Biofilms in an urban water distribution system: measurement of biofilm biomass, pathogens and pathogen persistence within the Greater Stockholm area, Sweden

J. Långmark*, M.V. Storey*, N.J. Ashbolt** and T.A. Stenström*

*Department of Parasitology, Mycology and Water Microbiology, Swedish Institute for Infectious Disease Control, 171 82 Solna, Sweden (E-mail: jlk@smi.ki.se)

**School of Civil and Environmental Engineering, University of New South Wales, UNSW-Sydney 2052, Australia

Abstract Distribution pipe biofilms can provide sites for the concentration of a wide range of microbial pathogens, thereby acting as a potential source of continual microbial exposure and furthermore can affect the aesthetic quality of water. In a joint project between Stockholm Water, the MISTRA “Sustainable Urban Water” program, the Swedish Institute for Infectious Disease Control and the Royal Technical University, Stockholm, the aim of the current study was to investigate biofilms formed in an urban water distribution system, and quantify the impact of such biofilms on potential pathogen accumulation and persistence within the Greater Stockholm Area, Sweden. When used for primary disinfection, ultra-violet (UV) treatment had no measurable influence on biofilm formation within the distribution system when compared to conventional chlorination. Biofilms produced within a model pilot-plant were found to be representative to those that had formed within the larger municipal water distribution system, demonstrating the applicability of the novel pilot-plant for future studies. Polystyrene microspheres (1.0 \text{\mu m}) and Salmonella bacteriophages demonstrated their ability to accumulate and persist within the model pilot-plant system, where the means of primary disinfection (UV-treatment, chlorination) had no influence on such phenomena. With the exception of aeromonads, potential pathogens and faecal indicators could not be detected within biofilms from the Stockholm water distribution system. Results from this investigation may provide information for water treatment and distribution management strategies, and fill key data gaps that presently hinder the refinement of microbial risk models.

Keywords Biofilm; drinking water distribution system; fluorescent polystyrene microspheres; model pathogen; Salmonella bacteriophages; UV-treatment

Introduction

Biofilms play an essential barrier role in conventional water treatment through the entrapment of particulate material as well as through nutrient removal. In distribution systems water practitioners have limited control over many of the processes that take place. Within these environments, distribution pipe biofilms can impact on the aesthetic (colour, taste and odour) quality of water and act as a reservoir of opportunistic microbial pathogens including members of the aeromonads, legionellae, mycobacteria and pseudomonads (Szewzyk et al., 2000). Other primary pathogens such as Campylobacter spp. (Buswell et al., 1998), enterohaemorrhagic E. coli (Szewzyk et al., 1994), Helicobacter pylori (Mackay et al., 1998), Salmonella typhimurium (Armon et al., 1997) as well as model enteric virions (Storey and Ashbolt, 2001, 2003) have been shown to accumulate and persist in model systems, and may therefore present an additional source of concern.

To minimise the formation of potentially hazardous disinfection by-products formed through the reaction of natural organic material with chlorine, Stockholm Water is currently trialling the efficacy of ultra-violet (UV) light as a primary disinfectant in place of conventional chlorination. What remains unknown is the effect that UV-treatment may
have on biofilm formation and the incidence, accumulation and persistence of microbial pathogens within a municipal water distribution system. This paper describes a project involving Stockholm Water, the MISTRA “Sustainable Urban Water” program, the Swedish Institute for Infectious Disease Control and the Royal Technical University, Stockholm with the primary aims being to:

- quantify biofilm biomass in a novel pilot-plant and large-scale municipal water distribution system, and subsequently evaluate the suitability of a pilot-plant for the analysis of large-scale systems;
- compare the influence that two primary disinfection methods (UV-treatment and chlorination) have on biofilm biomass within a water distribution system;
- identify whether such biofilms may have an impact on the accumulation and persistence of model microbial particles within biofilms formed within a water distribution system; and
- investigate if a range of primary and opportunistic bacterial pathogens and indicators occurred within the investigated systems.

**Materials and methods**

Two study sites within the Stockholm area were investigated. Hässelby district, which currently receives drinking water that has undergone UV-treatment, and Nockeby district, which receives water treated using chlorine as a primary disinfectant. Water in both systems was chloraminated prior to its distribution at a target total chlorine residual of 0.3 mg L$^{-1}$. A novel pilot-scale water distribution system comprising 1 km of 50 mm polyethylene (PE) tubing was connected directly to the Lovö water treatment plant post disinfection and prior to its distribution to Hässelby and Nockeby districts. Coupon devices were located immediately post conventional treatment (proximal site) and at the end of the distribution system (remote site) equating to residence times of 0.1 hours and 110 hours respectively within each full-scale municipal distribution system. A proximal site was chosen since it is more representative of water treatment processes, whilst distal sites may represent worst-case conditions within a water distribution system (Block et al., 1993). Biofilms were allowed to reach a “steady-state” (80–90 days) and were grown during the following periods; Sept.–Nov. (winter), Aug.–Oct. (autumn) and May–July (summer).

For biofilm biomass quantification and pathogen incidence studies, biofilms were grown on glass coupons located at proximal and distal sites in both the pilot-plant system as well as the full-scale municipal water distribution system. On sampling days, triplicate coupons were removed from biofilm sampling devices and placed in stomacher bags containing 20 mL of quarter strength Ringer solution. Bags were sealed and placed on ice prior to being processed in the laboratory (within 2 hours). Biofilm was removed from coupon surfaces using sterile cell-scrapers (TPP) then homogenised in a stomacher (Bag-Mixer, Interscience, St. Nom, France) for one minute. Biofilms formed in all systems were compared on the basis of their culturability (heterotrophic plate count, HPC on R2A agar) and directly by staining with 4',6-diamidino-2-phenylindole (DAPI; 1 μg mL$^{-1}$) using filter set 01 (365/397 nm; Zeiss) on a Zeiss Axioskop microscope. For this part, appropriate volumes of biofilm homogenate were filtered using a 0.2 μm black polycarbonate membrane (Millipore) and viewed directly by epifluorescence microscopy (EFM).

Total biofilm proteins content was measured using the procedure provided with the NanoOrange Protein Quantitation kit (Molecular Probes, Oregon, USA). Carbohydrate content was measured colorimetrically by a phenol-sulfuric assay (Dubois et al., 1956). The presence of both autochthonous (aeromonads and legionellae) and allochthonous (salmonellae, enterobacteria, enterococci) indicators was sought by fluorescence in situ
hybridisation (FISH) using rRNA oligonucleotide probes (Proligo, Paris) and modified protocols of Manz (1999), Kalmbach et al. (1997), Nordentoft et al. (1997) and Kempf et al. (2000). The presence of total coliforms, E. coli and enterococci were further sought by culture using Enterolert™ and Colilert™ DST® assays (Idexx Pty Ltd, Portland).

For model pathogen accumulation and persistence studies, biofilms were grown on glass coupons within the pilot-plant for 8 weeks, after which time they were challenged with blue 1.0 μm hydrophobic (excitation 365 nm, emission 415 nm) and green hydrophilic (505/515 nm) polystyrene fluorescent microspheres (Molecular Probes, Oregon), as well as Salmonella typhimurium 28B bacteriophages, each at a final concentration between $10^9$ – $10^{11}$ particles per millilitre. The inoculum was re-circulated throughout coupon devices by a peristaltic pump for a period of 24 hours (day 0), after which time each device was re-opened to single-pass flow to allow suspended model particles to be lost from the system. The persistence of each particle type was then measured over a 38-day experimental period, with results from day 1 (24 hours following the conclusion of the inoculation period) used for the purpose of quantifying particle accumulation.

On sampling days 1, 2, 6, 12 and 38, triplicate coupons were removed from all sampling devices and biofilms removed using procedures outlined previously. The day prior to dosing (day 0) triplicate glass coupons were removed from each chamber, and the numbers of biofilm bacteria were assessed by DAPI-staining. This procedure was repeated for biofilms at the conclusion of the experimental period (day 38). AOC analyses were performed according to the method described by van der Kooij et al. (1982) with the following alteration. Duplicate 500 mL samples were heated overnight at 60°C in Extran®-washed bottles. After this time samples were inoculated with Pseudomonas fluorescens strain P17 and incubated at 20°C. Colony counts were made on nutrient agar by drop-plate method after 3, 7 and 14 days at 20°C. AOC values were calculated using the yield coefficient for P. fluorescens of $4.1 \times 10^6$ cfu/μg C (as acetate).

Results and discussion
Influence of UV-treatment and chlorination on biofilm biomass
Water temperatures within the municipal water distribution system and pilot-plant are presented in Table 1. Water temperature was within the range of 5.2°C to 9.6°C within

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Site</th>
<th>Chlorine (mg.L⁻¹)</th>
<th>AOC (µg.L⁻¹)</th>
<th>Temperature (°C) (Biofilm type)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Winter</td>
<td>Autumn</td>
<td>Summer</td>
</tr>
<tr>
<td>Chlorination (Nockeby)</td>
<td>Proximal</td>
<td>0.26 ± 0.05</td>
<td>34 ± 10</td>
<td>6.6</td>
</tr>
<tr>
<td></td>
<td>Field-scale</td>
<td>0.24 ± 0.01</td>
<td>36 ± 16</td>
<td>8.5</td>
</tr>
<tr>
<td></td>
<td>Pilot plant</td>
<td>0.09 ± 0.05</td>
<td>15 ± 11</td>
<td>8.8</td>
</tr>
<tr>
<td></td>
<td>Distal</td>
<td>0.02 ± 0.02</td>
<td>27 ± 4</td>
<td>9.4</td>
</tr>
<tr>
<td>UV-treatment (Hässelby)</td>
<td>Proximal</td>
<td>0.24 ± 0.04</td>
<td>24 ± 10</td>
<td>5.2</td>
</tr>
<tr>
<td></td>
<td>Field-scale</td>
<td>0.26 ± 0.07</td>
<td>22 ± 7</td>
<td>8.6</td>
</tr>
<tr>
<td></td>
<td>Pilot plant</td>
<td>0.04 ± 0.01</td>
<td>38 ± 15</td>
<td>9.6</td>
</tr>
<tr>
<td></td>
<td>Distal</td>
<td>0.01 ± 0.00</td>
<td>27 ± 5</td>
<td>9.7</td>
</tr>
</tbody>
</table>
the municipal system and 8.5°C and 9.7°C within the pilot-plant during early winter (November), and 18.8°C to 20.8°C within the distribution system and 13.2°C and 15.2°C in the pilot-plant system during the summer study period (July). AOC concentrations were generally greatest at the beginning of each distribution system, measured at 24–34 μg.L⁻¹ and 36–38 μg.L⁻¹ at proximal sites within the field-scale and pilot-plant systems, respectively (Table 1). The concentration of AOC appeared to decrease with increasing water residence time (15–22 μg.L⁻¹ and 27 μg.L⁻¹ at distal sites within the municipal and pilot-plant systems respectively) though these trends could not be supported statistically. Similarly, the concentration of total chlorine was highest at the beginning of each distribution system (0.24–0.26 mg.L⁻¹ in the field- and pilot-scale systems), decreasing significantly with increasing water residence time, to less than 0.05 mg.L⁻¹ at distal sites within each the distribution system (p = 0.001). There was no significant difference between the pilot- and field-scale systems in AOC and chlorine concentrations though there was a significant difference between systems in water temperature, with the pilot-plant being less influenced by seasonality compared to the full-scale water distribution system.

At proximal sites within both the municipal and pilot-plant systems total (DAPI) counts of bacteria generally ranged in the order of 10⁴ to 10⁵ cells.cm⁻² (Figure 1). In the municipal (field-scale) distribution system, there were significantly (p < 0.005) higher bacterial numbers quantified at proximal sites in the UV-treated than the chlorinated system, though the opposite was observed for biofilms sampled during the wintertime. Within the pilot-plant, however only biofilms grown during the summertime showed significant differences, in which case there were significantly more bacteria in the chlorinated system than the UV-treated system at the proximal sites (Figure 1). At distal sites in the field-scale system there were significantly more bacterial cells in the UV-treated system than the chlorinated system, though no such difference was observed with biofilms formed during the summertime. Similar trends were observed in the pilot-plant system, with significantly more bacteria associated with the UV-treated system, though the opposite was observed with biofilms formed during the summertime, with more biofilm bacteria present in the chlorinated system than the UV-treated system (p = 0.001). Numbers of culturable heterotrophic bacteria (HPC) accounted for 0.01–0.1% of the number of bacteria quantified directly by EFM, and were generally low in proximal sites though varied seasonally between biofilms sourced from proximal and distal sites within the distribution system. Significantly higher numbers of culturable heterotrophs were found in the chlorinated Nockeby system when compared with the UV-treated Hässelby system during the winter and summertime (p < 0.05). UV-treatment, however, appeared to
support the growth of more culturable heterotrophs than chlorination during the autumn, with more HPC detected in the Hässelby system though this finding was not significant, and in any case should be interpreted cautiously as numbers were in the range of 10–15 cfu.cm⁻². The general trend however was that there was no difference between chlorinated and UV-treated drinking water at distal sites in both systems.

Reliable and reproducible quantification of biofilm biomass from field and laboratory systems is inherently problematic, largely because of the heterogeneity in biofilm coverage, thickness and composition (McFeters et al., 1999; Storey and Ashbolt, 2002). Furthermore, in biofilms formed in low-nutrient potable water distribution systems, the quantification of biofilms is even more problematic, given that analytical techniques are generally within one order of magnitude of the detection limit (Storey and Ashbolt, 2002). For this reason, quantification of biofilm biomass by protein and carbohydrate content was below methodological resolution and the assessment of biofilm biomass was therefore limited to total direct counts (TDC) of bacterial cells. Among other factors, protozoan grazing appeared to have an additional influence on the composition of biofilms and distribution of bacteria on coupon surfaces. Notwithstanding this, biofilms from pilot-plant when enumerated by direct bacterial counts indicated that the Lovö pilot-plant was a suitable model for the analysis of the field-scale system since trends were more of less representative of those formed within the municipal distribution system. Though often unable to directly replicate the conditions that are encountered within a municipal distribution system, the benefit of experimental biofilm coupon devices often lie in their simplicity and ability to show relative differences under varying conditions.

Accumulation of model microbial particles

The experimental conditions under which biofilms were produced prior to being challenged with model bacterial (fluorescent microspheres) and viral (bacteriophages) particles are presented in Table 2. Water temperature in the chlorinated (Nockeby) system was 5.2°C immediately post treatment and distribution (proximal site), increasing to 8.2°C at the end of the pilot-scale system (distal site). Values of 5.5°C and 7.6°C respectively were obtained in the UV-treated (Hässelby) system. Assimilable organic carbon (AOC) was measured at 37 ± 16 µg.L⁻¹ (± 1 SD) and 38 ± 16 µg.L⁻¹ at proximal and 27 ± 4 µg.L⁻¹ and 28 ± 5 µg.L⁻¹ at distal sites in chlorinated and UV-treated systems, respectively. Biofilms were generally sparse with total direct counts (TDC) of bacterial cells at day 0 measured at 1.4 × 10⁴ cells.cm⁻² at the proximal chlorinated site, and 3.3 × 10⁴ cells.cm⁻² at the corresponding UV-treated site. These values increased significantly to 9.5 × 10⁴ cells.cm⁻² and 1.0 × 10⁵ cells.cm⁻², respectively at the end of each distribution system, caused in part by the absence of a measurable disinfectant residual (<0.03 mg.L⁻¹). At the end of the experimental period (day 38) numbers of biofilm bacteria were not significantly different from those measured at the

Table 2 Water temperature (n = 4), total direct counts (TDC) of biofilm cells at day 0 (TDC₀) and day 38 (TDCₚₚ) (n = 3) expressed as cells per cm² of biofilm coverage on glass coupon surfaces ± 1 SD as well as assimilable organic carbon (AOC) (n = 3) at proximal and distal sampling sites at residence times of 0.1 and 110 hours respectively

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Site</th>
<th>Residence (hours)</th>
<th>Temperature (°C)</th>
<th>TDC₀ (× 10⁴.cm⁻²)</th>
<th>TDCₚₚ (× 10⁵.cm⁻²)</th>
<th>AOC (µg.L⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorination (Nockeby)</td>
<td>Proximal</td>
<td>0.1</td>
<td>5.2 ± 0.4</td>
<td>1.4 ± 0.2</td>
<td>1.7 ± 0.1</td>
<td>37 ± 16</td>
</tr>
<tr>
<td></td>
<td>Distal</td>
<td>110</td>
<td>8.2 ± 0.8</td>
<td>9.5 ± 2.3</td>
<td>10.4 ± 0.7</td>
<td>27 ± 4</td>
</tr>
<tr>
<td>UV-treatment (Hässelby)</td>
<td>Proximal</td>
<td>0.1</td>
<td>5.5 ± 0.4</td>
<td>3.3 ± 0.1</td>
<td>2.8 ± 0.3</td>
<td>38 ± 16</td>
</tr>
<tr>
<td></td>
<td>Distal</td>
<td>110</td>
<td>7.6 ± 0.7</td>
<td>10.0 ± 0.8</td>
<td>10.4 ± 0.8</td>
<td>28 ± 5</td>
</tr>
</tbody>
</table>
commencement of the study suggesting that biofilms had reached and maintained a “steady-state” during the experimental period.

The numbers of accumulated model particles (fluorescent spheres and bacteriophages) are presented in Table 3. At proximal sites there were three to four times more spheres accumulated in the chlorinated system than the UV-treated system, though no such pattern was observed at distal sites. In this case there were 2–3 times as many spheres accumulated by the UV-treated system than the chlorinated system. There were significantly fewer hydrophilic and hydrophobic spheres accumulated in the distal site when compared to the corresponding proximal site ($p = 0.001$). Since the numbers of accumulated spheres could not be positively correlated to bacterial counts in biofilms ($R^2 = 0.4$), accumulation of spheres was deemed to be influenced more by physicochemical properties of both the substratum and spheres than by the biofilm itself.

Bacteriophages were accumulated in the order of $10^4$ plaque forming units (pfu) per cm$^2$ of biofilm at proximal and distal sites within the chlorinated Nockeby and UV-treated Håsséby distribution systems (Table 3), though there was no significant difference in the accumulation of bacteriophages at each of these sites ($p > 0.05$). Given the total chlorine measured at the proximal (0.24–0.26 mg.L$^{-1}$) and distal ($<0.02$ mg.L$^{-1}$) sites, the inference is that bacteriophage accumulation was not affected by the concentration of the residual total chlorine disinfectant. Furthermore, the accumulation of bacteriophages could not be positively correlated with biofilm density, further suggesting that like

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Site</th>
<th>Hydrophobic spheres.cm$^{-2}$ (% total)</th>
<th>Hydrophilic spheres.cm$^{-2}$ (% total)</th>
<th>Bacteriophages pfu.cm$^{-2}$ (% total)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorination</td>
<td>Proximal</td>
<td>411 ± 311 (0.2)</td>
<td>2860 ± 1580 (1.1)</td>
<td>4 ± 6 ($&lt; 0.001$)</td>
</tr>
<tr>
<td>(Nockeby)</td>
<td>Distal</td>
<td>32 ± 8 (0.1)</td>
<td>110 ± 37 (0.3)</td>
<td>7 ± 2 ($&lt; 0.001$)</td>
</tr>
<tr>
<td>UV-treatment</td>
<td>Proximal</td>
<td>151 ± 31 (0.2)</td>
<td>713 ± 108 (0.6)</td>
<td>21 ± 13 ($&lt; 0.001$)</td>
</tr>
<tr>
<td>(Håsséby)</td>
<td>Distal</td>
<td>91 ± 66 (0.3)</td>
<td>172 ± 113 (0.5)</td>
<td>6 ± 2 ($&lt; 0.001$)</td>
</tr>
</tbody>
</table>

Table 3 The accumulation of model particles in biofilms located in the chlorinated (Nockeby) and UV-treated (Håsséby) pilot-plant distribution systems (day 1). Mean values of hydrophobic and hydrophilic spheres accumulated on day 1 of the experiment were normalised to $10^5$ of biofilm bacterial cells and expressed as units per cm$^2$ (± 1 SD) ($n = 3$). Accumulated particles are also presented as a percentage (in brackets) of the total number of dosed hydrophobic spheres ($2.8 \times 10^9$), hydrophilic spheres ($3.3 \times 10^9$) and 28B bacteriophages ($9.5 \times 10^{10}$) per total surface area available.

**Figure 2** Hydrophobic (black) and hydrophilic (grey) fluorescent microspheres at A) proximal and B) distal sites within chlorinated (Nockeby) and UV-treated (Håsséby) pilot-plant systems. Fluorescent microspheres were expressed per cm$^2$ of biofilm (y-axis) over the 38 day experimental period (x-axis). Error bars represent 1 SD.
Persistence of model microbial particles

There was no significant difference in the numbers of accumulated hydrophilic and hydrophobic spheres at proximal sites in chlorinated (Nockeby) and UV-treated (Hässelby) systems over the course of the 38 day experimental period ($p = 0.001$). Results from both systems were therefore combined (Figure 2). At distal sites there was a gradual and significant decline in both particle types over the duration of the experimental period ($p = 0.01$). Furthermore, there was no significant difference in this trend in chlorinated and UV-treated systems ($p < 0.05$). In a similar manner to their accumulation, the persistence of fluorescent spheres could not be positively correlated to biofilm density, suggesting that their persistence was influenced by electrostatic interactions between spheres and substrata surface and not the thin biofilm itself.

The loss of bacteriophages throughout the duration of the experiment was more pronounced than the loss of spheres (Figure 3). At the proximal site higher decay rates were recorded in the UV-treated than the chlorinated system ($k = 0.26$; $k = 0.06$ respectively), whereas at distal sites decay rates decreased and were identical ($k = 0.08$) for both systems. Generally higher numbers of bacteriophages were recorded at distal sites compared to proximal sites in the chlorinated Nockeby system. The absence of a significant difference in bacteriophage decay between proximal and distal sites in the UV-treated Hässelby pilot-plant system indicated that persistence of bacteriophages was governed by disinfection to some extent and factors other than biofilm density and disinfection played an additional role in bacteriophage persistence.

Opportunistic bacterial pathogens and indicators

With the exception of aeromonads (1–5% of total bacterial numbers) neither allochthonous (salmonellae, enterobacteria) nor autochthonous (legionellae) pathogens could be detected within the municipal water distribution system by FISH. Aeromonads are routinely isolated from distribution systems, though the exact human health significance of this is unknown (Szewzyk et al., 2000). What they may be indicative of though is the trophic state of the system. Total coliforms and enterococci were not detected by culture

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**Figure 3** Salmonella 28B bacteriophages recovered from biofilms at A) proximal and B) distal sites within the chlorinated (Nockeby, black) and UV-treated (Hässelby, grey) pilot-plant distribution systems. Infective bacteriophages were expressed as plaque-forming units (pfu) per cm$^2$ of biofilm ($y$-axis) over the 38 day experimental period ($x$-axis). Error bars represent 1 SD.
using Enterolert™ and Colilert™ DST® assays in any biofilm homogenate sampled (detection limit 0.1 cfu.cm⁻²).

**Conclusions**

Water practitioners face an ongoing challenge to provide water to the consumer that is on one hand aesthetically pleasing, whilst at the same time, microbiologically sound. Furthermore, they have limited control over what takes place within a water distribution system, and whilst many of the biofilm processes are at best poorly understood, most remain unknown. Data generated in this study therefore can have wide-reaching implications for water treatment and distribution strategies, and assist in the development of quantitative microbial risk assessment models. Furthermore, the current study has demonstrated that:

- a model pilot-plant system produced biofilms that were representative of those formed within a large-scale municipal water distribution system;
- the often incoherent relationship between the pilot- and field-scale systems could be accounted for by the variability that is encountered with the analysis of distribution pipe biofilms.
- when used as a primary disinfectant, UV-treatment did not enhance the growth of biofilms within a municipal water distribution system, and furthermore
- UV-treatment did not influence the accumulation and persistence of model microbial pathogens within a water distribution system; and
- with the exception of aeromonads, primary (salmonellae, enterobacteria) and opportunist (legionellae) pathogens as well as indicator bacteria (E. coli, coliforms, enterococci) could not be recovered from the greater Stockholm Area water distribution system.

**Acknowledgements**

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