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The monitoring of biofilm formation in a mulch biowall barrier and its effect on performance

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Abstract

Lab scale mulch-biofilm biowall barriers were constructed and tested to monitor the effect of biofilm formation on the performance of the biobarrier. Naphthalene, a two-ring polycyclic aromatic hydrocarbon (PAH), was used as the model compound. With column reactors, the amounts of viable naphthalene degraders and biofilm formation were monitored, as was the performance of the biobarrier.

The sorption capacity of the mulch, the increase in biomass and the extracellular polymeric substance (EPS) content of the biofilm created a strong affinity for naphthalene and induced an increase in the number of slowly growing hydrocarbon degraders, resulting in a higher degradation rate and more stable PAH removal.

Concentration profiles of pore water naphthalene and electron acceptors indicated that dissolved oxygen (DO) was preferentially used as the electron acceptor, and the greatest removal occurred at the inlet to the column reactor where DO was highest. However, when using nitrate as an alternative electron acceptor, both biofilm formation and continual degradation of naphthalene also occurred. Microprofiles of DO in the biofilm revealed that oxygen transport in the biofilm was limited, and there might be sequential utilization of nitrate for naphthalene removal in the anoxic zones of the biofilm. These results provide insight into the distribution of viable biomass and biofilm EPS production in engineered permeable reactive mulch biobarriers.

Keywords

Biofilm; EPS; Permeable Reactive Biobarrier; Microelectrode

1. Introduction

Soil and groundwater contaminated by hydrocarbons have been source of world wide environmental concern (Yerushalmi et al., 1999) and has spurred the development of different bioremediation strategies for their effective removal. Recently, *in-situ* remediation has received much attention and has been successfully applied in the treatment of contaminated soil and groundwater as cost effective methods (Alexander, 1999; Kao et al., 2001). Among in-situ bioremediation technologies, use of permeable reactive barriers within contaminated aquifers has grown as a way to prevent further migration of dissolved hydrocarbons from

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contaminated plumes with minimal maintenance costs (Kao et al., 2001). The permeable reactive barrier has been tested for various organic compounds, at both lab scale and full scale. Lamarche et al. (2001) reported the field testing of a funnel and gate system filled with a coarse-medium silica sand containing 1% by volume of granular activated carbon. A field scale system was tested on a naphthalene plume for over 2 years, and up to a 98% removal efficiency was obtained. Miller et al. (2001) also reported on a full scale biobarrier system that achieved a treatment efficiency of more than 99.9% for dissolved benzene-toluene-ethylene-xylene (BTEX).

Among many different types of support material for use in permeable reactive barriers, solid organic materials, such as organic mulch and peat moss, have commonly been used in bioremediation and bioretention systems in order to increase the efficiency of the permeable reactive biobarrier (Aziz et al., 1997; Yerushalmi et al., 1999). Organic mulch has many biopolymers, and among them lignin is known to have a high affinity and sorption capacity for nonionic organic compounds (Garbarini and Lion, 1986); they can also act as complex additive fertilizer for hydrocarbon degradation (Kastner and Mahro, 1996). However, in addition to the sorption capacity of supporting material in the biobarrier, microbial activity and biofilm formation play important roles in the performance of the permeable reactive biobarrier. An increased biomass and the extracellular polymeric substance (EPS) content of the biofilm can also create a strong affinity for the hydrophobic organic compounds (Ebihara and Bishop, 2002; Moretti and Neufeld, 1989). The increased mass of organic carbon sorbed to the biofilm matrix, as well as to the organic mulch itself, can induce an increase in the number of slowly growing hydrocarbon degraders, a higher degradation rate and more stable removal.

In this aspect, the formation of an active biofilm is crucial for the stable operation of the permeable reactive barrier. However, even though active biofilm formation is crucial for treatment efficiency and for preventing contaminant migration through the permeable reactive barrier, structural forms of the biofilm exposed to the hydrocarbons and distribution of hydrocarbon degraders are not well understood. Most studies have focused on the total removal of a target hydrocarbon with a variety of supporting materials, and only a few studies have considered the biofilm formation generated over long periods from growth on the hydrocarbons.

In this research, lab scale mulch-biofilm barriers were constructed and tested to evaluate the effect of biofilm formation on the performance of the biobarrier. Naphthalene, a two-ring polycyclic aromatic hydrocarbon, was used as the model compound. Viable naphthalene degrader populations and resultant biofilm formation were monitored, as was the performance of the biobarrier.

2. Materials and Methods

2.1. Organic mulch

Based on preliminary sorption tests and physicochemical analyses, hardwood bark mulch was selected as the model supporting material for the lab scale biobarrier (Seo et al., 2007). Based on element analysis using an element analyzer (Perkin Elmer 2400), the hardwood mulch was found to be composed of carbon (42.2%), oxygen (50.7%), hydrogen (5.4%) and nitrogen (0.68%). Physicochemical properties include a water content of $55.9 \pm 6\%$, a pH of 7.43 ± 0.28 , a cation exchange capacity of $42.3 \pm 3.4 \text{ meq } 100^{-1} \text{ g}^{-1}$, and a conductivity of $0.36 \pm 0.01 \text{ mS cm}^{-1}$ at 25°C .

2.2. Isotherm experiment and model simulation

In order to estimate the adsorption capacity of the hardwood mulch for dissolved naphthalene, isotherm tests were conducted using the bottle point method. Teflon-lined screw cap bottles (75 mL) were filled to the top with naphthalene solution to minimize headspace. When equilibrium was reached, the liquid phase naphthalene concentration (C_e , mg L⁻¹) was measured, and the concentration of naphthalene on the mulch (Q_e , mg g⁻¹) was calculated. All experimental data were correlated to the Freundlich isotherm equation: $Q_e = K_f C_e^{1/n}$ where K_f and n are the Freundlich empirical constants obtained using least-squares regression analysis.

Based on the isotherm results, the breakthrough curve for naphthalene in the columns was predicted using the constant pattern homogeneous surface diffusion model (CPHSDM) developed by Hand et al. (1982). The simulation was conducted using Adsorption Design Software (AdDesignSTM).

2.3. Column setup

Lab scale mulch-biofilm column reactors (Seo et al., 2007) were used to evaluate biofilm formation and the degradation of the model hydrocarbon, naphthalene, during simulated subsurface aerobic permeable biowall treatment. Two glass columns were constructed, each with a 300 mm length and 38 mm inner-diameter; each was equipped with five sample ports along the length, as well as four specially constructed flow cell ports for destructive biofilm analysis.

To obtain a naphthalene degrading biomass, activated sludge obtained from a wastewater treatment plant (Polk Run WWTP, Cincinnati, OH) was mixed with a suspension of PAH contaminated soil and was acclimated to naphthalene in a master culture reactor for 1 month. The resulting mixed consortia was inoculated into each column which contained 95 g of mulch. A 50 L mineral salts solution tank was connected to a naphthalene tank (200 mg l⁻¹). The mineral salts solution was composed of 64 mg L⁻¹ NaNO₃, 20 mg L⁻¹ NH₄Cl, 80 mg L⁻¹ Na₂HPO₄·7H₂O, 20 mg L⁻¹ KH₂PO₄, 2.8 mg L⁻¹ CaCl₂·H₂O, 7.6 mg L⁻¹ MgSO₄, 1.3 mg L⁻¹ FeCl₃·6H₂O, 0.0224 mg L⁻¹ MnSO₄, 0.0014 mg L⁻¹ CuSO₄, 0.0008 mg L⁻¹ Na₂MoO₄·2H₂O and 0.024 mg L⁻¹ ZnSO₄·7H₂O. The mineral salts solution was mixed in the naphthalene tank, and the combined solution was supplied to the bottom of each column at a constant flow rate of 2.55 mL min⁻¹ which simulated 1.6 m d⁻¹ linear velocity. The flow rate chosen was selected based on the particle size of the hardwood mulch and its hydro-conductivity (Hillel, 1998; Linsley and Franzini, 1972).

Four flow cells attached to each column were also operated under the same conditions as the main column, using a micro cassette pump, and were sacrificed for biofilm analysis in the middle of column operation in order not to disturb the main columns.

To prevent naphthalene particles from leaving the influent tank, filter fabric was attached to the outlet line of the naphthalene tank and a glass frit was also constructed at the inlet port of the column. Dissolved oxygen was supplied to the biofilm by pumping 0.8% hydrogen peroxide solution through the column at a 1.45 mL hr⁻¹ flow rate. The DO at each sampling port was monitored using a dissolved oxygen meter equipped with a flow-through mini-electrode (Microelectrodes, Inc. Londonderry, NH). For one month, two columns were operated in parallel under the same conditions in order to develop the necessary attached biofilm. After that, one column was converted to an abiotic-controlled system by supplying 1 g L⁻¹ of sodium azide. For the abiotic control column, only sodium azide was applied because decomposition of added hydrogen peroxide caused air entrainment and disturbance of flow in the column. Periodically, samples were obtained from the five column sampling ports; the samples were

filtered before HPLC analysis. Schematic diagrams of the column reactors and flowcells can be found in the supplementary material.

2.4. Instrumental analysis

Naphthalene was analyzed using reverse phase HPLC (Agilent 1100, Agilent Technologies, CA) with a C18 silica column (Supelcosil LC-18DB, Sigma-Aldrich, PA) and a diode array detector. A mixture of 40% Milli-Q water and 60% acetonitrile solution was used as the mobile phase. Using a 1.5 mLmin⁻¹ flow rate and an elevated temperature (55 °C), naphthalene was measured at an absorbance of 218 nm and a retention time of 2.8 min.

In order to monitor utilization of alternative electron acceptors, nitrite, nitrate, sulfate and chloride were monitored with a DIONEX DX-120 system, and concentrations of nitrite, nitrate, and sulfate were corrected with the chloride concentration.

2.5. Structural composition of biofilm

In situ monitoring of biofilm formation—A flow cell from the first sampling port was used to conduct biofilm analysis during the middle of column experiment runs. An upright confocal laser scanning microscope (CLSM) (Zeiss LSM 510) was used in order to monitor bacterial viability as well as to monitor the physical structure and heterogeneity of growth patterns of the multi-species biofilm grown on the organic mulch.

The LIVE/DEAD BacLight bacterial viability kit (Molecular Probes, Eugene, OR) was used to stain live and dead cells in the multi-species biofilm. This stain is composed of equal volumes of SYTO 9 and propidium iodide (PI). PI penetrates damaged cell membranes and stains damaged cells red, while SYTO 9 stains live cells green. Excitation/emission maxima were 480/500 nm for SYTO 9 and 490/635 nm for PI, respectively. Images were obtained using a 60×, 1.3 numerical aperture oil immersion lens. Green (SYTO 9) images were collected using a band pass 505-530 emission filter, and red (PI) images were collected using a long pass 560 emission filter.

In order to measure the biofilm thickness, DO microelectrodes were applied for determining the location of the biofilm surface and the bottom. In detail, using a light source from above the biofilm surface, as well as a video monitor connected to the microscope, the surface of biofilm was observed when the microelectrode touched the biofilm. After that, the microelectrode was slowly moved into the biofilm with a micro-manipulator. When the microelectrode hit the substratum of the biofilm, the microelectrode would bend slightly. The thickness of the biofilm could be measured by the distance the microelectrode moved inside the biofilm from the surface to the substratum.

Destructive biofilm analysis—To monitor biofilm formation and the amount of viable biomass in the presence of naphthalene, destructive biofilm analyses, including protein and carbohydrate as well as total lipid phosphate analysis for viable biomass, were conducted. The biofilm analyses was conducted three times (beginning of the column experiment, middle of column operation, and at the end of column operation). Flow cells attached to the columns were sacrificed to conduct the biofilm analyses in the middle of the column experiment without disturbing the main column. The protein content of the biofilm was determined by adaptation of the Bradford method, as described by the Coomassie Protein Assay Technical Handbook (Pierce Biotechnology, Rockford, IL). Total carbohydrate was measured using the phenol reaction method, described by Daniels et al. (1994), as an estimate of the polysaccharide content of the biofilm tested.

For viable biomass analysis, total lipid phosphate (LP) was measured using the modified procedure developed for the viable biomass in sediments (Findlay et al., 1989). Findlay et al. (1989) reported that phospholipids have a good correlation to cell biomass, and once a cell dies, the phospholipids have a short half life.

2.6. Measurement of oxygen transport with a microelectrode

In order to monitor oxygen transport and flux in the biofilm, oxygen profiles inside the biofilm under different flow rate conditions were measured using an oxygen microelectrode. The microelectrode fabrication procedure by Yu (2000) was followed. The oxygen microelectrodes had 3~5 μm tip diameters and were referenced against a Ag/AgCl reference electrode under an electric potential of -750 mV. Three point oxygen calibrations were performed using three DO concentrations (0%, 10%, and 21%) before and after measurements in order to monitor any response drift. The microelectrode was mounted on a motor-driven micromanipulator, and the oxygen profile in the biofilm on the mulch was measured at 10 or 50 μm intervals under three different flow rate conditions (5, 10, 15 m d^{-1}).

Naphthalene was dissolved using the same composition of mineral salts solution as for the column. The solution was aerated with 21% oxygen and recycled through the open channel chamber using a peristaltic pump. The naphthalene concentration in the mixed solution tank was around 25 mg L^{-1} and 17 mg L^{-1} before and after the experiment, respectively.

3. Results and discussions

3.1 Model simulation and performance of biobarrier

In order to determine the amount of naphthalene removal by sorption to organic mulch during column operation, adsorption isotherm testing of the naphthalene to the mulch was conducted. The Freundlich isotherm model was applied to define the relationship between solid phase loading and the aqueous phase solute concentration. The Freundlich parameters K_f and n^{-1} for the hardwood mulch were found to be 0.280 and 0.767, respectively.

Based on the Freundlich isotherm for the mulch, model simulation using the CPHSDM method was conducted to predict the naphthalene breakthrough curve. Figure 1 shows the performance of the mulch biobarrier, along with the mathematical model simulation. Based on the model simulation using the abiotic sorption capacity of mulch, the column containing hardwood mulch lost its sorption capacity, and breakthrough of naphthalene was reached, in 10 d. The mulch had been thoroughly washed to remove sand and fine debris. The relatively small sorption capacity might have been caused by the vigorous pretreatment of mulch as well as by the high naphthalene loading rate.

Lab scale mulch biofilm column reactors were operated continuously for 210 d. For 30 d, two columns were operated in parallel under the same conditions in order to generate attached biofilm formation on the mulch. Both columns were operated under biotic conditions, and during the initial 5 d of the operation period, over 99% naphthalene removal was achieved.

From d 5 to d 30, effluent concentrations varied around 2~5 mg L^{-1} , a 77-99% removal efficiency. During this operating period, there was degradation of naphthalene by the active biofilm, but it appeared that the sorption capacity of the mulch, which was providing most of the naphthalene removal, might already be losing some of its sorption capacity.

At d 30 of operation, one column was converted to an abiotic control system by supplying 1 g L^{-1} of sodium azide. From the point of sodium azide injection, the abiotic control column began to lose its ability to degrade naphthalene, and the effluent concentration reached 99% of the influent concentration in 16 d.

Considering the results of the model simulation and the loss of sorption capacity, the abiotic column showed a slower breakthrough of naphthalene than that predicted by the abiotic model simulation. The difference between observed concentrations and predicted concentrations might be related to resistance of the biofilm to the biocide. It has been reported that bacteria grown in EPS show sequestering effects and a high resistance to biocide (Bishop, 1997). Even under high concentrations of sodium azide, it appeared that the bacteria showed some resistance to the biocide for a while. Another possible reason might be the sorption capacity of the biofilm biomass. Many researchers have reported that PAHs have a high degree of affinity to microbial biomass (Stringfellow and Alvarez-Cohen, 1999; Ebihara and Bishop, 2002). Produced biomass attached to the mulch and its EPS might provide increased sorption capacity for aqueous naphthalene.

After one month, in the biotic column, the effluent naphthalene concentrations decreased again and became negligible for the remainder of the operation; a consistent removal of 94-99.99% was obtained. Considering the high loading rate, the mulch biofilm barrier showed very stable removal for long periods, due to sorption on the mulch as well as biodegradation within the biofilm.

Sorption of hydrophobic organic compounds (HOC) is important for stable operation in a permeable reactive biobarrier, because the biobarrier must contend with the low growth rate of the HOC-degrading microorganisms and the relatively short passage time for the organic pollutants through the biobarrier. In a mulch biofilm barrier, in addition to the sorption capacity of the mulch, the increased biomass and EPS of the biofilm can also provide additional sorption capacity. Therefore, the sorption capacity of the mulch and the biofilm EPS could prevent migration of naphthalene. Also, naphthalene adsorbed to the mulch could be desorbed and biodegraded by the attached biofilm, thus regenerating the sorption capacity.

In addition to the increased sorption capacity due to biofilm formation, the increased mass of hydrophobic organic compounds sorbed to the biofilm matrix, and the ability of the mulch to induce an increase in slowly growing hydrocarbon degraders, higher degradation rates and more stable removal result.

3.2. Structural composition of biofilm

In situ monitoring of biofilm formation—Because of the large particle sizes of the mulch and the small working distance afforded by the CLSM, the flowcells were opened and mulch biofilm samples were carefully removed from the flowcells for CLSM image analysis. Typically, 10 to 15 representative locations were examined.

A significant amount of direct bacterial cell attachment imbedded in EPS was observed on the organic mulch. In mulch biofilm barriers containing naphthalene in its pore water, a film type biofilm formation was observed overall.

The surface biofilm mostly consisted of a single layer of biofilm. However, in many places, large cluster-type biomass structures were also observed, as were protrusion type biofilm structures. Based on thickness measurements with microelectrodes, the monolayer biofilm had a thickness of 20 to 1100 μm .

The general characteristic structure of the biofilms observed in the mulch biofilm barrier studied here was similar to that of a toluene degrading biofilm growth on sand (Ebihara and Bishop, 1999). However, because of the larger mulch particle size, as well as the organic material present in the mulch, the biofilm thickness grown on mulch was greater, and more development of biofilm aggregates in coarse pore spaces was observed. A CLSM image of a typical biofilm formation on organic mulch can be found in the supplementary material.

Destructive biofilm analysis—The EPS composition of the biofilm and the amount of viable biomass utilizing naphthalene in the biobarrier were monitored during the experimentation. Biofilm EPS was characterized by monitoring the changes in carbohydrate and protein content, and the amount of viable biomass was measured by total lipid phosphate analysis.

Figure 2 shows the changes in viable biomass concentration during the biobarrier operation. The highest LP concentration ($908 \pm 111 \text{ nmol g}^{-1}$) was observed at the bottom of column. This was likely due to low linear flow velocity and the highest presence of oxygen and substrate at that point. After the column inlet, the highest amount of viable biomass was observed at the first sampling port. It was measured as $572 \pm 91 \text{ nmol g}^{-1}$ at 150 d and $800 \pm 180 \text{ nmol g}^{-1}$ at 210 d.

After the 150 d sampling point, LP concentrations increased overall (around 2 to 4.5 times higher than initial concentration) along the column, implying an increased number of naphthalene degraders per unit volume. After 210 d, an increased amount of viable biomass was also observed at the second sampling port (12 cm from the bottom of column) when compared with values from 150 d. However, LP concentrations decreased higher up in the column, from 483 to 282 nmol g^{-1} (at 18 cm), from 360 to 99 nmol g^{-1} (at 24 cm), and from 148 to 83 nmol g^{-1} (at 30 cm). This decrease might be related to increased growth of viable naphthalene degraders at the second sampling port. This increase in naphthalene degraders to the second sampling port might have caused a depletion of oxygen, substrate and micronutrients. Considering the significant increase of viable biomass at the first sampling port, it appears that the availability of DO plays an important role for the naphthalene degrading bacteria. However, even though measurable oxygen concentration became negligible after the first sampling port, an increased amount of viable biomass was also observed at the second and third sampling port. It seems there was still biomass growth under microaerophilic conditions.

In the abiotic control column, there were large LP concentration differences compared to the biotic column, as expected. LP content decreased continually, except at the inlet port to the column. A slight increase in viable biomass in the abiotic column at the inlet port might be caused by some growth of viable biomass in the naphthalene tank. Overall, LP concentrations decreased with time. However, LP concentrations did not decrease to zero; even 1 g L^{-1} of sodium azide didn't inactivate the bacteria completely.

Figure 3 shows the changes in protein content of biofilm biomass during the 210 d of biobarrier operation. After inoculation, the initial protein concentration was $180 \pm 56 \mu\text{g g}^{-1}$ and the protein content began to increase along the length of column. In the biotic column, the protein concentration was also highest at the inlet port of the column where oxygen and other micronutrients were most abundant. At the first sampling port, where oxygen was still present, the protein concentration increased from $180 \pm 56 \mu\text{g g}^{-1}$ to $1015 \pm 309 \mu\text{g g}^{-1}$ at d 150, and from 1770 to 74 $\mu\text{g g}^{-1}$ at d 210.

The protein content continually increased as time went on up to 12 cm from the bottom of the column. However, after the 12 cm point, the protein content became relatively constant or decreased slightly over time. Similarly to the LP results, this might be related to substrate depletion in the most biologically active zone of the column (up to 12 cm), with remaining viable cells utilizing mainly the EPS under these starvation conditions (Zhang and Bishop, 2003).

In the abiotic column, the protein concentration remained in the range of 142 to 398 $\mu\text{g g}^{-1}$. After sodium azide injection, even though the amount of viable biomass decreased

significantly, the protein content of the biofilm biomass remained relatively constant along the column, and overall the protein content was higher than the initial protein content. This might be caused by cell lysis material as well as by the existence of preformed EPS protein on the mulch before the sodium azide injection. The amount of protein in the killed control was $28 \pm 10\%$ of that in the biotic column in the biologically active zone (to 12 cm) and $57 \pm 11\%$ in the less active zone (12 to 30 cm).

The carbohydrate content of the biofilm EPS was measured using the phenol sulfuric reaction method. However, due to the high carbohydrate content of the mulch and the presence of small debris within the biofilm, the carbohydrate measurement was significantly inhibited and reliable results could not be obtained.

Overall, significant amounts of microbial protein were produced and increases in viable biomass were observed in the mulch biobarrier. The structural composition of the biofilm formation in the biobarrier indicated a great dependence on the presence of substrate and dissolved oxygen.

3.3 The effect of biofilm formation on naphthalene removal and electron acceptor concentration profiles

Figure 4 shows concentration changes of naphthalene and electron acceptors at various depths in the biotic column. H_2O_2 (0.8%) was supplied to provide DO inside the column. The oxygen concentration at the influent port ranged from 8.60 to 5.83 mg L^{-1} , but was consumed and reduced to 1.29 to 0.08 mg L^{-1} (generally less than 0.12 mg L^{-1} after 100 d) at the first column port. After the first port, the column operated under either microaerophilic or anoxic conditions. Along the length of the biotic column reactor, the naphthalene removal occurred within the first 6 cm of the column from the influent port where oxygen was most abundant.

This result is consistent with the results for the biofilm EPS and viable biomass composition. The alternative electron acceptor, nitrate decreased from 51.2 to 43.8 mg L^{-1} at the first port and then continually decreased to 38.8, 36.3, and 33.2 mg L^{-1} at the second, third, and fourth ports, respectively. Even at the first port, where the oxygen concentration and biofilm growth were the highest, the nitrate concentration decreased by 7.4 mg L^{-1} . It appears that microaerophilic conditions were created, but biodegradation of naphthalene still occurred through a combination of oxygen and nitrate utilization. After the first port, under microaerophilic or anoxic conditions, naphthalene was also removed continually using nitrate. Sulfate concentrations remained constant along the length of the column and were not utilized.

Recent studies show that low molecular weight PAHs can be degraded with the use of nitrate, and possibly sulfate, as the electron acceptors (Rockne and Strand 1998, Uribe-Jongbloed, 2006). It has also been reported that the presence of alternative electron acceptors; in addition to oxygen, can stimulate enhanced hydrocarbon degradation (Wilson and Bouwer, 1997). Yerushalmi et al. (1999) reported that, in their permeable reactive barrier study with biofilm grown on peat moss, high removal efficiencies of gasoline and BTEX could be achieved under microaerophilic conditions. This previous research suggested that it might be advantageous to apply nitrate along with oxygen to degrade recalcitrant aromatic hydrocarbons because of the high solubility of nitrate and its energy yield close to that of oxygen. Our results also indicate that nitrate was utilized for naphthalene removal as an alternative to oxygen when oxygen was depleted.

3.4. Measurement of oxygen transport in mulch biofilm

Oxygen flux and transport inside biofilm were monitored with an oxygen microelectrode after 120 d of column operation. For the microelectrode measurements, a biofilm aggregate with a

relatively large thickness (800 μm) was taken from a flowcell and used. Three different velocities (5, 10, 15 m d^{-1}) were considered as possible flow rates, based on the porosity and the hydraulic conductivity of the mulch biofilm barrier, in order to monitor the effect of velocity on oxygen transport.

Figure 5 shows the microprofiles of DO transport into the biofilm. The oxygen concentrations in the bulk solution were 7.4 mg L^{-1} to 8.2 mg L^{-1} , depending on the flowrate. Increased flow velocity changed the thickness of the mass transfer boundary layer and the resulting oxygen distribution in the biofilm. The thicknesses of the dissolved oxygen mass transfer boundary layers were 250, 100, and 60 μm for 5, 10, and 15 m d^{-1} flow velocities, respectively. Oxygen uptakes (oxygen flux, J , into the biofilm) were estimated using the measured oxygen profiles. Fick's first law, $J=D \text{dC/dX}$, where D = diffusivity of oxygen in water ($2.12 \times 10^{-5} \text{ cm}^2 \text{ s}^{-1}$) and dC/dX =measured concentration gradient at the boundary layer, was applied to calculate the oxygen uptake rates. They were found to be 0.269, 0.335, and 0.47 ($10^{-6} \text{ mg cm}^{-2} \text{ s}^{-1}$) at velocities of 5, 10, 15 m d^{-1} , respectively.

Even though higher flow rates gave increased oxygen transport near the biofilm / bulk solution interface, the increased oxygen flux was consumed inside the biofilm and oxygen was completely depleted before it reached the substratum. DO could not be transported more than 430 μm into the biofilm under any of the three flow rates, and there was a 400 μm thick anoxic zone in biofilm. Considering the thickest biofilm observed in our studies, the thickness of the anoxic zone could be as much as 800 μm .

In contrast to dispersed bacterial growth, mass transport greatly influences the rate of biotransformation in attached biofilm systems, and biofilm may be flux limited by electron donor and acceptor concentrations (Fu, 1993). Wilson and Bower (1997) reported that complex mechanisms involved in the regulation of electron transfer to oxygen or nitrate will be controlled by the critical oxygen concentration and associated transition range. However, in addition to these, mass transfer limitations such as oxygen diffusion and media pore space may also play important roles in determining whether oxygen and nitrate will be used simultaneously or sequentially during biodegradation in the subsurface.

Monitored results of the electron acceptor concentration in the column and the oxygen microprofile in the biofilm indicate that there might be simultaneous naphthalene degradation using both oxygen and nitrate in the biofilm.

4. Conclusions

The structural composition of biofilm formation when exposed to the polycyclic aromatic hydrocarbons and its effect on the performance of a biobarrier were observed. Film type biofilm formation, as well as large cluster types of structures, was observed. Destructive biofilm analysis revealed that significant amounts of EPS substances were produced as part of the increased viable biomass in the mulch biobarrier. Structural composition based on the protein content of the EPS and viable cells concentrations in the mulch biobarrier indicated a great dependence on substrate and dissolved oxygen.

The sorption capacity of the mulch prevented fast migration of hydrocarbons and helped the biobarrier to overcome its initial lag phase of bacterial biofilm formation, until it eventually increased the amount of viable biomass per unit area. Considering the high loading rate, the organic content of the mulch, the increased biomass and the extracellular polymeric substances also increased the sorption capacity of mulch biofilm barrier.

Concentration profiles of naphthalene and electron acceptors in mulch pore water and biofilms indicated that dissolved oxygen is preferentially used and resulted in the greatest removal in

the column. However, while using nitrate as the electron acceptor, the biofilm still continued to degrade the naphthalene. Microprofiles of DO in the biofilm revealed that oxygen transport in the biofilm was limited. This indicates there might be sequential utilization of oxygen followed by nitrate for naphthalene removal in the anoxic zone of the biofilm.

This study contributes to giving an insight into developing permeable biofilm barriers with new supporting materials, and the importance of biofilm formation for attenuation of the transport of hydrophobic contaminants through groundwater.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Performance of the mulch biofilm barrier for 210 days: Influent naphthalene concentration (●), Effluent concentration of biotic column (○), Effluent concentration of abiotic column (▼), Model simulation result (solid line). The insert shows model simulation results for days 1 to 10.

Fig. 2.
Lipid concentration changes in mulch biofilm barrier: (a) Biotic, (b) Abiotic condition.

Fig. 3. Protein concentration changes in mulch biofilm barrier: (a) Biotic, (b) Abiotic condition.

Fig. 4. Concentration profiles of naphthalene in pore water and electron acceptors in column. : Effluent concentration of naphthalene in biotic column (●), in abiotic column (○), Oxygen (▲), Nitrate (■), Sulfate(▼).

Fig. 5.
Microprofiles of dissolved oxygen in biofilm.