Identification of pentachlorophenol (PCP) tolerant bacterial communities in contaminated groundwater after air-sparging remediation

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Pentachlorophenol (PCP), a highly toxic and recalcitrant wood preservative, contaminates groundwater aquifers in many areas of the United States. Improper handling, storage, and disposal practices in the past have led to the contamination of groundwater at many wood treatment facilities. Air sparging, the injection of clean air under pressure into the groundwater system, has emerged as a viable in-situ treatment option for removal of this type of contamination. Previous studies have relied on morphological studies for identification of the bacterial community that is responsible for PCP degradation. However, molecular identification of DNA extracted from the bacterial community present in the groundwater will provide a more accurate description of the microbial community. Groundwater samples from eight biosparging wells were taken quarterly and analyzed for total PCP concentration, nutrient content, and monthly samples were used for microbial identification. Microbial counts were taken for each well on selective media, and changes over time were compared between wells within the sparging wells' zone of influence and wells not directly impacted by air sparging. PCP concentration was below 1 ppb and nutrient levels were within the normal range. Well 14 (above air injection) revealed *Burkholderia* sp., *Denitratisoma oestradiolicum*, *Thauera* sp., and *Rhodoanobacter thiooxydans*, along with >40 other species that were listed as "uncultured" in BLAST. Well 51 (below air injection), presented a greater variety of bacterial species than Well 14, including the known PCP degrader *Flavobacterium*, in addition to numerous "uncultured" species. DNA extracted from other wells is currently being sequenced and T-RFLP analysis is underway to provide a comparison over time of microbial communities between aerated and non aerated wells.

Key words: toxic substances, treatment, ground water, methods, remediation

Introduction

Pentachlorophenol (PCP, Penta) is a widely used wood treatment chemical that is highly resistant to degradation. In the United States, its use was restricted in 1997 when it was classified by the EPA as a probable human carcinogen. PCP is still used in the treatment of utility poles in the United States. Prior to regulation, there were a number of issues with disposal of excess chemical, disposal of wood wastes, leakage of stored chemical, and cleanup of spilled PCP. Because of PCP's strong resistance to degradation, it becomes a very recalcitrant contaminant when introduced to soil or water systems. The relatively recent introduction of PCP in 1936 means that indigenous microorganisms have likely developed PCP degradation mechanisms only in the last 70 years(Crawford 2007). A variety of remediation techniques have been applied to aid in the removal of PCP, and air sparging is one that has been particularly useful. Air sparging is the application of air under pressure into saturated zones to volatilize dissolved phase contaminants and increase oxygen levels in groundwater (Bass 2000). Air sparging increases both the physical removal and aerobic biodegradation of contaminants, and is especially successful on volatile organic compounds such as chlorinated solvents and petroleum hydrocarbons (Bass 2000). PCP's heavily chlorinated

structure makes this treatment an ideal solution for removal of the contaminant without the need to excavate the contaminated soil and without pumping a large volume of groundwater out for treatment off-site.

It is understood that indigenous microorganisms often have the ability to degrade PCP given ideal conditions such as ample nutrients and oxygen. What is not understood, however, is which members of the microbial community are actively involved in the degradation. The identification of the members of a bacterial community that has been exposed to a PCP contamination, and that has undergone a remediation treatment that is beneficial to the growth of the community, is therefore the primary goal of this study. By identifying specific members of the community that are responsible for PCP degradation, it is hoped that bioremediation of contaminants such as PCP can be enhanced.

Materials and Methods

Air Sparging Treatment

In 2000, a series of air sparging wells were installed at a wood treatment facility in Mississippi where a groundwater contamination of PCP had been identified. The wells were placed downstream of the contaminant, creating a "curtain" of aeration intended to prevent the spread of the contaminant stream into the neighboring aquifer. Monitoring wells were installed at the same time, and the site has been sampled quarterly since the installation. The air sparging wells were connected to a regenerative blower supplying

105 standard cubic feet of air per minute (scfm) at a pressure of 15 pounds per square inch (psi)(Borazjani 2005). Wells are made of 2-inch schedule 40 PVC pipe with a 5-foot slotted screen portion at the bottom of each well. The screened section was situated at installation within the base of the saturated zone. This ensures aeration of the entire depth of the contaminated area, providing a zone of influence with an approximate radius of 30 feet. Well depths range from 23 to 29 feet below the ground surface. Injection of air through sparging wells has been continuous to date. Figure 1 shows an aerial view of the study site.

Biological and Chemical Sampling

As stated, monitoring wells have been sampled quarterly since installation to test a variety of chemical and biological parameters. Chemical testing has included measurements of PCP levels, total Kjeldahl nitrogen (TKN), total organic carbon (TOC), total organic phosphorus (TOP), ortho-phosphate (Ortho-P), and chlorine ion (Cl). Water samples (1L) have been collected from each well at each sampling period. Chemical testing has been conducted according to EPA Standard Methods for the examination of water and wastewater. Each measured parameter is either an indicator of the contaminant or its breakdown, or a contributing factor to the health of the bacterial community. Bacterial populations have been counted monthly, using the same sampling method as with the quarterly samples. Bacteria were enumerated by diluting 1ml of water collected from each well by a factor of 100, and plating this dilution on nonrestrictive nutrient agar and selective nutrient agar containing PCP. Differences in the two plate counts indicate differences in the PCP-tolerant and non-PCP tolerant species of bacteria. Colony forming units (cfu) were counted and the cfu/mL for each well was calculated from these numbers.

Beginning in December 2009, 500ml water samples were taken monthly, before and after addition of liquid nutrients introduced after the first monthly sampling. The nutrient amendment was selected to increase the available nitrogen and phosphorus to active bacteria within the system. These samples were not subjected to chemical testing monthly, but reserved for more thorough microbiological examination to determine the composition of the community responsible for PCP degradation. Samples for quarterly chemical analysis were continued in addition to the monthly samples. Quarterly water samples were divided for analysis, with 200 ml for PCP concentration determination, 200 ml for direct extraction of DNA, and 100 ml for plating on selective media and growing in liquid culture. PCP concentration was determined by EPA Standard Method 3510C. DNA was extracted from the water samples using a WaterMaster DNA Purification Kit from Epicentre Biotechnologies.

DNA sequencing for identification

When poor quality DNA was produced from the direct extractions from water samples in December 2009 and January 2010, an alternative culturing step was added to increase concentration of viable bacteria. One milliliter of each water sample was added to sterile nutrient broth containing 1ppb (1 µg/L) PCP From these cultures, DNA was extracted using a NucleoSpin Plant II nucleic acid purification kit from Macherey-Nagel. The 16s region of extracted DNA was amplified using bacterial specific primers and polymerase chain reaction (PCR). Primers used in the amplification were 16SFOR (5'AGATCGATCCTGGCTCAG) and 16SREV (5'-GGTTACCTTGTTACGACTT). Amplified products were then cloned into *E.coli* cells containing pCR 4-TOPO vector using a TOPO TA Cloning Kit for Sequencing (Invitrogen). The resulting plasmids were extracted from the cells using a PureLink Plasmid Miniprep kit, also from Invitrogen, and sequenced on a Beckman-Coulter CEQ8000 Genetic Sequencer. Sequences obtained from the CEQ8000 were subjected to BLAST database searches, and matches with a greater than 96% identity match and 3 or fewer sequence gaps were accepted as identified species.

Results

Microbial counts

Variations were observed in total bacterial counts (cfu/mL) as well as in the PCP-tolerant bacterial counts (cfu/mL) between wells at each sampling period, and for the same well at different sampling periods. This fluctuation may be attributed to changes in subsurface water and nutrient availability, as weather changes impact the site. Figures 2 and 3 show the variation in the eight monitoring wells over the sampling period. There is a significant difference in the variation between PCP-tolerant and Total bacteria from the pre-amendment samples and the post-amendment samples. Pre-amendment total bacteria range from 0 to 450,000 cfu/mL, while the PCP-tolerant bacteria range from 0 to 220,000 cfu/mL. Post-amendment total bacteria range from 0 to 700,000 cfu/mL, while PCP-tolerant bacteria range from 0 to 620,000 cfu/mL.

All wells except well 44 showed no detectable bacterial colonies for the months of January and February. Well 44 is at the greatest distance above the line of air sparging wells, and is least impacted by the injection of air. Monitoring well 41, located furthest below the air injection wells, showed the least amount of bacterial growth over time. Monitoring wells 14, 51, 52, 42, and 43 are definitely impacted by the air injection, as they are within the 30-foot radius of the air injection wells. Wells 41, 17, and 44 are outside the zone of influence of the air injection wells.

PCP analysis

PCP was examined for each well at each monthly sampling point according to EPA Standard Method 3510C. EPA detectable limits of PCP in groundwater are currently set at 1ppb. (Federal Register 1999) Figure 4 shows the variation of PCP in the eight monitoring wells over a three month period in early 2010.

DNA sequencing and identification of species

Table 1 shows results from two of the eight monitoring wells. Wells 14 and 51 were chosen for sequencing first because the DNA produced from these two wells was of the highest quality and purity. Only sequences with a greater than 96% identity match and less than 3 sequence gaps were considered positive matches. There were a large number of "uncultured" strains that fit the criteria for positive matches, but were not included in the table because they could not be assigned to a particular genus.

Discussion

The quantity changes within the bacterial community may be influenced by natural fluctuations related to weather patterns, or water or nutrient availability within the soil system. Bacterial populations in a particular well varied in number from month to month, and between wells. The differences between bacterial populations for different wells are likely a result of the presence of increased oxygen from the air injection wells, the availability of the contaminant to be used as a carbon source

for bacterial growth, and the improved C:N ratio provided by the nutrient amendment. The last point is verified by the doubling of both the total bacteria and PCP-tolerant bacterial counts from pre-amendment samples to post-amendment samples. Wells that are further above the air injection site, closer to the original source of contamination, seem to have a more stable population of bacteria than wells that are far below the sparging line, further from the original contaminant source. Therefore, it seems that improving the C:N ratio, in conjunction with the air sparging remediation, is beneficial to bacterial community growth.

PCP concentration analysis shows that the levels of contaminant are relatively stable throughout the remediation area, varying from 0.1 to 1.4 ppb. Using the EPA recommendation of 1ppb as a guideline, and knowing that PCP levels as high as 3.60ppm were measured early in the remediation, we can reasonably conclude that the air sparging remediation has been beneficial to this area. Whether the remaining PCP in the system is available to the indigenous bacteria as a food source cannot be determined at this point.

As yet, only two known PCP degraders have been positively identified from sequence analysis. Flavobacterium and Burkholderia have the ability to degrade the chlorinated phenol (Saber 1985; Xun 1996). It is possible that Flavobacterium and Burkholderia are the dominant PCP-degrading species within the entire system. This may be determined as more samples are sequenced and analyzed for known bacterial species.

To further examine changes in bacterial communities by well location, the bacterial DNA collected from each well is being used in a terminal restriction fragment length polymorphism (T-RFLP) analysis. Terminal restriction fragments (T-RFs) from fluorescently-labeled, digested 16s PCR products are separated by capillary electrophoresis and visualized using the Fragment Analysis program of the Beckman-Coulter CEQ 8000 used in bacterial identification. After performing a peak ratio analysis, this data will show the relative distribution of fragment sizes throughout each sample. This analysis will show changes in the bacterial community at each sampling point, giving a picture of how the species distribution changes throughout the study site over time. Additionally, gene expression of enzymes specific to PCP degradation will be examined using real time PCR (RT-PCR). This will determine the community members capable of performing each step of PCP degradation, and provide a more thorough understanding of the mechanism of PCP breakdown. Research is continuing in this study.

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Table 1. Identification of bacteria from two monitoring wells. Species marked with an asterisk (*) are known PCP degrading bacteria.

Well 14 – February 2010	**Well 51 – February 2010**	
Burkholderia cepacia *	*Burkholderia sp.* *	*Herbaspirillum sp*
Rhodoanobacter thiooxydans	*Janthinobacterium lividum*	*Azospirillum irakense*
Thauera sp.	*Duganella sp.*	*Collimonas sp.*
Denitratisoma oestradiolicum	*Pedobacter insulae*	*Janthinobacterium agaricidamno-sum*
	Pedobacter duraquae	*Massilia dura*
	Flavobacterium sp. *	*Aquaspirillum arcticum*
	Oxalicibacterium faecigallinarum	

Figure 1. Aerial view of study site, indicating monitoring wells (MW- #), air sparging wells (unlabeled), and groundwater flow direction (arrows).

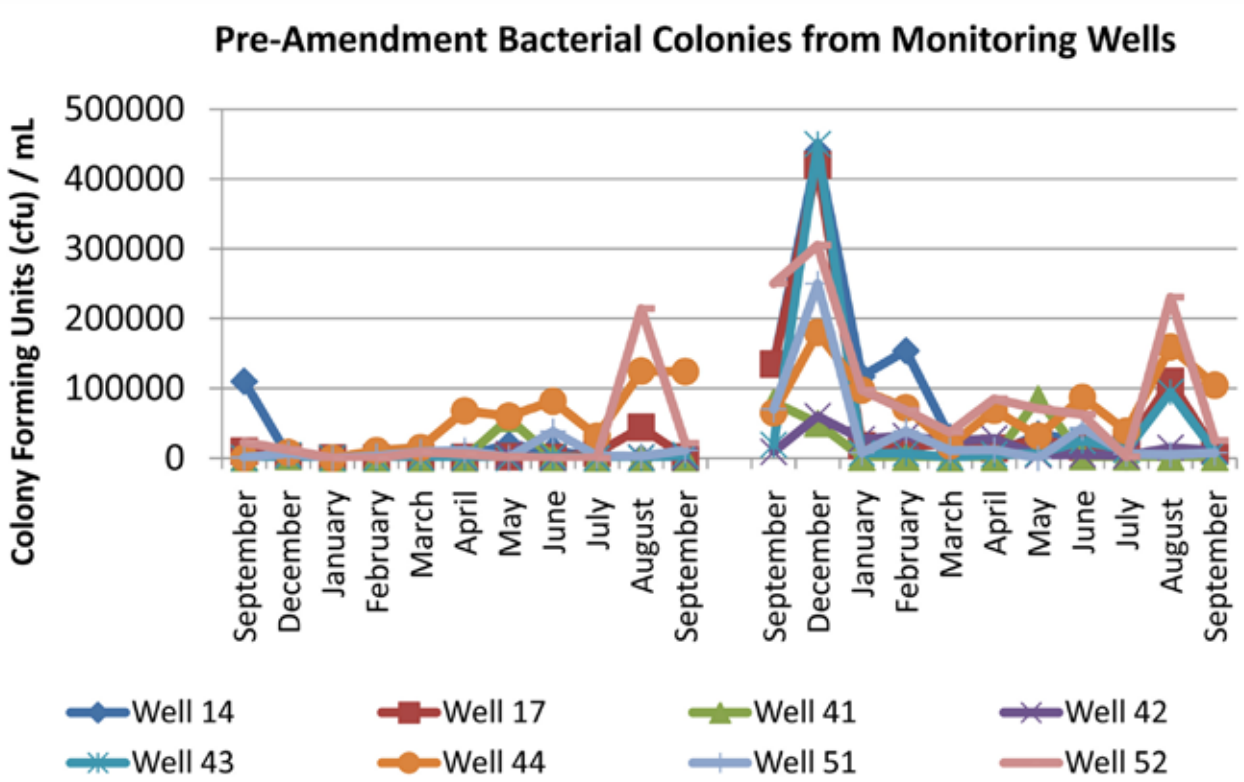

Figure 2. Bacterial enumeration from pre-amendment samples. PCP tolerant bacteria are shown on the left; Total bacteria on the right.

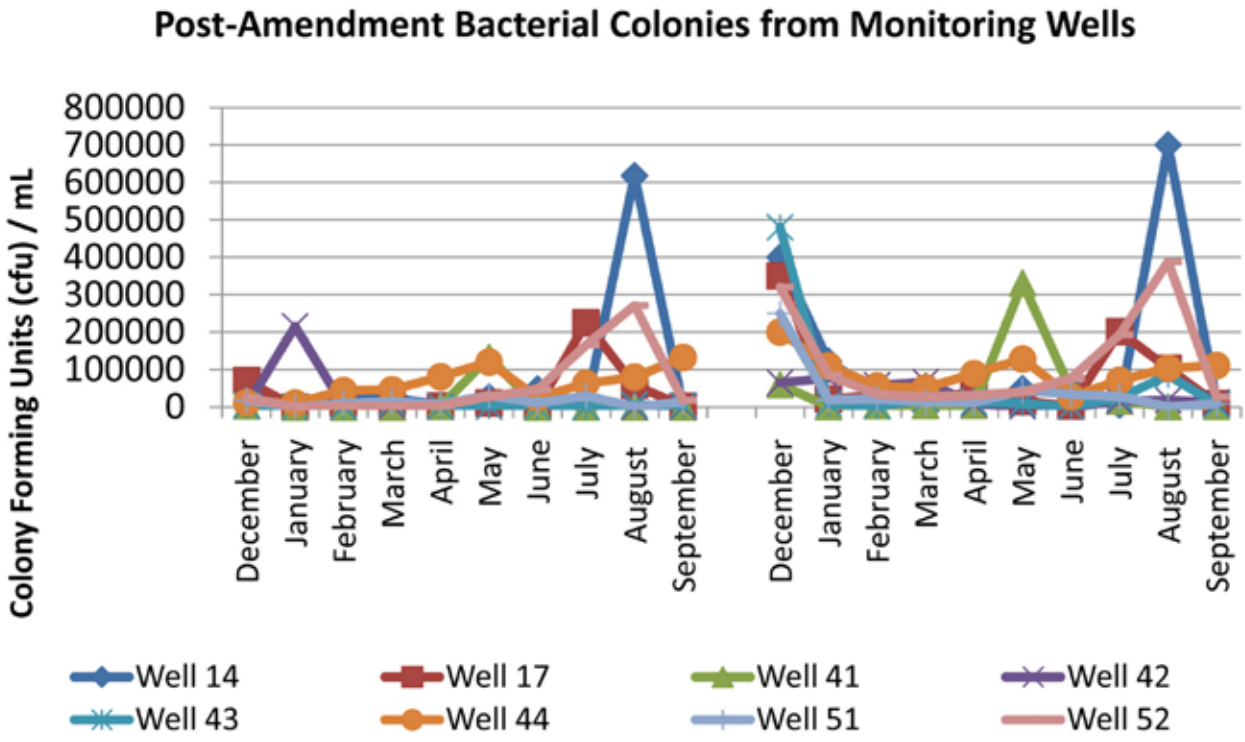

Figure 3. Bacterial enumeration from post-amendment samples. PCP-tolerant bacteria are shown on the left; Total bacteria on the right.

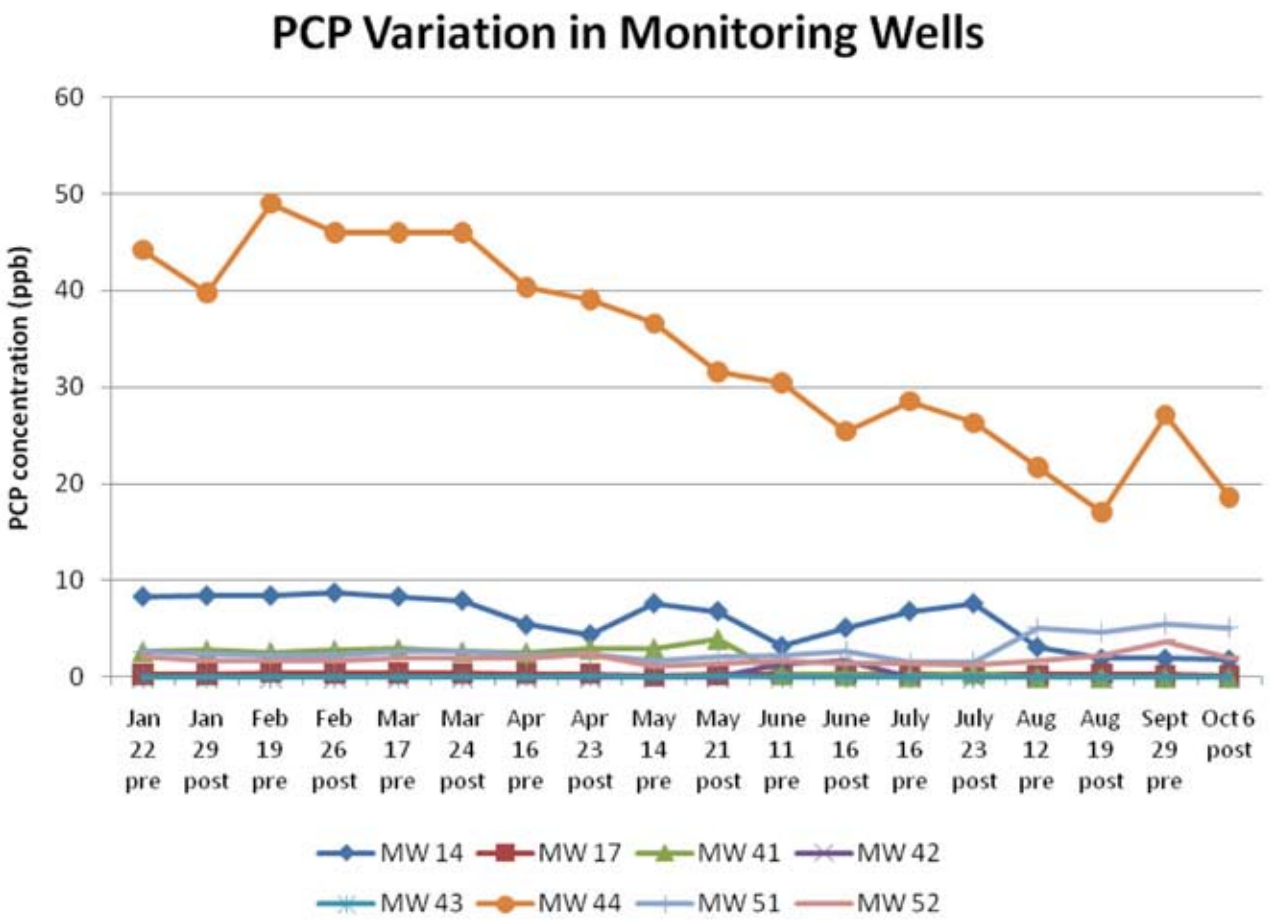

Figure 4. PCP variation over nine months. 1ppb is the maximum contaminant load (MCL) set by EPA for PCP in groundwater. Upstream wells – 14 and 44; Intermediate wells – 51 and 52; Downstream wells – 17, 41, 42, and 43.