Occurrence and persistence of bacterial and viral faecal indicators in wastewater biofilms


*Centre de Recherche Public – Gabriel Lippmann, Department of Environment and Agro-biotechnologies (EVA), 41 rue du Brill, L-4422 Belvaux, Luxembourg (E-mail: skraber@lippmann.lu)

**Laboratoire Chimie Physique et Microbiologie pour l’Environnement (LCPME), UMR 7564, CNRS/Université Henri Poincaré Nancy 1, Faculté de Pharmacie, 5 rue Albert Lebrun, F-54000 Nancy, France

Abstract Biofilms within wastewater treatment plants can capture enteric microorganisms initially present in the water phase immobilising them either definitively or temporarily. Consequently, fates of microorganisms may totally change depending on whether they interact or not with biofilms. In this study, we assessed the stability of wastewater biofilms comparing the evolution of the concentrations of bacteria (heterotrophic plate count [HPC], thermotolerant coliforms [TC]) and viral (somatic coliphages [SC] and F-specific phages [F+]) indicators in the biofilms and in the corresponding wastewaters at 4 and 20 °C. Additionally, we assessed the monthly occurrence of these bacterial and viral indicators as well as of pathogenic protozoa (Cryptosporidium oocysts and Giardia cysts) in three native wastewater biofilms for four months. Our results show that viral indicators (SC and F+) persist longer in biofilms than in the corresponding wastewaters at 4 °C as well as at 20 °C. In contrast, persistence of bacterial indicators (TC and HPC) depends on both the temperature and the matrix. Differences between viral and bacterial persistence are discussed. Monthly analysis of native wastewater biofilms shows that bacterial and viral indicators, as well as Cryptosporidium oocysts and Giardia cysts, attach to wastewater biofilms to a concentration that remains stable in time, probably as a result of a dynamic equilibrium between attachment and detachment processes.

Keywords Biofilms; F-specific phages; heterotrophic plate count; occurrence; persistence; somatic coliphages; thermotolerant coliforms; wastewater

Introduction

Pipes and surfaces within wastewater treatment plants are covered with biofilms offering adsorption sites to suspended solids and colloids, but also to enteric pathogens including viruses and protozoa (Quignon et al., 1997; Sibille et al., 1998). If the interaction of pathogens with biofilms would lead only to their attachment and/or inactivation, biofilms could be considered as a water treatment process that would remove pathogens from the water phase. However, biofilm-associated microorganisms may persist and further detach by erosion and sloughing leading to the potential release of entrapped infective pathogens. Survival and transport of microorganisms may totally change depending on whether microorganisms have or have not formerly interacted with biofilms. For instance, enteric pathogens may persist longer when associated with organic solids than when they are free in flowing water, as suggested by different authors (Smith et al., 1978; Chung and Sobsey, 1993; Karim et al., 2004). If so, the protective effect of the biofilm may, in fact, increase the risk for biofilm-associated pathogens to reach a potential host. The concentrations of viable/infectious microorganisms that are released from biofilms depend on: (i) their concentrations in the water phase; (ii) their attachment rates; (iii) their detachment rates (including phenomena of erosion and sloughing); and (iv) their persistence within the biofilm. The persistence results from the balance between multiplication and inactivation in the case of indicators but results only from inactivation in the case of...
pathogens that are not able to multiply outside a eukaryote host cell. Currently, no quantitative data describe the proportion of enteric pathogens that interact with wastewater biofilms before being released in the environment.

In this preliminary study, we compared the occurrence and the persistence of microorganisms in biofilms and in the corresponding wastewaters. Heterotrophic plate count (HPC) and thermotolerant coliforms (TC) were used as bacterial indicators whereas bacteriophages such as somatic coliphages (SC) or F-specific phages (F+), sharing similarities with human enteric viruses, were used as viral indicators. To achieve our objectives, we combined laboratory experiments with wastewater biofilm analyses. The aim of the laboratory experiments was to assess the persistence of bacterial and viral indicators in biofilms and in the corresponding wastewater at two extreme seasonal temperatures: 4 and 20°C. Biofilm and water samples were analysed weekly in duplicate for two months. Beside laboratory experiments, we assessed the occurrence of bacterial and viral indicators in native wastewater biofilms. Samples were collected monthly in two wastewater treatment plants and one wastewater stabilisation pond. A total of 12 wastewater biofilms were analysed. All biofilms were characterised regarding their morphology (Scanning Electron Microscopy), their bacterial populations (total and cultivable bacteria using SYBR Green staining and PCA culture media, respectively), the presence of faecal indicators (TC, SC, F+) and the amount of total proteins (adapted from Lowry’s method). Additionally, we looked for Cryptosporidium oocysts (4–6 μm) and Giardia cysts (10–15 μm) in these wastewater biofilms.

Materials and methods
Wastewater and biofilm sampling for the occurrence study and the persistence experiment
A set of 5 × 15 cm removable polyvinyl chloride (PVC) coupons was immersed in wastewaters at two wastewater treatment plants in Luxembourg (Pétange [raw and treated wastewater] and Schifflange [treated wastewater]) as well as in a wastewater stabilisation pond (Differdange). In both wastewater treatment plants, coupons were immersed in flowing waters while in the wastewater stabilisation pond, coupons were immersed in still water. After at least two months of setting, the PVC coupons colonised with wastewater biofilm were rinsed with sterile phosphate buffer saline (PBS) pH = 7.4 and collected in a 1-L bottle filled up with new PBS kept at 4°C. Treated wastewater biofilms from Pétange, Schifflange and Differdange, as well as the corresponding waters, were analysed monthly from December 2005 to March 2006 (n_total = 12) in order to assess the occurrence of culturable bacteria (HPC and TC) and infectious viral indicators (SC and F+). For persistence assessment, raw wastewater biofilms from Pétange were incubated in duplicate at 4 and 20°C and analysed weekly for two months. This experiment was conducted twice starting 19 October 2005 and 18 January 2006, respectively. During the second experiment, persistence of HPC, TC, SC and F+ was also assessed in wastewaters under continuous magnetic stirring (~100 rpm) at 4 and 20°C in duplicate.

Biofilm characterisation
Scanning electron microscopy (SEM). Biofilm morphology was examined by scanning electron microscopy (LEICA STEREOSCAN 430i). Briefly, coupons were rinsed with PBS (2 × 30 min) and fixed in 5% glutaraldehyde-PBS 0.05 M pH = 7 for 4 hours. After the fixative was removed, samples were dehydrated successively in 25% ethanol for 30 min, 50% ethanol for 30 min, 75% ethanol for 30 min and twice in 100% ethanol for at least 30 min and finally air dried in a desiccator for three days. Samples were then mounted on aluminium stubs with graphite paint and gold sputter coated (BAL-TEC MED 020 sputtering system) for 75 sec at 60 mA and 5 Pa of argon plasma.
Total protein quantification. Scrapped biofilm was resuspended in 1 mL of MilliQ water. Weight of analysed biofilm was estimated from a 2-mL tube weighted before and after the addition of biofilm. One millilitre of 0.1 M NaOH was added to the sample and mixed for 15 min at 80°C for protein extraction. Sample was centrifuged at 10,000 g for 10 min and the protein content was determined in the supernatant as described previously by Lowry et al. (1951) using bovine serum albumin as standard. Results are expressed in µg of proteins per mg of biofilm.

Cryptosporidium oocysts and Giardia cysts quantification. Scrapped biofilm was resuspended in 5 mL of PBS pH = 7.4 in a 15-mL centrifuge tube (Falcon). Weight of analysed biofilm was estimated from the tube weights before and after the addition of the biofilm. Biofilm suspension was full speed vortexed (2,600 rpm) for 1 min. Cryptosporidium oocysts and Giardia cysts were quantified according to the protocol EPA 1623 (USEPA, 2001).

Biofilm and wastewater analyses
Microbiological parameters. The elution protocol was adapted from Gilgen et al. (1997). Scrapped biofilm was resuspended in 5 mL of 1% pasta beef extract (Difco, 212610) and 0.4% glycine at pH = 9.5 in a 15-mL centrifuge tube (Falcon). Biofilm suspension was vortexed at full speed (2,600 rpm) for 1 min before neutralisation by ten-fold dilutions in PBS (pH = 7.4). Dilutions were kept at 4°C until characterisation which was performed within 4 hours.

Bacteria enumeration. Heterotrophic plate count (HPC) bacteria were assessed for wastewaters and biofilm eluates using plate count agar (PCA) media according to the norm ISO 6222 (International Standard, 1999).

Culturable thermotolerant coliforms (TC) were quantified for wastewaters and biofilm eluates using m-FC media after membrane filtration, according to norm ISO 9308-1 (International Standard, 1990).

Total bacteria (SYBR Green staining). Total bacteria (TB) were quantified on biofilms after SYBR Green staining. Briefly 1 mL of ten-times diluted biofilm suspension was mixed with 10 µL of SYBR Green I (Molecular Probes, S7563). After 10 min in the dark, the suspension was filtrated on 0.22 µm pore size polycarbonate filter (Millipore, GTBP02500). Total bacteria were enumerated under blue excitation light at 1000 X magnification using a Leica epifluorescence microscope (DMRB) equipped with an ocular grid.

Somatic coliphage and F-specific coliphage quantification. Infectious SC and F+ were quantified using the bacterial host strains Escherichia coli (WG5) and Salmonella typhimurium (WG49), respectively, according to the standardised methods described in ISO/FDIS 10705-2 (International Standard, 1999) and ISO/FDIS 10705-1 (International Standard, 1997) respectively.

Concentration calculations and statistical analyses. Concentrations of culturable bacteria and infectious phages are expressed respectively as colony forming unit (cfu) and plaque forming unit (pfu) per millilitre of water (mL) or per milligram of biofilm (mg). The weight of biofilm analysed varied from 24 to 80 mg for protein analysis (mean = 50 mg ± 20), from 65 to 302 mg for bacterial and viral enumeration (mean = 140 mg ± 70) and from 147 to 496 mg for Cryptosporidium oocysts and
*Giardia* cysts quantification (mean = 299 mg ± 106). Concentration values were log-transformed before calculation of the averages (\( C = \log (x + 1) \) in the case of F-specific phage concentrations). To compare the variations of microorganism density in the biofilm and in the wastewater, the concentrations obtained for each sampling date and for each sampling site were submitted to a standard two-way analysis of variance (ANOVA) using SigmaStat (v2.03) after testing for homogeneity of variance on log-transformed data.

For the persistence experiment, a linear regression was the model that fitted best the averages of log-transformed data (\( n_{\text{biofilm}} = 4 \) and \( n_{\text{water}} = 2 \) for each temperature condition). Calculated slopes (all were negative or not significantly different from 0) describe for each parameter the concentration decrease in log units per day (log.d\(^{-1}\)). Comparison of slopes was performed with an analysis of covariance (ANCOVA, Sokal and Rohlf, 1997) using the R Software (freely available at [http://www.r-project.org/](http://www.r-project.org/)).

**Results and discussion**

**Persistence of faecal indicators in wastewater and in biofilms**

Persistence of bacterial and viral indicators was assessed at 4 and 20°C in biofilms and in wastewaters. Evolutions of protein, total bacteria and faecal indicator concentrations in wastewater biofilms and in the corresponding waters are presented in Table 1.

During the two months of the experiment, concentrations of proteins and TB did not significantly vary in biofilms either at 4 or 20°C, showing a stability in the biofilm structure during this period (Table 1). F + and TC displayed a slight concentration decrease at 4°C of \(-0.025 \) and \(-0.030 \) log.d\(^{-1}\), respectively, while no significant decrease was observed for SC and HPC. At 20°C, viral indicators behaved the same way with a slight concentration decrease (\(-0.023 \) and \(-0.026 \) log.d\(^{-1}\)) while bacterial indicators exhibited faster decreases with \(-0.046 \) and \(-0.072 \) log.d\(^{-1}\) for HPC and TC, respectively. When comparing experimental conditions, differences between viral and bacterial indicators were also observed. In water, bacteriophage concentrations decreased faster when temperature increased while the opposite was observed for TC (Table 2). No significant difference was observed for HPC whatever the condition tested. Regarding the viral indicators, SC and F + concentrations decreased faster at 20°C than at 4°C (with the exception of F + in biofilms where no difference was observed) and always faster in water than in biofilm. According to our results, the average of SC or F + log reduction either at 4 or 20°C did not exceed 0.8 log pfu per month in biofilms while reaching 2–10 log pfu per month in water at 20°C. By comparison, Storey and Ashbolt (2001) reported a 2-log reduction of bacteriophage models (MS2 and B40-8) in diluted sewage biofilms after one month of experiment. In their experiment, biofilms were in a reactor under a constant flow rate of 0.32 L.s\(^{-1}\) which can explain the higher log reduction after one month. Also, in our experimental conditions, a linear inactivation model fits better the bacteriophage persistence than a biphasic model with rapid initial inactivation followed by slow inactivation (Storey and Ashbolt, 2001). A difference of susceptibility between bacteriophages close to the surface and bacteriophages located deeper in the biofilm may be accentuated when using circulating reused water (Storey and Ashbolt, 2001) compared with stationary PBS (our experiment). The higher persistence of SC and F + in biofilm compared with water may be associated with a protective effect of the biofilm rather than with the multiplication of the bacteriophages in the biofilm. Indeed, multiplication of F + at temperatures below 25°C is unlikely in wastewater (Havelaar and Pot-Hogeboom, 1988; Woody and Cliver, 1995). However, this remains to be investigated for both SC and F + in biofilms as bacteriophages may interact specifically with susceptible bacteria within the biofilm (Sutherland et al., 2004).
Table 1: Evolutions of protein, total bacteria and faecal indicator concentrations in biofilm and wastewater expressed in concentration log units per day (corresponding to the calculated slope obtained from a linear regression model applied to the plotted data of the persistence experiment)

<table>
<thead>
<tr>
<th></th>
<th>Biofilms</th>
<th></th>
<th>Wastewater</th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>4 °C</td>
<td>20 °C</td>
<td>4 °C</td>
<td>20 °C</td>
</tr>
<tr>
<td>Slope</td>
<td>r²</td>
<td>Test*</td>
<td>Slope</td>
<td>r²</td>
</tr>
<tr>
<td>Proteins (log µg/mg)</td>
<td>ns</td>
<td>0.22 a</td>
<td>ns</td>
<td>0.60 a</td>
</tr>
<tr>
<td>Total bacteria (log/mg)</td>
<td>ns</td>
<td>0.07 a</td>
<td>ns</td>
<td>0.45 a</td>
</tr>
<tr>
<td>HPC (log cfu/mg)</td>
<td>ns</td>
<td>0.20 a</td>
<td>−0.046</td>
<td>0.80 c</td>
</tr>
<tr>
<td>Thermotolerant coliforms (log cfu/mg)</td>
<td>−0.030</td>
<td>0.85 b</td>
<td>−0.072</td>
<td>0.94 d</td>
</tr>
<tr>
<td>Somatic coliphages (log pfu/mg)</td>
<td>ns</td>
<td>0.56 a</td>
<td>−0.026</td>
<td>0.96 b</td>
</tr>
<tr>
<td>F-specific phages (log pfu/mg)</td>
<td>−0.025</td>
<td>0.69 b</td>
<td>−0.023</td>
<td>0.53 b</td>
</tr>
</tbody>
</table>

Slopes and associated coefficients of determination (r²) are calculated on seven concentrations that correspond to days 0, 7, 14, 21, 28, 35 and 49 (each concentration is an average of four [in case of biofilm] and two [in case of water] values) except for e and f where slopes could only be calculated on four and two concentrations, respectively (afterwards concentrations were below the detection limit).

*Results of the ANCOVA slope comparison test where a, b, c, d represent parameters or group of parameters that exhibit no significant difference in slope value (p > 0.05)

ND, not determined

ns, Slope values that were not significantly different from 0 (ANCOVA, p > 0.05)
Concentrations of faecal bacteria (TC) decreased faster at 20°C than at 4°C in biofilms while the opposite was observed in waters. Maintaining faecal bacteria in water for a long period of time may reduce their cultivability faster at a low temperature than at 20°C. HPC, which is a more heterogeneous group of bacteria, seems not to be affected. It can be noted that the presence of grazing protozoa and metazoa may contribute to the decrease of both bacterial and viral concentrations in biofilms. Indeed, protozoa grazing activities may contribute to viral, bacterial and pathogenic protozoa removal during wastewater treatment, as suggested by different authors (Kim and Unno, 1996; Eisenmann et al., 2001; Stott and Tanner, 2005). Although the contribution of these mechanisms in wastewater biofilms remains to be quantified for viruses, bacteria and pathogenic protozoa, optimal grazing activity of protozoa is observed for temperatures ranging from 10 to 25°C (Pauli et al., 2001). According to our results, inactivation rate in biofilm was two to four times higher at 20°C compared with 4°C for TC and SC, respectively, but did not vary significantly for F+ or HPC despite sub-(4°C) and optimal temperatures (20°C) for predatory activities suggesting a low (if any) contribution of these mechanisms in our experimental conditions.

Occurrence of faecal indicators and pathogenic protozoa in native wastewater biofilms

Treated wastewater biofilms and the corresponding waters were collected from two wastewater treatment plants and one wastewater stabilisation pond. They were analysed monthly from December 2005 to March 2006. Results are presented in Figure 1. A two-way ANOVA test shows no sampling date effect (p > 0.05) while a site effect exists for all conditions with the exception of TC in wastewaters and F+ in biofilms. The significant difference of indicator concentrations in biofilms between sites can be explained by a difference in wastewater contamination but also by a difference in biofilm structures. As shown in Figure 2, wastewater biofilms are complex biological networks presenting a high microorganism diversity and may harbour very different communities, including diatoms (Figure 2a) and Vorticella-like organisms (Figure 2b).

The absence of sampling date effect seems to indicate an equilibrium between accumulation (attachment/multiplication) and loss (detachment/inactivation) of indicators in the biofilms regardless of the biofilm age varying from 2 to 5 months between the first and the last sampling date. In the absence of significant multiplication in the biofilm (as discussed above for bacteriophages), this can be explained either by a high turnover of

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**Table 2** Comparison of proteins, TB and indicator persistence in function of the experimental conditions.

When significant differences were observed (p < 0.05), the calculated ratio indicates the difference factor that exists between two experimental conditions.

<table>
<thead>
<tr>
<th>Indicator</th>
<th>Comparison between 20°C (a) and 4°C (b)</th>
<th>Comparison between water (a) and biofilm (b)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>In biofilm</td>
<td>At 4°C</td>
</tr>
<tr>
<td></td>
<td>Ratioa/b p</td>
<td>Ratioa/b p</td>
</tr>
<tr>
<td>Proteins</td>
<td>–</td>
<td>0.313 NA NA NA NA NA</td>
</tr>
<tr>
<td>Total bacteria</td>
<td>–</td>
<td>0.411 NA NA NA NA NA</td>
</tr>
<tr>
<td>HPC</td>
<td>–</td>
<td>0.080 – 0.884 – 0.058 – 0.637</td>
</tr>
<tr>
<td>Thermotolerant coliforms</td>
<td>2.4 0.002 0.2 0.003 4.8 0.008 0.3 &lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Somatic coliphages</td>
<td>4.2 &lt;0.001 2.6 &lt;0.001 3.7 &lt;0.001 2.3 &lt;0.001</td>
<td></td>
</tr>
<tr>
<td>F-specific phages</td>
<td>– 0.874 2.9 0.043 4.8 0.004 15 0.011</td>
<td></td>
</tr>
</tbody>
</table>

*, not significantly different (p > 0.05)
NA, not applicable since the parameter has not been monitored in water
#Slope ratio calculated from Table 1 when differences between experimental conditions are significant (ANCOVA, p < 0.05). Ratio > 1 means that inactivation rate was higher in the (a) condition than in the (b) condition whereas the opposite is true for a ratio  < 1
the biofilm contamination resulting from permanent attachments and detachments (independently of the inactivation rate) or, on the other hand, by a low turnover of the biofilm contamination resulting from low attachment and low inactivation rates. According to our preliminary results (persistence experiment) a low inactivation rate for viral indicators in biofilm occurs (inferior to 0.8 log pfu per month). However, although virus attachment to and detachment from biofilms have been reported (Storey and Ashbolt, 2001; for a review see Skraber et al., 2005), a lack in quantitative data precludes a conclusion. Attachment and detachment rates of viruses to/from wastewater biofilms are currently under investigation in our laboratory.

**Occurrence of Cryptosporidium and Giardia (oo)cysts in wastewater biofilms**

Giardia cysts were detected 11 times out of 12 (92%) at concentrations up to 63 cysts per gram of biofilm whereas Cryptosporidium oocysts were detected six times out of 12 (50%) at concentrations up to 14 oocysts per gram of biofilm (Table 3). The presence of these pathogens in wastewater biofilms shows the ability of biofilms to entrap microorganisms with a size up to 15 µm under constant flow rate (Pétange and Schifflange) as well as in still water (Differdange). (Oo)cyst concentrations did not increase during the four month monitoring period (data not shown) suggesting that either protozoa contamination of biofilms remains stable for a long period of time, or, similar to bacterial and viral indicators which are permanently present in wastewater, no accumulation of these parasites in wastewater biofilms occurs as a result of a constant turnover.

**Figure 1** Averages and standard deviations of indicator concentrations in biofilms and in the corresponding wastewaters in function of the sampling site (n = 4 sampling dates)

**Figure 2** SEM observations of wastewater biofilms from Differdange (a) and Schifflange (b). Wastewater biofilms from Pétange have similar structures to biofilms from Schifflange (data not shown) (scale bars = 10 µm)
Conclusions
In this study we assessed the persistence of bacterial (HPC and TC) and viral indicators (SC and F+ ) in wastewater biofilms at 4°C and 20°C as well as the occurrence of these indicators and pathogenic protozoa (Cryptosporidium oocysts and Giardia cysts) in three native wastewater biofilms. The main conclusions are as follows:

- Bacterial and viral indicators behave differently in biofilms. At 20°C, both viral indicators (SC and F+) persist significantly longer than bacterial indicators (HPC and TC).
- Bacterial and viral indicators, as well as Cryptosporidium oocysts and Giardia cysts, could be retrieved from wastewater biofilms at concentrations that remain stable for at least four months (time of our monitoring period), probably as a result of a dynamic equilibrium between attachment and detachment processes.

Although the role of biofilms in pathogen dissemination remains to be investigated, it cannot be excluded that a protective effect of the biofilm associated with biofilm sloughing and erosion may enhance the risk for a pathogen to reach a potential host.

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