

FINAL REPORT

Development of Slow Release Compounds for the Aerobic
Cometabolic Treatment of Complex Mixtures of COC Released
from Low Permeability Zones

SERDP Project ER-2716

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14. ABSTRACT A novel process was developed for the passive aerobic cometabolic treatment of contaminant mixtures in groundwater. <i>Rhodococcus rhodochrous</i> ATCC 21198 was utilized which can concurrently oxidize 1,4-dioxane (1,4-D) and diverse mixtures of chlorinated aliphatic hydrocarbons (CAHs) after growth on 1-butanol and 2-butanol. Methods were developed to co-encapsulate orthosilicates as slow-release compounds (SRCs) that hydrolyze to produce these alcohols and ATCC 21198 in gellan-gum hydrogel beads. ATCC 21198 grows within the hydrogel beads and upon diffusion into the beads the contaminants are transformed. In batch reactors containing the gellan-gum beads successive additions of a mixture of 1,1,1-trichloroethane (1,1,1-TCA), <i>cis</i> -dichloroethene (<i>cis</i> -DCE), and 1,4-D were transformed with rates of transformation correlated with the rates of alcohol release, oxygen consumption, and CO ₂ production. In continuous flow columns packed with the gellan-gum beads over 99% removal of a mixture of 1,1,1-TCA, <i>cis</i> -DCE, and 1,4-D was achieved with a hydraulic residence time of approximately 12 hours. The columns performance was negatively affected when <i>cis</i> -DCE was replaced by 1,1-dichloroethene (1,1-DCE), due to 1,1-DCE transformation product toxicity. A 3-D physical aquifer model using a funnel-and-gate treatment system containing co-encapsulated gellan-gum beads was used to demonstrate the concept of a passive cometabolic permeable reactive barrier (CPRB).				
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Abstract

The overall aim of ER-2716 was to develop a passive system of long-term cometabolic transformation of mixtures of Contaminants of Concern (COCs), by co-encapsulating a pure bacteria culture and slow releases compounds (SRCs) within hydrogel beads. The SRCs would slowly release alcohols upon abiotic hydrolysis with water, which serve as substrates for growth and maintenance of the bacteria culture while also promoting aerobic cometabolism. The hydrogel bead would create a protective environment where the pure culture can grow as the SRC hydrolyses within the bead and the COCs diffuse into the bead to be cometabolically transformed. The five specific aims of the project were met including: 1) determining the pure bacteria culture to be co-encapsulated that has the ability to cometabolize a broad range of COCs; 2) demonstrating that alcohols and the SRCs that produce them, would promote long-term cometabolic transformations with the selected bacteria culture; 3) developing an effective method for co-encapsulation of the bacteria culture and the SRC within the hydrogel beads; 4) evaluating the co-encapsulated hydrogel beads in batch kinetic tests and groundwater microcosms; and 5) evaluating the hydrogel beads in continuous flow 1-dimensional (1-D) packed columns and finally as a proof of concept experiment in a large scale physical aquifer model (PAM) where the beads were used to construct a cometabolic permeable reactive barrier (CPRB).

The bacterium *Rhodococcus rhodochrous* strain ATCC 21198 (*R. rhodochrous* ATCC 21198) was selected for use in this study, since it was very effective in transforming a broad range of COCs, including 1,4-dioxane (1,4-D), 1,1,1-trichloroethane (1,1,1-TCA), 1,1-dichloroethane (1,1-DCA), vinyl chloride (VC), *cis*-dichloroethene (*cis*-DCE), and 1,1-dichloroethene (1,1-DCE). Trichloroethene (TCE) however, was not effectively transformed. When grown on 1-butanol, 2-butanol, and 2-ethyl-1-butanol, *R. rhodochrous* ATCC 21198 was capable of cometabolic transforming the same range of COCs, but at slower rates than when grown on isobutane. *R. rhodochrous* ATCC 21198 grown on 2-butanol had the largest transformation capacity and fastest degradation rates and was able to transform a mixture of 1,4-D, *cis*-DCE, and 1,1,1-TCA to greater than 99%.

Three orthosilicates were tested as SRCs for *R. rhodochrous* ATCC 21198: tetrabutylorthosilicate (TBOS), tetra-sec-butylorthosilicate (T2BOS), and tetraisopropoxysilane (T2POS). The rates of hydrolysis in poisoned controls depended on the orthosilicate structure with TBOS, which produces 1-butanol, hydrolyzing more rapidly than T2POS and T2BOS that produce 2-butanol and 2-propanol, respectively. In batch tests with the orthosilicates, continuous rates of oxygen (O₂) consumption and carbon dioxide (CO₂) production confirmed alcohol metabolism by *R. rhodochrous* ATCC 21198 was occurring. The rates of metabolism, as indicated by O₂ consumption and CO₂ production in batch incubations (TBOS > T2POS > T2BOS) were consistent with the rates of alcohol release via abiotic hydrolysis. Both 1,4-D and 1,1,1-TCA were continuously transformed in their successive additions over 125 days, with the rates correlated with the rates of metabolism. Only a single addition of the orthosilicate was required. Alcohol concentrations in the biologically active reactors remained below the levels of detection, indicating they were metabolized rapidly after being produced. Much lower rates of O₂ consumption occurred in reactors containing T2BOS, which has benefits for in-situ bioremediation.

A process was developed to co-encapsulate *R. rhodochrous* ATCC 21198 in gellan-gum (GG) hydrogel macrobeads with orthosilicates as SRCs. Encapsulation with GG was found to be more effective than with alginate. TBOS and T2BOS, were encapsulated in GG hydrogels at mass loadings as high as 10% (w/w), along with *R. rhodochrous* ATCC 21198. In the GG beads, TBOS

hydrolyzed 27 times faster than T2BOS and rates were ~4 times higher in suspension than when encapsulated. Batch kinetic tests supported the aerobic cometabolism of a mixture of 1,1,1-TCA, *cis*-DCE, and 1,4-D at aqueous concentrations ranging from 250 to 1000 µg/L. In biologically active batch reactors, the co-encapsulated *R. rhodochrous* ATCC 21198 effectively utilized the SRC hydrolysis products (1- and 2-butanol) and cometabolized repeated additions of the mixture of 1,1,1-TCA, *cis*-DCE, and 1,4-D for over 300 days. The transformation followed pseudo-first-order kinetics. VC and 1,1-DCE were also effectively transformed. Appropriately scaled first-order rates indicate very high extents of contaminant transformation could be achieved with a residence time of a few days if co-encapsulated beads were densely packed in a column or CPRB. Studies in groundwater/sediment microcosms also demonstrated that long-term cometabolic treatment can be achieved under conditions that mimic the subsurface. Based on the lower rate of oxygen consumption while achieving similar long-term rates, T2BOS was found to be a more effective substrate than TBOS.

The continuous aerobic cometabolic treatment of a mixture of 1,1,1-TCA, *cis*-DCE and 1,4-D, each at a concentration of 250 µg/L, was studied in 1-D laboratory columns packed with the GG hydrogel beads co-encapsulated with *R. rhodochrous* ATCC 21198 and 8% (w/w) TBOS or T2BOS. Hydraulic residence times (HRT) in the columns ranged from approximately 2 days to 12 hours. In two columns containing GG beads with TBOS, over 99.5% removal of *cis*-DCE, 1,1,1-TCA, and 1,4-D was achieved within the first 25 days operation at an HRT of approximately 2 days. The development of aerobic conditions throughout the columns, through hydrogen peroxide (H₂O₂) addition (50 to 100 mg/L), resulted in biostimulation of *R. rhodochrous* ATCC 21198 within the beads throughout the columns, permitting a decrease in the HRT to 12 hours, while maintaining a contaminant removal efficiency of over 99.5%. With prolonged treatment only dissolved O₂ (DO) addition (15 mg/L) was required to maintain effective treatment. After ~ 240 pore volumes of flow (PV), *cis*-DCE was replaced by 1,1-DCE (100 µg/L), which imparts a high degree of transformation toxicity. 1,1-DCE was effectively transformed with over 98% removal achieved, while 1,4-D and 1,1,1-TCA continued to be effectively transformed. After ~ 300 PV, the influent concentration of 1,1-DCE was increased to 250 µg/L. Effective transformation of all three contaminants was achieved until ~450 PV, after which all three contaminants began to increase to the influent concentrations.

The third column was packed with GG beads with T2BOS. The column was fed groundwater that contained 14 mg/L DO and a mixture of *cis*-DCE, 1,1,1-TCA and 1,4-D, each at 250 µg/L. Due to the slower rate of hydrolysis of T2BOS, H₂O₂ addition was not required. Over 99% removal of 1,1,1-TCA, *cis*-DCE and 1,4-D was achieved with an HRT of 12 hours. After 90 PV of flow, when the contaminant concentrations were increased from 250 µg/L to 500 µg/L, the effluent concentrations of *cis*-DCE and 1,4-D began to immediately increase and reached the influent concentrations after 120 PV of flow, while 1,1,1-TCA only reached about 25% of the influent concentration. Upon reducing the influent concentrations to 250 µg/L, the cometabolic transformation was restored, with over 98% removal of the contaminants observed. *Cis*-DCE was then replaced by 1,1-DCE (100 µg/L), while continuing to add 1,4-D and 1,1,1-TCA. A rapid increase in all three contaminants in the column effluent was observed. The results suggest that 1,1-DCE transformation toxicity caused the cessation in the cometabolic activity in the column. At the end of the column studies the beads were sampled and assayed for the amount of TBOS and T2BOS remaining. Approximately 56% and 98.5% of the TBOS and T2BOS originally encapsulated remained, respectively. The estimated rate of hydrolysis of T2BOS in the columns

was factor of 20 lower than TBOS, which was consistent with the batch incubations. The results indicate that TBOS and T2BOS limitations were not responsible for the cessation in transformation activity in the columns, and it was likely caused from the cometabolism of 1,1-DCE.

Experiments were initiated in a 100-L physical aquifer model (PAM) packed with sand (20 to 30 mesh) to evaluate the creation of a Funnel-and-Gate CPRB. The Funnel-and-Gate CPRB was created by inserting a 1-L cylinder packed with GG beads with TBOS and *R. rhodochrous* ATCC 21198 into the PAM. Barrier walls were also inserted to direct flow into the CPRB, however flow could bypass the CPRB, as a result of clogging and a reduction in permeability. Isobutene was used a reactive surrogate for the COCs, since its cometabolism produces isobutene oxide that can be easily detected by gas chromatography. The initial phase of the study showed effective transformation of isobutene within the CPRB, with a significant fraction of isobutene converted to isobutene oxide. The effluent of the PAM is also being monitored to determine the amount of isobutene that is cometabolized. Overall, the initial results show the potential of creating a CPRB using the hydrogel technology that was developed in this project.

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List of Acronyms

µg/L – microgram per liter
1,1,1-TCA – 1,1,1-trichloroethane
1,1-DCA – 1,1-dichloroethane
1,1-DCE – 1,1-dichloroethene
1,2,3-TCP – 1,2,3-trichloropropane
1,2-DCA – 1,2-dichloroethane
1,4-D – 1,4-dioxane
17OD – 1,7-octadiyne
2HBA – 2-hydroxy benzyl alcohol
ABL – activity-based labeling
ABPP – activity-based protein profiling
AMO – ammonia monooxygenase
ATCC – American Type Culture Collection
AWWA – American Water Works Association
BTEX – benzene, toluene, ethylbenzene, xylene
C/N – carbon to nitrogen ratio
CAH – chlorinated aliphatic hydrocarbon
CDC – Center for Disease Control
CF – chloroform
cis – DCE – 1,2-*cis*-dichloroethene
CO₂ – carbon dioxide
COC – contaminants of concern
CYD – casein-yeast extract-dextrose
DCM – dichloromethane
DI – deionized
DMSO – dimethyl sulfoxide
DNAPL – dense non aqueous phase liquid
DO- dissolved oxygen
DoD – Department of Defense
ERSON – Environmental Restoration Statement of Need
GC-ECD – gas chromatography electron capture detector

GC-FID – gas chromatography flame ionization detector
GC-MS – gas chromatography mass selective detector
GG – gellan-gum
H₂O₂ – hydrogen peroxide
HPLC – high performance liquid chromatography
K_{ow} – octanol water partition coefficient
LNAPL – light non aqueous phase liquid
MCL – maximum contamination level
MSM – mineral salts medium
MTBE – methyl tert-butyl ether
NADH – nicotinamide adenine dinucleotide and hydrogen
NaOH – sodium hydroxide
NAPL – non aqueous phase liquid
NCSU – North Carolina State University
NDMA – N-Nitrosodimethylamine
NIR – near infrared spectroscopy
O₂ – oxygen
OD₆₀₀- optical density at 600 nm
ORC – oxygen releasing compound
OSU – Oregon State University
PCB – polychlorinated biphenyls
PCE – tetrachloroethene
PET – polyethylene terephthalate
PFC – perfluorochemicals
PHAS – polyhydroxyalkanoates
PHB – poly-3-hydroxybutyrate
PHV – polyhydroxyvalerate
ppb – parts per billion
ppm – parts per million
PRB – permeable reactive barrier
PRBBS – permeable reactive bio-barriers
RPM – revolutions per minute

RT – retention time
SCAM – short chain alkane monooxygenase
SDS-PAGE – sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SERDP – Strategic Environmental Research and Development Program
SON – Statement of Need
SRC – slow-release compound
T2BOS – tetra-sec-butylorthosilicate
T2MO – toluene 2-monooxygenase
TBOS – tetrabutylorthosilicate
TCE – trichloroethene
THF – tetrahydrofuran
TKEBS – tetrakis (2-ethylbutoxy) silane
TOL – the plasmid that specifies the degradation of aromatic hydrocarbons
TOM – toluene *ortho*-monooxygenase
TPhOS – tetraphenoxysilane
trans-DCE – *trans*-1,2-dichloroethylene
TSS – total suspended solids
USAF – United States Air Force
USEPA – United State Environmental Protection Agency
USGS – United States Geologic Survey
v/v – volume per volume
VC – vinyl chloride
VOA – volatile organics analysis
VOC – volatile organic compounds
ZVI – zero valent iron

Chapter 1. Executive Summary

This Final Report for ER-2716 presents the results of the development of hydrogel beads for achieving the long-term cometabolic transformation of mixtures of Contaminants of Concern (COCs), including 1,4-dioxane (1,4-D) and chlorinated ethanes and ethenes. Gellan-gum hydrogels beads co-encapsulated a pure bacteria culture, *Rhodococcus rhodochrous* American Type Culture Collection (ATCC) 21198 (*R. rhodochrous* ATCC 21198) along with orthosilicates, that serve as slow releases compounds (SRCs) that produce alcohols as growth substrates via hydrolysis. The report includes results from the following major objectives: selecting of the bacteria culture to be co-encapsulated; demonstrating the alcohols and the SRCs would promote long term cometabolism; developing the method of co-encapsulation to produce hydrogel beads; evaluating the co-encapsulated beads in batch kinetic tests and groundwater microcosms; and evaluating the hydrogel beads in continuous flow 1-dimensional (1-D) packed columns; and a proof of concept study in a large scale physical aquifer model (PAM) where the beads were used to construct a cometabolic permeable reactive barrier (PRB).

In Objective 1 we explored how effective isobutane-utilizing bacteria are at cometabolically degrading COC mixtures. This objective was covered in the latter part of our prior SERDP-2303 project “Evaluation of Branched Hydrocarbons as Stimulants for in situ Cometabolic Biodegradation of 1,4-D and its Associated Co-contaminants.” That work showed that *R. rhodochrous* ATCC 21198 was very effective in transforming 1,4-D and a broad range of chlorinated ethanes and ethenes. *R. rhodochrous* ATCC 21198 was one of the most effective cultures for transforming this broad range of contaminants, thus it became the model organism studied in Objectives 2 through 5, once we demonstrated that its growth on alcohols could support aerobic cometabolism.

In Objective 2 we found that when grown on 1-butanol, 2-butanol, and 2-ethyl-1-butanol, *R. rhodochrous* ATCC 21198 was capable of cometabolic transformation of 1,4-D and a variety of chlorinated aliphatic hydrocarbons (CAHs). The *R. rhodochrous* ATCC 21198 grown on 2-butanol had the largest transformation capacity and fastest rates and was able to transform the mixture of 1,4-D, *cis*-DCE (1,2-*cis*-dichloroethene), and 1,1,1-TCA (1,1,1-trichloroethane) to >99%. This shows 2-butanol provides the most efficient transformation results of the alcohols tested. In mixtures, *R. rhodochrous* ATCC 21198 grown on these alcohols preferentially transforms *cis*-DCE over both 1,1,1-TCA and 1,4-D.

We also explored the use of esters that hydrolyze to form an alcohol and an organic acid as SRCs. Sec-butyl acetate was investigated, which produces 2-butanol and acetate. When *R. rhodochrous* ATCC 21198 was grown on sec-butyl acetate, the harvested cells transformed a mixture of 1,1,1-TCA and 1,4-D at rates faster than 2-butanol-grown cells. Other pure cultures such as *Rhodococcus butanica* (ATCC 21197) and *Mycobacterium sp.* 4CI that were isolated on isobutane, were capable of transforming 1,1,1-TCA and 1,4-D when grown on acetate. However, these transformation rates were 17.7 and 2.9 times slower than isobutane grown *R. rhodochrous* ATCC 21198.

Since *R. rhodochrous* ATCC 21198 transformed TCE very slowly, we also evaluated the toluene utilizing culture *Burkholderia vietnamiensis* G4 for TCE cometabolism potential when grown on alcohols. Benzyl alcohol was assessed as a food-grade growth substrate for promoting the cometabolism of TCE, via the toluene-2-monooxygenase (T2MO) of *B. vietnamiensis* G4.

Benzyl alcohol was evaluated by comparing the level of induction of the T2MO to that seen in toluene-grown cells. The level of T2MO expression was evaluated by two methods: 1) Activity based labeling (ABL); a gel assay method used to identify catalytically active monooxygenases (e.g. T2MO), and 2) resting cell kinetic tests occurring over the course of days to weeks. Other substrates were also tested for their ability to support TCE cometabolism including phenol, benzyl acetate, benzyl butyrate, acetate, butyrate, and lactate. Both the ABL assay and resting cell tests demonstrated the level of T2MO expression in cells grown on benzyl alcohol was comparable to that observed with toluene, with similar levels of expression detected and similar TCE transformation rates and capacities. The benzyl esters induced T2MO expression, with benzyl butyrate-grown cells exhibiting the highest TCE transformation rate and capacity of all substrates tested. Acetate, butyrate, and lactate showed minimal levels of enzyme expression, verifying benzyl alcohol was responsible for T2MO expression in benzyl ester-grown cells. The ABL assay and resting cell kinetic tests largely agreed, demonstrating that ABL is a reliable method of determining suitable substrates to support cometabolism. Additionally, kinetic tests with either substrate benzyl alcohol or toluene and TCE were performed to determine whether, like toluene, benzyl alcohol competitively inhibits contaminant transformation by T2MO. Results suggest that benzyl alcohol inhibition occurs, but at concentrations greater than 10 mg/L and to a lesser degree than toluene at the same concentration. Finally, kinetics for *cis*-DCE, 1,1-DCE and VC were determined for cells grown on benzyl alcohol and compared to toluene-grown cells. Toluene-grown cells had higher initial transformation rates for VC, *cis*-DCE, and 1,1-DCE, however, benzyl alcohol-grown cells had approximately twice the transformation capacity for the same compounds.

In Objective 3 we investigated whether effective cometabolism of COCs could be driven by SRCs that generate alcohols. We found that long-term cometabolic transformation of 1,1,1-TCA and 1,4-D could be achieved using SRCs as growth substrates for *R. rhodochrous* ATCC 21198. Batch metabolism and transformation tests were performed in the presence of 1,1,1-TCA and 1,4-D with the growth of *R. rhodochrous* ATCC 21198 on alcohols produced by the hydrolysis of the orthosilicates. Three orthosilicates were tested: tetrabutylorthosilicate (TBOS), tetra-sec-butylorthosilicate (T2BOS), and tetraisopropoxysilane (T2POS). The measured rates of alcohol release in poisoned controls depended on the orthosilicate structure with TBOS, which produced a 1° alcohol (1-butanol), hydrolyzing more rapidly than T2POS and T2BOS that produced the 2° alcohols 2-butanol and 2-propanol, respectively. The orthosilicates were added as light non-aqueous phase liquids (LNAPLs) along with *R. rhodochrous* ATCC 21198 and formed dispersed droplets when continuously mixed in the batch reactors. Continuous rates of oxygen (O₂) consumption and carbon dioxide (CO₂) production confirmed alcohol metabolism by *R. rhodochrous* ATCC 21198 was occurring. The rates of metabolism (TBOS > T2POS > T2BOS) were consistent with the rates of alcohol release via abiotic hydrolysis. 1,4-D and 1,1,1-TCA were continuously transformed in successive additions over 125 days, with the rates correlated with the rates of metabolism. The metabolism of the alcohols was not inhibited by acetylene, while transformation of 1,4-D and 1,1,1-TCA was. This indicated that while monooxygenase activity was required for the cometabolic transformations, it was not required for alcohol utilization. Alcohol concentrations in the biologically active reactors were maintained below the levels of detection, indicating they were metabolized rapidly after being produced. Much lower rates of O₂ consumption were observed in the reactors containing T2BOS, which has benefits for in-situ bioremediation. The results illustrate the importance of the structure of the SRC when developing passive aerobic cometabolic treatment systems.

In Objective 4, we demonstrated that the SRCs and *R. rhodochrous* ATCC 21198 could be effectively co-encapsulated for delivery for in situ treatment. *R. rhodochrous* ATCC 21198 was successfully co-encapsulated in gellan-gum hydrogel beads with orthosilicates as SRCs to support aerobic cometabolism of a mixture of 1,1,1-TCA, *cis*-DCE, and 1,4-D at initial aqueous concentrations ranging from 250 to 1000 µg/L (micrograms per liter). The fabrication method for the co-encapsulated gellan-gum hydrogel beads is outlined in Chapter 7 of the report. O₂ consumption and CO₂ production showed the co-encapsulated cells utilized the alcohols that were released by the co-encapsulated SRCs. TBOS and T2BOS, which hydrolyze to produce 1- and 2-butanol, respectively, were encapsulated in gellan-gum at mass loadings as high as 10% (w/w), along with *R. rhodochrous* ATCC 21198. In the gellan-gum beads, TBOS hydrolyzed 27 times faster than T2BOS and rates were ~4 times higher in suspension than when encapsulated. In biologically active batch reactors, the co-encapsulated *R. rhodochrous* ATCC 21198 effectively utilized the SRC hydrolysis products (1- and 2-butanol) and cometabolized repeated additions of a mixture of 1,1,1-TCA, *cis*-DCE, and 1,4-D for over 300 days. The transformation followed pseudo-first-order kinetics. Vinyl chloride (VC) and 1,1-dichloroethene (1,1-DCE) were also transformed in the reactors. In the long-term treatment, the batch reactors with T2BOS gellan-gum beads achieved similar transformation rates, but at much lower O₂ consumption rates than those with TBOS. Reaction rates decreased with prolonged incubation, but slowed to a lesser extent with beads containing T2BOS. Appropriately scaled first-order rates indicate very high extents of contaminant transformation could be achieved with a residence time of a few days if co-encapsulated beads were densely packed in a column or CPRB. Studies in groundwater/sediment microcosms also demonstrated that long-term cometabolic treatment can be achieved under conditions that mimic the subsurface. Based on the lower rate of O₂ consumption while achieving similar long-term rates, T2BOS was found to be a more effective substrate than TBOS to co-encapsulate with *R. rhodochrous* ATCC 21198. The results demonstrate that the co-encapsulation technology can be a passive method for the cometabolic treatment of dilute groundwater plumes.

In Objective 5, we examined if effective treatment of COCs could be achieved using the co-encapsulation technology in a 2-D PAM. We also performed packed bed long-term 1-D column studies to test the concept of creating a cometabolic PRB. The continuous aerobic cometabolic treatment of a mixture of 1,1,1-TCA, *cis*-DCE and 1,4-D was studied in 1-D laboratory columns packed with the gellan-gum hydrogel beads co-encapsulated the with *R. rodochrous* ATCC 21198 and 8% (w/w) TBOS or T2BOS. A mixture of *cis*-DCE, 1,1,1-TCA, and 1,4-D was continuously fed the columns, each at a concentration of 250 µg/L. Hydraulic residence times (HRT) in the columns ranged from approximately 2 days to 12 hours. Biostimulation of *R. rhodochrous* ATCC 21198 occurred during Phase 1 of the column studies. Two columns (Column 1 and 2), packed TBOS beads, were operated nearly identically. During Phase 1, 1-butanol concentrations in the column effluent reached 30 mg/L and the influent dissolved O₂ (DO) concentration (15 mg/L) was reduced to below detection in the column effluent. The addition of hydrogen peroxide (H₂O₂) to the column influent groundwater (50 to 100 ppm) as an additional source of DO, resulted in the effective utilization of 1-butanol, with concentrations decreasing to below 1 mg/L in the columns effluent. Both columns achieved over 99.5% removal of *cis*-DCE, 1,1,1-TCA, and 1,4-D within the first 25 days operation at an HRT of approximately 2 days. The development of aerobic conditions resulted in the biostimulation of *R. rhodochrous* ATCC 21198 within the beads throughout the column. This permitted a decrease in the HRT to 12 hours, while maintaining a contaminant removal efficiency of ≥ 99.5%. In Phase 2 of the tests, the cometabolic transformation was confirmed by stopping H₂O₂ and DO addition, which resulted in an increase in the effluent

contaminant concentrations to the influent levels. Upon the reintroduction of DO (15 mg/L) the cometabolic treatment was reinitiated and contaminant concentrations decreased to levels achieved prior the termination of H₂O₂ and DO addition, with 95% removal of 1,1,1-TCA and > 99% removal of 1,4-D and *cis*-DCE.

In Phase III, after ~ 240 pore volumes of flow (PV), *cis*-DCE was replaced by 1,1-DCE (100 µg/L), which imparts a high degree of transformation toxicity. 1,1-DCE was effectively transformed with 98% removal achieved, while 1,4-D and 1,1,1-TCA continued to be effectively transformed. After ~ 300 PV the concentration of 1,1-DCE was increased to 250 µg/L. Effective transformation for all three contaminants was achieved until around 450 PV after which all three contaminants began to increase to the influent concentrations. After 550 PV of flow, complete breakthrough of three contaminants was observed. Column 2 show nearly identical responses as observed in Column 1 for all Phases of the tests.

Column 3 was packed with gellan-gum beads co-encapsulated with *R. rhodochrous* ATCC 21198 and T2BOS, which hydrolyzes at a much slower rate than TBOS. Column 3 was fed groundwater that contained 14 mg/L DO and a mixture of *cis*-DCE, 1,1,1-TCA and 1,4-D, each at 250 µg/L. During the biostimulation phase (Phase I) the maximum 2-butanol concentration observed in the column effluent was 3 mg/L, which were a factor of 10 lower than that observed in Columns 1 and 2, consistent with the slower rate of hydrolysis of T2BOS. 2-butanol concentrations gradually decreased, and after about 50 PV of flow were below 50 µg/L. Due to the slower rate of hydrolysis, H₂O₂ addition was not required. Over 99% removal of 1,1,1-TCA, *cis*-DCE and 1,4-D was achieved. After 90 PV of flow, Phase II was initiated and the contaminant concentrations were increased from 250 µg/L to 500 µg/L. The effluent concentrations of *cis*-DCE and 1,4-D began to immediately increase and reached the influent concentrations after 120 PV of flow, while 1,1,1-TCA only reached about 25% of the influent concentration. Upon reduction of the influent concentrations in Phase III to 250 µg/L, the cometabolic transformation was restored, with over 98% removal of the contaminants observed. In Phase IV, *cis*-DCE addition was replaced by 1,1-DCE (100 µg/L), while continuing to add 1,4-D and 1,1,1-TCA. A rapid increase in all three contaminants in the column effluent was observed. In Phase V, 1,1-DCE addition in the column effluent was stopped, however 1,4-D and 1,1,1-TCA, continued to breakthrough to the injected concentrations. A final test showed *cis*-DCE, when added as the only contaminant could not be transformed. The results suggest that 1,1-DCE transformation toxicity caused the cessation in the cometabolic activity in the column. An increase in the DO in the column effluent was also observed indicating the metabolic activity of *R. rhodochrous* ATCC strain 21198 also decreased.

At the end of the column studies the gellan-gum beads were sampled and assayed for the amount of TBOS and T2BOS remaining. Approximately 56% and 98.5 % of the TBOS and T2BOS originally encapsulated remained, respectively. TBOS and T2BOS limitations were not responsible for the cessation in transformation activity in the columns. The estimated rate of hydrolysis of T2BOS was factor of 20 lower than TBOS, which was consistent with the batch incubations. The results from the column tests indicate that a passive CPRB might be created using the co-encapsulated technology that was developed. In a barrier, consisting of packed beads, very high removal efficiencies might be achieved with residence times of less than one day. The co-encapsulated gellan-gum macrobeads could also mixed in-situ with contaminated aquifer solids to create a passive reactive cometabolic barrier or potentially applied in an in-well reactor.

Experiments were initiated in a 100-L PAM packed with sand (20 to 30 mesh) to evaluate the creation of a CPRB. The Funnel-and-Gate CPRB was created by inserting a 1-L cylinder

packed with gellan-gum beads with TBOS and *R. rhodochrous* ATCC 21198 into the PAM. Barrier walls were also inserted to direct flow into the CPRB, however flow could bypass the CPRB, as a result of clogging and a reduction in permeability. Isobutene was used as a reactive surrogate for the COCs, since its cometabolism produces isobutene oxide that can be easily detected by gas chromatography. Uniform flow in the PAM was created by having a manifold across the inlet of the PAM to distribute the continuous flow. An identical manifold was used to capture the groundwater exiting the PAM. The PAM was operated as a confined aquifer. H₂O₂ (50 mg/L) was added to synthetic groundwater along with bromide as a conservative tracer and isobutene (30 to 100 µg/L). In the initial phase of the study effective transformation of isobutene was observed within the CPRB, with a significant fraction of isobutene converted to isobutene oxide. The effluent of the PAM is also being monitored to determine the amount of isobutene that is cometabolized. Overall, the initial results show the potential of creating a CPRB using the hydrogel technology that was developed in this project.

The PI has recently received an award from National Institute of Environmental Health Science (NIEHS) to continue the further development of this technology. The PAM study that was initiated under SERDP funding will be continued under NIEHS funding. We plan to study the transformation of mixtures of 1,4-D and 1,1,1-TCA and *cis*-DCE as part of this ongoing study.

The results from the column and PAM tests indicate that a passive cometabolic treatment might be created using the co-encapsulated technology that was developed. In a CPRB, fabricated with the packed hydrogel beads, very high removal efficiencies might be achieved with residence times of less than one day. There are other forms of treatment, both in-situ and ex-situ, that might utilize this technology. For example, the co-encapsulated macrobeads could also be mixed in-situ with contaminated aquifer solids to create a CPRB. Removal rates are high enough to consider using a packed bed reactor of co-encapsulated beads within a recirculation well for in-situ treatment. Ex-situ treatment might also be achieved using above ground packed bed reactors.

We also demonstrated that *B. vietnamiensis* G4, when grown on benzyl alcohol or esters that produce benzyl alcohol, such as benzyl butyrate, can effectively cometabolize chlorinated ethenes, including TCE, *cis*-DCE, 1,1-DCE and VC. Benzyl alcohol was as effective as toluene in promoting the cometabolism of these compounds. Thus, there is potential for co-encapsulation of other microorganisms, such as *B. vietnamiensis* G4 along with SRCs, that can effectively treat chlorinated ethenes. A broad range of COCs might be treated in a CPRB that contains a mixture of gellan-gum beads with different microorganisms and different SRCs. Thus, mixing of gellan-gum beads that are effective for treating different contaminants would be easily implemented in practice to treat a wide range of COCs.

Chapter 2. Objectives

Mixtures of COCs are present at many Department of Defense (DOD) sites. A recent review indicated that 59% of DOD sites have more than one contaminant present, often in commingled plumes (SERDP, 2015). Most frequently observed COCs include chlorinated and non-chlorinated volatile organic compounds (VOCs), polychlorinated biphenyls (PCBs), metals, inorganics, perchlorate, perfluorochemicals (PFCs), NDMA, and munitions. 1,4-D is also often present in mixtures of CAHs, due to its use as a stabilizer in commercial solvent formulations. In a multisite survey of 2000 sites in California (Adamson et al., 2014), 1,4-D was detected at 194 sites with 95% of these containing one or more chlorinated solvent. In 76% of these sites both 1,4-D and 1,1,1-TCA were present, and in 59 sites 1,4-D was present with 1,1,1-TCA, TCE, and 1,1-DCE. While 1,1,1-TCA is thought to be the likely source of most 1,4-D plumes, 64% of 1,4-D detections at USAF installations were associated with only TCE (Anderson et al., 2012). Thus, the ability to treat mixtures of TCE and 1,4-D is of interest. Adamson *et al.* (Adamson et al., 2014) also found that in 62% of the plumes studied, the CAH plume was longer than the 1,4-D plume. Thus, zones where 1,4-D and CAHs co-occur and where *in-situ* treatment may be applied are common.

In situ bioremediation could potentially provide a large cost savings for treatment of COC mixtures, especially for mixtures that include 1,4-D. Aerobic cometabolism is a particularly attractive option, as specific microorganisms can be stimulated *in situ* using specific primary substrates. Microbial growth on these substrates leads to expression of oxygenase enzymes that can then oxidize and remediate contaminants to concentrations well below drinking water standards. Through our past SERDP project (ER2303) we found that diverse microorganisms grown on isobutane consistently biodegrade low ppb concentrations of 1,4-D *via* aerobic cometabolism. The model isobutane-metabolizing strain, *R. rhodochrous* ATCC 21198, degrades 1,4-D at very high rates and transformation capacities to concentrations approaching the drinking water screening level of 0.67 $\mu\text{g L}^{-1}$. This strain can also degrade a broad range of chlorinated solvents. *cis*-DCE and VC, the products of the anaerobic degradation of tetrachloroethene (PCE) and TCE, are also both readily degraded, as well as 1,1-DCE, the abiotic degradation product of 1,1,1-TCA. *R. rhodochrous* ATCC 21198 is also capable of degrading a broad range of chlorinated ethanes, as well as a slow degradation of 1,2,3-Trichloropropane.

The overall aim of the project was to develop novel aerobic cometabolic processes based on isobutane-metabolizing bacteria and slow-release compounds (SRCs) for the aerobic treatment of mixtures of 1,4-D and CAHs. The research aims to identify and characterize specific microorganisms and SRCs that can be co-encapsulated in hydrogel beads to treat COCs being released from low permeability zones. The technology that was developed can be applied to a broad range of hydrogeologic settings as information was obtained on a gaseous substrate (isobutane), liquid substrates (alcohols) and SRCs, which may be applied in different remediation schemes.

TECHNICAL OBJECTIVE: The overall aim of the project was to develop novel aerobic cometabolic processes based on co-encapsulation of SRCs and isobutane-metabolizing bacteria to treat COC mixtures of interest to DOD. While mixtures of 1,4-D and CAHs was our primary interest, the microorganisms, SRCs, and encapsulation technologies identified in these studies may

be suitable for treating COC mixtures containing NDMA and other DOD-relevant contaminants. There were five specific Objectives that addressed five key questions.

Q1. How effective are isobutane-utilizing bacteria at cometabolically degrading COC mixtures? In our previous research, rapid transformation of 1,4-D at low (<100 ppb) concentrations was achieved using *R. rhodochrous* ATCC 21198 grown on isobutane. A broad range of CAHs are also transformed by this strain. **In Objective 1** we evaluated a series of isobutane-oxidizing bacteria to determine whether similar cometabolic activities consistently occur with these strains and whether these activities can be attributed to specific monooxygenase enzymes. Batch kinetic studies were used to determine rates and extents of the cometabolic transformations of individual COCs and defined COC mixtures. Shotgun and activity-based protein profiling (ABPP)-based proteomic analyses were used to identify the monooxygenases involved in COC degradation in each strain.

Q2. Can alcohols be used as primary substrates to support cometabolism of COC mixtures? Previous studies by us and others indicate *R. rhodochrous* ATCC 21198 (Kuntz et al., 2003) and other isobutane-metabolizing bacteria (Smith et al., 2003b) can have broad COC-degrading activities when grown on alcohols. The ability to use alcohols to support cometabolic processes could provide an effective alternative substrate delivery approach for *in situ* remediation compared to gaseous substrates and will also enable development of SRCs. **In Objective 2** we characterized the ability of a range of 1° and 2° alcohols to support growth and biodegradation of individual COCs and COC mixtures by the bacteria studied in Objective 1. Proteomic analyses were used to characterize the ability of alcohols, COCs and COC mixtures to induce expression of COC-degrading monooxygenases in these strains.

Q3. Can effective cometabolism of COCs be driven by SRCs that generate alcohols? We have previously shown non-toxic silico-oxide-organics (tetraalkoxysilanes) can act as alcohol-releasing SRCs and promote the aerobic cometabolism of *cis*-DCE and TCE (Sempriani & Vancheeswaran, 2002; Vancheeswaran et al., 1999, 2003). Controlled rates of alcohol production can be achieved by varying the structure of the tetraalkoxysilanes. **In Objective 3**, we evaluated the ability of specific tetraalkoxysilanes SRCs to support cometabolism of COC mixtures based on the effects of alcohol and COC induction patterns observed in Objective 2

Q4. Can the SRC and the bacteria culture be effectively co-encapsulated for delivery for *in situ* treatment? The co-delivery of the SRC with an appropriate bacterial strain could provide an effective system for the passive treatment of COCs, especially when COCs are slowly released from low permeability zones. **In Objective 4** we further developed and tested this approach. Based on the results of Objective 3, we developed a co-encapsulation technology with the most promising SRCs and bacteria strain. We optimized the encapsulation approach to achieve long-term transformation of the COCs mixtures in studies involving both microcosms and continuous flow columns.

Q5. Can effective treatment of COCs be achieved using the co-encapsulation technology in a 2-D physical aquifer model? There are many practical issues impacting whether co-encapsulation technology can be applied to conditions encountered in a heterogeneous subsurface environment. As a proof-of-concept, **in Objective 5** we tested the co-encapsulation technology in 2-D physical aquifer model that includes zones of low and high permeability. We used the concept of a PRB treatment system and used hydrogel co-encapsulated beads to create

the barrier. 1-D continuous flow column studies were performed with the columns packed with co-encapsulated gellan-gum beads to test the concept of a CPRB.

The research in this project was response to ERSON-17-01 (SERDP, 2015). This SON aimed to support basic research to improve our ability to treat mixed COCs in groundwater. The research responded to several of the focus areas of the SON including: *developing a greater understanding of the effect of intrinsic physical, chemical, and biological properties and their associated impact on contaminant behavior and fate when contaminants are present as mixtures in lower permeability zones*; and *to develop a greater understanding of potential treatment synergies that could lead to cost savings and improved treatment strategies*. Of particular interest are mixtures of chlorinated compounds containing 1,4-D, the treatment which was a focus of the research. The SON also states an interest *in technologies and approaches that are applicable to a variety of hydrogeologic settings*. We investigated aerobic cometabolism with substrates that range from gases (isobutane), to alcohols (liquids), to SRCs, the technology can be applied to a range of hydrogeologic environments and using remediation approaches that have been previously applied in practice. In particular, the co-encapsulation technology we developed can be directed towards treating COCs that are being slowly released from zones of low permeability; a high priority in ERSON-17-01.

Chapter 3. Background

Mixtures of COC are present in groundwater at many DOD sites as well as industrial facilities (SERDP, 2015; Adamson et al., 2014; Anderson et al., 2012; Council, 1 C.E.; Squillace et al., 2002b). The contaminant problem nationwide is supported by USGS survey of 1255 domestic drinking water wells and 242 public supply wells in the United States that found 14 compounds (seven VOCs, six pesticides, and nitrate) represented 95% of the detected contaminant mixtures (Squillace et al., 2002b). The seven VOCs and their rank order of detection were: CF, (4); PCE (5); 1,1,1-TCA (8); MTBE (10); TCE (11); 1,1-dichloroethane (1,1-DCA) (12) and 1,1-DCE (13). Except for PCE, all of these compounds can be biodegraded to CO₂ *via* aerobic cometabolism. 1,4-D is also frequently present in mixtures of CAHs, due to its use as a stabilizer in commercial solvent formulations. A multisite survey of 2000 sites in California (Adamson et al., 2014), demonstrated that 1,4-D is frequently detected with both 1,1,1-TCA and TCE in contaminated groundwater. The ability to treat COC mixtures that contain both CAHs and 1,4-D is therefore of particular interest.

1,4-D and a limited number of the VOCs listed above can be utilized directly by aerobic microorganisms as growth-supporting substrates. However, remediation of these compounds in the environment typically requires specialized microorganisms with unusual metabolic capabilities. High concentrations of individual COCs are also usually needed to supply sufficient carbon and energy to support growth of these microorganisms. In contrast, with the exception of PCE, all of these compounds, as well as 1,4-D, can be biodegraded *via* aerobic cometabolism. In this process the growth and activity of microorganism is promoted by addition of an exogenous primary growth-supporting substrate. Target COCs are then fortuitously degraded by enzymes expressed to enable microbial growth on the primary substrate. As COC biodegradation then occurs in response to the primary substrate rather than the COC, cometabolic treatment processes can often degrade COCs at low, environmentally relevant concentrations. Cometabolic treatment processes can also be designed to treat specific COC mixtures, by varying the primary substrates used to stimulate microbial activity (Semprini, 1997). There is also the potential to bioaugment specific microorganisms to achieve effective cometabolic treatment (Semprini, 2013). Being an aerobic process, there is potential to maintain groundwater quality, compared to anaerobic treatment processes (Semprini, 2001).

The treatment of COC mixtures is often further complicated by the presence of zones of low permeability that can slowly release contaminants, especially when they are present as DNAPLs (Brown et al., 2012; Stroo et al., 2003). An important aim of the research was to develop cometabolic treatment methods that can remediate COC mixtures as they are slowly released from low permeability zones. To this end, we developed slow-release compounds (SRCs) as well as co-encapsulation methods combining both SRCs and specific bacteria to facilitate aerobic cometabolism of COC mixtures that slowly diffuse from zones of low permeability and potentially DNAPL source zones. The systems that were developed could be used in conjunction with recirculation well technology or PRB to create reactive biological treatment zones (McCarty et al., 1998; McCarty & Semprini, 1993). Here groundwater might be recirculated around a low permeability zone, and the COC mixture forced through the bioactive zone that is created.

Through our past SERDP project ER2303 we have discovered that diverse microorganisms grown on isobutane can typically biodegrade low (<100 ppb) concentrations of 1,4-D *via* aerobic

cometabolism. The best-characterized isobutane-metabolizing strain, *R. rhodochrous* ATCC 21198, degrades 1,4-D at very high rates and transformation capacities to concentrations approaching the drinking water screening level of $0.67 \mu\text{g L}^{-1}$. Resting cells of this strain can also degrade a broad range of chlorinated solvents (Rich, 2015). *cis*-DCE and VC, the products of the anaerobic degradation of PCE and TCE, are both readily degraded, as well as 1,1-DCE, the abiotic degradation product of 1,1,1-TCA. Isobutane-grown cells of *R. rhodochrous* ATCC 21198 can also degrade a broad range of chlorinated ethanes, as well as the slow degradation of 1,2,3-TCP. As less chlorinated ethenes and ethanes are degraded at faster rates than the more chlorinated forms, *R. rhodochrous* ATCC 21198 or similar organisms could potentially contribute to *in situ* treatment trains in which an aerobic process follows an anaerobic treatment process.

An underlying rationale for the research is that cometabolic degradation of mixtures of 1,4-D, CAHs, and potentially other COCs such as NDMA could be achieved by isobutane-utilizing microorganisms typified by *R. rhodochrous* 21198. *R. rhodochrous* ATCC 21198 was originally deposited with ATCC as one of a group of six fast-growing isolates (ATCC strains 21194-21199) patented for citrate and biomass production using gaseous *n*-alkanes ($\text{C}_2\text{-C}_4$) as growth substrates (Kimura & Nakanishi, 1975; Tanaka, 1973). The most extensive previous studies of bacterial isobutane metabolism have been conducted with *R. rhodochrous* ATCC 21198 (MacMichael & Brown, 1987). However, other organisms within this group appear to have very similar properties. For example, we have established ATCC strain 21194 also grows on isobutane and can also oxidize 1,4-D after growth on this gas. Propane-grown ATCC strain 21197 can cometabolically degrade a very similar range of CAHs as isobutane-grown *R. rhodochrous* ATCC 21198, and is also reported to degrade chloroform (CF) and dichloromethane (DCM) (Malachowsky et al., 1994). We have also isolated several isobutane-utilizing strains from environmental samples and have shown all of these strains can also oxidize low (<100 ppb) concentrations of 1,4-D as determined by a heated purge and trap method available at both NCSU and OSU (M. Sun et al., 2016). Collectively, our results therefore suggest that these bacteria are not only related by their common metabolic capability to grow on isobutane but they likely also have very similar cometabolic capabilities. A key aim of this project was to evaluate this possibility through a comprehensive analysis of the abilities of diverse isobutane-metabolizing bacteria to degrade 1,4-D, individual COCs and COC mixtures. The results of this work are presented in Section 4 of the report.

In this project an important aim was to identify which monooxygenase enzymes are responsible for the cometabolic activities observed in isobutane-metabolizing bacteria. We used an ABPP approach developed during our past SERDP project (Bennett et al., 2016) and shotgun proteomic analyses to identify and quantify these 1,4-D-degrading monooxygenases. In many previous applications of cometabolic biodegradation, microbial activity has been stimulated *in situ* using primary substrates such as methane, propane, or toluene (Semprini, 1997). The rationale for this is that utilization of these compounds requires the target microorganisms to express COC-degrading monooxygenases as these enzymes normally function to initiate primary substrate oxidation. The immediate products of aerobic microbial alkane oxidation are alcohols. Previous studies with the isobutane-metabolizing strain *Mycobacterium vaccae* JOB5 have shown this strain can still biodegrade COCs such as 1,4-D (Hand et al., 2015) and MTBE (Smith et al., 2003b) after growth on 1° and 2° alcohols. As this strain does not express COC-degrading activity after growth on substrates such as glucose, these observations indicate that expression of the COC-degrading monooxygenases in this model bacterium is induced by alcohols. A similar effect has previously been reported for *R. rhodochrous* ATCC 21198, which degrades VC after growth on isopropanol

(Kuntz et al., 2003). In other studies, conducted during our past SERDP project we have shown *R. rhodochrous* ATCC 21198 can oxidize 1,4-D and mixtures of c-DCE and 1,1,1-TCA during growth on isopropanol, butanol, and benzyl alcohol. In another experiment we have shown the effects of transcription and translation inhibitors to confirm 1,4-D acts as an inducer of monooxygenase expression. The ability of other individual COCs such as TCE and DCE to induce expression of bacterial monooxygenases is well established (Doughty et al., 2005; McClay et al., 1995; Parales et al., 2000; Ryoo et al., 2001; Shingleton et al., 1998; Yeager et al., 2004) and also occurs with COC mixtures (Kageyama et al., 2005). A key objective of this project was to characterize the abilities of both alcohols and COCs to act as inducers of monooxygenase expression in diverse isobutane-metabolizing strains. In Chapter 4 of this report, results of the ABPP approach are presented along with ability of *R. rhodochrous* ATCC 21198 and other strains to transform COC mixtures after growth on 1° and 2° alcohols.

In Chapter 5 of the report, results are presented where ABPP labeling approach was used to study the induction of monooxygenase activity the toluene utilizing microorganism *Burkholderia vietnamiensis* G4 on benzyl alcohol. *B. vietnamiensis* G4 has been extensively studied for its ability to transform chlorinated ethenes through aerobic metabolism by the toluene-2-monooxygenase (T2MO) when grown on aromatic growth substrates such as toluene and phenol (Folsom et al., 1990; Shields et al., 1989, 1995; Shields & Reagin, 1992). This microorganism was chosen as it has previously been shown that *B. vietnamiensis* G4 can cometabolically transform chlorinated ethenes to a greater extent than other microorganisms that aerobically transform TCE, in addition to having a high affinity and high toxicity threshold for TCE (Sun & Wood, 1996; Yeager et al., 2001). The SRCs looked at in this study for their ability to support cometabolism in *B. vietnamiensis* G4 were tetraphenoxysilane (TPhOS), and the esters benzyl acetate, and benzyl butyrate. As a tetra-alkoxysilane, for every mole of TPhOS, hydrolysis results in four moles of phenol and silicic acid. Whereas, the esters benzyl acetate and benzyl butyrate both hydrolyze to release benzyl alcohol and acetic and butyric acid, respectively. While research with this microorganism was not originally proposed, the result obtained broaden the COCs that might be treated using the alcohols as growth substrate along with SRCs that produce aromatic alcohols.

Collectively, these prior observations suggest COC-degrading activities of isobutane-utilizing microorganisms could be exploited in treatments using SRCs that release alcohols, especially if technologies can be developed that enable SRCs and appropriate microorganisms to be co-encapsulated and co-delivered to specific locations in treatment systems. Besides the ease of addition, a compelling benefit of using alcohols to promote cometabolic COC degradation is that these compounds can lead to more effective remediation by eliminating competitive interactions between gaseous growth substrates such as isobutane and COCs. We had previously demonstrated that tetraalkoxysilanes are effective alcohol-releasing SRCs that can support aerobic cometabolism of CAHs such as TCE and *cis*-DCE (Semprini & Vancheeswaran, 2002; Vancheeswaran et al., 1999, 2003). In this project we further developed these compounds as SRCs for specific isobutane-metabolizing bacteria and the degradation of COC mixtures. The results of this work are presented in Chapter 6 of the report.

Encapsulation of microorganisms is widely used for industrial production of specific products, and a wide range of encapsulating compounds and procedures have been investigated (Freeman & Lilly, 1998; Orive et al., 2004; Park & Chang, 2000). Alginate has been frequently used and enables cell encapsulation in a hydrogel. Other potential encapsulation materials include agarose, polyvinylalcohol, and polyethethylene glycol. To increase biocompatibility and

mechanical stability, alginate is often mixed with other immobilizing compounds, such as agarose, chitosan, poly-L-ornithine, and cellulose sulfate (Freeman & Lilly, 1998; Park & Chang, 2000). In previous studies we have successfully developed encapsulation methods for aerobic methane-oxidizing bacteria. In this project we extended these studies to develop effective co-encapsulation technology that allowed specific SRCs and microorganisms to be co-encapsulated. In Chapter 7 of the report we present the development of the co-encapsulation technology using gellan-gum to create hydrogel beads. The co-encapsulated technology was tested in batch systems with media and in microcosms created with groundwater and sediments from a DOD site.

In Chapter 8 of the report the co-encapsulated gellan-gum beads were tested in continuous flow column that were packed with the co-encapsulated gellan-gum beads to mimic treatment in a PRB system. Hydraulic residence times in the columns ranged from 2 days to 12 hours. While we have obtained compelling evidence for the feasibility of all of the objectives listed above, the use of SRCs co-encapsulated with specific microorganisms has not been tested under field-like conditions. The final component of this study involved proof of concept tests of co-encapsulated SRCs and isobutane-oxidizing bacteria to cometabolically degrade COCs mixtures in a laboratory-based physical aquifer model (PAM) that incorporates zones of high and low permeability. A passive treatment Funnel-and-Gate CPRB was evaluated. The results are presented in Chapter 9 of the report.

Chapter 4. *R. rhodochrous* ATCC 21198 Grown on Alcohols and Esters and Transformation of CAHS and 1,4-Dioxane

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Introduction

Historical use and disposal of chlorinated solvents has resulted in widespread groundwater contamination by a variety of carcinogenic CAH. These chemicals were primarily used for vapor degreasing, metal cleaning, and as a dry-cleaning reagent due to their ability to dissolve a variety of organic compounds both quickly and efficiently (Doherty, 2000). Due to extended and diverse use, contamination by mixtures of CAHs is pervasive in the environment (Carter et al., 2008; Moran et al., 2007). Chlorinated solvents are among the most common substances found at superfund sites and are often in landfill leachate and under military and industrial sites (M. Moran et al., 2007). 1,4-D, a suspected human carcinogen on the USEPA's contaminant candidate list for regulation (USEPA, 2017a), has been more recently identified as a common co-contaminant (Adamson et al., 2014) linked by its use as a stabilizer for commonly used solvent 1,1,1-TCA. 1,4-D contamination is pervasive in both drinking water (USEPA, 2017a) and domestic wastewater (Simonich et al., 2013) in the US.

Chlorinated solvents have low solubility in water and are highly volatile, and at high concentrations they exist as DNAPL. Most CAHs have octanol-water partitioning coefficients greater than 1 and are likely to sorb to aquifer material. Contaminant DNAPL can act as a persistent source diffusing into low permeability aquifer layers to be released slowly and at low concentrations creating large dilute plumes (Pankow, 1996). Chlorinated solvent remediation strategies commonly take advantage of the volatility of the compounds utilizing air stripping or adsorption to treat groundwater or excavation of the DNAPL source zone (Huang et al., 2014). The concurrence of mixtures of CAHs with 1,4-D complicates remedial efforts as the physical properties of 1,4-D are incompatible with the traditional methods to address CAHs, it is also miscible in water and thus highly mobile in the subsurface (USEPA, 2017b).

Bioremediation of these mixed contaminant plumes can be less resource and energy intensive and thus more cost effective than the traditional methods (Frasconi et al., 2015). A bacterial strain, *R. rhodochrous* ATCC 21198 (previously *Nocardia paraffinica*), has been reported to cometabolize a variety of CAHs as well as 1,4-D (P. Bennett et al., 2018; Krippaehne, 2018; Rolston et al., 2019). This cometabolic activity involves short chain alkane monooxygenase (SCAM) which fortuitously transforms non-growth substrates such as COCs (Dalton et al., 1982).

Although *R. rhodochrous* ATCC 21198 has been characterized as an isobutane-utilizer, this strain also grows on other short chain alkanes (Babu & Brown, 1984; Hou et al., 1983; Murnane et al., 2021) and can effectively transform 1,4-D and select CAHs under conditions when SCAM expression is highly induced. In addition to 1,4-D, alkane-grown *R. rhodochrous* ATCC

21198 can transform a variety of CAHS including vinyl chloride, 1,1-DCE, cis-DCE, 1,2 DCA, 1,1,1-TCA, and 1,1,2-TCA (Krippaehne, 2018, Rolston et al., 2019). This strain has also been successfully bioaugmented into microcosms to transform 1,4-D in the presence of TCE (Rolston et al., 2019).

In addition to isobutane and other gaseous alkanes, cometabolic activity in *R. rhodochrous* ATCC 21198 is also supported by 1-butanol and 2-butanol (Murnane et al., 2021). 1-Butanol has also been shown to induce cometabolism in several other bacterial strains (Hand et al., 2015; Kelley et al., 2001). For sustained cometabolic transformation, alcohol-releasing orthosilicates have been identified as suitable SRCs, inducing cometabolism in *R. rhodochrous* ATCC 21198 (Rasmussen et al., 2020).

Objectives set out for this study were as follows:

1. Evaluate compounds including alcohols, organic acids, and esters as potential growth substrates for *R. rhodochrous* ATCC 21198 and assess the contaminant-oxidizing ability of cells grown on these substrates.
2. Quantify and compare rates of cometabolism and transformation capacity of *R. rhodochrous* ATCC 21198 grown on alkanes and butane-based alcohols for COCs including a variety of CAHs and 1,4-D.
3. Investigate substrates that are available as slow-release compounds for their ability to stimulate cometabolism of COCs.
4. Assess alternative isobutane utilizing microbes grown on potential slow-release substrates for transformation of COCs.

Materials and Methods

Chemicals

The chemicals used in all the following experiments are analytical grade: Isobutane, 99.99% (Gas Innovations); 1-butanol, 99.4% (Acros, Fisher Scientific); 2-butanol, >99% (Sigma-Aldrich); 2-ethyl-1-butanol, 99% (Sigma-Aldrich); All additions of CAHs to reactors were done from a saturated stock solution. These solutions were made by adding 1mL of neat CAH to a 27 mL serum vial which was then filled with DI water and capped with a butyl septum and shaken to ensure equilibrium. The concentration of the aqueous phase was assumed to be the water solubility limit of the compound at room temperature. The neat 1,1,1-TCA is stabilized with 1,4-D when received from the manufacturer. To remove the 1,4-D from the saturated stock solution the aqueous phase was decanted off and disposed according to hazardous waste guidelines. The DI water replaced, and shaken again to return to equilibrium. This process was repeated once again at which point the aqueous concentration of 1,4-D was below limit of detection.

*Growth and maintenance of *R. rhodochrous* ATCC 21198*

Pure cultures of *R. rhodochrous* ATCC 21198 were grown and maintained for the experiments as follows. The bacterium was obtained from Dr. M. Hyman at North Carolina State University and was maintained by streak plating colonies onto MSM plates with an added fungicide, nystatin at 24 mL/L of a 10,000 unit/mL solution. The plates were stored in an airtight canister at 30°C with isobutane present at a minimum gas phase concentration of 5% (v/v)

For inoculation into experimental reactors, a solution of concentrated biomass was grown on a substrate of interest. A colony was transferred using a flame sterilized metal loop from

maintained MSM plates into Wheaton media bottles (500 mL) with butyl septa caps containing MV media (300 mL) and air in the headspace, (see appendix for MV media recipe). Substrate was added at varying concentrations considering flammability limits and oxygen availability. The growth reactors were kept at 30° C and shaken at 150 RPM. After 4 days, the reactor headspace was exchanged with air to replenish oxygen and a second addition of substrate was added. At this time cells were streak plated on TSA plates from each growth reactor to visually confirm the culture had remained uncontaminated. Reactors were stored for an additional day, then harvested.

The duration of incubation and optical density of growth reactors were kept consistent between cell growth batches and were determined in order to harvest cells during the exponential phase of growth within the reactor. Growth reactor contents were transferred to Nalgene centrifuge bottles (500 mL) and centrifuged (9000 rpm for 10 minutes). The resulting supernatant was discarded, and cell pellet was resuspended in sodium phosphate buffer (50 mM, pH 7). The cell solution was centrifuged again, and the final cell pellet was resuspended in phosphate buffer (5-10 mL) and then storage at 4° C before use in experiments.

The concentrated cell solution was quantified on a weight basis using a total suspended solids (TSS) test as described in AWWA standards (AWWA, 2017). Between 0.5 and 0.8 mL of the cell solution was vacuum filtered through a 0.45 µm Whatman ME 25/21 mixed cellulose filter then dried for 20 minutes in a 103° C oven. The dry weight of cells was calculated as the difference between the weight of the clean filter and the dried filter with cells, which was used to determine the concentration of cell mass in mg/mL of the solution.

Resting cell transformation tests

The ability of *R. rhodochrous* ATCC 21198 to oxidize propylene, tetrahydrofuran (THF), and 1,4-D was determined by performing rate tests with resting cells grown on either individual gaseous alkanes, their corresponding primary and secondary alcohols, acetate, propionate, or butyrate. Tests were done in triplicate with zero order rates of oxidation determined and normalized to mg protein, reported as an average nmoles/min-mg protein.

Single compound transformation rate tests of COCs, a variety of CAHs and 1,4-D, were performed with resting cells of *R. rhodochrous* ATCC 21198 grown on various substrates including alkanes and alcohols, as described by Rolston *et al* (2019) in sterile serum vials (27 mL) sealed with butyl septa and aluminum crimp tops. All tests were performed with resting cells, and no substrate was present in the reactors. Vials were filled with MSM (10 mL) before being sealed. The desired volume of contaminant and 5mg TSS of cells were added through the septa. See Table 4.1 for initial concentrations of each contaminant of interest and biomass added to each kinetic test. Abiotic and acetylene-containing controls were included with duplicates of each control type and triplicates of active reactors. To determine transformation capacity of cells, once the initial contaminant mass was transformed below its detection limit subsequent additions of the selected contaminant were made until contaminant transformation ceased. Transformation capacity was calculated as the total mass transformed normalized to biomass and reported as µmol/mgTSS.

The frequency of contaminant mixtures observed at sites establishes a need to evaluate the efficacy of cells to transform mixtures as well as single compounds. Compound mixture transformation rate tests were performed in larger reactors as described by Rasmussen *et al* (2020). Wheaton bottles (125 mL) containing MSM (100 mL) were sealed with a plastic screw cap containing a butyl rubber septum. A mixture of 1,1,1-TCA, cis-DCE, and 1,4-D was chosen and added to reactors at environmentally relevant concentrations of between 250 and 1000 µg/L.

Analytical methods

Volatile compounds were analyzed from headspace samples (100 μ L) taken with a Hamilton 1700 gas tight syringe which were immediately injected into gas chromatographs. Liquid samples were needed for 1,4-D and 2-butanol quantification. Depending on concentrations and analytical needs, samples (0.4 to 0.6mL), were taken through the septa using disposable syringes (1 mL) and filtered through a 0.2 μ m polyvinylidene fluoride syringe filter. These filtered samples were stored in 2mL microcentrifuge tubes at 4 °C for analysis.

Chlorinated compounds, except VC, were analyzed from headspace samples on a Hewlett Packard 6890 Series Gas Chromatograph with a micro electron capture detector (GC-ECD) and capillary column (Agilent Technologies DB-624 UI). Carrier gas was helium flowing at 15 mL/min and oven temperature was 50 °C isothermal. All other volatile compounds (isobutane, VC, pentane, methane, and acetylene) were analyzed on a Hewlett Packard 6890 Series Gas Chromatograph with a flame ionization detector (GC-FID) and a capillary column (Agilent Technologies 115-3432 GS-Q). Helium was used as the carrier gas at a flow rate of 15 mL/min and oven temperature was 150° C except for when methane and acetylene were present together, in which case a temperature ramp method was used (70°C-90°C 10°C/min, 90°C-155°C 30°C/min). O₂ and CO₂ were measured using Hewlett Packard 5890 Series Gas Chromatographs with thermal conductivity detectors (GC-TCD) and capillary columns (Supelco 60/80 Carboxen 1000). Carrier gas for oxygen measurements was helium, and carrier gas for CO₂ measurements was argon, both at flow rates of 30 mL/min. The oven temperatures were 40° C and 220° C respectively.

For 1,4-D analysis, liquid samples stored at 4° C in microcentrifuge tubes were used. A sub sample (0.2 mL) was diluted into 40mL VOA vials with DI water (25 mL) to reach concentrations of 1,4-D below 20 μ g/L. An internal standard of deuterated 1,4-D was added to each VOA vial from a concentrated stock solution for a final concentration of 5 μ g/L. For analysis, 5 mL of solution was taken from each VOA via a Teledyne Tekmar AQUATek100 autosampler and run through a Teledyne Tekmar Lumin PTC purge-and-trap before measuring on a Hewlett Packard 6890 Series Gas Chromatograph with a mass selective detector (GC-MS), (Hewlett Packard 5873), and a capillary column (Restk Rtx-VMS). The GC-MS was run in single ion mode for each type of sample, isolating regular and deuterated 1,4-D (m/z 88 and 96. Additional details of the purge-and-trap methods used are provided by Rolston et al. (2019).

An approximation of the limit of detection for the instrumentation and methods detailed above can be defined as a peak area of 5 in chromatograph results. For the CAHs monitored in the following experiments, limits of detection are less than one μ g/L in the aqueous phase for all compounds except cis-DCE which has a limit of 7 μ g/L. Samples for 1,4-D analysis were diluted 50-125 times resulting in an effective limit of 5-12.5 μ g/L.

Activity-based labeling

Fluorescent labeling of components of the SCAM enzyme was done based on procedures detailed in Bennett et al. (2016) and described here in brief. The first step includes the inactivation of monooxygenase enzymes in active cells using a 1,7-octadiyne probe (17OD) which reacts with amino acids in the enzyme's active sites irreversibly inactivating enzyme function. This is followed by a reaction between the probe and an azide-containing fluor. This results in polypeptides that are fluorescently labeled and can then be separated and visualized by SDS-PAGE and NIR scanning, methods also found in Bennett et al. (2016). Murnane et al. (2021) presents labeling results of ATCC 21198 after growth on isobutane and various alcohols.

Results

Growth substrates for R. rhodochrous ATCC 21198

R. rhodochrous ATCC 21198 has been shown to grow on a variety of gaseous alkanes including ethane, propane, *n*-butane and *iso*-butane (Babu & Brown, 1984; Hou et al., 1983; MacMichael & Brown, 1987). This bacterium also grows on a variety of substrates including organic acids, esters, dextrose and vanillin. The following is a comprehensive list of substrates that were tested and resulted in growth of this bacterium: acetate, lactate, butyrate, ethanol, 1-butanol, 2-butanol, 2-ethyl butanol, benzyl alcohol, dextrose, vanillin, isobutane, ethyl lactate, propionate, butyrate, 1-propanol, and 2-propanol. Rate tests have been performed for many of these substrates, detailed in the following sections.

The specific propylene-, THF- and 1,4-D- oxidizing activities were determined for cells grown on gaseous alkanes and their corresponding primary and secondary alcohols as well as three acids: acetate, propionate and butyrate (Figure 4.1). Results indicate that with the exception of cells grown on acetate, 2-propanol, and 2-butanol, cells grown on potential alkane-derived metabolites do not initially oxidize any of the three tested compounds (propylene, THF, or 1,4-D) at a significant rate compared to alkane grown cells. Consistent trends are seen between the rates of transformation of 1,4-D, THF, and propylene with THF most rapidly transformed and propylene the slowest for cells grown on all substrates. THF was transformed at a factor of 2 times greater than 1,4-D and 4-5 times greater than propylene. Growth on isobutane and *n*-butane yielded similar rates, the highest for all growth substrates, of the gaseous substrates, propane yielded the slowest rate. Similar oxidation rates were observed for cells grown on both 2-propanol and 2-butanol, these rates were about an order of magnitude lower than those observed with cells grown on isobutane or *n*-butane.

In addition to examining the specific activity of these different cell types, activity-based protein fluorescent labeling of cells grown on each of these substrates. These results are summarized in Figure 4.2. These results mirror those of the oxidizing activity tests. High levels of labeling was observed for all alkanes tested: ethane, propane, *n*-butane, and isobutane. Again, with the possible exception of cells grown on 2-butanol, no significant labeling of components of SCAM was observed in any cell type other than cells grown on alkanes. In combination, the ABL studies (Figure 4.2) and activity measurements using several different potential monooxygenase substrates (Figure 4.1) strongly suggest that SCAM is produced to significant levels in cells grown on alkanes, and more minimally in cells grown on 2-butanol, 2-propanol, and potentially acetate.

Resting cell transformations of CAHs and 1,4-D by R. rhodochrous ATCC 21198

Single compound rate tests were performed using *R. rhodochrous* ATCC 21198 grown on several alkanes: isobutane, ethane, and propane, as well as 2-butanol to evaluate primary substrates effective at inducing the cometabolic transformation of CAHs and 1,4-D. An example of the typical results of these rate tests is depicted in Figure 4.3 showing the total mass of 1,1-DCE (A) and 1,4-D (B) per mg TSS over time for active and control reactors of 2-butanol grown *R. rhodochrous* ATCC 21198. Experiments were done at varying contaminant concentrations but were kept consistent between cell types. In the rate test shown in Figure 4.3 the transformation of the initial mass of 1,1-DCE occurs relatively rapidly over the first two days of the experiment, the second addition is then transformed more slowly and not completely. For 1,4-D, 2-butanol grown cells were able to fully transform multiple additions for up to 10 days before transformation ceased. Acetylene was effective at blocking the monooxygenase enzymes responsible for cometabolism

as substrate concentrations in control reactors remained constant between acetylene and abiotic treatments. The minor reduction of mass in these controls are likely due to leakage over time and sorption to the septa. The total mass of contaminant transformed before cell activity ceased was normalized to the mass of cells used, in mg TSS, to estimate the transformation capacity of *R. rhodochrous* ATCC 21198 for each compound.

Rate tests for 2-butanol grown *R. rhodochrous* ATCC 21198 of cis-DCE, 1,1-DCA, 1,1,1-TCA, and VC had 4-8 hour lag phases before transformation was observed, while tests of 2-butanol grown *R. rhodochrous* ATCC 21198 of 1,1-DCE and 1,4-D exhibited no lag period. The cells used for the 1,1-DCE rate test was stored for 5 days before inoculation into reactors, in all other tests cells were used 0-1 days after harvesting. This extended storage may have contributed to the lack of lag time in 1,1-DCE compared to other CAHs. The 4-8 hour lag phase observed from *R. rhodochrous* ATCC 21198 grown on 2-butanol was not observed for other growth substrates: isobutane, propane, and ethane, where transformation appears to begin instantaneously.

A comparison of the zero order initial transformation rates for each CAH of interest and 1,4-D for isobutane, ethane, propane, and 2-butanol grown *R. rhodochrous* ATCC 21198 is shown in Figure 4.4. Zero order initial rates are calculated from regions exhibiting linear rates of degradation, when a lag was observed time points after the lag were considered. Zero order rates of transformation followed a general trend of less chlorinated compounds (1,1-DCE, cis-DCE) having a higher transformation rate as compared to more chlorinated compounds (1,1,1-TCA, and TCE) for all cell types. 2-butanol grown *R. rhodochrous* ATCC 21198 decreased as follows VC > cis-DCE > 1,1-DCE > 1,4-D > 1,1,1-TCA > TCE, isobutane grown cells deviated from this in that 1,1-DCE had the highest transformation rate. This follows a general trend of less chlorinated compounds having higher transformation rates as compared to more chlorinated compounds that has been observed in most studies of aerobic cometabolism (Semprini, 1997). The transformation rate of 1,4-D was comparable to the less chlorinated compounds, all of which were significantly higher than that of 1,1,1-TCA. Consistent with results presented in Figure 4.1, rates with propane grown cells are slower than rates obtained with isobutane- and ethane-grown cells.

The zero-order rates of 2-butanol grown compared to isobutane-grown cells are shown in Table 4.2. Transformation rates of 2-butanol grown cells are below 10% of the corresponding isobutane grown cells for each compound tested, with 1,1-DCE, 1,4-D and 1,1,1-TCA less than 1%. Ratios between 2-butanol- and isobutane-grown *R. rhodochrous* ATCC 21198 rates varies from 8% for cis-DCE to 0.04% for 1,1-DCE, which had the fastest rate when grown on isobutane. 2-butanol-grown *R. rhodochrous* ATCC 21198 exhibited less variability in zero order rates between compounds with 1-2 chlorines than isobutane-grown cells. 2-butanol-grown cells also had lower 1,1-DCE transformation rates relative to other compounds, possibly due to longer exposure to the toxic compound as a result of slower cometabolic ability.

The transformation capacity of 2-butanol grown 21198 for the same collection of contaminants is presented in Table 4.2. The transformation capacities of less chlorinated compounds, VC, cis-DCE, and 1,1 DCA, were similar to each other and were around two orders of magnitude higher than 1,1,1-TCA. The exception to this was 1,1 DCE which had one of the lowest transformation capacities, more similar to 1,1,1-TCA. This is likely due to the formation of an intermediate epoxide as a cometabolic product, this compound has been shown to have highly toxic effects compared to transformation products of other chloroethenes on bacteria using a variety of oxygenase enzymes systems (Doherty, 2000; Dolan & McCarty, 1995). The transformation capacities of cis-DCE and 1,1-DCE for isobutane-, propane-, ethane-, and 2-

butanol-grown 21198 are presented in Figure 4.5. It is interesting to note that the transformation capacity of cis-DCE is higher for 2-butanol-grown cells compared to cells grown on all three other substrates tested including isobutane. Generally the transformation capacity of cells grown on the various substrates decreased as follows ethane-grown > isobutane-grown > propane-grown.

The transformation of cis-DCE by 2-butanol-grown *R. rhodochrous* ATCC 21198 provides some additional insight into the mechanics of cometabolism. The cometabolic product, referred to here as cis-DCE epoxide, is easily measurable through the same analytical methods as cis-DCE. The data from this rate test is detailed in Figure 4.6(A) and narrowed to the first 1.5 days (B) revealing the details of early time points. The cis-DCE epoxide was measured 20 minutes after cells were added and before observable transformation of cis-DCE, after a 6 hour lag. This suggests SCAM was present at the start of the rate test, and that the lag in cis-DCE transformation observed may be due to an inability to detect a difference in mass in headspace measurements. Increased induction of the monooxygenase enzyme expression might have occurred with an acceleration in rates, however more research is needed to assess possible induction mechanisms. Following the second addition of cis-DCE, the transformation of the cis-DCE epoxide slowed and a more extended time period was required for the reduction in concentration. These results support the biotic transformation of the epoxide during the first addition, and as the cells reach exhaustion the transformation of both cis-DCE and the epoxide slow. The production of the epoxide provides strong evidence of the cometabolic transformation observed with 2-butanol-grown cells.

Transformation of mixtures of CAHs and 1,4-D by alcohol-grown R. rhodochrous ATCC 21198

Pure culture rates tests were performed with *R. rhodochrous* ATCC 21198 grown on three variations of butanol; 1-butanol, 2-butanol, and 2-ethyl-1-butanol to test their ability to cometabolize a mixture of contaminants in batch reactors. These three compounds were chosen as primary substrates as they can be produced by an orthosilicate as a slow-release compound, are relatively non-toxic, and are hypothesized to induce enzyme expression necessary for cometabolism. After growth on the alcohols, resting cells in a minimal growth media were exposed to a mixture of cis-DCE, 1,1,1-TCA, and 1,4-D. ATCC 21198 grown on all three alcohol substrates was able to transform the contaminants. Acetylene and abiotic controls showed minimal to no loss of mass, indicating cometabolism was the process responsible for contaminant loss. Cells grown on all substrates transformed cis-DCE at the highest first order transformation rate. Over 99% of cis-DCE was transformed by cells grown on all substrates, while 1,1,1-TCA and 1,4-D were only fully transformed by 2-butanol-grown cells. Due to co-elution of large masses of 1,1,1-TCA and cis-DCE epoxide in GC analysis, initial small epoxide peak areas were not detected.

Results from 1-butanol-grown *R. rhodochrous* 21198 are shown in Figure 4.7. The transformation of cis-DCE occurred most rapidly after a lag period of 8-12 hours with no change in concentration. The cis-DCE is transformed to below the detection limit of instrumentation, >99% after 6 days of incubation. The presence of cis-DCE epoxide is first observed at 8 hours of incubation before any change in mass of cis-DCE is measurable. Transformation of 1,1,1-TCA had a similar lag period of 8-12 hours, but occurred at a slower rate and complete transformation was not achieved, with an average of $79 \pm 10\%$ reduction in mass. Transformation of 1,4-D occurred at a similar rate to 1,1,1-TCA, but to the least extent with $69 \pm 3\%$ reduction in mass after an 8-12 hour lag phase. No further transformation of contaminants was observed after 5 days of incubation.

The results for 2-butanol-grown *R. rhodochrous* ATCC 21198 are shown in Figure 4.8. The transformation of both cis-DCE and 1,1,1-TCA lagged for between 8-12 hours. Cis-DCE is transformed the most rapidly, followed by 1,1,1-TCA then 1,4-D. Similar to observations with 1-butanol and 2-ethyl-1-butanol-grown cells, cis-DCE is transformed to below the detection limit of instrumentation in two out of three reactors, >99%. Transformation of 1,1,1-TCA and 1,4-D occurred to a similar extent, >99% and >99.9% respectively. Cis-DCE epoxide was first measured at 8 hours of incubation, similarly to 1-butanol-grown *R. rhodochrous* ATCC 21198. This is before measurable change in mass of cis-DCE. The formation of the cis-DCE epoxide peaked more rapidly compared to the test with cells grown on the other two substrates, with highest concentrations observed at 1.1 days compared to 2.0 and 3.1 days with 1-butanol and 2-ethyl-1-butanol-grown *R. rhodochrous* ATCC 21198. The cis-DCE epoxide was also transformed most rapidly by 2-butanol grown cells. The first-order transformation rate of 1,4-D was faster than 1,1,1-TCA in these reactors, with a slower linear phase followed by more rapid transformation that more closely resembles a first-order reaction. Results from 2-ethyl butanol-grown *R. rhodochrous* ATCC 21198 are included in the supplementary information. The 8-12 hour lag phase observed from *R. rhodochrous* ATCC 21198 grown on 1-butanol or 2-butanol was not observed for *R. rhodochrous* ATCC 21198 grown on other substrates isobutane, propane, isobutene, and ethane, where transformation appears to begin instantaneously.

As the batch reactors with 2-butanol-grown cells were able to fully transform the initial contaminants unlike other substrate grown cells, another addition of the same mass of 1,1,1-TCA and cis-DCE was added to investigate continued transformation capacity. In the second addition, transformation of cis-DCE occurs more rapidly at a rate of 0.462 1/days/mgTSS while 1,1,1-TCA was transformed at a similar rate to the first addition. Neither compound appears to lag before transformation of the second addition began, however less frequent data points were collected as compared to the first addition. This is particularly evident for 1,1,1-TCA where after 18 hours over 40% of the compound has been transformed compared to just 9% at a similar time point after the initial addition. This suggests continued enzyme induction once initial induction has occurred.

A comparison of first order rates of each contaminant for the three growth substrates is presented in Figure 4.9, rates were calculated after the lag period for each compound. Cells grown on 2-butanol had the fastest rates of transformation for each contaminant followed by cells grown on 1-butanol and then cells grown on 2-ethyl-1-butanol. Rates of cis-DCE transformation were highest for each growth substrate. Rates of transformation by 2-butanol grown cells were 4 times higher than 1-butanol grown for both 1,1,1-TCA and 1,4-D, but only 1.4 times higher for cis-DCE. Rates of 1,4-D and 1,1,1-TCA transformation were similar for each 1-butanol and 2-ethyl-1-butanol grown cells.

Transformation of 1,1,1-TCA and 1,4-D by R. rhodochrous ATCC 21198 and Alternative Microbes grown on Acetate and sec-Butyl Acetate

Rates of 1,1,1-TCA and 1,4-D transformation in mixtures were determined with three different strains; *R. rhodochrous* ATCC 21198, *Rhodococcus rhodochrous* ATCC 21197 and an isobutane-utilizing isolate, 4CI. *R. rhodochrous* ATCC 21198 was grown on three different substrates: isobutane, 2-butanol, and sec-butyl acetate, *Rhodococcus rhodochrous* ATCC 21197 and isolate 4CI were grown on acetate. sec-Butyl acetate is a potential slow-release substrate as it is an ester that undergoes hydrolysis to form 2-butanol and acetate. 1,1,1-TCA and 1,4-D were completely transformed by *R. rhodochrous* ATCC 21198 grown on isobutane or sec-butyl acetate within 24 hours. The rates of transformation by cultures grown on acetate were slower, with 90%

transformed within 120 hours Figure 4.10 (A-B). The initial transformation rates for each compound are presented in Table 4.3. The rates of 1,1,1-TCA and 1,4-D transformation by *R. rhodochrous* ATCC 21198 grown on isobutane were 0.319 $\mu\text{mol}/\text{mg}$ TSS-day and 0.17 $\mu\text{mol}/\text{mg}$ TSS-day, respectively (Table 4.3). When *R. rhodochrous* ATCC 21198 was grown on 2-butanol, rates of transformation for both 1,1,1-TCA and 1,4-D decreased when compared to isobutane-grown cells by factors of 7.6 and 6.8, respectively (Table 4.3). These tests, however, were conducted at lower aqueous concentrations than the tests using isobutane-grown cells. It is of interest to note that when *R. rhodochrous* ATCC 21198 was grown on sec-butyl acetate, the rates of 1,1,1-TCA and 1,4-D were reduced by factors of only 2.9 and 1.8 of those obtained with isobutane-grown cells (Table 4.3). In these tests, the aqueous concentrations were similar to the tests conducted with isobutane-grown cells.

Based on the transformation of 1,1,1-TCA and 1,4-D by the ester sec-butyl acetate grown *R. rhodochrous* ATCC 21198, two new cultures 4CI and *R. rhodochrous* ATCC 21197 were tested. When grown on acetate, slow rates of 1,1,1-TCA and 1,4-D transformation were achieved for both 4CI and *R. rhodochrous* 21197 strains (Figure 4.10). The 1,1,1-TCA and 1,4-D transformation rates were reduced by a factor of 17.7 and 2.9 respectively compared to those achieved by isobutane-grown cells (Table 4.3). Rates of single compound of 1,1,1-TCA and 1,4-D transformation with *R. rhodochrous* ATCC 21197 grown on acetate had similar rate for 1,1,1-TCA in the mixture, while 1,4-D rate decreased by a factor of 1.8 (Table 4.3). Without the addition of 1,4-D the 1,1,1-TCA transformation rate when *R. rhodochrous* ATCC 21198 culture was grown on 2-butanol is about an order magnitude higher (0.136 $\mu\text{mol}/\text{mg}$ TSS-day) compared to *R. rhodochrous* ATCC 21197 culture grown on acetate (0.018 $\mu\text{mol}/\text{mg}$ TSS-day) as presented in Table 4.3. There was no difference in the transformation rate of 1,1,1-TCA in the mixture with 1,4-D (0.018 $\mu\text{mol}/\text{mg}$ TSS-day) to that obtained with only 1,1,1-TCA (Table 4.3).

Overall, data shows acetate grown cultures were not as effective in transforming 1,1,1-TCA and 1,4-D compared to isobutane and 2-butanol-grown cultures. *R. rhodochrous* ATCC 21198 grown on sec-butyl acetate showed good potential for the transformation of mixtures of 1,4-D and 1,1,1-TCA. The tests show a broad mixture of CAHs and 1,4-D can be transformed by *R. rhodochrous* ATCC 21198 grown on 2-butanol. Based on the results of these tests sec-butyl acetate holds promise as a slow-release substrate to be encapsulated with *R. rhodochrous* ATCC 21198.

Discussion

The results of this study show *R. rhodochrous* ATCC 21198 is capable of cometabolic transformations when grown on a variety of butane-derived alcohols. The extended lag phase of 8-12 hours before transformation occurred in both 2-ethyl-1-butanol- and 1-butanol-grown cells indicates enzyme induction may not have been a result of the growth substrate. ABL showed primary alcohols including 1-butanol did not induce the SCAM enzyme in *R. rhodochrous* ATCC 21198, while secondary alcohols including 2-butanol did induce SCAM but to a lesser degree than isobutane. Rate comparisons confirm more SCAM activity in isobutane over 2-butanol-grown *R. rhodochrous* ATCC 21198. In kinetic tests, Murnane et al. (2021) observed an approximately 4-hour lag period in 1,4-D transformation by 1-butanol-grown cells and no lag period in 2-butanol-grown cells. The results of this current study demonstrated a longer lag phase for 1-butanol grown cells of 8-12 hours before transformation of 1,4-D occurred but confirms the earlier results that there was no lag for 2-butanol grown cells for 1,4-D transformation. While both studies report no lag in 1,4-D transformation by 2-butanol grown cells, a 4-8 hour lag was observed for CAHs; cis-

DCE, 1,1-DCA, 1,1,1-TCA, and VC. However cis-DCE epoxide was observed within minutes of cell inoculation and 1,1-DCE was the only CAH to be transformed without a lag by 2-butanol-grown cells. The cells used for this test were stored for 5 days compared to 0-1 days for other tests. These results indicate more studies investigating enzyme expression need to be done before induction mechanisms can be explained, and that conditions such as starvation, growth substrate, and exposure to contaminants or epoxides may play a role.

In long term transformation tests isobutane-grown *R. rhodochrous* ATCC 21198 co-encapsulated with TBOS remained cometabolically active for 200 days in medi. Similar long term activity was seen for this bacterium when it was co-encapsulated with T2BOS (Rasmussen et al., 2020) confirming both non enzyme inducing and enzyme inducing substrates can support extended cometabolic activity. Bacteria in carbon-limited conditions often express multiple enzyme systems (Egli, 2010), a starvation effect could be responsible for delayed enzyme expression in resting 1-butanol grown cells, and cells in the presence of low substrate concentrations like those co-encapsulated with TBOS or T2BOS.

Conclusions

- Growth of *R. rhodochrous* ATCC 21198 was found to be supported by various alcohols, organic acids, esters and various other organic compounds. However, only growth on 2-butanol resulted in significant labeling of SCAM enzymes and the ability to oxidize 1,4-D, propylene, and THF.
- In single compound rate tests, 2-butanol grown *R. rhodochrous* ATCC 21198 exhibited faster transformation rates for less chlorinated compounds (VC, cis-DCE, 1,1-DCE, 1,1-DCA) than more chlorinated compounds (1,1,1-TCA, TCE). The rates of transformation are ranked as follows from high to low: VC > cis-DCE > 1,1-DCA > 1,1-DCE > 1,4-D > 1,1,1-TCA > TCE. All rates were significantly lower than alkane grown cells and less than 10% of isobutane-grown cells for each compound.
- 2-butanol grown *R. rhodochrous* ATCC 21198 exhibited a lag of 4-8 hours before cometabolic transformation of most CAHs was observed including cis-DCE, however production of cis-DCE epoxide was measured with no lag. 1,4-D transformation also exhibited no lag phase before transformation began. The mechanism for potential enzyme induction in 2-butanol grown *R. rhodochrous* ATCC 21198 requires further investigation.
- 1-Butanol, 2-butanol, and 2-ethyl-1-butanol grown *R. rhodochrous* ATCC 21198 were capable of cometabolic transformation of 1,4-D and a variety of CAHs. Cells grown on 2-butanol had the largest transformation capacity and fastest rates and were able to transform the mixture of 1,4-D, cis-DCE, and 1,1,1-TCA to >99%. This demonstrates 2-butanol provides the most efficient transformation results of the alcohols tested. In mixtures, cells grown on these alcohols preferentially transforms cis-DCE over both 1,1,1-TCA and 1,4-D.
- Sec-Butyl acetate has a potential to be used as a slow-release substrate. *R. rhodochrous* ATCC 21198 grown on this ester transformed a mixture of 1,1,1-TCA and 1,4-D at rates faster than 2-butanol grown cells.
- Pure cultures of acetate grown *R. rhodochrous* ATCC 21197 and isolate 4CI were capable of transforming 1,1,1-TCA and 1,4-D, however, transformation rates were found to be a factor of 17.7 and 2.9 times slower than isobutane grown *R. rhodochrous* ATCC 21198.

Tables and Figures

Contaminant	Mass Added (μg)	Initial Liquid Concentration (μM)	Biomass (mgTSS)
VC	181	101	5.1
1,1 DCE	10.9	7	4.8
1,1 DCA	126	87	5.0
<i>cis</i> -DCE	280	645	11.4
1,1,1 TCA	129	23.24	10.9
TCE	12.8	12.7	12.1
1,4-D	3.4	38.2	5.4

Table 4.1. Single compound rate test experimental design parameters including mass of contaminant added (μg), measured initial liquid concentrations (μM), and biomass (mgTSS).

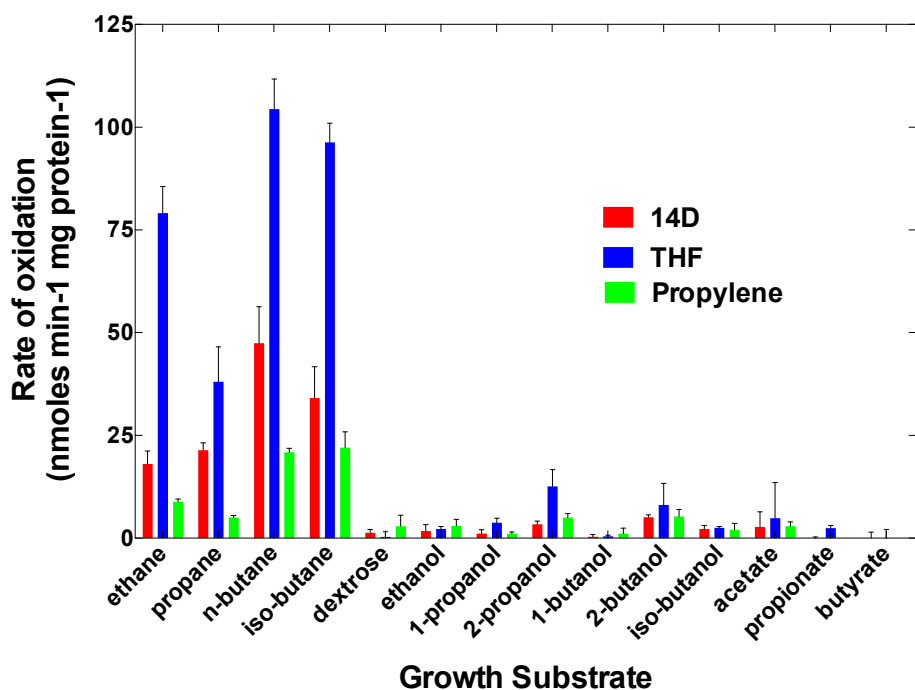


Figure 4.1. Specific rates of THF, 1,4-D, and propylene oxidation by cells of *R. rhodochrous* ATCC 21198 grown on gaseous alkanes and their corresponding 1° and 2° alcohols and acids. Results presented are the mean and SD of three biological replicates.

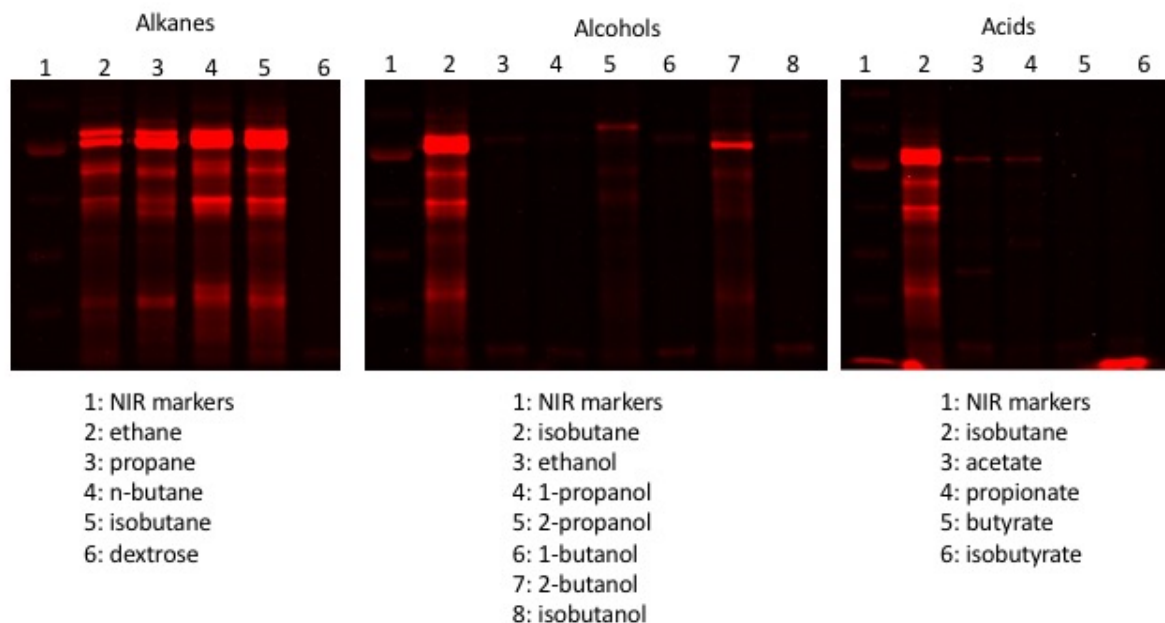


Figure 4.2. Activity-based labeling of *R. rhodochrous* ATCC 21198 after growth on the indicated substrates and subsequent activity-based labeling using 17OD as a probe for the SCAM.

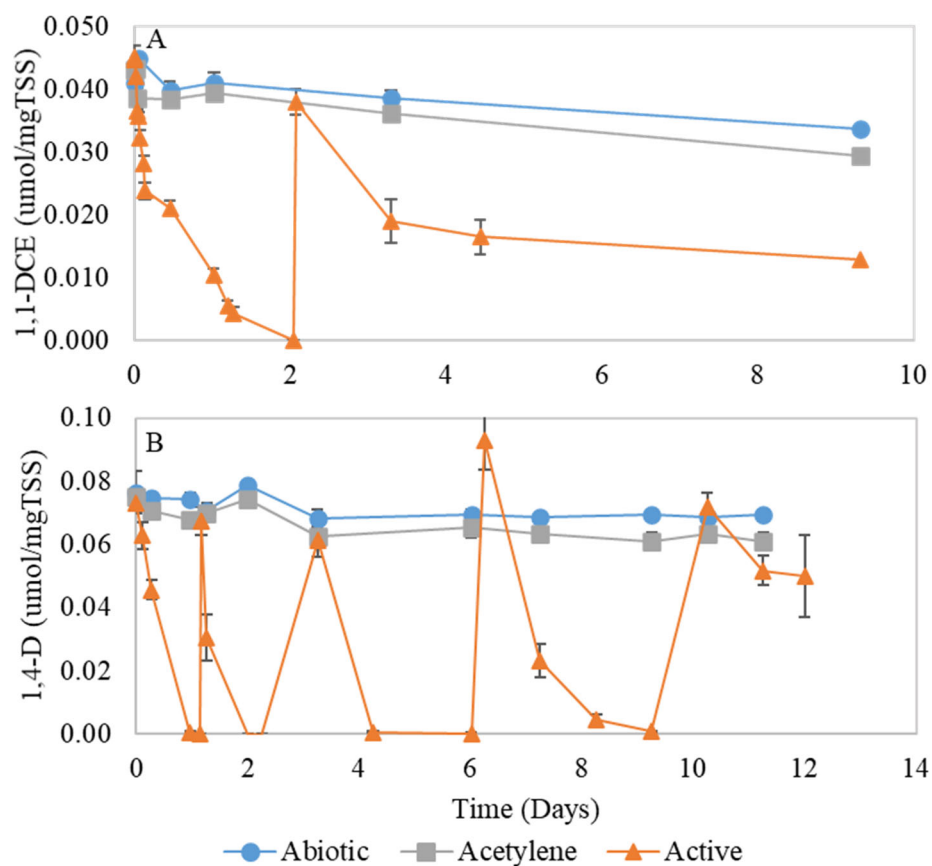


Figure 4.3. Cometabolic transformation of 1,1-DCE (A) and 1,4-D (B) by 2-butanol-grown resting *R. rhodochrous* ATCC 21198 in single compound tests. Total mass of 1,1-DCE and 1,4-D is normalized to initial biomass ($\mu\text{mol/mgTSS}$). Error bars indicate standard deviation of replicates.

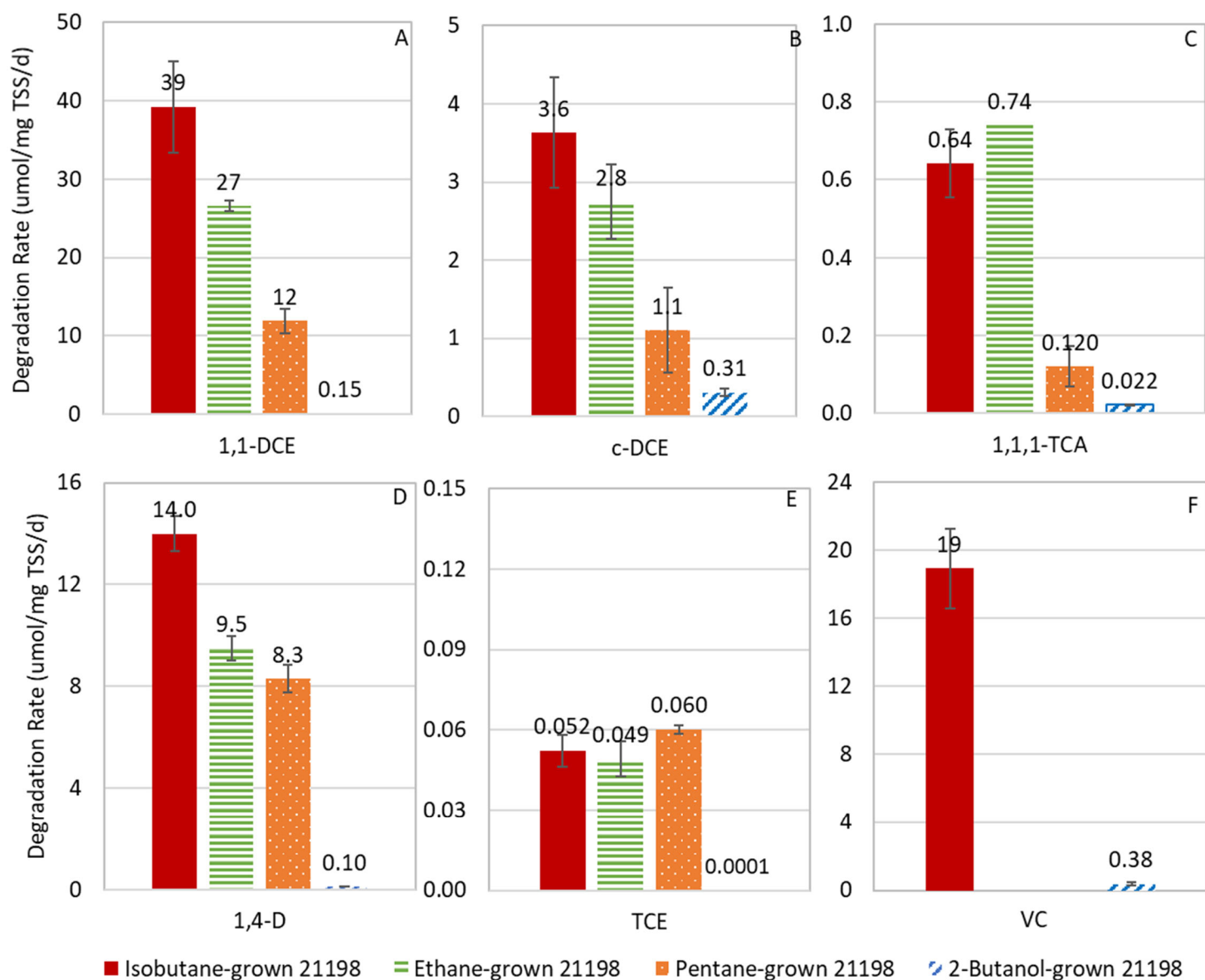


Figure 4.4. Zero order transformation rates of 1,1-DCE (A), *cis*-DCE (B), 1,1,1-TCA(C), 1,4-D (D), TCE (E), and VC (F) for isobutane-, ethane-, pentane-, and 2-butanol-grown *R. rhodochrous* 21198. Error bars indicate the standard deviation of a two or three sample average.

	Zero-order Transformation Rates ($\mu\text{mol}/\text{mgTSS}\cdot\text{day}$)		Transformation Capacity ($\mu\text{mol}/\text{mgTSS}$)	
	2-Butanol	Isobutane	2-butanol	Isobutane
VC	0.377	18.9	0.878	2.46
1,1 DCA	0.267	n/a	1.07	
<i>cis</i> -DCE	0.307	3.64	1.18	0.56
1,1-DCE	0.149	39.3	0.0674	0.658
1,4-D	0.102	22.2	0.0639	
1,1,1 TCA	0.022	0.642	0.0581	
TCE	0.0	0.052	0.00985	

Table 4.2 Zero-order transformation rates and transformation capacities for 2-butanol- and isobutane-grown *R. rhodochrous* ATCC 21198.

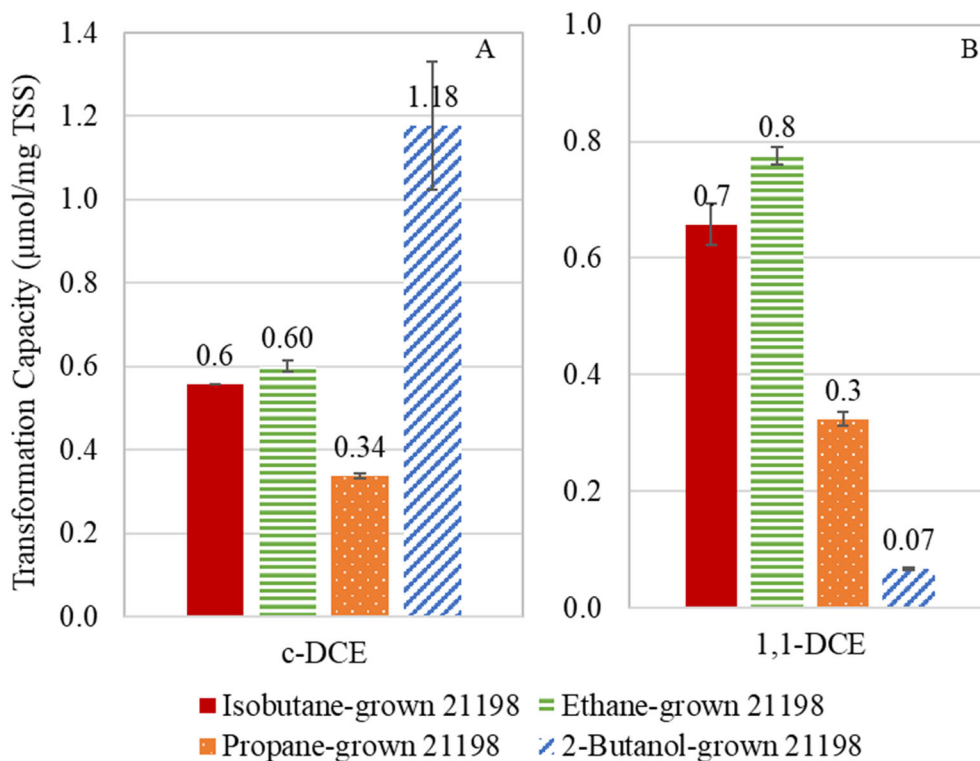


Figure 4.5. Transformation capacities of *cis*-DCE (A) and 1,1-DCE (B) for isobutane-, propane-, ethane-, and 2-butanol-grown *R. rhodochrous* 21198. Error bars indicate the standard deviation of a three sample average.

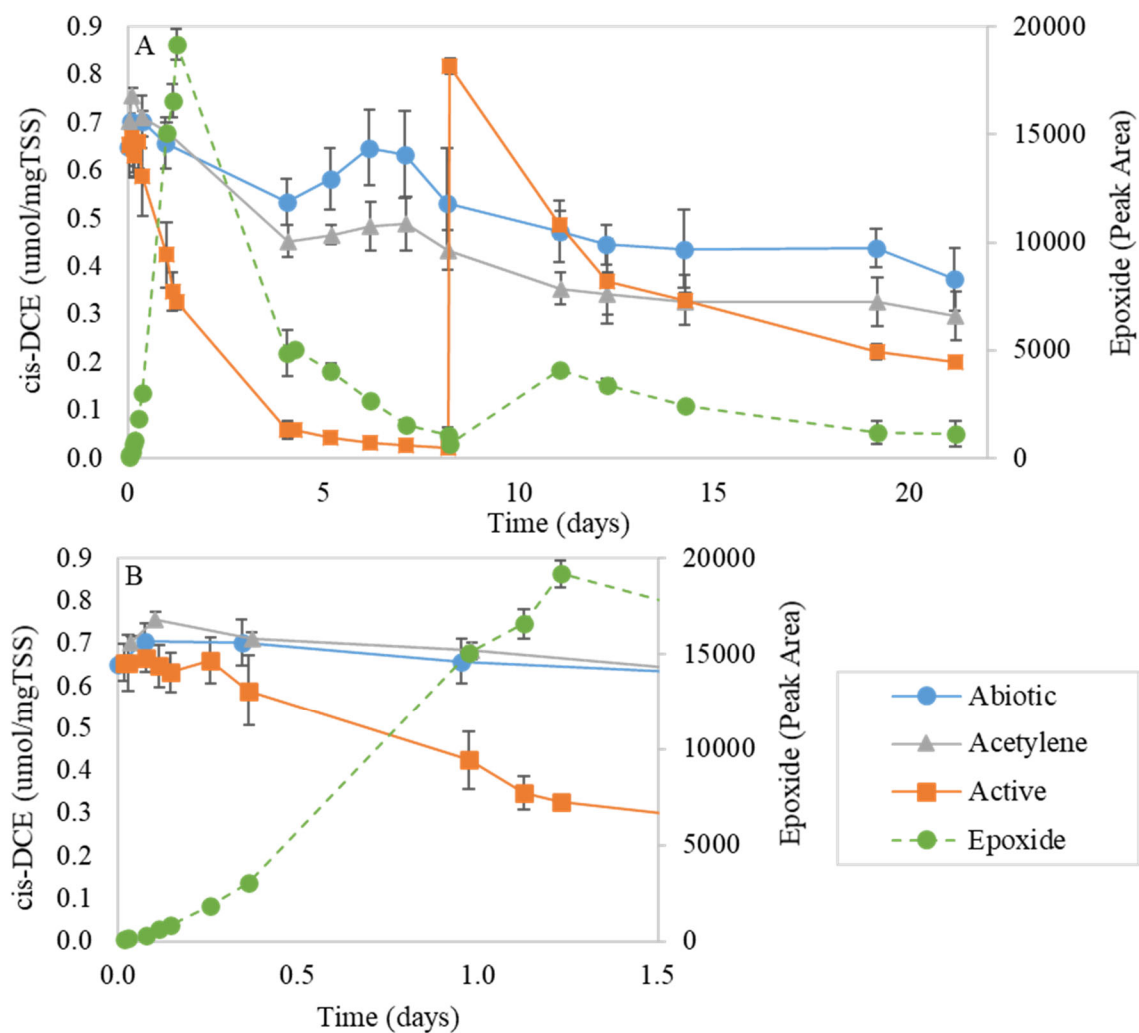


Figure 4.6. Single compound rate test results for 2-butanol-grown *R. rhodochrous* 21198 and *cis*-DCE. Full duration of the experiment to the cell's capacity (A) and narrowed to the first 1.5 days (B). Error bars represent standard deviation of replicates.

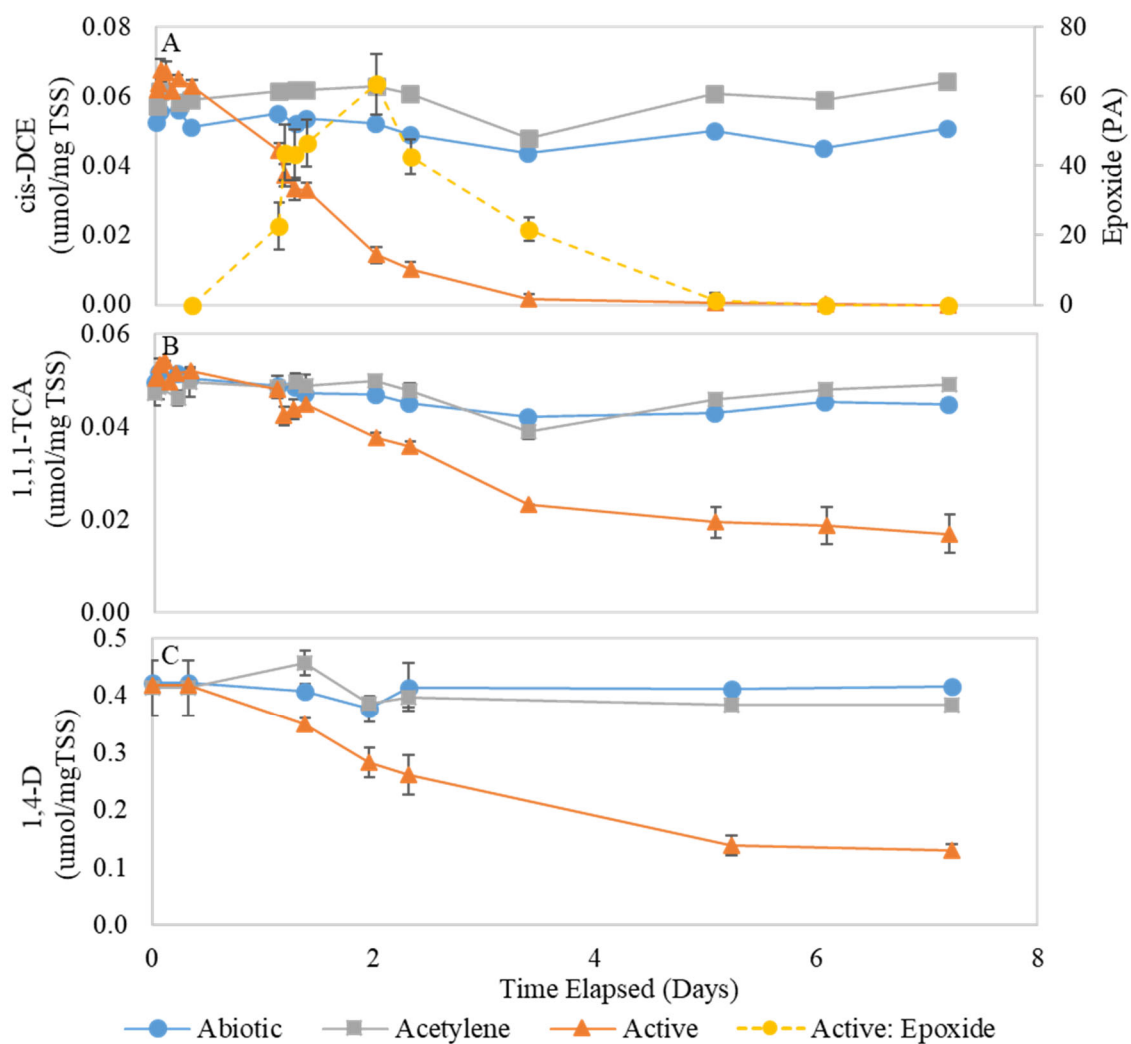


Figure 4.7. Transformation of a mixture of *cis*-DCE (A), 1,1,1-TCA (B), and 1,4-D (C) 5mg of 1-butanol-grown *R. rhodochrous* 21198. Data points and error bars represent the average and standard deviation of triplicates

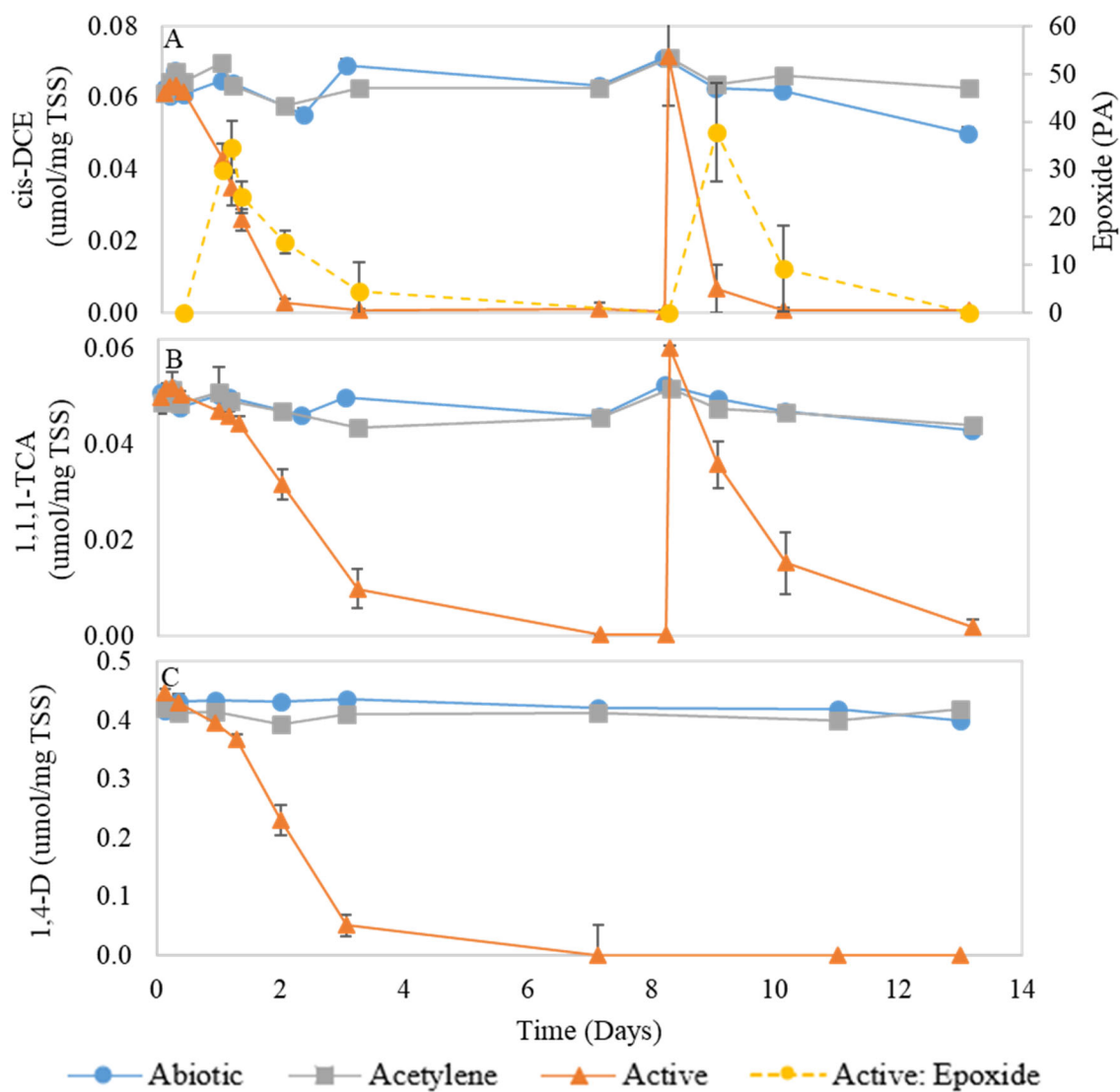


Figure 4.8. Transformation of a mixture of *cis*-DCE (A), 1,1,1-TCA (B), and 1,4-D (C) 5mg of 2-butanol-grown *R. rhodochrous* 21198. Data points and error bars represent the average and standard deviation of triplicates.

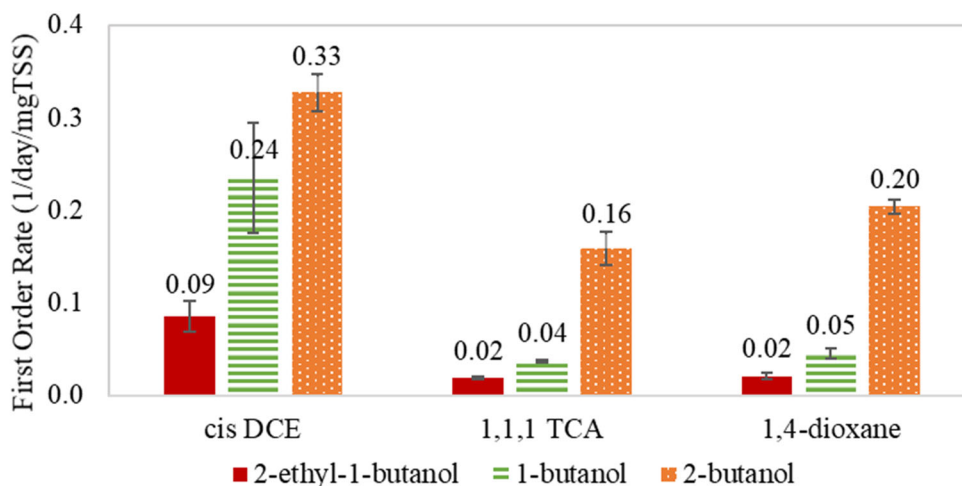


Figure 4.9. Comparison of first order transformation rates of *cis*-DCE, 1,1,1-TCA, and 1,4-D in a mixture by *R. rhodochrous* ATCC 21198 grown on various alcohols.

Culture and Growth Substrate	Contaminant	CL ₀ (uM)	Rate (μmol/mgTSS-day)
21198 Grown on Isobutane			
	1,1,1-TCA	12.2	0.319
	1,4-D	28.9	0.170
21198 Grown on 2-Butanol			
	1,1,1-TCA	2.80	0.042
	1,4-D	6.50	0.047
	1,1,1-TCA only	9.6	0.140
21198 Grown on sec-Butyl Acetate			
	1,1,1-TCA	12.2	0.109
	1,4-D	33.0	0.094
4CI Grown on Acetate			
	1,1,1-TCA	9.86	0.019
	1,4-D	30.9	0.021
21197 Grown on Acetate			
	1,1,1-TCA	9.86	0.018
	1,4-D	34.1	0.057
	1,1,1-TCA only	9.70	0.018
	1,4-D only	38.3	0.107

Table 4.3: Summary of transformation rates of a mixture of 1,1,1-TCA and 1,4-D by *R. rhodochrous* ATCC 21198, *R. rhodochrous* ATCC 21197, and 4CI grown on different substrates.

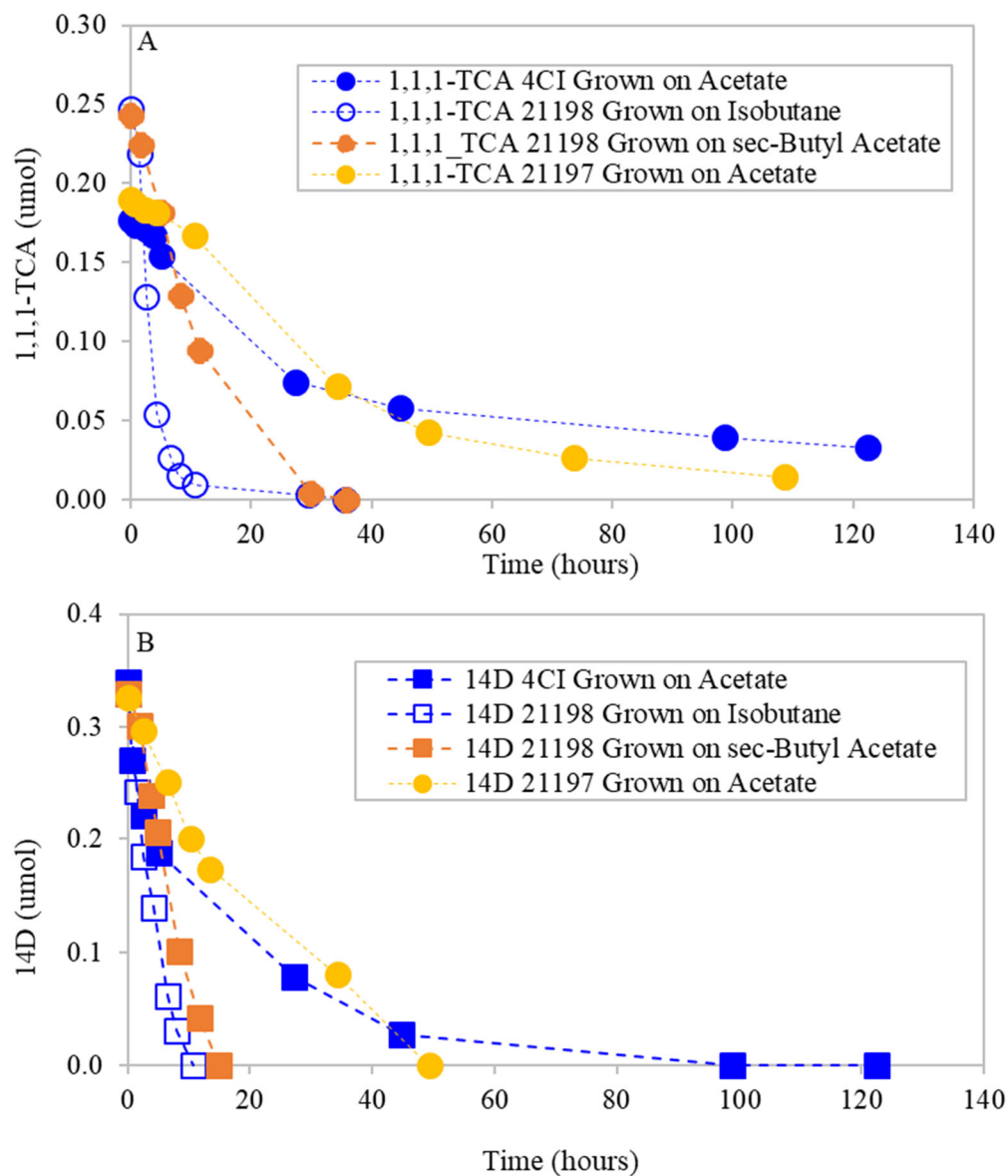


Figure 4.10. 1,1,1-TCA (A) and 1,4-D (B) transformation in a mixture of 1,1,1-TCA and 1,4-D by isobutane- and sec-butyl acetate-grown *R. rhodochrous* ATCC 21198 and acetate-grown cultures of 4CI and *R. rhodochrous* ATCC 21197.

Chapter 5. Growth of G4 on Alcohols and Esters and the Transformation of Chlorinated Ethenes

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Abstract

Benzyl alcohol was assessed as a food-grade growth substrate for promoting the cometabolism of TCE, via the toluene-2-monooxygenase (T2MO) of *Burkholderia vietnamiensis* G4. Benzyl alcohol was evaluated by comparing the level of induction of the T2MO to that seen in toluene-grown cells. The level of T2MO expression was evaluated by two methods: 1) ABL; a gel assay method used to identify catalytically active monooxygenases (e.g. T2MO), and 2) resting cell kinetic tests occurring over the course of days to weeks. Other substrates were also tested for their ability to support TCE cometabolism including phenol, benzyl acetate, benzyl butyrate, acetate, butyrate, and lactate. Both the ABL assay and resting cell tests demonstrated the level of T2MO expression in cells grown on benzyl alcohol was comparable to that observed with toluene, with similar levels of fluorescence detected and similar TCE transformation rates and capacities. The benzyl esters induced T2MO expression, with benzyl butyrate having the highest TCE transformation rate and capacity of all substrates tested. Acetate, butyrate, and lactate showed minimal levels of induction, verifying benzyl alcohol was responsible for T2MO expression in benzyl ester grown cells and growth on lactate could not support cometabolism. ABL assay and resting cell kinetic tests largely agreed, demonstrating that ABL is a reliable method of determining suitable substrates to support cometabolism. Additionally, kinetic tests with either substrate benzyl alcohol or toluene and TCE were performed to determine whether, like toluene, benzyl alcohol competitively inhibits contaminant transformation by T2MO. Results suggest that benzyl alcohol inhibition occurs, but at concentrations greater than 10 mg/L and to a lesser degree than toluene at the same concentration. Finally, kinetics for *cis*-DCE, 1,1-DCE, and VC were determined for cells grown on benzyl alcohol and compared to toluene-grown cells. Toluene-grown cells had higher initial rates for VC, *cis*-DCE, and 1,1-DCE, however, benzyl alcohol-grown cells had approximately twice the transformation capacity for the same compounds.

Introduction

Due to its extensive use in industry as a solvent and degreaser during the twentieth century, TCE, a known human carcinogen, is one of the most common organic groundwater contaminants and a priority pollutant by the USEPA (Moran, 2006). TCE and the fully chlorinated PCE, can also result in the production of *cis*-DCE and VC under anaerobic conditions through reductive dehalogenation with 1,1-DCE and *trans*-1,2-dichloroethene (*trans*-DCE) as minor products (Magnuson et al., 1998; Semprini et al., 1995). In situ bioremediation of chlorinated solvents via aerobic cometabolism is a cost-effective means of treatment and involves the metabolism of a primary substrate via an oxygenase enzyme, which results in the fortuitous transformation of the contaminants (Arp et al., 2001; Frascari et al., 2015; Semprini, 1997). In particular, toluene utilizers are a ubiquitous group of bacteria well studied for the cometabolism of TCE and dichloroethene isomers with toluene or phenol as primary substrates (Azizian et al., 2007; Hopkins & McCarty, 1995; Nelson et al., 1987).

To date, five distinct pathways by bacteria under aerobic conditions have been described for the oxygenase-catalyzed hydroxylation of toluene (Duetz et al., 1994; Laskin, 2008; Shields et al., 1989). Three of these pathways include toluene monooxygenases that hydroxylate toluene at the ortho-, meta-, and para positions, including respectively the toluene-2-monooxygenase of *Burkholderia vietnamiensis* G4, the toluene-3-monooxygenase of *Ralstonia pickettii* PKO1, and the toluene-4-monooxygenase of *Pseudomonas mendocina* KR1 (Laskin, 2008; Shields et al., 1995). The other two pathways include the toluene-2,3-dioxygenase of *Pseudomonas putida* F1 and the catabolic plasmid TOL (the plasmid that specifies the degradation of aromatic hydrocarbons) originally described in *Pseudomonas putida* mt-2 that oxidizes the methyl group of toluene to form benzyl alcohol (Burlage et al., 1989; Shields et al., 1995).

The culture *B. vietnamiensis* G4 has been studied for its ability to degrade TCE through the T2MO pathway when grown on inducing substrates such as phenol and toluene (Folsom et al., 1990; Landa et al., 1994; Leahy et al., 1996; Sun & Wood, 1996; Yeager et al., 2001). Interest in *B. vietnamiensis* G4 for bioremediation applications is due to its ability to sustain high rates of transformation, which is a considerable challenge given most cultures experience some degree of toxicity associated with TCE degradation, whether this is due to the compound itself or transformation products (Arp et al., 2001). It was previously thought that toxicity effects of TCE transformation were insignificant for *B. vietnamiensis* G4 (Folsom et al., 1990), however Yeager et al., 2001 indicates there exists a high toxicity threshold for TCE, beyond which culturability dramatically decreases. In a study examining the effect carbon starvation had on toluene degradation in the four model toluene monooxygenase-expressing bacteria, *B. vietnamiensis* G4 was superior with the lowest deactivation rate by a factor of approximately two (Johnson et al., 2006).

Biostimulation of aquifers with toluene or phenol for *in situ* treatment via aerobic cometabolism is controversial given these substrates are also contaminants. Both toluene and phenol are on the USEPA priority pollutant list with a USEPA maximum contaminant level (MCL) of 1 ppm for toluene and a lifetime health advisory level of 2 ppm for phenol (USEPA, 2018). Another concern is the chlorination of phenol and the corresponding formation of CF (Gallard & von Gunten, 2002). The use of phenol and toluene in pilot-scale field tests was successfully demonstrated at the Moffett field site for the cometabolic treatment of chlorinated ethenes, and at Edwards Air Force Base for treatment of TCE (Hopkins, Munakata, et al., 1993; Hopkins, Semprini, et al., 1993; Hopkins & McCarty, 1995; McCarty et al., 1998). In both instances, phenol

and toluene were degraded well below regulatory levels either at or below 1 ppb. Despite these successful field demonstrations, the use of these aromatic compounds in the field has met resistance due to concerns of using regulated compounds. Another obstacle is that these substrates often act as competitive inhibitors to contaminant oxidation, and a challenge in field applications is the balance of substrate addition to contaminant present (Alvarez-Cohen & McCarty, 1991b; Semprini et al., 1991). If excess substrate is present contaminant oxidation will be inhibited, but conversely if contaminant concentrations are high toxicity becomes a concern and there will be a net drain of reductant resulting in the loss of growth and cometabolism (Alvarez-Cohen & McCarty, 1991a; Arp et al., 2001; Hyman et al., 1995). Both of these phenomena were observed in a chemostat with *B. vietnamiensis* G4 that was fed TCE and toluene as the primary substrate. To test the resilience of the system cells were exposed to a high dose of TCE, which completely inhibited toluene degradation, stopped growth, and caused washout of cells. When the TCE concentration was decreased, degradation only resumed when the toluene concentration in the liquid phase decreased to 1.2 μM (Landa et al., 1994). These inhibition effects may be mitigated in the subsurface by alternate pulsing of substrate and DO, which has the added benefits of limiting biofouling at the injection well and distributing microbial growth (Semprini, 1995).

A biological method of addressing these challenges was through the development of a non-recombinant mutant strain of *B. vietnamiensis* G4 that can constitutively express toluene *ortho*-monooxygenase (TOM) — a large, self-transmissible plasmid where all the toluene degradation genes are found for *B. vietnamiensis* G4 — without aromatic induction (Shields et al., 1995; Shields & Reagin, 1992). *B. vietnamiensis* PR123, developed by Tn5 insertion, degraded TCE when grown on lactate and also degraded *cis*-DCE, *trans*-DCE, and 1,1-DCE (Shields & Reagin, 1992). One study bioaugmented two mutant strains of G4, *B. vietnamiensis* PR123 and PR1301 that could both constitutively express TOM, in small-column microcosms with lactate, though lactate-fed microcosms had a poorer performance than those with phenol additions (Munakata-Marr et al., 1996).

Another method undertaken to address the challenges of regulation and competitive inhibition of toluene and phenol was exploring alternative inducing substrates for cometabolism. A study examined the induction of toluene oxidation activity in various toluene utilizers by non-aromatic substrates including lactate, acetate, glucose, fructose, glutamate and LB broth. In this study *B. vietnamiensis* G4 did not degrade TCE while growing on any of these substrates tested, and it was determined to be the most susceptible to catabolite repression of the cultures examined (Yeager et al., 2004). Another study looked at an alternative aromatic compound—poplar leaf homogenate, which contains natural phenolic compounds—to support TCE degradation and found that the presence of the homogenate was able to promote TCE transformation for *B. vietnamiensis* G4 initially grown on glucose to a greater extent than toluene (Kang & Doty, 2014).

The exploration of alternative inducing substrates for the cometabolic transformation of TCE was investigated here, with the goal of determining whether benzyl alcohol can promote aerobic cometabolism in *B. vietnamiensis* G4. Not only is benzyl alcohol aromatic in structure, it was previously shown that a mixed culture grown on benzyl alcohol was capable of TCE, *cis*-DCE and VC cometabolism (Tejasen, 2003). Benzyl alcohol has the distinct advantages of being a non-toxic and food grade chemical, and hence non-regulated. In addition, benzyl alcohol is easy to handle as a non-volatile liquid. It is also a natural compound found in the essential oil of many plants and used commercially, particularly for cosmetics. The potential of using an ester that hydrolyzes to form benzyl alcohol may have advantages as described below.

This study also looked at the T2MO expression of *B. vietnamiensis* G4 grown on a variety of substrates. The substrates investigated include benzyl alcohol, toluene, phenol, benzyl acetate, benzyl butyrate, acetate, butyrate, and lactate. Given that multiple other studies looked at TCE kinetics for *B. vietnamiensis* G4 grown on phenol and toluene, these substrates were tested to verify rates in this study were comparable to literature values. In addition, toluene grown *B. vietnamiensis* G4 was used as the comparison benchmark for the other substrates. Benzyl butyrate and benzyl acetate, which both hydrolyze to produce benzyl alcohol, were investigated for their ability to support TCE cometabolism. Given that hydrolysis of the esters needs to occur for the release of benzyl alcohol, these slow-release compounds (SRC) have the potential to transport further in the aquifer before becoming bioavailable, resulting in a greater microbial population distribution and limited biofouling at the injection well. In addition, the corresponding carboxylic acids can serve as an additional energy source for the culture to drive cometabolism. These carboxylic acids, acetate and butyrate, were also tested as substrates to verify benzyl alcohol was responsible for transformation with the esters. Finally, lactate was tested as a substrate to compare TCE kinetics with that of the mutant strain of *B. vietnamiensis* G4 that can constitutively express TOM when grown on lactate.

The degree of T2MO expression of *B. vietnamiensis* G4 grown on the various substrates was first evaluated by ABL; a gel assay method used to identify catalytically active monooxygenases (e.g. T2MO). ABL is a recently developed assay method and has been used previously to identify ammonia monooxygenase (AMO) activity (Bennett et al., 2016). The ABP results were then compared to the TCE cometabolic degradation ability for resting cells grown on the same substrates. Based on the results seen with benzyl alcohol as a growth substrate, further TCE kinetic results were performed to determine if competitive inhibition of the T2MO occurs with benzyl alcohol. This was then compared to TCE kinetic tests with toluene present at the same concentrations, as competitive inhibition is a known concern with this substrate (Alagappan & Cowan, 2003; Landa et al., 1994). Finally, benzyl alcohol grown *B. vietnamiensis* G4 was investigated for TCE, 1,1-DCE, *cis*-DCE and VC cometabolic degradation and compared to toluene grown *B. vietnamiensis* G4.

Materials and Methods

Chemicals

TCE (99.5%), benzyl alcohol (100%), and sodium lactate (60% syrup) were purchased from J.T. Baker. Toluene (99.8%), VC (99.5%), 1, 1-DCE (99%), benzyl acetate (99%), sodium acetate (99%), and propyne ($\geq 99\%$) were purchased from Sigma-Aldrich. Benzyl butyrate (97%) and *cis*-DCE (99%) were purchased from TCI. Phenol (99%) and sodium butyrate (98%) were purchased from Alfa Aesar.

Bacterial strain and cell growth

B. vietnamiensis G4 was provided by Dr. Michael Hyman at North Carolina State University, Raleigh, NC. The culture was kept on mineral salt media plates with lactate (20 μ M) as the sole carbon and energy source. Culture was grown in batch in Wheaton serum glass bottles (500 mL) sealed with butyl septa caps containing sterile phosphate buffered mineral salt media (300 mL) (Yeager et al., 1999). The bottles were inoculated from culture kept on agar plates kept at 30°C. The culture was grown on toluene (1.9 mM) or other growth substrates on an equivalent electron donor basis and added as pure compounds, with the exception of acetate and butyrate. Neat liquids were added for toluene (61 μ L), benzyl alcohol (63 μ L), benzyl acetate (70 μ L), and

benzyl butyrate (65 μ L), while phenol was added as a solid (69.5 mg) and sodium lactate was present as 60% syrup (260 μ L). Stock solutions of sodium acetate (10 g/L) and sodium butyrate (5 g/L) were made, and cultures were grown at concentrations of 80 mg/L and 40 mg/L respectively. The growth reactors were incubated in the dark at 30°C and shaking at 200 rpm until an optical density at 600 nm [OD₆₀₀] of ~0.30 (approximately three days), which was determined using an Orion Aquamate 8000 UV-Vis Spectrophotometer (Thermo Scientific). At this point, the growth reactors were opened in a sterile laminar flow hood for twenty minutes to refresh the headspace with air, and additionally were refreshed with the same mass of substrate. A sample of each growth reactor was also streaked on mineral salt-lactate plates to confirm the purity of the culture. The culture was incubated overnight to a final OD₆₀₀ of ~0.6 and harvested by centrifugation at 7500 g for 10 min. Cells were washed with 100 mM potassium phosphate buffer (PPB) and centrifuged again, and the resulting cell pellet was re-suspended with buffer (5 mL, as above). Cells were quantified by a TSS test and related to protein through a standard Bradford protein assay.

Experimental procedures

(i) Solvent Stock Solutions. Saturated stock solutions of chlorinated solvents were made by adding 1 mL of neat solvent to a glass serum vial (27 mL) with DI water and minimal headspace and sealed with butyl septa. The day before use, the vial was vigorously shaken for 30 seconds to ensure saturation and allowed to settle overnight. It was reported that as short an incubation period as 1 hour is sufficient to achieve equilibrium (Gossett, 1987).

(ii) Rate of contaminant transformation. Resting cell kinetic rests were conducted in sterile glass serum vials (27 mL) with 10 mL of MSM and 17-mL air headspace sealed with sterile butyl septa and aluminum crimp tops. Using a 100 μ L liquid syringe (Hamilton, Reno, NV), the desired mass of contaminant, calculated based on the solubility limit, was added to active vials and controls from the saturated stock solution through the septa taking care to exclude the non-aqueous phase liquid. Vials were shaken vigorously at 200 rpm for at least 10 minutes to achieve equilibrium and an initial concentration of the contaminant was measured. A known mass of cells based on TSS analysis was then added to the vials through the septa using a sterile 1 mL leur-lok syringe in a sterile laminar flow hood. For the duration of the experiment, the batch reactor vials were kept on a shaker table at 200 rpm at 20°C. Shaking at 200 rpm assured equilibrium was maintained between the aqueous and headspace compartments, and dimensionless Henry's constants at 20°C used to calculate the total mass for each contaminant.

(iii) Extent of transformation. The transformation capacity, defined as the mass of substrate that can be transformed by a given mass of resting cells, was determined in the same experiments used to determine initial rates (Alvarez-Cohen & McCarty, 1991b). Once the contaminant had been approximately 100% degraded, more was added at the same or higher concentrations until transformation ceased. The mass degraded by resting cells was then compared to the mass lost in abiotic controls (MSM only) given these tests were done over the course of several days to weeks. In determination of the initial rates, which occurred on the timescale of hours, the aqueous contaminant concentration in the abiotic control remained essentially unchanged indicating loss was insignificant. In determination of the transformation capacity, the reduction of mass in the abiotic control was considered in calculating overall mass of contaminant transformed. The capacity was calculated as:

$$\frac{[\sum \text{initial mass (umol)} - \text{final mass (umol)}] - \text{Abiotic losses (umol)}}{\text{Biomass (mg}_{\text{TSS}})}$$

To verify transformation was due to T2MO activity, cells in medium with 2% propyne gas in the headspace was used as it is a mechanism-based inactivator of toluene *ortho*-monooxygenase enzymes (Yeager et al., 1999).

(iv) Substrate inhibition. Substrate inhibition tests with toluene and benzyl alcohol were performed at aqueous concentrations of 10 mg/L and 20 mg/L to determine whether benzyl alcohol competitively inhibits the T2MO in *B. vietnamiensis* G4. At 10 mg/L of substrate triplicate treatments consisted of 1) culture with no substrate and TCE present, 2) culture with substrate and TCE present, and 3) culture with substrate, TCE, and propyne (2% v/v) present. This experiment was conducted in sterile Wheaton serum glass bottles (125 mL) with 50 mL of MSM and 105 mL of headspace sealed with plastic screw caps and butyl septa. TCE was added in the same manner as above from a stock solution to achieve a liquid concentration of 550 ppb. Toluene was added from a 1000 mg/L aqueous stock solution to achieve liquid concentrations of 10 mg/L and 20 mg/L. Initial toluene concentrations were measured prior to the addition of culture. Benzyl alcohol was added from a stock solution of 5000 mg/L to achieve liquid concentrations of 10 mg/L and 20 mg/L. *B. vietnamiensis* G4 was grown on the respective substrate of interest and 1 mg_{TSS} was added as described above.

To determine if the T2MO in *B. vietnamiensis* G4 was needed for growth on benzyl alcohol, a growth test was performed with cells in the presence and absence of propyne. The experiment was conducted in sterile Wheaton serum glass bottles (250 mL) with 175 mL of MSM and 135 mL of headspace with plastic screw caps and butyl septa. The two treatments were performed in triplicate and consisted of a low biomass of *B. vietnamiensis* G4 (0.1 mg_{TSS}) in MSM with benzyl alcohol (0.50 mM), with or without propyne at 15% (v/v) in the headspace. Propyne at 15% (v/v) ensures inhibition of the T2MO, and is above the upper explosive limit of 11.7% (v/v) (Nassimi et al., 2017). Biomass growth was measured over time through OD₆₀₀ measurements using an Orion Aquamate 8000 UV-Vis Spectrophotometer (Thermo Scientific).

(v) Activity based profiling. Many alkynes are mechanism-based inactivators of various oxygenase enzymes. The T2MO activity of *B. vietnamiensis* G4 was inactivated by the mechanism-based inactivator 17OD, which was used as a probe. After *B. vietnamiensis* G4 was grown on the substrate of interest, they were incubated with 17OD for 30 minutes in a shaking water bath at 30C and 150 rpm, cells were centrifuged and washed then resuspended in a buffered solution. Cells were lysed mechanically by bead beating and a lysing matrix, the cell extract was reacted with AlexaFluor 647 to fluorescently label 235 monooxygenase components using a copper(I)-catalyzed alkyne-azide cycloaddition (CuAAC) reaction. SDS-PAGE analyses were conducted using 9% discontinuous polyacrylamide gels using the Bio-Rad 247Mini-PROTEAN electrophoresis system (Bio-Rad, Hercules CA). Protein samples ($\leq 100 \mu\text{g}$ total protein) were prepared by adding 6X sample buffer (375mM Tris-HCl, 9% SDS, 50% glycerol, 0.05% bromophenol blue, and 9% β -mercaptoethanol) at room temperature. Samples (5 μg total protein) were then loaded onto the gels and electrophoresed at room temperature for 90 minutes at a fixed current of 25-35 mA. To visualize fluorescently labeled polypeptides, immediately after electrophoresis the unfixed gel was scanned with an Li-Cor Odyssey 9120 IR scanner (Li-Cor Biotechnology, Lincoln, NE) and migration of fluorescently-labeled NIR marker proteins was used to estimate the mass of fluorescently labeled bacterial 4polypeptides (Bennett et al. 2016). The gel images were recorded and analyzed by Odyssey imaging system application software (Version 3.0). The protein concentration of all cell extracts was determined by Biuret protein assay (Gornall et al. 1949). In summary, samples were solubilized by incubation with 1.5 M NaOH (final

concentration) at 65°C for 30 minutes. The solubilized protein sample was briefly centrifuged (10000 $\times$ g, 5 min) to remove insoluble material was then assayed using bovine serum albumin as a standard.

Analytical procedures

(i) Culture. The culture density (mg_{TSS}/L) was determined gravimetrically through TSS analysis. A dry weight was obtained with a 0.45 μ m Whatman ME 25/21 mixed cellulose filter in a 5.1-cm aluminum foil dish, after which a known volume of suspended cells was added to the filter. The filtered cell mass was then dried at 103°C for 30 minutes, cooled, and the weight change was used to calculate TSS. Protein was determined through a standard protein assay for both toluene and benzyl alcohol grown cells and was 0.66 mg_{protein}/mg_{TSS} and 0.57 mg_{protein}/mg_{TSS}, respectively.

(ii) Volatile Chemicals. A 100 μ L gas sample was taken from the batch reactor headspace using a 100 μ L Hamilton 1700 Series gas-tight syringes. TCE, *cis*-DCE and 1,1-DCE samples were injected in a Hewlett Packard 6890 Series Gas Chromatograph with a micro electron capture detector (GC-ECD) and a capillary column (Agilent DB-624 UI 30m $\times$ 0.53mm). Helium was used as the carrier gas at 15 mL/min and a constant oven temperature of 50°C was used. VC and toluene samples were injected in a Hewlett Packard 6890 Series Gas Chromatograph with a Flame Ionization Detector (GC-FID) and capillary column (Agilent DB-624 UI 30m $\times$ 0.53mm). Helium was the carrier gas at 15 mL/min and a constant oven temperature of 150°C and 220°C was used for VC and toluene, respectively. Compounds were quantified by comparing peak areas to standard curves made from known standards. The total mass of contaminants was calculated assuming equilibrium between the liquid and headspace using dimensionless Henry's constants at 20°C (Sanders, 1999).

Results

TCE ABP

The fluorescent labeling of the T2MO in *B. vietnamiensis* G4 after growth on toluene, benzyl alcohol, benzyl acetate, benzyl butyrate, phenol, acetate, butyrate, and lactate was done and shown in Figure 5.1A. Noticeable fluorescence of a 62 kDa polypeptide is seen with cells grown on toluene, benzyl alcohol, benzyl acetate, benzyl butyrate and phenol, whereas little to no fluorescence is seen for butyrate, acetate, and lactate grown cells. Quantification of the fluorescence pixels was also done for each substrate (Figure 5.1B).

TCE kinetic test with various growth substrates

Resting cell TCE kinetic tests were performed with cells grown on the different substrates. The maximum rate at a specific contaminant concentration and transformation capacity were determined. Zero-order rates were calculated from the maximum slope for the initial mass of contaminant seen and normalized to biomass present. The transformation capacity was determined with repeated additions over time, with rates slowing until transformation ceased. Transformation capacity tests were a continuation of the initial rates tests.

TCE kinetic results obtained with benzyl alcohol grown cells were compared directly to toluene-grown cells as a benchmark, since the detected fluorescence was greatest for cells grown on this substrate. TCE transformation data used to obtain initial rates and capacities for both

substrates can be seen in Figures 5.2 and 5.3. Rates and capacities are summarized in Table 5.1. Very linear initial rates were observed, indicating zero-order kinetics at the concentration tested. Reported half saturation constant (K_s) values for TCE transformation by *B. vietnamiensis* G4 range from 3 – 6 μM , which is 3 – 4 times less than the initial aqueous concentrations tested here (Folsom et al., 1990; Landa et al., 1994; Sun & Wood, 1996). Kinetic results were comparable between the two substrates, with benzyl alcohol grown *B. vietnamiensis* G4 having a slightly higher rate and capacity for TCE. The TCE transformation rates for benzyl alcohol cells (2.2 $\mu\text{mol/day/mg}_{\text{TSS}}$) was 18% greater than toluene grown cells (1.8 $\mu\text{mol/day/mg}_{\text{TSS}}$). Based on the protein analysis of toluene and benzyl alcohol grown *B. vietnamiensis* G4, this corresponds to initial rates of 1.8 and 2.7 $\text{nmol/min/mg}_{\text{protein}}$. These experimental rate values for both toluene and benzyl alcohol grown *B. vietnamiensis* G4 agree very well with previously reported rates. At an initial TCE concentration of 20 μM , phenol grown *B. vietnamiensis* G4 had an initial rate of 2.5 $\text{nmol/min/mg}_{\text{protein}}$ at room temperature (Folsom, 1990) and at 40 μM TCE, toluene grown *B. vietnamiensis* G4 had an initial rate of 3.0 $\text{nmol/min/mg}_{\text{protein}}$ at 27°C (Leahy et al., 1996).

The TCE transformation capacity for benzyl alcohol grown cells (0.63 $\mu\text{mol/mg}_{\text{TSS}}$) was 20% greater than toluene grown cells (0.47 $\mu\text{mol/mg}_{\text{TSS}}$). Yeager et al. measured transformation capacities ranging from 0.61 – 0.95 $\mu\text{mol TCE/mg cells}$ (dry weight) over a 4-hour incubation time by *B. vietnamiensis* G4 grown on toluene at cell densities ranging from 0.42 – 4.2 mg cells/mL (Yeager et al., 2001). The 0.42 mg cells/mL biomass concentration was most similar to tests in this study, and had approximately two times greater capacity (0.95 $\mu\text{mol/mg cells}$) than measured here with toluene or benzyl alcohol grown cells. This difference in values may be due to how the tests were performed; Yeager had a 4 hour incubation by adding high masses of TCE (239 to 349 μM), whereas this study added smaller additions over the course of many days (Figure 5.2 and 5.3). Typically, the determination of the transformation capacity is done over the course of hours instead of days, so to our knowledge this is the first demonstration of long-term transformation by resting *B. vietnamiensis* G4 cells. The results presented in Figure 5.3 show TCE resting cell transformation continued for up to 15 days. The decline in transformation rates with time indicate a depletion of energy reserves needed to produce NADH and/or possible transformation toxicity. However, in this case depleted energy may be the dominating factor for the exhaustion of transformation. This is due to the phenomenon seen that the addition of toluene upon exhaustion promoted renewed T2MO-catalyzed oxidations (Figure S5.1). G4 was able to recover rapidly after TCE transformation ceased. Some toxicity by the chlorinated solvents or transformation products is a likely factor, as most bacteria that degrade TCE suffer toxic effects in some way (Arp et al., 2001). However, it has been shown that this culture has a high ability to recover from the toxic effects at the TCE concentrations used (Folsom et al., 1990; Yeager et al., 2001).

TCE transformation kinetics were also determined for G4 grown on phenol, benzyl butyrate, benzyl acetate, acetate, butyrate, and lactate (Table 5.1). The highest initial rates of TCE transformation were comparable between benzyl butyrate, benzyl alcohol and phenol grown cells, with average values of 2.2, 2.2, and 2.1 $\mu\text{mol/day/mg}_{\text{TSS}}$. The next highest rate was by toluene-grown cells followed by benzyl acetate. Cells grown on butyrate, acetate, and lactate had rates of transformation an order-of-magnitude lower than the aromatic substrates. This similar trend between the substrates was also seen for the transformation capacity, with the exception that benzyl butyrate grown cells had a considerably greater capacity (1.5 $\mu\text{mol/mg}_{\text{TSS}}$) than any other substrate. The next highest capacity measured was by benzyl acetate and phenol grown culture at 0.78 and 0.77 $\mu\text{mol/mg}_{\text{TSS}}$ respectively.

It is not known why the benzyl esters, particularly benzyl butyrate grown cells, had a greater capacity. The likely explanation is that upon hydrolysis, two substrates are available as sources of reducing power. The considerably greater capacity seen with growth on benzyl butyrate compared to benzyl acetate is likely due to the longer chain butyric acid. With two substrates available, cells potentially had a higher amount of stored polyhydroxyalkanoates (PHAs), which are biodegradable polyesters that act as intracellular carbon and energy reserves under limiting nutrient conditions while in the presence of excess carbon (Pan et al., 2012; Ramsay et al., 1989). *Burkholderia cepacia* can produce a variety of PHAs including homopolymers poly-3-hydroxybutyrate (PHB), polyhydroxyvalerate (PHV), and copolymers of PHB and PVB (PHB-co-PHV) (Keenan et al., 2004). It was shown that *Burkholderia cepacia* could accumulate significant amounts of PHA accounting for as much as 50% of the dry biomass weight (Keenan et al., 2004; Ramsay et al., 1989). Comparatively, analysis of a phenol-grown mixed culture showed PHB to account for 12% of cell dry weight (Hopkins, Semprini, et al., 1993). A factor in the PHB production is the carbon to nitrogen (C/N) ratio. A study exploring the optimization of PHB production by a mixed culture, observed that the amount of PHB produced was greatest at higher C/N ratios and saw PHB production increase linearly with substrate concentration (Serafim et al., 2004). The excess carbon present with benzyl butyrate, and thus a higher C/N ratio, could have resulted in higher accumulation of PHB to supply energy for cometabolic transformation by resting cells.

The effect of an increased extent of TCE degradation through the addition of a noncompetitive external substrate has been seen before with *B. vietnamiensis* G4 and lactate in a microcosm system, as well as in a phenol-grown mixed suspended culture with the addition of formate, lactate, and to a lesser extent acetate (Hopkins, et al., 1993; Munakata-Marr et al., 1996). Additionally, there is a high likelihood that *B. vietnamiensis* G4 contains an esterase enzyme to accelerate hydrolysis of the benzyl esters, as growth was seen after two days, and the abiotic hydrolysis half-life of benzyl acetate and benzyl butyrate at a pH of 7 are 1.0 year and 198 days, respectively (USEPA, 2009).

Linear regression analysis was performed on the ABP fluorescence versus the average initial TCE transformation rate for all the growth substrates tested (Figure 5.4). A fairly linear relationship was observed with the exception of phenol as an outlier. The very low amount of ABP labeling observed with acetate, butyrate and lactate grown cells correlates well with the very low rates of TCE transformation, while the high degree of ABP fluorescence correlates well with the highest rates observed with toluene and benzyl alcohol grown cells. The results indicate that ABP is an effective means of screening for cometabolic transformation potential.

Transformation was minimal by acetate and butyrate grown cells compared to cells grown on the other substrates, which reaffirms the induction of the T2MO is due to the release of benzyl alcohol upon hydrolysis. However, it is worth noting that acetate and butyrate grown cells were cometabolically active when compared to controls and were able to transform TCE to some degree. The results with the lactate grown *B. vietnamiensis* G4 also show minimal transformation of TCE compared to the other substrates tested. When Shields and Reagin were selecting a strain of *B. vietnamiensis* G4 for constitutive degradation of TCE, they tested wild type *B. vietnamiensis* G4 grown on lactate and saw no transformation of TCE over 19 hours, whereas *B. vietnamiensis* PR123 removed all detectable TCE in the same time (Shields & Reagin, 1992). This is somewhat similar to our results with lactate grown *B. vietnamiensis* G4, though over 19 hours approximately 25% of the TCE was degraded. Another study determined TCE kinetics for the mutant strain *B.*

vietnamiensis PR1₃₁ grown on glucose and observed a maximum specific TCE degradation rate of 0.8 nmol/min/mg_{protein} (Inguva & Shreve, 1999). Assuming protein mass accounts for 60% of dry cell mass for lactate grown cells, this rate is sixteen times the rate we observed with the wild type strain *B. vietnamiensis* G4 grown on lactate.

Substrate inhibition test

Given that APB and kinetic tests show growth on benzyl alcohol effectively supports the cometabolic activity in *B. vietnamiensis* G4, experiments were performed to see if benzyl alcohol competitively inhibited TCE transformation. Since T2MO is not required for the utilization of benzyl alcohol, minimal competitive inhibition was expected. TCE transformation rates were compared between two treatments: 1) resting cell suspensions of *B. vietnamiensis* G4 culture and 2) *B. vietnamiensis* G4 cell suspensions in the presence of benzyl alcohol. Toluene inhibition tests were also conducted for comparison, since toluene inhibition is known to occur. Rates were calculated as zero-order rates, and a lower biomass concentration of 0.02 mg_{TSS}/mL was used to achieve a longer observation period of the tests. These inhibition tests were conducted at benzyl alcohol concentrations of 10 and 20 mg/L. The same tests were done with toluene for contrast at similar liquid concentrations. The actual measured concentrations of toluene averaged 11.7 ± 0.5 and 20.1 ± 1 mg/L in the tests conducted in triplicate. Benzyl alcohol concentration is based on the known addition of the liquid standard.

Toluene Inhibition Tests

The inhibition test at initial toluene concentrations of 11.7 and 20.1 mg/L is shown in Figures 5.5 and 5.6, respectively. At 11.7 mg/L toluene, a lag in the transformation of TCE was observed for approximately 40 minutes, whereas the treatment with no toluene had lag of 20 min (Figure 5.5A). Comparatively, toluene consumption was monitored and there was no lag in transformation as there was with TCE (Figure S5.2). TCE rates were determined with data collected after the lag periods. As expected, at 11.7 mg/L toluene the TCE transformation rate for resting culture with no toluene present (2.4 ± 0.1 umol/day/mg_{TSS}) was greater than with toluene present (2.0 ± 0.4 umol/day/mg_{TSS}), which represents a 14% decrease in the rate (Figure 5.5B). At 20 mg/L of toluene, stronger inhibition was observed, with a lag in TCE transformation occurring for approximately 2.7 hours (Figure 5.6A). After 3.2 hours, the average toluene liquid concentration was 7.4 ± 0.1 mg/L, and at this time noticeable transformation of TCE occurred and a zero-order rate was determined. The transformation rate for resting culture without toluene was (1.3 ± 0.07 umol/day/mg_{TSS}), which was 40% greater than the rate for culture with 20 mg/L of toluene present (0.78 ± 0.02 umol/day/mg_{TSS}) (Figure 5.6B). This observed toluene inhibition is expected and consistent with previous observations of toluene inhibiting rates of TCE cometabolism (Alagappan & Cowan, 2003; Landa et al., 1994).

Benzyl Alcohol Inhibition Tests

The inhibition tests at initial benzyl alcohol concentrations of 10 mg/L and 20 mg/L can be seen in Figures 5.7 and 5.8. There was not any indication of a lag in TCE transformation at 10 mg/L benzyl alcohol (Figure 5.7A). There also is no difference in TCE transformation rates between the resting cell treatment and cells with 10 mg/L benzyl alcohol present. The average zero-order rates between resting culture (1.3 ± 0.5 umol/day/mg_{TSS}) and culture with (10 mg/L) benzyl alcohol present (1.3 ± 0.03 umol/day/mg_{TSS}) were the same, indicating benzyl alcohol did not inhibit TCE transformation.

However, when the benzyl alcohol concentration was increased to 20 mg/L, a short lag time in TCE transformation of 45 minutes was observed causing the two treatments to differ (Figure 5.8A). When benzyl alcohol was present, the average zero-order rate was (0.58 ± 0.04 $\mu\text{mol/day/mg}_{\text{TSS}}$), which was a 40% decrease from the rate for resting culture without benzyl alcohol (0.97 ± 0.01 $\mu\text{mol/day/mg}_{\text{TSS}}$) (Figure 5.8B). These results indicate that benzyl alcohol was inhibitory at the higher concentration. However, benzyl alcohol was less inhibitory compared to toluene at the similar concentration, where the TCE transformation lagged for approximately three hours.

Basu et al., (2003) identified two pathways involved in the metabolism of benzyl alcohol by *Pseudomonas putida* CSV86 (SI). These two pathways saw benzyl alcohol converted to benzaldehyde or 2-hydroxy benzyl alcohol (2HBA), which were subsequently converted to aromatic acids. These acids were further metabolized by a specific *ortho*- or *meta*- pathways to tricarboxylic acid (TCA) cycle. This study concluded that aromatic alcohols induced an upper regulon that codes aromatic alcohol- and aromatic aldehyde- dehydrogenases for conversion to acid, which in turn induced a specific lower regulon for metabolism via an *ortho*- or *meta*- cleavage pathway (Basu et al., 2003).

Tejasen, 2004 also observed the same two pathways for benzyl alcohol degradation in a benzyl alcohol mixed culture; the formation of benzaldehyde by an alcohol dehydrogenase and formation of 2HBA through hydroxylation by an *ortho*-monooxygenase. This study investigated inhibition effects in a benzyl alcohol grown mixed culture and found that when the *ortho*-monooxygenase was inhibited no TCE transformation or 2HBA production was observed, but benzyl alcohol degradation decreased only slightly. This same process potentially applies when *B. vietnamiensis* G4 is utilizing benzyl alcohol given that the T2MO hydroxylates at the *ortho* position. It is likely that the existence of the secondary pathway for benzyl alcohol degradation to form benzaldehyde could alleviate inhibition effects, unless concentrations are high as they were in the test with 20 mg/L of benzyl alcohol. The formation of benzaldehyde and 2HBA were not measured as these compounds could not be measured on the HPLC.

To determine whether the monooxygenase was required for benzyl alcohol metabolism, a growth test of *B. vietnamiensis* G4 was done in the presence of propyne. The experiment consisted of two treatments, growth with and without propyne to inhibit monooxygenase activity. If the T2MO is necessary for benzyl alcohol utilization, the propyne treated cells should show little/no signs of biomass growth. Figure 5.9 shows OD₆₀₀ measurements taken during growth for both treatments. Results show that cells not exposed to propyne enter exponential growth phase by day six, whereas no growth is seen by cells exposed to propyne over the course of ten days. This indicates that the monooxygenase is required for growth on benzyl alcohol. This is not surprising given the work of both Tejasen and Basu et al. suggest that the two pathways for benzyl alcohol utilization require an *ortho*-monooxygenase; the formation of 2HBA facilitated through an *ortho*-monooxygenase, and a second pathway, which forms benzaldehyde and is metabolized by the *ortho*-monooxygenase to the TCA cycle.

Kinetic Tests with VC, 1,1-DCE, and cis-DCE

Additional kinetic tests with toluene and benzyl alcohol grown *B. vietnamiensis* G4 were performed for the contaminants VC, 1,1-DCE, and *cis*-DCE and are summarized in Table 2. Figures 5.10 and 5.11 show the test results obtained with *cis*-DCE, and test results for VC and 1,1-DCE are provide in the Appendix, Figures S5.3-S5.6. Given the rate of transformation at the

contaminant concentrations tested, a zero-order rate fit the data well. Initial transformation rates with toluene grown cells were higher than benzyl alcohol grown cells for each compound except TCE; rates with toluene grown cells were 18% greater than benzyl alcohol grown *B. vietnamiensis* G4 for VC, 64% greater for *cis*-DCE and 39% greater for 1,1-DCE. It is of note that while rates are comparable between benzyl alcohol and toluene grown cultures for VC and TCE, there's a considerable difference in rates for 1,1-DCE and *cis*-DCE between the two substrates. For both growth substrates, initial rates were higher for VC and *cis*-DCE cometabolism compared to 1,1-DCE and TCE. This is likely due to the fact that these compounds are less chlorinated compared to TCE, and do not have the high toxic effects seen with the 1,1-DCE epoxide (Dolan & McCarty, 1995).

The transformation data for *cis*-DCE for both toluene and benzyl alcohol are shown in Figures 5.10 and 5.11, respectively. The data for this contaminant is shown because it again highlights the ability of *B. vietnamiensis* G4 to maintain long-term cometabolic ability over the course of weeks. Toluene grown *B. vietnamiensis* G4 continued to transform *cis*-DCE over the course of 25 days, whereas benzyl alcohol grown *B. vietnamiensis* G4 was active over 10 days. This difference in duration is due to the higher mass of *cis*-DCE added to the benzyl alcohol grown cells. The results of the VC tests are shown in Figures S5.3 and S5.4. VC transformation capacity was reached in two and twelve days for toluene and benzyl alcohol cells, respectively (Figures S5.3 and S5.4). The difference in mass additions does not account for the duration difference for VC, as toluene-grown cells received lower mass additions (~4 μmol) than benzyl alcohol grown cells (~6 μmol) (Figures S5.3 and S5.4). The only contaminant where cometabolic transformation was not seen over the timescale of days was 1,1-DCE indicating that transformation toxicity was the dominating factor in exhaustion. 1,1-DCE transformation capacity was reached in two and ten hours for toluene (0.048 $\mu\text{mol}/\text{mg}_{\text{TSS}}$) and benzyl alcohol grown cells (0.085 $\mu\text{mol}/\text{mg}_{\text{TSS}}$), respectively (Figures S5.5 and S5.6). As mentioned previously, aerobic cometabolism of 1,1-DCE is a toxic process to cells and causes rapid inactivation (Arp et al., 2001; Dolan & McCarty, 1995; Hopkins & McCarty, 1995). The ability to transform *cis*-DCE and VC over the course of many days by resting cells indicates that *B. vietnamiensis* G4 has a high toxicity threshold for these contaminants as it does for TCE.

The transformation capacity for each compound was greater for benzyl alcohol grown *B. vietnamiensis* G4 compared to the toluene grown *B. vietnamiensis* G4, which is the converse pattern seen with initial rates. Transformation capacities for benzyl alcohol grown cells were 59% greater than toluene grown cells for VC, 49% greater for *cis*-DCE, and 44% greater for 1,1-DCE. For both growth substrates, the highest transformation capacity was observed with VC followed by *cis*-DCE, TCE and lastly 1,1-DCE. (Shields & Reagin, 1992) looked at the degradation of 1,1-DCE, *cis*-DCE, and TCE by the mutant strain PR1 in terms of percent remaining after an overnight incubation; TCE had (2 ± 2) percent remaining, followed by *cis*-DCE (12 ± 9) and 1,1-DCE (50 ± 3) (Shields & Reagin, 1992). It is likely that the capacity for 1,1-DCE is the lowest of these compounds, which agrees with our results. Shim and Wood (2000) looked at the degradation of various contaminants individually and in mixtures by a recombinant strain of *Escherichia coli* constitutively expressing the toluene *ortho*-monooxygenase of *B. vietnamiensis* G4. They saw the greatest percent removal of TCE (62%), followed by 1,1-DCE (20%), *cis*-DCE (19%), and little removal of VC (2%) after 24 hours with resting cells (Shim & Wood, 2000). At the Moffett field test site, biotransformation of VC, *trans*-DCE, *cis*-DCE, and TCE by a mixed methanotrophic bacteria population was observed. VC and *trans*-DCE were the most effectively degraded with 95

and 90% removal, respectively, followed by *cis*-DCE and TCE at 50% and 20% (Semprini et al., 1991). At the same test site with phenol-grown bacteria, the greatest rate and extent of transformation was seen with *cis*-DCE, TCE, and lastly *trans*-DCE (Hopkins, Semprini, et al., 1993). Both of these field demonstrations with a similar mixture of contaminants, though done with a mixed culture, agree with the pattern seen here.

Toxicity may be a factor in the difference in transformation capacities seen between benzyl alcohol and toluene grown *B. vietnamiensis* G4. Toluene is known to exert toxicity on cells by permeabilization of the cell membrane at high concentrations (Heipieper et al., 1994; Sikkema et al., 1994). If cells were compromised at all while growing on toluene, and if this effect is not seen with benzyl alcohol, it could account for the difference in capacities and would be another benefit of using benzyl alcohol as a primary substrate. Another possibility for the difference in capacities is a difference in PHB accumulation in cells growing on benzyl alcohol compared to growth on toluene. The consistent trend of twice the transformation capacity for the DCEs and VC, and a higher capacity for TCE, indicates that benzyl alcohol grown cells may have a greater capacity for energy storage than toluene-grown cells. This may have been reflected in the lower protein mass per dry cell weight observed for the toluene grown cells compared to benzyl alcohol grown cells. This impact the growth substrate has on PHB production, and whether this affects the extent of transformation should be studied further.

Conclusions

This is the first report to our knowledge of benzyl alcohol supporting cometabolic activity in *B. vietnamiensis* G4 to a degree comparable to that of toluene-grown cells. ABP tests showed the level of T2MO expression in *B. vietnamiensis* G4 was greatest with benzyl alcohol, toluene, benzyl butyrate, and benzyl acetate as primary growth substrates. This matched TCE kinetic transformation results with benzyl alcohol, benzyl butyrate, phenol, and toluene grown cells having the highest rate of TCE transformation, and the greatest extent of transformation seen in the benzyl esters, phenol and benzyl alcohol. This agreement between ABL and kinetic results demonstrate that ABL is a valuable screening method for determining inducing substrates for cultures performing aerobic cometabolism. The higher transformation capacities seen with the benzyl esters indicates that the carboxylic acids available upon hydrolysis serve as a non-competitive carbon source during biomass growth. The minimal transformation rate and extent of transformation seen with acetate and butyrate support this conclusion and verify that the benzyl alcohol released from the esters is responsible for contaminant transformation. Further research should be done on slow release versions of benzyl alcohol, as done here with benzyl butyrate and benzyl acetate, as they proved comparable in initial TCE rates and superior for supporting a greater transformation capacity.

An important consideration here in determining the suitability of benzyl alcohol as a primary substrate for cometabolism was whether competitive inhibition for the T2MO occurs. Experimental results indicate benzyl alcohol is less inhibitory than toluene, as inhibitory effects were not observed at 10 mg/L, and only minor effects were exhibited at 20 mg/L. This is likely due to two metabolic pathways available for benzyl alcohol metabolism. Growth of *B. vietnamiensis* G4 on benzyl alcohol in the presence and absence of propyne indicate that if two pathways for benzyl alcohol utilization exist, the T2MO is required for both. However, more research needs to be done to determine the details of these metabolic pathways and where the T2MO is used. Future work should also be done exploring growth on benzyl alcohol by other toluene oxidizing bacteria capable of cometabolism. Given that mixtures of CAHs are often found

in the subsurface, it was important that we considered transformation of other contaminants by *B. vietnamiensis* G4 grown on benzyl alcohol. Data also showed that benzyl alcohol cells were able to transform 1,1-DCE, *cis*-DCE, and VC with results comparable to toluene-grown cells. While rates were higher for toluene-grown cells, benzyl alcohol grown cells had a greater transformation capacity for each contaminant. It is possible that this phenomenon is due to some degree of cell permeabilization by toluene and/or increased energy storage from growth on benzyl alcohol. Exploration of PHB accumulation for growth on various primary substrates should be explored further since utilizable carbon is limited in the subsurface and this could be an important factor when choosing a suitable microorganism and substrate for bioremediation. For both toluene and benzyl alcohol, VC and *cis*-DCE had considerably greater rates of transformation and transformation capacities than TCE and 1,1-DCE, which agrees with results seen in the field with methane and phenol mixed cultures (Semprini et al, 1991; Hopkins et al, 1993b). Transformation of all the contaminants (except 1,1-DCE) by resting cells was maintained over the course of multiple days. Most kinetic studies are over a shorter amount of time, but this extended transformation of contaminants by *B. vietnamiensis* G4 demonstrates that this culture has a high toxicity threshold for multiple chlorinated ethenes and highlights the energy storage capabilities of this microbe. Benzyl alcohol is food grade and easy to handle as a liquid, and results from this study indicate it has potential for in situ bioremediation via aerobic cometabolism.

Tables and Figures

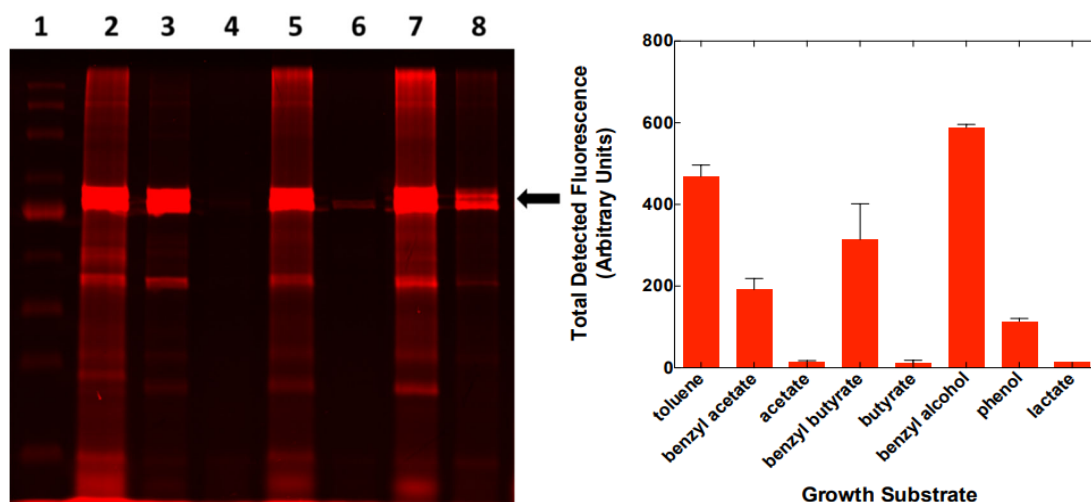


Figure 5.1. Whole cells of *B. vietnamiensis* G4 were pretreated with 170D and analyzed by SDS-PAGE and IR scanning as described in Materials and Methods section. (Left) Lane 1, NIR markers; lane 2, cells grown on toluene; lane 3, cells grown on benzyl acetate; lane 4 cells grown on acetate; lane 5, cells grown on benzyl butyrate; lane 6, cells grown on butyrate; lane 7, cells grown on benzyl alcohol; lane 8, cells grown on phenol. (Right) The average total IR fluorescence of the gel on the left was quantified in arbitrary units for each lane.

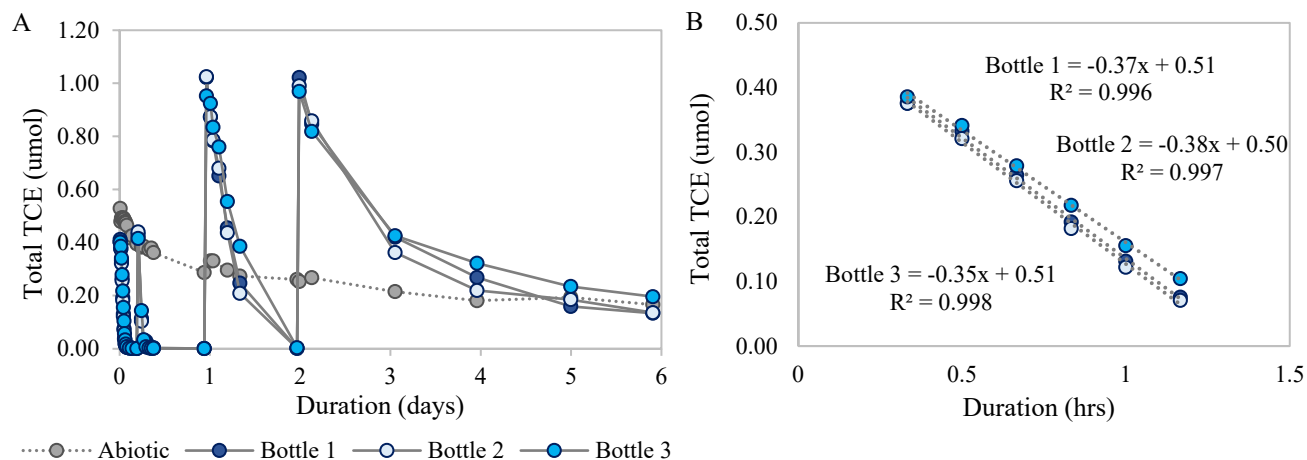


Figure 5.2. Kinetic degradation results of TCE by resting toluene grown *B. vietnamiensis* G4 (5 mgTSS) showing (A) TCE reinjections to determine total transformation capacity (umol/mgTSS) and (B) initial degradation of TCE to determine initial zero order rate (umol/day/mgTSS).

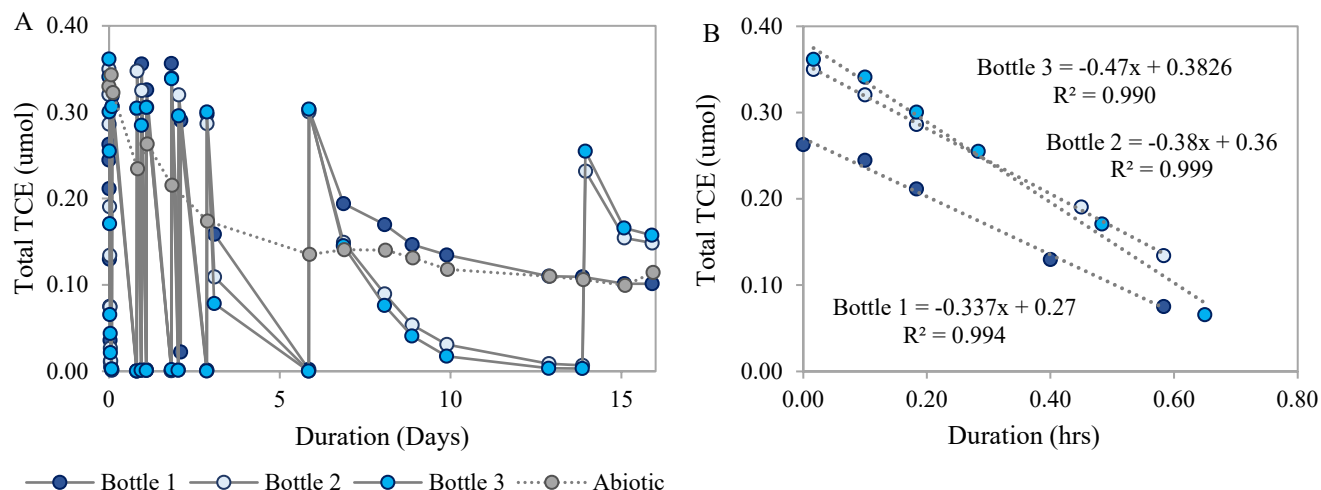


Figure 5.3. Kinetic degradation results of TCE by resting benzyl alcohol grown *B. vietnamiensis* G4 (4.2 mg_{TSS}) showing (A) TCE reinjections to determine total transformation capacity (umol/ mg_{TSS}) and (B) initial degradation of TCE to determine initial zero order rate (umol/day/mg_{TSS}).

Table 5.1. Comparison of initial rates and transformation capacities of TCE by *B. vietnamiensis* G4 grown on various substrates at the given initial liquid and biomass concentrations.

TCE Kinetic Comparison								
	Toluene	Benzyl Alcohol	Benzyl Acetate	Benzyl Butyrate	Phenol	Acetate	Butyrate	Lactate
Initial Rate (umol/day/mg _{TSS})	1.75 ± 0.08 ^b	2.18 ± 0.2 ^b	0.79 (0.79, 0.79) ^a	2.20 (2.19 - 2.22) ^a	2.12 ± 0.1 ^b	0.11 ± 0.03 ^b	0.14 ± 0.04 ^b	0.047 ± 0.009 ^b
T _C (umol/mg _{TSS})	0.47 ± 0.02 ^b	0.63 ± 0.03 ^b	0.78 (0.78, 0.78) ^a	1.46 (1.44, 1.48) ^a	0.77 ± 0.007 ^b	0.097 ± 0.001 ^b	0.14 ± 0.04 ^b	0.050 ± 0.007 ^b
Initial Liquid Conc. (uM)	25.7 ± 0.4 ^b	22.2 ± 3.6 ^b	16.1 (15.9 - 16.2) ^a	27.2 (27.2, 27.2) ^a	17.4 ± 0.8 ^b	16.1 ± 0.4 ^b	16.5 ± 0.4 ^b	15.2 ± 0.9 ^b
Biomass (mg _{TSS} /mL)	0.46	0.42	0.45	0.50	0.46	0.50	0.55	0.50

^aTest done in duplicate and values are means with the range of values given in parenthesis.

^bTest done in triplicate and values are means ± SD for *n* = 3

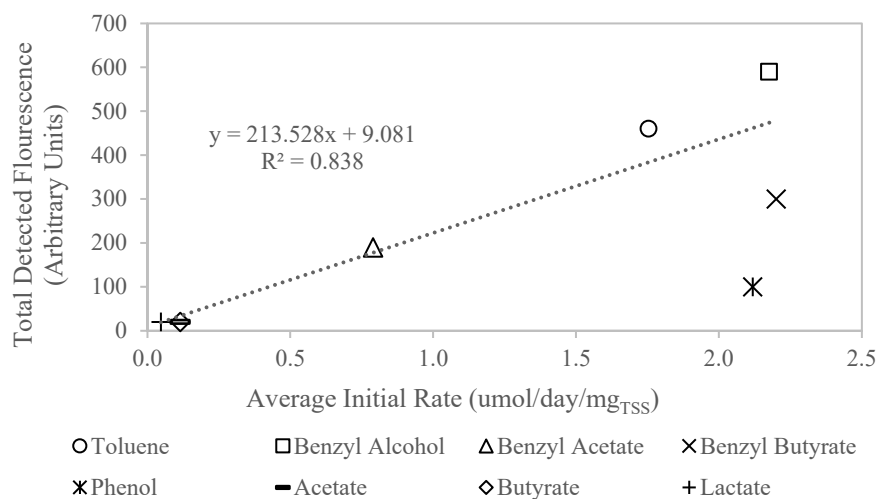


Figure 5.4. Regression analysis of total detected fluorescence and the average initial rate for toluene, benzyl alcohol, benzyl acetate, benzyl butyrate, phenol, acetate, butyrate, and lactate grown *B. vietnamiensis* G4. The linear regression is shown for the data with the exception of phenol as an outlier.

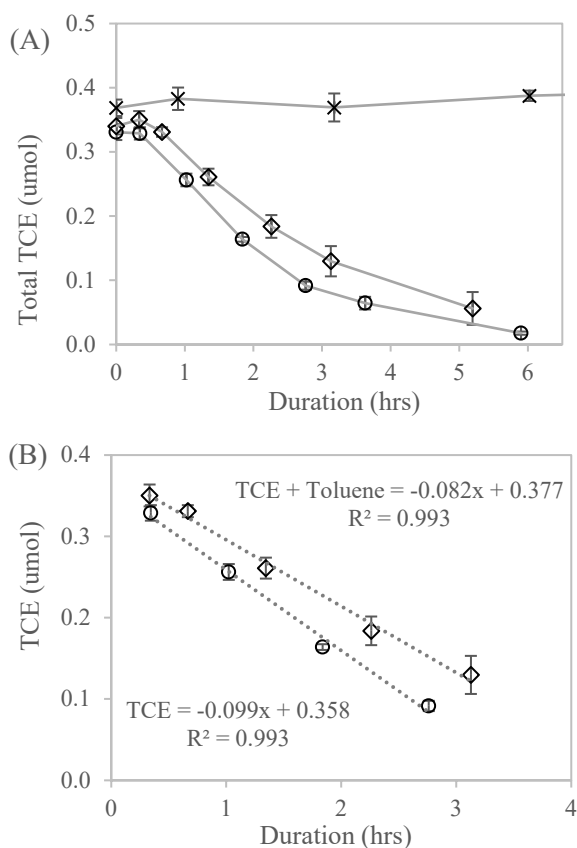


Figure 5.5. TCE disappearance by resting *B. vietnamiensis* G4 (○), with toluene at 12 mg/L (◇), and with propyne 2% (v/v) as a monooxygenase inhibitor (×). Shown is (A) raw data and (B) zero-order rate regression. Treatments were done in triplicate with error bars representing SD for $n = 3$.

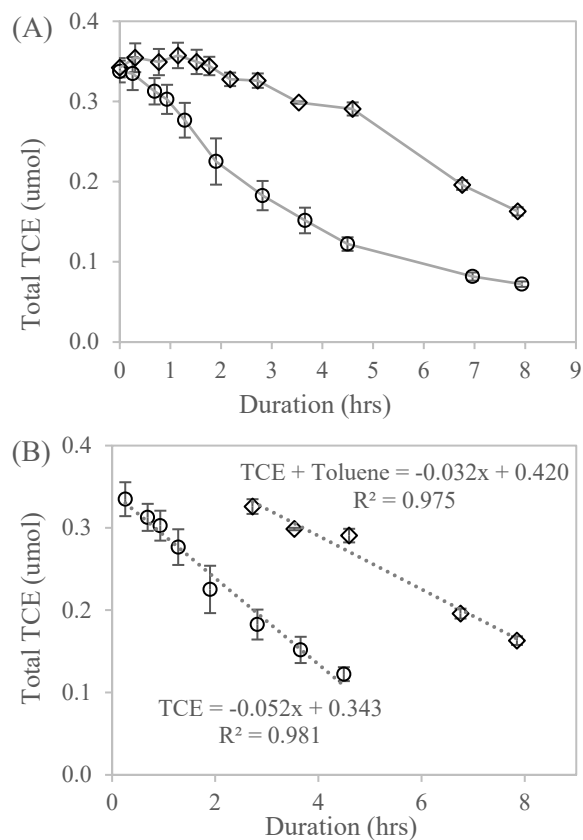


Figure 5.6. TCE disappearance by resting *B. vietnamiensis* G4 (○) and with toluene at 20 mg/L (◇). Shown is (A) raw data and (B) zero-order rate fit. Treatments were done in triplicate with error bars representing SD for $n = 3$.

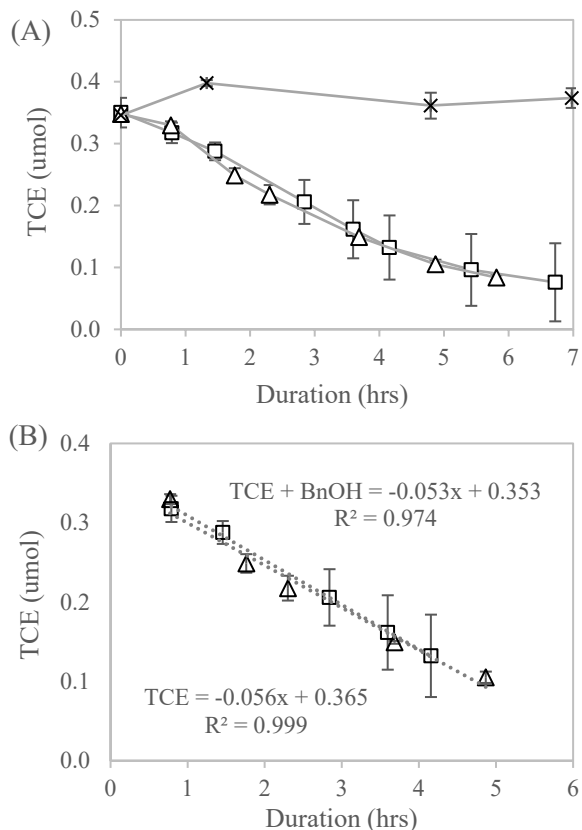


Figure 5.7. TCE disappearance by resting *B. vietnamiensis* G4 alcohol (□) and with benzyl alcohol at 10 mg/L (Δ), and with propyne 2% (v/v) present as a monooxygenase inhibitor (×). Shown is (A) raw data and (B) zero-order rate regression. Treatments were done in triplicate and values with error bars representing SD for $n = 3$.

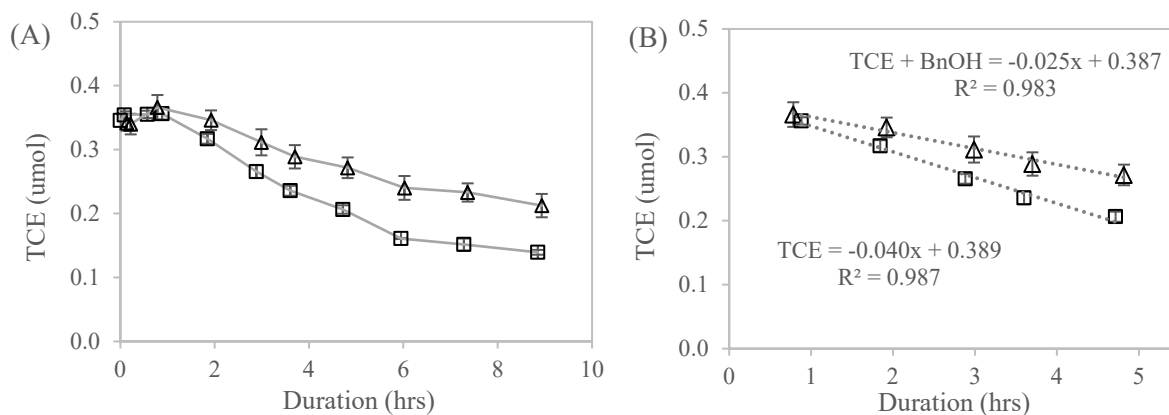


Figure 5.8. TCE disappearance by resting *B. vietnamiensis* G4 (□) and with benzyl alcohol at 20 mg/L (Δ). Shown is (A) raw data and (B) zero-order rate fit. Treatments were done in triplicate with error bars representing SD for $n = 3$.

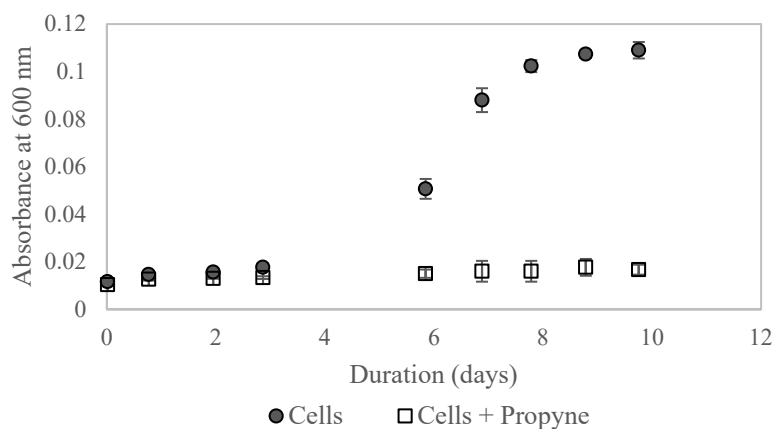


Figure 5.9. OD₆₀₀ measurements over time for *B. vietnamiensis* G4 grown on benzyl alcohol (A) and grown in the presence of propyne (15% v/v). Treatments were done in triplicate with error bars representing SD for $n = 3$.

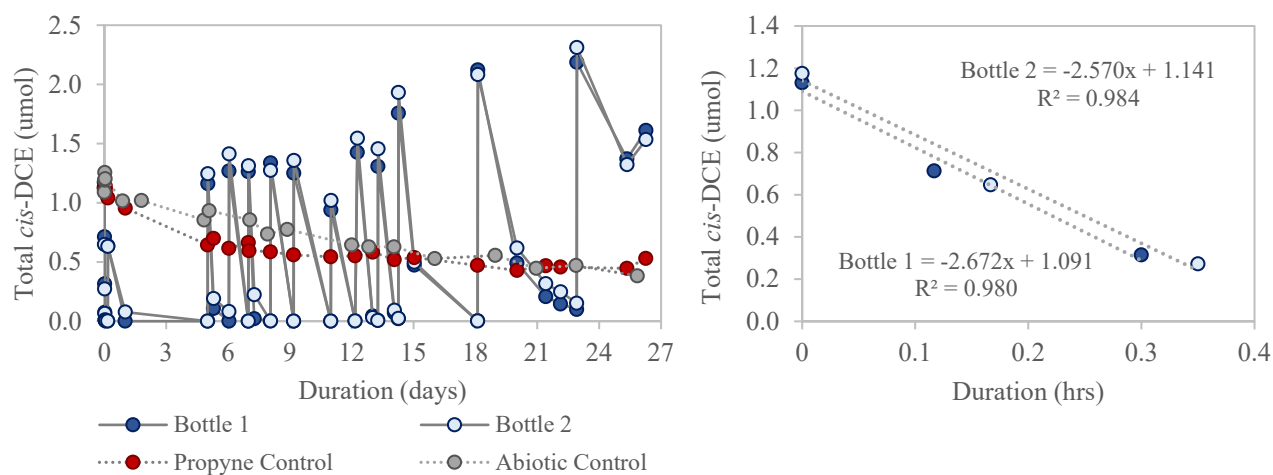


Figure 5.10. Kinetic degradation results of *cis*-DCE by resting toluene grown *B. vietnamiensis* G4 (5.5 mg_{TSS}) showing (A) *cis*-DCE reinjections to determine total transformation capacity (umol/ mg_{TSS}) and (B) initial degradation of *cis*-DCE to determine initial zero order rate (umol/day/mg_{TSS}).

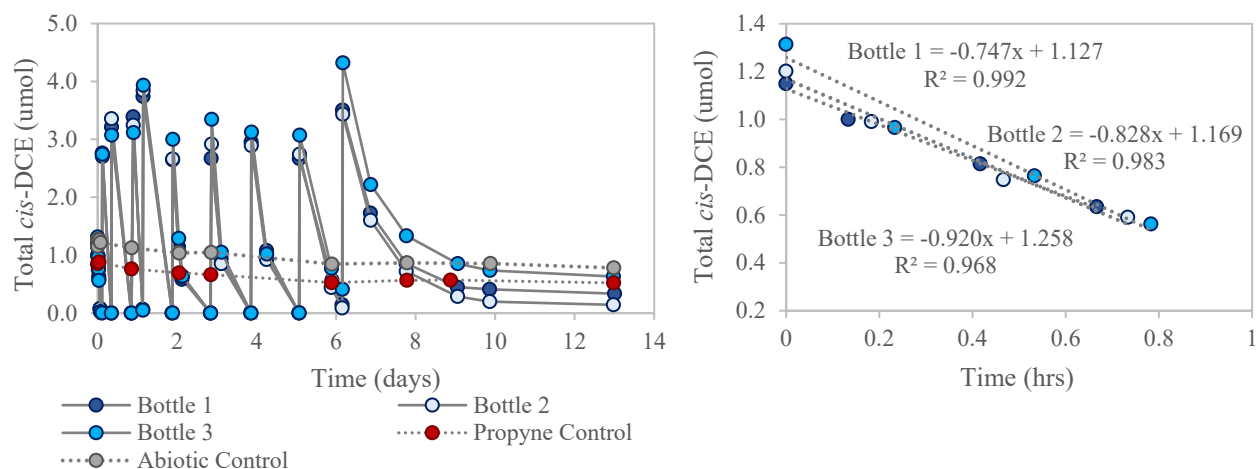


Figure 5.11. Kinetic degradation results of *cis*-DCE by resting benzyl alcohol grown *B. vietnamiensis* G4 (4.8 mg_{TSS}) showing (A) TCE reinjections to determine total transformation capacity (umol/ mg_{TSS}) and (B) initial degradation of TCE to determine initial zero order rate (umol/day/mg_{TSS}).

Table 5.2 Comparison of initial rates and transformation capacities at the given initial liquid and biomass concentrations with benzyl alcohol and toluene grown *B. vietnamiensis* G4.

Toluene Grown <i>B. vietnamiensis</i> G4				
	VC	<i>cis</i> -DCE	1,1-DCE	TCE
Initial Rate (umol/day/mg _{TSS})	9.7 (9.60 - 9.83) ^a	11.4 (11.22 - 11.66) ^a	2.4 (2.33 - 2.46) ^a	1.7 ± 0.1 ^b
T _C (umol/mg _{TSS})	3.08 (3.04 - 3.13) ^a	2.92 (2.84 - 3.00) ^a	0.048 (0.0477 - 0.0481) ^a	0.47 ± 0.02 ^b
Initial Liquid Conc. (uM)	24.2 (23.7 - 24.7) ^a	90.2 (88.4 - 91.9) ^a	22.4 (22.2 - 22.5) ^a	25.7 ± 0.4 ^b
Biomass (mg _{TSS} /mL)	0.10	0.55	0.55	0.46
Benzyl Alcohol Grown <i>B. vietnamiensis</i> G4				
	VC	<i>cis</i> -DCE	1,1-DCE	TCE
Initial Rate (umol/day/mg _{TSS})	7.98 ± 1.2 ^b	4.16 ± 0.4 ^b	1.45 ± 0.1 ^b	2.18 ± 0.2 ^b
T _C (umol/mg _{TSS})	7.67 ± 0.1 ^b	5.92 ± 0.2 ^b	0.085 ± 0.005 ^b	0.63 ± 0.03 ^b
Initial Liquid Conc. (uM)	31.8 ± 0.6 ^b	95.5 ± 6.6 ^b	22.3 ± 0.6 ^b	22.2 ± 3.6 ^b
Biomass (mg _{TSS} /mL)	0.96	0.48	0.48	0.42

^aTest done in duplicate and values are means with the range of values given in parenthesis.

^bTest done in triplicate and values are means ± SD for *n* = 3

Chapter 6. Growth of *R. rhodochrous* ATCC 21198 on Slow Release Compounds

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Abstract

Long-term cometabolic transformation of 1,1,1-TCA and 1,4-D was achieved using SRCs as growth substrates for pure cultures of *R. rhodochrous* ATCC 21198. Resting cells transformation tests showed 1,4-D transformation occurred without a lag phase for cells grown on 2-butanol, while an induction period of several hours was required for 1-butanol grown cells. These observations were consistent with ABL patterns for monooxygenase hydroxylase components and specific rates of tetrahydrofuran (THF) degradation. 1,1,1-TCA and 1,4-D degradation rates for alcohol-grown cells were slower than those for cells grown on gaseous alkanes such as isobutane. Batch metabolism and degradation tests were performed, in the presence of 1,1,1-TCA and 1,4-D, with the growth of *R. rhodochrous* ATCC 21198 on alcohols produced by the hydrolysis of orthosilicates. Three orthosilicates were tested: TBOS, T2BOS, and T2POS. The measured rates of alcohol release in poisoned controls depended on the orthosilicate structure with TBOS which produced a 1° alcohol (1-butanol) hydrolyzing more rapidly than T2POS and T2BOS that produced the 2° alcohols 2-butanol and 2-propanol, respectively. The orthosilicates were added as light non-aqueous phase liquids (LNAPLs) with *R. rhodochrous* ATCC 21198 and formed dispersed droplets when continuously mixed. Continuous rates of oxygen O₂ consumption and CO₂ production confirmed alcohol metabolism by *R. rhodochrous* ATCC 21198 was occurring. The rates of metabolism (TBOS > T2POS > T2BOS) were consistent with the rates of alcohol release via abiotic hydrolysis. 1,4-D and 1,1,1-TCA were continuously transformed in successive additions by *R. rhodochrous* ATCC 21198 over 125 days, with the rates correlated with the rates of metabolism. The metabolism of the alcohols was not inhibited by acetylene, while transformation of 1,4-D and 1,1,1-TCA was. This indicated that while monooxygenase activity was required for the cometabolic transformations, it was not required for alcohol utilization. Alcohol concentrations in the biologically active reactors were maintained below the levels of detection, indicating they were metabolized rapidly after being produced. Much lower rates of O₂ consumption were observed in the reactors containing T2BOS, which has benefits for in-situ bioremediation. The results illustrate the importance of the structure of the SRC when developing passive aerobic cometabolic treatment systems.

Introduction

Current sources of 1,4-D include paint strippers, antifreeze, degreasing and deicing fluids, deodorants, shampoos, contraceptives, and as a byproduct during the manufacture of polyethylene terephthalate (PET) (USEPA, 2017b). However, historically 1,4-D was most frequently used as a chemical stabilizer for chlorinated solvents such as 1,1,1-TCA, to prevent decomposition due to reactions from light, heat, oxygen, or acid-base chemistry (Mohr, 2001; USEPA, 2017b). 1,1,1-TCA formulas contain up to 2% - 18% 1,4-D (Adamson et al., 2014; Mohr, 2001) and due to poor solvent disposal practices, 1,4-D is now a commonly detected groundwater contaminant in areas with chlorinated solvent contamination (Adamson et al., 2014). For example, in an evaluation of over 2000 sites in California, 1,4-D was detected at 194 sites and at 95% of these sites a chlorinated solvent was also detected (Adamson *et al.* (2014), and 76% had both 1,4-D and 1,1,1-TCA present.

1,4-D has several characteristics that create challenges for remediation of contaminated aquifers. 1,4-D is fully miscible in water (infinitely soluble) and has a low octanol water partition coefficient (K_{ow}) (Mohr, 2001; USEPA, 2017b). 1,4-D is generally resistant to anaerobic transformation (McElroy et al., 2019). These characteristics result in 1,4-D having a high mobility in groundwater with the potential to form extended dissolved contaminant plumes (Mohr, 2001). 1,4-D is categorized as a B2 probable human carcinogen (Derosa et al., 1996; Mohr, 2001). There is currently no federal maximum contaminant level (MCL) for 1,4-D, but USEPA has determined an aqueous concentration of 0.35 $\mu\text{g/L}$ represents a 1/1,000,000 cancer risk and has established a screening level of 1,4-D in drinking water at 0.46 $\mu\text{g/L}$. State agencies have varying regulatory levels in drinking water and for groundwater cleanup ranging from 0.25 $\mu\text{g/L}$ in New Hampshire to 77 $\mu\text{g/L}$ in Alaska (USEPA, 2017b). 1,1,1-TCA was used for degreasing and in various adhesives and is currently classified as Group D carcinogen (USEPA, 1999). The maximum contaminant level (MCL) for 1,1,1-TCA in drinking water is 200 $\mu\text{g/L}$ (USEPA, 1999).

Both ex-situ and in-situ methods for treating 1,4-D have been reviewed by McElroy *et al.* (2019) and Zhang *et al.* (2017). In-situ bioremediation can potentially provide a large cost savings for treatment mixtures of chlorinated solvents that include 1,4-D. Direct metabolism of 1,4-D as a sole carbon and energy source has been demonstrated for a number of pure cultures of bacteria and fungi (McElroy et al., 2019; Zhang, Gedalanga, et al., 2017). Growth is fairly inefficient, with high whole-cell half-saturation constants ranging from 17.5 to 160 mg/L, slow growth rates, low biomass yields and low enzymatic activity (Barajas-Rodriguez & Freedman, 2018; McElroy et al., 2019). Thus, treating low concentrations (≤ 100 ppb) of 1,4-D is expected to be ineffective using microorganisms that directly metabolize and grow on 1,4-D. Aerobic cometabolism is a particularly attractive option, as specific microorganisms can be stimulated *in situ* using selective primary substrates to drive the transformation (Frasconi et al., 2015; Semprini, 1997). Microbial growth on the primary substrates leads to expression of oxygenase enzymes that can then fortuitously oxidize and remediate contaminants to low concentration levels needed for the in-situ treatment of 1,4-D. Cometabolic transformation of 1,4-D has been reported on a range of substrates for growth and/or induction of cometabolic enzymes as reviewed by Zhang *et al.* (2017) and McElroy *et al.* (2019). Common gaseous substrates include propane (Barajas-Rodriguez & Freedman, 2018; Freedman & Gossett, 1989; Lippincott, 2015), ethane (Hatzinger et al., 2017) and isobutane (Rolston et al., 2019). Recent *in-situ* studies for 1,4-D remediation have focused on aerobic cometabolism using microorganisms simulated on propane as a primary substrate using either sparging (Lippincott, 2015); or recirculation well technology (Chu et al., 2018).

Matrix diffusion or the diffusion of contaminants between high and low permeability zones can complicate remediation practices (Parker et al., 2008). Decades of matrix diffusion can cause the low-permeability zones to act as a storage for high concentrations of groundwater contaminants. Matrix diffusion causes the COCs to be released back into high permeability zones (Liu & Ball, 2002). An analytical modeling study by Adamson *et al.* (2014) investigated the behavior of 1,4-D and 1,1,1-TCA under various loading conditions in a dual media aquifer containing a high permeability (sand) and a low-permeability layer (silt). The study indicated that remediation methods, such as pump and treat are significantly hindered due to the release of 1,4-D and 1,1,1-TCA from the low permeability media after initial treatment of the high permeability zone.

Due to the existence of long plumes of low concentration and matrix diffusion over extended periods, there is interest in developing passive *in-situ* treatment systems. Passive treatment systems, such as PRBs, have the potential to reduce costs and eliminates the need to extract groundwater or continuous add substrates, acceptors and nutrients for biological treatment or chemical treatment. Scherer *et al.* (2008) provide a review of PRBs for groundwater treatment using chemical and biological methods. Cometabolic treatment systems require the continuous addition of a primary substrate and this can lead to competitive inhibition of the contaminant-transforming monooxygenase and a decrease in process efficiency (Frasconi et al., 2015; Semprini, 1997). This has led to strategies for in-situ treatment, such as the pulsed addition of the cometabolic substrate (McCarty et al., 1998; Semprini et al., 1991).

A goal of the research presented here is towards the development of more passive systems for the cometabolic treatment of 1,4-D and 1,1,1-TCA and other COCs. The objective was to evaluate SRCs that slowly hydrolyze to produce primary growth substrates that do not inhibit the monooxygenase enzyme but promote continuous and effective cometabolic treatment. An effective SRC would slowly supply the needed growth substrate needed for the cometabolically-active microorganisms while also promoting the production of the monooxygenase enzyme required for the contaminant transformation. The SRC would undergo abiotic hydrolysis with water to produce the substrate used by the microorganism and would be resistant to biotic hydrolysis to increase its long-term potential as a substrate. The SRC should have a low solubility in water permitting its addition as a non-aqueous phase liquid (NAPL) for in-situ bioremediation. This property would also permit its co-encapsulation at high concentrations with the selected microorganism in a hydrogel beads that could be used to create a PRB. An SRC that upon hydrolysis produces food grade substrate is also of interest to facilitate its in-situ use.

The microorganism evaluated in this study was *R. rhodochrous* ATCC 21198. When grown on isobutane, *R. rhodochrous* ATCC 21198 cometabolically transforms 1,4-D rapidly via first-order rates to low concentrations of ~1 µg/L (Rolston et al., 2019). Isobutane-grown cells of this organism can also transform a broad range of chlorinated ethanes including: 1,1,1-TCA, 1,2-dichloroethane (1,2-DCA), 1,1-DCA and chlorinated ethenes, including: *cis*-DCE, 1,1-DCE and VC (Krippaehne, 2018; Thankitkul, 2016). Mixtures of 1,4-D, 1,1,1-TCA, 1,1-DCE and 1,2-DCA are also effectively transformed by *R. rhodochrous* ATCC 21198 (Krippaehne, 2018).

Previous studies with *Mycobacterium vaccae* JOB5 and *Rhodococcus jostii* RHA demonstrated these bacteria can cometabolize 1,4-D and TCE when grown on 1-butanol (Hand et al., 2015). *R. rhodochrous* ATCC 21198 has also been reported to cometabolically degrade VC after growth on 2-propanol (Kuntz et al., 2003). Thus, there is the potential of supporting the cometabolic transformation by SRCs that produce alcohols. In earlier, but unrelated studies, it was

observed that aerobic cometabolism of TCE and *cis*-DCE could be achieved by a mixed culture maintained on orthosilicates that hydrolyze to form alcohols (Semprini & Vancheeswaran, 2002; Vancheeswaran et al., 1999). The orthosilicates have four organic groups linked to the central silicon atom via oxygen bonds and upon hydrolysis produce four alcohols. Figure 6.1 shows an example of an orthosilicate used in this study, tetra-*s*-butyl orthosilicate (T2BOS), that hydrolyzes to produce 2-butanol (Ihlenfeldt et al., 2009). Vancheeswaran *et al.* (1999) evaluated two orthosilicates, tetrabutyl orthosilicate (TBOS) and tetrakis (2-ethylbutoxy) silane (TKEBS) that hydrolyzed to form 1-butanol and ethylbutanol, respectively. Abiotic hydrolysis rates of TKEBS were an order of magnitude slower than TBOS, indicating the branched nature of the alcohol groups slowed the rates of hydrolysis. For the microbial community enriched on TBOS, the hydrolysis rates were higher than the abiotic rates, suggesting biotic hydrolysis of the orthosilicate ester linkages.

Rasmussen *et al.* (2020) presented results where *R. rhodochrous* ATCC 21198 was co-encapsulated in a gellan-gum hydrogel along with TBOS or T2BOS as SRCs. Long-term cometabolic transformation of a mixture of 1,1,1-TCA, *cis*-DCE and 1,4-D was maintained for over 300 days as the contaminants diffused into the beads and were cometabolically transformed. These results illustrated the promise of co-encapsulating

R. rhodochrous ATCC 21198 with orthosilicates to produce cometabolic gellan-gum beads that can be potentially used to create CPRBs. The work presented here was conducted in parallel with that of Rasmussen *et al.* (2020) and provides a different system of testing and potential direct use of the orthosilicates as SRCs. In this study orthosilicates were added as a neat LNAPL phase along with *R. rhodochrous* ATCC 21198 in batch reactors. The objectives were to determine what alcohols promoted the cometabolic transformation of 1,4-D by *R. rhodochrous* ATCC 21198. Once the potential alcohols were determined, the orthosilicates that produced the alcohol were selected for study.

In batch systems, *R. rhodochrous* ATCC 21198 was grown on the selected orthosilicates, and the simultaneous transformation of 1,4-D and 1,1,1-TCA was evaluated. The abiotic hydrolysis rates of the orthosilicate were also determined under the same conditions of the batch incubations, using poisoned controls. The rates of metabolism, via oxygen (O₂) consumption and CO₂ production, were compared with the rates of cometabolism and the abiotic rates of alcohol production. Three different orthosilicates were evaluated: TBOS; T2BOS and T2POS that produce 1-butanol, 2-butanol and 2-propanol, respectively. The tests were performed in batch reactors and monitored for up to 125 days to evaluate their long-term performance. These comparisons provided insight into how the structure of the orthosilicates are an important variable when selecting SRC for a passive cometabolic treatment system. The method provides a simpler system for evaluation of the SRC, since encapsulation is not required. The study also evaluated the potential for the direct addition of the SRC as a NAPL along with the desired microorganism to promote long-term cometabolic treatment.

Methods and Materials

Chemicals

All chemicals used were analytical grade: Isobutane, 99.99% (Gas Innovations, Texas); 1,4-D, 99% (Baker Analytical, Fisher Scientific); deuterated 1,4-D, >99%, 1-butanol, 99% (Acros (Fisher Scientific); 2-butanol, 99%; 2-propanol, 99% (Sigma-Aldrich, Missouri); tetra-butyl orthosilicate, 97% (Aldrich, St. Louis); tetra-*sec*-butyl-orthosilicate, 95%; tetrakispropoxysilane,

99% (Gelest Pennsylvania); 1,1,1-TCA, 95.5% (Baker Analytical, Fisher Scientific). Stock solutions of 1,4-D and deuterated 1,4-D, 1 mg/L and 100 mg/L, respectively, and 1,1,1-TCA were prepared in autoclaved deionized water. Acetylene was generated by the reaction of calcium carbide (Alfa Aesar, Tewksbury, MA) with water.

Growth and maintenance of R. rhodochrous ATCC 21198

For resting cell and cometabolism tests, *R. rhodochrous* ATCC 21198, obtained from the ATCC (Manassas VA) was grown on isobutane, 1-butanol, 2-butanol, and 2-propanol in 720-mL glass bottles containing 270 mL of phosphate-buffered mineral salts medium (MSM) (Kottegoda *et al.*, 2015) and 450 mL of air headspace, as described by Rolston *et al.* (2019). The cells were incubated at 30°C at 200 rpm and harvested at the late exponential growth phase by centrifugation at $15,000 \times g$ for 10 minutes. The cell pellet was resuspended in 50 mM phosphate buffer (25 mL) and centrifugation and washing was repeated. The biomass concentration was determined using a TSS test as described in AWWA standards (Rice *et al.*, 2017). Protein analysis was used to quantify the TSS protein ratio. Under the growth conditions described the ratio of protein to TSS was 0.45 mg protein.mg TSS (Rolston *et al.*, 2019).

For ABL studies *R. rhodochrous* ATCC 21198 was grown in batch culture in glass serum vials (160 mL) sealed with butyl rubber stoppers and aluminum crimp seals (Wheaton Scientific, Millville, N.J.). The vials contained mineral salts medium (MSM) (23 mL) (Wiegant & De Bont, 1980) and were inoculated to an initial optical density (OD₆₀₀) of ≤ 0.02 using cells previously grown on agar plates containing casein-yeast extract-dextrose (CYD) media. Isobutane (10% v/v gas phase) or alcohols (0.1% v/v liquid phase) were added as sole sources of carbon and energy and the cultures were grown in the dark in an environmental shaker operated at 30°C and 150 rpm. After growth for ~3 days, the culture vials were opened in a sterile laminar flow hood to replenish air in the vials. After equilibration for 10 minutes, the vials were re-sealed, and the original growth substrate was added again to the original initial concentration. Once the final culture OD₆₀₀ reached ~0.7, a sample (20 μ L) of the culture was plated on CYD plates to subsequently confirm the purity of the culture. The remaining cells were harvested by centrifugation ($10,000 \times g$, 5 min at 4°C). The supernatant was decanted, and the resulting cell pellet was resuspended in buffer (10 mL, 50 mM sodium phosphate, pH 7.0) and centrifuged again ($10,000 \times g$, 5 min at 4°C). The cell pellet was finally resuspended in buffer to a protein concentration of ~10 mg/mL and the washed cells were stored on ice and used for experiments within 4 hours.

Activity Based Labeling

The ABL method was based on procedures described by Bennett *et al.* (2016) to covalently fluorescently label components of AMO. In the first step of the procedure, cells were incubated with 17OD to inactivate monooxygenase enzymes. The reactions were conducted in glass serum vials (10 mL) sealed with butyl rubber stoppers and aluminum tear-off seals. The reactions contained 17OD (100 μ M) added from a stock solution (0.1 M) in DMSO and were initiated by the addition of harvested cells (1 mL) to the reaction vial which were then incubated for 30 minutes in a shaking water bath operated at 30°C and 150 rpm. After this incubation, the incubated cells were pelleted by centrifugation ($10,000 \times g$, 5 min), washed twice with buffer (1 mL, 50 mM sodium phosphate, pH 7.0) and finally resuspended with buffer (~0.3 mL). The resulting cell suspension was then disrupted by two cycles of bead beating (5.0 m/s, 55 sec) in tubes (2 mL) containing lysing matrix B (MP Biomedicals, Irvine, CA). The resulting cell extract was briefly centrifuged to remove unbroken cells and the resulting cell-free extract was stored on ice at 4°C

and used within 1 hour. In the second step of the procedure, the cell extract from 17OD-treated cells was reacted with AlexaFluor 647 to fluorescently label monooxygenase components using a copper(I)-catalyzed alkyne-azide cycloaddition reaction. In summary, a sample of the broken cell extract from 17OD-treated cells (30 µg total protein) was mixed with deionized water in a microfuge tube (500 µL) and SDS was added from an aqueous stock solution (10 % w/v) to final concentration of 0.13% (w/v). AlexaFluor 647 azide (8 µM) (1 µL of 0.6 mM stock solution in DMSO), CuSO₄ (2 mM) (1.5 µL 0.1 M aqueous solution) and sodium ascorbate (10 mM) (7.5 µL of 0.1 M aqueous solution) were added sequentially to give a final reaction volume of 75 µL. The vials were incubated at room temperature in a dark room for 1 hour and were then quenched by adding 3-butyn-1-ol (13 mM) (1 µL of a 1M aqueous solution). The resulting labeled cell extracts were stored in the dark at -20 °C prior to analysis by SDS-PAGE.

SDS-PAGE and IR scanning

SDS-PAGE analyses were conducted using 9% discontinuous polyacrylamide gels using the Bio-Rad Mini-PROTEAN electrophoresis system (Bio-Rad, Hercules CA). Protein samples (≤ 100 µg total protein) were prepared by adding 6X sample buffer (375mM Tris-HCl, 9% SDS, 50% glycerol, 0.05% bromophenol blue, and 9% β-mercaptoethanol) at room temperature. Samples (5 µg total protein) were then loaded onto the gels and electrophoresed at room temperature for 90 minutes at a fixed current of 25-35 mA. To visualize fluorescently labeled polypeptides, immediately after electrophoresis the unfixed gel was scanned with an Li-Cor Odyssey 9120 IR scanner (Li-Cor Biotechnology, Lincoln, NE) and migration of fluorescently-labeled NIR marker proteins was used to estimate the mass of fluorescently labeled bacterial polypeptides (K. Bennett et al., 2016). The gel images were recorded and analyzed by Odyssey imaging system application software (Version 3.0). The protein concentration of all cell extracts was determined by Biuret protein assay (Gornall et al., 1949). In summary, samples were solubilized by incubation with 1.5 M NaOH (final concentration) at 65°C for 30 minutes. The solubilized protein sample was briefly centrifuged (10000 × *g*, 5 min) to remove insoluble material was then assayed using bovine serum albumin as a standard.

Resting-cell 1,4-D transformation tests

Resting cell 1,4-D transformation tests were performed in sterile 27 mL batch vials sealed with butyl rubber septa and crimp tops, as described by Rolston *et al.* (2019). Phosphate-buffered nutrient medium (10 mL) was added along with cells (5 mg-TSS). Experimental controls included an abiotic control (no cells) and an acetylene control (10% v/v gas phase) to inactivate the monooxygenase enzyme (M. R. Hyman & Wood, 1985). Acetylene was found to be an effective inhibitor of 1,4-D cometabolism and isobutane utilization by *R. rhodochrous* ATCC 21198 (Rolston et al., 2019). Acetylene was generated from the reaction of water with calcium carbide (CaC₂) and then added to the reactor headspace. Acetylene was allowed to equilibrate with the cells for 1 hour on a shaker table (200 rpm) before 1,4-D, or isobutane, was added, which ensured the monooxygenase enzyme were fully inactivated. The batch reactors were incubated at 30 °C while shaking at 200 rpm to ensure equilibrium was maintained between the gas and liquid phases throughout the experiment. Cometabolism tests were performed at an aqueous concentration of 1,4-D of 17.6 mg/L.

The degradation of THF was determined in small-scale reactions conducted in glass serum vials (10 mL). The vials contained 50 mM phosphate buffer (900 µL) and were sealed with Teflon-lined Mininert valves. THF was added to the vials to an initial concentration of 1 mM using a

freshly prepared aqueous solution (~ 0.1 M). The vials were then placed in a heated shaking water bath (3°C, 150 rpm) for 10 min to allow equilibration of the THF between the gas and liquid phase. The reactions were initiated by adding aliquots (100 µL) of concentrated cell suspension. The reaction vials were then returned to the shaking water bath and samples were removed as required for analysis of residual THF by gas chromatography.

Cometabolism Tests

Methods presented by Vancheeswaran *et al.* (1999) were used to evaluate the growth of *R. rhodochrous* ATCC 21198 on TBOS, T2BOS, and T2POS as SRCs, and cometabolism of 1,1,1-TCA and 1,4-D. In summary, batch tests were performed in autoclaved glass serum bottles (178 mL) sealed with screw-on caps with butyl rubber septa and each experimental condition was conducted in duplicate. The batch reactors were constructed aseptically in a laminar flow hood and contained phosphate-buffer MSM (68 mL) with the remaining volume (110 mL) as air headspace. The reactor medium was inoculated with a small amount of isobutane-grown cells *R. rhodochrous* ATCC 21198 (0.1 mg TSS) to ensure that cell growth would be observed during the test. Tests were first performed to examine growth directly on alcohols by adding individual alcohols (45 µmol) to the batch reactors. For tests with orthosilicates as long-term sources of alcohols, the neat compounds were added directly to the medium to achieve a nominal concentration of 1500 mg/L. These concentrations are above the aqueous solubility limit; the orthosilicates were present mainly as dispersed LNAPLs, as described by Vancheeswaran *et al.* (1999). Thus, *R. rhodochrous* ATCC 21198 was exposed to both the dissolved and non-aqueous phase orthosilicate. 1, 4-D (59 µL) was added from a 1000 ppm aqueous stock solution, while 1,1,1-TCA (23 µL) was added from a saturated aqueous stock solution to achieve the desired mass additions. Several additions of 1,4-D and 1,1,1-TCA were made over the time course of the experiment.

In order to study the abiotic hydrolysis of the orthosilicates, batch reactors were constructed as described above with sodium azide (0.2% w/v) added from a 1M aqueous stock solution. To investigate the role of monooxygenases in the transformation of orthosilicates, 1,4-D and 1,1,1-TCA, control reactions were prepared as described above with acetylene (10% (v/v) added to the reaction headspace. To evaluate the ability of resting cells to transform the mixtures of 1,4-D and 1,1,1-TCA, controls tests were also constructed with the initial cell inoculum (0.1 mg-TSS) added, but with no orthosilicate present.

Analytical Methods

Details of the analytical methods for the headspace measurement of 1,1,1-TCA and the aqueous concentration of 1,4-D are described by Rolston *et al.* (2019). Briefly, 1,1,1-TCA was measured in a 0.1 ml gas headspace sample on a Hewlett Packard Series 6890 Gas Chromatograph equipped with an electron capture detector (ECD). Chromatographic separation was achieved using an Agilent Technologies Inc. DB-62941 column with helium as a carrier gas at 8 mL/min and the ECD received a flow of 95% argon and 5% methane at ultra-high purity (AIRGAS, Corvallis/Albany OR). The temperatures of the oven, injector, and detector were 100°C, and 250°C, and 250°C, respectively. The residence time of 1,1,1-TCA was 1.3 minutes. The method was quantified using external standards. The quantification limits for 1,1,1-TCA was around 1 µg/L at an equilibrated aqueous concentration in the reactor.

The headspace O₂ and CO₂ concentrations were used as indicators of metabolic activity. Headspace O₂ was quantified with a Hewlett Packard 5890 GC equipped with a Supelco 60/80 Carboxen 1000 column and a thermal conductivity detector (TCD). The temperatures of the oven,

injector, and detector were 40°C, 275 °C, and 275°C, respectively. Ultra-high purity helium (AIRGAS, Corvallis/Albany OR) was the carrier gas flowing at 30 mL/min. The O₂ residence time was 4.5 min. CO₂ was also quantified with a Hewlett Packard 5890 GC equipped with a Supelco 60/80 Carboxen 1000 column equipped with a TCD detector. The temperature of the oven, injector, and detector were 220°C, 275°C, and 275°C, respectively. Ultra-high purity argon was used (AIRGAS, Corvallis/Albany OR) as the carrier gas flowing at 15 mL/min. The residence time for CO₂ was 3 minutes. Both the O₂ and CO₂ headspace concentrations were quantified using external standards.

The concentration of the alcohols (1-butanol, 2-butanol, and 2-propanol) in active and poisoned reactors was determined by GC analysis of aqueous samples. To avoid sampling the non-aqueous phase orthosilicate, the aqueous samples were obtained by inverting the batch reactors so that the orthosilicate LNAPL floated to the top of the medium and aqueous sample was obtained from the middle of the reactor. The samples were filtered through a 0.45- μ m PVDF filter and 5 μ L was injected to a Hewlett Packard 5890 GC with a 0.2% CW-1500 80/100 Carbopack C column and FID detector. The temperatures of the oven, injector, and detector were 105°C, 150°C, and 175°C, respectively. Residence times for 1-butanol, 2-butanol and 2-propanol were 3.5, 3.5 and 1.2 minutes, respectively. The alcohol detection limit for this analysis was 1 mg/L.

1,4-D was analyzed from liquid samples on a Hewlett Packard Series 6890 Gas Chromatograph-Model 5973 Mass Spectrometer (GC-MS) preceded by a Tekmar Dohrmann Model 3100 heated purge-and-trap concentrator. Rolston et al. (2019) describe details of the method. The GC-MS was run in single ion mode for m/z 88 (1,4-D) and m/z 96 (deuterated 1,4-D run as an internal standard (Draper et al., 2000)). The 1,4-D quantification limit was 0.1 μ g/L with a 25-ml aqueous sample. Samples from the batch reactors (0.3 ml) were diluted 125 to 250 times in DI water when loaded onto the purge-and-trap concentrator.

For the analysis of THF degradation, aqueous samples (2 μ L) from the sealed reaction vials were immediately injected into a Shimadzu GC-8A gas chromatograph fitted with a flame ionization detector and a stainless-steel column (0.3 by 183 cm) filled with Porapak Q (60 to 80 mesh; Waters Associates, Framingham, Mass.). The analysis was conducted with a column temperature of 160°C, an injection port temperature of 200°C, and a detector temperature of 220°C. Nitrogen was used as the carrier gas at a flow rate of 15 ml min⁻¹. The gas chromatograph was interfaced with a Hewlett Packard HP3395 (Hewlett Packard, Palo Alto, Calif.) integrator for data collection. Rates of THF degradation were determined from plots of residual THF versus time for samples taken every 10 minutes after the addition of cells. The rates were determined by linear regression analyses. Quantification of THF was determined by the calibration plots developed using authentic standards of THF.

Mass balances

For the duration of each experiment, the batch reactors were stored at 20°C and shaken at 150 rpm on a rotary shaker table to maintain equilibrium partitioning between the headspace and liquid phases. The total mass, M_T , of each compound was calculated from Equation 5.1, where C_l , C_g , V_l , and V_g are the concentrations and volumes in the liquid and gas compartments, respectively, and H_{cc} is the dimensionless Henry's constant equal to the ratio of a compound in the gas and liquid phases. H_{cc} values were 49.2, 0.0002, 0.548, 31.5 and 1.20 for isobutane, 1,4-D, 1,1,1-TCA, O₂ and CO₂, respectively (Sander, 1999).

$$Mass_{total} = C_g \left(\frac{V_l}{H_{cc}} + V_g \right) \quad (\text{Equation 5.1})$$

Zero-order rates of O₂ consumption, CO₂ and alcohol production and first-order rates of 1,1,1-TCA and 1,4-D were determined via linear-regression of the mass histories using Microsoft Excel.

Experimental Results

Activity Based Labeling

The ABL approach used in this study targets the hydroxylase components of catalytically active monooxygenases and generates a covalent fluor:probe:enzyme adduct that can be visualized by NIR scanning of SDS-PAGE analyses of protein extracts from cells. In this study, we used whole cells assays to determine monooxygenase activity and these rates were then compared to the fluorescent ABL patterns and intensity for cells grown on the same substrates.

The activity of cells was initially determined by determining the specific rate of consumption of THF, a structural analog of 1,4-D. Cells grown on gaseous alkanes (C₂-C₄) all exhibited high rates of degradation of THF (40-110 nmoles/min-mg total protein) (Figure 6.2A) with the lowest rate observed with propane-grown cells and the highest rates observed with cells grown on *n*-butane or isobutane. Only very low rates of THF consumption (≤ 5 nmoles/min-mg total protein) were determined for cells grown on the 1° alcohols tested (ethanol, 1-propanol, 1-butanol, isobutanol) while low but readily detectable rates of THF consumption (~ 10 -15 nmoles/min-mg total protein) were detected for cells grown on 2° alcohols (2-propanol and 2-butanol). In the ABL assays, isobutane-grown were used to illustrate the labeling pattern and intensity for cells with high level of monooxygenase activity (Figure 6.2B). The labeling pattern included at least one intensely labeled polypeptide with a mass of ~ 60 kDa and several substantially less intensely labeled smaller polypeptides. In the case of alcohol-grown cells, no labeling was observed for cells grown on any of the primary 1° alcohols tested (ethanol, 1-propanol, 1-butanol and isobutanol) while low levels of labeling were observed for cells grown on either 2-butanol or 2-propanol. Cells grown on 2-butanol exhibited labeling of a ~ 60 kDa polypeptide while the lower level of labeling was associated with a larger (~ 70 kDa) polypeptide.

Resting cell transformation of 1,4-D by R. rhodochrous ATCC 21198

Resting cell transformation tests were performed with *R. rhodochrous* ATCC 21198 grown on either 1-butanol, 2-butanol, 2-propanol or isobutane to investigate whether the trends observed in the ABL experiments also occurred with 1,4-D. Both isobutane- (Figure 6.3A) and 2-butanol-grown cells (Figure 6.3C) immediately transformed 1,4-D (17.6 mg/L) without a lag phase although the rates of 1,4-D degradation of 2-butanol-grown cells were 5-fold lower than isobutane-grown cells. 2-propanol-grown cells also immediately transformed 1,4-D, but at rate ~ 9 slower than isobutane-grown cells (data not shown). In contrast, 1-butanol-grown cells only transformed 1,4-D after a lag-period of 3 to 4 hours, indicating an induction period was required before transformation occurred (Figure 6.3B). After the lag period, 1,4-D was transformed at rate ~ 14 times lower than isobutane-grown cells

Cometabolic transformation of 1,4-D and 1,1,1-TCA during growth on 2-butanol.

As cells grown on 2-butanol appeared to have readily detectable rates of monooxygenase-catalyzed reactions, we subsequently examined the longer-term degradation of contaminants (1,4-D and 1,1,1-TCA) by *R. rhodochrous* ATCC 21198 when growing on 2-butanol in batch reactors.

Our results (Figure 6.4) are presented for the second addition of 2-butanol to the reactors. The production of CO₂ and utilization of 2-butanol (Figure 6.4A), the increase in optical density (Figure 6.4B), all demonstrate that in this experimental system *R. rhodochrous* ATCC 21198 grew on 2-butanol within approximately 1 day. Following a second addition of 2-butanol, degradation of 1,4-D (Figure 6.4C) accelerated as microbial growth occurred, with most of the degradation occurring 0.5 to 1.5 days after the alcohol addition. 1,4-D transformation lagged the consumption of 2-butanol. 1,1,1-TCA (Figure 6.4C) transformation was consistent with 1,4-D, however, it was transformed at a slower rate. The slowing in rates after 1.5 days may result from 1,1,1-TCA concentrations being reduced below the half-substrate concentration, K_s value.

Based on the results presented in Figure 6.4, both zero-order and first-order rate estimates were made of 1,1,1-TCA and 1,4-D cometabolism, while zero-order rate estimates were made for CO₂ production and O₂ and 2-butanol consumption (Table S6.3). Similar rates estimates were obtained for the duplicate reactors and consistent increases in rates were observed with the second addition of 2-butanol. The first-order rates for 1,1,1-TCA increased by a factor of 2.1, while for 1,4-D by a factor of 2.3 between the first and second additions. The increased transformation rates are consistent with the increased rates of metabolism as indicated by CO₂ production and 2-butanol consumption. The zero-order rates of CO₂ production increased by a factor of 2.0, while that of 2-butanol was 3.4. The results are consistent with the increase in O.D. resulting from the increase biomass. The first-order rates of 1,4-D transformation were a factor of 2.3 and 2.5 greater than 1,1,1-TCA consistently, between the first and second edition, respectively.

Abiotic hydrolysis of orthosilicates.

In order to provide for a long-term source of alcohols, 1500 mg/L of three orthosilicates (TBOS, T2BOS and T2POS) were added to reactors as an LNAPL. Mixing on the rotary shaker table dispersed the LNAPL fine droplets and provided a high surface area for interaction with water. The time course of alcohol production from via abiotic hydrolysis of each orthosilicate in poisoned controls showed a near linear increase in each case, indicating the abiotic hydrolysis rate followed pseudo zero-order kinetics (Figure 6.5). The zero-order rates determined via linear regression were 11.1, 5.1, and 0.76 µmol/day for TBOS, T2POS, and T2BOS respectively. When normalized for the aqueous volume present, the rates were 62.4, 28.7, and 4.3 µmol/L-day, respectively.

Metabolism and Cometabolism on T2BOS, TBOS, and T2POS

Metabolism and Cometabolism with T2BOS

Metabolism in the batch reactors was determined through the monitoring of CO₂ production and O₂ consumption in the reactor headspace, while cometabolism was monitored by the concentration histories of 1,1,1-TCA and 1,4-D. The long-term observations from the T2BOS reactor are shown in Figure 6.6. The early rapid increase in CO₂, likely results from the 2-butanol present as an impurity in the neat T2BOS that was added. The initial 2-butanol concentration decreased rapidly in both the live and the acetylene-treated reactors (Figure 6.5B). A constant and gradual increase in CO₂ was then observed over 125 days. Between day 5 and day 125, approximately 30 µmol of CO₂ were produced in the reactor headspace. A significant amount of CO₂ produced would also be associated with bicarbonate ion (HCO₃⁻) in solution at the buffered pH of 7.0, which is not included in the mass reported. CO₂ production was also observed in the batch reactor containing acetylene (Figure 6.6A), which is consistent with 2-butanol utilization not requiring monooxygenase activity. No CO₂ production was observed in the poisoned control. The

gradual increases in CO₂ are consistent with the slow rates of hydrolysis observed in the poisoned control (Figure 6.5B). After the initial 2-butanol added as an impurity of the neat T2BOS was consumed, 2-butanol was not detected in the aqueous phase of the live reactors with and without acetylene, indicating that it was rapidly utilized as it was produced via hydrolysis (Figure 6.5B).

A slow, but gradual rate of O₂ consumption was observed compared to the poisoned and abiotic controls (Figure 6.6B). O₂ consumption occurred in all the reactors where CO₂ accumulated, including the acetylene-treated control reactors. The rate of O₂ consumption (Figure 6.5B) is in the range expected from the utilization of the 2-butanol produced via hydrolysis (Figure 6.5B). CO₂ production and O₂ consumption and 2-butanol concentrations being below detection, all support continuously metabolic activity over the 125 days of incubation. The acetylene live treatment tracked the live treatments without acetylene, demonstrating the monooxygenase activity was not required to metabolize 2-butanol.

The concentration histories of 1,1,1-TCA and 1,4-D for the batch tests with T2BOS are shown in Figure 6.6 C and D. Three additions of 1,1,1-TCA and 1,4-D were made to the biologically active reactors at 0, 21, and 60 days. The acetylene-treated, the poisoned, and cell only reactors received only one addition of 1,1,1-TCA and two additions of 1,4-D. Consistent with CO₂ production, as an indicator of metabolic activity (Figure 6.6A), 1,1,1-TCA and 1,4-D were both cometabolically transformed during the first 20 days of incubation. Minimal decreases in 1,1,1-TCA and 1,4-D were observed in the abiotic and acetylene-treated live control. 1,1,1-TCA and 1,4-D transformation not being observed in the live acetylene-treated control, indicates monooxygenase activity was responsible for their transformation. The rates of 1,1,1-TCA and 1,4-D transformation decreased slightly in the second addition. For the initial transformation test (day 0 to 20), the cells had access to the free aqueous 2-butanol from the impurity present in the neat T2BOS, which likely facilitated the more rapid cometabolic transformation.

A third addition of 1,1,1-TCA and 1,4-D was made to the active bottles around day 60. The same mass of 1,1,1-TCA and 1,4-D were added except for active bottle 1 (AB1). For AB1, a 10-fold greater mass of 1,4-D was added to investigate the transformation rate at higher 1,4-D concentrations (Figure. 6.6D). The increased 1,4-D concentration was effectively cometabolized and its higher concentration did not affect the first-order rate of 1,1,1-TCA transformation (Figure 6.6C). The results show the cometabolic transformation of 1,1,1-TCA and 1,4-D was maintained over a period of 125 days, with a single addition of T2BOS. As discussed above, the sustained metabolism was achieved over this period. The observations support sustained cometabolism of 1,1,1-TCA and 1,4-D driven by the continuous metabolism of 2-butanol produced from T2BOS hydrolysis.

Metabolism and Cometabolism with TBOS

Figure 6.7 shows the results from the incubation of *R. rhodochrous* ATCC 21198 with TBOS. Since the abiotic hydrolysis of TBOS is a factor of 15 higher than T2BOS (Figure 6.5), much higher rates of metabolism were expected. A continuous increase in CO₂ headspace concentration was observed over the 45 days of the test in both the live reactors and the acetylene-treated control. One of the duplicates had a slower rate of CO₂ accumulation, while the acetylene-treated control matched the other duplicate. The zero-order rate of CO₂ production in the TBOS reactors were factors of 7 to 14 times higher than the T2BOS reactors (Table S5) consistent with the higher rate of TBOS hydrolysis. Measurements of headspace O₂ concentrations were initiated on day 21. Over the period of 20 to 25 days, the duplicate with the slower rate of CO₂ production

had a slower rate of O₂ consumption. The slower rates might be associated with a lower initial biomass of *R. rhodochrous* ATCC 21198 in the reactor. Due to the high rate of consumption, O₂ was added to the reactors on days 28 and 34. After the third addition, the rate of O₂ consumption was similar in the all reactors, likely indicating the development of a similar biomass. The much higher utilization of O₂ in the TBOS reactors compared to the T2BOS reactors is evident. A buildup of 1-butanol was only observed in the poisoned control (Figure 6.5A). Concentrations remained near detection in the live and acetylene treated reactor indicating 1-butanol was rapidly consumed upon release from TBOS hydrolysis. The acetylene-treated control also showed similar rates of CO₂ production and O₂ consumption as the active bottles that lacked acetylene, consistent with the T2BOS results.

1,1,1-TCA and 1,4-D concentration histories for the TBOS reactors are shown Figure 6.7C and D. The duplicate reactor that more slowly produced CO₂ and consumed O₂ initially lags in 1,1,1-TCA and 1,4-D transformation, which likely results in lower biomass being present. The rates of 1,1,1-TCA and 1,4-D transformation increase in the second and third additions indicating an increasing biomass in that reactor. The duplicate reactor showed increasing cometabolic rates consistent with increase in the rate of oxygen uptake. The results support an increase in biomass concentration resulting from microbial growth. Continuous transformation of 1,1,1-TCA and 1,4-D was observed over the 45 day study. No transformation was observed in the acetylene treated control, consistent with the T2BOS reactor observations.

Metabolism and Cometabolism of T2POS.

Figure 6.8 presents the results from the incubation of *R. rhodochrous* ATCC 21198 with T2POS. T2POS has a rate of hydrolysis between that of T2BOS and TBOS (Figure 6.5C), thus the rate of metabolism and cometabolism is expected to be in between those of TBOS and T2BOS. Duplicate active bottles showed very reproducible rates of CO₂ production and O₂ consumption. The acetylene control showed a more rapid increase CO₂ production and O₂ consumption, possibly due to a higher initial biomass. Consistent with the rate of hydrolysis, the rates of CO₂ production and O₂ consumption were between those of the T2BOS and TBOS reactors (Table S6).

The continuous transformation of 1,1,1-TCA and 1,4-D was observed in the duplicate live reactors. Some resting cell transformation was observed inoculum control, however after 7 days, transformation of 1,1,1-TCA and 1,4-D slowed, whereas the active bottles transformation continued. Another addition of 1,1,1-TCA and 1,4-D was added on day 19 and the active bottles continued to transform 1,1,1-TCA and 1,4-D. Consistent with the TBOS and the T2BOS tests, transformation was not observed in the reactor containing acetylene.

Comparison of rate of metabolism and cometabolism observed in the batch tests

Data used to calculate rates for single reactors presented in Tables S6.4 to S6.6 and Figures S6.4 to S6.8. Both zero-order and first-order rate estimates were made of 1,1,1-TCA and 1,4-D cometabolism, while only zero-order rate estimates were made for CO₂ production and O₂ consumption. The first-order rate estimates were made over the full range of concentrations, while zero-order made over the periods of the highest rates of contaminant decrease.

In the T2BOS reactors, the first-order and zero-order rates of cometabolism were similar for the duplicates (Table S4). The first-order rates decreased with each addition of 1,1,1-TCA and 1,4-D. The initial rate was a factor of 5 greater for 1,1,1-TCA and approximately 10 higher for 1,4-D compared to the rate estimated for the third addition. A much higher concentration of 1,4-

D was added in the third addition, which resulted in a higher zero-order rate. Similar rates of CO₂ production and O₂ consumption were observed in the duplicates consistent with the similar rates of cometabolism.

It is interesting to compare the rates observed with T2BOS to those obtained with the growth directly on 2-butanol (Figure 6.4, Table S6.3). The first-order rates of 1,1,1-TCA and 1,4-D achieved with their first addition in the T2BOS reactors was 0.4 and 0.5, respectively of those obtained during the growth of 2-butanol. The rate of 1,4-D was also a factor of 2.5 times higher than 1,1,1-TCA during this period, consistent with observations during growth on 2-butanol. These high rates support early growth on 2-butanol that was present in the T2BOS. By the third addition of 1,1,1-TCA and 1,4-D to the T2BOS reactors the rates were reduced by over an order magnitude compared to those obtained during direct growth on 2-butanol.

In the TBOS reactors (Table S6.5) the first-order rates more than doubled for both 1,1,1-TCA and 1,4-D with the three additions, while zero-order rates increased by a factor three. While the first-order rate of the duplicates initially differed by a factor of three, they were very similar by the third addition. The first-order rates were higher than observed in the tests with T2BOS, consistent with the higher rates of metabolism and alcohol production. For example, both the rates of CO₂ production and O₂ consumption were approximately a factor of 20 higher in the TBOS reactors than the T2BOS reactor. The higher rates of metabolism are consistent with the rate of 1-butanol release, which was about a factor of 15 greater than 2-butanol. The first-order rates of cometabolism of 1,1,1-TCA and 1,4-D ranged from 10 to 20 times higher in the TBOS reactors, supporting a greater biomass developing in these reactors.

For the T2POS reactors (Table S6.6) first-order rates of 1,1,1-TCA and 1,4-D transformation were very similar in the duplicate reactors and remained fairly constant between the first and second additions. The rates of CO₂ production and O₂ consumption were also very similar between the duplicates. The estimated first-order rates for T2POS are slower than those observed with TBOS, but faster than observed with T2BOS.

Figure 6.9 provides a summary of the estimated rates provided in Appendix A (Figures S6.4 – S6.8). The results show a strong correlation between rates of metabolism, as indicated by CO₂ production and O₂ consumption (not shown), and the rates of 1,1,1-TCA and 1,4-D transformation. The rates of CO₂ production are also correlated with the rates of alcohol production. It is interesting to note that the first-order rates of 1,4-D transformation are a factor of 1.6 times those of 1,1,1-TCA for all three orthosilicates tested, despite the rates differing by a factor of 18 between TBOS and T2BOS reactors. The similar factor difference in rates indicate the same oxygenase enzyme is transforming the contaminant despite the growth on the different alcohol substrates.

Within a treatment, comparison of duplicate reactors indicate rate of cometabolism are also correlated with the rates of metabolism and likely biomass present. When the rates of metabolism were higher for a duplicate then the rates of cometabolism for both 1,1,1-TCA and 1,4-D were also higher. Both the rates of metabolism and cometabolism being correlated indicate that higher biomass concentrations were maintained in the TBOS reactor compared to the T2POS and T2BOS reactors.

A photo of the TBOS reactor at the start (Day 0) and end (Day 43) of the incubation is presented in Figure S6.9. The dispersion of the TBOS into colloidal liquid droplets is indicated on Day 0. At a later time, the orange color indicates the growth of *R. rhodochrous* ATCC 21198,

which when plated appears orange in color. The cells clump and tend to grow around particle of TBOS making measurements of biomass on a protein basis difficult. Figure S6.10 shows the photos for the reactors with growth on the different substrates. For the T2BOS and T2POS batch reactors a visual increase of *R. rhodochrous* ATCC 21198 was difficult to see, consistent with their lower metabolic activity. A greater extent of growth was observed in the reactor containing TBOS compared to the reactors containing T2POS and T2BOS, consistent with the higher rates of metabolism and cometabolism observed.

The zero-order estimated rates of oxygen utilization divided by the zero-order the rates of alcohol production can be used to estimate the molar ratios of O₂ utilized to substrate consumed. The ratios are 3.5, 2.5, 0.7 $\mu\text{mol O}_2/\mu\text{mol alcohol}$, for TBOS, T2BOS and T2POS incubations, respectively. These ratios compared with 6.5, 6.5, and 3.5 $\mu\text{mol O}_2/\mu\text{mol alcohol}$ for the complete oxidation of the respective alcohols to CO₂ and H₂O. The lower the ratios based on the utilization compared to ratios required for complete oxidation are consistent with substrate utilization for the generation of cell biomass (Rittman and McCarty, 2003). The results also indicate that biotic hydrolysis does not seem to be important, since a more rapid rate of alcohol production would have increased the oxygen demand.

Discussion of Results

In this study, ABL was shown to be a useful and simple method for determining the level of monooxygenase activity in cells and one that potentially provides more information than whole cell activity measurements alone. For example, the labeling intensity for isobutane-grown cells was much greater than cells grown on 2-butanol and this difference clearly reflected the substantial differences in the specific rates of THF (Figure 6.2) and 1,4-D (Figure 6.3) degradation. Conversely, the lack of measured monooxygenase activity in cells grown on 1° alcohols was also accurately reported by the ABL approach. The ABL approach also enabled discrimination between cells that express a 1,4-D-degrading monooxygenase during growth on alcohols (e.g. 2° alcohols) as opposed to expressing these enzymes after growth (e.g. 1° alcohols). This difference was confirmed by demonstrating that cells grown on 2-butanol degrade 1,4-D without a lag phase (Figure 6.3C) while cells grown on 1-butanol exhibit a lag phase in activity (Figure 6.3B) that likely represents the time required for the cells to induce and newly synthesize monooxygenase enzymes. Lastly, as the ABL method targets the active-site containing component of monooxygenases, the labeling pattern can potentially identify which enzyme is expressed. For instance, as a labeled polypeptide of the same mass (~60 kDa) was detected for cells grown on isobutane and 2-butanol, our results suggest that the same enzyme is likely induced during growth on these two substrates. While promising, more research is needed to use ABL to quantify monooxygenase expression levels and allow direct correlations between protein expression and the rates of cometabolism. A potential application of a quantitative ABL approach could be to monitor the time course of monooxygenase induction in 1-butanol-grown cells when they are exposed to 1,4-D. More research is also needed to exploit ABL for identifying specific monooxygenases. Although the labeled polypeptides we observed in this study were not identified at the molecular level, we have previously shown that the labeling targets the active-site containing polypeptides in bacterial monooxygenases (Bennett *et al.*, 2016). As unequivocal identification of labeled polypeptides can require extensive and expensive mass spectral approaches, the current ABL procedure can provide a simple and effective screening procedure where unequivocal protein identification is not an immediate priority.

The cometabolic transformation of TCA and 1,4-D was observed during growth on 2-butanol, with increases in rate corrected with higher rates of metabolism and increases in optical density associated with increases in biomass. This type of growth experiment provides a simple method of evaluating the potential of different alcohols produced by orthosilicates to promote cometabolism. Cometabolism was continuously observed with all the three of the orthosilicates tested, indicating continuous monooxygenase expression. Inhibition by the growth substrate on cometabolism was likely not occurring since monooxygenase activity is not needed for alcohol utilization, as indicated by the continuous metabolic activity with all three orthosilicates that occurred in the presence of acetylene.

The differences in the rates of hydrolysis are consistent with previous observations of Vancheeswaran *et al.* (1999) who evaluated TBOS that produces 1-butanol and TKEBS that produces 2-ethylbutanol, a branched alcohol. Abiotic hydrolysis rates for TKEBS were an order of magnitude slower than for TBOS, indicating that branching has an impact on the orthosilicate hydrolysis rates. Results presented here therefore suggest the rate of hydrolysis for T2BOS being a factor of 15 slower than TBOS was due to the branching effect previously observed with TBOS and TKEBS. The substituent chain length is also important as T2BOS that has an additional carbon atom hydrolyzed at a 7-fold slower rate than T2POS. The structure of the orthosilicate and the alcohol leaving group therefore are important factors that can be used in design of in-situ remediation systems. For example, if higher biomass and higher rate systems are needed TBOS would be chosen while for lower biomass and slow rate branched chained orthosilicates with longer chain lengths, like T2BOS would be used.

Another consideration is delivery of the O₂ that is needed for this aerobic process. O₂ consumption is correlated with the rates of hydrolysis and alcohol release. Much higher rates of oxygen consumption were observed in TBOS fed reactors compared to T2POS and T2BOS. Thus, there is a benefit of using a more slowly hydrolyzing orthosilicate.

The results presented here did not provide strong evidence for the biotic hydrolysis of the orthosilicates. Rates of alcohol production in the poisoned controls seemed to match the rates of metabolism estimated from O₂ consumption and CO₂ production. The zero-order rates of CO₂ production also remained constant with time consistent with the constant zero-order rates of alcohol production. An acceleration in rate did not occur as might be expected if biotic hydrolysis was occurring and the biomass increased. Vancheeswaran *et al.* (1999) suggested that biotic hydrolysis was likely in their studies with TBOS. They, however, were using a mixed culture in their studies, which may have contained microorganisms that possessed enzymes capable of hydrolyzing orthosilicates. More research is needed to determine whether *R. rhodochrous* ATCC 21198 has orthosilicate-hydrolyzing capabilities and enzymes. One benefit of using a pure culture system that does not promote biotic hydrolysis is having more control of the rate of hydrolysis via abiotic processes.

The results presented here agree with those presented by Rasmussen *et al.* (2020) where TBOS and T2BOS were co-encapsulated in gellan-gum along with *R. rhodochrous* ATCC 21198. Higher rates of hydrolysis, metabolism, and cometabolism were observed in the reactors containing gellan-gum bead-containing TBOS compared to T2BOS. Both studies were conducted with the same mass loading of orthosilicates to the batch reactors to permit comparison of the direct contact of the orthosilicate and *R. rhodochrous* ATCC 21198 and their co-encapsulation in the gellan-gum hydrogel. The abiotic rates of hydrolysis reported here were 8 to 15 times higher than observed with the encapsulated beads. The higher rates of hydrolysis likely resulted from

mass transfer issues into the gellan-gum beads and the high surface areas created by the colloidal droplets in solution observed in this study. The rates of oxygen consumption reported here were factors of 4 and 1.25 times higher for T2BOS and TBOS, respectively, compared to the estimates with the co-encapsulated beads. It is not known why greater differences were not observed. One possibility is that there was also an O₂ demand associated biodegradation of the gellan-gum beads. Both studies showed consistent results related to the cometabolic transformation of 1,1,1-TCA and 1,4-D. More rapid cometabolic transformation was observed with TBOS compared to T2BOS and 1,4-D was more rapidly transformed than 1,1,1-TCA. Comparing first-order rates between the two studies, shows much higher rates observed here compared to the co-encapsulated systems, consistent with the more rapid rates of hydrolysis. In the study of Rasmussen et al. (2020), cis-DCE was also present as a co-contaminant, which may have affected the transformation rates of 1,1,1-TCA and 1,4-D.

The present study was conducted in buffer growth media, thus representing more optimal conditions compared to those that might be encountered in-situ. Rasmussen *et al.* (2020) presented results where the co-encapsulated gellan-gum beads containing TBOS and *R. rhodochrous* ATCC 21198 were incubated in microcosms containing groundwater and aquifer solids. Continuous cometabolic transformation of cis-DCE, 1,1,1-TCA and 1,4-D was observed in the microcosms for over 220 days, with rates slower, but in the range of those in media. Similar studies are needed for the process reported here, where the orthosilicates are directly added along with *R. rhodochrous* ATCC 21198 to groundwater microcosms. In these tests, *R. rhodochrous* ATCC 21198 would be in competition with indigenous microorganisms for the alcohols that are released, but may not promote the cometabolic transformation. With the gellan-gum co-encapsulation system, the alcohols that are produced are consumed within the hydrogel by the microbial culture that is effective towards cometabolism of contaminants of interest. Thus, there is protection offered by the co-encapsulation of the microorganism and the orthosilicate within the gel.

The methods presented here provide means of investigating other microorganisms that have potential to cometabolize COCs when grown on orthosilicates that slowly release alcohols. The method used provides a means for growing microorganisms slowly at low substrate concentrations, which may be key to obtaining the long-term continuous cometabolic transformation. The methods provide for a simpler means of testing of SRCs that might be incorporated into co-encapsulated hydrogel systems.

Conclusions

The following conclusion can be drawn for the results that were presented:

1. Secondary alcohols, such as 2-butanol induce monooxygenase activity in *R. rhodochrous* ATCC 21198 during growth whereas primary alcohols, such as 1-butanol did not. However, cometabolism of 1,4-D was observed by 1-butanol grown cells after exposure to 1,4-D.
2. 1,1,1-TCA and 1,4-D transformation was achieved during direct growth of *R. rhodochrous* ATCC 21198 on 2-butanol, with the first-order rates of 1,4-D being a factor of 2.5 times greater than 1,1,1-TCA.
3. Alcohol-releasing orthosilicates continually promoted the cometabolic transformation of 1,1,1-TCA and 1,4-D by *R. rhodochrous* ATCC 21198, for up to 125 days.
4. The order of the rates of abiotic hydrolysis of alcohols from orthosilicates were as follows: TBOS > T2POS > T2BOS. The straight-chained orthosilicate TBOS was hydrolyzed more

quickly than the branched chained T2BOS. The shorter branched chained T2POS were hydrolyzed more quickly than the longer chained T2BOS.

5. There is a strong correlation between the rates of metabolism and cometabolism and the rates of hydrolysis of the orthosilicates. The correlation likely resulted from the higher biomass of *R. rhodochrous* ATCC 21198 being maintained by the more rapidly hydrolyzing orthosilicates.

Tables and Figures

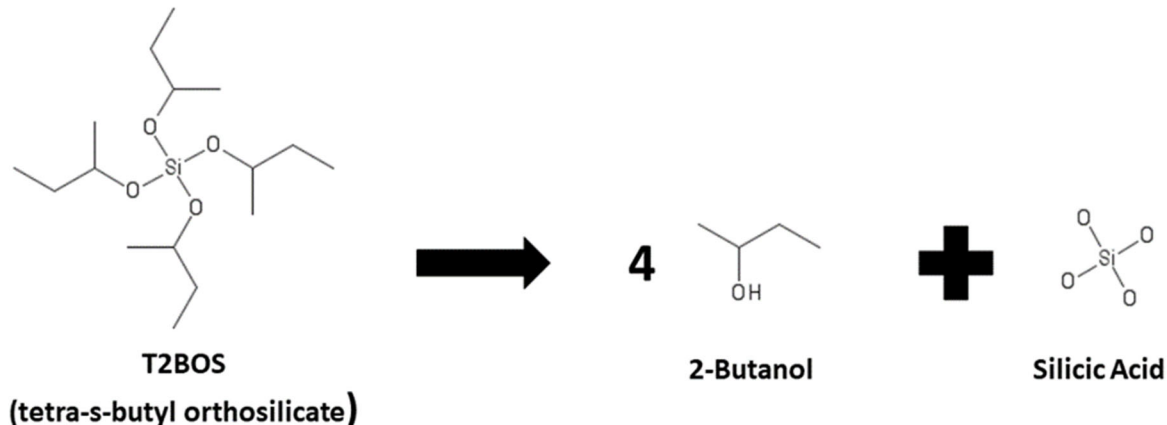


Figure 6.1. Tetra-s-butyl orthosilicate (T2BOS) hydrolysis reaction: The figure shows the hydrolysis reaction of the orthosilicate T2BOS to its respective alcohol and silicic acid.

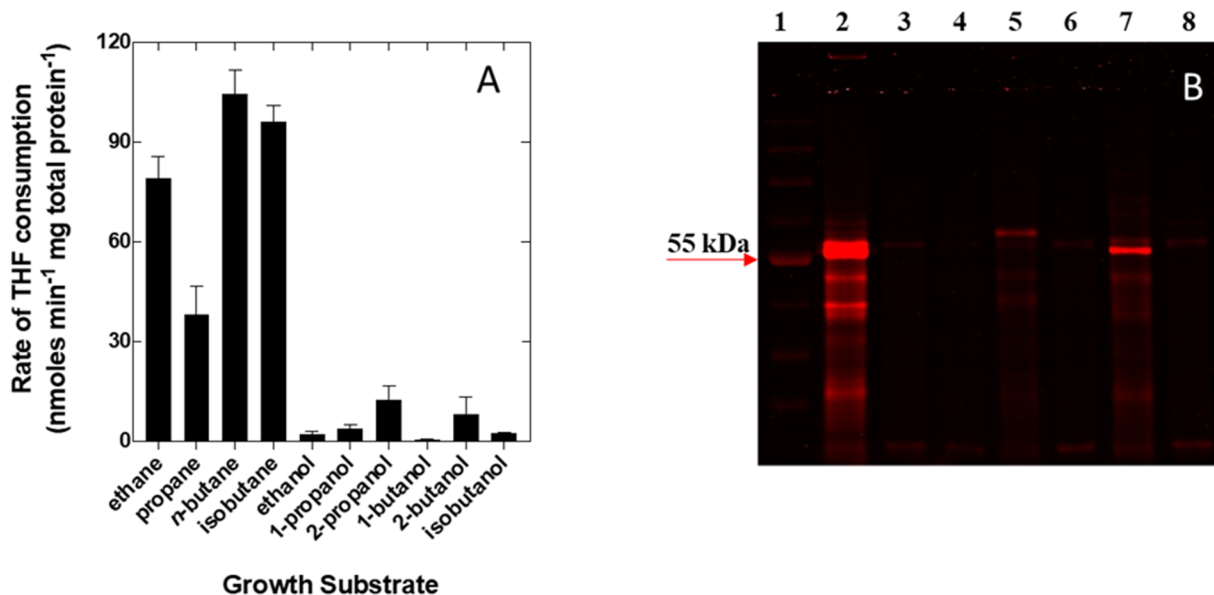


Figure 6.2. THF degradation and activity-based labeling of *R. rhodochrous* ATCC 21198. Specific rates of THF degradation for cells on *R. rhodochrous* ATCC 21198 after growth on the indicated substrates (A). The rates were determined as described in the Methods section and the results presented show the mean and standard deviation of three biological replicates. NIR fluorescence patterns for samples (5 μ g total protein) from 17OD-treated cells of *R. rhodochrous* ATCC 21198 after separation by SDS-PAGE on 9% gels (B). The samples correspond to NIR marker proteins (Lane 1) and cells grown on either isobutane (Lane 2), ethanol (Lane 3), 1-propanol (Lane 4), 2-propanol (Lane 5), 1-butanol (Lane 6), 2-butanol (Lane 7), or isobutanol (2-methyl-1-propanol) (Lane 8).

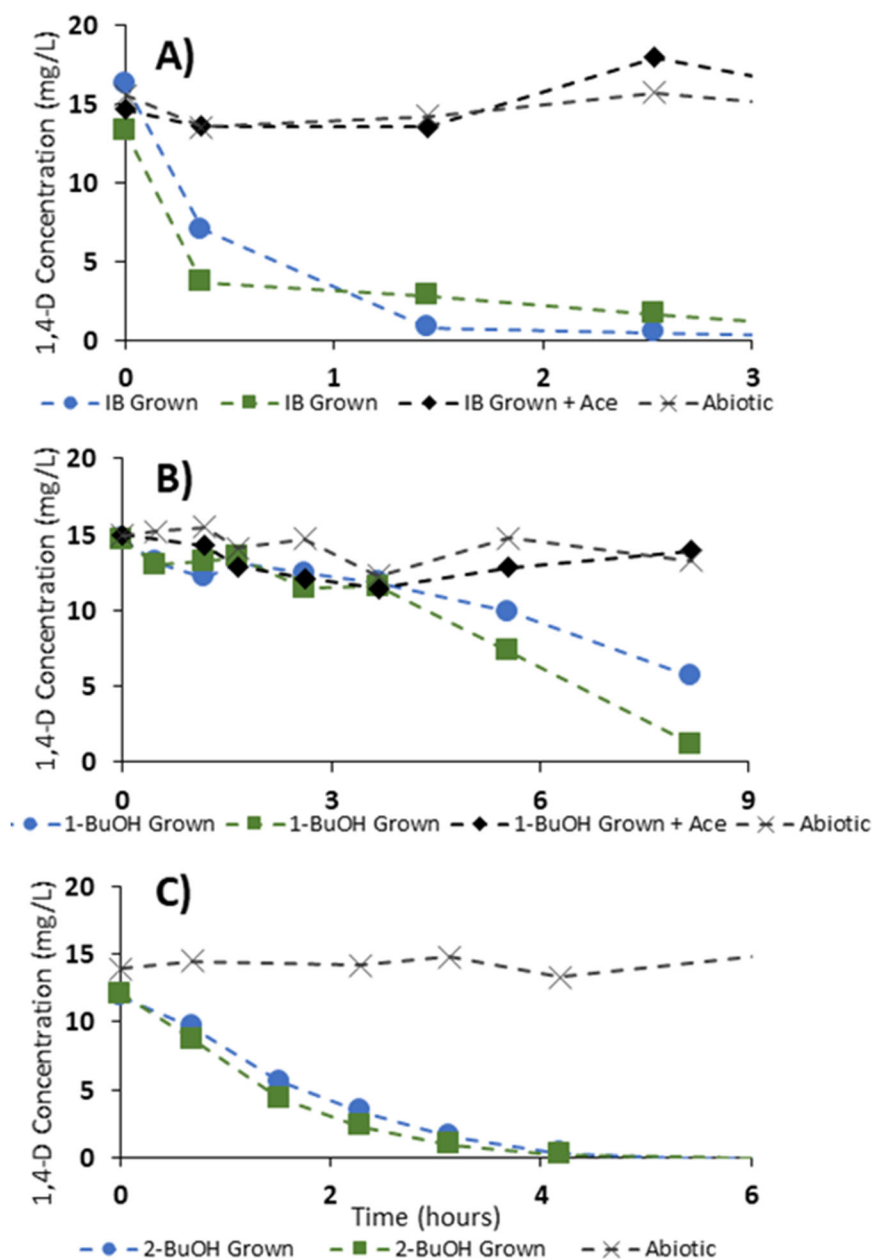


Figure 6.3. 1,4-D resting cell transformation with *R. rhodochrous* ATCC 21198 grown on different substrates: (A) isobutane grown cells (IB), (B) 1-butanol grown cells (1-BuOH), and (C) 2-butanol grown cells (2-BuOH). The results show duplicates for biologically active bottles, a control without cells (Abiotic), and a control for cells + acetylene (Ace) which is a known inactivator of bacterial monooxygenases.

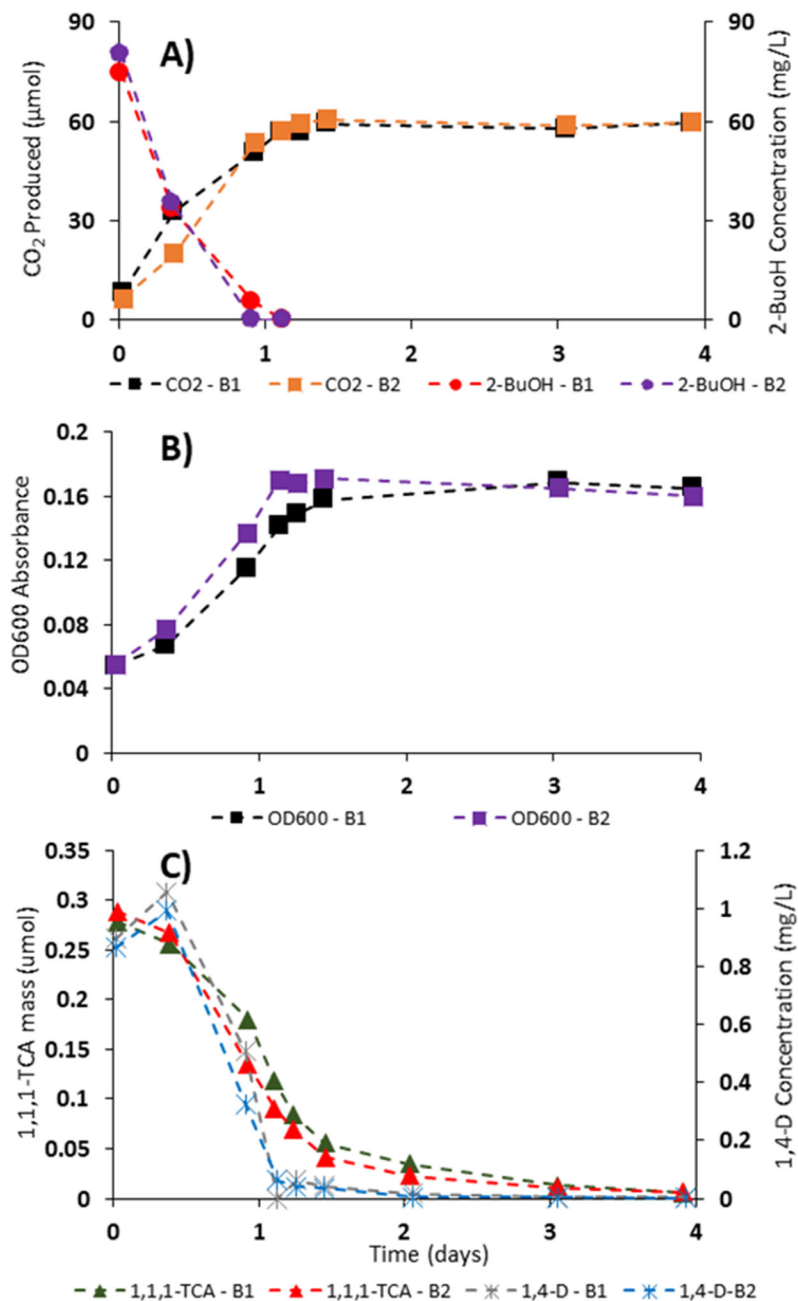


Figure 6.4. Transformation of 1,4-D and 1,1,1-TCA by *R. rhodochrous* ATCC 21198 during growth on 2-butanol in duplicate batch reactors: (A-B) evidence of metabolism of 2-BuOH by *R. rhodochrous* ATCC 21198 with an increase of headspace CO₂ mass (A), and aqueous OD₆₀₀ measurements (B) as well as a decrease of aqueous 2-BuOH concentration (A). (C) Transformation of 1,1,1-TCA and 1,4-D by *R. rhodochrous* ATCC 21198.

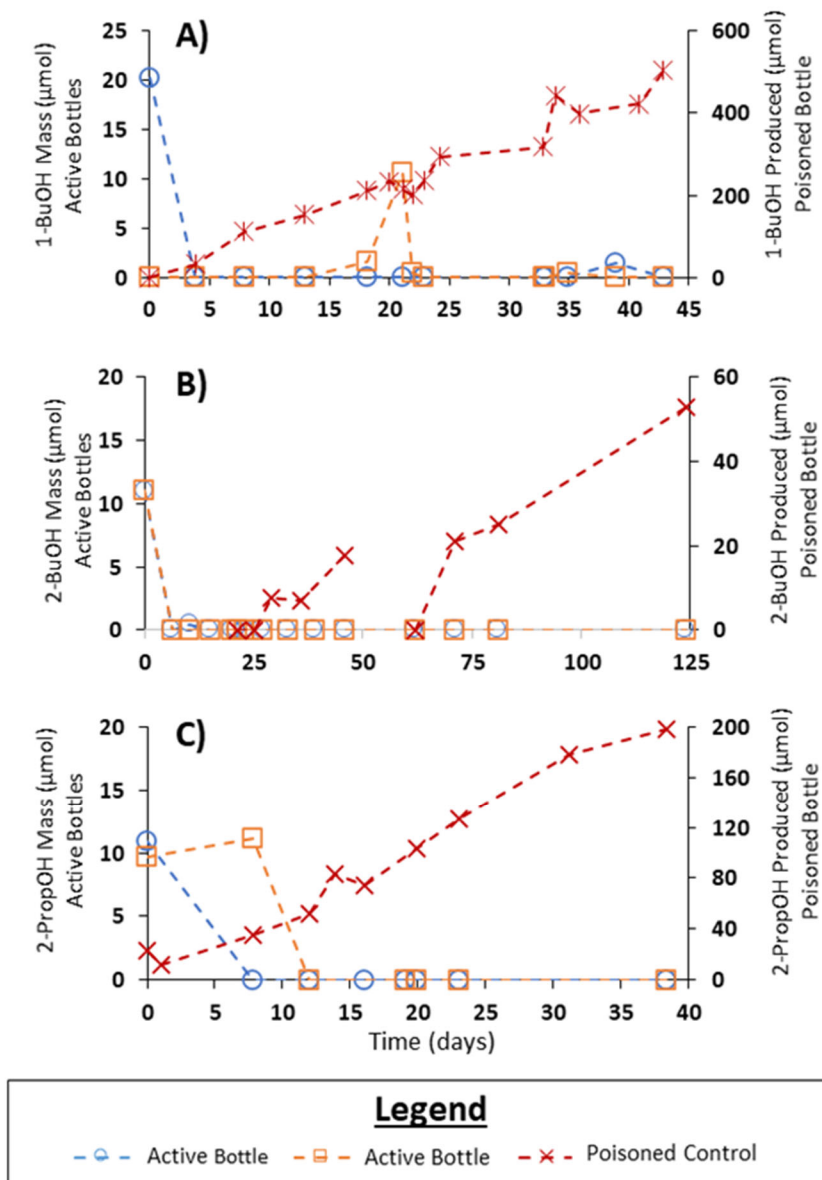


Figure 6.5. 1-BuOH, 2-BuOH, and 2-PropOH concentrations in batch reactors with TBOS (A), T2BOS (B) and T2POS (C). The poisoned controls show a linear increase in aqueous alcohol concentration. The initial concentration of alcohol is due to impurity in the added orthosilicate. The orthosilicate abiotic rates follow the trend TBOS > T2POS > T2BOS. Biologically active bottles show no build-up in alcohols after the initially impurities are utilized.

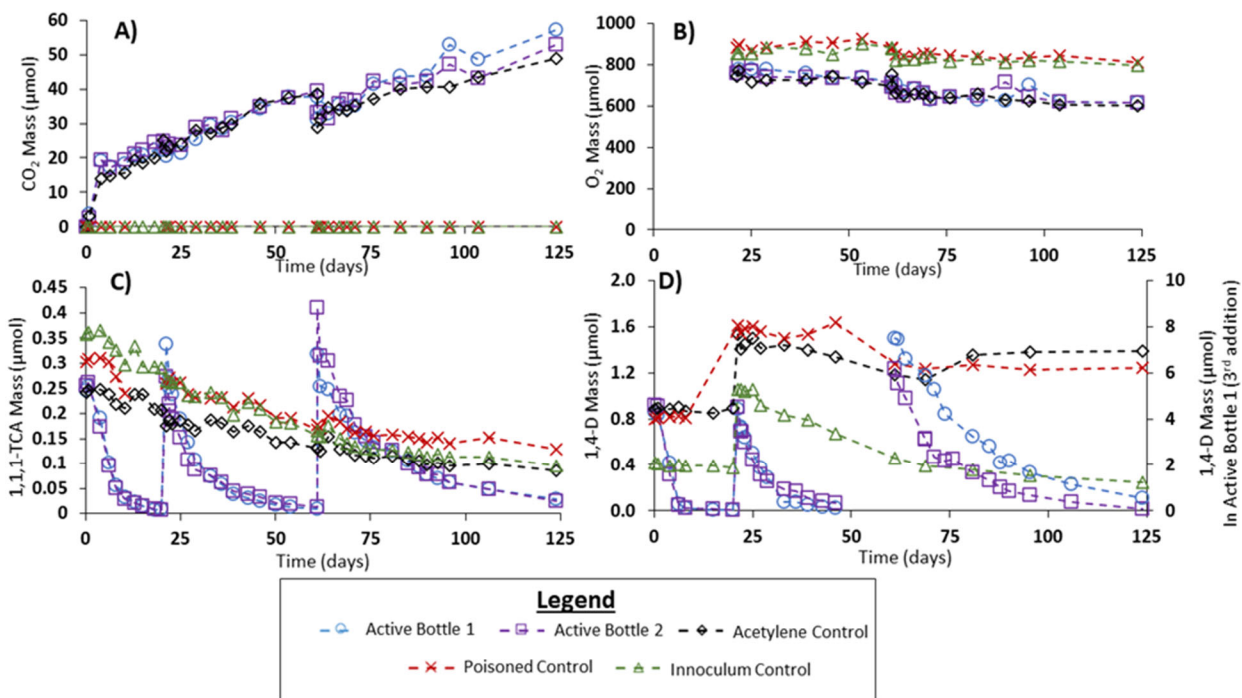


Figure 6.6. Cometabolism of 1,1,1-TCA and 1,4-D by *R. rhodochrous* ATCC 21198 during growth on 2-BuOH hydrolyzed from T2BOS: (A) CO₂ production; (B) O₂ consumption; (C) 1,1,1-TCA transformation, and (D) 1,4-D transformation. 1,4-D and 1,1,1-TCA were added three times. The third addition of 1,4-D was at a great mass and is displayed on the secondary axis.

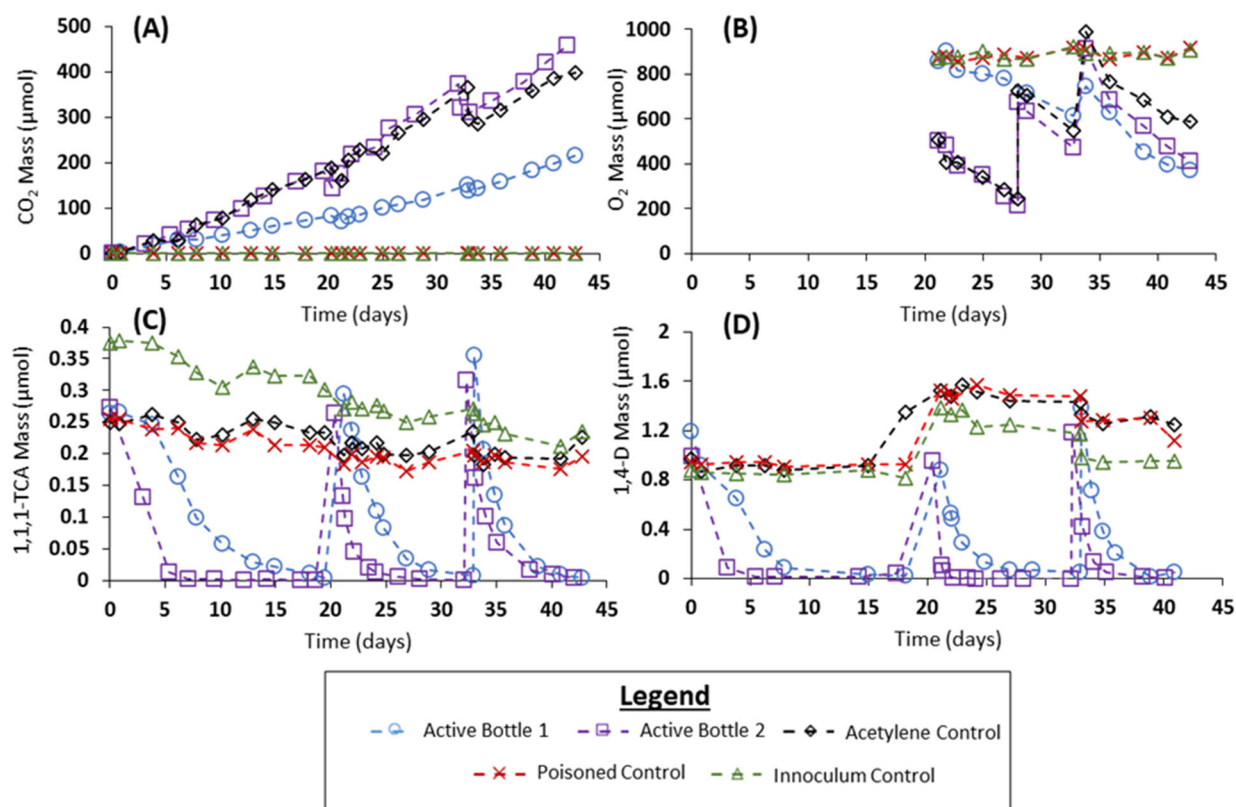


Figure 6.7. Cometabolism of 1,1,1-TCA and 1,4-D by *R. rhodochrous* ATCC 21198 during growth on 1-BuOH hydrolyzed from TBOS: (A) CO₂ production; (B) O₂ consumption; (C) 1,1,1-TCA transformation, and (D) 1,4-D transformation. 1,4-D and 1,1,1-TCA were added three times. Oxygen was measured starting day 20 and was re-added to the headspace three times. Metabolism indicators, O₂ consumption CO₂ production, indicate much more rapid hydrolysis of TBOS compared to T2BOS.

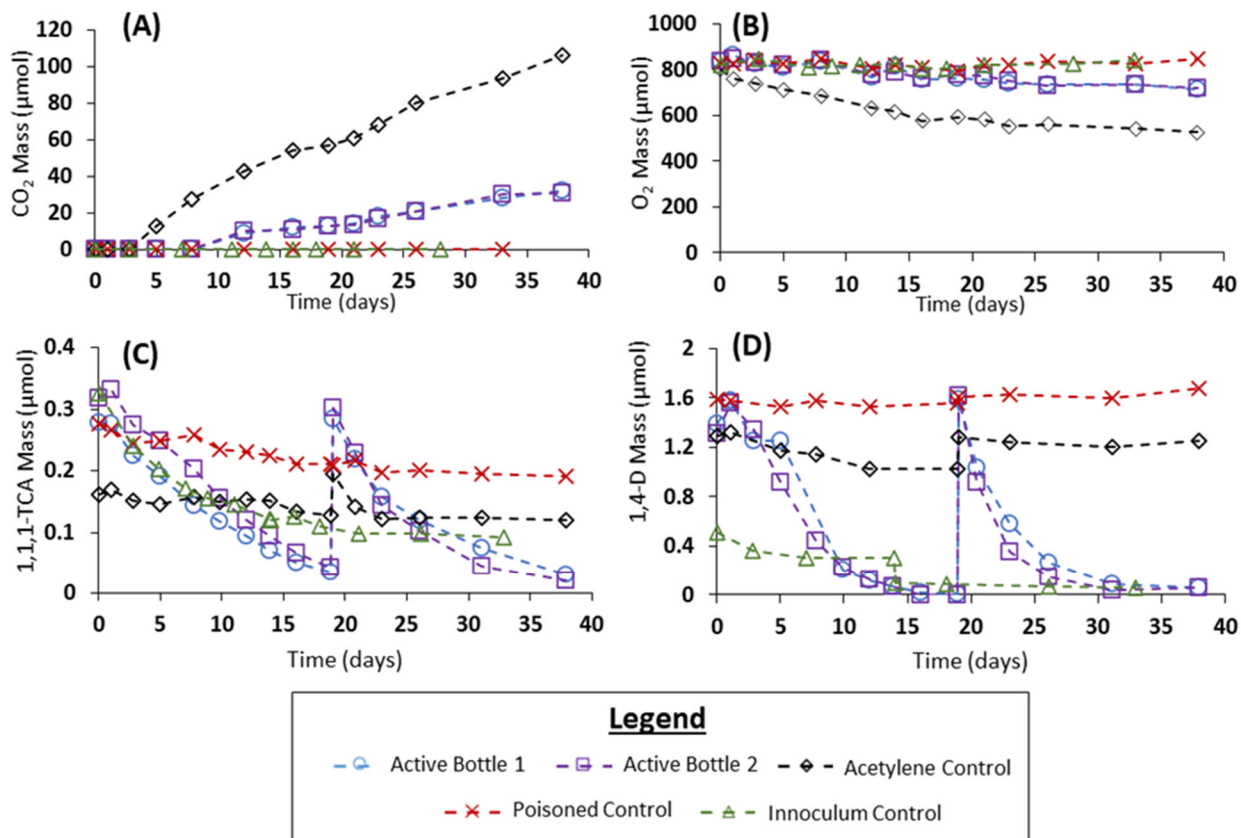


Figure 6.8. Cometabolism of 1,1,1-TCA and 1,4-D by *R. rhodochrous* ATCC 21198 during growth on 2-PropOH hydrolyzed from T2POS: (A) CO₂ production; (B) O₂ consumption; (C) 1,1,1-TCA transformation, and (D) 1,4-D transformation. 1,4-D and 1,1,1-TCA were added two times. The Acetylene control had much faster O₂ consumption and CO₂ production than the active bottles, likely due to a higher initial biomass. Metabolism indicators, O₂ consumption CO₂ production, indicate an intermediate hydrolysis rate of T2POS compared to TBOS and T2BOS.

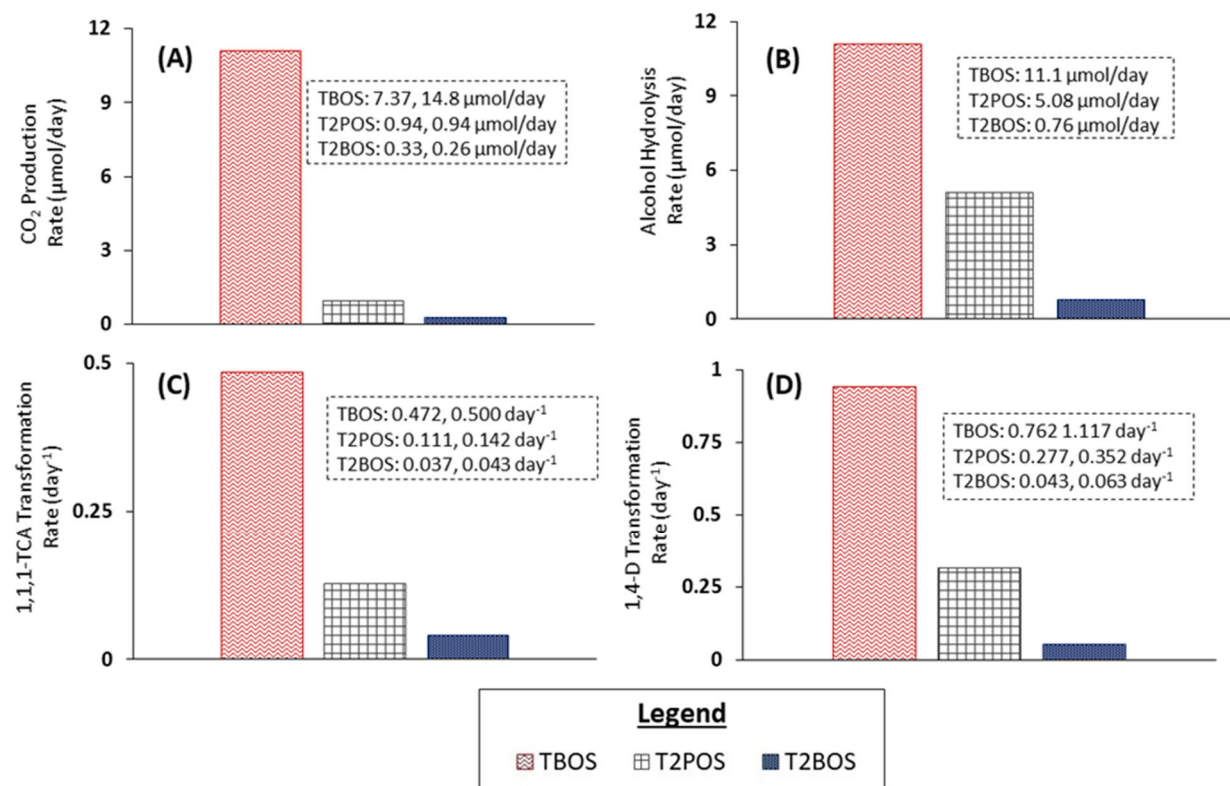


Figure 6.9. Comparison of zero-order rates of (A) CO₂ and (B) alcohol production (poisoned control) and first-order rates of (C) 1,1,1-TCA and (D) 1,4-D transformation during growth on TBOS, T2BOS, and T2POS. The rates of metabolic activity show the trend TBOS > T2POS > T2BOS. Rates plotted for CO₂, 1,1,1-TCA and 1,4-D are averages of duplicates.

Chapter 7. Development of Alginate and Gellan-Gum Hydrogels for the Co-Encapsulation of *R. rhodochrous* ATCC 21198 and Slow Release Compounds

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Abstract

R. rhodochrous ATCC 21198 was successfully co-encapsulated in gellan-gum beads with orthosilicates as SRCs to support aerobic cometabolism of a mixture of 1,1,1-TCA, *cis*-DCE, and 1,4-D at aqueous concentrations ranging from 250 to 1000 µg/L. O₂ consumption and CO₂ production showed the co-encapsulated cells utilized the alcohols that were released from the co-encapsulated SRCs. Two model SRCs, TBOS and T2BOS which hydrolyze to produce 1- and 2-butanol, respectively, were encapsulated in gellan-gum (GG) at mass loadings as high as 10% (w/w), along with *R. rhodochrous* ATCC 21198. In the GG encapsulated beads, TBOS hydrolyzed 27 times faster than T2BOS and rates were ~4 times higher in suspension than when encapsulated. In biologically active reactors, the co-encapsulated *R. rhodochrous* ATCC 21198 effectively utilized the SRC hydrolysis products (1- and 2-butanol) and cometabolized repeated additions of a mixture of 1,1,1-TCA, *cis*-DCE, and 1,4-D for over 300 days. The transformation followed pseudo-first-order kinetics. VC and 1,1-DCE were also transformed in the reactors. In the long-term treatment, the batch reactors with T2BOS GG beads achieved similar transformation rates, but at much lower O₂ consumption rates than those with TBOS. The results demonstrate that the co-encapsulation technology can be a passive method for the cometabolic treatment of dilute groundwater plumes.

Introduction

Groundwater contamination with VOCs is a widespread issue throughout the United States (Moran, 2006; Squillace et al., 2002b). Over 50% of more than 3500 groundwater samples collected from 98 major drinking water supply aquifers from 1985-2001 contained at least one anthropogenic contaminant with VOCs detected most frequently (Moran, 2006). Among the top 15 VOCs detected in this survey, eight were CAHs; CF, PCE, TCE, 1,1,1-TCA, *cis*-DCE, *trans*-DCE, dichloromethane (DCM), and 1,1-DCA. All of these CAHs are listed by the Center for Disease Control (CDC) as being likely human carcinogens as well as having other harmful environmental and human health concerns (NIOSH, 2013b). Of increasing concern is the high likelihood for CAH plumes to contain multiple CAHs as well as co-contaminants, such as 1,4-D, which is also listed as a likely human carcinogen (USEPA, 2017b). 1,4-D was most commonly used as a chemical stabilizer for chlorinated solvents, mainly 1,1,1-TCA to prevent decomposition due to reactions from light, heat, oxygen, or acid-base chemistry (Mohr, 2001; USEPA, 2017b). 1,1,1-TCA formulas can contain up to 2% - 8% by volume 1,4-D (Adamson et al., 2011; Mohr, 2001). 1,4-D is a commonly detected groundwater contaminant in areas with chlorinated solvent contamination (Adamson et al., 2011; Derosa et al., 1996). Of over 2000 sites evaluated in California, 194 contained 1,4-D, 95% of sites had at least one chlorinated solvent, and 76% had both 1,4-D and 1,1,1-TCA present. The median value of the range of maximal historical 1,4-D concentrations at these sites was 365 µg/L (Adamson et al., 2014).

The ubiquitous and long-term contamination of groundwater has allowed CAHs and co-contaminants to diffuse into saturated low permeability zones, such as clay layers, within aquifers (Liu & Ball, 2002). Common remediation techniques, such as pump-and-treat and soil vapor extraction, are less effective at treating compounds within low permeability zones due to diffusion limitations and inability to remove or target the contaminant directly (Parker et al., 2008). Also, current active remediation techniques often require long-term site occupation, are not economical, and are not capable or are ill-equipped for the treatment of mixtures of contaminants that have varying physical and chemical properties. These issues emphasize the need for exploration into long-term passive and economical remediation techniques such as bioremediation. 1,4-D, is fully miscible in water (infinitely soluble) and has a low octanol water partition coefficient (K_{ow}) (Mohr, 2001; USEPA, 2017b). 1,4-D is also generally resistant to anaerobic transformation (McElroy et al., 2019). These chemical characteristics result in 1,4-D having a high mobility in groundwater with the potential to form extended dissolved plumes (Mohr et al., 2010). 1,4-D is not effectively removed by air stripping or sorption onto activated carbon, which is commonly used for the ex-situ treatment of CAHs.

With the exception of PCE, all listed CAHs above as well as 1,4-D have the potential to be cometabolically transformed in-situ by aerobic microorganisms (Hand et al., 2015; T. K. G. Mohr et al., 2010; Semprini, 1995, 1997). Aerobic cometabolism is a process where microbes utilize a primary growth substrate for cellular growth and energy, and fortuitous degradation of contaminants occurs due to the expression of specific enzyme systems within active microbes (Frascari et al., 2015; Semprini, 1997). This study focused on the bacterium *R. rhodochrous* ATCC 21198 which has been shown to be capable of aerobic cometabolic transformation of a wide variety of CAHs and common co-contaminants such as 1,4-D (Krippaehne, 2018; Rich, 2015; Rolston et al., 2019).

Current bioremediation technologies for the remediation of mixtures of CAHs and 1,4-D have relied on the biostimulation of native microbes capable of aerobic cometabolism using

propane as a primary substrate, or bioaugmentation and then stimulation with propane (Chu et al., 2018; Lippincott et al. 2015). Though biostimulation with gaseous substrates, like propane, has been successful at generating biomass capable of transformation and mineralization of CAHs in-situ, there are concerns related to explosion hazards as well as the lack of sustainability due to the need for long-term site occupation for continuous injection of gaseous substrates to sustain populations of cometabolically active microbes (Azizian et al., 2007; Connon et al., 2005; Semprini, 1997).

Passive treatment systems, such as PRBs, have the potential to reduce costs and eliminate the need to extract groundwater or continuously add substrates, electron acceptors, and nutrients for biological treatment. Scherer et al. (2000b) provides a review of PRBs for groundwater treatment using chemical and biological methods. Upadhyay and Sinha (2018) review permeable reactive bio-barriers (PRBBS) for environmental clean-up. For aerobic treatment, barriers have focused on contaminants that can be metabolized, such as benzene, toluene, ethylbenzene, and xylene (BTEX) through the addition of slow release forms of O₂-releasing compounds (ORCs) such as calcium or magnesium peroxides. However, there has been little attention given to the development of PRBBS for aerobic cometabolism. Reported here is a novel system that was developed to promote long-term passive aerobic cometabolism that might be used to construct PRBBS.

The passive cometabolic system developed here involves encapsulating a pure microbial culture in a hydrogel along with a SRC that hydrolyze to produce an alcohol. The released alcohol maintains the activity of the co-encapsulated microorganisms and enables sustained cometabolic degradation of contaminants, which diffuse into the hydrogel. Natural hydrogel matrices such as alginate, agar, and agarose have been used to physically buffer microbes from environmental conditions by providing a diffusion layer that decreases toxic levels of ambient compounds, retards any changes in chemical and temperature conditions, and protects from predation by protozoa (Cassidy et al., 1996; Moslemy et al., 2003; Neufeld, et al., 2002; Petrich et al., 1998). Encapsulation has also been investigated for its ability to inhibit the production of biofilms, which may reduce well clogging and increase the transport distance of cells through aquifer material (Moslemy et al. 2003; Guiot, et al., 2002; Petrich et al., 1998). Also, by controlling bead size, encapsulation may provide a control for bead affixation to aquifer material around low permeability zones (Moslemy et al., 2003). Encapsulation for resting cell TCE cometabolism has also been reported (Chen et al., 2007).

Gellan-gum (GG) was used here to encapsulate *R. rhodochrous* ATCC 21198 with the SRCs, TBOS and T₂BOS. Gellan-gum is a natural gelling polysaccharide produced primarily by bacterium *Sphingomonas elodea*. It consists of chains of glucose, glucuronic acid, and rhamnose molecules (Doner & Becard, 1991; Osmalek et al., 2014) and similar to alginate, gelation of GG occurs via ionic crosslinking. Though GG gelation is ionically activated, the gelation process is more similar to the thermal hysteresis type gelation of agar and agarose (Grasdalen & Smidsrød, 1987; Norton & Lacroix, 1990). GG gelation temperatures are dependent on the type and concentration of crosslinking cations, concentration of GG, and concentration of chelating agents (Camelin et al., 1993; Doner & Becard, 1991; Grasdalen & Smidsrød, 1987). Gellan-gum has become a widely used hydrogel matrix replacing matrices like agar and alginate because it has superior rheological properties, chemical stability, temperature resistance and enzyme resistance (Camelin et al., 1993; Doner & Becard, 1991; Norton & Lacroix, 1990).

The cometabolic transformation of 1,4-D has been reported on a range of substrates for growth and/ or induction of cometabolic enzymes as reviewed by Zhang *et al.* (2017) and McElroy *et al.* (2019). Rolston *et al.* (2019) recently reported the cometabolic transformation of 1,4-D by *R. rhodochrous* ATCC 21198 in microcosms fed with isobutane as a primary substrate. *R. rhodochrous* ATCC 21198, is a soil-isolated microbe that expresses a SCAM capable of transforming 1,4-D. When grown on isobutane, *R. rhodochrous* ATCC 21198 rapidly cometabolically transforms 1,4-D at pseudo-first-order rates to low concentrations of ~1 ppb (Rolston *et al.*, 2019). This strain can also cometabolically transform a broad range of CAHs including: 1,1,1-TCA, 1,1,2-trichloroethane (1,1,2-TCA), 1,2-DCA, 1,1-DCA and chlorinated ethenes including: *cis*-DCE, 1,1-DCE and VC (Krippaehne, 2018; Rich, 2015; Thankitkul, 2016). Mixtures of 1,4-D, 1,1,1-TCA, 1,1-DCE and 1,2-DCA are also effectively transformed by this strain (Krippaehne, 2018).

The novel process investigated here was to co-encapsulate active microbial cells with a large molecular weight SRC that hydrolyzes to produce an organic electron donor that can support cometabolism. The SRCs encapsulated with *R. rhodochrous* ATCC 21198 in this study were silicon-based organic compounds known as tetra-alkoxysilanes. These compounds consist of a central silicon atom bound via ester linkages to four alkoxy groups that can vary in carbon chain length and structure. Examples of these compounds used in this study include TBOS and T2BOS. While tetra-alkoxysilanes are insoluble in water at room temperature (Arkles *et al.*, 1992), their ester bonds hydrolyze abiotically at varying rates which are determined by the pH of solution and the size or structure of the leaving group (Arkles *et al.*, 1992; Osterholtz & Pohl, 1992; Vancheeswaran *et al.*, 1999). For example, TBOS hydrolyzes and releases 1-butanol while T2BOS hydrolyzes more slowly and releases 2-butanol. The hydrolysis reaction can therefore be used to continuously generate alcohols over extended time periods.

While most studies of cometabolic biotransformations have focused on reactions involving unoxidized primary substrates such as methane, propane, or butanes, several studies have described aerobic cometabolic reactions supported by alcohols. For example, Semprini and Vancheeswaran (2002) described a mixed culture grown on 1-butanol produced from TBOS hydrolysis that could cometabolically oxidize TCE and *cis*-DCE (Vancheeswaran *et al.*, 1999). Previous studies with *Mycobacterium vaccae* JOB5 have shown that this strain can biodegrade 1,4-D (Hand *et al.*, 2015) and MTBE (Smith *et al.*, 2003a) after growth on either 1- or 2-propanol. Hand *et al.* (2015) also reported *Mycobacterium vaccae* JOB5 and *Rhodococcus* RHA1 can oxidize 1,4-D and TCE when grown on 1-butanol and *R. rhodochrous* ATCC 21198 has also been reported to oxidize VC after growth on isopropanol (Kuntz *et al.*, 2003). The potential to support cometabolic transformations by SRCs that produce alcohols is therefore strongly supported by prior physiological studies with diverse bacteria.

The main objectives of the study were to develop a method to co-encapsulate *R. rhodochrous* ATCC 21198 and TBOS or T2BOS in GG hydrogel beads, and to evaluate the performance for achieving long-term cometabolic transformations in aerobic batch reactor systems. Specific objectives were to: 1) optimize the co-encapsulation methods to produce mechanically stable GG macro-beads that maintained the microbial activity of *R. rhodochrous* ATCC 21198 and contained up to 10 % wt/wt TBOS and T2BOS; 2) measure the abiotic rate of hydrolysis of TBOS and T2BOS in aqueous suspension and in the co-encapsulated hydrogel GG macro-beads; 3) evaluate the ability of co-encapsulated *R. rhodochrous* ATCC 21198 to utilize 1-butanol and 2-butanol released from SRCs over an extended time period (300 days); 4) determine

the cometabolic transformation rates of mixtures of 1,1,1-TCA, *cis*-DCE, and 1,4-D by the co-encapsulated cells with successive additions over time, and determine how the rates changed over time; 5) and assess the performance of the GG macro beads in groundwater/sediment microcosms from a contaminated site.

Materials and methods

Materials

All CAHs were purchased from Tokyo Chemical Industry (>98% purity), 1,4-D was purchased from J.T. Baker (>99%), and TBOS and T₂BOS were purchased from Gelest Inc. (>95%). 1- and 2-butanol used for calibration standards were purchased from Sigma-Aldrich (>99%). Isobutane was used for initial batch growth of *R. rhodochrous* ATCC 21198 culture and purchased from Gas Innovations (>99.99%). Sodium alginate was purchased from Spectrum Chemical and Gellan-gum (Kelcogel) was donated by C.P. Kelco. All other chemicals used in the study were of reagent grade.

Growth of R. rhodochrous ATCC 21198 for encapsulation and suspended cell tests

R. rhodochrous ATCC 21198 was obtained from Dr. Michael Hyman, North Carolina State University and is commercially available from the ATCC. Details of the culture maintenance and batch growth are provided by Rolston *et al.* (2019). *R. rhodochrous* ATCC 21198 was grown on isobutane in 720-mL glass bottles containing 270 mL of phosphate-buffered mineral salts medium (MSM) (Kottegoda *et al.*, 2015) and 450 mL of air headspace with 45 mL of isobutane added. The culture was incubated at 30°C on a rotary shaker table at 200 rpm and harvested by centrifugation when in late exponential growth phase. The cell pellet was resuspended in phosphate buffer (50 mM, pH 7.0) and centrifuged again. The washing was repeated, and the resting cell pellet was finally resuspended in buffer. The biomass concentration was determined using TSS analysis as described in AWWA standards (Rice *et al.*, 2017). Under the growth conditions described above the ratio of protein to TSS was 0.45 mg protein/mg TSS (Rolston *et al.*, 2019).

Encapsulation and Co-encapsulation

R. rhodochrous ATCC 21198 was encapsulated separately in alginate and GG to enable comparisons between the two encapsulation methods and media. Details of the method used to encapsulate *R. rhodochrous* ATCC 21198 in alginate are provided by Rasmussen *et al.* (2020). The GG encapsulation method is more involved than alginate because gelation is a function of temperature as well as the crosslinking concentration (Camelin *et al.*, 1993; Grasdalen & Smidsrød, 1987). The gelation process requires heating of pre-gel solution to at least 60°C followed by a direct addition of crosslinking cation salts or solutions, and cooling to below 45°C to initiate gelation. The temperature-dependent crosslinking of GG allows for the development of more simple emulsification internal gelation procedures (Moslemy, Guiot, *et al.*, 2002; Tan *et al.*, 2011). The gelation method has been used to create highly stable spherical micro-beads ranging from below 20 µm to above 150 µm in size (Moslemy, Guiot, *et al.*, 2002). Methods developed here to co-encapsulate *R. rhodochrous* ATCC 21198 with the SRCs resulted in the production of macro-beads (~ 2 mm in diameter) that could potentially be used to create a PRB.

Encapsulation of R. rhodochrous ATCC 21198 in gellan-gum macrobeads

The following procedure was used to encapsulate *R. rhodochrous* ATCC 21198 in GG macrobeads that maintained high rates of microbial activity. The method developed follows a

similar procedure presented by Hamid et al. (2014) and Li et al. (1996) in which cylindrical macrobeads are created. A 0.75% (w/v) GG pre-gel solution was prepared in autoclaved phosphate buffer (~2mM, pH-7) at ~85°C. GG powder was added immediately after removing the solution from the autoclave. The pre-gel solution was shaken vigorously for 30 seconds and placed on a heated magnetic stir plate keeping the solution at ~85°C while mixing at 200 rpm for 30 minutes. A CaCl₂ solution was added to the pre-gel solution to achieve a final concentration of 0.06% (w/v) CaCl₂. The solution was allowed to cool to ~60°C and the pH was adjusted to 7 with dilute NaOH. The solution was then cooled to ~45°C and a known mass of *R. rhodochrous* ATCC 21198 from a concentrated slurry was added. *R. rhodochrous* ATCC 21198 that was added was grown on isobutane and harvested at late exponential growth phase, as described above. At this stage the pre-gel solution was ready for gelation. The pre-gel solution was drawn into a 1.5m section of flexible rubber tubing with an inner diameter of ~2mm using an attached 60-ml plastic syringe (Figures S7.3-S7.5). The pre-gel solution was cooled to ~15°C, by setting the tubing on ice solidifying the gel within the tubing. The gel-filled tubing was then placed in a laminar flow hood for 60 minutes to provide extra time for internal crosslinking prior to extrusion of the gel. In a laminar flow hood, the solidified GG was pushed from the tubing onto long sections of Parafilm using an attached 60 mL syringe filled with air. The extruded sections of hardened GG were ~2 mm in diameter by ~15-30 cm in length. A sterilized razor blade was used to cut the long sections into ~2 mm sections, such that the height of each cylinder was approximately the same as the diameter. The cylinders were allowed to cure for an additional 10 minutes. The cylinder beads were then crosslinked by transferring them to a 1L beaker containing 500 mL of 0.25% (w/v) CaCl₂ solution and allowed to react for 60 minutes. The macro-beads were separated from the external crosslinking solution using a vacuum pump fitted with a 70 mm plastic filter funnel. The filtered beads were washed three times with carbonate buffered MSM (pH 7) and damp dried using the vacuum pump filter funnel. To calculate the final mass loading of cells in beads (mg TSS/g bead), the assumption was made that 1 mL of pre-gel solution formed 1g of beads and that all cells added were encapsulated. Typically, microbially active beads were used for experimentation the day they were made; however, on occasion beads were stored overnight in pH 7 carbonate buffered MSM at 4°C.

Co-encapsulation of TBOS or T₂BOS and R. rhodochrous ATCC 21198 in gellan-gum

The encapsulation of TBOS or T₂BOS in GG macrobeads required an additional step to the microbial encapsulation method presented above. That step required the emulsification of SRCs within autoclaved GG pre-gel solution prior to adding CaCl₂ crosslinking solution. After complete hydration of GG powder (as described above), a known volume of warm pre-gel solution, typically 40-50 mL, was transferred to an autoclaved-125 mL wide mouth glass vial. Span-80 emulsifier was added to achieve a concentration of 0.1% (v/v). A known volume of TBOS or T₂BOS was then added to the pre-gel solution and the mixture was emulsified using an IKA RW 20 digital overhead impeller mixer at 2500 rpm for 10 minutes. Following emulsification, the pre-gel solution was heated back to ~80°C and transferred to a 50 mL Falcon tube. To initiate gelation, an appropriate volume of 10% CaCl₂ stock solution was added to make a final concentration of 0.06% w/v. The solution was vortexed for 30 seconds. The pre-gel solution was then left at room temperature to cool to ~60°C before the pH was adjusted to 7 with dilute NaOH, if necessary. If co-encapsulation of cells was desired, the pre-gel solution was again left at room temperature to cool to ~45°C before adding cells. To create macro-beads from the warm pre-gel solution the tubing cooling and extrusion method described above was used. Microbially active macrobeads

were separated from the external crosslinking solution using a vacuum pump fitted with a 70mm plastic filter funnel. To rinse any exogenous SRCs from the surface of beads, they were washed three times with a sterile and microbially safe 0.1% (v/v) Tween-80 soap. Finally, the beads were rinsed three times with carbonate buffered MSM (pH 7) and dried a final time using the vacuum pump.

In order to determine the SRC mass loading in beads as $g_{\text{SRC}}/g_{\text{bead}}$ and encapsulation efficiency of this process, 0.25 g samples of beads were transferred into 27 mL vials containing 10 mL of 2 mM sodium citrate solution. Sodium citrate was used to chelate calcium and help break apart the GG cylinders such that any encapsulated TBOS or T₂BOS was released into solution (Camelin et al., 1993; Doner & Becard, 1991). These vials were heated to ~80°C then placed on a shaker table shaking at 250 rpm for 120 minutes. The amount of encapsulated TBOS or T₂BOS was quantified using dichloromethane (DCM) extraction, and the known initial mass of beads broken down was used to determine a mass loading ($g_{\text{SRC}}/g_{\text{bead}}$). The amount of TBOS or T₂BOS in the extracted DCM was determined by GC analysis, as described in the analytical methods. The encapsulation process efficiency—the percent of added SRC that was successfully encapsulated—was then determined for the measured mass loading ($g_{\text{SRC}}/g_{\text{bead}}$), the measured final mass of beads (g_{bead}) and the known mass of added SRC.

Assessment of microbial activity after encapsulation

Short-term isobutane utilization tests were used to determine the immediate effect encapsulation had on cell viability. Batch reactor tests were conducted at 20°C in 27 mL crimp topped glass vials sealed with gray butyl rubber septa. Ten mL of carbonate buffered MSM (pH 7) was added to each sterile vial, followed by an addition of a known mass of suspended, encapsulated, or co-encapsulated bacterial cells ranging from 0.5-5mg cells as TSS. Isobutane (~8 μmol) was added to the vial headspace using a gas tight syringe. Vials were shaken rapidly on a rotary shaker table at 200 rpm to ensure equilibration of the headspace with the aqueous phase. Gas concentrations were determined by GC analyses of the headspace and were used to estimate the total dissolved mass using Henry's Law. Substrate utilization rates were calculated through linear regression of isobutane mass data *versus* time and the cell mass added to each reactor. Suspended cell substrate utilization rates were used as a benchmark to assess the effect encapsulation had on cellular viability.

Long-term cometabolic transformation experiments

Batch reactors were used to evaluate the long-term transformation performance of co-encapsulated *R. rhodochrous* ATCC 21198 with TBOS or T₂BOS. These tests were conducted in 125 mL and 250 mL glass Wheaton serum bottles with nominal volumes of 155 or 310 mL, respectively. The serum bottles (batch reactors) were sealed with screw on caps fitted with gray butyl rubber septa. The serum bottles were filled with 100 or 200 mL of carbonate-buffered MSM (pH 7) followed by an addition of suspended, encapsulated, or co-encapsulated cells to achieve an initial cell concentration of 10 mg-TSS/L. Encapsulated bead cell mass loadings as $g_{\text{TSS}}/g_{\text{bead}}$ were used to determine the mass of beads to add to each reactor. With initial encapsulated cell mass loadings of 0.5 $\text{mg}_{\text{TSS}}/g_{\text{bead}}$, 2 g of beads were added to the serum bottles for co-encapsulated treatments and 1 mg of cells to the suspended treatments so the cell concentrations were ~10 mg/L.

Due to mixtures being frequently observed at contaminated sites, the ability of *R. rhodochrous* ATCC 21198 to transform a mixture of 1,1,1-TCA, *cis*-DCE, and 1,4-D was evaluated. The reactors received initial and/or successive additions of environmentally-relevant

aqueous concentrations of each contaminant (~250-1000 µg/L). CAHs were introduced via additions of Nanopure water saturated with the CAH of interest and 1,4-D was added from a 1000 ppm stock solution. Controls without cells or beads were used to monitor abiotic losses from the reactors. To monitor the potential mass of alcohol being released from encapsulated SRCs, several reactors with co-encapsulated cells were poisoned with 2% (w/v) sodium azide to ensure cells would not consume the hydrolysis byproducts. These reactors were used to determine the rates of abiotic hydrolysis described below. Reactors were monitored over a period of 0 to 303 days for respiration data (headspace concentrations of O₂ and CO₂), alcohol and 1,4-D concentrations in the bulk aqueous phase, and headspace concentrations of 1,1,1-TCA and *cis*-DCE using analytical methods described below. The batch reactors were incubated at 20°C on a shaker table at 100 rpm. The shake speed was reduced from 200 rpm to 100 rpm to lessen the abrasion of the beads, however the long incubation time ensured Henry's Law equilibrium was maintained. The continuous shaking also helped to determine the long-term mechanical stability of the GG beads.

Microcosm experiments with groundwater and aquifer sediments and GG beads

Microcosm reactors were constructed using the same procedures as described in section 2.5, but media was replaced with groundwater and aquifer solids from a site contaminated with 1,4-D and a mixture of CAHs. Procedures for the microcosm construction are provided by Rolston et al. (2019). The microcosms were constructed in sterile, 125 mL glass Wheaton serum bottles sealed with reuseable screw caps fitted with gray butyl rubber septa. Each microcosm contained 15 mL of mixed core material obtained from three depths from the aquifer and 50 mL of site groundwater. Prior to the addition of the GG beads the microcosms were purged with N₂ gas for several hours to remove volatile site contaminants, which permitted the same contaminant mixture of 1,1,1-TCA, *cis*-DCE, and 1,4-D as tests in media (section 2.5) to be assessed. Beads co-encapsulated with *R. rhodochrous* ATCC 21198 and TBOS were then added at the same SRC mass loading and cell mass loading (2 gm of beads at 0.5 mg_{TSS}/g_{bead}) to the buffered media batch reactors permitting a direct comparison between the two treatments. Acetylene controls (a known SCAM inhibitor) were constructed by adding 1.0 mL of acetylene to a batch reactor headspace. Repeated additions of a mixture of 1,1,1-TCA, *cis*-DCE and 1,4-D were added as described above.

Abiotic hydrolysis experiments

The rates of hydrolysis of TBOS and T2BOS were determined using methods previously described by Varcheeswaran *et al.* (1999). Batch reactors that were poisoned, as described above, were used to determine the rate of hydrolysis both in suspension and released from GG co-encapsulated beads at different mass loadings in GG. These batch kinetic tests were conducted in 125 mL glass Wheaton serum bottles sealed with reusable screw caps fitted with gray butyl rubber septa. Reactors were filled with 100 mL of carbonate-buffered MSM (pH 7), followed by an addition of free suspended or encapsulated SRCs. The total mass of TBOS or T2BOS added to each reactor ranged from 1000-1500 mg/L. O₂ and CO₂ concentrations were monitored to demonstrate no microbial activity occurred in the poisoned controls and served as an additional control. The batch reactors were incubated at 20°C on a shaker table at 100 rpm.

Analytical methods

All volatile compounds (O₂, CO₂, isobutane, *cis*-DCE, 1,1,1-TCA, 1,1-DCE, and VC) were measured by sampling the gas headspace with 100 µL gas tight Hamilton syringes followed by injection into a Hewlett Packard (HP) 5890 or 6890 series gas chromatograph (GC). Isobutane was measured using HP 6890 series GC equipped with a flame ionization detector (FID). Isobutane

was separated using an Agilent GS-Q capillary column (30m x 0.53mm) with He as the carrier gas (15 mL/min) at 150°C, resulting in a retention time (RT) of 0.8 min. A HP 6890 series GC equipped with a micro-electron capture detector (ECD) was used to measure 1,1,1-TCA and *cis*-DCE. 1,1,1-TCA and *cis*-DCE which were separated with an Agilent DB-624 UI capillary column (30m x 0.53mm) with He as the carrier gas (15 mL/min) at 50°C resulting in *cis*-DCE and 1,1,1-TCA RT of 2.0 and 2.4 min, respectively. O₂ and CO₂ were measured using HP 5890 series GC equipped with thermal conductivity detectors (TCD). O₂ and CO₂ were separated using a Supelco 60/80 Carboxen-1000 packed stainless-steel column (15ft x 1/8in.). The O₂ method used He as the carrier gas (30 mL/min) at 40 °C, while CO₂ used argon as the carrier gas (30 mL/min) at 220 °C. The GC methods were calibrated using external standards. The total masses in the reactors were determined using the measured headspace concentrations and by applying Henry's Law. 1- and 2-butanol produced from the hydrolysis of TBOS and T₂BOS respectively, were measured using a HP 5890 series GC equipped with an FID. A 5µL liquid sample was injected onto a Supelco 80/100 Carboxen-C packed column (6 ft x 1/8 in) with N₂ as the carrier gas and chromatographic separation was achieved at 105°C.

1,4-D was analyzed from aqueous samples using a Hewlett Packard Series 6890 Gas Chromatograph-Model 5973 Mass Spectrometer (GC-MS) preceded by a Tekmar Dohrmann Model 3100 heated purge and trap concentrator. Details of the analytical method are described by Rolston et al. (2019). The GC-MS was run in single ion mode for *m/z* 88 (1,4-D) and *m/z* 96 (deuterated 1,4-D) run as an internal standard (Draper et al., 2000). The 1,4-D quantification limit was 1 x 10⁻⁴ mg/L, however 25-ml was required for each 1,4-D sample. Due to the large volume and the sensitivity of the instrument, most samples from the batch reactors were diluted 125 to 250 times.

The mass of TBOS and T₂BOS encapsulated in GG beads was determined in the liquid produced from GG beads after treatment with sodium citrate by liquid-liquid (dichloromethane-aqueous) extractions, as described by Vancheeswaran et al. (1999). The reactors were vigorously shaken for 30-60 seconds to ensure adequate homogenization of TBOS and T₂BOS and surrounding media. The sampled liquid (1 ml) was directly added to 1 mL DCM containing 3000 mg/L tetra-*n*-propoxysilane (TPOS) as an internal standard. These samples were then vortexed for 15 min in 4 mL gas tight glass vials.

The liquid DCM was separated from the aqueous sample and transferred into 2 mL gas tight glass auto-sampler vials with rubber septa. Vials were loaded onto a Hewlett Packard HP 6890 Series auto-sampler that automatically injected 5 µL DCM liquid samples onto the GC. The GC was equipped with a Restek RTX-20 capillary column (15m x 0.53 µm) and an FID detector. Helium was the carrier (12 mL/min) with an initial oven temperature of 100°C. The initial temperature was held for one minute followed by a 35°C/minute temperature ramp to 220°C, which was held to a final run time of 5 minute. DCM, TPOS, T₂BOS, and TBOS peaks had RTs of 0.8, 3.2, 3.9, and 4.3 minutes, respectively. Concentration measurements were converted to total mass in the beads.

Results

Cell viability after encapsulation

Cell viability experiments were performed after optimization of encapsulation methods to determine the activity of *R. rhodochrous* ATCC 21198 in both alginate and GG matrices. The utilization rates of isobutane in the beads were compared with those of suspended cells (Figure 7.1

and Table S7.1). Alginate macrobeads were spherical and ~2 mm in diameter (Figure S7.2) while GG macrobeads were cylindrical and ~2 mm in diameter by ~2 mm tall (Figure S7.6). The results show very similar isobutane utilization rates for encapsulated cells compared to suspended cells. Zero-order rates achieved in encapsulated cells were approximately 90% of those obtained by cells in suspension. Encapsulated cells often experience rate limitations in comparison to suspended cells due to slow O₂ or substrate diffusion into hydrogel beads (Gosmann & Rehm, 1986; Hiemstra et al., 1983). One explanation for the observed similarity in isobutane-utilization rates between encapsulated and suspended cells is the relatively low mass loading of cells within the beads (~0.5 mg_{TSS}/g_{bead}). In most hydrogel diffusion and utilization studies the cell mass loading are greater than 10 mg_{TSS}/g_{bead}. Hiemstra *et al.* (1983) investigated the influence of cell mass loadings in alginate macro-beads and found that the higher the mass loading of cells the greater diffusion inhibited O₂ utilization. Overall, the isobutane utilization rate tests demonstrated that *R. rhodochrous* ATCC 21198 could successfully be encapsulated in both alginate and GG hydrogels with minimal to no loss in metabolic activity.

Rates of abiotic hydrolysis of TBOS and T₂BOS encapsulated in alginate and GG beads

The rates of abiotic hydrolysis of TBOS and T₂BOS encapsulated in alginate and GG beads was determined in batch reactor tests. Previous research indicated that the non-aqueous phase concentration of TBOS in solution affected the rate of hydrolysis (Vancheeswaran et al., 1999). The mass loading of TBOS and T₂BOS in the aqueous phase of the batch reactors were therefore kept the same for the suspended and GG bead tests. The concentrations of TBOS and T₂BOS in abiotic poisoned reactors were the same as in the live biotic reactors containing GG beads (1000-1500 mg/L) and had the potential to produce 1300-1920 μmols of the alcohols

The initial abiotic hydrolysis tests were conducted with TBOS encapsulated in alginate macrobeads at a mass loading of 5 and 30% (wt/wt). The beads were added to 100 ml of carbonate-buffered media (pH 7), to achieve an initial TBOS concentration of 1000 mg/L; 2 grams of 5% beads and 0.33 grams of 30% beads were added. The reactors were shaken at 100 rpm on a rotary shaker table to provide good mixing, while avoiding abrasion of the beads. The reactors were monitored for 1-butanol production over 140 days (Figure 7.2 Top) and the zero-order production rates were determined via linear regression (Table 7.1). The hydrolysis reaction was well fit by a zero-order rate estimate for both suspended and encapsulated results. The rate of 1-butanol production was an order of magnitude greater when TBOS was in free suspension than when encapsulated (Table 7.1). One possible explanation for the higher hydrolysis rate of TBOS in suspension is that an emulsion formed in the aqueous phase while shaking the reactors at 100 rpm. This provided a large surface area for hydrolysis and mass transfer to occur. In the reactors containing alginate beads TBOS droplets are dispersed within the hydrogel and the hydrolysis reaction was likely mass transfer limited. The results also showed that when TBOS was encapsulated in alginate at ~30% (w/w) it hydrolyzed at half the rate of TBOS encapsulated in alginate at ~5% (w/w). However, the mass of beads present was 6 times lower for the 30% case. Based on the measured zero-order rate of hydrolysis and the assumption that the rate remained constant with time, the beads were estimated to produce 1-butanol for 5 and 10 years at loading rates of 5 and 30% at the mass loading of the reactors.

The rates of abiotic hydrolysis of TBOS and T₂BOS encapsulated in GG beds was also determined. Both SRCs were encapsulated in cylindrical GG macro-beads (Figure S7.6) at mass loadings of ~8% (w/w). Cells of *R. rhodochrous* ATCC 21198 were also co-encapsulated within the GG hydrogel at initial concentrations of ~0.5mg_{TSS}/g_{bead} with sodium azide (0.2% (w/v) added

as a cellular poison. Two grams of beads were added to each abiotic reactor such that a final concentration of ~1500 mg/L TBOS and T₂BOS was achieved. Reactors containing suspended T₂BOS at ~1500 mg/L were created for comparison.

The results of the hydrolysis rate tests with T₂BOS are presented in Figure 7.3. Some 2-butanol was present in the T₂BOS (95% purity) that was used for encapsulation resulting in 2-butanol being initially present. Zero-order rates of hydrolysis fit the results fairly well, as indicated by the linear regressions. The GG encapsulated T₂BOS hydrolyzed a factor of four slower than the suspended T₂BOS (Figure 7.3, Table 7.2), which is consistent with observations of TBOS encapsulated in alginate (Table 7.1). GG-encapsulated TBOS hydrolyzed ~2 times faster than alginate-encapsulated TBOS (Figure 7.2). The greater mass loading in GG of 8% wt/wt compared to 5% wt/wt in alginate may be partly responsible for the higher rates. Both encapsulation in alginate and GG resulted in factors of 4 to 10 lower rates of hydrolysis than observed in solution at the same mass loadings. The rate of hydrolysis of TBOS encapsulated in GG was 27 times greater T₂BOS, at mass loadings of ~8% (wt/wt) (Table 7.2), while in solution TBOS hydrolyzes ~30 times more quickly than T₂BOS. This is consistent with previous research where the more sterically hindered the central silicate is by the alcohol leaving groups, the less access water has to hydrolysis sites (Arkles et al., 1992; Osterholtz & Pohl, 1992). The slow rate of hydrolysis of T₂BOS in GG would potentially provide for a very long life of the substrate to promote aerobic cometabolism.

Long-term cometabolic transformation studies with GG beads co-encapsulated with R. rhodochrous ATCC 21198 and SRCs

Batch reactor experiments were conducted to determine if *R. rhodochrous* ATCC 21198 could maintain long-term cometabolic treatment of a mixture of 1,1,1-TCA, *cis*-DCE, and 1,4-D when co-encapsulated with TBOS or T₂BOS. The experiments were conducted with cylindrical GG macro-beads containing *R. rhodochrous* ATCC 21198 and TBOS or T₂BOS with biomass loadings of ~0.5mg_{TSS}/g_{bead} and SRC mass loadings of ~8% (w/w) SRC. Two grams of beads were added to the batch reactors, to achieve final cell concentrations of ~10 mg_{TSS}/L and SRC concentrations of ~1500 mg/L. A control with no addition of beads was used to monitor for abiotic losses. Suspended cell controls with the same biomass as encapsulated in the GG beads, but with no SRC added illustrated the effect of co-encapsulation on initial and long-term cell viability. Reactors were created in duplicate or triplicate.

Respiration activity of R. rhodochrous ATCC 21198 co-encapsulated with TBOS

Figure 7.4 presents respiration (O₂ and CO₂), cometabolic transformation (1,1,1-TCA, *cis*-DCE, and 1,4-D), and substrate (1-butanol) results for the batch reactors with GG beads that contained cells of *R. rhodochrous* ATCC 21198 co-Encapsulated with TBOS (CET), along with the abiotic control data. There was a lag in O₂ utilization and CO₂ production, with O₂ depleted during the first 30 days of incubation. After O₂ was depleted due to cellular respiration, pure O₂ was added to the reactor headspace. The results from the abiotic control and the suspended cell reactors (Figure S7.7) show little uptake of O₂ or change in the CO₂ concentration. The repetitive decrease in O₂ concentrations and constant increase in CO₂ within CET reactors, in comparison to suspended cell control reactors, demonstrates cellular respiration was occurring in the CET reactors. (Figure 7.4 A-B).

The O₂ utilization in the CET reactors increased by 3-4 times from the initial observed rate to the rate observed after the second addition of O₂. However, after a subsequent third and fourth

addition of O₂, the O₂ uptake rate remained fairly constant (Table S7.2). The observed increase in O₂ consumption rates indicates that microbial growth likely occurred within CET reactors with a plateauing of rates in subsequent additions over 60 days of incubation. The O₂ consumption results support a pseudo-steady-state biomass concentration being reached in the beads. The continuous increase in headspace CO₂ concentrations supports the O₂ utilization results. Results from the suspended cell control reactors are shown in Figure S7.7. Minimal O₂ utilization or CO₂ production was observed in the suspended cell controls.

The observed O₂ utilization and CO₂ production in CET reactors is due to cellular utilization of 1-butanol released from the hydrolysis of encapsulated TBOS. An elevated O₂ consumption rate was estimated for CET-B reactor compared to CET-A reactor (Table S7.2). It is not known why the duplicates behaved differently, but this would be expected in long-term incubations during which both microbial growth and decay occurs. The utilization of 1-butanol that resulted from TBOS hydrolysis is also supported by measurements of 1-butanol concentration in the bulk solution of active CET reactors compared to the poisoned control (Figure 7.4F). The 1-butanol concentrations in the bulk solution of the CET reactors were typically below the detection limit (~1 mg/L), while the continuous increase in 1-butanol was observed in the poisoned controls. The results support the utilization of 1-butanol produced by TBOS hydrolysis by *R. rhodochrous* ATCC 21198 co-encapsulated in the GG beads (Figure 7.4F).

Another line of evidence confirming that the production of 1-butanol from encapsulated TBOS is driving O₂ utilization and CO₂ production within CET reactors, is the comparison of O₂ and 1-butanol results around 30 days of incubation, when reactor CET-B went anoxic. Due to a vacuum that develops in the reactors, and the method of sampling used (that permits air to enter sampling syringe after it is withdrawn from the reactor), a reported O₂ mass of ~180 μmol represents anoxic conditions in the reactors. When the reactor went anoxic, 1-butanol was detected in the bulk solution (Figure 7.4F). After O₂ was added to the CET-B reactor headspace, the 1-butanol decreased back below the detection limit, providing evidence that O₂ utilization within these reactors is due to cellular oxidation of 1-butanol released from encapsulated TBOS. This period of going anoxic may partly be responsible for the deviation in performance of the duplicate reactors.

Stoichiometric analysis also indicates O₂ consumption and CO₂ production were due to cellular utilization of 1-butanol released from TBOS. The amount O₂ utilized within CET reactors over the first 70 days of incubation was 930 and 1320 μmol in the CET-A and CET-B reactors, respectively. The amount of 1-butanol released via abiotic hydrolysis in the poisoned control over 70 days, estimated from the abiotic hydrolysis rate of 1.3 μmol/day, (Table 7.2) is 91 μmol. Based on 6 μmol of O₂ required to oxidize 1 μmol of butanol to CO₂ and H₂O (assuming no cell yield), 550 μmol of O₂ would be required. CO₂ mass balances were also performed using the reactor headspace CO₂ concentrations and Visual Minteq (2005)(Gustafsson, 2012) calculations, and assuming a buffered pH of 7.0 to determine the aqueous mass of H₂CO₃ and HCO₃⁻ⁱ. The estimated mass of CO₂ produced is 550 μmol which is in good agreement with the amount expected to be formed, assuming no cell yield. The amount of O₂ consumed in the biotic reactors was about twice that predicted based on 1-butanol released by abiotic hydrolysis in the poisoned control. However, this estimate does not consider butanol utilized for cell yield and maintenance, which would decrease the amount of 1-butanol respired to CO₂ and H₂O. The O₂ required to oxidize the three contaminants (1,1,1-TCA, *cis*-DCE, and 1,4-D), would also contribute to the O₂ demand. Assuming the contaminants were oxidized to CO₂, H₂O, and Cl⁻, their transformation contributed

to on average 0.45%, 0.29%, and 2.9% of the total O₂ consumed in the CET-A and CET-B, over the initial 70 days of incubation. The O₂ respiration results indicate that the rate of hydrolysis in the live reactors was greater than expected from the rates of abiotic hydrolysis to produce 1-butanol, while CO₂ production was close to that expected. Both estimates indicate that the production of 1-butanol from the abiotic hydrolysis is supporting the microbial metabolism.

Cometabolic transformation by R. rhodochrous ATCC 21198 in the CET reactors

Based on the respiration data in CET reactors, it is apparent that the biomass is active and TBOS hydrolysis is supporting the biomass of *R. rhodochrous* ATCC 21198 in the GG beads. The observed microbial activity translated into cometabolic transformation of 1,1,1-TCA, *cis*-DCE and 1,4-D (Figure 7.4 C-E). The CET reactors received and transformed five additions of the contaminant mixture, three at the initial mass amount and two at approximately double the initial mass amount. In contrast, suspended cell control reactors that contained a similar initial biomass received multiple additions of contaminants (Figure S7.7) but only transformed the first addition. Limited resting cell transformation was achieved by the suspended biomass that had been grown on isobutane. A long-term slow decrease of *cis*-DCE was also observed that may have been cometabolic in nature. Continuous transformation of 1,1,1-TCA, *cis*-DCE and 1,4-D was observed in the CET reactors. *Cis*-DCE was transformed most rapidly, while 1,1,1-TCA and 1,4-D were transformed at similar first-order rates. The rates of transformation of 1,1,1-TCA, *cis*-DCE, and 1,4-D within CET reactors achieved in the four additions were similar over the duration of the experiment (Figure 7.4 C-E). In the reactors with suspended cells, but with no substrate added (Figure S7.7), the three contaminants were initially transformed, but the transformation ceased after the first addition. Of note is the lag in transformation of the contaminants observed in reactor CET-B, just after the third contaminant addition at 30 days when the reactor went anoxic. The absence of O₂ is consistent with the observed lack of transformation of 1,1,1-TCA and *cis*-DCE. After the addition of O₂ at ~45 days, cometabolic transformation of contaminants proceeds at rates similar to reactor CET-A.

The observations support continuous cometabolic transformation activity associated with the microbial activity in the GG beads. *R. rhodochrous* ATCC 21198 utilizes the 1-butanol released from encapsulated TBOS and uses oxygenase enzymes that are expressed under these conditions to enable cometabolic transformations. The data collected in CET reactors indicate that 1-butanol bulk aqueous concentrations are kept low by the combined effects of microbial activity and the slow hydrolysis of TBOS. The long-term transformation of the final addition will be discussed in a subsequent section.

Respiration activity of R. rhodochrous ATCC 21198 co-encapsulated with T₂BOS

Batch reactor tests were conducted with cells of *R. rhodochrous* ATCC 21198 co-encapsulated with T₂BOS in GG (CET₂) that replicated the tests with TBOS discussed above. Figure 7.5 presents respiration (O₂ and CO₂), cometabolic transformation (1,1,1-TCA, *cis*-DCE and 1,4-D), and substrate data (2-butanol) collected for CET₂ reactors (A, B, and C) that contained the GG beads co-encapsulated with T₂BOS and *R. rhodochrous* ATCC 21198. O₂ and CO₂ results for the CET₂ reactors show much lower cellular activity compared to CET reactors with encapsulated TBOS. Minor decreases in O₂ concentration were observed in relation to suspended cells (Figure S7.7) or abiotic reactors over the initial ~70 day period (Figure 7.5 A-B). However, linear regression of the O₂ mass of time-series, shows significant differences relative to suspended and abiotic controls. O₂ within the CET₂ reactors headspace decreased at a significant zero-order

rate compared to the controls (Table S7.3). The much lower rate of O₂ consumption in CET₂ reactors compared to CET reactors is consistent with the much lower abiotic hydrolysis rate of T₂BOS compared to TBOS (Table 7.2). The rate of O₂ utilization of 0.48 μmol/day (Table S7.3) in the CET₂ reactors, compared with 18 to 40 μmol/day for the CET reactors. The factor of 40 to 80 times higher rate O₂ utilization rate in the CET reactors is consistent with the 27 times higher rate of alcohol production when TBOS was encapsulated in GG beads compared to T₂BOS (Table 7.2).

Based on the estimated abiotic hydrolysis rate of the encapsulated T₂BOS of 0.05 μmol/day, (Table 7.2) after 70 days approximately 3.5 μmol of 2-butanol would be produced (Figure 7.5F). This would require ~21 μmol of O₂ to oxidize it to CO₂ and H₂O (assuming no cell yield). Based on a measured O₂ utilization rate of 0.48 μmol/day, ~34 μmols of oxygen were consumed in CET₂ reactors after 70 days of incubation. These results are consistent with observations with encapsulated TBOS, that observed O₂ utilization rates were about twice those estimated from the rates of hydrolysis, which might result from biotic hydrolysis of T₂BOS. The estimates support the observed utilization of O₂ due to microbial consumption of 2-butanol being slowly hydrolyzed from encapsulated T₂BOS. The analysis indicates that respiration due to cellular activity is occurring within CET₂ reactors when compared to suspended cell controls, with considerably slower activity in comparison to the CET reactors, which is consistent with the much slower rates of hydrolysis. Figure 7.5F shows some detection of 2-butanol in the bulk solution, but concentrations tend to be below 1 mg/L. Thus, the encapsulated cells are effective in utilizing the 2-butanol produced via hydrolysis.

Cometabolic transformations by R. rhodochrous ATCC 21198 in the CET₂ reactors

Respiration data for the CET₂ batch reactors indicate that there is much lower cellular activity occurring compared to the CET reactors. However, three consecutive additions of the 1,1,1-TCA, *cis*-DCE, and 1,4-D mixture were transformed in CET₂ reactors, all at concentrations similar to the CET reactors. CET₂ reactors transformed the majority of contaminants after 80 days of incubation, with *cis*-DCE most rapidly transformed and 1,1,1-TCA and 1,4-D more slowly transformed (Figure 7.5 C-E). In contrast to suspended cells stalling in transformation after the first addition when compared to the abiotic control (Figure S7.7), CET₂ reactors continued to transform all three contaminants at appreciable rates. This data indicates that in CET₂ reactors, the slow hydrolysis of T₂BOS and resulting consumption of 2-butanol produced, maintained microbial activity and cometabolic transformation of 1,1,1-TCA, *cis*-DCE, and 1,4-D. While the rates were slower than those observed in the CET reactors, the rate of O₂ consumption was much lower. The low O₂ demand observed in CET₂ reactors, in comparison to the mass of contaminants transformed, is a very positive result, since O₂ will likely be a limiting factor in contaminated aquifers.

Long-term cometabolic transformations in the CET and CET₂ Reactors

Figure 7.4 presents the continued monitoring of the CET reactors for an incubation period of 300 days. O₂ continues to be depleted due to cellular respiration, and in the headspace O₂ was added numerous times to ensure that aerobic respiration and cometabolism proceeded. The repetitive decrease in O₂ and constant increase in CO₂ within CET reactors is apparent. The rate of O₂ utilization decreased in the last two additions on days 150 and 180, consistent with the decreased rate of CO₂ production. Very consistent trends in both O₂ consumption and CO₂ production were observed in the duplicate reactors. O₂ consumption rates decreased by about a factor of two, from an estimated rate of 11.6 μmol/day over the period of 90 to 120 days to an

estimated 5.3 $\mu\text{mol/day}$ over the period of 180 to 260 days (Figure S7.9). This decrease is consistent with the decrease in rate of CO_2 accumulation in the reactors headspace. These O_2 consumption results combined with the continued CO_2 production indicate continuous microbial activity over the 300-day incubation period.

Continued cometabolic transformation of the last addition of 1,1,1-TCA, *cis*-DCE, and 1,4-D was observed in the duplicate CET reactors over the period of 90 to 300 days. First-order plots are presented in Figures S7.10 to S7.12 for an earlier time period and a later time period and are summarized in Figure 7.6. *Cis*-DCE was most rapidly transformed at a first-order rate about 10 times greater than 1,1,1-TCA, while 1,4-D was transformed at twice the rate of 1,1,1-TCA. All three contaminants were more rapidly transformed in one of the duplicate reactors, illustrating a consistent cometabolic processes. It is not known why the treatments deviated among the duplicates, since rates of O_2 utilization and CO_2 production of the duplicate reactors were similar over this period. The deviation occurred, however, after the CET-B went anoxic.

Transformation rates within CET reactors of 1,1,1-TCA, *cis*-DCE, and 1,4-D slowed with time (Figure 7.6). The decrease in these rates are consistent with the decreased rate of O_2 utilization and CO_2 production. The observations of maintained transformation abilities and continued cellular activity suggest that co-encapsulated microorganisms utilizing 1-butanol released from encapsulated TBOS are able to maintain cometabolic activity for over 300 days, however the rate of transformation slowed in the final addition.

Figure 7.5 presents the continued monitoring of the respiration data (O_2 and CO_2), cometabolic transformation data of 1,1,1-TCA, *cis*-DCE and 1,4-D, and substrate data (2-butanol) collected for live CET₂ reactors A, B, and C along with the abiotic control reactor data. CET₂ reactors continued to show very slow rates of O_2 consumption over the extended time period, and therefore, no additions of O_2 were made to the reactor headspace. Continued O_2 utilization was observed in CET₂ reactors compared to the control over the period of 90 to 230 days (Figure S7.8). CO_2 production above the abiotic control was also observed. Both the O_2 uptake and the CO_2 production provide evidence of slow cellular activity over the 300-day incubation period (Figure 7.5 A-B) with continued O_2 utilization and CO_2 production compared to the control.

Four consecutive additions of the chosen contaminant mixture were transformed by CET₂ reactors, all at levels similar to CET reactors. CET₂ reactors transformed the majority of the contaminants added, with the exception of 1,1,1-TCA, which has the lowest transformation rate of the mixture (Figure 7.5 C-E). The log plot of concentration *versus* time for the CET₂ for the earlier and extended time periods are shown in Figure S7.13 to S7.15 and rates are summarized in Figure 7.6. The results show the transformations followed first-order kinetics fairly well.

The co-encapsulated cometabolic transformation potential was maintained for over 300 days at very slow rates of O_2 utilization (Figure S7.8). A continuous long-term cometabolic transformation is indicated by the first-order plots shown in Figures S7.13 to S7.15. First-order transformation continued over the period of 80 to 300 days. For example, 1,1,1-TCA shows first-order kinetics up to around 300 days. *Cis*-DCE, which was transformed at the highest rate, fit first-order kinetics very well with a reduction in concentration by two orders of magnitude achieved in both of the time periods that were plotted. The first-order rate coefficients showed consistent decreases in the last transformation compared to the previous (Figure 7.6, Figures S7.13-S7.15). The first-order rate coefficient decreased by factors of 2.5, 2.6, and 1.8 for 1,1,1-TCA, *cis*-DCE, and 1,4-D, respectively. The decreases in the rates support the slowing of the same

cometabolic process. One likely explanation is that the biomass of *R. rhodochrous* ATCC 21198 that is being supported by the hydrolysis of T₂BOS decreased by about a factor of two. More work is needed to determine factors responsible for the decrease in rates.

It is interesting to note that the CET₂ reactors rates of transformation slowed to a lesser extent than the CET reactors, whose first-order rate coefficient decreased by factors of 6 to 7 (Figure 7.6). The results suggest that the higher biomass being supported by faster rates of TBOS hydrolysis were not being maintained as well as the lower biomass supported by T₂BOS. The fit of the first-order rates also generally were better for the CET₂ reactors compared to the CET reactors, with R² values ranging from 0.953 to 0.995 for the CET₂ reactors compared with 0.921 to 0.973 for the CET reactors. The CET reactors show some slowing in rates over the time period of the regression, which result in a poorer fit. An example is the *cis*-DCE in the CET reactor over the period of 75 to 160 days (Figure S7.11). A faster rate is shown early on with a slowing in the rate later, resulting in deviations for the first-order regression (R² = 0.947). Over the same time period in the CET₂ reactors (75 to 172 days) the first-order model fits the entirety of the *cis*-DCE time series very well (R² = 0.994) (Figure S7.15). The changes in rates of O₂ consumption with time support a greater slowing in the CET reactors versus the CET₂ reactors. Regression plots of zero-order O₂ mass decreases with time in the reactors are shown in Figures S7.8 and S7.9. In the CET reactors, the rate of O₂ consumption decreases by a factor of 4 over the three regression periods. Over the latter period the O₂ consumption rate was 5.3 μmol/day. The rates for the CET₂ reactors for the linear regression over 40 to 70 days show no observable uptake, while from 70 to 300 days the utilization rate increased to 0.62 μmol/day, a factor of 9 lower than the CET reactors.

VC and 1,1-DCE transformation in the CET and CET₂ reactors

To examine the cometabolic activity in the reactors with other CAHs, VC and 1,1-DCE was added to the CET and CET₂ reactors, on days 264 and 286, respectively. VC was transformed below the detection limit within a week in the TBOS (CET) and T₂BOS (CET₂) reactors (Figure 7.7A). The fastest rate was achieved in a reactor that contained twice as many beads (2X) as the CET₂ reactors. The reactor with 2X the amount of beads was constructed at the same time as the CET₂ (1X) reactors and was subjected to similar multiple exposures of the contaminants. Similar rates were achieved in the CET and CET₂ reactors that had the same mass of beads. In batch, resting cell transformation experiments with isobutane-grown cells of *R. rhodochrous* ATCC 21198, VC was transformed at a very high rate (~19 μmol/mg_{TSS}/day), compared to *cis*-DCE that was transformed at 3 μmol/mg_{TSS}/day (Rich, 2015). The high VC rate observed is consistent with the high rate observed with isobutane grown cells. 1,1-DCE transformation was slower than VC in both CET and CET₂ reactors, while the CET₂ reactor with twice the mass of beads, transformed 1,1-DCE at the highest rate, consistent with the VC test (Figure 7.7B). The results demonstrate 1,1-DCE transformation capability after 287 days of bead incubation. It should be noted that 1,1,1-TCA continued to be transformed during the period of the VC and 1,1-DCE tests (Figure S7.13.).

1,4-D Transformation of 1,4-D to Low Concentrations in the CET and CET₂ Reactors

After the slowdown of transformation rates of 1,1,1-TCA, *cis*-DCE, and 1,4-D, the concentrations of 1,4-D were monitored to ppb levels using a heated purge-and-trap GC/MS method. Lower detection limits were achieved by adding a larger volume of aqueous sample to the purge-and-trap. Figure 7.8 (A-C) shows 1,4-D concentration data in the CET and CET₂ reactors from 70-303 days of incubation on a log-scale. The decrease in concentration tracked first-order kinetics fairly well. Figure 7.8 (lower left) shows the log plot of 1,4-D concentration versus time

in the CET reactors with beads co-encapsulated with TBOS and *R. rhodochrous* ATCC 21198. The 1,4-D concentrations were reduced to 13.3 ppb, representing over a 2-order of magnitude decrease in concentration. Similar results were observed with the CET₂ reactors (Figure 7.8 – upper left), where the 1,4-D concentration was reduced to 5.5 ppb, though with a slightly higher first-order rate constant compared to the CET reactor. When the amount of beads containing T₂BOS was increased by a factor of 2, 1,4-D transformation rate increased by a factor of 1.45 and the 1,4-D concentration was reduced to 0.5 ppb (Figure 7.8 -upper left). Overall cometabolic transformation of 1,4-D fit a first-order rate model and was able to reduce the 1,4-D concentration to 0.5 ppb, which may be required for in-situ treatment.

Incubation of GG beads co-encapsulated with TBOS and R. rhodochrous ATCC 21198 in microcosms with aquifer material and groundwater from a contaminated site

One potential use of the technology is to create a PRB by mixing the co-encapsulated beads with aquifer solids. A batch microcosm study was performed to test this concept. The microcosms were constructed with groundwater and aquifer material from a contaminated site as described in the methods section. GG beads that were co-encapsulated with TBOS and *R. rhodochrous* ATCC 21198 were synthesized as described in methods and added to the microcosms in the same mass amount (2 g) as used in the tests previously described. TBOS was chosen as the SRC to provide a more rapid response in the microcosms. These beads were made separately than those used to generate the results presented previously. However, in order to make a direct comparison with the batch tests performed previously in media, treatments with media only were made that replicated those used to generate results presented in Figure 7.4. The media treatment was constructed and operated at the same time as the microcosms.

These tests also evaluated the reproducibility of the bead synthesis process. The results of this study are presented in Figure 7.9. The rates achieved in the media batch reactors were very similar to those obtained with GG beads that were synthesized 6 months earlier. The rate estimates are provided in Figure S7.18. These results illustrate the ability to reproducibly synthesize the GG beads co-encapsulated with TBOS and *R. rhodochrous* ATCC 21198. The long-term effectiveness was also reproduced when results in media (Figure S7.17) are compared with those presented in Figure 7.4. Acetylene treated controls in these batch reactors with beads, not included tests presented in Figure 7.4, show similar rates of O₂ uptake as non-treated reactors, consistent with the SCAM not needed for alcohol utilization. Acetylene, however, effectively inhibited the cometabolic transformation of *cis*-DCE, 1,1,1-TCA, and 1,4-D. Both the acetylene treated and the poisoned controls also show *cis*-DCE and 1,1,1-TCA were not strongly sorbed into the GG beads.

Initial rates in the groundwater/sediment microcosms were similar for all three contaminants to those obtained in media. Some slowing in the first-order rate was observed in the groundwater/sediment microcosms in the second and third additions of *cis*-DCE and 1,1,1-TCA. The single addition of 1,4-D was reduced by 2 orders of magnitude in the media batch reactors and one in the GW/sediment microcosms. Oxygen consumption rates were similar in the media and GW/sediment microcosm. The media and GW/sediments were operated for 220 days with repetitive additions of the three contaminants. Oxygen was also added when needed. The results of the long-term incubation are shown in Figure S7.17. Effective long-term treatment was achieved in both the media and the GW/sediment microcosms. The first-order rate estimates presented in Figure S7.18, shows the rate of 1,1,1-TCA in the second addition being about a factor of three lower in the microcosm compared to the media control, while the rate of *cis*-DCE in the microcosm was about a factor of four lower in the third addition. The additions of the contaminants

at 205 days were rapidly removed in the media reactors. While slower rates were achieved in the GW/sediment microcosm, the three contaminants were removed over a period of approximately 15 days. Over two orders of magnitude reduction in 1,4-D was achieved. The results show that the GG beaded technology promoted effective cometabolic transformation in a GW/sediment microcosm for over 200 days of incubation.

Discussion

A long-term, passive cometabolic transformation process was developed by co-encapsulating SRCs and an axenic bacterial culture in a GG hydrogel. TBOS or T₂BOS served as SRCs that hydrolyzed at different rates to produce 1-butanol and 2-butanol respectively. The GG method generated more durable beads due to both thermal and ionic cross linking. Deterioration of alginate beads was observed in the hydrolysis tests and microbially active alginate bead tests not reported here. Abiotic rates of hydrolysis of TBOS and T₂BOS measured in solution and in both alginate and GG hydrogels, showed the rates of hydrolysis were 4 to 10 times slower in the hydrogels, with TBOS hydrolyzing at a rate 27 times faster than T₂BOS. GG was selected from long-term tests since it was more durable than alginate. More detailed studies are needed to determine the factors and mechanisms affecting the rates of hydrolysis of TBOS and T₂BOS and other orthosilicates in hydrogels.

Isobutane-utilization activity tests demonstrated that upon encapsulation in either alginate or GG, cells of *R. rhodochrous* ATCC 21198 maintained high rates of activity. Long-term (300 day) incubation of co-encapsulated beads showed continued metabolism of the alcohols produced in the beads as indicated by O₂ consumption and CO₂ production. The rates of O₂ consumption were consistent with those expected from the abiotic rates of hydrolysis. Mass balances indicated that enzymatic hydrolysis may have occurred, consistent with observations of Vancheeswaran et al. (1999). Much higher rates of O₂ consumption were observed when TBOS was co-encapsulated compared to T₂BOS. Sustained by the alcohols produced, *R. rhodochrous* ATCC 21198 was able to cometabolize mixtures of 1,1,1-TCA, *cis*-DCE, and 1,4-D that diffused from the bulk solution into the beads. Prior work of Murnane et al. (2021) showed that when grown on these SRCs, *R. rhodochrous* ATCC 21198 can cometabolize mixtures of 1,1,1-TCA and 1,4-D, and that acetylene, a frequent inhibitor of monooxygenase enzymes, inhibited the cometabolic oxidation of 1,1,1-TCA and 1,4-D, but did not block alcohol utilization. Similar observations are presented in Figure 7.9 with GG beads. Taken together, these results indicated that continuous cell growth and maintenance could be achieved by the slow hydrolysis of the SRCs while achieving continuous cometabolic transformation.

The underlying biochemical basis for the effects of the alcohols released from the SRCs on *R. rhodochrous* ATCC 21198 is currently unclear. One effect appears to include a direct inducing effect of these alcohols on the expression and activity of one or both of the gaseous hydrocarbon and contaminant-oxidizing monooxygenases encoded in the genome of this bacterium. These two enzymes are a propane monooxygenase (PrMO) and another soluble di-iron monooxygenase, short chain alkane monooxygenase (SCAM). An inducing effect on oxygenase expression would be compatible with the various previous reports summarized in the Introduction, of cometabolic contaminant degradation reactions catalyzed by alcohol rather than alkane-grown bacteria, including *R. rhodochrous* ATCC 21198. Another possibility is that the expression of these monooxygenases also involves a response to carbon limitation or starvation conditions. It is a common response of bacteria under carbon-limited conditions to express multiple enzyme systems and catabolic pathways so they can scavenge and benefit from trace amounts of diverse carbon

sources.(Egli, 2010) In the case of *Rhodococcus jostii* RHA1, this starvation response includes highly elevated expression of PrMO (Patrauchan et al., 2012). While it may seem counterintuitive that cells may be carbon-limited when they are co-encapsulated with SRCs, certainly in the case of *R. rhodochrous* ATCC 21198 co-encapsulated with T₂BOS, measured signatures of cellular activity such as O₂ uptake and CO₂ production (Figure 7.5) suggest the rate of SRC hydrolysis is very slow. In the case of T₂BOS, it may be that the rate of 2-butanol release from abiotic T₂BOS hydrolysis is sufficiently rapid for the cells to maintain low levels of “maintenance metabolism” for extended periods of time, and yet sufficiently slow that the cells consume 2-butanol as fast as it is released from T₂BOS and are therefore effectively existing under carbon-limited conditions. Although we cannot exclude the possibility that there are direct effects on monooxygenase expression and activity arising from the encapsulation process itself, or the from the interactions of the cells with high concentrations of SRC within the beads, the possibility that the cells are carbon-limited in T₂BOS co-encapsulated beads is certainly supported by the observation that 2-butanol concentrations are near detection limits in the bulk solution (Figure 7.5F).

Irrespective of the mechanism by which SRCs sustained cometabolically active microorganisms in the co-encapsulated systems described in this study, this co-encapsulation system offers several potential advantages over conventional approaches to aerobic cometabolism. For example, an inhibitory effect of growth substrate on contaminant transformation is frequently observed in cometabolic systems in both laboratory and field studies, and this is due to competitive interactions between two or more substrates for the same oxygenase enzyme.(Criddle, 1993; Kim et al., 2002; Semprini & McCarty, 1992) When cometabolism is applied for bioremediation, one reason for alternate pulsing of the electron acceptor (O₂) and growth substrates such as methane or toluene, is to lessen this competitive interaction.(McCarty et al., 1998; Semprini, 1995; Semprini & McCarty, 1992) In the system developed here, competitive inhibition is avoided since the growth substrates and contaminants are not oxidized by the same enzyme. Evidence for a consistent cometabolic transformation process that is unhindered by a variable competitive interaction, was provided by the repeated transformation of the 1,1,1-TCA, *cis*-DCE, and 1,4-D in the batch reactors and the consistent fit of the temporal data to a first-order transformation model.

While using SRCs such as TBOS and T₂BOS avoids inhibitor competitive interactions, these compounds still enable the process of contaminant degradation and energy-generating metabolism to be decoupled. The decoupling of these two processes is fundamental to the ability of cometabolic processes to degrade contaminants to very low concentrations. For example, the ability to treat contaminants such as 1,4-D, which may have action level of 0.35 µg/L, is not practical with 1,4-D-metabolizing strains such as *Pseudonocardia dioxanivorans* CB1190 which use this compound as a sole source of carbon and energy.(Mahendra & Alvarez-Cohen, 2005) In contrast, the log-plots shown in Figure 7.7 show *R. rhodochrous* ATCC 21198 co-encapsulated with SRCs in GG beads are very capable of degrading 1,4-D to such low levels, and similar two to three orders of magnitude decreases in concentrations were also observed for 1,1,1-TCA and *cis*-DCE.

Another advantage of adding co-encapsulated bacteria with SRCs is that a much lower supply of oxygen would be required, especially when T₂BOS is used as a SRC. The rate of O₂ consumption in the CET₂ reactors containing beads co-encapsulated with T₂BOS was a factor of 50 lower than the CET reactors with TBOS beads. The transformation rates, however, in the CET₂ reactors were only a factor of three lower early on and were nearly the same later. Thus, T₂BOS

is a more efficient SRC with much lower rates of oxygen consumption while maintaining reasonable rates of contaminant transformation.

A final advantage of encapsulating a pure microbial culture within the hydrogel is that this approach not only represents a mechanism for highly selective and long-lived bioaugmentation, it also maximizes the amount of substrate directed to the desired, cometabolically active strain (or strains). For example, if SRCs were added directly to a contaminated aquifer without a co-encapsulated bacterium, competition for the resulting hydrolysis products would exist between diverse native microorganisms. Unless the hydrolysis product itself was highly selective for supporting the growth of cometabolically-active microorganisms, it is likely that growth of non-cometabolically active microorganisms would be promoted and the efficiency of the SRCs would be very limited. This type of population bias towards non-cometabolically active microorganisms would also be further amplified if the biostimulated cometabolically active microorganisms also suffered any toxicity effects arising from the oxidation of non-growth supporting substrates such as CAHs. Other advantages of the co-encapsulation approach include the possibility of including more than one microbial strain in a bead and the possibility of using a specific microorganism or microorganisms with cometabolic activities and oxygenase enzymes that are selected specifically to address site-specific suites of contaminants.

The batch reactor tests were performed with a very low mass of beads (2 g) per 100 ml of solution. When the mass of beads was doubled the first-order rate of 1,4-D transformation increased by a factor of 1.45. Thus, increasing the density of the bead packing would greatly enhance the rates of transformation. An estimate can be made of the potential rates if the co-encapsulated beads were densely packed to make a continuous flow column representative of a PRB. Assuming a porosity of 0.40 and a bead density approximately equal to 1 g/mL, the rate of reaction would be increased approximately 30-fold compared to the rates estimated in the batch reactors. The first-order rate determined in the batch reactor for TBOS of 0.15 day^{-1} (Figure 7.6) would scale to 4.5 day^{-1} in a packed bed configuration. The residence time required to achieve 99% transformation of the 1,4-D would be approximately 1 day. Using the slower rate determined with long-term incubation of 0.03 day^{-1} would yield a rate coefficient of 0.9 day^{-1} in a packed bed, which would require a 5-day residence time to achieve 99% transformation of 1,4-D. There are many factors that would influence these estimates. The contaminant concentrations $250 \mu\text{g/L}$ were low, which resulted in a reasonable first-order fit to the data. As concentrations increase, a first-order approximation would likely not apply. Also, transformation toxicity and transformation yields would become important. With the higher packing density, the rates of oxygen consumption would increase and a supply O_2 would be required. Studies are currently being conducted in densely packed columns to demonstrate that short residence times would be required to achieve very high extents of treatment. Detailed modeling studies are needed that incorporate mass transfer, hydrolysis, microbial process of growth and decay along with the complex process of cometabolism in order to better understand the results observed here and to simulate continuous flow experiments, such as packed columns. Modeling, for example, may help determine what factors contributed to the slowing in the rates of cometabolic transformation over the course of the batch incubations.

The range of contaminants transformed is the same as those observed when *R. rhodochrous* ATCC 21198 was grown on isobutane (Krippaehne, 2018; Rich, 2015). VC and 1,1-DCE were transformed in the later stage of the batch tests. Based on resting cell studies with *R. rhodochrous* ATCC 21198 grown on isobutane, 1,2-DCA, 1,1-DCA and 1,2,3-trichloropropane (1,2,3-TCP)

could also be treated by both TBOS and T2BOS co-encapsulated GG beads. The potential mixture of 1,1-DCE and 1,1-DCA, both abiotic and biotic transformation products of 1,1,1-TCA, respectively, along with 1,4-D could be treated by the GG beads that were tested. The co-encapsulated beads that were created would also be able to treat *cis*-DCE and VC, which are the products of the reductive dehalogenation of TCE.

For subsurface in-situ treatment, O₂ addition would likely be required. Oxygen could be added via air sparging or H₂O₂ addition. To create a completely passive system, solid slow release compounds that produce O₂ (Koenigsberg & Sandefur, 1999; Waite et al., 1999) could be used along with the GG bead system. Studies are needed to develop completely passive cometabolic systems using oxygen release compounds and the co-encapsulated bead technology presented here. In addition to subsurface systems, the co-encapsulated bead technology that was developed might also be used for ex-situ treatment of CAHs and 1,4-D in packed columns or fluidized bed reactors.

The structural stability of the hydrogel beads is important if long-term treatment is to be achieved. Figure S7.16 shows the CET reactors and the CET₂ reactors after 232 days of incubation on a shaker table at 100 rpm. As previously discussed, much higher rates of metabolism occurred in the CET reactors as a result of the higher rates of hydrolysis of TBOS compared to T₂BOS. The beads floating on top in the CET reactors (left) are orange in color that is associated with the higher biomass that developed. CET₂ reactors (right) co-encapsulated with T₂BOS have much lower metabolic activity resulting in lower biomass growth and are clearer in color. Also, there is a higher optical density in CET reactors, which is associated with deterioration of the beads as a result of continuous agitation and microbial growth in the beads. CET₂ reactors are much clearer, showing the beads remain intact even after an incubation of 232 days on the shaker table.

Conclusions

A method was developed to co-encapsulate *R. rhodochrous* ATCC 21198 in GG hydrogel beads with the organosilicates TBOS and T₂BOS that slowly hydrolyze to produce 1-butanol and 2-butanol, respectively. *R. rhodochrous* ATCC 21198 in the beads was able to continuously cometabolize a mixture of 1,1,1-TCA, *cis*-DCE and 1,4-D for over 300 days, and successive additions of a contaminant mixture were transformed. Rates of O₂ utilization and CO₂ production were much higher in batch reactors with beads containing TBOS compared to T₂BOS, which was consistent with its much greater rate of abiotic hydrolysis. Reaction rates decreased with prolonged incubation, but slowed to a lesser extent with beads containing T₂BOS. Appropriately scaled first-order rates indicate very high extents of contaminant transformation could be achieved with a residence time of a few days if co-encapsulated beads were densely packed in a PRB. Studies in groundwater/sediment microcosms also demonstrated that long-term cometabolic treatment can be achieved under conditions that mimic the subsurface. Based on the lower rate of oxygen consumption while achieving similar long-term rates, T₂BOS is a much more effective substrate than TBOS to co-encapsulate with *R. rhodochrous* ATCC 21198.

Tables and Figures

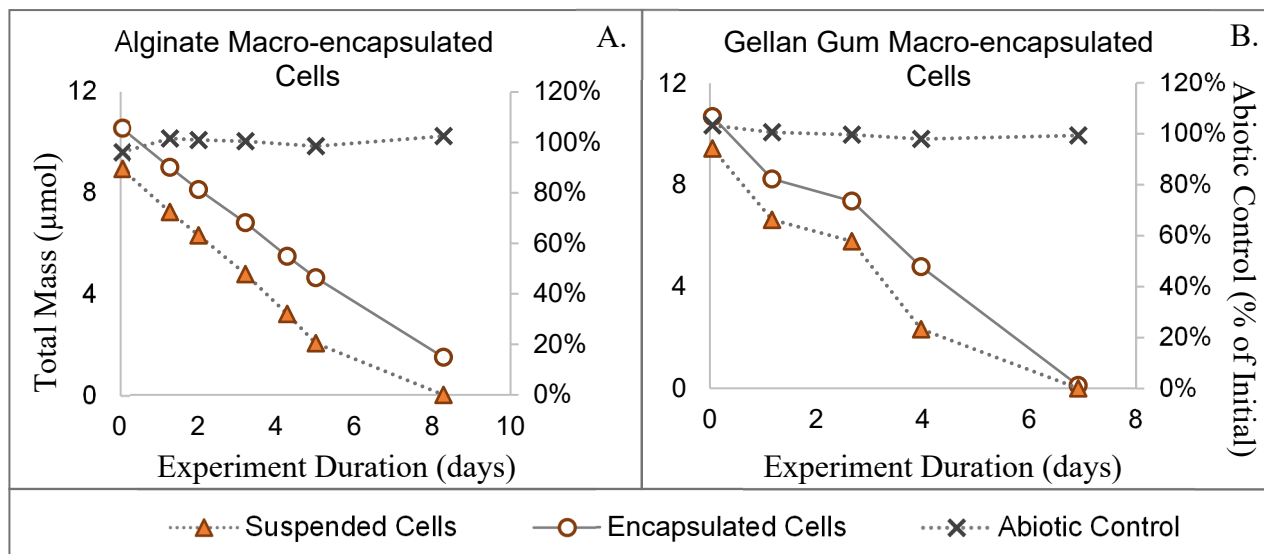


Figure 7.1 Isobutane utilization curves measured for *R. rhodochrous* ATCC 21198 suspended and encapsulated in (A) spherical alginate macro-beads ~2mm in diameter, (B) cylindrical GG macro-beads (~2 mm x 2 mm) and, (C) spherical GG micro-beads (~10-100 μm) in diameter. All data points are averages of duplicate reactors.

Table 7.1. Abiotic rates of hydrolysis of TBOS in alginate macrobeads and suspended in solution with estimated lifetimes of encapsulated TBOS.

Treatment	Added Initial TBOS Mass (μmol)	Maximum Possible Butanol Release (μmol)	Butanol Production Rate (μmol/day)	Estimated Exhaustion of TBOS (years)
Suspended TBOS	312	1250	5.9	0.6
Encapsulated TBOS (5% w/w)	326	1310	0.69	5.2
Encapsulated TBOS (30% w/w)	324	1300	0.35	10.1

Table 7.2 Abiotic rates of hydrolysis of T₂BOS and TBOS in gellan-gum macrobeads and suspended in solution with estimated lifetimes of encapsulated T₂BOS and TBOS.

Treatment	Added Initial TBOS Mass (μmol)	Maximum Possible Butanol Release (μmol)	Butanol Production Rate ($\mu\text{mol/day}$)	Estimated Exhaustion of TBOS (years)
Suspended T₂BOS	475	1900	0.20	26
GG Encapsulated T₂BOS (8% w/w)	441	1770	0.050	97
GG Encapsulated TBOS (8% w/w)	479	1920	1.3	4.0

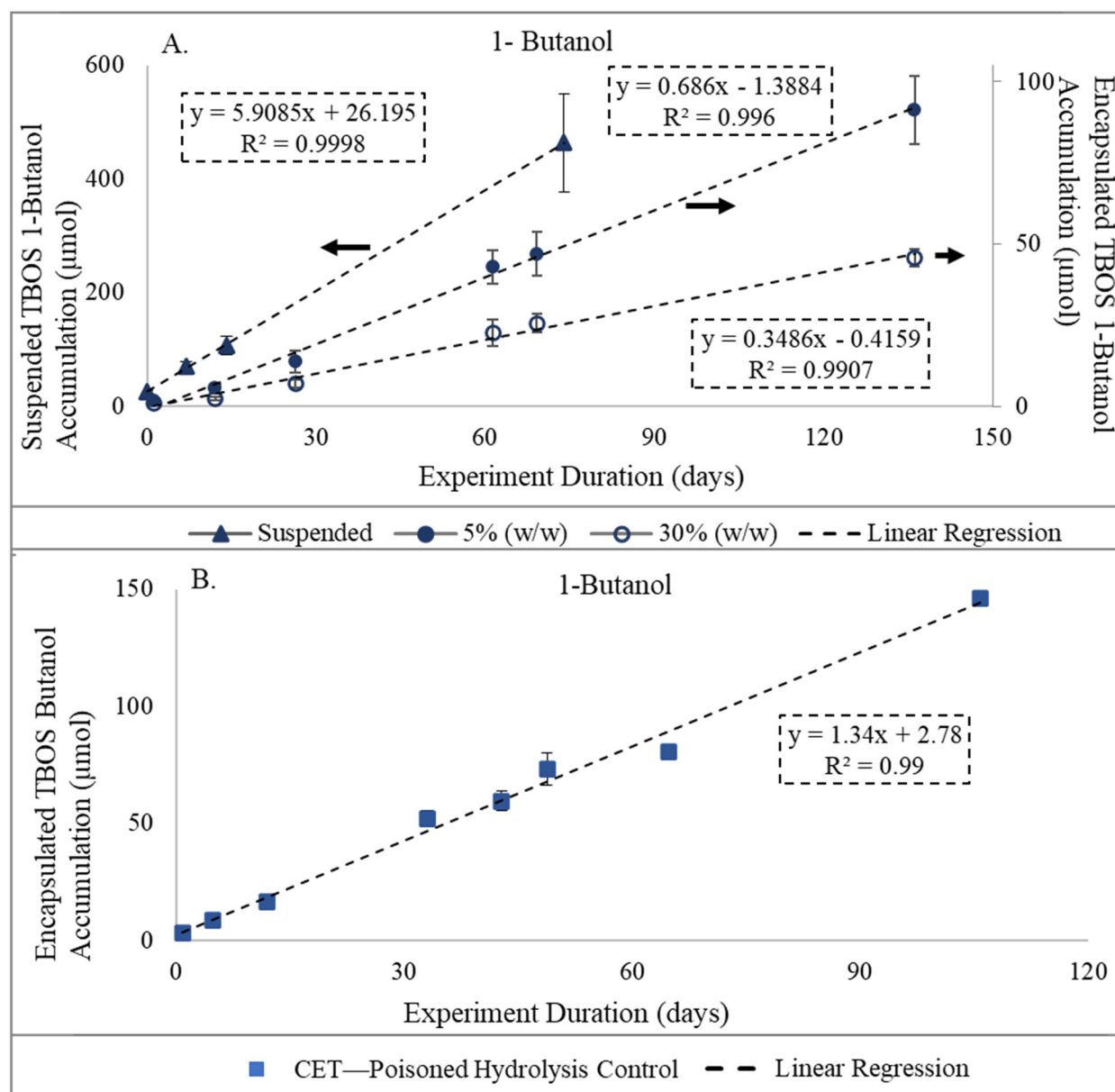


Figure 7.2. (A) Measured 1-butanol masses in solution from the abiotic hydrolysis of suspended (left-axis) and alginate encapsulated TBOS (right-axis). Total TBOS solution concentrations of 1000 mg/L and bead mass loading 5 and 30% (wt/wt). Excel linear trend-line functions are fit to the time-series and black-dashed boxes represent linear regression. Data points are averages between duplicate (suspended) or triplicate (encapsulated) reactors for each treatment, and errors bars are the range of duplicates or 95% confidence intervals for triplicates. (B) Measured 1-butanol masses in solution from the abiotic hydrolysis of duplicate GG encapsulated TBOS. Total TBOS solution concentration of 1500 mg/L and bead mass loading 8% (wt/wt). Error bars are range of the duplicates.

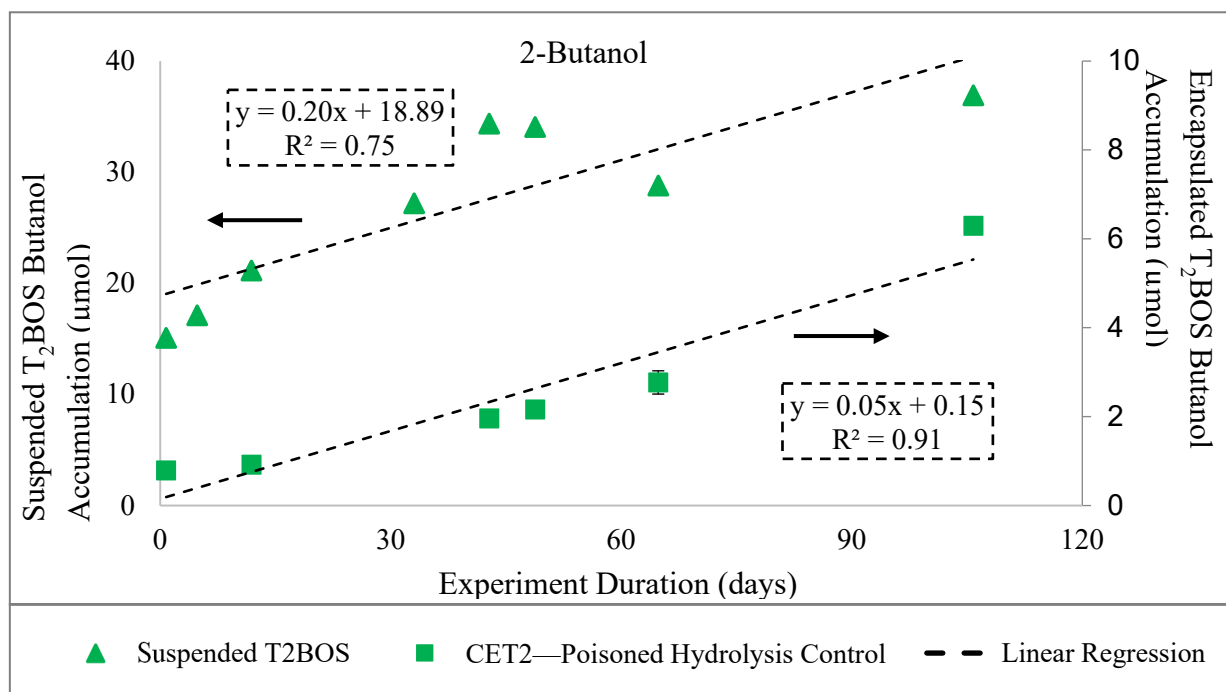


Figure 7.3. Measured 2-butanol masses in solution from the abiotic hydrolysis of suspended (left-axis) and GG encapsulated T₂BOS (right-axis). Total T₂BOS solution concentrations of 1500 mg/L and bead mass loading 8% (wt/wt). Black arrows signify the axis that time-series correspond to. Excel linear trend-line functions fit to time-series and the black-dashed boxes represent linear regression equations calculated by excel linear trend-line tool. Data are averages between duplicate reactors in each treatment and errors bars are the range of the duplicates.

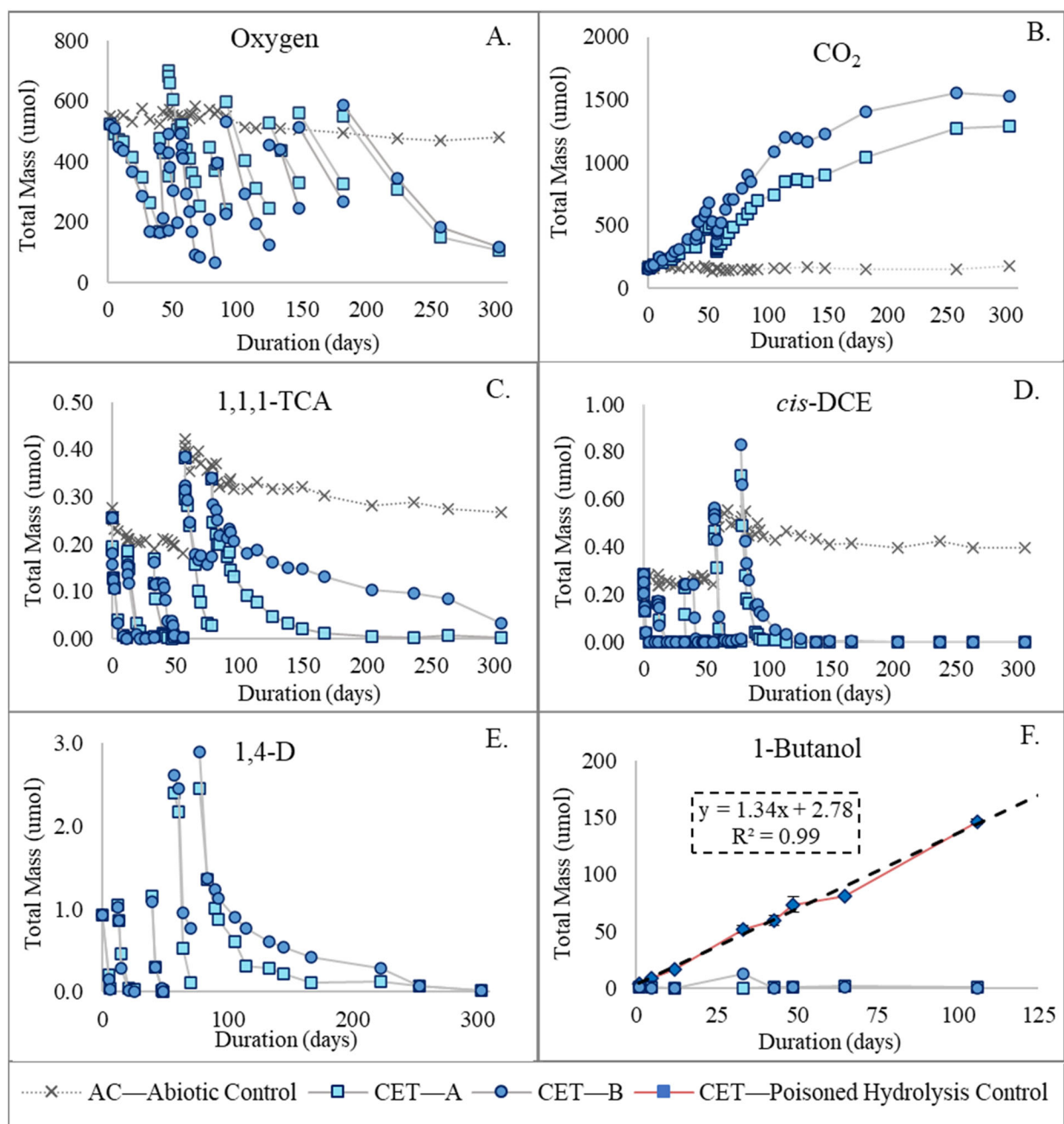


Figure 7.4. Longevity study of the cometabolism of 1,1,1-TCA, *cis*-DCE and 1,4-D in batch CET reactors containing co-encapsulated GG beads with TBOS and *R. rhodochrous* ATCC 21198. (A-B) O₂ and CO₂ respiration data. (C-E) Contaminant transformation data. (F) 1-butanol production data in sodium azide (2% w/v) poisoned hydrolysis control and live reactors. (AC) – Abiotic control. (CET) *R. rhodochrous* ATCC 21198 co-encapsulated with 8% (wt/wt) TBOS. Alphabetical designations CET-A and CET-B are for replicate live reactors. AC has a single reactor. The total mass was determined using Henry's Law. O₂ masses of ~ 180 μmol are assumed to represent anoxic conditions. Breaks in the time-series signify successive additions of O₂ and the contaminants.

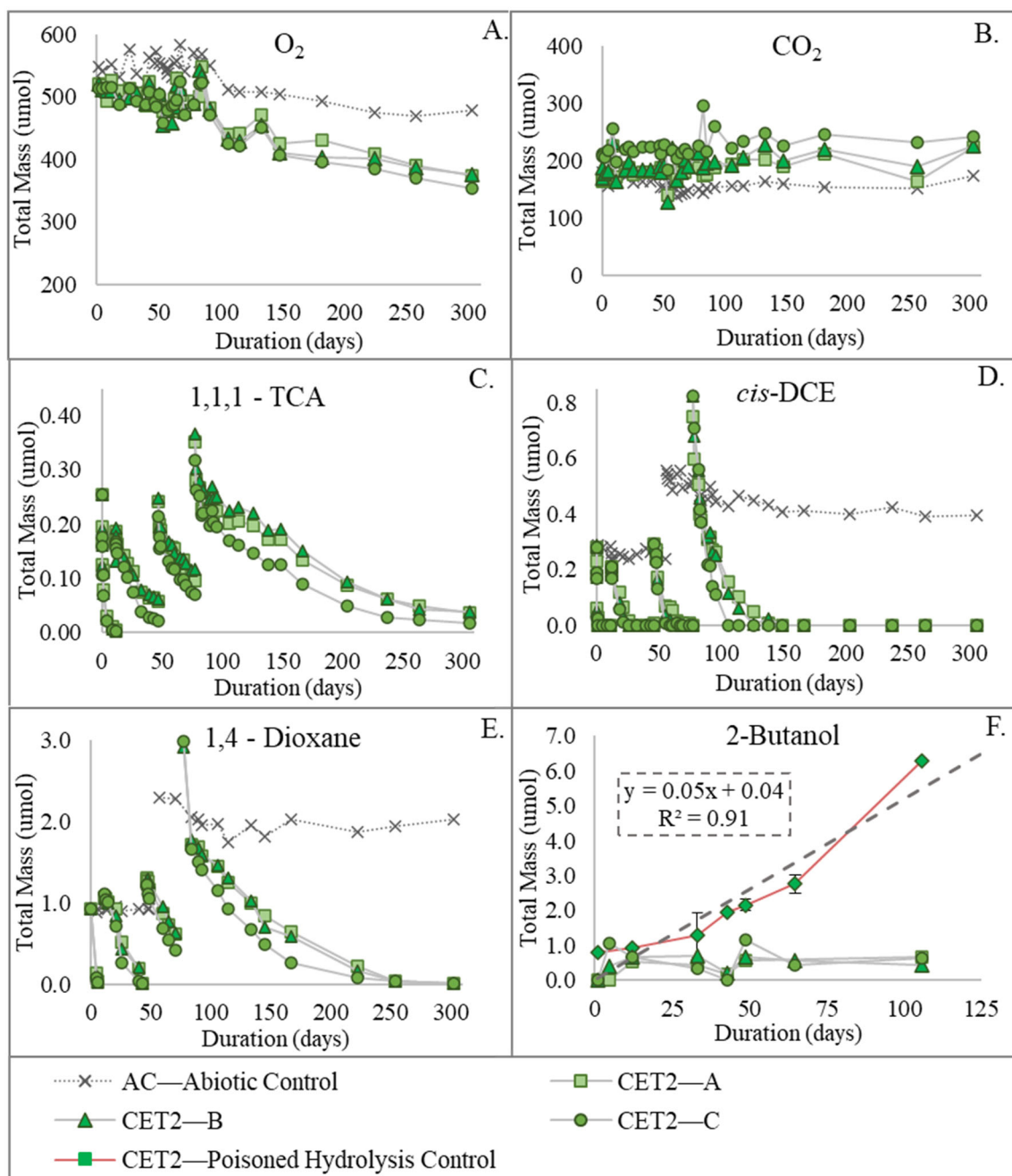


Figure 7.5. Longevity study of the cometabolism of 1,1,1-TCA, *cis*-DCE and 1,4-D in batch CET₂ reactors containing co-encapsulated GG beads with T₂BOS and *R. rhodochrous* ATCC 21198. (A-B) O₂ and CO₂ respiration data. (C-E) Contaminant transformation data. (F) 1-butanol production data in sodium azide (2% w/v) poisoned hydrolysis control and live reactors. (AC) – Abiotic control. (CET₂) *R. rhodochrous* ATCC 21198 co-encapsulated with 8% (wt/wt) TBOS. Alphabetical designations CET₂-A, CET₂-B, CET₂-C are for triplicate live reactors. AC has a single reactor. The total mass was determined using Henry's Law (Equation 1). Breaks in the time-series signify successive additions of O₂ and the contaminants.

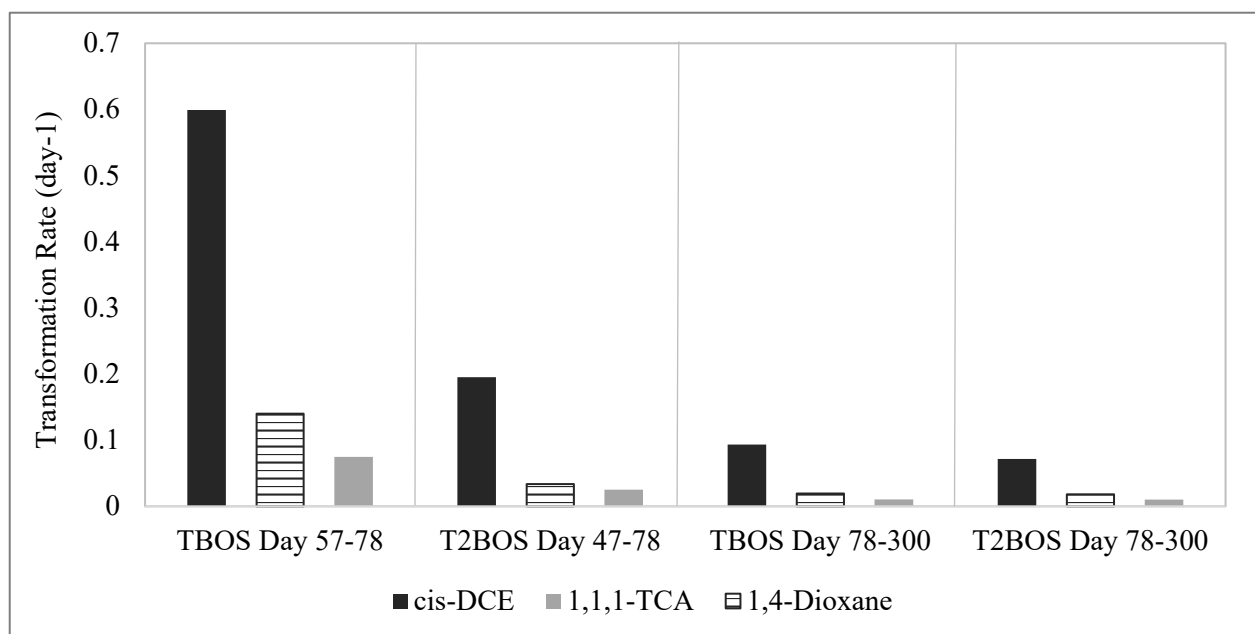


Figure 7.6. First-order transformation rates for successive transformations of 1,1,1-TCA, *cis*-DCE, and 1,4-D. The first-order plots are provided in Figure S7.10-S7.15 in Appendix A.

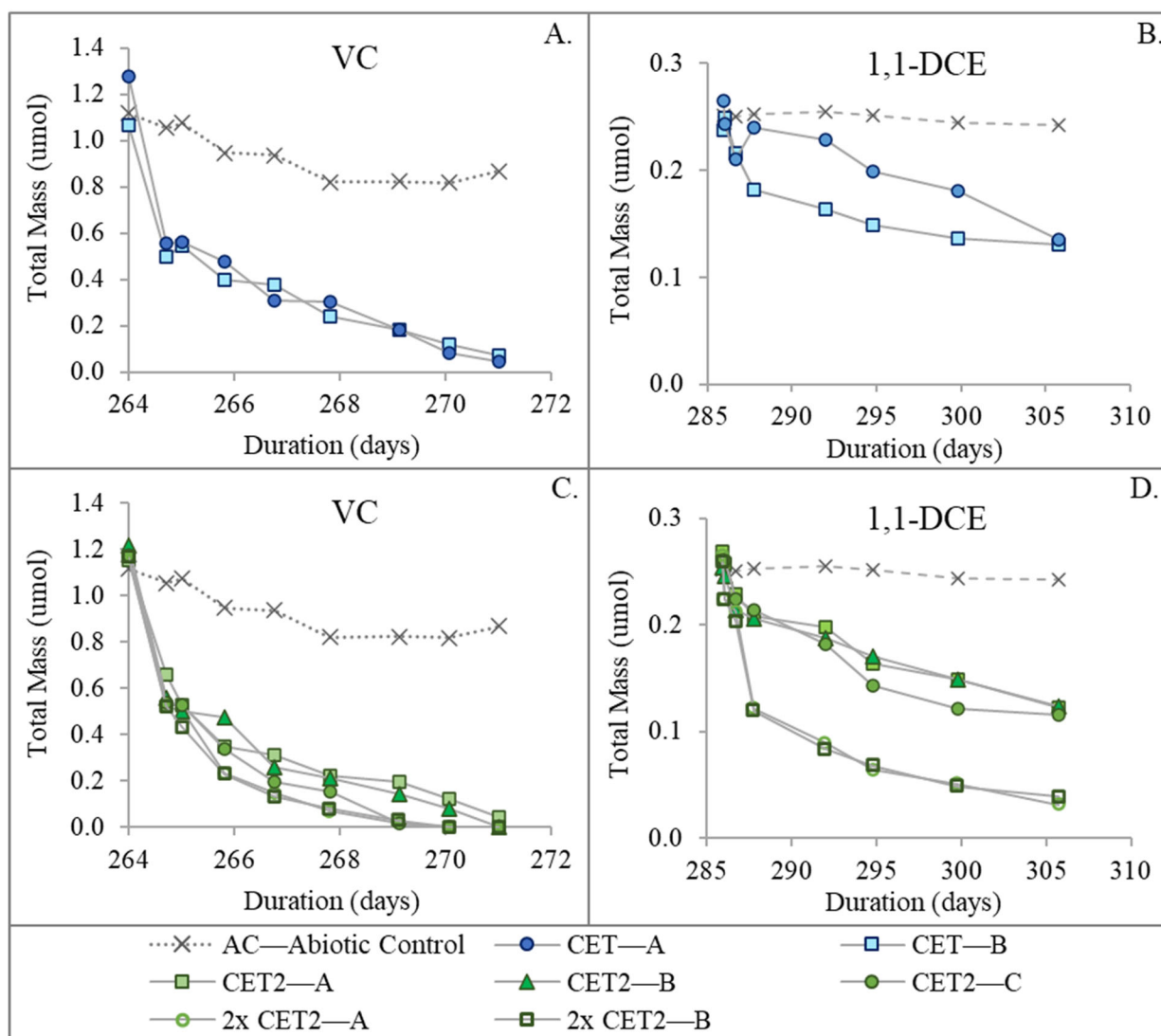


Figure 7.7. VC (A, C) and 1,1-DCE (B, D) Cometabolic transformation with TBOS and T₂BOS co-encapsulated with cells of *R. rhodochrous* ATCC 21198 after long-term (264 day) exposure to *cis*-DCE, 1,1,1-TCA, and 1,4D. (AC) - Abiotic control. (CET) - Cells co-encapsulated with 8% (wt/wt) TBOS. (CET₂) - Cells co-encapsulated with 8% (wt/wt) T₂BOS. (2X CET₂) addition of twice the mass of co-encapsulated beads (4 g) with 8% (wt/wt) T₂BOS.

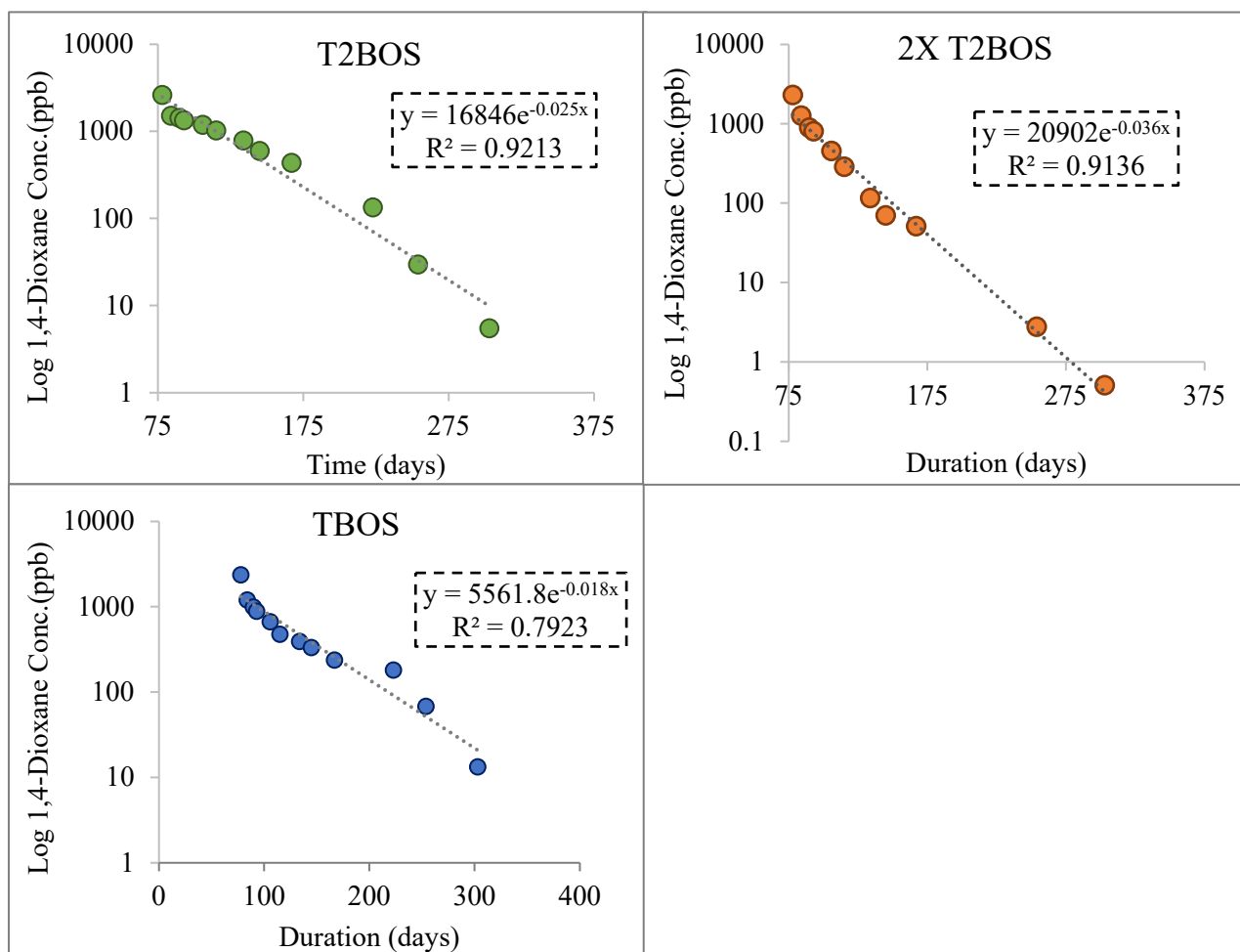


Figure 7.8. Long-term first-order transformation of the last addition of 1,4-D to the CET (TBOS/21198) and CET₂ (T₂BOS/21198) batch reactors (1X beads) shown in Figures 7.4 and 7.5. The 2X bead results are for a batch reactor that has 2 times the amount of GG beads as the CET₂ (1X) reactors.

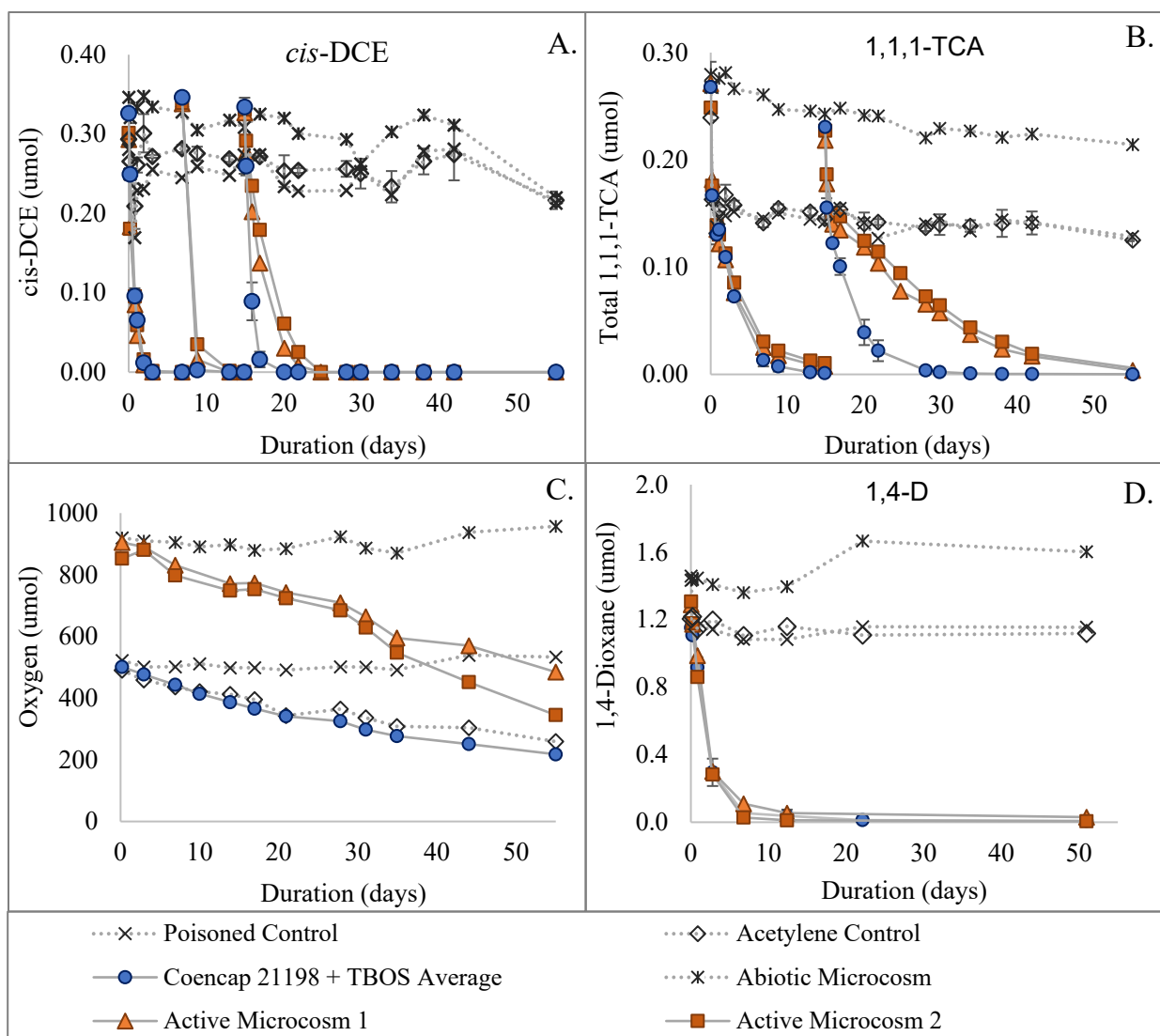


Figure 7.9. Comparison of co-encapsulated TBOS and *R. rhodochrous* ATCC 21198 GG beads in batch reactors with media and groundwater/ aquifer solid microcosms. (A) *cis*-DCE; B (1,1,1-TCA); (C) O₂; (D) 1,4-D. The media reactors replicate the conditions of the tests presented in Figure 4, with GG beads synthesized six months after those tests.

Chapter 8. Continuous Flow Column Studies Packed with Gellan-Gum Beads Co-Encapsulated with *R. rhodochrous* ATCC 21198 and TBOS or T2BOS as a Slow Release Compounds

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Abstract

The continuous aerobic cometabolic treatment of a mixture of CAHs and 1,4-D was studied in laboratory columns packed with hydrogel beads. Columns were packed with gellan-gum hydrogel beads that co-encapsulate the bacterium *R. rhodochrous* ATCC 21198 and 8% (w/w) TBOS or T2BOS as SRCs. TBOS and T2BOS slowly hydrolyze to produce 1-butanol and 2-butanol, respectively as a growth-supporting substrates for *R. rhodochrous* ATCC 21198. A mixture of *cis*-DCE, 1,1,1-TCA, and 1,4-D was continuously fed the columns, each at a concentration of 250 µg/L. Hydraulic residence times (HRT) ranged from approximately 2 days to 12 hours. Biostimulation of *R. rhodochrous* ATCC 21198 occurred during Phase 1 of the column studies. Two columns (Column 1 and 2) packed TBOS beads were operated nearly identically. During the initial stage of operation (Phase 1), 1-butanol concentrations in the column effluent reached 30 mg/L and influent DO concentration (15 mg/L) was reduced to below detection in the column effluent. The addition of H₂O₂ to the column influent groundwater (50 to 100 ppm) as an additional source of DO, resulting in the effective utilization of 1-butanol, with concentrations decreasing to below 1 mg/L in the columns effluent. Both columns achieved over 99.5% removal of *cis*-DCE, 1,1,1-TCA, and 1,4-D within the first 25 days operation at an HRT of approximately 2 days. The development of aerobic conditions resulted in the biostimulation of *R. rhodochrous* ATCC 21198 within the beads throughout the column. This permitted a decrease in the hydraulic residence time 12 hours, while maintaining a contaminant removal efficiency of over 99.5%. In Phase 2 of the tests, the cometabolic transformation was confirmed by stopping H₂O₂ and DO addition, which resulted in an increase in the effluent contaminant concentrations to the influent levels. Upon the reintroduction of DO (15 mg/L) the cometabolic treatment was reinitiated and contaminant concentrations decreased to levels achieved prior the termination of H₂O₂ and DO addition, with 95% removal of 1,1,1-TCA and over 99% removal of 1,4-D and *cis*-DCE.

In Phase III, after ~ 240 PV, *cis*-DCE was replaced by 1,1-DCE (100 µg/L), which imparts a high degree of transformation toxicity. 1,1-DCE was effectively transformed with over 98% removal achieved, while 1,4-D and 1,1,1-TCA continued to be effectively transformed. After ~ 300 PV the concentration of 1,1-DCE was increased to 250 µg/L. Effective transformation for all three contaminants was achieved until around 450 PV after which all three contaminants began to increase to the influent concentrations. After 550 PV of flow, complete breakthrough of three contaminants was observed. Column 2 show nearly identical responses as observed in Column 1 for all Phases of the tests.

Column 3 tests evaluated with gellan-gum beads co-encapsulated with *R. rhodochrous* ATCC 21198 and T2BOS, which hydrolyzes at a much slower rate than TBOS. Column 3 was fed groundwater that contained 14 mg/L DO and a mixture of *cis*-DCE, 1,1,1-TCA, and 1,4-D, each at 250 µg/L. During the biostimulation phase (Phase I) the maximum 2-butanol concentration

observed in the column effluent was 3 mg/L, which were a factor of 10 lower than that observed in Columns 1 and 2, consistent with the slower rate of hydrolysis of T2BOS. 2-butanol concentrations gradually decreased, and after about 50 PV of flow were below 50 µg/L. Due to the slower rate of hydrolysis, H₂O₂ addition was not required. Over 99% removal of 1,1,1-TCA, cis-DCE and 1,4-D was achieved. After 90 PV of flow, Phase II was initiated and the contaminant concentrations were increased from 250 µg/L to 500 µg/L. The effluent concentrations of cis-DCE and 1,4-D began to immediately increase and reached the influent concentrations after 120 PV of flow, while 1,1,1-TCA only reached about 25% of the influent concentration. Upon reduction of the influent concentrations in Phase III to 250 µg/L, the cometabolic transformation was restored, with over 98% removal of the contaminants observed. In Phase IV, cis-DCE addition was replaced by 1,1-DCE (100 µg/L), while continuing to add 1,4-D and 1,1,1-TCA. A rapid increase in all three contaminants in the column effluent was observed. In Phase V, 1,1-DCE addition in the column effluent was stopped, however 1,4-D and 1,1,1-TCA, continued to breakthrough to the injected concentrations. A final test showed cis-DCE, as the only contaminant that could not be transformed. The results suggest that 1,1-DCE transformation toxicity caused the cessation of the cometabolic activity in the column. An increase in the DO in the column effluent was also observed indicating the metabolic activity of *R. rhodochrous* ATCC 21198 also decreased.

At the end of the column studies the beads were sampled and assayed for the amount of TBOS and T2BOS that remained. Approximately 56% and 98.5% of the TBOS and T2BOS, respectively remained of that originally encapsulated. TBOS and T2BOS limitations were not responsible for the cessation in transformation activity in the columns, the estimated rate of hydrolysis of T2BOS was factor of 20 lower than TBOS, which was consistent with the batch incubations.

The results from the column tests indicate that a passive cometabolic permeable reactive barrier (CPRB) might be created using the co-encapsulated technology that was developed. In a barrier, consisting of packed beads, very high removal efficiencies might be achieved with residence times of less than one day. The co-encapsulated macrobeads could also mixed *in-situ* with contaminated aquifer solids to create a passive reactive cometabolic barrier or potentially applied in an in-well reactor.

Introduction

Groundwater contamination with VOCs is a widespread issue throughout the United States (Squillace et al., 2002a; Zogorski, 2006). Over 50% of more than 3500 groundwater samples collected from 98 major drinking water supply aquifers from 1985-2001 contained at least one anthropogenic contaminant with VOCs detected most frequently (Zogorski, 2006). Among the top 15 VOCs detected in this survey, eight were CAH; CF, PCE, TCE, 1,1,1-TCA, *cis*-DCE, *trans*-1,2-dichloroethylene (*trans*-DCE), dichloromethane (DCM), and 1,1-DCA. All of these CAHs are listed by the Centers for Disease Control and Prevention as being likely human carcinogens as well as having other harmful environmental and human health effects (NIOSH, 2013a). Of increasing concern is the high likelihood groundwater plumes containing multiple CAHs as well as co-contaminants, such as 1,4-D (Anderson et al., 2012), which is also listed as a likely human carcinogen (ASTDR, 2013; Derosa et al., 1996). 1,4-D was most commonly used as a chemical stabilizer for chlorinated solvents, mainly 1,1,1-TCA to prevent decomposition due to reactions from light, heat, oxygen, or acid-base chemistry (Mohr, 2001; USEPA, 2017c). 1,1,1-TCA formulas contain up to 2% - 8% by volume 1,4-D (Mohr, 2001). 1,4-D is a commonly detected groundwater contaminant in areas with chlorinated solvent contamination (Adamson et al., 2014;

Anderson et al., 2012; Mohr, 2001). Of over 2000 sites evaluated in California, 194 contained 1,4-D, 95% of sites had at least one chlorinated solvent, and 76% had both 1,4-D and 1,1,1-TCA present (Adamson et al., 2014). The median value of the range of maximal historical 1,4-D concentrations at these sites was 365 µg/L (Adamson et al., 2014). The ubiquitous and long-term contamination of groundwater has allowed co-contaminants to diffuse into saturated low permeability zones, such as clay layers, within aquifers (Adamson et al., 2011). Common remediation techniques, such as pump-and-treat are less effective at treating compounds within low permeability zones due to diffusion limitations and inability to remove or target the contaminant directly (Adamson et al., 2011). Also, current active remediation techniques often require long-term site occupation, are not economical, and are not capable or are ill-equipped to treat of mixtures of contaminants that have varying physical and chemical properties. These issues emphasize the need for exploration into long-term passive and economical remediation techniques such as bioremediation.

In situ bioremediation could potentially provide a large cost savings for treatment of contaminant mixtures, including 1,1-DCE, *cis*-DCE, 1,1,1-TCA, and 1,4-D. 1,1-DCE is often also present at 1,4-D contaminated sites due to its formation during the abiotic transformation of 1,1,1-TCA (Vogel & McCarty, 1987). *Cis*-DCE is often observed at sites resulting from the stall in anaerobic reductive dehalogenation of PCE and TCE (USEPA, 2000) while 1,4-D contamination has most notably been connected to the use of 1,1,1-TCA, it has also been found to be a common co-contaminant with TCE (Adamson et al., 2014).

Aerobic cometabolism is a particularly attractive since microbial growth is maintained on specific substrates that lead to expression of oxygenase enzymes that can then oxidize and remediate CoCs to very low concentrations (Frasconi et al., 2015; Semprini, 1997). The cometabolic transformation of 1,4-D has been reported on a range of substrates for growth and/or induction of cometabolic enzymes as reviewed by Zhang et al. (2017) and McElroy et al. (2019). Rolston *et al.* (2019) reported the cometabolic transformation of 1,4-D by *R. rhodochrous* ATCC 21198 in microcosms fed with isobutane as a primary substrate. This strain can also cometabolically transform a broad range of CAHs including: 1,1,1-TCA, 1,1,2-trichloroethane (1,1,2-TCA), 1,2-DCA, 1,1-DCA and chlorinated ethenes including: *cis*-DCE, 1,1-DCE and VC (Krippaehne, 2018; Rich, 2015; Thankitkul, 2016). Mixtures of 1,4-D, 1,1,1-TCA, 1,1-DCE and 1,2-DCA are also effectively transformed by this strain (Krippaehne, 2018).

Passive treatment systems, such as PRBs, have the potential to reduce costs and eliminate the need to extract groundwater or continuously add substrates, electron acceptors, and nutrients for biological treatment. Scherer et al. (2000b) provides a review of PRBs for groundwater treatment using chemical and biological methods. Upadhyay and Sinha (2018) review permeable reactive bio-barriers (PRBBS) for environmental clean-up. For aerobic treatment, barriers have focused on contaminants that can be metabolized, such as BTEX, through the addition of slow release forms of O₂-releasing compounds (ORCs) such as calcium or magnesium peroxides. However, there has been little attention given to the development of PRBBS for aerobic cometabolism.

Reported here is a novel system that was developed to promote long-term passive aerobic cometabolism that might be used to construct PRBBS. The work is based on the results of our previous study in batch systems of Rasmussen et al. (2020). In that study (presented in Chapter 7) the bacterium *R. rhodochrous* ATCC 21198 was successfully co-encapsulated in gellan-gum beads with orthosilicates as SRCs supporting the aerobic cometabolism of a mixture 1,1,1-TCA,

cis-DCE, and 1,4-D. That study showed the long-term transformation of the contaminants for over 300 days. Two model SRCs, TBOS and T2BOS, which hydrolyze to produce 1- and 2- butanol, respectively, were encapsulated in gellan-gum beads at mass loadings as high as 8% (w/w), along with *R. rhodochrous* ATCC 21198. These batch tests that were conducted at high volume ratio of water to hydrogel beads (50:1) resulted in overall first-order transformation rates of 0.6, 0.15 and 0.07 day⁻¹ for *cis*-DCE, 1,4-D, and 1,1,1-TCA respectively with beads containing TBOS. The first-order rates achieved with beads containing T2BOS were a factor of three lower. Much lower rates of oxygen consumption were observed in the reactors containing T2BOS beads, since the rate of hydrolysis was estimated to be a factor of 27 times slower beads with TBOS.

Presented here are the results of continuous flow experiments with laboratory columns packed with gellan-gum beads that co-encapsulate *R. rhodochrous* ATCC 21198 and either TBOS or T2BOS. The results from our batch experiments indicate that very effective treatment of the mixture of the *cis*-DCE, 1,1,1-TCA, and 1,4-D contaminants could be achieved in continuous flow columns that were densely packed with the co-encapsulated hydrogels beads. The first-order rates are estimated to be a factor of 50 times higher than observed in the batch systems due to the lower volume ratio of water to hydrogel beads, with a bulk porosity of ~ 50%. Thus, simple plug-flow calculations indicate over 99% treatment might be achieved with a column HRT of approximately 1-day.

There were multiple objectives for the study. One objective was to track the initial biostimulation of *R. rhodochrous* ATCC 21198 in the hydrogel bead pack, since the beads are fabricated with a low biomass concentration. A second objective was to determine the steady-state removals of a mixture of *cis*-DCE, 1,1,1-TCA and 1,4-D could be achieved with HRTs ranging from 2 days to 12 hours. A third objective was to demonstrate the removal results from aerobic cometabolism by removing dissolved DO from the column influent solution. In the later stages of the tests, 1,1-DCE was substituted for *cis*-DCE, to evaluate how effectively it could be removed and whether it imposes a high transformation toxicity when it is cometabolically transformed. A final objective was to determine the rates of hydrolysis of TBOS and T2BOS in the beads. The overall objective was to demonstrate long-term cometabolic treatment could be sustained in these packed columns that were supplemented only by sources of DO (including H₂O₂) and synthetic groundwater containing minor nutrients (N and P).

Material and Methods

Chemicals

All CAHs and other chemicals used in the study were reagent grade. 1,1,1-TCA (98.00%) was from Tokyo Chemical Industry. 1, 1-DCE (99%) and *cis*-DCE (99%) were purchased from TCI. 1,4-D (99.00%) was purchased from J.T. Baker. Isobutane used for initial batch growth of *R. rhodochrous* ATCC 21198 was purchased from Gas Innovations (>99.99%). Gellan-gum (GG) (Kelcogel) was donated by C.P. Kelco. TBOS, T2BOS, and TPOS were purchased from Gelest Inc. (>95%). 1- and 2-butanol used for calibration standards were purchased from Sigma-Aldrich (>99%).

Analytical Methods

All liquid samples (1 to 2-ml) were obtained from the columns for CAH analysis using the headspace method described by Rolston et al. (2019). A 1-mL aliquot of the liquid sample was transferred into a 2-mL GC vial for the headspace analysis. After equilibration was reached by

vortexing for 1 minute, a 100 μ L headspace sample was injected with a gas tight syringe to a Hewlett Packard 6890 Series Gas Chromatograph with a micro electron capture detector (GC-ECD) and a capillary column (Agilent DB-624 UI 30m x 0.53mm). Helium was used as the carrier gas at 15 mL/min and a constant oven temperature of 50°C was used. The method was calibrated using external standards. The aqueous concentration of the sample was then determined by mass balance using Henry's Law.

DO was measured in liquid samples by measuring O₂ using HP GC 5890 series II equipped with TCD. A 1-mL liquid sample from the column was added to a 2-mL GC vial that was purged with nitrogen to remove all the oxygen. After vortexing for 1 minute a 100 μ L sample was injected to the GC and chromatographic separation was achieved with a Supelco 60/80 Carboxen-1000 packed stainless-steel column (15 x 1/8 in.). The method used He as the carrier gas (30 mL min⁻¹) at 40° C. The DO concentration in the liquid sample was determined by mass balance using Henry's Law. The method permitted low DO concentration measurements.

SRCs, TBOS and T2BOS, were co-encapsulated into gellan-gum beads using methods described by Rasmussen *et al.* (2020). In order to determine the amount of TBOS or T2BOS that were encapsulated, two ~ 0.25g samples of beads were transferred into 27 mL vials containing 10 mL of 2 mM sodium citrate solution that was used to dissolve the hydrogel. The vials were placed on a shaker table and shaken at 250 rpm for 90 minutes. The amount of TBOS or T2BOS released into solution was determined via liquid-liquid (dichloromethane/aqueous) extraction method as described by Vancheeswaran *et al.* (1999). Briefly, 1 mL of a well shaken liquid sample was directly added to 1 mL DCM containing 100 μ g L⁻¹ tetra-n-propoxysilane (TPOS) as an internal standard. The liquid/DCM mixture was then vortexed for 5 min in 4 mL gas tight glass vials. DCM was separated from the aqueous sample and transferred into a 2 mL gas tight glass vial with Teflon septa. 1 μ L of DCM liquid was directly injected onto the GC-MS and the single ion m/z of 277, 165, and 235 for TBOS, T2BOS and TPOS, respectively, were monitored. An Agilent 6890 GC with Restek Rtx-VMS column (30m x 0.2mm) was used for separation and a signal was detected using a 5973 Mass Selective Detector. Concentration measurements were converted to total TBOS and T2BOS masses in the beads.

1,4-D, 1-butanol, and 2-butanol concentrations were determined using heated purge and trap and Gas Chromatography Mass Spectrophotometry (GC-MS) analysis. 1,4-D was analyzed from aqueous samples using a Hewlett Packard Series 6890 Gas Chromatograph-Model 5973 Mass Spectrometer (GC-MS) preceded by a Tekmar Dohrmann Model 3100 heated purge and trap concentrator. Details of the analytical method are described by Rolston *et al.* (2019). The GC-MS was run in single ion mode for m/z 88 (1,4-D) and m/z 96 (deuterated 1,4-D) run as an internal standard (Draper *et al.*, 2000). The 1,4-D quantification limit was 0.1 μ g/L, however 25-mL was required for each 1,4-D sample. Due to the large volume and the sensitivity of the instrument, most liquid samples from the columns were diluted 125 to 250 times. The same heated purge-and -trap method was used to measure 1-butanol and 2-butanol concentrations with the GC-MS SIM at m/z of 56 and 45, respectively. Deuterated 1,4-D at m/z was used as an internal standard at a concentration of 5 ppb. 1-butanol and 2-butanol peaks eluted at 5.68 and 6.08 minutes, respectively.

Growth of R. rhodochrous ATCC 21198 for encapsulation

Details of the culture maintenance and batch growth of *R. rhodochrous* ATCC 21198 are provided by Rolston *et al.* (2019). Prior to encapsulation *R. rhodochrous* ATCC 21198 was grown

on isobutane in 500 mL glass bottles containing 270 mL of phosphate-buffered mineral salts medium (MSM) (Kottegoda et al., 2015) and 450 mL of air headspace with 45 mL of isobutene added. The culture was incubated at 30° C on a rotary shaker table at 200 rpm and harvested by centrifugation when in late exponential growth phase. The cell pellet was resuspended in phosphate buffer (50 mM, pH 7.0) and centrifuged again. The washing was repeated, and the resting cell pellet was finally re-suspended in buffer. The biomass concentration was determined using TSS analysis as described in AWWA standards (Rice et al., 2017). Under the growth conditions described above, the ratio of protein to TSS was 0.45 mg protein/mg TSS (Rolston et al., 2019).

Co-Encapsulation of the SRCs and R. rhodochrous ATCC 21198 in Gellan-Gum Beads

The method used for the co-encapsulation of SRCs and *R. rhodochrous* ATCC 21198 in gellan-gum macro-beads is described in detail by Rasmussen et al. (2020) and is provided in Section 7 of the report. The beads fabricated for the column studies were the same as those used in the batch tests described in Section 7. They were fabricated to initially contain 0.5 mg_{TSS}/g_{bead} of *R. rhodochrous* ATCC 21198 and 8% by weight of TBOS or T2BOS. Since *R. rhodochrous* ATCC 21198 can potentially grow in the gellan-gum beads, this represents the initial biomass concentration at the start of the column study. The amount of TBOS or T2BOS was quantified using the DCM extraction method and measuring the mass of SRC release as described above. Prior to the packing of the column the beads were washed with growth media.

Column Set up and Operation

Three glass columns experiments were conducted to examine the aerobic co-metabolism of a mixture of contaminants, including *cis*-DCE, 1,1,1-TCA, and 1,4-D, each at a concentration of 250 µg/L. The columns were 14.5 cm in length with a cross sectional area of 4.9 cm² and were fitted with Teflon end caps. Details of the columns and the results of bromide tracer tests are provided in Table 8.1. The empty bed volumes ranged from 69 to 71 cm³ and the total mass beads added to the columns ranged from 49 to 64 grams (Table 8.4). The columns were wet-packed with beads, while vibrating the column to ensure an even bead pack.

The contaminant mixture of *cis*-DCE, 1,1,1-TCA, and 1,4-D (each at 250 µg/L) were fed from 1-liter collapsible mylar bag, while the synthetic groundwater containing saturated DO was fed from a 2-liter glass bottle with a 5-liter collapsible bag of pure oxygen attached to prevent negative pressure in the bottle and maintain a O₂ headspace. The DO concentration in the groundwater was 28 mg/L and after mixing with *cis*-DCE, 1,1,1-TCA, and 1,4-D solution at 50:50 ratio, resulted in an influent DO concentration of approximately 14 ppm. Influent samples (1 mL) were taken from the column injection point, where COCs and DO solution mixed, using 1 mL syringe. The column effluent samples were collected in a 2 mL GC vial through which in the effluent continuously flows.

Bromide ion (Br⁻) tracer tests were performed at a flow rate of 1.0 mL/hr to determine the porosity and dispersion coefficient of the bead pack. Details of the bromide tracer tests are provided in Table 8.1. The bromide breakthrough and elution curves for the three columns are shown Figure 8.1. Bromide tracer tests showed times for 50% breakthrough of the bromide tracer of 56, 49, and 64 hours in Column 1, Column 2 and Column 3, respectively, corresponding to porosity estimates of 0.58, 0.64, and 0.52. Figure 8.1 shows the good fit of the one-dimensional (1-D) advection-dispersion equation (Bear, 1979) to the experimental breakthrough. The high porosity estimates obtained from the modeling likely result from the diffusion of bromide into the

gellan-gum beads. Van Beinun et al. (2000) applied a mobile and immobile zone model to describe transport in columns packed with alginate hydrogels of similar size as our beads. Their results, that fit a mobile/immobile zone transport model, were conducted at much higher velocities compared to our tracer tests. Diffusion tests conducted with their beads show time scales of minutes for diffusion out of their beads. Our time scale for the bromide tests was on the order of hours, indicating there was ample time for diffusion into the beads, which likely resulted in the high porosity estimate. A fair amount of dispersion was also observed in the bromide breakthrough and elution curves. Similar dispersion coefficients were obtained for all three column tests (Table 8.1).

The contaminant mixture of *cis*-DCE, 1,1,1-TCA, and 1,4-D were fed from 1-liter collapsible Mylar bag. The synthetic groundwater containing saturated DO was fed from a 2-liter glass bottle with a 5-liter collapsible bag of pure oxygen attached to prevent negative pressure in the bottle and maintain a O₂ headspace. The DO concentration in the groundwater was 28 mg/L. A Masterflux peristaltic pump was used to pump the groundwater from each of the fed reservoirs at similar feed rates. The two groundwaters were then combined and mixed in a four-way port valve. After mixing with groundwater with *cis*-DCE, 1,1,1-TCA, and 1,4-D at 50:50 ratio, the resulting influent DO concentration was approximately 14 ppm. The 50:50 dilution resulted in influent *cis*-DCE, 1,1,1-TCA and 1,4-D concentrations of 250 µg/L.

Results

The column studies were conducted in different phases as summarized in Table 8.2. Presented first will be the results obtained for Columns 1 and 2 that were packed with gellan-gum beads that contained TBOS, followed by the study conducted in Column 3 packed with gellan-gum beads with T2BOS.

The studies in Columns 1 and 2 were conducted in three phases (Table 8.2). The objective of Phase I was to determine the conditions required to achieve effective biostimulation of *R. rhodochrous* ATCC 21198 in the gellan-gum beads. This was indicated by measuring of the 1-butanol that was released to the bulk solution, with effective biostimulation indicated when little 1-butanol observed in the column effluent. Biostimulation would also be indicated by pseudo-steady-state transformation of the COC mixture that was continuously being added. During the latter stages of *Phase I*, H₂O₂ was added as an additional source of DO, since high concentrations of 1-butanol were initially observed in the column effluent, indicating dissolved DO was limiting. H₂O₂ was added at concentrations ranging from of 50-100 mg/L. During Phase I, the hydraulic residence time was also decreased from 2-days to 12 hours, in both an attempt to supply more DO, but also to demonstrate that the COC mixture could be effectively transformed at a shorter HRT. Phase II demonstrated that the COC removal was due to aerobic cometabolism. This was demonstrated by stopping of H₂O₂ addition and decreasing the DO injected concentration to ~ 4 ppm. The low DO phase was then proceeded by the reintroduction of groundwater that contained 14 mg/L DO addition. The objective of Phase III was to replace *cis*-DCE with 1,1-DCE (100 µg/L) in the influent groundwater and in the latter stage it was increased to 250 µg/L.

Phase I – Biostimulation with H2O2 addition

The biostimulation of the column represents the initial period when the biomass of *R. rhodochrous* ATCC 21198 was potentially growing within the beads. Figure 8.2 show photographs of the column during different stages of biostimulation. Figure 8.2A shows column the after the initial packing. The bead color is white and does not show the orange color of *R. rhodochrous*

ATCC 21198 when it grows to high biomass concentration in the beads. This represents the initial low biomass added to the column and prior to H₂O₂ addition. Figure 8.2B shows the orange color appearing at the column entrance (bottom) after 50 PV of flow and the addition of H₂O₂ as an additional source of DO. The complete biostimulation of the *R. rhodochrous* ATCC 21198 in the gellan-gum beads throughout the whole column after 100 PV of flow and is indicated by the orange color throughout the column (Figure 8.2C).

Concentrations of 1-butanol and DO in the column effluent are presented for Phase I in Figures 8.3a and 8.6a, for Column 1 and 2, respectively. Very reproducible responses were observed in both columns. During the initial start-up, prior to the addition of H₂O₂, the DO concentration in the influent of the columns was approximately 16 mg/L. Effluent concentrations of DO were reduced to below 1 mg/L (Figure 8.3A and 8.6A). DO limitations resulted in limited consumption of 1-butanol within the beads. 1-butanol concentrations in the column effluent, resulting from TBOS hydrolysis, reached a maximum concentration of 32 mg/L (Figures 8.3A and 8.6a). 1-butanol concentrations increased along Column 1, from 10 mg/L at the first port to 30 mg/L in the column effluent (data not shown). These results are consistent with the rates of the abiotic production of 1-butanol that were observed in our batch experiments with TBOS in gellan-gum beads shown in Section 7. The results indicate 1-butanol was being released from the beads as a result of limited DO addition to support its utilization of 1-butanol within the beads.

An attempt was made to increase the DO concentration within the column, by doubling the flowrate from 1 mL/hr to 2 mL/hr (Table 8.2), resulting in a hydraulic residence time of approximately 1 days in both columns, which doubled the mass flux of DO into the column. Increasing flowrate, raised the DO concentrations at different ports of Column 2 (data not shown). However, the DO concentrations remained low at the effluent of both columns, indicated that there is still not enough DO being delivered to consume all the 1-butanol being produced.

In order to provide more DO to the column, H₂O₂ addition (50 mg/L) plus DO initially present in the influent groundwater, was started at 31 and 37 PV in Columns 1 and 2, respectively (Figure 8.3A and 8.6A). This resulted in decreases in the 1-butanol effluent concentrations in Columns 1 and 2, to below detection (50 µg/L) after 56 and 106 PV, respectively. The response indicates the biostimulation of *R. rhodochrous* ATCC 21198 within the hydrogel beads.

The DO concentration response to H₂O₂ addition is shown in Figures 8.3A and 8.6A. With 50 mg/L H₂O₂ addition the influent DO concentrations increased from 15 mg/L to 30 mg/L, while 100 mg/L of H₂O₂ increased DO to ~ 40 mg/L. The results indicate that some H₂O₂ remaining in the influent groundwater entering the column. After the addition of 100 mg/L of H₂O₂ the effluent DO concentration increased to ~ 18 mg/L in both columns. Thus, there was excess DO in the groundwater, resulting in 1-butanol being consumed within the gellan-gum beads. The effluent DO concentrations decreased to around 12 mg/L at 90 PV and 100 PV in Column 1 and Column 2, respectively. Effluent 1-butanol concentrations remained below detection with excess DO present in the column effluent. As shown in Figure 8.2C after 100 PV, biostimulation of *R. rhodochrous* ATCC 21198 that occurred within the beads throughout the column as indicated by the orange color.

Cometabolism of CAHs in Phase I

The contaminant mixture of 1,1,1-TCA, *cis*-DCE, and 1,4-D, each at a concentration of 250 µg /L was continuously added to the columns. The concentration histories normalized to the injected concentrations are shown for Columns 1 and 2 in Figures 8.3B and 8.6B, respectively. All

three contaminants were effectively removed, even during the early stages of the Phase 1 when DO was completely removed and 1-butanol was observed in the column effluent. Some breakthrough of *cis*-DCE to approximately 5% of the injected concentration was observed at around 30 to 40 PV of flow in both columns during the period when over 30 mg/L of 1-butanol was observed in the column effluent. With the addition of H₂O₂ and the decrease of 1-butanol to below detection, over 99% removal of all the contaminants was achieved in both columns. During the period of 60 to 100 PV (Column 1) and 70 to 140 PV (Column 2), with an HRT of 12 hours, steady-state effluent concentrations of TCA (0.2 µg/L), *cis*-DCE (0.3 µg/L) and 1,4-D (1.1 µg/L) were achieved, representing removals of 99.9%, 99.8%, and 99.6%, respectively (Table 8.3). The results show very high degrees of cometabolic treatment were achieved during the latter period of the Phase I test, when 1-butanol was being effectively utilized and DO was present throughout the column, as indicated by DO concentrations above 10 mg/L.

Response in Phase 2 Tests of Stopping H₂O₂ and limited DO addition

To demonstrate the observed contaminant removals resulted from an aerobic cometabolic transformation, anaerobic conditions were created in Phase II by stopping H₂O₂ addition and most of the DO addition to the column influent groundwater. After about 110 PV and 130 PV of flow to Column 1 and Column 2, respectively, the influent DO concentration was reduced from 40 mg/L to 4 mg/L by stopping H₂O₂ addition and lowering DO in the influent storage reservoir. Figures 8.4 and 8.7 show the response of the contaminants and 1-butanol and DO. After the reduction in DO, an immediate increase in the contaminant concentration was observed, with a dramatic increase observed after 160 PV in Column 1 and 180 PV in Column 2. The complete breakthrough of *cis*-DCE, 1,4-D and 1,1,1-TCA to the injected concentration occurred after 190 PV and 230 PV (Figures 8.4B and 8.7B). The lower influent DO concentration resulted in 1-butanol concentrations gradually increasing in both columns to as the breakthrough in the COCs occurred (Figures 8.4 and 8.7). The 1-butanol concentrations, however, were much lower (9 to 4 mg/L) than observed during the initial start-up phase prior to H₂O₂ addition and indicates the rate of 1-butanol production in the beads may have decreased with time. The DO concentration in the column effluent was reduced to below 1 mg/L during the period of low DO addition.

End of Phase II – Increase DO to 14 mg/L

At the end of Phase II, the influent DO concentration was increased to 14 mg/L at ~194 PV in Column 1 and at 230 PV in Column 2. H₂O₂ was not added at the end of Phase II. A decrease in *cis*-DCE, 1,1,1-TCA, and 1,4-D concentrations were immediately observed as a result of the resumption of DO addition. *Cis*-DCE and 1,4-D concentrations decreased more rapidly than 1,1,1-TCA. The effluent DO concentration in both columns increases to around 3 mg/L and the 1-butanol decreased to less than 0.5 mg/L. This low, but measurable concentration of 1-butanol, indicates potential DO limitations in the gellan-gum beads. When steady-state conditions were achieved, at ~210 and 260 PV of flow, in Column 1 and 2 respectively, around 99% removal of *cis*-DCE and 1,4-D was observed, while 1,1,1-TCA removal of 84 and 94% was achieved in Column 1 and Column 2, respectively (Table 8.3 and Figures 8.4B and 8.7B). The results show the recovery of the cometabolic transformation by the encapsulated *R. rhodochrous* ATCC 21198, and the ability of the beads system tolerate periods of anaerobic conditions. Results show that very effective cometabolic transformation of COCs could be achieved with a hydraulic residence time of 12 hours, when effluent DO concentrations above 4 mg/L were maintained in the columns. The response of the columns to the increase in DO concentration further supports a very effective cometabolic

transformation process was occurring within the gellan-gum beads. H₂O₂ addition was not required to maintain 4 mg/L of DO in the column effluent.

Phase 3 - Cis-DCE replaced by 1,1-DCE

In Phase 3, *cis*-DCE was replaced by 1,1-DCE, while continuing the addition of 1,1,1-TCA and 1,4-D. 1,1-DCE was of particular interest since it is an abiotic transformation product of 1,1,1-TCA and it also has been shown to promote very high transformation toxicity when it is cometabolically transformed (Dolan and McCarty, 1998). After 230 PV and 290 PV of flow in Column 1 and 2 (Figures 8.5B and 8.8B) respectively, *cis*-DCE (250 µL) was replaced by 1,1-DCE (100 µg/L). Breakthrough of 1,1-DCE was not observed upon its addition indicating it was being effectively cometabolically treated. 1,1-DCE was essentially completely transformed with greater than 99% of mass removed from the groundwater through each column. 1,1,1-TCA and 1,4-D continued to be effectively removed with 95 to 92% transformed, respectively (Figure 8.5B and 8.8B and Table 8.3). The results indicate that a mixture containing 100 µg/L 1,1,1-DCE, and 250 µg/L of 1,1,1-TCA and 1,4-D could be effectively cometabolically treated.

The metabolism of *R. rhodochrous* ATCC 21198 may have impacted by the transformation of 1,1-DCE. 1-butanol concentrations in the column effluent gradually increased from the time prior to 1,1-DCE addition of less than 0.5 mg/L to values of 1 to 1.5 mg/L after 1,1-DCE was added. Effluent DO concentrations also showed a gradual increase from 3 mg/L at the start of 1,1-DCE addition to around 5 mg/L at around 370 PV prior to the increase in 1,1-DCE concentrations to 250 µg/L. The increase in both 1-butanol and DO would support a decrease in microbial activity in the column.

In the latter stage of Phase III, to examine the effects of transforming higher concentrations of 1,1-DCE, the influent concentration of 1,1-DCE was increased from 100 µg/L to 250 µg/L during 300-550 PVs in Column 1 and 380-600 PVs in Column 2. As shown in Figures 8.5B and 8.8b, 1,1-DCE was initially transformed along with 1,1,1-TCA and 1,4-D. About 98% of 1,1-DCE and 1,4-D were removed and 90% of 1,1,1-TCA. These high degrees of transformation were maintained for about 150 PVs through the columns after which complete breakthrough of 1,1-DCE, 1,1,1-TCA, and 1,4-D occurred (Figures 8.5B and 8.8B).

During the period when the transformation of the contaminants ceased about 5 to 6 mg/L of 1-butanol appeared in the effluent of both columns and DO concentrations gradually increasing to 5 to 7 mg/L. These results indicate 1-butanol was continuously released from the TBOS that was co-encapsulated in the beads and cell metabolism may have ceased. The physical appearance of columns showed a faded orange color (not shown). The change in color compared to that observed in Phase I after biostimulation of the whole column, indicates a reduction of the active biomass of *R. rhodochrous* ATCC 21198 in the latter stage of the test. The lower biomass likely resulted from transformation toxicity caused by the cometabolic transformation of 1,1-DCE.

The results indicate that biomass being maintained via TBOS hydrolysis during the Phase III of the test was not sustainable. This likely from 1,1-DCE transformation toxicity and the inability to maintain an active biomass with the rate of TBOS hydrolysis that was occurring. The DO consumption observed in both columns during Phase III was approximately 8 mg/L, which corresponds to a rate of 1-butanol consumption of about 3 to 4 mg/L, if oxidation to CO₂ and H₂O and some cell yield is assumed. At the very end of Phase III, 5 to 6 mg/L of 1-butanol was observed in the column effluent. If we assume that this was the production rate of butanol at the time a transformation yield of 1,1-DCE can be estimated. Very effective transformation of 250 µg/L 1,1-

DCE was being achieved prior to the observed breakthrough of the contaminants. If around 4 mg/L 1-butanol was being consumed the transformation yield of 0.0625 mg 1,1-DCE/mg 1-butanol was being achieved. This is a very high burden of 1,1-DCE transformation to be maintained over extended periods of operation. Results from resting-cell transformation tests indicate 21198 has the ability to transform about 0.06 mg 1,1-DCE/mg isobutane. In addition, 250 µg/L of 1,1,1-TCA and 1,4-D were also being transformed, which would also decrease the transformation yield. The extended period when all three contaminants were effectively transformed likely resulted from the utilization of biomass that has built-up in the column prior to the addition of 1,1-DCE. This biomass was likely inactivated as a result of 1,1-DCE transformation toxicity.

Column 3 - Column pack with gellan-gum beads with T2BOS.

Column 3 was packed gellan-gum beads that were co-encapsulated with 8% T2BOS, as SRC of 2-butanol. As shown in Chapter 7, the rate of hydrolysis of T2BOS in gellan-gum beads is 20 times lower than TBOS (Rasmussen et al., 2020). Thus, we anticipated less 2-butanol would be observed in the column effluent during the start-up phase of the experiment as well as less DO consumption and less biomass growth in the gellan-gum beads. Based on the results of the batch experiments presented in Chapter 7, that showed cometabolic transformation rates only slightly lower than those achieved with TBOS, we expected very efficient removal at the bead density of the packed column. The Column 3 study was conducted in five Phases as shown in Table 8.2. Due to the lower rate of 2-butanol production, H₂O₂ addition to the column influent was not required. The column influent water was saturated with 14 mg/L of DO throughout the tests. The flowrate to the columns of 4 mL/hr was also held constant through all the phases resulting in a hydraulic residence time approximately 12 hrs. The same mixture of contaminants was evaluated as the tests in Columns 1 and 2. During Phase I, the influent contaminant concentrations of *cis*-DCE, 1,1,1-TCA and 1,4-D were 250 µg/L. During Phase II all the contaminant concentrations were all increased to 500 µg/L. In Phase III the contaminant concentrations were all reduced to 250 µg/L. During Phase IV, *cis*-DCE was replaced by 100 µg/L of 1,1-DCE. During Phase V of the study only *cis*-DCE was fed to the column an attempt to determine if any cometabolism potential remained after the exposure of the beads to 1,1-DCE.

Phase I – Biostimulation through DO addition

Phase I represents the initial biostimulation of *R. rhodochrous* ATCC 21198 in the column beads through the addition of 14 mg/L of DO to the column influent. Figure 8.9 shows the gellan-gum bead pack in Column 3 during the initial startup (Phase I) and in the later Phases. Even in the latter Phases the beads have only a faint shade of orange. This is in contrast to the deep orange color of the beads in Column 1 (Figure 8.2). This is consistent with TBOS producing 1-butanol at a much higher rate than T2BOS produces 2-butanol. The results indicate a much lower biomass developed in the beads of Column 3, consistent the much lower rate of 2-butanol production.

The effluent concentrations of 2-butanol and DO are shown in Figure 8.10A while the contaminant concentrations are shown in Figure 8.10B. The 2-butanol concentrations increased to a maximum concentration of around 3.5 mg/L after ~ 10 PV of flow and then began to gradually decrease to below 0.10 mg/L after about 40 PV of flow. During this biostimulation phase an increase in *R. rhodochrous* ATCC 21198 biomass was likely occurring within the gellan-gum beads. During the entire phase of the experiment the DO concentration in the column effluent was around 0.4 mg/L and indicates that DO may likely be limited within the gellan-gum beads towards

the effluent end of the column. However, the very limited production of 2-butanol in the column effluent indicates effective stimulation of biomass throughout the packed column.

It is interesting to note that the maximum 2-butanol concentration of 3.5 mg/L is an order of magnitude lower than the 30 mg/L maximum 1-butanol produced in Columns 1 and 2. Columns 1 and 2 were operating at a flowrate of 2 mL/hr when the maximum concentrations were measured, while the flow to Column 3 was 4 mL/hr. The results are consistent with batch tests where the rate of abiotic hydrolysis of T2BOS in gellan-gum beads was a factor of 26 times lower than TBOS.

Phase 1 results show essentially the complete transformation of all three COCs through 90 PV of flow (Figure 8.10B). Over 99% transformation of all three COCs was achieved, with *cis*-DCE being the most effectively treated, followed by 1,4-D. Unlike Columns 1 and 2, H₂O₂ was not needed, even during the start-up phase, to achieve very high extent of transformation of all three contaminants. The effluent DO concentration of ~ 0.5 mg/L stayed constant for over 90 PV of flow and likely resulted in DO limitations within the beads. However, very low 2-butanol concentrations in the column effluent corresponded to very high removals of *cis*-DCE, 1,4-D and 1,1,1-TCA.

Phase II: Increase in concentration of cis-DCE, 1,1,1-TCA, and 1,4-D to 500 µg/L

After 90 PV, the influent contaminant concentrations were doubled to 500 µg/L to test the ability to transform higher concentrations. Within about 10 PV of flow, complete breakthrough of *cis*-DCE and 1,4-D occurred, while 1,1,1-TCA breakthrough reached 25% (Figure 8.11B). The incomplete breakthrough for 1,1,1-TCA possibly indicates that it was preferentially treated at the higher concentrations. During this period when the high contaminant concentrations were not effectively treated, the concentrations of 2-butanol and DO in the column effluent remained very low at around 0.03 mg/L and 1 mg/L, respectively (Figure 8.11A). The results indicate that the metabolic activity was likely not affected by the increase in the contaminant concentrations. Since the monooxygenase enzyme is not required for the utilization of the 2-butanol, it is possible that while the high concentrations of the contaminants may have inhibited the monooxygenase enzyme, but they did not inhibit 2-butanol utilization.

Phase III: Decrease in concentration of cis-DCE, 1,1,1-TCA and 1,4-D to 250 µg/L

After 120 PV of flow, the contaminant concentrations were reduced to 250 µg/L in an attempt to recover the cometabolic transformation. The contaminant concentrations gradually decreased and after 180 PV the following extents of removal were achieved, 89%, 96%, and 92% for *cis*-DCE, 1,1,1-TCA, and 1,4-D, respectively. During this period DO concentration remained low with a slight increase in concentration to 3 mg/L at 190 PV. The 2-butanol concentrations remained very low at around 0.03 mg/L indicating effective metabolism was occurring.

The rapid initiation of the cometabolic process upon lowering the contaminant concentrations in Phase III is consistent with the observation that cell metabolism was likely not inhibited during Phase II when the concentrations were higher. The extents of removal, were reduced, ranging from 89 to 96%, compared to prior to the increase in concentrations, when removals were greater than 99%. Thus, it is possible that the high concentrations may have impacted the biomass of *R. rhodochrous* ATCC 21198 in the gellan-gum beads. The slight increase of effluent DO concentrations over the period of 160 to 190 PV may be an indicator of some loss in metabolic activity.

Phase IV: cis-DCE replaced by 1,1-DCE

During Phase IV, beginning at 190 PV, *cis*-DCE (250 µg/L) was replaced by 1,1-DCE (100 µg/L), similar to the operation of Column 1 and 2 tests. Fairly immediate breakthrough for 1,1-DCE occurred, with a gradual breakthrough of 1,4-D and 1,1,1-TCA as well (Figure 8.12B). Consistent with the Phase II tests, 1,1,1-TCA increased more gradually than 1,4-D. The results indicate that 1,1-DCE did impart toxicity to the *R. rhodochrous* ATCC 21198 in the beads even at the low concentration of 100 µg/L.

Phase V. 1,1-DCE replaced by cis-DCE and then only cis-DCE

In an attempt to recover transformation activity in the column, the feeding of 1,1-DCE was stopped at around 200 PV, and was replaced by *cis*-DCE (250 µg/L). Column 3, however, did not recover from the exposure to 1,1-DCE and the contaminant was not effectively transformed. Exposure to 1,1-DCE and its transformation had a more immediate effect on Column 3 compared to Columns 1 and 2, which transformed 1,1-DCE, 1,1,1-TCA and 1,4-D for longer times. As shown in Figure 8.12B, 1,1-DCE quickly eludes from the column after its injection terminated, while *cis*-DCE and 1,4-D rapidly raises to the injected concentration, while 1,1,1-TCA increases more slowly (Figure 8.13B). The DO concentration also slowly increases towards the injected concentration (Figure 8.13A). It is interesting to note the effluent 2-butanol concentration remains very low, despite the lack of DO uptake. The reason for lack in increase in 2-butanol is not known. Analysis of the beads at the end of the column test, that will be discussed later, shows T2BOS remained in the beads, thus 2-butanol production was not limited.

The results indicate little 1,1-DCE was transformed in Column 3, and if it was transformed, the transformation products were highly toxic. 1,1-DCE being effectively transformed for a much longer period in Columns 1 and 2 likely results from much greater biomass present during column operation of more than 240 PV without 1,1-DCE addition combined with the much higher rate of 1-butanol production. A lower biomass was present in Column 3, due to the slower rate of hydrolysis of T2BOS, as indicated by the beads in the column never developing a bright orange color (Figure 8.9).

At 260 PV, the flow to the column was stopped to see if decreases in contaminant concentration occurred with a longer residence time (Figure 8.13B). While temporal decreases in concentration occurred, they rapidly rose to the influent concentration after flow was resumed. After 300 PV, the addition of 1,1,1-TCA, 1,4-D and *cis*-DCE was terminated. Their elution from the column rapidly occurred. At 310 PV *cis*-DCE addition to the influent groundwater was reinitiated at a concentration of 250 µg/L. *Cis*-DCE breakthrough occurred after about 20-PV of flow as shown in Figure 8.13B. Results show no transformation activity in the column after 340 PV of flow. During this period the 2-butanol concentrations in the column effluent remained low and about 7 mg/L of DO consumption continued in the column.

Destructive Testing of the Columns

At the end of the column studies, the columns were destructively tested to determine the amount of TBOS or T2BOS remaining in the gellan-gum beads. Table 8.4 provides mass balances for TBOS and T2BOS that were added to the columns based on amount encapsulated in the beads used to pack the columns and the amount remaining in the beads at the end of the test. The amount TBOS and T2BOS in the beads at the start and the end of the column test was determined by bead digestion using citrate to liberate the encapsulated TBOS and T2BOS and then extraction into

DCM. The TBOS or T2BOS in DCM extract was determined via GC-MS analysis. The beads used to pack the column were 8% by weight TBOS and T2BOS. 56, 49 and 64 grams of beads were used to pack Columns 1, 2 and 3 respectively (Table 8.1). This corresponds to initial masses of TBOS of 4.5 g and 3.9 g in Column 1 and 2, respectively and 5.1 g of T2BOS in Column 3 (Table 8.4). This corresponds to 14.0, 12.2 mmol of TBOS in Column 1 and 2, respectively, and 20.5 mmol of T2BOS in Column 3. At the end of the column study the amount of TBOS or T2BOS remaining in the gellan-gum beads was 42% and 43% of the TBOS initially Column 1 and Column 2 beads, respectively, while in Column 3 98.6% of the T2BOS remained. The much greater amount of T2BOS is consistent with the much slow rate of T2BOS hydrolysis.

Based on the calculated loss of TBOS and T2BOS over the time course of the column studies the rate of hydrolysis was estimated. The overall rate of TBOS hydrolysis was very similar in both Column 1 and Column 2, 17.7 and 17.9 $\mu\text{mol/day}$. In Column 3 that rate of T2BOS was 0.9 $\mu\text{mol/day}$, which is a factor of 20 times slower than TBOS. This slower rate in the column test is in very good agreement with that observed in the batch tests presented in Chapter 7. In the batch tests the abiotic rate of hydrolysis of T2BOS in poisoned controls was a factor of 27 times slower than TBOS.

The rates of hydrolysis estimated in the columns can also be compared to those measured in batch tests on a mass of beads basis. The rate of TBOS hydrolysis in gellan-gum beads in the batch tests was 0.65 $\mu\text{mol/g bead-day}$ compared to 0.32 $\mu\text{mol/g bead-day}$ estimated in the column study. The rate of T2BOS hydrolysis was 0.025 $\mu\text{mol/g-bead-day}$ in the batch test compared to 0.014 $\mu\text{mol/g-bead-day}$ in the column study. The rates in the column were about a factor of 2 lower than measured in the batch systems. The rates being within a factor of two is quite surprising considering the differences in how the rates were estimated and operating conditions of the tests. For example, the rates in the batch tests were determined by directly measuring the rate of alcohol production in poisoned controls, while those for column were based on the mass TBOS and T2BOS lost from the gellan-gum beads over the course of the column study. In the column study the hydrolysis occurred in the presence of *R. rhodochrous* ATCC 21198. As discussed in Chapter 7, this also supports the hydrolysis being abiotic and not microbially catalyzed. The similar rates also support the use of batch tests to make estimates of rates of hydrolysis that will be achieved to flow through column tests.

Table 8.4 also provides mass balances on the amount of the alcohols as well as the amount of TBOS and T2BOS released in the column effluent. Only a small fraction of the alcohols (2 to 4%) formed via hydrolysis (2 to 4%) were measured in the column effluent supporting the microbial utilization of the alcohols. Most of the alcohols were observed in the column effluent during the initial phases of the column test prior to the growth of biomass in the beads. Also presented in Table 8.4 are estimates of how long the columns may have operated based on the rates of hydrolysis and the amount of TBOS and T2BOS remaining in the beads. For the TBOS columns the estimate is 2.3 years, while for the T2BOS column it is 62 years. The mass balances show TBOS and T2BOS limitations were not responsible for the cessation in transformation activity in the columns.

These estimates are only dependent on the rate of hydrolysis and do not include factors. For example, it is not clear how long the gellan-gum beads would remain intact or how long *R. rhodochrous* ATCC 21198 would continue to remain active within the bead. The estimates do indicate that the columns had the potential to remain cometabolically active for longer periods, if

only challenged with low concentrations of a mixture of *cis*-DCE, 1,1,1-TCA, and 1,4-D and not challenged with 1,1-DCE.

Discussion and Conclusions

The purpose of these column studies was to examine how the gellan-gum beads that co-encapsulated *R. rhodochrous* ATCC 21198 and 8% TBOS or T2BOS cometabolically treated contaminated groundwater under continuous flow conditions. The continuous transformation of a mixture of *cis*-DCE, 1,1,1-TCA and 1,4-D was achieved with over 99% removal obtained at hydraulic residence time of ~ 12 hours. The short HRT is consistent with the biostimulation of the beads throughout the column. Biostimulation of beads throughout the column was indicated by the orange bead color that was observed, which indicates a high biomass of *R. rhodochrous* ATCC 21198 in the gellan-gum beads. The biostimulation was also indicated by the very low concentrations of 1-butanol and 2-butanol observed in the column effluent.

As shown in Table 8.3, the percentage of transformation based the influent and effluent concentrations was very high for mixture of contaminants tested. Column 1 and 2 showed very consistent extents of transformation. Even during the latter part of the test when the mixture included 1,1-DCE the following extents of transformation were achieved in Column 1, 99.4%, 97.7% and 96.5% for 1,1,1-TCA, 1,4-D, and 1,1-DCE, respectively, while Column 2 removed 98.4%, 98.3% and 90%, until transformation ceased likely due to 1,1-DCE transformation toxicity.

Simple first-order transformation rates can be calculated with the assumption of a plug-flow transport model and an estimated residence time of 12 hours. A first-order transformation rate of 0.38/hr (9.2/day) is estimated for Column 1 and 2 when over 99% removal was being achieved for all the contaminants with a HRT of 0.5 days (Table 8.3). On the lower end of treatment, of around 90% removal, first-order rates of 0.191/hr (4.6/day) are estimated. As shown in Table 8.3, very little decrease in the extents of treatment were observed over the extended operation of Column 1 and 2 for up to 450 PV of flow, which corresponds to a period of continuous operation of around 250 days or almost 9 months. Column 3 maintained very effective transformation for around 200 PV of flow or for around 100 days. It would likely have performed much longer if it had not been exposed to 1,1-DCE.

The first-order rates observed in the columns can be compared to those reported by Rasmussen *et al.* (2020) in batch reactors presented in Chapter 7. First-order rates observed in batch reactors (Figure 8.6) were higher for TBOS beads compared to T2BOS, by about a factor of three. Rates were also higher by about a factor of three of *cis*-DCE compared to 1,1,1-TCA and 1,4-D, which were in a similar range. The extents of removal observed in the columns, however, were similar for all the compounds. For example, *cis*-DCE was not treated at a significantly higher overall rate than 1,1,1-TCA or 1,4-D, based on the extent of removal. It should be noted that when such high extents of removal are achieved (over 99%) it is difficult observe differences in rates.

There are numerous factors likely contributing to the observed extents of removal that might be explored through more detailed modeling. A spatial distribution in the transformation of the contaminants is likely occurring in the columns. For example, if *cis*-DCE is more rapidly transformed, it might be removed closer to the entrance of the column. Competitive inhibition may also exist between contaminants that would result in a spatial distribution of the treatment in the column. The preferential removal of a contaminant near the influent end of the column could lead to the transformation of the remaining contaminants closer to the effluent end. Mass transfer limitations also likely exist, since diffusion of the contaminants into the beads needs to occur.

Thus, the achievement of removals of over 99% is noteworthy and indicates major mass transfer limitations did not strongly limit the degree of treatment that was achieved.

Comparing the first-order rate estimates achieved in the column studies, to those achieved in the batch tests, shows the much higher overall rates achieved in the columns. This is expected due to the much higher bead volume to liquid volume in the columns. The rates obtained in batch tests with gellan-gum beads with TBOS and also T2BOS are presented in Figure 8.6 (Rasmussen et al., 2020). For TBOS beads, the highest rates were observed early in the incubation, 57-78 days and while for T2BOS beads 47-78 days. The highest first-order rates of *cis*-DCE, 1,4-D, and 1,1,1-TCA were 0.6, 0.14, and 0.065 per day, respectively in the reactors with the TBOS beads, while the highest rates for the reactors with T2BOS beads were 0.2, 0.034, and 0.025 per day for *cis*-DCE, 1,4-D and 1,1,1-TCA respectively. As shown in Figure 8.6, in the later stages of the batch incubations, the rates decreased for both TBOS and T2BOS and rates became more comparable. These lower first-order rates of *cis*-DCE, 1,4-D, and 1,1,1-TCA were 0.072, 0.019, and 0.011 per day, respectively in the reactors with the TBOS beads, reduced by factors ranging from 6 to 8 compared to the earlier time. The reduced rates for the reactor with T2BOS beads in the later stages were 0.071, 0.018, and 0.010 per day for *cis*-DCE, 1,4-D and 1,1,1-TCA respectively, reduced by factors ranging from 1.8 to 2.7 compared to the earlier stages.

In the column study there was little evidence of decreases in rates over similar time intervals. It is possible that the continuous flow system higher overall rates were maintained due to the spatial distribution in the cometabolic transformation in the column. The continuous removal of reaction products and the continuous supply of minor nutrients may have helped maintain the high rates in the columns. If similar reductions in reaction rates by factors of 6 to 8 were observed in the TBOS column with long term operation, then the extents of removal would have been expected to decrease to 54% to 42% from the 99% observed at early time. Such decreases were not observed as shown by the extents of transformation reported in Table 8.3. For the T2BOS column a reduction in rates by factors of 1.8 to 2.7, as was observed in the TBOS column, would have resulted in extents of removal decreasing to 92% and 82%, which were also not observed. Thus, the continuous operation of the column maintained higher overall rates than observed in the batch experiments.

Comparing the estimated overall rates of reaction at earlier times in the batch reactors to the rates in the continuous flow systems show much higher rates in the continuous columns. This is due to the higher ratio of beads per unit liquid volume of reactor. The batch reactor contained 2 mL of beads with a liquid volume of 100 mL. Thus, the ratio of bead to liquid volume at 0.02 mL/mL. In the packed column, the porosity was approximately 50%, which represents a bead to liquid volume of 1.0 mL/mL. Thus, overall the first-order reaction rates are expected to be approximately 50 times higher in the packed column. With over 99% removal being achieved in the column tests with an HRT of 0.5 days, an overall first-order reaction rate of 9.2 day^{-1} is estimated, assuming a simple plug-flow model. This rate is a factor of 14, 66, and 140 times higher than the highest rates observed for *cis*-DCE, 1,4-D, and 1,1,1-TCA in the batch reactors with TBOS beads. For the column packed with T2BOS beads the rates are factors of 46, 270 and 370 times faster in the column than the fastest rate observed in the batch tests. The overall higher rates in the column are in line with the increases in rate estimated by scaling of the ratio of bead to liquid volume from the batch reactors to the packed column.

The overall rates in the column tend to exceed the estimates based the scaling of the bead volume and liquid volumes in the batch reactor to the packed bead configuration. The bulk porosity

is likely lower than estimated from the bromide tracer test due to mass transfer into the hydrogel beads. Using a bulk porosity of lower than 50% would increase the rate estimates. Other factors might be associated with the higher rates in the column, such as spatial changes in the contaminant concentration within the column that might lower competitive inhibition among the contaminants. The continuous removal of reaction products, and the continuous supply of minor nutrients might also make the continuous flow columns maintain higher rates. However, the extrapolation of the highest rates observed in batch tests to the column tests, are in the range of those observed in the columns. The results indicate that the rates obtained in batch tests might be used to estimate the extents of treatment that might be achieved in the column tests.

DO consumption in the columns decreased as more PVs of groundwater flowed through the columns. At 240 PVs, the total DO consumed in Columns 1 and 2 was about 10-11 mg/L, while the initial consumption was much higher, requiring the addition of H₂O₂. This likely indicates a decrease in the overall rates of TBOS hydrolysis as time proceeded. In Column 3, packed with beads containing T2BOS, H₂O₂ addition was never required. It is interesting to note that the long term DO consumption in the T2BOS column was also about 10 to 12 mg/L. Thus, during the long-term performance similar DO uptake rates were observed in the columns packed with TBOS and T2BOS beads. This result differs greatly from the oxygen uptake observed in the batch reactors presented in Chapter 7. In the batch reactors the oxygen consumption rates were a factor of 50 higher in reactors containing TBOS compared to T2BOS. Rates of oxygen consumption also slowed by a factor of 4 to 5 with prolonged incubation in the batch reactors containing beads with TBOS, consistent with the decrease of DO consumption observed in the columns. Oxygen consumption remained fairly constant in the batch reactors with the T2BOS beads, consistent with the observed DO uptake in Column 3.

Since the rate of substrate utilization is difficult to determine and the biomass that developed in the beads is unknown, the assessment of the rates of oxygen utilization to the first-order rates of cometabolism is of interest. In the batch incubations zero-order rates in the TBOS reactors ranged between 18 and 34 $\mu\text{mol/day}$. During this period the highest first-order rates of *cis*-DCE, 1,4-D, and 1,1,1-TCA were 0.6, 0.14, and 0.065 per day, respectively. Dividing the first-order rate of cometabolism by the zero-order rate of oxygen utilization may also be a means of comparing the rates in the batch and column. The oxygen normalized rates are 0.02 μmol^{-1} , 0.005 μmol^{-1} , and 0.0025 μmol^{-1} , for *cis*-DCE, 1,4-D, and 1,1,1-TCA respectively. During the early stage of the column study 100 mg/L of O₂ were being consumed. An influent flowrate of 4 mL/hr corresponds to an oxygen utilization rate of 300 $\mu\text{mol/day}$. The first-order rate for all three compounds was 9.2/day to achieve 99% removal. The first-order rate normalized to the oxygen consumption rate was 0.03 μmol^{-1} , which is in the range of *cis*-DCE calculated for the batch reactors, but a factor of 10 higher than those for 1,4-D and 1,1,1-TCA. In the latter stages of the batch tests the rate of oxygen utilization decreased to around 5 $\mu\text{mol/day}$, while first-order transformation rates also decreased to 0.071, 0.018, and 0.010 per day for *cis*-DCE, 1,4-D and 1,1,1-TCA respectively. The first-order rates normalized to oxygen consumption were 0.014 μmol^{-1} , 0.0036 μmol^{-1} and 0.002 μmol^{-1} for *cis*-DCE, 1,4-D and 1,1,1-TCA, respectively. In the later time operation of the column around 10 mg/L of DO was consumed, which corresponds to an oxygen consumption rate of 30 $\mu\text{mol/day}$. 99% removal of all the contaminants continued during this period, thus the overall rate of 9.2/day was maintained. Thus, the oxygen consumption normalized rate is estimated to be 0.30 μmol^{-1} . These rates normalized to rates of oxygen utilization are a factor of 4 times higher than the batch tests and support very effective cometabolism in the

column on the basis of oxygen utilization. A similar result is obtained for Column 3, since 99% removal was being achieved and oxygen consumption of around 10 to 15 mg/L was observed during the column tests.

A mass balance approach is another way of determining the effectiveness of process by comparing the mass of oxygen consumed to the amount of COCs transformed. During the steady-state operating periods of the columns, when only DO needed to be added (15 mg/L) the effluent DO concentration was between 3 and 4 mg/L, which corresponds to about 12 mg/L of DO consumption. Essentially all *cis*-DCE, 1,1,1-TCA and 1,4-D and later 1,1-DCE were removed at concentrations of 250 µg/L. Thus, on a total contaminant basis 0.75 mg were continuously transformed. On an oxygen consumption basis, 0.0625 mg of the contaminant mixture was transformed per mg O₂ consumed, which represents a transformation yield on an oxygen basis. A similar yield would be estimated for Column 3.

This yield can be compared to those achieved on well documented field demonstrations where both substrate addition and DO consumption were measured. For example, in the field evaluation of the bioaugmentation of butane utilizers, which would be in the family of microorganisms studied here (Semprini et al., 2007), 1,1,1-TCA, and 1,1-DCA was evaluated in-situ at the Moffett Field test facility (Table 8.5). Approximately 100 µg/l and 65 µg/L of 1,1-DCA and 1,1-DCE were transformed, while approximately 16 mg/L of DO were consumed, which give of ratio of 0.01 mg of the contaminant mixture per mg O₂. In another study at Moffett Field, the transformation of TCE by phenol utilizing microorganisms was evaluated (Hopkins, Munakata, et al., 1993). This study is of interest since TCE concentrations were increased permitting a maximum O₂ transformation yield to be determined. The amount of oxygen consumed to phenol utilized was 2.0 mg DO/mg phenol and the highest TCE transformation yield achieved was 0.062 g TCE/g phenol. This results in an estimated oxygen transformation yield of 0.03 mg TCE/mg O₂. This transformation yield on an O₂ basis is about 50% less than estimated for the column study presented here.

In studies of TCE and 1,1-DCE transformation by phenol and toluene utilizing microorganisms at the Moffett Field test Facility, Hopkins and McCarty (1995) summarize the results of past studies with methane utilizers and their studies with phenol and toluene. They also report the DO consumed in these tests. Table 8.5 presents a summary of results of the field tests conducted the Moffett Field test facility including the different substrates used, an estimate of the DO consumed in the test leg and the degree of removal of the contaminants that were achieved. In the Moffett Field test groundwater was either sparged with pure oxygen or H₂O₂ was added so that high concentrations of the growth substrate could be added to achieve a high biomass in-situ. The DO consumption to substrate addition was in the range of 2 to 4 mg of DO per mg of substrate. DO consumption ranged from 20 to 30 mg/L in the treatment zone. In our column studies, DO consumption after the initial start-up ranged from 10 to 15 mg/L, a factor of 2 lower than observed typically in the field.

Moffett field cometabolic studies were conducted using four different primary substrates in attempts to transform different chlorinated ethenes and chlorinated ethanes (Dolan & McCarty, 1995; Hopkins, Munakata, et al., 1993; Hopkins & McCarty, 1995; Semprini et al., 1991, 2007, 2009; Semprini & McCarty, 1991). The highest degrees of removal achieved in the treatment zone were when toluene was used as a cometabolic substrate with over 98% removal of both *cis*-DCE VC, and 93% and 73% removal of TCE and *trans*-DCE respectively. In these studies, TCE concentration was 230 µg/L and *cis*-DCE and *trans*-DCE were added a 119 µg/L and 81 µg/L,

during different stages of the study along with TCE. This study demonstrated high degrees of treatment could be achieved using toluene as a substrate with DO consumption in the range of 30 mg/L. High degrees of removal were achieved for *cis*-DCE and VC of 92 and 98 percent when phenol was used as a substrate and with DO utilization of 30 mg/L at concentrations of 100 µg/L and 44 µg/L, respectively.

The ability to cometabolize 1,1-DCE were also studied in the field tests conducted with phenol and butane as substrates (Hopkins & McCarty, 1995; Semprini et al., 2007). The concentrations were typically low in the range of 65 µg/L. The extents of removal were 54 % and 83 % with phenol and butane, respectively, with DO consumption of 25 mg/L. In Columns 1 and 2 studied here, when 100 µg/L of 1,1-DCE was continuously fed 98% removal of 1,1-DCE was achieved, while continuing to remove 91% to 94% of the 1,1,1-TCA and 98% of the 1,4-D. In the field study with butane, while 83 % of the 1,1-DCE was treated, only 63% of 1,1-DCA and 31 to 70% of 1,1,1-TCA was achieved. While the transformation of 250 µg/L of 1,1-DCE eventually resulted in the loss of activity in the columns, during the 50 PV when transformation was achieved the removals of 1,1,1-TCA, 1,4-D, and 1,1-DCE were 90%, 98%, and 98% for 1,1,1-TCA, 1,4-D, and 1,1-DCE respectively.

While the field scale is much larger and the ability to implement is much more difficult, the comparison with the column study does have value. The results illustrate that the degrees of removal achieved in the columns are as high, if not higher, than those in field studies. Steady-state levels of DO consumption were about 50% of those observed in the field tests. In the field studies the growth substrate had to be continuously added and to alleviate the effects of competitive inhibition it was injected in alternating pulses with water containing DO or H₂O₂. The columns required only the continuous addition of oxygenated groundwater in the latter phases or groundwater with H₂O₂ during the start-up phase.

The high degrees of transformation observed in the column studies are consistent with estimates obtained in batch tests. More studies are needed to determine why such high rates of removal are achieved over extended time periods while supported by substrates that do not require the monooxygenase enzyme for their utilization. The continuous supply of energy from substrates that do not require the monooxygenase enzyme is one explanation. Competitive inhibition by the growth substrate is eliminated when growth is on alcohols, thus resulting in high rates of the cometabolic transformation. It is also possible that the hydrogel environment provides some protection from the toxicity that results from the cometabolic transformations. As shown in Figure 8.2, a continuous and high biomass concentration is maintained throughout the column, as long as DO is present. Thus, the ability to transform the contaminants is maintained through the whole spatial domain. For example, when the cometabolic substrate is continuously added to the injected groundwater, it is difficult to maintain an evenly distributed spatial biomass, even with alternate pulse injection of the substrate (Semprini & McCarty, 1991, 1992).

The results from the column tests indicate that a passive cometabolic permeable treatment barrier may be created using the co-encapsulated technology that was developed. A barrier consisting of packed beads can achieve very high removal efficiencies with residence times of less than one day. The co-encapsulated macrobeads could also be mixed *in-situ* with contaminated aquifer solids to create a passive reactive cometabolic barrier or potentially applied in an in-well reactor.

Tables and Figures

Table 8.1. Columns 1, 2, and 3 operation conditions as well as estimated parameters based on the bromide tracer tests.

	Column 1	Column 2	Column 3	Unit
Dispersion Coefficient	0.10	0.10	0.12	cm ² /hr
Column Length	15.9	14.1	15.9	cm
Cross Sectional Area	4.91	4.91	4.91	cm ²
Flow Rate	96	96	96	mL/day
Cross Sectional Area	4.91	4.91	4.91	cm ²
Superficial Velocity	4.89	4.89	4.89	cm/day
Avg. Linear Velocity	0.28	0.31	0.30	cm/hr
Mass of Packed Beads	57	49	64	g
Empty Volume of Column	78	69.2	78.0	mL
Hydraulic Residence Time	56	46	50	hr
Empty Bed Contact Time	19.5	17.3	19.5	hr
Porosity	0.58	0.64	0.52	

Table 8.2. Phases and parameters measured during the operation of more than 500 pore volumes flow through the Columns 1, 2, and 3 were packed with gellan-gum macrobeads co-encapsulated with *Rhodococcus rho* gellan-gum macrobeads.

	Pore Volume	Flow rate (mL/hr) (PVs)	H2O2 /DO (mg/L) (PVs)	Test Performed
Column 1				
Phase I	0 - 120	1.0 (0 – 10) 2.0 (10 - 57) 4.0 (57 - 120)	DO = 16 (0 - 30) H2O2 = 50 (31 - 41) H2O2 = 100 (41 - 76) H2O2 = 50 (76 - 120)	CAHs addition 250 µg/L & CAHs transformation
Phase II	120 – 220	4.0 (120 – 220)	DO = 4.2 (120 – 193) DO = 14 (193 - 220)	Stop H2O2 addition, CAHs breakthrough
Phase III	240 – 300 300 – 540	4.0 (220 – 540)	DO = 14.0	Replace Cis-DCE w/1,1-DCE 100 µg/L Increase 1,1-DCE to 250 µg/L
Column 2				
Phase I	0 - 140	1.0 (0 - 13.4) 2.0 (13.4 - 72) 4.0 (72 - 140)	DO = 16.2 (0 - 37) H2O2 = 50 (37 – 47) H2O2 = 100 (47 - 83) H2O2 = 50 (83 - 140)	CAHs addition 250 µg/L & CAHs transformation
Phase II	140 – 230 230 - 280	4.0 (140 – 280)	DO = 4.2 DO = 14.0	Stop H2O2 addition, DO 4.2 mg/L & CAHs breakthrough
Phase III	290 – 380 380 - 600	4.0 (280 – 600)	DO = 14.0	Replace Cis-DCE with 1,1-DCE 100 µg/L Increase 1,1-DCE to 250 µg/L
Column 3				
Phase I	0 - 90	4.0 (0 – 90)	DO = 14.0	CAHs addition 250 µg/L & CAHs transformation
Phase II	90 - 120	4.0 (90 – 120)	DO = 14.0	Increase CAHs to 500 µg/L, Cis-DCE & 1,4-D breakthrough
Phases III & IV	120 – 160 190 - 220	4.0 (120 – 220)	DO = 14.0	Decrease CAHs from 500 µg/L to 250 µg/L Replace Cis-DCE w/1,1-DCE 100 µg/L
Phase V	220 – 300 300 - 340	4.0 (220 – 340)	DO = 14.0	CAHs breakthrough due to 1,1-DCE Cis-DCE 250 ug/L addition only

Table 8.3. Mass balances of CAHs transformation in the Column # 3 at different pore volumes period where C/Co were close to zero.

Column Pore Volume	DO consumed mg/L	<i>cis</i> -DCE transformed (%)	1,1,1-TCA transformed (%)	1,4-D transformed (%)	1,1-DCE transformed (%)
Column 1					
10 - 20	>50	100	99.63	99.83	*NA
60- 112	>50	100	99.84	100	NA
240 - 289	>50	100	93.88	98.78	NA
400 - 450	11	0.0	95.08	91.78	99.9
Column 2					
10 - 20	>50	100	99.8	99.9	NA
60 – 113	>40	99.9	99.6	99.8	NA
240 – 290	>40	99.6	83.3	98.4	0.05
400 - 480	9	0.0	90.2	98.6	99.9
Column 3					
10 - 60	15	100	99.4	99.7	NA
60 – 90	15	99.0	99.4	99.7	NA
175 – 190	15	89.4	91.3	92.5	NA
315 - 350	15	0	92.8	97.7	NA

*NA – not added

Table 8.4. Mass balances on TBOS and T2BOS utilization at the end of column experiments

	Column 1 ^a	Column 2 ^a	Column 3 ^b
Mass of Beads (g)	56	49	64
TBOS/T2BOS added (8%) (g)	4.5	3.9	5.1
TBOS/T2BOS Total (mmol)	14.0	12.2	20.5
1- or 2-Butanol Collected @ Effluent (mmol)	0.4	0.4	0.05
TBOS/T2BOS Collected @ Effluent (mmol)	0.1	0.1	0.01
TBOS/T2BOS Remaining in Beads @ end of the Test (mmol)	7.9	6.8	20.2
TBOS/T2BOS Utilized for Co- metabolic Treatment (mmol)	6.0	5.3	0.3
TBOS/T2BOS Utilization Rate (μ mol/day)	17.7	17.9	0.9
TBOS/T2BOS Estimated Exhaustion (yrs)	2.3	2.3	61.7

^aColumn packed w/TBOS beads; ^bColumn packed w/T2BOS beads

Table 8.5. Summary of Moffett Field In-situ Experiments. Methane, Phenol and Toluene Tests Summarized by Hopkins and McCarty (1995) and Hopkins et al. 1993; Butane Substrate Tests from Semprini et al. (2007) and Semprini et al (2009); Methane Tests by Semprini et al. 1991, ^aMethane test CAH concentrations: from Semprini et al. 1991 (TCE (46 µg/L); cis-DCE (100 µg/L); *trans*-DCE(54 µg/L); and VC (44 µg/L); ^bPhenol test CAH concentrations from Hopkins et al. 1993 (TCE 500 µg/L; 1000 µg/L); ^cPhenol test CAH concentrations from Hopkins and McCarty 1995 (TCE 230 µg/L; 1,1-DCE 134; 65 µg/L); Toluene test CAH concentrations (TCE 230 µg/L; cis-DCE 119; and *trans*-DCE – 81 µg/L; VC not reported).

Primary Substrate	Primary Substrate mg/L	DO Consumed mg/L	% Removal TCE	% Removal 1,1-DCE	% Removal <i>cis</i> -DCE	% Removal <i>trans</i> -DCE	% Removal VC	% Removal 1,1-DCA	% Removal 1,1,1-TCA
Methane ^a	6.6	20	19	nd	43	90	95	nd	0
Phenol ^b	12.5	25	84	nd	nd	nd	nd	nd	0
Phenol ^c	12.5	30	94	54	92	73	98	nd	0
Toluene ^d	9.0	28	93	nd	>98	75	>98	nd	0
Butane ^e	8.0	25	nd	83	nd	nd	nd	66	70
Butane ^d	8.75	20	nd	81	nd	nd	nd	63	31

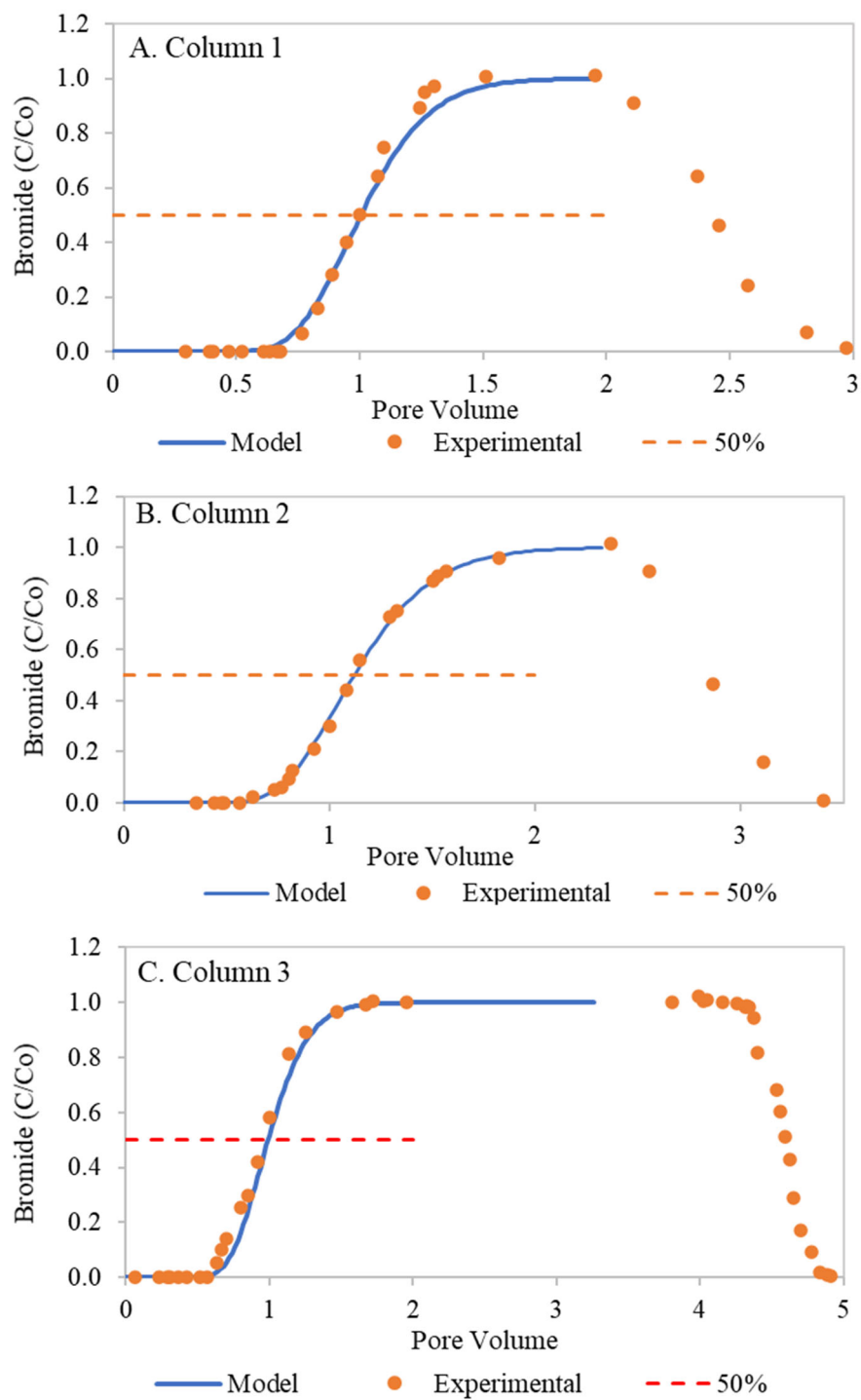


Figure 8.1. Bromide breakthrough and elution in Columns 1, 2, 3 at a flow rate of 1.0 mL/hr and the 1-D model fit.

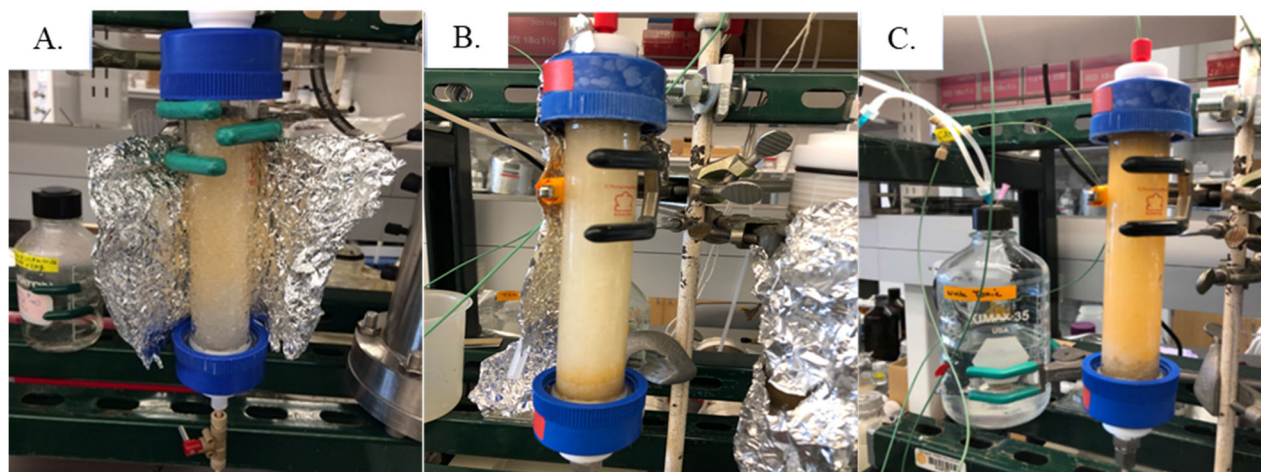


Figure 8.2. Column 1 packed with gellan-gum beads co-encapsulated with 21198 and TBOS; (a) initial column set up and before addition of H₂O₂, (b) after ~50 PV of flow and addition of H₂O₂, and (c) after ~100 PV of flow through the column with H₂O₂ addition and achieving biostimulation in the gellan-gum beads throughout the column.

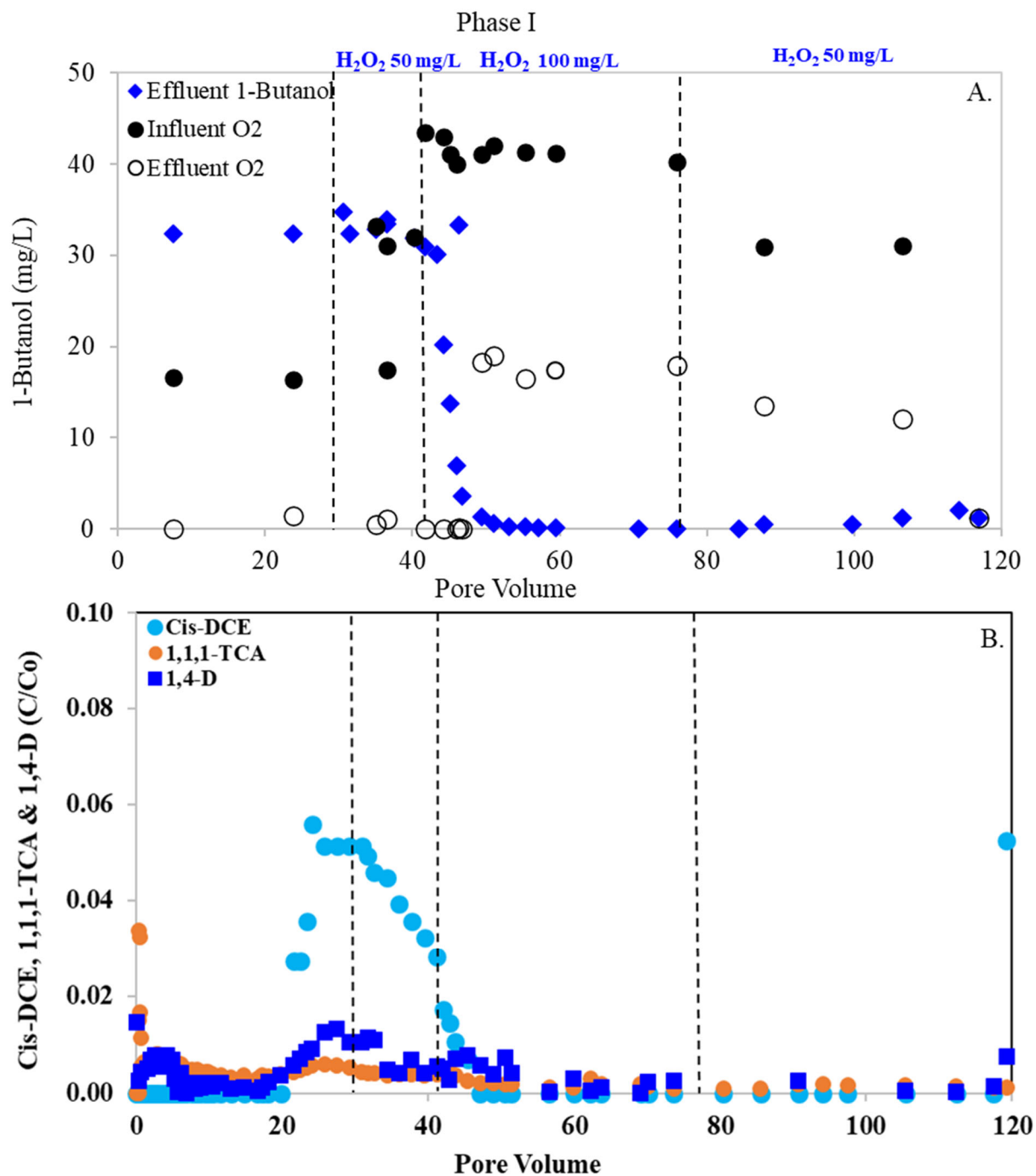


Figure 8.3. Effluent 1-butanol and DO concentrations (A) and cis-DCE, 1,1,1-TCA, 1,4-D concentrations normalized to the influent (B) during Phase I of the Column 1 study. Note the maximum scale normalized concentrations is 0.10 to illustrate the high degree of treatment being achieved.

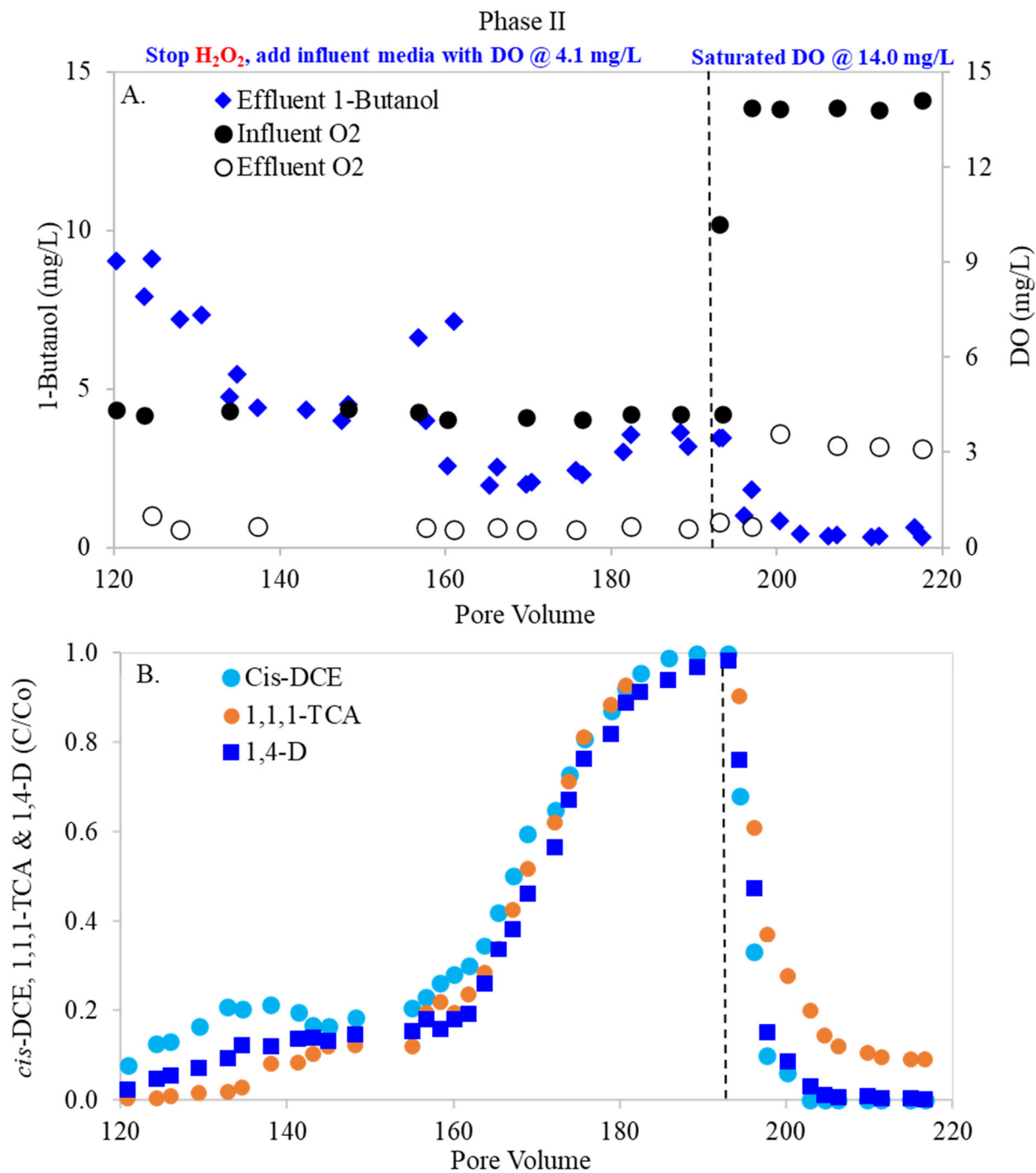


Figure 8.4. Effluent 1-butanol and DO concentrations (A) and *cis*-DCE, 1,1,1-TCA, 1,4-D concentrations normalized to the influent (B) during Phase II of the Column 1 study.

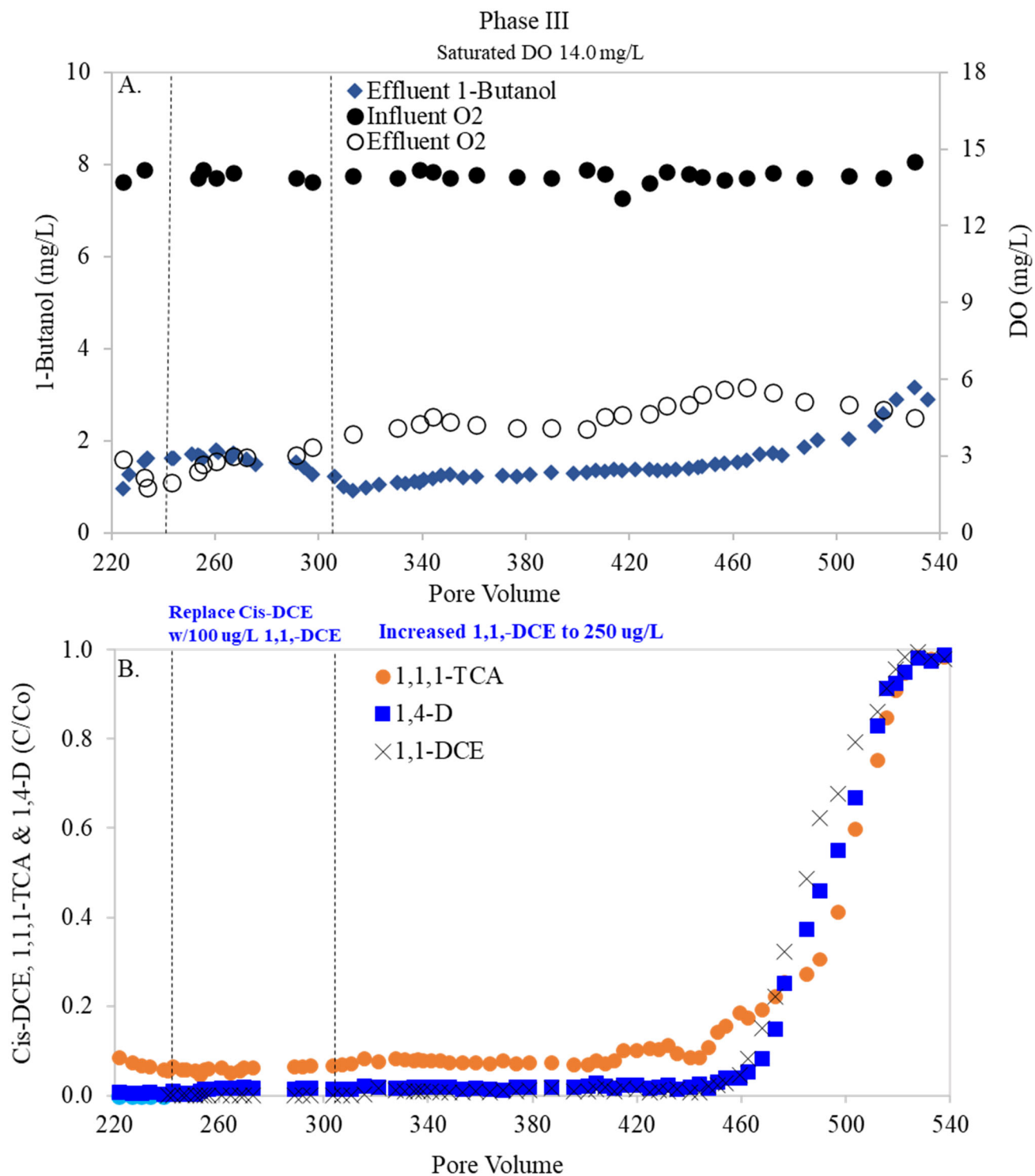


Figure 8.5. Effluent 1-butanol and DO concentrations (A) and cis-DCE, 1,1,1-TCA, 1,4-D concentrations normalized to the influent (B) during Phase III of the Column 1 study.

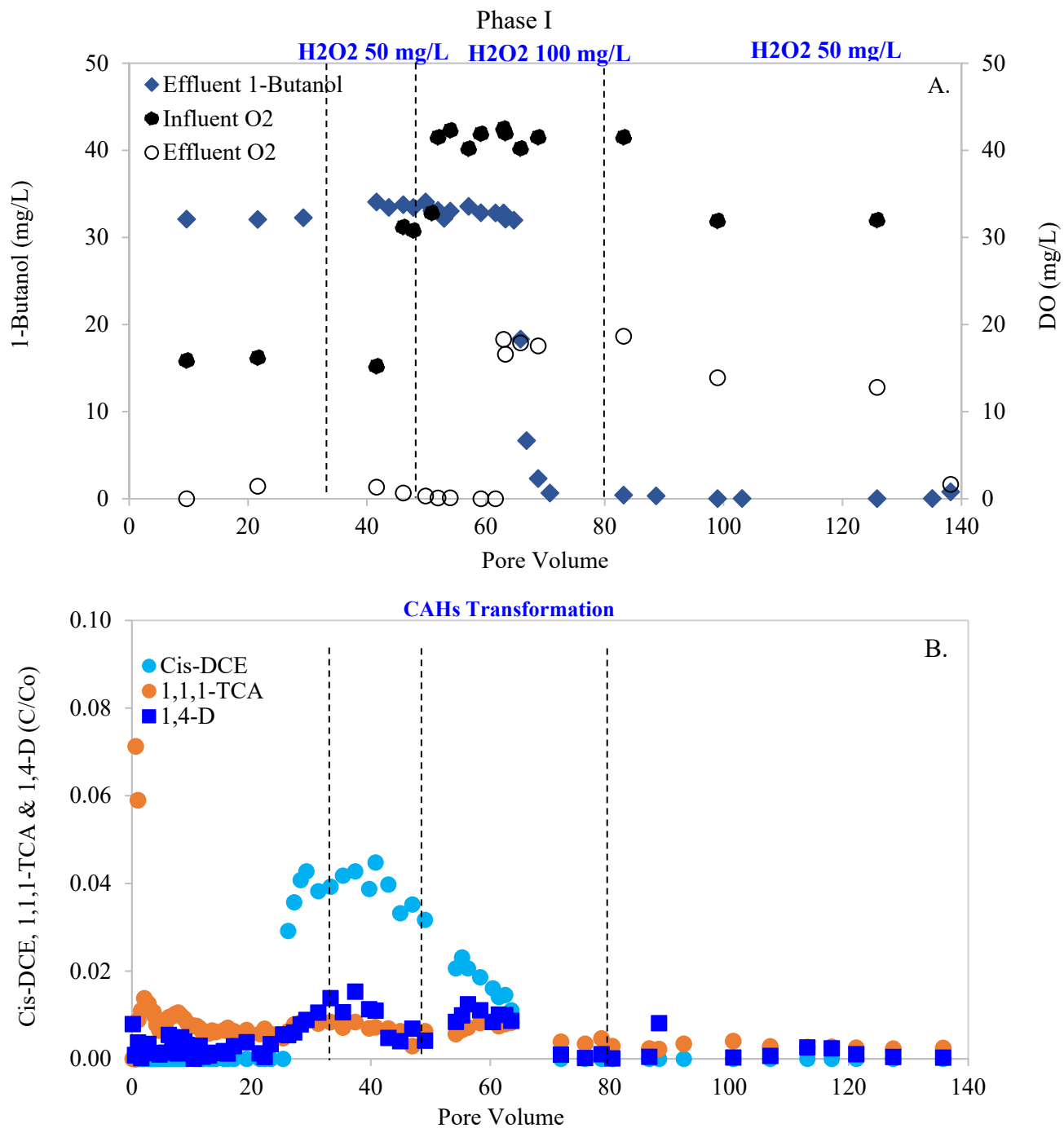


Figure 8.6. Effluent 1-butanol and DO concentrations (A) and *cis*-DCE, 1,1,1-TCA, 1,4-D concentrations normalized to the influent (B) during Phase I of the Column 2 study. Note the maximum scale normalized concentrations is 0.10 to illustrate the high degree of treatment being achieved.

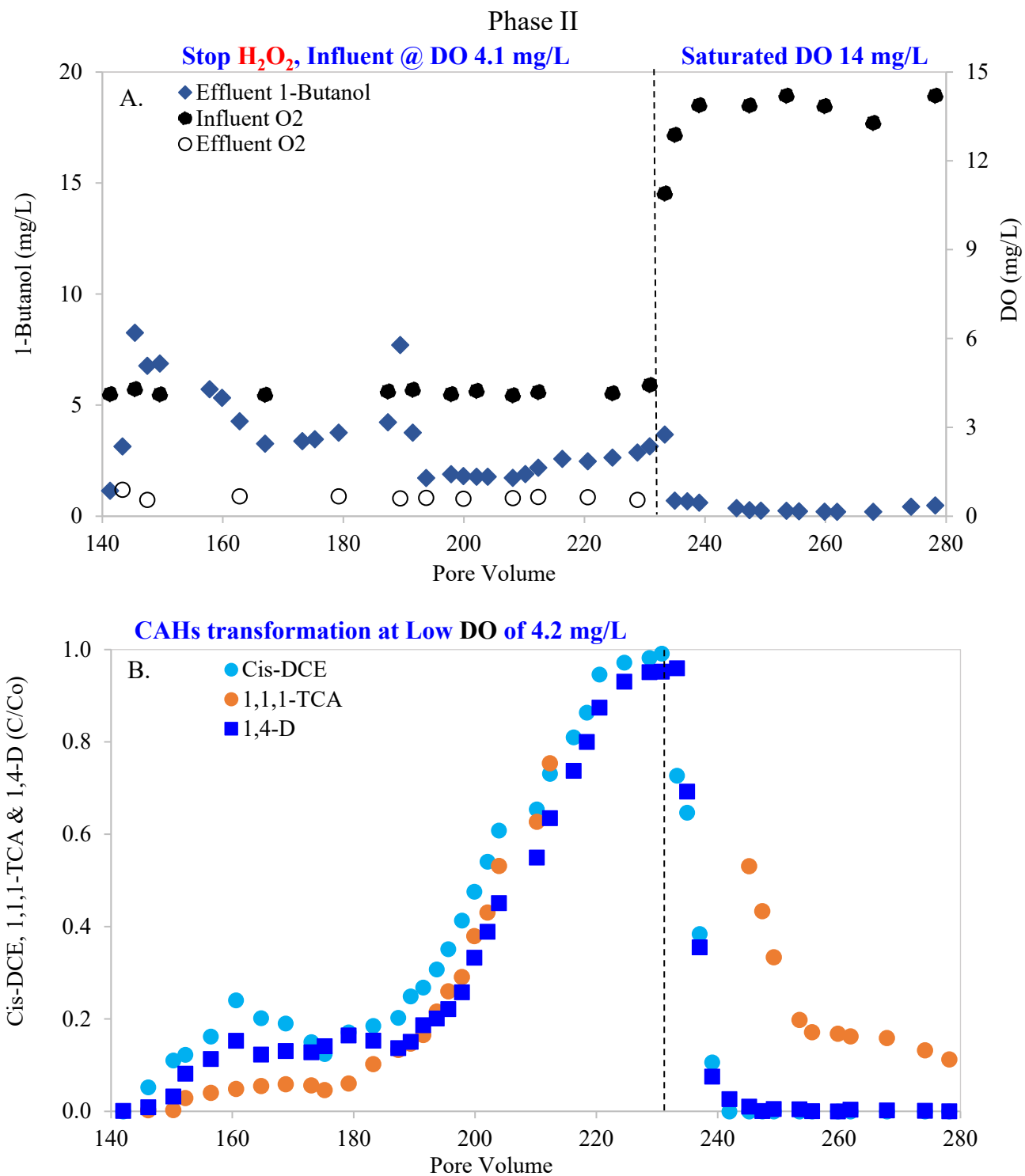


Figure 8.7. Effluent 1-butanol and DO concentrations (A) and *cis*-DCE, 1,1,1-TCA, 1,4-D concentrations normalized to the influent (B) during Phase II of the Column 2 study.

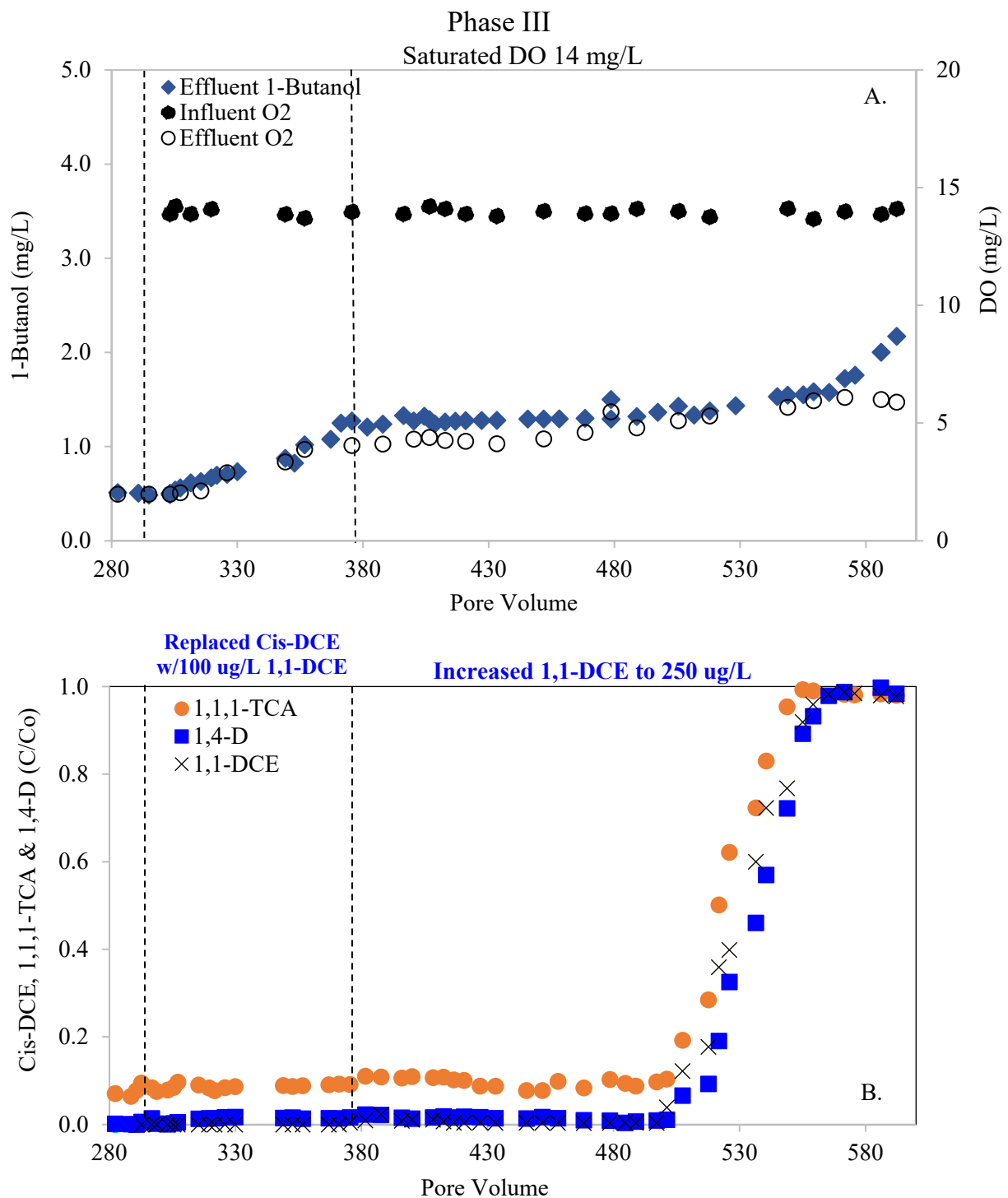


Figure 8.8. Effluent 1-butanol and DO concentrations (A) and *cis*-DCE, 1,1,1-TCA, 1,4-D concentrations normalized to the influent (B) during Phase III of the Column 2 study.

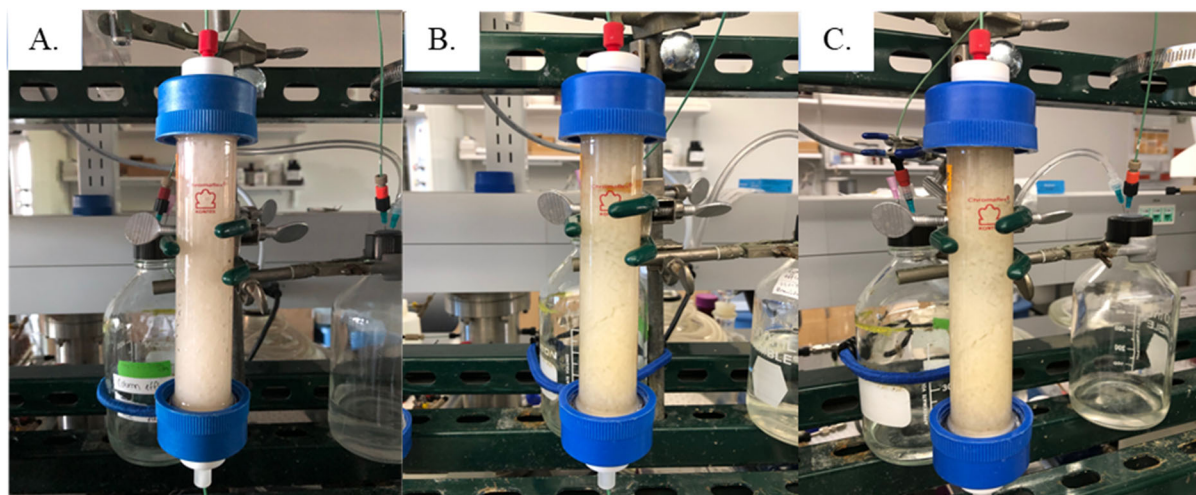


Figure 8.9. Column 3 packed with gellan-gum beads co-encapsulated with 21198 and T2BOS; (a) initial column set up and before addition of saturated O_2 , (b) after 250 PV of flow, and (c) after 480 PV of flow through the column, achieving less of an orange color indicating a lower biomass in the gellan-gum beads throughout the column compared to Columns 1.

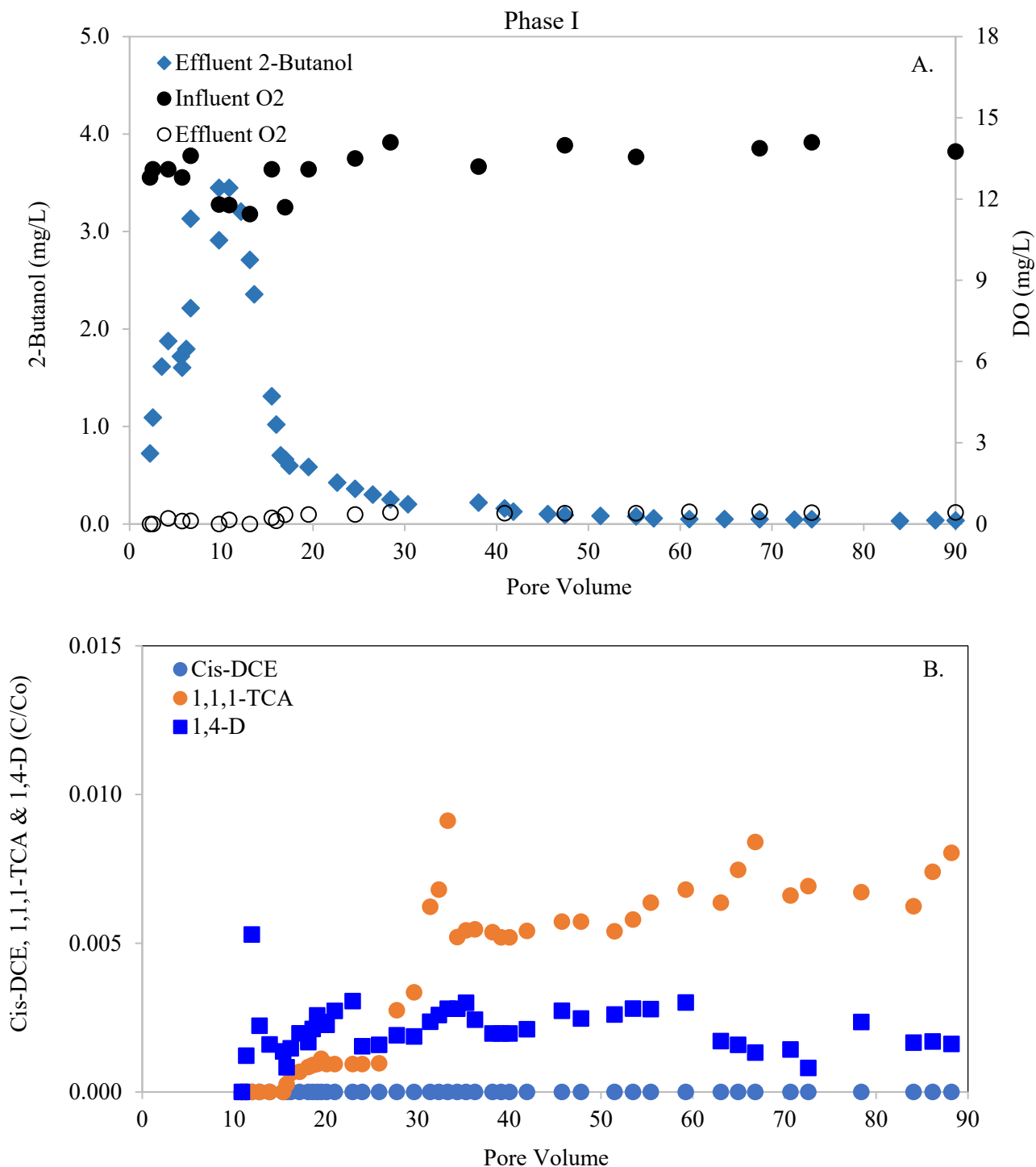


Figure 8.10. Effluent 1-butanol and DO concentrations (A) and *cis*-DCE, 1,1,1-TCA, 1,4-D concentrations normalized to the influent (B) during Phase I of the Column 3 study. Note the maximum scale normalized concentrations is 0.015 to illustrate the high degree of treatment being achieved.

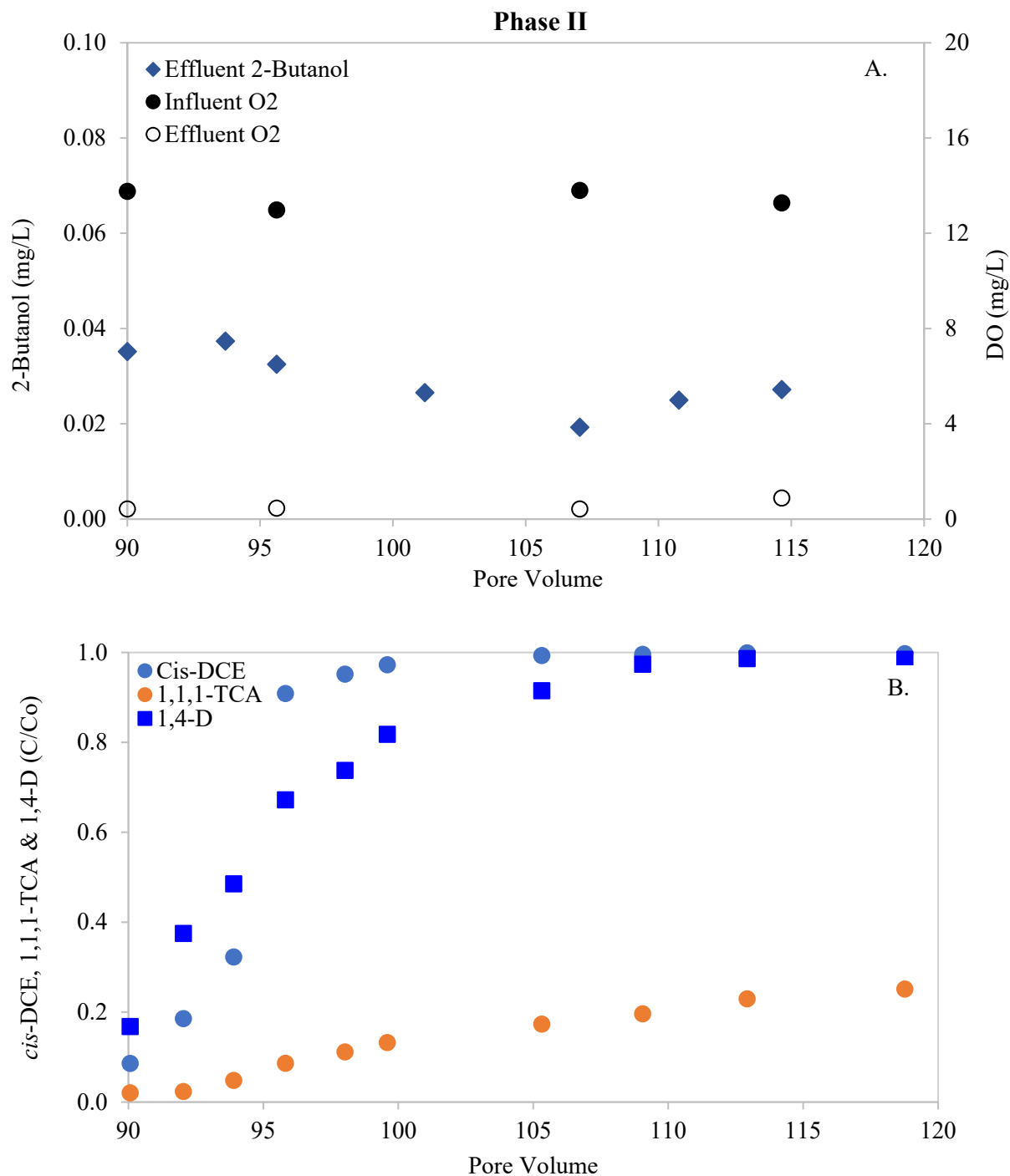


Figure 8.11. Effluent 1-butanol and DO concentrations (a) and *cis*-DCE, 1,1,1-TCA, 1,4-D concentrations normalized to the influent (b) during Phase II of the Column 3 study when the contamination concentrations were increased to 250 µg/L.

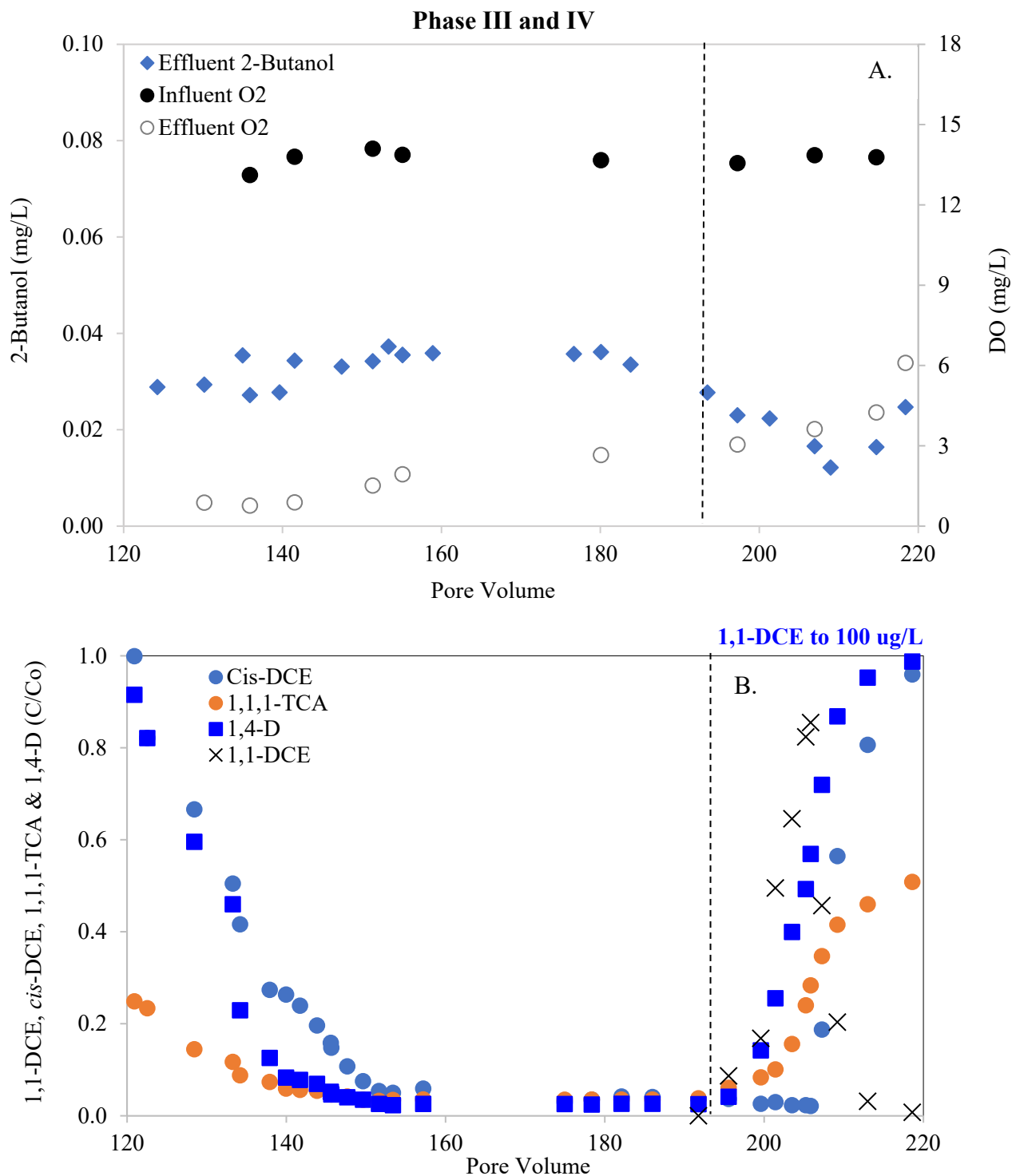


Figure 8.12. Effluent 1-butanol and DO concentrations (A) and *cis*-DCE, 1,1,1-TCA, 1,4-D concentrations normalized to the influent (B) during Phase III of the Column 3 study when the contamination concentrations were decreased to 125 µg/L and Phase IV of the Column 3 study when *cis*-DCE (125 µg/L) was replaced by 1,1-DCE (125 µg/L).

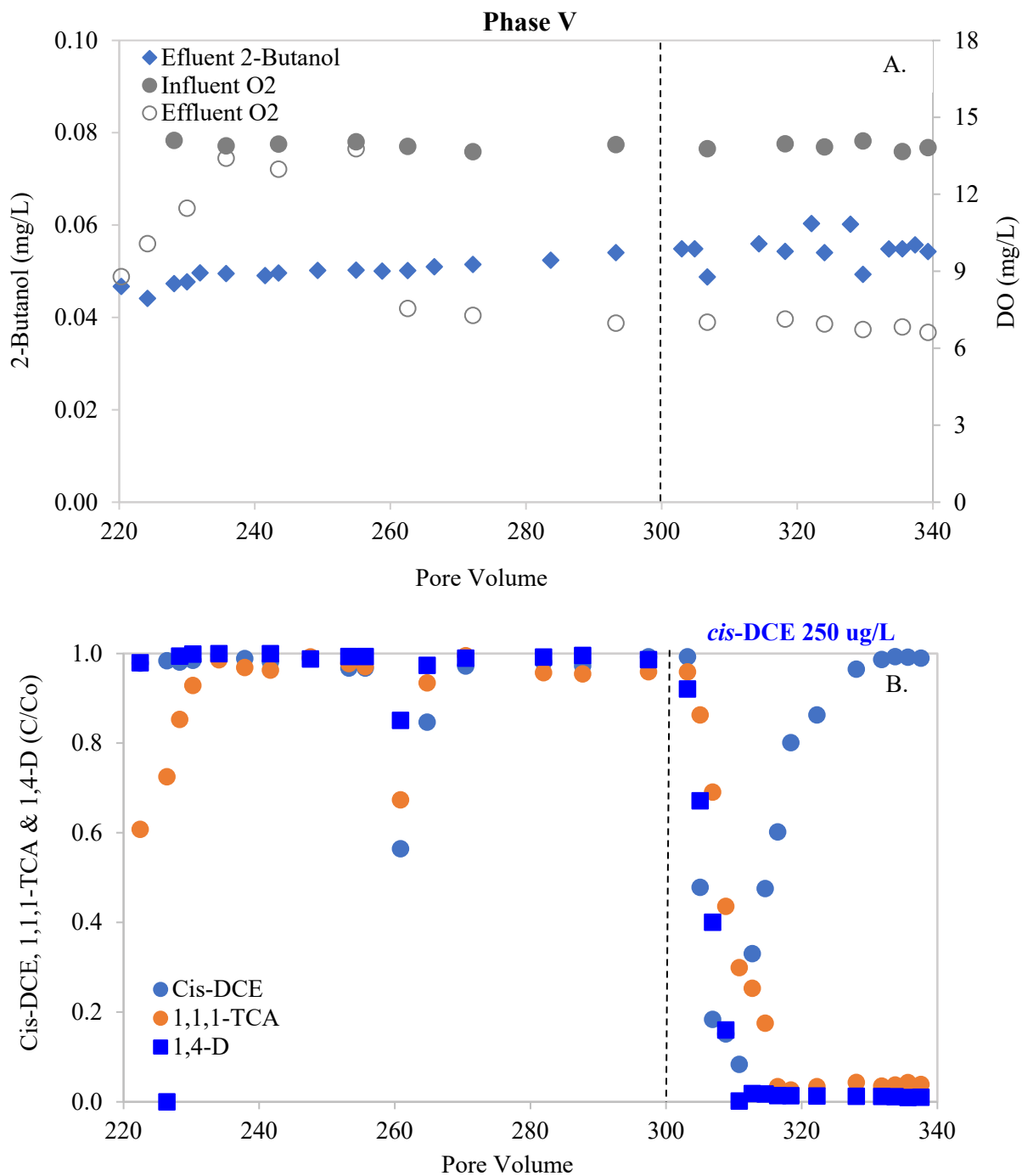


Figure 8.13. Effluent 1-butanol and DO concentrations (A) and *cis*-DCE, 1,1,1-TCA, 1,4-D, 1,1-DCE concentrations normalized to the influent (B) during Phase V of the Column 3 study when 1,1-DCE (125 $\mu\text{g/L}$) was replaced by *cis*-DCE (125 $\mu\text{g/L}$) at 205 PV and then only *cis*-DCE added to the influent at 310 PV. A stop flow kinetic test occurred at 260 PV.

Chapter 9. Evaluation of a Funnel-and-gate Cometabolic Permeable Reactive Barrier in a Physical Aquifer Model

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Abstract

The results from the PAM study indicate that a CBRB can be constructed using the co-encapsulated gellan-gum beads. A Funnel-and-Gate treatment system was created in a PAM to test this concept. In this initial test, only the first Bioreactor RB1 was packed with gellan-gum beads co-encapsulated with TBOS (8%) and *R. rhodochrous* 21198. Prior to packing in the PAM, the gellan-gum beads were stored several months at 4° C in a carbonate buffer. The gellan-gum beads were emplaced into the RB1 Bioreactor by the injecting a slurry to mimic how this might be performed in practice. The CPRB test was conducted by creating a uniform flow field through the injection and extraction of synthetic groundwater. H₂O₂ was added as a source of DO. Biostimulation of the gellan-gum beads was indicated by the gradual consumption of 1-butanol to below detection in both the Bioreactor RB1 and the effluent of the PAM. Bromide tracer tests indicated that some of the synthetic groundwater was by-passing the Funnel-and-Gate treatment system. Monitoring of 1-butanol in groundwater from the Bioreactor RB1 showed low concentrations present with long-term operation of the PAM test. Isobutene added as a reactive surrogate was effectively transformed to isobutene oxide during transport through the Funnel-and-Gate RB1 Bioreactor. Isobutene oxide concentrations, near the maximum isobutene concentration (100 µg/L) injected, were observed and isobutene was consistently below 1 µg/L. Little isobutene oxide was observed in the effluent of the PAM, which may indicate it was transformed downgradient of the RB1 Bioreactor. Some isobutene (~ 0 to 12 µg/L) was observed in the PAM effluent, which likely is associated with groundwater that by-passed the Funnel-and-Gate CPRB. Incubation of groundwater samples obtained from the Bioreactor RB1 showed significant isobutene cometabolic activity, indicating that some breakdown of the gellan-gum beads likely occurred. The high activity within the RB1 and funnel-and-gate indicates that the original beads or small particles of beads have remained cometabolically active for over 170 days. The PAM test will continue under an NIEHS grant awarded to the PI. Continued monitoring of the current experiment will help establish the long-term performance of Funnel-and-Gate CBRB. The amount of groundwater that is potentially by-passing the PAM will be determined through continued monitoring the PAM effluent.

Introduction

PRBs for the in-situ groundwater treatment of legacy and emerging contaminants can reduce low cost and be more sustainable than current remediation methods. In the method proposed here, costs would be eliminated to extract groundwater or continuously add substrates, electron acceptors, and nutrients for cometabolic treatment. Scherer et al. (2000a) reviewed PRBs for groundwater treatment using chemical and biological methods. Upadhyay and Sinha (2018) reviewed PRBBS for environmental clean-up. Scherer et al. (2000) discuss the three important characteristics for effective treatment by PRBs including: 1) the ability of the immobilized material to transform the contaminant at a rapid rate; 2) the ability of the barrier to maintain adequate

permeability and 3) the ability only to release benign compounds because of the treatment process. For the treatment of CAHs, they found that 45% of the PRBs were reductive processes using zero-valent iron (ZVI). However, ZVI alone is not effective for the remediation of 1,4-D (Mohr et al., 2010). 1,4-D treatment with ZVI requires using strong oxidizing agents including peroxides (Y.-G. Kang et al., 2018) and slow release persulfate (Kambhu et al., 2017). 1,4-D is not transformed under biologically enhanced reductive dehalogenation conditions created by ZVI barriers (McElroy et al., 2019).

PRBs for aerobic cometabolism have received little attention, since the continuous addition of substrate is required to drive the process along with oxygen. One potential method that was tried was the injection of resting cells for their cometabolic transformation capabilities (Duba et al., 1996), which had limited success and would require the constant addition of cells to the subsurface (Semprini, 2013).

Based on the results of the 1-D column studies presented in Chapter 8, we found that very high levels of treatment of a mixture of 1,4-D, 1,1,1-TCA, and *cis*-DCE was achieved in packed columns that contained gellan-gum beads co-encapsulated with *R. rhodochrous* ATCC 21198 and TBOS and T2BOS. Over 99% removal was achieved with an HRT of 12 hours in the columns, with long-term treatment observed for over 9 months. The results of the column studies indicated these hydrogel beads could be used to construct a CPRB. HRT within the CPRB of less than one day might only be needed to achieve continuous treatment of the COC mixture of interest.

The approach used here was to create a Funnel-and-Gate CPRB within a large-scale physical aquifer model (PAM). The PAM and Funnel-and-Gate treatment system was constructed so groundwater would be channeled in the CPRB, with the permeability of the gellan-gum bead pack being greater than the permeability of the PAM's sand pack. The Funnel-and-Gate would channel the contaminated groundwater into the gellan-gum bead pack. The Funnel-and-Gate, however, was not constructed over the entire width of the PAM so that groundwater could bypass the CPRB, and flow around it. If the CPRB permeability decreases with time then greater amounts of groundwater would be expected to bypass the Funnel-and-Gate treatment system.

The conceptual model of the Funnel-and-Gate CPRB treatment system is shown in Figure 9.1. The Funnel-and-Gate CPRB is placed towards the center of the PAM. The winged gates direct groundwater into the bioreactors packed with the co-encapsulated gellan-gum beads. The winged gates are placed so that there is space between the walls of the PAM so the groundwater could bypass the Funnel-and-Gate, resulting from the loss of permeability in the Bioreactors over time. Three 0.5-liter cylinder bioreactors were emplaced in the PAM. The cylinder configuration was used to represent large well bores that might be used to construct a PRB. To provide flexibility, three potential bioreactors were placed in series, however in the initial system only the first reactor was filled with gellan-gum beads co-encapsulated with *R. rhodochrous* ATCC 21198 and TBOS.

Only the upper half of the PAM was used in the experiments. A bentonite clay layer was emplaced that divided the PAM into an upper and lower aquifer. The bentonite clay would also potentially serve as a potential layer for the back diffusion of contaminants. Dividing the PAM aquifer into two layers also permits the evaluation of other forms of in-situ treatment, such as recirculation wells that circulate contaminated between the aquifers. An in-well reactor with the co-encapsulated gellan-gum beads could be tested in such a system.

A study was initiated to evaluate the potential of creating a CPRB using a PAM. There were several objectives for the study, including: creating a Funnel-and Gate- treatment system that

channels groundwater into the CPRB; creating a series of CPRBs that were filled with co-encapsulated beads; developing uniform flow conditions by continuously adding synthetic groundwater to the inlet of the PAM and continuously extracting groundwater from the end of the PAM; adding isobutene as a reactive surrogate for the COCs of interest that *R. rhodochrous* ATCC 21198 will cometabolize to the epoxide, isobutene oxide, which can be easily tracked; and in the later stages testing COC mixtures of 1,4-D, *cis*-DCE and 1,1,1-TCA. A long-term study would determine if high levels of treatment could be maintained in the CPRB. The monitoring concentrations within and outside of the Bioreactors, would determine how effective was the cometabolic treatment within the CPRB. Monitoring the effluent concentration will provide information on the degree of treatment achieved and the interaction of the contaminated groundwater with the CPRB. Temporal changes that occur would be an indication of groundwater bypassing the Funnel-and-Gate CPRB.

Methods

The PAM, shown in Figure 9.1 and 9.4, is 100 cm long, 50 cm tall and 20 cm wide, with a total volume of 100 L. It is constructed of anodized aluminum with inlet and outlet end plates engineered to help distribute flow and create a uniform flow field. The physical model was previously used in detailed fluid transport studies described by Vogler et al. (2018).

The PAM was packed with sand to construct two different aquifers separated by a bentonite clay layer. The upper aquifer was packed with 20 to 30 mesh accusand, while the bottom was packed with a 40 to 60 mesh accusand. Prior to packing, 100 mesh stainless steel screens were added to the influent and effluent walls of the PAM in order to prevent clogging of the influent and effluent lines. Accusand is a quartz-based sand of ASTM grade. The PAM was packed wet by slowly adding 250 ml of sand in vertical layers using a 500 ml beaker. The water level in the PAM was gradually raised to maintain a wet pack throughout the course of packing. After packing approximately 50 L of the bottom aquifer with 40 to 60 mesh sand, a ~ 0.5-inch-thick layer of bentonite clay layer was installed that covered the complete surface area of the PAM. The clay layer served to create two different aquifers and also to provide a low permeability layer from which contaminants could back diffuse back during prolonged experiments. After the addition, the clay was permitted to harden overnight prior to the packing of the upper aquifer with 20-30 mesh accusand that contained the funnel-and-gate treatment system. The top of the clay is shown in Figure 9.2.

The Funnel-and-Gate treatment system, as it was emplaced in the upper aquifer of the PAM, is shown in Figure 9.2. The Funnel-and-Gate consisted of winged panels to channel groundwater into the Bioreactors. The winged panels were essentially two HDPE walls at 45-degree angles that point into the bio-reactors. The Funnel-and-Gate is 10 inches high with an internal inlet (the gate) of 2 inches into the first Bioreactor (RB1). There was 1.5" of clearance on each side of the PAM that permits groundwater to by-pass the CPRB, if there was a loss in permeability in the bioreactors with prolonged treatment. Three bioreactors that could be packed with co-encapsulated gellan-gum beads were 2 inches in diameter and 10 inches high, with stainless-steel filter screens with HDPE housing (Grainger). Each-reactor has an internal volume of 0.5 L. The stainless filter screens provided a low cost means of creating the bioreactors, and potentially represented a large-wellbore method of installing the CPRB in the field.

Prior to installing the reactors in the PAM, they were tested in the lab to show that the screens kept the aquifer sands from entering the Bioreactors. Three reactors were placed vertically

in series. Having three reactors in series would potentially permit installing different gellan-gum co-encapsulated beads. For example, the first bioreactor could be packed with gellan-gum beads with TBOS, to treat high COC concentrations, followed by the second reactor containing gellan-gum beads with T2BOS, to act as a polishing step. The third bioreactor could be packed with beads encapsulated only with *R. rhodochrous* ATCC 21198. This reactor would remove alcohols that might be released from the first two reactors and could serve as a final polishing step for treating the COCs.

The Funnel-and-Gate CPRB was keyed into the bottom clay layer, so that contaminated groundwater would not flow under it. The top of the funnel-and-gate was also keyed into the top of the PAM, since the upper aquifer was operated as a confined aquifer. After the funnel-and-gate was emplaced in the PAM, the upper aquifer was then packed with 20 to 30 mesh accusand, using the same wet packing procedures. Figure 9.3 shows the PAM after packing and prior to placing the top lid onto the PAM. The sand was packed to the top so that upon placing the cover the sand would completely fill the PAM and a confined aquifer could be created with a uniform flow field prior to entering the Funnel-and-Gate CPRB. The completely packed PAM is shown in Figure 9.4. On the right side of the PAM is shown the manifold that was used to inject the synthetic groundwater and create a uniform flow field.

Gellan-gum beads co-encapsulated with *R. rhodochrous* ATCC 21198 and 10% (w/w) tetrabutyl - orthosilicate (TBOS) were packed into the first Bioreactor (RB1) in the series after the lid had been emplaced on the PAM. The PAM was completely saturated with synthetic groundwater, so the hydrogel beads were packed into the 0.5-L Bioreactor as a slurry through a ½ inch I.D. port in the top of the RB1 Bioreactor. The gellan-gum beads had been fabricated using the procedure previously described in Chapter 7. The method was scaled-up to produce the large quantity of beads (1L). In previous studies the maximum amount of beads fabricated was ~ 100 ml. The hydrogel gellan-gum beads were fabricated several months prior to the addition to the CPRB and stored in a carbonate buffer at 4° C.

The gellan-gum beads were added to the first Bioreactor (RB1) in the series as a slurry through 3/8" tygon tubing. A 100-ml plastic syringe was used to inject the slurry into the bioreactor. Figure 9.5 shows the process of packing the gellan-gum beads into RB1. Since the gellan-gum beads are slightly less dense than water the beads rose to the top of RB1, which was gradually packed from the top to the bottom. A high resistance to injection was achieved after the addition of about 1.5 liters of the slurry, indicating that the RB1 Bioreactor was completely packed. The ability to move the tygon tubing through the bead pack, however, indicated that we did not achieve a high packing density with the slurry method that was used.

Experiments were performed by continuous injection of synthetic groundwater to create a uniform flow field. The injection of synthetic groundwater was achieved using a continuous injection ISCO syringe pump (Teledyne ISCO) shown on the left of Figure 9.6. Flow rate through the PAM was established by injecting 2 mL/min or 2.88 L day of synthetic ground water with about 100 ppm calcium cation to help keep the gellan-gum macro beads from dissociation. The composition of the synthetic groundwater is provided in Table S9.1. It contained low concentrations of ammonia ion (0.68 mg/L) and nitrate (2.3 mg/L) and phosphorus (0.045 mg/L) as minor nutrients.

The synthetic groundwater was stored in a 25-L plastic carboy, which served as reservoir for the syringe pump. H₂O₂, as a source of DO, was added directly to the carboy to achieve an

influent concentration of 50 mg/L. A syringe pump, equipped with two 100-ml gas-tight syringes, was operated continuously at a flow of 0.5 mL/hr to add the bromide tracer and isobutene as a reactive surrogate. The syringe pump will also be used to add the 1,4-D, *cis*-DCE, and 1,1,1-TCA. The concentrations of the bromide and isobutene were adjusted, so that upon mixing, with the high flowrate ISCO syringe pump, the desired influent concentrations were achieved.

The synthetic groundwater that was amended with the bromide tracer, H₂O₂ and isobutene as a reactive surrogate was added to the PAM through a manifold to create a uniform flow field. This manifold is shown in the bottom of the left in Figure 9.6. Since only the top aquifer was used for the funnel-and-gate CPRB, the synthetic groundwater was added only to the upper aquifer. The effluent of the PAM had a similar manifold to collect the synthetic groundwater exiting the PAM (Figure 9.6 far right). Having identical influent and effluent manifolds helped establish a uniform flow field through the PAM.

The synthetic groundwater was sampled at selected locations in the PAM using dedicated sampling locations. Figure 9.2, shows the installation of sample tubing (green tubing) along the centerline of the PAM. On and off valves located outside the PAM permitted access to the sample tubing within the PAM. Sampling ports located in the top cover of the PAM permitted sampling within the Bioreactors. Sample ports on the outside of the wings of the funnel-and-gate permitted samples to be obtained of groundwater that bypassed the funnel-and-gate system. Groundwater samples were obtained by connecting a 2-mL or 5-mL plastic syringe and gently filling the syringe to obtain an all liquid sample. The sample lines were purged by discarding the first 1-mL of sample that was collected. The samples were added to 2-mL glass vials sealed with Teflon caps. The vials were stored on ice for transport to our analytical laboratory for analysis. DO was determined on site using an oxygen probe using a 2-ml sample.

Figure 9.7 shows sampling locations used in the PAM experiments. Samples include the influent groundwater, four locations along the center line of the PAM (10, 20, 28, and 35 inches from the influent, that includes a sample in the in Reactive Barrier 1 (RB1) at 20 inches, and at the sides of the wings (20 inches), to investigate short circuiting around the Funnel-and-Gate treatment system. An effluent sample from the PAM represents an integrated sample of the total flow through the PAM. All samples were obtained at mid-depth in the PAM at 6 inches depth.

Analytical methods previously described were used to monitor the aqueous concentrations of bromide (IC), isobutene (GC-FID headspace analysis, dissolved oxygen (DO probe); 1-butanol (GC-MS purge-and-trap) and isobutene oxide (GC-MS purge-and-trap). In later stages of the test 1,4-D, *cis*-DCE, and 1,1,1-TCA concentrations will be determined using method previously described.

Provided in Table 9.1 are continuous operating conditions of the PAM and the Funnel-and-Gate treatment system. At an operating flowrate of 2.88 L/day, the HRT in the PAM is estimated to be 5.21 days assuming a packed porosity of 0.30. The HRT in the Bioreactor RB1 is estimated to be 0.15 days (3.6 hours). This HRT estimate assumes all of the groundwater flow goes through the Funnel-and-Gate treatment system. Calculations of the HRTs are provided in the Table S9.2 in Appendix A.

Table 9.1. Continuous operating parameters of the physical aquifer model (PAM). Isobutene injection started on day 149. H₂O₂ is added to maintain DO levels.

Continuous Operating Parameters	
Active Model Volume [L]	50
Bio-Reactor Volume [L]	0.5
Model Flowrate [L/day]	2.88
Sand Porosity [-]	0.3
Gellan-gum Bead Pack Porosity [-]	0.7
Model Retention Time [days]	5.21
Bio-Reactor Retention Time [days]	0.15
Saturated Isobutene Injection [mL/day]	12
H ₂ O ₂ addition to feed stream [ppm]	50
Inlet Feed Pump	Teledyne ISCO D-Series 500(2) Syringe Pump
Isobutene Injection Pump	Thermo Sage M361 Syringe Pump
Sand Pack	20-30 mesh Accusand

Results

Batch kinetic tests

The study in the PAM used isobutene as a reactive surrogate for the COCs of interest. Studies with *R. rhodochrous* ATCC 21198 grown on isobutane showed that isobutene could be used as a reactive surrogate to study the cometabolism of the COCs of interest. Since the cometabolism of isobutene produces an epoxide, isobutene oxide, the production of isobutene oxide can be tracked, providing direct evidence that cometabolic transformation is occurring.

Shown in Figure 9.8 are the results of a batch resting cell test with *R. rhodochrous* ATCC 21198 grown on isobutane. A mixture of 1,4-D, 1,1,1-TCA, *cis*-DCE, 1,1-DCE and isobutene is shown to be cometabolically transformed. Isobutene is transformed to isobutene oxide at a rate similar to 1,1-DCE, over period of 0.02 days (0.5 hours). The graph at the right presents later time data showing the slower cometabolic transformation of 1,4-D, 1,1,1-TCA, and *cis*-DCE. The results also show that isobutene oxide is also being transformed. The rapid transformation of isobutene indicates it should be effectively transformed when transported through the Funnel-and-Gate CPRB system. Since it is more rapidly transformed than *cis*-DCE, 1,1,1-TCA, and 1,4-D it would likely over predict the transformation of these COCs in the CPRB system. However, it would likely predict the transformation of 1,1-DCE that might be achieved.

The Bioreactor RB1 was packed with gellan-gum beads that co-encapsulated TBOS and *R. rhodochrous* ATCC 21198. After fabrication, these beads were stored for several months at 4° C prior to emplacement in the RB1 Bioreactor. To test the activity of the beads used to create the Bioreactor in the PAM, a batch kinetic test was performed with a sample of the beads. The kinetic test was performed near the time the beads were emplaced in the PAM Bioreactor RB1. The batch kinetic tests were performed using the sample procedures as described in the methods of Chapter 7. The only difference was 3 grams of beads were added to each reactor, while 2 grams were used in previous studies. Each reactor contained a cell mass loadings of 0.5 mg TSS/gram bead and

initially contained 100 mL of carbonate buffered media, with an air headspace as a source of oxygen.

Figure 9.9 presents the results from the batch kinetic tests. The results indicate a fairly long period of incubation prior to the rapid transformation of *cis*-DCE and 1,4-D. When transformation was not observed after 15 days, the TBOS1 reactor was fed isobutane to stimulate the monooxygenase enzyme. This resulted in the earlier transformation in the reactor TBOS1 compared to TBOS2, which was not fed isobutane. However, with prolonged incubation effective transformation was also observed in the TBOS2 reactor. After 60 days of incubation very rapid transformation of both *cis*-DCE and 1,4-D was observed in both reactors. The production of *cis*-DCE epoxide was also observed indicating cometabolic transformation was occurring. The results support the growth of the *R. rhodochrous* ATCC 21198 within the beads, with rapid transformation after 60 days of incubation. The results indicate that there no need for stimulation of the bead pack added to the PAM with isobutane, since rapid cometabolism was observed in the T2BOS reactor without adding isobutane.

Results from the Funnel-and-Gate CPRB PAM tests.

After the gellan-gum beads were emplaced in the PAM, H₂O₂ addition to the influent synthetic groundwater (50 mg/L) was initiated and the injection flowrate was set to 2.88 liters/day. Monitoring of the DO and the 1-butanol in the PAM effluent and the bulk synthetic groundwater of the Bioreactor (RB1) commenced. Shown in Figure 9.10B is the DO and 1-butanol in the effluent of the PAM. DO gradually depletes over a period of about 30 days, while 1-butanol concentrations rise and reach a maximum concentration between 20 and 30 days, and then begin to decrease to below detection. The results are consistent what is expected with the biostimulation of *R. rhodochrous* ATCC 21198 within the RB1 Bioreactor. The peak concentration is delayed compared to the RB1 reactor Figure 9.10A, due to the required transport from RB1 to the effluent of the PAM. The reduction of the 1-butanol concentration to below detection at later time indicates very effective biostimulation of *R. rhodochrous* ATCC 21198 within the beads. The low DO in the effluent from the PAM indicates very effective utilization of DO that is being added to the influent groundwater.

Figure 9.10A shows the concentration history of 1-butanol and DO from the RB1 Bioreactor (RB1). Very high 1-butanol concentrations were observed soon after the emplacement of the gellan-gum beads in RB1, with a maximum concentration observed of 120 mg/L after 7 days. Concentrations began to gradually decrease over the next 30 days and were below detection after around 45 days. The decrease occurs sooner than that observed in the PAM effluent, since RB1 is located up gradient. DO is also shown to be reduced to very low levels as a result of DO consumption within the RB1. The results are consistent with the response observed in the column experiments present in Chapter 8. In the column studies the maximum 1-butanol concentration reached was approximately 30 mg/L and began to decrease over a period of about 10 PV of flow. The decrease of 1-butanol with time observed in the PAM is also consistent with the time scale for biostimulation observed in the column study. The higher maximum concentration observed in the PAM could have resulted from a number of factors including the storage of the beads for extended period resulting in a release of 1-butanol via hydrolysis, and slower flow through the bioreactor compared to the laboratory columns.

It is interesting to note that the maximum 1-butanol concentrations observed in the PAM effluent of 4 mg/L are much lower than the maximum observed of 120 mg/L in the RB1 Bioreactor

The results potentially indicate biological transformation is also occurring down gradient of the RB1 bioreactor. A dilution in concentration could also be occurring due to short circuiting of groundwater around the Funnel-and-Gate treatment system. However, dilution estimates would indicate that only around 3 to 4 % of the groundwater is flowing through the Funnel-and-Gate, which is not likely occurring. Observations from the later bromide tracer tests and isobutene reactive tracer indicate biostimulation likely occurred down gradient of the RB1 Bioreactor. The DO concentration is higher in the effluent of the PAM than in the RB1 bioreactor, which also indicates some short-circuiting of groundwater around the Funnel-and-Gate treatment system.

Isobutene Surrogate Reactive Tracer Test

The isobutene surrogate tracer test was initiated approximately 149 days after the gellan-gum beads were emplaced in the RB1 Bioreactor. During the period of 149 days synthetic groundwater was continuously added to the PAM along with H₂O₂ (50 mg/l). Bromide tracer tests were also initiated along with the addition of the isobutene.

Figure 9.11 presents the results of the bromide tracer tests and breakthrough observed around the Funnel-and-Gate treatment system. The locations of where the breakthroughs were measured is shown in the right of Figure 9.11. Bromide breakthrough was observed at all locations, with more rapid breakthrough observed at the Right sample location which is outside wing of the Funnel-and-Gate compared to the Left sample location, also outside the left wing. Bromide breakthrough at the RB1 location was in between the two locations. The results indicate that not all of the flow is going through the Funnel-and-Gate treatment system, and significant flow could be short circuiting on the Right side. The transport times for 50% breakthrough are 5.5 days (Right), 6 days (RB1) and 7.5 days (Left). The breakthrough times observed are consistent with the PAM packing having a higher porosity than the 30% used in HRT estimates (Table S9.2). It should also be noted that in bromide tracer tests in columns packed with beads (data not shown), a retardation in transport was observed, due to the diffusion of bromide into the beads, which represents an immobile transport zone. Thus, the slower breakthrough of the bromide tracers in RB1 compared to the Right sampling locations, could also be associated retarded transport through the Bioreactor (RB1).

Figure 9.12 presents the breakthrough in the bromide tracer in the effluent of the PAM. A longer time to breakthrough was observed as well as much more dispersion compared to the upgradient locations (Figure 9.11). The greater dispersion is likely a result of the mixing of groundwater from different paths through and around the Funnel-and-Gate treatment system. A longer HRT in the PAM is indicated by the bromide tracer test, compared to calculations that assumed a porosity of 30% (Table S9.2), indicating a higher porosity in the PAM.

Figure 9.13A presents the DO and 1-butanol concentrations in the RB1 during the isobutene surrogate tracer test. Low 1-butanol concentrations were maintained consistent with the observations presented in Figure 9.10 obtained in the earlier biostimulation phase. DO concentrations in the range of 2 to 4 mg/L were observed. The DO concentrations were also higher than concentrations observed in the earlier biostimulation phase of the experiment. This result is consistent with the column studies presented in Chapter 8, which showed less oxygen demand occurred with time. The results indicated significant DO utilization with flow through the Bioreactor RB1. The results show bioactivity in the RB1 reactor for 170 days since gellan-gum beads were placed in the RB1 Bioreactor.

Figure 9.13B shows the 1-butanol and DO in the PAM effluent over the same period. The DO is higher (6 to 8 mg/L) compared to those observed in the RB1 Bioreactor (3 to 6 mg/L), which is consistent with some groundwater bypassing the Funnel-and-Gate treatment system. The 1-butanol concentrations are below detection, lower than observed in RB1, indicating mixing is occurring and possibly utilizing down gradient of RB1 Bioreactor.

The results of the isobutene surrogate test are shown in Figure 9.14. The isobutene concentration of the influent synthetic groundwater (A) shows some variability in the influent groundwater added to the PAM. The experiment was designed to have an influent concentration of 100 $\mu\text{g/L}$, which was achieved during the early phase of the experiment. The average injection concentration during the first 5 days of the test was $\sim 85 \mu\text{g/L}$. Lower concentrations at later times were associated with a change in the influent that contained less isobutene.

The concentration history of isobutene and isobutene oxide in the Bioreactor RB1 is shown in Figure 9.14B. Isobutene oxide was first observed after around 6 to 7 days of addition, which is consistent with the breakthrough of the bromide tracer shown in Figure 9.11. The isobutene oxide concentrations increase to a maximum observed concentration of around 86 $\mu\text{g/L}$, and over the period of 163 to 170 days the average concentration was $\sim 64 \mu\text{g/L}$. Very low concentrations of isobutene were observed in the RB1 reactor indicating very effective cometabolism of isobutene to isobutene oxide was occurring. This is also supported by the high concentrations of isobutene oxide that were observed. Due to the variability in the measured injected concentration of isobutene, it is difficult to estimate the percent transformation that was being achieved. The very low concentrations of isobutene and the high concentrations of the isobutene oxide, however, indicate a fairly high degree of transformation was being achieved.

Figure 9.14C presents the isobutene and isobutene oxide concentrations in the effluent of the PAM. The experiment has not been conducted for a long enough period of time to draw strong conclusions. Some breakthrough of isobutene is being observed with a maximum concentration of around 12 $\mu\text{g/L}$ observed at 171 days. This isobutene likely results from the groundwater that does not flow through the Funnel-and-Gate treatment system. Some isobutene oxide has been observed, but concentrations tended to be low. The low concentrations might result from mixing of the isobutene oxide with groundwater that has bypassed the funnel-and-gate treatment. Isobutene oxide could also be transformed down gradient of the RB1 Bioreactor. Longer term operation will be required to draw stronger conclusions based on the effluent concentration history.

Isobutene batch kinetic tests with groundwater from the RB1 Bioreactor

Groundwater samples were obtained from the RB1 Bioreactor to determine if there was cometabolic activity. Activity in the groundwater samples would indicate that some degree of breakdown of the beads within the reactor, since small size particles of the beads may be in the groundwater samples. If cometabolic activity was observed, it would also indicate, that upon breakdown, small particles of beads may have been transported down gradient of the RB1 Bioreactor and created cometabolic treatment zones in the aquifer sand. The methods used for this test are described in previous chapters. 10-ml groundwater samples were incubated in 28-ml serum bottles with 18-ml air headspace as a source of oxygen. Isobutene was added as a reactive surrogate and the production of isobutene oxide was measured.

Figure 9.15 presents the results of a batch incubation of the groundwater sample from the RB1 Bioreactor taken during the isobutene surrogate test. The production of isobutene oxide was observed in duplicate samples, with rapid increase observed during the first 24 hours of the incubation. The results support some breakdown of the beads in the RB1 reactor likely occurred.

The results from the isobutene reactive surrogate PAM test indicate a high degree of cometabolic activity within the RB1 Bioreactor. The breakthroughs from the bromide tracer indicates some groundwater is by-passing the Funnel-and-Gate treatment system. The by-passing of the treatment system is supported by higher DO and lower 1-butanol concentrations in the PAM effluent compared to RB1 reactor. Longer term studies will permit estimates of the amount of by-pass that is occurring.

Summary

The results from the PAM study indicate that a CBRB can be constructed using the co-encapsulated gellan-gum beads. A Funnel-and-Gate treatment system was created in a PAM to test this concept. The test system that was created in the PAM permitted the synthetic groundwater to by-pass the Funnel-and-Gate treatment system. In this initial test, only the first Bioreactor RB1 was packed with gellan-gum beads co-encapsulated with TBOS (8%) and *R. rhodochrous* 21198. Prior to packing in the PAM, the gellan-gum beads were stored several months at 4° C in a carbonate buffer. The gellan-gum beads were emplaced into the RB1 Bioreactor by the injecting a slurry to mimic how this might be performed in practice.

The CPRB test was conducted by creating a uniform flow field through the injection and extraction of synthetic groundwater. H₂O₂ was added as a source of DO. Biostimulation of the gellan-gum beads was indicated by the gradual consumption of 1-butanol to below detection in both the RB1 Bioreactor and the effluent of the PAM. DO consumption was also observed, consistent the biostimulation of *R. rhodochrous* ATCC 21198. The biostimulation response was consistent with our observations in the continuous column studies.

Bromide tracer tests indicated that some of the synthetic groundwater was by-passing the Funnel-and-Gate treatment system. More rapid transport was observed in one of the sample locations along the side walls of the PAM, however, bromide breakthrough was observed in RB1 Bioreactor in a similar time frame, indicating the synthetic groundwater was flowing through the Funnel-and-Gate treatment system.

Monitoring of 1-butanol in groundwater from the Bioreactor RB1 showed low concentrations present with long-term operation of the PAM test. Concentrations of less than 0.5 mg/L were observed during the isobutene activity tests, which was initiated 149 days after the emplacement of the gellan-gum beads in the RB1 Funnel-and-Gate system. Isobutene was effectively transformed to isobutene oxide during transport through the Funnel-and-Gate RB1 Bioreactor. Isobutene oxide concentrations, near the maximum isobutene concentration (100 µg/L) injected, were observed and isobutene was consistently below 1 µg/L. Little isobutene oxide was observed in the effluent of the PAM, which may indicate it was transformed downgradient of the RB1 Bioreactor. Some isobutene (~ 0 to 12 µg/L) was observed in the PAM effluent, which likely is associated with groundwater that by-passed the Funnel-and-Gate CPRB.

Incubation of groundwater samples obtained from the Bioreactor RB1 showed significant isobutene cometabolic activity, indicating that some breakdown of the gellan-gum beads likely

occurred. The high activity within the RB1 and funnel-and-gate indicates that small particles of beads have remained cometabolically active for over 170 days.

The PAM test will continue under an NIEHS grant awarded to the PI. Continued monitoring of the current experiment will help establish the long-term performance of Funnel-and-Gate CBRB. The amount of groundwater that is potentially by-passing the PAM will be determined through continued monitoring the PAM effluent. COCs will be added along with isobutene. Since the cometabolic reaction rates of 1,1-DCE are rapid, and in the range of those of isobutene, we are considering evaluating its transformation in the Funnel-and-Gate treatment system. We also plan to add co-encapsulated beads to the second and third Bioreactors within the PAM to achieved higher degrees of treatment. With three reactors in the Funnel-and-Gate in place we would test the treatment of a COC mixture containing 1,1,1-TCA, *cis*-DCE and 1,4-D.

Figures

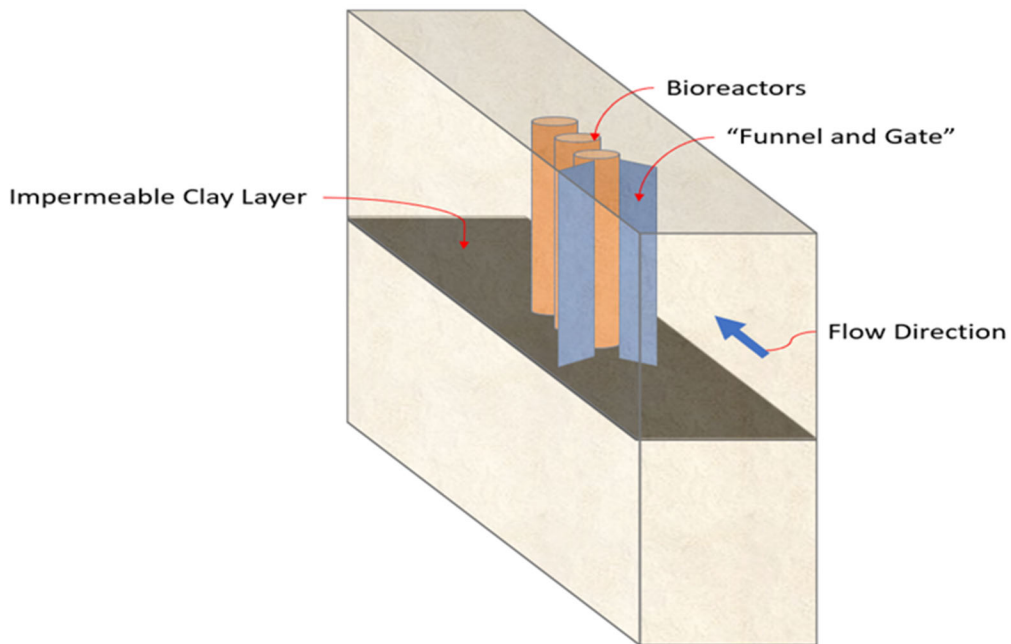


Figure 9.1. Conceptual model of the Funnel-and-Gate CPRB constructed in the Physical Aquifer Model. Three Bioreactors can be packed gellan-gum macrobeads co-encapsulated with *R. rhodochrous* ATCC 21198 strain with TBOS or T2BOS.

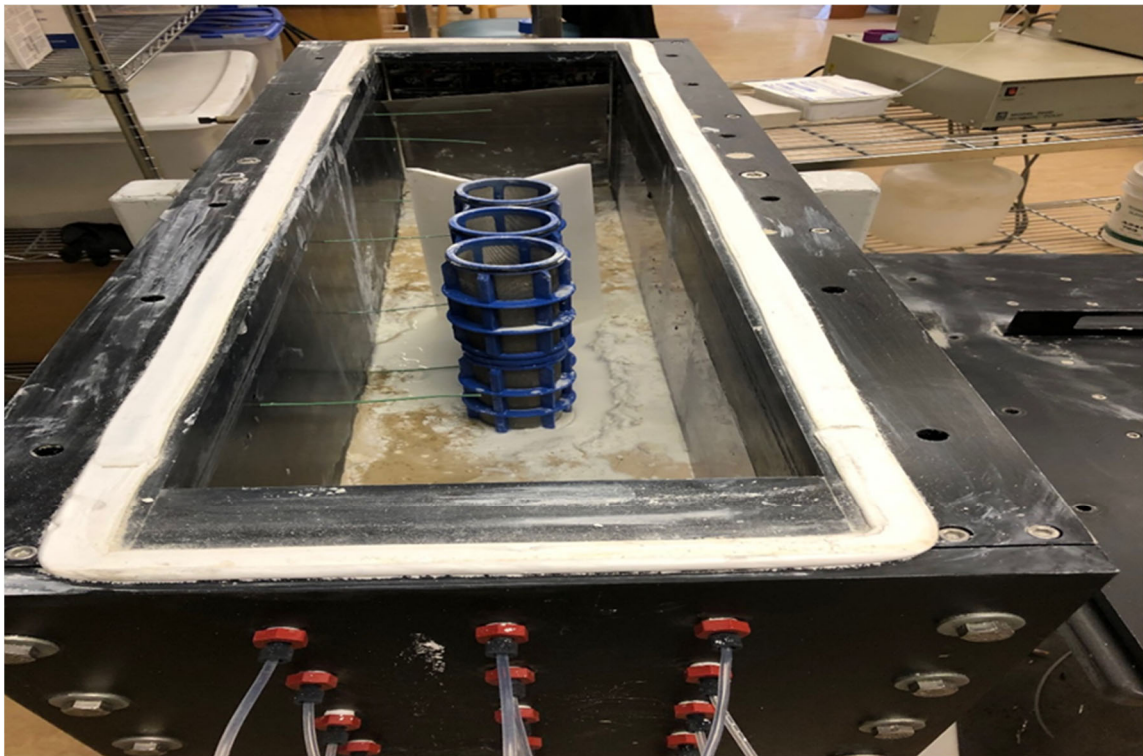


Figure 9.2. The upper-half of the PAM with the Funnel-and-Gate CPRB with three Bioreactors that can be packed with gellan-gum beads. The Funnel-and-Gate is keyed into the bentonite clay layer.



Figure 9.3. The PAM packed with accusand sand 20-30 mesh and three bioreactors which can be packed gellan-gum macrobeads co-encapsulated with *R. rhodochrous* ATCC 21198 and TBOS and T2BOS.

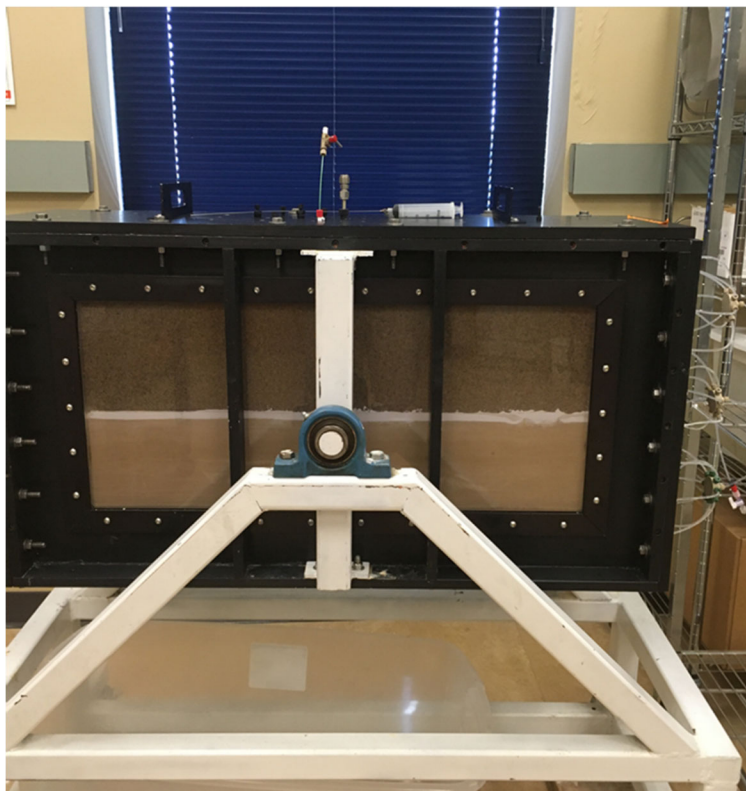


Figure 9.4. The PAM after packing to create a two aquifer model with a bentonite clay layer in between. The bottom aquifer contains 40 to 60 mesh sand, while the upper aquifer contains 20 to 30 mesh sand.

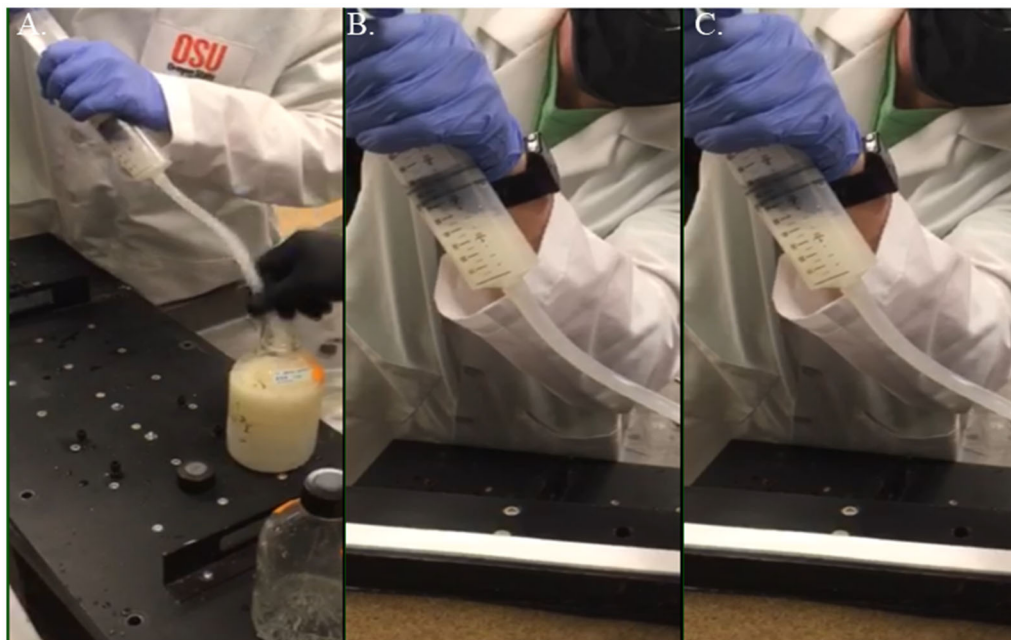


Figure 9.5. Slurry packing of gellan-gum beads co-encapsulated with TBOS (8%) and *R. rhodochrous* ATCC 21198 into Bioreactor 1 (RB1).

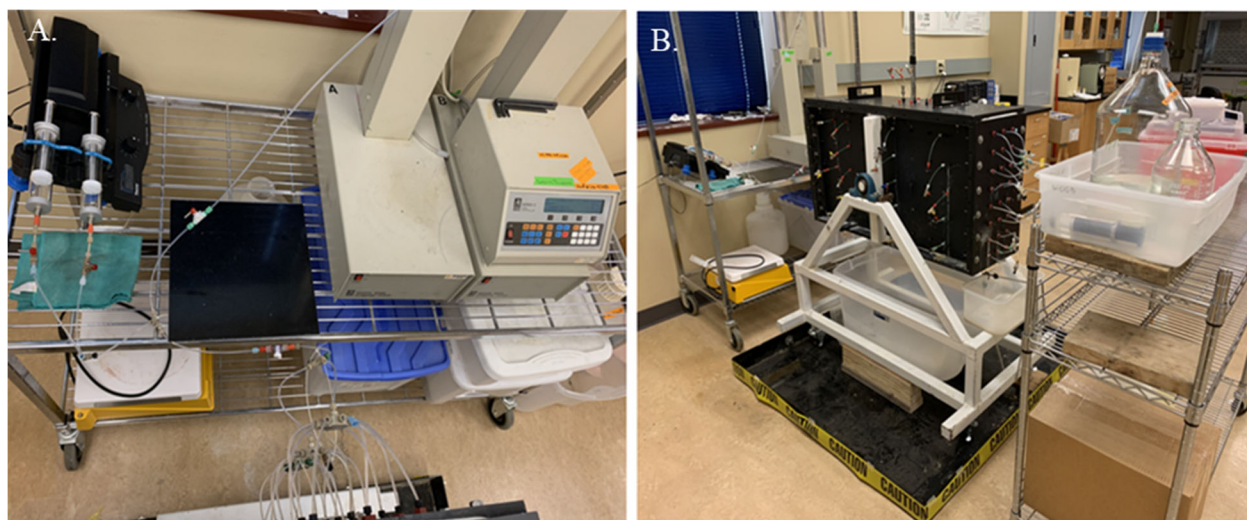


Figure 9.6. (A) Experiment configuration for the continuous injection of groundwater with isobutene and bromide using syringe pump (black) with two 100-mL syringes and the large-scale syringe pump (tan) which supplies most of the synthetic groundwater. The two syringe pump streams are mixed before entering the physical aquifer model. (B) the physical aquifer model experiment configuration with the flow direction from left to right.

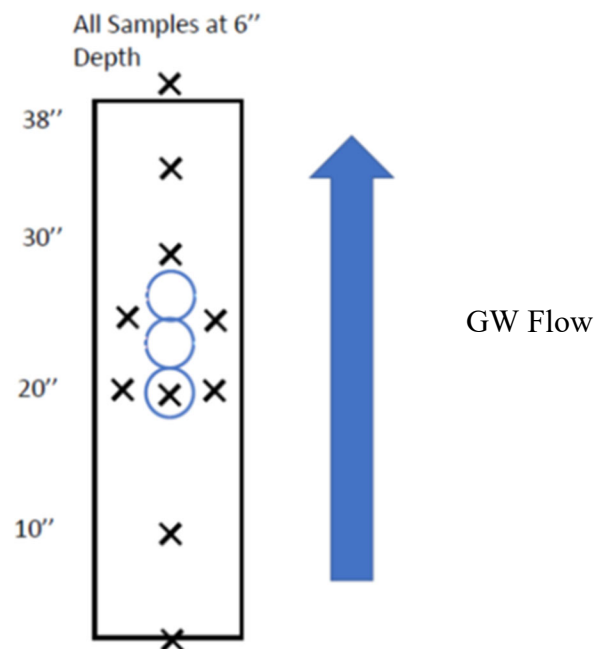


Figure 9.7. Sample locations used in the PAM Funnel-and-Gate CPRB experiment.

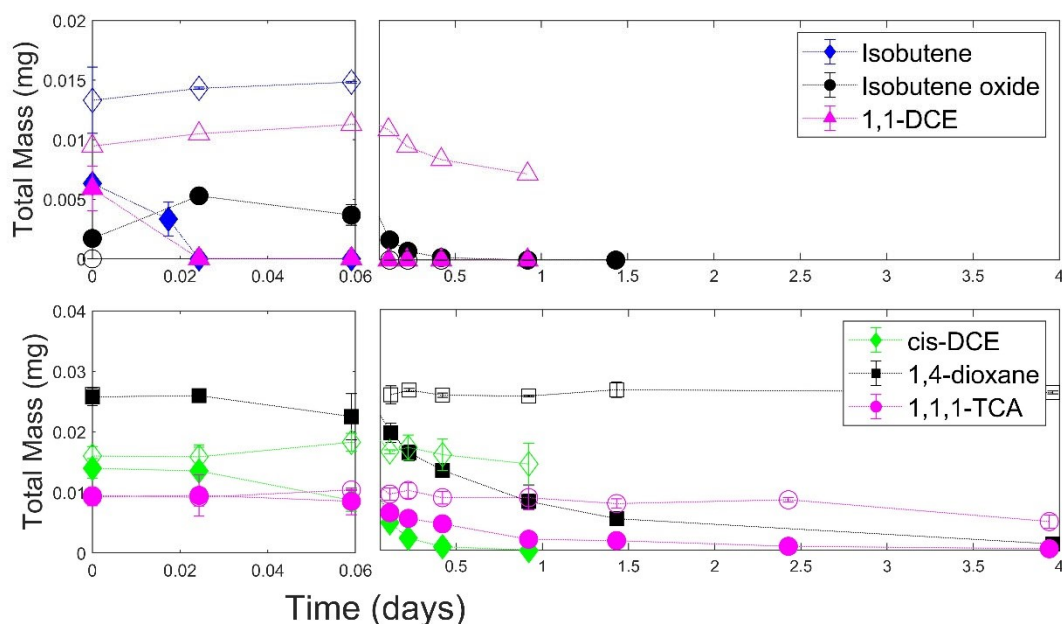


Figure 9.8. Cometabolic transformation of 1,4-D and chlorinated aliphatic hydrocarbons in groundwater microcosms bioaugmented with a mass of isobutane-grown *R. rhodochrous* ATCC 21198 approximately equal to the modeled quantity of isobutane-utilizers in biostimulated microcosms. Active reactors are designated by filled markers. Open markers are acetylene controls. Error bars show one standard deviation. The time scale is split into subplots to show rapid transformation of 1,1-DCE and isobutene and isobutene epoxide formation in less than 0.05 days.

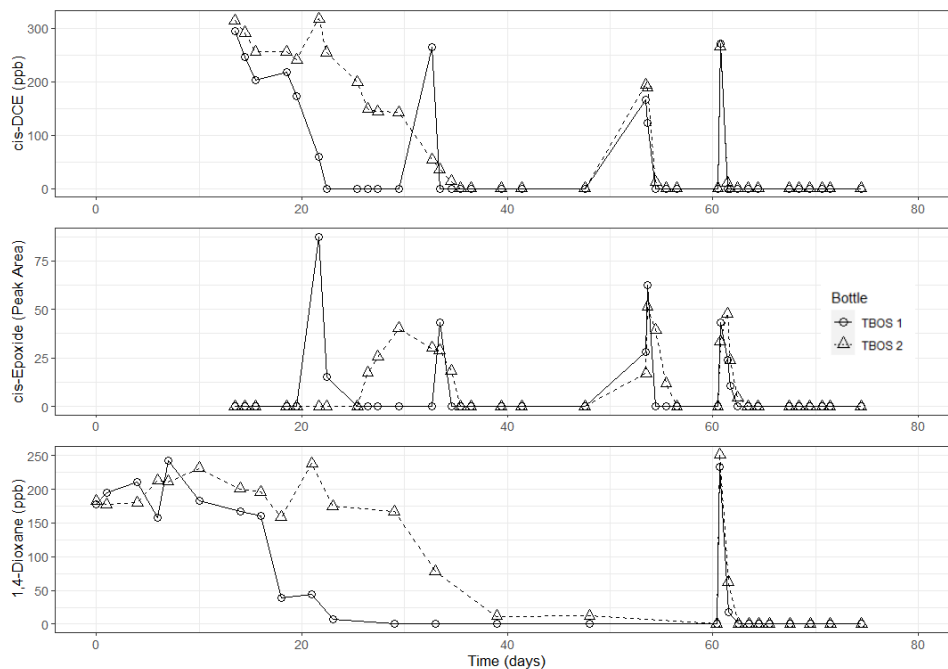


Figure 9.9. Results of batch kinetic test with the gellan-gum beads co-encapsulated with TBOS and *R. rhodochrous* ATCC 21198 that were used in the Funnel-and-Gate CPRB PAM experiment. The test was performed around the time the beads were packed into the PAM. Cometabolism of 1,4-D and *cis*-DCE was evaluated.

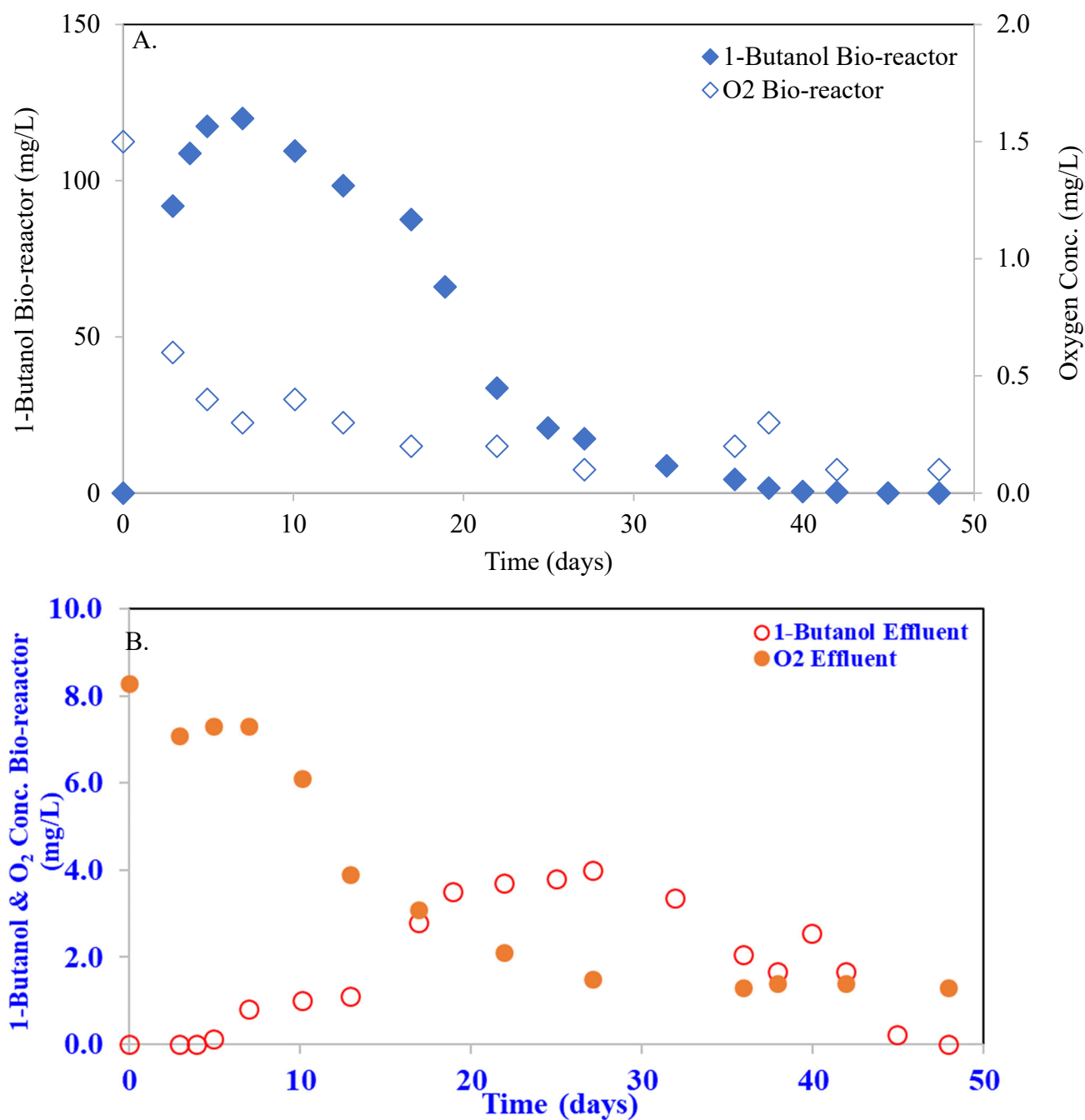


Figure 9.10. 1-Butanol and dissolved oxygen concentrations changes in the RB1 reactor (A) and effluent of the PAM (B) during the initial 50 days operation of the PAM Funnel-and-Gate CPRB.

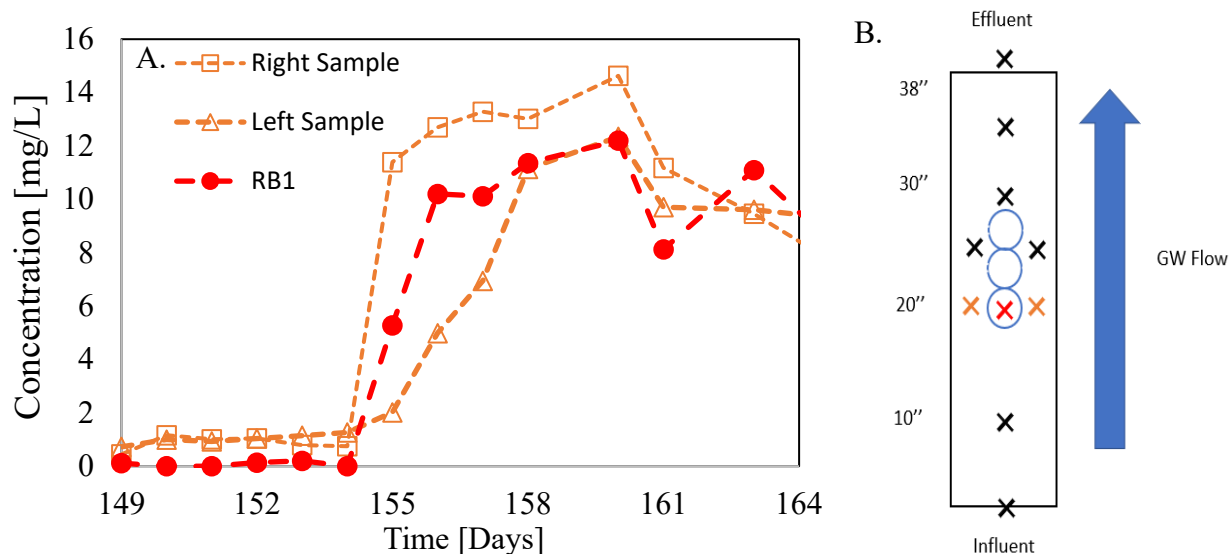


Figure 9.11. (A) Conservative bromide tracer concentration where the groundwater initially passes through the bio-reactor. Deviations from the bio-reactor concentration (RB1) indicate preferential flow away from the bio-reactor. (B) Diagram of the sample points in the physical aquifer model. All samples taken at 6'' depth.

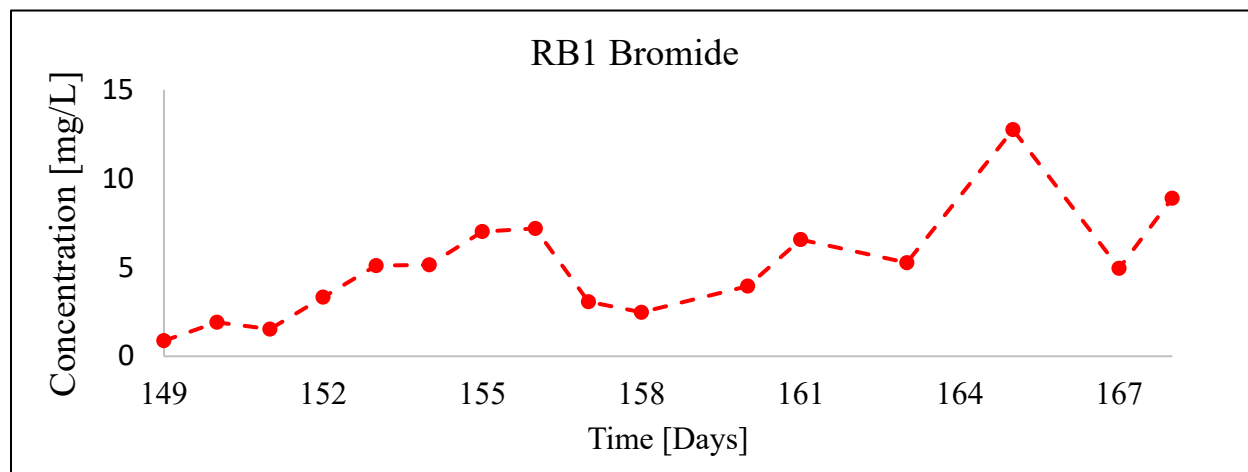


Figure 9.12. Effluent bromide concentrations from the PAM during the Funnel-and-Gate CPRB experiment.

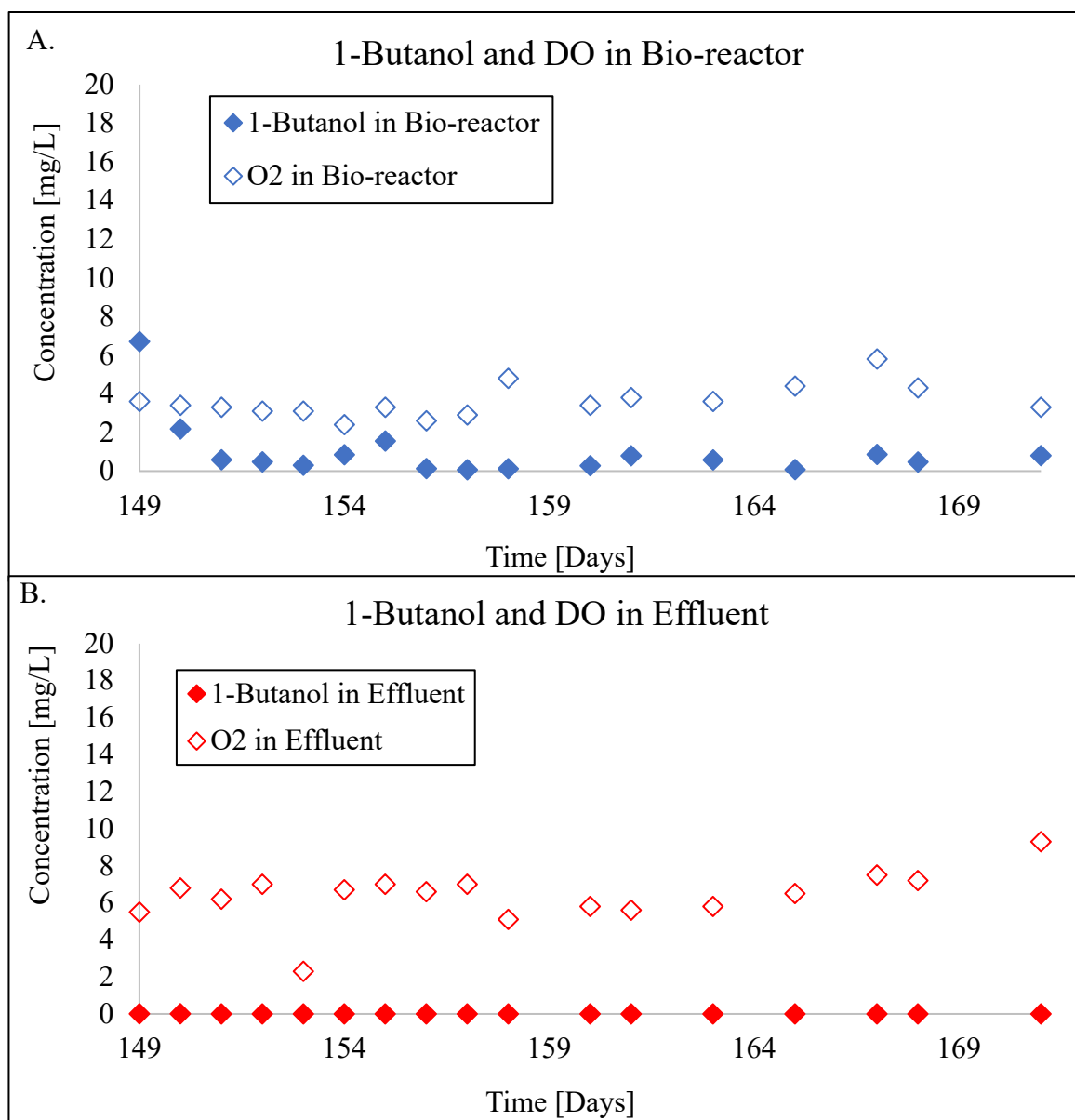


Figure 9.13. Concentration histories of DO and 1-butanol in the groundwater from Reactive Barrier 1 (RB1) (A) and PAM effluent (B) during the Funnel-and-Gate CPRB experiment.

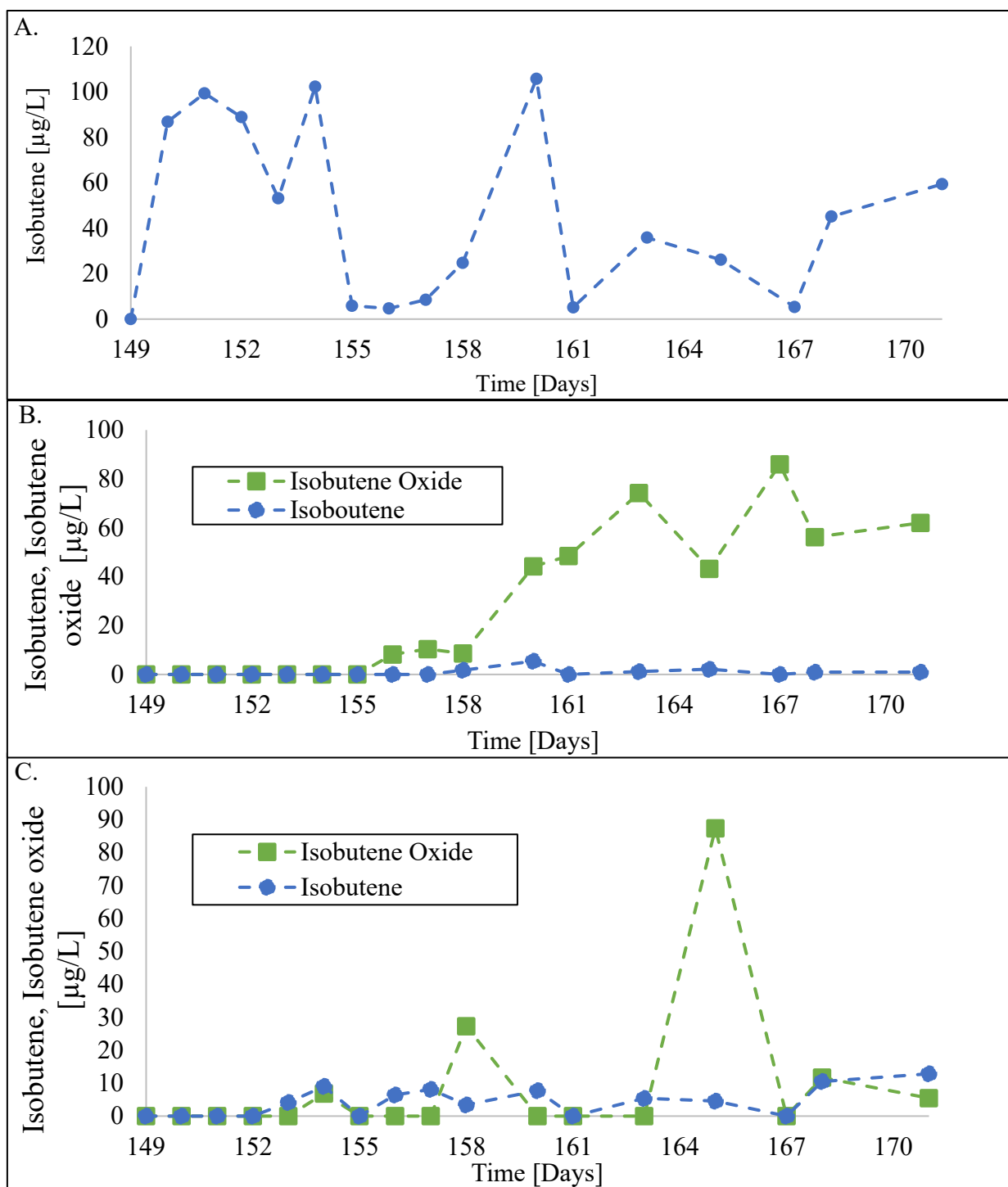


Figure 9.14. Isobutene and isobutene concentration histories in Funnel-and-Gate CPRB test. Concentration of isobutene in the influent groundwater (A). Concentration on isobutene and isobutene oxide in the reactive barrier 1 (RB1) (B); and Concentration of isobutene and isobutene oxide in the effluent of the PAM (C).

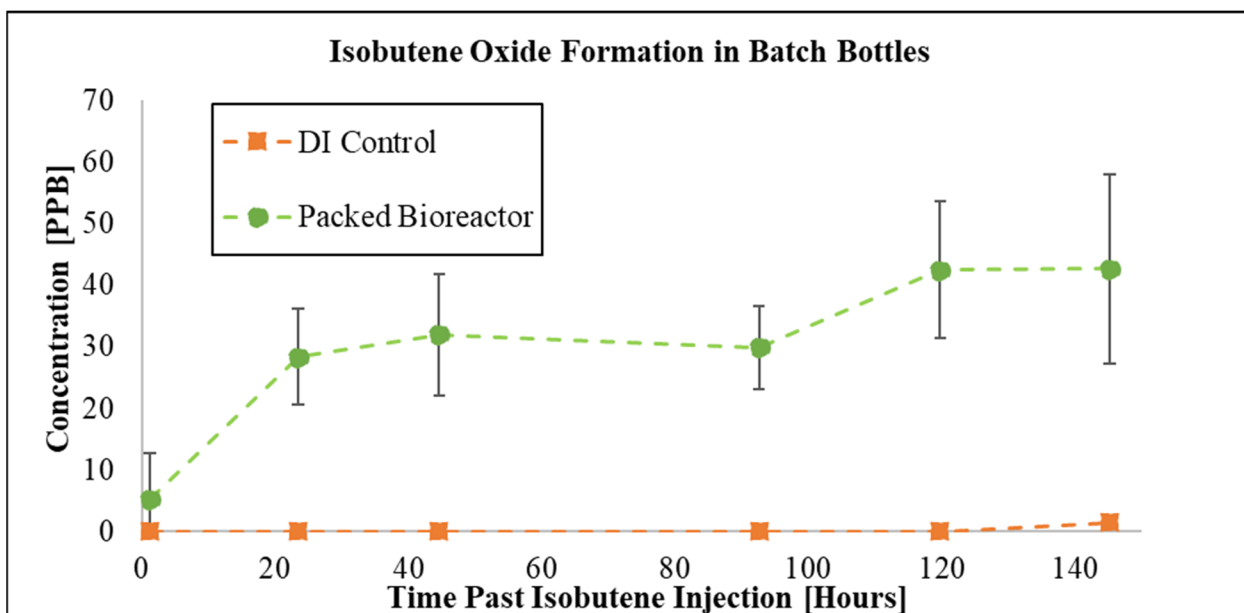


Figure 9.15. Isobutene oxide concentration in a batch kinetic test of isobutene transformation from 10 mL samples of groundwater taken from the gellan-gum bead packed bioreactor RB1 taken during the Funnel-and-Gate CPRB test. DI was also injected with isobutene as an abiotic control. The error bars indicate the range of the duplicate samples.

Chapter 10. Conclusions and Implications for Future Research and Field Applications

This Final Report presents the results of the development of hydrogel beads to achieve the long-term cometabolic transformation of COC mixtures including 1,4-D and chlorinated ethanes and ethenes. Gellan-gum hydrogels beads co-encapsulated a pure bacteria culture, *R. rhodochrous* ATCC 21198 with orthosilicates, that serve as SRCs that produce alcohols as growth substrates via hydrolysis. The report includes results from five objectives: selecting of the bacteria culture to be co-encapsulated; demonstrating the alcohols and the SRCs would promote long term cometabolism; developing the method of co-encapsulation to produce hydrogel beads; evaluating the co-encapsulated beads in batch kinetic tests, groundwater microcosms, continuous flow 1-D packed columns; and finally, as a proof of concept, an experiment in a large scale PAM was performed where the beads were used to construct a CPRB.

In Objective 1 we explored how effective isobutane-utilizing bacteria are at cometabolically degrading COC mixtures. This objective was covered in the latter part of our prior SERDP-2303 project “Evaluation of Branched Hydrocarbons as Stimulants for in situ Cometabolic Biodegradation of 1,4-Dioxane and its Associated Co-contaminants.” That work showed the bacteria culture *R. rhodochrous* ATCC 21198 was very effective in transforming 1,4-D and a broad range of chlorinated ethanes and ethenes. Of the cultures studied, *R. rhodochrous* ATCC 21198 was one of the most effective at transforming this broad range of contaminants, thus once we demonstrated that its growth on alcohols could support aerobic cometabolism, it became the model organism studied in Objectives 2 through 5.

In Objective 2 we found that the growth of *R. rhodochrous* ATCC 21198 was supported by alternative substrates including various alcohols, organic acids, esters, and various other organic compounds. However, only growth on 2-butanol resulted in significant labeling of SCAM enzymes and the ability to oxidize 1,4-D, propylene, and THF. In single compound rate tests, 2-butanol grown *R. rhodochrous* ATCC 21198 exhibited faster transformation rates for less chlorinated compounds (VC, cis-DCE, 1,1-DCE, 1,1-DCA) than more chlorinated compounds (1,1,1-TCA, TCE). The rates of transformation are ranked as follows from high to low: VC > cis-DCE > 1,1-DCA > 1,1-DCE > 1,4-D > 1,1,1-TCA > TCE. All rates of cells grown on these alternative substrates were significantly lower than alkane grown cells and less than 10% of

isobutane-grown cells. 2-Butanol grown *R. rhodochrous* ATCC 21198 exhibited a lag of 4-8 hours before cometabolic transformation of most CAHs including cis-DCE, however production of cis-DCE epoxide was measured with no lag. 1,4-D transformation also exhibited no lag phase before transformation began. The mechanism for potential enzyme induction in 2-butanol grown *R. rhodochrous* ATCC 21198 requires further investigation.

1-Butanol-, 2-butanol-, and 2-ethyl-1-butanol-grown *R. rhodochrous* ATCC 21198 were capable of cometabolic transformation of 1,4-D and a variety of chlorinated ethenes and ethanes. Cells grown on 2-butanol had the largest transformation capacity and fastest rates and were able to transform mixtures of 1,4-D, cis-DCE, and 1,1,1-TCA to >99%. The results indicate 2-butanol provides the most efficient transformation of the alcohols tested and has the potential to be used as a slow-release substrate. In mixtures, cells grown on all three alcohols preferentially transform cis-DCE over both 1,1,1-TCA and 1,4-D.

Sec-Butyl acetate was also found to have potential to be used as a slow-release substrate. *R. rhodochrous* ATCC 21198 grown on this ester transformed a mixture of 1,1,1-TCA and 1,4-D at rates faster than 2-butanol grown cells. Pure cultures of acetate-grown *R. rhodochrous* ATCC 21197 and isolate 4CI were capable of transforming 1,1,1-TCA and 1,4-D, however, transformation rates were found to be a factor of 17.7 and 2.9 times slower than isobutane-grown *R. rhodochrous* ATCC 21198.

Since *R. rhodochrous* ATCC 21198 transforms TCE very slowly, we also evaluated the toluene utilizing culture *Burkholderia vietnamiensis* G4 for TCE cometabolism potential when grown on alcohols. Benzyl alcohol was assessed as a food-grade growth substrate for promoting the cometabolism of TCE, via the T2MO of *B. vietnamiensis* G4. Benzyl alcohol was evaluated by comparing the level of induction of the T2MO to that seen in toluene-grown cells. The level of T2MO expression was evaluated by two methods: 1) ABL; a gel assay method used to identify catalytically active monooxygenases (e.g. T2MO), and 2) resting cell kinetic tests occurring over the course of days to weeks. Other substrates were also tested for their ability to support *B. vietnamiensis* G4 cometabolism of TCE including phenol, benzyl acetate, benzyl butyrate, acetate, butyrate, and lactate.

Both the ABL assay and resting cell tests demonstrated the level of T2MO expression in cells grown on benzyl alcohol was comparable to that observed with toluene, with similar levels

of expression detected and similar TCE transformation rates and capacities. The benzyl esters induced T2MO expression, with benzyl butyrate having the highest TCE transformation rate and capacity of all substrates tested. Acetate, butyrate, and lactate showed minimal levels of enzyme expression, verifying benzyl alcohol was responsible for T2MO expression in benzyl ester grown cells and growth on lactate could not support cometabolism. The ABL assay and resting cell kinetic tests largely agreed, demonstrating that ABL is a reliable method of determining suitable substrates to support cometabolism. Additionally, kinetic tests with benzyl alcohol or toluene and TCE indicate that benzyl alcohol inhibition occurs, but at concentrations greater than 10 mg/L and to a lesser degree than toluene at the same concentration. Finally, kinetics for *cis*-DCE, 1,1-DCE and VC were determined for cells grown on benzyl alcohol and compared to toluene-grown cells. Toluene-grown cells had higher initial transformation rates for VC, *cis*-DCE, and 1,1-DCE, however, benzyl alcohol-grown cells had approximately twice the transformation capacity for the same compounds.

In Objective 3 we determined whether orthosilicates could be used as SRCs to produce the desired alcohols via abiotic hydrolysis. Long-term cometabolic transformation of 1,1,1-TCA and 1,4-D was achieved using SRCs as growth substrates for pure cultures of *R. rhodochrous* ATCC 21198. Resting cells transformation tests showed 1,4-D transformation occurred without a lag phase for cells grown on 2-butanol, while an induction period of several hours was required for 1-butanol grown cells. These observations were consistent with ABL patterns for monooxygenase hydroxylase components and specific rates of THF degradation. 1,1,1-TCA and 1,4-D degradation rates for alcohol-grown cells were slower than those for cells grown on gaseous alkanes such as isobutane. Batch kinetic tests were performed where the orthosilicates were directly added along with *R. rhodochrous* ATCC 21198 in the presence of 1,1,1-TCA and 1,4-D. The hydrolysis of the orthosilicates releasing alcohols supported growth of *R. rhodochrous* ATCC 21198.

Three orthosilicates were tested: TBOS, T2BOS, and T2POS. The measured rates of alcohol release in poisoned controls depended on the orthosilicate structure with TBOS which produced a 1° alcohol (1-butanol) hydrolyzing more rapidly than T2POS and T2BOS that produced the 2° alcohols 2-butanol and 2-propanol, respectively. The orthosilicates were added to batch reactors as LNAPLs with *R. rhodochrous* ATCC 21198 and formed dispersed droplets when continuously mixed. Continuous rates of oxygen O₂ consumption and CO₂ production confirmed alcohol metabolism by *R. rhodochrous* ATCC 21198 was occurring. The rates of metabolism

(TBOS > T2POS > T2BOS) were consistent with the rates of alcohol release via abiotic hydrolysis. 1,4-D and 1,1,1-TCA were continuously transformed in successive additions by *R. rhodochrous* ATCC 21198 over 125 days, with the rates correlated with the rates of metabolism. The metabolism of the alcohols was not inhibited by acetylene, while transformation of 1,4-D and 1,1,1-TCA was inhibited. This indicated that while monooxygenase activity was required for the cometabolic transformations, it was not required for alcohol utilization. Alcohol concentrations in the biologically active reactors were maintained below the levels of detection, indicating they were metabolized rapidly after being produced. Much lower rates of O₂ consumption were observed in the reactors containing T2BOS, which has benefits for in-situ bioremediation. The results illustrate the importance of the structure of the SRC when developing passive aerobic cometabolic treatment systems.

In Objective 4 we demonstrated that the SRCs and *R. rhodochrous* ATCC 21198 could be effectively co-encapsulated in gellan-gum hydrogel beads. The fabrication method for the co-encapsulated gellan-gum hydrogel beads is presented in Chapter 7. The method involved several steps including the emulsification of the gellan-gum and SRC along with *R. rhodochrous* ATCC 21198; a thermal cross-linking step with cooling in tubing; cutting to make small cylinders; and finally, a chemical cross-linking step using calcium as a crossing linking agent. The method produces macro beads (small cylinders) with diameters of around 2 mm. The lengths could be varied depending on the cutting step, but those used in batch and column studies were typically were 2 mm to 4 mm in length. TBOS or T2BOS were typically loaded up to 8 to 10% (w/w) by wet weight of the gellan-gum hydrogel, with as high as 30% by weight achieved.

The fabricated co-encapsulated gellan-gum beads were evaluated in batch tests with media present and in microcosms with containing groundwater and sediment. Gellan-gum beads that encapsulated TBOS or T2BOS were evaluated at 8% (w/w). The aerobic cometabolism of a mixture of 1,1,1-TCA, *cis*-DCE, and 1,4-D at aqueous concentrations ranging from 250 to 1000 µg/L was evaluated in these batch tests. O₂ consumption and CO₂ production showed the co-encapsulated cells effectively utilized the alcohols that were released from the co-encapsulated SRCs. Very low bulk solution concentrations of the alcohols indicated that the alcohols were consumed within the gellan-gum beads. Encapsulated TBOS hydrolyzed 27 times faster than T2BOS and rates were ~4 times slower than when measured in suspension. In biologically active

batch reactors, the co-encapsulated *R. rhodochrous* ATCC 21198 effectively utilized the SRC hydrolysis products (1- and 2-butanol) and cometabolized repeated additions of a mixture of 1,1,1-TCA, *cis*-DCE, and 1,4-D for over 300 days. The transformation followed pseudo-first-order kinetics. VC and 1,1-DCE were also transformed in the reactors. In the long-term treatment, the batch reactors with T₂BOS gellan-gum beads achieved similar transformation rates, but at much lower O₂ consumption rates than those with TBOS. Reaction rates decreased with prolonged incubation, but slowed to a lesser extent with beads containing T₂BOS. Appropriately scaled first-order rates indicate very high extents of contaminant transformation could be achieved with a residence time of a few days if co-encapsulated beads were densely packed in a column or permeable reactive barrier. Studies in groundwater/sediment microcosms also demonstrated that long-term cometabolic treatment can be achieved under conditions that mimic the subsurface. The groundwater/sediment microcosms also indicated that by encapsulating *R. rhodochrous* ATCC 21198 within the gellan-gum bead with the TBOS/T₂BOS, it likely out competed indigenous bacteria for the alcohols that were released, thus resulting in maintained long-term cometabolism. Based on the lower rate of oxygen consumption while achieving similar long-term rates, T₂BOS was thought to be a more effective substrate than TBOS for co-encapsulation with *R. rhodochrous* ATCC 21198.

In Objective 5, the co-encapsulated gellan-gum beads were evaluated in packed bed 1-D reactor columns under continuous flow conditions. The continuous aerobic cometabolic treatment of a mixture of CAHs and 1,4-D was studied. Columns were packed with gellan-gum hydrogel beads that co-encapsulated *R. rhodochrous* ATCC 21198 and 8% (v/v) TBOS or T₂BOS as SRCs. A mixture of *cis*-DCE, 1,1,1-TCA, and 1,4-D was continuously fed to the columns, each at a concentration of 250 µg/L. Hydraulic residence times (HRT) ranged from approximately 2 days to 12 hours. Biostimulation of *R. rhodochrous* ATCC 21198 occurred during Phase 1 of the column studies. Two columns (Column 1 and 2) packed with TBOS beads were operated nearly identically. During the initial stage of operation (Phase 1), 1-butanol concentrations in the column effluent reached 30 mg/L and influent DO concentration (15 mg/L) were reduced to below detection limits in the column effluent. The addition of H₂O₂ to the column influent groundwater (50 to 100 ppm) as an additional source of DO, resulted in the effective utilization of 1-butanol, with concentrations decreasing to below 1 mg/L in the column's effluent. Both columns achieved over 99.5% removal of *cis*-DCE, 1,1,1-TCA, and 1,4-D within the first 25 days operation at an

HRT of approximately 2 days. The development of aerobic conditions resulted in the biostimulation of *R. rhodochrous* ATCC 21198 within the beads throughout the column. This permitted a decrease in the hydraulic residence time 12 hours while maintaining a contaminant removal efficiency of over 99.5%. In Phase 2 of the tests, the cometabolic transformation was confirmed by stopping H₂O₂ and DO addition, which resulted in an increase in the effluent contaminant concentrations to the influent levels. Upon the reintroduction of DO (15 mg/L) the cometabolic treatment was reinitiated and contaminant concentrations decreased to levels achieved prior the termination of H₂O₂ and DO addition, with 95% removal of 1,1,1-TCA and over 99% removal of 1,4-D and *cis*-DCE.

In Phase III, after ~ 240 PV, *cis*-DCE was replaced by 1,1-DCE (100 µg/L), which imparts a high degree of transformation toxicity. 1,1-DCE was effectively transformed with over 98% removal achieved, while 1,4-D and 1,1,1-TCA continued to be effectively transformed. After ~ 300 PV the concentration of 1,1-DCE was increased to 250 µg/L. Effective transformation for all three contaminants was achieved until around 450 PV after which all three contaminants began to increase in the effluent to the influent concentrations. After 550 PV of flow, complete breakthrough of all three contaminants was observed. Column 2 show nearly identical responses as observed in Column 1 for all phases of the experiment.

Column 3 evaluated gellan-gum beads co-encapsulating *R. rhodochrous* ATCC 21198 and T2BOS. Column 3 was fed artificial groundwater that contained 14 mg/L DO and a mixture of *cis*-DCE, 1,1,1-TCA and 1,4-D each at 250 µg/L. During the biostimulation phase (Phase I) the maximum 2-butanol concentration observed in the column effluent was 3 mg/L, which is a factor of 10 lower than that observed in Columns 1 and 2. This is consistent with the slower rate of hydrolysis of T2BOS compared to TBOS. 2-butanol concentrations gradually decreased, and after about 50 PV of flow were below 50 µg/L. Due to the slower rate of hydrolysis, H₂O₂ addition was not required. Over 99% removal of 1,1,1-TCA, *cis*-DCE, and 1,4-D was achieved. After 90 PV of flow, Phase II was initiated and the contaminant concentrations were increased from 250 µg/L to 500 µg/L. The effluent concentrations of *cis*-DCE and 1,4-D began to immediately increase and reached the influent concentrations after 120 PV of flow, 1,1,1-TCA only reached about 25% of the influent concentration. Upon reduction of the influent concentrations in Phase III to 250 µg/L, the cometabolic transformation was restored, with over 98% removal of the contaminants

observed. In Phase IV, cis-DCE addition was replaced by 1,1-DCE (100 µg/L), while continuing to add 1,4-D and 1,1,1-TCA. A rapid increase in all three contaminants in the column effluent was observed. In Phase V, 1,1-DCE addition in the column effluent was stopped, however 1,4-D and 1,1,1-TCA continued to breakthrough to the influent concentrations. A final phase showed cis-DCE, as the only contaminant, still could not be transformed. The results suggest that 1,1-DCE transformation toxicity caused the cessation of the combetabolic activity in the column. An increase in the DO in the column effluent was also observed indicating the metabolic activity of *R. rhodochrous* ATCC 21198 decreased.

At the end of the column studies the beads were sampled and assayed for the amount of TBOS and T2BOS that remained. Approximately 56% and 98.5 % of the TBOS and T2BOS originally encapsulated in beads remained, respectively. TBOS and T2BOS limitations were not responsible for the cessation in transformation activity in the columns, the estimated rate of hydrolysis of T2BOS was factor of 20 lower than TBOS, which was consistent with the batch incubations.

Another task in Objective 5 was to evaluate the performance of the co-encapsulated beads in a 3-D PAM. The tests were performed in a PAM with a volume of 100-L. The results from the column studies indicate that a CBRB might be constructed using the co-encapsulated gellan-gum beads. A Funnel-and-Gate treatment system was created in a PAM to test this concept. The PAM was packed with a medium grade sand with a bentonite clay layer installed in the middle of the of the PAM to represent a low permeability layer. The system created in the PAM permitted synthetic groundwater to by-pass the Funnel-and-Gate treatment system, if there was a loss in permeability in the section packed with the gellan-gum beads. In the initial test, only a single bioreactor was packed with gellan-gum beads co-encapsulated with TBOS (8% w/w) and *R. rhodochrous* ATCC 21198. The gellan-gum beads were stored several months at 4° C in a carbonate buffer prior to packing in the PAM. The gellan-gum beads were emplaced into the RB1 Bioreactor by injecting a slurry of beads and media to mimic how this might be performed in practice.

The CPRB test was conducted by creating a uniform flow field through the injection and extraction of synthetic groundwater. H₂O₂ was added as a source of DO. Biostimulation of the gellan-gum beads was indicated by the gradual consumption of 1-butanol to below detection limits in both the Bioreactor RB1 and the effluent of from the PAM. DO consumption was also observed,

consistent the biostimulation of *R. rhodochrous* ATCC 21198. The biostimulation response was consistent with our observations in the continuous column studies.

Bromide tracer tests indicated some of the synthetic groundwater was by-passing the Funnel-and-Gate treatment system. More rapid transport was observed in sample locations along the side walls of the PAM, however, bromide breakthrough was observed in RB1 Bioreactor in a similar time frame, indicating the synthetic groundwater was flowing through the Funnel-and-Gate treatment system.

Monitoring of 1-butanol in Bioreactor RB1 showed low concentrations, less than 0.5 mg/L, with long-term operation of the PAM experiment. The cometabolic treatment achieved in the PAM test was evaluated using isobutene as a surrogate for the CAHs and 1,4-D, to avoid using regulated contaminants in the initial tests. Isobutene is cometabolized to the epoxide, isobutene oxide, which was tracked during the tests. The isobutene activity tests were initiated 149 days after the emplacement of the gellan-gum beads in the RB1 Funnel-and-Gate system. Isobutene was effectively transformed to isobutene oxide during transport through the Funnel-and-Gate RB1 Bioreactor. Isobutene oxide concentrations near the maximum isobutene concentration (100 µg/L) injected were observed and isobutene was consistently treated to below 1 µg/L. Little isobutene oxide was observed in the effluent of the PAM, which may indicate it was transformed downgradient of the RB1 Bioreactor. Some isobutene (~ 0 to 12 µg/L) was observed in the PAM effluent, which likely is associated with groundwater that by-passed the Funnel-and-Gate CPRB.

Incubation of groundwater samples obtained from the Bioreactor RB1 showed significant isobutene cometabolic activity, indicating that some breakdown of the gellan-gum beads had likely occurred. The high activity within the RB1 Bioreactor and funnel-and-gate indicates that small particles of beads remained cometabolically active for over 170 days.

The PAM test will continue under an NIEHS grant awarded to the PI. Continued monitoring of the current experiment will help establish the long-term performance of Funnel-and-Gate CBRB. The amount of groundwater that is potentially by-passing the PAM will be determined through continued monitoring of the PAM effluent. COCs will be added along with isobutene. Since the cometabolic reaction rates of 1,2-DCA are rapid, and in the range of those of isobutene, we are considering evaluating its transformation in the Funnel-and-Gate treatment system. We also plan to add co-encapsulated beads to the second and third Bioreactors within the

PAM to achieved higher degrees of treatment. With three reactors in the Funnel-and-Gate in place we would test the treatment of a COC mixture containing 1,1,1-TCA, *cis*-DCE and 1,4-D.

Implications for Future Research

A better understanding is needed for the mechanisms that result in enzyme induction when *R. rhodochrous* ATCC 21198 is grown on alcohols. It would also be of interest to evaluate the induction of cometabolic enzymes with a broader group on microorganisms grown on 2-butanol, 1-butanol, and benzyl alcohol

Determining if there is an SRC that can be used to produce benzyl alcohol would also be of interest. The ester benzyl butyrate worked well, but being polar will too quickly diffuse from the hydrogel beads into solution to serve as an effective SRC. There is not a commercially available orthosilicate that hydrolyzes to produce benzyl alcohol. We would expect, if synthesized, that it would likely have a slow rate of hydrolysis. There is an orthosilicate, tetraphenoxysilane (TPhOS) that produces phenol, that may work if co-encapsulated *B. vietnamiensis* G4. More research is needed to determine if TPhOS would hydrolyze at a rapid enough rate to be useful as an SRC for to co-encapsulation with *B. vietnamiensis* G4.

The hydrogels that were explored in this project were alginate and gellan-gum. We did find that gellan-gum was superior to alginate for the co-encapsulation of the SRCs with *R. rhodochrous* ATCC 21198. However, we did observe some compaction of the gellan-gum beads in the long-term operation of the columns. The method used to fabricate the gellan-gum beads involved a thermal cross-linking step and a chemical cross-linking step, this would make the scale-up to produce large quantities of beads more difficult. Through our recently funded NIEHS project we are exploring making co-encapsulated hydrogel beads that are only chemically cross-linked, which would be easier to scale-up for mass production. We are exploring fabricating hydrogels beads using poly vinyl alcohol combined with alginate that are chemically cross-linked. We expect these hydrogel beads will have stronger mechanical properties and will maintain those properties under different groundwater geochemistries.

The column studies proved to be a very useful method for demonstrating the long-term performance of the hydrogel beads. Due to time limitations of the project we were not able to run the columns studies for time periods greater than one year. The columns were stressed by exposing the hydrogel beads to 1,1-DCE, which resulted in transformation toxicity to *R. rhodochrous* ATCC

21198. At the end of the column studies, it was determined that there was ample TBOS and T2BOS remaining in the gellan-gum beads, so the columns may have operated much longer, if they had not been exposed to 1,1-DCE. It would be of interest to conduct longer term studies of 1,4-D transformation, which does not impart transformation toxicity. It would also be of interest to conduct long-term transformation studies with groundwater from a DOD site that has a contaminant mixture that contains mainly 1,4-D but also low concentrations of CAHs that *R. rhodochrous* ATCC 21198 transforms well. Groundwater from the DOD site will also have native microorganisms that might attack the hydrogels. Also, longer term column tests are needed with geochemistries representative of DOD sites.

The PAM tests need to be continued to evaluate the concept of the CPRB for treating 1,4-D and CAHs of interest. This work will continue under our currently funded NIEHS project. Tracer tests will help determine the amount of groundwater that by-passes the Funnel-and-Gate treatment system. Like performed in the column study, a dissection of the PAM at the end of the tests will help determine the status of the hydrogels in the CPRB and if the beads broke down, did pieces of the hydrogels inoculate downgradient sections of the PAM. Multiple CPRBs should be installed to determine if effective and prolonged treatment can be achieved in the Funnel-and-Gate treatment system. For example, co-encapsulated gellan-gum beads with TBOS could be followed by gellan-gum beads that co-encapsulate T2BOS. The delivery of dissolved oxygen could also be addressed in the PAM. It would be of interest to determine if solid phase forms of oxygen-releasing compounds can be tested in the PAM in order to construct a completely passive system.

Numerical modeling needs to be continued to simulate the performance of the co-encapsulated beads. A reactive-transport model needs to be developed that includes the diffusion of contaminants, dissolved oxygen and nutrients into the hydrogel beads. The diffusive transport into the beads needs to be coupled with the hydrolysis of the orthosilicates within the beads, the growth and maintenance of the microbial culture within the hydrogel beads on the alcohols produced, along with the complex process of cometabolism. A numerical model should be developed that can be used to simulate the results of batch reactor incubations of the co-encapsulated gellan-gum hydrogel beads. The model could then be extended to include 1-D advective/dispersive transport to simulate the results of column studies. The model might then be

incorporated into a 3-D transport code to simulate the experiments conducted in the Funnel-and-Gate PAM experiments and can be extended to design passive in-situ treatment systems.

Directions for Field Applications

The results from the column tests indicate that passive CPRB might be created using the co-encapsulated technology that was developed. In a barrier consisting of packed beads, very high removal efficiencies might be achieved with hydraulic residence times of around one day. The CPRB might be included in the design of a Funnel-and-Gate treatment system. The co-encapsulated macro beads could also be mixed *in-situ* with contaminated aquifer solids to create a CPRB. If high enough rates are achieved with the contaminants of interest, packed bed reactors might be inserted into recirculation wells, to have the reaction occur within the wells. Above ground reactor (ex-situ) systems might also be considered, using packed bed reactors.

All of the above treatment scenarios will require some form of a treatability study to determine the potential for using this passive system for treatment. The forms of the treatability study would differ for the treatment being considered, but have similar features. The treatability tests should be able to address:

- How well the treatment might be used for the contaminants present at the site?
- Will the treatment be able to treat the contaminant concentrations that are present?
- What regions of the plume is the process is best suited to?
- What SRC should be selected to be co-encapsulated with the microbial culture?
- Are minor nutrients (N+P) available in the site groundwater able to sustain the microbial culture in the hydrogels, or is there a need to add minor nutrients to the groundwater?
- What are the levels of dissolved oxygen (DO) addition needed and the type of oxygen addition that is required?
- What is the durability of the hydrogel beads in the groundwater geochemistry of are site?
- What is the potential for long-term treatment?

Information obtained from the contaminated site is of interest along with laboratory studies can help to answer many of these questions. Information from the site should include the types and of contaminants present and their concentrations, this would help determine of treatment using this technology is applicable. *R. rhodochrous* ATCC 21198 has been shown to be effective in transforming of a broad range of CAHs and 1,4-D individually and in mixtures. The CAHs include

both chlorinated ethanes and chlorinated ethenes. In resting cell tests *R. rhodochrous* ATCC 21198 has transformed 1,1,1-TCA, 1,1,2-TCA, 1,1-DCA and 1,2-DCA, with less chlorinated ethanes transformed at higher rates than more chlorinated ethenes. In resting cell tests with chlorinated ethenes *R. rhodochrous* ATCC 21198 has been shown to cometabolize VC, cis-DCE, 1,1-DCE and very slowly transform TCE. As observed in our work, 1,1-DCE can be rapidly transformed, but it imparts high transformation toxicity, so high concentrations, above ~ 100 µg/L should be avoided. 1,4-D is easily transformed by *R. rhodochrous* ATCC 21198. In column studies a mixture of 1,1,1-TCA, cis-DCE, and 1,4-D was treated, each at a concentration of 250 µg/L. While more studies are needed, it appears a broad range of mixtures can be transformed by *R. rhodochrous* ATCC 21198. For example, the anaerobic transformation products of incomplete reductive dehalogenation of PCE and TCE, including cis-DCE and VC could be treated. The mixture that would result from the abiotic and biotic transformation of 1,1,1-TCA, to 1,1-DCE and 1,1-DCA along with 1,4-D could also be treated.

This treatment process should be considered for concentrations typically below 250 µg/L, but will vary depending in the mixture of contaminants present. It will also depend on which SRC compound is selected to be co-encapsulated. For example, in our column studies, the column packed with beads co-encapsulated with TBOS was more effective in treating 1,1-DCE, compared to the column packed with beads containing T2BOS. This resulted from a greater biomass that developed in the beads that contained TBOS.

Treatability Studies

Treatability studies can include batch tests and column tests. Methods for conducting these tests are provided in Chapters 7 and 8, therefore will not be discussed in detail here. Discussed here are important considerations related to designing the treatability tests for a specific site.

Testing in batch reactor groundwater microcosms

The testing of the hydrogel beads in batch reactor microcosms is described in Chapter 7. In these studies, the batch reactors contained a low volume of beads (2 mL) to liquid (100 mL) and a headspace with air (70 mL) as a source of oxygen. This type of batch system is fairly simple to operate, sample, and analyze, and since they are batch systems, multiple batch reactors can be constructed and tested simultaneously. As presented in Chapter 5, analysis of the headspace air

can be used to determine the concentration history of the CAHs, O₂, and CO₂. Samples of the liquid phase are used to determine the concentration history of 1,4-D and the alcohols that are being generated by the SRC, if they are released from the hydrogel beads. The batch reactors can also be poisoned to determine the rate of generation of the alcohols via abiotic hydrolysis of the SRC co-encapsulated in the hydrogel beads. Acetylene can also be added to inactivate the cometabolic process, but maintain the metabolic process, as demonstrated in Chapter 6.

An analysis should be performed of the contaminants present at a site, their concentrations, and location for logistical considerations as to where a CPRB might be emplaced at a site. Groundwater should be collected as close as possible to a chosen treatment location. Groundwater should be collected via methods that minimize the loss of contaminants of interest, since the batch tests will be evaluating the ability to treat the mixture of contaminants present. Samples for groundwater analysis should be obtained using standard methods at the same time larger volume samples are obtained for conducting batch groundwater microcosm tests. Since having native microorganisms present will be an important factor in the batch tests, no attempt should be made to filter the groundwater or to remove microorganisms. The sampled groundwater should be shipped on ice to location of the treatability study.

Methods for constructing the batch reactor groundwater microcosms are described in Chapter 7. Since the treatability study is directed towards determining the potential for implementing a CPRB consisting of co-encapsulated hydrogel beads, the batch reactors will be constructed using groundwater, and need not contain aquifer material from the site. Analysis of the groundwater should include determining the background geochemistry including major anions and cations and minor nutrients (N and P). The concentrations of the CAHs present along with other contaminants of interest, such as 1,4-D, should also be determined.

Gellan-gum hydrogel beads used for the treatability study should be fabricated using methods described in Chapter 7. The hydrogel beads used in the batch studies presented in Chapter 7 and the column studies of Chapter 8 contained ~ 8% v/v TBOS or T2BOS co-encapsulated with 0.5 mg *R. rhodochrous* ATCC 21198 per mL of hydrogel. Vendors to purchase TBOS and T2BOS are provided in Chapters 6 and 7. One design variable for the treatability study is the testing of beads that contain either TBOS or T2BOS. Another design variable could be the amount of TBOS

or T2BOS encapsulated in the hydrogel beads. We have fabricated gellan-gum beads that contain up to 30% TBOS.

The groundwater microcosm construction can follow procedures outlined in Chapter 7. 178-mL Wheaton Serum bottles sealed with butyl rubber caps were used. For studies using site groundwater with contaminants the hydrogel beads should be added first. The amount of hydrogel beads can be varied, for the studies performed in Chapter 7, 2 grams of hydrogel beads were added to 100 mL of water in the batch reactors. The groundwater containing the contaminants can be added by gently pouring the groundwater into the serum bottles to minimize the volatile loss of contaminants. The headspace of the microcosms should be immediately sampled to determine the initial concentrations of CAHs, CO₂, and O₂ in the headspace. Liquid samples should also be obtained for 1,4-D, 1-butanol, and 2-butanol analysis. Methods for the analysis headspace and liquid samples are provided in Chapters 6 and 7 of the report.

Samples of the beads used to construct the groundwater microcosms should be obtained for later analysis. For example, the amount of TBOS or T2BOS that was encapsulated in the beads can be determined using methods described in Chapter 7. The amounts can be compared to an analysis of the beads at the end of the incubations.

The types of controls and live microcosms are outlined in Chapter 7. Controls can include microcosms that contain the co-encapsulated beads that are poisoned, and live controls that contain acetylene which blocks the cometabolic transformation. Acetylene controls, however, will not block cellular metabolism, since SCAM is not required for the utilization of the alcohols that are produced from the SRCs. The acetylene controls will be of used to determine if the cometabolic process is promoting transformation toxicity, since the rates of oxygen consumption and CO₂ production can be compared with live microcosms where transformation is occurring. Measuring the 1-butanol or 2-butanol concentrations in the groundwater of the poisoned controls can be used to establish the rate of abiotic hydrolysis occurring in the hydrogel beads, as described in Chapter 7.

The groundwater microcosms can be incubated at 20° C on a rotary shaker table at 150 rpm. The microcosms can be periodically sampled to determine of concentration histories. As discussed in Chapter 7, it is important that the air headspace does not become depleted in O₂. The air headspace can be replenished by adding pure O₂ to replace the vacuum that develops in the

microcosm. An alternative method is to open the microcosm under a flume hood and to enable room air to replace the headspace. The direct addition of pure O₂ to replace the vacuum permits the continuation of a transformation experiment.

Multiple additions of the site groundwater would likely be needed to test the long-term performance of the groundwater microcosms. This can be accomplished at the end of a treatment by exchanging the treated groundwater with new groundwater containing the contaminants. This could be accomplished by draining the microcosms under a flume hood and replacing with new groundwater, or extracting the treated groundwater using a large volume syringe and replacing with new groundwater.

At the end of the study the beads in the microcosms can be sampled to determine how their properties have changed. For example, standard compression tests on the hydrogel beads can be performed to determine if the strength of the beads changed during the incubations. The amount of TBOS or T2BOS remaining in the beads can be determined using methods described in Chapters 7 and 8. If the cometabolic process failed, microbial based methods could be applied. For example, plating the incubation can be applied to determine if *R. rhodochrous* ATCC 21198 remains the dominant microorganisms in the system.

As conducted in Chapter 7, these batch tests would provide information for the estimation of transformation rates. Zero-order rates of O₂ consumption and CO₂ production from active microcosms can be estimated, along with the abiotic rate of alcohol production from the poisoned control. First-order rates of contaminant transformation for each of the successive additions of contaminated groundwater can be calculated in order to establish how the rates might change over time.

These rate estimates can be normalized to the bead volume and to liquid volume of the reactor. These rates might then be extrapolated to rates that might be achieved in a column study with a much higher bead volume to liquid volume ratio. For example, estimates of rates determined in batch tests presented in Chapter 7 indicated that first-order rates would increase by factors of 30 to 40 in column tests which were in reasonable agreement with rates estimated for the continuous column tests presented in Chapter 8.

Continuous Flow Column Studies

Continuous flow column studies can be performed using the procedures and methods described in Chapter 8. For these studies, the columns should be packed completely with hydrogel beads, to mimic the construction of a permeable reactive barrier. A major difference with the treatability studies for a contaminated site, is site groundwater would be used in the studies. In the studies described in Chapter 8 a diluted media mix was used as a synthetic groundwater. The size of the columns used for these studies can vary. For the size of the columns used in Chapter 7 tests (~ 70-mL), with the porosity measured in bromide tracer tests, the groundwater flow to the columns would be around 40 to 50 mL/day to achieve a hydraulic residence time of about 12 hours. This continuous flow can be achieved using syringe pumps equipped with 100-mL gas tight syringes that contain site groundwater and other amendments. H_2O_2 can be added to the groundwater as an easy means of adding oxygen to the groundwater fed. The addition of H_2O_2 would eliminate the need to aerate the groundwater to add DO. This would permit minimal losses of the contaminant mixture present in the site groundwater. Site groundwater needs would be minimal for the column studies of this size. Operating a rate of 50 mL/day, about 1.5 L of groundwater would be needed from the site of interest to conduct a single column test for about one month.

The operation of the columns continuously would provide needed information for the design of a CPRB in the field and would help answer many of the questions outlined above. For example, if two columns were employed it would help answer which of the two SRCs, TBOS or T2BOS, might provide the most effective treatment for the contaminant mixture present. For example, if low contaminant concentrations are present at site beads co-encapsulated with T2BOS, which would require less DO and would provide for a much longer-term substrate source, might be more effective than beads co-encapsulated with TBOS.

The continuous operation of the columns would help answer questions as to whether there are contaminants present in the site groundwater that might be inhibitory to the treatment process. They would also provide a means to assess the degree of treatment that can be obtained for the contaminant mixture present at the site. The required residence time to achieve the degree of required treatment can also be estimated. Information might be obtained on the amount of DO or H_2O_2 addition required during the biostimulation start-up phase of treatment, as well as how this

addition might change during the course of treatment. Long-term tests would help indicate if prolonged treatment can be maintained.

The continuous flow column tests would proceed in stages as outlined in Chapter 8. At the start-up of the column study measurements of the permeability of the packed column should be made. A constant head permeability test could be performed, where flowrates through the column are measured at a given pressure drops through the column. Similar tests could be performed at the end of the column study to determine if permeability changes over the course of column study.

The initial stage (Phase 1) of the column test is the biostimulation phase. In order to mimic the field CPRB process the column test should be conducted in the presence of the contaminated groundwater. This will help address whether the microorganisms can be stimulated within the hydrogel beads in the presence of the groundwater contaminants. Monitoring of DO consumption and alcohol concentrations in the column effluent will help determine if effective stimulation is achieved. As was observed in the Chapter 8 column studies, under O_2 limited conditions 1-butanol was observed in the column effluent. Upon the introduction of H_2O_2 1-butanol decreased to below detection limits, indicating biostimulation was occurring. Similar observations occurred in the column that contained beads co-encapsulated with T2BOS, but the maximum 2-butanol concentrations were an order of magnitude lower, due to the slower rate of hydrolysis. The end of Phase 1 would be when the alcohols reach the lower levels of detection in the column effluent. At the end of Phase 1, there should be measurable DO in the effluent of the column. For example, 2 mg/L of DO in the column effluent would indicate the presence of DO throughout the column.

Phase 2 would represent when steady-state cometabolic transformation of the contaminant mixture is being achieved. During this stage a range of HRTs can be tested to determine how the extents of transformation depend on the HRT. It is difficult to change only one variable at a time when HRT is changed. For example, increasing the flowrate to decrease the HRT would also result in a greater flux of DO or H_2O_2 to the column, this might change the distribution of DO in the column. The HRT tests should be conducted with enough time for steady-state conditions to be achieved. Since it is difficult to determine differences in pseudo-first order rates when very high extents of treatment are achieved, it would be of interest to conduct tests at low enough HRT so that difference in the extents of treatment can be observed. More experimental work is needed to determine how HRTs effect treatment extents, since for example, at low HRTs transformation

products might be transported throughout the columns that could impact the performance of the columns.

Phase 3 of the test could demonstrate the column's response to anoxic conditions created by stopping DO and/or H₂O₂ addition. This phase was used in the column studies presented in Chapter 8 to demonstrate that the contaminant decreases were due to cometabolic transformations. This phase of treatment would help determine how long it might take to recover the aerobic treatment after a period when DO or H₂O₂ addition is stopped in the field.

A final phase, Phase 4, of the treatability study would help determine if DO or H₂O₂ addition requirements change with time. DO to H₂O₂ addition might be adjusted to maintain aerobic conditions (> 2 mg/L) in the column effluent. Phase 4 tests would also help determine if the degree of treatment of the contaminant mixture remains constant for long time periods of operation. In our studies the column tests were performed for a period of almost one year.

At the end of the column test, a permeability test should be performed to determine if there was a loss of permeability during the test. The sample method should be employed as used at the start of the column study. The column can then be dissected and the beads sampled. Methods described in Chapters 7 and 8 could be used to determine the amount of TBOS or T2BOS that remained in the beads. The amounts can be compared with those determined in the batch tests as described in Chapter 8. Batch tests could also be performed with beads sampled from the column to determine the rates of metabolism and cometabolism. These rates can be compared with those obtained from batch tests with beads used to pack the column.

Pseudo-first-order rates presented in Chapter 8 were estimated for the continuous flow column tests using the assumption of plug-flow reaction kinetics. These pseudo-first order rates might be used to make preliminary estimates of the size of a CBRB required. Procedures for estimating the amount of Zero-Valent-Iron (ZVI) required to construct permeable reactive barriers based on first-order rate estimates are provided by USEPA (1998). For these estimates the amount of ZVI that is required to achieve a desired degree of treatment can be calculated (see Appendix C of the EPA report). Their model was based on laboratory measured first-order rate reaction rates, the groundwater velocity, porosity, and the degree of treatment required. The estimates also used a safety factor. Using Equation 43 in USEPA report an estimate of the amount of ZVI that is required normalized to the cross-sectional area for treatment can be made. It may be possible to

apply similar methods to determine the mass of co-encapsulated hydrogels that are needed to be applied per unit cross sectional area of a CRPB, using the first-order reaction rate coefficients that are determined in batch tests or continuous flow column studies. More studies are needed to determine if this simplified approach can be applied to the co-encapsulated hydrogel system that was developed in this project.

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Appendix A: Supplemental Information

4. Chapter 4 Supplementary Information

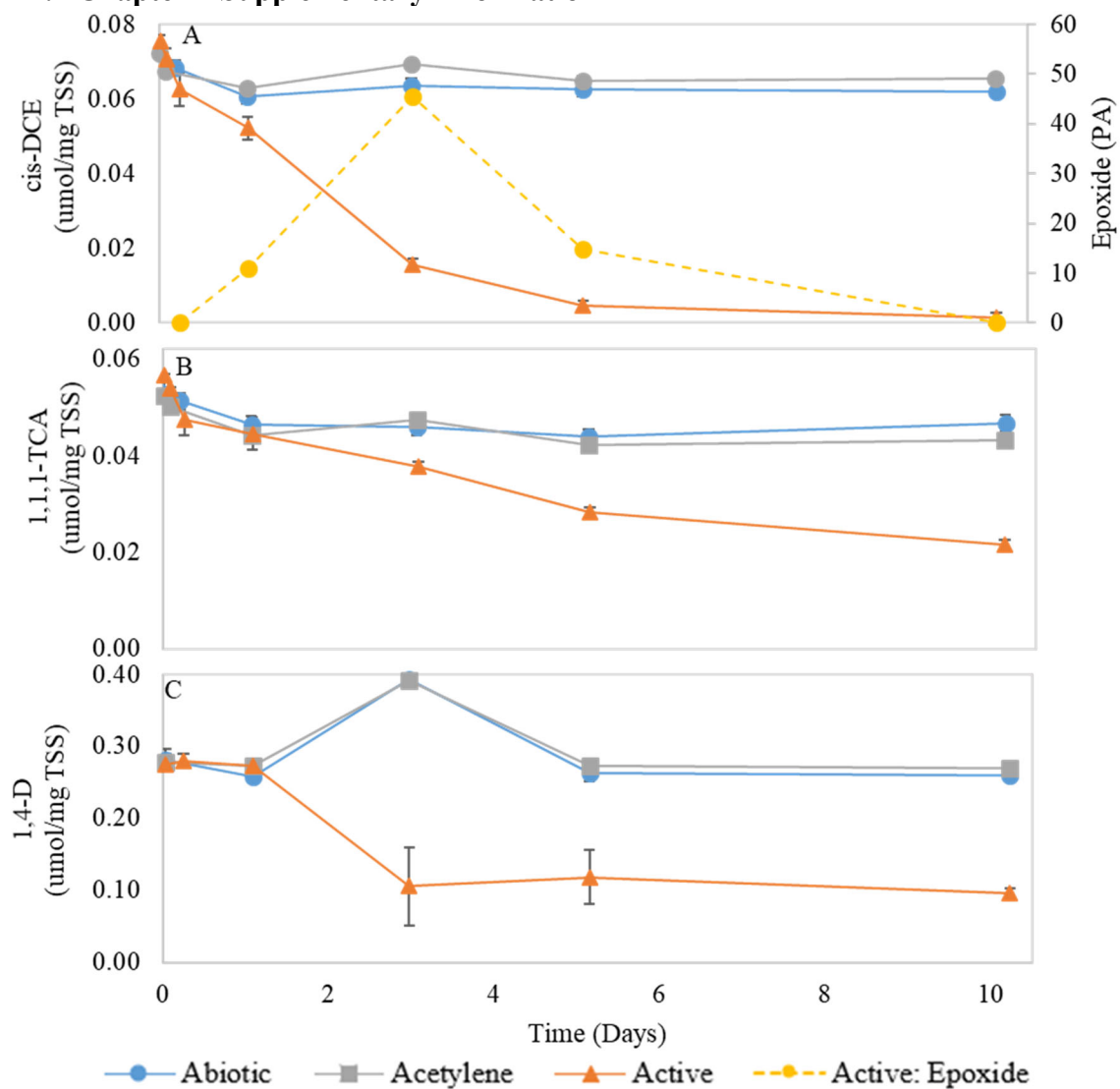


Figure S 4.1. Transformation of a mixture of cis-DCE (A), 1,1,1-TCA (B), and 1,4-D (C) 5mg of 2-ethyl-1-butanol grown *R. rhodochrous* ATCC 21198. Data points and error bars represent the average and standard deviation of triplicates

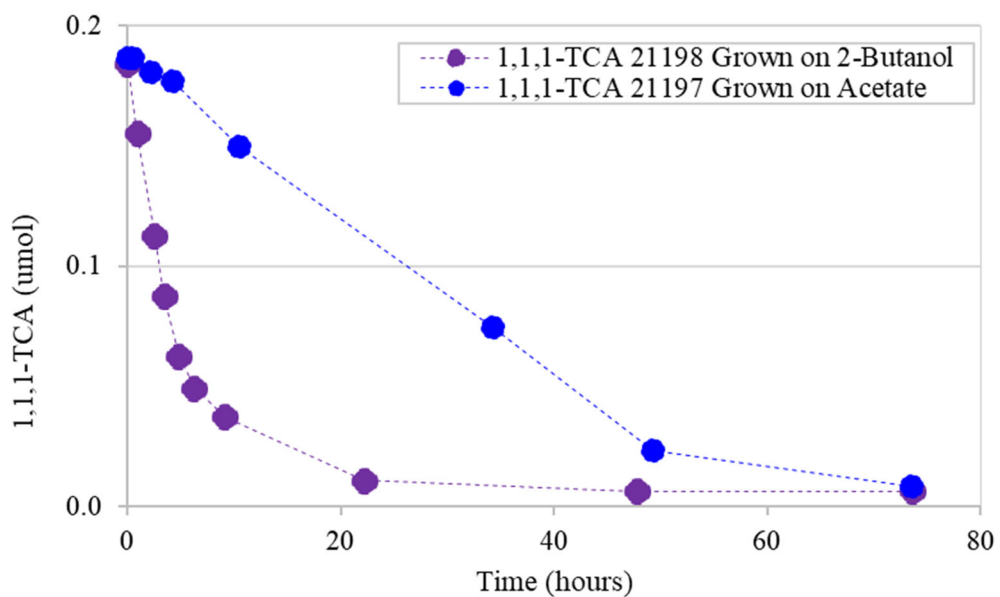


Figure S 4.2. Comparison rate of 1,1,1-TCA transformation with *R. rhodochrous* ATCC 21198 culture grown on 2-Butanol and ATCC 21197 grown on acetate.

5. Chapter 5 Supplementary Information

Table S 5.1. Dimensionless Henry's constants at 20°C used for calculating the total mass assuming equilibrium

CAH	Hcc
TCE	0.294
cis-DCE	0.121
1,1-DCE	0.848
VC	0.892

^aHenry's constants calculated at 20°C from Gossett (1987).

^bHenry's constant from EPI Suite™, USEPA.

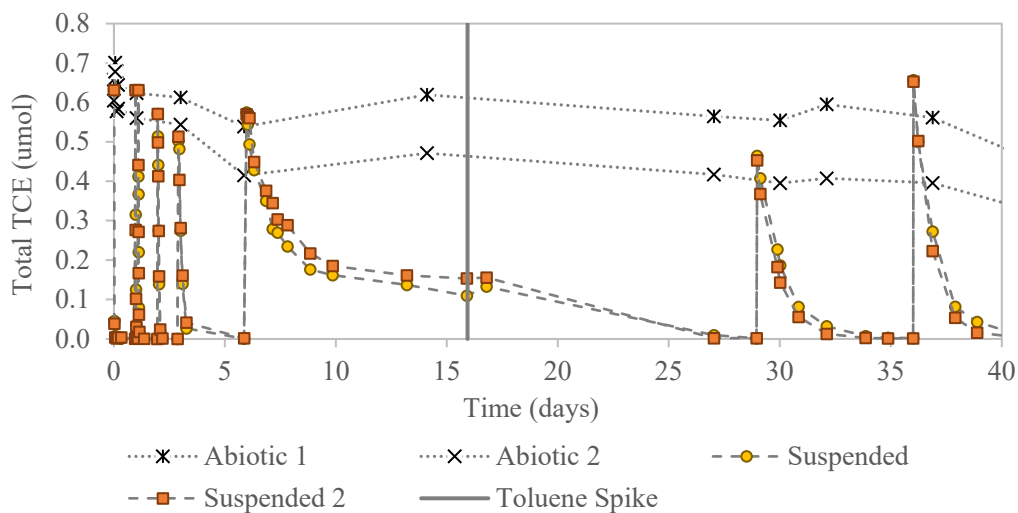


Figure S 5.1. Cometabolic transformation of TCE over the course of 40 days by *B. vietnamiensis* G4. Treatments consisted of duplicate treatments of suspended cells of *B. vietnamiensis* G4 at 16 mg_{TSS}/reactor, and duplicate abiotic control treatments. Toluene was added on day 16 to stimulate further cometabolic activity.

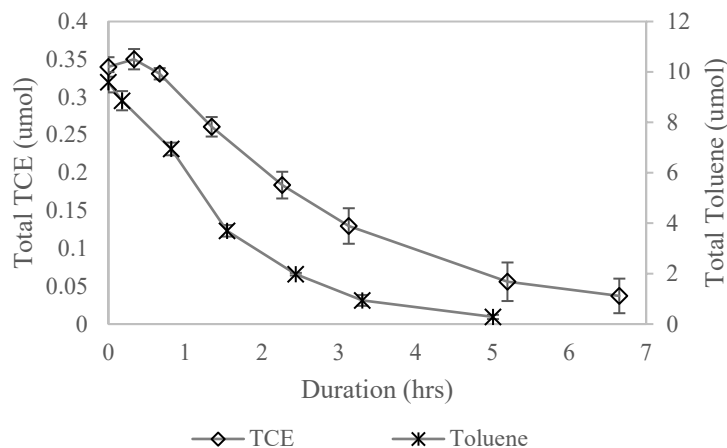


Figure S 5.2. Results of concurrent TCE and Toluene disappearance by *B. vietnamiensis* G4 (1 mg_{TSS}). Initial toluene liquid concentration was 11.7 mg/L. Treatment was done in triplicate with error bars representing SD for n = 3.

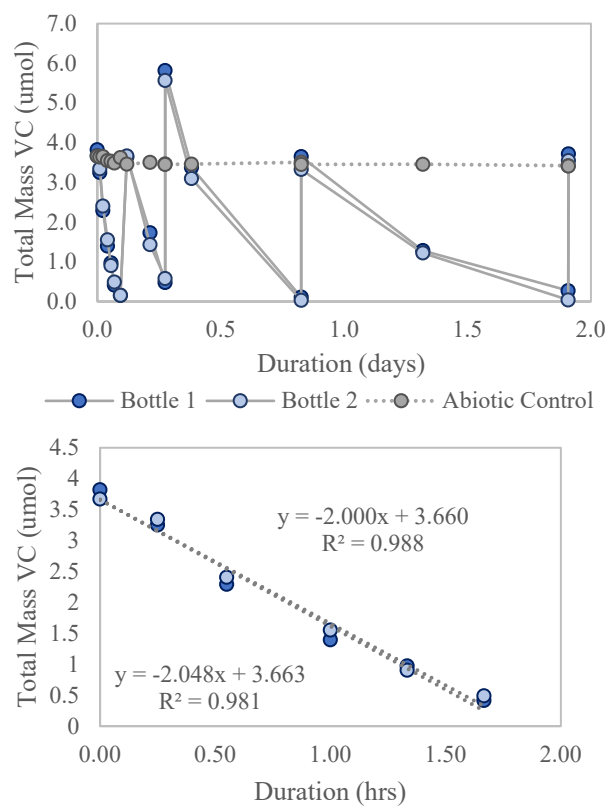


Figure S 5.3. Kinetic degradation results of VC by resting toluene grown *B. vietnamiensis* G4 (5.0 mgTSS) showing (A) VC reinjections to determine total transformation capacity ($\mu\text{mol}/\text{mgTSS}$) and (B) initial degradation of VC to determine initial zero order rate ($\mu\text{mol}/\text{day}/\text{mgTSS}$).

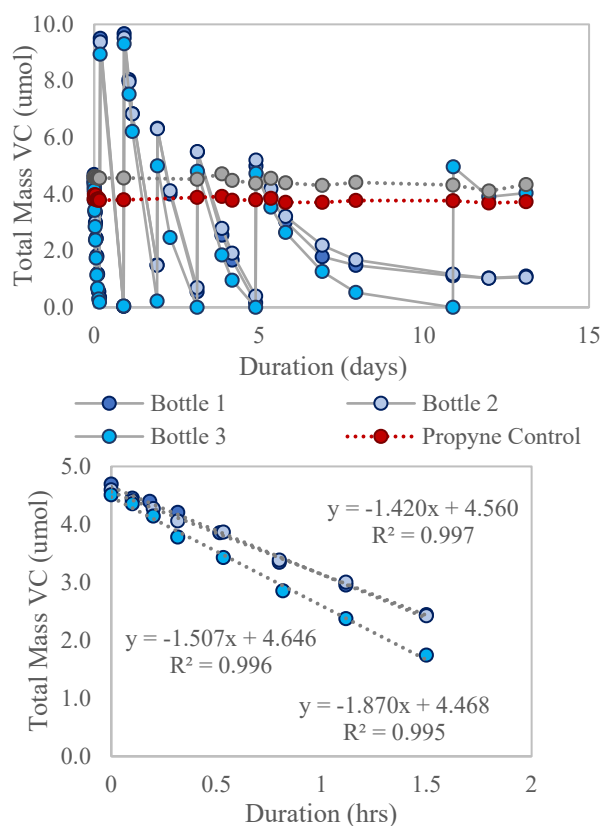


Figure S 5.4. Kinetic degradation results of VC by resting benzyl alcohol grown *B. vietnamiensis* G4 (5.0 mg_{TSS}) showing (A) VC reinjections to determine total transformation capacity (μmol/ mg_{TSS}) and (B) initial degradation of VC to determine initial zero order rate (μmol/day/mg_{TSS}).

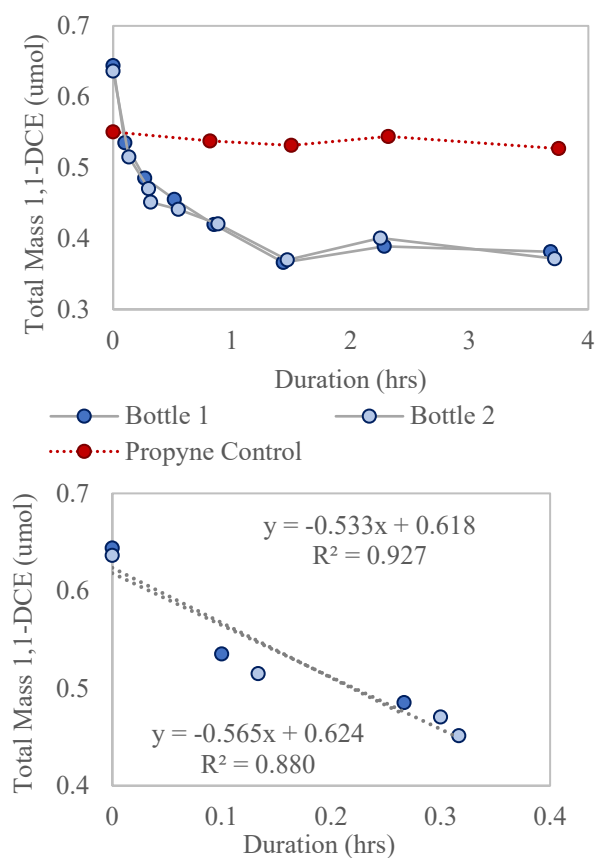


Figure S 5.5. Kinetic degradation results of 1,1-DCE by resting toluene grown *B. vietnamiensis* G4 (5.5 mg_{TSS}) showing (A) 1,1-DCE reinjections to determine total transformation capacity (μmol/ mg_{TSS}) and (B) initial degradation of 1,1-DCE to determine initial zero order rate (μmol/day/mg_{TSS}).

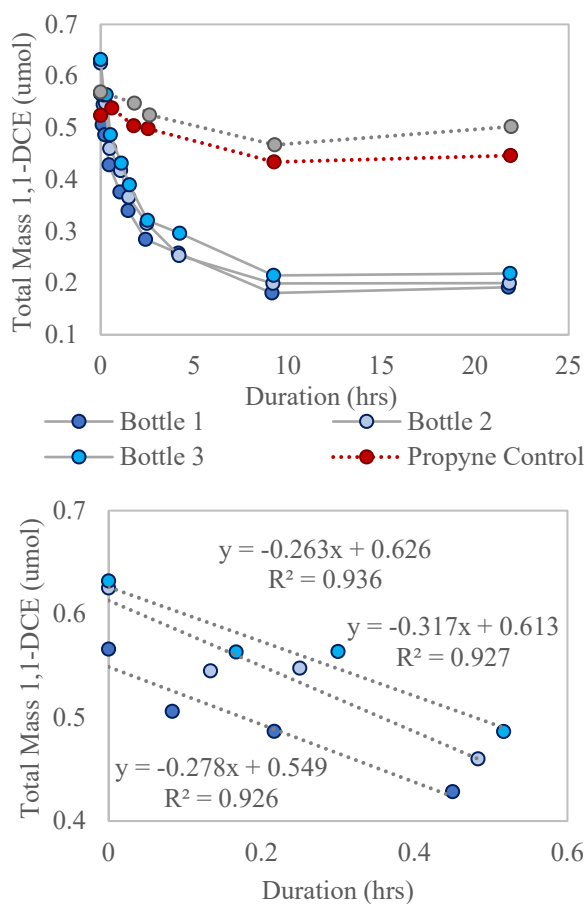
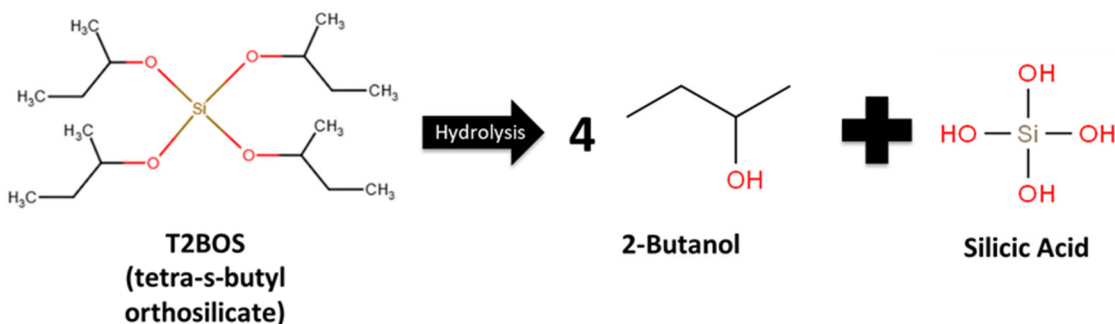


Figure S 5.6. Kinetic degradation results of 1,1-DCE by resting benzyl alcohol grown *B. vietnamiensis* G4 (4.8 mg_{TSS}) showing (A) 1,1-DCE reinjections to determine total transformation capacity (μmol/mg_{TSS}) and (B) initial degradation of 1,1-DCE to determine initial zero order rate (μmol/day/mg_{TSS}).

6. Chapter 6 Supplementary Information

SRC Evaluation – T2BOS and 21198



- Produces 2-butanol - which induces SCAM
- Branched group – slower rate of abiotic hydrolysis compared to linear TBOS

Figure S 6.1. T2BOS hydrolysis to 2-butanol.

SRC Evaluation – TBOS 21198 and TBOS Batch Incubation

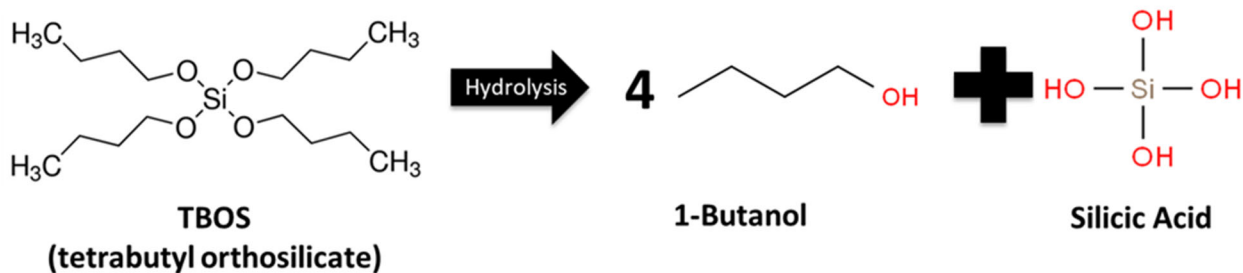
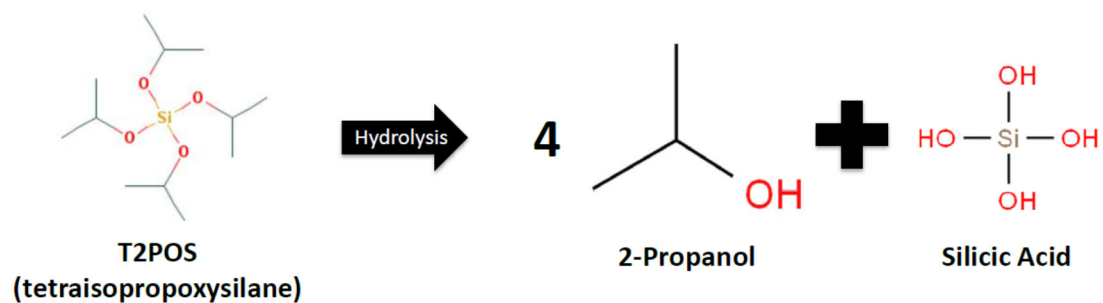


Figure S 6.2. TBOS and the hydrolysis to 1-butanol.



- Produces 2-propanol – Induction of SCAM?
- Branched group with shorted chain length than T2BOS – faster rate of hydrolysis than T2BOS

Figure S 6.3. T2POS and the hydrolysis to 2-propanol

Table S 6.1. List of chemicals used in the following experiments including CAS No., purity, and vendor.

List of Chemicals			
<i>Name</i>	<i>CAS No.</i>	<i>Manufacturer</i>	<i>Purity</i>
Sodium Hydroxide	1310-73-2	Fisher Scientific	50% w/v
Butyrate	156-54-7	Sigma-Aldrich	≥98.5%
Sodium Lactate	72-17-3	JT Baker	60% Syrup
Ethanol	64-17-5	Sigma-Aldrich	100%
1-Butanol	71-36-3	Acros	99.4% (GC)
2-Butanol	78-92-2	Sigma-Aldrich	≥ 99%
2-Propanol	67-63-0	Sigma-Aldrich	99.5%
sec-Butyl Acetate	105-46-4	Aldrich	98%
Tetra-butyl orthosilicate	4766-57-8	Aldrich	97%
Tetra-s-butyl orthosilicate	5089-76-9	Gelest	95%
Tetraisopropoxysilane	1992-48-9	Gelest	>99%
1,1,1-Trichloroethane	71-55-6	Baker Analytical	95.50%
1,4-Dioxane	123-91-1	Baker Analytical	100%
Calcium Carbide	75-20-7	Alfa Aesar	solid

Table S 6.2. Henry's law constants and solubility's for isobutane, 1,4-D, and 1,1,1-TCA used in the following experiments. The Henry's Constant is dimensionless.

List of Constants		
<i>Chemical</i>	<i>Henry's Constant, H_{cc}</i>	<i>Solubility, S_w (mg/L)</i>
Isobutane	49.2	48.9
1,4-Dioxane	0.000198	miscible
1,1,1-Trichloroethane	0.548	1,290

Table S 6.3. Summary of 2-BuOH growth cometabolism experiment. Rates of duplicate bottles are reported as AB1, AB2.

Summary of 2-Butanol Growth Cometabolism Experiment			
COC Transformation			
<i>COC Transformed</i>	<i>1st Order Rate (day⁻¹)</i>	<i>0 Order Rate (μmol/day)</i>	<i>Approx Lag Time (days)</i>
1,1,1-TCA - 1 st Addition	0.59, 0.44	0.20, 0.18	3
1,1,1-TCA - 2 nd Addition	1.09, 1.05	0.18, 0.25	1
1,4-D - 1 st Addition	1.06, 1.26	0.96, 1.09	3
1,4-D - 2 nd Addition	2.07, 3.33	0.92, 0.93	1
Growth Indicator Changes			
<i>Growth Indicator</i>	<i>Rate (units vary)</i>	<i>Approx Duration of Change (days)</i>	
CO ₂ Produced - 1 st Addition (μmol/day)	0.86, 0.98	4	
CO ₂ Produced - 2 nd Addition (μmol/day)	1.64, 1.95	1.5	
OD600 Increase - 1 st Addition (Abs/day)	0.0015, 0.001	4	
OD600 Increase - 2 nd Addition (Abs/day)	0.0043, 0.0033	1.5	
2-Butanol Consumption - 1 st Addition (μmol/day)	1.02, 0.86	3	
2-Butanol Consumption - 2 nd Addition (μmol/day)	3.66, 2.72	1	

Table S 6.4. Summary of T2BOS Growth and Cometabolism Test. Rates from duplicate bottles are reported in the two columns.

Summary of T2BOS Growth Cometabolism Experiment			
COC Transformation			
<i>COC Transformed</i>	<i>1st Order Rate (day⁻¹)</i>	<i>0 Order Rate (μmol/day)</i>	<i>Approx Lag Time (day)</i>
1,1,1-TCA - 1 st Addition	0.21, 0.20	0.03, 0.03	1
1,1,1-TCA - 2 nd Addition	0.12, 0.08	0.06, 0.08	0
1,1,1-TCA - 3 rd Addition	0.04, 0.04	0.02, 0.02	0
1,4-D - 1 st Addition	0.52, 0.55	0.15, 0.16	1
1,4-D - 2 nd Addition	0.15, 0.10	0.14, 0.18	0
1,4-D - 3 rd Addition	0.04, 0.06	0.34, 0.09	0
Growth Indicator Changes			
<i>Growth Indicator</i>	<i>Rate (units vary)</i>	<i>Duration of Change (day)</i>	
CO ₂ Production (μmol/day)	0.38, 0.34	Continuous	
O ₂ Consumption (μmol/day)	1.80, 1.64	Continuous	
2-Butanol Production 1 st Addition (μmol/day)	N/A	Broken Bottle	
2-Butanol Production 2 nd Addition (μmol/day)	0.71	Continuous	
2-Butanol Production 3 rd Addition (μmol/day)	0.76	Continuous	

Table S 6.5. Summary of TBOS growth cometabolism experiment. Rates from duplicate bottles are reported in the two columns.

Summary of TBOS Growth Cometabolism Experiment			
<i>COC Transformation</i>			
<i>COC Transformed</i>	<i>1st Order Rate (day⁻¹)</i>	<i>0 Order Rate (μmol/day)</i>	<i>Approx Lag Time (day)</i>
1,1,1-TCA - 1 st Addition	0.22, 0.66	0.036, 0.48	1
1,1,1-TCA - 2 nd Addition	0.38, 0.80	0.09, 0.19	0
1,1,1-TCA - 3 rd Addition	0.47, 0.50	0.12, 0.12	0
1,4-D - 1 st Addition	0.31, 0.80	0.13, 0.19	1
1,4-D - 2 nd Addition	0.43, 2.72	0.43, 1.00	0
1,4-D - 3 rd Addition	0.76, 1.09	0.54, 0.56	0
<i>Growth Indicator Changes</i>			
<i>Growth Indicator</i>	<i>Rate (units vary)</i>	<i>Duration of Change (day)</i>	
CO ₂ Production - 1 st , 2 nd , and 3 rd Additions (μmol/day)	6.33, 18.8	Continuous	
O ₂ Consumption - 1 st , 2 nd , and 3 rd Additions (μmol/day)	32.4, 45.8	Continuous	
1-Butanol Production (μmol/day)	11.1	Continuous	

Table S 6.6. Summary of T2POS growth cometabolism experiment. Rates from duplicate bottles are reported in the two columns.

Summary of T2POS Growth Cometabolism Experiment			
<i>COC Transformation</i>			
<i>COC Transformed</i>	<i>1st Order Rate (day⁻¹)</i>	<i>0 Order Rate (μmol/day)</i>	<i>Approx Lag Time (day)</i>
1,1,1-TCA - 1 st Addition	0.12, 0.11	0.02, 0.02	1
1,1,1-TCA - 2 nd Addition	0.11, 0.14	0.03, 0.04	0
1,4-D - 1 st Addition	0.32, 0.24	0.08, 0.16	1
1,4-D - 2 nd Addition	0.28, 0.35	0.24, 0.31	0
<i>Growth Indicator</i>	<i>Rate (units vary)</i>	<i>Duration of Change (day)</i>	
CO ₂ Production (μmol/day)	0.94, 0.94	Continuous	
O ₂ Consumption (μmol/day)	3.61, 3.53	Continuous	
2-Propanol Production (μmol/day)	5.08	Continuous	

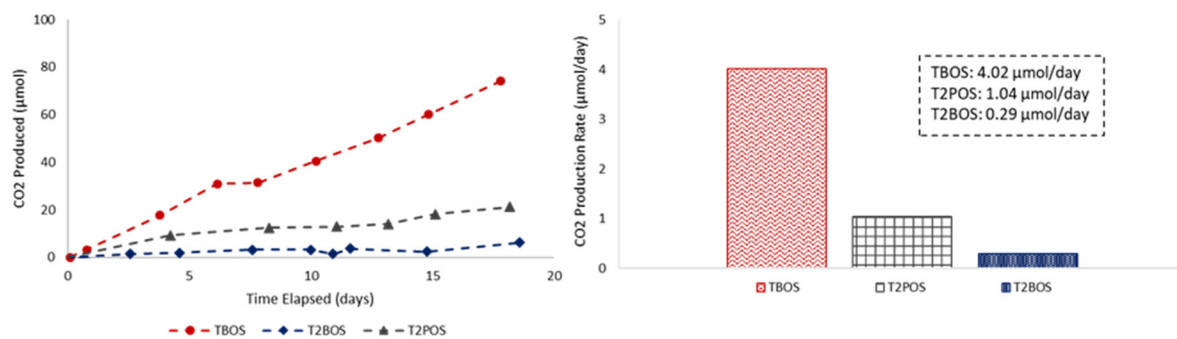


Figure S 6.4. Production of CO₂ from the release of alcohols from TBOS, T2BOS, and T2POS.

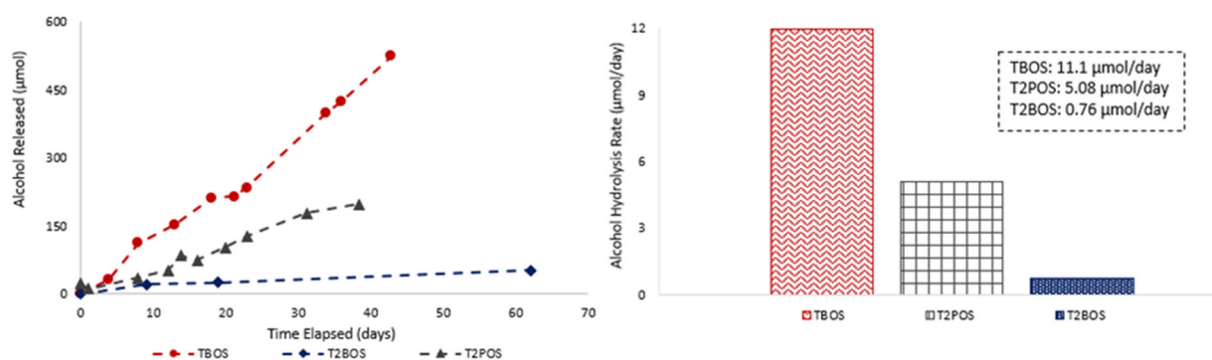


Figure S 6.5. Production of alcohols observed in the poisoned controls for TBOS, T2BOS, and T2POS.

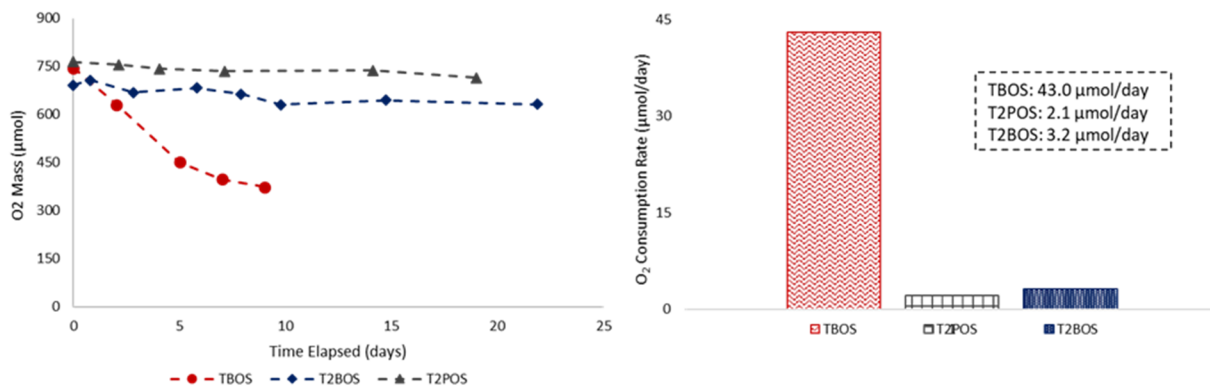


Figure S 6.6. Consumption of O₂ from the release of alcohols from TBOS, T2BOS, and T2POS.

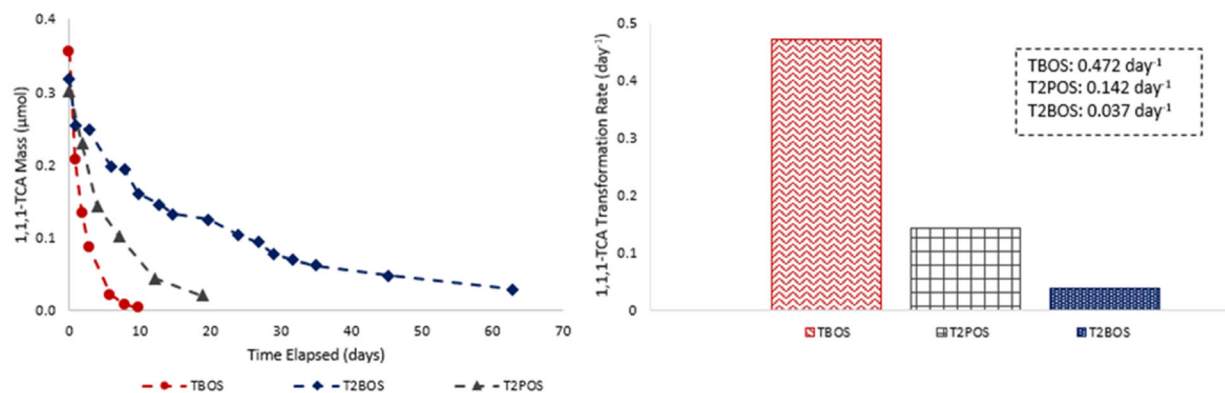


Figure S 6.7. Transformation of 1,1,1-TCA by *R. rhodochrous* ATCC 21198 grown on TBOS, T2BOS, or T2POS. These rates are for 1 active bottle on the third addition for TBOS and T2BOS and on the second addition of T2POS. TBOS provides the fastest transformation rates, followed by T2POS grown *R. rhodochrous* ATCC 21198.

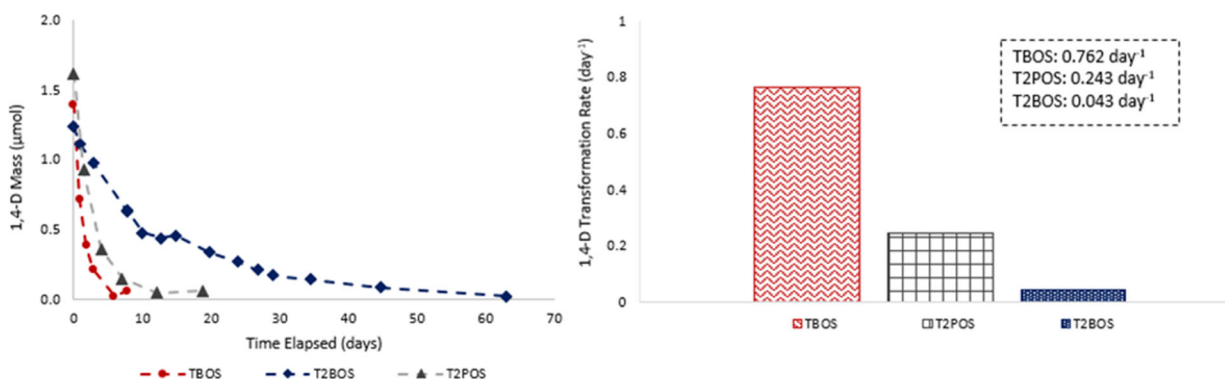


Figure S 6.8. Transformation of 1,4-D by *R. rhodochrous* ATCC 21198 grown on TBOS, T2BOS, or T2POS. These rates are for 1 active bottle on the third addition for TBOS and T2BOS and on the second addition of T2POS. TBOS provides the fastest transformation rates, followed by T2POS grown *R. rhodochrous* ATCC 21198.

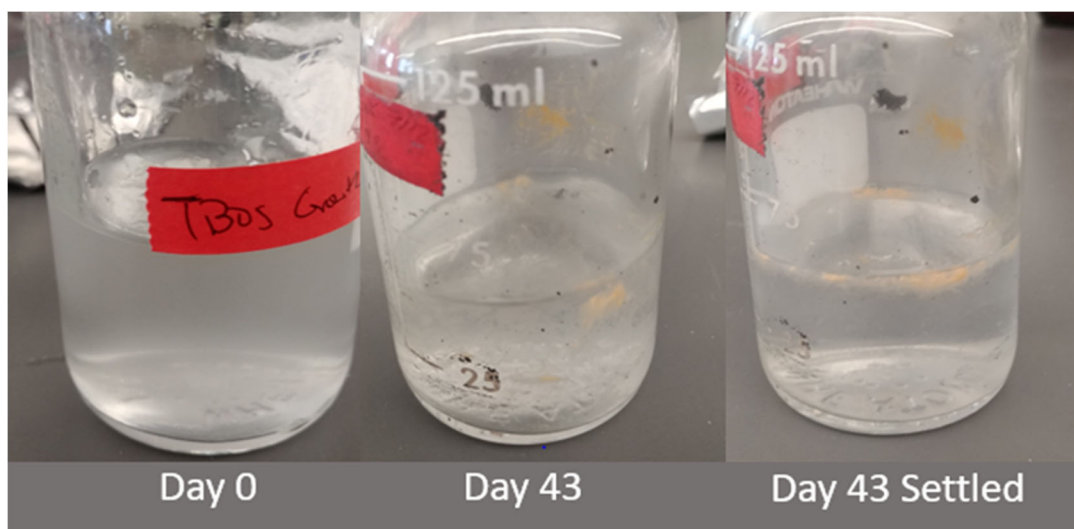
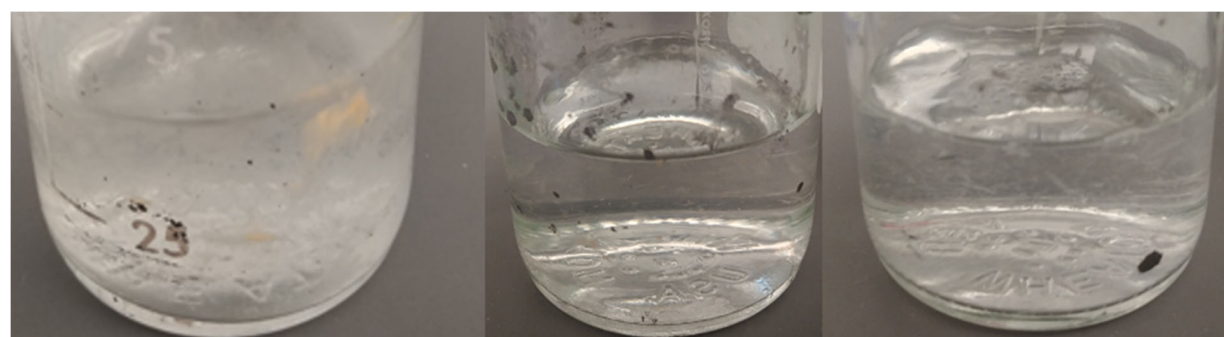


Figure S 6.9. Reactor containing TBOS and *R. rhodochrous* ATCC 21198 on day 0 and day 43 of incubation. The colloidal dispersion of TBOS is shown on day 0. The orange color of *R. rhodochrous* ATCC 21198 is shown on day 43. Growth around particles of TBOS are indicated.



TBOS Day 43

T2POS Day 61

T2BOS Day 145

Figure S 6.10. Reactor containing TBOS, T2POS and T2BOS after 43, 61, and 145 days of incubation, respectively. Note the denser and more orange color and TBOS reactor resulting from the higher biomass present compared to the T2POS and T2BOS reactors.

7. Chapter 7 Supplementary Information

Alginate SRC Encapsulation

The methods used to encapsulate LNAPL SRCs in alginate were adapted from Soliman et al. who developed an emulsification based method to encapsulate essential oils at mass loadings as high as 30% (w/w) in alginate microspheres, ~500um in diameter [93]. The final method followed the same approach to make 2% (w/v) alginate pre-gel solution as section 3.4.1 above. Alginate was added to heated Nanopure and mixed for 30 minutes to complete hydration, and was then autoclaved. After removing the alginate pre-gel solution from the autoclave a known amount, typically around 40-50mL, was transferred to a 125mL wide mouth glass vial and allowed to cool to ~70°C.

After the solution cooled, an addition of Span-80 emulsifier was made to achieve a concentration of 0.1% (v/v). A known volume of LNAPL SRC was then added to the pre-gel solution and the mixture was emulsified using an IKA RW 20 digital overhead impeller mixer at 2500rpm for 10 minutes. Following emulsification, the pre-gel solution was transferred to a 50mL Falcon tube and allowed to cool to ~45°C. The pH was then adjusted to seven by addition of pH-7 phosphate buffer to achieve a final concentration of 4mMol total phosphate. If co-encapsulation of cells was desired a known volume of concentrated cell slurry suspended in 50mMol phosphate buffer was added here to the SRC containing alginate pre-gel solution and vortexed for 30 seconds. Following the addition of cells or just after pH adjustment, the emulsified pre-gel solution was transferred to 5-20mL liquid syringes fitted with a 25 gauge x 1 inch needle (Figure S7.1). The pre-gel solution was then extruded into 900mL of 0.25% (w/v) CaCl₂ solution from a distance of 2-5cm above liquid surface. CaCl₂ solution was continuously mixed with a magnetic stir plate at 150-250rpm. The beads were allowed to crosslink for a total of 60 minutes measured from the time that the last bead was formed. Figure S7.2 shows the uniform size of the beads that were formed.

Soliman, E.A., El-Moghazy, A.Y., El-Din, M.S.M., and Massoud, M.A. (2013). Microencapsulation of Essential Oils within Alginate: Formulation and Evaluation of Antifungal Activity. *J. Encapsulation Adsorpt. Sci.* 03, 48–55.



Figure S 7.1. Calcium-alginate macrobead formation by extrusion into crosslinking solution.



Figure S 7.2. Gelated alginate macrobeads with a VWR ruler for scale.

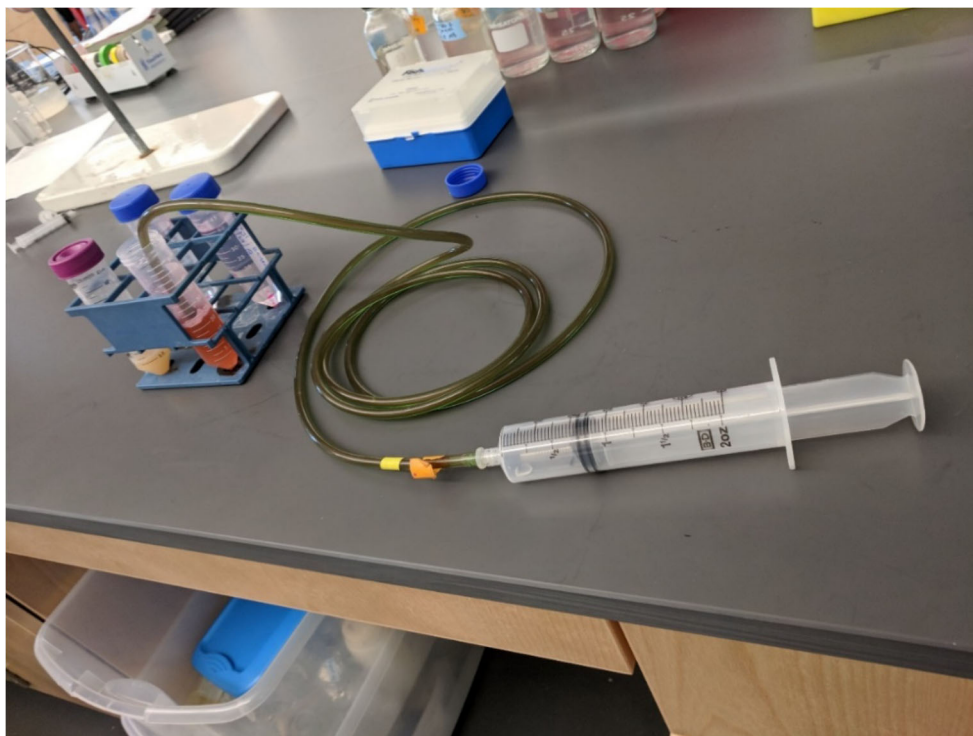


Figure S 7.3. Gellan gum macrobead generation step 1. Warm gellan-gum pre-gel solution is drawn into the green tubing with a 60mL syringe. For better visualization GG pre-gel solution was dyed orange.

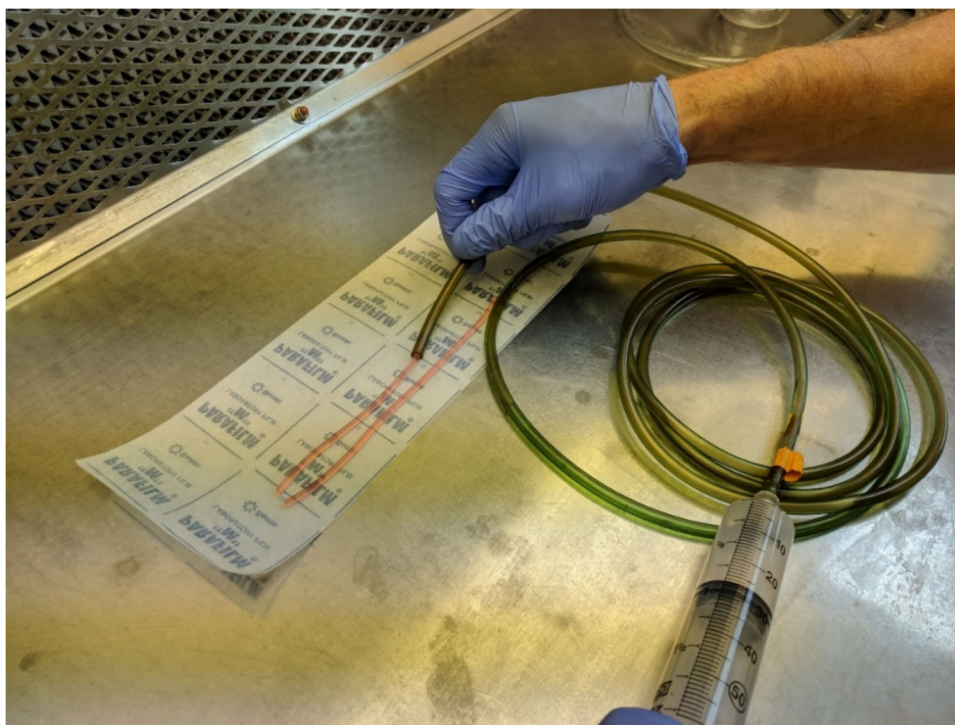


Figure S 7.4. Extrusion of solidified gellan-gum cylindrical sections from rubber tubing onto Parafilm. For better visualization GG pre-gel solution was dyed orange.



Figure S 7.5. Manual cutting of gellan-gum strands into 2mm right cylinders for experimental use. For better visualization GG pre-gel solution was dyed orange.



Figure S 7.6. Gellan-gum beads with *R. rhodochrous* ATCC 21198 and TBOS co-encapsulated 2 mm in diameter by 2 to 3 mm in length.

Table S 7.1. Encapsulated cell viability assessment. Percent difference is calculated as the percent change from suspended cell utilization rates to encapsualted cell utilization rates.

Encapsulation Matrix/Method	Benchmark		Encapsulated Cell Utilization Rate ($\mu\text{mol/day/mg}$)	Percent Difference
	Suspended Utilization ($\mu\text{mol/day/mg}$)	Cell Rate		
Alginate Macro-bead	15.78		14.19	-10.1%
Gellan-gum Macro-bead	13.33		13.25	-0.6%
Gellan-gum Micro-bead	11.11		11.03	-0.7%

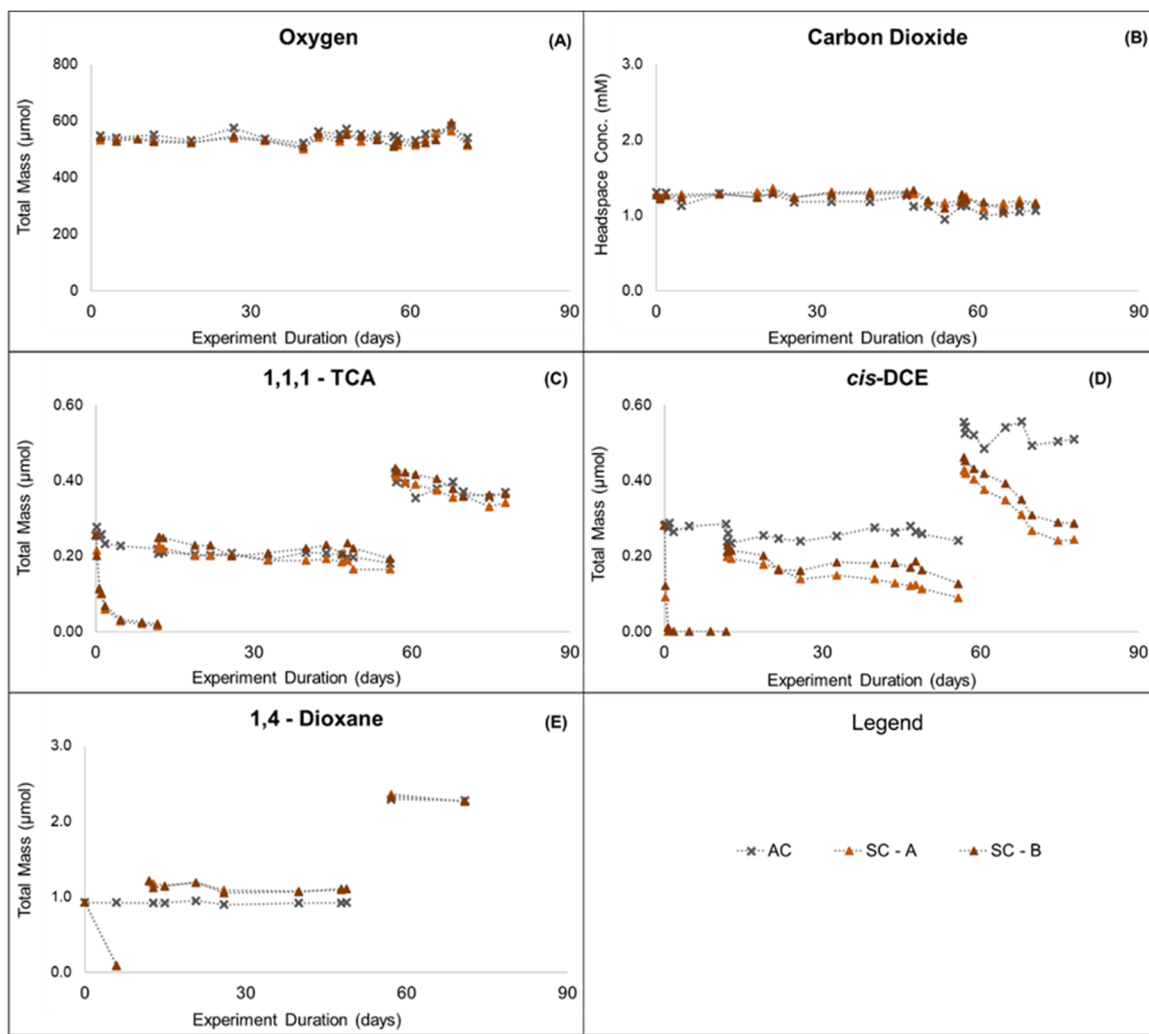


Figure S 7.7. Suspended Cell Control Reactor respiration data (A and B) and contaminant data (C-E). (AC) – Abiotic control. (SC) – Suspended cell control reactors. Alphabetical designations (A,B,C) are used to signify replicate reactors. AC has a single reactor, SC are duplicate reactors with suspended cells.

Table S 7.2. Rates of oxygen consumption in the CET reactors.

O2 Addition	Method	Time of Addition (days)	Estimated O2 Utilization Rate ($\mu\text{mol/day}$)	
			CET - A	CET - B
0	Initial O2	0	6.0	9.2
1	Pure O2 Addition 1	40	18.6	38.1
2	Pure O2 Addition 2	47	23.1	40.5
3	Atmosphere Headspace Equilibration	57	18.3	34.5

Table S 7.3. Rates of oxygen consumption in the CET₂ reactors.

Treatment	Abbreviation	O2 Utilization Rate ($\mu\text{mol/day}$)
Abiotic Control	AC	0.04 $\pm$ N/A
Suspended Cell Remediation Control	SCRC	0.09 $\pm$ 0.02
C-encapsulated T2BOS/ATCC 21198	CET2	0.48 $\pm$ 0.003

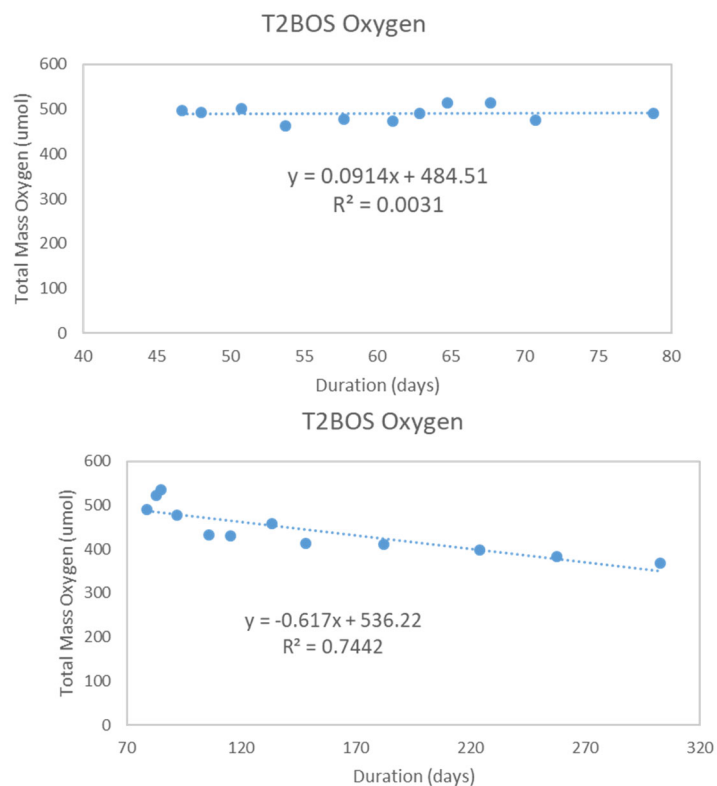


Figure S 7.8. Rates of O₂ consumption in the CET₂ reactors with GG beads co-encapsulated the *R. rhodochrous* ATCC 21198 and T2BOS. Rates were determined over the early and later time periods of the incubation.

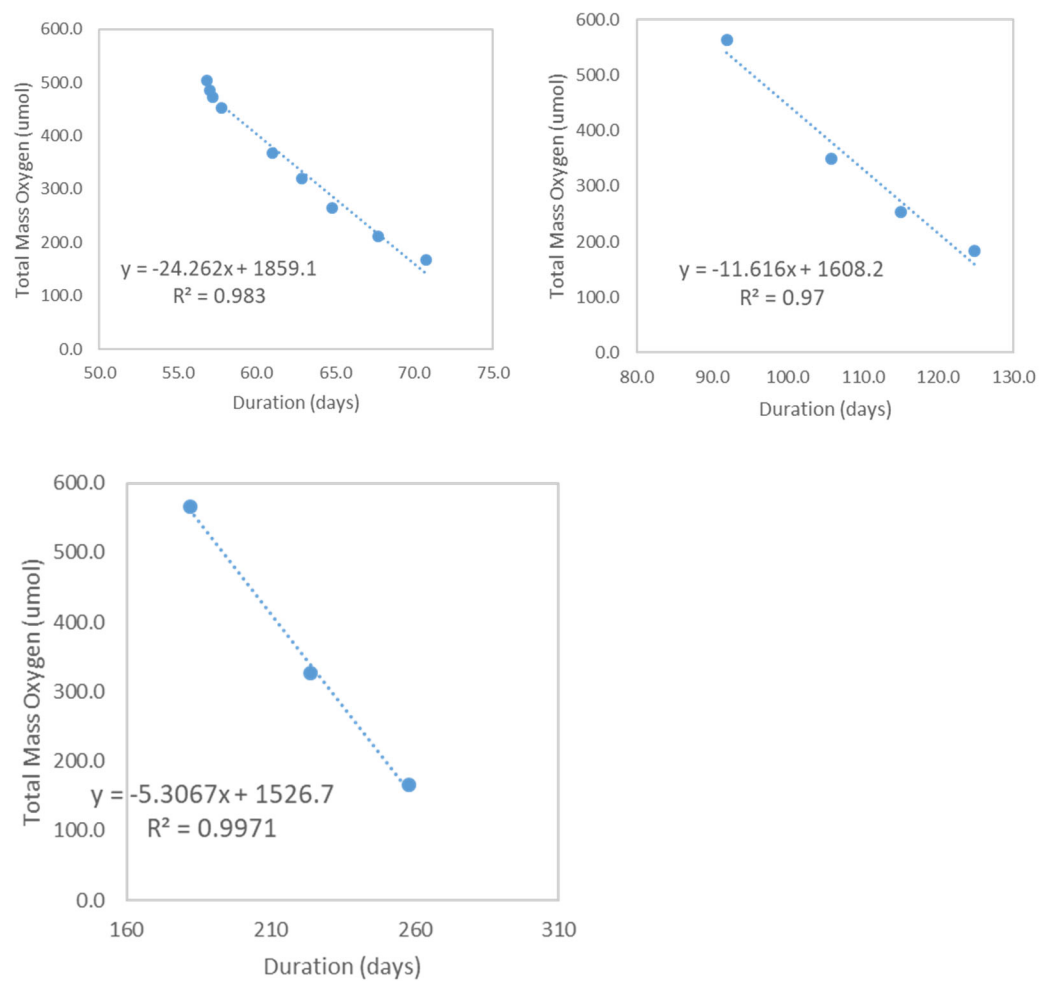


Figure S 7.9. Rates of O₂ consumption in the CET reactors. Rates were determined for different periods of 300 day incubation and show a gradual slowing in the rate of O₂ consumption.

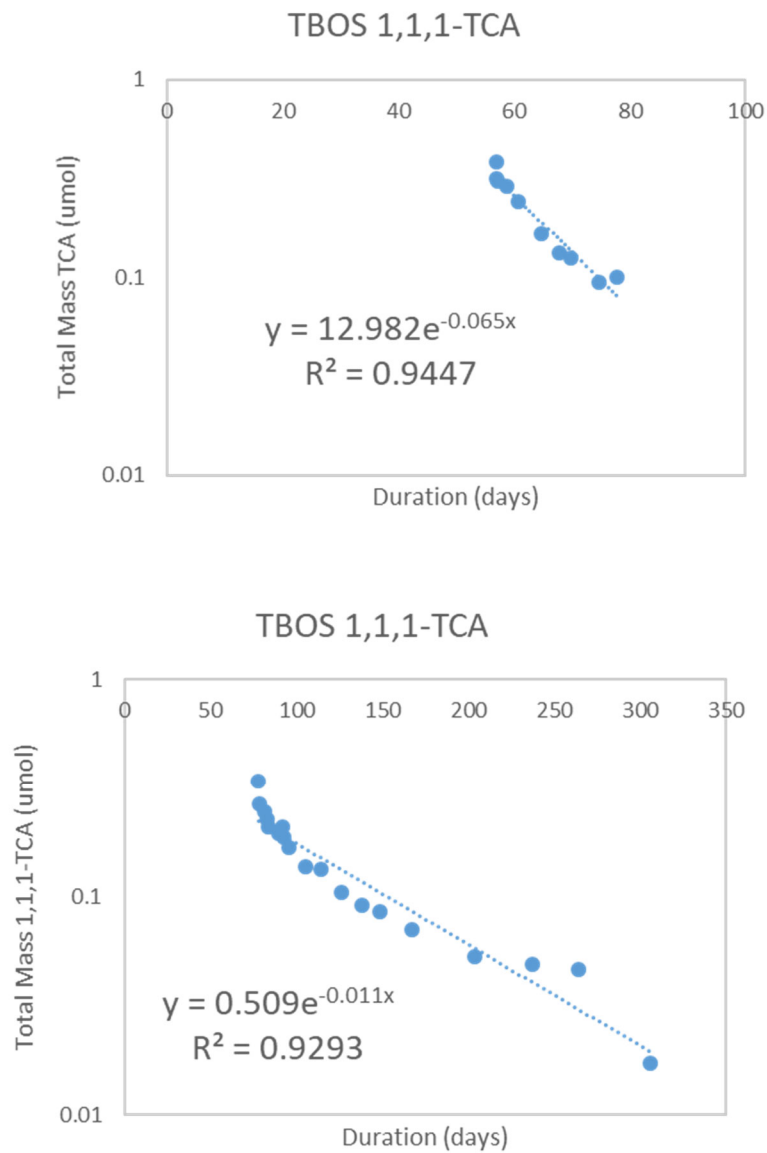


Figure S 7.10. First-order plots of 1,1,1-TCA concentration versus time in a CET reactor with GG beads co-encapsulated the *R. rhodochrous* ATCC 21198 and TBOS. Rates were determined for different periods of 300 day incubation and show a slowing in the rate of 1,1,1-TCA transformation with time.

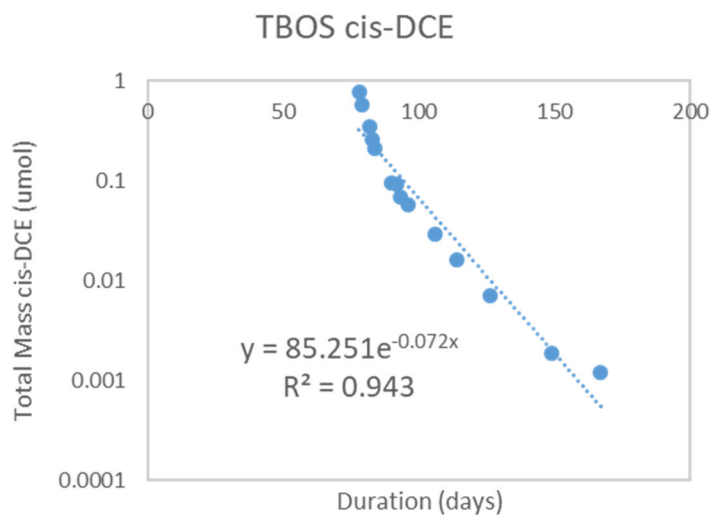
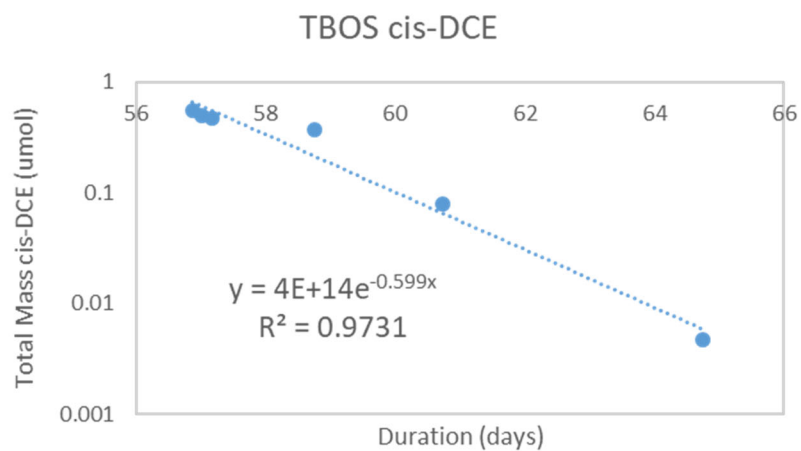


Figure S 7.11. First-order plots of cis-DCE concentration versus time in a CET reactor with GG beads co-encapsulated the *R. rhodochrous* ATCC 21198 and TBOS. Rates were determined for different periods of 300 day incubation and show a slowing in the rate of *cis*-CCE transformation with time.

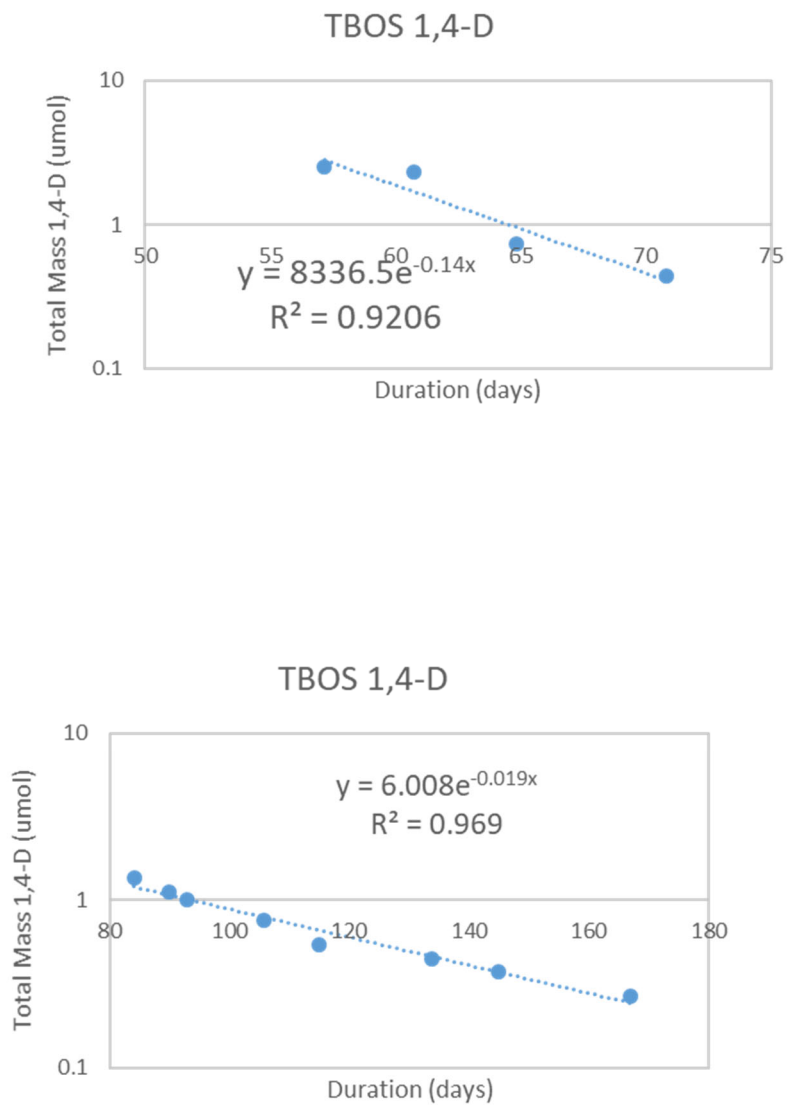


Figure S 7.12. First-order plots of 1,4-D concentration versus time in a CET reactor with GG beads co-encapsulated the *R. rhodochrous* ATCC 21198 and TBOS. Rates were determined for different periods of 300 day incubation and show a slowing in the rate of 1,4-D transformation with time.

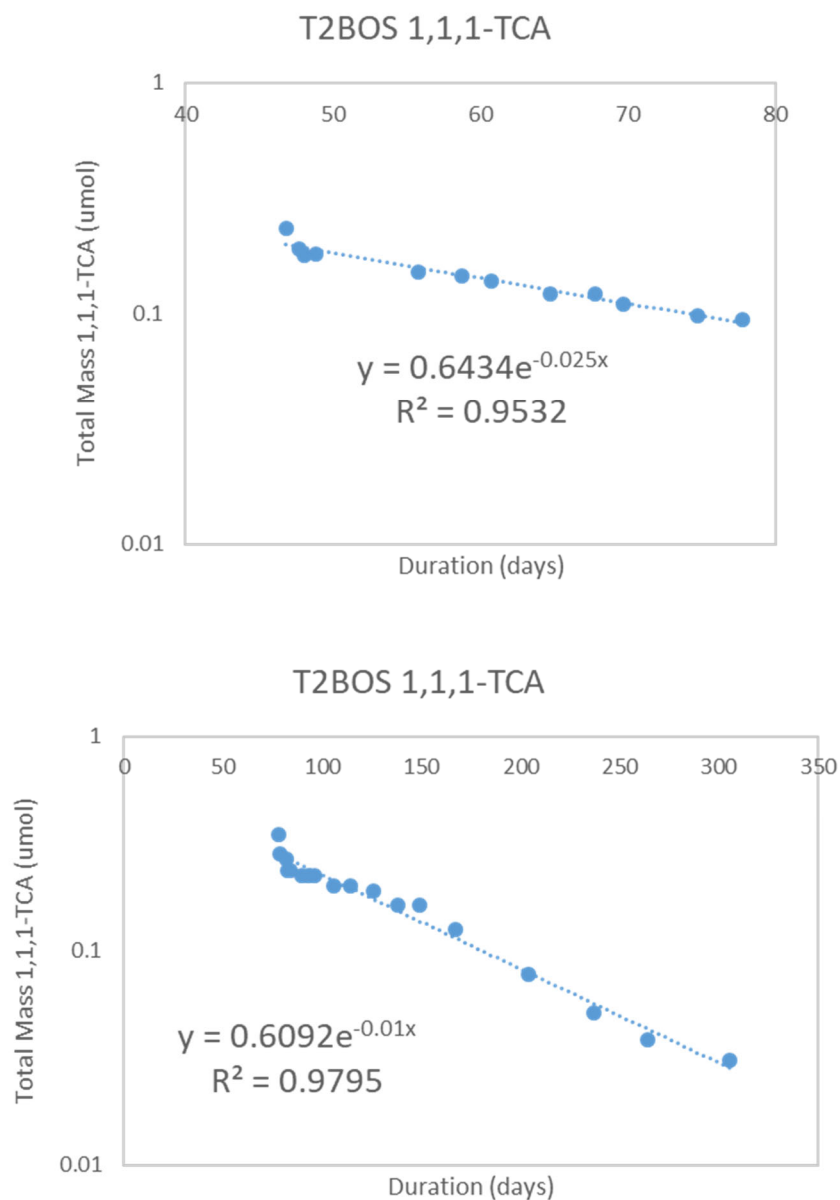


Figure S 7.13. First-order plots of 1,1,1-TCA concentration versus time in a CET₂ reactor with GG beads co-encapsulated the *R. rhodochrous* ATCC 21198 and T2BOS. Rates were determined for different periods of 300 day incubation and show a slowing in the rate of 1,1,1-TCA transformation with time.

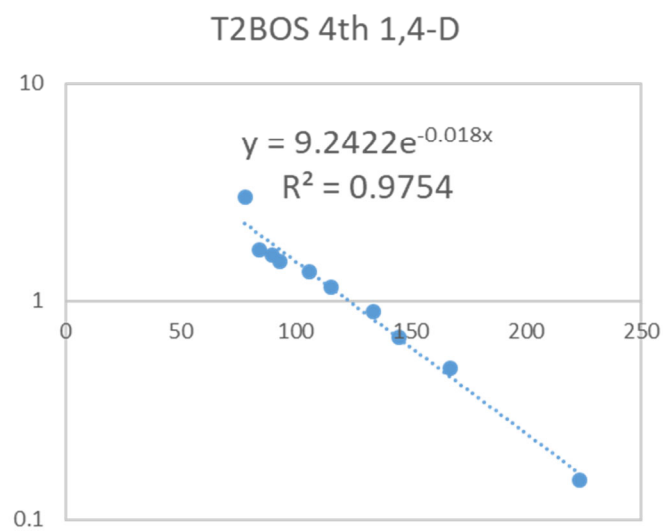
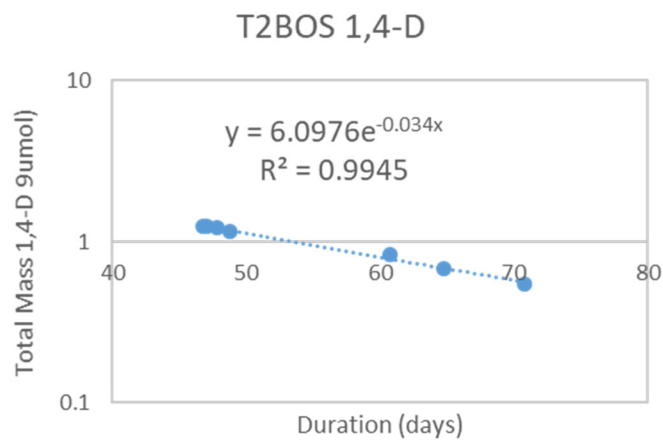


Figure S 7.14. First-order plots of 1,4-D concentration versus time in a CET2 reactor with GG beads co-encapsulated the *R. rhodochrous* ATCC 21198 and T2BOS. Rates were determined for different periods of 300 day incubation and show a slowing in the rate of 1,4-D transformation with time.

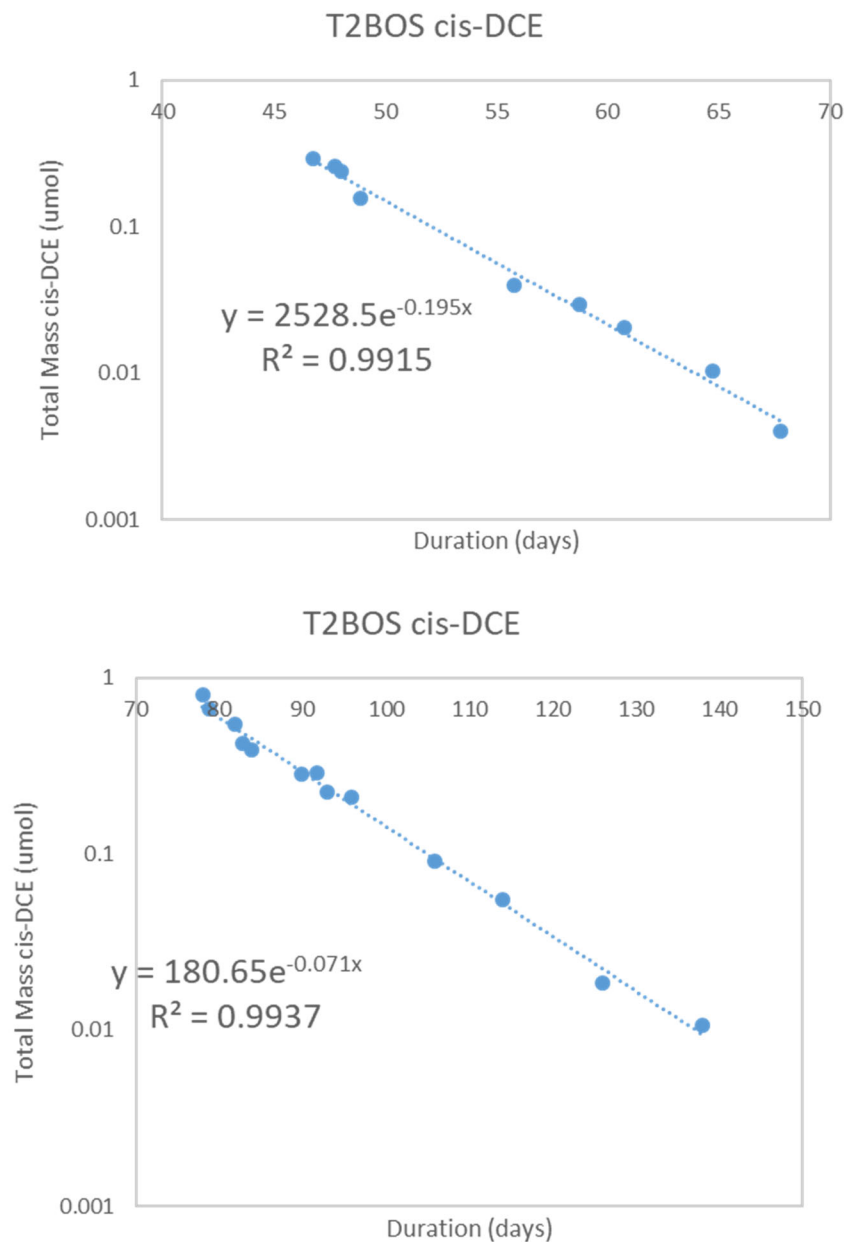
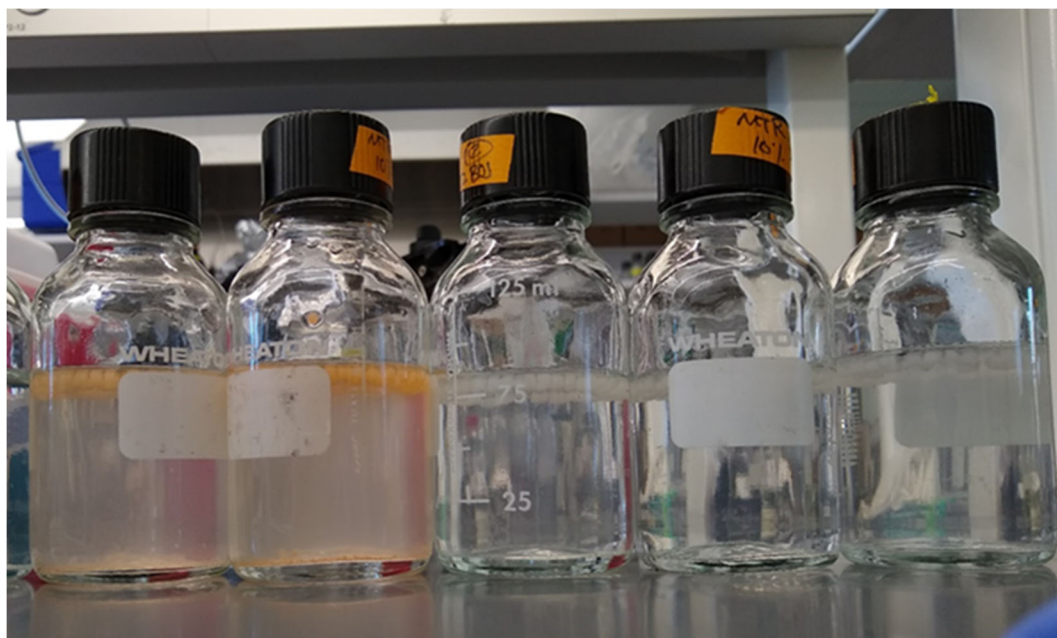


Figure S 7.15. First-order plots of *cis*-DCE concentration versus time in a CET₂ reactor with GG beads co-encapsulated the *R. rhodochrous* ATCC 21198 and T2BOS. Rates were determined for different periods of 300 day incubation and show a slowing in the rate of 1,4-D transformation with time.



TBOS/21198 (CET) Reactors

T2BOS/21198 (CET2) Reactors

Figure S 7.16. Two CET reactors with the TBOS/21198 GG beads (on the left) and three CET2 reactors T2BOS/21198 GG beads (CET₂ reactors on the right) after 232 days of incubation on a shaker table operating at 20° C and 100 rpm.

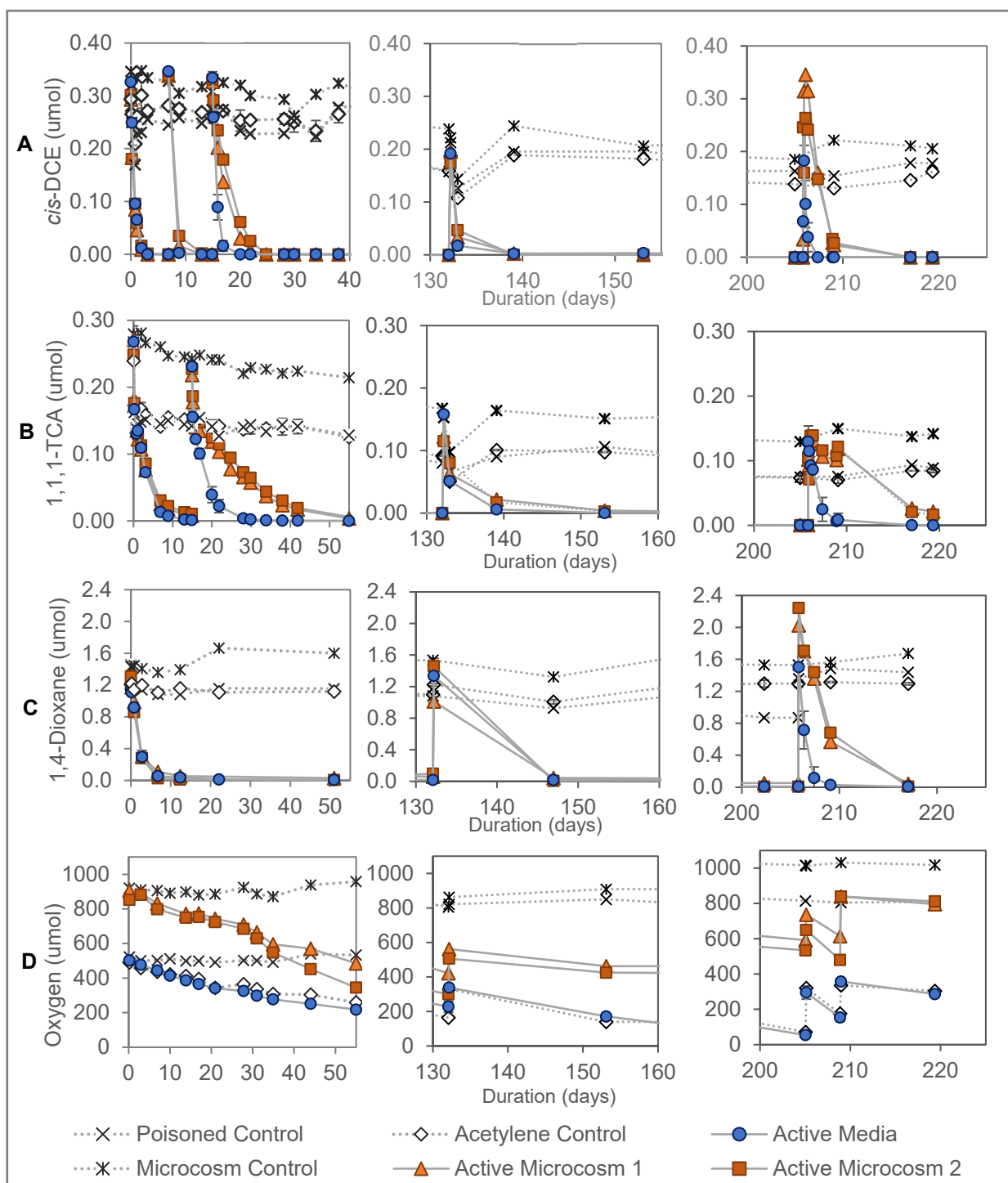


Figure S 7.17. Longer-term incubation of groundwater microcosms and media with TBOS and *R. rhodochrous* ATCC 21198 in GG beads. 2 grams of GG beads containing 8% TBOS by WT were added to the microcosm and media batch reactors. The panel on the left presents results presented in Figure 7.9.

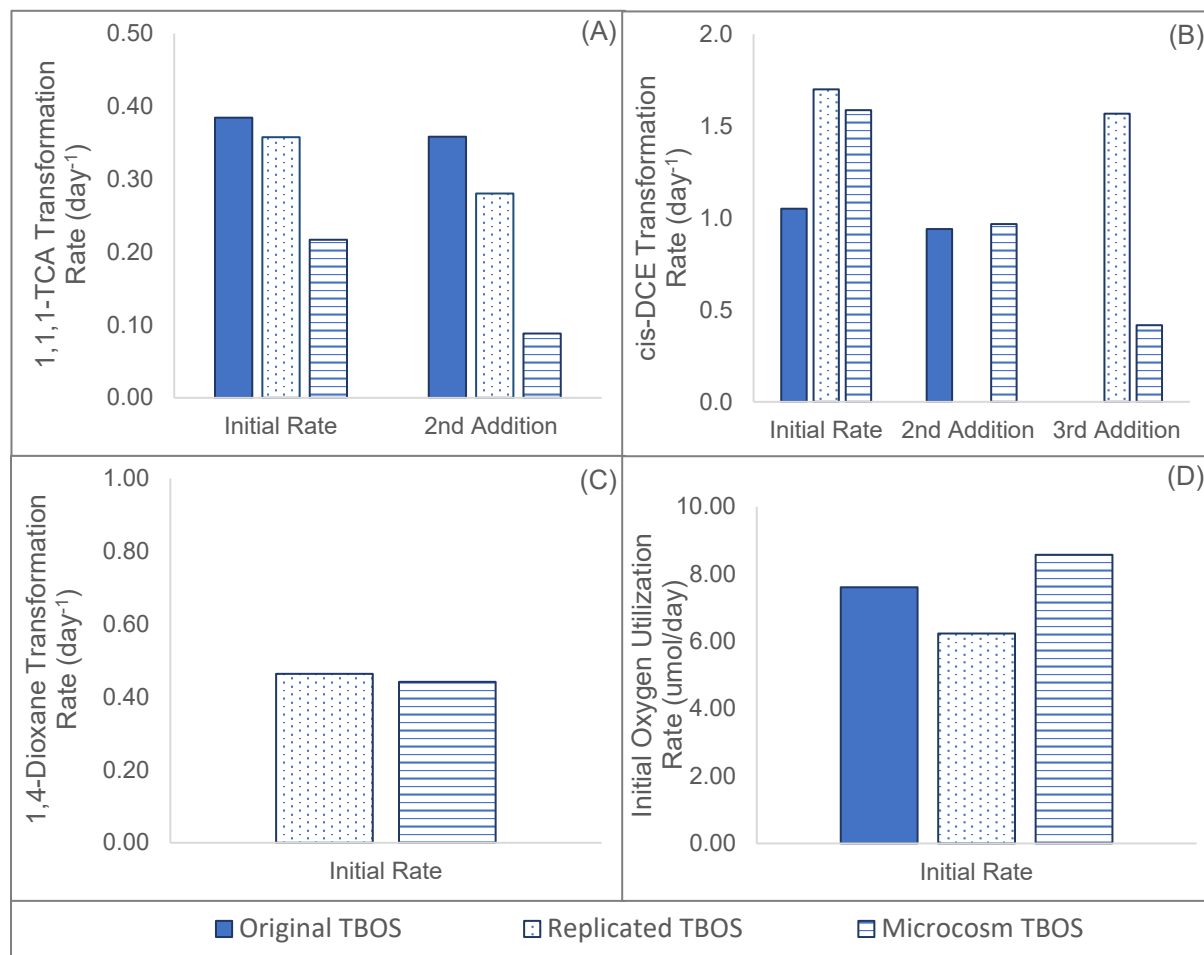


Figure S 7.18. Pseudo-first order rate comparison for 1,1,1-TCA, cis-DCE and 1,4-dioxane between gellan-gum coencapsulated beads containing 8% TBOS by WT. (Original TBOS) – Coencapsulated cells with 8% (wt/wt) TBOS, the data which is presented in Figure 4. (Replicated TBOS) - Coencapsulated cells with 8% (wt/wt) TBOS that were made separately to those used in Figure 4 to test replicability. (Microcosm TBOS) - Coencapsulated cells with 8% (wt/wt) TBOS in a microcosm system. Time of contaminant additions are approximately the same between treatments. Missing rate data for some additions of cis-DCE is due to inability to calculate a rate from the amount of data collected.

9. Chapter 9 Supplementary Information

Table S 9.1. Synthetic groundwater chemistry of the continuous physical model inlet feed stream.

Total Ion Concentration	
Ion	Concentration mg/l
Mg	100.9
Na	110.925
Ca	190.5
SO4	733.618
Cl	92.52113
CO3	285.71
K	3.147
Mn	0.182
NH4	0.675
NO3	2.324
F	0.792
NO2	0.667
Cr	0.00254
Cd	0.00307
P	0.045
Fe	0.084

Table S 9.2. Estimates of Flow through the Physical Aquifer Model

Dimensions:

Dimension of Model Reactor: H = 20 in., W = 8 in., L = 39.5 in.

Total Volume of the model = $20 \times 8 \times 39.5 \text{ in}^3 = 6320 \text{ in}^3$ or about 100 L

Dimension of Bio-reactor: R = 1 in., H = 10 in., 3X

Volume of the three screen reactors = 94.2 in^3 (1.5L) in total, about 0.5 L per bio-reactor.

Flow Calculations:

Assumptions/Operating Parameters:

- The effective volume in the model is about 30% due to the sand-pack.
- The effective volume in the bio-reactors is about 70%, since it is packed with gellan-gum beads.
- Groundwater flowrate of 2.88L/day and is evenly distributed across the model influent
- The isobutene experiment uses the top portion of the reactor (reactor is divided by clay layer) with a total volume of approx. 50 L.
- Velocity will be relatively constant at all heights of the bio-reactor.
- 80% of the flow will be directed into the bio-reactor by the Funnel/Gate

Hydraulic retention time in active Model:

$$\tau_{\text{model}} = V_{\text{eff, model}} / Q = \epsilon_{\text{sand}} V_{\text{model}} / Q_{\text{model}}$$

continuous

Hydraulic retention time in packed bio-reactor:

$$\tau_{\text{reactor}} = V_{\text{eff, model}} / Q = \epsilon_{\text{Gellan-gum}} V_{\text{reactor}} / Q_{\text{bio-reactor}}$$

$$Q_{\text{Bio-reactor}} = 0.8 Q_{\text{model}}$$

$$\tau_{\text{bio-reactor}} = (0.70)(0.5 \text{ L}) / (0.8)(2.88 \text{ L/day}) = \mathbf{0.15 \text{ days}}$$

$$\tau_{\text{model}} = (0.30)(50 \text{ L}) / (2.88 \text{ L/day}) = \mathbf{5.21 \text{ days}}$$

Appendix B: Publications

Publications

Rasmussen, M.T., A.M. Saito, M. R. Hyman, L. Semprini. Co-encapsulation of slow release compounds and *Rhodococcus rhodochrous* ATCC 21198 in gellan-gum beads to promote the long-term aerobic cometabolic transformation of 1,1,1-trichloroethane, cis-1,2-dichloroethene and 1,4-dioxane. *Environmental Science Processes & Impacts* Vol 22(3):771-791 (2020).

Murnane R.A, W. Chen, M. R. Hyman, L. Semprini. Long-Term Cometabolic Transformation of 1,1,1-Trichloroethane and 1,4-Dioxane by *Rhodococcus rhodochrous* ATCC 21198 Grown on Alcohols Slowly Produced by Orthosilicates. *Journal of Contaminant Hydrology* Vol 240:1-14: (2021) doi.org/10.1016/j.jconhyd.2021.103796

Saito, A., A. Del Pila Oyarzun Mejia, M. R. Hyman, L. Semprini. Chlorinated Ethene Cometabolic Degradation by *Burkholderia vietnamiensis* G4 Grown on Benzyl Alcohol as a Toluene Alternative. In preparation for submission to: *Applied and Environmental Microbiology*.

Mohammad, A., M.R. Hyman, L. Semprini. Cometabolic Treatment of Cis-dichloroethene, 1,1,1-Trichloroethane, and 1,4-Dioxane in Columns Packed Gellan-gum Beads Co-encapsulated with Slow Release Compounds and *Rhodococcus rhodochrous* ATCC 21198. In preparation for submission to: *Environmental Science and Technology*.

M.S. Thesis

Murnane, R.A. Long-Term Transformation of 1,4-Dioxane and 1,1,1-Trichloroethane by *Rhodococcus rhodochrous* in the Presence of Alcohol-Producing Slow-Release Compounds, M.S Thesis Environmental Engineering, Oregon State University, December 7, 2018.

Rasmussen, M.T. Co-Encapsulation of Slow Release Substrates and Microbial Cultures in Alginate and Gellan-gum Beads to Promote the Co-metabolic Transformation of 1,4-Dioxane and Chlorinate Aliphatic Hydrocarbons. , M.S Thesis Environmental Engineering, Oregon State University, September 14, 2018.

https://ir.library.oregonstate.edu/concern/graduate_thesis_or_dissertations/70795f07p

Saito, A.M. Cometabolism of Chlorinated Ethenes by *Burkholderia vietnamiensis* G4 Grown on Aromatic Substrates: Resting and Co-encapsulated Cell Kinetic Tests. M.S Thesis Environmental Engineering, Oregon State University, March 12, 2020.

https://ir.library.oregonstate.edu/concern/graduate_thesis_or_dissertations/k930c401h

Bealessio, A.D. Cometabolism of 1,4-Dioxane and Chlorinated Aliphatic Hydrocarbons by *Rhodococcus rhodochrous* ATCC 21198 and Coencapsulated with a Slow-Release Substrate in Microcosm and Column Studies. M.S Thesis Environmental Engineering, Oregon State University, March 18, 2021

https://ir.library.oregonstate.edu/concern/graduate_thesis_or_dissertations/8p58pm98t

Conference Abstracts and Presentations:

Rasmussen M., R. Murnane, L. Semprini, W. Chen, C. Smith, M. Hyman. Cometabolism of 1,4-Dioxane and 1,1,1-Trichloroethene by *Rhodococcus rhodochrous* 21198 Growing Slow-Release-Compounds that Produce 1-Butanol and 2-Butanol. SERDP-ESTCP Symposium, November 27-29, 2018, Washington, DC.

Semprini L., Invited Keynote Presentation, REMTEC, Cometabolism of 1,4-Dioxane and Chlorinated Solvent Mixtures by *Rhodococcus rhodochrous* 21198 Encapsulated in Hydrogels with Slow-Release-Compounds. REMTEC, Denver, Feb. 26-28, 2019.

Rasumussen M., R. Murnane, A. Sanito, M. Azizian and L. Semprini, Passive treatment of 1,4-Dioxane, cis-Dichloroethene and 1,1,1-Trichloroethene by *Rhodococcus rhodochrous* 21198 Growing on Slow-Release-Compounds, AEESP Research and Education Conference, ASU, May 14-16, 2019.

Semprini L., M. Rasumussen, R. Murnane, M. Azizian, C. Smith, Michael Hyman, Passive treatment of 1,4-Dioxane, cis-Dichloroethene and 1,1,1-Trichloroethane by *Rhodococcus rhodochrous* 21198 Growing on Slow-Release-Compounds, GQ 2119, Leige, Belgium Sept 9-12, 2019.

Semprini L., M. Rasumussen, R. Murnane, A. Saito, M. Azizian, W. Chen, C. Smith, M. Hyman, Development of Slow Release Compounds for the Aerobic Cometabolic Treatment of Complex Mixtures of COC Released from Low Permeability Zones. SERDP-ESTCP Symposium, Dec, 2-5, 2019 Washington, DC.

Semprini L., M. Rasumussen, R. Murnane, A. Saito, M. Azizian¹ C. Smith, M. Hyman, Cometabolic Treatment of 1,4-Dioxane, cis-Dichloroethene and 1,1,1-Trichloroethane by *Rhodococcus rhodochrous* 21198 Co-Encapsulated with Slow-Release-Compounds, AGU Fall Meeting, San Francisco, December 9 -13, 2019.

Azizian M., M.R. Hyman, L. Semprini, Passive Cometabolic Treatment of 1,4-Dioxane, cis-Dichloroethene, 1,1,1-Trichloroethane in continuous flow columns packed with gellan gum hydrogels bead co-encapsulated with *Rhodococcus rhodochrous* 21198 and Slow-Release-Compounds. ACS Fall 2020 Virtual Meeting and Expo, August 17-20.

Semprini L., M. Rasumussen, R. Murnane, A. Saito, M. Azizian, A. Bealesio, B. Jones, W. Chen, C. Smith, M. Hyman Development of Slow Release Compounds for the Aerobic Cometabolic Treatment of Complex Mixtures of COC Released from Low Permeability Zones. SERDP-ESTCP Symposium, Nov. 30-Dec. 4, 2020, Washington, DC.

Appendix C: Other Supporting Information

Patent Application. Bioremediation Using Co-metabolic Substrates. Semprini et al. US Patent Application: US 2020 01575523 A1, May 21, 2020.

Grant Award: Development of Passive and Sustainable Cometabolic Systems to Treat Complex Contaminant Mixtures by Encapsulating Microbial Cultures and Slow Release Substrates in Hydrogels. NIEHS-RO1. Semprini (Lead-PI); Rochefort (Co-PI); M. Hyman NCSU (Co-PI); \$1,440,000. Dec. 2020-Dec 2025.