

F+ RNA Coliphages as Source Tracking Viral Indicators of Fecal Contamination

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Submitted by

**Dr. Mark D. Sobsey,
David C. Love and Greg L. Lovelace
Department of Environmental Sciences and Engineering
University of North Carolina at Chapel Hill
Chapel Hill, North Carolina, 27599-7400**

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ABSTRACT

Fecal contamination of coastal recreational water and bivalve molluscan shellfish is a public health concern. Direct testing of water and shellfish for pathogens is technically challenging and costly, so regulatory programs and coastal managers rely on monitoring microbial indicators of fecal pollution. This study evaluates and applies methods for recovery, detection, and quantification of 6 indicator microbes, specifically F+ and somatic coliphages, enterococci, fecal coliforms, *Escherichia coli*, and *Clostridium perfringens*, in shellfish and estuarine water in 9 geographically diverse USA estuaries. It is also the first large-scale evaluation of the Reverse Line Blot (RLB) hybridization assay for FRNA coliphage detection and genotyping in environmental samples. Bacterial indicator methods and some F+ and somatic coliphage methods detected significantly more microbes in water at human-impacted sites than at non-impacted or pristine sites. Microbial indicator concentrations in water and shellfish were higher at sites with greater wastewater treatment plant discharges. For coliphages, a two-step enrichment was most sensitive, but direct membrane filtration (for water) and single agar layer (for water and shellfish) methods provided more diverse groups of field isolates for microbial source tracking. The sensitivity and specificity of the RNase test, FRNA coliphage reverse transcriptase PCR and RLB hybridization assay was assessed. Among 1033 FRNA isolates, 99.9% gave confirmed positive RT-PCR products and 98.3% were genotyped by RLB. The genotyping rates among estuaries ranged from 96.6% to 100%. Most estuaries had greater levels of group II (GA-like) F+ RNA coliphages in human-impacted sites than non-impacted sites. However, F+ RNA coliphage source tracking was not quantitatively reliable because high proportions of group I (MS2-like) coliphages were found in samples from both human and animal impacted sites. Eighteen field isolates were not typed by RLB, and a portion of their replicase gene region was sequenced for positive confirmation. A phylogenetic tree of leviviruses mapped 4 isolates to the JS subgroup with >40% sequence variation, which further confirms the existence of additional FRNA groups and shows that RT-PCR and sequencing may improve our knowledge of coliphage ecology beyond what is known from serological methods. RLB assay was a robust method for the detection and genotyping of FRNA coliphages from diverse geographic areas, and useful for microbial source tracking and total maximum daily load estimates. This information on the occurrence, levels, types, sources, and performance of detection techniques for microbial fecal indicators informs the design and conduct of human health effects studies on marine bathing waters and edible bivalve molluscan shellfish and choices of fecal indicators for management decisions.

INTRODUCTION

Growing coastal area populations and development bring increased human waste loads that need to be treated and managed. Human fecal wastes can harbor pathogenic human enteric viruses such as hepatitis A virus, enteroviruses, adenoviruses, and noroviruses, as well as bacterial and protozoan pathogens. Enteric viruses survive better than fecal indicator and pathogenic bacteria in wastewater treatment plants (WWTPs) and (Chung et al., 1998), and treatment is often inadequate to prevent contamination of water and shellfish (Shieh, 2003; Lodder and de Roda Husman, 2004). Contamination of coastal recreational water and bivalve molluscan shellfish by point and non-point source fecal waste is an important public health concern. Epidemiological studies found swimming in ocean water is associated with an increased risk of illness (Corbett et al., 1993; Prieto, et al., 2001; Colford et al., 2006), and specifically when swimming near storm drains and in water harboring enteric viruses (Haile et al., 1999). Links between consumption of bivalve shellfish and enteric viral diseases are also established (Shieh et al., 2000; Sanchez et al., 2002; Kingsley et al., 2002). Direct testing of water and shellfish for enteric viruses is expensive, time consuming, and technically difficult. Instead, regulatory programs and coastal managers use bacterial indicators of fecal pollution (enterococci and fecal coliforms) for waters and shellfish quality monitoring.

The Beaches Environmental Assessment and Coastal Health (BEACHES) Act of 2000 applies US EPA Enterococci criteria for bathing beaches (USEPA 1986), and the US Food and Drug Administration National Shellfish Sanitation Program regulates the microbiological quality of shellfish growing waters and shellfish meat using fecal coliforms (US FDA 2003). However, water and shellfish with acceptable fecal indicator bacteria levels can contain excessive levels of enteric viruses causing health risks (Chung et al., 1998; Dore et al., 2000; 2003; Formiga-Cruz et al., 2003). Fecal indicator bacteria are present in the gut of and are excreted by all warm-blooded animals, including birds. Because estuaries and coastal regions are prime areas for breeding bird populations and habitats for mammalian wildlife, reliance on fecal indicator bacteria criteria and standards in areas with known non-point source pollution by feral animal populations and migratory or resident bird populations may unnecessarily restrict molluscan shellfishing and recreational use by overestimating or misclassifying fecal contamination as human rather than animal.

F+ coliphage viral indicators have been used successfully to identify animal sources of fecal pollution in a Florida water body and a New York City reservoir, the latter finding having led to a bird deterrent program to reduce fecal inputs (Griffin et al., 2000; Alderisio et al., 1996). In these examples, F+ coliphage indicators were used as alternatives to fecal indicator bacteria for microbial source tracking. F+ coliphages are viruses infecting *E. coli* bacteria; their presence in water and shellfish indicates the presence of fecal waste (Contreras-Coll et al., 2002). Coliphages correlate with the presence of pathogenic human viruses in water and shellfish and increased viral illness risks (Chung et al., 1998; Havelaar, 1993; Dore et al., 2000; Jiang et al., 2001; Wade et al., 2003; Colford et al., 2006). The RNA group of F+ coliphages (FRNA coliphages) is used primarily as fecal indicator viruses because it resembles human enteric viruses (e.g., hepatitis A and E viruses, enteroviruses, noroviruses and astroviruses) in size, shape and

general composition (Havelaar 1993; Hsu 1995; Sobsey 1995). FRNA coliphages are also fecal source tracking microbes, because they distinguish human fecal waste from non-human fecal waste (Furuse et al., 1981; Osawa et al., 1981, Hsu et al., 1995; Vinjé et al., 2004).

This study evaluates and applies methods for the detection, quantification and identification of sources of microbial fecal contaminants in shellfish and marine/estuarine water using coliphage and bacterial indicators. Three assays for F+ or somatic coliphage recovery and detection, US EPA method 1601 and 1602, and Direct Membrane Filtration, were compared and validated in estuarine water and shellfish samples collected seasonally from 9 sites on the East, West, and Gulf coasts having diverse fecal waste sources and levels. The results of F+ and somatic coliphage assays were compared with those for a suite of fecal indicator bacteria (*E. coli*, fecal coliforms, enterococci, *Clostridium perfringens*) to determine if these coliphage analyses provide statistically equivalent results as the bacteria for the detection of fecal contamination. FRNA coliphage isolates underwent molecular genetic characterization by reverse transcription polymerase chain reaction (RT-PCR) and Reverse Line Blot hybridization (Vinjé et al., 2004) in an effort to substantiate their microbial source tracking potential and performance as indicators of fecal pollution in estuary water and shellfish.

The coliphage methods, if shown to work in a variety of areas containing water of varying physical and chemical characteristics and impacted by different sources of fecal contamination, will provide coastal zone managers and shellfish sanitation programs with reliable microbial tools to protect bathers and consumers of shellfish from human enteric virus exposures. These tools will also allow authorities to better assess and manage coastal development and its impacts. When used in conjunction with shoreline surveys, coliphage testing will more conclusively pinpoint sources of fecal contamination, allowing state and local agencies to better control those sources.

OBJECTIVES

The overall project objective is to evaluate and apply novel, cost-effective technologies and methods for the detection, quantification and identification of sources of microbial contaminants and the characterization of those sources as human or non-human. Specific objectives follow.

(1) A main objective is to validate and apply new and rapid methods for coliphage detection, quantification and grouping in estuarine water and shellfish samples from a variety of sites, consisting of representative National Estuarine Research Reserves (NERRs) having diverse fecal waste sources and levels. The results of coliphage assays will be compared with those for a suite of fecal indicator bacteria to show that these coliphage analyses provide statistically equivalent results for the detection of fecal contamination.

(2) Methods used to distinguish human from animal fecal contamination by coliphage isolate typing using nucleic acid hybridization biosensor technology will be validated and

applied. An effort will be made to substantiate that specific coliphage groups are indicators of human enteric viruses in estuary water and shellfish.

(3) Coliphage assay kits will then be developed which contain the necessary materials for coliphage analysis and F+ RNA coliphage grouping on samples of shellfish and estuarine water.

(4) Using the resources and expertise of the education office of the NCNERR, workshops and other dissemination tools will be developed, based on an audience needs assessment, to train state agencies, private labs and larger communities in the use of these methods and kits. Through implementation, these tools will provide new and better sources of information regarding levels and sources of fecal contamination to coastal management and shellfish sanitation programs, thereby reducing the risks of viral disease to the public who use estuaries for recreation and for shellfishing communities who draw their livelihoods from those waters.

METHODS & MATERIALS

Study sites, sample collection and processing. Sampling sites were chosen in 9 estuaries that are part of the National Estuarine Research Reserves (NERR) system. At each estuary two sites were sampled— one in an area approved for shellfish harvesting and one in a prohibited area where fecal contamination levels exceed those allowable for shellfish harvesting and/or primary contact recreation. Samples consisted of 10-12 oysters, 10-20 clams, and/or 10-20 mussels (at sites where shellfish were available) and 4-liter grab samples of estuarine water. Samples were shipped on ice by a commercial carrier and processed within 24 hours of collection. Oysters, clams, and mussels were rinsed, aseptically opened with sterilized shellfish shucking knives, and batches of shellfish were homogenized (Waring Blender; Torrington, CT) at high speed for 1 to 2 minutes. The resulting shellfish tissue homogenate was assayed for indicator bacteria and coliphages.

Indicator bacteria in shellfish. Fecal coliform and *E. coli* bacteria in the shellfish homogenates were enumerated by Multiple Fermentation Tube methods as previously described (APHA, 1998). Shellfish homogenate (5 replicates and tenfold-dilutions thereof) were added to lauryl tryptose broth with inverted vials, incubated for 24-48 hr at 35°C, and vials with gas production confirmed on fresh EC-MUG medium as fecal coliforms (growth and gas production) and *E. coli* (growth, gas production and blue fluorescence under long wavelength UV light). The combination of positive tubes was used to compute the Most Probable Number (MPN) of fecal coliforms and *E. coli* per 100 ml of homogenate (100 grams of shellfish meat). Enterococci were enumerated by direct plating of shellfish homogenate (replicate 1 ml and tenfold-dilutions) mixed with 15 ml molten mEnterococcus agar on 150x15mm petri dishes (Clesceri et al., 1998; Bordner et al.). The plates were incubated for 48 ± 3 hr at 35°C, with red colonies as presumptive positives. A representative number of dark blue presumptive colonies were confirmed by streaking on membrane filters placed on mEI plates, incubated for 24 ± 2 hr at 41°C and

observed for growth distinctive of enterococci. The colony forming units (CFU) of enterococci per 100 ml of homogenate (or 100 grams of shellfish meat) was based on % of total colonies confirmed. For *Clostridium perfringens* detection and enumeration, shellfish homogenates (1 ml and tenfold serial dilutions thereof) were inoculated into 10 ml Iron Milk medium, incubated overnight at 41-44.5°C (St. John et al., 1982), and presumptive positive tubes having “stormy fermentation” were confirmed by streaking on filter membranes on mCP agar plates (as described above). Iron Milk Medium contains 1 can of evaporated milk (12 oz Carnation evaporated milk, Nestlé), 950 ml sterile deionized water, and 50 ml of filter-sterilized 2% FeSO₄ solution. The combination of confirmed positive tubes was used to compute the MPN of *C. perfringens* per 100 ml of homogenate (or 100 grams of shellfish meat).

Indicator bacteria in estuary water. Estuary water samples were analyzed by the membrane filter techniques for fecal coliforms, *E. coli*, enterococci, and *C. perfringens* using duplicate volumes of 100 ml, 10 ml, and 1 ml as previously described (APHA, 1998). Briefly samples of water were vacuum filtered through a 47 mm diameter, 0.45 µm pore size, cellulose ester filters (Millipore, Billerica, MA), and filters were placed on mFC agar petri plates to detect fecal coliforms or mCP agar plates to detect *C. perfringens*. The mFC plates were incubated for 2-5 hours at 37°C for resuscitation of injured bacteria, transferred to 44.5°C for a total incubation of 24 ± 2 hours. Blue colonies (fecal coliforms) were enumerated and transferred to nutrient agar plates with MUG (4-methylumbelliferyl-β-D-glucuronide)(Sigma-Aldrich, St. Louis, MO), incubated for 4 to 6 hours at 44.5°C, and long-wave ultraviolet light exposure for enumerating fluorescent bright blue colonies (*E. coli*). Enterococci were detected (dark blue colonies surrounded by a dark blue halo of precipitate) and enumerated on mEI agar plates after 24 ± 3 hr incubation at 41°C. *C. perfringens* were detected (as bright pink colonies when exposed to ammonium hydroxide (NH₄OH) fumes) after 18 hr incubation at 44.5°C in anaerobic conditions on mCP agar plates.

Coliphage recovery assays. Samples of homogenized shellfish tissue and estuary water were spilt and assayed for both somatic and F+ coliphages by 2 methods for water samples (US EPA methods 1601 and 1602) and 3 methods for shellfish samples (US EPA methods 1601 and 1602, and Direct Membrane Filtration). The US EPA methods were originally validated for use with groundwater, but applied to new matrices in this study after confirming their effective performance in preliminary studies that compared them to standard, “benchmark” methods. All methods employ specialized strains of *E. coli* as the bacterial host for assay of either somatic or F+ coliphages. Strain CN13 (ATCC # 700609) was used to detect somatic coliphage and strain F_{amp} (ATCC # 700891) was used to detect F+ coliphages. Assays used antibiotic Nystatin for *E. coli* CN13 and Streptomycin sulfate and Ampicillin for *E. coli* F_{amp} to prevent competing bacterial growth. Positive controls and negative controls were run in parallel with field samples.

US EPA Method 1602- Single Agar Layer Assay was performed as described previously (EPA 2001b), using 10 replicates of 10ml aliquots of water and as a new application using 10 replicates of 1ml aliquots of shellfish homogenate. Plaques (zones of bacterial lysis) were enumerated and the titer of coliphages in the sample calculated as PFU (plaque-forming units) per 100 ml (or 100 grams of shellfish meat).

US EPA Method 1601- Two Step Enrichment Assay was performed as previously described (USEPA, 2001a), except a MPN assay was used to achieve quantitative results. Triplicate volumes of 300ml, 30 ml, and 3 ml for water, and in a new application, triplicate amounts of 3 grams, 0.3 grams, and 0.03 grams of shellfish homogenate were assayed. Coliphage presence/absence was scored for each dilution and replicate to give the MPN/100ml (or 100 grams of shellfish meat).

Direct Membrane Filtration (DMF) was applied to samples of estuary water as described previously (Sobsey et al., 1990), with some modifications. For each sample, 10 replicates of 100 ml of estuarine water were vacuum filtered through 47 mm diameter, 0.45 μ m pore size cellulose ester filters. Filters were then placed face-down on 60 x 15 mm petri dishes containing 0.75% TSA, log-phase *E. coli* F_{amp} (male-specific coliphages) or *E. coli* CN13 (somatic coliphages), 0.3% Tween-80 and 100 μ g/ml X-Gal (5-bromo-4-chloro-3-indolyl-beta-D-galactoside) (Sigma-Aldrich, St. Louis, MO) and IPTG (isopropylthio-beta-D-galactoside) (Sigma-Aldrich, St. Louis, MO). After inverted incubation at 35-37°C overnight, blue coliphage plaques were counted, with titers expressed as PFU (plaque-forming units) per 100 ml water.

Coliphage isolation and RNase testing. As many as 10 F+ coliphage plaques and somatic plaques per sample were randomly chosen from SAL, DMF or enrichment spot plates. Plaque material was enriched in 5 ml of TSB by EPA method 1601, clarified by 1200 *xg* centrifugation for 20 min, and frozen at -80°C for subsequent confirmation and genogrouping. F+ coliphage also underwent an RNase test as previously described (Hsu et al., 1995) by re-plating the isolates in the presence and absence of Ribonuclease A (Sigma-Aldrich, St. Louis, MO) to determine if the coliphage had DNA or RNA as its nucleic acid. Coliphages that grew in the presence of RNase were considered FDNA and those that did not were considered FRNA.

FRNA coliphage detection and genotyping by Reverse Line Blot hybridization. Approximately 1,500 F+ coliphage isolates were further subjected to a typing test to distinguish the 4 groups of F+ RNA coliphages (Groups I, II, III, and IV) by broadly-reactive RT-PCR amplification of a partial region of the replicase gene of both levi- and alloviviruses using biotinylated primers (Vinjé et al., 2004). RT-PCR products were then further characterized by reverse line blot hybridization (RLB) using a panel of group- and subgroup-specific probes in assays (Vinjé et al., 2004). FDNA coliphages were analyzed by PCR to confirm their presence in mixed isolates containing both FRNA and FDNA coliphages (Vinjé et al., 2004). Bound (RT)-PCR products on the RLB membranes were detected by chemiluminescence on a Biomax MS light X-ray film (Kodak, Rochester, NY) for 30 to 60 min. and the film was developed in a SRX-101A film processor (Konika, Wayne, NJ).

DNA sequencing and phylogenetic analysis. FRNA coliphage strains that generated RT-PCR products with appropriate size for leviviruses (266 bp) or alloviviruses (229 bp), but did not hybridize to any of the RLB probes were sequenced. FRNA RT-PCR products were purified using the QIAquick PCR Purification Kit (Qiagen, Valencia, CA) and sequenced at the Nucleic Acids Core Facility (UNC, Chapel Hill, NC). DNA sequences were edited and aligned using BioEdit (Hall, 1998) and

imported into TreeCon (V 1.3b) (van der Peer, 1994). Phylogenetic trees were derived using the Jukes and Cantor correction and the confidence values of the internal nodes were calculated by performing 100 bootstrap analyses.

Data Management and Analysis. Data were recorded in laboratory notebooks and entered into spreadsheets in Excel software (Microsoft, Redmond, CA). Summary statistics and statistical tests were performed with SPSS (Chicago, IL) and InStat (GraphPad Software Inc., San Diego, CA). In graphs, data was normalized using \log_{10} values, and both standard deviation bands and outliers were reported. Where appropriate, trendlines were fit to series of mean levels of microbes, with R-squared values reported. Comparisons between matched sets of samples were made for 2 pair using Wilcoxon Signed Rank test, and for 3 or more with Friedman's test and Dunn's multiple comparison test. Proportions of FRNA coliphage genogroups were compared between methods using a Z-test statistic with 2 tails and an alpha of 0.05, and Chi-squared analysis. Significance was set before analysis at an alpha of 0.05, and reported with P-values.

RESULTS

Sampling sites and fecal contamination sources. Water and shellfish were sampled in estuaries that had both sites proximal (impacted) and distant (non-impacted) to sources of human fecal contamination. Sanitary surveys, total maximum daily load (TMDL) analysis, published literature, and first-hand accounts were used to characterize existing sources of fecal waste. Human point source pollution in this study was primarily from waste water treatment plant (WWTP) treated effluent and possibly raw sewage leaks, while likely human non-point sources included urban runoff, seepage from septic tanks, and boat dumping (Table 1). Sites with non-human non-point fecal waste contained populations of wildfowl (goose, duck, gull), wild horses, other feral animals, agricultural animals, a dog park and urban pet waste (Table 1). At 4 estuaries the impacted sites included human point and non-point sources, while the non-impacted sites were pristine sites with wildlife refuges or were geographically separated from human populations (Table 1). In the Tijuana River Reserve in Southern CA human impacts were documented at all study sites, so in the absence of a truly pristine or non-impacted site, a site with only non-point source runoff from human development was compared to a more contaminated site at the mouth of the Tijuana River containing untreated sewage from Mexico (Table 1).

Comparing levels of fecal indicators in impacted and non-impacted waters. Levels of fecal indicators in water samples from fecally impacted and non-impacted sites were compared in Fig. 1. It was of interest to determine the extent to which coliphage and bacterial indicators were effective indicators of fecal pollution in water, based on the expectation that the levels of the indicator would likely be higher in samples of the station more impacted by (closer to) the fecal contamination source than in samples from stations remote from (less impacted by) the fecal contamination source or considered pristine. Statistical comparisons were performed for each microbial indicator with the

Wilcoxon Signed Rank Test, using 33 to 35 matched pairs of neighboring impacted/non-impacted sites (Table 2). *C. perfringens*, enterococci, *E. coli*, and fecal coliforms were all detected in higher concentrations at impacted sites than non-impacted sites (p values of <0.001, < 0.001, 0.001, 0.003, respectively) (Fig. 1a, Table 2). The consistent use of membrane filtration methods was the basis for allowing a direct comparison among bacterial indicators. However, three different assays were compared for the recovery of somatic and F+ coliphages, which could result in different results because of differences in the performance of the methods. For F+ coliphage, Two-Step enrichment and single agar layer recovered more F+ coliphage at impacted sites than non-impacted sites (p values = 0.002 and 0.045) (Fig 1a, Table 2). Using the direct membrane filter method, there was a not quite significant difference between F+ coliphages at impacted and non-impacted sites (p value = 0.06) (Fig. 1a, Table 2). For somatic coliphages, only two-step enrichment detected significantly more coliphages at impacted sites than at non-impacted sites (p value = 0.012) (Fig. 1a, Table 2). Coliphage concentrations were generally lower by direct membrane filtration than by the other two methods, and these lower numbers also could have been a factor in not being able to detect significant differences between impacted and non-impacted sites.

Comparing levels of fecal indicators in impacted and non-impacted shellfish.

In a similar manner as for water samples, levels of fecal microbes in shellfish from impacted and non-impacted sites were compared (Fig. 1b). All comparisons were made using the Wilcoxon Signed Rank Test on 33 or 34 sets of matched pairs (Table 2). The direct colony counting method for Enterococci detected a similar range of concentrations as was detected by multiple fermentation tube MPN type methods for *E. coli* and fecal coliforms (Fig. 1b, Table 2). However, only among MPN type methods, and only for *C. perfringens* and *E. coli*, were levels higher in impacted sites than non-impacted sites (p values = 0.014 and 0.023, respectively (Table 2). None of the somatic or F+ coliphage methods recovered more phages from contaminated sites than uncontaminated sites, but the Single Agar Layer method for F+ coliphage gave a not quite significant difference for the levels in impacted and non-impacted sites (p value = 0.073) (Fig. 1b, Table 2).

TABLE 1. Point and non-point sources of human and non-human fecal contamination at field sampling sites.

Site (samples)	Point and non-point sources of human and non-human fecal contamination
Elkhorn Slough, California (water; oysters; mussels)	Elkhorn Slough is a coastal marsh on Monterey Bay in central California. Inland is a hilly agricultural region with sandy soil (NOAA 2000). Hudson's Landing site is impacted by human and agricultural runoff from freshwater flows. South Marsh site at Whistlestop Lagoon does not have human or agricultural sources and receives no inland freshwater.
Tijuana River, California (water; mussels)	The impacted site is at the egress of the Tijuana River into the Pacific Ocean south of San Diego, CA. During the rainy season the Tijuana River contains untreated human sewage from Mexico, and 70% of the Tijuana River Basin is in Mexico where no secondary treatment of sewage is provided. The largest city near the Tijuana River is Tijuana, Mexico that has a population of 1.2 million people. An international WWTP ^a at the border of US and Mexico treats 25 MGD ^b of sewage from the river's dry-weather flows, but is not designed to treat large volumes of sewage during wet weather flows (Zuniga 2005). A less contaminated site is at Shelter Island in the San Diego Bay, which receives non-point-source input from human development. A San Diego WWTP serves a metropolitan population of 2.2 million treating 175 MDG, but the treatment plant effluent is discharged 4.5 miles out to sea, and far from sampling sites.
Delaware Bay, Delaware (water)	Scotton Landing site is around 6 miles downstream from the 30,000 person town of Dover, DE, and around 3 miles upstream from the terminus of the St. John's River into the Delaware Bay. Scotton Landing receives non-point-source pollution from the upstream portion of the river, most likely from Dover where public recreation and urban runoff occur. The non-human impacted site is a waterfowl impoundment that receives seasonal non-point-source fecal pollution from waterfowl. The town of Dover and New Kent County provide secondary treatment with chlorine disinfection at a WWTP, which discharge into the Murderkill River. The Murderkill River and the St. John's River are not connected.
Apalachicola Bay, Florida (water; oysters)	Apalachicola River empties into the Gulf of Mexico. The impacted site is near the mouth of the Apalachicola River, 1 mile south of the 9,000 person town of Apalachicola. It receives inputs from Apalachicola WWTP, an activated sludge system with chlorine disinfection that produced 0.3 MGD monthly flows in 2003 (Apalachicola TMDL 2005). Wastewater effluent flows into a holding pond, then to a receiving wetland swamp and on to Apalachicola Bay. The surrounding county, Franklin County, has low population density (10 people/mi ²), and 57% of residents use septic tanks which opens the possibility for non-point source human impacts (Apalachicola TMDL 2005). The non-impacted site is on a barrier island in Apalachicola Bay, 5 miles southwest of Apalachicola.

Waquoit Bay, Massachusetts (water; clams)	Sites are on the southern side of Cape Cod on the Atlantic Ocean. Eel Pond Forks, an impacted site, is a narrow tidal creek used by local homeowners with recreational boats to access the ocean. Eel Pond Forks receives non-point source impacts from boats, runoff from dense human development, and possible sewage leaks from household septic tanks and/or groundwater discharge sites. Sage Lot Pond is a pristine site surrounded by salt marsh that supports seasonal wildfowl populations of swans, geese and ducks, with few human impacts and low residential home density in the watershed. An intermediate site in Waquoit Bay contains a mix of residential homes and undeveloped land.
Rachel Carson, North Carolina (water; oysters)	Rachel Carson is in coastal NC bordering the Pamlico Sound. The impacted sites are on the north side of Carrot island facing the town of Beaufort, NC and receive point-source human fecal waste from the mainland WWTP outfall with 0.8 MGD flows from a 5,500 person service area, and non-point-sources from the boats which moor in the harbor. The non-impacted sites are on the ocean side (south side) of Carrot island, an island uninhabited by humans, and receive non-point-source animal inputs from avian and mammalian wildlife, including a herd of feral horses which roams the island.
Masonboro Island, North Carolina (water; oysters)	Masonboro Island is a pristine barrier island in eastern NC near the 75,000 person city of Wilmington. The impacted site, Whiskey Creek, is on the mainland and receives non-point-source input from extensive human development surrounding the creek. The uncontaminated site, Research Creek, is on the sound side of Masonboro Island and separated from the mainland by the Intercoastal Waterway. Research Creek may be impacted by sea birds and other wildlife. The Wilmington WWTP discharges into the Cape Fear River, distant from all sampling sites.
Great Bay, New Hampshire (water; oysters)	The impacted site, Oyster River, runs past the town of Durham, NH (around 13,000 people) and Strafford County (304 people/mi ²), which had non-point sources from urban runoff, houses on the shoreline with septic systems, a dog park, a buffalo farm, and feral animals and birds. The town of Dover processes 1.3 MGD of sewage in an activated sludge WWTP and discharges effluent into Oyster River near Durham and less than 1 mile upstream from the sampling site. Nannie Island, a non-impacted site, is a small uninhabited island in the middle of Great Bay that may have seasonal wildfowl impacts.
Narragansett Bay, Rhode Island (water; clams)	Sites were located on Dyer Island, a small (0.5 mi x 0.25 mi) island in Narragansett Bay which is positioned 0.5 mi west of the town of Melville (around 2,300 people) and Newport County (821 people/mi ²), and 1 mile east of an underdeveloped Prudence Island. Providence, RI, a 178,000 person city and Newport, RI, a 26,000 person city are each 12 miles away from Dyer Island on rivers confluent with Narragansett Bay. The impacted site on the east side of Dyer Island is the only permanently closed shellfishing site in the Narragansett Bay Research Reserve and receives non-point-source inputs from extensive human development from the town of Melville and surrounding areas. The east side of Dyer Island is the uncontaminated site, which receives few human impacts and possibly some impacts from wildlife on the small island.

^a WWTP = waste water treatment plant

^b MGD = million gallons per day

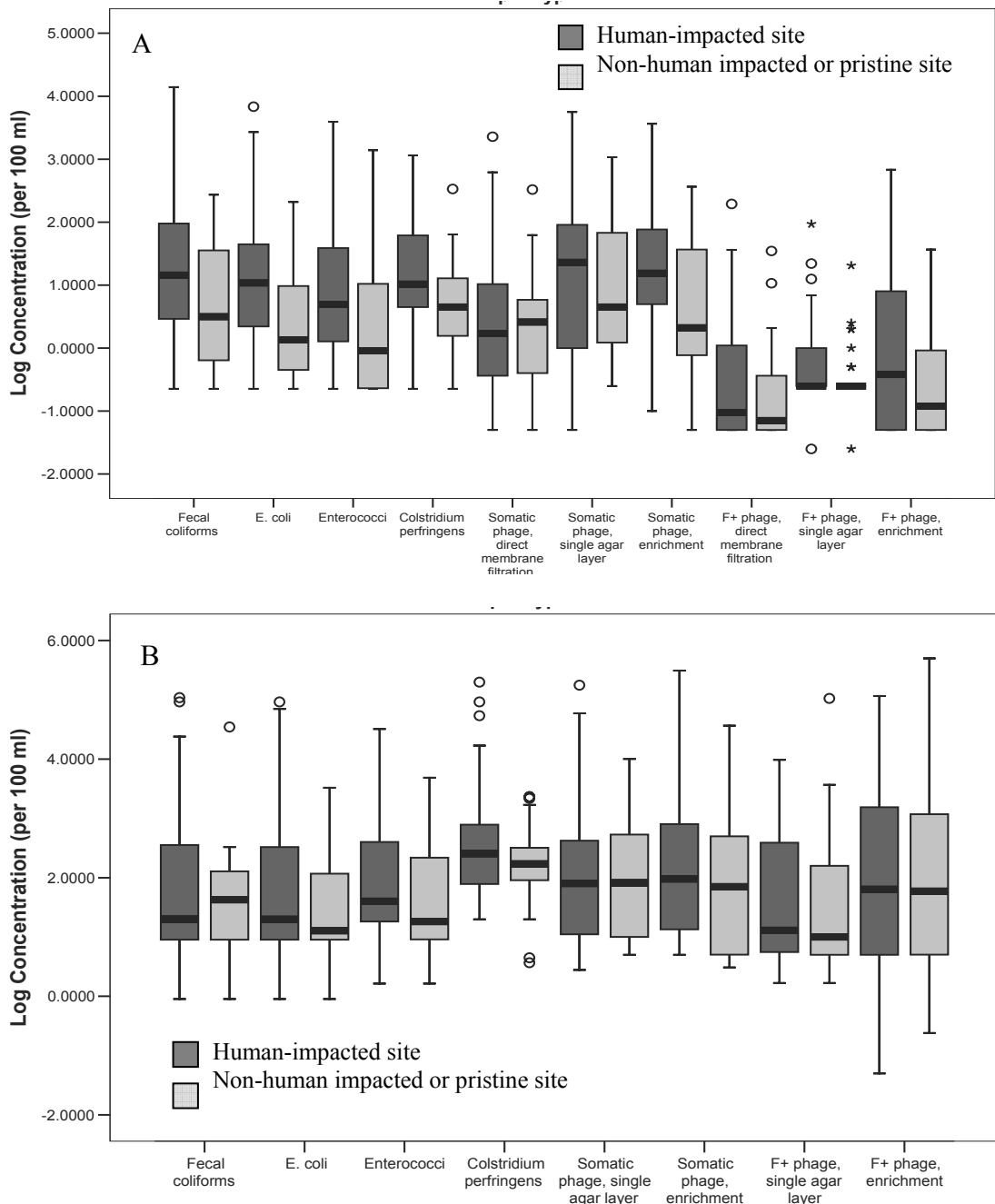


FIG 1. Box-and-whisker plots of log-levels of indicator bacteria and coliphage detected in estuarine water (a) and shellfish (b) at sites with human fecal impacts (dark grey) or pristine sites that may contain non-human fecal impacts (light grey). Data are from 9 estuaries with 2 to 4 sites per estuary and 3 to 5 sampling events per site. Box-and-whisker plot explained: lower and upper bands give minimum and maximum log-concentrations, the top and bottom of the box delineate the first and third quartiles, the horizontal black bar is the geometric mean concentration, and the open circles and stars are individual outliers.

TABLE 2. Comparison of indicator levels in human fecal impacted and non-human fecal impacted water and shellfish (Note: this table is a companion to Figure 1a and 1b)

Matrix	Indicator	Method	Impacted sites vs. non-impacted sites		
			Number of pairs	P value	Significant difference
Water	<i>C. perfringens</i>	MF	35	<0.001	yes, u < c ¹
	Enterococci	MF	35	<0.001	yes, u < c
	<i>E. coli</i>	MF	35	0.001	yes, u < c
	fecal coliform	MF	35	0.003	yes, u < c
	F+ coliphage	MF	34	0.060	no
	F+ coliphage	ENR	33	0.002	yes, u < c
	F+ coliphage	SAL	33	0.045	yes, u < c
	Somatic coliphage	MF	34	0.458	no
	Somatic coliphage	ENR	34	0.012	yes, u < c
	Somatic coliphage	SAL	35	0.518	no
Shellfish	<i>C. perfringens</i>	MFT	34	0.014	yes, u < c
	Enterococci	DP	33	0.062	no
	<i>E. coli</i>	MFT	34	0.023	Yes, u < c
	fecal coliform	MFT	34	0.183	no
	F+ coliphage	ENR	34	0.710	no
	F+ coliphage	SAL	34	0.073	no
	Somatic coliphage	ENR	33	0.710	no
	Somatic coliphage	SAL	34	0.782	no

¹The Wilcoxon signed rank test on matched pairs with level of significance set at alpha = 0.05. DP = Direct Plating; ENR = Two Step Enrichment; MF = Membrane Filtration; MFT = Multiple Fermentation Tube; SAL = Single Agar Layer

Comparison of three methods for F+ coliphage recovery from water and shellfish. Direct membrane filtration assay, single agar layer assay (US EPA Method 1602), and a quantitative two-step enrichment assay (US EPA Method 1601) were used in parallel to recover F+ coliphage from water, oysters, clams, and mussels. Based on volume of water assayed, two-step enrichment and direct membrane filtration both had theoretical lower detection limits of 0.1 plaque forming units (PFU) per 100 ml, while single agar layer detection method had a much higher lower detection limit of 5 PFU per 100 ml. In water samples, two-step enrichment assay detected F+ coliphage in 59% of samples (43/73), which was statistically similar (p value = 0.2442) to direct membrane filtration with 47% positives (36/75), while each was significantly more (two-step enrichment p value < 0.0001; direct membrane filtration p value = 0.0046) than single

agar layer with 24% positives (18/74) (Table 3). Two-step enrichment recovered more F+ coliphage isolates from water (n = 224) than single agar layer (n = 164) or direct membrane filtration (n= 38) (Table 3), although the number of unique strains was not analyzed. Genetic characterization of isolates by RLB genogrouping (for Groups I, II, III, and IV) revealed that a significantly higher percentage of FRNA Group I isolates were recovered by two-step enrichment than recovered by single agar layer and direct membrane filtration, respectively (p values = 0.028 and 0.0005) (Table 5). Similarly, single agar layer and direct membrane filtration both provided significantly higher percentages of FRNA Group II and III human-type isolates than two-step enrichment (p values = 0.028 and 0.0005) (Table 5).

In shellfish, two-step enrichment assay and single agar layer assay were compared in parallel. The geometric mean recovered by two-step enrichment was greater than by single agar layer for all shellfish and for clams only (Tables 3 and 5). The percentage of F+ coliphage positive samples by two-step enrichment was 66% (49/74), and was statistically equivalent (p value 0.3164) to the 54% (40/74) positive samples by single agar layer. Single agar layer yielded more FRNA coliphage isolates from all shellfish and from mussels than did two-step enrichment. After genogrouping all FRNA isolates, a significantly higher proportion of FRNA Group II/III isolates were recovered by single agar layer from mussels (p value <0.001), clams (p value <0.001), and all shellfish (p value = 0.001) than were recovered by two-step enrichment (Table 5). Single agar layer recovered a similar percentage of Group II/III isolates from clams, oysters and mussels, while two-step enrichment varied in the proportion of Group II/III it recovered with as few as 1% of clam isolates and as many as 19% of oyster (Table 3). The detection limit of each assay was approximately 4 PFU per 100 ml or 100 grams shellfish. Direct membrane filtration was not used on shellfish samples because shellfish tissue homogenates clog filters.

TABLE 3. F+ coliphage recovered by 3 methods from water, oysters, clams, and mussels, and the resulting FRNA coliphage genogroups isolated.

Matrix	Method	Recovered F+ coliphages		Recovered FRNA coliphages by genogroup		
		Geometric mean as log-PFU per 100 ml (\pm st dev)	# of samples (% below detection limit)	% Group I	% Group II & III	# FRNA isolates
Water ^a	ENR ^c	-0.36 (\pm 1.08)	73 (41%)	96% ^d	4%	224
	SAL ^c	-0.33 (\pm 0.62)	74 (76%)	84%	15% ^e	38
	DMF	-0.67 (\pm 0.86)	75 (52%)	85%	16% ^e	164
All ^b	ENR ^c	1.98 (\pm 1.43)	74 (34%)	90% ^d	9%	328
	SAL	1.59 (\pm 1.09)	74 (46%)	76%	24% ^e	351
Oysters ^b	ENR	1.81 (\pm 1.36)	34 (38%)	80%	19%	142
	SAL	1.65 (\pm 1.23)	34 (47%)	77%	24%	129
Clams ^b	ENR ^c	2.04 (\pm 1.43)	28 (36%)	99% ^d	1%	110
	SAL	1.40 (\pm 0.87)	28 (46%)	75%	24% ^e	93
Mussels ^b	ENR	2.34 (\pm 1.68)	12 (17%)	93% ^d	5%	76
	SAL	1.83 (\pm 1.16)	12 (42%)	77%	24% ^e	129

F+ coliphages recovery methods: DMF = Direct Membrane Filtration assay; SAL = Single Agar Layer Assay (US EPA Method 1602); and ENR = Two-Step Enrichment Assay (US EPA Method 1601). ^a Friedman's test non-parametric, and Dunn's multiple comparison test with matched comparisons for data in this row. ^b Wilcoxon matched-pairs signed-ranks test for data in these rows. ^c significantly different method with higher recoveries than other methods. ^d significant difference between the percentage of Group I genotypes recovered by methods (for water, all shellfish, oysters, clams, or mussels). ^e significant difference between the percentage of Group II and III genotypes recovered by methods (for water, all shellfish, oysters, clams, or mussels).

TABLE 4. Statistical analysis of 3 recovery methods for Male Specific (F+) coliphages from water and shellfish.

Matrix	Test	Male Specific (F+) coliphage					
		All matched pairs			Matched pairs without below detect values		
		Number of pairs	P value	Significant difference	Number of pairs	P value	Significant
Water ^a	ENR vs DMF	69	<0.05	yes DMF < ENR	12	>0.05	no *
	SAL vs DMF	69	<0.01	Yes DMF < SAL	12	>0.05	no *
	SAL vs ENR	69	>0.05	no	12	>0.05	no *
All Shellfish ^b	SAL vs ENR	73	<0.0001	yes SAL < ENR	37	0.0018	yes SAL < ENR
Oysters ^b	SAL vs ENR	34	0.252	no	16	0.4954	no
Clams ^b	SAL vs ENR	28	<0.0001	yes SAL < ENR	14	<0.0001	yes SAL < ENR
Mussels ^b	SAL vs ENR	11	0.083	no	7	0.2118	no

SAL = Single Agar Layer Assay (EPA method 1601); ENR = Two-Step Enrichment Assay (EPA method 1602); DMF = Direct Membrane Filtration method. ^a Friedman's test non-parametric, and Dunn's multiple comparison test for data in this row. ^b Wilcoxon matched-pairs signed-ranks test for data in these rows. * Significant differences (P values = 0.0458) exist among all 3 methods, but significant differences do not exist between any two methods.

TABLE 5. Comparison of the proportion of genogroups (Group I or Group II + III) recovered by FRNA coliphage methods.

Matrix	FRNA genogroup	Recovery method comparison (P value)		
		ENR vs. SAL	ENR vs. DMF	SAL vs. DMF
Water	I	ENR (0.028)	ENR (0.0005)	(0.429)
	II and III	SAL (0.028)	DMF (0.0005)	(0.429)
All Shellfish	I	ENR (<0.001)	NA	NA
	II and III	SAL (<0.001)	NA	NA
Oysters	I	(0.24)	NA	NA
	II and III	(0.21)	NA	NA
Clams	I	ENR (<0.001)	NA	NA
	II and III	SAL (<0.001)	NA	NA
Mussel	I	ENR (0.002)	NA	NA
	II and III	SAL (0.001)	NA	NA

Proportion of FRNA coliphage genogroups recovered were compared between methods using a Z test statistic with 2 tails and an alpha of 0.05. SAL = Single Agar Layer Assay (EPA method 1601); ENR = Two-Step Enrichment Assay (EPA method 1602); DMF = Direct Membrane Filtration method. NA = not available, because DMF was not performed on shellfish samples.

Comparison of somatic coliphage recovery by three methods in water and shellfish. Shellfish and water were also analyzed for somatic coliphages by 3 assays: direct membrane filtration assay, single agar layer assay (US EPA Method 1602), and quantitative two-step enrichment assay (US EPA Method 1601). Methods were successful at recovering somatic coliphage from 79.5% of shellfish samples and 90% of water samples (Table 6). In all shellfish or in oysters, clams, or mussels, there were no significant differences in the levels of somatic coliphages recovered by two-step enrichment assay and single agar layer assay (Tables 6 and 7). Both two-step enrichment and single agar layer methods had similar lower detection limits of approximately 4 PFU per 100 ml. In water, however, two-step enrichment and direct membrane filtration were more sensitive with lower detection limits of 0.1 PFU per 100 ml, compared to single agar layer, which was less sensitive at 5 PFU per 100 ml for a lower detection limit. In water, the theoretical lower detection limit alone did not predict the best recovery method, because two-step enrichment and single agar layer each recovered significantly higher levels of somatic coliphage in field samples than direct membrane filtration (Tables 6 and 7).

TABLE 6. Somatic coliphage recovered by 3 methods in water, oysters, clams, and mussels.

Matrix	Method	Somatic coliphages	
		Geometric mean as log-PFU per 100 ml (\pm st dev)	# of samples (% below detection limit)
Water ^a	ENR ^c	0.77 (\pm 1.11)	76 (4%)
	SAL ^c	0.93 (\pm 1.15)	78 (14%)
	DMF	0.54 (\pm 1.11)	76 (12%)
All Shellfish ^b	ENR	2.05 (\pm 1.23)	72 (21%)
	SAL	2.00 (\pm 1.13)	74 (20%)
Oysters ^b	ENR	1.95 (\pm 1.03)	33 (18%)
	SAL	1.86 (\pm 0.83)	34 (18%)
Clams ^b	ENR	1.99 (\pm 1.13)	28 (21%)
	SAL	1.91 (\pm 1.13)	28 (21%)
Mussels ^b	ENR	2.52 (\pm 1.89)	11 (27%)
	SAL	2.60 (\pm 1.65)	12 (25%)

Somatic coliphages recovery method acronyms same as for Table 3. ^a Friedman's test non-parametric, and Dunn's multiple comparison test with matched comparisons. ^b Wilcoxon matched-pairs signed-ranks test. ^c significantly different method with higher recoveries than other methods or values in the same column and matrix ($\alpha = 0.05$).

Table 7. Statistical analysis of 3 recovery methods for Somatic coliphages from water and shellfish. Note: This table is a companion to Table 6.

Matrix	Test	Somatic coliphage					
		All matched pairs			Matched pairs without below detect values		
		Number of pairs	P value	Significant Difference	Number of pairs	P value	Significant
Water ¹	ENR vs DMF	76	<0.001	Yes DMF < ENR	57	<0.001	Yes DMF < ENR
	SAL vs DMF	76	<0.001	Yes DMF < SAL	57	<0.001	Yes DMF < SAL
	SAL vs ENR	76	>0.05	No	57	>0.05	No
All Shellfish	SAL vs ENR	72	0.0739	No	44	0.0145	No

Clams ²	SAL vs ENR	28	0.3109	No	17	0.4307	No
Mussels ²	SAL vs ENR	11	0.7646	No	7	0.6875	No
Oysters ²	SAL vs ENR	33	0.074	No	24	0.0384	Yes SAL < ENR

SAL = Single Agar Layer Assay (EPA method 1601); ENR = Two-Step Enrichment Assay (EPA method 1602); DMF = Direct Membrane Filtration method. ¹ Friedman's test non-parametric, and Dunn's multiple comparison test matched comparisons (compares 3 or more columns). ² Wilcoxon matched-pairs signed-ranks test (compares 2 samples).

Fecal indicators recovered from estuarine water and shellfish in relation to impacts by waste water treatments plants. Of the 9 estuaries in the study, 4 were impacted by point source discharges of waste water treatment plant (WWTP) effluent. Trends between the levels of wastewater flows and F+ coliphages were explored in Fig. 2. WWTPs discharged up to 0.3 million gallons per day (MDG) of treated effluent into Apalachicola Bay, FL, 0.8 MGD in Rachel Carson Reserve, NC, 1.3 MGD into Oyster River in Great Bay, NH; 25 MGD into Tijuana River, CA. The general trend was that coliphage levels in water and shellfish tended to increase with increasing wastewater discharges (Fig. 2a). Linear regression trendlines fit to geometric mean levels of F+ coliphage predicts 90-98% of the variability in water samples and 72-92% in shellfish samples (Fig. 2a). Somatic coliphages levels also tended to increase as WWTP effluent volumes increased and the R-squared trendlines predict 94-99% of the variability in water and 99% in shellfish (Fig. 2b). Bacterial indicator levels (fecal coliforms, *E. coli*, Enterococci, *C. perfringens*) at WWTP impacted sites had similar trends as for coliphage, with 81-85% of the variability in shellfish and 65-96% in water predicted by a linear regression model (data not shown). Sites in estuaries distant from point-source wastewater impacts were not included in this analysis, because these sites were often physically separated from wastewater point sources, such as in a different water body or on the other side of an island. Sites near WWTP sewage effluent outfalls were sampled over several seasons and in total 3 or 4 times for water and 4 or 8 times for shellfish, which produced intra-site variability seen in the reported error bars for the trendlines (Fig. 2).

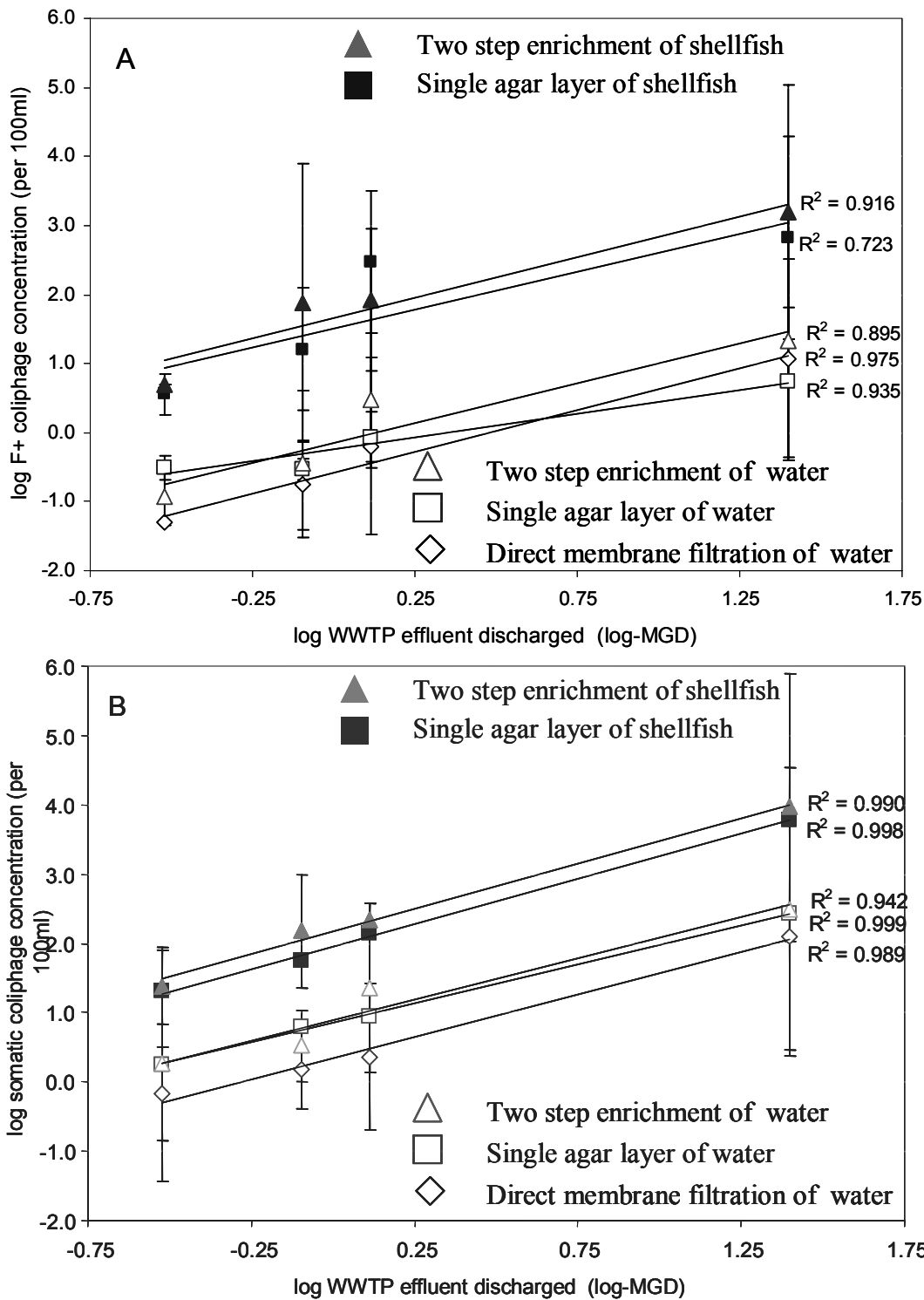


FIG. 2. F+ coliphage (a) and somatic coliphage (b) recovered in estuarine waters and shellfish impacted by WWTP discharges. WWTP and MGD acronyms in Table 1. Monitoring sites include Apalachicola Bay, FL at mouth of bay (n=3-4) (0.3 MGD from Apalachicola WWTP), Rachel Carson, NC on north shore of Carrot Island (n=4 water, n=8 shellfish) (0.8 MGD from Beaufort, NC WWTP), Great Bay, NH at Oyster River (n=4) (1.3 MGD from Dover, NH WWTP), and Tijuana River, CA (n=4) (25 MGD from an International WWTP on US-Mexico border).

FRNA genogroups detected in water and shellfish from coastal estuaries.

Four hundred thirty-six F+ coliphage isolates from water were genogrouped as FRNA group I, II, III, or IV by reverse line blot hybridization (Fig. 3a). FRNA group II (GA-like) phages were detected at 7 of 9 estuaries, with no more than 10 FRNA group II isolates detected at any estuary (Fig.3a). In 3 estuaries with the highest levels of group II phages, only moderate levels of MS2-like (group I) phages were detected. The majority of FRNA isolates from water were group I (MS2-like) phages. More group I isolates were detected in Delaware Bay, DE, in part due to the increased number of water sampling sites compared to other estuaries. Unlike other sites with high numbers of group I FRNA coliphages, only 3 group I phages was found in Waquoit Bay, MA and 1 group I was found in Masonboro, NC (Fig. 3a). FRNA group III (Qβ-like or M11-like) and group IV (SP-like or FI-like) phages were the least prevalent in water. Group III phages were detected at 3 of 7 estuaries and group IV phages were not detected in water at any estuaries (Fig. 3a).

F+ coliphage isolates from shellfish totaled 591. These isolates were genogrouped and results were arranged by estuary and shellfish type (clam, oyster, mussel) (Fig. 3b). When FRNA coliphages were found, group I (MS2-like) coliphages were the major type recovered from shellfish. Oysters and mussels from Elkhorn Slough, CA each lacked group I (GA-like) phages, which were present at all other estuaries, and each contained group IV (Sp-like) phages, which were rare elsewhere. Great Bay, NH oysters had more diverse proportions of group I, II, and III FRNA coliphage than shellfish at other estuaries. Apalachicola Bay, FL was the only estuary where no FRNA phages were detected, although FDNA coliphages and somatic coliphages were routinely found.

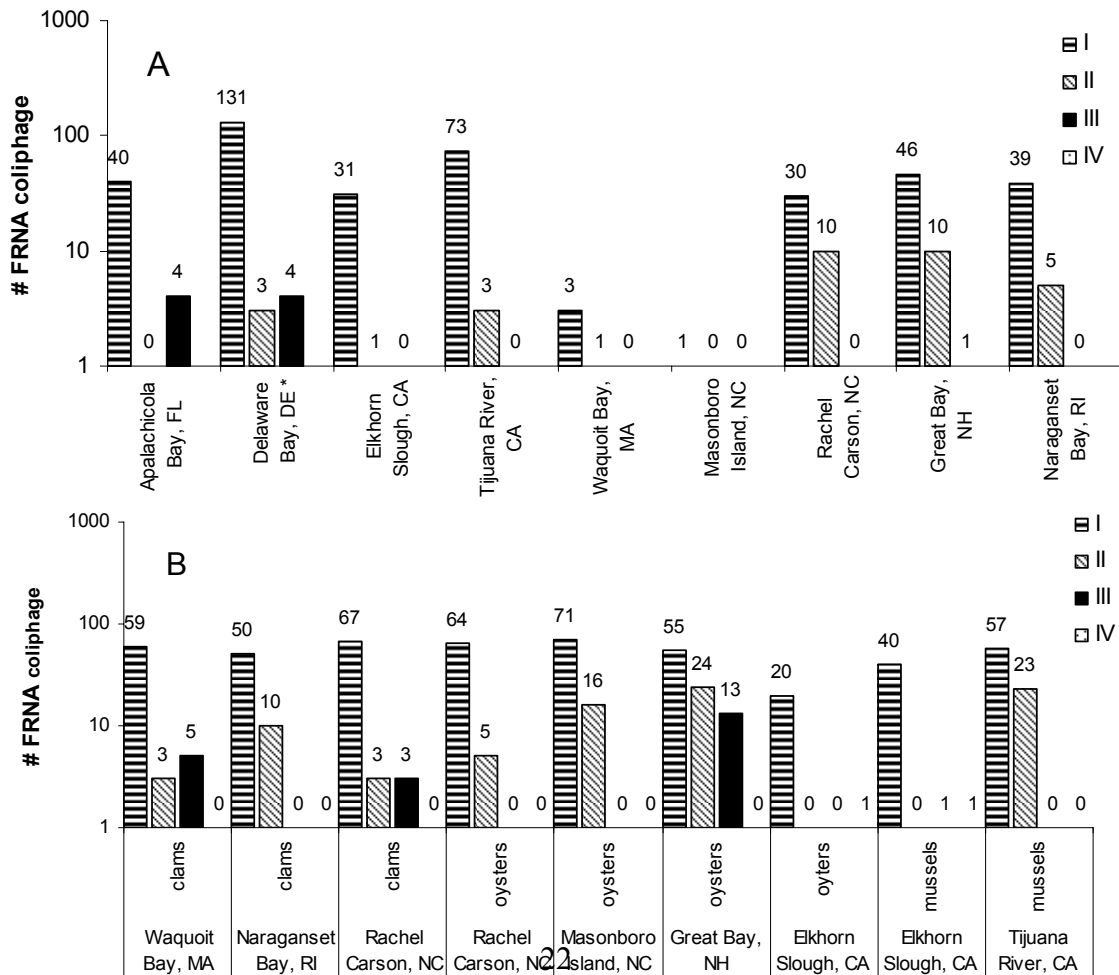


FIG. 3. F+ RNA coliphage genogroups (groups I, II, III, and IV) detected in estuarine water (a) and shellfish (b) from 9 coastal sites. Then number of genogrouped isolates is shown above each column. In Fig. 3a no group IV coliphages were detected. Fig. 4b. separates oysters, mussels, and clams by location and does not show Apalachicola Bay, FL where no FRNA coliphage were detected.

RNase testing to identify FRNA and FDNA coliphages. The RNase spot-plate test was used to classify 1033 F+ coliphage isolates by their viral nucleic acid type. The RNase test agreed with molecular typing in 919 of 921 (99.8%) FRNA isolates (Table 8). Of the two remaining isolates, one tested positive for FDNA and the other tested positive for both FRNA and FDNA using the spot plate test. Of the 112 isolates that were mixtures of FRNA and FDNA by molecular typing, 7 (6.3%) tested positive for FRNA, 85 (75.9%) positive for FDNA and 20 (17.8%) tested positive for both FRNA and FDNA by the RNase assay. Because FRNA coliphages were the major focus of the study, only isolates that were positive for both FRNA and FDNA using the spot plate test were tested by FDNA PCR.

TABLE 8. Comparison of RNase test with (RT)-PCR for the typing of F+ coliphages.

F+ Coliphage isolates	RNase Test ^a			Total
	FRNA positive	FRNA & FDNA positive	FDNA positive	
FRNA ^b	919 (99.8%)	1 (0.1%)	1 (0.1%)	921 (100%)
FRNA & FDNA ^c	7 (6.3%)	20 (17.8%)	85 (75.9%)	112 (100%)

^a RNase test using ribonuclease A for inhibition of RNA containing phages.

^b RT-PCR and RLB hybridization or sequencing for FRNA detection and confirmation.

^c PCR RLB for FDNA detection and confirmation

Evaluation of RT-PCR followed by RLB for FRNA coliphage typing. Of the 1033 FRNA spot plate positive isolates from diverse geographic locations, 1032 (99.9%) tested positive by RT-PCR, and of these 1014 (98.3%) could be typed by RLB into one of the 6 different RLB types: MS2 (genogroup [GG] I); GA (GG II); M11 (GG III); Q-Beta (GG III); Fi (GG IV); or SP (GG IV). All 18 RLB negative strains were nucleotide sequenced in the replicase gene and were confirmed as FRNA coliphage by phylogenetic analysis. RT-PCR - RLB was able to detect and confirm FRNA coliphages in all field isolates that also contained FDNA by the spot test (Table 9). A total of 877 (84.9%), 117 (11.3%), 4 (0.4%), 27 (2.6%), 2 (0.2%), and 0 (0%) strains hybridized with the MS2 (GGI), GA GG II), M11 (GG III), Q-Beta (GG III), Sp (GG IV) or Fi (GG IV) probes, respectively (data not shown).

TABLE 9. FRNA coliphages characterization by RT-PCR and Reverse Line Blot Hybridization.

	Detection of FRNA Coliphages		
	RT-PCR confirmed positive ^a	No RT-PCR amplicon	
FRNA	920 ^c	1 ^d	921
FRNA & FDNA ^b	112	0	112
Total no. (% of total)	1032 (99.9%)	1 (0.1%)	1033

^a RT-PCR confirmed positive by RLB or sequencing.

^b FRNA and FDNA mixed samples

^c 18 isolates were RT-PCR confirmed positive, but RLB negative and sequenced as F+ RNA.

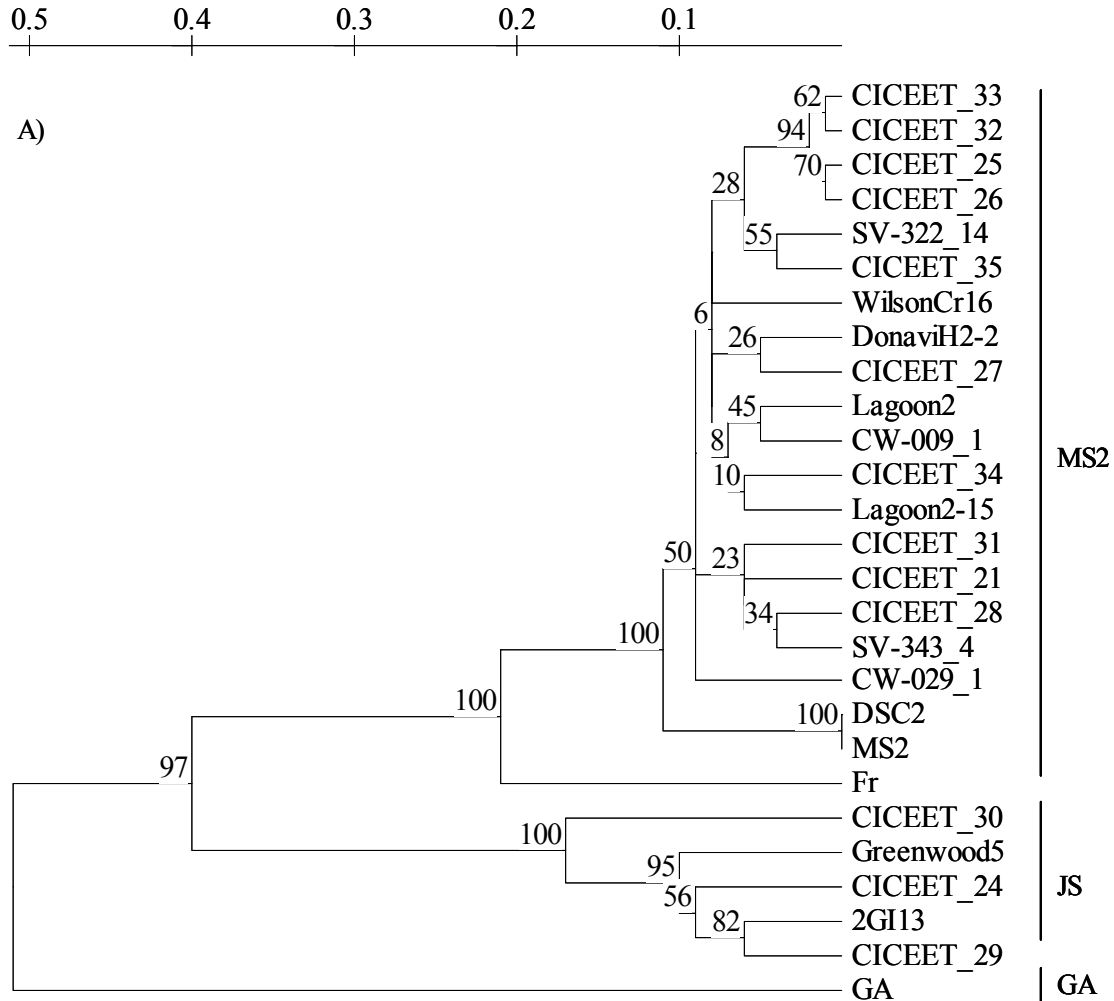
^d RNase test positive for an RNA containing phage, and serotyped to *Leviviridae* family

FRNA genotyping results using RLB hybridization. The majority (98.7% ± 1.5%) of the FRNA isolates from each estuary could be genotyped by RLB (Table 10). All isolates collected at the estuaries in California, (n=95) Massachusetts (n=71), and Florida (n=41) could be typed and at estuaries in Delaware and Rhode Island all but 1 isolate was confirmed and typed (Table 10). The average number of FRNA isolates assayed from an estuary was 114 ± 45 and ranged from 44 to 182 among estuaries.

TABLE 10. RLB hybridization of F+ RNA coliphage from 9 estuaries.

	Confirmed FRNA Coliphages		Total no.
	RLB positive (% positive)	RLB negative (% negative)	
Rachel Carson, NC	178 (97.8%)	4 (2.2%)	182
Tijuana River, CA	152 (97.4%)	4 (2.6%)	156
Great Bay, NH	144 (96.6%)	5 (3.4%)	149
Delaware Bay, DE	137 (99.3%)	1 (0.7%)	138
Narragansett Bay, RI	103 (99.9%)	1 (0.1%)	104
Elkhorn Slough, CA	95 (100%)	0	95
Masonboro Island, NC	85 (96.6%)	3 (3.3%)	88
Waquoit Bay, MA	71 (100%)	0	71
Apalachicola Bay, FL	44 (100%)	0	44

Sequencing and Phylogenetic analysis of FRNA coliphages. A partial region of the replicase gene of both RLB typeable (n=5) and untypeable (n=18) FRNA isolates was sequenced and typed by phylogenetic analysis along with prototype leviviruses and existing field strains. Eighteen isolates that did not react in repeated RLB hybridization assays were nucleic acid sequenced and phylogenetically sorted into 6 group I strains and 12 group II strains. Group I strains shown in Fig. 4a. clustered into 2 branches: one branch with about 90% similarity to MS2, the prototype strain, and another branch (JS subgroup) with about 60% similarity to MS2. Field isolates in the JS-subgroup were rare, but the few that were found came from geographically diverse estuaries. RLB positive FRNA field strains were either identical to the group I RLB probe, or had a 1 nucleotide (nt) mismatch that produced a weakly positive hybridization signal (Fig. 4b). Two or more nt mismatches between the 19 nt group I probe and FRNA group I targets resulted in a lack of RLB detection as was seen with strains in the group I, JS-subgroup (Fig. 4b).



B)	Genogroup I probe	5'	<u>GAGACGATAC</u>	<u>GATGGGAAC</u>	3'	<u>RLB result</u>
	MS2	CGTAGATGGC	<u>GAGACGATAC</u>	<u>GATGGGAAC</u>	TATTTTCCACA	+
	CICEET_30	--- C -- C -- G	C --- T T -- TG	AC --- C - C -	---- C ----- T	-
	CICEET_29	--- T -- C -- A	CGTG TAG - CG	AT --- C - C -	-----	-
	CICEET_24	--- T -- C -- A	CGT - T --- CG	AC --- C - C -	----- T	-
	CICEET_11	--- T -- CG - G	CGTGTA -- CG	A - - - C - T -	---- C -----	-
	CICEET_28	----- C -- A	----- T -----	- G -----	-----	-
	CICEET_21	----- C -- A	----- G -	- G -----	-----	-
	CICEET_31	----- C -- A	-----	- G -----	-----	+
	CICEET_26	----- G	-----	-----	-----	+
	CICEET_25	----- G	-----	-----	-----	+

FIG. 4. Phylogenetic tree (a) and sequence alignment (b) of genogroup I FRNA coliphage isolates that were and were not typed by Reverse Line Blot hybridization. Phylogenetic tree of a 189 nt section of the replicase gene of leviviruses with 100 bootstrap values.

DISCUSSION

The choice of microbial fecal indicators and microbial assays for monitoring marine/estuarine waters is not fully settled science, as shown in recent meta-analyses and health effects studies on the incidence of diseases in bathers (Prüss 1998; Wade et al., 2003; Wade et al., 2006). A recent study reported that “standard” bacterial indicators recommended by US EPA and the World Health Organization did not predict the risks for gastrointestinal illness from bathing in marine water impacted by non-point source fecal contamination (Colford et al., 2006). Only F+ coliphage, a non-traditional fecal indicator, was predictive of gastrointestinal illness risks from this bathing water.

Our study of 6 microbial fecal indicators (F+ and somatic coliphage, *E. coli*, fecal coliforms, enterococci, *C. perfringens*) provides critical comparative information on levels, types, sources, and best available techniques to recover and quantify microbial fecal indicators in estuarine water and shellfish over a broad geographic and temporal range in the USA. These data can be used to inform the choices of microbial fecal indicators of marine bathing water quality for health effects studies and for source tracking efforts. At each of 9 estuaries, sites were chosen to represent i) a point or non-point human fecal source; ii) a pristine or less impacted site with only animal non-point source fecal inputs; and iii) at some estuaries an intermediated site with human and animal non-point source fecal inputs. Fecal sources were identified at these estuaries from published data including TMDL reports, sanitary surveys, local knowledge of managers and other coastal stakeholders, and background research on population density, household sewerage, and WWTP permits (Table 1). The choice of indicator microbes for this study was based on past health effects studies in marine bathing water and shellfish, where positive associations of varying magnitude existed between levels of certain microbial indicators and risks of adverse health effects (Prüss 1998). In marine waters, *E. coli* and Enterococci correlate with gastrointestinal illness in bathers in several studies, while coliphages and enteroviruses also predict risks of gastrointestinal illness but need more validation due to only few available studies (Cabelli 1983, Dufor 1984, EPA 1986; NRC 2004; Wade 2003).

In this present study F+ coliphages somatic coliphages and bacterial indicators were found to be reasonable indicators of fecal pollution in water. Significantly more of these microbes were detected in human-impacted water than non-human impacted or pristine water using two-step enrichment assay for F+ and somatic coliphages, single agar layer assay for F+ coliphages, and membrane filtration methods for bacterial indicators (Fig. 1a). All bacterial indicators were recovered using membrane filtration methods, with a theoretical lower detection limit of 0.005 CFU/100ml, while somatic and F+ coliphages were recovered using 3 types of methods: membrane filtration followed by directly plating the filter on agar medium with host cells (Direct Membrane Filter method) (Sobsey et al., 1990), single agar layer assay (EPA method 1602), and a quantitative version of the two-step enrichment assay (EPA method 1601). The detection limits of the coliphage assays, due in part to their different sample volumes, and any other intrinsic biases within the methods (discussed later) would affect their performance.

In shellfish, only *C. perfringens* and *E. coli* were consistently recovered in higher levels at human impacted sites compared to non-impacted/pristine sites (Fig. 1b). United States shellfish sanitation regulations are based on fecal coliform levels. These bacterial

indicators are present in the gut of coastal mammals and birds which may unnecessarily close shellfishing beds when human fecal contamination is absent (US FDA 2003). In this study fecal coliform levels in shellfish were not predictive of human fecal impacts. Therefore, genetic or phenotypic discrimination or distinction of fecal indicator microbes by type and fecal source is needed to separate human from non-human impacts and better predict the level of disease risk associated with consuming shellfish grown in human fecal impacted waters.

Bivalve shellfish accumulate indicator microbes and pathogens by filter feeding overlying water and resuspended sediments containing persistent microbes (Smith et al., 1978; Landry et al., 1983; Jamieson et al., 2005). Shellfishing beds near WWTP discharges are typically closed for harvesting based on sanitary surveys. To control fecal impacts from microbial pathogens in WWTP discharges to ambient waters the federal Clean Water Act and the National Pollution Discharge Elimination System (NPDES) use permits that approve discharge magnitudes and quality and require effluent monitoring for fecal coliforms and other key indicators of wastewater quality. Permits are also based on the quality and use of the receiving water, but in general, waters near WWTP discharge sites must have a geometric mean of <200 fecal coliforms (FC)/100 ml from 5 consecutive samples in 30 days, and <20% of samples can exceed 400 FC/100ml. For discharges into unfiltered drinking water sources the levels must be <50 FC/100 ml (NCAC).

In this study exceedances of NPDES permitted levels may have occurred once at Tijuana River, CA (1,250 FC/100ml) and once at Nannies Island in Great Bay, NH (404 FC/100ml), if we assume that these levels in estuarine water are caused by wastewater discharges to these waters. Further investigation into all study sites impacted by WWTPs showed that concentrations of microbial indicators in water and shellfish tended to be higher with higher permitted volumes of WWTP daily discharge (Fig 3). In previous studies positive correlations were found between the presence of aggregates of fecal indicators and the presence of pathogens in the effluent of 6 WWTPs (Harwood et al., 2005). However, individual indicators did not significantly correlate with pathogens, probably due to small sample sizes (Harwood et al., 2005). Some proportions of both the pathogens and fecal indicators in sewage survive effluent disinfection (Bonadonna et al., 2002; Fleischer et al., 2000). The findings of this present study suggest that persistent microbial pathogens like *Cryptosporidium*, *Giardia*, and enteric viruses also could be present at higher concentrations in the effluent receiving waters of larger WWTP plants than from smaller plants. Because of their greater potential pathogen load to receiving waters, perhaps larger WWTPs should have stricter disinfection requirements to produce cleaner disinfected effluent with fewer pathogens and indicators than smaller plants that discharge less waste water. F+ coliphages have been detected at higher levels in disinfected effluent than bacterial indicators (Chung et al., 1998; Harwood et al., 2005), which may explain our finding that F+ and somatic coliphages had a strong association with the magnitude of the WWTP impacts, and were more predictive of WWTP discharge volumes than were fecal bacterial indicators.

Coliphages have been proposed as viral indicators of fecal pollution (Havelaar 1993). FRNA coliphages can be grouped by type of fecal source as human or animal in a library independent manner, with distinctive human (groups II and III) or animal (groups I and IV) source patterns in SE Asia. Similar patterns have been observed in North

America, but inconsistencies have been noted in terms of the extent to which the measured levels of the different FRNA groups predict the relative magnitudes of the human and animal fecal waste sources (Hsu et al., 1995; Cole et al., 2003; Stewart et al., 2006).

This study is one of the few to date that compares F+ and somatic coliphage recovery and detection assays in marine water and shellfish, and compares them for application to FRNA microbial source tracking. F+ and somatic coliphage were detected at higher levels in shellfish than overlying water (Table 2). Somatic and F+ coliphage were detected in about 90% and 60% of water samples, respectively, and about 80% and 60% of shellfish, respectively. When comparing assays, the quantitative two-step enrichment (EPA 1601) was best at a-specific F+ and somatic coliphage detection based on numbers of positive samples and concentration detected. Two step enrichment detected F+ coliphages in more water or shellfish samples than single agar layer method (EPA 1602) or direct membrane filtration. Two step enrichment was less effective based on recovery of discrete field isolates for microbial source tracking. Based on the genogroups of coliphages recovered by each method, two-step enrichment method recovered a statistically higher proportion of group I phages than other methods in water and in all shellfish (combined), clams, or mussels (Table 2). This is potentially explained by previous studies showing that group I phages have a larger burst size and when enriched they produce more progeny than FRNA coliphages from groups II, III, and IV (Furuse 1987; D. Love unpublished data). Hence, in enriched samples, group I FRNA coliphages will be present at far higher concentrations than FRNA coliphage of groups. This phenomenon of increased concentrations of group I FRNA coliphages compared to other groups in enrichment is further exacerbated in the coliphage isolation process by the fact that phages isolated from enriched samples were plaque purified at dilutions low enough to yield single plaques. As a result the procedure selected for the isolation of only the predominant coliphage group, to the exclusion of the minority coliphage groups. Typing coliphages in enrichment cultures without plaque purification can resolve this problem, as long as the typing method can resolve the presence of- and determine the identity of multiple coliphage groups. Microbial source tracking from a pool of isolates biased in relative presence due to their differential growth in enrichment cultures would provide skewed estimates of the magnitude of the different fecal sources. Although two-step enrichment has greater sensitivity in coliphage detection, direct membrane filtration for water and single agar layer for shellfish each provide a more accurate and unbiased representation of the minority FRNA coliphage groups present in the sample, such as groups II and III, because each coliphage replicates discretely to form individual plaques when directly cultured from the sample (Table 2).

Microbial source tracking with FRNA coliphage has been successfully applied in Florida and New York City to assess and then manage the impacts of human and animal waste sources (Alderisio et al., 1996; Griffin et al., 2000). In this study, about 150 water samples and pooled shellfish samples yielded over 1,500 F+ field isolates for source tracking. The major type of FRNA coliphage detected was group I (MS2-like), which is consistent with previous findings in fecally impacted surface waters (Cole et al., 2003). However, more than half (65 of 117) of group II (GA-like) phages and two-fifths (13 of 31) of group III (Q β -like) phages were recovered from areas with known human impacts (Table 1). These findings are consistent with those of previous studies linking groups II

and III FRNA coliphages to human fecal waste sources (Furuse et al., 1981, 1987; Osawa et al., 1981). Five of 9 estuaries had greater levels of group II (GA-like) phages in human impacted sites than in pristine sites or non-human impacted sites, thus providing further evidence of the association of this FRNA coliphage group with human fecal waste. Associations of human impacted sites with FRNA coliphage field isolate group identities were in some cases striking. For example, 100% (n = 16) of recovered group II (GA-like) from Masonboro Island, NC came from the human impacted site, 75% (3 of 4) of group III (QB-like) isolates were from an Apalachicola Bay, FL site with an upriver 0.3 MGD discharging WWTP, and 62% (21 of 34) of group II (GA-like) isolates in Great Bay, NH were from a site with non-point source runoff from development and septic tanks and an <1 km upriver 1.3 MGD discharging WWTP. Source tracking was not always so effective at identifying fecal contamination source type, especially when human and animal impacted sites had high levels of group I (MS2-like) coliphage. Better FRNA coliphage fecal source matches may be achievable with accurate assessment of known fecal waste sources (sanitary or shoreline survey), better characterization of the hydrological conditions in the receiving water, better understanding of the relative persistence of different F+ coliphages in estuaries, and better resolution of coliphage mixtures during detection and isolation. A FRNA nucleotide sequence library of coliphage isolates from known fecal sources may allow for greater resolution in identifying fecal waste sources and their impacts by sequence matching with isolates from shellfish and surface water (Stewart et al., 2006). However, these more technologically demanding, time consuming, and costly techniques may be less adaptable to the goal of rapid, simple and low cost field detection and fecal waste source tracking based on FRNA coliphage grouping as human or animal source.

The overall study findings were that bacterial indicator methods and some F+ and somatic coliphage methods detected significantly more microbes in water at human-impacted sites than at non-human impacted or pristine sites. Three F+ and somatic coliphage recovery methods were compared using estuarine water and shellfish, showing single agar layer method for shellfish and direct membrane filtration method for water to be most compatibility with FRNA microbial source tracking. Others can use this information on the occurrence, levels, types, sources, and performance of detection techniques for microbial fecal indicators to inform the design and focus of human health effects studies on marine bathing waters and edible bivalve molluscan shellfish, and affect choices of fecal indicators for management decisions

This study evaluated the RT-PCR – RLB coliphage detection and typing method to establish its robustness for use in further fecal waste source tracking and ecology studies. Of 1033 field isolates tested, 99.9% gave confirmed positive RT-PCR products, and 98.3% were genotyped by RLB. The RLB assay was a robust molecular method to detect and genotype F+ RNA coliphages from diverse geographic areas and watersheds, and can be used for large scale microbial source tracking studies.

The RNase test was used in this study as a screening method to rapidly separate FRNA from FDNA viruses. In our evaluation the RNase test performed reasonably well for FRNA isolates (99.8%), but the assay was unable to detect FRNA phages in 76% of mixed samples also containing FDNA coliphages, and therefore was biased towards FDNA phage detection. A likely reason for this bias is that FDNA coliphages enrich to 2 to 3 log₁₀ higher titers than FRNA coliphages (Love unpublished data), and on agar plates

the more numerous FDNA coliphage plaques obscure the observation of FRNA plaques. For more reliable nucleic acid screening methods, parallel DNase and RNase treatments should be performed. Alternatively, a FRNA-specific bacterial host could perhaps be made or FDNA coliphage neutralizing antisera could be used, which block FDNA adsorption on the host F-pilus tip while allowing FRNA infection along the length of the F-pilus (Manchak et al., 2002).

Coliphage RLB typing was evaluated using a large panel of F+ coliphage field strains from 9 geographically diverse estuaries in the United States. FRNA coliphages were detected using a recently described, broadly reactive duplex RT-PCR assay using degenerate primers targeting both levi- and alloviviruses (Vinje et al., 2004). A previous study in which the duplex RT-PCR assay was developed, it was used to detect and genotype 100% (107/107) of FRNA field strains (Vinjé et al., 2004). Because the previous study was conducted on a relatively small panel of known positive FRNA field strains, our goal was to further evaluate this assay on a larger, temporally and spatially diverse panel FRNA field strains for which there existed no a-priori geno- or serotyping data. The sensitivity of the duplex RT-PCR assay was 99.9% (1032/1033), which agrees with previous findings that this RT-PCR method is sensitive. Using temporally and spatially diverse field strains of FRNA coliphages, we further showed that the duplex RT-PCR method was robust and effective for microbial source tracking studies. One FRNA coliphage isolate could not be amplified by RT-PCR, although the titer was about 10^6 PFU/ml, it contained RNA nucleic acid, and it was neutralized by antisera against MS2 (GG I) and GA (GG II) in the levivirus genus.

RLB is a high throughput, cost-effective method to type up to 45 RT-PCR positive samples in one run using probes that are covalently bound to a nylon membrane. This membrane can be re-used up to 20 times without any decrease of sensitivity (data not shown). RLB hybridization has been used to successfully type norovirus from stool samples (Vinjé and Koopmans 2000), respiratory viruses from nasopharyngeal aspirates (Coirao et al., 2005), and antibiotic resistance genes of *Streptococcus agalactiae* (Zeng et al., 2006). In this study, RLB genotyped 98.4% of the FRNA coliphage isolates tested, which was a level of performance consistent with previous work (Vinjé et al., 2004). Nucleic acid sequencing of RLB negative strains improved genotyping rates to 99.9%, and was used only when RLB hybridization gave inconclusive results.

Based on genotyping, 84.9% of field isolates in this study were FRNA GG I. Field isolates were representative of the four main coliphage types or groups that could occur in shellfish and estuarine water samples impacted by human and/or animal fecal waste sources. However, a greater diversity in FRNA groups or types would have been preferred, especially for studying RLB GGs III and IV probe sensitivity.

The geographic location of FRNA coliphage isolates did not affect the genotyping rate of RLB hybridization (Table 3). An average of 114 FRNA coliphage isolates were assayed from each of 9 estuaries, and the genotyping rate ranged from 96.6% to 100% at any given estuary. RLB untypeable group II field strains clustered into 2 branches with 80% sequence similarity to each other (data not shown). Strains clustering with the JS-subgroup had >40% sequence difference with GGs I and II leviviruses, including mismatches in the region targeted by the GG I and GG II probes (Figure 2). Including a new and unique probe on the RLB membrane to specifically detect strains within this

cluster will improve the use of RLB for classifying FRNA strains without nucleotide sequencing.

Typing of coliphages isolates from surface water, groundwater, wastewater, animal feces, and meat processing plants has been routinely used as a tool to identify sources of fecal contamination. However, FRNA coliphage strains are rarely sequenced, and thus their ecology and genetic diversity is not well understood beyond the serogroup level of identification (Furuse et al., 1981; Hsu et al., 1995; Hsu et al., 2002; Cole et al., 2003). In this study, sequencing and phylogenetic analysis of a partial region of the replicase gene confirmed the existence of additional FRNA genogroups or subgroups. This finding suggests that more genogroups or subgroups may be found using sequencing methods instead of serological methods.

The findings of this study also document that genetically similar strains of FRNA coliphages can be found in different geographic locations. For example, this study detected a JS-subgroup field strain in East Coast estuaries in DE, NC, NH, and RI, but none from the Gulf or West Coasts. The reasons for such regional presence are unknown, but may be caused by migratory birds (waterfowl) carrying these coliphages along Atlantic migration routes. Stewart and colleagues previously demonstrated that a cluster of related FRNA strains can exist at specific sites, such as hog farms in North and South Carolina (2006). Sequencing FRNA coliphage from swine waste lagoons revealed distinct patterns among sequences from different lagoons, which could be used for source tracking fecal waste (Stewart et al., 2006). Further investigation into the ecology of FRNA coliphages is needed to further validate coliphage grouping for microbial source tracking and to better understand their diversity, host ranges, evolution and selection.

In *E. coli* spp, geographic distribution accounts for <20% of its genetic diversity (Hartel et al., 2002; Gordon and Lee 1999; Souza et al., 1999). Differences in diversity and distribution of *E. coli* ribotypes have been seen in wild and captive deer that have different diets (Hartel et al., 2003). In populations of cows, horses, and humans, *E. coli* had complex population dynamics that are host species specific, with *E. coli* ribotypes commonly shared within species of a population (Anderson et al., 2006). Bacterial source tracking is performed with typing libraries of known fecal sources that are temporally and geographically specific to an area (US EPA 2005). Bacterial source tracking with an under-represented library or a library generated from a different area will produce less accurate source tracking results (Jenkins et al., 2003; Parveen et al., 1999; Wiggins et al., 2003). Although FRNA coliphages replicate in *E. coli*, and selective pressures on gut bacteria may indirectly effect coliphage ecology, selection and emergence, these coliphages are for the most part spatially and temporally predictable (Furuse et al 1981; Osawa et al., 1981; Furuse 1987; Cole 2003; Hsu 1995), which obviates the need for expensive location-specific source tracking libraries.

FRNA coliphage typing can be done using a variety of molecular and serological methods. In addition to serotyping by neutralization of virus infectivity, a simple particle agglutination immunoassay has recently been developed in our lab for 60-second FRNA detection and serotyping (unpublished data). Another rapid method, based on nucleic acid detection is realtime RT-PCR, which was recently developed by Kirs and Smith for FRNA coliphage genotyping (Kirs, M. and D. C. Smith. Abstr. 105th Gen.Meeting at the Am. Soc. for Microbiol., 2005). Dot blot hybridization, another genotypic method, uses RNA probes in the maturation region (groups I or II) or the 5' end nontranslated region

(groups III or IV). This method was reported to have a sensitivity of 96.6% (196/203) for FRNA coliphages (Hsu et al., 1995). In dot blot hybridization, F+ coliphage plaques are lifted from agar plates onto 4 replicate membranes that are labeled with reusable oligonucleotide probe solutions for each of the 4 FRNA genogroups and detected with a colorimetric, immunoenzymatic signal (Hsu et al., 1995; Beekwilder et al., 1996). Unlike relatively new genotyping methods, FRNA serotyping by neutralization of coliphage infectivity has been performed for over 30 years using polyclonal antisera against the 4 FRNA coliphage groups (Furuse et al., 1973; Furuse et al., 1981). In one study, serological detection rates for FRNA coliphages from water, waste, and shellfish was 99.5% (202/203), although the assay could not always reliably differentiate group I from group II leviviruses (Hsu et al., 1995), thereby making source tracking sometimes problematic. Coliphage antisera required for serogrouping can be difficult and expensive to generate, serotyping assays by neutralization of coliphage infectivity are time consuming to perform, and serotyping cannot detect coliphages that do not form clear lysis zones on agar plates with host lawns. Until significant improvements in current serotyping methods for F+ coliphages, molecular methods to genotype F+ coliphages, such as by RLB hybridization, will continue to be the gold standard for FRNA coliphage fecal source tracking.

TECHNOLOGY COMMERCIALIZATION

A patent application is in progress for a rapid and field portable coliphage detection and typing assay, which was a side project of this larger study. We developed, optimized and validated the assay, which is performed by mixing a drop of virus enrichment with a drop of detection reagent on a cardboard card. Visual agglutination or clumping of positive samples occurred in less than 60 seconds. The assay has a sensitivity over 95% and a specificity higher than 97% for two families of coliphage fecal indicators. For technology application of this new assay, our group is preparing a proposal for the CICEET/SCCWRP 2007 RFP on beach water fecal monitoring. Private sector partnerships for the commercial production of coliphage detection kits are being sought out. The commercial product or kit would be a package of dry biological reagents for 2-3 hr coliphage culture and a dropper bottle with rapid coliphage detection and typing reagents. Our rapid and simple detection assay for fecal indicator viruses provides a new and improved tool to monitor the microbiological quality of drinking, recreational, shellfishing and other waters. This kit would overcome the perceived methodological barrier of coliphages, by providing simple, rapid and standardized coliphage detection and typing methods. Current groundwater monitoring regulations include coliphage indicators, and the Interstate Shellfish Sanitation group is discussing the use of coliphage indicators in shellfish.

ACHIEVEMENT AND DISSEMINATION

Manuscripts:

From this work, two manuscripts in draft form tentatively titled “Microbial Indicator Recovery, Detection, and FRNA Source Tracking in Estuarine Water and Shellfish from Geographically Diverse Estuaries” and “Methods Analysis of FRNA Coliphage Genogrouping with RT-PCR and Reverse Line Blot Hybridization” are and likely will be submitted to refereed journals in Summer or Fall 2006.

Graduate Students:

Jen Murphy, tentatively Spring 2006 for Ph.D in Environmental Science. Dr. Mark Sobsey Advisor. Title: “Evaluation of Fecal Contamination by Seagulls in an Urban Estuarine Environment Using Microbiological and Molecular Approaches.”

David Love, tentatively Spring 2008 for Ph.D in Environmental Science. Dr. Mark Sobsey Advisor. Tentative title: “Rapid Method to Detect FRNA Coliphage Fecal Indicators in Surface Waters and Shellfish.”

Conferences, Workshops, and Outreach:

The research program and progress of the laboratory was presented at the US EPA BEACHES Program “National Beaches Conference” held October 13-15, 2004 in San Diego, CA. Greg Lovelace presented a paper entitled: “Male-Specific Coliphages as Indicators of Fecal Pollution in Coastal Recreational Waters.” That presentation included data from the on-going research of the current project.

The results based on data collected as of February 2005 were presented at the meeting of the Gulf and South Atlantic Shellfish Meeting, May 22-25, in Myrtle Beach, SC. On May 23d, Dr. Mark Sobsey and Greg Lovelace gave a talk entitled “New Indicator Methods for Virus Detection in Shellfish” to an audience of state shellfish sanitation program managers.

The results based on data collected as of February 2005 were presented as a poster entitled “Improved Methods for Detection of Coliphages in Coastal Water and Molluscan Shellfish” by Greg Lovelace at the American Society for Microbiology annual meeting in Atlanta, GA on June 8, 2005. That poster is currently available on the CICEET Project Explorer website.

On June 8, 2005, the results of rapid coliphage detection were presented by David Love in a poster entitled “Development of a Rapid Method to Detect F+ Coliphage as an Indicator of Fecal Pollution” at the American Society for Microbiology annual meeting in Atlanta, GA.

On July 11, 2005, at the invitation of Dr. Roger Fujioka, Greg Lovelace presented the talk entitled “New Indicator Methods For Virus Detection in Coastal Water and Molluscan Shellfish” to an audience of students and faculty at the University of Hawaii in Honolulu.

Improved methods for coliphage detection were presented at the International Workshop on Molluscan Shellfish Safety on August 12, 2005 in connection with the biannual meeting of the Interstate Shellfish Sanitation Conference in Point Clear, AL. Greg Lovelace and Brian Robinson of the NOAA Center for Coastal Environmental Health and Biomolecular Research conducted the workshop at the invitation of the USFDA Shellfish Laboratory in Dauphin Island, AL. A document entitled "Methods to Detect and Genotype Coliphage in Water and Shellfish" was produced for distribution at the workshop. It is available online at <http://www.unc.edu/sobseylab/ISSCcoliphagedemo.pdf>.

On October 17, 2005, the results of the final study were presented by Greg Lovelace and David Love in a poster entitled “F+ RNA Coliphages as Viral Indicators of Fecal Contamination in Estuarine Water and Shellfish” at an Estuarine Research Federation Conference in Norfolk, VA.

On November 3, 2005, at an invited lecture to the North Carolina state Shellfish Quality and Safety Workshop, David Love presented at talk entitled “Shellfish Associated Infections and Illnesses from Bacterial and Viral Agents” in Pine Knoll Shores, NC.

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