Occurrence and correlations between coliphages and anthropogenic viruses in the Massachusetts Bay using enrichment and ICC-nPCR

Nicola A. Ballester, Justin H. Fontaine and Aaron B. Margolin

ABSTRACT

We evaluated a two-step enrichment procedure to detect coliphages and an integrated cell culture-nested polymerase chain reaction (ICC-nPCR) to detect human astrovirus, enteroviruses, rotavirus and adenovirus type 40 and 41 in marine water samples collected by the Massachusetts Water Resource Authority (MWRA). MWRA has been monitoring its receiving waters for coliphages, anthropogenic viruses and indicator bacteria in order to evaluate the impact of Boston's Deer Island Sewage Treatment Plant discharge. Coliphages and enteric viruses were originally assayed using single agar overlay and most probable number cell culture (MPN) methods, respectively. Reanalysis of these samples for enteric viruses by ICC-nPCR demonstrated that 46% were positive for at least one virus compared with 23% with the MPN method. Use of the enrichment method showed a 47% increase in the detection of male specific and somatic coliphages compared with the single agar overlay method. Correlations between the presence of coliphages, enteric viruses and indicator bacteria were based on proximity to the treatment plant discharge, seasonal variations and site levels. The presence of enteric viruses was significantly correlated to coliphages but not to indicator bacteria. Preliminary comparative results demonstrate that effective and efficient monitoring of anthropogenic contamination can be achieved using these more sensitive and specific techniques.

Key words | adenovirus, astrovirus, coliphages, enterovirus, ICC-nPCR, rotavirus

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INTRODUCTION

Beginning in 1995, the Massachusetts Water Resource Authority (MWRA) began an intensive monitoring programme designed to detect sources of fecal contamination in the Massachusetts Bay, Boston Harbor, and its tributaries. The primary objective of this research was to assess the presence of anthropogenic viruses and their indicators (coliphages and coliforms) in Massachusetts Bay. The secondary objective was to evaluate the impact of the Deer Island Sewage Treatment Plant's outfall pipe on the occurrence of anthropogenic viruses and viral indicators. The outfall pipe was designed to increase the level of disinfection while reducing the level of chlorination through a longer contact time. Because there is a poor understanding of how viruses and their indicators are inactivated during secondary treatment and disinfection, enteric viruses, coliphages and indicator bacteria were monitored before and after the start-up of the outfall pipe in the Massachusetts Bay.

Several previous studies have documented the presence of enteroviruses and adenoviruses in seawater (Tsai *et al.* 1993; Girones *et al.* 1993; Abbaszadegan *et al.* 1993; Puig *et al.* 1994; Enriquez *et al.* 1995; Enriquez & Gerba 1995). Enteroviruses (poliovirus, coxsackie virus types A and B, 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 greatly underestimated because of difficulty isolating them in cell culture. Enteric viruses survive longer than indicator bacteria in seawater (Melnick & Gerba 1980) and adenoviruses have been shown to survive longer than most enteric viruses in seawater (Enriquez *et al.* 1995). Indicator bacteria may be

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undetectable in a few days whereas enteric viruses may persist for several months (Wheeler 1990). Indicator bacteria (fecal coliforms and *Enterococcus*) were studied to achieve a better understanding of their role and suspected inadequacy as sole indicators of fecal pollution or as indicators of human viruses. The detection and presence of coliphages was also assessed for their potential use as an additional indicator for fecal contamination and viral presence.

Five sample sites in Massachusetts Bay were chosen based on their proximity to the outfall pipe (Figure 1). Two sample sites were located on either side of the outfall diffuser, one directly east and the other directly west of the diffuser head. Shore sites were chosen near the coastlines northwest and southwest of the diffuser. One of shore sites was of particular interest due to its use as a shellfish resource. The last site was chosen in the mouth of the bay to the far northeast of the diffuser head and acted as a control not influenced by sewage discharge. These five sites were sampled bimonthly over 7 years to study the variability of anthropogenic contamination in the bay.

From 1995 to 1999 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). However, other work done from our laboratory (Chapron *et al.* 2000) previously 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 to occur. Hence, many of the epidemiologically important enteric viruses go largely undetected when using only the TCVA-MPN method.

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

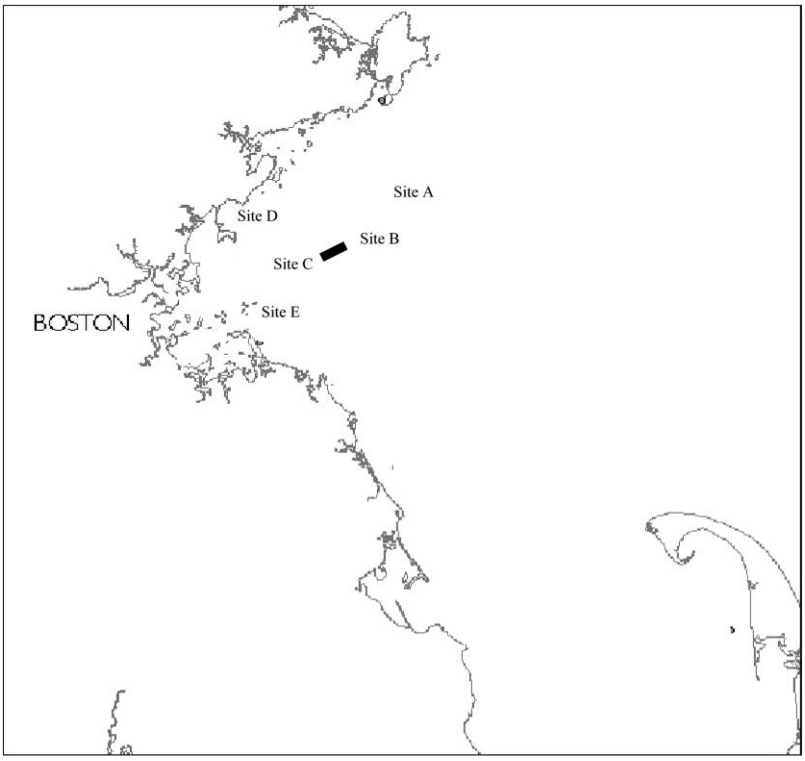

Figure 1 | MWRA outfall monitoring/sampling stations.

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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 products, thus enabling detection of very low numbers of specific viral particles. ICC-nPCR used with two cell lines (BGMK and CaCo-2) and the addition of a proteolytic enzyme may be a useful technique for the detection and confirmation of a wide variety of enteric viruses in environmental samples. Additionally the cell culture step provides a means for infectivity testing. With direct PCR, infectious and non-infectious viruses could be detected whereas with ICC-nPCR the infectious nature of the viruses can be determined by comparing viral levels in the cell lysates compared with the concentrate.

Routine monitoring could be an attractive alternative to the costly and labour-intensive ICC-nPCR method if a good indicator organism could be identified. The detection and evaluation of coliphages was important in this study because of their potential use as indicators of fecal contamination and other anthropogenic viruses. Because they have a similar structure and size to some enteric viruses, coliphages have been suggested as possible indicators of enteric viruses and may also aid in the detection of fecal pollution. Coliphages were originally detected using the single agar overlay method (USEPA 2001). This method, while being able to detect both male specific and somatic phages, only utilized a very small portion of the water sample. More recently, the modified two-step enrichment procedure (USEPA 2000) has been used to detect male specific and somatic phages. The two-step enrichment procedure is desirable because it utilizes a larger sample volume, vastly increasing its sensitivity to phage detection. While not only increasing sample volume, it is thought that the enrichment method is more sensitive to phage detection because of an initial incubation step, in which low numbers of target coliphages are allowed to replicate.

In this study we looked at the correlations between the detected levels of coliphages, indicator bacteria and human enteric viruses in relationship to seasonal variation and proximity to the diffuser.

MATERIALS AND METHODS

TCVA-MPN method

During 1998 – 1999 water samples of 114 – 151 litres (≅ 30 – 40 gallons) were collected using Zeta Plus MW (Cuno, Inc. Meriden, Connecticut) micro wound filters (Standard Methods 1998). These samples were then tested using the TCVA-MPN method (USEPA 1995; *Standard Methods* 1998). Viruses were eluted from the filters with a 1.5% beef extract solution (pH 9.5, BBL Sparks, MD beef extract powder, 0.375% glycine). Eluates were concentrated by organic flocculation (pH 3.5) followed by centrifugation (USEPA 1995). The pellet was resuspended with sodium phosphate buffer (0.15 M Na_2HPO_4, pH 9.5), 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°C with rocking every 15 minutes. Fifteen ml of serum-free maintenance cell culture media was added to each flask after incubation. Flasks were incubated at 37°C and examined daily for 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% 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/litre calculated.

Single agar overlay method

During the same period, coliphage analysis was done on 1-l grab samples by the single agar overlay method (USEPA 2001) and indicator bacteria (fecal coliforms and *Enterococcus*) were analysed by membrane filtration (*Standard Methods* 1998). Six 100 ml portions of water sample were analysed by the single agar overlay method. Three portions were used for male specific coliphage detection and three for somatic coliphage detection. One hundred ml of water sample with 0.5 ml 4 M magnesium chloride hexahydrate ($MgCl_2$-$6H_2O$) was warmed to 37°C. Ten ml of log phase bacterial host (*E.coli* CN-13 or F_{amp}) was added and mixed. The sample was warmed to 43°C and added to 100 ml of

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tryptic soy agar ($80\,g\,l^{-1}$). One ml of host appropriate antibiotic (0.04 M nalidixic acid or a mixture of 0.001 M streptomycin and 0.004 M ampicillin) was then added, mixed and poured evenly in Petri dishes. Plates were incubated for 24 hours at 37°C and observed for plaques.

ICC-nPCR method

All samples after 1999 were evaluated by ICC-nPCR. Four-litre grab samples were concentrated by mixing 40 grams of beef extract powder into the sample. Samples were brought to pH 3.5 and mixed for 30 minutes and centrifuged. Pellets were resuspended in 20 ml of 0.15 M 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). Two 3 ml portions of each sample concentrate were incubated for 30 minutes at 37°C with $5\,\mu g\,ml^{-1}$ or $10\,\mu g\,ml^{-1}$ of trypsin (Sigma St Louis, Missouri). Samples containing $5\,\mu g\,ml^{-1}$ were inoculated onto CaCo-2 cells and $10\,\mu g\,ml^{-1}$ onto BGMK cells. The flasks were incubated for 90 minutes at 37°C with rocking every 15 minutes. Trypsin concentrations of $5\,\mu g\,ml^{-1}$ were used for astrovirus and rotavirus and $10\,\mu g\,ml^{-1}$ for adenovirus and enteroviruses. Following incubation, 15 ml of serum-free media was added to each flask. The flasks were incubated for 5 days at 37°C. After 5 days flasks were freeze thawed and cell lysates were pooled. The cell lysates were analysed using the ICC/nPCR procedure for enteroviruses, astrovirus, rotavirus and adenovirus 40 and 41 (Chapron *et al.* 2000; Reynolds *et al.* 2001). 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 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) reaction 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, 30 s, 55°C, 30 s, 72°C, 30 s. 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′-GAAACACG-GACACCCAAAGTA-3′. Samples were run for 35 cycles of 95°C, 30 s, 55°C, 30 s, 72°C, 30 s, yielding a 138 bp amplicon. Twelve μl of each nested PCR product was run and sized on 1.8% agarose gels 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.

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′-GCCGCAGTGGTCTTACATG-CACATC-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′-GCCACC-GAGACGTACTTCAGCCTG-3′) and (5′-TTGTACGAGTAC-GCGGTATCCTCGCGGTC-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, 30 s, 56°C, 30 s, 72°C, 30 s, with a final extension at 72°C for 5 minutes. For nPCR, the procedure was the same as described above, but the primers

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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′-ATAGAAGACAGCGC-ACCGGATTTG-3′ and PCR primer 5′-ACAGACTTT-CATTTGCGTCCGCAA-3′. The PCR parameters were 95°C, 5 min hot start followed by 35 cycles of 95°C, 30 s, 52°C, 30 s, 72°C, 30 s, with a final extension at 72°C for 5 minutes. The nPCR procedure used the primers, 5′-GACGCATCA-ACTGAAATAATAAAC-3′ and 5′-TGCACCAGCGAACATA-CAGC-3′. These primers yielded a 300-bp amplicon. Rotavirus WA strain was used as a positive control.

Two-step enrichment method

Coliphage analysis was performed on 1-l grab samples by the modified two-step enrichment method (USEPA 2000) and indicator bacteria by membrane filtration (*Standard Methods* 1998). Aliquots of 500 ml of the water samples were analysed for male specific and somatic coliphages. Twenty-five ml of tryptic soy broth (300 g l^{-1}), 6.25 ml 4 M $MgCl_2$-$6H_2O$, 3 ml log phase bacterial host, and 5 ml host specific antibiotic (0.04 M nalidixic acid or a mixture of 0.001 M streptomycin and 0.004 M ampicillin) were added to the samples and mixed. Samples were incubated for 24 hours at 37°C. Between 10 and 20 μl of sample was then spotted onto pre-poured tryptic soy agar plates containing log phase host bacterium. Spot plates were incubated for 24 hours at 37°C and examined for lysis zones on the bacterial layer. Roughly 50% of the lysis zones were plucked and reconfirmed by enrichment followed by spot plate.

Statistical analysis

Pearson linear correlation was used to analyse the relationships between organism presence, proximity to the outfall and seasonal variation.

RESULTS

Overall occurrence at all sampling sites of enteric viruses detected by ICC-nPCR remained relatively consistent from 1998 to 2002. In the samples from 1998–1999 that were reanalysed by ICC-nPCR, 36 (46%) of 78 were positive for one or more enteric viruses and in the samples from 2000–2002, 55 (57.3%) of 96 samples were positive for one or more enteric viruses. Viral detection of the same 1998–1999 samples when done with the TCVA-MPN method demonstrated only 18 (23%) of 78 samples as positive. During this 5-year period the presence of adenovirus and astrovirus increased while rotavirus and enterovirus presence decreased (Figure 2). Several of the 1998–1999 samples that were originally positive by the TCVA-MPN method (14.75%) were negative when reanalysed by ICC-nPCR and several that were originally negative by the TCVA-MPN method (36.1%) were now positive by ICC-nPCR.

Coliphage detection changed significantly from 1998 to 2000 due to the use of the more sensitive two-step enrichment method. Detection of the male specific coliphages changed from 8.0% with the single agar overlay method to 58% with the two-step enrichment and somatic coliphages from 9.8% to 55%. Indicator bacteria remained below statistically significant counts (<30 cfu per plate) throughout 1998–1999 and 2000–2002 in the Massachusetts Bay.

Detection method comparisons showed that the two-step enrichment method was more sensitive than the single agar overlay method and that the ICC-nPCR method was more sensitive and specific than the TCVA-MPN method (Figure 3). Therefore the single agar overlay method and the TCVA-MPN methods were not used after 1999.

Correlations between the presence of coliphages, enteric viruses and indicator bacteria were based on presence, proximity to the outfall pipe diffuser and seasonal variations. The presence of enteric viruses was significantly correlated with the presence of male specific coliphages ($r = 0.682$) and somatic coliphages ($r = 0.573$), but not significantly correlated with the presence of indicator bacteria ($r = 0$). Both male specific and somatic coliphages presence were significantly correlated to one another ($r = 0.891$) and to the presence of adenovirus ($r = 0.651$ and $r = 0.672$ respectively), but not significantly correlated to the presence of astrovirus ($r = 0.122$ and $r = 0.060$ respectively). Male specific coliphages were also

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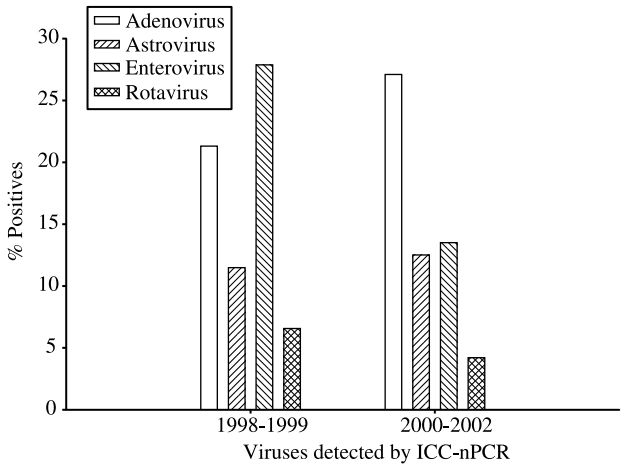

Figure 2 | Changes in viruses present in the Bay.

significantly correlated to the presence of rotavirus ($r = 0.692$) and enterovirus ($r = 0.608$), whereas somatic coliphages did not have a strong significant correlation ($r = 0.504$ and $r = 0.419$, respectively).

Between 2000 and 2002, seasonal levels of male specific coliphages and total enteric viruses were greatest in the autumn, whereas somatic coliphages concentration was highest in the spring and winter. Specifically rotavirus was most prevalent in the spring, enterovirus in the summer, adenovirus in the summer and autumn, and astrovirus in the autumn. Of interest is the absence of adenovirus and enterovirus in the spring and rotavirus in both the summer and autumn (Figure 4).

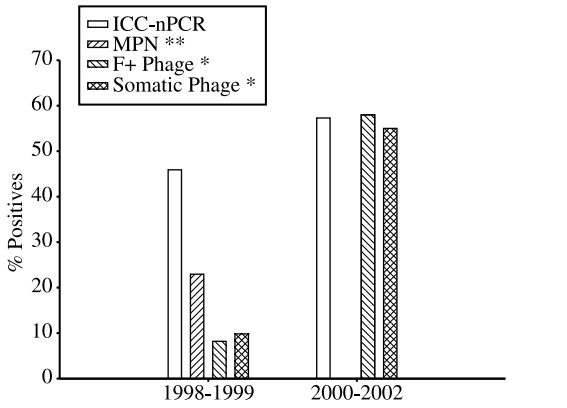

Figure 3 | Comparison of detection methods.

Data from 1998–1999 for coliphages obtained by the single agar overlay method and total enteric viruses by the TCVA-MPN method were done with the best available technology but should not be directly compared with the newer methods due to the lack of sensitivity of these assays. Reanalysis of these samples with ICC-nPCR demonstrated that the prevalence of total enteric viruses was highest in the spring. Specifically adenovirus, enterovirus and astrovirus were most prevalent in the spring, and rotavirus in the winter and summer. Adenovirus was not detected in the winter. Astrovirus and rotavirus were not detected in the autumn (Figure 4).

Prevalence due to proximity to the outfall pipe diffuser remained relatively similar from 1998–1999 to 2000–2002. In 1998–1999 male specific and somatic coliphages analysed by the single agar overlay method were most prevalent at the shores northwest and southwest of the diffuser. Adenovirus, enterovirus and astrovirus were most prevalent directly to the west of the diffuser and rotavirus directly to the east of the diffuser (Figure 5). In 2000–2002 male specific and somatic coliphages analysed by the enrichment method were most prevalent at both shore sites and directly to the west of the diffuser. Adenovirus was mostly prevalent directly east of the diffuser, rotavirus directly to the west of the diffuser, astrovirus at the shore southwest of the diffuser, and enterovirus at the farthest site in the mouth of the bay (Figure 5).

DISCUSSION

In this study we demonstrated the usefulness and benefits of the ICC-nPCR method compared with the TCVA-MPN method in marine samples. The results from reanalysis of samples with ICC-nPCR show a great underestimation of viral levels when using the TCVA-MPN method alone. Use of the ICC-nPCR assay with two cell lines indicated that almost twice as many samples were positive (46%) for one or more viruses compared with 23% by the TCVA-MPN method. Reasons for the difference in results between methods could be due to mixed viral populations. When environmental samples are inoculated onto cell lines it is likely that there is a competitive advantage for some viral groups to reach receptor sites for viral

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propagation before others and so detection of specific virus levels may be altered or absent.

The incorporation of nested PCR into this method achieved the increased sensitivity and specificity necessary to detect very low numbers of viruses. The ICC-nPCR method also provided a greater degree of certainty that the samples were either negative or positive for specific viruses due to the specificity of the internal primers rather than the subjectivity involved with the recognition of viral CPE. Of the samples reanalysed 14.75% that were originally positive by TCVA-MPN method were negative when analysed by ICC-nPCR. The TCVA-MPN method is not specific to any one virus and detects all culturable viruses that exhibit CPE. Therefore the discrepancies in the data may be due to the presence of reoviruses or other non-human mammalian viruses in the original sample. In contrast, 36.1% of the samples that were negative by TCVA-MPN were found to be positive when reanalysed by ICC-nPCR. This is primarily due to the addition of a second cell line (CaCo-2) and the addition of a proteolytic enzyme prior to the cell culture step for optimal propagation of rotavirus and astrovirus. Three of the four viruses detected by ICC-nPCR need the addition of a proteolytic enzyme to infect the cells; therefore these may have gone undetected with the TCVA-MPN method as well. Inhibition and non-CPE forming viruses may also play a large part in the variations between data from the TCVA-MPN method and ICC-nPCR method.

The increased sensitivity of the two-step enrichment method for coliphage detection compared with the single agar overlay method was also demonstrated. In the two-step enrichment procedure the target phage is propagated during the overnight incubation step allowing for very low numbers of phage to be detected, thus making it more sensitive than the single agar overlay method. The two-step enrichment method also uses a more representative sample volume and is simpler and more cost effective than the single agar overlay method. Only 8.2% of the samples analysed were positive for male-specific coliphages with the single agar overlay method whereas 58% of the same sites were positive using the two-step enrichment method. Similar results were found with somatic phage, 9.8% were positive with the single agar overlay method and 55% with the two-step enrichment method. The differences in detection are due to the use of a larger volume of sample and the pre-incubation step in the enrichment procedure. The pre-incubation step also allows for propagation of phage in a liquid medium that may otherwise be restricted in growth by the single agar overlay method's semi-solid medium.

Correlations between the presence of coliphages, enteric viruses and indicator bacteria were based on

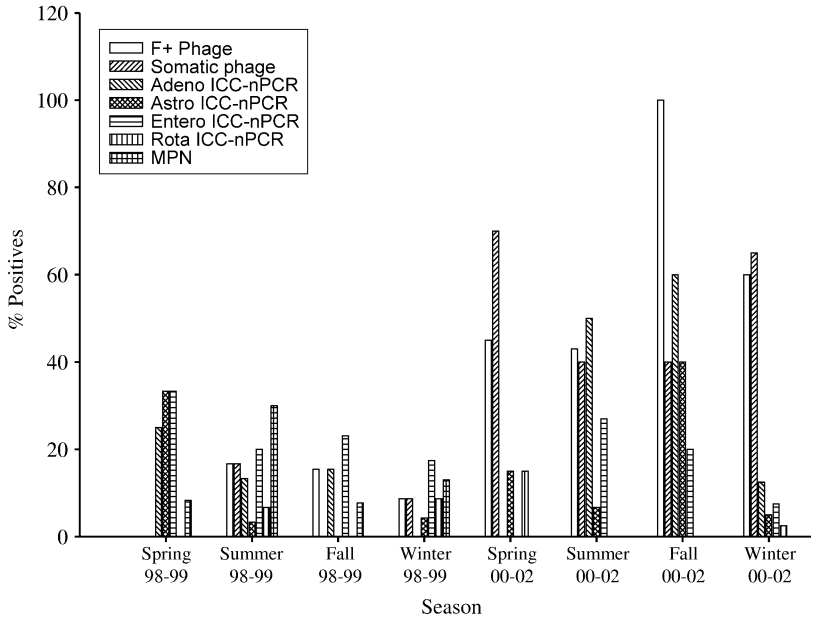

Figure 4 | Seasonal variation between 1998–1999 and 2000–2002.

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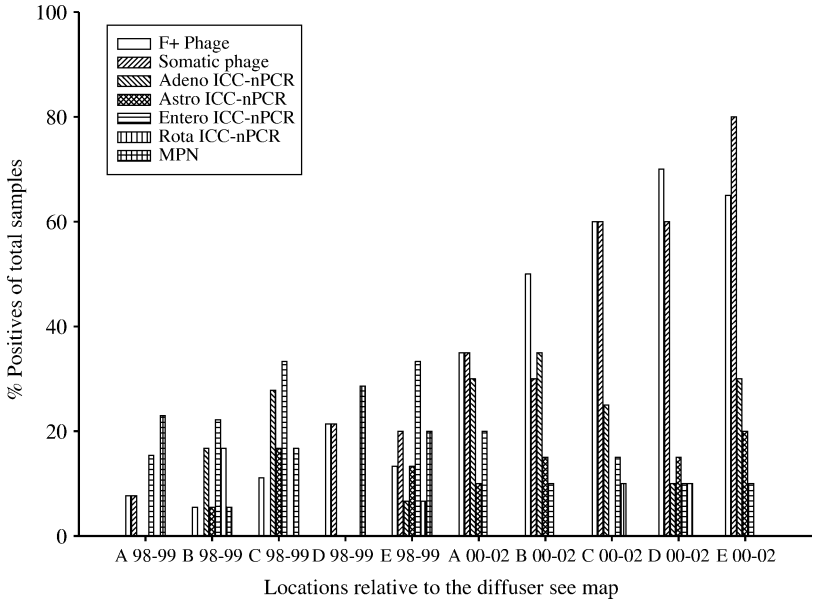

Figure 5 | Proximity variations to outfall diffuser 1998–1999 and 2000–2002.

proximity to the treatment plant and seasonal variations. The presence of total enteric viruses was significantly correlated with the presence of male specific coliphages and somatic coliphages. Both male specific coliphages and somatic coliphages were significantly correlated to each other and to the presence of adenovirus, but not significantly correlated to the presence of astrovirus. Male specific coliphages were also significantly correlated to the presence of rotavirus and enterovirus. Indicator bacteria were not significantly correlated to any of the virus or phage, further demonstrating that indicator bacteria should not be used as an indicator of viral presence. Seasonal relationships in 2000–2002 demonstrated that detection frequency of male specific coliphages and total enteric viruses were highest in the autumn. Somatic coliphages were highest in the spring and winter. Coliphage presence through the seasons indicates that monitoring of both male specific and somatic coliphages is needed for them to be considered good indicators of fecal pollution. From seasonal and proximity correlation data, it appears that coliphages are better indicators of viral presence, male specific coliphages having highest correlations. In fresh water studies male specific coliphages have been found to be more useful indicators of enteric virus presence (Sobsey *et al.* 1995).

Previous studies of coastal marine waters found that the presence of enteric viruses could not be meaningfully correlated to the presence of indicator bacteria (Hughes *et al.* 1992; Wyer *et al.* 1995; Muscillo *et al.* 1997; Vantarakis & Papapetropoulou 1998) and additionally that the numbers of enteroviruses and indicator bacteria vary spatially and temporally (Wyer *et al.* 1995; Vantarakis *et al.* 1998).

The four viral groups (enterovirus, adenovirus, astrovirus and rotavirus) detected showed a seasonal variation as well. Rotavirus was most prevalent in the spring, enterovirus in the summer, adenovirus in the summer and autumn, and astrovirus in the autumn. Of interest is the absence of adenovirus and enterovirus in the spring and rotavirus in both the summer and autumn. These seasonal changes in presence may be due to temperature differences, rain events and incidence of infection in the community. Also the absence of viruses could be due to the competitive attachment and replication encountered with mixed viral populations.

Samples from 1998–1999 reanalysed with ICC-nPCR compared with the samples from 2000–2002 showed that prevalence of total enteric viruses was highest in the spring compared with the autumn, respectively. The seasonal differences could have been due to climate changes for the Massachusetts Bay area and sample age. Samples from 1998–1999 were held for 2–3 years at −80°C before reanalysis. Specific viral presence when the older samples were reanalysed showed changes as well. Adenovirus, enterovirus and astrovirus were most prevalent in the

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spring, and rotavirus in the winter and summer. Adenovirus was not detected in the winter, and astrovirus and rotavirus were not detected in the autumn. No seasonal relationships can be concluded from the data for two reasons. First, the data demonstrates large variations in viral occurrence. These could be due to temperature differences, rain events, snowmelt, climate changes and human impact. Human impact probably plays a large part in the seasonal variation results. The bay is used extensively for recreation in the spring, summer and autumn. Second, the effects of mixed viral populations encountered with environmental samples are still poorly understood. With mixed viral cultures competitive growth rates and the ability for attachment depend largely on the number of viral particles and the growth conditions.

Prevalence of coliphage due to proximity to the outfall diffuser remained relatively similar from 1998–1999 to 2000–2002. Coliphages were most prevalent at the shores northwest and southwest of the diffuser and directly to the west of the diffuser. Presence at the shores was expected because of recreational use. The site to the west of the diffuser had coliphage positives as well as adenovirus, astrovirus and rotavirus positives but the indicator bacteria results were negative. This further demonstrates the limited use of bacterial indicators as indications of fecal pollution. No prevalence to proximity correlations could be made due to the erratic changes of viral presence. The 5-year span of this study may also not be long enough to see any cyclic events within the viral populations due to proximity and seasonal variation.

The prevalence of coliphages and adenovirus at the southwest shore site may be due to recreational use, and are of particular interest because it is a shell fishing resource area. Indicator bacteria numbers were below statistical significance (<30 cfu per plate) for this site, again indicating their inadequacy as indicators of fecal pollution. Coliform bacteria are routinely used as indicators of fecal pollution in water quality analyses. Using solely indicator bacteria to assess water quality is not adequate because of their lack of survivability in the environment. Coliphages would be a much more useful indicator, especially when detected by the enrichment method.

The site in the mouth of the bay, not influenced by sewage discharge, was to be used as a control to compare with the other sites. Male specific coliphage, somatic coliphage and adenovirus as well as the highest number of positives for enteroviruses were detected at this site in the 2000–2002 samples. Their occurrence is thought to be either a result of boats releasing fecal material into the water or from water currents and tidal changes.

While indicator bacteria have been shown not to be good indicators of viral presence, more research needs to be done on male specific and somatic coliphages and their role as viral indicators. Further prevalence and occurrence data needs to be compiled to elucidate the value of coliphages as viral indicators. Other work done in our laboratory (unpublished data) would indicate that site-specific factors that are not yet understood influence the reliability of coliphages as an indicator organism. Hence, until these factors have been further studied, use of phage as an indicator needs empirical demonstration, through monitoring, for a particular site. Additionally, reovirus, hepatitis A virus and other enteric viruses also need to be included to better establish their relationship with coliphages. In previous studies, reoviruses have frequently been detected in seawater and suggested as a possible indicator of fecal pollution (Muscillo *et al.* 1994; Tani *et al.* 1995; Patti *et al.* 1996). But since reoviruses are more common in the animal population, the relationship between reoviruses and coliphages, or their use as predictors of other anthropogenic viruses, is not currently well defined.

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