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

Final Report

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Statement of Critical Regional Water Problems

Groundwater is a valuable commodity not only in Mississippi and the region but the United States and worldwide because it impacts the health and diversity of all living organisms that comprise our ecosystems. Groundwater provides more that 90% of the drinking water supply in Mississippi. Approximately 2.6 billion gallons of water are pumped from aquifers in Mississippi each day of which 65% is used for irrigation, 15% for aquaculture and 11% for public supply. There are 1535 public water systems that use 3300 wells from 16 major aquifers and many minor aquifers throughout Mississippi that provide needed water. Therefore Mississippi and the world need to protect its groundwater quality from contamination.

Sources of groundwater contamination in Mississippi include: leaking underground storage tanks (USTs) that hold petroleum-based products and have faulty septic systems; localized brine (saltwater) contamination of shallow aquifers, agriculture practices and improper handling and storage of hazardous wastes at commercial and industrial facilities. Groundwater quality and cleanup of contaminated sites is overseen nationally by the Environmental Protection Agency (EPA) and in Mississippi by the Groundwater Assessment and Remediation Division (GARD) within the Department of Environmental Quality's Office of Pollution Control. Mississippi has four sites of groundwater contamination by wood preservatives that are listed on the EPA's Superfund National Priority List of hazardous waste sites. These sites are located in Flowood, Hattiesburg, Louisville and Picayune where pentachlorophenol (PCP) and creosote were used to treat utility poles and crossties. PCP and creosote are characterized as probable human carcinogens and their cleanup in contaminated groundwater at these sites in Mississippi is estimated to cost between $70 and $75 million.

Statement of Results, Benefits and/or Information

Results from this research are expected to reveal which of 3 consortiums of bioaugmented PCP degrading bacteria will increase PCP degradation in contaminated groundwater. The information that will be gained from this research should lead to customizing remediation methods based on the indigenous microbial community at a contaminated site. Not only could bacterial consortiums be used for PCP degradation, they could also be used to address other water quality issues such as high Biological Oxygen Demand (BOD) that impacts wastewater discharge from industries in Mississippi and nationwide such as the pulp and paper mills, oil spills in the Gulf Coast and excess nitrogen in agriculture runoff.

Nature, Scope and Objective of Research

PCP is a five chlorine containing aromatic phenolic compound that makes it not only a very effective wood preservative and herbicide but a persistent contaminant when it is placed in the environment (Cole et al. 1996). PCP works by disruptive oxidative phosphorylation of living cells. Chlorinated dioxins, extremely toxic compounds, are often present with PCP as a result of the manufacturing process increasing the urgency in remediating this contaminant. PCP has also been used for

over 60 years in many industrial settings including tanneries, distilleries, paint manufacturing and pulp and paper mills (Chandra et al 2006).

Because PCP is highly toxic and very recalcitrant it has been classified as a priority pollutant by the Environmental Protection Agency (EPA). As a result of the wood treating process, wood treating facilities have generated millions of gallons of PCP contaminated groundwater. Groundwater contamination by PCP has resulted because of poor disposal and usage practices and has become a major health and environmental concern (Langwaldt 1998).

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. 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.

Materials and Methods

Approximately 19 liters of PCP contaminated groundwater was collected from a monitoring well located nearest a PCP contaminated lagoon and outside of the air sparging impact zone. This research was conducted with four treatments and three replicates within each treatment. The four treatments were: groundwater + nutrients (Treatment 1), groundwater + nutrients + *Sphingobium chlorophenolicum* (Treatment 2), groundwater + nutrients + *Burkholderia cepacia* (Treatment 3), and groundwater + nutrients + *S. chlorophenolicum* + *B. cepacia* (Treatment 4). Each treatment contained 750 ml groundwater plus 2 teaspoons of Miracle Gro^{TM}. Bottles were capped with a plastic cap containing a 3 mm hole used for weekly air sparging. Treatment bottles were daily shaken manually for one minute. Treatments 3 and 4 were kept in a locked cooler at room temperature because Bio-Safety level 2 (BSL2) regulations are required for *B. cepacia*. A two hundred fifty milliliter groundwater sample was analyzed on day 0, 36 and 72 for pH, bacterial enumerations, PCP concentration (EPA method 3510C), and mRNA gene expression. Bacterial identification using DNA based techniques was performed on Day 0. A complete description of the experimental methods is found in Joshi et al. 2012.

Results and Discussion

The pH of the groundwater was initially acidic (3.8) but increased to 8.4 by the end of the study. Optimal growth for most microbial communities in groundwater is at neutral pH. The pH and PCP tolerant bacteria correlated strongly positive indicating that as the pH approached the neutral range the number of PCP tolerant bacteria increased (Figure 1).

The most frequently identified bacteria in the PCP contaminated groundwater were: *Burkholderia* sp. (35*%), Ralstonia eutropha* (20%) *Cupriavidus* sp (18%), *Bacillus cereus* sp (18%), *S. chlorophenolicum* (6%) and *Pseudomonas* sp (Figure 2). *Burkholderia sp*., *S chlorophenolicum sp*, *Bacillus cereus* and *Pseudomonas sp* have

been reported to degrade PCP in groundwater (He et al. 2008, Karn et al 2010 (a, b), Louie et al. 2002, Sanchez and Gonzalez 2007, Xun and Gisi 2003).

The initial concentration of PCP in the groundwater was 0.49 ppm and was higher than the maximum containment level of 1 ppb for drinking water set by the EPA. There was no significant reduction in PCP in treatment 1 which contained no added bacteria (Figure 3). However, the single bacterium amended treatments did show PCP reductions of 32% and 49% but no significant reduction was observed in the two bacteria amended treatment 4. The lack of PCP reduction in treatment 4 may have been due to antagonistic effects. Similar results have been reported in other studies involving other environmental contaminants (Slatter and Lovatt 1984, Wijngaard et al. 1993). A negative correlation (r= -0.62) resulted between the average PCP concentration and the average PCP tolerant bacteria in all treatments indicating that as the number of PCP tolerant bacteria increased the average PCP concentration decreased in all treatments.

mRNA gene expression analyses were performed in order to determine which members of the bacterial community were expressing genes for the production of enzymes that would degrade the PCP. Gene specific primers were developed for the dominant PCP degrading bacteria, *Burkholderia cepacia*, and a minor PCP degrading bacteria, *Sphingobium chlorophenolicum.* Treatment 1 (groundwater plus nutrients) was used as the control treatment and its gene expression was set to 1 for gene both the *S. chlorophenolicum* and *B. cepacia* PCP degrading genes. The relative expression of the *B. cepacia* gene in Treatment 2 (nutrients plus *S. chlorophenolicum*) on day 0 was 2X the expression in the control treatment, increased to approximately 8X the control on day 36 and deceased to approximately 5X the control on day 72 (Figure 4). In Treatment 3 (nutrients plus *B. cepacia*) on day 0 the relative gene expression of the *B. cepacia* PCP degrading gene was 2X the control, and like in Treatment 2 increased to approximately 8X at day 36 but continued to increase by 11X the control on day 72. The relative gene expression of the *B. cepacia* gene in the mixed culture Treatment 4 on day 0 was approximately 4X that in the control treatment, did not change at day 32 and increased only slightly by the end of the study. Overall, a strong negative correlation (r= -0.8139) between the *B. cepacia* PCP degrading gene expression and the PCP concentration indicated that as the gene expression increased the PCP concentration decreased (Figure 5). The lack of substantial increase in gene expression in treatment 4 over the study may support the concept that an antagonistic effect results from *S. chlorophenolicum* toward *B. cepacia* when these cultures are inoculated together in PCP contaminated groundwater.

The relative gene expression by the minor PCP degrading bacteria, *S. chlorophenolicum* on day 0 in Treatment 2 (nutrients plus *S. chlorophenolicum*) was less than the expression in the control treatment but increased to 4X the control at day 32 and 7X on day 72 (Figure 6). In Treatment 3 (nutrients plus *B. cepacia*) the relative gene expression of the *S. chlorophenolicum* PCP degrading gene was also less than in the control but increased to approximately the same as the control on day 36 and day 72. The relative *S. chlorophenolicum* gene expression in the *S. chlorophenolicum* and *B. cepacia* mixed treatment was also less than the control on day 0, increased to 7X the control on day 36 but decreased to 5X the control on day 72. The *S. chlorophenolicum* gene was not as affected by the presence of the *B. cepacia* as was seen by the B.

cepacia gene when mixed with *S. chlorophenolicum*. A weak negative correlation (r=-0.4105) resulted between the PCP degrading gene expression of *S. chlorophenolicum* and the PCP concentration (Figure 7).

In summary, the results of this study indicate that identification of the indigenous groundwater bacterial community before beginning treatment at a contaminated site would help to select known PCP degrading bacteria to be added to the groundwater in order to increase remediation of the contaminant. It is not only important to know the members of the indigenous community but also to know the members who are actively producing enzymes (gene expression) to degrade the contaminant. We conclude from this study that adding known PCP degrading bacteria (identified in the PCP contaminated groundwater) to an indigenous community along with the addition of nutrients and air sparging will increase the degradation of PCP and enhance the bioremediation effort.

Further studies, student training, information transfer, or source of funding

Further studies should include determining the activity of the PCP degrading enzymes from *Burkholderia cepacia*. In this study we assumed that because the genes for the PCP degrading enzymes were expressed, the corresponding enzymes were produced. However this needs to be verified. It is also possible that if these enzymes were produced, their activity was not sufficient to have caused the reduction in PCP and another enzyme was responsible for this reduction. Therefore the enzyme activity should be determined. Lastly, laboratory results do not always translate into field results and further studies should include a field study.

This project provided funding and training for one master student, Vaibhav Joshi, who has written and defended his thesis research, has completed the requirements for a master's degree and will graduate in May 2013. Also working with Mr. Joshi was Min Lee, another graduate student. Mr. Joshi presented the results from this research project to three professional conferences: Water Resources Research Institute, Forest Products Society and Environmental Science and Technology. Mr. Joshi will also present these results at the American Wood Preserver's Conference in Hawaii next month. One non-referred paper has been published (Proceedings of the Mississippi Water Resource Conference), one has been submitted to a referred journal for publication (The Journal of General and Applied Microbiology) and one more is in final stages of preparation for submission to another referred journal (Journal of Applied Microbiology).

This project was also supported in part by the Department of Forest Products. Results from this research should provide background information for seeking funding from industries associated with wood preservation or other environmental contaminants and the EPA.

References

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Figures

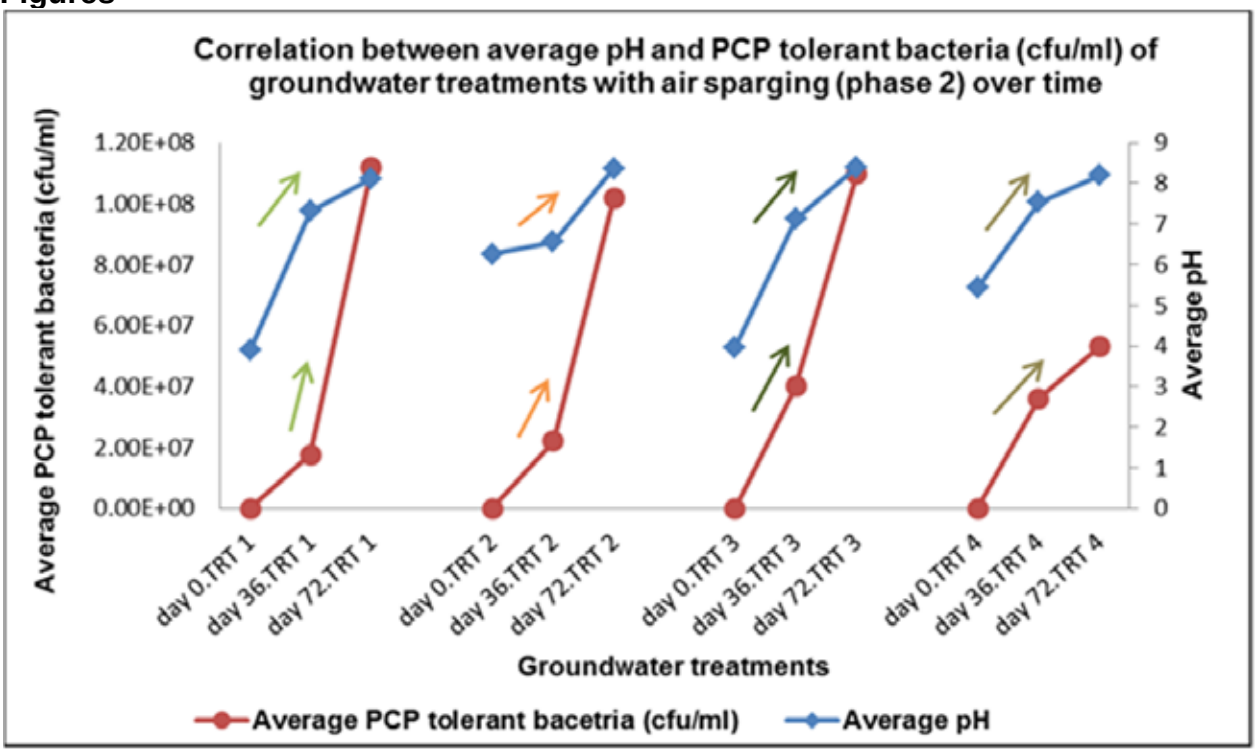

Figure 1 Correlation between average pH and PCP tolerant bacteria (cfu/ml) of groundwater treatments with air sparging (phase 2) over time. Treatments: TRT 1= with Miracle Gro ™, TRT 2= Miracle Gro ™ + *S. chlorophenolicum*, TRT 3= Miracle Gro™ + *B. cepacia* and TRT 4= Miracle Gro™ + *S. chlorophenolicum* + *B. cepacia*. There were three replicates per treatment. Correlation coefficient (r) values: TRT 1= 0.7521, TRT 2= 0.9970, TRT 3= 0.9219 and TRT 4= 0.9959. Average Correlation coefficient (r) value of all treatments over time= 0.9176.

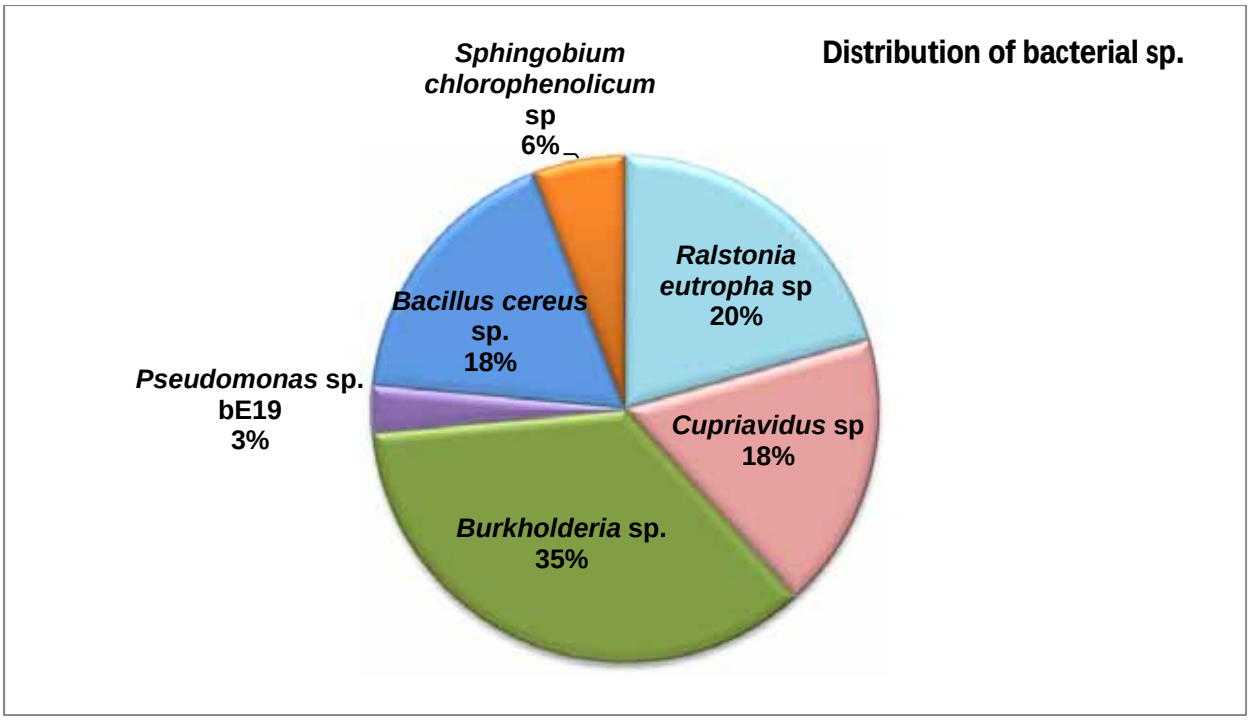

Figure 2 Composition of PCP tolerant bacterial species in PCP contaminated groundwater.

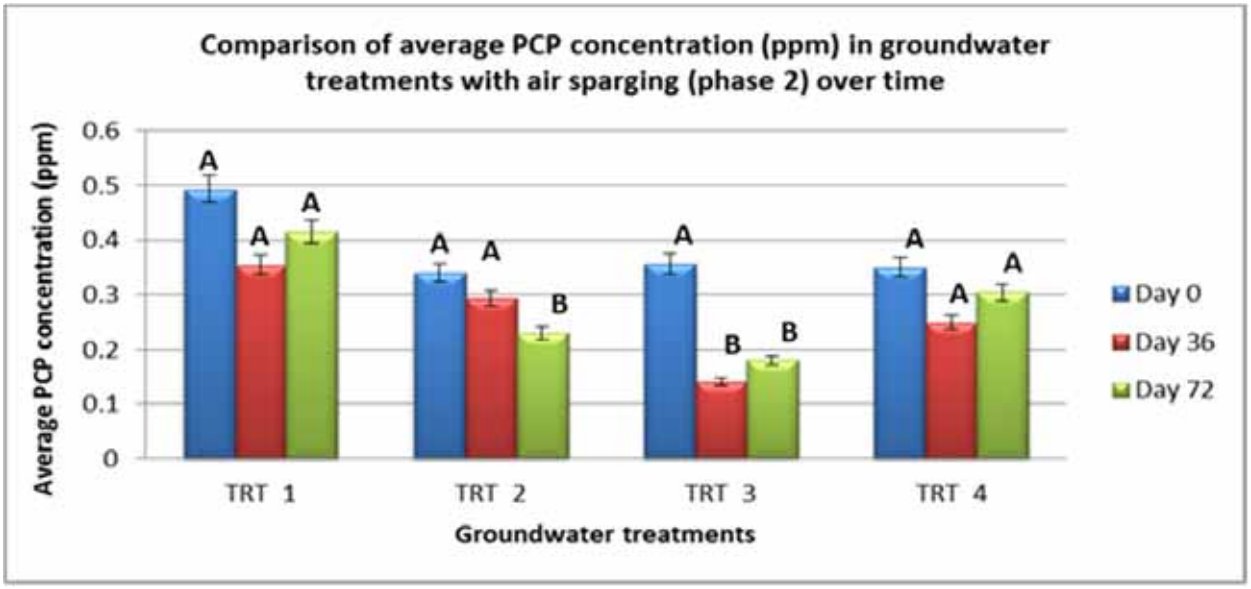

Figure 3 Comparison of average PCP concentration (ppm) in groundwater treatments with air sparging (phase 2) over sampling times day 0, 36 and 72. Treatments: TRT 1= with Miracle Gro ™, TRT 2= Miracle Gro ™ + *S. chlorophenolicum*, TRT 3= Miracle Gro™ + *B. cepacia*and TRT 4= Miracle Gro™ + *S. chlorophenolicum* + *B. cepacia*. There were three replicates per treatment. Letters A and B indicate statistically different average PCP concentration values of treatments over time. Statistical values: α= 0.05 for all treatments. TRT 1: F value= 3.9, F critical= 4.3 and P value= 0.07; TRT 2: F value= 19.6, F critical= 4.3 and P value= 0.01; TRT 3: F value= 294, F critical= 4.3, P value= 0.0001, TRT 4: F value= 3.4, F critical= 4.3, P value= 0.1.

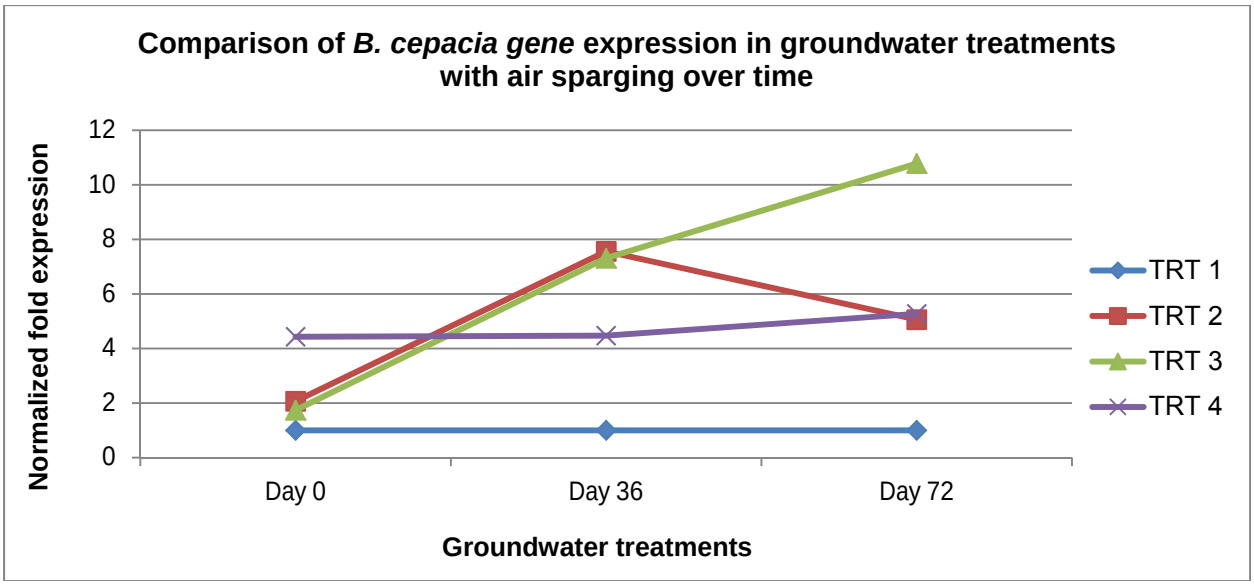

Figure 4. Comparison of *B. cepacia* gene expression in groundwater treatments with air sparging (phase 2) over sampling times day 0, 36 and 72. Treatments: TRT 1 = with Miracle Gro ™, TRT 2 = Miracle Gro ™ + *S. chlorophenolicum*, TRT 3 = Miracle Gro™ + *B. cepacia* and TRT 4 = Miracle Gro™ + *S. chlorophenolicum* + *B. cepacia*. There were three replicates per treatment.

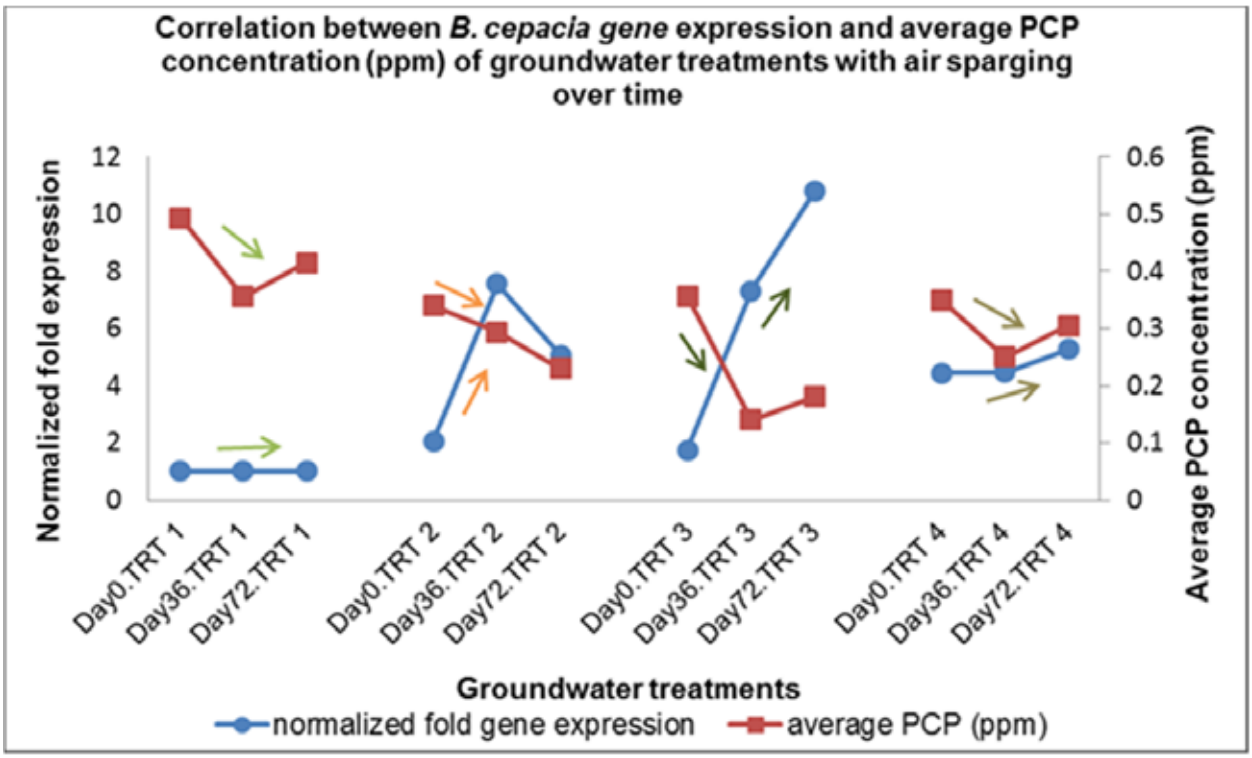

Figure 5 Correlation between *B. cepacia* gene expression and average PCP concentration (ppm) of groundwater treatments with air sparging (phase 2) over sampling days 0, 36 and 72. Treatments: TRT 1 = with Miracle Gro ™, TRT 2 = Miracle Gro ™ + *S. chlorophenolicum*, TRT 3 = Miracle Gro™ + *B. cepacia* and TRT 4 = Miracle Gro™ + *S. chlorophenolicum* + *B. cepacia*. There were three replicates per treatment. Correlation coefficient (r) = -0.8139.

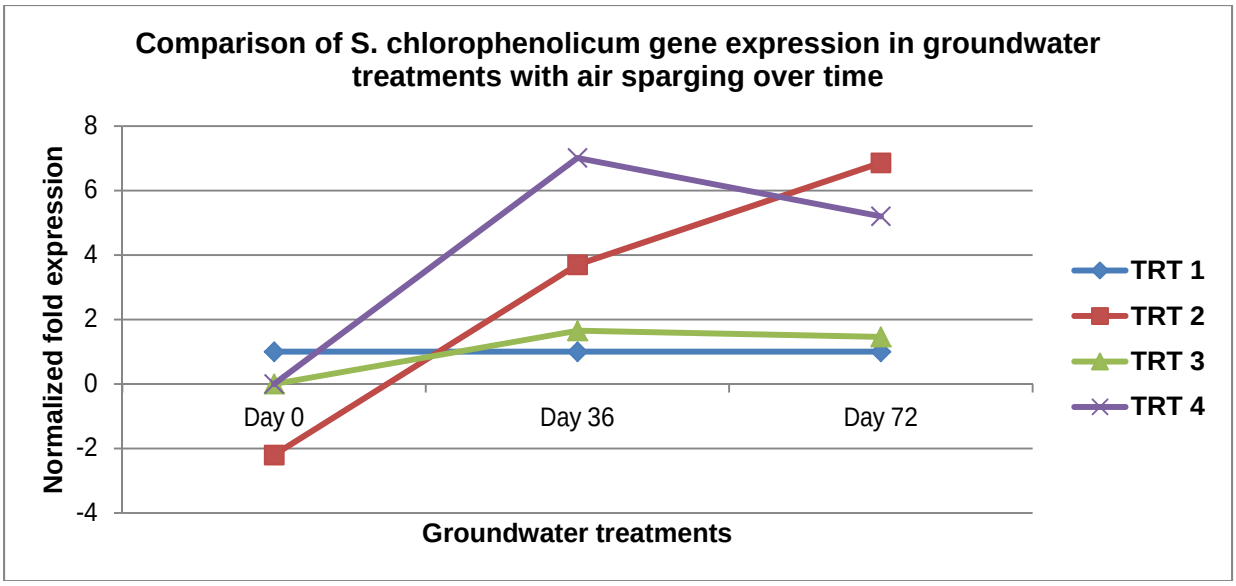

Figure 6 Comparison of *pcp*B (pentachlorophenol-4-monooxygenase) gene expression in groundwater treatments with air sparging (phase 2) over sampling days 0, 36 and 72. Treatments: TRT 1 = with Miracle Gro ™, TRT 2 = Miracle Gro ™ + *S. chlorophenolicum*, TRT 3 = Miracle Gro™ + *B. cepacia* and TRT 4 = Miracle Gro™ + *S. chlorophenolicum* + *B. cepacia*. There were three replicates per treatment.

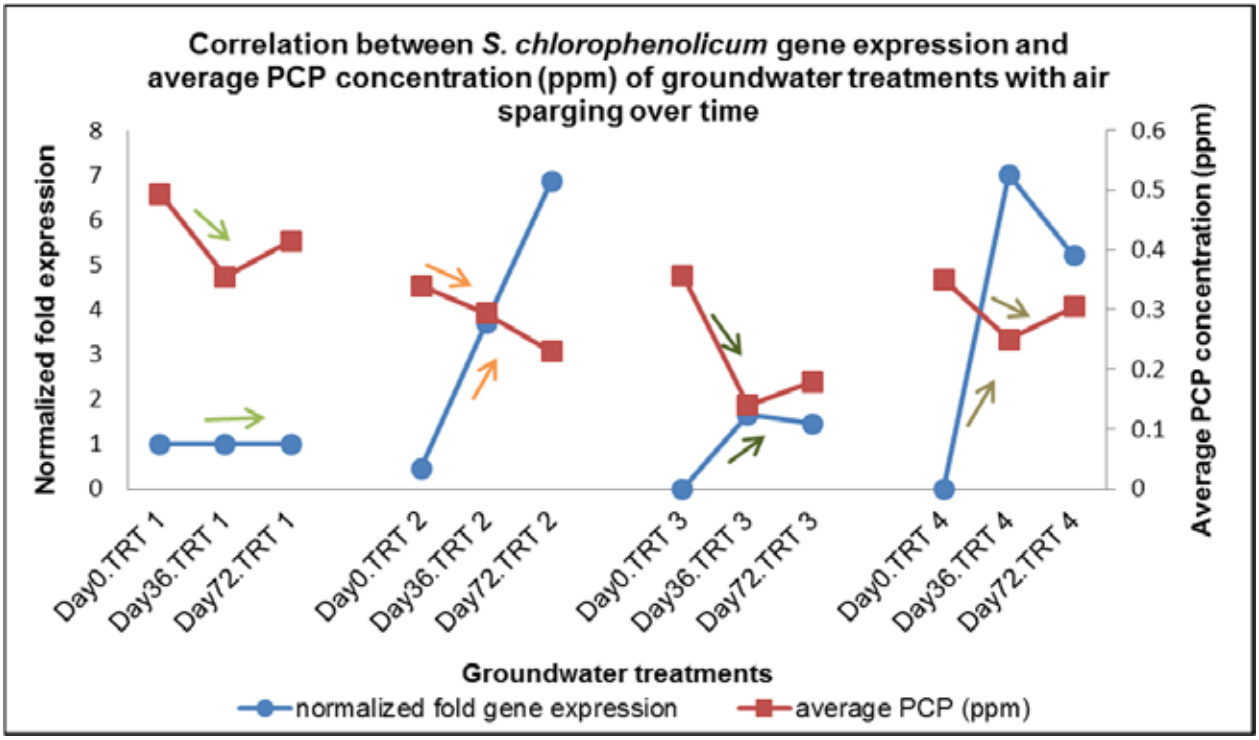

Figure 7 Correlation between *pcp*B expression and average PCP concentration (ppm) of groundwater treatments with air sparging (phase 2) over sampling days 0, 36 and 72. Treatments: TRT 1 = with Miracle Gro ™, TRT 2 = Miracle Gro ™ + *S. chlorophenolicum*, TRT 3 = Miracle Gro™ + *B. cepacia* and TRT 4 = Miracle Gro™ + *S. chlorophenolicum* + *B. cepacia*. There were three replicates per treatment. Correlation coefficient (r) = -0.4105.