NEW AND IMPROVED METHODS FOR F+ COLIPHAGE CULTURE, DETECTION, AND TYPING TO MONITOR WATER AND SHELLFISH FOR FECAL CONTAMINATION

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ABSTRACT

DAVID CLIFFORD LOVE: New and Improved Methods for F+ Coliphage Culture, Detection, and Typing to Monitor Water and Shellfish for Fecal Contamination
(Under the direction of Mark Sobsey)

Human fecal contamination of coastal recreational water and in shellfishing water is a public health concern because of disease risks to bathers and shellfish consumers, and resulting economic costs of illnesses and beach or shellfishing closures. Coastal managers monitor water and shellfish quality using microbial fecal indicators. In this study, six such indicator microbes (F+ and somatic coliphages, enterococci, fecal coliforms, *E. coli*, and *Clostridium perfringens*) were evaluated and compared in estuarine water and shellfish from nine United States estuaries. Bacterial indicator methods and three F+ or somatic coliphage methods detected significantly more microbes in water at human-impacted stations than at non-human impacted or pristine stations. In shellfish, fecal coliform levels were not predictive of human fecal impacts (p=0.183), unlike *E. coli* (p=0.023). F+ coliphages were nearly significant in predicting human fecal impacts in shellfish (p =0.073), and were detected in 66% of shellfish samples, using the two-step enrichment assay, the most sensitive F+ coliphage method for both water and shellfish. Genogrouping of F+ RNA isolates found 85.4% (n=877) group I, 11.4% (n=117) group II, 3.4% (n=31) group III, and 0.2% (n=2) group IV isolates in water and shellfish for microbial source tracking. The F+ RNA genotyping rates among estuaries

ranged from 96.6% to 100%. This information on the occurrence, levels, types, sources of microbial indicators and on the performance of methods informs the design of human health effects studies on marine bathing waters and choices of fecal indicators for management decisions.

Simple, rapid and reliable fecal indicator tests are needed to better monitor and manage waters and wastes. This study developed, optimized, and validated a coliphage culture latex agglutination and typing (CLAT) assay to detect individual F+ coliphage serogroups. CLAT had a sensitivity of 96.4% (185/192 samples) and 98.2% (161/164 samples), and a specificity of 100% (34/34 samples) and 97.7% (129/132 samples) for F+ RNA and F+ DNA coliphages, respectively. This particle agglutination technique for rapid and simple detection and grouping of F+ coliphages provides a new and improved tool to monitor the microbiological quality of drinking, recreational, shellfishing, and other waters.

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1 INTRODUCTION

Background

The microbial quality of beach water and shellfishing areas are topics of increasing public health concern, due to increased use and pollution of the United States (US) shorelines. Coastal populations make up slightly more than half of the US population (153 million people), and have grown by 28% in the last 25 years (Crossett et al., 2004). Water pollution by point-source (e.g. municipal waste water discharges) and non-point source impacts (e.g. urban runoff, stormwater runoff, boat waste dumping) may harbor human microbial pathogens. In waste water, enteric viruses are known to survive wastewater treatment better than fecal indicator and pathogenic bacteria (Chung et al., 1998). Often waste water effluent disinfection is inadequate to prevent contamination of estuarine water and pathogen bioaccumulation in shellfish (Shieh, 2003; Lodder and de Roda Husman, 2004), putting bathers and shellfish consumers at risk for acquiring diseases like gastroenteritis, respiratory illnesses, and skin infections. Non-point source pollution is harder than point source pollution to measure because of its inherently diffuse nature, but the disease risks are also substantial and documented (Colford et al., 2005).

Epidemiological studies found swimming in ocean water is associated with an increased risk of illness (Colford et al., 2005; Corbett et al., 1993; Prieto, et al., 2001;

(Haile et al., 1999). Epidemiological links between the consumption of bivalve shellfish and enteric diseases are also well established (Shieh et al., 2000; Sanchez et al., 2002; Kingsley et al., 2002). Large disease outbreaks attributed to shellfish in China (>300,000 cases of acute hepatitis in 1988) (Xu et al., 1992) and municipal water from Milwaukee, WI (estimated 400,000 cases of Cryptosporidiosis in 1993) (Kramer et al., 1996) and other smaller outbreaks have taught the public health community that management based on monitoring and warning systems for fecal pollution in recreational and shellfishing waters is critical in achieving acceptable levels of risk to water-contact users and those consuming bivalve molluscan shellfish.

Reducing disease risks among bathers and shellfish consumers undoubtedly requires national regulations for management systems and diligent monitoring and reporting on the part of government agencies or their representatives. Federal laws that address water quality include the Clean Water Act, the National Pollution Discharge and Elimination Systems (NPDES) permitting system, and the BEACHES Act of 2000, which monitor and attempt to control water quality levels based on water use levels and potential exposure risks. Other water quality management strategies or policies that are promising for control of microbes and other contaminants, but need more legislative clarification or regulatory action, include: riparian buffers and sedimentation basins or ponds for new and existing developments, limits on impervious surfaces in watersheds and coastal areas, public investment and upgrades for stormwater management and sewerage, management practices beyond animal waste effluent lagoons for concentrated animal feeding operations (CAFOs), and community watershed restoration programs. As a whole, these activities and the laws and policies that support them are intended to

manage water as a resource and to protect both the environment and human health. However, many of the root problems underlying poor water quality are not adequately addressed by existing regulations, management systems and water quality monitoring programs.

As a management option, direct testing of water and shellfish for human pathogens is currently considered too technically difficult, unreliable, time consuming and expensive for regular and routine use. As an alternative, water managers rely on the monitoring of non-pathogenic fecal indicator microbes to indicate the presence or magnitude of fecal pollution, pathogenic microbes, and prediction of disease risks. Other forward thinking programs also use stormwater models to prëmptively close beaches and shellfish harvesting waters, or community-assisted monitoring programs (e.g. Surfrider Foundation) to assist in management decisions. Current regulations use enterococci and *E. coli* for bathing waters, and fecal coliforms for shellfish waters and meats (US EPA 1986; FDA 2002) as bacterial indicators of fecal contamination that are predictive of human health risks. Regulations for water are based on health effects studies in which levels of candidate bacterial indicators positively correlated with incidence of gastrointestinal illness in bathers (Cabelli 1983, Dufour 1984, US EPA 1986). These regulations include action levels for one-time exceedances of standards and for 30-day geometric mean exceedances of standards based on the density or concentration of the bacteria in water.

Several fundamental problems exist with current bacterial indicators used by regulators of water and shellfish quality. For one, current microbial indicators are bacteria and many waterborne pathogens are enteric viruses for which bacterial indicators are

inadequate or unreliable due to greater virus and bacteriophage resistance to water and waste water treatment processes (Harwood et al., 2005; Jofre et al., 1995), and greater virus and bacteriophage survival and persistence in freshwater, seawater and shellfish than that of enteric bacteria (Contreras-Coll et al., 2002; Duran et al., 2002; Moce-Llivina et al., 2005). Water and shellfish with acceptable levels of fecal indicator bacteria can contain excessive levels of enteric viruses causing human health risks (Chung et al., 1998; Dore et al., 2000; 2003; Formiga-Cruz et al., 2003). For another, bacterial indictor assays used by regulators take one to four days for results, which causes delays in water quality decisions and warnings (NRC 2004), and results in posting and closures long after human exposure has occurred and by which time water quality conditions could have changed. Finally, routine fecal indicator bacteria assays used by managers cannot differentiate human and non-human fecal waste for tracking and controlling their sources. The ability to track fecal microbes in water and shellfish back to their sources is a potentially powerful tool for prevention and control measures intended to reduce releases and better protect water resources. Because of these problems with fecal indicator bacteria as used for water and shellfish management, there is a need for simple, reliable and rapid viral indicators and effective methods to detect, assay and characterize them.

Several promising viral indicators are different types of bacteriophages, or viruses infecting bacteria, that inhabit the gut of animals including humans. Coliphages (viruses of *Escherichia coli* and possibly other coliforms), *Bacteriodes fragilis* phages, and *Salmonella* phages have been measured in water and shellfish monitoring studies in the US and Europe (Colford et al., 2007; Dore et al., 2000; Mocé-Llivina et al., 2005). In

particular, F-pilus specific (F+) coliphages with RNA genomes (F+ RNA coliphages) look promising as fecal indicators in water and shellfish (Haavelar, 1993; Dore et al., 2000). However, the extent to which F+ coliphages are predictive of human health risks from recreational water exposures has not been extensively studied (Wade et al., 2003; Colford et al., 2007) and even less is known about the their predictability of human health risks from molluscan shellfish consumption (Dore et al., 2000; 2003). Furthermore, rigorous comparisons of candidate coliphage detection and assay methods have not been comprehensively performed in marine waters or shellfish from different geographic areas of the World or within the United States. This has led to the independent development and promotion of different coliphage detection methods in Europe and the United States and between the US EPA and the US FDA, and to uncertainties about which method(s) is best suited for these various sample matrices and geographic locations. For example, the US EPA Ground Water Rule includes coliphage as fecal indicator microbes, but promotes two very different coliphage methods (a presence/absence liquid culture method and an agar-host direct plating, plaque enumerative method) without acknowledging the inherent differences between methods.

In addition to being a fecal indicator, F+ RNA coliphages are used for microbial source tracking, because these viruses can be genotyped or serotyped into several distinct groups that typically differ from human or animal waste sources, with some exceptions (Furuse et al., 1981; Osawa et al., 1981; Hsu et al., 1995; Cole et al., 2004; Stewart et al., 2006). Source tracking is useful in Total Maximum Daily Load (TMDL) calculations to quantitatively account for various fecal sources and their magnitudes in a water body. A TMDL calculation is the maximum level of pollution a water body can sustain while still

meeting water quality standards including a margin of safety, and fecal source characterization and apportionment helps determine which fecal sources to include in the TMDL. TMDLs are required by section 303(d) of the 1972 Clean Water Act for all impaired waters (US EPA TMDL). Source tracking with F+ RNA coliphage has been used to identify and control animal sources of fecal pollution in surface water (Griffin et al., 2000; Alderisio et al., 1996). However, others found inconsistencies between human/animal F+ RNA groups that could be resolved only with further molecular analysis, which suggests the need for continued research to resolve inconsistencies and address uncertainties (Hsu et al., 1995; Cole et al., 2004; Stewart et al., 2006).

In laboratory studies, differential die-off was observed for F+ RNA coliphages in treated wastewater, groundwater and soil, which showed trends in persistence (from most persistent to least) of MS2 > Qβ > GA, SP and FI, with temperature (4°C or 25°C) but not matrix as a significant factor (Meschke 2001). These findings agree with high levels of MS2 and Qβ, and low levels of SP and FI detected in field studies (Brion et al., 2002; Cole et al., 2003), which can be the potential cause of problems in using the abundance of F+ RNA coliphage groups as an indicator of the source and magnitude of human or animal fecal contamination.

F+ DNA coliphages and somatic coliphages have also received attention as fecal and viral indicators, but that their ability to predict human health risks or to distinguish fecal contamination sources is either unknown or uncertain (Cole et al., 2003; Long et al., 2005; Hot et al., 2003). In freshwater, sunlight inactivation of fecal indicators follows (from most persistent to least) F+ RNA coliphages > somatic coliphages > *E. coli* > fecal coliforms > enterococci (Sinton et al., 2002), showing that somatic coliphages are, and

suggesting that F+ DNA coliphages are likely to be more persistent that bacterial fecal indicators.

Because of their potential effectiveness as viral indicators of fecal contamination, predictability of gastrointestinal illness risks from recreational exposures and shellfish consumption, and ability to distinguish human and animal sources of fecal contamination, F+ RNA coliphages and possibly other coliphages deserve further investigation as a management tool for recreational water and shellfish quality. Microbial water quality monitoring is in need of a simple, rapid and field-portable microbial detection assay that is predictive of the magnitude of fecal contamination and its sources and provides same-day results for decision-making prior to exposures. Coliphages, especially F+ RNA coliphages, offer considerable promise for such methods development and application in management systems..

Objectives

The general aim of this study is to examine coliphages as fecal indicators in coastal environments, and as easily and rapidly measurable microbial tools that provide information predictive of the risks of exposure to pathogens capable of causing infection and illness in recreational bathers and shellfish consumers. Three hypotheses have been formulated to address specific aspects of the general aim of this project:

I. It is hypothesized that the use of coliphage indicators can improve monitoring of fecal pollution in estuarine water and shellfish, just as recently proposed and promulgated coliphage rules are doing for groundwater. Research is needed in coastal environments to determine if coliphages are as useful, predictive and applicable as are the bacterial fecal indicators now used by regulators for monitoring and quantifying fecal pollution in estuarine water and shellfish. The ambient levels, types, and sources of coliphages (both F+ and somatic coliphages) will be compared to bacterial indicators at sites with a range of human and animal, and point and non-point fecal sources to better substantiate the assertion that coliphages are fecal contamination indicators in marine water and shellfish. F+ and somatic coliphage fecal indicators, and different coliphage recovery methods, specifically broth culture enrichment-spot plating by a modification of US EPA Method 1601 (2001a), single agar layer plaque assay by US EPA Method 1602 (2001b), and a direct membrane filtration methods (Sobsey et al., 1990) will be compared to determine the best available methods for coastal fecal contamination

monitoring of water and shellfish. Because few systematic comparisons of coliphage methods have been performed for estuarine water or shellfish and even fewer at such a geographically diverse number of field sites as investigated in this study, this information would be an important and timely contribution to our knowledge and understanding.

II. It is hypothesized that F+ RNA coliphages provide meaningful information about fecal inputs and their sources when these viruses are genotyped into their known distinct groups that are historically linked to and considered predictive of human or animal sources. Microbial fecal indicators used by regulators (fecal coliforms, *E.* coli, and enterococci) indicate the level of fecal pollution, but cannot characterize fecal sources. Fecal sources can be elucidated with extra analysis of phenotypic or genotypic traits of fecally-associated microbes as a process called microbial source tracking. In this study the source tracking potential as well as sensitivity, specificity and false-negative and positive rates of a new F+ RNA genotyping assay (Vinje et al., 2004) will be studied using a large, geographically diverse pool of F+ RNA and F+ DNA field isolates. This work intends to advance microbial source tracking by attempting to validate F+ RNA coliphage genogrouping patterns and their corresponding human/animal sources for microbial source tracking and by identifying potential new probes to capture and characterize previously undetected or suspect F+ RNA coliphages. Achieving these goals can improve the tools available to water quality manager to predict the source, amount, and impact of fecal pollution and thereby to better protect bathers and

shellfish consumers from pathogen exposures and their attendant disease risks, and also improve TMDL calculations.

III. It is hypothesized that a rapid (about 3 hours) and novel infectious F+ RNA coliphage recovery, detection/quantification, and serotyping assay can be developed in this study for same-day water and shellfish quality monitoring. One of the greatest challenges for water quality managers is to provide timely closings/advisories for fecally polluted surface waters and shellfishing areas. With current technology, water managers do not have timely information from which to make decisions about the microbial quality of water or shellfish based on the detection of culturable or infectious bacteria. This is because current bacterial indicators (fecal coliforms, *E. coli* and enterococci) require one to four days of culturing, sometimes successively, for definitive laboratory results (NRC 2004). It is proposed that existing microbiological analysis tools with critical improvements can be applied in novel ways for F+ RNA coliphage detection in about three hours, and that these methods might be field-ready or nearly so upon completion of this project. Specific goals are: i) to improve an existing coliphage broth enrichment culture method to be performed in 3 or less hours and that is compatible with coliphage detection in fresh, estuarine, and marine waters; ii) to develop a rapid (within minutes) F+ RNA coliphage detection and group identification method that is simple, inexpensive and field-portable; iii) to develop a simple and field-portable test system combining the coliphage enrichment culture step with the rapid detection and typing step. If achieved, this research will provide a new and novel

fecal indicator detection system that may be integrated as a field-ready kit or have convenient portions that are field-portable. The benefit of this work would be a useful and timely tool for rapid and timely management of the microbiological quality of water and shellfish.

Literature Review

Water and Shellfish-Borne Pathogens. The majority of known human microbial pathogens in water and shellfish come from point-source (e.g. sewage) or non-point source fecal pollution (e.g. runoff, stormwater, boat dumping), although some pathogens are endemic to marine and estuarine environments such as various Vibrio bacteria species such as *Vibrio vulnificus* and certain Aeromonas and Plesiomonas species (Harwood et al, 2004). Fresh and marine waters and bivalve mollusks also can become contaminated with marine algal toxins, toxic cyanobacteria and eukaryotes, such as toxic dinoflagellates (Van Dolan 2000; Sellner et al., 2003). However, these toxin-producing microbes are thought to be incapable of infecting and proliferating in humans, and therefore are not pathogens but instead toxicants. These microbial biotoxins are important to human health and aquatic ecology, but they are not the focus of this research and will not be further considered here.

A well-documented source of microbial pathogens is waste water effluent (Gilbert et al., 1976; Harwood et al., 2005). Waste water treatment is often inadequate to prevent contamination of estuarine water and pathogen bioaccumulation in shellfish (Shieh, 2003; Lodder and de Roda Husman, 2004), which puts bathers and shellfish consumers at risk for a host of diseases such as gastroenteritis, respiratory illnesses, infectious hepatitis and skin infections to name a few. Some pathogenic microbes of concern in recreational water and shellfish are bacteria such as *Salmonella* spp. *Pseudomonoas aeruginosa*, cholera and non-cholera *Vibrio* spp, *Shigella sonnei*, *Campylobacter jejuni, Legionella spp, E. coli* O157:H7, *Staphylococcus* spp, *Bacillus* spp; protozoan parasites such as

Cryptosporidium spp. and *Giardia intestinalis* of enteric origin and free-living amoeba such as *Naegleria fowleri,*; and viruses such as hepatitis A virus, enteroviruses, adenoviruses, and noroviruses (Lipp and Rose 1997; Yoder et al, 2004).

People at higher risks of more serious illness and death from pathogens acquired as water exposure illnesses or from shellfish consumption are children, the elderly, those who are imunocompromised, persons with other gastrointestinal disorders, regular users of recreational waters (e.g, surfers, swimmers) and consumers of raw bivalve mollusks. Water contact recreation in ocean water is associated with an increased risk of illness (Cabelli, 1983; Corbett et al., 1993; Haile et al., 1999; Prieto, et al., 2001; Wade et al., 2003; NRC 2004; Pond 2005).

Risk groups for shellfish-borne illnesses are similar to those for water, and especially those individuals who consume raw or partially cooked shellfish. Epidemiological links between the consumption of contaminated bivalve shellfish and enteric diseases are well established (Hackney and Pierson, 1994; Shieh et al., 2000; Sanchez et al., 2002; Kingsley et al., 2002; NCR 2004). Shellfish-related gastroenteritis outbreaks from Norovirus are directly related to levels fecal pollution in shellfishing areas (Table 1.1), and environmental monitoring of shellfish (oysters, mussels, clams, cockles) shows that many shellfishing grounds in North America, Europe, and Japan contain readily detectable levels of pathogenic viruses (Table 1.2).

TABLE 1.1. Outbreaks of Norovirus linked to sewage impacted shellfishing areas (Love 2004).

Reference	Location	Date	Source of Sewage	Cases
Appleton, 1977	England	1976	Unknown	33
Murphy, 1997	Australia	1978	Unknown	2000
Gill, 1983	England	1983	Unknown	137
Kohn, 1995	Louisiana, USA	1993	oyster harvester	190
CDC MMWR 1994	Florida, USA	1993	oyster harvester	30
CDC MMWR 1995	Florida, USA	1995	oyster harvester, recreational boaters	70
Berg 1997	Louisiana, USA	1996	Oyster harvester, oil rig	72
CDC, MMWR 1997	Louisiana, USA	1996	oyster harvester	153

Modified from (Berg, 2000)

TABLE 1.2. Pathogenic viruses detected in shellfish (Love 2004).

Reference	Country	Duration	Organism	Shellfish	% shellfish postive for virus by (RT)PCR					
		Months		Sample number	ADV	ASV	EV	HAV	NV	RV
Chung 1996	USA, NC	12	Oysters	31	-	-	50	17	-	-
Le Guyader 2000	France	36	Oysters	108	-	17	19	0	23	27
Le Guyader 2000	France	36	Mussels	73	-	50	45	13	35	52
Chironna 2002	Greece, Spain, Italy	11	Mussels	290	-	-	-	18	-	-
Formiga-Cruz 2002	Greece	18	oysters and mussels	144	33	-	15	4	2	-
Formiga-Cruz 2002	Spain	18	oysters and mussels	105	36	-	26	3	26	-
Formiga-Cruz 2002	Sweden	18	Mussels	54	33	-	24	0	41	-
Formiga-Cruz 2002	United Kingdom	18	oysters and mussels	173	46	-	14	1	10	-
Romalde 2002	Spain	--	Mussels	136	-	-	44	28	-	-
Romalde 2003	Spain	--	clams and cockles	28	-	-	43	25	-	-
Beuret 2003	Switzerland	3	Oysters	87	-	-	5	0	9	-
Nishida 2003	Japan	14	Oysters	191	-	-	-	-	9	-
Shieh 2003	Gulf of Mexico	18	Oysters	12	-	-	42	-	58	-

ADV = adenovirus; ASV = astrovirus; EV = enterovirus; HAV = hepatitis A virus; NV = norovirus; RV = rotavirus

Microbial Indicators for Recreational Water and Shellfish Monitoring. Water quality managers and shellfish sanitation managers currently rely on non-pathogenic enteric microbes to indicate the presence or magnitude of (i) fecal pollution, (ii) pathogenic microbes, or (iii) magnitude of disease risks. Direct testing of water and shellfish for human pathogens is often not feasible because assays are expensive, time consuming, technically difficult, and pathogens may be in low concentrations. Microbial indicator for pathogens and their risks are based on criteria of an ideal indictor (Gerba 1987) and the quantitative association between levels of an indicator and a specific health outcome. Quantifiable associations exist between microbial indicator levels and risks of adverse health effects (Prüss 1998), although these associations are different for different studies and study sites. In marine waters, *E. coli* and enterococci correlate with gastrointestinal illness in bathers, while coliphages and enteroviruses predict risks of gastrointestinal illness but need more validation due to the limited number of studies (Cabelli 1983, Dufour 1984, US EPA 1986; NRC 2004; Wade 2003). For other water-contact illnesses, the relative risk of skin disorders increased with fecal coliforms, enterococci, and *E. coli,* while no microbial indicators have been reported for respiratory illness in marine or freshwater (NRC 2004; Wade 2003). In shellfish, fecal coliform are the bacterial indicators of choice by US regulators (FDA 2002). An effort was begun the early 1990s to reevaluate microbial indicators of shellfish quality and disease risk in the USA, but this initiative was never completed and no new or alternative indicators were adopted for regulation. F+ RNA coliphages have shown associations with fecal pollution and increases in shellfish-related disease outbreaks in the UK (Doré et al., 2000). This

has led the European Commission to consider the possibility F+ RNA coliphages being used as candidate viral indicators of shellfish quality (Formiga-Cruz et al., 2003).

Recreational waters and shellfish are monitored by state or local agencies using bacterial indicators: enterococci, *E. coli*, and fecal coliforms. The US Food and Drug Administration's National Shellfish Sanitation Program uses fecal coliforms to regulate the microbiological quality of shellfish growing waters and shellfish meat (FDA 2002). Action levels for closings or advisories in shellfish use the geometric mean of fecal coliforms in overlying water (<14 CFU/ml) and/or shellfish meat (<230 CFU/ml). State Shellfish Sanitation Programs also have bacterial indicator limits for sites only harvestable after depuration in clean water, and for condemned sites.

The Beaches Environmental Assessment and Coastal Health (BEACHES) Act of 2000 applies US EPA enterococci bacterial criteria for bathing beaches (US EPA 1986). States may adopt EPA recommendations or develop their own equally or more conservative monitoring schemes. EPA recommended action levels in recreational water depend on whether the site is high use (Tier 1; <104 CFU/100ml single sample; <35 CFU/100ml monthly average of 5 or more samples), medium use (Tier 2; <276 CFU/ml single sample), or low use (Tier 3; <500 CFU/ml single sample). Recently promulgated BEACHES Act requirements are affecting the stringency with which states must monitor their surface waters, which is part of the reason for a yearly increase in beach closings and advisories from 1999 to 2004. In 2005, ocean beaches, bays, and lakes in the US had nearly 20,000 days with closings or advisories (NRDC, 2006). The majority of closures/advisories were caused by exceedances of bacterial indicator levels from unknown sources (NRDC 2006). Other closures/advisories were caused by

rain/runoff/stormwater and sewage spills (NRDC, 2006). Fecal pollution sources were unknown because current bacterial indicator assays cannot track fecal pollution to its specific sources. This inability to track sources of fecal contamination further confounds and severely limits management and control of water quality.

Of the conventional bacterial indicators (fecal coliforms, *E. coli*, enterococci), none are 100% feces-specific. Coliforms were introduced in sanitary studies in 1914 by the US Public Health Service (FDA 2002). Upon finding that coliforms reside in soil, water, and the gut of animals, more rigorous culture conditions (44.5°C for water and shellfish or 45.5°C for foods) were applied to samples to recover only coliforms "thermotolerant" to gut environments (synonymous with fecal coliforms) (FDA 2002). However, non-fecal thermotolerant bacteria such as *Klebsiella* species can still be recovered (Caplenas and Kanarek 1984). *E. coli* is used in place of fecal coliforms to monitor shellfish in Europe (EU 1991), on the premise that they are the most fecal-specific coliform bacteria. Hence, *E. coli* bacteria constitute a subset of coliforms such that total coliforms > "thermotolerant" fecal coliforms > *E. coli*, with corresponding progressively greater specificity for predicting fecal contamination

E coli was discovered by Theodore Escherich in 1885 (Escherich 1885), and first proposed as a fecal pollution indicator in 1892 (FDA 2002). Without extra characterization *E. coli* has low fecal source specificity, but *E. coli* is more specific to fecal pollution than fecal or total coliforms. *E. coli* can survive and grow in temperate soils (Ishii et al., 2006) and tropical soils and estuaries (Solo-Gabriele et al., 2000; Chandran et al., 2005), making a less than ideal fecal indicator in those environments.

Species of enterococci are another fecal indicator group used for monitoring recreational marine water. Enterococci are facultative anaerobes that include those of primarily both fecal (e.g., *E. faecalis* and *E. faecium*) and non-fecal (e.g., *E. casseliflavus*) origin in the *Enterococcaceae* family (US EPA 2002). Like total and fecal coliforms this group of microbes is not entirely feces specific either. Furthermore, there are more than two dozen species of enterococci, some primarily fecal and some not, and of those that are fecal, some primarily human and some primarily from other animals. Speciation is difficult based on simple biochemical properties that can be included in culture media. Reliable speciation requires advanced biochemical testing or nucleic analyses, making it impractical to distinguish fecal enterococci from non-fecal enterococci using routine culture methods (Harwood et al., 2005).

Problems with bacterial indicator source-specificity extend beyond fecal or non-fecal source differentiation. Fecal indicator bacteria are present in the gut of and are excreted by all warm-blooded animals, including birds (Abedon 1990). Since estuaries and coastal regions are prime areas for breeding bird populations and habitats for mammalian wildlife, reliance on fecal indicator bacteria 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.

The problem of distinguishing human from non-human fecal contamination by detecting and characterizing a simple indicator microbe were reportedly overcome with F+ RNA coliphage viral indicators. Serotyping or genotyping F+ RNA coliphages identified animal sources of fecal pollution in a Florida water body and a New York City

reservoir. The latter finding led to a bird deterrent program to reduce fecal inputs by keeping migratory birds off the water supply reservoir (Griffin et al., 2000; Alderisio et al., 1996). In these examples, F+ RNA coliphage indicators were used as alternatives to fecal indicator bacteria for microbial source tracking.

Coliphages. Bacteriophages (phages) infecting Enterobacteria, species of the genera *Caulobacter* and *Pseudomonas*, and other gram-negative bacteria are colloquially termed "coliphages" and formally fall in 6 formal taxonomic families: three families of double-stranded DNA phages *Myoviridae, Styloviriae, Podoviridae,* two families of single-stranded (ss) DNA phages *Microviridae* and *Inoviridae*, and the *Leviviridae* family of ss RNA phages (herein called F+ RNA coliphages) (Van Regenmortel et al., 2000). The phage site of infection on host bacteria is means for differentiating coliphages, with somatic coliphages infecting through the bacterial cell wall, and F+ coliphages infecting through the bacterial F-pilus (Van Regenmortel et al., 2000). When comparing attachment to host pili, *Inoviridae* (F+ DNA) phages attach to the tip of the F-pili, while *Leviviridae* F+ RNA phages attach to the sides of F-pili (Manchak et al.., 2002).

F+ coliphages in the *Leviviridae* family are small viruses, 23 nm diameter, possess a capsid of icosahedral shape, and contain a single-stranded RNA genome of 3500-4200 nucleotides. They can be grouped into two distinct genera, *Levivirus* and *Allolevivirus*, and three unclassified groups (Van Regenmortel et al., 2000). F+ RNA coliphages resemble the physical characteristics, environmental persistence, and disinfection properties of many enteric viruses in the *Picornaviridae* family such as hepatitis A virus and others in the *Enterovirus* genus, and *Noroviruses* genus in the *Caliciviridae* family (Allwood et al., 2004; Grabow 2001; Havelaar, 1993; Van

Regenmortel et al., 2000). F+ RNA coliphages reside in the gut of animals including humans and can be grouped as representative of human feces (group II and III) or animal feces (group I and IV) as shown in SE Asia and North America (Furuse et al., 1981; Osawa et al., 1981) and North America (Cole et al., 2003; Hsu et al., 1995). Genomic organization of *Levivirus* groups I and II and *Allolevivirus* groups III and IV share three genes: maturation protein, coat protein, and subunit II of replicase. However, they differ in that only *Levivirus* species have a lysis gene, while the genome of *Alloleviviruses* are ~700 nt longer and have a read-through frame (Crawford and Gesteland 1964; Van Regenmortel et al., 2000). From statistical predictions of phylogenetic trees, the *Allolevivirus* group may have evolved by gene expansion from historic *Levivirus* strains (Bollback and Huelsenbeck 2001)

As fecal indicators, coliphages correlate with the presence of pathogenic human viruses in water and shellfish and a subsequent increase in viral illness risk (Chung et al., 1998; Havelaar, 1993; Doré et al., 2000; Jiang et al., 2001; Wade et al., 2003). F+ RNA coliphages are also used as indicators of fecal pollution in foods like shellfish, agricultural produce and meat (Chung et al., 1998; Endley et al., 2003; Hsu et al., 2002) and as surrogates for human enteric viruses in water (Allwood et al., 2004), soils (Meschke 2001), and produce (Dawson et al., 2005).

In this author's estimation, coliphage recovery methods lack proper performance validation or methods comparison in many environmental matrices for which they are used or are being considered for use. This is because there have been no or few reports of spiked sample recovery efficiency studies or systematic recovery efficiency comparisons with other methods for samples such as agricultural produce (Endley et al.,

2003a, 2003b), soils, sediments, and biosolids, shellfish, and seawater. In contrast, coliphage recovery efficiency and inter-laboratory performance evaluation of certain coliphage recovery and detection methods has been done for artificially contaminated ground water and model waters (US EPA, 2003a, 2003b; Sobsey et al., 2004; Mooijmans et al., 2005)

Unlike recovery methods, detection and grouping methods (for genogrouping or source tracking) F+ RNA coliphages have advanced greatly in recent years with the advent of two oligonucleotide probe hybridization assays (Hsu et al., 1995; Vinjé et al., 2004) and two quantitative Taqman reverse transcriptase (RT)PCR assay (Kirs and Smith 2006; Ogorzaly and Gantzer 2006). These molecular methods have been studied for their ability to reliably type F+ RNA coliphages based on analysis of know type strains and by benchmarking performance for field strain typing against other reference methods. (Stewart et al., 2006; Stewart-Pullaro et al., 2006).

Microbial Source Tracking. Microbial source tracking is an analytical process for determining sources of fecal pollution by matching or grouping fecally-associated microbes found in the environment (e.g. surface water) with their original or statistically similar fecal source (human, specific animal, etc.) (US EPA 2005). Microbial grouping assays attempt to create genotypic or phenotypic distinctions among a population of target microbes, and in some cases a microbial library of known fecal sources is generated for comparison with microbial field samples. Microbial source tracking has gained importance through its use in Total Maximum Daily Load (TMDL) calculations required by the Clean Water Act (US EPA 2005) and because microbial source tracking

is a means for identifying non-point source fecal pollution, which cannot be as easily discerned as point-source pollution with traditional microbiological assays.

Initial screening methods require high logistic feasibility, broad applicability, and rapid results, while microbial assessment methods should be specific to a fecal source or origin and measure indicators with similar survival and transport as pathogens (NRC 2004). A question that science is attempting to answer is: which methods using what model organisms are best for microbial source tracking? As several authors have alluded to or stated outright, many microbial source tracking assays are effective, but no single assay works best for all situations (Scott et al., 2002; Noble et al., 2003; Stewart et al., 2003; US EPA 2005). The decision for one assay and against others should depend on site-specific circumstances, speed to obtain results, desired outcome, public health consequences and cost of a correct or incorrect answer (US EPA 2005). Promising microbial source tracking methods should be studied and applied in an effort to gauge their ability to improve microbial quality through fecal source characterization and management.

F+ RNA coliphage source tracking has some key advantages over bacterial and chemical source tracking methods that make it a good microbial source tracking candidate. Unlike bacterial source tracking methods (e.g. ribotyping, antibiotic resistance testing, and PFGE fingerprinting), F+ RNA coliphage methods do not require the generation of a watershed-specific source library. Creating and maintaining a large microbial source tracking library is expensive and time consuming. Animal-associated or human-associated F+ RNA coliphages are hypothetically the same regardless of the watershed or geographic area from which they are recovered. This makes F+ RNA

source tracking a broadly applicable approach for areas without existing bacterial source tracking libraries. F+ RNA MST has advantages over chemical source tracking (e.g. caffeine or corprostanol) because chemical detection methods are technically demanding, insensitive, expensive, and have not been correlated with health outcomes (Scott et al., 2002).

Genetic diversity at the nucleotide sequence level in the replicase gene of F+ RNA coliphages reveals as much as 50% differences between Leviviruses in F+ RNA groups I and II, and more than 60% sequence differences between Alloleviviruses in F+ RNA group III and IV (Vinje et al., 2004), allowing probes to target each individual F+ RNA genogroup. The weakness of F+ RNA source tracking is that it does not always produce clear distinctions between human and animal sources or the magnitude of those sources (Hsu et al., 1995; Cole et al., 2003; Stewart et al., 2006). These limitations should be investigated further (Scott et al., 2002) because the extent to which they compromise performance may vary with the site and environmental conditions. To overcome a lack of fecal source distinction, F+ RNA group III isolates from three hog farms in the Carolinas were compared by sequence analysis of a portion of the maturation gene showed clustering of isolates from each farm (Stewart et al., 2006). Stewart's findings are consistent with the hypothesis that RNA phage progeny are a "clustered spread of variant sequences" (Reanney 1982). From Stewart's findings, more work is warranted on F+ RNA coliphage genetic drift in a fragmented source population (such as in concentrated animal feeding operations [CAFOs]), because F+ RNA coliphages have a remarkably high mutation rate of 1 per genome per replication (Drake 1993) and based Fon current understanding F+ RNA genogroups are not absolute source specific (Hsu et

al., 1995; Cole et al., 2003). A better understanding of sub-group source differences and the extent to which there are source-specific differences among these groups would improve microbial source tracking with F+ RNA coliphages. Future efforts to investigate intra-group diversity of strains from various fecal sources might well use genomic regions with high variability such as the maturation gene instead of conserved regions such as the replicase gene.

The development of rapid, simple, and inexpensive source tracking methods are needed for realistic daily use in decision-making. While real-time quantitative PCR or RT-PCR has promise as a rapid source tracking procedure for a variety of microbes, including coliphages, there are a number of limitations and disadvantages with this approach. First, direct PCR or RT-PCR whether by the slower conventional or more rapid real-time quantitative methods does not distinguish infectious or culturable microbes from non-infectious, inactivated microbes. These methods have the potential to detect the nucleic acid of inactivated viruses and other microbes (Sobsey et al., 1998). Second, real-time quantitative PCR and other nucleic acid amplification methods are not yet conveniently and reliably field-portable, especially the sample processing steps for recovering and extracting nucleic acids from water and other environmental samples. Third, the microbial recovery and nucleic acid extraction and amplification methods are technically demanding, require skilled and trained analysts, are costly (for both needed hardware and consumable supplies) and can not be done easily and quickly in the field.

Rapid Indicator Detection. Water and shellfish sanitation managers do not have timely information from which to make decisions about the microbial quality of water or

shellfish because current bacterial standards (for fecal coliforms, E. coli and enterococci) cannot be performed in a timely manner. Bacterial indicator tests require one to four days, which cause delays in water quality warnings, shellfishing area openings and closings and approval or condemnation of previously harvested shellfish (NRC 2004). Contamination events in recreational waters and shellfish areas are intermittent and often return to below threshold levels in 24 hours, so any warnings or advisories are usually posted days after the contamination event clears (Boehm et al., 2002; Leecaster and Weisberg, 2001).

Another important deficiency of bacterial fecal indicators is their lack of predictability for enteric virus contamination. New technology for rapid detection of indicator bacteria has the potential to test water samples in less than 4 hours. However, these rapid bacterial tests fail other criteria, primarily due to poor sensitivity and small sample volumes (much less than 1 ml), and the inability of some tests to detect viable microbes and distinguish infectious or culturable from non-infectious or non-culturable ones (Noble and Weisberg, 2004; Alliance for Coastal Technologies, 2003). New technologies are being introduced for both bacterial and coliphage indicator tests using immunoassay techniques, nucleic acid techniques, and enzyme/substrate methods that would perhaps make rapid and sensitive detection a reality (Table 1.1) (Noble and Weisberg, 2004). None of these methods have been standardized and they have not been subjected to inter-laboratory performance validation for microbial detection in either recreational or shellfishing waters or in shellfish meats. Such collaborative studies are in progress in Europe to develop tested methods for the recovery and detection of adenoviruses and noroviruses in European bathing waters (SEMIDE website,

VIROBATHE). Furthermore, no rapid indicator tests using new technology have been approved by the EPA for beach water quality monitoring or by the Interstate Shellfish Sanitation Conference for shellfish sanitation monitoring.

Rapid and simple field kits are available for clinical microbiology and these assays are used in hospitals, clinical diagnostic labs, and are sold commercially. Some of these rapid field kits use antibody-antigen capture and "latex" (solid bead particle) agglutination methods to detect HIV in blood, rotavirus and adenovirus in stool, avian influenza, and *Streptococcus* for obstetric exams (Table 1.1) (Hughes et al., 1984; Yolken et al., 1986; Mortensson-Egnund, 1988; Quentin et al., 1993 Arai et al., 1999; Xu et al., 2005). These antibody tests are simple, and can be performed in less than 20 minutes, but have yet to be applied to water quality monitoring.

Novel approaches for rapid and field-portable coliphage detection are possible by combining aspects of coliphage field kits developed for use in developing countries (Dan et al., 1996; Loh, 1988) with new molecular technologies and/or clinical diagnostic immunoassay test kits. Eight candidate rapid coliphage indicator detection methods are listed in Table 1.3. The only published method for the rapid detection of coliphage among those in Table 1.3 is realtime RT-PCR (Kirs and Smith 2006; Ogorzaly and Gantzer 2006). Realtime RT-PCR and other molecular detection schemes are not readily available as field-portable units, and cannot be performed ad-hoc in the field because these assays require "molecular clean" techniques performed free of RNase and preferably in a laminar flow hood. They also require nucleic extraction and purification steps that are currently impractical or impossible to perform in non-laboratory settings. Furthermore, these methods require skilled and experienced analysts and can not be

reliably done by unskilled and untrained people. Several of the candidate methods in Table 1.3 use fluorescent signals to indicate hybridization of coliphage targets with synthetic oligonucleotide probes. Fluorescent signals might be detectable with a handheld fluorescent detector if the fluorescent signal is strong, but handheld fluorescent detectors are probably not sensitive enough and they have never been evaluated for this purpose. The fastest hypothetical method is nucleic acid sequence based amplification (NASBA),; however, this method is the most costly of those listed and it cannot be performed in the field (Table 1.3). The cheapest ($0.25/sample) and most user-friendly of the rapid detection methods is "latex" or particle agglutination (Singer and Plotz 1956) (Table 1.3). It is likely that a latex agglutination method for coliphage could be readily commercially produced for water quality monitoring labs, as such methods already are for clinical diagnostic microbiology tests (Hughes et al., 1984; Slotved et al., 2004).

TABLE 1.3. Rapid coliphage (and other microbe) detection technologies.

Technique	Coliphage culture step	Target	Signal	Simple and field-portable [1]	Estimated detection time (min)	Estimated unit price [2]	Materials and equipment [3]
NASBA [6]							
i) realtime probe	no	NA [4]	Fluorescent	No	60	$20	A, B, C, E, H
ii) antibody	no	CP [5]	Chromogenic	No	180	$20	A, B, C, G,
iii) antibody	no	CP	Electrochemical	No	180	$20	A, B, C, F, G
Realtime RT-PCR [7]	no	NA	Fluorescent	Maybe	60	$10	D, H
Molecular beacon	yes	NA	Fluorescent	Maybe	180	$5	A, B, E, H, K
Fluorescent nanoparticles							
i) realtime probe	no	NA	Fluorescent	Maybe	90	$10	B, C, D, I, H
ii) antibody	yes	CP	Fluorescent	Yes	120-180	$10	A, E, G, I, K
Latex agglutination	yes	CP	Visual clumping	Yes	120-180	$0.25	A, G, J, K

[1] assumes that assays with nucleic acid detection cannot be performed "molecular clean" in the field; [2] based on 25-50 ul sample, not including biological reagents and disposables. [3]A = water bath ($500 to $1k; Fischer), B = RNA extraction kit ($4/sample; Qiagen); C = RT-PCR amplification kits ($2.50 to $5/sample; Qiagen); NASBA amplification kit ($12.5/sample BioMerieux), D = realtime thermocycler ($30k to $40k); E= handheld fluorescent detector ($3,000); F= x-ray film developer ($5k to $10k; Kodak) ; G= antibodies- polyclonal rabbit IgG ($200/antibody ; UNC); H= molecular beacon ($500/10nM probe; IDT) (Tyagi and Kramer 1996) ; I= fluorescent nanoparticles ($500/1.5 ml tube; Q-dots); J = polystyrene “latex” particles ($125/15 ml tube; Seradyn); K = biological reagents and media. [4]NA= nucleic acids with probes; [5]CP=capsid proteins with antibodies. [6] NASBA= nucleic acid sequence based amplification. [7] RT-PCR = realtime polymerase chain reaction

Summary. The gap between the high art and professional practice of water and shellfish microbiological quality monitoring has, in this authors opinion, increased an irresponsible amount. The results of widening the gap of knowledge-to-practice will be greater inefficiencies in resource use and labor spent on water and shellfish monitoring, where modern methods could proved more accurate, timely, and more detailed results. Using methods and concepts that are over a century old, the US shellfish quality assessments for fecal coliforms remain a key example of the lack of technology uptake and acceptance among shellfish regulators. This paralysis by analysis is a problem not just for state shellfish sanitation offices and the ISSC, but for consumers' confidence in the shellfishing industry and ultimately the health of shellfish consumers.

Small inroads are emerging in regulatory use of new methods for water monitoring. The recreational water quality field is beginning to revisit existing regulations to improve predictions of exposure and disease risks for water contact users, where newer molecular biology methods are being compared with standard culture and plating methods with positive results (Wade et al., 2005). The new Ground Water Rule from the US EPA acknowledges that "old guard" methods for fecal indicator bacteria may not be sufficient indicators of enteric viruses in ground water, and thus have included coliphages for this purpose. The use of microbial source tracking for TMDLs, source apportionment, and detailed pollution studies is another field where regulators are introduced to a new generation of water and shellfish monitoring tools.

Where new technology uptake has occurred the mechanisms for that change should be studied and repeated for other areas of interaction between environmental microbiology research and practice. Certainly it appears to be the responsibility of both

the research community and regulators/practitioners to help bridge the gap between the state of science and government regulations.

References

1. **Abedon, S. T.** 1990. The ecology of Bacteriophage T4. Doctoral Disseratation. The University of Arizona.

2. **Alderisio, K. A., Wait, D. A., and M. D. Sobsey**. 1996 Detection and characterization of male-specific RNA coliphages in a New York City reservoir. *In* J. J. McDonnell, D. L. Leopold, J. B. Stribling, and L. R. Neville (ed.) Watershed Restoration Management New York City Water Supply Studies. American Water Resources Association, Herndon, Virginia. pp. 133–142.

3. **Allwood, P. B., Malik, Y. S.,, Maherchandani, S., Vought, K., Johnson, L. A., Braymen, C., Hedberg, C. W., and S. M. Goyal.** 2004. Occurrence of *Escherichia coli*, noroviruses, and F-specific coliphages in fresh market-ready produce. J. Food Prot. **67:**2387-2390.

4. **Arai, H., Petchclai, B., Khupulsup, K., Kurimura, T., and K. Takeda.** 1999. Evaluation of a rapid immunochromatographic test for detection of antibodies to human immunodeficiency virus. J. Clin. Microbiol. **7(2):**367-370.

5. **Boehm, A. B., Grant, S. B., Kim, J. H., Mowbray, S. L., McGee, C. D., Clark, C. D., Foley, D. M., and D. E. Wellman.** (2002) Decadal and shorter period variability of surf zone water quality at Huntington Beach, California.Environ Sci Technol. **36(18):**3885-3892.

6. **Bollback, J.P and J.P. Huelsenbeck.** 2001. Phylogeny, genome evolution, and host specificity of single-stranded RNA bacteriophage (family Leviviridae). J. Mol. Evol. **52:**117-128.

7. **Brion, G., Meschke, J.S., and M.D. Sobsey**. 2002. F-specific RNA coliphages: occurrence, types, and survival in natural waters. Wat. Res. **36(9):**2419-2425.

8. **Cabelli, V.J.** 1983. Health Effects Criteria for Marine Recreational Waters. Cincinnati, Ohio. EPA-600-1-80-031

9. **Chandran, A.., and A. A. Mohamed Hatha.** 2005. Relative survival of *Escherichia coli* and *Salmonella typhimurium* in a tropical estuary. Wat. Res. **39(7):**1397-1403.

10. **Chung, H., Jaykus, L.-A., Lovelace, G., and M. D. Sobsey.** 1998 Bacteriophages and bacteria as indicators of enteric viruses in oysters and their harvest waters. Wat. Sci. Tech., **38(12):**37-44.

11. **Cole, D., Long, S. D., and M. D. Sobsey**. 2003. Evaluation of F+ RNA and DNA coliphages as source-specific indicators of fecal contamination in surface waters. Appl Environ Microbiol. **69(11):**6507-6514.

12. **Colford Jr., J.M., Wade, T.J., Schiff, K.C, Wright, C, Griffith, J.F., Sandhu, S.K., and S.B. Weisberg.** 2005. Recreational Water Contact and Illness in Mission Bay, California. Southern California Coastal Water Research Project. Technical Report # 449.

13. **Colford, J. M. Jr, Wade, T. J., Schiff, K. C., Wright, C. C., Griffith, J. F., Sandhu, S. K., Burns, S., Sobsey, M., Lovelace, G. and S. B. Weisberg.** 2007.Water quality indicators and the risk of illness at beaches with nonpoint sources of fecal contamination. Epidemiol. **8(1):**27-35.

14. **Corbett, S. J., Rubin, G. L., Curry, G. K., and D. G. Kleinbaum.** 1993. The health effects of swimming at Sydney beaches. The Sydney Beach Users Study Advisory Group. Am J Public Health. **83(12):**1701-1706.

15. **Crawford, E.M. and R.F. Gesteland**. 1964. The adsorption of bacteriophage R17. Virol. **22:**165-167.

16. **Crossett, K. M., Culliton, T. J., Wiley, P. C., and T. R. Goodspeed.** 2004. Population Trends Along the Coastal United States: 1980- 2008. NOAA. [Online.] http://www.oceanservice.noaa.gov/programs/mb/supp_cstl_population.html

17. **Dan, F. S., Ngeow, Y. F, and C.W. Wang.** 1996. Coliphage Field Kit: Technical Final Report. International Development Research Center. [Online.] http://archive.idrc.ca/library/document/106339/index_e.html

18. **Dawson ,D. J., Paish, A., Staffell, L. M., Seymour, I. J., and H. Appleton.** 2005. Survival of viruses on fresh produce, using MS2 as a surrogate for norovirus. J Appl Microbiol. **98(1):**203-209.

19. **Dore, W.J., Henshilwood, K., and D. N. Lees.** 2000 Evaluation of F-specific RNA bacteriophage as a candidate human enteric virus indicator for bivalve molluscan shellfish. Appl. Environ. Microbiol. **66(4):**1280-1285.

20. **Dore, W. J., Mackie, M., and D. N. Lees.** 2003. Levels of male-specific RNA bacteriophage and Escherichia coli in molluscan bivalve shellfish from commercial harvesting areas. Lett. Appl. Microbiol. **36(2):**92-96.

21. **Drake J.** 1993. Rates of spontaneous mutation rates among RNA viruses. Proc Natl Acad Sci USA **90:**4171–4175.

22. **Endley, S., L. Lu, E. Vega, M. E. Hume, and S. D. Pillai.** 2003a. Male-specific coliphages as an additional fecal contamination indicator for screening fresh carrots. J. Food Prot. **66:**88-93

23. **Endley, S., E. Johnson, and S. D. Pillai.** 2003b. A simple method to screen cilantro and parsley for fecal indicator viruses. J. Food Prot **66:**1506-1509.

24. **Escherich, T.** 1885. Die darmbakterien des neugeborenen und sauglings. Fortshr. Med. **3:5-15-522**, 547-554.

25. **European Union (EU).** 1991. Council Directive 91/492/EEC of 15 July 1991 laying down the health conditions for the production and the placing on the market of live bivalve mollusks.

26. **FDA**. Bacteriological Analytical Manual (BAM). Ch 4. Enumeration of *Escherichia coli* and the coliform bacteria. [Online.] http://www.cfsan.fda.gov/~ebam/bam-4.html

27. **Formiga-Cruz, M., Allard, A. K., Conden-Hansson, A. C., Henshilwood, K., Hernroth, B. E., Jofre, J., Lees, D. N., Lucena, F., Papapetropoulou, M., Rangdale, R. E., Tsibouxi, A., Vantarakis, A., and R. Girones.** 2003. Evaluation

of potential indicators of viral contamination in shellfish and their applicability to diverse geographical areas. Appl. Environ. Microbiol., **69(3):**1556-1563.

28. **Furuse, K., Ando, A., Osawa, S., and I. Watanabe.** 1981. Distribution of ribonucleic acid coliphages in raw sewage from treatment plants in Japan. Appl. Environ. Microbiol. **41(5):**1139-1143.

29. **Gilbert, R. G., Rice, R. C., Bouwer, H., Gerba, C. P., Wallis, C., and J. L. Melnick.** 1976. Wastewater renovation and reuse: virus removal by soil filtration. Science. **192(4243):**1004-1005.

30. **Grabow, W. O. K**. 2001. Bacteriophages: Update on application as models for viruses in water. Water SA. **27(2):**251-268.

31. **Griffin, D. W., Stokes, R., Rose, J. B., and J. H. Paul III.** 2000. Bacterial indicator occurrence and the use of an F+ specific RNA coliphage assay to identify fecal sources in Homosassa Springs, Florida. Microb. Ecol. **39:**56-64.

32. Hackney, C. R. and M. D. Pierson. 1994. Environmental indicators and shellfish safety. New York, Chapman & Hall. 523.

33. **Haile, R. W., Witte, J. S., Gold, M., Cressey, R., McGee, C., Millikan, R. C., Glasser, A., Harawa, N., Ervin, C., Harmon, P., Harper, J., Dermand, J., Alamillo, J., Barrett, K., Nides, M., and G. Wang.** 1999. The health effects of swimming in ocean water contaminated by storm drain runoff. Epidemiol. **10(4):**355-63.

34. **Harwood, V. J., Gandhi, J. P., and A. C. Wright.** 2004. Methods for isolation and confirmation of Vibrio vulnificus from oysters and environmental sources: a review. J. Microb. Methods. **59(3):**301-316.

35. Harwood, V.J. N.C. Delahoya, R.M. Ulrich, M.F. Kramer, J.E. Whitlock, J.R. Garey, D.V. Lim (2004) Molecular confirmation of Enterococcus faecalis and E. faecium from clinical, faecal and environmental sources Letters in Applied Microbiology 38 (6), 476–482.

36. **Harwood, V. J., Levine, A. D., Scott, T. M., Chivukula, V., Lukasik, J., Farrah, S. R., and J. B. Rose**. 2005. Validity of the indicator organism paradigm for pathogen reduction in reclaimed water and public health protection. Appl. Env. Microbiol. **71(6):**3163-3170.

37. **Havelaar, A.H.** 1993. Bacteriophages as models of human enteric viruses in the environment. ASM News. **59(12):**614-619.

38. **Hot D, Legeay O, Jacques J, Gantzer C, Caudrelier Y, Guyard K, Lange M, Andreoletti L.** 2003. Detection of somatic phages, infectious enteroviruses and enterovirus genomes as indicators of human enteric viral pollution in surface water. Water Res. **37(19):**4703-4710.

39. **Hsu, F.-C.,. Shieh, Y.-S.C, van Duin, J., Beekwilder, M. J., and M. D. Sobsey.** 1995. Genotyping male-specific RNA coliphages by hybridization with oligonucleotide probes. Appl. Environ. Microbiol., **61(11):**3960-3966.

40. **Hsu, F. C., Shieh, Y. S., and M. D. Sobsey.** 2002. Enteric bacteriophages as potential fecal indicators in ground beef and poultry meat. J Food Prot **65:**93-99.

41. **Hughes, J. H., Tuomari, A. V., Mann, D. R., and V. V. Hamparian.** 1984. Latex immunoasssy for rapid detection of rotavirus. J. Clin. Microbiol. **20(3):**441-447.

42. **Ishii, S., Ksoll, W. B., Hicks, R. E., and M. J. Sadowsky.** 2006. Presence and Growth of Naturalized Escherichia coli in Temperate Soils from Lake Superior Watersheds. Appl. Env. Microbiol.**72(1):**612-621.

43. **Jiang, S., Noble, R. and W. Chu.** 2001. Human adenoviruses and coliphages in urban runoff-impacted coastal waters of southern California. Appl. Environ. Micro. **67(1):**179-184.

44. **Kingsley, D. H., Meade, G. K., G. P. Richards.** 2002. Detection of both hepatitis A virus and Norwalk-like virus in imported clams associated with food-borne illness. Appl Environ Microbiol. **68(8):**3914-3918.

45. **Kirs M., and D. C. Smith**. 2006. Multiplex quantitative real-time RT-PCR for the F+ specific RNA coliphages: a method for use in microbial source tracking. Appl. Environ. Microbiol. **73(3):**808-814.

46. **Kramer, M. H., Herwaldt, B. L., Craun, G. F., Calderon, R. L., and D. D. Juranek.** 1996. Surveillance for waterborne-disease outbreaks--United States, 1993-1994. MMWR. **45(1):**1-33.
47. **Lipp, E. K and J. B. Rose.** 1997. The role of seafood in foodborne diseases in the United States of America. Rev. Sci Tech. **16(2):**620-640.

48. **Leecaster, M. K., and S. B. Weisberg.** 2001. Effect of sampling frequency on shoreline microbiology assessments. Mar Pollut Bull. **42(11):**1150-1154.

49. **Lodder, W. J. and A. M. de Roda Husman.** 2005. Presence of noroviruses and other enteric viruses in sewage and surface waters in The Netherlands. Appl Environ Microbiol. **71(3):**1453-1461.

50. **Loh, C. L., Jangi, M. S., Ho, Y. C., Ngeow, Y. F., and C.W. Wang.** 1988. Development of a field test kit for coliphage detection in natural waters. Toxicity Assess. Int. J. **3(5)**: 451–460.

51. **Long, S. C., El-Khoury, S. S., Oudejans, S., Sobsey M. D., and J. Vinjé**. 2005. Assessment of Sources and Diversity of Male-Specific Coliphages for Source Tracking. Environ. Enginer. Sci. **22(3):**367-377.

52. **Love, D.** Masters Technical Report. 2004. The University of North Carolina at Chapel Hill, Chapel Hill, NC.

53. **Meschke, J. S.** 2001. Comparative adsorption, persistence, and mobility of Norwalk virus, poliovirus type I, and F+RNA coliphages in soil and groundwater. Doctoral Dissertation. University of North Carolina at Chapel Hill, Chapel Hill, NC.

54. **Manchak, J., Anthony, G., and L. S. Frost.** 2002. Mutational analysis of F-pillin reveals domains for pilus assembly, phage infection and DNA transfer. Mol. Microbiol. **(43)1:**195-205.

55. **Mortensson-Egnund K.** 1988. Rapid diagnosis of adenovirus gastroenteritis by a commercial latex agglutination test. A comparison with other methods. NIPH Ann. **11(2):**67-69.

56. **Noble, R.T. and S.B. Weisberg.** 2004. A review of technologies being developed for rapid detection of bacteria in recreational waters. J. Water and Health. **3:**381-392.

57. **Noble, R.T, Allen, S. M., Blackwood, W. C., Jiang, S. C., Lovelace, G. L., Sobsey, M. D., Stewart, J. R., and D. A. Wait.** 2003. Use of viral pathogens and indicators to differentiate between human and non-human fecal contamination in a microbial source tracking comparison. J. Water and Health. **1(4):**195-207.

58. **NRC.** 2004. Indicators of Waterborne Pathogens. National Research Council. The National Academies Press. Washington D.C.

59. **NRDC.** 2005. Testing the Waters 2005: A Guide to Water Quality at Vacation Beaches. National Resources Defense Council. [Online.] http://www.nrdc.org/water/oceans/ttw/titinx.asp

60. **Ogorzaly, L. and C. Gantzer**. 2006. Development of real-time RT-PCR methods for specific detection of F-specific RNA bacteriophage genogroups: Application to urban raw wastewater. J. Virol. Meth. **138(1-2):**131-139.

61. **Osawa, S., Furuse, K., and I. Watanabe**. 1981. Distribution of ribonucleic acid coliphages in animals. Appl Environ Microbiol. **41(1):**164-168.

62. **Pond, K.** 2005. Water recreation and disease. Plausibility of associated infections: Acute effects, sequelae and mortality. World Health Organization ISBN 92 4 156305 2. [Online.] http://www.who.int/water_sanitation_health/bathing/recreadis/en/index.html

63. **Prieto, M. D., Lopez, B., Juanes, J. A., Revilla, J. A., Llorca, J., and M. Delgado-Rodríguez**. 2001. Recreation in coastal waters: health risks associated with bathing in sea water. J. Epidemiol Community Health. **55:**442-447.

64. **Prüss, A.** 1998. A review of epidemiological studies on health effects from exposure to recreational water. Intnl. J. Epidemiol. **27(1):**1-9.

65. **Quentin, R., Dubarry, I., Gignier, C., Saulnier, M., Pierre, F., and A. Goudeau.** 1993. Evaluation of a rapid latex test for direct detection of Streptococcus agalactiae in various obstetrical and gynaecological disorders.Eur J Clin Microbiol Infect Dis. **12(1):**51-54.

66. **Reanney D. C.** 1982. The Evolution of RNA viruses. Annu. Rev. Microbiol. **36:**47-73.

67. **Sanchez, G., Pinto, R. M., Vanaclocha, H., and A. Bosch.** 2002. Molecular characterization of hepatitis a virus isolates from a transcontinental shellfish-borne outbreak. J Clin Microbiol. **40(11):**4148-4155.

68. **Scott, T.M., Rose, J.B., Jenkins, T.M., Farrah, S.R., and J. Lukasik**. 2002. Microbial Source Tracking: Current Methodology and Future Directions. Appl. Env. Microbiol. **(68)12:**5796–5803.

69. **Shieh, C. Y., R. S. Baric, Woods, J. W., and K. R. Calci.** 2003. Molecular surveillance of enteroviruses and Norwalk-like viruses in oysters relocated to a municipal-sewage-impacted gulf estuary. Appl. Environ. Microbiol. **69(12):**7130-7136.

70. **Shieh, Y., Monroe, S. S., Rankhauser, R.L., Langlois, G. W., Burkhardt III, W., and R. S. Baric.** 2000. Detection of norwalk-like virus in shellfish implicated in illness. J. Infect. Dis. **181(Suppl.2):**S360-366.

71. **Sellner KG, Doucette GJ, and GJ Kirkpatrick.** 2003. Harmful algal blooms: causes, impacts and detection. J. Ind. Microbiol. Biotechnol. **30(7):**383-406.

72. **SEMIDE website**. Methods for the concentration and detection of adenovirus and norovirus in European bathing waters with reference to the revision of the bathing water directive 76/160/EEC. [Online].
http://www.semide.net/initiatives/fol060732/proj123442

73. **Singer, J. M. and C. M. Plotz.** 1956. The latex fixation test. I. Application of the serologic diagnosis of rheumatoid arthritis. Amer. J. Med. **21:**888-892.

74. **Sinton, L.W., Hall, C.H., Lynch, P.A., and R.J. Davies-Colley**. 2002. Sunlight inactivation of fecal indicator bacteria and bacteriophages from waste stabilization ponds effluent in fresh and saline waters. Appl. Envrion. Microbiol. **68(3):**1122-1131.

75. **Slotved, H-C., Kaltoft, M., Skovsted, I.C., Derrn, M.B., and F. Espersen.** 2004. Simple, rapid latex agglutination test for serotyping of Pneumococci (pneumotest-latex). J. Clin. Microbiol. **42(6):**2518-2522.

76. **Sobsey, M. D., Schwab, K. J., and T. R. Handzel.** 1990. A simple membrane filter method to concentrate and enumerate male-specific RNA coliphages. Journal of American Water Works Association 82: 52-59.

77. **Sobsey, M.D., D. A. Battigelli, G.-A. Shin and S. Newland.** 1998. RT-PCR amplification detects inactivated viruses in water and wastewater. Water Sci. Technol. **38(12)**:91–94.

78. **Sobsey, M.D., M.V. Yates, F-C. Hsu, G. Lovelace, D. Battigelli, A. Margolin, S.D. Pillai and N. Nwachuku.** 2004. Development and evaluation of methods to detect coliphages in large volumes of water. Water Sci. Technol. **50(1):** 211–217.

79. **Solo-Gabriele, H. M., Wolfert, M. A., Desmarais, T. R., and C. J. Palmer.** 2000 . Sources of *Escherichia coli* in a coastal subtropical environment. Appl. Environ. Microbiol. **66**:230-237.

80. **Steinbergs, J., Kilchewska, K., Lazdina, U., Dishlers, A., Ose, V., Sallberg, M., and A. Tsimanis.** 1996. Short synthetic CDR-peptides forming the antibody combining site of the monoclonal antibody against RNA bacteriophage fr neutralize the phage activity. Hum Antibodies Hybridomas. **7(3):**106-112.

81. **Stewart, J.R., Vinjé, J., Oudejans, S.J.G., Scott, G.I., and M.D. Sobsey.** 2006. Sequence variation among group III F-specific RNA coliphages from water samples and swine lagoons. Appl. Environ. Microbiol. **72(2):**1126-1230.

82. **Stewart-Pullaro, J., Daugomah, J.W., Chestnut, D.E., Graves, D.A., Sobsey, M.D. & Scott, G.I.** 2006. F^+ RNA coliphage typing for microbial source tracking in surface waters. J. Appl. Microbiol. **101(5)**:1015-1026.

83. **Stewart, J. R., Ellender, R. D., Gooch, J. A., Jiang, S., Myoda, S. P. and S. B. Weisberg**. 2003.Recommendations for microbial source tracking: Lessons from a methods comparison study. J. Water and Health. **1(4):** 225-231.

84. **Tyagi, S. and F. R. Kramer.** 1996 Molecular beacons: probes that fluoresce upon hybridization. Nat. Biotechnol. **14:**303-308.

85. **US EPA.** 1986. Bacteriological ambient water quality criteria availability. Fed. Register. **51(45):**8012.

86. **US EPA Method 1601.** 2001a. Male-specific (F+) and somatic coliphage in water by two-step enrichment procedure. EPA-821-R-01-030, April, 2001, Washington, DC.

87. **US EPA Method 1602**. 2001b. Male-specific (F+) and somatic coliphage in water by single agar layer (SAL) procedure. EPA-821-R-01-029, April, 2001, Washington, DC.

88. **US EPA 2003a.** Results of the Interlaboratory Validation of EPA Method 1601 for Presence/Absence of male-specific (F+) and Somatic Coliphage in Water by Two-Step Enrichment. Pages: 19. US EPA, Office of Water, EPA-821-R-03-015, Washington.

89. **US EPA 2003b.** Results of the Interlaboratory Validation of EPA Method 1602 for Enumeration of Male-specific (F+) and Somatic Coliphage in Water by Single Agar Layer. Pages: 19. US EPA, Office of Water, EPA-821-R-03-016, Washington.

90. **US EPA.** 2005. Microbial Source Tracking Guide. U.S. EPA Office of Research and Development; Cincinnati, OH; EPA-600-R-05-064. pp 1-150.

91. **US EPA. TMDL**. Overview of current total maximum daily load –TMDL – Program and regulations. [Online.] http://www.epa.gov/owow/tmdl/overviewfs.html

92. **Van Dolah FM.** 2000 Marine algal toxins: origins, health effects, and their increased occurrence. Environ Health Perspect. **108-S(1):**133-141.

93. **Van Regenmortel, M. H. V., Fauquet, C. M., Bishop, D. H. L., Carstens, E. B., Estes, M. K., Lemon, S. M., McGeoch, D. J., Maniloff, J., Mayo, M. A., Pringle, C. R., and R. B. Wickner.** 2000. Virus Taxonomy, Classification and Nomenclature of Viruses. Seventh ICTV Report, Academic Press.

94. **Vinjé, J., Oudejans, S. J., Stewart, J. R., Sobsey, M. D., and S. C.Long.** 2004. Molecular detection and genotyping of male-specific coliphages by reverse transcription-PCR and reverse line blot hybridization. Appl. Environ. Microbiol. **70(10):**5996-6004.

95. **Wade, T. J., Pai, N., Eisenberg, J. N. S, and J. M. Colford, Jr.** 2003. Do U.S. Environmental Protection Agency Water Quality Guidelines for Recreational Waters Prevent Gastrointestinal Illness? A Systematic Review and Meta-analysis. Environmental Health Perspectives. **111(8):**1102-1109.

96. **Xu, X., Jin, M., Yu, Z., Li, H., Qiu, D., Tan, Y., and H. Chen.** 2005. Latex agglutination test for monitoring antibodies to avian influenza virus subtype H5N1. J Clin Microbiol. **43(4):**1953-1955.

97. **Yoder, J S., Blackburn, B. G., Craun, G. F., Hill, V., Levy, D. A., Chen, N., Lee, S. H., Calderon, R. L., M. J. Beach**. 2004. Surveillance for waterborne-disease outbreaks associated with recreational water--United States, 2001-2002. MMWR Surveill. Summary. **53(8):**1-22.

98. **Yolken RH, and R. J. Leggiadro.** 1986. Immunoassays for the diagnosis of viral enteric pathogens. Diagn. Microbiol. Infect. Dis. (3 Suppl.):61S-69S.

2 Microbial Indicator Recovery, Detection, and F+ RNA Coliphage Source Tracking in Estuarine Water from Nine Geographically Diverse United States Estuaries.

Abstract

Fecal contamination of coastal recreational water is a public health concern. Direct testing of water for pathogens is technically challenging and costly, so coastal managers rely on monitoring microbial indicators of fecal pollution. This study evaluates and applies methods for six indicator microbes: F+ and somatic coliphages, enterococci, fecal coliforms, *E. coli*, and *Clostridium perfringens* in estuarine water at nine geographically diverse United States estuaries. Bacterial indicator methods and several F+ and somatic coliphage methods detected significantly more microbes in water at human-impacted sites than at non-human impacted or pristine sites. By linear regression analysis, microbial indicator concentrations were higher in waters receiving greater volumes of wastewater treatment plant discharges. Positive correlations were found between $\log_{10}$ concentrations of somatic coliphages and enterococci or *E. coli* in paired samples. For F+ coliphages, the two-step enrichment method (EPA Method 1601) was most sensitive, but direct membrane filtration method provided more unbiased representation of the minority F+ RNA coliphage groups II and III for microbial source tracking. F+ RNA genotyping found 90.4% (n=394) Group I, 7.6% (n=33) Group II, 2.4% (n=9) Group III, and no Group IV isolates. F+ RNA coliphage source tracking with

Group I was not quantitatively reliable because high proportions of Group I coliphages were found in samples from both human and animal impacted sites. This information on the occurrence, levels, types, sources of microbial fecal indicators and on the performance of alternative methods for F+ coliphage analysis informs the design and conduct of human health effects studies on marine bathing waters and choices of fecal indicators for management decisions.

Introduction

Contamination of coastal recreational water and bivalve molluscan shellfish by point and non-point source fecal waste is an important public health concern. Growing coastal 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) (Chung et al., 1998; Payment et al., 2001), and treatment is often inadequate to prevent contamination of water and shellfish (Shieh, 2003; Lodder and de Roda Husman, 2004). Swimming in fecally contaminated recreational marine water is associated with measurable health risks, where the risk of illness is higher among swimmers than non-swimmers (Corbett et al., 1993; Prieto, et al., 2001; Colford et al., 2007; Haile et al., 1999).

Recreational water quality monitoring is intended to reduce exposures and illness risks from pathogenic microorganisms, but direct testing of water for pathogens is

expensive, time consuming, and technically difficult. Instead US EPA and the World Health Organization suggest water quality managers monitoring for fecally-associated non-pathogenic microbes (enterococci and *E. coli*) as indicators of the presence of fecal waste or pathogens (USEPA 1986; WHO 2003). Poor associations between bacterial fecal indicators and pathogenic enteric viruses in water have led some to question the use of bacterial indicators and use instead viral fecal indicators (Wait et al., 2001; Jiang et al., 2001; Fujioka and Yoneyama 2002). Coliphages are viruses of *E. coli* bacteria that in some studies 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., 2007).

Somatic coliphages infect the host cell wall, they are associated with fecal waste, and have been detected in higher levels than other fecal indicators in marine bathing waters (Mocé-Llivina et al., 2005). F+ coliphage infect the F-pili of host bacteria, and the RNA group of F+ coliphages (F+ RNA coliphages) is used primarily as fecal indicator because it superficially resembles human enteric viruses (e.g., hepatitis A and E viruses, enteroviruses, noroviruses and astroviruses) in size, shape and general composition (Havelaar 1993; Hsu et al., 1995; Sobsey et al., 1995). F+ RNA coliphages can be grouped or typed on the basis of human (Groups II and III) or animal (Groups I and IV) source patterns (Furuse et al., 1981, 1987; Osawa et al.., 1981), with some limitations of the extent to which the measured levels of the different F+ RNA 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).

In response to growing interest in marine recreational water quality in the United States, this study evaluates six microbiological fecal indicators in water from nine estuaries on the East, West, and Gulf coasts having diverse fecal waste sources and levels. Established methods or intended improvements of them were used for the detection, quantification and identification of sources of microbial fecal contaminants. Three assays for F+ or somatic coliphage recovery and detection, US EPA methods 1601 and 1602, and Direct Membrane Filtration, were compared and validated in parallel estuarine water samples. 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 and correlation with bacteria for the detection of fecal contamination. F+ RNA coliphage isolates underwent molecular genetic characterization by reverse transcription PCR (RT-PCR) and reverse line blot (RLB) hybridization (Vinjé et al., 2004) in an effort to substantiate their microbial source tracking potential and performance as indicators of fecal pollution in estuarine water.

Materials and Methods

Study sites, sample collection and processing. Sampling sites were chosen in nine estuaries that are part of the National Estuarine Research Reserves (NERR) system. At each estuary two stations were sampled— one in waters approved for shellfish harvesting or primary contact recreations and one in a prohibited area where fecal contamination levels exceed those allowable for shellfish harvesting and/or primary

contact recreation. If fecal contamination levels were not known, stations distant and proximal to human waste point sources were established by other means (see results). Samples consisted of 4-liter grab samples of estuarine water. Samples were shipped on ice by commercial carriers and processed within 24 hours of collection.

Indicator bacterial assays Estuary water samples were analyzed by membrane filter techniques for fecal coliforms, *E. coli*, enterococci, and *C. perfringens* using duplicate volumes of 100 ml, ten ml, and one ml as previously described (APHA, 1998). Bacteria concentrations were calculated as colony forming units (CFU) per 100 ml of water. Samples of water were vacuum filtered through a 47 mm diameter, 0.45 µm pore size, cellulose ester filters (type HA, 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^{o}C for resuscitation of injured bacteria, and then transferred to 44.5^{o}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-ß-D-glucuronide)(Sigma-Aldrich, St. Louis, MO), incubated for 4 to 6 hours at 44.5^{o}C, and exposed to long-wave ultraviolet light to visualize and enumerate fluorescent bright blue colonies (*E. coli*). Enterococci were detected and enumerated as dark blue colonies surrounded by a dark blue halo of precipitate on mEI agar plates after 24 ± 3 hr incubation at 41^{o}C. *C. perfringens* were detected as bright pink colonies when exposed to ammonium hydroxide (NH_4OH) fumes after 18 hr incubation at 44.5^{o}C in anaerobic conditions on mCP agar plates.

Coliphage assays. Water samples were spilt and assayed for both somatic and F+ coliphages by three methods (US EPA methods 1601 and 1602, and Direct Membrane

Filtration). The US EPA methods originally validated for use with groundwater, were used for marine waters in this study after confirming their effective performance in preliminary studies that compared them to standard, "benchmark" methods. *E. coli* strain CN13 (ATCC # 700609) was used to detect somatic coliphage and strain F_{amp} (ATCC # 700891) was used to detect F+ coliphages. Assays used the antibiotic nalidixic acid 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 (SAL) Assay was performed as described previously (EPA 2001b), using ten replicates of 10 ml volumes of water. Plaques were enumerated and the titer of coliphages in the sample calculated as PFU (plaque-forming units) per 100 ml.

US EPA Method 1601 Two Step Enrichment (ENR) Assay was performed as previously described (USEPA, 2001a), except a MPN assay was used to achieve quantitative results by analyzing triplicate volumes of 300ml, 30 ml, and 3 ml. Coliphage presence/absence was scored in each sample volume to estimate the MPN/100ml.

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 µm 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 µg/ml each of 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^{o}C overnight, blue coliphage plaques were counted, with titers expressed as PFU (plaque-forming units) per 100 ml of water.

Coliphage isolation and RNase testing. As many as ten F+ coliphage and somatic coliphage plaques per sample were randomly chosen from SAL, DMF or ENR plates. Plaque material was enriched in 5 ml of TSB by EPA method 1601, clarified by centrifugation at 1200 *xg* 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 (100 µg/ml; Sigma-Aldrich, St. Louis, MO) to determine if it had DNA or RNA as its nucleic acid. Coliphages that lysed hosts and formed plaques in the presence of RNase were considered F+ DNA and those that did not were considered F+ RNA.

F+ RNA coliphage detection and genotyping by Reverse Line Blot hybridization. Approximately 1,500 F+ coliphage isolates were further subjected to a nucleic acid hybridization typing test to distinguish the four groups of F+ RNA coliphages (Groups I, II, III, and IV). After broadly-reactive RT-PCR amplification of a partial region of the replicase gene of both levi- and allolevivirus using biotinylated primers (Vinjé et al., 2004), amplicons were then further characterized by reverse line blot hybridization (RLB) using a panel of group- and subgroup-specific probes (Vinjé et al., 2004). F+ DNA coliphages were analyzed by PCR to confirm their presence in mixed isolates containing both F+ RNA and F+ DNA coliphages (Vinjé et al., 2004). Bound (RT)-PCR products on the RLB membranes were detected by chemilumenescence

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).

Data Analysis. 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 a pair using Wilcoxon Signed Rank test, and for three or more with Friedman's test and Dunn's multiple comparison test. Proportions of F+ RNA coliphage genogroups were compared between methods using a Z-test statistic with two tails and a pre-determined alpha of 0.05, and Chi-squared analysis. Statistical significance was preset at an alpha of 0.05, and p values are reported

Results

Sampling sites and stations and fecal contamination sources. Water was sampled in estuaries that had stations both proximal (impacted) and distant (non-impacted) to sources of human fecal contamination. Sanitary surveys, 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) discharges of treated effluent and possibly raw sewage leaks, while likely human non-point sources included urban runoff, seepage from septic tanks, and boat dumping of sanitary wastes (Table 2.1). 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 2.1). At four estuaries impacts included human point and non-point sources, while non-impacted stations were pristine areas with wildlife refuges or were geographically separated from human populations (Table 2.1). In the Tijuana River Reserve in Southern CA human impacts were documented at all sampling stations, so in the absence of a truly pristine or non-impacted area, a stations with only non-point source runoff from human development was compared to a more contaminated station at the mouth of the Tijuana River that contained untreated sewage from Mexico (Table 2.1).

Comparison of fecal indicator levels in impacted and non-impacted waters. Levels of fecal indicator microbes in human fecally-impacted waters were higher than in non-human impacted or pristine stations (Fig. 2.1, Table 2.2). 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 (or pristine) stations (Table 2.2). *C. perfringens*, enterococci, *E. coli*, and fecal coliforms were all detected in higher concentrations at impacted stations than non-impacted stations (p values of <0.001, < 0.001, 0.001, 0.003, respectively) (Fig. 2.1, Table 2.2). Enumeration of bacteria by membrane filtration methods facilitated a direct comparison among bacterial indicators.

Three different assays (ENR, SAL, DMF) were compared for both somatic and F+ coliphage recovery, and the differences in the performance of the methods was examined. For F+ coliphage, ENR and SAL recovered more F+ coliphage at impacted than non-impacted stations (p values = 0.002 and 0.045) (Fig 2.1, Table 2.2). Using

DMF, there was a not quite significant difference between F+ coliphage levels at impacted and non-impacted stations (p value = 0.06) (Fig. 2.1, Table 2.2). For somatic coliphages, only ENR detected significantly more coliphages at impacted than at non-impacted stations (p value = 0.012) (Fig. 2.1, Table 2.2).

Relationships between different fecal indicators. Log_{10} levels of organism from 72-77 matched-pairs of water samples were compared using scatter plots and linear regression analysis with R^2 trendlines (Fig 2.2; Table 2.3). Strongest positive correlations were between fecal coliforms and *E. coli* (R^2 = 0.887), while weakest positive correlations were between *C. perfringens* and F+ coliphages with the ENR method (R^2 = 0.001) (Table 2.3). Positive correlations were observed among F+ coliphage methods (R^2 = 0.324 to 0.525) and among somatic coliphage methods (R^2 = 0.494 to 0.544) (Table 2.3). Somatic and F+ coliphages were somewhat predictive of levels of bacterial indicators in water, while bacterial indicator levels (except for *C. perfringens*) were strong predictive of each other (Table 2.3). Linear regression trendlines fit to log_{10} levels of enterococci predicted 69% and 60% of the variability in levels of *E. coli* and fecal coliforms in water (Fig. 2.2e, 2.2f). Similar linear regression models explained 43% and 47% of the variability in somatic coliphages vs. enterococci and somatic coliphage vs. *E. coli* plots (Fig. 2.2a, 2.2b). F+ coliphages levels were less strongly related to bacterial indicator levels, with linear regression trendlines predicting 28% and 34% of the variability in enterococci and *E. coli* levels, respectively (Fig. 2.2c, 2.2d).

Comparison of three methods for F+ coliphage recovery. DMF, SAL, and quantitative ENR were used in parallel to recover F+ coliphage from water. ENR assay detected F+ coliphage in 59% of samples (43/73), which was statistically similar (p value = 0.2442) to DMF with 47% positives (36/75), while each was significantly greater (ENR p value < 0.0001; DMF p value = 0.0046) than SAL with 24% positives (18/74) (Table 2.4). The geometric mean concentration of F+ coliphages was higher in ENR and SAL than DMF using Dunn's multiple comparison test with matched pairs. However, SAL values were perhaps artificially elevated by assigning below detection results of non-zero discrete values that were ½ the detection limit of the assay. This hypothesis was tested by re-analyzing using only the detectable values, and this showed there was a significant difference among all three methods (p values = 0.046), but no significant differences between any two pairs of methods (p values > 0.05) (Table 2.5). Based on volume of water assayed, the 1-liter sample volume volumes of both ENR and DMF methods had theoretical lower detection limits of 0.1 infectious units per 100 ml, while the 100 ml sample volumes of the SAL method had a much greater lower detection limit of 1 PFU per 100 ml.

When comparing recovery methods in the context of F+ RNA coliphage microbial source tracking, ENR recovered 3.1 isolates F+ RNA coliphage isolates per water sample (224 isolates/ 73 samples), which was more than SAL with 2.2 F+ RNA coliphage isolates per sample (164 isolates/ 74 samples) or DMF with 0.5 F+ RNA coliphage isolates per sample (38 isolates/ 75 samples) (Table 2.4). Genetic characterization of isolates by RLB genogrouping (for Groups I, II, III, and IV) revealed that a significantly higher percentage of F+ RNA Group I isolates were recovered by

ENR than recovered by SAL and DMF, respectively (p values = 0.028 and 0.0005) (Table 2.6). Similarly, SAL and DMF both provided significantly higher percentages of F+ RNA Group II and III human-type isolates than ENR (p values = 0.028 and 0.0005) (Table 2.6).

Comparison of three methods for somatic coliphage recovery. Water was analyzed in parallel for somatic coliphages by DMF, SAL, and quantitative ENR. Methods recovered somatic coliphage from about 90% of 76 to 78 water samples (Table 2.7). ENR and DMF had lower detection limits of 0.1 PFU per 100 ml and were thought to be more sensitive than SAL, which had 1 PFU per 100 ml for a lower detection limit. The theoretical lower detection limit alone did not predict the best recovery method, because ENR and SAL each recovered significantly higher levels of somatic coliphage in field samples than did the DMF method (p values <0.001 by Wilcoxon matched-pairs signed-ranks test) (Tables 2.7 and 2.8). The same statistical outcomes for ENR and SAL were obtained when re-running the analysis using only matched pairs with detectable levels of coliphages (excluding samples that were below detection). ENR and SAL were not significantly different from each other (p value >0.05) (Tables 2.7 and 2.8) despite their differences in sample volumes (1 and 0.1 liter, respectively)

Fecal indicators recovered from estuarine water in relation to impacts by waste water treatments plants. Four estuaries were impacted by waste water treatment plant (WWTP) effluent discharges, and coliphage levels at stations near receiving waters tended to be higher as a function of the magnitude of permitted discharge volumes (Fig.

2.3). Linear regression trendlines fit to geometric mean levels of F+ coliphage predicts 90-98% of the variability in water samples in relation to effluent discharge volume (Fig. 2.3a). Somatic coliphages levels also were higher when WWTP permitted effluent volumes were greater, with the R^2 values of trendlines predicting 94-99% of the variability (Fig. 2.3b). Bacterial indicator levels (fecal coliforms, *E. coli*, enterococci, *C. perfringens*) at WWTP impacted stations had similar trends in relation to effluent discharge volumes as for coliphages, with 65-96% of the variability in water predicted by linear regression models (data not shown). Stations distant from point-source wastewater impacts were not included in this analysis, because they were often physically separated from wastewater point sources, such as in a different water body or on the other side of an island.

F+ RNA genogroups detected in estuarine water. Four hundred thirty-six plaque purified F+ coliphage isolates were genogrouped as F+ RNA group I, II, III, or IV by RLB hybridization (Table 2.8). F+ RNA group II (GA-like) phages were detected at seven of nine estuaries and constituted 2.2% to 25% of isolates recovered at those estuaries (Table 2.9). Group III (Qβ-like or M11-like) isolates were detected in three of nine estuaries, comprising 1.4% to 6.8% of isolates recovered at those estuaries (Table 2.8). The majority of F+ RNA isolates were group I (MS2-like) (90.4%), followed by group II (7.6%), and group III (2.1%), with no group IV (SP-like or FI-like) isolates detected (Table 2.9). High levels of group I isolates were recovered from most samples (394 isolates from 234 sub-samples), although unexplainably only 3 group I coliphages

were found in 36 water samples from Waquoit Bay, MA 36 water samples and only 1 group I isolate was found in 24 Masonboro, NC water samples (Table 2.9).

Discussion

This study of six microbial fecal indicators (F+ and somatic coliphage, *E. coli*, fecal coliforms, enterococci, *C. perfringens*) provides critical comparative information on their levels, types, sources, and on the best available techniques to recover and quantify coliphages as fecal indicators in a broad geographic range of estuarine waters in the US, Similar studies have been done previously in Western Europe (Contreras-Coll et al., 2002). Overall, F+ coliphages, somatic coliphages, and bacterial indicators were found to be effective indicators of fecal pollution in estuarine water. Significantly more of these microbes were detected in human-impacted water than non-human impacted or pristine water using a quantitative ENR assay for F+ and somatic coliphages, SAL for F+ coliphages, and membrane filtration methods for the bacterial indicators of *E. coli*, fecal coliforms, enterococci, *C. perfringens*. The choice of microbial fecal indicators and microbial assays for monitoring marine and estuarine waters remains uncertain, as indicated by recent meta-analyses and health effects studies on the incidence of diseases in bathers (Prüss 1998; Wade et al., 2003; Wade et al., 2006; Colford et al., 2007) and by the availability of different methods to detect them.

The 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 in a recent study (Colford et al.,

2007; Schwab, 2007). Only F+ coliphage, a non-traditional, non-bacterial virus indicator of fecal contamination, was predictive of gastrointestinal illness risks from this bathing water. In marine waters with point-source human fecal contamination, *E. coli* and enterococci have positively correlated 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, Dufour 1984, EPA 1986; NRC 2004; Wade 2003). The information from this study can be used by others to inform the design and focus of human health effects studies on marine bathing waters, and make choices of candidate fecal indicators for management decisions.

When comparing levels of pairs of fecal indicators in positive water samples, positive correlations were observed between most types of fecal indicators. There was nearly parity for relationship between $\log_{10}$ concentrations of fecal coliform and *E. coli* in water (data not shown). This could be expected because *E. coli* is a predominant bacterium of fecal origin in the coliform group. In this work *E. coli* were distinguished from fecal coliforms by their ability to hydrolyze and ferment glucuronide substrates, a major biochemical marker unique to *E. coli* among the coliforms. Strong positive correlation was observed between $\log_{10}$ values of enterococci and fecal coliforms in water, and 88% of enterococci-fecal coliform matched-pairs of data were in agreement for water quality exceedances by both EPA bacterial criteria (200 CFU/100ml for fecal coliforms and 35 CFU/100ml for enterococci) (EPA 1986). Most disagreements among matched-pairs were from enterococci concentrations greater than regulatory level but fecal coliform concentration within regulatory levels .

There were moderate positive correlations in this study between $\log_{10}$ concentrations of somatic coliphages and enterococci or *E. coli*, which is consistent with results of previously observed European marine water and fresh water studies (Wiedenmann et al, 2006; Contreras-Cole et al., 2002). The high incidence (96%) of somatic coliphages in waters of this study suggests that these coliphages may be good candidate fecal indicators, especially if they are shown to predict risks of gastrointestinal and other illnesses in health-based epidemiological studies of marine bathing water. The levels of somatic coliphages that might be predictive of human health effects, consistent with current risk levels for recreational marine water based on enterococci, is probably between 10-100 PFU/100ml. This estimate was derived from the somatic coliphage concentrations present in samples containing 35 CFU/100ml of enterococci.

Weak correlations between $\log_{10}$ concentrations of F+ coliphage and other viral and bacterial indicators were most likely due to the large numbers of water samples without detectable levels of F+ coliphages (41%). These findings agree with previous ones by our group and others, which often show that F+ coliphages occur in lower concentrations in marine water than other fecal indicators (Chung et al., 1998; Mocé-Llivina et al., 2005). The degree to which low levels of F+ coliphage as compared to other fecal indicators signifies truly lower levels of fecal impacts in our study is not known.

Three F+ and somatic coliphage recovery and detection methods were compared in parallel for about 75 geographically diverse water samples. The results of this comparison showed that ENR is suitable for recovery of low levels of F+ and somatic coliphages in water. However, when comparing methods for application to microbial

source tracking, DMF and SAL provided a more unbiased representation of the minority F+ RNA coliphage groups (groups II and III) present in a water sample than ENR. This is because DMF and SAL are performed on agar plates where each coliphage replicates discretely to form individual plaques, while ENR is performed in broth cultures that favor strains replicating rapidly and to high concentrations. This type of coliphage enrichment bias is supported by previous studies showing that group I F+ RNA coliphages have a larger burst size and when enriched they produce more progeny than F+ RNA coliphages from groups II, III, and IV (Furuse 1987). As we and others have found with F+ RNA group I coliphages in ENR enriched samples, these coliphages were present at far higher concentrations than other F+ RNA coliphage groups, making it difficult to estimate the magnitudes of the different fecal sources (Stewart-Pullaro et al., 2006). This phenomenon of increased concentrations of group I F+ RNA coliphages compared to other groups in enrichment is further exacerbated in the coliphage plaque purification process where often only the predominant group is isolated, 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. Similar problems arise in bacterial source tracking where an under-represented typing library or a library generated from a different area produces less accurate source tracking results (Jenkins et al., 2003; Parveen et al., 1999; Wiggins et al., 2003). These examples highlight the need for assessment and comparison of the field performance of methods in relation to the first principles governing their design and performance

Our data suggest that two WWTPs may have exceeded their NPDES permitted levels of fecal coliforms, with 1,250 fecal coliforms/100ml in Tijuana River, CA and 404 fecal coliforms/100ml in Great Bay, NH, if these fecal coliform levels in were caused by the wastewater discharges to these waters. At study sites impacted by WWTPs, concentrations of bacterial and coliphage fecal indicators in receiving waters tended to be higher where WWTPs had larger permitted daily discharge volumes. F+ and somatic coliphages on average had a stronger association with the magnitude of the WWTP impacts and were more predictive of WWTP discharge volumes than were *E. coli,* enterococci, and *C. perfringens*, while only somatic coliphages were more strongly associated with WWTPs impacts and discharge volumes than fecal coliforms. This finding may be explained by the higher levels of F+ coliphages than bacterial indicators detected in disinfected WWTP effluent (Chung et al., 1998; Harwood et al., 2005). Along with fecal indicators, some proportion of pathogens also survive WWTP effluent disinfection (Bonadonna et al., 2002; Fleischer et al., 2000). It has been previously reported that the presence of aggregates of fecal indicators correlated with the presence of microbial pathogens in the effluent of six WWTPs (Harwood et al., 2005). The WWTPs of that study used similar disinfection methods as the WWTPs in our study. Taken together, these findings and conditions suggest that if more microbial indicators are discharged in effluent receiving waters of larger WWTP plants than from smaller plants, then persistent microbial pathogens like *Cryptosporidium*, *Giardia*, and enteric viruses could also be present at higher levels in waters receiving larger WWTP discharge volumes. Because of their greater potential pathogen load from larger WWTP effluent volumes, perhaps these plants should not only achieve Clean Water Act standards for

receiving water quality, but also have stricter disinfection requirements to produce disinfected effluent with fewer pathogens and indicators than smaller plants that discharge less waste water.

The ecology of F+ RNA coliphages, based on their likely sources, distribution, and types in estuarine water, was further explored using RT-PCR and RLB probe hybridization for 436 F+ RNA field isolates (Vinje et al., 2004). The majority of F+ RNA coliphage isolates were genogroup I (90.4%), which is consistent with proportions found previously in surface water (Cole et al., 2003). Low prevalence of F+ RNA Groups III and IV found in estuarine waters in this study may be explained by either low prevalence, or faster die-off rates of these groups, as documented in fresh water microcosm experiments, compared to F+ RNA Groups I and II (Long and Sobsey 2004; Brion et al., 2002; Schaper et al., 2002).

Genogroup II or III F+ RNA coliphages were found in 89% of all estuaries and may be related to human fecal waste sources. One hundred percent of Group III isolates in Apalachicola Bay, Florida were from the station upriver from a WWTP permitted to discharge 0.3 MGD, and 80% of Group III isolates in Waquoit Bay, Massachusetts came from stations with recreational boaters and waterside residences as diffuse sources of fecal contamination (Sobsey et al., 2003). These findings are consistent with those of previous studies linking groups II and III F+ RNA coliphages to human fecal waste sources (Furuse et al., 1981, 1987; Osawa et al.., 1981).

In this study, F+ RNA microbial source tracking was not entirely effective at quantifying fecal contamination source type because human and animal impacted sites had high levels of Group I F+ RNA coliphages. These viruses have been found in both

human source waste water and animal waste (Stewart-Pullaro et al., 2006; Cole et al., 2003). Because of such uncertainties about the sources of group I coliphages and differences in survival of the different F+ RNA coliphage groups, microbial source tracking with them cannot be performed reliably and accurately without supporting documentation of known fecal waste sources using sanitary or shoreline surveys. More studies are needed on the microbial fate and transport of F+ RNA coliphages, other fecal indicator microbes and enteric pathogens, such as human *Adenoviruses* or *Polyomaviruses*, in runoff and other non-point sources in order to provide a better understanding of the relative persistence of different F+ coliphages in relation to pathogens in estuaries. Greater resolution in identifying fecal waste sources and their impacts may be achieved by nucleotide sequence matching of F+ RNA coliphage isolates from impacted waters and those from known sources, collected in a coliphage sequence library (Stewart et al., 2006). However, these more technologically demanding, time consuming, and costly library-based techniques may be less adaptable to the goal of rapid, simple and low-cost field detection and fecal source tracking of F+ RNA coliphages. Despite certain limitations, F+ RNA coliphages are useful for microbial source tracking, and both F+ and somatic coliphages are as useful as microbial indicators for determining estuarine water quality and fecal waste impacts. The methods validated for coliphage detection and source tracking in this study should prove useful for future studies and are applicable on a practical basis to water quality management programs and systems.

References

1. **APHA** 1998. Standard methods for the examination of water and wastewater, 20th edition, American Public Health Association, Washington, DC

2. **Bonadonna L., Briancesco R., Cataldo C., Divizia M., Donia D., and A. Pana**. 2002. Fate of bacterial indicators, viruses and protozoan parasites in a wastewater multi-component treatment system. New Microbiol. **25(4):**413-420.

3. **Brion, G. M., Meschke, J. S., and M. D. Sobsey** 2002. F-specific RNA coliphages: occurrence, types, and survival in natural waters. Water Res. **36(9):**2419-2425.

4. **Cabelli, V.J.** 1983. Health effects criteria for marine recreational waters. Cincinnati, Ohio. EPA-600-1-80-031.

5. **Chung, H., Jaykus, L.-A., Lovelace, G., and M. D. Sobsey.** 1998. Bacteriophages and bacteria as indicators of enteric viruses in oysters and their harvest waters. Wat. Sci. Tech. **38(12):**37-44.

6. **Cole, D., Long, S. D., and M. D. Sobsey.** 2003. Evaluation of F+ RNA and DNA coliphages as source-specific indicators of fecal contamination in surface waters. Appl. Environ. Microbiol. **69(11):**6507-1654.

7. **Colford, J. M. Jr., Wade, T. J., Schiff, K. C., Wright, C. C., Griffith, J. G., Sandhu, S. K., Burns, S., Hayes, J., Sobsey, M., Lovelace, G., and S. B. Weisberg.** 2007. Water quality indicators and the risk of illness in non-point source impacted recreational waters. Epidemiol. **(1):**27-35.

8. **Contreras-Coll N., Lucena F., Mooijman K., Havelaar A., Pierz V., Boque M., Gawler A, Holler C., Lambiri M., Mirolo G., Moreno B., Niemi M., Sommer R., Valentin B., Wiedenmann A., Young V., and J. Jofre.** 2002. Occurrence and levels of indicator bacteriophages in bathing waters throughout Europe. Water Res. **36(20):**4963-4974.

9. **Corbett, S.J., Rubin, G.L., Curry, G.K., and D. G. Kleinbaum.** 1993. The health effects of swimming at Sydney beaches. the Sydney beach users study advisory group. Am J. Public Health. **83(12):**1701-1706.

10. **Dore, W.J., Henshilwood, K., and D. N., Lees.** 2000. Evaluation of F-specific RNA bacteriophage as a candidate human enteric virus indicator for bivalve molluscan shellfish. Appl. Environ. Microbiol. **66(4):**1280-1285.

11. **Dufour, A.P.** 1984. Health Effects Criteria for Fresh Recreational Waters. Cincinnati, Ohio. EPA-600-1-84-004.

12. **Fleischer, J., Schlafmann, K., Otchwemah, R., and K. Botzenhart.** 2000. Elimination of enteroviruses, other enteric viruses, F-specific coliphages, somatic coliphages and *E. coli* in four sewage treatment plants of southern Germany, J. Water Supply Res. Technol. **49(3):** 127–138.

13. **Fujioka R. S., and B. S. Yoneyama**. 2002. Sunlight inactivation of human enteric viruses and fecal bacteria. Water Sci Technol. **46(11-12):**291-295.

14. **Furuse, K., Ando, A., Osawa, S., and I. Watanabe.** 1981. Distribution of ribonucleic acid coliphages in raw sewage from treatment plants in Japan. Appl. Environ. Microbiol. **41(5):**1139-1143.

15. **Furuse, K.** Distribution of coliphages in the environment. *In* S. M. Goyal, C. P. Gerba, and G. Bitton. (ed.) Phage ecology.Wiley-Interscience. New York. 1987. pp. 87-124.

16. **Haile, R.W., Witte, J.S., Gold, M., Cressey, R., McGee, C., Millikan, R.C., Glasser, A., Harawa, N., Ervin, C., Harmon, P., Harper, J., Dermand, J., Alamillo, J., Barrett, K., Nides, M., and G. Wang.** 1999. The health effects of swimming in ocean water contaminated by storm drain runoff. Epidemiol. **10(4):**355-363.

17. **Harwood, V.J., Levine, A.D., Scott, T.M, Chivukula, V., Lukasik, J., Farrah, S.R., and J.B. Rose.** 2005. Validity of the indicator organism paradigm for pathogen reduction in reclaimed water and public health protection. Appl. Env. Microbiol. **71(6):**3163–3170.

18. **Havelaar, A.H.** 1993. Bacteriophages as models of human enteric viruses in the environment. ASM News. **59(12):**614-619.

19. **Hsu, F.-C., Y.-S.C. Shieh, J. van Duin, M.J. Beekwilder and M.D. Sobsey** 1995. Genotyping male-specific RNA coliphages by hybridization with oligonucleotide probes. Appl. Environ. Microbiol. **61(11):**3960-3966.

20. **Jenkins, M. B., Hartel P. G., Olexa, T. J., and J. A. Stuedemann.** 2003. Putative temporal variability of *Escherichia coli* ribotypes from yearling steers. J. Environ. Qual. **32:**305-309.

21. **Jiang S., Noble, R. and W. Chu.** 2001. Human adenoviruses and coliphages in urban runoff-impacted coastal waters of southern California. Appl. Environ. Microbiol. **67(1):**179-184.

22. **Long, S.C., and M. D. Sobsey**. 2004. A comparison of the survival of F+RNA and F+DNA coliphages in lake water microcosms. J. Water Health. **2(1):**15-22.

23. **Mocé-Llivina, L., Lucena, F., and J. Jofre.** 2005. Enteroviruses and Bacteriophages in Bathing Waters Appl Environ Microbiol. **71(11):**6838–6844.

24. **NOAA.** 2000. Polluted runoff: lessons learned from the national estuarine research reserve system. NOAA Estuarine Reserves Division. Silver Springs, MD. [Online.] http://www.ocrm.nos.noaa.gov/pdf/NERRLessonsLearned.pdf.

25. **NRC.** 2004. Indicators of waterborne pathogens. National Research Council. The National Academies Press. Washington D.C.

26. **Osawa, S., Furuse, K., and I. Watanabe.** 1981. Distribution of ribonucleic acid coliphages in animals. Appl. Environ. Microbiol. **41(1):**164-168.

27. **Parveen, S., Portier, K. M., Robinson, K., Edmiston, L., and M. L. Tamplin.** 1999. Discriminant analysis of ribotype profiles of *Escherichia coli* for differentiating human and nonhuman sources of fecal pollution. Appl. Environ. Microbiol. **65:**3142-3147.

28. **Payment P, Plante R, and P. Cejka.** 2001. Removal of indicator bacteria, human enteric viruses, Giardia cysts, and Cryptosporidium oocysts at a large wastewater primary treatment facility. Can J Microbiol. **47(3):**188-189.

29. **Prieto, M.D., Lopez, B., Juanes, J.A., Revilla, J.A., Llorca, J., and M. Delgado-Rodríguez.** 2001. Recreation in coastal waters: health risks associated with bathing in sea water. J. Epidemiol. Community Health. **55:**442-447.

30. **Prüss, A.** 1998. A review of epidemiological studies on health effects from exposure to recreational water. Intnl. J. Epidemiol. **27(1):**1-9.

31. **Schaper, M., A. E. Duran, and J. Jofre.** 2002. Comparative resistance of phage isolates of four genotypes of F-specific RNA bacteriophages to various inactivation processes. Appl. Environ. Microbiol. 68**:**3702-3707.

32. **Schwab KJ**. 2007. Are existing bacterial indicators adequate for determining recreational water illness in waters impacted by nonpoint pollution? Epidemiol. **18(1)**:21-22.

33. **Shieh, Y. C., Baric, R. S., Woods, J. W., and K. R. Calci.** 2003. Molecular surveillance of enterovirus and norwalk-like virus in oysters relocated to a municipal-sewage-impacted gulf estuary. Appl. Environ. Microbiol. **69(12):**7130-7136.

34. **Sobsey, M.D., D.A. Battigelli, T.R. Handzel and K.J. Schwab**. 1995. Male-specific Coliphages as Indicators of Viral Contamination of Drinking Water, 150 pp. American Water Works Association Research Foundation, Denver, Co.

35. **Sobsey, M. D., K. J. Schwab, et al.** 1990. A simple membrane filter method to concentrate and enumerate male-specific RNA coliphages. Journal of American Water Works Association **82:**52-59.

36. **Sobsey, M.D., R. Perdue, M. Overton and J. Fisher.** 2003. Factors influencing faecal contamination in coastal marinas. Wat. Sci. Technol., **47(3):**199–204.

37. **Stewart, J.R., Vinjé, J., Oudejans, S.J.G., Scott, G.I., and M.D. Sobsey.** 2006. Sequence variation among group III F-specific RNA coliphages from water samples and swine lagoons. Appl. Environ. Microbiol. **72(2):**1126-1230.

38. **Stewart-Pullaro, J., Daugomah, J. W., Chestnut, D. E., Graves, D. A., Sobsey, M. D. and G. I. Scott.** 2006. F^{+}RNA coliphage typing for microbial source tracking in surface waters. J. of Appl. Microbiol. **101(5):**1015-1026.

39. **TMDL Apalachicola.** Total Maximum Daily Load (TMDL) for Fecal Coliforms in Huckleberry Creek. March 2005. Florida Department of Environmental Protection, Division of Water Resource Management, Bureau of Watershed Management. [Online.] http://www.epa.gov/region4/water/tmdl/florida/documents/HUCKLEBERRYCRK_final_Fcoli_TMDL_000.pdf

40. **US EPA.** 1986. Ambient water quality criteria for bacteria - 1986. EPA 440/5/84-002.).

41. **US EPA Method 1601**: Male-specific (F+) and somatic coliphage in water by two-step enrichment procedure. EPA-821-R-01-030, April, 2001, Washington, DC.

42. **US EPA Method 1602**: Male-specific (F+) and somatic coliphage in water by single agar layer (SAL) procedure. EPA-821-R-01-029, April, 2001, Washington, DC.

43. **Vinjé J., Oudejans, S. J., Stewart, J. R., Sobsey, M. D., and S. C. Long .** 2004 Molecular detection and genotyping of male-specific coliphages by reverse transcription-PCR and reverse line blot hybridization. Appl. Environ. Microbiol. **70(10):**5996-6004.

44. **Wiedenmann, A.. Krüger, P., Dietz, K., López-Pila, J. M., Szewzyk, R., and K. Botzenhart.** 2006. A Randomized Controlled Trial Assessing Infectious Disease Risks from Bathing in Fresh Recreational Waters in Relation to the Concentration of *Escherichia coli*, Intestinal Enterococci, *Clostridium perfringens*, and Somatic Coliphages. Environ Health Perspect. **114(2):**228–236.

45. **Wade, T.J., Pai, N., Eisenberg, J.N.S, and J. M. Colford Jr.** 2003. Do U.S. Environmental Protection Agency Water Quality Guidelines for Recreational Waters Prevent Gastrointestinal Illness? A Systematic Review and Meta-analysis. Environ. Health Perspect. **111(8):**1102-1109.

46. **Wade, T. J., Calderon, R. L., Sams, E., Beach, M., Brenner, K. P,, Williams, A. H., and A. P. Dufour.** 2006. Rapidly measured indicators of recreational water quality are predictive of swimming-associated gastrointestinal illness. Environ. Health Perspect. **114(1):**24-28.

47. **Wait D. A., and M. D. Sobsey.** 2001. Comparative survival of enteric viruses and bacteria in Atlantic Ocean seawater.Water Sci Technol. **43(12):**139-142.

48. **Wiggins, B. A., Cash, P. W., Creamer W. S., Dart, S. E., Garcia, P. P., Gerecke, T. M., Han, J., Henry, B. L., Hoover, K. B., Johnson, E. L., Jones, K. C., McCarthy, J. G., McDonough, J. A., Mercer, S. A., Noto, M. J., Park, H., Phillips, M. S., Purner, S. M., Smith, B. M., Stevens, E. N., and E. N. Varner**. 2003. Use of antibiotic resistance analysis for representativeness testing of multiwatershed libraries. Appl. Environ. Microbiol. **69:**3399-3405.

49. **World Health Organization (WHO).** 2003. Guidelines for safe recreational water environments. Vol 1., coastal and fresh waters. [Online.] http://www.who.int/water_sanitation_health/bathing/srwe1/en/

50. **Zuniga, J.** As sewage spills into the ocean, solutions are sought to clean up pollution from Tijuana River." The San Diego Union-Tribune. 27, May 2005. [Online.] http://www.signonsandiego.com/uniontrib/20050527/news_6m27sewage.htm.

TABLE 2.1. 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)	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 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, because 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 about 6 miles from the 30,000 population town of Dover, DE, and about 3 miles upstream from the terminus of the St. John's River into Delaware Bay. Scotton Landing receives non-point-source pollution from the upstream portion of the river, most likely from Dover where urban runoff occurs and there is public recreation. 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 and discharges wastewater into another river, the Murderkill River. The Murderkill River and the St. John's River are not connected, but they are less than a mile apart when they flow into Delaware Bay.
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 population 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 population 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 population 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 population) 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 located 0.5 mi west of the town of Melville (around 2,300 population) and Newport County (821 people/mi^2), and 1 mile east of underdeveloped Prudence Island. Providence, RI, a 178,000 population city and Newport, RI, a 26,000 population city are each 12 miles away from Dyer Island on rivers' confluence 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

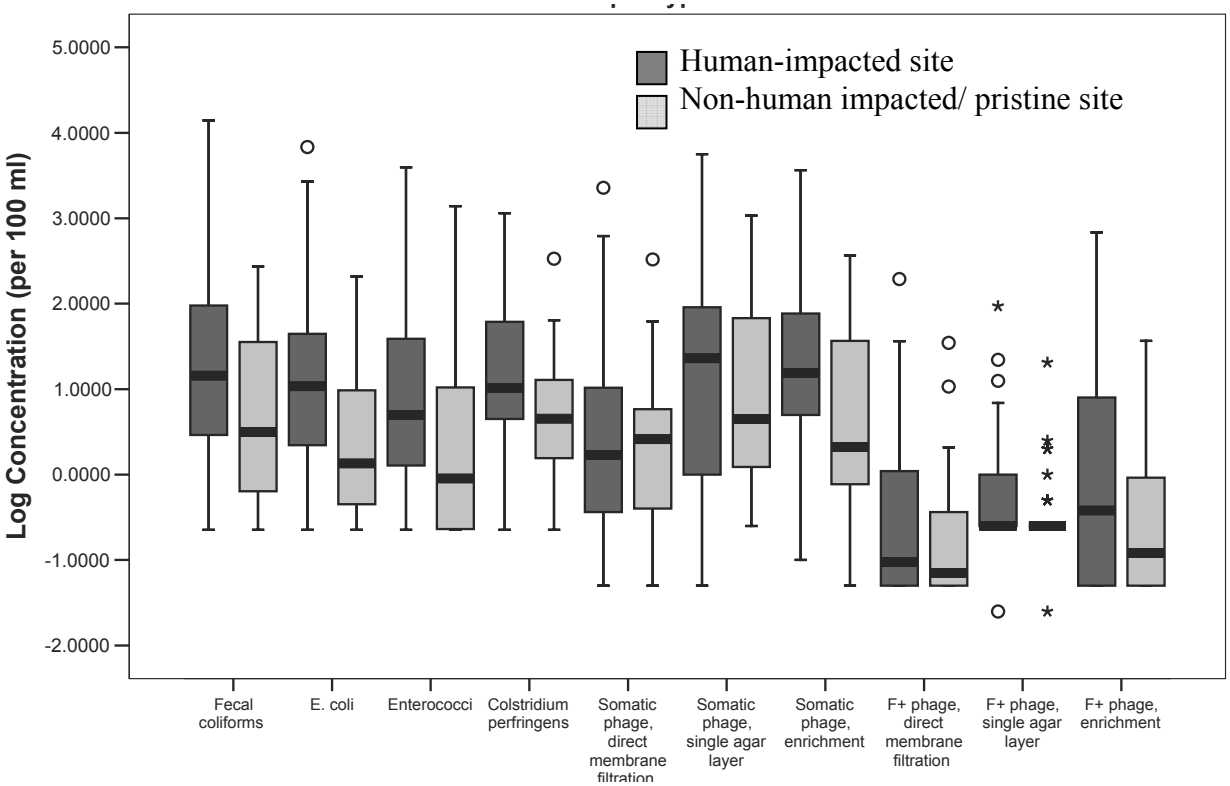


FIG 2.1. Box-and-whisker plots of $\log_{10}$ levels of indicator bacteria and coliphages in estuarine water at stations with human fecal impacts (dark grey; n = 39 for each organism) or pristine stations that may contain non-human fecal impacts (light grey; n = 35 for each organism). Lower and upper bands give minimum and maximum log10 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 2.2. Comparison of indicator levels in human fecal impacted and non-human fecal impacted water.

Indicator	Method	Impacted sites vs. non-impacted sites [a]	
		Number of pairs	p value
C. perfringens	MF	35	<0.001 *
Enterococci	MF	35	<0.001 *
E. coli	MF	35	0.001 *
fecal coliform	MF	35	0.003 *
F+ coliphage	MF	34	0.060
F+ coliphage	ENR	33	0.002 *
F+ coliphage	SAL	33	0.045 *
Somatic coliphage	MF	34	0.458
Somatic coliphage	ENR	34	0.012 *
Somatic coliphage	SAL	35	0.518

[a] The Wilcoxon signed rank test on matched pairs between non-impacted and impacted sites with an asterisks (*) by those with significant differences (alpha = 0.05). ENR = Two Step Enrichment; MF = Membrane Filtration; SAL = Single Agar Layer.

Table 2.3. Linear regression R^2 correlation analysis for matched pairs of fecal indicators in water.

Organism	Linear Regression R^2 estimate (# of matched pairs)								
	F+ coliphages (ENR)	F+ coliphages (SAL)	F+ coliphage (MF)	somatic coliphages (ENR)	somatic coliphages (SAL)	somatic coliphage (MF)	*E. coli*	fecal coliforms	enterococci
F+ coliphages (ENR)	-	-	-	-	-	-	-	-	-
F+ coliphages (SAL)	0.525 (72)	-	-	-	-	-	-	-	-
F+ coliphage (MF)	0.624 (72)	0.324 (73)	-	-	-	-	-	-	-
somatic coliphages (ENR)	0.279 (72)	0.235 (73)	0.291 (74)	-	-	-	-	-	-
somatic coliphages (SAL)	0.281 (72)	0.231 (73)	0.237 (74)	0.554 (75)	-	-	-	-	-
somatic coliphage (MF)	0.358 (72)	0.164 (73)	0.348 (74)	0.523 (74)	0.494 (74)	-	-	-	-
E. coli	0.307 (72)	0.307 (73)	0.219 (74)	0.394 (75)	0.467 (77)	0.291 (74)	-	-	-
fecal coliforms	0.329 (72)	0.321 (73)	0.214 (74)	0.393 (75)	0.456 (77)	0.317 (74)	0.887 (77)	-	-
enterococci	0.283 (72)	0.283 (73)	0.183 (74)	0.278 (75)	0.431 (77)	0.222 (74)	0.688 (77)	0.602 (77)	-
C. perfringens	0.01 (72)	0.0755 (73)	0.233 (74)	0.405 (75)	0.22 (77)	0.374 (74)	0.33 (77)	0.355 (77)	0.171 (77)

Abbreviations as in Table 2.2

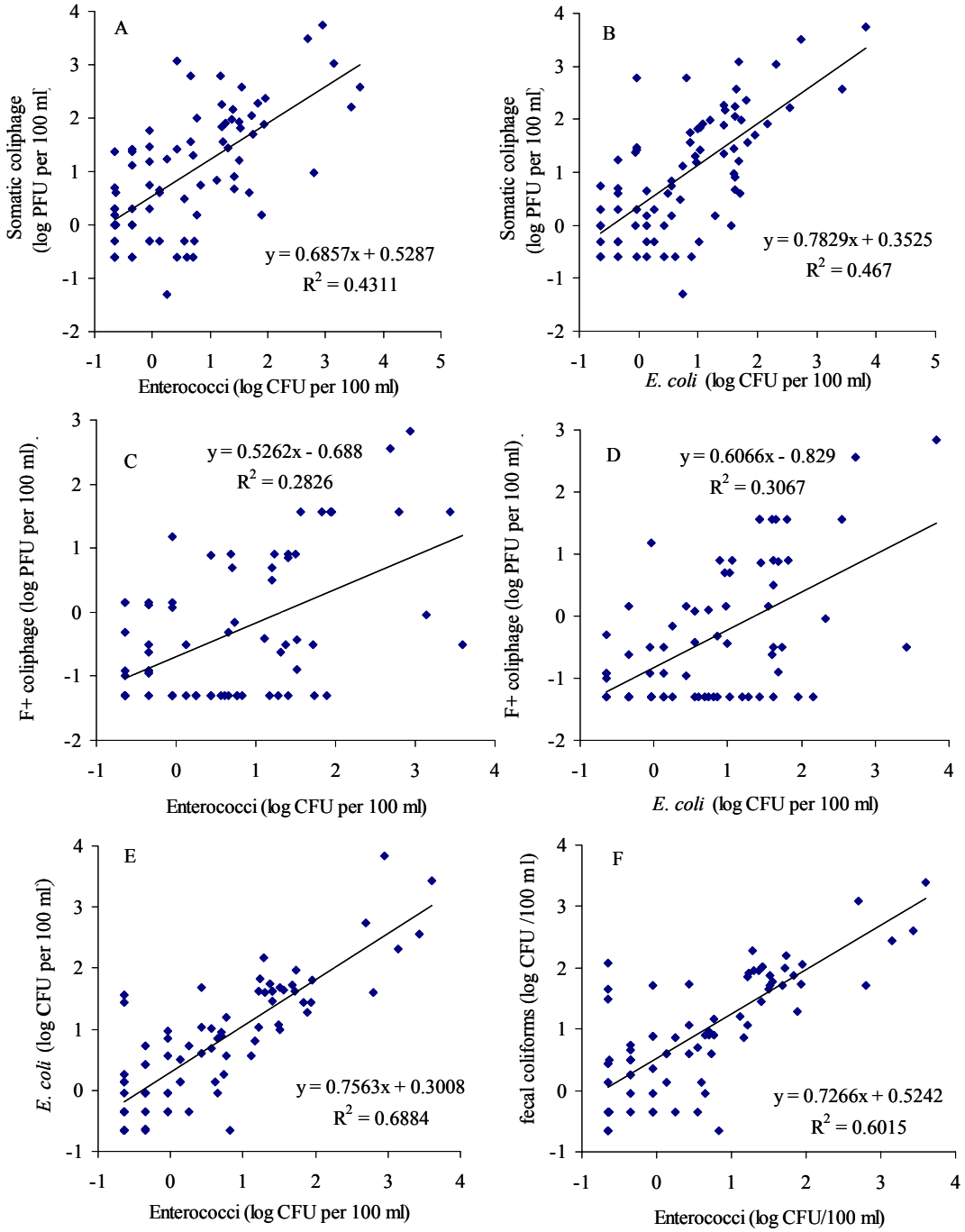


FIG. 2.2. Relationship between fecal microbes found in water. A) somatic coliphages vs. enterococci; B) somatic coliphages vs. *E. coli*; C) F+ coliphages vs. enterococci; D) F+ coliphages vs. *E.* coli; E) *E. coli* vs. enterococci; and F) fecal coliforms vs. enterococci. (n = 77-79 pairs) F+ coliphages were recovered by EPA Method 1601, and somatic coliphages by EPA Method 1602.

TABLE 2.4. F+ coliphage recovery by three methods and the resulting F+ RNA coliphage genogroups isolated.

Method [a]	Recovered F+ coliphages		Recovered F+ RNA coliphages by genogroup		
	Geometric mean as log-PFU per 100 ml (±st dev)	% of sample positives (total no. samples)	% Group I	% Group II & III	# F+ RNA isolates
ENR [b]	-0.36 (± 1.08)	59% (73)	96% [c]	4%	224
SAL [b]	-0.33 (± 0.62)	24% (74)	84%	15% [d]	38
DMF	-0.67 (± 0.86)	48% (75)	85%	16% [d]	164

Abbreviations as in Table 2.2. [a] Friedman's test non-parametric, and Dunn's multiple comparison test with matched pairs comparisons for methods in this column. [b] significantly different method with higher recoveries than DMF. [c] significant different method among the percentage of Group I genotypes recovered. [d] significant different method among the percentage of Group II and III genotypes recovered. Tables 2.5 and 2.6 are companions to this table.

TABLE 2.5. Statistical analysis of three methods for F+ coliphage recovery from water.

Test [a]	F+ coliphage					
	All matched pairs			Matched pairs without below detect values		
	Number of pairs	p value	Significant difference	Number of pairs	p value	Significant Difference
ENR vs DMF	69	<0.05	DMF < ENR	12	>0.05	no [b]
SAL vs DMF	69	<0.01	DMF < SAL	12	>0.05	no [b]
SAL vs ENR	69	>0.05	No	12	>0.05	no [b]

Abbreviations as in Table 2.2. [a] Friedman's test non-parametric, and Dunn's multiple comparison test for methods in this column. [b] Significant differences (p values = 0.0458) exist among all 3 methods, but significant differences do not exist between any two methods.

TABLE 2.6. Comparison of the proportion of F+ RNA coliphage genogroups (Group I or Group II + III) recovered by three different methods.

F+ RNA genogroup	Recovery method comparison (p value)		
	ENR vs. SAL	ENR vs. DMF	SAL vs. DMF
I	SAL<ENR (0.028)	DMF<ENR (0.0005)	not significant (0.429)
II + III	ENR<SAL (0.028)	ENR<DMF (0.0005)	not significant (0.429)

Abbreviations as in Table 2.2. Proportion of F+ RNA coliphage genogroups recovered were compared between methods using a Z test statistic with two tails and an alpha of 0.05.

TABLE 2.7. Somatic coliphage recovery from water by three methods.

Method [a]	Somatic coliphages	
	Geometric mean as Log_{10} PFU per 100 ml (± st dev)	% of sample positives (total no. samples)
ENR [b]	0.77 (±1.11)	96% (76)
SAL [b]	0.93 (±1.15)	86% (78)
DMF	0.54 (±1.11)	88% (76)

Abbreviations as in Table 2.2.. [a] Friedman's test non-parametric, and Dunn's multiple comparison test with matched comparisons for methods in this column. [b] significantly different method with higher recoveries than DMF (alpha = 0.05).

TABLE 2.8. Statistical analysis of somatic coliphage recovery by three methods.

Test [a]	Somatic coliphage					
	Matched pairs			Matched pairs without below detect values		
	Number of pairs	P value	Significant Difference	Number of pairs	P value	Significant
ENR vs DMF	76	<0.001	DMF <ENR	57	<0.001	DMF < ENR
SAL vs DMF	76	<0.001	DMF < SAL	57	<0.001	DMF < SAL
SAL vs ENR	76	>0.05	No	57	>0.05	No

Abbreviations as in Table 2.2.. [a] Wilcoxon matched-pairs signed-ranks test.

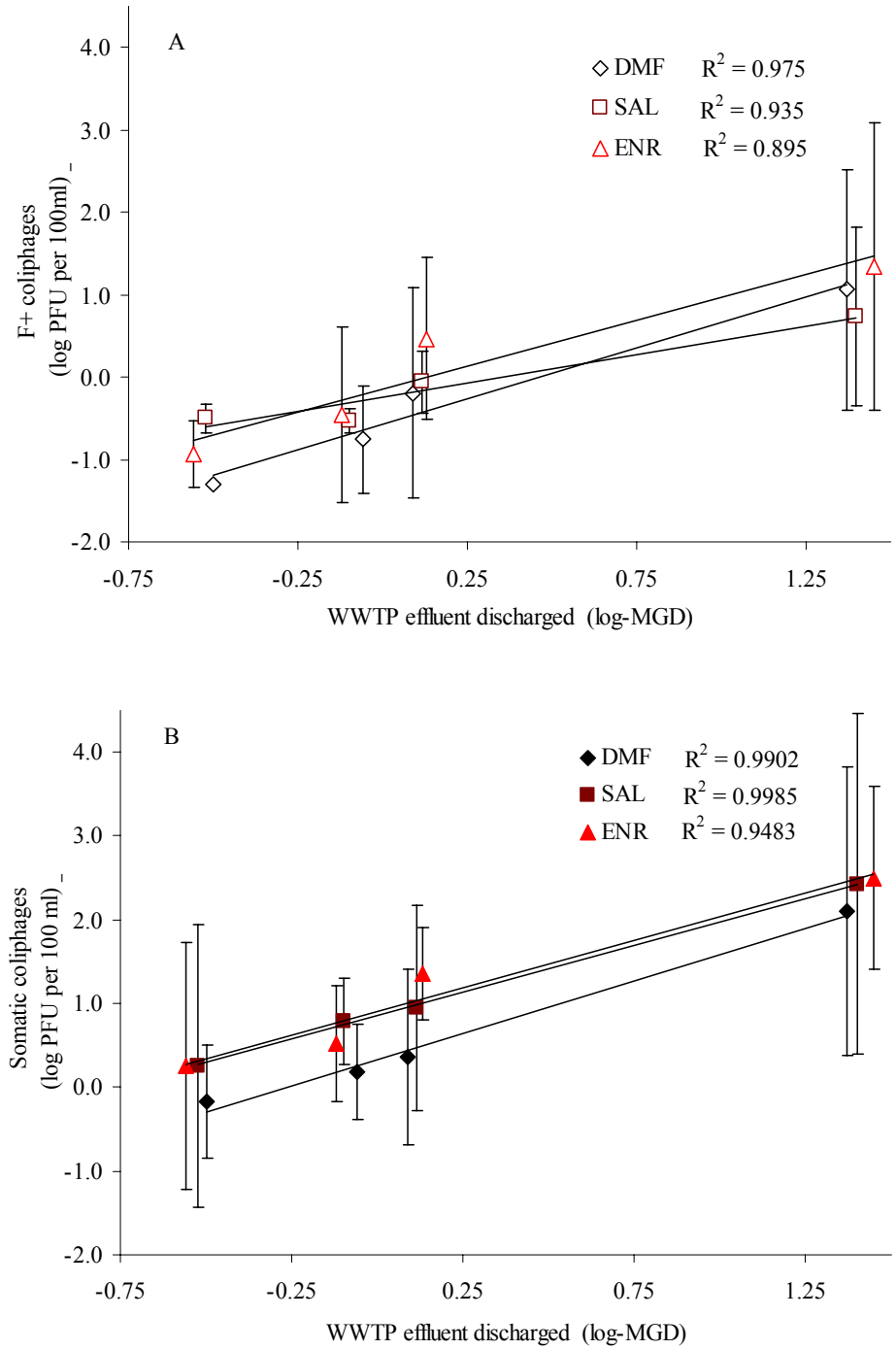


FIG. 2.3. F+ coliphage (a) and somatic coliphage (b) recovered in estuarine waters impacted by WWTP discharges. Acronyms in Tables 2.1 and 2.2. X-axis values offset slightly to show vertical standard deviation error bars. Monitoring sites include Apalachicola Bay, FL at mouth of bay (n=3-4) (0.3 MGD WWTP), Rachel Carson, NC on north shore of Carrot Island, Beaufort, NC (n=4) (0.8 MGD WWTP), Great Bay, NH at Oyster River (n=4) (1.3 MGD WWTP), and Tijuana River, CA (n=4) (25 MGD WWTP).

TABLE 2.9. F+ RNA genogroups detected in waters of nine US estuaries.

Estuary	No. water sub-samples [a]	Genogrouped isolates (% of total)					
		Group I (MS2)	Group II (GA)	Group III (Qβ)	Group III (M11)	Group IV (SP)	Group IV (FI)
Apalachicola Bay, FL	18	40 (90.9%)	-	3 (6.8%)	1 (2.3%)	-	-
Delaware Bay, DE	36	131 (94.9%)	3 (2.2%)	2 (1.4%)	2 (1.4%)	-	-
Elkhorn Slough, CA	24	31 (96.9%)	1 (3.1%)	-	-	-	-
Great Bay, NH	24	46 (80.7%)	10 (17.5%)	1 (1.8%)	-	-	-
Masonboro Island, NC	24	1 (100%)	-	-	-	-	-
Narragansett Bay, RI	18	39 (88.6%)	5 (11.4%)	-	-	-	-
Rachel Carson, NC	30	30 (75.0%)	10 (25.0%)	-	-	-	-
Tijuana River, CA	24	73 (96.1%)	3 (3.9%)	-	-	-	-
Waquoit Bay, MA	36	3 (75.0%)	1 (25.0%)	-	-	-	-
Total no. (% of total)	234	394 (90.4%)	33 (7.6%)	6 (1.4%)	3 (0.7%)	0	0

[a] Each water sample was split among three F+ coliphage recovery methods, so the actual number of sampling events is the number of sub-samples divided by three.

3 Comparison of Microbial Indicators of Fecal Contamination of Molluscan Shellfish from Nine Estuaries on East, West, and Gulf Coasts of the United States.

Abstract

Molluscan shellfish with acceptable levels of fecal indicator bacteria can contain enteric viruses and toxin-producing endemic microbes at levels causing health risks. This study compares somatic and F+ coliphages, enterococci, *E. coli*, and *Clostridium perfringens* to the standard fecal coliform indicator to determine if alternative indicators are suitable for monitoring shellfish quality. Oysters, mussels and clams were collected from sites at nine estuaries containing human and non-human fecal impacts from point and non-point contamination sources for a geographically diverse assessment of these fecal indicators in the United States. Fecal coliform levels in shellfish were not predictive of human fecal impacts ($p = 0.183$), unlike *E. coli* ($p = 0.023$) or *C. perfringens* ($p = 0.014$). Log_{10} *E. coli* levels explained 94% of the variability in log_{10} fecal coliform levels using linear regression. F+ coliphages were nearly significant in predicting human fecal impacts ($p = 0.073$), and were detected in 62%, 64%, and 83% of oysters, clams, and mussels, respectively, using the two-step enrichment assay, the most sensitive method. Both log_{10} somatic and F+ coliphages in shellfish correlated positively with wastewater discharge loads near shellfish grounds. To evaluate F+ RNA coliphages for microbial

source tracking, 591 isolates were genogrouped as 82% group I, 14% group II, 3.8% group III, and 0.3% group IV. Overall, F+ coliphages were readily detectable fecal indicators in shellfish and therefore can be used along with fecal coliforms for more accurate assessment of shellfish sanitary quality and enteric virus risks

Introduction

Bivalve molluscan shellfish (shellfish) are filter feeders that bioaccumulate microbial pathogens and fecal indicators from overlying water and from resuspended sediments (Shieh et al., 2003; Smith et al., 1978; Landry et al., 1983; Jamieson et al., 2005). Shellfishing areas near waste water treatment plant (WWTP) effluent discharges are typically closed for shellfish harvesting based on sanitary surveys, because these areas may contain pathogens that survive waste water disinfection (Payment et al., 2001). Eating contaminated shellfish has been linked to cases and outbreaks of viral gastroenteritis, acute viral hepatitis and other diseases (Shieh et al., 2000, 2007; Le Guyader et al., 2006).

In the early part of the 20th Century *Salmonella typhi* (typhoid fever) was the most common cause of shellfish-associated outbreaks in the US, and due to drastic improvements by US Public Health Service, the last reported typhoid fever outbreaks in shellfish was 1954 (Rippey 1994). Fecal coliform bacterial indicators are still used to monitor and control the sanitary quality of shellfish growing waters and shellfish meat (US FDA 2003), which is a large reason why bacterial pathogens of fecal origin have

constituted just 4% of shellfish-associated outbreaks in the last quarter century (Lipp and Rose, 1997; Rippey 1994).

However, fecal indicator bacterial monitoring cannot prevent all types of shellfish-associated diseases. It is well documented that shellfish with acceptable levels of fecal indicator bacteria can contain levels of human enteric viruses and toxin-producing endemic marine microbes such as *Vibrio* spp. causing health risks (Croci et al., 2000; Chung et al., 1998; Formiga-Cruz et al., 2003; Koh et al., 1994). Shellfish-associated outbreaks due to enteric viruses continue to occur despite fecal coliform monitoring (Lipp and Rose, 1997).

Other drawbacks of fecal indicator bacteria are their ubiquity in the gut and excreta of all warm-blooded and some cold blooded animals (Harwood et al, 1999), making source identification difficult without technically demanding microbial source tracking techniques (Scott et al., 2002). Coastal regions and their estuaries with shellfish are prime breeding areas for bird populations and habitats for mammalian wildlife. Reliance on fecal indicator bacteria criteria and standards in these areas may unnecessarily restrict molluscan shellfishing by overestimating or misclassifying fecal contamination as human rather than animal.

The future of shellfish monitoring will be to address the current, overwhelming causes of shellfish-associated outbreaks, namely those from fecally-associated enteric viruses such as norovirus and hepatitis A virus (Rippey 1994; Wallace et al., 1999; Lees, 2000), from chemical toxins produced by endemic marine bacteria such as *Vibrio* spp., and from dinoflagellates and diatoms causing paralytic and diarrheic shellfish poisoning (Wallace et al., 1999; Rippey 1994; Lipp and Rose, 1997).

This study compares the levels, types, and methods for detecting two viral fecal indictor viruses (somatic and F+ coliphages) and four bacterial fecal indicators (fecal coliforms, *E. coli*, enterococci, *Clostridium perfringens*) to determine if coliphages are suitable fecal indicators of shellfish sanitary quality. Shellfish were collected from sites impacted by known point and non-point sources of human and non-human fecal contamination of nine estuaries on the East, West and Gulf Coasts of the United States (US), thereby giving a representative and geographically diverse assessment of fecal indicators in shellfish. These findings provide shellfish monitoring programs with more comprehensive and presumably accurate information to assess the sanitary quality of shellfish and to reduce risks of viral disease to shellfish consumers.

Materials and Methods

Study sites, sample collection and processing. Sampling sites were chosen in nine estuaries that are part of the National Estuarine Research Reserves (NERR) system. At each estuary two stations were sampled: one in an area proximal to human waste sources and one in an area distant to human waste sources and considered pristine. Samples consisted of 10-12 oysters, 10-20 clams, and/or 10-20 mussels. Mussels were collected at two estuaries in California, clams at estuaries in Rhode Island, Massachusetts, and North Carolina, and oysters at estuaries in Florida, California, New Hampshire, and two estuaries in North Carolina. Samples were shipped chilled 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 homogenates were 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 of serial tenfold-dilutions) were added to lauryl tryptose broth with inverted vials, incubated for 24-48 hr at 35^{o}C, and vials with gas production were 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 confirmed positive and negative 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 pour plating of shellfish homogenate (replicate 1 ml volumes and tenfold-dilutions thereof) with 15 ml of molten mEnterococcus agar on 150x15mm petri dishes (Clesceri et al., 1998; Bordner et al., 1978). The plates were incubated for 48 ± 3 hr at 35^{o}C, with dark blue colonies as presumptive positives. A representative number of presumptive colonies were confirmed by streaking onto membrane filters placed on mEI plates, incubated for 24 ± 2 hr at 41^{o}C and observed for growth distinctive of enterococci. Estimated concentrations of colony forming units (CFU) of enterococci per 100 ml of homogenate (or 100 grams of shellfish meat) were based on the percent of total colonies confirmed. For *Clostridium perfringens* detection and enumeration, shellfish homogenates (1 ml and tenfold serial dilutions thereof) were inoculated into 10 ml volumes of Iron Milk medium, incubated overnight at 41-44.5^{o}C (St. John et al., 1982), and presumptive positive tubes having "stormy fermentation" were

confirmed by streaking on membrane filters on mCP agar plates (as described above). Iron Milk Medium contains one 12 oz can of evaporated milk (Carnation, Nestlé), 950 ml sterile deionized water, and 50 ml of filter-sterilized 2% $FeSO_4$ solution. The combination of confirmed positive and negative tubes was used to compute the MPN of *C. perfringens* per 100 ml of homogenate (or 100 grams of shellfish meat).

Coliphage recoveries and assays. Samples of homogenized shellfish tissue were assayed for both somatic and F+ coliphages by two methods (US EPA methods 1601 and 1602). 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 the antibiotic nalidixic acid 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, the Single Agar Layer (SAL) Assay, was performed as described previously (EPA 2001b) with minor modifications. Assays consisted of ten replicates of 1 ml aliquots of shellfish homogenate, each of which were combined with 20 ml of tryptic soy agar medium and host *E. coli* cells and then poured into individual 150 x 15 mm diameter Petri plates. After overnight incubation, plaques were enumerated and the titer of coliphages in the sample calculated as PFU (plaque-forming units) per 100 grams of shellfish meat.

US EPA Method 1601, the Two Step Enrichment (ENR) Assay, was performed as previously described (EPA, 2001a), except different samples volumes were analyzed in replicate to achieve quantitative results as an MPN assay 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/100 grams of shellfish meat.

Coliphage isolation and RNase testing. As many as ten F+ coliphage plaques and somatic plaques per sample were randomly chosen on agar plates. Plaque material was enriched in 5 ml of TSB by EPA method 1601, clarified by 1200 *xg* centrifugation for 20 min at 4°C, and frozen at -80°C for subsequent confirmation and genogrouping. F+ coliphages also underwent an RNase test as previously described (Hsu et al., 1995) by re-plating the isolates in the presence and absence of 100 µg/ml Ribonuclease A (Sigma-Aldrich, St. Louis, MO) to determine if its nucleic acid was DNA or RNA. Coliphages that grew in the presence of RNase were considered F+ DNA coliphages and those that did not were considered F+ RNA coliphages.

F+ RNA coliphage detection and genotyping by Reverse Line Blot hybridization. Approximately 600 F+ RNA coliphage isolates were further subjected to a genotyping test to distinguish the four 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 allolevivirus using biotinylated primers (Vinjé et al., 2004). RT-PCR products were then further characterized by reverse line blot (RLB) hybridization using a panel of group- and subgroup-specific oligonucleotide probes in assays (Vinjé et al., 2004). Bound RT-PCR products on the RLB membranes were detected as hybrids by chemilumenescence 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).

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 a pair using Wilcoxon Signed Rank test, and for three or more with Friedman's test and Dunn's multiple comparison test. Proportions of F+ RNA coliphage genogroups were compared between methods using a Z-test statistic with two 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

Comparing levels of fecal indicators in impacted and non-impacted shellfish. Levels of fecal indicator microbes in shellfish from paired stations considered impacted or not impacted by human fecal contamination sources are summarized and compared as box-and-whisker plots in Fig. 3.1. Summarized in Table 3.1 are the results of statistical analyses of these data, based on comparisons made using the Wilcoxon Signed Rank Test on 33 or 34 sets of matched pairs (Table 3.1). *C. perfringens* and *E. coli* levels in shellfish were significantly higher at impacted stations than non-impacted stations (p values = 0.014 and 0.023, respectively (Table 3.1). By both detection and assay method, neither somatic nor F+ coliphage levels were significantly different in shellfish from

paired stations classified as either human impacted non-human impacted. However, by the SAL method F+ coliphages were not quite significantly different when comparing levels from impacted and non-impacted stations (p value = 0.073) (Fig. 3.1, Table 3.1). Enterococci colony counts in shellfish from the pairs of sample stations classified as impacted or not impacted were not quite significantly different (p = 0.062) (Fig. 3.1, Table 3.1).

Relationship between fecal microbes found in shellfish. Scatter plots of $\log_{10}$ levels of pairs of microorganism in shellfish were made for all pairs of fecal indicators. Linear regression trendlines fit to geometric mean levels showed strongest positive correlations were between fecal coliforms and *E. coli* ($R^2 = 0.94$), while weakest positive correlations were between enterococci and F+ coliphages with the ENR method ($R^2 = 0.051$) (Table 3.2). Positive correlations were observed between somatic coliphage methods of ENR and SAL ($R^2 = 0.742$), and for F+ coliphage methods of ENR and SAL ($R^2 = 0.532$) (Table 3.2). Somatic and F+ coliphages were positively correlated with levels of bacterial indicators in shellfish to varying degrees, based on R^2 values. Somatic coliphage levels in shellfish explained 32% to 38% of the variability in fecal coliform and enterococci levels (Table 3.2; Fig. 3.2). F+ coliphages levels were less related to fecal coliform indicator levels with linear regression trendlines explaining just 6.2% to 6.4% of the variability in fecal coliform levels (Table 3.2; Fig. 3.2c). Bacterial indicators levels were moderate to strong predictors of other bacterial indicator levels in shellfish (R^2 = 0.36 to 0.94) (Table 3.2), where fecal coliforms explained 60% of the variability in levels of enterococci in shellfish (Fig. 3.2d).

Comparison of F+ coliphage recovery and detection in shellfish by enrichment and SAL methods. ENR and SAL methods were compared for nominal detection (+/- detection), for quantification of F+ coliphage levels, and with importance for microbial source tracking, the relative amounts and proportions of F+ RNA genogroups recovered by each method. For nominal detection, the percentage of F+ coliphage positive samples between the two methods was statistically equivalent ($p = 0.3164$ by Chi-squared test), with 66% (49/74) of shellfish samples positive by ENR and 54% (40/74) of shellfish samples positive by SAL. The geometric mean F+ coliphage levels recovered by ENR was greater than by SAL for all shellfish (p value <0.0001) and for clams only (p value <0.0001), while no statistically significant difference was seen in mussels and oysters (Tables 3.3). When below detection values were removed from the data set, the same statistical outcomes of ENR recovering significantly greater levels of F+ coliphage than SAL from all shellfish (p value = 0.0018) or from clams (p value <0.0001) remained (Table 3.4). The detection limit of each assay was approximately 4 PFU per 100 grams shellfish

For microbial source tracking purposes, SAL provided more F+ RNA coliphage isolates than ENR from all shellfish and from just mussels. After genogrouping all F+ RNA isolates, a significantly higher percentages of F+ RNA Group II/III isolates were recovered by SAL than by ENR from mussels (p value <0.001), clams (p value <0.001), and all shellfish (p value = 0.001) (Table 3.5). All shellfish types (clams, oysters and mussels) yielded similar percentages of Group II/III isolates by SAL, while using ENR the percentage of Group II/III isolates varied by shellfish type with the lowest percentage

in clams (Group II/III isolates comprised 1% of all isolates) and highest percentage in oysters (Group II/III isolates comprised 19% of all isolates) (Table 3.3 and 3.5).

Comparison of somatic coliphage recovery from shellfish by enrichment and SAL methods. Somatic coliphage were recovered from about 80% of shellfish samples with an average geometric mean ± standard deviation of 2.00 ± 1.13 PFU/100 grams for SAL and 2.05 ± 1.23 PFU/100 grams for ENR (Table 3.6). The sample positivity did not statistically differ between SAL (80% positive; 59/74 samples positive) and ENR (79% positive; 57/72 samples positive) for all shellfish (Chi-squared test, p value = 0.9329) (Table 3.6). In all shellfish or in oysters, clams, or mussels, there were no significant differences in the levels of somatic coliphages recovered by either method, ENR or SAL (Tables 3.6) using the entire data set, but when below detection values were removed from the data set ENR recovered significantly greater levels of somatic coliphage than SAL in all shellfish (p value = 0.0145) and in oysters (p value = 0.0384) but not in clams or mussels (Table 3.7) . Both ENR and SAL methods had similar lower detection limits of approximately 4 PFU per 100 grams shellfish

Fecal indicators recovered from shellfish in relation to impacts by waste water treatments plants. As shown in Figure 3a, somatic coliphage levels in shellfish tended to increase as WWTP effluent volumes increased and the R-squared linear regression trendlines explained 99-99.8% of the variability in coliphage levels in shellfish relative to WWTP discharge volumes (Fig. 3.3b). Similar trendlines fit to geometric mean levels of F+ coliphages in relation to WWTP discharges explained 72-92% of the variability in F+

coliphage levels in shellfish samples relative to WWTP discharge volumes (Fig. 4a). Bacterial indicator levels (fecal coliforms, *E. coli*, Enterococci, *C. perfringens*) at WWTP impacted sites had similar trends in their levels in shellfish relative to WWTP discharge volumes as for coliphage, with 81-85% of the variability in shellfish explained 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.

F+ RNA genogroups detected in shellfish. The sub-set of 591 F+ coliphage isolates with RNA genomes (F+ RNA coliphages) recovered from shellfish were genogrouped as F+ RNA group I, II, III, or IV by RLB hybridization (Table 3.8). F+ RNA group II (GA-like) phages were detected at six of eight estuaries and constituted 4.1% to 28.8% of isolates recovered at those estuaries (Table 3.8). Group III (Qβ-like or M11-like) isolates were detected in four of eight estuaries, making up 2.4% to 13% of isolates recovered at those estuaries (Table 3.8). The majority of F+ RNA isolates were group I (MS2-like) (81.7%), followed by group II (14.2%), group III (3.8%), and group IV (SP-like or FI-like) (0.3%) (Table 3.8). No F+ RNA shellfish isolates were found in Apalachicola Bay, FL (Table 3.8).

Discussion

This study compares the levels, types, and sources of fecal indicator bacteria (fecal coliforms, *E. coli*, *C. perfringens*, enterococci) and viruses (F+ and somatic

coliphages) in shellfish (oysters, mussels and clams) at nine geographically diverse estuaries across the US. In this work, fecal coliform levels in shellfish were not predictive of human fecal impacts based on proximity to municipal wastewater discharges, which suggests that basing shellfish sanitation on fecal coliforms, as is done in the US, inadequately predicts fecal impacts and disease risks to shellfish consumers. This finding adds to the body of work that questions the predictability fecal coliforms for fecal contamination impacts (Hendrick 1970; Adams 1972). Significant differences were observed for *E. coli* at human impacted sites compared to non-impacted or pristine sites, which is consistent with *E. coli* being a more feces-specific bacterial indicator than fecal coliforms. However, $\log_{10}$ *E. coli* levels explained only 94% of the variability in $\log_{10}$ fecal coliform levels using linear regression, with outlying samples having about ten-fold more fecal coliforms than *E. coli* (data not shown). Unfortunately, non-E. coli fecal coliforms were not speciated, so it is not possible to judge if the fecal coliforms detected at high levels when E. coli levels were much lower were of likely non-fecal origin, based on which species they were. Some fecal coliforms, such as various species of Klebsiella are often associated with woody vegetation sources (Bagley et al., 1978; Caplenas and Kanarek 1984).

The findings of this study suggest that *E. coli* may be a more accurate measure of fecal contamination than fecal coliforms, as has been previously reported by others (LeClerc et al., 2001). Some *E. coli* have non-fecal sources (Rivera et al., 1988), and they grow or regrow in tropical and sub-tropical sediments and soils (along with enterococci) (Solo-Gabriele et al., 2000; Desmarais et al., 2002. Byappanahalli and Fujioka 2004).. Although this makes E. coli less ideal for fecal monitoring in some

warmer latitudes, the results of the current study documented their predictability of fecal source impacts in the geographically diverse US waters of this study.

C. perfringens was also detected in significantly higher levels in shellfish at human impacted sites than non-impacted sites, which is similar to others findings in marine sediments (Cox et al., 2005). Our results may be explained by the presence of *C. perfringens* in disinfected effluent of WWTPs (Chung et al., 1998; Harwood et al., 2005) and their low prevalence in feral animals compared to domestic animals (Cox et al., 2005). Because *C. perfringens* tends to particle-associate and partition into sediments more than other fecal indicators (Characklis et al., 2005), and persists at undiminished levels in marine sediment for at least one year (Hill et al., 1996), we would expect that that bivalves living on or in sediments would be exposed to high levels of *C. perfringens* that could be bioaccumulated. In work reported by others, *C. perfringens* spores correlated with human enteric viruses, *Giardia lamblia* cysts, and *Cryptosporidium* spp. oocysts in river water (Brooks et al., 2005; Payment and Franco 1993), and have been proposed as a surrogate for human enteric viruses and parasite disinfection in drinking water (Payment and Franco 1993). The persistence and sedimentation of *C. perfringens* spores has been beneficial for source tracking a sewage leak in a tidal creek (D. C. Love unpublished data), and for tracing the impacts of point and and non-point sources on marine waters (Davies et al., 1995; Shibata et al., 2004). However, the great persistence of *C. perfringens* makes its ability to judge smaller and intermittent fecal contamination events in shellfish meat or growing water a challenge, as it tends to persist and accumulate rather than decline relatively quickly after transient fecal contamination

episodes. However, unlike some fecal indicator bacteria, it does not proliferate in water, sediments and soils of tropical climates.

Enterococci is a fecal indicator used widely in recreational marine water because of positive associations with disease risks among swimmers (US EPA 1986; Wade et al., 2003). However, enterococci is seldom used for assessing fecal contamination in shellfish meat or growing water. Consequently, this study is one of the few to compare enterococci to other fecal indicators in shellfish (Aulicino et al., 1979). In this work, enterococci was detected in shellfish at nearly significantly different levels in human impacted sites than non-impacted sites. Enterococci was more persistent than fecal coliforms in saltwater mesocosms (Anderson et al., 2005), and in this study $\log_{10}$ levels of enterococci explained 60% of the variability of $\log_{10}$ fecal coliforms, a stronger association than other fecal indicators except *E. coli*. Enterococci levels in shellfish were predictive of permitted WWTP effluent discharge volumes at four sites, but less so than were F+ and somatic coliphages.

Coliphages have been proposed as an alternative fecal indicator in shellfish (Dore et al., 2000, 2003), and this study represents one of the first comparisons of coliphage detection methods in field samples of shellfish from diverse geographic locations. For somatic coliphages, ENR recovered significantly higher levels than SAL from all shellfish and from oysters only, but there were no significant differences in the frequency of detection (as the proportion of positive samples) between ENR and SAL, which may be due to the greater sensitivity of ENR and shellfish with both high levels (about 2 $\log_{10}$ PFU/ml) and high prevalence (about 80%) of somatic coliphages. For F+ coliphages, ENR detected significantly more positive samples and recovered higher concentrations of

F+ coliphage than SAL, which agrees with previously reported findings (Hsu et al., 1998; Stewart-Pullaro 2006). F+ coliphages were detected in 62%, 64%, and 83% of oysters, clams, and mussels using ENR, which supports the view that F+ coliphages are prevalent and readily detectable fecal indicators in bivalve mollusks from diverse coastal sites in the US. In Spain, F+ coliphages were detected in 22% of mussels, much lower than in this study, while somatic coliphages were detected in similar numbers of mussels (86%) as in this study (Munianin-Mujka et al., 2003). In this study, F+ coliphages had nearly significant associations with human fecal impacted stations, even without genotypic or serological classification. In other studies, trends or associations with enteric viruses were seen for F+ coliphage in oysters and mussels from England and Whales (Dore et al., 2000, 2003), and for phages of *Bacteroides fragilis* in mussels from Spain (Munianin-Mujka et al., 2003). In this study, F+ coliphages had weakly positive correlation with fecal coliform or *E. coli* in shellfish by linear regression, which is in contrast to previously reported mild correlations in UK shellfish (Dore et al., 2003). In contrast, somatic coliphages did correlated well with fecal coliforms, E. coli and enterococci in this study, a type of analysis and result which has not been previously reported for shellfish..

F+ coliphage methods were compared for their ability to provide representative numbers and types of F+ RNA isolates for microbial source tracking. Using source tracking results as a gauge for F+ coliphage recovery methods, the SAL method provides a more accurate and unbiased representation of the minority F+ RNA coliphage groups present in shellfish sample, such as groups II and III. In contrast, the ENR method could potentially underestimates human fecal impacts when the growth of F+ RNA coliphage

groups II and III F+ RNA coliphages is overshadowed by group I during microbial source tracking efforts.

F+ RNA genotyping was applied to separate shellfish isolates from human fecal sources (genogroups II and III) and animal fecal sources (genogroups I and IV) to further validate their efficacy for microbial source tracking (Vinje et al., 2004; Hsu et al., 1995). The most common F+ RNA genogroup, group I, constituted 82% of all isolates, and these group I isolates were found in greater numbers in non-human impacted or pristine stations than human-impacted stations. Of the F+ RNA group II isolates detected in shellfish, 100%, 90%, 79%, and 58% came from the human-impacted stations at estuaries in Masonboro Island, NC, Narragansett Bay, MA, Great Bay, NH, and Tijuana River, CA, as consistent with previous findings for human or wastewater sources of group II isolates (Stewart-Pullaro 2006; Furuse 1981). These findings suggest F+ coliphages are useful fecal indicators of shellfish sanitary quality and F+ RNA coliphages are applicable to microbial source tracking studies to better represent proportions and relative amounts of human or animal fecal sources found in shellfish.

References

1. **Adams, J. C.** 1972. Unusual organism which gives a positive elevated temperature test for fecal coliforms. **23(1):**172-173.

2. **Anderson, K. L., Whitlock, J. E., and V. J. Harwood.** 2005. Persistence and differential survival of fecal indicator bacteria in subtropical waters and sediments. **71(6):**3041-3048.

3. **APHA.** 1998. Standard methods for the examination of water and wastewater, 20th edition, American Public Health Association, Washington, DC

4. **Aulicino, F. A., Zicarelli, M., Tosti, E., and L. Volterra.** 1979. Shellfish quality standards. Ann Ist Super Sanita. **15(4):**709-718.

5. **Bagley, S. T., Seidler, R. J., Talbot, H. W. Jr, and J. E. Morrow.** 1978. Isolation of Klebsielleae from within living wood. Appl Environ Microbiol. **36(1):**178-185.

6. **Bordner, R., J.A. Winter and P.V. Scarpino** (eds.). 1978. Microbiological methods for monitoring the environment, water and wastes, EPA-600/8-78-017. Office of Research and Development, US EPA

7. **Brookes, J. D., Hipsey, M. R., Burch, M. D., Regel, R. H., Linden, L. G., Ferguson, C. M., and J. P. Antenucci.** 2005. Relative value of surrogate indicators for detecting pathogens in lakes and reservoirs. Environ Sci. Technol. **39(22):**8614-8621.

8. **Byappanahalli, M., and R. Fujioka**. 2004. Indigenous soil bacteria and low moisture may limit but allow faecal bacteria to multiply and become a minor population in tropical soils. Water Sci. Technol. **50(1):**27-32.

9. **Caplenas N. R., and M. S. Kanarek**.1984. Thermotolerant non-fecal source *Klebsiella pneumoniae*: validity of the fecal coliform test in recreational waters. Am J Public Health.**74(11):**1273-1275.

10. **Characklis, G. W., Dilts, M. J., Simmons, O. D. 3rd, Likirdopulos, C. A., Krometis, L. A., and M. D. Sobsey.** 2005. Microbial partitioning to settleable particles in stormwater. Water Res. **39(9):**1773-1782.

11. **Chung, H., Jaykus, L.-A., Lovelace, G., and M. D. Sobsey.** 1998. Bacteriophages and bacteria as indicators of enteric viruses in oysters and their harvest waters. Wat. Sci. Tech. **38(12):**37-44.

12. **Clesceri, L. S., Greenberg, A. E., and A. D. Eaton** (eds.). 1998. Standard methods for the examination of water and wastewater. 20th Ed. United Book Press, Baltimore, MD.

13. **Cox, P., Griffith, M., Angles, M., Deere, D., and C. Ferguson.** 2005. Concentrations of pathogens and indicators in animal feces in the Sydney watershed. Appl. Environ. Microbiol. **71(10):**5929-5934.

14. **Croci, L., De Medici, D., Scalfaro, C., Fiore, A., Divizia, M., Donia, D., Cosentino, A. M., Moretti, P., and G. Costantini.** 2000. Determination of enteroviruses, hepatitis A virus, bacteriophages and Escherichia coli in Adriatic Sea mussels. J Appl. Microbiol. **88(2):**293-298.

15. **Davies, C. M., J. A. H. Long, M. Donald, and N. J. Ashbolt.** 1995. Survival of fecal microorganisms in marine and freshwater sediments. Appl. Environ. Microbiol. **61:**1888-1896.

16. **Desmarais, T. R., Solo-Gabriele, H. M., and C. J. Palmer.** 2002. Influence of soil on fecal indicator organisms in a tidally influenced subtropical environment. Appl. Environ. Microbiol. **68(3):**1165-1172.

17. **Dore, W. J., Henshilwood, K., and D. N., Lees.** 2000. Evaluation of F-specific RNA bacteriophage as a candidate human enteric virus indicator for bivalve molluscan shellfish. Appl. Environ. Microbiol. **66(4):**1280-1285.

18. **Dore, W. J., Mackie, M., and D. N. Lees.** 2003. Levels of male-specific RNA bacteriophage and *Escherichia coli* in molluscan bivalve shellfish from commercial harvesting areas. Lett. Appl. Microbiol. **36(2):**92-96.

19. **Formiga-Cruz M., Allard A. K. , Conden-Hansson, A.C., Henshilwood, K., Hernroth, B. E., Jofre J., Lees D. N., Lucena F., Papapetropoulou M., Rangdale R. E., Tsibouxi A., Vantarakis A., and R. Girones.** 2003. Evaluation of potential indicators of viral contamination in shellfish and their applicability to diverse geographical areas. Appl. Environ. Microbiol. **69(3):**1556-1563.

20. **Furuse, K., Ando, A., Osawa, S., and I. Watanabe.** 1981. Distribution of ribonucleic acid coliphages in raw sewage from treatment plants in Japan. Appl. Environ. Microbiol. **41(5):**1139-1143.

21. **Harwood, V. J., J. Butler, D. Parrish, and V. Wagner.** 1999. Isolation of fecal coliform bacteria from the diamondback terrapin (*Malaclemys terrapin centrata*). Appl. Environ. Microbiol. **65:**3698-3704.

22. **Harwood, V. J., Levine, A. D., Scott, T. M, Chivukula, V., Lukasik, J., Farrah, S. R., and J. B. Rose.** 2005. Validity of the indicator organism paradigm for pathogen reduction in reclaimed water and public health protection. Appl. Env. Microbiol. **71(6):**3163–3170.

23. **Hendrick, C. W.** 1970. Formic hydrogenlyase induction as a basis for the Eijkman fecal coliform concept. Appl. Micrbiol. **19(3):**441-445.

24. **Hill, R., W. Straube, A. Palmisano, S. Gibson, and R. Colwell.** 1996. Distribution of sewage indicated by *Clostridium perfringens* at a deep-water disposal site after cessation of sewage disposal. Appl. Environ. Microbiol. **62**:1741-1746.

25. **Hsu, F.-C., Shieh, Y.-S.C., van Duin, J., Beekwilder, M. J., and M.D. Sobsey** 1995. Genotyping male-specific RNA coliphages by hybridization with oligonucleotide probes. Appl. Environ. Microbiol. **61(11):**3960-3966.

26. **Hsu, F.-C., Handzel, T.R., Lovelace, G., Stewart, J.R., Sobsey, M.D., Thompson, S.S. and Yates, M.V.** 1998. Improved Methods to Detect low levels of Coliphages in Water by Enrichment Presence-Absence and Membrane Filter Methods. 1998 Water Quality Technology Conference. Denver, CO. American Water Works Association.

27. **Koh, E. G., Huyn, J. H., and P. A. LaRock.** 1994. Pertinence of indicator organisms and sampling variables to Vibrio concentrations. Appl Environ Microbiol. **60(10):**3897-3900.

28. **Landry, E. F., Vaughn, J. M., Vicale, T. J., and R. Mann.** 1983. Accumulation of sediment-associated viruses in shellfish. Appl Environ Microbiol. **45(1):**238-247.

29. **LeClerc, H., D. A. A. Mossel, S. C. Edberg, and C. B. Struijk.** 2001. Advances in the bacteriology of the coliform group: Their suitability as markers of microbial water safety. Annu. Rev. Microbiol. **55:**201-234.

30. **Le Guyader, F. S., Bon, F., DeMedici, D., Parnaudeau, S., Bertone, A., Crudeli, S., Doyle, A., Zidane, M., Suffredini, E., Kohli, E., Maddalo, F., Monini, M., Gallay, A., Pommepuy, M., Pothier, P., and F. M. Ruggeri.** 2006. Detection of multiple noroviruses associated with an international gastroenteritis outbreak linked to oyster consumption. J Clin Microbiol. **44(11):**3878-3882.

31. **Lees D.** 2000. Viruses and bivalve shellfish. Int J Food Microbiol. **25;59(1-2):**81-116.

32. **Lipp, E. K., and J. P. Rose** 1997. The role of seafood in foodborne diseases in the United States of America. Rev. Sci. Tech. **16(2):**620-640.

33. **Munianin-Mujka, I., Calvo, M., Lucena, F., and R. Girones.** 2003. Comparative analysis of viral pathogens and potential indicators in shellfish. Int J Food Microbiol. **83(1):**75-85.

34. **Payment, P., and E. Franco**. 1993. *Clostridium perfringens* and somatic coliphages as indicators of the efficiency of drinking water treatment for viruses and protozoan cysts. Appl. Environ. Microbiol. **59(8):**2418-2424.

35. **Payment P., Plante, R., and P. Cejka.** 2001. Removal of indicator bacteria, human enteric viruses, Giardia cysts, and Cryptosporidium oocysts at a large wastewater primary treatment facility. Can. J. Microbiol. **47(3):**188-1893.

36. **Rippey, S. R.** 1994. Infectious Diseases Associated with Molluscan Shellfish Consumption. Clin. Microbiol. Rev. **7(4)**419-425.

37. **Rivera, S. C., Hazen, T. C., and G. A. Toranzos.** 1988. Isolation of fecal coliforms from pristine sites in a tropical rain forest. Appl. Environ. Microbiol. **54(2):**513-517.

38. **Scott, T. M., Rose, J. B., Jenkins, T. M., Farrah, S. R., and J. Lukasik.** 2002. Microbial source tracking: current methodology and future directions. Appl. Environ. Microbiol. **68(12):**5796-5803.

39. **Shibata T., Solo-Gabriele H. M., Fleming L. E., and S. Elmir.** 2004. Monitoring marine recreational water quality using multiple microbial indicators in an urban tropical environment. Water Res.**38(13):**3119-3131.

40. **Shieh, Y, Monroe, S. S., Fankhauser, R. L., Langlois, G. W., Burkhardt, W. 3rd, and R. S. Baric.** 2000. Detection of orwalk-like virus in shellfish implicated in illness. J. Infect. Dis.181 Suppl **2:**S360-366.

41. **Shieh, Y. C., Baric, R. S., Woods, J. W., and K. R. Calci.** 2003. Molecular surveillance of enterovirus and Norwalk-like virus in oysters relocated to a municipal-sewage-impacted gulf estuary. Appl. Environ. Microbiol. **69(12):**7130-7136.

42. **Shieh, Y. C., Khudyakov, Y. E., Xia, G., Ganova-Raeva, L. M., Khambaty, F. M., Woods, J. W., Veazey, J. E., Motes, M. L., Glatzer, M. B., Bialek, S. R., and A. E. Fiore.** 2007. Molecular confirmation of oysters as the vector for hepatitis A in a 2005 multistate outbreak. J. Food Prot. **70(1):**145-150.

43. **Smith, E. M., Gerba, C. P., and J. L. Melnick**. 1978. Role of sediment in the persistence of enteroviruses in the estuarine environment. Appl. Env. Microbiol. **35(4):**685-689.

44. **Solo-Gabriele, H. M., Wolfert, M. A., Desmarais, T. R., and C. J. Palmer.** 2000 . Sources of *Escherichia coli* in a coastal subtropical environment. Appl. Environ. Microbiol. **66**:230-237.

45. **St. John, W., Matches, J., and M. Wekell.** 1982. Use of iron milk medium for enumeration of *Clostridium perfringens.* J. Assoc. Off. Anal. Chem. **65**:1129-1133.

46. **Stewart-Pullaro, J., Daugomah, J. W., Chestnut, D. E., Graves, D. A., Sobsey, M. D., and G. I. Scott.** 2006. F^{+}RNA coliphage typing for microbial source tracking in surface waters. J. of Appl. Microbiol. **101(5):**1015-1026.

47. **US EPA.** 1986. Ambient water quality criteria for bacteria – 1986. EPA 440/5/84-002.

48. **US EPA Method 1601**: Male-specific (F+) and somatic coliphage in water by two-step enrichment procedure. EPA-821-R-01-030, April, 2001, Washington, DC.

49. **US EPA Method 1602**: Male-specific (F+) and somatic coliphage in water by single agar layer (SAL) procedure. EPA-821-R-01-029, April, 2001, Washington, DC.

50. **US FDA.** 2003. National shellfish sanitation program guide for the control of molluscan shellfish 2003. [Online.] http://www.cfsan.fda.gov/~ear/nss2-toc.html

51. **Vinjé J., Oudejans, S. J., Stewart, J. R., Sobsey, M. D., and S. C. Long .** 2004 Molecular detection and genotyping of male-specific coliphages by reverse transcription-PCR and reverse line blot hybridization. Appl. Environ. Microbiol. **70(10):**5996-6004.

52. **Wallace, B. J., Guzewich, J. J., Cambridge, M., Altekruse, S., and D. L. Morse.** 1999. Seafood-associated disease outbreaks in New York, 1980-1994. Am. J. Prev. Med. **17(1):**48-54.

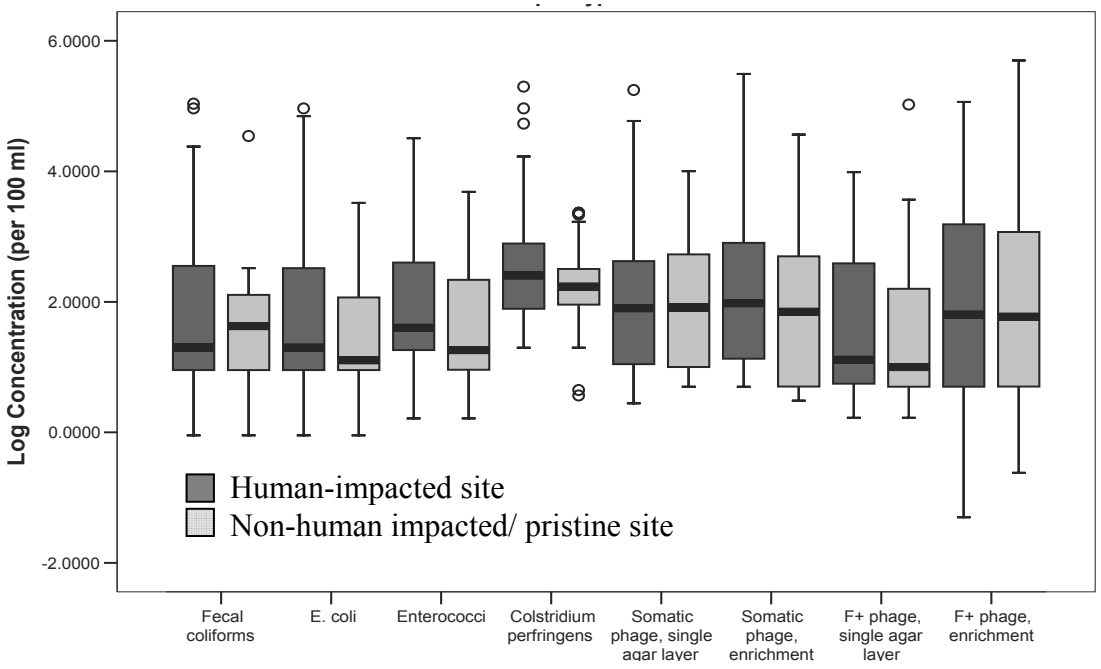


FIG 3.1. Box-and-whisker plots of log-levels of indicator bacteria and coliphage detected in shellfish at sites with human fecal impacts (dark grey; n = 38 for each microbe) or pristine sites that may contain non-human fecal impacts (light grey; n = 36 for each microbe). Lower and upper bands give minimum 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 are individual outliers.

TABLE 3.1. Statistical comparison of indicator levels in shellfish from paired stations impacted or not impacted by human fecal contamination sources.

Indicator	Method	Impacted sites vs. non-impacted sites	
		Number of pairs	P value [a]
C. perfringens	MFT	34	0.014 *
Enterococci	DP	33	0.062
E. coli	MFT	34	0.023 *
fecal coliform	MFT	34	0.183
F+ coliphage	ENR	34	0.710
F+ coliphage	SAL	34	0.073
Somatic coliphage	ENR	33	0.710
Somatic coliphage	SAL	34	0.782

[a] The Wilcoxon signed rank test on matched pairs between non-impacted and impacted sites with an asterisks (*) by those with significant differences (alpha = 0.05). DP = Direct Plating; ENR = Two Step Enrichment; MFT = Multiple Fermentation Tube; SAL = Single Agar Layer

TABLE 3.2. Linear regression R^2 correlation analysis for matched pairs of fecal indicators in shellfish.

Organism	Linear Regression R^2 estimate (# of matched pairs)						
	F+ coliphages (ENR)	F+ coliphages (SAL)	somatic coliphages (ENR)	somatic coliphages (SAL)	*E. coli*	fecal coliforms	enterococci
F+ coliphages (ENR)	-	-	-	-	-	-	-
F+ coliphages (SAL)	0.532 (74)	-	-	-	-	-	-
somatic coliphages (ENR)	0.141 (72)	0.179 (72)	-	-	-	-	-
somatic coliphages (SAL)	0.126 (74)	0.218 (74)	0.742 (72)	-	-	-	-
E. coli	0.0535 (74)	0.05 (74)	0.261 (72)	0.322 (74)	-	-	-
fecal coliforms	0.0641 (74)	0.0619 (74)	0.315 (72)	0.375 (74)	0.940 (74)	-	-
enterococci	0.051 (72)	0.0751 (72)	0.319 (72)	0.388 (72)	0.583 (72)	0.602 (72)	-
C. perfringens	0.0906 (74)	0.091 (74)	0.228 (72)	0.297 (74)	0.468 (74)	0.444 (74)	0.36 (72)

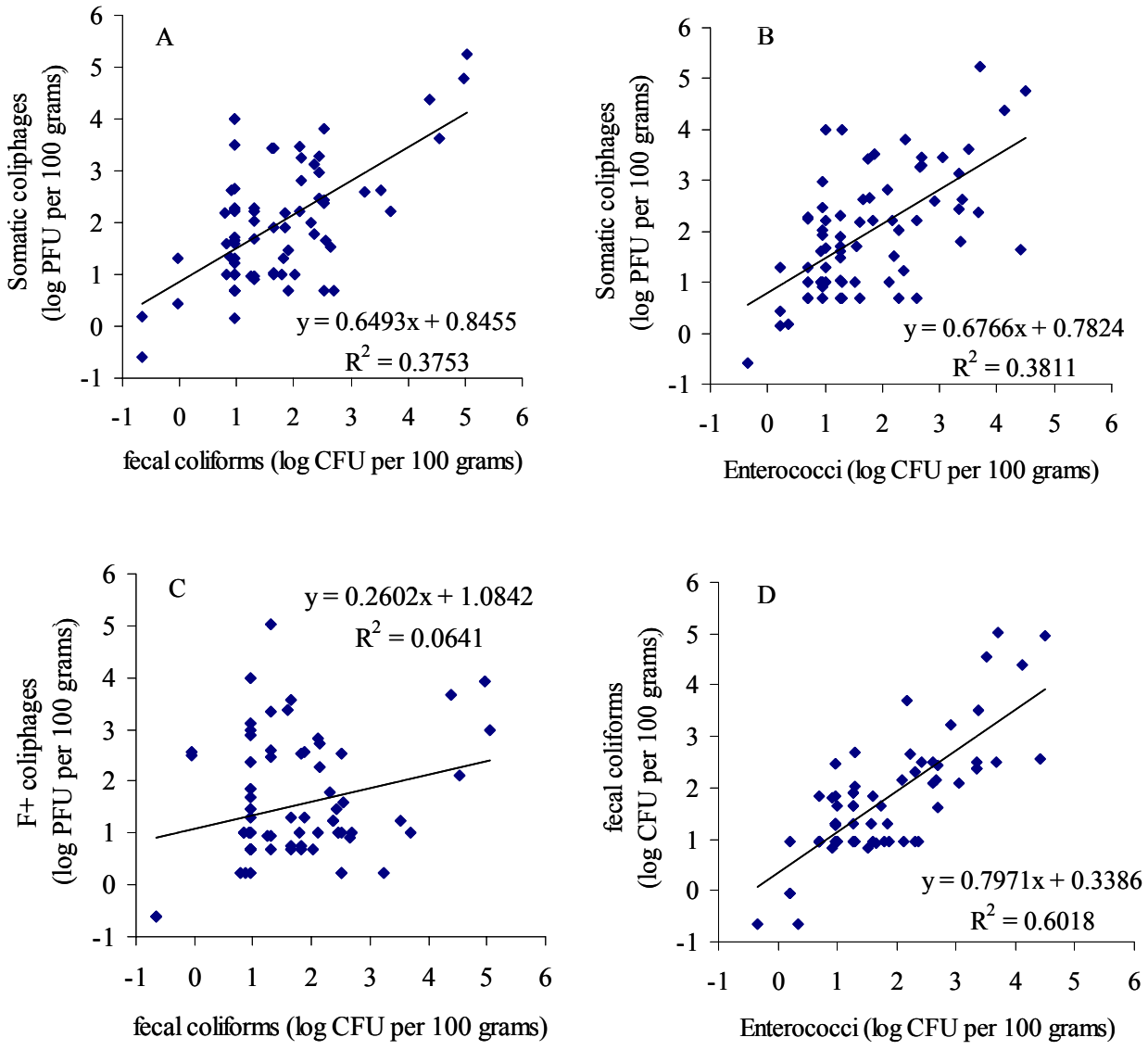


FIG. 3.2. Scatter plot of fecal microbes detected in shellfish: A) somatic coliphages vs. fecal coliforms; B) somatic coliphages vs. enterococci; C) F+ coliphages vs. fecal coliforms; and D) fecal coliforms vs. enterococci . n = 72 matched pairs of samples. F+ coliphages were recovered by EPA Method 1601; Somatic coliphages by EPA Method 1602. Linear regression, slope equation, and R-squared trendline reported. R^2 values from all fecal indicator comparisons are presented in Table 3.2.

TABLE 3.3. F+ coliphage recovery from oysters, clams, and mussels by two methods and resulting F+ RNA coliphage genogroups isolated.

Matrix	Method	Recovered F+ coliphages		Recovered F+ RNA coliphages by genogroup		
		Geometric mean as $\log_{10}$ PFU per 100 ml (±st dev)	% of sample positives (total # of samples)	% Group I	% Group II & III	# F+ RNA isolates
All [a]	ENR [b]	1.98 (± 1.43)	66% (74)	90% [c]	9%	328
Shellfish	SAL	1.59 (± 1.09)	54% (74)	76%	24% [d]	351
Oysters [a]	ENR	1.81 (± 1.36)	62% (34)	80%	19%	142
	SAL	1.65 (± 1.23)	53% (34)	77%	24%	129
Clams [a]	ENR [b]	2.04 (± 1.43)	64% (28)	99% [c]	1%	110
	SAL	1.40 (± 0.87)	54% (28)	75%	24% [d]	93
Mussels [a]	ENR	2.34 (± 1.68)	83% (12)	93% [c]	5%	76
	SAL	1.83 (± 1.16)	58% (12)	77%	24% [d]	129

Abbreviations as in Table 3.1. [a] Wilcoxon matched-pairs signed-ranks test between methods in these rows. [b] significantly different method with higher recoveries than SAL. [c] significant difference between the percentage of Group I genotypes recovered ENR than by SAL. [e] significant difference between the percentage of Group II and III genotypes recovered by SAL than by ENR. Tables 3.4 and 3.5 are companion tables.

TABLE 3.4. Statistical analysis of F+ coliphage recovery from oysters, clams, and mussels by two methods

.

Matrix	Test [a]	F+ coliphage					
		All matched pairs			Matched pairs without below detect values		
		Number of pairs	P value	Significant difference	Number of pairs	P value	Significant difference
All Shellfish	SAL vs ENR	73	<0.0001	SAL < ENR	37	0.0018	SAL < ENR
Oysters	SAL vs ENR	34	0.252	no	16	0.4954	no
Clams	SAL vs ENR	28	<0.0001	SAL < ENR	14	<0.0001	SAL < ENR
Mussels	SAL vs ENR	11	0.083	no	7	0.2118	no

Abbreviations as in Table 3.1. [a] Wilcoxon matched-pairs signed-ranks test.

TABLE 3.5. Comparison of the proportions of different F+ RNA coliphage genogroups (Group I or Group II + III) recovered by two methods.

Matrix	F+ RNA genogroup	Recovery methods comparison (P value)
All Shellfish	I	SAL<ENR (<0.001)
	II + III	ENR<SAL (<0.001)
Oysters	I	Not significant (0.24)
	II + III	Not significant (0.21)
Clams	I	SAL<ENR (<0.001)
	II + III	ENR<SAL (<0.001)
Mussel	I	SAL<ENR (0.002)
	II + III	ENR<SAL (0.001)

Abbreviations as in Table 3.1. Proportion of F+ RNA coliphage genogroups recovered were compared between methods using a Z test statistic with two tails and an alpha of 0.05. Sample numbers and proportions used in statistical tests are the same as in the far right columns in Table 3.3.

TABLE 3.6. Somatic coliphage recovery from oysters, clams, and mussels by two methods

Matrix	Method [a]	Somatic coliphages	
		Geometric mean as log-PFU per 100 ml (± st dev)	% of sample positives [b] (total no. samples)
All Shellfish	ENR	2.05 (±1.23)	79% (72)
	SAL	2.00 (±1.13)	80% (74)
Oysters	ENR	1.95 (±1.03)	82% (33)
	SAL	1.86 (±0.83)	82% (34)
Clams	ENR	1.99 (±1.13)	79% (28)
	SAL	1.91 (±1.13)	79% (28)
Mussels	ENR	2.52 (±1.89)	73% (11)
	SAL	2.60 (±1.65)	75% (12)

Abbreviations as in Table 3.1. [a] Wilcoxon matched-pairs signed-ranks test between methods gave no significant differences. [b] Chi-squared test between proportions of sample positives between methods for all shellfish, oysters, mussels, or clams gave no significant differences. Table 3.7 is a companion tables.

TABLE 3.7. Statistical analysis of somatic coliphage recovery from oysters, clams, and mussels by two methods

Matrix	Test [a]	Somatic coliphage					
		All matched pairs			Matched pairs without below detect values		
		Number of pairs	P value	Significant difference	Number of pairs	P value	Significant difference
All Shellfish	SAL vs ENR	72	0.0739	No	44	0.0145	SAL < ENR
Oysters	SAL vs ENR	33	0.074	No	24	0.0384	SAL < ENR
Clams	SAL vs ENR	28	0.3109	No	17	0.4307	No
Mussels	SAL vs ENR	11	0.7646	No	7	0.6875	No

Abbreviations as in Table 3.1. [a] Wilcoxon matched-pairs signed-ranks test between two methods.

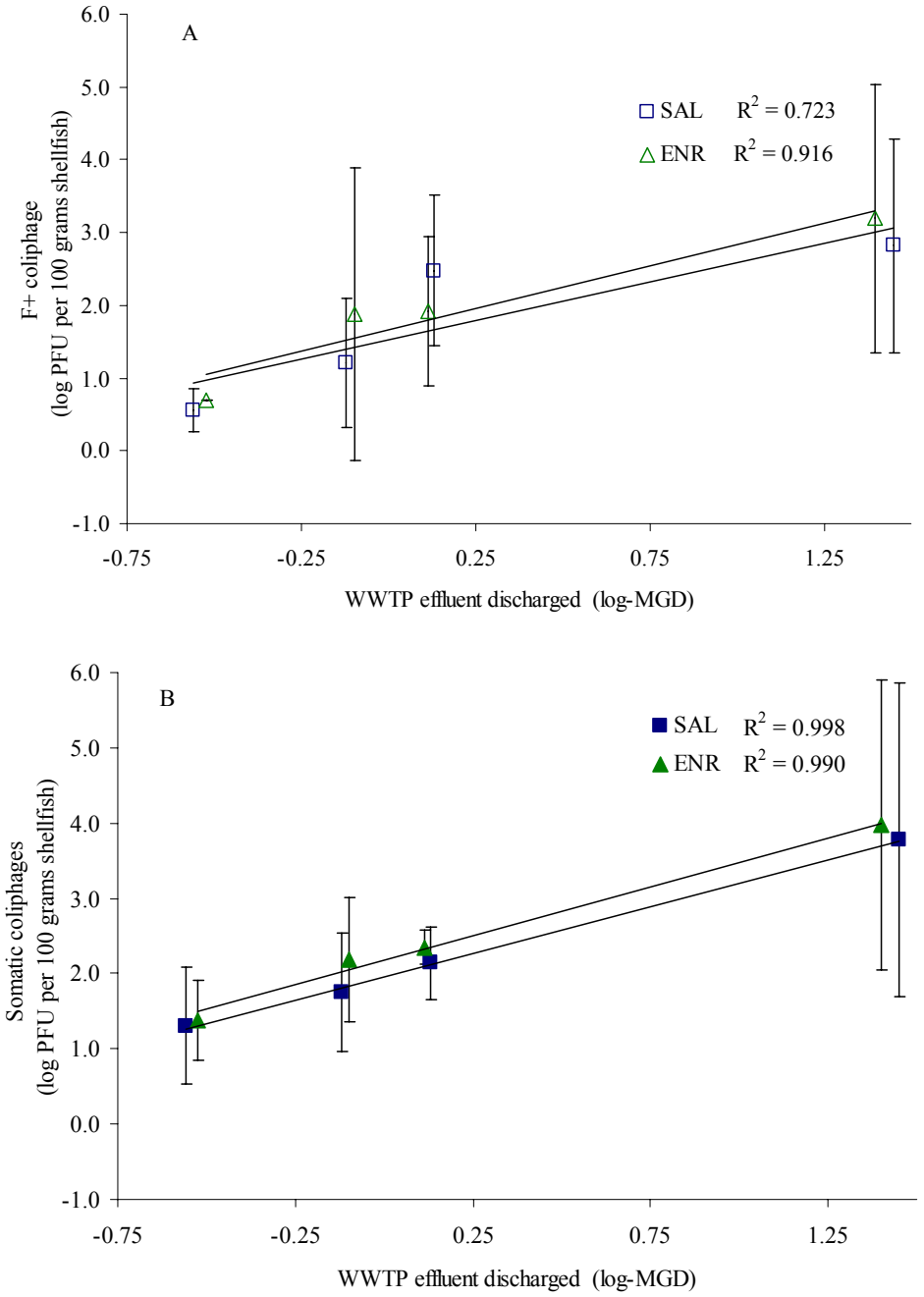


FIG. 3.3. Concentrations of F+ coliphage (a) and somatic coliphage (b) recovered in shellfish impacted by waste water treatment plant (WWTP) discharges. Abbreviations as in Tables 3.1. X-axis values offset slightly to show vertical standard deviation error bars. Monitoring sites include Apalachicola Bay, FL at mouth of bay [n=3-4; 0.3 million gallons per day (MGD) WWTP], Beaufort, NC on north shore of Carrot Island (n=8; 0.8 MGD WWTP), Great Bay, NH at Oyster River (n=4; 1.3 MGD WWTP), and Tijuana River, CA (n=4; 25 MGD WWTP).

TABLE 3.8. F+ RNA coliphage genogroups detected in shellfish at nine US estuaries.

Estuary	Shellfish type	No. shellfish sub-samples [a]	Number of genogrouped isolates (% of total)					
			Group I (MS2)	Group II (GA)	Group III (Qβ)	Group III (M11)	Group IV (SP)	Group IV (FI)
Elkhorn Slough, CA	oysters	4	20 (95%)	-	-	-	1 (5.0%)	-
Elkhorn Slough, CA	mussels	8	40 (95%)	-	1 (2.4%)	-	1 (2.4%)	-
Great Bay, NH	oysters	16	55 (60%)	24 (26%)	12 (13%)	1 (1.1%)	-	-
Masonboro Island, NC	oysters	16	71 (82%)	16 (18%)	-	-	-	-
Narragansett Bay, RI	clams	12	50 (83%)	10 (17%)	-	-	-	-
Rachel Carson, NC	oysters	20	64 (93%)	5 (7%)	-	-	-	-
Rachel Carson, NC	clams	20	67 (92%)	3 (4.0%)	3 (4.0%)	-	-	-
Tijuana River, CA	mussels	16	57 (71%)	23 (29%)	-	-	-	-
Waquoit Bay, MA	clams	24	59 (88%)	3 (4.5%)	5 (7.5%)	-	-	-
Apalachicola Bay	oysters	12	-	-	-	-	-	-
Total no. (% of total)		148	483 (82%)	84 (14%)	21 (3.6%)	1 (0.2%)	2 (0.3%)	-

[a] Each homogenized shellfish sample was split among two F+ coliphage recovery methods, so the actual number of sampling events is the number of shellfish sub-samples divided in half.

4 Evaluation of Reverse Transcriptase PCR and Reverse Line Blot Hybridization Assay for Detecting and Genotyping F+ RNA Coliphages from Estuary Waters and Molluscan Shellfish.

Abstract

Coliphages in the *Leviviridae* family (F+ RNA coliphages) are microbial indicators of fecal pollution and their serotyping or genotyping information is used for microbial source tracking. This study is the first large-scale evaluation of the reverse line blot (RLB) hybridization assay for F+ RNA coliphage detection and genotyping in environmental samples. From 2003 to 2005, 1033 F+ RNA coliphage field isolates were collected from water and shellfish in nine estuaries in the United States. The performance of the RNase test, F+ RNA coliphage reverse transcriptase PCR (RT-PCR) and RLB hybridization was assessed. Of 1033 F+ RNA 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%. 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 four isolates to the JS subgroup with >40% sequence variation, which further confirms the existence of an additional F+ RNA group. RT-PCR and sequencing improved knowledge of coliphage ecology beyond what is known from serological methods. RLB was a robust method for the detection

and genotyping of F+ RNA coliphages from diverse geographic areas, and useful for microbial source tracking and total maximum daily load estimates. Microbial source tracking with F+ RNA coliphages can better inform water quality managers and reduce risks of infectious diseases associated with exposures to waters containing human pathogens of fecal origin.

Introduction

Bacteriophages infecting *E. coli* and possibly other coliform bacteria are collectively termed "coliphages" and belong into six taxonomic families: three families of double-stranded DNA phages *Myoviridae, Styloviriae,* and *Podoviridae,* two families of single-stranded (ss) DNA phages *Microviridae* and *Inoviridae*, and the *Leviviridae* family of ss RNA phages (34). The site of infection on host bacteria is how coliphages can be differentiated, with somatic coliphages infecting via the bacterial cell wall, and F+ coliphages from the families *Leviviridae* and *Inoviridae* infecting via initial attachment to the F pilus (34).

Viruses in the family *Leviviridae* (F+ RNA coliphages) are icosahedral shaped, 23 nm in size, and possess a genome of approximately 3500-4200 nucleotides in length. F+ RNA coliphages have similar physical characteristics similar responses to environmental stressors and disinfectants as do many human enteric viruses in the *Picornaviridae* and *Caliciviridae* families (2,13,17,34). As a result, they are used as indicators of fecal pollution for these human enteric viruses in food (4,9,20), water (1,2,5,14,21), soils (25), and produce (7). Based on differences in genomic organization, the F+ RNA coliphages

can be further classified into two distinct genera: *Levivirus* and *Allolevivirus*, and three unclassified groups (34). The genus *Levivirus* contains group I and group II phages whereas the genus *Allolevivirus* contains group III and IV phages (34). In general, group II and III F+ RNA phages are found in environments impacted by human fecal sources whereas group I and IV are mostly associated with animal fecal sources (5,11,19,28).

Historically, methods to identify and group phages were based on their properties of morphology and composition, host cell lysis (8), plaque morphology (18), bacterial host range (16,23), or antigenic type based on infectivity neutralization by specific antisera (12). Antisera typing (serotyping) and host-range grouping are still widely used despite their drawbacks. These drawbacks include: host-range groupings not specific to individual F+ RNA coliphage groups, serotyping results in conflict with genotyping results (19), and a lack of availability of group-specific antisera. Based on nucleotide sequences of the well-established prototype strains, several F+ RNA genotyping methods have been developed as an alternative to serotyping, including direct hybridization of viral RNA to group-specific probes (3,19), reverse transcriptase PCR (RT-PCR) followed by reverse line blot (RLB) hybridization (36), and realtime RT-PCR (22,26). The design of broadly-reactive as well as group-specific primers and probes for these genotyping methods have been based on only a limited number of F+ RNA sequences.

The goal of this study was to evaluate the performance of RT-PCR - RLB as a routine detection, genotyping, and microbial source tracking method by applying the tests to 1033 F+ RNA coliphage field isolates collected at diverse coastal regions of the United States (US). F+ RNA strains from RLB negative samples were subsequently sequenced to determine the sequence variation within each individual genetic group and to establish

the robustness of this molecular typing method for further source tracking and ecological studies. This method validation was also part of a larger, three-year field study on coliphage and bacterial fecal indicator methods, and the occurrence, concentrations and types of these fecal indicator microbes in relation to fecal contamination sources at nine US estuaries

Materials and Methods

Bacterial host, virus stocks and environmental F+ coliphage isolates. F+ RNA prototype strains MS2 (serogroup I), GA (serogroup II), Qβ (serogroup III), M11 (serogroup III), SP, (serogroup IV) and Fi (serogroup IV), and F+ DNA strains M13, Fd and F1 were included as positive controls. From 2003 to 2005, a total of 78 1.5-liter water and 74 pooled bivalve molluscan shellfish samples (six to 12 shellfish per sample) were collected at nine National Estuarine Research Reserves in Florida, North Carolina, Delaware, New Hampshire, Massachusetts, Rhode Island, and California. The sampling sites included both open and restricted areas for shellfish harvesting. F+ RNA and F+ DNA coliphages were isolated by previously described methods (29,31,32,33) using permissive *E. coli* F_{amp} host (ATCC # 700891) grown on 0.75% tryptic soy agar plates (TSA; Difco, Sparks, MD). Up to ten F+ coliphage plaques were selected per sample for further overnight broth culture enrichment at 35°C in *E. coli* F_{amp} host. After clarification by centrifugation at 1,200 *xg* for 15 minutes the coliphage rich supernatant was mixed with 30% glycerol and stored -80ºC until further analyzed.

Spot plate assay to differentiate F+ RNA from F+ DNA coliphages. Each F+ coliphage isolate was re-plated to determine the type of viral nucleic acid (DNA or RNA) by spot plate assay. For this, 150 mm diameter petri plates of 0.75% TSA with antibiotics and log-phase *E. coli* F_{amp} host cells were prepared with and without RNase (100 µg/ml) (Ribonuclease A, Sigma-Aldrich, St. Louis, MO) as previously described (US EPA 2001c). Serial dilutions of coliphage isolates were spotted as 10 µl volumes on both RNase (+) and RNase (-) plates and incubated for six to 24 hours at 35°C. The patterns of lysis were recorded with DNA coliphages causing zones of lysis on both RNase (+) and RNase (-) plates and RNA coliphages causing zones of lysis only on the RNase (-) plates.

F+ RNA coliphage detection and genotyping by reverse line blot hybridization. F+ RNA coliphage strains were amplified by broadly-reactive RT-PCR of a partial region of the replicase gene of both levi- and allolevivirus using biotinylated primers as previously described (36). RT-PCR products were then further characterized by RLB hybridization assay using a panel of group- and subgroup-specific probes (36). F+ DNA coliphages were analyzed by PCR to confirm their presence in mixed isolates containing both F+ RNA and F+ DNA coliphages (36). Bound (RT)-PCR products on RLB hybridization membranes were detected by chemilumenescence on Biomax MS light X-ray film (Kodak, Rochester, NY) for 30 to 60 min exposures, followed by developing in a SRX-101A film processor (Konika, Wayne, NJ).

DNA sequencing and phylogenetic analysis. F+ RNA coliphage strains that generated RT-PCR products with appropriate size for leviviruses (266 bp) or allolevivirus (229 bp), but did not hybridize to any of the RLB probes were sequenced. F+ RNA RT-PCR products were purified using the QIAquick PCR Purification Kit (Qiagen, Valencia, CA) and sequenced at the Nucleic Acids Core Facility of UNC, Chapel Hill, NC. Sequences were edited and aligned using BioEdit (15) and imported into TreeCon (v 1.3b) (35). 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.

Results

RNase testing to identify F+ RNA and F+ DNA coliphages. The RNase spot-plate test was used to classify 1033 F+ coliphage isolates by their viral nucleic acid type as either RNA or DNA. The RNase test agreed with molecular typing in 919 of 921 (99.8%) F+ RNA isolates (Table 4.1). Of the two remaining coliphage isolates, one tested positive for F+ DNA and the other tested positive for both F+ RNA and F+ DNA using the spot plate test. Of the 112 coliphage isolates that were mixtures of F+ RNA and F+ DNA by molecular genotyping, only 20 (17.8%) tested positive for both F+ RNA and F+ DNA by the RNase assay, while 85 (75.9%) tested positive for F+ DNA and seven (6.3%) tested positive for F+ RNA by the RNase assay. Because F+ RNA coliphages were the major focus of the study, only isolates that were positive for both F+ RNA and F+ DNA using the spot plate RNase test were tested by F+ DNA PCR.

Evaluation of RT-PCR followed by RLB for F+ RNA coliphage typing. Of the 1033 F+ RNA 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 six different RLB genogroups or sub-groups: MS2 (Genogroup [GG] I); GA (GG II); M11 (GG III); Qβ (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 F+ RNA coliphage by phylogenetic analysis. RT-PCR - RLB was able to detect and confirm F+ RNA coliphages in all field isolates that also contained F+ DNA by the spot RNase test (Table 4.2). 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), Qβ (GG III), Sp (GG IV) or Fi (GG IV) probes, respectively (data not shown).

F+ RNA genotyping results using RLB hybridization. The majority (mean = 98.7% ± 1.5%) of the F+ RNA isolates from each estuary could be genotyped by RLB (Table 4.3). The average number of F+ RNA isolates assayed from an estuary was 114 and ranged from 44 to 182 among estuaries. All isolates collected at the estuaries in California (n=95), Massachusetts (n=71), and Florida (n=41) could be typed. At estuaries in Delaware and Rhode Island all but one isolate was confirmed and typed (Table 4.3).

Sequencing and Phylogenetic analysis of F+ RNA coliphages. A partial region of the replicase gene of both RLB typeable (n=5) and untypeable (n=18) F+ RNA 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 GG I strains and 12 GG II strains. GG I strains shown in Fig. 4.2a. clustered into two 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 separate estuaries. RLB positive F+ RNA field strains were either identical to the 19 nt GG I RLB probe, or had a one nucleotide (nt) mismatch that produced a weakly positive hybridization signal (Fig. 4.2b). Two or more nt mismatches between the 19 nt GG I probe and F+ RNA GG I targets resulted in a lack of RLB detection, as was seen with strains in the GG I, JS-subgroup (Fig. 4.2b).

Discussion

This study evaluated the RT-PCR – RLB coliphage detection and typing method in its largest field trial to date, in order to establish its robustness and performance in concurrent microbial source tracking and ecology studies at nine geographically representative US estuaries. Of 1033 F+ RNA field isolates tested, 99.9% gave confirmed positive RT-PCR products, and 98.3% were genotyped by RLB into GGs used to track human (GG II and III) and animal (GG I and IV) fecal sources. The observed robustness of RT-PCR – RLB suggests broad applicability of this method to detect and genotype diverse field strains of F+ RNA coliphages for microbial source tracking in all coastal regions of the US.

The RNase test was used in this study as a screening method to rapidly separate F+ RNA from F+ DNA viruses. In our evaluation, the RNase test performed reasonably

well for F+ RNA isolates (99.8%), but the assay was unable to detect F+ RNA phages in 76% of mixed samples also containing F+ DNA coliphages. Hence, the assay was biased towards F+ DNA phage detection. A likely reason for this bias is that F+ DNA coliphages enrich to two to three $\log_{10}$ higher titers than F+ RNA coliphages (Love unpublished data), and on agar medium-host lawn plates the more numerous F+ DNA coliphage plaques obscure the observation of F+ RNA plaques. For more reliable nucleic acid screening, parallel DNase and RNase treatments are recommended. Alternatively, a F+ RNA-specific bacterial host could perhaps be made, or F+ DNA coliphage neutralizing antisera could be used to block F+ DNA infectivity initiated by adsorption to the host F-pilus tip, while allowing F+ RNA infection along the length of the F-pilus (24).

The F+ RNA coliphages isolates from the 9 geographically diverse US estuaries were detected using a recently described, broadly reactive duplex RT-PCR assay based on degenerate primers targeting both levi- and allolevivirus (36). In the previous study in which the duplex RT-PCR assay was developed, it detected and genotyped 100% of 107 F+ RNA field strains tested (36). Our goal was to further evaluate this assay on a ten-fold larger, temporally and spatially diverse panel F+ RNA field strains for which there existed no a-priori geno- or serotyping data. The sensitivity of the duplex RT-PCR assay was 99.9%, which agrees with previous findings and further documents the method as robust and effective for microbial source tracking studies. Only one F+ RNA coliphage isolate could not be amplified by RT-PCR, even though it titered at about 10^6 PFU/ml, contained RNA, and was neutralized by antisera against MS2 (GG I) and GA (GG II) in the levivirus genus.

The RLB system is a high throughput and cost-effective method, typing 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 (36), respiratory viruses from nasopharyngeal aspirates (6), and antibiotic resistance genes of *Streptococcus agalactiae* (38). In this study, RLB genotyped 98.4% of the F+ RNA coliphage isolates tested, which was a level of performance consistent with previous work (36). A dot-blot hybridization assay for F+ RNA coliphage was reported to have a somewhat lower sensitivity than this study, with 96.6% of F+ RNA coliphages genotyped (19). In dot blot hybridization, F+ coliphage plaques are lifted from agar plates onto four replicate membranes that are labeled with reusable oligonucleotide probe solutions for each of the four F+ RNA GGs and detected with a colorimetric, immunoenzymatic signal (3,19). In this study, RT-PCR and not agar medium culture on host lawns in plates was used for coliphage amplification, which allowed for nucleic acid sequencing of RT-PCR positive but RLB negative strains. Nucleic acid sequencing improved the genotyping rates to 99.9%, and was only performed when RLB hybridization gave inconclusive results.

Based on genotyping, 84.9% of field isolates in this study were F+ RNA GG I, which is similar proportions others have found in surface water (5). 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 (10,11,12). However, more uniform representation of F+ RNA groups or types would

have been preferred, especially for studying probe sensitivity of the under-represented RLB GGs III and IV.

The genotyping rate of RLB hybridization did not vary appreciably by the estuary from which F+ RNA coliphages were isolated, averaging 114 F+ RNA coliphage isolates for the nine geographically diverse estuaries studies. Overall, F+ RNA coliphage detection and genotyping with RT-PCR RLB was robust, sensitive, and reproducible in each estuary. These findings suggest that F+ RNA coliphage diversity can be addressed by RLB hybridization, thus making it useful in future studies of F+ RNA coliphage ecology.

Among the few RLB untypeable strains, GG I strains were more varied that GG II strains, with 60% sequence similarity among GG I and 80% sequence similarity among GG II (data not shown). GG I strains clustered into the MS2-subgroup and the JS-subgroup, the latter resulting in mismatches in the region targeted by the GG I and GG II probes. Including a new and unique probe on the RLB membrane to specifically detect strains within the JS cluster will improve the use of RLB for genotyping F+ RNA 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, F+ RNA coliphage strains are rarely sequenced, and thus their ecology and genetic diversity is not well understood beyond the serogroup or genogroup level of identification (5,11,19,20). In this study, sequencing and phylogenetic analysis of a partial region of the replicase gene confirmed the existence of additional F+ RNA genogroups or subgroups. This finding suggests that

more genogroups or subgroups may be found using sequencing methods instead of direct nucleic acid hybridization and serological methods.

The findings of this study also suggest that genetically similar strains of F+ RNA coliphages can be found in some but not other geographic regions. For example, a JS-subgroup field strain was detected 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 such phenomena as migratory birds (waterfowl) carrying these coliphages along Atlantic migration routes. Stewart and colleagues previously demonstrated that a cluster of related F+ RNA strains can exist at specific sites, such as hog farms in North and South Carolina (30). Sequencing these F+ RNA coliphages revealed distinct patterns among sequences from different swine waste lagoons, thereby facilitating source tracking of fecal waste (30). Further investigation into the ecology of F+ RNA coliphages is needed to better validate coliphage grouping for microbial source tracking and understand their diversity, host ranges, evolution and selection.

Although F+ RNA 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 (5,10,11,19,28), which obviates the need for expensive location-specific source tracking libraries. Library independent F+ RNA coliphage typing can be performed using a variety of molecular and serological methods.

This study shows that RT-PCR - RLB is an improvement over previous F+ RNA coliphage serotyping and genotyping methods, as demonstrated by its success when used in a large-scale coastal water and shellfish monitoring study. A rapid method for F+

RNA coliphage genotyping based on nucleic acid detection by realtime RT-PCR has been reported (22, 27). The RLB method of this study could be used to further confirm the GG identities of F+ RNA coliphages detected by that method, without having to resort to nucleic acid sequencing.

References

1. **Alderisio, K. A., Wait, D. A., and M. D. Sobsey.** 1996. Detection and characterization of male-specific RNA coliphages in a New York City reservoir., p. 133–142. *In* J. J.McDonnell, D. L. Leopold, J. B. Stribling, and L. R. Neville (ed.), Watershed restoration management New York City water supply studies. American Water Resources Association, Herndon, Virginia.

2. **Allwood, P. B., Malik, Y. S., Hedberg, C. W., and S. M. Goyal.** 2003. Survival of F-specific RNA coliphage, feline calicivirus, and *Escherichia coli* in water: a comparative study. Appl. Environ. Microbiol. **69(9):**5707-5710.

3. **Beekwilder, J., Nieuwenhuizen, R., Havelaar, A. H., and J. van Duin.** 1996. An oligonucleotide hybridization assay for the identification and enumeration of F-specific RNA phages in surface water. J. Appl. Bacteriol. **80(2):**179-186.

4. **Chung, H., Jaykus, L. A., Lovelace, G., and M. D. Sobsey**. 1998. Bacteriophages and bacteria as indicators of enteric viruses in oysters and their harvest waters. Water Sci. Technol. **38:**37-44.

5. **Cole, D., Long, S. D., and M. D. Sobsey**. 2003. Evaluation of F+ RNA and DNA coliphages as source-specific indicators of fecal contamination in surface waters. Appl. Environ. Microbiol. **69(11):**6507-6514.

6. **Coiras, M. T., López-Huertas , M. R., López-Campos, J. C., Aguilar, G., and P. Pérez-Breña.** 2005. Oligonucleotide array for simultaneous detection of respiratory viruses using a reverse-line blot hybridization assay. J. Virol. Methods. **76(2):**256-264.

7. **Dawson, D. J., Paish, A., Staffell, L. M., Seymour, I. J., and H. Appleton.** 2005. Survival of viruses on fresh produce, using MS2 as a surrogate for norovirus. J. Appl. Microbiol. **98(1):**203-209.

8. **d'Herelle, F.** 1926. The bacteriophage and its behavior. Williams and Wilkins, Baltimore.

9. **Endley, S., Lu, L., Vega, E., Hume, M. E., and S. D. Pillai.** 2003. Male-specific coliphages as an additional fecal contamination indicator for screening fresh carrots. J. Food Prot. **66:**88-93.

10. **Furuse, K., Aoi, T., Shiba, T., Sakurai, T. Miyake, T., and I. Watanabe.** 1973. Isolation and grouping of RNA phages. IV. A survey in Japan. J. Keio. Med. Soc. **50:**363-376.

11. **Furuse, K., Ando, A., Osawa, S., and I. Watanabe.** 1981. Distribution of ribonucleic acid coliphages in raw sewage from treatment plants in Japan. Appl. Environ. Microbiol. **41(5):**1139-1143.

12. **Furuse, K.** Distribution of coliphages in the environment. *In* S. M., Goyal, , C. P. Gerba, and G. Bitton (ed.) Phage Ecology. Wiley-Interscience. New York. 1987.

13. **Grabow, W. O. K**. 2001. Bacteriophages: update on application as models for viruses in water. Water SA. **27(2):**251-268.

14. **Griffin, D. W., Stokes, R., Rose, J. B., and J. H. Paul III.** 2000. Bacterial indicator occurrence and the use of an F+ specific RNA coliphage assay to identify fecal sources in Homosassa Springs, Florida. Microb. Ecol. **39:**56-64.

15. **Hall, T. A.** 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl. Acids. Symp. Ser. **41:**95-98.

16. **Havelaar, A. H., Pot-Hogeboom, W. M., Furuse, K., Pot, R., and M. P. Hormann.** 1990. F-specific RNA bacteriophages and sensitive host strains in faeces and wastewater of human and animal origin. J. Appl. Bacteriol. **69(1):**30-37.

17. **Havelaar, A. H., van Olphen, M., and Y. C. Drost.** 1993. F-specific RNA bacteriophages are adequate model organisms for enteric viruses in fresh water. Appl. Environ. Microbiol. **59:**2956-2962.

18. **Hershey, A. D.** 1946. Mutation of bacteriophage with respect to type of plaque. Genetics. 31, 620.

19. **Hsu, F.C., Shieh, Y.-S. C., van Duin, J., Beekwilder, M. J., and M. D. Sobsey.** 1995. Genotyping male-specific RNA coliphages by hybridization with oligonucleotide probes. Appl. Environ. Microbiol. **61(11):**3960-3966.

20. **Hsu, F. C., Shieh, Y. S., and M. D. Sobsey.** 2002. Enteric bacteriophages as potential fecal indicators in ground beef and poultry meat. J. Food Prot. **65:**93-99.

21. **Jiang, S., Noble, R., and W. Chu.** 2001. Human adenoviruses and coliphages in urban runoff-impacted coastal waters of Southern California. Appl. Environ. Microbiol. **67(1):**179-184.

22. **Kirs M., and D. C. Smith**. 2006. Multiplex quantitative real-time RT-PCR for the F+ specific RNA coliphages: a method for use in microbial source tracking. Appl. Environ. Microbiol. [In press].

23. **Luria, S. E.** 1945. Mutations of bacterial viruses affecting their host range. Genetics. **30**:84.

24. **Manchak, J., Anthony, G., and L. S. Frost.** 2002. Mutational analysis of F-pillin reveals domains for pilus assembly, phage infection and DNA transfer. Mol. Microbiol. **(43)1:**195-205.

25. **Meshke, J. S., and M. D. Sobsey.** 2003. Comparative reduction of Norwalk virus, poliovirus type 1, F+ RNA coliphage MS2 and Escherichia coli in miniature soil columns.Water Sci. Technol. **47(3):**85-90.

26. **O'Connell K. P., Bucher J. R., Anderson P. E., Cao C. J., Khan A. S., Gostomski M. V., J. J. Valdes.** 2006. Real-time fluorogenic reverse transcription-PCR assays for detection of bacteriophage MS2. Appl. Environ. Microbiol. **72(1):**478-483

27. **Ogorzaly, L. and C. Gantzer**. 2006. Development of real-time RT-PCR methods for specific detection of F-specific RNA bacteriophage genogroups: Application to urban raw wastewater. J. Virol. Meth. **138(1-2):**131-139.

28. **Osawa, S., Furuse, K., and I. Watanabe.** 1981. Distribution of ribonucleic acid coliphages in animals. Appl. Environ. Microbiol. **41(1):**164-168

29. **Sobsey, M. D., Schwab, K. J. , and T. R. Handzel**. 1990. A simple membrane filter method to concentrate and enumerate male- specific RNA coliphages. J. Amer. Water Works Assoc. **82(9):**52-59.

30. **Stewart, J. R., Vinjé, J., Oudejans, S. J., Scott, G. I., and M. D. Sobsey.** 2006. Sequence variation among group III F-specific RNA coliphages from water samples and swine lagoons. Appl. Environ. Microbiol. **72(2):**1126-1230.

31. **US EPA.** 2001a. Method 1601: male-specific (F+) and somatic coliphage in water by two-step enrichment procedure. EPA Number: 821-R-01-030, April, 2001, Washington, DC.
32. **US EPA.** 2001b. Method 1602: male-specific (F+) and somatic coliphage in water by single agar layer (SAL) procedure. EPA Number: 821-R-01-029, April, 2001, Washington, DC.

33. **US EPA** 2001c. Manual of methods for virology. Chapter 16. EPA 600/4-84/013 (N16).

34. **Van Regenmortel, M. H. V., Fauquet, C. M., Bishop, D. H. L., Carstens, E. B., Estes, M. K., Lemon, S.M., McGeoch, D .J., Maniloff, J., Mayo, M. A., Pringle, C. R., and R. B. Wickner.** 2000. Virus taxonomy, classification and nomenclature of viruses. Seventh ICTV Report, Academic Press.

35. **Van de Peer, J., and R. De Wachter.** 1994. TREECON for Windows: a software package for the construction and drawing of evolutionary trees for the Microsoft Windows environment. Comput. Applic. Biosci. **10:**569–570.

36. **Vinjé, J., and M. P. G. Koopmans.** 2000. Simultaneous detection and genotyping of "norwalk-like viruses" by oligonucleotide array in a reverse line blot hybridization format. J. Clin. Microbiol. **38(7):**2595-2601.

37. **Vinjé J., Oudejans S. J., Stewart J. R., Sobsey M. D., and S. C. Long.** 2004. Molecular detection and genotyping of male-specific coliphages by reverse transcription-PCR and reverse line blot hybridization. Appl. Environ. Microbiol. **70(10):**5996-6004.

38. **Zeng, X., Kong, F., Wang, H., Darbar, A., and G. L. Gilbert.** 2006. Simultaneous detection of nine antibiotic resistance-related genes in *Streptococcus agalactiae* using multiplex PCR and reverse line blot hybridization assay. Antimicrob. Agents Chemother. **50(1):**204-209.

TABLE 4.1.Comparison of RNase test with (RT)-PCR for the typing of F+ coliphages.

F+ Coliphage isolates	RNase Test [a]			
	F+ RNA positive	F+ RNA & F+ DNA positive	F+ DNA positive	Total
F+ RNA [b]	919 (99.8%)	1 (0.1%)	1 (0.1%)	921 (100%)
F+ RNA & F+ DNA [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 reverse line blot (RLB) hybridization or sequencing for F+ RNA detection and confirmation.
[c] PCR RLB for F+ DNA detection and confirmation

TABLE 4.2. F+ RNA coliphages characterization by RT-PCR and reverse line blot hybridization.

F+ Coliphage isolates	Detection of F+ RNA Coliphages		Total no.
	RT-PCR confirmed positive [a]	No RT-PCR amplicon	
F+ RNA	920	1	921
F+ RNA & F+ DNA [b]	112	0	112
Total no. (% of total)	1032 (99.9%)	1 (0.1%)	1033

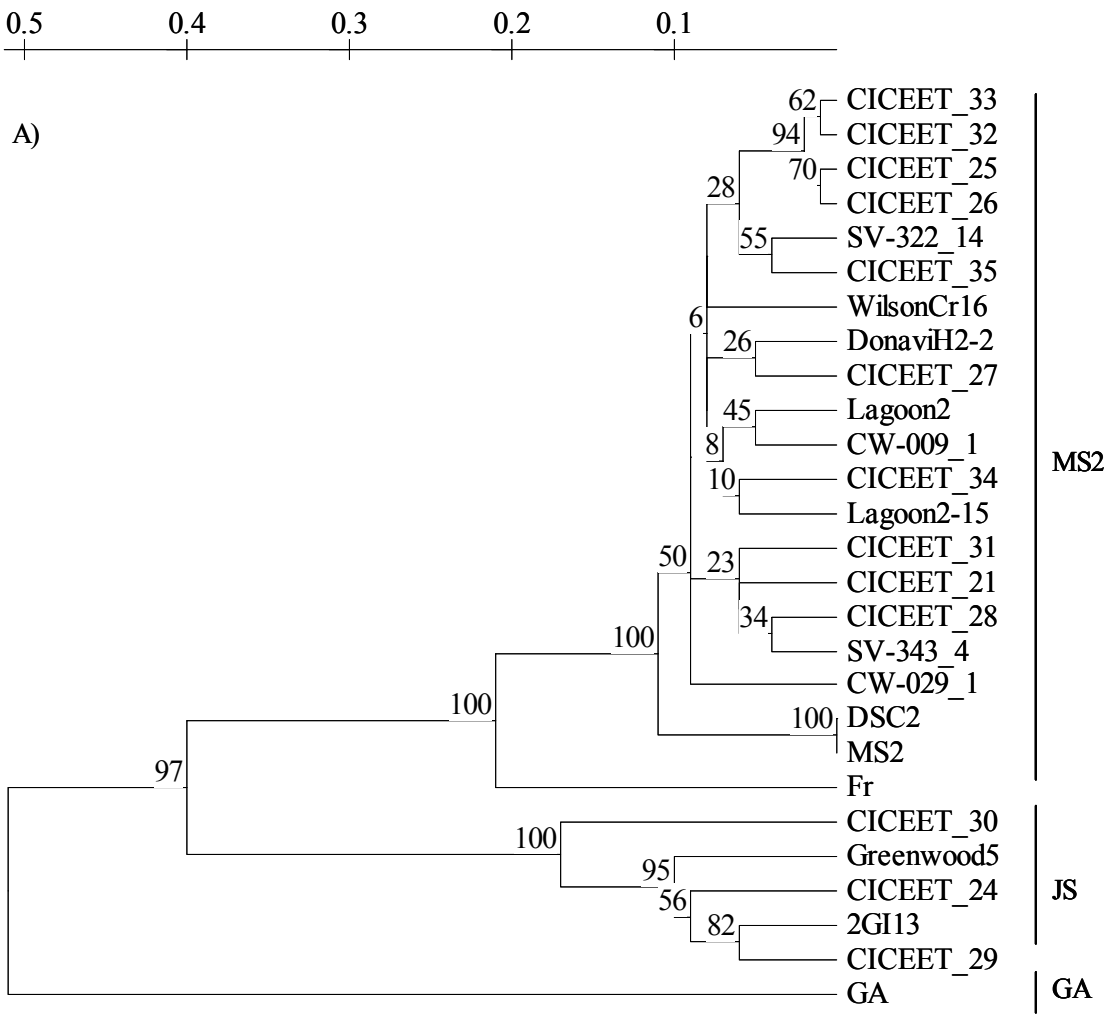
[a] RT-PCR confirmed positive by RLB or sequencing.
[b] F+ RNA and F+ DNA mixed samples

TABLE 4.3. RLB hybridization of F+ RNA coliphage from nine USA estuaries.

Estuary	Confirmed F+ RNA Coliphages		
	RLB positive (% positive)	RLB negative (% negative)	Total no.
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 (3.4%)	88
Waquoit Bay, MA	71 (100%)	0	71
Apalachicola Bay, FL	44 (100%)	0	44

1. Collect environmental samples of estuarine water and shellfish.
2. Recover F+ coliphage that infect and lyse *E. coli* F_{amp} hosts on agar plates using EPA methods 1601 and 1602 (31,32) or direct membrane filtration (29).
3. Isolate F+ coliphage by "picking" ten representative virus plaques.
4. Determine RNA or DNA nucleic acid content with RNase test (Table 1).
5. Perform duplex RT-PCR for F+ RNA coliphages in two genus (levivirus and allolevivirus).
6. Perform PCR for F+ DNA coliphages.
7. Ethidium bromide staining on 2% agarose gel for cDNA product visualization.
8. Genotype F+ RNA coliphage with RLB hybridization (37) (Table 2).
9. Nucleic acid sequence F+ RNA coliphage amplicons (Fig. 2).

FIG. 4.1. Flow diagram of sample processing for F+ RNA coliphage recovery, detection, and genotyping.



B) Group I probe 5′ GAGACGATAC GATGGGAAC 3′ RLB result

MS2	CGTAGATGGC	GAGACGATAC	GATGGGAAC	TATTTTCCACA	+
CICEET_30	- - - C - - C - - G	C - - - T T- - TG	AC - - - C - C -	- - - - C - - - - - T	-
CICEET_29	- - - T - - C - - A	CGTGTAG - 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.4.2. Phylogenetic tree (a) and sequence alignment (b) of group I F+ RNA 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 using Jukes and Cantor equations with 100 bootstrap values at tree nodes. Field strains from this study labeled as "CICEET_#."

5 Simple and Rapid F+ Coliphage Culture, Latex Agglutination, and Typing (CLAT) Assay to Detect and Source Track Fecal Contamination

Abstract

Simple, rapid and reliable fecal indicator tests are needed to better monitor and manage ambient waters and treated waters and wastes. Antibody-coated polymeric bead agglutination assays are potentially simple, rapid, specific, inexpensive, field-portable for non-lab settings, and their reagents can be stored at ambient temperatures for months. The goal of this study was to develop, optimize, and validate a rapid microbial water quality monitoring assay using F+ coliphage culture, latex agglutination and typing (CLAT) to detect F+ coliphage groups with antibody-coated particles. Rapid (180 minute) F+ coliphage culture was comparable to 16-24 hour culture time used in EPA Method 1601 and was amenable to CLAT detection. CLAT was performed on a cardboard card by mixing a drop of coliphage enrichment culture with a drop of antibody-coated polymeric beads as the detection reagent. Visual agglutination or clumping of positive samples occurred in <60 seconds. The CLAT assay had a sensitivity of 96.4% (185/192 samples) and 98.2% (161/164 samples), and a specificity of 100% (34/34 samples) and 97.7% (129/132 samples) for F+ RNA and DNA coliphages, respectively. CLAT successfully identified F+ RNA coliphages into

serogroups typically from human (groups II/III) and animal (groups I/IV) fecal sources, and in similar proportions as a nucleic acid hybridization assay. This novel group-specific antibody-based particle agglutination technique for rapid and simple detection and grouping of F+ coliphages provides a new and improved tool to monitor the microbiological quality of drinking, recreational, shellfishing, and other waters.

Introduction

Water quality is a global public health concern. In developing countries there is inadequate access to safe drinking water and its sources. Unsafe water, sanitation, and hygiene cause around 1.7 million deaths each year worldwide, mostly from infectious diarrhea in children of developing countries (55). Microbial pathogens causing gastrointestinal, dermal, and respiratory infections can be spread by drinking, bathing, or cleaning with water polluted with feces (56). In developed countries waterborne disease outbreaks and discrete disease cases continue to occur despite government regulations on wastewater and drinking water quality, treatment and monitoring-based warning systems for wastewater effluents, and recreational waters and shellfish growing waters (11, 29, 38). Fecal indicator microorganisms such as fecal coliforms, *E. coli*, and enterococci are used to measure the efficacy of water and wastewater treatment, drinking water quality and the sanitary quality of bathing and shellfishing waters (34). However, current microbial indicators are bacteria and many waterborne pathogens are enteric viruses for which bacterial indicators are inadequate or unreliable due to greater virus and bacteriophage resistance to water and waste water treatment processes (23, 28), and

greater virus and bacteriophage persistence in freshwater and seawater (10, 14, 33). Hence, there is a need for simple, reliable and rapid viral indicators and effective methods to detect and assay them.

United States (US) ambient water monitoring programs are just one example of the need for improved fecal indicator detection. Bacterial indictor assays used by regulators to monitor ambient water quality require 18- to 96-hours for results, which causes water quality decisions and warnings/advisories to be posted days after contamination events occur (34). Fecal pollution events in water are intermittent and often return to below threshold levels in 24 hours (5, 30). These same bacterial indicator assays cannot differentiate human and non-human fecal waste for tracking and controlling their sources, without extra and advanced steps, and they have a lack of predictability for enteric virus contamination (12). In 2005, regulators issued around 20,400 days of closures or advisories at US beaches and lakes due to exceedances of bacterial fecal indicators (35). About 75% of those 20,400 exceedances were caused by unknown sources of fecal pollution that could not be tracked, treated or managed (35).

Coliphages are alternatives to bacterial indicators. Coliphages are bacterial viruses that reside in the gut of animals, sometimes at titers similar to bacterial gut flora (1). Coliphages are obligate intracellular parasitic microorganisms that generally do not replicate in environments outside the gut, where host bacterial levels are $<10^4$ CFU/ml (50, 54), in nutrient poor environments that do not support host growth (54), and coliphage lysis only occurs in bacterial cultures undergoing exponential (logarithmic) growth (39). F+ coliphages infect the F pili of coliform bacteria, a pili that stop forming below 25°C (36, 53), further constraining the natural conditions needed for coliphage

replication. Coliphages are useful at indicating public health risks for water users and shellfish consumers because in some studies coliphages correlate with the presence of pathogenic human viruses in water and shellfish and the risks of viral illness (9, 12, 13, 27, 48). F+ coliphage can be divided into two families: *Leviviridae* containing RNA genomes (F+ RNA coliphage) or *Inoviridae* containing DNA genomes (F+ DNA coliphages) (46).

F+ RNA coliphages can be serotyped into distinct groups present in human fecal waste (groups II and III) or animal fecal waste (groups I and IV) (8, 17, 25). Microbial source tracking with F+ RNA coliphages has been used to identify and control human and animal sources of fecal pollution in surface waters (19, 44).

Current coliphage recovery and detection assays are as time consuming as culture-based bacterial indicator methods: taking one to three days for coliphage culture and plating methods (15, 16), one to two days for coliphage serotyping methods (25), or two days for molecular coliphage methods including reverse transcriptase PCR (RT-PCR) and probe-hybridization (47). The goals of this study were to develop a same-day microbial water quality monitoring assay using F+ coliphages and specifically to: (i) develop a simple and rapid assay to culture and detect F+ coliphages as fecal indicator microbes; (ii) distinguish between F+ RNA and F+ DNA coliphages; and (iii) concurrently sub-type F+ RNA coliphages into groups I-IV as microbial source tracking information to distinguish human or animal fecal origin.

This rapid coliphage detection assay is an antibody-based immunological approach commonly referred to as "latex agglutination," which was first performed in the mid-1950s by Singer and Plotz to detect rheumatoid arthritis (41). In this method,

particles are coated with antibodies (or antigens) and used to detect by visual means the binding and clumping of target antigens (or antibodies) with adjacent detector particles. Latex agglutination assays are generally rapid, simple, specific, and inexpensive, which makes them ideal for field or office diagnostic kits, such as those used to detect adenovirus and rotavirus in stool (18, 26), and the parasite *Leishmania* in urine (6). Agglutination tests are used in doctor offices, veterinarian offices, clinical diagnostic microbiology laboratories, other medical facilities, and virology laboratories to detect a number of different microbes, including *Herpes simplex virus* (22), *Tobacco mosaic virus* (45), *Staphylococcus aureus* (42), *Candida dublinienis* (32), antibodies against avian influenza virus subtype H5N1 (57), and antibodies against HIV (40). Unlike clinical samples with high titers of antigens, environmental samples usually have low levels of coliphage antigens which requires a culture step be used before coliphage detection by particle agglutination. This study describes the development and application of a rapid F+ coliphage enrichment culture and subsequent antibody-mediated particle agglutination test for rapid and simple recovery, detection, and grouping (typing) of F+ coliphages as a tool for monitoring the microbiological quality of drinking, recreational and shellfishing waters.

Materials and Methods

Virus strains, bacterial hosts, and environmental F+ coliphage isolates. F+ RNA prototype strains MS2 (serogroup I), GA (serogroup II), Qβ (serogroup III), M11 (serogroup III), SP (serogroup IV), and FI (serogroup IV), and F+ DNA prototype strains

Fd, F1, and M13 were used as positive controls. F+ coliphage field isolates were recovered from samples of shellfish tissue, water and bird feces at estuaries in Florida, North Carolina, Delaware, New Hampshire, Massachusetts, Rhode Island, and California by methods described previously (15, 16, 43) with permissive *E. coli* F_{amp} host (ATCC # 700891). F+ coliphage isolates were enriched under conditions described in EPA Method 1601 using a liquid broth culture to promote high phage titers. Enriched material was clarified by centrifugation at 1200 *xg* for 15 minutes, and the resulting supernatant was frozen at -80ºC in tryptic soy broth (TSB).

Rapid F+ coliphage culture. A rapid, two to three hour F+ coliphage culture enrichment was developed as a modified version of the 16-24 hour culture step of EPA Method 1601 (15). Rapid F+ coliphage culture conditions differed from EPA Method 1601 by using an optimized initial log-phase host concentration of 1 x 10^7 CFU *E. coli* F_{amp} per ml of culture and lasted two to three hours in a 35-37 ^{o}C water bath, at which time host bacteria entered stationary phase growth. Rapid F+ coliphage enrichments were compared for prototype F+ RNA coliphages (MS2, Qβ, SP, Fi) by inoculating 1-3 PFU into 333 ml broth cultures and tracking bacterial and coliphage levels at times throughout the culture period (zero, 30, 60, 90, 120, 180, and 360 minutes). F+ RNA coliphages were quantified on tryptic soy agar (TSA) spot plates containing host *E. coli* F_{amp} lawns, and *E. coli* were quantified before and after log-phase growth on TSA plates, and during log-phase growth by optical density at 520 nm in a spectrophotometer (Spectronic 1201; Milton Roy Company).

F+ Coliphage Enrichment Broths. Four nutrient broths were used for F+ coliphage enrichment: mineral salts with glucose (MSG), 0.5 x tryptic soy broth (TSB), 0.5x TSB supplemented with Instant Ocean (Aquarium Systems, Mentor, OH) to salinities of 11 parts per thousand (ppt), 23 ppt, and 35 ppt, and Colilert medium (IDEXX, Westbrook, ME). MSG medium contains 5.7 grams of Sodium Phosphate dibasic anhydrous (Na_2HPO_4), 1.5 grams of Potassium Phosphate monobasic (H_2PO_4), 0.5 grams of Sodium Chloride (NaCl), and 1 gram of Ammonium Chloride (NH_4Cl) per 1 liter of deionized water. Separately, a 50% wt/vol glucose solution is prepared and filter sterilized through a 0.22 μm filter and added to the autoclaved MSG media for a final glucose concentration of 0.2% wt/vol.

Spot Plate Method. The spot plate method was used to detect and enumerate F+ coliphage as described previously (15,58). Five replicate 0.01 ml spots of liquid from coliphage enriched samples or serial tenfold dilutions thereof were placed onto spot plates (150x15 mm petri dish) using a pipetman (P20, Gilson Co, France) for a total assay volume of 0.05 ml and a lower detection limit of 20 PFU/ml per sample. Positive and negative controls were included on each spot plate. Spot plates were air dried in a laminar flow hood for 30 minutes, then incubated inverted at 35 ºC for 8-14 hours. Spots were scored as positive/negative for lysis when using most probable number analysis, or their plaques were enumerated within the 0.01 ml spots, or dilutions there-of, for coliphage titers.

RNase test for detecting F+ RNA and F+ DNA coliphage. F+ coliphage field isolates were re-plated with and without RNase (Ribonuclease A, Sigma-Aldrich, St. Louis, MO) to distinguish viral nucleic acid content as DNA or RNA (25). RNase infectivity neutralization tests were performed on spot plates of 0.75% tryptic soy agar (TSA) containing log-phase *E. coli* F_{amp} host, streptomycin and ampicillin (each 15 µg/ml), and RNase (100 µg/ml).

F+ coliphage genogrouping. F+ coliphage isolates were also subjected to molecular typing to distinguish the four groups of F+ RNA coliphages (Groups I, II, III, and IV) by reverse transcription polymerase chain reaction (RT-PCR) of the replicase gene and F+ DNA coliphage analysis by PCR (47). Reverse line blot hybridization (RLB) assay was performed as previously described (47) to confirm (RT)-PCR amplified products. A new RT-PCR assay was developed to amplify the levivirus capsid region using DL10 and DL11 primers at 0.8 µM (Table 5.1). Capsid region amplification used reaction conditions previously reported (47), with the modification of RT and annealing steps increased to a temperature of 50ºC. F+ RNA and F+ DNA coliphage field isolates were also genogrouped by nucleic acid sequencing (UNC Nucleic Acids Core Facility, Chapel Hill, NC). Sequences were aligned using freeware software (Bioedit, Chromas lite v 2.0) (20) and phylogenetic trees were created using Jukes and Cantor distance estimation and 100 bootstrap values (TreeCon v 1.3b).

Rabbit antiserum production and collection. To generate polyclonal antibodies against F+ coliphages, New Zealand white rabbits were given intradermal inoculations with each

F+ RNA coliphage group (I = MS2, II = GA, III = Qβ, and IV = SP and Fi) and F+ DNA coliphages (Fd, F1, M13, Φ15, Φ16, Φ18). Initial virus inocula and a one-month booster were at titers of 10^{10}-10^{11} PFU/ml and had been partially purified and suspended in Freund's complete adjuvant (7). Antisera were collected from rabbits at 30, 45, 60, and/or 90 days post-immunization to obtain polyclonal rabbit immunoglobulins against coliphage antigens and stored at -20°C. No purification was performed to separate IgG or other Ig classes or other serum constituents. Anti-MS2 serum had a protein concentration of about 3 mg/ml, while other serum protein levels were not measured.

Antiserum labeling onto agglutinable particles. For the F+ coliphage latex agglutination and typing (CLAT) assay, polystyrene particles were first labeled with F+ coliphage antisera. A 1% suspension of 0.29 μm diameter polystyrene particles (OptiBind® particles; Seradyn Inc., Indianapolis, IN) was made from the commercial 10% stock solution of particles by diluting in either phosphate buffered saline (PBS) (0.136 M sodium chloride; 2.68 mM potassium chloride; 0.88 mM potassium phosphate monobasic; 3.4 mM sodium phosphate dibasic, pH 7.2 and 8.2) or citrate phosphate (CP) buffer (1.36 mM citric acid, 7.28 mM dibasic sodium phosphate, pH 6.2). Rabbit antisera against F+ coliphages or PBS (negative control) was added in equal volume as the polystyrene particles to the 1% polystyrene particle-buffer solution. The antibody-particle-buffer mixtures were agitated by pipeting up-and-down for several seconds (not vortexing) and then rocking at 150 rpm on a rotary platform (Orbit Shaker; Lab-Line Instruments; Melrose Park IL) for one hour at room temperature to facilitate hydrophobic adsorption of antibodies onto particles. Samples were then microcentrifuged at 14,000

rpm (Eppendorf Centrifuge 5415C; Brinkman Instruments; Westbury, NY) for five minutes, and the unbound antiserum in the supernatant was decanted from the antiserum-labeled particles in the pellet. The pellet was resuspended by pipeting (not vortexing) to give a 1% particle solution in either PBS-0.01% Bovine Serum Albumin (BSA; Sigma-Aldrich; St. Louis, MO) (pH 7.2 or 8.2) or CP-0.01% BSA (pH 6.2), to match the original buffer. BSA was use to block unbound particle binding sites and create a more stable solution for long-term storage. Labeled particles were stored at 4ºC or used directly. Five F+ RNA antiserum-labeled particle suspensions (anti-MS2, anti-GA, anti-QB, anti-SP, anti-Fi), and 6 F+ DNA antiserum-labeled particle suspensions (anti-Fd, anti-F1, anti-M13, anti-Φ15, anti-Φ16, anti-Φ18) were prepared.

F+ coliphage agglutination assay and optimization. Equal 2.5 µl volumes of antibody-labeled particles and coliphage enrichment cultures (or controls) were mixed on a black cardboard card (Agglutination Cards; Pro-Lab Diagnostics; Austin, TX) with a toothpick, and then rocked by hand for 30 seconds. Coliphage positive samples showed agglutination within 30-60 seconds as visualized by the naked eye for particles clumping together due to antibodies on different particles binding coliphages (Fig. 5.1). Negative samples where no coliphages were detected appear as a cloudy or "milky" liquid suspension of particles with no visible clumping.

To determine the appropriate types and concentrations of antisera for F+ coliphage typing and detection, a diverse panel of 32 F+ RNA and F+ DNA coliphage field isolates (confirmed by nucleic acid sequencing of the replicase gene of F+ RNA and gene IV of F+ DNA coliphages [47]), prototype strains (F+ RNA: MS2, GA, Qβ, SP, Fi;

F+ DNA: F1, Fd, M13), and negative controls (unlabeled particles; bacterial host cultures in TSB) was tested. Optimization experiments used a "checkerboard" titration system having combinations of varying amounts of antigen and antisera (serial 2-fold dilution of antisera from 1:4 to 1:128) per sample. The lower detection limit of the CLAT was determined using half-log dilution of prototype strains of F+ RNA and F+ DNA coliphage.

Protein assay for antisera. A protein detection assay (BCA Protein Assay Kit; Pierce; Rockford, IL) was used according to manufacturer's instructions to determine the levels of antisera adsorbed to polystyrene particles and in stocks of antisera. Briefly, the absorbance at 562 nm (spectrophotometer) of an albumin standard curve was generated, confirmed to have an R-squared value of >99%, and then compared to unknown samples. The amount of antisera labeled onto particles was taken to be the initial amount of protein added to particles minus the amount of unbound protein in the supernatant after centrifugation.

Statistical methods. Proportions of coliphage detected by CLAT and RLB were compared using a two-sided Z-test with a pre-set significance level of $\alpha = 0.05$ and p values reported. The Kruskal-Wallis Test, a nonparametric ANOVA, and Dunn's Multiple Comparisons Test were used to compare more than two variables, including different buffers for antibody binding efficiency and antibody dilutions. Statistics were calculated in Excel and InStat (v 3.06, GraphPad Software Inc.).

Results

Comparison of F+ Coliphage Enrichment Culture Broths. Three enrichment culture media (0.5x TSB, MSG, and Colilert) were compared for their ability to support rapid and sustained coliphage MS2 enrichment growth in log-phase *E. coli* F_{amp} host (Fig. 5.2). MS2 was added at initial levels of 1-5 PFU per sample, and after 180 minutes of enrichment at 37°C 0.5x TSB produced 5.5 x 10^8 PFU/ml progeny phage, which was 100-fold higher than MSG medium (5.3 x 10^6 PFU/ml) and 10,000-fold higher than Colilert medium (1.6 x 10^3 PFU/ml) (Fig. 5.2). The maximum viral titer in 0.5x TSB corresponds with the end of log-phase host growth at about 180 minutes (Fig. 5.2). In MSG medium, MS2 reached titers similar to 0.5x TSB media, but not within 180 minutes. Results for TSB and Colilert were based on three trials, while MSG results are based on only one trial, so more replicate experiments using MSG media are needed to better document MS2 growth kinetics (Fig. 5.2). Colilert medium was the least effective for rapid coliphage propagation and produced only 1.8 x 10^4 PFU/ml after 360 minutes of enrichment (Fig. 5.2). Subsequent enrichment experiments used 0.5 x TSB culture broth.

Optimizing Host Cell Levels for F+ Coliphage Enrichment. It was hypothesized that a more rapid coliphage enrichment could be achieved by increasing levels of host *E. coli* above the <10^5 CFU/ml suggested by EPA Method 1601. Low numbers of MS2 (1-5 PFU) were enriched at 37ºC in triplicate 1 ml volumes of 0.5 x TSB containing log-phase host levels of 7 x 10^5, 7 x 10^6, or 7 x 10^7 CFU/ml. The lowest starting titer (7 x 10^5 CFU/ml) produced about 100-fold to 1,000-fold less progeny phage after 45, 60, 75, and

120 minutes of incubation than did higher initial titers of host cells (Fig 5.2a). No significant difference was observed between starting host cell titers of 7 x 10^6 CFU/ml and 7 x 10^7 CFU/ml as seen in overlapping error bars of Fig 3a. MS2 infection (minute 15), eclipse (minute 30), and burst (minute 45) cycles are clearly visible during the enrichment process (Fig 5.3a). Higher host levels were further studied in larger volume enrichments to better simulate assay use for environmental water samples.

In larger 1/3 liter enrichments, initial log-phase titers of 1 x 10^6, 1 x 10^7, or 10^8 CFU/ml were compared in a single experiment for the rapid enrichment of about 3 PFU of MS2 (Figure 5.3b). Enrichment host levels starting at 1 x 10^7 CFU/ml produced 2.8 x 10^5 PFU/ml progeny MS2 in 90 minutes, 4.8 x 10^6 PFU/ml in 120 minutes, and 4.6 x 10^8 PFU/ml 180 minutes, which was greater than both lower and higher initial host levels. However, more replicate experiments are needed to better document phage yields and test for statistically significant differences among them (Figure 5.3b). Log-phase host levels starting at 10^8 CFU/ml reached stationary phase in about 120 minutes which facilitated MS2 enrichment in less time.

Rapid F+ coliphage culture. A modified version of EPA Method 1601 was used to rapidly enrich F+ RNA coliphage prototype strains in culture broths of host *E. coli* F_{amp} initially inoculated with 1-3 PFU of F+ RNA coliphages (MS2, Qβ, Sp, or Fi) and incubated at 35-37°C. Enrichment of these low levels of coliphage produced progeny coliphage at levels of 1.2 x 10^5 to 5.3 x 10^6 PFU/ml in 120 minutes and at levels of 4.3 x 10^6 to 5.5 x 10^8 PFU/ml in 180 minutes (Fig. 5.4). Rapid coliphage culture was achieved by increasing the concentration of log-phase *E. coli* F_{amp} host in broth cultures from about 10^4 CFU/ml in the overnight culture approach to as much as 10^7 CFU/ml in the new rapid

approach. *E. coli* F_{amp} reached stationary phase growth in 180 minutes with levels of 7.7 x 10^8 to 4.4 x 10^9 CFU/ml (Fig. 5.4).

Rapid Coliphage MS2 Enrichment in Simulated Marine and Estuarine Water. In applying the rapid coliphage enrichment to saline waters, it was hypothesized that the growth of *E. coli* F_{amp} would be an important rate limiting step. To explore this phenomenon, 0.5 x TSB was mixed with Instant Ocean (IO) to simulate water with 35 ppt (seawater), 23 ppt (estuarine water), 11 ppt salinity (brackish water), and a positive control of 0 ppt. As salinity increased, the rate of *E. coli* growth decreased and the enrichment of MS2 also decreased (Fig. 5a,b). Log-phase *E. coli* concentrations began at 4.6 x 10^6 CFU/ml and final stationary-phase concentrations ranged from 7.5 x 10^8 CFU/ml in 35 ppt water to 9.8 x 10^8 CFU/ml in 0 ppt water (data not shown). In 0 ppt and 11 ppt water samples, maximum or near-maximum MS2 enrichment occurred by 180 minutes, while enrichment in 23 ppt and 35 ppt samples took >180 minutes to reach maximum MS2 progeny levels (Fig. 5.5a).

Comparison of US EPA Method 1601 to a Modified Rapid Version of Method 1601 for F+ Coliphages. In an initial comparison, two 1-liter marine water samples and two 200 gram-pooled mussel samples from sites in Southern California were assayed for F+ coliphage by both overnight enrichment with US EPA Method 1601 and a modified version with 180-minute enrichment. F+ coliphage levels in samples were quantified using a 3-replicate by 6-dilution MPN assay. The 180-minute enrichment detected statistically similar levels of F+ coliphage in both mussel and water samples as the standard EPA Method 1601 with overnight enrichment as seen in overlapping error bars

in Fig 5.6. Mussels from Tijuana River, CA contained significantly more F+ coliphage than mussels from San Diego Bay, by both methods (Fig. 5.6).

Efficiency of adsorption of antisera onto polystyrene particles. Because adsorption of immunoglobulins varies with the isoelectric points of the antibodies in the sera, the pH of the adsorption buffer and electrolyte content were varied by employing three buffer pH levels, 6.2, 7.2, and 8.2, at 6 antisera dilutions (1:4 to 1:128) to examine their effects on anti-MS2 sera binding onto polystyrene particles. Binding of antisera to polystyrene particles was measured by a spectrophotometric protein detection assay. The saturation point for polystyrene particles with anti-MS2 sera was the 1:32 dilution, with a decreased binding efficiency both above and below this saturation point (Table 5.2). The highest binding efficiencies were in PBS at pH 7.2 with 106% ± 1% and 100% ± 2% binding of antisera at 1:64 and 1:32 antiserum dilutions, respectively (Table 5.2). PBS at pH 7.2 was significantly better than CP buffer at pH 6.2 or PBS at pH 8.2 at promoting adsorption of anti-MS2 sera to particles, and significant differences were seen among the three pH buffers at antiserum dilutions of 1:16 (p value = 0.0265), 1:32 (p value = 0.0036), and 1:64 (p value = 0.0379) (Table 5.2). Subsequent antiserum binding assays used PBS at pH 7.2 as the optimized adsorption buffer.

Optimizing particle agglutination by F+ coliphages. A series of experiments explored and optimized CLAT by varying types and concentration of antisera in "checkerboard" assays, based on true and false positive and true and false negative agglutination with a diverse panel of 32 nucleic acid sequenced F+ coliphage field isolates, F+ coliphage

prototype strains, and negative controls. Optimal concentrations of antisera were selected to detect true positives in the F+ coliphage panel while minimizing non-specific agglutination and false positive reactions. As shown in Table 5.3, CLAT detected and typed F+ RNA prototype strains into each of 4 serogroups, and gave true negative results for other F+ RNA and F+ DNA prototype strains, with the exceptions of anti-Fi sera cross-reacting with F+ RNA strain Sp (Table 5.3). No agglutination occurred when CLAT was performed with negative controls of TSB alone or stationary phase *E. coli* cultures in TSB (Table 5.3). For F+ DNA coliphage detection, CLAT could detect all F+ DNA reference strains but could not serotype F+ DNA field strains. Anti-Fd, anti-F1, anti-M13, and anti-Φ16 sera reacted with the three F+ DNA prototype strains, while no F+ DNA antisera reacted with F+ RNA coliphages or negative controls (Table 5.3). Anti-M13 serum at the 1:8 dilution was the most reactive, and the only antiserum to detect as positive all 16 F+ DNA field strains (data not shown). Anti-Φ15 and anti-Φ18 sera gave only weakly positive agglutination at the 1:4 dilutions, and therefore were not pursued further.

Lower detection limit of CLAT for F+ coliphages. The detection limit of the CLAT was determined using F+ coliphage prototype strains cultured overnight by EPA method 1601. Samples were scored as positive or negative by the presence of agglutination. The detection limit of F+ RNA coliphages ranged from 5×10^3 to 1×10^5 PFU depending upon the antisera used (Table 5.4). The detection limit for F+ DNA coliphage was 1×10^6 to 5×10^6 PFU (Table 5.4). F+ coliphages were assayed in 5 µl volumes and by half-log dilutions starting with enrichment concentrations as PFU/ml of 5.3×10^8 for MS2, 1 x

10^8 for GA, 8.4 x 10^9 for Qβ, 1.4 x 10^{10} for Sp, 1.0 x 10^{10} for Fi, 3.3 x 10^{11} for M13, and 3.4 x 10^{11} for Fd.

Application of CLAT to serotype F+ RNA coliphage field isolates. A diverse panel of F+ RNA and DNA field isolate were recovered from shellfish and water at ten estuaries on the East, West, and Gulf Coasts of the US by lysis zone isolation and overnight re-enrichment culture by EPA method 1601. These coliphage isolates were assayed by both CLAT serotyping and (for F+ RNA coliphages) by Reverse Line Blot (RLB) hybridization genotyping (47). Of the 192 F+ RNA field isolated tested, CLAT correctly serotyped 185 and RLB correctly genotyped 177. CLAT and RLB typed the same number of group I isolates, but CLAT typed significantly more group II isolates than did RLB (p value = 0.006) (Table 5.5). RLB typed 15 more F+ RNA group III isolates and four more F+ RNA group IV isolates than did CLAT, which were statistically significant differences (p values < 0.0002). The false negative rates were 4% for CLAT and 8% for RLB, a statistically significant difference (p value < 0.0002). Both typing methods gave no false positive results when challenged with 34 known F+ DNA field isolates (Table 5.5). Because CLAT serogrouping and RLB hybridization genogrouping provided different results for a small percentage of isolates, these differences were further explored.

Capsid analysis of discordantly typed F+ RNA leviviruses. The observed inconsistencies between serogrouping and genogrouping results in 24 of 192 F+ RNA coliphage field strains from Table 5.5 were further analyzed for nucleotide sequence in

the capsid genomic region. The capsid regions of the 24 problematic F+ RNA coliphage field strains were RT-PCR amplified, sequenced, and arranged in a phylogenetic tree alongside the CLAT and RLB grouping results (Fig. 5.7). Capsid sequence analysis showed 19 isolates clustered with F+ RNA group I at 90% sequence similarity, of which 17 were classified by CLAT as serogroup I and II (Fig. 5.7). Five isolates clustered as F+ RNA group II with slightly less than 90% sequence similarity, and CLAT serogrouping was in agreement for all five of these isolates (Fig. 5.7).

Application of CLAT to detect and type F+ DNA coliphage field isolates. A diverse panel of 164 F+ DNA field isolates and 132 F+ RNA field isolates were recovered from shellfish and water at ten estuaries on the East, West, and Gulf Coasts of the US by lysis zone isolation and overnight re-enrichment culture by EPA method 1601. Subsequently these coliphage isolates were assayed by both the CLAT and RNase infectivity neutralization assay for F+ DNA coliphages. The RNase infectivity neutralization assay scored coliphages as having either RNA or DNA nucleic acids, and was used as a standard for comparing to CLAT results. The CLAT detected 161 of 164 F+ DNA coliphage field isolates (98%), which was not statistically different from the 164 detections (100%) of the RNase assay (P value = 0.82) (Table 5.6). The CLAT failed to detect three of 164 F+ DNA isolates and gave false positive detection of three of 132 F+ RNA field isolates (2%) (Table 5.6). The detection rate of F+ DNA coliphages with M13 antiserum-coated particles was 83% (data not shown), which was improved to 98% detection by including a second level screening of all negative samples with Fd antiserum particles.

Discussion

The development and evaluation of a simple, rapid, and inexpensive F+ coliphage culture, latex agglutination and typing (CLAT) method is a new and novel tool to monitor the microbiological quality of water and other environmental media in both the developing and developed world, and to identify and track human and animal fecal waste sources. The CLAT is a novel application of the agglutination immunoassay originally developed for use in clinical medicine diagnostics. While clinical diagnostic samples typically have high titers of antigens and do not require a culture step before agglutination assays, water and other environmental samples have low levels of antigens (in our case coliphages) and require a culture step or other antigen enrichment step before detection by particle agglutination.

Other investigators have determined the lowest host concentration needed for bacteriophages attachment and replication (50, 54), while in this study host concentrations were increased to determine the highest level of host useful for rapid coliphage culture. This approach employed a 120-180 minute culture step, by modifying EPA Method 1601, to rapidly enrich both F+ RNA and DNA coliphages to levels amenable to particle agglutination.

Of the media compared, 0.5 x TSB outperformed MSG and Colilert media for rapid F+ coliphage enrichment, which supports the use 0.5 x TSB in US EPA coliphage standard methods documents (15,16). MSG was included in these trials because it is a clear broth that was hypothesized to interfere less than TSB with a coliphage detection

step using spectrophotometry or fluorometry readings. After initial experiments, coliphage detection of fluorescent molecular beacon signals was not pursued making the color of the broth media of less importance. The ease-of-use of broth media was another factor that was considered. Colilert dry medium is commercially available in sterile, pre-packaged containers aliquoted for use with 100 ml water samples, which simplifies and standardizes media formulation. If rapid coliphage enrichment kits were produced, similar sterile, pre-packaged TSB dry media aliquoted for use with 1 liter water samples would be desirable.

For rapid coliphage enrichment, our hypothesis was that modifying EPA Method 1601 by increasing initial *E. coli* levels in enrichments would decrease the total coliphage culture time from 16-24 hours to 60-360 minutes. The optimized rapid coliphage enrichment had starting levels of about 10^7 CFU/ml *E. coli* and enrichment incubation lasting 180 minutes in a 35-37°C water bath. US EPA Method 1601 uses much less initial *E. coli* host, with the starting log-phase *E. coli* titer of $<10^5$ CFU/ml, which is achieved by adding log-phase *E. coli* cultures that have: i) reached an optical density of 0.2 to 0.5 by a spectrophotometer set to 540 nm; and ii) are added at 1:200 vol/vol to sample enrichments. This study showed that by using higher initial titers of host cells, enrichment times could be reduced to 180 minutes while still producing high levels (4.6×10^8 PFU/ml) of progeny coliphage.

When coliphage enrichments in waters of varying salinity were compared, the rate of *E. coli* growth and MS2 enrichment kinetics decreased as water salinity increased. At 180 minutes, MS2 coliphage titers in 11 ppt and 23 ppt water samples were 4.2×10^7 PFU/ml and 1.6×10^7 PFU/ml, respectively, which is greater than the empirically

determined 1 x 10^7 PFU/ml lower detection limit for MS2 in the CLAT assay. MS2 titers in 35 ppt water samples were 6.5 x 10^4 PFU/ml after 180 minutes and 1.1 x 10^6 PFU/ml after 300 minutes, both lower than the CLAT lower detection limit. These findings suggest that a concentration step may be necessary for detection of MS2 and possibly other F+ RNA coliphages from marine waters but not from less saline waters. This prediction can be tested by applying the CLAT to enriched seawater containing or inoculated with coliphages (e.g., from sewage). In the work presented herein for mock-seawater experiments the CLAT assay had yet to be developed and enrichments were scored by enumerating coliphage plaques using spot plate assays. Though, further work showed the rapid F+ coliphage culture enrichment can be assayed directly by CLAT with no plaque purification or centrifugation.

Slow *E. coli* growth rates in highly saline waters were likely caused by osmotic up-shock of the bacteria that react to increased salinity in sample cultures. In an initial attempt to overcome or counteract this effect, two common osmoprotectants, betaine and trehalose, were each supplemented in 0.5 x TSB/seawater at levels of 0.05 mM, 0.1 mM, 0.5 mM or 1.0 mM, and inoculated with E. coli. Upon culture at 37ºC, neither osmoprotectant improved bacterial growth above that found in the negative control with no added osmoprotectants (data not shown). Others have used betaine or trehalose as osmoprotectants in much higher salinity environments and did see beneficial effects for bacterial growth (59,60). Their findings seem promising for the use of osmoprotectants to improve bacterial growth in marine waters assayed by coliphage enrichment methods. In future work it is suggested that *E. coli* be exposed to betaine or trehalose before adding the bacteria to enrichments containing marine water. Such pre-treatment may confer

more salt tolerance than seen in this work where betaine or trehalose was added at the time of enrichment and not during initial *E. coli* culture.

Preliminary trials with water and mussels from San Diego Bay, CA and Tijuana River, CA showed that rapid F+ coliphage cultures gave equivalent results as EPA Method 1601 with overnight enrichment. These preliminary findings are promising, but additional trials should be done to get more results for matched-samples comparisons of the two methods using waters of various salinities (marine, brackish, and fresh waters) to determine if there are relative differences in performance and to better document the benefits of the new rapid coliphage enrichment.

The CLAT was developed with the long-term goal of field-portable application, which necessitated the use of simple and easy methods, robust but non-sterile techniques, and inexpensive and stable detection materials. The presence of host bacteria in enrichment cultures did not adversely affect the detectablity of F+ coliphages by the CLAT (data not shown). Although in this study CAT results were scored as positive or negative, quantification is possible by a most probable number culture enrichment where replicate volumes in dilution are scored as positive or negative. Coliphage-enriched water samples analyzed by CLAT detected and sub-typed prototype F+ RNA strains accurately into serogroups I, II, III, and IV, and did not react with F+ DNA prototype strains or controls. Sub-grouping F+ RNA coliphages is useful for microbial source tracking (17, 25, 19, 37), but is not used routinely because it is time-consuming, more expensive than bacteriological analysis, and requires scientific knowledge and technical skill. This study improves access to F+ RNA coliphage detection and source tracking by

making it simpler, as affordable as bacteriological analysis, rapid, and potentially field-portable.

In validation studies of the F+ RNA CLAT for serotyping a large panel of F+ coliphage field strains, CLAT sensitivity was 96.4% and specificity was 100%. These findings are similar to those of previous F+ RNA characterization studies, where serotyping classified 99.5% of isolates (25), genotyping by probe-hybridization classified 96.6% of isolates (25), and RT-PCR followed by probe-hybridization correctly classified 97.8% isolates (47). The CLAT had similar performance and typing ability as a RT-PCR - probe-hybridization assay (47), when compared using the same panel of F+ coliphage field strains. Hsu et al. (25) also compared genotyping and serotyping outcomes using a common isolate panel and arrived at similar grouping outcome performance as reported in this study.

The few inconsistencies found between serogrouping and genogrouping results were further investigated in this study by examining virus capsid genes to better interpret CLAT results. It was hypothesized that studying the virus capsid gene of these problematic F+ RNA strains would provide a robust genetic approach consistent with antisera binding to distinct capsid (antigen) epitopes, and might reconcile inconsistencies between genogrouping and serogrouping (2). A new RT-PCR assay targeting the levivirus capsid gene was created for this purpose, which itself may be a stand-alone method for F+ RNA coliphage source tracking. When amplified levivirus capsid genes were sequenced and phylogenetically grouped, the findings did not agree 100% with either RLB genogrouping or CLAT serogrouping findings, which indicates that there may be multiple reasons for typing differences. Previous F+ RNA serotyping and genotyping

inconsistencies were shown to result from a change in three amino acids of the coat protein— causing a F+ RNA group II strain to be serotyped as group I but 95% genetically similar to group II (2, 21). In our study, environmental F+ RNA isolates may be serological intermediaries between groups I and II by sharing surface proteins for antibody binding as a result of prior genetic cross-over, by recombination events (antigenic shifts), or by progressive mutations common to single-stranded RNA viruses that occur at rates of 10^{-3} to 10^{-4} per incorporated nucleotide (genetic drifts) (24). As well, the group-specific antiserum that was generated against a single prototype strain may not be representative of the diversity of strains in the environment. Further analysis of levivirus epitopes by nucleic acid and protein microarrays, and by monoclonal antibody screening may give better insights into reasons for these discrepancies and the robustness of serogroup predictions from CLAT.

The developed F+ DNA CLAT provides a simple, robust, rapid and affordable means to facilitate detection of all F+ coliphages regardless of whether or not F+ RNA coliphages are present. F+ DNA coliphages as fecal indicator viruses have been isolated from wastewater treatment plants, swine, gull, and cattle waste (8). They have been found in higher proportions than F+ RNA coliphages in surface waters impacted by humans and animals, during storm events than during background flows, in warmer waters (8), and in epidemiological-microbiological studies of illness risks from recreational use of water contaminated by non-point fecal sources (9). Efforts to sub-type F+ DNA coliphage have not been as successful as for F+ RNA coliphage. In the CLAT, six F+ DNA polyclonal antisera were cross-reactive among the F+ DNA strains tested and thus could not be used for sub-typing, as has been previously observed (31).

Attempts at F+ coliphage genotyping have shown three genetic clusters called M13, Fd, and CH, based on >5% nucleotide sequence diversity in a 190 nucleotide region of Inovirus gene IV (47). However, analysis of other Inovirus genes is needed to confirm these distinct gene clusters and determine if a confirmed grouping method can be established for reliable and practical F+ DNA fecal source tracking (47).

The CLAT detection limit was sufficiently low for both F+ RNA and DNA coliphages that they can be readily detected after enriching water samples for 2-3 hours. While the lower detection limit was lower for F+ RNA coliphages than for F+ DNA coliphages, this difference does not pose a problem for F+ DNA coliphage CLAT detection, because these coliphages enrich to two to three $\log_{10}$ higher levels than F+ RNA coliphages. In this study all but three of 164 F+ DNA field isolates were detected by the CLAT assay. No difference was observed in the speed or strength of agglutination for the small, icosahedral RNA coliphages compared to long, rod-shaped DNA coliphages tested (data not shown), suggesting that virus morphology has little influence on CLAT detection. Antisera concentration (dilution) had an important role in the sensitivity and specificity of the CLAT, but the concentrations of the specific immunoglobulin types responsible for coliphage agglutination were not determined. Characterization of the anti-coliphage immunoglobulin types, their concentrations and agglutination reactivates would be informative for the development of standard reagents for the CLAT. Further efforts to create and test a coliphage monoclonal antibody library also may improve the sensitivity, specificity, and availability of the CLAT assay, if the monoclonal antibodies were as amenable to and effective in agglutination assays as were the polyclonal sera tested.

While agglutination assays are available for viruses of plants and animals (4, 26, 40), we were unable to find evidence of their existence for bacteriophages or specifically coliphages. Other fecal indicator viruses such as *Bacteroides fragilis* phages, *Salmonella* phages, and somatic coliphages as well as phages in terrestrial and marine environments could possibly be detected by agglutination assays. Phages in aquatic and terrestrial environments are not well characterized because often less than 1% of their natural hosts are culturable, resulting in the 'great-plaque-count-anomaly' (49, 51). Agglutination assays can potentially detect bacteriophage strains that infect bacterial hosts but do not form plaques, thereby obviating or circumventing the need for conventional serotyping methods based on neutralization of virus infectivity. Marine bacteriophages grow to titers as high as 10^8 PFU/ml of seawater (52), a titer that may be compatible with direct agglutination detection for further characterization and better understanding of their occurrence and ecology. The success of this newly developed CLAT for F+ coliphages suggests that additional applications of this assay to other bacteriophages also may be possible and provide useful information about coliphage occurrence, ecology, properties and public health risks.

References

1. **Abedon, S.T.** 1990. The ecology of bacteriophage T4. Ph.D. thesis. The University of Arizona.

2. **Adhin, M. R., Hirashima, A., and J. Van Duin.** 1989. Nucleotide sequences from the ssRNA bacteriophages JP34 resolves the discrepancy between serological and biophysical classification. Virol. **170:**238-242.

3. **Alderisio, K. A., Wait, D. A., and M. D. Sobsey**. 1996. Detection and characterization of male-specific RNA coliphages in a New York City reservoir. p.133–142. *In* J. J. McDonnell, D. L. Leopold, J. B. Stribling and L. R. Neville (ed.) Watershed restoration management New York City water supply studies. American Water Resources Association, Herndon, Virginia.

4. **Bercks, R., and G. Querfurth**. 1971. The use of the latex test for the detection of distant serological relationships among plant viruses. J. Gen. Virol. **12:**25-32.

5. **Boehm, A. B., Grant, S. B., Kim, J. H., Mowbray, S. L., McGee, C. D., Clark, C. D., Foley, D. M., and D. E. Wellman.** 2002. Decadal and shorter period variability of surf zone water quality at Huntington Beach, California. Environ. Sci. Technol. **36(18)**:3885-3892.

6. **Chappuis, F., Rijal, S., Jha, U. K., Desjeux., P., Karki, B. M. S., Koirala, S., Loutan, L., and M. Boelaert.** 2006. Field validity, reproducibility and feasibility of diagnostic tests for visceral leishmaniasis in rural Nepal. Tropical Med. and Intnl. Health. **11(1)**:31-40.

7. **Chung, H.-M.** 1993. F-Specific Coliphages and their Serogroups, and Bacteroides Fragilis Phages as Indicators of Estuarine Water and Shellfish Quality. Ph.D. thesis. The University of North Carolina at Chapel Hill.

8. **Cole, D., Long, S. D. and M. D. Sobsey.** 2003. Evaluation of F+ RNA and DNA coliphages as source-specific indicators of fecal contamination in surface waters. Appl Environ Microbiol. **69(11):**6507-6514.

9. **Colford, J. M. Jr., Wade, T. J., Schiff, K. C., Wright, C. C., Griffith, J. G., Sandhu, S. K., Burns, S., Hayes, J., Sobsey, M., Lovelace, G., and S. B. Weisberg.** 2007. Water quality indicators and the risk of illness in non-point source impacted recreational waters. Epidemiology. (in press).

10. **Contreras-Coll, N., Lucena, F., Mooijman, K., Havelaar, A., Pierz, V., Boque, M., Gawler, A., Holler, C., Lambiri, M., Mirolo, G., Moreno, B., Niemi, M., Sommer, R., Valentin, B., Wiedenmann, A., Young, V., and J. Jofre.** 2002. Occurrence and levels of indicator bacteriophages in bathing waters throughout Europe. Water Res. **(20):**4963-4974.

11. **Craun, G. F., Calderon, R. L. and M. F. Craun.** 2005. Outbreaks associated with recreational water in the United States. Intnl. J. Environ. Health Res. **15(4):**243-262.

12. **Dore, W. J., Henshilwood, K., Lees, D. N.** 2000. Evaluation of F-specific RNA bacteriophage as a candidate human enteric virus indicator for bivalve molluscan shellfish. Appl. Environ. Microbiol. **66(4):**1280-1285.

13. **Dufour, A. P.** 1984. Health Effects Criteria for Fresh Recreational Waters. Cinncinnati, Ohio. EPA-600-1-84-004.

14. **Duran, A. E., Muniesa, M., Mendez, X., Valero, F., Lucena F., and J. Jofre.** 2002. Removal and inactivation of indicator bacteriophages in fresh waters. J Appl Microbiol. **92(2):**338-347

15. **EPA Method 1601**. 2001. Male-specific (F+) and somatic coliphage in water by two-step enrichment procedure. EPA-821-R-01-030, April, 2001, Washington, DC.

16. **EPA Method 1602.** 2001. Male-specific (F+) and somatic coliphage in water by single agar layer (SAL) procedure. EPA-821-R-01-029, April, 2001, Washington, DC.

17. **Furuse, K., Sakurai, T., Hirashima, A., Katsuki, M., Ando, A., and I. Watanabe** 1978. Distribution of ribonucleic acid coliphages in south and east Asia. Appl Environ Microbiol. **35(6):**995-1002.

18. **Grandien, M., Pettersson, C.-A., Svensson, L., and I. Uhnoo.** 1987. Latex agglutination test for adenovirus diagnosis in diarrheal disease. J. Med. Virol. **23:**311-316.

19. **Griffin, D. W., Stokes, R., Rose, J. B., and J. H. Paul III.** 2000. Bacterial indicator occurrence and the use of an F+ specific RNA coliphage assay to identify fecal sources in Homosassa Springs, Florida. Microb. Ecol. **39:**56-64.

20. **Hall, T.A.** 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl. Acids. Symp. Ser. **41:**95-98.

21. **Harigai, H., Furuse, K., Inokuchi, Y., and A. Hirashima.** 1986. Characterization of an intergroup serological mutant from group II RNA phage GA. Microbiol. Immunol. **30(12):**1247-1257.

22. **Halstead, D. C., Beckwith, D. G., Sautter, R. L., Plosika, L., and K. A. Schneck.** 1987. Evaluation of a rapid latex slide agglutination test for herpes simplex virus as a specimen screen and culture identification method. J. Clin. Microbiol. **25(5):**936-937.

23. **Harwood, V. J., Levine, A. D., Scott, T. M., Chivukula, V., Lukasik, J., Farrah, S. R. and J. B. Rose**. 2005. Validity of the indicator organism paradigm for pathogen reduction in reclaimed water and public health protection. Appl. Environ. Microbiol. **71(6):**3163-3170.

24. **Holland, J. J.** 2006. Transitions in understanding of RNA viruses: a historical perspective. Curr Top Microbiol Immunol. **299:**371-401.

25. **Hsu, F. C., Y. S. Shieh, J. van Duin, M. J. Beekwilder, and M. D. Sobsey.** 1995.Genotyping male-specific RNA coliphages by hybridization with oligonucleotide probes. Appl Environ Microbiol **61:**3960-3966.

26. **Hughes, J. H., Tuomari, A. V., Mann, D. R., and V. V. Hamparian.** 1984. Latex immunoassay for rapid detection of rotavirus. J. Clin. Microbiol. **20(3):**441-447.

27. **Jiang S., Noble, R. and W. Chu.** 2001. Human adenoviruses and coliphages in urban runoff-impacted coastal waters of southern California. Appl. Environ. Microbiol.**67(1):**179-184.

28. **Jofre, J., Olle, E., Ribas, F., Vidal, A., and F. Lucena.**1995. Potential usefulness of bacteriophages that infect Bacteroides fragilis as model organisms for monitoring virus removal in drinking water treatment plants. Appl Environ. Microbiol. **61(9):**3227-31

29. **Koopmans, M.** 2005. Outbreaks of viral gastroenteritis: what's new in 2004? Curr. Opin. Infect. Dis. **18(4):**295-299.

30. **Leecaster, M. K., and S. B. Weisberg.** 2001. Effect of sampling frequency on shoreline microbiology assessments. Mar. Pollut. Bull. **42(11):**1150-1154.

31. **Long, S. C., El-Khoury, S. S., Oudejans, S., Sobsey M. D., and J. Vinjé**. 2005. Assessment of Sources and Diversity of Male-Specific Coliphages for Source Tracking. Environ. Enginer. Sci. **22(3):**367-377.

32. **Marot-Leblond, A., Beucher, B., David, S., Nail-Billaud, S., and R. Robert.** 2006. Development and evaluation of a rapid latex agglutination test using a monoclonal antibody to identify *Candida dubliniensis* colonies. J. Clin. Microbiol. **44(1):**138-142.

33. **Moce-Llivina, L., Lucena, F., and J. Jofre.** 2005. Enteroviruses and bacteriophages in bathing waters. Appl Environ Microbiol.**71(11):**6838-6844.

34. **National Research Council of the National Academies**. 2004. Indicators of waterborne pathogens. National Research Council. The National Academies Press. Washington D.C.

35. **National Resources Defense Council**. 2006. Testing the waters 2006: a guide to water quality at vacation beaches. [Online.] http://www.nrdc.org/water/oceans/ttw/titinx.asp

36. **Novotnym C, P**. **and K. Lavin.** 1971. Some effects of temperature on the growth of F pili. J. Bacteriol. **107:**671-682**.**

37. **Osawa, S., Furuse, K., and I. Watanabe.** 1981. Distribution of ribonucleic acid coliphages in animals. Appl Environ Microbiol. **41(1):**164-168.

38. **Poullis, D. A., Attwell, R. W., and S. C. Powell.** 2005. The characterization of waterborne-disease outbreaks. Rev. Environ. Health. **20(2):**141-149.

39. **Ricciuti, C. P.,** 1972. Host-virus interactions in *Escherichia coli*: effects of stationary phase on viral release from MS2-infected bacteria. J. Virol. **10(1):**162-165.

40. **Riggin, C. H., Beltz, G. A., Hung, C. H., Thorn, R. M. and D. J. Marciani.** 1987. Detection of antibodies to human immunodeficiency virus by latex agglutination with recombinant antigen. J Clin Microbiol. **25(9):**1772–1773.

41. **Singer J .M. and C. M. Plotz.** 1956. The latex fixation test. I. Application to the serologic diagnosis of rheumatoid arthritis. Am. J. Med. **21**:888-892.

42. **Smole, S. C., Aronson, E., Durbin, A., Brecher, S. M., and R. D. Arbeit.** 1998. Sensitivity and specificity of an improved rapid latex agglutination test for identification of methicillin-sensitive and –resistant *Staphylococcus aureus* isolates. J. Clin. Microbiol. **36(4):**1109-1112.

43. **Sobsey, M. D., Schwab, K. J., and T. R. Hanzel.** 1990. A simple membrane filter method to concentrate and enumerate male-specific RNA coliphages. J. Am. Water Works Assoc. **82:**52-59.

44. **Stewart-Pullaro, J., Daugomah, J. W., Chestnut, D. E., Graves, D. A., Sobsey, M. D., and G. I. Scott.** 2006. FRNA coliphage typing for microbial source tracking in surface waters. J. Appl Microbiol. **101(5):**1015-1026.

45. **Tsuda, S., Lameya-Iwaki, M., Hanada, K., Kouda, Y., Nikata, M. and K. Tomaru** 1992. A novel detection and identification technique for plant viruses: Rapid immunofilter paper assay (RIPA). Plant Disease. **76(5):**466-469.

46. **Van Regenmortel, M. H. V., Fauquet, C. M., Bishop, D. H. L., Carstens, E. B., Estes, M. K., Lemon, S. M., McGeoch, D. J., Maniloff, J., Mayo, M. A., Pringle, C. R., and R. B. Wickner.** 2000. Virus taxonomy, classification and nomenclature of viruses. Seventh ICTV Report, Academic Press.

47. **Vinjé J., Oudejans S. J., Stewart, J. R., Sobsey, M. D., and S. C. Long.** 2004. Molecular detection and genotyping of male-specific coliphages by reverse transcription-PCR and reverse line blot hybridization. Appl. Environ. Microbiol. **70(10):**5996-6004.

48. **Wade, T. J., Pai, N., Eisenberg, J. N. S., and J. M. Colford Jr.** 2003. Do U.S. environmental protection agency water quality guidelines for recreational waters prevent gastrointestinal illness? a systematic review and meta-analysis. Environ Health Perspect. **111(8):**1102-1109

.

49. **Weinbauer, M. G.** 2004. Ecology of prokaryotic viruses. FEMS Microbiol. Rev.**28:**127-181.

50. **Wiggins, B. A., and M. Alexander.** 1985. Minimum bacterial density for bacteriophage replication: implications for significance of bacteriophages in natural ecosystems. Appl. Environ. Microbiol. **49(1)**:19-23.

51. **Williamson, K. E., Radosevich, M., and K. E. Wommack.** 2005. Abundance and diversity of viruses in six Delaware soils. Appl. Environ. Microbiol. **71(6):**3119-3125.

52. **Wommack, K. E. and R. R. Colwell.** 2000. Virioplankton: viruses in aquaticecosystems. Microbiol. Mol. Biol. Rev. **64(1)**:69-114.

53. **Woody, M. A., and D. O. Cliver.** 1995. Effects of temperature and host cell growth phase on replication of F-specific RNA coliphage Qβ. Appl. Environ. Microbiol. **61(4):**1520-1526.

54. **Woody, M. A., and D. O. Cliver.** 1997. Replication of coliphage Qβ as affected by host cell number, nutrition, competition from insusceptible cells and non-FRNA coliphages. J. Appl. Microbiol. **82:**431-440.

55. **World Health Organization.** 2002. World Health Report 2002: reducing risks, promoting healthy life. Geneva, Switzerland. [Online.] http://www.who.int/whr/2002/en/whr02_en.pdf

56. **World Health Organization.** 2004. Guidelines for drinking-water quality, Vol 1, 3rd Ed. Geneva, Switzerland. P.121-144. [Online.] http://www.who.int/water_sanitation_health/dwq/gdwq3/en/

57. **Xu, X., Jin, M., Yu, Z., Li, H., Qiu, D., Tan, Y., and H. Chen.** 2005. Latex agglutination tests for monitoring antibodies to avian influenza virus subtype H5N1. J. Clin. Microbiol. **43(4):**1953-1955.

58. **Josephs, N. K., Couliette, A. D., Nappier, S. P., van Belle, G., Meschke, J. S., and M. D. Sobsey.** 2004. Development of a spot-titer culture assay for quantifying bacteria and viral indicators. Poster Presentation, 104th General Meeting of the American Society for Microbiology, New Orleans, LA., May 23-27 2004.

59. **Lucht, J. M. and E. Bremer.** 1994. Adaptation of Escherichia coli to high osmolarity environments: osmoregulation of the high-affinity glycine betaine transport system proU. FEMS Microbiol Rev. **14(1):**3-20.

60. **Strom, A. R., and I. Kaasen.** 1993. Trehalose metabolism in Escherichia coli: stress protection and stress regulation of gene expression. Mol Microbiol. **8(2):**205-10.

TABLE 5.1. Oligonucleotide primers for amplification of levivirus capsid region.

Oligonucleotide	Sequence (5'-3') [a]	Orien-tation	T_m [b] (ºC)	Location (based on MS2; NCC001417)
DL10	GTC GAY AAT GGC GGW AC	+	52	1365-1381
DL11	ATC GCG AGK RHG ATC HAT AC	-	53.3	1795-1814

[a] IUPAC codes for degenerate positions.
[b] Melting Temperature.

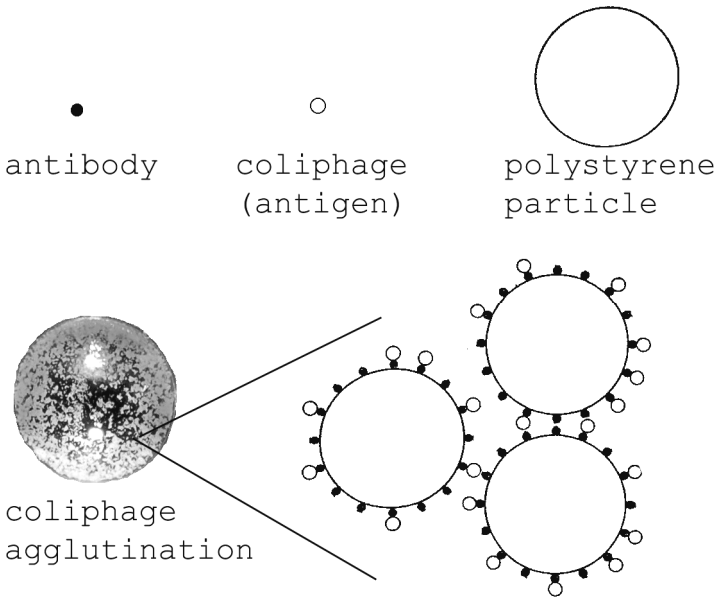


FIG. 5.1. Coliphage agglutination diagram. Coliphage agglutination visualized after mixing for 30 seconds equal volumes of coliphage enrichments with antibody-labeled polystyrene particles. Modified from Bercks and Querfurth (4).

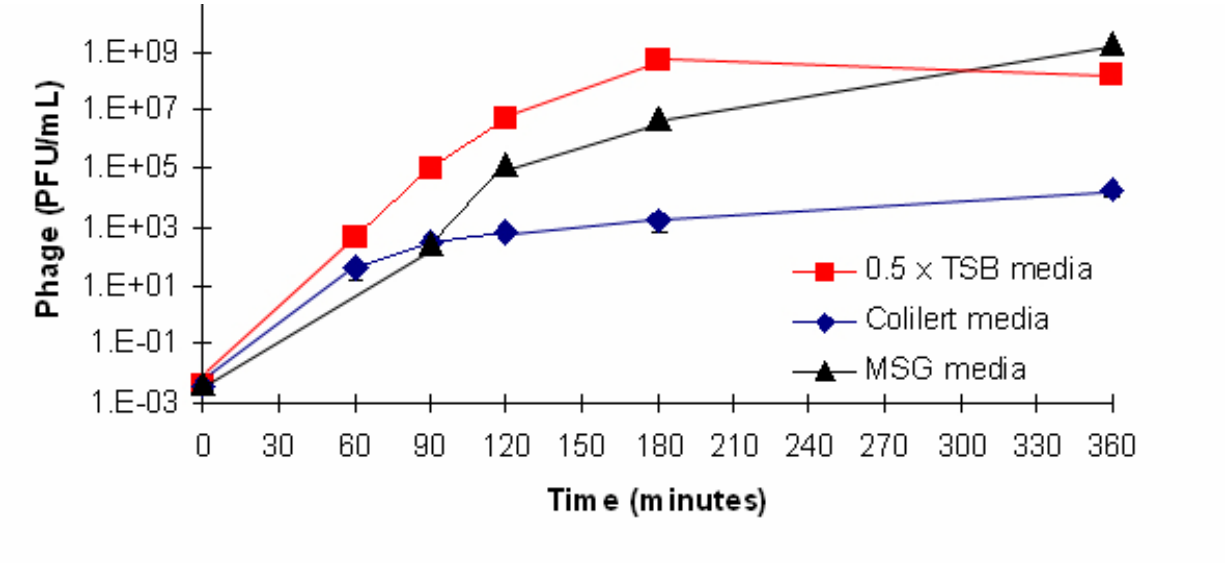


FIG 5.2. Coliphage MS2 enrichment in three enrichment broths with host *E. coli* F_{amp}. MSG = mineral salts with glucose media; TSB = tryptic soy broth. Data points for TSB (squares) and Colilert (diamonds) are three replicates with error bars (± standard deviation) that are sometimes smaller than data symbols. MSG (triangles) is based on values of one experiment and has no error bars.

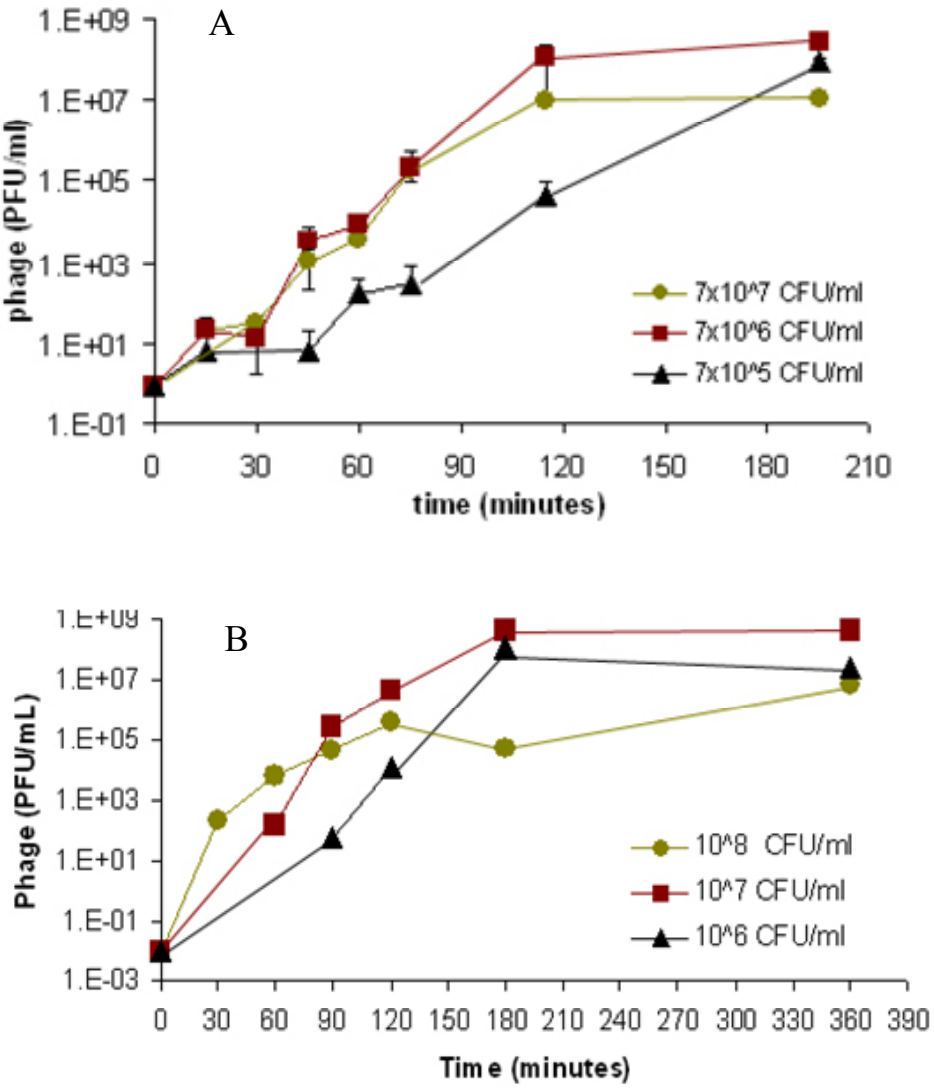


FIG 5.3. Coliphage MS2 enrichment in (A) 1 ml or (B) 333 ml volumes of 0.5 x TSB at three starting host cell concentrations. Starting levels of log-phase *E. coli* F_{amp} at levels of 10^5 to 10^8 CFU/ml were compared for rapid MS2 enrichment. Data in (A) is the mean of 3 replicates with error bars (± standard deviation) that are at times obscured by data symbols, while data in (B) is the value of one experiment.

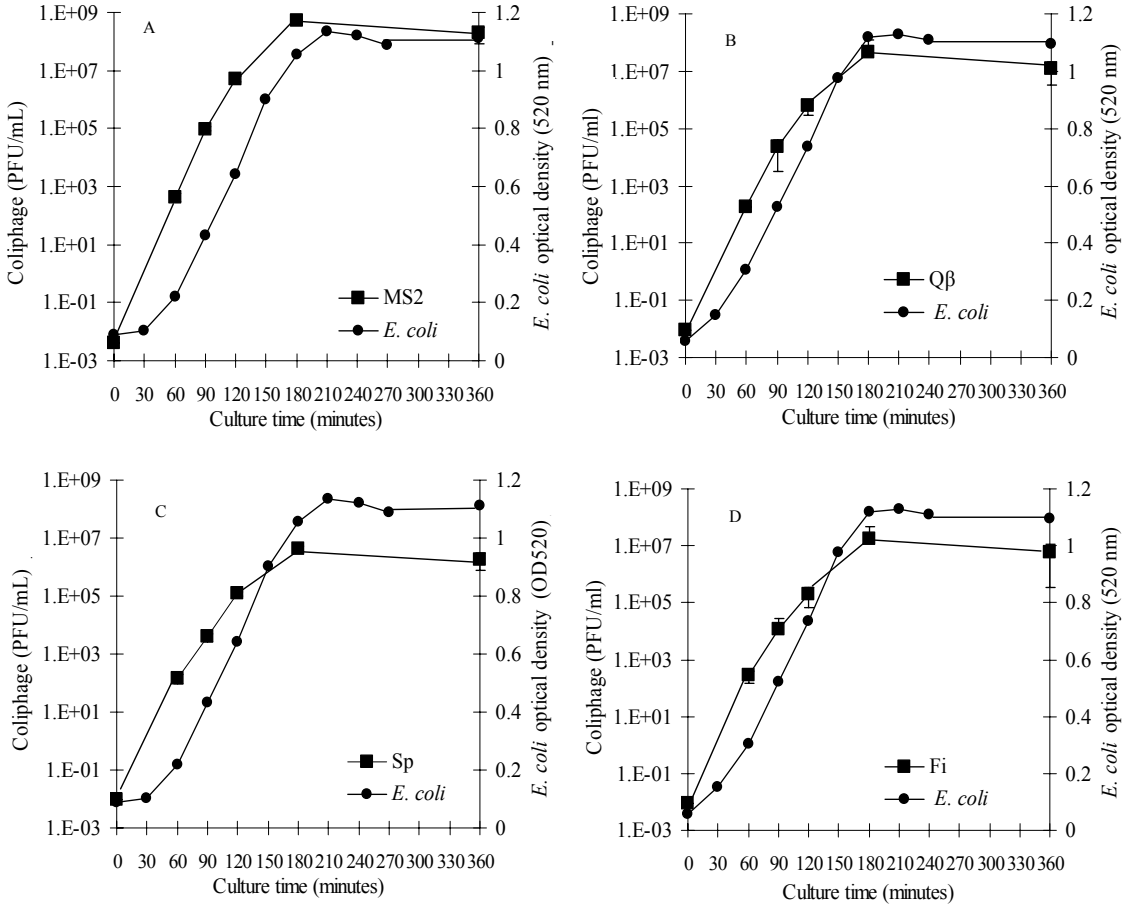


FIG. 5.4. Rapid culture enrichment of F+ RNA coliphage prototype strains (A) MS2, (B) Qβ, (C) Sp, (D) Fi (squares) in host *E. coli* F_{amp} (circles). Standard deviation error bars for coliphage (n = 3) are obscured by some square data points. Pre-culture levels of *E. coli* F_{amp} were 1 x 10^7 CFU/ml. *E. coli* levels during the experiment were measured by spectrophotometric absorbance at 520 nm.

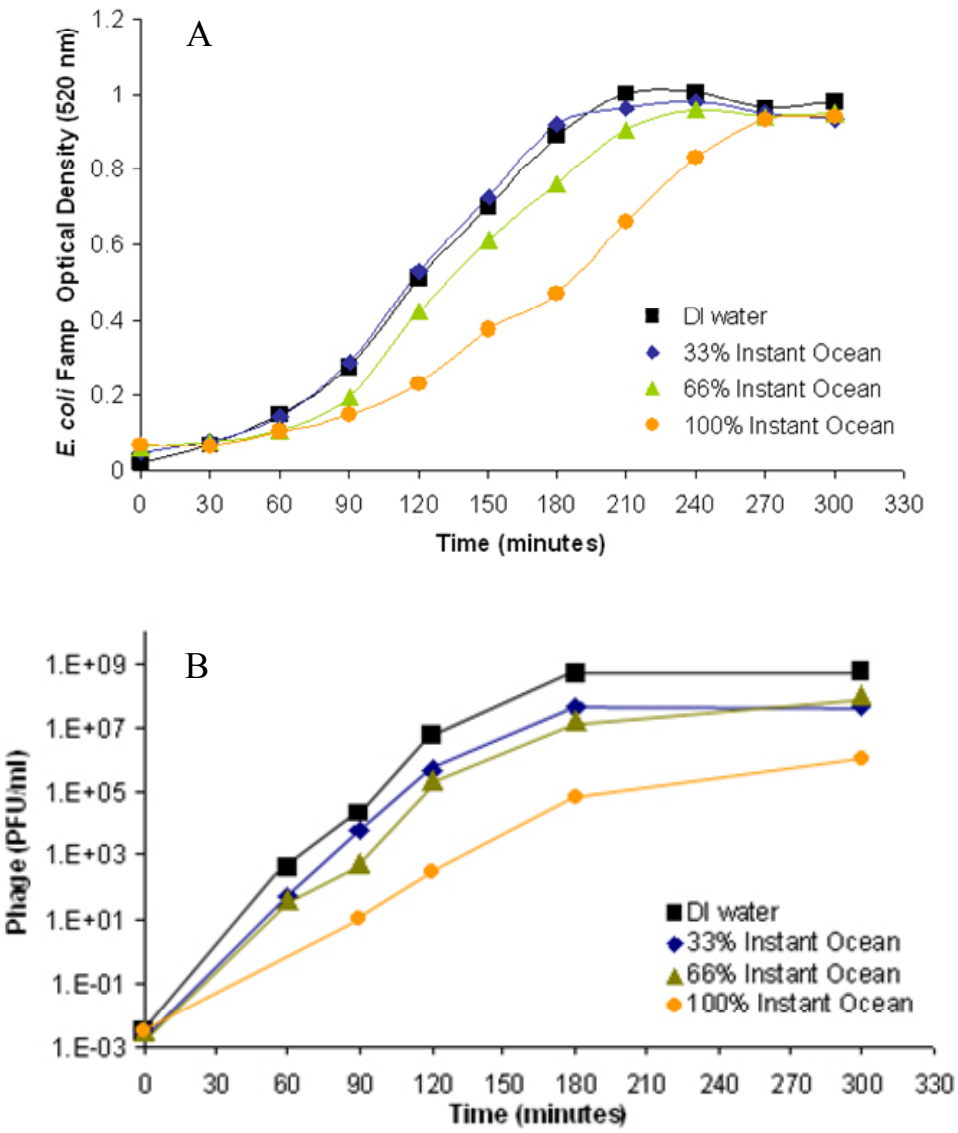


FIG 5.5. *E. coli* F_{amp} growth (A) and coliphage MS2 enrichment (B) in broth cultures simulating marine and estuarine water. Broth media was 0.5 x TSB supplemented with Instant Ocean (IO) to create 35 ppt (100% IO), 23 ppt (66% IO), and 11 ppt (33% IO) salinity, with a positive control using deionized water. Data points are mean values with 2 replicates for *E. coli*, and mean values of a single trial for MS2.

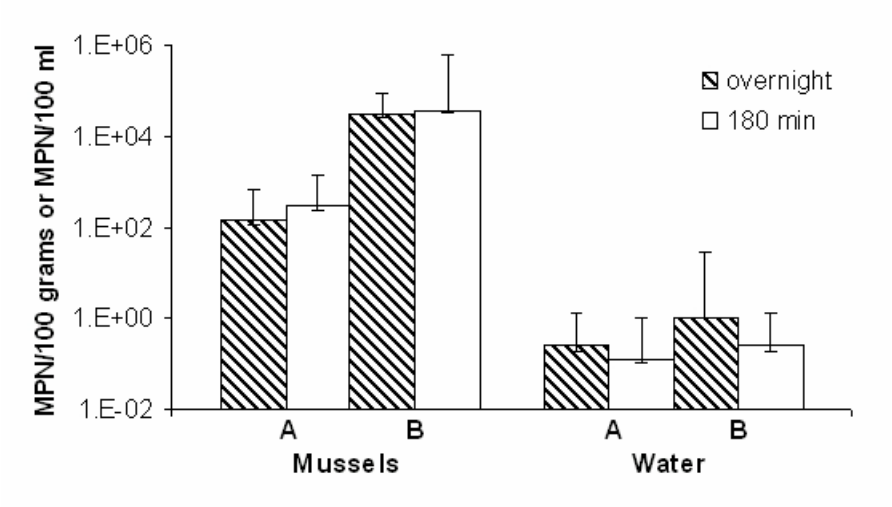


FIG 5.6. Enrichment and quantification of F+ coliphages from marine water and mussels by two methods. Samples from (A) Shelter Island in San Diego Bay, CA and (B) Tijuana River, CA were assayed for F+ coliphage by EPA Method 1601 with an overnight enrichment step (diagonal bars), or by a modified version of Method 1601 with a shorter 180 minute enrichment (white bars). Error bars are 95% confidence intervals.

TABLE 5.2. Binding efficiency of MS2 antiserum dilutions to polystyrene particles.

Buffer solution	Binding efficiency of antisera[a] (antiserum dilution)					
	(1:4)	(1:8)	(1:16) *	(1:32) *	(1:64) *	(1:128)
CP (pH 6.2) [b]	35% ± 6%	50% ± 4%	76% ± 4% *	90% ± 6%	71% ± 3% *	49% ± 9%
PBS (pH 7.2) [c]	40% ± 2%	50% ± 3%	71 ± 0.1%	100% ± 2% *	106% ± 1% *	39% [d]
PBS (pH 8.2)	38% ± 3%	46% ± 4%	54% ± 2% *	74% ± 3% *	78% ± 4%	50% ± 2%

[a] Average of 3 replicates ± standard deviation. * Statistically significant difference among 3 variables in a column (for asterisk on column headings) or between two variables within a column. Significance set at $\alpha = 0.05$.

[b] citrate phosphate buffer

[c] phosphate buffered saline

[d] Average of two replicates with no standard deviation

TABLE 5.3. Reaction matrix for testing agglutination of antiserum-coated particles with F+ coliphage antigens.

F+ coliphage prototype strains and controls		F+ coliphage antiserum labeled particles (antiserum dilution) [a]											
		F+ RNA antisera and dilutions					F+ DNA antisera and dilutions						negative control (no antisera)
		MS2 (1:16)	GA (1:32)	Qβ (1:8)	SP (1:16)	Fi (1:16)	Fd (1:16)	F1 (1:16)	M13 (1:8)	Φ15 (1:4)	Φ16 (1:16)	Φ18 (1:4)	
F+ RNA	MS2	+	-	-	-	-	-	-	-	-	-	-	-
	GA	-	+	-	-	-	-	-	-	-	-	-	-
	Qβ	-	-	+	-	-	-	-	-	-	-	-	-
	SP	-	-	-	+	+	-	-	-	-	-	-	-
	Fi	-	-	-	-	+	-	-	-	-	-	-	-
F+ DNA	Fd	-	-	-	-	-	+	+	+	+	+	-	-
	F1	-	-	-	-	-	+	+	+	-	+	-	-
	M13	-	-	-	-	-	+	+	+	+	+	+	-
negative controls	*E. coli* F_{amp} in TSB [b]	-	-	-	-	-	-	-	-	-	-	-	-
	TSB	-	-	-	-	-	-	-	-	-	-	-	-

[a] Checkerboard titration of antiserum-labeled particles (antiserum dilutions of 1:4, 1:8, 1:16, 1:32, 1:64, and 1:128) to empirically determine optimum antibody dilutions for detecting and typing a diverse panel of 32 nucleic acid sequenced F+ RNA and F+ DNA coliphage field isolates, 8 reference strains, and negative controls. This table gives a summary of agglutination results with F+ coliphage reference strains and controls at optimum antibody dilutions.

[b] TSB = tryptic soy broth.

TABLE 5.4. Lower detection limit of F+ coliphage prototype strains using antiserum-labeled polystyrene particles.

F+ coliphage prototype strains (PFU) [a]	F+ coliphage antiserum labeled particles (antiserum dilution)						
	F+ RNA antisera					F+ DNA antisera	
	MS2 (1:16)	GA (1:32)	Qβ (1:8)	SP (1:16)	Fi (1:16)	M13 (1:8)	Fd (1:16)
5×10^7	nd [b]	nd	nd	nd	nd	+	+
1×10^7	nd	nd	nd	nd	nd	+	+
5×10^6	nd	nd	nd	+	+	+	+
1×10^6	nd	nd	+	+	+	-	+
5×10^5	+	nd	+	+	+	-	-
1×10^5	+	nd	+	+	+	-	-
5×10^4	-	+	-	-	+	-	-
1×10^4	-	+	-	-	-	-	-
5×10^3	-	+	-	-	-	-	-
1×10^3	-	-	-	-	-	-	-
5×10^2	-	-	-	-	-	-	-

[a] F+ coliphage prototype strains (MS2, GA, Qβ, SP, Fi, M13, and Fd) were tested against their corresponding antisera.
[b] nd = not done

TABLE 5.5. CLAT detection and serotyping of F+ RNA coliphage field isolates.

FRNA detection and typing methods	F+ RNA field isolates [c] (n=192)					F+ DNA field isolate [c] false positives (n = 34)
	No. of sero/genogroup positives				No. of false negatives (% of total)	
	I	II	III	IV		
CLAT [a]	101	90	13	1	7 (3.6%)	0
RLB Hybridization [b]	101	67	28	5	15 (7.8%)	0

[a] CLAT assay serum: anti-MS2 serum at 1:16 dilution for group I; anti-GA serum diluted 1:32 for group II; anti-Qβ serum diluted 1:8 for group III; and anti-Sp and anti-Fi sera, both diluted 1:16 for group IV.
[b] RLB = Reverse Line Blot hybridization (47).
[c] Field isolates were plaque-purified enrichments of coliphages recovered from estuarine water and shellfish.

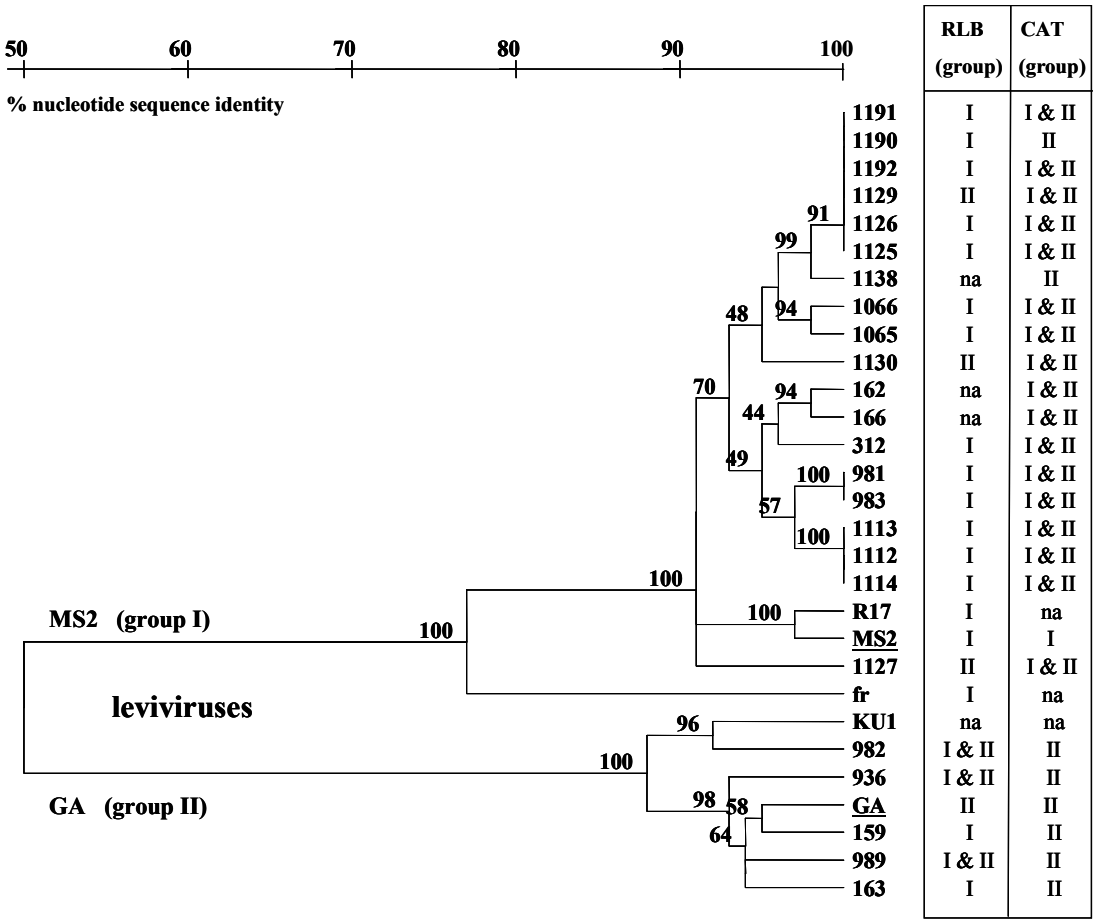


FIG. 5.7. Phylogenetic tree of the F+ RNA coliphage capsid region for 24 field isolates discordantly typed by RLB hybridization genogrouping and CLAT serogrouping. The phylogenetic tree is based on a 344-nt region of the 392-nt levivirus capsid gene using Jukes and Cantor distance estimations and 100 bootstrap values as indicated at tree nodes. Na = no typing data available

TABLE 5.6. CLAT detection of F+ DNA coliphage field isolates.

F+ DNA detection method	FDNA field isolates [c], (n= 164)		F+ RNA field isolate [c] false positives, (n = 132) (% of total)
	No. of true positives (% of total)	No. of false negatives (% of total)	
CLAT [a]	161 (97.7%) [b]	3 (2.3%)	3 (1.8%)
RNase Neutralization	164 (100%)	0	0

[a] CLAT for F+ DNA coliphage uses both M13 antiserum (1:8 antiserum dilution) and Fd antiserum (1:16 antiserum dilution).
[b] No significant difference between the proportion of true positive F+ DNA detected by the two methods (P value = 0.82).
[c] Field isolates were plaque-purified enrichments of coliphages recovered from estuarine water and shellfish.

6 GENERAL DISCUSSION AND CONCLUSIONS

Discussion

Questioning Assumptions about Water Quality, Fecal Indicators and Health. Anthropogenic sources of fecal pollution, such as agricultural runoff, septic system leaks, combined sewer overflow systems, and urban runoff have compromised water quality in many urban coastlines to the extent that bathing in these waters and harvesting shellfish from them constitutes a public health risk. To reduce the risks of disease among bathers the US EPA (EPA) introduced the Ambient Water Quality Criteria for Bacteria in 1986 (US EPA 1986), and Congress passed the BEACHES Act of 2000.

Risk-based health regulations require a consistent association between fecal indicators and disease symptoms in bathers (Prüss 1998) or consumers of bivalve mollusks. The extent to which fecal coliforms or other microbial indicators of fecal contamination are predictive of the human health risks from ingestion of raw mollusks has not been carefully or comprehensively studied based on linking the microbial quality of shellfish or their harvest waters to the risks of gastrointestinal illness or other illnesses from consuming specific quantities of such shellfish.

As indicators of disease risks to bathers, epidemiological studies have shown that enterococci and *E. coli* are better than other indicators for associations with skin disorders

in all waters, and GI illnesses in marine water, but no best indicator exists for GI illnesses in fresh water or for respiratory illnesses in any water (NCR 2004; Wade 2003). Although enterococci and *E. coli* may correlate with some disease symptoms in bathers, there remains a "black box" in the fecal indicator paradigm, which is the etiology of bathers' diseases. The unknowns in this paradigm are the etiologies of water contact diseases. Epidemiological bathing-associated disease risk studies often use a prospective cohort design with follow-up questionnaires that do not include efforts to identify disease causing pathogens. Therefore the etiology of water-contact diseases has not been adequately studied (Wade et al., 2003). Likewise, there is only limited information on the etiologies gastrointestinal illness and other illnesses associated with consumption of bivalve molluscan shellfish. Many shellfish-borne disease outbreaks are gastroenteritis, and at least some of these are known to be caused by noroviruses. However, there are only limited data on the range of etiologies of shellfish-borne gastrointestinal illness.

Similar disease symptoms of gastrointestinal illness can result from a variety of pathogens (viruses, bacteria, protozoa), but importantly these pathogens vary in their occurrence, persistence, virulence and infectious dose. The survival and persistence of fecal indicators and pathogens in water and shellfish varies. Hence, enterococci or *E. coli* are likely to be inadequate or unreliable indicators for at least certain pathogens such as enteric viruses in water and shellfish due to greater virus and bacteriophage resistance to water and waste water treatment processes (Harwood et al., 2005; Jofre et al., 1995), and greater virus and bacteriophage persistence in freshwater, seawater and bivalve mollosks (Contreras-Coll et al., 2002; Duran et al., 2002; Moce-Llivina et al., 2005). Fecal indicators that predict risks of GI illness may not be predicting risks of enteric viral GI

illnesses, or GI illnesses of other types of pathogens, such a enteric protozoan parasites, whose levels or persistence do not correlate with fecal bacteria indictors. Although current microbial fecal indicators make for pragmatic solutions, scientists and regulators must acknowledge and strive to reduce the inherent flaws in "black box" assumptions about microbial indicators of disease risks.

Fecal indicators also cannot predict the risks from certain naturally occurring, endemic waterborne pathogens, such as *Vibrio vulnificus* and other non-cholera *Vibrios* (Koh et al., 1994). These *Vibrios* are endemic to warm marine waters and caused 142 illnesses and nine deaths from 2003-2005 in the US (Dziuban et al., 2006). Other naturally occurring waterborne pathogens that fecal indicator microbes may not reliably predict are *Aeromonas hydrophila* and other *Aeromonas* species and various species of the genus *Plesiomoanas*, such as *Plesiomomas shigelloidee*s These bacteria have been associated human gastrointestinal illness from exposure to water and shellfish (San Joaquin, 1994; Soweid and Clarkson, 1995; Youssef et al., 1993).

The fecal origin (human or animal) and discharge source (point or non-point source) may also affect correlations between indicators and health outcomes. In marine waters with primarily human fecal pollution from point-sources, enterococci and *E. coli* correlate with GI illness in bathers (Cabelli 1983, Dufour 1984, EPA 1986; Wade 2003). Additional studies in other countries also document that enterococci or fecal streptococci are predictive of risks of gastrointestinal illness as well as respiratory illness from bathing in marine waters impacted by point sources of fecal contamination. Such studies done in the UK provided the basis of World Health Organization guidelines for recreational waters (WHO, 2003). However, similar associations among these bacteria and illness

were not seen in marine waters with non-point source non-human fecal pollution (Colford et al., 2007). These reasons for these differences in relationships between candidate fecal indicator microbes and risks of gastrointestinal illness and other diseases from water contact exposures are uncertain and have not been adequately elucidated. They may be related to the differential die-off or persistence of fecal indicator bacteria and human enteric virus or protozoan pathogens in marine waters and differences prevalence and concentrations of disease-causing pathogens and fecal indicator bacteria in waters impacted by non-point as opposed to point sources of fecal contamination.

It is recommended that future health-based water quality studies could include analysis for wider range of fecal indicator microbes, microbial source tracking and pathogens to control for different fecal sources, pathogen occurrence and indicator occurrence from fecal and non-fecal sources. Monitoring for harmful but not fecally-associated microbes, such a *Vibrio* species of bacteria is also recommended.

The EPA criteria for acceptable bathing water illness rates recommends beach managers post warnings or advisories when fecal indicator levels reach those corresponding to 19 illnesses per 1,000 people at marine beaches and 8 illnesses per 1,000 people at fresh water beaches (US EPA 1976; US EPA 1986). Comparing EPA's acceptable bathing water illness rates to illness rates found in epidemiology studies at marine beaches shows that the proposed EPA rates are on the low end of the range of diarrhea incidence observed in field studies (Cabelli et al.., 1979; Haile et al., 1996; Colford et al. 2007). The incidence of diarrhea among bathers in Santa Monica, California was 5-6% (Haile et al., 1996), 4-6% in Mission Bay, California (Colford et al. 2007), about 4% in New York (Cabelli et al., 1979), and 14% in the United Kingdom

(Fleisher et al., 1993). For fresh water, EPA accepted illness rates are an order of magnitude lower than observed diarrhea incidence in Lake Erie (10-14% incidence of diarrhea) and Lake Michigan (10% incidence of diarrhea) (Wade et al., 2006). These differences between observed and expected illness rates further suggest that current fecal indicator monitoring alone is not adequate for achieving public health standards set by EPA, and alternative fecal indicators should be explored.

A Better Approach to Fecal Pollution Monitoring and Management. As an alternative to the EPA approach for recreational water, the World Health Organization's "Annapolis Protocol" and "Guidelines for Safe Recreational Water Environments" are not based solely on absolute numbers of fecal indicator bacteria for regulation, and instead apply a management approach combining microbial water quality assessment and sanitary inspection to derive different levels of bathing water quality risk with provisional warnings for high risk events like rainfall (WHO, 1999; Bartram & Rees, 2000; WHO 2003). Risk assessment and risk management are key features in the WHO approach (WHO 2003; Bartram et al., 2001), while further applications of risk assessment could be to assess the costs and potential health benefits of hypothetical management options (Soller et al., 2006). The WHO approach also uses HACCP principles (hazard analysis; critical control points; critical limits; monitoring; management action; validation/verification; record keeping) taken from the food and beverage industry as applied to recreational water quality. As a whole, the WHO recreational water model integrates current thinking on the state of science for risk assessment and management in a more thorough and holistic way than the US EPA model.

When microbial water quality assessment is performed and analyzed by the WHO model, the critical control values are relational and not exact values like those used by US EPA, where in the WHO model the 95th percentile value of enterococci/100ml is used to determine water quality on a grading scale (e.g. A = <40 (95th percentile enterococci/100ml); B = 40-200; C 201-500; D = >500) (WHO 2003). Health targets are compared to public health outcomes so that feedback loops can refine management of water quality, and so more polluted areas can receive much needed priority attention, and less polluted areas can receive less attention by such means as reduced monitoring frequency. These feedback loops would optimize intervention actions and potentially reduce management costs.

Pollution interventions in WHO's "Annapolis Protocol" are not explicitly described, and in this author's view, an ecological approach would best address interventions that target the root causes poor water quality. Sites with repeated exceedances of fecal indicator microbes and poor results from sanitary surveys could trigger politically-achievable, comprehensively designed pollution prevention and control strategies that use an ecological approach. These approaches could include controlling amounts of impervious surfaces, planting riparian buffers to reduce runoff, regulating concentrated animal feeding operation wastes, controlling and monitoring sewage effluent discharges and encouraging alternatives to wet weather treatment plant bypasses to receiving waters, lengthening off-shore wastewater disposal pipes and removing or upgrading (e.g., with disinfection) combined sewer overflow systems, or other approaches directed at controlling source inputs and impacts. This system could be modeled after the transportation implementation plans (TIP) within the Clean Air Act, as

coastal implementation plans (CIPs) showing how future federal and state funding on coastal projects would be spent in a manner consistent with water quality goals.

Improved methods for forecasting or prëmptive water quality warnings and advisories are another important area for future work. Prëmptive closure due to rainfall and runoff accounted for 21% of US beach closings or advisories in the US in 2004 (NRDC 2005). Rainfall monitoring has increased each year in coastal and Great Lakes states since 2000 (NRDC 2005), which shows the increased interest and use for this monitoring technique. Newer rainfall models and related geohydrological models could integrate satellite imagery and other types of data (such as wastewater discharge flows from major point sources) for areas without rainwater gauges (Park and Stenstrom 2006) and integrate microbial fate and transport models for runoff entering surface waters (Liu et al., 2006).

The Role of this Study for Monitoring and Management. The role of this study in the scope of recreation water quality and shellfish quality monitoring is to address problems with current fecal indicators in marine water and shellfish by investigating basic associations among fecal microbe indicators, their levels, types, and sources in a geographically diverse set of estuaries in the US. This work tried to determine the best available techniques to recover and quantify coliphages as microbial fecal indicators for all coastal sites in the US, and helped evaluate what cold become a toolbox of effective fecal indicator microbes that provide flexibility as well as different time frame and levels of information, based on the speed of results, the impacts and cost of incorrect answers, and greater knowledge of existing fecal sources identified by

sanitary surveys. Critical comparative information on coliphages, fecal bacterial indicators and microbial source tracking can inform and improve water and shellfish monitoring and management methods and policies.

In this study, the water quality of strategically selected sampling stations at nine US estuaries on the East, West, and Gulf Coasts was analyzed for F+ coliphages, somatic coliphages, and bacterial indicators (*E. coli*, fecal coliforms, enterococci, *C. perfringens*). All of these microbes were found to be reasonable indicators of human fecal pollution in water, because significantly more of these microbes were detected in human-impacted water than non-human impacted or pristine water at each estuarine study site. In shellfish a different picture emerged. Fecal coliform bacteria levels in shellfish were not predictive of human fecal impacts. This is an ironic finding because fecal coliform bacteria are the regulatory indicator of the National Shellfish Sanitation program. Significant differences were observed in shellfish for *E. coli* levels at human impacted sites compared to non-impacted or pristine sites, a finding which is consistent with *E. coli* being more feces specific than fecal coliforms. These findings suggest *E. coli* may be a more accurate and reliable measure of fecal contamination in shellfish than fecal coliforms. Basing shellfish sanitation on fecal coliforms, as is done in the US, may under- or over-predict fecal source impacts and disease risks to shellfish consumers.

Correlations and levels were examined between pairs of fecal indicators, with strongest correlations between log-fecal coliforms and log-*E. coli* in both water and shellfish. This is to be expected because the *E. coli* group is a sub-set of fecal coliforms. Strong correlations between enterococci and fecal coliforms were observed with nearly identical correlations for shellfish (R^2 = 0.6018) and water (R^2 = 0.6015) by linear

regression, suggesting similar sources and/or responses to environmental factors. These correlations are partly explained by similar bacterial levels in shellfish (fecal coliforms: 1.71 $\log_{10}$ CFU/100ml and in 69% of 74 samples; enterococci: 1.74 $\log_{10}$ CFU/100ml and in 66% of 72 samples), while in water fecal coliforms outnumbered enterococci (1.02 $\log_{10}$ fecal coliforms/100ml to 0.62 $\log_{10}$ enterococci /100ml) and were more often detected (fecal coliforms in 94% of 78 water samples; enterococci in 81% of 78 water samples). Higher fecal coliform levels in water may be due to their lower specificity for feces, and general ubiquity in environmental waters. Strong correlations were also seen between log-*E. coli* and log-enterococci (R^2 = 0.688 in water; R^2 = 0.583 in shellfish). Such correlations have been observed previously in fresh water and marine water but no studies have reported such a comparison for shellfish (Wiedenmann et al, 2006; Contreras-Cole et al., 2002).

Coliphages also correlated with levels of bacterial indicators in shellfish (R^2 = 0.32 for somatic coliphages vs. *E. coli* in shellfish and R^2 = 0.47 for the same organisms in water) and water (R^2 = 0.05 for F+ coliphages vs. *E. coli* in shellfish and R^2 = 0.31 for the same organisms in water). Similar result to these have been previously reported for F+ coliphages vs. *E. coli* in shellfish or somatic coliphages vs. *E. coli* in water (Conreras-Coll et al., 2002; Dore et al., 2003), but no studies have reported correlations of somatic coliphages vs *E. coli* shellfish or F+ coliphages vs. *E. coli* in marine water. Levels of somatic coliphages (1.97 $\log_{10}$ PFU/100 grams) and F+ coliphages (1.96 $\log_{10}$ PFU/100 grams) in shellfish were similar to each other and to all bacterial indicators (1.85 $\log_{10}$ CFU/100 grams), while water samples showed similarities only between somatic coliphages (0.75 $\log_{10}$ PFU/100ml) and all bacterial indicators (0.82 $\log_{10}$ CFU/100ml)

with F+ coliphage levels much lower at -0.35 $\log_{10}$ PFU/100ml. Given the mildly positive correlations between somatic coliphages and bacterial indicators in waters of this study and others (Wiedenmann et al, 2006; Contreras-Cole et al., 2002), and our high incidence of somatic coliphages (96%) in water, these coliphage indicators deserve inclusion in other bathing water and shellfish studies both within and outside of Europe. F+ coliphages were also associated with fecal contamination sources and correlated with fecal indicator bacteria in shellfish, so they too deserve further consideration as fecal indicator viruses although their low prevalence in water is concerning.

Coliphages are promising indicators because they correlate with the presence of pathogenic human viruses in water and shellfish and increased viral illness risks in some studies (Chung et al., 1998; Havelaar, 1993; Dore et al., 2000; Jiang et al., 2001; Wade et al., 2003; Colford et al., 2006). Indeed, the value of F+ coliphages as fecal indicator viruses has been further documented in tropical waters where levels of naturally occurring *E. coli* and enterococci are not predictive of waste water impacts on surface waters (Luther and Fujioka, 2004). The documented growth of *E. coli* and enterococci in warm waters and sediments and on marine vegetation undermines their value as predictive indicators of fecal contamination (Desmarais et al., 2002, Lewis, 2006). Hence, coliphages are reasonable alternatives to *E. coli* and enterococci in tropical environments where these bacteria regrow and overestimate fecal impacts.

To help build a microbial toolbox of methods, coliphage methods were validated and compared for shellfish and estuarine water in this study. In a comparison of three coliphage detection and quantification methods (quantitative two-step enrichment, single agar layer [SAL], and direct membrane filtration [DMF] plaque assay, those that

recovered significantly higher levels of coliphages from water at human impacted than non-impacted sites included the quantitative two-step enrichment assay for F+ and somatic coliphages and the single agar layer assay for F+ coliphages. The difference in detection among coliphage methods is likely due in part to the differences in sample volumes assayed in each method and inherent limitations causing low sensitivity, such as inefficient coliphage transfer from membranes to agar medium-host lawns in the DMF method.

In shellfish from human impacted and non-impacted sites, using quantitative two-step enrichment and SAL, neither coliphage method recovered significantly more coliphages from human impacted than non-impacted sites, although the SAL assay for F+ coliphages (p value = 0.073) was nearly significant. ((compare 2-step to SAL)) From these results, it is recommended that future studies on fecal contamination use two-step enrichment for F+ and somatic coliphages in estuarine water, while shellfish studies use single agar layer assay— based on their sensitivity and relationship to human fecal impacts.

At four sites with WWTPs, fecal indicator concentrations in water and shellfish strongly correlated with an increase in treated effluent discharge volumes. Wastewater plants in 75% of study sites used activated sludge treatment with chloramine or chlorine disinfection. These findings suggest that large WWTP are either not achieving microbial removals that smaller WWTPs are achieving, or their microbial loading into receiving waters is greater. In this study it was not possible to analyze WWTP effluents or collect reliable data on discharge volumes, although EPA reports on NPDES permit exceedances for fecal coliforms show such events occurred at the Beaufort, NC WWTP during 3

months of the study, at the Dover, NH WWTP for 12 months of the study, and at the Tijuana River, CA WWTP for virtually the entire study period. Although it was not possible to determine exactly how much of the greater occurrence of coliphages at WWTPs was due to magnitude of discharge or quality of discharge with respect to coliphages and other fecal indicators, the findings of increasingly poor effluent receiving water near larger WWTPs is still valuable because receiving waters, like WWTP effluents are regulated under the Clean Water Act. In either case, larger WWTPs may need to reconsider effluent quality and disinfection strategies to prevent their effluent discharges from negatively impacting water recreational or shellfish users. In the long term, this information may inform policy decisions of how to better reduce microbial impacts by upgrading treatment or other alternative technological approaches. One solution is to plan for WWTP upgrades and seek financial support for them by increasing support for State Revolving Funds and reinstating previous infrastructure loan programs used so successfully in the 1960s and 1970s for upgrading and rehabilitating older WWTPs in need of upgrades.

Because not all fecal inputs came from point sources or just one of them, microbial source tracking was performed in this study to genotype 436 F+ RNA coliphages water isolates and 519 F+ RNA coliphage isolates from shellfish into genogroups associated with animal feces (groups I and IV) or human feces (groups II and III). Ninety percent of F+ RNA water isolates, and 82% of shellfish isolates were F+ RNA genogroup I, which is consistent with previous findings (Cole et al., 2003). Microbial source tracking with F+ RNA genogroup I was not quantitatively reliable because high proportions of these coliphages were found in samples from both human

and animal impacted sites. The finding of high proportions of genogroup I F+ RNA coliphages, as observed in previous studies, has been attributed at least in part to the greater persistence of many genogroup I coliphages in water compared to those of the other genogroups (Brion et al., 2001).

In shellfish and water for some estuaries, genogroup II (human-type) isolates were found more often in human impacted than non-impacted sites, consistent with past source tracking findings (Furuse et al., 1978; Hsu et al., 1995). Overall, microbial source tracking has been a specialized and expensive tool that required advanced and often additional methods of microbial analysis. However, it is a powerful and useful for detailed analysis of fecal pollution hot-spots, sources and mitigation priorities, especially in situations where more basic analyses such as routine microbial monitoring, even with expanded sampling gives inconclusive results. Ideally, microbial source tracking could be better integrated into routine indicator monitoring if techniques were rapid, simple, and cost-effective. In this present study coliphage source tracking to the group level of identification could be fully integrated into a simple, rapid and cost-effective assay system.

Benefits of Using Rapid Fecal Indicators. Conventional enterococci and *E. coli* methods give results in one to several days. This slow time to results causes delays in water quality warnings and advisories that are unable to provide information early enough to take preemptive actions to protect recreational users. Perhaps to the chagrin of water managers, these untimely monitoring methods were the main trigger for 75% of US beach closings or advisories in 2005 (NRDC 2006). Newer rapid monitoring

techniques with same-day results are needed to better manage recreational water quality and reduce exposure risks and health risks to bathers who unknowingly come in contact with contaminated water before conventional bacteriological results are obtained.

In one of the first trials of rapid fecal indicator methods, a Taqman PCR method for enterococci showed positive trends with GI illness among bathers in Lakes Michigan and Erie (Wade et al., 2006). However, such direct PCR results have been criticized by stakeholders because of their inability to distinguish between the nucleic acids of infectious or culturable microbes and that of inactivated, non-infectious microbes or their released nucleic acids. For example, Skraber et al. (2004) found that poliovirus genomes detected by RT-PCR persisted longer than poliovirus infectivity in river water. Similarly, Choi and Jiang (2005) reported that adenoviruses detected in rivers by PCR methods were largely non-culturable and probably non-infectious. They concluded that genome-based detection methods are inadequate for direct assessment of human health risk.

Our study provides an improved new tool for same-day, rapid water quality monitoring by detecting infectious F+ coliphages. The new method is based on short-term (< 3hours) liquid enrichment culture of different and multiple sample volumes in susceptible host cells, followed by <1 minute detection and typing of the coliphages enriched in these cultures by a simple particle agglutination immunoassay. The method provided comparable results to those obtained by conventional longer term enrichment followed by conventional genotyping based on direct nucleic acid hybridization or RT-PCR-hybridization.

This rapid F+ coliphage method has not yet been evaluated for its ability to predict human health risks to bathers in the context of epidemiological studies. However,

the method will be applied in the Summer of 2007 to a swimming-associated disease study in Southern California to validate its performance and the relationship of F+ coliphages to health outcomes from bathing exposures. In a previous study F+ coliphages in coastal bathing waters impacted by non-point sources of fecal contamination were predictive of the risks of gastrointestinal illness in exposed bathers (Colford et al., 2007).

The economic relationships between water and health underscore the importance of same-day water quality monitoring. The cost of water quality monitoring and lost tourist dollars from beach closings (Rabinovici et al., 2004) are often less than the costs of recreational bathing illnesses (Given et al., 2006). The effects of beach closings on the cost of bathing illnesses at two Great Lakes beaches was compared, where a same-day Taqman PCR enterococci methods closed beaches for 15 days that would otherwise have remained open using the standard two-to-three day enterococci methods (Tuteja et al., 2005). The same-day beach closures kept an estimated +3,000 people out of the water and saved some of those people from GI illnesses, saving them an estimated $202,000 (Tuteja et al., 2005). The value to tourism of avoiding 15 days of beach closures was estimated to be about $62,000, much less than the value placed on health (Tuteja et al., 2005).

Californians pay $1.3 million per year to monitor their +400 beaches (NRDC website), and sustain economic losses of $21 to $51 million per year from the estimated 0.6 to 1.4 million cases of GI illness at just Los Angeles and Orange County beaches (Given et al., 2006). The cost of GI illnesses across the state are estimated to be 10-100 times more than that of monitoring efforts. The causes of these recreational water

illnesses is not known. They could be caused by a lack of enteric virus risk predictability by fecal bacterial indicators, or similar to the Great Lakes example, that warnings and advisories are often posted days after fecal contamination and human exposures have occurred. These findings as a whole suggest that healthcare cost savings outweigh the costs of robust water quality monitoring, including those of rapid indicator detection methods.

Labor costs are also reduced with rapid fecal indicator monitoring. The costs of monitoring one beach for a year using a standard enterococcus culture method is about $5,700, but drops to about $1,050 per beach-year using a rapid enterococcus Taqman PCR (Haugland et al., 2005) as a result of the time-savings and reduced labor costs of the new method (Tuteja et al., 2005). Our CLAT assay can be performed in several hours, and also has the potential to reduce labor costs. It also used inexpensive materials with an estimated value of less than $2.50 per assay.

Drawbacks of using rapid Taqman PCR include the high equipment and reagent costs associated with this new technology. A major difference between the CLAT assay and enterococci Taqman PCR is that our method requires little specialized equipment and training. Another drawback of Taqman PCR is its lack of field portability of the current technology, so the driving times between beach sampling sites and the analytical lab where the tests have to be performed will probably be the deciding factor as to whether regulators using this tool can achieve same-day monitoring and management of water quality.

The CLAT assay was validated in the laboratory, but the goal is to use this assay in the field, similar to other latex agglutination tests that are routinely used outside of

microbiology labs, such as in doctors offices for *Streptococcus* throat tests, in people's homes for pregnancy tests, and in non-lab settings of developing countries for diarrheic viruses shed in stool. Future applied work on the evaluation and application of CLAT and Taqman PCR should be to develop field portable units for these assays.

Converting the CLAT assay into a field-portable method is possible in principle, and there are no major technical obstacles to overcome. However, like any new assay widespread availability and access to the test would require further production efforts to standardize the biological reagents, create a convenient form of *E. coli* host delivery, package pre-sterilized sample containers for MPN analysis using 3-replicate / 3-volume samples, and provide a field-portable water bath incubator capable of maintaining 35-37°C for three hours of coliphage culture. Initial results in the lab show promise for field-application of a < $200 water bath built from a 48-liter cooler, an aquarium heater and pump, and a marine duty battery as a source of electricity. Standardized reagents would ideally be pre-packaged as sterile unit quantities, as are Colilert reagents, for example. As proof-of-concept for a CLAT *Beta*-version, dry reagents were parsed out and stored in 50 ml conical tubes. To show that these dry biological reagents do not need to be autoclaved as liquids for their sanitization, experiments were performed on dry biological media using dry heat sterilization in an 80°C dessicator for 15 min. This achieved > 5-$\log_{10}$ reductions of experimentally inoculated *E. coli* and MS2 coliphages (data not shown).

The total cost of the materials for CLAT assay of F+ coliphages, including all steps for culture, detection, quantitation, and microbial source tracking, is about $2.50 per one-liter MPN sample (Appendix 1). This is appreciably less than Enterolert ™ (IDEXX,

Westbrook, ME), a popular commercially-available enterococci kit that costs $5.60 per 100 ml sample and cannot perform microbial source tracking. The estimated cost of the CLAT assay only includes materials purchased at retails by our laboratory, while the cost of Enterolert ™ kits probably includes their production or material sourcing costs, marketing costs, indirect costs and a profit margin. If CLAT assay materials were produced commercially the price may either decrease or increase, depending on the production and marketing plan and setting. Overall, it is expected that the CLAT assay can be used immediately by water and shellfish microbiology labs that have access to the required immunoassay reagents and that the costs of the test reagents and materials would be no more than and perhaps less than the costs of other microbial assays.

Research Summary and Conclusions

Summary. This research has provided data and its analysis and interpretation that provide greater understanding of coliphage and bacterial indicator levels, sources, types in United States coastal marine waters and bivalve molluscan shellfish. This research has also provided rigorous comparative performance information of methods for monitoring coliphages as fecal virus indicators of the sanitary quality of recreational marine water and shellfish in geographically representative coastal environments of the US. Another product of this research is a new coliphage assay and typing method, the coliphage latex agglutination and typing (CLAT) assay, which fulfills the need for a same-day, rapid fecal indicator detection, quantitation and source tracking method.

CLAT is a microbial source tracking tool that rapidly detects and distinguishes F+ RNA coliphages from human and animal fecal sources

Conclusions

- In coastal marine and estuarine waters of 9 geographically diverse sites of the coastal United States, significantly more bacterial fecal indicators (fecal coliforms, *E. coli*, *C. perfringens*, enterococci) and bacteriophage fecal indicators (F+ and somatic coliphages) were detected in human-impacted water than non-human impacted or pristine water using a quantitative enrichment assay for F+ and somatic coliphages, a single agar layer assay for F+ coliphages, and membrane filtration methods for bacterial indicators *E. coli*, fecal coliforms, enterococci and *C. perfringens*.

- In estuarine and sea water, enterococci and fecal coliforms were strongly correlated, and 88% of their matched-pairs agreed on EPA recreational water quality bacterial criteria for exceedances or allowances. These findings support enterococci as an effective replacement for fecal coliforms in bathing water monitoring criteria standards.

- In bivalve molluscan shellfish collected from these same coastal US sites, significant differences were observed for *E. coli*, but not fecal coliforms, at human impacted sites (near wastewater treatment plant discharges) compared to non-impacted or pristine sites. These findings are consistent with *E. coli* being more feces-specific than fecal coliforms and fecal coliforms being a poor indicator of fecal impacts and their associated human health risks from pathogens

- Based on results of this study in terms of sensitivity and relationship to human fecal waste source impacts, it is recommended that future studies on fecal contamination use the quantitative enrichment method (ENR) for F+ and somatic coliphage monitoring in estuarine and marine water, and for studies of coliphages in shellfish, the use of the single agar layer method (SAL).

- Based on the results of this study, it is recommended that when microbial source tracking of F+ RNA coliphages is to be used subsequent to their recovery, direct membrane filtration (DMF) be used instead of ENR for water and SAL be used instead of ENR for shellfish.

- F+ coliphages were detected in 62%, 64%, and 83% of oysters, clams, and mussels, respectively, using ENR. These findings support the view that F+ coliphages are prevalent and readily detectable fecal indicators in bivalve mollusks from diverse coastal sites in the US.

- F+ and somatic coliphages had a stronger association with the magnitude of the WWTP discharges and were more predictive of WWTP discharge volumes than were the fecal bacterial indicators Enterococci and *C. perfringens*.

- F+ RNA microbial source tracking was only somewhat effective at identifying fecal contamination source types at human and animal impacted sites because high levels

of Group I F+ RNA coliphages were often found. Because this group has been found in both waste water and animal waste, conclusive source attributions are not always possible when this group predominates. Therefore, supporting documentation, including the prevalence of other coliphage groups more indicative of human (groups III and II) and animal (group IV) sources and identification of known fecal waste sources using sanitary or shoreline surveys is needed as supporting information for more conclusive site assessments of microbial quality and potential human health risks.

- Of 1033 F+ RNA field isolates tested from study waters and shellfish, 99.9% gave confirmed positive RT-PCR products, and 98.3% were genotyped by RLB into groups used to track human (group II and III) and animal (groups I and IV) fecal sources. The robustness of RT-PCR – RLB at the nine geographically diverse US estuaries of this study suggests broad applicability of this method to detect and genotype diverse field strains of F+ RNA coliphages for microbial source tracking in all coastal regions of the US.

- Based on the results of this study, it is recommended that more reliable nucleic acid screening requires parallel DNase and RNase treatments instead of just RNase treatments. This is because of the bias towards DNA coliphages in RNase testing of RNA/DNA mixed samples observed in this study.

- The existence of an apparent new group or subgroup of F+ RNA coliphages was further documented by genetic analysis coliphage isolates from this study. Therefore, it is recommended that a new and unique oligonucleotide probe for this group be added to the probe set used for genogrouping by RLB hybridization. The goal would be to specifically detect strains within the new JS cluster of the levivirus group, which will improve the use of RLB for classifying F+ RNA strains without nucleotide sequencing.

- A rapid (180 minute) and quantitative F+ coliphage enrichment culture assay was developed by modifying EPA Method 1601, and this rapid variation was amenable to F+ coliphage typing by a newly developed rapid (<1 minute) particle agglutination immunoassay called the coliphage laxtex agglutination test or CLAT.

- The CLAT is a novel F+ coliphage group-specific, antibody-based, particle agglutination technique for rapid (<60 seconds) and simple detection and grouping of F+ coliphages. CLAT was found to have a sensitivity of 96.4% and 98.2%, and a specificity of 100% and 97.7% for F+ RNA and DNA coliphages, respectively. CLAT is an improved analytical tool for simple, rapid and affordable monitoring of the microbiological quality of drinking, recreational, shellfishing, and other waters to facilitate timely management decisions.

Planned and Recommended Future Work. Future work will assess CLAT and other rapid fecal indicators in conjunction with a 2007 epidemiological study of human health risks from exposure to marine beach water in Orange County, California. If successful, the CLAT will assay water samples in <3 hours on the beach, and will attempt to determine if there are positive associations or correlations of F+ coliphage occurrence, concentrations and groups with GI illnesses and other illnesses in bathers. Other uses of CLAT could be quantifying fecal impacts in drinking and source water in both developed developing countries, and for analysis of produce, irrigation water, meat and poultry to rapidly assess sanitary quality for management decisions.

References

1. **Bartram J, Rees G, ed**. 2000. *Monitoring bathing waters: a practical guide to the design and implementation of assessments and monitoring programmes*. London, E & FN Spon. Published on behalf of the World Health Organization, Commission of the European Communities and US Environmental Protection Agency.

2. **Bartram J, Fewtrell L, Stenström T-A** 2001. Harmonised assessment of risk and risk management for water-related infectious disease. An overview. In: Fewtrell L, Bartram J, ed. Water quality: *Guidelines, standards and health. Assessment of risk and risk management for water-relatedinfectious disease*. London, IWA Publishing, pp. 1–16.

3. **Blanch, A. R., Belanche-Munoz, L., Bonjoch, X., Ebdon, J., Gantzer, C., Lucena, F., Ottoson, J., Kourtis, C., Iversen, A., Kuhn, I., Moce, L., Muniesa, M., Schwartzbrod, J., Skraber, S., Papageorgiou, G. T., Taylor, H., Wallis, J., and J. Jofre.** 2006. Integrated analysis of established and novel microbial and chemical methods for microbial source tracking. Appl. Environ. Microbiol.**72(9):**5915-5926.

4. **Cabelli, V. J., Dufour, A. P., Levin, M. A., McCabe, L. J., and P. W. Haberman.** 1979. Relationship of microbial indicators to health effects at marine bathing beaches. Amer. J. Pub. Health. **69(7):**690-696.

5. **Cabelli, V.** 1983. Health Effects Criteria for Marine Recreational Waters. Cincinnati, OH: U.S. Environmental Protection Agency.

6. **Choi S, and S.C Jiang**. 2005. Real-time PCR quantification of human adenoviruses in urban rivers indicates genome prevalence but low infectivity. Appl Environ Microbiol. 71(11):7426-33.

7. **Colford, J. M. Jr, Wade, T. J., Schiff, K. C., Wright, C. C., Griffith, J. F., Sandhu, S. K., Burns, S., Sobsey, M., Lovelace, G. and S. B. Weisberg.** 2007.Water quality indicators and the risk of illness at beaches with nonpoint sources of fecal contamination. Epidemiol. **8(1):**27-35.

8. **Contreras-Coll N., Lucena F., Mooijman K., Havelaar A., Pierz V., Boque M., Gawler A, Holler C., Lambiri M., Mirolo G., Moreno B., Niemi M., Sommer R., Valentin B., Wiedenmann A., Young V., and J. Jofre.** 2002. Occurrence and levels of indicator bacteriophages in bathing waters throughout Europe. Water Res. **36(20):**4963-4974.

9. **Desmarais, T. R., Solo-Gabriele, H. M., and C. J. Palmer.** 2002. Influence of soil on fecal indicator organisms in a tidally influenced subtropical environment. Appl Environ Microbiol. **68(3):**1165-1172.

10. **Dziuban, E. J., Liang, J. L., Craun, G. F., Hill, V., Yu, P. A., Painter, J., Moore, M. R., Calderon, R. L., Roy, S. L. and M. J. Beach**. 2006. Surveillance for Waterborne Disease and Outbreaks Associated with Recreational Water – United States, 2003 -2004. Mor. Mort. Week. Rep. **55(SS12):**1-24.

11. **Fleisher, J. M., Jones, F., Kay, D., Stanwell-Smith, R., Wyer, M., and R. Morano.** 1993. Water and non-water-related risk factors for gastroenteritis among bathers exposed to sewage-contaminated marine waters. Int. J. Epidemiol. **22:**698–708.

12. **Given, S., Pendleton, L. H., and A. B. Boehm.** 2006. Regional public health cost estimates of contaminated coastal waters: a case study of gastroenteritis at southern California beaches. Environ. Sci. Technol. **40(16):**4851-8.

13. **Haile, R. W., Alamillo, J., Barrett, K., Cressey, R., Dermond, J., Ervin, C., Glasser, A., Harawa, N., Harmon, P., Harper, J.. McGee, C., Millikan, R. C., Nides, M., and J. S. Witte.** 1996 An Epidemiological Study of Possible Health Effects of Swimming in Santa Monica Bay: Final Report. Los Angeles, CA: Santa Monica Bay Restoration Commission.

14. **Harwood, V. J., Gandhi, J. P., and A. C. Wright**. 2004. Methods for isolation and confirmation of *Vibrio vulnificus* from oysters and environmental sources: a review. J. Microb. Methods. **59(3):**301-316.

15. **Haugland, R. A., Siefring, S. C., Wymer, L. J., Brenner, K. P., and A. P. Dufour.** Comparison of *Enterococcus* measurements in freshwater at two recreational beaches by quantitative polymerase chain reaction and membrane filter culture analysis. Water Res. **39(4):**559–568.

16. **Koh, E. G., Huyn, J. H., and P. A. LaRock.** 1994. Pertinence of indicator organisms and sampling variables to Vibrio concentrations. Appl Environ Microbiol. **60(10):**3897-3900.

17. **Liu L, Phanikumar MS, Molloy SL, Whitman RL, Shively DA, Nevers MB, Schwab DJ, and J. B. Rose.** 2006. Modeling the transport and inactivation of E. coli and enterococci in the near-shore region of Lake Michigan. Environ Sci Technol. **40(16):**5022-5028.

18. **Luther K, and R. Fujioka**. 2004. Usefulness of monitoring tropical streams for male-specific RNA coliphages. J Water Health. 2004 Sep;2(3):171-81.

19. **NRDC.** 2006. Testing the Waters 2006: A Guide to Water Quality at Vacation Beaches. National Resources Defense Council. [Online.] http://www.nrdc.org/water/oceans/ttw/titinx.asp

20. **Park, M. H. and M. K. Stenstrom.** 2006. Using satellite imagery for stormwater pollution management with Bayesian networks.Water Res **40(18):**3429-3438.

21. **Rabinovici, J., Bernknopf, R. L., Wein, A.M., Coursey, D.L., and R.L. Whitman.** 2004. Economic and Health Risk Trade-Offs of Swim Closures at a Lake Michigan Beach. Env. Sci. Technol. **38(10):**2742.

22. **Reeves, R. L., Grant, S. B., Mrse, R. D., Copil-Oancea, C. M., Sanders, B. F., A. B. Boehm.** 2004. Scaling and management of fecal indicator bacteria in runoff from a coastal urban watershed in southern California. Environ Sci Technol. 38(9):2637-2648.

23. **San Joaquin VH.** (1994) Aeromonas, Yersinia, and miscellaneous bacterial enteropathogens. Pediatr Ann. **23:**544-548.

24. **Skraber S, Gassilloud B, Schwartzbrod L,and C. Gantzer.** 2004. Survival of infectious Poliovirus-1 in river water compared to the persistence of somatic coliphages, thermotolerant coliforms and Poliovirus-1 genome. Water Res. **38(12):**2927-2933.

25. **Soweid AM and WK Clarkston WK.** 1995. *Plesiomonas shigelloides*: An unusual cause of diarrhea. Am J Gastroenterol **90:**2235-2236.

26. **Soller, J. A., Eisenberg, J. N., DeGeorge, J. F., Cooper, R. C., Tchobanoglous, G., and A. W. Olivier.** 2006. A public health evaluation of recreational water impairment. J Water Health. **4(1):**1-19.

27. **Tuteja, R., Hewitt, J., Clark, M., Wheeler, W., Sams, E., Wade, T., Brenner, K. and A. Dufour.** 2005. Economic Benefits of a New Beach Water Quality Monitoring Method. Poster. [Online.] www.epa.gov/scienceforum/2005/pdfs/ordposter/Hewitt_ORD11.pdf

28. **Youssef, N. M., El-Shamy, H. A., Abou-Donia, H. A., El-Barrawy, M. A., and Y. A. Abdel-Aal.** 1993. A study of Plesiomonas shigelloides from human, pets and aquatic environments. J Egypt Public Health Assoc. **68(3-4):**293-308.

29. **US EPA.** 1976. Quality Criteria for Water. PB-263-943. [Online.] http://www.epa.gov/waterscience/criteria/redbook.pdf.

30. **US EPA.** 1986. Ambient Water Quality Criteria for Bacteria – 1986. EPA440/5-84-002.

31. **Wiedenmann, A.. Krüger, P., Dietz, K., López-Pila, J. M., Szewzyk, R., and K. Botzenhart.** 2006. A Randomized Controlled Trial Assessing Infectious Disease Risks from Bathing in Fresh Recreational Waters in Relation to the Concentration of *Escherichia coli*, Intestinal Enterococci, *Clostridium perfringens*, and Somatic Coliphages. Environ Health Perspect. **114(2):**228–236.

32. **Wade, T.J., Calderon, R.L., Sams, E., Beach, M., Brenner, K.P., William, A.H. and A. P. Dufour.** 2006. Rapidly measured indicators of recreational water quality are predictive of swimming-associated gastrointestinal illness. Environ Health Perspect. **114(1):**24-28.

33. **WHO.** 1999. *Health-based monitoring of recreational waters: the feasibility of a new approach* (*the "Annapolis Protocol"*). Geneva, World Health Organization. Protection of the Human Environment, Water, Sanitation and Health Series. WHO/SDE/WSH/99.1 [Online.]

http://www.who.int/water_sanitation_health/Recreational_waters/Annapolis.pdf

34. **WHO**. 2003. *Guidelines for safe recreational waters. Vol 1 - Coastal and fresh waters*. Geneva, World Health Organization. ISBN 92 4 154580 1 [Online.] http://www.who.int/water_sanitation_health/bathing/srwe1/en/

APPENDICES

Appendix 1. Cost of materials for F+ coliphage culture and CLAT detection/serotyping.

Materials	Bulk cost	Reagent (per gram or ml)	Reagent cost (per gram or per ml)	Reagent per 3x3 MPN sample	Unit cost per 3x3 MPN sample
tryptic soy broth	$ 46	1000	$ 0.05	15	$ 0.70
magnesium chloride	$ 37	500	$ 0.07	10	$ 0.74
antibiotics 1	$ 47	50	$ 0.94	0.15	$ 0.14
antibiotics 2	$ 47	50	$ 0.94	0.15	$ 0.14
polystyrene beads	$ 150	150	$ 1.00	0.012	$ 0.01
toothpicks	$ 0.50	750	$ 0.00	6	$ 0.00
agglutination cards	$ 25	50	$ 0.50	1.000	$ 0.50
6 coliphage antisera	$ 3,000	900	$ 3.33	0.0008	$ 0.02
total	$ 3,353				$ 2.25