Comparison of Indigenous and Selected Pentachlorophenol (PCP) Degrading Bacterial Consortiums for Remediation of PCP Contaminated Groundwater

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Pentachlorophenol (PCP) is a toxic and recalcitrant wood preservative which has been classified as a priority pollutant by U. S. Environmental Protection Agency. Improper disposal and handling of PCP treating solutions has resulted in sites of soil and water contamination. Previous studies have reported on individual bacterial and fungal species that degrade PCP. However in the environment there are communities of microorganisms present at sites of PCP contamination and involved in degradation of PCP. Few studies have focused on the microbial community involved in PCP degradation. Therefore the objective of this work is to compare PCP degradation by the indigenous bacterial community from PCP contaminated groundwater and customized groups of known PCP degrading bacteria. The experimental setup included treatments of indigenous and known PCP degrading bacteria. Bacterial identification was performed by cloning and sequencing of 16S rRNA gene and expression analysis of the PCP degrading genes was done using real time RT-PCR. Many of the bacterial species identified from the PCP contaminated groundwater have been reported to degrade chlorinated phenols. *Burkholderia sp,* a PCP degrading bacterium, was the predominant species identified in this study. Statistical analysis of the data indicated significant differences between the average PCP concentrations of treatments between day 0 and 21, but there was no detectable expression of PCP degrading genes observed in the groundwater samples possibly indicating that the decrease in PCP may not be from the microbial community.

Introduction

Chlorophenolic compounds like Pentachlorophenol (PCP) are commonly used in a wide range of industrial and agricultural applications such as pesticides, paints, pulp bleaching, leather tanning and wood preservatives. Improper disposal of PCP containing materials and leakage of stored PCP into the environment have resulted in groundwater contamination (9, 12) which is a very serious health issue.

Prolonged exposure to PCP may lead to increased incidences of non-Hodgkin's lymphoma, multiple myeloma and cancer in humans (2). PCP has been listed as a priority pollutant by the U.S. Environmental Protection Agency (EPA) and its use is restricted (5). Currently EPA lists 173 PCP contaminated sites in the active superfund database, 3 within the state of Mississippi. Thus it is very important to effectively remediate PCP and its impurities causing contamination in the environment. Moreover PCP is very resistant to degradation due to the presence of a stable aromatic ring with high chloride content, which makes it a recalcitrant contaminant when introduced into soil or water (3). Many bacterial species, such as various strains of *Burkholderia cepacia, Sphingobium chlorophenolicum, Pseudomonas sp., Arthobacter sp.* and *Bacillus thuringensis* (7, 8) have been reported to actively degrade PCP. Previous studies have focused on degradation of PCP by individual bacterial species; however at sites of PCP contamination it is more likely that microbial communities are involved in

PCP degradation. The contribution of indigenous microbial communities in the degradation of PCP in groundwater is unknown and is therefore the objective of this study.

Methods and Materials

Bacterial strains and growth conditions:

Sphingobium chlorophenolicum strain L1 was obtained from American Type Culture Collection (ATCC). ATCC medium 1687 which contained (per liter) 0.65g of K_2HPO_4, 0.19g of KH_2PO_4, 0.1g of $MgSO_4.7H_2O$, 0.5g of $NaNO_3$, 4g of sodium glutamate ($C_5H_8NNaO_4$) and 2 ml of 0.01M $FeSO_4$ was used to grow the strain. Cells were grown in a 30°C shaker at 200 revolutions per minute.

Sample collection:

Twenty-five liters of PCP contaminated groundwater sample was collected from a 20 foot deep monitoring well (number 19AO) at a site located near a former wood treatment facility in central Mississippi (Figure 1).

Experimental setup:

The following treatments with three replications per treatment were used in this study:

1) Groundwater (with no amendments)
2) Groundwater amended with Miracle Gro
3) Groundwater inoculated with a pure culture of *S. chlorophenolicum* and amended with Miracle Gro.

Groundwater samples were collected on days 0 and 21.

Chemical analysis:

PCP concentration in groundwater samples (200ml) on day 0 and 21 was determined by EPA standard method 3510C (EPA 1996, separatory funnel liquid-liquid extraction) followed by analysis with gas chromatography electron capture detection (GC-ECD).

Bacterial Identification:

One milliliter of groundwater sample from each treatment was added to 100 ml of sterile nutrient broth containing 8 ppb of PCP. Genomic DNA was extracted from these cultures using a Nucleospin nucleic acid purification kit from Macherey-Nagel. Gene encoding 16S rRNA was amplified from isolated genomic DNA samples by PCR using two gene specific primers, 16S F (5'-AGATCGATCCTGGCTCAG) and 16S R (5'-GGTTACCTTGTTACGACTT). These amplified products were cloned into pCR 2.1-TOPO vector using TOPO TA cloning kit (Invitrogen). Recombinant plasmids were extracted from *E. coli* cells using PureLink plasmid miniprep kit (Invitrogen) and sequenced using Beckman-Coulter CEQ8000 Genetic Sequencer. Sequence analysis was performed using BLAST database searches.

RNA quality and gene expression:

RNA was extracted from bacterial cultures using the standard protocol of the RNAqueous kit (Ambion inc.) and treated with TURBO DNA-*free* kit (Ambion Inc.) to remove DNA contamination. RNA quality from extracted bacteria was determined using 1.5% agarose gel electrophoresis and Experion RNA StdSens chip (Bio-Rad) analysis (Figure 4). The presence of clear and distinct bands for 16s and 23s regions indicated good quality of RNA free from DNA contamination. Purified RNA samples with good quality were then converted into cDNA using two step iScript cDNA synthesis kit (Bio-Rad). The cDNAs were amplified using gene specific primers designed for PCP degrading enzymes (Table 1). SYBR green master mixture and 16s housekeeping gene was used for real time PCR of these cDNA samples. Real time PCR was performed using the following program: initial denaturation step of 5 minutes at 95°C, followed by 28 cycles of denaturation at 95°C for 50s, annealing at 63°C for 60s and extension at 72°C for 50s with a final extension at 72°C for 10 minutes (4). These amplified cDNA products were analyzed by electrophoresis on a 1.5% agarose gel.

Statistical analysis:

Significant differences in PCP concentration among treatments at day 0 and 21 were determined using PROC ANOVA and Tukey's Studentized Range

(HSD) test in SAS V. 9.2. Statistical values were: a =0.05, F value + 10.64, P value = 0.0022, F critical = 3.89.

Results and discussion

Analysis of PCP concentration:

PCP concentration was determined from treatments 1, 2 and 3 on day 0 and 21 (Figure 2). Average PCP concentration ranged from 0.8ppm to 1ppm, which was higher than EPA detectable limits of PCP in groundwater (1 ppb) (6). There were significant differences in average PCP concentrations between treatments (1 and 2; 1 and 3) with and without Miracle Gro at each of the two sampling times. Average PCP concentration (ppm) in treatment 1 (0.9972) on day 0 and 21 was different from that of treatment 2 (0.8015) and treatment 3 (0.7683), indicating decrease in PCP concentration. There were no significant differences between the average PCP concentrations of treatments 2 and 3 (without and with *S. chlorophenolicum*) on day 0 and 21. Also there were no significant differences between the average PCP concentrations on day 0 and 21 of each treatment. Therefore this may indicate that differences among treatments can be attributed to the addition of Miracle Gro, stimulating bacterial growth and degradation of PCP in the groundwater.

Bacterial identification:

Sequences obtained from CEQ8000 sequencer were analyzed using BLAST database searches. Sequences with greater than 98% identity match and less than 2 sequence gaps were selected as positive matches. Figure 3 represents the identification and composition of PCP tolerant bacterial species at day 0 and day 21. Bacterial species identified were *Burkholderia sp., S. chlorophenolicum, Pseudomonas sp., Bacillus cereus sp., Ralstonia eutropha sp., Cupriavidus sp.* and they have the ability to degrade chlorinated phenols in the environment (7, 8, 10, 11, and 13). Among these *S. chlorophenolicum* and *Burkholderia sp.* are known PCP degrading bacteria, and a proposed mechanism by which they degrade PCP has been reported (14). Our data indicates that *Burkholderia sp.* is a dominant bacteria present in this study.

Gene expression:

Results indicated that there was no gene expression obtained in RT-PCR, as there was no cDNA amplification observed, except in the case of 16s housekeeping gene (Figure 5) which indicates the presence of bacterial species in the groundwater. Average cycle threshold (Ct) value for 16s housekeeping gene was 22.2, which indicated strong positive gene expression for that gene, but there were no Ct values obtained for PCP degrading genes (Figure 6). Thus quantification of the gene expression was not possible for PCP degrading genes used in this study. This may indicate that bacterial genes encoding PCP degrading enzymes are not expressed or at very low level at these growth conditions and PCP concentrations.

Conclusions

Many bacterial species identified were potent chlorophenol degraders and *Burkholderia sp.* was a predominant PCP degrading bacterium present in the study. There were significant differences between average PCP concentrations among treatments over time period on day 0 and 21, but there was no gene expression observed for bacterial genes encoding for the PCP degrading enzymes. Therefore this may indicate that the decrease observed in the PCP concentration in the groundwater samples was due to the volatilization of PCP rather than microbial degradation. This site is undergoing biosparging bioremediation of PCP contaminated groundwater.

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Table 1. List of gene specific primers used for amplification of cDNA in this study (1, 13).

#	Name of gene	Forward Primer	Reverse Primer
1	16s	TAGGGTTGGCGATGGCTGAT	TTCTTCACACACGCGGCATT
2	PCP-B	CGGGTTCACGTTCAACTTC-GAGAA	GATCGTCGAAGGAACTGAGA-TAGC
3	PCP-C	CTATGACGACAAGCAGGTGGA-CAT	CATCCGCTGATAATAAGCGAG-CAG
4	PCP- A	CGAACCATATCACCAGTCTG-CATC	CATGAAGAAGTCCATGTCCTC-CAG
5	PCP-E	TCCATATCGGGTTATCTTCGGTCC	ATCGGGATCGTAGACCAC-GATCTT
6	PCP-D	GGAGACCCGTCATAT-GACAAACCCGT	GTCGATCTCGAGGATGTCCAG-CACCA
7	Chlorophenol-4- monooxy-genase	CGGAGGTGGTCGCACGGAAC	CCAGACAACGCGGCCGTCAT
8	*S. chlorophenolicum* "pcp" suite of genes	TGGTGACGTCGGCATTCGCC	CCCGGCGTCGCCTTCCATTT

Figure 1. Aerial photograph of study site, including locations of biosparging wells and monitoring wells. Water sample for this study was collected from MW44 circled in red. Study site is located in central Mississippi and had initial PCP groundwater concentrations of ~3ppm(Stokes 2011)

Figure 2. Statistical analysis of Average PCP concentration in three treatments on sampling day 0 and 21, by Gas Chromotography ECD. Statistical Values:a- 0.05, F value= 10.64, P value= 0.0022, F critical= 3.89

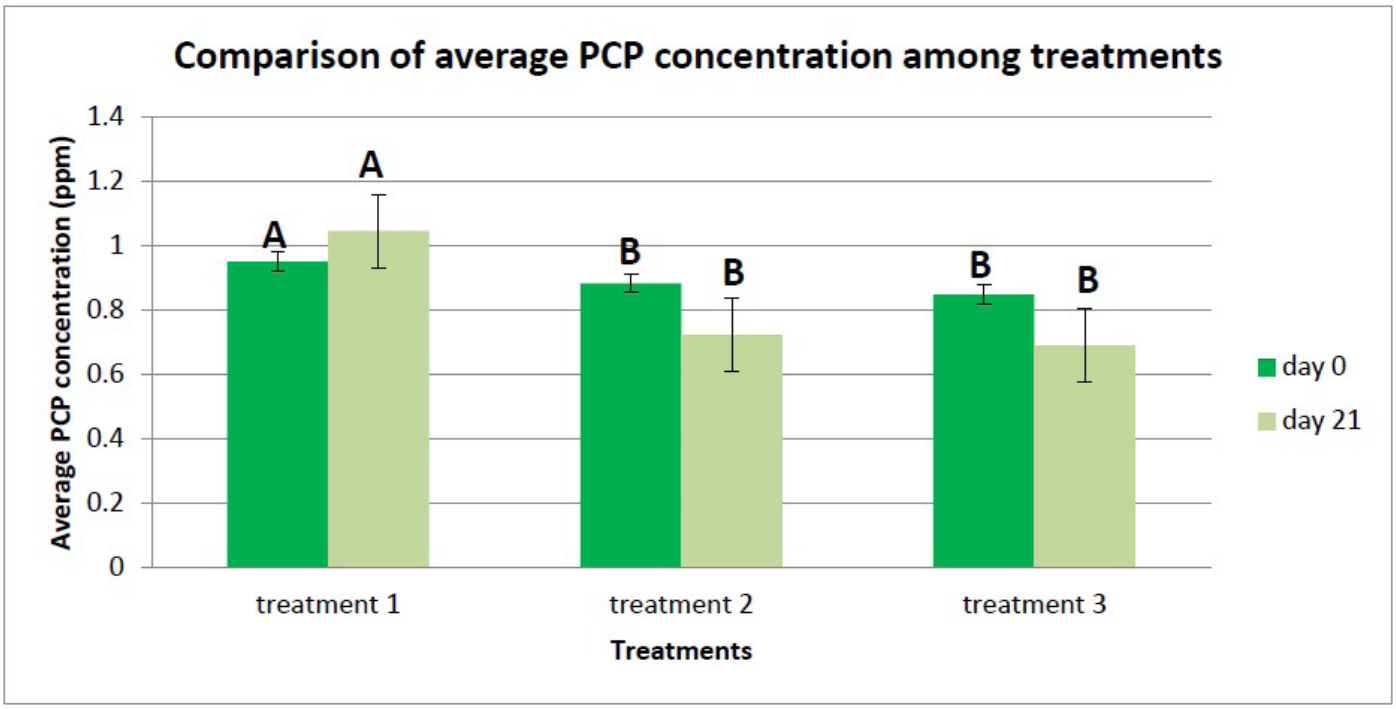


Figure 3. Identification and composition of idigenous bacterial species in PCP contaminated groundwater sample.

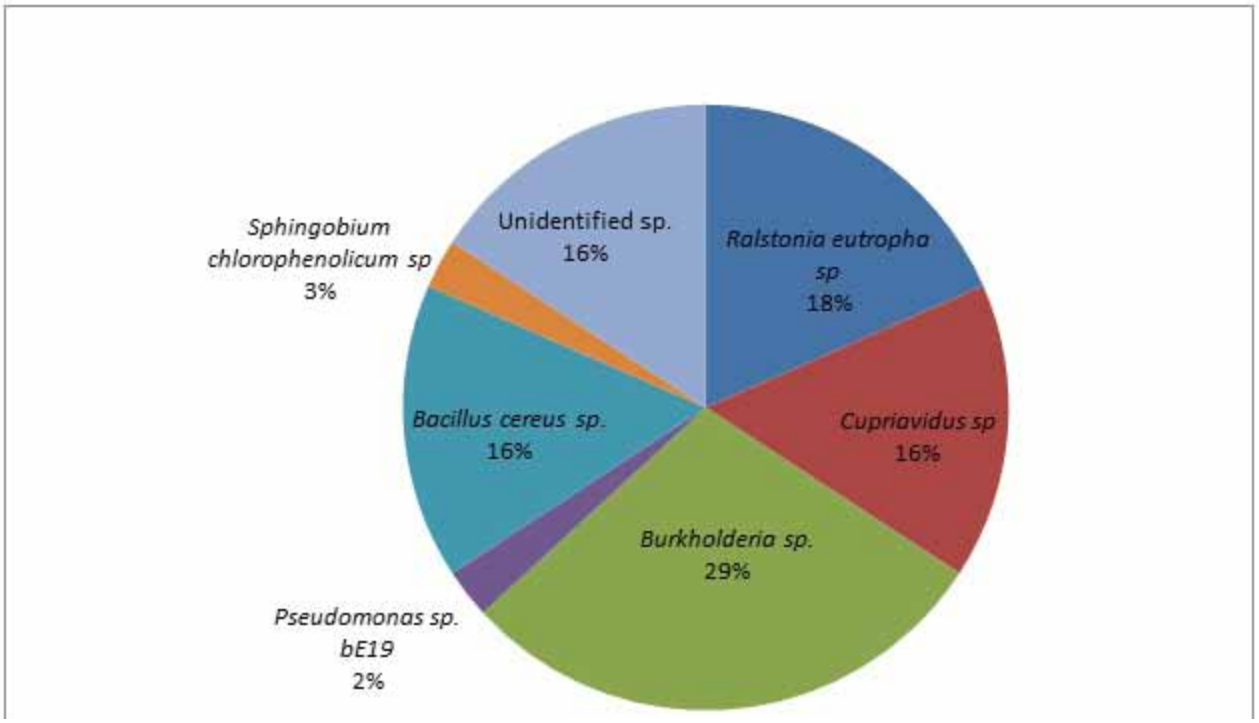


Figure 4. Experion RNA StdSens chip analysis of RNA samples. Lane L=RNA ladder, lane 1= day0_TRT1. rep2, lane 2=day0_TRT2.rep1, lane 3=day0_TRT2.rep2, lane 4=day0_TRT2.rep3, lane 5=day1_TRT1.rep1, lane 6+day1_TRT1.rep3, lane 7=day1_TRT2.rep1andlane8=day1_TRT2.rep2

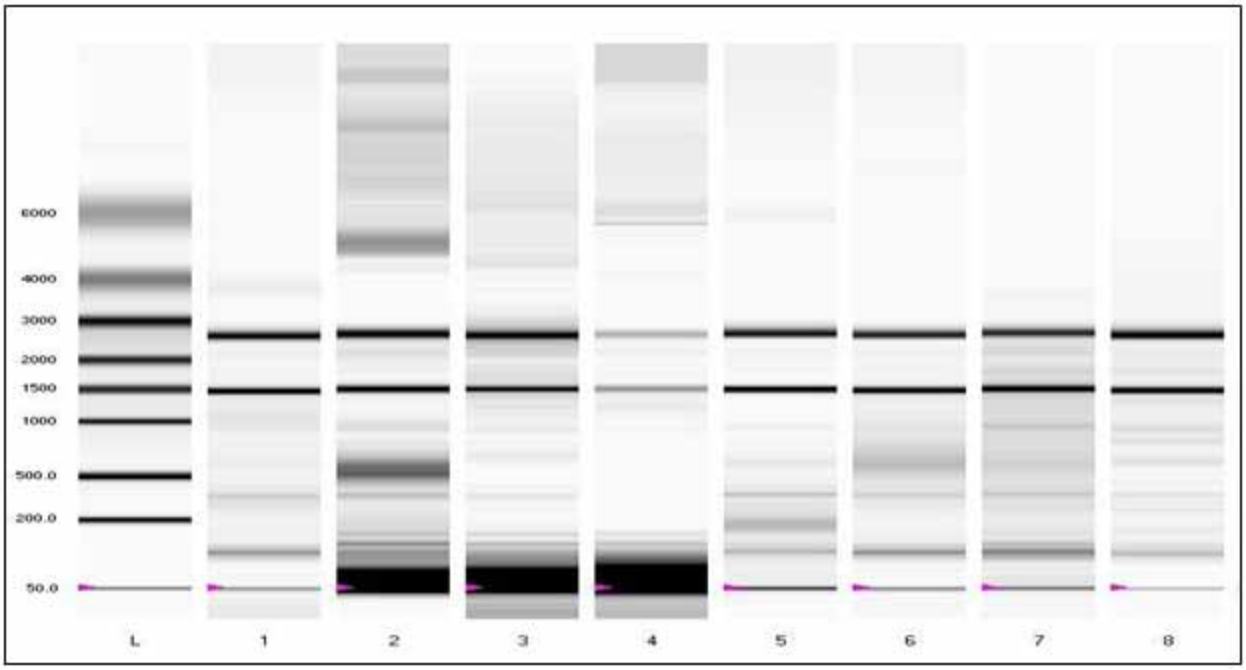
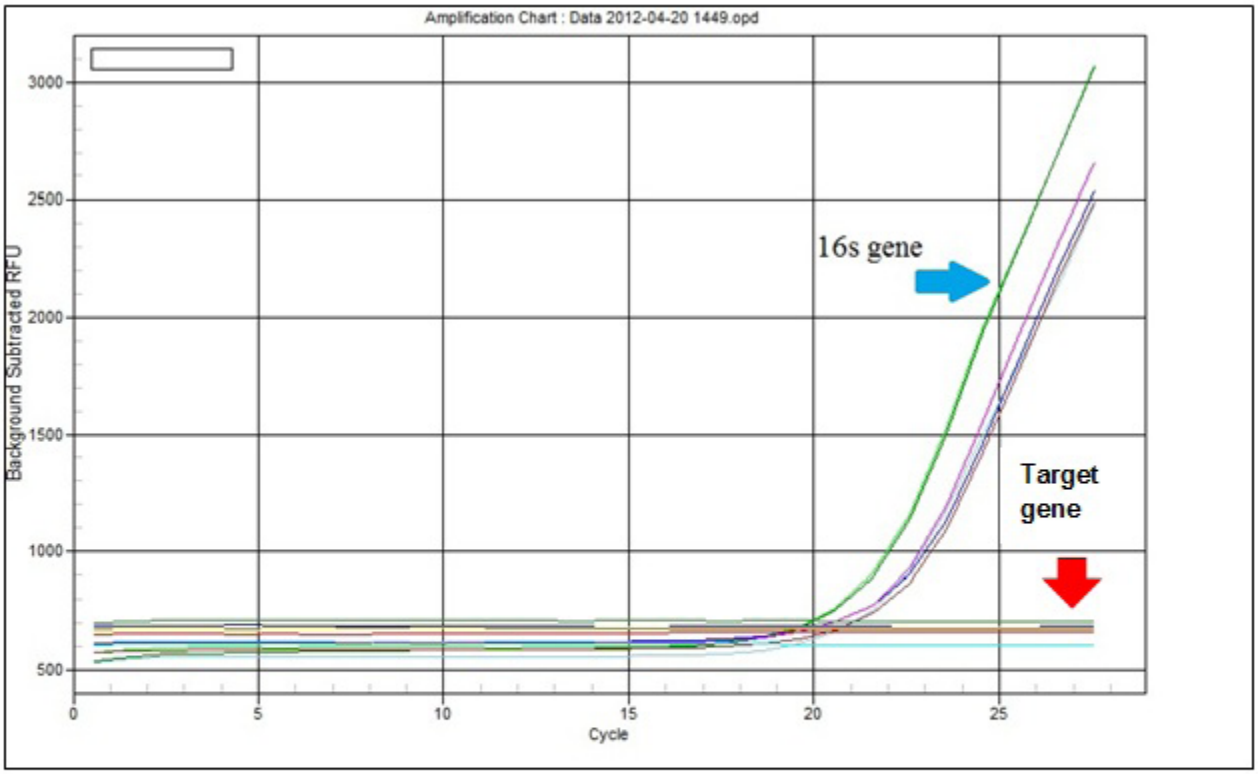


Figure 5. Amplification of cDNA of Day 21 TRT2.rep1 using gene specific primers. Lane 1-16s lane 2-chlorophenol 4 monooxygenase, lane 3- pentachlorophenol 4 monooxygenase, lane 4=pcpA, lane 5= pcpB, lane 6=pcpC, lane 7=pcpD, lane 8= pcpE and lane9-1Ko plus ladder

Figure 6. RT-PCR analysis of gene expression using gene specific primers in samples day0_TRT1.rep1 and day1_TRT2.rep1. Blue arrow indicates peaks for housekeeping gene 16s with average Ct value=22.2, while red arrow indicattes that there are no peaks observed for target gene chlorophenol-4-monooxygenase indicating no gene expression.



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