Water Science and Technology: Water Supply **Vol 2 No 3 pp 23–28** © IWA Publishing 2002

Hygienic risk assessment by monitoring pathogens in municipal sewage

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Abstract The purpose of this study was to monitor the levels of human enteric viruses and enteric protozoa and to relate their presence to the microbes used as hygienic quality indicators in domestic sewage from a small community in Finland during a period of one year. Genome-based sensitive detection methods for the pathogens selected (astro- and Norwalk-like viruses, *Giardia* and *Cryptosporidium*) have become available only recently and thus no earlier data was available. The effluent sewage is delivered into a river that serves as raw water for a larger town and the pathogens therefore constitute a health risk. The results showed that all the monitored pathogens could be detected, and that enteric viruses were present at considerable concentrations in sewage. High concentrations of astrovirus in raw sewage were observed during a diarrhea epidemic in the local day-care centre. The presence of viruses did not correlate with the monitored bacterial indicators of faecal contamination (coliforms, *E. coli* and enterococci) or with bacteriophages (somatic coliphages, F-specific RNA phages and *B. fragilis* phages). *Giardia* cysts and *Cryptosporidium* oocysts were detected from one sample (1/10) each.

Keywords Astroviruses; bacteriophages; caliciviruses; *Cryptosporidium*; *Giardia*; indicators; sewage

Introduction

Gastroenteritis is the second most common infection affecting man. The etiological agents are transported in sewage to treatment plants and further to the receiving water bodies. These pathogens include different bacteria, viruses and protozoa. The vast diversity of pathogens challenges the hygienic monitoring of waters because of the temporal occurrence of pathogens, variations in contamination levels and differences in survival characteristics. The conventional monitoring of hygienic water quality is based on faecal indicator bacteria, mainly coliform bacteria and enterococci. Recent developments in biotechnology now offer feasible and highly sensitive methods for the specific detection of many pathogens, even those not presently cultivable. Questions have been raised concerning the need to supplement the indicator system with direct monitoring of pathogens (Metcalf *et al*., 1995).

Monitoring of different pathogens in municipal sewage could be used as a tool to assess the health status of the community. In Finland this procedure has been applied to the surveillance of enteroviruses, especially poliovirus, for many years (Hovi *et al*., 1996). The purpose of this study was to monitor the hygienic quality of influent wastewater in a small community in Finland over a period of one year. We analysed astroviruses and NLV (Norwalk-like virus) caliciviruses and the protozoans *Giardia* and *Cryptosporidium*, together with faecal indicator bacteria and bacteriophages.

Materials and methods

Study area and sampling

The influent of the Aura treatment plant consists of the wastewater of 2,200 inhabitants and occasional storm water. The discharge is 500 m^3 d^{-1}. Industrial wastewaters are collected separately to the same wastewater plant with tertiary treatment. After treatment the wastewater is discharged to the river Aurajoki, which serves as the raw water source for the town of Turku with 172,000 inhabitants.

Time-based composite samples of incoming municipal sewage collected during 24 hours were taken every two weeks for a period of 18 months (November 1998–June 2000). The ambient temperature during collection was below 10°C. Samples of 1 litre were transported within 24 hours in disposable polyethylene bottles to the laboratories performing the analyses.

Viruses

For virus determinations 5 ml was aliquoted from each 1 litre sewage sample. The samples were kept at +4°C until analysed. The human enteric viruses selected as targets were Norwalk-like viruses (NLVs) and astroviruses. The NLVs were analysed as described by Maunula *et al*. (1999). Briefly, RNA was extracted from a 100 µl sample and the RNA was divided into portions for three PCRs; two separate portions for (polymerase region of) genogroup G1 and G2 NLVs, respectively, and one for astroviruses. The primers of Mitchell (Mitchell *et al*., 1995) were used in astrovirus RT-PCR. Amplification products were confirmed by hybridisation using a selection of one, three and four probes for astrovirus, G1 and G2 NLVs, respectively.

Semiquantitative determinations were performed from 10-fold end-point dilutions of the original sample. In addition, a method for quantitative measurements of astrovirus genomes was developed for the human astroviruses taking advantage of the specific conserved region in the 5′-end just prior to the polyA tail. The PCR was performed in a Perkin Elmer AbiPrism 7700 (Yang and Bonsdorff, unpublished). Samples from the first six months of the follow-up period were run in parallel with the above method.

Protozoa

Cryptosporidium and *Giardia* were analysed from 10 ml samples of influent wastewater by the immunomagnetic separation technique (Dynabeads GC-Combo, Dynal, Oslo, Norway) followed by further release and purification of DNA with 25% Chelex 100 (BioRad, USA) and five freeze–thaw cycles (Rimhanen-Finne *et al*., 2001). The primers cry15 and cry9 were used for amplification of a 550-bp fragment specific for *Cryptosporidium* oocyst wall protein (Spano *et al*., 1997). The primers GDH1 and GDH4 were used for amplification of a 768-bp fragment from the glutamate dehydrogenase gene of *Giardia* (Homan *et al*., 1998). The specificity of the amplified products was confirmed by probing with DIG-labelled probes.

Bacteriophages

F-specific RNA phages and somatic coliphages were enumerated with standard methods (ISO 10705-1, 1995 and ISO 10705-2, 2000) from 40 samples and *B. fragilis* RYC2056 phages from 12 samples with a proposed standard method (ISO/CD 10705-4, 1999). Subsamples of 4.5 ml were transferred from the composite sample to sterile polypropylene tubes and after the addition of 0.5 ml glycerol the subsamples were stored at –20°C. A reference sample of phage was analysed in duplicate simultaneously with the analysis of wastewater samples in duplicate. Deep frozen reference samples of phages were prepared as described by Mooijman *et al*. (1999). For the somatic coliphages Φ×174, for the RNA

phages MS2 and for *B. fragilis* phages HSP40_8, dilutions stored at –65°C were used. About 20% variation was observed between counts of reference phage samples between cultivations. The counts for wastewater samples were corrected for efficiency of plating (correction factor = average count divided by the count of each cultivation period) and for the dilution due to the addition of glycerol.

Indicator bacteria

Faecal indicator bacteria, coliforms, *Escherichia coli* and enterococci were determined in duplicate after dilution in phosphate buffer with Colilert and Enterolert (IDEXX, USA) immediately after the samples reached the laboratory. Colilert trays were incubated at 37 ± 1°C and Enterolert at 41 ± 0.5°C for 24 hours.

Results

The occurrence of the enteric viruses in the sewage samples followed a rather clear pattern (Figure 1). Each of the analysed viruses showed distinct temporal peaks. The astroviruses (Figure 1a) occurred as two prominent peaks, one in December 1998–January 1999 and the second in April–May 2000. The concentrations of virus in the samples as determined with the AbiPrism quantitative PCR were well in accordance with the semiquantitative values in Figure 1b (comparison data not shown). The earlier peak correlated directly with a severe outbreak of gastroenteritis in the local municipal day-care centre. G2 NLVs were present in most of the samples, whereas GI NLVs were less abundant. Noteworthy is the consistent presence of NLVs during the period June–October 1999.

Cryptosporidium and *Giardia* were studied from ten samples from December 1999 to June 2000. One of the samples was positive for *Cryptosporidium* and another for *Giardia*. The detection limit for both organisms was estimated from spiked samples to be 13 oocysts/cysts per ml.

Somatic coliphages (Figure 1c) and faecal indicator bacteria were permanently present in raw sewage. Numbers of somatic coliphages were at the same level as enterococci (10^2–10^4 per ml) but the numbers of *E. coli* were tenfold (Figure 1d). Coliforms, *E. coli* and enterococci were intercorrelated and *E. coli* correlated with somatic coliphages but no other correlations were observed between bacteria and phages (Table 1).

The correlations calculated for astroviruses and NLVs were approximate because only the levels of contamination, but not the exact numerical values, were estimated. However, relatively high numbers of coliforms and enterococci occurred simultaneously with high numbers of astroviruses. Numbers of coliphages tended to be higher (median 6250 PFU/ml) than the numbers of RNA phages (1070 PFU/ml) whereas the numbers of phages infecting *B. fragilis* RYC2056 were still lower (geometric mean of 12 samples, 200 PFU/ml). The calculation of numbers of RNA phages included subtraction of DNA

Table 1 Correlations between viruses and indicators in raw sewage (Pearson; log transformed data, $n = 21$)

	Astro	NLV-G1	NLV-G2	Coliforms	*E. coli*	Entero-cocci	RNA phages
NLV-G1	–0.22						
NLV-G2	0.16	–0.13					
Coliforms	0.41	0.14	–0.07				
E. coli	0.25	0.26	–0.26	0.71			
Enterococci	0.59	–0.11	–0.19	0.77	0.71		
RNA phages	–0.06	0.11	0.22	0.04	0.18	–0.01	
Somatic coliphages	0.16	0.30	–0.20	0.24	0.51	0.21	0.14

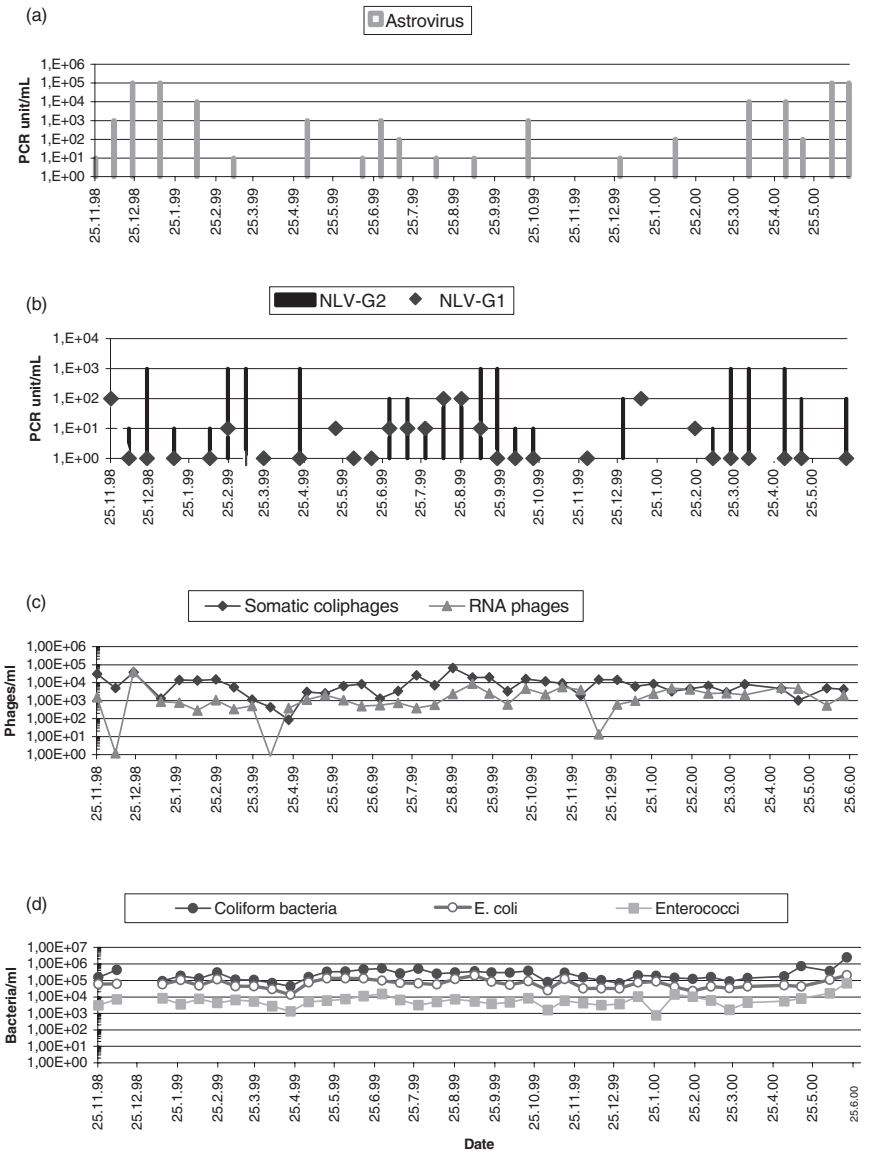

Figure 1 The concentrations of: (a) astroviruses, (b) NLV-G1 and NLV-G2, (c) somatic coliphages and RNA phages, and (d) indicator bacteria in sewage

phages from the total plaque count. Sometimes this led to negative values due to the uncertainty of measurement.

Discussion

Monitoring of viruses in sewage has mainly been directed towards cultivable enteroviruses. Especially for the surveillance of the poliovirus it still is a powerful method (Hovi *et al.*, 1996). When considering the health risks posed by viruses spread via sewage, hepatitis A and NLVs clearly stand out as medically significant; the former due to the serious clinical illness, and the latter due to the wide extent of outbreaks among adults. Widespread population immunity to many other entric viruses, such as group A rotaviruses, astro- and adenoviruses means that these viruses are rarely able to cause outbreaks except in children.

In the present material the concentration of astroviruses in the sewage tended to be somewhat higher than that of NLVs. We do not know whether this finding is reliable or whether the heterogeneity of NLVs affects the sensitivity of their determination. No significant correlations were detected between astroviruses, NLVs and bacteriophages.

The presence of NLVs and astroviruses in the sewage suggests that individual outbreaks can be discerned in a population of the size of Aura. Ideally, it should be possible to estimate the extent of the outbreak by the amount of virus seen in the sewage. Although NLVs cause epidemics most frequently during the winter (winter vomiting disease), NLVs were detected in sewage throughout the year in this study. The G2 NLVs appeared to be more prevalent than the G1 NLVs, in accordance with their prevalence in the outbreaks analysed (Maunula *et al*., 1999, Lappalainen *et al*., 2001). The consistent occurrence of NLVs during the late summer–early autumn of 1999 is noteworthy. It indicates that the NLVs circulate in the population although outbreaks are rarely recorded at this time of the year.

The results of our limited study from a small community of 2,200 inhabitants suggest that the incidence of infections caused by *Cryptosporidium* and *Giardia* is at the same level as shown in other studies (Bukhari *et al*., 1997). Due to limitations of the short study period and too few sampling sites, more studies are needed for risk assessment of the significance of *Cryptosporidium* and *Giardia* released from wastewater treatment plants to the receiving river Aurajoki.

If a reduction of phages of 90–99% can be assumed during activated sludge treatment, the level of *B. fragilis* phages in raw sewage would be too low for reliable detection in treated sewage. Furthermore, the low initial level and further decrease of RNA phages in the receiving river both by dilution and inactivation does not encourage their use for monitoring of faecal contamination. On the other hand, somatic coliphages allow a somewhat more pronounced dilution and their possible multiplication during activated sludge treatment may increase the safety margin when contamination caused by treated wastewater is of interest. When the reduction of somatic coliphages was measured four times in an activated sludge treatment plant using temporally coordinated influent and effluent samples, the reduction was only 53–89% (Niemi, unpublished).

The median value of *E. coli* was ten times higher than the medians of enterococci and somatic coliphages and almost 60 times the median of F-specific RNA phages, which is in good agreement with the findings of Contreras-Coll *et al*. (submitted) in raw sewage Europe wide. Contreras-Coll and coworkers obseved that the relative numbers of somatic coliphages compared with *E. coli* numbers in bathing waters were higher than in raw sewage.

When using PCR-based methods for the enumeration of virus it is not possible to measure the efficiency of wastewater treatment in inactivating viruses. If a reduction of 90–99% can be assumed, the observed high concentrations will result in viral contamination of the downstream river. This poses an increased infection risk and a challenge for the drinking water treatment. On the basis of long-term monitoring (Niemi *et al*., 1994) at the abstraction site of the river Aurajoki, the median of thermotolerant coliforms was 650 and for enterococci 266 in 100 ml. This indicates 10^3–10^4 fold decrease and dilution compared to raw sewage.

Monitoring of wastewater for specific enteric pathogens apparently functions as a reliable indicator of the occurrence of gastrointestinal infections in the population. It can be extended to comprise several more gastrointestinal pathogens. Although monitoring of pathogens can serve as a good indicator of the epidemiological situation it cannot yet substitute routine monitoring of faecal indicators in recipients, due to the seasonality of many enteric illnesses, the random occurrence of epidemics caused by enteric pathogens and the high diversity of different pathogens in wastewater.

Conclusions

Quantitative monitoring of sewage for certain enteric pathogens may serve as a reliable measure for the infection burden in the community.

Human enteric viruses reach high concentrations in sewage and therefore pose a contamination risk for the receiving water bodies.

The indicators monitored were present in high concentrations throughout the observation period, showing that they serve well for the detection of faecal contamination but not as indicators of the presence of enteric viruses.

The present results suggest that for risk assessment monitoring the studied pathogens themselves are required rather than any of the used indicators.

Acknowledgements

This study was supported by grants from the Academy of Finland (42675/98) and the National Technology Agency (40328/00) for the Finnish Research Programme on Environmental Health. We thank Ms Niina Torvela, Ms Tuula Ollinkangas, Ms Riitta Tarkiainen and Ms Jaana Eskelinen for skillful technical assistance and Mr Michael Bailey for the correction of the language.

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