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Earthworm Enhanced Bioaugmentation of PCB Contaminated Soil

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Abstract

In a recently developed strategy for in-situ treatment of polychlorinated biphenyls (PCB), bioaugmentation was used in conjunction with a surfactant, sorbitan trioleate, as a carbon source for the degrader bacteria, along with the monoterpene, carvone, and salicylic acid as inducing substrates. Two bacteria were used for soil inoculants, including *Arthrobacter* sp. st. B1B and *Ralstonia eutrophus* H850. This methodology achieved 60% degradation of PCBs in Aroclor 1242 after 18 weeks in soils receiving 34 repeated applications of the degrader bacteria. However, an obvious limitation was the requirement for soil mixing after every soil inoculation. In the research reported here, bioaugmentation and biostimulation treatment strategies were modified by using the earthworm, *Pheretima hawayana*, as a vector for dispersal and mixing of surface-applied PCB-degrading bacteria and soil chemical amendments. Changes in microbial biomass and microbial community structure due to earthworm effects were examined using DNA extraction and PCR-DGGE of 16S rDNA. Results showed that earthworms effectively promoted biodegradation of PCBs in bioaugmented soils to the same extent previously achieved using physical soil mixing, and had a lesser, but significant effect in promoting PCB biodegradation in biostimulated soils treated with carvone and salicylic acid. The effects of earthworms were speculated to involve many interacting factors including increased bacterial transport to lower soil depths, improved soil aeration, and enhanced microbial activity and diversity.

Introduction

Bioremediation of soil contaminants using bacterial inoculants is presently limited by problems associated with inoculum delivery, poor bioavailability, and difficulty in maintaining high population densities of degrader organisms. Prior research has examined various methods to circumvent these problems by using repeated inoculation of soils, and the use of plant microbial systems to provide a niche for maintaining degrader microorganisms in the plant rhizosphere. Automated field fermenters also have been developed to facilitate repeated inoculation of soils (3), but soil treatment may still be hindered by poor inoculum dispersal and inadequate mixing of the inoculum with the contaminant. This problem can be ameliorated to some extent by addition of surfactants to solubilize chemicals with low water solubility. Still another approach to improve soil mixing is the use of earthworms to promote incorporation of surface applied bacteria into the soil matrix. Earthworms enhance soil mixing by continuously ingesting microbes, organic matter, and soil particles that are then excreted as organic rich casts. Earthworms may also facilitate bacterial transport for microorganisms that adhere to their body surface (5) or by bulk flow in water that drains through the earthworm burrows (8, 14). These channels may also provide for increased aeration and thereby promote aerobic degradation processes.

Among the various organic contaminants that have been targeted for bioremediation are polycyclic aromatic hydrocarbons (PAH) and polychlorinated biphenyls (PCB). When present at high concentrations, these compounds are typically treated by soil excavation and landfilling, or by thermal desorption and incineration. However, when the contaminant is present at low concentrations over large land areas, these treatment methods are prohibitively expensive, and in situ treatment methods are preferred (13). This may involve plants or soil amendments to achieve

enhanced natural attenuation of the contaminant, or more intensive methods involving bioaugmentation or biostimulation to increase the population size of degrader organisms. Biostimulation and bioaugmentation are particularly important for enhancing degradation of substances by cometabolic processes, such as PCBs that are not readily degraded for use as a carbon and energy source. Induction of the requisite enzymes requires the addition of a cometabolite that in some instances may also serve as a growth substrate. Biostimulation strategies attempt to induce the catabolic enzymes by addition of co-substrates to the indigenous microflora. When this does not provide specific induction, bioaugmentation can be used, in which case the inoculum can be induced during growth in pure culture prior to soil inoculation.

Cometabolism of PCBs may be induced by addition of biphenyl, various monoterpenes, and naphthalene. Biphenyl is the best studied of these substances and is known to serve both as a growth substrate and to induce biphenyl dioxygenases that bring about dechlorination and ring cleavage of PCB congeners. Although biphenyl degrading bacteria are ubiquitous in soils, these bacteria vary widely in their abilities to degrade PCB congeners that include 209 different compounds. The mode of action of monoterpenes is not yet known, but for substances such as carvone, involves the induction of enzymes that are used to detoxify this substance rather than being used as a carbon source for growth (7). In comparison to biphenyl, monoterpenes have many desirable chemical properties, including their relatively high water solubility, low mammalian toxicity, and their efficacy in inducing cometabolism at very low concentrations.

In a recently developed treatment strategy for polychlorinated biphenyls (PCB), bioaugmentation was achieved using a surfactant, sorbitan trioleate, as a carbon source for the degrader bacteria, along with the monoterpene, carvone, and salicylic acid as inducing substrates. This methodology achieved 60% degradation of PCBs in Aroclor 1242 after 18 weeks in soils receiving 34 repeated applications of the degrader bacteria. An obvious limitation of this method was the requirement for soil mixing after every soil inoculation. In the research reported here, bioaugmentation and biostimulation treatment strategies were further modified by using the earthworm, *Pheretima hawayana*, as a soil animal vector for dispersal and mixing of surface-applied PCB-degrading bacteria. Changes in soil microbial biomass due to earthworm activity were determined by measuring soil DNA, which is correlated with the population density of the soil microflora. Changes in microbial community structure due to earthworm effects were examined using PCR-DGGE of 16S rDNA. The overall aim of this study was to examine the extent to which earthworms might replace physical mixing of the soil after soil inoculation, and how this would affect PCB degradation.

Materials and Methods

General Procedures. Soil columns were prepared by packing 600 g of coarse loamy soil (0.21% carbon, 0.01% nitrogen, pH 7.5) into 30 cm long, 2" diameter polyvinyl chloride (PVC) tubes. The soil contained 100 ppm PCBs, previously added as Aroclor 1242 (AccuStandard, Inc., New Haven, CT). Soils were contaminated 1 month prior to the experiment by spraying PCB dissolved in hexane over a thin layer of the soil in a tray, which was then allowed to dry and mixed with uncontaminated fresh soil to restore the indigenous microflora. At the beginning of the experiment, each column was amended with 0.5X minimal salts medium (MSM; (7)) sufficient to establish 12% water content. The experiment employed a 2 x 3 factorial design with 24 columns. Twelve columns were prepared with and without earthworms. Ten adult earthworms, *Pheretima hawayana*, (approximately 0.5 g worm^{-1}) were added into each of the 12 columns with worms. These two sets of columns were then used for 3 bioremediation treatments with 4 replicate columns per treatment. The treatments included bioaugmentation with induced bacteria, biostimulation with

carvone and salicylic acid, and a control set that was watered with minimal salts medium. The earthworms were fed approximately 1 g of rolled oats at the same time as the inoculum application.

Bacterial inoculants used for bioaugmentation were obtained from overnight cultures of *Ralstonia eutrophus* H850 or *Rhodococcus* sp. ACS, which were applied alternately to the soil at every other inoculation. *Ralstonia eutrophus* H850 was grown on 500 ppm salicylic acid (Fisher Scientific, Inc., Pittsburgh, PA) and 500 ppm of the surfactant, sorbitan trioleate (Sigma-Aldrich Co., Saint Louis, MO). *Rhodococcus* sp. ACS was grown on 500 ppm sorbitan trioleate and 100 ppm carvone (Aldrich Chemical Co., Milwaukee, WI). Before application, an additional 500 ppm of sorbitan trioleate was added to each culture flask to maintain the critical micelle concentration (90 mg l^{-1}). Soil inoculation was performed biweekly, using an inoculum volume that was sufficient to replenish the water loss between amendment intervals (average 10 ml). Amendments began 1 week after the introduction of the earthworms and continued for 18 weeks. Control soil columns that had no earthworm were amended with minimal salts medium instead of the inoculum solution.

The effects of the various treatments on biodegradation of PCBs was determined after 18 weeks. Earthworms were collected from soil columns before the sampling. Ten-gram soil samples were removed for microbiological analysis from each of the following depths: 0-2, 2-6, and 6-20 cm. PCBs were analyzed in soil from the 2-6 cm depth. PCB degradation was analyzed by extraction and analysis of PCB congeners using previously established procedures (Gilbert and Crowley 1998), with slight modifications. In brief soils were extracted with 1% Triton X-100, acetone, and hexane (4:1:10; vol:vol:vol) for 24 hours. At which time an aliquot of the hexane fraction was transferred to another vial where it was mixed with sodium sulfate and subsampled for analysis on GC-FID.

Microbial community analyses. Total soil DNA was extracted from 1 g soil samples using a FastDNA SPIN Kit for soil (Bio 101, Vista, CA). DNA was quantified using a DU-640 spectrophotometer (Beckman Instruments, Inc., Fullerton, CA) by calculating the absorbance ratios at 260 and 280 nm wavelengths with background correction at 320 nm. The amount of extractable DNA was used to quantify microbial biomass in soil as described by Marstorp and Witter (9). The V3 region of 16S rDNA genes was amplified from total soil using PRBA338f and PRUN518 universal eubacterial primers (12). The PCR reaction contained 40 ng DNA, 5 pmol of each primer, PCR bead (Pharmacia Biotech, Piscataway, NJ), and sterilized distilled water to a final volume of 25 µl. Amplification was performed on PTC-200 thermocycler (MJ Research, Inc., Watertown, MA) as previously described (16). DGGE was performed with a DCodeTM system (Bio-Rad Laboratories, Hercules, CA). PCR samples were applied to 8% polyacrylamide gels (bisacrylamide gel stock solution, 37:5:1: Bio-Rad Laboratories, Inc., Hercules, CA) with denaturing gradients ranging from 20-50% (where 100% denaturant contains 7 M urea and 40% formamide). DGGE was run in TAE buffer at 60 °C for 4 h at a constant voltage of 200 V.

Digital image analysis of the DNA bands representing the microbial communities was conducted using Scion Image (Scion Corporation, Frederick, MD) as described in Yang and Crowley (2000). Community similarities were compared based on the incidence and abundance of DNA bands using correspondance analysis (CANOCO 4.0; Microcomputer Power, Ithaca, NY). The DNA band profile data for the microbial communities were also used to estimate diversity of the predominant bacteria, calculated using the Shannon-Weaver index of diversity (6).

Results and Discussion

This study investigated the effect of earthworms on inoculated bacteria as well as indigenous bacteria in a model system that used the anecic earthworm, *Pheretima hawayana* as a biological vector for dispersal of surface-applied PCB-degrading bacteria to uninoculated soil.

Earthworms were used as part of an integrated bioaugmentation methodology that utilizes a surfactant, sorbitan trioleate, for enhanced bioavailability of soil-bound PCB, and two inducing substrates, salicylic acid and carvone, used to induce PCB-degrading enzymes in the inoculum, *Rhodococcus* sp. ACS and *Ralstonia eutrophus* H850, respectively. The bacteria were repeated inoculated to overcome problems associated with the long term survival of introduced bacterial inoculants, which typically decline rapidly in population size and activity after addition to soils (15). This strategy is readily facilitated under field conditions by use of an automated fermenter that injects bacteria into irrigation water (11). Previous experiments had demonstrated the efficacy of this treatment strategy for PCBs, but required physical mixing of the soil (7).

Results of this experiment showed that earthworms were highly effective for causing enhanced soil mixing and biodegradation of PCBs. After 18 weeks of bioaugmentation, 75% of the PCB originally added to the soil was recovered in soil columns without earthworms (Figure 1). In comparison, 35% of the added PCB was recovered in bioaugmented columns containing earthworms. Similar beneficial results were observed in columns treated by biostimulation of the indigenous microflora with carvone, salicylic acid, and surfactant, although biodegradation rates were lower than those observed in the bioaugmented treatments. In biostimulated treatments without earthworms, 74% of the PCB was recovered, whereas 58% of the PCB was recovered in columns with earthworms. In the control treatment, earthworms had no beneficial effect on biodegradation of PCBs, such that 62 and 58 ppm PCB were recovered from columns with and without earthworms, respectively.

The effects of earthworms and the three bioremediation treatments on microbial biomass and bacterial community structure were analyzed by molecular techniques. When used as a measure of soil biomass, quantities of extractable DNA were consistently higher in earthworm treated soil than in the non-earthworm and control soils at all depths (Table 1). The addition of inoculum onto the surface of the soil resulted in higher levels of extractable DNA at the surface as compared to amounts extracted at lower depths (Table 1). This DNA gradient was more distinct in the treatment without earthworms, in which the amount of DNA at 0 to 2 cm was 3 and 6 times more than at 2 to 6 and 6 to 20 cm, respectively. These data further suggest mixing of the bacterial inoculum and rolled oat earthworm food throughout the soil matrix. Previously, it has been shown that bacterial populations for soil inoculants may decline as they pass through the earthworm gut (2). The research conducted here did not specifically determine the survival of the inoculated bacteria after passage through the earthworms. Nonetheless, with repeated inoculations of high cell densities, slight reductions in population size may be tolerated and are offset by the increased soil mixing that is promoted by earthworm activity.

Changes in microbial community structures can be readily observed using PCR-DGGE of 16S rDNA, which has the advantage of revealing the predominant bacteria without the requirement for culture and enumeration on agar media (10). In this research, analysis of DGGE profiles of amplified 16S rDNA fragments showed shifts in bacterial community structure and complexity associated with both bioaugmentation and the addition of earthworms. Relatively simple communities comprised of a few predominant bacteria represented by a few DNA bands were found in control soil. Communities shifted with respect to depth, and increased in complexity in treatments with earthworms. This was observed as a smear of indistinct bands, which indicated complex communities resulting from earthworm activity, and was particularly prevalent at the 0-2 cm depth (data not shown). Several common bands were present at all depths in the earthworm treatment. In soils without earthworms, the banding profiles were more variable between different soil depths.

Community similarities based on the DGGE band 16S rDNA band profiles were determined by correspondance analysis, which takes into account both the overall similarity in

community structure as represented by the occurence of specific DNA bands (rf values) and their staining intensity. Using this statistical analysis method, the large variable set for all of the 16S rDNA bands are reduced to a smaller number of hypothetical variables or factors that co-vary. The first two factors that explain most of the variation in the data set can then be ordinated using an x/y plot. Approximately 50% of the variation between communities could be described by the first two factors. As shown by the clustering of the band profiles from the replicate columns for each treatment, each of the treatments resulted in consistent effects in shaping microbial community structures. Bioaugmented soils with and without earthworms were similar with regard to the first factor plotted on the x-axis, but separated when analyzed with respect to factor 2 plotted on the y-axis. This effect was particularly evident for soils sampled at the 0 – 2 cm, and 2 - 6 cm depths, but not at the deeper soil level from 6 – 12 cm. This suggests that most of the bioaugmentation and earthworm effects on microbial community structure were exerted in the upper soil profiles.

Changes in bacterial community diversity due to the different treatments and the presence or absence of earthworms were quantified using the Shannon diversity index, H, which was calculated from the DGGE banding patterns based on the number and relative intensity of bands on each lane (sample) of the DGGE profiles (6). The results showed that earthworms significantly increased bacterial diversity at all soil depths, but the effect was decreased at lower depths (Table 1). Bioaugmentation also caused a significantly increase in bacterial diversity as compared to control soil at all soil depths. However, the effect of inoculum alone was less than the combined effect of inoculum and earthworm.

In conclusion, earthworms promoted the biodegradation of PCBs in soils that were treated with bioaugmentation or by biostimulation, and effectively substituted for mechanical mixing of soil. The reasons for this enhanced biodegradation activity are likely to be complex and involve many different interactions. Earthworms increased the bacterial biomass and diversity. Although not studied here, this in itself may have helped to facilitate the removal of PCB degradation products such as chlorobenzoates that can accumulate and inhibit PCB degradation. Earthworm channels provide increased aeration that may promote the biodegradation of PCBs by aerobic processes involving monooxgenases that are used to catabolize PCBs. Lastly, earthworm casts and waste may enrich the soil with ammonia and other nutrient rich substances that may increase microbial activity (1, 4). These benefits may greatly enhance the efficacy of bioaugmentation and biostimulation treatments used for in situ cleanup of contaminated soils.

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Table 1. Effects of earthworm on bacterial communities were showed as changes in amount of extracted DNA per gram of soil and diversity index (H).

Treatments	Amount of DNA (μg/g soil)			Diversity Index (H)		
Depths	0-2 cm	2-6 cm	6-20 cm	0-2 cm	2-6 cm	6-20 cm
Bioaugmented-earthworms	5.0±0.3	2.0±0.7	1.3±0.3	1.24±0.01	1.20±0.02	1.17±0.01
Bioaugmented-no earthworms	3.1±0.7	1.1±0.6	0.5±0.1	1.13±0.04	1.07±0.03	1.08±0.04
Minimal salts medium-no earthworms	6.9±0.2	0.6±0.2	0.6±0.1	0.90±0.08	0.96±0.06	0.93±0.08

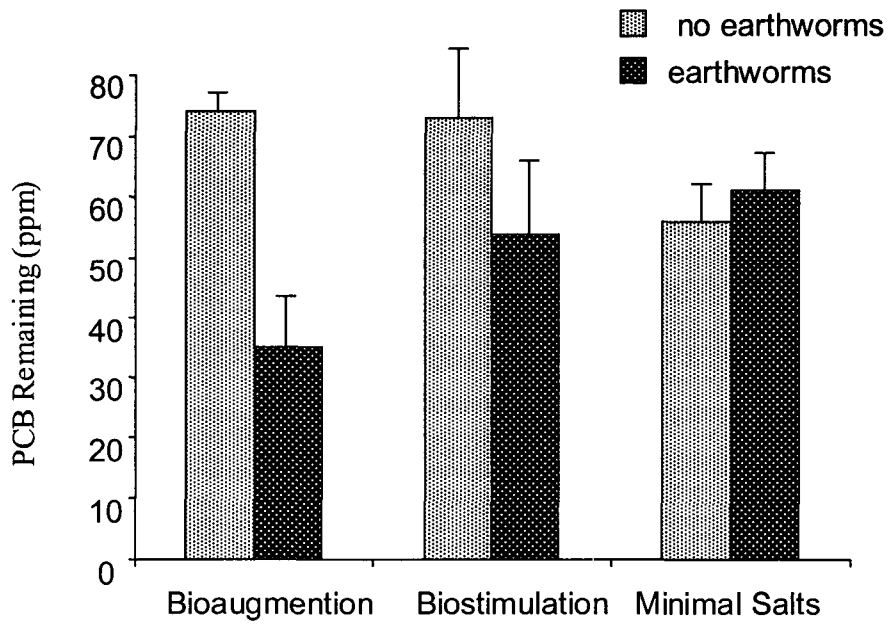

Figure 1. Influence of earthworms on amount of recovery of PCB from soil microcosms amended with 100 ppm Aroclor 1242 after treatment for 18 weeks using bioaugmentation, biostimulation, or addition of minimal salts medium (control).

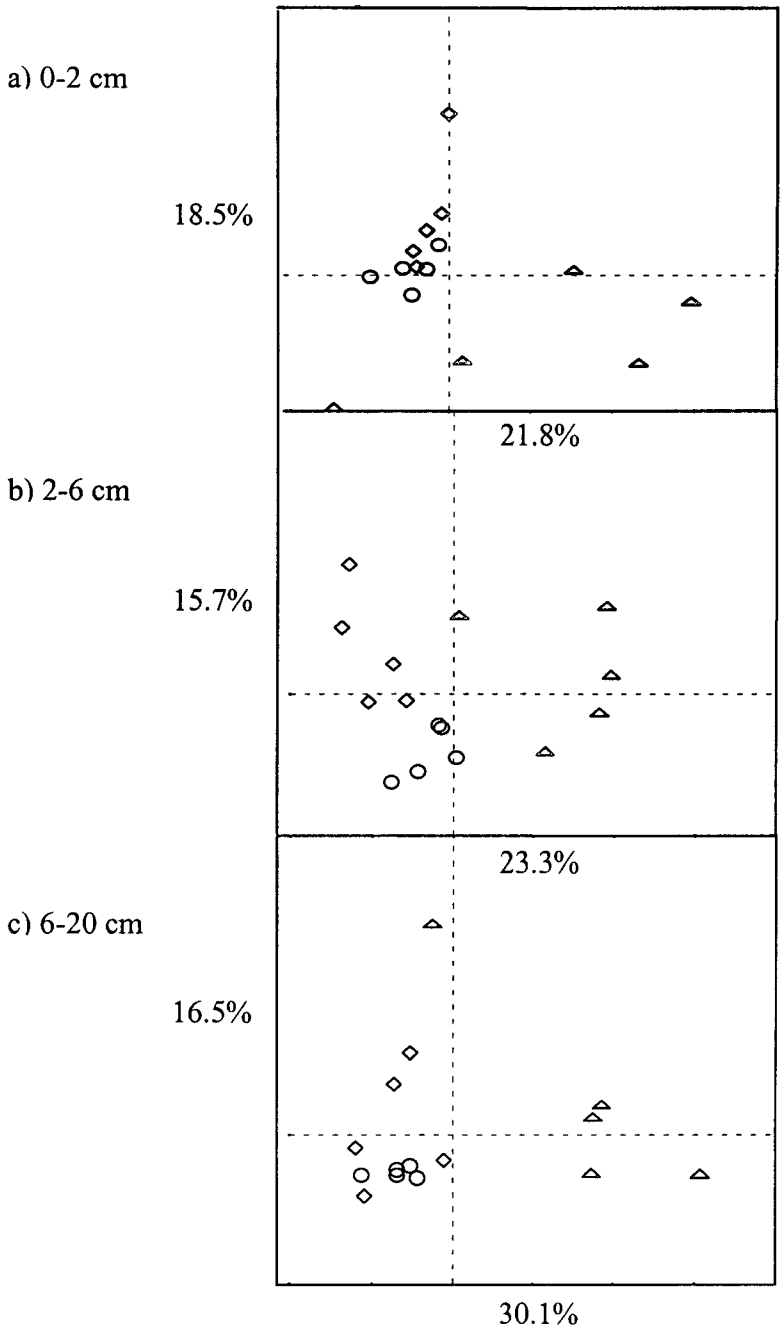

Figure 2. Correspondance analysis of DGGE data showed the relative similarities of microbial communities associated with depth of soil columns as affected by the presented of earthworms. (a) 0-2 cm depth. (b) 2-6 cm depth. (c) 6-20 cm depth. Symbols: ○, bioaugmented-earthworms; ◇, bioaugmented-no earthworms; △, minimal salts medium-no earthworms.