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Field and Laboratory Tests of a Multi-Process Phytoremediation System for Decontamination of Petroleum and Salt Impacted Soils

Bruce M. Greenberg (greenber@uwaterloo.ca), Xiao-Dong Huang, Karen Gerhardt, Bernard R. Glick (Department of Biology, University of Waterloo, Waterloo, Ontario, Canada and Waterloo Environmental Biotechnology, Hamilton, Ontario, Canada)
Jola Gurska, Wenxi Wang, Mark Lampi, Aaron Khalid, David Isherwood, Pearl Chang, Haitang Wang, Shan Shan Wu, Xiao Ming Yu, and D. George Dixon (Department of Biology, University of Waterloo, Waterloo, Ontario, Canada)
Perry Gerwing (Earthmaster Environmental Strategies Inc., Calgary, Alberta, Canada)

ABSTRACT: There is an overwhelming need for versatile *in situ* technologies that can efficiently remediate persistent contaminants from soil in a cost-effective, environmentally responsible way. Phytoremediation is gaining acceptance as a *bona fide* strategy for remediating sites impacted by organics, metals and/or salt. One challenge that has hindered widespread use of phytoremediation is that chemical toxicity, nutrient deficiency and/or water stress in impacted soils often result in the production of stress ethylene. This, in turn, can lead to growth inhibition and diminished plant biomass, resulting in unacceptable rates of remediation. We developed a multi-process phytoremediation system (MPPS) that utilizes plant/PGPR (plant growth promoting rhizobacteria) interactions to mitigate stress ethylene effects, thereby greatly increasing plant biomass, particularly in the rhizosphere. The MPPS degrades a variety of organic contaminants in soils with accelerated remediation kinetics. Over the last two years at a petroleum impacted site in Sarnia, ON, a decrease of ~ 50 % in CCME fractions 3 and 4 was observed. At a site in Turner Valley, AB, 30 % remediation of total petroleum hydrocarbons was achieved in 3.5 months. Recently, we tested the MPPS in salt-impacted soils in greenhouse experiments, with promising preliminary results.

INTRODUCTION

Large amounts of hazardous waste have been released into all phases of the environment largely from anthropogenic sources. Many contaminants are toxic, mutagenic, carcinogenic and/or persistent, posing threats to the environment, human health, and agricultural productivity. Examples include total petroleum hydrocarbons (TPHs), polycyclic aromatic hydrocarbons (PAHs), halogenated hydrocarbons, pesticides, solvents, metals and salt. Due to their environmental impacts, remediation of these compounds from contaminated land is a high priority. However, it can be costly and time-consuming to remediate such sites (Pilon-Smits, 2005). Contaminated land must be remediated prior to being returned to productive use or for changes in land use (Alberta Environment, 2001; SPIGEC, 2000). As such, research and development of remediation technologies, especially *in situ* systems that are efficient and cost effective, are critical priorities.

One group of widespread organic contaminants are petroleum hydrocarbons, often introduced into the environment via the oil and gas industry. Crude oil is a complex

mixture of aliphatic, aromatic, heterocyclic and asphaltene/tar hydrocarbons, ranging in size from C6 to > C50. Total petroleum hydrocarbons (TPHs) are classified into fractions. Fractions 1 (C6 – C10) and 2 (C10 – C16) are volatile or semi-volatile, whereas fractions 3 (C16 – C33) and 4 (C34 – C50) are hydrophobic and recalcitrant. Compounds from fractions 3 and 4 can be highly toxic and are regulated due to their mutagenicity and carcinogenicity (Adams et al., 2000; CCME, 2000). These hydrocarbons can be degraded by various processes, including photooxidation, microbial action, and reactions that occur naturally in the rhizosphere.

Salt-impacted sites can occur as a result of geologic formations (natural salinity), or they can be the result of anthropogenic activities such as road salt application, agriculture and industrial spills. Salt-impacted soils are an unavoidable problem in Western Canada as a result of upstream oil and gas production. This is because brine and hydrocarbons often occur together geologically (Alberta Environment, 2001). Brine water contains sulphates, bicarbonates and chlorides of Na, Ca and Mg, with NaCl being the most prevalent brine salt (Alberta Environment, 2001). Although salt is neither mutagenic nor carcinogenic, and is not generally considered toxic to animals, there are numerous environmental impacts associated with excess salt in soil. These include degradation of chemical and physical properties of the soil, diminished groundwater quality and impaired plant growth. Plants are perhaps the most vulnerable sector of the biosphere to salt. They often exhibit drought stress symptoms in salt-impacted soils despite the presence of adequate water because the resultant increases in osmotic pressure diminish water uptake by the plants (Qadir et al., 2003). Furthermore, the presence of excess ions in the soil can interfere with nutrient availability and numerous plant metabolic processes (Qadir et al., 2003). Unlike TPH, salt can not be degraded; therefore, remediation of salt-impacted sites is achieved only by removing salt ions from the soil.

Various strategies have been employed for remediation of impacted environmental and industrial sites (Saleh et al., 2004). Most traditional remediation strategies of terrestrial sites involve excavating contaminated soil and removing it to landfill, with subsequent processes such as chemical soil washing. These methods can provide a rapid solution to the immediate problem, but are very costly, leave unsightly blemishes on the landscape, and often have limited success. *In situ* biologically-based techniques have been tried as an alternative to “dig and dump” systems. Tilling can aerate the soil and bring organic contaminants to the soil surface, thereby promoting oxidation and photooxidation, respectively. (Huang et al., 2004a, 2004b, 2005). This method tends to remove only small and/or volatile organics (Huang et al., 2004b). Many organic compounds can be metabolized by microbes found in, or added to, bulk soil (Dua et al., 2002; Stapleton et al., 1998). Microbial bioremediation is rarely exploited successfully under field conditions because microbial populations in contaminated soils do not achieve sufficient biomass for acceptable rates of remediation (Brookes and McGrath, 1984). One widely used method for salt remediation is chemical amendment and leaching (Alberta Environment, 2001). However, this process is also costly and has limited effectiveness.

Clearly, *in situ* technologies are needed that can remediate petroleum and salt impacted soils in an environmentally responsible and cost effective way. Phytoremediation (defined as the use of plants for the extraction, immobilization, containment and/or degradation of contaminants) is an emerging technology that holds

great promise for the decontamination of a broad range of environmental pollutants, including salt (Glick, 2003; Greenberg et al., 2006a). Phytoremediation provides *in situ* treatment with numerous advantages over chemical amendments and *ex situ* remediation. These advantages include lower cost, greater environmental stewardship and ease of application. One criteria that is essential for successful phytoremediation is high plant biomass production. Unfortunately, plants growing in salt impacted soils are prone to the combined detrimental effects of water stress and nutritional deficiencies. This results in the production of stress ethylene, which leads to plant growth inhibition (Glick, 2004).

One strategy for increasing plant biomass in impacted soils is to treat seeds with plant growth promoting rhizobacteria (PGPR); a group of natural soil rhizosphere bacteria (25). The relationships that exist between plants and microbes in the rhizosphere play key roles in enhancing the efficacy of phytoremediation (Pilon-Smits, 2005; Glick, 2003; Chaudhry et al., 2005; Kuiper et al., 2004; Huang et al., 2004b). Root exudates can stimulate the growth of PGPR, which in turn can alleviate plant stress by lowering stress ethylene, by facilitating nutrient uptake by plants, and/or by degrading/sequestering soil contaminants (Chaudhry et al., 2005; Huang et al., 2004b; Hontzeas et al., 2004). These bacteria are nourished and carried through the soil by plant roots (Kuiper et al., 2004). In soil containing large volumes of roots, the microbial population can reach concentrations of ~10^{12} microbes per gram of soil (Whipps, 1990). This translates to a microbial biomass of ~500 kg ha^{-1} (Metting, 1992), localized primarily in the rhizosphere. By promoting root growth with PGPR, greater volumes of soil are accessed, potentially accelerating salt remediation. A key characteristic of many PGPR is that they naturally express 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase (Glick, 2004); PGPR use the ACC as an N source. PGPR with ACC deaminase can decrease the synthesis of stress ethylene by consuming ACC, the precursor in plants to ethylene. By preventing ethylene synthesis, tolerance to stress has been observed (Glick, 2004; Mayak et al., 2004; Sergeeva et al., 2006). Some PGPR also produce auxin, which further promotes root growth.

We have developed a multi-process phytoremediation system (MPPS) for the removal of persistent and recalcitrant contaminants from soil (Huang et al., 2004a, 2004b, 2005). The MPPS uses different techniques to target specific groups of contaminants: land farming (aeration and light exposure), microbial remediation (innoculation of contaminant-degrading bacteria) and enhanced phytoremediation (combined use of tolerant plant species and plant growth promoting rhizobacteria [PGPR]). This strategy has been extensively tested on a variety of impacted soils and was shown to effectively remove and degrade PAHs, including larger, recalcitrant PAHs (Huang et al., 2004a). Pb, Cu and Cd were removed from soils into plant tissue (Burd et al., 2000) showing that plants can remove ions from soils. TPH-impacted soils were successfully remediated in both the greenhouse and the field (Greenberg, 2006, Greenberg et al., 2006a, 2006b; Huang et al., 2004a, 2004b, 2005). In the greenhouse, 90% of TPHs from oil sludge contaminated land farm soil were removed in eight months using the MPPS (Huang et al., 2005). Notably, the MPPS was able to remediate the hydrophobic, recalcitrant TPH. Field tests of the MPPS were subsequently carried out at a TPH-impacted site in Sarnia, ON from 2004 to 2006 (Greenberg et al., 2006a, 2006b; Greenberg 2006), and TPH-impacted sites in Alberta in 2005 and 2006 (Greenberg et al., 2006a). The field applications of the MPPS were very successful, both in terms of plant growth on petroleum impacted soil

and remediation of the TPH (Greenberg, 2006; Greenberg et al., 2006b). Several lines of evidence were used to show that the MPPS degrades TPHs in the rhizosphere of the soil (ref).

The primary element for success of the MPPS is the use of PGPR-treated seeds, which increases the plant and root biomass in contact with impacted soil. We found that two strains of PGPR (*Pseudomonas putida* UW3 and UW4) were very effective for TPH remediation (Greenberg et al., 2006b). Over the past year, we have been employing the MPPS to remediate salt-impacted soils in greenhouse experiments. While salt cannot be degraded during phytoremediation, we have found that PGPR confers plant tolerance to salt stress and that NaCl is assimilated into foliar regions of grasses at a rate that suggests remediation is possible in the field (Mayak et al., 2004). If plants grow vigorously, and salt it taken up into the foliar regions of the plants, we assert that salt can be removed from the soil during successive growing seasons to achieve remediation.

MATERIALS AND METHODS

Preparation of plant growth promoting rhizobacteria (PGPR). Two strains of PGPR, *Pseudomonas putida* UW3 and *P. putida* UW4 were used to promote plant growth and increase tolerance to contaminants (Glick, 2003, 2004; Hontzeas et al., 2004). Both strains are naturally occurring and express 1-amino-cyclopropane-1-carboxylic acid (ACC) deaminase, an enzyme that consumes the precursor to ethylene, a plant stress hormone. They also both produce siderophores and indoleacetic acid (an auxin) that aids in the development of the host plant roots (Patten and Glick, 2002). *P. putida* is not pathogenic to animals or plants, and can therefore be used safely for environmental applications (Timmis, 2002).

Prior to seed treatment, *P. putida* UW3 *and P. putida* UW4 were cultivated in tryptic soy broth at room temperature for 24 h. A Hege Liquid Seed Treater was used to coat the seeds with a mixture of UW3 and UW4. Use of a colorant and polymer (methyl cellulose) in the seed treatment mix facilitates adhesion of PGPR to the seeds and satisfies the safety regulations requiring all treated seeds to be visibly colored. Seeds were dried and stored for a maximum of 30 days prior to sowing.

Field sites. Field trials were performed for 3 consecutive years (2004 - 2006) at TPH impacted site in Sarnia, ON, Canada. This site is contaminated with petroleum hydrocarbons from refinery oil sludge. The TPH level is ~150 g/kg (15%), consisting mostly of fractions 3 and 4. A field trial was performed for 2 consecutive years (2005 – 2006) on a petroleum-contaminated biopile in Turner Valley, AB, Canada. It is approximately 8 g/kg (0.8%) TPH, consisting mostly of fractions 3 and 4.

Greenhouse experiments. We were supplied with soils from salt impacted sites in Alberta (AB) and Saskatchewan (SK). These soils represented a range of salt levels. The AB soils were lower in salt (Cl^- = 260 - 1600 mg/kg, SAR = 11 - 27, ECe = 2 - 10 dS/m) and SK soils were higher in salt (Cl^- = 1.88 - 15 g/kg, SAR = 10 - 12, ECe = 13 - 50 dS/m). Oats, barley, ryegrass, slender wheatgrass, tall fescue, red fescue and Fall rye (all ± PGPR) were grown in small pots or seeding trays using salt impacted soils in the greenhouse. Plants grown in Promix served as a reference. Plants were harvested after 45

days growth and dried at 70 °C for 3 days. Replicates of each treatment were mixed and sent to ALS for sodium and chloride analysis. Only shoot samples were analyzed.

MPPS. Land farming has been performed regularly at both field sites for many years. Prior to planting, land was tilled for 1 – 2 weeks, with a minimum of 3 times per week prior to planting seeds ± PGPR. Tall fescue (*Festuca arundinacea*) and annual ryegrass (*Lolium multiflorum*) (Ontario Seed, Waterloo, ON, Canada) were planted together for phytoremediation in field experiments. Seed planting density (300 seeds/m^{-2}) was approximately two times the normal density. Plants were allowed to grow for the entire plant growth season.

Extraction and analysis of TPH. TPH levels in field soils were determined by assaying for total hydrocarbons. Soil samples were collected and stored at 4ºC until analysis. Samples were air dried at room temperature in the dark. 4 g of soil was extracted by ultrasonication into 20 ml of hexane/acetone (1:1 v:v) (U.S. EPA, 1998). Extracts were dried by completely evaporating the solvent under a stream of nitrogen gas. The amount of extracted sludge was then determined gravimetrically. To determine levels of CCME fractions 3 and 4, part of each soil sample was sent to Maxim Laboratories (Waterloo, ON, Canada) for analysis.

RESULTS AND DISCUSSION

Field remediation of TPH-impacted sites in Sarnia, ON and Turner Valley, AB. One plot at the Sarnia, ON site was planted over a three year period with ryegrass and tall fescue treated with PGPR. Plant growth and remediation were excellent. Based on fraction analysis, more than 50% remediation has been realized (Fig. 1A). The site in Turner Valley, AB was planted for a 2nd year with ryegrass and tall fescue. We performed ± PGPR treatments. Once again a positive PGPR effect on plant growth was observed. Similar to Year 1, good remediation was observed with 9% remediation after 2 months, and 30 % remediation in 3.5 months of plant growth (Fig. 1B).

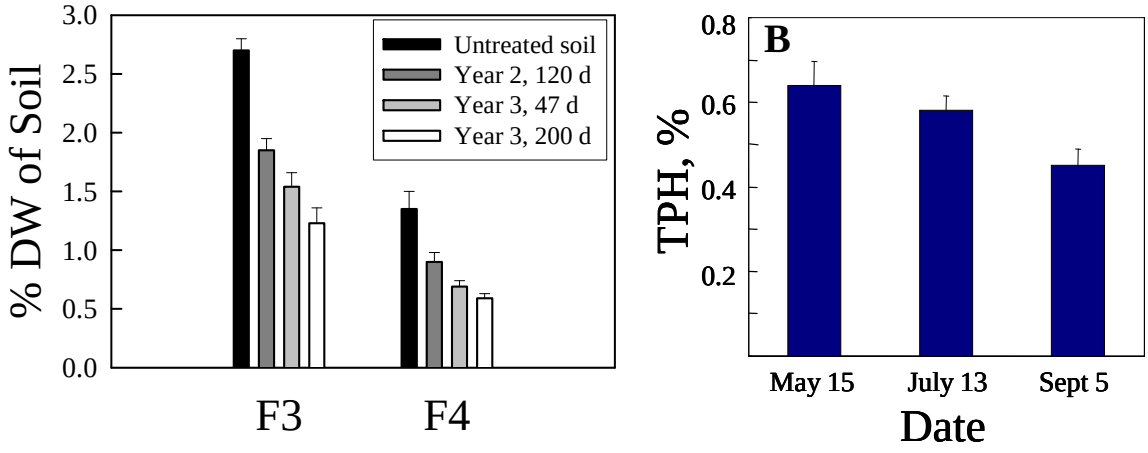

FIGURE 1. TPH removal from field soils using a mix of PGPR-treated ryegrass and tall fescue. Panel A: Sarnia, ON, field site. This plot was remediated for 3 consecutive years. Panel B: Turner Valley, AB, field site. This was the second year of remediation at this part of the site.

Greenhouse experiments using salt-impacted soils from AB and SK. Oats, barley, ryegrass, slender wheatgrass, tall fescue, red fescue and Fall rye all grew on the salt impacted soils in the greenhouse, with positive PGPR effects on plant growth in each case (Fig. 2). The strongest effects were observed for oats and Fall rye. In general, due to PGPR treatment we are observing at least a 100 % increase in plant germination and total plant biomass in salt impacted soils (data not shown). For this work we have tested 7 PGPRs to date. The naturally occurring strains of PGPR that we have used for petroleum remediation (UW3 and UW4) worked well on salt impacted soil. We have also isolated 5 native PGPR strains from salt impacted sites in AB and SK. These strains have the ability to consume the ethylene precursor (ACC), and promote plant growth under stress conditions. The identities of each of these PGPRs are currently being refined via 16S rRNA gene sequence analyses.

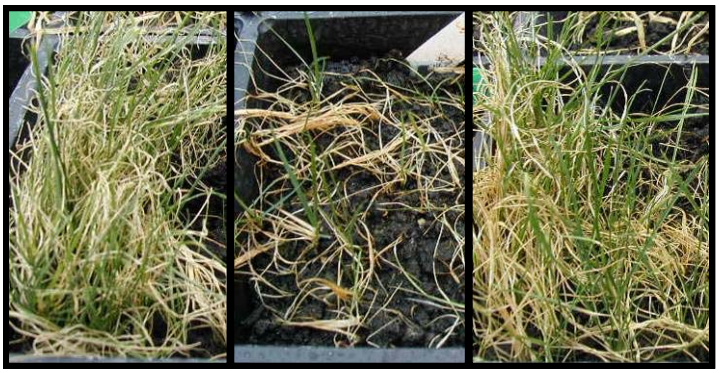

FIGURE 2. Ryegrass ± PGPR grown in salt impacted (SAR, 11 – 15) clay-loam soil from Fenn-Big Valley, AB.

We have performed preliminary assays of salt remediation, with very promising results. On a dry weight basis, NaCl uptake was ~ 80 g/kg DW in oat plants and ~ 60 g/kg DW in barley plants treated with PGPR (data not shown). In both cases, uptake of NaCl was greater in PGPR treated plants than in untreated plants. This implies that the more vigorous plant growth due to PGPR treatments resulted in greater salt assimilation. Based on our encouraging results, we commenced a field trial at a field site in Carlyle, SK in 2006. Thus far we have observed good plant growth showing that plants can grow on different parts of this site that vary in salt levels.

CONCLUSIONS

Remediation of petroleum and salt impacted land using single process techniques has met with limited success due, in large part, to slow rates of remediation. In the case of phytoremediation it is often difficult or impossible to establish plant growth in contaminated soils, which are often nutrient poor as well as toxic. Stress ethylene is produced under these conditions, frequently leading to diminished plant growth. A key feature of our MPPS is the alleviation of plant stress via PGPR that can decrease the levels of stress ethylene. This results in more vigorous plant growth in contaminated soils and leads to more rapid rates of remediation. Three consecutive years of field trials at a TPH impacted site in ON, and two consecutive years at a TPH impacted site in AB showed that remediation continues to occur with successive plantings of ryegrass/tall fescue. In both studies, a positive PGPR effect was observed, highlighting the importance

of the plant/PGPR interactions. Application of the MPPS in greenhouse experiments greatly improved plant performance in salt impacted soils, and improved salt uptake in these plants relative to plants without PGPR. Thus, we should be able to use the MPPS in the field to remediate salt impacted soils.

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