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Functional establishment of introduced chlorobenzoate degraders following bioaugmentation with newly activated soil

Enhanced contaminant remediation via activated soil bioaugmentation

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Abstract

Introduced degraders often do not survive when applied to polluted sites; however, the potential for successful bioaugmentation may be increased if newly activated soil (containing indigenous degrader populations recently exposed to the contaminant) or potentially active soil (containing indigenous degrader populations not previously exposed to the contaminant) is used as the inoculant. To investigate this concept, Madera and Oversite soils were amended with 0 or 500 micrograms of 2-, 3-, or 4-chlorobenzoate per gram soil. The Madera degraded 2-chlorobenzoate while the Oversite degraded 3- and 4-chlorobenzoate. After 22 days of incubation, non-active soils that had not degraded chlorobenzoate were bioaugmented with the appropriate activated soil that had been exposed to and degraded chlorobenzoate. Thus, Oversite soil that had not degraded 2-chlorobenzoate was bioaugmented with Madera soil that had degraded 2-chlorobenzoate. Likewise, Madera soil that had not degraded 3- or 4-chlorobenzoate was bioaugmented with the Oversite soil that had degraded 3- or 4-chlorobenzoate. Additionally, the non-active soils were bioaugmented with the corresponding potentially active soils. The Oversite soil amended with activated Madera soil degraded the 2-chlorobenzoate within 3 days of bioaugmentation. The Madera soil amended with activated Oversite soils degraded the 3- and 4-chlorobenzoate within 20 and 6 days, respectively. Large degrader populations developed in microcosms bioaugmented with activated soil, and shifts in the 3- and 4-CB degrader community structures occurred following bioaugmentation. In contrast, bioaugmentation with potentially active soil did not impact degradation. The results indicate the potential for bioaugmentation with newly activated soil to enhance contaminant degradation.

Introduction

Bioaugmentation with activated soil is a relatively unheralded technique for the introduction of microorganisms into contaminated sites even though studies have indicated its potential for remediation (Barbeau et al. 1997; Dejonghe et al. 2001; Gentry et al. 2003b; Runes et al. 2001). Barbeau et al. (1997) successfully utilized a pentachlorophenol (PCP)-contaminated soil, containing developed degrader populations, to inoculate and enhance PCP-degradation in another contaminated soil. Runes et al. (2001) found similar results using atrazine spill-site soil to bioaugment atrazine-contaminated sediment. The use of activated soil may initially appear to be less scientific than other methods of bioaugmentation (Pepper et al. 2002), but it has the potential advantages of: (1) introduction of naturally-developed degrader populations that may be composed of several members or even consortia that would not be as effective if they were isolated and applied to the site as pure cultures; (2) the degraders are not cultured outside of the soil and thus do not lose their ability to compete in the environment as is often observed for lab-cultured strains; and (3) potential inclusion of poorly culturable degraders that would be missed in

attempts to isolate and culture an organism from one site in order to introduce the organism to another site.

Previous studies have focused on the use of historically contaminated soil as the activated soil inoculant (Barbeau et al. 1997; Runes et al. 2001). One possible downside to the use of activated soil from previously contaminated sites is that the soil inoculant may contain other pollutants that may even be more hazardous that the contaminant(s) existing at the target site. However, if previously uncontaminated soil, with naturally developed populations of microorganisms that could degrade a chemical of interest were used as the inoculant, it would alleviate the concerns of adding additional contaminants to the site. In this study, we investigated the potential of bioaugmentation with either newly activated or potentially active soil to enhance contaminant degradation in non-active soil. Our definition of activated soil for this experiment includes previously uncontaminated soil that has been freshly exposed to and subsequently degraded anthropogenic levels of the contaminant immediately prior to use of the soil for bioaugmentation. We have defined potentially active soil as the same activated soil prior to contaminant exposure – it contains naturally developed degrader populations, but has not been exposed to the anthropogenic contaminant. We also define non-active soil as soil that has been contaminated, but either does not contain degrader populations or the degrader populations are not active under existing conditions, thus precluding degradation of the contaminant.

For this study, we used two similar soils and 2-, 3-, and 4-chlorobenzoate (CB) as contaminants. The CB degraders are normally widely distributed and diverse (Fulthorpe et al. 1998; Suzuki et al. 2001; Yi et al. 2000); however, in some soils, CB degraders are either not present or are not active under existing conditions (Focht and Shelton 1987; Gentry et al. 2001). The CB concentrations and degrader numbers were determined during this experiment. Additionally, past studies have demonstrated the potential for degraders to become established and significantly increase in numbers following bioaugmentation with activated soil, but to our knowledge the composition of the degrader populations with respect to those in the initial activated soil have not been characterized (Barbeau et al. 1997; Runes et al. 2001). Therefore, we isolated CB degraders in the bioaugmented soil, and compared the resultant populations to those occurring in the activated soil used for the inoculant.

Materials and methods

A. Soils

Soils were collected from the surface horizons of pristine, forested sites in Madera Canyon and Oversite Canyon of the Coronado National Forest near Tucson, AZ. The Madera and Oversite soils had pH values of 6.5 and 6.8, respectively. Percent organic C, N, sand, silt, and clay values were 2.6, 0.17, 76, 20 and 4 for the Madera soil and 2.8, 0.23, 74, 21, and 5 for the Oversite soil, respectively. Field-moist soil was passed through a 2-mm sieve and 100 g dry weight equivalent added to polypropylene jars. Sterile deionized water was added to bring the soils to the same water potential (140% of –0.3 MPa) slightly below field capacity. Microcosms were incubated for 7 d at 27 °C to allow for acclimation of indigenous microorganisms prior to imposing treatments.

B. Initial chlorobenzoate exposure to soils

In a concurrent experiment, soils were initially amended with 2-, 3-, or 4-CB (Gentry et al. 2003a). Stock solutions of 2-CB (98%, Aldrich Chemical Co. Inc., Milwaukee, WI), 3-CB (99%, Sigma Chemical Co. Inc., St. Louis, MO), and 4-CB (99%, Aldrich) were prepared similar to the method described by Di Giovanni et al. (1996) for 2,4-dichlorophenoxyacetic acid (2,4-D). Microcosms, containing Madera or Oversite Canyon soil, were amended with 0 or 500 μg of the respective CB g^{-1} dry soil. All treatments were conducted in triplicate. Sterile, deionized water was then added to both soils bringing the soil water potential to the same level (175% of –0.3 MPa) at approximately field capacity. Microcosms were incubated at 27 °C. The Madera soil degraded only the 2-CB within 21 d of incubation, in contrast to the Oversite soil that degraded only the 3- and 4-CB within 21 d (Gentry et al. 2003a).

C. Bioaugmentation with activated or potentially active soil

After 22 d of incubation, treatments from each non-active soil that had not degraded CB were bioaugmented with the appropriate activated soil that had been exposed to, and degraded, the chemical of concern (Figure 1) (Table 1). Twenty g (dry wt.) of each respective non-active soil was amended with 1 g (dry wt.) of the appropriate activated soil. Thus, Madera

Table 1. Numbers of CB degraders in the initial Madera and Oversite microcosms after amendment with 0 or 500 μg 2-, 3-, or 4-CB g^{-1} soil and in the originally non-active microcosms following bioaugmentation with activated soil

Soil	Contaminant	Number of CB degraders	
		Initial microcosms *	Bioaugmented Microcosms**
		CFU g^{-1}	
Madera	None	$<3.0 \times 10^3$	NB‡
Madera	2-CB	7.3×10^6 a†	NB
Madera	3-CB	$<3.0 \times 10^3$	4.1×10^6 a
Madera	4-CB	$<3.0 \times 10^3$	2.6×10^8 c
Oversite	None	$<3.0 \times 10^3$	NB
Oversite	2-CB	$<3.0 \times 10^3$	1.2×10^8 b
Oversite	3-CB	4.9×10^6 a	NB
Oversite	4-CB	8.9×10^8 b	NB

*Degraders were enumerated after 21 d of incubation. Numbers are listed as less than the limit of detection ($\sim 3.0 \times 10^3$ CFU g^{-1}) for treatments where no degraders were detected.
**Microbial numbers were assessed when $\geq$90% of the contaminant was degraded (3 d after Oversite/2-CB soil was bioaugmented and 6 and 20 d after the Madera/4-CB and Madera/3-CB soils, respectively, were bioaugmented).
†Numbers in a column followed by the same lower case letter are not significantly different at the 5% level.
‡Some soil/CB combinations were not bioaugmented (NB) either because CB was not added to the initial microcosms or the contaminant degraded in the initial microcosms before the bioaugmentation experiment began.

soil that had not degraded 3-CB or 4-CB was bioaugmented with the Oversite soil that had degraded 3-CB or 4-CB. Likewise, Oversite soil that had not degraded 2-CB was bioaugmented with Madera soil that had degraded 2-CB. Additionally, non-active soils were bioaugmented with the potentially active soils (not exposed to CB) corresponding to the activated soils used above (Figure 1).

D. Assessment of chlorobenzoate degradation

The CB extraction was performed by placing 1.2 g moist soil into 9.5 ml of extracting solution (6 μM Zwittergent detergent and 0.2% sodium hexametaphosphate) (Brendecke et al. 1993) and mixing at high setting (280 osc min^{-1}) on a horizontal shaker for 5 min. Following mixing, a 1.0-ml aliquot of the soil-extraction solution was placed in a 1.5-ml microcentrifuge tube and centrifuged at $16,000 \times g$ for 10 min. Supernatants were passed through 0.22 μm pore-size polypropylene filters prior to HPLC analysis. The concentration of CB was determined using HPLC as previously described (Gentry et al. 2001).

E. Measurement of culturable heterotrophic and chlorobenzoate-degrading bacteria

Culturable bacterial and CB degrader numbers were determined after >90% of the respective CB had been degraded in a microcosm. The soil extraction solution from the CB quantification was serially diluted in 0.85% NaCl solution. Numbers of culturable CB degraders were determined by spread-plating 0.1 ml of the appropriate dilution onto Bushnell-Haas Agar (BH) containing 500 mg of the appropriate CB l^{-1} as a C source (Atlas 1993). Fifty mg of bromthymol blue l^{-1} was added to BH as a pH indicator. Noble agar (Difco, Detroit, MI) was utilized as the solidifying agent in BH in order to reduce the presence of undesirable C compounds. Heterotrophic bacteria were enumerated on R2A (Difco). Plates were counted after incubation at 27 °C for 6 d (R2A) or 14 d (BH).

F. Characterization of dominant chlorobenzoate degraders

Dominant CB degraders were isolated and characterized during the study. About 40 bacteria were randomly selected from plates at the highest (most dilute) dilution that produced a countable number of bacterial colonies. Isolated bacteria were streaked to purity on the appropriate BH plates prior to a final streak onto R2A. Approximately one colony of bacterial cells was harvested, placed into 20 μl of sterile, molecular-grade water, and stored at –20 °C until analysis. Isolated degraders were grouped based on DNA fingerprints generated using enterobacterial repetitive intergenic consensus sequence (ERIC) PCR (Versalovic et al. 1991). Fingerprints were performed using the FailSafe™ PCR System (Epicentre, Madison, WI). Primers from Versalovic et al. (1991) (0.1 μM in reaction) were added to FailSafe™ PCR 2X PreMix F and Enzyme Mix according to the manufacturer's instructions. The PCR reaction was conducted in a GeneAmp® PCR System 2700 (Applied Biosystems, Foster City, CA) with an initial denaturation step for 7 min at 95 °C followed by 35 cycles of denaturation for 30 s at 94 °C, annealing for 30 s at 52 °C, and extension for 4 min at 68 °C. The PCR products were visualized on a 1.2% agarose gel following electrophoresis and ethidium bromide staining. The ERIC fingerprints of isolated degraders were compared with those of degraders isolated from the initial activated soil microcosms when approximately 90% of the CB had been degraded in the original microcosms (days

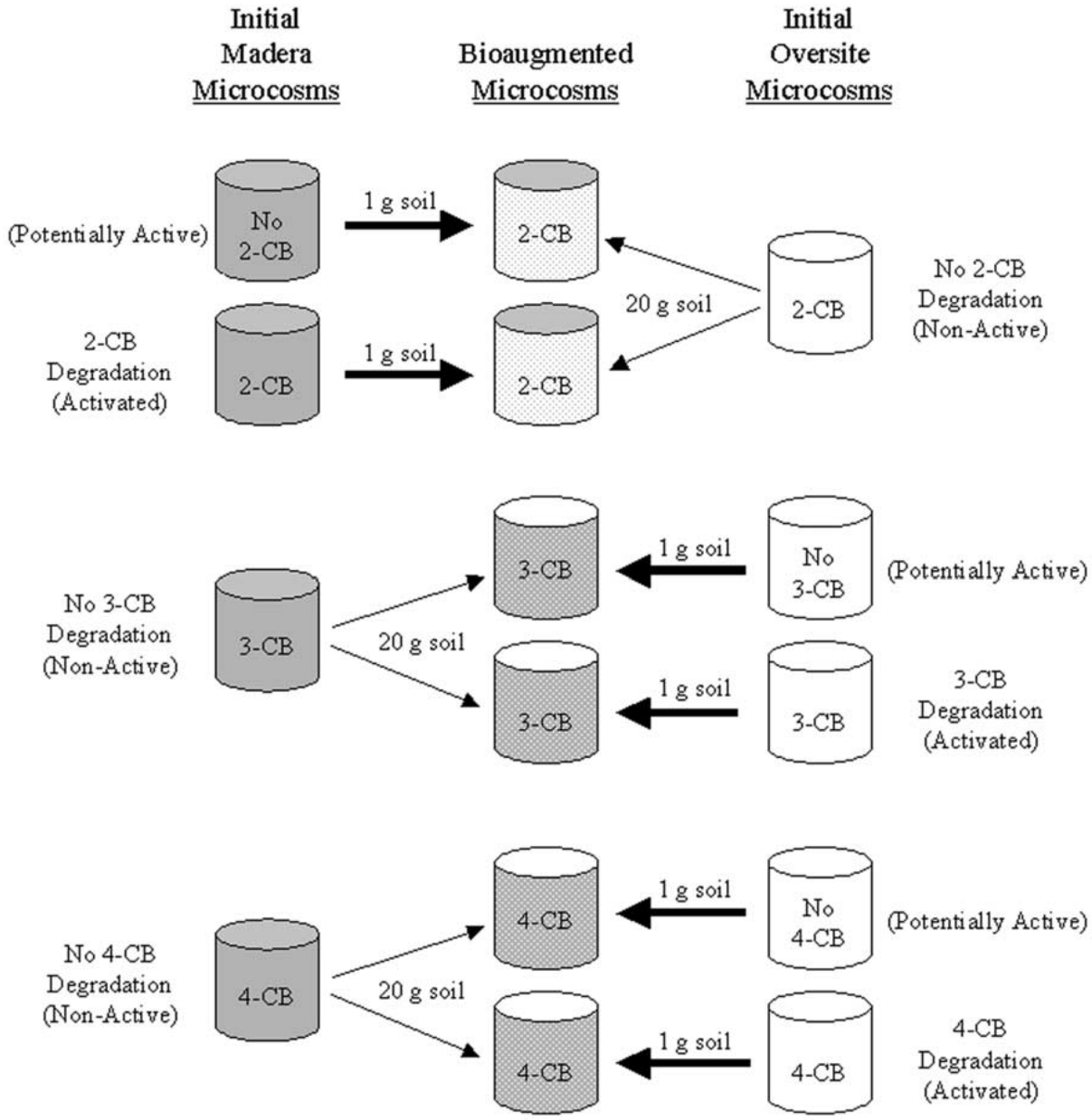

Figure 1. Diagram of procedures used to bioaugment non-active Madera and Oversite soils with activated or potentially active soil. Bold arrows indicate addition of activated or potentially active soil.

11, 14, and 21 for 2-, 3-, and 4-CB, respectively) (Gentry et al. 2003a).

Selected bacteria from each ERIC profile were further characterized by PCR amplification and sequencing of an approximately 440 bp region of the 16S rDNA (Gentry et al. 2001). The sequences were compared to those in GenBank with BLASTN 2.2.5 (Altschul et al. 1997). Isolated degraders were also screened for the presence of large plasmids according the method of Kado and Liu (1981). *Comamonas testosteroni* BR60 containing an 85-kb plasmid was used for the positive control in the plasmid screen (Nakatsu et al. 1997). Representative degrader isolates were also inoculated into modified M9 broth (without glucose or thiamine) (Atlas 1993) containing 100 mg of the respective CB l^{-1} in order to confirm the abil-

ity to degrade CB. The CB levels in the M9 broth were measured by UV spectrophotometry (Gentry et al. 2003a).

G. Statistical analysis

Microbial numbers were compared as log10-transformed values using single-factor analysis of variance (ANOVA) (Parkin & Robinson 1994). Calculated P values ≤ 0.05 were considered significant. Microsoft Excel 2000 version was used to perform all tests.

Results

A. Chlorobenzoate degradation following bioaugmentation

Bioaugmentation with activated soil increased the degradation of each 2-, 3-, and 4-CB. In contrast, bioaugmentation with the corresponding, potentially active soil did not impact CB degradation (Figure 2). The 2-CB in the Oversite soil was virtually eliminated within 3 d after bioaugmentation with activated soil. Conversely, over 350 μg of 2-CB g^{-1} soil remained at the end of the experiment in the microcosms bioaugmented with potentially active soil and in the initial, non-bioaugmented microcosms (Figure 2a). Likewise, the 3-CB in the Madera soil was mostly degraded within 20 d of bioaugmentation with activated soil, but over 360 μg of 3-CB g^{-1} soil remained at the end of the experiment in the microcosms bioaugmented with potentially active soil and in the initial, non-bioaugmented microcosms (Figure 2b). In contrast, the 4-CB was degraded in all tested microcosms (Figure 2c).

B. Enumeration of chlorobenzoate degraders and culturable bacterial populations

Approximately 10^8 CFU of 2-and 4-CB degraders g^{-1} developed in the Oversite and Madera soils, respectively, that were bioaugmented with activated soil (Table 1). Large populations (about 10^6 CFU of g^{-1}) of 3-CB degraders were also detected in the Madera microcosms that were bioaugmented with activated soil although the numbers were significantly lower than those of the 2- and 4-CB degraders ($P < 0.001$). Culturable bacterial numbers were approximately 10^8 CFU g^{-1} in all tested microcosms. No 4-CB degraders were detected in the Madera microcosms bioaugmented with potentially active soil or the initial, non-bioaugmented Madera microcosms even though 4-CB degradation occurred. Degraders and culturable bacteria were not assessed in the microcosms that did not degrade CB: (1) initial, non-bioaugmented Oversite soil with 2-CB; (2) Oversite soil with 2-CB, bioaugmented with potentially active soil; (3) initial, non-bioaugmented Madera soil with 3-CB; and 4) Madera soil with 3-CB, bioaugmented with potentially active soil.

C. Characterization of dominant chlorobenzoate degraders

The ERIC fingerprints of isolated 2- and 3-CB degraders matched those found in the original, activated soil microcosms (Figure 3a and 3b). All of the 2-CB degraders isolated from the Oversite soil had a single ERIC fingerprint (a) and were characterized as a *Burkholderia* sp. based on their partial 16S sequence. The majority of 3-CB degraders from the Madera soil (fingerprints g, m, and $s2$) were characterized as a *Burkholderia* sp., and a smaller portion were characterized as a *Microbacterium* sp. (f) (fingerprint g was detected in the initial, activated soil at an earlier sampling time which is not included in Figure 3b) (Gentry et al. 2003a). Although no new 3-CB degraders were detected, there were shifts in their relative proportions following bioaugmentation (Figure 3b). Many of the isolated 4-CB degraders were characterized as a *Bradyrhizobium* sp. ($ag3$) as in the initial, activated soil, but new degraders with unique fingerprints were detected including an *Alcaligenes* sp. (ba), *Bradyrhizobium* sp. (d), and two *Microbacterium* spp. ($ag5$ and bc) (Figure 3c). The partial, 16S rDNA sequences for these unique 4-CB degraders were submitted to GenBank and assigned the accession numbers AY267533–AY267536. No plasmids were detected in isolated 2-CB or 4-CB degraders. In contrast, a large plasmid ($\sim$85 kb) was detected in several of the isolated 3-CB degraders (fingerprints g, m, and $s2$). This plasmid was also detected in isolates from the initial activated soil microcosms having the same fingerprints (Gentry et al. 2003a).

Discussion

Large populations of CB degraders developed in the soils bioaugmented with activated soil indicating that the soils which previously did not harbor a functional degrader population could develop and maintain degrader populations if the appropriate microorganisms

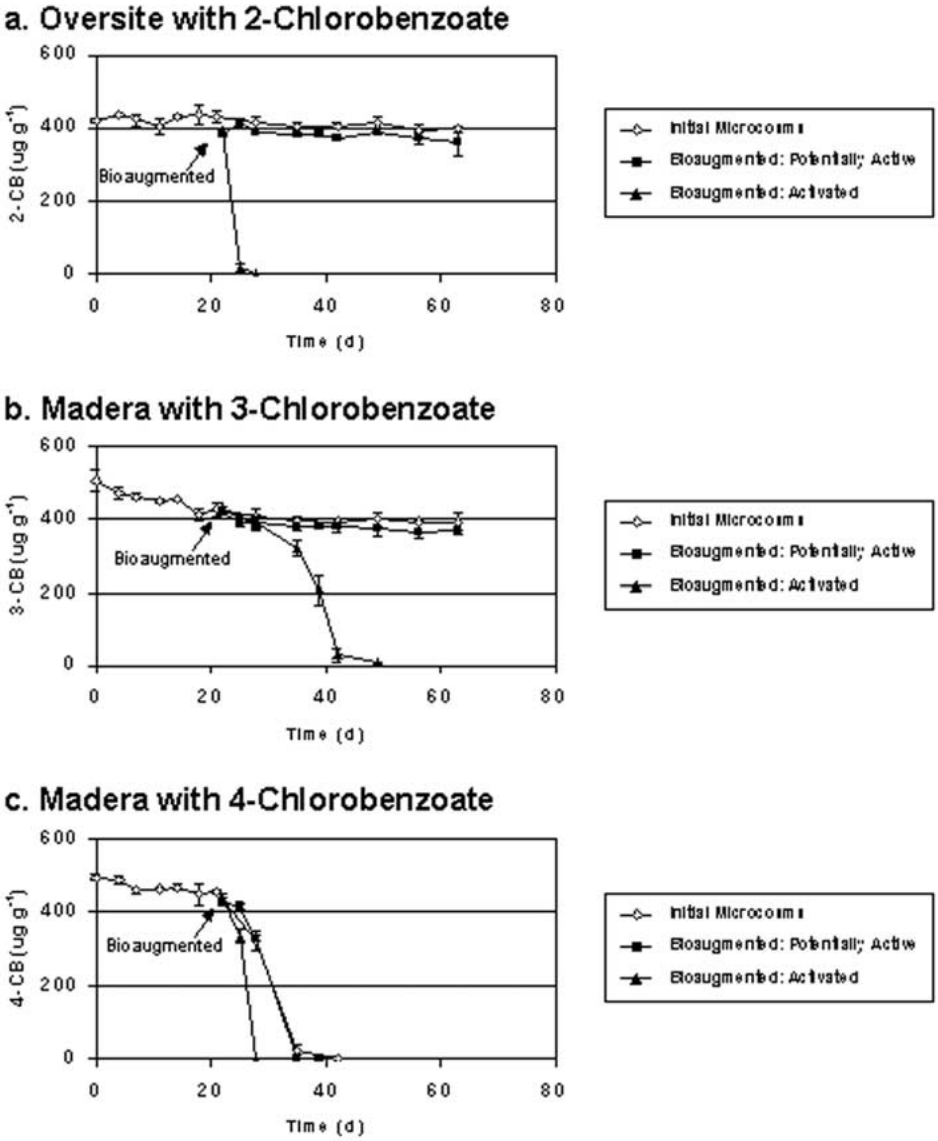

Figure 2. Levels of (a) 2-CB in Oversite soil, (b) 3-CB in Madera soil, and (c) 4-CB in Madera soil either not bioaugmented, bioaugmented with potentially active soil, or bioaugmented with activated soil. Error bars represent the standard deviation of three replicate microcosms.

were added. In contrast, no CB degraders were detected in soils bioaugmented with potentially active soil (the same soil as the activated soil but without CB exposure). Accordingly, bioaugmentation with activated soil resulted in enhanced CB degradation, but no effect was seen following bioaugmentation with the potentially active soil. The most likely explanation for this difference is the larger number of degraders in the activated soil as compared to the potentially active soil. It is possible that the microcosms bioaugmented with potentially active soils would have eventually degraded the CB if the experiment were carried out for a longer duration. The experiment was carried out for 42 d after bioaugmentation, but this may have not been long enough for the small, initial degrader populations in the potentially active soil to significantly increase following addition to the non-active soil, especially after diluting their numbers 20-fold.

Even though the two soils were very similar chemically and physically, they appeared to select for different degrader populations. The new populations of 4-CB degraders detected in the previously non-active

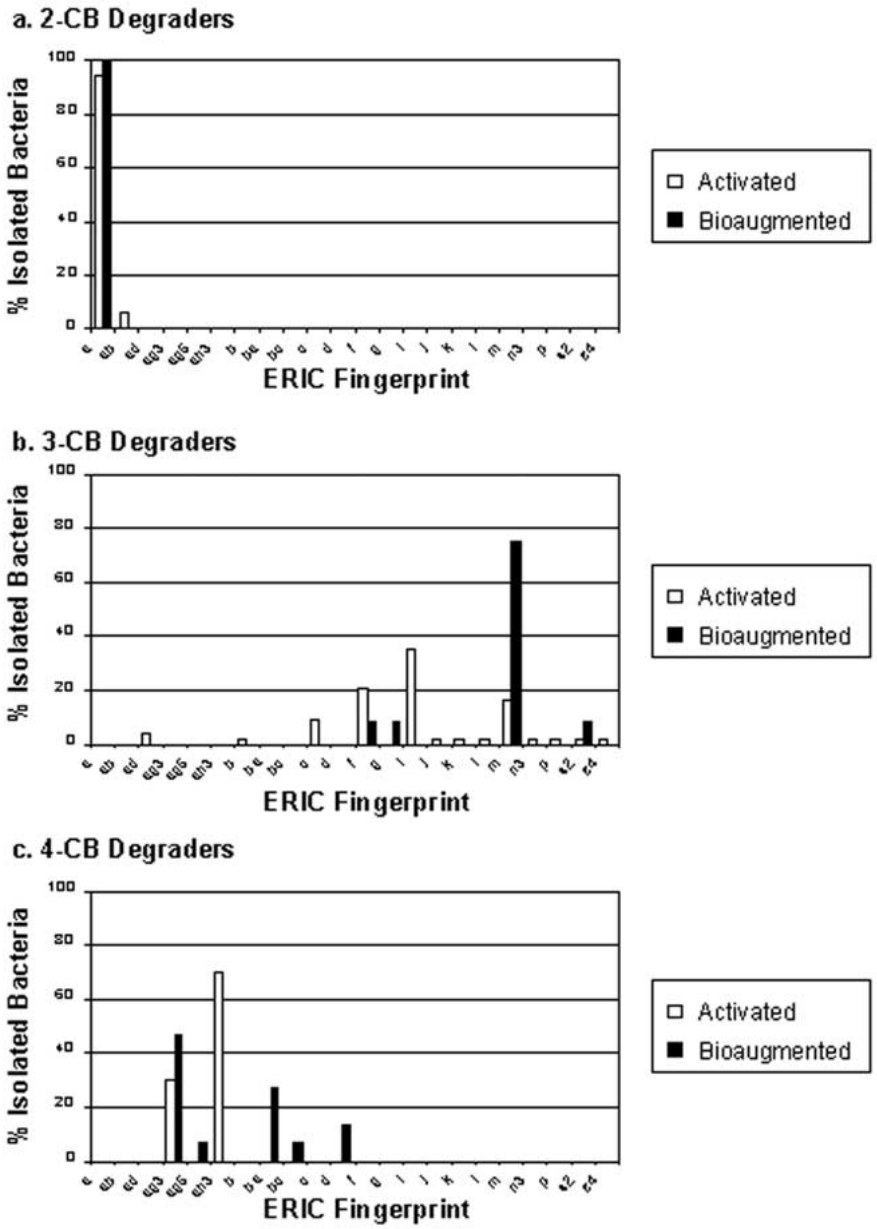

Figure 3. Semiquantitative analysis of (a) 2-, (b) 3- and (c) 4-CB degraders isolated from the initial, activated soils and the corresponding microcosms bioaugmented with activated soil. The ERIC fingerprints of isolated degraders are plotted as percentage of the total number of isolated degraders.

Madera soil, after bioaugmentation with activated Oversite soil, were similar to those degraders isolated from the initial, activated Oversite soil based on their partial 16S rDNA sequences. This similarity indicates that the new degraders probably represented minor populations in the Oversite soil that were more competitive in the Madera soil, and thus became dominant following bioaugmentation. Even though the 3-CB degraders isolated following bioaugmentation had the same ERIC fingerprints as those in the initial microcosms, the relative proportion of the different 3-CB degraders appeared to shift. We found no indication that horizontal transfer of CB degradative genes, as evidenced by plasmid transfer, contributed to the changes in degrader community structure following bioaugmentation. However, it is possible that

genes were transferred via other means such as transposons (Nakatsu et al. 1997; Sentchilo et al. 2003). In contrast to the 3- and 4-CB degraders, the 2-CB degraders in the original microcosms were dominated by one *Burkholderia* sp. thus limiting the capacity for shifts in degrader community composition following bioaugmentation. As illustrated by the shifts in the 3- and 4-CB degrader populations, an activated soil containing diverse degrader populations is likely to have a more adaptable community structure and thus greater functional stability potentially enabling its application to a wider variety of soils and environments (Fernandez et al. 2000; Müller et al. 2002). All of the 2-, 3-, and 4-CB degraders that were isolated in this experiment belonged to bacterial genera commonly reported to degrade CBs or complex organics (Arrault et al. 2002; Gentry et al. 2001; Song et al. 2000; Suzuki et al. 2001).

The differences between the Madera and Oversite soils are also indicated by the diversity of 2-, 3-, and 4-CB degraders isolated from the two soils in the initial, activated microcosms (Gentry et al. 2003a). Only one major 2-CB degrader population was detected in the initial, activated Madera soil. In contrast, several different 3- and 4-CB degraders were isolated from the initial, activated Oversite soil amended with 3- or 4-CB. The Madera soil may have been more selective since it produced only one population of dominant 2-CB degraders; however, this is not known for certain since there may have just been more natural diversity in the 3- and 4-CB degraders. The Madera soil also produced lower numbers of 2-, 3-, and 4-CB degraders in most cases than the Oversite soil did (data not shown). Since the two soils were very similar chemically and physically, we theorize that these differences are due to microbial competition and not abiotic factors.

These results suggest that it may be necessary to activate the degrader populations in previously uncontaminated, potentially active soils by pre-exposing the populations to anthropogenic contaminant levels prior to using the soil for bioaugmentation. If the site to be remediated contains multiple pollutants, the activated soil would need to contain degrader populations for each of the contaminants. Finding such a previously uncontaminated, potentially active soil would become more difficult with any increase in the number and/or complexity of contaminants present at a site. It may be possible to utilize several different activated soils, each containing different populations, in order to remediate the multiple pollutants. However, the use of newly activated soil as a remediation inoculant is probably more applicable to sites containing only a single compound or a few related contaminants.

Conclusion

This research demonstrates that previously uncontaminated soil that contains specific, activated indigenous degrader populations can be successfully used as a bioaugmentation inoculant following exposure to the contaminant of interest. Based on our research, it is unclear if the same uncontaminated soil containing potentially active indigenous degrader populations can be successfully used for bioaugmentation without pre-exposure to the contaminant. Large populations of CB degraders developed in microcosms inoculated with activated soil indicating the ability of degraders to become functionally established in the bioaugmented soils. The Madera soil appeared to be more suppressive to the development of CB degrader populations than was the Oversite soil. For each 2-, 3-, and 4-CB, the degraders that developed in microcosms bioaugmented with activated soil appeared to have originated from the activated soil inoculant. However, bioaugmentation did appear to alter the community structures of the introduced 3- and 4-CB degraders.

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