Molecular Identification of Bacterial Communities Associated with Biodegradation of Pentachlorophenol in Groundwater

Final Report

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Abstract

Pentachlorophenol (PCP) is a toxic and recalcitrant compound used predominately as a wood preservative to protect wood from decay caused by insects and microorganisms. Past storage, treatment and disposal practices of PCP have resulted in groundwater contamination near wood treating sites in Mississippi and nationwide. Because of PCP's recalcitrant nature and toxicity, it has been listed as a priority pollutant by the Environmental Protection Agency. Methods to remediate PCP in groundwater include pump and treat, filtration, and biosparging. Of these methods biosparging is the only in-situ method which substantially should reduce the remediation costs. Biosparging forces clean air under the groundwater table stimulating the indigenous microorganisms to degrade the pollutant. In this study eight biosparging wells were installed at a wood treating site in central Mississippi with contaminated groundwater. Two wells (#14 and #44) were located above and 6 wells (#42, #52, #43, #51, #41 and #17) were located beneath the air sparging lines. Water samples were collected quarterly for nutrient analysis, PCP concentration and microbial identification. In addition water samples were also collected monthly before and after nutrient amendment for microbial enumerations. Nutrients added were nitrogen, phosphorus, and potassium. After nutrient addition the largest increase in nutrient levels occurred for nitrogen and ortho-phosphorus in well numbers 52 and 17 both located near and far respectfully below the air sparging lines. Wells 52 and 17 also showed greater changes in Total Organic Phosphorus (TOP), Total Organic Carbon (TOC) and chloride ion (Cl^-) over time than the other wells. Total bacteria and PCP tolerant bacteria were highest in well # 14 located slightly above the sparge lines after eight monthly nutrient additions. PCP concentrations varied during the sampling period but did not decrease. Identification of PCP tolerant bacteria based on molecular methods revealed 17 bacterial species of which two were known PCP degraders, *Burkholderia cepacia* and *Flavobacterium* sp.

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Introduction

Groundwater quality is an important issue that affects not only the health and well being of all living things but also the economic growth and development of the state and region. More than 80% of Mississippi's total water supply is from groundwater and more than 93% of the potable water supply is extracted from water wells that tap available aquifers. (Mississippi Ground Water Resources) Approximately 2.6 billion gallons of water are pumped from aquifers in Mississippi each day of which 65% is used for irrigation, 15% used for aquaculture and 11% used for public supply. However there are no comprehensive national monitoring programs that exist to measure the full extent of groundwater contamination. State agencies indicate that groundwater contamination is a localized problem. Some reports indicate that 10% of rural domestic wells contain at least one pesticide or pesticide metabolite. One of the pesticides found in groundwater in the Mississippi Delta region is pentachlorophenol (PCP). 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, disposal of excess PCP, disposal of PCP treated wood waste, leakage of stored PCP, and cleanup of spilled PCP were a few issues that were of environmental concern. Because of PCP's strong resistance to degradation, it becomes a very recalcitrant contaminant when introduced to soil or water systems. The introduction of PCP in 1936 means that indigenous microorganisms may have likely developed PCP degradation mechanisms over the last 70 years (Crawford et al. 2007).

One of the most promising methods for remediation of PCP contaminated groundwater is Biosparging. Biosparging utilizes the indigenous microorganisms found in contaminated groundwater to biodegrade organic pollutants such as PCP. Clean air is injected into the contaminated zones increasing the oxygen concentration in the groundwater thereby enhancing aerobic biodegradation of the pollutant (Bass et al. 2000). Nutrients such as nitrogen, phosphorus and potassium may be added to also stimulate biodegradation. This technology can reduce the cost of remediation of contaminated sites and control the migration of contaminants into the subsurface.

The indigenous microbial community associated with the biodegradation of PCP in contaminated groundwater has not been established. This is due in part to a lack of accurate and reliable identification methods. Traditional microbial identification methods include isolation and culturing on selective media, morphological characterization, immunological responses and chemical assays (Jellison and Jasalavich 2000, Clausen 1997). However these methods have proven to be time consuming, inaccurate and incomplete. The principle limitation to the culturing of these microorganisms is the very low percentage (~ 1%) of the

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total microbial population that will grow on any one specific media (Buckley, 2004). Therefore the microorganisms that are enumerated and identified from growth media under-represent the microbes present in the soil or in the water.

The development of polymerase chain reaction (PCR, Mullis, 1987) was a critical turning point for microbial identification because it led to the development of culture-independent methods for identification of microorganisms to the species level. The power of PCR is its ability to make billions of copies of these unique DNA sequences in a short time period (Valasek and Repa, 2005). Subsequent DNA methods were developed which made use of the amplified DNA fragments generated by PCR. As a result, "DNA fingerprints" were created from the amplified DNA and used to identify microorganisms to the genus and species levels. Molecular based methods for microbial identification include Random Amplified Polymorphic DNA (RAPD), Amplified Ribosomal DNA Restriction Analyses (ARDRA), Restriction Fragment Length Polymorphism (RFLP), rDNA sequencing, Sequence-Specific Oligonucleotide Probe (Akopyanz et al., 1992, Adair et al., 2002, Jensen 1993, Jasalavich et al., 2000, Oh et al., 2003) and others. In these methods ribosomal DNA was used to study different taxonomic levels of bacteria and fungi. rDNA is a nuclear, multi-copy gene family arranged in tandem arrays that codes for the RNA subunits of the ribosome molecule. The small subunit (16S) rDNA has been shown to be highly effective for identification of bacteria. Primers designed to target the conserved regions of microbial rDNA have been used to amplify sequence variable fragments of genes or the intervening noncoding regions (Turene et al., 1999) increasing the sensitivity and selectivity for species identification. These methods work best for isolated cultures. Molecular methods for identification of mixed cultures include Terminal Restriction Fragment Length Polymorphisms (T-RFLP), Denaturing Gradient Gel Electrophoresis (DGGE), Temperature Gradient Gel Electrophoresis (TGGE), Single-Strand Conformation Polymorphisms (SSCP), or cloning coupled with DNA sequencing (Dickie, et al., 2002, Anderson and Cairney, 2004, Smit et al., 1999, Borneman and Hartin, 2000, Valinsky et al. 2002a, Valinsky et al., 2002b, O'Brien 2005).

The hypothesis for this project was that PCP degrading bacteria are present during biosparging of PCP contaminated groundwater. The objective of this proposal was to determine the bacterial community associated using cloning and sequence molecular methods for identification.

Materials and Methods

Eight biosparging wells were installed at a wood treating site in Mississippi (Figure 1). The wells consisted of 2 inch PVC pipe with a slotted screen section at the bottom of the well and positioned within the base of the saturated zone. The wells extended twenty-nine feet below ground surface. A regenerative blower was used to supply air up to 15 pounds per square inch.

Water samples (500ml) were collected quarterly for one year and

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analyzed for PCP using Gas Chromatography according to EPA Standard Methods. Beginning in December 2009, water samples were taken monthly, before and after addition of liquid nutrients (1 liter) containing 15% nitrogen, 30% phosphorus and 15% potassium and analyzed for microbial enumeration. Water samples, made to the appropriate dilutions if needed, were inoculated onto nutrient agar and nutrient agar amended with PCP and incubated for 48 hours at 28^{o}C to determine microbial enumeration. DNA was extracted from the water samples according to the protocol of the WaterMaster DNA extraction kit (Epicenter Biotechnologies, Madison WI). If the quality or quantity of DNA was not adequate for processing, microorganisms were then cultured in nutrient broth by adding 1 milliliter of water sample into 5 ml of nutrient broth while shaking overnight at 28^{o}C. From these cultures, DNA was extracted using a NucleoSpin Plant II nucleic acid purification kit from Macherey-Nagel (Bethlehem PA). The extracted DNA was amplified using bacterial 16s forward and reverse primers (5'-AGATCGATCCTGGCTCAG and 5"-GGTTACCTTGTTACGACTT). Verification of the mixed population amplified fragment was done using gel electrophoresis. The mixed fragments were cloned in *E. coli* competent cells using the TOPO TA cloning kit for sequencing (Invitrogen, Carlsbad CA). The clones were cultured in Luria broth media overnight, extracted using the Pure Link Quick Plasmid Mini Prep kit and the insert was verified by ECOR I enzyme digest. Sequencing was performed according to the Beckman Coulter DTCS Quick Start Kit (Beckman Coulter, Brea CA) and analyzed on a Beckman CEQ 8000 DNA Analysis System. The sequences were aligned using the Clustal W Multiple Sequence Alignment Program version 1.7 and analyzed data were identified using BLAST search of NCBI (Thompson et al. 1994). Sequences with a greater than 96% identity match and 3 or fewer sequence gaps were accepted as identified species.

Groundwater samples were collected quarterly and analyzed by a certified independent laboratory for total organic carbon according to EPA Method 9060 (U.S. Environmental Protection Agency), total Kjeldahl nitrogen according to EPA Method 351.4, total organic phosphorus according to EPA Method 365.3 and ortho-phosphate according to EPA Method 258.1

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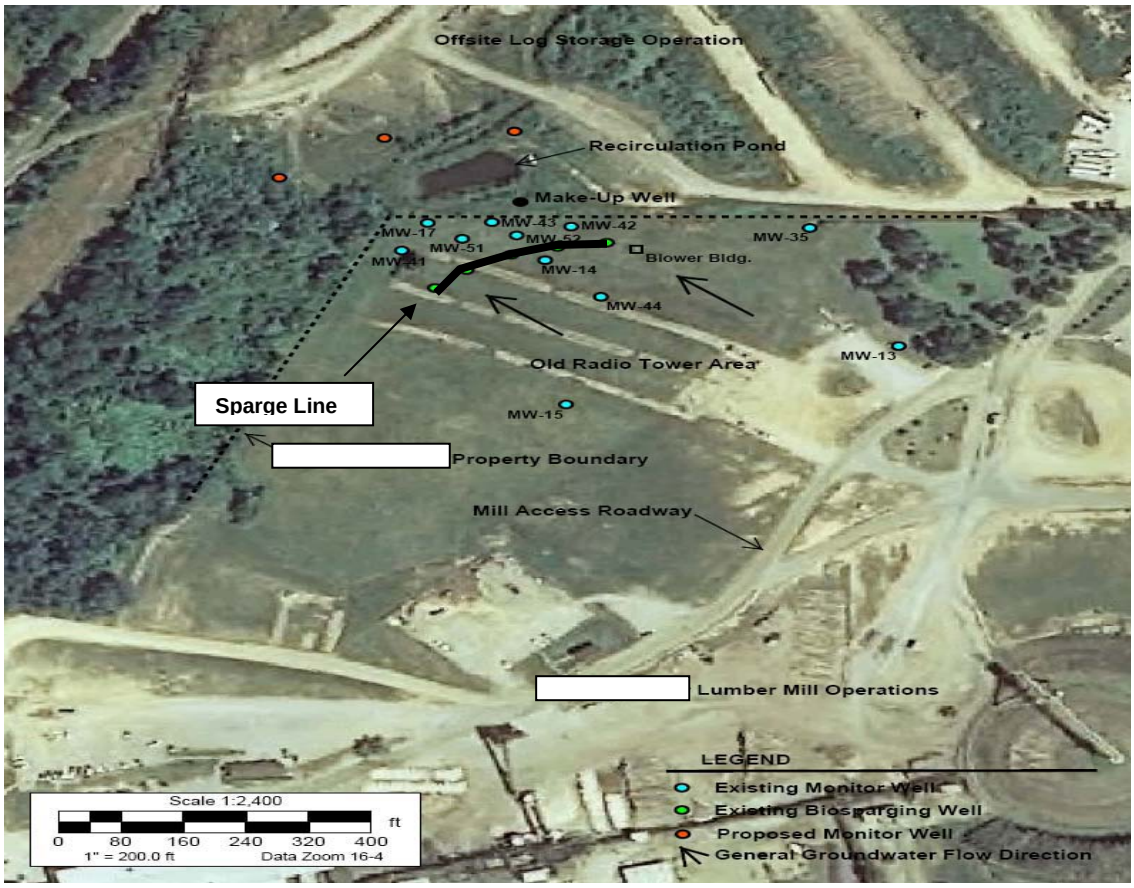

Figure1. Topographic map showing well locations. Used with permission from Lybrand Consulting, LLC.

RESULTS and DISCUSSION

Monthly nutrient additions resulted in an increase in the TKN in wells #17,# 42 and #52 (Figure 2) which were located below the sparge line. Ortho-phosphorus, and TOP were also highest in wells #17 and #52 after nutrient addition (Figures 3, 4). TOC and chloride ions (Figures 5, 6) were highest in wells #17, #51, and #52 all located below the sparge line. Total bacteria and PCP tolerant bacteria were monitored pre and post nutrient amendments (Figures 7,8). In general there was an increase in the colony forming units (cfu) of both general bacteria and PCP tolerant bacteria in each well after nutrient addition over the sampling times. For example in well # 14, located above the sparge line, there were no detectable PCP tolerant bacteria before nutrient addition and 23,000 cfu of PCP tolerant bacteria after nutrient addition. In the well below the sparge line, #51, there were 2800 cfu and 9700 cfu of PCP tolerant bacteria present before and after the nutrient addition respectfully. Extraction of DNA and cloning revealed different patterns of DNA fragments found in the groundwater samples (Figures 10,11). Four PCP tolerant bacteria were detected in well #14 compared to thirteen PCP tolerant bacteria detected in well #51(Figure 12, Table 1). Two

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bacteria, *Burkholderia cepacia* (Xun 1996) and *Flavobacterium sp* have been reported to be known PCP degraders (Topp and Hanson 1990) . *B. cepacia* is a common human pathogen that is often found in water and soil and survive for long periods of time. Some of the other organisms detected produce nitrifying and sulfur oxidizing enzymes. PCP concentration did not in general decrease over time (Figure 9) probably due to insufficient populations of PCP degrading organisms in the groundwater.

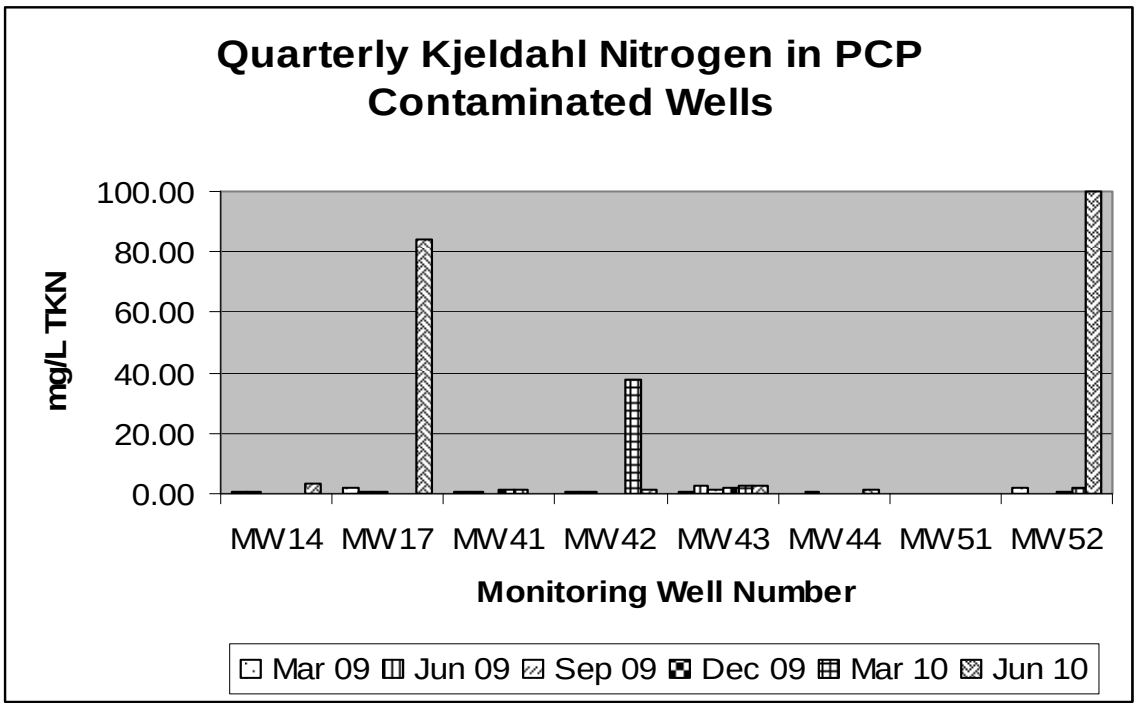

Figure 2. Kjeldahl Nitrogen in PCP contaminated groundwater collected in from eight monitoring wells over a 15 month period.

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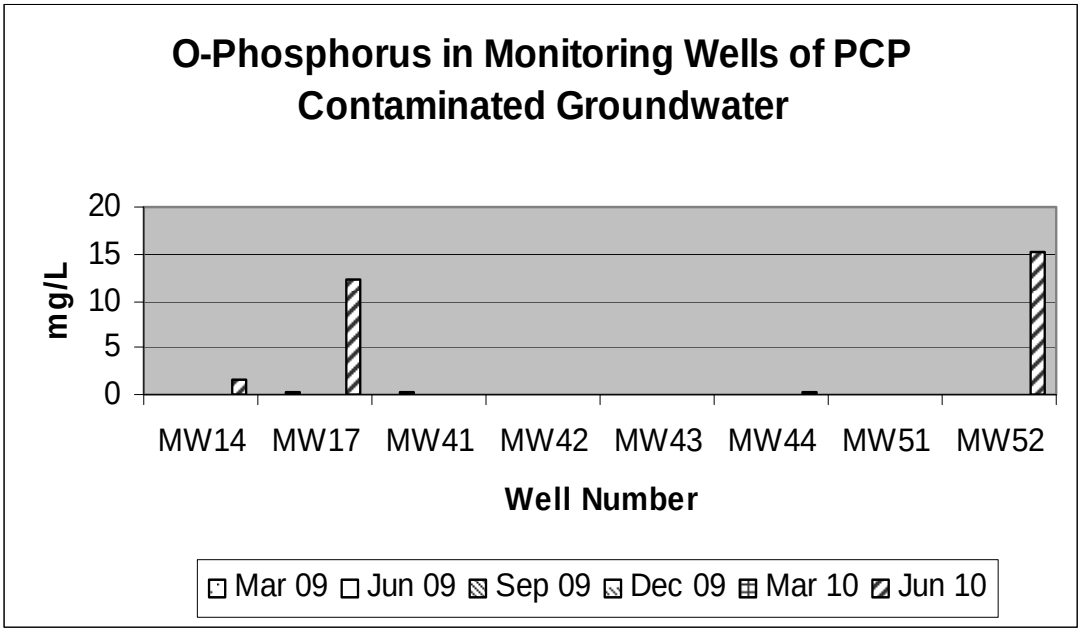

Figure 3. Ortho-phosphorus in PCP contaminated groundwater collected from eight monitoring wells over a 15 month period.

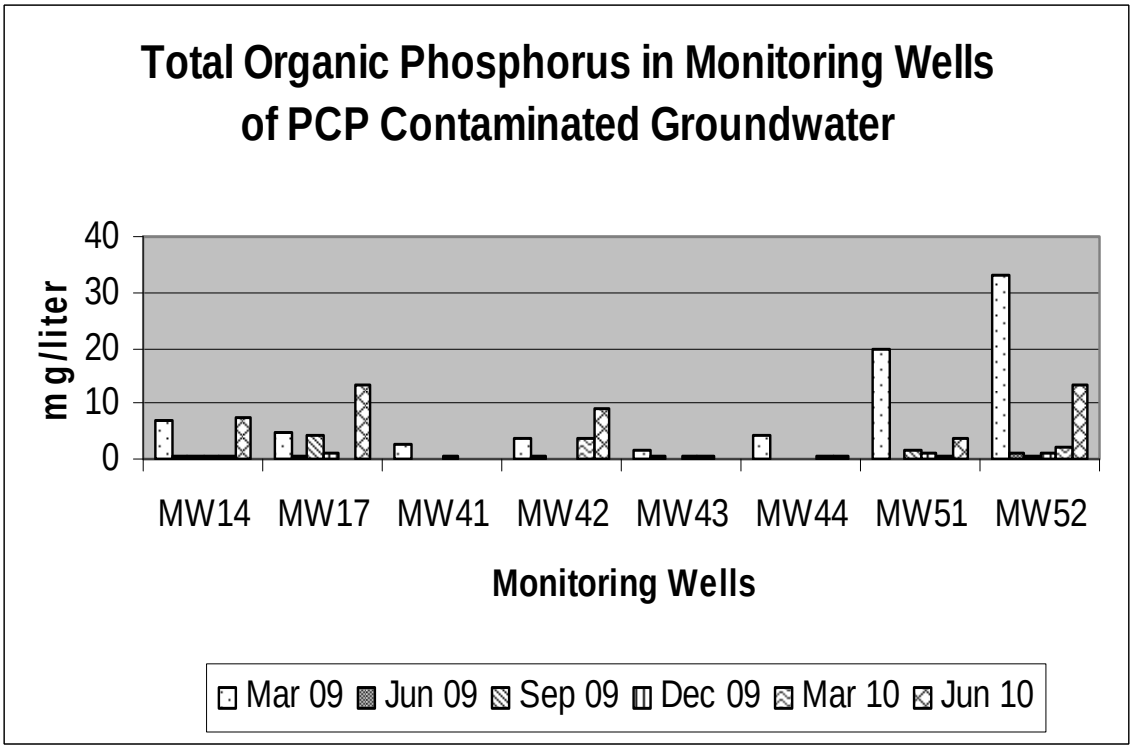

Figure 4. Total organic phosphorus in PCP contaminated groundwater collected from eight monitoring wells over a 15 month period.

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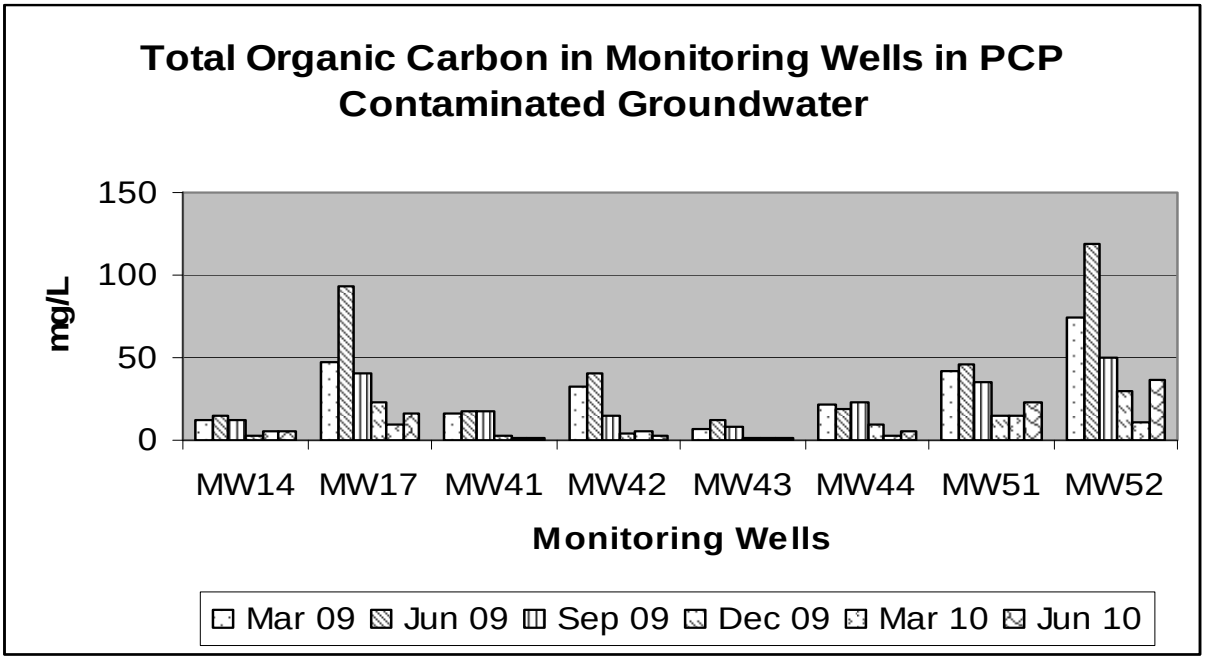

Figure 5. Total Organic Carbon in PCP contaminated groundwater collected in from eight monitoring wells over a 15 month period.

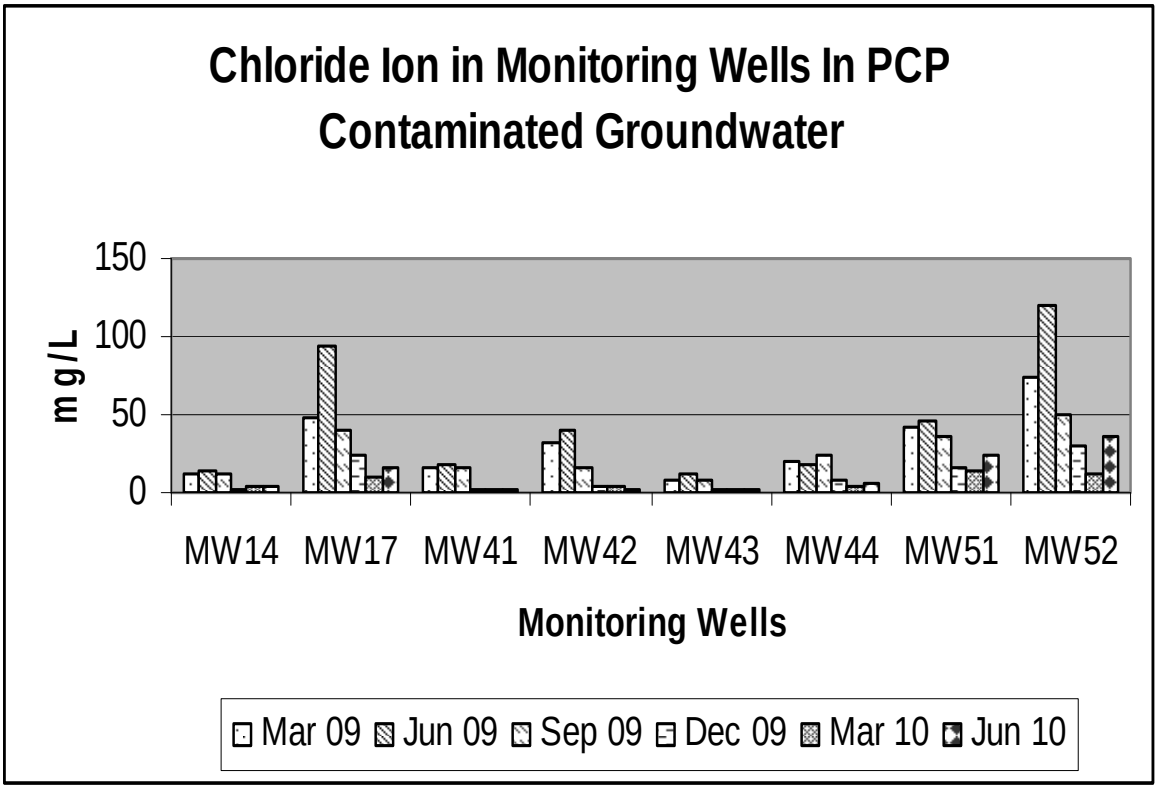

Figure 6. Chloride ion in PCP contaminated groundwater collected from eight monitoring wells over a 15 month period.

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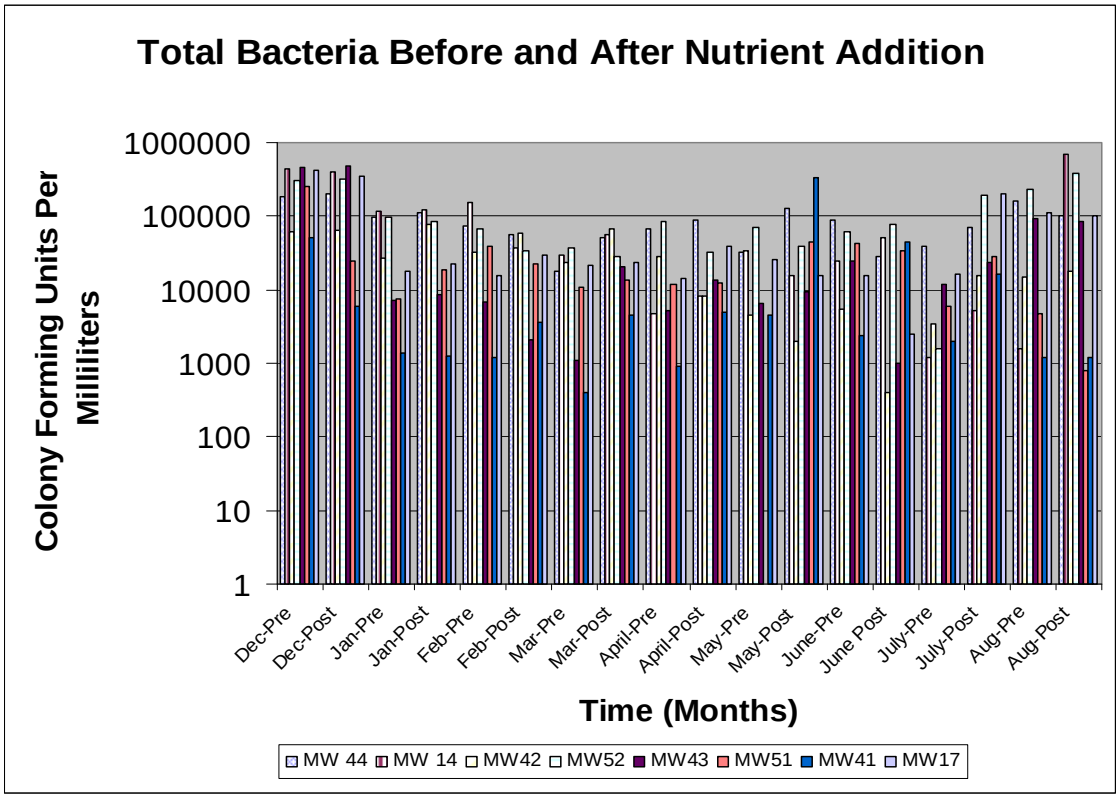

Figure 7. Total bacteria in PCP contaminated groundwater collected from eight monitoring wells.

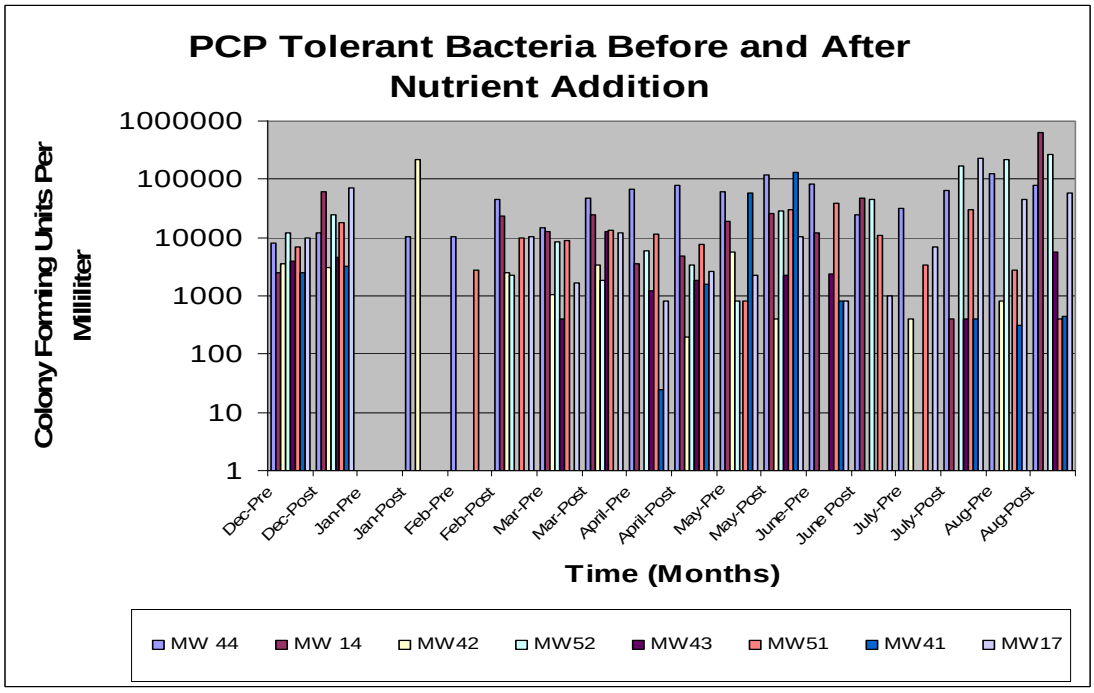

Figure 8. PCP tolerant bacteria PCP contaminated groundwater collected from eight monitoring wells.

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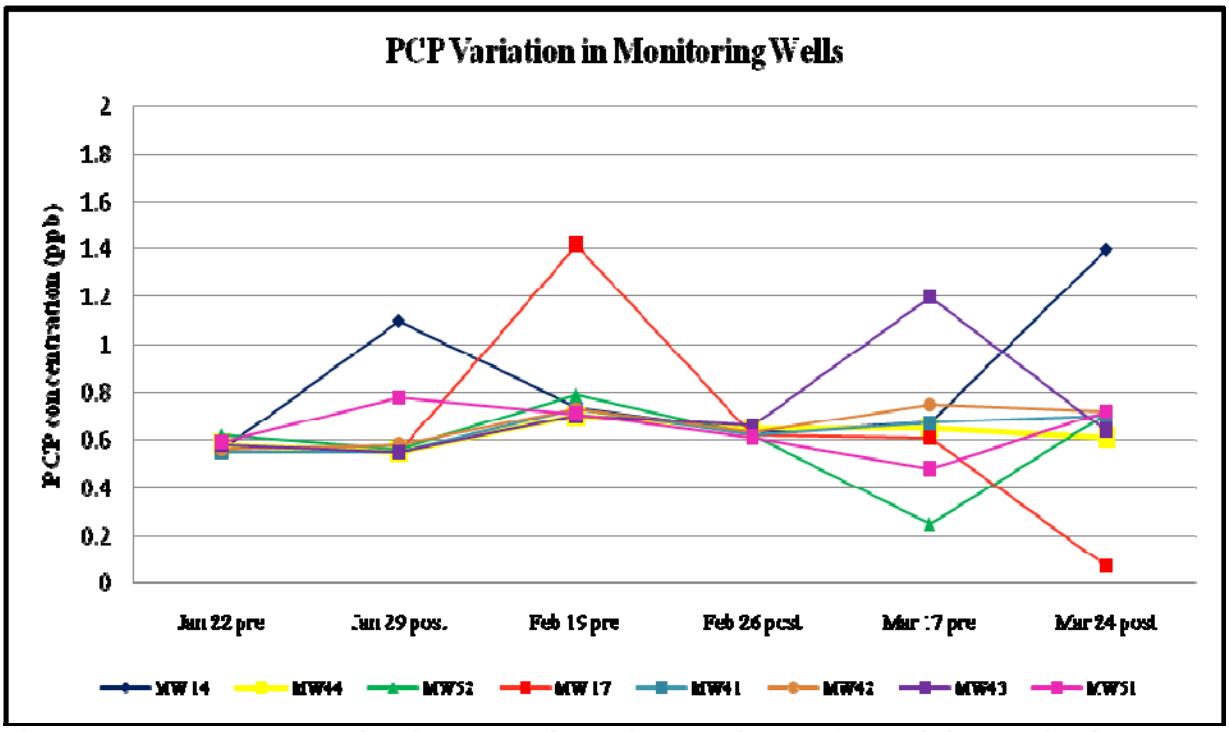

Figure 9. PCP concentration in contaminated groundwater from eight monitoring wells at selected time periods before and after nutrient addition.

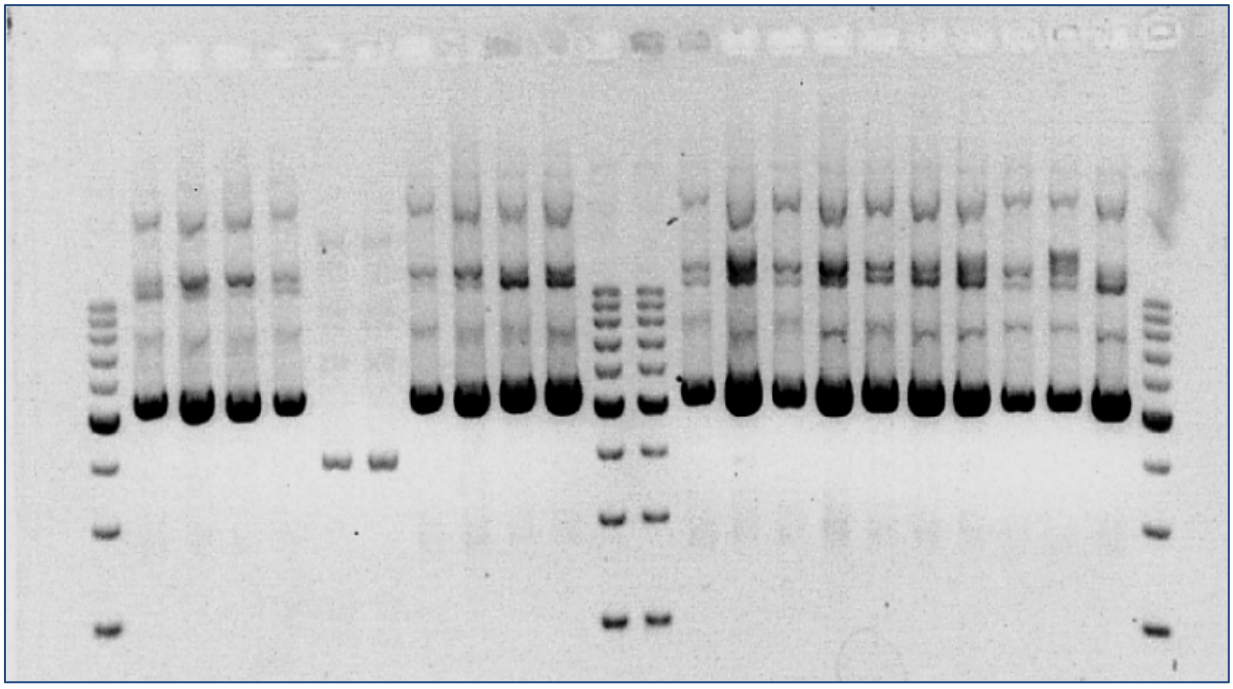

Figure 10. Extracted plasmid from clones containing the 16s region of interest for identification by sequencing. (Supercoiled DNA marker – lanes 1, 12, 13, & 24; well # 14 – lanes 2-11; well #51 – lanes 14-23)

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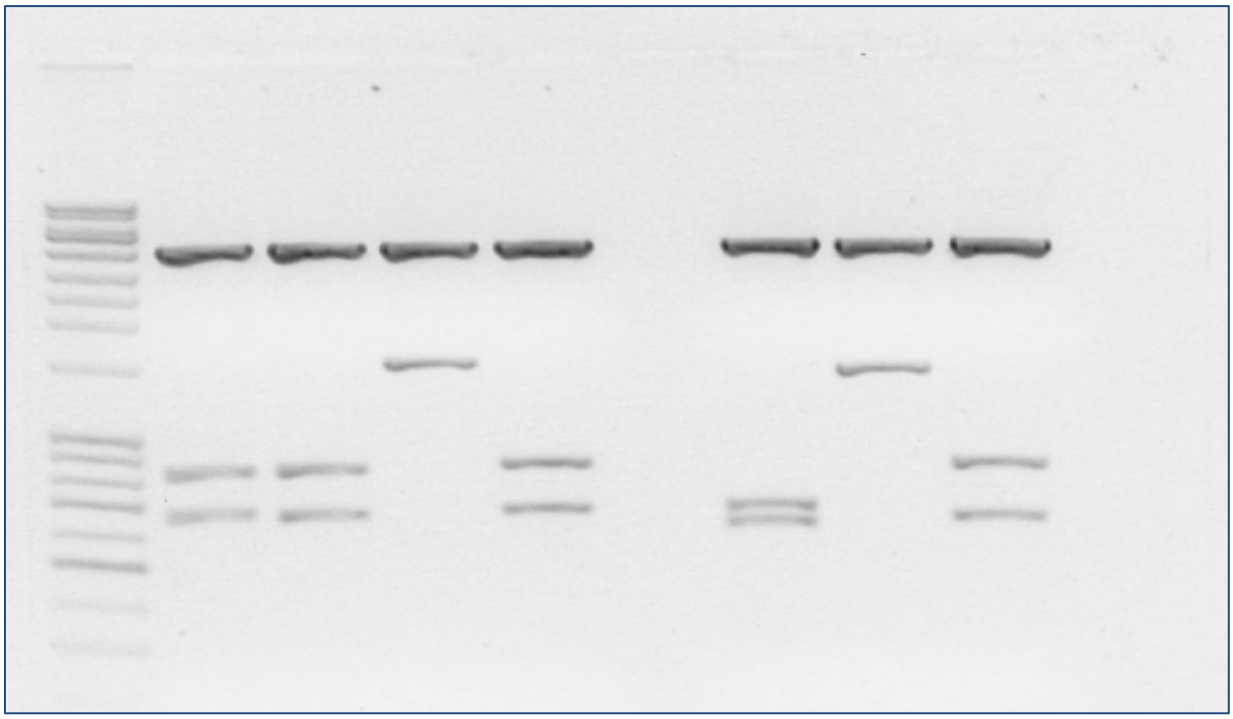

Figure 11. Digested plasmid and inserted 16s DNA fragment. (1kb DNA marker – lane 1; wells # 14– lanes 2-5; well #51 – lanes 7-9)

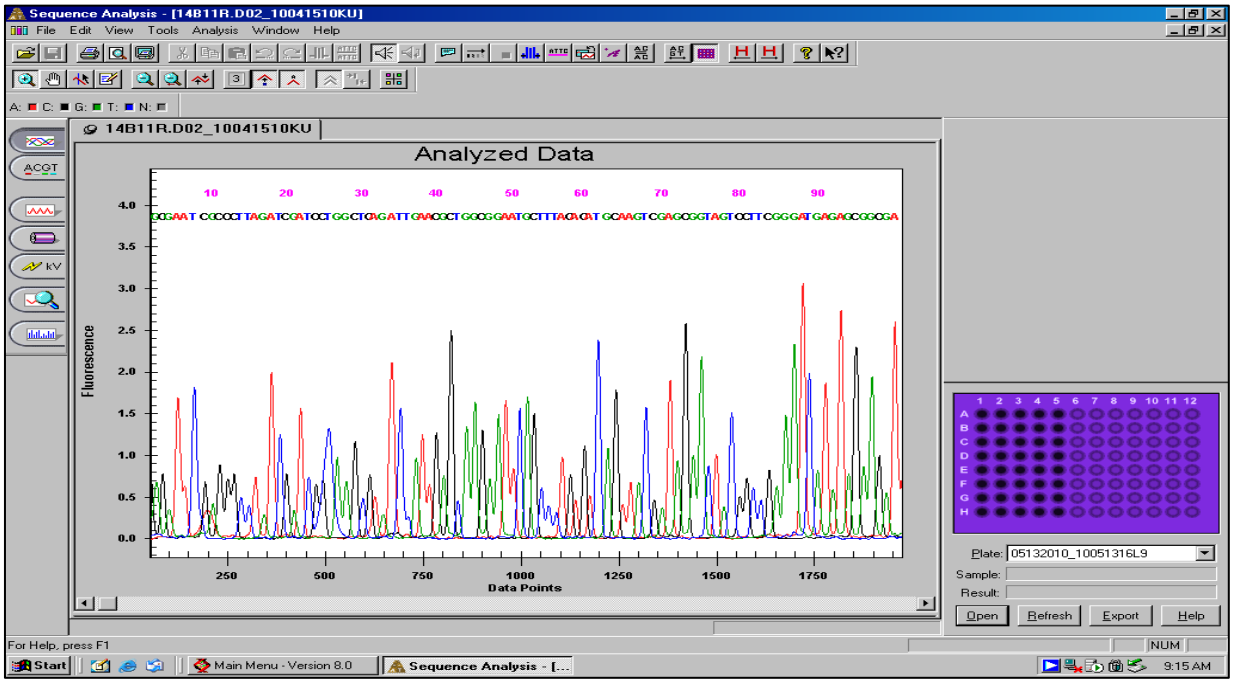

Figure 12. Sequence analysis of clone 16s fragment for identification of a PCP-degrading bacterium, *Flavobacterium sp* found in PCP contaminated groundwater**.**

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Table 1. Bacteria identified from two monitoring wells, with greater than 96% identity match and three or less sequence gaps.

Well 14 – February 2010	**Well 51 – February 2010**	
Burkholderia cepacia	*Burkholderia sp.*	*Pedobacter insulae*
Rhodanobacter thiooxydans	*Janthinobacterium lividum*	*Pedobacter duraquae*
Thauera sp.	*Duganella sp.*	*Herbaspirillum sp*
Denitratisoma oestradiolicum	*Azospirillum irakense*	*Janthinobacterium agaricidamnosum*
	Collimonas sp.	*Massilia dura*
	Flavobacterium sp.	*Aquaspirillum arcticum*
	Oxalicibacterium faecigallinarum	
** >40 Uncultured bacterial strains also found in each sample		

Conclusions

This study evaluated biosparging as a remediation tool to reduce the PCP concentration in groundwater at a wood treating site. The addition of nutrients was required in order to obtain sufficient bacteria for identification. However the bacterial population was very low and may have been insufficient to degrade PCP. Two PCP degrading bacteria were identified, *Burkolderia cepacia* and *Flavobacterium*. More studies are needed to determine if these bacteria are responsible for PCP degradation and if more nutrients will increase the population of these bacteria.

Literature Cited

Akopyanz, N., N.O. Bukanov, T.U. Westblom, S. Kresovich and D.E. Berg. 1992 "DNA diversity among clinical isolates of *Heliobacteri pulori* detected by PCR-based RAPD fingerprinting." Nucleic Acids Research. **20(19)**: 5137-5142.

Adair, S., S.H. Kim, and C. Breuil. 2002. "A molecular approach for early monitoring of decay basidiomycetes in wood chips." FEMS Micro. Letters. **211**:117-122.

Anderson, I.C. and J. W.G. Cairney. 2004. "Diversity and ecology of soil fungal communities: increased understanding through the application of molecular techniques". Environmental Microbiology. **6(8**): 769-779.

Bass, D. H., N. A. Hastings, and R. A. Brown. 2000. "Performance of air sparging systems: a review of case studies". J. Hazardous. Materials: **72,** 101–119.

Borneman J. and R. J. Hartin. 2000 "PCR primers that amplify fungal rRNA genes from environmental samples." Applied and Environmental Microbiology." **66(10):** 4356-4360.

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Buckley, M.R. 2004. “The Global Genome Question: Microbes as the key to As the key to understanding evolution and ecology”. American Society for Microbiology.

Clausen, C.A. 1997. “Immunological detection of wood decay fungi-an overview of techniques developed from 1986 to the present”. International Biodeterioration and Biodegradation. **39(2-3):** 133-143.

Crawford R.L., C.M. Jung and J.L. Strap. 2007. The Recent Evolution of Pentachlorophenol (PCP)-4-monooxygenase (PcpB) and Associated Pathways for Bacterial Degradation of PCP. Biodegradation 18: 525-539.

Dickie, I.A., B. Xu and R.T. Koide. 2002. “Vertical niche differentiation of ectomycorrhizal hyphae in soil as shown by T-RFLP analysis.” New Phytologist. **156(3**):527-535.

Jasalavich, C. A., A. Ostrofsky, and J. Jellison. 2000. “Detection and Identification of Decay Fungi in Spruce Wood by Restriction Fragment Length Polymorphism Analysis of Amplified Genes Encoding rRNA.” Applied Environmental Microbiology. **66(11):** 4724-4734.

Jellison,J. and C. Jasalvich. 2000 “A review of selected methods for the detection of degradative fungi.” International Biodeterioration and Biodegradation. **46**:241-244.

Jenson, M. A., J.A. Webster and N. Straus. 1993 “Rapid identification of bacteria on the basis of polymerase chain reaction-amplified ribosomal DNA spacer polymorphisms.” Applied and Environmental Microbiology. **59(4):** 945-952.

Mississippi Groundwater Resources. 2010. http://www.gwpc.org/e-library/documents/state_fact_sheets/mississippi. pdf

Mullis, K. B., F.A. Faloona, S.J. Scharf, R.K. Saiki, G.T. Horn, and H.A. Erlich. 1987. “Specific enzyme amplification of DNA in Vitro: The polymerase chain reaction.” Cold Spring Harbor Symp. Quant. Bio. **51**: 263-273. 1987.

O’Brien, H. E., J.L. Parrent, J.A. Jackson, J.M. Moncalvo and R. Vilgalys. 2005. “Fungal community analysis by large-scale sequencing of environmental samples”. Applied and Environmental Microbiology. **71(9):** 5544-5550.

Oh, S., D.P. Kamdem. D. E. Keathley and K.H. Han. 2003 “Detection and speces identification of wood-decaying fungi by hybridization of immobilized sequence-specific oligonucleotide probes with PCR-Amplified fungal ribosomoal DNA internal transcribed spacers”. Holzforschung. **57**:346-352..

``

Smit, E., P. Leeflang, B. Glandorf, J. D. van Elsas, K. Wernars. 1999. “Analysis of fungal diversity in the wheat rhizosphere by sequencing of cloned PCR-amplified genes encoding 18S r RNA and temperature gradient gel electrophoresis.” Applied and Environmental Microbiology. **65(6**). 2641-2621.

Thompson, T.D. Higgins, D.C., Gibson, T. J. 1994. “Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Research 11, 4673-4746.

urene, C.Y., S.E. Sanche, D.J. Hpban, J.A. Karlowsky, A.M. Kanani. 1999 “Rapid identification of fungi by using the ITS2 enetic region and an automated fluorescent capillary electrophoresis system.” Journal of Clinical Microbiology. 37:1846-1851.

Topp, E. and R.S. Hanson. 1990 “Degradation of Pentachlorophenol by a *Flavobacterium* SpeciesGrown in Continuous Culture under Various Nutrient Limitationst”. Applied and Environmental Microbiology. **56(2):**541-544.

Valasek, M.A. and J.J. Repa. 2005 “The power of real time PCR”. Advances in Physiology Education. American Physiological Society. **29**:151-159.

Valinsky, L., G. D. Vedova, A.J. Scupham, A. Alvey, A. Figueroa, B. Yin, R.J. Hartin, M. Clarobak, D.E. Crowley, T. Jian and J. Borneman. 2002a “Analysis of bacterial community composition by oligonucelotide fingerprinting of rRNA genes.” Applied and Environmental Microbiology. **68(7)**:3243-50. 2002a.

Valinsky, L., G.D. Vedova, T. Jiang and J. Borneman. 2002b “Oligonucleotide fingerprinting of rRNA genes for analysis of fungal community composition.” Applied and Environmental Microbiology. **68(12):** 5999-6004.

Xun, L. “Purification and Characterization of Chlorophenol 4-Monooxygenase from *Burkholderia cepacia* AC1100”. Journal of Bacteriology. **178(9**) 2645-2649

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Min Lee, undergraduate student, Forestry, May 2011 graduation date

Publications

Proceedings of the American Wood Protection Association –in press

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Presentations

American Wood Protection Association, Savannah, GA, May 24-26, 2010
Water Resources Research Institute, Tunica MS, August 5, 6 2009.
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