FINAL REPORT

Biodegradation of CVOCs and 1,4-Dioxane Mixtures by Engineered Microbial Communities

SERDP Project ER-2713

JANUARY 2020

Shaily Mahendra, PhD
Alexandra LaPat Polasko, MS
Ivy Kwok, MS
University of California, Los Angeles

Rula Deeb
Geosyntec Consultants

SERDP
DOD ▪ EPA ▪ DOE

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
31/01/2020	SERDP Final Report	9/8/2017 - 9/7/2021

4. TITLE AND SUBTITLE

Biodegradation of CVOCs and 1,4-Dioxane Mixtures by Engineered Microbial Communities

5a. CONTRACT NUMBER
17-C-0009

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Shaily Mahendra, PhD
Alexandra LaPat Polasko, MS
Ivy Kwok, MS
University of California, Los Angeles

Rula Deeb
Geosyntec Consultants

5d. PROJECT NUMBER
ER-2713

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of California, Los Angeles
5732C Boelter Hall
Los Angeles, CA 90095

8. PERFORMING ORGANIZATION REPORT NUMBER
ER-2713

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Strategic Environmental Research and Development Program (SERDP)
4800 Mark Center Drive, Suite 16F16
Alexandria, VA 22350-3605

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

11. SPONSOR/MONITOR'S REPORT NUMBER(S)
ER-2713

12. DISTRIBUTION/AVAILABILITY STATEMENT

DISTRIBUTION STATEMENT A. Approved for public release: distribution unlimited.

13. SUPPLEMENTARY NOTES

14. ABSTRACT

The overarching objective of this limited-scope project was to understand and apply potential treatment synergies to ultimately achieve biodegradation of multiple contaminants in source zones as well as distal plumes. Specifically, we formulated a microbial community to simultaneously or sequentially degrade chlorinated volatile organic compounds (CVOCs) and 1,4-dioxane across changing redox environments. Some CVOCs have used 1,4-dioxane as a solvent stabilizer. Consequently, 1,4-dioxane is detected as a co-occurring contaminant with CVOCs at many contaminated sites. Anaerobic biological reduction is a common remediation approach for CVOCs like trichloroethene (TCE). However, under some conditions, intermediate daughter products, such as cis-1,2-dichloroethene (cDCE) and vinyl chloride, are accumulated, which may be more toxic than their parent compounds. Aerobic cometabolism of CVOCs requires additional amendments of primary substrates. 1,4-Dioxane is mainly biodegraded under aerobic conditions.

15. SUBJECT TERMS

Biodegradation, chlorinated volatile organic compounds, CVOCs, 1,4-Dioxane Mixtures, Engineered Microbial Communities

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			Shaily Mahendra
UNCLASS	UNCLASS	UNCLASS	UNCLASS	39	**19b. TELEPHONE NUMBER** *(Include area code)* 310-794-9850

TABLE OF CONTENTS

LIST OF FIGURES

ABSTRACT

Introduction and Objectives

The overarching objective of this limited-scope project was to understand and apply potential treatment synergies to ultimately achieve biodegradation of multiple contaminants in source zones as well as distal plumes. Specifically, we formulated a microbial community to simultaneously or sequentially degrade chlorinated volatile organic compounds (CVOCs) and 1,4-dioxane across changing redox environments. Some CVOCs have used 1,4-dioxane as a solvent stabilizer. Consequently, 1,4-dioxane is detected as a co-occurring contaminant with CVOCs at many contaminated sites. Anaerobic biological reduction is a common remediation approach for CVOCs like trichloroethene (TCE). However, under some conditions, intermediate daughter products, such as cis-1,2-dichloroethene (cDCE) and vinyl chloride, are accumulated, which may be more toxic than their parent compounds. Aerobic cometabolism of CVOCs requires additional amendments of primary substrates. 1,4-Dioxane is mainly biodegraded under aerobic conditions. The opposing redox conditions favored by CVOC- and 1,4-dioxane-degrading bacteria pose challenges for concurrent bioremediation of both contaminant classes. By combining anaerobic and aerobic microbes, CVOC transformation products are less likely to persist, and can even be biodegraded aerobically, thereby mitigating their inhibition of 1,4-dioxane degrading bacteria.

Technical Approach

In this research project, we developed a microbial community composed of the anaerobic culture, KB-1®, and the aerobic strain, *Pseudonocardia dioxanivorans* CB1190 (CB1190), in a modified medium to degrade CVOCs and 1,4-dioxane. The concept of combining organisms that typically cannot survive together was initially counterintuitive. However, after rigorous trial and error, we were able to assemble a mixed community that successfully biodegraded TCE as well as 1,4-dioxane over changing redox conditions. Additionally, aerobic degradation of cDCE was carried out by CB1190 in the presence of 1,4-dioxane.

Results

We report that CB1190 is uniquely equipped with the ability to withstand a variety of environments usually favored by anaerobic microorganisms. For example, CB1190 was able to survive over 35 days of anaerobic incubation and subsequently degrade 1,4-dioxane when oxygen became available. During the anaerobic phase, KB-1 reductively dechlorinated TCE to cDCE via *tceA*-coded enzymes, while CB1190 down regulated the *dxmB* and *aldH* genes, which serve as biomarkers for the degradation of 1,4-dioxane and its downstream products. However, when oxygen was supplied, the genes necessary for 1,4-dioxane biodegradation were

upregulated. CB1190 degraded 1,4-dioxane even with as low as 3 mg/L dissolved oxygen. We demonstrated that bioaugmented CB1190 was viable and active in wells at a site previously treated with enhanced reductive dechlorination (ERD). *These data confirm CB1190's versatility and ability to withstand extended periods with limited availability of 1,4-dioxane as electron donor as well as oxygen as electron acceptor, and successfully biodegrade 1,4-dioxane in the presence of inhibitory CVOCs.*

Benefits

Biological treatment of mixed contaminations is often limited by the fact that certain microbes can only biodegrade a subset of compounds and are sensitive to prevailing geochemical conditions. Formulation of aerobic and anaerobic contaminant-degrading bacteria will increase the ability to remove pollutant mixtures in varying redox zones across contaminated sites. Costs associated with injecting contaminant-degrading microorganisms, carbon substrates, and nutrient amendments are among the highest considerations for *in situ* bioremediation methods. This co-culture reduces the number of injections that would otherwise be needed to transform CVOCs as well as 1,4-dioxane into benign end products. Results from this project will help the Department of Defense (DoD) with the decision process of when and how to transition from active ERD to enhanced attenuation of CVOCs as well as dioxane. In certain conditions, this approach could limit the cost and timeframe of the ERD phase (if already implemented) or replace ERD as the primary remedial technology. Our approach of using engineered microbial communities could reduce the cost, energy, and substrates required as well as expand the number of sites where *in situ* bioremediation is considered to be a viable remedy for these compounds.

OBJECTIVES AND RATIONALE

Mixtures of chlorinated volatile organic compounds (CVOCs) and 1,4-dioxane are commonly found as groundwater co-contaminants at many DoD sites.[1] Biological degradation of CVOCs[2-4] and 1,4-dioxane[5, 6] have been previously reported, but occur independently in reductive and oxidative processes, respectively. Moreover, our recent work indicates that certain CVOCs actually inhibit aerobic biodegradation of dioxane.[7] To mitigate the CVOCs inhibition and efficiently cleanup CVOCs as well as dioxane, we proposed designing engineered microbial communities to achieve rapid biodegradation rates of multiple contaminants in source zones as well as distal plumes.

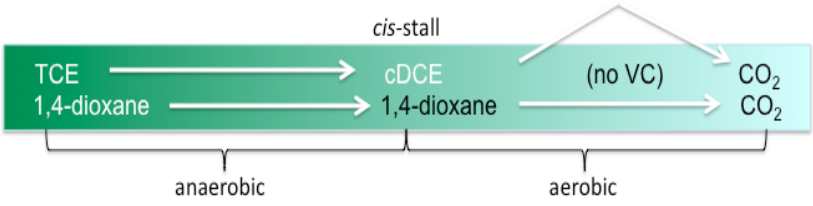


Figure 1. Conceptual design of anaerobic/aerobic co-culture. Partial reductive dechlorination of CVOCs followed by aerobic biodegradation of daughter products as well as 1,4-dioxane by engineered microbial communities in various redox regimes in groundwater plumes.

This limited scope research project tested the hypothesis that "*aerobic-anaerobic co-cultures mineralize 1,4-dioxane as well as dichloroethenes following incomplete reductive dechlorination of TCE*". The objectives of this proposal were to: **1)** Design and test microbial communities composed of previously identified anaerobic and aerobic contaminant degrading bacteria to simultaneously or sequentially degrade CVOCs and 1,4-dioxane mixtures; **2)** Elucidate the influence of O_2 dose and timing, on the individual biodegradation rates and overall contaminant mass removal by the engineered microbial community; **3)** Test the biodegradation of contaminant mixtures by engineered microbial communities under transitioning redox conditions in environmental samples.

Anaerobic conditions prevalent in subsurface plumes of 1,4-dioxane-contaminated groundwater or those created for enhanced reductive dechlorination of co-occurring CVOCs limit achieving complete *in situ* biodegradation of 1,4-dioxane. An anaerobic/aerobic microbial community able to adapt to a broad range of redox regimes will enhance CVOC and 1,4-dioxane biodegradation as well as reduce the need for multiple rounds of inoculation with different strains (Figure 1). This culture will be a valuable transition step from laboratory pure culture studies towards complex *in situ* microbial communities involved in natural and enhanced bioremediation of contaminant mixtures. A microbial community designed to survive and function in dynamic biogeochemical environments, will also be advantageous for remediation of various other contaminant mixtures.

BACKGROUND AND SERDP RELEVANCE

Chlorinated volatile organic compounds (CVOCs) are among the most frequently detected groundwater contaminants. Nearly 95% of the identified 1,4-dioxane plumes are comingled with one or more CVOCs[1], and the number of sites with mixtures of CVOCs and 1,4-dioxane are expected to increase due to the DoD's recent efforts to better characterize their sites for 1,4-dioxane. The divergent physical and chemical properties make complete degradation of 1,4-dioxane difficult using traditional physical-chemical water treatment technologies. While CVOCs are volatile and less soluble in water, 1,4-dioxane has a hydrophilic, cyclic, ether structure. Selection and application of treatment strategies to address mixed contaminants of concern (COCs) in groundwater is challenging and expensive.[8] Indeed, natural attenuation and engineered bioremediation was the most commonly used technology (43%) for cleaning up polluted soil and groundwater[8, 9] because of energy-efficiency and cost-effectiveness.

Enhanced reductive dechlorination (ERD) is one of the most common reductive treatment technologies applied at CVOCs sites,[3, 10] but 1,4-dioxane is not biodegraded under anaerobic conditions. While the goal of using ERD is to attain complete dechlorination of tetrachloroethene (PCE) and trichloroethene (TCE) to ethene[11], the reaction sometimes stops or slows down resulting in the accumulation of cis-1,2-dichloroethene (cDCE) or vinyl chloride (VC) (Figure 2), which can be even more toxic than its parent compound. Fortunately, cDCE and VC can also be metabolically or cometabolically treated under aerobic conditions by monooxygenase-expressing bacteria (Figure 2), a few of which can also degrade 1,4-dioxane.[5, 12-15]

A variety of microbial strains have been reported to metabolically or cometabolically degrade 1,4-dioxane under aerobic conditions only.[6] In most microbes characterized to date, a bacterial

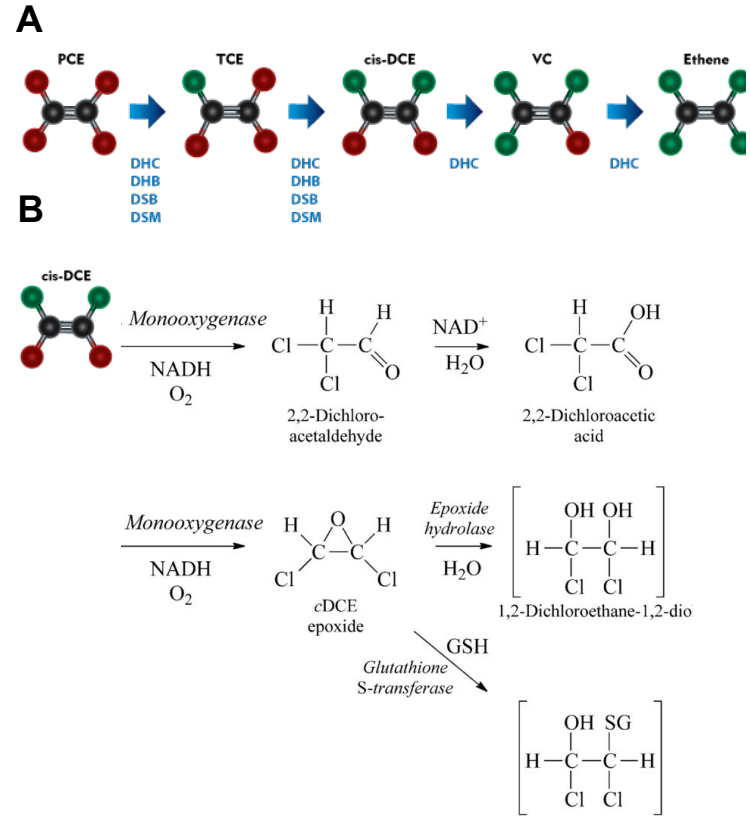


Figure 2. TCE Degradation Pathway. (A) TCE anaerobic biodegradation pathway[6] and (B) cDCE aerobic biotransformation pathway.[11]

multicomponent monooxygenase responsible for initiating 1,4-dioxane degradation is the tetrahydrofuran (THF)/dioxane monooxygenase.[16] The genes coding for this enzyme, *thmADBC*/*dxmADBC*, along with the gene coding for aldehyde dehydrogenase, *aldH*, serve as biomarkers to verify 1,4-dioxane biodegradation.[17] Some actinomycetes, especially members of *Pseudonocardia*, are capable of mineralizing 1,4-dioxane via initial monooxygenase-catalyzed hydroxylation, followed by common cellular metabolic pathways (Figure 3).[16, 18]

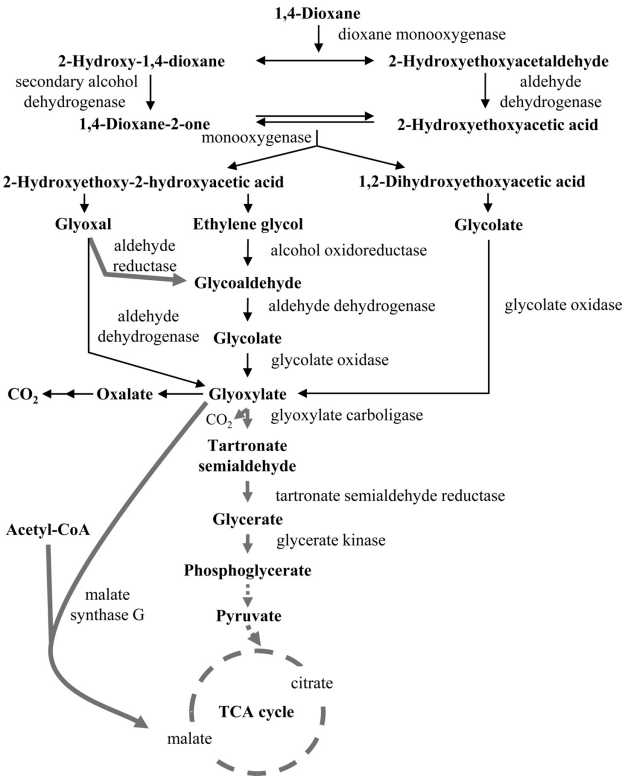


Figure 3. Pathway and enzymes involved in 1,4-dioxane degradation by CB1190.[13,15]

Identification of environmental factors that may influence 1,4-dioxane biodegradation plays an important role in the transition from the laboratory to field remediation. The inhibitory effects of chlorinated solvents (TCE, TCA, 1,1-DCE and cDCE) and metals (Cu, Cd, Ni, Zn, Cr) on 1,4-dioxane biodegradation have been identified in previous work[7, 19-21]. Although CVOCs are reported to negatively impact 1,4-dioxane biodegradation, with 1,1-DCE being the most inhibitory[7], growth mode (biofilm vs. planktonic)[22] and CVOC sequestration[23] or biodegradation can ultimately mitigate these effects.

MATERIALS AND METHODS

Bacterial Cultures and Growth Conditions

The widely used CVOC-degrading mixed consortium KB-1®, containing *Dehalococcoides*, was grown in UCLA Modified Medium,[24, 25] with 20 mg/L of TCE (aqueous) as the electron acceptor and 60 mg/L of lactate as the carbon source.[25] Sterile 160 mL serum bottles containing media were sparged for 3 min with filtered (0.2 μm) N_2 to achieve anaerobic conditions and sealed with 20 mm butyl rubber stoppers. Titanium (III) citrate was also used as the reductant.

P. dioxanivorans CB1190 seed cultures were prepared by transferring 50 mL of a pure culture undergoing log-phase growth in ammonium mineral salts medium (AMS) to 500 mL of fresh UCLA Modified Medium in a baffled flask. 1,4-dioxane was added to achieve an initial concentration of 100 mg/L, and the entire system was kept under aerobic conditions in the

incubator with 150 rpm rotation at 30℃. For the mixed culture studies, CB1190 was also grown aerobically in a lactate-free medium separately that was in the same formulation used for growing KB-1®. Experimental bottles were inoculated with stock cultures in their late exponential or early stationary phase after TCE (KB-1®) or 1,4-dioxane (CB1190) was degraded to below 1000 µg/L.

Design of Batch Experiments

Bacterial cultures were prepared in sterile 500 mL glass bottles and maintained with 1:5 liquid to headspace ratios with the following conditions: (1) KB-1® + CB1190 that transitioned from anaerobic to aerobic (Figure 4), (2) CB1190 only that transitioned from anaerobic to aerobic, and (3) CB1190 only that remained anaerobic throughout. Experimental bottles were inoculated with 10% (v/v) CB1190 and 5% (v/v) KB-1®. Figure 4 shows the sequence of oxygen adjustment over time, and once TCE was degraded to cDCE by KB-1® (left in Figure 4), 60 mL of filtered, high purity oxygen was added to transition the bottles from anaerobic to aerobic conditions (middle in Figure 4), to let CB1190 function aerobically (right in Figure 4). All bottles were incubated upright at 30°C with 150 rpm shaking. TCE and 1,4-dioxane concentrations were amended as liquid concentrations. At each time point, 200 µL of filtered liquid sample and 10-100 µL of headspace were collected for quantification of 1,4-dioxane and TCE, respectively.

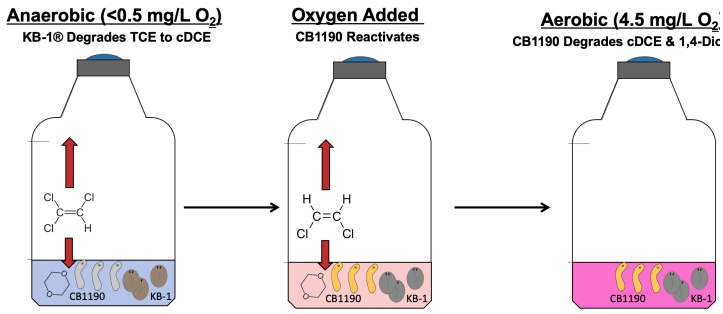


Figure 4. Conceptual Design of Co-Culture Batch Experiments. Evaluation of growth and viability in engineered microbial communities under transitioning redox conditions.

Batch experiments containing pure strain CB1190 were performed in 500 mL sterile glass bottles containing UCLA Modified Media, AMS, or BAV1[25] with a 1:5 liquid to headspace ratio. Sodium sulfide, titanium citrate, and lactate concentrations were filter (0.2 µm) sterilized into experimental bottles from liquid stock solutions. Varying dissolved oxygen was achieved via sparging with filtered (0.2 µm) N_2 gas at varying time intervals ranging from 5 seconds to 3 minutes. Dissolved oxygen was measured using an Orion Versa Star Probe (Thermo Fisher Scientific, Waltham, MA) and calibrated using air saturated water (high oxygen concentrations) and a 50 g/L sodium sulfite solution for the zero point (low oxygen concentrations).

Analytical Methods

Chlorinated ethenes were measured by direct headspace injections (10 or 100 µL, depending on concentration) using gas chromatography−mass spectrometry (GC-MS; Agilent 6890 GC and

5973 MS). 1,4-Dioxane concentrations higher than 1000 µg/L were measured using a GC equipped with a flame ionization detector in 2 µL aqueous samples, while 1,4-dioxane samples containing <1000 µg/L were measured by GC-MS after samples were prepared by a frozen microextraction technique. The detection limits for chlorinated ethenes and 1,4-dioxane were 2 and 5 µg/L, respectively.[26]

Total Nucleic Acids Extraction, Quantitative Polymerase Chain Reaction, and cDNA Synthesis
Total nucleic acids were extracted from cell pellets using a phenol-chloroform extraction method.[17] The effect of anaerobic incubation on CB1190's gene expression in pure and mixed cultures was determined by amplification of 1,4-dioxane biomarker targets (*dxmB* and *aldH*)[17]. CB1190 and KB-1®'s cellular growth over time was estimated using the *dxmB*, *tceA,* and *vcrA* gene copy numbers.[17, 27] Gene expression was quantified using the $2^{-\Delta\Delta CT}$ method, as described by Livak and Schmittgen.[28] Gene expression data were first normalized to the housekeeping gene RNA polymerase σ subunit D (*rpoD*), followed by normalization to the values obtained at the end of the anaerobic incubation period and after 1,4-dioxane was degraded below 1000 µg/L. Quantitative Polymerase Chain Reaction (qPCR) using the SYBR-green-based detection reagents were utilized to quantify gene copy numbers of CB1190 and KB-1® as well as gene transcripts of CB1190.

Bio-Trap® Parameters and Deployment
In order to validate the feasibility of the mixed culture containing CB1190 and KB-1®, Bio-Traps® made of polyvinyl chloride that contained Nomex® Powdered Activated Carbon (PAC) beads, were tested at an environmental site. This environmental site was previously an aluminum pipe manufacturing facility and has historically contained chlorinated ethenes (tetrachloroethene, TCE, 1,1,1-TCA, cDCE, 1,1-DCE, VC) and 1,4-dioxane. Additionally, the site is located in a groundwater discharge zone where groundwater moves not only laterally along fractures and bedding planes but also upward from depth. Evaluation of the pumping test results identified a minimum of two separate water-bearing zones, separated by a micaceous siltstone and shale layer identified at a depth of approximately 118 to 152 feet below ground surface (bgs). The groundwater temperature ranged from 14.9°C to 24°C, dissolved oxygen ranged from 2-6 mg/L and the pH remained circumneutral. Bio-Traps® (Figure 5) were seeded with CB1190 and grown in accordance with the above bacterial cultures and growth conditions section. Previously, the environmental site implemented enhanced reductive dechlorination; therefore, *Dehalococcoides* was already present in the groundwater and only CB1190 was seeded into the Bio-Traps®. After the 1,4-dioxane was degraded to below the detection limit (5 µg/L) in the Bio-Traps®, they were injected into multiple wells containing 350-3,500 µg/L 1,4-dioxane, 1,000-5,000 µg/L TCE, 200-2,000 µg/L cDCE, and 2-200 µg/L VC. After approximately three months, the Bio-Traps® were removed and gene abundances for CB1190 and *Dehalococcoides* were analyzed as well as contaminant concentrations.

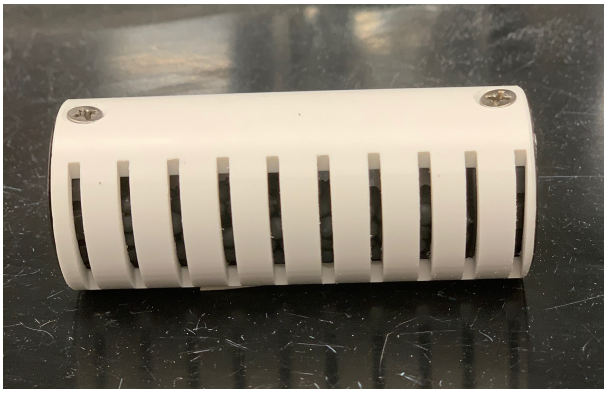


Figure 5. Representative image of the Bio-Traps® used in this study.

Bio-Traps® contained PAC beads, which were seeded with CB1190 and then deployed to an environmental site with 1,4-dioxane and CVOCs. The shell is made of polyvinyl chloride and contains PAC beads.

RESULTS AND DISCUSSION

Biodegradation Kinetics of CVOCs and 1,4-Dioxane Mixtures by the Engineered Microbial Community

Chlorinated Ethene Biodegradation by the Mixed Microbial Community

TCE was reductively dechlorinated under anaerobic conditions to cDCE only in the KB-1® + CB1190 anaerobic/aerobic bottles. KB-1® + CB1190 anaerobic/aerobic and CB1190 anaerobic/aerobic bottles remained anaerobic for 16 hours. This anaerobic incubation period corresponded to the time it took KB-1® to biodegrade TCE to cDCE. After the transition to aerobic conditions, CB1190 cells within the mixed microbial community oxidatively biodegraded cDCE. Since there were no other aerobic organisms in the community, the aerobic degradation was attributed to CB1190, and that is a novel result in this study.

After oxygen was added, the bottles with KB-1® + CB1190 degraded 1300 µg/L of cDCE at a rate of 0.206±0.002/day (Figure 6A). Higher concentrations of TCE and 1,4-dioxane were also successfully tested with the mixed microbial community (Supplementary Figures S1 and S2). Oxygen additions resulted in cessation of KB-1®'s growth (mainly *Dehalococcoides*)[29], as confirmed by no further increase in *tceA* copies/mL (Figure 6A). This was likely due to the oxidative stress on the enzymes that make up alternative respiratory chain in anaerobes, as well as the production of reactive species, such as superoxide radicals and hydrogen peroxide.[30-32] Additionally, VC remained below 2 µg/L in anaerobic as well as aerobic incubation periods. The CB1190-only anaerobic/aerobic bottles and CB1190-only anaerobic bottles did not show significant TCE biodegradation (Figure 6A).

1,4-Dioxane Biodegradation and Microbial Growth in the Mixed Community

During the anaerobic condition, 1,4-dioxane was not degraded significantly in all experimental designs. After the transition to aerobic conditions, CB1190 in pure and mixed culture experienced a short lag phase before beginning to biodegrade 1,4-dioxane. KB-1® + CB1190 anaerobic/aerobic degraded 3,000 µg/L of 1,4-dioxane at a rate of 0.195±0.002/day (Figure 6B), and CB1190 only anaerobic/aerobic bottles at a rate of 0.199±0.001/day (Figure 6B). CB1190-mediated 1,4-dioxane degradation rates were calculated using the end of the anaerobic phase and after 1,4-dioxane was degraded to below detection (5 µg/L). All bottles had an average initial CB1190 cell density of 1.21±0.2 x 10^{11} copies/mL and the KB-1® + CB1190 bottles had an average initial KB-1® cell density of 5.9±2.1 x 10^{8} *tceA* copies/mL (Figure 6B and Supplementary Figures S3 and S4). The KB-1® + CB1190 anaerobic/aerobic grew at 1.56±0.7 x 10^{12} *dxmB* copies/mL/day (Figure 6B). The CB1190 anaerobic/aerobic cultures grew at 5.22±0.02 x 10^{11} *dxmB* copies/mL/day but the CB1190 that was always anaerobic did not show significant growth (Supplementary Figure S4).

Previous studies have cultured aerobic microbes from anaerobic environments as well as combined aerobic and anaerobic bacteria to enhance the biodegradation of recalcitrant pollutants such as polychlorinated biphenyls, chlorobenzenes, dinitrotoluenes, and azo dyes.[33-36] Although these studies developed consortia containing anaerobes and aerobes, some were conducted in spatially and functionally separate bottles using immobilized cells. In our study, all microbes were added together into the same medium with chlorinated ethenes and 1,4-dioxane. Another report of degradation of TCE and 1,4-dioxane under changing redox conditions utilized a microbially driven Fenton reaction using the facultative anaerobe, *Shewanella oneidensis*, to drive the production of HO• radicals.[37] By contrast, our study relies on the TCE-reductase enzymes in *Dehalococcoides* [38] to break down TCE by replacing a covalently-bonded chlorine with a hydrogen and the monooxygenase enzyme in CB1190 to cleave the carbon-oxygen bond in 1,4-dioxane [16], leading to benign end products for both compounds, since *Dehalococcoides* can ultimately reductively dechlorinate TCE to ethene and CB1190 can ultimately convert 1,4-dioxne to CO_2. These previous studies serve as a foundation for considering this approach for removing multiple contaminants with bacteria that favor diverse redox conditions. The present study is the first report of biodegradation of 1,4-dioxane and chlorinated ethenes by a mixed microbial consortium containing aerobic and anaerobic bacteria, and describes its potential implications for bioremediation strategies for sites contaminanted with pollutant mixtures.

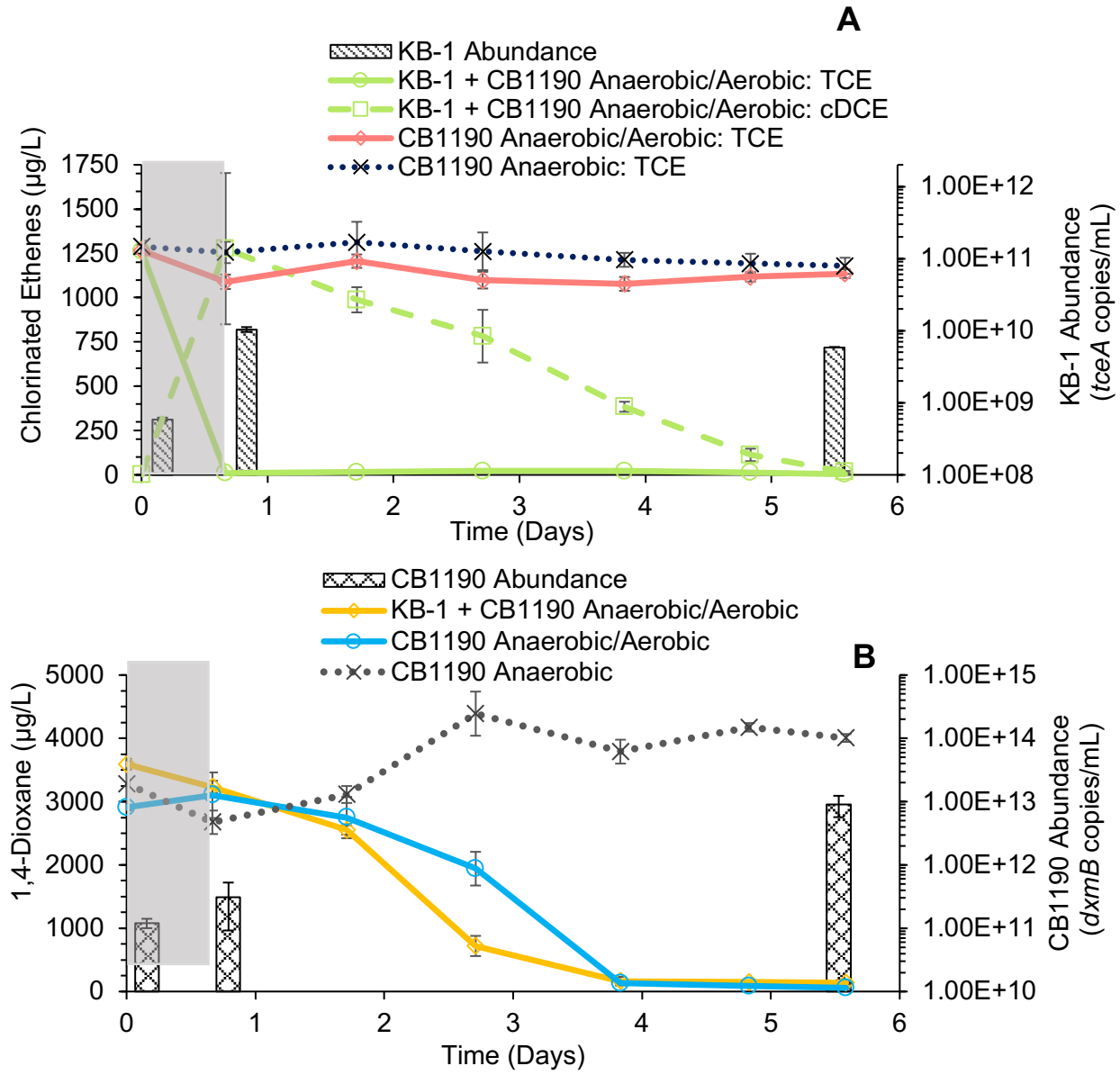


Figure 6. Biodegradation of CVOCs and 1,4-dioxane in pure CB1190 and KB-1® + CB1190. (A) Chlorinated ethene (TCE and cDCE) concentrations. cDCE was degraded by CB1190 during the aerobic phase. VC was not detected. (B) 1,4-Dioxane concentrations. KB-1® + CB1190 anaerobic/aerobic and CB1190 anaerobic/aerobic degraded 1,4-dioxane. The *dxmB* and *tceA* genes were used to quantify CB1190 cell numbers and the mixed culture cell abundance results are shown on secondary y-axis). Error bars indicate the standard deviation of triplicates, and the shaded gray areas indicate the anaerobic phase.

Parameters for Effective and Accelerated Biodegradation of CVOCS and 1,4-Dioxane with the Engineered Mixed Culture

In order for CB1190 to survive prolonged incubation in this mixed culture with *Dehalococcoides*, it was crucial to identify geochemical parameters that would inhibit or

promote CB1190's survivability such as reductants, additional carbon sources, nutrients components, and dissolved oxygen. It is widely known that *Dehalococcoides* are sensitive to dissolved oxygen and requires a carbon source such as lactate or acetate.[39] Iron sulfide, sodium sulfide, and titanium (III) citrate are commonly used as reductants in anaerobic cultures containing *Dehalococcoides* to reduce traces amounts of oxygen[25, 40-42] and lactate is often used as a carbon source and electron donor. Therefore, we tested CB1190's ability to degrade 1,4-dioxane in the presence of sulfide and lactate. Although chlorinated-ethene-respiring organisms such as *Dehalococcoides* are not inhibited by sulfide (<1 mM)[43, 44] and are often exposed to lactate to enhance chlorinated ethene degradation, it was unknown how these compounds would affect CB1190. Figure 7 demonstrates the inhibitory effect of sulfide on CB1190 1,4-dioxane biodegradation. Sodium sulfide (96 mg/L) completely inhibited CB1190's ability to biodegrade 1,4-dioxane.[45] Conversely, the presence of lactate (450 mg/L) did not significantly enhance or diminish CB1190's ability to degrade 1,4-dioxane. These data led to testing the effects of other suitable reductants for *Dehalococcoides* on CB1190.

We also tested CB1190's ability to biodegrade 1,4-dioxane in the presence of titanium (III) citrate and lower concentrations of sodium sulfide (7.5 mg/L) (Figure 8). Surprisingly, lower concentrations of sodium sulfide continued to cause complete inhibition of 1,4-dioxane degradation. However, titanium (III) citrate did not cause significant inhibitory effects compared to the control. CB1190 was also grown in an aerobic modified BAV1 media, which can also be used for *Dehalococcoides*-containing cultures, to ensure there were not trace minerals or nutrients that would impair CB1190's ability to degrade 1,4-dioxane (Figure 9).

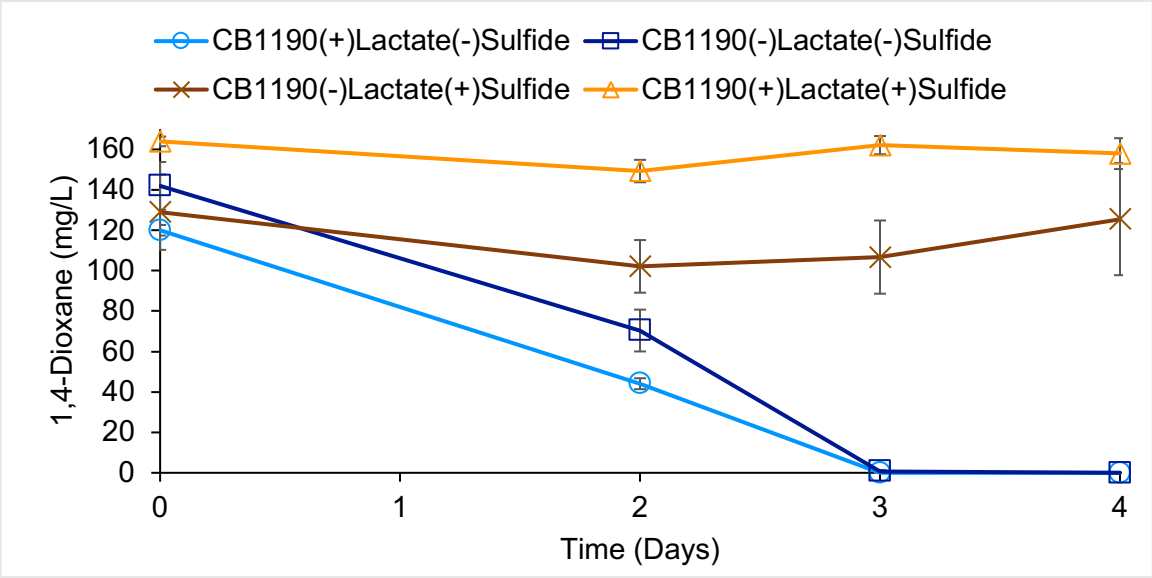


Figure 7. CB1190 degrades 1,4-dioxane at the same rate with or without lactate but is completely inhibited in the presence of sulfide. CB1190 was exposed to 450 mg/L of lactate and 96 mg/L of sodium sulfide. Error bars represent the standard deviation of triplicates.

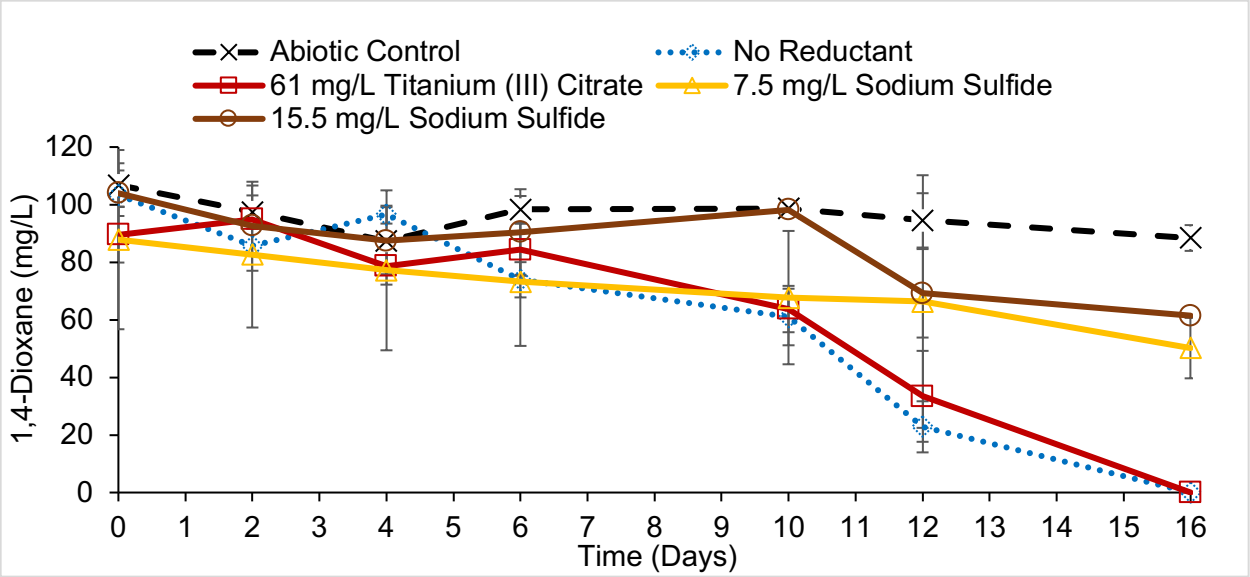


Figure 8. Titanium (III) citrate has a less inhibitory effect on CB1190's ability to biodegrade 1,4-dioxane than sodium sulfide. Titanium (III) citrate resulted in the least inhibition and sodium sulfide resulted in the most inhibition on CB1190's ability to biodegrade 1,4-dioxane. Error bars indicate standard deviation of 6 replicates.

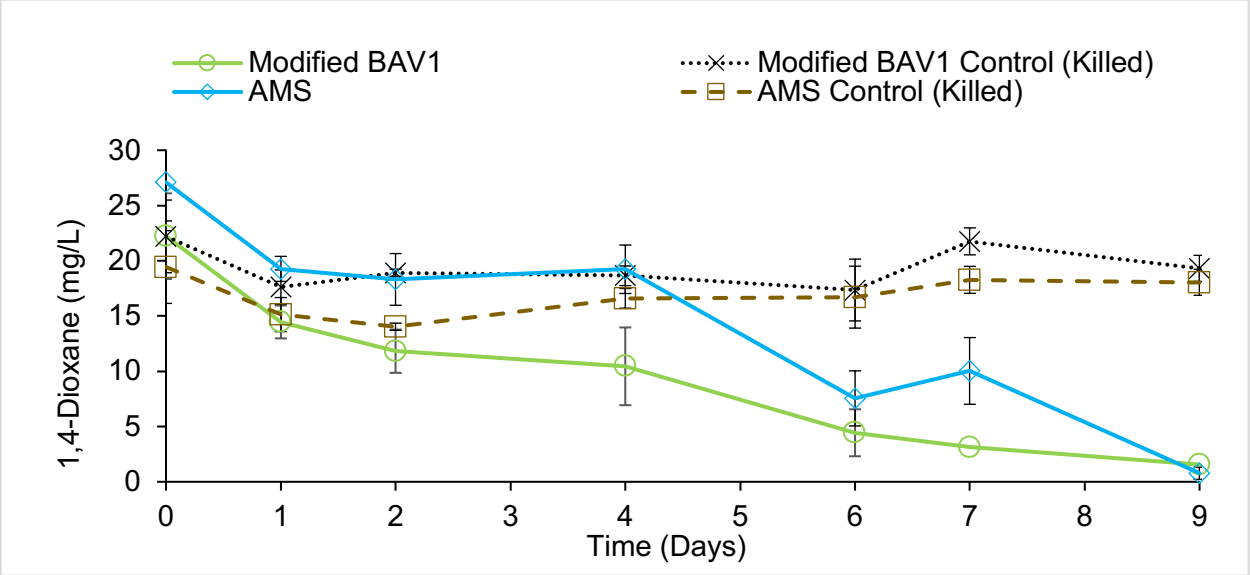


Figure 9. 1,4-Dioxane degradation by CB1190 grown in aerobic ammonium mineral salts medium (AMS) and modified BAV1 medium. There was a similar trend in 1,4-dioxane biodegradation between CB1190 grown in AMS[46] and CB1190 grown in modified BAV1 medium.[47] Error bars indicates standard deviation of 6 replicates.

Viability of CB1190 under Varying Dissolved Oxygen Concentrations

After optimizing the reductant and media components, the dissolved oxygen threshold for which CB1190 can degrade 1,4-dioxane was determined. Figure 10 shows CB1190 incubated under varying oxygen concentrations ranging from 1 mg/L to 7 mg/L. The CB1190 incubated with 1 mg/L and 2 mg/L dissolved oxygen did not degrade 1,4-dioxane. However, the bottles amended with 3 mg/L did significantly degrade 1,4-dioxane with a 2-day lag phase compared to the control bottles that were amended with 7 mg/L dissolved oxygen. These data are important because if CB1190 were amended to an environmental site where the water contained 3 mg/L of dissolved oxygen, CB1190 would still be able to degrade over 50 mg/L of 1,4-dioxane.

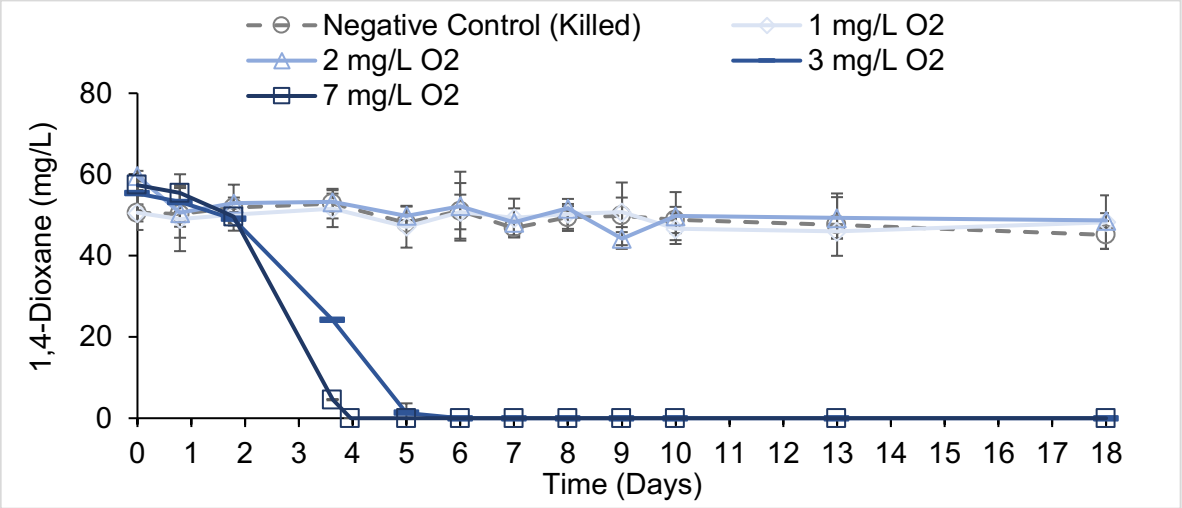


Figure 10. CB1190 maintains viability and activity in the presence of 3 mg/L of dissolved oxygen or higher. CB1190's oxygen scavenging capabilities demonstrate its need for less oxygen than other aerobic microorganisms relevant for bioremediation. Error bars represent the standard deviation of triplicates.

Viability and Gene Expression of CB1190 After Prolonged Periods of Anaerobic Incubation

Strain CB1190 Survivability and 1,4-Dioxane Biodegradation After Anaerobic Incubation

After 7, 14, 21, 28, 35, and 56 days of anaerobic incubation, strain CB1190 was able to biodegrade 1,4-dioxane to below the detection limit after oxygen addition (Figure 11). Strain CB1190 was initially inoculated into anaerobic media and amended with 1,4-dioxane for time periods ranging from 7 to 56 days. The aerobic bottles were continually amended with 1,4-dioxane and maintained in an aerobic environment as positive controls. The 56 days anaerobic bottles did not receive oxygen and served as anaerobic controls throughout the experiment. There was minimal lag phase for 1,4-dioxane biodegradation in each set of bottles that were amended with oxygen (Figure 11A). The bottles, which, were anaerobic for 35 days, had a slightly longer lag phase than bottles that were anaerobic for 7 days. The 1,4-dioxane biodegradation rate for each set was as follows: 0 Days Anaerobic (Aerobic Control): 0.211±0.009/day, 7 Days

Anaerobic: 0.162±0.003/day, 14 Days Anaerobic: 0.139±0.003/day, 21 Days Anaerobic: 0.140±0.001 day, 28 Days Anaerobic: 0.139±0.0001/day, and 35 Days Anaerobic: 0.105±0.0002/day. Bottles were amended with uniform cellular densities and had average initial CB1190 concentrations of 4.93±1.5 x 10^{11} *dxmB* copies/mL. During the anaerobic phase, the percent oxygen in the headspace averaged 2.5±0.7%, and oxygen reduction potential (ORP) in aqueous phase was -53±4.1 mV, which is considered a reducing environment.[48] After the anaerobic phase, the oxygen averaged 11.6±0.9% in the headspace (Supplementary Figure S5).

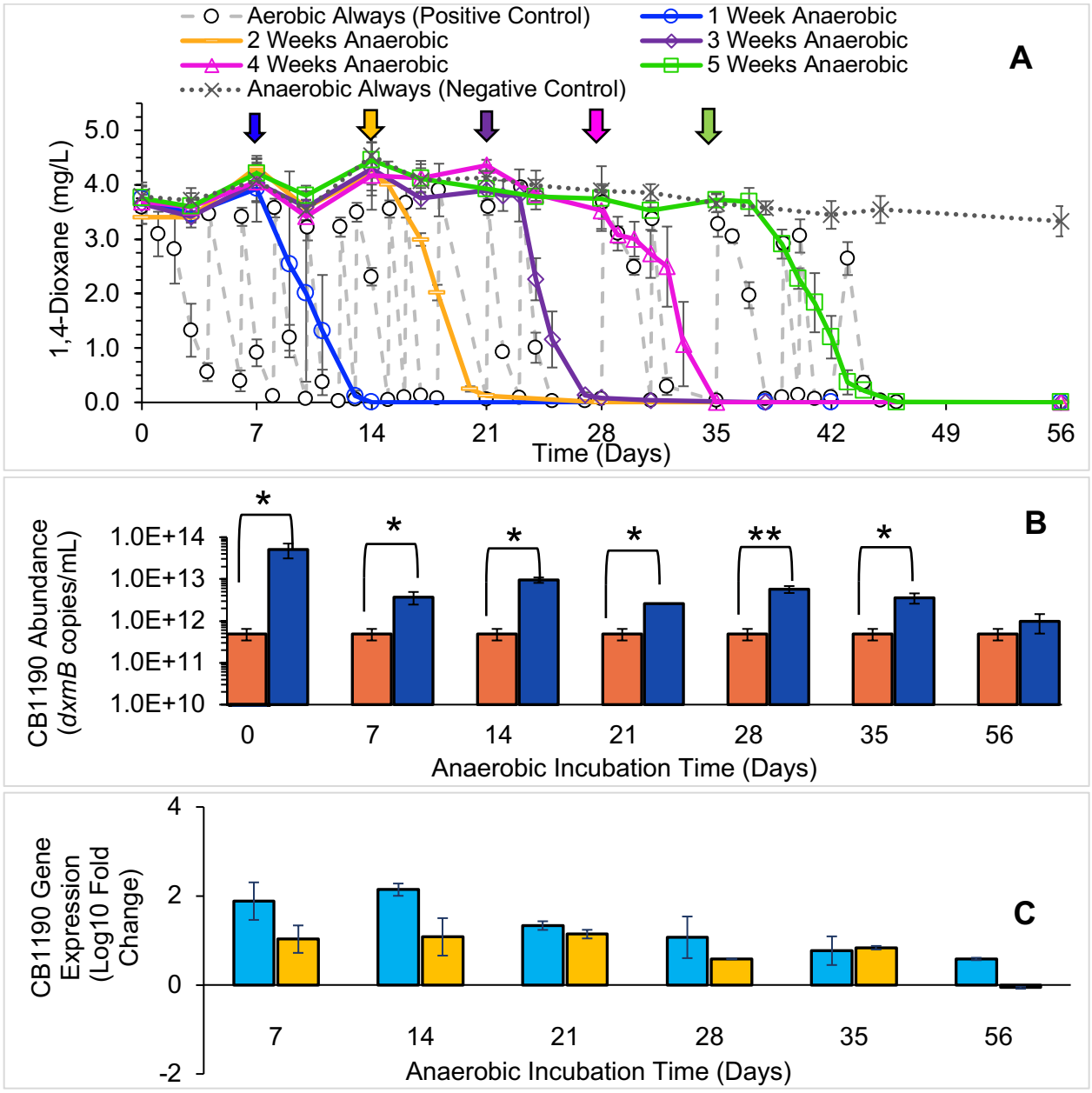


Figure 11. 1,4-Dioxane biodegradation, gene abundance, and gene expression in strain CB1190 after various anaerobic incubation periods. CB1190 was able to persist alone under anaerobic conditions and completely degrade 1,4-dioxane upon addition of O_2. Error bars indicate standard deviations of triplicates. (A) 1,4-Dioxane biodegradation by CB1190. Every week only one set of triplicate bottles was amended with O_2 (indicated by arrows). (B) CB1190 gene abundance on day 0, the last day of anaerobic incubation, and after 1,4-dioxane was degraded. *p-value <0.05 and **p-value< 0.01 (C) CB1190 gene expression. All cDNA copy numbers were first normalized to *rpoD* housekeeping gene and then to gene target concentrations at the end of the anaerobic phase. The 56 days anaerobic bottles were sampled on day 42 and 56 for gene expression.

CB1190 Cell Growth and Gene Expression After Anaerobic Incubation

1,4-Dioxane biodegradation that occurred after the transition to aerobic conditions produced a carbon and energy source for CB1190 growth. Significant CB1190 growth was observed in bottles that were kept anaerobic for 7, 14, 21, 28, and 35 days and then provided with oxygen amendments (Figure 11B). The aerobic bottles were replenished with 1,4-dioxane to demonstrate CB1190 biodegradation rates in the presence of continual oxygen and carbon substrate availability. Upregulation of *dxmB* and *aldH* genes were observed in days 7 through 35 demonstrating that 1,4-dioxane biodegradation was initiated and continued as the 1,4-dioxane degradation intermediates were produced (Figure 11C). The bottles that were maintained under anaerobic conditions were sampled at the beginning of day 42 and 56. The *dxmB* gene was upregulated in the anaerobic bottles, but the numbers of *aldH* gene transcripts were mostly unchanged. This could be explained by the fact that the *dxmB* gene is induced by 1,4-dioxane and can be expressed even if oxygen levels are insufficient. The *aldH* gene, which is induced by 1,4-dioxane biodegradation intermediates, was not upregulated likely because 1,4-dioxane had not been degraded.

CB1190's apparent dormancy under prolonged oxygen limited conditions could be explained by its ability to quickly adapt to stress and to utilize other substrates for fermentative growth . Similar aerobic Actinobacteria, *Mycobacterium tuberculosis* and *Mycobacterium bovis*, were previously grown in microaerophilic and anaerobic conditions.[49] Those cells appeared to produce a thickened cell wall to withstand oxygen deficient conditions *in vivo*. Specifically, the heat shock protein played a role in stabilizing the cell structure in the long-term by creating a multi-layered, protective casing of peptidoglycan.[17, 49, 50] CB1190 could be utilizing a hydrogenase enzyme to survive anaerobic incubation. Traditionally, hydrogenase enzymes have been associated with anaerobic metabolism because a majority are deactivated in the presence of oxygen. However, they are often present among aerobic bacteria and rarely studied among obligate aerobes.[51] CB1190 can fix CO_2 when provided hydrogen and maintain very slow cellular growth.[52] The hydrogenase enzyme in CB1190 was reported to utilize H_2 coupled with trace amounts of CO_2 or biocarbonate to remain active.[52] At low pH values, which are commonly found in anaerobic waters [53], the PHREEQC computer model showed that the chemical components of the modified media could produce H_2 in micromolar to nanomolar concentrations. We postulate that CB1190 could be utilizing the low amounts of hydrogen to remain dormant but quickly revive when sufficient oxygen becomes available for aerobic metabolism.

Reactivation of the *dxmB* and *aldH* in CB1190 was crucial in validating that CB1190 could endure oxygen restrictions in the subsurface. As our findings with CB1190 demonstrate, bacteria that harbor the ability to maintain cellular viability and metabolism when exposed to divergent redox conditions could provide a wider variety of solutions for chlorinated ethene and 1,4-dioxane bioremediation. The results of this study demonstrate that an intrinsic or bioaugmented

mixed microbial community can withstand the changing redox conditions that may be experienced by a plume and biodegrade chlorinated ethenes as well as 1,4-dioxane.

CB1190's Ability to Biodegrade cDCE With and Without 1,4-Dioxane Under Aerobic Conditions

Aerobic biodegradation of cDCE by CB1190 with and without 1,4-dioxane was first recorded in the present study (Figure 12-13). The presence of 1,4-dioxane did not inhibit or slow CB1190's ability to degrade cDCE. Killed controls containing CB1190 did not show significant degradation of cDCE or 1,4-dioxane (Figure 12-13). Biodegradation of cDCE by CB1190 can mitigate cDCE's inhibition of 1,4-dioxane biodegradation [7, 19, 22] as well as prevent VC production. This reduces the need to amend additional microorganisms and their primary substrates to oxidatively cometabolize cDCE.

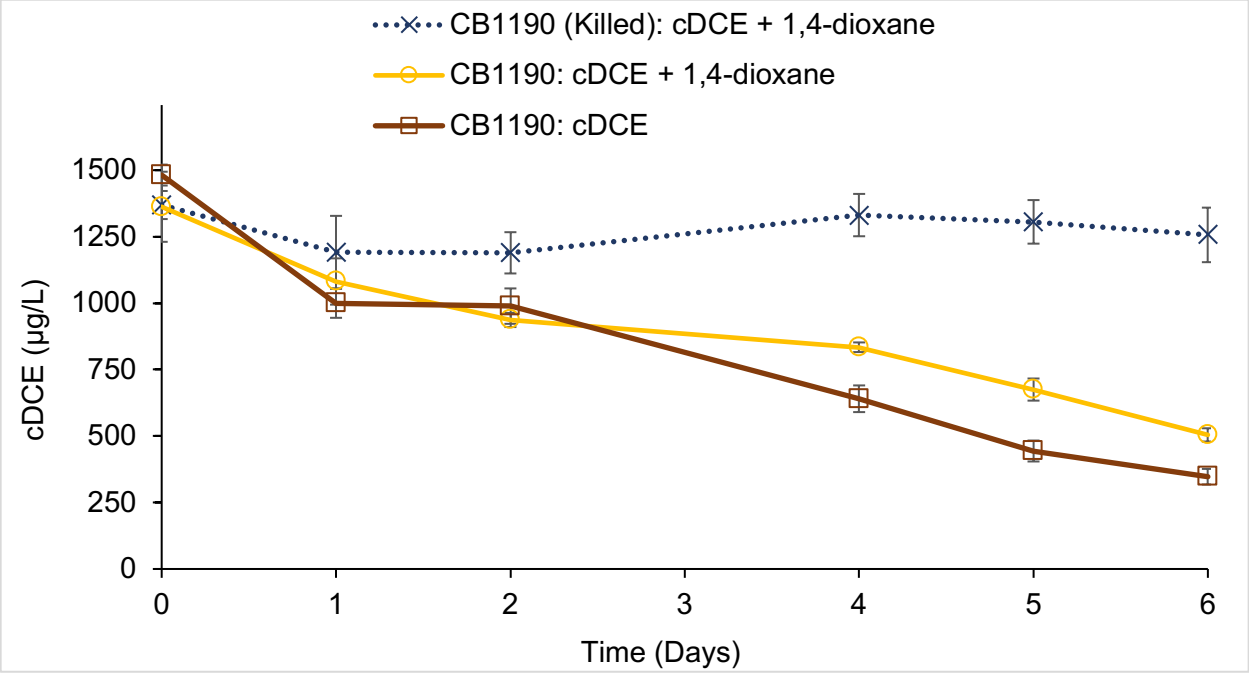


Figure 12. CB1190 aerobically degrades cDCE with and without 1,4-dioxane. There was no significant difference in degradation rates of cDCE when CB1190 was amended with and without 1,4-dioxane. Error bars represent the standard deviation of triplicates.

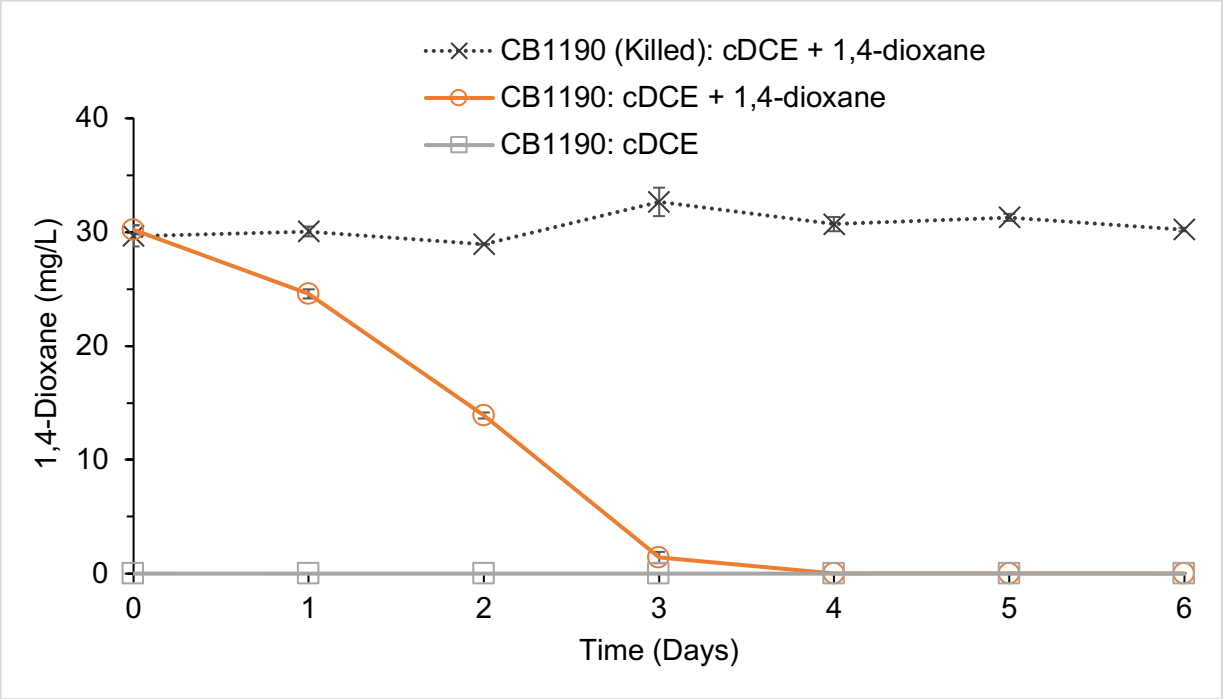


Figure 13. CB1190 aerobically degrades 1,4-dioxane in the presence of cDCE. There was no significant 1,4-dioxane transfer from the seed culture in CB1190 amended with only cDCE. Error bars represent the standard deviation of triplicates.

Validation of CB1190's Viability at an Environmental Site Previously Exposed to Enhanced Reductive Dechlorination

Bio-Trap® beads bioaugmented with CB1190 were deployed for 3 months to two wells containing CVOCs and 1,4-dioxane. Prior to bioaugmentation, the site had a significant *Dehalococcoides* population due the previous use of enhanced reductive dechlorination. The first well contained low levels of CVOCs and 1,4-dioxane (Figure 14) and over the course of the three months that the Bio-Traps® were in the well, cDCE and VC did not significantly change. However, 1,4-dioxane decreased by 40% and TCE increased by 34%. Gene targets for *Dehalococcoides* and CB1190 abundance were also measured during this time period. Results showed that the *tceA* gene abundance did not significantly change but that the *vcrA* gene abundance decreased. This could be from the fact that a significant amount of TCE is present in the well, thus making it advantageous for dechlorinating bacteria to have this gene and contrastingly less advantageous to have the *vcrA* gene because of the low concentrations of VC (5-9 µg/L). Both the *dxmB* and *aldH* gene abundances increased thus showing that CB1190 remained attached to the beads over prolonged periods in the field and maintain cellular biomass of 10^4-10^5 copies/bead. In the well with higher CVOCs and 1,4-dioxane (Figure 15), a similar trend was seen where cDCE and VC did not significantly change,1,4-dioxane decreased by 48%, and TCE increased by 33%.

Interestingly, in the well with high CVOCs and 1,4-dioxane, the *tceA*, *vcrA*, *dxmB*, and *aldH* gene targets decreased approximately half an order of magnitude. This could be because the CVOC and 1,4-dioxane concentrations were significantly higher in this well compared to the other well and could have caused inhibition to *Dehalococcoides'* or CB1190's cellular functions, which has been shown to be significant at these concentrations in the laboratory.[7] For example, the TCE and VC concentrations were much higher in the second monitoring well (Figure 15) (TCE = 5,000 µg/L, VC = 77 µg/L) compared to the first monitoring well (Figure 14) (TCE = 1,000 µg/L, VC = 7 µg/L). Although there was no increase in the *dxmB* or *aldH* abundance, it still remained around 10^3 copies/bead even after being exposed to high concentrations of chlorinated solvents and field conditions thus showing CB1190's affinity to remain attached to the PAC bead.

These Bio-Traps® showed that CB1190 is resilient when exposed to harsh environmental elements such as low dissolved oxygen (2-6 mg/L), inhibitors (e.g., CVOCs), and colder water temperatures. This field experiment demonstrates that CB1190 could be used in a larger pilot scale test with similar environmental conditions and be able degrade 1,4-dioxane and potentially cDCE.

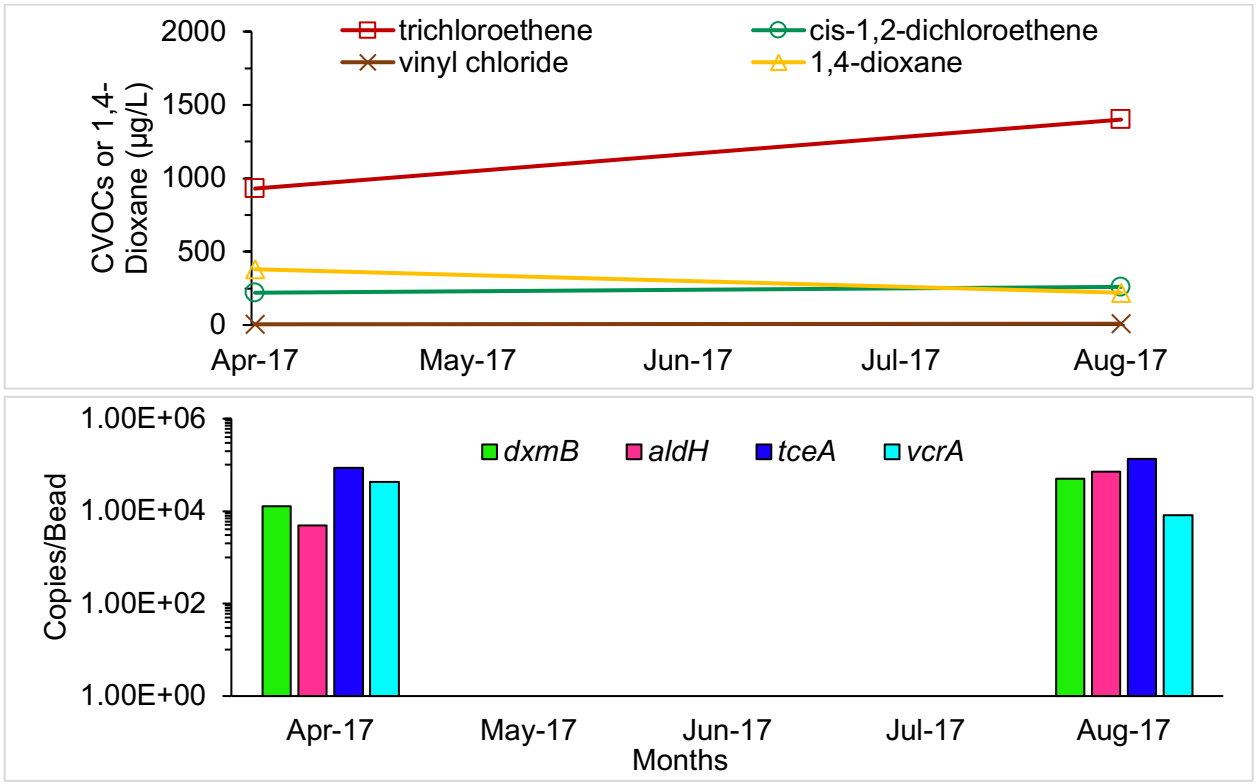


Figure 14. CB1190 remained attached to PAC beads after 3 months in the field with low 1,4-dioxane and CVOC concentrations. Gene abundances were measured via Bio-Trap® PAC beads immediately before deployment and after 3 months in the monitoring well.

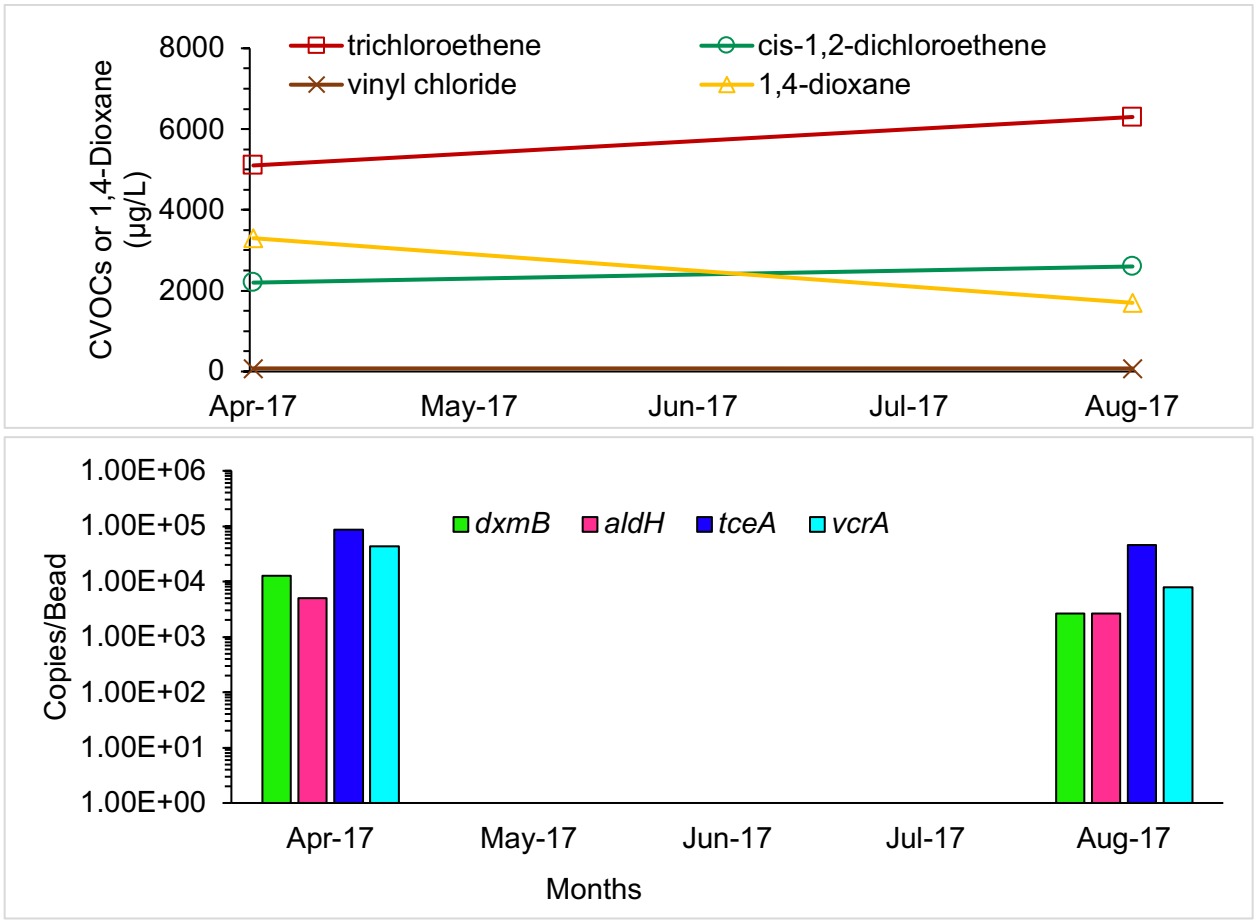


Figure 15. CB1190 remained attached to PAC beads after 3 months in the field with high 1,4-dioxane and CVOC concentrations. Gene abundances were measured via Bio-Trap® PAC beads immediately before deployment and after 3 months in the monitoring well.

CONCLUSIONS

Conclusion #1: Engineered microbial communities can subsist under changing redox conditions for and biodegrading contaminant mixtures.
The opposing redox conditions favored by CVOC- and 1,4-dioxane-degrading bacteria pose a difficult problem for concurrent bioremediation of both contaminants. We successfully engineered a microbial community composed of the previously identified anaerobic culture, KB-1®, and the aerobic bacterium, CB1190, to simultaneously and sequentially degrade CVOCs and 1,4-dioxane. Furthermore, geochemical parameters were optimized to extend CB1190's survival. For example, titanium (III) citrate and a modified BAV1 medium were found to support both CB1190 and *Dehalococcoides'* degradation capabilities. Lactate, which can serve as a carbon source for *Dehalococcoides*, does not significantly reduce CB1190's 1,4-dioxane biodegradation rate and can therefore be used for this KB-1® + CB1190 co-culture. The results of this project demonstrate that the engineered microbial community can withstand changing redox conditions and biodegrade both chlorinated ethenes and 1,4-dioxane. This approach could reduce the cost, energy, and substrates required for *in situ* bioremediation for chlorinated ethenes and dioxane.

Conclusion #2: CB1190 biodegrades cDCE aerobically without generating VC.
We report CB1190's ability to biodegrade cDCE aerobically in the presence of 1,4-dioxane. This is beneficial because sites previously treated with enhanced reductive dechlorination can have increased levels of cDCE. CB1190 is able to biodegrade 1,4-dioxane and cDCE simultaneously thus removing the federally regulated contaminant, cDCE, and 1,4-dioxane. This has the additional benefit of limiting cDCE's inhibition of 1,4-dioxane biodegradation.

Conclusion #3: CB1190 can withstand prolonged anaerobic incubation and grow when conditions turn aerobic.
CB1190's ability to withstand over 35 days of anaerobic incubation and subsequently degrade 1,4-dioxane once sufficient oxygen became available is critical for the mixed microbial communities' application in the field. CB1190 could be potentially exposed to anaerobic conditions for weeks to months as TCE is degraded to cDCE and would need to survive this period without an electron acceptor. Our results show that CB1190 is resilient to these sub-optimal conditions and can maintain the ability to degrade 1,4-dioxane. With minimal lag phase, CB1190 upregulates the genes responsible for 1,4-dioxane degradation and the degradation of its downstream intermediates. The monooxygenase enzymes that are induced in the CB1190 + KB-1® culture can biodegrade 1,4-dioxane with minimal lag.

Conclusion #4: 1,4-Dioxane degradation occurs in oxygen levels as low as 3 mg/L.
CB1190 is an oxygen scavenging microorganism. It can degrade up to 60,000 µg/L of 1,4-dioxane with as little as 3 mg/L dissolved oxygen in 5 days. Surprisingly, when provided with 9 mg/L dissolved oxygen CB1190 degraded 1,4-dioxane only marginally faster (4 days). Thus,

CB1190 has demonstrated its ability to mineralize 1,4-dioxane even under the limited availability of oxygen as terminal electron acceptor.

Conclusion #5: CB1190 remains viable at sites previously treated with enhanced reductive dechlorination.

CB1190 cells that were seeded onto the Bio-Trap® PAC beads remained attached and active even after 3 months of incubation in a field site well with sub-optimal dissolved oxygen levels, high CVOCs and 1,4-dioxane concentrations, and low water temperatures. This shows that CB1190 would be a promising microorganism to use for *in situ* biological remediation of CVOCs and 1,4-dioxane in plumes where redox conditions transition from anaerobic to aerobic.

ACKNOWLEDGEMENTS

We thank Dr. Phillip Gedalanga, Dr. Yu Miao, Dr. Nicholas Johnson, Jiahui Wang, Alessandro Zulli, and Dominic Robolino for their support with the laboratory experiments and data analyses. Ms. Sandra Dworatzek of SiREM, Guelph, ON, Canada provided the KB-1® culture. Dr. Laurie LaPat-Polasko of Matrix New World Engineering, Phoenix, AZ and her team collaborated on the *in situ* studies involving CB1190-seeded Bio-Traps®.

PUBLICATIONS AND TECHNOLOGY TRANSFER

Papers

1. Polasko A., A. Zulli, P. B. Gedalanga, P. Pornwongthong, and S. Mahendra (2019). A Mixed Microbial Community for the Biodegradation of Chlorinated Ethenes and 1,4-Dioxane. *Environmental Science and Technology Letters* 6 (1): 49-54. ACS Editor's Choice
2. Chen, R., Y. Miao, Y. Liu, L. Zhang, M. Zhong, J. M. Adams, Y. Dong, and S. Mahendra (2021). Identification of Novel 1,4-Dioxane Degraders and Related Genes from Activated Sludge by Taxonomic and Functional Gene Sequence Analysis. *Journal of Hazardous Materials* 412: 125157.
3. Polasko, A., Y. Miao, I. Kwok, K. Park, J. Park, and S. Mahendra (2021). Vinyl Chloride and 1,4-Dioxane Metabolism by *Pseudonocardia dioxanivorans* CB1190. *Journal of Hazardous Materials Letters* 2: 100039.

Patent

1. Mahendra, S. and A. Polasko. Anaerobic-Aerobic Bioremediation of Contaminated Water (62/590,030, filed November 21, 2017).

Thesis

1. Polasko, A. (2017). Sequential Anaerobic/Aerobic Biodegradation of Trichloroethene and 1,4-Dioxane. MS Thesis. UCLA Civil and Environmental Engineering.

Conference Presentations

1. Mahendra, S. (2019). Removal of 1,4-Dioxane from Water: Adsorption, Anaerobic/Aerobic Biodegradation, and Insights from Molecular Biological Tools. *1,4-Dioxane Summit*. Minneapolis, MN.
2. Mahendra, S. (2019). Molecular Diagnostic Tools to Estimate Field-based Degradation Rates. *University Consortium for Field Focused Groundwater Research Fall Focus Meeting*. Fort Collins, CO.
3. Polasko, A., D. Robolino, A. Zulli, and S. Mahendra (2019). Building an Unconventional Community: Training Environmental Engineers and Microbes for Bioremediation Applications. *Association of Environmental Engineering and Science Professors (AEESP) Conference,* Tempe, AZ.
4. 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.
5. Polasko, A., A. Zulli, S. Dworatzek, E. Mack, C. Walecka-Hutchinson, and S. Mahendra (2018). Optimizing a Mixed Microbial Community to Biodegrade Chlorinated Ethenes and

1,4-Dioxane. *Eleventh International Conference on Remediation of Chlorinated and Recalcitrant Compounds*, Palm Springs, CA.

6. 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.
7. Polasko, A., A. Zulli, and S. Mahendra (2018). Biodegradation of CVOCs and 1,4-Dioxane by an Engineered Microbial Community. *Emerging Contaminants Summit*. Westminster, CO.
8. Polasko, A., A. Zulli, and S. Mahendra (2017). Optimizing a Mixed Microbial Community for Biodegradation of CVOCs and 1,4-Dioxane. *Strategic Environmental Research & Development Program Annual Symposium*, Washington, DC.
9. Mahendra, S. (2017). Biodegradation of 1,4-Dioxane: Co-Contaminant Effects and Monitoring Tools. *SERDP & ESTCP Webinar #52*, Arlington, VA.
10. 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.

REFERENCES

(1) Adamson, D.T., S. Mahendra, K.L. Walker, S.R. Rauch, S. Sengupta, and C.J. Newell. A Multisite Survey to Identify the Scale of the 1,4-Dioxane Problem at Contaminated Groundwater Sites. *Environmental Science & Technology Letters*, **2014**, 1, 254-258.

(2) Maymó-Gatell, X., T. Anguish, and S.H. Zinder. Reductive Dechlorination of Chlorinated Ethenes and 1,2-Dichloroethane by "*Dehalococcoides ethenogenes*" 195. *Applied and Environmental Microbiology*, **1999**, 65, 3108-3113.

(3) Löffler, F.E., J. Yan, K.M. Ritalahti, L. Adrian, E.A. Edwards, K.T. Konstantinidis, J.A. Mueller, H. Fullerton, S.H. Zinder, and A.M. Spormann. *Dehalococcoides mccartyi* gen. nov., sp nov., Obligately Organohalide-Respiring Anaerobic Bacteria Relevant to Halogen Cycling and Bioremediation, Belong to a Novel Bacterial Class, *Dehalococcoidia* classis nov., Order *Dehalococcoidales* ord. nov and Family *Dehalococcoidaceae* fam. nov., within the Phylum *Chloroflexi. International Journal of Systematic and Evolutionary Microbiology*, **2013**, 63, 625-635.

(4) DiStefano, T.D., J.M. Gossett, and S.H. Zinder. Reductive Dechlorination of High Concentrations of Tetrachloroethene to Ethene by an Anaerobic Enrichment Culture in the Absence of Methanogenesis. *Applied and Environmental Microbiology*, **1991**, 57, 2287-2292.

(5) Mahendra, S. and L. Alvarez-Cohen. Kinetics of 1,4-Dioxane Biodegradation by Monooxygenase-Expressing Bacteria. *Environmental Science & Technology*, **2006**, 40, 5435-5442.

(6) Zhang, S., P.B. Gedalanga, and S. Mahendra. Advances in Bioremediation of 1,4-Dioxane-Contaminated Waters. *Journal of Environmental Management*, **2017**, 204, 765-774.

(7) Zhang, S., P.B. Gedalanga, and S. Mahendra. Biodegradation Kinetics of 1,4-Dioxane in Chlorinated Solvent Mixtures. *Environmental Science & Technology*, **2016**, 50, 9599-9607.

(8) Stroo, H.F., A. Leeson, and C.H. Ward, *Bioaugmentation for Groundwater Remediation*. Vol. 5. 2012: Springer Science & Business Media.

(9) Stroo, H.F. and C.H. Ward, *In Situ Remediation of Chlorinated Solvent Plumes*. 2010: Springer Science & Business Media.

(10) Yang, Y., M. Pesaro, W. Sigler, and J. Zeyer. Identification of Microorganisms Involved in Reductive Dehalogenation of Chlorinated Ethenes in an Anaerobic Microbial Community. *Water Research*, **2005**, 39, 3954-3966.

(11) Vogel, T.M. and P.L. McCarty. Biotransformation of Tetrachloroethylene to Trichloroethylene, Dichloroethylene, Vinyl Chloride, and Carbon Dioxide under Methanogenic Conditions. *Applied and Environmental Microbiology*, **1985**, 49, 1080-1083.

(12) Mattes, T.E., N.V. Coleman, J.C. Spain, and J.M. Gossett. Physiological and Molecular Genetic Analyses of Vinyl Chloride and Ethene Biodegradation in *Nocardioides* Sp. Strain JS614. *Archives of Microbiology*, **2005**, 183, 95-106.

(13) Coleman, N.V. and J.C. Spain. Distribution of the Coenzyme M Pathway of Epoxide Metabolism among Ethene-and Vinyl Chloride-Degrading *Mycobacterium* Strains. *Applied and Environmental Microbiology*, **2003**, 69, 6041-6046.

(14) Aziz, C., G. Georgiou, and G. Speitel. Cometabolism of Chlorinated Solvents and Binary Chlorinated Solvent Mixtures Using *M. trichosporium* OB3b PP358. *Biotechnology and Bioengineering*, **1999**, 65, 100-107.
(15) Nishino, S.F., K.A. Shin, J.M. Gossett, and J.C. Spain. Cytochrome P450 Initiates Degradation of Cis-Dichloroethene by *Polaromonas* Sp. Strain JS666. *Applied and Environmental Microbiology*, **2013**, 79, 2263-2272.
(16) Mahendra, S., C.J. Petzold, E.E. Baidoo, J.D. Keasling, and L. Alvarez-Cohen. Identification of the Intermediates of in Vivo Oxidation of 1,4-Dioxane by Monooxygenase-Containing Bacteria. *Environmental Science & Technology*, **2007**, 41, 7330-7336.
(17) Gedalanga, P.B., P. Pornwongthong, R. Mora, S.-Y.D. Chiang, B. Baldwin, D. Ogles, and S. Mahendra. Identification of Biomarker Genes to Predict Biodegradation of 1,4-Dioxane. *Applied and Environmental Microbiology*, **2014**, 80, 3209-3218.
(18) Grostern, A., C.M. Sales, W. Zhuang, O. Erbilgin, and L. Alvarez-Cohen. Glyoxylate Metabolism Is a Key Feature of the Metabolic Degradation of 1,4-Dioxane by *Pseudonocardia dioxanivorans* Strain CB1190. *Applied and Environmental Microbiology*, **2012**, 78, 3298-3308.
(19) Mahendra, S., A. Grostern, and L. Alvarez-Cohen. The Impact of Chlorinated Solvent Co-Contaminants on the Biodegradation Kinetics of 1,4-Dioxane. *Chemosphere*, **2013**, 91, 88-92.
(20) Pornwongthong, P., A. Mulchandani, P.B. Gedalanga, and S. Mahendra. Transition Metals and Organic Ligands Influence Biodegradation of 1,4-Dioxane. *Applied Biochemistry and Biotechnology*, **2014**, 173, 291-306.
(21) Johnson, N.W., P.B. Gedalanga, L. Zhao, B. Gu, and S. Mahendra. Cometabolic Biotransformation of 1,4-Dioxane in Mixtures with Hexavalent Chromium Using Attached and Planktonic Bacteria. *Science of the Total Environment*, **2020**, 706, 135734.
(22) Zhao, L., X. Lu, A. Polasko, N.W. Johnson, Y. Miao, Z. Yang, S. Mahendra, and B. Gu. Co-Contaminant Effects on 1,4-Dioxane Biodegradation in Packed Soil Column Flow-through Systems. *Environmental Polutionl.*, **2018**, 243, 573-581.
(23) Liu, Y., N.W. Johnson, C. Liu, R. Chen, M. Zhong, Y. Dong, and S. Mahendra. 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*, **2019**, 53, 14538-14547.
(24) Alvarez-Cohen, L. and P. McCarty. Effects of Toxicity, Aeration, and Reductant Supply on Trichloroethylene Transformation by a Mixed Methanotrophic Culture. *Applied and Environmental Microbiology*, **1991**, 57, 228-235.
(25) He, J., V.F. Holmes, P.K.H. Lee, and L. Alvarez-Cohen. Influence of Vitamin B12 and Cocultures on the Growth of *Dehalococcoides* Isolates in Defined Medium. *Applied and Environmental Microbiology*, **2007**, 73, 2847-2853.
(26) Li, M., P. Conlon, S. Fiorenza, R.J. Vitale, and P.J.J. Alvarez. Rapid Analysis of 1,4-Dioxane in Groundwater by Frozen Micro-Extraction with Gas Chromatography-Mass Spectrometry. *Ground Water Monitoring & Remediation*, **2011**, 31, 70-76.
(27) Waller, A.S., R. Krajmalnik-Brown, F.E. Loffler, and E.A. Edwards. Multiple Reductive-Dehalogenase-Homologous Genes Are Simultaneously Transcribed During Dechlorination by *Dehalococcoides*-Containing Cultures. *Applied and Environmental Microbiology*, **2005**, 71, 8257-8264.

(28) Livak, K.J. and T.D. Schmittgen. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods*, **2001**, 25, 402-408.
(29) Lee, P.K.H., D.R. Johnson, V.F. Holmes, J. He, and L. Alvarez-Cohen. Reductive Dehalogenase Gene Expression as a Biomarker for Physiological Activity of *Dehalococcoides* Spp. *Applied and Environmental Microbiology*, **2006**, 72, 6161-6168.
(30) Rocha, E.R. and J.C. Smith. Role of the Alkyl Hydroperoxide Reductase (Ahpcf) Gene in Oxidative Stress Defense of the Obligate Anaerobe *Bacteroides fragilis*. *Journal of Bacteriology*, **1999**, 181, 5701-5710.
(31) Miao, Y., N.W. Johnson, P.B. Gedalanga, D. Adamson, C. Newell, and S. Mahendra. Response and Recovery of Microbial Communities Subjected to Oxidative and Biological Treatments of 1,4-Dioxane and Co-Contaminants. *Water Research*, **2019**, 149, 74-85.
(32) Miao, Y., N.W. Johnson, T. Phan, K. Heck, P.B. Gedalanga, X. Zheng, D. Adamson, C. Newell, M.S. Wong, and S. Mahendra. Monitoring, Assessment, and Prediction of Microbial Shifts in Coupled Catalysis and Biodegradation of 1,4-Dioxane and Co-Contaminants. *Water Research*, **2020**, 173, 115540.
(33) Brown, D. and B. Hamburger. The Degradation of Dyestuffs: Part Iii - Investigations of Their Ultimate Degradability. *Chemosphere*, **1987**, 16, 1539-1553.
(34) Fathepure, B.Z. and T.M. Vogel. Complete Degradation of Polychlorinated Hydrocarbons by a Two-Stage Biofilm Reactor. *Applied and Environmental Microbiology*, **1991**, 57, 3418-3422.
(35) Beunink, J. and H.-J. Rehm. Synchronous Anaerobic and Aerobic Degradation of DDT by an Immobilized Mixed Culture System. *Applied Microbiology and Biotechnology*, **1988**, 29, 72-80.
(36) Anid, P.J., B.P. Ravest-Webster, and T.M. Vogel. Effect of Hydrogen Peroxide on the Biodegradation of PCBs in Anaerobically Dechlorinated River Sediments. *Biodegradation*, **1993**, 4, 241-248.
(37) Sekar, R., M. Taillefert, and T.J. DiChristina. Simultaneous Transformation of Commingled Trichloroethylene, Tetrachloroethylene, and 1,4-Dioxane by a Microbially Driven Fenton Reaction in Batch Liquid Cultures. *Applied and Environmental Microbiology*, **2016**, 82, 6335-6343.
(38) Magnuson, J.K., R.V. Stern, J.M. Gossett, S.H. Zinder, and D.R. Burris. Reductive Dechlorination of Tetrachloroethene to Ethene by a Two-Component Enzyme Pathway. *Applied and Environmental Microbiology*, **1998**, 64, 1270-1275.
(39) Maymó-Gatell, X., Y.-t. Chien, J.M. Gossett, and S.H. Zinder. Isolation of a Bacterium That Reductively Dechlorinates Tetrachloroethene to Ethene. *Science*, **1997**, 276, 1568-1571.
(40) He, J., K.M. Ritalahti, M.R. Aiello, and F.E. Löffler. Complete Detoxification of Vinyl Chloride by an Anaerobic Enrichment Culture and Identification of the Reductively Dechlorinating Population as a *Dehalococcoides* Species. *Applied and Environmental Microbiology*, **2003**, 69, 996-1003.
(41) Rosenthal, H., L. Adrian, and M. Steiof. Dechlorination of PCE in the Presence of Fe0 Enhanced by a Mixed Culture Containing Two *Dehalococcoides* Strains. *Chemosphere*, **2004**, 55, 661-669.

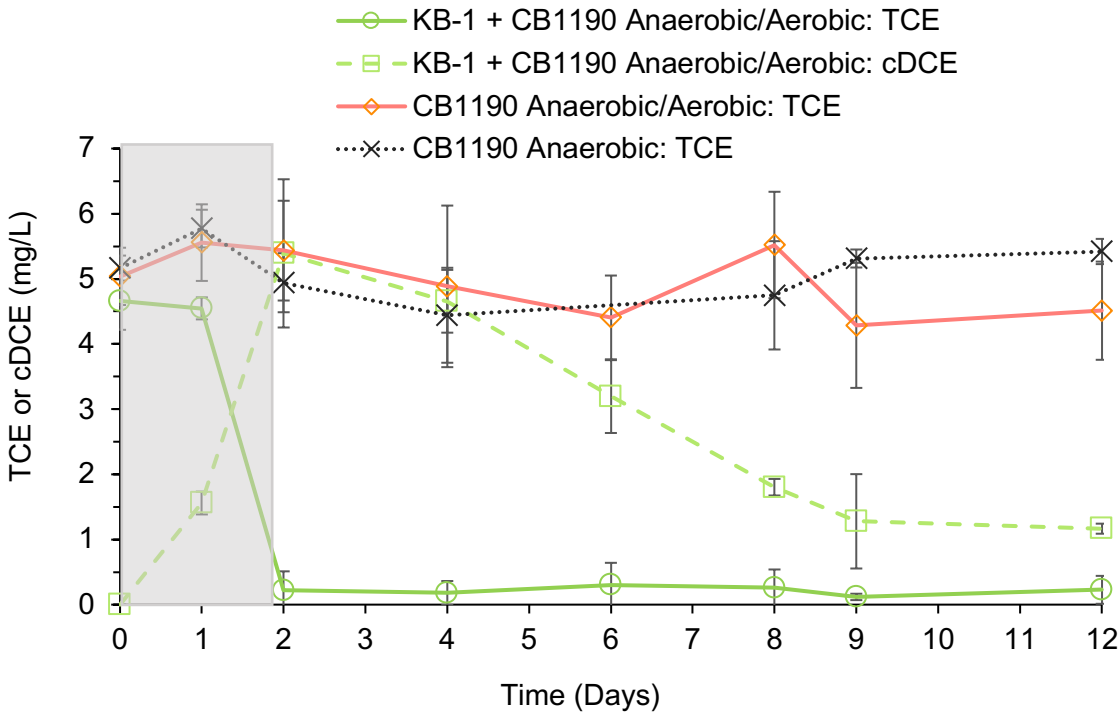
(42) Lesage, S., S. Brown, and K. Millar. A Different Mechanism for the Reductive Dechlorination of Chlorinated Ethenes: Kinetic and Spectroscopic Evidence. *Environmental Science & Technology*, **1998**, 32, 2264-2272.
(43) Mao, X., A. Polasko, and L. Alvarez-Cohen. Effects of Sulfate Reduction on Trichloroethene Dechlorination by *Dehalococcoides*-Containing Microbial Communities. *Applied and Environmental Microbiology*, **2017**, 83, 1-13.
(44) He, J., Y. Sung, R. Krajmalnik-Brown, K.M. Ritalahti, and F.E. Löffler. Isolation and Characterization of *Dehalococcoides* Sp. Strain FL2, a Trichloroethene (TCE)- and 1,2-Dichloroethene-Respiring Anaerobe. *Environmental Microbiology*, **2005**, 7, 1442-1450.
(45) Puentes Jácome, L.A., P.-H. Wang, O. Molenda, Y.X. Li, M.A. Islam, and E.A. Edwards. Sustained Dechlorination of Vinyl Chloride to Ethene in *Dehalococcoides*-Enriched Cultures Grown without Addition of Exogenous Vitamins and at Low pH. *Environmental Science & Technology*, **2019**, 53, 11364-11374.
(46) Parales, R.E., J.E. Adamus, N. White, and H.D. May. Dedegradation of 1,4-Dioxane by an Actinomycete in Pure Culture. *Applied and Environmental Microbiology*, **1994**, 60, 4527-4530.
(47) Cole, J.R., A.L. Cascarelli, W.W. Mohn, and J.M. Tiedje. Isolation and Characterization of a Novel Bacterium Growing Via Reductive Dehalogenation of 2-Chlorophenol. *Applied and Environmental Microbiology*, **1994**, 60, 3536-3542.
(48) Parsons, F., G.B. Lage, and R. Rice. Biotransformation of Chlorinated Organic Solvents in Static Microcosms. *Environmental Toxicology and Chemistry*, **1985**, 4, 739-742.
(49) Cunningham, A.F. and C.L. Spreadbury. Mycobacterial Stationary Phase Induced by Low Oxygen Tension: Cell Wall Thickening and Localization of the 16-Kilodalton A-Crystallin Homolog. *Journal of Bacteriology*, **1998**, 180, 801-808.
(50) Masuda, H. Identification and Characterization of Monooxygenase Enzymes Involved in 1,4-Dioxane Degradation in *Pseudonocardia* sp. Strain ENV478, *Mycobacterium* Sp. Strain Env421, and *Nocardia* Sp. Strain ENV425. PhD. Thesis *Rutgers The State University of New Jersey-New Brunswick and University of Medicine and Dentistry of New Jersey.*, **2009**.
(51) Michael, B., G. Chris, H. Kiel, C. Desmond, and C.G. M. Three Different [Nife] Hydrogenases Confer Metabolic Flexibility in the Obligate Aerobe *Mycobacterium smegmatis. Environmental Microbiology*, **2014**, 16, 318-330.
(52) Grostern, A. and L. Alvarez-Cohen. Rubisco-Based Co2 Fixation and C1 Metabolism in the Actinobacterium *Pseudonocardia dioxanivorans* Cb1190. *Environmental Microbiology*, **2013**, 15, 3040-3053.
(53) De, A.K., *Environmental Chemistry: Fifth Edition*. 2003, Daryaganj, New Delhi: New Age International.

APPENDICES

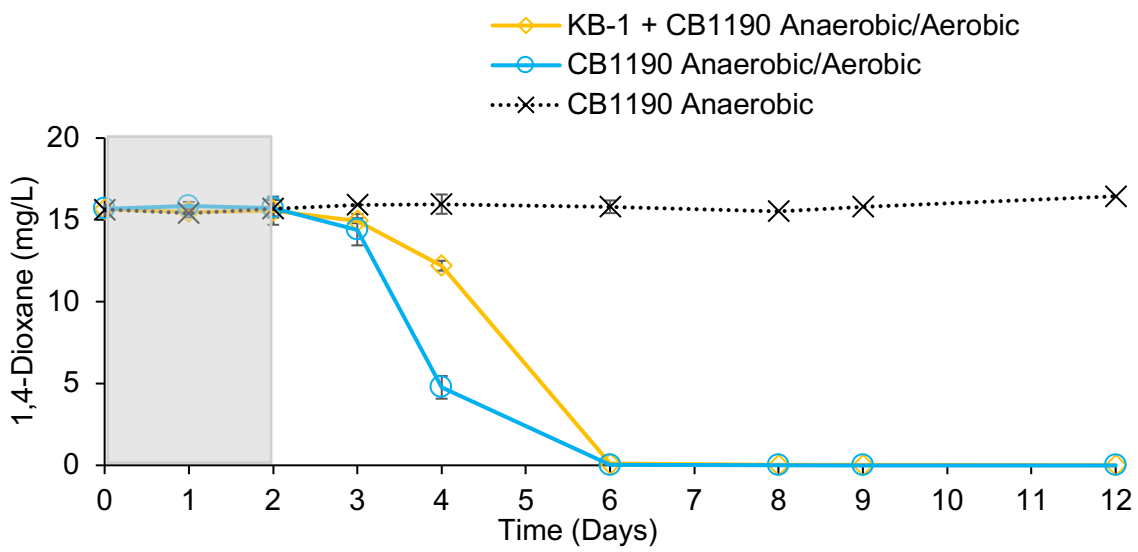
Appendix A: List of Acronyms

aldH Gene coding for Alcohol Dehydrogenase (ALDH)
cDCE *cis*-1,2-Dichloroethene
CB1190 *Pseudonocardia dioxanivorans* CB1190
CVOCs Chlorinated Volatile Organic Compounds
DNA Deoxyribonucleic Acid
DoD Department of Defense
dxmB Gene coding for 1,4-Dioxane Monooxygenase (DXMO)
ESTCP Environmental Security Technology Certification Program
GC-FID Gas Chromatography Flame Ionization Detector
GC-MS Gas Chromatography Mass Spectrometry
KB-1 Kitchener Breslau trichloroethene dechlorinating microbial culture
PAC Powder Activated Carbon
PCR Polymerase Chain Reaction
PI Principal Investigator
qPCR Quantitative Polymerase Chain Reaction
rpoD RNA Polymerase subunit D housekeeping sigma factor
RNA Ribonucleic Acid
SERDP Strategic Environmental Research and Development Program
TCE Trichloroethylene
tceA Gene coding for trichloroethene reductase alpha subunit
UCLA University of California, Los Angeles
VC Vinyl Chloride
vcrA Gene coding for vinyl chloride reductase alpha subunit

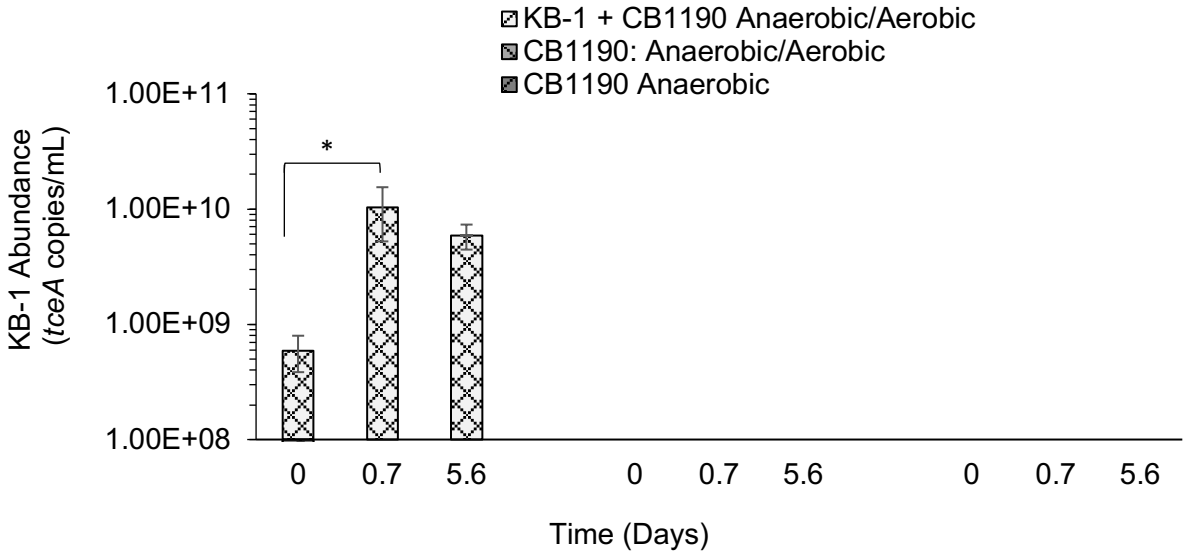
Appendix B: Supplemental Figures



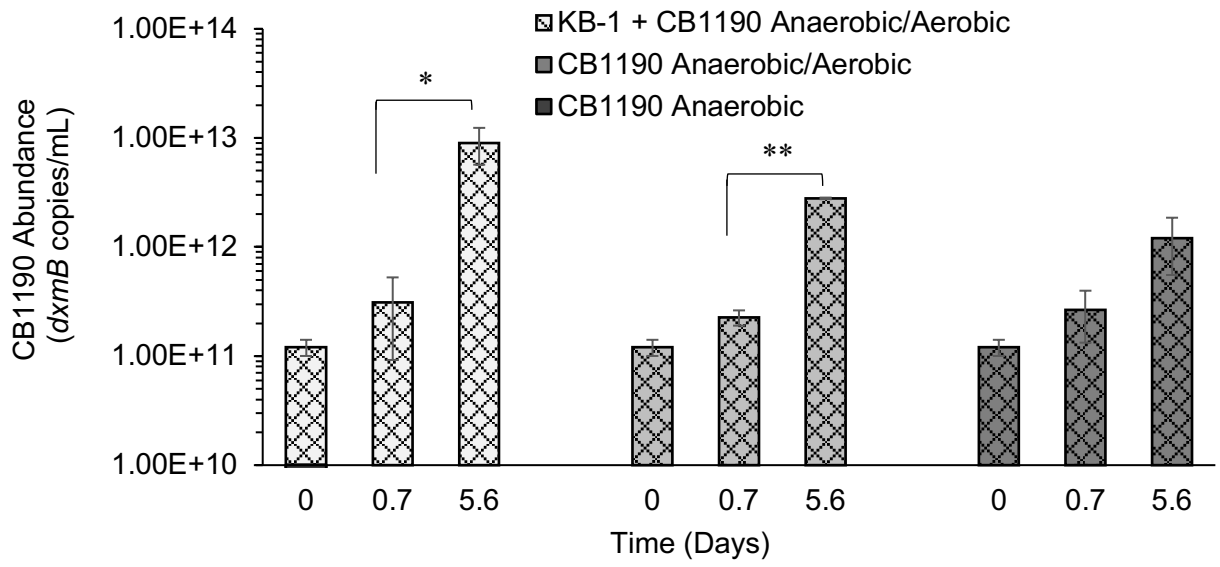
Supplementary Figure 1. Chlorinated ethene biodegradation in pure CB1190 and mixed cultures. The CB1190 anaerobic/aerobic pure culture did not degrade TCE under anaerobic or aerobic conditions, while KB-1® + CB1190 anaerobic/aerobic biodegraded TCE anaerobically and its transformation product, cDCE, aerobically. cDCE was degraded by CB1190 at a rate of 0.078±0.004/day. Error bars indicate the standard deviation of triplicates, and the shaded grey area indicates the anaerobic phase.



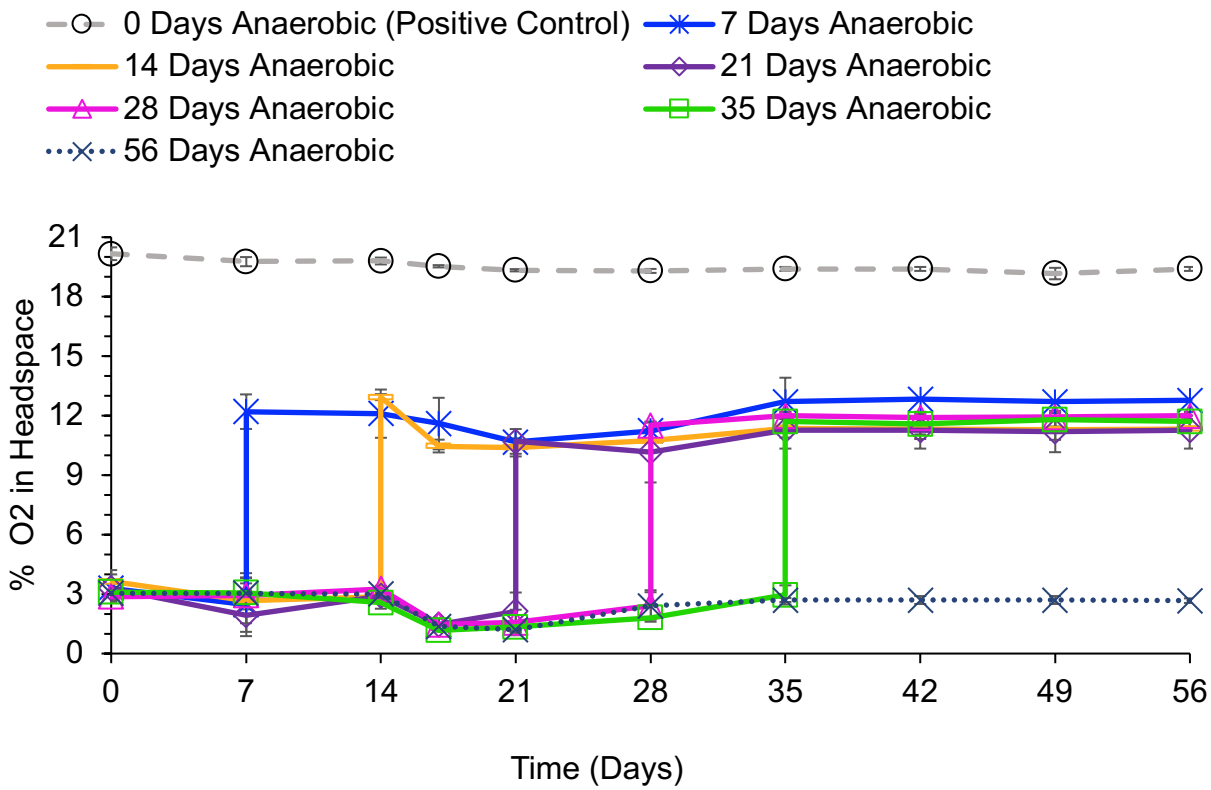
Supplementary Figure 2. 1,4-Dioxane biodegradation in pure CB1190 and mixed cultures. KB-1® + CB1190 anaerobic/aerobic and CB1190 anaerobic/aerobic biodegraded 1,4-dioxane aerobically. CB1190 bottles that were not amended with oxygen did not degrade 1,4-dioxane. KB-1® + CB1190 anaerobic/aerobic degraded 15,000 µg/L of 1,4-dioxane at a rate of 0.249±0.0008/day, and CB1190 only anaerobic/aerobic bottles at a rate of 0.250±0.00002/day. CB1190-mediated 1,4-dioxane degradation rates were calculated using the end of the anaerobic phase and after 1,4-dioxane was degraded to below detection. Error bars indicate the standard deviation of triplicates, and the shaded grey area indicates the anaerobic phase.



Supplementary Figure 3. KB-1® grows during anaerobic phase and does not grow during the aerobic phase. Bottles contained 1,250 µg/L TCE and 3,000 µg/L 1,4-dioxane. The *tceA* gene was used to quantify cell number. KB-1® grew under anaerobic conditions in the KB-1® + CB1190 anaerobic/aerobic bottles and did not grow under aerobic conditions (* $p < 0.05$, ** $p < 0.01$). Error bars indicate the standard deviation of triplicates.



Supplementary Figure 4 CB1190 grows during aerobic phase after anaerobic incubation. Bottles contained 1,250 µg/L TCE and 3,000 µg/L 1,4-dioxane. The *dxmB* gene was used to quantify cell number. CB1190 + KB-1® anaerobic/aerobic and CB1190 anaerobic/aerobic grew after the anaerobic phase and during aerobic conditions, (* $p < 0.05$, ** $p < 0.01$). The CB1190 anaerobic bottles did not grow significantly during the anaerobic phase. Error bars indicate the standard deviation of triplicate samples.



Supplementary Figure 5. Dissolved oxygen concentrations after prolonged anaerobic incubation. CB1190 was able to biodegrade 1,4-dioxane after anaerobic incubation and ~12% oxygen in the headspace. Error bars indicate standard deviation of 6 replicates.