

Role of rhizosphere microorganisms in phytoremediation of biphenyl in a contaminated groundwater plume

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Abstract:

The microbial degradation of biphenyl in a pilot phytoremediation project, executed by Malroz Engineering Inc, Kingston, Ontario was studied. Aerobic biphenyl degraders were enriched at $23\pm 1^\circ\text{C}$ from the rhizosphere soil obtained from the study site. Four enriched cultures degraded 2000 ppm biphenyl in two weeks to a final biphenyl concentration of 0.4 ppm. However, at 8°C , only the two cultures enriched from an engineered soil showed significant biphenyl degradation. The two major members of the consortium were identified as *Burkholderia xenovorans* LB400 and another strain of *Burkholderia xenovorans*.

Anaerobic biphenyl degraders, enriched from the willow and poplar rhizospheres, degraded biphenyl more slowly when nitrate, sulphate or CO_2 were provided as the terminal electron acceptor (TEA). One enriched culture under sulphate-reducing conditions showed complete degradation of biphenyl. From GC/MS analysis, no significant metabolic intermediates were detected at concentrations of concern. Although sulphate was provided as the sole TEA, biphenyl degradation was not directly linked to its consumption but the rate of degradation was improved.

SO_4^{2-} or HCO_3^- (as TEA), poplar root exudate, and increasing fertilizer concentration enhanced biphenyl mineralization when rhizosphere soil was used as the inoculum. Based on a factorial design and analysis, the nitrogen source in a defined medium had the most significant effect on biphenyl degradation, and is consistent with the results obtained from an enriched culture when biphenyl disappearance was monitored.

Introduction:

Biphenyl is an aromatic organic chemical that occurs naturally in the environment in trace amounts, and has been produced industrially for a number of applications. In Canada, its production as a by-product in the hydroalkylation of toluene to benzene is a major industrial source (Health Canada, 2005). It is also used as a dye carrier in the textile industry and as a heat transfer agent. In the past, biphenyl has been used as an intermediate in the production of polychlorinated biphenyls (PCBs), and as a fungicide treatment for citrus fruit packaging. However, these uses have since been prohibited in Canada (Health Canada, 2005).

Biphenyl is a health and environmental risk. It is an irritant to the eyes, the respiratory system and skin (Pohanish, 2002). Long term exposure may cause damage to the nervous system and liver. Biphenyl is highly toxic to aquatic life and may cause long term adverse effects in aquatic environments. It is fairly persistent in the environment partly because of its low water solubility, and consequently its poor accessibility to microorganisms. In Ontario, the standard for non-potable groundwater is $4.3\ \mu\text{g/g}$ in soil and $1700\ \mu\text{g/L}$ in

groundwater, and for potable groundwater 0.89 µg/g in soil and 350 µg/L in ground water (Environmental Protection Act, 2004).

An industrial site contaminated with biphenyl is currently remediated by pump and treat. Since it can be very expensive in the long term, Malroz Engineering (Kingston, Ontario) propose phytoremediation as an alternate treatment, and rows of poplar and willow trees were planted in the path of groundwater flow. Since biphenyl is very hydrophobic, direct transpiration by the trees is not significant. On the other hand, soil microorganisms can degrade many organic compounds and their population is usually higher in the rhizosphere than in the non-rhizosphere soil. Therefore, biphenyl biodegradation in the rhizosphere might play an important role in its removal from that site.

The aerobic biodegradation of biphenyl has been well studied. Several Gram-negative bacteria were enriched using biphenyl as the sole carbon source in the early 1970s (Lunt and Evans 1970, Catelani et al., 1970). Growth of fungi and Gram-positive bacteria on biphenyl are also reported. The early development of microbial degradation of biphenyl and PCB has been reviewed (Higson, 1992). The aerobic biphenyl degradation pathway is known (<http://umbbd.msi.umn.edu>).

Although the aerobic process is fairly well understood, groundwater is normally in anaerobic. The anaerobic degradation of biphenyl is less well documented. Rockne and Strand (1998) were the first to show clear evidence of anaerobic biphenyl degradation. Using a Fluidized Bed Reactor (FBR), a consortium of polyaromatic hydrocarbon (PAH) degrading bacteria was enriched from coal-tar creosote contaminated sediment containing biphenyl. Biphenyl was degraded under both sulfate and nitrate reducing conditions but the anaerobic degradation rates were much slower than what had been reported for aerobic processes. The sulfate-reducing culture completely degraded approximately 5.5 ppm biphenyl within 20 weeks. The complete degradation of biphenyl was achieved by the nitrate-reducing culture within 15 days.

Further studies by Rockne and Strand (2001) demonstrated the dependence of biphenyl degradation with the terminal electron acceptor (TEA). The biodegradation of biphenyl stopped when nitrate was exhausted, and recommenced when nitrate was re-supplied. The fastest rates observed in this study was complete removal of approximately 3 ppm biphenyl within 10 to 20 days. Studies with ¹⁴C-biphenyl showed about 55% mineralization to carbon dioxide.

Grishchenkov et al. (2001) studied the anaerobic biphenyl degradation by a Gram negative, facultative anaerobe, *Citrobacter freundii*, under nitrate reducing conditions. Approximately 10% of the biphenyl disappearance was due to mineralization, while the remainder was transformed to intermediates.

Methanogens also degraded biphenyl when it was the sole carbon source with 75% degradation within 50 days with the production of methane (Natarjan et al. 1999). Radiolabelled experiments confirmed that the carbon in methane came from biphenyl

with carbon dioxide and methane produced in a ratio of 1:2. The production of methane indicates the significance of methanogens in the biphenyl degrading consortium.

In this study, the microbial degradation of biphenyl by the indigenous microorganisms was investigated at both aerobic and anaerobic conditions but with special interests in anaerobic degradation. The potential biphenyl degraders were enriched and identified. Various factors were studied to enhance anaerobic biphenyl degradation.

Materials and Methods:

Medium Composition

Cells were grown in Basal Salts Medium (BSM) containing (g/L): K_2HPO_4 , 3.24; $NaH_2PO_4 \cdot H_2O$, 1.0; NH_4Cl , 2.0; as well as trace elements including (mg/L): Nitrilotriacetic acid, 15; $MgSO_4 \cdot 7H_2O$, 30; $MnSO_4 \cdot H_2O$, 5.0; $NaCl$, 10; $FeSO_4 \cdot 7H_2O$, 1.0; $CoCl_2 \cdot 6H_2O$, 1.0; $CaCl_2 \cdot 2H_2O$, 1.32; $ZnSO_4 \cdot 7H_2O$, 1.0; $CuSO_4 \cdot 7H_2O$, 0.0874; $AlK(SO_4)_2 \cdot 12H_2O$, 0.10; H_3BO_3 , 0.10; $Na_2MoO_4 \cdot 2H_2O$, 0.10; $NiCl_2 \cdot 6H_2O$, 0.271; $Na_2WO_4 \cdot 2H_2O$, 0.20 (Modified from Gerhardt et al, 1994).

Aerobic biphenyl enrichment

Microbial consortia were enriched from rhizosphere soil of poplar and willow trees planted in the path of a contaminated groundwater plume. 10 g of soil was added to 125 ml Erlenmeyer flasks containing 50 mL BSM and 2 g/L biphenyl as carbon and energy source and incubated at $23 \pm 1^\circ C$ and 200 rpm on an Innova rotary shaker. After biphenyl disappearance was observed, 10 mL was transferred to fresh BSM medium with 1 g/L biphenyl and biphenyl degrading consortia were obtained by serial transfer.

Anaerobic biphenyl enrichment

Microcosms to investigate the anaerobic biphenyl degradation of the rhizosphere microorganisms were prepared using Hungate technique and kept in an anaerobic glove box. Rhizosphere soil was added to BSM with 1 g/L biphenyl as the sole carbon and energy source and 100 mg/L of NO_3^- , SO_4^{2-} , or CO_2 was supplied as terminal electron acceptors (TEA). To provide anaerobic conditions, the microcosm bottles containing BSM medium with biphenyl were sparged with nitrogen gas, then placed in the glove box (Nexus One; Vacuum Atmospheres Company, Hawthorn, Calif.). After about 2 months, the cultures were transferred to fresh medium. The microcosms showing biphenyl degradation were chosen for further studies.

Anaerobic biphenyl mineralization

In the mineralization experiment, soil from the rhizosphere of a willow tree at a depth of 40~60 cm below the surface were used directly as the source of inoculum. Typically, 20 g of soil, 30 mL deionized water, 50 mg/L biphenyl and an initial activity of 100,000 cpm ^{14}C -biphenyl were added to a 100 mL serum bottle then capped with a butyl rubber stopper and an aluminum crimp. A 5-mL test tube containing 1 mL of 1 mol/L KOH was placed in each serum bottle to trap volatile ^{14}C , and the radioactivity was measured with a Beckman LS 6500 Scintillation Counter using the Optiphase "HiSafe 3" liquid scintillation cocktail (Wallac Scintillation Products, Turku, Finland). The CIL Tree and

Hedge Feed fertilizer, which was used at the site to promote the tree growth, was evaluated at concentrations of 0.01, 0.05, 0.1, 0.5 g/g soil. A concentrated BSM stock solution (X10) was added to poplar and willow root exudates obtained from hydroponic cultures, and filter sterilized with a 0.22 μm nitrocellulose membrane (Millipore).

Chemical Analysis

Biphenyl and its degradation intermediates were analyzed using gas chromatography and mass spectrometry (GC/MS) by Testmark (Garson, Ontario). After filtering through a 0.45 μm nitrocellulose membranes (Millipore) or centrifuging at $8000 \times g$ for 2 min to remove biomass and biphenyl crystals, culture samples were extracted with an equal volume of ethyl acetate at both neutral and acidic conditions (pH 2). The organic phase was collected and dried with sodium sulfate and 4 mg/L of 2-fluorobiphenyl was added as an internal standard. The concentration of biphenyl and degradation products were determined with a GC (Varian CP-3800) equipped with a VF-1 MS column (15 m \times 0.25 mm) coupled to a MS (Varian 1200) operated at EI 70 eV. The injector temperature was 250°C and the oven temperature was 70°C for 1 min, with a 20°C ramp to 260°C, and a final hold at 260°C for 1 min. Biphenyl was also analyzed by a UNICAM UV 1, UV/Vis spectrophotometer at a wavelength of 247 nm.

Sulfate was analyzed by the Turbidimetric method (Clesceri et al., 1998) where samples were diluted with water to be in the analytical range for this method (less than 40 ppm). Seven millilitres of diluted sample and 1.4 mL of Buffer A (30 g/L MgCl_2 , 5g/L $\text{CH}_3\text{COONa}\cdot 3\text{H}_2\text{O}$, 1.0 g/L KNO_3 , and 20 mL/L CH_3COOH , 99%) were stirred in a 20 mL glass vial. After a small scoop of barium chloride was added, stirring was continued for 60 sec. The solution was then analyzed spectrophotometrically at 420 nm. Samples were quantified using a calibration curve. Soil samples were filtered prior to analysis to remove turbidity that would interfere with the absorbance.

Microbial identification

The consortium from the poplar rhizosphere was characterised using polymerase chain reaction and denaturing gradient gel electrophoresis (PCR-DGGE) (Spiegelman et al., 2005). Total DNA was extracted using a bacterial DNA extraction kit (E.Z.N.A). The 16S rDNA was amplified using a T1 thermal cycler (Biometra), with an initial 11 cycles of 1 min denaturation at 94°C, 1 min annealing at 65°C stepping down 1 degree each cycle to 55°C, and 3 min extension at 72°C followed by 20 cycles of 1 min at 94°C, 1 min at 55 °C, and 3 min at 72°C. Each 50 μL PCR reaction contained 1 μL of extracted total DNA, 8 μL of 1.25 mM dNTPs, 1 μL of each primer U341-GC2 and U758, 4 μL 25 mM MgCl_2 , 5 μL of 10X reaction buffer and 2.5 units of Taq polymerase (Fisher Scientific Ltd., Canada). The amplified DNA was separated on an 8% (w/v), 0-80% denaturing gradient acrylamide gel in 1X TAE buffer. The gel was run at 80 V for 16 h at 60°C and stained for 10 min in 1X TAE containing SYBR Gold (Invitrogen) (Fortin et al., 2004). To identify the consortium, 16S DNA bands were excised and eluted with water at 4 °C overnight, precipitated with ethanol, re-amplified for 25 cycles of 1 min at 94°C, 1 min at 55 °C and 1 min at 72 °C using U341 and 758 primers, and sequenced (Cortec). The 16S DNA sequences were matched using BLAST.

Results:

Aerobic biphenyl enrichments at room temperature

To enrich for biphenyl degrading microorganisms, four rhizosphere soil samples from various locations at the site were used as inocula, and 2 g/L of biphenyl crystal was added as the primary carbon and energy source. Since the water solubility of biphenyl is low, most of the biphenyl crystals settled to the bottom of the flasks. Sterile Ottawa sand was used as an abiotic control. After one week, the white biphenyl particles disappeared from the four flasks with rhizosphere soil but not from the sand control. The turbidity in the four flasks also increased. A typical time course growth curve after three serial transfers is shown in Fig 1. The optical density at a wavelength of 600 nm increased quickly in the first four days and was almost constant thereafter. The concentration of biphenyl at the end of one week incubation was 0.4 mg/L, indicating that biphenyl was almost completely degraded.

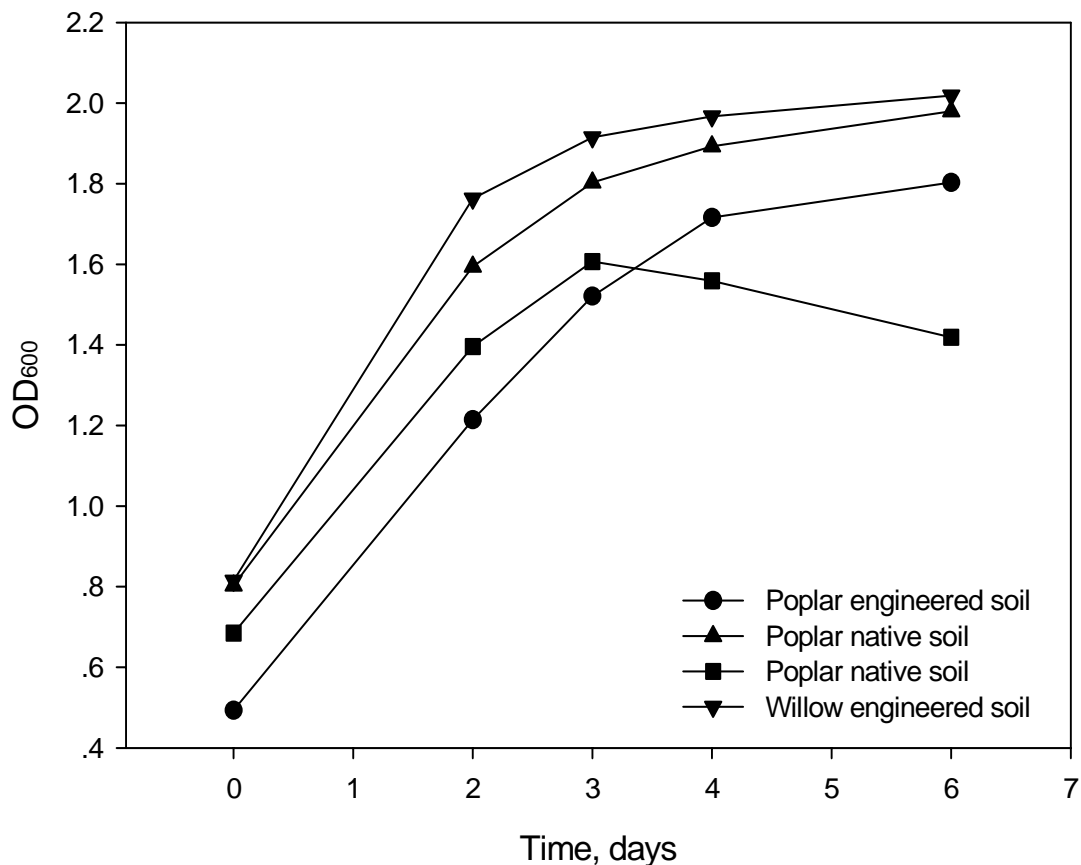


Fig 1: Growth of four aerobic enrichment cultures on biphenyl after the third serial transfer at $23 \pm 1^\circ\text{C}$. Trees were planted either in native soil which had not been modified or in engineered soils which had been modified to a proprietary formulation.

Aerobic biphenyl degradation at groundwater temperature

Since *in situ* biphenyl degradation is of interest, and the groundwater temperature is typically low (8 to 10°C), the four cultures enriched at room temperature were grown as described earlier but at 8°C (Fig. 2). Interestingly, the growth of the two enriched cultures from the engineered soil was similar at both temperatures but the growth of the consortia enriched from native soils was drastically decreased at 8°C. When the microbial population of the four enriched cultures was investigated by PCR-DGGE, four different DGGE patterns were obtained (data not shown). This might explain why the temperature had different effects on biphenyl degradation. In a culture with the fastest biphenyl degradation, two dominant bacteria were present: an exact match with *Burkholderia xenovorans* LB400 and the other was closely related to *Burkholderia xenovorans*. There were also 3-4 other minor members of the consortium.

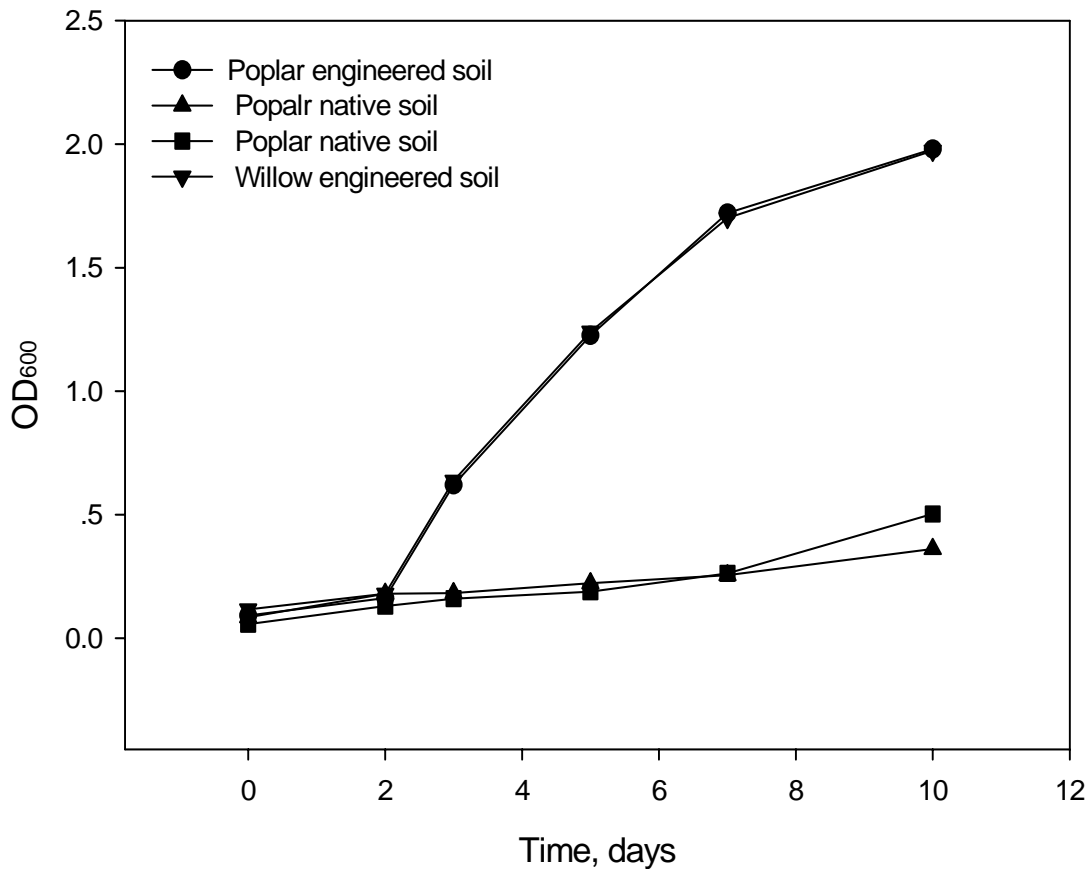


Fig 2: Growth of aerobic biphenyl degraders at 8°C.

Anaerobic biphenyl enrichments

To enrich for anaerobic biphenyl degraders, a total of 48 microcosms were set up with NO_3^- , SO_4^{2-} , or CO_2 as TEA, and inoculated with the rhizosphere soil of poplar or willow

trees planted in either the native or engineered (i.e. amended to a proprietary formulation), at different depths (i.e. shallow ~30cm, or deep ~70cm). Biphenyl was used as the sole carbon and energy source. Abiotic controls were prepared by autoclaving microcosms once a day at 121°C for 20 min over three days. The biotic microcosms were transferred to fresh medium every 2 months. After two transfers, a qualitative assessment of the disappearance of biphenyl showed that anaerobic biphenyl degraders were widely distributed on the site (Table 1). Of the three TEAs provided, nitrate appeared to be used more often by the microorganisms from the shallow depth, and carbon dioxide by the microorganisms in the deeper soil.

Table 1: Anaerobic biphenyl degradation in the third transfer

		NO ₃ ⁻		SO ₄ ²⁻		CO ₂	
		Deep	Shallow	Deep	Shallow	Deep	Shallow
Poplar	Engineered soil	--	++	--	+	--	+
	Native soil	++	+++	+	+++	+++	+++
Willow	Engineered soil	--	+++	+++	+++	+++	--
	Native soil	--	+	+	+++	+++	--
Control	Sterile soil	--		--		--	

(--) 0-25% degradation (+) 25-50% degradation (++) 50-75% degradation (+++) >75% degradation

Eleven microcosms showing the highest biphenyl degradation in Table 1 were transferred to fresh medium with 1 g/L biphenyl for further studies. After two months, two microcosms with the best biphenyl degradation, one using nitrate and the other using sulphate as TEA, were analyzed by GC/MS. The final concentration of biphenyl was 0.65 ppm and 39.6 ppm under sulphate reducing and nitrate reducing conditions respectively, representing 99 and 96% biphenyl degradation. No significant metabolic intermediates were accumulated in either culture. Two metabolites under sulphate reducing conditions, o-hydroxybiphenyl and o-hydroxybiphenyl, are not present in the common aerobic biphenyl degradation pathway indicating that anaerobic biphenyl degraders may have a different pathway.

Anaerobic biphenyl mineralization

Since biphenyl is poorly soluble in water, measuring biphenyl concentration in soil samples can be very difficult. ¹⁴C-biphenyl was used in mineralization experiments and the ¹⁴CO₂ produced was used to monitor biphenyl mineralization. Instead of using an enriched consortium, rhizosphere soil was used as inocula in these experiments to provide an environment similar to the site conditions. The effect of TEAs, fertilizer and root exudates on anaerobic biphenyl degradation was investigated. The mineralization of biphenyl was compared with BSM only and the normalized results are shown in Table 2.

Table 2 Factors affecting anaerobic biphenyl degradation in microcosms containing basal salts medium (BSM), 20 g of willow rhizosphere soil, 50 mg/L biphenyl and 100,000 cpm ^{14}C -biphenyl.

	Biphenyl Mineralization
Water	0.7
BSM only	1.0
Fertilizer	1.8
NO_3^-	0.9
SO_4^{2-}	1.3
CO_2	1.5
Poplar root exudates	1.4
Willow root exudates	1.0

Three TEAs, NO_3^- , SO_4^{2-} and HCO_3^- , were investigated and 100 ppm of each was added to the microcosms. Addition of SO_4^{2-} or HCO_3^- enhanced the extent of anaerobic biphenyl degradation while NO_3^- did not. Since the soil was taken from the deeper location, these results agree with the anaerobic enrichment experiments (Table 1) in which the microorganisms in the willow rhizosphere used SO_4^{2-} or HCO_3^- as TEA but not NO_3^- . The 80% enhancement on biphenyl mineralization was obtained at a fertilizer concentration of 0.5 g/g soil.

Poplar root exudate enhanced biphenyl degradation by about 40%, while willow root exudates did not. Since the soil inocula were obtained from the rhizosphere of willow trees, the microorganisms might be adapted to willow root exudation. Therefore no significant effect was seen.

When BSM was compared with water only, BSM had higher biphenyl mineralization. The components in BSM can be categorized into three groups; nitrogen source, phosphorous source and trace elements. Factorial design was used to investigate the effect of these components in BSM on biphenyl mineralization. In this study, only the nitrogen source had a significant effect on biphenyl mineralization, while phosphate and trace elements did not (data not shown).

Discussion and conclusion:

In this study, biphenyl degrading microorganisms were enriched from poplar and willow rhizosphere soil under both aerobic and anaerobic conditions. The enriched

microorganisms degraded biphenyl at 8°C, and under anaerobic conditions indicating that the *in situ* biodegradation by the indigenous microorganisms at that site could happen naturally. Complete biphenyl degradation occurred under both aerobic and anaerobic conditions with the rhizosphere soil, proving that biodegradation of biphenyl is a potential means of biphenyl removal in phytoremediation.

The addition of TEAs might enhance biphenyl remediation if specific microorganisms are present at the site, and in deeper soils sulphate and carbon dioxide were used. Fertilizer addition had the best stimulatory effect on biphenyl degradation and is probably a better choice because of the lower price. The major component enhancing biphenyl degradation in the BSM medium was the NH₄ -nitrogen source and this could be obtained from an appropriate fertilizer. These results can be exploited to accelerate the *in situ* biphenyl removal.

Root exudates were demonstrated to stimulate microbial growth and enhance biphenyl degradation. Root exudates containing many organic carbon source can promote microbial growth and stimulate contaminant degradation. The interaction between plant and soil microorganism is very important to success of a phytoremediation project.

This research provides the knowledge to enhance removal of biphenyl from the subsurface allowing Malroz to treat the current site with greater efficiency, and the technology can be transferred to other projects with similar site conditions.

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