Real time monitoring of biofilm development under flow conditions in porous media

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Biofilm growth can impact the effectiveness of industrial processes that involve porous media. To better understand and characterize how biofilms develop and affect hydraulic properties in porous media, both spatial and temporal development of biofilms under flow conditions was investigated in a translucent porous medium by using Pseudomonas fluorescens HK44, a bacterial strain genetically engineered to luminesce in the presence of an induction agent. Real-time visualization of luminescent biofilm growth patterns under constant pressure conditions was captured using a CCD camera. Images obtained over 8 days revealed that variations in bioluminescence intensity could be correlated to biofilm cell density and hydraulic conductivity. These results were used to develop a real-time imaging method to study the dynamic behavior of biofilm evolution in a porous medium, thereby providing a new tool to investigate the impact of biological fouling in porous media under flow conditions.

Keywords: biofilm; bioluminescence; porous medium; biofouling; hydraulic conductivity; imaging

Introduction

Biofilms are composed of dynamic communities of microbial cells enclosed in structured, self-produced matrices of hydrated extracellular polymeric substances (EPS) that adhere to inert or living surfaces (Costerton 1995). It is believed that more than 90% of all bacteria live within biofilms (Characklis et al. 1990). The stages that lead to the development of a mature biofilm, including attachment of planktonic bacterial cells to a solid surface, colonization, growth, and EPS production, have been well investigated (Costerton 1995; Costerton and Lappin-Scott 1995).

Biofilms are encountered frequently in medical applications, as well as in industrial and environmental processes. Biofouling of porous structures such as filters, soils, and petroleum reservoirs by biofilm can reduce the size of pores over time. This in turn can impact permeability of the porous medium, a phenomenon known as bioclogging. For example, bioclogging has been found to adversely affect the performance of sand filters used to treat wastewater (Nicoletta et al. 2000) and drinking water (Sharp et al. 2001; Urfer and Huck 2001). Biofouling in porous media leads to reduced hydraulic conductivity, and thus, a full understanding of interactions between microbes and their environment is critical to better design, operate, and control these systems (Thomas and Ward 1989; Clement et al. 1996; Singh et al. 2006; Bishop 2007).

The relationships between biofilm distribution in a porous medium, hydraulic conductivity and the consequent impact on metabolic reactions are not fully understood. In most studies, biofilm growth is detected indirectly by monitoring the pressure drop (at fixed flow rate) across a system, or the flow rate (at fixed pressure drop) through the system (Taylor and Jaffe 1990a, 1990b; Kildsgaard and Engesgaard 2002; Seki et al. 2006). Also, most studies destructively sample the porous medium at the end of the experiment to measure biofilm cell density (Komlos et al. 2004; Seki et al. 2006). Here, the authors report on a method to simultaneously visualize biofilm development and evaluate cell density, hydraulic conductivity, and fraction of void space occupied by biofilm (ie biofilm saturation).

Background

At present, imaging methods to visualize the growth and impact of biofilm in porous media systems include direct optical imaging by UV or visible light (Niemet and Selker 2001; Huang et al. 2002), dual-energy gamma radiation (Oostrom et al. 1998), magnetic resonance imaging (Seymour et al. 2004), and X-ray microtomography (Davit et al. 2011). In addition, many studies have demonstrated the effect of biofilm on hydraulic conductivity by using

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standard soil methods (Cunningham et al. 1991, 1997; Vandevivere and Baveye 1992), dye tracer experiments (Kildsgaard and Engesgaard 2002; Thullner et al. 2002; Seki et al. 2006), breakthrough curves (Wollum and Cassel 1978; Harvey et al. 1989; Sharp et al. 1999), light microscopy (Dupin and McCarty 2000; Kim and Fogler 2000), and mathematical models (Vandevivere et al. 1995; Clement et al. 1996; Eberl et al. 2000; Thullner et al. 2004; Bozorg et al. 2011). Light microscope based visualization has been used to investigate biofilm structure and cell interactions at pore-scale (Dupin and McCarty 2000; Kim and Fogler 2000). Electron microscopy (e.g. SEM and TEM) has also been used to determine biofilm thickness on solid particles in porous media (Rinck-Pfeiffer et al. 2000; Hand et al. 2008). However, the destructive nature of sampling, as well as the time needed to prepare the samples for analysis by electron microscopy, makes SEM and TEM visualization techniques inappropriate for real-time visualization of biofilm development in porous media.

In recent years, bioluminescence exhibited by natural and engineered microorganisms has been used to monitor microbial processes (Bellage et al. 1990; Sanseverino et al. 1993; Ripp et al. 2000; Uesugi et al. 2001; Sharp et al. 2005). Sharp and coworkers (2005) used bioluminescent bacteria in an etched glass flat-plate flow chamber to study biofilm growth in a pore network. In this experiment, bacterial bioluminescence was used to track biofilm development, but no quantification was made in terms of the detected bioluminescence intensities. Also, green fluorescent protein (GFP) has been expressed in bacterial cells as a reporter gene to detect bacterial colonies (Chalifet et al. 1994; Tombolini et al. 1997; Tresse et al. 1998). Constitutive production of GFP makes the gfp-gene an excellent marker to detect bacteria in complex substrata such as soil. Moreover, the growth, fate and transport of microorganisms in natural environments has been monitored by cloning and expressing luminescence (lux) genes in a variety of host microbial species (Burlage et al. 1990; Uesugi et al. 2001; Shaw et al. 1992; Oates et al. 2005; Troegl et al. 2007). However, experimental methods described in the literature involving bioluminescent microorganisms can result in inconsistent induction of lux genes due to issues related to the mass transfer of induction agents. Here, a method to address these mass transfer issues is described, thereby allowing consistent bioluminescence of microbial species containing inducible lux genes in porous media. Using this method, the authors have been able to nondestructively observe how biofilms grow and disperse in a porous medium while simultaneously measuring the hydraulic conductivity.

Materials and methods

Bacterial strain

The bacterial strain used here was the bioluminescent reporter strain Pseudomonas fluorescens HK44 obtained from the University of Tennessee Center for Environmental Technology (Knoxville, Tennessee). HK44 is a rod-shaped, Gram-negative bacterium originally isolated from soil heavily contaminated with polyaromatic hydrocarbons and heavy metals (Sanseverino et al. 1993). It carries the naphthalene catabolic plasmid pUTK21 and was genetically modified by transposon insertion of the salicylate-inducible nah-G-luxCDABE (bioluminescent) gene cassette and a tetracycline resistance marker (King et al. 1990). This strain emits luminescent light in the presence of naphthalene, salicylate, 4-methyl salicylate, and other aromatic hydrocarbons (Sanseverino et al. 1993; Ripp et al. 2000).

Media

Three different media were used: one to maintain and expand cell populations in planktonic culture, a second one for flow chamber studies, and a third medium to induce bioluminescence for visualization. The pH of each medium was adjusted to 7.2 ± 0.05 with 1 M NaOH or HCl and all media were autoclaved at 121°C for 20 min prior to being used. Stock solutions were stored at 4°C for up to 2 weeks. Also, to ensure plasmid maintenance, all media used were amended with 30 mg l⁻¹ (final concentration) of tetracycline (EMD Chemicals, OmniPur® EM-8990). Tetracycline solution was prepared in 50% ethanol (v/v with water) and added through a 0.2 μm filter to the autoclaved medium.

Stock culture growth medium

Oxygen-saturated nitrate-free growth medium was used to maintain a stock of cells for use in this study. All cell stocks were generated in a shaken, batch culture vessel where they maintained a planktonic phenotype. One litre of growth medium was generated by combining: (i) 333.3 ml of mineral based medium consisting of MgSO₄ (1.2 g l⁻¹), CaCl₂-2H₂O (0.3 g l⁻¹), NH₄Cl (1.2 g l⁻¹); (ii) 332.4 ml of phosphate buffer saline (PBS) solution consisting of NaCl (24 g l⁻¹), KCl (0.6 g l⁻¹), Na₂HPO₄ (3.45 g l⁻¹), K₂HPO₄ (0.78 g l⁻¹); (iii) 1.0 ml of trace element solution containing HCl (3.66 g l⁻¹), FeSO₄·7H₂O (21 g l⁻¹), H₂BO₃ (0.3 g l⁻¹), MnCl₂·4H₂O (1 g l⁻¹), CoCl₂·6H₂O (1.9 g l⁻¹), NiCl₂·6H₂O (0.24 g l⁻¹), CuCl₂·2H₂O (0.02 g l⁻¹), Na₂EDTA·2H₂O (10 g l⁻¹), ZnSO₄·7H₂O (1.44 g l⁻¹), Na₂MoO₄·2H₂O (0.36 g l⁻¹); and (iv) 333.3 ml
of glucose solution (3 g l$^{-1}$) as the main carbon source.

Medium for biofilm growth in flow chamber

The growth medium described above for stock culture was also used in all experiments carried out in the porous medium flow chamber. However, when used in the flow chamber, the glucose concentration was lowered to 0.25 g l$^{-1}$; high concentrations were not required since the spent medium was continuously replenished with fresh medium containing glucose (unlike in the batch cultures). For clarity, this medium will be referred to as biofilm growth medium.

Induction medium

The induction medium was generated by using the same components as listed for the stock culture growth medium, with the exception of phosphate sources. The elimination of phosphate from the medium allowed available nutrients to be dedicated to bioluminescence, and minimized other cell activities including cell division. The medium was also supplemented with 0.1 g l$^{-1}$ (final concentration) of salicylate to induce bioluminescence. Continuous bioluminescence requires a considerable amount of cellular energy, with ATP levels in bacterial cells dropping by as much as an order of magnitude (DeLuca and McElroy 1978). In addition, the preliminary results (not shown) indicated that low oxygen concentrations negatively affected HK44 bioluminescence levels. Thus, to have constant and continuous bioluminescence, the induction medium was supplemented with 1.0 g l$^{-1}$ glucose (instead of 0.25 g l$^{-1}$ in the flow chamber medium used for biofilm development in the porous medium) and fully saturated with oxygen prior to injection into the chamber.

Chamber inoculation

A stock culture of bacterial strain HK44 (0.1% v/v) was prepared at room temperature in oxygen-saturated growth medium. Following overnight incubation (shaken at 150 rpm) at room temperature, 0.1 ml of the culture (4.13 × 10$^8$ cells ml) was inoculated into 30 ml of fresh growth medium. After 24 h, cells were harvested by centrifugation (Beckman Coulter®, X-22R) at 5000 rcf for 20 min, washed in PBS, centrifuged again and resuspended in growth medium to 6 × 10$^8$ cells ml. This culture was used as the flow chamber inoculum. All population densities were determined by measuring light absorbance at 550 nm in a spectrophotometer (DU 730, UV/Vis Spectrophotometer, Beckman Coulter®) which had been calibrated previously by using a cell counting chamber (Hemacytometer Set, Hausser Scientific).

Setup and operation of the flow chamber

All flow chamber experiments were conducted in a dark box to ensure minimal interference between bacterial bioluminescence and other light sources. To avoid contamination of tubes and inlet/outlet ports, three way valves were used at each connection port to facilitate alcohol disinfection as needed. Autoclaved medium was aerated with an aquarium air pump (Whisper® Tetra Holding, US) connected to a diffuser. Figure 1 displays the custom-designed flow chamber used in the present studies. It consisted of a 9.0 cm by 14.0 cm by 1.0 cm aluminum spacer with two 10 cm by 1 cm by 1 cm regions which had been removed so they could house porous medium, and was equipped with inlet and outlet ports. The aluminum spacer was sandwiched between two transparent acrylic plates sealed to the spacer by fluorocarbon rubber (Viton®, Dupont Dow Elastomers). The bottom plate was drilled with five holes in a row, located 1, 3, 5, 7, and 9 cm from the inlet (see Figure 1) to monitor the pressure distribution by using a calibrated high accuracy micro-machined silicone sensor pressure transducer (PX409-100GUSB, Omega). These ports were also used to inoculate the porous medium at different locations within the chamber.

Each void space in the aluminum spacer was filled with spherical acid-washed glass beads ranging in diameter from 425 to 600 μm (30–40 US sieve) (Sigma–Aldrich, G8772), with particle density equal to 2.6 g cm$^{-3}$. The chamber was fitted with 0.1 mm nylon mesh screens at both the inlet and outlet of the porous medium. Also, fluid fluxes under different pressure gradients were measured to determine the hydraulic conductivity of the packed porous medium as follows:

$$Q_w = -\frac{AKh}{L}$$

where $Q_w$ is volumetric flow rate of the water phase, $A$ is cross-sectional area of the porous medium, $K$ is hydraulic conductivity, and $h$ is hydraulic head difference applied over a length $L$.

A Mariotte tube (SMS®, Arizona, USA) was used to maintain a constant pressure difference between chamber inflow and outflow, thereby enabling the hydraulic conductivity of the porous medium to be determined. After inoculation and during the biofilm growth and development period, a constant hydraulic pressure head of 7 cm was applied across the porous medium resulting in an average hydraulic gradient of 0.7 m m$^{-1}$. 


Prior to use, the chamber was autoclaved after assembly and allowed to dry and equilibrate to room temperature for 24 h in a laminar flow biosafety cabinet (Forma Class II A2; Thermo Electron Corporation). After drying, the chamber was sealed and all autoclaved fittings and tubing connectors were installed, taking care to ensure sterility. The porous medium was then filled with sterile deionized water via a Gilson Minipuls 3 peristaltic pump while the chamber was maintained in a vertical position. After filling, the chamber was placed horizontally in the dark box and a tracer test was conducted to ensure uniform flow across the porous medium. Movement of the tracer pulse indicated that the chamber was packed uniformly, as revealed by a flat leading edge profile, and breakthrough studies confirmed that the chamber was homogeneously packed and no preferential flow path existed (data not shown). Following the tracer test, the chamber was flushed with biofilm growth medium and subsequently, the experiment was initiated by inoculation of 0.25 ml of cell culture inoculum (6 × 10⁶ cells ml⁻¹) into the porous medium via the center port on the bottom plate of the chamber. This resulted in a 0.5 cm radius inoculation region around the injection port with a bulk volume of approximately 0.8 cm³. The inoculation region was visualized using Brilliant Blue FCF dye (ACROS Organics) which had been included in the inoculum. To prevent bacterial washout, continuous background flow of biofilm growth medium was not initiated until 24 h after inoculation to provide sufficient time for bacterial attachment to the porous matrix. The fluid flow rate was monitored by using calibrated in-line flowmeters (BEL-ART Riteflow⁸) at both the inlet and outlet of the chamber.

Bioluminescence imaging

Images of bioluminescence emitted from biofilms within the chamber were taken once every 24 h. Prior to taking images, the medium flowing through the inlet port was switched from biofilm growth medium to induction medium for a period of 90 min. All inlet flow was then blocked and a bioluminescence image was taken using a 14-bit digital CCD camera (Progres MFcool CCD camera, Jenoptik, Germany) with an interline 2/3” Sony ICX285AL 1.4 megapixel progressive scan monochrome CCD sensor (encased by a hermetically sealed, nitrogen-flushed capsule cooled by a Peltier element, a heat sink and a fan). This camera was fitted with a Computar Megapixel lens with

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*Figure 1.* Experimental setup showing the custom designed flow chamber. The aluminum spacer was sandwiched between two transparent acrylic plates with gaskets serving to ensure a tight seal. The bottom acrylic plate was drilled with five holes in a row, located at 1, 3, 5, 7, and 9 cm from the inlet to monitor the local distribution of pressure along the chamber. The chamber was operated in a horizontal orientation. A Mariotte tube was used to house the biofilm growth medium used in the chamber. The inlet valves were used to ensure that, at any time, the medium only entered one porous medium channel, and not the other.
35 mm focal length and f/1.4 focal ratio. Images were taken with a longpass UV filter (Edmund Optics, 25.5 mm) which permitted the passage of all fluorescent light while protecting the lens.

To take images, the camera was located 40 cm above the chamber and controlled by the CapturePro software (ProgRes® CapturePro 2.7.7, Jenoptik, Germany). In all the experiments, a 5 min exposure time with full aperture (f/4) was used. The conditions and procedures used to obtain each image were consistent for all experiments making it possible to compare different bioluminescence images on the basis of bioluminescence intensity (BI), and therefore, evaluate the impact of different parameters on cell bioluminescence. A background image was taken prior to induction and then subtracted from each image recorded after induction (using CapturePro software) to minimize the effects of surrounding optical noise.

**Image processing**

*Assignment of bioluminescence intensity values*

Each image was taken in grayscale, with a direct relationship between BI and pixel intensity. Seven distinct gray levels could be electronically detected in the bioluminescence images. Thus, each pixel in a grayscale image was assigned an integer value from 0 (no bioluminescence detected) to 6 (maximum bioluminescence detected). This assignment allowed each 2D image to be treated as a 2D matrix of gray level values based on the scale used by the camera. Each 2D matrix was then analyzed by using MATLAB® Image Processing Toolbox Version 7.8 (MATLAB® R2009a, MathWorks Inc.). However, the differences in darkness between pixels in the raw images were difficult to discern with the naked eye. To improve visual quality, advantage was taken of the 256 gray levels (8 bit grayscale color map) in the MATLAB® software package. The camera-based integer values in each 2D matrix were scaled to the entire MATLAB® grayscale color map by a common practice in image processing known as gray contrast enhancement with a linear contrast stretch (Schowengerdt 1983; Al-amri et al. 2010). Thus, the original gray scale was expanded to the scale shown in Figure 2 where a value of 0 again refers to no bioluminescence and a value of 6 refers to maximum bioluminescence. Rescaling the gray levels allowed for small differences in gray level intensities detected by the camera to be visually discerned by the naked eye in images produced by the image processing software.

*Image segmentation*

To analyze bioluminescence images and verify whether the detected BI could be related to hydraulic conductivity, the average BI in each zone was required. However, to have statistically meaningful outcomes from a series of images, a consistent method should be utilized to determine these average values. Based on results reported by Sezgin and Sankur (2004), the Otsu approach (Otsu 1979) was selected to obtain optimum threshold values (ie the average BI in each bioluminescence image). Thresholding is an image segmentation method that can be used to separate an image into distinct components (Yang et al. 2001; Sezgin and Sankur 2004). In this technique, target elements are isolated from background regions based on gray level distribution amongst image elements. The method separates data by grouping all pixels with intensities greater than a certain gray level into objects and all other pixels into background (Otsu 1979). In this technique, the procedure iterates over all possible thresholds to determine an optimal value based on minimization of within-class variances (Sezgin and Sankur 2004) as follows: (1) An intensity level is selected as the threshold value ($\tau_i$). (2) The bioluminescence image is segmented into object (O) and background (B) classes, and then the average BI for each zone in the porous medium chamber (see Figure 2 for the locations of the zones) is computed. The average object bioluminescence intensity ($\text{BI}_O$) is calculated as:

![Figure 2](image-url)
A dissolved oxygen test kit (CHEMetrics, Inc.) was used to determine the amount of dissolved oxygen in the chamber effluent. In this method, the dissolved oxygen oxidizes the leuco-base of indigo carmine to produce a blue-green dye, the intensity of which can be measured spectrophotometrically. Here, measured absorbance values (wavelength 470 nm) were compared against standard solutions ranging from 0 to 10 mg l\(^{-1}\) oxygen (provided by CHEMetrics, Inc.).

**Quantification of bacterial cell populations**

The bacterial population density in each porous medium sample was determined by measuring the mass of total DNA based on the selective binding of DNA to a silica-based membrane. Proteinase K and chaotropic salt were used to lyse the cells and degrade the protein, allowing DNA in the chaotropic salt to be easily bound by the silica-based membrane (Qubit dsDNA Assay Kit, Invitrogen, Oregon, USA). In addition, RNase A was added to the lysate to degrade RNA and minimize RNA contamination of the purified DNA samples.

To correlate the total DNA collected in a sample to the number of cells present in that sample, it was necessary to first calculate the average DNA content per cell. By summing the mass of the PUTK21 plasmid used in HK44 and the published mass of DNA in wild type *P. fluorescens*, the total DNA content per cell was calculated to be equal to 7.843 \(\times 10^{-15}\) g (King et al. 1990). A correlation between mass of DNA and corresponding number of bacterial cells was generated by manual cell counting (at least 360 bacterial cells per slide were counted in triplicate for statistical relevance) using a counting chamber (Hemacytometer Set, Hausser Scientific).

**Protein measurement**

Protein can be found both within cells and in the EPS. The total protein concentration in porous medium samples was determined by using the bicinchoninic acid (BCA) method (Smith et al. 1985). Bacterial protein extraction lysis buffer (Bacterial–PELB, GBio-siences\(^{\circledR}\)) was used to extract intracellular proteins. The absorbance of light (wavelength 560 nm) against the fresh reagent was measured by the same spectrophotometer described above and compared to a standard curve generated by using bovine serum albumin (BSA). Also, to differentiate between EPS and intracellular proteins, a liquid batch culture of HK44 was grown in stock culture growth medium on a rotary shaker (Heidolph Unimax 2010, Germany) at 150 rpm and room temperature. Three samples (5 ml each) were collected at different growth stages and bacteria were harvested from each sample by centrifugation (5000 rcf, 4\(^\circ\)C for 20 min) and washed twice.
with PBS. The total intracellular protein was then measured by using the same method as described above. In addition, the protein content of individual cells, obtained from the biofilm samples, was evaluated to identify differences in protein content between planktonic and biofilm bacterial cells.

**Direct visualization**

Biofilm samples collected from each zone were visualized by phase-contrast microscopy (Axio Observer.A1, Zeiss, Germany) to investigate the effect of environmental conditions on the biofilm structure.

**Results and discussion**

**Spatiotemporal development of biofilm in porous medium**

Figure 2 shows bioluminescence images obtained for the evolving biofilm under flow in a porous medium over a period of 8 days. This experiment, with identical conditions, was repeated five times with very similar results (see Figures S1 and S2 in Supplementary information for data from other trials) [Supplementary material is available via a multimedia link on the online article webpage]. The bioluminescent response of the cells was recorded after injection of induction medium from the left side through the chamber inlet (ie one-way induction). The first image (Day 1) of the chamber was taken 24 h after being inoculated, but prior to injection of growth medium. This image was used to visualize the region that was initially inoculated (marked ‘Inoculation region’ in Figure 2). The batch experiments showed that maximum HK44 bioluminescence occurred after approximately 90 min of induction and remained constant for at least 30 min in the oxygen-saturated induction medium (results not shown). Thus, in further experiments, injection of the induction medium was maintained for 90 min.

The bioluminescence images revealed that, during the first 3 days, biofilm growth was confined to the inoculation region, and spatial spread of biofilm throughout the chamber was negligible. However, on Day 4, once biofilm growth had grown sufficiently within the inoculation region, it extended almost symmetrically into Zones 2 and 3. From the Day 4 image in Figure 2, small independent areas of bioluminescence downstream of the inoculation zone are evident. The biofilm distribution in the downstream sections (Zones 3 and 4) can be explained by convective transport of detached biomass (detached cells and biofilm clusters) from upstream zones which reattach in downstream regions. The discontinuous and non-homogeneous growth pattern of biofilm in downstream sections could have resulted from random localization of detached cells and non-homogeneous flow (ie nutrient availability) due to biofilm growth and hydraulic conductivity changes in upstream zones.

After Day 4, biofilm continued to grow and spread throughout the chamber, with BI indicating that biofilm saturation (fraction of pore space occupied by biofilm) was highest upstream of the inoculation site towards the nutrient inlet port. Similar growth patterns have been reported in other biofilm studies indicating that biofilm tends to migrate primarily from initially inoculated regions toward inlet ports (DeLeo and Baveye 1997; Kildsgaard and Engesgaard 2002; Seifter and Engesgaard 2012). Migration of cells against a pressure gradient cannot be explained simply by convective transport, and therefore, dynamic interactions between biofilm and fluid flow must be taken into account. One possible explanation for this observation is that as the flowing liquid approaches the biofilm mass, it decelerates and forms a stagnation point at the nose of the mass, thereby creating slow flow conditions just upstream of the biomass. This creates an active growth zone immediately upstream of the biofilm mass due to lower flowrates and the presence of fresh growth medium which promotes biofilm growth towards the inlet. Also, bacterial movement, and consequently biofilm growth pattern, may be influenced by bacterial chemotaxis in response to nutrient concentration gradients.

Another important feature of the images was emergence of dark regions on Days 6 and 8 in sections previously shown to be occupied by biofilm on Day 5. Quantification of cells obtained by destructive sampling at the end of the experiment (Figure 3A; see also Figure S2 in Supplementary information) [Supplementary material is available via a multimedia link on the online article webpage] indicated relatively high cell population densities within regions of Zones 3 and 4 even though these regions appeared dark in the images on Day 8 (Figure 2) and had earlier emitted detectable bioluminescence. For example, the region peripheral to the inoculation port changed from being highly luminescent on Day 5 to less so on Day 6 (Figure 2). Low flow rates and moderately mature biofilm in these sections suggested that the reduction in the detected BI was not caused by cell loss. Rather, as biofilm saturation increased, penetration of induction medium into these sections was reduced and redirected around these low permeability regions, thereby not reaching the cells in sufficient quantities needed for them to bioluminesce. Thus, unlike those microbes that contain constitutively expressed fluorescent proteins, bioluminescence intensity variations in microbial populations containing inducible lux genes can be used to represent varying degrees of biofouling in biofilm containing regions. Biofouling is typically diagnosed indirectly in experimental investigations or biological processes by...
the impact that it has on hydraulic conductivity or product quality/quantity (Flemming and Wingender 2010).

To verify that cells within dark regions that were previously luminescent retained their ability to emit bioluminescence, they were sampled at the conclusion of the flow experiment and cultivated in fresh stock culture growth medium. Bioluminescence activities recorded after exposure to induction medium confirmed the presence of active lux-genes in the culture obtained using cells from dark regions (Figure 2, Zones 3 and 4). This suggests that the lack of bioluminescence in the flow chamber was not due to a change within the cells, but rather the absence of one or more factors that induce bioluminescence. Within biofilm-saturated zones, mass transfer is dominated by diffusion and dispersion. Consequently, fluids do not readily penetrate into biofilm-saturated regions due to their lower permeability. Thus, during HK44 induction, it is likely that induction medium bypassed these biolegged regions, instead penetrating regions with higher permeabilities. The relative lack of induction agent within biofilm-saturated regions negatively impacts the ability to visualize cells in these regions by using the described induction method.

Metabolism related to bioluminescence requires high levels of molecular oxygen (Uesugi et al. 2001; Oates et al. 2005). During the induction phase on Day 8, dissolved oxygen measurements at the chamber outlet revealed a reduction to less than 3 mg l$^{-1}$ from its inlet concentration of approximately 9 mg l$^{-1}$. This relatively large reduction in oxygen concentration is explained by the high oxygen consumption required for bioluminescence. Dissolved oxygen in the chamber effluent was detected as high as 7–8 mg l$^{-1}$ prior to switching the background flow to induction medium which indicates that HK44 bioluminescence depends upon oxygen availability. Thus, lack of bioluminescence in regions with high biofilm saturation may have been due, in part, to low oxygen concentration in addition to lack of induction agent, consistent with other studies on the bioluminescent response of lux-genes (Oates et al. 2005).

**Two-way induction**

To reduce the negative effects of oxygen depletion and lack of inducer availability on bioluminescence, especially downstream of biolegged regions, another set of experiments was conducted in which images were first taken as described above, and then again with the flow reversed. That is, after being fed through the inlet and flowing towards the outlet as shown in Figure 1, the induction medium was fed through the outlet and allowed to flow back through the chamber towards the inlet. The method of injecting fluids into the chamber was identical for both one-way (described in the Materials and methods section) and two-way induction. This was done to overcome downstream oxygen limitations that would occur if the medium was only fed through the inlet, which in turn would enable improved imaging of cells in Zones 3 and 4. These results are summarized in Figure 4. According to the results obtained from the inlet side induction (Figure 4A), practically the same bioluminescence behaviors as
recorded in the previously described experiment (Figure 2) were observed. However, injection of induction medium from the outlet side (Figure 4B) revealed a different bioluminescence pattern throughout the chamber. Similar results were obtained in a duplicate experiment (see Figure S3 in Supplementary information) [Supplementary material is available via a multimedia link on the online article webpage]. Compared to injection of induction medium from the inlet side, Zones 1 and 2 now exhibited lower BIs, whereas Zones 3 and 4 exhibited much higher BIs. Again, these results were obtained because bioclogging affected the flow of induction agent in Zones 1 and 2. Even when induction medium was able to penetrate areas in these two zones, oxygen would have been relatively low due to consumption by cells in Zones 3 and 4. In contrast, feeding induction medium through the outlet resulted in relatively high concentrations of induction agent and oxygen in Zones 3 and 4.

To obtain a better understanding of overall biofilm growth and development throughout the porous medium, images obtained from inlet and outlet side inductions were merged together. A merged image was constructed by comparing the BI of a given pixel in the inlet side induction image to the BI of the corresponding pixel in the outlet side induction image. The higher of the two intensities was used to generate the final merged image (Figure 5A; see also Figure S3 in Supplementary information) [Supplementary material is available via a multimedia link on the online article webpage]. This merged image was a more accurate representation of biofilm growth in the chamber than either of the two images alone from which it was constructed.

**Analysis of bioluminescence images**

To correlate the measured BI of a merged image to biofilm characteristics and hydraulic conductivity, it was necessary to develop methods to accurately estimate the overall amount of biofilm within each zone of the chamber. To accomplish this, bioluminescence images were first subjected to an image segmentation process to distinguish biofilm from void space that did not contain biofilm. Figure 5B shows the binary image obtained by segmenting the final merged bioluminescence images by using a threshold value determined using the Otsu approach. Also, threshold values, representing the average BI in the corresponding region, were calculated for each zone to show average BI variations throughout the 8 day experiment (Figure 6A). Note that the threshold values were normalized by the maximum gray level because MATLAB® does not accept threshold values larger than unity.

**Biological analyses**

From a qualitative analysis of the bioluminescence images, it appeared that bacteria tended to grow and form biofilm preferentially from the inoculation site towards the inlet rather than downstream into Zones 3 and 4. To determine if the images could be used to quantitatively assess the properties of the biofilm, assays were carried out in an attempt to correlate BIs to cell numbers and protein concentrations within the chamber. The results of these assays, summarized in Figure 3A and 3B, respectively, show that in upstream Zones 1 and 2, with higher BI, the average numbers of cells were higher than those in the downstream zones. Also, microscopic investigation of biofilm samples revealed a denser bacterial population upstream of the inoculation site compared to downstream sections (Figure 3C). The measured cell numbers were plotted...
against calculated BIs for each zone, as shown in Figure 6B. A power function was determined to be the most accurate expression ($R^2 = 0.97$) to describe the relationship between these two variables. Based upon Figure 6B (see also Figure S3 in Supplementary information for more data) [Supplementary material is available via a multimedia link on the online article webpage], the scaling exponent was calculated as 3.11, meaning that biomass accumulation affected microbial bioluminescence activity in the porous medium.

West and Brown (2005) conducted a theoretical investigation to explain allometric scaling relationships between biological activities and biomass accumulation in biological systems. They hypothesized that in any biological system, microbial populations could be sustained by transport of materials through linear networks that branch to supply metabolic substrates and remove waste products from all parts of a biomass. Accordingly, during the initial stages of biomass accumulation, it would be expected that the metabolic activity of microorganisms (such as emission of bioluminescence) would increase isometrically with an increase in the number of cells. However, evolution of the biomass over time would put geometric constraints on the accessibility of network branches, thereby restricting substrate (including oxygen and induction factor) distribution, which in turn would cause the relationship between metabolic activity and cell number to shift from being linear to sub-linear. West and Brown (2005) showed that for most biological activities, the allometric scaling exponent would be in the range of 1.3 to 4, which was consistent with the scaling exponent of 3.11 found in the present study for microbial bioluminescence activity. In addition, a second possible contributor to the observed nonlinearity could be that at higher cell numbers, the emitted bioluminescence from cells within a biomass could be increasingly masked by the cells and EPS that make up the outer layers of the biomass.

Figure 5 (A) Overall bioluminescence image on Day 8 obtained by merging images captured following both inlet and outlet induction. Scale indicates increasing bioluminescence intensity from 0 to 6. (B) Binary image obtained by applying the Otsu approach to determine a threshold value.

Figure 6 (A) Calculated average bioluminescence intensity for each zone during the course of an 8-day experiment. (B) Number of cells vs average bioluminescence intensity calculated on day 8 showing a power function relationship between cell numbers and average bioluminescence intensities at each zone.
Protein concentration measurements from the four zones, did not correlate directly to cell numbers, with the ratio of protein mass to cell numbers being higher in Zones 3 and 4 compared to Zones 1 and 2 (Figure 3B). In early biofilm studies, EPS was believed to be composed mostly of polysaccharides. However, more recent investigations suggest that protein is also present in EPS in considerable amounts. For example, Nielsen et al. (1997) quantified the EPS fraction of biofilms in trickling filters and biofilters and concluded that protein is abundant in biofilm EPS. Other investigations on the EPS of active sludge biofilms have also revealed that protein is present in large quantities (Dignac et al. 1998; Zhang and Bishop 2003; Metzger et al. 2009; Flemming and Wingender 2010). The high protein-to-cell ratio measured in Zones 3 and 4, therefore, could be explained by the fact that in the present studies, total protein (i.e., intracellular and EPS protein) had been measured, and not just intracellular protein. Since cell densities in Zones 3 and 4 were lower than in Zones 1 and 2, but total proteins levels were much greater, it suggests that conditions in downstream regions promoted greater EPS secretion and less cellular proliferation than the prevalent environmental conditions in upstream regions.

Given that the number of cells within biofilm samples was measured and it was found the average intracellular protein content was $1.12 \times 10^{-13}$ g of protein per cell (regardless of whether the cells were suspended in a planktonic manner or within a biofilm), the proportion of total protein that was actually extracellular (i.e., from the EPS) in each sample could be calculated. These results are summarized in Figure 3B. It has been shown that under adverse environmental conditions, bacteria consume available nutrients to secrete EPS rather than proliferate in order to provide mechanical stability to their surroundings, thereby enabling better protection against environmental stresses (Flemming and Wingender 2010). In addition, biofilm EPS acts as an extracellular nutrient store which can later be biodegraded by bacterial cells when needed (Zhang and Bishop 2003). In the present studies, the high ratio of EPS to cell count in downstream regions is explained by unfavourable environmental conditions with respect to nutrient and oxygen availability. High biofilm content in upstream regions significantly interfere with penetration of biofilm growth medium flow to downstream regions, thereby creating stressful conditions known to promote EPS production.

**Hydraulic properties of the porous medium**

Based on mass measurements and density of solid particles, the total porosity of the medium was calculated to be 38% at the beginning of the experiment. Also, based on flux measurements at different pressure head values, the hydraulic conductivity of the system was estimated at $6.05 \times 10^{-5}$ m s$^{-1}$ (Equation 1).

Over 8 days, under constant pressure head, the measured water flow rate through the chamber decreased gradually from 0.248 ml min$^{-1}$ at the beginning of the experiment to 0.110 ml min$^{-1}$ by the end (Figure 7A), indicating a reduction in hydraulic conductivity. As biofilm develops and occupies a greater proportion of pore space, the remaining pore space available for fluid flow decreases. Consequently, the reduction in hydraulic conductivity at constant pressure head leads to a drop in the fluid flux through the porous medium. Figure 7B (see also Figure S3 in Supplementary information) [Supplementary material is available via a multimedia link on the online article webpage] shows that hydraulic conductivity, represented by fluid flow rates, were

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**Figure 7.** (A) Fluid flow rate through the porous medium measured over the course of 8 days. (B) Flow rates through the porous medium vs overall bioluminescence intensities calculated for the entire porous medium showing a linear correlation.
correlated to the overall measured BI in an image, calculated as threshold values by the Otsu approach, over the entire porous medium. Based upon the allometric scaling relationship between BI and number of cells (Figure 6B), the obtained linear correlation between BI and flow rate (Figure 7B) implies a nonlinear relationship between hydraulic conductivity and the number of cells in porous media. One possibility for this nonlinear relationship can be diffusion of nutrients to the biofilm saturated zones which sustains microbial growth in such regions. However, higher biomass accumulation in these clogged regions will have trivial effects on overall hydraulic conductivity, thereby resulting in a nonlinear relationship between these two parameters.

To confirm the relationship between BI and hydraulic conductivity, after each bioluminescence image was recorded, the accompanying hydraulic head was recorded along the porous medium via the pressure ports at the bottom of the flow chamber (Figure 8A). Based on the hydraulic head profiles, the highest pressure drop observed after 6 days was in the vicinity of the initially inoculated region, as revealed by a sharp pressure gradient across Zones 2 and 3 (3 to 7 cm from the inlet). The overall hydraulic head reduction observed throughout the porous medium

![Graphs showing hydraulic head profiles and hydraulic conductivity variations over time.](https://example.com/graph.png)

Figure 8. (A) Hydraulic head profiles measured along the porous media via pressure ports at the bottom of the flow chamber at 1, 3, 5, 7, and 9 cm from the inlet. (B) Hydraulic conductivity variations during the course of an 8-day experiment combined with average bioluminescence intensities calculated for each zone showing possible correlations between average BI and porous medium hydraulic properties.
on Day 8 could be explained by clogging of the inlet screen. In addition, based on pressure gradients across each zone and measured fluid flow rates, Equation (1) was used to calculate hydraulic conductivity changes and these values were plotted against the time dependent variations in BI in each zone (Figure 8B). The correlation revealed between hydraulic conductivity and the average BI indicated that the overall BI could be used to evaluate hydraulic conductivity in each zone. Such a correlation was not unexpected as the development of biofilm restricts fluid flow and mass transfer, in turn causing a drop in BI.

Conclusions

It has been shown that it is possible to simultaneously evaluate biofilm development and its corresponding effects on the hydraulic conductivity of porous media by using real time imaging in conjunction with a model bioluminescent organism. Microbial bioluminescence intensities, calculated through image analysis, were correlated to the number of cells and hydraulic conductivity changes in corresponding regions. Such correlations can be used to determine important characteristics in porous medium based biofilm systems (such as cell density, biofilm saturation, and hydraulic conductivity) in real-time, and provide an opportunity to better understand, predict and control interactions between biofilm growth and hydraulic properties, thereby facilitating the development of porous medium based biofilm applications.

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