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Mineralization of 1,4-dioxane in the presence of a structural analog

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Abstract

A mixed culture with the ability to aerobically biodegrade 1,4-dioxane in the presence of tetrahydrofuran (THF) was enriched from a 1,4-dioxane contaminated aquifer. This consortium contained 3–4 morphologically different types of colonies and was grown in mineral salts media. Biodegradation of 1,4-dioxane began when THF concentrations in batch experiments became relatively low. No biodegradation of 1,4-dioxane was observed in the absence of THF and the measured cell yield was similar during degradation of 1,4-dioxane with THF or with THF alone. However, when the consortium was grown in the presence of ^{14}C-1,4-dioxane plus THF, 2.1% of the radiolabeled 1,4-dioxane was present in the particulate fraction. The majority of the ^{14}C (78.1%) was recovered as $^{14}CO_2$, while 5.8% remained in the liquid fraction. This activity is interesting since the non-growth substrate is mineralized, yet only minimally assimilated into biomass. Using THF as the growth substrate, the consortium also degraded 1,3-dioxane, methyl *t*-butyl ether, ethyl *t*-butyl ether and *t*-amyl methyl ether.

Introduction

1,4-Dioxane is a cyclic ether that is present in certain industrial wastewater streams. Commonly formed as a byproduct during the production of organic fibers from terephthalic acid and ethylene glycol (Popoola 1991), 1,4-dioxane is also used as a stabilizer for chlorinated solvents and as a wetting agent in textile processes. Unfortunately, heterocycles and ethers are generally persistent chemicals and there are numerous reports on the recalcitrance of 1,4-dioxane (Alexander 1973; Mills and Stack 1954). Thus, 1,4-dioxane can be transported through biological wastewater treatment plants and released to surface and groundwater. In 1997, approximately 324 tons of 1,4-dioxane were released to the environment in the United States (Toxics Release Inventory (TRI) 1997). Because it is generally difficult to degrade, the continued release of 1,4-dioxane has resulted in its presence as a groundwater pollutant (Francis et al. 1980; Weimar 1980; DeWalle & Chian 1981; Lesage et al. 1990; Fetter 1993; Taylor et al. 1997). In a recent study, Abe (1999) detected 1,4-dioxane contamination at 24 sites in the Kanagawa Prefecture of Japan.

The Environmental Protection Agency (EPA) classifies 1,4-dioxane as a probable human carcinogen. Due to its cyclic structure and ether linkages, 1,4-dioxane is also highly soluble in water. The combined effects of its environmental release, solubility, and recalcitrance have made 1,4-dioxane a formidable challenge for groundwater remediation (Burmaster 1982; DeWalle & Chian 1981; Francis et al. 1980; Taylor et al. 1997) and biological wastewater treatment (Sock 1993).

Recently, 1,4-dioxane biodegradation has been reported in both pure (Parales et al. 1994; Burback & Perry 1993; Bernhardt & Diekmann 1991) and mixed culture (Sock 1993). A pure culture of *Mycobacterium vaccae* was shown to partially degrade 1,4-dioxane, but its biodegradation did not support growth (Burback & Perry 1993). Bernhardt & Diekmann (1991) reported the biodegradation of 1,4-dioxane as the sole carbon and energy source by a *Rhodococcus* strain. Parales et al. (1994) observed a nocardioform actinomycete strain CB1190 that was capable of sustained

growth and mineralization of 1,4-dioxane. This microorganism was later reported as belonging to the genus *Amycolata* (Kelley et al. 1999). A mixed culture containing several *Rhodococcus* species was also capable of mineralizing 1,4-dioxane as the sole carbon source in a continuous flow bioreactor (Cowan et al. 1994; Sock 1993). In this study, a consortium was enriched from 1,4-dioxane contaminated aquifer sediment with the ability to mineralize 1,4-dioxane in the obligate presence of THF as the growth substrate.

Materials and methods

Chemicals

THF was obtained from Fisher Scientific (Pittsburgh, PA) and had a purity of 99.9%. 1,4-Dioxane, 1,3-dioxane, tetrahydropyran, 3-hydroxytetrahydrofuran (3-THF), methyl *t*-butyl ether (MTBE), ethyl *t*-butyl ether (ETBE), *t*-amyl methyl ether (TAME), diisopropyl ether (DIPE) and *t*-butyl alcohol (TBA) were obtained from Aldrich Chemical (Milwaukee, WI) at 99.9% purity. Uniformly labeled ^{14}C-1,4-dioxane was obtained from Moravek Biochemicals (Brea, CA) at a purity of >97%.

Consortium and growth conditions

The consortium was isolated from an aquifer that had been contaminated with 1,4-dioxane. A sample of the aquifer sediment was retrieved and incubated aerobically with native groundwater, 200 mg/L 1,4-dioxane, and 200 mg/L THF at 35 °C in triplicate bottles. After measurement of 1,4-dioxane disappearance, samples were taken from each soil microcosm and transferred to a flask containing mineral medium L (Leadbetter & Foster 1958). Mineral medium L had the following composition: $NaNO_3$, 2.0 g; $MgSO_4$, 0.2 g; Na_2HPO_4, 0.1 g; NaH_2PO_4, 0.15 g; KCl, 0.04 g, $CaCl_2$, 0.015 g; $FeSO_4$—$7H_2O$, 1 mg; $CuSO_4$-$5H_2O$, 20 μg; H_3BO_3, 57 μg; $MnCl_2$-$4H_2O$, 36 μg; $ZnSO_4$—$7H_2O$, 4 μg; Na_2MoO_4—$2H_2O$, 177 μg; and deionized water, 1L.

A continuous flow rotating biological contactor (RBC) was inoculated with samples from each soil microcosm bottle and operated in a temperature-controlled room at 35 °C. The RBC was utilized to maintain the consortium and to provide inocula for subsequent experiments. The RBC was continuously fed (6 L/day) 25% mineral medium L solution containing 20 mg THF/L and 30 mg 1,4-dioxane/L.

1,4-Dioxane Mineralization

Uniformly ^{14}C-labeled 1,4-dioxane was utilized to monitor mineralization and assimilation. Assays were conducted in 160 mL serum bottles containing 100 mL of mineral medium L and sealed with black butyl rubber stoppers (Geo-Microbial Technologies, Ochelata, OK). Five bottles were inoculated with a small amount (<0.1 mg TSS) of live biomass from the RBC, while one uninoculated bottle served as an abiotic control. Radiolabeled 1,4-dioxane (1.6 μCi) was added to each bottle. Non-labeled 1,4-dioxane and THF were added in sufficient quantity to achieve a final concentration of 200 mg/L each.

Dissolved 1,4-dioxane and THF were monitored until concentrations fell below the gas chromatograph (GC) detection limits (0.8 mg/L). Bottles were then acidified with 2 mL of 12N HCl. The liquid in each bottle was sparged with 50 mL/min of oxygen for 30 min to transfer CO_2 and any volatile organics into a series of 3 NaOH (15 mL, 2N) traps. After removal of a sample from the traps for analysis of total dissolved ^{14}C, 1 mL of trap liquid was added to 9 mL of 0.2M $CdSO_4$ to precipitate CO_2 (aq). This cadmium-NaOH solution was then stored at 4 °C overnight, centrifuged at 1500 rpm, and filtered through a 0.45 μm filter and analyzed for residual radioactivity. The amount of $^{14}CO_2$ in each trap was calculated as the difference between the total counts in the trap and the counts after Cd treatment (Ress et al. 1998). The counts remaining in the traps after Cd treatment was assumed to be due to volatile biotransformation products.

After acidification and sparging, the liquid remaining in the serum bottles was analyzed for total suspended solids (TSS). The filtered liquid from this procedure was also analyzed for radioactivity to quantify soluble, non-volatile biotransformation products. Once filtered, the particulates retained on the filter were flushed 5 times with 20 mL of deionized water to remove soluble radioactivity. The liquid from the last washing was analyzed to verify the absence of radioactivity. The particulates remaining on the filter were then combusted at 800 °C in a Tekmar–Dohrmann Model 183 TOC Boat Sampler (Cincinnati, OH) followed by a 700 °C platinum catalyst tube furnace (Thermolyne, Dubuque, IA) to ensure complete combustion. The exhaust gas was routed through 3 NaOH vials (15 mL, 2N) to trap CO2. Samples were combusted for 45 minutes with an oxygen flowrate of 100 mL/min. Traps were analyzed by scintillation counting.

Batch tests

Several batch experiments were conducted to determine the consortium's dependence on THF, the effect of temperature on biodegradation, the effect of 1,4-dioxane on biomass production, and the ability to biodegrade other ethers. For the THF dependence experiment, inocula were taken from the RBC and placed in nine separate flasks. Three flasks (1–3) contained 200 mg/L of 1,4-dioxane, while six (4–9) contained 200 mg/L of both 1,4-dioxane and THF. Another three (10–12) flasks containing 200 mg/L of both chemicals were not inoculated and served as abiotic controls. Flasks were incubated at 35 °C and monitored for ether disappearance.

The temperature and cell yield experiments were conducted in flasks containing either 200 mg/L THF or 200 mg/L each of both THF and 1,4-dioxane. The temperature experiments were conducted at 20, 25, 30, 35, and 40 °C and compound disappearance was monitored during incubation. The resulting depletion curves for each temperature were compared by calculating the slope of a plot of the natural log (ln) of concentration versus time for the linear portion of the decay curve. For cell yield experiments, flasks contained 200 mg/L THF or 200 mg/L of both THF and 1,4-dioxane. This experiment was conducted at 35 °C. All batch tests were conducted in triplicate using mineral medium L.

The ability of the consortium to biodegrade other cyclic and alkyl ethers was tested in the presence and absence of THF. Ethers tested included: methyl *t*-butyl ether (MTBE), ethyl *t*-butyl ether (ETBE), *t*-amyl methyl ether (TAME), diisopropyl ether (DIPE), tetrahydropyran, 1,3-dioxane, 3-hydroxytetrahydrofuran (3-THF), and *t*-butyl alcohol (TBA). Tests were conducted at 35 °C in single bottles containing approximately 200 mg/L of the target chemical in 100 mL of mineral medium L.

Analytical methods

Dissolved 1,4-dioxane, THF, 1,3-dioxane, tetrahydropyran, 3-THF, MTBE, ETBE, TAME, DIPE and TBA were analyzed by direct aqueous injection of 0.5 μL samples into a Hewlett-Packard 5890 GC (Wilmington, DE) equipped with a flame ionization detector (FID) and a 75-meter DB-VRX column (J&W Scientific, Folsom, CA). The column was held at 70 °C for 2 min, raised to 160 °C at 15 °C/min, then raised to 220 °C at 70 °C/min and held at 220 °C for 2 min. The detection limits for all chemicals was approximately 0.8 mg/L.

Dissolved organic carbon (DOC) was analyzed after filtration through a 0.45 μm filter by using a Shimadzu 5000A Total Carbon Analyzer (Columbia, MD). Total suspended solids (TSS) were used as a surrogate for biomass and were determined according to Standard Method 2540D (APHA et al. 1995). Radioactivity was monitored by using a Packard TRICARB 2100TR Liquid Scintillation Analyzer (Downers Grove, IL). Two mL of liquid sample was placed in 18 mL of Ultima Gold (Packard) scintillation cocktail.

Results

Soil microcosms

Disappearance of THF and 1,4-dioxane was observed after 3 months in 1 microcosm and after 10 months in the remaining two. No 1,4-dioxane biodegradation was observed in parallel microcosms incubated without THF (Zenker et al. 1999). 1,4-Dioxane was respiked into one replicate, but no biodegradation was observed. When THF was re-spiked into this bottle, both compounds were degraded within 75 days (Figure 1). After ten months, liquid samples from all three bottles were transferred to L-salts medium containing 200 mg/L of both THF and 1,4-dioxane and were incubated at 35 °C. Disappearance of both chemicals was observed in two weeks, along with the appearance of white, filamentous colonies floating on the water surface.

After THF and 1,4-dioxane disappearance was measured in L-salts medium, samples were T-streaked onto 10% tryptic soy agar and incubated at 35 °C. The formation of 3–4 morphologically different types of colonies was observed within 1 week. Each type of colony was then restreaked onto solid L-salts media containing THF. Unfortunately, none of the cultures isolated from the tryptic soy agar plates were capable of growing on L-salts medium with THF. There are several potential explanations for this absence of growth including (1) the consortium's ether-degrading ability was lost upon growth on rich media, (2) the ether-degrading bacteria did not develop into visible colonies on the agar plates, or (3) syntrophic relationships in the consortium precluded its separation into pure cultures with THF as the carbon source.

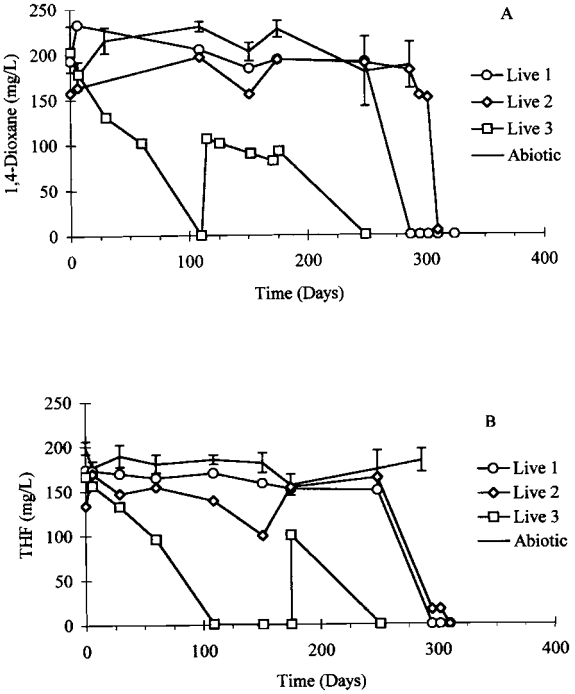

Figure 1. Biodegradation of 1,4-dioxane (A) and THF (B) in soil microcosms. Error bars in abiotics represent 1 standard deviation. Live 3 was respiked with 1,4-dioxane at 115 days, and respiked with THF at 175 days.

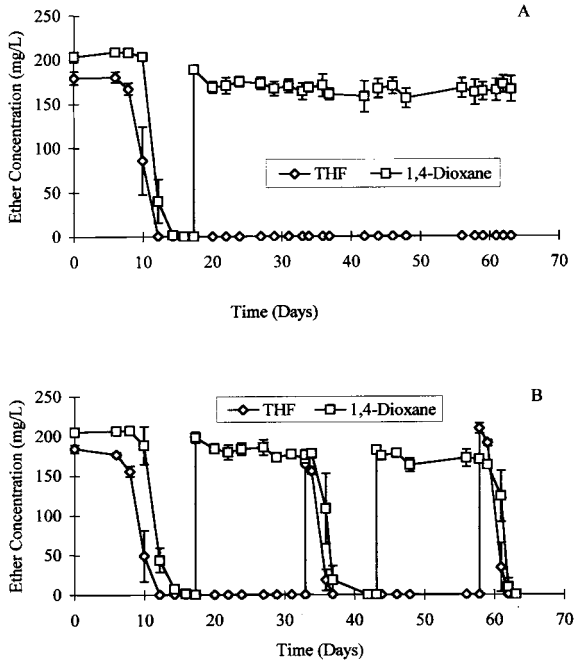

Figure 2. Biodegradation of 1,4-dioxane in liquid culture (L-salts medium) in the presence and absence of THF. Figure 2A presents average concentrations from bottles 4-6. Figure 2B presents average concentrations from bottles 7-9. Error bars represent 1 standard deviation.

Dependence on THF

In batch tests inoculated with the consortium as maintained in the RBC, biodegradation of both 1,4-dioxane and THF was observed in live flasks containing both chemicals (flasks 4–9) within 19 days of incubation. However, as illustrated in Figure 2, 1,4-dioxane biodegradation only occurred in the presence of THF. On day 19, 200 mg/L of 1,4-dioxane was respiked into flasks 4–9. No biodegradation was observed for approximately 16 days. THF was then respiked into flasks 7–9, which resulted in the biodegradation of both chemicals within 9 days. 1,4-Dioxane was again respiked into flasks 7–9, but its disappearance was not observed for 15 days. THF was then added into flasks 7–9 and both chemicals were again biodegraded within 5 days. The 1,4-dioxane respiked into flasks 4–6 did not biodegrade for approximately 46 days after re-addition (Figures 2A,B). In all tests, 1,4-dioxane degradation ceased within a few hours after THF was depleted, and did not resume until additional THF was added.

First order decay constants for THF and 1,4-dioxane as a function of temperature are presented in Figure 3. Maximum decay rates for THF and 1,4-dioxane do not occur at the same temperature. Biodegradation of both ethers was observed at all temperatures tested, but biodegradation activity was greatly diminished at 40 °C.

Mineralization and assimilation of 1,4-dioxane

The cell yield was measured to further investigate the role of 1,4-dioxane in cell growth. Cell growth in the presence of 200 mg/L of THF and 200 mg/L of both 1,4-dioxane and THF was measured as total suspended solids (TSS). In triplicate bottles containing only THF, the cell yield was 0.668 (s.d. = 0.104) mg TSS/mg C as THF, while the yield for bottles containing both THF and 1,4-dioxane was 0.750 (s.d. = 0.062) mg TSS/mg C as THF. Since one set of bottles did not contain 1,4-dioxane, the cell mass values for each treatment were normalized by using only the amount of THF added. While the measured yield was about 12% higher in the presence of 1,4-dioxane plus THF, this difference was not significant at the 90% confidence level.

Based on DOC measurements, less than 5% of the carbon added to the bottles as THF and 1,4-dioxane behaved as soluble, non-volatile material in

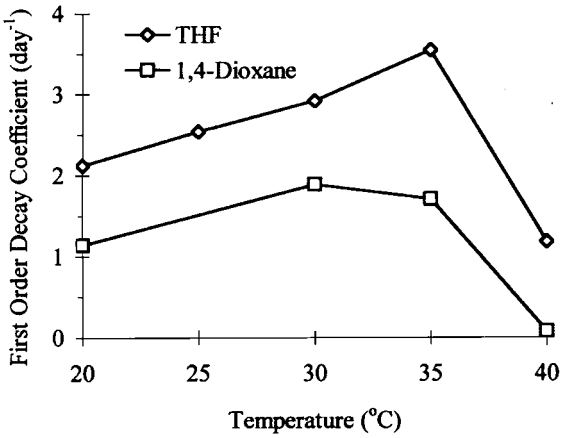

Figure 3. Effect of temperature on THF and 1,4-dioxane biodegradation. Decay coefficients were determined using the steepest section of substrate depletion curve. Biomass was assumed constant throughout the test period. THF coefficients were determined in the absence of 1,4-dioxane

Table 1. Fate of 1,4-dioxane during biodegradation.

	Percent recovered[a]
CO_2	78.1 ± 2.3%
Liquid	
Non-volatile	5.8 ± 1.3%
Volatile	nd
Biomass	2.1 ± 0.65%
Total recovery	86.0 ± 2.1%

[a] Values are the average of 5 replicates ± one standard deviation and represent the percent of original radioactivity added.

the cell yield experiment, suggesting that both compounds were being mineralized. To further study the fate of 1,4-dioxane, tests were conducted with ^{14}C-1,4-dioxane and the results are presented in Table 1. These data document that the enrichment culture can completely oxidize 1,4-dioxane, and show that a small amount of ^{14}C-dioxane was present in the particulate fraction, which we consider to be cell mass. This fractional ^{14}C incorporation is consistent with the small increase in cell mass reported above. The majority of the 1,4-dioxane (78.1%) was converted to CO_2, while 5.8% remained in the liquid. Cadmium treatment of all NaOH traps effectively removed all traces of radioactivity, confirming that the radioactivity present in the NaOH traps was $^{14}CO_2$ and not volatile biotransformation products. Additionally, no radioactivity was detected in water used to rinse filtered biomass.

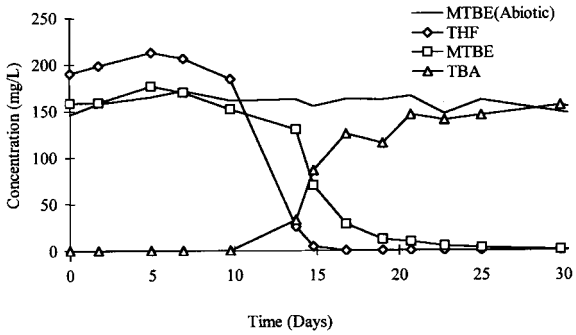

Figure 4. Biodegradation of MTBE in the presence of THF.

Biodegradation of other ethers

This consortium is capable of biodegrading a number of ethers in the presence and absence of THF. With the exception of *tert*-butyl alcohol (TBA) and diisopropyl ether (DIPE), each chemical (MTBE, ETBE, TAME, tetrahydropyran, 3-hydroxy THF, 1,3-dioxane) was biodegraded in the presence of THF. Tetrahydropyran and 3-hydroxytetrahydrofuran were biodegraded in the absence of THF. The biodegradation of MTBE in the presence of THF is illustrated in Figure 4. It is interesting to note that the consortium formed the biotransformation product TBA, but was unable to biodegrade it further. TBA accumulation was also observed during the biodegradation of ETBE.

Discussion

It is evident from Figure 2 that the biodegradation of 1,4-dioxane is dependent on THF as the repetitive addition of 1,4-dioxane in the absence of THF failed to stimulate biodegradation. Repeated attempts were made to determine if our culture had developed the ability to biodegrade 1,4-dioxane as its sole carbon and energy source. Over the course of RBC operation (400 days), samples were periodically placed in mineral medium L containing only 1,4-dioxane. Neither 1,4-dioxane disappearance nor microbial growth was observed during the course of these experiments. This contrasts with research in which Sock (1993) reported the development of a mixed culture with the ability to biodegrade 1,4-dioxane as the sole carbon and energy source in an attached growth reactor previously operated with THF and 1,4-dioxane.

Although THF is required for 1,4-dioxane biodegradation, it is evident that the consortium is capable of mineralizing 1,4-dioxane. 1,4-Dioxane

was repeatedly biodegraded to below detection limits (<0.8 mg/L) in all batch tests (with THF addition). Inspection of chromatograms during biodegradation did not show the formation of unknown compounds. Furthermore, DOC analysis of the residual liquid from the cell yield experiment showed that >95% of the added organic carbon was degraded and these observations were corroborated with the use of ^{14}C-1,4-dioxane (Table 1).

Based on these observations, it is reasonable to assume that 1,4-dioxane is biodegraded via a cometabolic process. Cometabolism is traditionally defined as "the transformation of a non-growth substrate in the obligate presence of a growth substrate or another transformable compound" (Dalton & Stirling 1982). The culture enriched here clearly shows a continued dependence on THF to maintain 1,4-dioxane biodegradation. However, 1,4-dioxane does contribute to a small increase in biomass, as evidenced by the ^{14}C experiment. This increase in biomass could be attributed to the formation of storage compounds synthesized using carbon from 1,4-dioxane. This observation may seem contradictory to the Dalton & Stirling (1982) definition, which implicitly assumes that transformation of the non-growth substrate yields no nutritional benefit to the micro-organism. Although the formation of additional biomass may be beneficial to the consortium, it seems unlikely that this incorporation of 1,4-dioxane yields viable biomass. Strictly interpreted, the Dalton & Stirling (1982) definition is therefore applicable to this situation.

Despite these observations, it is interesting that only 2.1% of the organic carbon from 1,4-dioxane was incorporated into biomass. Bernhardt & Diekmann (1991) hypothesized that the catabolic pathway of THF would yield short chain alcohols and carboxylic acids. Assuming the biodegradation of 1,4-dioxane yields similar products, it is unusual that these products were not assimilated to a larger extent. It is also possible that one or more members of the consortium were able to incorporate small amounts of $^{14}CO_2$ into biomass via the phosphoenolpyruvate carboxylase pathway.

Another important observation is the fact that product toxicity does not seem to be occurring in this consortium. Although product toxicity does not occur in all cases of cometabolism, it has important consequences in some situations. For example, in the widely reported cometabolic process for trichloroethene (TCE) biotransformation by methanotrophs, declines in utilization rates of growth substrates following, or concurrent with, the biotransformation of non-growth substrates have been reported (Alvarez-Cohen & McCarty 1991; Chang & Alvarez-Cohen 1995). This product toxicity is due to the formation of epoxides that can denature enzymes or other cellular components. Therefore, the continued biodegradation of the non-growth substrate is limited by the effects of product toxicity. In contrast, 1,4-dioxane biodegradation did not decrease the observed THF biodegradation rate (Figure 2). This is not surprising since transient biotransformation products were not detected based on inspection of chromatograms and DOC analysis.

Competitive inhibition is a commonly observed phenomenon in cometabolic processes, (Chang et al. 1993; Saez & Rittman 1991) and appears to be occurring here (Figure 2). 1,4-Dioxane disappearance does not begin until THF has reached relatively low levels. Cometabolism can result when a nonspecific enzyme fortuitously transforms a substrate other than the one for which it was synthesized. Therefore, the growth and non-growth substrates typically compete for the same enzyme.

Despite evidence that 1,4-dioxane is mineralized, produces no product toxicity, and is partially assimilated into biomass; biodegradation activity is lost in the absence of THF. A possible explanation for the loss of activity can be attributed to the depletion of enzymatic cofactors. The cofactor NADH, for example, is depleted in cultures that biotransform TCE using methane as the growth substrate (Chang & Alvarez-Cohen 1995; Alvarez-Cohen & McCarty 1991). It is possible that the biodegradation of 1,4-dioxane exerts an abnormally high demand on the resources of the consortium. For example, the consortium may invest more energy/cofactors in the biodegradation of 1,4-dioxane than is ultimately yielded. Loss of activity could also be due to rapid turnover of the active enzyme once THF is depleted.

The consortium was also capable of biodegrading several other cyclic and alkyl ethers. Figure 4 illustrates the biodegradation of MTBE in the presence of THF. It is interesting to note that this activity produces a dead-end product (TBA). A biotransformation product, possibly *tert*-amyl alcohol, was also formed during the biodegradation of TAME, but it was not conclusively identified.

The broad catabolic activity of this consortium has interesting possibilities for biological treatment of water contaminated with ethers. 1,4-Dioxane and MTBE are very soluble ethers, and thus not amenable to treatment via physical separation processes.

Biological treatment of these chemicals presents a cost-effective alternative for removal of ethers from aqueous solutions. THF can be added to the influent of a bioreactor, for example, to stimulate the growth of a microbial community with the ability to biodegrade 1,4-dioxane. The temperature response of the consortium indicates that it can retain ether-degrading activity at lower temperatures (Figure 3). Further research is needed, however, to determine the kinetics of THF/1,4-dioxane biodegradation.

The use of THF as a growth substrate, however, may limit the potential for this consortium to be utilized for *in situ* bioremediation as THF is a toxic chemical and would cause concern if it were not completely removed from the treatment process. Further research may focus on either eliminating the dependence of a growth substrate, or identifying a less-toxic alternative to THF. Since the consortium has the ability to biotransform a variety of non-growth substrates, it is possible that a less toxic growth substrate can be found to induce similar activity.

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