FINAL REPORT

In Situ Bioremediation of 1,4-Dioxane by Methane Oxidizing Bacteria in Coupled Anaerobic-Aerobic Zones

SERDP Project ER-2306

FEBRUARY 2016

Charles Schaefer, PhD
CDM Smith

Paul Koster van Groos, PhD
CB&I

Paul Hatzinger, PhD
CB&I

SERDP
DOD ▪ EPA ▪ DOE

Page Intentionally Left Blank

Page Intentionally Left Blank

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
11-02-2016	Final	June 2013 - February 2016

4. TITLE AND SUBTITLE
In Situ Bioremediation of 1,4-Dioxane by Methane Oxidizing Bacteria in Coupled Anaerobic-Aerobic Zones

5a. CONTRACT NUMBER
W912HQ-13-C-0009

5b. GRANT NUMBER
NA

5c. PROGRAM ELEMENT NUMBER
NA

6. AUTHOR(S)
Schaefer, Charles, Ph.D.
Hatzinger, Paul B., Ph.D.

5d. PROJECT NUMBER
ER-2306

5e. TASK NUMBER
NA

5f. WORK UNIT NUMBER
NA

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
CB&I Federal Services, LLC.
17 Princess Road
Lawrenceville, NJ 08648

8. PERFORMING ORGANIZATION REPORT NUMBER
NA

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Environmental Security Technology Certification Program
4800 Mark Center Drive, Suite 17D08
Alexandria, VA 22350-3605

10. SPONSOR/MONITOR'S ACRONYM(S)
ESTCP

11. SPONSOR/MONITOR'S REPORT NUMBER(S)
NA

12. DISTRIBUTION/AVAILABILITY STATEMENT
Distribution Statement A: Approved for Public Release, Distribution is Unlimited

13. SUPPLEMENTARY NOTES
None

14. ABSTRACT

Microcosm tests, microbial enrichments, pure culture studies, and column experiments were performed to evaluate the co-metabolic biodegradation of 1,4-dioxane (1,4-D) by methane-, ethane- and propane-oxidizing bacteria. Results obtained during this limited scope study showed that methane-oxidizing bacteria were not effective for promoting the biodegradation of 1,4-D. For all conditions examined, including use of copper chelators, various nutrients, pure and enrichment cultures, and soil collected from 3 different sites, 1,4-D biodegradation was not observed with methane as a cosubstrate. However, ethane- and propane-oxidizing bacteria were shown to biodegrade 1,4-D. Michaelis-Menten kinetic parameters were determined for both 1,4-D and ethane during degradation by an enrichment culture. Regression of the model parameters Vmax and Km for 1,4-dioxane were $4.6 \pm 1.7 \times 10(-5)$ mg/mg/cell/h and 0.23 ± 0.07 mg/L, respectively; Vmax and Km for ethane were $1.8 \pm 0.6 \times 10(-3)$ mg/mg/cell/h and 0.064 ± 0.02 mg/L, respectively. Overall, the data suggest that ethane and propane are more effective cosubstrates than methane for stimulating 1,4-D biodegradation.

15. SUBJECT TERMS
biodegradation, aquifer, 1,4-dioxane, methane, propane, ethane, cometabolism, kinetics,

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			Dr. Charles Schaefer
U	U	U	UU		19b. TELEPHONE NUMBER *(Include area code)* 732-590-4633

Page Intentionally Left Blank

Table of Contents

List of Figures

List of Tables

List of Acronyms and Abbreviations

AFCEC – Air Force Civil Engineer Center
AFP3 – Air Force Plant 3
BAA – Broad Agency Announcement
bgs – below ground surface
BSM – basal salt medium
^{o}C – degrees Celsius
CB&I – CB&I Federal Services, LLC
Cu – copper
CVOC – chlorinated volatile organic compound
DAP – diammonium phosphate
DO – dissolved oxygen
DoD – Department of Defense
EPA – Environmental Protection Agency
ESTCP – Environmental Security Technology Certification Program
ft – foot/feet
g – gram(s)
GC-FID – gas chromatograph-flame ionization detector
GC-MS – gas chromatograph-mass spectrometer
GC-TCD – gas chromatograph-thermal conductivity detector
h – hour(s)
HCl – hydrochloric acid
2HEAA – 2-hydroxyethoxyacetic acid
H_2O – water
KH_2PO_4 – potassium phosphate
L – liter(s)
MBAFB – Myrtle Beach Air Force Base
µg – microgram(s)
mg – milligram(s)
µM – micromolar
µmols – micromoles
$MnSO_4$ – manganese sulfate
N – nitrogen
NaCl – sodium chloride
$NaHCO_3$ – sodium bicarbonate
NaH_2PO_4 – sodium phosphate, monobasic
$NaNO_3$ – sodium nitrate
$NaSO_4$ – sodium sulfate
NDMA – n-nitrosodimethylamine
ng – nanogram(s)
$(NH_4)_2HPO_4$ – diammonium phosphate
NMS – nitrate mineral salts
OD – optical density
P - phosphate
pMMO – particulate methane monooygenase

PCE – tetrachloroethene
SERDP – Strategic Environmental Research and Development Program
sMMO – soluble methane monooygenase
TCA – trichloroethane
TCE – trichloroethene
THF – tetrahydrofuran
UV – ultraviolet
VAFB – Vandenberg Air Force Base
VC – vinyl chloride
VOCs – volatile organic compounds

1.0 ABSTRACT

A series of microbial enrichments, batch biodegradation testing, pure culture studies, and column testing was performed to evaluate the co-metabolic biodegradation of 1,4-dioxane by methane-, ethane-, and propane-oxidizing bacteria. Results obtained during this limited scope study showed that methane-oxidizing bacteria were not effective for promoting the biodegradation of 1,4-dioxane. For all conditions examined, including use of copper chelators, various nutrients, pure and enrichment cultures, and soil collected from 3 different sites, 1,4-dioxane biodegradation was not observed with methane as a cosubstrate. However, ethane- and propane-oxidizing bacteria were shown to biodegrade 1,4-dioxane. Because ethane often is present at 1,4-dioxane sites (due to dechlorination processes associated with co-mingled chlorinated solvents), our research efforts with respect to kinetic and column studies shifted to assessing ethanotrophs for biodegradation of 1,4-dioxane. To this point, ethane has rarely been considered as a co-metabolic substrate in aquifers with 1,4-dioxane or other contaminants-of-concern.

Michaelis-Menten kinetic parameters were determined for both 1,4-dioxane and ethane using a mixed culture obtained from former Myrtle Beach Air Force Base (MBAFB) in South Carolina. Regression of the model parameters V_{max} and K_m for 1,4-dioxane were 4.6 ± 1.7 x 10^{-5} mg/mg/cell/h and 0.23 ± 0.07 mg/L, respectively; V_{max} and K_m for ethane were 1.8 ± 0.6 x 10^{-3} mg/mg/cell/h and 0.064 ± 0.02 mg/L, respectively. Ethane inhibition of 1,4-dioxane biodegradation was well described by assuming the inhibition coefficient was equal to the ethane half-saturation coefficient.

Using these regressed parameters, as well as the observed rates of ethane biodegradation in MBAFB soil, the estimated half-life for 1,4-dioxane was approximately 1.9 years. This value is in excellent agreement with published rates of 1,4-dioxane biodegradation observed in the field (Adamson et al., 2015). In addition, results from MBAFB in this study show that ethane is present within the 1,4-dioxane plume at concentrations on the order of 20 µg/L. Others have shown ethane at concentrations on the order of 0.06 µg/L within 1,4-dioxane plumes (e.g., Chiang et al., 2012). Thus, results of this research suggest that ethane-oxidizing bacteria, sustained by the presence of ethane at sites with co-mingled 1,4-dioxane and chlorinated solvent plumes, may be responsible for slow yet sustained 1,4-dioxane biodegradation at some DoD facilities. Further laboratory and field studies are necessary to confirm this hypothesis and to develop an improved understanding of the occurrence and types of ethane-degrading bacteria in groundwater aquifers and the enzyme(s) they utilize for degrading ethane and 1,4-dioxane, as well as their potential role in the fate of other DoD contaminants of concern.

2.0 OBJECTIVE

The overall goal of this limited scope SERDP effort was to measure and assess the extent to which 1,4-dioxane can be biodegraded by methane-oxidizing bacteria under conditions representative of a co-mingled chlorinated solvent plume. For this effort, our focus initially was on the co-metabolic biodegradation of 1,4-dioxane by methanotrophs. The methane required for this process is a typical byproduct of organic substrate addition to aquifers, the bioremediation approach most commonly used for chlorinated solvents. The ability of methanotrophs to biodegrade 1,4-dioxane was evaluated in a series of laboratory batch and column experiments under geochemical conditions representative of those observed downgradient of typical solvent plumes that have undergone biological treatment via substrate addition. To attain this overall goal, the following specific objectives were proposed:

- Provide a preliminary screening assessment to determine the extent to which methanotrophs capable of degrading 1,4-dioxane are present at 1,4-dioxane contaminated sites;

- Determine the conditions, with respect to methane, dissolved oxygen, and chlorinated solvent concentrations, for which effective biodegradation of 1,4-dioxane by methanotrophs will occur;

- Determine the rates and extents of 1,4-dioxane degradation by indigenous methanotrophs; and

- Evaluate the overall potential for natural attenuation of 1,4-dioxane by methanotrophs in the downgradient plume following and during bioremediation of co-mingled chlorinated solvents.

As discussed in the Methods and Results sections of this report, early results indicated that methanotrophs were not effective at co-metabolic biodegradation of 1,4-dioxane under the conditions tested in our study. However, ethanotrophs were shown to be effective, thus ethanotrophs became the focus of this research effort. Ethane is generated in many chlorinated solvent plumes, and has previously been shown to support the microbial co-metabolism of 1,2-dibromoethane to below 20 ng/L (Hatzinger et al., 2015); thus, we believe that the use of ethanotrophs for natural attenuation and enhanced treatment of 1,4-dioxane warrants further evaluation.

3.0 BACKGROUND

1,4-Dioxane Treatment Challenges

Few treatment methods have proven successful and economically feasible for removing 1,4-dioxane from groundwater *in situ*. Because of its low K_{oc} and Henry's Law constant, traditional remediation technologies such as carbon adsorption and air stripping are inefficient and costly. Chemical oxidants such as Fenton's reagent and persulfate have been shown to be effective for treating 1,4-dioxane (Klečka and Gonsior, 1986, Félix-Navarro et al., 2007), but the technical and economic feasibility of applying these *in situ* oxidation technologies under site specific conditions have not been fully examined. Only a few *ex situ* technologies, including chemical oxidation with combined addition of ozone and hydrogen peroxide (Adams et al., 1994) or hydrogen peroxide and UV light (Stefan and Bolton, 1998) have been utilized commercially to destroy 1,4-dioxane, but the cost of applying these technologies can be prohibitive.

Bioremediation

Relatively few studies have thoroughly evaluated the biological degradation of 1,4-dioxane in natural environments, but current data suggest that indigenous microorganisms at contaminated sites often are not able to degrade this compound under ambient conditions (Fincher and Payne, 1962; Lesage et al., 1990; Sei et al., 2010). During the past several years, however, 1,4-dioxane biodegradation has been reported by both pure cultures (Parales et al., 1994; Burback and Perry, 1993; Bernhardt and Diekmann, 1991) and mixed cultures of bacteria (Klečka and Gonsior, 1986; Zenker et al., 2000). For example, a pure culture of the propane oxidizing bacterium *Mycobacterium vaccae* JOB5 was shown to degrade 1,4-dioxane, but not to grow on the compound (Burback and Perry, 1993). Bernhardt and Diekmann (1991) reported the biodegradation of 1,4-dioxane by a *Rhodococcus* strain, and Parales et al. (1994) isolated a bacterium (strain CB1190) that is capable of sustained growth and mineralization of 1,4-dioxane, albeit at low rates. Strain CB1190 has recently been re-classified as *Pseudonocardia dioxanivorans* strain CB1190 (Mahendra and Alvarez-Cohen, 2005). In other studies, mixed cultures of bacteria were able to degrade 1,4-dioxane, but only in the presence of the co-substrate tetrahydrofuran (THF) (Zenker et al., 2000).

The first microbial pathway of 1,4-dioxane degradation was described in our laboratory (Vainberg et al., 2006) in a study of propane and THF-oxidizing bacteria. Results from this study revealed that propanotrophs can oxidize 1,4-dioxane after growth on propane, but are not able to use the ether as a sole carbon and energy source, either because of poor induction of the propane oxidation genes or due to an inability to grow on 2-hydroxyethoxyacetic acid (2HEAA), a product of 1,-4 dioxane degradation by these bacteria. Using propane as a co-substrate, however, propanotrophs were able to degrade 1,4-dioxane in aquifer microcosms for extended periods, and presumably other bacteria in the environmental samples were able to metabolize the resulting 2HEAA. This approach was recently demonstrated in the field at Vandenberg AFB using air and propane biosparging combined with bioaugmentation (Lippincott et al., 2015). In the pure culture studies of Mahendra and Alvarez-Cohen (2006), the methane oxidizing laboratory strain *Methylosinus trichosporium* OB3b was also reported to biodegrade 1,4-dioxane, albeit at high concentrations (500 mg/L).

Two Phase Treatment Approach for 1,4-Dioxane

The cometabolic biodegradation of 1,4-dioxane via methane oxidizing bacteria is of particular interest for *in situ* remedial purposes for two reasons.

(1) Cometabolic mechanisms allow for treatment of contaminants to very low levels; even levels below those required to sustain the growth and survival of the degradative organisms (Fournier et al., 2006; Hatzinger et al., 2011, 2015). When a target pollutant is a growth substrate for a degradative organism, the amount of carbon and the energy available to the organism becomes depleted as the contaminant concentration is reduced to very low levels. With cometabolic treatment, the growth substrate required for the organisms (e.g., methane) can be in abundant supply to support the degradative bacteria while the organisms degrade the target pollutant to low levels. This was clearly shown in a recent study of NDMA degradation by Hatzinger et al., (2011), where a propane-oxidizing bacterium was able to consistently biodegraded NDMA to < 10 ng/L (parts-per-trillion) in a propane-fed bioreactor. Similarly, in an *in situ* field demonstration using propane-oxidizing bacteria, 1,4-dioxane was biodegraded to <2 µg/L (Lippincott et al., 2015).

(2) Second, methane is often present in the chlorinated solvent source zones associated with the 1,4-dioxane plumes. This methane may be present as a result of *in situ* biostimulation to treat the chlorinated solvents, anaerobic biodegradation of other organic co-contaminants (e.g., hydrocarbons), or decomposition of organic matter by native bacteria. Results from several field studies of anaerobic bioremediation of chlorinated solvents, including our recently completed ESTCP project ER-0515 that examined the impacts of bioaugmentation dosage on clean-up times, indicate that methane groundwater concentrations in the low mg/L range, and up to 14 mg/L, can be expected within the treatment zone (Ellis et al., 2000; Hood et al., 2008; Scheutz et al., 2010). Downgradient of this anoxic source zone, aquifer conditions often are characterized as microaerophilic to aerobic, as anoxic water from the treatment area mixes with aerobic water in the bulk aquifer. Aerobic conditions also can be generated artificially via biosparging, oxygen release compounds, passive oxygen diffusion devices, or other means. If appropriate methanotrophs are present in the aquifer in the microaerophilic/aerobic zone (along with methane from upgradient treatment of VOCs), then the biodegradation of 1,4-dioxane may be favored. This conceptualized two- phase treatment approach is shown in Figure 3-1. ***It is also noted that other short chain hydrocarbons, such as ethane and ethene also are often present in chlorinated solvent source zones associated with 1,4-dioxane plumes (e.g., Chiang et al., 2012), thus these compounds also may serve as potential co-metabolites for 1,4-dioxane biodegradation.***

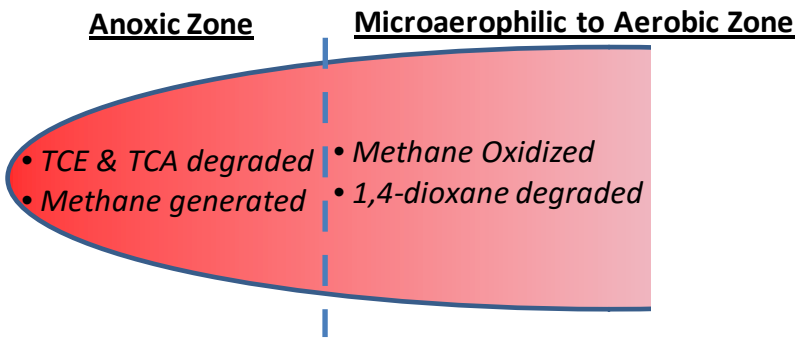

Figure 3-1. Schematic of two-phase treatment approach for 1,4-dioxane.

Approaches similar to that presented in Figure 3-1 have been employed for PCE, TCE, and their reductive dechlorination daughter products. Studies have shown that biological treatment of PCE and TCE occurs in the anoxic zone after addition of electron donor, while vinyl chloride is biodegraded under microaerophilic to aerobic conditions downgradient of the source (Beeman and Bleckmann, 2002; Witt et al., 2002). ***Gossett (2010) recently showed that only very low levels of oxygen (0.02 mg/L) were needed to promote the direct oxidation of VC, suggesting that aerobic biodegradation processes needed for the co-metabolic treatment of 1,4-dioxane also may be possible in microaerophilic regions in close proximity to anoxic source zones.***

A similar mechanism to that described in the above paragraphs, and in Figure 3-1, was observed by Freedman et al. (2001) for the co-metabolic biodegradation of vinyl chloride by methane and ethene downgradient of a TCE and TCA plume. Their work showed that the methane (and ethene) generated during the reductive dechlorination of TCE and TCA in the source area could facilitate the co-metabolic biodegradation of vinyl chloride in the downgradient plume where microaerophilic to aerobic conditions exist. The methane concentration used to stimulate co-metabolic treatment of vinyl chloride was only 0.2 mg/L in groundwater.

While a laboratory study revealed that a methanotroph was able to oxidize 1,4-dioxane at a high concentration (Mahendra and Alvarez-Cohen, 2006), studies have not been performed to measure and assess the extent to which indigenous methanotrophs in aquifers can degrade 1,4-dioxane (or other short chain hydrocarbons) in sequenced anoxic-aerobic aquifer materials, similar to the process shown in Figure 3-1, and similar to processes demonstrated for vinyl chloride. Key questions regarding this process include:

- Are methanotrophs capable of 1,4-dioxane biodegradation typically present in the environment?
- Does the presence of chlorinated solvents or their degradation products impact the ability of methanotrophs to degrade 1,4-dioxane?
- To what extent might methane inhibit co-metabolic 1,4-dioxane degradation *in situ*?
- What dissolved oxygen (DO) levels are needed to facilitate the biodegradation of 1,4-dioxane (i.e., are trace levels of DO sufficient and/or are high DO levels inhibitory)?
- What is the rate and extent of 1,4-dioxane biodegradation that can be achieved under typical methane-oxidizing conditions in an aquifer?

4.0 MATERIALS AND METHODS

4.1 OVERALL APPROACH AND RATIONALE

The overall approach employed for this study consisted of first collecting soil from 1,4-dioxane and chlorinated solvent impacted sites. These soil samples were then used in screening experiments to determine if methane (or other short-chain hydrocarbons such as propane or ethane) oxidizing bacteria were present, and if these bacteria could facilitate the oxidation of 1,4-dioxane. After preparing enrichment cultures from selected samples, batch experiments were performed to assess the co-metabolic biodegradation kinetics of 1,4-dioxane. Finally, column experiments were performed to simulate the co-metabolic biodegradation of 1,4-dioxane in the downgradient plume.

4.2 SITE EVALUATION, SELECTION, AND SAMPLE COLLECTION.

Contaminant and geochemical data were collected from 3 sites where co-mingled chlorinated solvents and 1, 4-dioxane are known to exist. Sites included Myrtle Beach AFB, SC (MBAFB), (2) Vandenberg AFB, CA (VAFB) and (3), Air Force Plant 3, OK (AFP3). Aquifer and groundwater samples from sites 1 and 2 were available in CB&I's laboratory in Lawrenceville, NJ as part of work conducted under AFCEC BAA Project #769 "Demonstration of Enhanced Biodegradation of 1,4-Dioxane Under Methane-Oxidizing Conditions" (Paul Hatzinger, PI).

Site FT-11 from Myrtle Beach AFB was selected, and has a low concentration of 1, 4-dioxane (~ 1 to 50 μg/L), and residual vinyl chloride. Aquifer solids were retained from two shallow zones and an intermediate zone. The samples collected from these areas were used for microcosm studies and enrichments to evaluate the potential for methanotrophs to biodegrade 1,4-dioxane in these zones. The ability to enhance degradation of 1,4-dioxane using ethane and propane also was evaluated.

Vandenberg AFB (VAFB) was selected as a second site for evaluation. This site was also in conjunction with work conducted under AFCEC BAA Project #518 "*In Situ* Bioremediation of 1,4-Dioxane-Contaminated Aquifers" (Rob Steffan, PI), in which the application of propane gas for enhancing 1,4-dioxane biodegradation was being evaluated in the field. Later, this site was evaluated for potential for 1, 4-dioxane degradation as part of Project #769.

The third site for evaluation was former Air Force Plant 3 in Tulsa, OK. Aquifer solids and groundwater were provided by Dr. Borden and colleagues at Solutions IES, who also have an AFCEC project focused on 1,4-dioxane biodegradation (AFCEC Contract FA8903-12-C-0007: "Novel Substrate Application for Bioremediation of Comingled 1,4-Dioxane and Chlorinated Solvent Plumes"). Dr. Borden confirmed that the concentrations of 1,4-dioxane at this site are in the range of 200 μg/L, with TCE present at up to 26,000 μg/L. Soil and groundwater from the AFP3 site in Tulsa, OK were received at the CB&I laboratory the week of November 11, 2013. Five liters of site groundwater (MW-280), and one soil core (SB-13) obtained from between 12.5 ft bgs and 15 ft bgs, consisting of mostly silty/sandy clay, was received.

Some of the initial microcosm studies described herein were conducted using funding from the AFCEC projects, but are presented because they support our transition to the study of ethane-degrading bacteria.

4.3 MICROBIAL ENRICHMENT AND CHARACTERIZATION

Microcosms were prepared from each site outlined in Section 4.2 to determine whether the addition of methane and air to samples stimulated native methanotrophs to biodegrade the 1,4-dioxane that was present in the collected soil and groundwater samples. Enrichments were performed from the most active site to isolate native methanotrophs and further assess their ability to degrade 1, 4-dioxane. In addition to methane, select microcosms also were amended with propane or ethane to determine if propane- or ethane-oxidizing bacteria were present to facilitate the degradation of 1,4-dioxane. Enrichments were also conducted from active microcosms receiving each of these gases.

4.3.1 MBAFB Microcosms

Microcosms were prepared from site samples using aquifer solids and groundwater that was homogenized by hand, with clay material removed. Soil samples for this study were collected from boring location FT11-14D-DPT-3A, from 16 to 28 feet below ground surface. Twenty five grams of aquifer solids were placed into each of 20 serum bottles (total volume = 160 mL). Each microcosm received 80 mL of site water. The total occupied volume was 96 mL, with a headspace of 64 mL in each bottle. The bottles were sealed with Teflon®-lined butyl rubber stoppers and crimp caps. Duplicate bottles of the following treatments were prepared:

1. *No addition.* These bottles received no amendments. Bottle headspace consisted of room air. This treatment served as a control to monitor 1,4-dioxane loss in the absence of any amendments;

2. *Methane.* These bottles received methane gas such that the final concentration in the headspace was 10%, with room air comprising the balance of the headspace gas;

3. *Methane, nitrate, and phosphate.* These bottles received methane gas such that the final concentration in the headspace was 10%, with room air comprising the balance of the headspace gas. These bottles also received 20 mg/L N in the form of sodium nitrate ($NaNO_3$) and 5 mg/L P in the form of potassium phosphate (KH_2PO_4). Nitrate was used as a source of N in these bottles based on a study showing that ammonium can potentially inhibit methanotrophs (Chu and Alvarez-Cohen, 1999).

4. *Methane, nitrate, phosphate, and allylthiourea.* These bottles received methane gas such that the final concentration in the headspace was 10%, with room air comprising the balance of the headspace gas. These bottles also received 20 mg/L N in the form of sodium nitrate ($NaNO_3$) and 5 mg/L P in the form of potassium phosphate (KH_2PO_4). These bottles also received 15 μM allylthiourea as a copper chelators (Yu et al., 2009) to promote the growth of organisms that express sMMO rather than pMMO (see Section 2.2.2 and Results and Discussion).

5. Methane, nitrate, phosphate, and tetrathiomolybdate. These bottles received methane gas such that the final concentration in the headspace was 10%, with room air comprising the balance of the headspace gas. These bottles also received 20 mg/L N in the form of sodium nitrate ($NaNO_3$) and 5 mg/L P in the form of potassium phosphate (KH_2PO_4). These bottles also received 15 μM tetrathiomolybdate as a copper chelator (Medici and Sturniolo, 2008) for the reason described in Treatment 4.

6. Methane, ammonium, and phosphate. These bottles received methane gas such that the final concentration in the headspace was 10%, with room air comprising the balance of the headspace gas. These bottles also received diammonium phosphate ($(NH_4)_2HPO_4$) to yield 20 mg/L N and 18 mg/L P;

7. Low methane, low oxygen. These bottles received methane gas such that the final concentration in the headspace was 0.1%. The headspace gas consisted of 25% room air and 75% nitrogen gas. No nutrients were added to these bottles as the nitrogen gas was intended for use as a source of nitrogen;

8. Ethane, ammonium, and phosphate. These bottles received ethane gas such that the final concentration in the headspace was 10%, with room air comprising the balance of the headspace gas. These bottles also received diammonium phosphate ($(NH_4)_2HPO_4$) to yield 20 mg/L N and 18 mg/L P;

9. Propane, ammonium, and phosphate. These bottles received propane gas such that the final concentration in the headspace was 10%, with room air comprising the balance of the headspace gas. These bottles also received diammonium phosphate ($(NH_4)_2HPO_4$) to yield 20 mg/L N and 18 mg/L P;

10. *Bioaugmentation.* These bottles received methane gas such that the final concentration in the headspace was 10%, with room air comprising the balance of the headspace gas. These bottles also received diammonium phosphate ($(NH_4)_2HPO_4$) to yield 20 mg/L N and 18 mg/L P. These bottles also received the methanotrophic bacterial strain *M. trichosporium* OB3b to a final optical density (OD_{550}) of 0.1. Bacterial culture was only added after the microcosms were spiked with 1, 4-dioxane (see below).

Bottles were incubated on a rotary shaker at room temperature (22-23^oC). Once per week for eight weeks the headspace was flushed and fresh headspace gases were added. After eight weeks of this pre-incubation phase, microcosms were spiked with 1,4-dioxane to a final concentration of 500 μg/L in the aqueous phase. At this time, *M. trichosporium* OB3b was added to the bioaugmentation bottles. Bottles were then returned to the shaker overnight to allow the 1,4-dioxane to equilibrate, and initial (i.e., time zero) samples were taken the next day. Microcosms were sampled by removing 3 mL groundwater through the septa using a 5 mL glass gastight syringe equipped with a 25 gauge needle. Samples were placed in 3 mL amber glass vials preserved with hydrochloric acid (HCl) and sealed with Teflon®-lined septa and aluminum crimp seals and analyzed for 1,4-dioxane, methane, ethane, and propane concentrations.

4.3.2 VAFB Microcosms

Soil from boring location 24-SW-2B (located from a depth interval of 86-87 feet below ground surface) was manually homogenized. Microcosms were prepared in glass serum bottles. Approximately 30 g of homogenized soil and 125 mL of site groundwater (also from 24-SW-2B) was added to each of the bottles, leaving approximately 21 mL of headspace in each bottle. The bottles were sealed with Teflon®-lined butyl rubber stoppers and crimp caps. Three sets of microcosm treatments were prepared in triplicate as follows:

1. *No addition.* These bottles received no amendments. Bottle headspace consisted of room air. This treatment served as a control to monitor 1,4-dioxane loss in the absence of any amendments;

2. *Methane*. These bottles received methane gas such that the final concentration in the headspace was 10%, with room air comprising the balance of the headspace gas. These bottles also received diammonium phosphate ($(NH_4)_2HPO_4$) to yield 20 mg/L N and 18 mg/L P;

3. *Killed control*. These bottles received no amendments except for 1% (vol/vol) formaldehyde solution to inhibit microbial activity.

Microcosm bottles were incubated with gentle shaking on an orbital shaker at 15°C. At each sampling event, liquid samples were removed through the septa, placed into vials preserved with hydrochloric acid (HCl), and analyzed for 1,4-dioxane and methane concentrations. Diammonium phosphate was re-added to bottles with methane on Day 140.

4.3.3 AFP3 Microcosms

Microcosms bottles were prepared with soil and groundwater from APF3 using the same methods as described for the MBAFB study (Section 4.3.1). The following treatments were prepared in triplicate:

1. *No addition.* These bottles received no amendments. Bottle headspace consisted of room air. This treatment served as a control to monitor 1, 4-dioxane loss in the absence of any amendments;

2. *Methane Biostimulation with Nitrate as N Source*. These treatments received methane gas in the headspace (10% methane by volume) and inorganic nutrients (20 mg/L N as $NaNO_3$ and 5 mg/L P as KH_2PO_4);

3. *Methane Biostimulation with Ammonium as N Source*. These treatments received methane gas in the headspace (10% methane by volume) and inorganic nutrients (20 mg/L N in the form of diammonium phosphate ($(NH_4)_2HPO_4$);

4. *Ethane Biostimulation*. These treatments received ethane gas in the headspace (10% methane by volume) and inorganic nutrients (20 mg/L N in the form of diammonium phosphate ($(NH_4)_2HPO_4$);

5. *Ethene Biostimulation*. These treatments received ethene gas in the headspace (10% ethene by volume) and inorganic nutrients (20 mg/L N in the form of diammonium phosphate ($(NH_4)_2HPO_4$);

6. *Propane Biostimulation*. These treatments received propane gas in the headspace (10% propane by volume) and inorganic nutrients (20 mg/L N in the form of diammonium phosphate ($(NH_4)_2HPO_4$);

7. *Methane Biostimulation + Copper Chelator*. These treatments received methane gas in the headspace (10% methane by volume) and inorganic nutrients (20 mg/L N as $NaNO_3$ and 5 mg/L P as KH_2PO_4). Bottles also received 15 mM ammonium tetrathiomolybdate as a copper chelator.

8. *Killed Controls*. These treatments received 300 mg/L mercuric chloride to inhibit microbial activity. These treatments also received methane gas in the headspace (10% by volume).

Bottles were sealed with Teflon®-lined butyl rubber stoppers and aluminum crimp seals, and incubated on their sides at 15˚C on a rotary shaker. Once per week for eight weeks the headspace was flushed and replaced with fresh headspace gases. After eight weeks of this pre-incubation phase, microcosms were spiked with 1,4-dioxane to a final concentration of 500 µg/L in the aqueous phase. Bottles were then returned to the shaker overnight to allow the 1,4-dioxane to equilibrate, and initial (i.e., time zero) samples were taken the next day. Microcosms were sampled by removing 3 mL groundwater through the septa using a 5 mL glass gastight syringe equipped with a 25 gauge needle. Samples were placed in 3 mL amber glass vials preserved with hydrochloric acid (HCl) and sealed with Teflon®-lined septa and aluminum crimp seals and analyzed for 1,4-dioxane by modified EPA 8260 (GC-MS with heated purge-and-trap).

4.3.4 Microbial Enrichments (Mixed Cultures)

In order to isolate and characterize a 1, 4-dioxane degrading bacteria, enrichment cultures were initiated. All enrichments consisted of 0.5 g homogenized site soil in 100 mL sterile media in a 250 mL shake flask. The enrichments consisted of the following media: (1) basal salts medium (BSM; Hareland et al., 1975) (2) nitrate mineral salts medium (NMS; Chu and Alvarez-Cohen, 1999); and (3) OB3b medium (Fox et al., 1990). Headspace was supplemented with 10% (v/v) methane, 3% (v/v) ethane, or 3% (v/v) propane as the electron donor. Enrichments were placed on a rotary shaker operating at room temperature, and the headspace was purged and replaced with fresh gases weekly. Once four weeks of incubation was completed, cultures were passed (1:100) into fresh media of the same type with the same headspace gases. Cultures were checked weekly for turbidity, and any cultures that grew turbid were again passed into fresh medium. Once these cultures exhibited turbidity, they were then tested for the ability to degrade 1,4-dioxane as described in Section 4.3.5.

Following evidence of rapid methane consumption in the microcosms prepared with MBAFB site soil, additional enrichments were prepared using a small aliquot of groundwater removed from MBAFB microcosm treatments 2 through 6. All enrichments were prepared using basal salt medium (BSM) (Hareland et al., 1975) and were supplied with a filter-sterilized headspace consisting of 10% methane/90% room air. All enrichments exhibited turbid growth after three passes as described above and were subsequently tested for 1, 4-dioxane degradation.

Enrichments from soil and microcosms subsequently were assessed for their ability to degrade 1,4-dioxane. Cultures were grown to high density in each medium, washed, and then re-suspended in the same medium. Individual 60 mL glass serum bottles with each culture (approximately 30mL) were then amended with 20 μM 1,4-dioxane and/or 20 μM TCE and then amended with the appropriate headspace gas (i.e., 10% methane, 3% ethane, or 3% propane). No cell controls also were prepared. TCE was used as a positive control to demonstrate that the target oxygenase enzyme was active. Samples were taken at time 0 to serve as an initial condition and again at 48hrs and 1 week. Samples were removed through the septa using a 5 mL glass gastight syringe equipped with a 25 gauge needle, and were placed into 3 mL amber serum bottles preserved with HCl for analysis by modified EPA 8260 (GC-MS with heated purge-and-trap).

4.3.5 Batch Kinetic Testing to Determine Kinetic Parameters

Methane was not effective as a cometabolic substrate for 1,4-dioxane degradation in any of the studies, so it was not further evaluated. However, ethane was observed to support 1,4-dioxane biodegradation. ***Based on the effectiveness of MBAFB microcosms that received ethane as the cometabolite (discussed in in Section 5.1), 1,4-dioxane kinetic experiments were prepared using mixed and pure enrichment cultures grown on ethane.*** The purpose of these experiments was to determine kinetic parameters describing cometabolic biodegradation of 1,4-dioxane with ethane as a primary substrate.

Mixed and pure colony enrichment cultures were grown as explained previously with 3% (v/v) ethane to an optical density (OD) of 0.5. From these enriched cultures, batch experiments were prepared in duplicate or triplicate using 60 mL clear glass serum bottles with 30 mL initial headspace volume (air, with or without ethane), Teflon-lined septa, and aluminum crimp seals. Samples were collected as a function of time to determine decreases in 1,4-dioxane and ethane, as described further below. 1,4-Dioxane analyses were performed using modified EPA Method 8260 (GC-MS with heated purge-and-trap), and ethane concentrations were determined via headspace analysis using a GC-FID (EPA Method 3810). Headspace samples also were used to measure dissolved oxygen using a GC-TCD. Experiments focused on the use of the mixed enrichment culture, although one experiment was performed using the pure culture for comparison.

Batch kinetic testing was performed in three phases. The first phase focused on the biodegradation of 1,4-dioxane in the absence of ethane, using the resting cells to assess treatment kinetics. These experiments focused on measuring the rate of 1,4-dioxane biodegradation using a range of initial 1,4-dioxane concentrations of 0.2 to 500 mg/L. Experiments were performed in duplicate or triplicate using 60 mL glass serum bottles with 30 mL of basal salt medium (BSM)

(Hareland et al., 1975). Bottle headspace consisted of room air. An initial cell density (mixed or pure cultures) of approximately 1.5 g dry cell/L was used (optical density of 0.5). Data from this first phase were used to determine the maximum degradation rate coefficient and the half saturation constant for 1,4-dioxane by ethanotrophs, as described in Section 4.5.

The second phase of the batch kinetic testing was used as an initial screening to assess the impacts of ethane inhibition on 1,4-dioxane biodegradation. Experiments were prepared as described for Phase 1, except that bottles were amended with initial aqueous ethane concentrations ranging from 0 to 6,000 µg/L (calculated equilibrium aqueous concentrations based on Henry's Law partitioning with gas phase ethane). 1,4-Dioxane concentrations were monitored as a function of time, and initial and final ethane headspace concentrations were measured. Aqueous ethane concentrations were determined using Henry's Law.

The third phase of the batch kinetic testing used the same experimental procedures described above for Phases 1 and 2 and evaluated the rate of ethane biodegradation. The rate of 1,4-dioxane biodegradation also was measured while monitoring ethane concentrations during the duration of the experiment. These data were used to determine the ethane biodegradation kinetic parameters, as described in Section 4.5. The initial ethane concentration used in the Phase 3 experiments was approximately 350 µg/L, which was determined based on the results of the Phase 2 testing. A 1,4-dioxane concentration of approximately 600 µg/L was used in the third phase testing.

4.4 COLUMN EXPERIMENTS

Column experiments were performed to assess the biodegradation of 1,4-dioxane by ethane-oxidizing bacteria in aquifer materials. Homogenized soil collected from MBAFB was used for the column experiments. A synthetic groundwater consisting of deionized water amended with $MnSO_4$-H_2O (1 mg/L), $NaSO_4$ (14 mg/L), NaCl (34 mg/L), $NaHCO_3$ (40 mg/L) and HCl to bring the final pH to 6.5 was used for the columns.

Columns (2 inch diameter and 12 inches long) were constructed in duplicate using stainless steel. Water flow into the column was directed upwards using a peristaltic pump at a rate of approximately 5.5 mL/hour. Tracer testing using sodium bromide showed that the residence time in the column at this flow rate was 40 hours. Influent water was amended with ethane (dissolved concentration of 700 µg/L), nutrients (14 mg/L $NaNO_3$ and 6.5 mg/L NaH_2PO_4), and 1,4-dioxane (500 µg/L). Influent dissolved oxygen concentrations were approximately 8 mg/L. Both influent and effluent samples were routinely monitored for ethane, oxygen, and 1,4-dioxane. After 102 days of operation, the columns were bioaugmented using the enriched mixed culture from MBAFB. A total of 0.045 g dry cell weight of the mixed culture was injected into each column via a 30 mL enriched solution.

4.5 BIODEGRADATION MODEL AND PARAMETER DETERMINATION

The cometabolic biodegradation of 1,4-dioxane by ethanotrophs was modeled assuming Michaelis-Menten kinetics. It was assumed that biodegradation occurred with excess oxygen present, and with no nutrient limitations. Because the kinetic experiments were performed within short time frames (i.e., <24 hours), ethanotroph concentrations were assumed constant during the

kinetic testing, which is consistent with the observed doubling time of 10 days measured during enrichment testing. The equations used for describing 1,4-dioxane and ethane biodegradation are as follows:

$$\frac{dC_D}{dt} = \frac{XV_{max,D}C_D}{C_D + K_D\left(1+\frac{C_E}{K_{I,E}}\right)} \quad \text{Eq. 1}$$

$$\frac{dC_E}{dt} = \frac{XV_{max,E}C_E}{C_E + K_E} \quad \text{Eq. 2}$$

where C_D and C_E are the 1,4-dioxane and ethane concentrations [mg/L], t is time [days], X is the inoculated ethanotroph concentration [1.5 mg cell/L], $V_{max,D}$ and $V_{max,E}$ are the maximum degradation rate coefficients for 1,4-dioxane and ethane [mg/mg cell/h], and K_D and K_E are the half saturation coefficients [mg/L]. It is noted that Eq. 1 assumes that 1,4-dioxane is inhibited by the presence of ethane, and that the inhibition coefficient can be approximated by the ethane half saturation constant. The assumption that the inhibition coefficient can be approximated by the half saturation coefficient has been employed in previous co-metabolic studies using short-chain hydrocarbons (Strand et al., 1990). For purposes of this study, no inhibition by 1,4-dioxane on ethane biodegradation was assumed.

For ethane degradation, equilibrium between headspace gas and aqueous phase was assumed at each time point with a Henry's constant of 20.8 L-water/L-air. Changes in gas and liquid volumes due to sampling were incorporated into the modeling.

The maximum degradation rate coefficient and half saturation constant for 1,4-dioxane (Eq. 1) were regressed to the 1,4-dioxane biodegradation data obtained in the first phase of the batch kinetic testing (no ethane, and a range of 1,4-dioxane concentrations). The regression was performed using the methodology previously employed (Smith et al., 1998; Schaefer et al., 2009), where a 4th order Runge-Kutta method with a time step of 1.5 minutes was solved within Microsoft Excel®. The uncertainty weighted sum-square of relative errors between observed and modeled concentrations was minimized using the Solver function of Microsoft Excel®, and initial 1,4-dioxane concentrations, ethane concentrations, K_E, and the ratio of $V_{max,D}/K_E$ were varied in a nonlinear least-square analysis similar to that described by Smith et al. (1998). The ratio $V_{max,D}/K_E$ was utilized rather than k_{max} because the two kinetic parameters can be highly correlated at lower concentrations as noted by Smith et al. (1998), potentially leading to underestimation of parameter uncertainty. Kinetic parameters for ethane (Eq. 2) were determined in a similar fashion.

5.0 RESULTS AND DISCUSSION

5.1 MICROCOSMS

5.1.1 MBAFB Microcosms

A wide variety of different treatments were applied to the samples from Myrtle Beach in order to maximize the potential for degradation of 1,4-dioxane by indigenous methanotrophs. As

described in the methods, these treatments included the testing of three different N sources for growth, ammonium, nitrate, and nitrogen gas (Whitenbury et al., 1970; Chu and Alvarez-Cohen, 1996, 1999). Methanotrophs can utilize a wide variety of nitrogen sources, and in some instances, ammonium has been hypothesized to slow reactions of MMO by acting as a competitive inhibitor, so alternate sources including nitrate and nitrogen gas (to stimulate nitrogen fixing methanotrophs) were tested in this study. In addition, two different copper chelators (tetrathiomolybdate and allylthioyurea; Yu et al., 2009; Medici and Sturniolo, 2008) were used in order to reduce available copper in the environmental samples to the extent possible. These additions were designed to promote the activity of soluble methane monooxygenase (sMMO), which has been reported to catalyze 1,4-dioxane degradation (Mahendra and Alvarez-Cohen, 2006) over the copper-containing particulate methane monooxygenase (pMMO). Copper is a key factor regulating the relative expression of sMMO and pMMO (many methanotrophs contain both enzymes), with sMMO being expressed only in the absence of available copper (> 5.6 µmols Cu/g protein) (Semrau et al., 2010). Different initial methane concentrations also were tested to avoid inhibition at high methane levels.

The initial concentrations of anions, dissolved gases, 1,4-dioxane, and VOCs measured in the microcosms receiving "*No addition*" (the first treatment described in in Section 4.3.1) are provided in Table 5-1. Both methane and ethane were naturally present, as well as trace levels of ethene**.** The 1,4-dioxane concentration was increased to ~ 400 µg/L in all microcosms after the pre-incubation period to facilitate measurement of 1,4-dioxane biodegradation.

Table 5-1. Concentrations of anions, dissolved gases, 1,4-dioxane, and volatile organic compounds in the treatment receiving no additions (Treatment 1) for MBAFB. Qualifiers: U indicates that the sample was below the detection limit; J indicates that the value quantity was estimated (below reporting limit); D indicates the value was determined in a diluted sample.

Sample ID		MBZ0304-IW122	
Date Sampled		03/05/2013	
1,4-Dioxane	µg/L	**28.0**	
Fluoride	mg/L	0.2	U
Chloride	mg/L	**31.1**	D
Nitrite as N	mg/L	0.2	U
Sulfate as SO4	mg/L	**4.65**	
Bromide	mg/L	**21.5**	
Nitrate as N	mg/L	0.2	U
Chlorate	mg/L	0.2	U
Phosphate as P, ortho	mg/L	0.2	U
Methane	µg/L	**2840**	
Ethane	µg/L	**153**	
Ethene	µg/L	**7.71**	
Propane	µg/L	6.0	U
Acetylene	µg/L	7.0	U
Benzene	µg/L	**3.6**	J
Ethylbenzene	µg/L	5.0	U
1,1-Dichloroethane	µg/L	**3.8**	J
cis-1,2-Dichloroethene	µg/L	5.0	U
Toluene	µg/L	5.0	U
Chlorobenzene	µg/L	5.0	U
Xylenes (meta/para)	µg/L	5.0	U
o-Xylene	µg/L	5.0	U
Isopropyl Benzene	µg/L	5.0	U
1,3,5-Trimethylbenzene	µg/L	5.0	U
1,2,4-Trimethylbenzene	µg/L	5.0	U
1,4-Dichlorobenzene	µg/L	5.0	U
Naphthalene	µg/L	5.0	U

Degradation was not observed in any of the bottles that received methane with various treatments, including bioaugmentation with *M. trichosporium* Ob3B and addition of various chelators (Figure 5-1). In this case, however, good methane consumption was observed in all bottles with methane, suggesting the presence of indigenous methanotrophs in all treatments (Figure 5-2). ***Degradation of 1,4-dioxane was observed in bottles that received ethane or propane with ammonium as a nutrient source.*** The bottles with propane were bioaugmented with *R. ruber* ENV425 on Day 43, and degradation commenced shortly thereafter.

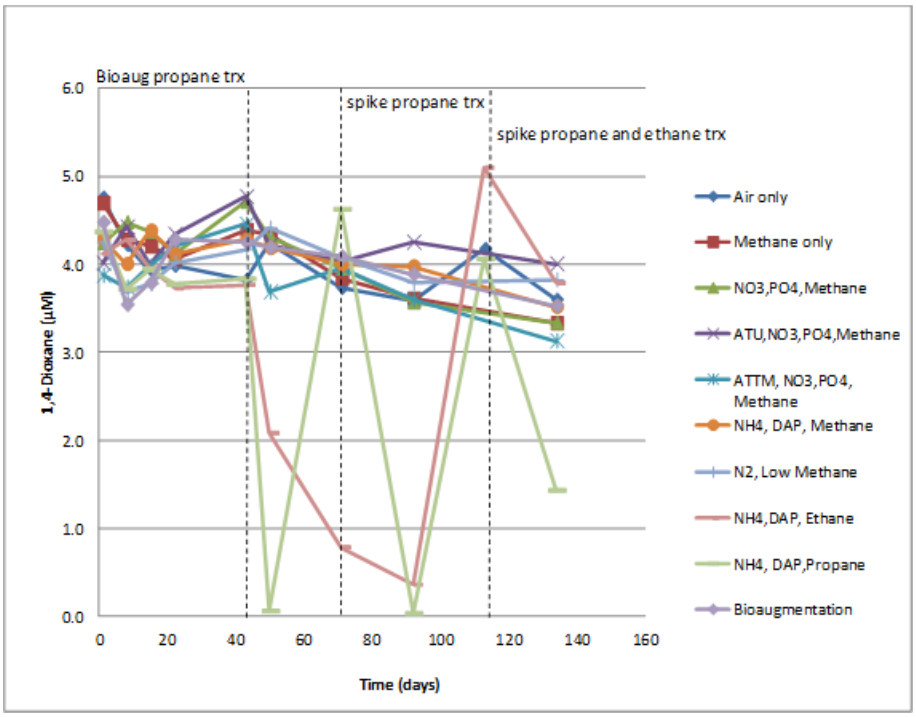

Figure 5-1. Biodegradation of 1,4-dioxane in microcosms from MBAFB. 1,4-dioxane was re-added to propane and ethane microcosms where indicated. DAP = diammonium phosphate.

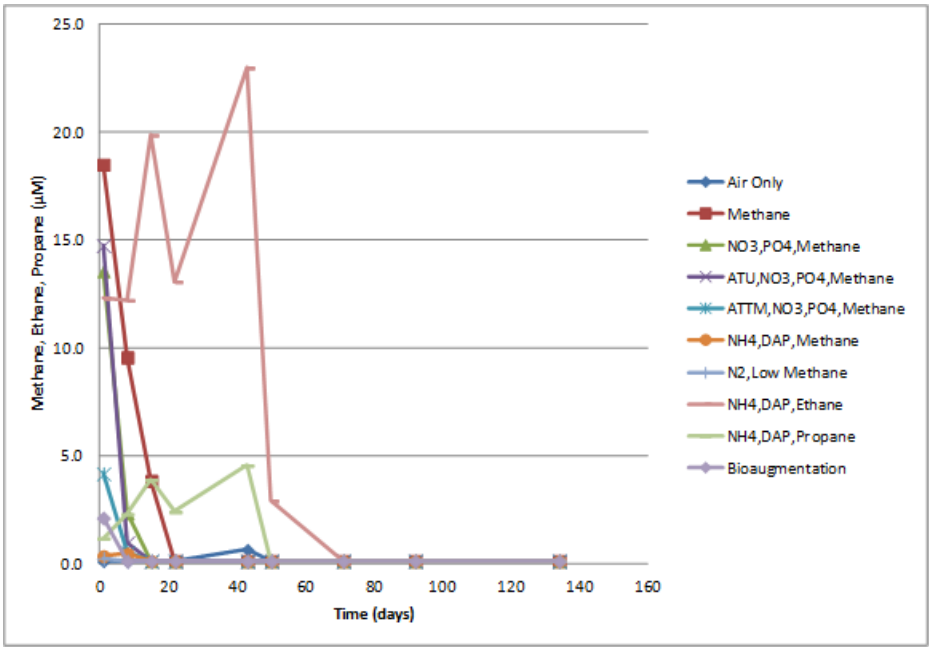

Figure 5-2. Biodegradation of methane, ethane, or propane in microcosms from MBAFB. DAP = diammonium phosphate.

5.1.2 VAFB Microcosms

Biodegradation of 1,4-dioxane in microcosms from VAFB was not observed in any of the treatments throughout the entire 220 day duration of the study, although biodegradation of methane was observed. These results suggest that native methane-oxidizing bacteria where not effective for degrading 1,4-dioxane at this site, or other site specific conditions prevented this degradation reaction from occurring.

5.1.3 AFP3 Microcosms

1,4-Dioxane results for the AFP3 microcosms are shown in Figure 5-3. No measureable biodegradation of 1,4-dioxane was observed in any of the treatments. In addition, with the exception of ethene, losses of the hydrocarbon gases was minimal (Figure 5-4), suggesting substantial biodegradation of these gases was not occurring. Because gases were not added to the killed control, it is not possible to quantify gas losses due to sample collection or other routes versus losses due to biodegradation. However, gas concentrations other than ethene remained relatively constant over more than 100 days. It is unclear why the AFP3 microcosms showed such low levels of microbial activity with respect to both 1,4-dioxane and the hydrocarbon gases compared to the MBAFB and VAFB microcosms.

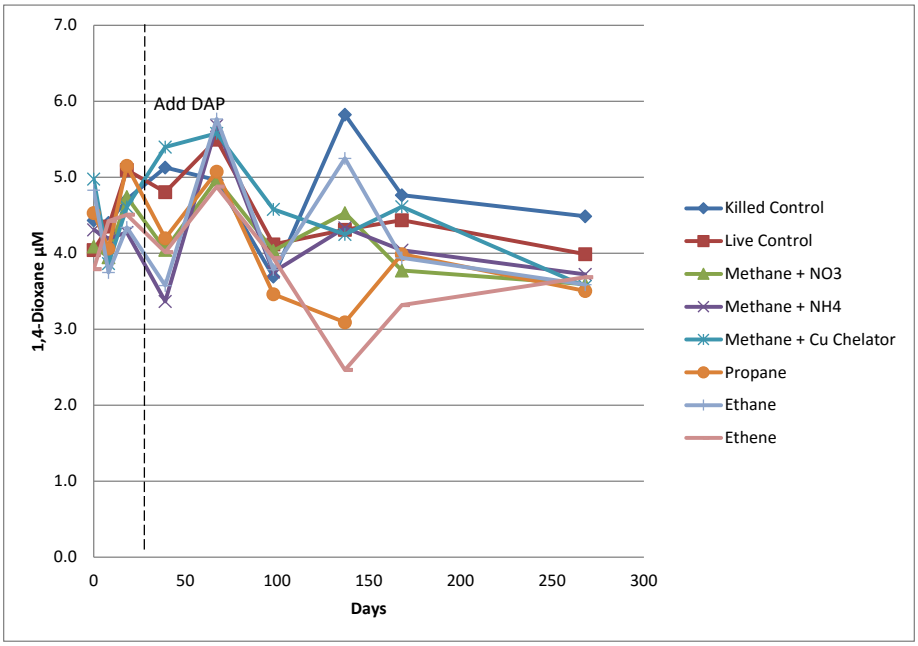

Figure 5-3. Biodegradation of 1,4-dioxane in microcosms from AFP3. The dashed vertical line indicates when diammonium phosphate (DAP) was added.

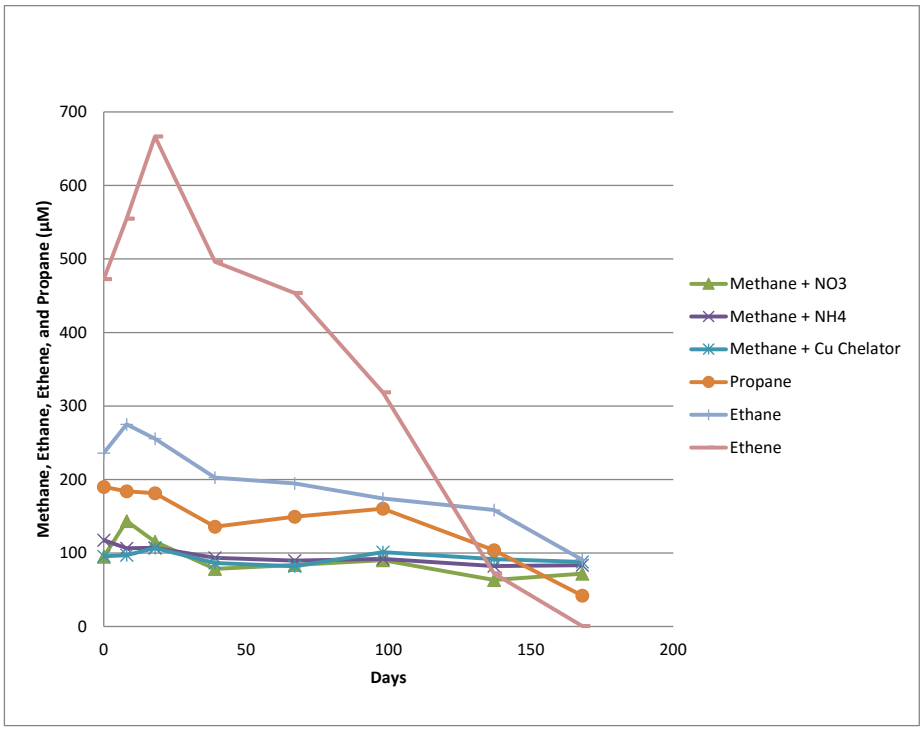

Figure 5-4. Biodegradation of hydrocarbon gases in the AFP3 microcosms.

5.2 MICROBIAL ENRICHMENTS

5.2.1 Mixed cultures

Enrichment cultures capable of growing on methane, ethane, and propane were isolated from MBAFB as described in Section 4.3.4. Experiments were conducted with each of these mixed enrichment cultures to determine if they could biodegrade 1,4-dioxane. The degradation of 1,4-dioxane by the various cultures is shown in Figure 5-5. ***The propane and ethane enrichment cultures were each capable of rapidly degrading 1,4-dioxane in the presence or absence of added propane and ethane in incubations, but the methane enrichment did not degrade 1,4-dioxane, regardless of whether methane was added to the incubations.*** The media used for the methanotrophs did not contain copper in order to preferentially stimulate sMMO. Thus, the enrichment data support the data from site microcosms, indicating that propanotrophs and ethanotrophs were capable of degrading 1-4-dioxane, but that indigenous methanotrophs were not capable of similar rates or extents of 1,4-dioxane degradation.

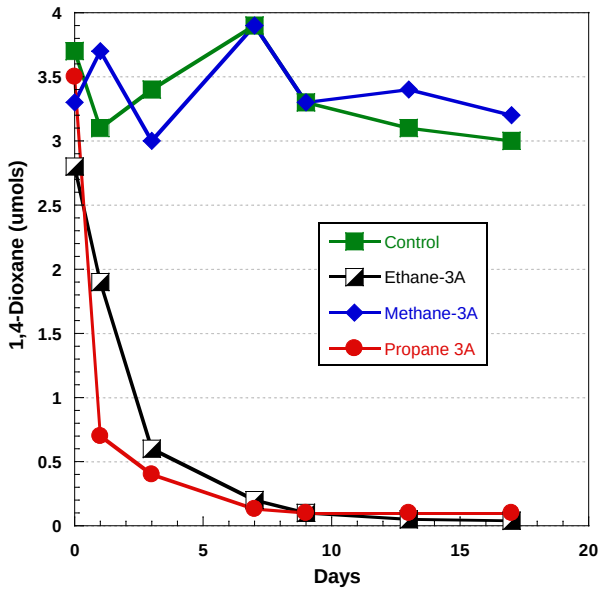

Figure 5-5. Biodegradation of 1,4-dioxane using a mixed enrichment culture from MBAFB. Enrichments grown on methane, ethane, and propane were evaluated.

Much like the MBAFB enrichment, 1,4-dioxane was not biodegraded by the VAFB mixed enrichment culture grown on methane, in the presence or absence of copper (Figure 5-6). TCE was readily degraded by this culture during the same study (data not shown), indicating that the culture and the sMMO enzymes were active. A previous study by Mahendra and Alvarez-Cohen, (2006) suggested that the methanotroph *M. trichosporium* OB3b degraded 1,4-dioxane using sMMO. However, the 1,4-dioxane concentration used in their study (500 mg/L) was much higher than is typically found in environmental samples or was used herein. We previously evaluated 1,4-dioxane biodegradation with strain OB3b at low concentrations ($\leq$ 10 mg/L), and 1,4-dioxane degradation was not observed, but we did not test higher concentrations (*Hatzinger, unpublished data*). Little is known about the capacity for other methanotrophs to biodegrade this oxygenate, or the optimal conditions for this degradative process to occur. Thus, it is possible that the mixed methanotrophic culture enriched in the Vandenberg samples did not include an isolate capable of degrading 1,4-dioxane, even though TCE was degraded. These results were similar to those observed for enrichments from MBAFB.

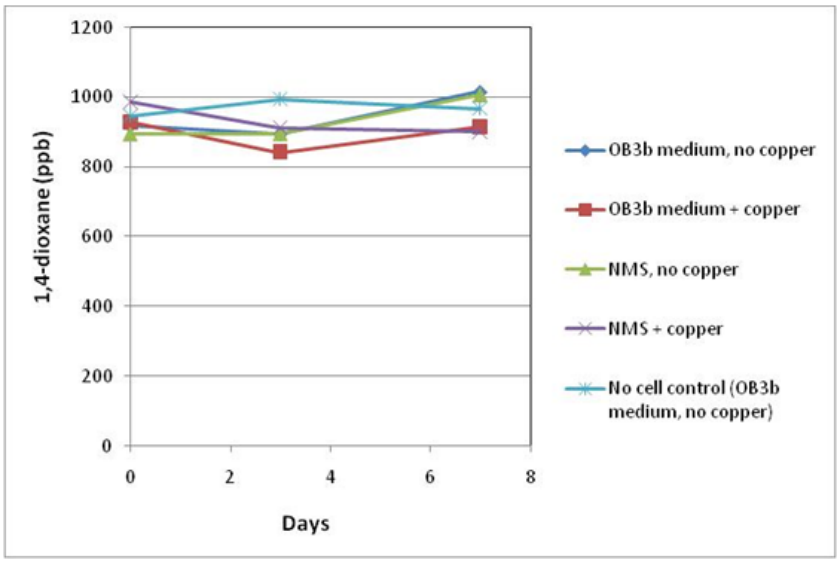

Figure 5-6. Biodegradation of 1,4-dioxane by the VAFB enrichment culture in different media with or without copper. The mixed culture was grown on methane.

5.2.2 Pure Culture Testing – Ethanotroph *Mycobacterium sphagni* ENV482

The bacterium *Mycobacterium sphagni* ENV482 was originally isolated from a groundwater aquifer on Joint Base Cape Cod in Massachusetts that contained trace levels of 1,2-dibromoethane. This pure culture, which was isolated from a mixed enrichment culture growing on ethane and oxygen (Hatzinger et al., 2015), was observed to be capable of cometabolically degrading 1,2-dibromoethane as well as some other halogenated organic compounds (data not shown). During the current project, this strain was observed to biodegrade 1,4-dioxane after growth on ethane (Figure 5-7). To our knowledge, this is the first report of ethane-supported 1,4-dioxane biodegradation by a pure culture. Further studies are warranted to evaluate the degradation pathway and the enzymes used by this strain for degrading 1,4-dioxane and other contaminants.

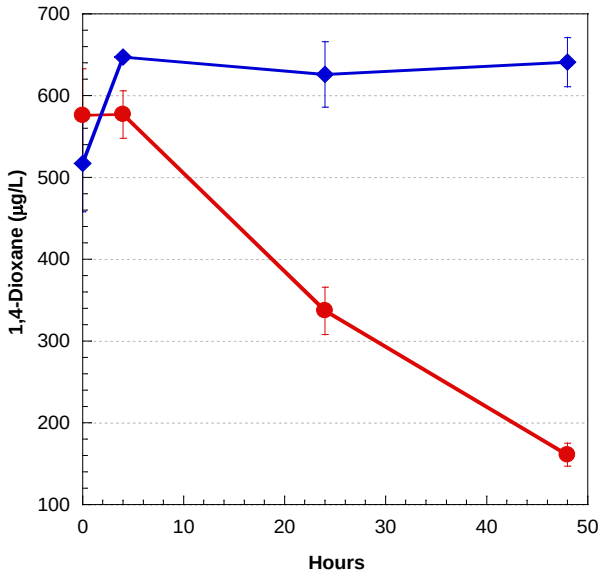

Figure 5-7. Biodegradation of 1,4-dioxane by the ethanotroph *Mycobacterium sphagni* ENV482. The blue symbols represent an uninoculated control and the red symbols represent bottles with ENV482. Error bars are form duplicate samples.

5.3 KINETIC TESTING

Kinetic tests were performed with the ethane-degrading enrichment culture from MBAFB to quantify and model 1,4-dioxane biodegradation. Results of the first phase of kinetic testing, performed using resting cells (no ethane) and with varying initial 1,4-dioxane concentrations, are shown in Figure 5-8. Biodegradation of 1,4-dioxane was evident for initial concentrations of approximately 2,000 µg/L or less. Slow relative degradation rates at higher concentrations and analytical uncertainties made assessing trends problematic when initial 1,4-dioxane concentrations exceeded 2,000 µg/L.

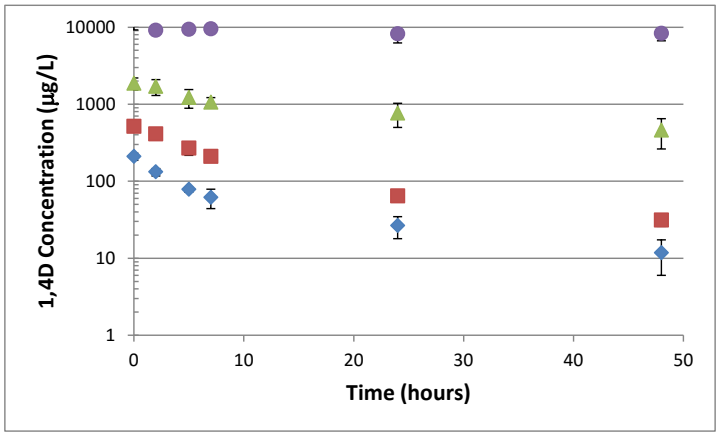

Figure 5-8. Biodegradation of 1,4-dioxane by the MBAFB enrichment culture under resting conditions. Experiments with initial 1,4-dioxane concentrations ranging from approximately 10,000 to 200 µg/L are shown. The averages of triplicate samples are shown. Error bars represent 95% confidence intervals. Abiotic controls showed no decreasing trend in 1,4-dioxane concentrations (data not shown).

The impacts of initial ethane concentration on the observed 1,4-dioxane biodegradation is shown in Figure 5-9. Results show that no 1,4-dioxane biodegradation was observed when ethane concentrations remained above 110 µg/L. At an initial ethane concentration of 250 µg/L, 1,4-dioxane degradation was inhibited through the first 3 to 4 hours of the experiment, after which ethane concentrations likely decreased to less than 110 µg/L and biodegradation of 1,4-dioxane commenced.

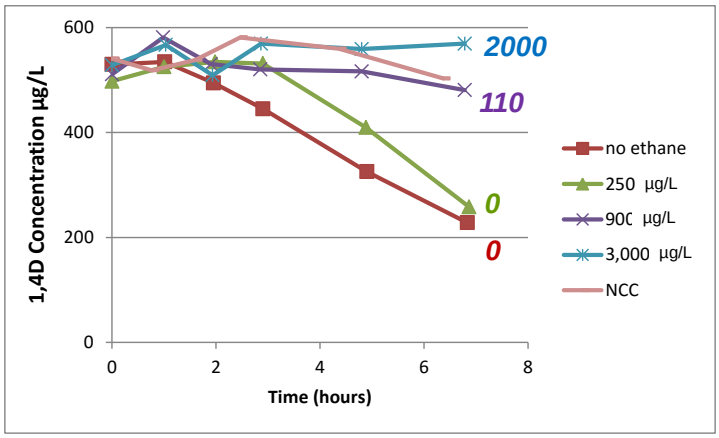

Figure 5-9. Biodegradation of 1,4-dioxane by the enrichment culture for a range of initial ethane concentrations. Final ethane concentrations also are shown in the figure. Average of duplicate samples are shown. NCC= no cell control.

Regression of the model parameters V_{max} and K_m for 1,4-dioxane to the data in Figure 5-10, and regression of V_{max} and K_M for ethane to the ethane data in Figure 5-11, resulted in the kinetic values shown in Table 5-2. It is noted that the model fits only were sensitive for data through 7 hours in Figure 5-10, as the transformation capacity of the resting cells is approached beyond this time and no significant biodegradation was observed. The curves shown in Figures 5-10 and 5-11 represent the model, and provide a reasonable description of the data. The simulation in Figure 5-11 representing the biodegradation of 1,4-dioxane in the presence of ethane was not used in the regression, but represents the model prediction of ethane inhibition on 1,4-dioxane biodegradation by ethane-oxidizing bacteria. The model was able to predict this small but significant impact of the low ethane concentrations on 1,4-dioxane degradation.

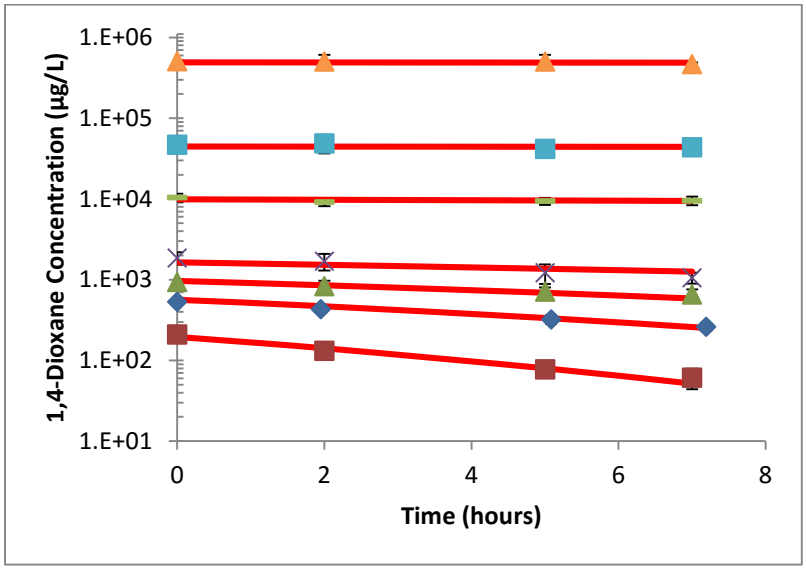

Figure 5-10. Model regression to the 1,4-dioxane initial rate data. The solid lines represent the regressed kinetic model. The averages of replicate samples are shown. Error bars represent 95% confidence intervals. The solid lines represent the model regression.

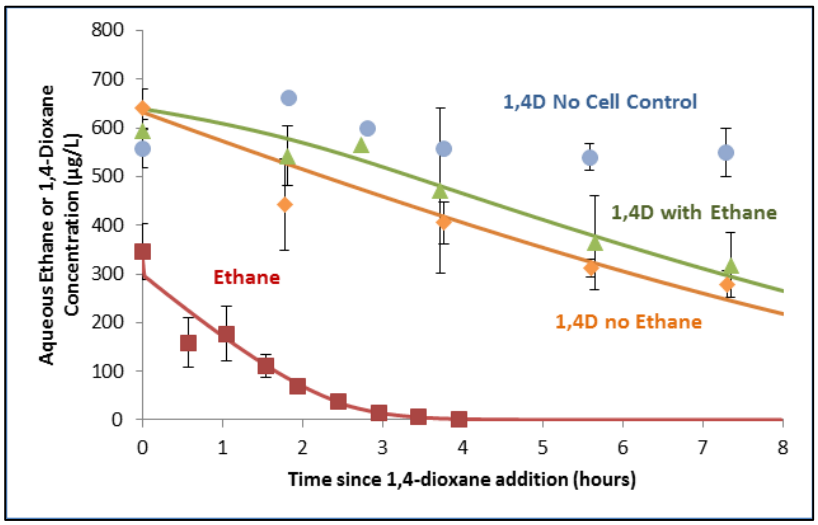

Figure 5-11. Coupled biodegradation of ethane and 1,4-dioxane. The solid lines represent the regressed kinetic model. The averages of triplicate samples are shown for 1,4-dioxane, and the averages of duplicate samples are shown for ethane. Error bars represent 95% confidence intervals.

Table 5-2. Regressed kinetic model parameters. The ethane inhibition coefficient for 1,4-dioxane degradation was assumed equal to K_m for 1,4-dioxane.

Parameter	Units	Regressed Value
1,4-Dioxane		
V_{max}	mg/mg cell/hr	$4.6 \pm 1.7 \times 10^{-5}$
K_m	mg/L	0.23 ± 0.07
Ethane		
V_{max}	mg/mg cell/hr	$1.8 \pm 0.6 \times 10^{-3}$
K_m	mg/L	0.064 ± 0.02

The maximum degradation rate obtained for ethane is similar to that obtained by Chang and Alvarez-Cohen (1995) for methane, where a V_{max} of 3.9×10^{-2} mg/mg cell/hr was observed; however, the half saturation constant calculated by Chang and Alvarez-Cohen was 1.1 mg/L, which is approximately 20 times greater than the value for ethane in Table 3. Joergensen and Degn (1983) obtained V_{max} and K_{max} values for methane of 1.5 mg/mg cell/hr and 0.013 mg/L, respectively. Thus, the values for ethane obtained in this current study are within the same order of magnitude as the reported ranges for methane.

The V_{max} value determined for 1,4-dioxane is approximately 3 orders of magnitude less than the V_{max} value determined by Mahendra and Alvarez-Cohen (2006) using methane-oxidizing

bacteria to biodegrade 1,4-dioxane. The reason for this large discrepancy is unclear, but might be due to different activity of the relevant enzymes or due to the fact that the previous study used pure cultures, whereas a mixed culture was used in the current study. We should also note that we were previously unable to stimulate 1,4-dioxane degradation by strain OB3b at the concentrations that we tested, although TCE was readily degraded by the strain in the same studies, suggesting that the MMO enzyme(s) were active in studies (*Hatzinger, unpublished data*).

The data shown in Figure 5-9, as well as the low value of the inhibition coefficient for 1,4-dioxane biodegradation, indicates that, for this mixed culture, relatively low levels of ethane in groundwater slow the biodegradation of 1,4-dioxane. However, additional research beyond these preliminary studies is required to better understand and document the relative inhibition of ethane on the degradation of 1,4-dioxane and other contaminants-of-concern by ethane-degrading strains. Degradation kinetics of contaminants can be highly variable among co-metabolic strains growing on a given substrate. For example, Sharp et al., (2010) observed that the concentration of propane resulting in 50% inhibition of the rate of N-nitrosodimethylamine degradation by two propane-degrading strains varied from 120 µg/L to 7,700 µg/L. Very little is currently known about the degradation of trace ethane in the groundwater or its effect on the degradation of 1,4-dioxane. However, we believe that this could be an important contributor to 1,4-dioxane degradation at groundwater sites.

For example, based on the rate of ethane consumption observed in the MAFB microcosms (Figure 5-2), and assuming a doubling time of 10 days for the ethane-oxidizing bacteria (based on the rate of growth observed in our enrichment cultures) during the 60-day period of ethane consumption, the initial concentration of ethanotrophs in the microcosms was 0.016 mg/cell/L. Assuming a 13-fold dilution of bacteria in the microcosms due to the increased water:soil ratio, the ambient groundwater cell concentration of ethanotrophs in the field at MBAFB is estimated at 0.21 mg dry cell/L. Applying this cell density to the kinetic equation (Eq. 1 and Table 5-2), and assuming the 1,4-dioxane concentration is much less than the half-saturation constant, a 1,4-dioxane biodegradation half-life of 1.9 years is estimated. ***While this approach is considered a very rough (order of magnitude) estimate of potential 1,4-dioxane biodegradation due to ethane-oxidizing bacteria, it is interesting to note that it falls within the range of intrinsic 1,4-dioxane degradation observed by Adamson et al. (2015).***

5.4 COLUMN TESTING

Results of the column testing are shown in Figure 5-12. Prior to bioaugmenting with the MBAFB enrichment culture, ethane biodegradation appeared nominal, at best, although scatter in the data due to variability in the influent concentrations made determining the extent of ethane biodegradation difficult. However, shortly after bioaugmenting, concentrations of ethane decreased to below the analytical detection limit, suggesting that bioaugmentation was effective for increasing the rate of microbial ethane oxidations in the columns. Despite the increase in ethane-oxidizing microbial activity, 1,4-dioxane did not show any measureable biodegradation before or after bioaugmentation.

The absence of any measureable 1,4-dioxane biodegradation in the columns is likely due to several factors. First, dissolved oxygen levels were consistently <2 mg/L in the column effluent,

which may have limited both the growth of ethane-oxidizing bacteria within the column and the rate of 1,4-dioxane biodegradation itself. Such low oxygen levels would likely have diminished the already slow rate of ethane biodegradation observed in the native MBAFB soil (see Figure 5-2). Second, the extent to which the bioaugmentation culture migrated through the column in unknown, and thus the extent of the enrichment culture distribution throughout the column may have been limited to near the column influent, thereby limiting the residence time for treatment of 1,4-dioxane. Finally, the ethane concentration profile within the column was unknown, and elevated ethane concentrations (approaching 100 µg/L – see Figure 5-9) may have persisted throughout a large extent of the column, inhibiting 1,4-dioxane biodegradation by the enrichment culture, as observed with the batch study. It is possible that, with a longer residence time in the columns, allowing more complete degradation of ethane, subsequent 1,4-dioxane degradation may have been observed. This is more representative of a typical field site where much longer residence times are typical.

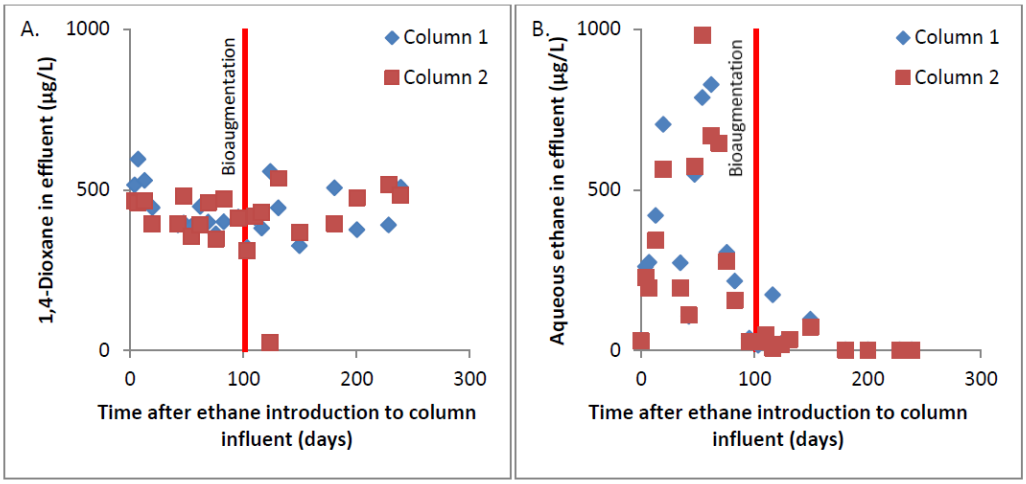

Figure 5-12. 1,4-Dioxane and ethane results from the effluent of the duplicate columns (Column 1 and Column 2). The vertical line indicates when the columns were bioaugmented with the MBAFB ethane-oxidizing enrichment culture. Influent 1,4-dioxane and ethane concentrations were prepared et 500 and 700 µg/L, respectively.

6.0 CONCLUSIONS AND IMPLICATIONS FOR FUTURE RESEARCH

6.1 CONCLUSIONS

Overall, results obtained during this limited scope study showed that methane-oxidizing bacteria were not effective for promoting the biodegradation of 1,4-dioxane. For all conditions examined, including use of copper chelators, various nutrients, pure and enrichment cultures, and soil collected from 3 different sites, no 1,4-dioxane biodegradation was observed by methanotrophs. However, ethane- and propane-oxidizing bacteria were shown to be generally effective for biodegrading 1,4-dioxane. Because ethane often is present at 1,4-dioxane sites (due to dechlorination processes associated with comingled chlorinated solvents), our research efforts with respect to the kinetic and column shifted to assessing ethanotrophs for biodegradation of 1,4-dioxane. An example showing the presence of ethane at a chlorinated solvent site in New Jersey (Raritan Arsenal) after *in situ* bioremediation is provided in Figure 5-13.

Figure 5-13. Average concentrations of chlorinated volatile organic compound (CVOCs), ethene and ethane in groundwater at Raritan Arsenal Area 18C after in situ bioremediation. Figure from Shaw Environmental, Inc. (2010).

Michaelis-Menten kinetic parameters were determined for both 1,4-dioxane and ethane with a mixed culture from MBAFB. Using these regressed parameters, as well as the observed rates of ethane biodegradation in MBAFB soil, the estimated half-life for 1,4-dioxane was approximately 1.9 years. This value is in excellent agreement with published rates of 1,4-dioxane

biodegradation observed in the field. In addition, results from MBAFB in this study show that ethane is present within the 1,4-dioxane plume at concentrations on the order of 20 µg/L. Others have shown ethane at concentrations on the order of 0.06 µg/L within the 1,4-dioxane plume (Chiang et al., 2012). Thus, results of this research suggest that ethane-oxidizing bacteria, sustained by the presence of ethane at many sites with comingled 1,4-dioxane and chlorinated solvent plumes, may be responsible for slow yet sustained 1,4-dioxane biodegradation at DoD facilities.

6.2 FUTURE RESEARCH

Results attained in this limited scope study suggest that ethane-oxidizing bacteria may play a significant role in the intrinsic biodegradation of 1,4-dioxane at DoD sites. Key remaining questions include the following:

- ***Are all bacteria capable of biodegrading ethane also capable of degrading 1,4-dioxane, and what genes/enzymes are involved in this process?*** Unlike methane and propane, there is relatively little data on the organisms/enzymes that biodegrade ethane in nature and whether these organisms are also capable of biodegrading 1,4-dioxane or other contaminants-of-concern. Laboratory studies with pure cultures can be conducted using traditional enzyme assays, molecular methods, and proteomic methods to evaluate which enzymes are responsible for ethane and 1,4-dioxane degradation. Similarly, stable isotope probing methods with ^{13}C-lableled ethane can be applied to develop a better understanding of the organisms and microbial communities in soils that biodegrade ethane, and whether these organisms also typically are capable of biodegrading 1,4-dioxane.

- ***Are the low levels of ethane observed within comingled chlorinated solvent and 1,4-dioxane plumes really responsible for the observed 1,4-dioxane aerobic biodegradation observed at some DoD sites?*** While the laboratory studies presented herein have clearly demonstrated that 1,4-dioxane is amendable to biodegradation via ethane-oxidizing bacteria, the sustained biodegradation of 1,4-dioxane by this cometabolic process in the presence of very low levels of ethane (discussed in Section 6.1) has yet to be verified. Long term laboratory batch experiments, maintained with low concentrations of ethane using gas permeation tubes so that gas levels remain consistent with field-measured levels (i.e, 0.1 to 10 µg/L), are needed to demonstrate that ethane-oxidizing bacteria are capable of sustained biodegradation of 1,4-dioxane. Both intrinsic ethanotroph levels present in site soils (e.g., MBAFB, VAFB), as well as soil bioaugmented with an enrichment culture capable of oxidizing 1,4-dioxane, should be examined to demonstrate that ethane-oxidizing bacteria can biodegrade 1,4-dioxane in the presence of low ethane levels at a rate that is meaningful with respect to the natural attenuation of 1,4-dioxane.

- ***Can this relatively slow 1,4-dioxane biodegradation by ethane-oxidizing bacteria be verified and quantified in the field?*** While the long-term laboratory testing with low sustained ethane concentrations described above is essential for demonstrating and quantifying the rate that ethane-oxidizing bacteria are capable of biodegrading 1,4-dioxane, *in situ* verification of this co-metabolic biodegradation process is needed to verify that this is in fact occurring in the field. Demonstration of this co-metabolic

process is complicated by the fact that other 1,4-dioxane degradation processes also may be occurring at slow rates (e.g., direct oxidation of 1,4-dioxane). A potential approach to determine if ethane-oxidizing bacteria are responsible for observed 1,4-dioxane degradation in the field is to employ the use of baited BioTraps. Deployed BioTraps set in wells within aerobic zones can then be used to determine the rate of 1,4-dioxane biodegradation 1) under ambient conditions, 2) in the absence of ethane/methane (by gently sparging the borehole with air to remove dissolved gases from the groundwater), and 3) in the presence of additional ethane (by sparging the borehole with air amended with ethane). Microbial communities in the BioTraps also can be assessed to further evaluate the potential role of ethane-oxidizing bacteria with respect to 1,4-dioxane biodegradation *in situ*.

7.0 REFERENCES CITED

Adams, C.D., P.A. Scanlan, and N.D. Secrist. 1994. Oxidation and biodegradability enhancement of 1,4-dioxane using hydrogen peroxide and ozone. *Environ. Sci. Technol.* 28:1812-1818.

Adamson, D.T., R.H. Anderson, S. Mahendra, and C.J. Newell. 2015. Evidence of 1,4-dioxane attenuation at groundwater sites contaminated with chlorinated solvents and 1,4-dioxane. *Environ. Sci. Technol*. 49:6510-6518.

Beeman, R.E. and Bleckmann, C.A. 2002. Sequential anaerobic-aerobic treatment of an aquifer contaminated by halogenated organics: field results. *J. Contam. Hydrol*. 57, 147-159.

Bernhardt, D., and H. Diekmann. 1991. Degradation of dioxane, tetrahydrofuran and other cyclic ethers by an environmental *Rhodococcus* strain. *Appl. Microbiol. Biotechnol.* 36:120-123.

Burback, B.L., and J.J. Perry. 1993. Biodegradation and biotransformation of groundwater pollutant mixtures by *Mycobacterium vaccae*. *Appl. Environ. Microbiol.* 59:1025-1029.

Chiang, S.D., R. Mora, W.H. Diguiseppi, G. Davis, K. Sublette, P. Gedalanga, and S. Mahendra. 2012. Characterizing the intrinsic bioremediation potential of 1,4-dioxane and trichloroethene using innovative environmental diagnostic tools. *J. Environ. Monitor*. 14:2317-2326.

Chu, K.-H. and L. Alvarez-Cohen. 1996. TCE degradation by methane-oxidizing cultures grown with various nitrogen sources. *Water Environ. Res.* 68:76-82.

Chu, K.-H., and L. Alvarez-Cohen. 1999. Trichloroethylene degradation by methane-oxidizing cultures grown with various nitrogen sources. *Water Environ. Res*. 68:76-82.

Joergensen, L., and H. Degn. 1983. Mass spectrometric measurements of methane and oxygen utilization by methanotrophic bacteria. *FEMS Microbiology Letters* 20:331-335.

Ellis, D.E. et al. Bioaugmentation for accelerated in situ anaerobic bioremediation. 2000. *Environ. Sci. Technol*. 34:2254-2260.

Félix-Navarro, R.M., S.W. Lin-Ho, N. Barrera-Díaz, and S. Pérez-Sicairos. 2007. Kinetics of the degradation of 1,4-dioxane using persulfate. *J. Mex. Chem. Soc*. 51:67-71.

Fincher, E.L., and W.J. Payne. 1962. Bacterial utilization of ether glycols. *Appl. Microbiol.* 10:542-547.

Fournier, D., J. Hawari, A. Halasz, S.H. Streger, K. McClay, and P.B. Hatzinger. 2006. Biotransformation of n-nitrosodimethylamine by Pseudomonas mendocina KR1. *Appl. Environ. Microbiol.* 72:6693-6698**.**

Fox, B.G., W.A. Froland, D.R. Jollie, and J.D. Lipscomb. 1990. Methane monooxygenase from Methylosinus trichosporium OB3b. *Methods Enzymol.* 188:191-202.

Freedman, D.L, A.S. Danko, and M.F. Verce. 2001. Substrate interactions during aerobic biodegradation of methane, ethene, vinyl chloride, and 1,2-dichloroethenes. *Water Sci. Technol.* 43:333-340.

Gossett, J.M. Sustained aerobic oxidation of vinyl chloride at low oxygen concentrations. 2010. *Environ. Sci. Technol*. 44:1405-1411.

Hareland, W.A., R.L. Crawford, P.J. Chapman, and S. Dagley. 1975. Metabolic function and properties of 4-hydroxyphenylacetic acid 1-hydroxylase from Pseudomonas acidovorans. *J Bacteriol*. 121:272–285.

Hatzinger, P. B., S.H. Streger, and J. Begley. 2015. Enhancing aerobic biodegradation of 1,2-dibromoethane in groundwater using ethane or propane gas and inorganic nutrients. *J. Contam. Hydrol.* 172: 61-70.

Hatzinger, P.B., C.W. Condee, K.R. McClay, and A.P. Togna. 2011. Aerobic treatment of N-nitrosodimethylamine in a propane-fed membrane bioreactor. *Water Res.* 45:254-262.

Hood, E.D., D.W. Major, J.W. Quinn, W.-S.Yoon, A. Gravaskar, and E.A. Edwards. 2008. Demonstration of enhanced bioremediation in a TCE source area at Launch Complex 34, Cape Canaveral Air Force Station. *Ground Water Monit. Rem*. 28:98-107.

Joergensen, L., and H. Degn. 1983. Mass spectrometric measurements of methane and oxygen utilization by methanotrophic bacteria. *FEMS Microbiol. Letters* 20:331-335.

Klečka, G.M., and S.J. Gonsior. 1986. Removal of 1,4-dioxane from wastewater. *J. Haz. Mat.* 13:161-168.

Lesage, S., R.E. Jackson, M.W. Priddle, and P.G. Riemann. 1990. Occurrence and fate of organic solvent residues in anoxic groundwater at the Gloucester landfill, Canada. *Environ. Sci. Technol.* 24:559-566.

Lippincott, D., S.H. Streger, C.E. Schaefer, J. Hinkle, J. Stormo, and R.J. Steffan. 2015. Bioaugmentation and propane biosparging for *in situ* biodegradation of 1,4-dioxane. *Ground Water Monit. Rem.* 35:81-92.

Mahendra, S., and L. Alvarez-Cohen. 2005. *Pseudonocardia dioxanivoran*s sp. Nov., a novel actinomycete that grows on 1,4-dioxane. *Int. Syst. Evol. Microbiol.* 55:593-598.

Mahendra, S., and L. Alvarez-Cohen. 2006. Kinetics of 1,4-dioxane biodegradation by monooxygenase-expressing bacteria. *Environ. Sci. Technol*. 40:5435-5442.

Medici V., and G.C. Sturniolo. 2008. Tetrathiomolybdate, a copper chelator for the treatment of Wilson disease, pulmonary fibrosis and other indications. *i drugs.* 11:592-606.

Parales, R.E., J.E. Adanus, N. White, and H.D. May. 1994. Degradation of 1,4-dioxane by an actinomycete in pure cultures. *Appl. Environ. Microbiol.* 60:4527-4530.

Schaefer, C.E., C.W. Condee, S. Vainberg, and R.J. Steffan. 2009. Bioaugmentation for Chlorinated Ethenes using *Dehalococcoides* sp.: Comparison between batch and column experiments, *Chemosphere* 75:141-148.

Scheutz, C. et al. 2010. Field evaluation of biological enhanced reductive dechlorination of chloroethenes in clayey till. *Environ, Sci. Technol*. 44:5134-5141.

Sei, K., T. Kakinoki, D. Inoue, S. Soda, M. Fujita, and M. Ike. 2010. Evaluation of the biodegradation potential of 1,4-dioxane in river, soil and activated sludge samples. *Biodegradation.* 21:585-591.

Semrau, J.D., A.A. DiSpirito, and S. Yoon. 2010. Methanotrophs and copper. *FEMS Microbiol. Rev.* 34:496-531.

Sharp, J.O., C.M. Sales, and L. Alvarez-Cohen. 2010. Functional characterization of propane-enhanced N-nitrosodimethylamine degradation by two actinomycetales. *Biotech. Bioeng.* 107:924-932.

Shaw Environmental Inc., 2010. Final Remedial Action Report: Building 165 and Area 18C Groundwater Treatment Pilot Systems; Former Raritan Arsenal Project, Edison, NJ. US Army Corps of Engineers, Omaha District, Final Report: Contract # DACA45-03-D-0022, Task Order 0018.

Smith, L.H., P.L. McCarty, and P. Kitanidis. 1998. Spreadsheet method for evaluation of biochemical reaction rate coefficients and their uncertainties by weighted nonlinear least-squares analysis of the integrated Monod equation. *Appl. Environ. Microbiol*. 64:2044-2050.

Stefan, M.I., and J.R. Bolton. 1998. Mechanism of the degradation of 1,4-dioxane in dilute aqueous solutions using the UV/hydrogen peroxide process. *Environ. Sci. Technol.* 32:1588-1595.

Strand, S.E., M.D. Bjelland, and H.D. Stensel. 1990. Kinetics of chlorinated hydrocarbon degradation by suspended cultures of methane-oxidizing bacteria. *Research J. WPCF* 62;124-129.

Yu, Y., J.A. Ramsay, and B.A. Ramsay. 2009. Use of allylthiourea to produce soluble methane monooxygenase in the presence of copper. *Appl. Microbiol. Biotechnol.* 82:333-339.

Vainberg, S., K. McClay, H. Masuda, D. Root, C. Condee, G.J. Zylstra, and R.J. Steffan. 2006. Biodegradation of ether pollutants by *Pseudonocardia* sp. strain ENV478. *Appl. Environ. Microbiol.* 72:5218-5224.

Whitenbury, R. 1970. Enrichment, isolation and some properties of methane-utilising bacteria. *J. Gen. Microbiol.* 61:202-218.

Witt, M.E. et al. 2002. Natural attenuation of chlorinated solvents at Area 6, Dover Air Force Base: groundwater biogeochemistry. *J. Contam. Hydrol*. 57:61-80.

Zenker, M.J., R.C. Borden, and M.A. Barlaz. 2000. Mineralization of 1,4-dioxane in the presence of a structural analog. *Biodegradation.* 11:239-246.