Enteric virions and microbial biofilms – a secondary source of public health concern?

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Abstract Through their many sorption sites, microbial biofilms can accumulate both organic and inorganic particulate and colloidal material from bulk water environments. An application of such first principles to the ecology of “biocolloidal” enteric virions would suggest that they too may be concentrated by biofilms in a similar way. Though previous studies have isolated human gastrointestinal (enteric) virions from microbial biofilms, the exact human health significance of this has been neither fully investigated nor completely understood. Through an assessment of the location, accumulation and persistence of model enteric virions (φX174, MS2 and B40-8 bacteriophages as well as 20 nm fluorescent latex microspheres) within biofilms, the aim of the current study was to investigate whether the interaction of enteric virions with distribution pipe biofilms could provide a secondary source of public health concern to consumers. Model enteric virions were found to be incorporated into biofilms at concentrations representing 1% of those present in the adjacent bulk water environment. A sub-population (0.01%) of these persisted throughout an experimental period of 30 days, inferring their potential to accumulate over time. Furthermore, model enteric virions were partitioned into bacterial microcolonies, environments where biofilm bacteria can persist and re-grow, even in the presence of “acceptable” levels of disinfection. A risk model for enteric virion accumulation and release from distribution pipe biofilms suggested that associated risks may exceed USEPA benchmark values. These findings could have wide-reaching implications in water treatment and distribution strategies, and necessitate a re-appraisal of current water guideline values.

Keywords φX174; MS2; B40-8; bacteriophages; biofilm ecology; distribution water; enteric virus; quantitative microbial risk assessment; virion

Introduction Gastrointestinal illness presents a critical socioeconomic concern to industrialised nations such as the United States, where it is estimated to be responsible for the loss of US$23 billion annually in medical costs and productivity (Garthright et al., 1988). Furthermore, enteric pathogens pose a greater concern to immunocompromised individuals worldwide, who are thought to comprise as many as 20% of the total population of an industrialised nation (Gerba et al., 1996). Enteric virions are the aetiological agents responsible for the majority of gastrointestinal illness, and therefore research that could lead to their risk reduction in water systems holds immense public interest and value. Nonetheless, research into the presence of enteric virions within distribution pipe biofilms, whilst appreciated for some time, has been neglected in the monitoring of water distribution systems and in public health microbiology in general. Biofilms that form ubiquitously on pipe surfaces within a distribution system may act as a reservoir for microbial pathogens to persist for prolonged periods of time and even recolonise the bulk water environment (Szewzyk et al., 2000). Problems can subsequently arise when concentrated numbers of biofilm-associated pathogens become detached from substrata and are mobilised into the bulk water where they have the potential to reach consumers through the ingestion of contaminated water (Howe et al., 2002), or food that has come in contact with contaminated water, through the
inhalation of aerosols, or through breaks in the skin (Ashbolt, 1995). Pathogenic bacterial species that have been isolated from distribution pipe biofilms include *Aeromonas* spp. (Holmes and Nicolls, 1995), *Legionella pneumophila* (Rogers and Keevil, 1992; Walker et al., 2000), *Mycobacterium* spp. (Schwartz et al., 1998; Walker et al., 2000) and *Pseudomonas aeruginosa* (Walker et al., 2000). Other emerging waterborne pathogens such as *Helicobacter pylori* (Mackay et al., 1998), enterohaemorrhagic *E. coli* (EHEC) (Szewzyk et al., 1994), *Salmonella typhimurium* (Armon et al., 1997), and *Campylobacter* spp. (Buswell et al., 1998), although not having been isolated from real-world distribution systems, have been shown to persist within biofilms formed in experimental laboratory systems and are therefore thought to have the potential to accumulate and persist within pipe biofilms in a municipal distribution system. What remains to be investigated however is the integration and persistence of perhaps the most important of the microbial waterborne pathogens, the enteric virion.

Bacteriophages share many fundamental properties and characteristics with human enteric virions (Table 1) such as their rates of decay, and behaviour in, and resistance to conventional unit water treatment processes (Havelaar et al., 1991) and for this reason, were chosen as model enteric virions for use in this study. This paper therefore presents an overview of an investigation into the ecology of human enteric virions within distribution pipe biofilms and can be broadly divided into three main parts, a) virion persistence studies, b) *in situ* detection of model enteric virions within biofilms, and c) development of a quantitative microbial risk assessment (QMRA).

Despite initial investigations of the accumulation of enteric virions in distribution pipe biofilms (Quignon et al., 1997a; Quignon et al., 1997b), such work has fallen short of quantifying virion persistence within such a system. Other studies have demonstrated the potential of model enteric virions and virion-sized particles to accumulate and persist within microbial biofilms (Flood and Ashbolt, 2000), though the mechanisms that permit this phenomenon are only known in general terms (Gerba, 1986). In an effort to address such a deficiency of research, the primary aim of this study was to ascertain whether the interaction of

<table>
<thead>
<tr>
<th>Virion</th>
<th>Virus type</th>
<th>Genera (family)</th>
<th>Morphology (size)</th>
<th>Isoelectric point</th>
<th>Genome</th>
<th>Host</th>
</tr>
</thead>
<tbody>
<tr>
<td>B40-8</td>
<td>Somatic bacteriophage</td>
<td>‡ (Siphoviridae)</td>
<td>Tailed phage (30 nm)</td>
<td>Unknown</td>
<td>ds DNA</td>
<td><em>B. fragilis</em></td>
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<td></td>
<td></td>
<td></td>
<td>(26 nm)</td>
<td></td>
<td></td>
<td><em>E. coli</em></td>
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<tr>
<td>MS2</td>
<td>F-RNA coliphage</td>
<td><em>Levirivirus</em> (Levirviridae)</td>
<td>Icosahedral (24 nm)</td>
<td>3.9</td>
<td>ss RNA</td>
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<td></td>
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<td></td>
<td>(26–32 nm)</td>
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<td></td>
<td><em>E. coli</em></td>
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<tr>
<td>φX174</td>
<td>Somatic coliphage</td>
<td><em>Microvirus</em> (Microviridae)</td>
<td>Icosahedral (65–80 nm)</td>
<td>6.6</td>
<td>circular</td>
<td></td>
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<tr>
<td>Adenovirus</td>
<td>Enteric virus</td>
<td><em>Atadenovirus</em>, Mastadenovirus (Adenoviridae)</td>
<td>Icosahedral (27 nm)</td>
<td>2.8</td>
<td>ss RNA</td>
<td>humans†</td>
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<td>(35–39 nm)</td>
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<td>Hepatitis A virus</td>
<td>Enteric virus</td>
<td><em>Hepatovirus</em> (Picornaviridae)</td>
<td>Icosahedral (27 nm)</td>
<td>2.8</td>
<td>ss RNA</td>
<td>humans†</td>
</tr>
<tr>
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<td><em>Norwalk-like virus</em> (Caliciviridae)</td>
<td>Icosahedral (35–39 nm)</td>
<td>Unknown</td>
<td>ss RNA</td>
<td>humans†</td>
</tr>
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<td>Poliovirus</td>
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<td>Icosahedral (28–30 nm)</td>
<td>7.2</td>
<td>ss RNA</td>
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<td>Unknown</td>
<td>ds RNA</td>
<td>humans†</td>
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</tbody>
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† different species may also infect other vertebrates
‡ not classified at genus level
human enteric virions with distribution pipe biofilms could provide an additional source of health concern to consumers receiving water that is designated for domestic potable and non-potable use. This aim was addressed through an investigation of the persistence of model enteric virions (φX174, MS2 and B40-8 bacteriophages) (Table 1) within biofilms. Biofilms formed in potable and “recycled” water in urban and laboratory-scale distribution systems (3 months old) were challenged with bacteriophages and their persistence recorded over a 30 day experimental period. To fully appreciate the potential health concern associated with microbial pathogens, it is imperative to have an appreciation of the ecology of these microorganisms (Szewzyk et al., 2000). Subsequently, the spatial distribution of B40-8 bacteriophages and 20 nm fluorescent latex microspheres within biofilms formed in “recycled” water pipes were investigated to establish the ecological niche that virions occupy within biofilms that may facilitate their accumulation and persistence within a municipal distribution system. What also remains unknown is whether such phenomena can present an underlying potential waterborne health risk to consumers connected to a municipal distribution system. A simplistic model based on the maximum risk dose-response curve (Teunis and Havelaar, 2000) was therefore developed to estimate the magnitude that such a health risk could present to more sensitive sub-populations. Risk models traditionally assume an engineering approach, focusing on pathogen removal rates through unit treatment processes and pathogen survival in water. The current model takes into consideration the potential interaction of enteric virions with the biofilms that form ubiquitously on the surfaces of distribution systems and their release to a water distribution system.

Methods

Virion persistence study
Biofilms were allowed to accumulate on stainless steel (SS) and unplasticised (u)PVC coupons in a modified Robbins device (MRD) within a potable water distribution system for a period of three months, after which time, coupons were removed and placed in a Biofilm Reactor™ (BR™) (Storey and Ashbolt, 2001). The BR™ was challenged with PEG-precipitated MS2 coliphages and B40-8 bacteriophages, each at a final concentration of approximately 10^8 plaque forming units (PFU).mL^{-1}. Bacteriophages were re-circulated through the device for a period of 24 hours to facilitate the maximum accumulation of virions (Flood and Ashbolt, 2000), after which time the reactor was run in a single pass, which allowed planktonic virions to be washed from the system. On days 0, 1, 2, 3, 6, 10, 15, 22 and 30, triplicate coupons were removed from the BR™ using sterile forceps and biofilm removed from coupons by a combination of sonication and stomaching. A multi-parametric analysis of biofilm biomass that included the enumeration of infective bacteriophages from the same homogenate using ISO methods was then carried out on coupons (Storey and Ashbolt, 2002). Free and total chlorine and pH were measured throughout the duration of the experimental period by Dulcotest® sensors (ProMinent® Dosiertechnik, GmbH, Heidelberg).

In situ detection of model enteric virions within biofilms
B40-8 bacteriophages were prepared in large quantities and then purified by a combination of PEG precipitation and CsCl ultracentrifugation (Sambrook et al., 1989). Polyclonal antisera were raised against purified B40-8 bacteriophages in a New Zealand White rabbit which had been injected with an emulsion containing equal volumes of CsCl-purified B40-8 bacteriophages (10^{11}.mL^{-1}) and Freund’s incomplete adjuvant (Sigma Chemical Co., Missouri) at monthly intervals over a 3 month period. Rabbit anti-B40-8 bacteriophage (R α B40-8) antisera was collected, aliquoted and stored at –20°C using methods described
Biofilms formed on SS coupons in artificial recycled water in a BR™ (total organic carbon: 68 mg.L⁻¹; total nitrogen: 40.3 mg.L⁻¹; total phosphorous: 24.8 mg.L⁻¹) for a period of 3 months were later challenged with CsCl-purified B40-8 bacteriophages and 20 nm carboxylate-modified blue fluorescent latex microspheres (Fluospheres®) (Molecular Probes Inc., Oregon), each at final concentrations of 10¹⁰ particles.mL⁻¹. Water was re-circulated in the BR™ for a period of 24 hours to facilitate the maximum accumulation of particles (Flood and Ashbolt, 2000) after which time it was run in single pass for a further 48 hours to flush unbound bacteriophages and microspheres from the system. Infective sessile B40-8 bacteriophages were extracted from biofilms and enumerated by plaque assays using ISO methods as mentioned previously. SS coupons were also examined by scanning confocal laser microscopy (SCLM) for model enteric virions. Coupon surfaces were gently rinsed with 0.22 µm-filtered tap water and then immersed in primary antibody (R α B40-8 antisera) diluted 1 in 100 in blocking agent (2% skim milk powder in PBS, pH 7.5) and incubated for 60 minutes at 37°C in a humidified chamber. Biofilms were gently rinsed with PBS, then covered with Cy-3®-conjugated sheep anti-rabbit (S α R) secondary antibody (Sigma Chemical Co., Missouri) diluted 1 in 50 in Milli-Q. Biofilms were incubated with the secondary antibody for a further 60 minutes at 37°C, rinsed thoroughly and counterstained with SYTO-9 (Molecular Probes Inc., Oregon). Biofilm preparations were then scanned at 5.0 µm intervals with a Leica TCS SP Spectral Confocal Microscope (Leica Microsystems, Heidelberg) (SCLM) fitted with a 488 nm argon and 543 nm helium/neon lasers and a 1.2 ps 2-photon imaging system (Tsunami, Spectra-Physics).

Risk model
A quantitative microbial risk assessment (QMRA) using the maximum risk dose-response relationship (Teunis and Havelaar, 2000) for a water distribution system was undertaken based on results obtained in an experimental pipe loop in the laboratory. Three month-old biofilms formed on SS coupons in artificial recycled water in a lab-scale pipe loop (total organic carbon: 68 mg.L⁻¹; total nitrogen: 40.3 mg.L⁻¹; total phosphorous: 24.8 mg.L⁻¹) were challenged with PEG-precipitated φX174, MS2 and B40-8 bacteriophages, and their persistence recorded over a 30 day experimental period. A number of scenarios were used to examine the sensitivity of the QMRA model using a range of influent concentrations of enteric virions into a distribution system based on sub-optimal performance events in a water treatment system (Storey and Ashbolt, 2003). The probability of infection (Pᵢ) resulting from the ingestion of a certain number of organisms (D) over a number of exposure events (n) was calculated using the maximum risk curve Pᵢ = 1 – e⁻ʳD, with r = 1 (Teunis and Havelaar, 2000). The annual risk of infection was calculated for a daily exposure to one millilitre of water using the formula: Pᵢ (annual) = 1 – (1 – Pᵢ)ⁿ (Haas et al., 1993). A range of assumptions were also made for the risk model (Storey and Ashbolt, 2003). The beginning of the distribution system was chosen as it has been shown to have the highest flux of assimilable organic carbon and ultimately the largest amount of biofilm growth, and is thought to be the most representative of the efficacy of a treatment process (Block et al., 1993).

Results and discussion
Virion persistence study
MS2 and B40-8 bacteriophages were initially incorporated into biofilms in a ratio representing 1% of those present in the bulk water phase (PFU.cm⁻²:PFU.mL⁻¹). When a simple linear regression was fitted to the bacteriophage decay data that had been normalised to biofilm protein content, deviation from first order kinetics was observed, indicating that a
more complex description of the data was necessary to describe the inactivation and potential loss of bacteriophages. Nonetheless, extrapolation of the simple linear regression suggested that B40-8 and MS2 bacteriophages may persist within the experimental system for a period of approximately 28 days (B40-8: $k = 0.23 \text{ day}^{-1}$; MS2: $k = 0.21 \text{ day}^{-1}$) (Figure 1a). The data however, better fitted a bi-phasic inactivation or loss with an initial rapid decline in bacteriophage numbers (B40-8: $k_1 = 0.5 \text{ day}^{-1}$; MS2: $k_1 = 0.95 \text{ day}^{-1}$) being followed by a more constant, gradual decline (Figure 1b). The data therefore supported the presence of a more persistent sub-population (approximately 0.01%) of bacteriophages that have the potential to persist over an extended period of time, potentially in excess of 100 days for B40-8 bacteriophages ($k_2 = 0.096 \text{ day}^{-1}$) (Figure 1b) and even longer for MS2 ($k_2 = 0.009 \text{ day}^{-1}$). Free chlorine was measured at a concentration of $0.19 \pm 0.29 \text{ mg.L}^{-1}$ ($n = 5,900$) and $pH 8.7 \pm 0.13$ ($n = 5,900$) during the experimental period. Sessile heterotrophic and total bacterial cells were shown to be unaffected by the presence of a total chlorine residual of $0.56 \pm 0.12 \text{ mg.L}^{-1}$, which was measured in the system ($n = 5,900$). Such an observation suggested that model enteric virions may occupy a similar ecological niche to biofilm bacteria, inferring that aspects of their ecology within biofilms may too sequester them from the biocidal action of chlorination.

**In situ detection of model enteric virions within biofilms**

The majority of biofilm bacteria were localised in microcolonies surrounded by dense matrix material, and where the matrix was less dense, water channels or pores were observed throughout the depth of biofilms. Water channels were generally in the range of 20–100 µm, and often emptied into large voids or cavities; areas of minimal or no bacterial growth. Biofilms were punctuated by these voids, which were as large as 250 µm in diameter. Biofilms accumulated both model enteric virions (B40-8 bacteriophages and 20 nm blue fluorescent latex microspheres). B40-8 bacteriophages were found to be present in the biofilm in the order of $10^8 \text{ PFU.cm}^{-2}$, representing 1:100 of those present in the adjacent bulk water phase (cm$^{-2}$:mL) as observed in the earlier virion persistence study. Single fluorescent 20 nm microspheres could neither be resolved nor quantified by EFM as they were below the limit of resolution, though were visible when aggregated or when viewed by SCLM. Both CY-3 – conjugated SαRαB40-8 complexes and 20 nm blue latex microspheres could be visualised by SCLM throughout the depth of microbial biofilms, and were found to transverse water channels and localise in voids within biofilms. Both model bacteriophages

![Figure 1](https://iwaponline.com/wst/article-pdf/48/3/97/423124/97.pdf)

**Figure 1** Persistence of B40-8 bacteriophages within potable water biofilms. Data was normalised against biofilm protein content, and expressed as plaque forming units (PFU).cm$^{-2}$:μg protein$^{-1}$. When a single line of regression was fitted to the data, deviation from 1st order kinetics was observed ($k = 0.23 \text{ day}^{-1}$) (Figure 1a), though a better fit occurred if a bi-phasic inactivation or loss was assumed ($k_1 = 0.5 \text{ day}^{-1}$, $k_2 = 0.096 \text{ day}^{-1}$) (Figure 1b). Total chlorine was measured at $0.56 \pm 0.12 \text{ mg.L}^{-1}$ during this time ($n = 5,900$). Error bars represent 1 SD ($n = 10$).
Enteric virions were also shown to penetrate biofilm micropores and accumulate within bacterial microcolonies. Whilst previous studies have shown bacteriophages to infect bacterial cells immobilised within biofilms (Doolittle et al., 1995, 1996), an ecological niche that virions could potentially occupy within a biofilm has not been demonstrated prior to this study. Microcolonies of bacteria are less susceptible to the effects of disinfection, thereby suggesting that biocides can be transported readily through biofilm water channels and voids, but are readily consumed prior to, or excluded from bacterial microcolonies, thereby creating an environment in which enteric virions can be sequestered from chlorination within a distribution system. The public health significance of the results obtained in the current study suggests that this paucity of research may warrant further attention and that a simplistic quantitative risk model investigating the accumulation within, and subsequent release of enteric virions to, a distribution system is warranted.

Risk model
Bacteriophages (φX174, MS2 and B40-8) were recovered from biofilms throughout the duration of the pipe loop experimental period using simulated reclaimed wastewater, and were accumulated in the same proportions observed in earlier potable water studies (1%). All bacteriophages were detected in sediment and sloughed biomass material recovered from the base of the pipe loop after 70 days. When a single line of regression was fitted to B40-8 (k = 0.40 day⁻¹), MS2 (k = 0.36 day⁻¹) and φX174 (k = 0.43 day⁻¹) data, the decay was clearly seen to deviate from first order kinetics as observed earlier. Extrapolation of the simple linear regression for each plot indicated that under the conditions of the study, bacteriophages would remain infectious within the biofilms for 44, 43 and 30 days for B40-8, MS2 and φX174 bacteriophages, respectively, though bi-phasic decay better described the data as observed earlier. Of the bacteriophages that were incorporated into biofilms, a sub-population of approximately 0.01% was assumed to persist and remain infectious within biofilms. Highlighting the significance of this model is the fact that if 1% of virions present in the bulk water were sequestered into pipe biofilms per metre length of the distribution system, ignoring the effects of erosion and sloughing, virions would generally remain undetected in the bulk-water over long distances, potentially masking treatment failures that are traditionally assessed through microbiological analysis of the bulk water phase. Whilst it is apparent that distribution pipe biofilms may therefore play an important ecological role in the removal of enteric virions from distribution systems, during a worst-case scenario (10 virions.L⁻¹) for example, virions would not be detected in the bulk water after 1.5 km, subsequently masking a potential health concern to consumers that could arise should biofilm be sloughed from pipe surfaces.

The introduction of one virion per 100 L into a reclaimed pipe biofilm (300 µm thick, with a 90% sloughing event) presented a Pi of 1.4 × 10⁻⁴ (1.4 illnesses in 10,000 people) for the accidental consumption of 100 mL and 1.4 × 10⁻³ (14 illnesses in 10,000 people) for the consumption of 1 L of recycled water. Given that a tolerable level of waterborne risk is deemed to be one infection in 10,000 (i.e. 10⁻⁴) in the general population (Regli et al., 1991), it is possible that even under normal plant operating conditions the probability of infection may exceed this benchmark value for sensitive members of a population. The daily consumption of one millilitre of water under worst-case operating conditions (10 virions.L⁻¹, 300 µm thick biofilm and a 90% sloughing event) led to a Pi of 1.4 × 10⁻³ (14 illnesses in 10,000 people) and a Pi (annual) of 0.40 (4 illnesses in 10 people). This finding could have important implications for consumers given the potential infection of enteric virions through the inhalation of aerosols. Notwithstanding the limitations of the simplistic model used, the current study has elucidated a poorly recognised or at best, underestimated potential health risk associated with the distribution of water.
Whilst an ever-increasing attention is being paid to the accumulation and persistence of emerging waterborne bacterial pathogens within microbial biofilms (Szewzyk et al., 2000), an understanding of the possible interaction that enteric virions may have with biofilms has generally been ignored or dismissed altogether. It is widely accepted that both the physical and microbiological quality of water deteriorates in distribution systems. To compound this, the routine monitoring and surveillance of potable water for enteric virions is not recommended in any guideline due to the technical complexity, unreliability and extensive time and cost associated with their testing. Furthermore, the low numbers of enteric viruses generally associated with finished potable and recycled waters means that assaying enteric viruses is impractical and generally does not produce the level of sensitivity required to assess current benchmark values (Regli et al., 1991). Thus there is a substantial need to better understand the functioning of a distribution system in order to protect the microbiological quality of water after it leaves a treatment plant (Olson and Nagy, 1984). An appreciation of enteric virion ecology within distribution pipe biofilms therefore provides invaluable information for water treatment and distribution strategies.

Conclusions

Whilst it is important to establish a balance between the production of water that is on one hand microbiologically safe and at the same time cost-effective to both the provider and consumer, water meeting current water guideline values could potentially pose an additional health risk to consumers. The results obtained in this study may therefore infer the need for a more holistic approach to the formulation of risk models and routine sampling protocols, taking into consideration the accumulation of enteric virions (and other microbial pathogens) within distribution pipe biofilms. Furthermore, the results of this study may necessitate a re-appraisal of current potable and recycled water guidelines, with the inclusion of microbial pathogen ecology, the significance of which has been either underestimated or dismissed in the past.

References


