Effect of biofilm on colloid attachment in saturated porous media
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ABSTRACT
Biofilm plays an important role in controlling the transport of colloids in a porous media. Biofilms are formed when microorganisms come in contact with substrates, and are able to attach and grow with availability of nutrients. The microorganisms get embedded in a matrix of the substrate and extracellular polymeric substances which are responsible for the morphology, physico-chemical properties, structure and coherence of the biofilm. In this study, the effect of biofilm and its aging on colloid removal was studied on a glass bead column. Oocysts, polystyrene microspheres and inorganic colloids were used as colloidal particles. Pseudomonas aeruginosa was used as a model biofilm-forming microorganism. Presence of biofilm significantly enhanced colloid removal in the column. After 3 weeks, almost complete colloid removal was observed. The formation of biofilm was confirmed by various physical characterization techniques. During the extended aging study, biofilm sloughed off under shear stress. The loss of biofilm was higher during the early stage of its growth, and subsequently slowed down probably due to the formation of a more rigid biofilm. This research indicates that biofilm formation, maturation and sloughing-off play a critical role in colloid removal through porous media.

Key words | biofilm, colloid removal, extracellular polymeric substances, microsphere, oocysts, porous media

INTRODUCTION
Transport processes of colloids passing through a porous subsurface environment are of great importance due to their impact on human health. Studies have been carried out to understand colloidal transport through various substrates like sand, glass beads, and polystyrene beads (Yao et al. 1971; Rajagopalan & Tien 1977; Elimelech 1992; Tufenkji et al. 2005). Various factors such as solution chemistry (ions, pH), and hydrodynamic, physical and physico-chemical properties of colloids and substrates play an important role in colloidal transport (Elimelech et al. 1995; Li & Johnson 2005; Foppen et al. 2007; Chattarjee & Gupta 2009). The transport behaviour of bacteria and viruses through various saturated porous media (e.g. silica sand) and the influence of organics on their transport were studied (Sinton et al. 2012; Weaver et al. 2013). The results suggested that the removal of microbial contaminants was lower in presence of organics. The effect of changing hydraulic conditions during flood to predict virus removal through river bank filtration was also studied (Derx et al. 2013). The effects of fluctuations in river water level on virus transport during riverbank filtration, considering 3-D transient groundwater flow and virus transport, were investigated. The simulations showed that, in comparison with steady flow conditions, fluctuations in river water level caused viruses to be transported further at higher concentrations into the riverbank. Recent studies have also investigated the transport behaviour of engineered nano particles through natural soils with varying physical and chemical properties (Cornelis et al. 2013).

Interestingly, biofilms also play an important role in the transport of colloids in the subsurface. Biofilm is formed when the subsurface comes into contact with microorganisms and water. The surface is covered with
a film which consists of macromolecules such as humic substances, polysaccharides and proteins that are present in trace amounts in water. The microorganisms are embedded in a matrix of extracellular polymeric substances which are responsible for the morphology, physico-chemical properties, structure and coherence of the biofilm (Flemming & Wingender 2002). Biofilm growth is limited either by nutrient scarcity or by shear forces. The growth of biofilm tends to remain in equilibrium between external mechanical stress and internal mechanical stability (Sutherland 2001; Shafani & Vafai 2009). A rigid and dense biofilm is not sloughed off easily by normal shear forces (Mayer et al. 1999; Kim et al. 2010). In biofiltration, biofilm is allowed to form on filter media to couple biological treatment with the filtration process (Characklis & Marshall 1990). Evaluation of biofiltration in drinking water treatment has gained scientific interest due to its ability to reduce organic carbon in water (Hozalski et al. 1995). However the role of biofilm formation on substrate surface on colloid removal is not well understood. A few papers have reported that biofilm on a substrate could enhance colloid removal efficiency due to biochemical, physical characteristics and stability of biofilm (Morales et al. 2007; Tong et al. 2010), whereas lowering of particle removal in biofilm-coated filter compared to a filter media without a biofilm is also reported. This was attributed to reduction in fluid flow area or pore clogging with biofilm growth and short-circuiting (Dai & Hozalski 2002).

Thus there is a genuine need to develop better understanding on the effect of biofilm on colloid removal efficiency through saturated porous media. The transport behaviour of Cryptosporidium parvum oocysts, polystyrene latex microspheres and inorganic particles through a packed bed at various stages of biofilm growth has been studied in this paper. The formation of a biofilm was confirmed by various physical characterization techniques. Effect of aging of biofilm on colloid filtration is also reported.

**MATERIALS AND METHODS**

**Materials**

**Colloidal particles**

Cryptosporidium parvum oocysts, polystyrene latex microsphere and inorganic particles were used for the study. Cryptosporidium parvum oocysts were procured from BTF (Australia). The master stock suspension (10 mL vials) of the oocysts was supplied as gamma-irradiated inactivated cysts (sized 4–5 μm) and had a concentration of \(4 \times 10^5\) oocysts/mL in standard phosphate buffered saline (PBS) solution. Fluorescent carboxylated polystyrene-based latex microspheres (oocysts surrogate) were procured from Polysciences Inc. as a 2.5 wt% aqueous suspension (~1.68× \(10^9\) microspheres/mL, average size 4.5 μm). The inorganic particle was procured from Powder Technology Inc., USA. The particle size distribution of inorganic particle in deionized water was \(d(0.5) \sim 2.34\), \(d(0.1) \sim 1.16\), and \(d(0.9) \sim 4.93\ μm\).

**Filtration media**

Spherical glass beads of size range 300–355 μm were used as filtration media and procured from Mo-Sci. Speciality Products (Rolla, MO, USA). Prior to the experiments, glass beads were soaked in alkaline water overnight followed by rinsing with Milli-Q water until there was no change in pH and conductivity of rinse water.

**Bacterial strains**

Pseudomonas aeruginosa was used as a model biofilm-forming microorganism. P. aeruginosa is a mucoid, Gram-negative, motile bacterium. This strain was isolated from reverse osmosis membranes used in a home water treatment device.

**Methods**

**Media column preparation**

Colloid deposition experiments were carried in two 0.75 m long borosilicate glass columns, with internal diameter of 3.0 cm and with a single output at the bottom. The columns were first filled with Milli-Q water to a certain height and then 500 g of glass beads was added to it. The clean bed porosity was 0.39 with a clean bed pore volume of 138 mL. The path length was 49.5 cm. One of the columns was maintained in sterile condition and the biofilm was grown on the other. The colloidal removal experiments were carried by pumping the colloidal suspension using a peristaltic pump to the top of the glass column. A constant head of 10 cm water was maintained above the glass bead column in all experiments. The interstitial flow velocity was kept between 0.06 and 0.4 cm/s.
The samples were air dried and processed further for SEM. 

Bacterial inoculums and biofilm formation

The organism was maintained as a glycerol stock in a −70 °C deep-freezer and a working stock was maintained on tryptic soy agar and stored in a refrigerator. Fresh cultures were grown on tryptic soy broth. First, the column was disinfected by holding one pore volume of 5 ppm sodium hypochlorite solution for 1 h. Twenty-five pore volumes of Milli-Q water were then passed through it to replace all residual chlorine from the column bed. The column was then spiked with three pore volumes of log-phase culture of P. aeruginosa at a concentration of ∼10^9 cfu/mL after passing three pore volumes of tryptic soy broth. This system was left undisturbed overnight to allow initial adhesion of the cells to the beads and initiate biofilm formation. Thereafter the growth medium in the column was replaced every day with an alginate-promoting medium (alginate is the primary polysaccharide produced by P. aeruginosa), i.e. APM 50, to initiate biofilm formation and maintenance of the biofilm. The compositions of tryptic soy broth and APM 50 in Milli-Q water are as reported in literature (Morales et al. 2007).

Quantification of bacterial population on filtration media and the effluent

Bacterial counts in the effluent and on the glass beads were determined using the plate count method (Donnelly et al. 1976). The glass beads were first subjected to an extraction procedure (Morales et al. 2007) using an extraction buffer to detach the biofilm and then analyzed for total cell counts.

Scanning electron microscopy

Scanning electron microscopy (SEM) of the glass beads was carried out to view the presence of biofilm on the outer surface. The samples were prepared by drawing a small amount of the beads at regular intervals (once a week) from the column and placing them on one side of double-sided adhesive conductive carbon tape and air drying them. After air drying, all unattached beads were removed by inverting the strips and then washing the strips with PBS. The strips were then treated with 0.25% glutaraldehyde for around 30 min and washed once with PBS. The samples were then dehydrated by treating with different concentrations of ethanol in water (30, 50, 70, 80, 90 and 100%). The samples were air dried and processed further for SEM.

Fluorescence microscopy

Lectins are a group of diverse proteins which bind to specific configurations of sugar residues. As lectins can bind to the exo-polysaccharides of biofilms, they are being widely used to investigate the formation of biofilms on surfaces. The glass beads were treated with a fluorescent lectin conjugate and then checked for fluorescence. Glass beads were drawn from the column and placed on one side of a small strip made from the double sided adhesive conductive carbon tape. This tape was then treated with 0.1% lectin (concanavalin A)-fluorescein isothiocyanate conjugate solution in PBS for 15 min in the dark and then washed with PBS. Thereafter the samples were treated with 100 μL of 20 mM stock solution of propidium iodide in PBS for 15 min and washed with PBS. Propidium iodide stained the bacteria red, and green fluorescence was observed wherever the lectin was present. The samples were then observed in a fluorescence microscope (model no. AX 70, supplied by Olympus).

Colloid removal experiments

Removal performance was evaluated in presence and absence of biofilm on the columns. The columns were first preconditioned by passing 25 pore volumes of Milli-Q water with 1 mM MgSO_4·7H_2O (≥99% pure from Merck). For the oocyst removal study, oocyst concentration of 50,000/L was prepared by adding required numbers of oocysts to Milli-Q water containing 1 mM MgSO_4·7H_2O. The effluent was sampled at different pore volumes. The influent and the effluent oocyst concentration were determined by filtering through a 0.45 μm Millipore filter paper (as per NSF 53 norms). The oocysts were counted using an Olympus fluorescence microscope (model no. AX 70).

For polystyrene latex microspheres, a similar procedure was followed. To study removal of inorganic particles, the concentration was ~20 mg/L. Effluent sample was collected at different pore volumes. The influent and effluent concentration was measured in terms of turbidity (NTU) using a Merck Turbiquant 1,500 T turbidity meter.

RESULTS AND DISCUSSION

Biofilm formation on spiked column of glass beads

Formation of biofilm on the spiked column was confirmed by three methods.
Evidence 1: plate counts of *Pseudomonas aeruginosa* on glass beads

Glass beads from the column were sampled at different time points and the bound bacteria was extracted and measured as per the method described previously. The concentration of *P. aeruginosa* was found in the range of $10^8$ to $10^9$ cfu/g of glass beads at all time points.

Evidence 2: SEM of glass beads

Samples of glass beads from sterile and *Pseudomonas* spiked columns were analysed using SEM. The images are shown in Figure 1. Clusters of *Pseudomonas* were observed from Day 1 in the spiked column. With time, the extent of growth and number of biofilm-like structures increased. Day 21 sample showed almost the entire surface of the glass beads covered by the bacteria embedded in a matrix. No bacterial growth was observed in the sterile column.

Evidence 3: fluorescence microscopy of glass beads

Lectin (concanavalin A)-fluorescein isothiocyanate conjugate was used to stain the polysaccharides (one of the constituents of biofilm) present on the glass beads. The pictures of the beads are shown in Figure 2.

The surface coverage of glass beads with polysaccharides was observed to increase with time for the *Pseudomonas*-spiked column. By Day 14, glass beads were completely coated with polysaccharide, which was confirmed by fluorescence of lectin bound with polysaccharides (shown as bright white in Figure 2, green in online version of the figure, available at http://www.iwaponline.com/wst/toc.htm). Glass beads from the sterile column did not stain indicating absence of any polysaccharide on the surface.

Colloidal removal performance

Removal of *Cryptosporidium parvum* oocysts

Removal of *Cryptosporidium parvum* oocysts through sterile and biofilm-coated columns at various stages of biofilm growth was studied. The results are shown in Table 1.

It was observed that the oocyst removal increased with growth of biofilm. In sterile condition, the log oocyst removal was 1.34, which remained unchanged, whereas complete oocyst removal was achieved after 14 days in the biofilm column. This result coincides with the observation that a stable biofilm is formed on the glass beads after 14 days as indicated by SEM and fluorescence microscopy.

Removal of microspheres

Removal of microspheres through sterile and biofilm-coated columns after 21 days of biofilm growth was studied. The results are shown in Table 2.
Complete microsphere removal was observed in the biofilm-coated column compared to 1.78 log removal in a sterile column after 21 days.

**Table 1** | Performance of *Cryptosporidium parvum* oocyst removal through a sterile and a biofilm-coated glass bead column at various time points

<table>
<thead>
<tr>
<th>Type of column</th>
<th>Input of <em>Cryptosporidium parvum</em> oocysts (Log)</th>
<th>Removal of <em>Cryptosporidium parvum</em> oocysts (Log)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sterile (Day 1)</td>
<td>4.3</td>
<td>1.34</td>
</tr>
<tr>
<td>Biofilm (Day 1)</td>
<td>4.3</td>
<td>2.0</td>
</tr>
<tr>
<td>Biofilm (Day 7)</td>
<td>4.3</td>
<td>2.0</td>
</tr>
<tr>
<td>Biofilm (Day 14)</td>
<td>4.3</td>
<td>4.3</td>
</tr>
<tr>
<td>Biofilm (Day 21)</td>
<td>4.3</td>
<td>4.3</td>
</tr>
</tbody>
</table>

**Table 2** | Performance of microsphere removal through a sterile and a biofilm coated columns after 21 days

<table>
<thead>
<tr>
<th>Type of column</th>
<th>Input of microspheres (Log)</th>
<th>Removal of microspheres (Log)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sterile (Day 1)</td>
<td>4.5</td>
<td>1.78</td>
</tr>
<tr>
<td>Biofilm (Day 21)</td>
<td>4.5</td>
<td>4.5</td>
</tr>
</tbody>
</table>

**Removal of inorganic particles**

The removal of inorganic particles through the sterile and biofilm-coated columns after 21 days of biofilm growth was studied. The results are plotted in **Figure 3**.
In Figure 3, the removal is represented in terms of fraction of survivors (C/C₀) in the effluent. Higher survival fraction in the effluent indicates lower removal efficiency. The spiked column showed higher removal of inorganic particles due to formation of biofilm compared to the sterile column, as in the case of oocysts and microspheres.

It was also observed that formation of biofilm on the packed bed reduced the flow rate of water through the column. This could possibly be due to reduction in the bed porosity. The flow rate in the sterile column was 60 mL/min and in the column with biofilm was 40 mL/min after 21 days. To check the effect of flow rate on particle removal, removal of inorganic particles was studied in another sterile column at 40 mL/min. The removal profile is shown in Figure 4.

Particle removal efficiency increased (represented by reduction in C/C₀ from ∼0.28 to 0.2) with the reduction in flow rate in the sterile column. Still, the efficiency of removal of inorganic particles in the presence of biofilm was significantly higher compared to the sterile column at the same flow rate.

Effect of aging of biofilm

The biofilm-coated column was aged up to 105 days. The effect of aging was studied on flow rate and colloid removal.

Effect of aging on flow rate and removal

Effect of aging on the flow rate of water passed through the column is shown in Figure 5. It was observed that the flow rate of water dropped with time possibly due to the growth of biofilm within the column and not due to the deposition of inorganic particles on the glass beads, as the input concentration of the particles was very low (∼20 ppm). The particle removal efficiency dropped from 98% to 90% on the 45th day, there was a sudden increase in the flow rate from 16 mL/min to 30 mL/min. Later, the trend of drop in flow rate with time was found to be same as in the initial phase. Similar observations are reported in the literature (Sutherland 2001; Shafani & Vafai 2009), the reason being the sloughing-off of the excess amount of biofilm. Similar phenomena were not observed subsequently, probably due to formation of a more rigid and dense biofilm which is difficult to slough off by normal shear (Mayer et al. 1999; Kim et al. 2010). To validate the hypothesis of sloughing of the biofilm, the concentration of P. aeruginosa was measured in the output water. It was interesting to note that Pseudomonas concentration was relatively higher in the output water on the 45th day when the flow rate was suddenly increased. This supported our hypothesis of dislodging of biofilm from the column, resulting in an increase of flow rate on that day.

Porosity of glass bead column

The head loss due to clogging resulting in decrease in the porosity of the column was predicted using the Kozeny–Carman equation at various time points.

$$\frac{\Delta P}{\Delta x} = \frac{180 \mu u (1 - \varepsilon)^2}{d_c^2 \varepsilon^3}$$  \hspace{1cm} (1)

ΔP is pressure drop (Kg/m-sec²), Δx is bed length (m), μ is dynamic viscosity (N-s/m²), u is superficial velocity (m/s), d_c is collector diameter (m) and ε is bed porosity.

The predicted porosity of the column is shown in Figure 6, the trend of which was similar to the flow rate.
Based on predicted porosity at different stages of biofilm growth, mass of biofilm per gram of glass beads was calculated by measuring the change in bulk density of the column, also shown in Figure 6. It was observed that the predicted growth of biofilm increased with time.

CONCLUSIONS

The effect of biofilm on colloid removal was studied in the presence and absence of biofilm. Effect of aging of column on flow rate and colloid removal was also studied. The following are the conclusions from the study:

1. The presence of biofilm significantly enhanced colloid removal efficiency compared to a sterile column for all the colloids used in this work.
2. After 3 weeks, almost complete colloid removal was observed for all the three colloids.
3. In the aging study, it was observed that a part of biofilm sloughed off under shear. The slough-off was confirmed by the analysis of bacteria from effluent water.
4. The sloughing of biofilm resulted in lower colloid removal and was not observed again probably due to formation of a more rigid biofilm.
5. This work indicates that biofilm formation, maturation and sloughing-off play a critical role in colloid removal through porous media.
6. The Kozeny–Carman equation was used to predict the decrease in the porosity of the column with time.

REFERENCES


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