The influence of substrate and electron acceptor availability on bioactive zone dynamics in porous media

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Abstract

Two approaches were used to investigate the influence of dissolved oxygen (DO) and substrate availability on the formation and dynamics of “bioactive zones” in a water-saturated porous medium. A bioactive zone is defined as a region where a microbial community is sufficiently active to metabolize bioavailable substrates. In the first approach, microbial activity was characterized by monitoring the spatial and temporal variability of DO and aqueous substrate (salicylate and naphthalene) concentrations during miscible-displacement experiments. In the second approach, microbial activity was monitored using multiple fiber optics emplaced in the porous medium to detect luminescence produced by *Pseudomonas putida* RB1353, a bioluminescent reporter organism that produces light when salicylate (an intermediate of naphthalene degradation) is present. The results of both approaches show that the location and size of the bioactive zones were influenced by in situ DO and substrate availability. When DO was not a limiting factor (i.e., lower substrate input concentrations), the bioactive zone encompassed the entire column, with the majority of the microbial activity occurring between the inlet and midpoint. However, as the availability of DO became limiting for the higher substrate input

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experiments, the size of the bioactive zone shrank and was ultimately limited to the proximity of
the column inlet.

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1. Introduction

Contamination of soil and groundwater by hazardous organic chemicals resulting from improper disposal, accidental spills, or leaking underground storage tanks is recognized as a major environmental and health concern. In situ bioremediation has gained widespread interest for remediating organic-contaminated soils and aquifers due to its cost effectiveness and applicability to a wide range of pollutants (National Research Council (NRC), 1993). In situ bioremediation applications often involve the addition of limiting nutrients and electron acceptors to stimulate biodegradative processes. The injection of these essential substances into contaminated soils or aquifers creates regions called bioactive zones wherein the microbial community becomes sufficiently active to metabolize bioavailable substrates (Odencrantz et al., 1990; Wookeun et al., 1990; Holden and Firestone 1997; Keijzer et al., 1999; Mahinthakumar et al., 1999). Successful in situ bioremediation relies on the formation of these microbiologically active zones (NRC, 1993). The location and size of these bioactive zones are controlled by both physicochemical (e.g., subsurface heterogeneity, environmental conditions, the nature and concentrations of substrates, electron acceptors, and nutrients) and microbiological (e.g., the dynamics and physiological status of the microbial community) factors. Because many of these factors are in a state of flux in the subsurface environment, and because the subsurface environment is generally heterogeneous, the formation of bioactive zones is expected to be a temporally and spatially variable process.

Despite their importance, relatively few studies have focused specifically on characterizing the formation and dynamics of bioactive zones. For example, Odencrantz et al. (1990) demonstrated that the location and size of bioactive zones can be successfully controlled through the location-specific injection of electron acceptors. In an additional study, Wookeun et al. (1990) showed that greater removal rates of halogenated compounds are achieved with an increased contaminant residence time within the bioactive zone. Keijzer et al. (1999) characterized the performance of in situ bioremediation using an analytical model that incorporated both the contaminant removal rate and biologically active zones. This study showed that a decrease in flow rate or the injected electron acceptor concentration resulted in a smaller, more efficient bioactive zone and therefore an improvement in bioremediation performance.

The primary goal of this research was to examine the spatial and temporal distribution of a biologically active zone, as well as the degree of microbial activity within the zone, in response to local changes in substrate and electron acceptor availability. Two methods were employed to accomplish this goal. The first method involves monitoring of substrate
(salicylate and naphthalene) and dissolved oxygen concentrations at various locations within a column system. The second method involves noninvasive, real-time monitoring of in situ reporter gene activity in the column system. Reporter gene activity, reflecting substrate (naphthalene) degradation, was monitored by employing a multiple fiber-optic detection system to collect luminescence produced by *Pseudomonas putida* RB1353, a bioluminescent reporter organism.

### 2. Materials and methods

#### 2.1. Solutions and sorbent

Sodium salicylate (C\(_7\)H\(_5\)NaO\(_3\); Sigma, St. Louis, MO) and naphthalene (C\(_{10}\)H\(_8\); EM Science, Gibbstown, NJ) were chosen as the model substrates. Salicylate (160.1 g mol\(^{-1}\)) has a low vapor pressure (0.44 Pa) and a high aqueous solubility (1100 g l\(^{-1}\)), which allows investigation of the influence of a large range of substrate input concentrations on system behavior. In addition, salicylate is not sorbed by the porous medium used in this study, which eliminates bioavailability constraints. Conversely, naphthalene has a relatively large vapor pressure (11 Pa), small aqueous solubility (31 mg l\(^{-1}\)), and is slightly sorbed by the porous medium used in this study. Naphthalene was used as a representative hydrocarbon contaminant.

Mineral salts broth (MSB) was used as a nutrient/electrolyte solution for the column experiments. MSB contains (per liter): 1.5 g KH\(_2\)PO\(_4\), 0.5 g Na\(_2\)HPO\(_4\), 0.2 g MgSO\(_4\)-7H\(_2\)O, 2.5 g NH\(_4\)Cl, 0.3 mg FeCl\(_3\), and 13.2 mg CaCl\(_2\)-2H\(_2\)O, and is adjusted to pH 7.0. A well-sorted (20/30 mesh) quartz sand was used as the model porous medium (North Kato Supply, Mankato, MN). Based on sieve analysis, 99% of the sand is comprised of particle diameters in the range of 600–850 \(\mu\)m, with a mean particle diameter of 700 \(\mu\)m. The sand has an organic carbon content of 0.04% and a cation exchange capacity of 5.7 mEq kg\(^{-1}\).

#### 2.2. Bacterial strain

*P. putida* RB1353, provided by Dr. Robert Burlage (University of Wisconsin, Milwaukee, WI), was used in all experiments. This strain contains plasmids NAH7 and pUTK9 (kanamycin resistance). The NAH7 plasmid contains the genes for naphthalene degradation in two operons referred to as the upper and lower pathways, both regulated by the nahR gene product. The upper pathway degrades naphthalene to salicylate while the lower pathway is responsible for the degradation of salicylate to acetylaldehyde and pyruvate (Schell, 1990; Yen and Serdar, 1988). In the presence of salicylate, the regulatory protein NahR activates the upper and the lower pathways by binding to the upper and lower pathway promoters (Burlage et al., 1990). Naphthalene does not directly induce the upper or lower pathway. However, when naphthalene is present, constitutive low levels of expression of the upper pathway result in conversion of naphthalene to salicylate, at which time full induction occurs (Burlage et al., 1990; Schell, 1990; Yen and Serdar, 1988).
The reporter plasmid, pUTK9, contains a subclone in which the NAH7 upper-pathway promoter is fused with the luxCDABE genes from *Vibrio fischeri* (Burlage et al., 1990). When salicylate is present, induction of the lux genes results in production of luminescence. Thus, naphthalene biodegradation can be monitored by measuring the luminescence produced upon conversion of naphthalene to salicylate. Neilson et al. (1999) indicated that the presence of the pUTK9 plasmid containing the lux genes does not deleteriously impact the growth and biodegradation potential of the organism. In previous studies, Burlage et al. (1990) demonstrated that light production by RB1351, an identical sister clone to RB1353, was directly correlated to naphthalene catabolism. In addition, Neilson et al. (1999) and Yolcubal et al. (2000) observed a linear relationship between salicylate concentration and luminescence for RB1353 in batch and column studies, respectively.

The bacterial strains were cultured and maintained in Luria Broth media (LB) supplemented with kanamycin to select for pUTK9. LBK medium consists of (per liter): 10 g tryptone, 5 g yeast extract, 10 g NaCl, 100 mg kanamycin sulfate, and is adjusted to pH 7. Agar plates were made by adding 15 g l\(^{-1}\) Bacto-agar (Difco Laboratories, Detroit, MI) to the LBK medium. The bacterial strains were stored frozen in 12% glycerol. Fresh cultures were inoculated from a frozen stock for each experiment to avoid plasmid loss. Precultures were inoculated in a 250-ml flask containing 25 ml of LB media and 250 µl of filter-sterilized 10 mg ml\(^{-1}\) kanamycin solution, and placed on a shaker (120 rpm, 24 °C) for 24 h. Following serial dilutions, growth cultures were prepared at a cell density of 10\(^5\) colony forming units (CFU)/ml from the preculture, placed on a shaker, and allowed to grow to stationary phase, which required approximately 48 h (Neilson et al., 1999). A 20-ml aliquot of cell solution was taken from the growth culture and centrifuged for 10 min at 9000 rpm to pellet the cells. The pelleted cells were washed once with 20 ml of 0.85% NaCl and then resuspended in 20 ml of sterile MSB solution. This resulted in a final suspension of approximately 10\(^9\) CFU ml\(^{-1}\). Following cell harvesting, serial dilutions were prepared to obtain a final concentration that would produce a cell density of approximately 10\(^7\) CFU g\(^{-1}\) dry sand upon inoculation of the porous medium.

### 2.3. Column experiments

A stainless steel column (ModCol, St. Louis, MO) with dimensions of 5 cm inner diameter by 10 cm in length was used for the column experiments. The column has ports near the inlet and the midpoint of the column for collecting in situ aqueous samples for measurement of substrate and DO concentrations. The column effluent was also monitored for substrate, DO, and cell density. The column has five luminescence measurement ports along the column axis (0.2, 2.1, 3.2, 5.0, and 7.5 cm from the inlet) into which optical fibers were inserted for continuous data acquisition. The fibers were inserted such that their tips were at the center of the column. The column was connected to a single-piston pump (SSI Accuflow series II, Deerfield, IN) using stainless steel tubing with a two-way valve to switch between MSB and substrate solutions (Fig. 1).

Prior to each experiment, a 2% bleach solution was used to sterilize the apparatus. After treatment with bleach, the system was flushed with 0.01% sterile sodium-thiosulfate to
neutralize the bleach. Finally, the system was flushed with sterile deionized, distilled water. The glassware and solutions were also sterilized.

For each column experiment, 420 g of sterile sand was inoculated with *P. putida* RB1353. The inoculated sand was mixed thoroughly under a laminar flow hood to ensure a homogeneous distribution of bacteria. Subsamples were plated in triplicate on LBK plates for determination of initial cell density. The column was then packed in incremental steps with the inoculated sand under sterile conditions to obtain uniform bulk density. After packing, the column was saturated from the bottom for 17 h (≈ 15 pore volumes) with sterile MSB solution at a flow rate of 1 ml min$^{-1}$. The MSB and salicylate solution reservoirs were continuously sparged with oxygen during both saturation and substrate injection to reduce oxygen limitations. To prevent naphthalene volatilization during the experiment, naphthalene solutions were prepared using a pre-sparged MSB solution in a custom-made tedlar bag with no headspace. Following saturation, a substrate pulse at the concentration of interest was injected into the column. After the substrate pulse was completed, the column was flushed with two pore volumes of MSB at a flow rate of 1 ml min$^{-1}$. Samples were collected from the reservoir prior to and after each experiment to ensure that salicylate and naphthalene concentrations remained constant.

Column experiments were conducted using salicylate input concentrations ($C_o$) of 4.6, 19.3, 21.8, 62.4, 64.2, and 89.7 mg l$^{-1}$ and a naphthalene input concentration of 30 mg l$^{-1}$, and an average pore-water velocity of approximately 9 cm h$^{-1}$ (Table 1). This velocity is equivalent to a hydraulic residence time of about 1.2 h. Sterile column experiments were conducted to characterize the retention and transport behavior of salicylate and naphthalene in the porous medium. Pentafluorobenzoic acid (PFBA) was
used as a nonreactive, conservative tracer to characterize the hydrodynamic properties of the packed columns.

The initial total biomass was calculated as the product of the initial soil-phase cell density (CFU g\(^{-1}\) dry soil) and the mass of dry sand packed in the column. The amount of biomass remaining in the sand before the substrate pulse was injected was calculated by subtracting the total amount of cells eluted during initial MSB saturation from the initial total soil-phase biomass. The total amount of cells eluted during the substrate pulse was calculated by integrating the area under the cell elution curve. All this information was used to calculate the population growth during substrate injection. An estimate of population growth was also calculated based on the total mass of substrate degraded. The mass degraded for each column experiment was calculated by subtracting the total mass of substrate eluted from the total input mass. The estimated biomass produced was determined using a cell yield coefficient of 0.22 g-cells-produced/g-salicylate-consumed obtained from batch studies (Sandrin et al., 2001). This cell mass was then converted to cell numbers (CFU) assuming an individual cell weight of 9.5 \(\times\) 10\(^{-10}\) mg (Neidhardt et al., 1990).

### 2.4. Quantification of luminescence and substrate and dissolved oxygen concentrations

Solution samples were periodically collected from the three sampling locations for analysis of salicylate, naphthalene, and DO concentrations. The DO was measured using an oxygen microelectrode connected to an oxygen meter (Microelectrodes, Bedford, NH). Immediately after the DO measurements were taken, 100 µl of 2.75 M NaOH was added to each 1 ml salicylate sample to inhibit microbial activity. Solution samples were stored at 4 °C until analyzed for salicylate. Prior to salicylate analysis, samples were centrifuged at 10,000 \(\times\) g for 10 min to pellet cell debris. Naphthalene samples (2 ml) were collected using a luer-lock glass syringe to prevent volatilization and then analyzed immediately. Salicylate and naphthalene concentrations were determined using UV/VIS spectrophotometry (UV-1601, Shimadzu) at 231 and 276 nm, respectively.

Luminescence was measured using a multiple fiber-optic detection system that allows noninvasive, real-time in situ monitoring of the spatial and temporal distribution of

<table>
<thead>
<tr>
<th>Experiment number</th>
<th>Input concentration (mg l(^{-1}))</th>
<th>Initial cell density (CFU g(^{-1}) dry soil)</th>
<th>Pore-water velocity (cm h(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4.6</td>
<td>2.3 (± 0.74) (\times) 10(^7)</td>
<td>8.4</td>
</tr>
<tr>
<td>2</td>
<td>19.3</td>
<td>1.6 (± 0.33) (\times) 10(^7)</td>
<td>9.0</td>
</tr>
<tr>
<td>3</td>
<td>21.8</td>
<td>2.0 (± 0.28) (\times) 10(^7)</td>
<td>8.7</td>
</tr>
<tr>
<td>4</td>
<td>62.4</td>
<td>2.8 (± 0.59) (\times) 10(^7)</td>
<td>8.5</td>
</tr>
<tr>
<td>5</td>
<td>64.2</td>
<td>1.4 (± 0.38) (\times) 10(^7)</td>
<td>7.8</td>
</tr>
<tr>
<td>6</td>
<td>89.7</td>
<td>2.6 (± 0.27) (\times) 10(^7)</td>
<td>8.8</td>
</tr>
<tr>
<td>7</td>
<td>30.0</td>
<td>1.3 (± 0.66) (\times) 10(^7)</td>
<td>8.9</td>
</tr>
</tbody>
</table>

Salicylate was used for experiments 1–6 and naphthalene for experiment 7. The values in the parentheses represent the standard deviation (1σ) of 6–9 plate counts. Bulk density and porosity of the packed column for these experiments were 1.75 g cm\(^{-3}\) and 0.34, respectively.
microbial activity under dynamic conditions in porous media. The design, calibration, and operation of the fiber optic system are described in detail elsewhere (Yolcubal et al., 2000; Yolcubal, 2001). The setup of the luminescence detection system is shown in Fig. 1. The light collected by the optical fiber is routed to a gated photomultiplier tube (PMT) that converts the light signal to an electrical signal. The current leaving the PMT is sent to a conditioning circuit for noise filtration and signal amplification. The output current from the conditioning circuit is then routed to a signal processor for data acquisition and the processed data is stored on a PC.

2.5. Data analysis

Moment analyses of salicylate and naphthalene breakthrough curves were performed to obtain the retardation factors of the compounds in the system (sterile experiments) and the total amount of substrate degraded (inoculated experiments). To measure the retardation factor, zeroth and first moments were calculated for salicylate and naphthalene breakthrough curves obtained from the column experiments conducted using noninoculated sand. The spatial and temporal distribution of salicylate and naphthalene biodegradation was determined from the breakthrough curves obtained for each sampling location (near the inlet and midpoint of the column, and in the effluent) during the experiments conducted with the inoculated sand. Using these substrate breakthrough curves, the percentage of total substrate degradation in the vicinity of the sampling locations was calculated. In addition, the total amount of substrate degraded between sampling locations was determined. For this calculation, the cumulative substrate degraded per pore volume was first calculated using the zeroth moments of the breakthrough curves obtained at each sampling location. The total amount of substrate degraded between sampling locations was then taken as the difference between the results for the respective sampling locations.

3. Results and discussion

3.1. Hydrodynamic characterization of the system

Breakthrough curves obtained for PFBA, the nonreactive tracer, were sharp and symmetrical, indicating ideal transport (Fig. 2). The results of sterile column experiments conducted with salicylate and naphthalene are also presented in Fig. 2. Mass recovery was complete, indicating no abiotic loss mechanisms. The retardation factors for salicylate and naphthalene were 1.1 and 1.4, respectively. Therefore, sorption of both salicylate and naphthalene is minimal in this system. Based on these results, it is unlikely that biodegradation of these two compounds will be significantly influenced by substrate bioavailability constraints.

3.2. Spatial variability of substrate biodegradation

For the first 15 pore volumes, salicylate biodegradation occurred throughout the entire column for all experiments. This is indicated by the fact that the salicylate concentrations
are lower for each successive sampling location (see Figs. 3 and 4). During this time, the majority of the biodegradation occurred between the inlet and midpoint of the column. After 10–15 pore volumes, behavior of the various systems began to diverge, reflecting the influence of substrate and O₂ concentrations on bioactive zone dynamics. For example, for the lowest- and intermediate-\(C_0\) experiments (4.6, 19.3, 21.8 mg l\(^{-1}\)), the bioactive zone persisted throughout the entire column for the duration of the experiments (Figs. 3 and 5). Conversely, such behavior was not observed for the large-\(C_0\) experiments (Figs. 4 and 5). The behavior for each of these systems is discussed in detail below.

For the lowest-\(C_0\) experiment, microbial activity in the system reached steady state at approximately 10 pore volumes. Steady-state conditions are indicated by constant aqueous concentrations (Fig. 3a) as well as a constant rate of biodegradation (uniform increase in cumulative salicylate degraded per pore volume) (Fig. 6). For this experiment, the rate of salicylate degradation after 10 pore volumes was 0.01 mg/PV \((R^2 = 0.98; N = 23)\) near the inlet of the column; 0.12 mg/PV \((R^2 = 0.99; N = 14)\) between the inlet and midpoint of the column, and 0.04 mg/PV \((R^2 = 0.99; N = 14)\) between the midpoint and outlet of the column. These data indicate that the majority of the degradation (~70%) took place between the inlet and midpoint of the column (Fig. 5). However, appreciable degradation (~25% of total) was still occurring between the midpoint and outlet of the column by the end of the experiment (Fig. 5c). The bioactive zone persisted throughout the column for the entire experiment because of two factors. First, DO did not become a limiting factor as indicated by the fact that the DO concentration consistently remained above 20 mg l\(^{-1}\) in the column effluent. Second, the substrate concentration present for this experiment was not high enough to support a significant increase in the microbial population. This is supported by the growth estimate based on the amount of salicylate degraded, which indicates that the microbial population increased by only 24% throughout the course of the

Fig. 2. Salicylate, naphthalene, and PFBA breakthrough curves obtained from sterile column experiments.
In a repeat experiment conducted under similar initial conditions, the cell mass balance calculations and growth estimate based on the salicylate degraded showed 12% and 22% total microbial growth, respectively (data not shown).

For the intermediate-$C_0$ experiments, salicylate degradation between the inlet and midpoint of the column began to decrease after 10 pore volumes, and stabilized after approximately 28 pore volumes for the 21.8 mg l$^{-1}$ $C_0$ experiment (Fig. 3b) and 36 pore volumes for the 19.3 mg l$^{-1}$ $C_0$ experiment (Fig. 3c). For the latter experiment, the rate of salicylate degradation decreased from 0.52 mg/PV ($R^2 = 0.99$, $N=6$) to 0.3 mg/PV.

Fig. 3. Salicylate breakthrough curves obtained at three sampling locations for the lowest- and intermediate-$C_0$ experiments. (a) $C_0 = 4.6$ mg l$^{-1}$, (b) $C_0 = 21.8$ mg l$^{-1}$, (c) $C_0 = 19.3$ mg l$^{-1}$. Arrows represent the initiation of MSB flushing. 1 PV = 1.2 h.
between 10 and 36 pore volumes (Fig. 6). After 36 pore volumes, the majority of degradation (~70%) was confined to the vicinity of the column inlet (Fig. 5a), wherein the rate of salicylate degradation became 0.91 mg/PV \( (R^2 = 1, N = 6) \). Conversely, only about 20% of the total degradation was taking place between the midpoint and outlet of the column at this time (Fig. 5c), and the rate of salicylate degradation in this zone was 0.03 mg/PV \( (R^2 = 0.99, N = 5) \) (Fig. 6). This change in the distribution of microbial activity throughout the bioactive zone is related to population growth and the resultant increased capacity for salicylate degradation near the inlet. Population
growths of approximately 220% and 204% were determined based on the cell mass balance and the total salicylate degraded, respectively. These results illustrate the impact of salicylate availability and attendant population growth behavior on bioactive zone dynamics.

For the larger-$C_o$ experiments (62.4, 64.2, and 89.7 mg l$^{-1}$), DO became a limiting factor despite the fact that the solution reservoirs were continuously sparged with

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Fig. 5. Percentage of the total salicylate degradation associated with each sampling domain for experiments conducted with different salicylate input concentrations. 1 PV = 1.2 h.
oxygen. As a consequence, the distribution of salicylate biodegradation in the column was influenced by DO availability. For the first 15 pore volumes, the salicylate concentration decreased between the inlet, midpoint, and effluent sampling locations (Fig. 4). This indicates that biodegradation was occurring throughout the entire column. However, after 15 pore volumes, the bioactive zone began to decrease in size due to decreasing DO availability coupled with population growth (Fig. 5). After approximately 17–20 pore volumes, there was no measurable biodegradation occurring between the
midpoint and outlet of the column, as indicated by the coincident plateau concentrations for the midpoint and effluent breakthrough curves (Fig. 4). After approximately 23–25 pore volumes, there was no measurable biodegradation occurring between the inlet and midpoint, and essentially all salicylate biodegradation was taking place in the vicinity of the column inlet. The growth estimates based on the total salicylate degraded for the 64.2 and 89.7 mg l$^{-1}$ experiments indicate that microbial population increased 460% and 280%, respectively. This difference in the growth rate mainly results from dissimilarity in the total biomass present in the column before the salicylate pulse was initiated.

In the larger-$C_0$ experiments, dissolved oxygen concentrations in the midpoint and effluent samples decreased from approximately 40 to 2 mg l$^{-1}$ between approximately 12–15 PVs, and remained relatively constant thereafter (Fig. 7). The observed reduction in oxygen concentration was due to increasing microbial activity near the inlet of the column. The rebound in salicylate concentration observed for the midpoint and effluent breakthrough curves for the larger-$C_0$ experiments is an indication of the influence of oxygen constraints on salicylate degradation. The rebound of substrate concentrations may have been caused by one of several processes. One hypothesis involves the accumulation and degradation of an intermediate compound that competes for oxygen in the system. This hypothesis may be valid for the naphthalene experiment, wherein a significant amount of salicylate ($\sim 3$ mg l$^{-1}$), which is an intermediate in the degradation pathway of naphthalene, was detected in the samples. However, in a batch experiment conducted with salicylate, there was no evidence of intermediate compounds being present at levels that would provide significant competition for oxygen utilization. Another hypothesis involves temporal variability in the metabolic status of the microbial population, which concomitantly may cause a change in metabolic oxygen requirements with time. For example, it has been shown that substrate degradation to CO$_2$ requires more oxygen than production of biomass precursors (Bouchez et al., 1995). Thus, it is possible that the oxygen requirements increased as biodegradation became more constrained as metabolic activity shifted from biomass production to CO$_2$ production. The salicylate-rebound behavior was not observed for the lower-$C_0$ experiments, most likely because overall oxygen demand was lower, thus, preventing DO from becoming a limiting factor.

For the larger-$C_0$ experiments where DO influenced the size of the bioactive zone, a correlation between the rate of biodegradation and the size of the bioactive zone was observed. As the bioactive zone decreased in size, the rate of biodegradation within the bioactive zone initially increased and eventually reached a steady rate. For example, between approximately 2 and 20 pore volumes, the average rate of biodegradation increased from 0.24 to 1.4 mg/PV at the near-inlet location for the larger-$C_0$ experiments (Fig. 6). After approximately 20 pore volumes, the average rate of biodegradation remained the same (2.5 mg/PV). Furthermore, the larger-$C_0$ experiments all exhibited a similar rate of biodegradation after 20 pore volumes, indicating that the maximum biodegradation potential had been attained under the given conditions within the bioactive zone (Fig. 6).

Comparable results were obtained for a naphthalene experiment conducted under conditions equivalent to those of the salicylate experiments (Table 1). Fig. 8 shows the
naphthalene breakthrough curves obtained at all sampling locations. For the first 5 pore volumes, 25% of the total naphthalene degradation (0.32 mg/PV, Fig. 9) in the column occurred near the inlet of the column. During the same time, the percentage of naphthalene degradation occurring between the inlet and midpoint of the column increased from approximately 25% to 70%, while the percentage of total naphthalene degradation between the midpoint and outlet of the column decreased from approximately 50% to 5% (Fig. 9).

After 5 pore volumes, naphthalene degradation between the inlet and midpoint of the column began to decrease and eventually ceased after approximately 9 pore volumes.
volumes (Fig. 9a). Conversely, naphthalene degradation between the midpoint and outlet of the column started to increase. This temporal variability in microbial activity reflects the nonuniform distribution of DO within the column. For example, at 9 pore volumes, the DO concentrations at the midpoint sampling location and in the effluent were approximately 2–3 and 14 mg l\(^{-1}\), respectively (See Fig. 8 insert). After the low concentration DO front reached the outlet of the column at approximately 13 pore volumes, naphthalene degradation ceased between the midpoint and outlet. Thereafter, degradation occurred only near the column inlet at a steady rate of 0.48 mg/PV (Fig. 9).

3.3. Monitoring in situ microbial activity

As shown above for the larger-C\(_0\) experiments, the bioactive zone initially encompassed the entire column, but after several pore volumes it decreased in size, ultimately shrinking to the immediate vicinity of the column inlet (~ 0.2 cm from inlet). These results clearly illustrate that substrate and electron acceptor availability influence the location and size of bioactive zones. Monitoring the formation of bioactive zones in porous media requires intensive sampling and measurement of substrate and DO concentrations at various locations. In addition, breakthrough curves represent an integration of the impacts of microbial activity for the entire region between sampling locations, rather than at a specific sampling location. In this study, an additional approach that employs bioluminescent bacteria and a multiple fiber-optic detection system was used to gain insight into the spatial and temporal distributions of microbial activity in porous media in response to changes in environmental conditions (e.g., DO and substrate concentrations). The advantages of this method...
are that it is rapid, noninvasive, and provides localized, in situ measurements of microbial activity.

Luminescence was continuously measured at five locations (i.e., 0.2, 2.1, 3.1, 5.0, and 7.5 cm from the inlet of the column) during the naphthalene experiment. The spatial and temporal distribution of luminescence emitted by *P. putida* RB1353 in response to changes in naphthalene and DO concentrations are shown in Fig. 10. The luminescence response was greatest near the substrate source and decreased with increasing distance from the inlet of the column, as would be expected given that the microorganisms near the inlet of the column were exposed to higher naphthalene concentrations.

![Graph showing naphthalene degradation](image)

**Fig. 9.** Naphthalene degradation associated with each sampling domain (a) percentage of total naphthalene degradation and (b) the total amount of naphthalene degraded. 1 PV = 1.1 h.
and DO concentrations. For the first 5 h (1 pore volume = 1.12 h), the luminescence response was detectable at all fiber-optic locations, indicating that microbial activity was occurring throughout the entire system. The magnitude of the light response at the 0.2-, 2.1-, 3.1-, and 5-cm locations were similar and approximately three times larger than the light response at the 7.5-cm location. These results suggest that the majority of the microbial activity occurred between the inlet and midpoint of the column for the first 5 h. This is consistent with the results discussed above for the substrate concentration data.

After approximately 5 h, the light response started to drop at the 7.5-cm location, and shortly thereafter at the midpoint of the column (5-cm location). The slow decrease in the light response observed between 5 and 9 h at the 5-cm location correlates well with the rebound in the naphthalene breakthrough curve observed for the column midpoint location (Fig. 11). The decrease in naphthalene degradation rate (i.e., indicated by the decrease in slope of the cumulative naphthalene degradation curve between 5 and 10 pore volumes, Fig. 9b), and therefore in microbial activity, at this location was a result of the rapid decline in DO (16 to 2 mg l$^{-1}$) that occurred in this zone. The decrease in DO concentration is attributed to an increase in microbial activity near the inlet of the column (see Fig. 8 insert) as noted above.

The effect of the increase in DO demand near the inlet of the column was also observed at upgradient locations (i.e., at 2.1 and 3.1 cm), where the luminescence response rapidly dropped to background levels between 6 and 7 h and remained constant thereafter. Similarly, after a 1–2 h delay, the luminescence response at the 5- and 7.5-cm locations rapidly completed their drop to background levels, indicating that the low concentration DO front reached these measurement locations. This delay
created a momentary nonuniform luminescence response distribution where luminescence, and therefore microbial activity, was only observed at the sampling locations near the inlet (i.e., 0.2 cm) and between the midpoint and outlet of the column (i.e., 5 and 7.5 cm).

At approximately 9–10 h, the luminescence response at the 5- and 7.5-m locations reached background levels, while the luminescence response near the inlet of the column (i.e., 0.2 cm) remained significantly above background. During this time, the rapid drop in the luminescence response at the midpoint of the column corresponds well to the convergence of plateau concentrations for the inlet and midpoint breakthrough curves (Fig. 11). These results suggest that the microbial activity was confined to the vicinity of the column inlet after approximately 15 h, and agree well with the conclusions drawn using the substrate concentration data discussed above (Figs. 8 and 9).

4. Summary

Two approaches were used to characterize the formation and dynamics of bioactive zones in response to changes in local substrate and DO concentrations. The first approach involved frequent monitoring of the spatial and temporal variability of DO and aqueous substrate (salicylate and naphthalene) concentrations. The second approach involved real-time in situ monitoring of microbial activity via detection of the luminescence response generated by a reporter organism at various locations in the porous medium. The results obtained from both approaches illustrate that the location and size of bioactive zones in porous media are influenced by substrate and DO availability. The application of luminescent reporter organisms in real-time in situ monitoring of microbial activity in porous media can provide insight concerning how microbial populations respond to...
changes in local environmental conditions. The correlation of the luminescence response with changes in DO and substrate availability suggest that this approach is useful for monitoring changes in the specific environmental conditions necessary to maintain optimal in situ biodegradation in bioactive zones.

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References