Title
Sequential Anaerobic-Aerobic Biodegradation of Trichloroethylene and 1,4-Dioxane

Author
Polasko, Alexandra LaPat

Publication Date
2017

Peer reviewed|Thesis/dissertation

UNIVERSITY OF CALIFORNIA

Los Angeles

Sequential Anaerobic-Aerobic Biodegradation of Trichloroethylene and 1,4-Dioxane

A thesis submitted in partial satisfaction

of the requirements for the degree Master of Science

in Civil Engineering

by

Alexandra LaPat Polasko

2017

ABSTRACT OF THE THESIS

Sequential Anaerobic-Aerobic Biodegradation of Trichloroethylene and 1,4-Dioxane

by

Alexandra LaPat Polasko

Master of Science in Civil Engineering

University of California, Los Angeles, 2017

Professor Shaily Mahendra, Chair

Chlorinated ethenes, such as trichloroethylene (TCE) and their stabilizers, such as 1,4-dioxane, are widespread groundwater contaminants. Bioremediation can be an effective approach, but opposing redox conditions favored by chlorinated ethane-and 1,4-dioxane-degrading bacteria pose a challenge for their concurrent bioremediation. We engineered a microbial community composed of the anaerobic chlorinated ethene-degrading consortium (KB-1®) and aerobic (*Pseudonocardia dioxanivorans* CB1190 (CB1190)) bacterial strain, which uses 1,4-dioxane. After anaerobic incubation and TCE reduction, CB1190 + KB-1® coculture was viable, and rapidly biodegraded 1,4-dioxane in the presence of oxygen. Aerobic biodegradation of cis-1,2-dichloroethylene (cDCE) by CB1190 was also described. As a plume disperses downgradient, the redox conditions change from anaerobic (source zone) to aerobic (leading edge). The results from this study demonstrate that the engineered microbial community can

survive redox changes and biodegrade chlorinated ethenes and 1,4-dioxane. This approach could reduce the cost, energy, and substrates required for in-situ bioremediation of contaminant mixtures.

The thesis of Alexandra LaPat Polasko is approved.

Jennifer A. Jay

Michael K. Stenstrom

Shaily Mahendra, Committee Chair

University of California, Los Angeles

2017

Table of Contents

List of Acronyms

Chlorinated Volatile Organic Compounds	CVOCs
Trichloroethylene	TCE
cis-Dichloroethene	cis-DCE
Vinyl Chloride	VC
1,4-Dioxane (Dioxane)	Dx
Gas Chromatography-Flame Ionizing Detector	GC-FID
Gas Chromatography-Mass Spectrometer	GC-MS
High Purity Oxygen	HPO
Quantitative Polymerase Chain Reaction	qPCR
Dehalococcoides	Dhc
Pseudonocardia dioxanivorans CB1190	CB1190

List of Figures

List of Tables

Acknowledgements

I am so grateful for all of the support and guidance I have had throughout my Master's at UCLA. First and foremost, I would like to express my gratitude and admiration for my advisor, Dr. Shaily Mahendra. Her patience and direction made it possible to achieve my academic goals. In times of doubt, she was always an uplifting, encouraging, and many times humorous force that enabled me to further my research. I would like to thank my committee members, Professor Jennifer Jay and Professor Michael Stenstrom for providing me the tools to better understand environmental chemistry and biological treatment processes. Also, I would like to acknowledge their advice in times of frustration with the analytical machines (e.g. gas chromatographs). I am grateful for Professor Irwin Suffet and Professor Shane Que Hee, who are in the Environmental Health Science department, for building my background in the fate and transport of organic chemicals and analytical methods in engineering.

During my first year of graduate school, Dr. Phillip Gedalanga, Dr. Shu Zhang, and Yu Miao spent hours teaching me every technique needed to perform excellent research. I am so appreciative of their time and this thesis would not have been possible without their support. Also, I would like to thank the undergraduates that helped make this research possible as well--Sophia (Jiahui) Wang and Alessandro Zulli. They were my co-pilots in the lab as we spent hours running samples on the gas chromatographs, performing DNA extractions, and designing experiments.

Lastly, I would like to thank my parents (Laurie and Ken), brother (Max), and partner (Mike) for providing technical insight (thanks mom!) as well as emotional support through the trials and tribulations of research. I look forward to many more years in this field.

Introduction

1,4-Dioxane and Trichloroethylene Relevance and Co-occurrence in Water

1,4-Dioxane and trichloroethylene (TCE) are two common and often comingled groundwater contaminants (1, 2). 1,4-Dioxane was first used as a solvent for a variety of raw commodities such as, plastics manufacturing, dye production, printing, degreasing, cellulose acetate, varnishes, paints, cosmetics, preservatives, deodorants, glues, and fumigants (3). By the mid-1940s it started to be used as a solvent stabilizer for methyl chloroform (1,1,1-Trichloroethane), as well as antifreeze for military aircrafts and machinery (3). Trichloroethylene was also introduced to the market in the early 1900s as an anesthetic before being replaced in the 1950s by halothane, today's general anesthetic. It was soon repurposed and popularized for oil extraction and metal degreasing (4). Historical failures in handling, disposing and storing industrial waste containing trichloroethylene, trichloroethane, and 1,4-dioxane underground has led to widespread groundwater contamination (1). 1,4-Dioxane sites are rarely found independent of chlorinated solvents, and 64% of all 1,4-dioxane sites are detected with TCE (1), even though 1,4-dioxane was not used as a solvent for TCE.

Microbial Biodegradation of 1,4-Dioxane and Trichloroethylene

Biological remediation has been reported as an effective technology for breaking down these compounds in various laboratory and field studies (5, 6). However, the success of bioremediation many times hinges on the geochemical properties of the sub-surface. The redox environment is one of the largest factors that can limit natural attenuation or enhanced biodegradation (7). Anaerobic biological reduction is a common remediation strategy for chlorinated ethenes. Members of the bacterial genus, *Dehalococcoides*, have been widely reported to degrade chlorinated ethenes via reductive dechlorination. This stepwise process

involves the transfer of an electron from hydrogen to the chlorinated compound and results in the substitution of a chlorine atom with a hydrogen atom (8). Reductive dechlorination occurs most optimally under strict anaerobic conditions. However, a potential challenge to this process is the formation and accumulation of intermediate daughter products, such as cis-1,2-dichloroethene (cDCE) and vinyl chloride (a known human carcinogen) (9). Aerobic biodegradation of chlorinated ethenes can occur cometabolically. The microorganisms biodegrade their primary substrates, such as methane, ethane, or toluene, by producing oxygenase enzymes, which can also transform TCE to a TCE epoxide and then to Cl^-, CO_2, and H^+ (10). Although, aerobic cometabolic remediation may be useful for plumes under oxidizing conditions, it can be burdensome due to the additional amendments of electron donors and electron acceptors required by microbial populations (11, 12).

To date, there are no reliable reports of 1,4-dioxane biodegradation by anaerobic microbes (6). However, aerobic microbes have been shown to effectively biodegrade 1,4-dioxane even in the presence of co-contaminants, such as metals and chlorinated volatile organic compounds (CVOCs) (6, 13-15). Actinomycetes, especially, members of *Pseudonocardia*, are capable of mineralizing 1,4-dioxane via initial monooxygenase-catalyzed hydroxylation, followed by common cellular metabolic pathways (13, 16). Cometabolism of 1,4-dioxane has been reported for microbes utilizing methane, propane, or toluene for their growth. Also, toxic intermediates are not expected to accumulate during these processes (17).

Numerous in situ bioremediation projects have been performed that use biostimulation along with bioaugmentation to promote biodegradation of chlorinated ethenes (18-21). Many of these projects have involved enhancing reductive dechlorination via a carbon source amendment and have focused on promoting highly anaerobic conditions with very low redox conditions

(oxidation reduction potential (ORP) < -200 millivolts). However, few studies have evaluated divergent redox conditions that may be required to biodegrade the CVOCs and 1,4-dioxane as co-contaminants.

Objectives

The purpose of this study was to engineer a mixed microbial community composed of previously identified anaerobic and aerobic contaminant-degrading bacteria to simultaneously or sequentially degrade CVOCs (e.g. TCE) and 1,4-dioxane.

Materials and Methods

Chemicals

1,4-Dioxane (99.8%, ACS grade), TCE (≥99.5%, ACS grade), cDCE (97%), sodium lactate (60% w/w), and titanium trichloride ($TiCl_3$) (≥99.9%), were obtained from Sigma-Aldrich. Saturated chlorinated stock solutions were prepared in accordance with previous methods (22).

Bacterial Cultures and Growth Conditions

The mixed culture KB-1®, containing mostly *Dehalococcoides sp.*, was grown in 160 mL batch reactors in a defined medium with N_2 headspace (23), 1 mg/L TCE as electron acceptor, and 0.9 g L^{-1} lactate as carbon source. The defined media contained: 2.3 g L^{-1} of TES, 2.25 g L^{-1} of $NaCO_3$, 10 mL L^{-1} of 100x salt stock solution (100x stock solution: 100 g L^{-1} NaCl, 30 g L^{-1} NH_4Cl, 30 g L^{-1} KCl, 20 g L^{-1} KH_2PO_4, 50 g L^{-1} $MgCl_2$-$6H_2O$ 1.5 $CaCl_2$-$2H_2O$), 1 mL L^{-1} of the Trace Element Solution A (Trace Element Solution A: 10 g L^{-1} HCl (25% w/w), 0.006 g L^{-1} H_3BO_3, 0.5 NaOH, 0.1 g L^{-1} $MnCl_2$-$4H_2O$, 1.5 g L^{-1} $FeCl_2$-$4H_2O$, 0.19 g L^{-1} $CoCl_2$-$6H_2O$, 0.07 g L^{-1} $ZnCl_2$, 0.002 g L^{-1} $CuCl_2$-$2H_2O$, 0.024 g L^{-1} $NiCl_2$-$6H_2O$, 0.036 g L^{-1} $NaMoO_4$-$6H_2O$), and 1

mL L^{-1} of Trace Element Solution B (Trace Element Solution B: 0.006 g L^{-1} Na_2SeO_4-$5H_2O$ and 0.008 g L^{-1} Na_2WO_4-$5H_2O$). After the above ingredients were added, the medium's pH was adjusted to 7.2-7.3 and then autoclaved for 30 minutes at 121°C. After the media were added to sterile serum bottles, they were sparged for 3 minutes with filtered N_2 gas to achieve anaerobic conditions. Bottles were sealed with 20 mm butyl rubber stoppers. CB1190 stock cultures were prepared using a 1% (v/v) transfer from an actively growing, pure culture that was incubated at 30 °C at 150 rpm to maintain aerobic conditions in the same defined media as KB-1®. Cultures were used to inoculate experimental bottles after stock cultures achieved late exponential or early stationary phase and biodegraded TCE or 1,4-dioxane to below <1.0 mg L^{-1}. $TiCl_3$ was amended to reduce any trace amounts of oxygen in anaerobic cultures.

Batch Reactor Design

Microcosms were prepared in sterile 160 mL or 500 mL glass bottles fitted with butyl rubber stoppers (Chemglass, Vineland, NJ). All reactors were maintained with 1:5 ratio of liquid to headspace. The microcosms were developed with the following conditions: 1) CB1190 + KB-1® that transitioned from anaerobic to aerobic 2) CB1190 only that transitioned from anaerobic to aerobic conditions, and 3) CB1190 only that remained anaerobic. Experimental bottles according to their condition were inoculated with 10% (v/v) CB1190 and 5% (v/v) KB-1®. Once TCE was degraded to cDCE by KB-1®, filtered, high purity oxygen was amended to specific microcosms in sets 1 and 2 as described above, until reactors achieved atmospheric conditions. Microcosms were incubated at 30°C with 150 rpm shaking. At each time point, 200 µL of liquid sample were collected and filtered for 1,4- dioxane quantification. High 1,4-dioxane and TCE concentration (hereafter high concentration) reactors were inoculated with 17 mg L^{-1} 1,4-dioxane

and 5 mg L^{-1} TCE. Low 1,4-dioxane and TCE concentration reactors (hereafter low concentration) were inoculated with 3 mg L^{-1} 1,4-dioxane and 1.3 mg L^{-1} TCE.

Analytical Methods

Chlorinated ethenes were measured in 10 or 100 µL headspace samples using gas chromatography-mass spectrometry (Agilent 6890 GC and 5973 MS system). 1,4-Dioxane concentrations higher than >1 mg/L were measured using a GC equipped with a flame ionization detector using 2 µL directly injected liquid samples, while 1,4-dioxane concentrations <1 mg/L were measured in samples prepared by a frozen microextraction technique using the GC-MS (24). The detection limits for chlorinated ethenes and 1,4-dioxane were 1 µg L^{-1} and 20 µg L^{-1}, respectively. The mass of each compound was calculated based on Henry's law (25) (dimensionless constant at 30 °C for: TCE = 0.523 and cDCE = 0.202) regarding gas-liquid equilibrium constants at 30°C. Dissolved oxygen and pH were measured using a Orion 5-Star Plus electrodes and multiparameter meter (Thermo Scientific, Waltham, MA), and were measured in the anaerobic as well as aerobic incubation conditions. The dissolved oxygen probe was calibrated using air saturated water (high oxygen concentrations) and a 50 g L^{-1} sodium sulfite solution for the set zero (low oxygen concentrations).

Total Nucleic Acids Extraction and cDNA Synthesis

Total nucleic acids were extracted from cell pellets using a phenol-chloroform extraction method (26). For cell density measurements, 500µL liquid samples were collected during incubation, with cells harvested via centrifugation (21,000 x g, 10 min at 4°C) and the supernatant was discarded. Cells were lysed by adding 250 µL of lysis buffer (50 mM sodium acetate, 10 mM EDTA [pH 5.1]), 100 µL 10% sodium dodecyl sulfate, 1.0 mL pH 8.0 buffer-

equilibrated phenol, and 1 g of 100 μm-diameter zirconia−silica beads (Biospec Products, Bartlesville, OK), followed by heating at 65 °C for 2 min, bead beating for 2 min with a Mini-Beadbeater 16 (Biospec Products, Bartlesville, OK), incubating for 8 min at 65 °C, and bead beating again for 2 min. The lysate was collected by centrifugation at 13000 g for 5 min, followed by phenol−chloroform−isoamyl alcohol purification (1 volume) and chloroform−isoamyl alcohol purification (1 volume). Precipitation of total nucleic acids was performed by the addition of 3 M sodium acetate (0.1 volume) and isopropanol 91 volume) followed by incubation at −20 °C overnight. Nucleic acid pellets were collected by centrifugation at 4 °C for 30 min at 20000 g. The precipitate was washed with 70% ethanol and resuspended in 100 μL of DNase- and RNase-free water. The purity of DNA and RNA were determined by a Nanodrop 2000C spectrophotometer (Thermo Scientific, Wilmington, DE). For gene expression analyses, RNA was isolated from total nucleic acid extracts using a RapidOUT DNase Kit (Thermo Scientific, Waltham, MA). The cDNA was synthesized from purified total RNA using a Maxima First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, MA). All samples were stored at −80°C until further amplification and analyses.

Quantitative Polymerase Chain Reaction

The effect of anaerobic incubation on CB1190's gene expression in pure and mixed cultures was determined by amplification of CB1190's 1,4-dioxane biomarker targets (*dxmB* and *aldH*). These biomarker targets were monitored because of their relevance to the 1,4-dioxane biodegradation pathways, such as the monooxygenase and dehydrogenase enzymes as previously described (26). CB1190's cellular growth over time was estimated using the 16S rRNA gene copy numbers. Gene expression was quantified using the $2^{-\Delta\Delta CT}$ method, as described by Livak and Schmittgen (27). 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. 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 expression of CB1190. Primer sequences are listed in Table 1. All reactions were run on a StepOnePlus thermocycler (Life Technologies, Carlsbad, CA) using a total volume of 20 µL containing 1× Luminaris Color HiGreen−HiROX qPCR Master Mix (Thermo Scientific, Waltham, MA), 0.3 mM primers, and 2.5 µL of DNA (1−10 ng/µL) template. The cycling parameters to amplify the gene fragment included sample holds at 50 °C for 2 min and 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. Melt-curve analyses that were within the ranges of 78.1−80.5 °C (*rpoD*) and 81.5−83.6 °C (*dxmB*, *aldH*, and 16S rRNA) were considered specific to each target gene (15, 26, 28, 29).

Live/Dead Viability Assay

Post anaerobic incubation, 20 µL cell culture samples were collected and fixed to a glass slide. Bacteria were stained using the LIVE/DEAD BacLight Bacterial Viability Kit L13152 (Invitrogen, Molecular Probes, Inc. Eugene, OR, USA) for 15 minutes to determine the proportion of live/dead cells. Samples were then rinsed with sterilized deionized water and fixed with a 1 mm glass cover slip. The SYTO 9 and propidium iodide solution was prepared by dissolving the contents of component A or B of the kit in 30 mL of sterile, deionized water. The kit deciphers live/dead cells based on their cell membrane (30). Bacterial cells with intact cell membranes displayed green fluorescence whereas dead or damaged bacterial cells displayed red fluorescence. Samples were imaged using a 4x CCD camera (Axiocam MRm System) attached to a Zeiss Axioskop 2 microscope with a 40x objective, fluorescent lamp, and a blue excitation

filter. During observation, the images were taken at an excitation range of 450-490 nm. Fluorescence images were analyzed using ImageJ software.

Statistical Analysis

All experiments were performed in biological triplicates and presented as the means ± standard deviations. 1,4-Dioxane and CVOC inter and intragroup differences were estimated using a paired two sample for means t-Test. Intragroup biomass differenes were estimated using a one-way paired t-Test. Values were considered statistically significant when the *p* values were < 0.05.

Results

1,4-Dioxane and CVOC Biodegradation Before and After Anaerobic Incubation

In the bottles with transitioning redox conditions, high concentration reactors remained anaerobic for 20 hours and low concentration reactors remained anaerobic for 16 hours. Once TCE was biodegraded to cDCE by KB-1® in the CB1190 + KB-1® reactors, high purity oxygen was added to achieve atmospheric oxygen concentrations. Vinyl chloride was also monitored and remained below 1 μg L^{-1}. Oxygen amendments resulted in cessation of KB-1®'s activity. This was likely due to the oxidative stress on the enzymes that make up their alternative respiratory chain, as well as, the production of partially reduced reactive species such as, superoxide radicals and hydrogen peroxide (31). After the transition to aerobic conditions, CB1190 experienced a lag phase before beginning to biodegrade 1,4-dioxane as well as cDCE. Biodegradation of cDCE by CB1190 was first recorded in the present study.

Table 2 shows the biodegradation of TCE, cDCE, and 1,4-dioxane under various microcosm conditions. The high concentration bottles with KB-1® + CB1190, degraded 17 mg L^{-}

[1] 1,4-dioxane at a rate of 7,199±980 μg L^{-1} hr^{-1} (Figure 1) and cDCE at a rate of 1,737±120 μg L^{-1} hr^{-1} (Figure 2) after oxygen was added. CB1190 only transition bottles degraded 1,4-dioxane at a rate of 2,625±1300 μg L^{-1} hr^{-1} (Figure 1) under aerobic conditions. The low concentration bottles with KB-1® + CB1190 degraded 3 mg L^{-1} 1,4-dioxane at a rate of 619±20 μg L^{-1} day^{-1} (Figure 3) and cDCE at a rate of 263±90 μg L^{-1} day^{-1} (Figure 4), and the CB1190 only transition bottles degrded 1,4-dioxane at a rate of 511±10 μg L^{-1} day^{-1} (Figure 3). The results are consistent with previous reports of biodegradation kinetics of TCE and 1,4-dioxane being first-order with respect to the initial concentrations of these compounds (13, 32). Susrprisingly, the biodegradation rates of 1,4-dioxane by pure cultures of CB1190 were lower than those by KB-1® + CB1190. This suggests the mitigation of CVOC toxicity or availability of additional reducing power to CB1190 cells, even though the initial culture densities were similar for both microcosm conditions. The CB1190 only transition bottles did not show significant TCE biodegradation in both the high and low concentration bottles (Figures 2 & 4).

CB1190 Biomass Growth Before and After Anaerobic Incubation

In the redox-transitioning high concentration bottes, CB1190 + KB-1® total biomass grew at a rate of 7.68±0.7x10^9 cells mL^{-1} day^{-1} (Figure 5), while the CB1190 only bottles grew at a rate of 6.31±0.9x10^9 cells mL^{-1} day^{-1} (Figure 5). In the low concentration bottles, CB1190 + KB-1® bottles grew at a rate of 1.81±0.9x10^9 cells mL^{-1} day^{-1} (Figure 6), and the CB1190 only transition bottles grew at a rate of 9.95±0.39x10^9 cells mL^{-1} day^{-1} (Figure 6). CB1190 anaerobic bottles did not show significant growth (Figures 5 & 6).

Bacterial Viability Assay

Cell culture samples were collected in the low concentration bottles at time 4.8 days. This time point was post anaerobic incubation and 97% of the 1,4-dioxane was biodegraded. CB1190 + KB-1® (Figure 7) had 2.5 times more live cells than the CB1190 anaerobic bottles (p value < 0.05) (Figure 7). The CB1190 only transition bottles (Figure 7) had 3.5 times more live cells than the CB1190 anaerobic bottles (p value < 0.05) (Figure 7). Figure 8 shows the live cell count of these images.

Discussion

The inability of a single microbial culture to biodegrade groundwater pollutants under varying redox conditions can be a major limitation to field applications. Mixed cultures capable of biodegrading co-contaminants that can survive changing redox environments will significantly improve the effectiveness of bioremediation. Previous studies have focused on culturing aerobic microbes from anaerobic environemnts as well as combine aerobic and anaerobic bacteria to enhance the biodegradation of recalcitrant pollutants such as polychlorinated biphenyls, chlorobenzenes, dinitrotoluene, and azo dyes (33-36). These previous studies serve as a foundation for conducting research approaches for removing multiple contaminants with bacteria that favor diverse redox conditions. However, the present study is the first report of simultaneous biodegradation of 1,4-dioxane and TCE by a mixed microbial consortia containing aerobic and anaerobic bacteria. Figure 1 demonstrates that the mixed culture of CB1190 + KB-1® was able to successfully biodegrade 1,4-dioxane within three days after being transitioned from anaerobic to aerobic conditions. Likewise, Figure 2 shows that the mixed culture was able to biodegrade the high concentration of TCE within 20 hours. Whereas, an additional 50 hours were needed to aerobically biodegrade cDCE to non-detect levels by CB1190. However, when the mixed culture

was exposed to lower concentrations of 1,4-dioxane and TCE, the degradation rates for 1,4-dioxane and cDCE were lower as illustrated in Figures 3 and 4 and Table 2.

Recent research that complements this study is a paper by Sekar and coworkers, 2016. They developed a microbially driven Fenton reaction using the facultative anaerobic bacteria, *Shewanella oneidensis*, to drive the production of HO• radicals that can then attack chlorinated solvents and 1,4-dioxane. By toggling anaerobic and aerobic environments, *Shewanella oneidensis* was able to utilize microbial O_2 respiration to produce H_2O_2 and reduce Fe(II). This method lessened the need for continual addition of H_2O_2 and Fe(II) to drive HO• production. *S. oneidensis* cultures were incubated with very high concentrations of 1,4-dioxane (176 mg L^{-1}) and chlorinated solvents (13.1 mg L^{-1}) and produced comparable biodegradation rates to this study (Table 3). This aforementioned approach produced similar contaminant reduction times; however, the mechanisms through which it breaks down the pollutant was vastly different. Sekar et al, (2016) did not utilize bacterial enzymes to directly breakdown the contaminants, but production of excess free radicals. This approach can be difficult for *in situ* remediation treatments. The free radicals produced by *Shewanella oneidensis* are only useful if they come in to direct contact with the co-contaminants; otherwise, they will react with the other sub surface compounds. By contrast, this study relies on the TCE-RDase enzyme in *Dehalococcoides* (37) to break down trichloroethylene by replacing a covalently-bonded chlorine with a hydrogen and the monooxygenase enzyme (17) in CB1190 to break down the carbon-oxygen bond in 1,4-dioxane, leading to subsequent mineralization of both compounds. This study also demonstrated that even when CB1190 was provided with high or low concentrations of TCE and 1,4-dioxane, and had been exposed to anaerobic conditions for an extended period followed by aerobic incubation, it was viable, and demonstrated significant increase in biomass during aerobic incubations. These

results demonstrate the survivability of this microbial consortium and potential to be used in diverse contaminated groundwater sites.

CB1190's apparent dormancy under oxygen limited conditions could be explained by its ability to quickly adapt to stressful conditions and utilizing other substrates for fermentative growth (16). Similar aerobic bacteria, *Mycobacterium tuberculosis* and *Mycobacterium bovis*, were grown in microaerophilic and anaerobic conditions. The cells appeared to produce a thickened cell wall to withstand oxygen deficient conditions *in vivo*. Specifically, the heat shock protein may play a role in stabilizing the cell structure in the long-term by creating a multi-layer, peptidoglycan protective casing (38). CB1190 could be exhibiting similar mechanisms to protect itself and reactivate when sufficient oxygen becomes present. It was unclear how CB1190 would interact with 1,4-dioxane after prolonged anaerobic incubation. Its ability to not only metabolize and grow on 1,4-dioxane, but also upregulate the enzymes responsible for dioxane and its intermediate products was surprising. Reactivation of the *dxmB* and *aldH* (genes coding for enzymes capable of biodegrading 1,4-dioxane and intermediates) in CB1190 were crucial in supporting the hypothesis that CB1190 could withstand oxygen restrictions in the ground.

Comingled contaminants, such as, trichloroethylene and 1,4-dioxane, have proven to be challenging groundwater contaminants that require innovative approaches to successfully remediate. They are both suspect carcinogens and are widespread throughout aquifers. In fact, a multisite survey was conducted in 2014 that characterized contaminated groundwater plumes in Calfornia. The survey indicated that there were 108 sites that contained 1,4-dioxane and of those 108 sites, 103 contained one or more chlorinated solvents, thus confirming the prevelance and importance of developing methods to biodegrade these pollutants simultaneously (39). If bioremediation is a viable option, it becomes difficult for practitioners to adjust redox conditions

to prioritize biodegradation of one contaminant over another and until both contaminants are removed from the water, it remains unsafe for drinking as well as most other uses. This research successfully demonstrated the use of a co-culture containing both anaerobic and aerobic bacteria to survive in the redox changes within a plume over time and space allowing for anaerobic biodegradation of trichloroethylene and then aerobic biodegradation of TCE degradation products as well as 1,4-dioxane. Many of the current technologies for 1,4-dioxane and trichloroethylene groundwater remediation rely on expensive pump-and-treat methods or have to perform multiple well injections. Engineering a mixed microbial consortium that is optimized for both redox conditions can be a cheaper, more effective remediation alternative that could address both co-contaminants.

Summary and Future Work

In this study, the metabolism, growth, and cellular viability was explored with the goal of creating a co-culture to bioremediate trichloroethylene and 1,4-dioxane. Pure CB1190 and CB1190 + KB-1® co-cultures were able withstand anaerobic incubation to then biodegrade higher (17 mg L^{-1}) and lower (3 mg L^{-1}) concentrations of 1,4-dioxane. CB1190 was also able to withstand dissolved oxygen limitations, which is beneficial for practitioners to know when evaluating the potential for bioaugmentation into aquifers with low dissolved oxygen. This culture would be optimized for various sites contaminated with a range of concentrations of 1,4-dioxane, trichloroethylene and the common geophysical properties that occur as plume spreads down gradient. It would also allow for a single injection into the well, as opposed to, bioaugmenting multiple times in different places to achieve contaminant removal across the entire plume.

Future work must include testing the length of time in which CB1190 can remain in anaerobic conditions before receiving sufficient dissolved oxygen concentrations to biodegrade 1,4-dioxane. The longer the anaerobic incubation period CB1190 can withstand, the more applicable this method will be at field sites where it may take weeks or months to reach a sufficiently oxygenated section of the contaminant plume. Also, analyses of biomarkers for confirming activation of 1,4-dioxane-degrading enzymes in CB1190-like bacteria will provide insights into how changing redox conditions can control the onset and activity of cDCE and 1,4-dioxane-degrading enzymes.

List of Figures

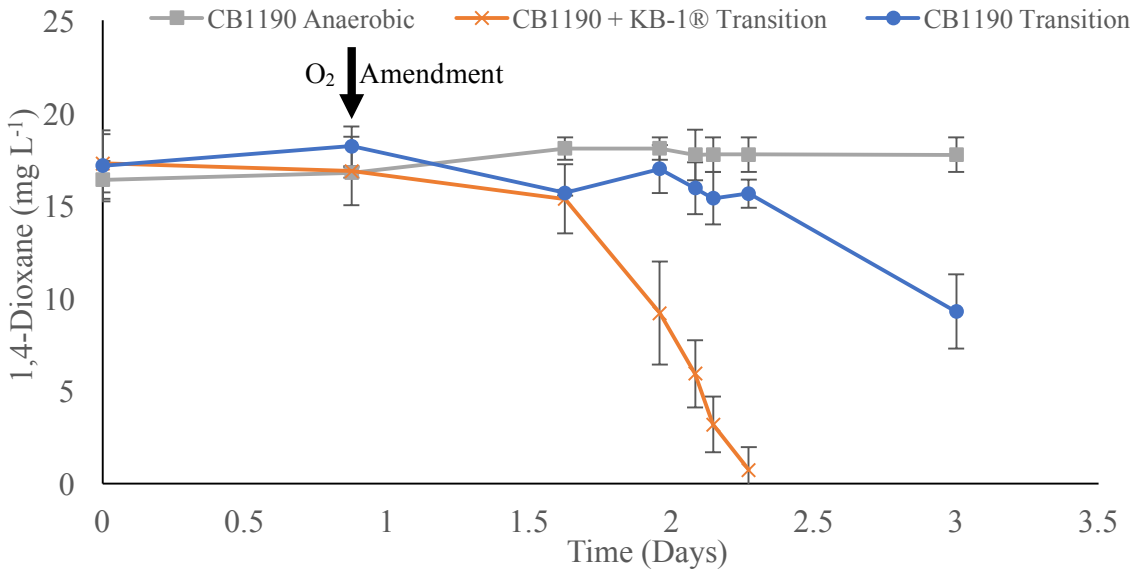

Figure 1. Biodegradation of high concentrations of 1,4-dioxane by CB1190 in pure and mixed cultures. CB1190 + KB-1® degraded 1,4-dioxane at a faster rate than CB1190 Transition in pure culture. No 1,4-dioxane degradation occurred under anaerobic conditions.

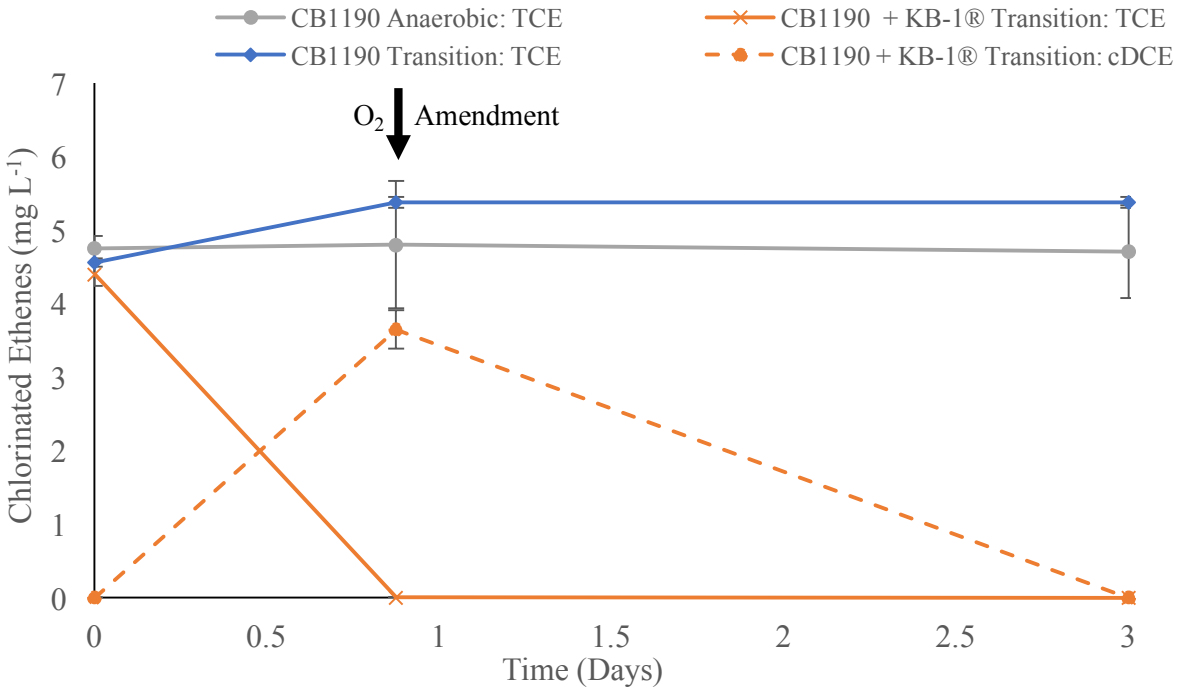

Figure 2. Biodegradation of high concentrations of TCE by KB-1® and cDCE biodegradation by CB1190 in pure and mixed cultures. Pure culture of CB1190 did not degrade TCE under anaerobic or aerobic conditions, while CB1190 + KB-1® biodegraded TCE anaerobically and its product, cDCE, aerobically.

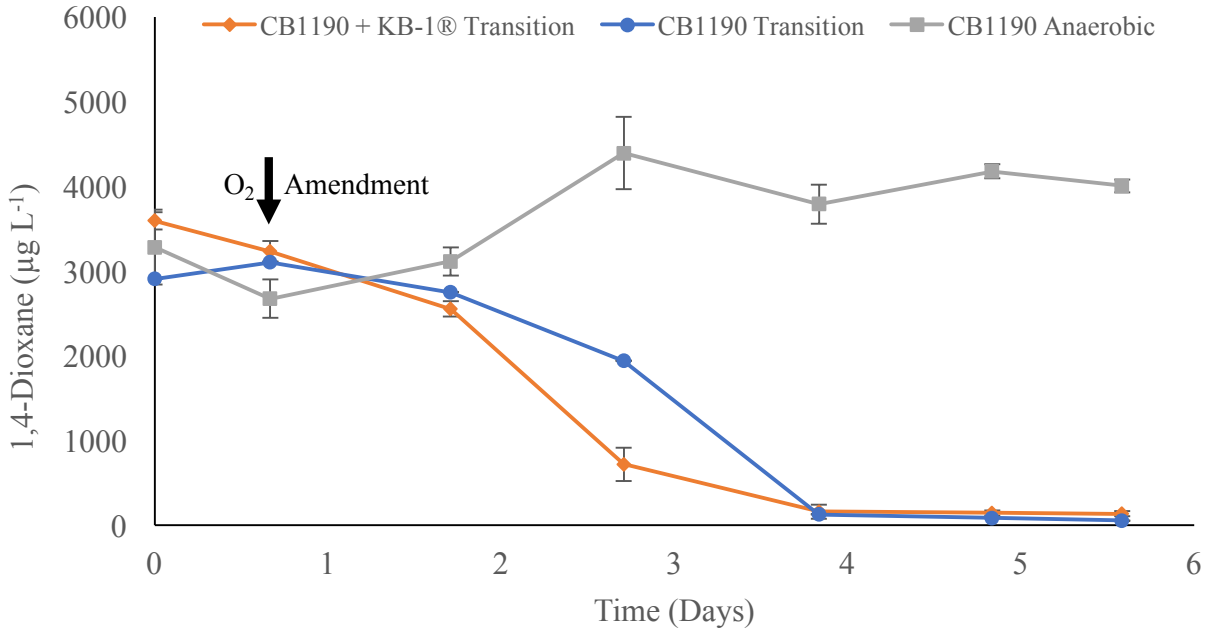

Figure 3. Biodegradation of low concentrations of 1,4-dioxane by CB1190 in pure and mixed cultures Both CB1190 + KB-1® and CB1190 pure culture biodegraded 1,4-dioxane at similar rates after anaerobic incubation. No 1,4-dioxane degradation occurred under anaerobic conditions.

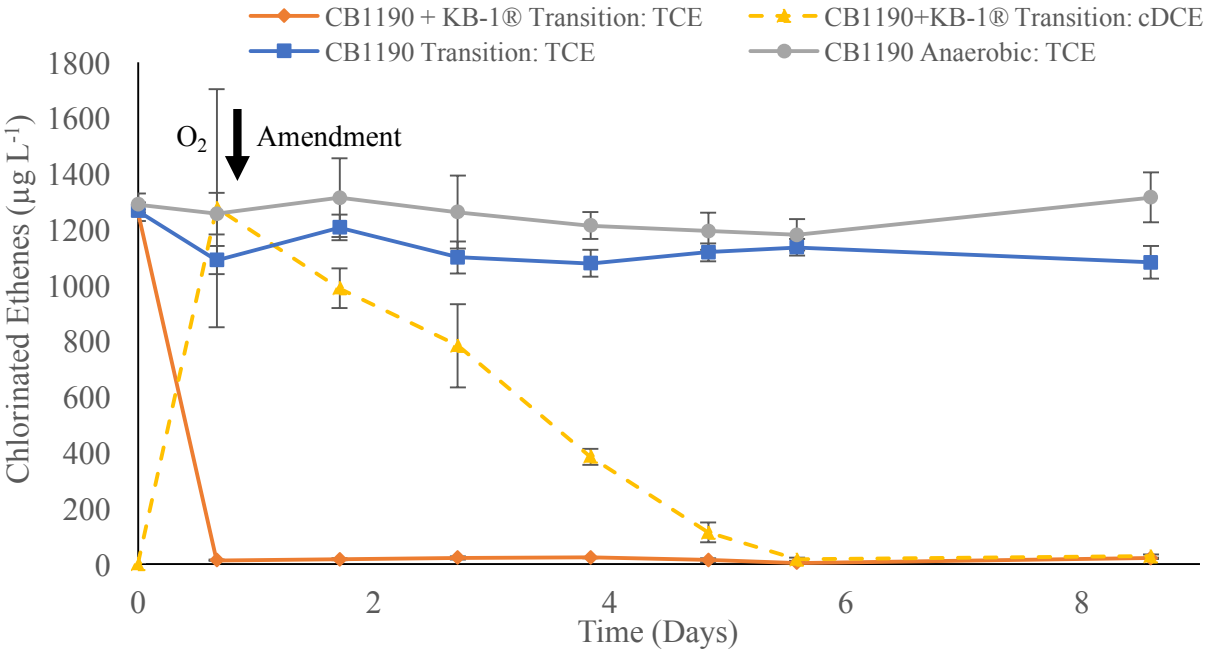

Figure 4. Biodegradation of low concentrations of TCE by KB-1® and cDCE biodegradation by CB1190 in pure and mixed cultures. Pure cultures of CB1190 did not degrade TCE under anaerobic or aerobic conditions, while CB1190 + KB-1® biodegraded TCE anaerobically and its product, cDCE, aerobically. cDCE was biodegraded at a lower rate for low (1.3 mg L^{-1}) initial concentrations of TCE.

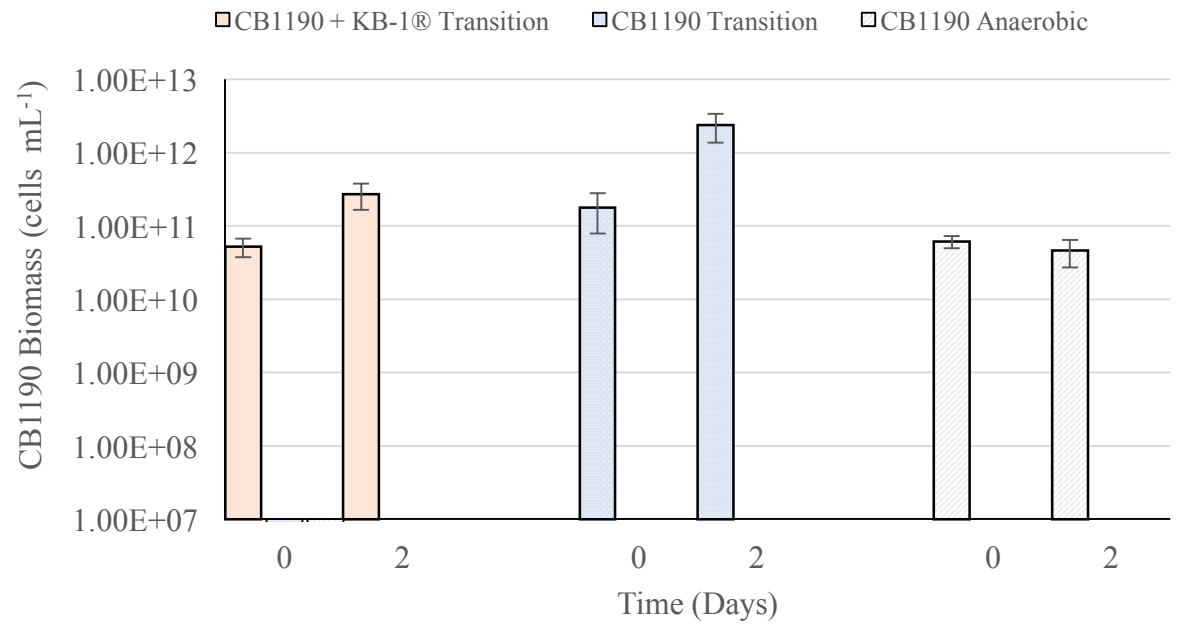

Figure 5. CB1190's growth before and after oxygen amendments in high TCE and 1,4-dioxane concentration bottles. The *dxmB* gene was used to quantify cell number. CB1190 pure culture did not grow under anaerobic conditions, but CB1190 + KB-1® and CB1190 Transition grew significantly compared to CB1190 Anaerobic (p value < 0.05).

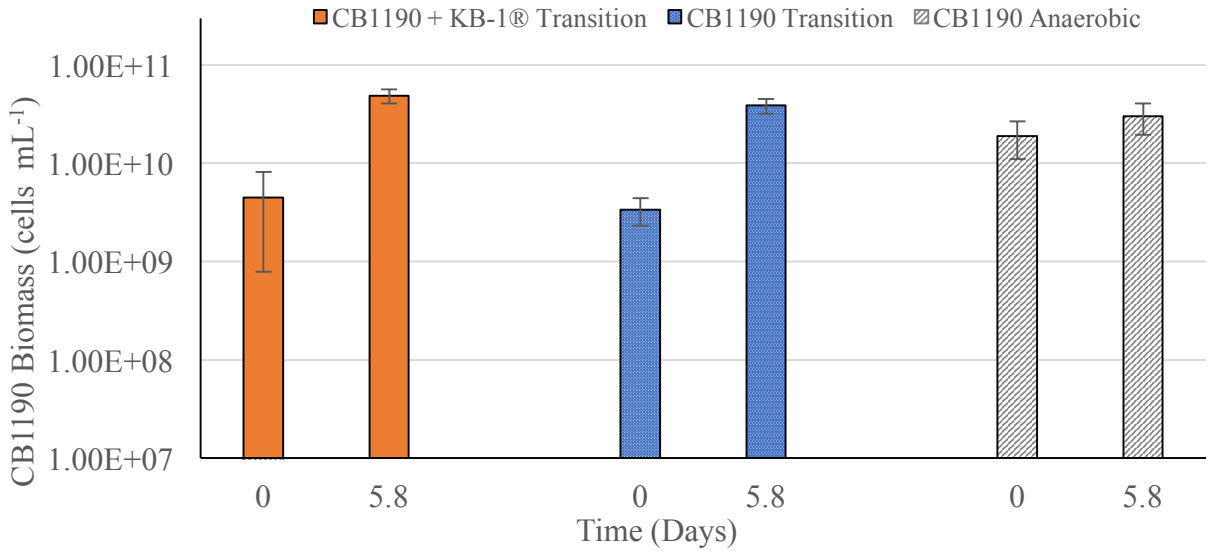

Figure 6. CB1190's growth before and after oxygen amendments in low TCE and 1,4-dioxane concentration bottles. The *dxmB* gene was used to quantify cell number. CB1190 + KB-1® and CB1190 Transition grew nearly an order of magnitude as compared to CB1190 Anaerobic (p value < 0.05).

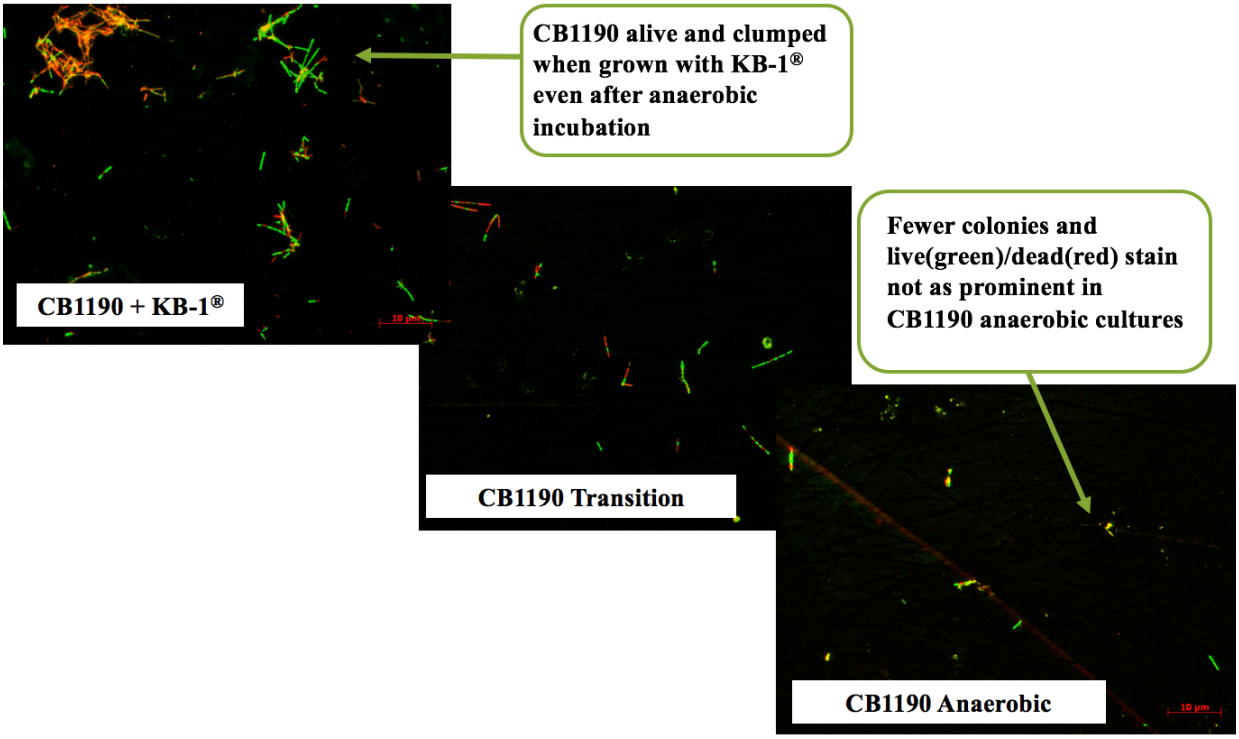

Figure 7. Live/Dead microscopy image of low concentration bottles. From left to right: CB1190 + KB-1®, CB1190 Transition, and CB1190 anaerobic. CB1190 + KB-1® showed more live cells in clusters than CB1190 Transition and CB1190 Anaerobic bottles.

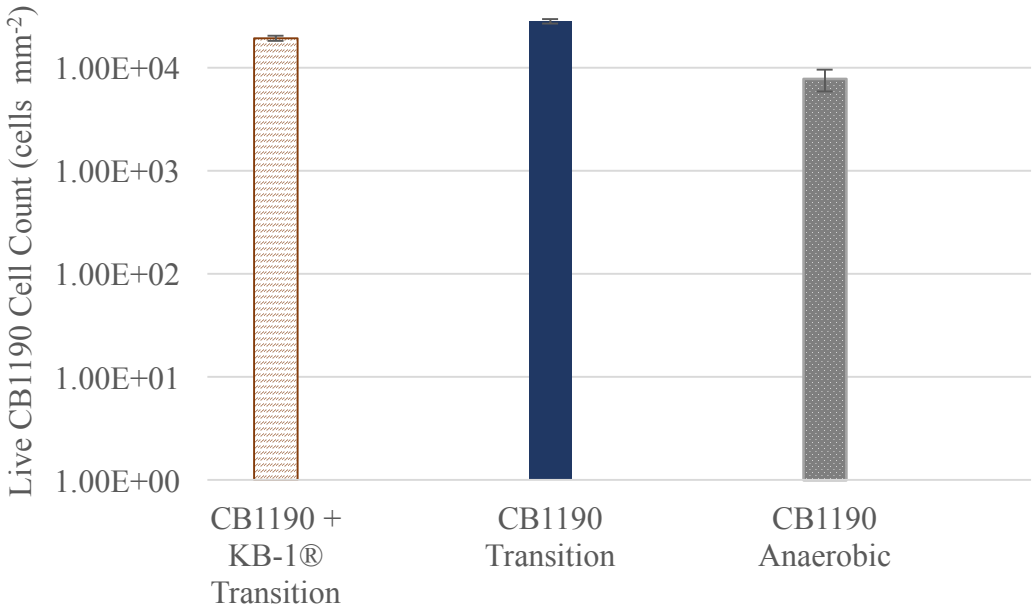

Figure 8. Live cell count of CB1190 in low TCE and 1,4-dioxane concentration bottles after anaerobic phase. CB1190 + KB-1 and CB1190 Transition had significantly more lives cells than CB1190 Anaerobic (p value < 0.05).

List of Tables

Table 1. Primer sequences used for amplification of genes by qPCR

Primers	Sequence	Reference
CB1190 16S rRNA for CB1190 16S rRNA rev	5'-ACGGTCTCGCAGCCCTCTGT -3' 5'-AGCGGGTTATGCCGGGGACT-3'	Zhang et al, 2016
tceA for *tceA* rev	5'-TAATATATGCCGCCACGAATGG-3' 5'-AATCGTATACCAAGGCCCGAGG-3'	Waller et al, 2005
rpoD for *rpoD* rev	5'-GGGCGAAGAAGGAAATGGTC-3' 5'-TCATTAACGGCAGCAAACGC-3'	Grostern et al, 2012
dxmB for *dxmB* rev	5'-CCAAACGGGCGTCAGTCAT-3' 5'-AGAACGTGCGCTCCCAAAG-3'	Gedalanga et al, 2014
aldH for *aldH* rev	5'-GCCGACGCTTTTAGCAGATG-3' 5'-TCATTAACGGCAGCAAACGC-3'	Gedalanga et al, 2014

Table 2. Biodegradation rates of 1,4-dioxane and cDCE in mixed and pure cultures containing CB1190

Experimental Condition	1,4-Dioxane Concentration (mg L^{-1})	1,4-Dioxane Biodegradation Rate (µg L^{-1} day^{-1})	TCE Concentration (mg L^{-1})	cDCE Biodegradation Rate (µg L^{-1} day^{-1})
CB1190 + KB-1®	17	7,199±980	5	cDCE: 1,737±120
CB1190 only	17	2,625±1300	5	N/A
CB1190 + KB-1®	3	619±20	1.3	cDCE: 263±90
CB1190 only	3	511±10	1.3	N/A

Table 3. Biodegradation rates of 1,4-dioxane by CB1190 and *Shewanella oneidensis* post anaerobic incubation

Reference	1,4-Dioxane Concentration (mg L^{-1})	TCE Concentration (mg L^{-1})	1,4-Dioxane Biodegradation Rate (µg L^{-1} day^{-1})	
This Study[1,2]	17	5	CB1190 + KB-1® CB1190 only	7,199±980 2,625±1300
This Study[1,2]	3	1.3	CB1190 + KB-1® CB1190 only	619±20 511±10
*Sekar et al, 2016	176	13		23,000±8,800 (1) 47,000±7,000 (2) 58,000±14,000 (3)
*Sekar et al, 2016	176	N/A		65,000±17,000 (1) 77,000±8,700 (2) 60,000±7,100 (3)

*() indicate the Fenton reaction cycle number. Biodegradation rates increased with increasing cycles.
[1]Values rounded to two significant figures
[2]The TCE concentration in CB1190 + KB-1 bottles represent the initial concentration. Over time, TCE was converted to cDCE.

References

1. Anderson RH, Anderson JK, Bower PA. Co-occurrence of 1,4-dioxane with trichloroethylene in chlorinated solvent groundwater plumes at US Air Force installations: fact or fiction. Integrated Environmental Assessment and Management. 2012;8(4):731-7.

2. Adamson DT, Anderson RH, Mahendra S, Newell CJ. Evidence of 1,4-dioxane attenuation at groundwater sites contaminated with chlorinated solvents and 1,4-dioxane. Environmental Science & Technology. 2015;49(11):6510-8.

3. Mohr TKG. Environmental investigation and remediation: 1,4-dioxane and other solvent stabilizers: CRC Press; 2010. 1-503 p.

4. USEPA. Trichloroethylene technical fact sheet. 2016.

5. McCarty PL. In situ bioremediation of chlorinated solvents. Current Opinion in Biotechnology. 1993;4(3):323-30.

6. Zhang S, Gedalanga PB, Mahendra S. Advances in bioremediation of 1,4-dioxane-contaminated waters. Journal of Environmental Management. 2017;204(Part 2):765-74.

7. Christensen TH, Bjerg PL, Banwart SA, Jakobsen R, Heron G, Albrechtsen H-J. Characterization of redox conditions in groundwater contaminant plumes. Journal of Contaminant Hydrology. 2000;45(3):165-241.

8. Janssen DB, Oppentocht JE, Poelarends GJ. Microbial dehalogenation. Current Opinion in Biotechnology. 2001;12(3):254-8.

9. Vogel TM, McCarty PL. Biotransformation of tetrachloroethylene to trichloroethylene, dichloroethylene, vinyl chloride, and carbon dioxide under methanogenic conditions. Applied and Environmental Microbiology. 1985;49(5):1080-3.

10. Shukla AK, Upadhyay SN, Dubey SK. Current trends in trichloroethylene biodegradation: a review. Critical Reviews in Biotechnology. 2014;34(2):101-14.

11. Suttinun O, Luepromchai, E. & Müller, R. Cometabolism of trichloroethylene: concepts, limitations and available strategies for sustained biodegradation. Environmental Science and Technology. 2013;12(1):99-114.

12. Frascari D, Zanaroli G, Danko AS. In situ aerobic cometabolism of chlorinated solvents: A review. Journal of Hazardous Materials. 2015;283(Supplement C):382-99.

13. Mahendra S, Alvarez-Cohen L. Kinetics of 1,4-dioxane biodegradation by monooxygenase-expressing bacteria. Environmental Science & Technology. 2006;40(17):5435-42.

14. Pornwongthong P, Mulchandani A, Gedalanga PB, Mahendra S. Transition metals and organic ligands influence biodegradation of 1,4-dioxane. Applied Biochemistry and Biotechnology. 2014;173(1):291-306.

15. Zhang S, Gedalanga PB, Mahendra S. Biodegradation kinetics of 1,4-dioxane in chlorinated solvent mixtures. Environmental Science & Technology. 2016;50(17):9599-607.

16. Mahendra S, Alvarez-Cohen L. *Pseudonocardia dioxanivorans* sp nov., a novel actinomycete that grows on 1,4-dioxane. International Journal of Systematic and Evolutionary Microbiology. 2005;55:593-8.

17. Mahendra S, Petzold CJ, Baidoo EE, Keasling JD, Alvarez-Cohen L. Identification of the intermediates of in vivo oxidation of 1, 4-dioxane by monooxygenase-containing bacteria. Environmental Science & Technology. 2007;41(21):7330-6.

18. Ellis DE, Lutz EJ, Odom JM, Buchanan RJ, Bartlett CL, Lee MD, et al. Bioaugmentation for accelerated in situ anaerobic bioremediation. Environmental Science & Technology. 2000;34(11):2254-60.

19. Major DW, McMaster ML, Cox EE, Edwards EA, Dworatzek SM, Hendrickson ER, et al. Field demonstration of successful bioaugmentation to achieve dechlorination of tetrachloroethene to ethene. Environmental Science & Technology. 2002;36(23):5106-16.

20. Lendvay JM, Loffler FE, Dollhopf M, Aiello MR, Daniels G, Fathepure BZ, et al. Bioreactive barriers: a comparison of bioaugmentation and biostimulation for chlorinated solvent remediation. Environmental Science & Technology. 2003;37(7):1422-31.

21. Hood ED, Major DW, Quinn JW, Yoon WS, Gavaskar A, Edwards EA. Demonstration of enhanced bioremediation in a TCE source area at Launch Complex 34, Cape Canaveral Air Force Station. Ground Water Monitoring & Remediation. 2008;28(2):98-107.

22. Alvarez-Cohen L, McCarty P. Effects of toxicity, aeration, and reductant supply on trichloroethylene transformation by a mixed methanotrophic culture. Applied and Environmental Microbiology. 1991;57(1):228-35.

23. He J, Holmes VF, Lee PKH, Alvarez-Cohen L. Influence of vitamin B12 and cocultures on the growth of dehalococcoides isolates in defined medium. Applied and Environmental Microbiology. 2007;73(9):2847-53.

24. Li M, Conlon P, Fiorenza S, Vitale RJ, Alvarez PJJ. Rapid analysis of 1,4-dioxane in groundwater by frozen micro-extraction with gas chromatography/mass spectrometry. Ground Water Monitoring & Remediation. 2011;31(4):70-6.

25. Wilson JL, Conrad, S. H., Mason, W. R., Peplinski, W., and Hagan E. Laboratory Investigation of Residual Liquid Organics. In: Agency EP, editor. 1990.

26. Gedalanga PB, Pornwongthong P, Mora R, Chiang S-YD, Baldwin B, Ogles D, et al. Identification of biomarker genes to predict biodegradation of 1, 4-dioxane. Applied and Environmental Microbiology. 2014;80(10):3209-18.

27. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 2001;25(4):402-8.

28. Waller AS, Krajmalnik-Brown R, Loffler FE, Edwards EA. Multiple reductive-dehalogenase-homologous genes are simultaneously transcribed during dechlorination by Dehalococcoides-containing cultures. Applied and Environmental Microbiology. 2005;71(12):8257-64.

29. Grostern A, Sales CM, Zhuang W-Q, Erbilgin O, Alvarez-Cohen L. 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(9):3298-308.

30. Stiefel P, Schmidt-Emrich S, Maniura-Weber K, Ren Q. Critical aspects of using bacterial cell viability assays with the fluorophores SYTO9 and propidium iodide. BMC Microbiology. 2015;15:36.

31. Rocha ER, Smith CJ. Role of the alkyl hydroperoxide reductase (ahpCF) gene in oxidative stress defense of the obligate anaerobe Bacteroides fragilis. Journal of Bacteriology. 1999;181(18):5701-10.

32. Alvarez-Cohen L, Speitel G, Jr. Kinetics of aerobic cometabolism of chlorinated solvents. Biodegradation. 2001;12(2):105-26.

33. Fathepure BZ, Vogel, T. M. Complete degradation of polychlorinated hydrocarbons by a two-stage biofilm reactor. Applied and Environmental Microbiology. 1991;57:3418-22.

34. Beunink J, Rehm H-J. Synchronous anaerobic and aerobic degradation of DDT by an immobilized mixed culture system. Applied Microbiology and Biotechnology. 1988;29(1):72-80.

35. Brown D, Hamburger B. The degradation of dyestuffs: Part III - Investigations of their ultimate degradability. Chemosphere. 1987;16(7):1539-53.

36. Anid PJ, Ravest-Webster BP, Vogel TM. Effect of hydrogen peroxide on the biodegradation of PCBs in anaerobically dechlorinated river sediments. Biodegradation. 1993;4(4):241-8.

37. Magnuson JK, Stern RV, Gossett JM, Zinder SH, Burris DR. Reductive dechlorination of tetrachloroethene to ethene by a two-component enzyme pathway. Applied and Environmental Microbiology. 1998;64(4):1270-5.

38. Cunningham AF, Spreadbury CL. Mycobacterial stationary phase induced by low oxygen tension: cell wall thickening and localization of the 16-kilodalton α-crystallin homolog. Journal of Bacteriology. 1998;180(4):801-8.

39. Adamson DT, Mahendra S, Walker KL, Rauch SR, Sengupta S, Newell CJ. A multisite survey to identify the scale of the 1,4-dioxane problem at contaminated groundwater sites. Environmental Science & Technology Letters. 2014;1(5):254-8.