RICE UNIVERSITY

Microcosm Assessment of Aerobic Intrinsic Bioremediation and Mineralization Potential for three 1,4 Dioxane-Impacted Sites

by

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A THESIS SUBMITTED
IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE

Master of Science

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August 2013

Abstract

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1,4-Dioxane (dioxane) is a potential carcinogen widely used as a stabilizer for chlorinated solvents, and it exhibits high mobility in groundwater. Dioxane is recalcitrant to biodegradation, and its physicochemical properties preclude effective removal by volatilization or adsorption. Through this long-term microcosm study, we have assessed the natural attenuation potential of dioxane for multiple sediment and groundwater samples collected from three sites located in Los Angeles, CA. Groundwater and sediment samples were taken from three locations at each plume, representing the source zone, middle and leading edge. A total of 13 monitoring wells were sampled to prepare the microcosms and subsequently assess the indigenous potential to biodegrade dioxane. The microcosms were spiked with ^{14}C-labeed dioxane to assess mineralization potential (per $^{14}CO_2$ recovery).

No dioxane loss and less than 8% CO_2 recovery was observed in the negative controls, indicating that dioxane removal (and mineralization) was due to biodegradation. Positive control microcosms amended with the dioxane degrader *Pseudonocardia dioxivorans* CB1190 exhibited dioxane degradation activity statistically indistinguishable from observed batch incubations prepared with

mineral media, indicating an absence of inhibitory compounds in source zone samples. Complete dioxane removal, exhibiting linear (zero-order) kinetics (indicative of saturated enzymes), was observed during 24 weeks incubation in all biologically active, unaugmented microcosms. Up to 43% mineralization as CO_2 and 5% to 7% biomass growth was observed in unaugmented microcosms experiencing rapid dioxane loss. Degradation activity decreased with increasing distance from the contaminant source zone, presumably due to less acclimation. Source-zone microcosms from Site 1 exhibited relatively high biodegradation activity (323.9 ± 7.6 µg/L/day) and were respiked with dioxane for confirmatory purposes. The respike (2 ppm dioxane) was degraded faster within four weeks, suggesting a higher level of acclimation (possibly due to the growth of indigenous dioxane degraders) after the initial 24 week study. Source-zone microcosms from Site 2 and 3 exhibited biodegradation activities of 1.4 ± 0.09 µg/L/day and 47.1 ± 1.8 µg/L/day, respectively. Overall, these results show that indigenous microorganisms capable of degrading dioxane are present at the three sites considered, and suggest that monitored natural attenuation should be considered as a remedial response.

Acknowledgments

I would like to thank my advisor and mentor, Dr. Pedro J. Alvarez, for guiding me in my greatest academic challenge: becoming an engineer. Thank you to David DeVries and AMEC for the funding, samples, and data critiques which made this research possible. I also thank Dr. John Novak for opening the door to the field of Environmental Engineering and taking on the role of advisor during my first semester of graduate school at Virginia Tech. Thank you to my mentors and role models: Drs. Roberta Rudnick, William McDonough, John Merck, Tom Holtz, Alan Jay Kaufman, Sujay Kaushal and Andrew Campbell, from the Department of Geology at University of Maryland. You cultivated and nurtured the scientist in me and I will be forever grateful for the experience of studying under you. I would like to thank the graduate students from Rice University and University of Maryland who served as colleagues, advisors, and friends during my academic studies and research. I would like to specifically thank Mengyan Li who trained and mentored me as a friend and supervisor during my Masters Thesis research. Thank you to Ambassador Robert Jordan for your letter of recommendation and thank you to my Grandpa Jerry Jones for your ongoing support in my life. Thank you to my parents, Julie and Dan Van Orden. I am grateful for your unconditional love and support. I believe that my success is solely because of the values with which you raised me. Adam Harden, thank you for taking me off of my parents' hands, stress and all. I am grateful for your love and guidance and I look forward to returning the favor.

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Nomenclature

Dioxane	1,4-Dioxane
MNA	Monitored Natural Attenuation
DO	Dissolved Oxygen
THF	Tetrahydrofuran
DCM	Dichloromethane
GC/MS	Gas Chromatograph/ Mass Spectrometer
LC	Liquid Scintillation Counter
TCA	1, 1, 1 Trichloroethane
MTBE	Methyl Tertiary Butyl Ether
HEAA	2-Hydroxyethoxyacetic Acid
BO-LS	Biological Oxidizer – Liquid Scintillation

Chapter 1

Problem Statement

1.1. Health Risks and Treatment Challenges

1,4 Dioxane, or 1,4-diethylene oxide (further referred to as dioxane), is a flammable substance classified by the EPA as a class B2 probable human carcinogen. This classification is based on research indicating an increased occurrence of nasal carcinomas in rats, liver carcinomas in mice, and gall bladder carcinomas in guinea pigs (National Cancer Institute, 1978). The EPA has established a drinking water health advisory for dioxane with a lifetime cancer risk of 1:10,000 for a water concentration of 0.3 mg/L (USEPA, 2000). Some states have independently established stricter guidelines for dioxane with drinking water and groundwater with limits ranging from 3 to 85 ug/L (Mohr, 2001).

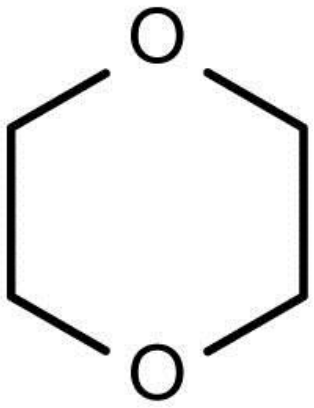

Figure 1. 1,4 dioxane molecular structure

Dioxane is a cyclic organic compound with two ether linkages forming para-substitutions (Figure 1). The dioxane chemical structure makes the compound fully miscible in water, highly hydrophyllic, with a log K_{ow} = -0.27, and is highly mobile in water (Table 1) (Scharzenbach et al, 2003). In 1985, 90% of dioxane was produced as a stabilizing agent for 1,1,1-trichloroethane (TCA) and other chlorinated solvents (Hazardous Substances Data Bank, 1995). Dioxane is still used for TCA stabilization and is considered a co-contaminant (Mohr, 2001), however, contamination directly related to TCA stabilization has decreased due to an increase in strict air quality emissions related to TCA use which decreased use of the compound (Doherty, 2000). Due to limited EPA regulation and persistence of dioxane in water, some sites currently or previously contaminated with associated organic solvents are still contaminated with dioxane. Occurrences of dioxane extend past organic solvents, as it has been found in municipal wastewater streams, rivers and river beds, groundwater and coastal marine environments (Abe, 1999).

Dioxane poses a current and future threat to human health due to its extensive industrial and commercial use. This thesis describes a single microcosm study from a broad environmental site investigation. The following information is not only intended for consideration during site-specific remedial design, but is also intended to contribute to future research addressing natural attenuation and remediation of 1,4 dioxane.

Table 1. Chemical and physical properties of 1,4 dioxane

Property	*Value*	*Reference*
Chemical Formula	$C_4H_8O_2$	-
Molecular Weight	88.1	Schwarzenbach et al 2006
Density	1.03 g/cm^3	Schwarzenbach et al 2006
Solubility	Fully Miscible	Schwarzenbach et al 2006
Henry's Law Constant	4.88×10^{-6} atm m^3/mol	Howard 2003
log Kow	-0.27	Howard 2003

Chapter 2

Objective and Hypothesis

Research has indicated that 1,4-dioxane (dioxane) can biodegrade in groundwater (Li et al 2010; Sun et al, 2011; Young et al, 1976; Grady et al, 1997; Roy et al, 1994; Sock et al, 1993; Cowan et al, 1994). However, the rate and of dioxane biodegradation must be determined on a site specific basis because the presence and expression of dioxane biodegradation capabilities are not ubiquitous. Recent findings by our lab and others suggest that indigenous bacteria that can degrade dioxane might be more widespread than previously assumed.

2.1. Objective Statement

We proposed to conduct a microcosm study that mimics *in situ* conditions at Honeywell (Location: Los Angeles, CA) (i.e. use local groundwater and aquifer material, and incubate in the dark at site-specific temperature and pH) using ^{14}C-labeled 1,4-dioxane to answer two questions:

- Is dioxane biodegradation occurring naturally?
- At what rate is dioxane biodegradation occurring?

Specific tasks to answer these questions and assess the feasibility of intrinsic bioremediation and Monitored Natural Attenuation (MNA) included:

1. Determine if dioxane is removed in biologically active microcosms, but not in sterile controls. This is important to discern biodegradation from potential abiotic losses. Negative controls would be run using background samples to obtain a baseline to determine if microbes from the plumes are acclimated and degrade dioxane faster or with a shorter lag time.
2. Discern the fate of ^{14}C-labeled dioxane, with focus on quantifying its mineralization ($^{14}CO_2$ recovery) and ^{14}C assimilation into biomass. This is important not only to obtain unequivocal evidence of biodegradation, but also to infer if the biodegradation process is metabolic (i.e. faster and more sustainable) rather than co-metabolic (which depends on the presence of a primary substrate.
3. Quantify dioxane biodegradation rates, by fitting the concentration versus time data using exponential decay (first order kinetics) or linear decay (zero order kinetics) models. This exercise is important to provide insight into the characteristics of the naturally occurring dioxane degraders.

2.2. Hypothesis

Although recent studies on dioxane attenuation have reported biodegradation to take place in groundwater, this biological activity has been historically uncommon. Recent studies reporting dioxane degradation have primarily used pure culture assays or mixed cultures capable of cometabolic biodegradation. These cases are not ubiquitous and do not directly resemble to field conditions present in this study, thus I hypothesize:

1. No significant ($p < 0.05$) mineralization or dioxane loss will be observed in the sterile negative controls. Methods of autoclaving and poisoning will be applied in order discern abiotic losses. Abiotic dioxane removal mechanisms such as precipitation or adsorption are unlikely to occur given the water chemistry and log K_{ow} value of dioxane. The closed microcosm design will prevent significant losses from evaporation and the absence of strong oxidants in the water precludes dioxane removal by oxidation.
2. Positive controls, amended with *P. dioxivorans* (CB1190) will experience 100% dioxane loss over the 1 year study period with significant mineralization and similar degradation patterns as in batch incubations prepared with mineral medium thus inferring the absence of inhibitory conditions in site microcosms.
3. Although recent studies on dioxane biodegradation have identified microbes capable of dioxane degradation, the laboratory study

conditions and media used to cultivate these microbes do not explicitly corroborate that native dioxane degraders will be ubiquitously present at dioxane-contaminated sites. If degradation is observed, a higher rate and extent of ^{14}C-dioxane metabolism (assessed by $^{14}CO_2$ evolution and ^{14}C assimilation into biomass) will be observed in soure zone microcosms that are presumably more acclimated. This acclimation is defined as increased capability of degrading dioxane.

Chapter 3

Literature Review

Currently, three EPA regions and 14 states have implemented concentration guidelines addressing dioxane in various matrices (US EPA, 2006). Minimum water concentration requirement from these locations vary widely and reach as low as 3 µg/L for groundwater and drinking water. The sites addressed in this study must meet a Health-Based Advisory Level of 3 µg/L for drinking water,

3.1. Physicochemical Treatment Studies

Numerous methods of physicochemical treatment of dioxane have been explored in response to screening levels set forth at the state level and by EPA region. Distillation was proposed as a potential removal mechanism, but it was ruled out due to the uneconomical, energy intensive process of reaching the 101°C boiling point of dioxane. Air stripping was deemed insufficient and prohibitively expensive

(Zenker et al, 2003) (Table 2). Adsorption was also preliminarily concluded ineffective due to dioxane's high affinity for water. However, studies have indicated dioxane removal above 90% (GRAC, 2003; Curry, 2012). Effective dioxane removal by GAC adsorption was achieved for influent concentrations up to 10^3 µg/L in water (GRAC, 2003). The GAC treatment system was originally intened to treat chlorinated solvents present in groundwater and the reason for this unexpected result remains unknown. A recent study of *ex situ* dioxane removal achieved a similar result of up to 96% removal (Curry, 2012). This adsorption reached an asymptotic minimum limit of `4.5 µg/L where dioxane was no longer removed. It was concluded that coconut base GAC was an optimal matrix treating 112 µg/L dioxane to <3 µg/L in a period of 96 days. Past these initial discoveries of dioxane adsorption, further studies need to be performed to address the fate, effect of environmental conditions on adsorption, and effective concentration range of dioxane in groundwater.

Table 2. Chemical and physical treatment methods for 1,4 dioxane. Based on Young et al 1976

Method	Relevant Property	Description	Reference
Air Sparging	Henry's Law Constant	Dioxane is not volatile enough for air sparging to be effective.	Howard 2003
Activated Carbon Adsorption	Log K_{ow}	The compound is unlikely to partition out of water	Howard 2003
Membrane Filtration	Molecular weight	Molecule is very small and may not be captured by the membrane.	Kishimoto et al 2008
Chemical Oxidation	-	Effective but expensive	Young et al 1976

Advanced oxidation and photocatalysis studies have produced results of successful dioxane degradation, particularly in combined treatments. Early studies of dioxane oxidation found that H_2O_2 and ozone were, by themselves, incapable of dioxane oxidation (Adams et al, 1994; Hoigne et al, 1983). However, the combined oxidants were found to degrade dioxane, although the reaction produced undesirable byproducts (Adams et al, 1994). Dioxane was found to degrade in wastewater by 97% in 10 hours using Fenton's reagent (Klecka and Gonsoir , 1986). Marino et al (1997) successfully demonstrated dioxane degradation by phtotcatalysis and compared the degradation efficiency of 0.2 g/L of TiO_2 at λ>340 nm to efficiency of H_2O_2+UV with 0.1M H_2O_2 at λ>295 nm. The photocatalytic process was found to be more efficient at 30 ppm (dioxane in water) than the oxidative system.

A recent study by Coleman et al (2007) addressed the optimization of photocatalysis and H_2O_2 + UV teatments. Degussa P25 TiO_2, lab-synthesized magnetic photocatalyst and sol-gel were studied as methods of photocatalysis under a variety of loading conditions. Complete dioxane removal was achieved (C/C_0) in all treatments where P25 photocatalyst demonstrated the highest degradation rates, up to 2.42 ± 0.14 μgC/min. The magnetic photocatalyst reached a maximum rate of 0.24 ± 0.02 μgC/min. The addition of H_2O_2 to P25 decreased the efficiency of degradation and increased the efficiency of magnetic photocatalyst. H_2O_2 and UVC degraded dioxane at a rate similar to the sol-gel system. The reported degradation rates from this study support advance oxidation as an effective method of dioxane removal.

Although complete degradation of dioxane by photocatalytic treatment would presumably leave no intermediates, the risk associated with these degradation byproducts suggests sole photocatalysis to be an insufficient remediative approach. Marino et al (1997) and Hill et al (1997) revealed a photocatalytic pathway of dioxane degradation in water, where ethylene glycol diformate (EGDF) was identified as a dominant intermediate of the reaction. Although non-toxic, EGDF is an irritant and is highly flammable (US EPA IRIS). Other heterogeneous photocatalytic studies found minor byproducts of the reaction to include formaldehyde as well as formic, glycolic, and oxalic acids (Marino et al, 1997; Mehrvar et al, 2000; Mehrvar et al, 2002; Lam et al, 2007). These acids have a corrosive nature, are a strong irritant, and are identified as potential carcinogens with risk of developmental toxicity (US EPA IRIS). It was also found that if contaminated groundwater contains bromide, the ozidizing agent will react to form bromate, another probable human carcinogen (Horst 2005). Despite potential risk, the treatment benefit of dioxane remediation for advanced oxidation prevailed. Advanced oxidation is currently the dominant method for remediation of dioxane in groundwater.

Beckett and Hua (2000) reported the absence of the EGDF intermediate pathway when sonolysis was used to degrade dioxane. Not only did this study reduce the risk related to intermediates, but also eliminated the rate limiting reaction of degrading EGDF (Marino et al, 1997; Hill et al 1997). Becket and Hua concluded a combination of sonication, UV, and HF treated TiO_2 yielded the fasted degradation rate (100% removal by 160 minutes). EGDF was produced by this

process but peaked in concentration at 160 minutes and was completely removed by 260 minutes. This preliminary study of sonication successfully degraded dioxane, however, the complete chemical pathway of this process has not been described and unknown risks may exist. Additionally, the scale-up of this treatment method may not be cost effective given the energy required for UV and sonication as well as the TiO_2 dose required to treat a range of dioxane concentrations.

Overall, a knowledge gap exists in the field of *in situ* remediation. Physicochemical methods of dioxane treatment exist; however, the cost and risk associated with bioremediation is lower and therefore more desirable if the method is achievable. As a result, numerous studies have taken place addressing the existence, isolation, and metabolic capability of dioxane degraders.

3.2. Bioremediative Studies

Despite efforts to improve physicochemical treatment methods, gaps still exist which may be addressed by recent research in bioremediation. Advantages of successful biodegradation include the ability to remediate *in situ*, substantially reducing the treatment cost in comparison to pump-and-treat methods; biodegradation often results in harmless byproducts that do not require further treatment or disposal. However, bioremediation is marginally effective for recalcitrant compounds such as dioxane. Dioxane is historically reported as recalcitrant to biological treatment methods; however, numerous attempts have been made to isolate bacterial strains capable of dioxane degradation (Abe, 1999;

Zenker et al, 2003; Parales et al, 1994; Adams et al, 1994). When successfully isolated, microcosms or temporary assays are inoculated with the isolated culture and incubated to determine degradation capacity.

Demonstrating clear mass loss and kinetics of degradation at the bench scale is most meaningful when designed with native culture, controls, and mass balances. Several articles have been published, describing mixed and pure cultures of bacteria, capable of aerobically degrading dioxane. Of those studies, profound and clear conclusions came from experimental methods utilizing negative and positive controls as well as triplicates and radiolabelled carbon sources to demonstrate mass balances. In the following illustrative studies, negative controls were used to establish an abiotic baseline and triplicates were used in most cases as a reference for degree of experimental error. In the cases of Zenker et al 2000 and Parales et al 1994, CO_2 evolution was used to track experimental mass balances and unequivocally demonstrate biodegradation.

Early publications describing dioxane degradation described cometabolism in the presence of tetrahydrofuran (THF), a dioxane analogue. Zenker et al 2000 conducted batch tests of degradation dependence on THF presence and temperature. Microbes were cultured from sediment and water from a contaminated site, and once enriched, the culture was mixed with mineral media, THF and dioxane. Cell yield was measured in a separate radiolabelled dioxane mass balance microcosm study where NaOH traps were used to measure CO_2 mineralization and suspended solids were combusted to calculate biomass. Two sets of triplicate microcosms were created: three with solitary dioxane and three

with equal portions dioxane and THF. Temperature dependence was evaluated in a separate batch test of triplicate flasks where solitary dioxane and THF/dioxane mixtures were exposed to 20, 25, 30, 35 and 40 degrees Celsius. When added alone, dioxane was consistently not degraded while the THF and dioxane mixture exhibited higher dioxane degradation at 30 and 35 degrees Celsius while 40°C was inhibitory (Zenker et al, 2000). This study unequivocally established THF as primary substrate for dioxane cometabolism using a native cell culture, where the biomass yield was approximately 2% and 30°C stimulated dioxane and THF degradation (Zenker et al, 2000). The role of temperature in degradation as it relates to cell activity and growth was confirmed and new insight was illustrated by the unexpectedly low percent of radiolabelled carbon incorporated into biomass.

A study on the ability of *Flavobacterium* to degrade dioxane was conducted by Sun et al (2011). Microbes cultured from soil samples, mineral media, and dioxane or THF as the sole carbon source were incubated in an assay for 9 months. Only one microcosm of triplicates containing THF was found to degrade THF and dioxane. The demonstrated metabolic relationship does not adequately fit the trend of cometabolism, and ultimately the published data are inconclusive. PCR and DGGE were used to isolate and tentatively classify the degrader as *Flavobacterium*. Due to the irreproducibility of this degrader in the other two THF assays, it is unclear if this degrader originated from the soil sample or if this is an artifact of cross contamination. Other publications have described a relationship between analogous co-substrates (THF, propane and toluene) (Parales et al, 1994; Bernhardt and

Diekmann, 1991), although in the case of Sei et al 2010 the data did not unequivocally demonstrate cometabolism with THF.

Parales et al 1994 isolated a strain of Actinomycete (CB1190) capable of utilizing dioxane as a sole carbon source. The pure culture came from an industrial aerobic activated sludge and was first grown on THF media before transferring to dioxane as the sole carbon source. Growth was measured by optical density and CO_2 mineralization was measured against a negative control to ensure biodegradation was taking place. The data unequivocally indicate pure culture CB1190 is capable of degrading dioxane as a sole carbon source, and is the first capable culture reported in literature. Further studies have been conducted with CB1190, including a study of bioaugmentation, biostimulation, and natural attenuation in Artic conditions. Microcosms prepared with contaminated aquifer sediment and groundwater were incubated in triplicate at 4 and 14°C. CB1190 degraded dioxane under low temperature conditions, qualified by negative and positive controls, with a higher degradation rate at 14°C (Li et al, 2010), indicating temperature dependence previously reported. This study offers insight into the range of temperatures at which CB1190 is capable of dioxane degradation.

Novel insight from these and other microcosm studies support CB1190 as the first microbe to utilize dioxane as a sole carbon source, while other strains degrade dioxane cometabolically or by some other metabolic relationship. Microbes of close phylogeny to CB1190 and microbes capable of degrading a dioxane structural analogue may be capable of transforming dioxane mass in some metabolic capacity. Further research on dioxane degrading microbes has been aided by understanding

the metabolic pathway of dioxane degraders and the enzymes related to this utilizing this carbon source.

3.3. Degradation Pathway Mapping

Pathway mapping can be used as an independent line of evidence in studying microbial degradation of dioxane. Previously, negative and positive controls coupled with radiolabeled mass balances were the only methods of confirming biodegradation, however mass spectrometry and solid phase extraction have enabled researchers to identify degradation intermediates and ultimately a complete metabolic pathway of dioxane degradation.

Young et al 1976 and Woo et al 1977 introduced an incomplete mammalian, dioxane biodegradation pathway by analyzing urine. Vainberg et al 2006 used a Pseudonocardia strain to study microbial metabolism of dioxane. These three studies concluded 2-hydroxyethoxyacetic acid (HEAA) to be a terminal product of dioxane degradation carried out by monoxygenase. Mahendra et al 2007 proposed a complete metabolic pathway by bacterial monoxygenase (Figure 2).

Through use of GC-FID, Solid Phase Extraction, MS technology and ^{14}C mineralization, intermediates of the dioxane metabolic and cometabolic pathways were described, indicating HEAA as a major intermediate and ultimately concluding the pathway will not directly cause accumulation of toxic compounds. This proposed low risk of the dioxane metabolic pathway supports bioremediation as a viable method of dioxane treatment.

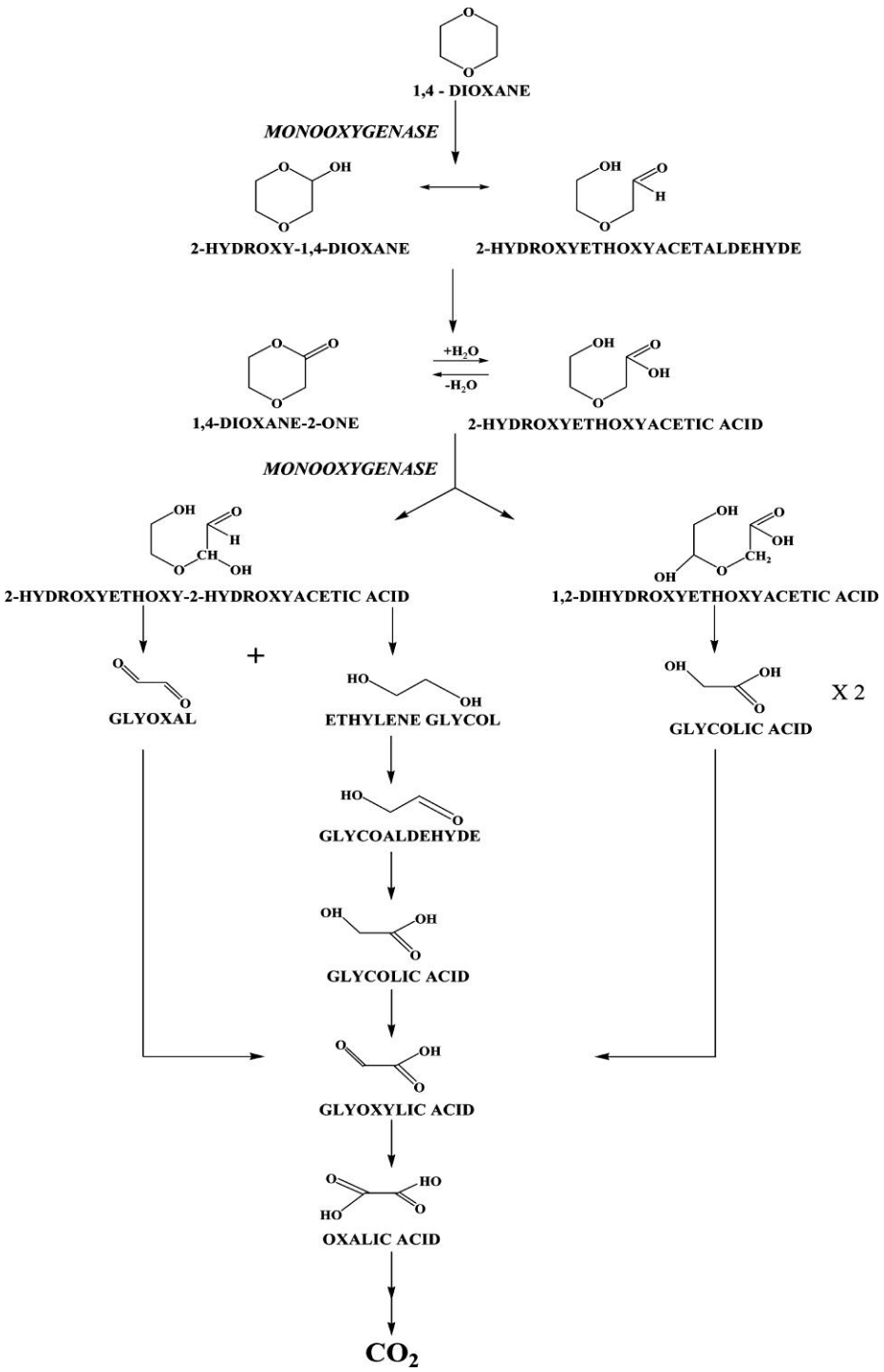

Figure 2. Biodegration pathway of 1,4 dioxane by monoxygenase enzyme. Proposed by Mahendra et al 2007.

Mahendra et al also presented mass balance data in the 2007 study indicating a recurring concept that dioxane degradation does not yield high biomass, reported as 5% in this study. The source of this proposed metabolic pathway and recurring information on low biomass yield are further discussed in studies addressing the enzyme that facilitates dioxane degradation.

3.4. Enzymes, Inducers, and Inhibitors

Quantification of enzyme activity and kinetics are the next approach to understanding the metabolic capacity of microbes concluded to degrade dioxane. The proposed metabolic pathway suggests monoxygenase is the facilitating enzyme, but the degree of degradation capability is unclear.

Mahendra and Alvarez-Cohen 2006 compiled a list of bacterial strains reported as a dioxane degrader or a dioxane analogue degrader, and tested each strain for dioxane degradation capability. Each strain was grown on a substrate favorable for the associated oxygenase expressed in previous literature, and were then isolated and exposed to dioxane as the sole carbon source. They found that CB1190 and B5 were the only strains capable of utilizing dioxane as a sole carbon source; B5 had not previously been reported to grow on a dioxane substrate. Other strains (Table 3) cometabolically degraded dioxane in the presence of substrate analogues, including LB broth, toluene, propane, soluble methane and THF. Soluble methane served as a successful substrate, only in the absence of copper salts. Acetylene gas was found to irreversibly inhibit monooxygenases, and MTBE was determined not to be an inducer, contrary to its analogous structure.

Table 3. **Primary and cometabolic dioxane degraders, specific oxygenase and activation substrate. Based on Mahendra and Alvarez-Cohen 2006**

Bacterial Strain	Metabolism	Substrate	Oxygenase	Degradation Rate (mg/hr/ mg protein)
P.dioxanivorans (CB1190)	Primary	Dioxane	Unknown	0.19 ± 0.007
P.benzenivorans (B5)	Primary	Dioxane	Unknown	0.01 ±0.003
Pseudonocardia (K1)	Cometabolic	THF	THF MO	0.26 ± 0.013
Pseudonocardia (K1)	Cometabolic	Toluene	THF MO	0.16 ± 0.006
M. trichosporium (Ob3b)	Cometabolic	Methane	Soluble methane MO	0.38 ± 0.02
My. vaccae (JOB5)	Cometabolic	Propane	Propane MO	0.40 ± 0.06
Rhodococcus (RR1)	Cometabolic	Toluene	Unknown	0.38 ± 0.03
B. cepacia (G4)	Cometabolic	Toluene	Toluene 2 MO	0.10 ± 0.006
R. picketti (PKO1)	Cometabolic	Toluene	Toluene p MO	0.31 ± 0.007
Pn. Mendocina (KR1)	Cometabolic	Toluene	Toluene 4 MO	0.37 ± 0.04
E.coli (TG1)	Cometabolic	LB broth	Toluene 2 MO	0.06 ± 0.008
E. coli (TG1)	Cometabolic	LB broth	Toluene p MO	0.17 ± 0.01
E. coli (TG1)	Cometabolic	LB broth	Toluene 4 MO	0.26 ± 0.03

Toluene dioxygenases was also tested using *Psedomonas* JS150 and F1 which were not capable of facilitating dioxane degradation (Mahendra and Alvarez-Cohen, 2006). This study clearly demonstrates the variety of monoxygenase enzymes capable of facilitating dioxane degradation as well as the substrates that induce monoxygenase.

These studies on microbial strains, substrate growth, metabolic pathways and effect of environmental conditions indicate bioremediation may be an effective method of dioxane removal. Further research into MNA of dioxane is needed in order to formulate a cost and resource effective method of dioxane remediation. This study is one of few to illustrate MNA of dioxane in groundwater at a range of concentrations (10^{-1} to 10^{1} mg/L).

Chapter 4

Methods and Materials

4.1. Chemicals

1,4-Dioxane (99.9%, stabilized with 10 mg/L sodium diethyldithiocarbonate) was purchased from EM Science, Cherry Hill, NJ. 1,4-Dioxane-d_8 (99.9%) was obtained from Sigma Aldrich, St. Louis, MO. Dimethylene chloride (99.9%) was obtained from Fisher Scientific, Fair Lawn, NJ. Anhydrous sodium sulfate was purchased from Thermo Fisher Scientific, Waltham, MA. Anhydrous mercury chloride was purchased from Thermo Fisher Scientific, Waltham, MA.

One liter of AMS contained 100 mL of 10x salts solution, 1.0 mL of AMS trace elements, 1.0 mL of stock A, and 20 mL of 1.0 M phosphate buffer (added after sterilization). The AMS 10x salt solution contained 6.6g of $(NH_4)_2SO_4$, 10.0 g of $MgSO_4 \cdot 7H_2O$, and 0.15g of $CaCl_2 \cdot 2H_2O$. The AMS trace elements contained, per liter, 0.5 g of $FeSO_4 \cdot 7H_2O$, 0.4g of $ZnSO_4 \cdot 7H_2O$, 0.02g of $MnSO_4 \cdot H_2O$, 0.015g of H_3BO_3,

0.01g of $NiCl_2 \cdot 6H_2O$, 0.05g of $CoCl_2 \cdot 2H_2O$, and 0.25 of EDTA. AMS stock A contained, per liter, 5.0g of Fe-Na EDTA and 2.0g of $NaMoO_4 \cdot 2H_2$). The 1M phosphate buffer contained 113.0 g of K_2HPO_4 and 47.0 g of KH_2PO_4. All reagents used in medium preparation were of ACS or better.

4.2. Laboratory Strains

One reference strain, *Pseudonocardia dioxanivorans* CB1190 (ATCC #55486) was selected as the bioaugmentation candidate. CB1190 is a well characterized dioxane degrader (Parales et al., 1994; Kelley et al., 2001). CB1190 was grown on R2A medium in a sterile petri dish at 24 °C. Cells were harvested by scraping with a sterile plastic cell applicator and inoculated into AMS medium where it was incubated for 5 days at 24°C and shaken at 150 rpm.

4.3. Microcosm Preparation

Microcosms were prepared using sediment and groundwater samples collected in June of 2012 from three industrial sites in Los Angeles, CA. Site 1 aquifer contained a nearly 1 mile-long plume (Figure 3) in a sandy/silty rock layer. Downhole dissolved oxygen (DO) ranged from 0 to 2.9 mg/L with no discernible relationship between DO concentration and distance from the contaminant source. Site 2 aquifer contained a plume of unknown size (data not provided by client) in a sandy/silty rock layer. Downhole DO was 2.32 mg/L. Site 3 aquifer contained a 0.2 mile plume (Figure 4) in a sandy/silty rock layer. Downhole DO ranged from 0.06 to

3.07 mg/L with no discernible relationship between DO and distance from the contaminant source. Groundwater at all sites was of neutral pH.

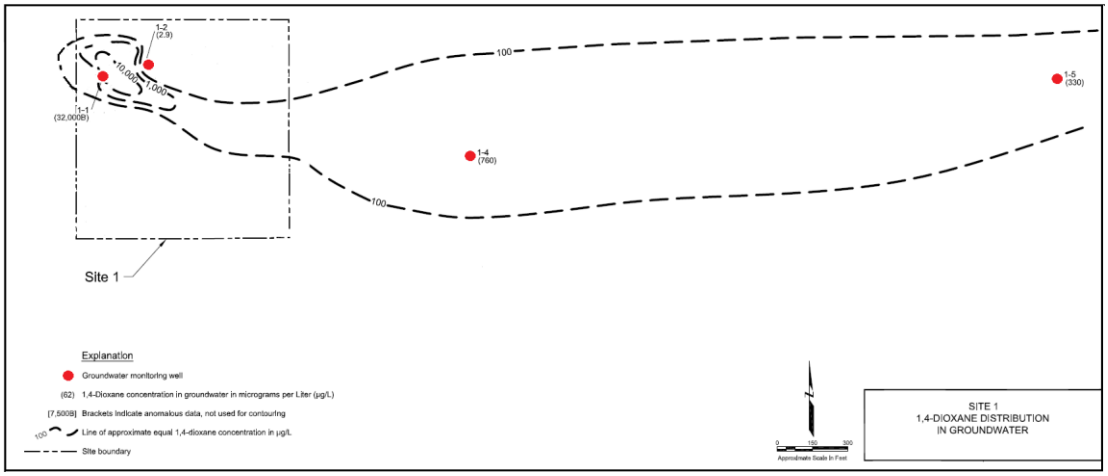

Figure 3. Site 1 map. Monitoring wells marked by red dots, dioxane contour lines marked as black dashed line.

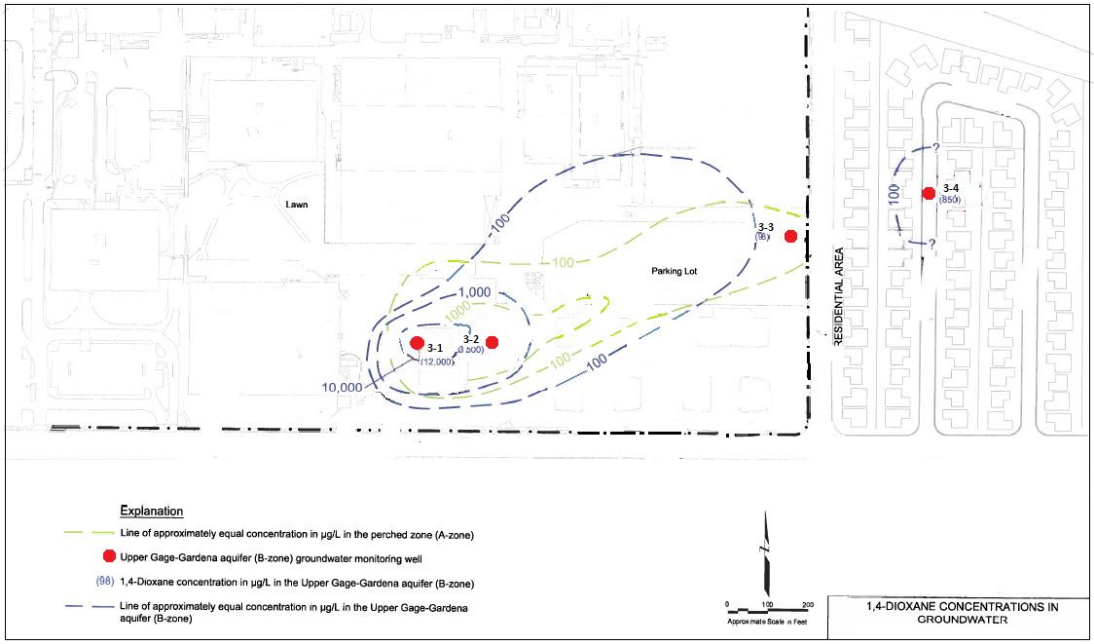

Figure 4. Site 3 map. Monitoring wells marked as red dots, dioxane concentrations marked as dashed green or blue lines.

4.3.1. Initial Microcosm Preparation

In early July 2012, from 7/10 to 7/18, a total of seventy-two (72) microcosms were prepared, marking the beginning of the 12 month bench-scale study for Honeywell (Figure 5). Three treatment groups (Table 4a) were created in triplicate. Positive controls were created using sediment and water from the monitoring well containing the highest contamination level, for each site. This resulted in three positive control groups, totaling nine (9) microcosms. Negative controls were created likewise using sediment and groundwater from the wells of highest concentration, plus triplicated for microcosms which did not contain the uniform 50g of sediment (two additional wells). This resulted in five (5) negative control groups, totaling fifteen (15) microcosms. Unaugmented microcosms were created in triplicate for each monitoring well, totaling forty-eight (48) microcosms. All microcosm bottles were autoclaved prior to addition of sediment and groundwater. See Table 4b for complete preparation checklist.

Figure 5. 1,4 dioxane microcosm study. Microcosm count: 72.
Photo taken: 8-3-2012

Table 4a. Treatments for microcosms spiked with 1 uCi/mL of radiolabeled dioxane.

	Water Component	*Sediment Component*	*Additional Treatment*
Positive Control	120 mL groundwater	50 g sediment	30 mL CB1190 innocula
Negative Control	150 mL groundwater	50 g sediment	Sediment and water autoclaved 200 mg/L $HgCl_2$
Unaugmented	150 mL groundwater	50 g sediment	None

In order to obtain solid sediment mass from the suspended solid samples sent to us, volumes of these samples were centrifuged and measured for each microcosm. Sixty of the microcosms were amended with 50g of sediment and 150 mL of groundwater. For monitoring wells 1-1 and 1-2, we obtained three layers of soil samples, including an upper sand layer (S), middle silt layer (M), and lower sand layer (D). No groundwater was sampled for the middle layers. Hence, 75 mL groundwater from both the S and D layers were added to these microcosms (1-1M and 1-2M). Three of the remaining microcosms, triplicates of 1-5, received 10g of sediment per bottle due to the low concentration of suspended solid in the sample we received. Triplicates of 1-6 and 3-5 received only groundwater because no sediment sample was sent. Each microcosm was also spiked with 1 μCi of radiolabeled ^{14}C-1,4-dioxane (purity > 99.9%) for improved determination of the fate of 1,4-dioxane (e.g., % mineralization to CO_2). Sterile controls were autoclaved separately and poisoned with $HgCl_2$ (200 mg/L), to discern biodegradation from

Table 4b Microcosms Preparation Checklist

		Microcosm #	150 mL GW	50 g Sediment	1μCi ^{14}C labeled Dioxane	No additional treatment	Note
Bench Test	Site 1	1-1S	+	+	+		
		1-1D	+	+	+		
		1-2S	+	+	+		
		1-2D	+	+	+		
		1-1M	*	+	+		75 ml 1-1S, 75 ml 1-1D
		1-2M	*	+	+		75 ml 1-2S, 75 ml 1-2D
		1-4	+	+	+		
		1-5	+	*	+		10g of sediment per bottle
		1-6	+	-	+		sediment not provided
	Site 2	2-1	+	+	+		
		2-2	+	+	+		
	Site 3	3-1	+	+	+		
		3-2	+	+	+		
		3-3	+	+	+		
		3-4	+	+	+		
		3-5	+	-	+		sediment not provided

		Microcosm #	125 mL GW	50 g Sediment	1μCi ^{14}C labeled Dioxane	25 mL AMS containing CB1190	Note: site samples used for control conditions
Positive Control	Site 1	+1-C	+	+	+	+	use 1-1D
	Site 2	+2-C	+	+	+	+	use 2-1
	Site 3	+3-C	+	+	+	+	use 3-1

		Microcosm #	150 mL GW	50 g Sediment	1μCi ^{14}C labeled Dioxane	Autoclave sediment, poisoned & GW w/ $HgCl_2$	Note
Negative Control	Site 1	(-)1-C	+	+	+	+	use 1-1D
		(-)1-C-M	+	+	+	+	use 1-1M
		(-)1-C-6	+	+	+	+	use 1-6
	Site 2	(-)2-C	+	+	+	+	use 2-1
	Site 3	(-)3-C	+	+	+	+	use 3-1

+/- indicates the composition was added or not while preparing microcosms;

* indicates special adjustment referring to the note.

potential abiotic losses. Microcosm caps were exposed to UV disinfection prior to microcosm construction.

The NaOH mineralization trap was constructed in the microcosm by epoxying a keck clip to the inside-center of each jar lid in order to hold a sterile 1.7 mL Phenix Ultraclear Microtube allowing for trap replacement (Appendix A). 1 mL of 1 M NaOH (purity > 99.9%) was pipetted into each vial during microcosm construction and was subsequently replaced during every sampling period during the study.

4.3.2. Respiking of microcosm 1-1D

Two experimental strategies were planed and performed to confirm our observation that relatively fast dioxane degradation occurred in the microcosms, including i) respiking the microcosms in which dioxane were fully depleted and ii) making new sets of microcosms prepared with freshly collected groundwater in March, 2013. Treatment 1-1D was chosen for respiking, because the triplicates exhibited rapid dioxane loss at room temperature within 20 weeks and no dioxane remained prior to replication. Thus, we respiked these microcosms with 150 μL of pure dioxane. Based on previous dioxane studies, we expected the rate of degradation during this experiment to be higher than before due to enhanced microbial acclimation.

4.3.3. Replication of microcosm 1-1D

A new water sample was provided from sampling location 1-1D which was used to create new microcosms, in order to establish reproducibility of previous results indicating biodegradation. Prior to creating these microcosms, four new microcosm designs were tested to improve the mass balance results of this study. A new design (see Appendix A) was selection that utilizes a larger NaOH trap (Figure 6) and a glass container (Figure 7) with a lid capable of sealing gas generating media to eliminate the leakage of generated CO_2. The larger trap is a 20 mL capless glass vial that accommodates 10 mL of NaOH and offers easy access for trap sampling and NaOH replacement during each sampling. The trap is epoxied to the inside-bottom of the jar to prevent the trap from floating and spilling when water is added to the microcosm. The company providing our jars no longer makes amber jars, so the jars were covered with tape to prevent light penetration.

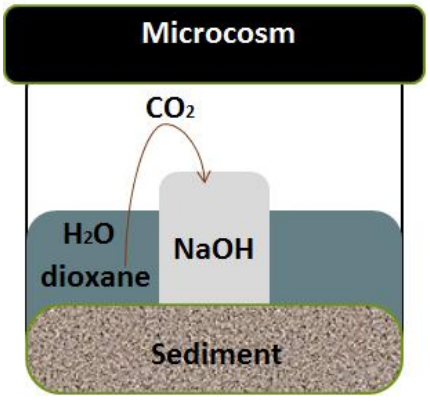

Figure 6. Diagram of new microcosm design.

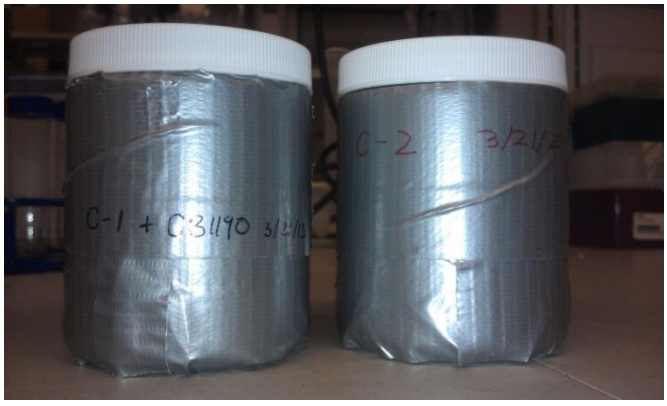

Figure 7. Photo of new 1-1D microcosms.

New microcosms were prepared with 25 g sediment, 75 mL of water, and 6.6 uCi/L of radiolabeled dioxane; these are proportional to the water, sediment, and dioxane added in the previous study. The sediment was previously sampled by AMEC in sealed pipes and stored at 4°C. Sediment from previously unopened containers was used in order to avoid potential previous contamination. Use of sediment was deemed necessary for the purpose of reproducibility and is suspected to contain biomass.

Four microcosms were prepared with the newly sampled water: three treatment replicates and one negative autoclaved control. All jars were autoclaved and caps were exposed to UV disinfection prior to microcosm construction. Water and sediment used for the negative control were autoclaved and poisoned with 200 mg/L of $HgCl_2$. Sterile pipets and weight station materials were used to measure and introduce sediment, water, NaOH and dioxane to the microcosms. All open-microcosm construction was conducted in the presence of an open flame in order to reduce contamination by air.

4.4. Sampling and Analytical Methods

Water samples (0.5 mL) were taken by 1 mL sterile syringes at week 0, 1, 2, 4, 6, 8, 12, 16, 20 and 24. For the repiked 1-1D microcosms, water samples were collected at 0, 12, and 28 days. The replicated 1-1D microcosms were sampled at 0, 2, 4, 8 and 12 weeks. All water samples were frozen in capped 1.7 mL Phenix Ultraclear Microtubes prior to microextraction and GC/MS injection. During water

sampling for every microcosm set, the NaOH traps were simultaneously sampled (0.5 mL) and replaced. All open-microcosm sampling was conducted within 1 ft of an open flame and new syringes were used to sample every microcosm in order to reduce risk of cross contamination.

A novel method of GC/MS sample preparation was used for all water samples during the study. This method or frozen microextraction can be found in Li et al 2011. Three calibration standards were created over the 1 year study period (Appendix B). A series dilution on a range from 800ppb to 12.5 ppb was used for each calibration. During sample preparation for analysis, samples of predicted concentrations exceeding the 800ppb standard were diluted to $1/10^{th}$ or $1/100^{th}$ to achieve a more precise mass analysis. The injection volume of all samples was 1 µL.

A combined HP Agilent mass spectrometer Model 5973 and HP Agilent gas chromatograph unit with HP-5 column 30m x 0.25mm i.d., 0.25 um film thickness and electronic pressure control system was used. An HP Agilent autosampler Model 7683 was installed on the front inlet to be controlled by Chemstation Version 3.0. The inlet temperature was 200°C and the inlet pressure was 10.0 psi with inlet pulse pressure set to 40 psi for 2.0 minutes. Total flow was 53 mL/min, with septum purge set at 3.0 mL/min for 1.0 minutes. In order to reduce the loss of active analytes by extended residence time in the liner, pulsed splitless injection was used with a 40:1 split ratio. The total gas flow was 1.4 mL/min with helium (purity > 99%). The oven method was set to1) hold at 35°C for 5.0 minutes, 2) run with a 20°C/min ramp to reach 100°C, 3) run with a 50°C/min ramp to reach 275°C, and 4)

hold at 275°C for 1.0 minute. A single run takes 12.75 minutes and is followed by an 8 minute cool-down, totaling 20.75 minutes per sample. A solvent delay of 5.0 minutes and EM offset of 200 were set and the SIM parameters were divided into two groups and each ion was assigned a dwell time of 100 us. The ratio of peak area of the internal standard ion, d8-dioxane, to the target ion was used to calculate the mass concentration. See Table 5 for the target ion retention times and mass to charge ratios.

An OX600 R.J. Harvey Instrument Biological Oxidizer was used to analyze ^{14}C in bound residue (e.g. bacterial cells) by solids combustion. A flow rate of 315 CC/minute was used and the combustion temperature was 915° Celsius. The nitrogen and oxygen pressures were 350 psi. Glassware is washed in methanol prior to combustion. Solids are combusted for 2 minutes and the effluent vapor is effervesced through 15 mL of ^{14}C cocktail. During sample preparation, solids samples were acquired by first mixing the microcosm contents and transferring 40 mL of homogeneous mixture by pipette to a sterile, plastic 50 mL centrifuge vial. Vials were centrifuged at 5000 rpm for 5 minutes at 20° Celsius to separate solids from water. Water was removed by pipette and solids were rinsed with Millipore water three times and centrifuged. After combustion, the ^{14}C cocktail is measure by Liquid Scintillation Counter (LSC) using a Beckman LS 6500. This combined process is henceforth referred to as BO-LS.

Table 5. 1,4 dioxane and 1,4 dioxane-d8 retention times and selection ions for GC/MS-SIM analysis.

Compound	*Retention Time (minutes)*	*SIM ions Mass:Charge*
1,4 dioxane-d8	4.9	64, 96
1,4 dioxane	5.1	58. 88

Chapter 5

Results and Discussion

5.1. Evidence of Dioxane Biodegradation

The removal of dioxane in biologically active microcosms but not in sterile controls provided evidence of biodegradation (Figures 9-13). First order kinetics were observed in positive controls (i.e., bioaugmented with *Pseudonocardia dioxivorans* CB1190) for site 1, 2, and 3, respectively, with significant extent of mineralization (30% ±<0.05%, 17% ±0.1%, and 17% ±0.3%, respectively). The degradation and mineralization patterns by CB1190 in the positive controls were similar to the patterns observed for CB1190 in AMS medium (Figure 8). This suggests the absence of inhibitory compounds at the three sites. No dioxane loss and less than 5% ± 0.01% mineralization was observed: in the three negative controls (Figure 8).

Zero order kinetics was observed in 11 of 16 microcosm sets (Figures 9-11). The overall degradation trends of microcosms 1-1 and 1-2 were consistently linear with $R^2 > 0.90$. An exception was 1-1S which had an R^2 of 0.76. Dioxane was degraded by week 24 in microcosm sets 1-1 and 1-2 while microcosms 1-4, 1-5 and 1-6 (site control) (Figure 9b) exhibited no detectable dioxane degradation (<0.2 ppb/week). This absence of degradation in microcosms 1-4 and 1-5 suggests the absence of dioxane degraders down gradient from the source zone. Mineralization extents of 44% ±0.5%, 38% ± 1.0%, and 20% ± 0.4% were observed for microcosms, 1-1S, 1-1M, and 1-1D, respectively. Mineralization extents of 20% ± 0.1%, 31% ± 0.1%, and 15% ± 0.3% were observed for microcosms, 1-2S, 1-2M, and 1-2D, respectively (Figures 9a and b). Zero order kinetics were observed in microcosms 2-1 (R^2= 0.97) and 2-2 (R^2= 0.83) (Figure 10). Dioxane was degraded by week 24 in these two microcosms. Mineralization of 20% ±0.2%, 38% ± 1.0%, and 10% ± 0.5% were observed for microcosms, 2-1 and 2-2, respectively.). Zero order kinetics were observed in microcosms 3-1 (R^2= 0.97) and 3-2 (R^2= 0.96) (Figure 11). Dioxane was degraded by week 24 in these two microcosms. Mineralization of 11% ±0.5% and 14% ± 1.4% were observed for microcosms, 3-1 and 3-2, respectively. Microcosm 3-4 experienced 30% ± 1% dioxane loss over 24 weeks and exhibited 16% ± 0.3% mineralization. Microcosms 3-3 and 3-5 did not experience dioxane loss and recovered 16% ±<0.05% and 11% ±0.1% as $^{14}CO_2$, respectively. These degradation trends and subsequent $^{14}CO_2$ recovery suggest saturated enzyme kinetics (low K_s value), coupled with low significant microbial growth (Table 6). A small K_s value suggests a high affinity for the primary substrate, which is typical of

oligotrophic bacteria (Atlas and Bartha, 1997). This zero order trend can be compared to the previously mentioned first order trend exhibited in the positive controls which were inoculated with CB1190. This exogeneous strain exhibits unsaturated enzyme kinetics, indicative of high K_s values that are characteristic of r-strategists (Li et al, 2010). ^{14}C was also recovered in the $^{14}CO_2$ trap in microcosms 1-4, 1-5, 1-6, 3-3 and 3-5, which did not degrade dioxane (Figure 9b and Figure 11). Therefore, that recovery was unlikely due to mineralization; rather, it is likely the result of some evaporation and condensation/deposition of radiolabelled dioxane in water vapor into the uncapped NaOH trap.

In order to verify the biodegradation observed in this study, microcosm set 1-1D was respiked with 2 mg/L of dioxane after complete dioxane removal was confirmed by analytical methods. The respiked dioxane was degraded even faster, and no dioxane was detected after 28 days (Figure 12). This suggests that the microcosms had become more acclimated to dioxane after the first stage, possibly due to the growth of indigenous dioxane degraders (Li et al 2010). A new set of 1-1D microcosms was prepared using freshly sampled groundwater and sediment from site 1. A new microcosm design utilizing a larger mineralization trap and an improved microcosm cap (Appendix A) was used for this experiment which ultimately yielded a more complete mass balance. Dioxane removal reached 64% ± 0.2% by week 12 with 53% ± 0.2% mineralization (Figure 13). No dioxane loss and 14% ±<0.05% $^{14}CO_2$ recovery was observed in the 1-1D Replicate negative control. There is a discrepancy between the ^{14}C recovery observed the original negative controls (~5%) and this replicate negative control (14%). This is likely a result of

the higher (6.6 μCi/L) concentration of radiolabeled dioxane present in the replicate compared to the originals which only contained 3.3 μCi/L. When evaporation and deposition to the NaOH trap took place in the new microcosm, the ^{14}C recovery was twice that of the original controls.

Mineralization of more than 50% has been reported in a previous study (Mahendra et al 2007), where approximately 5% of the ^{14}C mass was incorporated into biomass and 30% was attributed to volatile acids and non-volatile intermediates. Biologically active microcosms in this current study exhibited cumulative mineralization up to 43% total mineralization and up to 8% incorporation into biomass. No radiolabeled impurities or residual intermediates remained in the water once all of the dioxane was removed (Figure 13). The total ^{14}C recovery for all of the microcosms was lower than 95% (Table 6) due to apparent volatile losses (e.g., $^{14}CO_2$) that the microcosm design could not prevent (Appendix A). Leakage was minimized by increasing the surface area of the mineralization trap and using a gas-sealing microcosm lid for the subsequent 1-1D replication study where the total ^{14}C mass accounted after 12 weeks was 94% ± 0.2% (Figure 14).

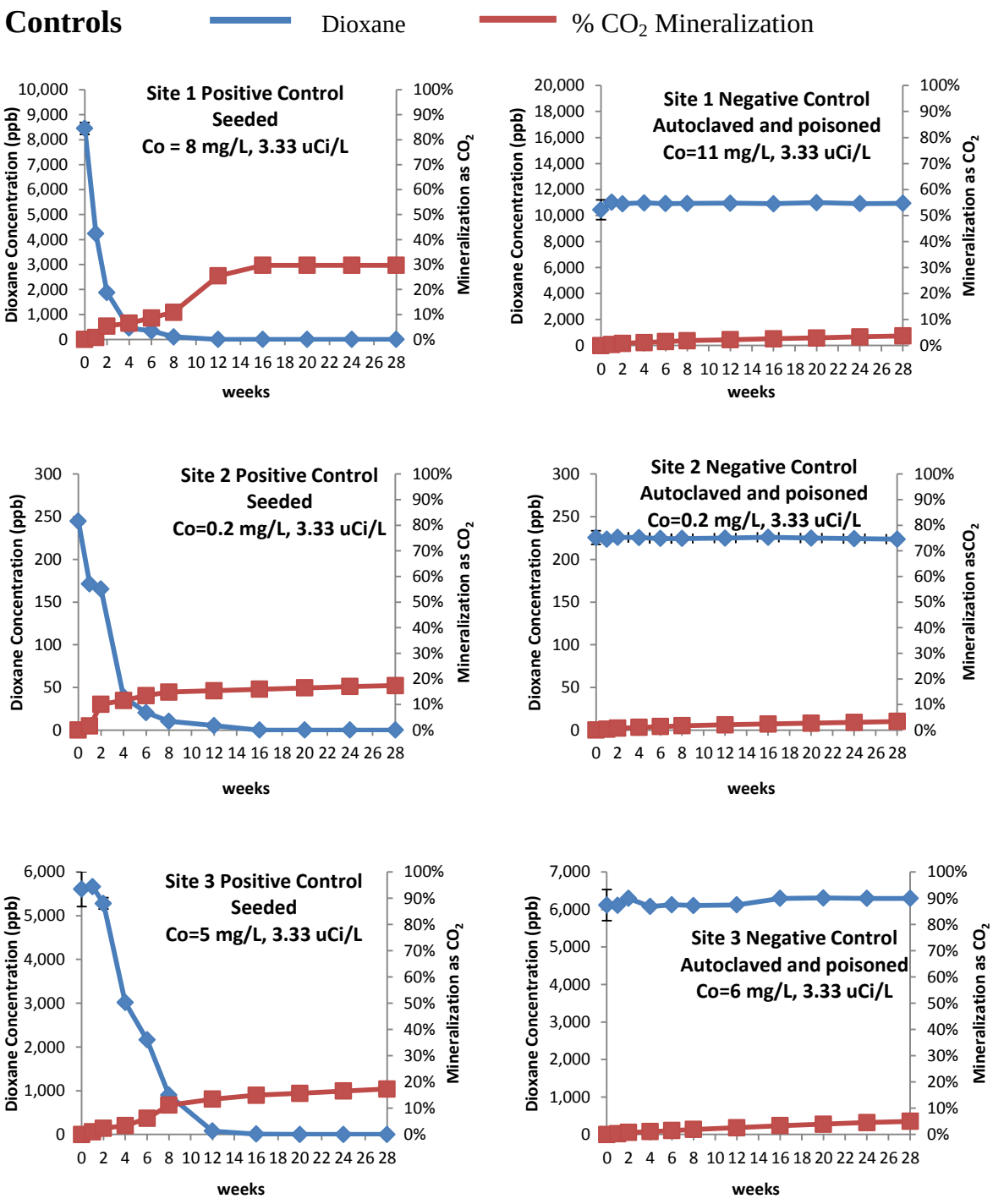

Figure 8. Dioxane and CO_2 mineralization mass over time for positive and negative (autoclaved) controls for sites 1, 2, and 3. Positive controls were inoculated with 30 mL of AMS containing suspended CB1190. Microcosms were stored quiescently at 20°C and neutral pH.

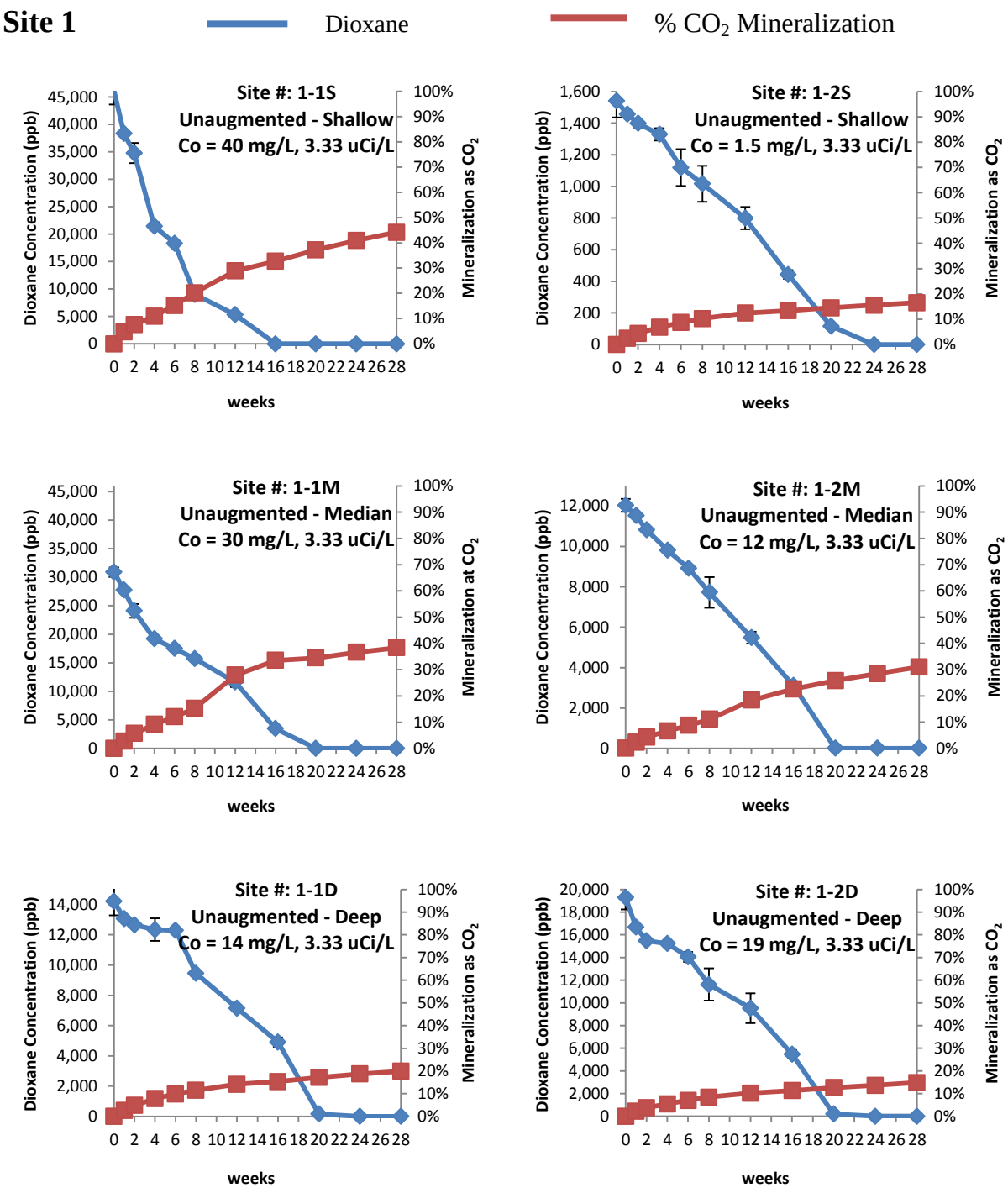

Figure 9a. Dioxane and CO2 mineralization mass over time for monitoring wells 1-1 and 1-2, at three depths at Site 1. Due to the stratigraphic nature of the aquifer, water and sediment samples were taken at three depths: shallow groundwater, sandy median, and deep groundwater. Quantitative depths were not provided with the samples. Microcosms were stored quiescently at 20°C and neutral pH.

Site 1 continued

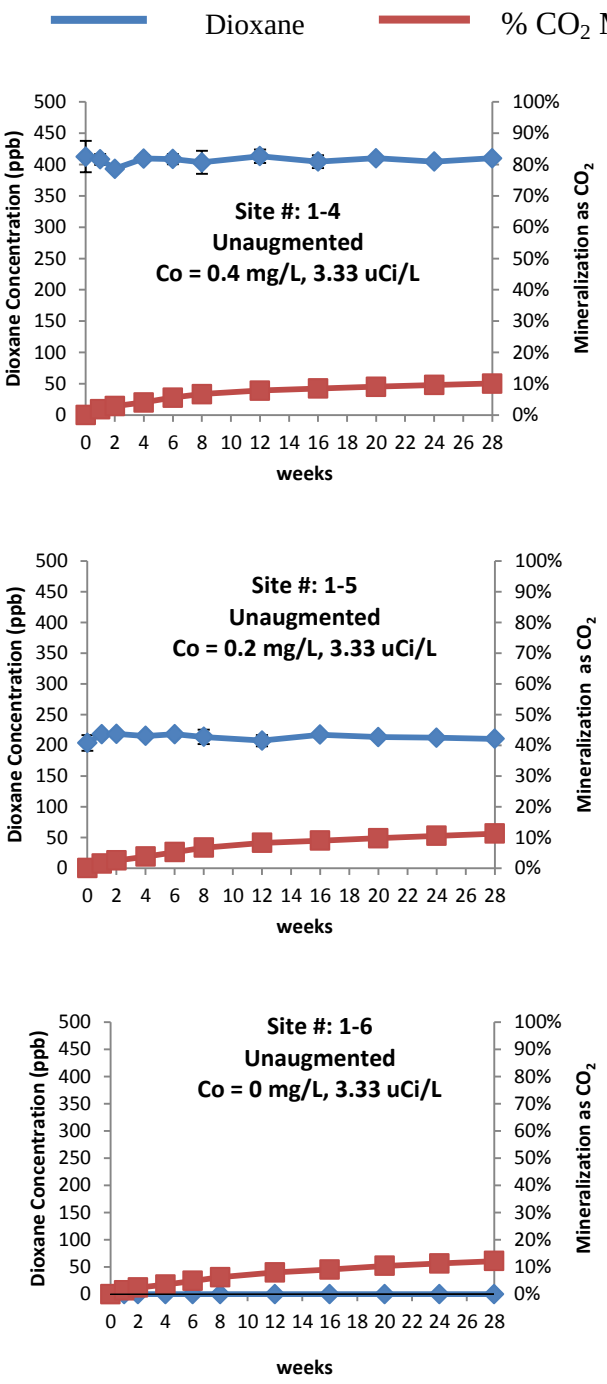

Figure 9b. Dioxane and CO_2 mineralization mass over time for monitoring wells 1-4, 1-5 and 1-6 for Site 1. Microcosms were stored quiescently at 20°C and neutral pH.

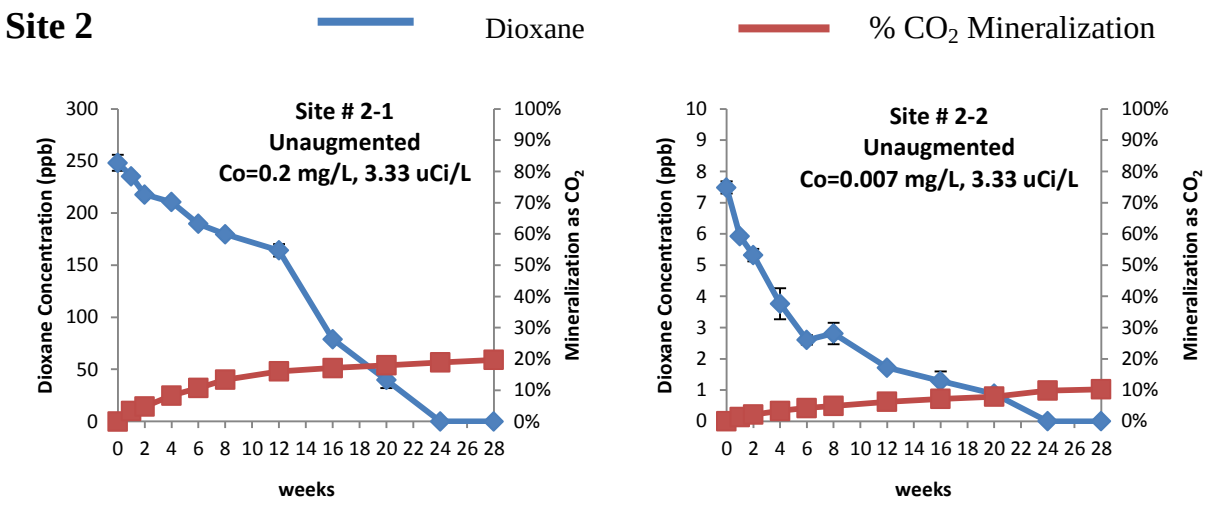

Figure 10. Dioxane and CO_2 mineralization mass over time for monitoring wells 2-1 and 2-2 for Site 2. Microcosms were stored quiescently at 20°C and neutral pH.

5.2. The Presence of Indigenous Dioxane Degraders is Further Suggested by ^{14}C Assimilation into Suspended Solids.

Sediment and biomass-associated ^{14}C was recovered by analysis of the suspended solids using a biological oxidizer with LC at the end of the experiment. Since dioxane has limited tendency to adsorb (Howard 2003), most of this radiolabel is presumed to be associated with biomass growth, and despite the fact that some biomass would have decayed during the 28-week incubation period, ^{14}C BO-LS data represent an additional line of evidence of the presence of indigenous dioxane degraders at these sites. Note that no biomass-associated ^{14}C

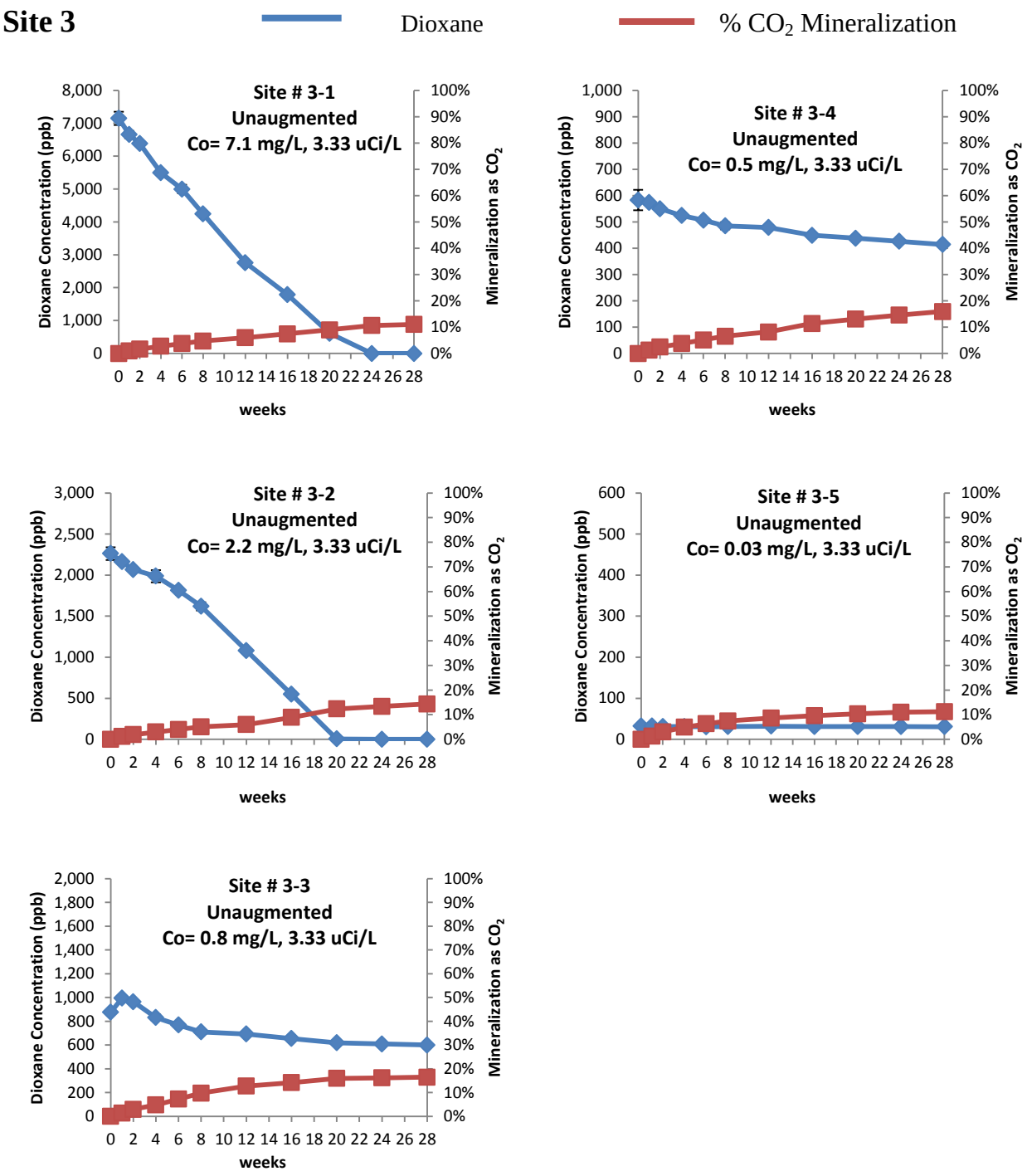

Figure 11. Dioxane and CO2 mineralization mass over time for monitoring wells 3-1, 3-2, 3-3, 3-4 and 3-5 for Site 3. Microcosms were stored quiescently at 20°C and neutral pH.

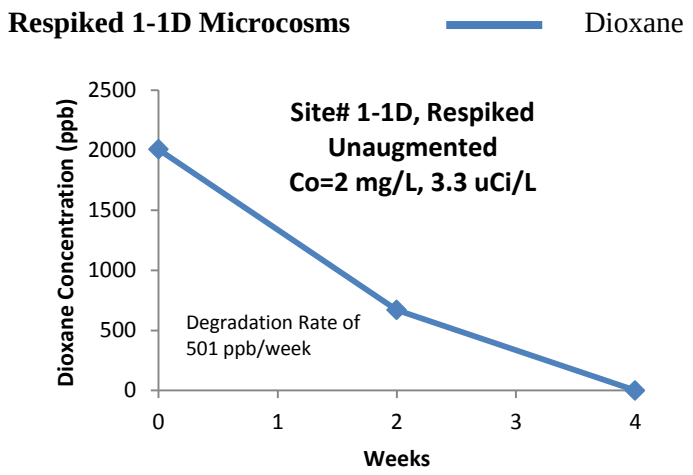

Figure 12. Dioxane loss over time for the respiked 1-1D triplicate microcosms.

Table 6. Mass Balances of Unaugmented Microcosms. Percentages are calculated as fraction of total ^{14}C (initially as radiolabeled dioxane). Carbon sinks categorized as: mineralization as $^{14}CO_2$, incorporation of ^{14}C into biomass, and remaining radiolabeled dioxane.

Treatment	Initial Concentration (ppb)	^{14}C Incorporated to Biomass	Mineralized as $^{14}CO_2$	Radiolabeled Dioxane	Total
1-1S	**46,000**	8%	44%	0%	**52%**
1-1M	**31,000**	7%	38%	0%	**46%**
1-1D	**14,000**	7%	20%	0%	**27%**
1-2S	**1,500**	3%	17%	0%	**19%**
1-2M	**12,000**	3%	31%	0%	**34%**
1-2D	**19,000**	7%	15%	0%	**22%**
1-4	**400**	0%	10%	88%	**98%**
1-5	**200**	0%	11%	89%	**100%**
1-6	**0**	0%	12%	92%	**104%**
2-1	**200**	7%	20%	0%	**27%**
2-2	**7**	2%	10%	0%	**12%**
3-1	**7,000**	7%	11%	0%	**18%**
3-2	**2,000**	5%	14%	0%	**19%**
3-3	**900**	4%	16%	85%	**102%**
3-4	**600**	3%	16%	29%	**49%**
3-5	**30**	0%	11%	90%	**101%**
1-1D Replicate	**46,000**	6%	53%	35%	**94%**

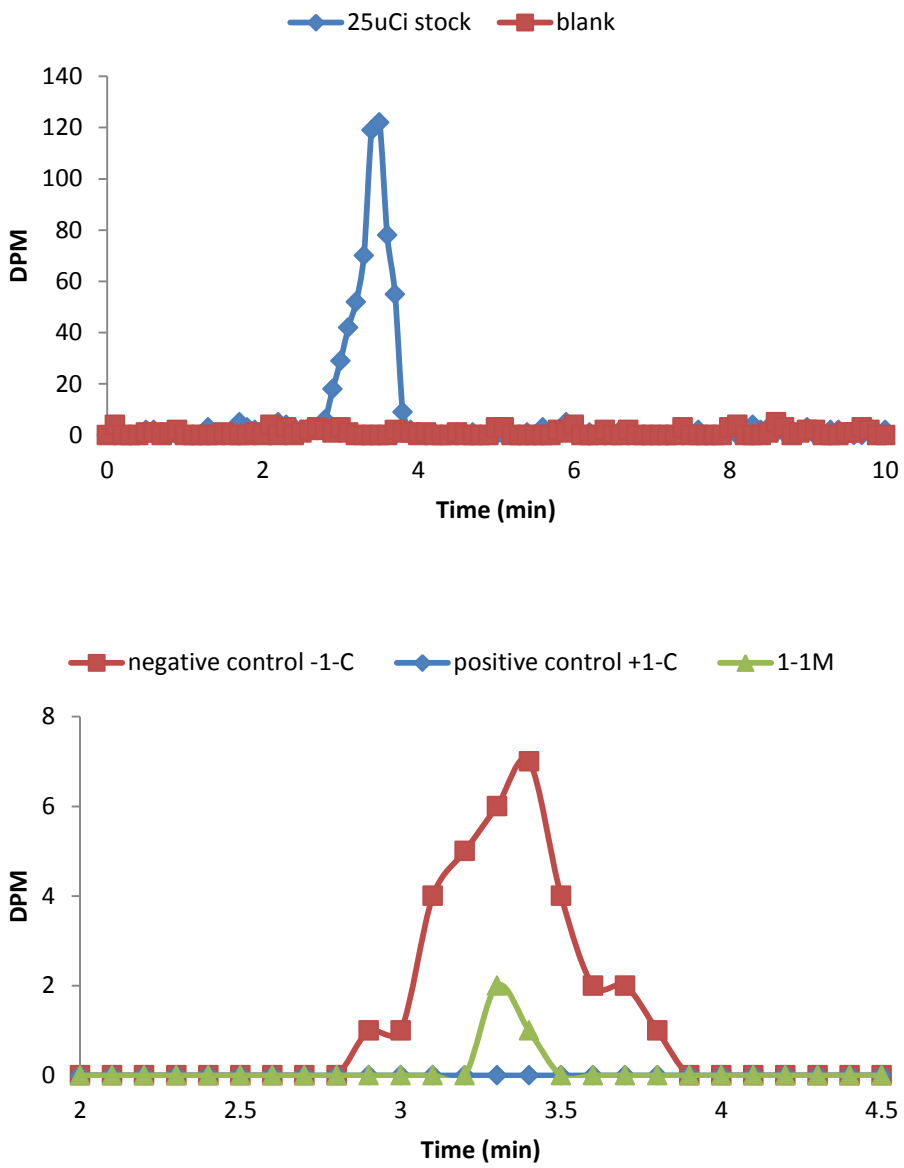

Figure 13. (Top) HPLC Radiochromatogram for ^{14}C labeled dioxane stock of 25 µCi/mL. The redline represents the baseline. The single peak indicates the initial purity. (Bottom) HPLC Radiochromatogram for filtered samples collected from microcosms for location 1-1 after 20 weeks' incubation, including treatment 1-1M, which experienced the fastest dioxane attenuation, as well as the corresponding positive and negative controls. The retention time for ^{14}C labeled dioxane is around 3.3 min. Background noise peaks were removed by subtracting the blank output.

was detected in microcosms experiencing no dioxane degradation (Table 7), which confirms limited adsorption and corroborates that most of the ^{14}C recovered by BO-LS was associated with biomass. Site 1 microcosms experienced between 3% and 8% biomass growth, determined by solids combustion at 12 months. Site 2 experienced a maximum of 7% and site 3 experienced a maximum of 7% (Table 6).

5.3. ^{14}C-Dioxane that Was Presumably Assimilated by Biomass Correlated with the Observed Biodegradation Activity.

The apparent biomass yield found in this study is slightly higher than that reported in a previously study that reported 5% of radiolabeled dioxane was incorporated into CB1190 biomass (Mahendra et al 2007). The difference between observed and reported biomass growth may be explained by metabolic differences; of CB1190 appears to be an r-strategist, capable of rapid and less-efficient nutrient uptake and storage (lower biomass yield). The native microbes in this study are likely oligotrophs, as discussed previously based on the zero- order degradation patterns that indicate saturated enzymes and thus high affinity (low Ks values) for dioxane. Accordingly, the indigenous bacteria would tend to degrade and store nutrients more efficiently, resulting in a higher total accumulation of ^{14}C. An alternative explanation is that some of the ^{14}C recovered by biological oxidation of suspended solids was associated with adsorbed dioxane or inorganic precipitates (e.g., $CaCO_3$).

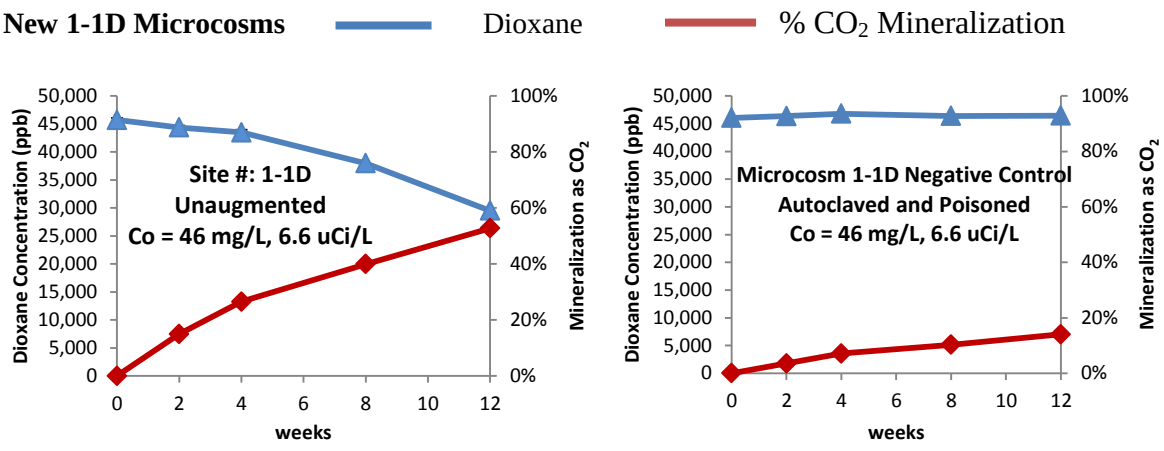

Figure 14. Dioxane and CO2 mineralization mass over time for the 1-1D replicates and control.

Table 7. Comparison of degradation rate to percent of ^{14}C incorporated into native microbe biomass.

Treatment	*Degradation Rate (ppb/week)*	*Incorporation of ^{14}C into Biomass*
1-1S	2267.5	8%
1-1M	1463.4	7%
1-1D	654.2	7%
1-2S	69.6	3%
1-2M	584.2	3%
1-2D	848.6	7%
1-4	0.15	0%
1-5	0.01	0%
1-6	0.01	0%
2-1	9.9	7%
2-2	0.3	2%
3-1	326.5	7%
3-2	112.1	5%
3-3	13.4	0%
3-4	5.8	4%
3-5	0.03	0%

However, this outcome is not expected given the mobility (K_{oc} = 1.23) and hydrophyllic nature (log K_{ow} = -0.27) of dioxane.

A significant (p < 0.05) correlation was observed between the degradation rates and the extent of ^{14}C accumulation in biomass (Figure 15), further supporting the notion that dioxane was metabolized by the indigenous microflora. Biomass growth was significantly higher in microcosms which exhibited a degradation rate higher than 5 ppb/week (Figure 15). Biomass assimilation was also significantly higher in microcosms prepared with samples from the source zone (Figure 16). Microcosms 1-1, 1-2, and 1-3 which contain source zone material, experienced the highest overall biomass assimilation at 7%-8% (Table 7 and 8).

5.4. Mineralization Activity Correlated to Degradation Rates

Biodegradation of dioxane was observed in six of the nine unaugmented Site 1 microcosm sets, as well as in the two Site 2 microcosm sets, and three of the five Site 3 unaugmented microcosm sets. This is confirmed by the absence of dioxane loss in the autoclaved negative controls and the simultaneous $^{14}CO_2$ recovery observed in the biologically active microcosms. With the exception of samples from well 3-3, the well samples demonstrating biodegradation are within approximately 200 ft of the dioxane source. The overall mineralization observed in the biologically active microcosms (positive controls and degrading) is significantly greater (p < 0.05) than that of the inactive microcosms (negative controls and those exhibiting no degradation) (Figure 17).

Table 8. List of microcosm degradation rate constants, initial concentrations, and sample distances from source.

Treatment	*Rate (ppb/week)*	*Distance from Source Zone (ft)*	*Initial Concentration (mg/L)*
1-1S	2267.5 ± 53.2	0	40
1-1M	1463.4 ± 19.4	0	30
1-1D	654.2 ± 12.6	0	14
1-2S	69.6 ± 2.1	200	1.5
1-2M	584.2 ± 18.3	200	12
1-2D	848.6 ± 11.9	200	19
1-4	0.15 ± 0.02	1640	0.4
1-5	0.01 0.001	3200	0.2
1-6	0.01 ± 0.0004	-	0
2-1	9.9 ± 0.6	0	0.2
2-2	0.3 ± 0.006	-	0.007
3-1	326.5 ± 12.5	0	7.1
3-2	112.1 ± 8.2	200	2.2
3-3	13.4 ± 0.1	1000	0.8
3-4	5.8 ± 0.09	1350	0.5
3-5	0.03 ± 0.0002	-	0.03

Degradation Rates	
Negative Control	Rate (ppb/week)
Site 1	-5.1 ± 0.03
Site 2	+0.02 ± 0.0006
Site 3	-6.9 ± 0.01

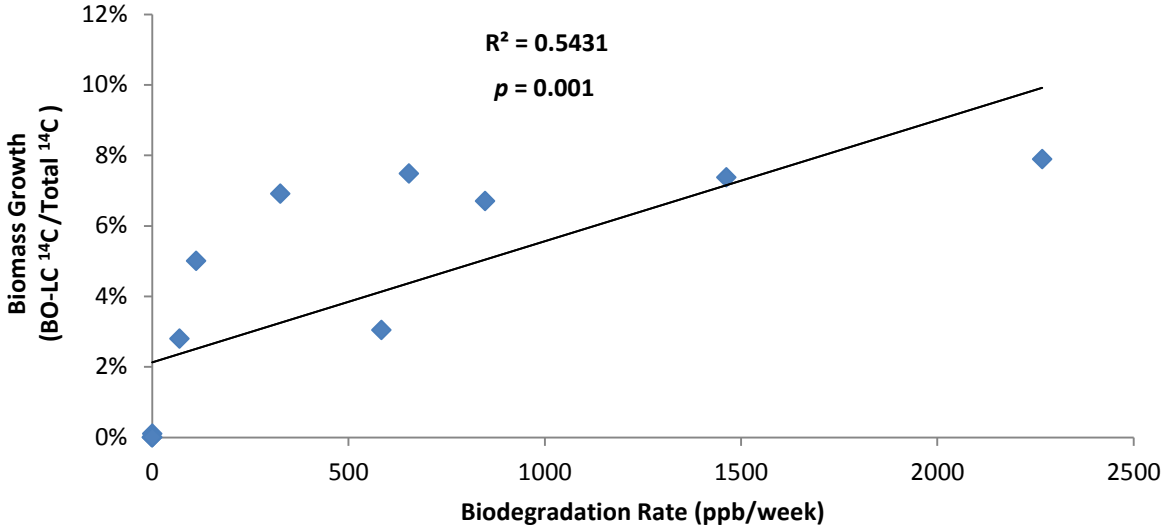

Figure 15. Correlation between ^{14}C incorporation into biomass and biodegradation rate.

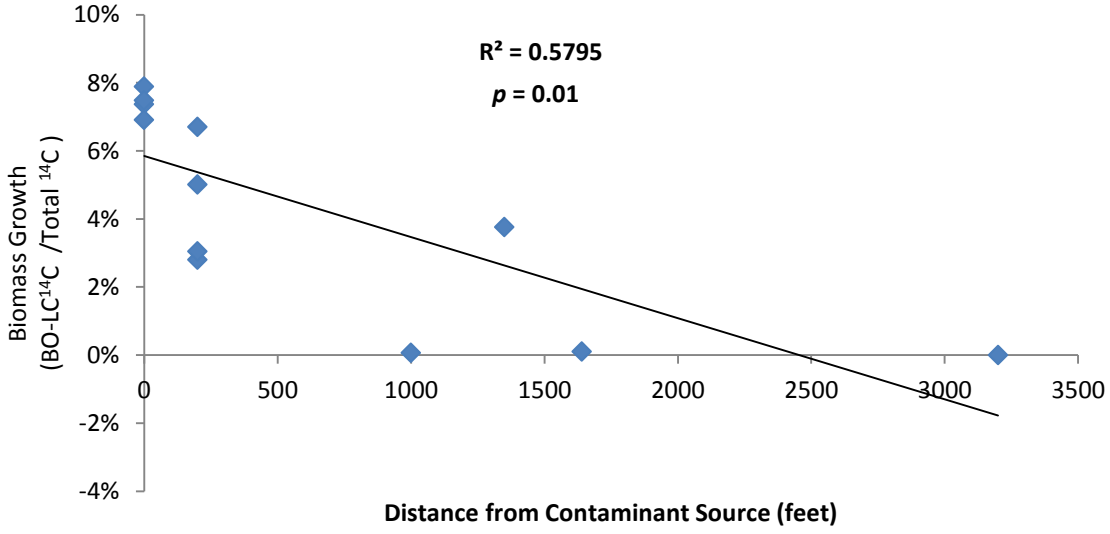

Figure 16. Inverse correlation between ^{14}C incorporation into biomass and proximity to source zone.

5.5. Degradation Rates Were Significantly Higher in Source-Zone Samples, Possibly Reflecting Higher Acclimation

A relationship exists ($p < 0.08$) between the degradation rates and distance from the source zone from which microcosm constituents were collected (Figure 18). For example, microcosm sets 1-4 and 1-5 exhibit no degradation (0.15 ± 0.02 and 0.01 ± 0.001 ppb/week) at the same site where the highest degradation, 1-1S (2267.5 ±53.2 ppb/week), was observed. Additionally, microcosms 2-1 and 2-2 exhibited rates of 9.9 ± 0.6 and 0.3 ± 0.006 ppb/week, respectively, while 3-4 and 3-5 exhibited rates of 5.8 ± 0.09 and 0.03 ±<0.005 ppb/week, respectively. The initial concentrations for these three sets are similar (Table 8) but the degradation rates are higher for site 2. It is possible that shorter distance from the source is associated with longer exposure time. It has been previously reported that exposure to high concentration actually results in a longer acclimation time due to toxicity whereas long exposure time results in higher acclimation and more rapid metabolic response over time (Chong et al, 2012).

The degradation rates observed in this study are comparable to *in situ* degradation rates of other relatively recalcitrant organic contaminants found in groundwater. Prior MNA studies have reported a wide range of aerobic degradation rates in water for common contaminants such as DCA, DCA, vinyl chloride and CCl_4. DCA has been reported to degrade at 7021 ppb/week while DCE has been reported to degrade between 2.9 and 7022 ppb/week (Nobre and Nobre, 2004; Broholm et al, 2005; Clement et al 2000). CCl_4 has been reported to degrade at 7.5 ppb/week

(Devlin et al, 2004) and vinyl chloride has been reported to degrade between 11.3 ppb/week and 7008 ppb/week (Broholm et al, 2005; Clement et al, 2000). These previous studies that concluded MNA would be a feasible component to remedial response base on the degradation rates observed for the contaminant of concern. Accordingly, the comparable degradation rates observed in this microcosm study suggest that MNA may also be a feasible component of the remedial response at these sites. However, before MNA is selected, further studies to validate these results with in situ observations and ensure that migration rates do not exceed degradation rates (e.g., assess plume stability) are recommended.

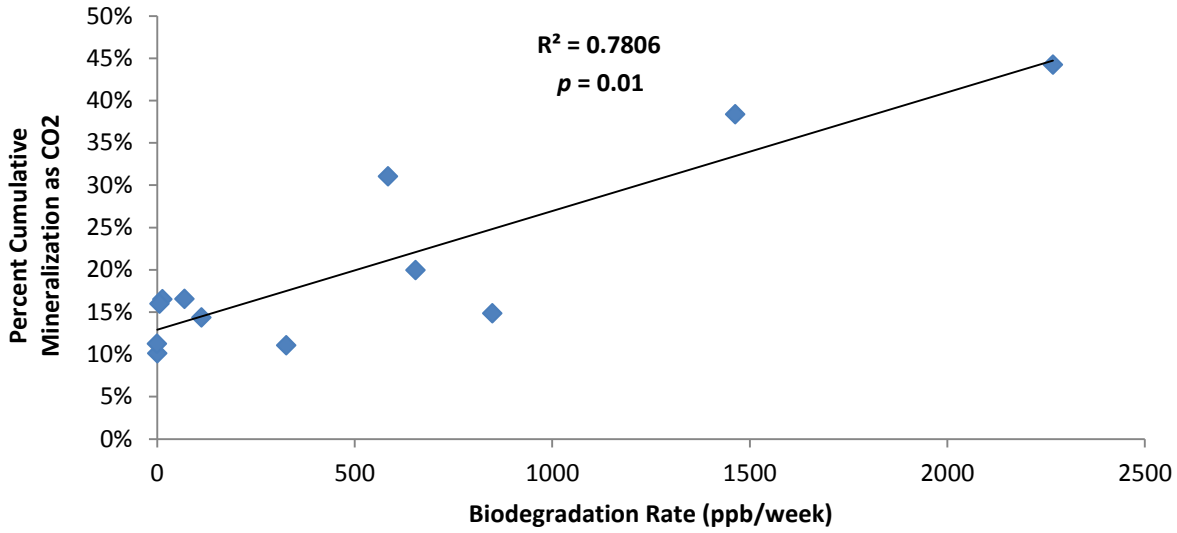

Figure 17. Correlation between biodegradation rate and cumulative mineralization.

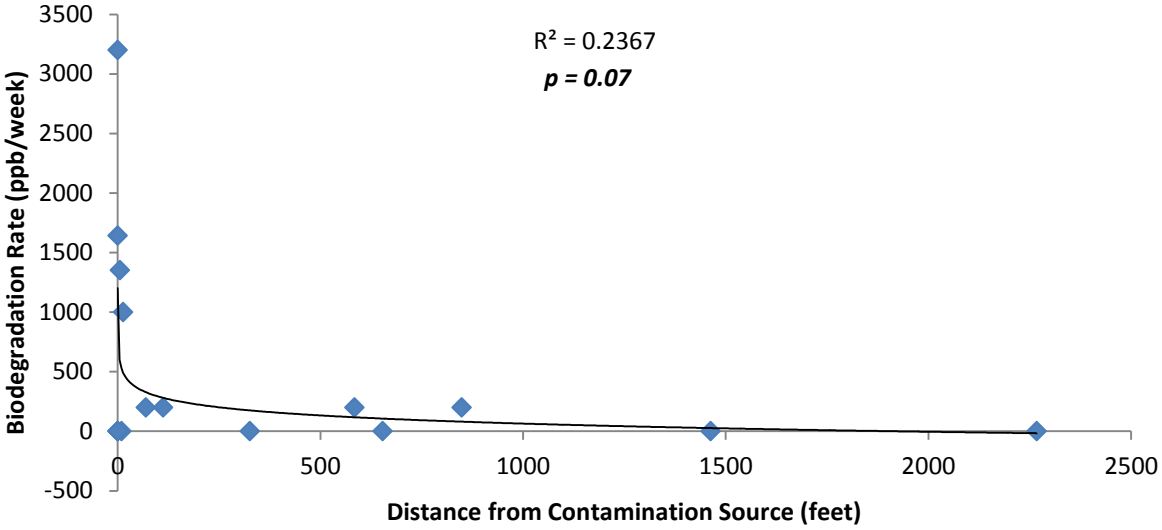

Figure 18. Exponential correlation between degradation rate and proximity to source zone

Chapter 6

Conclusions

This study is one of few to unequivocally demonstrate natural attenuation of 1,4 dioxane in groundwater microcosms. Three independent lines of evidence indicate dioxane utilization as a sole carbon source: (1) dioxane removal in biologically active microcosms but not in sterile controls; (2) ^{14}C dioxane was oxidized to $^{14}CO_2$ and a higher extent of mineralization was observed for (presumably more acclimated) source-zone samples exhibiting higher degradation activity; and (3) ^{14}C was incorporated into biomass only in microcosms where dioxane was biodegraded. Replication of a microcosm set from site 1 confirmed relatively rapid dioxane degradation (i.e., 100% removal by 20 weeks).

The presence and expression of indigenous dioxane degradation capabilities suggests that MNA may be a feasible component of the remedial response at these sites. Prior to fully recommending MNA, further characterization of plume dynamics (e.g., ensure it is stable or receding) and quantification of migration rates in relation

to biodegradation rates is recommended. This is necessary to ensure the plume migration and dilution rates do not exceed degradation rates.

Future research on dioxane natural attenuation should investigate the usefulness of chemical markers of dioxane degraders in groundwater, particularly known degradation intermediates such as HEAA (Mahendra et al 2007). As a terminal intermediate of the dioxane degradation pathway, HEAA might be present in higher concentration where degradation is taking place. Preliminary HEAA analysis only takes days, whereas a microcosm study can take a year to complete. This alternative would save time in obtaining negative results by indicating substantial degradation is not taking place and a microcosm study is not necessary. For sites where HEAA concentrations suggest degradation is taking place, a microcosm study would still be necessary to characterize the degradation. Ultimately, remedial planning could be more deliberately directed and cost effective by utilizing a biomarker method of assessing natural attenuation potential.

Another approach to reliably and quickly demonstrate the presence and activity of dioxane degraders is to develop gene probes that target conserved regions in functional genes (e.g., the active site of soluble di-iron dioxygenase genes, ref). Such genetic biomarkers should be enriched enriched in dioxane plumes undergoing degradation (due to proliferation of dioxane degraders) relative to background samples, and biomarker concentrations could be correlated to biodegradation activity. Ultimately, remedial planning could be more deliberately

directed and cost effective by utilizing selective biomarker analysis to assess natural attenuation potential.

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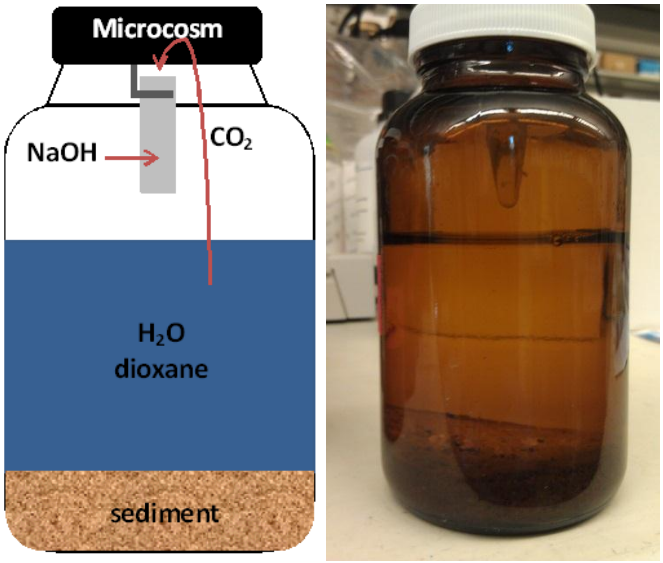
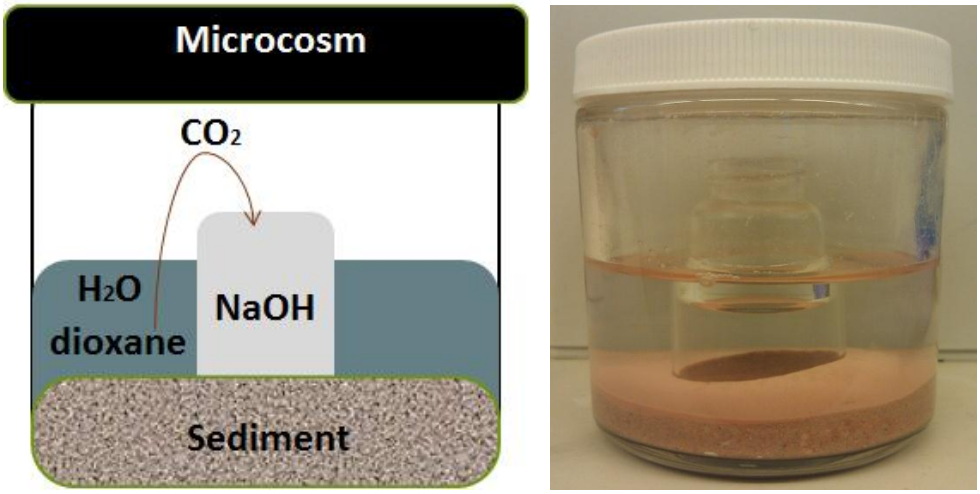
Appendix A

Microcosm Design #1: Microcosm is a 200mL amber bottle with a Teflon seal in the cap. The NaOH trap is a 1.7 mL Phenix ultraclear microtube attached by an epoxied keck clip inside the microcosm cap. This microcosm did not yield a good mass balance and leaked CO_2. A solution was attempted by 1) wrapping tape around the screw-top threading to tighten the seal 2) wrapping Teflon tape around the cap itself to contain the gas. Neither of these solutions were successful.



Appendix A cont'd

Microcosm Design #2: The microcosm is a 150mL clear bottle with a PTFE membrane seal in the cap. The NaOH trap is a 20 mL glass epoxied to the bottom of the jar to prevent floating and tipping. This microcosm yielding 90% mass recovery after a four day incubation containing radiolabelled glucose. This design was selected as the design for the replicate 1-1D study.



Appendix B

Appendix B

Calibration 1

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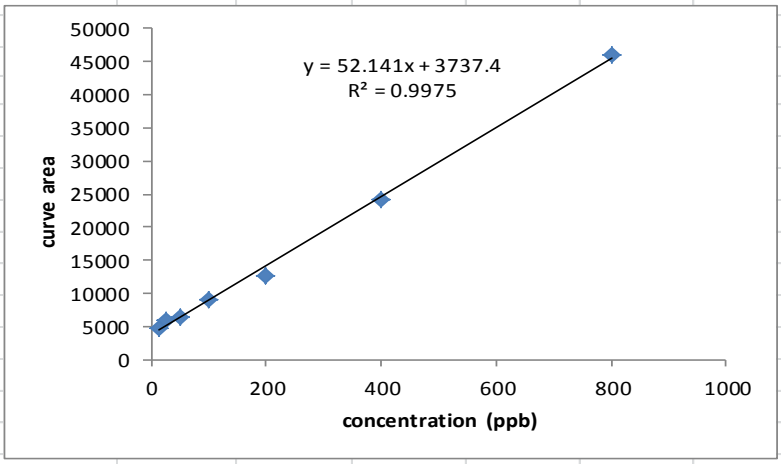
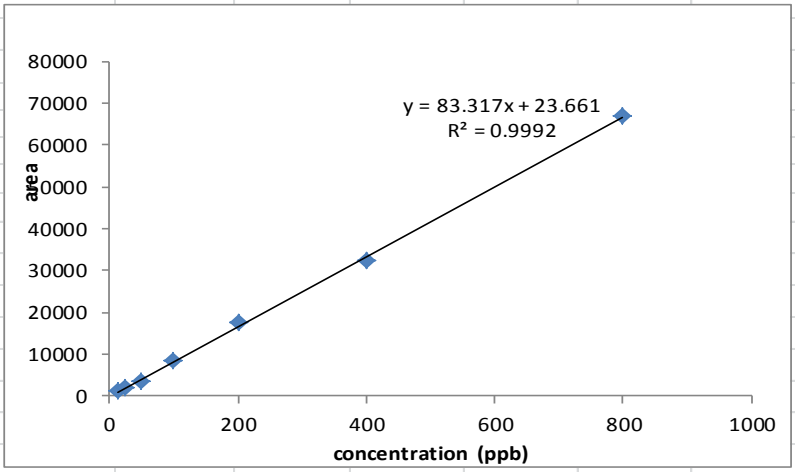
25	5897
50	6331
100	9123
200	12709
400	24211
800	45953



Calibration 2

standard	Diox
0	0
12.5	1376
25	2018
50	3669
100	8315
200	17782
400	32314
800	66957



Calibration 3

standard	Diox
0	0
12.5	7238
25	14081
50	18628
100	41655
200	97406
400	174323
800	338922