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BIODEGRADATION OF 1,4-DIOXANE IN PLANTED AND UNPLANTED SOIL: EFFECT OF BIOAUGMENTATION WITH *AMYCOLATA* sp. CB1190

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Abstract—1,4-dioxane is one of the most recalcitrant and toxic contaminants in the subsurface. This study investigated the potential to enhance dioxane biodegradation in both planted and unplanted soil, by adding the dioxane-degrading actinomycete, *Amycolata* sp. CB1190. Dioxane was not removed within 120 days in sterile controls or in viable microcosms not amended with CB1190. Poplar root extract (40 mg/L as COD) stimulated dioxane degradation in bioaugmented soil, and 100 mg/L dioxane were removed within 45 days. Other co-substrates that enhanced dioxane degradation by CB1190 include tetrahydrofuran (THF) and 1-butanol, while glucose and soil extract did not affect dioxane degradation. The stimulatory effect of THF was partly due to enhanced enzyme induction, while that of root extract and 1-butanol was attributed to additional growth of CB1190. In another experiment with dioxane added at 10 mg/kg-soil, reactors planted with hybrid poplar trees removed (by evapotranspiration and biodegradation in the root zone) more dioxane within 26 days than unplanted reactors, regardless of whether CB1190 was added. Nevertheless, CB1190 enhanced mineralization of [^{14}C]-dioxane in all experiments. This enhancement was more pronounced in unplanted soil because plant uptake reduced the availability of dioxane for microbial degradation. These results suggest that bioaugmented phytoremediation is an attractive alternative to remove dioxane from shallow contaminated sites. © 2001 Published by Elsevier Science Ltd.

Key words—mineralization, phytoremediation, root extract, substrate interactions, tetrahydrofuran

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INTRODUCTION

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1,4-Dioxane (dioxane) is a cyclic ether that is widely used as a stabilizer for chlorinated solvents. Dioxane is also used as a solvent in paints, lacquers, cosmetics, deodorants, fumigants and detergents, and is also an undesirable byproduct of some industrial processes such as polyester synthesis (Howard, 1990; US EPA, 1995). Dioxane has been detected in surface and groundwater supplies across the United States (Burmaster, 1982), which is of concern because of its persistence and potential carcinogenicity (Klaasen *et al.*, 1986). Clean up of dioxane-contaminated sites is a very difficult task because this xenobiotic is recalcitrant to microbial degradation, has a low tendency to volatilize from water, and is highly mobile in groundwater due to its low tendency to be retarded by sorption (Adams *et al.*, 1994; Howard, 1990).

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Phytoremediation, the use of plants to remove environmental pollutants, shows great promise as an approach to clean up dioxane-contaminated sites (Aitchison *et al.*, 2000). In addition to stabilizing soils and reducing vertical percolation, some plants can enhance the removal of such toxic chemicals by at least two mechanisms (Schnoor, 1997). These are: (1) direct contaminant uptake and, in some cases, in-plant transformations to less toxic metabolites or volatilization to the atmosphere (Burken and Schnoor, 1996; Nair *et al.*, 1992; Shimp *et al.*, 1993; Simonich and Hites, 1995; Thompson *et al.*, 1998), and (2) stimulation of microbial activity and biochemical transformations in the rhizosphere through the release of root exudates and plant enzymes (Table 1) (Anderson *et al.*, 1993; 1994; Newman, 1995; Reilley *et al.*, 1998; Vaughan *et al.*, 1994; Walton and Anderson, 1990). This latter mechanism, however, is relatively ineffective for removing dioxane because microorganisms with the potential to synthesize dioxane-degrading enzymes are rare in nature, and root exudates can dioxane degradation only if appropriate genotypes are present. Thus, activating

Table 1. Compounds detected in root exudates

Compounds	Example of compound	Reference
Carbohydrates	Glucose, fructose, sucrose, maltose, galactose, xylose, oligosaccharides	[16]Curl and Truelove, 1986
Amino acids	Glycine, glutamic acid, aspargine, serine, alanine, lysine, arginine, threonine, homoserine	[16]Curl and Truelove, 1986
Aromatics	Phenols, l-carvone, p-cymene, limonene, isoprene	[19]Fletcher and Hegde, 1995; [20]Gilbert and Crowley, 1997
Organic acids	Acetic acid, propionic acid, citric acid, butyric acid, valeric acid, malic acid	[16]Curl and Truelove, 1986
Volatile compounds	Ethanol, methanol, formaldehyde, acetone, acetaldehyde, propionaldehyde, methyl sulfide, propyl sulfide, allyl sulfide	[16]Curl and Truelove, 1986
Vitamins	Thiamine, biotin, niacin, riboflavin, pyridoxine, pantothenic acid	[16]Curl and Truelove, 1986
Enzymes	Phosphatase, dehydrogenase, peroxidase, dehalogenase, nitroreductase, laccase, nitrilase	[16]Curl and Truelove, 1986; [42]Vaughan *et al.*, 1994

this removal mechanism might require inoculating the rhizosphere with specialized strains (i.e., bioaugmentation).

Microorganisms have been previously added to the rhizosphere as pest control agents or plant-growth promoting rhizobacteria to enhance agricultural practices, with survival of the added bacteria being a common limiting factor (Colbert *et al.*, 1993; Walter, 1987). The concept of enhancing phytoremediation through the addition of specialized degrader bacteria to the root zone is new, and has been practised only at the bench scale. Two studies have been reported on this topic, both of which were conducted by the same group. Crowley *et al.* (1996) used *P. fluorescens* 2-79RLD to enhance phytoremediation of 2,5-dichlorobenzoate (DCB). This strain contains a chromosomal *lux* marker for tagging as well as the catabolic plasmid pPB111, which codes for DCB degradation. The rhizosphere of bean plants (*Phaseolus vulgaris*) promoted rapid degradation of DCB and provided a niche that enhanced maintenance of plasmid pPB111 in the degrader bacterium. The other study (Alvey and Crowley, 1996) used a mixed culture of atrazine-mineralizing bacteria to inoculate the rhizosphere of corn plants. Bioaugmentation greatly enhanced the rate of atrazine mineralization, and the rhizosphere enhanced the long-term survival of the added microorganisms. To our knowledge, no previous studies have focused on bioaugmentation for the specific purpose of enhancing biodegradation of dioxane in either rhizosphere or unplanted soil.

To rationally exploit bioaugmented phytoremediation as a hazardous waste management approach, we need to improve our understanding of the interactions between the specific degraders and plant roots. In this work, we evaluated the potential to enhance dioxane removal in both planted and unplanted soil, by inoculating batch reactors with a dioxane-degrading bacterium. Hybrid poplar trees, which are widely used as phytoremediation tools (Schnoor, 1997), were used in the planted-soil experiments. The potential for poplar trees to affect dioxane mineralization in the root zone was also addressed.

MATERIALS AND METHODS

Microbial seed

Amycolata sp. CB1190 is the dioxane-degrading microorganism that was used for bioaugmentation. This bacterium was isolated from dioxane-contaminated industrial sludge by Parales *et al.* (1994), and was deposited with the American Type Culture Collection as strain 55486. CB1190 is an actinomycete, of the family *Pseudonocardiaceae*. The original enrichment degraded dioxane only cometabolically, with tetrahydrofuran (THF) as the primary substrate. Consequently, it was suggested that CB1190 is a mutant THF-degrading organism with a broader substrate range (Parales *et al.*, 1994). CB1190 can grow aerobically on dioxane as the sole carbon and energy source (doubling time 30 h at 30°C), although it grows much faster on THF (doubling time 11 h) (Parales *et al.*, 1994). CB1190 was also reported to grow well on 1-butanol and other cyclic and linear ethers such as 1,3-dioxane, tetrahydropyran, 2-methyl-1,3-dioxolane, butyl methyl ether, and diethyl ether. Interestingly, CB1190 grows slowly on glucose (doubling time 32 h) and cannot grow on some Kreb's cycle intermediates (e.g., citrate, fumarate, and succinate) or on other easily degradable compounds such as lactose, fructose, maltose, starch, and the amino acids asparagine and glutamate. Little is known about the enzyme system that CB1190 utilizes to degrade dioxane, and if it is inducible, which substrates can serve as inducers.

For bioaugmentation purposes, CB1190 was grown in basal salts medium amended with 1000 mg/L THF. The growth medium is described elsewhere (Parales *et al.*, 1994). Cells were harvested in late exponential growth phase and washed 3 times by centrifuging at 13,000 rpm in a Marathan 21K/BR apparatus (Fisher Scientific). Cells were resuspended in basal salts medium prior to inoculation. CB1190 concentrations were estimated based on optical density measurements at 600 nm (OD_{600}), using a conversion factor of 1 OD_{600} unit is equal to 400 mg/L of volatile suspended solids (VSS) (Koch, 1994).

Microcosm and pure-culture experiments

Microcosms were used to evaluate the potential to enhance dioxane removal from contaminated soil by adding CB1190. Microcosms were also used to determine if poplar root extract enhanced dioxane degradation, and if so, to assess the nature of the stimulatory effect.

Microcosms were prepared in duplicate using 250 mL bottles amended with 20 g of Nodeway uncontaminated soil ($f_{oc} = 0.02$) and 200 mL of the basal salts medium used to grow CB1190. All microcosms were fed dioxane (100 mg/L) and capped with Mininert valves. Bioaugmented microcosms were inoculated with 2 mL of CB1190 suspension

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(OD_{600} = 0.015). No-treatment controls were prepared without CB1190, and sterile controls were poisoned with 170 mg/L $HgCl_2$. To evaluate the stimulatory effect of plant-exuded substrates, one additional set of bioaugmented microcosms was prepared with poplar root extracts, added at 40 mg/L as chemical oxygen demand [COD]. This concentration is within the typical range found in the rhizosphere (Curl and Truelove, 1986) and is representative of poplar root exudates (Kelley, 1999). Although root extracts do not necessarily have the same composition as root exudates, extracts were used in this study because it is difficult to obtain exudates in sufficient quantities for microbial growth and induction studies. In addition, root extracts contain many of the same organic substrates that induce and support microbial activity (Gilbert and Crowley, 1997).

To prepare the root extract, clean roots were combined with DI water (200 mL/g) and ground in a Waring Lab Blender for 3 min. The blend was vacuum-filtered through a series of filters of decreasing pore size were: Whatman 4 filter paper, Whatman GF/C glass fiber filter, and Whatman GF/F glass fiber filter. The extract was then filtered–sterilized with a Millex-GS 0.22 μm syringe filter. The COD of the extract was measured using a Hach kit (Hach Co., Loveland, CO), diluted to the desired COD concentration, and used within 1 day.

To ensure that O_2 was not limiting, the headspace of all microcosms was periodically flushed with sterile air introduced through a needle. The pH of the microcosms was monitored periodically, and was always between 6.5 and 6.7. All microcosms were incubated on a Lab-Line Orbit Shaker at 150 rpm and about 20°C.

Batch experiments were also conducted to study how root extracts and other potentially co-occurring substrates affect dioxane degradation by CB1190. These experiments were set up similarly in 250 mL bottles, except that dioxane was added at about 25 mg/L, CB1190 was inoculated at a higher concentration (OD_{600} = 0.1), and no soil was added. Dioxane was fed alone (no-treatment control) or together with either THF, root extract, or soil extract at about 40 mg/L as COD. The soil extract represents naturally occurring background organic matter, and was prepared by autoclaving 1 kg of the same Nodeway soil used in the microcosms with 1 L of distilled water. Centrifugation and vacuum filtration were used to separate the soil extract prior to dilution to the desired COD. THF, root extract, or soil extract was fed at about. This experiment was repeated under similar conditions with 1-butanol as co-substrate, with dioxane fed at 60 mg/L and 1-butanol at about 100 mg/L as COD.

Additional batch experiments were set up to determine if the dioxane-degrading enzyme system in CB1190 is constitutive or inducible, and if inducible, which growth substrates induce it best. CB1190 was grown on either dioxane, THF, 1-butanol, glucose, root extract, or soil extract. Cells were harvested in late exponential phase, centrifuged, and resuspended in basal salts medium at equal concentrations on a protein basis (50 mg/L protein). The protein concentration was measured with a Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA) using the Bradford method (Bradford, 1976). Enzyme activity was then measured with whole cells as the rate of dioxane degradation (added at 30 mg/L) normalized to the protein concentration. These degradation assays were conducted in the presence of chloramphenicol (350 mg/L) to inhibit *de novo* enzyme synthesis (Cundliffe and McQuillen, 1967). Chloramphenicol prevents cell growth and ensures a constant enzyme concentration during the assay, which simplifies data interpretation (Costura and Alvarez, 2000).

Mineralization experiments with poplar trees

^{14}C-labeled dioxane (Moravek Biochemical, purity >98%) was used as a tracer to determine whether adding CB1190 to the poplar rhizosphere or to unplanted soil could enhance dioxane mineralization and removal kinetics. The primary mechanisms influencing the fate of dioxane (*i.e.*, plant uptake and accumulation, volatilization through evapotranspiration, microbial degradation, and adsorption onto soil) were studied interactively using plant bioreactors that compartmentalized the root zone separately from the rest of the plant, as described elsewhere (Aitchison *et al.*, 2000) (Fig. 1).

For planted reactors, hybrid poplar cuttings (200 mm), identical male clones from adult Imperial Carolina hybrid poplar trees (*Populus deltoides* × *nigra*, DN34) were allowed to root hydroponically for approximately 2 weeks in half-strength Hoagland's nutrient solution. This solution contained (in mg/L of distilled water) KH_2PO_4 (208), $CaNO_3$ (161), $CaSO_4$ (289) KNO_3 (137) $MgSO_4$, and (469) K_2SO_4 (161). A predrilled screw cap and Teflon-lined septum (Weaton Scientific, Milville, NJ) were fit snugly around each of the cuttings using rubber sealant. The cuttings were then planted in 280 mL glass screw-top flasks containing 185 g uncontaminated topsoil ($f_{oc} = 8.4\%$) mixed with about 90 mL of the nutrient solution. A modified 1-L Erlenmeyer flask was placed over the aerial portion of the cutting to capture any dioxane volatilized through the leaves (Aitchison *et al.*, 2000), and the top flasks were sealed to the bottom flasks with Parafilm to compartmentalize the root zone. The reactors were weighed initially and every third day, and water was added periodically to keep soil moisture at about 70% field capacity. The field capacity, which is the soil water-holding capacity after gravity drain, was estimated by saturating the soil, allowing it to drain, and drying it for 24 h at 100°C to determine soil moisture content. Three out of six planted reactors were inoculated with CB1190 at about 10^7 cells/g-soil, assuming a dry cell weight of 10^{-12} g (Bitton, 1996). CB1190 was added in liquid culture (16 mL, OD_{600} = 0.6) to the bottom compartment to achieve this concentration.

Unplanted reactors were prepared similarly but without a top reactor compartment, and were not watered during the course of the experiment. Sterile controls were also prepared, by amending the nutrient solution with 10 g/L $HgCl_2$. Similar to the planted reactors, two out of four unplanted reactors were bioaugmented with CB1190 at about 10^7 cells/g-soil. Experiments were also conducted with excised-tree reactors that offered a root zone (exuding potentially stimulatory substrates) but could not remove much dioxane by plant evapotranspiration. Three "excised-tree" reactors were prepared by cutting off the top of the tree just above the caps before the top flask was added, and one was bioaugmented also at about 10^7 cells/g-soil.

All reactors were spiked with about 10 mg cold dioxane plus 5 μCi [^{14}C] dioxane per kg of soil. Air was pulled into the bottom portion of the reactor through an Orbo (activated carbon) tube (32 large, Supelco) at 2 L/min, and out through another Orbo tube to capture any dioxane volatilized from dry soil (removed by air suction). The air was also passed through two NaOH traps (1 N, 15 mL) in series to capture any $^{14}CO_2$ produced by microbial degradation. Air was also pulled through the top portion of the planted reactors through two Orbo tubes in series to capture transpired dioxane, and into a 200 mL NaOH trap to capture any potential $^{14}CO_2$ released by the plant. The NaOH traps were sampled and changed approximately every 2 to 3 days. The Orbo tubes were also changed every 2 to 3 days to prevent saturation of the binding sites, which could lead to dioxane breakthrough.

A walk-in fume hood equipped with 16 Vita-Lite growth lights was used as a growth chamber. All reactors were incubated for 26 days with a light intensity that varied between 110 and 165 μmol/m^2 s, for 16 h per day. The temperature was kept at about 24°C.

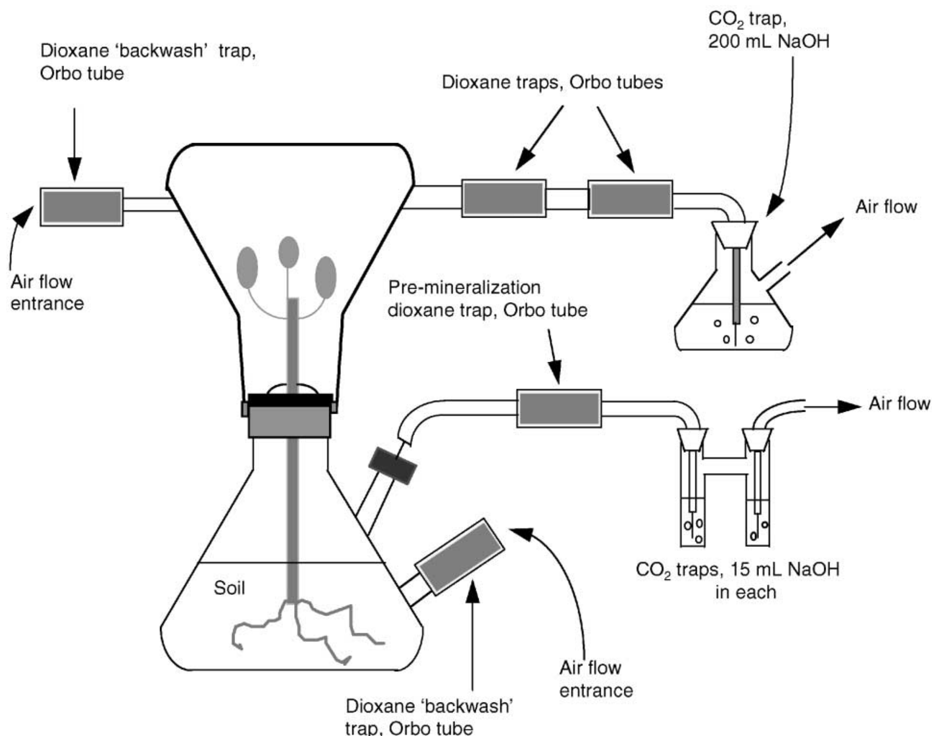

Fig. 1. Planted bioreactors. Bottom compartment was sealed to separate microbial mineralization in the rhizosphere from evapotranspiration from leaves. Air flowed through bottom compartment into two 15 mL NaOH traps in series, and through top compartment into one 250 mL NaOH trap to capture $^{14}CO_2$. Activated carbon Orbo tubes captured [^{14}C]-dioxane released through evapotranspiration or volatilization from soil.

Analytical methods

Cold dioxane was analyzed using a Hewlett-Packard 5890A gas chromatograph equipped with a DB-WAX capillary column (J&W Scientific, Fossom, CA), a flame ionization detector, and a Hewlett-Packard autosampler. The limit of detection was 1 mg/L. A Beckman 6000IC liquid scintillation counter (LSC) was used to determine the concentration of ^{14}C compounds. The LSC counted for 15 min or until a 95% confidence level was reached. At the end of the mineralization experiment, soil and plant tissue were oxidized with a RJ Harvey OX-600 Biological Oxidizer to convert any residual [^{14}C]-dioxane to $^{14}CO_2$. After the samples were oxidized, a 10 mL scintillation cocktail sample was removed from the trap and analyzed on the LSC. To analyze for [^{14}C]-dioxane trapped in the Orbo tubes, the activated carbon was poured into 4 mLvials and submerged in 2 mL of acetone for 24–48 h. Acetone samples (100 µl) were added to 15 mL Ultima Gold scintillation cocktail for LSC analysis. To quantify $^{14}CO_2$ generation, 1 mL samples were transferred daily from the NaOH traps to 15 mL of scintillation cocktail for counting on the LSC.

RESULTS AND DISCUSSION

Microcosm and pure-culture experiments

Dioxane removal in batch reactors, but not in sterile controls, provided evidence of biodegradation. Positive controls seeded with CB1190 ($OD_{600} = 0.01$) degraded 100 mg/L dioxane in incubations without soil within one month (data not shown). Dioxane degradation resulted in CB1190 growth, although the cell yield ($Y = 0.06$ g-VSS/g-COD) was relatively small for an aerobic heterotrophic processes (Metcalf and Eddy, 1993). Dioxane concentrations above 500 mg/L were apparently inhibitory to CB1190 because little degradation (or growth) occurred within 4 months.

Dioxane was not removed in sterile controls or in microcosms not amended with CB1190 within 120 days (Fig. 2), indicating that the indigenous soil population did not have the propensity to degrade dioxane. Nevertheless, the indigenous consortium adapted after this long lag time and removed about 60% of the added (100 mg/L) dioxane after 6 months. It is unknown if biodegradation was the result of a genetic mutation in response to dioxane exposure, or if the long lag reflects the time required by indigenous specific degraders to grow to a critical concentration capable of exerting measurable degradation rates (Alexander, 1999). Regardless of the responsible adaptation mechanisms, bioaugmentation significantly shortened the acclimation period, which is important for rapid degradation of contaminants that are briefly exposed to microorganisms before transport into groundwater. In this work, all microcosms with CB1190 degraded dioxane faster and without an apparent acclimation period, suggesting the feasibility of bioaugmentation to degrade this recalcitrant compound.

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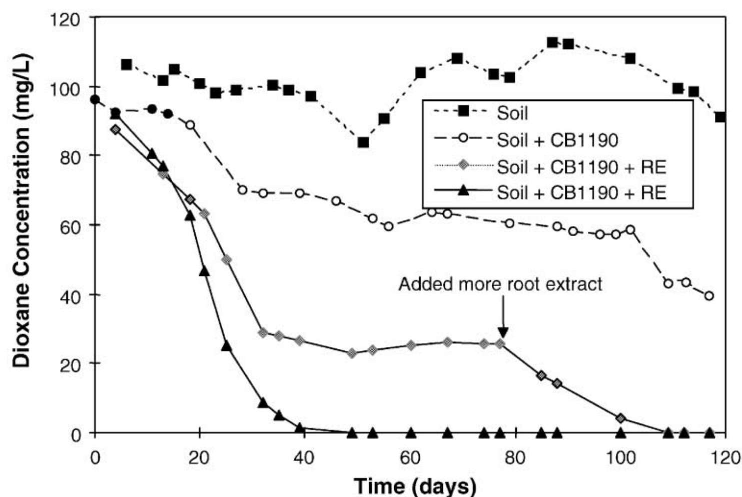

Fig. 2. Dioxane degradation in soil microcosms amended with CB1190, root exudate (RE), both, or none.

Root extract stimulated dioxane degradation in microcosms with CB1190, and 100 mg/L was removed within 45 days. Interestingly, a duplicate bioaugmented microcosm stopped degrading dioxane once it decreased to 25 mg/L, and spiking this reactor with additional root extract stimulated further degradation of the remaining dioxane (Fig. 2). Because some root exudates are known to contain enzymes that can degrade environmental pollutants (Table 1), batch tests were also conducted with filter-sterilized root extract (but without CB1190 or soil) to investigate if poplar enzymes contributed to dioxane degradation. Nevertheless, dioxane (10 mg/L) was not removed in these reactors within 1 month (data not shown), suggesting that the beneficial effect of root extracts was related to its effect on microbial growth and/or activity.

Enzyme induction assays

Some aromatic compounds exuded by plant roots (e.g., phenols, *l*-carvone, and *p*-cymene) induce microbial dioxygenase enzymes that have relaxed specificity and degrade polychlorinated biphenyls (Donnelly *et al*., 1994; Fletcher and Hedge, 1995; Gilbert and Crowley, 1997). Thus, some compounds exuded by plants could serve as primary substrates for the co-oxidation of some priority pollutants (Anderson *et al*., 1993). Nevertheless, roots also exude easily degradable compounds such as sugars and aminoacids (Table 1) that could hinder bioremediation by repressing enzyme induction or serving as preferential substrates (diauxy). Therefore, additional experiments were conducted with pure CB1190 cultures to discern the overall effect of root extracts on catabolic enzyme induction.

CB1190 was grown on root extracts and other potentially co-occurring substrates, and washed cells were subsequently assayed for dioxane-degradation activity in the presence of chloramphenicol. Glucose-grown cells were used as a negative control to ensure that chloramphenicol prevented *de novo* enzyme synthesis when cells were exposed to dioxane during the assay. Dioxane-grown cells were used as positive controls to ensure that chloramphenicol (350 mg/L) did not adversely affect the activity of induced cells. These cells degraded dioxane at similar rates (*ca*. 1 g-dioxane g-protein^{-1} day^{-1}) in the presence or absence of chloramphenicol. Cells grown on root extract, soil extract, glucose, or 1-butanol degraded dioxane without chloramphenicol, verifying that the cells were viable and could synthesize appropriate degradative enzymes when exposed to dioxane. Nevertheless, cells grown on these substrates did not degrade dioxane when chloramphenicol was present (Table 2). This indicates that CB1190's dioxane-degrading enzyme system is inducible, and that root extract, soil extract, 1-butanol, and glucose did not induce it.

Table 2. Induction of dioxane degradation activity in CB1190 by different growth substrates

Growth substrate	Specific activity (g-dioxane/(g-protein day))
1,4-Dioxane	0.92 ± 0.29
Tetrahydrofuran	1.53 ± 0.26
1-Butanol	0.00 ± 0.01
Glucose	0.00 ± 0.01
Poplar root extract	0.00 ± 0.01
Soil extract	0.00 ± 0.01

Of the tested substrates, only dioxane and THF induced dioxane-degrading activity in CB1190. Interestingly, the highest specific activity was obtained for THF as the growth substrate (Table 2), which supports the notion that CB1190 might be a THF-degrading mutant organism with broader substrate range (Parales *et al*., 1994). We also determined that

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the cell yield is higher for THF than for dioxane ($Y = 0.22$ vs. 0.06 g-VSS/g-COD). Since CB1190 also grows faster on THF than on dioxane (Parales *et al.*, 1994), these results suggest that THF would be a good substrate to grow and induce CB1190 for bioaugmentation purposes.

Overall effect of potentially co-occurring substrates on dioxane degradation by CB1190

Additional pure-culture experiments were conducted with binary mixtures to study substrate interactions between dioxane and either root extract, soil extract, THF, 1-butanol, or glucose. Incubations were also prepared with dioxane alone to establish a baseline for determining the effect of the other co-substrates. These experiments confirmed that root extract and THF can enhance dioxane degradation by CB1190. Dioxane degradation was also enhanced by 1-butanol, while soil extract and glucose had no significant effect (Fig. 3). These experiments were repeated, and the same trends were observed.

The reason(s) for the beneficial effect of root extract is unclear, although as shown previously, it cannot be attributed to enhanced enzyme induction or to the presence of plant enzymes. Because root extract can serve as an energy source to stimulate additional (coincidental) growth of CB1190, its beneficial effect could be due to increasing the concentration of CB1190. This would be conducive to faster degradation rates. In theory, root extract could also be providing co-factors that enhance the viability of CB1190, such as vitamins or amino acids. In addition, many oxygenase enzymes are NADH-dependent (Zylstra and Gibson, 1989), and their activity can be exhausted when NADH is depleted faster by other metabolic reactions than regenerated by catabolism of the target pollutant (Henry and Grbič-Galič, 1991). Therefore, if dioxane degradation were mediated by such an enzyme system, root extract could serve as an electron donor to regenerate NADH and indirectly sustain dioxane degradation activity. This would explain, in part, the restoration of dioxane degradation activity in the microcosm respiked with root extract (Fig. 2).

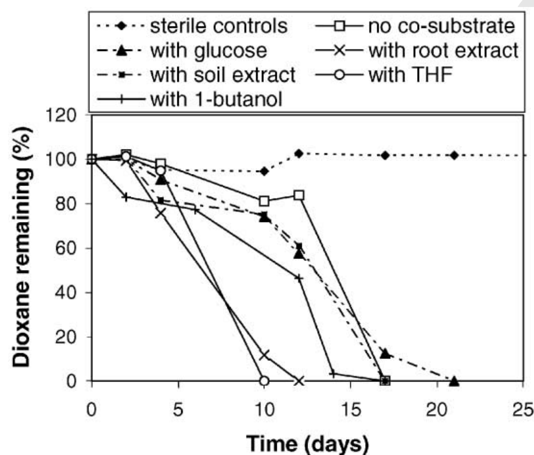

Fig. 3. Dioxane degradation by CB1190 in mineral medium fed different co-substrates. Dioxane was fed at about 25 mg/L, and the other substrates were added at about 40 mg/L as COD (except reactors with 1-butanol, which were fed 60 mg/L dioxane and 100 mg/L 1-butanol as COD). Reactors were seeded with CB1190 at $OD_{600} = 0.1$ and incubated at 20°C while shaking at 150 rpm. Data points show the average concentration from duplicate reactors.

The beneficial effect of THF could be attributed to both increasing the concentration of CB1190 and enhancing enzyme induction, while the favorable effect of 1-butanol was probably related only to supporting additional growth of CB1190. As discussed previously, CB1190 grows relatively fast on THF and on 1-butanol, but grows poorly on glucose (Parales *et al.*, 1994). In addition, soil extract lacks the rich auxiliary substrates present in root extract (Curl and Truelove, 1986). Therefore, glucose and soil extract are not as effective as 1-butanol or root extract in stimulating the growth of CB1190, which may explain their relative inability to enhance dioxane degradation (Fig. 3).

None of the added substrates had an adverse effect on dioxane degradation. This is contrary to the common observation that, when carbon is not limiting, easily degradable substrates such as glucose are preferentially utilized and repress and delay the degradation of target pollutants (Alexander, 1999; Egli, 1995). Apparently, CB1190 does not behave as a typical (heterotrophic) r-strategist since it cannot grow on common Kreb's cycle intermediates or other easily degradable substrates (Parales *et al.*, 1994). This suggests that the auxiliary substrates encountered in the poplar rhizosphere should not readily repress the ability of CB1190 to degrade dioxane.

Mineralization studies with planted and unplanted reactors

Planted and unplanted reactors were spiked with ^{14}C-labeled dioxane to (1) evaluate the effect of poplar trees on dioxane removal from contaminated soil, and (2) determine whether bioaugmentation with CB1190 enhances dioxane mineralization in planted or unplanted soil. The radiolabel recoveries ranged from 44% to 67%, with higher recoveries corresponding to unplanted reactors (Table 3). Considering the volatility of dioxane from dry soil, the continuous-air flow design of the reactors, and the extended time over which the experiments were run, it is likely that most of the unrecovered radiolabel leaked from the system. Overall, the mass balances for this experiment compare favorably with published recoveries of less than 50% for phytoremediation studies with volatile organic compounds (Anderson and Walton, 1995; McFarlane *et al.*, 1990; Strand *et al.*, 1995).

Bioaugmentation significantly enhanced dioxane mineralization in unplanted soil ($p < 0.05$). Reactors

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Table 3. Fate of ^{14}C-dioxane in various reactors[a]

	Unplanted $n=2$	Unplanted + CB1190 $n=2$	Planted $n=3$	Planted + CB1190 $n=3$	Excised tree $n=2$	Excised tree + CB1190 $n=1$
Remaining in soil	17 ± 4	12 ± 3	4 ± 1	11 ± 6	19 ± 1	19
Mineralized in soil	8 ± 1	25 ± 5	3 ± 2	17 ± 14	6 ± 1	35
Evapotranspired by plant	—	—	33 ± 9	21 ± 17	3 ± 1	0
Accumulated in plant tissue	—	—	1 ± 1	2 ± 1	3 ± 1	1
Removed by air suction	40 ± 4	30 ± 5	8 ± 7	5 ± 7	13 ± 4	1
Total recovery	65 ± 8	67 ± 2	49 ± 1	56 ± 4	44 ± 7	56

[a] Numbers reflect percentages of the added radiolabel, as the average ± one standard deviation. Each reactor initially contained 10 mg/kg dioxane, and was incubated for 18–26 days. Bioaugmented reactors had initially about 10^7 cells/g-soil.

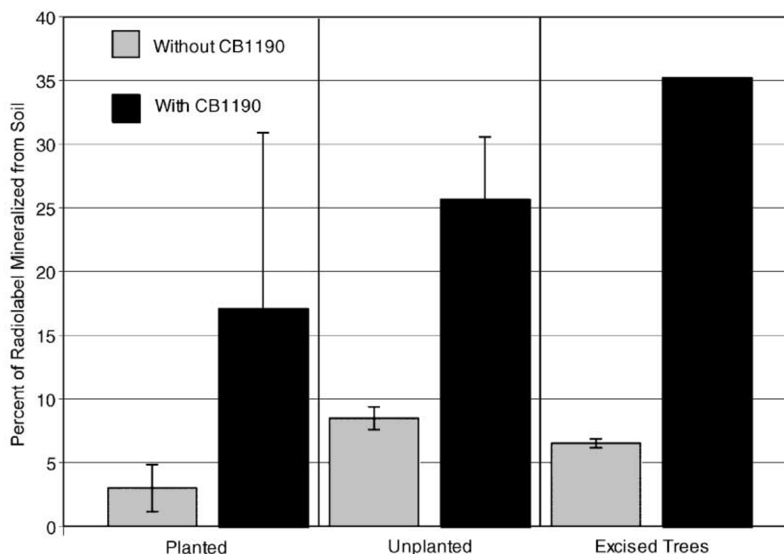

Fig. 4. Percentage of radiolabel mineralized from soil. Each reactor initially contained 10 mg/kg 1,4-dioxane, and was incubated for 18–26 days. Bioaugmented reactors had initially about 10^7 cells/g-soil.

amended with CB1190 (about 10^7 cells/g-soil) mineralized 25 ± 5 % of the added ^{14}C-labeled dioxane to $^{14}CO_2$, compared to 8 ± 1% for no-treatment controls (Fig. 4).

Excised-tree reactors were used to evaluate the "rhizosphere effect" on biodegradation while eliminating confounding effects from dioxane uptake by plants. Excised trees do not take up dioxane readily since this process is driven by evapotranspiration (Aitchison *et al*., 2000), and release root exudates possibly to a greater extent that normal trees because they are injured (Clarkson *et al*., 1974; Curl and Truelove, 1986; Lynch, 1990). Bioaugmentation of excised-tree reactors significantly enhanced dioxane mineralization, from 6 ± 1% to 35%. In the absence of evapotranspiration, roots also enhanced the performance of CB1190, which mineralized more dioxane in the excised-tree reactor (35%) than when added to unplanted soil (25 ± 5%). However, the beneficial effect of CB1190 was not statistically significant for planted soil. This was attributed to the fact that plant uptake was relatively fast compared to biodegradation, and it decreased dioxane availability for microbial mineralization.

Although CB1190 significantly enhanced dioxane mineralization in unplanted and excised-tree reactors, it did not significantly reduce the residual dioxane concentration in those soils (Fig. 5). The lack of enhancement of dioxane removal from soil was more pronounced in planted reactors, where more dioxane remained in bioaugmented soil (11 ± 6%) than in soil without CB1190 (4 ± 1%). These effects are not statistically significant and are due, in part, to an experimental artifact. Reactors without CB1190 had a higher percentage of dioxane removed by air suction (13% vs. 1% for excised-tree reactors, 40% vs. 30% for unplanted reactors, and 8% vs. 5% for planted reactors), which was determined by analysis of [^{14}C]-dioxane trapped in the lower-compartment Orbo tubes (Fig. 1). The reason for this artifact is unclear, and appears to be related to higher airflow in reactors without CB1190.

This experiment verified that poplars can take up and transpire dioxane, as shown by analysis of activated carbon (Orbo tube) traps connected to the top compartment (Fig. 1), and that in-plant accumulation is relatively small (Table 3) (Aitchison *et al*., 2000). Plant and activated carbon trap samples were

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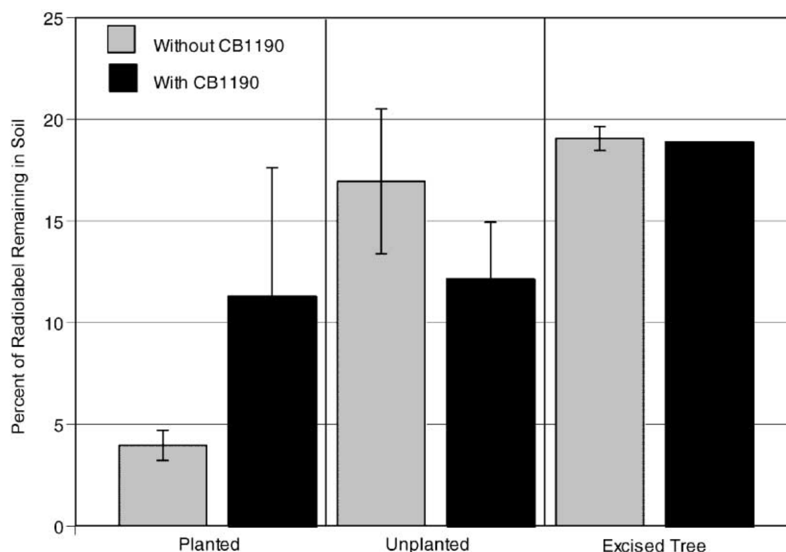

Fig. 5. Percentage of radiolabel remaining in soil. Each reactor initially contained 10 mg/kg 1,4-dioxane, and was incubated for 18–26 days. Bioaugmented reactors had initially about 10^7 cells/g-soil.

analyzed side-by-side on the LSC and GC to compare ^{14}C label recoveries with pure dioxane recoveries. This side-by-side sampling was performed because the LSC cannot differentiate between ^{14}C-dioxane and potential ^{14}C metabolites. Both methods gave comparable results, indicating that radiolabel transpired from leaves and found in plant tissue was primarily parent dioxane. Consistent with the notion that microorganisms compete with plants for dioxane removal, more radiolabel was volatilized through plant evapotranspiration in reactors without CB1190 (34% vs. 23%).

Trees significantly enhanced dioxane removal from soil, regardless of whether CB1190 was added or not (Fig. 5). For example, the percent dioxane remaining in the soil was significantly lower ($p < 0.05$) in the planted reactors without CB1190 ($4 \pm 1\%$) than in the unplanted controls ($17 \pm 4\%$), even though more dioxane was removed by air suction from the unplanted reactors (40% vs. 8%).

The fate of CB1190 was not investigated in this work. However, the higher dioxane-mineralization activity in bioaugmented reactors shows that CB1190 can survive and compete with the indigenous soil and rhizosphere microflora. Indeed, research by Leval and Remacle (1969) suggests that actinomycetes such as CB1190 can successfully colonize the poplar rhizosphere, which suggests that bioaugmenting poplar roots with CB1190 might enhance the remediation process.

Practical considerations for growing CB1190 for full-scale bioaugmentation projects

Obtaining large quantities of CB1190 for full-scale bioaugmentation is a major challenge because cells grow slowly and the cell yield is relatively low. Yet, the cell quantities needed for bioaugmentation are relatively large. For example, to bioaugment one acre of contaminated soil (over a one-foot thickness), at 10 mg dry cell weight (dcw) per kg-soil (i.e., about 10^7 cells/g), requires 22 kg dcw, or about 15,000 gallons of cell suspension at $OD_{600} = 1.0$. These calculations assume a soil bulk density of 1800 kg/m^3 and that one unit of $OD_{600} = 400$ mg/L.

To maximize the growth rate and minimize the opportunity for microbial contamination, fermentors at The University of Iowa's Center for Biocatalysis and Bioprocessing were utilized to grow CB1190 at high concentrations under controlled growth conditions. A trial run was conducted using a 10-L BioStat E fermentor (B. Braun, Allentown, PA) that was inoculated with a culture of CB1190 at an $OD_{600} = 0.44$; this resulted in an initial OD_{600} of 0.06 for the fermentor. The temperature was maintained at 30°C, the dissolved oxygen concentration was monitored and adjusted to remain above 20% during the entire run, and the pH was controlled at 6.0. THF, which was previously shown effective in growing and inducing CB1190, was supplied as primary carbon source (1000 mg/L) along with dioxane (200 mg/L). THF has a higher vapor pressure than dioxane (25 vs. 5 kPa) which represents a greater potential for volatile losses and associated health hazards to lab personnel. Therefore, only dioxane was fed to the culture after the optical density increased to 1.0 (after 5 days). The optical density increased subsequently to 13.6 after 13 days. During this time, ammonium chloride was added periodically to replace the nitrogen used from the medium. For harvesting, the temperature was decreased to 10°C and the culture was centrifuged in a Beckman MCJ2 apparatus (3 cycles at 10,000 g for 10 min at 4°C) to separate the cells.

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Freeze-drying was evaluated as an approach to facilitate cell storage and transportation to field sites. Freeze drying is widely used in the pharmaceutical and food industry to allow for longer shelf life of the product with storage at ambient temperature, and it represents a minor cost element in the overall process. In our case, it required 13 days of fermentor use compared to 3 days of freeze-drying. CB1190 was freeze-dried, half as a cell paste and the other half in 15% milk to determine if this cryoprotectant is effective in protecting cells from ice crystal damage (Hunter-Cevera and Belt, 1996). The cells were freeze-dried in a Virtis Genesis 35 EL apparatus. The freeze dryer held the temperature at −25°C for 30 min and then began applying vacuum at 250 mTorr while increasing the temperature to −10°C. The later cycles gradually increased the temperature to 10°C and decreased the vacuum to 100 mTorr. The freeze drying cycle lasted 42 hours. The post heat cycle held the temperature at 15°C, 100 mTorr for 960 min before the cycle was complete. The result was approximately 75 g dry cells.

Freeze-dried cells were rehydrated (15 mg dcw in 50 mL mineral medium) and assayed for dioxane degradation activity at different time intervals. The degradation activity decreased with storage time, and this effect was more pronounced for cells freeze-dried without the cryoprotectant (Fig. 6). For example, 1 month old, cryoprotected cells (with 15% skim milk) degraded 10 mg/L dioxane in about two days (Fig. 6b), while it took about 4 days for similar cells without a cryoprotectant (Fig. 6a). In addition, only cells freeze-dried with skin milk retained the ability to degrade dioxane after 4 months of storage, showing that this cryoprotectant can lengthen the shelf life of CB1190.

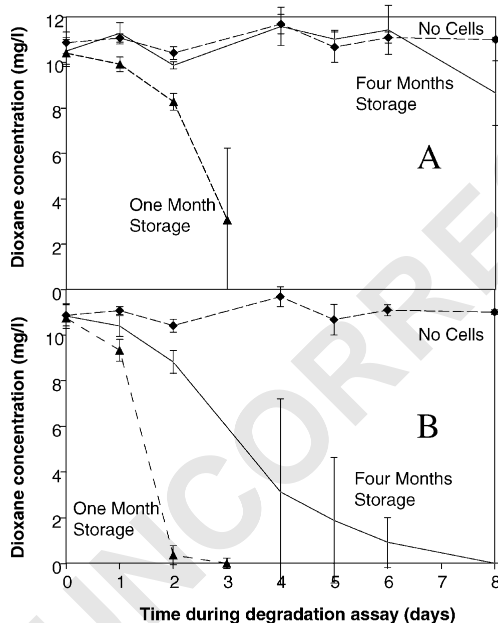

Fig. 6. Performance of freeze-dried CB1190 after different storage times. Panel (A) corresponds to cells with no preservative and (B) for cells preserved with 15% milk as a cryoprotectant. All experiments were conducted with 15.5 mg cells in 50 mL medium, shaking at 150 rpm at 20°C. Error bars represent ±1SD from the mean of triplicate incubations.

These experiments suggest that high-cell density fermentation and freeze-drying may be an effective approach to grow and transport large quantities of bacteria necessary for bioremediation, especially when growing specialized strains on-site is not feasible. Nevertheless, bioaugmentation with CB1190 needs to be more thoroughly evaluated before widespread large-scale implementation is recommended.

SUMMARY AND CONCLUSIONS

- Laboratory experiments showed that bioaugmentation with CB1190 enhances the removal of 1,4-dioxane from planted and unplanted contaminated soil.
- Tetrahydrofuran was a more effective substrate than dioxane for both growing and inducing CB1190, and should be considered to grow CB1190 for field application when volatile emissions do not pose a problem.
- Although root extracts did not induce microbial enzymes that degrade dioxane, the auxiliary substrates encountered in the poplar rhizosphere apparently enhanced the growth of CB1190 and increased the dioxane degradation activity.
- High-cell density fermentation and freeze-drying may be an effective approach to grow and transport large quantities of CB1190 necessary for bioremediation.
- Rapid uptake and volatilization of dioxane by hybrid poplar trees coupled with mineralization by CB1190 in the rhizosphere makes bioaugmented phytoremediation an attractive alternative to clean up (shallow) dioxane contaminated sites.

6. UNCITED REFERENCE

Bitton 1994; Burken and Schnoor 1998; Metcalf and Eddy, 1991; Reilley et al. 1996; Schnoor et al. 1995; Walter 1997.

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