Effect of temperature and pipe material on biofilm formation and survival of *Escherichia coli* in used drinking water pipes: a laboratory-based study

J. Silhan***, C.B. Corfitzen* and H.J. Albrechtsen*

*Institute of Environment & Resources, Technical University of Denmark, Bygning 115, 2800 Lyngby, Denmark (E-mail: cbc@er.dtu.dk, hja@er.dtu.dk)

**Faculty of Mechanical Engineering, Brno University of Technology, Technicka 2896/2, 616 69 Brno, Czech Republic (E-mail: jsilhan@centrum.cz)

Abstract Segments of used drinking water pipes of galvanised steel (GS), cross-linked polyethylene (PEX), copper pipes (Cu) or new medium-density polyethylene (PE) were investigated for the formation of biofilm and survival of *E. coli* in biofilm and in the water phase. Pipes were filled with water and incubated at 15 °C or 35 °C under static conditions. Biofilm formation was followed during 32, 40 and 56 (58) d. The most dense biofilm was formed on GS, reaching approximately 4.7 × 10^5 CFU/cm² measured as heterotrophic plate count (HPC), and at the other materials the density reached 3 × 10^3 CFU/cm² on PE and PEX and 5 × 10^1 and 5 × 10^2 CFU/cm² on Cu pipes after 58 d at 15 °C. Biofilm HPC values were higher at 35 °C than at 15 °C, with only slightly higher values on the metals, but 100-fold higher on PE and PEX. Adenosine triphosphate (ATP) measurements confirmed the general trends observed by HPC. Higher temperature was seen to be an important factor reducing *E. coli* survival in the water phase in drinking water pipes. At 15 °C *E. coli* survived more than 4 d in GS and Cu pipes and 8 d in PE pipes, but was not detected after 48 h at 35 °C. The *E. coli* survived longer at both temperatures in the glass control bottles than in the drinking water pipes. Despite the obvious biofilm formation, *E. coli* was not detected in the biofilm at any of the investigated surfaces.

Keywords Biofilm; *E. coli*; pipe material; survival

Introduction

In water distribution systems bacteria are mainly present on internal surfaces as attached bacteria (as a biofilm), with a minor part in the water phase (Percival et al., 1998; Bagh et al., 2003). Biofilms may deteriorate the water quality by microbial activity, and in some cases, constitute a serious health risk for consumers, either due to pathogenic bacteria growing in the biofilm or since biofilms may provide a safe haven for intruding pathogens such as enteropathogenic *Escherichia coli* or *Campylobacter* (LeChevallier et al., 1987; Percival and Walker, 1999). Biofilm formation in water distribution systems depends on a variety of factors, e.g. water composition, amount and type of nutrient, disinfectant residuals, thermal and hydraulic conditions, but also on the type and composition of the pipe material as a substratum. Composition of pipe material affects the attachment rate of bacteria (LeChevallier et al., 1990), but certainly also, e.g., by release of compounds, which may be excellent substrates for bacterial growth. However, several investigations have shown no significant difference in biofilm formation on various materials over several months (Wingender and Flemming, 2004) contrary to the findings of others (Rogers et al., 1994; Niquette et al., 1999; Hallam et al., 2001; Lehtolla et al., 2004) showing that pipe material considerably influences biofilm formation, at least in the short term. Temperature is considered to be an important regulator of biofilm growth,
especially in non-disinfected water systems (Rogers et al., 1994; Hallam et al., 2001; Bagh et al., 2002).

Many of the investigations conducted so far have either used laboratory model systems or test coupons. Such systems are unable to mimic the growth conditions and surface structures of materials, which have been in use in house installation for long periods (decades), and where scaling and leaching from the materials may have changed the surface completely.

The aim of this study was to investigate how biofilm formation in non-disinfected water was affected by different used pipe material cut-out from real distribution systems and by temperature (15 °C and 35 °C). Furthermore, to study the interaction between the biofilm and enteric bacteria, survival of *Escherichia coli* was also studied in the water phase as well as in the biofilm.

**Methods**

Generally, all glassware used during this study for water preserving, sampling, etc. was acid washed and subsequently heated to 550 °C. All procedures requiring sterile conditions (HPC plates, biofilm sampling) were carried out in sterile LAF Bench (HERA Safe, Kendro Laboratory Products).

**Experimental pipe set-ups**

Used pipes (except PE) were collected from house installations from the area of Kgs. Lyngby, in the vicinity of Copenhagen, Denmark, which is supplied by aerated, filtered groundwater without disinfection. Four different materials of drinking water pipes were compared: used galvanised steel pipes (GS, some collected from the cold water (GS CW) and some from the hot water installation (GS HW), new medium density polyethylene (PE), used cross-linked polyethylene (PEX) and used copper pipes (Cu) of inner diameter 12 mm (Cu12 mm) and 16 mm (Cu16 mm). Pipes were cut to segments of approximately 1-m length, containing a volume of 200 mL of water, and closed with brass fittings equipped with syringe adapters for water phase sampling. Tightness of all joints was ensured using VITRON O-rings and teflon tape. Prior to use all pipes were flushed for 5 min with demineralised, distilled water and then placed horizontally in holders in thermostated rooms. One set of pipe duplicates was kept at 15 °C (CW set-up) and filled with freshly produced cold drinking water, and one set at 35 °C (HW set-up) and filled with hot tap water; both set-ups were supplemented with duplicates of glass control bottles (CB, 250 mL Pyrex bottle closed with cap with teflon inlayer) as a negative control (no contact to materials). Pipe set-up is shown in Figure 1.
Drinking water (aerated, filtered groundwater without disinfection) for the cold water set-up was collected directly at the local municipal water works (Lyngby) in sterile glass bottles on the day of use and kept at 4°C (less than 6 h) before filling or refilling the CW set-up. The water was adjusted to 15°C before filling of set-up. For filling and refilling the hot water set-up water was collected from the hot water tap in the laboratory (Technical University of Denmark, Bldg 115/2nd floor), which was supplied by water from Lyngby municipal water works.

Sampling procedure
A sterile syringe was inserted on the adapters at each end of the pipes for sampling of water phase. To collect a sample (5–50 mL), freshly collected water was slowly injected into the pipe by the syringe in the inlet to create a plug flow, which pushed out a water sample into the syringe at the outlet of the pipe.

Biofilm was sampled after cutting off a pipe length with an inner surface area of 20 cm². The inner surface of the pipes was swabbed with five to seven sterile cotton sticks, which were transferred to the first of five glass vials containing 10 mL of sterilised, filtered water (0.2 μm, Millipore) and vortexed vigorously for 1 min in each vial to release the bacteria from the sticks. These solutions were combined and homogenised (Bagh et al., 1999). Subsamples were analysed by HPC and ATP. Biofilm was collected after 32, 40 and 56 d (58 d for CW).

During a 32-d long preconditioning of biofilm in pipes, water samples were collected every 3–7 d and were analysed within 2 h. The total volume of refilled water was approximately 50 mL, corresponding to one-quarter of the total pipe volume. Hereafter, the set-ups were filled with water inoculated with Escherichia coli ATCC 25922. Inoculation carried out on day 32 was followed by every day subsampling of the water phase (5–50 mL). Set-ups were thereafter filled with fresh test water, which was completely exchanged 8 d later by inoculated test water. Daily sampling of water phase was repeated, but without refilling (pipe set-ups placed vertically).

Bacterial strain and growth conditions
Prior to inoculation of the water in the pipes, Escherichia coli ATCC 25922 strain was grown at 30°C in nutrient-rich media Tryptic Soy Broth (TSB, Fluka BioChemika, Germany). Subsequently cells were harvested in the early stationary phase (18 h) and washed twice with sterilised, filtered tap water (0.22 μm, Millipore), centrifuged for 12 min at 12,000 rpm at 15–25°C (Sorvall RC-5B Refrigerated Superspeed Centrifuge). The bacterial pellet was resuspended and cell density was enumerated in the microscope (Olympus BH-2, Japan) by microscope counting chamber. According to the cell count (10^7–10^8 CFU/mL), a suspension was produced to fill the CW and HW set-ups to reach an initial concentration of approximately 10^3 E. coli CFU/mL. The suspension was homogenised by thoroughly mixing, whereafter pipe set-ups were filled. Time interval between first centrifugation and completed filling of the pipes was approximately 5 h.

Bacteria enumeration techniques
Heterotrophic plate counts (HPC). Biofilm and water samples were analysed by HPC on R2A agar (Fluka BioChemika, Germany) and incubated for 7 d. Plates with samples from the CW set-up were incubated at 20°C, and plates from the HW set-up at 35°C. HPC bacteria in solutions from biofilm sampling were enumerated by the same technique.
Adenosine Triphosphate (ATP). Selected biofilm and water samples were analysed by ATP using Lumin(ATE)/Lumin(EX) reagents (Celsis No. 92687) and a luminometer (Advance coupe, Celsis, Landgraaf, The Netherlands). ATP content was calculated from an ATP standard curve prepared from ATP standard salt (Celsis No. 92521) dissolved in Lumin(PM) buffer (Celsis No. 92588) and diluted in sterile tap water. Measurements were performed by use of internal standard addition.

E. coli enumeration. E. coli was enumerated by membrane filtration method (MF, cellulose nitrate filter, pore size 0.45 µm, Sartorius) combined with cultivation on MLS Agar (Membrane Lauryl Sulphate Agar, Fluka BioChemika, Germany) for 21 h at 44°C.

Results and discussion
Bacterial population in water phase
Microbial colonisation of pipes and formation of the biofilm on the inner surface of the pipes was under way within the first 32 d of study. The microbial population in the water phase increased rapidly in all the pipes and in glass control bottles. In the CW set-up at 15°C, an initial variation in HPC between the pipe duplicates during the first 15 d (especially for GS and Cu pipes) was probably due to the different origin of pipes and the different initial state of the pipe surface (age, composition of precipitates, time between cut-out from installation and start of the experiment). However, after 15 d the water phase HPC values were quite stable. In the HW set-up at 35°C, 8 d were generally needed to reach relatively steady-state HPC in the water phase, but the HPC values continued increasing slowly, and reached levels slightly higher than in the cold water set-up. Generally, the HPC values were very similar between pipe duplicates in both temperature set-ups, always within one order of magnitude, with highest values in plastic pipes – PEX and PE.

Several studies show that plastic materials can release biodegradable compounds (e.g. Corfitzen et al., 2004) and thus enhance bacterial growth. Surprisingly, copper pipes, that are considered to inhibit bacterial growth due to their toxicity, were populated more than glass control bottles and the HPCs were the lowest in the galvanized steel pipes.

During the whole experimental period of (56) 58 d the water was completely exchanged three times, and the HPC values in the water phase always showed a rapid growth within 8 d after the exchange of water, reaching approximately the same HPC numbers or slightly higher as before water exchange (Figure 2).

ATP measurements of selected pipes, in general, confirmed the trends seen by HPC measurements (Figure 2), with values in the range 10¹ pg ATP/mL. Exceptions in the consistency between trends observed by HPC and ATP were seen in the HW set-ups from day 32 for PEX, Cu and CB, which may be explained by a change in biomass activity, since ATP detects the whole microbial population without the selectivity of the HPC.

Bacterial population in biofilm
Incubation at 15°C and 35°C confirmed findings by Bagh et al. (2002), that higher temperature increases biofilm growth, since biofilm formed much more rapidly in the HW set-up than in the CW set-up (Figure 3). In the HW set-up the biofilm formation was fastest in the plastic pipes, especially the virgin PE, where 32 d was a sufficiently long period to form a rather stable biofilm with around 1 × 10⁵ CFU/cm², which remained during the rest of the study. For the other materials, biofilm density increased over the 56 (58) d period in both HW and CW set-ups, indicating that this period was not sufficient for biofilm to mature. The mature state of biofilm has been described to occur within 3 weeks (Block et al., 1993; Hallam et al., 2001), 200 d (Boe-Hansen et al., 2002) in laboratory-based
studies, and 12–18 months in real distribution systems (Wingender and Flemming, 2004). The complete exchange of water after day 32 did not show any considerable changes in the biofilm density in the HW set-up, with only slight decreases in biofilm densities. Complete exchange of water on day 32 affected the growth of biofilm in all the pipes in the CW set-up, since the biofilm density increased considerably, especially in GS and copper. The water exchange might have introduced nutrients that were depleted, thus supporting the bacterial activity. Oxygen concentration in CW in plastic pipes remained almost unchanged at the original concentration of 8.5 mg/L, but decreased to 2.6–2.9 mg/L in GS and copper pipes during 30 d. A similar decrease was also observed in the HW set-up.

**Figure 2** Development of microbial population in water phase as HPC and as ATP

**Figure 3** Formation of biofilm at different pipes measured by R2A
The pipe material affected the biofilm formation considerably. First, 32 d of incubation were not sufficient to form biofilm at Cu12 mm in both set-ups, in contrast to the biofilm formed on Cu16 mm. Generally, the highest HPC values in HW set-up were reached on plastic pipes around $1 \times 10^5$ CFU/cm$^2$, the lowest in copper pipes, Cu12 mm with $1.3 \times 10^2$ and on Cu16 mm with values eight times lower. GS CW, steel pipe without any scaling, had the highest bacteria density with $4.8 \times 10^5$ CFU/cm$^2$, 16 times higher than in the other GS pipe, of which the surface was covered with 1–2 mm thick layer of precipitates, tubercles, etc. Biofilm formation in CW set-up was highest on GS pipes. The surface in both pipes was covered with a 1–2 mm thick layer of precipitates and with HPCs of almost identical values: $4.7 \times 10^5$ CFU/cm$^2$. PE and PEX reached values in the range $2.5–4 \times 10^3$ CFU/cm$^2$. HPC on Cu12 mm considerably dropped to 1/80 of previous result and reached $5 \times 10^1$ CFU/cm$^2$, which is exactly one order of magnitude lower than the second copper pipe. ATP measurements of the biofilm at the end of the experimental period generally confirmed the ranking of materials (Figure 4), with values in the $10^{-1}$–$10^2$ pg ATP/cm$^2$ range.

In long-term established distribution systems the majority of microbial growth is found in biofilm (76% reported by Bagh et al., 2003). Similar proportions or even higher were observed in GS pipes by the end of this study. The biofilm–water phase ratio changed during the incubation. An increasing proportion of bacteria in biofilm is displayed in Table 1. Preferential accumulation of bacteria in biofilm is caused by better access of cells to nutrients that accumulate at surfaces. This applies especially to reactive metal surfaces (e.g. GS) which enhance microbial growth by adsorption of organic compounds on iron oxides.

**Survival of Escherichia coli**

Two different sampling regimes were applied. A regime with replacement of sampled water was practised between days 32 and 40. No addition of refilling water after sampling was introduced between days 48 and 56 (58). The survival of *E. coli* in the water phase differed depending on water sampling regime (refilling vs. no refilling), pipe material and incubation temperature, which had the main impact. Generally, *E. coli* survived for a shorter time at both temperatures during the regime with refilling.

With refilling, in the HW set-up, *E. coli* was not detected by membrane filtration (detection limit 2 CFU/100 mL) in the water phase after 48 h in any of the materials. In the CW set-up no *E. coli* was detected in copper pipes, ranking the length of survival in the remaining materials: GS < PEX < PE < control bottles.
During the regime without refilling of water, no *E. coli* was observed after 48 h in the HW set-up in all materials; observations in the CW set-up resulted in survival for 4 d in GS, 5 d in Cu, over 10 d in plastic-based pipes, and more than 37 d in glass control bottles.

Other studies under different experimental conditions have indicated that coliforms, including *E. coli*, are able to persist and multiply in biofilms (Fass et al., 1996; Parent et al., 1996). Despite the presence of biofilm, culturable *E. coli* was not detected on the surfaces of the pipe materials at any of the temperatures. Also, an extensive field study by Wingender and Flemming (2004) showed sporadic occurrence of coliform bacteria in drinking water biofilm, but no detection of *E. coli*, either on pipe surfaces several years old, or test coupons of different materials exposed in distribution systems for several months. This could be due to several reasons. First of all, in complex multispecies biofilm in drinking water, the indigenous and competitive microflora might have been too hostile for *E. coli* to be able to survive, especially to cultures grown in nutrient-rich media that are very sensitive to any external effects, as reported by Camper et al. (1996). Furthermore, *E. coli* hidden in the biofilm could be in a non-viable state, and could thus not be detected by cultivating methods. Several authors described injury of coliform bacteria in (chlorinated) water distribution systems, which thus escape detection on standard selective media (McFeters et al., 1986).

**Conclusion**

The colonisation of water phase and biofilm in drinking water pipes was very rapid. HPC values in the water phase reached a relatively steady state after 15 d, but periods longer than 60 d are needed for biofilm to mature fully. Biofilm colonised all the investigated materials but its formation rate depended on the type of material and temperature. Formation of biofilm in CW on plastic-based pipes, PE and PEX, was much alike, with no considerable difference between the virgin surface of new PE and used PEX pipes. This was not confirmed in the HW set-up. Roughness of plumbing material also affected biofilm formation. Biofilm formed rapidly on galvanised steel pipes. In comparison with other materials, the proportion of the total bacteria population that resided in the biofilm was highest in GS and the least on copper pipes. Copper pipes had lower bacteria density than other materials, and biofilm formation was unstable, accompanied with considerable drops and growths. This study also showed that survival of *E. coli* in drinking water pipes was very temperature dependent. Temperature around 15°C was more favourable for *E. coli* surviving in water distribution pipes than 35°C, despite the fact that this temperature is typical in their natural environment. Generally, survival was longest in plastic-based pipes and shortest in galvanised steel pipes. *E. coli* ATCC 25922 could not be detected in any of the biofilms formed on any of the surfaces investigated.

<table>
<thead>
<tr>
<th>Ratio of bacteria in biofilm/total bacterial population [%]</th>
<th>P1 GS HW</th>
<th>P2 GS CW</th>
<th>P3 PE</th>
<th>P4 PE</th>
<th>P5 PEX</th>
<th>P6 PEX</th>
<th>P7 Cu12 mm</th>
<th>P8 Cu16 mm</th>
</tr>
</thead>
<tbody>
<tr>
<td>CW 32 days</td>
<td>17.9</td>
<td>93.3</td>
<td>0.1</td>
<td>2.4</td>
<td>0.6</td>
<td>0.3</td>
<td>nd*</td>
<td>63.8</td>
</tr>
<tr>
<td>40 days</td>
<td>73.6</td>
<td>71.2</td>
<td>2.1</td>
<td>4.1</td>
<td>0.2</td>
<td>1.2</td>
<td>28.3</td>
<td>17.4</td>
</tr>
<tr>
<td>58 days</td>
<td>89.4</td>
<td>82.2</td>
<td>9.0</td>
<td>2.6</td>
<td>0.8</td>
<td>0.8</td>
<td>28.3</td>
<td>17.4</td>
</tr>
<tr>
<td>HW 32 days</td>
<td>72.1</td>
<td>40.4</td>
<td>71.7</td>
<td>62.1</td>
<td>13.4</td>
<td>0.5</td>
<td>nd*</td>
<td>2.8</td>
</tr>
<tr>
<td>40 days</td>
<td>4.6</td>
<td>0.2</td>
<td>23.2</td>
<td>26.9</td>
<td>3.2</td>
<td>2.7</td>
<td>0.5</td>
<td>5.6</td>
</tr>
<tr>
<td>56 days</td>
<td>93.3</td>
<td>79.7</td>
<td>46.7</td>
<td>48.6</td>
<td>7.9</td>
<td>78.8</td>
<td>2.1</td>
<td>0.3</td>
</tr>
</tbody>
</table>

*Biofilm not detected*
Acknowledgements
The authors wish to thank Mona Refstrup for her assistance in the laboratory. The work was partly funded by the BAKMAT-project. Nordisk Wavin A/S is acknowledged for providing the PE-pipes.

References