# Phytoremediation of heavy metal and PAH-contaminated brownfield sites

Roy, Sébastien; Labelle, Suzanne; Mehta, Punita; Mihoc, Anca; Fortin, Nathalie; Masson, Claude; Leblanc, René; Châteauneuf, Guy; Sura, Christine; Gallipeau, Christine; Olsen, Caroline; Delisle, Serge; Labrecque, Michel; Greer, Charles W.



**Publisher's version / Version de l'éditeur:**
https://doi.org/10.1007/s11104-004-5295-9
*Plant and Soil, 272, 1-2, pp. 277-290, 2005-05*

Plant and Soil (2005) 272: 277–290
DOI 10.1007/s11104-004-5295-9


# Phytoremediation of heavy metal and PAH-contaminated brownfield sites

Sébastien Roy[1,2], Suzanne Labelle[1], Punita Mehta[1], Anca Mihoc[1], Nathalie Fortin[1], Claude Masson[1], René Leblanc[3], Guy Châteauneuf[3], Christine Sura[3], Christine Gallipeau[4], Caroline Olsen[5], Serge Delisle[1], Michel Labrecque[4] & Charles W. Greer[1,6]
[1]*Biotechnology Research Institute, National Research Council, Montreal, Qc, Canada H4P 2R2.* [2]*Present address: Institut de Pharmacologie de Sherbrooke, Université de Sherbrooke, Sherbrooke, Qc, Canada J1H 5N4.* [3]*Inspec-Sol Inc., St-Laurent, Qc, Canada H4S 1C7.* [4]*Institut de recherche en biologie végétale, Montréal, Qc, Canada H1X 2B2.* [5]*COREM, Québec, Qc, Canada G1N 1X7.* [6]*Corresponding author**

Received 6 July 2004. Accepted in revised form 24 October 2004

*Key words:* bioremediation, hydrocarbons, Indian mustard, phytoextraction, rhizosphere

## Introduction

Intense industrial activity in the 20th century has been particularly deleterious to our environment, resulting in a large number and variety of contaminated sites. In urban areas, many sites must now be rehabilitated to prevent migration of contaminants or to allow re-development. Inorganic and organic contaminants typically found on these sites are heavy metals and petroleum derived products. The presence of both types of contaminants on the same site presents technical and economic challenges for decontamination strategies.

Heavy metals, with soil residence times of thousands of years, present numerous health dangers to higher organisms (Garbisu and Alkorta, 2001). They are also known to decrease plant growth, ground cover and have a negative impact on soil microflora (McGrath et al., 2001). Polycyclic aromatic hydrocarbons (PAHs) are contaminants generated from many sources such as the combustion of coal and fossil fuels for energy production and are potential carcinogens that can induce mutations. As lipophilic compounds, they present a significant health risk if they enter the food chain (Henner et al., 1997; Reilley et al., 1996). These compounds can be used by soil microorganisms as an energy and carbon source, although four-, five-, and six-ring PAHs are more resistant to biodegradation. Bacteria initiate PAH degradation via dioxygenase attack, increasing PAH chemical reactivity and solubility (Harvey et al., 2002; Reilley et al., 1996).

One *in situ* decontamination approach showing promise for addressing both organic and inorganic contaminants is phytoremediation, a field of study that has grown considerably over the last decade (Glass, 2000). Central to this field are the plants and their specific capabilities in regards to metal accumulation and resistance, as well as their impact on rhizosphere microflora diversity, density and metabolic activity. There are roughly 400 known species of plants characterized as metal hyperaccumulators and about 75% of these accumulate nickel and come from ultramafic ("serpentine") soils (McIntyre and Glennis, 1997). By definition, hyperaccumulators used in continuous phytoextraction are capable of accumulating over 1000 mg/kg of dry-weight plant of a specific heavy metal. The main limitations of hyperaccumulator species are their low-biomass and specific growth needs (Gleba et al., 1999). An alternative approach is that of induced phytoextraction. High-biomass plants are allowed to grow to a substantial size, then a metal

* FAX No: +1-514-496-6265. E-mail: charles.greer@nrc-cnrc. gc.ca.

chelating compound such as EDTA is applied to the soil, rendering soil metals more available (hydrosoluble) to the plant. There ensues an intense period of metal absorption, which may result in plant death. The overall efficiency in metal extraction is thus a function of plant biomass and metal concentration in plant tissues. This degree of efficiency will determine the number of sequential harvests needed to extract the mass of metal necessary to reach target (i.e., regulatory) levels and in turn, the number of harvests will determine the total cost of the operation, including biomass disposal through landfill, incineration, or composting (Ensley, 2000; Salt et al., 1998).

PAH degradation enhancement by plants was also investigated since it is known that plant exudates are a significant source of organic nutrients and inorganic compounds. In terms of energy, the amount of exudates can be equivalent to 10–20% of the plant's annual photosynthesis (Schnoor et al., 1995). It is widely recognized that these compounds improve the nutrient status of soils and produce a rich microenvironment capable of promoting microbial proliferation, and improving xenobiotic-degrading capabilities of rhizosphere microorganisms (Harvey et al., 2002; Liste and Alexander, 2000; Reilley et al., 1996; Salt et al., 1998; Schnoor et al., 1995; Siciliano and Germida, 1999; Wiltse et al., 1998).

In the work presented here, we addressed the particular context of an alkaline soil contaminated with heavy metals and PAHs. The objective of this treatability study was to assess heavy metal phytoextraction performance from alkaline soils and the impact of the selected plants on rhizosphere microbial communities. These experiments were conducted toward the end of a 3-year project that included greenhouse trials, *in situ* trials, and an economic feasability study. The screening of effective plant species was performed prior to this study. Out of the 10 plant species screened, higher-biomass species were chosen, such as *Brassica juncea*, *Salix viminalis*, and *Festuca arundinacea* (Indian mustard, willow and fescue, respectively). These species were chosen because of their ability to extract heavy-metals and to grow on the tested soils. Of the selected species, none were hyperaccumulators. The willow was also chosen for its ability to grow from a stump, its higher hydraulic pumping pressure, and greater depth of prospection (Glass, 2000). Other advantages offered by these woody plants are soil aeration and fertilization (Kopponen et al., 2001).

## Materials and methods

### *Experimental design*

The soil used in this experiment was excavated from the top metre of a brownfield site in Montréal (Québec, Canada) contaminated with heavy metals and low levels of PAHs (Table 1). This site was a rail marshalling yard used throughout most of the 20th century. These alkaline soils (pH 8–8.5) were excavated during fall 2001, mixed and sieved to 5 mm, then amended with commercial creosote (7 mL/kg). This resulted in a PAH contamination level of approximately 154 mg/kg. This soil was allowed to age, covered, outdoors over the winter. In May, before the greenhouse trial, the soil was mixed again and

*Table 1.* Initial chemical characterization of soil

| Contaminant | Total (mg/kg) | Bioavailable Exchangeable | Carbonates | Oxides | Others |
|---|---|---|---|---|---|
| Copper (Cu)[a] | 1760 | 1.40 | 182 | 254 | 1320 |
| Lead (Pb)[a] | 6840 | 1.03 | 1500 | 1520 | 3820 |
| Zinc (Zn)[a] | 3560 | 3.12 | 870 | 1400 | 1290 |
| Petroleum hydrocarbons ($C_{10}$–$C_{50}$)[b] | 1030 | N/A | N/A | N/A | N/A |
| PAHs | 154 | N/A | N/A | N/A | N/A |

[a]Average results for three samples analyzed prior to adding creosote to soil.
[b]Average results for nine samples taken at T0 before potting, after creosote addition and aging.

*Table 2.* Experimental design and sampling plan during the trial

| Sampling time plant: weeks of growth at harvest | Chemical analyses (plant tissues and/or soil) | Microbiological analyses |
|---|---|---|
| Prior to EDTA amendment (Wk 0)<br>Indian mustard: 10, willow: 14, fescue: 17 | Six replicate plants sampled and analyzed (Wk 0, w/o EDTA)<br>No soil analysis | Three replicate plants sampled and analyzed (Wk 0, w/o EDTA) |
| One week (Wk 1) after EDTA amendment<br>Indian mustard: 11, willow: 15, fescue: 18 | Three replicate plants sampled and analyzed (Wk 1, w EDTA)<br>No soil analysis | Three replicate plants sampled and analyzed (Wk 1, w EDTA) |
| Two weeks (Wk 2) after EDTA amendment<br>Indian mustard: 12[a], willow: 16, fescue: 19[1] | Three replicate plants of two treatments sampled and analyzed (Wk 2, w and w/o EDTA)<br>Soil analyses conducted | Three replicate plants of two treatments sampled and analyzed (Wk 2, w and w/o EDTA) |

[a]On these two dates, three control (i.e., unplanted and untreated) pots were sampled and their soils were analyzed.

nine samples were taken for hydrocarbons ($C_{10}$–$C_{50}$) and PAH analysis, which were performed throughout the study by Maxxam Analytique Inc. (Montreal, QC) using GC-FID and GC-MS. Three (3) soil samples were also analyzed for Cu, Pb and Zn prior to adding creosote (Table 1). Simultaneously, the speciation of these metals was estimated using the Tessier sequential extraction method (Tessier et al., 1979). Five fractions were obtained that included exchangeable (fraction 1), bound to carbonate (fraction 2), bound to iron and manganese oxides (fraction 3), bound to organic matter (fraction 4) and residual (fraction 5). The exchangeable and bound to carbonate fractions were considered bioavailable for the purposes of this study. Metal analyses were performed at COREM using ICP-MS (inductively coupled plasma-mass spectrometry) as described subsequently.

The pot trial was conducted by planting five seeds per pot of fescue (*Festuca arundinacea Schreb.*), eight seeds per pot of Indian mustard (*Brassica juncea* (L.) Czern.), and 1 cutting per pot of willow (*Salix viminalis* L.) in 20, 25, and 30 cm plastic pots containing approximately 3.2, 7.3, and 14 kg contaminated soil, respectively. To reduce PAH volatilization and retain soil humidity, activated charcoal granules were applied to the surface of the soil in all pots. Moderate watering was performed 2–3 times a week, as needed. Since root systems were needed both for microbiological, as well as tissue analyses, a double series of pots were used. For each plant species, treatments, samplings and replicates were as shown in Table 2. Parallel to these pots with plants, "control pots" without plants, and containing only contaminated soil (20 cm pots) were also regularly watered, and none of these were treated with $H_4$EDTA. The pots were arranged randomly in the greenhouse and the experiment was conducted between 15 May and 25 September 2002.

### *Nutrient amendments*

In these trials, minimal nutrient amendments were made. Based on a C:N ratio of 100:10, 350 mg/kg N delivered as ammonium nitrate was added at the beginning of the experiment (Roy, 2000). Other nutrients used during the experiment were in response to leaf chlorosis on the willows. Following leaf tissue analysis it was determined that the symptoms were likely due to P deficiency. During week 9, 5 weeks before the first willow harvest, pots with willows received 750 mL ferrous sulphate (2 g/L) to readjust pH temporarily and allow better P availability. The following day, 500 mL of 250 mg/L P solution (Plant Prod® 20:20:20 fertilizer:N as nitrate, ammonium and urea; P as phosphoric acid; K as soluble potash) was added, as well as foliar nutrients (3.5 g 5.8% chelated Mg, 1.25 g 13% chelated Mn, and 3.25 g 7% EDTA-chelated iron in 4 L water) (Plant Prod®).

*Chelating agent used*

In an initial experiment (results not shown), the relative efficiency of metal extraction from soil using disodium dihydrate EDTA ($Na_2EDTA{\cdot}2H_2O$), and the free acid of EDTA ($H_4EDTA$) were compared. We found that in our alkaline test soil (pH 8–8.5), $H_4EDTA$ (subsequently referred to as EDTA) was more efficient and thus used at a concentration of 5 mmol/kg (dry mass) soil, based in part on work from Blaylock et al. (1997). In treated pots, the chelating agent was thoroughly mixed with the first 3 cm of soil, followed by watering.

*Heavy metal analysis of plant material*

Plants for chemical analyses were harvested by separating the aerial (stem, leaves and flowers) and root system, and these two parts were analyzed separately. The fresh weight of the aerial material was determined, then it was dried 48 h at 70 °C to obtain the dry mass, which was analyzed for heavy metals. Soil was removed from roots by rinsing under tap water, using a stainless steel sieve (2 mm) to collect rootlets. Roots were then blotted to obtain an approximate fresh weight, then dried and processed as described for the aerial tissue. Heavy metals in dry plant tissues were solubilized using an oxidation step with hydrogen peroxide and nitric acid. Dried plant material (500 mg) was placed in a 75 mL graduated flask, 4 mL of hydrogen peroxide was added, the flask was covered with a glass dish and heated at 150 °C for 30 min with occasional mixing on a vortex. Then 7.5 mL of concentrated nitric acid was slowly added and heating was continued for an additional 30 min. An additional 3.7 mL nitric acid was added and heating was continued for 1 h (final concentration of nitric acid was 15%). The flask was cooled for 15–20 min and brought to 75 mL using ultrapure demineralized water. Metal analysis and quantification were conducted using inductively coupled plasma-atomic emission spectrometry (ICP-AES).

*Microbial enumerations*

Microbial counts were carried out in 96-well plates using the MPN method (Haines et al., 1996; Wrenn and Venosa, 1996). Phenanthrene-degrading microbial counts were carried out in Bushnell-Haas mineral broth (Difco, Detroit, MI), supplemented (following inoculation and dilution steps) with 50 mg/kg phenanthrene delivered as 4 $\mu$L/well of 2.5 mg/mL stock in ethanol. Naphthalene-degrading microorganism counts were carried out in Bushnell-Haas mineral broth, with naphthalene as the sole carbon source delivered in the gaseous phase, using a saturated chamber (Roy et al., 2003). MPN counts were incubated 14 days at room temperature, then 25 $\mu$L *p*-iodonitrotetrazolium violet (INT, Sigma, St. Louis, MS) and 25 $\mu$L sodium succinate (1 *M* in 1 *M* PBS, pH 7.2, filter sterilized) was added to each well. INT (filter sterilized) was prepared by dissolving 0.3 g INT in 10 mL methanol, then adding 40 mL $H_2O$. Succinate was used to accelerate and enhance INT-based coloration (Gribbon and Barer, 1995). Positive wells were visually scored after a 24-h incubation at room temperature and MPN was calculated using the program developed by Klee (1993).

Microbial extractions performed on bulk soil samples (from plantless control pots) were carried out by extracting 5 g (fresh weight) of soil with 15 mL extraction buffer (0.1% w/v $Na_4P_2O_7 \cdot 10H_2O$ in water, pH 7.0) in a $25 \times 150$ mm sterile screw cap culture tube containing approximately 2.5 g glass beads (3 mm). The tube was vortexed at high speed for 2 min and allowed to settle for 30 s. Total rhizosphere extractions were performed on roots by blender extraction using a ratio of 9:1 extraction buffer to root sample ratio (v/w). The samples consisted of the entire root system and closely associated soil. Minimum and maximum volumes of buffer used were 50–450 mL, and the maximum weight of sample that could be processed was 50 g. Samples were vortexed at high speed for 1 min and allowed to settle for 1 min. These extracts were used to inoculate MPN plates and the remainder was stored at −80 °C for molecular characterization. All MPN results were calculated on a CFU/g basis.

*Total community DNA extraction and DGGE analysis*

Total bacterial community DNA was extracted from 13 samples; three soils and 10 soil

or rhizosphere (roots and soils) extracts. These extracts had been generated for MPN purposes using sodium pyrophosphate buffer as described previously. The extracts were centrifuged at 10,000 × *g* in order to recover the totality of the bacteria and solids present in the liquid sample, and supernatants were discarded. DNA extractions were performed on 0.5 g of all thirteen samples according to the method described by Fortin et al. (2004). Samples were washed twice with buffer 1 (Tris, NaCl, EDTA, Triton buffer), to remove the high concentrations of heavy metals present in the samples. In the chemical lysis method, ammonium acetate was used to precipitate proteins and humic acids. The microbial diversity of bulk soils and rhizosphere was characterized by PCR amplification of the 16S rDNA gene and denaturing gradient gel electrophoresis (DGGE) as described by Fortin et al. (2004). The amount of 16S rDNA loaded on the gel was 700 and 500 ng for lanes 1–5 and 6–13, respectively (Figure 6). Sequencing of the DGGE fragments was performed as described in Lawrence et al. (2004). Sequences were assigned GenBank accession numbers AY649333 to AY649358, which correspond sequentially to bands a to *z*.

*Mineralization assay*

Microbial PAH degradation capabilities were determined using mineralization experiments with $^{14}$C-labeled PAHs. One (1) gram soil samples were spiked with a methanol solution containing either $^{14}$C-naphthalene or $^{14}$C-phenanthrene (to provide a final concentration of substrate of 10 mg/kg and 10 000 dpm), in 25-mL serum bottles sealed with teflon/rubber septa. These bottles contained 0.5 mL of 1.0 N KOH in tube ($CO_2$ trap), which was sampled and replaced on a regular basis. The quantitation of $^{14}CO_2$ removed by the trap was achieved using a Canberra–Packard scintillation counter. No nutrients or water were added to the tested soils, and all analyses were conducted in triplicate. The soils used in these trials were all collected at the time of final harvest of each plant species (see Table 2). Harvested root systems were shaken to remove bulk soil, and rhizosphere soils were collected by shaking off the remaining, closely associated soil, over a surface-sterilized stainless steel pan. In the case of Indian mustard and fescue, control (i.e. unplanted) soils were also sampled on the same dates.

*Statistical analyses*

The statistical significance of differences between treatments and plants was verified using the *t*-test for independent samples (Sokal and Rohlf, 1995).

## Results

*Phytoextraction of heavy metals*

The difference in tissue-specific metal accumulation for the Indian mustard is shown in Figure 1a. In the absence of EDTA treatment (Wk 2, w/o [EDTA]), the aerial plant parts (stems, leaves and flowers) accumulated Zn to a greater extent than Pb and Cu ($P = 0.01$). A hyperaccumulation of metals was observed after EDTA amendment. The root system contained the vast majority of accumulated Pb. More importantly, there was an almost 4-fold ($P = 0.10$) and 5-fold ($P = 0.25$) increase in total metals accumulated in aerial, and root tissues, respectively, when plants were harvested 2 weeks (Wk 2, W [EDTA]) rather than one week (Wk 1, W [EDTA]) following the EDTA treatment.

Little or no accumulation of Cu or Pb was observed in the willow aerial tissues, and EDTA had no significant impact on the overall concentration of heavy metals accumulated in either aerial or root tissues (Figure 1b). In fescue (Figure 1c), the concentration of Zn and Pb accumulated in aerial tissue was increased 5-fold with EDTA (Wk 1 w [EDTA] treatment, $P = 0.02$) and root tissues seemed to accumulate metals non-specifically. Two weeks after EDTA addition (Wk 2 w [EDTA]), root metal concentrations (mainly Zn) also increased. The metal concentrations in the aerial tissue one and 2 weeks after EDTA amendment did not differ significantly, indicating that it would not be necessary to wait for 2 weeks before harvesting the aerial parts of this species.

The ratios of the metal concentrations in aerial and root tissues are presented in Figure 2a–c. These translocation factors quantify the degree of heavy metal translocation to aerial parts as a function of treatment. In Figure 2a–c the relative

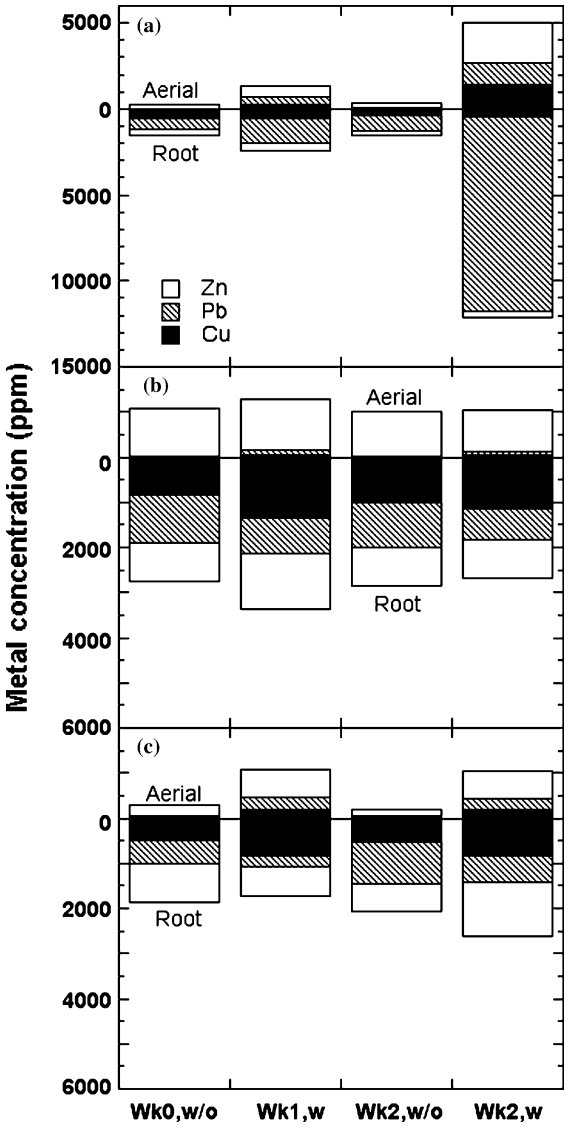


*Figure 1.* Heavy metal accumulation in *B. juncea* (a), *S. viminalis* (b), and *F. arundinacea* (c) aerial and root tissues. *B. juncea*, *S. viminalis* and *F. arundinacea* designate Indian mustard, willow, and fescue, respectively. Plants were harvested at week 0 (Wk 0), at 1 week (Wk 1) and 2 weeks (Wk 2) without treatment (w/o) or following the addition of EDTA (w).

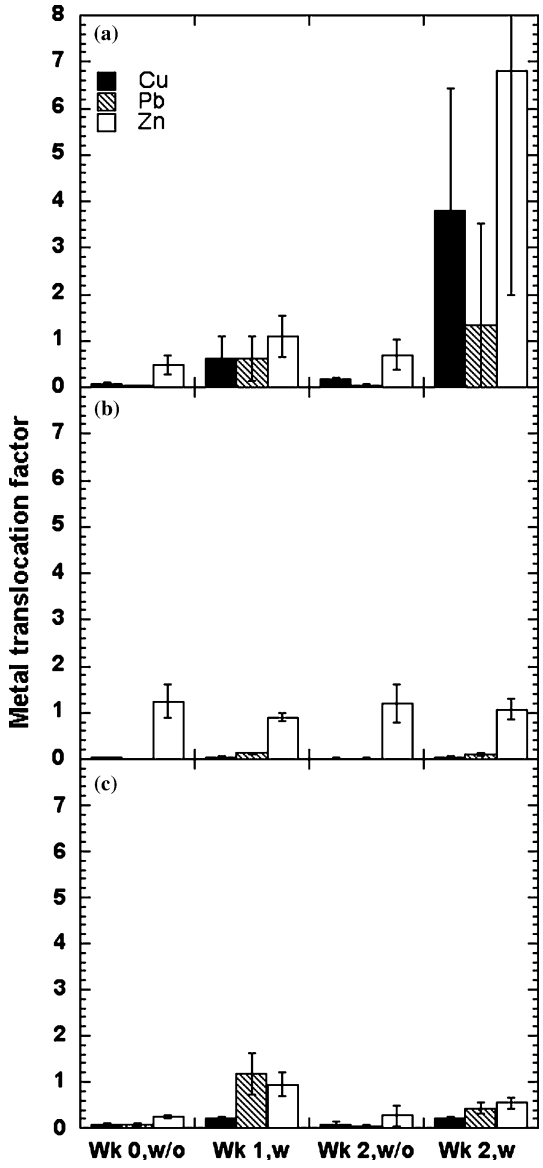


*Figure 2.* Heavy metal translocation factors (mg/kg in aerial tissue/mg/kg in roots) for *B. juncea* (a), *S. viminalis* (b), and *F. arundinacea* (c). *B. juncea*, *S. viminalis* and *F. arundinacea* designate Indian mustard, willow, and fescue, respectively. Plants were harvested at week 0 (Wk 0), at 1 week (Wk 1) and 2 weeks (Wk 2) without treatment (w/o) or following the addition of EDTA (w).

increase in these ratios ($y$-axes) is larger for Indian mustard than for willow or fescue. This indicates that the presence of EDTA has a greater influence over translocation of these heavy metals in Indian mustard than in the two other plant species. Furthermore, the ratio of heavy metals in the Indian mustard aerial tissue increased to an even more significant degree (13-fold) if harvested 2 weeks after EDTA amendment (Figure 2a, Wk 2, w [EDTA]), and translocation to aerial tissues is improved to a greater degree for Zn and Cu than for Pb. The overall influence of EDTA is negligible for the willow (Figure 2b), however in the case of fescue (Figure 2c), there is a slight effect of EDTA

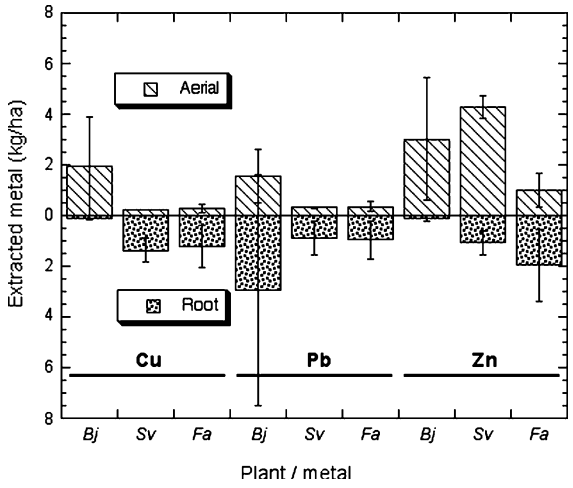


*Figure 3*. Projected heavy metal extraction at first harvest by each plant species with optimal treatments for each; *B. juncea* treated with EDTA and harvested two weeks later, *S. viminalis* without EDTA treatment, and *F. arundinacea* treated with EDTA and harvested two weeks later. *Bj*, *Sv*, and *Fa* designate *B. juncea* (Indian mustard), *S. viminalis* (willow), and *F. arundinacea* (fescue), respectively.

for Zn, Pb, and Cu. Finally, in fescue, the increase was greater when plants were harvested 1 week after amendment, as opposed to 2 weeks after amendment. This was the result of a greater increase in metal concentrations in root tissues compared to aerial tissues, when plants were exposed to EDTA for 2 weeks (Figure 1c, Wk 2, w [EDTA]).

From these phytoextraction results, it is possible to extrapolate the quantity of metal that could be extracted per hectare. Figure 3 illustrates these results, which were calculated from the optimal treatments for each plant species, as determined above, and from planting densities, which were determined in a field trial to be 90,000, 20,000, and 270,000 plants/ha for Indian mustard, willow, and fescue, respectively. If only the aerial part of the plants are harvested, the willow would yield a lower Cu, Pb, and Zn extraction than Indian mustard, however it could extract more Zn than fescue (1.7 kg per ha) (Figure 3). Furthermore, Zn extraction for the first harvest at least, could be accomplished without the need for EDTA treatment. Indian mustard was the most effective for Pb extraction although most of the metal remained in the roots. In the case of the fescue, since harvesting of the total plant would be considered, a total of 1.5 kg per ha of Cu could be extracted, and thus could be used in conjunction with Indian mustard (capable of extracting 1.9 kg Cu per ha) on a Cu-contaminated site. Furthermore, fescue's total Zn extraction capacity with a total plant harvest is comparable to that of the aerial parts of Indian mustard (2.9 kg Zn per ha versus 3.0 kg Zn per ha, respectively). At the time of final harvest for the three plant species, total dry biomass for willows was highest, followed by Indian mustard, and then fescue (60–150, 17–25, 5–25 g per plant, respectively). When taking into account the planting densities given above, harvests of aerial parts over one hectare would generate approximately 1.4, 1.7, and 1.7 tons dry plant material for disposal of Indian mustard, willow, and fescue, respectively.

### *Microbial PAH-degrading activity and residual hydrocarbon contamination*

Phenanthrene-degrading activity was superior to naphthalene with all plant species (Figure 4). The mineralization of naphthalene and phenanthrene in plant rhizosphere soil and in the bulk soil (without plants) did not differ significantly (data not shown). Also, the ability of rhizosphere microflora to degrade PAHs did not seem to be affected by EDTA treatments. Soil samples were analyzed for total hydrocarbons and PAH content, at the beginning of the experiment and following the final harvest of each plant species. In Table 3, results show that the degradation of trace levels of alkane hydrocarbons occurred with or without plants. Similarly, it was shown that there was no significant difference ($P = 0.05$) in PAH degradation in the presence or absence of plants. This was the case if the sum of all PAHs was taken into account. Table 4 shows individual PAH concentration differences in bulk soils from pots with plants and in soils without plants. Taking into account the variability in results for replicate plants and soils, there was no statistically significant ($P = 0.05$) difference between any treatment or plant.

### *Soil and rhizosphere microbial populations*

At the begining and end of the experiment, microbial populations in bulk soil and/or in plant rhizosphere were monitored. MPN analyses were

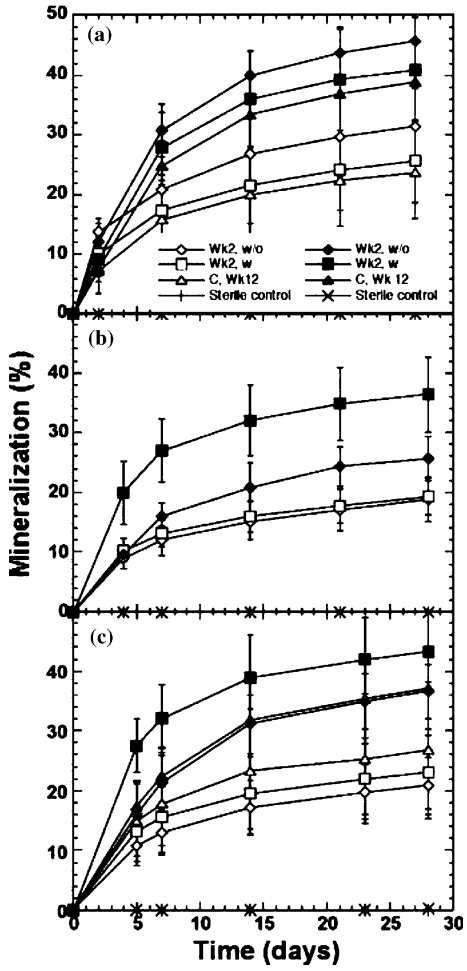


*Figure 4*. Naphthalene (open symbols) and phenanthrene (closed symbols) mineralization capabilities of rhizosphere microflora of *B. juncea* (a), *S. viminalis* (b), and *F. arundinacea* (c). Soils tested were from pots at week 0 (Wk 0), at 1 week (Wk 1) and 2 weeks (Wk 2) without treatment (w/o) or following the addition of EDTA (w), and control soils (untreated, unplanted) from Wk 12 (C, Wk 12).

used to enumerate three functional populations: total heterotrophs, naphthalene- and phenanthrene-degrading microorganisms. Rhizosphere counts were similar for all three plants. Total microbial numbers in the fescue rhizosphere remained high ($5 \times 10^7$ to $1 \times 10^8$ CFU/g) throughout the experiment and microbial population density did not seem to be influenced by treatment with EDTA (Figure 5). Naphthalene and phenanthrene-degrading microorganisms were of the same order of magnitude and typically comprised 2–10% of the total heterotrophic population.

DGGE analysis of selected samples was also undertaken to determine the microbial community diversity in contaminated bulk soils and plant rhizospheres. The 13 samples chosen were: two bulk soil samples taken at the begining of the experiment (T0), two samples taken at the end of the experiment in control pots without plants, one bulk soil sample from a pot of Indian mustard (final harvest time week 12, without EDTA), two rhizosphere extracts from pots of Indian mustard (final harvest time week 12, without EDTA), two rhizosphere extracts from pots of Indian mustard (final harvest time week 12, with EDTA), two rhizosphere extracts from pots of willow (final harvest time week 16, without EDTA), and two rhizosphere extracts from pots of fescue (final harvest time week 19, without EDTA). These samples were chosen for further characterization based on PAH mineralization results. Significant differences were observed between the DGGE banding profiles of the T0 bulk soils, the potted soils, and rhizospheres (Figure 6). Immediately after the addition of creosote (T0), the bulk soil microbial community showed very little diversity (lanes 1 and 2). Its composition consisted of species related to uncul-

*Table 3*. Hydrocarbon concentrations prior to, and following the trial (mg/kg)

| | *Sv* w/o | *Sv* w | *Bj* w/o | *Bj* w | *Fa* w/o | *Fa* w | *C* wk 12 | *C* wk 19 | T0* |
|---|---|---|---|---|---|---|---|---|---|
| $C_{10}$–$C_{50}$ (mg/kg) | N.D. | 33.3 | 305.0 | 320.0 | 253.3 | 280.0 | 453.3 | 246.7 | 1027.8 |
| SD(mg/kg) | N.D. | 57.7 | 117.6 | 60.8 | 5.8 | 85.4 | 28.9 | 37.9 | 71.7 |

*Bj*, *Sv*, and *Fa* designate *B. juncea* (Indian mustard), *S. viminalis* (willow), and *F. arundinacea* (fescue), respectively.
*C* designates control soils (pots without plants).
w and w/o designate with and without EDTA amendment, respectively.
T0*: These values represent the average $C_{10}$–$C_{50}$ levels at the begining of the experiment of nine samples taken, as opposed to triplicate samples for all other values presented.
SD designates the standard deviation.

*Table 4*. PAH concentrations prior to, and following the trial (mg/kg)

| Compound | *Sv* w/o | *Sv* w | *Bj* w/o | *Bj* w | *Fa* w/o | *Fa* w | *C* wk12 | *C* wk19 | T0* |
|---|---|---|---|---|---|---|---|---|---|
| Anthracene | 0.7 | 1.4 | 1.2 | 0.3 | 0.8 | 0.8 | 0.4 | 0.5 | 1.4 |
| Benzo(a)anthracene | 2.3 | 4.3 | 4.3 | 0.0 | 0.0 | 0.0 | 0.0 | 0.1 | 0.1 |
| Benzo(a)pyrene | 2.5 | 4.0 | 5.9 | 0.0 | 0.1 | 0.0 | 0.0 | 0.1 | 0.0 |
| Benzo(b + j + k)fluoranthene | 6.1 | 9.4 | 14.8 | 0.2 | 0.2 | 0.2 | 0.1 | 0.2 | 0.2 |
| Benzo(c)phenanthrene | 0.4 | 0.7 | 0.9 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| Benzo(*ghi*)perylene | 1.8 | 2.6 | 4.6 | 0.0 | 0.0 | 0.0 | 0.0 | 0.1 | 0.0 |
| Chrysene | 2.9 | 4.8 | 6.0 | 0.1 | 0.1 | 0.0 | 0.1 | 0.1 | 0.2 |
| Fluoranthene | 3.5 | 10.0 | 6.6 | 0.3 | 0.5 | 0.5 | 0.2 | 0.4 | 0.7 |
| Fluorene | 0.1 | 0.3 | 0.5 | 0.7 | 3.1 | 3.3 | 0.8 | 0.9 | 8.8 |
| Indeno(1,2,3-*cd*)pyrene | 1.7 | 2.5 | 4.2 | 0.0 | 0.0 | 0.0 | 0.0 | 0.1 | 0.0 |
| Naphthalene | 0.6 | 0.9 | 2.4 | 1.7 | 4.5 | 9.7 | 3.4 | 4.8 | 12.5 |
| Phenanthrene | 1.7 | 3.6 | 2.2 | 0.6 | 1.8 | 1.5 | 0.6 | 1.1 | 2.4 |
| Pyrene | 3.4 | 8.4 | 6.4 | 0.7 | 0.9 | 0.8 | 0.6 | 0.8 | 1.0 |
| Total PAHs | 31.9 | 58.5 | 85.4 | 21.9 | 62.1 | 87.8 | 33.9 | 49.5 | 154.3 |

*Bj*, *Sv*, and *Fa* designate *B. juncea* (Indian mustard), *S. viminalis* (willow), and *F. arundinacea* (fescue), respectively.
*C* designates control soils (pots without plants).
w and w/o designate with and without EDTA amendment, respectively.
T0* : PAH concentrations at the beginning of the experiment.

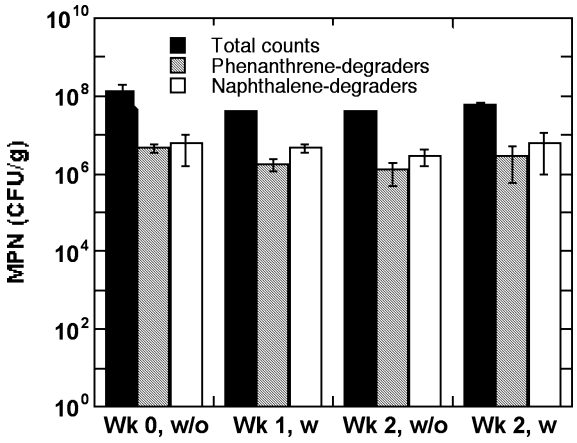


*Figure 5*. Microbial enumerations in the rhizosphere of *F. arundinacea* (fescue). Analysis was performed on rhizosphere soil at week 0 (Wk 0), at 1 week (Wk 1) and 2 weeks (Wk 2) without treatment (w/o) or following the addition of EDTA (w).

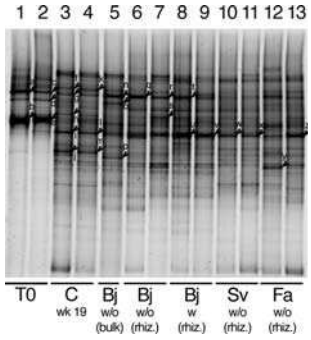


*Figure 6*. DGGE analysis of soil and rhizosphere microflora. *Bj*, *Sv*, and *Fa* designate *B. juncea* (Indian mustard), *S. viminalis* (willow), and *F. arundinacea* (fescue), respectively. Lanes 1–2, bulk soil from the begining of the experiment; lanes 3–4, bulk soil from control pots without plants (taken at the end of the experiment); lane 5, bulk soil from an Indian mustard pot (final harvest time week 12, w/o EDTA); lanes 6–7, rhizosphere extracts from Indian mustard (final harvest time week 12, w/o EDTA); lanes 8–9, rhizosphere extracts from Indian mustard (final harvest time week 12, w EDTA); lanes 10–11, rhizosphere extracts from willow (final harvest time week 16, w/o EDTA); lanes 12–13, rhizosphere extracts from fescue (final harvest time week 19, w/o EDTA).

tured proteobacteria, various *Sphingomonas* and *Pseudomonas* strains (Table 5). The two predominant bands (b and e) found in lanes 1 and 2 showed similarity to an uncultured gamma proteobacterium and several *Pseudomonas* species, respectively. These nearest relatives were observed in environments contaminated with oil, petroleum hydrocarbon, and creosote. The addition of 7 mL/kg creosote resulted in a substantial

*Table 5*. 16Sr DNA DGGE fragments, their percents similarity and nearest relatives

| DGGE band | Nearest relatives | % identity | Characteristics of nearest relatives |
|---|---|---|---|
| a, b, c | Uncultured gamma proteobacterium | 92–100 | Found in petroleum hydrocarbon contaminated soils |
| d, o: | *Sphingomonas* (5 spp.) | 97–98 | PAHs degradation (e.g., phenanthrene, chrysene, and acenaphthene). |
| | Uncultured alpha proteobacterium | 97 | Found in uranium mining waste piles (interactions with heavy metals) |
| e: | *Pseudomonas* (5 spp.) | 95 | Phenanthrene, phenyl acetic acid, 4-amyl-phenol and 4-hexyl-phenol degradation; Isolated from creosote contaminated soil; Found in oil and meta-toluate contaminated rhizospheres |
| f*, k: | *Sphingomonas* (3 spp.) | 95–97 (90*) | PAHs degradation; |
| | Uncultured bacterium | 96 (89*) | Found in heavy metal contaminated rhizosphere of the metal-hyperaccumulating plant (*Thlaspi caerulescens*) |
| g* | *Ochrobactrum* (9 spp.) | 87* | Isolated from oil-contaminated rhizosphere of *Galega orientalis*; Benzo-a-pyrene and phenol degradation |
| | Uncultured bacterium | 87* | Found in heavy metal contaminated rhizosphere of the metal-hyperaccumulating plant (*Thlaspi caerulescens*) |
| h* | Uncultured bacterium | 90* | Found in heavy metal contaminated rhizosphere of the metal-hyperaccumulating plant (*Thlaspi caerulescens*) |
| | *Sinorhizobium* sp. | 89* | Implicated in pollutant transport in soils (PAHs, lead and cadmium) |
| i, u, v, w*, z: | *Arthrobacter* (3 spp.) | 98–100 (92*) | Phenanthrene degradation; Copper and lead resistance |
| | Uncultured bacterium and *Actinobacteria* | 98–99 (92*) | Found in heavy metal contaminated environments and uranium mining waste piles (interactions with heavy metals) |
| j* | *Cycloclasticus* (4 spp.) | 91* | PAHs degradation (creosote (Superfund site) and PAHs contaminated sediments) |
| l* | *Streptomyces* sp. | 88* | Heavy metal resistance |
| m* | Uncultured alpha proteobacterium | 90* | Found in uranium mining waste piles (interactions with heavy metals) |
| | Uncultured gold mine bacterium | 90* | Found in heavy metal contaminated mine tailings |
| n: | *Sphingomonas* sp. | 97 | PAHs degradation (e.g., phenanthrene and acenaphthene) |
| p: | *Nocardioides* sp. | 97 | Phenanthrene degradation |
| q, r, t: | *Phaeospirillum* sp. | 93–98 | Phenol-degrading denitrifier |
| s* | Uncultured alpha proteobacterium | 90* | Found in heavy metal contaminated environments |
| x* | Uncultured *Actinomycete Streptomyces* sp. | 89* | Found in petroleum hydrocarbon contaminated soil |
| | | 88* | Heavy metal resistance |
| y: | Uncultured bacterium | 90 | 4-methylbenzoate degradation |

*Closest match obtained from sequencing results of one strand only.

decrease in these predominant species by week 19 (lanes 3 and 4), suggesting that the addition of high concentrations of creosote had a detrimental effect on these specific bacteria. However, the overall microbial diversity in the bulk soil increased substantially after the addition of creosote.

Several DGGE bands showed similarity to PAH-degrading bacteria. These included several *Sphingomonas* (bands f and k), *Ochrobactrum* (band g) and *Cycloclasticus* (band j) species. Band i showed a good identity with various *Arthrobacter* species and uncultured bacteria. The *Arthrobacter* relatives had the ability to degrade phenanthrene or showed resistance to heavy metals such as Cu and Pb. Several other DGGE bands in the bulk soil, were similar to species that demonstrated heavy metal resistance or that were found in heavy metal contaminated environments. The relatives included *Streptomyces* (bands l and *x*), *Sinorhizobium* (band h), and various uncultured bacteria (bands f, g, h, i, and m). The various *Arthrobacter*/uncultured bacteria (band i) observed in the bulk soil contaminated with creosote were also present in the rhizosphere of the three plants under study (lanes 8, 9 10–13, bands u, v, w, z). These species were clearly enriched in the rhizosphere of Indian mustard after the addition of EDTA (bands u and v). Two of the relatively abundant species identified in the bulk soil from the Indian mustard pot (lane 5), that showed strong similarities to PAH-degrading *Sphingomonas* (band o) and *Nocardioides* species (band p), were absent from the corresponding rhizosphere soils (lanes 6–9). The predominant bands q, r, and t, (lanes 6–8) were related to a phenol-degrading and denitrifying *Phaeospirillum* and were specific to that rhizosphere. The Indian mustard (lanes 6 and 7) and fescue (lanes 12 and 13) rhizospheres had a similar DGGE banding profile. Unique banding patterns were observed in the microbial community structure of the willow rhizosphere (lanes 10 and 11).

## Discussion

### *Phytoextraction of heavy metals*

In this study, the use of EDTA for the extraction of heavy metals in alkaline soils (Tejowulan, 1999) and the exposure time of the plant to the chelating agent before harvest were evaluated. In the akaline soil tested, the use of the free-acid form of EDTA and exposure time of one to 2 weeks before harvesting, increased the concentration of metals translocated to plant tissues. Total heavy metal accumulation (Cu, Pb, and Zn) was in the range of 0.1–0.25% of aerial and root tissues for both willow and fescue. Indian mustard accumulated these metals to an extent of 0.5% and 1.2% of aerial and root biomass, respectively, after a 2 week exposure time to EDTA. The phytoextraction results were lower (i.e., 0.12%) than those obtained by Blaylock et al. (1997) in a pot trial (soil pH 7.3, and using 5 mmol/kg EDTA), where Indian mustard (cv. 426308) accumulated 1% Pb in aerial tissues. Direct comparison of these two studies is difficult however, due to notable differences in the soils used. The soil used by Blaylock et al. (1997) was a clean silt loam soil amended with heavy metals, whereas the soil used in this study was collected from the site, without metal amendment or pH readjustment. To our knowledge, this is the first phytoextraction study focussing on alkaline soils.

The use of chelating agents in heavy metal-contaminated soils could promote leaching of the contaminants into the soil. In our study, we found no significant difference in heavy metal concentrations in higher and lower soil horizons between EDTA treated and untreated soils (results not shown). Since the bioavailability of heavy metals in soils decreases above pH 5.5–6 (Blaylock and Huang, 2000; Blaylock et al., 1997), the use of a chelating agent is warranted, and may be required, in alkaline soils. We found that exposing plants to EDTA for a longer period (2 weeks) could improve metal translocation in plant tissue as well as overall phytoextraction performance. This is an important observation, as it sheds light on the operational parameters that should be applied in the field. In the case of the Indian mustard, it would be preferable to harvest plants 2 weeks after EDTA amendment. The fescue plants should clearly be harvested 1 week after EDTA treatment to avoid metal release from decaying roots. It is noteworthy that the fescue could be the best candidate for a complete (i.e., aerial and root) harvest.

The extrapolation of field phytoextraction performances from pot trial data is imprecise, but by integrating total plant biomass and

planting density parameters, an approximation of what could be observed on site can be attempted. Our results showed that using a mixture of different plant species could hold promise. Woody species such as willows are capable of regenerating stools after coppicing from left over stumps. Sequential harvests could allow better soil prospection by root systems left to develop over a number of years. We found the willow can accumulate significant levels of Zn in its aerial parts in the absence of EDTA treatment, corroborating the findings of Labrecque et al. (1995). In a soil with a high concentration of bioavailable metals, this may contribute to reduce phytoremediation costs.

*Rhizosphere microbial population diversity, numbers, and activity*

It is generally recognized that microbial populations in rhizosphere soils can be 10–100 times higher than in the surrounding bulk soil and have greater xenobiotic degrading capabilities (Boyle and Shann, 1998; Nedunuri et al., 2000; Pichtel and Liskanen, 2001; Reilley et al., 1996; Walton et al., 1994). In the present study we found no significant changes with time in microbial population sizes (total heterotrophs, naphthalene- and phenanthrene-degrading micro organisms) in the bulk or rhizosphere soils. Mineralization assays suggested that the addition of 5 mmol/kg EDTA to enhance the phytoextraction of heavy metals would not hinder PAH degradation activity. DGGE results demonstrated that EDTA amendments could also have a positive impact on some microbial populations of the Indian mustard rhizosphere. Indian mustard is known to remove Pb, Cu, Cd, Ni and Zn from metal contaminated soils (Salt et al., 1998). In this study, the hyperaccumulation of Pb observed in the roots of the Indian mustard plant was concomitant with the enrichment in the rhizosphere, of *Arthrobacter* relatives that showed resistance to Pb and Cu. The Indian mustard plant also supported the enrichment of a bacterium that was closely related to a phenol-degrading and denitrifying *Phaeospirillum*. Phenol is one of the major chemicals in creosote. The presence of this type of bacterium in the root zone may have protected the plant and other bacteria from the toxic effects of phenol.

In this study, based on the PAH concentrations measured in soil, it is difficult to conclude whether the presence of these plants accelerated or hindered PAH degradation. However, microbial enumerations and mineralization assays demonstrated that indigenous microbial PAH-degrading activity was already well established in the soil (results not shown) and DGGE analyses revealed that PAH-degrading bacteria represented a significant fraction of the community. Predominant species that shared strong identities with bacteria that have the ability to degrade several of the PAHs found in coal tar creosote as well as phenol derivatives were identified in the bulk soil. One of the predominant species also shared identity with a bacterium that was isolated from a creosote contaminated soil.

## Conclusions

The use of phytoremediation on any site entails a feasibility study to determine the expected performance of such a bioremediation strategy. In this study, a compounding issue in regards to metal phytoextraction was the high soil pH. At pH 8.0–8.5, metals (Cu, Pb, Zn) were almost insoluble and thus not very bioavailable. The application of a synthetic chelating agent (EDTA) at 5 mmol/kg yielded positive results. The assessment of phytoremediation in a heavy metal and PAH contaminated soil has led us to conclude that this technology can offer a technically feasible alternative for the restoration and management of sites with alkaline soils. However, our study also shows that the phytoextraction of total heavy metals from alkaline soils that also contain organic contaminants would take a long time and require many harvests, indicating that additional efforts would be required to optimize such an approach. Currently, two regulatory issues further complicate the adoption of phytoextraction for the decontamination of soils. Firstly, bioavailability of metals in a given soil is not a parameter used in establishing the risk posed by a site and/or in establishing the environmentally acceptable contamination endpoint. Secondly, to date, contaminated soil can still be disposed of by landfilling in Quebec, rendering phytoremediation less economically interesting as a primary remediation technology. Nonetheless,

use of vegetation on contaminated sites remains an important tool for the control of contaminant migration through the mechanisms of hydraulic control, soil stabilization, and the reduction of dispersal by wind (Schnoor et al., 1995).

## Acknowledgements

We thank Inspec-Sol Inc., the Montreal Centre of Excellence in Brownfields Remediation (MCEBR), and the Ministère de l'environnement et de la faune du Québec for financial support of this work. We also thank Danielle Ouellette for assistance with the DGGE analysis.

## References

Blaylock M J and Huang J W 2000. Phytoextraction of Metals. Eds. I Raskin and B D Ensley. pp. 53–70. John Wiley & Sons, Toronto.

Blaylock M J, Salt D E, Dushenkov S, Zakharova O, Gussman C, Kapulnik Y, Ensley B and Raskin I 1997 Enhanced accumulation of Pb in Indian mustard by soil-applied chelating agents. Environ. Sci. Technol. 31, 860–865.

Boyle J J and Shann J R 1998 The influence of planting and soil characteristics on mineralization of 2,4,5-T in rhizosphere soil. J. Environ. Qual. 27, 704–709.

Ensley B D 2000. Rational for Use of Phytoremediation. Eds. I Raskin and B D Ensley. pp. 1–11. John Wiley & Sons, Toronto.

Fortin N, Beaumier D, Lee K and Greer C W 2004 Soil washing improves the recovery of total community DNA from polluted and high organic content sediments. J. Microbiol. Meth. 56(2), 181–191.

Garbisu C and Alkorta I 2001 Phytoextraction: a cost-effective plant-based technology for the removal of metals from the environment. Biores. Technol. 77, 229–236.

Glass D J 2000 Economic Potential of Phytoremediation. In Phytoremediation of Toxic Metals – Using Plants to Clean Up the Environment. Eds. I Raskin and B D Ensley. pp. 15–31. John Wiley & Sons, Toronto.

Gleba D, Borisjuk N V, Borisjuk L G, Kneer R, Poulev A, Skarzhinskaya M, Dushenkov S, Logendra S, Gleba Y Y and Raskin I 1999 Use of plant roots for phytoremediation and molecular farming. Proc. Natl. Acad. Sci. USA 96, 5973–5977.

Gribbon L T and Barer M R 1995 Oxidative metabolism in nonculturable *Helicobacter pylori* and *Vibrio vulnificus* cells studied by substrate-enhanced tetrazolium reduction and digital image processing. Appl. Environ. Microbiol. 61, 3379–3384.

Haines J R, Wrenn B A, Holder E L, Strohmeier K L, Herrington R T and Venosa A D 1996 Mesurement of hydrocarbon-degrading microbial populations by a 96-well plate most-probable-number procedure. J. Indust. Microbiol. 16, 36–41.

Harvey P J, Campanella B F, Castro P M L, Harms H, Lichtfouse E, Schaffner A R, Smrcek S and Werck-Reichhart D 2002 Phytoremediation of polyaromatic hydrocarbons, anilines and phenols. Environ. Sci. Pollut. Res. 9, 29–47.

Henner P, Schiavon M, Morel J L and Lichtfouse E 1997 Polycyclic aromatic hydrocarbon (PAH) occurrence and remediation methods. Analusis 25, M56–M59.

Klee A J 1993 A computer program for the determination of most probable number and its confidence limits. J. Microbiol. Meth. 18, 91–98.

Kopponen P M, Utriainen M, Lukkari K, Suntioinen S, Karenlampi L and Karenlampi S 2001 Clonal differences in copper and zinc tolerance of birch in metal-supplemented soils. Environ. Poll. 112, 89–97.

Labrecque M, Teodorescu T I and Daigle S 1995 Effect of wastewater sludge on growth and heavy metal bioaccumulation of two Salix species. Plant Soil 171, 303–316.

Lawrence J R, Chenier M R, Roy, R, Beaumier D, Fortin N, Swerhone G D W, Neu T R and Greer C W 2004 Microscale and molecular assessment of impacts of nickel, nutrients, and oxygen level on structure and function of river biofilm communities. Appl. Environ. Microbiol. 70, 4326–4339.

Liste H H and Alexander M 2000 Accumulation of phenanthrene and pyrene in rhizosphere soil. Chemosphere 40, 11–14.

McGrath S P, Zhao F J and Lombi E 2001 Plant and rhizosphere processes involved in phytoremediation of metal-contaminated soils. Plant Soil 232, 207–214.

McIntyre T and Glennis M L 1997 The advancement of phytoremediation as an innovative environmental technology for stabilization, remediation, or restoration of contaminated sites in Canada: a discussion paper. J. Soil Contam. 6, 227–241.

Nedunuri K V, Govindanaraju R S, Banks M K, Schwab A P and Chen Z 2000 Evaluation of phytoremediation for field-scale degradation of total petroleum hydrocarbons. J. Environ. Eng. 126, 483–490.

Pichtel J and Liskanen P 2001 Degradation of diesel fuel in rhizosphere soil. Environ. Eng. Sci. 18, 145–157.

Reilley K A, Banks M K and Schwab A P 1996 Dissipation of polycyclic aromatic hydrocarbons in the rhizosphere. J. Environ. Qual. 25, 212–219.

Roy S 2000 Évaluation et stimulation du potentiel biodégradateur de la microflore du sol dans les cours de stockage de bois traité au pentachlorophenol. Ph.D. Thesis. Département de Biologie, Université de Sherbrooke. 320 pp.

Roy S Gendron J, Delhoménie M C, Bibeau L, Heitz M and Brzezinski R 2003 *Pseudomonas putida as* the dominant toluene-degading bacterial species during air decontamination by biofiltration. Appl. Microbiol. Biotechnol. 61(4), 366–373.

Salt D E, Smith R D and Raskin E 1998 Phytoremediation. Annu. Rev. Plant Physiol. Plant Mol. Biol. 49, 643–668.

Schnoor J L, Licht L A, McCutcheon S C, Wolfe N L and Carreira L H 1995 Phytoremediation of organic and nutrient contaminants. Environ. Sci. Technol. 29, 318A–323A.

Siciliano S D and Germida J J 1999 Enhanced phytoremediation of chlorobenzoates in rhizosphere soil. Soil Biol. Biochem. 31, 299–305.

Sokal R R and Rohlf F J 1995 Biometry. W H Freeman and Company, NewYork, USA. 850 pp.

Tejowulan R S 1999 Remediation of trace metal contaminated soils. Ph.D. Thesis. Department of Natural Resource Sciences, McGill University. 172 pp.

Tessier A, Campbell P G and Bisson M 1979 Sequential extraction procedure for the speciation of particulate trace metals. Anal. Chem. 51, 844–851.

Walton B T, Guthrie E A and Hoylman A M 1994 Toxicant degradation in the rhizosphere. *In* Bioremediation through rhizosphere technology. Eds. T A Anderson and J R Coats. pp. 11–26. American Chemical Society, Washington.

Wiltse C C, Rooney W L, Chen Z, Schwab A P and Banks M K 1998 Greenhouse evaluation of agronomic and crude oil-phytoremediation potential among alfalfa genotypes. J. Environ. Qual. 27, 169–173.

Wrenn B A and Venosa A D 1996 Selective enumeration of aromatic and aliphatic hydrocarbon degrading bacteria by a most-probable number procedure. Can. J. Microbiol. 42, 252–258.

*Section editor: Y.-G. Zhu*