

Genetic Testing Prerequisites for Effective Tree-Based Phytoremediation Systems

D. L. Rockwood (dlock@ufl.edu), B. Becker, A. Lindner, A. Pacheco, and L. Ma
(University of Florida, Gainesville, FL, USA)
C. Lin and N. Brown (Ecology & Environment, Tallahassee, FL, and Chicago, IL, USA)
T. Spriggs and R. Seip (CH2M Hill, Tampa, FL, and Montgomery, AL, USA)
J. Isebrands (Environmental Forestry Consultants, New London, WI, USA)
R. Hall (Iowa State University, Ames, IA, USA)
R. Lange (Illinois EPA, LaSalle, IL, USA)
L. Licht (Ecolotree, North Liberty, IA)
B. Kanzler (Reichhold Chemicals, Research Triangle Park, NC, USA)

ABSTRACT: Effective phytoremediation of heavy metal- or chlorinated solvent-contaminated sites by fast-growing trees is strongly influenced by the choice of tree species and genotypes (e.g., clones, progenies). As evidenced in ongoing phytoremediation systems, genetic testing is necessary to match species and genotypes within species to site, microclimate, and contaminant conditions. Tree growth and arsenic uptake varied considerably between and within three species. At a toluene-contaminated site, two species were initially similar in vigor and emitted contaminants, but one species was eventually superior and had contaminants in leaf and branch tissues. At a trichloroethylene (TCE)-contaminated site, locally adapted clones greatly surpassed a clone widely planted for phytoremediation in more temperate regions. While 16 clones survived and grew rapidly in perchloroethylene (PCE)-contaminated soil, the five best clones were as much as twice the average size and supported more methanotrophs. At a hydrocarbon-contaminated site, even five local clones varied considerably. In a landfill cap, 16 progenies and 32 local clones had comparable survival and tree size that exceeded two non-adapted clones. These and similar results elsewhere illustrate the considerable importance of field testing species and genotypes for appropriate prerequisites in order to reach the phytoremediation potential of fast-growing trees.

INTRODUCTION

Effective phytoremediation of heavy metal- or chlorinated solvent-contaminated sites by fast-growing trees such as *Eucalyptus amplifolia* (EA), *E. grandis* (EG), eastern cottonwood (*Populus deltoides*, PD), and *Salix* spp. appears to be strongly influenced by the choice of tree genotypes (Rockwood et al. 2003, Licht and Isebrands 2005). Genetic testing results from six ongoing phytoremediation systems are reviewed with respect to matching species and genotypes (e.g., clones, progenies) within species to site, microclimate, and contaminant conditions.

The role of microorganisms in the root zone of phytoremediating trees is poorly understood, but they may be essential in oxidizing contaminants such as TCE. Methane-oxidizing bacteria (methanotrophs) are supported by this microenvironment (Brigmon et al. 1999) and are capable of cometabolically oxidizing TCE (Little et al. 1988; Fox et al. 1990). However, rates of TCE oxidation are dependent on many variables, including

the type of methanotroph, interactions with heterotrophic bacteria capable of metabolizing multiple-carbon substrates, and environmental conditions (Uchiyama et al. 1992). Types II and X methanotrophs express the soluble form of methane monooxygenase (sMMO) under low-to-nonexistent levels of copper, which can oxidize a wider variety of organic compounds, including alkanes, alkenes, and aromatics, than its particulate form (pMMO). Consequently, the bioremediation potential of these types of methanotrophs has been widely recognized (Hanson and Hanson 1996).

MATERIALS AND METHODS

In Study 1 (Table 1), Chinese brake fern (*Pteris vittata*, *PV*) and selected *EA*, *EG*, and *PD* genotypes were established in reconstructed portions of a former CCA wood treatment facility (Rockwood et al. 2003). A randomized complete block design (RCBD) with two replications of split plot arrangements of compost and no compost amendments with *PV*, *PD*, *EA*, *PD-PV*, *EA-PV*, and *EG-PV* plots containing 18 single tree plots at 1.0 × 0.5 m spacing was employed. Tree growth and arsenic uptake were assessed periodically.

TABLE 1. Details of six field studies involving *EA*, *EG*, *PD* and other poplars, and willows at arsenic-, toluene-, TCE-, PCE-, hydrocarbon-, and/or methane-contaminated sites.

Study	Location	Estab. Date	Contaminant	No. of Genotypes: Trees
1	Archer, FL	4/00;3/01;8/03	Arsenic	6 <i>EA</i> , 7 <i>EG</i> , 11 <i>PD</i> : 705
2	St. Augustine, FL	8/00	Toluene	15 <i>PD</i> , 15 <i>EA</i> : 630
3	LaSalle, IL	3/02-4/03	TCE, PCE	18 poplar, 24 willow: 1,074
4	Gulfport, MS	3/03	Hydrocarbons	5 <i>PD</i> : 150
5	Tallahassee, FL	5/04	Methane	16 <i>EA</i> , 32 <i>PD</i> : 432
6	Seattle, WA	2/00	Landfill Cap	4 poplar: 10,000

For Study 2, after land clearing, 315 rooted *PD* cuttings and 315 *EA* seedlings were planted in alternating rows of six-tree row plots at 10 × 3-ft spacing within three replications of a RCBD. Within each row plot, three of the six trees were randomly planted in 6-inch diameter plastic “training” tubes of 2-, 3-, and 4-ft lengths. A drip irrigation system was installed by tapping an existing treatment effluent system averaging 15 gallons per minute. Drip lines in the “training” tubes were periodically lowered to below the estimated root depth to facilitate downward root growth. Tree height, diameter breast height (DBH), and survival were measured periodically. Leaf and branch samples were collected from six trees in different sections of the study area on November 19, 2003.

Study 3 consists of a PCE-contaminated northwest study (NW) and a TCE-contaminated Groundwater Treatment Unit study (GTU) (Rockwood et al. 2003). Tree height, DBH, and survival were measured annually. Samples were taken in July 2003 and July and November 2004 over both low- and high-concentration zones and a range of depths in the root zones of poplar clone I 45/51 and other *PD* clones in the NW and I 45/51 and willow clones SX61, S365, and 94014 in the GTU, as well as from a non-vegetated control, and kept on ice until aseptically separated into soil and root samples. Most probable number (MPN) and colony-forming unit (CFU) counts estimated the

abundance of the methanotroph and heterotroph populations, respectively. Methane and TCE oxidation activity potentials of methanotroph-heterotroph mixed cultures enriched from the soil and root samples were determined using oxygen uptake measurements using a Clarke-type electrode and an automated data acquisition system (Lindner et al. 2000).

Study 4 at a hydrocarbon-contaminated site near Gulfport, MS, had five local *PD* clones (Ken8, S7C1, S13C20, ST163, ST244) planted as 6-ft unrooted whips and 18-inch cuttings in March 2003 within a larger dendroremediation project. In adjacent rows (10 ft apart) of 75 cuttings or whips planted 3 ft apart, five replications of a RCBD were installed using three-tree row plots. After 24 months, the trees were measured for height, DBH, and survival.

Study 5 established 16 *EA* progenies and 32 local *PD* clones as a landfill cap at the Leon County landfill near Tallahassee, FL, in May 2004. Within species blocks of 18 rows spaced 6 ft apart of 12 trees spaced 4 ft apart and divided equally into lined and unlined sections, 7 *EA* progenies and 6 *PD* clones, respectively, were systematically allocated to single tree plots in seven replications. In the *PD* block, three cuttings each of DN-21 and DN-34 were included in, and planted adjacent to, two replications. In the remaining positions in each species block, additional progenies and clones were planted in 1-, 2-, 3-, or 12-tree plots. Tree height and survival were measured in August 2004.

Study 6 at the Duvall Landfill near Seattle was prepared with a modified ripper and planted with 5 ft whips of four poplar clones (TD15-29, TD52-225, TD-184-411, and OP367). A microspray irrigation system applied water during 2000 and 2001. Growth, precipitation, and site hydraulic conditions — especially percolation — were monitored.

RESULTS AND DISCUSSION

Tree growth and arsenic uptake in Study 1 varied considerably between and within *PD*, *EA*, and *EG*. After three months, compost had increased arsenic concentration in the soil by some two-fold but lowered arsenic concentration in *PV* by nearly 40% (Table 2). *PV* still demonstrated its capability to hyperaccumulate arsenic, with *PV* concentrations of as much as 80 times soil levels. Compost had not caused more rapid tree growth through three months, but *PD* was more vigorous than *EA* and *EG*. A tendency for fast-growing clones to have lower arsenic concentrations suggests that total arsenic uptake

TABLE 2. Three-month *PD*, *EA*, and *EG* tree heights (m) and arsenic concentrations (mg/kg) in *PV* and soil with and without compost for various fern-tree mixes in Study 1.

Mix	No Compost			Compost		
	Height	<i>PV</i>	Soil	Height	<i>PV</i>	Soil
<i>PD</i>	1.66	-	39.0	1.58	-	91.6
<i>PD-PV</i>	1.59	2355	43.9	1.29	1457	79.2
<i>EA</i>	0.65	-	33.4	0.74	-	69.6
<i>EA-PV</i>	0.60	2917	36.5	0.60	2096	73.8
<i>EG</i>	1.02	-	-	1.39	-	-
<i>EG-PV</i>	0.70	3139	41.4	0.73	1810	56.1
<i>PV</i>	-	3010	45.5	-	1923	71.5

will be a balance between biomass production and arsenic concentration. However, certain *PD* clones, such as ST-229, that combine fast growth with high concentration would maximize uptake.

Within tree variation for arsenic concentration was substantial in previous analyses at the Study 1 site (Rockwood et al. 2003). Arsenic was highest in the leaves, next highest in branch bark, followed by stem bark, then branch wood, and lowest in stem wood. Leaves from the lowest part of the crown had more than twice the concentration of leaves from the upper crown. Bark from upper branches tended to concentrations some 6 mg/kg less than upper leaves. Crown or stem position had little influence on arsenic concentrations in branch wood, stem bark, or stem wood in October.

Toluene concentrations throughout Study 2 have steadily decreased since mid-2003. As of July 2004, decreases in toluene concentrations were in a range of 4-fold to two orders of magnitude. The most significant reduction (from 68,000 µg/L to ~500 µg/L) was observed near a former recovery well close to an *EA* tree whose leaf sample had by far the highest toluene concentration. The rates of contaminant concentrations reduction seem proportional to the initial contaminant concentrations, and the contaminant concentrations at different parts of the study area are converging to the low hundreds range. Tree height and DBH results suggest that survival and growth rates are not affected by the initial contaminant concentrations. Species and genotype differences are evident, as *EA* was nearly twice as vigorous as *PD*, and an *EA* progeny was the most productive genotype after 40 months (Table 3).

TABLE 3. Number, mean, range, and significance (non-significant – NS, significant at 5% - *) of genotype means for representative traits (height – H (m), diameter – DBH (cm), and/or tree size index – D2H (cm²m)) at various ages (months) by species in components of five studies.

Study	Comp.	Trait	Age	Species	No.	Mean	Range	Significance
2	No Tubes	D2H	40	<i>PD</i>	15	33.5	2.2 – 60.7	*
				<i>EA</i>	15	72.2	13.1 – 199.5	*
3	NW	H	8	<i>PD</i>	18	1.46	0.73 – 2.00	*
		H	20			4.1	2.3 – 5.1	*
		D2H	20			4.60	1.36 – 10.28	*
	GTU	H	13	<i>Salix</i>	24	2.4	2.0 – 3.2	NS
4	Cuttings	D	24	<i>PD</i>	5	2.8	2.3 – 3.2	*
	Whips					2.5	1.9 – 3.1	*
5	Unlined	H	3	<i>PD</i>	34	1.49	0.80 – 1.90	*
	Lined					1.20	0.76 – 1.78	*
	Unlined			<i>EA</i>	16	0.83	0.66 – 1.08	NS
	Lined					0.82	0.64 – 1.05	NS

After 40 months, the “training” tubes considerably inhibited above ground growth of *PD* and *EA* (Table 4) and presumably root growth and access to groundwater. Trees planted in 2-ft, 3-ft, and 4-ft tubes had 10% less survival and were 0.8 m shorter and from 0.4-0.8 cm less in DBH.

TABLE 4. Number of trees, means, and significance for tube depths by *PD* and *EA* height and D2H at 40 months and survival at 54 months in Study 2.

Depth	Species	Height (m)			D2H (cm ² m)			Survival (%)		
		No.	Mean	Signif.	No.	Mean	Signif.	No.	Mean	Signif.
0	<i>PD</i>	145	3.29	a	126	41.0	a	180	65.6	a
	<i>EA</i>	161	3.33		135	72.2		180	76.1	
2	<i>PD</i>	34	2.04	b	25	8.7	ab	45	55.6	ab
	<i>EA</i>	36	2.24		26	43.5		45	66.6	
3	<i>PD</i>	30	2.13	b	21	9.8	b	45	46.7	b
	<i>EA</i>	33	2.11		25	12.5		45	55.5	
4	<i>PD</i>	33	2.02	b	22	7.6	b	45	55.6	ab
	<i>EA</i>	34	2.15		24	17.3		45	62.2	

At a TCE- and PCE-contaminated site in Orlando (Rockwood et al. 2003), *PD* whips deep planted 6 ft into sandy soil averaged 18 ft in height in six months. In the operational planting of some 2,000 trees, locally adapted *PD* clones typically grew more than poplar clone DN-34, which is widely planted for phytoremediation in more temperate regions. In whip-cutting comparisons in an area of fluctuating groundwater, whips of ST-261 were more than twice as large as cuttings, but whips and cuttings had similar root biomass allocations. *PD* clone ST-261 was slightly taller than DN-34, but DN-34 had proportionately more root biomass at some 72%. Roots on the bottom 3 ft of all whips had died due to anaerobic conditions. However, in the drier site, root development went deeper than the original 6 ft planting depth, and the root biomass allocation of DN-34 was even larger.

Two-year-old poplar clones in the NW component of Study 3 ranged widely around an average height of 4.1 m (Table 3) and DBH of 3.3 cm. Survival was excellent except for a few frost susceptible clones. The largest poplar clone, ISU 25-R4, a cottonwood, averaged 5 m in height and 4.5 cm in diameter. The five best clones were as much as twice the average size and supported more methanotrophs. Nine clones exceeded the growth of the standard clone, Eugenei. Thus, these new clones may have enhanced dendroremediation potential compared to the industry standard.

The soil and root samples from both contaminated sites contained more heterotrophs (e.g., $2 \times 10^6 \text{ g}^{-1}$ dry soil to $3 \times 10^9 \text{ g}^{-1}$ fresh root) than methanotrophs ($3 \times 10^2 \text{ g}^{-1}$ dry soil to $6 \times 10^6 \text{ g}^{-1}$ fresh root). Additionally, methanotrophs were less numerous in higher PCE- and TCE-contaminated samples. Tree roots had more heterotrophs than soil ($3 \times 10^9 \text{ g}^{-1}$ fresh root vs. $5 \times 10^8 \text{ g}^{-1}$ dry soil). Conversely, the numbers of methanotrophs did not show as clear a trend and were influenced by the contaminant type. Methanotrophs were higher in roots than in soil from the low- and high-concentration TCE zones, but comparable in both concentration zones of the PCE site.

Below-ground biomass increased from July 2003 to November 2004. While heterotroph counts at either site did not change significantly, methanotrophs at the PCE site declined. In particular, at high PCE concentrations, counts decreased from 1×10^6 to $3 \times 10^2 \text{ g}^{-1}$ dry soil from 2003-2004. In general, microbes were higher at the TCE site, perhaps because the trees were grown in pots containing planting material (mulch) that may have enriched thriving methanotrophs. In contrast, the PCE trees were established in native soil where methanotrophs, not capable of oxidizing PCE, may be nutrient-limited,

not as active, and more susceptible to the toxic effects of PCE compared to those present at the TCE site. Contrary to methanotrophs in the poplar and two of the willow clones' rhizospheres, soils of clone SX61 contained more methanotrophs in the high-concentration TCE zone compared to the low-concentration TCE (1×10^5 and $3 \times 10^5 \text{ g}^{-1}$ dry soil, respectively). These results imply that SX61 may possess greater resistance to higher TCE.

Methanotroph enriched mixed cultures from 2003 and 2004 root and soil samples of all tree clones were analyzed for their oxidative potential in the presence of their primary substrate, methane. The oxidative potential of the control was always the highest (Figure 1), reflecting the effect of the contaminant over methanotrophs, as rhizosphere samples are exposed to higher contaminant concentrations than the control.

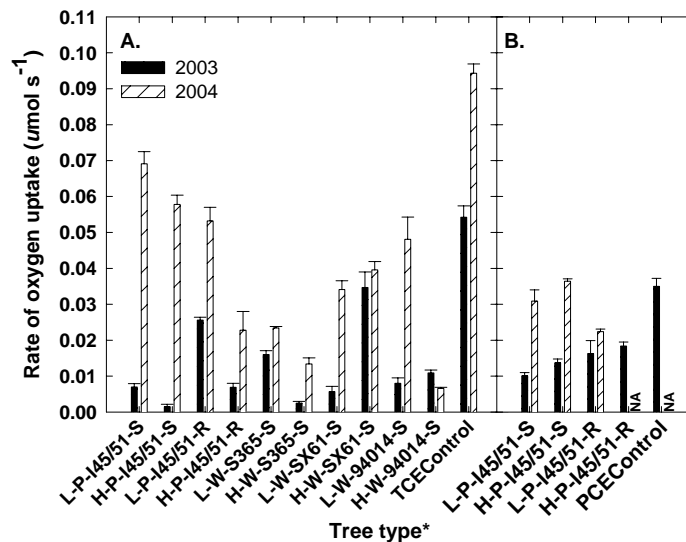


FIGURE 1. Oxygen uptake for enriched mixed cultures from rhizosphere samples of different contaminant concentration (L-low/H-high), species (P-poplar/W-willow), clone, and rhizosphere (S-soil/R-root) at the TCE (A) and PCE sites (B), LaSalle, IL.

At the TCE site (Figure 1A), oxygen uptake rates in the presence of methane increased with time, and poplars showed the most dramatic increase from 2003 to 2004. Cultures from the 2003 poplar roots showed higher oxidative potential than the soils, whereas this trend was reversed in 2004 (with higher methane oxidation displayed by cultures from soils). However, higher rates were observed with cultures from the lower TCE-contaminated sites for both years. In contrast, the PCE site (Figure 1B) showed similar oxygen uptake rates between roots and soils from each clone, and between high and low contaminant concentration. As observed in the TCE site, rates increased with time but were lower than those obtained from the rhizosphere enrichments from the poplars at the TCE site (Figure 1A).

Oxidative patterns for the willow enrichments (Figure 1A) were not as clear. SX61 enrichment was positively affected by high contaminant concentration, and S365 enrichment was negatively affected, in accordance with the counts of methanotrophs discussed

above. However, 94014 enrichments showed different results. The highest oxidation rate obtained from all 2003 enrichments was observed with SX61 cultures from samples taken from the *high* contaminant concentration. However, the 94014 enrichments from *low* TCE showed the higher rate of methane oxidation in 2004. These results emphasize differences in willow clones that may lead to the selection of the best clone for these particular phytoremediation conditions.

In Study 4, cuttings and whips were of equal size after two years, and five local *PD* clones varied considerably (Table 3). Although the cuttings that grew to be slightly taller and larger in DBH than the whips suggest that planting whips is unnecessary, the 15% lower survival of the cuttings suggests that whips may be more stress tolerant. While the five clones did not differ significantly in tree size (D2H), clone S7C1 was notably shorter and less in DBH than Clone S13C20, and Clone ST244 performed poorly when planted as a whip.

In Study 5 after three months, 16 *EA* progenies and 32 local *PD* clones varied in survival and tree height but surpassed two non-adapted poplar clones (Table 3), by from 0.3 to 1.0 m in height. *EA* tree size and survival tended to be similar in the unlined and lined sections of the landfill cap, whereas *PD* was typically taller in the unlined section.

In Study 6 through 2005, remarkable growth rate differences were observed among the poplar clones. Response to compost-added fertility was best for TD15-29, and all TD clones grew more than OP367. Mountain vole infestation was worse on OP367 and 184-411. Plant diversity is increasing by the maintenance program — especially including more conifers that increase winter evapotranspiration during the deciduous dormant season. As the phyto forest matures, the diversified habitat benefits wildlife populations.

All genotypes in these studies were derived from regional tree improvement programs. Because the *PD* clones in Studies 1, 2, 4, and 5 did not include the best 100 of over 1,000 new clones that surpassed standard clones in a Southeast-wide *PD* improvement program, more productive *PD* clones may be available for phytoremediation in the Southeast. All poplar and willow genotypes in Study 2 were derived from Midwest and Lake States regional tree improvement programs. The genetic variability detected compared with regional performance data suggests that poplar clones ISU 25-R4, ISU 25-21, 51-5, ISU 25-R5, and 80x01107 are most suitable for achieving the remediation goals at LaSalle, IL, and similar sites.

These and similar results elsewhere (e.g., Coyle et al. 2005, Licht and Isebrands 2005) illustrate the considerable importance of field testing species and genotypes for appropriate prerequisites in order to reach the phytoremediation potential of fast-growing trees.

CONCLUSIONS

Effective phytoremediation of heavy metal- or chlorinated solvent-contaminated sites by fast-growing trees such as *EA*, *EG*, and *PD* is strongly influenced by the choice of tree genotypes. As evidenced in ongoing phytoremediation systems, genetic testing is necessary to match species and genotypes (e.g., clones, progenies) within species to site, microclimate, and contaminant conditions. These results illustrate the considerable importance of field testing species and genotypes for appropriate prerequisites, as suggested by Westphal and Isebrands (2001), in order to reach the phytoremediation potential of

fast-growing trees. Methanotroph abundance and activity with poplars were less at high contaminant concentrations, but higher with a willow clone.

ACKNOWLEDGMENTS

This paper reports results of research supported by the Florida Department of Environmental Protection, Reichhold Chemicals, the Illinois Environmental Protection Agency, King County Solid Waste Department, and the NIEHS UF Superfund Basic Research Program.

REFERENCES

- Brigmon R.L., T.A. Anderson, C.B. Fliermans. 1999. Methanotrophic bacteria in the rhizosphere of trichloroethylene-degrading plants. *International Journal of Phytoremediation* 1:241-253.
- Coyle D.R., M.D. Coleman, J.A. Durant, and L.A. Newman. 2005. Survival and growth of 31 *Populus* clones in South Carolina. *Biomass and Bioenergy*. (in press).
- Fox B.G., J.G. Borneman, L.P. Wackett, J.D. Lipscomb. 1990. Haloalkene oxidation by the soluble methane monooxygenase from *Methylosinus trichosporium* OB3b: mechanistic and environmental implications. *Biochemistry* 29:6419-6427.
- Hanson R.S., T.E. Hanson. 1996. Methanotrophic bacteria. *Microbiological Reviews* 60:439-471.
- Licht L., and J.G. Isebrands. 2005. Linking phytoremediated pollutant removal to biomass economic opportunities. *Biomass and Bioenergy* 28:203-218.
- Lindner A.S., P. Adriaens, J.D. Semrau. 2000. Transformation of *ortho*-Substituted Biphenyls by *Methylosinus trichosporium* OB3b: Substituent Effects on Oxidation Kinetics and Product Formation. *Archives of Microbiology* 174:35-41.
- Little C.D., A.V. Palumbo, S.E. Herbes, M.E. Lidstrom, R.L. Tyndall, P.J. Gilmer. 1988. Trichloroethylene biodegradation by a methane-oxidizing bacterium. *Applied and Environmental Microbiology* 54:951-956.
- Rockwood, D.L., G.R. Alker, R.W. Cardellino, C. Lin, N. Brown, T. Spriggs, S. Tsangaris, J.G. Isebrands, R.B. Hall, R. Lange, and B. Nwokike. 2003. Fast-growing trees for heavy metal and chlorinated solvent phytoremediation. In: V.S. Magar and M.E. Kelley (Eds.), *In Situ and On-Site Bioremediation—2003*. Proceedings of the Seventh International In Situ and On-Site Bioremediation Symposium, June 2-5, 2003, Orlando, FL, Paper F-12, Battelle Press, Columbus, OH. CD format.
- Rockwood, D.L., C.V. Naidu, D.R. Carter, M. Rahmani, T. Spriggs, C. Lin, G.R. Alker, J.G. Isebrands, and S.A. Segrest. 2004. Short-rotation woody crops and phytoremediation: Opportunities for agroforestry? In: *New Vistas in Agroforestry, A Compendium for the 1st World Congress of Agroforestry 2004*, P.K.R. Nair, M.R. Rao, and L.E. Buck (Editors), Kluwer Academic Publishers, Dordrecht, The Netherlands. p. 51-63.
- Uchiyama H., T. Nakajima, O. Yagi, T. Nakahara. 1992. Role of heterotrophic bacteria in complete mineralization of trichloroethylene by *Methylocystis* sp. strain M. *Applied and Environmental Microbiology* 58:3067-3071.
- Westphal L.M., and J.G. Isebrands. 2001. Phytoremediation of Chicago's brownfields – consideration of ecological approaches and social issues. Proceedings of Brownfields 2001 Conference. Chicago, IL. BB-11-02. <http://www.brownfields2002.org/proceedings2001/BB-11-02.pdf>.