

FINAL REPORT

In Situ Biodegradation of 1,4-Dioxane: Effects of Metals and Chlorinated Solvent Co-Contaminants

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LIST OF ACRONYMS AND ABBREVIATIONS

ALDH	Alcohol Dehydrogenase
<i>aldH</i>	Alcohol dehydrogenase gene
AMS	Ammonium Mineral Salts
ATP	Adenosine Triphosphate
BDL	Below Detection Limit
BG	Background
<i>cop</i>	Copper resistance genes
<i>chr</i>	Chromate resistance genes
cDCE	<i>cis</i> -1,2-Dichloroethene
CB1190	<i>Pseudonocardia dioxanivorans</i> CB1190
Cr(VI)	Hexavalent Chromium
Cu(II)	Divalent (Cupric) Copper
DCE	1,1-Dichloroethene
DNA	Deoxyribonucleic Acid
DoD	Department of Defense
DOE	Department of Energy
<i>dxmB</i>	Dioxane monooxygenase subunit B gene
DXMO	Dioxane Monooxygenase
ERD	Enhanced Reductive Dechlorination
ESTCP	Environmental Security Technology Certification Program
FA	Fulvic Acids
GAC	Granular Activated Carbon
GC-FID	Gas Chromatography-Flame Ionization Detector
GC-ECD	Gas Chromatography-Electron Capture Detector
GC-MS	Gas Chromatography-Mass Spectrometry
HA	Humic Acids
ICP-OES	Inductively Coupled Plasma Optical Emission Spectrometry
JOB5	<i>Mycobacterium austroafricanum</i> (formerly <i>vaccae</i>) JOB5
MNA	Monitored Natural Attenuation
NMS	Nitrate Mineral Salts
NOM	Natural Organic Matter
ORNL	Oak Ridge National Laboratory
ORP	Oxidation Reduction Potential
PCoA	Principal Coordinates Analysis
PCR	Polymerase Chain Reaction
PI	Principal Investigator
qPCR	Quantitative Polymerase Chain Reaction
rDNA	Ribosomal Deoxyribonucleic Acid
<i>rpoD</i>	RNA Polymerase subunit D housekeeping sigma factor
rRNA	Ribosomal Ribonucleic Acid
RNA	Ribonucleic Acid
SERDP	Strategic Environmental Research and Development Program
SIP	Stable Isotope Probe

sMMO	Soluble Methane Monooxygenase
SON	Statement of Need
SSA	Species-Species Associations
TCA	1,1,1-Trichloroethane
TCE	Trichloroethylene or trichloroethene
THF	Tetrahydrofuran
UCLA	University of California, Los Angeles

KEYWORDS

1,4-Dioxane, dioxane, solvent, stabilizer, chlorinated, trichloroethane, trichloroethene, trichloroethylene, dichloroethene, dichloroethylene, metal, trace, heavy, copper, chromium, hexavalent, iron, inhibition, toxic, bacteria, microbe, enzyme, monooxygenase, aerobic, propane, cometabolism, degradation, biodegradation, biotransformation, bioremediation, natural attenuation, biostimulation, bioaugmentation, biofilm, extracellular polymeric substances, exopolymeric substances, quartz, activated carbon, zeolite, column, soil, sediment, sorption, adsorption, desorption, groundwater.

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ABSTRACT

Objectives and Rationale

1,4-Dioxane is a possible human carcinogen and a contaminant of emerging concern in groundwater at many military and industrial contaminated sites. We hypothesized that the inhibitory effects of co-contaminants on microbes might be responsible for limited in situ 1,4-dioxane biodegradation in contaminated environments. Additionally, while biodegradation by planktonic cultures is well established, the biodegradation capabilities of biofilms, a preferred growth mode in much of the natural and built environment, are poorly understood. This research project evaluated the suspected limiting effects of co-contaminants and their remedial technologies on 1,4-dioxane biodegradation through the following technical objectives:

1. To characterize the effects of chlorinated solvents trichloroethene (TCE) and 1,1,1-trichloroethane (TCA) and their degradation products on 1,4-dioxane biodegradation;
2. To determine the effects of metals on 1,4-dioxane biodegradation in bacterial pure cultures;
3. To examine the ability of 1,4-dioxane to be absorbed by abiotic adsorbents and be biodegraded by bioaugmented adsorbents;
4. To evaluate co-contaminant effects on biodegradation of 1,4-dioxane by bacterial biofilms;
5. To assess the effects of enhanced reductive dechlorination (ERD) on 1,4-dioxane biodegradation in columns constructed with media from an impacted site, with and without select co-contaminants; and
6. To analyze microbial composition and dynamic changes during the chemical oxidation and biological processes.

Technical Approach

1,4-Dioxane biodegradation was investigated in the presence of chlorinated solvents or metals, which are known to co-exist with 1,4-dioxane at many contaminated DoD sites. Differences in degradation kinetics (rates and affinity constants) provided insights into potentially synergistic or competitive interactions between chlorinated solvents, metal ions, and 1,4-dioxane. The biodegradation potential of metabolic and cometabolic 1,4-dioxane-degrading biofilms in batch and column reactors was examined and compared with planktonic cultures. Subsequently, 1,4-dioxane biodegradation by biofilms was evaluated under a variety of flow regimes for various co-contaminant mixtures. Column studies were expanded to evaluate the impact of co-contaminants and ERD treatment on 1,4-dioxane biodegradation in environmental samples. Changes in gene abundance and expression indicated mechanisms of toxicity, inhibition, or regulation associated with exposure to the co-contaminants. In addition, microbial community analysis was conducted to examine composition and dynamics changes during a treatment train consisting of chemical oxidation by hydrogen peroxide followed by biological treatment.

Results

Rates of 1,4-dioxane metabolism by *Pseudonocardia dioxanivorans* CB1190 and cometabolism by propanotroph *Mycobacterium austroafricanum* JOB5 are sensitive to chlorinated solvent (with VC being the greatest inhibitor) and trace metal (with Cu(II) being the greatest inhibitor) co-contaminants. While chlorinated solvents negatively impacted 1,4-dioxane biodegradation, CB1190 was found to be capable of biodegrading both VC and cDCE concurrently with 1,4-dioxane. Biofilms were able to resist chlorinated solvent and metal inhibition to greater extents than planktonic cultures. GAC and zeolite were both found to be capable of adsorbing 1,4-

dioxane, and, when used as a growth support for biofilms, helped mitigate chlorinated solvent inhibition. Lastly, chemical oxidative/reductive remedial technologies commonly applied to chlorinated solvent plumes were found to have minor effects on subsequent 1,4-dioxane biodegradation due to the resilience of the microbial community.

Benefits and SERDP Relevance

Our research project responded to the Statement of Need ERSON-13-01: In Situ Remediation of 1,4-Dioxane-Contaminated Groundwater. This research directly supports SERDP's mission to reduce the DoD's liabilities by developing sustainable, cost-effective technologies for expedited site cleanup and closure by *in situ* remediation of contamination in soil, sediments, and water. Specifically, this project determined the effects of typical co-contaminants and their common treatment technologies on 1,4-dioxane bioremediation. The identification of pure or mixed cultures as well as biogeochemical conditions, which generate desirable enzyme activities in multiple organic-contaminated, metal-stressed environments, will guide the design and optimization of bioaugmentation and biostimulation efforts thereby developing the optimum long-term remedial strategies for 1,4-dioxane. These results will also provide fundamental understanding as well as quantitative tools for natural and enhanced bioremediation of 1,4-dioxane-contaminated soil and groundwater.

EXECUTIVE SUMMARY

Introduction

1,4-Dioxane is a possible human carcinogen that can distribute rapidly in the lungs, kidneys, spleen, liver, colon, and skeletal muscle tissue (Stickney, 2003). It is often found as a by-product in consumables (e.g. paint strippers, antifreeze fluids, dyes, greases, cosmetics, deodorants, solvents, and shampoos) and is most widely known as a solvent stabilizer for 1,1,1-trichloroethane (TCA), a common metal degreaser and dry-cleaning solvent (Mohr et al., 2010). 1,4-Dioxane is readily miscible in water and found widespread in ground water and surface water across the United States (Adamson et al., 2014; Sun et al., 2016). Currently, there is no federal regulation for 1,4-dioxane, but many states have set recommended limits for drinking water that range from 0.25 µg/L (New Hampshire) and to 77 µg/L (Alaska) (Suthersan et al., 2016). Biodegradation of 1,4-dioxane has been reported in several laboratory-based and field-based studies (Zhang et al., 2017). However, its persistence in the environment remains quite high, and is often found comingled with TCA, trichloroethene (TCE), and their degradation products, such as 1,1-dichloroethene, cis-1,2-dichloroethene, vinyl chloride as well as trace metals (Adamson et al., 2015; Adamson et al., 2014; Anderson et al., 2012). There is a critical need to understand microbial populations and processes in response to changing biogeochemistry and contaminant concentrations, and to provide support for the optimal remediation strategies for comingled contaminant plumes.

Objectives

The specific technical objectives of this project were:

1. *To characterize the effects of chlorinated solvents such as TCE, TCA, and their degradation products on 1,4-dioxane biodegradation rates.* Due to the close association of these two prevalent groundwater contaminants, it is important to understand the inhibitory effects they can have on 1,4-dioxane degrading bacteria.
2. *To determine the effects of metal ions on 1,4-dioxane biodegradation by bacterial pure cultures amended into column and batch reactors.* Because many bacteria are sensitive to levels of heavy metals that may co-occur with 1,4-dioxane, it is essential to understand how they impact biodegradation so that bioremediation strategies may be effectively employed.
3. *To test the ability of bacterial biofilms to degrade 1,4-dioxane in batch and flow-through column reactors.* Even though attached-growth tends to be the preferred mode of growth in the natural and built environment, the majority of 1,4-dioxane biodegradation experiments have been conducted using planktonic bacteria. Understanding the differences in biotransformation between planktonic and attached-growth bacteria is critical for accurately understanding 1,4-dioxane biodegradation kinetics in natural and engineered systems.
4. *To evaluate co-contaminant effects on biodegradation of 1,4-dioxane by bacterial biofilms.* The inhibition constants of chlorinated solvents on 1,4-dioxane degrading bacteria in columns are expected to be different from those obtained from planktonic culture experiments. A comprehensive understanding of these interactions will yield accurate and representative 1,4-dioxane biodegradation rates in batch and flow-through systems.
5. *To assess the effects of chlorinated solvent remediation technologies on 1,4-dioxane biodegradation in columns constructed with media from an impacted site.* The co-occurrence of chlorinated solvents and 1,4-dioxane has been widely characterized (Adamson et al., 2014;

Anderson et al., 2012) and as the concern for 1,4-dioxane rises, 1,4-dioxane treatment approaches must consider the impact of previous or ongoing chlorinated solvent treatment activities. The results of this study will determine if 1,4-dioxane biodegradation can be implemented following enhanced reductive or oxidative treatment operations.

6. *To analyze the microbial compositions and dynamic changes during the chemical oxidation and biological processes.* We will test the hypothesis that the oxidants deplete co-contaminants and provide oxygen to stimulate biodegradation of 1,4-dioxane, as well as influence the microbial ecology. Microbial community analysis in samples collected from treatment processes will help identify key microbes and functional genes for successful 1,4-dioxane biodegradation in the presence of co-contaminants, and track dynamic trends responding to co-contaminants, as well as evaluate post-treatment microbial ecology.

Technical Approach

1,4-Dioxane biodegradation to support the growth of *Pseudonocardia dioxanivorans* CB1190 as well as propane-induced cometabolism by *Mycobacterium austroafricanum* JOB5 was investigated in the presence of chlorinated solvents or metals, which are known to co-exist with 1,4-dioxane at many contaminated DoD sites. Differences in degradation kinetics (rates and affinity constants) provided insights into potentially synergistic or competitive interactions among chlorinated solvents, metal ions, and 1,4-dioxane. Chlorinated solvent concentrations ranged from 0.05-100 mg/L in the aqueous phase and the metals investigated were cadmium(II), copper(II), iron(II), nickel(II), zinc(II) and chromium(VI). Two adsorbent types, carbon (Norit 1240) and the pentasil zeolite ZSM-5, were also tested in abiotic and bacterially-bioaugmented modes as a treatment strategy for 1,4-dioxane with and without chlorinated solvents.

Biofilms were established on solid media in batch as well as column systems to test the abilities of metabolic and cometabolic 1,4-dioxane-degrading microbes to degrade 1,4-dioxane. and the results were compared to planktonic cultures. 1,4-Dioxane biodegradation in columns was determined under a variety of flow regimes using contaminant mixtures relevant to contaminated aquifer environments.

Column studies were expanded to evaluate the impact of co-contaminants and enhanced reductive dechlorination (ERD) treatment on 1,4-dioxane biodegradation in environmental samples. Four column conditions were tested: 1) untreated soil with oxic influent, 2) untreated soil with anoxic influent, 3) soil bioaugmented with CB1190 under oxic conditions, and 4) sterilized soil with oxic influent.

A subset of the column samples was analyzed for changes in 1,4-dioxane biodegradation potential using biomarkers for the 16S rRNA and genes coding for 1,4-dioxane-degrading enzymes (dioxane monooxygenase, aldehyde dehydrogenase, and propane monooxygenase). Changes in gene abundance and expression determined using quantitative polymerase chain reaction (qPCR) would indicate mechanisms of toxicity, inhibition, or regulation associated with exposure to the co-contaminants.

Microbial community analyses were used to identify relationships among the microbial compositions, contaminant removals, and process-specific features. Specifically, treatment process that combined oxidation and biodegradation were tested. Five sets of microcosms were established, which included 1) high 1,4-dioxane high CVOCs, 2) high 1,4-dioxane low CVOCs, 3) low 1,4-dioxane low CVOCs, and 4) low 1,4-dioxane-only, and bioaugmentation under high 1,4-dioxane low CVOCs. Illumina Miseq platform was used to sequencing 16S rRNA gene, coupling with bioinformatic and statistical tools, to explore the influence of key variables and biological

factors that shape overall microbial community assembly and functions.

Results and Discussion

Planktonic CB1190 cells were able to biodegrade 1,4-dioxane in the presence of chlorinated solvents at low concentrations. Metabolic biodegradation of 100 mg/L 1,4-dioxane was not inhibited by concentrations up to 3 mg/L TCE and 30 mg/L TCA (Zhang et al., 2016). However, the degradation byproducts of those chlorinated solvents, VC, 1,1-DCE, and cDCE produced the greatest inhibition to metabolic biodegradation by CB1190 (Table 1).

Table 1. Effects of various chlorinated solvents on the 1,4-dioxane biodegradation capability of *Pseudonocardia dioxanivorans* CB1190.

Chlorinated Solvent	Initial CVOC Level	Initial 1,4-Dioxane ^a Biodegradation Rate ^{b,c,d}	1,4-Dioxane ^a Pseudo First Order Rate Constant ^{d,e}	Normalized 1,4-Dioxane ^a Degradation ^{c,d} (t=10 h)
	μg/L	μmol/gene copy·h	L/gene copy·h	μmol/gene copy
TCA	5,000	(5.4±0.95) x 10 ⁻¹²	(1.6±0.39) x 10 ⁻¹²	(2.8±0.32) x 10 ⁻¹¹
TCE	5,000	(6.6±0.59) x 10 ⁻¹²	(1.5±0.23) x 10 ⁻¹²	(3.7±0.25) x 10 ⁻¹¹
cDCE	5,000	(5.6±0.24) x 10 ⁻¹²	(8.4±0.44) x 10 ⁻¹³	(2.3±0.30) x 10 ⁻¹¹
1,1-DCE	5,000	(3.3±1.5) x 10 ⁻¹²	(4.1±1.6) x 10 ⁻¹³	(1.6±7.1) x 10 ⁻¹²
VC	4,200	(6.1±1.5) x 10 ⁻¹³	(1.0±0.25) x 10 ⁻¹³	(6.1±1.5) x 10 ⁻¹²

^a1,4-Dioxane levels were initially 1000 μg/L.

^bRates determined by linear regression of first 3 time points.

^cError is the standard deviation of triplicate experimental reactors.

^dNormalization was done using initial biomass as universal 16s rRNA gene abundance.

^eFirst order rates were calculated by linear regression of -ln(C_t/C₀) for the first 3 time points.

Inhibitory effects of heavy metals on 1,4-dioxane metabolic biodegradation were measured to be in the following order: Cu(II) > Cd(II) ≥ Ni(II) > Zn(II) (Pornwongthong et al., 2014). Inhibitory effects of heavy metals on 1,4-dioxane cometabolic biodegradation were measured to be in the following order: Cu(II) > Cd(II) ≥ Ni(II) > Fe(II). 1,4-Dioxane biodegradation in cultures exposed to 2 mg/L Cu(II) was restored in the presence of 0.005, 0.05, and 0.5 mM alginin, 0.05, and 0.5 mM cysteine, and 0.005 mM tannin. These results indicated that specific ligands bind with transition metals and alleviate bacterial toxicity. Thus, monooxygenase-catalyzed biodegradation rates by 1,4-dioxane metabolizing or cometabolizing bacteria were subject to interactions among transition metals and natural organic ligands in the environment. Our results also suggest that bioaugmentation strategies for 1,4-dioxane are suitable for environments that contain Cr(VI) at levels as high as 100 mg/L.

Initial studies with abiotic sorbents, granular activated carbon (GAC) and zeolite, demonstrated that while 1,4-dioxane can be sorbed, the process is easily reversible (Figure 1). Because microbes can irreversibly biodegrade 1,4-dioxane, we found that by bioaugmented adsorbents, the effective capacity of the adsorbent could be extended (Figure 2). As the microbes on the surface of the adsorbent biodegrade 1,4-dioxane in bulk solution, a concentration gradient is formed that causes 1,4-dioxane to desorb from the adsorbent, thus becoming available for biodegradation. In general, the inhibition due to chlorinated solvents and metals was diminished when 1,4-dioxane-biodegrading bacteria were established as biofilms. This can primarily be explained by the nature of the extracellular polymeric substance (EPS) matrix of the biofilms that

reduce penetration of antimicrobial substances (such as chlorinated solvents and metals). In addition to the protection that the biofilm growth mode provides to cells, the adsorbents themselves protect the cells by adsorbing the inhibitory chlorinated solvents (Figure 3). The only instance where the solvents proved completely inhibitory for bioaugmented adsorbents was in the case of bio-zeolite for any mixture including 1,1-DCE. Single solute isotherms show that the order of maximum adsorption capacity for the zeolite was $\text{TCE} > \text{cDCE} > 1,1\text{-DCE} = 1,4\text{-dioxane}$, suggesting that the 1,4-dioxane adsorbed during the bioaugmentation of the zeolite would impede adsorption of 1,1-DCE. This competitive adsorption phenomenon was not observed for bio-GAC, likely due to GACs typically higher affinities for higher molecular weight and more non-polar compounds (Urano et al., 1991).

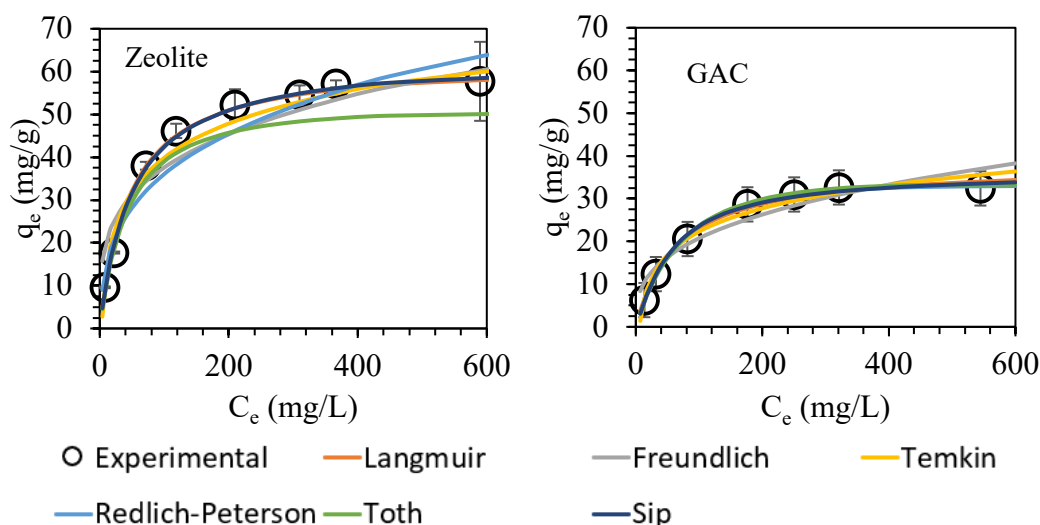


Figure 1. ZSM-5 zeolite and Norit 1240 GAC isotherms conducted at 30°C. Zeolite and GAC were found to have a maximum adsorption capacity of 60 and 34 mg/g, respectively. Error bars represent the standard deviation of triplicate reactors.

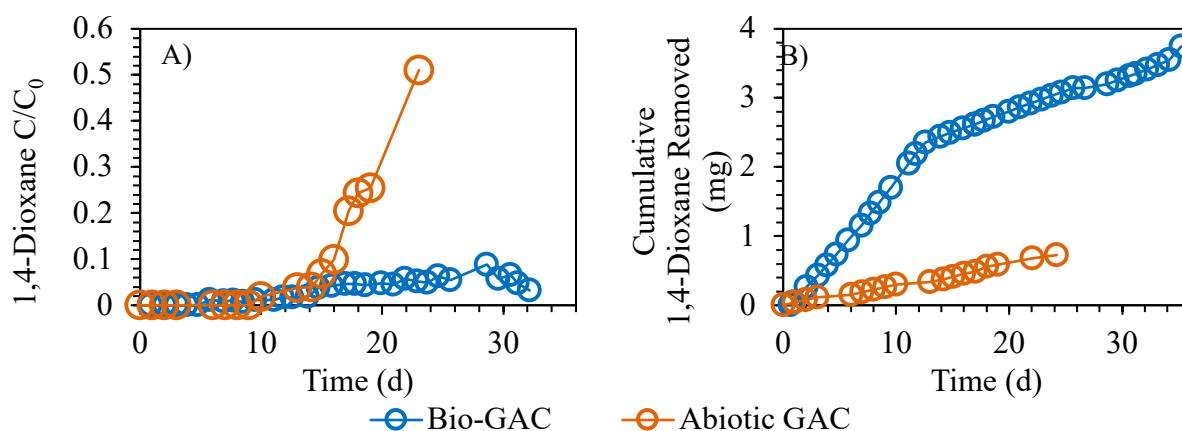


Figure 2. The bio-GAC flow-through column outperformed its abiotic GAC counterpart. A) Bio-GAC maintained greater than 90% removal rates during 36 days of operation while abiotic GAC rapidly broke through as removal rates decreased from ~90% on day 16 to ~50% removal by day 23. B) Bio-GAC removed more than 3 mg 1,4-dioxane by day 24, while abiotic GAC only removed 0.7 mg in the same time period.

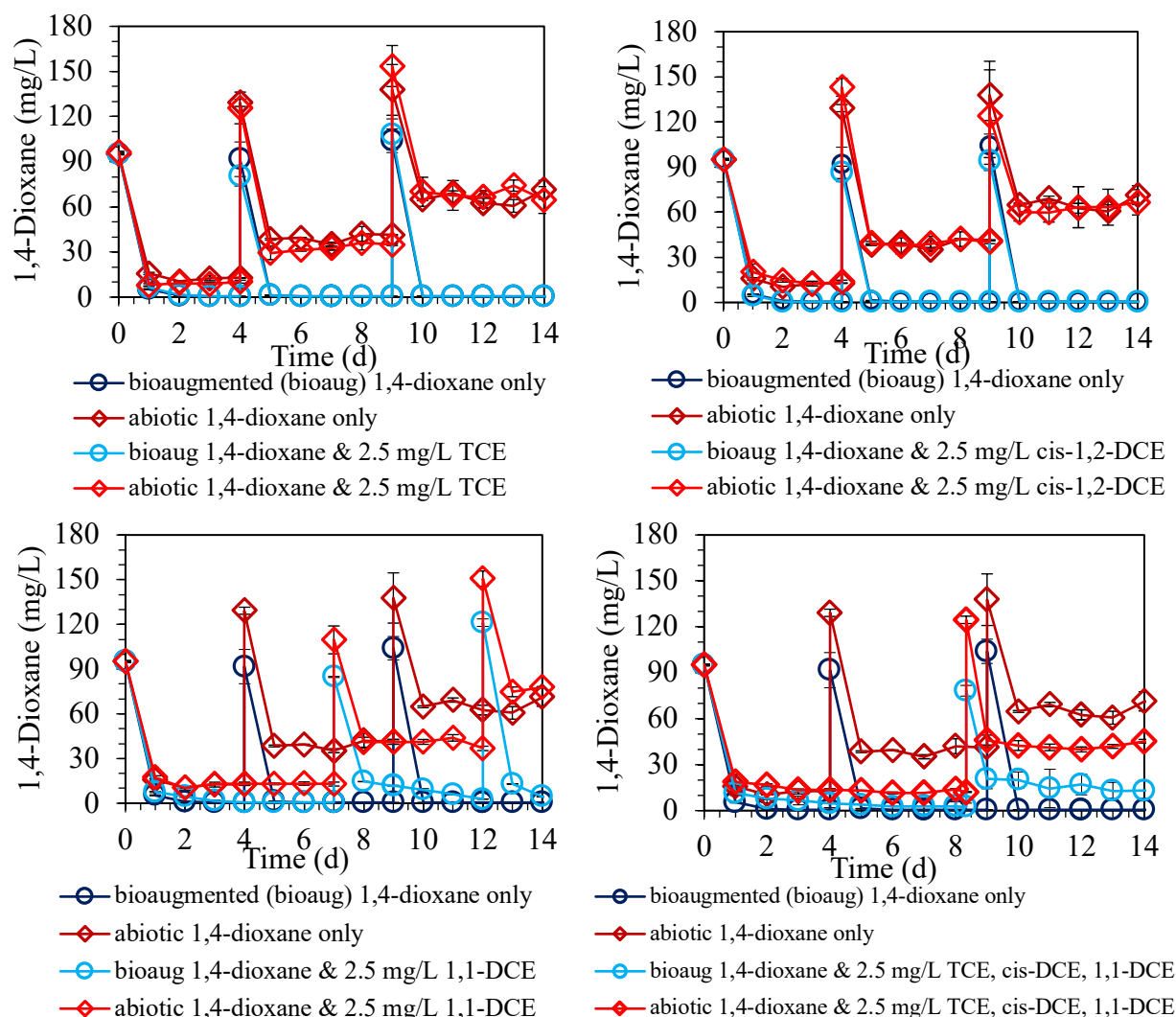


Figure 3. Batch CB1190 bio-GAC reactors degraded 1,4-dioxane in the presence of CVOCs. Reactors were initially amended with 100 mg/L 1,4-dioxane and 0 or 2.5 mg/L (total reactor concentration) of TCE, cDCE, 1,1-DCE or all three chlorinated solvents. Error bars represent half the range of duplicate reactors.

1,4-Dioxane removal was tested in columns packed with enhanced reductive dechlorination treated soil, and those bioaugmented with CB1190 consistently removed 80-100% 1,4-dioxane under varying influent concentrations and flow rates, and untreated soil under oxic conditions removed approximately 25-50% 1,4-dioxane only with 0.01 mg/L flow rate (Figure 4). In the presence of 1,1-DCE and Cu(II), untreated soil under oxic conditions did remove 15-50% 1,4-dioxane in the 100 day experiment, but 5 and 10 mg/L 1,1-DCE decreased 1,4-dioxane removal efficiency. Soil bioaugmented with CB1190 kept 90-95% 1,4-dioxane removals with the addition of 5 and 10 mg/L 1,1-DCE, as well as 10 mg/L Cu(II) (Zhao et al., 2018). This observation demonstrates that biofilm growth mode significantly affects the ability of CB1190 to resist inhibition by co-contaminants.

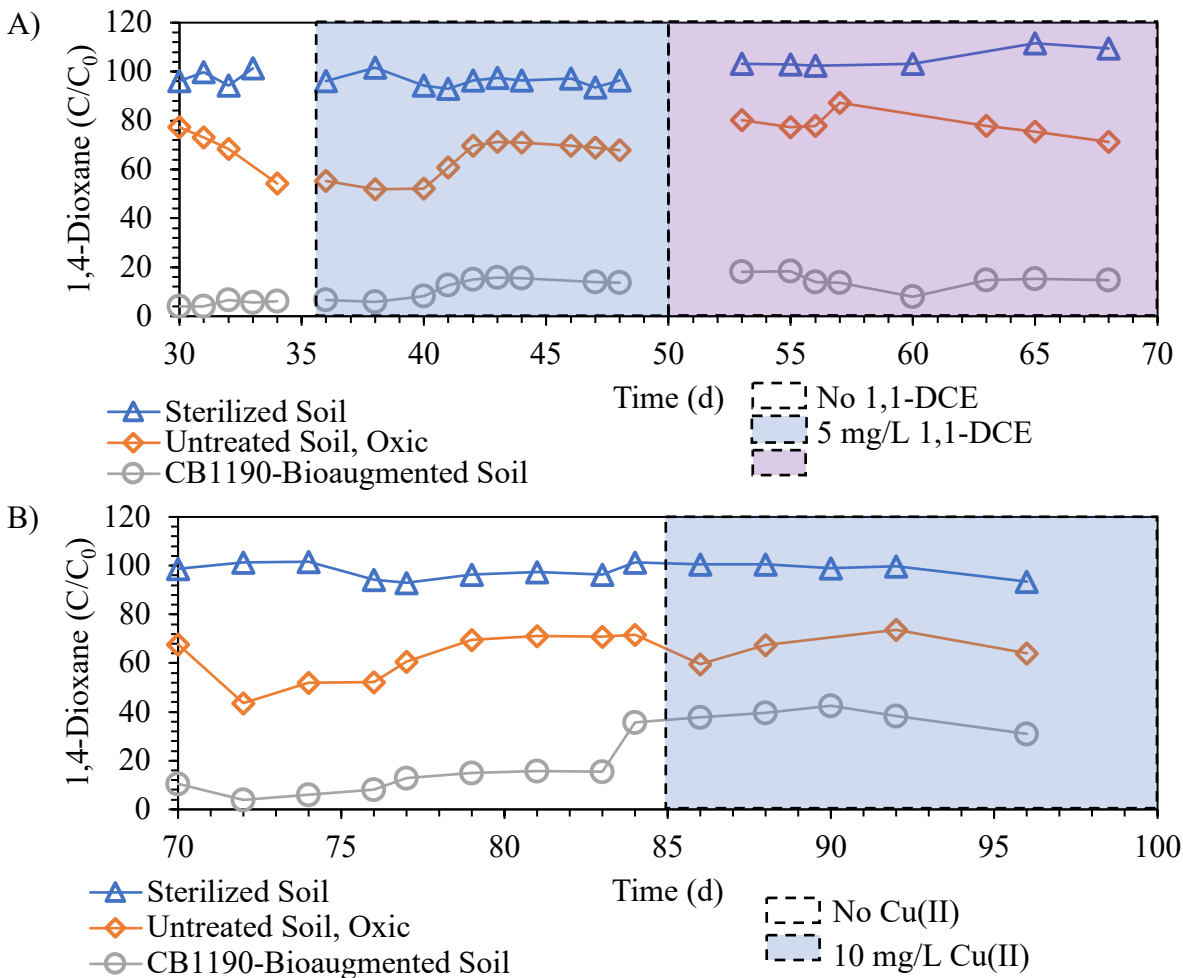


Figure 4. Inhibitory effects of co-contaminants A) 1,1-DCE and B) Cu(II) on 1,4-dioxane biodegradation in flow-through column systems packed with ERD-treated soil. Soil columns included a sterilized soil column, an untreated soil column under oxic condition, and a bioaugmented soil column. All columns were operated at a flow rate of 0.01 mL/min (0.3 bed volume/d; residence reaction time 78 h) with 10 mg/L 1,4-dioxane in the influent.

Microbial community was analyzed in the sequential oxidation and biodegradation treatment processes for 1,4-dioxane in the presence of co-contaminants. The richness and biodiversity were inhibited after oxidation by hydrogen peroxide. However, after 10 weeks inoculation, the microbial community was resilient, with even higher level of diversity with multiple species related to hydrocarbon degradations, that could be attributed to less chlorinated stress, higher bioavailability and higher oxygen level. Moreover, even with the different levels of 1,4-dioxane and co-contaminants, the microbial community showed similar dynamic change along with the operation time, and the contaminants only differentiated communities at the specific time point, indicating that the community developed in the same direction but via varying paths. This deterministic process was related to the microbial interactions, competitions, and niche differentiations (Miao et al., 2019).

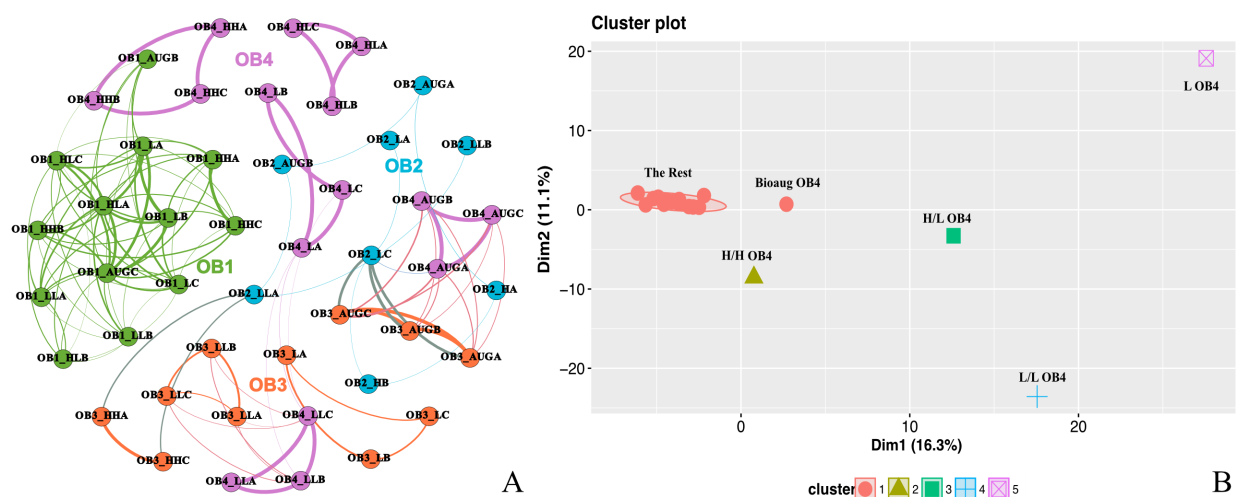


Figure 5. Network and cluster analyses among samples at each time point under all conditions. (A) The node in network are colored by each time point, and the connections represented a strong ($R > 0.6$) and significant ($p\text{-value} < 0.01$) correlations. The thickness of each edge is proportional to the p -value. (B) Cluster analysis pools all samples at different time points under different conditions and shows integral shift among conditions and time points.

Implications for Future Research and Benefits

This project investigated the effects of typical co-contaminants (chlorinated solvents and metals) and their common treatment technologies (chemical oxidation or reduction) on 1,4-dioxane bioremediation, thus supporting SERDP's mission to reduce the DoD's liabilities by developing sustainable, cost-effective technologies for in situ remediation of groundwater.

Conclusion 1: Although chlorinated solvents negatively impact 1,4-dioxane biodegradation, with vinyl chloride being the most inhibitory, growth mode (biofilm vs. planktonic) and solvent biodegradation can ultimately mitigate these effects.

Dose-dependent inhibition of 1,4-dioxane biodegradation by individual chlorinated solvents, TCA, TCE, 1,1-DCE, cDCE, as well as their mixtures on 1,4-dioxane biodegradation at two initial concentrations of 1,4-dioxane (1 mg/L and 100 mg/L) was quantified. The co-contaminant effects were studied using planktonic cultures of *Pseudonocardia dioxanivorans* CB1190, which uses 1,4-dioxane as growth substrate, and *Mycobacterium austroafricanum* JOB5, which cometabolizes 1,4-dioxane after growth on propane, so that the results would be valuable for considering natural attenuation, biostimulation, or bioaugmentation as groundwater remediation strategies. The inhibition was reversible in all cases, was strongest in CB1190 due to VC, followed by 1,1-DCE, cDCE, TCE, and TCA. CB1190 was able to degrade VC and cDCE but not any other solvents. In contrast, JOB5 was able to cometabolize all of the chlorinated solvents examined, and the rapid removal of these solvents suggests cometabolism might provide a robust strategy for 1,4-dioxane as well as its chlorinated solvent co-contaminants.

Conclusion 2: 1,4-Dioxane biodegradation was influenced to varying degrees by individual exposure to five transition metals, with Cu(II), an essential nutrient, being the most inhibitory, while Cr(VI), a toxic oxyanion, had little effect at environmentally relevant levels. Surprisingly, bioavailability and toxicity of Cu(II) and other metals can be alleviated by organic ligands, which resulted in improved biodegradation rates.

Cd(II), Cu(II), Cr(VI), Fe(II), and Ni(II) also inhibited 1,4-dioxane biodegradation, whereas Zn(II) had no measurable effect on biodegradation rates. Surprisingly, Cu(II) was the strongest inhibitor, even at low levels, while Cr(VI) was only considerably inhibitory at highly elevated levels (250 mg/L for planktonic JOB5 and only slightly at 10 mg/L for JOB5 biofilms). 1,4-Dioxane biodegradation in cultures exposed to 2 mg/L Cu(II) was restored in the presence of 0.005, 0.05, and 0.5 mM alginin, 0.05, and 0.5 mM cysteine, and 0.005 mM tannin. These results indicated that specific ligands bind with transition metals and alleviate bacterial toxicity. Thus, monooxygenase-catalyzed biodegradation rates by 1,4-dioxane metabolizing or cometabolizing bacteria were subject to interactions among transition metals and natural organic ligands in the environment. Interestingly, the addition of copper was found to induce EPS production even with high concentrations of 1,4-dioxane remaining. These findings suggest that more EPS were produced by CB1190 as a stress response to starvation and metal ions, so CB1190 biofilms would be viable, and perhaps protected, in contaminated environments. Additionally, our results also imply that bioaugmentation strategies at sites with environmentally relevant levels of Cr(VI) are appropriate due to the surprisingly low impact of Cr(VI) on 1,4-dioxane biodegradation.

Conclusion 3: GAC and zeolites are capable of adsorbing 1,4-dioxane with relatively large maximum capacities. Although desorption can occur, bioaugmenting the sorbent greatly increases their capacity and treatment effectiveness.

Bioaugmented adsorbents were examined first as a means of testing the ability of biofilms to degrade 1,4-dioxane with and without chlorinated solvent co-contaminants. Due to the adsorbents' own abilities to adsorb both 1,4-dioxane and chlorinated solvents, they may be beneficial in the treatment of 1,4-dioxane and chlorinated solvent mixtures. Bio-GAC outperformed abiotic GAC in the removal of 1,4-dioxane from industrial wastewater and groundwater samples. Additionally, bioaugmentation of GAC mitigated the amount of 1,4-dioxane adsorbed by sequential washes with DI water when compared to abiotic GAC. Bio-zeolite outperformed abiotic zeolite in the removal of 1,4-dioxane in the presence of CVOCs, except in the case of 1,1-DCE, which fully inhibited the system, due to zeolite's similar adsorption affinities for 1,1-DCE and 1,4-dioxane. This highlights the importance of understanding the competition effects between adsorbates in bioaugmented adsorbent systems.

Conclusion 4: Attached microorganisms outperform planktonic cells for the biodegradation of 1,4-dioxane in the presence of chlorinated solvents and metals such as Cr(VI) and Cu(II).

Biofilms resist chlorinated solvent and metal inhibition better than planktonic cultures. CB1190 biofilms were capable of resisting Cu(II), 1,1-DCE, cDCE, and TCE inhibition better than planktonic cells. JOB5 biofilms were also able to resist Cr(VI) inhibition, but it was similar to the resistance of the planktonic cultures examined, likely due to the ability of EPS to bind with Cr(VI) oxyanions. Growing microbes on different solid supports differentially enhanced mitigation of chlorinated solvent inhibition. CB1190 bio-GAC was able to degrade 1,4-dioxane (initial concentration of 100 mg/L) in the presence of individual and mixtures of chlorinated solvents (TCE, cDCE, and 1,1-DCE). CB1190 bio-zeolite was able to treat 1,4-dioxane in the presence of TCE and cDCE. Due to the competitive adsorption effects (the somewhat equal affinities of 1,4-dioxane and 1,1-DCE for adsorption on zeolite), bio-zeolite was inhibited by mixtures containing 1,1-DCE. Bio-GAC flow-through column systems lasted considerably longer than abiotic columns. This suggests that bioaugmented adsorbents may be an effective in countering some of the drawbacks of both abiotic adsorption (finite capacities, desorption

potential, non-destructive nature of the treatment) and biodegradation only (co-contaminant inhibition) for the removal of 1,4-dioxane with and without CVOC co-contaminants.

Conclusion 5: Microbial community can be resilient even after oxidant stress, along with bacterial interactions in treatment processes, and it developed time but independent of the contaminant levels.

Based on the microbial community analysis, shifts in microbial community structures occurred in the course of treatment, and were influenced by contaminant concentrations at individual time points. Various microbial groups recovered after exposure to peroxide, a microbicide, and evolved during the biodegradation with deterministic factors, including competition, interaction and niche differentiation, that could be also attributed to higher bioavailability and oxygen. Biodiversity and richness were inhibited right after oxidation process, but they increased again during subsequent biodegradation process with multiple species related to hydrocarbon degradation (e.g., *Afipia*, *Pseudolabrys*, *Sphingomonas*, and *Cupriavidus*), helping further evaluation of remediation work. It is important to consider the impact of abiotic and biological remediation technologies on the microbial ecology to predict success of short-term active treatment as well as long-term passive attenuation processes.

Implication 1: Treatment trains are crucial to remediation efforts that involve complex geochemistry and contaminant mixtures.

Remediation of large and complex groundwater plumes of contaminant mixtures can rarely be achieved with single remedies. Chlorinated solvents (especially VC and 1,1-DCE) and metals (especially Cu(II)) can greatly inhibit aerobic 1,4-dioxane biodegradation. To this challenge, our findings offer hope in the form of treatment trains or hybrid remedies that can either synergize or help mitigate drawbacks of individual treatment technologies for addressing complex contaminant mixtures (e.g. chemical oxidation/reduction followed by biodegradation or bioaugmented adsorbents). More work is needed to identify other promising combinations of treatment technologies that can effectively handle complex remediation challenges.

Implication 2: Understanding the responses of microbial communities to abiotic treatments is important when considering concurrent or subsequent biological treatment strategies.

Treatment trains that include biodegradation can only be effective by understanding how the abiotic remedies may impact the microbes. Microbial community analysis offers powerful tools that can provide insights into the way that abiotic treatment technologies affect microbial community composition and dynamics, which can have direct impacts on community functions. Just as additional research is needed to identify promising treatment trains, we need more work to understand how the microbial communities respond when placed in these treatment systems so that we may optimize them to be as effective as possible.

Implication 3: Characterization of the effects of additional co-contaminants on 1,4-dioxane biodegradation is needed to improve the effective deployment of bioremediation strategies.

Other chlorinated solvents, such as 1,1-dichloroethane (1,1-DCA) or 1,2-dichloroethane (1,2-DCA), commonly used in the production of VC and other industrial processes, are also reported to co-occur with 1,4-dioxane. Understanding the implications of environmentally relevant concentrations of these compounds on 1,4-dioxane metabolizing and cometabolizing bacteria are critical for accurate degradation rates and timelines. As shown by our results and

previous reports, chlorinated solvents can initially inhibit 1,4-dioxane degradation, but their effects can be mitigated when they are concurrently or sequentially degraded. Further characterization of the effects of additional co-contaminants will increase our ability to clean up sites contaminated with various mixtures of chlorinated solvents and 1,4-dioxane.

Project Outputs:

The results of this research were disseminated in 10 peer-reviewed papers, 5 theses, and 45 presentations. Other avenues for technology transfer and demonstration are being explored.

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OBJECTIVE

Having performed substantial research confirming 1,4-dioxane biodegradation mechanisms and rates in the laboratory, we hypothesize that 1,4-dioxane biodegradation rates are sensitive to the interrelationships between chlorinated solvent and trace metal co-contaminant concentrations, intrinsic microbial communities, and aquifer geochemical conditions, such that the apparent 1,4-dioxane degradation rate can be inhibited or enhanced with variation in these parameters. While bacterial and fungal degradation of 1,4-dioxane under laboratory-controlled conditions has been well established (Zhang et al., 2017), biodegradation of 1,4-dioxane is less frequently observed in contaminated aquifers with commingled plumes. In addition to limitations from inadequate microbial communities, dissolved oxygen, and aquifer biogeochemistry, the inhibitory effects of co-contaminants might be responsible for hindering *in situ* 1,4-dioxane biodegradation. Lastly, reductive remedial technologies commonly applied to chlorinated solvent plumes may have no effect, or worse yet, may have an inhibitory effect on 1,4-dioxane biodegradation. We thus proposed a 3-year research project that evaluated these hypotheses through the following technical objectives:

1. To characterize the effects of chlorinated solvents (TCE and TCA) and their degradation products on metabolic and cometabolic biodegradation of 1,4-dioxane in planktonic cultures. Since 1,4-dioxane is found associated with chlorinated solvents in affected aquifers, it is important to understand how chlorinated solvents and their degradation byproducts influence 1,4-dioxane biodegradation. Our preliminary data show that metabolic biodegradation of 1,4-dioxane in the presence of TCA follows Michaelis-Menten kinetics with mixed inhibition whereas cometabolism by toluene-oxidizing bacteria exhibits competitive inhibition between 1,4-dioxane and TCA. The effects of biodegradation products of these compounds have not yet been evaluated. These findings warrant a thorough investigation into the effects of other chlorinated solvents and their degradation products on 1,4-dioxane degrading bacteria.
2. To determine the effects of metal ions (copper, iron, cadmium, nickel, chromium) on metabolic and cometabolic biodegradation of 1,4-dioxane in planktonic pure cultures. Since monooxygenases involved in the metabolic or cometabolic degradation of chlorinated solvents and/or 1,4-dioxane are metallo-enzymes that are highly sensitive to metal concentrations, it is critical to measure and model degradation rates in the presence of relevant metals. Data collected by the team indicate that metals are generally found at 1,4-dioxane-contaminated sites, and that metals such as copper can influence microbial growth and inhibit 1,4-dioxane biodegradation to a significant extent. This effort identified metal threshold concentrations that produce either no observable effect or are microbicidal and will form the basis of subsequent experiments.
3. To evaluate 1,4-dioxane biodegradation by bacterial biofilms in batch and column reactors. While most research in microbiology has involved planktonic cultures, the majority of environmental microbes live in biofilms in soil and sediments (Costerton, 1995; Schembri et al., 2003). Not only is it established that bacteria express distinct sets of genes when growing as a biofilm (Schembri et al., 2003; Yamamoto et al., 2011), attached communities may experience different bioavailability and biotransformation rates of organic and inorganic compounds. To date, most studies of 1,4-dioxane biodegradation were performed in batch reactors containing liquid cultures. Consequently, it is important to investigate biodegradation by biofilms grown on solid

supports in both batch and column systems to understand both mechanism and performance. We tested the hypothesis that degradation pathways and kinetics of 1,4-dioxane by bacteria grown attached to solid supports will be different from those observed in planktonic cultures, and it will depend greatly on support type.

4. To evaluate co-contaminant effects on metabolic and cometabolic biodegradation of 1,4-dioxane in flow-through columns. The inhibition constants of chlorinated solvents and metal ions on 1,4-dioxane degrading bacteria in columns are expected to be different from those obtained from planktonic culture experiments. Furthermore, the organic content in column packing material might influence the bioavailability of hydrophobic chlorinated solvents by sorption and alleviate metal-derived toxicity by complexing metal ions. Indeed, our data suggest that rates of 1,4-dioxane degradation by bacteria growing in attached communities are slower than those in planktonic cultures. Furthermore, the inhibition of 1,4-dioxane by copper ions is characteristically different (Appendix D: Figures 3 and 4). A comprehensive understanding of these interactions will yield accurate and representative 1,4-dioxane biodegradation rates.
5. To assess the effects of chlorinated solvent remedial technologies on 1,4-dioxane degradation under realistic environmental conditions. Reductive dechlorination has been widely adopted as the optimal remedial technology for source or dissolved plume treatment for chlorinated solvent contamination. During the remedial technology selection process for chlorinated solvents, the treatment of 1,4-dioxane is typically not considered due to insufficient investigation data or lower risk concern as compared to the chlorinated solvents. As the concern of 1,4-dioxane rises, 1,4-dioxane treatment approaches must consider the impact of previous or ongoing chlorinated solvent treatment activities. At contaminated sites undergoing ERD for chlorinated solvents, those activities will alter biogeochemical conditions, increase the concentrations of daughter compounds, and possibly mobilize metals. As a result, the biodegradation rates of 1,4-dioxane in these columns will be different from sites where no chlorinated solvent biogeochemical remedies have been implemented. The results of this study will determine if 1,4-dioxane treatment would be best implemented in parallel or subsequent to reductive treatment operations.
6. To analyze microbial compositions and dynamic changes during the chemical and biological processes treating 1,4-dioxane with oxygen altered by hydrogen peroxide under various co-contaminants conditions. We will test the hypothesis that the oxidants reduce co-contaminants and provide oxygen to stimulate biodegradation of 1,4-dioxane, as well as influence the microbial ecology. Microbial community analysis in samples collected from treatment processes will help identify key microbes and functional genes for successful 1,4-dioxane biodegradation in the presence of co-contaminants, and track dynamic trends responding to co-contaminants, as well as evaluate post-treatment microbial ecology. This analysis will pinpoint specific microbial populations in response to changing contaminant (1,4-dioxane, TCA/TCE/DCE) concentrations and treatment processes, and provide support for the optimal remediation strategy for commingled contaminant plumes.

BACKGROUND

1,4-Dioxane is an emerging groundwater contaminant that is often accompanied by chlorinated solvents and metal ions in aquifers found at many contaminated DoD sites (Bannon et al., 2009; Hunter et al., 2006). While 1,4-dioxane is present in small amounts as a stabilizer of TCA, its concentrations may be significantly higher in spent TCA. For example, the average concentration of 1,4-dioxane in unused TCA is 5% whereas dioxane concentrations of 10% have been reported in waste TCA from the electronics industries, and 30% in still bottom sludges of vapor degreasers (Jackson and Dwarkanath, 1999; Mohr et al., 2010). Furthermore, many facilities use activated carbon for TCA reclamation before waste disposal, and due to differential adsorption characteristics, 1,4-dioxane is further enriched in waste streams. The hydrophilicity of 1,4-dioxane allows it to mix rapidly with groundwater, such that 1,4-dioxane plumes can expand well beyond TCA plumes. Consequently, a wide variety of 1,4-dioxane/solvent concentration ratios may occur in aerobic aquifer zones where monooxygenase-mediated natural or engineered bioremediation would potentially occur.

While TCA is widely accepted as a major source of 1,4-dioxane in contaminated aquifers, TCE is more commonly found as its co-contaminant. The reasons for mixed plumes of 1,4-dioxane and TCE can be explained by the substitution of TCA for TCE as the preferred solvent in industrial processes and the instability and rapid degradation of TCE in natural environments (Doherty, 2000). Certainly, anaerobic processes are commonly used to remediate chlorinated solvent contamination (Berlendis et al., 2010; Lee et al., 2006; Scheutz et al., 2011); however anaerobic reductive dechlorination of TCA and TCE results in the accumulation of daughter products such as 1,1-DCE, and cDCE (Major et al., 2002; Smidt and de Vos, 2004). The physical and chemical properties of 1,1-DCE, cDCE and 1,4-dioxane indicate high mobility in water suggesting these compounds will likely migrate to areas away from the source where aerobic conditions may exist. Therefore, it is important to characterize the effects these daughter products may have on the kinetics of 1,4-dioxane biodegradation.

The quantification of interactions between 1,4-dioxane and associated co-contaminant degradation kinetics will provide valuable inputs into reactive fate and transport models and facilitate design of remediation strategies. While 1,4-dioxane may not affect chlorinated solvent degradation processes, the presence of these solvents certainly inhibits 1,4-dioxane degradation (Mahendra et al., 2013). Therefore, 1,4-dioxane bioremediation efforts will need to consider the presence of chlorinated solvents. Furthermore, the same enzymes in aerobic bacteria may cometabolize 1,4-dioxane and its associated chlorinated solvents. The differences in affinities of those enzymes for 1,4-dioxane, chlorinated compounds, as well as any primary growth substrates will have implications for designing biostimulation systems for site remediation.

A lack of comprehensive understanding of site biogeochemical influences has prevented the successful transition of 1,4-dioxane biodegradation in the laboratory to the field. Co-contaminant metals can potentially serve as inhibitors to microbes carrying out *in situ* biodegradation of 1,4-dioxane. Several metals including hexavalent chromium [Cr(VI)] and divalent copper [Cu(II)] are prevalent contaminants at DoD facilities (Bannon et al., 2009; Hunter et al., 2006). A study conducted on the prevalence of metal contamination on DoD sites found that concentrations of Cr(VI) in soil samples exceeded the risk-based screening criteria for human health and

ecological concerns in a large percentage of sites tested (Salatas et al., 2004). A recent study found significant co-occurrence between 1,4-dioxane, CVOCs, and Cr(VI) across California (Hausladen et al., 2018). In addition, elevated concentrations of divalent metals such as copper, nickel, iron, and zinc have major implications for biological processes. A number of studies have been conducted on the sensitivity of enzymatic processes to metal concentrations as the primary mechanism for inhibition. As cofactors of metalloenzymes and proteins, trace metals are involved in all general metabolic processes of microorganisms, including photosynthesis, respiration and major nutrient assimilation (Morel et al., 1991). They play an essential role by maintaining conformation and tertiary structures in some proteins. In some enzymes, metals occur in the catalytic site and facilitate oxidation-reduction reactions, transferring electrons from one redox couple to another (Alvarez-Cohen and Speitel, 2001).

While metals are essential elements for biological activity, they may also be toxic at elevated concentrations, attacking nucleic acids and enzymatic pathways (Baath, 1989). Several microorganisms have developed a variety of mechanisms to protect against metal toxicity, including decreased uptake, binding of the metal by proteins or extracellular polymers, formation of insoluble metal sulfides, volatilization and enhanced export from the cells (Giller et al., 1998). Copper, nickel and zinc have been shown to inhibit the soluble methane monooxygenase (sMMO) activity in purified enzyme assays (Jahng and Wood, 1996). This enzyme is responsible for the cometabolic degradation of 1,4-dioxane by methanotrophic bacteria. According to Grosse et al. (Grosse et al., 1999), Cu(II), Ni(II), and Zn(II) ions inhibited the reductase, and Cu(II) may also influence other components of the sMMO. In the presence of 1 mM concentration of Cu(II), Ni(II), and Zn(II) ions, the loss of *in vitro* sMMO activity was 95, 40 and 80% respectively (Grosse et al., 1999).

It is unknown which forms of trace metals are available to bacteria for uptake. Metal speciation (the distribution of metals in free, chelated and precipitated forms) is governed by total metal concentrations, pH and complex interactions between the metals, ligands, major ions, and particles (Xue et al., 1995). Trace metals tend to be complexed by organic and inorganic ligands in natural waters and soils (Morton et al., 2000). Copper, iron, and zinc can also be complexed by biologically produced organic ligands (DiSpirito et al., 1998; Morel et al., 1991; Tellez et al., 1998). Copper forms stronger organic complexes than most other divalent metals (Xue and Sigg, 1993). Nickel and zinc can possibly replace iron or copper in the MMO and influence the enzyme regulation and catalytic rates. There have also been reports of competition of copper and zinc for strong ligands in the environment (Xue et al., 1995), and metal substitutions in enzymes *in vivo* (Price and Morel, 1990; Yee and Morel, 1996). The total quantities of copper, nickel or zinc available to methanotrophs, as well as their speciation, have the potential to strongly affect MMO expression and subsequent methane and 1,4-dioxan oxidation reactions.

The literature assessing metal toxicity is well established with a growing body of evidence using *in vivo* and *in vitro* models indicating that exposure to copper contamination causes various adverse responses in eukaryotic organisms including mice (Szabova et al., 1990), mammalian cell lines (Grillo et al., 2009; Seth et al., 2004), bacterial strains (Hanson and Hanson, 1996; Shuttleworth and Unz, 1991), fish (Erickson et al., 1996; Pagenkop.Gk et al., 1974), and invertebrates (Korthals et al., 1996; Lage et al., 1996; McIntosh and Kevern, 1974). Metal ions cause cytotoxicity by binding with the thiol groups of the respiratory and transport proteins,

leading to the interruption of cell functions (Holt and Bard, 2005; Liao et al., 1997; Petering, 1976). Even sub- micromolar concentrations of silver ions were reported to inhibit NADH dehydrogenase causing disruption of the respiratory chain (Holt and Bard, 2005). Silver ions were also able to induce proton leakage and collapse of proton motive force through binding with specific proton transport proteins (Dibrov et al., 2002; Lok et al., 2007). Additionally, it has been found that DNA mutation frequencies during polymerase chain reactions increased in the presence of silver and copper ions (Chen et al., 2006; Yang et al., 2009; Yoon et al., 2007). Thus, understanding the effects of metal co-contaminants on bacteria and enzymes catalyzing 1,4-dioxane biodegradation will lead to an improved understanding of field-scale 1,4-dioxane bioremediation.

MATERIALS AND METHODS

Chemicals

1,4-Dioxane (99.8%, ACS grade) (1,4-DX), 1,1-dichloroethene ($\geq 99.9\%$) (1,1-DCE), cis-1,2-dichloroethene (97%) (cDCE), trichloroethene ($\geq 99.5\%$, ACS grade) (TCE), vinyl chloride ($\geq 99.9\%$) (VC), copper (II) chloride dihydrate (ACS grade), nickel (II) chloride hexahydrate (ACS grade), zinc (II) sulfate heptahydrate (ACS grade), tannic acid, alginic acid, and L-cysteine were purchased from Sigma-Aldrich (St. Louis, MO). Bovine serum albumin (BSA) protein, ACS grade cadmium sulfate 8/3 hydrate, and sodium dichromate was purchased from Fisher Scientific (Fair Lawn, NJ). Suwannee River natural organic matter (SRNOM) was obtained from the International Humic Substances Society (Saint Paul, MN). 1,1,1-Trichloroethane ($>95\%$) (TCA) was obtained from Ultra Scientific (North Kingstown, RI). Propane (99.9%) was supplied by Matheson (Basking Ridge, NJ). Stock solutions of saturated chlorinated solvents were prepared as previously described (Alvarez-Cohen and McCarty, 1991). Transition metal and organic ligand stock solutions were prepared by dissolving required masses of metal salts or organic ligands in deionized water. Metal and organic ligand stock solutions were filter-sterilized and stored at 4°C prior to use.

Bacterial Strains and Growth Conditions

1,4-Dioxane metabolism and cometabolism were evaluated using pure cultures of *Pseudonocardia dioxanivorans* CB1190 (hereafter CB1190) and *Mycobacterium austroafricanum* (vaccae) strain JOB5 (hereafter JOB5), respectively. CB1190 was maintained in ammonium mineral salts medium (Parales et al., 1994) as previously described (Mahendra and Alvarez-Cohen, 2006) and JOB5 was cultured in nitrate mineral salts medium (Whittenbury et al., 1970) supplemented with propane as described in Lan et al (2013) with the exception that 25% (vol/vol) propane was supplied to the headspace. All cultures were incubated at 30°C with 150 rpm agitation.

Task 1: Characterizing the Effects of Chlorinated Solvents (TCE and TCA) and their Degradation Products on Metabolic and Cometabolic Biodegradation of 1,4-Dioxane under Planktonic Growth Conditions.

Kinetics of 1,4-Dioxane Metabolism by Pseudonocardia dioxanivorans CB1190 in the Presence of Individual Chlorinated Solvents

Experiments were conducted to evaluate biodegradation of 1,4-dioxane at high (100 mg/L) and low (1 mg/L) initial concentrations. For high 1,4-dioxane concentrations, CB1190 was inoculated into fresh AMS medium amended with 1,4-dioxane at a total volume of 30 mL in a 160 mL serum bottle or 200mL in a 1000mL serum bottle with an aluminum crimp sealed butyl septum. Sample bottles were exposed to 0.05 – 100 mg/L of TCE, TCA, 1,1-DCE, cDCE or VC. Abiotic controls and solvent-free controls were employed. All experiments were conducted in triplicate bottles and were incubated at 30°C and 150 rpm agitation. Aqueous samples were collected for 1,4-dioxane and ATP analysis. Solvents were analyzed by Gas Chromatography equipped with a Flame Ionization Detector (GC-FID) or Electron Capture Detector (GC-ECD).

For low 1,4-dioxane concentrations, CB1190 was grown in AMS medium as described above with the exception that cells were allowed to completely degrade 150 mg/L of 1,4-dioxane. 1,4-

Dioxane was then spiked to a final concentration of 1-2 mg/L and chlorinated solvents were added to the bottles at concentrations of 0.5 – 50 mg/L. Gas Chromatography-Mass Spectrometer Detector (GC-MSD) was used to quantify low levels of 1,4-dioxane. In addition, oxygen and carbon dioxide content in the headspace was quantified and samples were collected for nucleic acid extraction followed by gene abundance and expression analyses.

Investigating the Influence of Chlorinated Solvent Mixtures on 1,4-Dioxane Metabolism by *Pseudonocardia dioxanivorans* CB1190

To mimic the concentrations of 1,1-DCE, cDCE, TCE and TCA commonly detected in the environment, variable amount of solvents were added in 3 different mixtures of solvents. Mixture 1 contained 5000 µg/L 1,1-DCE, 500 µg/L cDCE, 700 µg/L TCE and 120 µg/L TCA. Mixture 2 had 500 µg/L cDCE, 700 µg/L TCE and 120 µg/L TCA. Mixture 3 had only 700 µg/L TCE and 120 µg/L TCA was added. The concentration of volatile solvents in the aqueous phase was calculated based on Henry's Law constant (US.EPA, 1991b) and are typical concentrations found in the environment. Each bottle was closed with a butyl rubber stopper and crimp-sealed with an aluminum cap. At every time point (0, 2, 4, 6, 8, 10, 12, 14, 16, and 24 hours), 200 µL aqueous samples were collected by using 1 mL syringe equipped with sterile needle (23 gauge, 0.64 mm O.D., 1.5 in. length) for GC-MS analysis and stored at -20 °C; 500 µL samples were collected and stored at -80 °C for total nucleic acids extraction to analyze for gene abundance and gene expression. Gene expression data were first normalized to the housekeeping gene, *rpoD*, followed by normalization to time 0. 100 µL headspace samples in each bottle were used to test the solvent's concentration at time 0, 1, 7, 10 days by GC-FID and/or GC-MS.

Kinetics of 1,4-Dioxane Cometabolism by *Mycobacterium austroafricanum* (vaccae) JOB5 in the Presence of Individual Chlorinated Solvents

The influence of chlorinated solvents on cometabolism 1,4-dioxane was investigated using the propanotroph, JOB5. JOB5 cells were grown on propane as described above to an optical density of 1.0 at 600nm. Cells were transferred to either 160 mL or 1000mL serum bottles with an aluminum crimp sealed butyl septum and supplemented with 1-2 mg/L of 1,4-dioxane, and chlorinated solvents ranging between 0.05 – 100 mg/L to a total volume of 30 mL per bottle. Samples were collected and analyzed for 1,4-dioxane, nucleic acids, and chlorinated solvents.

Task 2. Quantifying the Impacts of Metal Ions on 1,4-Dioxane Biodegradation

1,4-Dioxane Biodegradation by CB1190 in the Presence of Individual Metals and/or Organic Ligands

Biodegradation of 1,4-dioxane by CB1190 was carried out in sterile 100 mL glass bottles with screw caps (Fisher Scientific, Fair Lawn, NJ). The culture medium was kept at 20% of total volume of the container (i.e., 20 mL in 100 mL media bottle) to prevent oxygen mass transfer limitations. Cultures were incubated at 30 °C and 150 rpm agitation, and aseptic conditions were maintained to prevent contamination. In biodegradation studies, initial concentration of 1,4-dioxane was 120 mg/L in the bacterial cultures, which were inoculated with 1% v/v CB1190 and exposed to individual transition metals. Metal salts were added from the stock solutions to yield Cd(II), Cu(II), Ni(II), and Zn(II) final concentrations of 0, 1, 10, or 20 mg/L. Organic ligands were added in the presence of each metal in the bacterial cultures to identify the role of free ions. In addition, ligands were independently investigated to determine their influence on 1,4-dioxane

biodegradation in metal-free cultures. Alginic acid, tannic acid, and L-cysteine were added to obtain final concentrations of 0, 0.005, 0.05, and 0.5 mM, whereas BSA protein and SRNOM were amended to a final concentration of 0, 0.01, 0.1, and 1 mg/L. Concentrations of 1,4-dioxane were monitored by GC-FID, and biodegradation rates were estimated by calculating the change of 1,4-dioxane concentration in each sample over a period of time. Aliquots of 0.5 mL culture samples were also collected and stored at -20 °C for DNA extraction and analyses of gene abundance. All experiments were conducted in duplicate bottles. Duplicate abiotic controls in each condition were maintained throughout this study.

1,4-Dioxane Cometabolism by JOB5 in the Presence of Individual Divalent Metals

JOB5 was cultured as described above to an OD₆₀₀ of 1.0. Prior to the addition of dioxane, the gaseous growth substrate was removed to prevent continual bacterial growth and ensure cometabolism of 1,4-dioxane. Initial concentration of 1,4-dioxane was 100 mg/L in bacterial cultures, which were exposed to transition metal salts individually. Metal salts were added from stock solutions to yield final concentrations of 0, 1, 50, 100, 250, and 500 mg/L for Cu(II) and Fe(II) experiments and 0, 10, 50, 100, 250, and 500 mg/L for Cd(II) experiments. Duplicate bottles were prepared for each concentration. Concentrations of 1,4-dioxane were monitored by gas chromatography, and biodegradation rates were determined by calculating the change of dioxane concentrations over time. Samples were collected at 0, 6, 12, 24, 36, 48, 60, 72, 96, 120, and 144 h for Cu(II) and Fe(II) experiments and 0, 6, 12, 24, 36, 48, 60, 72, 96, 120, 144, and 168 h for Cd(II) experiments. At each time point, aliquots of 500 µl culture samples were collected and stored at -80°C for nucleic acid extraction to analyze for gene abundance and gene expression.

Determining the Effect of Hexavalent Chromium on Metabolism and Cometabolism of 1,4-Dioxane

CB1190 and JOB5 were cultured as described above to high biomass concentrations. A 1% (v/v) transfer of CB1190 in AMS medium was supplied 3 cycles of 100 mg/L 1,4-dioxane and JOB5 was cultured in NMS medium to an OD₆₀₀ of 1.0 before starting the experiments involving hexavalent chromium. Hexavalent chromium was added to cultures at concentrations ranging from 0 – 500 mg/L. 1,4-Dioxane was supplemented to each bottle at 100 mg/L and 1 mg/L for metabolic and cometabolic experiments, respectively. 1,4-Dioxane concentrations were monitored on a GC-FID or GC-MS and samples were collected for gene abundance and expression analyses.

Task 3. Biofilm Development for 1,4-Dioxane Biodegradation

Batch Experiments for Growth of CB1190 on Quartz and Granular Activated Carbon

Batch reactors were prepared in order to quantify the reaction kinetics of CB1190-bioaugmented Norit 1240 granulated activated carbon (GAC). Sterile 100 ml pyrex bottles were filled with 20 ml AMS media, 0.4 g of Norit 1240 GAC, 100 mg/L 1,4-dioxane, and 1% (v/v) seed culture of CB1190. Samples were collected throughout the experiment and filter sterilized by using 0.2 µm-pore nylon syringe filter and stored at -20° C before being analyzed on a GC-FID. 1,4-dioxane was added to 100 mg/L when the concentrations became undetectable using a GC-FID. These concentrations were compared to concurrently run abiotic Norit 1240 batch reactors, two

with 100 mg/L 1,4-dioxane added every other day after day 5, and two with no additional 1,4-dioxane added.

Additional batch reactors were prepared with bioaugmented Norit 1240 granulated activated carbon (GAC) in a similar manner as described above except with an initial 1,4-dioxane concentration of 400 mg/L. 1,4-Dioxane was added to achieve a level of 400 mg/L when the concentrations became less than 5% or 0.5% for bioaugmented Norit 1240 GAC. These concentrations were compared to concurrently run abiotic sorbent batch reactors, two with 400 mg/L 1,4-dioxane with their respective bioaugmented sorbent counterpart, and two with no additional 1,4-dioxane.

Batch Experiments for the Adsorption of 1,4-Dioxane by GAC and Zeolite

Batch experiments were conducted to determine the adsorption isotherm parameters and adsorption kinetics of GAC and zeolite (pentasil type, ZSM-5, Si/Al = 200). For GAC experiments, 0.4 g of GAC was added to 20 mL of AMS medium in sterile 100 mL bottles containing 1,4-dioxane concentrations ranging from 12.5-800 mg/L and placed in shaking incubators (30°C, 150 rpm). Samples collected periodically for GC-FID analysis were processed and stored as previously described. Desorption from the equilibrated GAC was tested by removing the supernatant from the bottles and adding 20 mL of sterile, 1,4-dioxane-free AMS. The bottles were again placed in shaking incubators with the same settings and samples were taken after 1 day for GC-FID analysis. Zeolite experiments were conducted in a similar fashion, but instead 0.02 g of zeolite were placed in 30 mL of AMS medium within 150 mL serum bottles (under sterile conditions) and incubated as before with a range of 1,4-dioxane concentrations. Samples were taken as before and analyzed by GC-FID.

Batch Experiments for Biodegradation of 1,4-Dioxane by CB1190 and JOB5 on Granular Activated Carbon in Environmental Matrices

Additional batch experiments were conducted to test the specific ability of CB1190 or JOB5 biofilms grown on GAC to metabolically or co-metabolically biodegrade 1,4-dioxane in environmental samples. The complete methodology can be found as we have previously described in Myers et al. (2018). Two environmental matrices were used: 1,4-dioxane-impacted groundwater from a contaminated site and industrial wastewater.

Recirculating Column Experiments for 1,4-Dioxane Metabolism by CB1190

Recirculating columns were used in order to gauge the feasibility of 1,4-dioxane biodegradation by CB1190 in biofilms established on quartz or carbon surfaces. Two different configurations were tested, a top-feed gravity flow and a packed bottom-feed pump column apparatus. The top-feed gravity flow column used a 2.54 cm diameter column with 12.7 cm, or 27.9 g, Norit 1240 and 10 cm of AMS medium, or 50.6 mL, maintained. This system was fed with a 2 L reservoir with an initial 1,4-dioxane concentration of 100 mg/L. An aliquot of 20 mL CB1190 culture was added directly to the sorbent in the column. The top packed bottom-feed pump column used 1 cm diameter column with an 8 mL capacity. This column was packed with 8 mL, or 4.4 g, Norit 1240 GAC, and was fed with a 250 mL reservoir with an initial 1,4-dioxane concentration of 400 mg/L. 2.5 mL CB1190 culture was added directly to the sorbent in the column, with a filter preventing the introduction of CB1190 into the reservoir. Each column had an abiotic counterpart for comparison. All samples for both configurations have samples taken throughout

the experiment and were filter sterilized using 0.2 μm -pore nylon syringe filters and stored at -20° C before being analyzed on a GC-FID.

Flow-Through Column Experiments for 1,4-Dioxane Metabolism by CB1190

Flow-through columns were used to determine the ability of CB1190-bioaugmented GAC to remove 1,4-dioxane. Columns were constructed using glass chromatographic columns with polytetrafluoroethylene (PTFE) endcaps (30 cm L. x 2.5 cm D.), PTFE tubing, polypropylene or PTFE fittings, and silicone tubing for the peristaltic pump and to make connections between fittings and tubing where necessary. Additionally, a pre-wetted PTFE filter was installed near the inlet to prevent contamination of the column, biofilm growing down the influent tubing, and the need for sterilization of the influent solution. CB1190 was grown on GAC and acid-washed silica sand in sterile beakers. Prior to packing, the bioaugmented GAC and sand were washed several times with sterile, 1,4-dioxane-free AMS medium. The bioaugmented GAC (1.5 cm bed height) was first wet packed into the column, which had previously been acid sterilized (with 10% vol. conc. HCl/vol. DI water), followed by the backfilling of the remaining column volume with bioaugmented sand. This was done to prevent abiotic control experiments from being exceedingly long while providing appropriate contact time between the influent solution and the biofilms for biodegradation to occur. The influent consisted of AMS medium initially amended with 4 mg/L 1,4-dioxane. This influent solution was later replaced with one containing 1 mg/L 1,4-dioxane. The influent solution was aerated using silicone tubing to bubble in air prior to the addition of 1,4-dioxane. Samples were collected for 1,4-dioxane analysis from the influent reservoir, from a valve just before the inlet of the column, and from a valve at the column outlet. Samples were filtered as previously discussed and stored for later analysis by GC-MS following a previously discussed frozen microextraction procedure. The influent was pumped in the column from the bottom (upflow) at a rate of 0.03 mL/min to mimic groundwater seepage velocities and ensure enough contact time for biodegradation to proceed.

Task 4. Co-contaminant Effects on Biodegradation of 1,4-Dioxane by Biofilms

Flow-Through Column Experiments for 1,4-Dioxane Metabolism by CB1190 in the Presence of TCE and 1,1-DCE

Flow-through column experiments using CB1190 grown on quartz sand was conducted according to our methodology as published in Zhao et al. (2018). In brief, CB1190 was grown on packed sand in sterile, acid washed columns (2.5 cm I.D. x 10.5 cm L.). Recirculation was used to achieve biofilm formation and then the columns were switched to flow-through mode. Influent containing only 1,4-dioxane (100 mg/L) was first tested as a CVOC-free control followed by 1,4-dioxane (100 mg/L) with either TCE (25 mg/L) or 1,1-DCE (1 mg/L). Samples were collected periodically for 1,4-dioxane analysis by GC-FID. Column experiments used flow rates of 0.1 mL/min.

Batch Experiments for 1,4-Dioxane Metabolism by CB1190 in the Presence of CVOCs

Two sets of batch experiments were conducted with CB1190 grown on GAC or zeolite to determine 1,4-dioxane removal extents in the presence of CVOCs. GAC experiments were conducted with 1 g GAC placed initially in 50 mL of pure CB1190 culture within a 250 mL serum bottle. The culture was incubated with 3 sequential additions of 100 mg/L 1,4-dioxane at 30°C and 150 rpm shaking. After the final addition of 1,4-dioxane was degraded to below GC-

FID detection, the GAC was washed with 3 equal volumes of 1,4-dioxane-free AMS to remove planktonic cells before finally being resuspended in AMS containing 100 mg/L 1,4-dioxane. CVOCs were added to select bottles in the following conditions: 0 mg/L CVOCs, 2.5 mg/L TCE, 2.5 mg/L cDCE, 2.5 mg/L 1,1-DCE, and 2.5 mg/L each. Duplicate reactors were used and samples were collected for 1,4-dioxane analysis as previously described. Once 1,4-dioxane levels dropped below detection or degradation began to slow, 1,4-dioxane levels were increased by 100 mg/L. Bioaugmented reactor results were compared against those from reactors containing only abiotic GAC for each condition.

Similar experiments were conducted with zeolite. 0.02 g of zeolite was bioaugmented with CB1190 in the same way as the GAC except the solution and bottle volumes were changed to 30 and 150 mL, respectively. At the start of the experiment, AMS containing 1 mg/L of 1,4-dioxane was added. CVOCs were added according to table 1. Each condition was conducted in triplicate. Periodic 1,4-dioxane samples were collected for GC-MS analysis as previously described. When 1,4-dioxane levels dropped below GC-MS detection, they were increased by 1 mg/L. Bioaugmented zeolite data was compared against reactors containing abiotic zeolite with only 1,4-dioxane.

Experiments were also conducted to compare the relative affinities of 1,4-dioxane and the CVOCs for adsorption on the zeolite. Single solute and bisolute (1:1 molar ratio of solutes) systems were tested over a range of concentrations to generate isotherms and gain insight into the order of adsorption affinities of the solutes for adsorption.

Table 1. Bioaugmented Zeolite Batch Conditions Including 1,4-Dioxane and CVOCs

1,4-Dioxane	TCE	cDCE		1,1-DCE	
mg/L	mmol/L	mg/L	mmol/L	mg/L	mmol/L
1	0		0		0
1	0.05	6.57	0		0
1	0		0.05	4.84	0
1	0		0		0.05
1	0.5	65.7	0		0.05
1	0		0.05	4.84	0.05
1	0.05	6.57	0.05	4.84	0.05
1	0.01	1.31	0		0
1	0.1	13.14	0		0
1	0.5	65.7	0		0
1	0		0.01	0.97	0
1	0		0.1	9.69	0
1	0		0.5	48.47	0
1	0		0		0.01
1	0		0		0.1
1	0		0		0.5

Task 5. Effects of Chlorinated Solvent Remedial Technologies on 1,4-Dioxane Biodegradation in Columns Constructed with Media from an Impacted Site

Column experiments were conducted as we previously described in Zhao et al. (2018). Soil that had been previously exposed to enhanced reductive dechlorination (ERD) treatment was used as the solid matrix for the experiments. The soil was then subjected to the following treatments: autoclave sterilized, untreated under oxic conditions, untreated under anoxic conditions, and bioaugmented with CB1190. Columns were conducted under sequential conditions starting with groundwater containing only 1,4-dioxane, followed by the additions of 5 and then 10 mg/L 1,1-DCE. The columns were then again fed groundwater containing only 1,4-dioxane followed by the addition of Cu(II) (10 mg/L).

Task 6. Analysis of microbial compositions and dynamic changes during the chemical and biological processes treating 1,4-dioxane by hydrogen peroxide under various co-contaminants exposure conditions.

Synergistic oxidation-biodegradation treating process was conducted to alter oxygen by hydrogen peroxide in 1,4-dioxane degradation process. Five sets of microcosms were established, including high 1,4-dioxane high CVOCs (H-DX/H-CV), high 1,4-dioxane low CVOCs (H-DX/L-CV), low 1,4-dioxane low CVOCs (L-DX/L-CV), low 1,4-dioxane-only (L-DX), and bioaugmentation under high 1,4-dioxane low CVOCs (Bioaug). Oxidation treatment involved

amending site materials with hydrogen peroxide, and microcosm bottles were prepared with 50 ml groundwater and 10 g soil, as well as 100 mg $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$. 10% sulfuric acid was added to achieve a pH at 4.5. Bottles were incubated at room temperature and rotated with 150 rpm. After 12 hours, the H_2O_2 oxidation process was quenched with NaOH (Miao et al., 2018). Then, same bottles were maintained in a shaking incubator at 30°C and 150 rpm shaking for more than 10 weeks. During this process, 1,4-dioxane was spiked to ensure concentrations were consistent with site conditions. Aseptic techniques were used throughout the experiment to prevent contamination. In addition to active microcosm treatments, a sterile control to correct for abiotic losses of 1,4-dioxane, and a positive control bioaugmented with *Pseudonocardia dioxanivorans* CB1190 was also tested (Bioaug). Slurry samples were collected before (OB1) and after (OB2) oxidation, as well as after first time bioaugmentation (OB3), and after 10 weeks (OB4). The change in 1,4-dioxane concentration over time was measured during the treatment train. Gene abundance was quantified using qPCR for different bacterial populations and qPCR for monooxygenase genes involved in 1,4-dioxane biodegradation. Microbial community analysis was used to reveal whole community structure shift during the treatment, and dynamic trends over time.

Analytical Methods

Quantification of 1,4-Dioxane Concentrations

1,4-Dioxane concentrations greater than 1 mg/L were quantified using a GC-FID as previously described (Pornwonthong et al., 2014). Concentrations of 1,4-dioxane below 1 mg/L were quantified using a Hewlett-Packard 6890 chromatograph equipped with a mass spectrometer detector and a Supelco SPB-1 Sulfur column (30 m x 0.32 mm id x 4 μm) (GC-MSD). Prior to injection, the collected aqueous sample was extracted using a frozen microextraction procedure (Li et al., 2011). 5 μL of processed sample was injected into the GC-MSD fitted with a pulsed splitless injection with an inlet temperature of 150°C, 77 kPa and a pulsed pressure of 170 kPa for 2 min, purge flow of 15.0 mL min^{-1} . The oven temperature program was as follows: initial hold at 35°C for 5 minutes, then increased to 100°C at a rate of 20°C min^{-1} and further raised to 275 °C at a rate of 50°C min^{-1} . The MSD was operated to use an EM offset of 400 and EM voltage of 2000 V. The MS quadrupole was programmed at 150°C and source at 230°C.

Quantification of Chlorinated Solvent Concentrations

Headspace concentrations of TCE, TCA, 1,1-DCE and cDCE were individually quantified by Hewlett-Packard 6890 GC-FID (Hewlett-Packard, Atlanta, GA) with a Restek® Stabilwax-DB capillary column (30 m $\times$ 0.53 mm $\times$ 1 μm ; Restek, Bellefonte, PA, USA). Injector and detector temperatures were set at 220°C with 7.4 mL min^{-1} of carrier gas flow rate, and 250°C, respectively. The oven was initially set at 100 °C for 1 min then increased at 25 °C/min to 150 °C and held constant for 30 seconds. In the mixture experiments, 3500 Capillary gas chromatograph equipped with electron capture detector (GC-ECD) was used to quantify 0-5000 $\mu\text{g/L}$ TCE, TCA and 1,1-DCE, with an Agilent DB-5 capillary column (30m $\times$ 0.25mm $\times$ 0.25 μm ;, USA). 50 μL headspace samples was injected to GC-ECD that injector was set at 260°C and detector was set at 310 °C. The oven temperature was held at 35 °C for 30 min with 9.6 mL min^{-1} of column flow rate. The cDCE concentration from 0-5000 $\mu\text{g/L}$ was measured by GC-FID with constant oven temperature at 130 °C for 7 min. The flow rate was 1.7 mL min^{-1} and other parameters were same as described for individual solvents. Headspace concentrations of VC was also quantified by Hewlett-Packard 6890 GC-FID (Hewlett-Packard, Atlanta, GA) with a Restek® Stabilwax-DB capillary column (30 m $\times$ 0.53 mm $\times$ 1 μm ; Restek, Bellefonte, PA, USA). Injector

and detector temperatures were set at 220°C with 10.2 mL min⁻¹ of carrier gas flow rate, and 250°C, respectively. The oven was initially set at 100 °C for 1 min then increased at 25 °C/min to 150 °C and held constant for 30 seconds.

Quantification of Hexavalent Chromium Concentrations in Aqueous Solutions

Hexavalent chromium was quantified using a spectrophotometric method modified from EPA Method 7196a. Briefly, 95 µL of the aqueous sample was collected in 1.7 mL microcentrifuge tubes. 2 µL of 1,5-diphenylcarbazide solution (5 mg 1,5-diphenylcarbazide per 1 mL acetone) was added to the sample. Samples were acidified with H₂SO₄ to a pH of 2 ± 0.5 and the volume was adjusted to 100 µL with sterile water. Samples were then incubated at room temperature for 5 minutes to allow color development and absorbance was determined at 540 nm. Concentrations were determined by comparison to standards of known concentration.

Total Nucleic Acid Extraction, RNA Purification and cDNA Synthesis

To extract total nucleic acids from samples, a modified phenol-chloroform extraction method was used as described previously (Gedalanga et al., 2014). RNA was purified from total nucleic acid extractions by treatment with DNase I (Life Technologies, Carlsbad, CA) following the manufacturer's protocol. The cDNA was synthesized from purified RNA using a Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Scientific, Waltham, MA) following the manufacturer's suggestion. Total nucleic acids and synthesized cDNA were stored at -80°C until ready for use.

Quantitative Polymerase Chain Reaction (qPCR)

Gene abundance analysis was performed using primers targeting a universally conserved region of the 16S rRNA gene and/or a region of the 16S rRNA gene specific to CB1190 and/or JOB5. Gene expression analysis included quantification of a housekeeping gene (*rpoD*), the 1,4-dioxane biomarkers (DXMO and ALDH) and universal stress protein gene (*usp*) by qPCR of synthesized cDNA as previously described (Gedalanga et al., 2014). In addition, copper and cadmium resistance genes were identified and evaluated for gene expression in the metal experiments with JOB5. All qPCR and RT-qPCR reactions were analyzed with an Applied Biosystems StepOne Plus (Life Technologies, Carlsbad, CA) using a total volume of 20 µL containing: 1× Luminaris Color HiGreen qPCR Master Mix (Thermo Scientific, Waltham, MA), 0.3 µM of primers, and 2 µL of DNA template. Primers for all gene targets are listed in Table 1. The cycling parameters to amplify the gene fragment included 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 45 s. All reactions were accompanied by a melt curve analysis to confirm the specificity of qPCR products. Gene expression was normalized to housekeeping gene and initial time point.

Table 2. Sequences of primers used in this study.

Primers	Sequence
Universal 16S rRNA for	5'-ATGGCTGTCGTCAGCT-3'
Universal 16S rRNA rev	5'-ACGGGCGGTGTGTAC-3'
CB1190 16S rRNA for	5'-ACGGTCTCGCAGCCCTCTGT-3'
CB1190 16S rRNA rev	5'-AGCGGGTTATGCCGGGGACT-3'
JOB5 16S rRNA for	5'-AATTCCTGGTGTAGCGGTGG-3'
JOB5 16S rRNA rev	5'-CCCAAGGAAGGAAACCCACA-3'
rpoD for	5'-GGGCGAAGAAGGAAATGGTC-3'
rpoD rev	5'-CAGGTGGCGTAGGTGGAGAA-3'
DXMO for	5'-CCAAACGGGCGTCAGTCAT-3'
DXMO rev	5'-AGAACGTGCGCTCCCAAAG-3'
ALDH for	5'-GCCGACGCTTTTAGCAGATG-3'
ALDH rev	5'-TCATTAACGGCAGCAAACGC-3'
usp A for	5'-GACAGCCCAGTACCAGCAG-3'
usp A rev	5'-ATGGGAATGACCACGCTCG-3'
ALKMO for	5'-GCCCTGATGTTCTCCTCGTGAT-3'
ALKMO rev	5'-CTCCACAACCAGCACGCC-3'
JOB5 czc for	5'-ATGTTTCATGGGCCTGACCG-3'
JOB5 czc rev	5'-TCGAAGGCCTCGTAGAGGAT-3'
JOB5 copD for	5'-TTCATCGTCGGGTTCTACGG-3'
JOB5 copD rev	5'-CTGAGCAGGAAGTGCAGGTT-3'

Illumina Miseq Sequencing and Microbial Community Analysis

The hypervariable V3–V4 regions of 16S rRNA gene was chosen to be sequenced on the Illumina Miseq platform (Miao et al., 2019). Each condition had triplicate DNA samples from triplicate microcosm bottles, respectively. The three DNA samples were mixed and analyzed via duplicate PCR reactions. Thus, each condition had two PCR products. The PCR reaction system was 50 μ L containing 2 $\times$ EasyTaq[®] PCR SuperMix (25 μ L), forward primer (2 μ L, 10 μ mol/L) and reverse primer (2 μ L, 10 μ mol/L), 40 ng sample genomic DNA and 21 μ L double deionized H₂O, with annealing at 55°C. PCR products were sent to Laragen, Inc. (Culver City, California, USA) for further purification and additions of sample specific dual-index barcodes, then pooled and run Illumina Miseq sequencing, using MiSeq v3 reagents to generate paired 300-bp reads. The generated 16S rRNA gene raw reads were pooled together and preprocessed in Mothur (v 1.39.5) (Schloss et al., 2009). The contigs were assembled from paired-end reads by using “make.contigs”. Then the successfully formed contigs were trimmed by using “trim.seqs” with zero ambiguous bases and an average quality cutoff at 30 (Miao et al., 2018). The trimmed contigs were aligned and classified with Silva database, but taxa in unknown, mitochondria, chloroplasts, or Eukaryota were removed by command “remove.lineage”. The chimera cross samples were detected based on de novo and referenced by using “chimera.vsearch”. Finally, high quality reads in each sample were normalized at the same sequencing depth of 30000 reads. The normalized reads were clustered into operational taxonomic units (OTUs) by “cluster.split” at 97% identity, resulting in a total of 3412 OTUs, and each OTU was represented by at least 10 reads on average across all samples. The α -diversity indices (rarefaction) and β -diversity

(weighted UniFrac distances) metrics based on the normalized OTUs table were calculated by the “summary.single” and “unifrac.weighted” commands, respectively. The similarity of different samples was shown by cluster analysis (CA) and principal coordinates analysis (PCoA), which classified samples belonged to different conditions into multiple groups by using OTU abundances on the normalized OTUs table.

Network analysis based on correlations, which was previously applied to study microbial co-occurrence/co-exclusion species-species associations (SSA) patterns (Chao et al., 2016; Ju et al., 2017), was calculated using an average relative abundance of 97% similarity OTUs (0.97-OTUs). The algorithm was based on the non-parametric Spearman’s rank correlation with strict coefficients at ± 0.6 and significant cutoff p-value at 0.01 (Barberan et al., 2012).

RESULTS AND DISCUSSION

Effects of Chlorinated Solvents on Metabolism of 1,4-Dioxane at High Concentrations

Planktonic CB1190 cells were able to biodegrade 1,4-dioxane in the presence of chlorinated solvents at low concentrations. Metabolic biodegradation of 100 mg/L 1,4-dioxane was not inhibited by concentrations up to 3 mg/L TCE, 0.05 mg/L 1,1-DCE, 0.5 mg/L cDCE, and 30 mg/L TCA (Table 2). However, the degradation byproducts of those chlorinated solvents, 1,1-DCE and cDCE produced the greatest inhibition to metabolic biodegradation by CB1190.

Further investigation determined that exposure to chlorinated solvents extends the lag phase of growth by CB1190 and decreases the biodegradation rates. For instance, in Figure 1, the biodegradation rate was not significantly different between TCE-Free and 10 mg/L TCE conditions, while the lag phase was delayed 2 days in the presence of 10 mg/L TCE. Increasing chlorinated solvent's concentration produced stronger inhibition both in extended lag phases and lower degradation rates. After 1,4-dioxane was depleted in the cell culture, ~ 100 mg/L 1,4-dioxane was added to the culture to examine whether the solvents kill bacteria, inactivate the biodegradative enzyme, or compete with 1,4-dioxane in CB1190 cells. Interestingly, the inhibition was determined to be reversible.

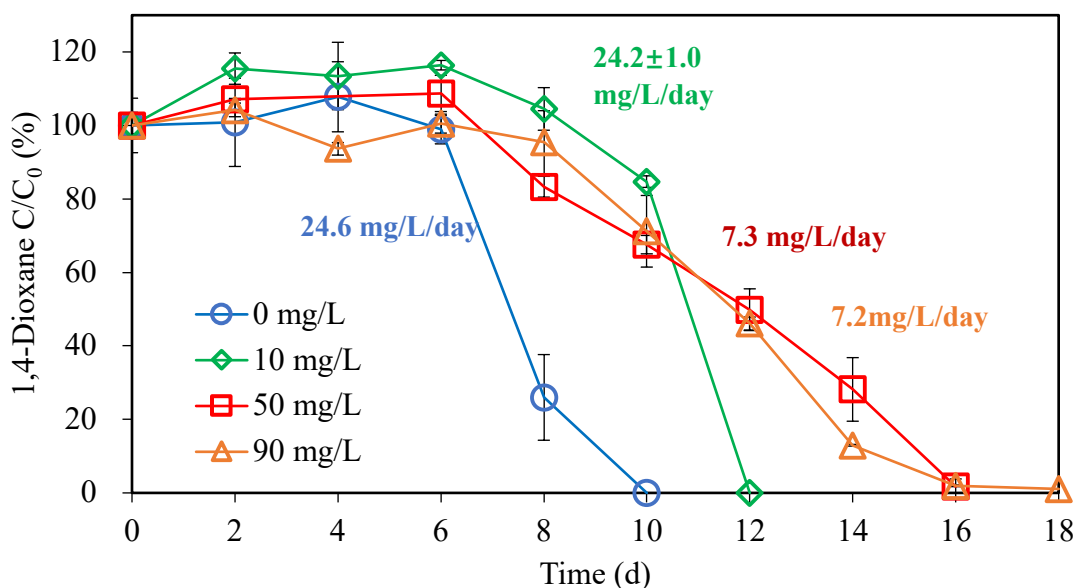


Figure 1. Effects of TCE on 1,4-dioxane biodegradation by CB1190. Increasing concentrations of TCE resulted in extended lag phases and slower biodegradation rates. Error bars represent the range of duplicates.

Table 3. Rates of 1,4-dioxane biodegradation by CB1190 in the presence of chlorinated solvents (100 mg/L initial 1,4-dioxane; N=2)

Solvent Level (mg/L)	1,4-Dioxane Degradation Rate (mg/L·d)			
	TCE	1,1-DCE	cDCE	TCA
0	24.6 ± 0.7	15.5 ± 0.04	25.9 ± 0.02	18.1 ± 0.9
0.05	-	14.5 ± 0.2	20.3 ± 2.7	12.4 ± 2.4
0.5	-	9.2 ± 0.7	20.9 ± 0.23	-
1	-	× ^d	-	12.7 ± 2.2
2.5 ^a	10.8 ± 0.5	× ^d	14.9 ± 0.4	-
5	-	× ^d	11.9 ± 1.0	-
10	24.2 ± 1.0	× ^d	11.1 ± 3.4	16.4 ± 3.1
25 ^c	-	× ^d	11.7 ± 3.1	12.7 ± 3.8
50	7.3 ± 1.3	× ^d	6.5 ± 0.3	11.1 ± 0.9
100 ^b	7.3 ± 0.5	× ^d	× ^d	-
125	-			3.7 ± 4.4
Inhibition	Non-competitive	Non-competitive	competitive	Non-competitive

^aThe actual TCE concentration detected by GC-FID was 3 mg/L

^bThe actual TCE concentration detected by GC-FID was 90 mg/L

^cThe actual TCA concentration detected by GC-FID was 20 mg/L

^dNo biodegradation was observed during the 30 day study.

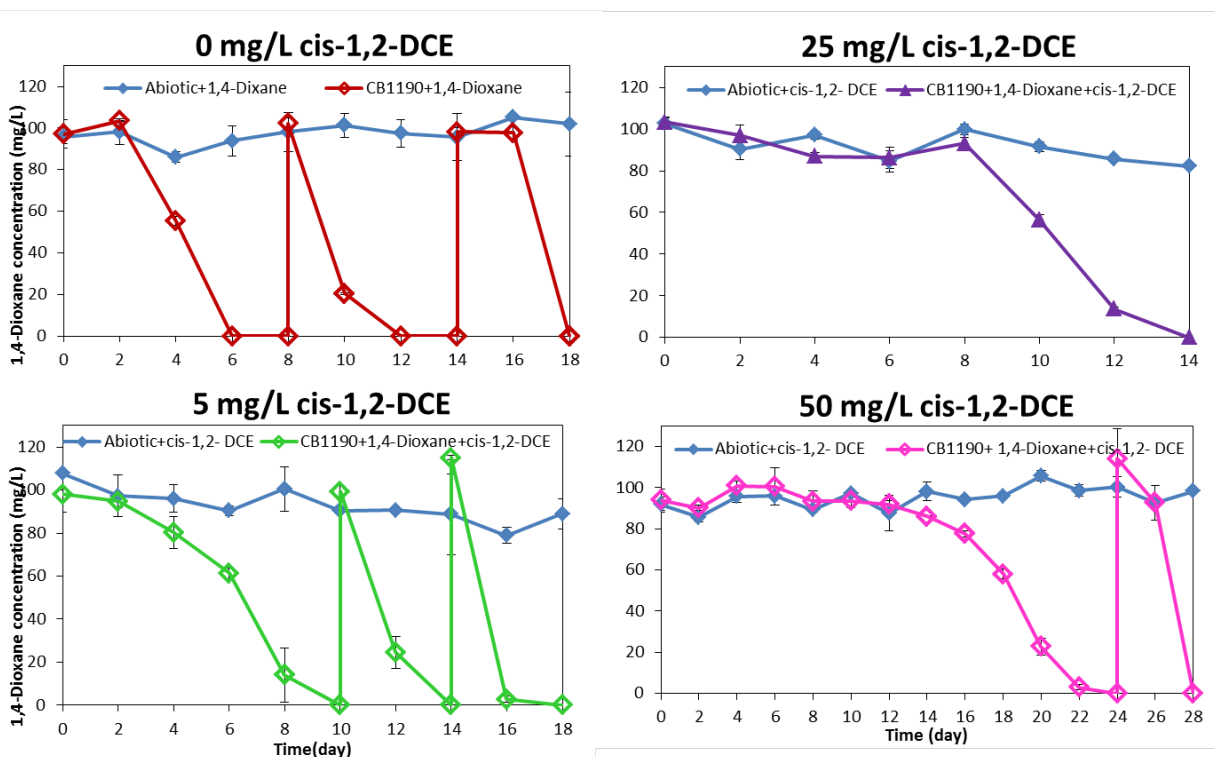


Figure 2. Effects of cDCE on 1,4-dioxane biodegradation by CB1190. The presence of higher concentrations of cDCE elongated lag phase, however inhibition was reversible. Error bars represent the range of duplicates.

During 1,4-dioxane biodegradation, concentrations of chlorinated solvents were monitored to identify whether solvents were potentially competing with 1,4-dioxane as substrates for CB1190. Our results indicated that CB1190 was unable to metabolize or cometabolize TCA, TCE, and 1,1-DCE, however cDCE concentrations were diminished over time (Figure 16). Concentrations as low as 5 mg/L cDCE increased the time to completely remove 100 mg/L 1,4-dioxane. During the first 2 days of the experiment, intermediate and high cDCE exposed bottles remained near initial concentrations of 5 and 50 mg/L, respectively. cDCE was significantly removed in the intermediate and high cDCE conditions by day 7 and after day 17, respectively. In both treatments, 1,4-dioxane biodegradation was rapid when cDCE concentrations were removed to less than 4 mg/L (Figures 2 and 3). Subsequent spikes with 1,4-dioxane indicated that the inhibition was reversible and likely to be competitive inhibition (Figure 3). These results highlight the potential for CB1190 to concurrently remove both cDCE and 1,4-dioxane from contaminated aquifers.

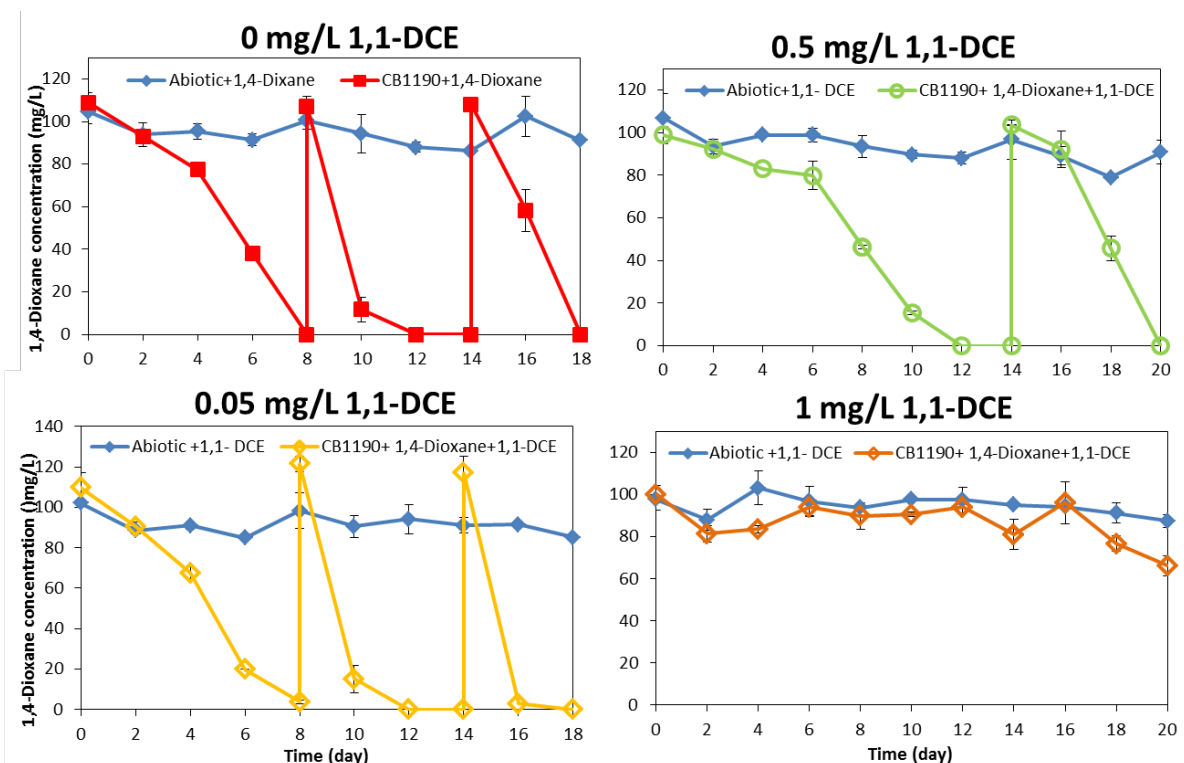


Figure 3. Effects of 1,1-DCE on 1,4-dioxane biodegradation by CB1190. 1,4-Dioxane biodegradation was strongly inhibited in the presence of 1 mg/L 1,1-DCE. All experiments were performed in triplicate and error bars represent the standard deviation.

The average 1,4-dioxane biodegradation rate at high concentrations of 1,4-dioxane was 14.5 mg/L/d in the absence of chlorinated compounds. This rate was decreased to 9.2 mg/L/d when the cells were exposed to 0.5 mg/L 1,1-DCE (Figure 3). TCA did not inhibit 1,4-dioxane metabolism at concentrations up to 30 mg/L (Figure 4) displaying average biodegradation rates of 12.1 mg/L/d. This rate was decreased to 7.4 mg/L/d when exposed to 50 mg/L TCA. Unlike cDCE, TCA was not biodegraded by CB1190 for the duration of this study.

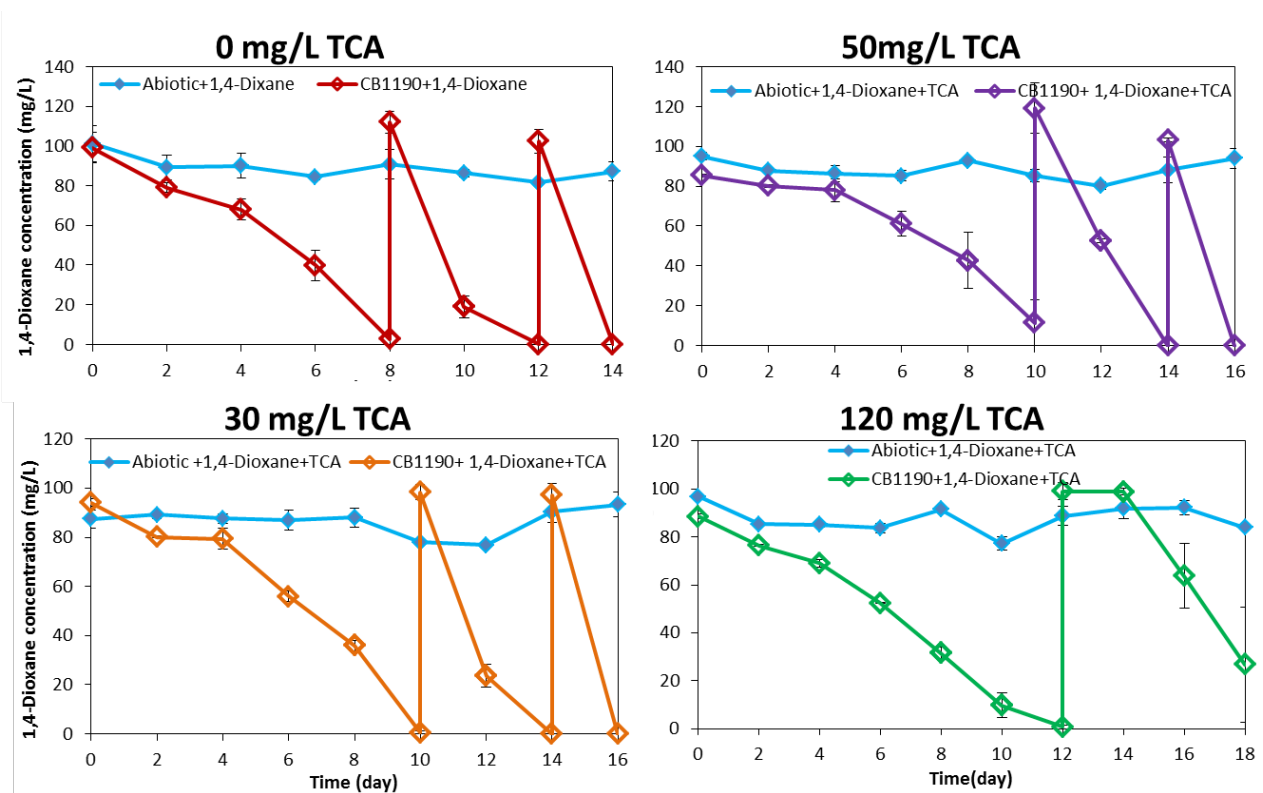


Figure 4. Effects of TCA on 1,4-dioxane biodegradation by CB1190. 1,4-Dioxane biodegradation was somewhat inhibited in the presence of 120 mg/L 1,1,1-TCA. All experiments were performed in triplicate and error bars represent the standard deviation.

Two experiments with different 1,4-dioxane and VC concentration ranges were conducted to explore degradation efficiency by CB1190. The first batch was set with specific 2 mg/L 1,4-dioxane and 40, 280, and 780 $\mu\text{g/L}$ VC. The bottles were monitored over 12 days, and VC with 40 $\mu\text{g/L}$ initial concentration was completely removed in 48 hours (Figure 5), regardless of the presence of 1,4-dioxane, and 99% 1,4-dioxane in this condition was removed at the same time (Figure 6). However, the VC degradation rate decreased when ~ 40 $\mu\text{g/L}$ VC was spiked again after 48 hours, and the complete removals were achieved over 72 hours. In other two VC conditions, VC was initially degraded faster in the first 48 hours and tended to be flat afterwards. The presence of 1,4-dioxane had little to no impact on 280 $\mu\text{g/L}$ VC degradation when comparing bottles with and without 1,4-dioxane, while 1,4-dioxane seemed to stimulate 780 $\mu\text{g/L}$ VC degradation after first 72 hours because of the potential cometabolism of VC by CB1190. Furthermore, the higher VC level showed inhibitory impacts on 1,4-dioxane degradation, that 22.5% and 42.5% 1,4-dioxane were removed in first 48 hours, under 780 and 280 $\mu\text{g/L}$ VC condition, respectively. The qPCR result indicated that the addition of VC could reduce the biomass based on total 16S rRNA gene abundance, except for the 780 $\mu\text{g/L}$ VC with 2 mg/L 1,4-dioxane condition. However, the gene abundances rebounded after 48 hours, especially in the conditions with 1,4-dioxane spikes, where the gene abundances were even higher than initial numbers (Figure 7). Besides, the first-order degradation of VC was calculated based on

first 6-hour results and normalized to 16S rRNA gene copy numbers, and the simulated degrading rate constant demonstrated that the higher VC had relatively higher initial degradation rates, while the additions of 1,4-dioxane had no clear impacts on that (Table 4).

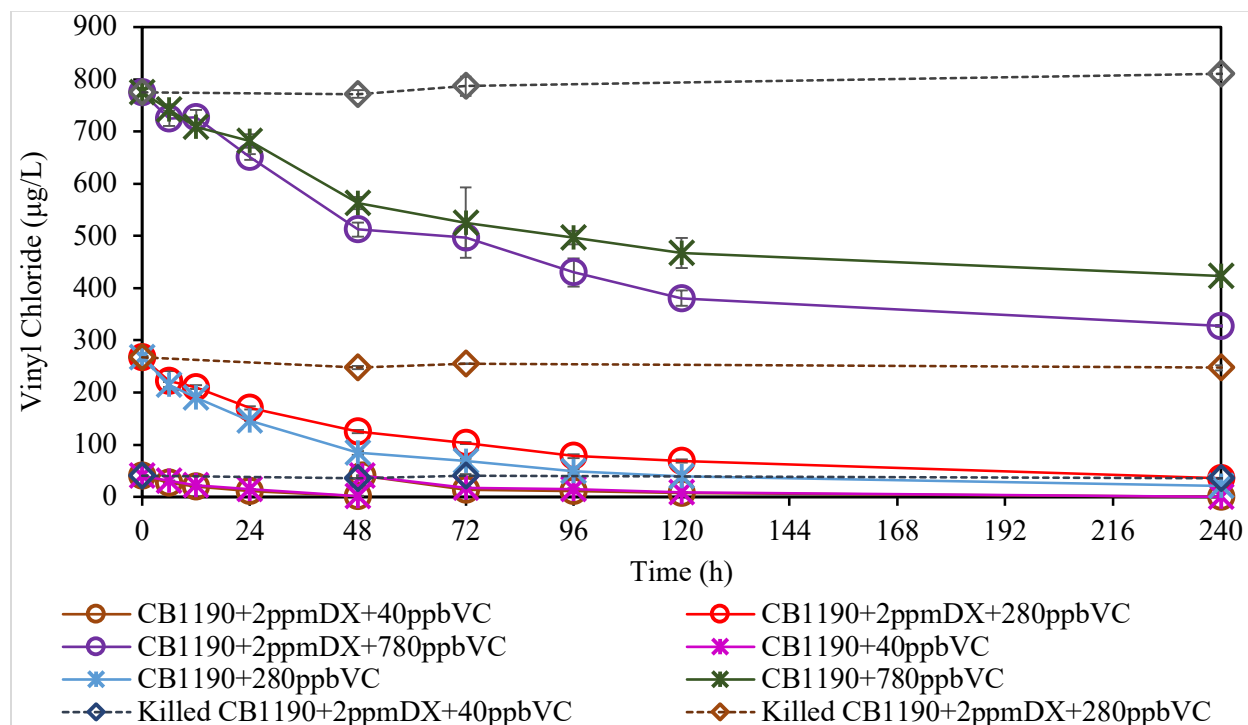


Figure 5. CB1190's ability to biodegrade varying concentrations of VC under 0 and 2 mg/L 1,4-dioxane. Higher VC concentrations corresponded with decreasing VC degradation rates. All experiments were performed in triplicate and error bars represent the standard deviation.

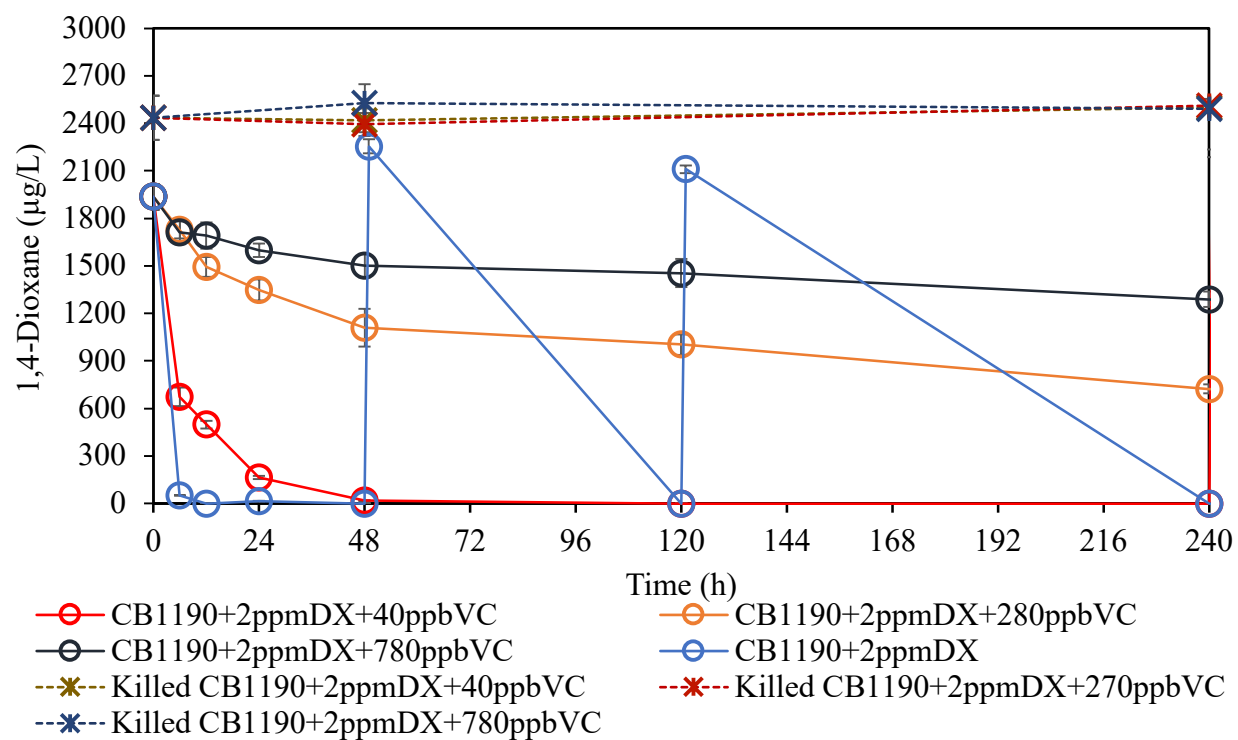


Figure 6. 1,4-Dioxane biodegradation by CB1190 under various VC concentrations. Higher VC demonstrated greater inhibition on 1,4-dioxane degradation. All experiments were performed in triplicate and error bars represent standard deviation.

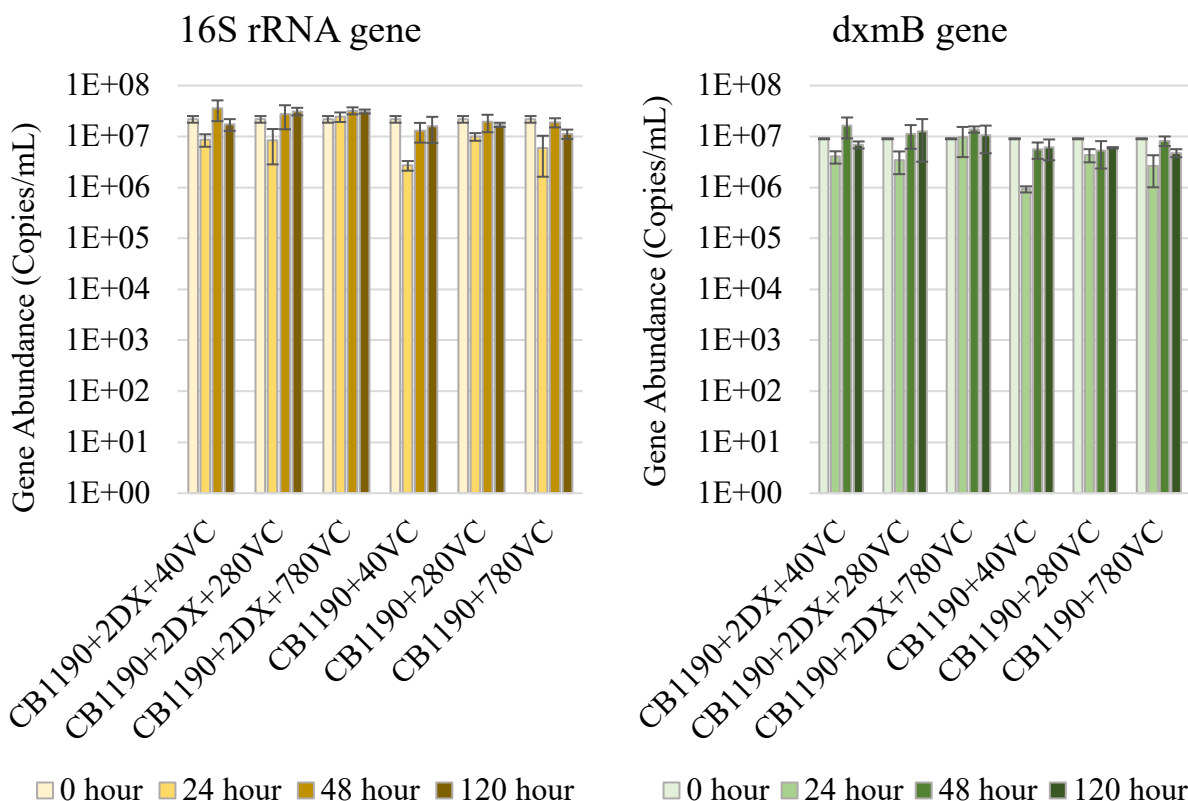


Figure 7. Abundances of total bacterial gene (16S rRNA) and dioxane monooxygenase gene (*dxmB*) under different conditions. Genes were reduced after VC firstly added, then rebounded after 24 hours.

The second batch experiment included more 1,4-dioxane concentrations at 1, 5, and 10 mg/L, with multiple initial VC concentrations at 85, 420, 1400, 4200, and 8500 $\mu\text{g/L}$. The bottles experienced the lag phase in the presence of VC and 1,4-dioxane started to degrade after 10 hours. Same to the previous experiment, the higher 1,4-dioxane degradation rates were achieved under lower VC concentrations, and the more different degradation rates were observed under higher 1,4-dioxane condition (Figure 8). Regarding to VC degradations, the lowest (85 $\mu\text{g/L}$) showed 20-27% degradation, higher than other conditions, while no clear impact of 1,4-dioxane was found on VC degradation (Figure 9). When focused on 1,4-dioxane degradation functional genes, the abundance was around 10^8 copies/mL in all conditions (Figure 10), while only *dxmB* gene up-regulated under 5 and 10 mg/L 1,4-dioxane conditions (Figure 11). These observations suggest that while simultaneous degradation of 1,4-dioxane and VC by CB1190 is possible, high VC concentrations inhibit 1,4-dioxane degradation, while 1,4-dioxane could feed CB1190 to enhance cometabolism of high level of VC.

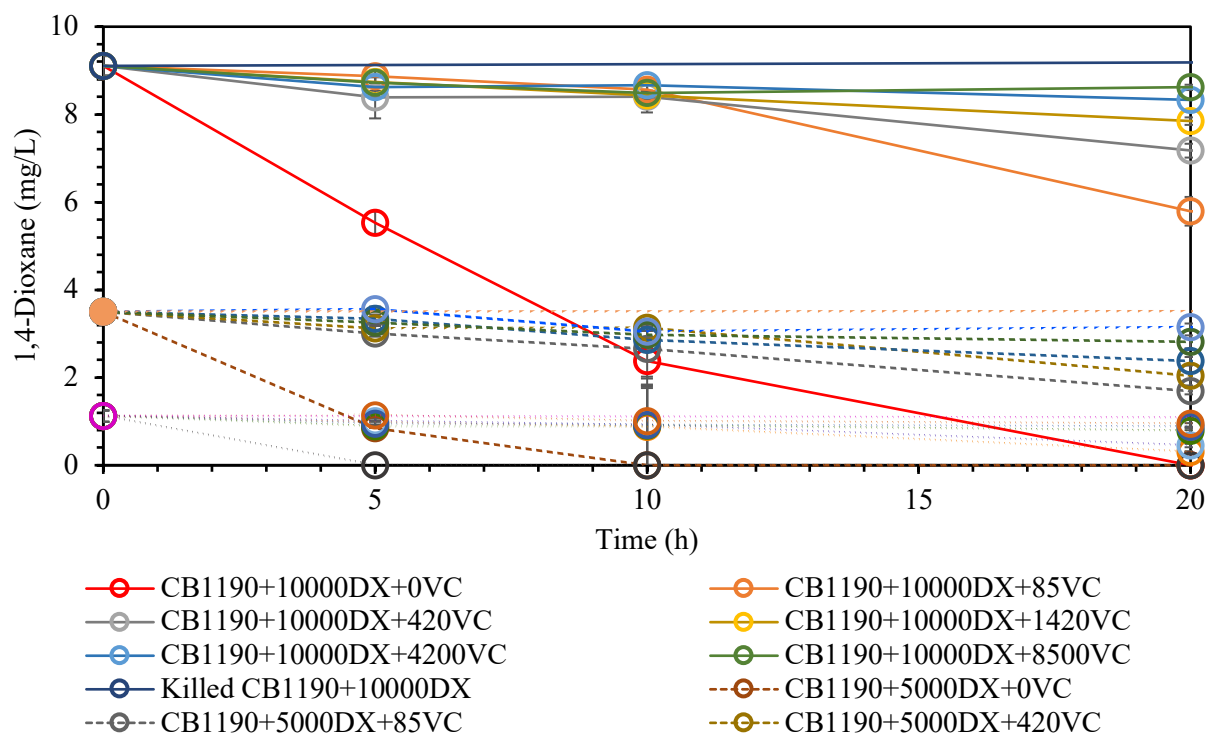


Figure 8. 1,4-Dioxane biodegradation by CB1190 under varying VC concentrations. The higher the VC concentration, the greater the inhibition on 1,4-dioxane biodegradation.

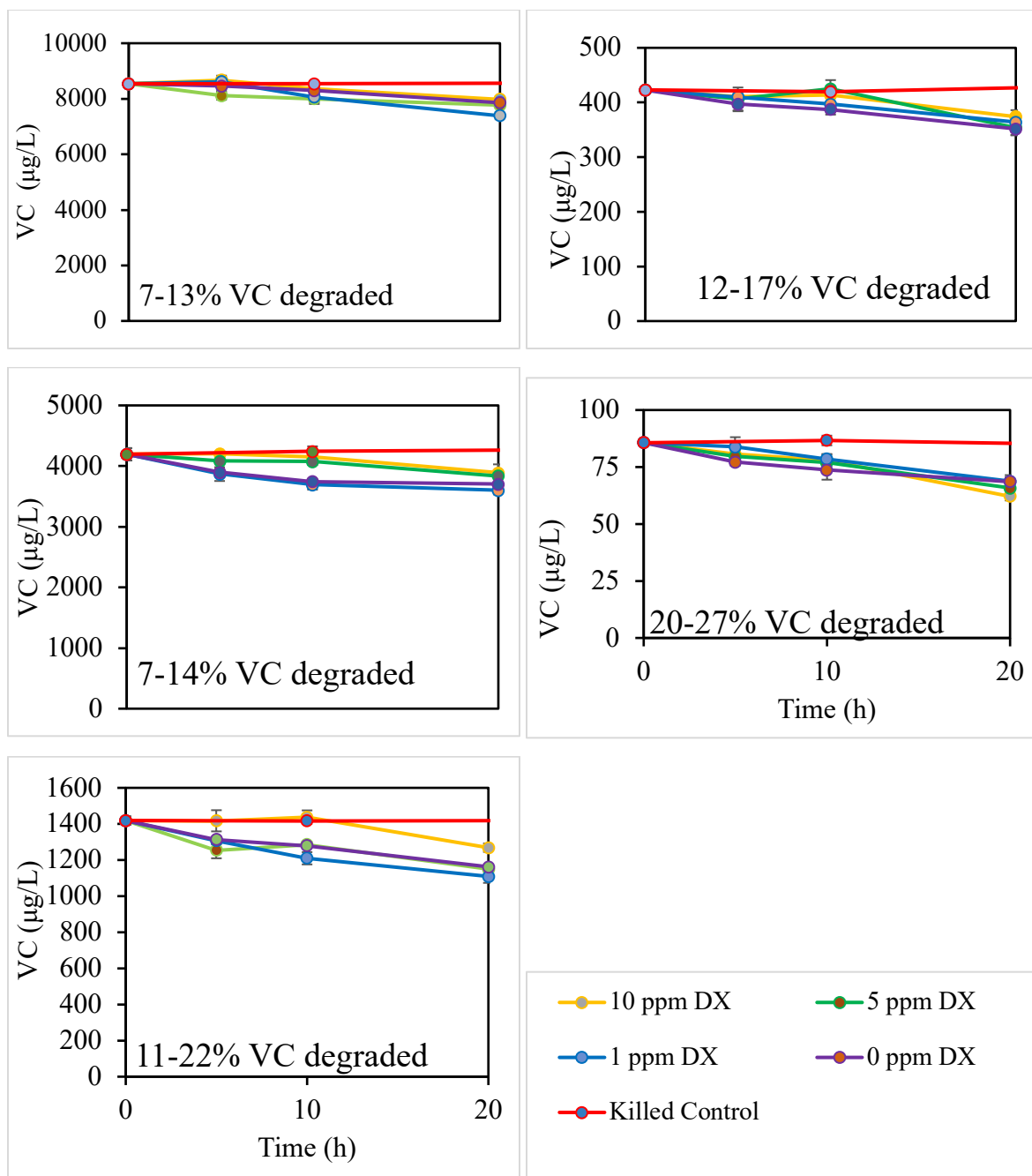


Figure 9. Biodegradation of high and low VC by CB1190 under varying 1,4-dioxane concentrations. Lower VC concentrations showed higher degradation rates without a direct 1,4-dioxane impacts.

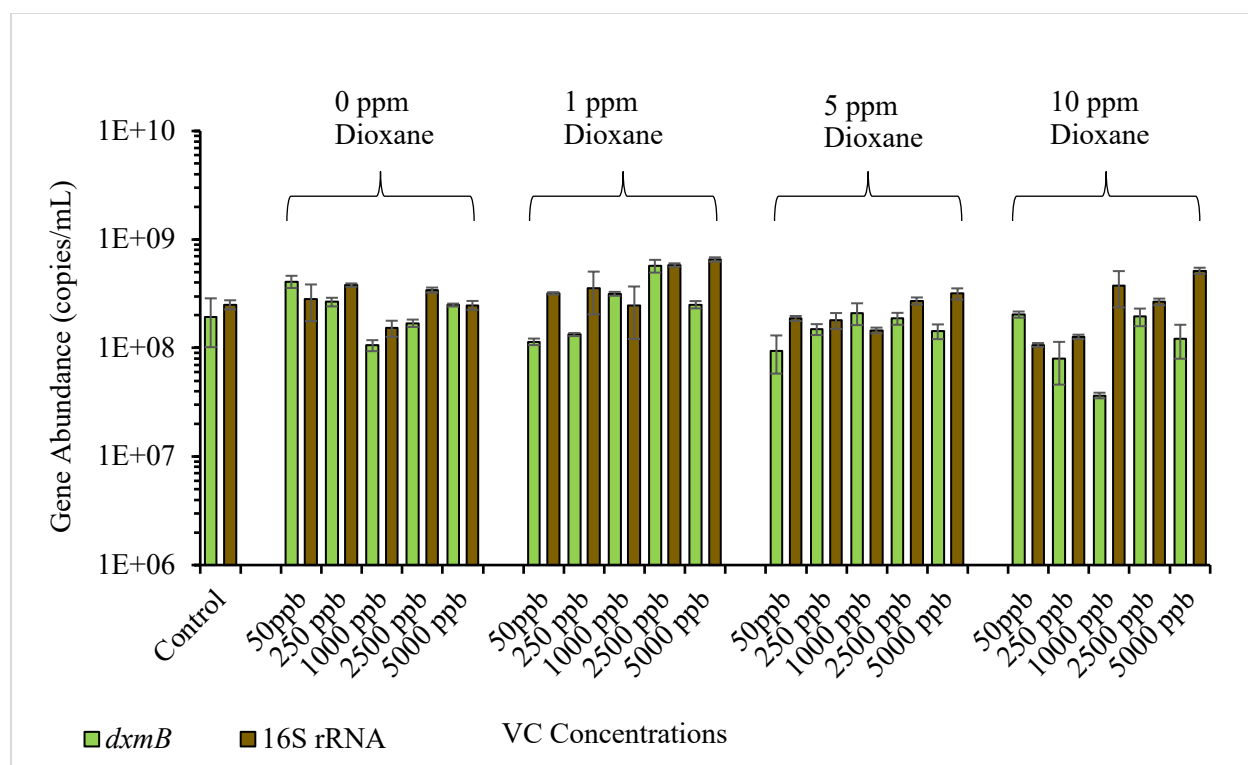


Figure 10. Abundances of total bacterial gene (16S rRNA) and dioxane monooxygenase gene (*dxmB*) under high and low VC and 1,4-dioxane conditions. There was no significant difference in gene abundance between the control or experimental conditions. All experiments were performed in triplicate and error bars represent the standard deviation.

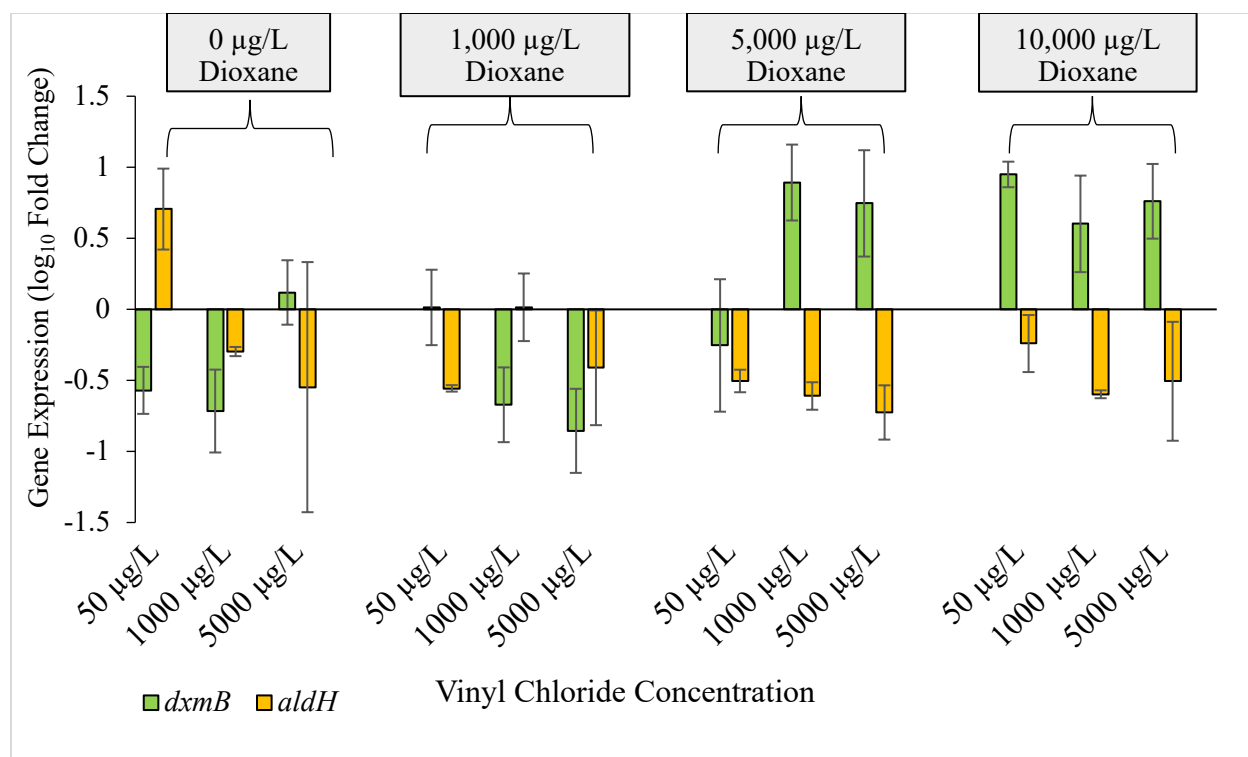


Figure 11. Expression of dioxane monooxygenase gene (*dxmB*) and aldehyde dehydrogenase gene (*aldH*) under high and low VC and 1,4-dioxane conditions. The *dxmB* gene was only upregulated under higher 1,4-dioxane conditions regardless of the VC concentration. Samples were normalized to day 0 and the housekeeping gene, *rpoD*. All experiments were performed in triplicate and error bars represent the standard deviation.

Table 4. Vinyl Chloride Degradation Rates by CB1190

VC (ppb)	Rate Constant (1/d)	
	No 1,4-Dioxane	2000 µg/L 1,4-Dioxane
40	1.18±0.06	1.42±0.05
280	0.58±0.02	0.38±0.03
780	0.15±0.01	0.20±0.01

Glucose fed CB1190 was used to test potential 1,4-dioxane monooxygenase in VC degradation, since glucose was a general carbon source that unable to induce monooxygenase. In this test, two levels of VC at 400 and 4000 µg/L were spiked in same bottles with glucose fed CB1190, and no VC degradation was achieved in 60 hours (Figure 12), indicating that VC degradation by CB1190 might be monooxygenase relevant process. Moreover, VC degradation by CB1190 could be a cometabolic process, since both *dxmB* and *aldH* remained stable in the table (Figure 13), meaning that CB1190 could not grow on VC to induce monooxygenase and dehydrogenase.

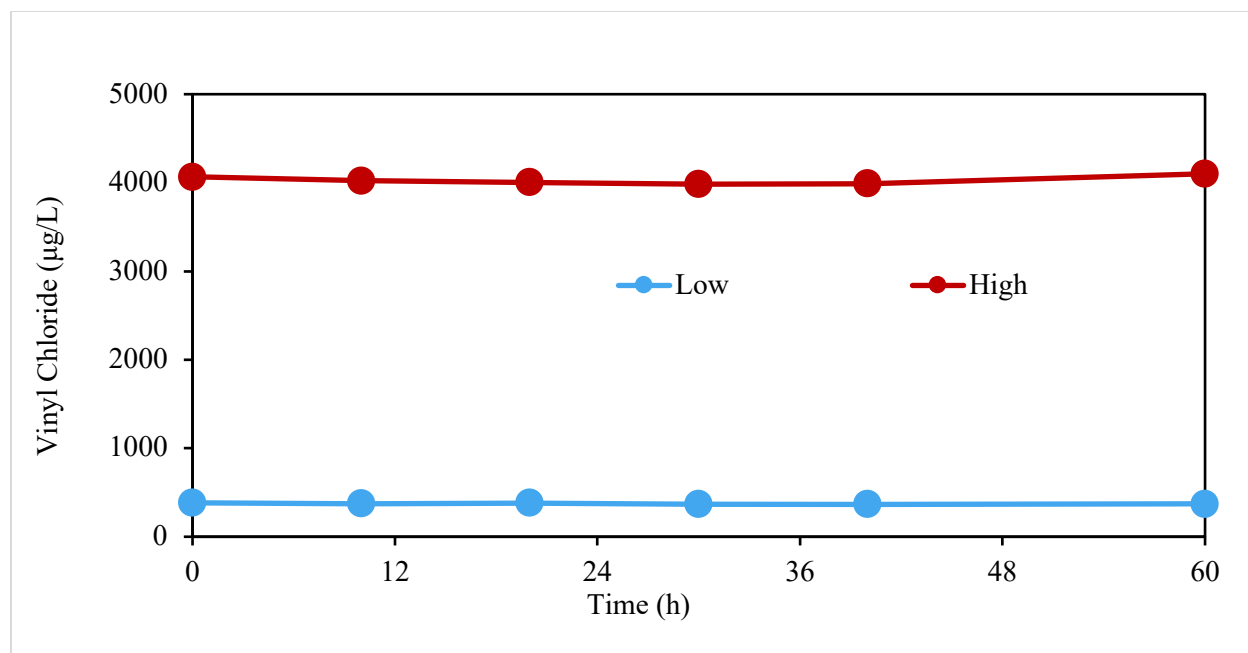


Figure 12. VC biodegradation by CB1190 grown on dextrose. No significant VC degradation was achieved by non-dioxane monooxygenases in CB1190.

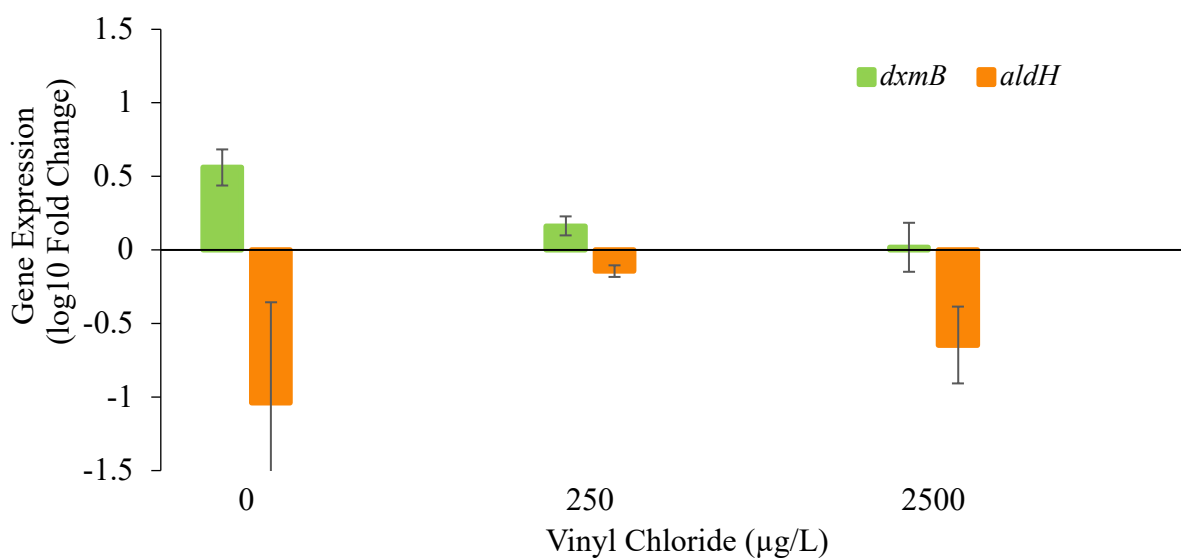


Figure 13. Expression of dioxane monooxygenase gene (*dxmB*) and aldehyde dehydrogenase gene (*aldH*) under 250µg/L and 2500µg/L VC by glucose fed CB1190. Increasing VC resulted in decreased upregulation of *dxmB*. Samples were normalized to day 0 and the housekeeping gene, *rpoD*. All experiments were performed in triplicate and error bars represent the standard deviation.

Effect of Chlorinated Solvents on Biodegradation of Low 1,4-Dioxane Concentrations

Table 5. 1,4-Dioxane biodegradation rates by CB1190 exposed to chlorinated solvents at low 1,4-dioxane concentrations (1 mg/L initial 1,4-dioxane; n=3) (Zhang et al., 2016)

Solvent Level (mg/L)	1,4-Dioxane Degradation Rate (mg/L·d)		
	TCE	1,1-DCE	cDCE
0	100.3 ± 10.0	100.3 ± 10.0	100.3 ± 10.0
0.5	95.4 ± 1.4	79.4 ± 3.7	93.9 ± 8.2
5	75.4 ± 6.3	10.0 ± 6.2	38.9 ± 2.0
50	86.4 ± 12.8	10.8 ± 2.9	21.6 ± 17.4
Inhibition	Non-competitive	Non-competitive	competitive

The results of our comprehensive analysis on the effects of TCE, 1,1-DCE, and cDCE on 1,4-dioxane metabolism are summarized in Table 5. At low concentrations of 1,4-dioxane, TCE and 1,1-DCE non-competitively inhibit metabolic biodegradation, whereas cDCE is the only evaluated solvent to show competitive inhibition. Even at low concentrations and relatively high biomass, there was inhibition of 1,4-dioxane biodegradation was similar to the high 1,4-dioxane condition (Table 2). One exception was the increased sensitivity to cDCE at 50 mg/L. Previously we found that 50 mg/L cDCE displayed partial inhibition at high 1,4-dioxane concentrations, however at low concentrations, complete inhibition was observed at this concentration. This difference can be attributed to the duration of the experiments as the low 1,4-dioxane experiments were conducted within a 24-hour period, and limited amount of 1,4-dioxane and energy available for cellular growth. During this time, biomass concentrations were confirmed to be relatively unchanged for the first 12 hours of the experiment (Figure 14). Initial CB1190 concentrations in all bottles ranged from 3.08×10^7 – 4.17×10^8 cells/L. At low initial 1,4-dioxane concentrations, 50 mg/L TCA partially inhibited 1,4-dioxane biodegradation such that greater than 50% remained after 24 hours of incubation. In addition, complete inhibition was observed at 5 mg/L 1,1-DCE and 50 mg/L cDCE.

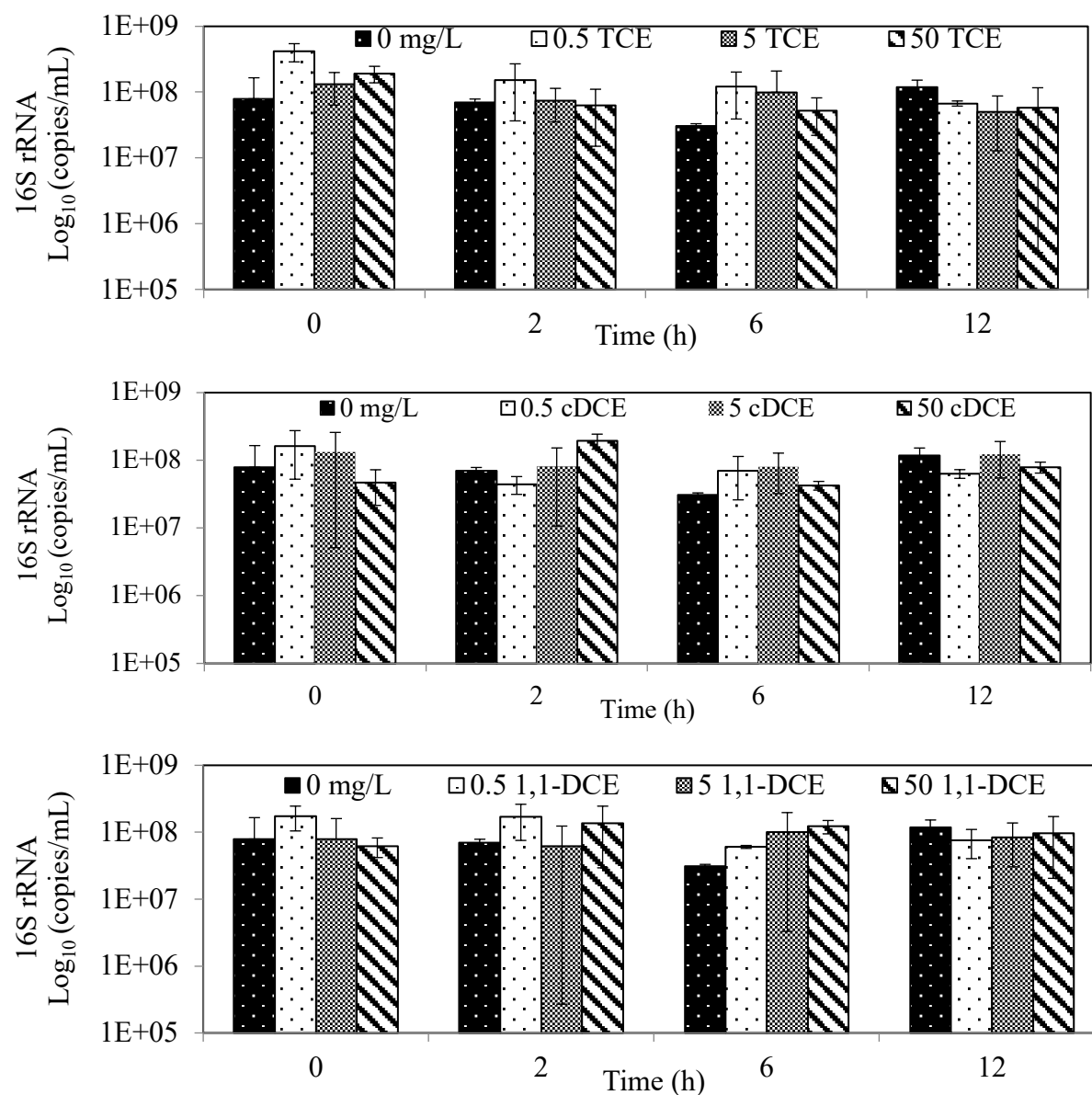


Figure 14. Abundance of CB1190 over a 12-hour period during biodegradation of 1 mg/L 1,4-dioxane in the presence of chlorinated solvents. Error bars represent standard deviation of experimental triplicates (Zhang et al., 2016).

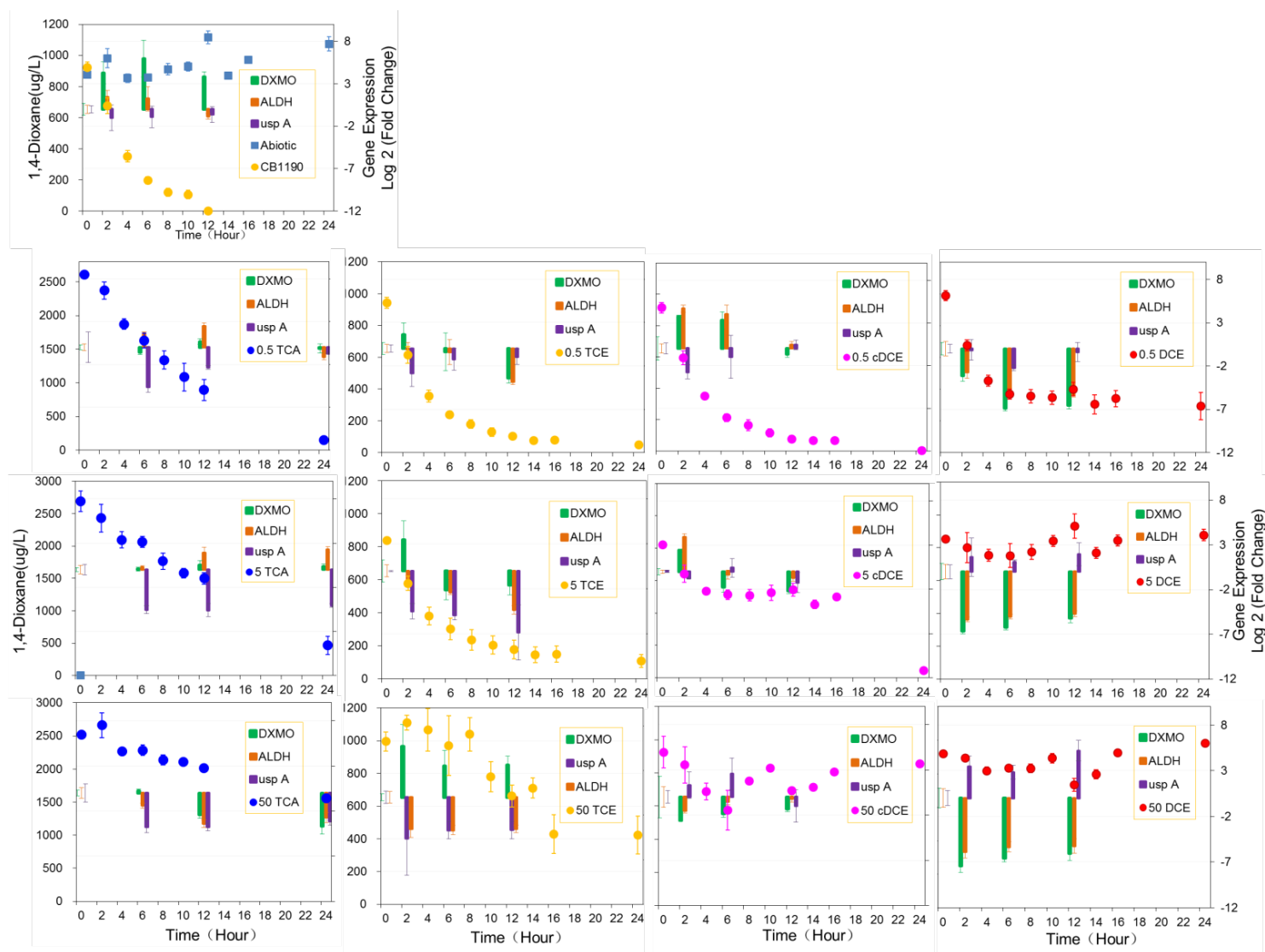


Figure 15. Effects of TCE, 1,1-DCE, and TCA on biodegradation at low 1,4-dioxane (1-2 mg/L) by CB1190. 1,4-Dioxane concentrations as well as cDNA copy numbers are shown. Error bars represent standard deviation of experimental triplicates.

Expression of 1,4-dioxane biomarker gene targets demonstrated either no change or increased expression of *dxmB* and *aldH* relative to their initial time point when 1,4-dioxane concentrations were between 1 – 2 mg/L 1,4-dioxane except for high concentrations of cDCE and concentrations greater than 5 mg/L 1,1-DCE (Figure 16). At these concentrations of cDCE and 1,1-DCE, the *uspA* gene was upregulated indicating a universal stress response to these solvents at these concentrations. In all other conditions and concentrations of solvents, expression of the *uspA* gene was lower for the 12-hour duration of monitoring. These results suggest that the *uspA* gene may be a promising indicator for inhibition of 1,4-dioxane biodegradation related to the presence of cDCE and 1,1-DCE. Additionally, biomarker gene expression in the presence of chlorinated solvents was complicated without further validation by increased expression of *uspA*. DXMO expression was induced even at 1 mg/L in the absence of chlorinated solvents, however the addition of 5 mg/L 1,1-DCE significantly lowered expression. This finding could be attributed to the increased expression of *uspA*. These results suggest that 1,1-DCE triggers a stress response in CB1190 and might influence regulation of the dioxane monooxygenase enzyme. Studies are ongoing to reveal the mechanisms of inhibition and identify optimal conditions for implementing bioremediation strategies to remove 1,4-dioxane in contaminated complex environments.

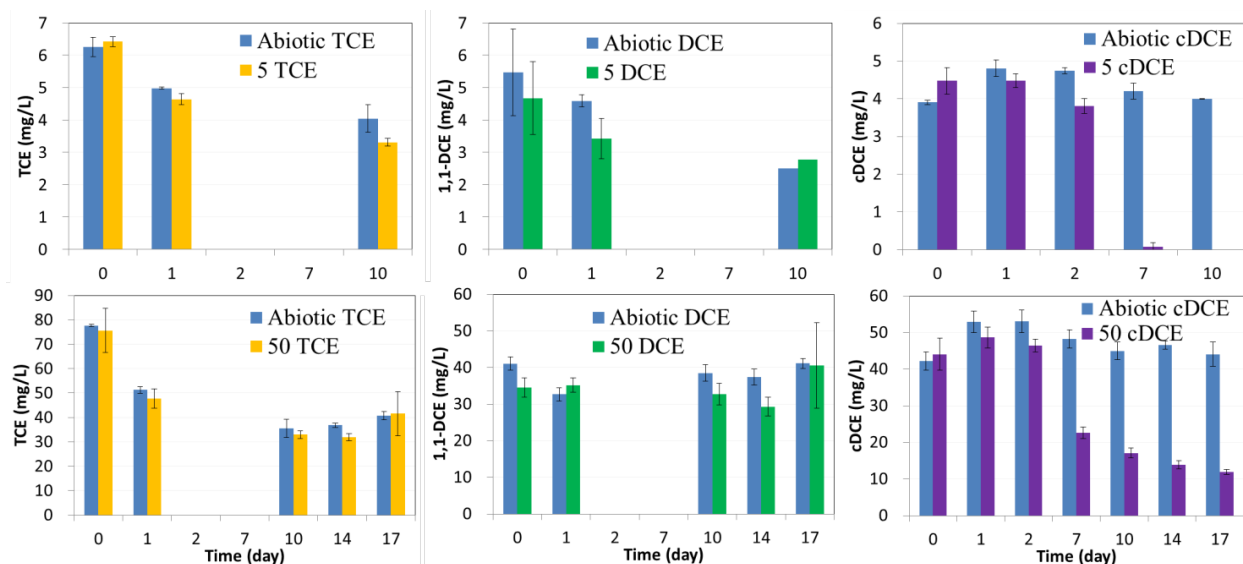


Figure 16. Chlorinated solvents concentrations in the headspace of bottles containing CB1190 in AMS medium supplemented with 1 mg/L 1,4-dioxane. CB1190 could biodegrade cDCE at all concentrations evaluated in this study. All experiments were performed in triplicate (Zhang et al., 2016).

Influence of Chlorinated Solvent Mixtures on 1,4-Dioxane Metabolism at Low Initial Concentrations of 1,4-Dioxane

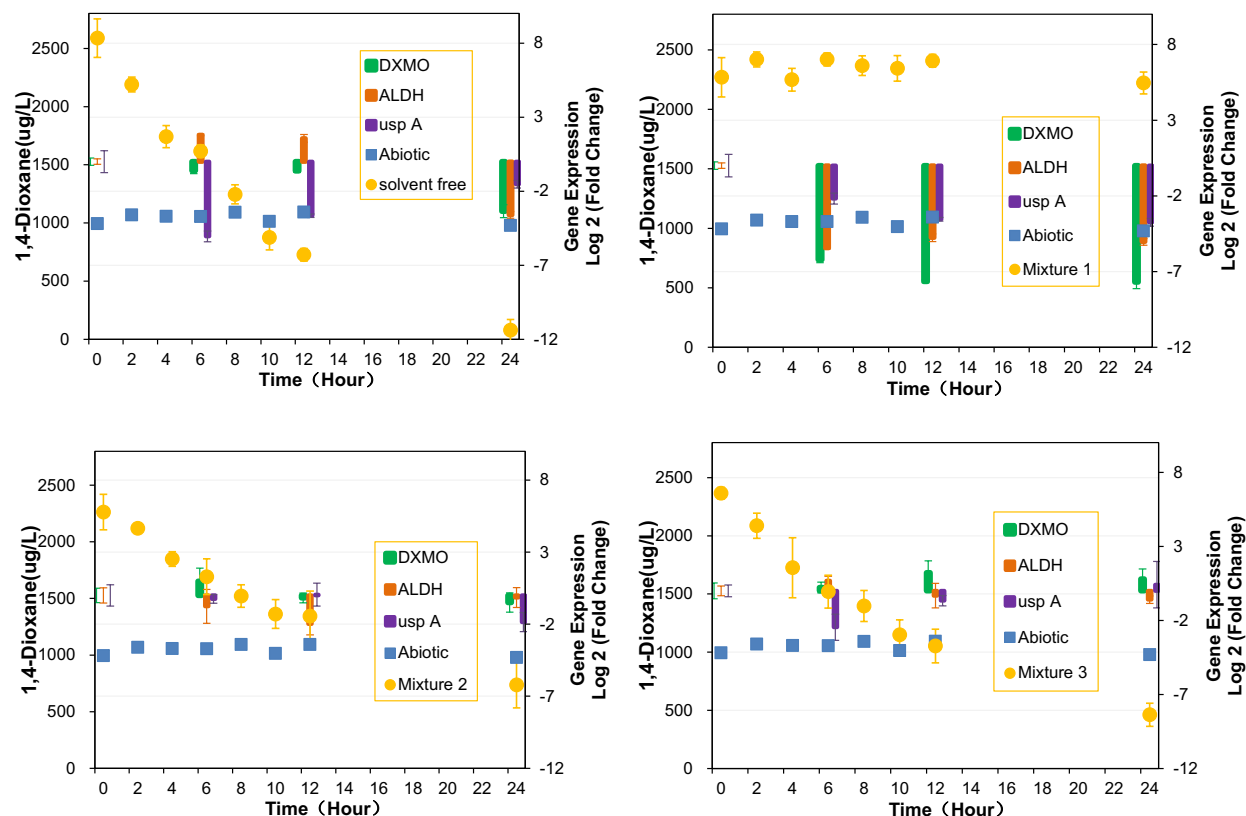


Figure 17. Solvent mixtures indicated that 1,1-DCE produced the greatest inhibition to 1,4-dioxane biodegradation. 1,4-Dioxane concentrations as well as cDNA copy numbers of genes coding for 1,4-dioxane-degrading enzymes as well as universal stress protein are shown. All experiments were performed in triplicate and error bars represent the standard deviation (Zhang et al., 2016).

Solvents are typically found in mixtures at 1,4-dioxane contaminated sites (Anderson et al., 2012), therefore we chose to evaluate the effects of different solvent mixtures on 1,4-dioxane metabolism by CB1190. Three mixtures were investigated containing: (Mixture 1) 5000 µg/L 1,1-DCE, 500 µg/L cDCE, 700 µg/L TCE, 120 µg/L TCA, (Mixture 2) 500 µg/L cDCE, 700 µg/L TCE, and 120 µg/L TCA, (Mixture 3) 700 µg/L TCE and 120 µg/L TCA in the aqueous phase. These concentrations are representative of solvent mixtures found in the environment. Our results showed that the presence of 1,1-DCE in solvent mixtures inhibited 1,4-dioxane metabolism (Figure 17), which was expected from our results on individual exposures. There was no significant difference between 1,4-dioxane biodegradation rates in bottles exposed to solvent mixtures 2 and 3 suggesting that CB1190 is only partially inhibited by solvents at these concentrations. Chlorinated solvent concentrations in mixtures was constant throughout the

experiment, however because of the sample collection at each time point (0.7 mL cell culture) and each solvent's partitioning efficiency factor, the loss of TCE and TCA in the headspace were inevitable (Figure 18).

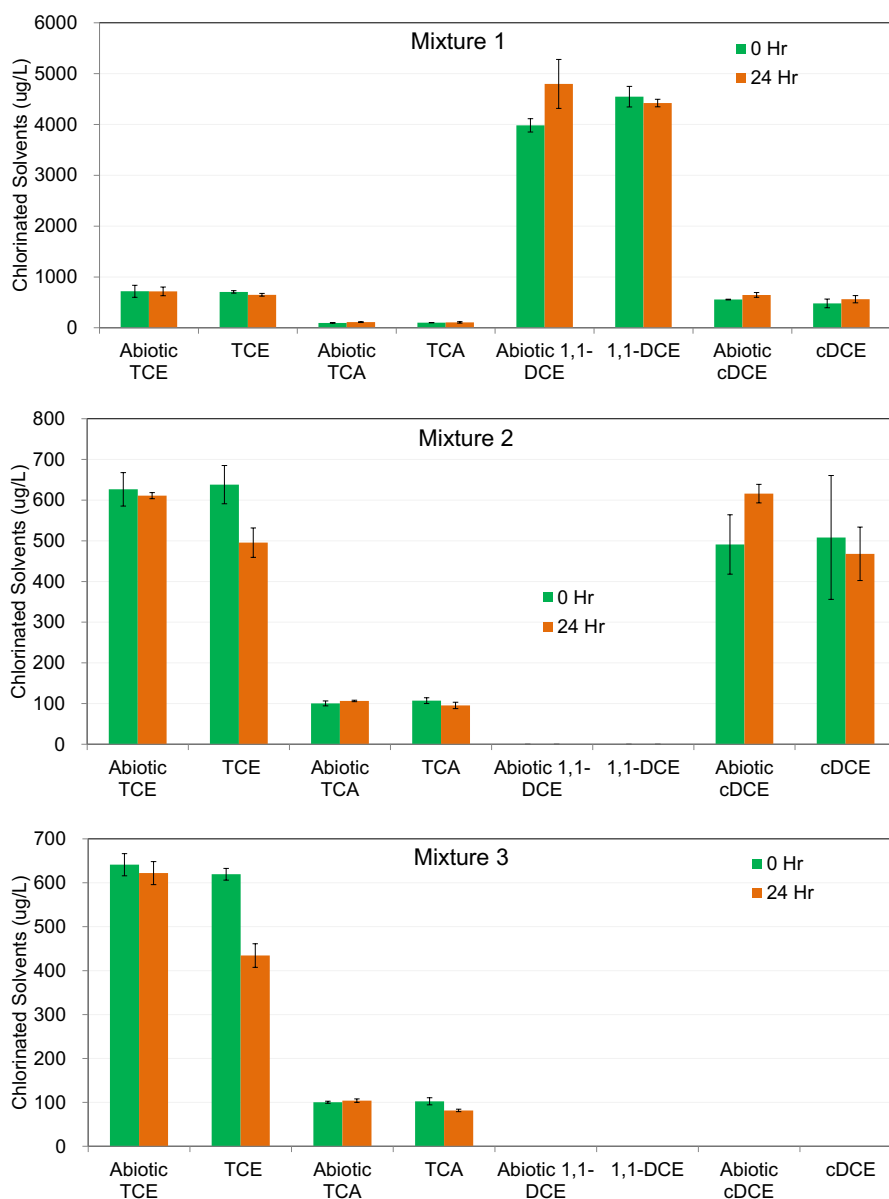


Figure 18. TCA, DCE, and cDCE concentrations in mixtures remained relatively constant for the duration of the experiment. Triplicates of solvent-exposed bottles and duplicates of abiotic bottles were used in the experiment and error bars represent the standard deviation and value range, respectively.

Effects of Chlorinated Solvents on Cometabolism of 1,4-Dioxane by JOB5

Cometabolism of cyclic ethers including 1,4-dioxane by the propanotroph JOB5 has been previously studied (Lan et al., 2013). However, our approach provides significantly novel information that focuses on the impacts of chlorinated solvent co-contaminants on this process from a kinetic and genetic perspective. Interestingly, cometabolism of 1,4-dioxane by JOB5 demonstrated similar sensitivities to chlorinated solvents compared to metabolic biodegradation by CB1190 (Figures 19 and 20). Both pathways were most sensitive to the presence of 1,1-DCE (Figure 20). Our study determined that 1,4-dioxane biodegradation by JOB5 was inhibited by chlorinated solvents in the order of: DCE > cDCE > TCE > TCA. In addition, chlorinated solvent inhibition was partial even at the highest concentrations of cDCE and DCE. This result contrasts the inhibition by high concentrations of cDCE experienced for the metabolic pathway by CB1190. Concurrent cometabolism of chlorinated solvents by the alkane monooxygenase may explain this result. In contrast to CB1190, JOB5 was able to cometabolize all of the chlorinated solvents examined. JOB5 had a high affinity for cDCE and DCE and the rapid removal of these solvents in the 5 mg/L exposure experiments suggests cometabolism may provide a robust strategy for comingled contaminants.

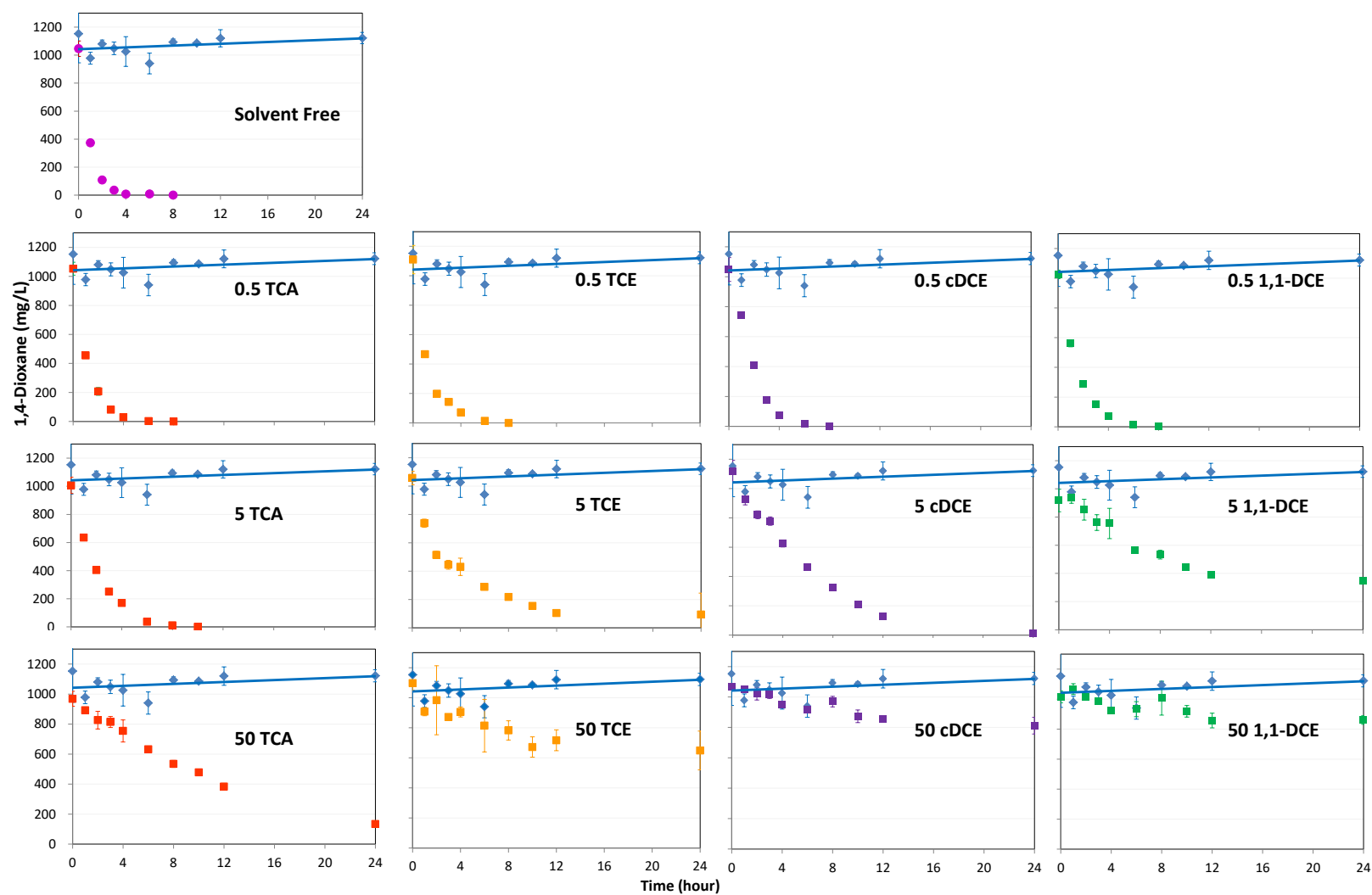


Figure 19. Effect of individual chlorinated solvents on 1,4-dioxane cometabolism by *Mycobacterium austroafricanum* JOB5. Concentrations of chlorinated solvents are shown in mg/L. All experiments were performed in triplicate and error bars represent the standard deviation.

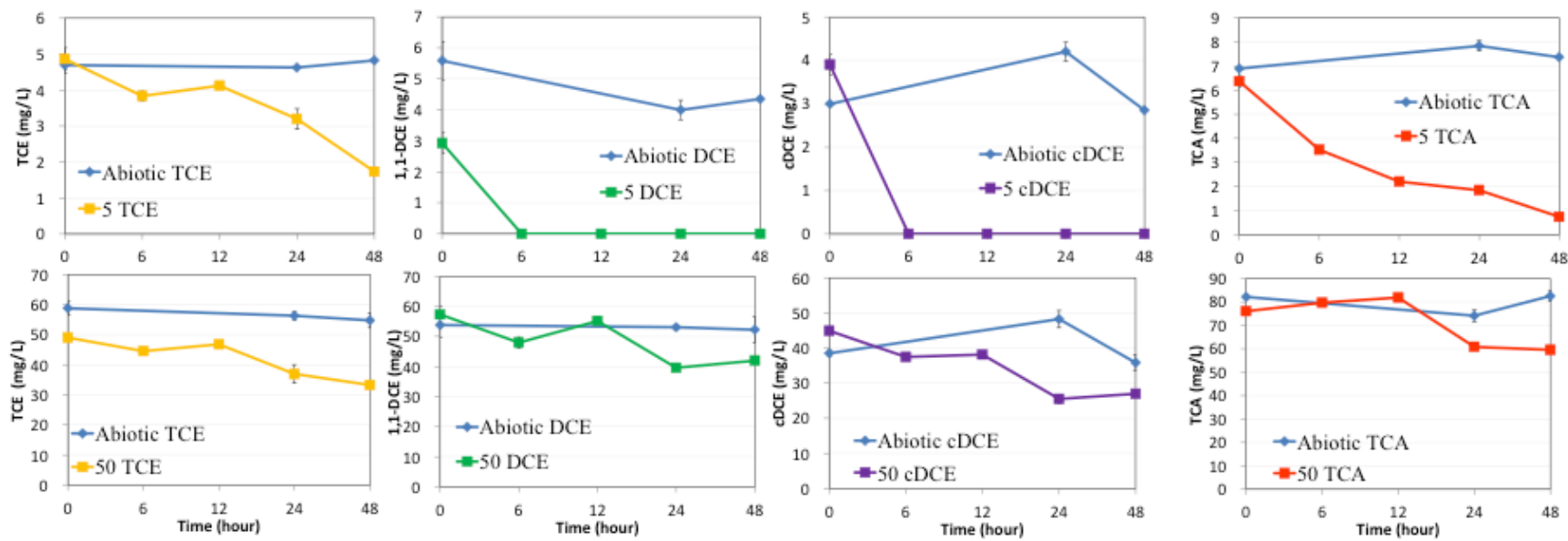


Figure 20. Biodegradation of individual chlorinated solvents by *Mycobacterium austroafricanum* JOB5. Triplicates of exposure bottles and duplicates of abiotic bottles were used in the experiment. Concentrations of chlorinated solvents are given in mg/L; error bars represent the standard deviation and value range, respectively.

Moreover, we investigated the simultaneous cometabolism of 1,4-dioxane and VC by JOB5. The bottles were monitored over 15 days, and VC with 40 $\mu\text{g/L}$ initial concentration was completely removed in first 12 hours, with 95% 1,4-dioxane removals at the same time (Figure 21). In other conditions, VC was degraded relatively faster in the first 24 hours, and tended to be flat afterwards in the microcosms with 1420 $\mu\text{g/L}$ initial concentrations, while slow degradation continued over time under 280 and 780 $\mu\text{g/L}$ VC initial concentrations (Figure 22). However, 1,4-dioxane degradation achieving ~ 500 $\mu\text{g/L}$ removals were only observed in first 12 hours, that ceased, while slow VC degradation continued over 15 days. Moreover, JOB5 could keep degrading when five cycles of 40 $\mu\text{g/L}$ VC were added in the bottles without additional spikes of propane. The presence of 1,4-dioxane addition had little to no impact on 1420 $\mu\text{g/L}$ VC degradation when comparing bottles with and without 1,4-dioxane. Furthermore, the first-order degradation of VC was calculated based on first 48-hour results, and the simulated degrading rate constant demonstrated that the lower VC had higher initial degradation rates (Table 6). These observations suggest that while simultaneous cometabolism of 1,4-dioxane and VC by JOB5 is possible, JOB5 has a much higher affinity for VC, and high VC concentrations inhibit both VC and 1,4-dioxane degradation by propanotrophic bacteria.

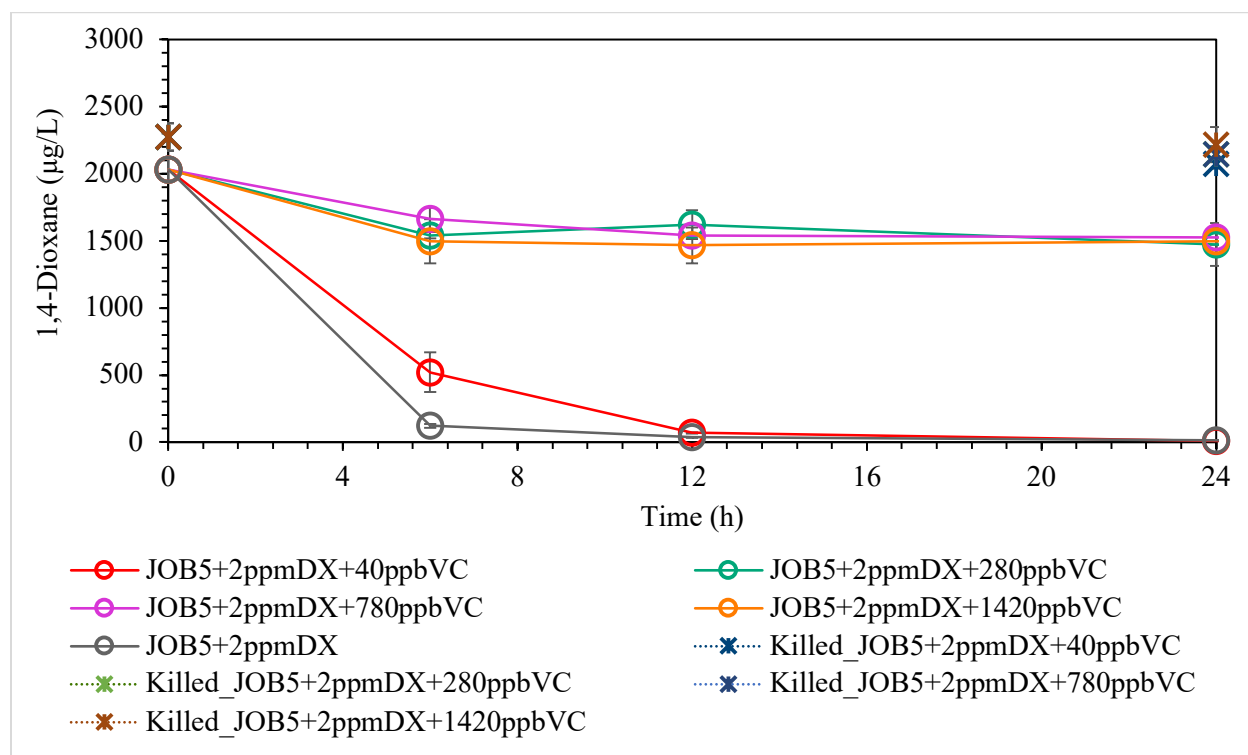


Figure 21. 1,4-Dioxane biodegradation by JOB5 under varying VC concentrations. Only 40 $\mu\text{g/L}$ VC showed partial inhibition, and the higher VC completely stalled 1,4-dioxane degradation. All experiments were performed in triplicate and error bars represent the standard deviation.

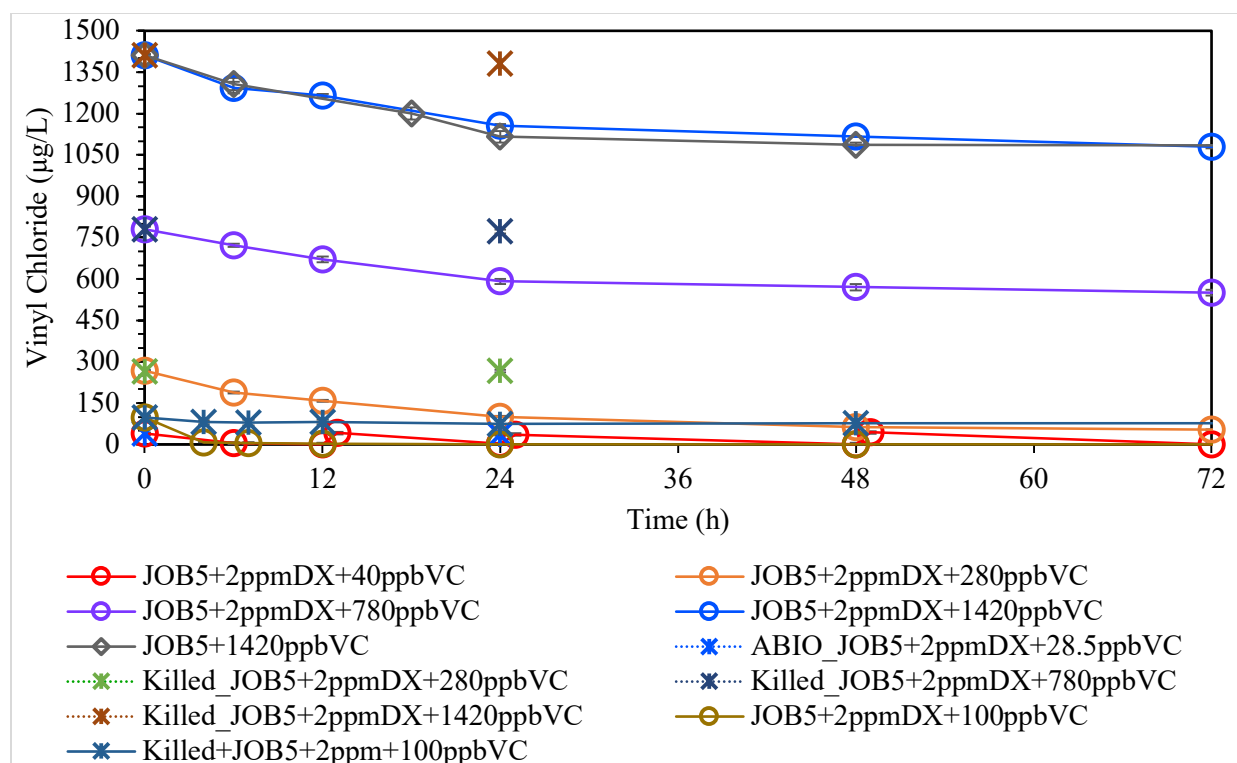


Figure 22. VC biodegradation by JOB5 in the presence of 1,4-dioxane. Lower VC corresponded to higher degradation rates and 2 mg/L 1,4-dioxane demonstrated no impact on 1420 µg/L VC degradation. All experiments were performed in triplicate and error bars represent the standard deviation.

Table 6. Cometabolic Degradation Rates of Vinyl Chloride by JOB5

Vinyl Chloride (µg/L)	1,4-Dioxane (µg/L)	Rate constants (1/d)
40	2000	9.18±0.71
280	2000	0.88±0.06
780	2000	0.17±0.02
1420	2000	0.12±0.01
1420	0	0.13±0.02

Impact of Metals on Metabolic Biodegradation of 1,4-Dioxane by CB1190

Biodegradation of 1,4-dioxane was influenced to varying degrees by individual exposure to the four transition metals Cd(II), Cu(II), Ni(II), and Zn(II) (Figure 23). In this study, percent biodegradation rates were computed and compared (Figure 24). In the absence of any added transition metal beyond AMS recipe (referred to as metal-free or positive control), CB1190 biodegraded 120 mg/L 1,4-dioxane to below detection limit within four days with an average

biodegradation rate of 30.23 ± 2.86 mg/L·d. Biodegradation of 1,4-dioxane by cultures containing 1 mg/L Cd(II) or Ni(II), or 1, 10, or 20 mg/L Zn(II) was indistinguishable from the metal-free controls. Cultures with addition of 1 mg/L Cu(II), 10 or 20 mg/L Cd(II), or 10 or 20 mg/L Ni(II) demonstrated longer initial lag, but ultimately degraded 1,4-dioxane to below detection limit within six days. Addition of 10 and 20 mg/L Cu(II) decreased 1,4-dioxane biodegradation rates by nearly 90% to 2.84 ± 1.42 and 2.79 ± 0.07 mg/L·d, respectively.

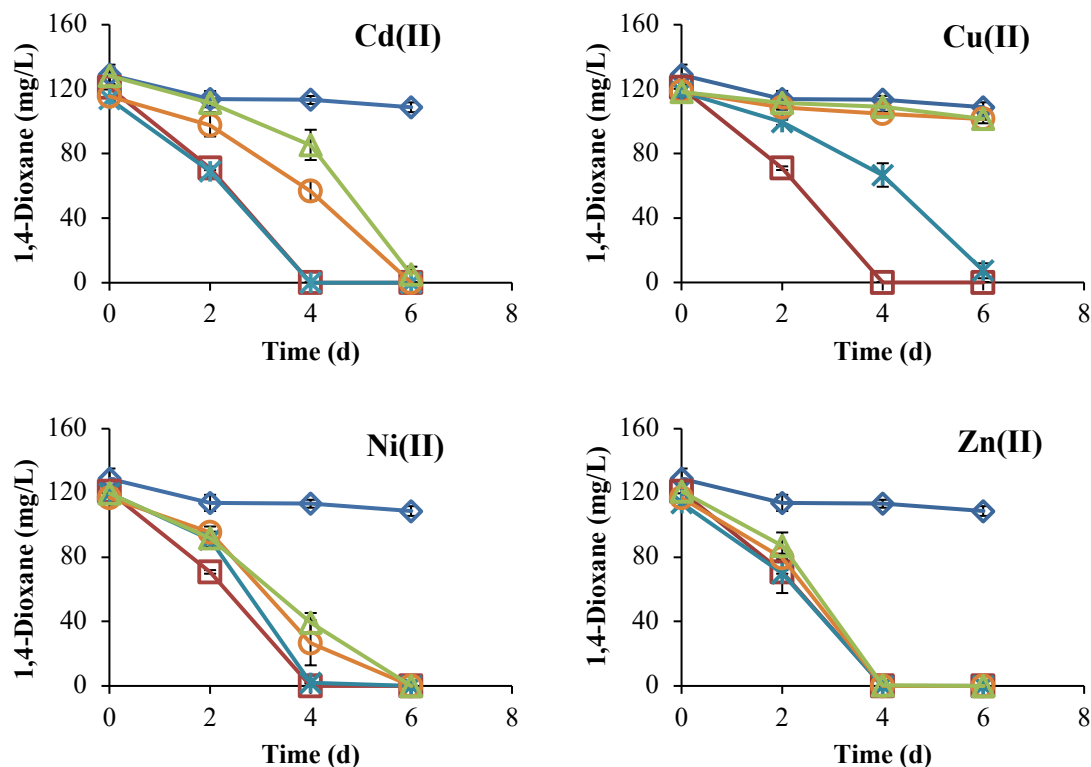


Figure 23. 1,4-Dioxane biodegradation by *Pseudonocardia dioxanivorans* CB1190 in the presence of four different metals. Biodegradation of 1,4-dioxane by cultures with three different concentrations of 1 mg/L (crosses), 10 mg/L (circles) and 20 mg/L (triangles). Diamonds represent abiotic control as negative control and squares represent metal-free control as positive control. Inhibitory effect of heavy metals on 1,4-dioxane biodegradation was measured to be in the following order: Cu(II) > Cd(II) ≥ Ni(II) > Zn(II). The error bars correspond to the range of duplicate samples (Pornwongthong et al., 2014).

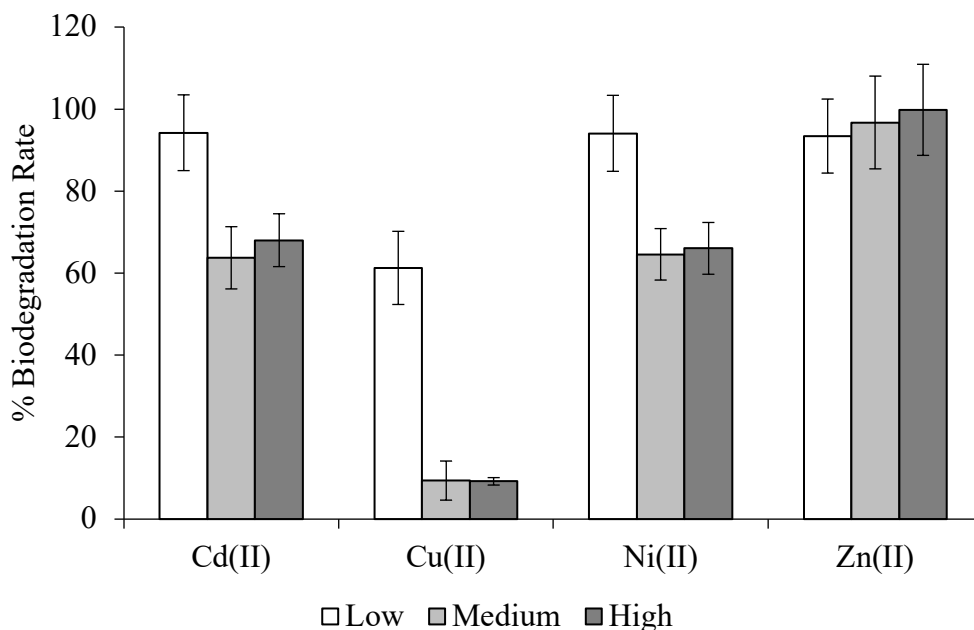


Figure 24. Effects of four different heavy metals at three different concentrations on the rate of 1,4-dioxane biodegradation by *Pseudonocardia dioxanivorans* CB1190. Biodegradation rate in y-axis is a normalized biodegradation rate of tested conditions with that established for metal-free controls. White columns, 1 mg/L heavy metal; light grey columns, 10 mg/L heavy metal; dark grey column, 20 mg/L heavy metal. Addition of Cd(II) to 10 and 20 mg/L, Cu(II) to all tested concentrations, and Ni(II) to 10 and 20 mg/L could significant affect 1,4-dioxane biodegradation rate. The error bars correspond to the range of duplicate samples (Pornwongthong et al., 2014).

Interactive Effects of Cu(II) and Organic Ligands on 1,4-Dioxane Biodegradation

Bioavailability and toxicity of Cu(II) could be alleviated by some organic ligands resulting in improved biodegradation rates (Figure 25). While metal-free controls degraded 1,4-dioxane to below detection within 8 days, at biodegradation rates between 12.57 ± 0.15 and 17.09 ± 0.24 mg/L·d, cultures with addition of 2 mg/L Cu(II) produced biodegradation rates between 0.35 ± 0.51 and 1.15 ± 0.80 mg/L·d, but could not completely biodegrade 1,4-dioxane. 1,4-Dioxane biodegradation rates were higher when 0.05 or 0.5 mM L-cysteine, or 0.005 mM tannic acid were added to the cultures in the presence of 2 mg/L Cu(II). Under these conditions, 100%, 100%, and 44% of the initial 1,4-dioxane was degraded at rates of 15.19 ± 2.60 , 16.14 ± 0.18 and 7.54 ± 1.03 mg/L·d, respectively. These biodegradation rates were significantly different from those determined for cultures exposed to 2 mg/L Cu(II) ($p < 0.05$) without ligands. Biodegradation rates with addition of 2 mg/L Cu(II) were higher at all concentrations of alginic acid, but they were not significantly different from Cu(II) controls ($p = 0.18 - 0.25$). Addition of BSA or SRNOM in cultures with 2 mg/L Cu(II) did not improve biodegradation rates (Figure 25).

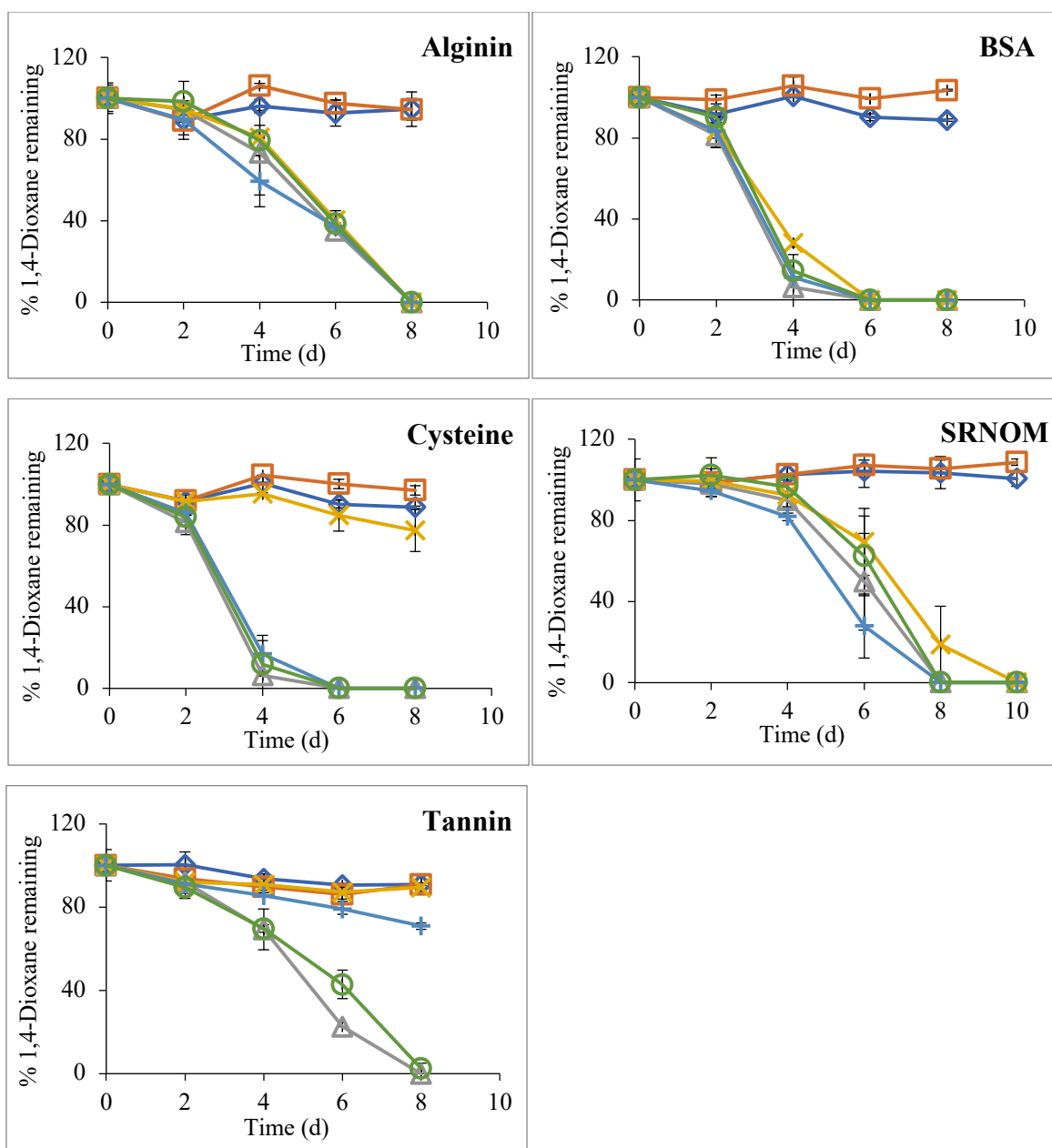


Figure 25. 1,4-Dioxane biodegradation by *Pseudonocardia dioxanivorans* CB1190 in presence of the five different organic ligands. Low, medium, and high concentrations are represented by circles, plus signs and X signs, respectively. In cultures amended with alginin, L-cysteine and tannin, the concentrations of the organic ligands tested are 0.005, 0.05 and 0.5 mM. In cultures with addition of BSA and SRNOM, the concentrations of organic ligands tested are 0.01, 0.1 and 1 mg/L. Diamonds, open squares and triangles represent abiotic controls as negative control, ligand-amended abiotic controls and ligand-free control as positive control, respectively. Addition of organic ligands at various concentrations did not affect biodegradation of 1,4-dioxane except when tannic acid at 0.05 and 0.5 mM and L-cysteine at 0.5 mM were amended. The error bars correspond to the range of duplicate samples (Pornwongthong et al., 2014).

Influence of Metals on Cometabolism of 1,4-Dioxane by JOB5

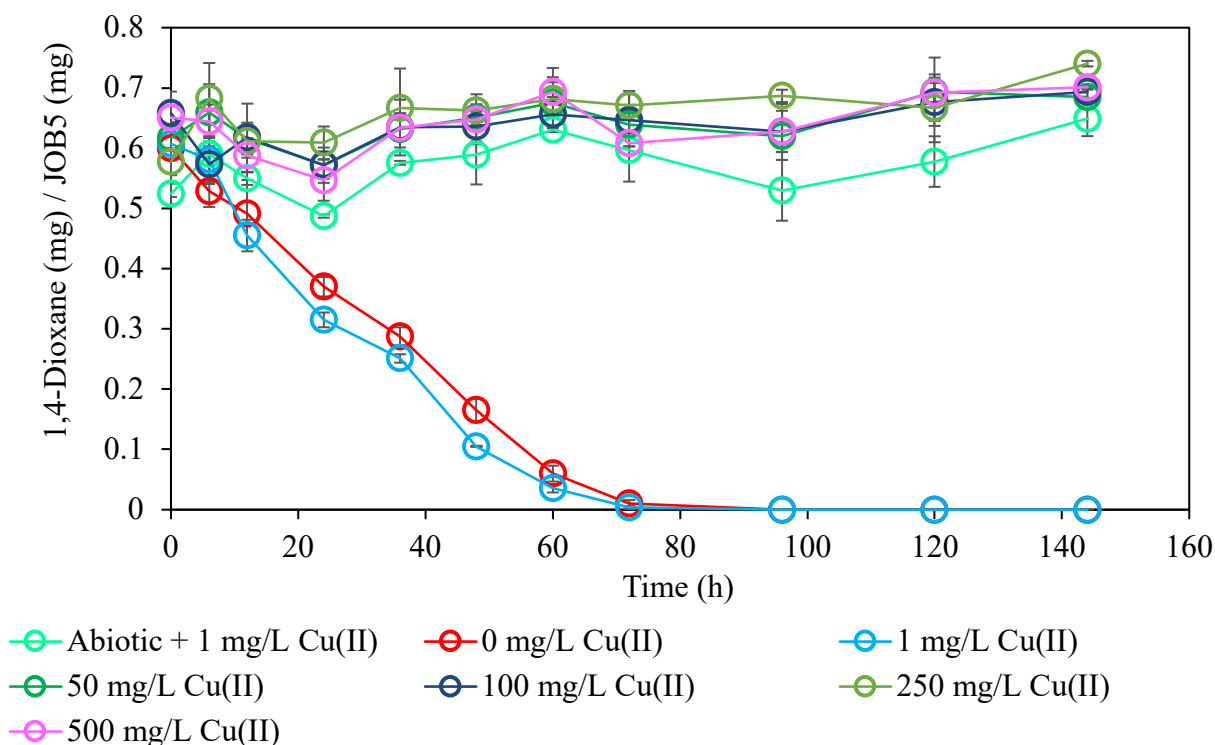


Figure 26. JOB5 cometabolic degradation of 1,4-dioxane in the presence of divalent copper. Cu(II) inhibits 1,4-dioxane biodegradation at concentrations ≥ 50 mg/L. Error bars correspond to the standard deviation of duplicate experimental samples and their analytical duplicates ($n=4$).

Biodegradation of 1,4-dioxane by JOB5 was sensitive to inhibition by Cu(II) and Cd(II). The average cell density of JOB5 was 1.34×10^{11} ($sd=1.50 \times 10^{10}$) cells/L and 2.23×10^{12} cells/L ($sd=4.30 \times 10^{10}$) cells/L for Cu(II) and Cd(II) exposure conditions, respectively. There was no significant difference between cell densities for the duration of the experiments confirming 1,4-dioxane was not used as a carbon or energy source by JOB5.

For the Cu(II) experiments at high concentrations of 1,4-dioxane, JOB5 degraded 100 mg/L of dioxane below detection limit within 3 days at an average degradation rate of 1.13 mg/L/h. Rates were calculated between 12 to 36 h, representing a linear degradation of dioxane. Biodegradation of 1,4-dioxane was completely inhibited with the addition of 50 mg/L, 100 mg/L, 250 mg/L, and 500 mg/L Cu(II) (Figure 26). Addition of 1 mg/L Cu(II) had no impact on 1,4-dioxane biodegradation. Cu(II) is an essential trace metal and concentrations in the growth medium may not have been optimal for this biomass concentration.

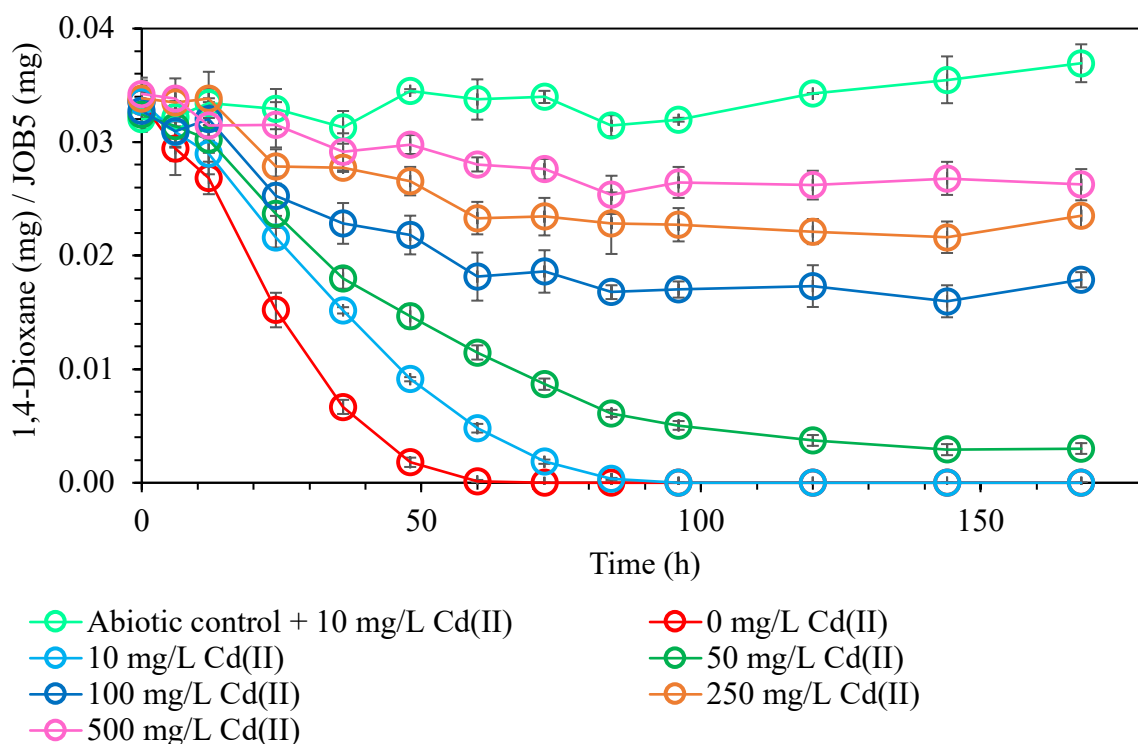
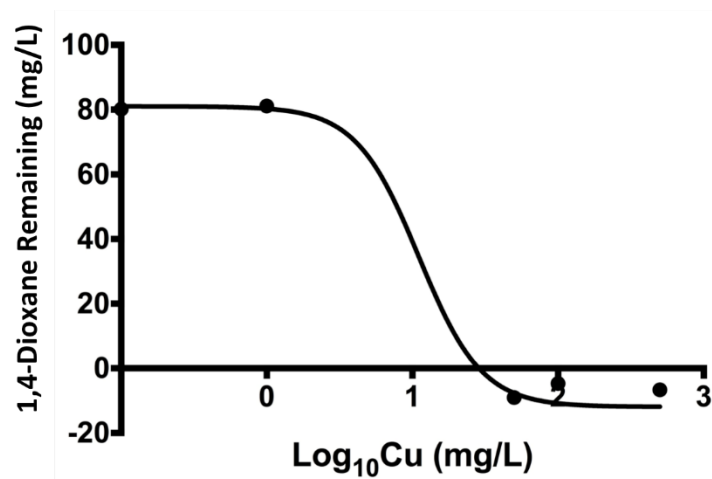


Figure 27. JOB5 cometabolic degradation of 1,4-dioxane in the presence of divalent cadmium. Cd(II) inhibits cometabolism of 1,4-dioxane in a dose dependent manner: 500 mg/L > 250 mg/L > 100 mg/L > 50 mg/L > 10 mg/L > 0 mg/L. Error bars correspond to the standard deviation of duplicate experimental samples and their analytical duplicates (n=4).

In the Cd(II) experiments, cometabolism of 1,4-dioxane by JOB5 was inhibited in a dose-dependent manner. Metal-free controls degraded 100 mg/L dioxane within 2.5 days at an average degradation rate of 1.88 mg/L/h (Figure 27). Cultures with 10 mg/L Cd(II) slowed complete cometabolic degradation of dioxane to 4 days at an average degradation rate of 1.29 mg/L/h. Cultures with 50 mg/L Cd(II) further decreased the degradation rate of dioxane to 1.14 mg/L/h. At 50 mg/L Cd(II), JOB5 did not completely degrade dioxane to below detection level compared to metal-free control; nevertheless, 73.2% of dioxane was degraded by 2.5 days at this Cd(II) concentration. Compared to metal-free controls at 2.5 days, cultures with either 100 mg/L, 250 mg/L, or 500 mg/L Cd(II) demonstrated inhibitory characteristics by slowing down dioxane degradation rates by 43.2%, 30.8%, and 19.4%, respectively. Doyle and coworkers (1975) indicated that at 40 and 80 $\mu\text{g/mL}$, *Escherichia coli* and *Bacillus cereus* successfully grew while *Lactobacillus acidophilus*, *Staphylococcus aureus*, *Streptococcus faecalis*, and *Actinomyces niger* were growth repressed by cadmium.(Doyle et al., 1975) In our study, cadmium toxicity impacted the rate of dioxane biodegradation across a range of concentrations (10 mg/L—500 mg/L, IC_{50} : 74.77 mg/L) at time point 36 h.

A)



B)

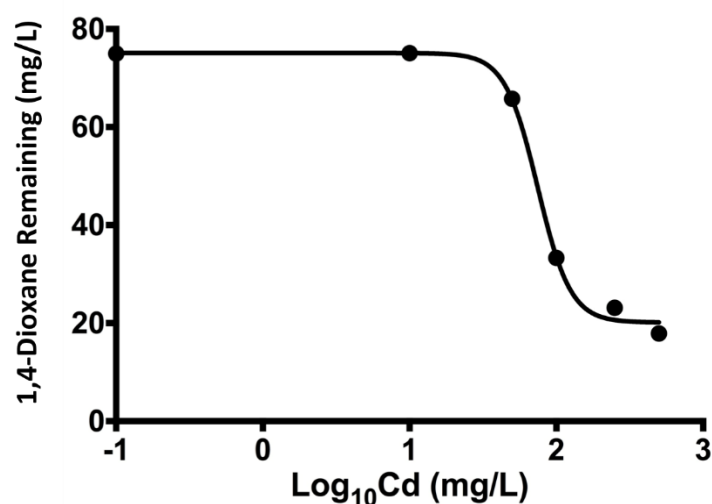


Figure 28. IC₅₀ curves for cometabolic 1,4-dioxane degradation activity by JOB5 in the presence of increasing concentrations of transition metals. A) The Cu(II) concentration required to reduce 50% of 1,4-dioxane degradation (normalized to unexposed control) was 11.09 mg/L. B) As for Cd(II), the IC₅₀ concentration was 74.77 mg/L.

Figure 28 exhibits the IC₅₀ curves of 1,4-dioxane in the presence of either Cu(II) or Cd(II). The Cu(II) concentration required to reduce 50% of dioxane-degrading activity was 11.09 mg/L. As for Cd(II), the IC₅₀ was 74.77 mg/L. These values were generated based on a fixed time point of 36 h. Depending on the exposure level of metal, dioxane was degraded at different rates during this time point. These results indicate that 1,4-dioxane cometabolism is more sensitive to Cu(II) than Cd(II). This is similar to the results represented by Pornwongthong et al. (2014), which showed that the effect of Cu(II) on dioxane metabolic biodegradation was more inhibitory than that of Cd(II). Zhang and colleagues reported that copper had an acute effect on indigenous microorganism population in soils (IMPS) with an IC₅₀ equal to 1.76 mg/L (Zhang et al., 2003).

In contrast, our study described the influence of copper on a cometabolic process whereby the primary substrate has been removed and no microbial growth exists which could explain the differences in the IC_{50} values obtained in our study. Zhang and coworkers also determined that Cu(II) stimulated the growth of IMPS at concentrations lower than 1.40 mg/L, but had a toxic effect at greater concentrations (Zhang et al., 2003). As shown in Figure 26, our results also confirmed that JOB5 was more efficient in degrading 1,4-dioxane in the presence of 1 mg/L Cu(II).

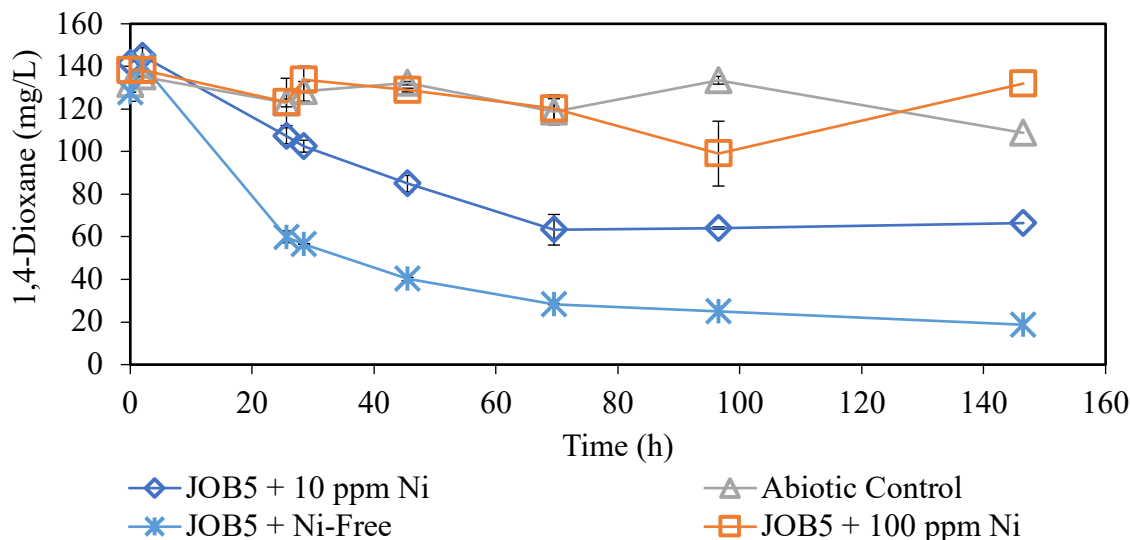


Figure 29. Effect of divalent nickel on cometabolism of 1,4-dioxane by *Mycobacterium austroafricanum* JOB5. Inhibition was observed at concentrations as low as 10 mg/L Ni(II) with complete inhibition at exposure to 100 mg/L Ni(II). All experiments were performed in triplicate and error bars represent the standard deviation.

Investigating the effect of divalent nickel (Ni(II)) on 1,4-dioxane cometabolism revealed a dose-dependent relationship for the examined concentrations (Figure 29). 10 mg/L Ni(II) partially inhibited biodegradation resulting in approximately 50% 1,4-dioxane remaining at 146.5 h. 100 mg/L Ni(II) completely inhibited cometabolism. These results illustrate that Ni(II) is an important metal contaminant to consider prior to implementing bioremediation strategies for metabolic and cometabolic biodegradation of 1,4-dioxane. In the presence of Ni(II), CB1190 was able to completely degrade 1,4-dioxane, however incomplete removal was observed for JOB5 at all concentrations tested. This is important because Ni(II) is naturally found in the environment and has also been identified as a contaminant at different small arms ranges (Bannon et al., 2009).

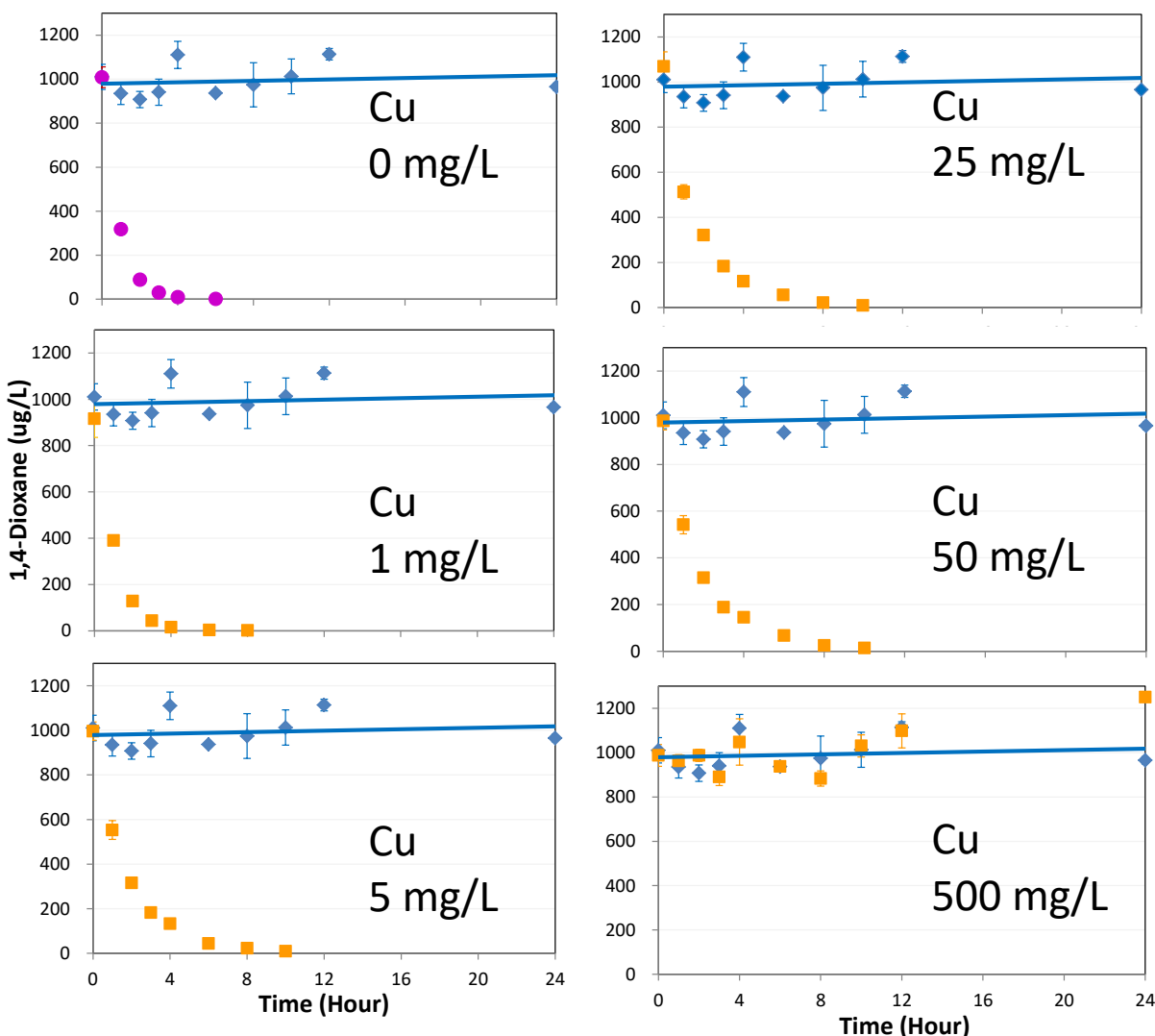


Figure 30. Effect of divalent copper on cometabolism of low 1,4-dioxane concentrations by JOB5. All experiments were performed in triplicate and error bars represent the standard deviation.

The inhibition effects of Cu(II), Cd(II), and Fe(II) on cometabolism of low 1,4-dioxane concentrations was studied to determine the impacts of these transition metals at environmentally relevant concentrations of 1,4-dioxane contamination (Figures 30-32). While both Cu(II) and Cd(II) produced a similar dose-dependent inhibition compared to high 1,4-dioxane concentration experiments, the inhibitory effects were less severe at low 1,4-dioxane concentrations. At high 1,4-dioxane levels, cometabolism was completely inhibited at 50 mg/L Cu(II). However, complete biodegradation was observed in the low 1,4-dioxane conditions at this concentration of Cu(II) (Figure 30). Complete inhibition did not occur in CB1190 growing on low 1,4-dioxane concentration until exposure to 500 mg/L Cu(II).

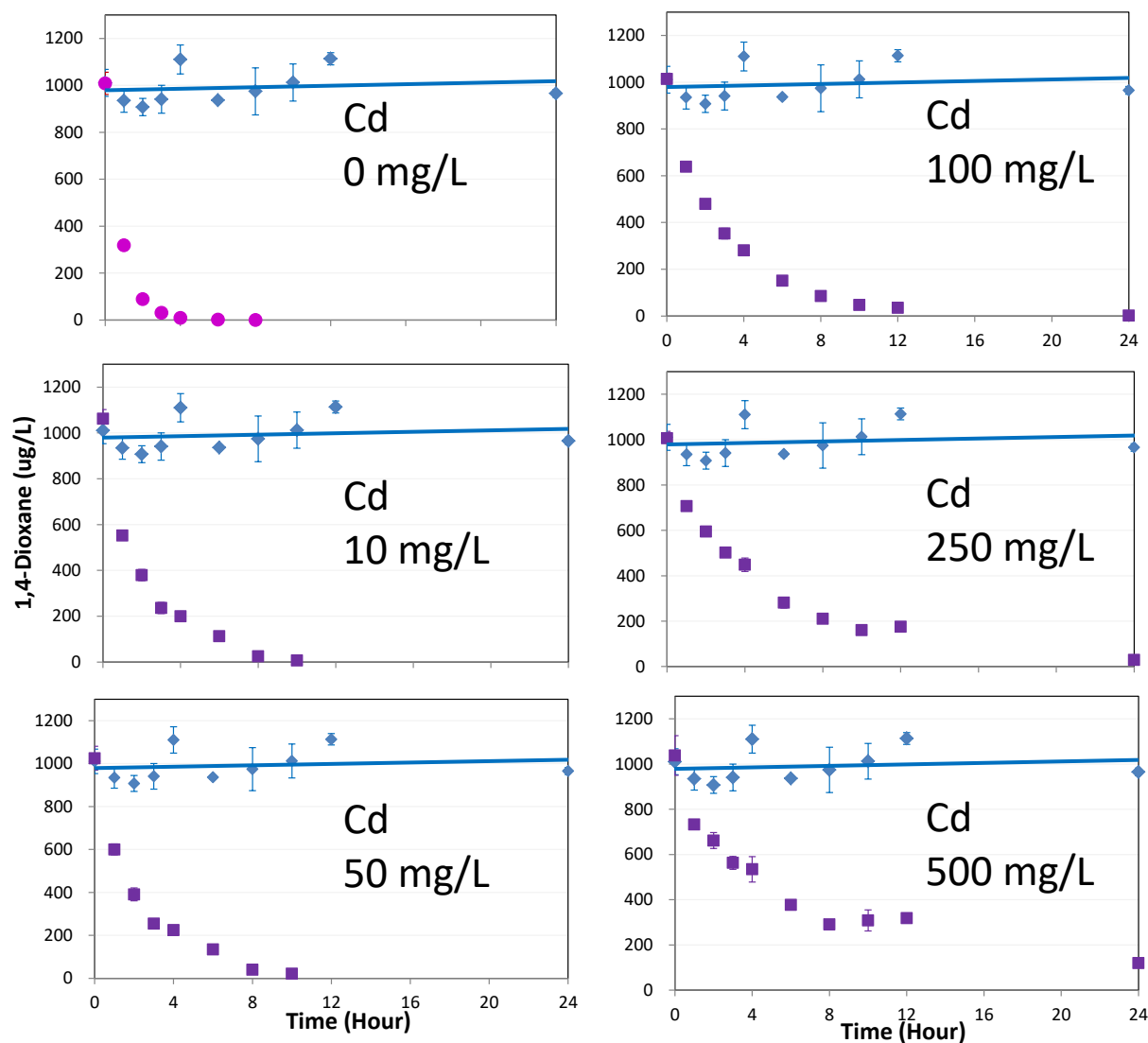


Figure 31. Effect of divalent cadmium on cometabolic biodegradation of low 1,4-dioxane concentrations by JOB5. All experiments were performed in triplicate and error bars represent the standard deviation.

Cd(II) inhibition was also mitigated at low 1,4-dioxane concentrations. 1,4-Dioxane was cometabolized by over 80% at all Cd(II) concentrations evaluated (Figure 31). This is in stark contrast to the high 1,4-dioxane condition where 100 mg/L Cd(II) inhibited cometabolism by approximately 50%. These differences are likely to the higher enzyme to substrate ratio in the different experiments which further supports cometabolism as a promising approach for bioremediation. Somewhat similarly to Cu(II) with low levels of 1,4-dioxane, Fe(II) only inhibited 1,4-dioxane cometabolism (initial concentration of 1000 $\mu\text{g/L}$) at levels ≥ 250 Fe(II) (Figure 32).

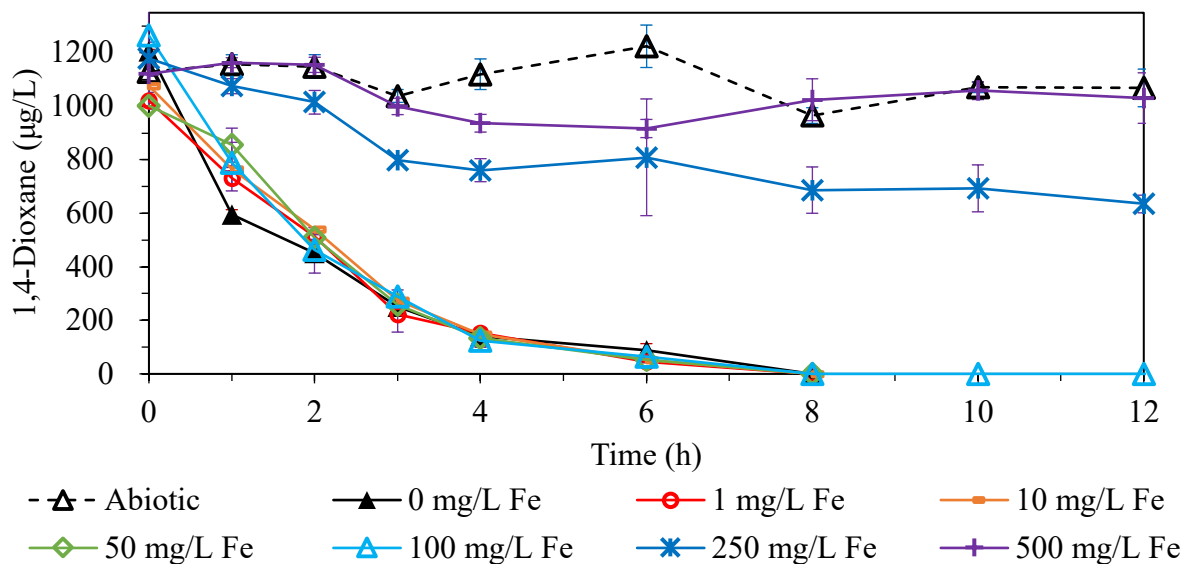


Figure 32. Fe(II) did not greatly impact 1,4-dioxane cometabolism by JOB5. Biodegradation extents and rates did not differ between the no Fe(II) condition and the conditions ranging from 1-100 mg/L Fe (II). Levels ≥ 250 mg/L Fe(II) greatly inhibited 1,4-dioxane cometabolism. Error bars represent the standard deviation of triplicate reactors.

Effects of Hexavalent Chromium on Metabolic and Cometabolic Biodegradation of 1,4-Dioxane

Hexavalent chromium is a known carcinogen and an important groundwater contaminant. Many military installations contaminated with 1,4-dioxane may be contaminated with hexavalent chromium as a result of chrome plating and vapor degreasing operations. In addition, many sites throughout California have been identified that contain both elevated levels of 1,4-dioxane and Cr(VI) (Hausladen et al., 2018). . Thus, it is important to determine the influence of Cr(VI) on 1,4-dioxane metabolism. Cr(VI) was found to inhibit 1,4-dioxane metabolism by CB1190. Concentrations as low as 5 mg/L extended the time to completely remove 100 mg/L by 2 days and 50 mg/L Cr(VI) resulted in less than 50% removal in 11 days (Figure 33).

To obtain a higher resolution of the inhibitory concentration of Cr(VI) on CB1190, experiments were designed to model the half maximal inhibitory concentration (IC_{50}) (Figure 34). These results demonstrated that CB1190 has a relatively high tolerance for Cr(VI). The IC_{50} of Cr(VI) on 1,4-dioxane degradation by CB1190 was determined to be 195.5 mg/L ($R^2 = 0.92$; $p < 0.01$). Additionally, we determined that slower biodegradation rates might be explained by regulation of the dioxane monooxygenase. We found that expression of the *dxmB* and *aldH* gene targets showed decreased expression in the presence of 5 mg/L Cr(VI) relative to the unexposed control.

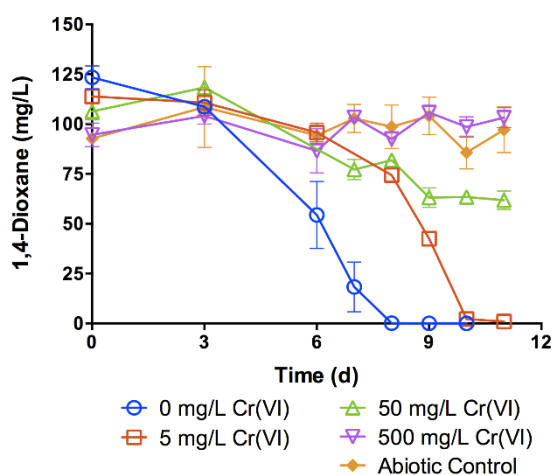


Figure 33. Influence of Cr(VI) on 1,4-dioxane biodegradation by CB1190. 1,4-Dioxane metabolism by CB1190 was inhibited at concentrations ≥ 5 mg/L Cr(VI).

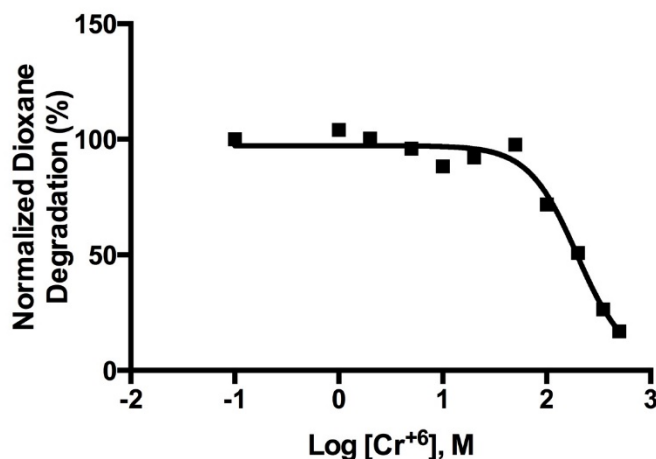


Figure 34. Half maximal inhibitory concentration (IC_{50}) for hexavalent chromium on the 1,4-dioxane degradation by CB1190. An IC_{50} value of 195.5 mg/L ($R^2 = 0.92$; $p < 0.01$) was determined based on the concentrations of 1,4-dioxane remaining after a 12 hour exposure to hexavalent chromium ranging from 0 – 500 mg/L.

The impact of Cr(VI) on cometabolism of environmentally relevant concentrations of 1,4-dioxane are shown in Figure 35. Similar to CB1190, JOB5 was able to biodegrade 1,4-dioxane in the presence of 5 and 50 mg/L Cr(VI). However, when exposed to 500 mg/L Cr(VI), cometabolism was completely inhibited. Because attached growth modes are preferred in the natural and built environment, the effects of Cr(VI) on JOB5 biofilms were tested to more accurately predict potential effects in an in situ field setting. Figure 36 shows the column setup used to evaluate the effects of Cr(VI) on JOB5 biofilm biodegradation of 1,4-dioxane (results presented in Figure 37). Cr(VI) was not removed from bulk solution by the biofilms (Figure 38). Table 7 contains the calculated rates and rate constants for both the JOB5 planktonic and biofilm experiments containing both Cr(VI) and 1,4-dioxane. Biofilm biodegradation rates were noticeably slower than those from planktonic reactors. Additionally, greater rate constant differences were found between 0 and 10 mg/L Cr(VI) conditions for biofilm rather than planktonic reactors. Our lab has observed that planktonic JOB5 tends to produce more soluble extracellular polymeric substances (sEPS) than biofilm JOB5 and sEPS was observed to bind to Cr(VI), which may mitigate its inhibitory potential. However, in the cases of both JOB5 and CB1190, typical groundwater concentrations of Cr(VI) (0.1-10 mg/L) did not appreciably affect 1,4-dioxane biodegradation, suggesting that biodegradation is an effective 1,4-dioxane treatment alternative even in the presence of elevated levels of Cr(VI).

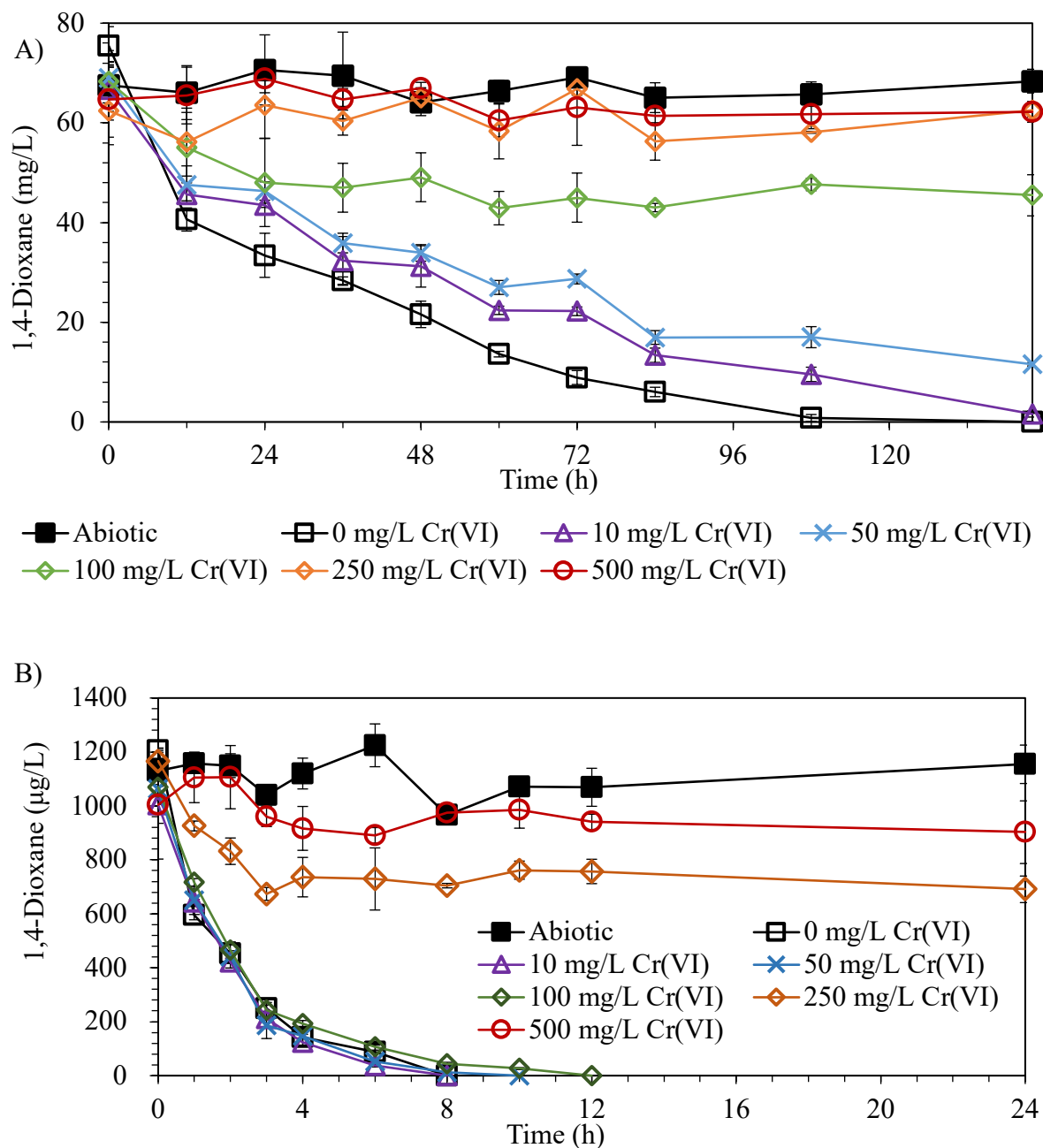


Figure 35. Effect of hexavalent chromium on cometabolism of high and low 1,4-dioxane concentrations by JOB5. A) Cr(VI) levels ≥ 250 mg/L severely inhibit cometabolism of high levels of 1,4-dioxane by JOB5. Moderate inhibition was also observed for 10 and 50 mg/L Cr(VI). B) Only 250 and 500 mg/L Cr(VI) inhibits cometabolism of low levels of 1,4-dioxane by JOB5. Error bars correspond to the standard deviation of triplicate experimental reactors.

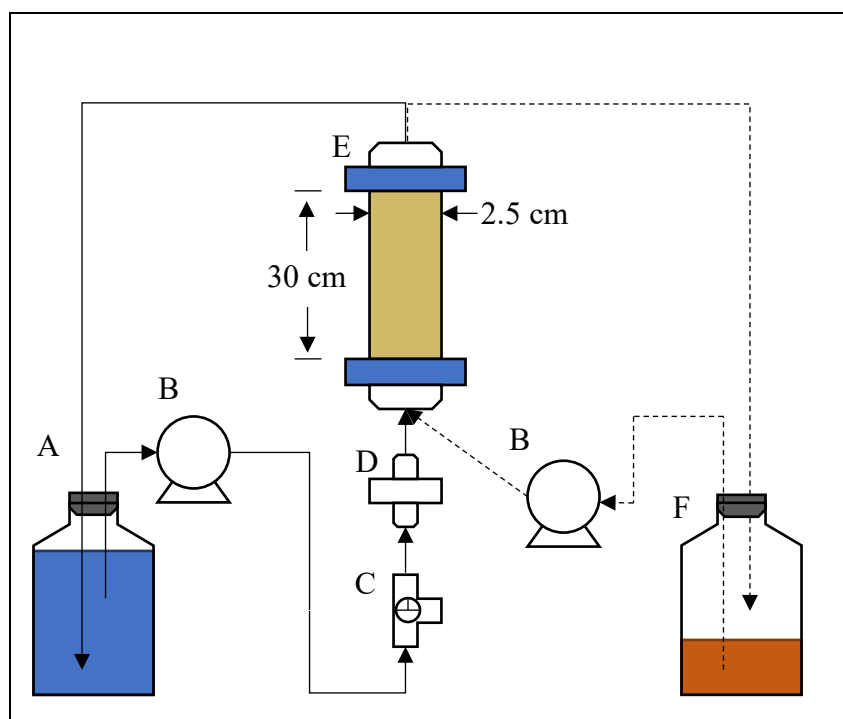


Figure 36. JOB5 biofilm column schematic for 1,4-dioxane and Cr(VI) experiments. A) experimental column reservoir, B) peristaltic pump, C) sample valve, D) column influent filter, E) packed column, F) culture recirculation vessel. Dashed lines denote inoculation flow scheme and solid lines denote the experimental flow scheme.

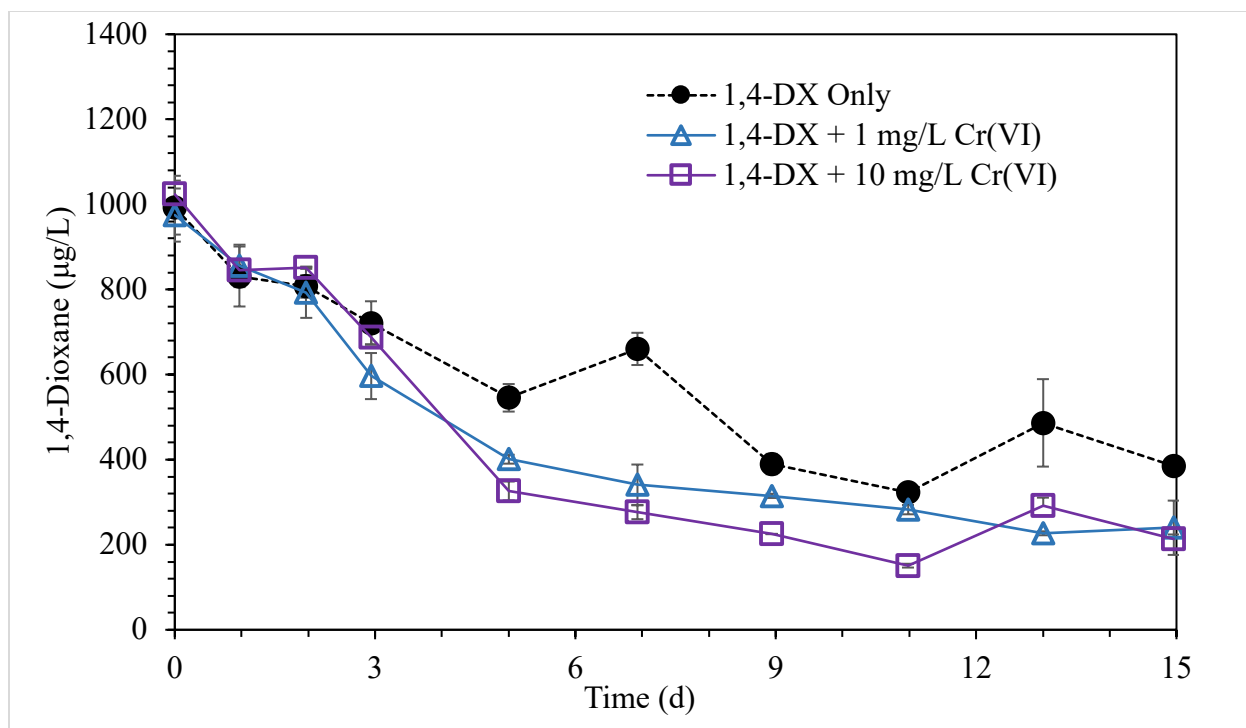


Figure 37. JOB5 biofilms grown on acid-washed silica sand resisted inhibition by Cr(VI) and biodegraded 1,4-dioxane. Single column experiments were conducted for each experimental condition. Error bars represent half the range of duplicate analytical measurements.

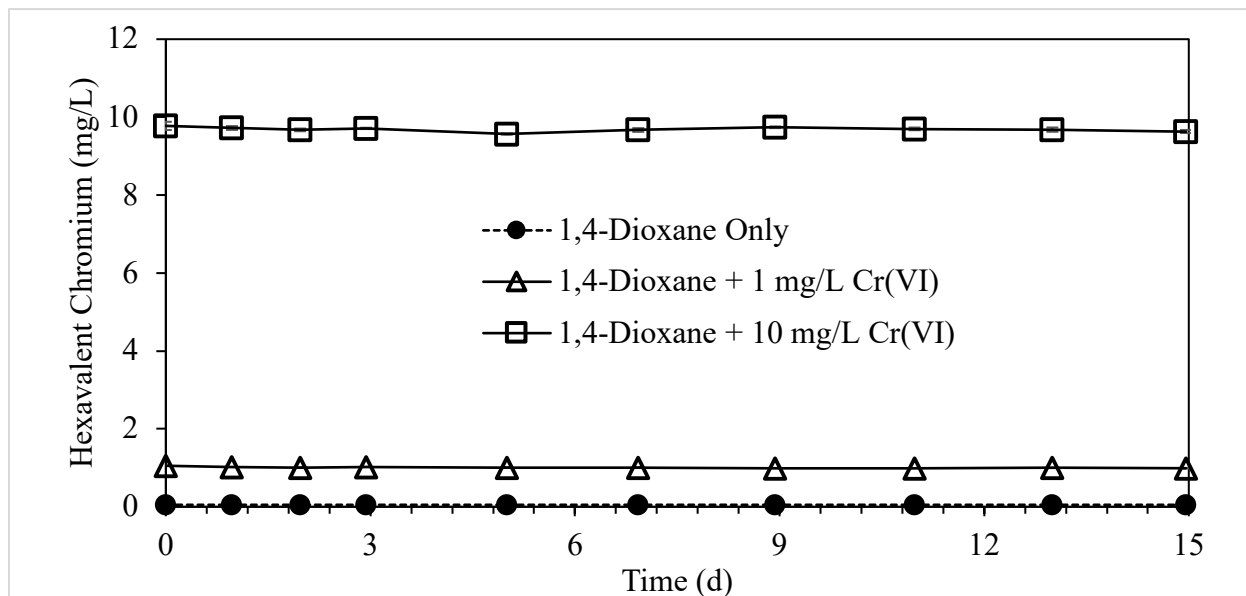


Figure 38. JOB5 biofilms and associated EPS did not remove Cr(VI). No significant reduction in Cr(VI) was seen in either the low or high Cr(VI) conditions. Error bars represent the standard deviation of triplicate analytical measurements.

Table 7. Initial 1,4-Dioxane Cometabolic Degradation Rates

Growth Mode	Initial 1,4-Dioxane	Initial Cr(VI)	Rate $\pm$ SE ^a	Apparent ^b Rate Constant $\pm$ SE ^a	Modeled ^c Rate Constant $\pm$ SE ^d
	$\mu\text{g/L}$	mg/L	$\mu\text{g/copy}\cdot\text{d}$	1/d	1/d
Biofilm	1000	0	$(4.2 \pm 0.57) \times 10^{-11}$	0.13 ± 0.0059	3.1 ± 0.66
Biofilm	1000	1	$(3.8 \pm 0.38) \times 10^{-11}$	0.18 ± 0.011	3.6 ± 0.55
Biofilm	1000	10	$(2.0 \pm 0.17) \times 10^{-11}$	0.20 ± 0.025	4.7 ± 1.3
Planktonic	1000	0	$(5.5 \pm 0.065) \times 10^{-7}$	12 ± 0.59	-
Planktonic	1000	10	$(6.3 \pm 0.29) \times 10^{-7}$	12 ± 0.51	-
Planktonic	1000	100	$(6.4 \pm 0.23) \times 10^{-7}$	11 ± 0.38	-
Planktonic	1000	250	$(3.1 \pm 0.35) \times 10^{-7}$	3.5 ± 0.34	-
Planktonic	1000	500	$(8.5 \pm 9.8) \times 10^{-8}$	0.070 ± 1.2	-

^aSE = Standard Error^bApparent rate constants determined directly using degradation data^cModeled rate constants determined using degradation data and reactor modeling. Rate constants were calculated using the first 5 time points. These 4 rate constants were then averaged.^dStandard error calculated for the 4 calculated rate constants.

Biodegradation of 1,4-Dioxane by Biofilms

To confirm biofilm formation by CB1190, experiments were conducted to determine whether or not extracellular polymeric substances were formed during growth on 1,4-dioxane. Figure 39 shows that EPS production occurred after 1,4-dioxane was completely degraded by CB1190. Interestingly, the addition of copper was found to induce EPS production even with high concentrations of 1,4-dioxane remaining. These findings suggest that more EPS were produced by CB1190 as a stress response to starvation and metal ions, so CB1190 biofilms would be viable, and perhaps protected, in contaminated environments. Furthermore, the production of EPS supports the formation of biofilms by CB1190 because it is an integral component of bacterial biofilms.

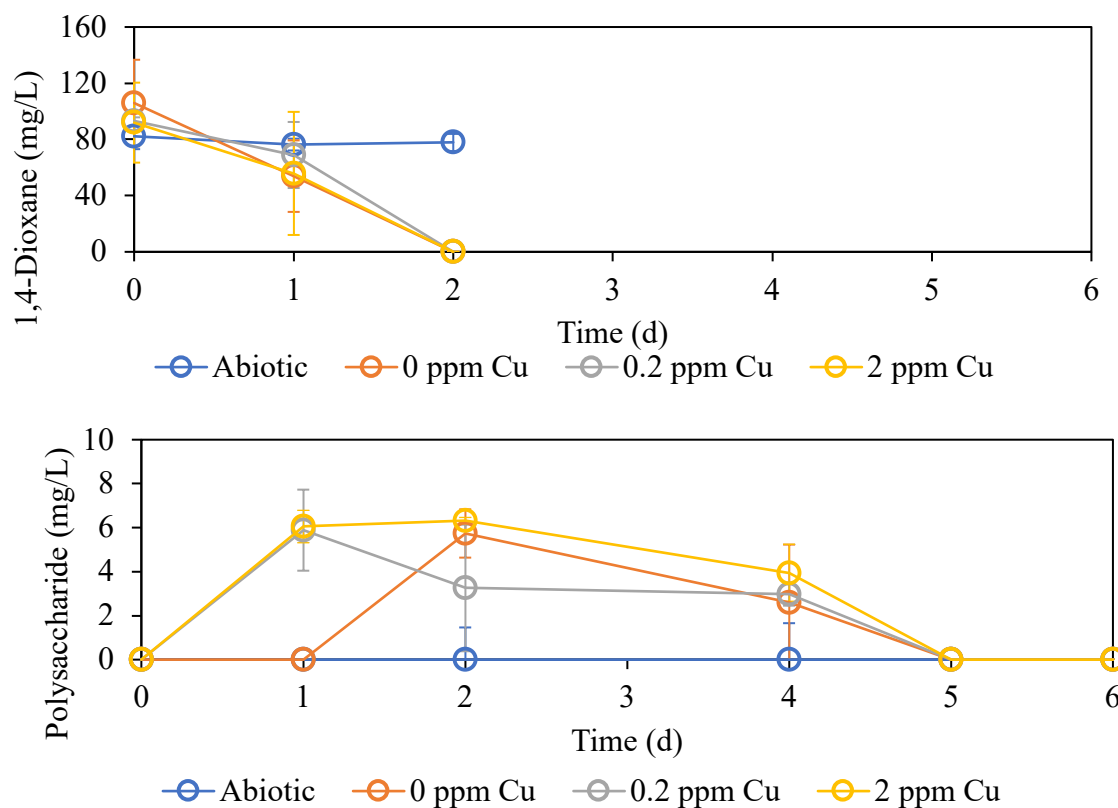


Figure 39. Extracellular polymeric substances (EPS) production by CB1190 using 1,4-dioxane for growth. EPS was produced earlier upon starvation and exposure to copper indicating the potential for biofilm formation even under stress.

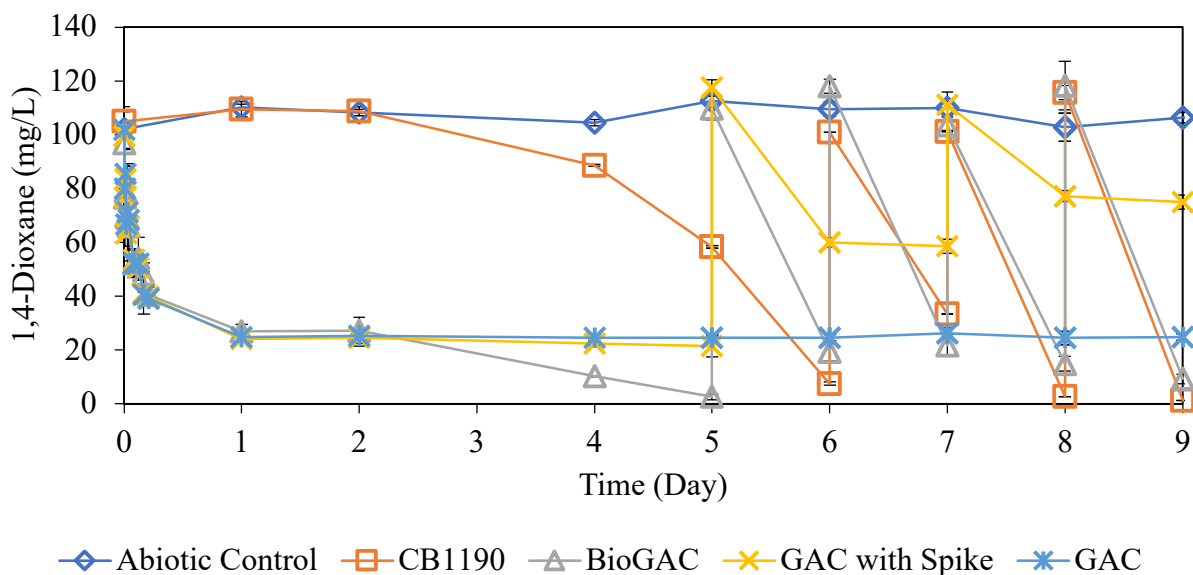


Figure 40. Bioaugmented and abiotic GAC reactors with 100 mg/L initial 1,4-dioxane concentration. Three conditions were tested: CB1190 bioaugmented Norit 1240 GAC with 1,4-dioxane added when the 1,4-dioxane was degraded below 25mg/L after initial degradation, abiotic GAC with 1,4-dioxane added every other day after day 5, and abiotic GAC with no additional 1,4-dioxane added. Abiotic and planktonic CB1190 controls were also tested for reference.

Biodegradation by CB1190 was successfully achieved in batch reactors and flow-through columns (Figures 40-43). Schematics for flow-through columns are shown in Figure 44. In CB1190 bioaugmented Norit 1240 GAC, 100 mg/L 1,4-dioxane initial concentration degraded to below detection limit in 5 days, requiring approximately one day thereafter to degrade to the 25 mg/L 1,4-dioxane level (Figure 40). Abiotic GAC with additional 1,4-dioxane added showed an increase in aqueous concentration of 1,4-dioxane, due to the limited adsorption capacity of GAC. This was confirmed by the abiotic GAC with no additional 1,4-dioxane added, which showed no change in aqueous concentration after equilibrium was reached after 1 day. Initial concentrations of 400 mg/L 1,4-dioxane were degraded by CB1190 bio-GAC in 15.5 days to below the set limit of 5% of added 1,4-dioxane remaining (Figure 41). Abiotic GAC with additional 1,4-dioxane added showed an increase in aqueous concentration of 1,4-dioxane, due to the limited sorption capacity of the GAC. Even though 1,4-dioxane biodegradation is first order with respect to 1,4-dioxane, the difference in time required for initial degradation between Figure 40 and 41 was likely due to the higher concentration added (Figure 41). For the batch reactors described, biodegradation from bio-GAC appeared to be effective between 100 and 400 mg/L initial 1,4-dioxane.

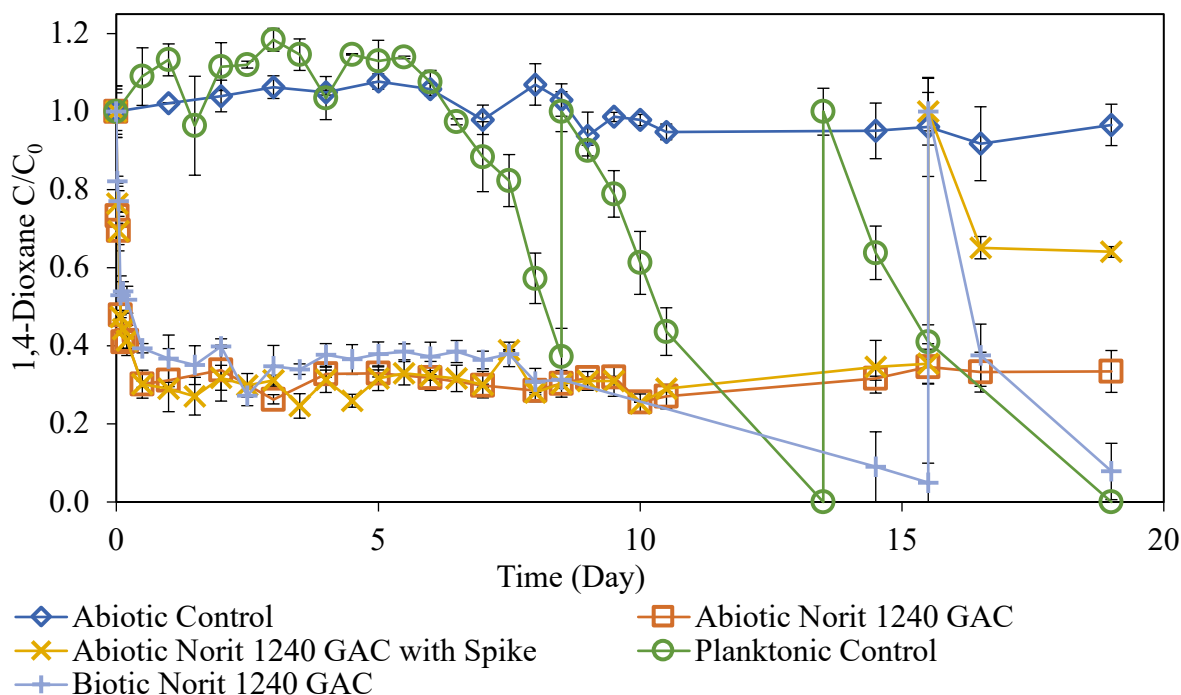


Figure 41. Bioaugmented and abiotic GAC batch reactors with 400 mg/L initial 1,4-dioxane concentration. CB1190 bioaugmented and abiotic Norit 1240 GAC with 1,4-dioxane added at 15.5 days and abiotic GAC with no additional 1,4-dioxane added. Abiotic and planktonic CB1190 controls were also tested for reference.

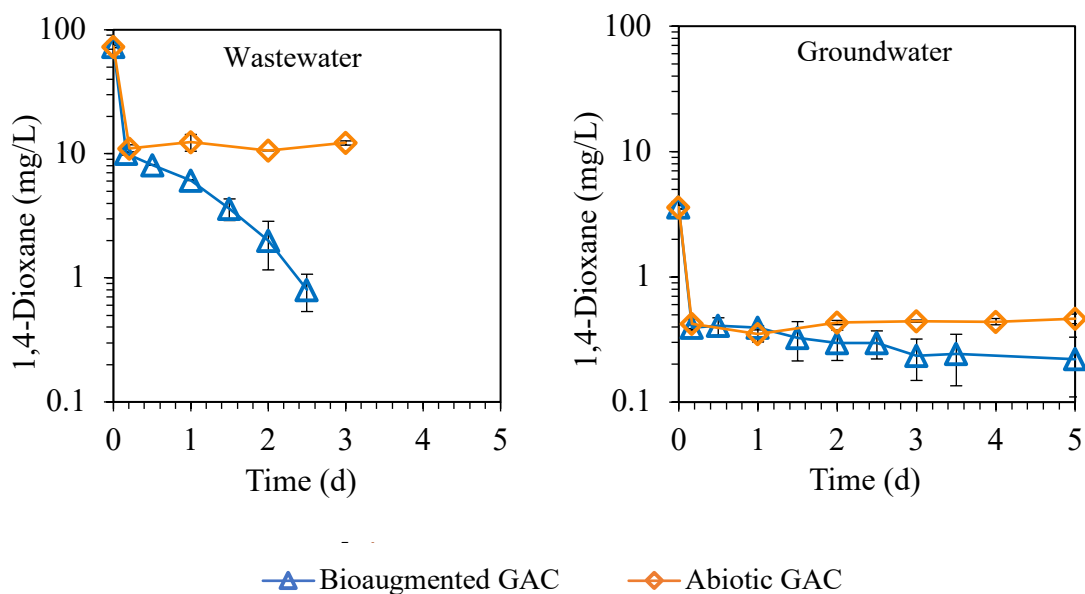


Figure 42. CB1190-bioaugmented GAC removed more 1,4-dioxane than abiotic GAC from environmental samples. Error bars represent half the range of duplicate reactors (Myers et al., 2018).

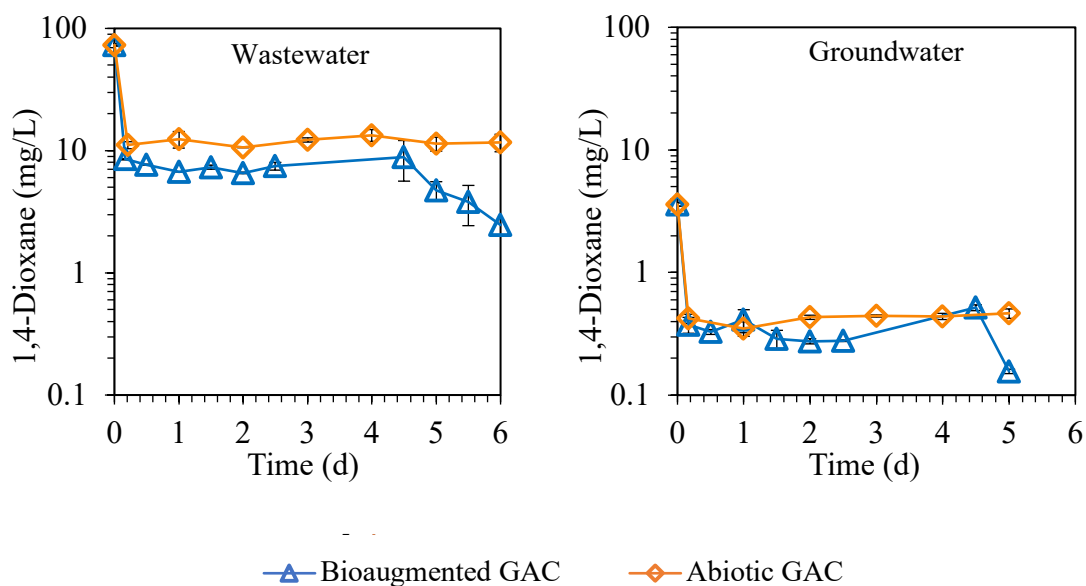


Figure 43. JOB5-bioaugmented GAC removed more 1,4-dioxane from environmental samples than abiotic GAC. Error bars represent half the range of duplicate reactors (Myers et al., 2018).

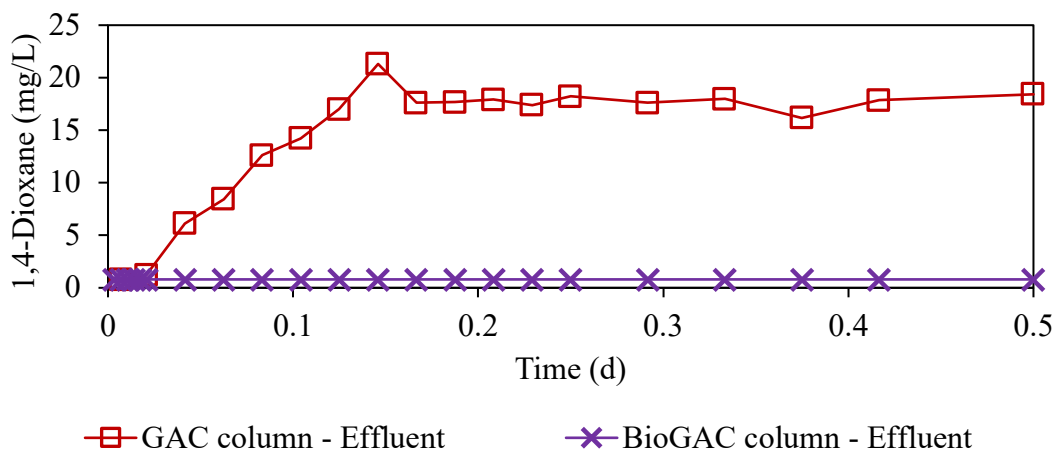


Figure 44. Column effluent 1,4-dioxane of a top-feed gravity recirculating reactor with an initial reservoir 1,4-dioxane concentration of 100 mg/L. The abiotic GAC column reached and maintained an effluent equilibrium level of 17 mg/L 1,4-dioxane, while the bioaugmented Norit 1240 GAC column maintained effluent concentrations below the limit of detection for the GC-FID.

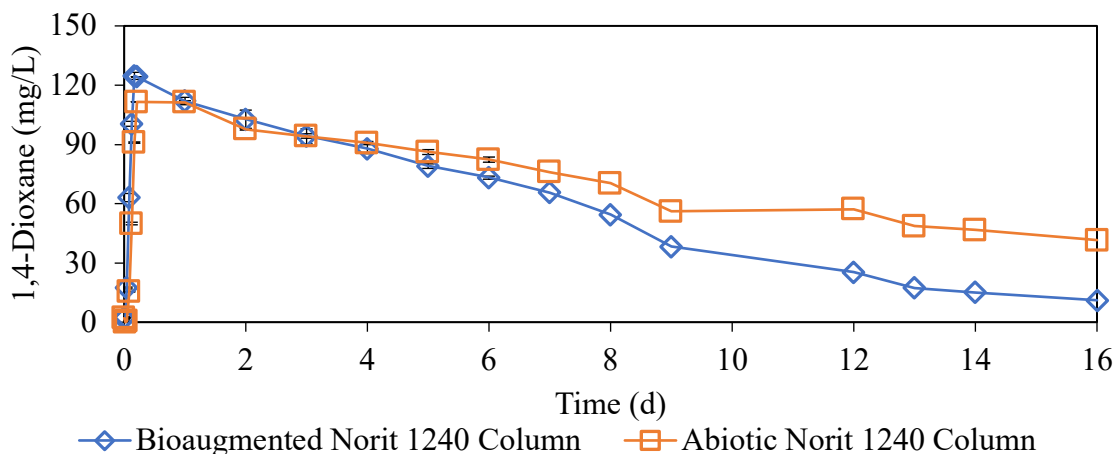


Figure 45. Influent levels of a recirculating column reactor with an initial reservoir 1,4-dioxane concentration of 400 mg/L. Divergence is observed at day 3, with the bioaugmented Norit 1240 column showing higher rates of 1,4-dioxane degradation.

Metabolic and cometabolic bio-GAC were tested in batch with 1,4-dioxane-impacted industrial wastewater and groundwater. Metabolic (figure 42) and cometabolic (figure 43) bio-GAC removed more 1,4-dioxane than abiotic GAC from contaminated industrial wastewater and groundwater. CB1190 bio-GAC removed 98.9% of 1,4-dioxane (73.4 mg/L down to 0.803 mg/L in 2.5 d) from industrial wastewater but only 93.8% (3.595 mg/L down to 0.220 mg/L in 5 d) from groundwater. Cometabolic bio-GAC also outperformed abiotic GAC. Cometabolic bio-GAC removed 96.6% (73.4 mg/L down to 2.5 mg/L in 6 d) and 95.6% (3.595 mg/L down to 0.157 mg/L in 5 d) from industrial wastewater and groundwater, respectively. The differences between metabolic and cometabolic bio-GAC performance can be explained by the nature of metabolic and cometabolic degradation. Metabolic degradation will lead to increased biomass (especially with higher substrate levels), while cometabolic degradation will have relatively constant biomass without the addition of primary substrate. CB1190 metabolism has been observed to kinetically respond to the level of primary substrate (in this case 1,4-dioxane), with lower levels leading to lower degradation rates. At certain low concentrations, the level of 1,4-dioxane is not enough to sustain 1,4-dioxane biodegradation by CB1190 and degradation will slow and eventually cease.

Cometabolic degradation is less dependent on contaminant concentration at the low end of the concentration range (as evidenced by the greater extent of removal in the groundwater than metabolic bio-GAC). However, cometabolic bio-GAC was unable to remove as much 1,4-dioxane from the more concentrated industrial wastewater than metabolic bio-GAC. This is consistent with the phenomenon of finite cometabolic biodegradation transformation capacity. Transformation capacity is the finite extent of degradation that cells are capable of before the energy requirements, product toxicity, and starvation of the cells slows and ceases biodegradation activity (Zenker et al., 2002).

For continuous flow columns, the difference between abiotic and bioaugmented GAC was observed as well. Schematics and pictures for two configurations of the recirculating column configurations are presented in Figures 46-47. Bioaugmented top-feed gravity flow column with

an initial concentration of 100 mg/L 1,4-dioxane had no measurable effluent 1,4-dioxane concentration throughout the experiment, while the abiotic top-feed gravity flow column achieves and maintains an equilibrium effluent concentration of approximately 17 mg/L 1,4-dioxane (Figure 44). Bioaugmented packed bottom-feed pump column with an initial concentration of 400 mg/L 1,4-dioxane demonstrated divergence from the abiotic packed bottom-feed pump column at day 3 (Figure 45). Bioaugmented sorbent in this configuration showed a steeper degradation rate than its abiotic counterpart. It is likely that the differences in feed configuration and the initial 1,4-dioxane concentrations resulted in the dramatic differences in bioaugmented Norit 1240 effluent concentrations. The difference between the bioaugmented Norit 1240 GAC in both columns and batch reactors was likely due to the biodegradation of 1,4-dioxane by CB1190. 1,4-Dioxane biodegradation was also carried out by CB1190 biofilms established on inorganic quartz packed in recirculating columns (Figure 48). Measured 1,4-dioxane rates were lower possibly because of lower CB1190 population and attachment on quartz surface area, but also limited oxygen availability in columns at lower recirculation rate. However, 1,4-dioxane degradation rates were not significantly inhibited in the presence of Cu(II) ions, likely because of longer incubation periods, lower per cell body burden, and decreased bioavailability of Cu(II) to CB1190 cells due to EPS in biofilms.

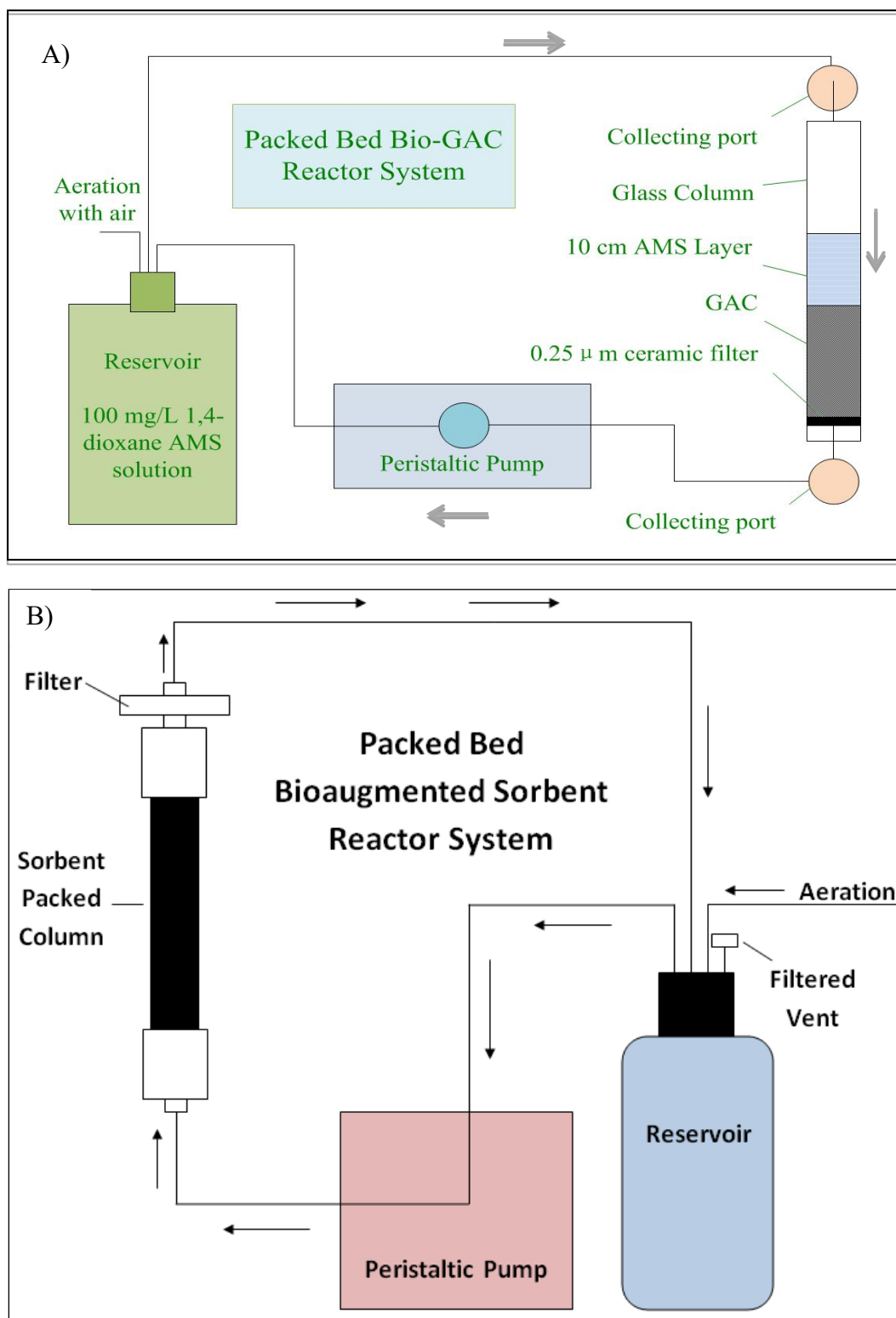


Figure 46. Schematics of columns used to study 1,4-dioxane biodegradation biofilms. (A) top-feed gravity and (B) packed bottom-feed recirculation.

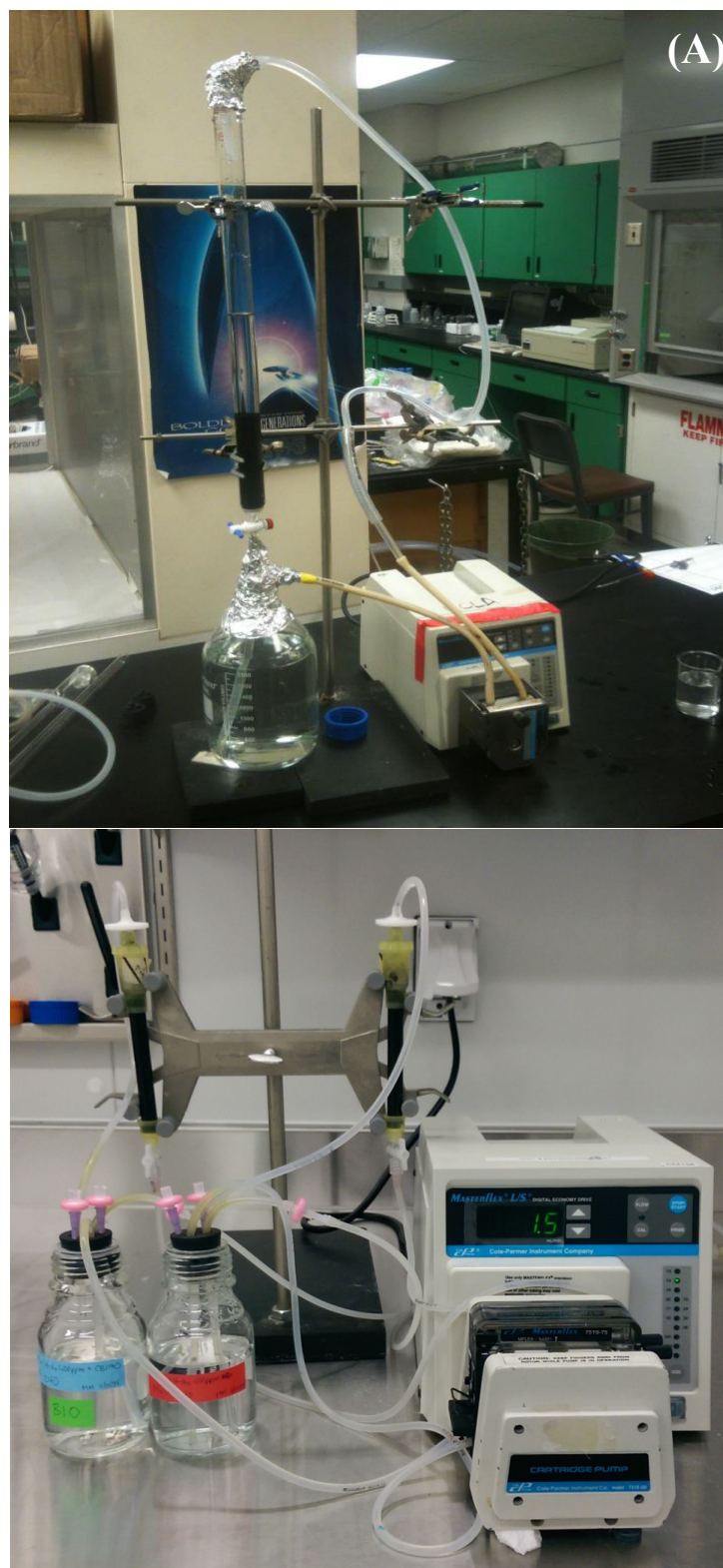


Figure 47. Photographs of columns used to study 1,4-dioxane biodegradation biofilms. (A) top-feed gravity and (B) packed bottom-feed recirculating columns.

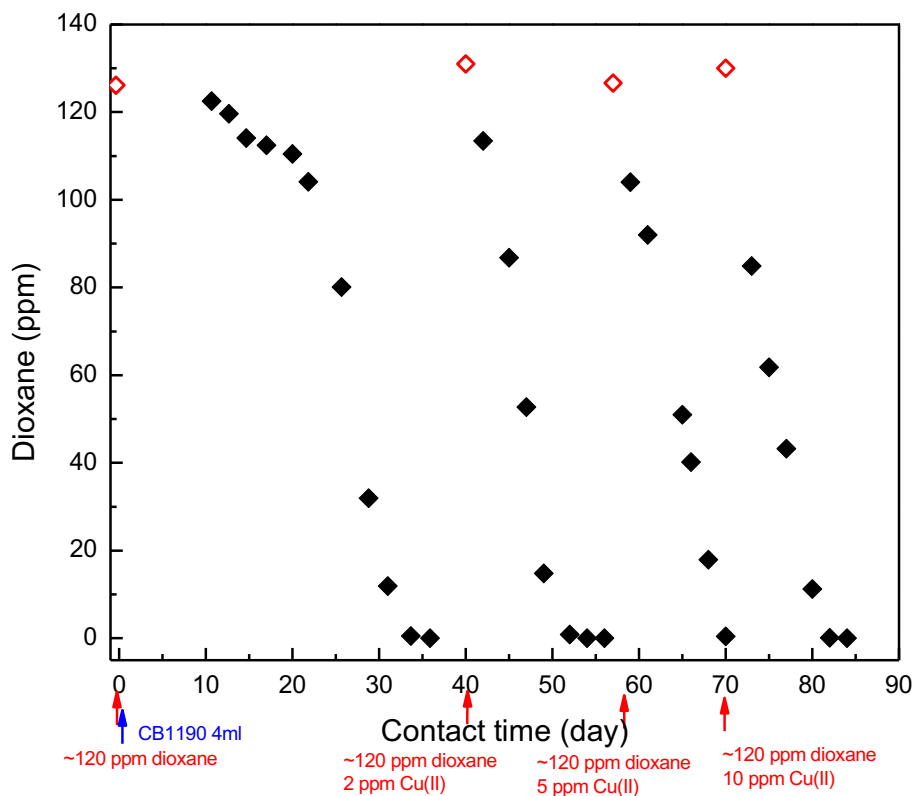


Figure 48. Recirculating column with 120 mg/L 1,4-dioxane added at day 0, day 40(with 2 mg/L Cu), day 58 (with 5 mg/L Cu), and day 70 (with 10 mg/L Cu).

Flow-through column reactors were constructed with abiotic and bio-GAC (figures 49-50). During the first 12 days of operation, the bio-GAC column removed 1,4-dioxane to levels below 100 µg/L with influent 1,4-dioxane levels between 3162 and 4040 µg/L. On day 13, the influent 1,4-dioxane was adjusted to 1000 µg/L. Effluent concentrations remained below 100 µg/L (except for one time point on day 28). The data is also presented in terms of relative effluent concentrations and mass removed for better comparison with abiotic GAC columns (Figure 50). During all 36 days of operation, removal efficiency remained greater than 90% for bio-GAC. Abiotic GAC rapidly broke through starting on day 10 (98.1% removal) with only about 48% removal efficiency by day 23. Over the course of 36 days of operation, the bio-GAC column removed 3.74 mg 1,4-dioxane. The abiotic GAC column removed 0.726 mg 1,4-dioxane in 24 days, while bio-GAC removed 2.97 mg in the same amount of time. It should be noted that the abiotic column was only exposed to influent concentration of 1000 µg/L over the course of the experiment. However, if it had been exposed to the initially higher loading of the bio-GAC column, it would have broken through even more quickly. This clearly demonstrates the significant advantage of bioaugmented GAC with biofilms. Bio-GAC has a considerably larger removal capacity than abiotic GAC, which means longer times between changing the column media.

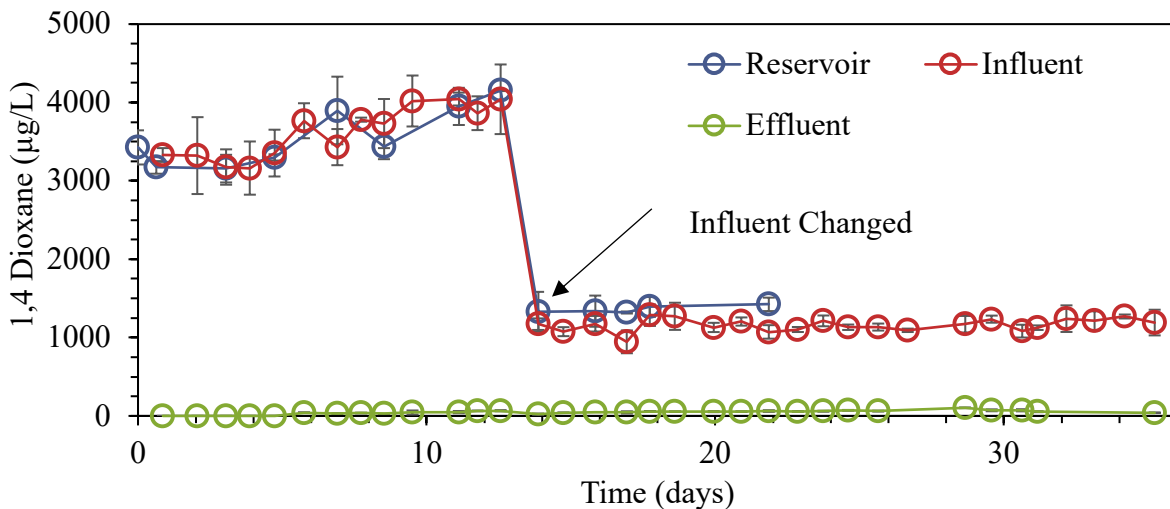


Figure 49. CB1190 bio-GAC flow-through column reactors removed more than 90% of influent 1,4-dioxane during 36 days of operation. Even when the influent concentration decreased from 3000-4000 µg/L down to approximately 1000 µg/L, effluent concentration remained below 100 µg/L. Error bars represent half the range of analytical replicates (n=2).

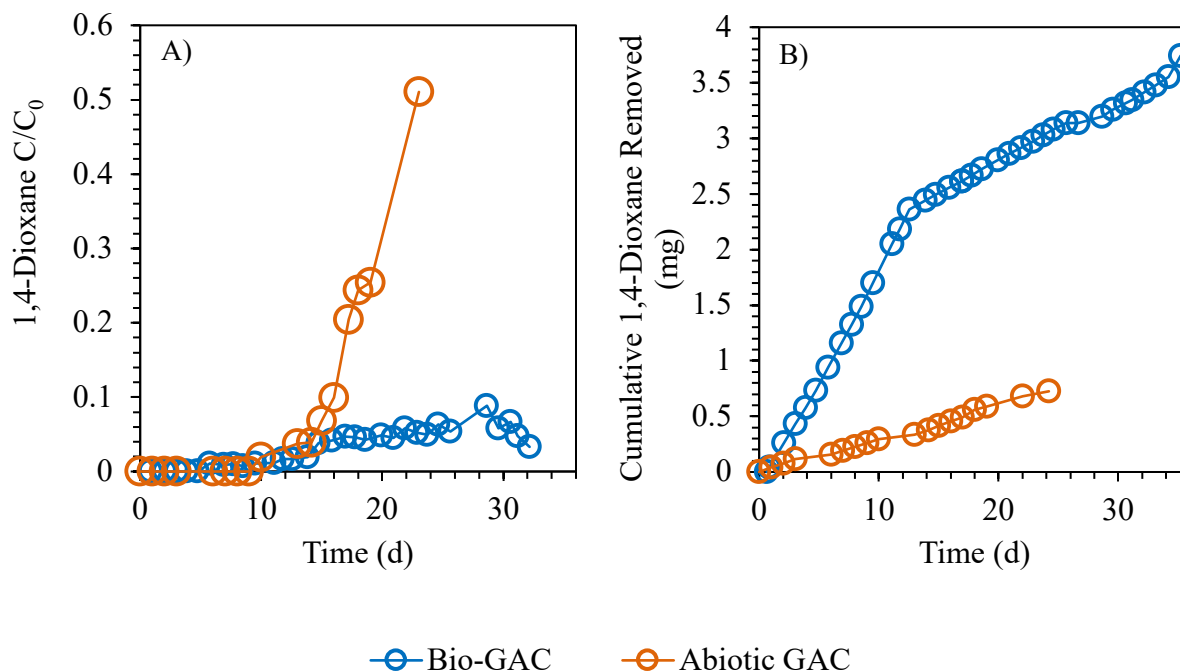


Figure 50. The bio-GAC flow-through column outperformed its abiotic GAC counterpart. A) Bio-GAC maintained greater than 90% removal rates during 36 days of operation while abiotic GAC rapidly broke through as removal rates decreased from ~90% on day 16 to ~50% removal by day 23. B) Bio-GAC removed more than 3 mg 1,4-dioxane by day 24, while abiotic GAC only removed 0.7 mg in the same time period.

Adsorption of 1,4-Dioxane

For many years, adsorption has not been considered as a viable method for removing 1,4-dioxane from water due to its low K_{oc} value. However, during our attempts to use Norit 1240 GAC as a solid support for biofilm growth, we found that GAC is in fact capable of adsorbing amounts of 1,4-dioxane. This has led to additional investigation into other potential adsorbents for removing 1,4-dioxane from water. It should be noted that in the years since the start of this project, a synthetic adsorbent under the trade name Ambersorb 560 has been developed which has a relatively large maximum adsorption capacity for 1,4-dioxane (40 mg/g at an equilibrium concentration of 80 mg/L) (Woodard et al., 2014). One other promising adsorbent beyond Ambersorb 560 that we have investigated is the pentasil adsorbent ZSM-5, which has primarily been used as a catalyst for hydrocarbon cracking (Rahimi and Karimzadeh, 2011). We found during isotherm investigation that ZSM-5 (hereafter referred to as zeolite) has an even higher maximum adsorption capacity than GAC (65 and 38 mg/g, respectively; figure 51).

While GAC can rapidly adsorb 1,4-dioxane, this process is also reversible. The adsorption and desorption kinetics are presented in Figure 52. Norit 1240 GAC reaches equilibrium with 1,4-dioxane within 10 hours, but can also desorb 22-39% of 1,4-dioxane when exposed to 1,4-dioxane-free, DI water and allowed time for equilibration. In a separate desorption experiment (Figure 53), CB1190-bioaugmented GAC and abiotic GAC were first amended 5 and 9 times, respectively, with 1,4-dioxane to increase reactor levels to 100 mg/L each time. Following 1,4-dioxane loading, the solids were washed 5 times with 25 mL AMS medium and deionized water, respectively. Each wash consisted of allowing batch reactors to equilibrate for 10 minutes with the added supernatant before the supernatant was removed for 1,4-dioxane quantification.

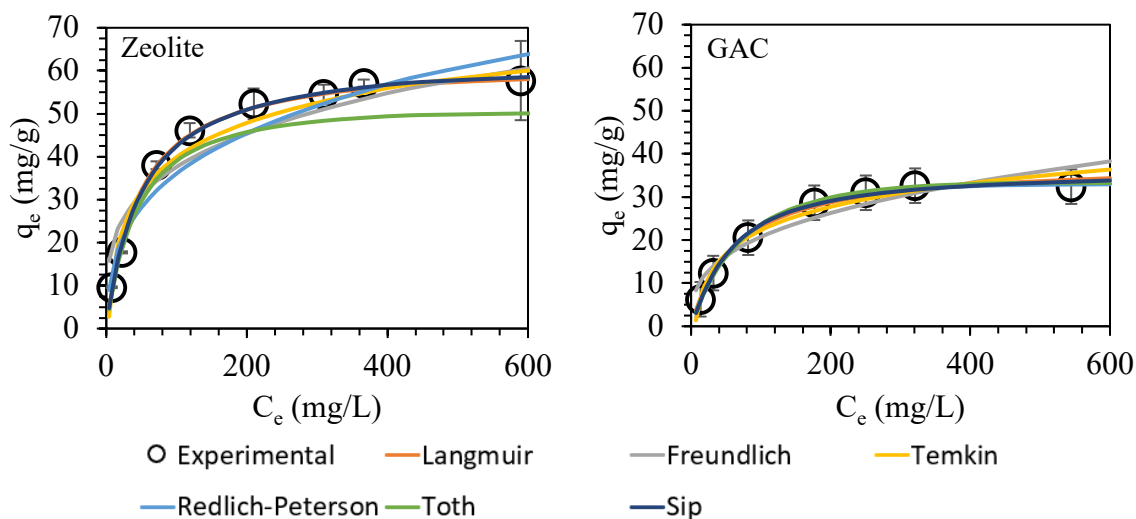


Figure 51. ZSM-5 zeolite and Norit 1240 GAC isotherms conducted at 30°C. Zeolite and GAC were found to have a maximum adsorption capacity of 65 and 38 mg/g, respectively. Error bars represent the standard deviation of triplicate reactors (Myers et al., 2018).

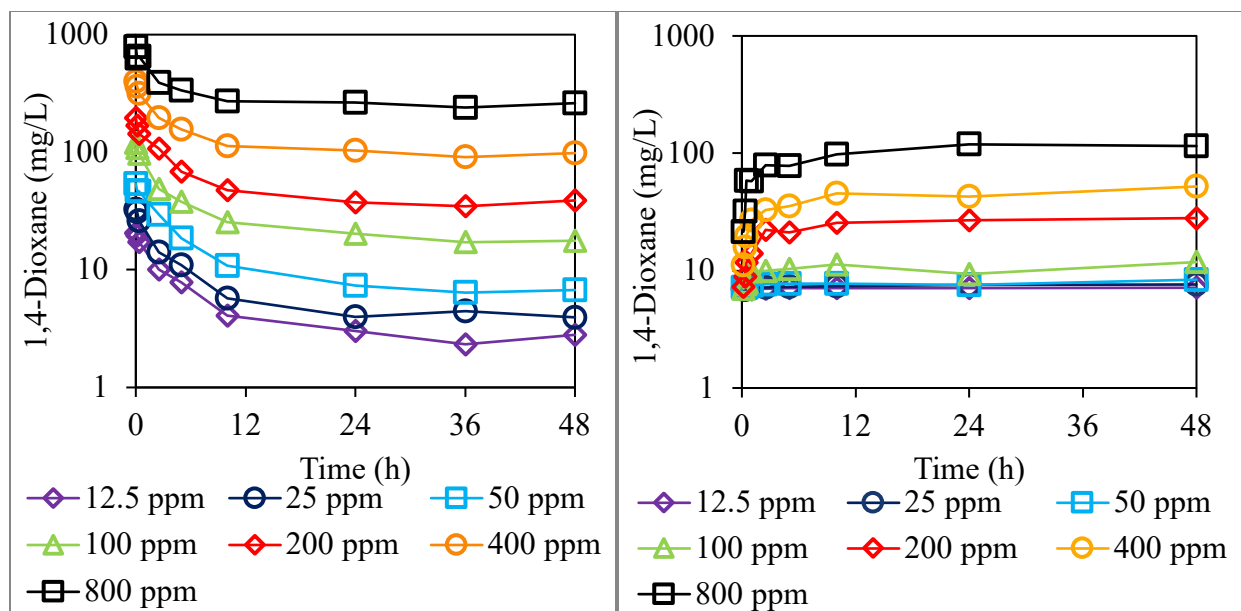


Figure 52. 1,4-Dioxane easily adsorbs and desorbs from GAC. Adsorption equilibrium was achieved after 10 h. After submerging equilibrated GAC in 1,4-dioxane-free DI water, desorption equilibrium was achieved in 24 h.

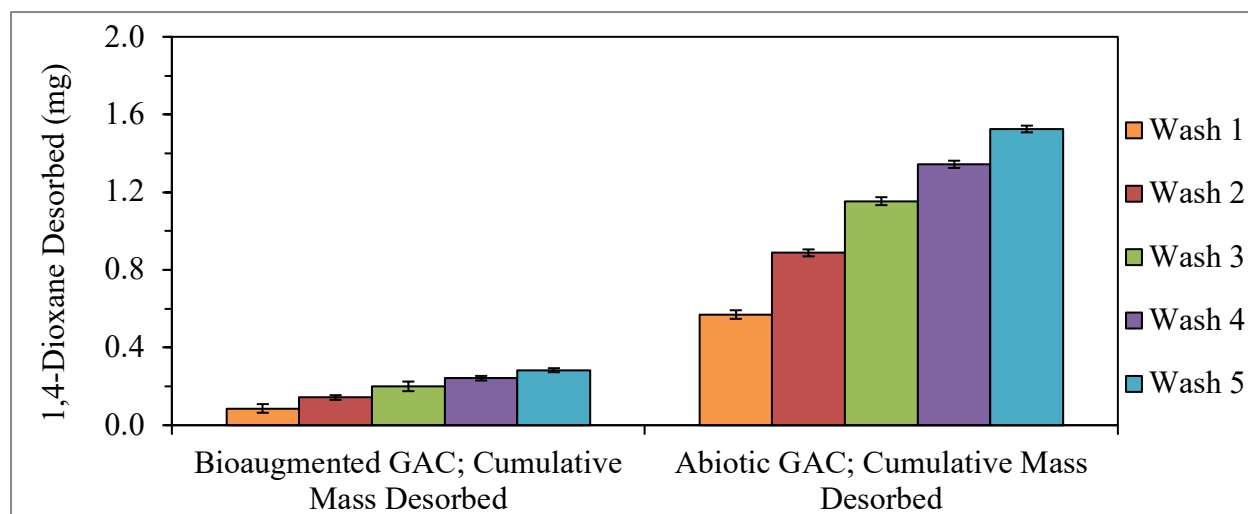


Figure 53. Bioaugmenting GAC mitigated the extent of 1,4-dioxane desorption when compared with abiotic GAC. Over 5 successive washes, abiotic GAC desorbed 538% more 1,4-dioxane than CB1190-bioaugmented GAC. Error bars represent the standard deviation of analytical replicates (n=4) (Myers et al., 2018).

Biodegradation of 1,4-Dioxane in Chlorinated Solvent Mixtures by Biofilms

CB1190 grown on quartz sand was first used to test the ability of metabolic biofilms to degrade 1,4-dioxane in CVOC mixtures. 25 mg/L TCE did not significantly inhibit 1,4-dioxane biodegradation by biofilms when compared with a TCE-free control, while 1 mg/L 1,1-DCE

reduced 1,4-dioxane removal by approximately 20% relative to the 1,1-DCE-free control (figure 54). This is expected given our data that identifies 1,1-DCE as a more potent inhibitor compared to TCE. However, it is important to note that at these levels, 1,4-dioxane biodegradation was completely inhibited in planktonic biodegradation studies.

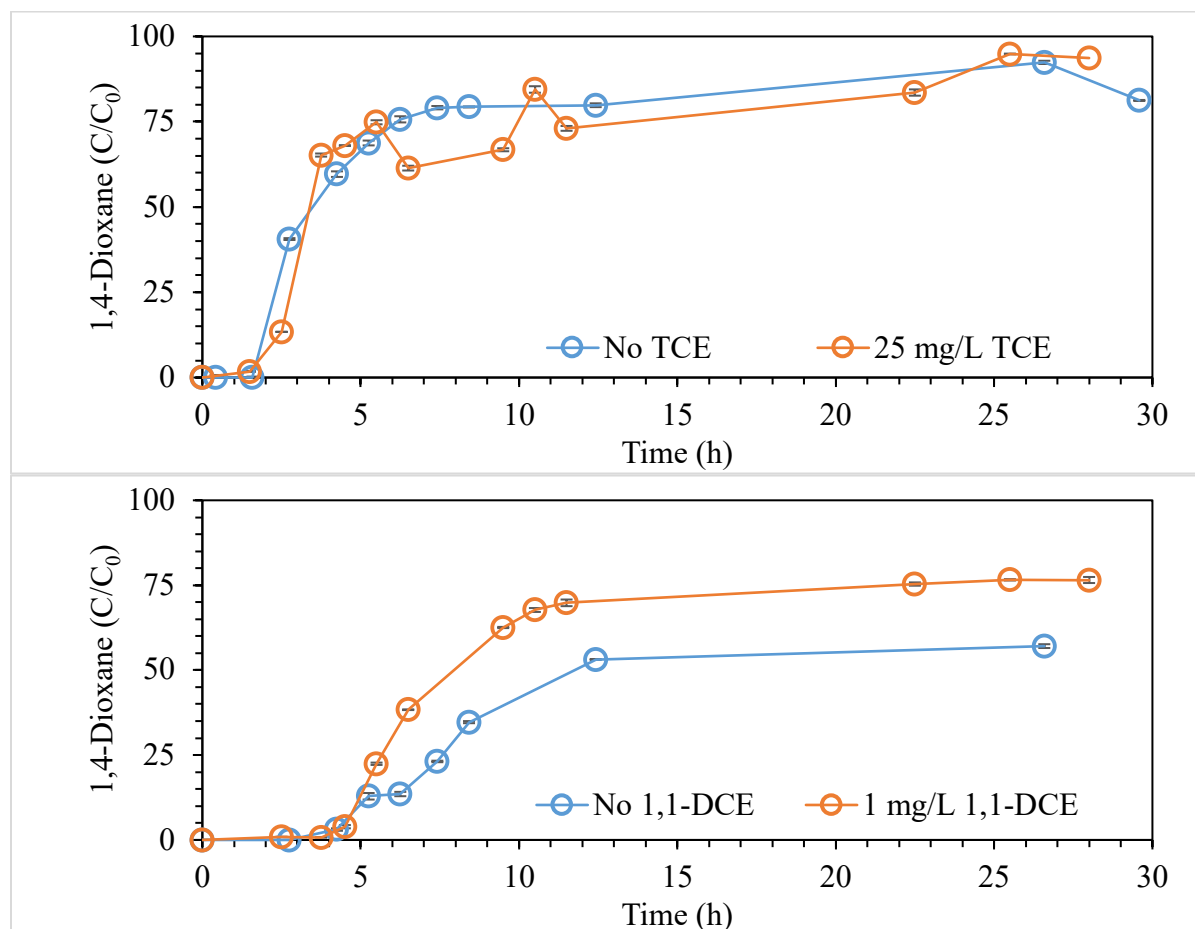


Figure 54. Effect of chlorinated solvents on the degradation of 1,4-dioxane in quartz columns inoculated with *P. dioxanivorans* CB1190. The columns were operated in flow-through mode at a flow rate of 0.1 mL/min (4.2 bed volumes/d; residence reaction time of 5.7 h). Columns were first run with only 1,4-dioxane (100mg/L) as controls (chlorinated solvent-free). Columns were then run with influent solution containing (A) 100mg/L 1,4-dioxane and 25mg/L TCE and (B) 100mg/L 1,4-dioxane and 1 mg/L 1,1-DCE. 1,4-dioxane degradation was expressed as the ratio of the influent concentration to the influent concentration of 1,4-dioxane (C/C_0) (Zhao et al., 2018).

Next, we investigated the potential for CB1190 bioaugmented GAC and zeolite to remove 1,4-dioxane in CVOC mixtures. The results are presented in figures 55 and 56 for batch reactors containing bio-GAC and bio-zeolite, respectively. Bio-GAC reactors tested solutions of AMS medium amended with 100 mg/L 1,4-dioxane with the addition of 0 or 2.5 mg/L (reactor concentration) TCE, cDCE, 1,1-DCE, or all three solvents. In all cases, bio-GAC removed 1,4-dioxane to lower levels than abiotic GAC (even with an additional amendment of 1,4-dioxane spiked in partway through the experiments). TCE and cDCE did not cause appreciable inhibition

when compared with 1,4-dioxane-only in bio-GAC reactors. 1,1-DCE did somewhat inhibit bio-GAC removal of 1,4-dioxane as evidenced by the noticeable slope after the initial rapid removal due to adsorption. Mixtures containing all 3 CVOCs further inhibited bio-GAC.

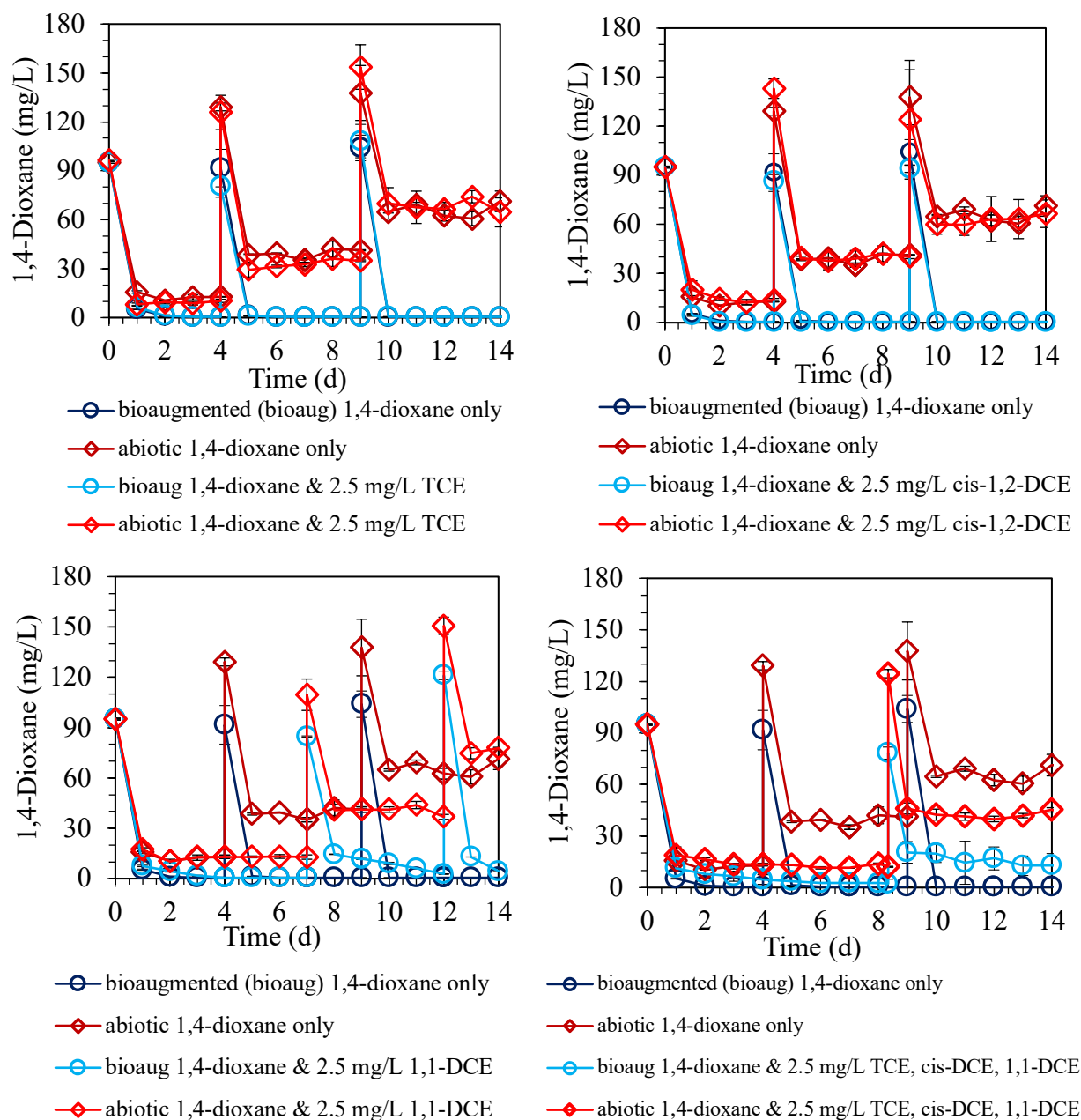


Figure 55. Batch CB1190 bio-GAC reactors degraded 1,4-dioxane in the presence of CVOCs. Reactors were initially amended with 100 mg/L 1,4-dioxane and 0 or 2.5 mg/L (total reactor concentration) of TCE, cDCE, 1,1-DCE or all three chlorinated solvents. Bio-GAC reactors consistently outperformed abiotic GAC in terms of the extent of 1,4-dioxane removal. Error bars represent half the range of duplicate reactors.

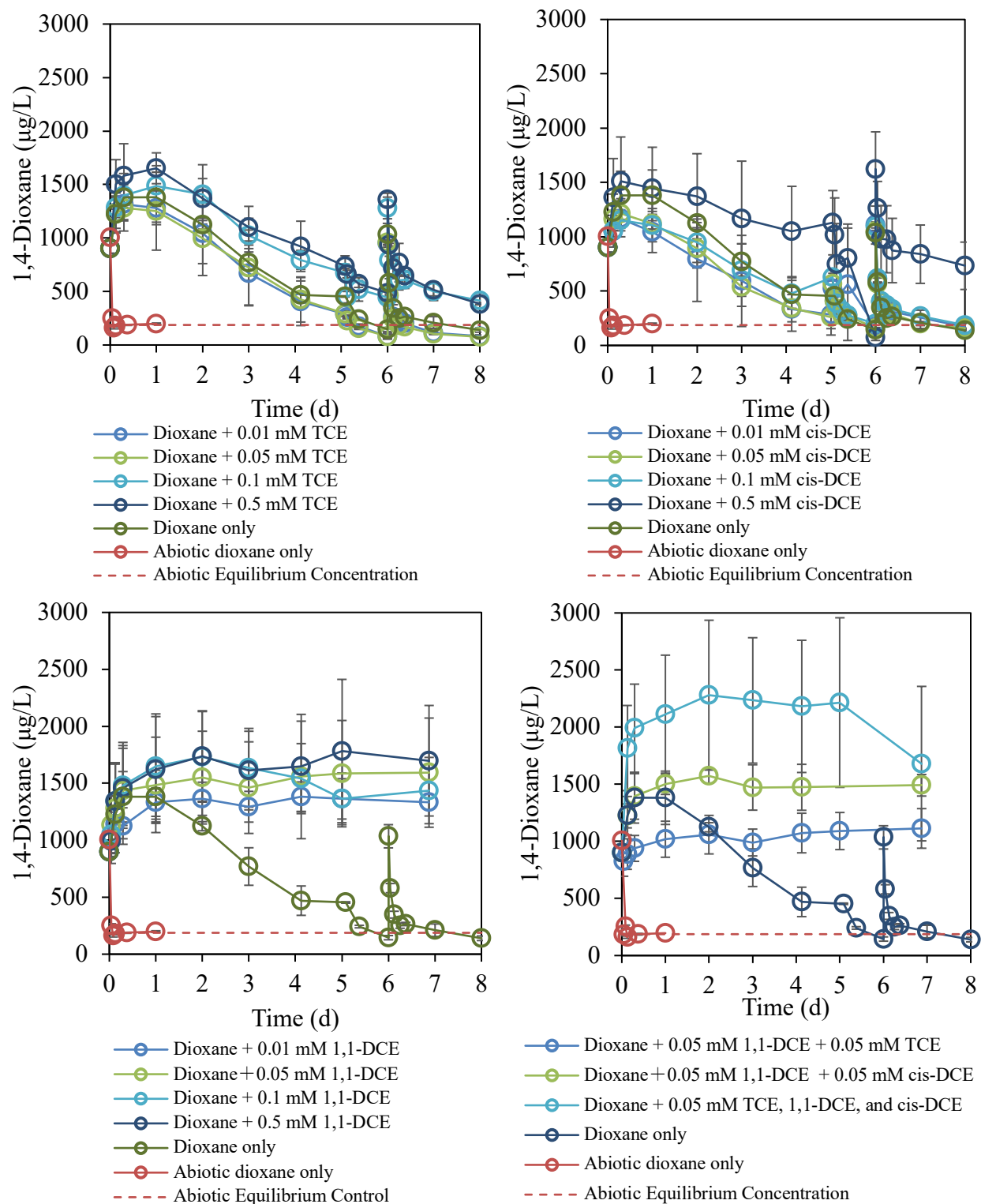


Figure 56. Batch CB1190 bio-zeolite reactors degraded 1,4-dioxane in the presence of TCE and cDCE. Batch reactors consistently outperformed abiotic zeolite for 1,4-dioxane in the presence of both TCE and cDCE. 1,1-DCE and mixtures containing 1,1-DCE inhibited 1,4-dioxane degradation. Error bars represent the standard deviation of triplicate reactors.

Bio-zeolite batch reactors were also able to remove 1,4-dioxane in the presence of CVOCs. Batch reactors containing bio-zeolite and mixtures of 1,4-dioxane, TCE, cDCE, 1,1-DCE, and CVOC mixtures were compared against abiotic zeolite with only 1,4-dioxane. Abiotic zeolite reactors were only tested with 1,4-dioxane because that condition would have the best removal compared with abiotic zeolite reactors that also contained CVOCs. TCE and cDCE did not inhibit 1,4-dioxane removal rates at all levels tested, ranging from 0.01-0.5 mmol/L. However, these CVOCs did cause some desorption of 1,4-dioxane adsorbed during the bioaugmentation step prior to the start of the experiment. This can be clearly seen in Figure 55 as the initial 1,4-dioxane level of 1000 µg/L increases to 1500 µg/L within the first day of the experiment. Reactors containing TCE and cDCE were able to recover and degrade 1,4-dioxane to low µg/L levels. The reactors were able to repeatedly biodegrade 1,4-dioxane to low levels after 1,4-dioxane was spiked to 1000 µg/L on day 6.

Reactors containing 1,1-DCE at levels ranging from 0.01-0.5 mmol/L completely inhibited 1,4-dioxane removal. Additionally, reactors containing 1,1-DCE in mixtures with either TCE, cDCE, or both completely inhibited 1,4-dioxane removal, suggesting that 1,1-DCE dominates as an inhibitor in mixtures of CVOCs. To help explain why 1,1-DCE was such a potent inhibitor for 1,4-dioxane removal by bio-zeolite when bio-GAC was able to degrade 1,4-dioxane in the presence of 1,1-DCE, competitive adsorption batch studies were conducted with 1,4-dioxane and all 3 CVOCs (Figure 57). Isotherm experiments were conducted with single and bi-solute systems in 1:1 molar ratios of 1,4-dioxane and TCE, cDCE, or 1,1-DCE. Single solute isotherms show that the order of maximum adsorption capacity for the contaminants is in the order TCE > cDCE > 1,1-DCE = 1,4-dioxane. This is further supported by the isotherms of the bi-solute systems. This suggests that the 1,4-dioxane adsorbed during the bioaugmentation of the zeolite would impede adsorption of 1,1-DCE. This 1,1-DCE would then remain in bulk solution and inhibit 1,4-dioxane biodegradation by the bacteria grown on the surface of the zeolite granules.

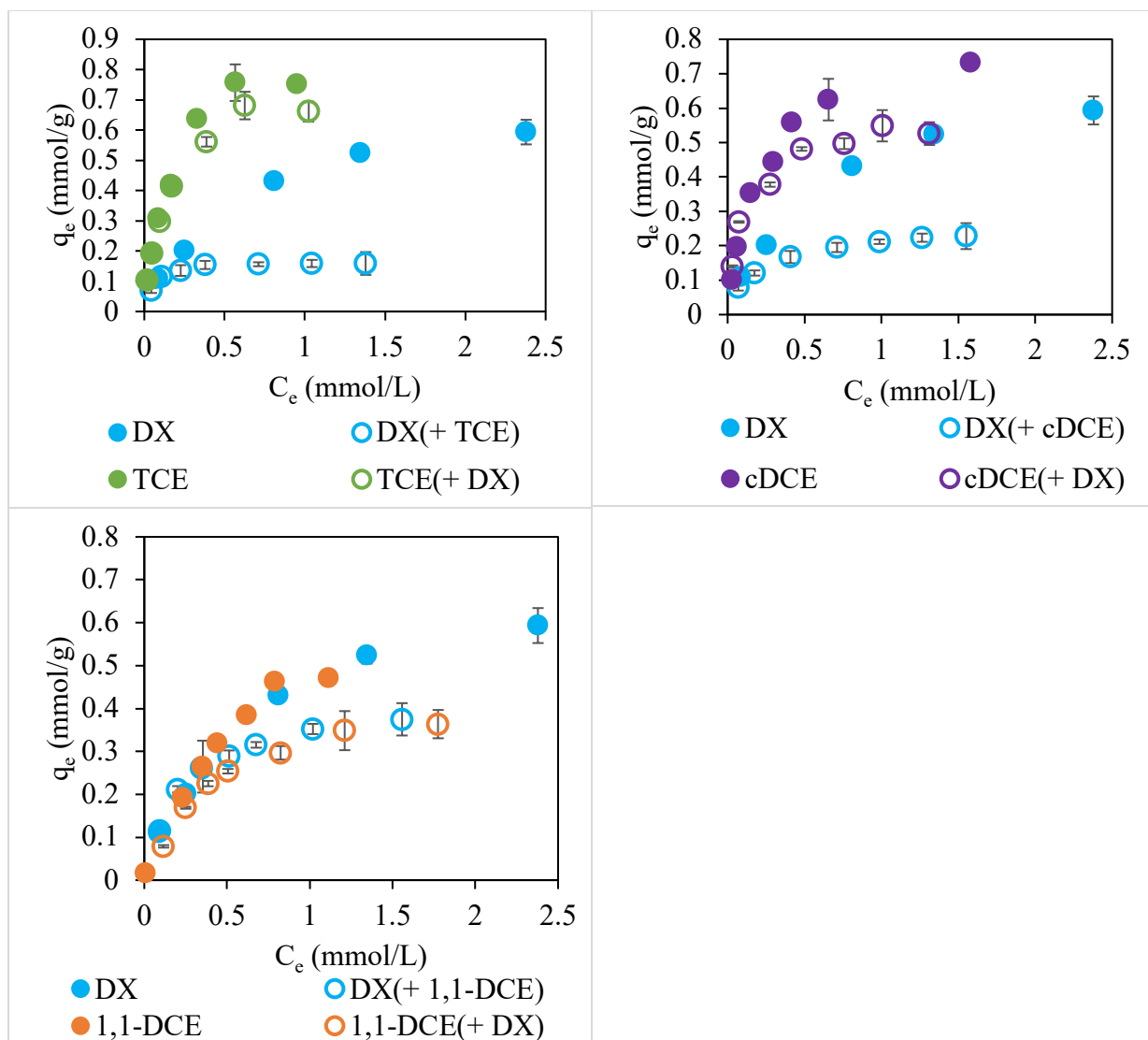


Figure 57. Zeolite competitive isotherms. The order of affinity according to competitive isotherm experiments is TCE > cDCE > 1,1-DCE = 1,4-dioxane. Error bars represent the standard deviation of triplicate reactors.

Biodegradation of 1,4-Dioxane by Biofilms in ERD Treated Soil

The potential for the application of bioremediation to treat 1,4-dioxane in ERD treated soil was tested in pack soil columns using soil and groundwater from a site previously treated by enhanced reductive dichlorination (Figure 58). 1,4-Dioxane removal was tested under 4 conditions including autoclaved sterilized soil, untreated soil with oxic influent, untreated soil with anoxic influent, and soil bioaugmented with CB1190. The soil and groundwater were both sourced from Air Force Plant (AFP) 44. As expected, the soil column bioaugmented with CB1190 consistently removed 80-100% 1,4-dioxane under varying influent concentrations and flow rates. Little to no removal was observed in the 3 other conditions when influent 1,4-dioxane was 3 mg/L. When influent 1,4-dioxane was increased to 10 mg/L and flow rates were reduced to 0.01 mL/min, untreated soil under oxic conditions removed approximately 25-50% 1,4-dioxane after the transient response due to restarting the column after the influent change. Autoclave sterilized and anoxic untreated soil conditions exhibited no appreciable 1,4-dioxane removal.

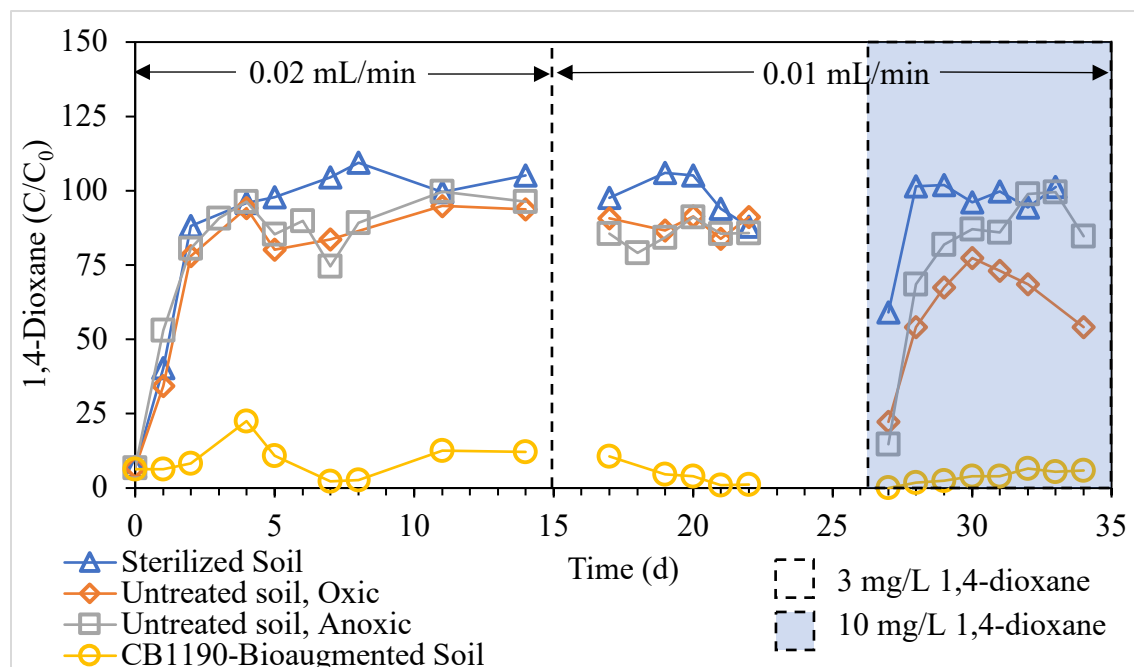


Figure 58. Biodegradation of 1,4-dioxane in flow-through column systems packed with ERD-treated soil. These columns consisted of: a sterilized soil column (control), untreated (native) soil column under oxic conditions, untreated soil column under anoxic conditions, and soil column bioaugmented with CB1190. The columns were all operated at a flow rate of 0.02 mL/min (0.6 bed volumes/d; residence reaction time 39 h) for 14 d, followed by the first 14 d. At day 14 the rate was decreased to 0.01 mL/min (~ 0.3 bed volumes/d; residence reaction time 78 h) and held constant for the duration of the run. The remainder of the experiment. Over the course of the experiment, the influent groundwater was spiked with 1,4-dioxane levels were kept at either 3 or 10 mg/L 1,4-dioxane (without co-contaminants) (Zhao et al., 2018).

After testing 1,4-dioxane removal capabilities without co-contaminants, new columns were constructed with the same soil and groundwater and used to test the potential for 1,4-dioxane biodegradation in the presence of 1,1-DCE and Cu(II) (Figure 59). These experiments contained

the same conditions except the anoxic condition. Autoclave sterilized soil did not remove any 1,4-dioxane under all conditions. Untreated soil under oxic conditions did remove 15-50% 1,4-dioxane of the course of the entire 100-day experiment. Noticeable decreases in removal efficiency with the addition of 5 and 10 mg/L 1,1-DCE (approximately 35 and 25% removal, respectively) can be observed relatively to the initial condition containing just 1,4-dioxane (approximately 50% removal). Soil bioaugmented with CB1190 followed similar trends, though no real difference was observed between 5 and 10 mg/L 1,1-DCE (average of approximately 85% removal) conditions, while the initial removal efficiency was approximately 90-95%.

The second phase of the experiment consisted of changing the influent back to groundwater containing only 1,4-dioxane on day 70 followed by the addition of Cu(II) at around day 84. No clear changes in the untreated soil under oxic conditions with the addition of Cu(II). Again, little to no removal was observed for the autoclave sterilized soil condition. The CB1190 bioaugmented condition presented a sharp decrease in removal efficiency with the addition of 10 mg/L Cu(II) (85-90% removal down to approximately 60-70% removal).

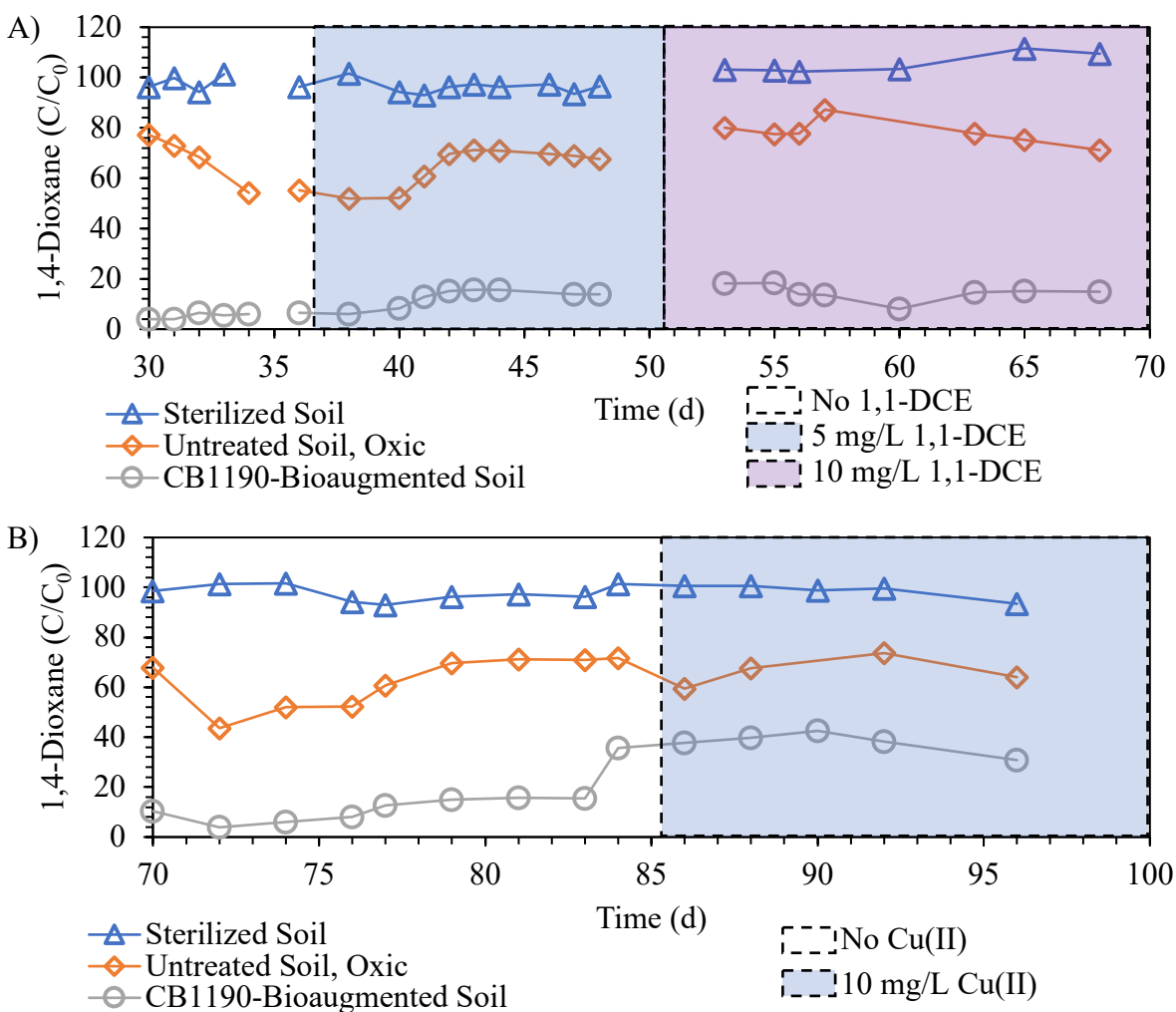


Figure 59. Inhibitory effects of co-contaminants A) 1,1-DCE and B) Cu(II) on 1,4-dioxane biodegradation in flow-through column systems packed with ERD-treated soil. Soil columns correspond to those used in Figure 58 (without co-contaminants), including a sterilized soil column, an untreated soil column under oxidic condition, and a bioaugmented soil column. All columns were operated at a flow rate of 0.01 mL/min (0.3 bed volume/d; residence reaction time 78 h) with 10 mg/L 1,4-dioxane in the influent (Zhao et al., 2018).

Only partial inhibition was observed for 5 or 10 mg/L 1,1-DCE and 10 mg/L Cu(II). However in planktonic cultures, these concentrations of co-contaminants (5 mg/L 1,1-DCE and 10 mg/L Cu(II)) completely inhibited 1,4-dioxane biodegradation by CB1190. This observation demonstrates that biofilm growth mode significantly affects the ability of CB1190 to resist inhibition by co-contaminants. This is true even when CB1190 is grown on previously ERD-treated soil.

Microbial Community Analysis

The objective of microbial community analysis was to identify the relationships among the microbial compositions, contaminant removals, and process-specific features as a result of combining chemical oxidation and biodegradation. To achieve this goal, we investigated the influence of key variables (initial contaminant concentrations and operational duration) and biological factors (microbial interactions and co-occurring/co-excluding patterns) that shape overall microbial community assembly and functions.

Both Spearman's rank and Pearson's linear correlations showed no significant connections among biodiversity, richness, and 1,4-dioxane concentrations (Table 8). However, the Shannon index indicated that biodiversity under all conditions was reduced significantly (p -value < 0.05) during the oxidation phase, except for the H-DX/H-CV condition. Afterwards, during the first stage of the biodegradation phase from OB2 (day 0) to OB3 (day 17), the biodiversity was relatively stable under H-DX/H-CV, H-DX/L-CV and L-DX/L-CV, but increased significantly under L-DX and Bioaug conditions. Rapid and significant increases of biodiversity were observed after 9-week incubation of biodegradation from OB3 to OB4 (day 63) under all conditions.

The richness metrics represented by the Chao1 index and observed species (0.97 OTU) depict the change of species along with treatment trains, both of which kept decreasing from OB1 to OB3 under all conditions with CVOCs. Under low 1,4-dioxane-only condition, the richness of microbial community started to recover from OB2 to OB3, which occurred earlier than in microcosms with CVOCs.

Table 8. Pearson and Spearman correlations between richness/diversity and 1,4-dioxane under different conditions.

Method s	Indices	HH		HL		L		LL		Bioaug	
		R	P-value	R	P-value	R	P-value	R	P-value	R	P-value
Pearson	invsimpson	0.412	0.588	0.229	0.771	-0.294	0.706	-0.017	0.983	0.540	0.46
	shannon	0.318	0.682	0.196	0.804	-0.016	0.984	0.102	0.898	-0.070	0.93
	Chao1	-0.096	0.904	-0.063	0.937	-0.084	0.916	-0.225	0.775	0.102	0.898
	sob	0.014	0.986	-0.027	0.973	-0.097	0.903	-0.179	0.821	-0.276	0.724
Spearman	invsimpson	0.4	0.6	0.4	0.6	0.4	0.6	0.6	0.4	-0.4	0.6
	shannon	0	1	0.8	0.2	0.4	0.6	0.6	0.4	0.4	0.6
	Chao1	0.4	0.6	0	1	0.8	0.2	0	1	0.2	0.8
	sob	0	1	0.6	0.4	0.8	0.2	0.6	0.4	0.8	0.2

The microbial community shifted rapidly along with time during the whole treatment train incubation. Results indicate that time was a better variable for explaining the dynamics of the microbial community (Figure 60B, 60D, color-coded) than the presence and/or concentration of co-contaminants (Figure 61). PCoA biplots based on Unifrac distances described that both microbial membership (unweighted) and abundance (weighted) developed along with treatment trains under different conditions. Furthermore, the oxidation phase clearly influenced the microbial community both in terms of membership and abundance. The bacterial membership (unweighted, Figure 60C, 60D) seemed to separate more apparently than bacterial abundance (weighted, Figure 60A, 60B) during the biodegradation phase, and this was consistent with the Venn diagram (Miao et al., 2019) showing that more species were identified at OB4.

When taking the conditions into consideration, the community in H-DX/H-CV condition (triangles in Figure 60A and 60C) changed more slowly than in other conditions, while the community in the L-DX condition (cross in Figure 60A and 60C) evolved relatively faster compared to other conditions. Moreover, the members at OB3 tended to merge with OB2 (Figure 60D), while the abundances were in relative proximity to OB4 (Figure 60C), indicating the unstable impacts under different conditions. Interestingly, microbiomes at OB4 showed more clear boundaries among conditions, and the linear connections-based Pearson correlation showed that samples at OB4 only had solid connections within same conditions (purple in Figure 62A). Additionally, impact from oxidation reduced the connections among samples under different conditions when comparing between OB1 (green in Figure 62A) and OB2 (thinner edge of blue in Figure 62A), while the signs of recovery could be found at OB3 (orange in Figure 62A), since more connections were re-built. The cluster analyses based on unsupervised classification (ungrouped) depicted the relative distance of each sample, and microbial community structure showed deviations under different conditions at OB4 with relatively further shifts than the rest of the samples (Figure 62B). In clustered samples collected at OB1, OB2 and OB3, samples belonging to OB2 and OB3 were closely related regardless of the conditions (Figure 63).

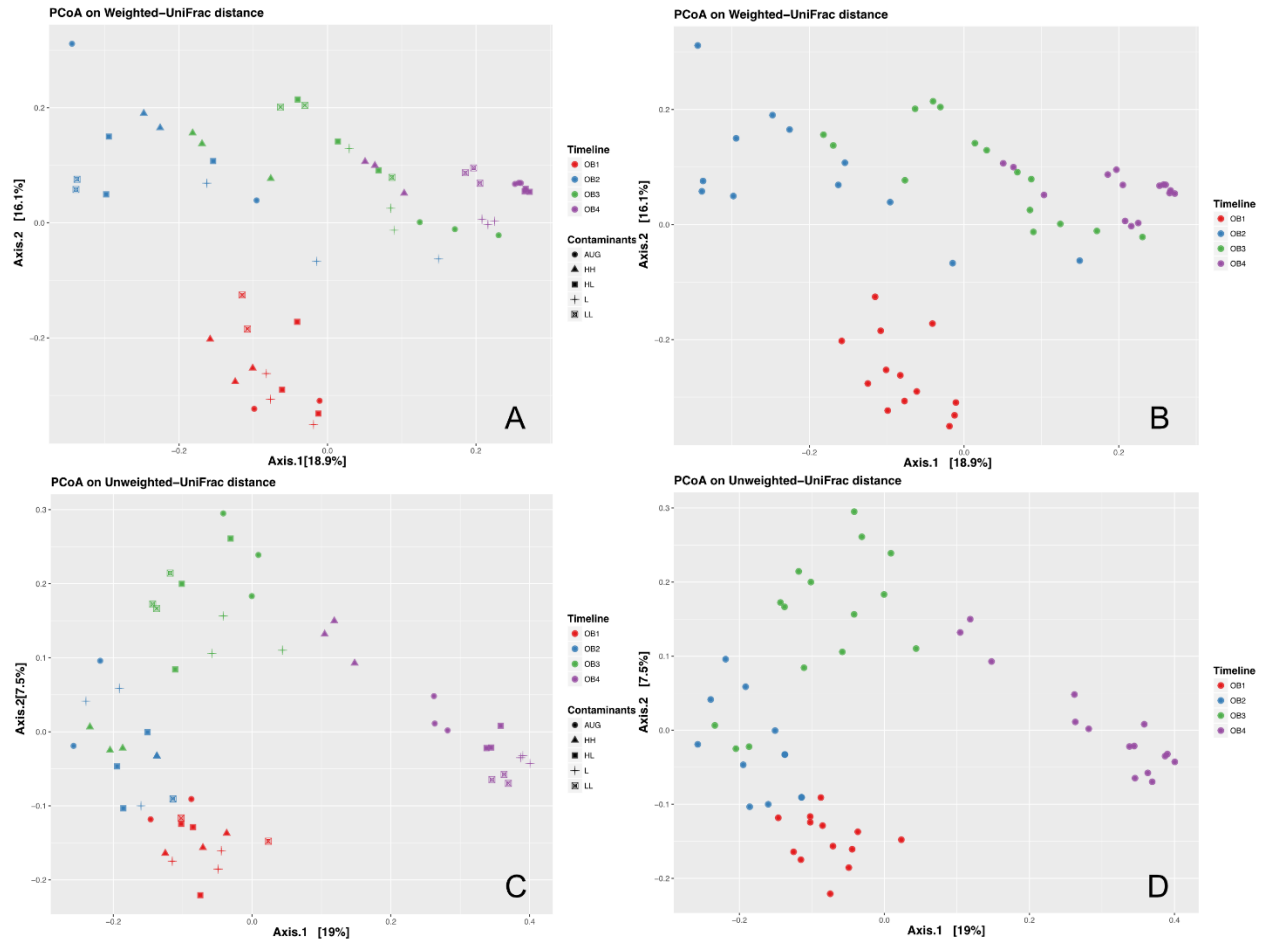
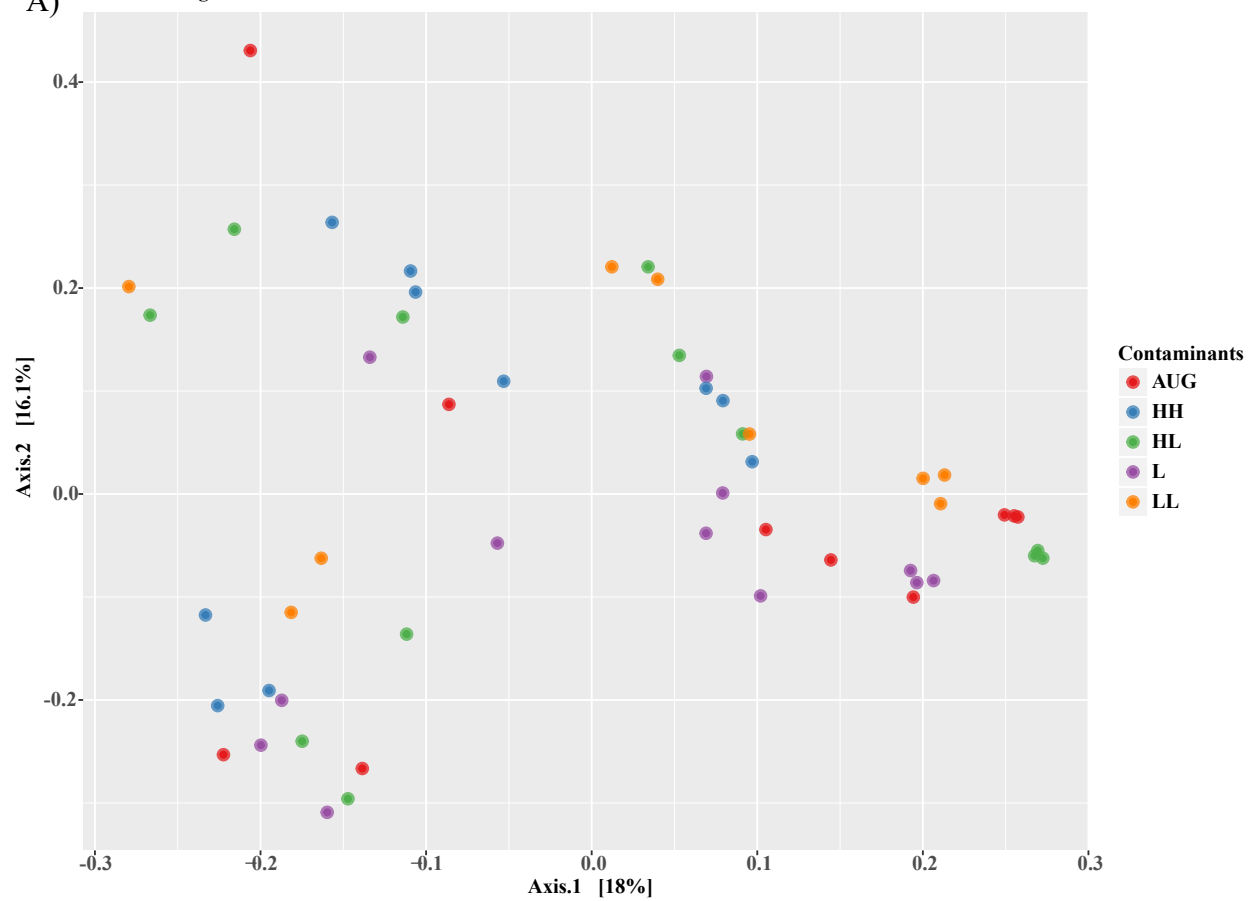


Figure 60. Differences of microbial composition and abundance on each sampling time point under all contaminant conditions at the 0.97-OTUs level, showing the development of community in the function of time. PCoA biplots show weighted (A and B) and unweighted (C and D) UniFrac distances were namely qualitative (i.e., phylogeny) measures of microbial dynamics with and without quantitative (i.e., abundance) measures. (A and C) The samples were colored by time and shaped by conditions. (B and D) The samples were only colored by time.

A) PCoA on weighted-UniFrac distance



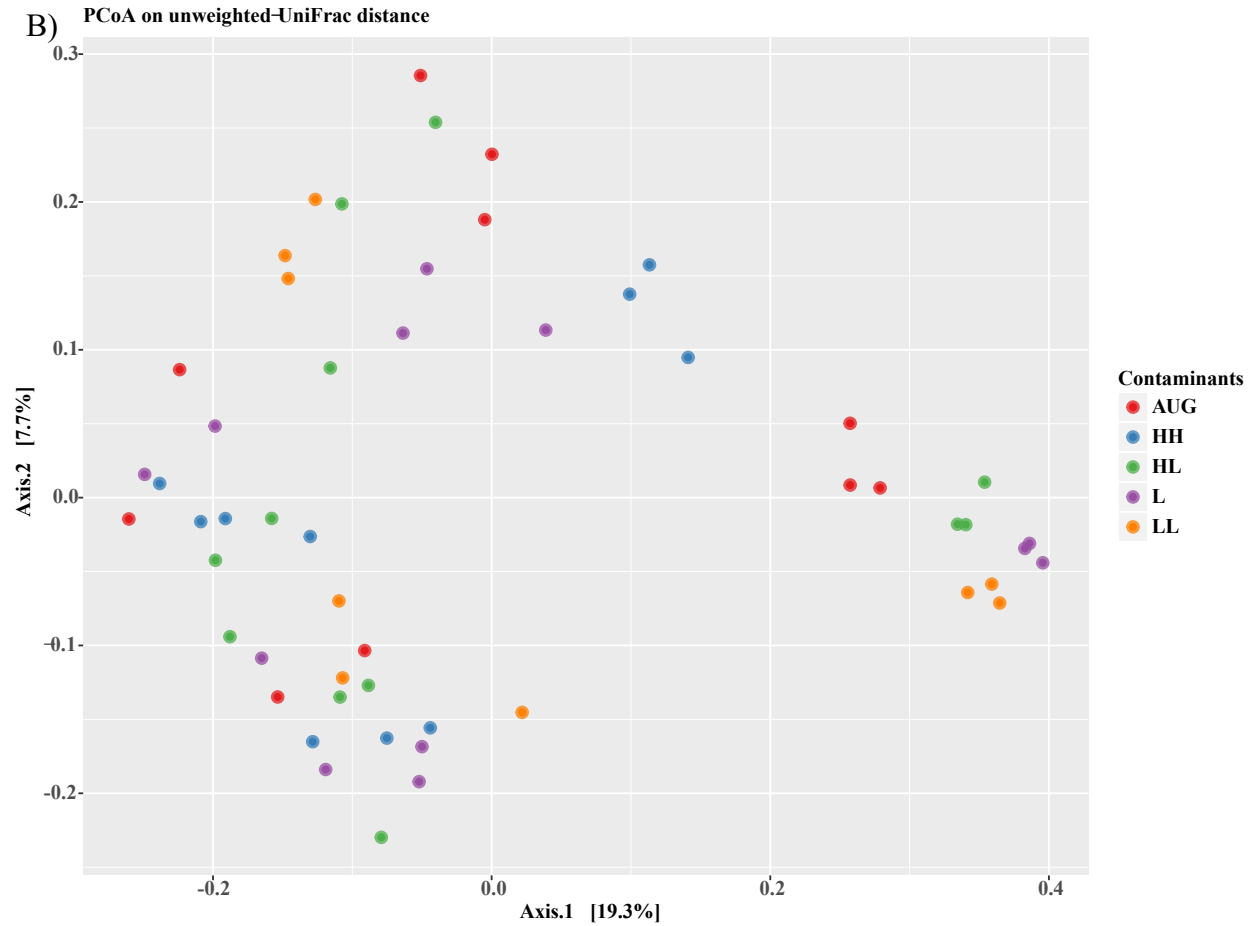


Figure 61. Dynamics of microbial community at each time point under all conditions at the 0.97-OTUs level. PCoA biplots showed A) weighted and B) unweighted UniFrac distances were namely qualitative (i.e., phylogeny) measures of microbial dynamics with and without quantitative (i.e., abundance) measures. All samples were colored by conditions.

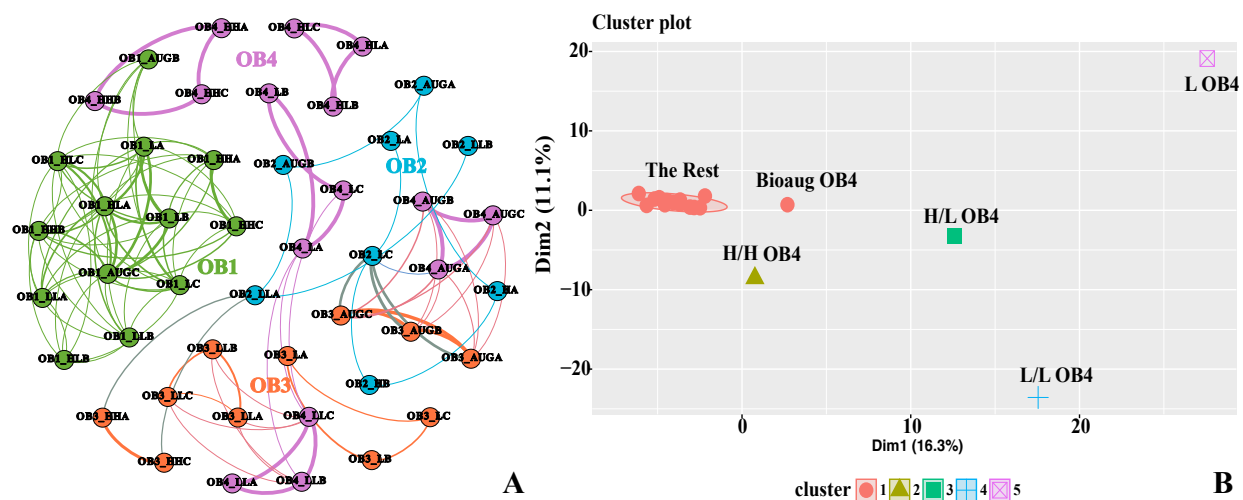


Figure 62. Network and cluster analyses among samples at each time point under all conditions. (A) The node in network are colored by each time point, and the connections represented a strong ($R > 0.6$) and significant ($p\text{-value} < 0.01$) correlations. The thickness of each edge is proportional to the p -value. (B) Cluster analysis pools all samples at different time points under different conditions and shows integral shift among conditions and time points.

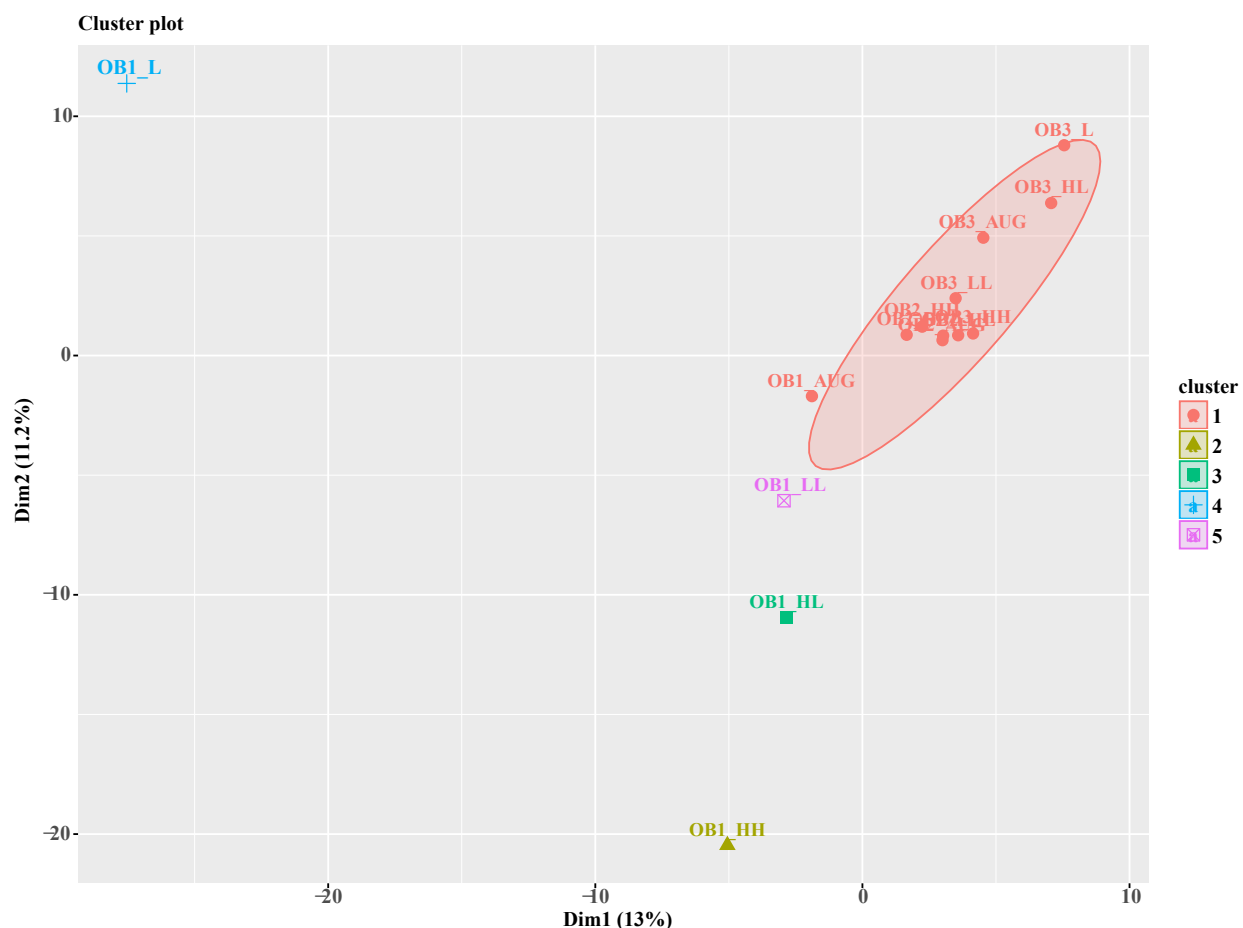


Figure 63. Cluster analysis based on all samples at OB1, OB2 and OB3 under all conditions.

In networks constructed based on the OTUs that totaled over 1% in all samples, only the strong (coefficients > 0.6 or < -0.6) and significant ($p < 0.01$) correlations were presented, which generally indicated the role of cooperative and competitive relations in guiding community dynamics. The output positive network contained 711 pairs of Spearman correlations between 168 OTUs (Figure 64), while the negative had 69 strong pairs between 51 OTUs (Figure 65). The OTUs in the positive network (co-occurrence) were mainly distributed into 12 phyla, which was consistent with the trend of taxonomic classification that Proteobacteria (54.17%), Actinobacteria (13.10%), Firmicutes (14.29%), Cyanobacteria (2.98%), and Bacteroidetes (8.33%) were the top five most abundant phyla. Among them, Proteobacteria had 220 connections and 142 of 220 were intra-phylum (within phylum) pairs, and this ratio were 22 of 83 and 11 of 76 for Firmicutes and Actinobacteria, respectively (Figure 64A). When compared against the network grouped by phyla (Figure 64A), most niches were occupied by multiple interacted phyla, except for module III where mostly matched a whole part of Proteobacteria. For example, phyla Proteobacteria and Cyanobacteria (OTU13, 25, 69, 88, and 95, yellow in Figure 64A) shared in same niche (Module V), and module II contained Proteobacteria, Firmicutes (OTU32, 48, 50, 58, 70, green in Figure 64A), and Actinobacteria (OTU9, 28, 37, 87, 153, orange in Figure 64A). Moreover, the co-exclusion pattern showed unmodularized distribution of

phyla, demonstrating the inter- or intra-phylum competitions within community. OTU60 (Comamonadaceae_unclassified), 72 (*Ramlibacter*), 73 (*Hydrogenophaga*), and 138 (Comamonadaceae_unclassified) belonging to Proteobacteria showed competitions with Proteobacteria OTU23 (*Pelomonas*), respectively, occupying the Module II in negative networks (blue in Figure 65B). Moreover, OTU6 (*Pseudomonas*) and 19 (*Janthinobacterium*) in Proteobacteria had more dense negative connections (large nodes in Figure 65B) with inter- or intra-phylum OTUs in Module III (purple in Figure 65B).

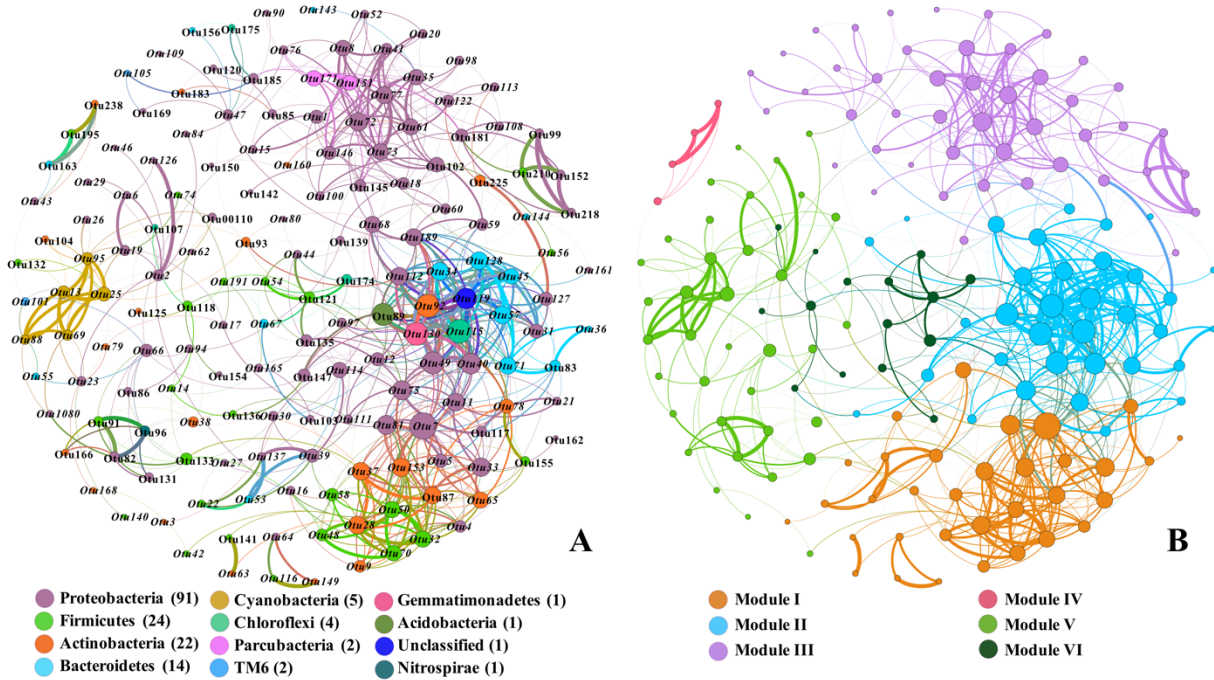


Figure 64. Correlation-based network analysis of species co-occurrence patterns in all samples, with significant (p -value < 0.01) and strong correlations ($R > 0.6$) between 0.97-OTU. The size of each node (0.97-OTUs) is proportional to the number of connections, and the thickness of each edge between two nodes is proportional to the strength of correlation. Left: network colored by phylum, number of OTUs follows label; Right: network colored by modules calculated by Gephi modularity toolkit, and nodes within the same module were more densely connected together than to the rest of the network.

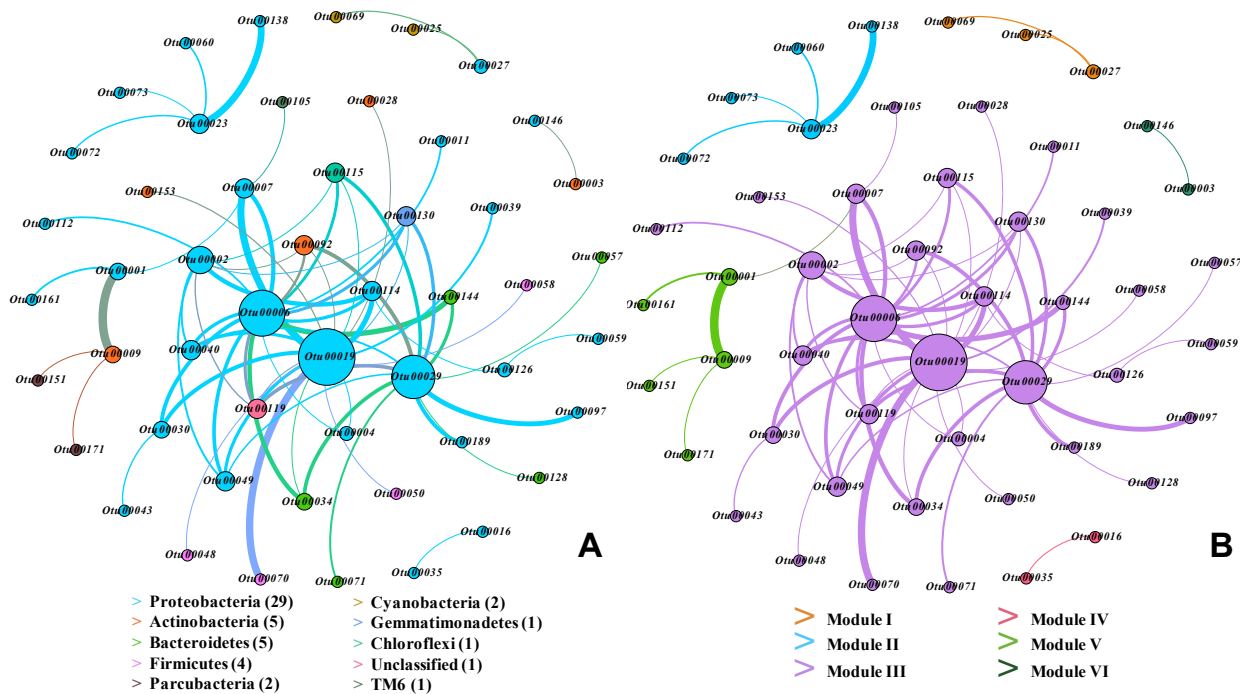


Figure 65. Correlation-based network analysis of species co-exclusion patterns in all samples, with significant (p -value < 0.01) and strong correlations ($R < -0.6$) between 0.97-OTU. The size of each node (0.97-OTUs) is proportional to the number of connections, and the thickness of each edge between two nodes is proportional to the strength of correlation. Left: network colored by phylum, number of OTUs follows label; Right: network colored by modules calculated by Gephi modularity toolkit, and nodes within the same module were more densely connected together than to the rest of the network.

CONCLUSIONS AND IMPLICATIONS FOR FUTURE RESEARCH

Conclusion 1: Although chlorinated solvents negatively impact 1,4-dioxane biodegradation, with vinyl chloride being the most inhibitory, growth mode (biofilm vs. planktonic) and solvent biodegradation can ultimately mitigate these effects.

Dose-dependent inhibition of 1,4-dioxane biodegradation by individual chlorinated solvents, TCA, TCE, 1,1-DCE, cDCE, as well as their mixtures on 1,4-dioxane biodegradation at two initial concentrations of 1,4-dioxane (1 mg/L and 100 mg/L) was quantified. The co-contaminant effects were studied using planktonic cultures of *Pseudonocardia dioxanivorans* CB1190, which uses 1,4-dioxane as growth substrate, and *Mycobacterium austroafricanum* JOB5, which cometabolizes 1,4-dioxane after growth on propane, so that the results would be valuable for considering natural attenuation, biostimulation, or bioaugmentation as groundwater remediation strategies. The inhibition was reversible in all cases, was strongest in CB1190 due to VC, followed by 1,1-DCE, cDCE, TCE, and TCA. CB1190 was able to degrade VC and cDCE but not any other solvents. In contrast, JOB5 was able to cometabolize all of the chlorinated solvents examined, and the rapid removal of these solvents suggests cometabolism might provide a robust strategy for 1,4-dioxane as well as its chlorinated solvent co-contaminants.

Conclusion 2: 1,4-Dioxane biodegradation was influenced to varying degrees by individual exposure to five transition metals, with Cu(II), an essential nutrient, being the most inhibitory, while Cr(VI), a toxic oxyanion, had little effect at environmentally relevant levels..

Surprisingly, bioavailability and toxicity of Cu(II) and other metals can be alleviated by organic ligands, which resulted in improved biodegradation rates.

Cd(II), Cu(II), Cr(VI), Fe(II), and Ni(II) also inhibited 1,4-dioxane biodegradation, whereas Zn(II) had no measurable effect on biodegradation rates. Surprisingly, Cu(II) was the strongest inhibitor, even at low levels, while Cr(VI) was only considerably inhibitory at highly elevated levels (250 mg/L for planktonic JOB5 and only slightly at 10 mg/L for JOB5 biofilms). 1,4-Dioxane biodegradation in cultures exposed to 2 mg/L Cu(II) was restored in the presence of 0.005, 0.05, and 0.5 mM alginin, 0.05, and 0.5 mM cysteine, and 0.005 mM tannin. These results indicated that specific ligands bind with transition metals and alleviate bacterial toxicity. Thus, monooxygenase-catalyzed biodegradation rates by 1,4-dioxane metabolizing or cometabolizing bacteria were subject to interactions among transition metals and natural organic ligands in the environment. EPS production occurred after 1,4-dioxane was completely degraded by CB1190. Interestingly, the addition of copper was found to induce EPS production even with high concentrations of 1,4-dioxane remaining. These findings suggest that more EPS were produced by CB1190 as a stress response to starvation and metal ions, so CB1190 biofilms would be viable, and perhaps protected, in contaminated environments. Additionally, our results also suggest that bioaugmentation strategies at sites with environmentally relevant levels of Cr(VI) are appropriate due to the surprisingly low impact of Cr(VI) on 1,4-dioxane biodegradation.

Conclusion 3: GAC and zeolites are capable of adsorbing 1,4-dioxane with relatively large maximum capacities. Although desorption can occur, bioaugmenting the sorbent greatly increases their capacity and treatment effectiveness.

Bioaugmented adsorbents were examined first as a means of testing the ability of biofilms to degrade 1,4-dioxane with and without chlorinated solvent co-contaminants. Due to the adsorbents' own abilities to adsorb both 1,4-dioxane and chlorinated solvents, they may be

beneficial in the treatment of 1,4-dioxane and chlorinated solvent mixtures. Bio-GAC outperformed abiotic GAC in the removal of 1,4-dioxane from industrial wastewater and groundwater samples. Additionally, bioaugmentation of GAC mitigated the amount of 1,4-dioxane adsorbed by sequential washes with DI water when compared to abiotic GAC. Bio-zeolite outperformed abiotic zeolite in the removal of 1,4-dioxane in the presence of CVOCs, except in the case of 1,1-DCE, which fully inhibited the system, due to zeolite's similar adsorption affinities for 1,1-DCE and 1,4-dioxane. This highlights the importance of understanding the competition effects between adsorbates in bioaugmented adsorbent systems.

Conclusion 4: Attached microorganisms outperform planktonic cells for the biodegradation of 1,4-dioxane in the presence of chlorinated solvents and metals such as Cr(VI) and Cu(II).

Biofilms resist chlorinated solvent and metal inhibition better than planktonic cultures. CB1190 biofilms were capable of resisting Cu(II), 1,1-DCE, cDCE, and TCE inhibition better than planktonic cells. JOB5 biofilms were also able to resist Cr(VI) inhibition, but it was similar to the resistance of the planktonic cultures examined, likely due to the ability of EPS to bind with Cr(VI) oxyanions. Growing microbes on different solid supports differentially enhanced mitigation of chlorinated solvent inhibition. CB1190 bio-GAC was able to degrade 1,4-dioxane (initial concentration of 100 mg/L) in the presence of individual and mixtures of chlorinated solvents (TCE, cDCE, and 1,1-DCE). CB1190 bio-zeolite was able to treat 1,4-dioxane in the presence of TCE and cDCE. Due to the competitive adsorption effects (the somewhat equal affinities of 1,4-dioxane and 1,1-DCE for adsorption on zeolite), bio-zeolite was inhibited by mixtures containing 1,1-DCE. Bio-GAC flow-through column systems lasted considerably longer than abiotic columns. This suggests that bioaugmented adsorbents may be an effective in countering some of the drawbacks of both abiotic adsorption (finite capacities, desorption potential, non-destructive nature of the treatment) and biodegradation only (co-contaminant inhibition) for the removal of 1,4-dioxane with and without CVOC co-contaminants.

Conclusion 5: Microbial community can be resilient even after oxidant stress, along with bacterial interactions in treatment processes, and it developed time but independent of the contaminant levels.

Based on the microbial community analysis, shifts in microbial community structures occurred in the course of treatment, and were influenced by contaminant concentrations at individual time points. Various microbial groups recovered after exposure to peroxide, a microbicide, and evolved during the biodegradation with deterministic factors, including competition, interaction and niche differentiation, that could be also attributed to higher bioavailability and oxygen. Biodiversity and richness were inhibited right after oxidation process, but they increased again during subsequent biodegradation process with multiple species related to hydrocarbon degradation (e.g., *Afipia*, *Pseudolabrys*, *Sphingomonas*, and *Cupriavidus*), helping further evaluation of remediation work. It is important to consider the impact of abiotic and biological remediation technologies on the microbial ecology to predict success of short-term active treatment as well as long-term passive attenuation processes.

Implication 1: Treatment trains are crucial to remediation efforts that involve complex geochemistry and contaminant mixtures.

Remediation of large and complex groundwater plumes of contaminant mixtures can rarely be achieved with single remedies. Chlorinated solvents (especially VC and 1,1-DCE) and

metals (especially Cu(II)) can greatly inhibit aerobic 1,4-dioxane biodegradation. To this challenge, our findings offer hope in the form of treatment trains or hybrid remedies that can either synergize or help mitigate drawbacks of individual treatment technologies for addressing complex contaminant mixtures (e.g. chemical oxidation/reduction followed by biodegradation or bioaugmented adsorbents). More work is needed to identify other promising combinations of treatment technologies that can effectively handle complex remediation challenges.

Implication 2: Understanding the responses of microbial communities to abiotic treatments is important when considering concurrent or subsequent biological treatment strategies.

Treatment trains that include biodegradation can only be effective by understanding how the abiotic remedies may impact the microbes. Microbial community analysis offers powerful tools that can provide insights into the way that abiotic treatment technologies affect microbial community composition and dynamics, which can have direct impacts on community functions. Just as additional research is needed to identify promising treatment trains, we need more work to understand how the microbial communities respond when placed in these treatment systems so that we may optimize them to be as effective as possible.

Implication 3: Characterization of the effects of additional co-contaminants on 1,4-dioxane biodegradation is needed to improve the effective deployment of bioremediation strategies.

Other chlorinated solvents, such as 1,1-dichloroethane (1,1-DCA) or 1,2-dichloroethane (1,2-DCA), commonly used in the production of VC and other industrial processes, are also reported to co-occur with 1,4-dioxane. Understanding the implications of environmentally relevant concentrations of these compounds on 1,4-dioxane metabolizing and cometabolizing bacteria are critical for accurate degradation rates and timelines. As shown by our results and previous reports, chlorinated solvents can initially inhibit 1,4-dioxane degradation, but their effects can be mitigated when they are concurrently or sequentially degraded. Further characterization of the effects of additional co-contaminants will increase our ability to clean up sites contaminated with various mixtures of chlorinated solvents and 1,4-dioxane.

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- Zhao, L., Lu, X., Polasko, A., Johnson, N.W., Miao, Y., Yang, Z., Mahendra, S., Gu, B., 2018. Co-contaminant effects on 1,4-dioxane biodegradation in packed soil column flow-through systems. *Environmental Pollution* 243, 573-581.

Appendix A: Supporting Data

None.

Appendix B: List of Scientific Publications

Peer-reviewed Papers:

1. Pornwongthong, P., A. Mulchandani, P. B. Gedalanga, and S. Mahendra (2014). Transition Metals and Organic Ligands Influence Biodegradation of 1,4-Dioxane. *Applied Biochemistry and Biotechnology* 173 (1): 291-306.
2. Zhang, S., P. B. Gedalanga, and S. Mahendra (2016). Biodegradation Kinetics of 1,4-Dioxane in Chlorinated Solvent Mixtures. *Environmental Science & Technology* 50 (17): 9599-9607. Author-paid Open Access
3. Gedalanga, P. B., A. Madison, Y. Miao, T. Richards, J. Hatton, W. H. Diguiseppi, J. Wilson, and S. Mahendra (2016). A Multiple Lines of Evidence Framework to Evaluate Intrinsic Biodegradation of 1,4-Dioxane. *Remediation Journal* 27 (1): 93-114.
4. Zhang, S., P. B. Gedalanga, and S. Mahendra (2017). Advances in Bioremediation of 1,4-Dioxane-Contaminated Waters. *Journal of Environmental Management* 204 (2): 765-774.
5. Myers, M., N. W. Johnson, E. Zerecero-Marin, P. Pornwongthong, Y. Liu, P. B. Gedalanga, and S. Mahendra (2018). Abiotic and Bioaugmented Granular Activated Carbon for the Treatment of 1,4-Dioxane-Contaminated Water. *Environmental Pollution* 240 (9): 916-924.
6. Zhao, L., X. Lu, A. Polasko, N. W. Johnson, Y. Miao, Z. Yang, S. Mahendra, and B. Gu (2018). Co-contaminant Effects on 1,4-Dioxane Biodegradation in Packed Soil Column Flow-through Systems. *Environmental Pollution* 243 (A): 573-581.
7. Miao, Y., N. W. Johnson, P. B. Gedalanga, D. T. Adamson, C. J. Newell, and S. Mahendra (2019). Response and Recovery of Microbial Communities Subjected to Oxidative and Biological Treatments of 1,4-Dioxane and Co-contaminants. *Water Research* 149 (2): 74-85.
8. Johnson, N. W., P. B. Gedalanga, L. Zhao, B. Gu, and S. Mahendra (2019). Cometabolic Biodegradation of 1,4-Dioxane in Mixtures with Hexavalent Chromium Using Attached and Planktonic Bacteria. *Science of the Total Environment* (in preparation).
9. Liu, Y., N. W. Johnson, C. Liu, R. Chen, Y. Dong, and S. Mahendra (2019). Mechanisms of 1,4-Dioxane Biodegradation and Adsorption by Bio-zeolite in the Presence of Chlorinated Solvents: Experimental and Molecular Dynamics Simulation Studies. *Environmental Science & Technology* (in preparation).
10. Miao, Y., N. W. Johnson, T. Phan, K. Heck, P. B. Gedalanga, D. T. Adamson, C. J. Newell, M. S. Wong, and S. Mahendra (2019). Monitoring, Assessment, and Prediction of Microbial Shifts in Coupled Catalysis and Biodegradation Treatment for 1,4-Dioxane and Co-contaminants. *ISME Journal* (in preparation).

Posters and Presentations:

1. Gedalanga, P. B., and S. Mahendra (2013). Molecular Biological Tools for Monitoring Biodegradation of 1,4-Dioxane. *Association of Environmental Health Sciences 23rd Annual International Conference on Soil, Water, Energy, and Air*, San Diego, CA.
2. Mahendra, S. (2013). Bioremediation of Contaminated Soil and Groundwater. *Education for Sustainable Living Program*, UC Los Angeles, CA.
3. Mahendra, S. (2013). Biological Degradation of Emerging Contaminants in Ground Water. *Environmental Health Sciences and Molecular Toxicology Program*, UC Los Angeles, CA.
4. Mahendra, S. (2013). 1,4-Dioxane Degradation by Monooxygenase-expressing Bacteria. *Microbial Insights, Inc.* Webinar.
5. Mulchandani, A., P. Pornwongthong, and S. Mahendra (2013). Heavy Metals Hamper Pollution-Eating Bacteria. *UCLA Henry Samueli School of Engineering & Applied Science Tech Forum*, Los Angeles, CA.
6. Mahendra, S. (2013). Biological Mitigation of Industrial Contamination in Groundwater Aquifers. *UCLA Henry Samueli School of Engineering and Applied Science Tech Forum*, Los Angeles, CA.
7. Chiang, D. S-Y., R. Mora, M. Zenker, S. Mahendra, and P. Dombrowski (2013). Remediation for 1,4-Dioxane: Updates, Possibilities, and Challenges. *Association of Environmental Health Sciences 29th Annual International Conference on Soils, Sediments, Water, and Energy*, Amherst, MA.
8. Mahendra, S. (2014). Characterization and Treatment of 1,4-Dioxane in Groundwater. *3rd GRACast Web Seminar in the Series on Compounds of Emerging Concern in Groundwater*. Groundwater Resources Association of California, Sacramento, CA.
9. Pornwongthong, P., A. Mulchandani, P. B. Gedalanga, and S. Mahendra (2014). Get Back in the Game with Bioremediating Dioxane. *American Water Works Association Spring Conference*, Anaheim, CA.
10. Pornwongthong, P., A. Mulchandani, T. Phan, P. B. Gedalanga, and S. Mahendra (2014). Influence of Transition Metals and Organic Ligands on Monooxygenase-Expressing Bacteria. *American Society for Microbiology 114th General Meeting*, Boston, MA.
11. Pornwongthong, P., A. Mulchandani, R. Mora, D. Chiang, B. Gu, and S. Mahendra (2014). Heavy Metals Influence Biodegradation of 1,4-Dioxane. *Ninth International Conference on Remediation of Chlorinated and Recalcitrant Compounds*, Monterey, CA.
12. Mahendra, S. (2014). Biodegradation of Contaminants of Emerging Concern in Water: Polyfluorinated Compounds and 1,4-Dioxane. *Geosyntec Consultants*, Oakland, CA.
13. Pornwongthong, P., A. Mulchandani, T. Folker, T. Phan, P. B. Gedalanga, and S. Mahendra (2014). Mechanistic Toxicology of Copper in a 1,4-Dioxane Degrading Bacterium. *American Chemical Society 248th National Meeting & Exposition*, San Francisco, CA.
14. Mahendra, S. (2015). Biodegradation Kinetics of 1,4-Dioxane in Contaminant Mixtures. *RemTec Summit*, Westminster, CO.

15. Zhang, S., P. B. Gedalanga, and S. Mahendra (2015). Simultaneous Degradation of Chlorinated Solvents and 1,4-Dioxane by Propane-oxidizing Bacteria. *RemTec Summit*, Westminster, CO.
16. Zhang, S., R. Bansal, P. B. Gedalanga, and S. Mahendra (2015). Killing Two Birds with One Stone: Bioremediation of Dichloroethene and 1,4-Dioxane in Water. *UCLA Henry Samueli School of Engineering and Applied Science Tech Forum*, Los Angeles, CA.
17. Mahendra, S. (2015). Biodegradation of 1,4-Dioxane: Pathways, Co-contaminant Effects, Monitoring Tools, and Treatment Trains. *1,4-Dioxane Summit*. Detroit, MI.
18. Mahendra, S. (2015). Biomarker-based Tools to Validate 1,4-Dioxane Biodegradation. *EOS Remediation, Inc. Webinar*, Raleigh, NC.
19. Zhang, S., S. Guo, P. B. Gedalanga, and S. Mahendra (2016). The Impact of Hexavalent Chromium on 1,4-Dioxane Biodegradation by Propane Oxidizing Bacteria. *Emerging Contaminants Summit*. Westminster, CO.
20. Mahendra, S. (2016). Bioaugmented Sorbents for Removing 1,4-Dioxane and CVOCs from Water. *American Chemical Society 251st National Meeting & Exposition*. San Diego, CA.
21. Zhang, S., S. Guo, P. B. Gedalanga, and S. Mahendra (2016). Bioprocesses for Simultaneously Removing Hexavalent Chromium and 1,4-Dioxane. *American Chemical Society 251st National Meeting & Exposition*. San Diego, CA.
22. Zhang, S., P. B. Gedalanga, S. Guo, and S. Mahendra (2016). Simultaneous Removal of 1,4-Dioxane and Hexavalent Chromium from Water. *Tenth International Conference on Remediation of Chlorinated and Recalcitrant Compounds*, Palm Springs, CA.
23. Mahendra, S. (2016). Degradation of 1,4-Dioxane – State of Knowledge. *The University Consortium for Field-Focused Groundwater Contamination Research Annual Meeting*, Guelph, ON.
24. Mahendra, S. (2016). Emerging Contaminants – Overview. *The University Consortium for Field-Focused Groundwater Contamination Research Annual Meeting*, Guelph, ON.
25. Zhang, S., P. B. Gedalanga, and S. Mahendra (2016). Biodegradation of 1,4-Dioxane in Chlorinated Solvent Mixtures. *American Chemical Society 252nd National Meeting & Exposition*. Philadelphia, PA.
26. Mahendra, S. (2016). 1,4-Dioxane in Groundwater: Occurrence, Regulation, Remediation and Monitoring. *Golder Associates Inc.*, Mt. Laurel, NJ.
27. Mahendra, S. (2016). Biodegradation of Emerging Contaminant 1,4-Dioxane: Bacteria, Pathways, Co-contaminant Effects, and Monitoring Tools. *Science Advisory Board for Contaminated Sites in British Columbia*, Vancouver, BC.
28. Miao, Y., N. W. Johnson, P. B. Gedalanga, D. T. Adamson, C. J. Newell, and S. Mahendra (2017). Changes in *in-situ* Microbial Communities Exposed to Oxidative Treatment of Contaminant Mixtures. *RemTec Summit*, Denver, CO.

29. Mahendra, S. (2017). Biodegradation of 1,4-Dioxane: Co-Contaminant Effects and Monitoring Tools. *SERDP & ESTCP Webinar #52*, Arlington, VA.
30. Gedalanga, P. B., Y. Miao, A. Madison, T. Richards, W. G. Gierke, H. Holbrook, R. Mora, D. S.-Y. Chiang, and S. Mahendra (2017). Biomarkers for Validating Natural and Enhanced Biodegradation of 1,4-Dioxane in Groundwater. *American Chemical Society 253rd National Meeting & Exposition*. San Francisco, CA.
31. Madison, A., T. Richards, P. B. Gedalanga, and S. Mahendra (2017). Evaluation and Enhancement of Intrinsic 1,4-Dioxane Biodegradation. *Fourth International Symposium on Bioremediation and Sustainable Environmental Technologies*, Miami, FL.
32. Madison, A., T. Richards, P. B. Gedalanga, and S. Mahendra (2017). Evaluation and Enhancement of Intrinsic 1,4-Dioxane Biodegradation. *Fourth International Symposium on Bioremediation and Sustainable Environmental Technologies*, Miami, FL.
33. Miao, Y., N. W. Johnson, P. B. Gedalanga, D. T. Adamson, C. J. Newell, and S. Mahendra (2017). Response and Recovery of Microbial Communities Subjected to Oxidative Treatment of Contaminant Mixtures. *Association of Environmental Engineering and Science Professors (AEESP) Conference*, Ann Arbor, MI.
34. Mahendra, S. (2017). Chlorinated Ethene Mixtures Influence Metabolic and Cometabolic Biodegradation of 1,4-Dioxane. *Strategic Environmental Research & Development Program Annual Symposium*, Washington, DC.
35. Madison, A. S., T. Richards, R. Illes, Y. Miao, and S. Mahendra (2018). Enhancement of Intrinsic 1,4-Dioxane Biodegradation. *Eleventh International Conference on Remediation of Chlorinated and Recalcitrant Compounds*, Palm Springs, CA.
36. Johnson, N. W., Y. Liu, P. Pornwongthong, E. Mack, C. Walecka-Hutchison, S. Mohanty, and S. Mahendra (2018). Treatment of 1,4-Dioxane and CVOCs by Bioaugmented Granular Activated Carbon and Bioaugmented Synthetic Zeolite. *Eleventh International Conference on Remediation of Chlorinated and Recalcitrant Compounds*, Palm Springs, CA.
37. LaPat-Polasko, L., D. Taggart, S. Rosolina, K. Clark, B. R. Baldwin, A. Polasko, and S. Mahendra. (2018). Field-Scale Evaluation of In Situ Biodegradation of 1,4-Dioxane via Bioaugmentation with *Pseudonocardia dioxanivorans* CB1190. *Eleventh International Conference on Remediation of Chlorinated and Recalcitrant Compounds*, Palm Springs, CA.
38. Mahendra, S. (2018). Biodegradation-Sorption Treatment Trains for 1,4-Dioxane and Chlorinated Solvent Mixtures. *The University Consortium for Field-Focused Groundwater Contamination Research Annual Meeting*, Guelph, ON.
39. Mahendra, S. (2018). Environmental Microbiomes: Characterization of Pollutant Toxicology and Remediation Applications. *UCLA Microbiome Center Seminar Series*. Los Angeles, CA.
40. Polasko, A., N. W. Johnson, Y. Miao, C. Clark, S. Zhang, X. Lu, L. Zhao, Z. Yang, R. Mora, D. S.-Y. Chiang, P. B. Gedalanga, P. Pornwongthong, B. Gu, and S. Mahendra (2018). Biodegradation Approaches for Mixtures of CVOCs, Heavy Metals, and 1,4-Dioxane.

Strategic Environmental Research & Development Program Annual Symposium,
Washington, DC.

41. Johnson, N.W., S. Zhang, Y. Liu, P. B. Gedalanga, L. Zhao, B. Gu, and S. Mahendra (2019). Treatment of 1,4-Dioxane in Mixtures with Chlorinated Solvents and Metals Using Bacterial Biofilms. *RemTEC Summit*, Denver, CO.
42. Mahendra, S. (2019). Oxidation-Biodegradation Treatment Trains for 1,4-Dioxane and Chlorinated Solvents: Response and Recovery of Microbial Communities. *RemTec Summit*, Denver, CO.
43. Mahendra, S. (2019). Treatment Trains for 1,4-Dioxane and Chlorinated Solvent Mixtures: Response and Recovery of Microbial Communities. *American Chemical Society 257th National Meeting & Exposition*. Orlando, FL.
44. Miao, Y., N. W. Johnson, P. B. Gedalanga, K. Heck, D. T. Adamson, C. J. Newell, M. S. Wong, and S. Mahendra (2019). Metagenomics in Environmental Engineering: Experimental, Statistical, and Educational Approaches. *Association of Environmental Engineering and Science Professors (AEESP) Conference*, Tempe, AZ.
45. Miao, Y., N. W. Johnson, P. B. Gedalanga, K. Heck, D. T. Adamson, C. J. Newell, M. S. Wong, and S. Mahendra (2019). Monitoring, Assessment, and Prediction of Microbial Shifts in Coupled Abiotic-Biological Treatment for Contaminant Mixtures in Soil and Groundwater. *American Society for Microbiology (ASM) Microbe*, San Francisco, CA.

Theses and Dissertations:

1. Pornwongthong, Peerapong (2014). Stable Isotopic and Molecular Biological Tools to Validate Biodegradation of 1,4-Dioxane. UCLA PhD Thesis.
2. Myers, Michelle (2016). 1,4-Dioxane Biodegradation using Bioaugmented Granular Activated Carbon. UCLA MS Thesis.
3. Zhang, Shu (2017). Biodegradation of 1,4-Dioxane in Co-contaminant Mixtures. UCLA PhD Thesis.
4. Polasko, Alexandra Lapat (2017). Sequential Anaerobic-Aerobic Biodegradation of Trichloroethylene and 1,4-Dioxane. UCLA MS Thesis.
5. Miao, Yu (2019). Microbial Ecology Insights into Natural and Engineered Biodegradation Processes for 1,4-Dioxane. UCLA PhD Thesis.