Study of anthropogenic viruses in Boston Harbor, Charles River, Cottage Farm CSO Treatment Facility and Deer Island Treatment Plant 1995-2003

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Study of anthropogenic viruses in Boston Harbor, Charles River, Cottage Farm CSO Treatment Facility and Deer Island Treatment Plant: 1995-2003

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EXECUTIVE SUMMARY

In 1995, MWRA undertook the first studies ever conducted of viral pathogens in natural waters within the Boston Harbor watershed. There are currently no regulatory requirements (at either the Massachusetts state level or federal level) or standards for monitoring viruses in wastewater treatment facilities or in receiving waters. However, there is concern that conventional bacterial indicators such as fecal coliform and *Enterococcus*, while appropriate for monitoring the presence of many bacterial pathogens, may not be adequate indicators of the presence of viruses. Viral pathogens are difficult to detect and measure, but more practical methods are evolving. In addition, viral indicators, such as coliphage, have been proposed as potentially appropriate indicators for viral pathogens. Goals of this exploratory study were to:

- learn if evolving virus monitoring methods are practical monitoring tools yet;
- explore if human enteric viruses and viral indicators (coliphages) could be detected in the study area and at what levels;
- develop correlative data among bacterial sewage indicators, anthropogenic viruses, viral indicators and environmental parameters (e.g. antecedent rainfall); and
- satisfy requirements of the Charles River variance and the MWRA Outfall Ambient Monitoring Plan.

There were three distinct project areas which have different aquatic environments and different potential sources of pathogenic viruses: (1) Boston Harbor, (2) Charles River and Cottage Farm combined sewer overflow treatment facility, and (3) Deer Island treatment plant. Samples were collected in wet weather after sufficient rain had fallen to activate at least one CSO facility, and in dry weather, when no rain had fallen for at least 72 hours. Testing was done for the most common water-born viruses—adenovirus, enterovirus, astrovirus, and rotavirus; and two viral indicators—male-specific coliphage and somatic coliphage as well as the bacterial indicators *Enterococcus* and fecal coliform. During the first five years of the study, pathogenic viruses were counted, but after 2000, a more sensitive but non-quantitative technique was used.

Boston Harbor

A total of 138 samples were collected at five different locations in Boston Harbor; 76 samples were after heavy rain, 62 in dry weather. Three sites in Boston's inner harbor were chosen to reflect impacts of CSO: at the mouth of the Charles River near the Prison Point CSO Facility outfall; at the mouth of the Mystic River near the Somerville Marginal CSO Facility outfall; and at the mouth of Fort Point Channel, which is affected by untreated CSO and other sources. Two sites were on beaches, one affected by untreated CSO and stormwater (Carson Beach) and one affected by stormwater (Wollaston Beach).

The analyses detected the presence of pathogenic viruses in about one-third of samples collected. The abundance of pathogens was very low; the highest count was about one virus per 10 liters of water and the overall average for all samples was about one virus per 100 liters of water. Enteroviruses were detected most frequently, followed by rotavirus, astrovirus, and adenovirus. The beach locations had the lowest virus prevalence (25%) and the Mystic River mouth had the highest (39%). Viral prevalence in Boston Harbor is similar to, or somewhat lower than, viral prevalence reported by investigators studying other geographic areas in the U.S. and the world.

Surprisingly, we found no significant difference in the prevalence of pathogenic viruses in wet and dry weather. Near the two CSO treatment facilities there was no difference in pathogen prevalence in samples

collected in wet weather compared to samples collected in dry weather. The viral indicators (non-pathogens) had a different pattern with respect to weather: viral indicators were detected significantly more often after rainstorms than in dry weather at three locations: the mouth of the Charles River, the mouth of Fort Point Channel, and at Carson Beach.

There were no statistically significant relationships among pathogenic virus presence and any of the bacterial or coliphage indicators presumed to predict pathogen presence. Although we had anticipated that coliphages would better correlate with the presence of pathogenic viruses than the traditional bacteria indicators, our data did not confirm this in Boston Harbor. Our inability to relate the presence of viral pathogens to either rainfall or other indicators may be a consequence of the very low abundance of viral pathogens detected and of the episodic nature of the presence of these pathogens in the human population.

Charles River and Cottage Farm CSO Treatment Facility

A total of 91 samples (58 in wet weather, 33 in dry) were collected at six locations in the lower Charles River from the Newton Yacht Club to the "basin (the "basin" is the wider area of the lower Charles used for sailing). Pathogenic viruses were detected in 31% of samples collected in the Charles River. Pathogenic virus counts were low—the highest count was approximately 1 virus per 10 liters of water and the average was about 1.6 virus per 100 liters of water, slightly higher than found in Boston Harbor.

In contrast to Boston Harbor, in the Charles River there was an effect of wet weather on pathogenic virus prevalence (18% dry, 38% wet). There were spatial differences in viral prevalence within the Charles segment, with the lowest prevalence upstream, the greatest prevalence at the BU Bridge, and slightly less prevalence in the "basin." Wet weather detection of viral pathogens at the BU Bridge station was about the same with or without antecedent Cottage Farm discharges, that is, Cottage Farm activations did not increase the prevalence of pathogenic virus in wet weather. Viral pathogens were detected in both wet and dry weather in the "basin," presumably reflecting the effect of the Stony Brook. Adenovirus and enterovirus were the only types of viral pathogens detected in the river, in contrast to the harbor, where all the types of virus tested for were found.

Several samples of Cottage Farm CSO treatment facility influent and effluent were analyzed quantitatively for viral pathogens; the results suggest that treatment at Cottage Farm reduces enterovirus in CSO by about 90%. With the caveat of having only a few samples, coliphage correlated with viral pathogens better than did indicator bacteria in Cottage Farm wastewater. The levels of pathogenic viruses measured in Cottage Farm effluent (about 4 virus MPN per liter) are similar to those found in secondary-treated and disinfected effluent from the Deer Island Treatment Plant (about 5 virus MPN per liter, see below). Three types of pathogenic viruses were found in Cottage Farm combined sewage: adenovirus, enterovirus, and astrovirus. The Cottage Farm Facility assessment study detected pathogenic viruses at the outfall and downstream during one of the two storms studied, none were detected immediately upstream. During the second storm, no pathogenic viruses were detected at any of the three locations. The effluent results for this study did not agree with the receiving water sampling: during the first storm, no pathogenic viruses were detected in the effluent, and in the second storm viruses were detected. These results are consistent with a low level of pathogens, present only sporadically.

Taken together, pathogenic virus sampling results in the Cottage Farm facility and the lower Charles River are consistent with multiple wet weather sources of virus to the upstream reach of this section of the river, likely including stormwater and CSO. Pathogenic viruses were detected in the basin portion of the river in both dry and wet weather, consistent with a more continuous source, probably the Stony Brook.

Deer Island Treatment Plant

Virus sampling was conducted at the Deer Island Treatment Plant (DITP) to learn which pathogenic viruses or viral indicators could be detected in DITP wastewater and at what levels, and to determine the

effectiveness of wastewater treatment processes to remove or inactivate infectious virus and their indicators. Concentrations of cultivatable viruses in DITP wastewater are low, with an arithmetic mean of 5.24 MPN/L and geometric mean of 3.1 MPN/L in final effluent. Decreases in cultivatable virus concentrations in each successive phase of treatment are more modest than for pathogen indicators. Coliphage were most reduced by primary treatment, and least reduced by disinfection. Unlike for Cottage Farm CSO wastewater all the pathogen indicators including coliphage, fecal coliform and *Enterococcus* were poor predictors of virus concentrations in all phases of wastewater treatment at DITP**.**

Summary

Overall, this virus monitoring study confirmed, as expected, that pathogenic viruses could be detected in the Charles River and in Boston Harbor. The types of viruses detected and prevalence of viruses (about 30%) were similar to those reported in other water bodies studied by other investigators. The concentrations of pathogenic viruses were similar to or lower than those reported in other water bodies (including beaches). There are no standards for virus concentrations in Massachusetts waters, but all the MWRA samples collected in the Charles River and in Boston Harbor had virus counts well below Arizona's standards for reclaimed water for partial contact, and the average counts in the Charles River and Boston Harbor were well below Arizona's full-body contact standard for reclaimed water. The data are consistent with multiple sources of pathogens, for example the wet-weather prevalence of viral pathogens near CSO treatment facility discharge locations was the same whether or not an antecedent discharge from the facility occurred, and pathogens were detected in the harbor at about the same prevalence in wet and dry weather.

Pathogenic viruses in wastewater were significantly reduced by treatment at the Cottage Farm CSO treatment facility and at the Deer Island Treatment Plant; on average treated CSO and final secondary effluent had equivalent levels of viruses.

Finally, none of the viral or bacterial indicators correlated well with the presence of pathogenic viruses in wastewater or ambient water, which probably reflects the episodic, highly variable presence of pathogens in human populations. The two viral coliphage indicators were more resistant to disinfection, and appeared to be detectable longer in the environment than were both the bacterial indicators. Therefore, the coliphage indicators may be useful in the Boston area as conservative tracers of wastewater. Because the levels of viral pathogens in this environment are low and difficult and expensive to measure, it is difficult to develop quantitative data. MWRA does not recommend that pathogen monitoring be conducted routinely.

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1.0 INTRODUCTION

Water quality in Boston Harbor has greatly improved over the past fifteen years, as the Massachusetts Water Resources Authority (MWRA) has dramatically reduced pollution from treatment plants and combined sewer overflows. Although this improvement is well documented (Rex *et al.* 2002, Taylor 2004), it is likely that standard methods using bacterial indicators to monitor the potential risk to public health from contaminated waters provide an incomplete picture. MWRA routinely monitors several bacterial indicators in its wastewater and receiving waters—fecal coliform, *Escherichia coli*, and *Enterococcus*. Monitoring these fecal pollution indicators is an important part of protecting public health; the methods have been standardized for many years and are cost-effective.

There is increasing interest within the public health community in measuring the pathogens themselves, rather than pathogen indicators like fecal coliform, particularly given the ongoing development of methods for virus detection and quantification.[1] The bacterial indicators are reasonably effective predictors of the presence of bacterial pathogens. However, viral diseases are a common cause of illness from contaminated recreational waters. Measuring pathogens in natural waters remains difficult and costly, however. There are currently no regulatory requirements (at either the Massachusetts state level or federal level) or standards for monitoring viruses in wastewater treatment facilities or in receiving waters. Methods to detect viral pathogens are continually evolving, making consistent and repeatable measures over time a challenge. Even if reliable methods are developed, the presence of pathogens in human populations—and is thereby in human waste—varies greatly over time as epidemics come and go.

Despite these drawbacks, MWRA undertook pathogen monitoring as a special study to complement its bacterial monitoring, since bacterial indicators alone may not be sufficient to characterize health risk. For example, indicator bacteria may be undetectable in a few days, whereas enteric viruses may persist for several months (Wheeler 1990, Melnick *et al*. 1980), and adenoviruses have been shown to survive longer than most enteric viruses in seawater (Enriquez *et al*. 1995). On the other hand, the presence of commonly used bacterial indicators does not mean that a human source of fecal contamination is necessarily present, because the bacteria are commonly found in many warm-blooded animals (Noble *et al*. 2003). If anthropogenic pathogens were not found in waters where bacterial indicator counts are elevated, this would suggest that sources other than human waste are causing elevated counts.

Other investigations have found pathogenic viruses, including enteroviruses and adenoviruses, in areas including urban rivers, coastal waters, and seawater (Tani *et al*. 1995, Castingnolles *et al*. 1998, Chapron *et al*. 2000, Tsai *et al*. 1993, Girones *et al*. 1993, Abbaszadegan *et al*. 1994, Puig *et al*. 1994, Enriquez *et al*. 1995, Enriquez & Gerba 1995). Enteroviruses (poliovirus, coxsackie virus types A and B, and echoviruses) can cause gastroenteritis, myocarditis and aseptic meningitis (Melnick 1990). Adenoviruses type 40 and 41 can also cause gastroenteritis, but their presence in seawater is typically greatly underestimated because of difficulty isolating them in cell culture. Adenoviruses have been suggested as a good indicator of human fecal pollution since they have been detected in numerous samples contaminated with fecal material (Pina *et al*. 1998). Similarly, the human enterovirus family (poliovirus, coxsackie virus types A and B, and echoviruses) has been found in many waters associated with human fecal contamination (Kopecka *et al*. 1993, Reynolds *et al*. 1998, Griffin *et al*. 1999, Noble & Fuhrman 2001, Jiang *et al*. 2001).

[1] "Pathogens" are microorganisms (bacteria, viruses, fungi, or protozoans) that cause disease. Many waterborne pathogens are spread by human or animal feces. Because the technology to detect pathogens is complicated and costly, their presence is usually inferred by monitoring pathogen indicators. Microbes present in sewage, like *E. coli*, *Enterococcus*, and coliphage are commonly used as indicators.

In 1995, MWRA began the first studies ever conducted of viral pathogens in natural waters within the Boston Harbor watershed. This project focuses on the potential for hazards to public health from anthropogenic viruses contained in combined sewer overflows (CSOs) which discharge into Boston Harbor and the Charles River; and in the Deer Island Treatment Plant discharge to Massachusetts Bay, and assess pathogenic virus levels in wastewater at different stages of treatment. Goals of this exploratory study were to:

- learn if evolving virus monitoring methods are practical monitoring tools yet;
- explore if human enteric viruses and viral indicators (coliphages) could be detected in the study area and at what levels;
- develop correlative data among bacterial sewage indicators, anthropogenic viruses, viral indicators and environmental parameters (e.g. antecedent rainfall); and
- satisfy requirements of the Charles River variance and the MWRA Outfall Ambient Monitoring Plan.

Table 1 shows the targeted human enteric viruses (poliovirus, echovirus, and coxsackie virus), adenovirus, rotavirus, and astrovirus, which are important etiological agents of waterborne diseases (Wyer *et al*. 1995, Noble & Fuhrman 2001, Noble *et al*. 2003).

Coliphages (male specific and somatic) were included to evaluate their potential as indicators. Because coliphages have a structure and size similar to some enteric viruses they have been suggested as indicators of enteric viruses and fecal pollution. Although coliphages occur in fewer numbers in wastewater than do bacterial indicators, they are hardier and persist longer in the environment (Havelaar *et al*. 1990, 1993, IAWPRC 1991, Sobsey 1995, Brion 2003). Coliphages have been routinely detected in domestic, hospital, and treated wastewaters (Funderburg & Sorber 1985). Male specific coliphages are found more commonly in fecally polluted waters (Borrego *et al*. 1987) and somatic coliphages are relatively more prevalent in natural waters, but both male-specific and somatic coliphages are found in human and animal fecal material. Therefore, similar to indicator bacteria, additional analyses would be required to confirm an anthropogenic source if coliphages are detected in the environment.

Finally, the study measured fecal coliform, *E.coli* and *Enterococcus* to better understand how well they indicate the presence of human pathogenic viruses in the Boston Harbor watershed and in wastewater. To evaluate the correlation between the presence of viruses and fecal bacterial indicators, samples for bacteriological and viral analysis were collected concurrently.

Collection of samples for both viral and bacterial indicator analysis in Boston Harbor and its tributaries was conducted by MWRA staff. Bacterial analyses were the responsibility of MWRA. The Waterborne Disease Laboratory at the University of New Hampshire conducted viral analyses, including those for coliphage and human pathogenic viruses.

Table 1. Description of pathogenic viruses and viral indicators monitored.

Pathogenic Virus[1]	Potential Health Effects	Environmental Source	Monitoring Importance
Adenovirus	Respiratory Diseases Conjunctivitis Gastroenteritis Cystitis	Human waste	Common cause of respiratory and gastrointestinal illnesses. Adenoviruses are typically detected at higher levels than enteroviruses in polluted waters and are the most resistant to environmental conditions of the four viruses listed in this table (Gerba *et a*l. 2002, Mahl *et al*. 1975, Enriquez *et al*. 1995).
Enteroviruses	Gastroenteritis Upper Respiratory Disease Meningitis Myocarditis Encephalitis	Human waste	Commonly found in human waste. Second only to rhinoviruses (common cold) in infectious agents to humans (Melnick 1990).
Rotavirus	Gastroenteritis	Human waste	Most common cause of severe diarrhea in children, with 600,000 dying worldwide per year (cdc.gov). Commonly found in human waste and polluted waters.
Astrovirus	Gastroenteritis	Human waste	Astrovirus associated gastroenteritis of children and adults are the second most common cause of viral gastroenteritis (Willcocks *et al.* 1995). Commonly found in human excrement and have been associated with the consumption of shellfish (Cubitt 1991).
Virus Indicator[2]	**Potential Health Effects**	**Environmental Source**	**Monitoring Importance**
Male Specific Coliphage	Non-infectious to humans	Human or animal waste	There are no standards for coliphage, but their relative abundance can be used to indicate fecal contamination from stormwater, CSO, and other sources. They survive longer in the environment than bacterial indicators, and closely mimic enteroviruses in disinfection studies and in environmental persistence. In particular male specific coliphage mimics poliovirus very closely (Sobsey 1995). Other investigators have suggested that coliphages be used as indicators of human viruses (Havelaar *et al.* 1990).
Somatic Coliphage	Non-infectious to humans		

[1]All the pathogenic viruses listed can infect humans by the fecal-oral route, by inhalation, or by direct contact. We attempted to measure norovirus, a cause of gastroenteritis, but never detected it, and stopped monitoring for it because the methods are deemed inadequate. Likewise, a PCR test for hepatitis A was used early in the project. However, the virus was detected extremely rarely, and the test does not allow determination of the viability of the virus. Therefore, we stopped monitoring for hepatitis A.

[2]Coliphages are viruses that infect coliform bacteria, and are typically abundant wherever coliforms are present, e.g. wastewater or stormwater. Coliphages are not infectious to humans, but like bacterial indicators, they have potential for use as a tracer for fecal contamination from multiple sources – though not exclusively human sources. They can multiply in wastewater (while the infected coliform bacteria are still alive) and persist in the environment long after the coliform bacteria have died off. Coliphages therefore function as a highly conservative and sensitive indicator of the presence of fecal contamination.

2.0 LABORATORY AND FIELD METHODS

2.1 Evolution of Laboratory Methods

The monitoring program assessed human enteric viruses, viral indicators, and fecal indicators in wastewater and receiving waters. However, virology methods are rapidly changing. During the course of this multi-year project, our study methods changed as better methodologies emerged. The methods used originally for the analysis of viruses and coliphages in 1995-1999 were at the time the best available technology in environmental microbial detection. The methods changed in 2000 due to the advent of more sensitive environmental detection methods. A summary of parameters measured and methods is below.

Water quality parameters useful to support interpretation of the data (i.e. dissolved oxygen, salinity, turbidity, etc.) were monitored throughout the course of the study.

Analyses of samples from 1995-1999 included:

- Quantification of cultivatable strains of viruses (poliovirus, coxsackie virus, and echovirus) using the total cultivatable virus assay most probable number method (TCVA-MPN) with buffalo green monkey kidney (BGMK) cells. Results were reported as most probable number per liter (MPN/L);
- Additional screening of strains not readily detected by the TCVA-MPN method (Hepatitis A virus and rotavirus) using the direct polymerase chain reaction (PCR) method, with results reported individually for each virus as presence/absence;
- Pan-enterovirus direct PCR screening for enteroviruses;
- Enumeration of both male specific and somatic coliphages, with results reported as plaque forming unit per milliliter (PFU/mL); and
- Enumeration of fecal coliform and *Enterococcus,* with results reported as colony forming units per milliliter (CFU/100 mL).

Analyses of samples from 2000-2003 included:

- Detection of cultivatable strains of viruses (enteroviruses [poliovirus, coxsackie virus, and echovirus], adenovirus 40 & 41, rotavirus, and astrovirus) using the Integrated Cell Culture Nested Polymerase Chain Reaction (ICC-nPCR) assay, with results reported as presence/absence for each specific virus;
- Detection of both male specific and somatic coliphages, with results reported as presence/absence, except for wastewater sample which were enumerated as PFU/ml;
- Enumeration of fecal coliform, *E. coli* and *Enterococcus* bacterial densities. (In 2001 MWRA replaced fecal coliform measurements with *E. coli* in receiving water monitoring.)

From 1995-99 enteric viruses were detected and enumerated by the Total Culturable Virus MPN Assay (TCVA-MPN) (USEPA 1995). This method required samples to be inoculated onto buffalo green monkey kidney cells (BGMK) and then evaluated for virus by visualization of cytopathic effects (CPE). CPE includes rounding, lysis, and vacuolation of the cells when viewed microscopically. However, other work done from the Waterborne Disease laboratory at UNH (Chapron *et al*. 2000) demonstrated that the level of viral contamination was greatly underestimated when using the BGMK cell line alone. Several enteric viruses do not exhibit CPE during their replication cycle, while others such as astrovirus and

rotavirus cannot replicate in this cell line. Both adenovirus and astrovirus require the addition of a proteolytic enzyme for infection of the cells to occur. Hence, many of the epidemiologically important enteric viruses went largely undetected when only the TCVA-MPN method was used.

In 2000, analyses of samples were performed with a more sensitive cell culture method. The integrated cell culture nested polymerase chain reaction (ICC-nPCR) assay incorporates a cell culture step prior to viral detection by PCR followed by nested PCR. The incorporation of a cell culture step permits viral replication resulting in an increase in the number of target nucleic acid copies (Pinto et. al 1995, Chapron *et al*. 2000, Reynolds *et al*. 2001). The cell culture step also reduces the amount of inhibition typically seen in molecular techniques used with environmental samples. ICC-nPCR amplifies target viral nucleic acid sequences with the reverse transcriptase polymerase chain reaction (RT-PCR) for RNA viruses or PCR for DNA viruses followed by a nested polymerase chain reaction (nPCR). The incorporation of nPCR into the assay increases sensitivity and specificity due to the use of primers internal to the RT-PCR or PCR nucleic acid products, thus enabling detection of very low numbers of specific viral particles. ICC-nPCR used two cell lines, BGMK and CaCo-2[2] cells, as well as the addition of a proteolytic enzyme. This was a useful technique for the detection and confirmation of a wide variety of enteric viruses. In addition, the cell culture step provided a means for infectivity testing. Direct PCR detects both infectious and non-infectious viruses and cannot discriminate between them, whereas ICC-nPCR can determine the infectious nature of the viruses by comparing viral levels in the cell lysates to direct PCR on the concentrate.

The ICC-nPCR method is costly and labor intensive, therefore an alternative for routine monitoring is desirable. The detection and enumeration of coliphages (male specific and somatic) was important in this study because of their potential use as inexpensive and technically simple indicators of fecal contamination and other anthropogenic viruses. In this study, coliphages were first (1995-1999) detected using the double agar overlay method on a portion of the sample concentrate (USEPA 2001). This method, while being able to detect both male specific and somatic coliphages, could only use a very small volume of water sample, therefore the detection limit was relatively high. During 2000-2003, the study changed to the modified two-step enrichment procedure (USEPA 2000) to detect male specific and somatic coliphages. The two-step enrichment procedure used a larger sample volume, vastly increasing its sensitivity to detect low numbers of coliphages. In addition, the enrichment method is more sensitive because an initial pre-incubation step allows low numbers of target coliphages to replicate, and the liquid medium is less likely to restrict growth than is the single agar overlay method's semi-solid medium.

When the methods were changed to the more sensitive methods, the form for reporting data also changed. In 1995-1999 all the viral data were reported as a density per liter and in 2000-2003 all the viral results were reported as presence/absence.

[2] CaCo-2 cells (Colorectal adenocarcinoma colon cells) are of human origin and are typically used for propagation of astroviruses. A protolytic enzyme (e.g. trypsin) is added to modify surface proteins on the virus to enable attachment to the MA-104 cells.

2.2 Parameters Measured

Table 2. Monitoring Parameters for Anthropogenic Virus Surveys.

Parameter (Laboratory)	Sample Container	Preservation	Analysis Method	Holding Time	Units
Cultivatable Viruses (UNH) 1995-99	Filtered in field	4°C (cooler with ice)	BGMK	elute within 48 hrs, indefinite at –80°C	MPN/L
Viral Genome Screening (UNH) 1995-99	Filtered in field		PCR	elute within 48 hrs, indefinite at –80°C	Presence/absence
Cultivatable Viruses (UNH) 2000-03	4-L Cubitainer		ICC-nPCR	Process within 72 hrs, indefinite at –80°C	Presence/absence
Coliphages (UNH) 1995-99	Filtered in field		SM9211 D	elute within 48 hrs, indefinite at –80°C	PFU/L
Coliphages (UNH) 2000-03	1-L Sterile Bottle		EPA 1601	Process within 72 hrs, indefinite at –80°C	Presence/absence
Fecal coliform (MWRA)	Sterile 250 mL LDPE bottle		SM9222D or	6-12 hrs	Fecal coliform colonies per 100mL
			SM9222E	72 hrs	
E.coli (MWRA)			EPA1603	6-12 hrs	*E. coli* colonies per 100mL
Enterococcus (MWRA)			SM9230C	6-12 hrs	Enterococci colonies per 100mL
Temperature [a,]	In-situ	NA	probe[b]	NA	°C
pH[f]					Standard pH units
Conductivity[a,]					mS/cm
Salinity[a,c,]					PSU
Dissolved Oxygen[a,]					mg/L
Secchi Depth			[e]		m
Transmissometry[a,]			probe[b]		m-1
Depth[a,]					m
Chlorine Residual[d]	Sterile polypropylene container		Colorimetric method	NA	mg/L

Notes: [a] Boston Harbor hydrographic data measured using Hydrolab Data Sonde 4.
[b] Probes are described in Albro *et al*., 1998.
[c] Density is reported as a calculated value.
[d] Deer Island and Cottage Farm
[e] Lind 1974

NA Not applicable
BGMK Buffalo Green Monkey Kidney cell method
PCR Polymerase Chain Reaction method
SOP Standard Operating Procedure
MPN Most Probable Number
PFU Plaque Forming Unit
PSU Practical Salinity Unit
SM Standard Methods
mS micro Siemens
UNH Processed at the University of New Hampshire
MWRA Processed by MWRA's Department of Laboratory Services

2.3 Sample Collection

Sampling locations are described in Section 3 Results and Discussion, for each study area: Boston Harbor, Charles River and the Cottage Farm CSO Treatment Facility, and Deer Island Treatment Plant.

Surface receiving waters (0-12 inches deep) were collected by either the concentration of 30-40 gallons in the field by microporous filtration (1995-1999) or grab sample in sterile 4-L cubitainers and 1-L bottles (2000-2003) for virus and coliphage analysis. Grab samples for bacteriological analyses were concurrently collected in sterile specimen cups. Samples were shipped on ice to the Waterborne Disease Laboratory at UNH for analyses of human pathogenic viruses and coliphages and to the MWRA Department of Laboratory Services on Deer Island for bacteriological analysis. Specific details on sampling and analytical procedures are provided in Section 12 of the Combined Work Quality Assurance Project Plans for Anthropogenic Virus Surveys (MWRA 1998, 2002).

Dry weather surveys were done when no more than 0.01 inches of rain occurred during the previous 72 hours. Wet weather surveys were done when at least 0.5 inches of rainfall had occurred within a 48-hour period, and when at least one CSO treatment facility was known to have activated.

Harbor and river samples were collected from 0-12 inches (30cm) below the surface. Wastewater effluent samples were dechlorinated with sodium thiosulfate.

2.4 Bacterial Methods

All bacterial sampling and detection methods complied with Standard Methods. Table 2 references the bacteria-specific methods (APHA 1998).

2.5 Viral Analysis 1995-1999

During 1995-1999, water samples of 30-40 gallons (≅ 114-151 liters) were collected using Zeta Plus MW (Cuno, Inc. Meriden, CT) micro wound filters (APHA 1995). These samples were then tested using the TCVA-MPN method (USEPA 1995, APHA 1995). Viruses were eluted from the filters with a 3 % beef extract solution (pH 9, BBL Sparks, MD beef extract powder, 90mM glycine). Eluates were concentrated by organic flocculation (pH 3.5) for 30 minutes followed by centrifugation, 10,000x g for 10 minutes (USEPA 1995). The pellet was resuspended (total volume 20-30 mL) with sodium phosphate buffer (0.1N Na_2HPO_4, pH 9), centrifuged and the supernatant was adjusted to pH 7 for archiving and analysis. Each sample concentrate was passed through a beef extract-treated 0.22-μm syringe filter to remove any microbial contaminants prior to inoculation on BGMK cells. Four 3-mL portions of filtered sample concentrate were each inoculated onto 75 cm^2 flasks of confluent BGMK cells. Flasks were incubated for 90 minutes at 37^{o}C with rocking every 15 minutes. Fifteen mL of serum-free maintenance cell culture media were added to each flask after incubation. Flasks were incubated at 37^{o}C and examined daily for cytopathic effects (CPE) and cytotoxicity for the first three days and then every other day for a total of 14 days. At the end of 14 days flasks were freeze thawed and 10 percent of the first passage was put onto a new cell culture flask of confluent BGMK cells for a second passage. Flasks that exhibited CPE were scored and the MPN/L calculated.

Hepatitis A virus detection, pan-enterovirus screening, and rotavirus detection were done by direct polymerase chain reaction (PCR). One to three mLs of concentrate were purified through a sephadex/chelex spin column. The purified sample was then analyzed according to Abbaszadegan *et al*. (1993). Briefly, 40-100 μL were added to three separate reaction tubes for detection of each specific

virus (HAV, pan-enterovirus, rotavirus). The samples were analyzed by PCR and visualized by agarose gel electrophoresis. Results were reported as presence/absence for each individual virus.

2.6 Coliphage Analysis 1995-1999

Coliphages were enumerated using a portion of the eluted sample for the TCVA-MPN method. The coliphages were detected according to Standard Methods 9211D with the following exceptions: both male specific and somatic coliphages were detected separately by the double agar overlay method (Adams 1959). Samples were added to warm overlay agar containing two mL of log phase bacterial host (*E.coli* CN-13 for somatic or F_{amp} for male specific) and mixed. The overlay mixture was then poured onto tryptic soy agar plates. Plates were incubated for 24 hours at 37^{o}C and observed for plaques. Coliphage density was reported as plaque forming unit per mL (PFU/mL).

2.7 Viral Analysis 2000-2003

All samples from 2000-2003 were evaluated by ICC-nPCR. Four-liter grab samples were concentrated by mixing 40 grams of beef extract powder into the sample. Samples were brought to pH 3.5, mixed for 30 minutes and centrifuged. Pellets were resuspended in 20 mL of 0.15M Na_2HPO_4 (pH 9.5) buffer and centrifuged again. The supernatant was then adjusted to pH 7. Concentrates were filtered through beef extract pre-treated 0.22 µm filters prior to inoculation onto BGMK and CaCo-2 cells (Chapron *et al*. 2000). In 2002 an additional cell line, Ma104's [3] was added. This cell line was used for the propagation of rotavirus. Two 3-mL portions of each sample concentrate were incubated for 30 minutes at 37^{o}C with 5µg/mL or 10 µg/mL of a proteolytic enzyme trypsin (Sigma St. Louis, MO). Samples containing 5 µg/mL trypsin were inoculated onto CaCo-2 (for astrovirus) and Ma-104 (for rotavirus) cells and 10 µg/mL onto BGMK (for adenovirus and enterovirus) cells. The flasks were incubated for 90 minutes at 37°C with rocking every 15 minutes. Trypsin concentrations of 5 µg/mL were used for astrovirus and rotavirus and 10 µg/mL for adenovirus and enteroviruses. Following incubation, 15 mL of serum-free media were added to each flask. The flasks were incubated for 5 days at 37°C. After five days flasks were freeze thawed and cell lysates were pooled. The cell lysates were analyzed using the ICC-nPCR method for enteroviruses, astrovirus, rotavirus and adenovirus 40 and 41 (Chapron *et al*. 2000, Reynolds *et al*. 2001). Results were reported as presence/absence for each individual virus. The laboratory procedures that were followed have been previously published (Chapron *et al*. 2000). Changes to the methods are described below:

Enterovirus RT-PCR/nPCR. Enterovirus RNA was detected by reverse transcriptase polymerase chain reaction (RT-PCR) using an RT primer (5'-ACCGGATGGCCAATCCAA-3') and a PCR primer (5'-CCTCCGGCCCCTGAATC-3') (Puig *et al*. 1994). A 10-µl sample of cell lysate and denature reaction mixture was run at 99°C for 8 minutes and then placed on ice. The reverse transcriptase (RT) mixture was added and run for 42 min at 42°C and 5 min at 95°C. The polymerase chain reaction (PCR) mixture was then added and run at 95°C for 5 minutes, taq polymerase was added, and then subjected to 35 cycles of 95°C, 30s; 55°C, 30s; 72°C, 30s. Final extension was achieved at 72°C for 5 minutes. For nested PCR, 1 µl from each RT-PCR reaction was added to a new tube containing 90 µl of a nested PCR reaction mixture which contained the primers 5'-TCCGGCCCCTGAATGCGGCTA-3' and 5'-GAAACACGGACACCCAAAGTA-3'. Samples were run for 35 cycles of 95°C, 30s; 55°C, 30s; 72°C, 30s yielding a 138 bp amplicon. Twelve µl of each nested PCR product was run and sized on 1.8% agarose gel and stained with ethidium bromide. Molecular weights were determined by comparison with a 1 Kb DNA ladder (Life Technologies). Poliovirus LsC-1-2ab was used as a positive control.

[3] Ma-104's (Monkey African green kidney cells) were used for the propagation of rotavirus. A protolytic enzyme (e.g. trypsin) is added to modify surface proteins on the virus to enable attachment to the Ma-104 cells.

Adenovirus PCR/nPCR. The primers used were specific for Adenovirus type 40 and 41. Changes to the procedure described above included omission of the RT step and the primers (5'-GCCGCAGTGGTCTTACATGCACATC-3') and (5'-CAGCACGCCGCGGATGTCAAAGT-3') (Puig *et al*. 1994). A 10-µl sample of cell lysate was denatured at 99°C for 8 min. A 90-µl (final volume) PCR mixture was added to the denatured sample. The PCR parameters were the same as described above. The nested procedure used was the procedure described above. The primers utilized were (5'-GCCACCGAGACGTACTTCAGCCTG-3') and (5'-TTGTACGAGTACGCGGTATCCTCGCGGTC-3). These primers yield a 142-bp amplicon. Adenovirus type 40 and 41 were used as positive controls.

Astrovirus RT-PCR/nPCR. The primers used were specific for human astrovirus, RT primer 5'-GTAAGATTCCCAGATTGGT-3' and PCR primer 5'-CCTGCCCCGAGAACAACCAAG-3'. A 10-µl sample of cell lysate was denatured with 0.5 µl each of 0.05 M EDTA and downstream primer at 99°C for 8 min. The RT mixture was added and run for 42 min at 42°C and 5 min at 95°C. After addition of PCR mixture the parameters were 95°C, 5 min hot start, followed by 35 cycles of 95°C, 30s; 56°C, 30s; 72°C, 30s; with a final extension at 72°C for 5 minutes. For nPCR, the procedure was the same as described above, but the primers used were 5'-CCTTGCCCCGAGCCAGAA-3' and 5'-TTGTTGCCATAAGTTTGTGAATA-3'. These primers yield a 143- and/or 183-bp amplicon. Astrovirus serotype 2 was used as a positive control.

Rotavirus RT-PCR/nPCR. The primers used were specific for Rotavirus WA strain. The procedure was the same as the astrovirus RTPCR/nPCR with the primers, RT primer 5'-ATAGAAGACAGCGCACCGGATTTG-3' and PCR primer 5'-ACAGACTTTCATTTGCGTCCGCAA-3'. The PCR parameters were 95°C, 5 min hot start followed by 35 cycles of 95°C, 30s, 52°C, 30s, 72°C, 30s, with a final extension at 72°C for 5 minutes. The nPCR procedure used the primers, 5'-GACGCATCAACTGAAATAATAAAC-3' and 5'-TGCACCAGCGAACATACAGC-3'. These primers yielded a 300- bp amplicon. Rotavirus WA strain was used as a positive control.

2.8 Coliphage Analysis 2000-2003

Analyses for coliphages were done on 1-L grab samples by the modified two-step enrichment method (USEPA 2000). Aliquots of 500 mL portions of the water samples were analyzed for male specific and somatic coliphages. Each sample was analyzed for male specific coliphage with *E.coli* $_{\text{Famp}}$ and for somatic coliphage with *E.coli* CN-13. Twenty-five mL of concentrated tryptic soy broth (300g/L), 6.25 mL 4M MgCl2-6H2O, 3 mL log phase bacterial host, and 5 mL host specific antibiotic (0.04M nalidixic acid for CN-13 or a mixture of 0.001M streptomycin and 0.004M ampicillin for Famp) were added to the samples and mixed. Samples were incubated for 24 hours at 37^{o}C. Between 10-20 µl of sample were then spotted onto pre-poured tryptic soy agar plates containing log phase host bacterium. Spot plates were incubated for 24 hours at 37^{o}C and examined for lysis zones on the bacterial layer. Roughly 50% of the lysis zones were plucked and reconfirmed. Coliphage results were reported as presence/absence.

3.0 RESULTS AND DISCUSSION

3.1 Boston Harbor

Sampling locations in Boston Harbor were chosen by MWRA to provide data on areas where the highest potential for health risks occurs, such as bathing beaches and shellfish resources and/or proximity to treated or untreated CSOs and stormwater.

These sites had additional selection criteria that included: historically high levels of indicator bacteria (CDC 1991), resource areas; and background water quality. Antecedent rainfall, rainfall depth and intensity, time lag prior to initiation of sampling, and maximum allowable sampling window after rainfall were all considered with the development of the sampling logistics (Rex 1989,1993, Leo *et al*. 1994). Sampling was conducted in both wet and dry weather. The sites are listed in Table 3; locations are mapped on Figure 1.

Table 3. Virus sampling locations in Boston Harbor.

MWRA Location Code	Coordinates		Location Description*
	Latitude Degrees	Longitude Degrees	
014	42.3705	-71.0515	Inner Harbor, at mouth of Charles River
052	42.3938	-71.0758	Inner Harbor, Mystic River mouth, below Earhart Dam
019	42.3590	-71.0448	Inner Harbor at Mouth of Fort Point Channel
036	42.3265	-71.0458	Carson Beach (near McCormack Bathhouse)
047	42.2689	-71.0011	Wollaston Beach (in Quincy Bay)

*All locations are affected by stormwater and CSO discharges except Wollaston Beach, which is affected by stormwater discharges only.

Inner Harbor at Mouth of Charles River. The Inner Harbor is designated SB(CSO) - fishable/swimmable/CSO. Recreational boating is the primary exposure route. Swimming standards are exceeded sometimes after rainfall events (Coughlin, in prep). The Charles and Mystic Rivers, drainage from Charlestown, East Boston, and the North End, and untreated CSOs and storm sewers, discharge into this area. The largest discharge is from the Prison Point CSO Treatment Facility (MWR203), which screens, chlorinates, and dechlorinates combined sewage during wet weather before discharging immediately downstream of the Charles River Dam. Station 014, located in the upper Inner Harbor near the Charles River mouth, characterizes the effects of this treated CSO discharge, untreated CSO discharge as well as contributions from the nearby Mystic and Chelsea Rivers.

Inner Harbor, mouth of Mystic River. The lower Mystic River (tidal segment below Amelia Earhart Dam to the upper Inner Harbor) is classified SB(CSO) - fishable/swimmable/CSO. Much of the waterfront is industrial and it is a heavily used shipping channel. The swimming standard and the boating standard can be exceeded during larger storms (Coughlin, in prep). The Somerville Marginal CSO Treatment Facility discharges below the Earhart Dam at MWR205 and provides screening, chlorination and dechlorination prior to discharge. Sampling at station 052, located just below the Earhart Dam near the MWRA 205 outfall, was conducted to assess the influence from the CSO and sources upstream in the Mystic River.

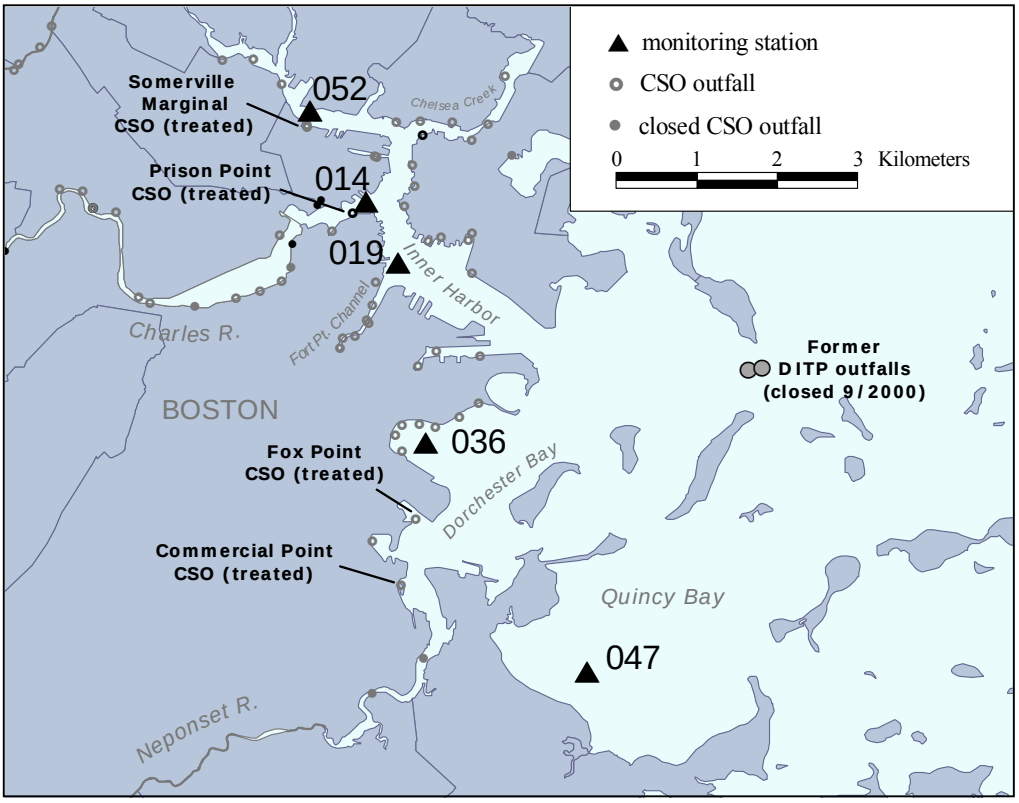

Figure 1. Boston Harbor sampling locations.

Inner Harbor, Fort Point Channel. Fort Point Channel is on the south side of the Inner Harbor and separates South Boston from downtown Boston and the North End. It is also classified SB(CSO). Potential risks to human health are associated primarily with recreational boating. The swimming standard can be exceeded in both wet and dry conditions, and the boating standard is exceeded after storms. This segment is heavily impacted by untreated CSO flows and storm water. It is the receiving water for the largest untreated CSO in the system (BOS070), which discharges at the head of the channel. Dilution of the CSO flow from BOS070 is approximately 10:1 (Ayuso & Adams 1994). Residence time of water in the channel has been estimated to be between 1 and 2.5 days. Although a few samples were collected at the head of the channel, these samples are not included in the data analysis because construction activities prevented continued sampling at that location. Sampling for Fort Point channel was done at the mouth of the channel at Station 019.

Northern Dorchester Bay, Carson Beach. Northern Dorchester Bay is classified SB (fishable swimmable) with restricted shellfishing. A large portion of the shoreline is public beach. The potential exposure pathways for human health risk are from ingestion of water during primary and secondary recreational contact, and from consumption of shellfish. Both swimming and shellfishing standards are generally met under dry weather conditions, but are exceeded after some rain events. Seven untreated CSOs discharged subtidally into Northern Dorchester Bay during this study (one CSO, BOS 087, has since been closed). Station 036 was included because of its proximity to untreated CSO and storm water discharges and because of heavy recreational use.

Quincy Bay, Wollaston Beach. Most of Quincy Bay is classified SB, a portion of Quincy Bay along the northerly shore of Hough's neck is classified SA. Quincy Bay is actively shellfished, and Wollaston

Beach is a popular recreational area. The potential exposure pathways for human health risk are from ingestion of water during primary and secondary recreational contact, and from consumption of shellfish. Water quality at Wollaston Beach is affected by eight storm drains, which discharge intertidally. The beach often does not meet swimming standards in both wet and dry weather. Sampling was conducted near Wollaston Beach at Station 047 to assess whether viruses can be found in recreational waters primarily affected by storm discharges.

Boston Harbor Results

We used quantitative methods for cultivatable virus and coliphage during the first five years of this study, before switching to more sensitive but non-quantitative methods in 2000. Table 4 contains the descriptive statistics for Boston Harbor from 1995-2000 for viruses, from 1995-2000 for fecal coliform and *Enterococcus*, and from 2001-2003 for *E. coli,* overall and for wet and dry weather. The table illustrates several characteristics of the data: cultivatable virus counts were consistently low, with a maximum of less than 1 virus per 10 L, and the error for quantifying cultivatable virus (especially at such low levels) is relatively high because of the low number of viruses present. Therefore it was deemed reasonable to change virus methods to the more sensitive and specific presence/absence methods that were used after 2000.

Table 4. Average concentrations of cultivatable anthropogenic virus, coliphage, and bacteria indicators, in Boston Harbor in wet and dry weather.

Parameter		N	Range	Mean (SD)	Geometric Mean
Cultivatable virus (MPN PFU/L)	Overall	65	0-0.096	0.011 (0.026)	0.0022
	Dry	33	0-0.090	0.016 (0.029)	0.0031
	Wet	32	0-0.096	0.007 (0.022)	0.0016
Male Specific Coliphage (PFU/L)	Overall	65	0-10.5	0.51 (1.8)	0.027
	Dry	33	0-2.2	0.07 (0.38)	0.012
	Wet	32	0-10.5	0.96 (2.4)	0.063
Somatic Coliphage (PFU/L)	Overall	65	0-150	7.2 (25.4)	0.066
	Dry	33	0-100	3.1 (17.4)	0.019
	Wet	32	0-150	11.3 (31.5)	0.24
Enterococcus (CFU/100mL)	Overall	134	0-11,000	260 (1,200)	17
	Dry	57	0-8,000	180 (1,100)	8
	Wet	77	0-11,000	320 (1,300)	30
E.coli (CFU/100mL)	Overall	28	0-31,000	1,300 (5,900)	24
	Dry	14	0-400	55 (120)	11
	Wet	14	0-31,000	2,500 (8,300)	50
Fecal Coliform (CFU/100mL)	Overall	109	0-6,700	340 (1,100)	34
	Dry	44	0-6,600	420 (1,400)	14
	Wet	65	0-6,700	290 (870)	60

MPN= Most probable number
PFU= Plaque-forming units (each PFU represents at least one viral particle)
CFU = Colony-forming units (each CFU represents at least one bacterial cell)
Viral results only 1995-2000; Bacterial results from 1995-2003
For comparison, Arizona reclaimed water quality standard limit for partial body contact was 125 PFU/40L (3.125 PFU/L) and the limit for full body contact was 1 PFU/40L (0.025 PFU/L) (Arizona, 2001).
"Wet" is ≥ 0.5 inches in previous 48 hours; "Dry" ≤ 0.01 inches of rainfall in previous 72 hours.

The remaining results and analyses presented below are based on presence-absence data. Tables 5 and 6 and Figure 2 show the proportion of positive results for the presence of human pathogenic viruses and coliphages in Boston Harbor from 1995-2003. For pathogenic viruses, "positive" means that at least one of the tests for any of the pathogens tested was positive, including tests that do not discriminate between viable and non-viable viruses. Overall, 29% of the 138 samples collected from Boston Harbor tested positive for pathogenic virus presence (Table 5). Overall there was no significant difference between the percent of positive samples found in dry vs. wet weather (chi-square = 0.29, p = 0.59) for anthropogenic viruses.

At individual locations (Table 6, Figure 2), the two beaches had the fewest positive tests for pathogenic viruses (25%), and the Mystic River site (Station 052) had the most (39%). Wet weather samples collected near the discharge locations for the Somerville Marginal CSO Treatment Facility (Station 052) and the Prison Point CSO Treatment Facility (Station 014) were further categorized by whether or not an antecedent discharge from the facility had occurred. Most of the wet weather samples at these sites were collected after a discharge (13 of 16), while most of the positive tests for pathogenic viruses and coliphages occurred in this group.. A contingency table analysis showed no significant differences between wet and dry weather samples for pathogenic viruses at any station.

Indicator viruses (coliphages) were detected more often than pathogenic viruses. This would be expected for indicators, which are more abundant. The two beaches (Stations 036 and 047) had the lowest proportion of positive samples for coliphage. Table 7 summarizes the results of contingency table analyses for the effect of wet weather for both types of coliphage. In contrast to the pathogenic viruses, both types of coliphages at two Inner Harbor stations (014 and 019) had significantly more positive tests in wet weather than in dry weather. Carson Beach, Station 036, had significantly more positive tests for male specific coliphage in wet weather. These results are consistent with stormwater and/or CSO sources of coliphage; it is not possible to distinguish which wet weather source is predominant.

Table 5. Overall proportion of positive virus samples in Boston Harbor, wet vs. dry weather.

Parameter	Overall	Wet Weather	Dry Weather
Viral Pathogens	41/138 (29%)	22/76 (29%)	19/62 (31%)
Male Specific Coliphage	80/138 (58%)	54/76 (71%)	26/62 (42%)
Somatic Coliphage	81/138 (59%)	48/76 (63%)	33/62 (52%)

"Wet weather" is $\geq$ 0.5 inches in previous 48 hours; "Dry weather" is $\leq$ 0.01 inches of rainfall in previous 72 hours.

Table 6. Proportions of positive virus samples in Boston Harbor by sampling station, wet vs. dry weather.

Station	Parameter	Overall (Positive /Total)	Wet Weather (Positive/Total)		Dry Weather (Positive/Total)
014 Inner Harbor, at Charles River Confluence	Pathogenic virus	8/28 (28%)	All 4/16 (25%)		4/12 (33%)
	Coliphage	20/28 (71%)	All 14/16 (87%)		6/12 (50%)
052 Inner Harbor, Mystic River mouth at MWR205 (Somerville Marginal CSO Facility)	Pathogenic virus	11/28 (39%)	All 6/16 (37%)	MWR205 activation: 5/13 No MWR205 activation: 1/3	5/12 (42%)
	Coliphage	21/28 (75%)	All 12/16 (75%)	MWR205 activation: 11/13 No MWR205 activation: 2/3	9/12 (75%)
019 Inner Harbor, mouth of Fort Point Channel	Pathogenic virus	8/27 (30%)	4/15 (27%)		4/12 (36%)
	Coliphage	19/27 (70%)	13/15 (87%)		6/12 (50%)
036 Carson Beach (McCormack Bathhouse, near BOS086)	Pathogenic virus	7/27 (26%)	4/14 (29%)		3/13 (23%)
	Coliphage	18/27 (67%)	11/14 (79%)		7/13 (54%)
047 Wollaston Beach	Pathogenic virus	7/28 (25%)	4/15 (27%)		3/13 (23%)
	Coliphage	15/28 (54%)	8/15 (53%)		7/13 (54%)

"Wet weather" is $\geq$ 0.5 inches in previous 48 hours; "Dry weather" is $\leq$ 0.01 inches of rainfall in previous 72 hours.

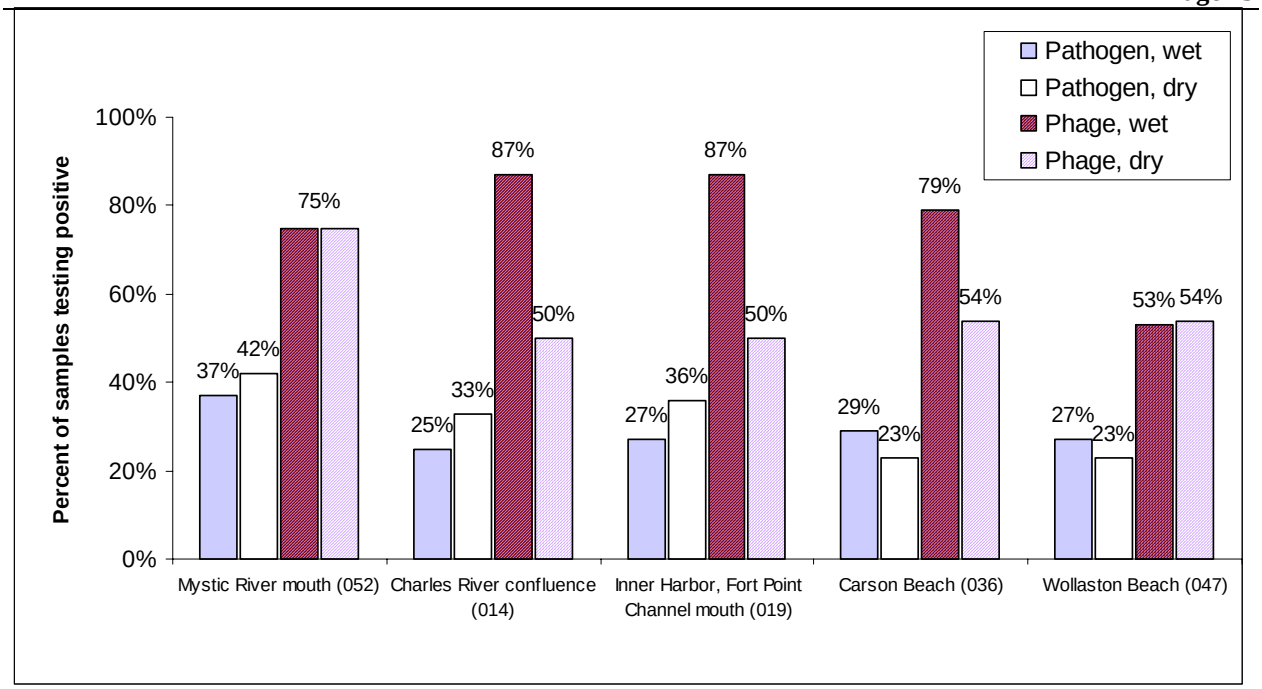

Figure 2. Percent of samples testing positive in wet and dry weather for pathogenic virus and coliphage in Boston Harbor, 1995-2003.

Table 7. Comparison of coliphage results in wet vs. dry weather by station, Boston Harbor.

Station	Coliphage type	Chi-square	P (Positive wet > Positive dry)
014 Inner Harbor, at Charles River Confluence	Male-specific	6.1	0.013
	Somatic	4.0	0.045
052 Inner Harbor, Mystic River mouth at MWR205	Male-specific	1.6	NS
	Somatic	<1	NS
019 Inner Harbor, mouth of Fort Point Channel	Male-specific	5.3	0.02
	Somatic	5.1	0.02
036 Carson Beach near CSO BOS086	Male-specific	5.4	0.02
	Somatic	<1	NS
047 Wollaston Beach	Male-specific	<1	NS
	Somatic	<1	NS

Statistical tests of association among parameters. One objective was to learn if any of the bacterial or viral indicators could predict the likelihood of the presence of a viral pathogen. Spearman's rank order correlations (nonparametric) were done among pairs of variables, with data from all harbor stations and weather conditions included. (Analysis results are shown in Appendix A.) None of the indicator variables

were strong predictors of the pathogenic virus. The strongest relationship with pathogenic virus was with *Enterococcus*, but even that correlation was very weak ($r_s = 0.28$, $t = 2.25$). The correlation between somatic coliphages and pathogenic virus was weak and barely significant at $\alpha=0.05$, ($r_s= -0.21$, $t = 1.73$)—because the relationship was negative, the correlation is likely spurious. Correlations between pathogenic virus and male-specific coliphage, and fecal coliform were not significant. The strongest correlation was between the two types of coliphage ($r_s = 0.72$). Contingency table analyses were used to assess whether any of the indicator variables were associated with the test for any pathogenic virus. Chi squared distributions ($\alpha=0.05$) were used for analysis of all Boston Harbor results from 1995-2003, incorporating both viral methods (Appendix A). Chi squared analysis found no relationship between human virus presence/absence and the presence/absence of either coliphage ($\chi^2 = 6.899$). Similar analyses were carried out for the presence of each type of coliphage separately, *Enterococcus* at levels greater than 104 col/100 mL, *Enterococcus* levels greater than 35 col/100 mL, and fecal coliform greater than 200 col/100 mL. None of these indicators were significantly associated with the presence of human pathogenic viruses in Boston Harbor samples.

Types of viruses detected. Figure 3 shows the variation in prevalence of different viruses as percentages of positive tests for different tests for viruses. BGMK and ICC methods detect living virus, while PCR detects genetic sequences in the target virus and cannot tell whether the virus is potentially infectious. The three different tests for enteroviruses (cultivatable virus BGMK, enterovirus ICC, and pan-enterovirus screen) were most frequently positive, while norovirus was never detected during the period it was monitored, and hepatitis A was found in only 3% of samples. Because of these low detection rates, testing for norovirus and hepatitis A was discontinued in September 1995 and December 1999, respectively. Less than 10% of samples detected adenovirus, astrovirus, and rotavirus by ICC. Rotavirus was detected by PCR in 14% of samples—it is likely that the differences in the detection rates by the two methods reflect the fact that PCR will detect both infectious and non-infectious virus.

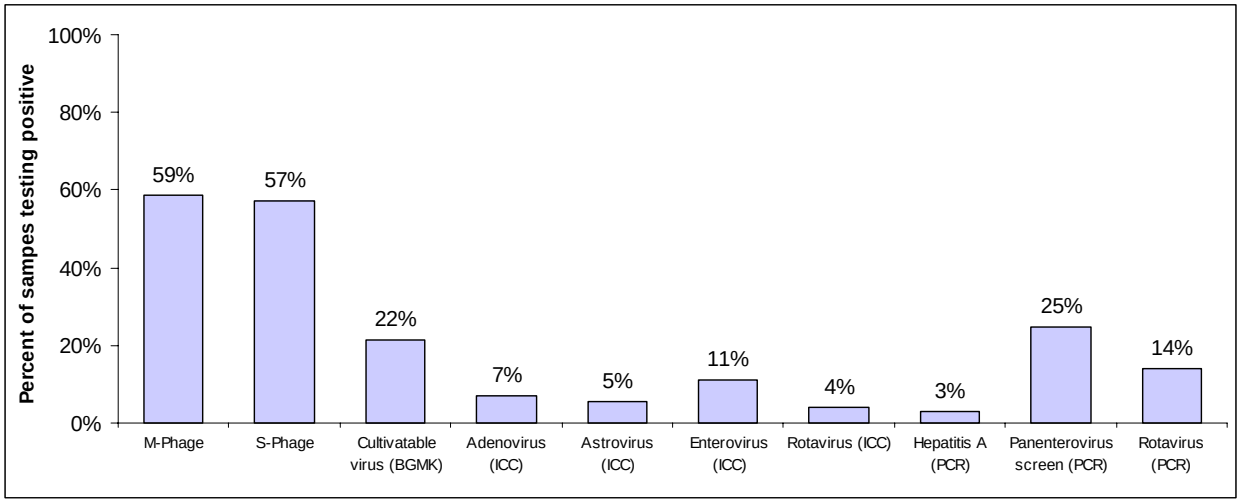

Figure 3. Types of viruses present in Boston Harbor, and percent of samples that tested positive by different analyses for viruses, 1995 - 2003.

Summary of Boston Harbor virus monitoring

The study design specifically targeted the locations and weather conditions (heavy rain) thought to be most likely to yield positive results for pathogenic viruses. The locations were in areas known to be impacted by CSO and/or contaminated stormwater. Dry weather samples were also collected.

Pathogenic virus counts were low—the highest MPN count was less than 1 PFU/10L (0.096 PFU/L), and the average was 0.011 PFU/L (about 1 virus PFU/100 L). The authors are aware of only one state, Arizona, that has had regulatory limits for virus levels in recreational waters; those limits were for reclaimed water. Before 2001, (when Arizona eliminated its pathogenic virus monitoring requirement for reclaimed waters), the Arizona reclaimed water quality standard limit for partial body contact was 125 PFU/40L (3.125 PFU/L) and the limit for full body contact was 1 PFU/40L (0.025 PFU/L) (Arizona, 2001). All the MWRA samples collected in Boston Harbor had counts well below Arizona's previous standard for partial contact, and the average count in Boston Harbor was well below Arizona's previous full-body contact standard for reclaimed water. Compared to studies in other areas, the Boston Harbor data are at the lower end of the ranges of human virus counts, with a high of 0.1 PFU/L. Griffin *et al* 2003 reported that counts ranged from <0.01/L at a beach to 13,000/L near a sewage outfall in Hawaii; and at most locations counts were between 0.01/L to 20/L).

Overall, pathogenic viruses were detected in less than one-third (29%) of samples. This is similar to virus prevalence rates reported at beaches in Italy and California, although higher than the 8% found at beaches in Hawaii. Virus prevalence higher than we found in Boston Harbor has been reported in Galveston Bay, Texas (40-59%); at beaches in Patras, Greece (83-90%); and in the Florida Keys (79-93%) (Griffin *et al*, 2003). Of the human viruses tested, enteroviruses were most frequently detected—in up to 26% of harbor samples.

There were no significant differences between the rates of viral pathogen detection or viral pathogen densities in dry and wet weather. This is in contrast to typical patterns of indicator bacteria in Boston Harbor, which are generally found at higher levels in wet weather. The typically "spiky" distribution of pathogenic viruses in time, the relatively low numbers of pathogenic viruses present, coupled with strong tidal mixing in the harbor and the perhaps slower rates of die-off or settling of pathogenic viruses may obscure the expected pattern of greater prevalence in wet weather.

Two harbor sampling locations (Stations 014 and 052) were near discharges from CSO treatment facilities (Prison Point and Somerville Marginal, respectively). Pathogenic virus prevalence in wet weather was not higher than in dry weather, implying that there are multiple sources of pathogens.

Unlike pathogenic viruses, coliphages at three harbor locations (mouth of Charles River, mouth of Fort Point Channel, and Carson Beach) had significantly higher counts in wet weather. There were no significant relationships among viral pathogen presence and any of the bacterial or coliphage indicators presumed to predict pathogenic virus presence. Although we had anticipated that coliphages would better correlate with the presence of viral pathogens than the traditional bacteria indicators, our data did not confirm this.

3.2 Charles River and Cottage Farm Combined Sewer Overflow Treatment Facility

This section discusses the virus monitoring of the lower Charles River and MWRA's Cottage Farm CSO Treatment Facility which discharges into that segment. Exploratory virus monitoring was conducted to:

1. learn whether pathogenic viruses and indicator viruses (coliphage) could be detected in the lower Charles River and in Cottage Farm influent and effluent, and if so at what levels;
2. explore the relationship between conventional bacterial indicators and/or coliphage indicators to the presence of viruses in the Charles River and in the Cottage Farm effluent; and
3. evaluate whether newer methods for monitoring for pathogenic viruses and indicator viruses in natural waters and in treated combined sewage are practicable for routine monitoring.

Also, this portion of MWRA's Virus Synthesis Report was prepared to satisfy condition in B.(3) in the Variance for the MWRA CSO Control Plan in the Charles River Basin: "…MWRA's analysis shall…assess pathogens to the extent such information is available or developed."

Virus sampling was conducted in the Charles River during a period when overall water quality in the Charles River was improving, due to improvements in MWRA's sewage system that reduced CSO volume and to community efforts to reduce CSOs and address illegal sewage discharges to the river. In addition, implementation of MWRA's CSO Plan for the Charles River is significantly reducing the volume and frequency of CSO discharges.

MWRA's Cottage Farm CSO Treatment Facility contributes approximately 60% of the CSO flow to the lower basin (MWRA 1997 FEIR). The discharge is screened, chlorinated and, since 2002, dechlorinated prior to discharge. Cottage Farm effluent is diluted approximately 1.5:1 within about 250 meters of the outfalls (Ayuso & Adams 1994). Samples were collected in influent and effluent at the Cottage Farm CSO Treatment Facility to assess the presence of viruses and virus indicators in treated and untreated CSO. The Cottage Farm Facility Assessment Study surveyed the presence of viruses in CSO before and after treatment and in the receiving water during Cottage Farm discharges (MWRA 2004 SEIR).

3.2.1 Charles River

The lower Charles River Basin is designated Class B, with uses defined as fishable/swimmable. The principal exposure pathway for human health risk is from ingestion of water during secondary contact recreation, primarily sailboarding and boating. There are no designated beaches in the segment.
This study measured anthropogenic viruses, coliphages, and bacterial indicators during wet and dry weather. Parameters measured are listed in Table 2. Sampling locations included one upstream of all CSOs for background levels, four in areas affected by untreated CSOs, and one at a site affected by the Cottage Farm treated CSO. The sites chosen are shown in Figure 4 and listed in Table 8.

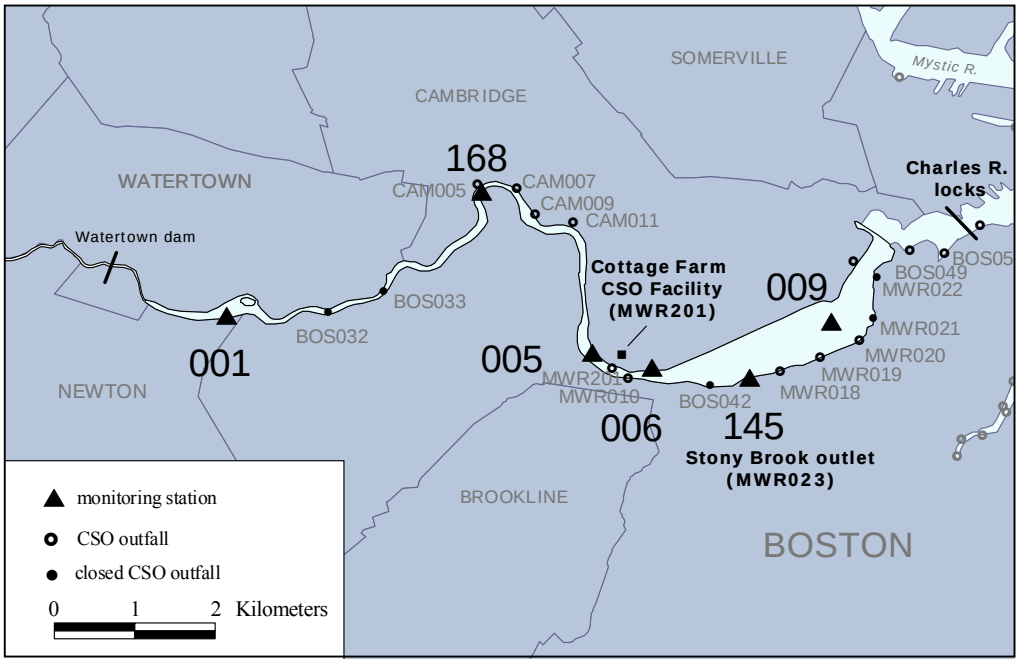

Figure 4. Charles River Sampling Locations.

Upstream. Station 001 is upstream of CSO influences, but does receive stormwater runoff. We used it as an indication of "background" levels of viruses.

CSO CAM-005. **Station 168** attempted to measure the effect of untreated CSOs in the Charles that would activate only rarely after CSO planned conditions were in place.

Magazine Beach. Station 005 is downstream of several CSOs, but upstream of the Cottage Farm CSO facility.

Boston University Bridge. Station 006 is downstream of the Cottage Farm CSO treatment facility and opposite an untreated CSO discharge, MWR 010, and generally reflects the combined effects of Cottage Farm, upstream stormwater and CSO, and MWR 010. MWR010 rarely activates, but its discharge may affect water quality in the region of the Cottage Farm outfalls during heavy storms. Station 006, also called CFDA, was also one of three locations sampled during the Cottage Farm Facility assessment study.

Charles River at Stony Brook. Station 145 was thought to reflect a "worst case," the location in the lower Charles where pathogenic viruses were most likely to be detected. MWR023, the Stony Brook outfall, discharges a large volume of Stony Brook base flow, which in wet weather includes combined sewage and storm water. This location has elevated bacteria counts in dry weather, indicating dry weather sources of contamination (Coughlin, in prep).

Mid-Charles River Basin. Station 009 was sampled to measure water quality in the area most frequently used for recreation, but not immediately adjacent to a pollution source. Typically, this is the area with the best bacterial water quality in the lower Charles (Coughlin, in prep).

Table 8. Charles River sampling locations.

MWRA Location Code	Latitude Degrees	Longitude Degrees	Location Description
001	42.3592	-71.1701	Upstream of CSOs at Community Rowing Dock, near Newton Yacht Club
168	42.3735	-71.1332	At CAM005 outfall
005	42.3551	-71.1155	Magazine Beach downstream of CAM005, CAM007, CAM009, CAM011, upstream of Cottage Farm outfall
006	42.3525	-71.1085	BU Bridge, downstream of Cottage Farm MWR201 outfalls; across the river from MWR010
145	42.3519	-71.0920	Stony Brook outfall, MWR023
009	42.3575	-71.0822	Lower Basin

Charles River results

We used quantitative methods for cultivatable virus and coliphage during the first five years of this study, before switching to more sensitive but non-quantitative methods in 2000. Table 9 contains descriptive statistics for the Charles River from 1995-2000 for cultivatable viruses and coliphages, from 1995-2000 for fecal coliform and *Enterococcus*, and from 2001-2003 for *E. coli,* overall and for wet and dry weather. Cultivatable virus counts were consistently low, with a maximum of slightly more than 1 virus per 10 L. Therefore it was reasonable to change virus methods to the more sensitive and less specific presence/absence methods that were used after 2000.

Table 9. Average concentrations of cultivatable anthropogenic virus, coliphage, and bacteria indicators, in Charles River in wet and dry weather.

Parameter		N	Range	Mean (SD)	Geometric Mean
Cultivatable virus (MPN/L)*	Overall	38	0-0.12	0.016 (0.030)	0.0033
	Dry	18	0-0.12	0.013 (0.031)	0.0024
	Wet	20	0-0.11	0.019 (0.019)	0.0044
Male Specific Coliphage (PFU/L)	Overall	38	0-2,800	91 (460)	2.4
	Dry	18	0-4.4	0.44 (1.1)	1.3
	Wet	20	0-2,800	170 (620)	4.5
Somatic Coliphage (PFU/L)	Overall	38	0-270	12 (47)	1.9
	Dry	18	0-100	6 (23)	1.4
	Wet	20	0-270	18 (60)	2.3
Enterococcus (CFU/100mL)	Overall	75	0-5,100	710 (1,200)	120
	Dry	33	0-560	74 (130)	22
	Wet	42	0-5,100	1,200 (1,400)	440
E.coli (CFU/100mL)	Overall	19	30-12,000	1,700 (2,900)	550
	Dry	7	30-520	260 (190)	170
	Wet	12	30-12,000	2,600 (3,400)	1,100
Fecal Coliform (CFU/100mL)	Overall	56	0-9,400	1,500 (2,300)	360
	Dry	26	0-1,400	220 (300)	90
	Wet	30	15-9,400	2,600 (2,700)	1,200

MPN= Most probable number; PFU= Plaque-forming units (each PFU represents at least one viral particle)
CFU = Colony-forming units (each CFU represents at least one bacterial cell)
Viral results only 1995-2000; Bacterial results from 1995-2003
For comparison, Arizona reclaimed water quality standard limit for partial body contact was 125 PFU/40L (3.125 PFU/L) and the limit for full body contact was 1 PFU/40L (0.025 PFU/L) (Arizona, 2001).
"Wet" is ≥ 0.5 inches in previous 48 hours; "Dry" is ≤ 0.01 inches of rainfall in previous 72 hours.

The results and analyses that follow are based on presence-absence data collected from 1995-2003. For viral pathogens, "positive" means that at least one of the tests for any of the pathogenic viruses tested was positive. Overall, 31% of the 91 samples collected from the Charles River tested positive for viral pathogen presence (Table 10). Although the overall prevalence of pathogenic viruses was similar to that in Boston Harbor, the pattern with respect to rainfall was different. Unlike in Boston Harbor, we found an increase in viral pathogen prevalence in wet weather in the Charles, with 38% of samples testing positive in wet weather compared to 18% in dry weather. Table 11 shows the virus and coliphage prevalence by station, and, in wet weather, what proportion of samples were taken shortly after a Cottage Farm activation. Five complete wet weather surveys of all the Charles River stations were performed. Additional wet weather sampling was done totaling 16 samples near the Stony Brook outfall, and nine at the BU Bridge. Of the five complete surveys, two were done subsequent to Cottage Farm activations, and three were done during or after heavy rain events where no Cottage Farm activation occurred prior to sampling. (Four additional samples were taken at the BU Bridge during Cottage Farm activations.)

Table 10. Overall proportion of Charles River samples testing positive for presence of any viral pathogen or coliphage (1995-2003).

Parameter	Overall	Wet Weather	Dry Weather
Viral Pathogens	28/91 (31%)	22/58 (38%)	6/33 (18%)
Male Specific Coliphage	60/91 (66%)	41/58 (71%)	19/33 (58%)
Somatic Coliphage	60/91 (66%)	41/58 (71%)	17/33 (52%)

"Wet weather" is ≥ 0.5 inches in previous 48 hours; "Dry weather" is ≤ 0.01 inches of rainfall in previous 72 hours.

Coliphage was usually present (as are indicator bacteria). The prevalence of coliphage was slightly higher in wet weather than in dry weather, and there was not a great deal of variation in prevalence along the length of the river segment.

Table 11. Proportion of samples collected in the Charles River testing positive for presence of a viral pathogen or coliphage, by sampling station, 1995-2003.

Station	Parameter	Overall	Wet Weather			Dry Weather
			Total	Antecedent Cottage Farm activation? [1]		
				Yes	No	
001 Newton Yacht Club	Pathogenic Virus	1/10 (10%)	1/6 (17%)	-- N/A[1]		0/4 (0%)
	Coliphage	6/10 (60%)	4/6 (67%)	- N/A		2/4 (50%)
168 Near CSO CAM005	Pathogenic Virus	2/9 (22%)	2/5 (20%)	- N/A		0/4 (0%)
	Coliphage	7/9 (78%)	4/5 (80%)	- N/A		3/4 (75%)
005 Magazine Beach	Pathogenic Virus	1/9 (11%)	1/5 (20%)	- N/A		0/4 (0%)
	Coliphage	6/9 (67%)	3/5 (60%)	- N/A		3/4 (75%)
006 Downstream of Cottage Farm at BU Bridge[2]	Pathogenic Virus	5/13 (38%)	5/9 (56%)	3/6	2/3	0/4 (0%)
	Coliphage	9/13 (69%)	7/9 (78%)	5/6	2/3	2/4 (50%)
145 Stony Brook Outlet	Pathogenic Virus	12/28 (43%)	8/16 (50%)	3/8	5/8	4/12 (33%)
	Coliphage	21/28 (75%)	14/16 (87%)	7/8	7/8	7/12 (58%)
009 Mid-Charles River Basin	Pathogenic Virus	4/10 (40%)	2/5 (40%)	1/2	1/3	2/5 (40%)
	Coliphage	7/10 (70%)	3/5 (60%)	1/2	2/3	4/5 (80%)

[1] Stations 001, 168 and 005 are upstream of the Cottage Farm outfalls and are not affected by the facility's discharge
[2]Includes data from 4 Cottage Farm Facility assessment study surveys (Station CFDA) discussed below.

The pathogenic virus prevalence data are graphed in Figure 5. In dry weather, anthropogenic virus was detected at only the most downstream stations—near the Stony Brook outfall and in the mid-basin. This is consistent with the presence of a dry weather source of viral pathogens in this area that is unrelated to upstream CSO or stormwater sources—this is likely the Stony Brook. In wet weather, viral pathogens were detected at all stations; there was a general trend for the prevalence of pathogenic viruses to increase from the most upstream site, Station 001, to below the BU Bridge at Station 006. Wet weather pathogenic virus prevalence decreases slightly in the wider "lower basin" portion of the river. Thus in the more

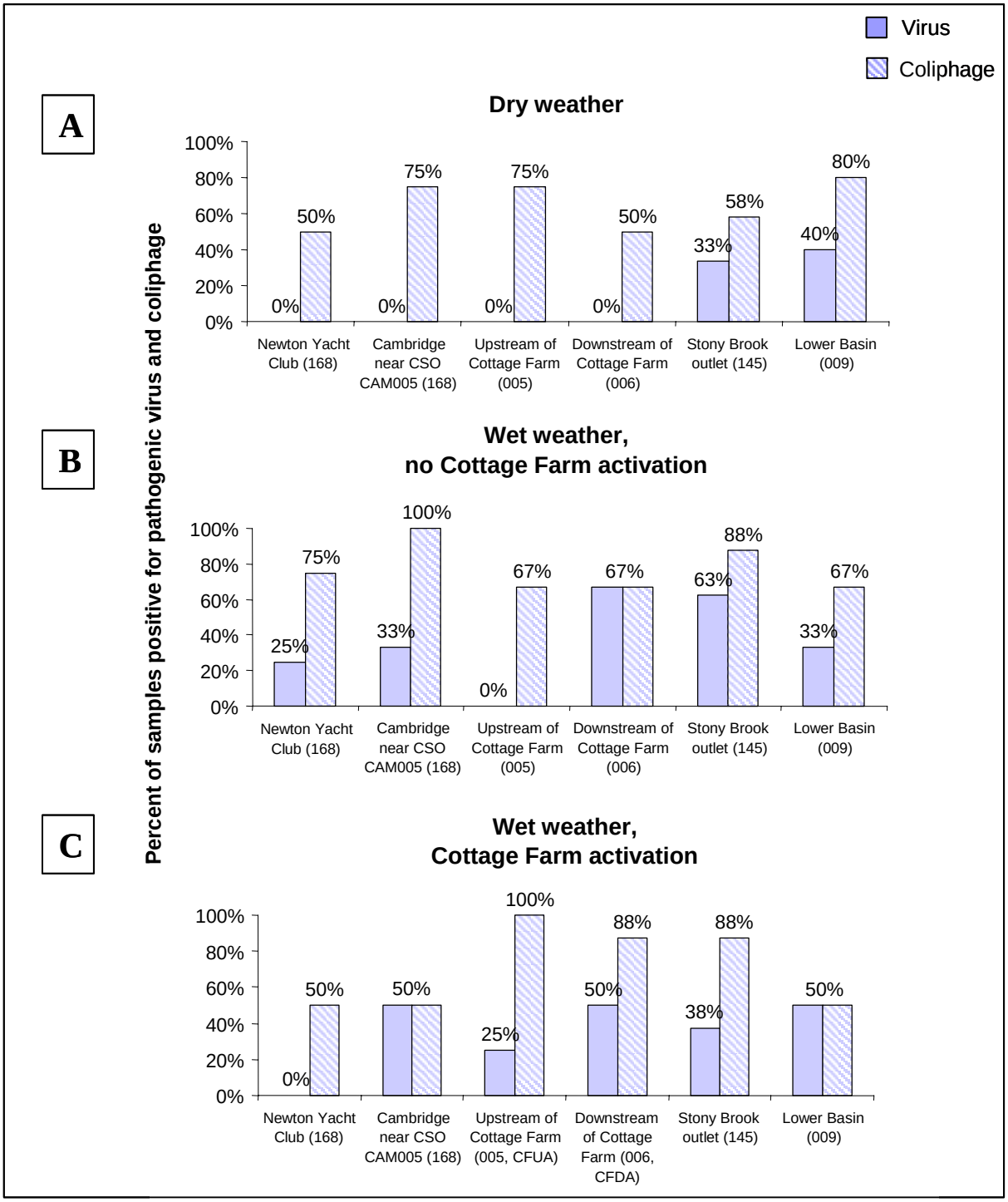

Figure 5. Percent of samples positive for pathogenic virus and coliphage for locations upstream and downstream of Cottage Farm CSO facility, for (A) Dry Weather; (B) Wet weather when Cottage Farm did not activate (stormwater and other CSO could be present); and (C) Wet weather when Cottage Farm activated (stormwater and other CSO were likely present). "Dry weather" is ≤ 0.01 in. rainfall in the previous 72 hours; "Wet weather" is ≥ 0.5 in. rainfall in previous 48 hours. Note that for both graphs B and C, untreated CSOs and stormwater may have discharged. Pathogenic virus and coliphage results from the Cottage Farm facility assessment study (see page 30) are included in graph C. Stations upstream of the Cottage Farm facility (001, 168, and 005) are not affected by facility discharges; they are included to show the results obtained in the three rainfall conditions - including conditions that triggered a CSO activation at Cottage Farm.

upstream reach of this river segment, pathogenic virus presence appears to be associated with wet weather sources (stormwater and CSO). Figure 5 illustrates the presence of viral pathogens and coliphage just upstream and downstream of the Cottage Farm outfall, and further downstream in the lower Charles basin. Although pathogenic virus was only detected in wet weather upstream and downstream of the Cottage Farm outfall, Cottage Farm activations did not increase the prevalence of pathogenic virus. At the lower basin location, the prevalence of pathogens was similar in dry and wet weather – with a slight increase in prevalence for conditions that caused a Cottage Farm discharge.

Types of viruses detected. Figure 6 shows the variation in prevalence of different viruses as percentages of positive tests for different tests for viruses. BGMK and ICC methods detect living virus, while PCR detects genetic sequences in the target virus and cannot tell whether the virus is potentially infectious. Unlike Boston Harbor, where all the virus tests except for norovirus were positive, only three types of tests in the Charles River were positive: adenovirus was most frequently detected, followed by two different tests for enteroviruses—cultivatable virus BGMK (which tests for enterovirus), and enterovirus ICC.

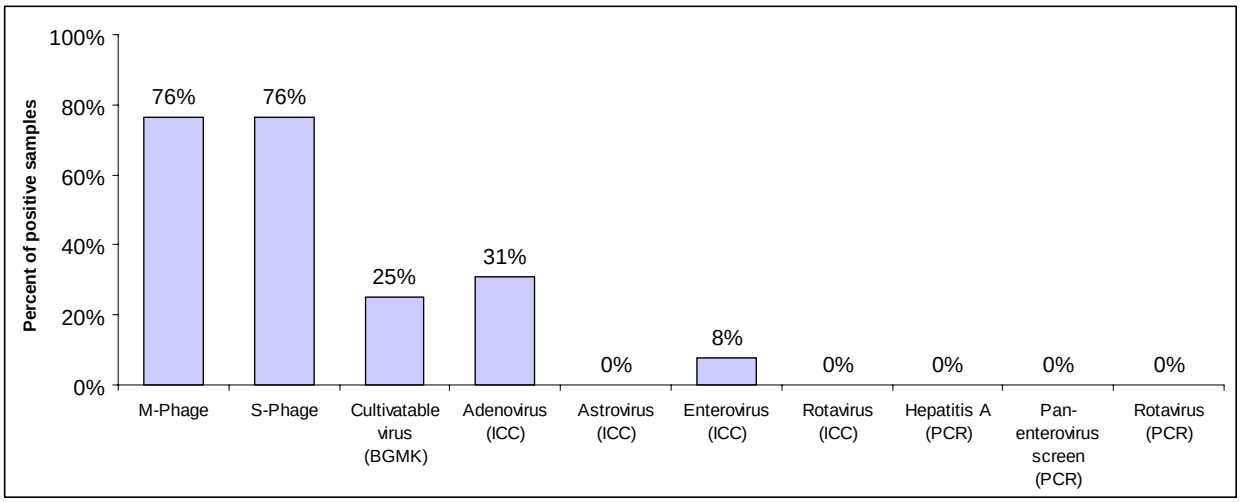

Figure 6. Types of viruses detected during Charles River monitoring, 1995 - 2003.

Summary of Charles River virus monitoring

The study design specifically targeted the locations and weather conditions (heavy rain) presumed to be the most likely to yield positive results for viral pathogens. Sampling locations were in areas known to be affected by CSO, treated CSO, and/or contaminated stormwater. Dry weather samples were also collected for comparison.

Riverine pathogenic virus counts were low—the highest count was approximately 1 PFU/10L (0.11 PFU/L), and the average was 0.016 PFU/L (about 1.6 virus PFU/100 L). As noted above, these numbers can be put into context by comparison to Arizona's (previous) regulatory limits for reclaimed water for discharge to recreational waters. The Arizona reclaimed water quality standard limit for partial body contact was 125 PFU/40L (3.125 PFU/L) and the limit for full body contact was 1 PFU/40L (0.025 PFU/L) (Arizona, 2001). All the MWRA samples collected in the Charles River had counts well below the standard for partial contact, and the average count in the Charles River was well below the full-body contact standard for reclaimed water. Overall, pathogenic viruses were detected in 30% of samples. Compared to studies in other areas, the Charles River data are within or at the lower end of the ranges of prevalence of human virus counts reported in surface waters. For example, Chapron et al. (2000) reported that of 29 randomly selected archived surface water samples, 19 (65.5%) contained infectious viruses, and Loge and Thompson (2002) reported that 22 of 58 (37%) of storm water samples were positive for *Salmonella*, enteropathogenic *E. coli*, or adenovirus. Jiang and Chu (2004) in a study of southern California urban rivers found that approximately 50% of sites sampled were positive for adenoviruses.

In contrast to Boston Harbor, in the Charles River there was an effect of wet weather on viral pathogen prevalence, doubling the prevalence rate from 18% to 37%. This may reflect the relatively greater loading from stormwater and/or CSO on the relatively smaller volume of receiving water in the river, compared to the harbor. There were spatial differences in viral prevalence within the Charles segment, with the lowest prevalence upstream, the greatest prevalence at the BU Bridge, and slightly less prevalence in the "basin." Wet weather detection of pathogenic viruses at the BU Bridge station was about the same with or without antecedent Cottage Farm discharges. Surprisingly, although the basin generally has the lowest bacteria counts (Coughlin, in prep) pathogenic viruses were detected there more consistently (in wet and dry weather) than upstream.

Adenovirus and enterovirus were the only types of viral pathogens detected in the river, in contrast to the harbor, where all the types of virus tested were found.

Finally, measuring coliphages as presence/absence in the Charles River does not give enough resolution to help understand sources, as most samples are positive for coliphage. If coliphages are used to monitor water quality in the future, they should be enumerated.

3.2.2 Cottage Farm CSO Treatment Facility

Virus sampling conducted during wet weather activations at the Cottage Farm CSO Treatment Facility included measurements of anthropogenic viruses and both types of coliphages. Bacterial indicators were measured when virus samples were collected, and also are collected repeatedly each year as part of MWRA's permit-required monitoring. Anthropogenic virus samples collected from 1997-2002 were analyzed by the TCVA-MPN method and direct PCR (Hepatitis A virus, enteroviruses, rotavirus). Samples collected in 2002-2003 were analyzed by the more sensitive ICC-nPCR method. Coliphage analysis methods remained consistent (double agar overlay method).

Results of quantitative sampling of wastewater at the Cottage Farm Facility are in Table 12. The table includes all *Enterococcus* and fecal coliform bacteria results from 1997-2004, and *E.coli* measurements that were added after 2002, in addition to results collected during the virus monitoring project sampling. The results underscore the extremely large variation in microbial quality of combined sewage entering the facility, with influent bacteria and coliphage counts ranging over several orders of magnitude, even in these relatively few samples.

Table 12. Quantitative statistics for viruses and bacteria in Cottage Farm CSO facility influent and effluent (1997-2004).

Parameter	Location	N	Range	Mean (SD)	Geometric Mean
Cultivatable virus (MPN/L)	Influent	2	9.2-59.7	34.5 (35.7)	23.5
	Effluent	2	3.6-4.6	4.1 (0.7)	4.1
Male Specific Coliphage (PFU/L)	Influent	7	21,000-1,260,000	409,000 (440,000)	224,000
	Effluent	8	9,900-655,000	180,000 (209,000)	93,000
Somatic Coliphage (PFU/L)	Influent	7	1,600-815,000	480,000 (360,000)	166,000
	Effluent	8	300-90,000	28,000 (30,000)	10,000
Fecal coliform (col/100mL)	Influent	10	1,000-3,600,000	1,700,000 (2,200,000)	1,100,000
	Effluent	188	10-98,000	1,100 (7,800)	39
Enterococcus (col/100 mL)	Influent	13	140,000-2,000,000	570,000 (480,000)	450,000
	Effluent	78	10-89,000	2,800 (10,000)	250
E.coli (col/100 mL)	Influent	7	210,000-2,800,000	1,200,000 (940,000)	850,000
	Effluent	8	100-3,000	1,300 (1,000)	770

MPN= Most probable number; PFU= Plaque-forming units (each PFU represents at least one viral particle)
CFU = Colony-forming units (each CFU represents at least one bacterial cell)
Although Cottage Farm influent and effluent samples were collected in 1995-1996, these data are considered invalid because the sample collection method (concentration on a wound fiber filter) yielded very poor recovery of viruses. After 1997 unconcentrated grab samples were collected, which yielded better recovery.
For comparison, Arizona reclaimed water quality standard limit for cultivatable viruses for partial body contact was 125 PFU/40L (3.125 PFU/L) and the limit for full body contact was 1 PFU/40L (0.025 PFU/L) (Arizona, 2001).

Counts of all the microbes studied were substantially reduced by treatment. Mean cultivatable virus was reduced approximately 90%, mean male-specific coliphage was reduced approximately 60%, and mean somatic coliphage was reduced approximately 90%, while all the bacterial indicators which were reduced by several orders of magnitude (>99.9%). This result is consistent with previous work by other investigators suggesting that coliphage may better mimic the behavior of viruses during treatment than do the commonly used indicator bacteria (Havelaar et al. 1990, 1993).

Association between indicators and cultivatable virus in Cottage Farm wastewater. Although the total number of samples collected for enumeration of cultivatable virus was only four, over the comparatively large range of values obtained in Cottage Farm wastewater when influent and effluent data are combined, positive associations between cultivatable virus and both somatic and male specific coliphages are apparent (Figure 7). However, there were only relatively weak associations between cultivatable virus and the bacteria indicators.

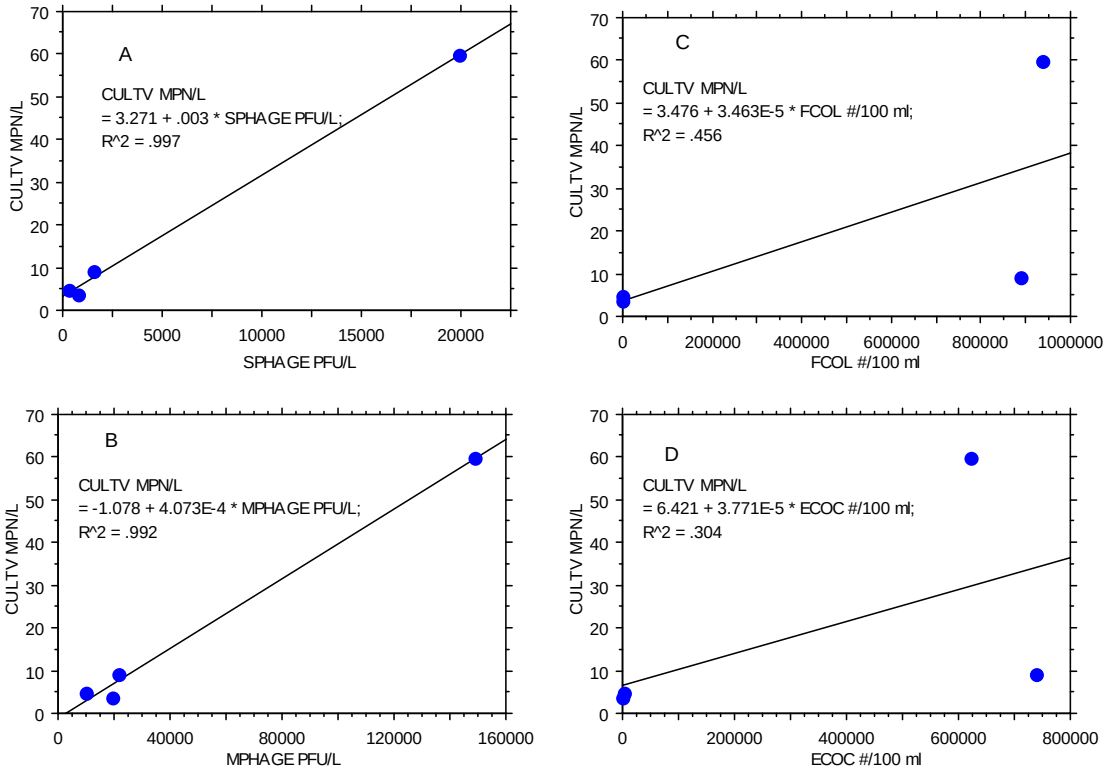

Figure 7. Relationship of somatic coliphage (A), male-specific coliphage (B), fecal coliform (C), and *Enterococcus* (D) with cultivatable virus in samples of Cottage Farm CSO treatment facility wastewater. With the caveat that the sample size is small, both coliphage indicators have a better relationship with cultivatable virus than either bacterial indicator.

Types of viruses detected. Figure 8 shows the variation in prevalence of different viruses as percentages of positive tests for different tests for viruses. BGMK and ICC methods detect living virus, while PCR detects genetic sequences in the target virus and cannot tell whether the virus is potentially infectious. The pattern of viruses found in Cottage Farm effluent is similar to but not identical to the pattern in the river. In the Charles River, adenovirus was most frequently detected, followed by cultivatable virus BGMK, and enterovirus ICC. In Cottage Farm effluent, cultivatable virus BGMK and adenovirus were most often found, followed by astrovirus and enterovirus ICC. Both types of coliphage were always present.

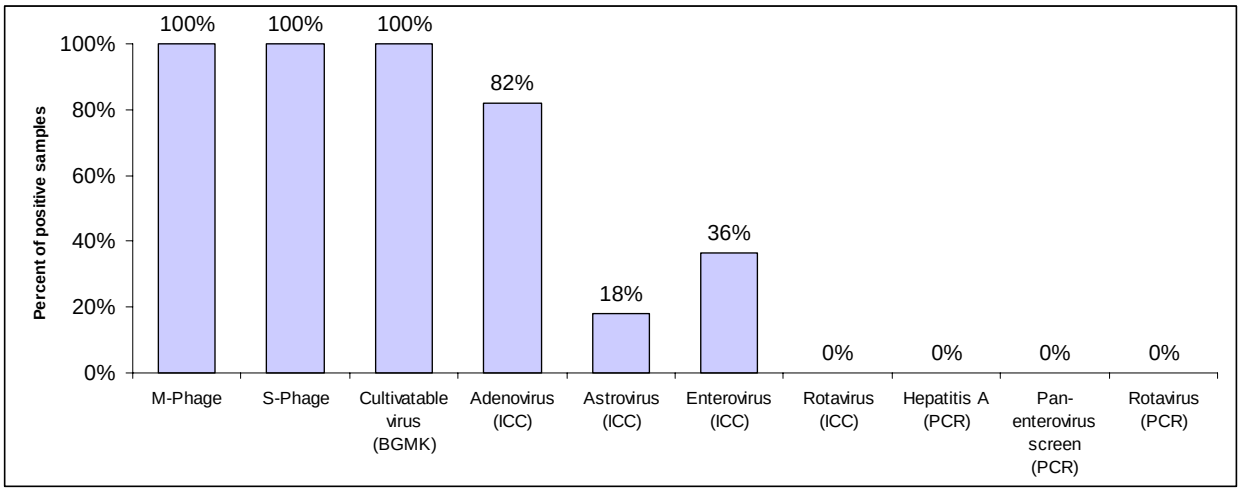

Figure 8. Types of viruses detected during Cottage Farm effluent monitoring, 1995 - 2003.
Enterovirus, adenovirus, and astrovirus were detected.

Cottage Farm CSO facility assessment study. Three receiving water locations listed in Table 13 and shown in Figure 9, upstream of Cottage Farm, at the outfall, and downstream of the outfall, were sampled for viruses during two wet weather events for a study that evaluated the performance of the Cottage Farm CSO treatment facility and its effect on the receiving water. These samples, as well as influent and effluent samples, were collected while the facility was discharging (the other receiving water virus monitoring discussed above occurred as soon as logistically feasible after learning that a discharge occurred).

Table 13. Sampling locations for Cottage Farm assessment.

MWRA Location Code*	Latitude Degrees	Longitude Degrees	Location Description
CFUA	42.355081	-71.11551	Upstream of Cottage Farm outfall: Facility assessment study
CFOA	42.354214	-71.11228	At Cottage Farm outfall: Facility assessment study
CFDA	42.3525	-71.1085	Downstream of Cottage Farm outfall: Facility assessment study
CF_INF	NA	NA	Cottage Farm Influent
CF_EFF	NA	NA	Cottage Farm Effluent
* Only midstream "A" samples were collected for the virus study			

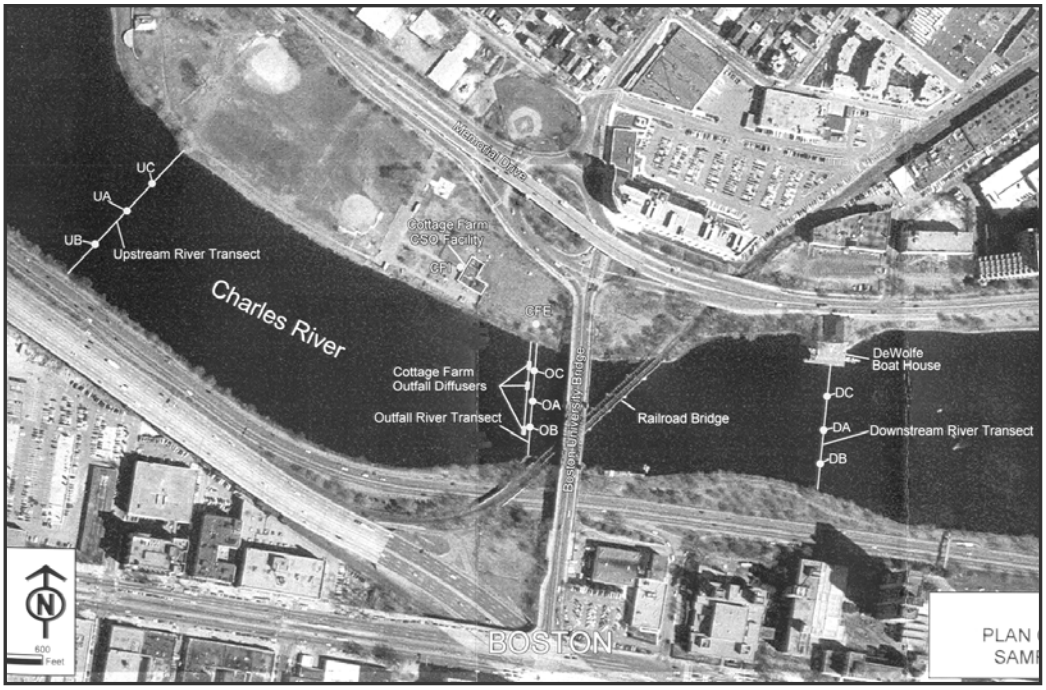

Figure 9. Receiving water sampling locations for Cottage Farm Facility Assessment Study.

Results for receiving water are in Table 14. The data reflect the variability in virus presence already noted. In the river, adenovirus was the only pathogenic virus detected, and that was detected only during the first storm—no pathogenic viruses were detected at any of the river locations during the second storm

in this study. Results for Cottage Farm influent and effluent are in Table 15. In Cottage Farm wastewater, adenovirus was detected in three influent samples, and enterovirus in two influent samples. In Cottage Farm effluent, no viral pathogens were detected in two samples collected during the first storm, but viruses were detected in effluent during the second storm. This result is consistent with the observed relatively low abundance of pathogenic virus in Cottage Farm effluent (see Table 12)—the lower the abundance, the less likely it will be that any given sample will contain a viral pathogen.

Table 14. Charles River virus results for Cottage Farm CSO treatment facility assessment.

Sample Location	Results 10/15/03				Results 10/29/03			
	Pathogenic Virus	Male Coliphage (PFU/L)	Somatic Coliphage (PFU/L)	Fecal coliform (CFU /100mL)	Pathogenic Virus	Male Coliphage	Somatic Coliphage	Fecal coliform (CFU /100mL)
Charles River CFUA Upstream of Cottage Farm	None detected	1,500	9,000	44,500	None detected	Present	Present	2,800
Charles River CFOA At Cottage Farm Outfall	Adenovirus present in one sample None in second sample	17,000	4,500	7,400	None detected	Present	Present	7,800
Charles River CFDA Downstream of Cottage Farm	Adenovirus present in both samples	29,000	7,000	8,900	None detected	Present	Present	9,500

Two storms were sampled, and two samples for pathogenic virus and coliphage were collected at each location during each storm. For the 10/15/03 storm, coliphage were enumerated, results are the mean of two samples. For the 10/29/03 storm, coliphage results are reported as presence/absence. Pathogenic virus results were reported as presence/absence for each type of virus monitored. Fecal coliform are the mean of the two samples collected synoptically with the virus samples.

Table 15. Influent and effluent virus results for Cottage Farm CSO treatment facility assessment.

Sample Location	Results 10/15/03				Results 10/29/03			
	Pathogenic Virus	Male Coliphage (PFU/L)	Somatic Coliphage (PFU/L)	Fecal coliform (CFU/ 100 mL)	Pathogenic Virus	Male Coliphage (PFU/L)	Somatic Coliphage (PFU/L)	Fecal Coliform (CFU/ 100 mL)
Cottage Farm CSO Influent	Adenovirus and enterovirus present in both samples	162,000	490,000	1,300,000	Adenovirus in one sample None detected in second sample	552,000	765,000	4,300,000
Cottage Farm CSO Effluent	None detected in either sample	89,000	11,000	<100	Adenovirus and astrovirus in both samples, enterovirus in one sample	223,000	33,000	<100

Two storms were sampled, and two samples for pathogenic virus and coliphage were collected at each location during each storm. Coliphage were enumerated, results are the mean of two samples. Fecal coliform are the mean of the two samples collected synoptically with the virus samples.

Summary of Cottage Farm Facility virus monitoring

Overall, a total of 7 influent and 8 effluent samples were collected for virus testing from the Cottage Farm CSO Treatment Facility. Not surprisingly, all but one influent and one effluent sample tested positive for the presence of pathogenic viruses and coliphage. Two rounds of sampling Cottage Farm influent and effluent included enumeration of enterovirus; the highest count in influent was almost 60 virus MPN per liter, and in effluent was about 5 MPN per liter. The number of samples was small, but the results suggest that CSO treatment at Cottage Farm reduces enterovirus by about 90%. Three types of pathogenic viruses were found in Cottage Farm combined sewage: adenovirus, enterovirus, and astrovirus. As expected, the bacteria indicator results for Cottage Farm influent were consistent with relatively dilute wastewater. The levels of pathogenic viruses measured in Cottage Farm effluent (about 4 MPN per liter) are similar to those found in secondary-treated and disinfected effluent from the Deer Island Treatment Plant (about 5 MPN per liter, see next section). There was a positive correlation between pathogenic viruses and both types of coliphage in Cottage Farm wastewater, but only a poor relationship between pathogenic viruses and indicator bacteria in Cottage Farm wastewater. Coliphages show promise as a conservative indicator for the presence of pathogens, but like indicator bacteria, they must also be used with the understanding that they are not specific to human waste.

3.3 Deer Island Wastewater Treatment Plant

Virus sampling was conducted at the Deer Island Treatment Plant (DITP) to determine the effectiveness of wastewater treatment processes to remove or inactivate infectious virus and their indicators. MWRA reduced the level of chlorination in its effluent when the Massachusetts Bay outfall went online (2000) because of the greater dilution available in Massachusetts Bay. While the levels of bacterial indicators such as fecal coliform are easily measured in wastewater, the understanding of virus concentrations, virus inactivation during secondary treatment and disinfection, and the ultimate fate of viruses in receiving water is poor. This sampling was performed to assess the presence of anthropogenic viruses and their indicators in different stages of wastewater treatment at DITP. Sampling sites are listed in Table 16.

Table 16. Deer Island Treatment Plant sampling locations.

Sampling Location	MWRA Location Code	Location Description
DITP Influent	AB00 or AD00	South System Influent North System Influent
DITP Primary Effluent	One of the following BA13, BB13, BC13, BD13	Primary effluent battery A, B, C, or D (battery selected for sampling was dependent upon operating conditions)
DITP Secondary Effluent	One of the following SAEL, SBEL, SCEL	Secondary effluent battery A, B, C, or D (battery selected for sampling was dependant upon operating conditions)
DITP Final Effluent	One of the following: BCEF, DEFF or FEFF	Final effluent, chlorinated and dechlorinated Final effluent, chlorinated (dechlorinated manually after sample collection)

Quantitative statistics for DITP influent are shown in Table 17. The results demonstrate a reduction of pathogenic viruses and coliphage with each successive stage of treatment. Male and somatic coliphage concentrations fell by >99% from influent to final effluent. Mean cultivatable pathogenic virus concentrations fell by nearly 90%. Bacterial indicators were reduced by >99.99%.

Table 17. Quantitative statistics for viruses and bacteria in DITP influent and effluent, 1998 - 2002.

Parameter		N	Range	Mean (SD)	Geometric Mean	Percent change from prior stage
Cultivatable virus (MPN/L)	Influent	3	17.53-89.58	43.77 (39.81)	33.62	-
	Primary Effluent	5	0-26.7	70.45 (128)	34.89	0%
	Secondary Effluent	4	1.92-17.91	11.64 (7.63)	8.63	-75%
	Final Effluent	4	0.92-13.86	5.24 (5.97)	3.10	-64%
Male Specific Coliphage (PFU/L)	Influent	7	81900-4800000	1,985,929 (1,924,947)	950,676	-
	Primary Effluent	9	263-4,300,000	1,260,001 (1,772,693)	77,308	-92%
	Secondary Effluent	7	720-600,000	187,186 (246,393)	29,177	-62%
	Final Effluent	9	90-500,000	176,267 (188,035)	41,183	0%
Somatic Coliphage (PFU/L)	Influent	7	47,400-3,600,000	1,529,486 (1,650,911)	467,917	-
	Primary Effluent	9	340-1,500,000	362,959 (530,778)	22,230	-95%
	Secondary Effluent	7	130-1,150,000	317,506 (431,960)	37,690	0%
	Final Effluent	9	67-1,050,000	158,187 (337,604)	15,110	-60%
Enterococcus (CFU/100mL)	Influent	8	260,000-1,660,000	645,000 (465,342)	536,469	-
	Primary Effluent	10	10,000-840,000	305,000 (221,372)	210,647	-61%
	Secondary Effluent	8	2,600-178,000	48,700 (57,741)	25,435	-88%
	Final Effluent	11	2.5-17,400	3,327 (5,768)	234	-99.1%
E.coli (CFU/100mL)	Influent	2	1,300,000-5,070,000	3,185,000 (2,665,793)	2,567,294	-
	Primary Effluent	2	1,200,000-1,600,000	1,400,000 (282,843)	1,385,641	-46%
	Secondary Effluent	2	112,000-1,100,000	606,000 (698,621)	350,999	-75%
	Final Effluent	2	25-155	90 (91.9)	62	- 99.99%
Fecal Coliform (CFU/100mL)	Influent	8	1,160,000-6,800,000	3,770,000 (2,088,143)	3,243,903	-
	Primary Effluent	10	10,000-6,300,000	3061000 (1670092.8)	1,793,551	-45%
	Secondary Effluent	8	23,000-1,390,000	293,375 (455,878)	135,812	-92 %
	Final Effluent	11	1.5-300	66.04 (115.06)	17	- 99.99%

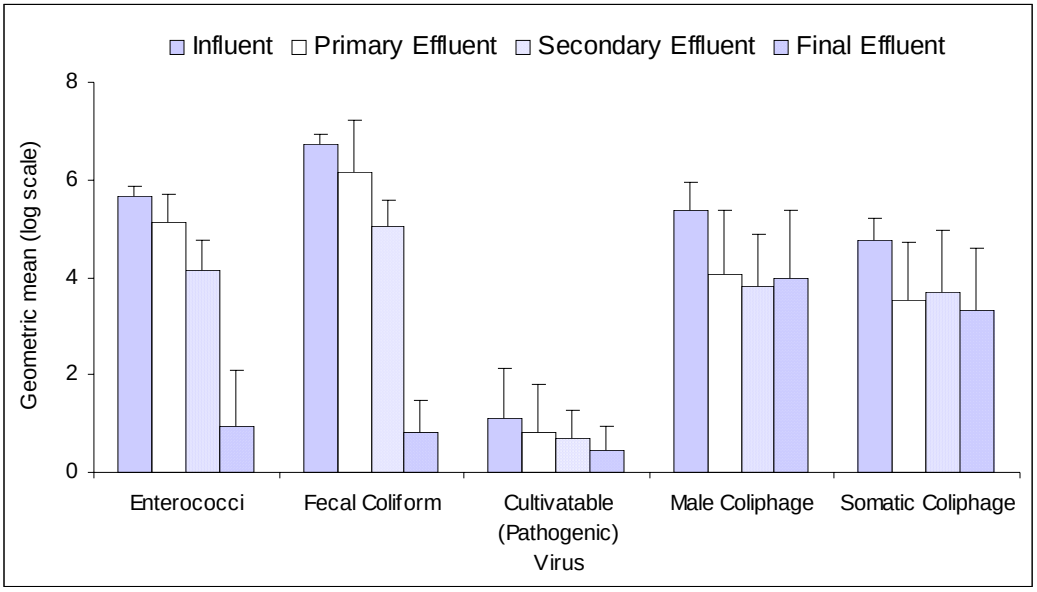

Figure 10. Geometric mean concentrations of pathogenic viruses and pathogen indicators, by phase of treatment, DITP 1998 – 2002. Units for *Enterococcus* and fecal coliform are in colonies per 100 ml. Units for cultivatable virus, male specific coliphage, and somatic coliphage are MPN/L.

The change in concentrations of pathogen indicators and cultivatable virus in different stages of wastewater treatment is shown in Figure 10. Bacterial indicators *Enterococcus* and fecal coliform show dramatic decreases from secondary effluent to final effluent, demonstrating the effectiveness of chlorination. On the other hand, the viruses (pathogenic virus, male specific coliphage, and somatic coliphage) are removed most effectively by primary and/or secondary treatment, and comparatively less removal by subsequent disinfection. These findings are consistent with other research (Havelaar *et al.* 1990, Tree *et al.* 1997), which has found that chlorination is relatively less effective on some pathogenic virus and on coliphage.

We used t-tests to evaluate the effect of treatment phase on the reduction in microbial parameters. Somatic coliphage ($t = 2.007$) and *Enterococcus* ($t = 2.049$) significantly ($\alpha=0.05$) decreased from the influent to the primary effluent. *Enterococcus* ($t = 3.171$) and fecal coliform ($t = 4.528$) significantly ($\alpha=0.05$) decreased from the primary effluent to the secondary effluent. *Enterococcus* ($t = 2.616$) and fecal coliform ($t = 2.157$) significantly ($\alpha=0.05$) decreased from secondary effluent to the final effluent. For subsequent treatment stages, male specific coliphage ($t = 2.831$), somatic coliphage ($t = 2.450$), *Enterococcus* ($t = 4.624$) and fecal coliform ($t = 6.054$) significantly ($\alpha=0.05$) decreased from influent to final effluent.

Figure 11 shows percent of samples positive for different virus tests in DITP final effluent. Most prevalent were, in descending order, adenovirus, cultivatable virus, astrovirus, enterovirus, rotavirus and hepatitis A. (The test for hepatitis A cannot distinguish between infectious and non-infectious virus.)

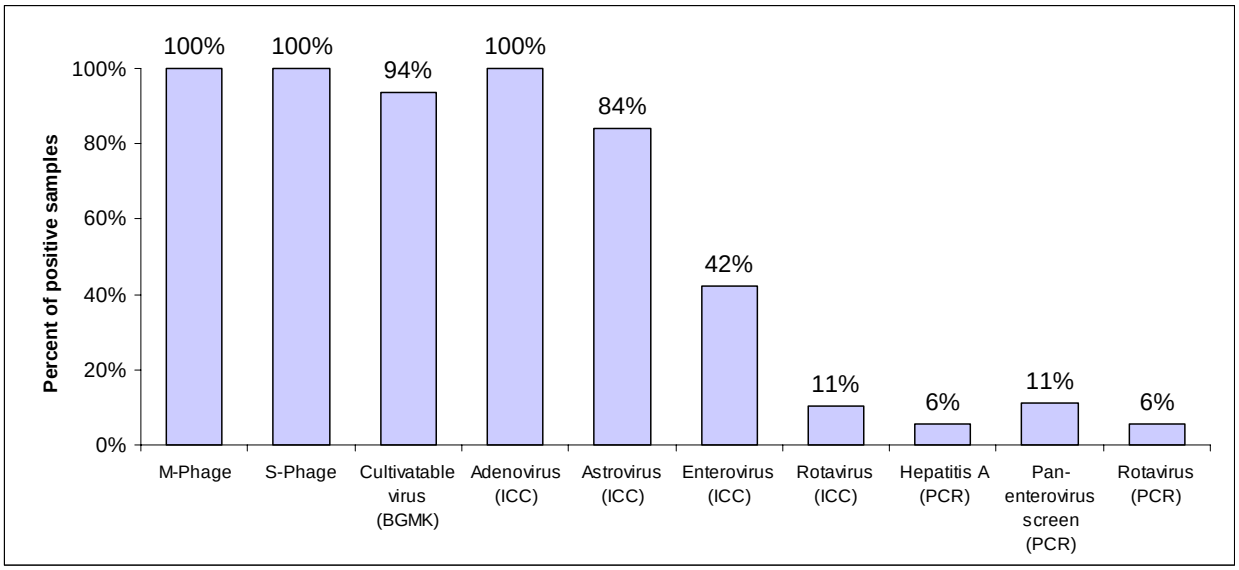

Figure 11. Percent of samples testing positive by viral test, DITP effluent 1998-2002.

Virus methods changed in 2000 from TCVA-MPN with results reported in MPN/L to the ICC-nPCR method, with results reported as presence/absence (unlike the other regions sampled in this project, the coliphage method has remained the same for wastewater throughout the monitoring period). Due to the increased sensitivity of the ICC-nPCR method, all samples collected after 2000 were positive for pathogenic virus presence. Table 18 shows all data from 1998-2002 from DITP as proportions of positive virus samples collected.

Table 18. Proportion of samples collected at Deer Island testing positive for presence of viruses.

Location	Pathogenic Virus	Male Specific Coliphage	Somatic Coliphage
Influent	100% (6/6)	100% (6/6)	100% (6/6)
Primary Effluent	78% (7/9)	100% (9/9)	100% (9/9)
Secondary Effluent	100% (8/8)	100% (8/8)	100% (8/8)
Final Effluent	100% (11/11)	100% (11/11)	100% (11/11)

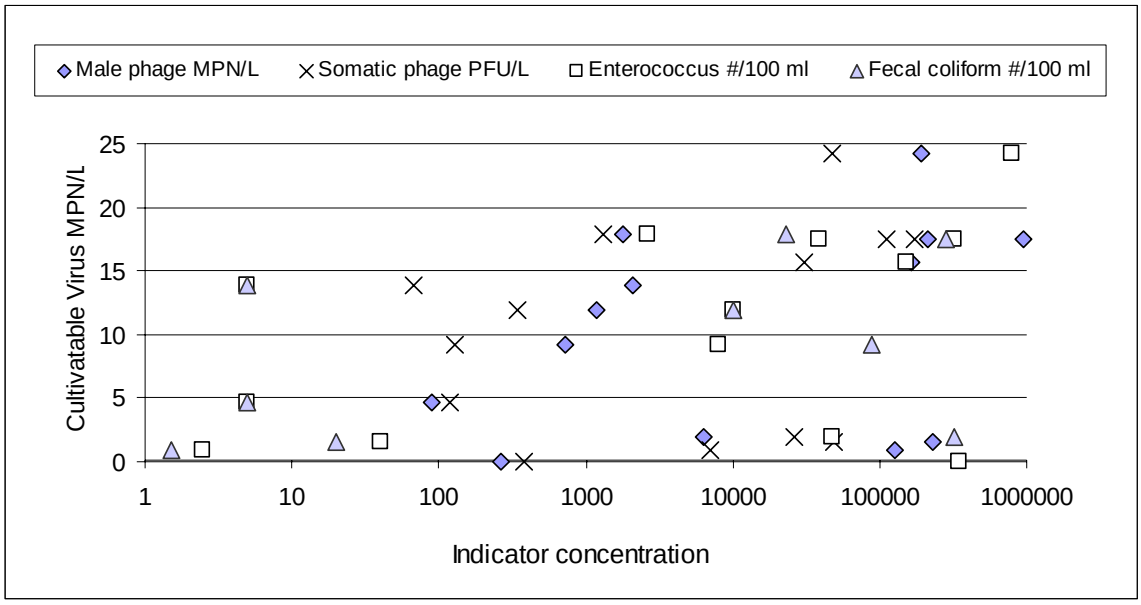

Figure 12. Relationship of pathogen indicators with cultivatable virus in DITP wastewater.
The graph includes data from wastewater at all stages of treatment, including influent.

While significant trends are evident for coliphage and indicator bacteria at different stages of wastewater treatment, there is a poor correlation between pathogen indicators and cultivatable virus concentrations in general, shown in Figure 12. Regression analyses yielded insignificant relationships between cultivatable virus for all indicators, both for all stages combined, and for individual treatment stages. However, in samples where cultivatable virus counts were most elevated, the two viral indicators and *Enterococcus* were also relatively elevated.

Summary of Deer Island Treatment Plant monitoring

Overall, concentrations of cultivatable viruses in wastewater effluent are low, with a geometric mean in final effluent of 3.1 MPN/L. The DITP treatment process effectively removed the bacterial indicators (*Enterococcus* and fecal coliform), the viral indicators (male specific coliphage and somatic coliphage), and the pathogenic viruses studied. However, the effectiveness of the three different stages of treatment (primary, secondary, disinfection) varies among the types of microbes. Cultivatable pathogenic viruses were most reduced by secondary treatment (75%), with disinfection providing a further 60% reduction. Both types of coliphages were most reduced by primary treatment (about 95%) with secondary treatment having less effect. Disinfection provided another 0 - 60% reduction. The three indicator bacteria (*Enterococcus*, *E. coli*, and fecal coliform) all responded to treatment similarly: primary treatment reduces by about 50 – 60%, secondary treatment another 75 – 90%, and disinfection by a further 99.99%.

4.0 SUMMARY AND CONCLUSIONS

This exploratory survey of pathogenic viruses in Boston Harbor, the Charles River, and wastewater treatment facilities found several types of anthropogenic viruses in all areas. During the study, several methodologies were used to test for anthropogenic virus and for coliphage, as this is an area of rapid technological development.

In Boston Harbor and the Charles River, levels of viral pathogens were consistently low, well below Arizona's partial-body contact standard, and at the low end of the range of levels reported by other investigators. On average, pathogenic viruses were detected in about one-third of samples collected, within or at the low end of results reported in other studies. The group of anthropogenic viruses most frequently detected was enterovirus. In the harbor, there was no difference in prevalence of anthropogenic virus in wet vs dry weather, while in the Charles River the prevalence in wet weather was about double that in dry weather. Spatial and temporal patterns of viral prevalence in both the river and the harbor are consistent with multiple sources of viruses, including dry weather and stormwater sources as well as treated and untreated CSO. The sampling did not find an increased prevalence of viruses in wet weather near CSO treatment facilities when a discharge had occurred compared to when a discharge had not occurred. In Boston Harbor, the lowest viral prevalence was near beaches, and the prevalence of viruses was similar at beaches affected by CSO discharges and/or stormwater discharges.

In wastewater treatment facilities, viruses were almost always detected in both treated CSO and secondary-treated and disinfected wastewater. Treatment appeared to remove about 90% of viruses from influent in both CSO and secondary treated wastewater. At DITP, the biggest removal was accomplished by secondary treatment. Levels in both types of effluents were similar, averaging about 5 viruses per liter. Enterovirus and adenovirus were most frequently detected.

None of the indicators studied were well correlated with the presence of viruses, including both types of coliphages. However, coliphages were found to be more persistent through wastewater treatment and in the environment than were the bacterial indicators, which means they could be useful in this region as relatively conservative sewage tracers (with the caveat that coliphage, like coliform and *Enterococcus*, are not specific to human waste but are also found in animal waste.) We would, however recommend that the enrichment coliphage methods be developed into MPN methods to enable enumeration.

Despite many method developments, the methods for detecting, enumerating, and identifying pathogenic viruses from ambient water samples remain difficult, time consuming, and expensive (cost is about 20-fold higher than for bacterial indicators). During this study, the sample collection method was greatly simplified; we began by using micro-wound filtration to filter many liters of water in the field, but later found that a grab sample (5 liters) actually gave better recovery. However, laboratory analysis remains difficult. Despite the promising development of PCR techniques, it still remains necessary to grow the virus in tissue culture to determine viability. Also, for environmental samples with low numbers of virus, as found in the Charles River and Boston Harbor, enumeration is accompanied by large error (measurement variability). This means that a prohibitively large number of samples would have to be analyzed to determine statistically meaningful comparisons of virus counts either over time or geographically.

Through this study we learned that pathogenic viruses can be detected in about 30% of samples, and when detected are present at very low abundance in the Charles River and in Boston Harbor. There appear to be multiple sources of pathogenic virus, including stormwater and dry weather sources. Also, treatment at the Cottage Farm facility and at DITP reduces the numbers of virus by about 90%. None of the indicators

studied reliably predict the presence or absence of pathogenic virus in this area. Given the cost and technical difficulty, and large error in quantifying them, routine ambient water monitoring for pathogenic virus is not recommended. However, if quantitative and sensitive methods for measuring coliphages were developed, they would likely be useful conservative indicators in concert with bacterial indicators. Using several different indicators with different characteristics will give the best overall picture of the sources and levels of microbial pollution.

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APPENDIX A. Statistical Analysis

Spearman's Rho Analysis

Charles River 1997-2000

Variable	By Variable	Spearman's Rho	T-test
Male Specific Coliphages	Virus	0.0995	0.4358
Somatic Coliphages	Virus	-0.1318	0.5795
Somatic Coliphages	Male Specific Coliphages	0.2527	1.1384
Fecal Coliforms	Virus	-0.0496	0.2165
Fecal Coliforms	Male Specific Coliphages	0.0161	0.070
Fecal Coliforms	Somatic Coliphages	0.2959	1.3502
Enterococcus	Virus	0.0719	0.3142
Enterococcus	Male Specific Coliphages	0.1144	0.5019
Enterococcus	Somatic Coliphages	0.3027	1.3843
Enterococcus	Fecal Coliforms	0.8610	7.379

(n= 21, d.f. = 19, $\alpha = 0.05$, T_{tab}= 1.729)

Boston Harbor 1995-2000

Variable	By Variable	Spearman's Rho	T-test
Male Specific Coliphages	Virus	-0.2055	1.666
Somatic Coliphages	Virus	-0.2133	1.7328
Somatic Coliphages	Male Specific Coliphages	0.7166	8.1547
Fecal Coliforms	Virus	-0.0877	0.6987
Fecal Coliforms	Male Specific Coliphages	0.2719	2.243
Fecal Coliforms	Somatic Coliphages	0.3300	2.775
Enterococcus	Virus	0.282	2.254
Enterococcus	Male Specific Coliphages	0.479	3.864
Enterococcus	Somatic Coliphages	0.469	3.780

(n= 65, d.f. = 63, $\alpha = 0.05$, T_{tab}= 1.669)

Contingency Table and Chi Squared Analysis

Charles River All Sites 1997-2003

Data

Coliphage	Human viruses		
	Yes	No	Total
Yes	17	36	53
No	6	17	23
Total	23	53	76

Expected

Coliphage	Human viruses		
	Yes	No	Total
Yes	16.03	36.9	52.93
No	6.95	16.0	22.95
Total	22.98	52.9	75.9

Explanation- Of the 23 samples with human viruses present 16 would be expected to contain coliphage as well and 6.95 would not. Of the 53 samples without human viruses present 36.9 would be expected to contain coliphage as well and 16 would not. Chi Squared ($\alpha = 0.05$) $\chi^2 = 0.39857$, $\rho > 0.001$ $\rho < \alpha$. Conclusion: there is not a relationship between human virus presence and coliphage presence.

Boston Harbor All Sites 1995-2003

Data

Coliphage	Human viruses		
	Yes	No	Total
Yes	24	61	85
No	16	31	47
Total	40	92	132

Expected

Coliphage	Human viruses		
	Yes	No	Total
Yes	25.77	59.24	85.01
No	8.19	32.76	40.95
Total	33.96	92	125.96

Explanation- Of the 40 sites with human viruses present 25.7 would be expected to contain coliphage as well and 8.19 would not. Of the 92 sites without human viruses present 59.2 would be expected to contain coliphage as well and 32.76 would not. Chi squared analysis ($\alpha = 0.05$) $\chi^2 = 6.899$, $0.010 < \rho < 0.001$, $\rho < \alpha$. Conclusion: there is not a relationship between human virus presence and coliphage presence.

Boston Harbor All Sites, Male Specific Coliphage 1995-2003

Chi-square = 5.9, p = 0.015 (negative relationship)

Data

Male-specific coliphage	Human viruses		
	Yes	No	Total
Yes	19	50	69
No	19	18	37
Total	38	68	106

Expected

Male-specific coliphage	Human viruses		
	Yes	No	Total
Yes	24.7	44.3	69
No	13.3	23.7	37
Total	38	68	106

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