Particles matter: Transformation of suspended particles in constructed wetlands
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Chapter 5

Trapping of bacterial cells and latex micro-spheres in natural and cultured phototrophic biofilms

Abstract

Phototrophic biofilms play a vital role in aquatic ecosystems by trapping and processing abiotic and biotic particles. To study the effects of both particle and biofilm characteristics on particle trapping, incubation experiments were conducted using both natural and cultured mono-culture phototrophic biofilms and several different traceable particles. In a first experiment we observed substantial trapping of particles smaller than 15 µm, with highest trapping efficiencies for particles in the size range 5.0 - 8.0 µm (49% in 150 minutes). In a second experiment field grown natural biofilms and *Achnantes lanceolata* (diatom) biofilms rapidly accumulated micro-spheres and *P. putida* cells, while *Nitzschia perminuta* (another diatom species) and the cyanobacterial biofilms trapped only few particles. Trapping of *P. putida* was up to one order of magnitude higher than the trapping of micro-spheres, which indicates selective trapping of particles. These results showed that the trapping of particles by phototrophic biofilms in aquatic ecosystems can be substantial, but is a highly variable process strongly dependent on both species composition of the biofilms and the size and nature of particles.
Introduction

Phototrophic biofilms cover almost all illuminated surfaces in aquatic systems and consist of consortia of phototrophic and associated heterotrophic micro-organisms mechanically stabilized to the substratum by a mucous matrix of extracellular polymeric substances (EPS) (Stewart and Franklin 2008). The microbial community in biofilms play an important role in biochemical cycles of aquatic ecosystems (Battin et al. 2007; Woodruff et al. 1999; Costerton et al. 1995; Sutherland 2001; Hall-Stoodley et al. 2004) and the abundance of phototrophic and heterotrophic micro-organisms make biofilms hotspots of biological activity (Romani et al. 2004; Vadeboncoeur et al. 2008; Heck and Hesslein 1995). One of the mechanisms through which biofilms influence biochemical cycles is the adhesion and trapping of particles from the water column (Bouwer 1987), which can then be retained for substantial periods of time, serve as food for the heterotrophic community or become part of the biofilm microbial community. The capacity of biofilms to trap particles is considered sufficiently high to determine water clarity in aquatic ecosystems, even in running waters (Kadlec and Wallace 2008; Jowett and Biggs 1997; Gantzer et al. 1988). This purifying role of biofilms has long been recognized and is utilized in rotating biological contactors (Alleman et al. 1982), sequencing batch reactors (Wilderer and McSwain 2004), other technical reactors and constructed wetlands for the removal of organic matter and pathogens from polluted waters (Kadlec and Wallace 2008). Several studies have investigated the trapping of particles by phototrophic biofilm and have shown that the trapping of particles by phototrophic biofilms is affected by particle properties and biofilm structure (Okabe and Kuroda et al. 1998; Stott and Tanner 2005; Eisenmann et al. 2001; Chabaud et al. 2006), but effects of species composition of phototrophic biofilms on the trapping of particles with different characteristics is not well understood.

Therefore the aims of this study were to 1) compare phototrophic biofilm trapping efficiency of different particle size classes, 2) compare the trapping capacity of biofilms dominated by different species of diatoms and cyanobacteria and 3) compare the trapping capacity of biofilm between model particles (latex micro-spheres) and bacteria (with Pseudomonas putida as a model organism). For this purpose laboratory incubation experiments were conducted using both natural mixed species biofilms and mono-culture biofilms of diatoms and cyanobacteria. Microspheres and Pseudomonas putida cells were added as suspensions and particle concentrations and Pseudomonas putida abundance were monitored over time in the water column and in the biofilm.

Materials and methods

Experiment I: Particle size; Phototrophic biofilms

Natural biofilms were collected from an experimental rainwater pond at the University of Amsterdam, Science Park, The Netherlands (N52 21.286 E4 57.471). Following Barranguet et al. (2004) 1.5 cm² glass discs were mounted in vertical racks suspended in the water for six weeks and mature biofilms (dominated by green algae) were collected and incubated on clean support racks in cone shaped vessels (1.7 L) in WC Medium (Guillard and Lorenzen 1972). The biofilms were kept at
18°C and left to acclimate for 2 days before the start of the experiments. Water in the cones was mixed by pumping water from the bottom of the cones back to top, creating a vertical water flow to minimize particle loss by sedimentation.

**Experiment I: Particle size; Biofilm, particle incubations**

After 2 days of acclimatisation, medium was drained from the cones to remove any accumulated particles and replaced with particle-free WC medium. Three cones containing glass slides with attached biofilm were used as experimental vessels. To quantify the particles present in the biofilm or released into the water by the biofilm a control cone contained biofilms, but no added particles. To quantify the loss of particles over time not induced by the presence of biofilm another control cone was used which contained no biofilm, but with added particles. The experiment started one hour after refreshment of the medium, by addition of a mixture of particles with difference sizes. The mixture consisted of micro-spheres (Polysciences Inc., Polybead® Polystyrene, Germany) of five different nominal sizes (2 μm; 4.5 μm; 6 μm; 10 μm and 25 μm). The slight negative surface charge of the micro-spheres correspond to the surface charge of many microbial organisms found in aquatic environments with a pH above 7 (Stevik et al. 2004; Ongerth and Pecoraro 1996; Gerba 1984). Water samples were collected on several time points during incubation to quantify the concentration of particles in the water in the size ranges of 1.5 - 3.0 μm, 3.0 - 5.0 μm, 5.0 - 8.0 μm, 8.0 – 15 μm and 15 - 35 μm. To this purpose, 2 mL water samples (three technical replicates) were taken from each cone and stored in 10 mL tubes for further analyses. Water samples were then digested to remove organic matter by adding 1 mL 10% HCl, 1 mL 10% HNO₃ and 4 drops of H₂O₂ for 24 h. Next, samples were diluted with MilliQ water to a total volume of 30 mL and particles in these samples were counted using a particle counter (PAMAS® WaterViewer; Sensor HCB-LD-50/50, Germany). The experiment lasted for 150 min and at that time the glass slides with attached biofilm (six per cone) were taken from each aquarium and transferred into tubes (three replicates per cone) and sequentially digested and counted as described above.

Data was not normally distributed for all samples and differences between treatments were therefore analysed with Kruskal-Wallis tests using PAST (Hammeret et al. 2001). Particle removal rates (linear regression) and first order removal rates (k_d; exponential decrease) were calculated with MS Excel. The k_d values determined using:

\[
k_d = -\frac{\ln\left(\frac{N_t}{N_0}\right)}{t}
\]

Where \(N_0\) is the particle concentration after addition of the particles and \(N_t\) the particle concentration at time point t (h).

**Experiment II: Biofilm composition and particle type; Phototrophic biofilms**

Two types of phototrophic biofilms were used. First, natural biofilms were collected from lakes in the infiltration dunes of Leiduinen, The Netherlands (N52 20.726 E4 31.653) as described in
Barranguet et al. (2004). Glass discs were pre-colonized in the field for 7 days at ca. 10°C at two sites (further referred to as Natural biofilm I and II). Although both sites received the same water from the dune aquifer, these biofilms, both diatom dominated, developed slightly different (Barranguet et al. 2005). Secondly, mono-culture biofilms of diatoms (Achnantes lanceolata and Nitzschia perminuta) and cyanobacteria (Cylindrospermum stagnale and Leptolyngbya foveolarum), isolated from floodplains from the River Rhine (van der Grinten et al. 2004), were grown by incubation of glass discs in monocultures of these different species in 2 L aquaria with WC medium (Guillard and Lorenzen 1972) for 10 days at 20°C. After colonization, the biofilms were transferred to 8 L aquaria with WC medium that was mixed by aeration, and left for three days to acclimatize at 20°C.

Experiment II: Biofilm composition and particle type; Biofilm, particle incubations

After acclimatisation of the biofilm, the experiment started by addition of particles to the 8 L aquaria. Two types of particles were used: 1) fluorescent micro-spheres with a nominal diameter of 1.0 μm (with a slightly negative surface charge; Polysciences Inc., Fluoresbrite® BB Carboxylate Microspheres) and 2) living bacteria (P. putida, which have rod shaped cells of approximately 1μm wide and 3μm long). The stock solution of micro-spheres contained 5.4×10⁸ particles mL⁻¹ and for a final concentration of 10⁷ particles mL⁻¹, 15 mL of stock solution was added to the aquaria. P. putida was cultured on liquid LB medium to a concentration of 5×10⁸ cells mL⁻¹ and concentrated by centrifugation to a concentration of 1.2×10⁹ cells mL⁻¹. From this stock of P. putida, 6.7 mL was added to the aquaria resulting in a final concentration of 10⁷ cells mL⁻¹ in the aquaria. After the addition of micro-spheres and bacteria, samples were taken at several time points during incubation to monitor the concentration of particles (micro-spheres and P. putida) in the water and the amount of particles trapped in the biofilms. For determination of particles in the water 4×10 mL of water was sampled from each aquarium, fixated by addition of 10 mL 96% EtOH and stored at 4°C prior to further analyses. For determination of trapped particles in the biofilms, four glass discs (surface area 1.5 cm² per disc) were removed from each aquarium; the biofilm was removed from the glass slide using a razor blade and transferred into four separate tubes. The biofilm samples were fixated with 10 mL 50% EtOH, homogenised with an ultra-blender and stored prior to further analyses. Samples were divided for the analyses of micro-spheres and P. putida, which resulted in duplicate measurements of each aquarium for both micro-spheres and P. putida. Fluorescence micro-spheres concentrations in the water and in the biofilm were determined using flow-cytometry (Epics Elite instrument, Coulter Corporation, Hialeah, FL). For P. putida concentrations in the water and in the biofilm samples were filtered over 0.2 μm poly carbonate membranefilters (Whatman, Ø25 mm), the filters were labelled with a P. putida specific fluorescence rRNA probes (DuTéau et al. 1998) using fluorescence in situ hybridization (FISH) according to Glockner et al. (1996). After labelling the filters were mounted on microscope slides and P. putida cells were counted using an inverted fluorescence microscopy (Olympus). First order removal rates (k_d, exponential decrease) were calculated using MS Excel (eq.1).
Results

**Experiment I: Particle size**

The control treatment with only particles showed no significant (p>0.05) decrease in concentrations of particles for all sizes during the experimental period (Fig. 5.1; dotted grey lines). The treatment with biofilm but without addition of particles showed release of particles by the biofilms into the water, but the concentration of released particles remained the same over the experimental period for all particle sizes (data not shown). This resulted in higher concentrations of suspended particles in the treatment with both biofilm and micro-spheres compared with the control treatment with micro-spheres only. Suspended particle concentrations released by the biofilm were on average 19 ±3% lower than the concentrations of added micro-spheres for each particle size. Average removal rates over 150 min of incubation were of 140 ±25, 39 ±16, 18 ±4, 8 ±2 and 2 ±1 particles mL⁻¹ min⁻¹ (± s.e.) for particles size classes of 1.5 - 3.0 μm, 3.0 - 5.0 μm, 5.0 - 8.0 μm, 8.0 - 15 μm, and 15 - 35 μm respectively (Fig. 5.1a-e). The corresponding first order removal rates (Kₐ constants) were 0.188 h⁻¹ (1.5 - 3.0 μm), 0.225 h⁻¹ (3.0 - 5.0 μm), 0.246 h⁻¹ (5.0 - 8.0 μm), 0.244 h⁻¹ (8.0 - 15 μm) and 0.231 h⁻¹ (15 - 35 μm) (Fig. 5.6).

In the biofilm, a significant (p<0.05) increase in particle concentrations was observed after 150 min for the size classes 1.5 - 3.0 μm, 3.0 - 5.0 μm, and 5.0 - 8.0 μm. A similar, but not significant, increase was observed for particles of 8.0 - 15 μm (Fig. 5.2). The trapping efficiency differed strongly between the different size classes: the percentage of trapped particles was highest for particles in the size class of 5.0 - 8.0 μm, (49 ±12% of the particles was trapped after 150 min) while less than 5% trapping was observed for the smallest and biggest size classes (Fig. 5.3).
Fig. 5.1 Experiment 1; Average particle concentrations (particles mL⁻¹) in solution for several particle size classes (a-e), over an incubation period of 150 minutes, after addition of a mixture of micro-spheres at t=0, in the presence (solid black line with open circles; n=8-9) or absence (dotted grey line with solid squares; n=3) of a natural mature biofilm.
**Fig. 5.2** Experiment 1; Average particle concentrations (particles cm$^{-2}$) in natural mature biofilm for several particle size classes, after an incubation period of 150 minutes with (white bars; n=8) or without (grey bars; n=3) addition of a mixture of micro-spheres differing in sizes at t=0. Error bars represent the standard error and stars indicate significant difference (P<0.05).

**Fig. 5.3** Experiment 1; Average calculated trapping efficiency of micro-spheres by natural mature biofilms after an incubation period of 150 minutes. Error bars represent the standard error (n=8) and letters indicate similarity (P<0.05).

**Experiment II: Biofilm composition and particle type**

Fifteen minutes after addition of the particles, the average concentration of micro-spheres in the water was $1.2 \times 10^7 \pm 1.9 \times 10^6$ particles mL$^{-1}$ (± s.d.) (Fig. 5.4). In the blank (aquarium with glass slides without attached biofilm) the micro-spheres concentration remained similar to the starting concentration for the first 8 h (Fig. 5.4a). After 8 h the concentration of micro-spheres in water of the blank treatment steadily decreased to a concentration of $2.0 \times 10^6 \pm 6.6 \times 10^4$ particles mL$^{-1}$ after 72 h.
During the same period of time the number of particles found in the biofilms increased to $1.9 \times 10^5 \pm 0.3 \times 10^4$ particles cm$^{-2}$. The decrease of micro-spheres from the water was lower in the presence of cyanobacterial biofilms (Fig. 5.4b,c) and after 72 h micro-spheres concentration were $2.2 \times 10^6 \pm 1.2 \times 10^5$ and $7.8 \times 10^5 \pm 1.9 \times 10^4$ particles mL$^{-1}$ respectively. The trapping of particles by *L. foveolarum* after 72 h was high compared with the blank (Fig. 5.4b; $6.8 \times 10^6 \pm 4.6 \times 10^5$), whereas particle numbers in *C. stagnale* biofilm were similar to the blank treatment (Fig. 5.4c; $1.5 \times 10^7 \pm 2.5 \times 10^6$). The natural biofilms and cultured diatom biofilms all showed a stronger decrease in micro-sphere concentrations in the water compared with the blank treatment with concentrations of $2.0 \times 10^6 \pm 2.3 \times 10^5$, $2.4 \times 10^6 \pm 4.4 \times 10^5$, $1.3 \times 10^6 \pm 4.7 \times 10^4$ and $9.2 \times 10^4 \pm 4.0 \times 10^1$ particles mL$^{-1}$ after 72 h for *N. perminuta, A. lanceolata*, Natural biofilm I and Natural biofilm II, respectively (Fig. 5.4d-g). Simultaneously the number of particles in the biofilm increased ranging from $6.1 \times 10^5 \pm 1.2 \times 10^5$ particles cm$^{-2}$ (Fig. 5.4d; *N. perminuta*) to $1.8 \times 10^6 \pm 7.0 \times 10^4$ particles cm$^{-2}$ (Fig. 5.4f; Natural biofilm I) after 72 h. The first order removal constants ($K_{d}$) were 0.025 h$^{-1}$, 0.025 h$^{-1}$, 0.006 h$^{-1}$, 0.019 h$^{-1}$, 0.023 h$^{-1}$, 0.032 h$^{-1}$ and 0.068 h$^{-1}$ for the blank, *L. foveolarum, C. stagnale, N. perminuta, A. lanceolata*, Natural Biofilm I and Natural Biofilm II respectively (Fig. 5.6).

In the blank treatment (no biofilm present), *P. putida* had a maximum abundance in the water of $1.9 \times 10^6 \pm 4.3 \times 10^5$ cells mL$^{-1}$ after 30 min of incubation and then gradually decreased to $9.9 \times 10^2 \pm 4.0 \times 10^3$ cells mL$^{-1}$ after 72 h (Fig. 5.5a). A small fraction of *P. putida* cells attached to the empty glass slides during the first 8 h of incubation and decreased to zero again after 48 h incubation. In biofilms consisting of cyanobacteria (*L. foveolarum* and *C. stagnale*) numbers of *P. putida* cells also decreased to zero after 72 h (Fig. 5.5b,c). The *P. putida* abundance found in the *C. stagnale* biofilm did however show substantial increase of *P. putida* abundance in the biofilm after 24 h of incubation (Fig. 5.5c). Incubation with biofilms composed of *N. perminuta* removed *P. putida* cells in the water phase almost completely within 48 h of incubation, although this decrease did not result in a substantial increase in *P. putida* cells in the *N. perminuta* biofilm (Fig. 5.5d). Biofilms of *A. lanceolata* caused a very rapid decline of *P. putida* cells in the water phase within the first 24 h of incubation, which coincided with a strong increase of *P. putida* cells in the biofilm (Fig. 5.5e). Both natural biofilms, especially Natural biofilm I, showed a strong decrease of *P. putida* cells in the water phase, which completely disappeared within the first 48 and 24 h of incubation with respectively Natural biofilm II and Natural biofilm I (Fig. 5.2f,g). In Natural biofilm II an increase of *P. putida* cells in the biofilm were observed short after addition of *P. putida*, but rapidly decreased again within the 24 h (Fig. 5.5g). This rapid disappearance of *P. putida* cells in the biofilm was also observed in Natural biofilm I were *P. putida* almost directly disappeared (Fig. 5.5f).
**Fig. 5.4** Experiment 2; development of micro-sphere concentration (1µm; particles mL⁻¹) in suspension (solid black line with open circles) and number of micro-spheres in biofilm (particles cm⁻²; dotted grey line with solid squares) over an incubation period of 72 h after addition of micro-spheres at t=0 h.
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Fig. 5.5 Experiment 2; development of *P. putida* abundance (cells mL\(^{-1}\)) in suspension (solid black line with open circles) and numbers of *P. putida* in biofilm (cells cm\(^{-2}\); dotted grey line with solid squares; attachment to glass disk in blank) over an incubation period of 72 h after addition of micro-spheres at t=0 h.
Chapter 5

Discussion

Substantial trapping of particles was observed for particles smaller than 15 µm, while bigger (>15 µm) particles were hardly removed from suspension by the biofilms present. The efficiency of particle trapping by phototrophic biofilms was, however, found to be strongly dependent on the size of the suspended particles and an optimum particle trapping efficiency (almost 50% removal from the suspension in a short period of time) was observed for particles between 5.0-8.0 µm diameter. These findings of substantial particle trapping partially corroborate a study of Stott and Tanner (2005), who described increasing trapping efficiency by natural mixed species biofilm grown in constructed wetlands with increasing particle sizes ranging from viruses (0.1 µm), bacteria (1 µm) to parasitic protozoa (4.5 µm). The trapping of particles does not only occur at the surface of the biofilm, but also deeper in the biofilm depending on the transport of particles via water channels in biofilms (Okabe, Yasuda and Watanabe, 1997). The abundance and dimensions of these water channels may affect the penetration, collision chance, surface area and consequently affect the possibility for particle attachment in biofilms. Trapping of large particles may be hindered by lack of penetration possibilities, whereas small particles may have a relative lower chance of coming into contact with the biofilm, resulting in an optimum trapping efficiency of medium sized particles.

The removal rates ($k_d$) of the 1 µm diameter micro-spheres by the two natural biofilms (0.32 h$^{-1}$ and 0.68 h$^{-1}$) observed in experiment 2 of this study are comparable to previously observed removal rates (Stott and Tanner 2005). In our first experiment, however, much higher removal rates (ranging from 0.188 h$^{-1}$ and 0.246 h$^{-1}$) were observed. This difference could be explained by differences in the composition and maturity of the biofilms (resulting from differences in the colonization period) and

Fig. 5.6 The first order removal rates of particles from suspension in the first (left) and second (right) experiment for microspheres (grey bars) and pseudomonas putida (white bars). Microspheres used in the first experiment had a nominal size of 1 µm, in the second experiment size classes of particles were measured, but microspheres added had a nominal size of 2.0 µm, 4.5 µm, 6.0 µm, 10 µm and 25 µm (left to right).
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by differences in the experimental setup (mainly the acclimatization period in experiment 2 could have cleaned the biofilms before the addition of particles). Although the removal rates are based on the initial trapping of particles in clean biofilms, and are therefore probably an overestimation of long-term trapping rates, in general the biofilms showed a high capacity to strongly reduce the suspended particle concentrations in the water.

The second experiment showed that the composition of the phototrophic biofilm, which can differ greatly in growth form, mucus production and associated heterotrophic bacteria, is an important factor in the trapping of suspended particles. Diatom biofilms were more effective in trapping micro-spheres from the surrounding water compared to cyanobacteria biofilms which virtually trapped no particles. Stott and Tanner (2005) also showed differences in particle trapping depending on the species composition, where phototrophic biofilms removed more particles compared to heterotrophic biofilm.

Biofilms that were capable of trapping high numbers of micro-spheres also showed significant trapping of \textit{P. putida} cells with removal rates of \textit{P. putida} cells being even up to one order of magnitude higher than removal rates of the micro-spheres. This difference between micro-spheres and \textit{P. putida} cells could indicate the occurrence of selective particle trapping by the biofilms, but since \textit{P. putida} generally prefers a sessile lifestyle and actively attach to any solid surface, it cannot be excluded that this difference could also (partly) be caused by attachment to the walls of the aquaria. In addition, the biofilms seemingly kill off the majority of trapped \textit{P. putida} cells. In the natural biofilms, the rapid depletion of suspended \textit{P. putida} cells was accompanied with only a relative small increase and sequential rapid decrease of \textit{P. putida} cells in the biofilm. This disappearance of \textit{P. putida} cells was probably caused by protozoa commonly associated with natural biofilm (Curds 1982; Fenchel 1986). In a study of Chabaud \textit{et al.} 2006 the capacity of removal of particles from septic effluent was subscribed for 60% to biofilm associated protozoa. Eisenmann \textit{et al.} (2001) also subscribed a substantial contribution to the trapping of suspended particles from wastewater by ciliates and feed activity by ciliates in biofilm have been suggested to increase particles trapping (Okabe \textit{et al.} 1997; Eisenmann \textit{et al.} 2001). Moreover, certain phototrophic species inhabiting the biofilms could express antibiotic effects and this may also contribute to different killing rates of \textit{P. putida}.

These experiments have shown that the trapping of particles from suspension by phototrophic biofilms in aquatic ecosystems can be very substantial for particles smaller than 15 µm. The efficiency of particle trapping is however highly variable and is dependent on both the size and nature of the particles as the species composition of the phototrophic biofilm.

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