC) and B40-8 (host HSP) in seawater compared to coliphages, an experiment was performed using flasks of sterile filtered natural seawater incubated in the dark at 10°C, 20°C, and 30°C. Filtered-sterilized (Millipore PVDF 0.22 µm filter) seawater (100 ml) was placed in sterile 250-ml flasks (salinity: 35‰, water temperature: 26°C, source: Gulf of Mexico Pier, Ft. DeSoto County Park, St. Petersburg, Florida) and preincubated at

the appropriate temperature for 24 h. Dilutions of MS2 phage and phages B56-3 (host RYC) and B40-8 (host HSP) were added to the flasks at each temperature to achieve a final phage concentration of 10^6 ml^{-1}. Flasks were incubated in the dark and sampled once a day for 12 d, then at 18 and 24 d. Aliquots were diluted and assayed in duplicate using the standard overlay protocol. The entire experiment was run in duplicate.

The results were analyzed as suggested by Yates et al. (1985). Phage concentrations at each sampling were log transformed and plotted against the corresponding day of the experiment. Best fit linear regression lines were applied to the curves, and the slope of the line was used to determine the decay rate of the virus by using the following equation: Decay rate = $-[(\log_{10}\ \text{pfu})\text{d}^{-1}]$.

Domestic Wastewater Survey

Influent and effluent samples were taken from three different domestic wastewater treatment plants in the Tampa Bay area of west central Florida. Plant 1 had a flow rate capacity of 98 million gallons per day (mgd). In addition to activated sludge, this plant design also included a denitrification filter and chlorination step followed by dechlorination prior to the effluent discharge into Tampa Bay. The contact time for the chlorine basin was 30 min. Plant 2 was rated for a flow capacity of 12 mgd. This plant did have a chlorination step, but the water was not dechlorinated. The discharge of the plant was routed to the reclaimed water distribution system. The contact time of the chlorine basin was 15 to 30 min. Plant 3 was rated for a 12 mgd flow. Activated sludge and a chlorination step were included in the plant design. The contact time of the chlorine basin was 15 to 30 min, and the discharge from the plant was routed to injection wells. There was no dechlorination step.

Plants 1 and 2 were sampled in triplicate, and plant 3 was sampled twice for a total of 8 influent samples; for effluent samples, plants 1 and 3 were sampled in duplicate, and plant 2 was sampled in triplicate for a total of 7 samples. Each sample set was taken on a different day. Influent samples from all three plants were taken at the intake port at the head of each treatment plant. Effluent samples from plant 1 were taken after dechlorination from the discharge line leading into Tampa Bay, effluent samples from plant 2 were taken after the chlorination step from the line leading to the reclaimed water distribution system, and effluent samples from plant 3 were taken after the chlorination step from the discharge line leading to the injection wells. All chlorinated samples were dechlorinated with sodium thiosulfate upon collection before analysis of fecal indicators.

Both influent and effluent samples were prefiltered through a 0.45-micron Millipore PVDF filter to remove background flora. Influent samples were assayed using the standard overlay method in triplicate for phages infecting the hosts RYC and HSP and the MS2 coliphage. Effluent samples were also assayed as described above using the standard overlay method (10 replicates), as well as the enrichment procedure using both hosts of *B. fragilis*. All unfiltered samples were assayed for fecal coliforms, enterococci, and *C. perfringens* using the methods previously described.

Results

Environmental Survey

All 22 sites in the Tampa Bay area were analyzed monthly for 3 mo using the enrichment method as well as an agar overlay for the *B. fragilis* phage on both hosts (62 samples total) to determine the levels and prevalence of these phages for comparison to the analysis for traditional and alternative indicators, including fecal coliforms, enterococci, *C. perfringens*, and coliphages. Table 1 shows the geometric mean and minimum and maximum values of the indicators at all sites, as well as the phage results. The geometric average of fecal coliforms ranged from <4 cfu 100 ml^{-1} at the control site TB22 to 8,180 cfu 100 ml^{-1} at TB6. Enterococci ranged from 1 cfu 100 ml^{-1} at the control site to 10,955 cfu 100 ml^{-1} at TB7. *C. perfringens* levels were below the detection limit for TB10, 16, 19, 20, and 22 and were as high as 9 cfu 100 ml^{-1} at TB1 and 3. Coliphage levels ranged from below the detection limit at TB13, 16, 20, and 22 to 2,998 pfu 100 ml^{-1} at TB4.

The Florida standard of 200 cfu 100 ml^{-1} geometric monthly mean for fecal coliforms in ambient waters was exceeded in 9 of the 22 sites. The USEPA suggested guidelines of 33–35 cfu 100 ml^{-1} for enterococci in ambient waters was exceeded in 17 of the 22 sites. Fujioka suggested a guideline level of 50 cfu 100 ml^{-1} for *C. perfringens* in freshwater for Hawaii and 5 cfu 100 ml^{-1} in marine waters (Fujioka and Shizumura 1985; Fujioka 2001). Three of the marine sites geometric means exceeded the suggested guideline. Although some of the sites exceeded the recommended level at individual samplings, the geometric means for all the freshwater sites were below 50 cfu 100 ml^{-1}. There is currently no suggested USEPA standard for coliphage levels in ambient waters; 8 of the 22 sites had means above 100 pfu 100 ml^{-1}, a number associated with the presence of enteric viruses in other studies (Lipp et al. 2001).

TABLE 1. Geometric mean and range of values of fecal indicators, salinity, and *Bacteroides fragilis* phage results in waters of Tampa Bay, Florida. Indicator numbers shown are the geometric mean (n = 3), the minimum, and the maximum results from August through October of 1999 reported in cfu or pfu100 ml^{-1}. Positive (+) and Negative (Neg) results were obtained using the enrichment assay. na = results not available due to contamination of enrichment bottle or nongrowth of host. Results reported in presence or absence in 100 ml of water.

						Host ATCC 51477-HSP40			Host RYC2056		
Site	Fecal coliforms (min–max)	Enterococci (min–max)	*C. perfringens* (min–max)	Coliphages (min–max)	Salinity range (‰)	Aug	Sept	Oct	Aug	Sept	Oct
TB1	966[a] (225–9,050)	1,650[b] (476–12,300)	9 (7–15)	1,242 (180–7,560)	0–5	Neg	+	Neg	Neg	+	Neg
TB2	133 (15–555)	83[b] (6–496)	5 (4–8)	37 (10–90)	8–24	Neg	na	Neg	20[d]	na	+
TB3	2,891[a] (440–16,350)	825[b] (90–17,200)	9[c] (2–20)	93 (20–1,850)	0–21	Neg	Neg	Neg	Neg	+	+
TB4	7,143[a] (800–174,900)	10,759[b] (2,030–135,650)	8 (<2–62)	2,998 (530–28,180)	0–5	Neg	Neg	+	+	+	+
TB5	1,278[a] (485–5,300)	2,954[b] (1,550–6,350)	6 (2–12)	326 (100–980)	0–5	Neg	Neg	Neg	Neg	+	+
TB6	8,180[a] (1,210–110,200)	1,945[b] (300–41,700)	7 (<2–46)	1,961 (520–22,920)	0–5	Neg	Neg	Neg	+	+	+
TB7	5,100[a] (1,890–8,450)	10,955[b] (5,450–31,650)	4 (2–12)	425 (60–3,680)	0–2	Neg	+	Neg	Neg	Neg	Neg
TB8	215[a] (90–1,035)	1,083[b] (115–17,850)	4 (<2–16)	57 (<10–1,680)	0–5	Neg	Neg	Neg	Neg	+	+
TB9	1,420[a] (185–10,250)	4,361[b] (1,110–17,200)	4 (<2–16)	601 (370–1,420)	0	Neg	na	Neg	Neg	na	+
TB10	297[a] (52–985)	106[b] (10–2905)	<2	51 (10–1,080)	10–19	Neg	na	Neg	Neg	na	+
TB11	96 (56–200)	44[b] (24–102)	2 (<2–2)	14 (10–20)	15–29	Neg	na	Neg	Neg	na	Neg
TB12	290[a] (55–940)	183[b] (65–310)	2 (<2–22)	69 (20–260)	0–15	+	Neg	Neg	+	+	Neg
TB13	473[a] (40–9,550)	34[b] (6–125)	2 (<2–12)	<10	20–28	Neg	Neg	Neg	Neg	Neg	Neg
TB14	5,249[a] (1,850–12,600)	1,353[b] (960–1,850)	8 (2–54)	392 (240–540)	0–8	Neg	+	Neg	+	+	+
TB15	305[a] (250–350)	85[b] (40–236)	3 (<2–8)	14 (10–20)	0–2	Neg	Neg	Neg	Neg	Neg	Neg
TB16	41 (2–390)	20 (2–557)	<2	<10	30–33	Neg	Neg	Neg	Neg	Neg	Neg
TB17	1,537[a] (160–23,700)	257[b] (45–720)	6[c] (<2–20)	61 (20–120)	18–21	+	+	Neg	+	+	Neg
TB18	429[a] (280–850)	98[b] (78–124)	6[c] (<2–30)	157 (90–220)	2–14	Neg	Neg	Neg	+	+	Neg
TB19	335[a] (34–13,240)	6 (2–28)	<2	2 (<10–10)	31–36	Neg	Neg	Neg	Neg	Neg	Neg
TB20	122[a] (<10–10,900)	11 (2–77)	<2	<10	32–36	Neg	Neg	Neg	Neg	Neg	Neg
TB21	396[a] (180–1,050)	149[b] (50–1,270)	2 (<2–18)	38 (10–230)	18–24	Neg	Neg	Neg	+	+	Neg
TB22	<4	1 (0–2)	<2	<10	26–31	Neg	Neg	Neg	Neg	Neg	Neg

[a] Exceeds USEPA guidelines for fecal coliforms for ambient waters (200 cfu 100 ml^{-1} geometric monthly mean).
[b] Exceeds USEPA guidelines for enterococci for ambient waters (33 cfu 100 ml^{-1} freshwater, 35 cfu 100 ml^{-1} marine water geometric monthly mean).
[c] Exceeds Hawaii's recommended guidelines of 5 cfu 100 ml^{-1} for marine water.
[d] Result of 20 cfu 100 ml^{-1}, this is the only sampling event that resulted in a phage level high enough to be detected by the standard overlay method.

In a previous 2-mo period of all 22 sites, 44 other samples were analyzed for phages with both bacterial hosts HSP and RYC using the standard overlay procedure and no enrichment procedure. Phage levels were consistently below the detection limit of the overlay procedure (10 pfu 100 ml^{-1}) for all sampling sites. The presence-absence *B. fragilis* enrichment assay was used in addition to the overlay method for 62 more samples during the remaining 3 mo of the study (Table 1). Positive enrichment results can be considered to be between 1 and 10 pfu 100 ml^{-1} for all sample sites, when plaque forming units using agar overlay methods were negative.

Table 1 shows the presence-absence enrichment results for both hosts of the *B. fragilis* phage for all 22 sampling sites during the 3-mo period. For the host HSP, 62 total enrichments were performed during the months of August, September, and October; 7 samples, or 11%, tested positive for the presence of phages infecting the host HSP. Of the 22 sites, 6 sites tested positive for the phages infecting this host and one site, TB17, tested positive for two of the three sampling dates. The host RYC was also used for a total of 62 enrichments during the months of August, September, and October; 28 samples, or 45%, tested positive using this host. Phages infecting the host RYC were detected at 14 of the 22 sites. TB4, 6, and 14 tested positive for phages infecting the host RYC during all 3 mo of the study. Only one site, TB2, during the month of August, gave a result high enough to be detected with the standard overlay method (20 pfu 100 ml^{-1}).

The sampling sites can be divided into three distinct groups, the rural sites (characterized by more septic tanks and agriculture), the urban sites (characterized by high density land use and storm water control), and the beach sites. For both the rural and urban sites, the range of salinity varied greatly (Table 1) between sites, with some sites showing a wide range depending on the time of day sampled. For the rural sites, the indicators were high, with fecal coliforms ranging from 96 to 8,180 cfu 100 ml^{-1}, enterococci ranging from 44 to 10,955 cfu 100 ml^{-1}, and coliphages ranging from 14 to 2,998 pfu 100 ml^{-1}. The urban sites showed slightly less indicator values, with fecal coliforms ranging from 290 to 5,249 cfu 100 ml^{-1}, enterococci ranging from 85 to 1,353 cfu 100 ml^{-1}, and coliphages ranging from 14 to 392 pfu 100 ml^{-1}. At the beach sites very low indicator levels were found during the 3-mo study period. While the traditional indicator data showed a difference in the overall indicator levels for the rural and urban sites, the presence-absence *B. fragilis* phage data showed little difference between the two areas. The rural sites had 10% and 59% of the samples positive using the host HSP and RYC, respectively, and urban sites had 19% and 52% of the samples positive using the hosts HSP and RYC, respectively. No *B. fragilis* phages were detected at the beach sites during the 3 mo of the study.

TABLE 2. Binary logistic regression results comparing fecal indicators to *Bacteroides* phage (n = 62).

Response Organism	Model Organism	Concordant %	Discordant %	Tie %
Phages detected by host RYC2056 (RYC)	Fecal coliforms	75.6	24.1	0.2
	Enterococci	85.1	14.7	0.2
	C. perfringens	67.0	16.9	16.1
	Coliphages	89.8	8.9	1.4
	All indicators	92.9	6.9	0.2
Phages detected by host ATCC 51477-HSP40 (HSP)	Fecal coliforms	67.2	31.8	1.0
	Enterococci	66.4	32.3	1.3
	C. perfringens	55.2	29.7	15.1
	Coliphages	69.0	28.6	2.3
	All indicators	72.7	27.3	0.0

Statisical analysis of the *B. fragilis* phage results from the environmental survey was performed to determine if the presence of the bacteriophage would correlate to the other indicator organisms. Because of the differing nature of the two data sets (indicator data as numerical concentrations and the *B. fragilis* phage as presence or absence), binary logistic regressions were performed to compare the presence of *B. fragilis* phages to the levels of fecal coliforms, enterococci, *C. perfringens*, and coliphages (Lipp 1999). Binary logistic regression first determines if the slope of the regression line is different from zero, then goodness of fit tests evaluate how well the data fit the modeled expected frequencies. Results of the regression are presented as the percentage of concordant pairs (signifying that the model predicted the outcome of both presence and absence), percentage of discordant pairs (model did not successfully predict the outcome), and percentage of tied pairs (model predicted only one of the parameters).

Table 2 shows the results of the binary logistic regression. When the *B. fragilis* phage on host RYC was used as the outcome or response organism, the highest concordant percentage was from using coliphage as the model organism, with 89.8% concordance. Enterococci were the second highest, with 85.1%, and fecal coliforms and *C. perfingens* showed low values, with 75.6% and 67%, respectively. Combining the use of all the indicators gave a concordant percentage of 92.9%. The combined concordant percentage when using the phage isolated on host HSP as the response or outcome organism was only 72.7%, with the concordant

percentages for the individual indicator organisms all falling below 67.2%. There was not an apparent strong correlation between the presence of the human specific *B. fragilis* phage (host HSP) and the levels of the other indicator organisms, likely as a result of the low levels of this phage in the environmental samples.

In order to confirm that the bacteriophage forming plaques on the *B. fragilis* phage host in the enrichment procedure were morphologically consistent with the known *B. fragilis* bacteriophages, transmission electron micrographs were taken of the control bacteriophages and of phages isolated from plaques from the environmental samples. All environmental phages screened exhibited the consistent morphology of icosahedral heads with long flexible segmented tails (Fig. 2). This morphology places the isolated phage in the virus family Siphoviridae, which is the family identified by Ackermann and DuBow (1987) as including the *B. fragilis* bacteriophages (Lasobras et al. 1997).

Survival in Seawater

The bacteriophage MS2 shows the highest decay rate for each of the three temperatures compared to the two *B. fragilis* phages. Temperature appears to have little effect on the survival of phages B56-3 (host RYC) and B40-8 (host HSP). Both *B. fragilis* phages exhibited a slightly lower decay rate at 30°C than at 20°C. This occurrence was seen in the duplicate experiment as well, but it is unclear why. The MS2 phage has the highest decay rate at 30°C, surviving in the seawater flask less than 36 h. Figures 3 through 5 show the linear regression curves for the three phages at each of the experimental temperatures, plotting survival against time to achieve the decay rate. Table 3 shows the resulting equations of the regression lines, and the decay rate calculated for each phage at the different temperatures. The decay rates for MS2 were 0.239 $\log_{10}$ d^{-1} at 10°C, but increased to 0.896 at 20°C and 2.615 $\log_{10}$ d^{-1} at 30°C. The two *B. fragilis* phages persisted much longer in the seawater compared to the coliphages and showed little variation between the temperatures. The phage B40-8 decay rates were 0.047 at 10°C, 0.204 at 20°C, and 0.084 $\log_{10}$ d^{-1} at 30°C. The decay rates for the phage B56-3 were 0.140 at 10°C, 0.258 at 20°C, and 0.054 $\log_{10}$ d^{-1} at 30°C.

Treatment Plant Indicator Levels

In order to compare the levels of *B. fragilis* bacteriophages in domestic sewage found in the Tampa Bay area to those levels found in other areas, samples were taken from three area wastewater treatment plants and analyzed for phages detected

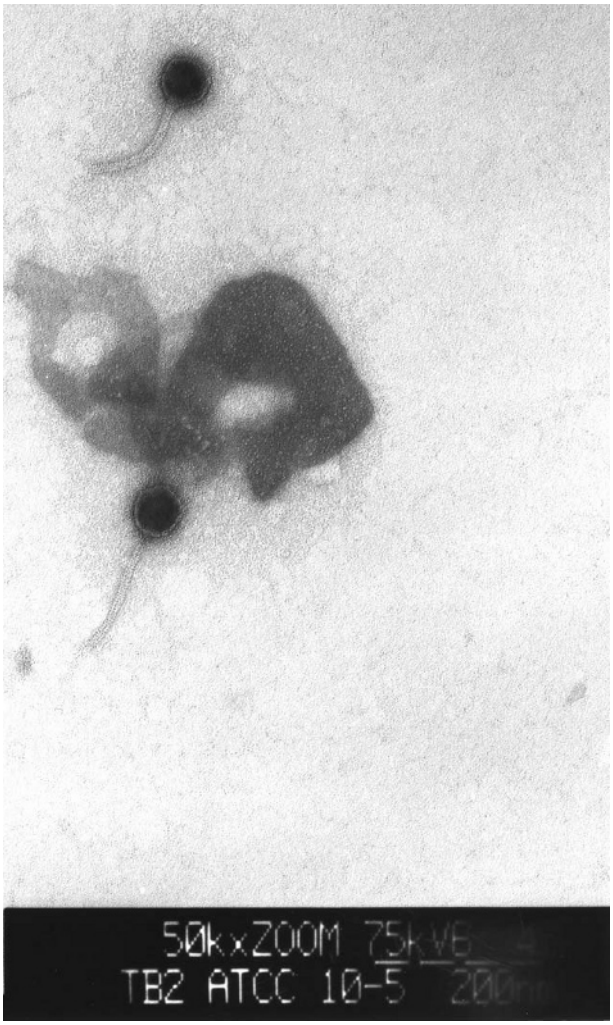

Fig. 2. Transmission electron microscopy (TEM) micrograph of phage isolated using *B. fragilis* host HSP from site TB2 in the Alafia River.

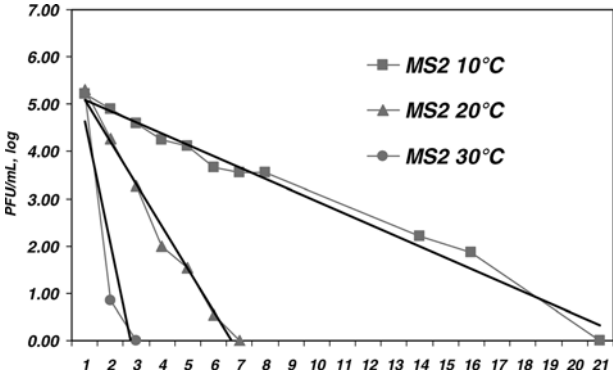

Fig. 3. Survival curves of *Escherichia coli* phage MS2 in seawater at 10°C, 20°C, and 30°C.

TABLE 3. Survival study of decay rates and linear regression line equations.

Phage	Temperature	Decay Rate	Equation	R^2 value
MS2	10°C	0.239	$y = -0.2386x + 5.3362$	0.983
	20°C	0.896	$y = -0.8959x + 5.9994$	0.9869
	30°C	2.62	$y = -2.6152x + 7.2556$	0.8675
B40-8	10°C	0.047	$y = -0.047x + 6.2399$	0.8751
	20°C	0.204	$y = -0.2037x + 5.9164$	0.8714
	30°C	0.084	$y = -0.0837x + 5.2492$	0.4965
B56-3	10°C	0.140	$y = -0.1394x + 6.1132$	0.9442
	20°C	0.258	$y = -0.2575x + 6.2852$	0.9539
	30°C	0.054	$y = -0.0542x + 5.3586$	0.6674

by the *B. fragilis* phage hosts HSP and RYC. The samples were also analyzed for the presence of fecal coliforms, enterococci, *C. perfringens*, and coliphages.

The range of indicator levels of all 3 plants appear in Table 4. For the *B. fragilis* phage results, both the standard overlay procedure and the enrichment method were performed. Fecal coliforms in the influent ranged from 1.6 to 4.1 $\times$ 10^6 cfu 100 ml^{-1} for the three treatment facilities. Enterococci levels were 1.5 to 6.2 $\times$ 10^5 cfu 100 ml^{-1}, *C. perfringens* ranged from 1.3 to 4.9 $\times$ 10^4 cfu 100 ml^{-1}, and coliphage levels ranged from 1.0 to 3.0 $\times$ 10^5 pfu 100 ml^{-1} of influent. The phages infecting the host *B. fragilis* RYC ranged from 1.2 $\times$ 10^4 to 1.1 $\times$ 10^5 plaque forming units (pfu) 100 ml^{-1}, while the phages infecting the host HSP were much lower, with the results ranging from 67 to 350 pfu 100 ml^{-1} for all three treatment facilities.

Indicator organisms were below the detection limit (nondetectable) in the disinfected effluent with the exception of fecal coliforms and coliphages at plants 2 and 3, and *C. perfringens* at plants 1 and 2. Positive results using enrichment procedures with the host RYC occurred in the effluent at 2 of the 3 facilities sampled. The phages infecting host HSP were not detected in any of the effluent samples analyzed. For each sample where a positive enrichment result for host RYC was found in the effluent, coliphages were also detected.

Discussion and Conclusions

The search for alternative indicators of fecal pollution in ambient waters focusing on the *B. fragilis* phage has been undertaken primarily in Europe and South Africa. Most of these studies concentrated on detecting only phage B40-8 (host HSP), which has been shown to be very specific to humans and rarely included the phages infecting the host RYC. The sampling sites of these studies generally included heavily affected or polluted waters directly receiving untreated sewage or nondisinfected sewage. The methods used were either the standard overlay method or the presence-absence assay in an most probable number (MPN) format (Allsop and Stickler 1984; Tartera et al. 1988, 1989; Lucena et al. 1996; Sun et al. 1997). Detection of the phages infecting the host HSP in the environment varied greatly depending on the sample source. Lucena et al. (1996) found 7 to 5,300 pfu 100 ml^{-1} in polluted river water, 1.2 $\times$ 10^2 100 g^{-1} in sediment, and 2–3 100 ml^{-1} in groundwater. Araujo et al. (1997) found waters receiving recent sewage input showed levels of 2.3 $\times$ 10^3 pfu 100 ml^{-1}, waters receiving intermediate sewage input showed levels of 23 pfu 100 ml^{-1}, and waters with persistently low levels of pollution averaged 1.67 pfu 100 ml^{-1}. Waters that were considered low impact, or those not receiving urban sewage and runoff, consistently fell below the detection limit of the assay for the phage B40-8 (host HSP).

TABLE 4. Detection of traditional fecal indicators and *Bacteroides fragilis* phage in raw and treated wastewater. All values are expressed as ranges of cfu or pfu 100 ml^{-1} of sample. [a] n = 3, [b] n = 2. Pos =positive.

	Influent			Effluent		
Indicators	Plant 1[a]	Plant 2[a]	Plant 3[b]	Plant 1[b]	Plant 2[a]	Plant 3[b]
Fecal coliforms	1.6 to 2.3 $\times$ 10^6	2.7 to 4.1 $\times$ 10^6	2.7 to 3.0 $\times$ 10^6	<1	1 to 6	<1 to 1
Enterococci	1.5 to 1.7 $\times$ 10^5	3.0 to 3.2 $\times$ 10^5	2.9 to 6.2 $\times$ 10^5	<1	<1	<1
C. perfringens	2.3 to 2.7 $\times$ 10^4	2.5 to 4.9 $\times$ 10^4	1.3 to 2.0 $\times$ 10^4	4	8 to 24	<1
Coliphage	1.0 to 3.0 $\times$ 10^5	1.2 to 3.0 $\times$ 10^5	1.2 to 2.0 $\times$ 10^5	<10	100 to 1.2 $\times$ 10^3	<10 to 30
Phages infecting *B. fragilis* host RYC2056	1.2 to 2.4 $\times$ 10^4	3.8 $\times$ 10^4 to 1.1 $\times$ 10^5	2.6 $\times$ 10^4	<1	Pos (1–10)	<1 to Pos (1–10)
Phages infecting *B. fragilis* host ATCC 71477-HSP40	67 to 167	100 to 350	233*	<1	<1	<1

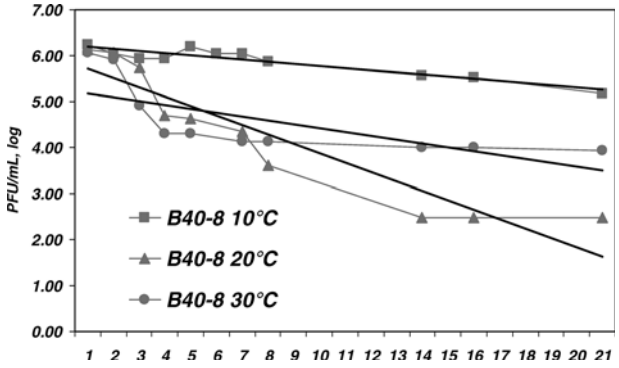

Fig. 4. Survival curves of *Bacteroides fragilis* phage B56-3 (host RYC) in seawater at 10°C, 20°C, and 30°C.

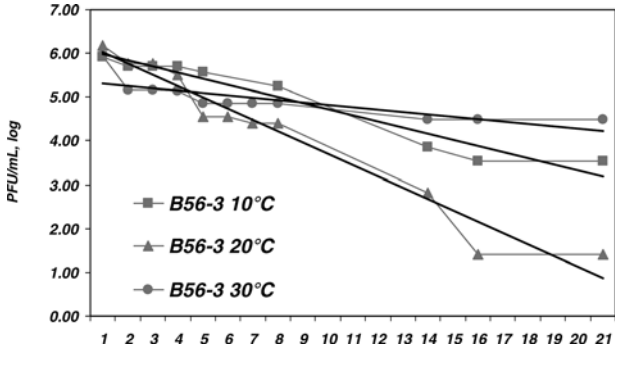

Fig. 5. Survival curves of *Bacteroides fragilis* phage B40-8 (host HSP) in seawater at 10°C, 20°C, and 30°C.

In our geographic location, *B. fragilis* phage infecting the hosts HSP and RYC were found in the environment at very low numbers. This differs from the findings in Europe and perhaps is expected as the U.S. sites were not directly receiving untreated domestic sewage discharges. The levels found in untreated sewage in the Tampa Bay area of phages infecting the host HSP were lower when compared to the European studies. Human specific *B. fragilis* phages (host HSP) detected in Tampa Bay wastewater treatment plants ranged from 66.7 to 350 pfu 100 ml^{-1}, and they were detected in 100% of the sewage influent samples tested. Influent samples taken in Spain and France showed mean levels of 6.2×10^3 pfu 100 ml^{-1}, 5.3×10^3 pfu 100 ml^{-1}, and 4.4×10^4 MPN 100 mL^{-1} for the phages of host HSP (Tartera and Jofre 1987; Tartera et al. 1989; Sun et al. 1997). Puig et al. (1999) reported finding phages of host RYC at a level of 2.4×10^4 pfu 100 ml^{-1} in sewage influent, which was similar to the levels found in our study of sewage influent (1.19×10^4 to 1.11×10^5 pfu 100 ml^{-1}).

In a study by Contreras-Coll et al. (2002) *B. fragilis* phage and coliphage concentrations and other indicators were examined in bathing waters throughout Europe. The RYC2056 was used and only 6 to 20 samples per location were collected and assayed, resulting in 28% to 100% of the samples positive for the *B. fragilis* phage in the 13 areas, averaging 67% positive using an agar overlay method with a tested volume of 10 ml. Their concentrations averaged approximately 5 pfu 100 ml^{-1} (extrapolated from Fig. 4). In our study 45% were positive for this phage, were below 10 pfu 100 ml^{-1}, and were very similar to this geographically diverse survey.

The data clearly show a distinct geographic difference in not only the numbers but the types of phage found in human populations. In other more recent studies focused on the *B. fragilis* phage (host RYC) determined by agar overlay methods in raw sewage, septage, and environmental waters (10 rivers from 22 sampling sites in Argentina, Colombia, France, and Spain; Lucena et al. 2003) reported that the indicators studied maintained the same relative densities in the raw sewage from the different geographical areas. Levels of the *B. fragilis* phage infecting host RYC in raw sewage were found on average at 6.3×10^4 pfu 100 ml^{-1}, which is similar to what was found in our untreated sewage study in Tampa Bay. Levels in the rivers in Argentina, Colombia, France, and Spain averaged 39 pfu 100 ml^{-1}, but again untreated sewage discharges were affecting the rivers. The septage in these studies averaged 398 pfu 100 ml^{-1}, indicating that septic tanks could be a source of these fecal indicators.

The low numbers of the phage infecting host HSP in Florida sewage, which should be primarily from human sources, may explain the low numbers in the ambient waters in this geographic location. The phage of host RYC do have environmental sources other than humans (pigs and poultry), but were also found at high levels in the domestic sewage in the Tampa Bay region, which may indicate that humans are a significant source in this area. Seabirds may be a consideration as a possible environmental source in Tampa Bay, but no studies have been published on the presence of phages infecting the host RYC in seabird fecal samples. It also appears that sewage treatment and disinfection can reduce the numbers of the *B. fragilis* phages to nondetection and untreated sewage, septic tank effluents, and direct fecal inputs may be suspected when these phages are detected in ambient waters.

The subtropical climate of Florida makes it a unique ecosystem when compared to the remainder of North America. The warm marine and estuarine waters make it difficult to apply bacterial indicator assays that may be used with great success in temperate zones and freshwater systems. Survival of pathogenic microorganisms and bacterial and viral indicator microorganisms may differ in regards

to the warm climate and saline waters. According to our study, the MS2 coliphage would not be a good model indicator for survival in warm marine waters. Detection of RNA F-specific phages in Florida waters likely indicates fairly recent pollution. In a study by Griffin et al. (1999), no coliphages were detected in the canal systems of the Florida Keys, but the sites did consistently show the presence of enteroviruses. In our study, the two *B. fragilis* phages showed persistence in warm marine waters and perhaps would have been superior indicators in the Keys study. In a study by Duran et al. (2002), the MS2 coliphage showed the highest inactivation rate in survival studies in river water, while phages of the host HSP and other Siphoviridae bacteriophages showed the lowest inactivation rates.

The published literature in Europe concludes a greater concentration of coliphage than the *B. fragilis* phage and a greater and more consistent number of the RYC phage compared to the HSP phage, and suggests that the change in ratios of coliphage and *B. fragilis* phage between sewage and receiving waters may be due to difference in survival. We corroborate these findings in the first study in the U.S. and demonstrate in particular that in warm tropical marine waters, differential survival between coliphages and *B. fragilis* phages is indeed a factor. Our studies also demonstrate the low detection of phages in treated and disinfected wastewater in the U.S., unlike Europe or other countries that do not routinely disinfect wastewater prior to discharge.

Our study showed very weak associations between the *B. fragilis* phage and the other indicator organisms, yet interestingly enough, the highest relationship was to the other virus indicator coliphage, and secondarily to enterococci, which has been recommended by USEPA for marine waters. *B. fragilis* phages may be useful in future water quality studies of Florida waters. The presence of the phage may indicate poorly treated sewage entering ambient waters, as *B. fragilis* phages were detected in only 3 of the 7 effluent samples using the host RYC and in none of the effluent samples using the host HSP. Even with untreated sewage inputs in areas with increased dilution, phages (host HSP) may be present in very low numbers and be undetectable without concentration. Studies in France corroborated this finding and reported that the density of *B. fragilis* phages was too low for unambiguous determination of their fate in river water compared to the more traditional bacterial indicators and coliphages (Skraber et al. 2002). This illustrates the need to use enrichment procedures when applying this assay to environmental monitoring. New methods that allow for a greater concentration of all types of phages have recently been suggested and have demonstrated greater sensitivity (Mooijman et al. 2005). We recommend that this method be tested for use in U.S. waters. Other recommendations for use of the *B. fragilis* phage assay as an additional indicator of fecal pollution in waters in the U.S. include use when untreated sewage is suspected, use when there is a need to strengthen the evidence of the presence of human fecal inputs, and use when traditional indicators may be regrowing or dying off quickly due to warm temperatures and the tropical nature of the environment.

Acknowledgments

This work was funded in part by the Tampa Bay Healthy Beaches Project in association with the Pinellas County Department of Health, the Florida Environmental Protection Agency, the Southwest Florida Water Management District, and the Tampa Bay Estuary Program. A special thanks to the Hillsborough County Environmental Protection Commission for providing monthly control samples and to Dr. Juan Jofre, Dr. Francisco Lucena, and Dr. Javier Mendez at the University of Barcelona for providing host and phage organisms and for technical support throughout the project.

Literature Cited

Ackermann, H.-W. and M. S. DuBow. 1987. Viruses of Prokaryotes, Volume 1. CRC Press, Boca Raton, Florida.

Allsop, K. and D. J. Stickler. 1984. The enumeration of *Bacteroides fragilis* group organisms from sewage and natural waters. *Journal of Applied Bacteriology* 56:15–24.

American Public Health Association (APHA). 1998. American Water Works Association and the Water Environmental Federation, p. 9-1–9131. *In* A. E. Greenburg, L. S. Clesceri, and A. D. Eaton (eds.), Standard Methods for the Examination and Water and Wastewater, 21st edition. American Public Health Association, Washington, D. C.

Araujo, R. M., J. Lasobras, F. Lucena, and J. Jofre. 1993. Methodological improvements for the recovery of *B. fragilis* phages and coliphages from environmental samples. *Water Science and Technology* 27:119–122.

Araujo, R. M., A. Puig, J. Lasobras, F. Lucena, and J. Jofre. 1997. Phages of enteric bacteria in fresh water with different levels of faecal pollution. *Journal of Applied Microbiology* 82:281–286.

Bisson, J. W. and V. J. Cabelli. 1979. Membrane filtration enumeration method for *Clostridium perfringens*. *Applied and Environmental Microbiology* 37:55–66.

Bosch, A., C. Tartera, R. Gajardo, J. M. Diez, and J. Jofre. 1989. Comparative resistance of bacteriophages active against *Bacteroides fragilis* to inactivation by chlorination or ultraviolet-radiation. *Water Science and Technology* 21:221–226.

Byappanahalli, M. N. and R. S. Fujioka. 1998. Evidence that tropical soil environment can support the growth of *Escherichia coli*. *Water Science and Technology* 38:171–174.

Chung, H. and M. D. Sobsey. 1993. Comparative survival of indicator viruses and enteric viruses in seawater and sediments. *Water Science and Technology* 27:425–428.

Contreras-Coll, N., F. Lucena, K. Mooijman, A. Havelaar, V. Pierzo, M. Boque, A. Gawler, C. Holler, M. Lambiri, G. Mirolo, B. Moreno, M. Niemi, R. Sommer, B. Valentin, A. Wiedenmann, V. Young, and J. Jofre. 2002. Occurrence and levels of indicator bacteriophages in bathing waters throughout Europe. *Water Research* 36:4963–4974.

Duran, A. E., M. Muniesa, X. Mendez, F. Valero, F. Lucena, and J. Jofre. 2002. Removal and inactivation of indicator bacteriophage in fresh waters. *Journal of Applied Microbiology* 92:338–347.

EDBERG, S. C., H. LECLERC, AND J. ROBERTSON. 1997. Natural protection of spring and well drinking water against surface microbial contamination. II. Indicators and monitoring parameters for parasites. *Critical Reviews in Microbiolog* 23:179–206.

FLEISHER, J. M., D. KAY, R. L. SALMON, F. JONES, M. D. WYER, AND A. F. GODFREE. 1996. Marine waters contaminated with domestic sewage: Nonenteric illnesses associated with bather exposure in the United Kingdom. *American Journal of Public Health* 86:1228–1234.

FUJIOKA, R. S. 2001. Monitoring coastal marine waters for spore-forming bacteria of faecal and soil origin to determine point from non-point source pollution. *Water Science and Technology* 44:181–188.

FUJIOKA, R. S. AND L. K. SHIZUMURA. 1985. Clostridium perfringens: A reliable indicator of stream water quality. *Journal of Water Pollution Control Federation* 57:986–992.

GALLAGHER, T. P. AND D. F. SPINO. 1968. The significance of numbers of coliform bacteria as an indicator of enteric pathogens. *Water Research* 2:169–175.

GRABOW, W. O. K., T. E. NEUBRECH, C. S. HOLTZHAUSEN, AND J. JOFRE. 1995. *Bacteroides fragilis* and *Escherichia coli* bacteriophages: Excretion by humans and animals. *Water Science and Technology* 31:223–230.

GRIFFIN, D. W., C. J. GIBSON III, E. K. LIPP, K. RILEY, J. H. PAUL, AND J. B. ROSE. 1999. Detection of viral pathogens by reverse transcriptase PCR and of microbial indicators by standard methods in the canals of the Florida Keys. *Applied and Environmental Microbiology* 65:4118–4125.

GRIFFIN, D. W., E. K. LIPP, M. R. MCLAUGHLIN, AND J. B. ROSE. 2001. Marine recreation and public health microbiology: Quest for the ideal indicator. *BioScience* 51:817–825.

HAVELAAR, A. H., K. FURUSE, AND W. M. HOGEBOOM. 1986. Bacteriophages and indicator bacteria in humans and animal feces. *Journal of Applied Bacteriology* 60:255–262.

HOLDEMAN, L. V., I. J. GOOD, AND W. E. C. MOORE. 1976. Human fecal flora: Variation in bacterial composition within individuals and a possible effect of emotional stress. *Applied and Environmental Microbiology* 31:359–375.

INTERNATIONAL STANDARDS PROTOCOL. 2001. Water Quality—Detection and Enumeration of Bacteriophages, Enumeration of Bacteriophages Infecting Bacteroides fragilis. ISO protocol CD 10705-4 (ISO/TC147/SC 4WG 11 N36), International Organization for Standardization, ISO Central Secretariat, 1 rue de Varembe, Case postale 56, CH-1211, Geneva 20, Switzerland, www.iso.org.

JOFRE, J., M. BLASI, A. BOSCH, AND F. LUCENA. 1989. Occurrences of bacteriophages infecting *Bacteroides fragilis* and other viruses in polluted marine sediments. *Water Science and Technology* 21: 15–19.

JOFRE, J., A. BOSCH, F. LUCENA, R. GIRONES, AND C. TARTERA. 1986. Evaluation of *Bacteroides fragilis* bacteriophages as indicators of the virological quality of water. *Water Science and Technology* 18: 167–173.

KREADER, C. A. 1995. Design and evaluation of Bacteroides DNA probes for the specific detection of human fecal pollution. *Applied and Environmental Microbiology* 61:1171–1179.

LASOBRAS, J., M. MUNIESA, J. FRIAS, F. LUCENA, AND J. JOFRE. 1997. Relationship between the morphology of bacteriophages and their persistence in the environment. *Water Science and Technology* 35:129–132.

LIPP, E. K. 1999. The occurrence, distribution and transport of human pathogens in coastal waters of southwest Florida. Ph.D. Dissertation, Department of Marine Science, University of South Florida, Tampa, Florida.

LIPP, E. K., R. KURZ, R. VINCENT, C. RODRIQUEZ-PALCOIS, S. R. FARRAH, AND J. B. ROSE. 2001. The effects of seasonal variability and weather on microbial fecal pollution and enteric pathogens in a subtropical estuary. *Estuaries* 24:266–276.

LUCENA, F., R. ARAUJO, AND J. JOFRE. 1996. Usefulness of bacteriophages infecting *Bacteroides fragilis* as index microorganisms of remote faecal pollution. *Water Research* 30:2812–2816.

LUCENA, F., X. MENDEZ, A. MORON, E. CALDERON, C. CAMPOS, A. GUERRERO, M. CARDENAS, C. GANTZER, L. SHWARTZBROOD, S. SKRABER, AND J. JOFRE. 2003. Occurrence and densities of bacteriophages proposed as indicators and bacterial indicators in river waters from Europe and South America. *Journal of Applied Microbiology* 94:808–815.

LUCENA, F., M. MUNIESA, A. PUIG, R. ARAUJO, AND J. JOFRE. 1995. Simple concentration method for bacteriophages of *Bacteroides fragilis* in drinking water. *Journal of Virological Methods* 54:121–130.

MOOIJMAN, K. A., Z. GHAMESHLOU, M. BAHAR, J. JOFRE, AND A. H. HAVELAAR. 2005. Enumeration of bacteriophages in water by different laboratories of the European Union in two interlaboratory comparison studies. *Journal Virological Methods* 127: 60–68.

NATIONAL RESOURCE DEFENSE COUNCIL REPORT (NRDC). 2004. Testing the Waters 2004: A Guide to Water Quality at Vacation Beaches. M. Dorfman (ed.), 14th edition, National Resource Defense Council Publications Department, New York.

PUIG, A., J. JOFRE, AND R. ARAUJO. 1997. Bacteriophages infecting various *Bacteroides fragilis* strains differ in their capacity to distinguish human from animal faecal pollution. *Water Science and Technology* 35:359–362.

PUIG, A., N. QUERALT, J. JOFRE, AND R. ARAUJO. 1999. Diversity of *Bacteroides fragilis* strains in their capacity to recover phages from human and animal wastes and from fecally polluted wastewater. *Applied and Environmental Microbiology* 65:1772–1776.

SINTON, L. W., R. K. FINLAY, AND D. J. HANNAH. 1998. Distinguishing human from animal faecal contamination in water—A review. *New Zealand Journal of Marine and Freshwater Research* 32: 323–348.

SKRABER, S., C. GANTZER, A. MAUL, AND L. SCHWARTZBROD. 2002. Fates of bacteriophages and bacterial indicators in the Moselle river (France). *Water Research* 36:3629–3637.

SUN, Z. P., Y. LEVI, L. KIENE, N. DUMOUTIER, AND F. LUCENA. 1997. Quantification of bacteriophages of *Bacteroides fragilis* in environmental water samples of Seine River. *Water Air and Soil Pollution* 96:175–183.

TARTERA, C., R. ARAUJO, T. MICHEL, AND J. JOFRE. 1992. Culture and decontamination methods affecting enumeration of phages infecting *Bacteroides fragilis* in sewage. *Applied and Environmental Microbiology* 58:2670–2673.

TARTERA, C. AND J. JOFRE. 1987. Bacteriophages active against *Bacteroides fragilis* in sewage polluted waters. *Applied and Environmental Microbiology* 53:1632–1637.

TARTERA, C., J. JOFRE, AND F. LUCENA. 1988. Relationship between numbers of enteroviruses and bacteriophages infecting *Bacteroides fragilis* in different environmental samples. *Environmental Technology Letters* 9:407–410.

TARTERA, C., F. LUCENA, AND J. JOFRE. 1989. Human origin of *Bacteroides fragilis* bacteriophages present in the environment. *Applied and Environmental Microbiology* 55:2696–2701.

U.S. ENVIRONMENTAL PROTECTION AGENCY (USEPA). 1986. Ambient Water Quality Criteria for Bacteria. Document number EPA 440/5-84-002. USEPA, Washington, D. C.

YATES, M. V., C. P. GERBA, AND L. M. KELLEY. 1985. Virus persistence in groundwater. *Applied and Environmental Microbiology* 49:778–781.

YATES, M. V. AND R. SCOTT. 1988. Modeling microbial fate in the subsurface environment. *CRC Critical Reviews in Environmental Control* 17:307–344.

Received, June 1, 2005
Revised, November 28, 2005
Accepted, December 7, 2005