

EFFECT OF HYBRID POPLAR TREES ON MICROBIAL POPULATIONS IMPORTANT TO
HAZARDOUS WASTE BIOREMEDIATIONJAMES L. JORDAHL, LESLEY FOSTER, JERALD L. SCHNOOR and PEDRO J.J. ALVAREZ*
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(Received 21 June 1996; Accepted 20 December 1996)

Abstract—Microbial concentrations of denitrifiers, pseudomonads, and monoaromatic petroleum hydrocarbon (BTX) degraders were significantly higher ($p < 0.1$) in soil samples from the rhizosphere of poplar trees than in adjacent agricultural soils, and atrazine degraders were found only in one rhizosphere sample. The relative abundance of these phenotypes (as a fraction of total heterotrophs) was not significantly different between rhizosphere and surrounding soils. Therefore, the poplar rhizosphere enhanced the growth of microbial populations that participate in natural bioremediation without exerting selective pressure for them.

Keywords—Atrazine BTX Nitrate Phytoremediation Rhizosphere

INTRODUCTION

Agricultural chemicals such as atrazine and nitrate, and monoaromatic petroleum hydrocarbons such as benzene, toluene, and xylenes (BTX) are ubiquitous groundwater pollutants that threaten public health. Phytoremediation, the use of plants to remove such environmental pollutants from contaminated sites, holds great promise as a low-cost remedial approach [1]. Plants can enhance the removal of xenobiotics by at least two mechanisms: (1) direct uptake and, in some cases, in-plant transformations to less toxic metabolites [2-4], and (2) stimulation of microbial activity and biochemical transformations in the root zone through the release of exudates and enzymes [5-7].

Poplar trees (*Populus* spp.) are commonly used as phytoremediation tools because they are perennial, hardy, tolerant to high concentrations of organics, highly tolerant of flooding, fast growing, easily propagated, and have a wide range of adaptation [1,3,8,9]. A key attribute of the poplar as related to bioremediation is the large quantity of contaminated water that it can take up from the soil. Because poplar is a phreatophyte (its roots can extend to the water table) it can withdraw water from great depths, up to 3 m (15 ft) [1]. Additional reasons for the widespread use of poplars in phytoremediation include ease of planting and growth from deep-planted cuttings, ability to transfer oxygen to the root zone for potential aerobic mineralization of organics, and build-up of organic carbon in the rhizosphere due to root necromass, which retards the movement of hydrophobic organics.

Numerous studies with agricultural and herbaceous plants have found increased biodegradation of pesticides, trichloroethylene (TCE), and petroleum products in the rhizosphere (for reviews, see Anderson and coworkers [10,11]). However, little is known about the microbiology of the poplar rhizosphere, particularly as it pertains to bioremediation. While the enumeration of specific microorganisms responsible for xenobiotic degradation is a logical step toward understanding pollution fate and transport, such data are very scarce for plant

root systems. To this end, this paper reports the first evaluation of the relative abundance of indigenous microorganisms in the poplar rhizosphere that can degrade selected priority pollutants.

MATERIALS AND METHODS

Experimental approach and sampling

Microbial populations from the rhizosphere of 7-year-old hybrid poplar trees (*Populus deltoides* × *nigra* DN-34, Imperial Carolina) were characterized by concentration and catalytic capacity. Most probable number (MPN) techniques were used for the enumeration of five specific phenotypes: total heterotrophs, denitrifiers, pseudomonads, BTX degraders, and atrazine degraders. The MPN techniques were used to allow for a greater chemical definition of the medium and to better account for the presence of fungi and nonculturable bacteria that participate in bioremediation of specific pollutants. The genus *Pseudomonas* has been recently subjected to taxonomic revision and is now restricted to the species of Group I as defined by ribosomal RNA-DNA reassociation experiments of Palleroni et al. [12]. Nevertheless, many of its current and former members can denitrify and are among the most catalytically versatile of gram-negative bacteria [13]. Therefore, the enumeration of pseudomonads was considered important for evaluating the potential for the poplar rhizosphere to enhance the removal of priority pollutants.

The MPN concentrations of the selected phenotypes were measured in three rhizosphere samples and compared to those in three (control) soil samples taken from an adjacent corn field. Student's *t*-test [14] was used to determine whether differences were statistically significant. The fractions of the heterotrophic community belonging to these phenotypic groups were also compared.

Soil samples were collected from a poplar tree plot near Lily Lake at Amana, Iowa, USA in May, 1995. The soils at the site were silty loam to silty clay loam alluvium (Nodaway-Ely complex—fine silty, mixed, mesic Typic Udifluent and fine silty, mixed, mesic Aquic Cumulic Hapludoll [15]; *soe* = 0.025). Holes were excavated using a backhoe immediately

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adjacent to three poplar trees, and at three different locations (devoid of roots) in an adjacent cornfield. Samples were taken from each site at the phreatic surface, approximately 1.2 m (4 ft) below the ground surface. Rhizosphere soil was scraped from within 2 to 10 mm of the root surface into sterile glass jars and stored at 4°C until analysis. Based on local agricultural practices, the tested soils had previous exposure to nitrate and atrazine but no known exposure to BTX compounds.

Exudate samples were collected from hybrid poplar trees grown in silica sand in a plant incubator (pH Environmental) with a 12-h photoperiod and a light intensity of 300 μ Einsteins/cm²-h. Soluble exudate was collected from drainage during watering in 300-ml glass bottles and analyzed for total organic carbon (TOC), biochemical oxygen demand (BOD) and chemical characteristics using standard methods [16]. To evaluate the carbon produced by photosynthesis, the plant biomass was measured gravimetrically at the beginning and end of the plant incubation period after oven drying to constant weight at 105°C. The carbon content was calculated as 50% of total dry matter. To evaluate the fraction of photosynthesized organic carbon released into the rhizosphere, the mass of TOC in exudates was calculated as a fraction of the total carbon produced by plant biomass during the incubation.

Microbial enumeration procedures

The MPN phenotypic enumerations were based on serial dilutions of soil extracts adapted from Alexander [17]. A basal mineral medium [18] was used to buffer all dilutions at pH 7. For each phenotype, 10-fold dilutions were prepared with five replicates per dilution. For serial dilution preparation, 10 g of soil were placed in an autoclaved 233-ml serum bottle (Qorpak) containing 100 ml of sterile mineral medium and capped with Mini-nert valves (Supelco). This 1:10 dilution was shaken for 1 h on a Burrell wrist-action shaker to detach microorganisms from soil particles. Ten milliliters of this slurry were subsequently transferred with a disposable sterile pipette into equal serum bottles containing 90 ml of mineral medium and shaken for approximately 5 min on the wrist-action shaker. This procedure was repeated until final dilutions of 10⁻¹¹ g of soil per ml of medium were reached.

The culture medium for total heterotroph and denitrifier enumeration was prepared by dissolving 8 g/L of tryptic soy broth powder and KNO₃ (0.5 mg/L) in mineral medium. The headspace contained 10 ml of acetylene (C₂H₂), which inhibits nitrous oxide reductase and results in the accumulation of N₂O. Dilutions were incubated for 2 weeks at 23°C in an anaerobic glove box. The reduction of greater than 20% of the added nitrate to N₂O was taken as a positive presence of denitrifiers [19]. The presence of total heterotrophs was inferred by growth-induced turbidity in these bottles.

A selective medium was used for pseudomonad enumeration. Pseudomonad isolation medium was prepared using 1 L of mineral medium. Tryptic soy broth was added at 8 g/L, along with 9 mg/L of paraosaniline (dissolved in 2 ml of methanol) and 140 mg/L of 2,3,5-triphenyl tetrazolium chloride to inhibit gram-positive microorganisms. This solution was autoclaved at 245°C for 20 min. Cycloheximide (900 mg/L), nalidixic acid (23 mg/L), and nitrofurantoin (10 mg/L) were then added to inhibit fungi, gram-negative cocci, and enteric organisms, and other gram-negative rods, respectively [20]. The medium was filter sterilized using a 0.2- μ m autoclaved membrane filter. The presence of pseudomonads was

inferred by growth-induced turbidity after 2 weeks of incubation.

For BTX degrader enumeration, the culture medium was aerobic and contained approximately 5 mg/L (each) of benzene, toluene, and *o*-xylene. An additional set of six replicates was prepared similarly and autoclaved for use as controls. Following 2 weeks of incubation, the removal of any of the three added BTX compounds from viable incubations but not from autoclaved controls was taken as a positive indication of BTX degraders. The same approach was used to enumerate atrazine degraders, except that dilutions were fed 100 μ g/L atrazine and were incubated for 30 d.

Chemical analyses

Atrazine was analyzed by solid-phase extraction with ethyl acetate using PrepSep[®] C₁₈ extraction columns, followed by gas chromatography using a Hewlett Packard 5890 Series II GC equipped with a nitrogen phosphorus detector [21]. Nitrate was analyzed in a Dionex 4500i ion chromatograph using an AS4A ion-exchange column for separation followed by chemical suppression and conductivity detection. The concentration of N₂O was measured by headspace injection into a Hewlett Packard 5890 Series II gas chromatograph fitted with a molecular sieve and thermal conductivity detector. The BTX were analyzed with a Hewlett Packard 5890 Series II GC equipped with a Hewlett Packard 19395A headspace autosampler and flame-ionization and photoionization detectors in series. Separation was achieved using a J&W Scientific DB-WAX column. The BOD of root exudates was analyzed using standard methods (5210 B) [16]. The ultimate BOD and the BOD decay coefficient (*k*) were determined using BOD values after 1, 3, 5, 7, 10, and 20 d of incubation and the nonlinear curve fit function provided by Sigma Plot[®]. TOC was also analyzed using standard methods (5310 B) [16] and a Dohrmann DC-80 analyzer.

RESULTS AND DISCUSSION

Although there are no reports in the literature about the effect of the rhizosphere on the concentration of the specific degraders tested in this study, increased concentration of microorganisms near plant roots is a well-known phenomenon, often referred to as the "rhizosphere effect." Thus, it is not surprising that the concentrations of all types of microbial populations investigated were higher in the poplar rhizosphere than in the surrounding soil (Fig. 1). Interestingly, poplars planted at a depth of 1.6 m (5 ft) developed beneficial rhizosphere populations relatively deep in the soil profile. In all cases, the increase was significant at the 90% level, and the rank in concentration was: total heterotrophs > denitrifiers > pseudomonads > BTX degraders > atrazine degraders. Figure 1 does not include atrazine degrader concentrations because this herbicide was not degraded in any of the soil samples, except for one out of three rhizosphere samples where atrazine was degraded in four out of five replicate incubations at the 10⁻¹ dilution.

Considerable organic carbon is leaked into the rhizosphere by poplars, which could enhance microbial growth. For example, poplars grown in the plant incubator under controlled conditions released 0.25 \pm 0.18% (*n* = 5) of their biomass produced as soluble exudates. Analysis of these exudates reflects that it is rich in biodegradable organic macromolecules (Table 1). The measured BOD decay coefficient (*k* = 0.4/d) was high, indicating that such substrates would be rapidly

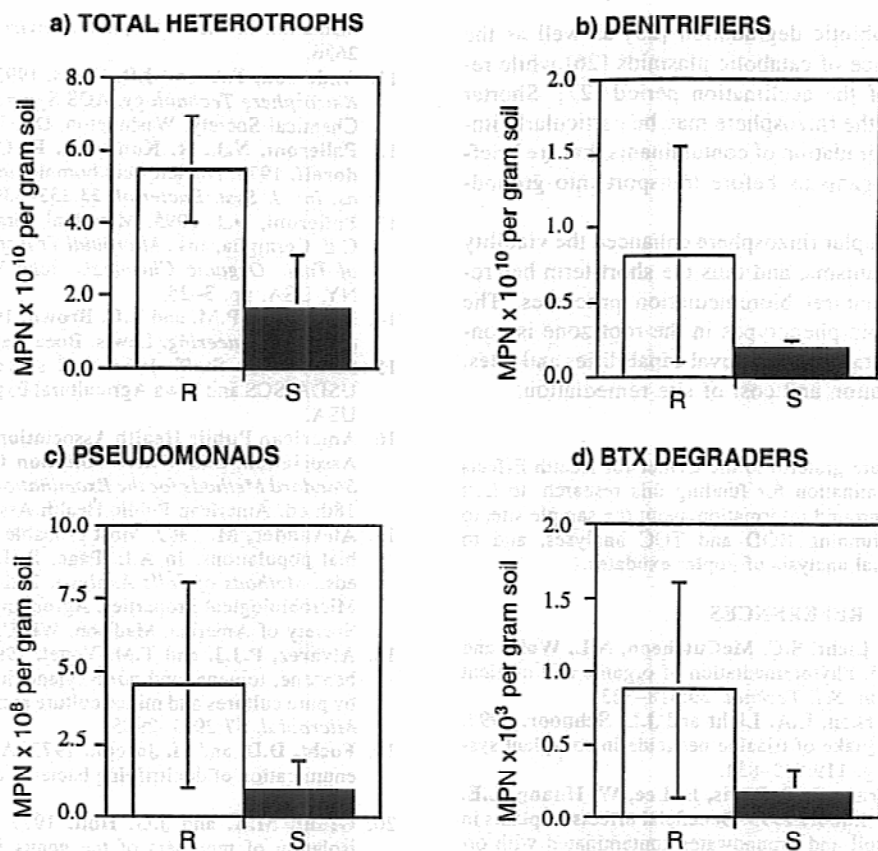


Fig. 1. Effect of poplar rhizosphere on microbial populations important for pollution control. The MPN concentrations for various phenotypes are depicted for rhizosphere (R) and surrounding soil (S) samples. Error bars represent \pm one standard deviation from the mean of three soil samples.

biodegraded. In theory, some of these substrates might enhance the degradation of the target contaminants by gratuitously enhancing catabolic enzyme induction, serving as primary substrates for cometabolism, or just supporting the growth of specific degraders. Conversely, some compounds in root exudates could have negative effects on xenobiotic degradation by exerting catabolite repression and diauxy or by increasing the sorption capacity of the soil to a point where bioavailability of the target contaminants is hindered. While the overall effect is probably system specific, the literature suggests that root exudates are likely to have a beneficial impact in contaminant degradation [5,11].

The "root-to-soil ratio" (R/S), which refers to the concentration of microorganisms in the rhizosphere divided by the concentration in the background surrounding soil, ranged from 3.4 to 5.0 for various specific degraders (Table 2). These ratios are within the (lower) range of values commonly reported for undefined microbial consortia in agricultural and herbaceous plant roots [22]. The higher concentration of pseudomonads in the poplar rhizosphere corroborates studies by Rovira and

Davey [23] who found that other plant roots selectively stimulated gram-negative bacteria, including *Pseudomonas*, spp. Because pseudomonads tend to have a broad degradative capacity toward priority pollutants, including those tested in these study, their proliferation in the poplar rhizosphere could enhance natural bioremediation processes.

To investigate whether the poplar rhizosphere selected for microbes that can degrade priority pollutants, the concentrations of specific degraders present in each sample were calculated as a fraction of the corresponding total heterotrophic concentrations (calculations not shown). Although the relative abundance of the tested phenotypes was generally higher in the rhizosphere than in surrounding soils, the differences were not statistically significant. This suggests that the poplar rhizosphere enhanced the growth of the tested desirable phenotypes without exerting selective pressure for them.

It should be kept in mind that limitations to mass transfer (e.g., bioavailability) and lack of gene expression may preclude correlating microbial population densities in soils with biodegradation activity, even when the microbial measure is indicative of the active biomass as is the MPN [24]. Nevertheless, a higher concentration of active biomass may enhance

Table 1. Summary of poplar root exudate characteristics

Component	Concentration
Dissolved organic carbon	17 \pm 8 mg/L
Ultimate biochemical oxygen demand	36 mg/L
BOD decay coefficient	0.4/d
Median molecular weight ^a	1,100 Da

^a Analysis by size-exclusion chromatography run by Yu-Ping Chin, Ohio State University.

Table 2. Ratio of microbial concentrations in the poplar rhizosphere to surrounding soil (R:S ratio)

	Total heterotrophs	Pseudomonads	BTX degraders	Denitrifiers
R:S ratio	3.4	4.9	5.0	4.1

the adaptation to xenobiotic degradation [25] as well as the transfer and maintenance of catabolic plasmids [26] while reducing the duration of the acclimation period [27]. Shorter acclimation periods in the rhizosphere may be particularly important for rapid biodegradation of contaminants that are briefly exposed to microorganisms before transport into groundwater.

In conclusion, the poplar rhizosphere enhanced the viability of beneficial microorganisms, and thus the short-term heterotrophic potential for natural bioremediation processes. The proliferation of desirable phenotypes in the root zone is conducive to enhanced contaminant removal capabilities and rates, and thus, reduced duration and cost of site remediation.

Acknowledgement—We are grateful to the Center for Health Effects of Environmental Contamination for funding this research, to Lou Licht for providing background information about the sample site, to Tanya McDermott for running BOD and TOC analyses, and to Yu-Ping Chin for chemical analysis of poplar exudates.

REFERENCES

- Schnoor, J.L., L.A. Licht, S.C. McCutcheon, N.L. Wolfe and L.H. Carreira. 1995. Phytoremediation of organic and nutrient contaminants. *Environ. Sci. Technol.* 29:318-323.
- Nair, D.R., J.G. Burken, L.A. Licht and J.L. Schnoor. 1992. Mineralization and uptake of triazine pesticide in soil-plant systems. *J. Environ. Eng.* 119:842-854.
- Shimp, J.F., J.C. Tracy, L.C. Davis, E. Lee, W. Huang, L.E. Erickson and J.L. Schnoor. 1993. Beneficial effects of plants in the remediation of soil and groundwater contaminated with organic materials. *Crit. Rev. Environ. Sci. Technol.* 23:41-77.
- Simonich, S.L. and R.A. Hites. 1995. Organic pollutant accumulation in vegetation. *Environ. Sci. Technol.* 29:2905-2914.
- Anderson, T.A., E.L. Kruger and J.R. Coats. 1994. Enhanced degradation of a mixture of three herbicides in the rhizosphere of a herbicide-tolerant plant. *Chemosphere* 28:1551-1557.
- Newman, A. 1995. Plant enzymes set for bioremediation field study. *Environ. Sci. Technol.* 29:18A.
- Walton, B.A. and T.A. Anderson. 1990. Microbial degradation of trichloroethylene in the rhizosphere: potential application to biological remediation of waste sites. *Appl. Environ. Microbiol.* 56:1012-1016.
- Heilman, P.E., R.F. Stettler, D.P. Hanley and R.W. Carkner. 1990. High yield hybrid poplar plantations in the Pacific Northwest. PNW356. Pacific Northwest Regional Extension Bulletin. Washington State University Cooperative Extension Service, Pullman, WA, USA.
- Shrive, S.C., R.A. McBride and A.M. Gordon. 1994. Photosynthetic and growth responses of two broad-leaf tree species to irrigation with municipal landfill leachate. *J. Environ. Qual.* 23: 534-542.
- Anderson, T.A., E.A. Guthrie and B.T. Walton. 1993. Bioremediation in the rhizosphere. *Environ. Sci. Technol.* 27:2630-2636.
- Anderson, T.A. and J.R. Coats. 1993. *Bioremediation Through Rhizosphere Technology*. ACS Symposium series 563. American Chemical Society, Washington, DC, USA.
- Palleroni, N.J., R. Kunisawa, R. Contopoulou and M. Doudoroff. 1973. Nucleic acid homologies in the genus *Pseudomonas*. *Int. J. Syst. Bacteriol.* 23:333-339.
- Palleroni, N.J. 1995. Microbial versatility. In L.Y. Young and C.E. Cerniglia, eds., *Microbial Transformation and Degradation of Toxic Organic Chemicals*. John Wiley & Sons, New York, NY, USA, pp. 3-25.
- Berthouex, P.M. and L.C. Brown. 1994. *Statistics for Environmental Engineering*. Lewis, Boca Raton, FL, USA.
- Soil Survey Staff. 1967. Soil survey of Iowa County, Iowa. USDA-SCS and Iowa Agricultural Experiment Station, Ames, IA, USA.
- American Public Health Association, American Water Works Association, and Water Pollution Control Federation. 1992. *Standard Methods for the Examination of Water and Wastewater*, 18th ed. American Public Health Association, Washington, DC.
- Alexander, M. 1982. Most probable number method for microbial populations. In A.L. Page, R.H. Miller and D.R. Keeney, eds., *Methods of Soils Analysis*, 2nd ed., Part 2—Chemical and Microbiological Properties. Agronomy Monograph 9. Agronomy Society of America, Madison, WI, USA, pp. 815-820.
- Alvarez, P.J.J. and T.M. Vogel. 1991. Substrate interaction of benzene, toluene, and *para*-xylene during microbial degradation by pure cultures and mixed culture aquifer slurries. *Appl. Environ. Microbiol.* 57:2981-2985.
- Focht, D.D. and H. Joseph. 1973. An improved method for the enumeration of denitrifying bacteria. *Soil Sci. Soc. Am. Proc.* 37: 698-699.
- Grant, M.A. and J.G. Holt. 1977. Medium for the selective isolation of members of the genus *Pseudomonas* from natural habitats. *Appl. Environ. Microbiol.* 33:1222-1224.
- Paterson, K.G. and J.L. Schnoor. 1992. Fate of alachlor and atrazine in a riparian zone field site. *Water Environ. Res.* 64:274-283.
- Atlas, R.M. and R. Bartha. 1992. *Microbial Ecology: Fundamentals and Applications*. Benjamin/Cummings, Menlo Park, CA, USA.
- Rovira, A.D. and C.B. Davey. 1974. Biology of the rhizosphere. In E.W. Carson, ed., *The Plant Root and its Environment*. University Press of Virginia, Charlottesville, VA, USA, pp. 153-204.
- Hickman, G.T. and J.T. Novak. 1989. Relationship between subsurface biodegradation rates and microbial density. *Environ. Sci. Technol.* 23:525-532.
- Nishimo, S.F. and J.C. Spain. 1993. Cell density-dependent adaptation of *Pseudomonas putida* to biodegradation of p-nitrophenol. *Environ. Sci. Technol.* 27:489-494.
- Alvey, S. and D.E. Crowley. 1996. Survival and activity of an atrazine-mineralizing bacterial consortium in rhizosphere soil. *Environ. Sci. Technol.* 30:1596-1603.
- Alvarez, P.J.J., P.J. Anid and T.M. Vogel. 1994. Kinetics of toluene degradation by denitrifying aquifer microorganisms. *J. Environ. Eng.* 120:1327-1336.