AN ABSTRACT OF THE THESIS OF

Alyssa M. Saito for the degree of Master of Science in Environmental Engineering presented on March 12, 2020.

Title: Cometabolism of Chlorinated Ethenes by *Burkholderia vietnamiensis* G4 Grown on Aromatic Substrates: Resting and Co-encapsulated Cell Kinetic Tests

Abstract approved: _____

Lewis Semprini

The cometabolic ability of *Burkholderia vietnamiensis* G4 grown on aromatic substrates was first assessed by resting suspended cells, and subsequently for cells encapsulated with and without slow release compounds (SRCs). In Chapter 3, benzyl alcohol was assessed as a food-grade growth substrate for promoting cometabolism of trichloroethene (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) Activity based labeling (ABL); a gel assay method used to identify catalytically active monooxygenases (*e.g.* T2MO), and 2) resting suspended cell kinetic tests. 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 kinetic 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 results 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 benzyl alcohol or toluene and TCE were performed to determine whether, like toluene, benzyl alcohol competitively inhibits contaminant transformation by the 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 1,2-*cis*-dichloroethene (*cis*-DCE), 1,1-dichloroethene (1,1-DCE), and vinyl chloride (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.

In Chapter 4, the cometabolic activity of encapsulated cells of *Burkholderia vietnamiensis* G4 was first determined through kinetic tests in batch systems over time. A singlet bottle of encapsulated *Burkholderia vietnamiensis* G4 saw transformation of TCE over 400 days with two external additions of toluene to re-stimulate cometabolic activity after the transformation capacity of resting cells was reached. Encapsulated cells at a higher biomass loading of 8 mg_{TSS}/g_{bead} verified that long-term transformation of a mixture of TCE and 1,1-DCE could be achieved over 100 days through external additions of toluene to recommence cometabolism. Given these results, possible SRCs were investigated for a co-encapsulated system with both cells and SRC present in the hydrogel bead. The two SRCs assessed were benzyl butyrate and tetraphenoxysilane (TPhOS), which hydrolyze at the ester bonds to produce benzyl alcohol and phenol, respectively. Though it was shown in the resting cell kinetic tests that benzyl butyrate is a promising substrate for supporting cometabolism, when co-encapsulated, it hydrolyzed too rapidly indicated by a high oxygen uptake rate, making it an unsuitable SRC for *in situ* use. TPhOS, which hydrolyzes to produce phenol, was co-encapsulated at 1.9% (w/w) and 0.5 mg_{TSS}/g_{bead}. TCE transformation was achieved for 180 days by the co-encapsulated cells with a minimal oxygen uptake rate for the majority of transformation. This setup was repeated (1.9% TPhOS and 0.5 mg_{TSS}/g_{bead}) in a microcosm system with a

TCE and *cis*-DCE mixture, and while TCE rates were lower and the oxygen uptake rate was higher than in media, TCE and *cis*-DCE were transformed for 100 and 175 days, respectively. TPhOS as a SRC provided proof of concept that a co-encapsulated system with *Burkholderia vietnamiensis* G4 could promote long-term transformation of chlorinated ethenes in both batch and microcosm systems.

Cometabolism of Chlorinated Ethenes by *Burkholderia vietnamiensis* G4 Grown on Aromatic Substrates: Resting and Co-encapsulated Cell Kinetic Tests

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I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

Alyssa M. Saito, Author

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ABBREVIATIONS

ABL	Activity Based Labeling
CR	Carbon tetrachloride
CAHs	Chlorinated Aliphatic Hydrocarbons
CET	Co-encapsulated Treatment
DNAPL	Dense Non-Aqueous Phase Liquid
cis-DCE	1,2-*cis*-Dichloroethene
trans-DCE	1, 2-trans-Dichloroethene
1,1-DCE	1,1-Dichloroethene
ET	Encapsulated Treatment
GC	Gas Chromatography
2HBA	2-hydroxy benzyl alcohol
LPZ	Low Permeability Zone
MCL	Maximum Contaminant Level
MCLG	Maximum Contaminant Level Goal
MSM	Mineral Salt Media
17OD	1,7-Octadiyne
OD_{600}	Optical Density at 600 nm
PCE	Perchloroethene
PB	Phosphate Buffer
SRC	Slow Release Compound
TBOS	Tetrabutoxysilane
T2BOS	Tetra-*sec*-butoxysilane
TKEBS	Tetrakis(2-ethylbutoxy)silane
TPhOS	Tetraphenoxysilane
T2MO	Toluene-2-monooxygenase
TSS	Total Suspended Solids
1,1,1-TCA	1,1,1-Trichloroethane
TCE	Trichloroethene
US EPA	United States Environmental Protection Agency
VC	Vinyl Chloride
VOC	Volatile Organic Compound

CHAPTER 1 — INTRODUCTION

As the demand for drinking water increases due to a growing global population, groundwater as a drinking water source is critical. However, many aquifers across the nation are contaminated and require remediation in order to reach established drinking level standards. Chlorinated solvents are pervasive groundwater contaminants found in aquifers across the country, and largely consist of chlorinated aliphatic hydrocarbons (CAHs). Four chlorinated solvents—carbon tetrachloride (CT), tetrachloroethene (PCE), 1,1,1-trichloroethane (1,1,1-TCA) and trichloroethene (TCE)—were the most prevalent in industry historically, and hence are the four most ubiquitous chlorinated solvents in aquifers across the nation (McCarty & Semprini, 1993). The primary contaminant of interest in this study is TCE, which was the most frequently detected volatile organic carbon (VOC) at concentrations of potential human-health concern measured in over 3500 groundwater samples from various wells across the country from 1985-2001 (Zogorski, 2006). Other CAHs included in this study are the dichloroethene isomers, 1,1-dichloroethene (1,1-DCE) and 1,2-*cis*-dichloroethene (*cis*-DCE), as well as vinyl chloride (VC). 1,1-DCE and VC were the third and eighth most detected VOCs at concentrations of potential health concern (Zogorski, 2006). The CAHs of interest in this study are shown in ***Figure 1.1***.

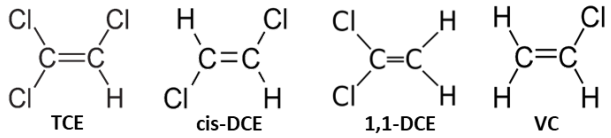

Figure 1.1 *Contaminants of interest in this study including trichloroethene (TCE), 1,2-cis-dichloroethene (cis-DCE), 1,1-dichloroethene (1,1-DCE), and vinyl chloride (VC).*

Contaminants of Interest: Use and Production

TCE has been used for industrial purposes since the early twentieth century primarily in the dry-cleaning industry and for vapor degreasing, with peak production in the late 1960s (ATSDR, 2019b). The current predominant use of TCE is for the manufacturing of hydrofluorocarbon (HFC-134a)—an alternative refrigerant to CFC-12—accounting for 83.6% of use in 2015 (US EPA, 2017). TCE was also used historically in textile processing, food processing, medical applications, as a chemical intermediate, and in

a variety of consumer products (US EPA, 2000). As for the other contaminants in this study, VC was also used as a refrigerant and is currently used primarily to produce polyvinyl chloride (*i.e.* PVC) (ATSDR, 2006). Additionally, both *cis*-DCE and 1,1-DCE were used as a refrigerant, extraction solvent, and as an intermediate in chemical synthesis (ATSDR, 1996, 2019a). During the twentieth century, disposal of waste into landfills, lagoons, waste pits, and storage tanks have led to the ubiquitous presence of these CAHs in aquifers today (Stroo & Ward, 2010).

In addition to industrial sources, various CAHs may be formed as degradation by-products under biotic and abiotic processes. Sequential reduction of PCE can occur under anoxic conditions by microorganisms to produce TCE, *cis*-DCE (or *trans*-DCE and 1,1-DCE to a lesser extent), and VC (Freedman & Gossett, 1989; Semprini et al., 1995). A relevant abiotic process is the transformation of 1,1,1-TCA to 1,1-DCE in groundwater (Vogel & McCarty, 1987). Given the prevalence of 1,1,1-TCA in aquifers, the formation of 1,1-DCE is of concern given that it forms an extremely toxic epoxide (Dolan & McCarty, 1995). These degradation by-products can lead to complex mixtures of CAHs in the subsurface difficult to remediate.

Toxicity and Regulation

The CAHs in this study are toxic and have been related to various health problems, particularly affecting the liver. Additionally, TCE and VC are classified as carcinogenic and 1,1-DCE is classified as a possible carcinogen. Under the Clean Water Act all are regulated with Maximum Contaminant Levels (MCL) and Maximum Contaminant Level Goals (MCLG), which are shown in ***Table 1.1*** (US EPA, 1995a-d)

Table 1.1 *Regulatory levels and summarized toxicity effects (US EPA, ATSDR).*

Contaminant	MCL (ppb)	MCLG (ppb)	Health	Carcinogenicity
TCE	5	0	Associated with liver, kidney, immune system, central nervous system problems	Carcinogenic
cis-DCE	70	70	Associated with liver problems	Inadequate info to assess
1,1-DCE	7	7	Associated with liver, central nervous system, and lung problems	Possibly carcinogenic
VC	2	0	Liver problems	Carcinogenic

Fate and Transportation

Sources of these CAHs into the environment include air emissions and deposition, landfill leachate, improper disposal practices, leaking storage tanks, and industrial waste (ATSDR, 1996, 2006, 2019a&b; McCarty & Semprini, 1993) In 2017, the estimated release of TCE to the soil was 52.5 metric tons, primarily from industrial discharges to surface waters and through landfill leachate (ATSDR, 2019b). The physical and chemical properties of TCE and the other CAHs of interest are shown in ***Table 1.2***.

Table 1.2 *Physical and chemical properties including molar mass, specific gravity, solubility, octanol-water partitioning coefficient, vapor pressure, and dimensionless Henry's constant (Gossett, 1987) of relevant CAHs in this study including TCE, 1,1-DCE, cis-DCE, and VC. Solubility, vapor pressure and log KOW obtained from (US EPA, 1995a-d).*

CAH	Molecular Weight (g/mol)	Specific Gravity at 20°C	Solubility at 25°C (mg/L)	H_{CC}	$LogK_{ow}$	Vapor Pressure (mmHg at 20°C)
TCE	131.39	1.46	1100	0.294	2.29	57.8
cis-DCE	96.95	1.26	3500	0.121	1.86	202
1,1-DCE	96.94	1.22	2500	0.848	1.48	591
VC	62.5	0.91	2700	0.892	0.6	2600

The chlorinated solvents in this study, except for VC, are dense non-aqueous phase liquids (DNAPL). As DNAPLs, they have a greater density than water and are only slightly soluble in water. As a DNAPL, vertical downward migration through the subsurface occurs until movement is impeded. DNAPL sources in the subsurface can ultimately become a long-term source of contamination resulting in extensive plume formation, as passing groundwater becomes saturated equal to the solubility limit. Transport may be slowed by sorption depending on the properties of the aquifer and the contaminant, particularly the organic carbon content of the soil and the octanol-water partitioning coefficient (K_{ow}) of the contaminant. As seen in ***Table 1.2***, the K_{ow} values, which is the distribution of a chemical between water and an organic phase, are above one and represent the degree of hydrophobicity. Hence, as non-polar organic compounds, sorption is an important process to consider for DNAPLs.

In addition, due to the historical use and ubiquitous contamination, diffusion into low-permeability zones (LPZ), such as clay layers, creates a long-term source of contamination difficult to remediate. As clean

groundwater flows over a LPZ contaminated with DNAPL, back-diffusion due to the concentration gradient, results in low-level contamination that can persist for years (Einarson & Mackay, 2001a). The last few decades have seen a shift from typical approaches of physical treatment for plumes and sources, such as pump-and-treat, that pumps contaminated groundwater to be treated ex situ, to more cost-effective plume treatment technologies (Stroo et al., 2012). Pump and treat requires long-term site occupation and could take decades or even centuries to treat source zones trapped in LPZs (Einarson & Mackay, 2001b).

Bioremediation of CAHs

A biological approach may be used to remediate CAHs utilizing microbial processes to treat contaminants in the subsurface. Multiple different strains of microorganisms have been studied for their ability to degrade TCE, both anaerobically through reductive dechlorination, and aerobically through aerobic cometabolism (Pant & Pant, 2010). As mentioned previously, reductive dechlorination involves sequential replacement of the chlorine atoms for hydrogen. This process can be enhanced for in situ treatment through the addition of organic substrates such as lactate, which produces hydrogen from fermentation to serve as electron donors. However, this process is often stalled at vinyl chloride in groundwater systems as reduction becomes energetically unfavorable for the microbes, as well as from severe competition from sulfate reducers and methanogens for hydrogen (Borden & Rodriguez, 2006; Semprini et al., 1995).

Another biological approach that results in mineralization of CAHs is aerobic cometabolism. Aerobic cometabolism is the fortuitous oxidation of contaminants by an oxygenase enzyme expressed in microorganisms due to growth on a substrate, and was the process used in this study (Arp et al., 2001). The degree of induction of the oxygenase enzyme is dependent on the growth substrate. This study focused on the microorganism *Burkholderia vietnamiensis* G4, which 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; M S Shields et al., 1995; Malcolm S Shields et al., 1989). 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; C. M. Yeager et al., 2001).

Typical bioremediation approaches for the treatment of contaminated aquifers include biostimulation and bioaugmentation. Biostimulation is the addition of amendments in the subsurface in order to stimulate native microorganisms capable of degrading contaminants, whereas bioaugmentation is the addition of a specific culture into the subsurface capable of degrading contaminants (McCarty & Semprini, 1993). Bioaugmentation is often used if a native culture is not able to be stimulated for the degradation of the desired contaminant(s), as native populations are already acclimated to site conditions.

Current biostimulation approaches for cometabolism of TCE have used methane, phenol, propane, or toluene as primary substrates (Arp et al., 2001). Effective TCE transformation using methanotrophs requires low copper concentrations to result in the production of the soluble methane monooxygenase instead of the particulate form produced at higher copper concentrations. Contrarily, the aromatics phenol and toluene have higher transformation capacities under less stringent conditions (Semprini, 1997). However, the use of these aromatics as growth substrates has met resistance, as both are toxic and regulated compounds. Thus, there exists a need to find alternative growth substrates to support TCE cometabolism in toluene utilizers. Chapter 3 of this study explores benzyl alcohol as an alternative growth substrate to toluene, as well as benzyl esters that release benzyl alcohol, for their ability to support cometabolism of chlorinated ethenes in *B. vietnamiensis* G4.

Slow Release Compounds

With traditional bioremediation approaches, repeated or continuous additions of the inducing growth substrate are needed to maintain cometabolic activity, since transformation is limited to the finite capacity of the added cells from energy limitations. An approach that is more passive and can reduce operational costs, is the use of slow release compounds (SRCs) that have at least one hydrolysable organic group

capable of forming an alcohol or organic acid to drive transformation of contaminants (Vancheeswaran et al., 1999). Traditional biostimulation approaches can encounter the problem of an aerobic population growing close to the location where oxygen and substrate are added forming a near-well bioreactor. This could result in biofouling and limit the area where treatment occurs, possibly requiring transport or recirculation of the contaminated plume (Semprini, 1997). The delay in substrate delivery dependent on the rate of hydrolysis could result in greater transport of the substrate before becoming bioavailable, and thus a greater distribution of the microbial population. The controlling factor here is the rate of hydrolysis, which ideally is slow enough to keep respiration rates low, but fast enough to maintain the microbial population and sustain cometabolism. Having low respiration rates is critical, as oxygen is a limited resource in the subsurface. A high concentration of substrate available would result in high metabolic activity, and possibly result in anoxic conditions. If this occurred, oxygen would have to be supplied, which would increase operational costs.

Studies have been performed exploring silicon-based organic compounds known as tetra-alkoxysilanes that hydrolyze abiotically, to produce alcohols to enhance bioremediation. Vancheeswaran et al., (1999) determined the abiotic hydrolysis of tetrabutoxysilane (TBOS) and tetrakis(2-ethylbutoxy)silane (TKEBS), which hydrolyze to produce 1-butanol and 2-ethylbutanol, respectively, and silicic acid. Vancheeswaran et al., (1999) also enriched an aerobic culture on TBOS and TKEBS, which was able to cometabolize TCE and *cis*-DCE. It was also seen that hydrolysis was biologically mediated by this aerobic mixed culture (Vancheeswaran et al., 1999). Additionally, cometabolism was demonstrated with the pure culture *Rhodoccoccus rhodocrus* ATCC 21198 while growing on 1- and 2-butanol released by TBOS and tetra-*sec*-butoxysilane (T2BOS) (Murnane, 2018; Rasmussen et al., 2020). These SRCs can also be used under anaerobic conditions, as TBOS was able to enhance reductive dechlorination of PCE and TCE from the fermentation of 1-butanol to butyrate and/or acetate (Yu & Semprini, 2002; Seungho Yu & Semprini, 2009).

One possible class of SRCs explored in this study are esters that hydrolyze to produce a carboxylic acid and an alcohol. The concept of using an ester as a SRC has been explored before. Kumari and Gupta (2014) investigated methyl ester (methyl oleate), which hydrolyzed to produce methanol to serve as a carbon source and for induction of the pAOX1 promoter in *Pichia pastoris* X33, and a fatty acid which served as a carbon source for biomass maintenance when methanol concentrations were low (Kumari & Gupta, 2014). They saw that a single dose of methyl ester supported higher production of proteins than repeated methanol induction.

The SRCs looked at in this study for their ability to support cometabolism in *B. vietnamiensis* G4 were tetraphenoxysilane (TPhOS), 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.

Encapsulation

A drawback to traditional bioaugmentation are problems associated with introducing non-native culture to the subsurface. Successful bioremediation through bioaugmentation requires the establishment of a population and the expression of the necessary enzymatic processes. The establishment of the bioaugmented community is affected by multiple factors including site pH, temperature, soil moisture content, nutrient availability, and predation by protozoa (Cassidy et al., 1996). One approach to mitigate these factors that varies depending on the site, is immobilizing microorganisms in a polymer gel-matrix. Studies have shown that there the advantages associated with encapsulation compared to suspended cells including protection from toxic substances, predation, and changes in pH and temperature. These advantages result from the microenvironment for the cells provided by encapsulation from the soil macroenvironment (Cassidy et al., 1996; Moslemy et al., 2002).

Chapter 4 explores the effect encapsulation in a polymer gel-matrix has on the ability of *B. vietnamiensis* G4 to perform cometabolism. Chapter 4 presents results of resting encapsulated cell cometabolic ability,

as well as investigates potential SRCs to create a co-encapsulated system where both cells and a SRC are encapsulated. This co-encapsulated system would have the benefits of both encapsulation and SRCs to support cometabolism as previously discussed. An additional benefit of a co-encapsulated system, is that encapsulation of the desired culture and SRC together likely limits competition if the substrate is not selective. The polymer gel-matrix used was gellan gum, which is a natural gelling polysaccharide produced by *Sphingomonas elodea* that has the repeating chain of glucose, glucuronic acid, and rhamnose. Gelation of gellan gum occurs both thermally and via ionic crosslinking and has superior rheological properties, chemical stability, temperature resistance and enzyme resistance compared to alginate (Rasmussen et al., 2020).

This is not the first study to investigate cometabolic activity by immobilized *B. vietnamiensis* G4. Radway et al. (1998) embedded *B. vietnamiensis* G4 grown in yeast-glucose medium in polyurethane prepolymer foam at 3 g dry wt. bacteria per 100 g wet wt., and induced cells with additions of phenol (2 mM) before harvesting (as opposed to growing on the inducing substrate) (Radway et al., 1998). Embedded and unembedded *B. vietnamiensis* G4 removed ~80% of TCE at 3.1 mg/L in solution, showing that G4 cells immobilized in foam could treat TCE. This study also showed that TCE cometabolism could be induced by phenol amendments after cells were already embedded, and the authors suggests that immobilized cells could be stored and used later and induced at the bioremediation site.

A more recent study compared immobilization of *B. vietnamiensis* G4 in polyethylene glycol (PEG) polymer to suspended cells exposed to TCE (0.1, 0.5, 1, and 5 mg/L) and toluene (10 and 50 mg/L). This study found that the TCE transformation rate decreased as the concentration was increased for suspended cells, but this effect was not seen with encapsulated cells. Additionally, it was seen that the removal efficiency greatly increased at the higher substrate concentration of 50 mg/L toluene compared to 10 mg/L. The authors state that the transformation yield by the encapsulated treatment of 0.427 mg-TCE/mg-toluene is higher than what has been previously reported in literature (Hamid et al., 2014). The higher

yield of encapsulated *B. vietnamiensis* G4 with substrate present, is promising for a co-encapsulated system with cells and a SRC.

The novel idea of a co-encapsulated system was first presented in Rasmussen et al (2020) and investigated two different SRCs—tetrabutylorthosilicate (TBOS) and tetra-s-butylorthosilicate (T_2BOS)—co-encapsulated with the model microbe *Rhodococcus rhodochrous* ATCC 21198. These batch kinetic tests showed transformation of multiple additions of contaminants over 300 days of 1,1,1-TCA, *cis*-DCE, and 1,4-dioxane, an emerging contaminant of concern used primarily as a chemical stabilizer for 1,1,1-TCA (Rasmussen et al., 2020). The oxygen consumption results showed that the alcohol released from the orthosilicates was utilized by the co-encapsulated strain to maintain cometabolic activity, and the higher hydrolysis rate of TBOS compared to T_2BOS corresponded to higher oxygen consumption and microbial growth. However, while this microbial strain has been shown to transform a variety of CAHs and co-contaminants by cometabolism, it does not transform TCE very well. Additionally, while it can transform 1,1-DCE at high rates, the toxicity of the epoxide greatly decreases system performance and cell viability (Krippaehne, 2018). One of the objectives of this thesis was to develop a co-encapsulated system with *B. vietnamiensis* G4 that could successfully transform chlorinated ethenes, TCE in particular, for long-term treatment.

The overall objectives of this study were divided into two chapters. Chapter 3 is written in a manuscript format and will be submitted for publication, whereas Chapter 4 is not. CHAPTER 3 performed studies with suspended cells and explored benzyl alcohol as an alternative substrate for supporting cometabolism in *B. vietnamiensis* G4. CHAPTER 4 performed studies with encapsulated *B. vietnamiensis* G4 with the goal of developing a co-encapsulated system that could effectively treat a variety of chlorinated ethenes, and consequently testing in a microcosm system.

Detailed objectives of this thesis were to:

1. Evaluate benzyl alcohol as an inducing substrate to promote aerobic cometabolism of TCE by comparison to toluene-induced cells through activity based profiling (ABL) and kinetic transformation results. *(CHAPTER 3)*

2. Compare TCE kinetic results between resting *B. vietnamiensis* G4 grown on benzyl alcohol, toluene, phenol, benzyl acetate, benzyl butyrate, acetate, butyrate, and lactate to results from ABL. *(CHAPTER 3)*

3. Determine if substrate inhibition of the T2MO by benzyl alcohol occurs during TCE transformation by comparison to toluene inhibition exhibited under the same conditions. Also determine if the T2MO is required by *B. vietnamiensis* G4 for benzyl alcohol utilization. *(CHAPTER 3)*

4. Evaluate cometabolic ability of *B. vietnamiensis* G4 encapsulated in gellan gum macrobeads by comparison to suspended cells. Also, demonstrate whether external additions of substrate can maintain long-term cometabolic activity. *(CHAPTER 4)*

5. Assess potential SRCs for co-encapsulation with *B. vietnamiensis* G4 and their ability to extend cometabolic transformation of TCE. *(CHAPTER 4)*

6. Assess the ability of co-encapsulated *B. vietnamiensis* G4 to transform contaminants in a microcosm system. *(CHAPTER 4)*

CHAPTER 2 — METHODS

2.1 Chemicals

All chemicals were chemical grade and listed below in ***Table 2.1*** along with CAS number, manufacturer and purity.

Table 2.1 *Chemicals used with CAS number, manufacturer and purity.*

Chemical	CAS Number	Manufacturer	Purity
TCE	79-01-6	J.T. Baker	99.5%
1,1-DCE	75-35-4	Sigma-Aldrich	99%
cis-DCE	156-59-2	TCI	99%
VV	75-35-4	Sigma-Aldrich	99.5%
1,1,1-TCA	71-55-6	J.T. Baker	99.5%
Toluene	108-88-3	Sigma-Aldrich	99.8%
Benzyl Butyrate	103-37-7	TCI	97%
TPhOS	1174-72-7	Gelest	95-100%*
KELCOGEL® Gellan Gum	71010-52-1	CP Kelco	--
Canola Oil	--	Kroger	--
Phenol	108-95-2	Alfa Aesar	99%
Benzyl Alcohol	100-51-6	J.T. Baker	100%
Propyne	74-99-7	Alfa Aesar	≥99%

*Product information presents a range for purity

2.1.1 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. It was reported that as short an incubation period as 1 hour is sufficient to achieve equilibrium (Gossett, 1987).

2.2 Analytical Methods

2.2.1 Chlorinated Aliphatic Hydrocarbons

Chlorinated compounds were analyzed via 100 uL headspace samples taken by a 100 uL Hamilton 1700 Series gas-tight syringe. Samples were measured on a Hewlett Packard 6890 Series Gas Chromatograph with an micro-electron capture detector (GC-ECD) and capillary column (Agilent DB-624 UI 30m x 0.53mm). Helium was used as the carrier gas at a flow rate of 15 mL/min and a constant oven temperature of 50°C. If TCE was being measured as a single compound, the oven temperature was increased to 100°C. All other chlorinated compounds including 1,1-DCE, *cis*-DCE, 1,1,1-TCA, and mixtures were run at 50°C. Individual external standards were made for each compound from saturated solutions for calibration.

2.2.2 Volatile Substrates

Toluene and VC were measured via headspace samples on a Hewlett Packard 6890 Series Gas Chromatograph with a Flame Ionization Detector (GC-FID) and capillary column (Agilent DB-624 UI 30m x 0.53mm). Helium was the carrier gas at 15 mL/min and at a constant oven temperature of 220°C was used for toluene and 150°C for VC.

2.2.3 Non-volatile Aromatics

To measure phenol, 1 mL liquid samples were taken through the butyl septa, filtered through a 0.2 um PVDF filter, and stored in a microcentrifuge tube at 4°C for later analysis. Liquid samples were then analyzed for phenol on a Dionex-500 HPLC chromatograph with a C18 column equipped with a Dionex AS40 Automated Sampler. The UV detector was operated at 275 nm for phenol. The eluent composition was 25 mM KH_2PO_4 with 20% acetonitrile, which was pumped through the column at 1 mL/min. An external standard was made for calibration.

2.2.4 Oxygen and Carbon Dioxide

For oxygen and carbon dioxide, headspace samples were measured using a Hewlett Packard 5890 Series II Gas Chromatograph with a thermal conductivity detector (GC-TCD) and Supelco 60/80 Caroboxen 1000 stainless steel capillary column (15 ft x 1/8 in.) Helium was the carrier gas to measure oxygen flowing at 30 mL/min at a constant oven temperature of 40°C, and argon was the carrier gas to measure carbon dioxide at 30 mL/min and 220°C.

2.3 Calculations and Constants

For gasses and volatile compounds, a mass balance was done on the liquid and gas phase to calculate total mass. Equilibrium was assumed between the two phases using Henry's Law with dimensionless Henry's constants listed in ***Table 2.2***.

$$m_{total} = C_g V_g + C_l V_l \qquad \text{Equation 1.}$$

$$H_{cc} = \frac{C_g}{C_l} \qquad \text{Equation 2.}$$

Table 2.2 *Dimensionless Henry's constants at 20°C calculated from Gossett (1987) and from EPI Suite™.*

Compound	H_{CC}
TCE	0.296
cis-DCE	0.164
1,1-DCE	0.857
1,1,1-TCA	0.656
PCE	0.549
Oxygen	31.45
Carbon Dioxide	1.20
Toluene	0.243

2.4 Culture Growth and Quantification

2.4.1 Inoculation

B. vietnamiensis G4 culture was obtained from Dr. Michael Hyman at North Carolina State University and kept on mineral salt media plates (recipe in Appendix B). Culture was grown in 500 mL Wheaton serum bottle that had caps with butyl septa. The bottles contained 300 mL of phosphate buffered mineral salt media (MSM) (recipe in Appendix A). The growth reactors were inoculated from plates, and 61 uL of toluene or another growth substrate at an equivalent electron donor basis was added. The amount of toluene added (61 μL) was in excess based on available oxygen in the 420 mL air headspace and the following reaction:

$$C_7H_{18} + 9O_2 \rightarrow 4\,H_2O + CO_2 \qquad \text{Equation 3.}$$

Growth bottles were kept on a shaker table at 30°C. Once the cells were in the exponential growth phase, which was typically three or four days ($OD_{600} \sim 0.3$), the bottles were refreshed with oxygen by opening in a sterile laminar flow hood for 15-20 minutes. The bottles also received another addition of toluene or other growth substrate before placing back on the shaker table at 30°C. During this stage, plates were streaked from each individual growth reactor on a MSM agar plate to verify purity of the culture. All pure culture work was done in a sterile laminar flow hood or biosafety cabinet.

2.4.2 Harvesting Culture

The day after growth reactors were refreshed, which corresponded to cells in the late stage of exponential growth and doubling of the optical density (OD_{600}), they were harvested for experimental use. Cells were harvested by centrifuging at 7500 rpm for 10 minutes, after which the supernatant was poured off and cells were washed with 100 mM phosphate buffer (PB). The cells were centrifuged again and concentrated in 5 or 10 mL of the 100 uM PB. Cells were typically used within 1 - 2 days of harvesting for experimental use and stored at 4°C.

2.4.3 Quantification

For cell quantification, a total suspended solids (TSS) analysis was performed as described in AWWA standards on the concentrated cells. This quantified cells on a dry weight basis and was reported as (mg_{TSS}/mL) (Rice et al., 2017). A dry weight with a 0.45 µm Whatman ME 25/21 mixed cellulose filter on a 5.1 cm aluminum foil dish was first obtained, after which a volume between 0.3 and 0.5 mL of the cell slurry was added and vacuum filtered. The filtered cell mass was then dried at 103°C for 20 minutes and a dry weight was obtained. TSS was calculated based on the weight difference and volume of cells added.

2.5 Suspended Culture Studies

2.5.1 Rate Test

Resting cell kinetic tests were performed in sterile glass serum vials (27 mL), to which 10 mL of MSM was added. Vials were sealed with sterile butyl septa and aluminum metal crimp tops. Using a 100 µL 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. Once the initial concentration was determined, a known mass of cells (typically between 4-5 mg_{TSS} based on TSS analysis) was added through the septa using a sterile 1 mL leur-lok syringe in a sterile laminar flow hood. The time cells were added was considered the start of the rate test. Active treatments were in either duplicate or triplicate, and vials were kept on a shaker table at 200 rpm at 20°C. Abiotic controls with MSM and the same mass of contaminant were used to verify transformation was biotic in nature and to determine sorption effects. Controls with propyne, a monooxygenase inhibitor, present at 2% in the headspace verified transformation was due to T2MO activity (Yeager, 1999).

2.5.2 Transformation Capacity Test

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 and McCarty, 1991). Once the contaminant had been approximately 100% degraded, more was added at the same or higher concentrations until transformation ceased. 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. However, in determination of the transformation capacity, the reduction of mass in the abiotic control was considered in calculating overall mass of contaminant transformed, given these tests were done over the course of several days to weeks.

The capacity was calculated as:

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

2.6 Microbial Encapsulation

2.6.1 Macro Encapsulation

The method for the macro encapsulation of a microbial culture in gellan gum cylindrical beads (2 mm diameter x 2 mm height) was developed previously (Rasmussen et al., 2020). In summary, the method entails heating a 0.75% gellan gum solution to its hydration temperature of 80 – 90°C, and activating crosslinking by the addition of $CaCl_2$ at 0.08% (v/v). At this step a minor modification to optimize bead integrity and to increase the microbial activity of G4 was included, which was the addition of a 1.6 M PB solution at 1% (v/v) (recipe in Appendix C). The reason for this modification is discussed in Appendix D. Cells were added between 45-50°C (typically 47°C) and vortexed. Cooling below 45°C set the gel, so immediately after cells were added and vortexed, the gel solution was drawn into flexible rubber tubing with an inner diameter of ~2 mm to cool. The gel was then extruded in a sterile laminar flow hood and cut into cylindrical beads ~2 mm in length, which was then transferred to a 0.25% $CaCl_2$ solution to finish crosslinking. After an hour in the crosslinking solution, the beads were washed three times with MSM

and filtered using a vacuum pump fitted with a 70 mm plastic filter funnel. It was assumed that all cells added were encapsulated, and that 1 mL of the pregel solution formed 1 g of beads. Beads were used either the day they were made or stored at 4°C in 100 mM PB and used the next day.

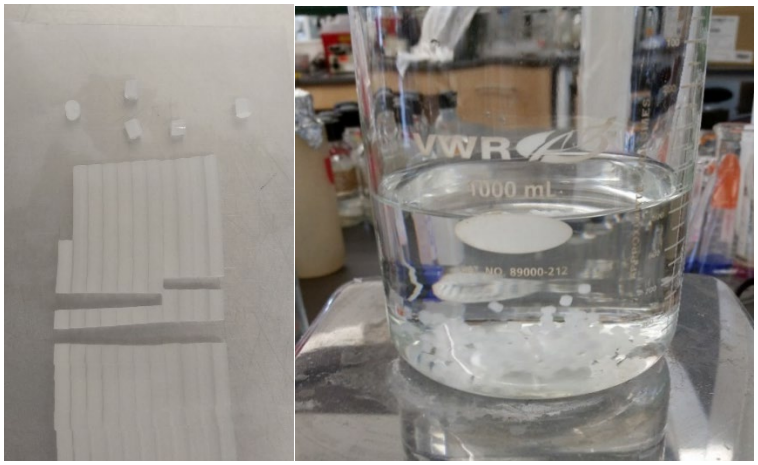

Figure 2.1 *(Left) Strands of gellan gum extruded from 2 mm diameter rubber tubing and cut into cylindrical pellets in a sterile laminar flow hood. (Right) After cutting, beads were transferred to a 0.25% CaCl2 crosslinking solution.*

2.6.2 Macro Co-encapsulation

The method for macro co-encapsulation including both culture and a SRC, was developed previously (Rasmussen et al., 2020). A single modification is made for the inclusion of the SRC; the emulsification of the desired liquid substrate and heated gellan gum at 2500 rpm for 7 minutes using an IKA RW-20 digital overhead impeller mixer, prior to initial $CaCl_2$ crosslinking. One of the SRCs in this study, TPhOS, is solid at room temperature and has a melting point of 40°C. During the emulsification stage, the pregel was heated to 90°C, which allowed emulsification to occur, though likely not for the entire 7 minute duration due to cooling of the solution. Other than handling the compound in a ventilated hood, no other changes were made during the co-encapsulation process for TPhOS.

2.7 Batch Kinetic Studies

Batch kinetic studies were performed with either encapsulated or co-encapsulated *B. vietnamiensis* G4 gellan gum macrobeads in phosphate buffered MSM. *B. vietnamiensis* G4 was grown on toluene unless stated otherwise.

2.7.1 Long-term Encapsulation/Co-encapsulation Kinetic Studies

Kinetic batch studies over the duration of weeks or months were performed to determine the long-term ability of both encapsulated and co-encapsulated beads to transform contaminants. These tests was conducted in 125 mL Wheaton glass serum bottles with 100 mL of phosphate buffered MSM and sealed with plastic screw caps with butyl septa. A known mass of beads were added at a known biomass loading (mg_{TSS}/g_{bead}). Using a 100 uL liquid syringe, a known mass of contaminant(s) were added from saturated stock solutions through the septa. Contaminants between each long-term kinetic study varied—sometimes present as simple compounds or as mixtures—but each experiment included TCE.

Shortly after contaminant addition, an initial concentration was measured and considered the time zero datum point. Respiration data was also monitored throughout the duration of the experiments. Liquid samples were collected if co-encapsulated beads were used and stored in 1.5 mL centrifuge tubes at 4°C. Reactors were kept shaking at 100 rpm at 20°C to maintain equilibrium. Treatments varied between experiments, but typically consisted of either duplicate or triplicate of active (co-)encapsulated treatments in media, suspended cell treatment, abiotic control treatment, and co-encapsulated poisoned control treatments using sodium azide at 2% (v/v). Specifics for each experiment including contaminants concentration, biomass loading, mass of beads added, and types of treatments are detailed in the results.

2.8 Microcosm Studies

Microcosms were made with aquifer solids of fine-grained clay silts from Fort Carson, a site with 1,4-dioxane and TCE contamination. Artificial groundwater was made to replicate groundwater quality at the site (Rolston et al., 2019). Solids and artificial groundwater were combined for a concentration of 52 g aquifer solids/L groundwater and mixed as a slurry before distributing (55 mL) in a 125 mL Wheaton glass bottle. The groundwater mixture was transferred quickly in an attempt to minimize loss of volatile compounds. Reactors were sealed with plastic screw caps with butyl septa and kept on a shaker table at 100 rpm at 20°C. Treatments include one abiotic control, one control with co-encapsulated beads and propyne (2% v/v in the headspace) as a monooxygenase inhibitor, and triplicates of active co-

encapsulated treatments. Toluene grown *B. vietnamiensis* G4 was co-encapsulated at 0.85 mg_{TSS}/g_{bead} with 2% (w/w) TPhOS. All treatments except the abiotic control had 2 g beads added for a total biomass of 1.7 mg_{TSS} per reactor. The initial TCE associated with the solids were low, therefore TCE from a saturated solution was added for an initial liquid concentration of ~660 ppb.

CHAPTER 3 —Chlorinated Ethene Cometabolic Degradation by *Burkholderia vietnamiensis G4* Grown on Benzyl Alcohol as a Toluene Alternative

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Abstract

Benzyl alcohol was assessed as a food-grade growth substrate for promoting the cometabolism of trichloroethene (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) 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 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 1,2-*cis*-dichloroethene (*cis*-DCE), 1,1-dichloroethene (1,1-DCE), and vinyl chloride (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, trichloroethene (TCE), a known human carcinogen, is one of the most common organic groundwater contaminants and a priority pollutant by the USEPA (Zogorski, 2006). TCE and the fully chlorinated tetrachloroethene (PCE), can also result in the production of *cis*-1,2-dichloroethene (*cis*-DCE) and vinyl chloride (VC) under anaerobic conditions through reductive dehalogenation with 1,1-dichloroethene (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; Gary D. 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 et al., 2003; 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 et al., 2003; M S Shields et al., 1995). The other two pathways include the toluene-2,3-dioxygenase of *Pseudomonas putida* F1 and the catabolic plasmid TOL 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; C. M. 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., 2000). 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 US EPA 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 (US EPA). Another concern is the chlorination of phenol and the corresponding formation of chloroform (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 et al., 1993a + 1993b; 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, 1991; 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, 1991; Arp et al., 2000.; 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 uM (Landa et al., 1994). These inhibition effects may be mitigated in the subsurface by alternate pulsing of substrate and dissolved oxygen, 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 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* $PR1_{23}$, developed by Tn5 insertion, degraded TCE when grown on lactate and also degraded *cis*-DCE, *trans*-DCE, and 1,1-DCE (Shields and Reagin, 1992). One study bioaugmented two mutant strains of G4, *B. vietnamiensis* $PR1_{23}$ and $PR1_{301}$ 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 Activity Based Labeling (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 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 (≥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 μM) 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 µL), benzyl alcohol (63 µL), benzyl acetate (70 µL), and benzyl butyrate (65 µL), while phenol was added as a solid (69.5 mg) and sodium lactate was present as 60% syrup (260 µL). 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 [OD_{600}] 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 OD_{600} 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 total suspended solids test (TSS).

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 µL 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 were 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, 1991). 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 abiotic loss was insignificant over this timescale. 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:

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

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) ensured inhibition of the T2MO, and was above the upper explosive limit of 11.7% (v/v) (Nassimi et al., 2017). Biomass growth was measured over time through OD_{600} 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 1,7-octadiyne (17OD), which was used as a probe. *B. vietnamiensis* G4 was grown on the substrate of interest, as described previously. Activity based profiling was performed by Dr. Michael Hyman at North Carolina State University as described in (Bennett et al., 2016).

Analytical procedures.

(i) TSS. The culture density (mg_{TSS}/L) was determined gravimetrically through TSS analysis. A dry weight was obtained with a 0.45 µm 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.

(ii) Volatile Chemicals. A 100 µL gas sample was taken from the batch reactor headspace using a 100 µL 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 an 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. 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 x 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 (Table S1).

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 3.1***. Noticeable flourescence of a 62 kDa polypeptide is seen with cells grown on toluene, benzyl alcohol, benzyl acetate, benzyl butyrate and phenol, whereas little to no fluorescense is seen for butyrate, acetate, and lactate grown cells. Quantification of the fluorescence pixels was also done for each substrate (***Figure 3.1***).

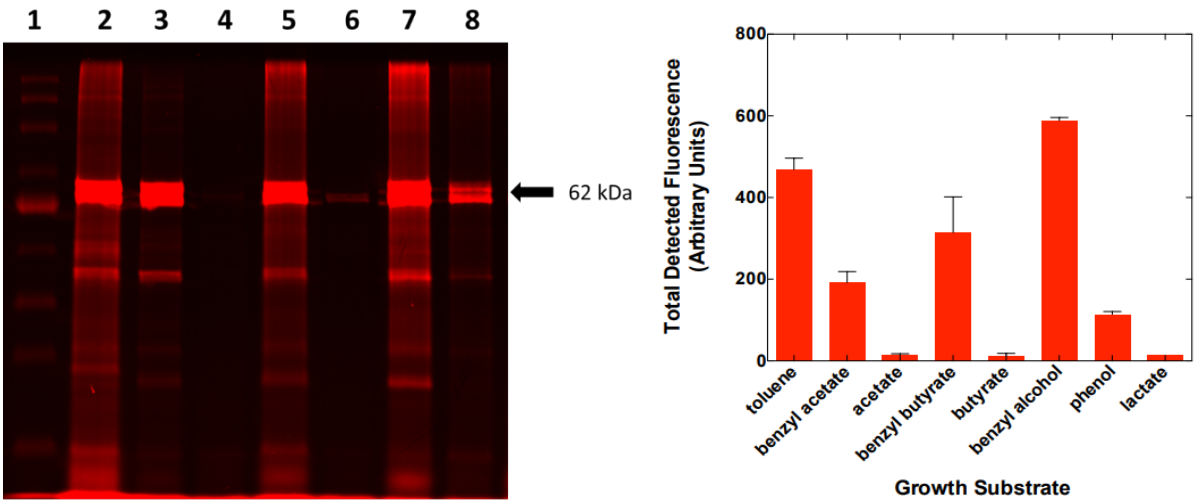

Figure 3.1 *Whole cells of B. vietnamiensis G4 were pretreated with 17OD and analyzed by SDS-PAGE and IR scanning as described in Materials and Methods section.* ***(Left)*** *Lane 1, NIR markers; lane 2, cells grown on toluene; lane 3, cells grown on benzyl acetate; lane 4, cells grown on acetate; lane 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.*

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 toluene has been extensively studied for supporting cometabolism of TCE. TCE transformation data used to obtain initial rates and capacities for both substrates can be seen in ***Figure 3.2*** *&* ***Figure 3.3***. Rates and capacities are summarized in ***Table 3.1***. 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 uM, 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 umol/day/mg_{TSS}) was 18% greater than toluene grown cells (1.8 umol/day/mg_{TSS}). The TCE transformation capacity for benzyl alcohol grown cells (0.63 umol/mg_{TSS}) was 20% greater than toluene grown cells (0.47 umol/mg_{TSS}). Assuming that protein mass accounts for 50% of dry cell mass, 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 uM and assuming 50% of dry mass is protein, phenol grown *B. vietnamiensis* G4 had an initial rate of approximately 1.8 umol/day/mg_{TSS} at room temperature (Folsom et al., 1990), and at 40 uM, TCE toluene grown *B. vietnamiensis* G4 had an initial rate of approximately 2.2 umol/day/mg_{TSS} at 27°C (Leahy et al., 1996).

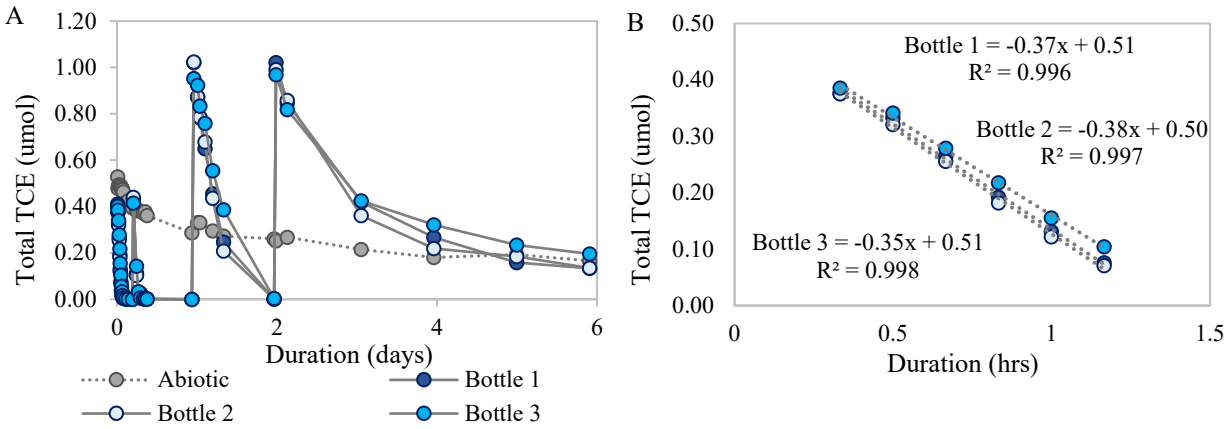

Figure 3.2 *Kinetic degradation results of TCE by resting toluene grown B. vietnamiensis G4 (5 mg_{TSS}) showing (A) TCE reinjections to determine total transformation capacity (umol/mg_{TSS}) and (B) initial degradation of TCE to determine initial zero order rate (umol/day/mg_{TSS}).*

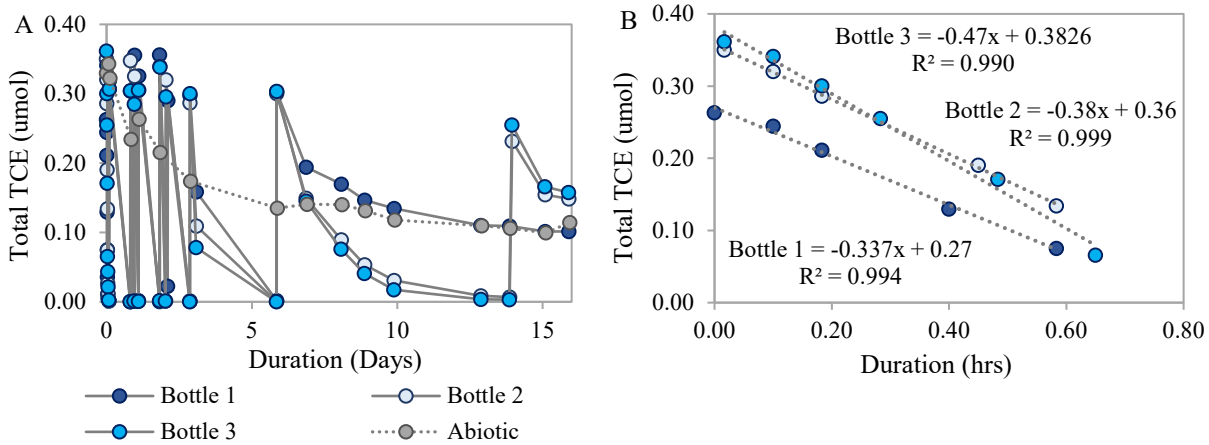

Figure 3.3 *Kinetic degradation results of TCE by resting benzyl alcohol grown B. vietnamiensis G4 (4.2 mg_{TSS}) showing (A) TCE reinjections to determine total transformation capacity (umol/mg_{TSS}) and (B) initial degradation of TCE to determine initial zero order rate (umol/day/mg_{TSS}).*

Yeager et al. measured transformation capacities ranging from 0.61 – 0.95 umol 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 umol/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 3.2*** *&* ***Figure 3.3***). 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 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 S1). 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, 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 3.1***). 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 umol/day/mg_{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 umol/mg_{TSS}) than any other substrate. The next highest capacity measured was by benzyl acetate and phenol grown culture at 0.78 and 0.77 umol/mg_{TSS} respectively.

Table 3.1 *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

[a]*Test done in duplicate and values are means with the range of values given in parenthesis.*
[b]*Test done in triplicate and values are means ± SD for n = 3*

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 were 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 (Ramsay et al., 1988; Pan et al., 2012). *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 et al., 1993a). 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., 1993a; 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 (EPISuite, USEPA).

Linear regression analysis was performed on the ABP fluorescence versus the average initial TCE transformation rate for all the growth substrates tested (***Figure 3.4***). 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 agreement between ABP and kinetic results indicate that ABP is an effective means of screening substrates for cometabolic transformation potential.

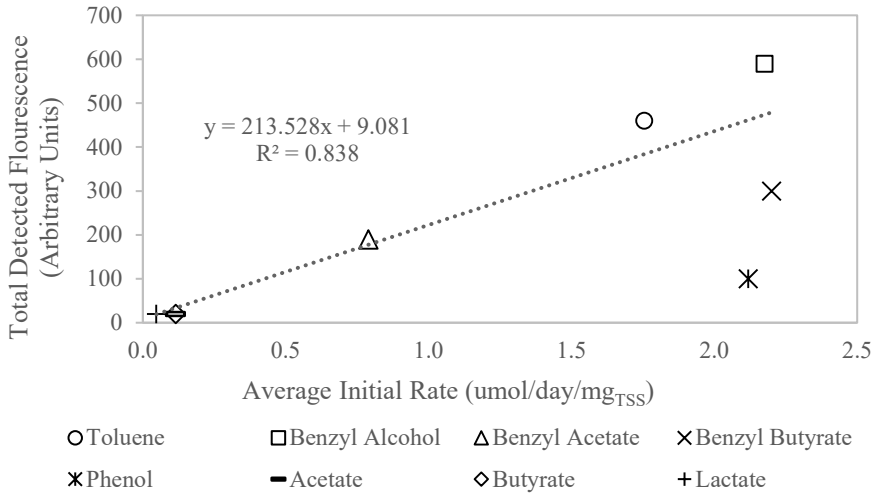

Figure 3.4 *Regression analysis of total detected fluorescence and the average initial rate for toluene, benzyl alcohol, benzyl acetate, benzyl butyrate, phenol, acetate, butyrate, and lactate grown B. vietnamiensis G4. Linear regression is shown for the data with the exception of phenol as an outlier.*

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* PR1$_{23}$ 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* PR1$_{31}$ grown on glucose, and assuming protein mass accounts for 50% of dry cell mass, observed a maximum specific TCE degradation rate of 0.6 umol/day/mg$_{TSS}$ (Inguva & Shreve, 1999). This rate is over twelve 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 and 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 3.5.A)***. Comparatively, toluene consumption was monitored and there was no lag in transformation as there was with TCE (Figure S2). 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 3.5.B***). At 20 mg/L of toluene, stronger inhibition was observed, with a lag in TCE transformation occurring for approximately 2.7 hours (***Figure 3.6.A***). 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 3.6.B***). This observed toluene

inhibition is expected and consistent with previous observations of toluene inhibiting rates of TCE cometabolism (Landa et al., 1994; Alagappan and Cowan, 2003).

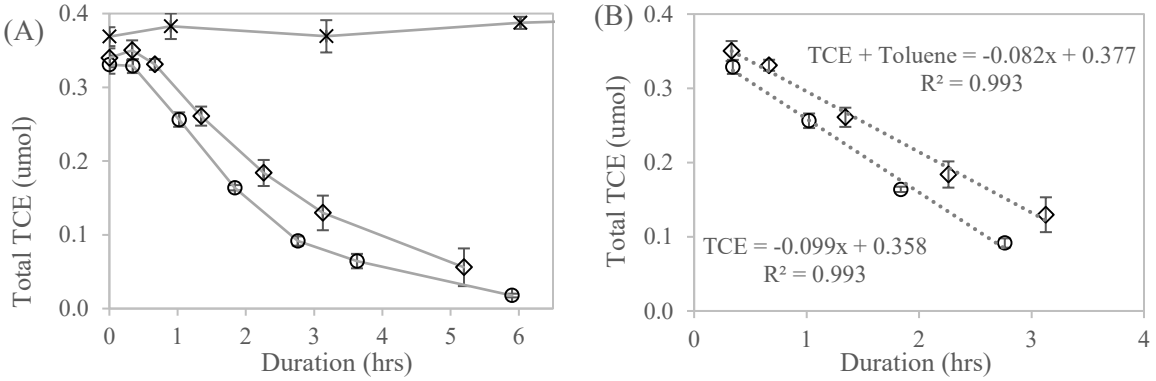

Figure 3.5 *TCE disappearance by resting B. vietnamiensis G4 (○), with toluene at 11.7 mg/L (◇), and with 2% (v/v) propyne as a monooxygenase inhibitor (×). Shown is (A) raw data and (B) zero-order rate regression. Treatments were done in triplicate with error bars representing SD for n = 3.*

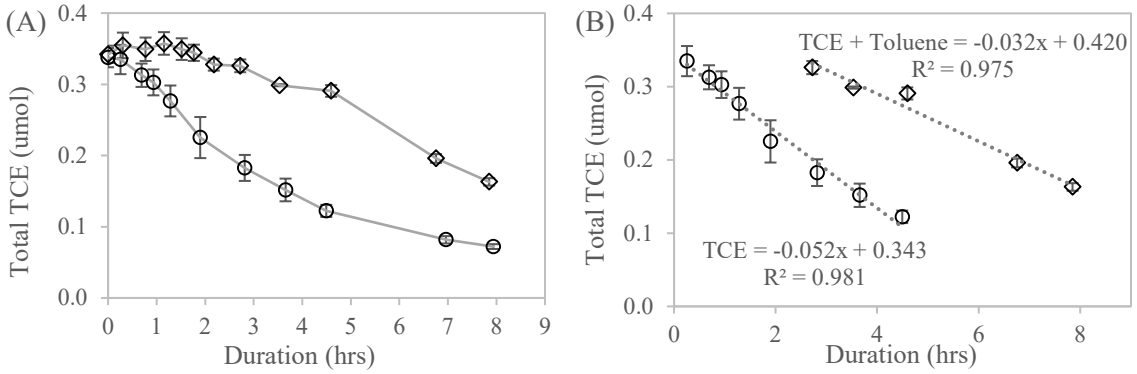

Figure 3.6 *TCE disappearance by resting B. vietnamiensis G4 (○) and with toluene at 20 mg/L (◇). Shown is (A) raw data and (B) zero-order rate fit. Treatments were done in triplicate with error bars representing SD for n = 3.*

Benzyl Alcohol Inhibition Tests. The inhibition tests at initial benzyl alcohol concentrations of 10 mg/L and 20 mg/L can be seen in ***Figure 3.7 & 3.8***. There was not any indication of lag in TCE transformation at 10 mg/L benzyl alcohol (***Figure 3.7.A***). 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 (***Figure 3.7.B***).

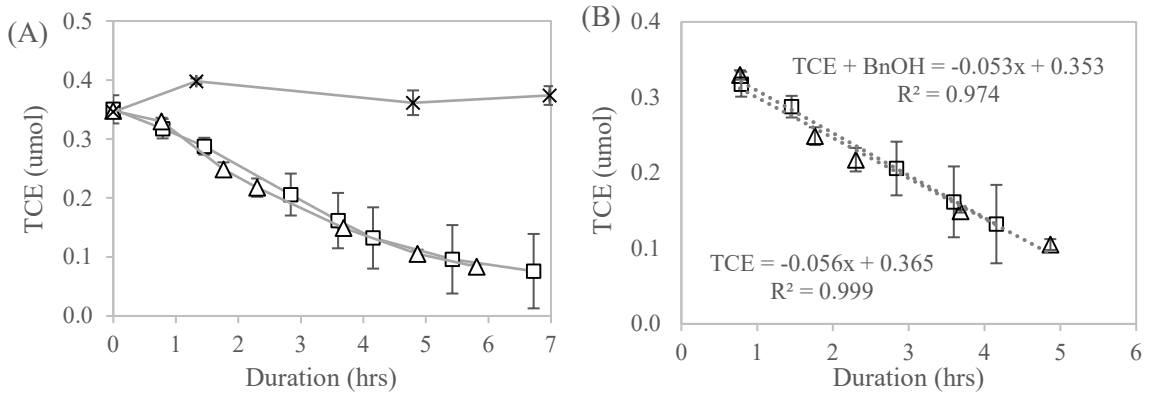

Figure 3.7 *TCE disappearance by resting B. vietnamiensis G4 alcohol (□) and with benzyl alcohol at 10 mg/L (△), and with 2% (v/v) propyne as a monooxygenase inhibitor (×). Shown is (A) raw data and (B) zero-order rate regression. Treatments were done in triplicate with error bars representing SD for n = 3.*

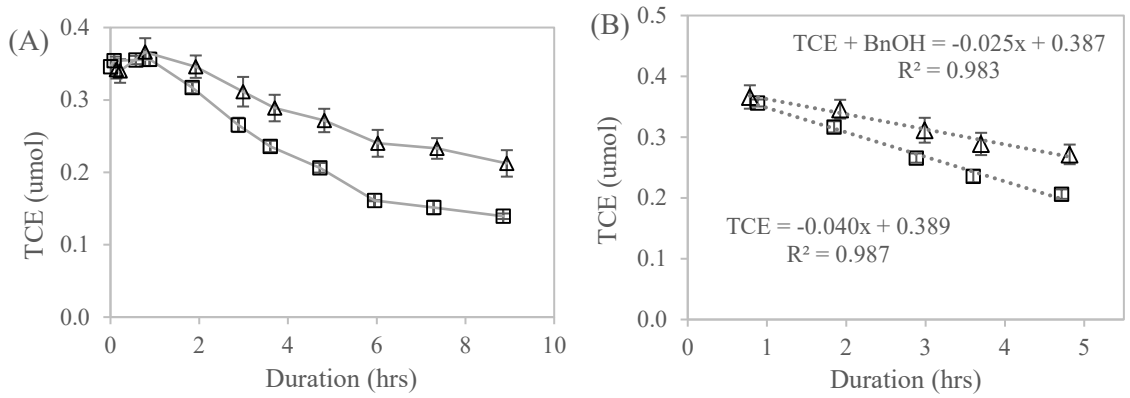

Figure 3.8 *TCE disappearance by resting B. vietnamiensis G4 (□) and with benzyl alcohol at 20 mg/L (△). Shown is (A) raw data and (B) zero-order rate fit. Treatments were done in triplicate with error bars representing SD for n = 3.*

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 3.8.A***). When benzyl alcohol was present, the average zero-order rate was (0.58 ± 0.04 umol/day/mg_{TSS}), which was a 40% decrease from the rate for resting culture without benzyl alcohol (0.97 ± 0.01 umol/day/mg_{TSS}) (***Figure 3.8.B***). 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. It was 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 2-hydroxy benzyl alcohol (2HBA) through hydroxylation by an *ortho*-monooxygenase. Tehasen, 2004 investigated nhibition effects in a benzyl alcohol grown mixed culture and found that when the *ortho*-monoxygenase was inhibited no TCE transformation or 2HBA production was observed, but benzyl alcohol degradation decreased only slightly. This same process described by these two studies potentially applies for benzyl alcohol utilization in *B. vietnamiensis* G4 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.

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 9 shows OD_{600} 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.

These results indicate that the monooxygenase is required for growth on benzyl alcohol. This is not surprising given the work of both Tejasan 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.

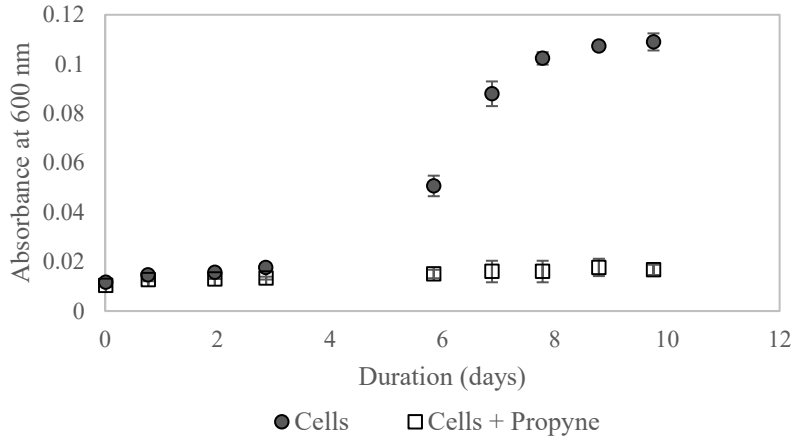

Figure 3.9 *OD_{600} measurements over time for B. vietnamiensis G4 grown on benzyl alcohol (A) and grown in the presence of propyne (15% v/v). Treatments were triplicates with error bars representing SD for n = 3.*

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 3.2***. ***Figure 3.10*** and ***Figure 3.11*** show the test results obtained with *cis*-DCE, and test results for VC and 1,1-DCE are provide in the SI, Figures S3-S6. 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).

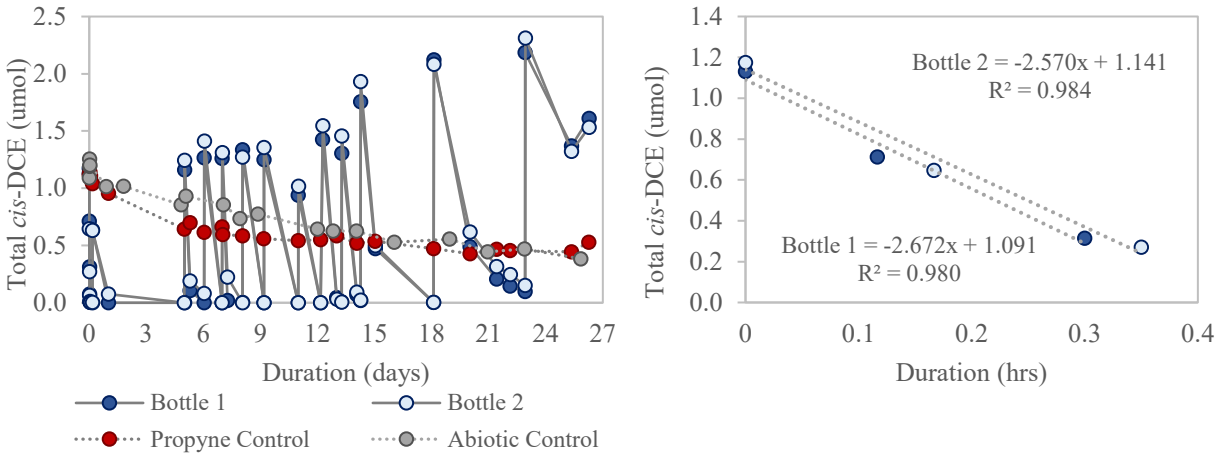

Figure 3.10 *Kinetic degradation results of cis-DCE by resting toluene-grown B. vietnamiensis G4 (5.5 mg_{TSS}) showing (A) cis-DCE reinjections to determine total transformation capacity (umol/mg_{TSS}) and (B) initial degradation of cis-DCE to determine initial zero order rate (umol/day/mg_{TSS}).*

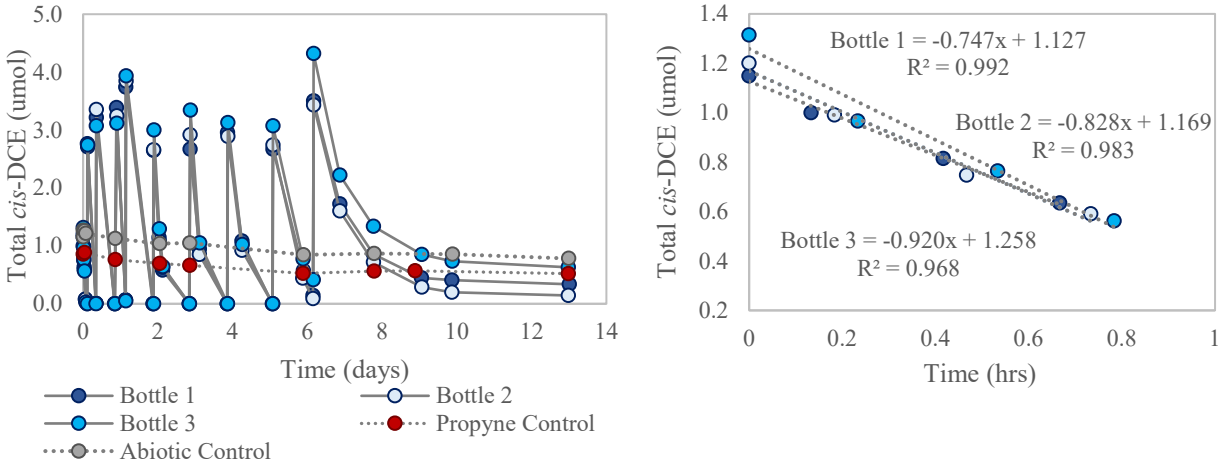

Figure 3.11 *Kinetic degradation results of cis-DCE by resting benzyl alcohol grown B. vietnamiensis G4 (4.8 mg_{TSS}) showing (A) TCE reinjections to determine total transformation capacity (umol/mg_{TSS}) and (B) initial degradation of TCE to determine initial zero order rate (umol/day/mg_{TSS}).*

The transformation data for *cis*-DCE for both benzyl alcohol and toluene are shown in ***Figure 3.10*** and ***Figure 3.11***. 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 S3 and S4. VC transformation capacity was reached in two and twelve days for toluene and benzyl alcohol cells, respectively (Figures S3 and S4). The difference in mass additions does not account for the duration

difference for VC, as toluene-grown cells received lower mass additions (~4 umol) than benzyl alcohol grown cells (~6 umol) (Figures S3 and S4). 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 umol/mg_{TSS}) and benzyl alcohol grown cells (0.085 umol/mg_{TSS}), respectively (Figures S5 and S6). As mentioned previously, aerobic cometabolism of 1,1-DCE is a toxic process to cells and causes rapid inactivation (Arp et al., 2000; Dolan & McCarty, 1995; Gary D. 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.

Table 3.2 *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 *B. vietnamiensis* G4				
	VC	*cis*-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 *B. vietnamiensis* G4				
	VC	*cis*-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

[a]Test done in duplicate and values are means with the range of values given in parenthesis.
[b]Test done in triplicate and values are means ± SD for $n = 3$

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 and 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 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 energy stores than toluene-grown cells. This impact the growth

substrate has on PHB production, and how this affects the extent of transformation should be studied further.

Conclusion

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. However, more research needs to be done to determine the details of these metabolic pathways of benzyl alcohol in *B. vietnamiensis* G4, as well as 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 (Hopkins et al., 1993; Semprini et al., 1991). 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.

Supplementary Information to:

Chlorinated Ethene Cometabolic Degradation by *Burkholderia vietnamiensis* G4 Grown on Benzyl Alcohol as a Toluene Alternative

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Table S-1 *Dimensionless Henry's constants at 20°C used for calculating the total mass assuming equilibrium (Gossett, 1987).*

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

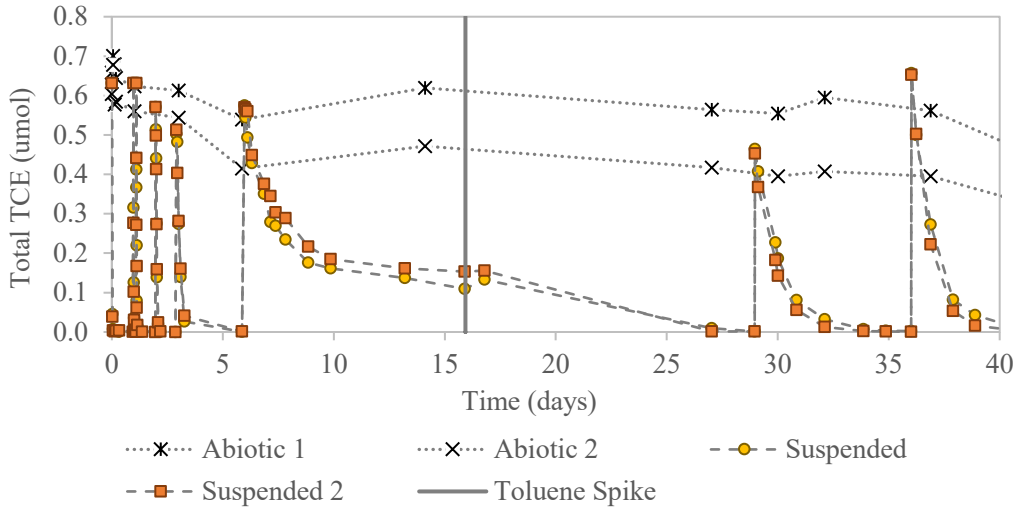

Figure S- 1 *Cometabolic transformation of TCE over the course of 40 days by B. vietnamiensis G4. Treatments consisted of duplicate treatments of suspended cells of B. vietnamiensis G4 at 16 mg$_{TSS}$/reactor, and duplicate abiotic control treatments. Toluene was added on day 16 to stimulate further cometabolic activity.*

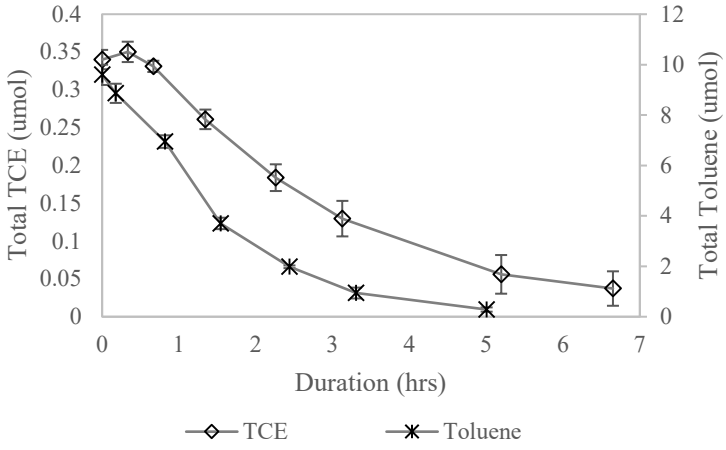

Figure S- 2 *Results of concurrent TCE and Toluene disappearance by B. vietnamiensis G4 (1 mg$_{TSS}$). Initial toluene liquid concentration was 11.7 mg/L. Treatment was done in triplicate with error bars representing SD for n = 3.*

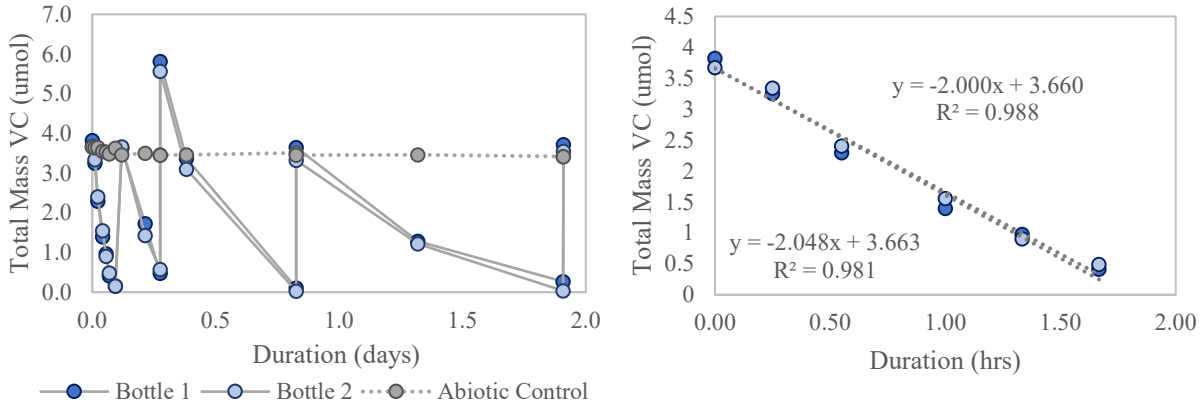

Figure S- 3 *Kinetic degradation results of VC by resting toluene grown B. vietnamiensis G4 (5.0 mg$_{TSS}$) showing (A) VC reinjections to determine total transformation capacity (umol/ mg$_{TSS}$) and (B) initial degradation of VC to determine initial zero order rate (umol/day/mg$_{TSS}$).*

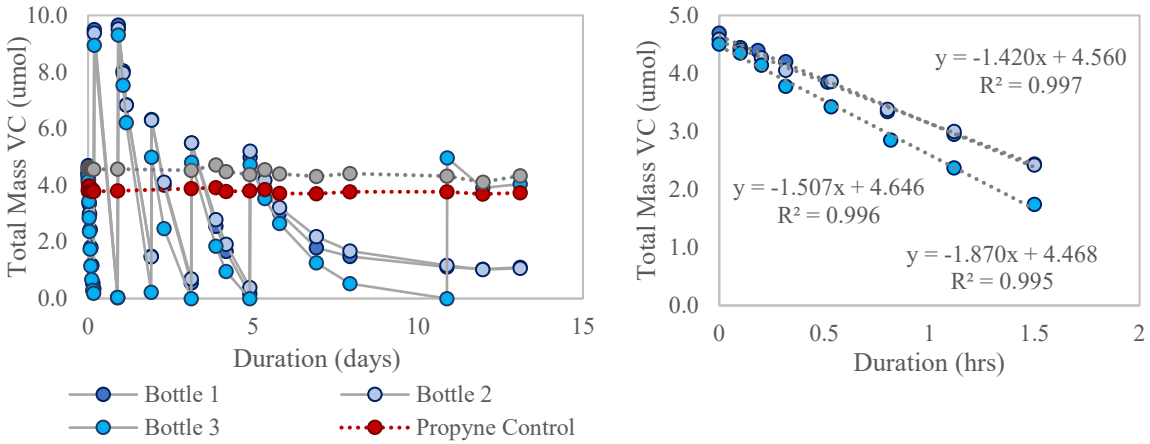

Figure S- 4 *Kinetic degradation results of VC by resting benzyl alcohol grown B. vietnamiensis G4 (5.0 mg$_{TSS}$) showing (A) VC reinjections to determine total transformation capacity (umol/ mg$_{TSS}$) and (B) initial degradation of VC to determine initial zero order rate (umol/day/mg$_{TSS}$).*

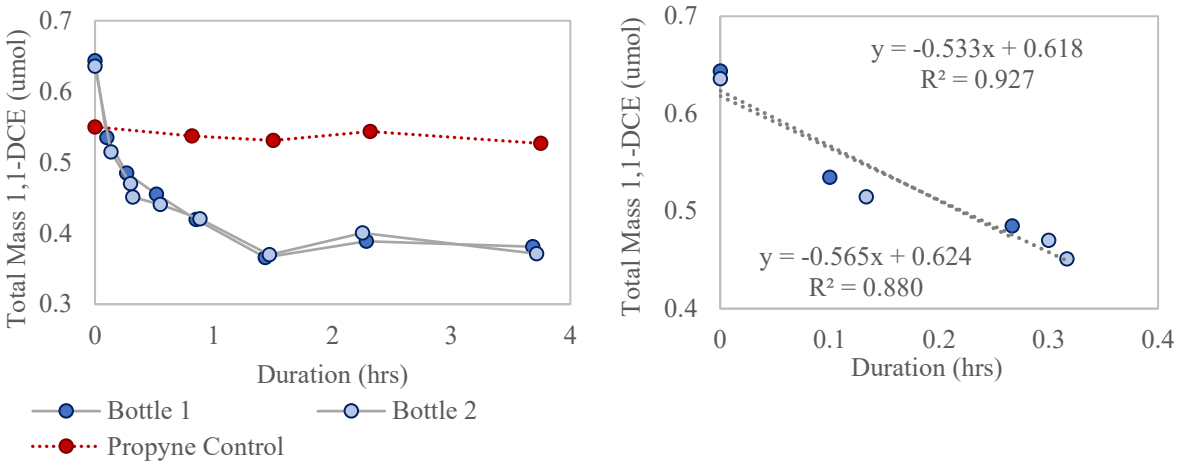

Figure S- 5 *Kinetic degradation results of 1,1-DCE by resting toluene grown B. vietnamiensis G4 (5.5 mg$_{TSS}$) showing (A) 1,1-DCE reinjections to determine total transformation capacity (umol/ mg$_{TSS}$) and (B) initial degradation of 1,1-DCE to determine initial zero order rate (umol/day/mg$_{TSS}$).*

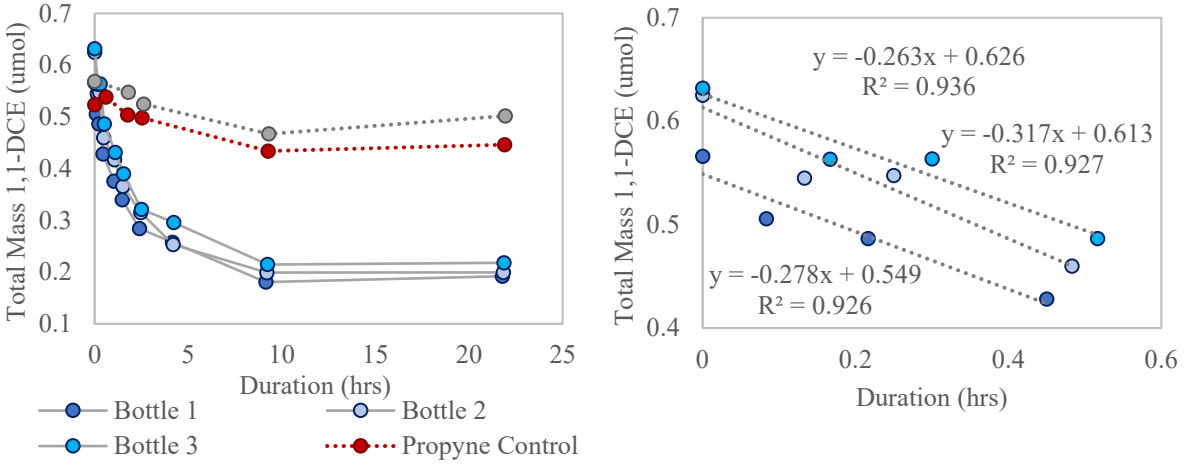

Figure S6. *Kinetic degradation results of 1,1-DCE by resting benzyl alcohol grown B. vietnamiensis G4 (4.8 mg$_{TSS}$) showing (A) 1,1-DCE reinjections to determine total transformation capacity (umol/ mg$_{TSS}$) and (B) initial degradation of 1,1-DCE to determine initial zero order rate (umol/day/mg$_{TSS}$).*

CHAPTER 4 — Immobilization of *Burkholderia vietnamiensis G4* in Gellan Gum Macrobeads for Long-term Cometabolic Transformation

The results from Chapter 3 demonstrated that benzyl alcohol, as well as the slow release compounds (SRCs) benzyl acetate and butyrate, were suitable substrates for induction of the T2MO in *B. vietnamiensis* G4. In this chapter, the overall objective was to develop a co-encapsulated system with *B. vietnamiensis* G4 and a SRC to promote long-term cometabolic treatment of TCE with minimal oxygen uptake. This exploration started by determining how the cometabolic activity of *B. vietnamiensis* G4 was affected by encapsulation by comparing the resting cell transformation potential to that of suspended cells. This was done at a lower biomass with single compound tests of TCE and 1,1-DCE, as well as at a higher biomass with TCE and 1,1-DCE as a mixture. Given the promising results of encapsulated resting cells able to maintain cometabolic activity and the high capacity observed (Chapter 3), benzyl butyrate was explored as a possible SRC for co-encapsulation. The other SRC explored was tetraphenoxysilane (TPhOS), which hydrolyzes to release phenol. This substrate was explored given the success of other orthosilicates as SRCs for co-encapsulation as discussed previously.

RESULTS

4.1 Encapsulation Batch Kinetic Studies

Before attempts were made to co-encapsulate *B. vietnamiensis* G4 with a SRC, experiments were performed with encapsulated resting *B. vietnamiensis* G4 (i.e. no SRC was present). The objectives of these experiments were as follows:

1. Verify *B. vietnamiensis* G4 can be encapsulated in gellan gum macrobeads and perform cometabolism. Additionally, verify that the gellan gum was not bioavailable, which would compromise the structure of the bead over time.
2. Compare the cometabolic transformation ability of encapsulated *B. vietnamiensis* G4 to suspended cells exposed to TCE and 1,1-DCE as single contaminants and as a mixture.

3. Determine whether external additions of substrate can maintain long-term transformation of a mixture of TCE and 1,1-DCE

4.1.1 Low Biomass Encapsulation

An experiment was performed with the goal of determining the resting capacity of encapsulated *B. vietnamiensis* G4 to transform TCE and 1,1-DCE. In this experiment, these contaminants were looked at separately and not as a mixture. Gellan gum macrobeads at a biomass loading of 1 mg_{TSS}/g_{bead} were added to each reactor (2 g per reactor). For each contaminant, treatments consisted of duplicate abiotic controls, duplicate encapsulated treatments, and duplicate suspended controls with an equivalent biomass. TCE and 1,1-DCE were added at concentrations of approximately 450 and 40 ppb respectively, with 1,1-DCE added at lower levels due to the toxicity of the epoxide formed, and the low capacity seen in the suspended rate tests in Chapter 3 (***Table 3.2***).

Both suspended and encapsulated treatments transformed 1,1-DCE at similar rates and quickly reached their transformation capacity by the first day (***Figure 4.1***). However, encapsulated treatments had ~2 times the average transformation capacity (0.029 umol/mg_{TSS}) than suspended treatments (0.016 umol/mg_{TSS}), indicating the polymer matrix may have provided some level of protection from 1,1-DCE toxicity. However, one of the encapsulated reactors (ET 1) started at a slightly higher initial 1,1-DCE concentration, which could have affected the higher average transformation capacity observed.

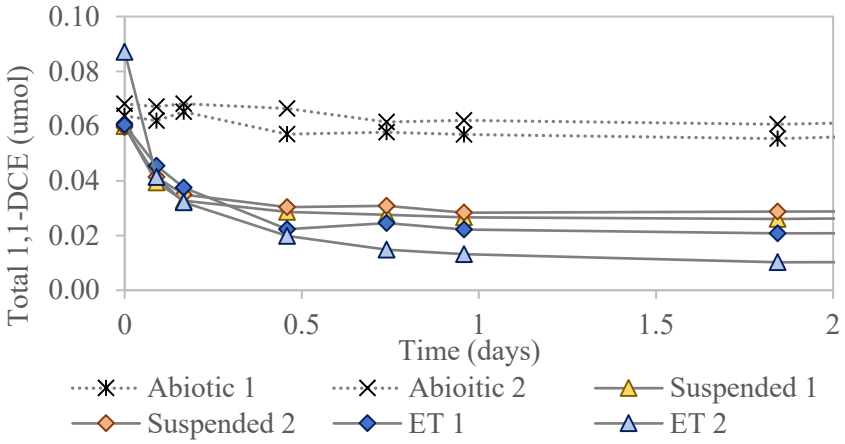

Figure 4.1 *Cometabolic transformation of (A) 1,1-DCE by immobilized G4 in gellan gum macrobeads (2 g beads/reactor) at a biomass loading of 1 mg_{TSS}/g_{bead}. Encapsulated treatment—ET*

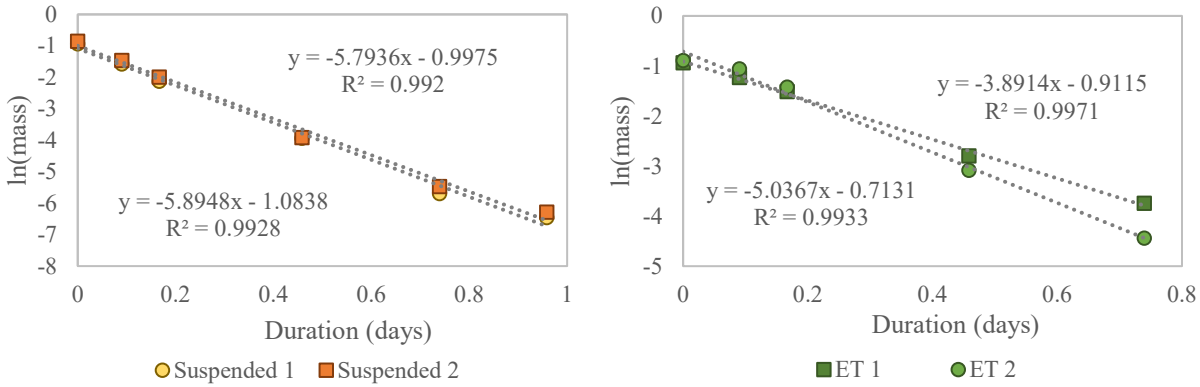

Figure 4.2 *First-order plots of initial TCE transformation over time for (A) suspended reactors and (B) encapsulated reactors (1 mg_{TSS}/g_{bead}) of B. vietnamiensis G4 at biomass of (2 mg_{TSS}/reactor). Reactors were in duplicate.*

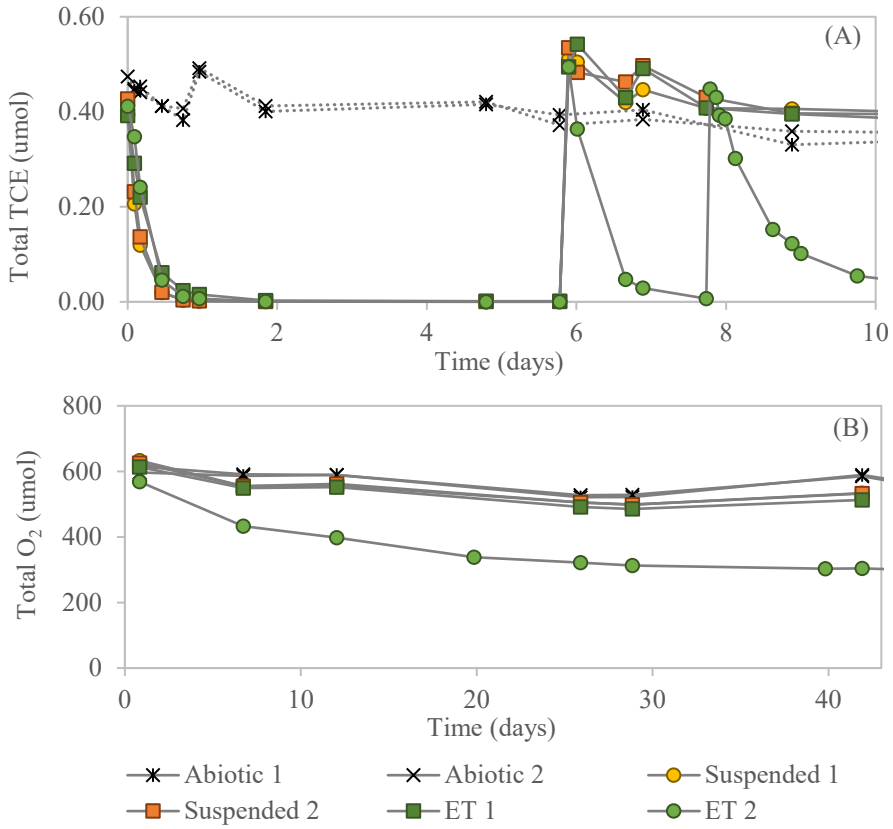

Figure 4.3 *Cometabolic transformation of (A) TCE and (B) O_2 utilization by immobilized G4 in gellan gum macrobeads (2 g beads/reactor) at a biomass loading of 1 mg_{TSS}/g_{bead}. One of the encapsulated reactors transforming TCE diverged on day 6. Data shown is for duplicates of each treatment. Encapsulated treatment—ET*

The suspended and encapsulated treatments that transformed TCE had similar psuedo-first order average rates at 5.8 and 4.5 day^{-1} respectively (***Figure 4.2***). On day six when a second addition of TCE was added, one of the encapsulated reactors, ET 2, continued TCE transformation (***Figure 4.3.A***). This reactor, for a reason unknown, differed from all other treatments from the beginning of the experiment by having a greater oxygen demand (***Figure 4.3.B***). This was unusual as the oxygen demand by resting cells is minimal as is seen with the other reactors compared to the abiotic controls. Considering abiotic losses, the oxygen uptake rate in ET 2 was ~3 times greater than ET 1 and the suspended treatments. One possible explanation for the greater activity is that the biomass distribution in the beads was not homogenous, and a greater initial biomass was present in ET 2. However, if a higher initial biomass were present, this is not reflected in the TCE transformation rate, which is similar to all other treatments.

ET 2 continued to be monitored separately from the other treatments (***Figure 4.4***). In addition, 1,1,1-TCA was added on day six to this bottle as an inidcator for abiotic losses as *B. vietnamiensis* G4 does not transform it via cometabolism. This singlet bottle continued to transform TCE, which was added continuously once it was degraded, for 86 days without any external substrate added (***Figure 4.4.A*)** The amount of TCE transformed until day 86 was 5.5 umol TCE, which corresponded to a capacity of 2.7 umol TCE/mg_{TSS} based on the initial amount of biomass added. Compared to the TCE capacity observed in Chapter 3 (0.47 umol TCE/mg_{TSS}) for toluene-grown *B. vietnamiensis* G4, this value is 5.7 times greater. It should be noted that this mass of TCE was transformed with minimal oxygen consumption; during this time of resting cell transformation, the ratio of TCE transformed to oxygen consumed was approximately 60 (umol/umol).

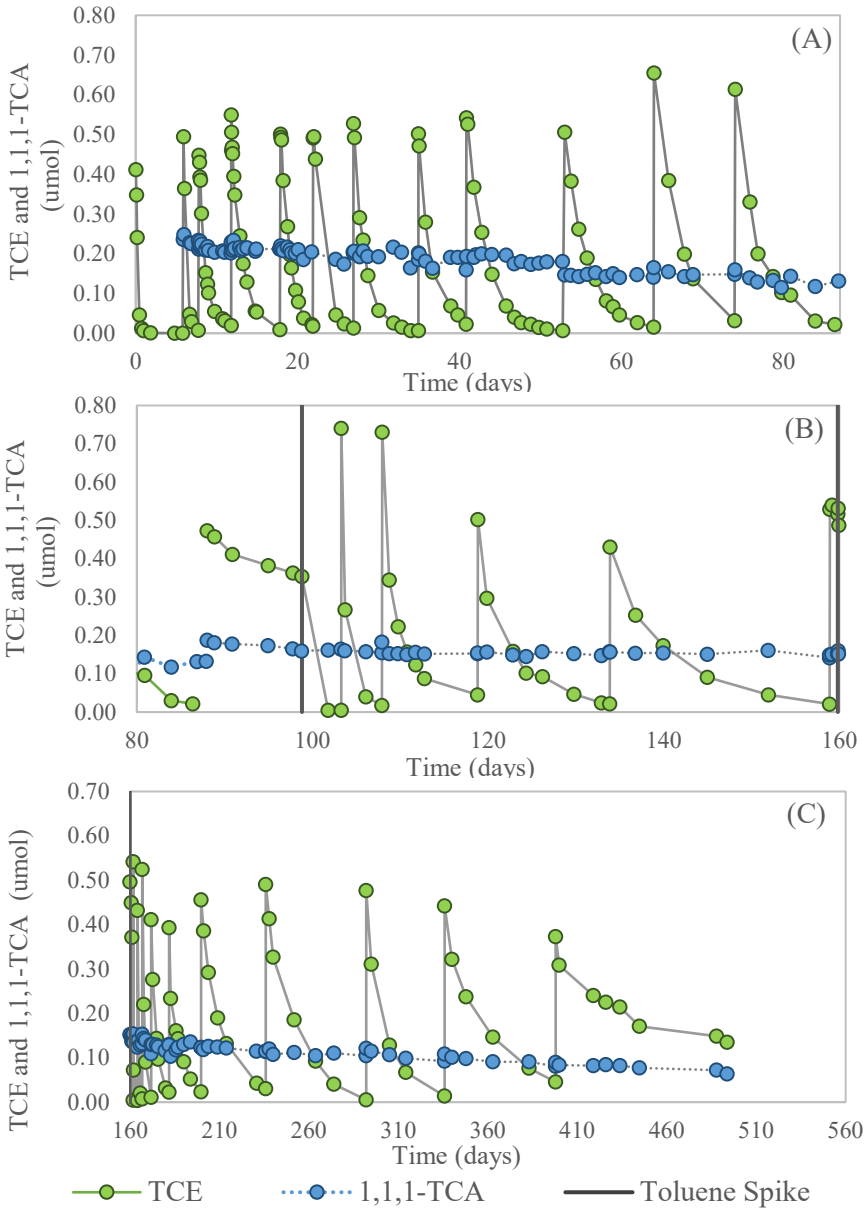

Figure 4.4 *Cometabolic transformation of TCE by G4 in gellan gum macrobeads (2 g beads/reactor) at a biomass loading of 1 mg_{TSS}/g_{bead}. Data shown for singlet that deviated. Beads (~20%) were removed on day 88 and the media was replaced. Toluene was added (74 umol) on day 99.*

On day 99, toluene was added to the singlet reactor to see if transformation would recommence. This indeed was the case, and TCE was degraded at a pseudo-first order rate of 0.77 day^{-1}, which was the same rate as the sixth addition of TCE added on day 22 (***Figure 4.5***). An additional 2.8 umol of TCE was transformed before stalling again on day 160. Based on the amount of toluene added—74 umol—this resulted in a transformation yield of 0.037 umol TCE/umol toluene (53 ug TCE/mg toluene). This yield is

almost four times the yield seen with *B. vietnamiensis* G4 grown on toluene in a chemostat system at 14 ug TCE/mg toluene (Landa et al., 1994). Another addition of toluene of the same mass was added on day 160, which sustained cometabolic activity for an additional 285 days (until day 445). This corresponded to a yield of ~0.064 umol TCE/umol toluene (91 ug TCE/mg toluene). During this period an additional 4.7 umol of TCE was degraded for a cumulative total mass of 13 umol (1.7 mg) of TCE.

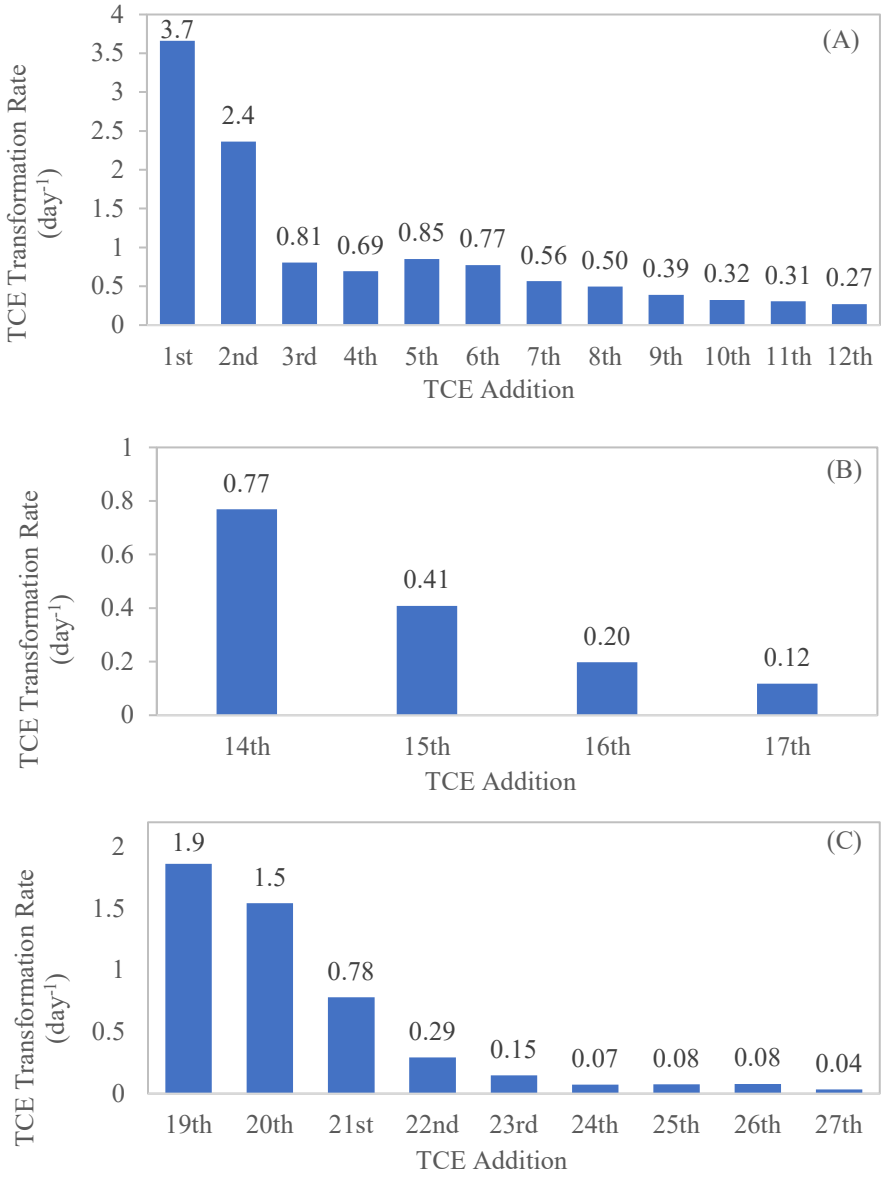

Figure 4.5 *Pseudo-first order TCE transformation rates for each addition of TCE by singlet encapsulated B. vietnamiensis G4 reactor (1 mg_{TSS}/g_{bead}) with an initial biomass of 2 mg_{TSS}. (A) Rates for resting cells, (B) rates after first addition of toluene on day 99, (C) rates after second addition of toluene on day 160.*

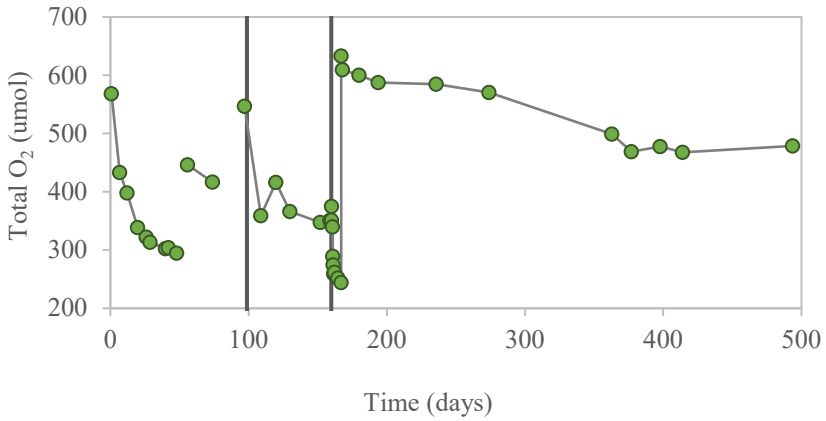

Figure 4.6 *Oxygen utilization by singlet reactor that diverged containing encapsulated G4 in gellan gum macrobeads. Toluene additions signified by lines at days 99 and 160.*

The oxygen uptake data for this singlet reactor was recorded over the 500 days of activity (***Figure 4.6***). The first discontinuity in the data was due to the addition of oxygen on day 55. The second discontinuity was from opening the reactor to take out 20% of the beads for analysis. At the time that the toluene was added (day 99), it can be seen that there was an oxygen demand of 188 umol over the course of at least ten days and then no change in oxygen until day 160. The second addition of toluene was similar except the oxygen demand was 107 umol over the course of seven days.

This initial and rapid oxygen demand seen when substrate was provided would ideally be circumvented through using a slow release substrate. These results would indicate that *B. vietnamiensis* G4 can be re-stimulated and continue cometabolism if substrate is provided, however, this phenomenon was only tested in a singlet reactor. Regardless, given how long this singlet reactor has been able to maintain cometabolic activity with two external additions of substrate and the considerable amount of TCE transformed, these results are very promising for a co-encapsulated version of this system.

4.1.2 High Biomass Encapsulation

An experiment was conducted with encapsulated resting cells to verify cometabolism by *B. vietnamiensis* G4 can be re-stimulated by external additions of substrate for long-term transformation. This experiment also encapsulated a higher biomass of *B. vietnamiensis* G4 that were exposed to a mixture of TCE and 1,1-DCE. A higher biomass was encapsulated because of the higher toxicity associated with transforming

1,1-DCE. Again, suspended cell treatments were created at an equivalent biomass to compare how encapsulation affected transformation rates and capacities.

Gellan gum macrobeads were made at a high biomass loading of 8 mg_{TSS}/g_{bead}. Two grams of beads were added to each batch reactor for encapsulated treatments for a total biomass of ~16 mg_{TSS}. TCE was added at ~700 ppb, and 1,1-DCE was added at a lower concentration of ~20 ppb, both of which were measured by headspace samples using methods outlined in Section 2.2.2. Additionally, respiration data was collected to verify cellular activity was occurring. The experiment consisted of duplicate active encapsulated and suspended treatments, in addition to duplicate abiotic controls.

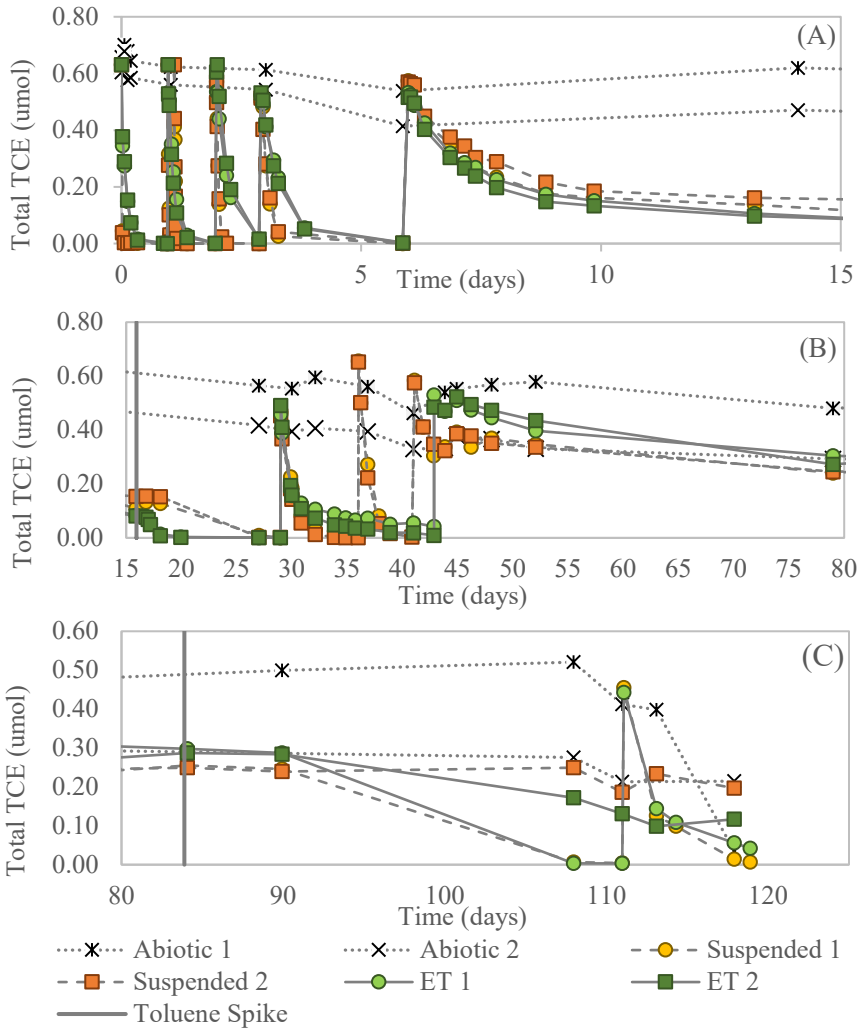

Figure 4.7 *Cometabolic transformation of TCE by resting encapsulated and suspended immobilized B. vietnamiensis G4 at 16 mg_{TSS}/reactor (A) resting cell transformation, (B) transformation after 1st toluene addition (day 16), (C) transformation after 2nd toluene addition (day 84). Treatment: duplicate encapsulated G4 (8 mg_{TSS}/g_{bead}), duplicate suspended G4, and duplicate abiotic controls.*

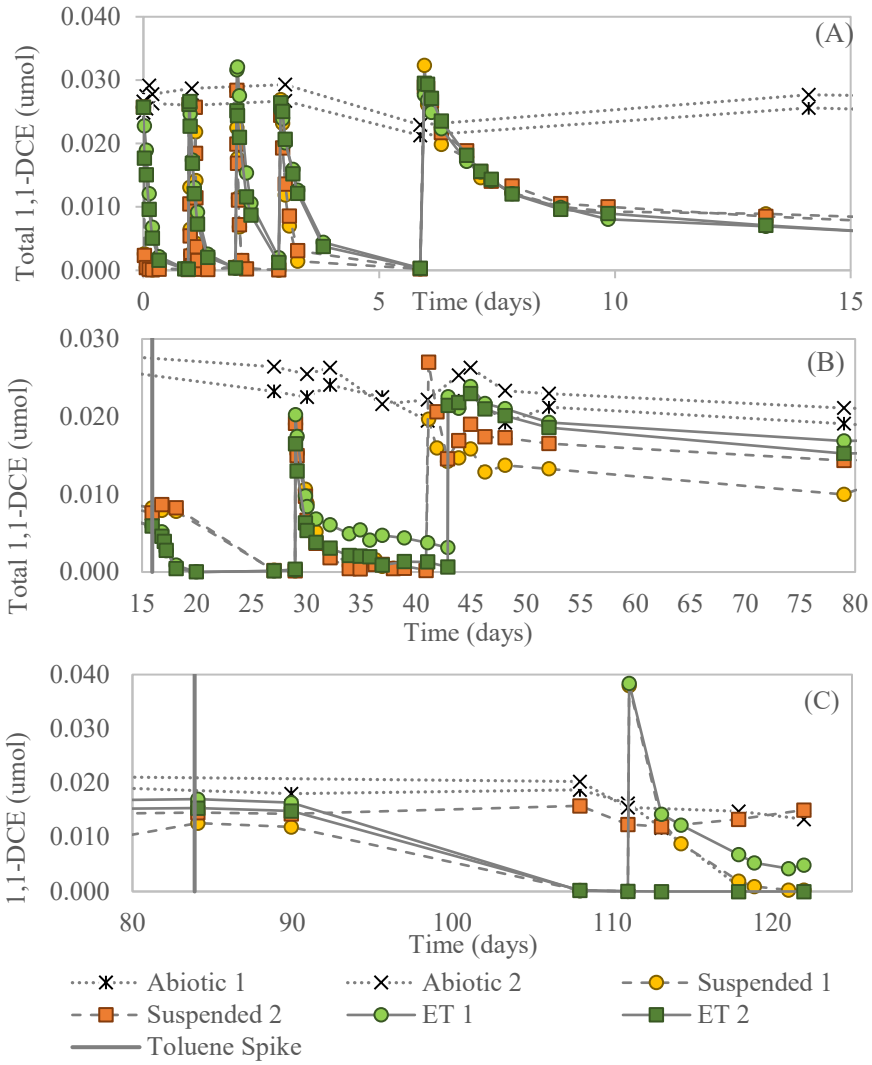

Figure 4.8 *Cometabolic transformation of 1,1-DCE by resting encapsulated and suspended immobilized B. vietnamiensis G4 at 16 mg_{TSS}/reactor (A) resting cell transformation, (B) transformation after 1st toluene addition (day 16), (C) transformation after 2nd toluene addition (day 84). Treatment: duplicate encapsulated G4 (8 mg_{TSS}/g_{bead}), duplicate suspended G4, and duplicate abiotic controls.*

The cometabolic transformation of TCE and 1,1-DCE can be seen in (***Figure 4.7*** *&* ***Figure 4.8)*** with the figures separated at day 15 and 80 for better visualization of the data. The rate of degradation of the suspended treatment was greater than encapsulated for the first four days when both treatment methods transformed the total mass of contaminants within a day. One explanation for the higher transformation rates seen with suspended cells are rate limitations due to the hydrogel creating a diffusion barrier. Previous studies observed a higher biomass loading in beads resulted in greater diffusion limited oxygen uptake rates (Gosmann & Rehm, 1986; Hiemstra et al., 1983).

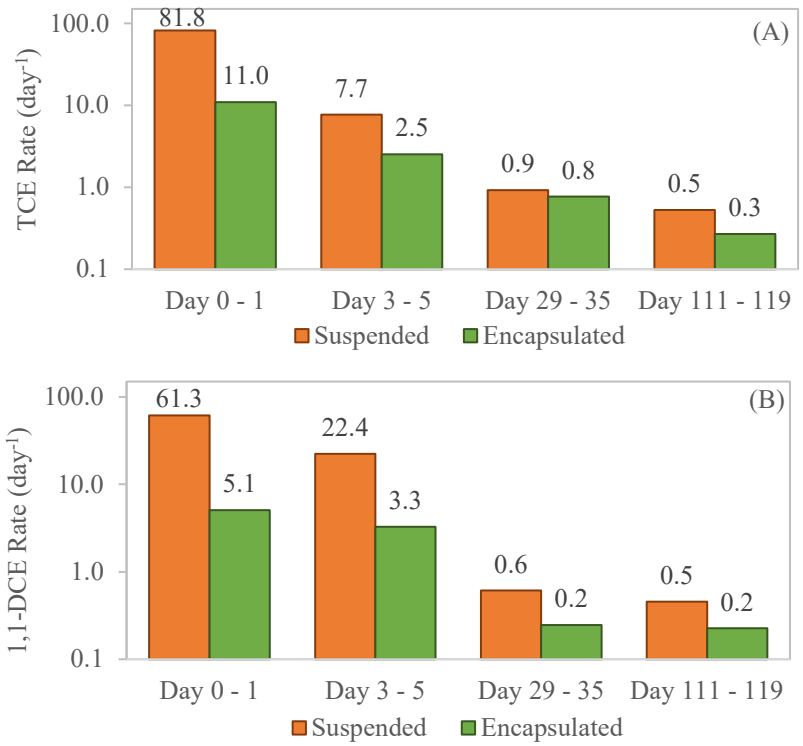

Figure 4.9 *Comparison of average pseudo-first order rates for (A) TCE and (B) 1,1-DCE in suspended and encapsulated treatments of B. vietnamiensis G4 (16 mg_{TSS}/reactor) at various time intervals: initial rates (Day 0 - 1), before resting transformation capacity is reached (Day 3 – 5), after an addition of toluene (Day 29 – 35), and after a second addition of toluene (Day 111 – 119).*

Pseudo-first order rates for the second (Day 0-1) and fourth (Day 3-5) contaminant mixture are presented (***Figure 4.9***). Transformation rates for suspended cells between these two periods decreased by a factor of approximately 10 and 3 for TCE and 1,1-DCE, respectively. Whereas, in the encapsulated treatments, the rate decreased by a factor of ~4 and 1.5 for TCE and 1,1-DCE, respectively. This reduction in rates for encapsulated cells is ~2 times less than is seen with suspended cells. Though suspended treatments had a greater reduction in rates between contaminant additions, this was because initial rates for both TCE and 1,1-DCE were almost ten times greater than encapsulated treatments. Since rates of transformation were greater in suspended treatments, it received one more addition of contaminants than encapsulated treatments resulting in a greater transformation capacity compared to encapsulated cells. Transformation slowed considerably with the fifth contaminant addition on day six, with both treatments unable to degrade the contaminant mixture over ten days (***Figure 4.7*** & ***Figure 4.8***). The transformation capacity until this point for both contaminants and treatments are shown in ***Table 4.1***.

Since both treatment methods had reached their transformation capacity for the TCE and 1,1-DCE mixture, toluene was added (~64 umol) as a substrate on day sixteen to determine if cells were still viable and if degradation could recommence. The addition of toluene to all treatments, which was done in a singlet reactor (***Figure 4.4***), would verify that *B. vietnamiensis* G4 could resume cometabolic activity after the resting cell transformation capacity was reached. Toluene uptake did not lag for either treatment (***Figure 4.10***), though the average toluene uptake rate was ~7 times faster in the encapsulated reactors at 38 umol/day than the suspended reactors at 5 umol/day (***Figure E.1***). Additionally, transformation of both 1,1-DCE and TCE resumed in the encapsulated treatments after one day, whereas the suspended treatments lagged in transforming contaminants for at least two days. The lag in transformation of contaminants but not substrate, indicates that substrate inhibition was occurring. Most notably the greater toluene uptake rate and transformation in the encapsulated treatments indicates that encapsulation alleviated the inhibition affects. It is also of note that cells metabolized toluene in the presence of 1,1-DCE and the toxic 1,1-DCE epoxide (***Figure 4.8.B***).

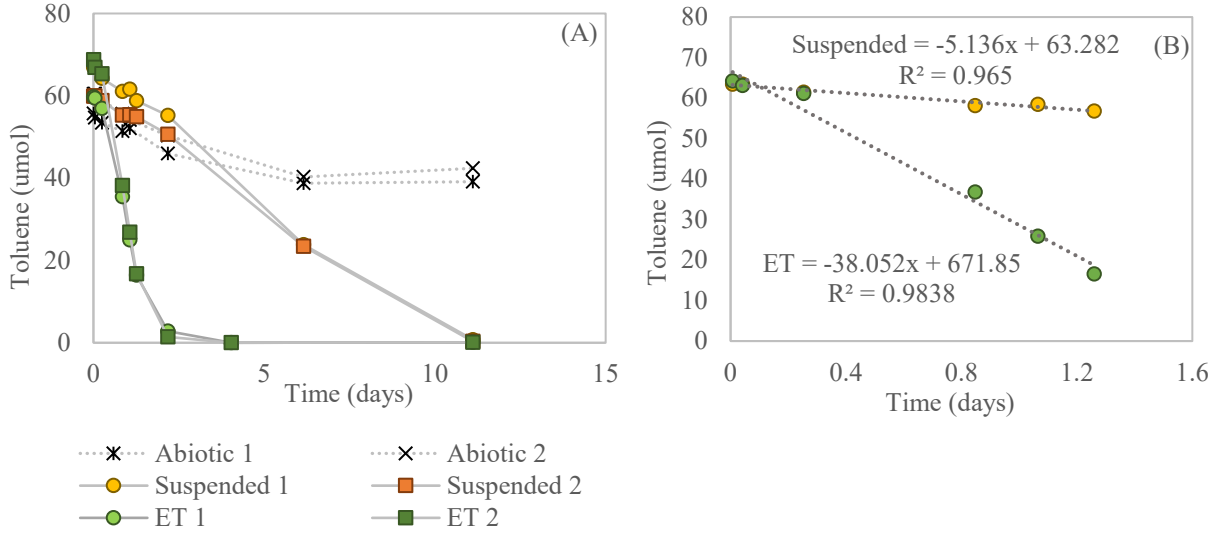

Figure 4.10 *(A) Toluene consumption after an addition made on day 16 in all treatments. Encapsulated treatments completely transformed toluene in four days, whereas suspended treatments transformed it in eleven days. (B) Average zero-order toluene uptake rates for suspended and encapsulated treatments of B. vietnamiensis G4 (16 mg$_{TSS}$/reactor)*

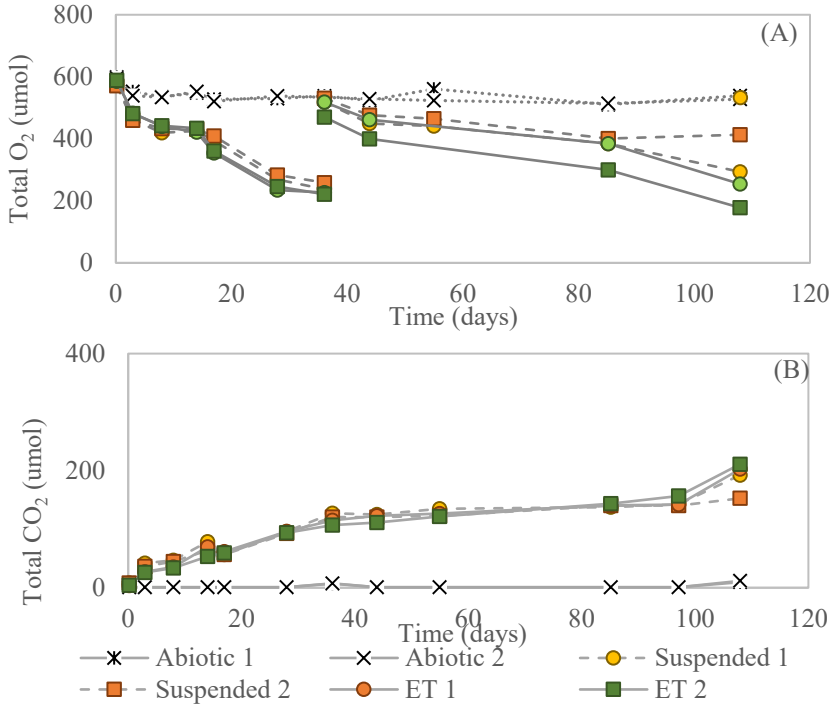

Figure 4.11 *(A) O_2 utilization and (B) CO_2 production by encapsulated G4 macrobeads (2 g beads/reactor) at a biomass loading of 8 mg_{TSS}/g_{bead}. Treatments consisted of duplicate encapsulated and suspended treatments at approximately equivalent biomass loadings. Pure oxygen was added to the headspace on day 26 for both encapsulated and suspended treatments. ET—encapsulated treatment.*

Respiration data shows similar oxygen uptake and carbon dioxide production between the two treatments indicating that a similar biomass was present. The similar oxygen uptake rate also indicates that there were not diffusion limitations of oxygen into the bead from the high biomass loading as was seen in literature (Hiemstra et al., 1983). Before substrate addition on day 16, there was an oxygen demand associated with the resting cell biomass in both treatments; an average of 144.5 umol of O_2 consumed in suspended reactors and 158.5 umol of O_2 consumed in encapsulated. Carbon dioxide production is similar with an average of 0.46 and 0.42 umol produced in suspended and encapsulated treatments, respectively during the same time. After the addition of toluene on day 16, a discernible increase in the oxygen uptake rate can be seen in both treatments, whereas the carbon dioxide production rate stays the same for both treatments (2.8 umol CO_2/day) (***Figure 4.11***). Between days 14-36, encapsulated reactors utilized an average of 204 umol oxygen compared to 180 umol in suspended treatments for the same mass of toluene added.

Once toluene was consumed and the remaining contaminants transformed, another addition of TCE and 1,1-DCE were added to both treatments on day 29 (***Figure 4.7.B & Figure 4.8.B***). Both contaminants were completely transformed, but at considerably decreased rates (rate comparison shown in ***Figure 4.9***). Though encapsulated reactors had a higher rate of toluene consumption, suspended reactors had a greater transformation rate and higher yield (umol contaminant transformed/mmol substrate added) for both contaminants (***Table 4.1***). In suspended reactors, the yield for 1,1-DCE was similar and the yield for TCE was twice that of encapsulated because they received one more addition of TCE (***Figure 4.7.B***). If both treatments were treated the same, the yields may have been closer as they were for 1,1-DCE.

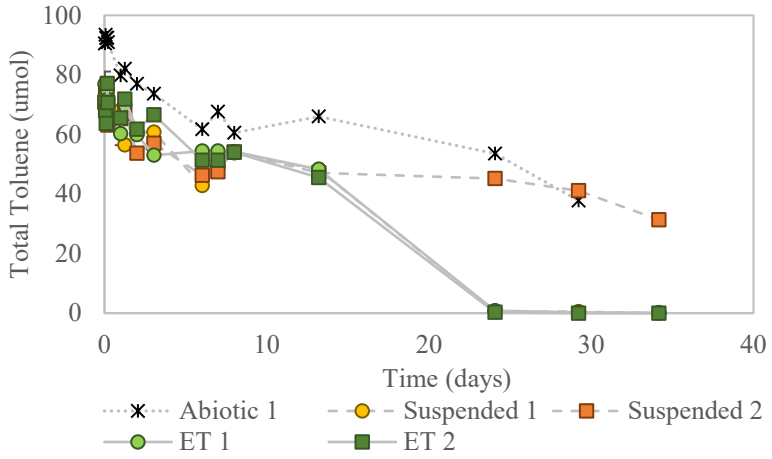

Figure 4.12 *Toluene consumption by encapsulated and suspended B. vietnamiensis G4 after an addition made on day 84 in all treatment. It took at least 24 days for all treatments (except Suspended 2) for complete toluene uptake.*

When transformation stalled again, all reactors were set aside for one month before another addition of toluene at approximately the same mass as was added before (~ 70 umol) on day 84 (***Figure 4.7.B & Figure 4.8.B***). During this month period (days 52 – 84), there was no change in the mass of contaminants compared to abiotic controls and minimal respiration indicators, thus little to no microbial activity was occurring prior to the toluene addition. Complete toluene consumption was seen in both encapsulated treatments, but in only one of the suspended treatments (Suspended 1) (***Figure 4.12***). This suggests that encapsulation provides some protection to the cells during long-term transformation of contaminants and increased cell viability. Literature also observed a greater log reduction in suspended cell count compared

to encapsulated cells. The lack of toluene uptake in Suspended 2 and no change in respiration during this time compared to the other reactors, indicates cells were no longer culturable in this reactor.

All reactors except Suspended 2 transformed the remaining 1,1-DCE, and only reactors Suspended 1 and Encapsulated 1 transformed the remaining TCE. The reactors that transformed the remaining 1,1-DCE and TCE received another addition of contaminants, which were also transformed (***Figure 4.7C, Figure 4.8.C***). It is also notable that both of these reactors, Suspended 1 and Encapsulated 1, had similar levels of cometabolic activity with the same transformation rates and yield for both contaminants (***Table 4.1***). This has not been the case until this point, with suspended treatments typically having higher transformation rates and a higher yield.

Table 4.1 *1,1-DCE and TCE average transformation capacity of resting cells, and average yields for initial and subsequent toluene additions for suspended and encapsulated treatments.*

	Suspended		Encapsulated	
	1,1-DCE	TCE	1,1-DCE	TCE
Resting Capacity (umol/mg_{TSS})	0.0095	0.21	0.0078	0.17
Yield[1] (umol/mmol toluene)	0.54	25	0.40	12
Yield[2] (umol/mmol toluene)	0.71	10	0.72	9.9

[1]Yield from the first addition of toluene added (~64 umol) for all reactors
[2]Yield from second addition of toluene added (~70 umol) for "Suspended 1" and "Encapsulated 1"

This study showed that cometabolism of a mixture of TCE and 1,1-DCE was able to resume in resting *B. vietnamiensis* G4 cells once the transformation capacity had been reached, through an external addition of substrate. This was even the case when cells were exposed to the contaminant mixture for a month without a carbon source present. This indicates that transformation stopped primarily due to lack of energy reserves rather than toxicity. This study also indicates that encapsulation alleviates substrate inhibition affects, given that encapsulated cells demonstrated greater toluene uptake. It is also possible encapsulation protected cells from toxicity effects by demonstrating greater cell viability after one month of inactivity. Responses to substrate addition and cometabolic transformation would likely have been

more robust if the culture had not spent as long in the presence of the contaminant mixture without an energy source. For application, this implies that cometabolism by *B. vietnamiensis* G4 could be regenerated ex situ or possibly in place for in situ treatment. These results are also promising for a co-encapsulated version of this system, which would provide a substrate at a slow rate over time to maintain cometabolic transformation.

4.2 Co-encapsulation Batch Kinetic Studies

Given the long-term transformation seen in encapsulated reactors with an external addition of a substrate, investigations were made into possible slow-release compounds (SRC). Based on the results seen with benzyl alcohol grown *B. vietnamiensis* G4 able to support aerobic cometabolism for all chlorinated ethene contaminants of interest (***Table 3.2***), attempts were made to find a suitable SRC that hydrolyzed to produce benzyl alcohol. Previous success was seen using an orthosilicate SRC that released alcohols for strain ATCC 21198, so exploring possible orthosilicates for *B. vietnamiensis* G4 was also of interest (Murnane, 2018; Rasmussen et al., 2020). Consequently, the two SRCs explored here were: 1) benzyl butyrate, an ester that hydrolyzes to produce benzyl alcohol and butyric acid; and 2) tetraphenoxysilane, an orthosilicate that hydrolyzes to produce phenol.

4.2.1 Benzyl Butyrate Co-encapsulation

Previously it was shown that benzyl alcohol grown *B. vietnamiensis* G4 was able to support cometabolic degradation of TCE, *cis*-DCE, 1,1-DCE, and VC (***Table 3.2***). It was also shown that benzyl butyrate grown *B. vietnamiensis* G4 supported high rates of TCE transformation and a greater transformation capacity than toluene or phenol grown cells (***Table 3.1***). Due to these promising results, benzyl butyrate was tested as a possible SRC. For this experiment, *B. vietnamiensis* G4 was grown on toluene and co-encapsulated with benzyl butyrate at ~10% (v/v) with a biomass loading of 0.5 mg_{TSS}/g_{bead}. Six reactors were made: one abiotic control, triplicate co-encapsulated treatments, and duplicate poisoned co-encapsulated bottles with sodium azide (0.2% w/w). Reactors contained 2 g beads/reactor for a total biomass of 1 mg_{TSS}. TCE was added at approximately 400 ppb to each reactor.

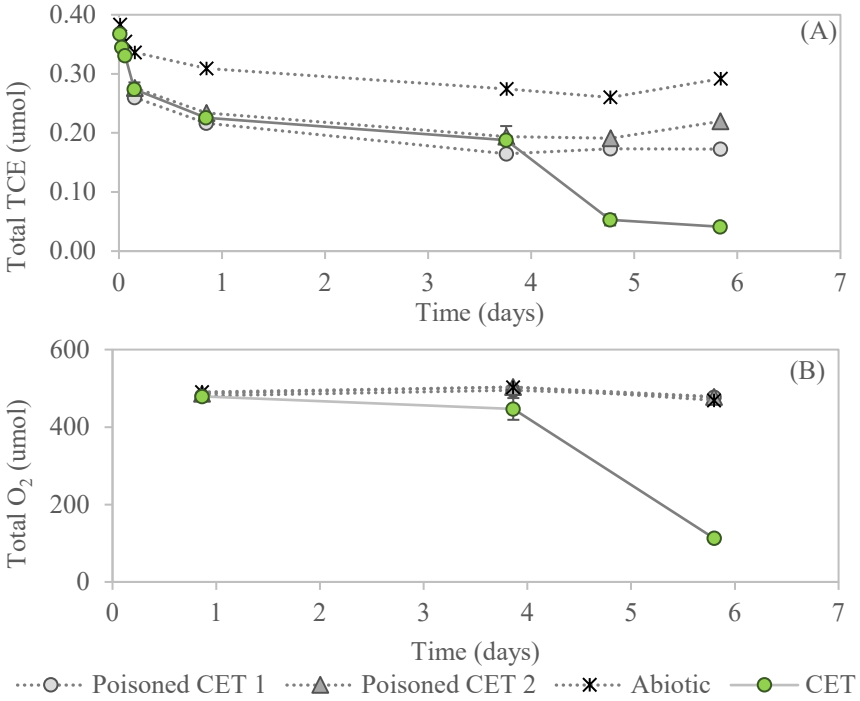

Figure 4.13 *(A) Cometabolic transformation of TCE and (B) oxygen utilization by co-encapsulated benzyl butyrate and G4 beads (2 g beads/ reactor). Beads were made at a biomass loading of 0.5 mg_{TSS}/g_{bead} and ~10% (v/v) benzyl butyrate. Treatments consisted of duplicate sodium azide (2% v/v) poisoned controls, a singlet abiotic control, and triplicate active co-encapsulated treatments in media (CET). CET values are means with error bars representing standard deviation (n=3).*

In this experiment, approximately 50% of added TCE was transformed in the first day, which represented the resting cell capacity. The remaining TCE was not transformed until day four in the active treatments. Concurrently though, oxygen was depleted in the active bottles and carbon dioxide was produced. According to EPI Suite, the hydrolysis half-life of benzyl butyrate at a pH of 7 is 1.047 years, suggesting *B. vietnamiensis* G4 may have an esterase enzyme capable of accelerating hydrolysis of the ester. These results were expected from observations of growing cells on benzyl butyrate for the resting cell kinetic tests in Chapter 3. Since oxygen was depleted over such a short time frame of two days, this experiment was stopped, as oxygen would have to have been constantly resupplied. Because of this, benzyl butyrate is not a suitable SRC for long-term, passive in situ treatment, as oxygen is a limited resource in the subsurface. If oxygen was supplied externally (such as through air sparging or with hydrogen peroxide) a treatment approach of bioaugmenting co-encapsulated *B. vietnamiensis* G4 with benzyl butyrate or biostimulating native culture capable of aerobic cometabolism, may be a possible treatment method. This

could be tested with co-encapsulated benzyl butyrate beads in a continuous flow system with oxygen supplied instead of a batch system.

4.2.2 Separated Benzyl Butyrate Encapsulation

An experiment was performed in an attempt to address the high respiration rates due to the accelerated hydrolysis of benzyl butyrate. Instead of co-encapsulated beads with benzyl butyrate and *B. vietnamiensis* G4 together, a test was performed to see if encapsulating the substrate and culture separately would slow the rate of hydrolysis. Two sets of beads were made: beads encapsulated with *B. vietnamiensis* G4 at a biomass loading of 0.5 mg_{TSS}/g_{bead}, and beads encapsulated with ~10% benzyl butyrate. The same loadings of biomass and substrate were done as in Section 4.2.1 so direct comparisons could be made. Treatments consisted of: 1) triplicate reactors with two grams of beads with encapsulated G4 beads and encapsulated benzyl butyrate beads (4 g beads total), 2) duplicate reactors with two grams of G4 beads (no substrate present), 3) duplicate reactors with two grams of encapsulated benzyl butyrate beads and suspended G4, 4) duplicate reactors with both type of beads with propyne (2% v/v) present, and 5) an abiotic control. A control with encapsulated benzyl butyrate and suspended *B. vietnamiensis* G4 was created as another means of observing the effects of separating components spatially.

It can be seen from ***Figure 4.14.A*** that there was minimal difference in TCE transformation between the three active treatments, which all reached the resting cell transformation capacity by day two. The treatment with suspended G4 may have had a higher rate and transformation capacity, followed by the separated encapsulated treatments. Most notable is the respiration data in ***Figure 4.14.B&C,*** particularly the oxygen uptake rates. In all treatments with substrate and *B. vietnamiensis* G4 present—the separated encapsulated treatments, the suspended *B. vietnamiensis* G4 and encapsulated benzyl butyrate, and the propyne controls—there was no difference between the oxygen uptake and carbon dioxide production. We see here that separating the substrate from cells did not slow oxygen uptake.

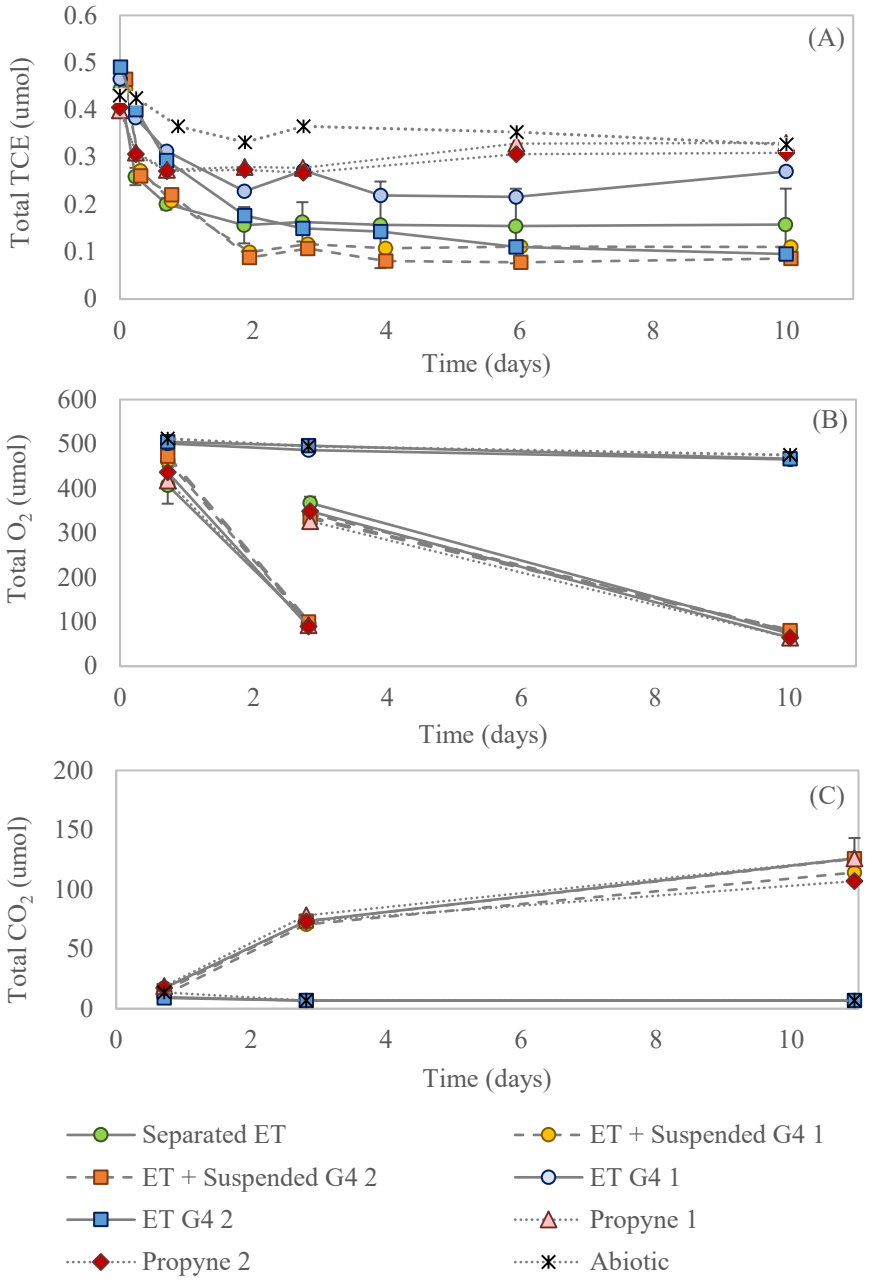

Figure 4.14 *(A) Cometabolic transformation of TCE, (B) Oxygen utilization and (C) carbon dioxide production by encapsulated benzyl butyrate and encapsulated G4 beads (2 g beads/ reactor). Beads with G4 were made at a biomass loading of 0.5 mg_{TSS}/g_{bead} and beads with benzyl butyrate were ~10% (v/v). Treatments consisted of triplicate separated encapsulated beads, duplicate encapsulated benzyl butyrate and suspended G4, duplicate encapsulated G4, duplicate encapsulated G4 with propyne (2% v/v), and a singlet abiotic control. Separated ET values are means with error bars representing standard deviation (n=3).*

A likely explanation for the similar oxygen uptake between treatments is that an amount of benzyl butyrate leached out of the beads. Based on Equation 4, it would take 37 umol of benzyl butyrate to consume the initial amount of oxygen present in the reactors (500 umol).

$$C_{11}H_{14}O_2 + 13.5O_2 \rightarrow 7H_2O + 11CO_2 \qquad \text{Equation 4.}$$

If encapsulation of benzyl butyrate was 100% successful, the mass of encapsulated benzyl butyrate would have been 0.2 g (1122 umol). If benzyl butyrate were present in solution, enough to maintain the solubility limit of 136 mg/L (McGinty et al., 2012), which would be a mass of 76 umol of benzyl butyrate, this would provide twice the mass needed to deplete the oxygen present. If this was the case, even separately encapsulated, benzyl butyrate is not a suitable substrate for our purposes.

4.2.3 Tetraphenoxysilane Co-encapsulation

As discussed previously, tetraalkoxysilanes such as tetrabutoxysilane (TBOS) and tetra-sec-butoxysilane (T2BOS) have been shown to support growth of cultures capable of aerobic cometabolism (Murnane, 2018; Vancheeswaran et al., 1999). These substrates are a group of silicon-based compounds that contain four ether linkages from the central silicon atom to the corresponding alkoxy group. It was also shown that these tetraalkoxysilanes can be co-encapsulated in gellan gum macrobeads to support transformation of CAHs over the course of 300 days (Rasmussen et al., 2020). Given the success with these compounds as SRCs, and the possibility to vary the alkoxy group, tetraphenoxysilane (TPhOS) was tested as a SRC for *B. vietnamiensis* G4. TPhOS hydrolyzes abiotically to produce phenol and silicic acid. The equation for complete hydrolysis of TPhOS is as follows

$$\mathrm{Si(O_4C_6H_5)_4 + 4H_2O \rightarrow 4C_6H_5OH + Si(OH)_4} \qquad \text{Equation 5.}$$

(TPhOS + Water → 4 Phenol + Silicic Acid)

Phenol was chosen as the alkoxy group as it supports cometabolism in *B. vietnamiensis* G4, and was readily available. Thus, the goal of this experiment was to provide proof of concept that a co-encapsulated system with *B. vietnamiensis* G4 could promote long-term transformation of chlorinated ethenes.

For this experiment, gellan gum beads were co-encapsulated at a biomass loading of 1.45 mg_{TSS}/g_{bead} and ~1.9% (w/w) TPhOS. Cells were grown on benzyl butyrate due to the higher capacity for TCE seen compared to toluene-grown cells. The set up included two co-encapsulated treatments and two co-encapsulated bottles poisoned with sodium azide (0.2% w/w) with 2 g beads per reactor (2.9 mg_{TSS}/ reactor). TCE was the only contaminant initially added at concentrations of ~400 ppb.

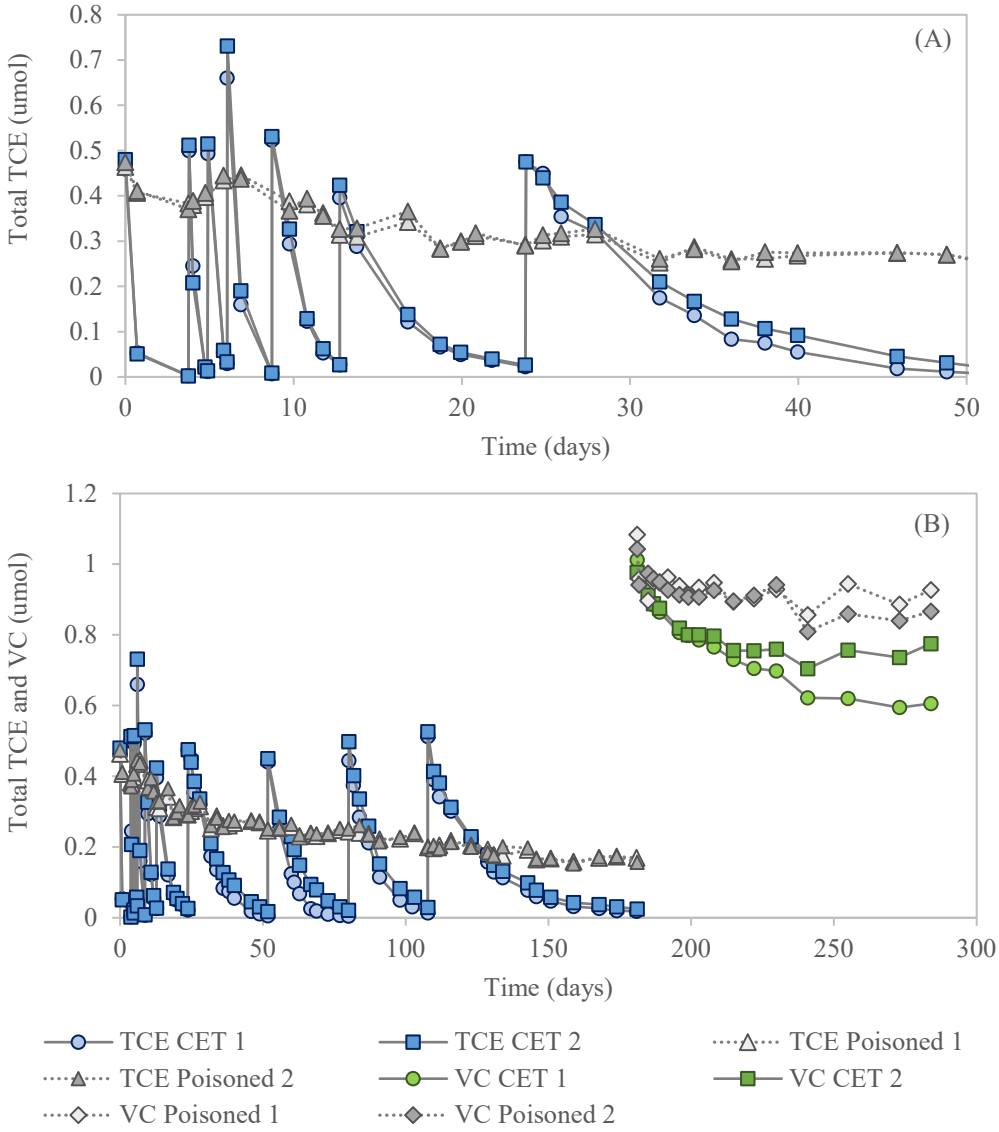

Figure 4.15 *(A) TCE cometabolic transformation data for the initial fifty days and (B) TCE and VC cometabolic transformation data over the entire duration by co-encapsulated TPhOS and G4 beads (2 g). Treatments only saw TCE until VC was added on day 180 once TCE was fully degraded. Beads were made at a biomass loading of 1.45* mg_{TSS}/g_{bead} *and 1.9% (w/w) TPhOS. Active treatments were opened on day 181, and 15% of beads were removed. Treatments consisted of duplicate sodium azide (2% v/v) poisoned controls with co-encapsulated beads, and duplicate active co-encapsulated treatments in media. (CET)—Co-encapsulated Treatment.*

It can be seen from ***Figure 4.15*** that TCE transformation rates were high, with continuous TCE exposure and transformation occurring over the course of 180 days. TCE rates gradually decreased until the period of day 24 – 108, where they appeared to have reached steady state at a pseudo-first order rate of ~0.12 day^{-1} (***Figure 4.16***). During this period of steady state, three additions of TCE (~1.4 umol) were transformed. The attainment of steady state rates appeared to indicate that during this time, the rate at which phenol was released sustained a constant biomass of encapsulated *B. vietnamiensis* G4 capable of transforming TCE at that rate. However, from day 108 - 181, the rate decreased to 0.05 day^{-1} (***Figure 4.16***). The total mass of TCE transformed was 4.5 umol TCE (0.6 mg TCE). Based on the original biomass encapsulated this would represent a transformation capacity of 1.6 umol/mg_{TSS}, which is 3.4 times greater than the capacity observed for TCE by toluene-grown cells (***Table 3.1***). Because of the decrease in activity, VC was added to both treatments on day 181. VC was chosen as it had the highest transformation rate by *B. vietnamiensis* G4 from the suspended kinetic tests done with various chlorinated ethenes (***Table 3.2***). As can be seen by ***Figure 4.15***, transformation of VC occurred slowly, with active bottles diverging on day 200. Active CET 1 stalled on day 240, whereas active CET 2 stalled on day 215.

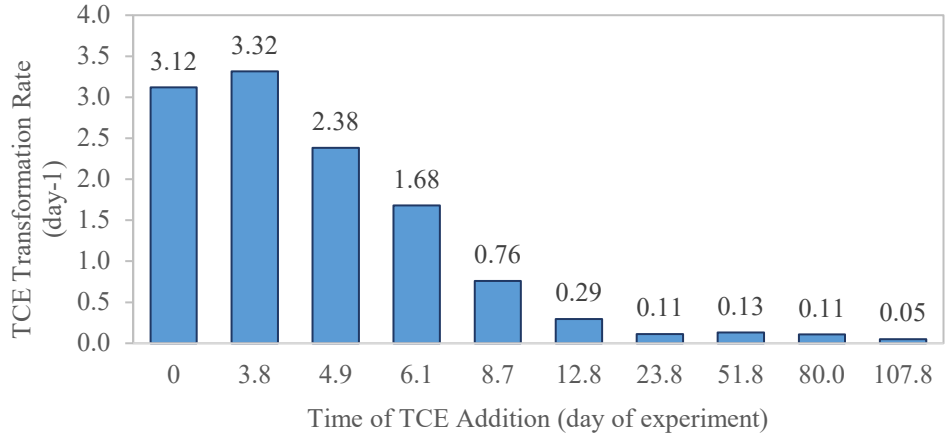

Figure 4.16 *Average psuedo-first order TCE transformation rates for each addition of TCE added to active treatments of co-encapsulated TPhOS (1.8%) and G4 (2 g of beads/reactor). Beads were made at a biomass loading of 1.45 mgTSS/g_{bead} and 1.8% (w/w) TPhOS. The rate of transformation decreased over time and was stable from day 23.8 to day 107.8 before decreasing on the next TCE addition.*

From the respiration data (***Figure 4.17***)**,** it is clear during the first four days the microbial population in the beads was active, due to the lower O_2 when compared to the poisoned controls. In the first four days, an average of 255 umol of oxygen was consumed. Carbon dioxide was not measured until day 50. This

initial high oxygen uptake is believed to be due to exposure to phenol; TPhOS purity is given as a range of 95-100% by the manufacturer. After this initial period though, low O_2 consumption rates are seen in the active CETs until day 173 at an average rate of 0.76 umol/day and carbon dioxide production appears to stall after day 110 (***Figure 4.17.A***). This rate is comparable to the oxygen uptake rate seen by co-encapsulated strain ATCC 21198 with T2BOS at 0.48 umol/day (Rasmussen et al., 2020), and is ~4 times less than the initial rate seen in resting encapsulated cells at a similar biomass of 2 mg_{TSS}/reactor (***Figure 4.3***).

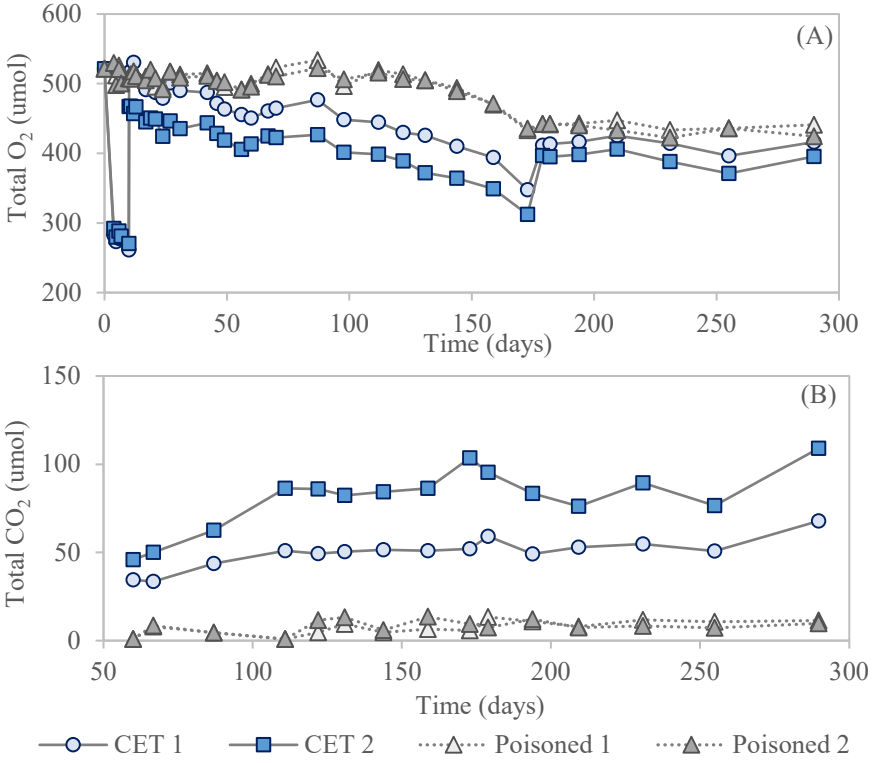

Figure 4.17 *(A) Oxygen utilization and (B) carbon dioxide production by co-encapsulated TPhOS and G4 beads (2 g). Beads were made at a biomass loading of 1.45 mg_{TSS}/g_{bead} and 1.9% (w/w) TPhOS. Treatments consisted of duplicate sodium azide (2% v/v) poisoned controls with co-encapsulated beads, and duplicate active co-encapsulated treatments in media. Active treatments were opened on day 181, and 15% of beads were removed. (CET)—Co-encapsulated Treatment.*

Despite evidence of this slow cellular activity past day four, high cometabolic activity is seen for TCE transformation (***Figure 4.15***). This efficient oxygen use to support cometabolic activity is ideal for in situ passive treatment. The small increase in oxygen on day 173 is due to opening active bottles to take out 15% of beads for future microbial analysis. After day 173, oxygen uptake seems to stall, which would

approximately coincide to when cometabolic activity stopped. The average mass of oxygen consumed in the CETs until this point is 330 umol considering abioitic losses.

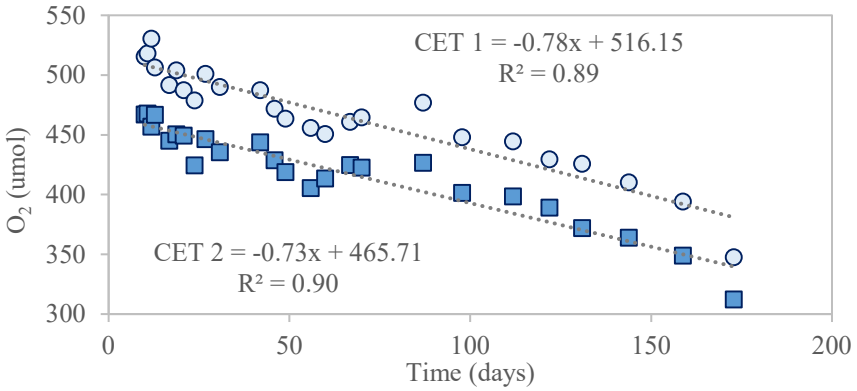

Figure 4.18 *Zero-order oxygen utilization rates for duplicate CET active reactors from day 10 – 173.*

The transformation rate declined over time, which may have resulted from the transformation capacity being exhausted. Transformation may have become limited if the rate of phenol released could not sustain the encapsulated microbial population. Given that transformation rates continued to decline and that respiration activity apparently ceased, it would suggest that substrate was potentially not being released to promote continuous cometabolism. To determine if substrate was being released over time and at what rate, liquid samples from both active and poisoned reactors were taken to determine the liquid concentration of phenol. Methods of how liquid samples were taken and how phenol was measured is described in Section 2.2.3. However, the data collected is sparse due to issues with the HPLC instrument used to analyze liquid samples and a hydrolysis rate was not determined.

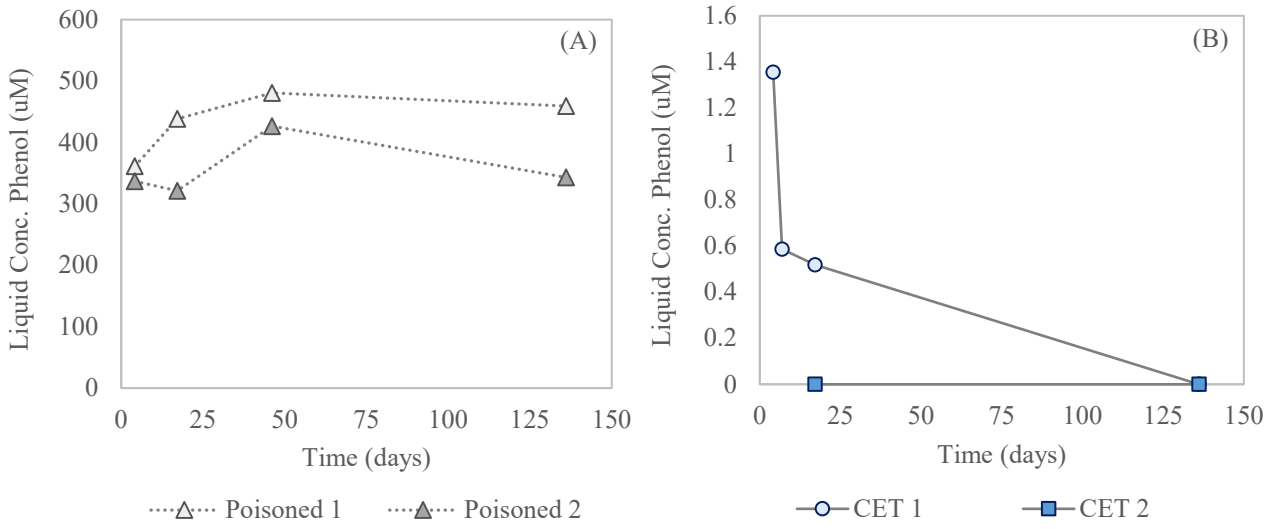

Figure 4.19 *Phenol liquid concentrations (uM) in (A) duplicate poisoned controls and (B) duplicate active co-encapsulated treatment (CET).*

Of the liquid samples collected and analyzed for phenol, the data for co-encapsulated active treatments is as expected, with little to no phenol detected (***Figure 4.19.B***). Only two samples were analyzed for CET 2, both of which were below the level of detection for phenol. Unexpectedly, the phenol concentrations in the two poisoned controls do not seem to change considerably between day 4 and 136, and are approximately 400 uM. Since phenol does not significantly change over 130 days, it is likely this mass of phenol (~40 umol in 100 mL) was from the percentage of impurity in TPhOS. The product information gives a range of 95-100% for purity. Based on the 2 grams of beads in each reactor and the theoretical mass loading of TPhOS—1.9% (m/m)—if 5% impurity is assumed, this would theoretically result in ~1.9 mg phenol encapsulated. Given the high solubility of phenol (26.16 g/L at 25°C); it would have existed in solution at a liquid concentration of approximately 200 uM. This is in range of the liquid concentration measured in the poisoned controls in ***Figure 4.19***.

Based on the mass of phenol measured, a mass balance can be done on oxygen. Based on the stoichiometry for the oxidation of phenol to carbon dioxide and water, 7 umol of oxygen is required to oxidize 1 umol of phenol; for 40 umol of phenol, 280 umol of oxygen would have been required. The time that oxygen stalls corresponds to the end of TCE transformation (~175 days), during which 330

umol oxygen is consumed and 4.5 umol of TCE is transformed. Phenol and TCE oxidation would account for ~290 umol of oxygen, which is consistent with the mass of oxygen utilized. A mass balance can also be performed on carbon dioxide. Based on a pH of 7 and the corresponding partial pressure of carbon dioxide based on the average mass produced (~60 umol), an estimate of the aqueous mass of H_2CO_3* and $HCO3^-$ was determined in Visual MINTEQ (Gustafsson, 2010). This corresponds to a total estimated mass of carbon dioxide of 540 umol, which is in decent agreement to the estimated mass based on stoichiometry.

Based on the mass of phenol, the initial biomass can also be estimated by the following equation,

$$X = X_0 + Y_g(M_0 - M) \qquad \text{Equation 6.}$$

where X is the final biomass, X_0 is the initial biomass, Y_g is the growth yield and represents the mass of biomass produced/mass of growth substrate, M_0 is the initial mass of substrate, and M is the final mass of substrate. Alagappan & Cowan, 2003 observed a Y_g of 0.58 and 0.59 (mg biomass/mg substrate) for toluene and benzene, respectively. If we assume a Y_g of 0.6 mg/mg for phenol, 40 umol of phenol consumed, and the initial biomass of 2.9 mg_{TSS}, this would result in a biomass of ~5.2 mg_{TSS}. Using the transformation capacity of 0.77 umol of TCE/mg_{TSS} observed for phenol grown *B. vietnamiensis* G4 in Chapter 3 (***Table 3.1***), this would correspond to 4 umol of TCE transformed. This is extremely close to the mass transformed observed here, which makes the case that the long-term transformation over 175 days seen resulted from the biomass that grew up in the beads initially with the phenol present. The transformation yield (T_y) calculated from the mass of TCE transformed and the initial mass of 40 umol of phenol results in an average T_y of 0.11 umol TCE/umol phenol.

The constant phenol liquid concentration in conjunction with the cometabolic and respiration data results, strongly suggest that the TPhOS hydrolysis rate is extremely low. One factor for the slow hydrolysis may be due to steric hindrance of the aromatic group. This effect was also observed with TBOS and T2BOS,

with the more branched T2BOS having a hydrolysis rate ten times less than TBOS. Benzyl alcohol, the ideal substrate in this case, would likely experience greater steric effects given the additional carbon; hence, an orthosilicate version may also have a slow hydrolysis rate.

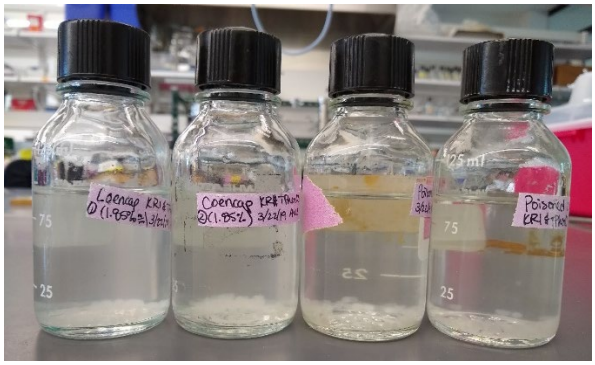

Figure 4.20 *From left to right: duplicates of active and poisoned treatments of co-encapsulated KR1 and 1.9% (w/w) TPhOS at day 112. Macro beads are at the bottom of the bottles. It is believed the majority of biomass is immobilized in the beads, as no visible growth in suspension is seen in the active treatments compared to the poisoned controls.*

Since TPhOS appears to have a low hydrolysis rate, it is an unsuitable SRC for our purposes. Yet, it should not be overlooked that a high initial mass of substrate supported cometabolic transformation for roughly 200 days; the majority of that time with cells likely in a state of starvation given phenol was consumed rapidly in active co-encapsulated treatments. This same phenomena was seen in Sections 4.1.1 and 4.1.2 with the single additions of toluene supporting long-term cometabolic activity. It is also of note that there was no initial lag in transformation of TCE, which is notable given phenol was shown to be inhibitory over phenol concentrations of 50 uM (Folsom et al., 1990).

4.3 Fort Carson Microcosm

Before it was realized that hydrolysis rates were likely low for TPhOS, a separate experiment was conducted with co-encapsulated TPhOS and *B. vietnamiensis* G4 beads in a microcosm system. The possible issues in a microcosm system not seen in a batch reactor with media, is competition with native culture, unknowns associated with site material, and possible nutrient limitations. The objective of this experiment was to see how co-encapsulated beads performed in the presence of native microorganisms in a less than ideal system for the culture.

The setup of the microcosms is described in Section 2.8. Toluene grown *B. vietnamiensis* G4 was co-encapsulated at 0.85 mg_{TSS}/g_{bead} with 1.9% (w/w) TPhOS. Treatments include one abiotic control, one control with co-encapsulated beads and propyne (2% v/v in the headspace) as a monooxygenase inhibitor, and triplicates of active co-encapsulated treatments. All treatments except the abiotic control had 2 g beads added for a total biomass of 1.7 mg_{TSS} per reactor. The initial TCE sorbed to the solids was low; therefore, TCE was added to bring concentrations up to a liquid concentration of ~660 ppb.

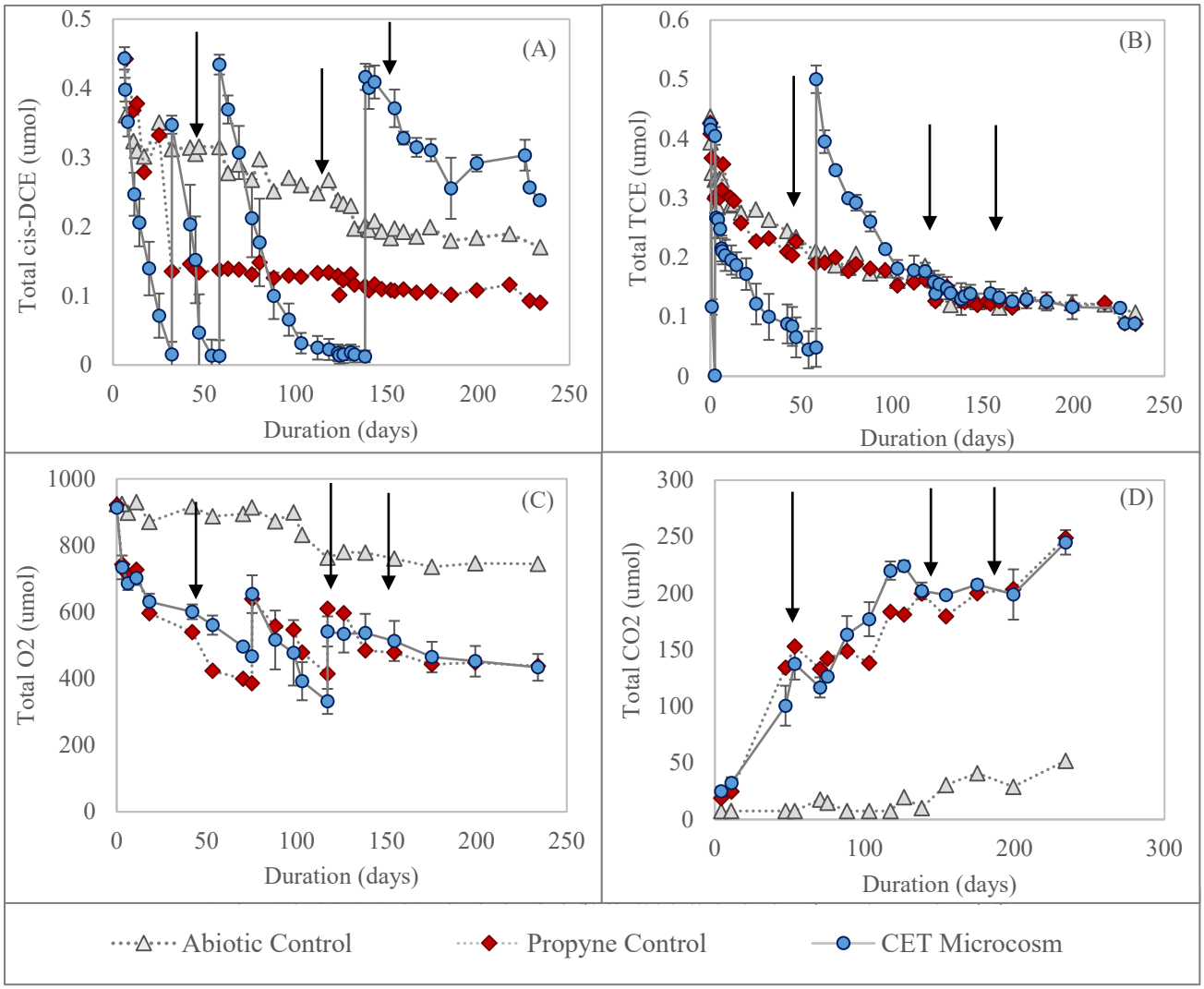

Figure 4.21 *Cometabolic transformation of (A) cis-DCE, (B) TCE, (C) O_2 utilization, and (D) CO_2 production in Fort Carson microcosms. (CET)—Co-encapsulated treatments with G4 and TPhOS at 1.9% (w/w) were in triplicate and abiotic and propyne controls were singlets. Arrows indicate nutrient additions.*

TCE was completely transformed in 2.5 days with no signs of an initial lag period **(*Figure 4.21.B*)** After this, TCE transformation slowed considerably and abruptly stalled. At this time, *cis*-DCE was added at a liquid concentration of ~600 ppb to all treatments to determine if cometabolic activity had stopped, given that it has a higher rate of transformation by *B. vietnamiensis* G4 (***Table 3.2***). Complete transformation of initial *cis*-DCE, however, was observed over the course of 20 days (***Figure 4.21.A***)

Though it is clear from ***Figure 4.21*** that cometabolic activity was occurring, slower rates were seen in the microcosms compared to the pure media experiment in Section 4.2.3. This is believed to be due to nutrient limitations. No additional nutrients were added to the microcosms other than what was in the artificial groundwater. It was not until day 45 that nutrients were added, when a concentrated phosphate buffer (1 mL of 2 M stock solution) was amended to all bottles. Immediately after adding the phosphate buffer, rates of *cis*-DCE transformation increased and continued TCE transformation was seen with a third addition of TCE. Transformation rates stalled again around ~120 days, at which time an IC analysis showed complete depletion of nitrate, nitrite, and phosphate. On day 122, two mL of MSM were added to each reactor, which did not affect TCE transformation rates. Given that activity resumed upon the initial addition of phosphate buffer on day 45, this same procedure was repeated on day 153 to verify that reactors did not stop due to nutrient limitation. Yet, this did not stimulate noticeable cometabolic activity. The total mass of TCE and *cis*-DCE transformed were 0.84 and 1.1 umol, respectively. The mass of TCE transformed was approximately five times lower than in the batch media reactors (4.5 umol). This is not directly comparable, however, as this is a microcosm system transforming a mixture of contaminants.

Though it appears that the microcosms were nutrient limited, from parts (C) and (D) of ***Figure 4.21***, the respiration data shows considerable oxygen uptake and carbon dioxide production compared to what was observed in media batch reactors (***Figure 4.17***). In the microcosms, total oxygen uptake was twice that of the batch media reactors on a mass basis and carbon dioxide production was 2.5 times as much. Given that both respiration indicators were greater for the microcosm system, which was bioaugmented with a lower initial biomass (1.7 mg_{TSS}/reactor compared to 2.9 mg_{TSS}/reactor), it is possible that a native culture

was stimulated and metabolically active. Microcosms made in the same method with aquifer material from Fort Carson were previously stimulated with isobutane previously and were cometabolically active, thus it is assumed native microbes are present with the sediments. Additionally, nutrient limitation was also seen (Rolston et al., 2019).

Upon close inspection, the oxygen uptake data indicates that rates were slowing and possibly stalling before the phosphate buffer addition on day 45, after which the oxygen uptake rate increased (***Figure 4.21.C***). Phosphate is often a limiting nutrient in environmental systems, thus the addition could have stimulated native microbes at this point. Whether a native culture was able to utilize any phenol (the only carbon source added besides gellan gum) released or initially present is unknown. A full suite of nutrients was not added until day 122 with the MSM addition, therefore prior to this, any native culture would have been stimulated by the nutrients available in the artificial groundwater and the phosphate buffer added.

Though the respiration data shows the co-encapsulated treatments were metabolically active, it also indicates the propyne treatment was too, whereas the abiotic treatment (which has received the same nutrient amendments as the other treatments) comparably is not. That fact that the oxygen uptake in the propyne treatment is the same as the co-encapsulated is of note. As a monooxygenase inhibitor, the propyne treatment serves to demonstrate that the transformation of contaminants is due to cometabolic activity, in this case the T2MO. Some amount of *cis*-DCE transformation was initially seen in the propyne treatment, but ceased when more propyne was added on day 32. Additionally, no TCE transformation was seen relative to the abiotic control. The oxygen results indicate that any phenol released was metabolized despite the blocked monooxygenase. This suggests either that the monooxygenase is not needed for phenol metabolism, or that a native phenol utilizer was stimulated in the microcosm and consumed any available phenol. However, this latter explanation seems unlikely given oxygen consumption in the propyne control occurred immediately and directly mirrors the co-encapsulated treatments (***Figure 4.21.C)***.

Since the same mass loading of TPhOS was used in this experiment as the last (1.9%), one assumption is that a similar concentration of phenol (~400 uM) was initially present due to the impurity of TPhOS. The oxygen uptake data suggests that phenol was initially available in the microcosms, given that in only two days 180 umol of oxygen was consumed. This is similar to the amount of oxygen initially consumed in three days in the media batch reactors (~260 umol) (***Figure 4.17***). The lower oxygen uptake here is likely due to the lower initial starting biomass. There could have also been differences in the amount of phenol encapsulated due to the impurity range of TPhOS, or differences in the encapsulation process, such as how well the TPhOS melted and was emulsified in the gellan gum or heterogeneities in the distribution of TPhOS in the bead after solidification. However, the oxygen uptake rate slowed considerably after the initial consumption in the batch media reactors, whereas this was not the case in the microcosms.

An interesting observation is that the similarity in the duration that the batch microcosm and media systems were cometabolically active. The experiment in Section 4.2.3 transformed contaminants for approximately 200 days (***Figure 4.15***), where this microcosm has exhibited transformation of TCE for 100 days and *cis*-DCE for approximately 175 days (***Figure 4.21***). The time scale for activity is similar, especially given that the microcosm was exposed to a contaminant mixture of both *cis*-DCE and TCE and transformed them concurrently until day 100.

Though we concluded previously that TPhOS is not a suitable SRC given the possible low hydrolysis rate, this experiment shows that this co-encapsulated system does support long-term transformation in an unideal system more representative of subsurface conditions that included nutrient limitation and possible native competition.

4.4 Chapter 4 Conclusion

Results are presented where *B. vietnamiensis* G4 was encapsulated in gellan gum macrobeads, and possible SRCs were tested to create a co-encapsulated system with both cells and substrate present. Experiments of encapsulated resting *B. vietnamiensis* G4 cells were performed at a low biomass loading

of 1 mg_{TSS}/g_{bead} with cells transforming TCE and 1,1-DCE as single contaminants, and a higher biomass of 8 mg_{TSS}/g_{bead} transforming a mixture of TCE and 1,1-DCE. At the low biomass loading, ~50% and 80% of the initial mass of the 1,1-DCE was transformed, which is slightly greater than the ~30% transformed at the Moffett field site where phenol and toluene were tested as primary substrates (Hopkins & McCarty, 1995). However, the greater value is expected as this test was done in an ideal environment with a pure culture. Given the low capacity seen for 1,1-DCE as a single compound at the lower biomass, a higher biomass was used for the TCE/1,1-DCE mixture. Both experiments saw re-stimulation of cometabolic activity after the transformation capacity was reached via external additions of toluene, resulting in long-term transformation of the contaminant(s). Re-stimulation of cells was also shown one month after the transformation capacity was reached. The long-term cometabolic activity achieved with external additions of substrate implies cometabolism by *B. vietnamiensis* G4 could be regenerated ex situ or possibly in place for in situ treatment.

One of the goals of this work was to create a co-encapsulated system with *B. vietnamiensis* G4 and a SRC that could sustain long-term cometabolism. The two SRCs investigated were benzyl butyrate, which hydrolyzes to form benzyl alcohol and butyric acid, and TPhOS, which hydrolyzes to produce phenol. Though benzyl butyrate promoted the highest rates and capacity in G4 (Chapter 3), the availability of the two substrates consumed all available oxygen in the batch reactors within two days. It is likely that hydrolysis was biologically mediated, as the abiotic hydrolysis half-life of benzyl butyrate is ~1 year. When it was attempted to encapsulate benzyl butyrate separately from culture, similar results were seen indicating diffusion limitations into the hydrogel were not enough to slow the biological hydrolysis. The high oxygen demand over such a short period makes benzyl butyrate an unsuitable SRC.

Given the success seen with orthosilicates as slow release compounds in Rasmussen, 2020, TPhOS was tested for proof of concept of a co-encapsulated system with *B. vietnamiensis* G4 transforming TCE long-term. TPhOS, had the opposite problem seen with benzyl butyrate; the data suggests the rate of hydrolysis is extremely slow and minimal over 125 days. However, cometabolism of TCE occurred for 180 days

transforming 4.5 umol of TCE. Between days 24-108, the pseudo-first order transformation rate for TCE was constant at 0.12 day^{-1}, but transformation subsequently slowed. VC was then added given the high transformation rates seen (Chapter 3), but minimal VC was transformed before stalling. Based on limited analysis of liquid phenol concentrations, it appears that a high concentration of phenol was present in the poisoned controls as early as day four, whereas minimal phenol was detected in the co-encapsulated treatments at this time. The phenol detected may be due to the impurity of TPhOS, assumed to be 95% based on the product information (provided as a range of 95-100%). This phenol, which was consumed by active cells within four days, may have sustained the transformation seen for over 200 days. It is possible the initial phenol measured generated a higher initial biomass in the beads, which supported the long-term TCE transformation observed.

When it appeared TPhOS was a promising SRC for supporting continuous cometabolic activity, co-encapsulated beads made with the same mass of TPhOS and biomass loading of 0.85 mg_{TSS}/g_{bead} were bioagumented to microcosms made with sediments from Fort Carson and artificial groundwater. TCE and *cis*-DCE transformation occurred for 100 and 175 days, respectively. The system was likely nutrient limited, indicated by an increase in transformation rate and oxygen consumption after an amendment of phosphorous. Additionally, there may have been stimulation of a native culture sometime after the addition of phosphorous, given the oxygen uptake and carbon dioxide production was greater than the batch media reactors. In the batch media reactors, it was hypothesized that a mass of phenol was initially present due to the impurity of TPhOS, which may also be the case in the microcosms given that beads were made to be have the same mass of TPhOS. This is supported given a similar high oxygen demand was seen in the first 2-3 days (180 and 260 umol).

Both cases of resting and co-encapsulated *B. vietnamiensis* G4 showed that a considerable mass of chlorinated ethenes could be transformed with the addition of substrate. Whether the substrate is added externally once the transformation capacity has been reached, or initially encapsulated with the cells, transformation of contaminants was observed for over 100 days. This shows that *B. vietnamiensis* G4 has

a high toxicity threshold for chlorinated ethenes, which was also concluded from long-term resting cell kinetic tests performed in Chapter 3. The tests with encapsulated and co-encapsulated cells also indicated that encapsulation could protect cells from substrate inhibition and possibly from toxicity. Encapsulated resting cells had greater toluene uptake rates and commenced contaminant transformation more quickly, despite respiration data indicating a similar level of biomass existed between treatments. Furthermore, both encapsulated treatments were culturable after one month sitting idle, whereas both suspended treatments were not. In the batch kinetic test with co-encapsulated TPhOS, the concentration of measured phenol (400 uM) in poisoned reactors was well above the inhibitory value reported in literature of 50 uM (Folsom et al., 1990), yet active reactors demonstrated a high level of cometabolic ability. The long-term transformation results of TCE or a TCE contaminant mixture by encapsulated *B. vietnamiensis* G4 with a substrate source, warrants further investigation and may have potential for in situ bioremediation via aerobic cometabolism.

CHAPTER 5 — OVERALL CONCLUSION

The work presented here addressed multiple research questions that pertained to different aspects of bioremediation of trichloroethene and other chlorinated ethenes, with the pure culture *B. vietnamiensis* G4. The relevant conclusions from both chapters are summarized below.

Summary of results presented in Chapter 3:

1. Benzyl alcohol can be used as an alternative substrate to toluene to support aerobic cometabolsim. ABP showed similar induction of the T2MO, and TCE kinetic tests were comparable with toluene-grown cells. Benzyl alcohol cells were also able to transform 1,1-DCE, *cis*-DCE, and VC. While rates were higher for toluene-grown cells, benzyl alcohol grown cells had a greater transformation capacity for each contaminant investigated.
2. 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, and minimal for acetate, butyrate, and lactate. This generally matched TCE kinetic transformation results, which demonstrates that ABL is a valuable screening method for determining inducing substrates for cultures performing aerobic cometabolism. ABL performed by Dr Michael Hyman at North Carolina State University, has now been successfully used for three different microorganisms expressing different monooxygenase systems; T2MO in *B. vietnamiensis* G4, the short-chain alkane monooxygenase (SCAM) in *Rhodococcus rhodochrous* ATCC 21198 (Murnane, 2018), and the ammonia monooxygenase in *Nitrosomonas europaea* (Bennett et al., 2016) .
3. Benzyl acetate and benzyl butyrate, which hydrolyze to produce benzyl alcohol supported the highest TCE transformation capacities seen. This is likely due to the carboxylic acids available upon hydrolysis to serve as a non-competitive carbon source for biomass maintenance. Having two substrates available, and hence a higher C/N ratio, may result in higher PHB accumulation during growth, resulting in the higher transformation capacities observed.

4. Results indicate benzyl alcohol is less inhibitory than toluene, as inhibitory effects were not observed at 10 mg/L benzyl alcohol, and only minor effects were exhibited at 20 mg/L. This is possibly due to two metabolic pathways available for benzyl alcohol metabolism; the formation of either 2-hydroxybenzyl alcohol (2HBA) or benzaldehyde. However, an experiment showed growth on benzyl alcohol was not possible when the monooxygenase enzyme is blocked. Thus, if two pathways for benzyl alcohol utilization exist, the results indicate both require a monooxygenase.
5. Transformation of all the contaminants (except 1,1-DCE) by resting cells was maintained over the course of multiple days. Most kinetic studies in literature are over the course of hours, 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.

Summary of results presented in Chapter 4:

1. Re-stimulation of cometabolic activity in *B. vietnamiensis* G4 after the transformation capacity is reached can occur via external additions of toluene for long-term transformation of the contaminant(s). This can occur even after one month after the transformation capacity is reached, though the time for growth of an active population is longer. This can also occur with 1,1-DCE, which forms a toxic epoxide, and for which a low transformation capacity was seen.
2. Though benzyl butyrate promoted the highest rates and capacity in G4 in Chapter 3, hydrolysis was likely biologically mediated, and the availability of the two substrates consumed all available oxygen in co-encapsulated batch reactors within two days. When it was attempted to encapsulate benzyl butyrate separately from culture, similar results were seen indicating diffusion limitations into the hydrogel were not enough to slow the biological hydrolysis.
3. Encapsulation of *B. vietnamiensis* G4 with a substrate can promote long-term transformation in a batch media system. Co-encapsulated TPhOS and *B. vietnamiensis* G4 supported transformation

of TCE, and subsequently VC, for 200 days in a batch reactor. However, data suggests the rate of hydrolysis of TPhOS, is extremely slow. Phenol was likely initially present due to the impurity of TPhOS, and generated a greater biomass than initially present in the beads, resulting in the transformation seen. This is supported by the high oxygen consumption that initially occurred, and the transformation capacity from the estimated biomass produced by the amount of phenol released.

4. Encapsulation of *B. vietnamiensis* G4 with a substrate can promote long-term transformation in a microcosm system. Co-encapsulated TPhOS and *B. vietnamiensis* G4 supported transformation of TCE and *cis*-DCE in microcosms, where TCE and *cis*-DCE transformation occurred for 100 and 175 days, respectively. Nutrient limitation occurred, shown by an increase in transformation rate and oxygen consumption after an amendment of phosphorous. A native culture may have been stimulated, from the overall greater oxygen uptake and carbon dioxide production compared to batch media reactors. A mass of phenol may have initially been present like in the batch reactors, due to the high oxygen uptake in the first two days (180 umol).
5. Encapsulation likely protects cells from substrate inhibition and possibly toxicity affects. Greater toluene utilization rates were observed in encapsulated reactors compared to suspended, despite respiration data indicating a similar level of biomass. In addition, cells in both encapsulated treatments were culturable after sitting idle for one month. In the batch kinetic test with co-encapsulated TPhOS, if a high mass of phenol was initially present, this did not inhibit TCE transformation.

In summary, benzyl alcohol as a biostimulant for cometabolism of chlorinated ethenes merits further study and consideration for use at field scale given the results presented here. Benzyl alcohol is a food-grade, non-volatile liquid that is not regulated, and can serve as an alternative aromatic growth substrate to toluene or phenol. Both suspended and encapsulated kinetic batch tests indicate that *B. vietnamiensis* G4 has a high toxicity threshold for chlorinated ethenes, and demonstrate the ability of resting cells to

transform contaminants for long periods. Encapsulation results showed that an addition of substrate, either initially or when the transformation capacity is reached, promote continuous cometabolism in *B. vietnamiensis* G4. This should be investigated further for field application as to whether this phenomenon can occur in situ, and explored for a variety of inducing substrates, such as benzyl alcohol.

CHAPTER 6 — FUTURE WORK

The work presented in this thesis can be expanded on, in both understanding the kinetics and mechanisms of *B. vietnamiensis* G4, and in further encapsulation and co-encapsulation studies. Continuing from the results presented in Chapter 3, future work should be done to understand the enzymatic pathway for benzyl alcohol utilization and the involvement of the T2MO. Additionally, it should be explored if benzyl alcohol can be utilized by other model cultures capable of utilizing toluene, such as *Pseudomonas mendocina* KR1 and *Ralstonia pickettii* PKO1, which express the T4MO and T3MO, respectively. Transformation of contaminants was seen over the course of multiple days to weeks for *B. vietnamiensis* G4, which was hypothesized to be due to the amount of PHB accumulation. Literature indicates that PHB accumulation can account for a high mass percentage in *B. vietnamiensis* G4, and it should be explored if this phenomenon differs for growth on various substrates, such as benzyl alcohol and benzyl butyrate.

Based on the results in Chapter 4, future work could be done exploring possible SRCs as well as with resting encapsulated cells. In order to achieve passive treatment for field application, it would be optimal to find a SRC that hydrolyzes at a fast enough rate to sustain a cometabolically active culture, but not so fast that the system becomes anoxic. Eventually, if a successful SRC were found for a co-encapsulated system with *B. vietnamiensis* G4, the concept of mixing different co-encapsulated cultures (for example, co-encapsulated strain ATCC 21198 and T2BOS) to treat a complex mixture of CAHs such as chlorinated ethanes and 1,4-dioxane should be explored. Alternatively, the less passive approach of supplying substrate externally to resting encapsulated cells could be further explored. The research presented here re-stimulated cometabolic activity with toluene, but this same phenomenon should be explored with benzyl alcohol, or a benzyl alcohol releasing substrate. This could be explored in a continuous flow column with resting encapsulated cells, either packed in such a way to resemble a permeable reactive barrier or packed with sediment. Prior to a column study, this could be investigated by adding external additions of benzyl alcohol to the co-encapsulated TPhOS batch and microcosm systems to see if cometabolism could recommence. Benzyl alcohol is not as selective a substrate as phenol or toluene, thus

the ability to sustain a microbial population capable of cometabolism, should be examined in a system with other competing microorganisms.

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CHAPTER 8 — APPENDICES

A. Recipe for KR1 mineral salts media (growth media)

Table A- 1 *Mineral Salt Media MV 10X Solution*

Compound	Amount Added to 1 L
NH_4NO_3	5 g
$MgSO_4 * 7\ H_2O$	2 g
$CaCl_2 * 2\ H_2O$	0.5 g
Disodium EDTA * 2 H_2O	0.1123 g
$Fe_2(SO_4)_3 * nH_2O$ (81.3% purity)	0.0941 g

Table A- 2 *Mineral Salt Media Potassium Phosphate Buffer Solution (2M)*

Compound	Amount Added to 1 L
KH_2PO_4	136.1 g
K_2HPO_4	174.2 g

Table A- 3 *Mineral Salt Media Trace Elements 10X Solution*

Compound	Amount Added to 1 L
H_3BO_3	1.43 g
$MgSO_4 * 7\ H_2O$	1.02 g
$ZnSO_4 * 7\ H_2O$	0.32 g
$CoSO_4 * 5\ H_2O$	0.2165 g
$CuSO_4 * 5\ H_2O$	0.08 g
$Na_2MoO_4 * 2\ H_2O$	0.05 g

1. Autoclave MV 10X solution, phosphate buffer solution, and trace element solutions separately.
2. To 270 mL of autoclaved DI water add 31.8 mL of MV 10X solution, 16 mL of phosphate buffer, and 0.32 mL of trace metals.

B. Mineral Salt Media Agar Plate Recipe

Table B- 1 *Mineral salt media agar plate recipe for growth of B. vietnamiensis G4. Prepares 1 L of agar.*

Compound	**Amount Added for 1 L**
Difo Agar	15 g
MV 10X Solution	100 mL
Phosphate Buffer	50 mL
Trace Elements*	1 mL
15% Sodium Lactate Solution*	25 mL

* Autoclave separately

1. Add MV solution, phosphate buffer, and agar to 850 mL of autoclaved DI water in a 1 L bottle.
2. Autoclave nutrient mixture, trace metals, and lactate solution for one hour
3. Let cool to 'hand hot' and just before pouring add trace metals and lactate solution
4. Gently mix and pour plates

C. Solution 2 (100X)—Phosphate Buffer (1.6M)

Table C- 1 *Phosphate buffer used during the encapsulation process.*

Compound	**Amount Added to 500 mL**
$NaH_2PO_4 \cdot H2O$	48.9 g
K_2HPO_4	7.5 g

D. Encapsulation Optimization

Given this is the first time to our knowledge that *B. vietnamiensis* G4 has been encapsulated in gellan gum, attempts were made to optimize the process for this culture. Two variables were examined for their impact on cellular activity: the temperature cells were temporarily exposed to and the buffer concentration added to the pregel solution. To examine the effect of temperature, a 27 mL vial with 10 mL of MSM was placed in a water bath set to the temperature of interest, to which a mass of suspended cells (0.5 mg_{TSS}) was added. The vial was then taken out of the water bath, sealed with butyl septa and aluminum crimp top, and allowed to come to room temperature. This temporary exposure of cells to a higher temperature followed by an immediate decrease closely mimics the changes in temperature that would be experienced during the encapsulation process. A known mass of TCE was then added from a saturated stock solution through the septa and monitored in the headspace following methods outlined in Section 3.2.2. The concentration of TCE was approximately 500 ppb. This test was done in duplicates and the cell exposure temperatures tested were 20°C (room temperature), 40°C, 45°C, and 50°C. This range of temperatures was chosen as the gel solidifies below 45°C and the method developed for strain ATCC 21198 calls for adding cells at 45°C (Rasmussen et al., 2020). A treatment at room temperature was used as a benchmark given that the long-term batch studies are performed at this temperature. Vials were kept shaking at 200 rpm and kept at 20°C for the duration of the rate test.

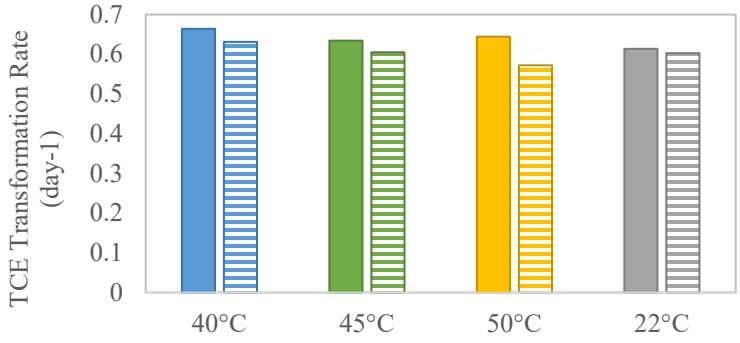

Figure D-1 *Comparison of pseudo-first order rates of TCE transformation for suspended B. vietnamiensis G4 culture exposed to MSM at 20°C, 40°C, 45°C, and 50°C. Data shown is for duplicates of each treatment.*

The results of this test show that this culture maintains similar rates of activity at all temperatures tested, and that the temporary exposure to temperatures as high as 50°C does not greatly impact cellular activity (***Figure D- 1***). This indicates that any differences between suspended and encapsulated activity is not attributed to temperature effects during the encapsulation process.

The second variable examined for its impact on cellular activity during encapsulation was the concentration of phosphate buffer added. The original method outlined in (Rasmussen et al., 2020) added 0.1% (v/v) of a 1.6 M PB and required the pregel solution to be pH adjusted to 7 with dilute NaOH. Encapsulated *B. vietnamiensis* G4 in gellan gum macrobeads were made at the original buffer concentration of 0.1%, as well as 0.2%, 0.5%, and 1% (v/v). The TCE transformation rate of these encapsulated cells were then compared to suspended cells, which served as the benchmark. The bead making process is time consuming, thus this test was divided into two days. Day one tested beads made at 0.1% and 0.2% (v/v) 1.6 M PB, and compared rates to that of suspended cells (***Figure D- 2***). The second day tested 0.5%, 1%, and a new treatment of suspended cells in the event that cellular activity decreased. TCE was added through the septa and was approximately 400 ppb on day one and 550 ppb on day two.

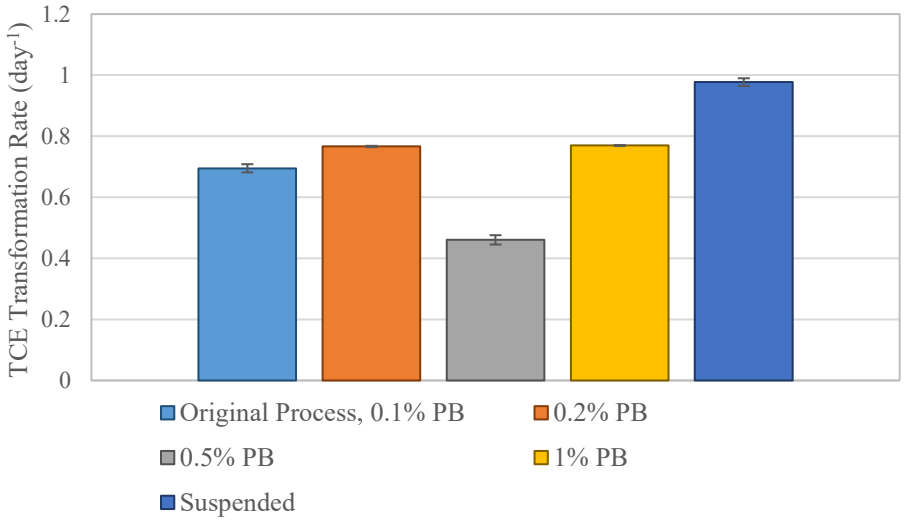

Figure D-2 *Comparison of pseudo-first order rates of TCE transformation for suspended and encapsulated B. vietnamiensis G4 in gellan gum macrobeads with 1.6 M phosphate buffer (PB) at 0.1%, 0.2%, 0.5%, and 1% (v/v). For 0.1, 0.2, 0.5, and 0.1% PB, error bars represent the range of duplicate reactors. Error bars for suspended treatment represent standard deviation with n=4.*

Results show that the original encapsulation process of 0.1% (v/v) phosphate buffer had the second lowest TCE transformation rate and was 28% less than the average suspended rate. In comparison, the beads made with 0.2% of the phosphate buffer had an average rate that was 21.0% less than the average suspended rate. Results also show that beads made with 0.5% (v/v) and 1% (v/v) of buffer solution had an average rate 53.2% and 21.8% less than suspended cells respectively. The beads made with PB at 0.5% (v/v) appears to be an outlier, but otherwise it appears that an increased concentration of buffer solution results in slightly increased cellular activity compared to the original process of PB at 0.1% (v/v). Though both 0.2% and 1% (v/v) phosphate buffer resulted in similar level of activity, the higher concentration of buffer eliminated the need to pH adjust the pregel, while maintaining the structural integrity of the beads. Based on these results, during the encapsulation process, cells were added at ~47C and 1% (v/v) of the 1.6 M PB was added.

At 1% PB (v/v), approximately 80% of encapsulated cell activity was maintained compared to suspended cell activity, and the encapsulation process was streamlined by the elimination of pH adjustment. This shows that *B. vietnamiensis* G4 is a robust culture that can undergo encapsulation while maintaining a high level of activity. In these tests, encapsulated rates were somewhat lower than suspended cells. One possible explanation is that contaminant diffusion into the beads slows the rate of transformation compared to suspended cells. Zero-order rates of isobutane utilization by encapsulated strain ATCC 21198 were able to achieve as high as 90% the rates achieved by cells in suspension, however this was by a different culture. It was hypothesized that this similar utilization rate was achieved because the low biomass loading decreased diffusion limitation effects into the hydrogel bead (Rasmussen et al., 2020). This was based on results seen where higher cell mass loadings in hydrogels led to greater diffusion limitations of oxygen (Hiemstra et al., 1983). It is also of note that the transformation rate of the suspended treatments was approximately the same between the two days. This demonstrates that culture can be stored at 4°C for at least 24 hours without losing cellular activity.

E. Encapsulation Shelf-Life

A short-term encapsulation kinetic test was performed when beads were made and at a later period, to determine the ability to store beads at 4°C and maintain activity. The viability of encapsulated cells was determined by comparing activity of encapsulated cells to that of suspended cells (the same harvested culture that was used for encapsulation). Treatments consisted of an abiotic control and duplicate suspended and encapsulated reactors. These tests were performed at 20°C in 27 mL glass vials with 10 mL of MSM and sealed with butyl septa. Before sealing, a known mass of beads were added at a biomass loading of 1 mg_{TSS}/g_{bead} for a total initial biomass of 4 mg_{TSS}. An equivalent mass of suspended cells were added to suspended treatments.

Activity was measured by substrate utilization rates, which were determined through linear regression and normalized to biomass (umol substrate/day/mg_{TSS}).Vials were kept shaking at 200 rpm to ensure equilibrium of toluene between the gas and liquid phases was maintained. Toluene was used as the substrate and ~20 umol was added through the septa. Toluene gas concentrations were monitored using GC methods described in Section 3.2.2 Tests were conducted until substrate was consumed, which was on the magnitude of hours or days. When tests were completed, reactors were stored at 4°C. This test was then repeated later in the same manner to determine the shelf life of the beads, with the addition of oxygen if needed.

Toluene was added the day after these beads were made and consumed in all treatments in approximately five days (***Figure E- 1.A***). The average initial mass of toluene added to encapsulated reactors was 22 umol and in suspended reactors was 26 umol. Rates for individual reactors can be seen in ***Figure E-2*** for both treatments. The average toluene-uptake rate normalized to biomass for encapsulated and suspended reactors, were approximately the same at 38 and 40 umol/day/mg_{TSS} respectively. This indicates that encapsulation was successful and the biomass added during this process remained active in the beads. All reactors were then stored at 4°C until they were taken out 237 days later.

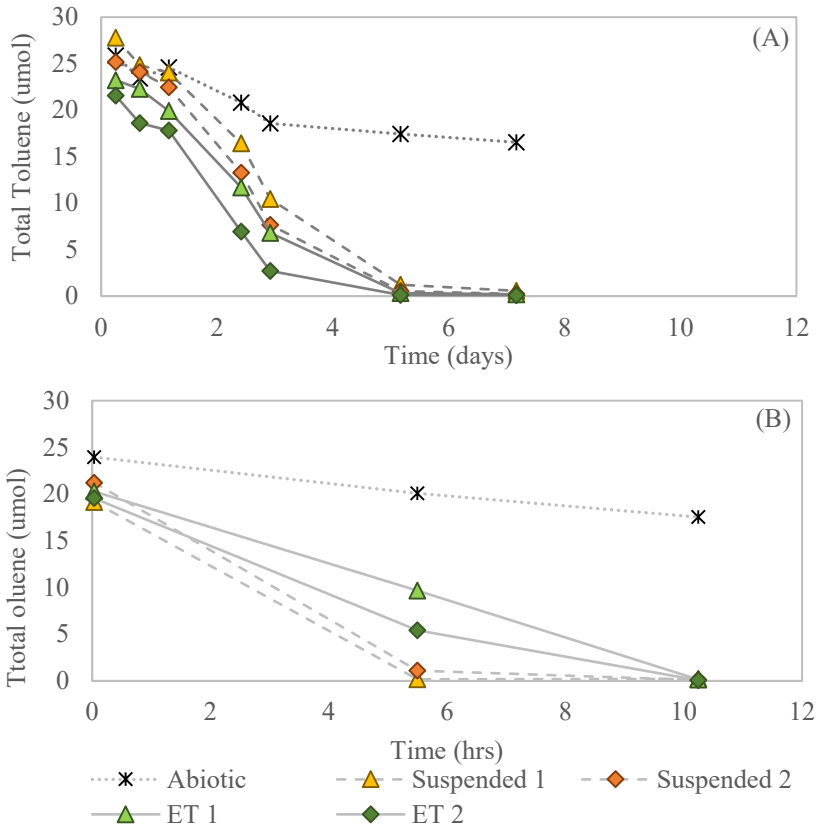

Figure E- 1 *Toluene utilization by suspended and encapsulated in gellan gum macrobeads B. vietnamiensis G4 at approximately the same biomass (4 mg_{TSS}) (A) after beads were made and (B) after 237 days stored at 4°C. Beads had a biomass loading of 1 mg_{TSS}/g_{bead}. Data shown for duplicates of each treatment and a single abiotic control.*

Before adding toluene again, it was verified that there was sufficient oxygen available for cometabolism. A similar mass of toluene was added (~20 umol) as previously, and was consumed in all treatments (***Figure E-1.B***) in 10 hours or less. However, whereas both treatments initially had the same toluene uptake rate initially, the rate in the encapsulated reactors is lower than in suspended reactors. This test proves that encapsulated G4 was able to remain active after 230 days stored at 4°C, with no lag in substrate uptake, though the toluene uptake rate was approximately half that of stored suspended cells.

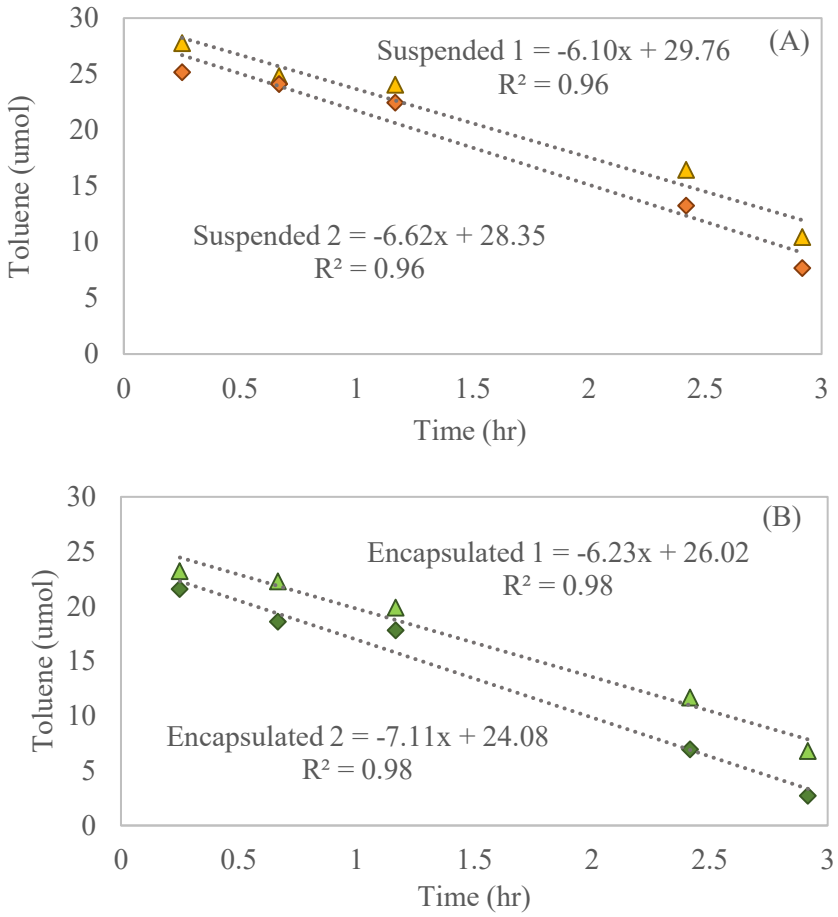

Figure E-2 *Initial zero-order substrate utilization rates for (A) suspended and (B) encapsulated B. vietnamiensis G4 at 4 mg$_{TSS}$/reactor.*