CHARACTERIZATION OF RHIZOSPHERE MICROBIAL CONSORTIA INVOLVED IN THE ANAEROBIC DEGRADATION OF ETHYLENE GLYCOL

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ABSTRACT

Phytoremediation continues to gain acceptance as a viable approach to treat contaminated soil and groundwater. Malroz Engineering Inc. installed a phytoremediation test plot to evaluate its effectiveness as an alternative to pump and treat. Utilizing bench top studies and molecular biology techniques, such as denaturant gradient gel electrophoresis, it was shown that rhizosphere microorganisms isolated from the test plot are able to degrade ethylene glycol under anaerobic conditions. Factors with the potential to influence and improve degradation were evaluated. It was determined that phytoremediation may serve as a viable alternative to pump and treat.

Le redressement << phytoremediation >> atteint continuellement reconnaissance comme étant une démarche viable pour le traitement du sol et de l'eau contaminés. La companie Malroz Engineering Inc. a testé le redressement << phytoremediation >> pour évaluer son efficacité en tant qu'alternative pour pomper et traiter. En employant des études de << bench top >>, ainsi que des méthods de la biologie moléculaire tel que le gel gradient d'électrophorèse dénaturant, la companie a découvert que les micro-organismes rhizosphères, qui ont été isolés pour l'analyse, sont capable de dégrader le glycol glycol éthylénique dans des conditions anaérobies. Les éléments qui ont une possibilité d'influencer et d'améliorer cette dégradation ont aussi été évalués. La companie a conclu que le redressement << phytoremediation >> est une alternative viable pour pouvoir pomper et traiter.

1 INTRODUCTION

Numerous properties have been contaminated by poor past management practices. Remediation of such properties is of interest to protect human health and the environment. However, the application of techniques facilitating remediation are relatively new and poorly understood. Sites with contamination extending into the subsurface and water table pose significant challenges due to poor accessibility, complex chemical interactions between contaminants and soil, flow of groundwater and, the heterogeneity of the soil composition and strata. These factors may contribute to increased remediation costs and treatment times. Pump and treat is commonly used to remediate contaminant plumes, though the process is costly and requires long term management.

Malroz Engineering was contracted to control a contaminant plume leaching from waste lagoons on an active industrial site. The plume is managed by a pump and treat system and consists of multiple chemicals with varied properties and concentrations. Phytoremediation has been suggested as a viable alternative to pump and treat. It has increased public acceptance and lower operating costs (Erickson, 1997).

Chemicals such as fuel oil, polychlorinated biphenyls (PCBs) and tetrachloroethylene (TCE) have been

successfully remediated by a combined approach of phytoremediation and microbial degradation (Tandlich et al., 1992; Shim et al., 2000). A combined approach has been shown to enhance the remediation process, though the means by which it occurs remains poorly understood. Given the promise of a lower maintenance and more cost effective approach, Malroz Engineering Inc. planted a test plot of poplar and willow trees in the path of the contaminant plume.

Ethylene glycol is a major contaminant of the plume. It is a highly water soluble compound and approaches concentrations of 120,000 mg/L in portions of the test plot. It is commonly utilized in aircraft and runway deicing operations, manufacturing processes and as a coolant for vehicles. Ethylene glycol can negatively impact the environment be increasing oxygen demand and promoting eutrophication. Toxicological evidence suggests that chronic exposure to the compound may result in kidney damage, brain damage and teratogenesis.

Ethylene glycol is readily degraded by microorganisms under aerobic conditions (Staples et al., 2001; Klecka et al., 1993) and has been demonstrated to be degraded under anaerobic conditions by microorganisms from sewage sludge (Dwyer and Tiedje, 1983). Research involving the degradation of ethylene glycol has primarily focused on runway runoff (Child and Willets, 1978; Klecka et al., 1993) or manufacturing waste streams (Schonberg et al., 1997). However, little is known about its subsurface degradation under anaerobic conditions at contaminated sites.

It has been demonstrated that during degradation of anthropogenic compounds, the composition of the rhizosphere microbial consortia involved in degradation might be influenced by the tree species (Marschner et al., 2001; Siciliano and Germida, 1998; Graff and Conrad, 2005; Leigh et al., 2006). The same research also suggests that successful degradation may be determined by the relationship between rhizosphere microorganisms and tree species.

The purpose of our laboratory research activities was to demonstrate that the microbial consortia associated with the phytoremediation test plot were able to anaerobically degrade ethylene glycol and to identify factors influencing degradation. A better understanding of the processes involved will improve site management and application of the technique elsewhere.

In the past, cultivation techniques were used to determine what microorganisms might be involved in a degradation process. This had serious limitations as it is generally accepted that only 10% of soil microbes are culturable. In addition, these techniques are unable to demonstrate complex and/or delicate community interactions such as those found within microbial consortia.

The use of molecular biology allows for a more complete picture of soil microbial communities without the need for cultivation. Denaturant gradient gel electrophoresis (DGGE) can be used to monitor shifts in microbial communities during adaptation to chemical exposure, nutrient amendments and other changes in environmental conditions. The technique isolates bands of DNA based on differences in base pairing, thereby allowing the theoretical isolation of differing species present in the sample (Muyzer et al., 1993). The application of molecular biology techniques provides a means to better integrate laboratory research into field applications and could lead to improved management of bioremediation/phytoremediation solutions.

2. MICROBIAL ENRICHMENT

Soil samples were taken from under the crowns of poplar and willow trees in the test plot to obtain a representation of the rhizosphere region believed to be the most active zone in terms of interaction between the trees and rhizosphere microbial consortium. Samples were frozen until needed.

Microcosms were set up with soils taken from bulk soil (unassociated with rhizosphere), willow and poplar rhizospheres. A basal salts medium (Gerhardt et al., 1994) and trace element solution (Gerhardt et al., 1994) were added as growth media and ethylene glycol was added as a sole carbon source. The provision of ethylene glycol as a sole carbon source was to promote the development of microorganisms able to degrade the compound.

Anaerobic microcosms were provided with either nitrate, sulphate or iron (III) as a terminal electron acceptor. The headspace of anaerobic microcosms was purged with oxygen free nitrogen gas and capped with a butyl stopper. The microcosms were then placed inside an anaerobic chamber with an oxygen free nitrogen atmosphere.

Degradation of ethylene glycol was monitored by gas chromatographic analyses so that the relationship between microbial species identified in DGGE analysis to degradation rates and soil source could be determined. Degradation of ethylene glycol was demonstrated to occur under aerobic, and anaerobic conditions (with nitrate, sulphate or iron (III) provided as a terminal electron acceptor). Fertilizer was added to selected anaerobic microcosms to study the effects of its addition on microbial degradation of ethylene glycol. It was demonstrated in microcosm studies that greater than 95% of an initial ethylene glycol concentration of 6000 mg/L could be degraded in less than 14 days.

3. CHARACTERIZATION OF MICROBIAL CONSORTIA

Soil samples isolated from the test plot and microcosm enrichments were analyzed using DGGE analysis. This was done to identify differences in the microbial composition resulting from enrichment, potential influences from association with tree species, terminal electron acceptor availability and addition of fertilizer.

The characterization of microbial composition necessary for successful degradation and how they relate to the aforementioned variables could serve as means for identifying sites where *in situ* treatment may not be possible using a native population or act as a troubleshooting mechanism should degradation not be progressing in the field.

3.1 Extraction and Amplification of DNA

DNA was extracted from the samples using a soil buffer wash technique previously described by Fortin et al., (2004). Reamplification of extracted DNA, using polymerase chain reaction (PCR), was required to obtain the concentrations required for analyses. A series of trial PCR runs were carried out to establish ideal conditions for amplification. Amplified DNA was loaded onto a DGGE gel. The gel was prepared with a 0-80% denaturant gradient and run at 80 V for 16 h at 60°C.

3.2 DGGE Analyses

When comparing the DGGE gels of microbial populations at different times and site conditions, different banding patterns are indicative of the presence or absence of species resulting from applied selective pressures. Each band (labeled A-G, Figure 1) theoretically represents a unique microbial species within a consortium. Comparison of willow rhizosphere and poplar rhizosphere soils suggested that the trees species were influencing the composition of the microbial consortia. Poplar (Figure 1, lane 1) produced a different banding pattern than willow (Figure 1, lane 8). Enrichment of willow associated soil with ethylene glycol as a sole carbon source reduced the population diversity to three major conserved species (Figure 1, lanes 5-7 and 9 bands D-F) relative to the test plot willow and poplar (lanes 1 and 8 respectively). There were also apparent differences in species dominance through a comparison of band intensities (Table 1).

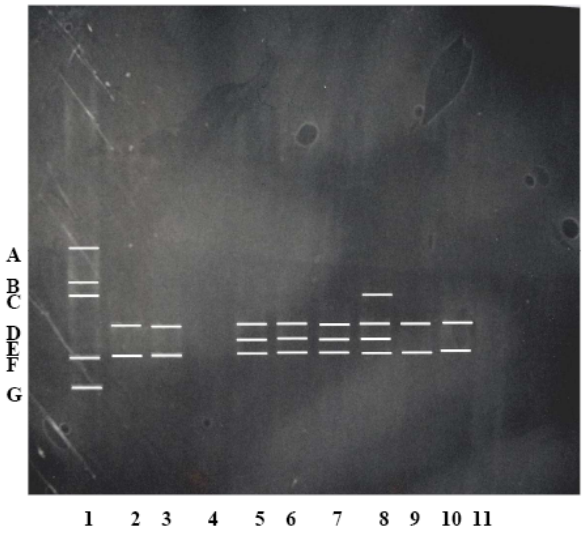

Figure 1. DGGE analysis of (1) test plot poplar 2004, (2) control plot willow, (3) bulk soil, (4) test plot willow 2005, (5) willow aerobic microcosm, (6) willow microcosm with nitrate TEA, (7) willow microcosm with fertilizer, (8) test plot willow 2004, (9) willow microcosm with sulphate TEA, (10) bulk soil anaerobic with nitrate, (11) negative control

A more intense band theoretically represents a greater concentration of the associated species in the original sample and thus greater dominance. Species that are better adapted to the use of ethylene glycol or other factors such as TEA availability will be more likely to outcompete other bacteria and will increase in prevalence. It was noted in a comparison of different terminal electron acceptors and a fertilized sample(Table 1), that there was some relationship between band intensity (Table 1, Bands D, E and F) and relative degradation rate. A comparison of anaerobic microcosms amended with either nitrate,

Table 1: Composite representation of Figure 1 relative intensity of bands corresponding to distinct species (A-G) expressed as (+) very faint, (++) faint, (+++) moderate, (++++) prominent. Relative rates of degradation were excellent (>>>>), good (>>>), moderate (>>) and (>) limited.

Samples	Organism							Relative Rate
	A	B	C	D	E	F	G	
Bulk soil				++		++		n/a
Bulk w/nitrate				++		+++		>
Test plot poplar (2004)	++	+++	++			+++	++	n/a
Test plot willow (2004)			++	++	+++	++		n/a
Control plot willow (2004)				++++		+		n/a
Willow aerobic microcosm				+++	++	+++		>>>>
Willow microcosm w/nitrate				+++	++	+++		>>>
Willow microcosm +fertilizer				++	++	++		>>
Willow microcosm w/sulphate				+		++		>

fertilizer or sulphate showed the nitrate amended sample having the greatest rate of degradation, followed by fertilized and sulphate. Comparing the intensity of bands D, E and F (Table 1) resulted in the same ordering of the aforementioned samples. This suggests that a greater concentration of species D, E and F is linked to better degradation and that they respond most favourably to increased nitrogen concentrations.

Differences in intensity were also apparent between test plot samples and enriched samples (Table 1). The degradation rates were listed as “n/a” for test plot samples as they were not enriched in microcosms and thus could not be monitored for ethylene glycol loss. However, a comparison of band intensities between test plot and enriched conditions, facilitates an understanding of how each species responds when ethylene glycol is the sole carbon source and different amendments are applied (Table 1). Addition of nitrate increased D and F relative to the field sample while fertilization produced no change and sulphate resulted in a reduction of band D (Table 1). Band E was most prevalent in the field sample and least prevalent in the sulphate sample. The consistent reduction in band E relative to enriched samples suggest that this species is competitively disadvantaged under anaerobic conditions where ethylene glycol is provided as a sole carbon source.

The test plot has a control plot which mirrors the preparation of the test plot but is positioned outside the path of the contaminant plume. The control plot willow had one band that was particularly dominant and present in all of the evaluated samples (Band D), suggesting that this species is naturally dominant, regardless of the influence of tree or ethylene glycol concentration. The lack of Band E in the control plot may represent a microorganism dependent on ethylene glycol as it appeared in most of the willow soils exposed to ethylene glycol in the test plot and microcosms.

Bands were not recovered in the 2005 willow test plot sample (Figure 1). This was either due to an inadequately small amount of DNA, or the environmental conditions at the time of sampling were not ideal for supporting a diverse consortium (the 2005 sampling, the soil was very dry).

It is possible to extract DNA from individual bands and identify the species which it represents. This would provide additional information about the composition of a microbial community and could lead to an improved understanding of the degradative enzymes involved in the remediation process. Despite isolating DGGE bands and attempting to extract DNA from them, no DNA was successfully reamplified for sequencing. Subsequent DGGE analysis was done with fewer samples, but double the concentration of DNA (1,200 ng). A similar pattern of consortial diversity was apparent and one band was successfully isolated and reamplified (Figure 2, lane band C).

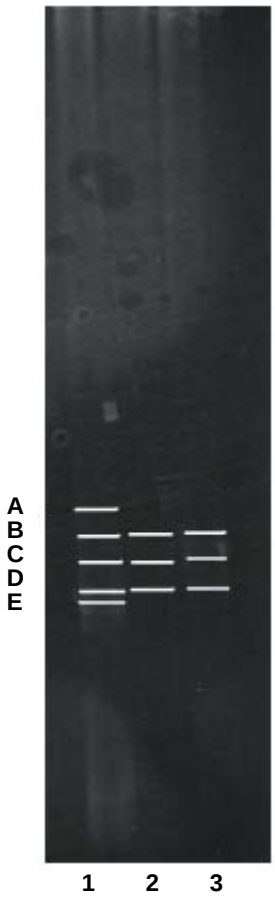

Figure 2: Analysis of higher DNA concentration for (1) test site control plot willow, (2) site poplar 2004, (3) site willow 2004.

4. CONCLUSIONS AND APPLICATIONS

The microcosms demonstrated less microbial diversity suggesting that they had successfully been enriched. Despite enrichment of the microcosms, which encourages a shift in consortial composition, common species (identified by common banding) were identified in microcosm and test plot samples. This suggests that test plot consortia are undergoing a degree of natural enrichment and that laboratory analyses can provide a representation of what may be expected in the field.

The comparison of enriched microcosms and variables driving natural selection demonstrated that it was possible to enrich for, and identify, microorganisms with a dominant role in ethylene glycol degradation under aerobic and anaerobic conditions. The technique further demonstrated a means by which the influence of terminal electron acceptors, and fertilizer amendments on species in microbial communities could be evaluated.

Comparison of microbial consortia associated with poplar and willow suggested that there are potential differences in their respective species compositions as a result of rhizosphere source. Identifying tree species which increase the presence of “degraders” allows for improved site management as it may accelerate the enrichment of the indigenous consortia for the purposes of remediation.

The use of DGGE in conjunction with laboratory studies, designed to develop a better understanding of the test plot and degradation of ethylene glycol, demonstrated the technique’s potential value to future projects. DGGE may be used to identify important species involved in the degradation of contaminants, regardless of whether they can be cultured or not, and may point to factors promoting/inhibiting their survival.

The potential benefits of such an approach are improved confidence in the management of phytoremediation applications and improved financial savings. This would result from substituting trial and error in the field for bench top studies which may evaluate the effectiveness of planned test plot manipulation, such as nutrient amendments, at a smaller scale.

The use of DGGE also makes it possible to isolate individual species from a community and apply additional molecular biology techniques. These techniques can allow for identification of unique species which may be used to “seed” other test plots and improve degradation rates or to identify and isolate genes coding for the enzymes which allow degradation to occur. These unique sections of DNA may then be hybridized into the indigenous bacteria of other sites which are adapted to the site conditions but otherwise lacking the ability for degradation.

The demonstrated technique and applications are not limited to ethylene glycol and associations with poplar and willow. It represents a means by which companies like Malroz Engineering Inc may develop a unique remedial “tool kit”, allowing for a customized application of phytoremediation in which tree species, bacterial species and amendments would be selected based on the target contaminant and site conditions.

While the development of biologically driven technologies appears onerous, they offer the promise of simplified long term management and lower operating costs. A successfully established system

would continue to improve with time as the trees grew and microorganisms continued to become better adapted.

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