Persistence of biofilm-associated *Escherichia coli* and *Pseudomonas aeruginosa* in groundwater and treated effluent in a laboratory model system

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This study was based on the hypothesis that groundwater-derived biofilms may provide a reservoir for coliform or pathogenic bacteria as has been observed in drinking water distribution systems. *Escherichia coli*, labelled with green fluorescent protein, was found to colonize all layers of mixed-population biofilms developed in association with indigenous groundwater micro-organisms in a laboratory-scale reactor. Biofilm-associated *E. coli* was removed at a slower rate from the reactor flasks than planktonic *E. coli* under a continuous flow regime. During flow-through of groundwater, planktonic *E. coli* removal was slower in flasks containing coverslips for enhanced biofilm development compared to a control flask without coverslips. Conversely, during flow-through of treated effluent, planktonic *E. coli* removal was faster in flasks with coverslips compared to without. Removal of attached *E. coli* was also fastest in the coverslip-containing flasks with effluent flow-through. This suggests that an increase in available nutrients may reduce *E. coli* survival potential due to either enhanced competition for nutrients or enhanced antagonism by the indigenous microbial population. Under identical conditions, GFP-labelled *Pseudomonas aeruginosa* was found to persist in the biofilms for longer than *E. coli*, most notably when exposed to flow-through of treated effluent. However, prolonged persistence of *P. aeruginosa* in the effluent could not be attributed to an association with the biofilms. This study has shown that under certain conditions the presence of mixed-population biofilms may limit the survival potential of enteric bacterial pathogens introduced into groundwater.

INTRODUCTION

In aquatic environments, the interaction of pathogens with biofilms has predominantly been a concern in man-made water systems and in particular, drinking water distribution systems (Szewzyk et al., 2000; Block, 1992). Drinking water utilities commonly use coliform bacteria such as *Escherichia coli* as an indicator of the presence of pathogens from faecal contamination. Frequent occurrences of increased coliform numbers in the distribution system without a contamination event led researchers to believe that biofilms on the surfaces of pipes and fittings may act as a reservoir for coliforms and other pathogenic bacteria (Van der Wende et al., 1989; LeChevallier et al., 1987; Camper et al., 1985). Subsequent laboratory-based studies have demonstrated the colonization of heterogeneous biofilms developed from tapwater micro-organisms by pathogens such as *E. coli* (Buswell et al., 2001; Camper et al., 1996; Robinson et al., 1995), *Aeromonas hydrophila* (Walker et al., 1995), *Legionella pneumophila* (Murga et al., 2001; Rogers et al., 1994; Colbourne et al., 1984), *Campylobacter jejuni* (Buswell et al., 1999) and *Helicobacter pylori* (Mackay et al., 1999). Prolonged persistence of biofilm-associated pathogens has been attributed to access of attached cells to nutrients that accumulate at surfaces and to the enhanced resistance of biofilm micro-organisms to disinfection by chlorination or other inimical agents (Gilbert & Brown, 1995; Vess et al., 1993; vanLoosdrecht, 1990). It has been proposed that biofilm communities in natural aquatic environments such as intertidal marine systems (Decho, 2000), hydrothermal springs (Marrao et al., 1993) or groundwater (Momba et al., 1999) may also provide favourable microenvironments for introduced pathogens. However, there is little experimental data on the conditions under which the biofilms may provide protection for introduced pathogens and the extent to which this phenomenon may occur outside of water distribution systems.

The attenuation of bacterial pathogens introduced into subsurface environments has been observed in both field
and laboratory studies and has been modelled using first-order decay kinetics (Pavelic et al., 1998; Dowd & Pillai, 1997; Yates & Yates, 1988). Factors that commonly limit the survival of bacterial pathogens introduced into groundwater include the low level of available nutrients, the lack of oxygen (for obligate aerobes) as well as the competitive, antagonistic and predatory activities of the indigenous microbial population (Gerba & Goyal, 1985). The practice of aquifer storage and recovery (ASR), which involves the injection of surface water into an aquifer, can create an environment which allows the rapid growth of indigenous and/or introduced bacteria in the aquifer, particularly when the injected water is high in nutrients (Pavelic & Dillon, 1997; Vecchioli, 1970). ASR provides a means of storing reclaimed wastewaters prior to re-use for purposes such as irrigation. During an ASR pilot project in South Australia using treated sewage effluent, bacterial growth during injection resulted in biofilm formation within the aquifer matrix immediately surrounding the injection well (Rinck-Pfeiffer, 2000).

This study was undertaken to determine whether the biofilms developed in an aquifer during ASR could potentially provide a reservoir for pathogenic bacteria. If the recovered water contains a greater number of pathogens than expected, its re-use may pose a potential public health risk. Furthermore, water from the initial stages of recovery is often enriched in dislodged biofilm material and may require special disposal if it contains large numbers of pathogens. In this study, *E. coli* was used as a representative of the enteric bacterial pathogens and its survival compared to the ubiquitous water-borne micro-organism and opportunistic pathogen *Pseudomonas aeruginosa*.

**METHODS**

**Biofilm reactor design.** The reactor set-up shown in Fig. 1 was duplicated to enable two holding reservoirs and eight reactor flasks to be run simultaneously. Glass was chosen to provide a surface for attachment as it is an inert material that simulates natural silicon oxides such as sand (Murgel, 1991). Nitrogen gas was constantly bubbled through the water in the holding reservoirs to create anaerobic conditions similar to those found in many aquifers. The outflow line from each reactor flask passed directly to a multi-channel peristaltic pump, which controlled the gravity-induced flow, and subsequently to a waste container. The mean flow rate was 7.5 ± 0.2 ml h⁻¹ [325 ml flask working volume; dilution rate (D) = 0.023 h⁻¹]. Homogeneous conditions in each flask were established by mixing using a cylindrical stirrer bar (4 × 15 mm) and placing each flask on a multi-point magnetic stirrer plate (Variomag HP 15) set at 200 r.p.m. for the duration of the experiment. All components of the reactor were sterilized at 121 °C for 15 min prior to use. The reactor was run at room temperature (23 ± 2 °C) in the dark. To sample from the reactor, flow-through was stopped, a flask detached and the lid unscrewed. Nitrogen gas was passed over the liquid surface during sampling to prevent exposure to oxygen. Coverslips were removed using flame-sterilized stainless steel forceps. The total surface area (SA) available for attachment in the reactor flasks containing coverslips was 541 cm². Each sampling occasion reduced the SA by 29 cm² (equivalent to three coverslips). The SA in reactor flasks without coverslips was 256 cm².

**Bacterial strains.** The type strain of *E. coli* (ATCC 11775) was transformed with the pEGFP vector (Clontech). This vector contains an ampicillin-resistance determinant and a green fluorescent protein (GFP) gene (GFPmut1) that has been mutated for improved fluorescence (Cormack et al., 1996). The type strain of *P. aeruginosa* (ATCC 10145) was transformed with the pSMC21 vector, a derivative of pSMC2 described by Bloemberg et al. (1997). This vector encodes an enhanced GFP (GFPmut2), a kanamycin-resistance determinant and a *Pseudomonas* ‘stabilizing’ fragment that allows the plasmid to replicate in *Pseudomonas* species as well as *E. coli*. A constitutive lac promoter drove expression of the GFP gene in these plasmids. Maintenance and expression of the GFP plasmids in their respective host cells in the absence of antibiotic selection was tested during successive subculture in Luria–Bertani (LB) broth for a minimum of 40 generations as well as incubation of the cells in sterile, anaerobic groundwater and effluent microcosms using previously described methods (Banning et al., 2002). For inoculation into the biofilm reactor, cells were grown overnight at 37 °C in LB broth containing 100 µg ampicillin ml⁻¹ for growth of *E. coli* (pEGFP) or 400 µg kanamycin ml⁻¹ for growth of *P. aeruginosa* (pSMC21). The cells were harvested by centrifugation (15 000 g 10 min at 4 °C) and washed three times in sterile groundwater with sequential reductions in volume. The final cell concentrations, determined on LB agar with the appropriate antibiotic were 1 × 10⁸ c.f.u. ml⁻¹ (*E. coli*) and 8 × 10⁶ c.f.u. ml⁻¹ (*P. aeruginosa*). Colony counts on LB without antibiotic were also performed and no non-fluorescent colonies were detected. The concentrated cell suspensions were stored at 4 °C prior to use.

**Quality of water used.** Anaerobic groundwater was collected from the superficial aquifer on the Swan Coastal Plain (Perth, Western Australia) using a submersible electric pump. When required, a sterile 10% (w/v) peptone (Oxoid) solution in distilled H₂O was added to 5 l groundwater to give a final concentration of 0.01% (w/v). Effluent was collected from a clarification pond, used for sedimentation processing after primary and activated sludge treatment, at the Subiaco Wastewater Treatment Plant, Western Australia. Particulates were removed from the effluent by passing through glass microfibre filter paper (1–2 µm pore size; Whatman). Some chemical properties of the waters used in the reactor experiments are given in Table 1.

**Experimental design and sampling.** The reactor experiments with *E. coli* and *P. aeruginosa* were performed on separate occasions following the protocol described below. Both holding reservoirs of the reactor were connected to four flasks, three of which contained coverslips. Peptide-amended groundwater in the holding reservoirs was allowed to flow into each reactor flask, which had been previously flushed with nitrogen gas, until the water level reached the

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**Fig. 1.** Diagram of biofilm reactor set-up.
Table 1. Mean water quality of groundwater and treated effluent used in the reactor

<table>
<thead>
<tr>
<th>Parameter (mg l⁻¹)</th>
<th>Groundwater*</th>
<th>Treated effluent</th>
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<tbody>
<tr>
<td>Total organic carbon</td>
<td>4.25 ± 1.3</td>
<td>13.3 ± 2.1</td>
</tr>
<tr>
<td>Ammonia-N</td>
<td>&lt;0.01 ± 0.0</td>
<td>0.34 ± 0.2</td>
</tr>
<tr>
<td>Nitrate-N</td>
<td>0.042 ± 0.04</td>
<td>10.7 ± 7.0</td>
</tr>
<tr>
<td>Nitrite-N</td>
<td>&lt;0.02 ± 0.0</td>
<td>0.17 ± 0.1</td>
</tr>
<tr>
<td>Phosphorus</td>
<td>0.01 ± 0.0</td>
<td>3.10 ± 2.9</td>
</tr>
<tr>
<td>Calcium</td>
<td>66 ± 5.7</td>
<td>51.3 ± 8.1</td>
</tr>
<tr>
<td>Magnesium</td>
<td>13 ± 1.4</td>
<td>13.7 ± 3.8</td>
</tr>
<tr>
<td>Dissolved oxygen</td>
<td>3.33 ± 1.9</td>
<td>6.3 ± 3.1</td>
</tr>
<tr>
<td>pH</td>
<td>7.16 ± 0.2</td>
<td>7.05 ± 0.33</td>
</tr>
</tbody>
</table>

*Amendment with peptone resulted in an approximate 10-fold increase in the concentration of total organic carbon.

Pathogen survival in aquatic biofilms

top of the flask. The reactor was operated in batch mode for 24 h. For the following 6 days, three of the flasks connected to each holding reservoir (six in total) were injected twice per day with 0.5 ml of the washed GFP-labelled E. coli or P. aeruginosa concentrated cell suspension. During this biofilm development period the reactor was operated under continuous flow conditions. The peptone-amended groundwater in the holding reservoirs was then replaced with unamended groundwater in one reservoir and clarified effluent in the other. This was designated day 0 and flow-through was continued for 3 weeks (E. coli) or 7 weeks (P. aeruginosa). Therefore, connected to each holding reservoir were duplicate test flasks used to measure E. coli or P. aeruginosa removal in the presence of biofilms in either groundwater or effluent, a third flask without coverslips used to measure E. coli or P. aeruginosa removal in the absence of biofilms in either groundwater or effluent and a fourth flask containing biofilms without inoculated GFP-labelled cells. The flask with uninoculated biofilms were used to provide negative controls for detection of GFP-conferred fluorescence.

Three coverslips and a water sample were removed from the reactor flasks on days 0, 3, 7, 10, 15 and 21 during the E. coli experiment and on days 0, 7, 14, 26 and 40 during the P. aeruginosa experiment. Coverslip samples were immediately rinsed three times in PBS (24 g NaCl l⁻¹; 0.6 g KCl l⁻¹; 4.32 g Na₂HPO₄ l⁻¹; 0.72 g KH₂PO₄ l⁻¹ at pH 7.0) to remove unattached or loosely adhered cells prior to further processing (described below). Viable E. coli numbers in the outflow from each flask were monitored during the E. coli experiment. At the end of the P. aeruginosa experiment (day 47), each flask that had been inoculated with P. aeruginosa was destructively sampled to determine the extent of biofilm development and numbers of attached P. aeruginosa on the remaining coverslips, the coverslip holder and wall of each reactor flask.

Culturable cell counts. Coverslip samples were placed in 12 ml PBS immediately after rinsing. Cells were removed from the surface by scraping with a sterile disposable cell scraper (17 mm blade length; Sarstedt), followed by sonication in a 50 kHz water-bath (10 min) and vortexing (1 min). The same procedure was followed to remove cells, after rinsing, from the coverslip holders and reactor flasks at the end of the P. aeruginosa experiment, using 20 ml or 100 ml volumes of PBS, respectively. Culturable counts of GFP-labelled cells in water or processed biofilm samples were performed by either the drop-on-plate method (with six replicate 10 μl drops of the appropriate dilutions) or spread-plate method (triplicate plates using undiluted 100 μl or 200 μl volumes) using LB agar containing the appropriate antibiotic. The number of colonies showing green fluorescence under blue light illumination was recorded.

Epifluorescence microscopy. For determination of fluorescent cell numbers, aqueous samples were filtered onto black polycarbonate filters (0.2 μm pore size, 25 mm diameter; Millipore) and mounted on glass slides as described by Hobbie et al. (1977). Coverslip samples were air-dried after rinsing and mounted on a glass slide using nail varnish. Mounted coverslips were overlaid with mineral oil and a clean coverslip before viewing. Filters and coverslip samples were viewed under oil immersion with a 100 x Plan objective on a Leitz Diaplan microscope fitted with a Leitz Pleomopak fluorescence attachment. GFP-conferred fluorescence was visualized under illumination with blue light (excitation 450–490 nm; suppression 515 nm). The number of cells in a minimum of 20 randomly chosen fields of view was determined for each filtered aqueous sample and each coverslip sample.

The SA coverage of the biofilms was determined by staining the coverslip samples with the nucleic acid stain DAPI (4′,6-diamidino-2′-phenylindole dihydrochloride) at a concentration of 0.5 μg ml⁻¹ for 10 min in the dark. Ten photomicrographs from each coverslip were taken under UV light (excitation 340–380 nm; suppression 430 nm) at 1000 x magnification with a cooled slow scan PXL CCD camera (Photometrics). The area of DAPI-conferred fluorescence (in μm²) was measured in each photomicrograph using IPLab Spectrum version 3 (Scalancis). The SA values were converted to a percentage of the photomicrograph area, which was 6143 μm², and the mean value for each coverslip sample determined.

Confocal scanning laser microscopy (CSLM). After rinsing, coverslip samples were mounted in screw-top circular stainless steel chambers with a viewing area of 7.5 mm radius. The chambers were filled with PBS to prevent the biofilm drying during viewing and the underside of each coverslip was cleaned with 70% ethanol. Biofilms were viewed using a Bio-Rad MRC1000/1024 UV confocal scanning laser microscope, mounted on a Nikon Diaphot 300 with a Nikon 60 x water-immersion PlanApo objective lens (numerical aperture of 1.2). A correction collar allowed adjustment for the thickness of the coverslip. The microscope was controlled by the COMOS software (Bio-Rad Microscience) and images were collected using a Kalman mathematical filter (where n=3) to reduce background noise. All images were collected using a confocal pinhole size of 2.5 mm. For GFP detection, 488 nm argon laser light was used to excite the specimen and fluorescence was collected through a 522/35 nm emission filter. Power and gain settings were chosen to supply the most sensitivity without picking up autofluorescence in the negative control biofilms (i.e. from flask that were not inoculated with GFP-labelled cells). A z-axis stepping motor, which allows movement of the focal plane in precise increments (minimum of 0.1 μm), was used to collect a z-series of images for each field of view. Confocal microscope images were processed using Confocal Assistant Software version 4.02 (Bio-Rad).

Data analysis. Removal rates were determined by calculating the slope and correlation coefficient (r²) of the linear regression of log-transformed cell concentration data according to the first-order decay relation given by: C = C₀e⁻kt where C is the microbial concentration at time t, C₀ the initial concentration on day 0 and k the removal rate. The concentration data were mean values from replicate culture-based or direct cell counts from a single water sample or coverslip sample. Where reactor flasks were run in duplicate (i.e. test flasks containing coverslips), the removal rate associated with each flask was calculated first and then the mean value and standard deviation of the two rates determined. The cell removal rates derived from direct GFP-fluorescent cell counts are referred to as ‘total’ and those from GFP-fluorescent colony counts are referred to as ‘viable’. 
A Student’s t-test (one-tailed distribution for two samples with unequal variance) was used to determine if differences between test flasks with flow-through of the different water types were significant.

RESULTS

GFP-conferred fluorescence

Transformation of E. coli and P. aeruginosa with the high-copy-number GFP plasmids pEGFP and pSMC21, respectively, resulted in the production of brightly fluorescent cells that were easily detectable by epifluorescence microscopy. Although the fluorescence intensity of GFP-labelled P. aeruginosa was visibly less than that of E. coli, they could still be distinguished from the indigenous microbial population. GFP has an intrinsic property of fluorescing without the addition of any co-factors or substrates and as it is a cytoplasmic protein, it has been found not to affect bacterial cell surface properties (Olofsson et al., 1998). The only requirement for GFP fluorescence is the presence of oxygen, although GFP-labelled cells grown under anaerobic conditions can develop fluorescence upon exposure to air (Scott et al., 1998).

The GFP plasmids were found to be stable in the type strains of E. coli and P. aeruginosa over 40 generations of growth in LB broth without the addition of antibiotic when incubated at 28 °C in a static incubator (data not shown). During incubation of E. coli(pEGFP) in sterile groundwater microcosms for 50 days, the number of c.f.u. on LB with or without antibiotic did not significantly differ (P > 0.05), demonstrating maintenance of plasmid expression in the culturable cell population. In addition, no significant differences between the total number of cells (enumerated using the nucleic acid stain DAPI) and the number of GFP-fluorescent cells were detected during this time (Fig. 2a). Incubation of E. coli(pEGFP) in sterile effluent microcosms gave similar results (data not shown). Maintenance of fluorescence in P. aeruginosa(pSMC21) in sterile groundwater microcosms was also demonstrated in the culturable cell population although the proportion of the total cell population with GFP-conferred fluorescence decreased over time (Fig. 2b). These results are in agreement with previous studies demonstrating that intact cells may maintain GFP-conferred fluorescence even if non-viable but that some intact dead or dying cells may lose GFP-conferred fluorescence under conditions of stress such as nutrient limitation (Banning et al., 2002) or heat (Lowder et al., 2000). It is likely that this loss of fluorescence is linked to leakage of the protein though a damaged cell membrane. Although the GFP plasmid–host constructs used in this study displayed different patterns of fluorescence loss, both GFP plasmids were considered as suitable cellular markers (but not viability indicators) for use under non-selective, anaerobic, nutrient-limited conditions.

Biofilm development

Flow of peptone-amended groundwater through the reactor flasks resulted in the formation of biofilms with large multi-layered cell clusters separated by sparsely populated areas. This heterogeneous colonization of the surface was a consistent feature on all coverslips within each flask and is typical of aquatic biofilm structure (Costerton et al., 1995). On day 0 of the E. coli experiment, the mean biofilm SA coverage in all flasks was 5.5 ± 2%. After 15 days, the biofilms in flasks with treated effluent flow-through had a greater mean SA coverage (16 ± 1%) than the flasks with groundwater flow-through (6.3 ± 2%). Combined fluorescence/transmission CSLM images revealed that E. coli had attached as single cells to the surface, had colonized clusters of other cell types and had also formed microcolonies (Fig. 3). Analysis of CSLM fluorescence images (taken as five z-sections in 0.2 μm increments) of biofilms from each of the four test flasks sampled on day 3 revealed that higher numbers of E. coli were attached near to the coverslip surface. The mean number of E. coli per field of view on all coverslips (n = 40) in the first z-section (closest to the coverslip surface) was 60 ± 14 compared to 31 ± 10, 17 ± 7, 8.2 ± 3 and 3.7 ± 2 in the second to fifth z-sections, respectively. Conversely, for E. coli associated with cell clusters, the highest numbers were in the second or third z-section from the surface. The mean number of E. coli per cell cluster on all coverslips (n = 56) was 5.2 ± 2, 6.2 ± 2,
4.8 ± 1, 3.1 ± 1, 1.3 ± 1 in the first to fifth z-sections, respectively.

Similar biofilm SA coverages were measured on coverslips during the P. aeruginosa experiment but the majority of attached P. aeruginosa cells were distributed as single cells with only a small number of P. aeruginosa-dominated microcolonies observed. The vertical distribution of P. aeruginosa cells was not analysed. Destructive sampling at the end of the P. aeruginosa experiment revealed that the mean percentage SA coverage of biofilm on the wall of the reactor flasks was either similar to (flasks with effluent flow) or less than (flasks with groundwater flow) the coverage on the coverslips sampled on the same day. The concentration of attached P. aeruginosa (c.f.u. cm⁻²) on the wall of the test flasks was significantly less than that on the coverslips (P < 0.05). This indicates that there was no preferential association of cells with the walls of the reactor flasks over the coverslips. There was no significant difference between the mean P. aeruginosa concentration detected on the coverslip holders and the mean concentration on coverslips from the same flask (P > 0.05). The concentration of P. aeruginosa attached to the wall of the control flasks was also less than the concentration on coverslips in the test flasks, indicating that the lack of coverslips did not promote attachment of the cells to the flask wall.

Removal rates of GFP-labelled cells

The rates of removal of total and viable E. coli(pEGFP) and P. aeruginosa(pSMC21) cells from biofilms and from the water phase are shown in Table 2. The time periods of days 0–10 for E. coli and 0–14 for P. aeruginosa were chosen for determining removal rates as cell numbers in all samples between these periods were above detection limits. Persistence of GFP-labelled cells inoculated into the reactor was a result of the interplay between the dilution or washout effect of continuous flow, cell growth and death rates and attachment/detachment processes. It was not possible to confirm whether any of the viable inoculated cells or their progeny had lost the GFP plasmid or expression of the plasmid during the reactor experiments due to the presence

Table 2. Mean E. coli removal rates between day 0 and 10, and mean P. aeruginosa removal rates between days 0 and 14

Units are log₁₀ (cells or c.f.u.) ml⁻¹ day⁻¹ (water phase) or log₁₀ (cells or c.f.u.) coverslip⁻¹ day⁻¹ (attached).

<table>
<thead>
<tr>
<th></th>
<th>Water phase*</th>
<th>Attached†</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Control flask</td>
<td>Test flasks†</td>
</tr>
<tr>
<td></td>
<td>E S</td>
<td>P S</td>
</tr>
<tr>
<td>Groundwater</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>−0.61</td>
<td>−0.42</td>
</tr>
<tr>
<td>Viable</td>
<td>−0.87</td>
<td>−0.39</td>
</tr>
<tr>
<td>Treated effluent</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>−0.33</td>
<td>−0.35</td>
</tr>
<tr>
<td>Viable</td>
<td>−0.44</td>
<td>−0.31</td>
</tr>
</tbody>
</table>

*<i>r</i>² for all rates was 0.8.
†<i>r</i>² for all rates was 0.7 except viable rates in flasks with groundwater where <i>r</i>² was 0.4.
‡These are mean rates derived from duplicate flasks with standard deviations given in parentheses.
$p$, E. coli(pEGFP); Pₛ, P. aeruginosa(pSMC21).
of the mixed microbial population. However, the results from the sterile microcosms, discussed above, suggest that this is unlikely to have occurred over the 21 or 40 day time period.

During the E. coli experiment, the numbers of attached E. coli cells increased over the first 3 days following the end of the inoculation period but subsequently declined by 2–3 log units, indicating that death or detachment processes were dominating the overall dynamics (Fig. 4). Nonetheless, interaction with the biofilms did appear to slow the washout effect as removal of attached E. coli was significantly slower than removal of planktonic E. coli (P<0.05). This was particularly evident in flasks with flow-through of groundwater in which removal of attached E. coli was slower than in flasks with flow-through of effluent.

The removal rates for GFP-labelled E. coli from the water phase of each flask were all faster than the theoretical washout rate of \(-0.24 \log_{10} \text{cells ml}^{-1} \text{day}^{-1}\), suggesting that cell death was contributing to the observed removal. Removal rates of planktonic E. coli measured using direct cell counts were generally faster than rates measured using viable counts, again suggesting cell death was occurring. The only exception was in flasks containing coverslips with groundwater flow-through in which the total and viable removal rates were very similar. In all flasks, the removal dynamics of the total E. coli population were not sufficiently different from the culturable population to suggest the emergence of 'viable but non-culturabe' cells during the course of the experiment. In flasks with groundwater flow-through, E. coli removal from the water phase was slower in test flasks with coverslips and associated biofilm compared to the control flask without coverslips. In these flasks viable cells may have detached from the biofilms, boosting numbers in the water phase. Conversely, in flasks with effluent flow-through, E. coli removal was faster in the test flasks compared to the control flask. It was confirmed that the faster removal of E. coli from the test flasks was not due to greater dilution through monitoring of the flow rates, which were the same for each flask, and monitoring of viable E. coli concentrations in the outflow, which were lower in samples from the flasks with faster removal rates. Thus, it appears that in the presence of effluent, the biofilms were expediting the removal of both water-phase and attached E. coli.

For P. aeruginosa, there was no significant difference between the removal rates of attached cells in flasks with groundwater or effluent flow-through and both rates were slower than that measured for E. coli. There was also little difference in removal rates (total or viable) of planktonic P. aeruginosa between control and test flasks. However, there was a pronounced difference between the behaviour of planktonic P. aeruginosa in flasks with flow-through of groundwater and those with effluent. After day 14 the number of viable P. aeruginosa in the flasks with groundwater-flow-through fell below detection limits whereas there were still detectable levels in the flasks with effluent flow-through by day 40. The rate of planktonic P. aeruginosa removal in flasks with effluent flow-through measured between days 0 and 40 was \(-0.12 \log_{10} \text{c.f.u. ml}^{-1} \text{day}^{-1}\), with or without biofilms, which was slower than the theoretical washout rate.

**Prolonged persistence of P. aeruginosa compared to E. coli**

A comparison of the length of time taken for the GFP-labelled cells in reactor flasks to fall below the detection limits also reveals that the most pronounced difference between E. coli and P. aeruginosa behaviour occurred in flasks with effluent flow-through (Fig. 5). In these flasks, P. aeruginosa persisted in an attached state and in the water phase for longer than E. coli and for longer than the theoretical time for cells to fall below the detection limits as a result of dilution.

**DISCUSSION**

The results from this study demonstrated that E. coli was able to persist in reactor flasks with groundwater flow-through beyond the time at which cells would have fallen...
E. coli viable attached (a) or planktonic (b) GFP-labelled floating bars represent the times between which the number of limit by day 40. Detection limits were 60 c.f.u. coverslip squared (a) indicates that the number had not fallen below the detection time at which cell numbers would have fallen below detection limits as a result of washout and control flasks with groundwater (diagonally striped bars) or groundwater (black bars) or effluent (white bars) flow-through. Analysis of the distribution of E. coli populations from flasks with effluent showed a close ‘intermingling’ of and control flasks without coverslip.

Comparison of survival potential of GFP-labelled E. coli and P. aeruginosa cells in groundwater and wastewater. The floating bars represent the times between which the number of viable attached (a) or planktonic (b) GFP-labelled E. coli or P. aeruginosa fell below the detection limits in test flasks with groundwater (black bars) or effluent (white bars) flow-through and control flasks with groundwater (diagonally striped bars) or effluent (horizontally striped bars) flow-through. The theoretical time at which cell numbers would have fallen below detection limits as a result of washout is also shown (i). The arrow in (a) indicates that the number had not fallen below the detection limit by day 40. Detection limits were 60 c.f.u. coverslip$^{-1}$ for biofilm samples and 5 c.f.u. ml$^{-1}$ for water samples.

Below detection limits as a result of washout only. Although E. coli did not accumulate in the biofilms over the entire length of the experiment, the dynamics of planktonic cell removal suggested that at least some of the cells detaching from the biofilms were viable and therefore capable of ‘re-infecting’ the water phase. Control flasks without coverslips were used in this study to measure the removal rates of GFP-labelled cells from flasks with limited biofilm development by the indigenous micro-organisms. The SA available for attachment in the control flasks was just under half that of the test flasks and it was confirmed that cells did not preferentially associate with the wall of the reactor flasks over the coverslips. Removal of E. coli from the control flask with treated effluent flow-through was slower than the groundwater control flask (although still faster than the theoretical washout rate), suggesting that E. coli die-off was slower in the effluent. However, the presence of biofilms appeared to expedite the removal of the attached and planktonic E. coli populations from flasks with effluent flow-through. In these flasks, the increase in mean biofilm SA coverage over the length of the experiment was approximately 10-fold greater than in the flasks with groundwater flow-through. Analysis of the distribution of E. coli in fully hydrated biofilms revealed a close ‘intermingling’ of E. coli with other bacterial surface colonizers, indicating the potential for interaction (either beneficial or detrimental) between the E. coli and indigenous microbial population. Thus, it is likely that the enhanced biofilm development in flasks exposed to effluent, a result of the higher level of nutrients in this water, increased the competition for nutrients and attachment space and the E. coli were out-competed by the indigenous microbial population. The enhanced biofilm development may also have increased the antagonistic activity of the indigenous micro-organisms.

Established biofilms developed from indigenous river water bacteria have been shown previously to reduce persistence of introduced E. coli and other enteric pathogens (Camper et al., 1985). Furthermore, changing biofilm dynamics and pathogen persistence as a result of an increase in nutrient levels has been reported elsewhere. The survival of a C. jejuni strain in heterogeneous tap-water biofilms was shown to be significantly reduced by the addition of serine, a carbon source known to be favoured by C. jejuni, during which time the number of indigenous biofilm microflora increased (Buswell et al., 1998, 1999). These studies demonstrate that under certain conditions biofilms may represent sites of intensified competition for limiting nutrients.

By comparison, P. aeruginosa persisted in the biofilms in the flasks with effluent flow-through for much longer than E. coli. In both the test flasks and the control flask with effluent flow-through, the rate of P. aeruginosa removal from the water phase slowed dramatically after day 14 to a rate slower than the theoretical washout rate, suggesting that P. aeruginosa was growing in the treated effluent. It is unlikely that there was enough oxygen available in the airtight reactor flasks to sustain growth of P. aeruginosa, which is an obligate aerobe. Although the mean concentration of dissolved oxygen in the treated effluent was higher than the groundwater upon collection (Table 1), the oxygen levels in the water decreased during the course of the experiment due to constant bubbling of water in the holding reservoirs with nitrogen gas. Thus, growth of P. aeruginosa most likely occurred through utilization of nitrate as the terminal electron acceptor, which was present at much higher concentrations in the effluent than the groundwater (Table 1). P. aeruginosa also has the ability to utilize a wider range of organic molecules as carbon and energy sources compared to Enterobacteriaceae (Bergey et al., 1984). As there was no difference in P. aeruginosa removal rates between the control and test flasks, the increased persistence of P. aeruginosa in effluent could not be attributed to an interaction with the biofilms.

The laboratory reactor used in this study was not a perfect simulation of aquifer conditions during ASR. Thus, survival times of the micro-organisms reported here may vary considerably from survival times in the field. Nonetheless, this study has shown that addition of a high-nutrient water to a low-nutrient environment may stimulate biofilm development but have a detrimental effect on survival of bacteria such as E. coli. Thus, the biofilm development which
has been found to occur during ASR using reclaimed sewage effluent (Rinck-Pfeiffer, 2000) may not pose a health risk with respect to the persistence of enteric bacterial pathogens in the aquifer during storage of the water. On the other hand, growth of the opportunistic pathogen *P. aeruginosa*, which is able to compete more effectively with the indigenous microbial population for available nutrients, may occur during ASR.

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