Biofouling
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713454511

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First Published on: 27 January 2009

To cite this Article Bauman, W. J., Nocker, A., Jones, W. L. and Camper, A. K.(2009)'Retention of a model pathogen in a porous media biofilm',Biofouling,25:3,229 — 240
To link to this Article: DOI: 10.1080/08927010802688566
URL: http://dx.doi.org/10.1080/08927010802688566

Biofouling
Vol. 25, No. 3, April 2009, 229–240

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Retention of a model pathogen in a porous media biofilm

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(*Received 1 August 2008; final version received 11 December 2008*)

The inadvertent or the deliberate introduction of pathogens into drinking water can lead to public health consequences. Distribution system sampling strategies are needed to provide information on the identity, source and fate of the introduced pathogens. Porous media biofilm reactors conditioned with undefined drinking water biofilms were tested for their ability to immobilize *Escherichia coli* 0157:H7. Biofilms were established by applying continuous flow of biologically activated carbon treated water with natural microflora and supplemented nutrient solution (0.5 mg l^{-1} C) for 2 or 3 weeks. Control reactors were clean and were not colonized with biofilm. All reactors were injected with slug doses of $\sim 1 \times 10^9$ cfu *E. coli* O157:H7. On the basis of the plate count enumeration of the introduced pathogen, reactors pre-colonized for 2 or 3 weeks retained significantly more cells (0.75 and 9.37% of the introduced spike dose, respectively) compared with uncolonized control reactors (0.22%). Compared with cultivation, microscopic direct counts and quantitative PCR suggested significantly higher and lower numbers of pathogens, respectively. Plate counts were thus considered as the method of choice for pathogen enumeration in this study. In addition to providing general insights into interactions between pathogens and drinking water biofilms, the study concluded that engineered biofilm systems may be considered as a device to capture pathogens from the bulk flow for monitoring purposes.

Keywords: biofilm; drinking water distribution system; pathogen capture; contaminant monitoring

Introduction

Drinking water distribution systems (DWDS) deliver finished water from treatment facilities to consumers. These dispersed systems make up a majority of the physical infrastructure of water utilities. The multitude of accesses, however, makes these systems vulnerable to contamination by pathogenic microbes (GAO 2004). Risks are linked to unintentional introduction of microbes by natural contamination and to intentional contamination, as in a bioterrorist attack. Although deliberate contamination of drinking water systems is a novel risk, unintentional microbial contamination has been a persistent and significant challenge for drinking water utility managers. Resulting outbreaks have been linked to water treatment failures and problems with distribution system integrity.

To protect public health, functional early-warning systems for water distribution system monitoring would be highly desirable. In the case of pathogens, such systems would ideally allow rapid location of the source, determination of the pathogenic species and their associated virulence, and their distribution within the system. The problem in DWDS lies primarily in concentrating and sampling highly diluted pathogens. The subsequent methods for microbial detection are evolving quickly.

One approach to trap pathogens in DWDS might be the use of biofilms. Biofilms in DWDS are heterogeneous, multi-species microbial communities that colonize the pipe surfaces (LeChevallier et al. 1987). They are typically characterized by extracellular polymers, entrained inorganic and organic materials and byproducts of microbially influenced corrosion, as well as a diversity of microorganisms (Beech 2004). The microbes associated with biofilms are ubiquitous in the water environment and normally pose little threat to public health. However, established biofilms have been shown to interact with externally introduced microbes including pathogens (Donlan 2002). Coliforms (Camper et al. 1991; McMath et al. 1999), *Legionella* (Rogers et al. 1994; Längmark et al. 2005; Liu et al. 2006), *Cryptospridium* oocysts (Searcy et al. 2006), *Helicobacter pylori* (Mackay et al. 1998), *Escherichia coli* (Camper et al. 1996; Banning et al. 2003; Li et al. 2006), *Pseudomonas aeruginosa* (Banning et al. 2003) and *Mycobacterium* (September et al. 2004) have all been found in distribution system biofilms.

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ISSN 0892-7014 print/ISSN 1029-2454 online

DOI: 10.1080/08927010802688566
http://www.informaworld.com

Trapping organisms can be easily imagined, considering the complex biofilm structure. Biofilms have been described as a 3-dimensional assembly of 'mushroom' shaped towers and strings (Costerton 1995), and as a porous framework of intertwined filamentous biomass (Okabe et al. 1998). The immobilization of microbes within this matrix is influenced by both physical and chemical properties. The physical structure of biofilms can contribute to the immobilization by reducing the shear force on cells and by increasing the number of adsorption sites. Additionally, extracellular polymers associated with the biofilm phenotype, often polysaccharides, can contribute to the irreversible adhesion of bacteria through hydrogen bonding and dipole–dipole interactions (Fletcher and Floodgate 1973; Costerton 1984). Once trapped in biofilms, pathogens can be protected from disinfectants and antimicrobials through mass transfer resistance (Stewart et al. 1996) or the adoption of a persister state (Roberts and Stewart 2005). Consequently, interactions between biofilms and pathogens have traditionally been viewed as contributing to public health risks. Trapped pathogens have the potential to become concentrated in biofilms or even to replicate. Subsequent release of pathogens could potentially result in severe consequences for microbial water quality. Whereas the harbouring of pathogens from the bulk flow is undesired in case of a system contamination, the same properties might make biofilms a useful tool for concentrating pathogens and for their subsequent monitoring. A 'biofilm trap' could possibly provide rapid sampling opportunities at critical points within a distribution network.

This study addressed the question whether an engineered system with pre-established biofilm provides sufficient retention to qualify as a pathogen detection device. A first step was made to characterize the capacity of a porous media biofilm, grown under drinking water conditions, to immobilize a model pathogen from bulk flow.

Materials and methods

A porous media biofilm reactor was constructed by filling a clear, cylindrical polycarbonate container with glass beads (Figure 1A). The reactor dimensions were 6 cm in diameter and 6 cm in height. A peristaltic pump was used to meter the continuous flow through the reactor. Flow entered through the base, directed tangential to a conical recess in the reactor bottom (Figure 1B). A stir bar was spun at 200 rpm to ensure the inlet region of the system and was well-mixed. A fine stainless steel screen separated the stir bar from the reactor media. Reactors were packed with 220 g of 3-mm borosilicate beads (Chemglass Vineland, NJ, USA, cat. No. CG-1101-02) for each experiment. Beads were autoclaved, rinsed with nanopure water (Barnstead NANOpure^{TM} Water Purification Systems, Boston, MA) and then baked at 550°C for 5 h before packing and subsequent sterilization by autoclaving within reactors.

Flow conditions

Reactors for biofilm experiments were colonized under the following flow conditions: Reactors were fed at 24.75 ml min^{-1} from a series of two activated carbon columns fed with Bozeman tap water. The first removed chlorine and the second was biologically active to remove the bioavailable organic carbon and produce an indigenous mixed population inoculum to the porous medium reactors. The concentration of heterotrophic bacteria in this biologically activated carbon (BAC) effluent was between 3.0×10^3 and 1.9×10^4 colony forming units (cfu) ml^{-1} as determined by plate counting on R2A medium. The BAC water was supplemented with 50 mg l^{-1} organic carbon at 0.25 ml min^{-1} for a total flow of 25 ml min^{-1} and a total organic carbon concentration of 0.5 mg l^{-1} (Figure 2). The organic carbon supplement stock

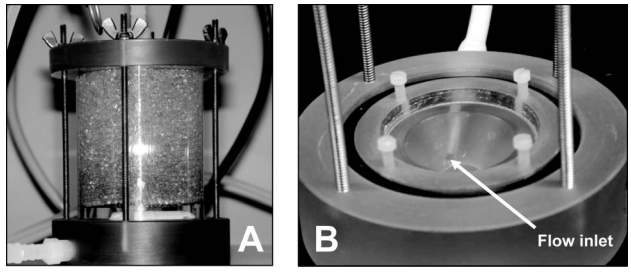

Figure 1. Packed porous media biofilm reactor (A), and close-up of the reactor base (B).

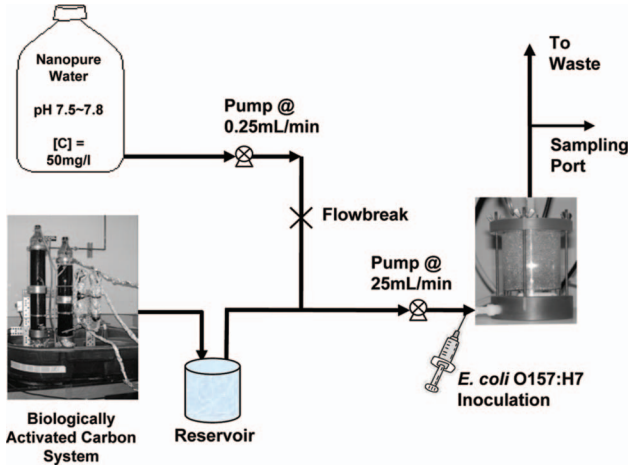

Figure 2. Flow schematic for biofilm experiments.

solution was a combination of amino acids and carbohydrates with the following concentrations: 4.7 g l^{-1} glutamic acid, 5.3 g l^{-1} aspartic acid, 5.5 g l^{-1} serine, 4.7 g l^{-1} alanine, 4.8 g l^{-1} glucose, 4.8 g l^{-1} galactose and 4.8 g l^{-1} arabinose (Sigma, St. Louis, MO, USA) (Ellis et al. 2000). This recipe was used to make a 13,000 mg l^{-1} carbon concentrated stock solution, which was diluted in sterile nanopure water. Before diluting, the exact concentration of the stock solution was measured using a Dohrmann DC80 organic carbon analyzer and the pH was adjusted to 7.5–7.8 with 6 N sodium hydroxide. Nitrogen (2.48 g l^{-1}), provided by the nutrient supplement, and phosphorus (in the range between 0.10 and 0.12 mg l^{-1}) from the BAC water, were sufficiently available to ensure that carbon was the limiting nutrient. Total dissolved phosphorus levels were measured by inductively coupled plasma optical emission spectrophotometry (ICP-OES, ARL 3500). Control reactors (no bacterial colonization) were fed at 25 ml min^{-1} with nutrient-enriched BAC water (as described above) that was filter-sterilized. Continuous flow for control reactors was initiated $\sim$2 h before inoculation to allow thorough wetting of the reactor media and internal surfaces.

Bacteria

E. coli O157:H7 (strain 932), a human isolate (obtained from the Department of Microbiology, Montana State University), was used as a model pathogen. To make plate counting possible in the presence of a biofilm background and to allow for microscopic direct counts, the strain was transformed with genes encoding for antibiotic resistance and a fluorescent protein. These genes were chromosomally integrated with a delivery plasmid, miniTn7(Km,Sm)$_{PA1/04/03}$-ecfp-a, and a helper plasmid, pUX-BF13 isolated from an *E. coli* carrier strain using a QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA, USA). The delivery plasmid contained genes responsible for antibiotic resistance to kanamycin, streptomycin and chloramphenicol, as well as the gene encoding for the cyan fluorescent protein (CFP). The helper plasmid ensured that the genes inserted into the desired location and orientation within the genome. Electroporation was done in an Electroporator 2510 (Eppendorf, Westbury, NY, USA) for 5 ms at 2400 V, as recommended by the manufacturer. Electrocompetent cells were prepared based on a protocol from the University of Washington (http://depts.washington.edu/bakerpg/electrocompetent.html).

Inoculum preparation

Full strength Luria Bertani (LB) broth (EMD, Gibbstown, NJ, USA) supplemented with 6 mg l^{-1} chloramphenicol (Cm) (EMD) was used to grow 10 ml cultures of transformed *E. coli* O157:H7 in 50 ml culture tubes. After 16 h, at 37°C and 180 rpm, stationary phase cells were harvested by centrifugation for 5 min at 5000*g*. Cell pellets were re-suspended into 10 ml filter-sterilized BAC effluent. This wash step was repeated once. The final cell suspension was shaken at room temperature at 150 rpm for 24 h before being introduced into reactors. The purpose of this procedure was to allow the possible changes in physiology or size that may be caused by low nutrient concentrations (Bjergbaek and Roslev 2005). Cell concentrations ranged from 4.87×10^8 to 1.82×10^9 cfu ml^{-1} according to the triplicate plate counts done on LB agar. This relatively high number was chosen so that any potential error introduced by having organism numbers at the level of detection of the three enumeration methods could be minimized.

Inoculation and sampling procedure

For biofilm experiments, reactors were colonized for either 2 or 3 weeks with organisms originating from BAC water. During that time, continuous flow at 25 ml min^{-1} was applied. BAC water was supplemented with nanopure water with 0.5 mg l^{-1} organic carbon (see above and Figure 2). Seven experiments were performed with uncolonized control reactors, five experiments with reactors containing 2-week old biofilms and two experiments with reactors containing 3-week old biofilms. Reactors were inoculated with 1 ml of *E. coli* O157:H7 suspension (prepared as described earlier). Mean spike concentrations (C_0) were 1.27×10^9 cells for control reactors, 1.07×10^9 cells for reactors with 2-week old biofilms and 1.09×10^9 cells for reactors with 3-week old biofilms. A 26-gauge needle was used to pierce the tubing immediately upstream from where flow entered the reactor to introduce *E. coli* O157:H7 cells to the bulk flow. The procedure for adding and sampling of the *E. coli* O157:H7 was performed in the same manner for non-biofilm control experiments and biofilm experiments. Simultaneous to inoculation, the reactor effluent was diverted to sterile flasks and sampled. Effluent samples were collected on 82 s intervals for 820 s, yielding 10 samples of $\sim$34 ml. Hydraulic residence times, and therefore, the number of pore volumes collected over the 820 s varied for each experiment as free pore spaces were reduced by biofilm accumulation. For control reactors without preconditioned biofilm with a pore volume of 89.4 ml, the 820 s sampling period represented 3.9 hydraulic residence times. For the reactors with 2- and 3-week biofilms, the 820 s sampling period corresponded to 5.9 and 12.9 hydraulic residence times.

Once all effluent samples were collected, flow to the reactor was stopped, samples were placed on ice, and the reactor was relocated from its stir plate to a laminar flow hood. In the laminar flow hood, the pore fluid remaining in the reactor was drained into a sterile graduated cylinder to measure its volume. Biofilm volumes were calculated as the difference of the freely-flowing pore fluid drained from the reactor and the pore fluid from a clean reactor without biofilm. The difference in the volume was assumed to be attributable to the biofilm accumulation. For each experiment, the pore fluid was homogenized for 30 s (Bio homogenizer M133/1281, Biospec Products, Bartlesville, OK, USA) on ice (to prevent heating the sample). Approximately 35 ml of this pore fluid sample were transferred to a sterile flask and placed in an ice bath for further analysis.

After each experiment, the reactor was disassembled and the glass beads were transferred into a sterile wide-mouth 250-ml flask using a sterile spatula and funnel. Fifty milliliters of filter-sterilized BAC effluent was added to the flask to assist in washing any *E. coli* O157:H7 or biofilm from the beads. This solution was vortexed (30 s) and sonicated (30 s, model fs15, Fisher Scientific). The vortex and sonication steps were repeated once before transferring the volume through a fine-mesh steel screen into a second sterile flask. For a final rinse, an additional 50 ml were added to the flask still containing the glass beads followed by vortexing (2 min). The second 50 ml were added to the first by transferring through the same screen to obtain a combined volume of 100 ml rinse water plus any recovered biofilm. For biofilm-conditioned experiments, the 100 ml sample was homogenized for 30 s, before transferring $\sim$35 into a 50-ml tube placed in an ice bath.

To recover the biological material associated with the internal reactor surfaces, filter-sterilized BAC effluent was used to rinse the surfaces before and after scraping with a rubber policeman (GSC International No. 1835, Fort Collins, CO, USA). Surfaces were initially rewetted with 5–10 ml and then scraped, followed by a second rinse. The following volumes were used to rinse the respective surfaces: 15 ml top, 30 ml sides, 20 ml screen, 10 ml stir bar and 25 ml bottom before combining into one 100 ml sample. Harvested material was homogenized for 30 s before transferring $\sim$35 ml into a 50-ml tube and storing in an ice bath until further processing.

Regarding the samples, the term 'pore fluid' refers to cells that were recovered from the freely-flowing pore fluid drained from the reactor before destructive sampling. 'Bead surface' refers to cells either attached to glass beads for control experiments, or recovered from biofilm that was harvested from glass beads for biofilm experiments. 'Reactor surface' refers to cells either attached to reactor surfaces or recovered from biofilm that was harvested from reactor surfaces.

Non-reactive tracer test

The hydrodynamics of a clean and sterile reactor were described using fluorescein dye as a tracer for breakthrough curves (Sharp et al. 1999). Under the same flow conditions as described above for uncolonized control reactors, 1 ml of 100 ppm fluorescein dye (Sigma F-6377, St Louis, MO, USA) was injected into a porous media reactor. The optical density at 490 nm was 7.4 as measured on a spectrophotometer (Thermo Scientific Spectronic Genesys 5, Hampton, NH, USA). Effluent was collected at 82 s intervals for a total time period of 820 s, and each sample was analyzed spectrophotometrically.

Plate count analysis

E. coli O157:H7 were enumerated by spread plating in triplicate. Samples were serially diluted in filter-sterilized BAC effluent such that 100 μl spread on an agar plate produced 30–300 colonies. All samples were plated on LB agar (Fisher Scientific, cat. No. BP1425-2) supplemented with 6 mg l^{-1} chloramphenicol and incubated at room temperature for 3 days. Room temperature was used to prevent temperature shock; the starvation period and reactor operation were also at room temperature. The percentage recovery of the inoculum was calculated for each experiment from the ratios between the total number of cells recovered (from the effluent samples and the various surfaces) and the total number of cells in the inoculum.

Direct count analysis

Selected samples were serially diluted in filter-sterilized BAC effluent. After vortexing for 30 s, 1 ml was filtered through a 0.44-μm black polycarbonate filter membrane (GE Water and Process Technologies, Watertown, MA, USA). Appropriate dilution factors were chosen to obtain between 20 and 200 cells per field of view for enumeration. Filters were mounted with oil on microscope slides. Twenty fields of view were captured using a black and white camera mounted to a Nikon E800 microscope with a 100 $\times$ 1.4 NA oil objective and CFP fluorescence filters (excitation 426–446 nm, dichromic mirror 455 nm and emission 460–500 nm). MetaVue, Version 6.1, (Universal Imaging, Downington, PA, USA) was used for capturing images and MetaMorph, Version 6.1r, was used to count the CFP fluorescent *E. coli* O157:H7 cells.

Genomic DNA extraction and quantitative PCR

Genomic DNA was extracted using the PowerSoil™ DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA). The manufacturer's 'Experienced User Protocol' was followed with the following exceptions: (1) 200 μl of homogenously suspended cells were added to the lysis matrix as opposed to soil, (2) cell lysis was achieved by bead beating in a FastPrep machine (MP Biomedicals, Aurora, OH, USA) for 30 s at a speed setting of 5.0 m s^{-1} and (3) only 1350 μl were loaded on to the spin filter. *E. coli* O157:H7 DNA was detected by quantitative PCR (qPCR) using a Smart-Cycler II (Cepheid, Sunnyvale, CA, USA). Multiple protocols and reagents were used in an effort to improve repeatability and optimize efficiency. In all PCR assays, 2 μl of extracted genomic DNA were added to 23 μl of PCR mixture containing 0.3 μM of primer *stx*1 – forward (5′-GACTGCAAAGACGT ATGTAGATTCG-3′) (Sharma and Dean-Nystrom 2003) and 0.3 μM of primer *stx*1 – reverse (5′-ATCTATCCCTCTGACAACTGC-3′) (Sharma and Dean-Nystrom 2003). The *stx*1 gene encodes for shiga toxin 1 and has been shown to successfully distinguish *E. coli* O157:H7 within complex biological matrices (Sharma and Dean-Nystrom 2003). In attempts to optimize the qPCR method, the assays were either based on SYBR® Green or on Taqman™ chemistry. Two commercially available PCR master mixes were used for SYBR Green based assays – SYBR Green PCR Master Mix and Power SYBR Green PCR Master Mix (both Applied Biosystems, Foster City, CA, USA). Both were used according to the manufacturer's protocol. In the case of Taqman assays, an *stx*1-probe was used in conjunction with 1 × AmpliTaq GOLD Master Mix (Applied Biosystems, Foster City, CA, USA) and had a 6-FAM fluorescent label attached to the 5′–end of the *stx*1-probe and a BHQ1 quencher attached to the 3′–end. The PCR reaction chemistries used in different experiments are summarized in Table 1.

Cycling parameters for Taqman assays were: 570 s at 95°C followed by 45 cycles of 20 s at 95°C, 30 s at 57°C and 25 s at 72°C. For SYBR Green based assays, the cycle parameters were: 8 min at 95°C followed by 50 cycles of 20 s at 95°C, 25 s at 55°C and 25 s at 72°C. For assays using SYBR Green, melt curve analyses were performed following PCR cycling by heating PCR products from 65 to 95°C in increments of 0.2 deg s^{-1}. Threshold cycles were only considered valid for those reactions showing the correct melt temperatures. Initially, correct product lengths were verified by loading an aliquot of qPCR product on 2% agarose gels stained with ethidium bromide. Threshold cycle values (C_t) were determined for all samples by the primary curve method with background subtraction, using the SmartCycler II software. The C_t values represented the PCR cycle at which fluorescence, as a surrogate measure of target DNA concentration, rose above a threshold of 30 fluorescent units. DNA templates for creating standard curves were obtained in one of the two ways: (1) DNA was extracted from a series of cell suspensions generated from serially diluting the inoculum, or (2) genomic DNA extracted from the undiluted inoculum was serially diluted. Standard curves were produced for each experiment and the concentration of *E. coli* O157:H7 cells in unknown samples were calculated by fitting their C_t values to this standard curve.

Mass balance on E. coli *O157:H7*

As will be shown in the results, the plate count data proved to be the most reliable. Therefore, a mass balance on *E. coli* O157:H7 was performed on the basis of the plate count data to compare the total number of cells added to the reactor with the number recovered. Recovered cells included all cells in the effluent and pore fluid, in addition to those that were immobilized within the reactor. Cell concentrations from each sample were multiplied by the total final volume in which they were suspended. The mean and the standard error (SE) of the log differences between input and recovered cells were calculated. A 95% confidence interval was then determined based on the mean and the SE and the 13 degrees of freedom ($n = 14$).

Statistics

To determine if differences in the fraction of inocula immobilized between the three reactor conditions was

Table 1. PCR reaction conditions.

Reactor condition	qPCR Reaction chemistry	Reactor condition	qPCR Reaction chemistry
Control no. 1	SYBR® Green	2-week biofilm no. 3	Power SYBR® Green
Control no. 3	Taqman™	2-week biofilm no. 4	Power SYBR® Green
Control no. 5	Taqman™	2-week biofilm no. 5	Power SYBR® Green
Control no. 7	Power SYBR® Green	2-week biofilm no. 1	SYBR® Green
2-week biofilm no. 1	SYBR® Green	2-week biofilm no. 2	SYBR® Green

statistically significant, a two sample, one-sided *t*-test was used. The null hypothesis for this test was that the geometric means of the percentage of cells immobilized was equal for all conditions. The mean and SE of log differences between *E. coli* O157:H7 cells input and *E. coli* O157:H7 cells immobilized were calculated. Degrees of freedom were computed using the Welch-Satterwaithe approximation (Ames and Webster 1991). The null hypothesis was considered invalid when *p*-values were < 0.05.

Results

A porous media reactor was colonized with an undefined drinking water biofilm. After 2 weeks of continuous flow, there was a visually obvious gradient in biofilm accumulation, which decreased along the direction of flow with no biofilm visible near the effluent port. One additional week of continuous flow was sufficient to close this gradient such that biofilm appeared along the entire flow path. The average biofilm volumes (pore space of reactor with biofilm minus pore space of clean reactor without biofilms) were 20.8 $\pm$ 1.92 ml (23% of available pore space) for 2-week-old biofilms and 52.5 $\pm$ 3.54 ml (59% of available pore space) for 3-week-old biofilms. The pore volume of the corresponding clean reactor without biofilm was 89.4 ml.

Distribution of E. coli *O157:H7 after passage through reactors*

The percentage recoveries of cells from the reactors (numbers recovered *vs*. numbers added) ranged from 69 to 150% (Table 2). For each condition (control, 2-week and 3-week biofilm experiments), the 95% confidence interval had a narrow range. From these data, plate count data were shown to be acceptable for closing the mass balances of the added pathogen. As will be discussed later, direct counts and qPCR data were not as reliable and were not deemed acceptable for interpreting pathogen capture.

Figure 3A shows the total number of *E. coli* O157:H7 cells remaining in different reactor compartments as determined by plate counting after continuous flow for 820 s. *E. coli* O157:H7 numbers in pore fluid samples were nearly identical for the three reactor conditions, whereas pathogen numbers recovered from bead surfaces and reactor surfaces showed biofilm-specific differences. For reactors with 2-week biofilms, the numbers of *E. coli* O157:H7 cells on the bead surfaces and on the reactor surfaces were $\sim$0.5 and 1.5 orders of magnitude higher, respectively, than on control bead surfaces and control reactor surfaces. For reactors with 3-week biofilms, the increase was around 2 orders of magnitude for both bead and reactor surface samples compared with the identical compartments of the control reactor.

Ten effluent samples were taken in 82 s intervals for each reactor type. The average total number of *E. coli* O157:H7 cells in each effluent sample as enumerated by plate counts was plotted *versus* the sample number (Figure 3B). The three reactor types showed a similar trend for pathogen-washout with cell numbers peaking early after inoculation and decreasing gradually over the 820 s sampling period. It appeared that the pathogen cells left the reactor earlier when biofilm was present, resulting in lower *E. coli* O157:H7 numbers in later effluent samples.

Breakthrough curves

The rates at which *E. coli* O157:H7 cells passed through reactors were described using breakthrough curves. A breakthrough curve is defined here as the average concentrations (C) of *E. coli* O157:H7 observed in effluent samples for each reactor condition,

Table 2. Percentage recoveries for *Escherichia coli* O157:H7 from control reactors (without biofilm) and from reactors with 2-week and 3-week biofilms.

Reactor condition	Recovered (cfu $\times$ 10^9)	Inoculum (cfu $\times$ 10^9)	Percentage recovered (%)	Reactor condition	Recovered (cfu $\times$ 10^9)	Inoculum (cfu $\times$ 10^9)	Percentage recovered (%)
Control	1.56	1.22	127	2-week biofilm	0.76	1.11	68
Control	1.01	0.73	138	2-week biofilm	0.95	1.11	85
Control	1.54	1.82	85	2-week biofilm	1.15	1.05	109
Control	0.53	0.46	116	2-week biofilm	1.30	1.00	130
Control	2.07	1.38	150	2-week biofilm	1.74	1.78	98
Control	1.41	1.70	83	Average	1.18	1.21	98
Control	1.09	1.06	103	SD	0.33	0.29	21
Average	1.31	1.20	115	3-week biofilm	1.40	1.40	100
SD	0.49	0.49	26	3-week biofilm	0.86	0.77	111
				Average	1.13	1.08	105

Percentage recoveries were calculated for each experiment by taking ratios between the total number of cells recovered and the total number of cells in the inoculum. Average values and SDs (SD, where appropriate) are shown in shaded boxes.

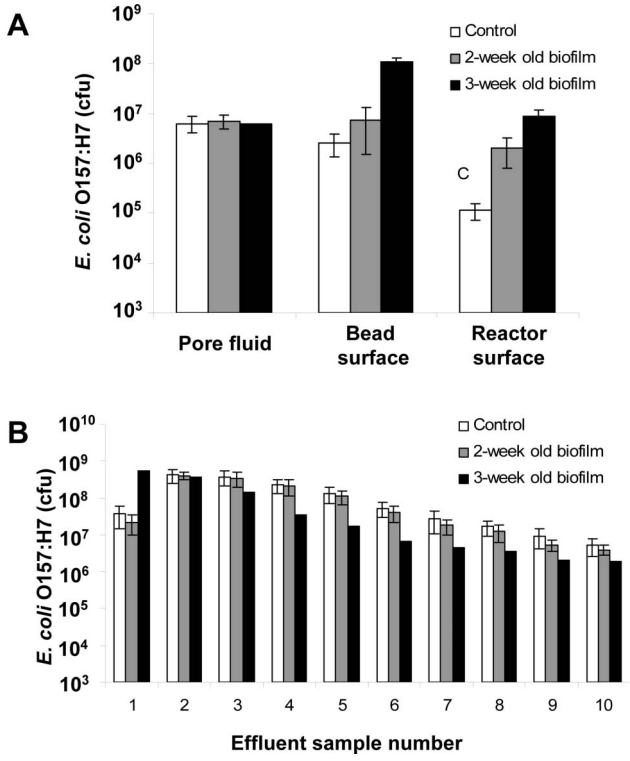

Figure 3. Total number of *E. coli* O157:H7 cells remaining in reactor compartments after 820 s of continuous flow following the spike event (A) and in 10 effluent samples (collected at 82 s intervals) from different reactor types (B). Cells were enumerated by plate counting on R2A medium. The term 'pore fluid' refers to cells that were recovered from the freely-flowing pore fluid drained from the reactor before destructive sampling. 'Bead surface' refers to cells either attached to glass beads for control experiments, or recovered from biofilm that was harvested from glass beads for biofilm experiments. 'Reactor surface' refers to cells either attached to reactor surfaces or recovered from biofilm that was harvested from reactor surfaces. Bars represent the mean of all experiments of each type with error bars displaying inter-experiment SDs. Number of experiments: $n = 7$ for control reactors, $n = 5$ for reactors with 2-week biofilms and $n = 2$ for reactors with 3-week biofilms.

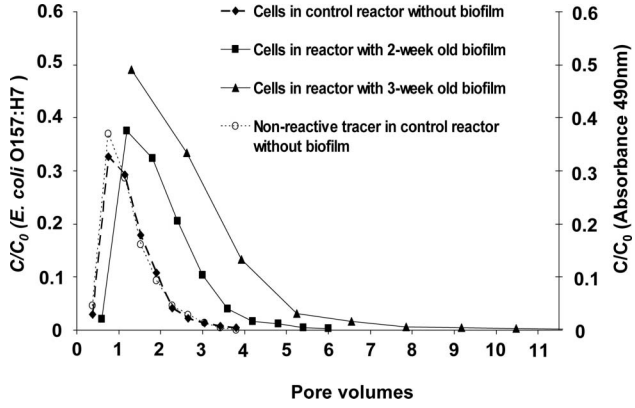

Figure 4. Breakthrough curves for *E. coli* O157:H7 cells and a non-reactive tracer. Pathogens were passed through an uncolonized control reactor and through reactors with 2- and 3-week biofilms. The non-reactive tracer was passed through an uncolonized reactor. Relative cell concentrations are based on plate counting (left axis), the relative concentrations of the non-reactive tracer are based on absorbance measurements (right axis). The C_0 values refer to the initial concentrations of the cells and the tracer in the inocula used for spiking.

normalized to average inocula concentrations (C_o), plotted *versus* the pore volume in which they were recovered (Figure 4). Because the free pore space within the reactors decreased as biofilm accumulated, each 34 ml effluent sample ($V_S = 25\ \text{ml min}^{-1} \times 82\ \text{s}$) contained a different fraction of one pore volume for each reactor condition. The average fraction of a pore volume (PV_{Frac}) was calculated for each biofilm condition using the following equation, where the sample volume (V_s) was constant and the pore volume (V_p) was measured during each experiment and averaged for each reactor condition:

$$\frac{V_S}{\bar{V}_P} = PV_{Frac}$$

On the basis of this formula, each effluent sample contained 0.39 pore volumes for control reactors, 0.59 pore volumes for reactors with 2-week biofilms and 1.29 pore volumes for reactors with 3-week biofilms.

The breakthrough curve for *E. coli* O157:H7 cells travelling through the control reactors was nearly identical to the breakthrough curve observed for a non-reactive tracer. However, reactors colonized with biofilm exhibited greater dispersion as well as a prolonged wash-out of *E. coli* O157:H7 relative to the control reactors. The degree to which cells were slowed with respect to the bulk fluid can be quantified using retardation factors (R_f).

Retardation factors

A retardation factor (R_f) is defined here as the ratio of the mean cell residence time to the hydraulic residence time (Johnson et al. 1998) and quantifies the attenuation of cells as they travel through porous media (Table 3). Average hydraulic residence times (θ_H) were calculated for each reactor condition using the following equation (with Q being the flow rate):

$$\frac{\bar{V}_P}{Q} = \theta_H$$

Average mean cell residence times (θ_C) (Table 3) were calculated for each reactor condition using the following equation:

$$\frac{\sum_{i=1}^{N}\left(\frac{\sum([E.coli]\times\text{time})}{\sum E.coli}\right)}{N} = \theta_C$$

Table 3. Average hydraulic residence times, mean cell residence times and retardation factors for the reactor with non-reactive tracer, the control reactor (without biofilm) and reactors with 2-week and 3-week biofilms.

	Average hydraulic residence times θ_h (s)	Average mean cell residence times θ_c (s)	Average retardation factors $R_f = \theta_c/\theta_h$
Non-reactive tracer	212	223.1	1.05
Control	212	234.9	1.11
2-week-old biofilm	139.7	226.9	1.62
3-week-old biofilm	63.6	110.7	1.74

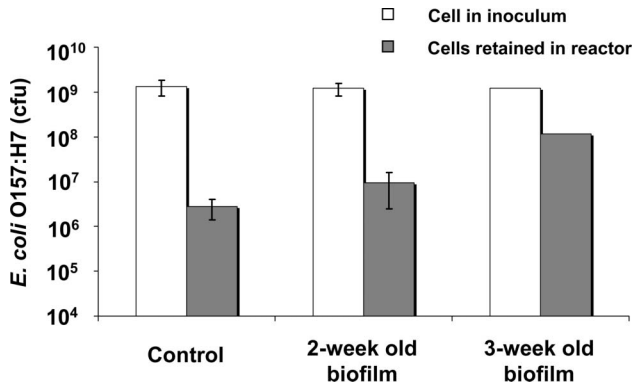

Figure 5. Average total number of *E. coli* O157:H7 cells in inocula and the average immobilized fraction retained in the reactor. Error bars = inter-experiment SDs. The values for the reactors with 3-week biofilms represent the mean from two duplicates.

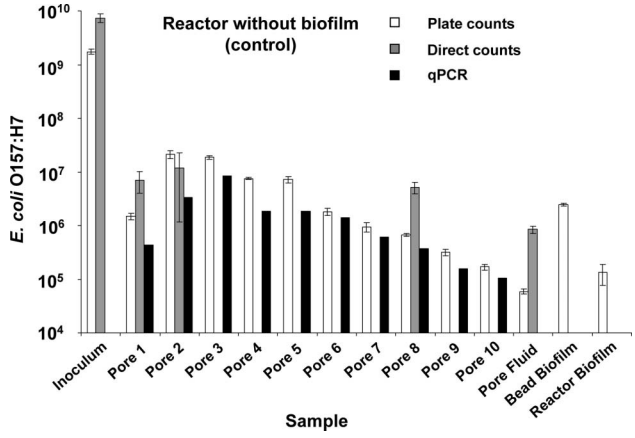

Figure 6. Comparison of *E. coli* O157:H7 concentrations determined by plate counts, direct counts and qPCR for control experiment No. 5. Error bars for plate counts and direct counts represent intra-experiment SDs. No error bars are given for the qPCR data as the measurement was performed only in duplicate.

Residence times and retardation factors were very similar for control reactors with *E. coli* O157:H7 and non-reactive tracers. The presence of biofilms resulted in a reduction of residence times and an increase in retardation factors for pathogen experiments.

Retention efficiency

The total number of cells remaining in the reactors after continuous flow for 820 s was considered to be the sum of cells from bead surfaces and reactor surfaces. The average numbers of retained cells from different experiments were calculated for each reactor condition and compared with the average number of cells in the corresponding inocula (Figure 5). On average, control reactors (without biofilm) retained 0.22% of cells from the inoculum, reactors with 2-week biofilms retained 0.75% and reactors with 3-week biofilms retained 9.37%. The differences in retention between the three reactor conditions are statistically significant ($p = 0.02$ between control reactors and reactors with 2-week biofilms, $p < 0.001$ between control reactors and reactors with 3-week biofilms and $p < 0.001$ between reactors with 2- and 3-week biofilms).

Direct counts and qPCR

Direct counts of *E. coli* O157:H7, based on fluorescence microscopy, and qPCR, based on detection of *stx1* genes, were compared with the corresponding plate counts (considered the standard). Typical examples of pathogen concentrations in reactor effluent samples are presented for all three reactor conditions in Figures 6 (control reactor), 7 (reactor with 2-week biofilm) and 8 (reactor with 3-week biofilm) (Figures 6–8). Whereas direct counts typically suggested higher pathogen numbers than plate counts, qPCR typically suggested lower numbers. These trends were independent of reactor conditions. The average log difference between direct counts and cultivation was 0.6. Direct microscopy was complicated by the presence of the other biofilm organisms, which made counting the target cells difficult. The *E. coli* was masked by the biofilm matrix, focusing was problematic, and autofluorescence occurred. In the case of qPCR, the discrepancy with plate count data was less predictable and even greater, especially for bead surface and reactor surface samples (data not shown). Because of the problems associated with the qPCR and the inability to optimize the method, it was not considered to be the optimal detection method.

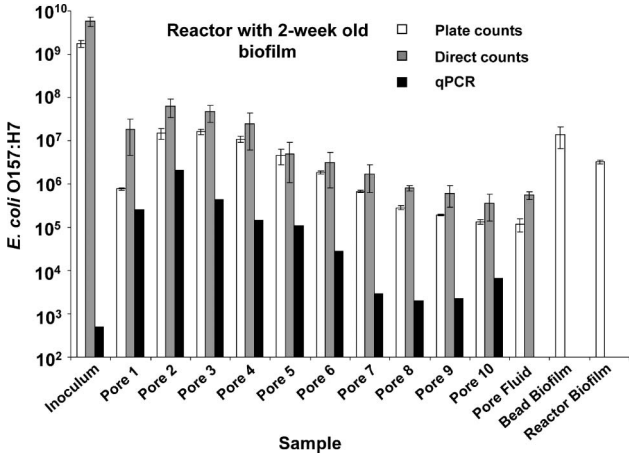

Figure 7. Comparison of *E. coli* O157:H7 concentrations determined by plate counts, direct counts and qPCR for a reactor with a 2-week biofilm (experiment No. 5). Error bars represent intra-experiment SDs. No error bars are given for the qPCR data as the measurement was performed only in duplicate.

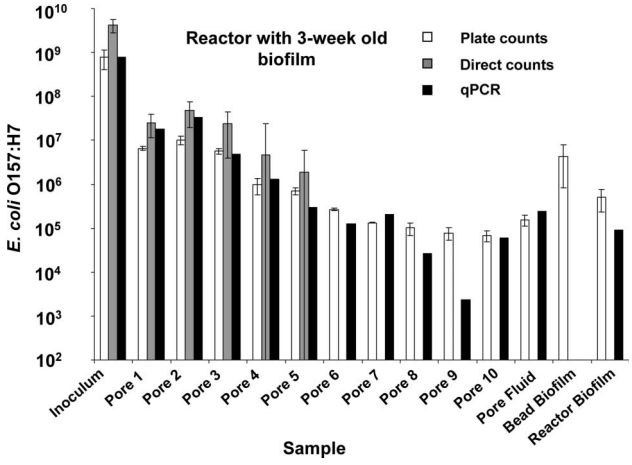

Figure 8. Comparison of *E. coli* O157:H7 concentrations determined by plate counts, direct counts and qPCR for a reactor with a 3-week biofilm (experiment No. 2). Error bars represent standard intra-experiment SDs. No error bars are given for the qPCR data as the measurement was performed only in duplicate.

Discussion

The results presented in this study suggest that porous media colonized with drinking water biofilms were able to retain significantly more *E. coli* O157:H7 cells than clean porous medium, which was not precolonized with biofilm. The fraction of retained cells increased with increasing biofilm biomass in the reactor. Biofilms also contributed to greater dispersion and retardation of *E. coli* O157:H7, which prolonged the washout of cells.

Changes in the hydrodynamic properties of porous media systems caused by the accumulation of biofilm have been investigated by a number of researchers. These investigations describe dynamic channel formation (Sharp et al. 2005), reduced porosity (Tiwari and Bowers 2001; Dunsmore et al. 2004), reduced permeability (Tiwari and Bowers 2001) and increased friction factors (Cunningham et al. 1991). A similar investigation, which aimed to describe flow in biofilm-colonized porous media, observed increased dispersion and accelerated breakthrough of a non-reactive tracer (Sharp et al. 1999). In contrast to the transparent reactor used in the study reported here, the laboratory-scale reactor system used in the prior cited experiments did not allow direct observations of biofilm thickness. As a result, hydrodynamics were described using a non-reactive tracer. An effort was made here to describe the transport of *E. coli* O157:H7 relative to the tracer and the influence of biofilm on *E. coli* O157:H7 transport.

The transport of *E. coli* O157:H7 through porous media reactors was described using breakthrough curves with plate count data to normalize the differences in hydraulic residence times between reactor conditions (Figure 4). Results suggested that the fluorescein tracer used is a good surrogate for bacteria in the absence of biofilms. Moreover, the graph demonstrates two possible influences of biofilm on the transport of *E. coli* O157:H7 through the porous media matrix – preferential channelling and prolonged washout. Prolonged washout refers to the number of pore volumes required for the concentration of eluted *E. coli* O157:H7 cells to approach zero and is affected by both dispersion (the flattening of the breakthrough curve) and retardation (the shifting of the centroid of breakthrough curves to the right). Prolonged washout was observed with 2- and 3-week biofilm experiments, with dispersion and retardation increasing with greater biofilm biomass within reactors. Prolonged washout was interpreted as a consequence of interactions with biofilm. Preferential channelling occurs as biofilm accumulates heterogeneously within reactors, blocking or slowing flow through some areas of the reactor whereas increasing flow in other areas (Sharp et al. 1999), accelerating breakthrough. Preferential channelling may explain why the breakthrough of *E. coli* O157:H7 occurred at a higher concentration in 3-week biofilm experiments relative to control and 2-week biofilm experiments.

These results suggest that *E. coli* O157:H7 transport through reactors with 3-week biofilms may be dualistic, with cells either washed out rapidly through preferential channelling or being significantly retarded via interactions with biofilm. This dualistic transport was also observed by Sharp et al. (1999) using porous media reactors and a fluorescein dye. This observation

is important, as cells that are retarded by biofilm remain in the reactors longer, per pore volume and may be more likely to be immobilized.

Enumeration of antibiotic-resistant *E. coli* O157:H7 using plate counting succeeded in closing the mass balance around the reactor. On the basis of the narrow range of the 95% confidence interval around 100% for percentage recoveries and the inherent problems with direct microscopy and qPCR, this method was considered as the standard for analyses. Because experiments typically took about 2 h to perform and samples were stored on ice following collection, the proliferation of *E. coli* O157:H7 cells was not considered a source of error in the mass balance.

By colonizing a porous media system with biofilm over 3 weeks under drinking water conditions, it was possible to retain, over 820 s (five residence times), up to ~9% of *E. coli* O157:H7 introduced as a slug dose. Interactions with biofilm within the reactor are believed to have contributed significantly to this retention. These interactions may be characterized by frequent and occasionally reversible attachment events caused by decreases in average pore size, protection from fluid shear and an increase in available sorption sites (Stevik et al. 2004). Mechanisms of attachment may include adsorption, as well as physical straining or filtration. Factors that may affect the physical straining of pathogens in similar experiments include porous media diameter and pathogen size (Stevik et al. 2004).

Comparing different methods for pathogen enumeration, direct counts typically suggested higher numbers of *E. coli* O157:H7 cells than plate counting on selective medium and qPCR in most cases led to lower numbers. In the first case, a certain degree of loss of culturability during the preparation of the inoculum would explain the difference. The facts that (1) cells were grown in full strength LB medium to stationary phase, (2) were subsequently resuspended in filter-sterilized BAC water with very low nutrient content and (3) incubated for 24 h prior addition to the reactor system, could all contribute to a loss of culturability by part of the pathogen population. This in turn would lead to lower plate counts compared with direct counts. Another reason might be insufficient dispersion of cell clumps with multiple cells forming one colony. Overestimation by direct counts, on the other hand, might result from the difficulty to differentiate cells from co-stained background material. In the case of qPCR, inhibition of amplification is the most likely explanation for reduced numbers. Spiking experiments with plasmid DNA that was otherwise not present in the samples showed a significant amount of inhibition for different sample types (data not shown). Many substances originating from water and biofilms are known to be co-extracted with DNA and to inhibit subsequent PCR amplification (Tsai and Olson 1992; Wilson 1997). The degree of inhibition varied greatly with the sample type with bead and reactor surface samples showing the greatest degree of inhibition. Variations of the genomic DNA extraction method, of PCR conditions, of PCR master mixes and template dilution were not successful to relieve inhibition from all sample types. The inability to find optimal conditions for all samples was the reason this approach was not further pursued.

The presented data corroborate observations from previous studies showing that microorganisms and inorganic beads can be retained in biofilms and on surfaces in pilot-scale and laboratory-scale distribution systems (Rogers et al. 1994; Långmark et al. 2005; Searcy et al. 2006; Szabo et al. 2007). As seen in a pilot scale biological treatment filter where *E. coli* was retained in biofilm found at the surface of the reactor and not lower in the filter where biofilm was not as abundant (Li et al. 2006), the results of the current work demonstrated that biofilm colonized reactors retained significantly more *E. coli* O157:H7 than clean control reactors. Furthermore, the biofilm caused retardation of *E. coli* O157:H7 cells and prolonged their washout from the reactors.

The usefulness of the immobilization of pathogens in a biofilm as a potential diagnostic will depend upon long-term immobilization of pathogens in such a system. Although this was not evaluated directly in the present study, previous reports about long-term interaction between microbes and biofilms give rise to optimism in this respect. For example, Szabo et al. (2006) showed that *Klebsiella pneumoniae* persisted for 9–17 days on corroded iron surfaces in both chlorinated and dechlorinated drinking water biofilms grown in annular reactors. Persistence was even longer with *Bacillus atrophaeus* subsp. *globigii* spores, which were present for up to 70 days in the presence of high doses of chlorine (Szabo et al. 2007). In the biofilm of a simulated DWDS, 1-μm hydrophilic and hydrophobic microspheres, *Salmonella* bacteriophages 28B and *Legionella pneumophila* persisted in drinking water biofilms for over 38 days (Långmark et al. 2005). Searcy et al. (2006) showed that more *Crytposporidium parvum* oocysts were trapped by biofilm than were retained on a clean glass surface, and the biofilm-entrained organisms were not released within 24 h of continuous flow. All of these observations suggest that the use of a biofilm-coated system for pathogen retention has promise for the interrogation of drinking water systems after a contamination event.

Further research on the validity of the approach should include inoculation with lower concentrations of pathogens, the impact of time on the recovery of the

pathogens retained in the biofilm and optimization of recovery methods. These studies would give more credibility to the use of biofilms for sequestering pathogens for subsequent detection of a contamination event in drinking water.

Acknowledgements

This research has been supported by a grant (DAAD 19-03-1-0198) from the Army Research Office. Any opinions, findings and conclusions or recommendations expressed in this material are those of the authors and do not necessarily reflect the views of the Army Research Office.

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