CHLORINATED SOLVENT COMETABOLISM BY BUTANE-GROWN MIXED CULTURE

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ABSTRACT: A survey of aerobic cometabolism of chlorinated aliphatic hydrocarbons by a butane-grown mixed culture was performed. The transformation of 1,1-dichloroethylene (1,1-DCE) and cis-1,2-dichloroethylene (c-DCE) required O_2 and was inhibited by butane and inactivated by acetylene, indicating that a monooxygenase enzyme was likely involved in the transformations. The initial transformation rates and the quantities of chlorinated aliphatic hydrocarbons transformed were inversely proportional to the chlorine contents within each group of chlorinated methanes, ethanes, and ethenes. Lower quantities of chloroform were transformed than chloromethane and dichloromethane, but chloroform transformation resulted in much higher cell inactivation. For the ethane group, chloroethane was most effectively transformed but caused significant cell inactivation. Di- or trichloroethanes that have all chlorines on one carbon were more effectively transformed and caused less cell inactivation than the isomers that have chlorine on both carbons. For chlorinated ethenes, 1,1-DCE was most rapidly transformed, whereas trans-1,2-dichloroethylene was not transformed. Vinyl chloride was transformed to the greatest extent, and very limited transformation of trichloroethylene was observed. The 1,1-DCE transformation caused greater cell inactivation than the transformation of the other chlorinated ethenes. Chloride release studies showed nearly complete oxidative dechlorination of chlorinated methanes and chloroethane, vinyl chloride and c-DCE (86% ~ 100%), and incomplete dechlorination of 1,1-dichloroethane, 1,2-dichloroethane, 1,1,1-trichloroethane, 1,1,2-trichloroethane, and 1,1-DCE (37% ~ 75%) was observed.

INTRODUCTION

Aerobic cometabolism is a potential method for remediating aquifers contaminated with chlorinated aliphatic hydrocarbons (CAHs) (McCarty and Semprini 1993). Microorganisms grown on a variety of substrates express oxygenase enzymes that are capable of transforming CAHs. The range of CAHs that can be transformed by microbes grown on different substrates is of interest to determine the potential for bioremediation. Surveys have been performed with *Methylosinus trichosporium* OB3b (Oldenhuis et al. 1989), *Nitrosomonas europaea* (Rasche et al. 1991), a propylene-grown *Xanthobacter* strain (Ensign et al. 1992), and methanotrophic mixed cultures (Dolan and McCarty 1995; Chang and Alvarez-Cohen 1996). The quantity of CAHs transformed and the inactivation of cells caused by CAH transformations were evaluated in these surveys.

McCarty and Semprini (1993) ranked the relative ability of specific oxygenase systems to cometabolically transform different CAHs. The rank was based on maximum transformation rates, resting cell transformation capacities (T_c, the mass of CAHs ultimately transformed/mass of cells), and transformation yields (T_y, the maximum mass of CAHs transformed/mass of growth substrates degraded). Chloroform (CF), 1,1,1-trichloroethane (1,1,1-TCA), and 1,1-dichloroethylene (1,1-DCE) were all shown to have limited potential for aerobic cometabolism by the systems known at that time. Thus, identification of cometabolic systems that perform well on these compounds are of interest.

In long-term microcosm studies with aquifer core material from Hanford, Wash., butane was found to be an effective substrate for aerobic cometabolism of CF and 1,1,1-TCA (Kim et al. 1997a). An enrichment culture, CF8, isolated from the Hanford DOE site microcosms transformed CF at rates comparable to those of *Methylosinus trichosporium* OB3b (Hamamura et al. 1997). Soil microcosm studies by Kim et al. (1997b) showed that butane utilizers effectively transformed the mixtures of 1,1,1-TCA and 1,1-DCE when added at aqueous concentrations of 50 μg/L.

This work evaluates how effectively a butane-grown enrichment culture could transform a broad range of chlorinated methanes, ethanes, and ethenes in single contaminant tests. The CAHs were transformed in resting cell tests in the absence of exogenous energy sources. The effects of butyrate and formate, as exogenous energy sources, on the transformation of 1,1-DCE and cis-1,2-dichloroethylene (c-DCE) also were evaluated. Loss of butane uptake ability after exposure to the compounds was used as a measure of cell inactivation due to the CAH transformation. Chloride release was measured as an indicator of the extent of dehalogenation achieved. This study provides the first detailed evaluation of the range of CAHs that can be transformed by a butane-grown enrichment culture.

MATERIALS AND METHODS

Butane-Utilizing Mixed Culture

The butane grown-enrichment was obtained from Hanford soil microcosms described by Kim et al. (1997a). The enrichment was batch grown in 750-mL capped bottles containing 10% butane [volume-to-volume ratio (vol/vol)] in air and 250 ml of *Xanthobacter* Py2 medium (Wiegant and de Bont 1980) with the pH adjusted to 7.3, except NH_4NO_3 replaced by $NaNO_3$. The bottles were rotary shaken at 200 rpm at 30°C and harvested at an optical density (OD_{600}) of 1.3, with a cell yield of 0.8-mg total suspended solids (TSS)/mg butane. Cells were harvested by centrifugation (6,000 × *g* for 15 min), washed, and resuspended in a chloride-free phosphate buffer (adjusted pH 7.3; 2-mM KH_2PO_4, and 2-mM $Na_2HPO_4 \cdot 7H_2O$) to give a final cell density of 2,000 mg/L (on a TSS basis). Resting cell transformation tests were performed within 2 h of harvesting. Resting cell activity was stable for 30 h after harvesting, based on butane uptake activity. Cell activities for different batches of cells were measured by determining butane uptake, 1,1-DCE transformation rates, and the T_c of 1,1-DCE. The 1,1-DCE was used to examine cell activities because it was rapidly transformed, and its transformation led to

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complete cell inactivation (measured as a loss in butane uptake activity).

Chemicals

Methane (99%), butane (≥99%), and acetylene (99.6%) were purchased from AIRCO (Vancouver, Wash.). Chloromethane (CM) (99.5%) was obtained from Liquid Carbonic Inc. (Chicago, Ill.). Butane (10% in nitrogen), carbon tetrachloride (CT) (99.9%), chloroethane (CA) (99.7%), 1,2-dichloroethane (1,2-DCA) (99%), 1,1,2-trichloroethane (1,1,2-TCA) (98%), 1,1,1,2-tetrachloroethane (1,1,1,2-TeCA) (99%), vinyl chloride (VC) (≥99.5%), 1.1-DCE (99%), trans-1,2-dichloroethene (t-DCE) (98%), c-DCE (97%), trichloroethylene (TCE) (≥99.5%), and perchloroethylene (PCE) (99%) were purchased from Aldrich Chemical Co. (Milwaukee, Wis.). The 1,1-dichloroethane (1,1-DCA) (≥99%), 1,1,2,2-tetrachloroethane (1,1,2,2-TeCA) (98.5%), pentachloroethane (PCA) (99%), and hexachloroethane (HCA) (99%) were obtained from Acros Organics (Pittsburgh, Pa.). Dichloromethane (DCM) (99.9%) and CF (99.9%) were obtained from Mallinckrodt Specialty Chemical Co. (Paris, Ky.). The 1,1,1-TCA (95.5%) was purchased from J. T. Baker Inc. (Phillipsburg, N.J.).

Saturated aqueous stock solutions of CAHs were prepared at room temperature by adding specific amounts of the liquid or a solid compound to 125-mL serum bottles containing autoclaved deionized water. This procedure eliminated the use of carrier solvents, such as methanol. The bottles were shaken for 6 h prior to use to ensure saturation and then allowed to settle for 6 h before use. Gaseous compounds were directly transferred to the batch bottles.

Analysis

Gaseous concentrations were determined by a headspace analysis (Kampbell et al. 1989). The total compound mass in each test bottle was calculated using the headspace and solution volumes and published Henry's constants (Mackay and Shiu 1981; Gossett 1987). All the experiments were conducted with vigorously shaken batch reactors to avoid mass transfer limitations. Calibration curves for all compounds were developed using external standards. Headspace concentrations of methane and butane were determined on a HP5890A series gas chromatograph (GC) using a 3.2 mm × 1.2 m HayeSep D80/100 mesh, packed column (Alltech Associates, Deerfield, Ill.), operated at 130°C, and a flame ionization detector. The CAH analysis was conducted by injecting headspace samples onto a HP 5890 series II GC with a 0.25 mm × 30 m HP-624 capillary column operated at 140°C and Model 5220 electrolytic conductivity detector (OI Analytical, College Station, Tex.).

Qualitative analysis of the c-DCE epoxide produced during c-DCE transformation was conducted using solid-phase microextraction of 1-mL aqueous samples with an 85-μm acrylate fiber (Supelco Inc., Bellefonte, Pa.). The GC equipped with mass spectrometer analysis was conducted as previously reported by Vancheeswaran et al. (1999).

Culture density was determined as TSS [American Public Health Association (APHA) 1985], using a 0.1-μm membrane filter (Micro Separation Inc., Westboro, Mass.). The optical density (OD_{600}) of cultures was measured at 600 nm using a HP8453 UV-Visible spectrophotometer.

Transformation of CAHs

The transformation of each CAH was monitored for 30 h. Autoclaved phosphate buffer solution (58 mL) was added to autoclaved 125-mL amber serum bottles that were crimp sealed with Teflon-lined rubber septa (Kimble, Vineland, N.J.). The CAH was added, and the initial CAH concentration was determined after 15 min of shaking. Washed and resuspended cells (4–6 mg on a TSS basis) were then added, and bottles were shaken at 180 rpm during the 30-h incubation. Each CAH test included duplicate bottles with active cells, a bottle with acetylene-treated cells, and a bottle without cells. For the CAHs that were effectively transformed (CM, DCM, CA, and 1,1-DCA), multiple additions were made over the 30-h incubation period. Acetylene blocking studies were performed on butane and CAH amended bottles. Acetylene inactivates activity of methane monooxygenase (MMO) and ammonia monooxygenases (AMO) (Prior and Dalton 1985; Bedard and Knowles 1989) and was shown to inactivate butane utilization and CAH transformation in butane utilizing pure cultures (Hamamura et al. 1997). The harvested cells were exposed to acetylene [23 ml, 35% (vol/vol) gas phase] with rapid shaking for 30 min, the bottles were purged with N_2, air was reintroduced, and the CAHs were added.

Cell Inactivation after Exposure to Compounds

Cell inactivation was determined from butane uptake measurements after CAH exposure. After the 30-h incubation, the bottles were purged with N_2 to remove the CAH, and air was then reintroduced. The bottles were recapped, and 2 mL of 10% butane in N_2 was added. Butane also was added to control bottles of cells incubated for 30 h without any CAH exposure. The headspace butane concentration was monitored to determine rates of butane uptake. Butane uptake rates of cells exposed to the CAH were compared to the control bottle of unexposed cells.

To evaluate the loss of butane uptake activity due to the 30-h period of shaking, the initial rates of butane uptake were measured before and after shaking. Butane uptake rates after 30 h of shaking were 82–93% of those with 0 h of shaking. This small loss was accounted for by reporting the percentage of butane uptake rates for cells exposed to individual CAH for 30 h, normalized by the butane uptake rates of cells shaken for 30 h in the absence of CAH.

Chloride Release Study

To evaluate the degree of dechlorination of the CAHs, the amount of chloride released was measured. Aqueous chloride concentrations at the beginning and end of the 30-h incubation were determined using a colorimetric method (Bergnam and Sanik 1957). This method was used to determine CAH dechlorination by *M. trichosporium* OB3b (Oldenhuis et al. 1989; van Hylckama Vlieg et al. 1996).

c-DCE or 1,1-DCE Transformation and Cell Inactivation

Duplicate batch bottles were prepared with combinations of c-DCE or 1,1-DCE, butane [0, 0.5, or 30% (vol/vol) gas phase], butyrate (2 mM), formate (20 mM), and with ambient air or no O_2 to evaluate the effects of exogenous energy source, butane concentration, and O_2 on c-DCE or 1,1-DCE transformations. Preincubation effects of exogenous energy sources were evaluated by incubating resting cells with butyrate (2 mM) or formate (20 mM) for 1 h before the addition of c-DCE. In a cometabolic process, the presence of physiological substrate at sufficiently high concentrations is expected to inhibit the transformation of another substrate (Colby and Dalton 1976). To provide a qualitative evaluation of inhibition (competing for active site of enzyme), butane was added into batch bottles with active cells at two concentrations of either 0.5 or 30% (vol/vol, gas phase) along with c-DCE or 1,1-DCE.

The O_2 depleted bottles were constructed with three vacuum cycles and N_2 gas purging. Four different kinds of cell activity control bottles were constructed: (1) No substrate with cells; (2) butyrate with cells; (3) formate with cells; and (4) c-DCE or 1,1-DCE with acetylene-treated cells.

RESULTS AND DISCUSSION

Cell Activities for Different Cell Preparation Batches

Rates of butane uptake and 1,1-DCE transformation and the T_c of 1,1-DCE were measured to evaluate the cell activities for different cell batches. The average initial transformation rates with standard deviations (SDs) for 10 cell batches were 0.35 ± 0.14-μmol 1,1-DCE transformed/mg TSS/h and 0.61 ± 0.31-μmol butane degraded/mg TSS/h. The average T_c with SD was 0.92 ± 0.23-μmol 1,1-DCE/mg TSS. These rates and transformation capacities were of sufficient reproducibility for the surveys of CAH transformation abilities assessed in the study.

Transformations of 1,1-DCE and c-DCE and Effects of Their Transformation on Cell Inactivation

The effects of O_2, butane, butyrate, formate, or cell treatment with acetylene [35% (vol/vol) gas phase] on the transformation of 1,1-DCE and c-DCE were examined [Figs. 1(a and b)]. The results with duplicate bottles were essentially identical; thus, the results from only one bottle are presented in Fig. 1. No transformation of 1,1-DCE or c-DCE was observed in the absence of O_2. Acetylene-treated cells transformed <15% of the amount of 1,1-DCE or c-DCE achieved by untreated cells. Untreated cells rapidly transformed 1,1-DCE, with most of the transformation occurring within the first 4 h of exposure. The c-DCE was transformed more slowly than 1,1-DCE, with an initial transformation rate being 0.20 μmol/mg TSS/h compared with 0.34 μmol/mg TSS/h for 1,1-DCE. The transformation of c-DCE decreased significantly after 12 h of incubation.

The 1,1-DCE and c-DCE transformation rates in the presence of butane [30% (vol/vol) gas phase] were about 22 and 13% of the rate in the absence of butane, respectively. The amount of 1,1-DCE and c-DCE transformed decreased by 50 and 60%, respectively. However, 0.5% butane did not inhibit the transformation. Butane degradation also was inhibited by both c-DCE and 1,1-DCE, with 1,1-DCE being a stronger inhibitor (data not presented).

The transformation of c-DCE resulted in the production of c-DCE epoxide. Mass spectrometry confirmed the presence of a compound with mass-to-charge-fragment ratios of 112, 83, 48, and 35. The values are in agreement with the spectra of a chemically synthesized DCE epoxide (Janssen et al. 1988).

A monooxygenase enzyme was likely involved in the transformation of the CAHs, based on the lack of transformation in the absence of O_2 and the inactivation of CAH transformation by acetylene and inhibition of CAH transformation by butane. The possible involvement of butane monooxygenase in the transformation of CAHs is consistent with the results obtained with pure butane-utilizing cultures and an enrichment of the culture tested here (Hamamura et al. 1997). More de-

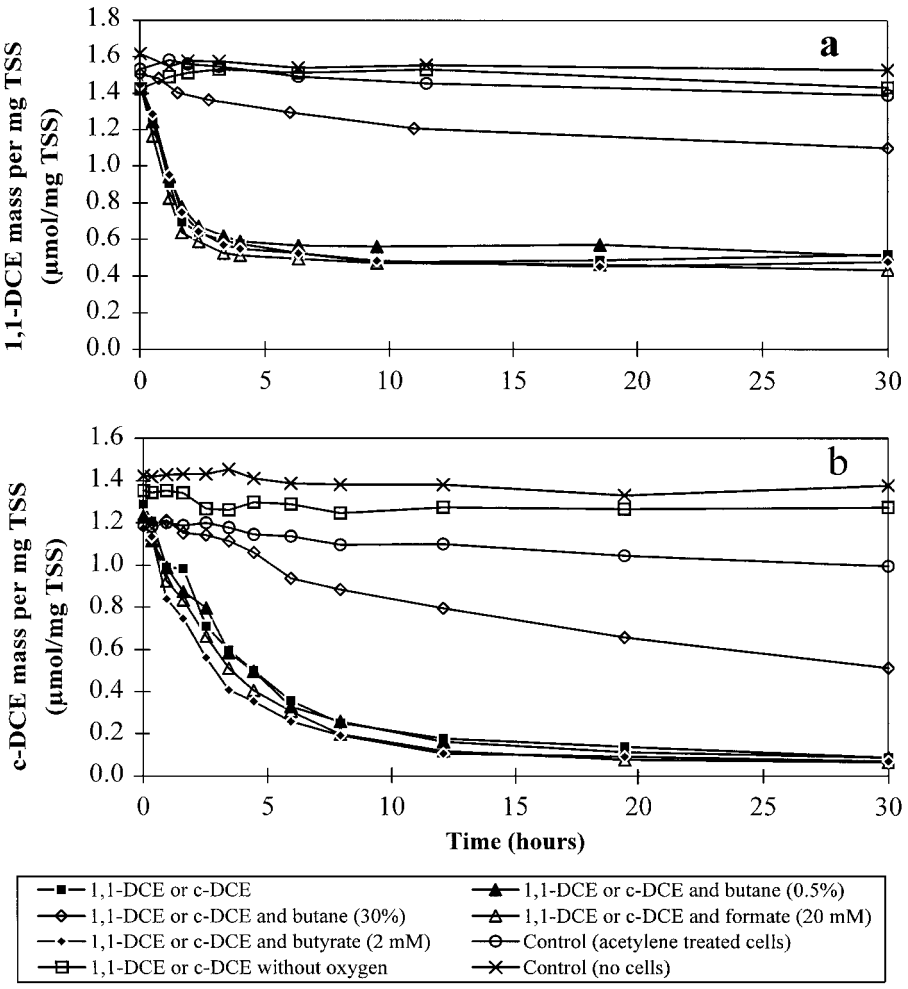

FIG. 1. Resting Cell Transformation of: (a) 1,1-DCE (3.4-mg TSS used); (b) c-DCE (4.5-mg TSS used) in Presence or Absence of O_2, Butane, Butyrate, or Formate

tailed enzyme analyses are needed to verify the involvement of a monooxygenase enzyme.

Butyrate and formate addition had no effect on transformation of 1,1-DCE and c-DCE. Transformation rates and extents and cell activation with or without preincubation or incubation with butyrate and formate were similar. Cells incubated with 1,1-DCE or c-DCE in the absence of O_2 retained 80 or 91% of butane-uptake activity, respectively, after 30 h of exposure, whereas cells incubated with 1,1-DCE or c-DCE in the presence of O_2 retained <1% activity. Cells also were highly inactivated after the transformation of each compound in the presence of O_2 and butyrate or formate, with <1% activity remaining. The rapid inactivation of 1,1-DCE and c-DCE transformation likely resulted from transformation product toxicity. The results indicate that either butyrate and formate are not effective exogenous energy sources or the butane-grown mixed culture is using internal energy reserves such as poly-β-hydroxybutyrate (PHB).

Based on our resting-cell transformation results, internal energy reserves such as PHB are likely driving the CAH transformation. A positive correlation between PHB content of methanotrophs and the TCE transformation rate and capacity was reported (Henry and Grbić-Galić 1991; Henrysson and McCarty 1993; Chu and Alvarez-Cohen 1996; Shah et al. 1996). The synthesis of PHB by *Nocardia* 107-332 grown on butane was reported, and the PHB and other polymers constituted about 12–14% of the cell mass (Davis 1964).

For both c-DCE and 1,1-DCE, similar extents of cell inactivation occurred in the absence or presence of 0.5% butane, with <2% activity remaining. However, with 30% butane, very different cell inactivation was observed. For 1,1-DCE, only 10% activity remained, and for c-DCE, 96% remained. These results indicate that toxicity resulting from c-DCE transformation could be greatly reduced in the presence of butane, whereas the toxicity resulting from 1,1-DCE transformation was only slightly reduced.

Transformation of Chlorinated Methanes, Ethanes, and Ethylenes

Transformation tests were performed for chlorinated methanes, ethanes, and ethylenes in the absence of butane using

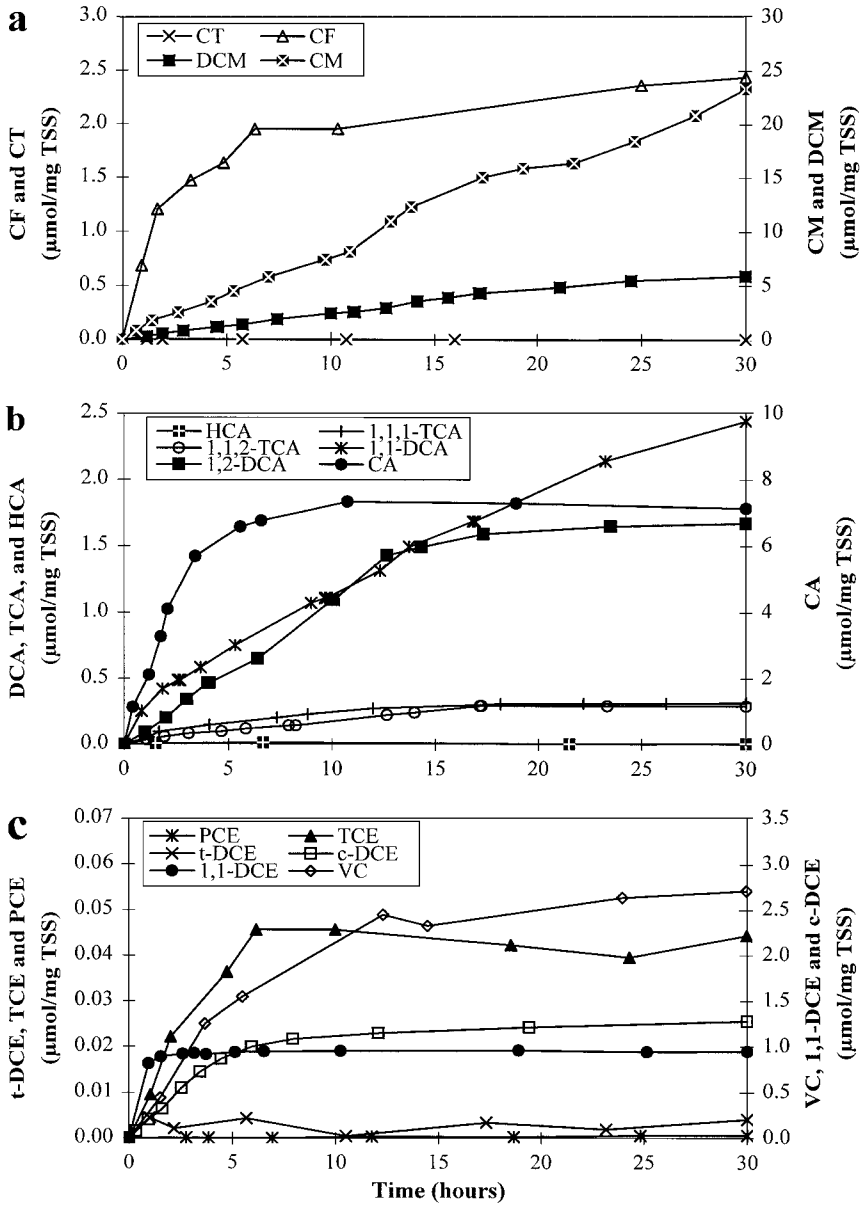

FIG. 2. Resting Cell Transformation of: (a) Chlorinated Methanes; (b) Chlorinated Ethanes; (c) Chlorinated Ethylenes [CM, DCM, CA, and 1,1-DCA; Values (Scale on Right *y*-Axis) Are Cumulative Amounts Following Multiple Additions of CAH; Addition Times in Hours Are As Follows: CM (9.8, 22), DCM (4.6, 11, 17), CA (1.2 and 3.4), and 1,1-DCA (2.7, 9.8, and 17)]

the same procedures as the 1,1-DCE and c-DCE tests. Butyrate and formate were not added as external energy sources. The tests, therefore, relied on the internal energy reserves to drive the CAH transformations. The transformations of all the compounds tested were inhibited by the treating cells with acetylene.

The results for the chlorinated methanes (a), ethanes (b), and ethenes (c) are presented in Fig. 2 where the amounts of CAH transformed per TSS mass versus time are plotted. For CAHs that were effectively transformed (CM, DCM, CA, and 1,1-DCA), the compounds were successively added to the batch reactors. The cumulative transformation amounts are presented (Fig. 2). Single-chlorine substituted CAHs (CM, CA, and VC), were transformed to the greatest extent in each of the three groups. Generally, the molecules with more chlorine atoms showed decreased transformation amounts.

TABLE 1. Average Initial Transformation Rates of Chlorinated Methanes, Ethanes, and Ethenes

CAH (1)	Initial rates[a] (μmol/mg TSS/h) (2)
(a) Chlorinated Methanes	
CM	0.95
DCM	0.28
CF	0.11
CT	≈0.00
(b) Chlorinated Ethanes	
CA	1.68
1,1-DCA	0.19
1,2-DCA	0.11
1,1,1-TCA	0.03
1,1,2-TCA	0.03
TeCA	≈0.00
PCA	≈0.00
HCA	≈0.00
(c) Chlorinated Ethenes	
VC	0.29
1,1-DCE	0.34
c-DCE	0.20
TCE	0.01
t-DCE	≈0.00
PCE	≈0.00

Note: Average initial aqueous concentrations (μm) were CM (428), DCM (90), CF (84), CT (4.0), CA (302), 1,1-DCA (47), 1,2-DCA (52), 1,1,1-TCA (25), 1,1,2-TCA (16), 1,1,1,2-TeCA (8.6), 1,1,2,2,-TeCA (28), PCA (39), HCA (9.3), VC (129), 1,1-DCE (37), c-DCE (87), TCE (19), and PCE (6.5).

[a]Results from duplicate bottles were used in calculating average initial transformation rates.

As expected, no transformation of fully chlorinated CAHs (CT, HCA, and PCE) was observed. The transformations of CM, DCM, and 1,1-DCA continued during the 30-h time course, while transformations of all other compounds ceased. For chlorinated methanes, the transformation rate of CF quickly decreased and CF transformation ceased.

No transformation of 1,1,1,2-TeCA, 1,1,2,2-TeCA, and PCA was observed (data not shown). The relative amounts of chlorinated ethane transformed per unit mass of TSS in the order of the highest to lowest were CA, 1,1-DCA, 1,2-DCA, 1,1,1-TCA, and 1,1,2-TCA. The amount transformed decreased in proportion to chlorine content and also depended on the location of the chlorine substitution. The transformation rate of CA decreased more quickly than that of 1,2-DCA, 1,1,1-TCA, and 1,1,2-TCA.

The amounts transformed per unit mass of TSS for the chlorinated ethenes in the order of the highest to lowest was VC > c-DCE > 1,1-DCE > TCE. The transformation of the DCE isomers differed, with no observable transformation of t-DCE, whereas similar amounts of c-DCE and 1,1-DCE were transformed but at different rates. Transformation rates decreased most rapidly for 1,1-DCE, followed by TCE, c-DCE, and VC.

The initial transformation rates of each CAH are presented in Table 1. For all three classes of compounds, the trend is toward decreasing rates of transformation with increasing number of chlorines. Whether this trend reflects differences in maximum transformation rates k_{max} or half-saturation coefficients K_s or both is not yet known. A more detailed kinetic study is needed to evaluate whether a direct relationship exists between chlorine content and the k_{max} of the butane-grown mixed culture.

CAHs Transformation Effects on Cell Inactivation

To compare the degree of CAH transformation and the transformation effect on cell inactivation, CAHs were divided into classes based on the amount of CAH transformed and the degree of cell inactivation that occurred. Cell inactivation was based on the rate of butane-uptake after 30-h exposure to the CAH. Cell inactivation is presented as the ratio of rates for

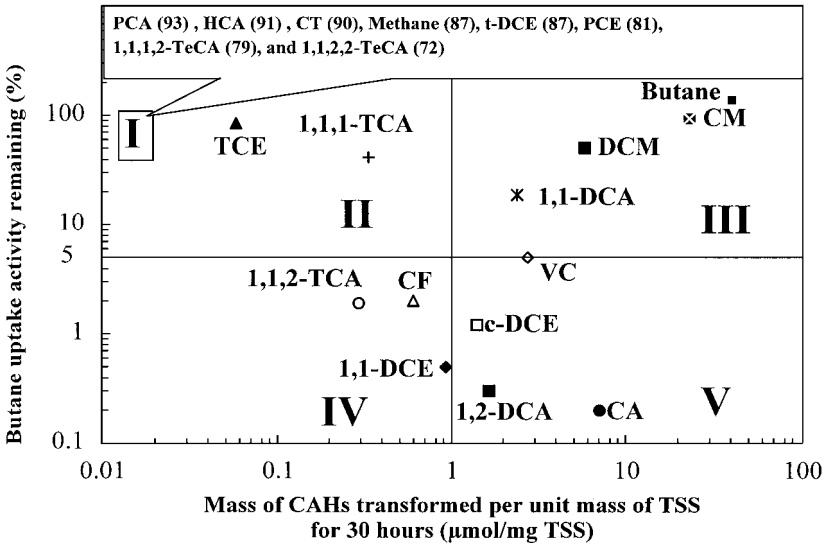

FIG. 3. Mass of CAHs Transformed per Milligram TSS after 30 h of Incubation versus Butane Uptake Activity after Exposure [Values in Class I Box (No Transformation) Indicate Percentage of Butane Uptake Activity Remaining after Exposure to Each Compound]

CAH exposed cells to those of controls with no exposure, which also were incubated for 30 h.

The compounds were divided into five classes, ranging from Class I (no transformation and minor inactivation) to Class V (major transformation and major inactivation). Values <1-μmol CAHs transformed/mg TSS after 30 h of incubation and >5% of cell activity remaining were considered ''minor'' for classification purposes, and values above these benchmarks were considered ''major.'' These criteria are based on the range for practical use in remediation purposes and the ability of cells likely to recover with 5% butane activity remaining. The results of this comparison are shown in Fig. 3.

Class I (≈0-μmol transformed/mg TSS and >70% of Activity Remaining)

Exposure to fully chlorinated aliphatic hydrocarbons (CT, HCA, and PCE), chlorinated ethanes with four or more chlorines, methane, and t-DCE did not greatly reduce cell activity. These compounds were not effectively transformed and general transformation product toxicity was not observed. Exposure to 1,1,2,2-TeCA resulted in loss of some activity; however, transformation was not detected.

The lack of transformation of methane by butane-grown microorganisms is consistent with previous observations of 15 isolated butane-grown bacteria (McLee et al. 1972) and *Nocardia* TB1 grown on butane (Vanginkel et al. 1987). In contrast, ammonia oxygenase, which can oxidize butane, also can oxidize methane (Hyman et al. 1988).

Class II (<1-μmol transformed/mg TSS and >5% of Activity Remaining)

TCE and 1,1,1-TCA were classified as Class II compounds, with limited transformation and limited cell inactivation. In studies with *M. trichosporium* OB3b, high cell inactivation followed TCE transformation; however, the amount transformed was 20 times that of the butane enrichment. Inactivation was attributed to nonspecific covalent binding of transformation products to cellular proteins (Oldenhuis et al. 1991).

Class III (>1-μmol transformed/mg TSS and >5% of Activity Remaining)

Class III compounds have the highest potential for cometabolic treatment due to relatively high transformation amounts and low cell inactivation—CM, DCM, and 1,1-DCA are in this class, with CM most effectively transformed among the CAHs tested.

The CM transformation caused little loss in cell activity in studies with *N. europaea* (Rasche et al. 1991) and methanotrophs (Chang and Alvarez-Cohen 1996). It is plausible that monooxygenase-mediated transformation of CM may produce formaldehyde through an oxidative dechlorination pathway (Rasche et al. 1991). Its transformation with little loss in rate and with essentially no loss in activity indicates that it may, upon being transformed, serve as an energy source for butane utilizers. In contrast, less DCM was transformed and more inactivation occurred.

The 1,1-DCA was effectively transformed and caused moderate cell inactivation. Rasche et al. (1991) suggested that AMO-turnover-dependent inactivation (loss in ammonia-dependent O_2 uptake activity) of ammonia oxidation by *N. europaea* resulted from the transformation of compounds having dichlorinated carbons. They suggested that the production of alkylating agents may provide the basis for inactivation by 1,1-DCA transformation. Such alkylating agents could account for the relatively higher cell inactivation resulting from transformation of 1,1-DCA than from transformation of the other compounds in Class III.

Class IV (<1-μmol transformed/mg TSS and <5% of Activity Remaining)

Compounds in Class IV (CF, 1,1,2-TCA, and 1,1-DCE) are problematic for this enrichment due to the relatively low amounts transformed and the high degree of cell inactivation. Among the chlorinated methanes, only CF was in this class. Previous observations with a methane-utilizing mixed culture suggested toxicity of transformation products of CF cometabolism (Alvarez-Cohen and McCarty 1991). Alvarez-Cohen and McCarty (1991) proposed phosgene as a potential intermediate product of the CF transformation, and Bartnicki and Castro (1994) confirmed this pathway with *M. trichosporium* OB3b. A similar pathway could account for high inactivation by CF transformation for the butane enrichment.

For *M. trichosporium* OB3b expressing soluble MMO (sMMO), 1,1-DCE transformation activity rapidly decreased during transformation and cell viability was greatly reduced (van Hylckama Vlieg et al. 1997). The 1,1-DCE transformation resulted in loss of O_2 uptake ability in *N. europaea* (Rasche et al. 1991). Similar transformation products and toxicity mechanisms likely affected the butane enrichment. The transformation of 1,1,2-TCA caused much greater cell inactivation than 1,1,1-TCA. In the case of TCA isomers, distribution of chlorine on the two carbons results in greater toxicity.

Class V (>1-μmol transformed/mg TSS and <5% of Activity Remaining)

Class V compounds (CA, 1,2-DCA, c-DCE, and VC) are problematic contaminants due to the high cell inactivation; however, relatively large amounts are transformed. Transformation of VC and c-DCE resulted in a high degree of cell inactivation. Cells exposed to CA were essentially completely inactivated. Despite less transformation, exposure to 1,2-DCA caused much higher cell inactivation than 1,1-DCA. As observed with 1,1,1-TCA and 1,1,2-TCA, the isomer with chlorine on each carbon caused more inactivation.

During the transformation of CA, less inactivation occurred with *N. europaea* (Rasche et al. 1991) and methanotrophs (Chang and Alvarez-Cohen 1996), with exposure times of 1 and 4 h, respectively. The major CA transformation product with *N. europaea* was acetaldehyde, and greater inactivation occurred with longer exposures (Rasche et al. 1990; 1991). The 30-h exposures of the butane culture may have resulted in greater inactivation, especially if acetaldehyde was present.

Cell inactivation resulting from CAH transformation was based on the loss of butane uptake ability. This assay provides a good measure of the impacts on the cell of CAH transformation, because butane consumption requires an active butane monooxygenase and an intact electron transport chain. The immediate and stable response to butane indicated that induction and de novo enzyme synthesis were not required. Because reductant is required for the monooxygenase reaction, measurement of butane consumption cannot distinguish between damage to the monooxygenase and to the flow of reductant. Activity studies with a readily degraded CAH also can be used to assay oxygenase enzyme activity and could be used in conjunction with the butane assay.

Chloride Release

The degree of dechlorination of the CAHs was determined by measuring the amount of chloride released after 30 h of incubation. The observed chloride release was compared with stoichiometric release of chloride required for the amount of CAH transformed. The dechlorination extent is presented on a percentage basis (Fig. 4).

Chlorinated methanes and ethylenes were more highly de-

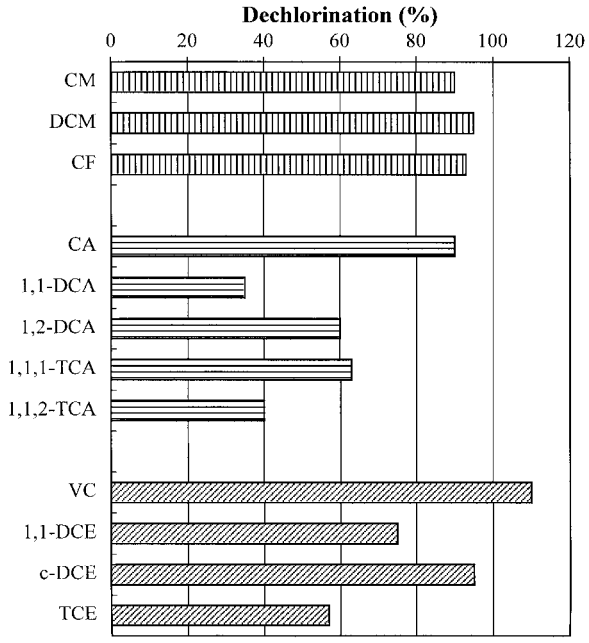

FIG. 4. Average Percent Dechlorination of CAHs after 30 h of Incubation

chlorinated than the chlorinated ethanes, except CA. Nearly complete dechlorination of chlorinated methanes (90–95%) indicates that transformations proceeded past an initial oxidation. Oldenhuis et al. (1989) reported 100 and 76% dechlorination of CF by *M. trichosporium* OB3b expressing sMMO and particulate MMO after 24 h of incubation, respectively, whereas after 1 h of incubation 73% dechlorination of CF by *N. europaea* was reported (Rasche et al. 1991). The degree of dechlorination was likely affected by the experimental protocol used here, where excess CAH was added during a 30-h exposure period. If the CAHs were permitted to be completely transformed, by adding lesser amounts, biotic transformation of products may have occurred, resulting in more complete dechlorination.

Partial dechlorination of 1,1-DCA, 1,2-DCA, 1,1,2-TCA, and 1,1,1-TCA indicates that chlorinated transformation products exist. The 1,1-DCA and 1,2-DCA were completely dechlorinated by *M. trichosporium* OB3b expressing sMMO (Oldenhuis et al. 1989). The 1,1-DCA was completely dechlorinated by *N. europaea*, but 1,2-DCA was only partially de chlorinated (Rasche et al. 1991). The ability to transform products of chlorinated ethanes therefore differs between butane utilizers and the MMO and AMO systems.

Essentially complete oxidative dechlorination of VC and c-DCE was observed, and 75% dechlorination of 1,1-DCE was achieved. The c-DCE was completely dechlorinated by *M. trichosporium* OB3b expressing sMMO, but incomplete 1,1-DCE dechlorination was observed (Oldenhuis et al. 1989). Dolan and McCarty (1995) speculated that methanotrophic transformation of 1,1-DCE may produce 1,1-DCE epoxide that rearranges to form chlorinated products of acyl chlorides (strong alkylating agents). It is plausible that chlorinated products of 1,1-DCE may result in high cell inactivation consistent with observations with other systems.

van Hylckama Vlieg et al. (1996) found that c-DCE epoxide produced from c-DCE transformation could be biologically transformed by *M. trichosporium* OB3b expressing sMMO. The c-DCE epoxide was formed by the butane enrichment, as previously discussed. As shown in Fig. 4, 95% Cl^- release occurred after 30 h of incubation of c-DCE. The half-life of c-DCE epoxide is approximately 72 h (Janssen et al. 1988). The nearly complete oxidative dechlorination of c-DCE within 30 h of incubation suggests c-DCE epoxide transformation by the butane utilizers. The high degree in inactivation of the butane utilizers potentially resulted from the biotic transformation of the epoxide.

SUMMARY AND CONCLUSIONS

An enrichment culture grown on butane transformed chlorinated methanes (CM, DCM, and CF), chlorinated ethanes (CA, 1,1-DCA, 1,2-DCA, 1,1,1-TCA, and 1,1,2-TCA), and chlorinated ethylenes (VC, 1,1-DCE, and c-DCE). A butane monooxygenase enzyme is likely involved in the transformation of CAHs, based on the lack of transformation in the absence of O_2 and the inactivation of CAH transformation by acetylene and inhibition of CAH transformation by butane. More detailed enzyme analyses are needed to verify the involvement of a monooxygenase enzyme.

Many sites are contaminated with mixtures of 1,1,1-TCA, 1,1-DCE, and 1,1-DCA as biotic and abiotic transformation products of 1,1,1-TCA (Vogel and McCarty 1987). The 1,1,1-TCA and 1,1-DCE were problematic CAHs for aerobic cometabolism. Limited 1,1,1-TCA transformation was observed with methane-grown cultures (Henson et al. 1989; Strand et al. 1990). During in situ studies with methane-utilizing microorganisms (Semprini et al. 1990) or with phenol-utilizing microorganisms (Hopkins et al. 1993; Hopkins and McCarty 1995), 1,1,1-TCA was not transformed, despite effective transformation of chlorinated ethenes. The 1,1-DCE also can be cometabolized by microorganisms grown on methane (van Hylckama Vlieg et al. 1996) and ammonia (Rasche et al. 1991). The transformation of 1,1-DCE was shown to be toxic to nitrifying bacteria and methane-utilizing microorganisms (Rasche et al. 1991; Dolan and McCarty 1995; van Hylckama Vlieg et al. 1997).

The butane culture has good potential for transforming 1,1,1-TCA, 1,1-DCE, and 1,1-DCA. Butane utilizers may have advantages over methanotrophs for these contaminants. Our butane-grown culture had a greater ability to transform 1,1,1-TCA on a basis of amount transformed per unit mass cells than a methane-grown mixed culture (Chang and Alvarez-Cohen 1996). The initial transformation rates of 1,1-DCA were comparable with that achieved by *M. trichosporium* OB3b expressing sMMO (Oldenhuis et al. 1991), and the T_c was a factor of 4–9 higher than achieved with methanotrophs (Chang and Alvarez-Cohen 1996). Thus butane utilizers may have better potential for remediating 1,1-DCE contamination than other oxygenase systems, despite being more toxic to the culture than other CAHs.

The CAH transformations by the butane-utilizing culture were achieved without the addition of an exogenous energy source, whereas the results with methanotrophs were typically achieved with the addition of 20-mM formate (Oldenhuis et al. 1991; Chang and Alvarez-Cohen 1996). A higher percentage of butane uptake activity was retained after 30-h exposure to 1,1,1-TCA than the percentage of methane uptake activity retained by methanotrophs exposed to 1,1,1-TCA for 4 h (Chang and Alvarez-Cohen 1996). The T_y values of the butane enrichment for 1,1-DCE and 1,1,1-TCA were also higher than those obtained by Chang and Alvarez-Cohen (1996) with chemostat-grown methanotrophs, likely because the cell yield of the butane enrichment was a factor of 2.5 higher than the methanotrophs. The 1,1-DCA also was very effectively transformed by the butane enrichment and showed a low degree of inactivation. The enrichment results are consistent with the ground-water/soil microcosm results (Kim et al. 1997b), which indicated that butane is an effective substrate for treating mixtures of 1,1,1-TCA, 1,1-DCE, and 1,1-DCA, as well as chlorinated methanes, VC, and c-DCE. The enrichment, how-

ever, did not effectively transform TCE, which is a common ground-water contaminant.

Effective transformation with methanotrophs is usually induced under copper-limited nutrient conditions, so that sMMO is expressed. Copper was not limiting (0.1 μM as $CuCl_2 \cdot 2H_2O$) in the media formulation used here. Thus butane utilizers might also have an advantage for in situ remediation, where it is difficult to limit copper available.

The results presented are for one butane enrichment that shows a broad range of CAH transformation abilities. More studies are needed with pure cultures and enrichments from other sites to determine how CAH transformation abilities differ among cultures and growth conditions.

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APPENDIX. REFERENCES

Alvarez-Cohen, L. M., and McCarty, P. L. (1991). ''Product toxicity and cometabolic competitive inhibition modeling of chloroform and trichloroethylene transformation by methanotrophic resting cells.'' *Appl. Envir. Microbiology*, 57(4), 1031–1037.

American Public Health Association (APHA). (1985). *Standard methods for the examination of water and wastewater*, 16th Ed. New York.

Bartnicki, E. W., and Castro, C. E. (1994). ''Biodehalogenation: Rapid oxidative metabolism of mono- and polyhalomethanes by *Methylosinus trichosporium* OB-3b.'' *Envir. Toxicology and Chem.*, 13(2), 241–245.

Bedard, C., and Knowles, R. (1989). ''Physiology, biochemistry, and specific inhibitors of CH_4, NH_4^+ and CO oxidation by methanotrophs and nitrifiers.'' *Microbiology Rev.*, 53(1), 68–84.

Bergnam, J. G., and Sanik, J. (1957). ''Determination of trace amounts of chloride in naphtha.'' *Analytical Chem.*, 29(2), 241–243.

Chang, H. L., and Alvarez-Cohen, L. M. (1996). ''Biodegradation of individual and multiple chlorinated aliphatic hydrocarbons by methane-oxidizing cultures.'' *Appl. Envir. Microbiology*, 62(9), 3371–3377.

Chu, K.-H., and Alvarez-Cohen, L. (1996). ''Trichloroethylene degradation by methane-oxidizing cultures grown with various nitrogen sources.'' *Water Envir. Res.*, 68(1), 76–82.

Colby, J., and Dalton, H. (1976). ''Some properties of a soluble methane mono-oxygenase from *Methylococcus capsulatus* strain bath.'' *Biochemistry J.*, 157, 495–497.

Davis, J. B. (1964). ''Cellular lipids of a *Nocardia* grown on propane and n-butane.'' *Appl. Microbiology*, 12(4), 301–304.

Dolan, M. E., and McCarty, P. L. (1995). ''Methanotrophic chloroethene transformation capacities and 1,1-dichloroethene transformation product toxicity.'' *Envir. Sci. and Technol.*, 29(11), 2741–2747.

Ensign, S. A., Hyman, M. R., and Arp, D. J. (1992). ''Cometabolic degradation of chlorinated alkenes by alkene monooxygenase in a propylene-grown *Xanobacter* strain.'' *Appl. Envir. Microbiology*, 58(9), 3038–3046.

Gossett, J. M. (1987). ''Measurements of Henry's law constants for C1 and C2 chlorinated hydrocarbons.'' *Envir. Sci. and Technol.*, 21(2), 202–208.

Hamamura, N., Page, C., Long, T., Semprini, L., and Arp, D. J. (1997). ''Chloroform cometabolism by butane-grown CF8, *Pseudomonas butanovora*, and *Mycobacterium vaccae* JOB5 and methane-grown *Methylosinus trichosporium* OB3b.'' *Appl. Envir. Microbiology*, 63(9), 3607–3613.

Henry, S. M., and Grbić-Galić, D. (1991). ''Influence of endogenous and exogenous electron donors and trichloroethylene oxidation toxicity on trichloroethylene oxidation by methanotrophic cultures from a groundwater aquifer.'' *Appl. Envir. Microbiology*, 57(1), 236–244.

Henrysson, T., and McCarty, P. L. (1993). ''Influence of the endogenous storage lipid poly-β-hydroxybutyrate on the reducing power availability during cometabolism of trichloroethylene and naphthalene by resting methanotrophic mixed cultures.'' *Appl. Envir. Microbiology*, 59(5), 1602–1606.

Henson, J. M., Yates, M. V., and Cochran, J. W. (1989). ''Metabolism of chlorinated methanes, ethanes and ethylenes by a mixed bacterial culture growing on methane.'' *J. Industrial Microbiology*, 4(1), 29–35.

Hopkins, G. D., and McCarty, P. L. (1995). ''Field evaluation of in situ aerobic cometabolism of trichloroethylene and three dichloroethylene isomers using phenol and toluene as primary substrates.'' *Envir. Sci. and Technol.*, 29(6), 1628–1637.

Hopkins, G. D., Semprini, L., and McCarty, P. L. (1993). ''Microcosm and in situ field studies of enhanced biotransformation of trichloroethylene by phenol-utilizing microorganisms.'' *Appl. Envir. Microbiology*, 59(7), 2277–2285.

Hyman, M. R., Murton, I. B., and Arp, D. J. (1988). ''Interaction of ammonia monooxygenase from *Nitrosomonas europaea* with alkanes, alkenes, and alkynes.'' *Appl. Envir. Microbiology*, 54(12), 3187–3190.

Janssen, D. B., Grobben, G., Hoekstra, R., Oldenhuis, R., and Witholt, B. (1988). ''Degradation of trans-1,2-dichloroethylene by mixed and pure cultures of methanotrophic bacteria.'' *Appl. Microbiology Biotechnology*, 29(4), 392–399.

Kampbell, D. H., Wilson, J. T., and Vandergrift, S. A. (1989). ''Dissolved oxygen and methane in water by a GC headspace equilibration technique.'' *J. Envir. Anal. Chem.*, 36, 249–259.

Kim, Y., Semprini, L., and Arp, D. J. (1997a). ''Aerobic cometabolism of chloroform and 1,1,1-trichloroethane by butane-grown microorganisms.'' *Bioremediation J.*, 2(1), 135–148.

Kim, Y., Semprini, L., and Arp, D. J. (1997b). ''Aerobic cometabolism of chloroform, 1,1,1-trichloroethane, and the other chlorinated aliphatic hydrocarbons by butane-utilizing microorganisms.'' *In situ and on-site bioremediation*, Vol. 3, B. C. Alleman and A. Leeson, eds., Battelle, Columbus, Ohio, 107–112.

Mackay, D., and Shiu, W. Y. (1981). ''A critical review of Henry's law constants for chemicals of environmental interest.'' *J. Phys. Chem. Ref. Data*, 10(4), 1175–1199.

McCarty, P. L., and Semprini, L. (1993). ''Ground-water treatment of chlorinated solvents.'' *Handbook of bioremediation*, Lewis, Chelsea, Mich., 87–116.

McLee, A. G., Kormendy, A. C., and Wayman, M. (1972). ''Isolation and characterization of n-butane utilizing microorganisms.'' *Can. J. Microbiology*, 18(8), 1191–1195.

Oldenhuis, R., Oedzes, J. Y., van der Waarde, J. J., and Janssen, D. B. (1991). ''Kinetic of chlorinated hydrocarbon degradation by *Methylosinus trichosporium* OB3b and toxicity of trichloroethylene.'' *Appl. Envir. Microbiology*, 57(1), 7–14.

Oldenhuis, R., Vink, R. L. J. M., Janssen, D. B., and Witholt, B. (1989). ''Degradation of chlorinated aliphatic hydrocarbons by *Methylosinus trichosporium* OB3b expressing soluble methane monooxygenase.'' *Appl. Envir. Microbiology*, 55(11), 2819–2826.

Prior, S. D., and Dalton, H. (1985). ''Acetylene as a suicide substrate and active site probe for methane monooxygenase from *Methylococcus capsulatus* (Bath).'' *FEMS Microbiol. Lett.*, 29(1–2), 105–109.

Rasche, M. E., Hicks, R. E., Hyman, M. R., and Arp, D. J. (1990). ''Oxidation of monohalogenated ethenes and n-chlorinated alkanes by whole cells of *Nitrosomonas europaea*.'' *J. Bacteriology*, 172(9), 5368–5373.

Rasche, M. E., Hyman, M. R., and Arp, D. J. (1991). ''Factors limiting aliphatic chlorocarbon degradation by *Nitrosomonas europaea*: Cometabolic inactivation of ammonia monooxygenase and substrate specificity.'' *Appl. Envir. Microbiology*, 57(10), 2986–2994.

Shah, N. N., Hanna, M. L., and Taylor, R. T. (1996). ''Batch cultivation of *Methylosinus trichosporium* OB3b:V. Characterization of poly-β-hydroxybutyrate production under methane-dependent growth conditions.'' *Biotechnology and Bioengineering*, 49(2), 161–171.

Semprini, L., Roberts, P. V., Hopkins, G. D., and McCarty, P. L. (1990). ''A field evaluation of in-situ biodegradation of chlorinated ethenes: Part 2. The results of biostimulation and biotransformation experiments.'' *Ground Water*, 28(5), 715–727.

Strand, S. E., Bjelland, M. D., and Stensel, H. D. (1990). ''Kinetics of chlorinated hydrocarbon degradation by suspended cultures of methane-oxidizing bacteria.'' *Res. J. Water Pollution Control Fedn.*, 62(2), 124–129.

Vancheeswaran, S., Halden, R. U., Williamson, K. J., Ingle, J. D., and Semprini, L. (1999). ''Abiotic and biological transformation of tetraalkoxysilanes and trichloroethene/cis-1,2-dichloroethene cometabolism driven by tetrabutoxysilane-degrading microorganisms.'' *Envir. Sci. and Technol.*, 33(7), 1077–1085.

Vanginkel, C. G., Welten, H. G. J., Hartmans, S., and de Bont, J. A. M. (1987). ''Metabolism of trans-2-butene in *Nocardia* TB1.'' *J. General Microbiology*, 133(7), 1713–1720.

van Hylckama Vlieg, J. E. T., de Koning, W., and Janssen, D. B. (1996). ''Transformation kinetic of chlorinated ethenes by *Methylosinus tri-*

chosporium OB3b and detection of unstable epoxides by on-line gas chromatography.'' *Appl. Envir. Microbiology*, 62(9), 3304–3312.
van Hylckama Vlieg, J. E. T., de Koning, W., and Janssen, D. B. (1997). ''Effect of chlorinated ethene conversion on viability and activity of *Methylosinus trichosporium* OB3b.'' *Appl. Envir. Microbiology*, 63(12), 4961–4964.
Vogel, T. L., and McCarty, P. L. (1987). ''Abiotic and biotic transformations of 1,1,1-trichloroethane under methanogenic conditions.'' *Envir. Sci. and Technol.*, 21(12), 1208–1213.
Wiegant, L. P., and de Bont, J. A. M. (1980). ''A new route for ethylene glycol metabolism in *Mycobacterium* E44.'' *J. General Microbiology*, 120(2), 325–331.