Decontamination of a drinking water pipeline system contaminated with adenovirus and *Escherichia coli* utilizing peracetic acid and chlorine

Ari Kauppinen, Jenni Ikonen, Anna Pursiainen, Tarja Pitkänen and Ilkka T. Miettinen

ABSTRACT

A contaminated drinking water distribution network can be responsible for major outbreaks of infections. In this study, two chemical decontaminants, peracetic acid (PAA) and chlorine, were used to test how a laboratory-scale pipeline system can be cleaned after simultaneous contamination with human adenovirus 40 (AdV40) and *Escherichia coli*. In addition, the effect of the decontaminants on biofilms was followed as heterotrophic plate counts (HPC) and total cell counts (TCC). Real-time quantitative polymerase chain reaction (qPCR) was used to determine AdV40 and plate counting was used to enumerate *E. coli*. PAA and chlorine proved to be effective decontaminants since they decreased the levels of AdV40 and *E. coli* to below method detection limits in both water and biofilms. However, without decontamination, AdV40 remained present in the pipelines for up to 4 days. In contrast, the concentration of cultivable *E. coli* decreased rapidly in the control pipelines, implying that *E. coli* may be an inadequate indicator for the presence of viral pathogens. Biofilms responded to the decontaminants by decreased HPCs while TCC remained stable. This indicates that the mechanism of pipeline decontamination by chlorine and PAA is inactivation rather than physical removal of microbes.

Key words | adenovirus, biofilm, decontamination, distribution network, microbial contamination

Ari Kauppinen (corresponding author)
Jenni Ikonen
Anna Pursiainen
Tarja Pitkänen
Ilkka T. Miettinen
Department of Environmental Health,
Water and Health Unit,
National Institute for Health and Welfare,
P.O. Box 95,
FI-70701 Kuopio,
Finland
E-mail: *ari.kauppinen@thl.fi*

ABBREVIATIONS

AdV40	human adenovirus 40
cfu	colony forming units
DAPI	4,6-diamidino-2-phenylindole
DBPs	disinfection by-products
E. coli	*Escherichia coli*
GC	genome copies
HPC	heterotrophic plate counts
PAA	peracetic acid
PVC	polyvinyl chloride
qPCR	quantitative polymerase chain reaction
TCC	total cell counts

INTRODUCTION

The contamination of the drinking water distribution network with waterborne pathogens poses a great threat to public health. Pipelines in the distribution network are a favourable environment for microbial growth and therefore the occurrence of biofilms on the inner surfaces of the pipelines is a common phenomenon. Biofilms are considered as a complex collection of living, nonviable and dead microorganisms together with extracellular polymeric substances as well as organic and inorganic matter (Costerton *et al.* 1995). Biofilms provide attachment sites and shelter for microbes against disinfectants and are thus considered a

doi: 10.2166/wh.2012.003

health risk to the consumer (Quignon *et al.* 1997). In addition, the consumption of disinfectant by reactions with biofilms can complicate the management of the contaminated pipelines.

There are many options for purification of drinking water, UV irradiation, chlorination and ozonation being some of the most common techniques. Chemical decontaminants conferring a residual disinfection effect are widely used in the purification of the contaminated drinking water and distribution network. However, usually a large quantity of chemical is needed, especially if the network has been contaminated with viruses or if the pipes contain large amounts of deposit. The most widely used chemical in the management and cleaning of the drinking water pipes is chlorine (Schoenen 2002). The disadvantage of using chlorine is its potential to form harmful disinfection by-products (DBPs) when it reacts with organic matter (Rook 1974). Another chemical believed to be suitable for water purification is peracetic acid (PAA). PAA is an oxidizing agent that can be used for the deactivation of a large variety of micro-organisms (Koivunen & Heinonen-Tanski 2005; Zanetti *et al.* 2007). PAA is widely used in various industries, such as the pharmaceutical and food industries, for sterilization of surfaces and equipment (Kitis 2004). Moreover, PAA is included in the US Environmental Protection Agency (USEPA) list of candidate disinfectants for use in sewer overflows (USEPA 1999) and it has already been introduced in some wastewater treatment plants. One of the benefits of PAA is that it is completely biodegradable and does not produce significant amounts of harmful DBPs (Monarca *et al.* 2002). Thus, PAA is an attractive choice for the disinfection of matrices of high organic load, e.g. wastewater.

Adenoviruses comprise a diverse group of viruses including pathogenic strains causing several serious conditions, such as respiratory disease, conjunctivitis and gastroenteritis. Enteric adenoviruses 40 and 41 (type F) can cause gastroenteritis, especially in young children. Adenoviruses can spread via contaminated water and they have been linked to waterborne outbreaks of gastroenteritis (Kukkula *et al.* 1997; Divizia *et al.* 2004; Maunula *et al.* 2009). Adenoviruses are also included in the USEPA list of Drinking Water Candidate Contaminants (USEPA 2009). Their ubiquity in wastewater has been recognized, making them good indicators of human sewage pollution (Pina *et al.* 1998). *Escherichia coli* is the most commonly used and best known bacterial indicator of the hygienic quality of drinking water and the presence of *E. coli* is an indication of fresh faecal pollution (Edberg *et al.* 2000; Tallon *et al.* 2005).

Numerous studies have tested various methods for the inactivation of microbes. These studies have revealed differences in the persistence of different microbes. In general, enteric viruses are known to be more resistant to disinfection procedures than bacteria (Sobsey 1989). Adenoviruses can survive for long periods in aqueous environments and exhibit good thermal stability (Enriquez *et al.* 1995). They are also extremely resistant to UV light and adenovirus 40 is the most UV-resistant waterborne pathogen known (Hijnen *et al.* 2006). The survival of cultivable *E. coli* is considered to be negligible compared to the survival of enteric viruses (Leclerc *et al.* 2001). However, more information is needed to understand microbial behaviour in aquatic environments and systems, e.g. distribution networks, in relation to decontamination practices. In the present study, the persistence of human adenovirus 40 (AdV40) and *E. coli* was evaluated in drinking water and biofilms in an experimental pipeline system. In addition, the effects of PAA and free chlorine against biofilms and selected microbes spiked into this system were assessed.

MATERIALS AND METHODS

Preparation of the contaminant

The persistence of faecal microbes in drinking water pipelines was studied employing enteric human AdV40 and faecal bacterium *E. coli*. Besides the stock of AdV40 strain Dugan (ATCC VR-931), the strain was also propagated in 293 cells as described by Mautner (1998). Briefly, 293 cells were grown in 75-cm^2 cell culture flasks in monolayers at 37 °C and 5% CO_2. The cells were infected with a 10-fold virus dilution and the viruses were allowed to adsorb for 1 h at 37 °C before medium was added. Infected cells were pooled and harvested after incubation for 5–7 days. Viruses were released by three freeze-thaw cycles and collected by centrifugation (1,500 g for 2 min). AdV40 serotype was confirmed by sequencing.

An environmental *E. coli* strain isolated from faecally contaminated drinking water during a waterborne outbreak in the town of Nokia, Finland, in December 2007 (Laine *et al.* 2010) was used. The *E. coli* strain was cultured aerobically at 36 ± 2 °C on tryptone soya agar (Oxoid), and the contaminant suspension was prepared in nutrient broth, washed and centrifuged as previously described (Lehtola *et al.* 2007). The strains were stored at −75 °C or lower.

Pipeline set up and sampling

Two parallel pipeline systems were constructed for the study, one for decontamination and the other as a non-decontamination control (Figure 1). Each pipeline system was constructed from 24 individual polyvinyl chloride (PVC) pieces (diameter 10 mm, length 10 cm) which were connected in line and could be collected separately. Before use, the pipes were chlorinated (20 mg/L, 20 min) and rinsed with distilled water and they were replaced by new ones between the experiments. The inlet water of the pipeline system was the tap water of the city of Kuopio, Finland, which contained 0.05 mg/L chlorine on average (standard deviation of 0.03 mg/L). The chlorine was not quenched before the pipeline systems to provide as authentic conditions as possible. Water flow through the pipelines was kept as 0.2 L/min by rotameters located before both pipeline systems. Biofilms in the pipelines were allowed to grow for 4 weeks by running systems with tap water to attain the conditions corresponding to the steady state. This stabilization of biofilm growth was checked by heterotrophic plate counts (HPC) and total cell counts (TCC), which were analysed every week before the start of the experiment. At every sampling point, single water samples and two to four replicate 10 cm PVC pieces for biofilm analyses were taken from both pipeline systems at the same time. Biofilms were detached using glass beads as previously described (Zacheus *et al.* 2000). Briefly, glass beads were added into the pipe and then vortexed for 10 min. The pipe was rinsed with 5 ml of sterile distilled water, which was combined with the vortexed water sample.

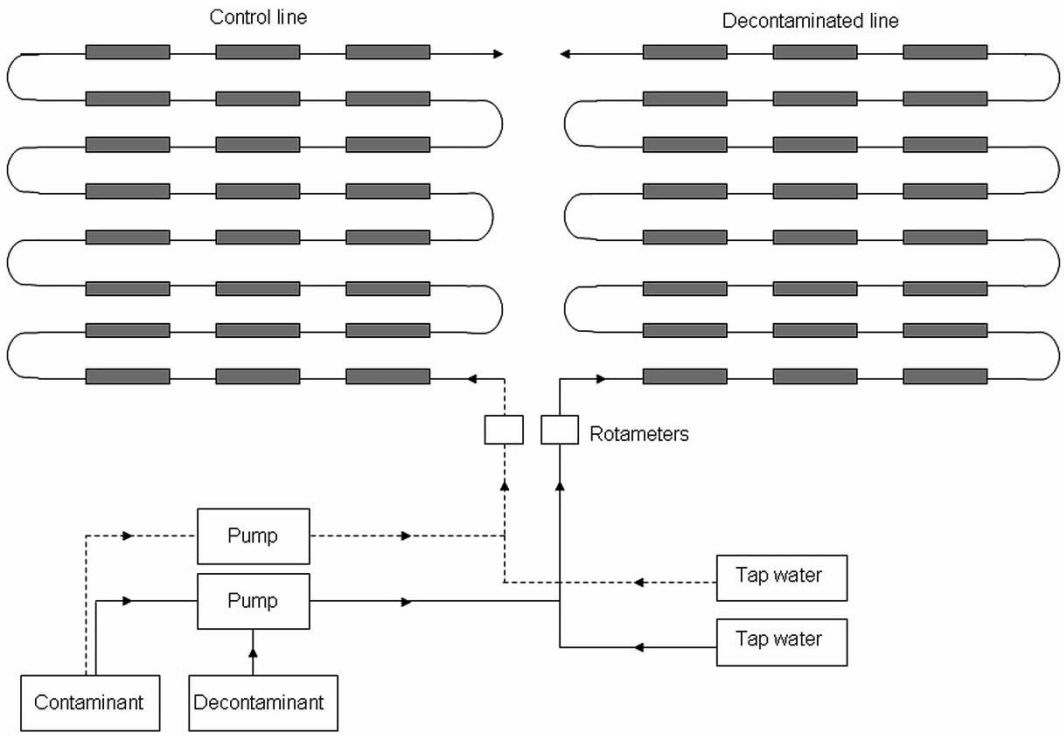

Figure 1 | The experimental setup. The grey boxes illustrate the PVC pieces. Every sampling point included two to four replicate PVC pieces for biofilm analyses.

Inoculation of the microbes

After 4 weeks' propagation of the biofilm, the spiking of the contaminant into pipeline systems was performed. The volume of the contaminant solution was 5 L of tap water and it was pumped with two peristaltic pumps (Grundfors, Denmark) equally to two separate pipelines immediately after preparation. The contaminant was added at flow rate of 0.1 L/min into the main stream and the overall water flow was kept as 0.2 L/min by rotameters. The concentration of AdV40 in the contaminant was approximately 3×10^8 genome copies (GC)/L (ATCC stock) and 5×10^8 GC/L (ATCC stock + propagated AdV40) in PAA and chlorine experiments, respectively. The *E. coli* concentrations in the contaminant were 7×10^8 cfu/L in the PAA experiment and 3×10^{10} cfu/L in the chlorine experiment. The concentrations of microbes, especially *E. coli*, were raised in the chlorine experiment due to the rapid decontamination results obtained with PAA. The spiking was conducted over a time period of 25 min after which only tap water was run into the pipelines.

Decontamination

In these experiments, decontaminant was pumped into one line and the other line was used as the control. Two separate decontamination experiments were conducted with PAA and chlorine. PAA and chlorine as sodium hypochlorite were dosed to achieve final concentrations of 1 mg/L in outlet water. Decontamination was started after 24 h from the spiking of the contaminant to rule out the high initial removal of microbes by washout and continued until the end of the experiment. The concentrations of the decontaminants were monitored in outlet water during the study. The decontaminant was quenched from the samples using sodium thiosulfate (20 mg/L) (ISO 19458 2006; Zanetti *et al.* 2007).

Virological analysis

Sample preparation

Adenoviruses were concentrated from the water samples by the adsorption–elution method described by Gilgen *et al.* (1997). Briefly, 1 L sample was run through a positively charged membrane (diameter 47 mm, pore size 45 μm; AMF-Cuno, Zetapor, Meriden, CO, USA). Viruses were eluted in 50 mM glycine buffer, pH 9.5, containing 1% beef extract and the eluate was rapidly neutralized with HCl. The volume was further reduced to about 200 μl with a microconcentrator (Vivaspin 2, Vivascience AG, Hannover, Germany). This concentrated sample was used for nucleic acid extraction.

Nucleic acid extraction

Viral DNA was extracted directly from the harvested biofilm suspensions and from concentrated water samples using the High Pure Viral Nucleic Acid Kit according to the manufacturer's instructions (Roche Molecular Biochemicals Ltd, Mannheim, Germany). Extracted nucleic acids were stored at −75 °C or lower.

Plasmid standard for quantitative real-time PCR

A 379-bp fragment of the hexon region of human AdV40 (Dugan-strain) was amplified using the forward primer 5′-TGGCCACCCCCTCGATGA-3′ and reverse primer 5′-TTTGGGGGCCAGGGAGTTGTA-3′ (Jothikumar *et al.* 2005). The insert was cloned into a pGEMT-Easy vector and transformed into JM109 competent cells using the pGEMT-Easy Vector System II kit (Promega) according to the manufacturer's instructions. The concentration of PstI-linearized plasmid was determined using the NanoDrop ND-1000 spectrophotometer (Thermo Scientific) and a standard curve generated with triplicate reactions of dilutions ranging from 10^1 to 10^8 genome copies.

Gene amplification

The primer and probe sequences that recognized the adenovirus hexon gene region were according to Jothikumar *et al.* (2005). Real-time polymerase chain reaction (PCR) assays were carried out in a Rotor-Gene™ 3000 real-time rotary analyser (Qiagen) as previously described (Maunula *et al.* 2009). All samples were run in duplicate and with 10-fold dilutions. Negative and positive controls were included in each assay. The amount of DNA was defined as the average of the data obtained. The theoretical detection limit for adenovirus in

water samples was 10 GC/L and in biofilm samples 22 GC/cm^2. In log removal calculations, the theoretical detection limit values were used when a result that was below detection limit was observed.

Bacteriological analysis

E. coli was analysed on Chromocult® Coliform Agar (CC, Merck, Germany) at 36 ± 2 °C for 21 ± 3 h according to the manufacturer's instructions and the international standard ISO 8199 (2005) using spread plating and membrane filtration techniques. The detection limits for *E. coli* were 1 cfu/0.5 L in water samples and 0.1–0.2 cfu/cm^2 in biofilm samples. In log removal calculations, the theoretical detection limit values were used when a result that was below detection limit was observed.

HPC in water and detached biofilms were analysed with a spread plating method on R2A-agar (Difco, USA). R2A medium was incubated for 7 days at 22 ± 2 °C before colony counting. Total cell counts of water and biofilm samples were analysed with 4′,6-diamidino-2-phenylindole (DAPI) staining: the sample was filtered on black 0.2 μm Nuclepore membranes (Whatman, UK) and stained with 5 μg/ml DAPI for 15 min prior to counting by epifluorescence microscopy (Olympus BX51, Olympus Co. Ltd, Japan).

Statistical analyses

The statistical differences were calculated using the Independent-Samples Mann–Whitney U Test with the SPSS 19.0 program for Windows. The method detection limit values were used when the number of organisms was considered to be below the detection limit. Differences were considered significant if p-value was < 0.05.

RESULTS

PAA experiment

Water samples

Figure 2(a) illustrates the fate of AdV40 and *E. coli* in outlet waters of PAA experiment. The concentration of AdV40 decreased by 2.0 and 4.0 logs in the decontamination and in non-decontamination control pipelines, respectively, after the first day from the contamination, before the dosage of the chemical. Within the same time, *E. coli* concentration declined 5.5 and 6.3 logs. The added decontaminant further lowered the concentrations of AdV40 and *E. coli* in the decontamination line yielding 2.5 and >2.9 logs decrease within the first hour of decontamination, respectively. *E. coli* was no longer detectable in the water after 1 h decontamination and AdV40 declined to below detection limit of the method after 24 h from the starting point of the decontamination. The total decrease of the microbes in the decontaminated line was over 5.3 logs for AdV40 and over 8.4 logs for *E. coli*. The concentration of PAA was 0.2–0.3 mg/L during the first day after the start of decontamination. At the end of the experiment, the concentration of PAA was 1.8 mg/L (Figure 2(a)). The control pipeline without PAA treatment remained contaminated with AdV40 and *E. coli*. The concentration of AdV40 was even elevated after the first day, exhibiting a 1.6 log increase by the end of the experiment. The total decrease of the AdV40 in the control line during 3 days' experimentation was 2.4 logs and the concentration of *E. coli* decreased 6.9 logs.

The contamination increased HPC of the outlet water in both pipelines but the concentrations decreased rapidly to near the initial level (Figure 3(a)). The PAA treatment had a clear effect on the HPC decreasing the count by 2.4 logs by the end of the experiment. TCC remained rather stable, showing no notable alteration between PAA-treated and control pipelines (Figure 3(a)).

Biofilm samples

The findings of AdV40 and *E. coli* in biofilms are presented in Table 1. Both microbes were effectively removed from the PAA-treated biofilms. Only one sample was detected as being positive for AdV40 after 1 h PAA decontamination. In the PAA-treated line, the difference in concentrations between the samples before ($n = 6$) and after ($n = 9$) the treatment was statistically significant for both AdV40 ($p = 0.002$) and *E. coli* ($p = 0.005$). In the control line, all parallel biofilm samples were positive for AdV40 until the end of the experiment, though *E. coli* was not detectable 48 h following contamination. In the control line, the average log removal

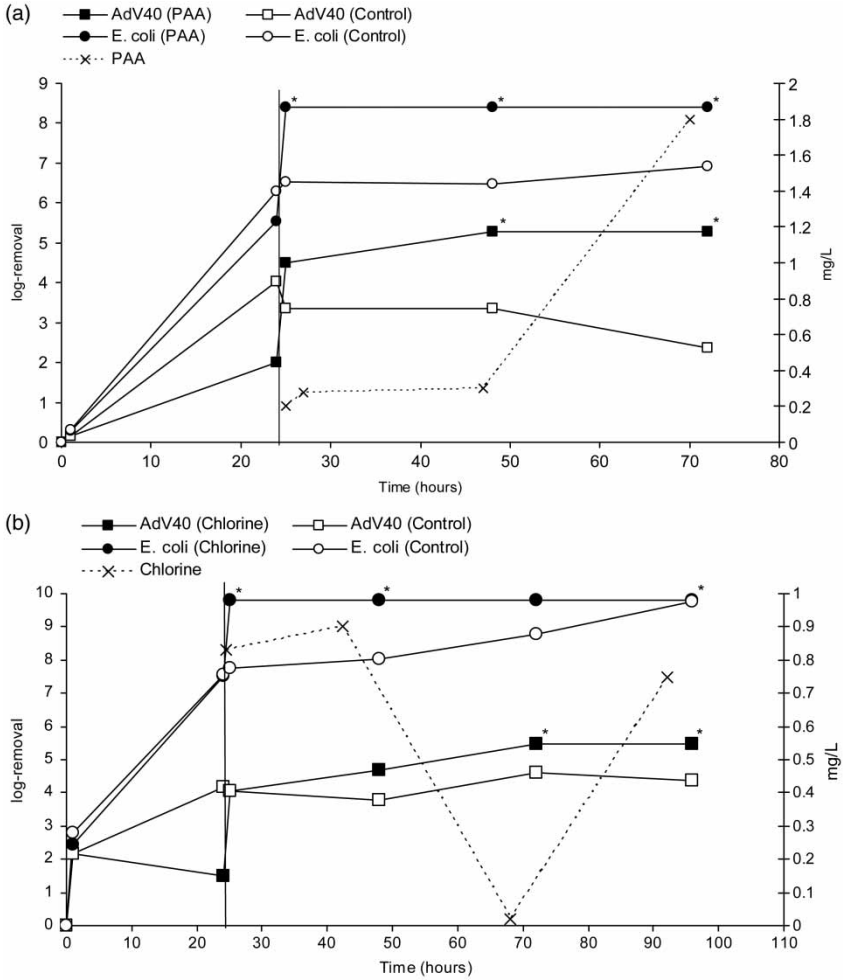

Figure 2 | Log removals of AdV40 and *E. coli* and decontaminant concentrations (mg/L) in outlet water during: (a) PAA experiment and (b) chlorine experiment. The perpendicular line at 24 h indicates the start of the decontamination. An asterisk above the measurement point indicates that the observation was below the detection limit.

of AdV40 over the course of the experiment was 0.3, compared to >3.3 log removal for *E. coli*; the difference in concentrations in the samples taken before ($n = 6$) and after ($n = 8$) the time of decontamination were not significant for AdV40 ($p = 0.49$) or *E. coli* ($p = 0.35$).

PAA decreased the HPC in biofilms by 2.1 logs during the first day and attained an approximately 3.4 log decrease between the start and the end of the experiment (Figure 4(a)). The effect of PAA was statistically significant between the samples before ($n = 6$) and after ($n = 9$) the decontamination ($p < 0.001$). In the control line, HPC remained constant showing no significant difference in concentrations between the samples before ($n = 6$) and after ($n = 9$) the time of decontamination ($p = 0.53$). Again, the TCC in biofilms were stable in both the PAA decontaminated and control line and only slight increase in concentrations were noted in both lines during the experiment (Figure 4(a)). This small increase between the samples before ($n = 6$) and after ($n = 9$) the time of decontamination was statistically significant in the control line ($p = 0.04$), but not in the decontaminated line ($p = 0.08$).

Chlorine experiment

Water samples

In the chlorine experiment, the level of AdV40 decreased by 1.5 and 4.2 logs in the outlet water of decontamination and

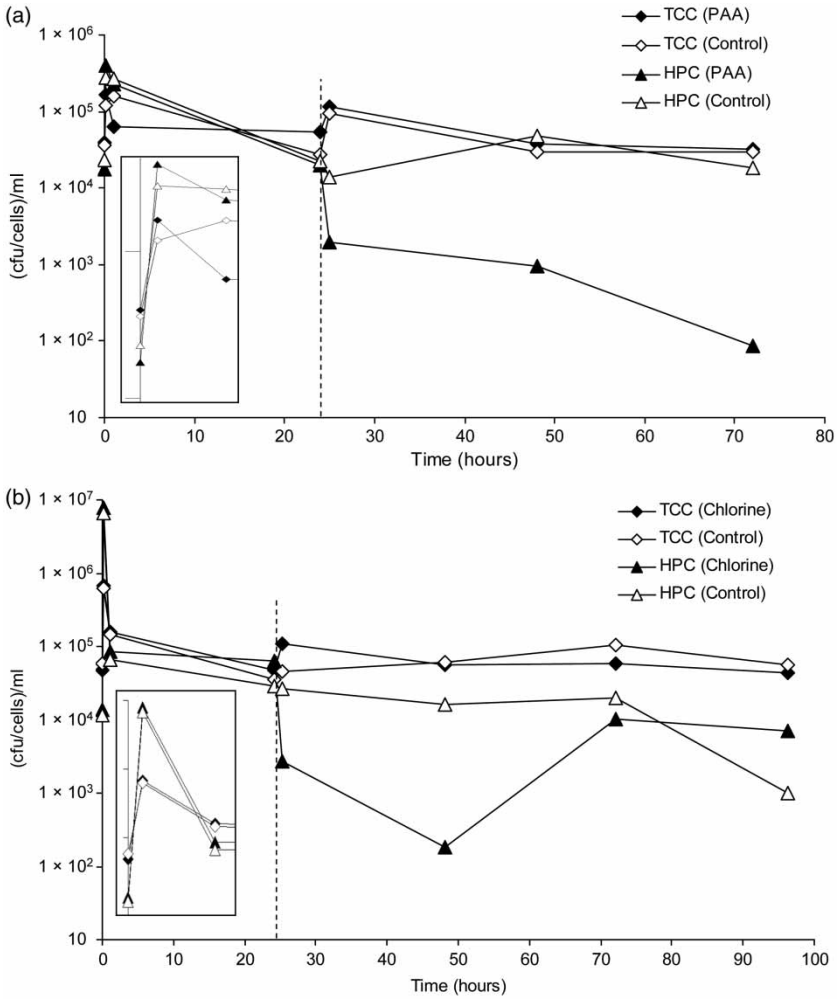

Figure 3 | The impact of the decontaminants on the HPC and TCC of the outlet water: (a) PAA experiment and (b) chlorine experiment. Inserts clarify the first three measuring points. The dashed perpendicular line indicates the start of the decontamination.

Table 1 | Recovery of AdV40 and *E. coli* from decontaminated and control biofilm samples in the PAA experiment

	AdV40				*E. coli*			
Sample	**PAA**	**Range (GC/cm²)**	**Control**	**Range (GC/cm²)**	**PAA**	**Range (cfu/cm²)**	**Control**	**Range (cfu/cm²)**
Before contamination	0/1[a]	BDL[b]	0/1	BDL	0/3	BDL*	0/3	BDL*
After contamination (1 h)	3/3	126–1060	3/3	22–340	3/3	44–1980	3/3	65–358
After contamination (24 h)	3/3	174–419	3/3	406–1548	2/3	BDL–0.4	0/3	BDL
After decontamination (25 h)	1/3	BDL–187	3/3	22–584	0/3	BDL	1/3	BDL–1
After decontamination (48 h)	0/3	BDL	2/2	693–1605	0/3	BDL	2/2	4–15
After decontamination (72 h)	0/3	BDL	3/3	41–157	0/3	BDL	0/3	BDL

The sampling time is counted from the start of the contamination.

[a]Number of positive samples/no. experiments.

[b]Below detection limit, AdV40: <22 GC/cm², AdV40 quantitation is indicative due to extensive variation between parallel samples, *E. coli*: <0.1 cfu/cm², * < 0.2 cfu/cm².

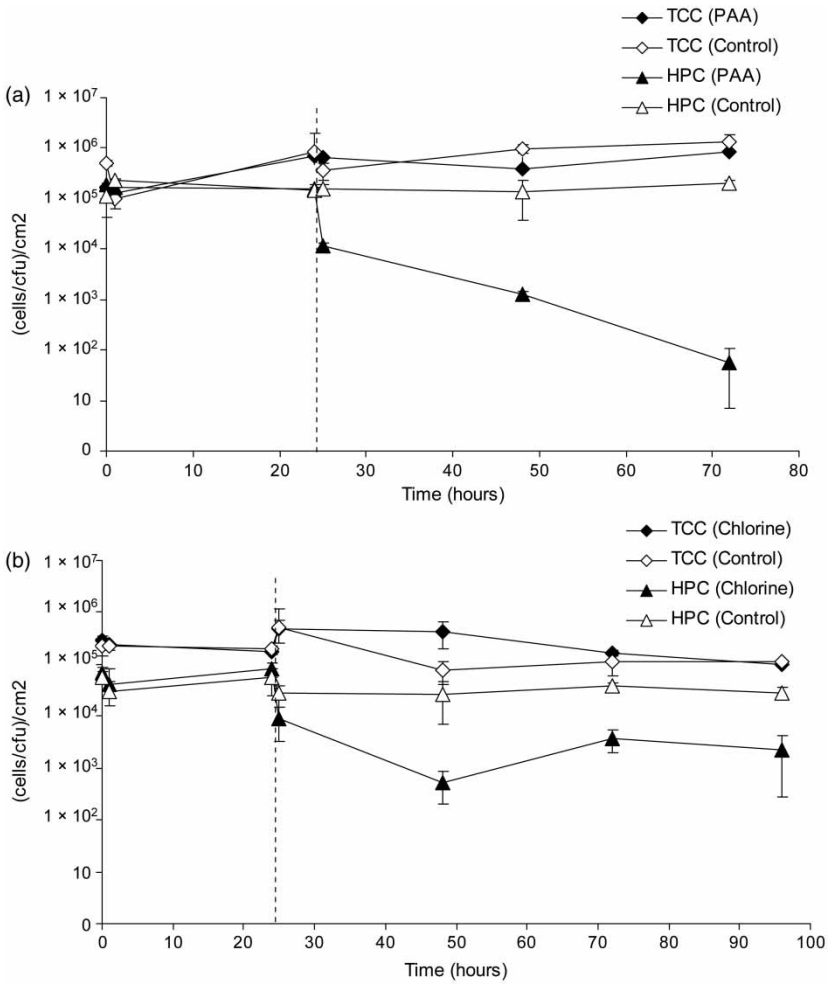

Figure 4 | The impact of the decontaminants on the HPC and TCC of the biofilm: (a) PAA experiment and (b) chlorine experiment. The dashed perpendicular line indicates the start of the decontamination. In the biofilm analyses, there is a mean of three replicate samples with standard deviations shown as error bars.

non-decontamination control pipelines, respectively, during the first day (Figure 2(b)). Within the same period of time, *E. coli* decreased by 7.5 and 7.6 logs. The chemical added into the decontamination line further lowered the concentration of AdV40 during the first hour by 2.6 logs, while *E. coli* was decreased to below the detection limit of the method. Finally, after 48 h from the starting point of the decontamination, AdV40 was no longer detected and over 4.0 logs removal was achieved. The total decrease of the virus in the decontaminated line was over 5.5 logs whereas for *E. coli*, it was over 9.8 logs. The concentration of chlorine was 0.8–0.9 mg/L during the first day after the start of decontamination. A problem in chlorine dosing between 51 h and 69 h, causing an 18 h interruption in chlorination (Figure 2(b)), occurred before the 72 h sample point when a low *E. coli* count was found from the water sample. Without decontamination, the level of AdV40 remained fairly constant till the end of the experiment. The total decrease of the AdV40 in the control line was 4.4 logs. *E. coli* concentration decreased clearly also in outlet water of the control line and *E. coli* was no longer detected after 96 h from the contamination, resulting in a total log removal of over 9.8 logs.

HPC of the outlet water at the chlorine experiment was elevated in both pipelines after contamination but declined rapidly to near to the starting point levels (Figure 3(b)). The chlorine treatment had a clear effect on the HPC after the first day of decontamination decreasing the amount by

2.5 logs. On the other hand, chlorine did not appear to have an effect on the TCC of the outlet water. The total effects of chlorine on HPC and TCC could not be accurately determined since the chlorination problem occurred.

Biofilm samples

Table 2 illustrates the results of the biofilm samples in the chlorine experiment. AdV40 and *E. coli* were readily removed from the decontaminated biofilms except for one of three parallel biofilm samples when *E. coli* was found 1 h after chlorine treatment. In the chlorine-treated line, the difference in concentrations between the samples before ($n = 7$) and after ($n = 12$) the treatment was statistically significant for both AdV40 ($p < 0.001$) and *E. coli* ($p < 0.001$). In the control line, almost all parallel samples were positive for AdV40 and this was still detectable at the end of the experiment as was *E. coli*, which was, however, detected less frequently and only in low concentrations. In the control line, the average log removal for AdV40 was 1.1 during the experiment, as compared to over 2.5 log removal for *E. coli*. Statistically significant differences were noted in concentrations between the samples before ($n = 7$) and after ($n = 11$–12) the time of treatment for both AdV40 ($p = 0.002$) and *E. coli* ($p = 0.04$).

The chlorine treatment decreased the HPC level of the biofilms initially by 2.2 logs between the sampling points of 0 and 48 h. At the end of the experiment, the HPC level in the decontaminated line remained 1.6 logs lower than that in the control line (Figure 4(b)). The effect of chlorine on HPC was statistically significant between the samples before ($n = 7$) and after ($n = 12$) the decontamination ($p < 0.001$). HPC in the control line did not change significantly between the sampling points of before ($n = 7$) and after ($n = 12$) the time of decontamination ($p = 0.2$). Again, no substantial change was detected in the level of TCC of biofilms between the decontaminated and the control line at the end of the experiment.

DISCUSSION

It is well recognized that contaminated water and pipe biofilms pose a potential health risk to the consumer but the exact importance of biofilms for water safety is still poorly understood (Skraber *et al.* 2005). There is also lack of information on best practices to manage contaminated distribution networks. In this study, a relatively short pulse-like contamination and subsequent decontamination was modelled in a preconditioned laboratory-scale pipeline system. The pathogenic human AdV40 and faecal bacterium *E. coli* were shown to be susceptible to both PAA and chlorine decontamination. The data revealed that AdV40 and *E. coli* can be removed effectively by the experimental concentrations of these decontaminants since they were not detected in biofilm and water samples after measuring points of 1 h (biofilm) and 48 h (water) from the start of the decontamination. However, without decontamination

Table 2 | Recovery of AdV40 and *E. coli* from decontaminated and control biofilm samples in the chlorine (Cl_2) experiment

	AdV40				*E. coli*			
Sample	Cl_2	Range (GC/cm^2)	Control	Range (GC/cm^2)	Cl_2	Range (cfu/cm^2)	Control	Range (cfu/cm^2)
Before contamination	0/3[a]	BDL[b]	0/3	BDL	0/3	BDL	0/3	BDL
After contamination (1 h)	3/3	273–1596	3/3	114–667	3/3	12–99	3/3	9–44
After contamination (24 h)	4/4	26–19621	4/4	22–222	4/4	0.4–2	2/4	BDL–2
After decontamination (25 h)	0/3	BDL	2/3	BDL–22	1/3	BDL–0.7	2/3	BDL–1
After decontamination (48 h)	0/3	BDL	3/3	38–47	0/3	BDL	1/3	BDL–0.2
After decontamination (72 h)	0/3	BDL	3/3	22–43	0/3	BDL	0/3	BDL
After decontamination (96 h)	0/3	BDL	3/3	22–31	0/3	BDL	1/3	BDL–0.1

The sampling time is counted from the start of the contamination.
[a]Number of positive samples/no. experiments.
[b]Below detection limit, AdV40: < 22 GC/cm^2, AdV40 quantitation is indicative due to extensive variation between parallel samples, *E. coli*: <0.1 cfu/cm^2.

the concentrations of AdV40 and *E. coli* in water were rather constant after the initial washout occurred during the first 24 h. Emphasizing the persistence of viruses in the water environments, AdV40 was detectable in both the water and biofilms of control lines till the end of the experiments, i.e. up to 4 days.

Adenoviruses are resistant to different decontamination procedures. However, the effectiveness of free chlorine towards adenovirus is comparable to other enteric viruses (Thurston-Enriquez *et al*. 2003; Baxter *et al*. 2007; Cromeans *et al*. 2010). Their common occurrence in wastewater and inherent persistence make adenoviruses a very suitable agent for assessing the microbiological purity of drinking water. In this study, the potency of both decontaminants was evident. Some differences were noted between the decontamination capacity of PAA and chlorine against AdV40 though the observed log removals did not differ substantially. With chlorine, it took from 24 to 48 h to decontaminate outlet water whereas with PAA, the water was decontaminated within 24 h. On the other hand, with PAA one parallel biofilm sample was AdV40 positive after 1 h decontamination whereas at that same time, the chlorine-treated biofilm was decontaminated.

The counts of cultivable *E. coli* declined in water even without decontamination and *E. coli* was detected only sporadically in biofilms of control lines. This may be due to weaker attachment of *E. coli* on biofilms or higher sensitivity of *E. coli* to residual chlorine present in the inlet water, as compared to AdV40. However, these findings are consistent with previous studies highlighting the negligible survival of *E. coli* in water environments compared to certain enteric microbes (Leclerc *et al*. 2001). It is important to take this limitation into consideration when waterborne outbreaks or contamination situations need to be investigated as the absence of *E. coli* is often misinterpreted as a proof of water safety (Tallon *et al*. 2005). Due to rapid removal of *E. coli*, a detailed comparison between PAA and chlorine could not be conducted but some differences were observed. There were no findings of culturable *E. coli* in PAA experiment after the start of decontamination. In contrast, *E. coli* was detected in one of three parallel biofilm samples after 1 h of chlorine treatment and also a small amount of *E. coli* was detected in the water after 48 h of chlorine decontamination. However, this may be a result of the higher concentration of *E. coli* in the contaminant of the chlorine experiment than in the PAA experiment. In addition, the problem in chlorine dosing which only occurred before the 72 h measure point may have influenced *E. coli* survival.

Previous studies have shown that microbes can accumulate in biofilms (Storey & Ashbolt 2003; Langmark *et al*. 2005; Lehtola *et al*. 2007). In this study, the findings from outlet water corresponded well to the incidence of microbes in biofilms and AdV40 was almost invariably detected in both positive water and concurrently collected biofilm samples. This is evidence that these viruses are bound in biofilms and are constantly shed from these sites into the water in small amounts. This can prolong the duration of water contamination episodes and thus careful and sufficiently long cleaning of the pipes is required since the slowly detaching microbes can still pose a risk to the consumer. Therefore, it is important to define the kinetic properties of different microbes in attachment on pipe biofilms and to estimate the persistence of these microbes in biofilm accumulations. In a previous study, it was demonstrated that feline caliciviruses were able to survive in biofilms and outlet waters for at least 4 weeks, whereas cultivable *E. coli* survived for only 4 days in biofilm and 8 days in water (Lehtola *et al*. 2007). In the present study, we observed that adenoviruses remained in both the outlet water and biofilms of the drinking water pipeline for at least 4 days after the contamination if decontaminant had not been added. Other studies have also noted the long persistence of enteric viruses in drinking and wastewater biofilms (Storey & Ashbolt 2003; Skraber *et al*. 2009). All of these studies together clearly suggest that allowing time to pass is not an appropriate way to manage contamination events. Instead, as our results showed, active cleaning procedures are necessary to decontaminate the distribution network.

The decontaminants used in this study lowered the amount of AdV40 eventually to below the detection limit of the method, which was relatively high, especially in biofilm samples. The sensitivity of the method was challenged due to the limited biofilm sample area and also microbes were seen to attach unevenly, producing dispersion among parallel samples. However, it was not reasonable to increase the number of viruses in this laboratory-scale experiment

since the densities used are already appropriate to model heavily contaminated drinking water. The lack of replicates in water samples may have influenced the reliability of some individual measures since the shedding of microbes can be inconsistent. This may explain, e.g., the increase of numbers for AdV40 observed in the PAA experiment control line at 72 h. However, the reproducibility of the study design was demonstrated by the comparable results of the control lines of two experiments.

Modern molecular techniques have mostly replaced conventional methods in surveillance of viruses and case-specific measures are thereby commonly based on PCR results. However, the use of quantitative PCR (qPCR) in disinfection studies has been criticised because it may overestimate the number of infectious microbes. On the other hand, traditional cultivation is time consuming and many enteric viruses are difficult or impossible to propagate in cells. Moreover, cultivation is known to underestimate the results (Blackmer *et al.* 2000). In this study, we used qPCR to determine absolute removals of AdV40, i.e. to ensure that the pipe is clean of all traces of viruses, including genetic material. The results of this study will add valuable practical information supporting the work of health authorities and form the basis for decision making in different water contamination cases. In these cases, often the only method for virus detection is qPCR and no surrogate exists that can perfectly model the real pathogen. Thus, as long as new approaches for PCR are being developed to include the assessment of infectivity (Rodriguez *et al.* 2009), direct qPCR with its inherent uncertainties offers the best available method for the interpretation of the virus removal results in this study.

Biofilms can be considered as a multilevel community of microbes, with the outermost layers in contact with water phase being the most important determinants of their surface binding properties. In this study, we used HPC to describe viable microbes and DAPI staining for the determination of the total cell counts in biofilms. Together these values represent biofilm integrity. Despite the fact that there are many methods to control biofilms (USEPA 2002), the best practices for effective removal and/or purification of biofilms in particular cases are poorly known. In this study, it was demonstrated that biofilms responded to decontaminants by decreased HPCs while the TCC remained stable. We also observed a clear connection between contaminant removal and the decrease in number of viable microbes in biofilms. This suggests that the contamination is restricted to the active surfaces of the biofilms consisting mainly of viable microbes. In addition, the constant count in TCC indicates that biofilms remained bound to the decontaminated pipes. In the light of these results, it seems that also the bound microbial contaminants are destroyed in biofilms. This can reduce the necessity for total removal and purification of the inner surfaces of the pipes, e.g. by mechanical methods. Instead, the use of the decontaminants tested in this study with their residual effects in the network could represent an adequate option for the effective removal of microbial contaminants. This is particularly important with respect to chlorine treatment since the penetration of chlorine into biofilms is known to be poor (De Beer *et al.* 1994). This study was conducted with relatively young biofilm and older pipes could be expected to contain more diverse and extensive biofilm accumulations. It still remains to be determined if these older pipes can provide even more binding sites and shelter for microbes.

Our results showed that both PAA and chlorine were effective decontaminants against HPC of the biofilms. The calculated log removals were comparable after the first day of decontamination before the difficulties in chlorine dosing. The inlet water of the pipeline system was tap water of Kuopio city with a low concentration of chlorine (0.05 mg/L). Thus, the microbial community of the biofilms produced might have been adapted to the chlorinated water environment lowering the effectiveness of chlorine compared to PAA. As this is the situation in most Finnish distribution networks, the chlorine was not quenched from the inlet water. Nonetheless, the effect of additional chlorine on biofilm HPCs was evident and consistent with the values reported by Chu *et al.* (2003) who showed two and three orders of magnitude lower HPCs of biofilms for low chlorine water (0.3–0.5 mg/L) and high-chlorine water (1.2–1.5 mg/L) than for chlorine-free water. With PAA there was a notable lag-time before the concentration of the chemical rose to the target level. This consumption of the chemical is possible due to rapid reactions with organic matter inside the pipelines. After these reactions, a sharp rise in the concentration of PAA was measured emphasizing the challenges of precise dosing of the chemical. Although

the degradation of PAA does not produce significant amounts of DBPs, the formation of acetic acid, which is an excellent nutrient for microbes, must be controlled through proper dosing. Nevertheless, the results obtained with PAA are promising and support its use in water decontamination procedures. However, its use in the water processing industry is not common and as far as we are aware there are no examples of its use in large-scale drinking water production.

CONCLUSIONS

The results of this study demonstrate that PAA and chlorine are powerful distribution network decontaminants and good purification effects were noted in the water and biofilms of the contaminated pipeline systems. However, without added decontaminant, AdV40 remained detectable over the whole experiment (up to 4 days) in both the water and biofilm samples. Cultivable *E. coli* was removed more efficiently than AdV40 from the pipeline systems, implying that the sole use of *E. coli* as a water quality parameter may underestimate the presence of viruses in water and biofilms. The removal of AdV40 and *E. coli* was linked to the decreased HPC levels in decontaminated pipes while TCC remained stable leading to a higher proportion of nonviable and dead micro-organisms than viable micro-organisms. This indicates that after the initial washout, the mechanism of pipeline decontamination is inactivation rather than physical removal of microbes.

ACKNOWLEDGEMENTS

This work was partly carried out as part of a national Sävel project, which is funded by the Finnish Funding Agency for Technology and Innovation (TEKES). The work was also part of the research project SECUREAU (Nr. 217976) which is supported by the European Union within the 7th Framework Programme. There hereby follows a disclaimer stating that the authors are solely responsible for the work. It does not represent the opinion of the Community and the Community is not responsible for any use that might be made of data appearing herein. We acknowledge the help of the personnel of National Institute for Health and Welfare in Kuopio.

REFERENCES

Baxter, C. S., Hofmann, R., Templeton, M. R., Brown, M. & Andrews, R. C. 2007 Inactivation of adenovirus types 2, 5, and 41 in drinking water by UV light, free chlorine, and monochloramine. *J. Environ. Eng.* **133**, 95–103.

Blackmer, F., Reynolds, K. A., Gerba, C. P. & Pepper, I. L. 2000 Use of integrated cell culture-PCR to evaluate the effectiveness of poliovirus inactivation by chlorine. *Appl. Environ. Microbiol.* **66**, 2267–2268.

Chu, C., Lu, C., Lee, C. & Tsai, C. 2003 Effects of chlorine level on the growth of biofilm in water pipes. *J. Environ. Sci. Health A Tox Hazard. Subst. Environ. Eng.* **38**, 1377–1388.

Costerton, J. W., Lewandowski, Z., Caldwell, D. E., Korber, D. R. & Lappin-Scott, H. M. 1995 Microbial biofilms. *Annu. Rev. Microbiol.* **49**, 711–745.

Cromeans, T. L., Kahler, A. M. & Hill, V. R. 2010 Inactivation of adenoviruses, enteroviruses, and murine norovirus in water by free chlorine and monochloramine. *Appl. Environ. Microbiol.* **76**, 1028–1033.

De Beer, D., Srinivasan, R. & Stewart, P. S. 1994 Direct measurement of chlorine penetration into biofilms during disinfection. *Appl. Environ. Microbiol.* **60**, 4339–4344.

Divizia, M., Gabrieli, R., Donia, D., Macaluso, A., Bosch, A., Guix, S., Sanchez, G., Villena, C., Pinto, R. M., Palombi, L., Buonuomo, E., Cenko, F., Leno, L., Bebeci, D. & Bino, S. 2004 Waterborne gastroenteritis outbreak in Albania. *Water Sci. Technol.* **50**, 57–61.

Edberg, S. C., Rice, E. W., Karlin, R. J. & Allen, M. J. 2000 *Escherichia coli*: the best biological drinking water indicator for public health protection. *J. Appl. Microbiol.* **88**, 106S–116S.

Enriquez, C. E., Hurst, C. J. & Gerba, C. P. 1995 Survival of the enteric adenoviruses 40 and 41 in tap, sea, and waste water. *Water Res.* **29**, 2548–2553.

Gilgen, M., Germann, D., Luthy, J. & Hubner, P. 1997 Three-step isolation method for sensitive detection of enterovirus, rotavirus, hepatitis A virus, and small round structured viruses in water samples. *Int. J. Food Microbiol.* **37**, 189–199.

Hijnen, W. A. M., Beerendonk, E. F. & Medema, G. J. 2006 Inactivation credit of UV radiation for viruses, bacteria and protozoan (oo)cysts in water: a review. *Water Res.* **40**, 3–22.

ISO_8199 2005 *Water quality – general guidance on the enumeration of micro-organisms by culture*. International Organization for Standardization (ISO), Geneva, Switzerland.

ISO_19458 2006 *Water quality – sampling for microbiological analysis*. International Organization for Standardization (ISO), Geneva, Switzerland.

Jothikumar, N., Cromeans, T. L., Hill, V. R., Lu, X., Sobsey, M. D. & Erdman, D. D. 2005 Quantitative real-time PCR assays for detection of human adenoviruses and identification of serotypes 40 and 41. *Appl. Environ. Microbiol.* **71**, 3131–3136.

Kitis, M. 2004 Disinfection of wastewater with peracetic acid: a review. *Environ. Int.* **30**, 47–55.

Koivunen, J. & Heinonen-Tanski, H. 2005 Peracetic acid (PAA) disinfection of primary, secondary and tertiary treated municipal wastewaters. *Water Res.* **39**, 4445–4453.

Kukkula, M., Arstila, P., Klossner, M. L., Maunula, L., Bonsdorff, C. H. & Jaatinen, P. 1997 Waterborne outbreak of viral gastroenteritis. *Scand. J. Infect. Dis.* **29**, 415–418.

Laine, J., Huovinen, E., Virtanen, M. J., Snellman, M., Lumio, J., Ruutu, P., Kujansuu, E., Vuento, R., Pitkanen, T., Miettinen, I., Herrala, J., Lepisto, O., Antonen, J., Helenius, J., Hanninen, M. L., Maunula, L., Mustonen, J. & Kuusi, M. 2010 An extensive gastroenteritis outbreak after drinking-water contamination by sewage effluent, Finland. *Epidemiol. Infect.* **15**, 1–9.

Langmark, J., Storey, M. V., Ashbolt, N. J. & Stenstrom, T. A. 2005 Accumulation and fate of microorganisms and microspheres in biofilms formed in a pilot-scale water distribution system. *Appl. Environ. Microbiol.* **71**, 706–712.

Leclerc, H., Mossel, D. A. A., Edberg, S. C. & Struijk, C. B. 2001 Advances in the bacteriology of the Coliform Group: their suitability as markers of microbial water safety. *Annu. Rev. Microbiol.* **55**, 201–234.

Lehtola, M. J., Torvinen, E., Kusnetsov, J., Pitkanen, T., Maunula, L., von Bonsdorff, C. H., Martikainen, P. J., Wilks, S. A., Keevil, C. W. & Miettinen, I. T. 2007 Survival of Mycobacterium avium, *Legionella pneumophila, Escherichia coli*, and caliciviruses in drinking water-associated biofilms grown under high-shear turbulent flow. *Appl. Environ. Microbiol.* **73**, 2854–2859.

Maunula, L., Klemola, P., Kauppinen, A., Soderberg, K., Ngujen, T., Pitkänen, T., Kaijalainen, S., Simonen, M. L., Miettinen, I. T., Lappalainen, M., Laine, J., Vuento, R., Kuusi, M. & Roivainen, M. 2009 Enteric viruses in a large waterborne outbreak of acute gastroenteritis in Finland. *Food Environ. Virol.* **1**, 31–36.

Mautner, V. 1998 *Methods for Growth and Purification of Enteric Adenovirus Type 40*. Humana Press, Totowa, NJ, USA.

Monarca, S., Richardson, S. D., Feretti, D., Grottolo, M., Thruston Jr., A. D., Zani, C., Navazio, G., Ragazzo, P., Zerbini, I. & Alberti, A. 2002 Mutagenicity and disinfection by-products in surface drinking water disinfected with peracetic acid. *Environ. Toxicol. Chem.* **21**, 309–318.

Pina, S., Puig, M., Lucena, F., Jofre, J. & Girones, R. 1998 Viral pollution in the environment and in shellfish: human adenovirus detection by PCR as an index of human viruses. *Appl. Environ. Microbiol.* **64**, 3376–3382.

Quignon, F., Sardin, M., Kiene, L. & Schwartzbrod, L. 1997 Poliovirus-1 inactivation and interaction with biofilm: a pilot-scale study. *Appl. Environ. Microbiol.* **63**, 978–982.

Rodriguez, R. A., Pepper, I. L. & Gerba, C. P. 2009 Application of PCR-based methods to assess the infectivity of enteric viruses in environmental samples. *Appl. Environ. Microbiol.* **75**, 297–307.

Rook, J. J. 1974 Formation of haloforms during chlorination of natural waters. *Water Treat. Exam.* **23**, 234–243.

Schoenen, D. 2002 Role of disinfection in suppressing the spread of pathogens with drinking water: possibilities and limitations. *Water Res.* **36**, 3874–3888.

Skraber, S., Ogorzaly, L., Helmi, K., Maul, A., Hoffmann, L., Cauchie, H. M. & Gantzer, C. 2009 Occurrence and persistence of enteroviruses, noroviruses and F-specific RNA phages in natural wastewater biofilms. *Water Res.* **43**, 4780–4789.

Skraber, S., Schijven, J., Gantzer, C. & de Roda Husman, A. M. 2005 Pathogenic viruses in drinking-water biofilms: a public health risk? *Biofilms* **2**, 105–117.

Sobsey, M. D. 1989 Inactivation of health-related microorganisms in water by disinfection processes. *Water Sci. Technol.* **21**, 179–195.

Storey, M. V. & Ashbolt, N. J. 2003 Enteric virions and microbial biofilms – a secondary source of public health concern? *Water Sci. Technol.* **48**, 97–104.

Tallon, P., Magajna, B., Lofranco, C. & Leung, K. T. 2005 Microbial indicators of faecal contamination in water: a current perspective. *Water Air Soil Poll.* **166**, 139–166.

Thurston-Enriquez, J. A., Haas, C. N., Jacangelo, J. & Gerba, C. P. 2003 Chlorine inactivation of adenovirus type 40 and feline calcivirus. *Appl. Environ. Microbiol.* **69**, 3979–3985.

USEPA 1999 *Combined Sewer Overflow Technology Fact Sheet: Alternative Disinfection Methods*. Office of Water Office of Water, Washington, DC, USA. EPA 832-F-899-033.

USEPA 2002 *Health Risks From Microbial Growth and Biofilms in Drinking Water Distribution Systems*. Office of Water, Washington, DC, USA.

USEPA 2009 Announcement of the drinking water contaminant candidate list 3, pp. 51850–51862, Federal Register.

Zacheus, O. M., Iivanainen, E. K., Nissinen, T. K., Lehtola, M. J. & Martikainen, P. J. 2000 Bacterial biofilm formation on polyvinyl chloride, polyethylene and stainless steel exposed to ozonated water. *Water Res.* **34**, 63–70.

Zanetti, F., De Luca, G., Sacchetti, R. & Stampi, S. 2007 Disinfection efficiency of peracetic acid (PAA): inactivation of coliphages and bacterial indicators in a municipal wastewater plant. *Environ. Technol.* **28**, 1265–1271.

First received 21 December 2011; accepted in revised form 30 April 2012. Available online 16 July 2012