RICE UNIVERSITY

Source Zone Bioremediation of Chlorobenzene DNAPLs: Performance Assessment using Real Time Quantitative Polymerase Chain Reaction.

by

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A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE Master of Science

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ABSTRACT

Source Zone Bioremediation of Chlorobenzene DNAPLs: Performance Assessment using Real Time Quantitative Polymerase Chain Reaction.

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Aquifer flow-through columns were operated for 12 weeks to evaluate the benefits of oxygen addition on the biodegradation of a Chlorobenzene (CB) DNAPL source zone. Quantitative PCR was used to measure total bacteria (16S rDNA) and several aromatic oxygenase genes. CB removal was faster in the oxygen-amended columns compared to a control column, and qPCR showed that whereas the biphenyl and toluene dioxygenase biomarkers were most abundant, increases in the biomarker concentration for the phenol hydroxylase gene reflected best the higher CB removal due to aerobic biostimulation. DGGE analyses of the soil amended with oxygen revealed the dominant presence of *Rhodococcus erythropolis* (89% sequence similarity), which belongs to a genus known for its ability to degrade many priority pollutants, including CBs.
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INTRODUCTION

Chlorobenzenes (CBs) represent a group of chemicals that are widely used as industrial solvents and degreasers, and are commonly encountered in the subsurface near industrial areas where they have been manufactured or used (Howard, 1989). CBs include monochlorobenzene (MCB) and the dichlorobenzene (DCB) isomers (1,2-DCB; 1,3-DCB; and 1,4-DCB). These compounds have low federal drinking water standards (i.e., maximum contaminant levels [MCLs] ranging from 0.075 mg/L for 1,4-DCB to 0.6 mg/L for 1,2-DCB) and relatively high water solubility, ranging from ~75 mg/L for 1,4-DCB to ~500 mg/L for MCB.

Bioremediation and monitored natural attenuation (MNA) are among the most cost-effective approaches to manage soil and groundwater contamination by organic pollutants (Alvarez and Illman, 2005; McDade et al., 2005). Past research shows that CBs can be degraded by a variety of both aerobic (Adrian et al., 2000; Dermietzel and Vieth, 2002; Lorbeer et al., 2002; Vogt et al., 2002; Vogt et al., 2004; Wenderoth et al., 2003) and anaerobic bacteria (Adrian et al., 2000; Kao and Prosser, 1999; Kaschl et al., 2005; Wenderoth et al., 2003). Biodegradation generally proceeds faster aerobically (Wenderoth et al., 2003), particularly in the case of MCB and 1,4-DCB (Dermietzel and Vieth, 2002), and oxygen availability is a common rate-limiting factor for microbial-mediated CB transformation. Several strategies have been developed that oxygenate the subsurface for biostimulating aerobic microorganisms to metabolize CBs, including the addition of hydrogen peroxide (Vogt et al., 2004) and air sparging (Balcke et al., 2004). However, most CB bioremediation efforts have focused on plume management (Dermietzel and Vieth, 2002; Lorbeer et al., 2002; Vogt et al., 2002; Vogt et al., 2004;...
Wenderoth et al., 2003) rather than on treating the source zones where CBs occur as a dense non-aqueous phase liquid (DNAPL) that slowly dissolve over many years and emanate groundwater plumes. Thus, plume-treatment technologies address the symptom rather than the cause, and there is a need to develop practical approaches to remove CB DNAPLs.

DNAPLs represent a major remediation challenge because of the inherent difficulties in their detection, characterization and delineation in the subsurface. CB DNAPLs tend to be persistent due to low aqueous solubility and slow dissolution kinetics, resulting in a long-term source of contamination. The effectiveness of traditional remedial approaches, such as groundwater extraction (i.e., “pump-and-treat”) is limited due to organic partitioning and wetting characteristics of these hydrophobic phases. Early bioremediation research did not focus on directly treating DNAPLs due to concerns about the potential microbial toxicity of high contaminant concentrations. However, recent research has shown that microorganisms can significantly enhance DNAPL dissolution (and thus DNAPL removal) by consuming dissolved pollutants near the DNAPL interface and increasing the concentration gradient and dissolution flux (Adamson et al., 2003; Cope and Hughes, 2001; Zheng et al., 2001). This has promoted the acceptance of source zone bioremediation (McGuire, 2006), which aims to increase the local flux of contaminants from DNAPLs into the aqueous phase through biodegradation and the production of more soluble metabolites that can be more easily detoxified in situ or removed by alternative (plume management) technologies. The potential efficacy of DNAPL source zone bioremediation has been recently demonstrated under anaerobic conditions for chlorinated ethenes at the pilot scale (Adamson et al.,
2003; Da Silva et al., 2006; Sleep et al., 2006) and field scales (Lendvay et al., 2003). However, there are no published reports on the use of bioremediation to enhance the removal of DNAPL source zones containing MCB and the DCB isomers.

A rigorous performance assessment of bioremediation requires documentation that contaminant removal is due to microbial rather than abiotic processes such as dissolution, dilution and volatilization. This is of particular importance in aerobic environments because of the difficulty in monitoring end-products (e.g., CO₂) and the complications presented by quantifying contaminant loss in complex media where multiple phases are present. Molecular microbial ecology techniques and specific biomarkers are increasingly being used to obtain supporting evidence of bioremediation (Abraham et al., 2005; Alfreider et al., 2002b; Balcke et al., 2004; Beller et al., 2002; Da Silva and Alvarez, 2004; Da Silva et al., 2006; Futumata et al., 2001; Wenderoth et al., 2003), including chlorocatechols (Alfreider et al., 2002b). Such efforts can be used to establish that specific microorganisms that can degrade the target pollutants (and their associated biomarkers) are present, and that their concentrations are higher in the treatment zone compared to background samples. Numerous catabolic biomarkers have been used to quantify the presence of organisms that degrade aromatic compounds (Baldwin et al., 2003; Beller et al., 2002; Suzuki et al., 2000). These include the genes coding for toluene dioxygenase, ring hydroxylating monooxygenase, naphthalene dioxygenase, biphenyl dioxygenase, and phenol hydroxylase. These biomarker assays are relatively straightforward but powerful tools that are ideally suited for determining the degree of biostimulation within a source zone. However, it is unknown whether such biomarkers could be applicable for a performance assessment of CB source zone
bioremediation. Given that chlorinated benzene degradation is initiated by oxygenase-promoted hydroxylations prior to ring cleavage, these represent promising candidates for further study.

In this thesis, I report the results of a biostimulation experiment to evaluate the efficacy of aerobic CB source zone bioremediation and characterize microbial community changes associated with the treatment process. Genotypic shifts associated with the proliferation of genes that code for various enzymes that initiate aerobic biodegradation of aromatic compounds (i.e., oxygenases) were also quantified to establish candidate biomarkers for assessment of CB source zone biodegradation.
MATERIALS AND METHODS

Biostimulation Experiment

Flow-through column studies were performed using aquifer material collected from a former chemical manufacturing facility located in the Midwestern United States. Environmental Visualization System software (EVS, Version 7.92) was used to identify the highest concentrations of MCB and DCB in the saturated zone source area to establish suitable locations for collection of soil samples. Approximately 18 kg (40 lbs) of soil for the biostimulation experiment were collected using rotasonic drilling techniques. The soil sample was divided into nine even sections and each section was split evenly among nine half-gallon containers leaving minimal headspace. After sealing, the gallons were cooled and shipped at 4°C to Rice University for homogenization and separation into bench-test aliquots. To provide baseline characterization data prior to biostimulation, additional sample aliquots were shipped at 4°C to Seven Trent Laboratories in Savannah, Georgia in laboratory-provided, method-specific containers for chemical and geotechnical analyses. This included soil samples used to characterize background microbial conditions, which were collected from a soil boring located approximately 700 m (cross-gradient) from the CB source zone. This background soil sample was collected from the same stratigraphic horizon as the samples collected for the biostimulation experiment (12 to 15 below ground surface). The boring was located adjacent to a separate mixed-waste disposal site, but chemical analysis of the soil indicated that the horizon used for background characterization did not contain detectable concentrations of volatile or semi-volatile organic compounds. The aquifer material used in these experiments (both biostimulated and background samples) uniformly consisted
of dark gray course sand and gravel. The porosity of the soil ranged between 41 and 43%. The bulk density ranged between 1.49 and 1.57 g/mL, and the grain density was between 2.64 and 2.65 g/mL.

Flow-through aquifer columns were constructed using custom made (Specialty Glass, Houston, TX) 7.6 cm diameter and 15.2 cm long columns (total volume 695 mL) that were packed with site soil. Fitted caps at both ends were constructed to ensure gastight, non-reactive conditions. Water was introduced in an upflow mode via peristaltic pumps at a rate designed to match the groundwater velocity at the site (8.7 cm/d). The influent solution was a bicarbonate-buffered mineral medium with geochemistry similar to the site groundwater, using deionized water supplemented with the following constituents (in mg/L): MgSO₄·7H₂O (50), FeSO₄·7H₂O (3), (NH₄)₂SO₄ (500), K₂HPO₄ (1750), KH₂PO₄ (1380), NaHCO₃ (500), MnSO₄·H₂O (2), H₃BO₃ (0.1), CaSO₄·5H₂O (0.05), ZnSO₄·7H₂O (0.05), Na₂MoO₄·2H₂O (0.05), H₃BO₃ (0.1), and CoSO₄·7H₂O (0.7). After a two-week equilibration period, industrial grade pure oxygen (100% v/v) was sparged continuously into the influent reservoir providing oxygen-saturated water (~48 mg/L) for the duration of the experiment. The influent reservoir was maintained in a refrigerated environment to simulate the average site groundwater temperature of 13 to 16°C.

A total of 8 columns underwent aerobic biostimulation (i.e., fed with oxygen-saturated medium). In addition to these oxygen-amended columns, a control column was fed from a separate reservoir with N₂-sparged medium (DO < 2 mg/L) amended with sodium azide (10 mg/L). Azide selectively inhibits Gram-negative aerobic bacteria (Snyder and Lichstein, 1940). The azide concentration used in the study was sublethal
(Lichstein and Soule, 1943) and resulted in bacteriostatic (rather than bactericidal) effects that decreased aerobic respiration and biodegradation activity. The azide-amended control column did not serve as a sterile control; it was used to reflect decreased biological activity that would occur under oxygen-limited conditions. Sodium azide was selected over other bacterial inhibitors because it does not change the structure and properties of the soil and because of its affordability and safe disposal.

The experiment was carried out over a 12-week period (following two weeks of equilibration). The effluent end of each column was fitted with an in-line sample collection reservoir, and weekly aqueous samples were collected for analysis of volatile organic compounds (VOC) by USEPA Method 8260 at Severn Trent Laboratories (Savannah, Georgia, USA). Dissolved oxygen (Oakton DO 110), oxidation-reduction potential (Cole-Parmer), temperature, pH, and specific conductivity (Hanna HI 991301) were measured directly by insertion of parameter-specific probes through a cap in the in-line reservoir. At two week intervals, a biostimulated (aerobic) column was sacrificed to obtain soil samples for posterior chemical and molecular analysis. The control column was sacrificed after 12 weeks. Column soil samples were collected in accordance with USEPA Method 3035 and shipped at 4°C to Seven Trent Laboratories (Savannah, Georgia, USA) for VOC analysis by USEPA Method 8260. A composite soil sample was also collected for molecular analysis performed at Rice University as described below.

**DNA extraction**

DNA was extracted from soil samples using MoBio power Soil DNA isolation kit (Carlsbad, CA, USA) according to manufacturer protocol. A bead-beating device (Model Mini Beadbeater-8; Biospec, Bartlesville, OK, USA) was utilized for cell lysis. As a
result, 100 µL of DNA were recovered from each sample and collected in a 1.5-mL Eppendorf vial and stored in a freezer (Isotemp® Basic, Fischer Scientific, Rockville, MD, USA) at -75°C. The concentration and purity of the DNA was measured based on the wavelength absorbance ratio (absorbance of 260 nm for DNA and 280 nm for protein) \( A_{260}/A_{280} \) using a spectrophotometer (Amersham Biosciences, Model Ultraspec 2100 Pro, Piscataway, NJ, USA). Each sampled was spiked with a 2-µL aliquot of bacteriophage \( \lambda \) DNA 500 bp (Sigma-Aldrich, St. Louis, MO, USA) prior to DNA extraction to serve as an internal standard for the determination of DNA efficiency recovery. When recovery was lower than 100%, gene copy numbers were normalized to the fraction recovered.

**Real-time quantitative PCR (qPCR)**

qPCR was used to quantify catabolic genes coding for toluene dioxygenase, naphthalene dioxygenase, ring hydroxylating monooxygenase, phenol hydroxylase, and biphenyl dioxygenase using the primers TOD, NAH, RMO, PHE, and BPH3, respectively, designed by Baldwin et al. (Baldwin et al., 2003) (Table 1).

These genes were selected as biomarkers because i) to date, no qPCR primers have been designed to quantify CB degradation genes, and the chlorobenzene dioxygenase sequences in the NCBI database are highly similar and often indiscernible from the toluene and biphenyl dioxygenase genes (Figure 1). In fact, previous studies have demonstrated that genes involved in the degradation of CBs are evolutionarily linked to toluene/benzene and biphenyl dioxygenases (Beil et al., 1998; Van der Meer, 1998) and utilize similar metabolic routes (Gibson and Parales, 2000); ii) the targeted oxygenases are know to have broad substrate specificity and attack a wide variety of aromatic compounds (Wackett and Hershberger, 2001) and iii) these primers target a
conserved region of the gene, permitting the detection of specific aromatic catabolic genotypes without excluding related but uncharacterized genes (Baldwin et al., 2003).

Table 1. Primers and probe sequences used in this study.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Targets gene coding for</th>
<th>Sequence</th>
<th>Probe</th>
</tr>
</thead>
<tbody>
<tr>
<td>PHE-F</td>
<td>Phenol hydroxylase&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5'-GTGCTGAC(G/C)AA(C/T)CTG(C/T)TGTC-3' 5'-CGCCAGAACCACC(T/C)TT(A/G)TC-3'</td>
<td>SYBR-Green</td>
</tr>
<tr>
<td>PHE-R</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TOD-F</td>
<td>Toluene dioxygenase&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5'-ACCGATGA(A/G)QA(C/T)CTGTACC-3' 5'-CTTCGTC(A/C)AGTAGCTGGTG-3'</td>
<td>SYBR-Green</td>
</tr>
<tr>
<td>TOD-R</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NAH-F</td>
<td>Naphthalene dioxygenase&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5'-CAAAA(A/G)CACTGAT(T/C)ATGG-3' 5'-A(C/T)(A/G)CG(A/G)(G/C)GACTTCTTTCAA-3'</td>
<td>SYBR-Green</td>
</tr>
<tr>
<td>NAH-R</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RMO-F</td>
<td>Ring hydroxylating monooxygenase&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5'-TTC(A/C/G)AGCAT(T/C)CAGAC(A/C/G)GACG-3' 5'-T(T/G/T)TCGTGAT(C/G/T)AC(A/G)TCCA-3'</td>
<td>SYBR-Green</td>
</tr>
<tr>
<td>RMO-R</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BPH3-F</td>
<td>Biphenyl dioxygenase&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5'-CCGAGAAGAACGGCAGGATC-3' 5'-TGCTCCGCTCGAATCTC-3'</td>
<td>SYBR-Green</td>
</tr>
<tr>
<td>BPH3-R</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BACT1369F</td>
<td>Bacteria (16S rDNA)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5'-GGTGAATACGTTCYGCG-3' 5'-GCCAGACCAACC(C/T)TT(A/G)TC-3'</td>
<td>FAM-5'&lt;br&gt;CTTGTACACAC&lt;br&gt;CGCCGTC-3'&lt;br&gt;BHQ</td>
</tr>
<tr>
<td>PROK1492R</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>λ</td>
<td>Bacteriophage λ&lt;sup&gt;c&lt;/sup&gt;</td>
<td>5'-ACGCCACCGGATGAC-3' 5'-AGAGACACGAAACGCCGTTC-3'</td>
<td>TET-5'&lt;br&gt;ACCTGTGGCAT&lt;br&gt;TTGTGCTGCCG-E-TAMRA</td>
</tr>
</tbody>
</table>

<sup>a</sup> Primers designed by Baldwin et al.(2003)
<sup>b</sup> Primers and probe developed by Suzuki et al.(2000)
<sup>c</sup> Primers and probe as in Beller et al. (2002)
**Figure 1.** Neighbor-joining tree obtained with EBI web-based tool ClustalW illustrating phylogenetic relationships among the aromatic dioxygenases \( \alpha \) (large) subunit, which is responsible for substrate specificity. The amino acid sequences compared are those of \textbf{TcbAa} and \textbf{CbzAa} (chlorobenzene dioxygenase), \textbf{BnzA} (toluene dioxygenase), \textbf{BphA1} (biphenyl dioxygenase), and \textbf{NdoB} (naphthalene dioxygenase) as obtained from NCBI database. The strains from which the sequences were obtained are \textit{Pseudomonas putida} (Pp), \textit{Pseudomonas putida} \textit{GJ31} (Pp GJ31), \textit{Burkholderia} sp LB400 (Bsp LB400), \textit{Pseudomonas} sp B4 (Psp B4), \textit{Pseudomonas aeruginosa} (Pa), \textit{Pseudomonas fluorescens} (Pf), and \textit{Pseudomonas putida} C18 (Pp C18). The scale bar represents 0.05 amino acid changes per position.

All primers and probes were obtained from Integrated DNA Technologies (Coralville, IA, USA). PCR mixtures contained 1×Taqman PCR Master Mix or SYBR GREEN (Applied Biosystems, Foster City, CA, USA); 500 nM forward and reverse primers, 250 nM of the probe (for reactions using Taqman) and sterile DNAase-free water to make up a final volume of 25 μL. PCR reactions were performed using a Sequence Detector (Model ABI 7500, Applied Biosystems, Foster City, CA, USA) with the following temperature conditions: 50°C for two min, followed by 95°C for 10 min and 40 cycles at 95°C for 15 s, and 60°C for one min. The genomic DNA sequences of various reference strains were utilized to prepare calibration curves for the targeted oxygenase genes. \textit{Pseudomonas putida} F1 was used for toluene dioxygenase, \textit{Pseudomonas putida} G7 for naphthalene dioxygenase, \textit{P. pseudoalcaligenes} KF707 for
biphenyl dioxygenase, *R. picketti* PK01 for ring hydroxylating monooxygenase, and *Pseudomonas putida* CF600 for phenol hydroxylase. Dilutions (10^1 to 10^8 gene copies/μL) were prepared for all calibration curves, yielding \( r^2 \) values ≥ 0.99.

Gene copies in each of the dilutions were estimated based on the following equation:

\[
\text{Gene copies/μL} = (μg \text{ DNA}/μL) \times (9.1257\times10^{14} \text{ bp/μg DNA}) \times \frac{\text{genome}}{6.18\times10^6 \text{ bp}} \times \# \text{ of gene copies/genome}
\]

This approach assumes that the approximate size of the bacterial genome used as the standard in the calibration curves was 6.18×10^6 base pairs (bp) (with approximately 9.12576 × 10^{14} bp/μg of DNA, equivalent to the size of the *P. putida* genome (http://www.genomesonline.org), and that there are seven gene copies for 16S rDNA per genome (1 copy for oxygenases) (http://rrndb.cme.msu.edu). The detection limits were on the order of 10^2 copy numbers/g-soil for oxygenase genes and 10^3 copy numbers/g-soil for 16S rDNA.

**Denaturing gradient gel electrophoresis (DGGE)**

DGGE analyses were conducted to determine changes in microbial community structure over time. PCR-based DGGE was conducted using a Biometra® thermocycler (T-gradient Thermoblock, Goettingen, Germany). The PCR reaction mix consisted of 1×Quiagen Taq PCR buffer (Valencia, CA, USA), the bacterial forward (5’-ATGGCTGTCGTACGCT-3’) and reverse (5’-GCCGGCCCGGCCGCACGCGGTGTGAC-3’) primers (0.5 μM each) (Ferris et al., 1996), 8 μL of the extracted sample DNA, and DNAase-free water to make up a final volume of 100 μL. The pair of primers was
used to amplify a 323-bp section of the 16S rDNA genes of the members of the domain *Bacteria*. The specific PCR temperature reaction for this set of primers was: 94°C 1 min, 65°C 1 min, 72°C 1 min (total of 20 cycles), 94°C 1 min, 55°C 1 min, 72°C 1 min (total of 20 cycles) and a final extension of 72°C for 7 min. Once the PCR reaction was finished, the presence of PCR products were verified on an agarose gel prior to DGGE analysis to ensure sufficient amplified DNA. The PCR gel consisted of 1% agarose containing 1×TAE running buffer and ethidium bromide dye (0.5 μg/mL). The presence of the PCR products were visualized under UV light (EpiChemi UVP BioImaging System, Upland, CA, USA).

DGGE was performed using a DCODE™ Universal mutation detection system (Bio-Rad, Hercules, CA, USA). The acrylamide gel consisted of a high denaturant (80%) and low denaturant (30%) gradient. Electrophoresis was performed at 113 V for 5 hours. The polyacrylamide gel containing the PCR-amplified 16S rDNA of the bacterial community provided an indicator of the difference among communities, with each band observed in the gel representing one bacterium. Several bands were excised and sequenced (Lone Star Labs, Inc. Houston, TX, USA), and phylogenetic affiliations were determined by comparing the DNA sequences retrieved to known bacterial sequences in the Ribosomal Database Project II (http://rdp.cme.msu.edu/index.jsp).

Changes in microbial population diversity were quantified using the Shannon-Weaver Diversity Index (H):

\[ H = \frac{2.3}{N} \left( N \log N - \sum n_i \log n_i \right) \]

where \( N \) is the total mass of the DNA bands, and \( n_i \) is the mass of the \( i^{th} \) band (taken as the area of the bands using a densitometer (EC3 Imaging System) connected to a
EpiChemi 3 Darkroom and LabWorks 4.5 Software), and $S$ is the number of DNA bands in the DGGE gel. The Shannon-Weaver Diversity Index is a general index that increases with the number of species and is higher when the mass is distributed more evenly over the species; hence the Shannon-Weaver Diversity Index considers both richness and evenness.
RESULTS AND DISCUSSION

Source zone bioremediation

Aerobic biostimulation was tested as a method for treating MCB and DCBs in source-zone soil where DNAPL was likely present. A baseline characterization of unamended, homogenized soil indicated an average initial concentration of 420 mg/kg MCB, 2,000 mg/kg 1,2-DCB, 470 mg/kg 1,3-DCB, and 1,700 mg/kg 1,4-DCB. These concentrations are consistent with historical high concentrations that have been measured at the site.

![Graph 1](image1)

**Biostimulation weeks**

![Graph 2](image2)

**Biostimulation weeks**

**Figure 2.** MCB and DCB effluent concentrations average values plotted for biostimulated columns and inhibited control column (azide-amended, no oxygen); error bars represent minimum and maximum.
MCB concentrations in column effluents were stable or increased slightly over the equilibration period (Figure 2). During the period of oxygen addition, concentrations in both the biostimulated columns and control column decreased exponentially from initial maximum concentrations of about 40 mg/L. The rate of MCB decrease was slightly faster in biostimulated columns, with concentrations falling below a 0.5 mg/L detection limit after six weeks of oxygen addition, compared to nine weeks for the control column. MCB concentrations in two of four biostimulated columns were below detection limits after five weeks of oxygen addition. The MCB concentration decrease over the test period was greater than 99% for both biostimulated and control columns, indicating that dissolution and advective flushing of this relatively soluble compound (500 mg/L water solubility) was an important removal mechanism.

In contrast to MCB, effluent concentrations of the DCB isomers remained relatively constant throughout the test in biostimulated columns, with the total DCB concentration near 80 mg/L (Figure 2). A slight increase was noted in the control, from an initial concentration of about 70 mg/L to near 100 mg/L at the end of the test. Total DCB concentrations in the effluent were consistently comprised of about 50% 1,2-DCB (~ 40 mg/L); 10% 1,3-DCB (~ 8 mg/L); and 40% 1,4-DCB (~ 32 mg/L). Based on the initial mole fractions measured in soil samples, Raoult's law would predict effective solubilities of 60 mg/L for 1,2-DCB; 12 mg/L for 1,3-DCB; and 28 mg/L for 1,4-DCB. Thus, the observed effluent concentrations were generally near or exceeding the estimated effective solubility, suggesting that DCBs were likely present in the columns as DNAPL, even though no free-phase product was visible during the duration of the sampling process.
**Figure 3.** MCB and DCB total mass reduction in each column. Numbers in x axis reflect the time (weeks) when biostimulated columns were sacrificed for analysis, and C denotes the inhibited control column (analyzed after 12 weeks).

Soil data from before, during, and after aerobic biostimulation provide evidence for mass removal (**Figure 3**). Soil samples collected from the water-saturated columns contained CBs sorbed to the soil and present in pore water, as well as any non-aqueous phase mass that may have been present. The overall MCB mass reduction for the 12-week test period was greater than 99% in the biostimulated columns. A high degree of MCB removal was also observed in the control column (96%), consistent with effluent sampling data. DCB total mass removal in biostimulated columns was also high. At the end of the 12-week oxygenation period, total DCB mass removal was 89% for 1,2-DCB; 83% for 1,3-DCB; and 90% for 1,4-DCB. These removal efficiencies were much higher
than those observed in the control column (34%, 29% and 36% respectively), demonstrating the benefits of aerobic biostimulation for this less-soluble class of compounds. Oxygen consumption observed in the biostimulated columns (from an influent dissolved oxygen concentration of 48.6 mg/L to an effluent value of approximately 3 mg/L) supported the finding aerobic biodegradation contributed to enhanced mass removal. Based on the degree of mass removal over 12 weeks, the biostimulation enhancement factor (relative to the inhibited control) was 2.6-fold for 1,2-DCB, 2.9-fold for 1,3-DCB and 2.5-fold for 1,4-DCB.

**Molecular Analyses**

DGGE was used to study changes in the community structure due to biostimulation. A reduction in the number of DGGE bands was observed as the experiment progressed, indicating a decrease in microbial diversity (**Figure 4**).

![Biostimulated (weeks) vs Control](image)

**Figure 4.** DGGE analysis shows that a bacterium was enriched as a result of the selective pressure exerted by CBs. The dominant bacterium (arrow) was identified as *Rhodococcus erythropolis* (89% similarity index) a putative CB degrader.
One band became dominant, as indicated by the marked increase in light intensity (Figure 4). This band was excised and gene sequencing associated it with a CB degrader (*Rhodococcus erythropolis*, 89% sequence identity) (Kaschl et al., 2005). *Rhodococcus* spp. are Gram positive bacteria known for their versatility in degrading a wide range of priority pollutants (Wackett and Hershberger, 2001), including CBs (Field and Sierra, 2001; Rehfuss and Urban, 2005; Zaitsev et al., 1995).

**Table 2.** Changes in Shannon-Weaver Diversity Index.

<table>
<thead>
<tr>
<th>Days</th>
<th>Biostimulation</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>2.02</td>
<td>ND*</td>
</tr>
<tr>
<td>42</td>
<td>1.83</td>
<td>ND*</td>
</tr>
<tr>
<td>56</td>
<td>1.90</td>
<td>ND*</td>
</tr>
<tr>
<td>84</td>
<td>1.79</td>
<td>1.73</td>
</tr>
</tbody>
</table>

*Not determined

The Shannon-Weaver diversity index decreased accordingly over the 12 weeks of biostimulation (Table 2). The decrease in the diversity index was likely due to the proliferation of bacteria that exploited CB degradation as a metabolic niche, which is a common response to bioremediation (Roling et al., 2002). A decrease in diversity index was also observed in the control column, probably due to the inhibitory effect of sodium azide, known to primarily affect gram negative aerobes (Lichstein and Soule, 1943).
Figure 5. Biomarker gene concentrations measured by qPCR. Genes coding for naphthalene dioxygenase and ring hydroxylating monoxygenase were not detected. * denotes statistically significant increase (p<0.05) relative to background control, and ** denotes statistically significant increase (p<0.05) relative to both background and initial conditions (t = 0). Error bars depict the standard deviation from the mean of triplicate measurements. The inhibited control column (azide-amended, oxygen-free) was not considered a sterile control.

The total bacteria population (measured by qPCR as 16S-rDNA gene copies) was one order of magnitude higher in the source zone sample than in the non-contaminated (background) soil, and increased by an additional order of magnitude following aerobic biostimulation (12 weeks) to about $10^7$ to $10^8$ copies/g-soil (Figure 5). A comparison of background versus source zone samples prior to biostimulation suggests that the presence of CBs promoted significant growth ($p < 0.05$) of bacteria harboring biphenyl
dioxygenase, toluene dioxygenase and phenol hydroxylase genes. These genes also experienced an increase following aerobic biostimulation, but only the phenol hydroxylase gene was significantly enriched ($p < 0.05$) (Figure 5). The biphenyl and toluene dioxygenase genes were already present at relatively high concentrations in the background soil ($\sim 10^4$ copies/g-soil), making them less sensitive biomarkers with respect to monitoring changes following biostimulation. The possibility that their initial abundance was due to the proximity of the background sample location to a landfill for mixed waste (which might have promoted the growth of such genotypes) could not be ruled out. Nonetheless, naphthalene dioxygenase and ring hydroxylating monooxygenase genes were not detected in either the background or source zone samples before or after biostimulation, indicating that these were not appropriate biomarkers for this soil and contaminant profile.

DNA-based catabolic biomarkers such as those used in this work cannot provide unequivocal evidence of biodegradation activity because the presence of a gene does not guarantee its expression. Thus, instantaneous activity can be better inferred by mRNA (gene expression) rather than DNA (gene presence) analysis. Nevertheless, DNA gene copy numbers should be temporally quite responsive to biodegradation activity because bacterial growth supported by utilization of the target pollutant during bioremediation increases the number of pertinent catabolic genes relative to background levels. In addition, DNA analysis is generally more sensitive and easier to perform than mRNA analysis, primarily because mRNA is relatively unstable and its quantification is subject to variable reverse transcriptase efficiency and lower qPCR recovery.

Although an unequivocal etiology between biphenyl or toluene dioxygenase and
CB degradation was not established, the notion that these enzymes played a role in CB biodegradation is supported by circumstantial evidence from previous studies. Specifically, regarding biphenyl dioxygenase, i) bacteria harboring this enzyme have been reported to abound at sites contaminated with CBs (Abraham et al., 2005), ii) biphenyl dioxygenase is known to have a relaxed substrate specificity and attack chlorobiphenyls (http://www.brenda.uni-koeln.de), which are structural analogues of CBs, iii) *Rhodococcus* spp., commonly harbor biphenyl dioxygenase (http://www.ncbi.nlm.nih.gov; http://www.ebi.ac.uk) (Baldwin et al., 2003), and iv) the degradation of CB in this study coincided with the enrichment of *Rhodococcus* sp. (Figure 4), a putative CB degrader. Therefore, biphenyl dioxygenase may either initiate the degradation of CBs or participate in the degradation of one or more of its byproducts. A similar argument can be postulated for toluene dioxygenase, because its gene is evolutionarily linked to biphenyl and chlorobenzene dioxygenase (Figure 1). In fact, the toluene dioxygenase gene sequence is difficult to discern from that of the chlorobenzene dioxygenase gene (Beil et al., 1998; Van der Meer, 1998).

The significant enrichment of the phenol hydroxylase gene following aerobic biostimulation suggests two possibilities: (a) phenols or chlorophenols, which are substrates for phenol hydroxylases, were produced following an initial attack on the CB molecule by another oxygenase, and some of these phenolics were subsequently degraded by organisms harboring phenol hydroxylase; or (b) phenol hydroxylase catalyzed both the first and second hydroxylation of the aromatic ring prior to oxidative cleavage. Regardless of whether the participation of organisms harboring phenol hydroxylase was direct or commensal, this biomarker was the most sensitive indicator of CB
biodegradation in this work. This suggests the potential value of this biomarker (as well as those for biphenyl and toluene dioxygenases) for assessment of CB bioremediation potential.
CONCLUSION

Although the efficacy of bioremediation and natural attenuation has been previously demonstrated for treating soil and groundwater contamination by hazardous organic pollutants, these environmental biotechnologies are not universally applicable and may be marginally effective when the necessary microbial catabolic capacity is not present or expressed. Demonstrating the role of microorganisms in contaminant removal is therefore important.

This research is the first to demonstrate the potential efficacy of aerobic bioremediation to treat CB source zones, and that quantification of specific catabolic DNA biomarkers can provide valuable insight as one of several converging lines of circumstantial evidence that bioremediation is working.

DNA-based catabolic biomarkers such as those used in this work do not provide unequivocal evidence of biodegradation activity because the presence of a gene does not guarantee its expression. Instantaneous activity can be better inferred by mRNA (gene expression) rather than DNA (gene presence) analysis. Nevertheless, DNA gene copy numbers should be temporally quite responsive to biodegradation activity because bacterial growth on the target pollutant during bioremediation results in significant increase in gene copy numbers relative to background levels. In addition, DNA analysis is generally easier to perform and data obtained this way is more reliable that mRNA analysis, because mRNA is relatively unstable and its quantification is subject to variable reverse transcriptase efficiency and lower qPCR recovery. Thus, DNA-based catabolic biomarkers hold great potential as simple forensic tools to assess source zone bioremediation in situ.

Since the universal applicability of the oxygenase biomarkers tested in this work
is unknown, further tests with samples from other contaminated sites are recommended to ascertain their reliability (specificity, selectivity and adequacy) and evaluate their broad applicability as forensic tools to assess CB source-zone bioremediation performance.
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Appendix I.

Effective Solubilities of CB and DCB
Effective Solubilities of Chlorobenzene and Dichlorobenzene congeners.

<table>
<thead>
<tr>
<th>Constituent</th>
<th>MW</th>
<th>Solubility (20°C)</th>
<th>Average Initial Conc.</th>
<th>Average Initial Mass</th>
<th>Mol Fraction</th>
<th>Effective Solubility</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorobenzene</td>
<td>112.6</td>
<td>500</td>
<td>421</td>
<td>4.12</td>
<td>0.12</td>
<td>58.4</td>
</tr>
<tr>
<td>1,2-Dichlorobenzene</td>
<td>146.9</td>
<td>140</td>
<td>2000</td>
<td>14.97</td>
<td>0.42</td>
<td>59.4</td>
</tr>
<tr>
<td>1,3-Dichlorobenzene</td>
<td>146.9</td>
<td>123</td>
<td>474</td>
<td>3.55</td>
<td>0.10</td>
<td>12.4</td>
</tr>
<tr>
<td>1,4-Dichlorobenzene</td>
<td>146.9</td>
<td>77</td>
<td>1686</td>
<td>12.62</td>
<td>0.36</td>
<td>27.6</td>
</tr>
</tbody>
</table>
Appendix II

Calibration curves for Quantitative Real Time Polymerase Chain Reaction Analysis
Standard curve 16S rDNA (Total Bacteria)

\[ y = 2 \times 10^e^{-0.5914x} \]
\[ R^2 = 0.9912 \]

Standard curve Toluene dioxygenase

\[ y = 2 \times 10^e^{-0.629x} \]
\[ R^2 = 0.9824 \]

Standard curve Biphenyl dioxygenase

\[ y = 4 \times 10^e^{-0.9919x} \]
\[ R^2 = 0.9977 \]
Standard curve Phenol hydroxylase

\[ y = 1E+12 e^{-0.971x} \]
\[ R^2 = 0.9991 \]

ct

Standard curve Ring hydroxylating monoxygenase

\[ y = 7E+09 e^{-0.6422x} \]
\[ R^2 = 0.9763 \]

ct

Standard curve Bacteriophage

\[ y = 4.6702 e^{-0.513x} \]
\[ R^2 = 0.9863 \]

dilutions

cr
Appendix III.

Pathways of Aerobic Biodegradation of Aromatic Compounds
(From: The University of Minnesota Biocatalyst/Biodegradation Database
http://umbbd.msi.umn.edu/)
The first step of the aerobic biodegradation of biphenyl is catalyzed by biphenyl dioxygenase.
The first step of the aerobic biodegradation chlorobenzene is catalyzed by chlorobenzene dioxygenase.

\[
\text{Chlorobenzene} \xrightarrow{\text{chlorobenzene dioxygenase}} \text{3-Chloro-cis-1,2-dihydroxycyclohexa-3,5-diene} \xrightarrow{\text{cis-1,2-dihydrobenzene-1,2-diol dehydrogenase}} \text{2,3-dihydroxybiphenyl 1,2-dioxygenase} \xrightarrow{} \text{3-Chloro-2-hydroxymuconic semialdehyde}
\]

\[
\text{3-Chlorocatechol} \xrightarrow{\text{catechol 1,2-dioxygenase}} \text{2-Chloro-cis,cis-muconate} \xrightarrow{\text{chloromuconate cycloisomerase}} \text{trans-4-Carboxymethylenebut-2-en-4-olide} \xrightarrow{\text{dienelactone hydrolase}} \text{Maleylacetate} \xrightarrow{\text{maleylacetate reductase}} \text{3-Oxoadipate}
\]
The first step of the aerobic biodegradation of chlorobiphenyl is catalyzed by biphenyl dioxygenase.
The first step of the aerobic biodegradation of 1,4-dichlorobenzene is catalyzed by chlorobenzene dioxygenase.
The aerobic biodegradation of toluene (Methylbenzene) is initiated by two successive monooxygenase attacks or by one benzene dioxygenase (a.k.a. toluene dioxygenase).
Appendix IV.

Graphic of the reaction
Chlorobenzene $\xrightarrow{\text{chlorobenzene dioxygenase}}$ 3-Chloro-cis-1,2-dihydroxycyclohexa-3,5-diene

1,4-Dichlorobenzene $\xrightarrow{\text{chlorobenzene dioxygenase}}$ 3,6-Dichloro-cis-1,2-dihydroxycyclohexa-3,5-diene

Toluene $\xrightarrow{\text{toluene dioxygenase}}$ Toluene-cis-1,2-dihydrodiol

Biphenyl $\xrightarrow{\text{biphenyl-2,3-dioxygenase}}$ cis-2,3-Dihydro-2,3-dihydroxybiphenyl