Accumulation of enteric bacteriophage in fresh water sediments

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ABSTRACT

Our study aimed to assess the accumulation of bacteriophages in sandy and clayey fresh water sediments. All of the 24 natural fresh water sediments were positive for somatic and F-specific phages, though their concentrations in the overlying water were undetectable in 1 and 11 samples, respectively, out of 24, corresponding to 4 and 46% for somatic and F-specific phages, respectively. Based on the sediment-to-water ratios, F-specific phages accumulate over 100 times more than the somatic coliphages in clayey sediments. Inactivation of bacteriophages in clayey and sandy sediments over a 1-month period at 15°C was negligible. Our data suggest that persistence of deposited viruses in fresh water sediments leads to accumulation and the findings call for additional investigations on the fate of entrapped pathogenic viruses.

Key words | F-specific phages, fresh water sediment, inactivation, somatic coliphages

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INTRODUCTION

Enteric viruses such as norovirus, rotavirus, enterovirus, hepatitis A virus, astrovirus and adenovirus are well recognized as pathogens associated with waterborne diseases (Leclerc *et al.* 2002). These viruses contaminate surface waters that are in use for recreational purposes, drinking water production or shellfish harvesting, mainly by discharges of raw and treated wastewater (Lodder & Roda Husman 2005; van den Berg *et al.* 2005). Studies have shown that a portion of these viruses present in wastewater and surface water are associated with solids (Hejkal *et al.* 1981; Rao *et al.* 1984; Schijven & Hassanisadeh 2000). Free virus particles may be trapped in river sediment through diffusion and advection, whereas solid-associated viruses may settle. Human pathogenic viruses, mainly enteroviruses, have been isolated from sediments (Gerba *et al.* 1977; Smith *et al.* 1978; LaBelle *et al.* 1980; Schaiberger *et al.* 1982; Rao *et al.* 1984, 1986; Lewis *et al.* 1985; Jofre *et al.* 1989; Botero *et al.* 1992; Lucena *et al.* 1996). It is noticeable that most of the investigations on the presence of enteric viruses in sediments were performed before 2000 and mainly in the 1980s. Occurrence of infectious viruses in sediment is controlled by factors such as their association with the sediment, inactivation, migration and/or release.

Once viruses are entrapped or associated with sediment, they are immobilized (reversibly or irreversibly), which may lead to accumulation of viruses in the sediments (De Flora *et al.* 1975). Viruses may persist longer in sediments than in water (Smith *et al.* 1978; Liew & Gerba 1980; LaBelle *et al.* 1980; Chung & Sobsey 1993; Sakoda *et al.* 1997; Schijven & Hassanisadeh 2000; Karim *et al.* 2004) because in sediment, they are protected from sunlight (Wilhelm *et al.* 2002; Heaselgrave *et al.* 2006) and the temperature may be lower than in the water (Liew & Gerba 1980). However, changes in both redox potential and pH of the sediments may accelerate oxidation of organic contaminants (Eggleton & Thomas 2004) which in turn may result

doi: 10.2166/wh.2009.098

in enhanced viral inactivation. Other phenomena such as predation of viruses (e.g. by protists) may occur in sediment (Gonzalez & Suttle 1993; Bettarel *et al.* 2005). Natural events or human activities may re-suspend sediment-associated viruses (LaBelle & Gerba 1979). This hypothesis has been proposed to explain increased virus concentrations in river water when flow rates increased (Wilkinson *et al.* 1995; Skraber *et al.* 2004*a*; Schijven & Husman 2005). In recreational waters, oyster harvesting waters or drinking water resources, release of virus from sediment may lead to short concentration peaks increasing public health risk (Westrell *et al.* 2006). Although the release of chemical contaminants from sediment has been extensively studied (Eggleton & Thomas 2004), release of viruses from sediment has never been quantified.

No epidemiological studies exist that relate outbreaks to resuspended sediment-associated virus. Data are available on concentrations of enteric viruses, mostly enteroviruses, in water or in sediment but the transfer of virus particles between the two phases is still unclear.

The culture of human viruses, when feasible, remains complex and time consuming. In contrast, bacteriophages of enteric origin such as somatic or F-specific coliphages are rapid and easy to cultivate and can therefore be useful to investigate the fate of viruses in sediment. These phages are similar to human enteric viruses in terms of size, shape and surface charge (Ferguson *et al.* 2003). Bacteriophages are non-pathogenic, easy to quantify and commonly at higher concentrations in the aqueous environment than enteric viruses. For these reasons, somatic coliphages and F-specific phages have frequently been studied (Skraber *et al.* 2002; Contreras-Coll *et al.* 2002; Duran *et al.* 2002; Lodder & Roda Husman 2005; Schets *et al.* 2008). Nevertheless, data on the presence of bacteriophages of enteric origin in sediments are scarce.

The main objective of our work was to gain insight into the accumulation of viruses in fresh water sediments. To that aim, somatic and F-specific coliphages were studied as models of viruses. Their concentrations in sandy and clayey sediments were determined and compared with their concentrations in the overlying surface water. In addition, their inactivation in sediments was investigated in order to evaluate the importance of inactivation for their potential accumulation in sediments.

MATERIALS AND METHODOLOGY

Water and sediment samples

From February to August 2004, sediments with different composition and faecal contamination level were sampled at six locations in the Netherlands. Four of these sediments were clayey and were collected in Amsterdam from the Herengracht (H) and the Prinsengracht (P) canals, from the Amstel (A) river and from the IJmeer (I) lake. Two sandy sediments were collected from ditches in Utrecht (U) and Bilthoven (B). One litre of surface water from each location was collected prior to the sediment sampling. Sediments were collected with an Ekman dredge at a depth varying between approximately 1 and 3 m depending on soil penetrability. Four samples of approximately 40 cm in length and 5 cm in diameter of top-sediment were mixed together in sterile containers. Both sediments and water samples were stored at 4°C and analysed the next day. Twenty-four samples of sediment and 24 samples of the corresponding overlying water ($n_{\text{total}} = 48$) were analysed for somatic coliphages and F-specific phages at locations H ($n = 5$), A ($n = 5$), I ($n = 5$), P ($n = 5$), U ($n = 2$) and B ($n = 2$).

Sediment dry matter content

The dry matter (DM) content of each sediment sample was determined in duplicate. To that aim, a portion of each sample was weighed before and after overnight desiccation at 105°C.

Elution protocols

The elution protocol was adapted from Ahmed & Sorensen (1995) who used it to extract bacteriophage f2 phages from biosolids. Briefly, 20 ml of 10% paste beef extract (Difco, Amsterdam, The Netherlands) at pH 9 was added to wet sediment that contained 4.44 g of dry matter. Thus, each sediment elution was performed the day following the sampling day in order to take into account the dry matter content. The mixture was shaken at 500 rpm for 30 min at 4°C, sonicated on ice (Branson, Danbury, Connecticut, model 5210; nonadjustable setting) for 5 min and centrifuged at 5,000 g for 30 min. The supernatant, adjusted to pH 7.2 with HCl 1M, constituted the eluate.

Recovery assessment of eluted viruses

Bacteriophages ϕX174 (ATCC 13706-B1), MS2 (ATCC 15597-B1) and PRD1 were used to determine the recovery efficiency of eluted viruses. Somatic coliphage ϕX174 is a ssDNA *Microviridae* with an icosahedral capsid of 27 nm and a isoelectric point (pI) of 6.6–6.8 (Fujito & Lytle 1996; Dowd *et al.* 1998). F-specific coliphage MS2 is a ssRNA *Leviviridae* with an icosahedral capsid of 27 nm and a pI of 3.5 (Penrod *et al.* 1996). Finally *Salmonella* phage PRD1 has a 62 nm icosahedral capsid with a pI estimated between 3 and 4 (Loveland *et al.* 1996).

In order to assess the recovery of eluted viruses, an experiment was conducted in which 300 ml of 10% paste beef extract at pH 9 was spiked with 300 μl of phages ϕX174, MS2 and PRD1 giving a final concentration of 60 pfu ml^{-1} for each phage. Then, 20 ml of this spiked elution buffer was mixed with 4.44 g equivalent of dry matter (DM) sediments previously sterilized at 121°C for 15 min in order to inactivate autochthonous bacteriophages that could have interfered with ϕX174, MS2 and/or PRD1 enumeration. Sediments from the different locations were dispersed in 20 ml of spiked buffer and each mix was shaken at 500 rpm for 30 min at 4°C. Each sample was further sonicated, centrifuged and the pH was neutralized as describe in the elution protocol. The concentration of bacteriophages was determined in each eluate. A control without sediment was used to evaluate the inactivation of each bacteriophage in the elution buffer.

Bacteriophages enumeration

Somatic coliphages and ϕX174 were enumerated using the bacterial host *E. coli* WG5 according to the standardized method (ISO/FDIS 10705-2 2001). F-specific bacteriophages as well as MS2 and PRD1 were enumerated using the bacterial hosts *Salmonella typhimurium* WG49 (for F-specific phages and MS2) and LT2 (for PRD1) according to the standardized method (ISO/FDIS 10705-1 2001). Nalidixic acid was not used in the case of PRD1 detection as *Salmonella typhimurium* LT2 strain is sensitive to that antibiotic. Stocks of bacteriophages ϕX174, MS2 and PRD1 were prepared at high concentration ($>10^9$ pfu ml^{-1}) as described in norms ISO/FDIS 10705-1/2. Diluted in de-ionized water, ϕX174, MS2 and PRD1 suspensions were used as controls and as viral models for spiked experiments.

Concentrations of phages in water are expressed in pfu per millilitre whereas concentrations in sediment were expressed in pfu per gram of dry matter (DM) according to the following equation:

$$S = [C \times (\text{Vw} + \text{Ve})]/\text{DM}$$

where S is concentration of phages in the sediment (pfu g^{-1} DM); C is concentration of phages after elution (pfu ml^{-1}); Vw is volume of water in the sediment (ml); Ve is volume of eluate (ml); and DM is weight of dry matter (g).

Phage inactivation in sediment

The inactivation of naturally occurring bacteriophages was measured in different sediments (H, A, I, P and B). Samples were stored in a dark incubator at 15.0 ± 0.2°C (day 0) and analysed at days 0, 2, 7, 14, 21 and 29 in duplicate for both somatic and F-specific coliphages. Virus inactivation in relatively mild conditions, such as temperatures below 20°C and near neutral pH, commonly proceeds as a first order rate with inactivation rate coefficient μ $[\text{T}^{-1}]$ (Schijven & Hassanisadeh 2000):

$$\log_{10}(C_t) = \log_{10}(C_0) - \frac{1}{\ln(10)}\mu t$$

where C_0 and C_t are the initial virus concentration and that after time t, respectively. The value of the inactivation rate coefficient μ was estimated by means of linear regression analysis.

RESULTS

Elution protocol

Firstly, we assessed the recovery rates of spiked ϕX174, MS2 and PRD1 applying our elution protocol. All recovery rates were high (Table 1). Bacteriophage recovery rates varied little but were lower with the clayey sediments H,

Table 1 | Recovery of spiked bacteriophages ϕX174, MS2 and PRD1 from sediment after elution. Percentages correspond to the number of phages retrieved from the eluate of sediments from different locations compared with the number of phages initially spiked. Percentages were calculated on raw data

	Sediment*	ϕX174 (%)	MS2 (%)	PRD1 (%)
With sterile sediment	H	79	83	93
	A	82	94	99
	I	69	92	89
	P	91	81	69
	B	100	100	94
	U	105	99	92
	Average ($n = 6$)	88	92	89
	SD	14	8	11
	Min	69	81	69
	Max	105	100	99
Without sediment	Controls ($n = 1$)	103	107	103

*Clayey sediments were collected from Amsterdam city canals at Herengracht (H) and Prinsengracht (P), from the river Amstel (A) and from the lake IJmeer (I). Sandy sediments were collected from ditches in Utrecht (U) and Bilthoven (B).

A and I than with sandy sediments B and U (*t*-test, $p < 0.05$). The control without sediment showed that there was no inactivation of the bacteriophages in the elution buffer during the time of the experiment.

Concentration of bacteriophages in sediments and in overlying waters

Dry matter contents in sediment ranged from 23% to 53% in clayey sediments (H, P, I, A) and from 58% to 75% in sandy sediments (U, B). Table 2 shows the concentrations of bacteriophages in the overlying water ($\log \text{pfu ml}^{-1}$) and in the sediments ($\log \text{pfu g}^{-1}$) and their ratios. Somatic coliphages and F-specific phages were detected in all sediment samples whereas their concentrations in the overlying water were under the limit of detection ($<0.1 \text{ pfu ml}^{-1}$) 11 times for F-specific phages (46%) and once for somatic coliphages (4%).

In the water phase, as in sandy sediments (U, B), concentrations of somatic coliphages were always higher than the concentrations of F-specific phages. In clayey sediments (A, H, I, P), the opposite was observed.

The sediment-to-water ratios for somatic coliphages were lower for the sandy sediments (0.2–2) than for the clayey sediments (4–11). Similarly, the sediment-to-water ratios for F-specific phages were lower for the sandy sediments (0.1–2), and a hundred to a thousand times higher for clayey sediments (>161–595).

Inactivation

Table 3 presents the inactivation rates of both somatic coliphages and F-specific phages in sediment from five locations at 15.0 ± 0.2°C. It can be noticed that the concentrations of F-specific phages in sample B were under the limit of detection precluding the assessment of the corresponding inactivation rate. Our results show that, for both phage groups, inactivation was negligible (not different from zero) during the experimental period with the exception of somatic coliphages in sample A where an inactivation of $0.025 \log_{10} \text{day}^{-1}$ was observed.

DISCUSSION

In our study, somatic coliphage concentrations were at least 10 times higher than F-specific phage concentrations in surface water regardless of the sampling site. This is in agreement with other data reporting ratios of somatic coliphages/F-specific phages greater than 1 in wastewater and in surface water (Skraber *et al.* 2002; Lucena *et al.* 2003; Schets *et al.* 2008). If somatic coliphages and F-specific phages behaved in the same way, the same ratio of the two bacteriophage groups would be expected in the sediment. Although in the sandy sediments the number of somatic coliphages outnumbered the F-specific phages, which is in agreement with previous results (Araujo *et al.* 1997), in clayey sediments, the opposite was observed.

The replication of F-specific bacteriophages in clayey sediments could explain our results. However, according to Woody & Cliver (1995, 1997), F-specific bacteriophages are unlikely to replicate in nutrient-poor environments, at temperatures below 25°C. Also, a higher replication of F-specific bacteriophages in the environment compared with somatic coliphages has never been reported. Finally, we did not observe an increase of F-specific phage concentrations during the month of sediment monitoring. Although it has to be confirmed, our results, in combination

Table 2 | Average concentrations and standard deviations (SD) of bacteriophages calculated on log-transformed data in overlying water, sediment and the corresponding ratio

		Overlying water (log pfu ml^{-1})		Sediment (log pfu g^{-1})		
Sediment type	**Sediment location***	**Average**	**SD**	**Average**	**SD**	**Ratios sediment/water (g/ml)†**
Somatic coliphages						
Clayey	A ($n = 5$)	0.9	0.6	1.5	0.3	4
	H ($n = 5$)	0.6	0.7	1.2	0.4	4
	I ($n = 5$)‡	0.1	0.7	1.0	0.5	9
	P ($n = 5$)	0.5	0.8	1.6	0.6	11
Sandy	U ($n = 2$)	1.3	1.0	1.5	0.4	2
	B ($n = 2$)	2.5	0.0	1.9	0.3	0.2
F-specific phages						
Clayey	A ($n = 5$)§	-0.4	0.8	2.4	0.3	595
	H ($n = 5$)§	-0.1	1.3	2.1	0.3	>161
	I ($n = 5$)§	<-1.0	NA	1.3	0.6	>200
	P ($n = 5$)§	-0.3	1.0	2.3	0.3	400
Sandy	U ($n = 2$)	0.2	1.0	0.4	0.4	2
	B ($n = 2$)	1.4	0.3	0.1	0.1	0.1

*Abbreviations for sediments are the same as those used in Table 1.

†Ratios were calculated as follows: ratio $= 10^{CS}/10^{CW}$ where CS and CW are the concentrations in sediment (log pfu g^{-1}) and in water (log pfu ml^{-1}), respectively.

‡Somatic coliphage concentration in water was once under the limit of detection. For calculation, the value was set to the limit of detection (-1.0 log pfu ml^{-1}).

§In water samples, F-specific phage concentrations were under the limit of detection 1, 2, 3 and 5 times in A, P, H and I, respectively. For calculation, values under the limit of detection were set to the limit of detection (-1.0 log pfu ml^{-1}).

NA: Not Applicable.

with reported data, tend to show that significant replication of F-specific bacteriophages does not occur in fresh water sediments.

A lower inactivation rate of F-specific phages compared with somatic coliphages in clayey sediment could explain the difference in concentrations since our data are based on infective particles. However, our results show that both somatic coliphages and F-specific phages were stable at 15°C in three out of four clayey sediments for one month. By comparison, Karim *et al.* (2004) reported an inactivation rate of 0.11 $\log_{10}$ day^{-1} for somatic coliphages in sediment, which is approximately four times higher than the higher

Table 3 | Inactivation rate coefficients μ ($\log_{10}$ day^{-1}) of somatic coliphages and F-specific phages in sediment from different locations determined in a one month survival experiment ($n = 6$)

		Sediment location*				
		H	**A**	**I**	**P**	**B**
Somatic coliphages	μ ($\log_{10}$ day^{-1})	0.009	0.025	0.022	0.006	0.015
	p-value	0.33	0.01†	0.12	0.46	0.23
	Lower 95%	0.033	0.040	0.053	0.025	0.044
	Upper 95%	0.014	0.011	0.009	0.013	0.014
F-specific phages	μ ($\log_{10}$ day^{-1})	0.001	0.010	0.007	0.004	–
	p-value	0.84	0.08	0.33	0.07	–
	Lower 95%	0.008	0.022	0.011	0.008	–
	Upper 95%	0.009	0.002	0.026	0.001	–

*Abbreviations of sediments are the same as those used in Table 1.

†Significant at a confidence level of 95%.

die-off rate we measured (0.025 $\log_{10}$ day^{-1}). In their study, sediments were stored at 22.5°C. The higher temperature can explain these results since temperature is known to affect virus inactivation in the environment (Liew & Gerba 1980; Chung & Sobsey 1993; Skraber *et al.* 2004*a*,*b*). In summary, inactivation seems not to be the main parameter influencing the ratio of F-specific/somatic coliphages in clayey sediments at temperatures lower than 15°C.

A higher detachment rate of F-specific compared with somatic coliphages from clayey sediment during the elution protocol (resulting in a higher recovery rate) would lead to an apparent difference in concentrations in the sediment. Johnson *et al.* (1984) observed that poliovirus recovery from sediment decreases as the concentration of clay increases. Although a difference in virus recovery with spiked sediment has been reported (LaBelle & Gerba 1979), no study has been found that shows a difference of 2 $\log_{10}$ that could explain our observations. Further investigations (e.g. using molecular tools) should determine to what extent a difference of detachment rates exists (if any) between F-specific and somatic coliphages from clayey sediments.

Finally, a higher attachment rate of F-specific phages to clayey sediment compared with somatic coliphages may explain the difference in concentrations. Clay is known for its ability to bind virus. For instance, Schiffenbauer & Stotzky (1982) showed that coliphages T1 and T7 attached more when the concentration of clay increased and Gantzer *et al.* (2001) reported more attachment of F-specific phages than somatic coliphages to clayey soil, which is in agreement with our findings. In addition, differences of surface properties between somatic and F-specific coliphages may explain the difference in attachment.

Because we found negligible inactivation rate coefficients, our results suggest that viral concentration in sediment depends mainly on attachment and detachment rates. Considering favourable attachment to clayey sediments and commonly less detachment, we may conclude that both types of bacteriophage, F-specific more than somatic coliphages, may accumulate in clayey sediments. In contrast, attachment to sandy sediment seems to be less favourable. In this study, we confirm that the viral contamination of sediments depends on virus types and strains (Gerba *et al.* 1980; Schijven & Hassanisadeh 2000) and that the selection of viruses in sediments relies on the sediment composition (e.g. sand/clay ratio). From June 2003 to June 2004, rotavirus, norovirus and enterovirus RNA were detected in the canal waters of Amsterdam (Schets *et al.* 2008). As with the bacteriophages, these pathogenic viruses may accumulate in the underlying sediments. Further investigations should elucidate whether enteric viruses entrapped in different types of sediment still represent a potential health risk by recontaminating the overlying water. For that purpose, bacteriophages and especially F-specific phages, for which complementary molecular detection techniques are already available (Vinje *et al.* 2004; Ogorzaly & Gantzer 2006), appear to be promising tools.

CONCLUSIONS

The inactivation of somatic and F-specific coliphages in sediments at 15°C over a one-month period appeared to be negligible, contributing to the potential accumulation of these viruses in the sediments. Based on the sediment-to-water ratios, F-specific phages accumulate over 100 times more than the somatic coliphages in clayey sediments. Our results suggest that fresh water sediments can potentially accumulate pathogenic viruses pointing out the need for additional investigations on the fate of these viruses, including their potential transfer from the sediment to the water phase (surface or ground waters).

ACKNOWLEDGEMENTS

This work was funded by the Ministry for Housing, Special Planning and the Environment. The authors would like to acknowledge OMEGAM Water (Amsterdam, The Netherlands) for their assistance in sediment sampling. The authors also thank Helmi Karim for critical review of the manuscript.

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First received 11 September 2008; accepted in revised form 11 November 2008. Available online May 2009