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Biotransformation of Persistent Groundwater Contaminants: Trichloroethene and Poly- and Perfluoroalkyl Substances (PFASs)

By

Katie Colleen Harding

A dissertation submitted in partial satisfaction of

the requirements for the degree of

Doctor of Philosophy

in

Engineering - Civil and Environmental Engineering

in the

Graduate Division

of the

University of California, Berkeley

Committee in charge:

Professor Lisa Alvarez-Cohen, Chair
Professor David L. Sedlak
Professor Mary K. Firestone

Fall 2014

Biotransformation of Persistent Groundwater Contaminants: Trichloroethene and Poly- and Perfluoroalkyl Substances (PFASs)

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Abstract

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Trichloroethene (TCE) is one of the most commonly detected groundwater contaminants in the United States and has been characterized by the U.S. Environmental Protection Agency as carcinogenic to humans. Past TCE storage and disposal procedures following use in dry-cleaning operations and metal degreasing has resulted in numerous contaminated sites where TCE and chlorinated transformation products, such as dichloroethene (DCE) and vinyl chloride (VC), are detected in soil, groundwater, and air. Poly- and perfluoroalkyl substances (PFASs) are key constituents of aqueous film-forming foams (AFFFs) and are responsible for the surface tension reduction properties that facilitate rapid foam spreading over ignited liquid fuels. Various PFASs have been detected in the soil and groundwater of AFFF-impacted sites, while certain PFASs, such as the eight-carbon homologs perfluorooctanoic acid (PFOA) and perfluorosulfonic acid (PFOS), have been linked to adverse human health effects.

The use of AFFF to extinguish chlorinated solvent-fueled fires has led to the co-contamination of TCE and PFASs at sites where foam wastewater and fuel were allowed to infiltrate the subsurface. Historically, groundwater and soil remediation at these sites was optimized for achieving TCE reductive dechlorination to ethene. However, due to recent increases in activities for measuring and characterizing PFAS contamination, particularly in groundwater and soils beneath firefighter training sites, greater attention is being paid to the fate and transformation of PFASs, as well as their effects on TCE-dechlorinating microbial communities. The *in situ* biotransformation of AFFF-derived PFAS compounds and the effects of AFFF and PFAS transformation products on TCE bioremediation must be understood.

The biotransformation of a principle PFAS compound used in multiple AFFF formulations, fluorotelomer thioether amido sulfonate (FtTAoS), was investigated under aerobic conditions in soil microcosms amended with AFFF. The aerobic biotransformation pathways for 4:2, 6:2, and 8:2 FtTAoS were determined by direct LC-MS/MS quantification of intermediate and end products and through the characterization of previously unidentified intermediate products with high resolution MS measurements. FtTAoS was biotransformed under aerobic conditions to a fluorotelomer sulfonate, two fluorotelomer carboxylic acids, and a suite of

perfluorinated carboxylic acids. The detection of two intermediate compounds, corresponding to singly- and doubly-oxygenated species of FtTAoS, suggest that the first two reactions in the biotransformation pathways are sequential oxygen additions to the thioether group. This is likely followed by a third oxygenation and cleavage between the resulting sulfonate and the amidosulfonate group to form a fluorotelomer sulfonate. The perfluorinated carboxylic acids appear to be the end products of FtTAoS biotransformation and were persistent in live microcosms. An oxidative assay employing PFAS oxidation by hydroxyl radical was applied to microcosm samples to indirectly quantify the total concentration of polyfluorinated compounds present during FtTAoS biotransformation for closure of the mass balance. The assay produced a full mass recovery of PFAS oxidation products before and after complete FtTAoS biotransformation even though only 10% (mol/mol) of the initial amended FtTAoS was accounted for by directly-measured PFASs.

The effects of AFFF and various PFASs on anaerobic TCE dechlorination were investigated in a *Dehalococcoides* (Dhc)-containing microbial community that dechlorinated TCE to ethene. When AFFF formulations from three different manufacturers: 3M, National Foam, and Ansul were amended to the cuture's growth medium as the sole carbon and energy source, varying yet sufficient quantities of hydrogen and acetate were produced to support dechlorination during all three foam-amendments. However, TCE dechlorination only occurred under 3M AFFF amendment, while no dechlorination was observed under National Foam and Ansul AFFF amendments. All PFAS compounds were persistent in the anaerobic communities and did not transform biologically or abiotically. The degradation of diethylene glycol butyl ether (DGBE), the primary glycol ether solvent in most AFFFs, produced less hydrogen and acetate when amended alone than in AFFF-amended microcosms, suggesting that smaller quantities of other organic carbon substances in the foams, such as hydrocarbon surfactants, may be more easily fermentable. Amendment of 16 mg/L 6:2 fluorotelomer sulfonamido betaine (6:2 FtSaB) slowed TCE dechlorination while 32 mg/L FtSaB completely inhibited dechlorination, suggesting dechlorination did not occur in the National Foam AFFF-amended experiment due to the presence of its most abundant PFAS. In cultures amended with perfluroalkyl acids, 110 mg/L total perfluorosulfonic acids did not inhibit TCE dechlorination, while 110 mg/L total perfluorocarboxylic and perfluorosulfonic acids did, suggesting that inhibition is dependent on PFAS structure as well as concentration.

Carbon stable isotope analysis is a frequently employed tool used to confirm and quantify *in situ* bioremediation of PCE and TCE to ethene. The impact of growth condition on the carbon isotope fractionation of TCE by Dhc was investigated by quantifying fractionation while Dhc was grown in pure and co-cultures as well as in mixed communities. Enrichment factors were not significantly affected by changes in any of the tested growth conditions for the pure cultures, co-cultures or the mixed communities, indicating that despite a variety of temperature, nutrient, and co-factor-limiting conditions, carbon isotope fractionations remain consistent for given Dhc cultures. However, the fractionation factors for the pure and co-cultures were outside the range of those quantified for the mixed communities, indicating that the fractionation may be strain-dependant.

dedicated to my Dad

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Acknowledgements

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I am grateful for the opportunity to have worked with Jennifer Field at Oregon State University, who was an important collaborator and source of technical advice on fluorochemical analyses. Without her the PFAS biotransformation work would not have been possible. I would also like to thank Tess Weathers and Josh Sharp at Colorado School of Mines for their collaboration and contributions to the TCE and PFAS co-contamination studies presented in Chapter 3.

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I am immensely grateful to my mom and dad for their unconditional love and support. My dad's passion for science is infectious and likely played a significant role in my brothers and I pursuing graduate degrees in science and engineering. I am grateful for the countless ways my

parents have supported us and every sacrifice they made in order to ensure us an education. It seems nearly impossible to express sufficient gratitude for their selflessness.

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Katie C. Harding
September 2014

CHAPTER 1: Introduction and Objectives

1.1 Motivation

Trichloroethene (TCE) and poly- and perfluoroalkyl substances (PFASs) are environmental contaminants that have been associated with adverse human health effects after exposure through contaminated water supplies [Emmett *et al.* 2006, Vieira *et al.* 2013, ATSDR 1997]. TCE occurence in groundwater was documented as early as the 1960s [Middleton and Walton 1961] and its widespread contamination at sites with historic spills and underground releases is now recognized [Westrick 1984, ATSDR 1997, USGS 2006]. The study and characterization of naturally occurring *Dehalococcoides* (Dhc) organisms that reductively dechlorinate TCE to its innocuous ethene end product has led to the implementation of stimulated anaerobic bioremediation as a cost-preferred and sustainable treatment strategy for TCE remediation in groundwater. The development of molecular and isotopic tools to monitor stimulated chloroethene bioremediation has aided efforts to further quantify *in situ* TCE dechlorination and to predict the activities of complex microbial communities [Lovely *et al.* 2003, Stroo *et al.* 2006, Hunkeler *et al.* 2005]. This has allowed practitioners to more accurately model the subsurface remediation rate at a given site.

In contrast, relatively little is known about the environmental fate and transformation of poly- and perfluoroalkyl substances (PFASs), particularly those found in aqueous film-forming foams (AFFF). Although perfluoroalkyl acids (PFAAs) have been in use since the 1940s [Prevedouros *et al.* 2006, Paul *et al.* 2009], reporting of their detection and quantification in environmental samples and biota gained momentum only in the late 1990s [Schultz *et al.* 2006, Boulanger *et al.* 2005, Houde *et al.* 2006, Kato *et al.* 2011, Glynn *et al.* 2012]. The absence of authentic analytical standards for many compounds has also precluded their routine monitoring in environmental media. Furthermore, the structures of many of the PFAS compounds present in AFFF were only recently elucidated, and commercial analytical standards for some of these compounds are not currently available [Place *et al.* 2012, D'Agostino *et al.* 2014].

There is significant potential for the co-contamination of TCE and PFASs to occur in areas where AFFF was used to extinguish chlorinated solvent fires. Firefighter training activities conducted on U.S. military bases have led to widespread groundwater and soil contamination by TCE and PFASs after AFFF wastewater and fuels were left to infiltrate unlined training pits. As increased monitoring of PFASs in environmental samples has occurred in recent years, sites with well-delineated chlorinated solvent plumes are now know to possess PFAS contamination [McGuire *et al.* 2014]. Remediation activities at these sites have typically been aimed at removing chlorinated solvents and hydrocarbons with little regard for the impact of AFFF components on performance outcomes. Additionally, PFAS biotransformation in these environments and the impacts of biotransformation products on TCE remediation have not been well characterized. Understanding PFAS biotransformation pathways and their expected product formations is of high importance for the successful remediation of PFASs, while understanding the impacts of PFASs on dechlorinating microbial communities is needed for the effective remediation of TCE. The application of an *in situ* remediation strategies that account for the fate and transformation processes of both target contaminants are essential for the successful co-remediation of TCE and PFASs.

1.2 Research Objectives

The goals of this research were to understand the transformation of TCE and PFASs under conditions representative of AFFF-impacted field sites. It was first hypothesized that the fluorotelomer compounds in AFFF biotransform aerobically in surficial soil and oxic groundwater to form PFAAs. It was then hypothesized that certain AFFF components, including PFAA biotransformation products, may impact TCE dechlorinating microbial communities following infiltration into or generation of anaerobic, subsurface zones. Finally, it was investigated whether the stable carbon isotope fractionation of TCE, a widely used monitoring tool to assess biodegradation, will vary under different Dhc growth conditions. If AFFF impacts Dhc metabolism and growth, and isotopic fractionation proves to vary under various Dhc growth conditions, there would be implications for the use of carbon isotope measurement to quantify TCE bioremediation at AFFF-impacted sites.

The following listed objectives sought to address these hypotheses with the aim of acquiring a holistic understanding of the varying processes that occur in aerobic and anaerobic groundwater contaminated with TCE and AFFF. Each objective represents a subsequent chapter of this thesis.

1) Determine the capability of AFFF-derived PFAS compounds to biotransform under aerobic conditions that are representative of the surficial soil and oxic groundwater in which the compounds can be found. **(Chapter 2)**

2) Characterize the effects of PFAAs on TCE dechlorination in anaerobic mixed microbial communities in order to describe the impact of PFAS biotransformation products on the dechlorination activity of Dhc. **(Chapter 3)**

3) Assess the impacts of AFFF and high concentrations of PFASs on TCE-dechlorinating microbial communities to characterize the effects of infiltration of both un-reacted AFFF and biotransformed AFFF-derived PFASs, into anaerobic aquifers. **(Chapter 4)**

4) Evaluate whether sub-optimal and varying growth conditions impact the TCE stable carbon isotope fractionation by Dhc to determine if isotope fractionation can be applied at AFFF-impacted sites where Dhc growth and dechlorination may be negatively impacted. **(Chapter 5)**

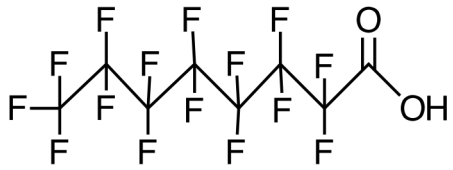
1.3 Background on poly- and perfluoroalkyl substances (PFASs)

1.3.1 Fluorochemical types and uses

Poly- and perfluorinated alkyl substances (PFASs) represent a large class of fluorinated compounds that all possess a fluoroalkyl chain ranging in length from three (C_3) to eighteen (C_{18}) carbons and a non-fluorinated moiety varying in size, structure, and charge. PFASs have been manufacturered for a variety of industrial and commercial uses, including textile stain repellants, food contact paper coatings, pesticides, and active ingredients in aqueous film-forming foams (AFFF) [Prevedouros *et al.* 2006, Paul *et al.* 2009, Moody et al. 2000]. Due to the strength of the C-F bond and rigidness imparted by the conjugation of multiple CF_2 groups, the fluoroalkyl chains of the compounds are resistant to transformation and degradation under a variety of chemical and thermal conditions [Schröder and Meesters 2005, Kissa et al. 2001]. Unlike their hydrocarbon analogues, PFASs are both hydrophobic and oleophobic, making them useful for water and oil-repellant applications [Kissa *et al*. 2001]. Although there are many types of PFASs that have been historically manufactured and are in current production [Prevedouros *et al.* 2006], three primary classes are relevant to the research conducted in this study due to their presence in AFFF or their potential to be biotransformation products of AFFF-derived PFASs: perfluoroalkyl acids (PFAAs), fluorotelomers, and perfluorosulfonamides. PFAAs and perfluorosulfonamides are considered to be "per"-fluoroalkyl substances, while fluorotelomers are considered to be "poly"-fluoroalkyl substances due to molecular structural differences that are described by Buck *et al*. [Buck *et al*. 2011].

PFAAs: Perfluoroalkyl acids refer to a family of compounds that contain a perfluorinated alkyl chain directly adjacent to a charged functional moiety, which is a carboxylic, sulfonic, or phosphonic group. Perfluorinated carboxylates (PFCAs) and perfluorinated sulfonates (PFSAs) are the most widely used and detected PFAAs [Prevedouros *et al.* 2006, Paul *et al.* 2009], and in this study the term PFAA refers to PFCAs and PFSAs only. The eight carbon-based (C_8) PFAAs, perfluorooctanoic acid (PFOA) and perfluorooctane sulfonic acid (PFOS) (Figure 1.1), are the most abundant and well known of the alkyl acids, although shorter (C_3-C_7) and longer (C_9-C_{18}) chains are also produced. PFAAs have been shown to be highly resistant to chemical and biological transformation [Rayne *et al*. 2009], making them persistent in the environment and potentially able to undergo long-term transport through certain ecosystems. PFAAs have been measured globally in environmental samples and wildlife, with some detections occurring in remote, unpopulated regions far from known point sources [Armitage *et al.* 2009a-b, Ellis *et al.* 2004, Houde *et al.* 2010]. They have been widely detected in surface waters and urban runoff [Murakami *et al*. 2009b, Houtz *et al*. 2010], groundwaters and soil [Murakami et al. 2009a, Moody *et al*. 2003], wildlife [Giesy and Kannan 2001, Houde *et al.* 2006], and humans [Kato *et al.* 2011, Olsen *et al.* 2012], and as such have gained the most regulatory and research attention of all the PFAS compound classes.

A



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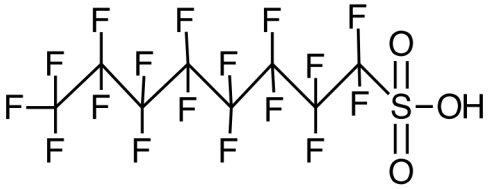
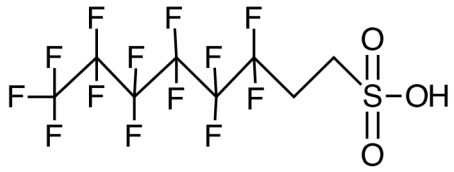


Figure 1.1 Structures of the protonated forms of perfluorooctanoic acid (A), and perfluorooctane sulfonic acid (B).

Fluorotelomers: Fluorotelomer compounds are related to PFAAs in structure, however they contain a non-fluorinated alkyl linkage of two carbons between their perfluoralkyl chains and ionic functional groups (Figure 1.2), and are thus designated as "poly"-fluoroalkyl substances. They are named after the telomerization synthesis process that utilizes the sequential addition of C_2F_4 groups (taxogens) to C_2F_5I (a telogen) to produce even-numbered and linear fluoroalkyl groups [D'Eon and Mabury 2011]. The name of a fluorotelomer compound is typically preceded by an n:2 numeric designation, where n is the number of fluorinated carbons in the fluoroalkyl backbone and 2 refers to the non-fluorinated carbons in the alkyl linkage. The ionic functional groups of fluorotelomer compounds can resemble those of PFAAs, such as fluorotelomer sulfonate (FtS) and fluorotelomer alcohol (FtOH), or they may have varying lengths and structures depending on the compound's desired application. For instance, some fluorotelomer compounds in AFFF, such as fluorotelomer thioamido sulfonate (FtTAoS), contain sulfonate and amide moieties, while certain flurotelomers with phosphonate groups, such as polyfluoroalkyl phosphate ester (PAP), are often used for food-contact papers [Place *et al*. 2012, D'Agostino *et al.* 2014, D'Eon and Mabury 2007].

A



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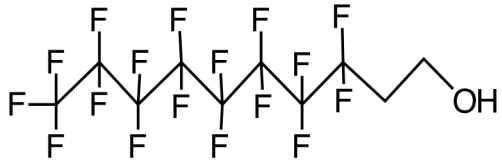


Figure 1.2 Protonated forms of two fluorotelomer compounds with different fluoroalkyl chain lengths and functional groups: 6:2 FtS (A) and 8:2 FtOH (B).

Perfluorosulfonamides: Perfluorosulfonamide compounds are defined by a $-SO_2N$ group that links a perfluoroalkyl chain of varying length to an ionic functional group (Figure 1.3). The perfluorinated chains of perfluorosulfonamides are typically produced through electrochemical fluorination by which an alkyl sulfonyl chloride precursor is electrolyzed in an aqueous hydrogen fluoride solution to produce fluorinated analogs that are a mixture of linear (70%) and branched (30%) isomers [Prevedouros *et al.* 2006]. Many of the perfluorosulfonamide compounds that have been detected and quantified in human and environmental samples, such as *N*-methyl-perfluoro-1-octanesulfonamideoacetic acid (*N*-MeFOSAA) and 2-*N*-ethyl-perfluoro-1-octanesulfonamido ethanol (*N*-EtFOSE), are unreacted residual building blocks in the production of fluoroalkyl polymers [Lee and Mabury 2011]. The 3M Company has historically been the primary producer of perfluorosulfonamides and until 2002 predominantly manufactured the compounds with C_8 perfluorinated chains. 3M reportedly switched to C_4-based manufacturing by using perfluorobutane sulfonamidoethanol building blocks instead [Martin *et al.* 2010, Olsen *et al.* 2007].

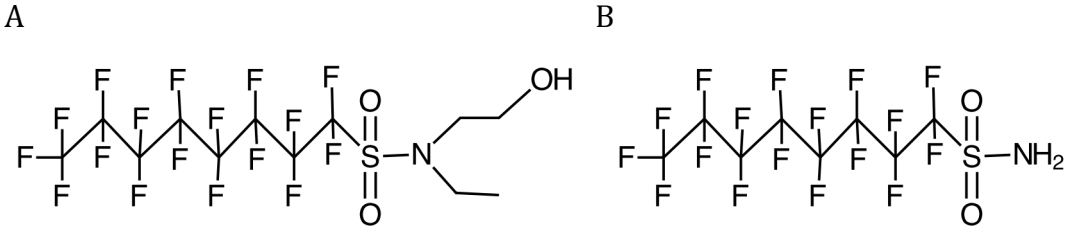


Figure 1.3 Two C_8 perfluorosulfonamide compounds with different functional groups: *N*-EtFOSE (A), and perfluorooctane sulfonamide (FOSA) (B).

1.3.2 Human Health Effects and Toxicology

Most human health and toxico-kinetic studies of PFASs have focused on the effects of PFAAs, particularly PFOA and PFOS. Longer-chained PFAAs (C_8 and greater) tend to bioaccumulate to greater degrees than shorter-chained PFAAs [Conder *et al.* 2008], leading to the migration and biomagnification of longer-chained compounds through food webs [Conder *et al.* 2008, Houde *et al.* 2010]. Both PFCAs and PFSAs show an affinity for binding to β-lipoproteins and liver fatty acid binding proteins (L-FABP) through site-specific interactions [Rand *et al*. 2014, Rand *et al*. 2013], and thus tend to accumulate in the protein-rich areas of human bodies and animals, including the blood, liver, and kidneys [Olsen *et al.* 2005, Kannan *et al*. 2004]. A recent study has pointed to the potential for 8:2 fluorotelomer unsaturated aldehyde (8:2 FTUAL), a metabolic transformation product of 8:2 FtOH, to form covalent bonds with proteins in rat liver microsomes [Rand *et al*. 2013]. It is thought that the PFAS-protein adducts may have greater cellular toxicity than proteins associated with, but not covalently bound to, PFASs due to the potential disruption of protein function and protein inactivation in the adducts [Rand *et al*. 2013].

Adverse human health effects have been documented in populations exposed to PFAAs and associated with whole blood, plasma, or serum concentrations of PFOA and PFOS [Vieira *et al*. 2013, Fei *et al*. 2007, Gallo *et al*. 2012]. High levels of exposure to PFOA from contaminated drinking water was positively associated with kidney and testicular cancer, and likely associated with non-Hodgkin lymphoma, ovarian, and prostate cancer in a population living near a Teflon® manufacturing plant in southeastern Ohio/West Virginia [Vieira *et al*. 2013]. Higher levels of total cholesterol and uric acid were positively correlated to PFOA serum concentrations in both occupationally-exposed adults and the general population [Costa *et al*. 2009, Lin *et al.* 2009, Nelson *et al.* 2009], indicating that fluorochemicals could be a risk factor for metabolic disorders. The plasma concentrations of PFOA in pregnant women have been associated with greater reported times to pregnancy [Fei et al. 2009], while PFOA concentrations were correlated to decreased birth weight of infants [Fei *et al.* 2007]. The health effects of non-PFAA PFAS compounds, such as those found in AFFF (Figure 1.5) have not been examined.

To date, the U.S. EPA does not regulate PFOA or PFOS through a federally mandated maximum contaminant level (MCL) in drinking water. However, due to rising concerns regarding their adverse affects on humans and increased detection in land-applied biosolids [Lau et al. 2007, Renner *et al*. 2009], the EPA issued Provisional Health Advisories (PHAs) of 200 and 400 ng/L for PFOS and PFOA, respectively, to represent acceptable levels of short-term exposure [U.S. EPA 2009]. The Minnesota Department of Health issued its own health risk limits (HRLs) for drinking water of 300 ng/L for PFOS and PFOA, and 7 μg/L for perfluorobutanoic acid (PFBA) and perfluorobutane sulfonic acid (PFBS). Minnesota has been heavily impacted by the fluorochemical manufacturing activities of the 3M Company since the early 1950s, and PFAAs have been detected in multiple private and public drinking water supplies throughout the state [MPCA 2008]. Additionally, Canada instituted a ban on the manufacture and use of PFOS in 2006 under the Canadian Environmental Protection Act (CEPA) of 1999, with 5-year exemptions given for stockpiled PFOS-based AFFF and PFOS-based fume suppressants used for electroplating [CEPA 1999]. PFOS was also added as a restricted chemical to the Stockholm Convention on Persistent Organic Pollutants in 2009.

1.3.3 Composition of AFFF and PFASs in AFFF

AFFF formulations are complex water-based mixtures used to extinguish fires involving flammable fuels. They were developed in the 1960s as a tool for combating potential hydrocarbon fires at military sites, industrial facilities, and municipalities where large quantities of ignitable fuels are stored and used [Moody *et al*. 2000]. Although the primary components of most formulations include hydrocarbon surfactants, fluorinated surfactants (PFASs), and a glycol-ether based solvent, published MSDS information and recent foam characterization studies [Place *et al.* 2012, D'Agostino *et al.* 2014] indicate that the foams vary according to manufacturer and year of production (Table 1.1 and 1.2). Diethylene glycol butyl ether (DGBE, also sold as Butyl Carbitol™) constitutes the major organic solvent of most formulations (Figure 1.4); however, its concentration in neat foam products also appears to vary by manufacturer

(Table 1.2), ranging from 6-8% (mass/mass) in AFFF manufactured by Chemguard to 20% (mass/mass) in 3M AFFF. Ethylene glycol reportedly constitutes up to half of the organic carbon solvent (by weight) in foams manufactured by National Foam [Aer-O-Water 3EM, MSDS No. MNS210].

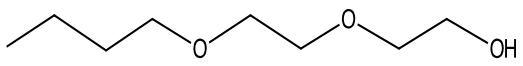


Figure 1.4 Structure of diethylene glycol butyl ether, an organic solvent present in AFFF at 6-20% by weight.

Table 1.1 Composition of five AFFF formulations (3% concentrates) according to published MSDS information. Empty cells indicate the ingredient was not reported in the formulation.

	Composition of AFFF concentrate (% by weight)				
Ingredient	3M Lightwater FC-203CF (3%)[c]	Ansul Ansulite AFC-5-A (3%)[d]	National Foam Aero-O-Water 3EM (3%)[e]	Chemguard (3%)[f]	Buckeye (3%)[g]
Water	69 - 71	75 - 80	62 - 79	70 - 80	> 56
Diethylene glycol butyl ether (DGBE)	20	17	8 - 12	6 - 8	< 19
Ethylene glycol			6 - 8		
Hexylene glycol		0.5			
1-propanol		0.4			
Perfluoroalkyl sulfonate salts (*i.e.* PFSAs)	0.5 - 1.5				
Hydrocarbon surfactants (proprietary)	1 – 5[a]	5 - 10	6 - 11	proprietary	< 25
Fluorinated surfactants (proprietary)	1 – 5[b]		0.5 - 2	proprietary	
Magnesium sulfate				0.5 – 1.5	
Triethanolamine	0.5 – 1.5				
Ethylenediaminetetraacetic acid tetrasodium salt				0.5 – 1.5	

[a]Reported as "alkyl sulfate salt"
[b]Reported as "amphoteric fluoroalkylamide derivative"
[c][FC-203CF, MSDS No CKQCB], [d][Ansulite 3% AFFF AFC-5-A, MSDS]
[e][Aer-O-Water 3EM, MSDS No. MNS210], [f][Chemguard 3% AFFF C-301MS, MSDS], [g][BFC-3MS, MSDS]

Table 1.2 DGBE and total organic carbon concentrations in AFFF formulations obtained from various U.S. military bases (Data obtained in this study).

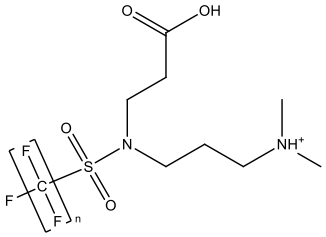
AFFF Product	Estimated manufacturer year	DGBE (g/L)	Total organic carbon (g/L)	% Total organic carbon from DGBE
3M Lightwater (3%)	1989	244	307	47
3M Lightwater (3%)	1993	214	176	72
3M Lightwater (3%)	1998	226	179	75
Ansul Ansulite (3%)	2008	223	163	81
Ansul Ansulite (3%)	2010	230	166	82
Buckeye (3%)	2003	57	90	37
National Foam Aer-O-Water 3EM (3%)	2003	133	217	36
National Foam Aer-O-Water 3EM (3%)	2008	160	204	46

Although most commercial formulations are proprietary, their compositions are driven by performance criteria, such as spreadability, extinguishment time, corrosion rate, and environmental impact as described by biological and chemical oxygen demand (BOD and COD, respectively) [Scheffey *et al.* 1994]. For AFFF purchased by the U.S. military, the largest consumer of AFFF products in the United States, the performance criteria are established by the military specification (Mil-Spec) [MIL-F-24385F 1994]. All AFFF products used for this study are "3% concentrates" (designed for dilution to a 3% AFFF-water mixture vol/vol before application) and meet the U.S. Mil-Spec requirements. The fluorinated and hydrocarbon surfactants in AFFF are important for reducing the air-foam surface tensions and foam-fuel interfacial tensions, which effectively increase the foam's spreading coefficient and allows for enhanced fire suppression and vapor sealing [Scheffey *et al.* 1994].

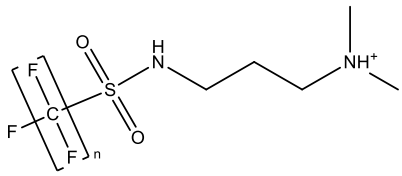
Recent studies that have characterized the PFAS components of various AFFF formulations by elucidating their chemical formulas with mass spectrometry (MS) and inferring molecular structure with patent information [Place *et al.* 2012, D'Agostino *et al.* 2014] have indicated that PFAS composition in the foams also varies according to manufacturer and year of production. The predominant PFASs in the products of several major AFFF manufacturers were identified [Place *et al.* 2012, D'Agostino *et al.* 2014] (Table 1.3), and key PFAS structural differences were observed among the formulations. PFASs with perfluoroalkyl chains ranging from 4 to 12 carbons were identified in AFFF manufactured by 3M, National Foam, Ansul, and Buckeye. Perfluoroalkyl sulfonamide amino carboxylates (PF_nSAmA) and perfluoroalkyl sulfonamido amines (PF_nSAm) with C_4, C_5, and C_6 perfluorinated chains were identified in 3M

AFFF, while varying perfluorinated chain lengths of certain fluorotelomer compounds, including a fluorotelomer betaine, fluorotelomer thioamido sulfonate (FtTAoS), and fluorotelomer sulfonamide betaine (FtSaB) were identified in Buckeye, Ansul, and National Foam AFFF products, respectively (Table 1.3) [Place *et al.* 2012]. PFCAs and PFSAs were generally not detected or detected in very low concentrations in Buckeye, Ansul, Chemguard, and National Foam AFFF formulations, indicating that the fluorotelomer compounds were likely the singular dominant PFASs in the foams [Houtz *et al.* 2013]. In some 3M formulations, however, PFOS constituted up to 40-50% (mol/mol) of the total PFAS concentration, with PFnSAmA and PF_nSAm comprising approximately half and minor amounts of PFHxS, PFBS, PFOA, and PFNA also being detected in some formulations [Houtz *et al.* 2013].

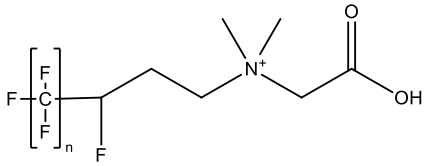
Figure 1.5 Structures of PFAS compounds found in various AFFF formulations (Adapted from Houtz *et al.* 2013)



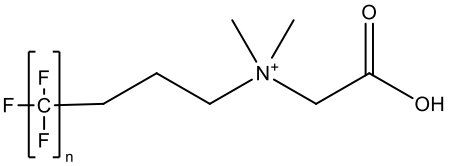
3M: Perfluoroalkyl sulfonamide amino carboxylates (PFnSaAmA). n=4,5,6.



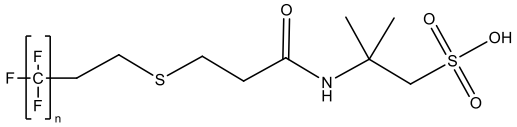
3M: Perfluoroalkyl sulfonamido amines (PFnSaAm). n= 4,5,6.



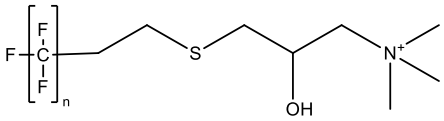
Buckeye: n:1:2 Fluorotelomer betaine. n=5,7,9.



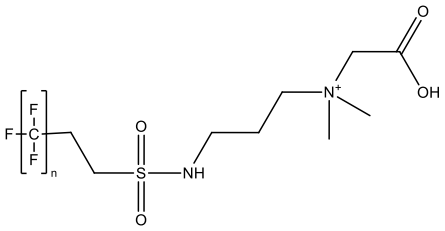
Buckeye: n:3 Fluorotelomer betaine. n=4,7,9.



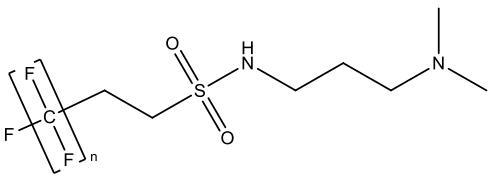
Ansul, Chemguard: Fluorotelomer thioamido Sulfonates (n:2 FtTAoS). n=4,6,8.



Ansul: Fluorotelomer thio hydroxy ammonium (n:2 FtTHN+). n=6.



National Foam (NF): Fluorotelomer Sulfonamido Betaines (n:2 FtSaB). n=6,8,10,12.



NF: Fluorotelomer Sulfonamido Amines (n:2 FtSaAm). n=6,8.

1.3.4 Environmental Occurrence of PFASs Due to AFFF contamination

PFAS contamination has been associated with environments where the accidental release or intentional deployment of AFFF occurred [Moody *et al.* 2002, Weiss *et al.* 2011, Karrman *et al.* 2011, Awad *et al.* 2011, Schultz *et al.* 2004, Moody *et al.* 2003, Backe *et al*. 2013, Houtz *et al.* 2013, McGuire *et al*. 2014]. Particularly, the repeated application of AFFF for firefighter training purposes has led to the detection of high concentrations of PFAAs in the groundwater and soil beneath training pits on some U.S. military bases [Moody *et al.* 2003, Schultz *et al.* 2004, Backe *et al*. 2013, Houtz *et al.* 2013, McGuire *et al*. 2014]. The concentrations in groundwater are often well above the U.S. EPA's PHAs for PFOS and PFOA, and in multiple cases exceed 1 mg/L. C_4-C_6 and C_8 PFSAs and C_6-C_8 PFCAs were detected in groundwater at Naval Air Station Fallon and Tyndall Air Force Base (up to 6.5 mg/L PFOA and 2.3 mg/L PFOS, respectively), while C_6 PFSA (PFHxS) and PFOS and PFOA were detected at the Wurtsmith Air Force Base (up to 105 μg/L PFOA and 110 μg/L PFOS) [Moody *et al.* 2003]. At two other U.S. military bases (denoted Site A and B), up to 220 μg/L PFOA and 78 μg/L PFOS were detected in groundwater at A, while up to 57 μg/L PFOA and 65 μg/L PFOS were detected at B [Backe *et al*. 2013]. At the Ellsworth Air Force Base, (EAFB) PFAA concentrations in groundwater reached 190 and 100 μg/L for PFOA and PFOS, respectively, while in surficial soils concentrations approached 20 mg/kg PFOS and 5.2 mg/kg PFOA [Houtz *et al.* 2013, McGuire *et al*. 2014]. Notably, in many cases the aqueous concentration of shorter-chained PFAAs (*i.e*. < C_8) were greater than PFOA and PFOS; for instance at Site A, 350 μg/L PFHxA and 360 μg/L PFHxS were detected [Backe *et al*. 2013], while at Ellsworth, 320 μg/L PFHxA and 530 μg/L PFHxS were detected in groundwater [Houtz *et al.* 2013, McGuire *et al*. 2014].

In addition to the presence of PFAAs at AFFF-impacted sites, some fluorotelomer and perfluorosulfonamide compounds have also been detected in the groundwater, soil, and aquifer sediments of military base firefighter training areas [Moody *et al.* 2003, Backe *et al*. 2013, Houtz *et al.* 2013]. 4:2, 6:2, and 8:2 FtS were detected in the groundwater of Tyndall, Wurtsmith, and Sites A, and B with maximum concentrations of 14 mg/L 6:2 FtS quantified at TAFB [Moody *et al.* 2003, Backe *et al*. 2013]. 6:2 and 8:2 FtS were detected in groundwater, surficial soil, and aquifer sediments extracted from EAFB, with maximum concentrations of 6:2 FtS at 6200 μg/kg in surface soil, 370 μg/kg in aquifer solids, and 270 μg/L in groundwater. 8:2 FtS concentrations at Ellsworth were generally an order of magnitude lower in all three media. 4:2 and 6:2 FtTAoS were detected in groundwater at Site A (maximum concentrations of 490 ng/L and 6.9 μg/L for 4:2 and 6:2, respectively), while C_4-C_6 PF_nSaAm were also detected at Site A (maximum concentration 54 ng/L PFBSaAm). PFHxSaAm was detected in surficial soil and aquifer sediments at EAFB (maximum concentrations of 1600 and 14 μg/kg in soil and sediment, respectively). 6:2 FtSaB was also detected in several soil samples collected from airports in Norway where the application of AFFF occurred [Moe *et al.* 2012].

Generally, the concentrations of PFAAs and FtSs at all sites were significantly higher than measured AFFF-derived PFASs. Even when the concentrations of FtSaB, FtTAoS, and PFnSaAmA were measured in soil and sediments, their expected partitioning phase in an aquifer system, they did not exceed method detection limits [Houtz *et al.* 2013, McGuire *et al*. 2014]. With the exception of some 3M AFFF formulations manufactured in the 1960s and 1970s, no

modern AFFF formulations (>1980) are reported to contain significant quantities of PFAAs and FtSs [Place *et al.* 2012, D'Agostino *et al.* 2014, Houtz *et al.* 2013]. This suggests that AFFF-derived PFASs may have been subjected to *in situ* abiotic or biological transformation to various FtS and PFCA compounds at these sites.

1.3.5 Biological Transformation of PFASs

PFAAs: PFAAs have not currently been reported to undergo any significant abiotic or biological transformation under naturally-occurring conditions, making them highly persistent in the environment and mobile within various ecological systems [Schultz *et al*. 2003, Parsons *et al*. 2008, Lau *et al*. 2007]. PFOA biotransformation was tested with soil and sewage-sludge inocula cultured under a range of redox conditions (nitrate-, iron-, sulfate-reducing, and methanogenic) and carbon substrates (acetate, lactate, ethanol, and/or hydrogen), including incubation with TCE to determine if co-metabolic degradation could occur by dehalorespiring organisms, yet, PFOA was persistent in the cultures up to 260 days [Lou *et al*. 2010]. PFOS degradation was also investigated in aerobic and anaerobic sewage sludge, but did not biotransform after 60-day incubations [Remde and Debus 1996]. In pure culture studies, *Pseudomonas* sp. strain D2 was able to completely defluorinate difluoromethane sulfonate and partially defluorinate 1H,1H,2H,2H-perfluoroctane sulfonate (H-PFOS) under aerobic, sulfur-limiting conditions, but showed no activity with respect to PFOS [Key *et al*. 1998]. PFOA transformation has been observed in artificial reaction systems employing the catalytic activity of horseradish-peroxidase enzyme (HRP) in the presence of a co-substrate (4-methoxyphenol) [Colosi *et al.* 2009]. HRP reacted with 4-methoxyphenol to form certain reactive phenolic species that oxidized PFOA to shortened perfluoroalkyl compounds, fluoride, and carbon dioxide [Colosi *et al.* 2009]. This has pointed to the potential for enzymatic defluorination of PFAAs under the natural conditions that might be encountered in environments laden with phenol-containing organic matter, such as humified soil, but to date none have been reported.

Partial reductive dehalogenation of PFOS was achieved at 30°C and pH 7 with a Ti(III) reducing agent (36 mM) and a vitamin B_{12} catalyst (260 μM) [Ochoa Herrera *et al.* 2008], prompting hypotheses that anaerobic reductive dechlorination might occur in biological systems by organisms harboring vitamin B_{12}-dependant reductive dehalogenase enzymes. However, the microbial biotransformation of a PFAA has not yet been reported under anaerobic or aerobic culture conditions.

Thermodynamic estimations suggest that the reductive defluorination of a fluoroalkyl chain is an energetically favorable reaction that microorganisms could theoretically gain energy from under anaerobic conditions. Gibb's free energy values (ΔG°) for the reductive dechlorination and defluorination of halobenzoates and halomethanes indicate that although energy obtained from a defluorination reaction is slightly less than that obtained from dechlorination, there is still substantial energy yield potential from defluorination (-156 and -164 kJ/mol acquired from the defluorination and dechlorination of halomethane, respectively, at 25°C, 1 atm, and pH 7) [Parsons *et al*. 2008]. Although there are few reported thermodynamic properties for PFAAs (*i.e.* $\Delta G^\circ_{formation}$), estimated ΔG°' for the hydrogenolytic defluorination of perfluoro-propane and -ethane to heptafluoro-propane and hexafluoro-ethane (respectively, with

H_2 electron donor) are negative and similar to those of hydrogenolysis of lesser fluorinated propanes and ethanes [Parsons *et al*. 2008, Dolfing *et al*. 2003, Yamada *et al*. 1999], suggesting that the reductive defluorination of longer-chained PFAAs may also be thermodynamically favorable. Because microbial reductive defluorination has not been reported as it has for reductive dechlorination [McCarty 1997, Quensen *et al*. 1988, Smidt and de Vos 2004] and debromination [He *et al*. 2006, Lee and He 2010b], it is possible that certain enzymatic kinetic limitations or steric hindrances are responsible for a lack of reactive activity on fluoroorganic molecules. Although some naturally-occurring fluorinated organic substances exist (monofluoroacetate produced by plants of *Dichapetalum* genus and tetrafluoroethene production in volcanic gases [Key *et al*. 1997]), microbial metabolic systems may not have sufficiently evolved to capture energy from carbon-fluorine bond cleavage, especially carbons with multiple fluorine substituents.

Activated carbon and membrane treatment have been used to remediate PFAA-contaminated groundwater after pumping to the surface [Yu *et al*. 2009, Tang *et al*. 2006]. Although other *ex-situ* treatment methods, such as subcritical elemental iron reduction, sonolysis, or photocatalysis may eventually become competitive with physical treatment methods, they exhibit little potential for cost effective *in-situ* application [Rayne and Forest 2009, Vecitis *et al*. 2009, Hori *et al*. 2006]. Recent studies found that PFOA can be electrochemically mineralized by a boron-doped diamond film electrode and a Ce-doped nanocrystalline PbO_2 film electrode, suggesting a potential use of electrochemical oxidation as an *in situ* remediation strategy for PFAS-contaminated groundwater [Niu *et al*. 2012].

Fluorotelomers and Persulfonamides: Some fluorotelomer compounds, such as FtOH, FtS, and polyfuorinated phosphate esters (PAPs), have been reported to biotransform in microbial incubations [Dinglasan *et al.* 2004, Wang *et al.* 2005, Liu *et al.* 2010, Lee *et al.* 2010a, Wang *et al.* 2011], mammals [Martin *et al.* 2005, D'Eon and Mabury 2007, Nilsson *et al.* 2013, Rand *et al.* 2014], and fish [Butt *et al.* 2010, Brandsma *et al.* 2011]. The transformation occurs primarily on the nonfluorinated functional group of the telomer through oxidative cleavage reactions that result in partial defluorination of the perfluoroalkyl tail. Most microbial studies have examined the aerobic biotransformation of 6:2 and 8:2 FtOH, which transforms in an array of mixed cultures (*i.e*. sludge [Wang *et al.* 2005], soil [Liu *et al*. 2010], microbial enrichment culture [Dinglasan *et al.* 2004] and bacterial isolates [Kim *et al.* 2012, Kim *et al.* 2014]) to produce C_4 through C_9 PFCAs and several other fluorocarboxylic acid intermediates. 6:2 FtS was also reported to biotransform in an aerobic sewage sludge through a 6:2 fluorotelomer unsaturated acid (6:2 FtUCA) and 5:3 fluorotelomer carboxylic acid (5:3 FtCA) intermediate to form C_4 to C_6 PFCA end products (Figure 1.6) [Wang *et al.* 2011]. The production of a 5:2 ketone and 5:2 FtOH were also reported (Figure 1.6). In most reported studies, the biotransformation of n:2 fluorotelomers results in the formation of n:2 fluorotelomer unsaturated carboxylic acid, n:2 fluorotelomer saturated carboxylic acid, (n-1):3 fluorotelomer carboxylic acid, and C_n, C_{n-1}, and C_{n-2} PFCAs. Partial defluorination is achieved through these transformation pathways as 2 moles fluoride are released for every mole C_n PFCA produced (1 $-CF_2$ group removed), while 4 and 6 moles fluoride are released for every mole C_{n-1} and C_{n-2} PFCA produced (2 and 3 $-CF_2$ groups removed), respectively. The molar recovery of PFCA products was generally less than 10% of the amended fluorotelomer substrate in most systems and the rate of PFCA formation was slower than fluorotelomer disappearance, indicating the transformation of certain intermediates, such as

fluorotelomer carboxylic acids, likely were rate limiting in the biotransformation pathways [Dinglasan *et al.* 2004, Wang *et al.* 2005, Liu *et al.* 2010, Lee *et al.* 2010a, Wang *et al.* 2011].

When 6:2 FtOH biotransformation was examined in three species of alkane-degrading bacteria (*Mycobacterium vaccae* JOB5, *Pseudomonas oleovorans*, and *Pseudomonas butanovora*) and one fluoroacetate-degrading bacterium (*Pseudomonas fluorescens* DSM 8341), different biotransformation pathways were preferred among organisms [Kim *et al.* 2012, Kim *et al.* 2014]. All species oxidized 6:2 FtOH to 5:3 acid, 6:2 FtUCA, PFHxA, and PFPeA, while only JOB5, *P. oleovorans*, and DSM 8341 were capable of oxidizing 6:2 FtUCA to PFBA, and DSM 8341 was only capable of PFBA production in the presence of 1 mM formate, an external reducing energy source [Kim *et al.* 2014]. Furthermore, *P. oleovorans* showed 6-fold greater 6:2 FtOH degradation and PFPeA production in the presence of 1 mM formate or dicyclopropylketone, an alkane hydroxylase inducer [Kim *et al.* 2014]. These studies suggest that fluorotelomer biotransformation may be dependent on a range of conditions, including bacterial strain, enzyme inducing substances, and reducing agents.

One microbial study has been conducted to examine perfluorosulfonamide biotransformation [Rhoads *et al*. 2008]. *N*-EtFOSE was found to aerobically biotransform within ten days in undiluted activated sludge through a series of volatile perfluorinated intermediates to PFOS (yields ranged from 4-50% per mole of amended intermediate) [Rhoads *et al*. 2008] Although *N*-EtFOSE is volatile and likely partitions into the atmosphere of a wastewater treatment activated sludge basin, its biotransformation pathway and the hypothesized transformation pathways of other perfluorosulfonamides may explain increases of PFOS concentrations measured in some wastewater treatment plans [Schultz *et al.* 2006].

It is currently unclear whether microorganisms are utilizing the fluorotelomers as a growth and energy substrate or the biotransformation is occurring co-metabolically with the degradation of other organic carbon sources in the growth medium. 8:2 FtOH biotransformation by an enriched microbial community occurred only under ethanol amendment [Dinglasan *et al.* 2004], while transformation studies with soil, sludge, and wastewater were likely to have alternative carbon forms present [Wang *et al.* 2005, Liu *et al.* 2010, Lee *et al.* 2010a, Wang *et al.* 2011]. All pure culture studies of 6:2 and 8:2 FtOH transformation were conducted with co-substrate amendment, such as n-octane and 1-butanol [Kim *et al.* 2012, Kim *et al.* 2014]. Determining the potential for metabolic transformation of fluorotelomers, particularly AFFF-derived PFASs, may be important for assessing their fate in oligotrophic aquifer systems where the organic constituents of AFFF have already degraded in surficial soils and unreacted fluorotelomer compounds infiltrate the groundwater table.

6:2 FtSaB was found to biotransform *in vivo* in *Mytilus edulis*, a marine bivalve, and *Scophthalmus maximus*, a species of flatfish, when the organisms were exposed to artificial aerated seawater with 40 to 1000 mg/L 6:2 FtSaB. A fluorotelomer sulfonamide, a deacetylated fluorotelomer betaine compound, and two demethylated species of the deacetylated betaine were formed in the mussel and fish tissue following exposure [Moe *et al.* 2012]. No defluorination of 6:2 FtSaB occurred in this biotransformation pathway, as the transformation reactions were limited to the telomer's non-fluorinated functional group only [Moe *et al.* 2012]. However, this study was the first to report the biological transformation of a PFAS fluorotelomer known to constitute the major fluorosurfactant component in AFFF (Figure 1.5).

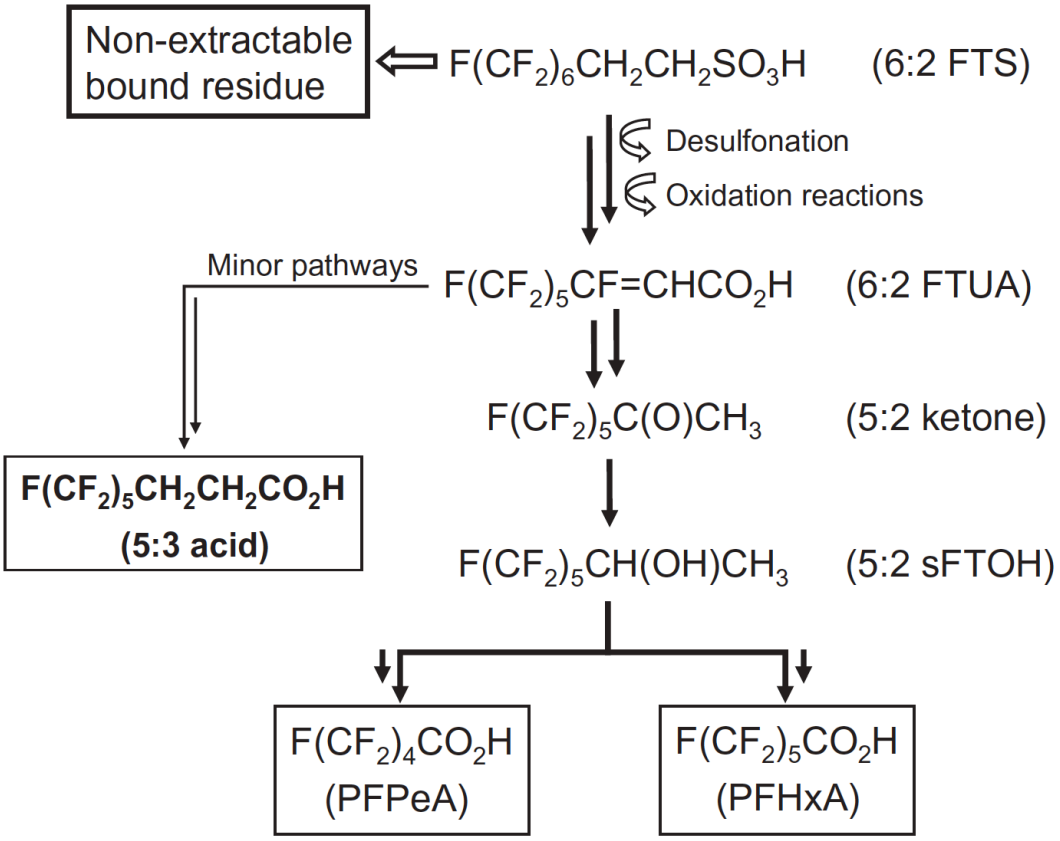


Figure 1.6 Proposed biotransformation pathway of 6:2 FtS in activated sludge from Wang *et al*. 2011.

1.4 Background on Trichloroethene

1.4.1 TCE Uses and Human Health Effects

Trichloroethene (TCE) is a volatile chlorinated organic compound and industrial solvent that has been used in dry-cleaning operations, metal-degreasing, and in the formulation of paints, varnishes, and adhesives [Doherty 2000a, Doherty 2000b] (Figure 1.7). It was first produced commercially in the United States in the 1920s for use in vapor degreasing processes and then introduced into dry-cleaning fluids in the 1930s; it was subsequently phased out of dry-cleaning in the 1950s and largely replaced with tetrachloroethene (also known as perchloroethene, PCE) [Doherty 2000a, Doherty 2000b]. Currently, most worldwide TCE production and use is for metal degreasing operations in industries that fabricate, assemble, or operate metal parts, such as aircraft and automotive manufacturing, oil production and refining, and electronics assembly [U.S. EPA 2011]. TCE production in the United States increased significantly during and after World War II and reached a peak of approximately 600 million pounds per year in 1970. Production began declining after growing environmental and health concerns in the early 1970s and has declined steadily since that time to under 300 million pounds per year in the late 1990s [Doherty 2000a, Doherty 2000b]. 1,1,1-trichloroethane (TCA) was widely adopted as a replacement solvent for degreasing operations until it became recognized as an atmospheric pollutant and ozone depleting substance. TCA was subsequently phased out under the international Montreal Protocol agreement and production began to decrease correspondingly in the 1990s [Doherty 2000a, Doherty 2000b, Stroo and Ward 2010, U.S. EPA 2001].

Table 1.3 Chemical and physical properties of TCE

TCE Properties	Value	
Molecular weight	131.39	Yaws *et al*. 1999
Density, 20˚C (g/cm^3)	1.46	Yaws *et al*. 1999
Boiling point ˚C	87.2	Lide *et al*. 1998
Henry's Law Constant K_H (atm/M)	12	Yaws *et al*. 1999
Aqueous solubility, 25˚C (mg/L)	1,100	Mackay *et al*. 1993
Vapor pressure, 25˚C (torr)	74.2	Mackay *et al*. 1993
Octanol/Water Partition Coefficient (log K_{ow})	2.53	Mackay *et al*. 1993

The predominant human exposure routes of TCE are inhalation and ingestion [U.S. EPA 2011]. TCE is a lipophilic compound that readily crosses biological membranes and preferentially partitions into fatty tissues. Studies conducted to examine the carcinogenicity and toxicity of TCE in both animals and human populations that have been exposed occupationally or unknowingly have found a large array of adverse health effects [Chiu *et al*. 2006, Wartenberg *et al*. 2000, McLaughlin and Blot 1997, Weisel and Jo 1996, Fisher *et al*. 1989, Herren-Freund *et al*. 1987, Stott *et al*. 1982, U.S. EPA 2011] Until 2011, TCE was designated by the U.S. EPA as a "possible human carcinogen;" however following the completion of their *Toxicological Review* in September 2011 [U.S. EPA 2011], which examined the weight of evidence for TCE's detrimental human health effects from published epidemiologic, animal, and mechanistic studies, the health risk designation was updated to "carcinogenic in humans through all routes of exposure" [U.S. EPA 2011]. Substantial evidence acquired from a multitude of human epidemiological studies supports a clear causal association between TCE exposure and kidney cancer, mediated through the body's metabolism of TCE and subsequent production of toxicologically active intermediate compounds [Brauch *et al*. 1999, McLaughlin and Blot 1997]. Exposure has also been strongly linked to non-Hodgkin Lymphoma, liver, and biliary tract cancer, while it is suspected to be associated with bladder, esophageal, prostate, cervical, and breast cancer, and childhood leukemia [Wartenberg *et al*. 2000, U.S. EPA 2011]. Toxicokinetic modeling and tumor growth following cancer bioassays in rat and mice have supported the labeling of TCE as a known carcinogen in humans [Fisher *et al*. 1989, Stott *et al*. 1982].

Non-cancer effects include central nervous system depression, decreased auditory and motor function, decreased fertility, prenatal death or impaired growth, kidney and liver failure, and a potential increase in risk of certain autoimmune diseases, such as scleroderma. A review of TCE's toxicity effects and carcinogenicity can be found in Chiu *et al*. 2013.

The concentration of TCE in drinking water is regulated through the U.S. EPA as an enforceable MCL, which is currently set at 5 μg/L and applies to public water systems that serve 25 or more people [U.S. EPA 1987 / 40 CFR 141.61]. As this drinking water standard became effective in 1989, prior to the 2011 *Toxicological Review,* some have questioned whether the 5 μg/L allowed concentration provides adequate drinking water protection in light of the most recent evidence for TCE's toxicity and carcinogenicity. EPA has stated that it would review the MCL and consider it for regulatory revision as part of the Safe Drinking Water Act's (SDWA) six-year review program [U.S. EPA 2010]. Several states have issued or recommended lower MCLs, such as Florida and New Jersey who have implemented TCE drinking water standards of 3 μg/L and 1 μg/L, respectively.

1.4.3 Environmental Occurrence and Co-Contamination with AFFF

Due to its relatively high vapor pressure (Table 1.3), most TCE released to topsoil and surface waters volatilizes into the atmosphere where it is present as a vapor phase [U.S. EPA 2011]. Volatilization from surface sources and direct release into the atmosphere have contributed to the detection of TCE in the ambient air in a variety of locations across the U.S., primarily those in industrial and urban centers [ATSDR 1997]. However, the downward migration of some TCE into the subsurface, through vadose zones and into aquifer systems has led to the widespread detection of TCE in groundwater. Its relatively high density and low aqueous solubility (Table 1.3) facilitate the formation of dense non-aqueous phase liquids (DNAPLs) that pool on impermeable clay layers and sorb to subsurface solids, leading to long-term sources of dissolved TCE in groundwater plumes.

To date, TCE is one of the most frequently detected organic contaminants in groundwater in the U.S. [Stroo and Ward 2010, Williams *et al*. 2002, ATSDR 1997, CDHS 1986, USGS 2006, Westerick *et al*. 1984]. In 2006 the United States Geological Society (USGS) completed a national assessment of 55 volatile organic compounds (VOCs), including TCE, PCE, 1,1,1-TCA, and methylene chloride, in thousands of domestic and public well samples collected from 1985-2001 [USGS 2006]. TCE was detected in the water of approximately 1% of domestic wells and over 4% of public well samples at > 0.2 μg/L [USGS 2006]. TCE concentrations were above the 5 μg/L MCL in six domestic wells and nine public wells, while TCE had the highest median concentration of the four listed chloroethenes in all wells [USGS 2006]. These results confirmed an earlier monitoring study by the U.S. EPA that found that 2.6% of public water supply systems in 16 states in the U.S. had at least one sample exceed the 0.5 μg/L TCE minimum reporting level, while 0.65% of the systems had a sample that exceeded 5 μg/L [U.S. EPA 2003].

TCE groundwater contamination has been extensively detected in California, a state with one of the highest rates of detection in public and private sources [CA SWRCB 2010, Williams *et al*. 2002, CDHS 1986]. The California Department of Health Services, which also enforces a 5 μg/L MCL, found that concentrations exceeded the MCL in approximately 245 active and standby public groundwater sources throughout the state in samples acquired prior to 2008 [CA SWRCB 2010]. Wells with the highest TCE concentrations were generally centered in urban and industrial areas, with Los Angeles County reporting the largest number of contaminated wells, followed by San Bernardino and Fresno Counties [CA SWRCB 2010]. As severe drought has persisted in the western U.S. since the beginning of 2013 and a significant deficit in groundwater supplies has been reported (~63 trillion gallons) [Borsa *et al*. 2014], chloroethene contamination in California's aquifers is a pressing concern.

Hazardous waste sites on the National Priorities List (NPL) maintained by the EPA under the Superfund program pose the greatest risk for contaminating potable water supplies. TCE has been reported at over 1000 of the 1689 NPL sites in 2007 [ATSDR 2007]. As the U.S. military has historically used large quantities of TCE and PCE for their operations and equipment maintenance, substantial contamination also exists in the soil and groundwater beneath military bases where the solvents were improperly stored or dumped after use and many closed and operating military installations are designated Superfund NPL sites. Additionally, the U.S. Air

force has a history of conducting regular firefighter-training exercises on base as part of their emergency preparedness plans. Waste chlorinated solvents, such as TCE or PCE or jet fuel (3000 L/week), were ignited in un-lined flooded pits and subsequently extinguished with diluted AFFF solutions (1200-3200 L) [Moody and Field 2000]. Typically, after the training exercises were concluded, fuels and AFFF were left to dissipate on the ground, leading to TCE and AFFF infiltration into soil, sediments, and groundwater [Moody and Field 2000]. While the chlorinated solvent plumes at many contaminated military bases have been well characterized as part of the site's remediation efforts, less is known regarding the extent of AFFF and PFAS contamination. Increased monitoring at some sites has pointed to the potential for significant co-contamination of TCE and PFASs in groundwater and soil [McGuire *et al.* 2014].

1.4.4 Anaerobic Biodegradation by Dehalococcoides organisms

Several chemical and physical groundwater treatment strategies have been used for the remediation of chloroethenes. Conventional pump-and-treat technologies that employ *ex situ* air stripping or granular activated carbon (GAC) absorption are widely used to control plume migration [Stroo and Ward 2010, NRC 1994]. Similarly, *in situ* air sparging coupled to soil vapor extraction (SVE) has also been used to strip VOCs from groundwater into an extractable vapor phase [Pankow et al. 1993] that can be further treated with GAC. *In situ* chemical oxidation (ISCO) that relies on the activity of ozone [Glaze and Kang, 1988], permanganate [Yan and Schwartz 1999, Schnarr *et al*. 1998] or Fenton's reagent [Teel *et al*. 2001, Chen *et al*. 2001] is a contaminant destruction process often applied to DNAPL-containing aquifers. Alternativey, *in situ* abiotic reductive dehalogenation of TCE with zero valent iron [Farrell *et al*. 2000, Gillham *et al*. 1994, Wang *et al*. 1997], most commonly in permeable reactive barriers [Puls *et al*. 1999, Phillips *et al*. 2010], has demonstrated potential for effective plume remediation and control. Many of these abiotic and physical removal strategies have notable disadvantages and limitations in chloroethene removal, however. Conventional pump-and-treat systems are typically inadequate for remediating sites with neat TCE, as DNAPL dissolution commonly occurs on a significantly longer time scale than the pumping operation. Of 77 pump-and-treat operations reviewed by the National Research Council (NRC) in 1994, 69 had not met clean-up goals, with some facilities operating for more than two decades [NRC 1994]. *In situ* air-sparging and in-well vapor stripping does not yield contaminant destruction, while chemical processes can be expensive and impractical for treating large, low-concentration plumes especially when the presence of non-target co-contaminants consumes oxidants or reductants.

In situ bioremediation through natural attenuation or engineered bioaugmentation and/or biostimulation can offer several operational advantages for the remediation of TCE, including reduced costs, enhanced site flexibility, minimized impact on existing site infrastructure, and the potential for complete chloroethene conversion to ethene [Stroo and Ward 2010]. Specifically, employing the anaerobic reductive dehalogenation of TCE by native or augmented microorganisms in anoxic aquifer systems has proven to be a cost efficient and sustainable remediation technology for the detoxification of chloroethenes to the innocuous end product ethene.

The biological reductive dechlorination of TCE to ethene occurs in a sequential reaction pathway whereby each chlorine atom is removed through a two-electron transfer and replaced with a hydrogen atom (Figure 1.7). This transformation pathway leads to the production of the lesser-chlorinated intermediate products DCE and VC prior to the formation of ethene. Although TCE dechlorination has been shown to occur under Fe(III)-reducing and SO_4^{2-}-reducing conditions, the dechlorination of DCE and VC to ethene appears to be associated with more highly-reducing and methanogen conditions [Ballapragada *et al*. 1997, Maymó-Gatell *et al*. 1995). *Dehalococcodies mccartyi* (Dhc) is currently the only species of microorganisms known to perform the complete reductive dechlorination of TCE to ethene [Löffler *et al.* 2013] (Figure 1.7). They catalyze these reactions with reductive dehalogenase enzymes (RDases) that require corrinoid co-factors for catalytic activity. While many other genera of bacteria, including *Dehalobacter* and *Dehalospirillum,* are capable of PCE and TCE dechlorination to *cis*DCE, they are unable to catalyze DCE dechlorination to VC and ethene [Smidt *et al.* 2004].

Dhc are obligate anaerobes and strict organohalide-respiring bacteria in the phylum Chloroflexi [Löffler *et al.* 2013]. They require hydrogen as an electron donor, acetate as a carbon source, an organohalide as an electron acceptor, and vitamin B_{12} (cobalamin) as an essential RDase co-factor in order to support their growth and metabolism. Various Dhc isolates representing distinct bacterial strains (16S rRNA gene sequence similarity > 98%) have been grown in pure culture and characterized; the most well-studied of these strains are 195, CBDB1, BAV1, VS, FL2, and GT [Löffler *et al.* 2013]. Chloroethene utilization appears to be unique to certain strains and likely results from the differing substrate ranges of the strains' RDase enyzmes (Figure 1.7). Most strains are known to possess one of three functionally confirmed RDases: TceA, VcrA, and BvcA [Löffler *et al.* 2013]. The RDases share structural similarity in that most of them contain two iron-sulfur clusters that are corrinoid dependent. Strain 195 harbors the TceA enzyme and is capable of the metabolic dechlorination of TCE, 1,1-DCE, and *cis*DCE, and slower, co-metabolic dechlorination of VC to ethene [Lee *et al.* 2006]. Strain BAV1 possesses the BvcA RDase and can dechlorinate 1,1-DCE, *cis*- and *trans*DCE and VC, but not TCE, while strains VS and GT possess VcrA and can dechlorinate *cis*DCE and VC, but no other chloroethene [Krajmalnik-Brown *et al*. 2004, Müller *et al*. 2004, Sung *et al*. 2006]. However, chloroethene usage may also depend strictly on the strain: although strain FL2 possesses TceA, it cannot utilize 1,1-DCE as a metabolic electron acceptor [He *et al.* 2005]. It is also possible that the numerous other putative RDase-encoding genes held by many of the strains contribute to certain chloroethene degradation patterns through mechanisms not currently well understood.

Several Dhc-containing mixed microbial communities, such as "ANAS" [Holmes et al. 2006], "KB1" [Waller et al. 2005], and an enriched groundwater culture [Men *et al.* 2013], have also been characterized according to their metabolic dechlorination activity. Although the isolation of specific Dhc strains has enabled a detailed examination of the organisms' genomic content [Lovley *et al.* 2003, Brisson *et al.* 2012], carbon metabolism [Tang *et al.* 2009], physiology [Löffler *et al.* 2013], and nutritional requirements, the growth of Dhc in pure culture is generally slow, with cell doubling rates on the order of 20 to 60 hours [Cupples *et al.* 2003, Maymó-Gatell *et al.* 1997, Sung *et al.* 2006]. Conversely, Dhc growth in mixed microbial communities or defined consortia appears to be more robust with faster growth and dechlorination rates [Duhamel and Edwards 2006, Duhamel *et al.* 2002, Maymó-Gatell *et al.* 1997, Men *et al*. 2013]. This suggests that co-existing microorganisms in the communities play

important roles in the growth and metabolism of Dhc. It is well established that Dhc's carbon source (acetate) and electron donor (hydrogen) are provided by fermenting bacteria that degrade labile carbon sources, such as lactate, methanol, or molasses [Duhamel and Edwards 2006, Freeborn *et al.* 2005, Lee *et al.* 2006, Holmes *et al.* 2006]. Recent studies have also elucidated syntrophic roles played by carbon monoxide (CO)-metabolizing bacteria growing with Dhc that produces CO as a by-product of acetyl-CoA cleavage in a modified Wood-Ljungdhal pathway [Zhuang *et al.* 2014]. CO accumulation inhibits Dhc growth in pure culture as Dhc lacks a CO dehydrogenase enzyme, however it is consumed in anaerobic mixed cultures by CO-degrading bacteria [Zhuang et al. 2014], facilitating more robust Dhc growth. Other studies have highlighted the important relationships established among Dhc and corrinoid-producing microorganisms [Yi *et al.* 2012, Men *et al.* 2014]. As Dhc is not capable of *de novo* corrinoid biosynthesis, they must obtain and/or remodel the co-factors from their environment from corrinoid-producing organisms [Yi *et al.* 2012, Men *et al.* 2014]. When grown in pure culture, cobalamin must be amended to Dhc's growth medium [Löffler *et al.* 2013]. The synergistic relationships that occur between Dhc and other microorganisms and play a controlling role in the TCE dechlorination rate are an area of active research.

In addition to these cross-species cooperative relationships, multiple Dhc strains have been shown to work in concert within a single community to achieve complete dechlorination of chloroethenes. Mixed communities frequently contain multiple Dhc strains that each possess different functional RDase enzymes and chloroethene utilization preferences. For instance, TCE to ethene dechlorination in the ANAS enrichment is likely catalyzed by both the TceA and VcrA RDases [Holmes *et al.* 2006], which are present in distinct Dhc strains (strains ANAS1 and ANAS2) [Brisson *et al.* 2012]. Additionally, a study examining the dechlorination and community structure of enriched subcultures derived from a PCE-to-ethene dechlorinating parent culture found that two of three subcultures lost the ability to dechlorinate PCE while retaining the ability to dechlorinate *cis*DCE and VC [Flynn *et al.* 2000]. Subsequent community structure analyses of the subcultures using16S rDNA sequencing, denaturing gradient gel electrophoresis (DGGE) and terminal restriction fragment length polymorphism (T-RFLP) suggested that at least two microbial populations were responsible for the dechlorination of PCE to ethene in the parent culture [Flynn *et al.* 2000]. Investigating the factors that control TCE dechlorination within Dhc-containing communities will provide the most practical insights into field-relevant remediation processes.

In situ anaerobic bioremediation of TCE to ethene by natural attenuation, biostimulation of native Dhc populations (*i.e.* addition of fermentable organic carbon source), and/or bioaugmentation with laboratory-grown cultures has been successfully executed at contaminated field sites [MacBeth *et al.* 2004, Lee *et al.* 2008]. Biostimulation and bioaugmentation materials are often easily administered using on-site injection wells and can produce relatively quick dechlorination results. However, monitoring the long-term performance and quantifying the effectiveness of these systems can be a complicated task. Typically, chloroethene concentrations and various geochemical parameters, such as dissolved oxygen and methane concentrations, are measured in groundwater samples and used to assess the occurrence of dechlorination and the establishment of the appropriate redox conditions. Varying groundwater flow paths, seasonal fluctuations, and hydrologic changes introduced by the remediation system can mask true concentration profiles of these analytes [Stroo and Ward 2010]. Additional lines of evidence for

successful bioremediation, like molecular-based diagnostic tools, such as Dhc 16S rRNA and RDase gene and transcript screening, may be required to monitor Dhc populations and activities [Lee *et al.* 2008]. Stable isotope analysis is another tool frequently utilized by practitioners seeking evidence of *in situ* TCE bioremediation [Morrill *et al.* 2005, Song *et al.* 2002].

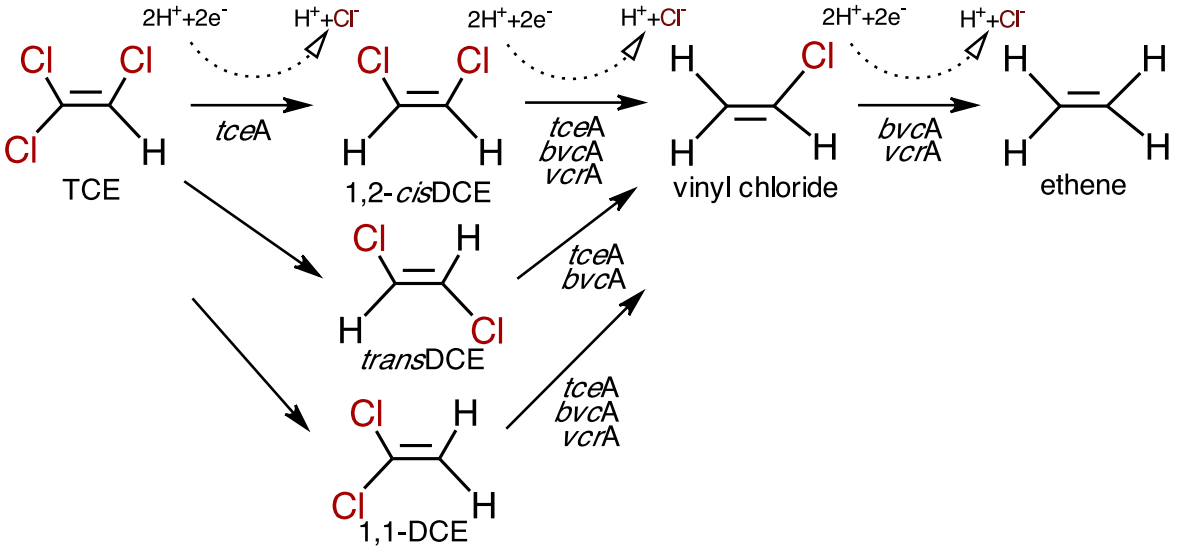


Figure 1.7 TCE reductive dechlorination pathways in *Dehalococcoides mccartyi*

1.4.5 Stable Carbon Isotope Fractionation

Stable isotope fractionation, also known as compound-specific isotope analysis (CSIA), is a tool that has been used to provide evidence of a compound's physical or chemical transformation. Isotope fractionation refers to an observed difference in isotope distribution (*i.e.* ^{12}C vs. ^{13}C) in a compound before and after it undergoes a transformation. Biological and abiotic chemical reactions can cause significant shifts in the isotope distribution of a compound as the reaction rates slightly differ between heavy- and light isotope-containing compounds. The molecular bonds formed with heavier isotopes (^{13}C or ^{2}H) are stronger and less reactive than those formed with lighter isotopes (^{12}C or ^{1}H), causing a dynamic accumulation of heavy isotopes in the reactant and lighter isotopes in the product [Galimov 1985]. Previous studies have shown that the physical processes acting on organic contaminants in groundwater systems, such as adsorption, volatilization, and dissolution, cause only small isotopic shifts that are typically within the analytical uncertainty of an isotope ratio measurement (±0.5 ‰ for carbon isotopes and ±5 ‰ for hydrogen isotopes). However, biological oxidation and reduction reactions can produce shifts of one to two orders of magnitude larger [Galimov 1985]. Thus, detecting a shift in a contaminant's isotope ratio can provide evidence of its *in situ* biotransformation, which can be further quantified using laboratory relationships established between transformation pathways and fractionation patterns [Elsner *et al*. 2005, Liang *et al*. 1997, Hunkeler *et al*. 2005].

Kinetic isotope fractionation is modeled using the Rayleigh equation, which describes the relationship between a compound's isotope ratio and its concentration for a given reaction pathway [Mariotti *et al*. 1981]. The equation was originally developed to model the fractional distillation of mixed liquids, and in this application the principles are related: an exponential relationship describes the irreversible partitioning of a species (*i.e*. an isotope) between two reservoirs where one reservoir, a homogeneous mixture of the species, is decreasing in size.

The foundation of the isotope-applied Rayleigh model is the establishment of a fractionation factor, α, which describes the ratio of the reaction rate constants, k_h and k_l, for the transformation of the heavy and light isotope-containing compounds, respectively (Equation 1.1) [Mariotti *et al*. 1981, Elsner *et al.* 2005]. The fractionation factor can be alternatively expressed as an enrichment factor, ε, in units of per mil, as defined in Equation 1.2.

$$\alpha = \frac{k_h}{k_l} \qquad \textit{Equation 1.1}$$

$$\varepsilon\ ‰ = (\alpha - 1) * 1000 \qquad \textit{Equation 1.2}$$

The changes in concentrations of the heavy and light isotopes (d[^{13}C] and d[^{12}C], for carbon isotope concentrations, respectively) can then be expressed according to the relationship in Equation 1.3. Integration of Equation 1.3 from time zero (subscript "0") to time *t* (subscript "t") then yields Equation 1.4, the most widely-utilized and simplest form of the Rayleigh equation (see supporting information of Elsner *et al.* 2005 for integration details), where R = [^{13}C]/ [^{12}C] and *f* describes the concentration fraction of compound remaining at time *t*.

$$\frac{d[^{13}C]}{d[^{12}C]} = \alpha \frac{[^{13}C]}{[^{12}C]} \qquad \textit{Equation 1.3}$$

$$\frac{R_t}{R_0} = \left[f \cdot \frac{1+R_0}{1+R_t}\right]^{\alpha-1} \qquad \textit{Equation 1.4}$$

After applying the ε and δ notations (Equations 1.3 and 1.5, respectively) and utilizing the assumption that [^{13}C]<<[^{12}C] at natural isotopic abundances (Equation 1.6), Equation 1.4 can be rearranged into the isotope fractionation equations that are presented in Chapter 5 and used in the data analysis of that study. $R_{standard}$ used for carbon isotopes is the Vienna Pee Dee Belemnite (VPDB) international standard.

$$\delta^{13}C\ (‰) = \left(\frac{R_{sample}}{R_{standard}} - 1\right) \cdot 1000 \qquad \textit{Equation 1.5}$$

$$\frac{1 + R_t}{1 + R_0} \cong 1 \qquad \textit{Equation 1.6}$$

The enrichment factors, ε, acquired from the application of the above equations and those in Chapter 5 to isotope ratios obtained from biological transformation reactions have been found to vary according to reaction pathways [Elsner *et al.* 2005], and microorganisms and enzymes [Fletcher *et al.* 2013, Lee *et al.* 2007, Nijenhuis *et al.* 2005] associated with the degradation of chlorinated solvents. Although the mechanisms that produce differences in ε due to varying transformation pathways have predominantly been established [Elsner *et al.* 2005], the factors that control differences in ε observed among microorganisms executing the same transformation pathway are not well understood. Kinetic processes associated with microbial metabolism, such as substrate transport across membranes or substrate-enzyme binding, are thought to contribute to differences in enrichment factors. These effects are usually recognized in studies that observe different isotope fractionations in cultures with varying cell integrities (*i.e.* growing cells, cell extracts, and purified enzymes) [Nijenhuis *et al.* 2005] However, the large range of enrichment factors that have been reported for reductive dechlorination of TCE, DCE, and VC by *D. mccartyi* (Table 1.4), including among physiologically similar strains harboring structurally homologous RDases, indicates that isotopic fractionation may be controlled by a multitude of subtle factors. The impact of varying organism growth conditions on TCE isotope fractionation by *D. mccartyi* strains has not been previously investigated and is the subject of Chapter 5.

Table 1.4 Compound-specific enrichment values (ε) for the reductive dechlorination of chlorinates ethenes by Dhc pure cultures and Dhc-containing mixed communities. Table adapted from Fletcher *et al.* 2013.

culture	TCE		1,1-DCE		*cis*-DCE		*trans*-DCE		vinyl chloride	
	ε (‰)	R^2	ε (‰)	R^2	ε (‰)	R^2	ε (‰)	R^2	ε (‰)	R^2
Dhc pure cultures										
195 (*tce*A)	-13.7±1.8[a]	0.95[a]	NR		NR		NA		NA	
	-9.6±0.4[b]	0.99[b]	-5.8±0.5[b]	0.97[b]	-21.1±1.8[b]	0.97[b]				
BAV1 (*bvc*A)	NA		-8.4±0.3[b]	0.99[b]	-16.9±1.4[b]	0.98[b]	-21.4±0.9[b]	0.99[b]	-24.0±2.0[b]	0.97[b]
			-5.1±0.3[e]	0.97[e]	-14.9±0.5[e]	0.99[e]	-20.8±2.8[e]	0.92[e]	-23.2±1.8[e]	0.96[e]
FL2 (*tce*A)	-8.0±0.4[e]	0.98[e]	NA		-15.8±1.1[e]	0.97[e]	NR		NA	
GT (*vcr*A)	NR		NR		-21.6±1.3[e]	0.97[e]	NA		-23.8±1.1[e]	0.99[e]
VS (*vcr*A)	NR		NR		-17.6±2.7[e]	0.89[e]	NA		-22.1±1.2[e]	0.97[e]
Dhc containing consortia										
ANAS[b]	-16.0±0.6	0.99	-23.9±1.2	0.98	-29.7±1.6	0.99	-28.3±1.4	0.99	-22.7±0.8	0.99
BDI	-15.3±0.8[d]	NR	NR		-25.3±1.0[e]	0.99[e]	NT		-19.9±1.6[e]	0.96[e]
KB-1[c]	-2.5 to -13.8	0.91	NR		-14.1 to -20.4	0.92	NA		-21.5 to -26.6	0.96

[a]Data from Cichocka *et al.* 2008, [b]Data from Lee *et al.* 2007, [c]Data from Bloom *et al.* 2000, Duhamel *et al.* 2002, and Slater *et al.* 2003, [d]Data from Liang *et al.* 2007, [e]Data from Fletcher *et al.* 2013
NA indicates fractionation is not reported because the chloroethene does not support growth and respiration by tested culture
NR indicates fractionation is not reported however the chloroethene does support growth and respiration

CHAPTER 2: Aerobic biotransformation of fluorotelomer thioamido sulfonate in AFFF-amended microcosms

2.1 Introduction

Aqueous film-forming foams (AFFFs) are water-based chemical mixtures applied to liquid-fuel fires, such as ignited petroleum hydrocarbons or chlorinated solvents, in order to extinguish flames and prevent fuel re-ignition [Moody *et al.* 2000, Tuve *et al.* 1966]. Although AFFF is typically a proprietary mixture that varies according to manufacturer and year of production, most formulations consist of a glycol ether-based solvent, hydrocarbon surfactants, and various poly- and perfluoroalkyl substances (PFASs) [Moody *et al.* 2000]. AFFF use at military, industrial, and municipal sites has led to widespread groundwater contamination with PFASs, and as well as their occurrence in soils, surface waters, and aquatic organisms [Moody *et al.* 2002, Oakes *et al.* 2010, Awad *et al.* 2011, Karrman *et al.* 2011, Moody *et al.* 2003, Weiss *et al.* 2012, Schultz *et al.* 2004]. Repeated AFFF application at fire-training facilities where fire-fighting exercises were conducted in unlined pits has led to particularly high environmental contamination of PFASs [Karrman *et al.* 2011, Moody *et al.* 2003, Schultz *et al.* 2004, Houtz *et al.* 2013, Backe *et al.* 2013, McGuire *et al.* 2014].

Perfluorocarboxylates (PFCAs) and perfluorosulfonates (PFSAs) are persistent [Awad *et al.* 2011, Moody *et al.* 2003, Houtz *et al.* 2013], resistant to biodegradation [Liu *et al.* 2013], and cause adverse human health effects [Nelson *et al.* 2009, Lau *et al.* 2007, Fei *et al.* 2007, Sakr *et al.* 2007]. The 3M company manufactured AFFF with eight-carbon (C_8) perfluorosulfonamides, perfluorosulfonic acid (PFOS), and perfluorooctanoic acid (PFOA) until 2001 when rising concerns over their health and environmental effects led them to be phased out of production [Kissa *et al.* 2001, CMER 2011]. The PFASs in formulations manufactured by other companies, such as Ansul, Chemguard, National Foam, Angus, and Buckeye, consisted of fluorotelomer-based compounds containing C_4 to C_{10} perfluorinated chains linked to an ionic alkyl group by two to three non-fluorinated carbons [Place *et al.* 2012]. The non-fluorinated alkyl groups may have carboxylic acid, tertiary amine, dimethyl quarternary amine, and/or sulfide moieties [Place *et al.* 2012, D'Agostino and Mabury 2014]. Despite growing knowledge of the composition of AFFF, little is known about the environmental fate of these PFASs, including their potential to biotransform under conditions representative of the soils, surface waters, and aquifer systems in which they are found.

Fluorotelomer thioamido sulfonate (FtTAoS, also sold under the trade name Lodyne™) is a PFAS present in several widely used AFFF formulations currently made by at least three manufacturers (*i.e.* Ansul, Chemguard, and Angus) and was used as early as 1984 [Place *et al.* 2012]. While the 6:2 fluorotelomer appears to be the most abundant FtTAoS homolog in many formulations, 4:2, 8:2, 10:2, 12:2, and 14:2 FtTAoS have also been detected in some AFFFs [Place *et al.* 2012, D'Agostino and Mabury 2014]. Although FtTAoS has been measured in groundwater and soil collected from a variety of firefighter-training sites [Houtz *et al.* 2013, Backe *et al.* 2013], and at one U.S. military base, FtTAoS was not detected in soil, despite extensive use of AFFF and detection of other PFASs [Houtz *et al.* 2013]. At two different sites where the application of FtTAoS-containing AFFF was known to occur, concentrations of 4:2 and 6:2 FtTAoS in groundwater were low relative to the measured concentrations of flurotelomer sulfonates (FtSs) and PFCAs [Backe *et al.* 2013]. Together, the FtS/FtTAoS and PFCA/FtTAoS ratios were much greater than the ratios measured in the AFFF formulations applied at the site, suggesting that FtTAoS may have been transformed to FtS and PFCAs after its release [Backe *et al.* 2013].

Although PFCAs and PFSAs have been shown to be completely resistant to microbial biotransformation under a variety of growth conditions [Liu *et al.* 2013], the larger non-fluorinated alkyl groups of the AFFF-derived PFASs, including FtTAoS, are likely amenable to biotransformation, especially by the diverse metabolic capacity of soil microbial communities. Some fluorotelomer compounds with more simple non-fluorinated functional groups, such as 4:2, 6:2, and 8:2 fluorotelomer alcohol (FtOH), 6:2 FtS, and 6:2 polyfluoroalkyl phosphate esters (mono- and di-PAP) undergo biotransformation to terminal C_4-C_9 PFCAs as well as a number of other products [Dinglasan *et al.* 2004, Wang *et al.* 2005, Lee *et al.* 2010a, Liu *et al.* 2010, Wang *et al.* 2011, Kim *et al.* 2012, Kim *et al.* 2014]. However, slow rates of transformation and poor mass recovery have made it difficult to differentiate PFAS loss due to physical mechanisms (*e.g.* sorption) [Rand *et al.* 2013] from loss due to biotransformation. Thus, given that the potential conversion of AFFF-derived PFASs, like FtTAoS, may constitute a significant future source of PFCAs in subsurface environments and that PFCAs have varying toxicities [Lau *et al.* 2007] and mobility [Guelfo *et al.* 2013], understanding the biotransformation potential of PFASs and their expected product formation under environmentally-relevant conditions is a topic of high importance.

Here the aerobic biotransformation of 4:2, 6:2, and 8:2 FtTAoS was investigated in soil microcosms constructed with AFFF-impacted topsoil and amended with an FtTAoS-containing Ansul AFFF formulation. Although 6:2 FtTAoS removal was previously observed in activated sludge [Weiner *et al.* 2013], it is unknown if other FtTAoS congeners are capable of transforming and whether their presence in an AFFF solution affects their transformation. Additionally, in the previous study, the disappearance of 6:2 FtTAoS was observed in autoclaved control cultures, indicating that sorption or abiotic transformation may have constituted a significant portion of the FtTAoS mass loss in the experiments. In this study, the transformation and sorption of three different FtTAoS congeners were examined and differentiated with live, sterile, and soil-free microcosms and quantitative mass balances of amended FtTAoS recovery were enabled using a newly developed assay. Two previously hypothesized biotransformation intermediate compounds were also confirmed in this study. By amending a native soil microbial community with a historically used AFFF formulation, FtTAoS transformation reactions can be described under conditions that represent contaminated soil and groundwater systems, and the fate of other AFFF organic solvents can be assessed.

2.2 Materials and Methods

2.2.1 Chemicals and Standards

Neat AFFF manufactured by Ansul, Inc. with an estimated 2008 manufacture date was obtained from a U.S. military base as previously described [Place *et al.* 2012]. It was stored in the laboratory in a sealed polyethylene tube at room temperature in the dark. The sample was previously confirmed to contain 4:2, 6:2, and 8:2 FtTAoS [Houtz *et al.* 2013], and the concentration of diethylene glycol butyl ether (DGBE) was 220 g/L, which constituted over 80% of the total organic carbon in the sample. A commercial source material containing 6:2 FtTAoS was provided by the Fire Fighting Foam Coalition, which served as a quantitative analytical standard [Backe *et al.* 2013]. C_4-C_{10} perfluorocarboxylates, 4:2 FtS, 6:2 FtS, 8:2 FtS, 5:3 FtCA, 7:3 FtCA, 6:2 FtCA, 8:2 FtCA, 6:2 FtUCA, and 8:2 FtUCA for use as LC/MS-MS analytical

standards were purchased from Wellington Laboratories (Guelph, Ontario, CA). All isotopically-labeled internal standards used for LC/MS-MS analysis were also purchased from Wellington as previously described [Houtz *et al.* 2013, Houtz *et al.* 2012] Oxygen (>99.5%) and DGBE were obtained from Sigma Aldrich (St. Louis, MO), while all other chemicals were purchased from either Fisher Scientific (Waltham, MA) or Sigma Aldrich at the highest purity possible.

2.2.2 Microcosm Set-up and Growth Conditions

The microcosms consisted of 250 mL glass bottles containing 60 mL of a 30 mM bicarbonate-buffered mineral medium [Lee *et al.* 2006], 60 µL of neat Ansul AFFF, and 5 grams of soil collected from a firefighter training area at the Ellsworth Air Force Base, South Dakota, U.S. The medium was modified from that described previously [Lee *et al.* 2006] by preparing aerobically and omitting cysteine sulfide, resazurin, and TES buffer, which is a potential microbial carbon source under oxic conditions. A set of sterile controls was prepared similarly, but with soils that were subjected to three cycles of autoclaving followed by overnight freezing at -20º C, and medium amended with 0.5 g/L sodium azide. Additionally, soil-free medium controls containing only sterile medium, sodium azide, and AFFF were also prepared to assess PFAS sorption as well as potential abiotic reactions. All experiments were performed with triplicate microcosms shaken at 100 rpm and incubated at 30º C for 60 days with samples periodically collected from the headspace or liquid slurry with syringes. Oxygen was measured by injecting 250 µL of microcosm headspace into a Hewlett Packard 5890 GC-TCD equipped with an Alltech HAYESEP Q column (80/100 mesh, 0.85" ID x 6') operated isothermally at 30ºC. Headspace oxygen was maintained between 15 and 25% (v/v) in live microcosms by periodically amending the bottles with pure oxygen using a 0.2 µm-filtered gas-tight syringe and then depressurizing the bottles for several seconds with a sterile needle inserted into the cap valve. The oxygen concentrations in all microcosms are provided in the Supporting Information (SI), Figure A2.1. Two, 60-µL aliquots of AFFF were added to the live microcosms over the course of the experiment: on day 0 and again on day 18 after the 6:2 FtTAoS had been depleted, while only one aliquot of AFFF was added to autoclaved and medium controls (day 0). On day 40 approximately 300 mg/L DGBE was added to live microcosms to provide 175 mg/L of additional organic carbon. Dissolved organic carbon was measured in all bottles by diluting 200 µL of microcosm supernatant (slurry previously centrifuged at 15,000 x g for 10 minutes) in deionized water and analyzing on a Shimadzu TOC-V analyzer. A lack of microbial activity in the autoclaved and medium controls was confirmed when no significant oxygen or organic carbon consumption was observed after 60 days (Figures A2.1 and A2.2).

All microcosms were equilibrated with AFFF for 24 hours prior to collection of the first sample and after addition of AFFF. At each time point, between 0.5 and 1.5 mL of a well-mixed soil and media slurry was removed with a sterile syringe and stored in a 2-mL polypropylene centrifuge tube. Approximately 200 µL of the slurry was immediately diluted in an equal volume of methanol and reserved for LC/MS-MS analysis, while 100 µL was aliquoted into a 7-mL HDPE vial for the total oxidizable precursor assay. The remaining slurry was centrifuged at 15,000 x g for 10 minutes, aliquoted for dissolved organic carbon analysis, and subsequently stored at -20ºC.

2.2.3 LC/MS-MS and HRMS Analyses

The 50:50 methanol:aqueous samples were vortexed for 30 minutes at room temperature and then centrifuged at 15,000 x g for 10 minutes. The supernatant was then amended with 50 µL of an internal standard stock containing 20 to 40 µg/L of mass-labeled internal standards [Houtz *et al.* 2013, Houtz *et al.* 2012] and diluted with HPLC-grade methanol and water to produce 500 µL of a 50:50 methanol:aqueous mix containing a target analyte concentration in the range of the established calibration curves. FtTAoS, FtS, and PFCA compounds were quantified on an Agilent 6410 LC/MS-MS operating in negative electrospray ionization mode as described previously [Houtz *et al.* 2013, Houtz *et al.* 2012], except for the addition of 4:2 FtS. The MS parameters and monitored ion transitions for 4:2 FtS are provided in Table S1. Because a commercial source material was not available for 4:2 and 8:2 FtTAoS, the 6:2 FtTAoS calibration curve was used to estimate their respective concentrations after normalization to the mass-labeled 6:2 FtS internal standard (described in greater detail in the supporting information).

All fluorotelomer carboxylic acid (FtCA) compounds were measured on a Shimadzu Nexera X2 UHPLC /ABSciEX 5500 Triple Quad MS system operating in negative ionization mode. The same 50:50 methanol:sample mix was first spiked with 2 µg/L of the internal standard stock and diluted with methanol and water to yield target analyte concentrations in the range of calibration curves. A gradient solvent program was operated with 2 mM ammonium acetate in water and 2 mM ammonium acetate in methanol and a flow rate of 0.5 mL/min (Table S3). Chromatographic separation was achieved on a Waters ACQUITY UPLC BEH C18 column, 130 Å, 1.7 µm, 2.1 mm x 50 mm following a sample injection of 10 µL. One or two MRM transitions were measured per FtCA compound and were quantified with isotope dilution (Table S2).

To identify biotransformation products of the polyfluorinated compounds in AFFF, the methanol:sample mix containing internal standard stock was analyzed on the Agilent 6410 LC/MS-MS operated in a molecular ion scanning mode (m/z 50 to 1000) in both positive and negative electrospray ionization modes. Prominent molecular ions were identified and their instrumental responses were normalized to the mass labeled-6:2 FtS internal standard response to evaluate their relative production and consumption in the microcosms. The LC/MS-MS product ion mass spectra of two of the prominent apparent biotransformation products were acquired to help elucidate their molecular structures. The compounds were also analyzed by high resolution mass spectrometry (HRMS) to confirm their molecular compositions. HRMS analysis was conducted on live-microcosm samples from day 38 of the incubation that had undergone solid phase extraction (SPE) using Oasis WAX SPE cartridges (6 cm^3, 150 mg, 30 µM; Waters, Milford, MA) with the conditioning and elution methods previously described [Houtz *et al.* 2013]. 50 µL of the extracted sample was directly infused onto a Thermo Scientific Finnigan LTQ FT HRMS operating in negative electrospray ionization over a scan range of 150 to 850, and the exact masses of the ions were confirmed within 5 ppm accuracy.

2.2.4 Total Oxidizable Precursor Assay

To assess the PFAS mass balance in the microcosms before and after biotransformation and to ensure that observed FtTAoS disappearance was not due to physical losses (*e.g.* volatilization), an oxidative precursor assay [Houtz *et al.* 2012] was applied to microcosm samples. Briefly, the oxidative precursor assay employs the reaction of polyfluorinated compounds with hydroxyl radicals to generate corresponding PFCAs of related perfluorinated chain length through the chemical oxidation of the non-fluorinated functional group [Houtz *et al.* 2012]. The samples were exposed to an excess of hydroxyl radicals produced through persulfate thermolysis at pH >12 and the molar quantity of resultant PFCAs was then quantified. For C_6 fluorotelomer compounds, approximately 70-75% (mol/mol) of the compound was recovered as PFCAs, indicating that some of the C_6 telomer compounds were oxidized to C_2 and C_3 PFCA products which were not measured by the LC/MS-MS method used in this study [Houtz *et al.* 2012]. For C_8 fluorotelomers, greater than 90% of the initial mass is recoverable as PFCAs after oxidation [Houtz *et al.* 2012]. For the microcosm samples in this study, the reaction was conducted in 7-mL HDPE vials containing 100 µL of a soil-media slurry, 3 mL HPLC-grade water, and 3 mL of 120 mM potassium persulfate in 0.25 M NaOH. The vials were submerged in an 85ºC water bath for approximately 12 hours. After cooling, the base was neutralized with HCl and amended with 1 mL methanol before LC/MS-MS analysis.

2.3 Results and Discussion

2.3.1 Organic Carbon Disappearance and FtTAoS Biotransformation

The amended AFFF and DGBE constituted the majority of the organic carbon present in live microcosms (Figure A2.2). The dissolved organic carbon disappeared within 3 to 5 days in live microcosms, while no change in concentration was observed in autoclaved or medium controls (Figure A2.2). Correspondingly, the headspace oxygen and carbon dioxide concentrations decreased and increased, respectively, in live microcosms (Figure A2.1), suggesting that the degradation of the AFFF organic solvents, including DGBE, occurred rapidly by the soil microbial community.

Complete disappearance and biotransformation of 4:2, 6:2, and 8:2 FtTAoS occurred in live microcosms when two aliquots of Ansul AFFF were added to the cultures (Figure 2.1). Approximately 45 μM of 6:2 FtTAoS, the most abundant FtTAoS congener, was biotransformed within 45 days (Figure 2.1A) in live microcosms, while concentrations in autoclaved cultures and medium controls remained nearly constant over the incubation period. Because analytical standards are not available for 4:2 and 8:2 FtTAoS, their concentrations were determined semi-quantitatively by calibrating their internal standard-normalized LC/MS-MS responses to the 6:2 FtTAoS calibration curve. Complete disappearance of these compounds was observed in live microcosms, while concentrations in control experiments remained nearly constant (Figures 2.1B and 2.1C). Approximately 80 nM of 8:2 FtTAoS and 10 nM of 4:2 FtTAoS were biotransformed in live microcosms after two AFFF doses (Figure 2.1B). The apparent lack of an observable increase in 4:2 FtTAoS concentrations following the second AFFF amendment (Figure 2.1C) is likely attributable to the very low concentration of the compound.

6:2 FtS was the most abundant biotransformation product detected in live cultures (Figure 2.2A), accounting for 8% of the total mass of FtTAoS biotransformed on day 60. 4:2 and 8:2 FtS were also detected in live cultures; however, together they accounted for less than 1% of total FtTAoS biotransformed (Figure A2.3). The production of 5:3 FtCA and 6:2 fluorotelomer unsaturated carboxylic acid (6:2 FtUCA) was also observed in live microcosms, accounting for approximately 0.5% and 0.18% of total FtTAoS biotransformed, respectively (Figure 2.2B). Some production of 8:2 FtUCA appeared to have occurred in live microcosms relative to autoclaved controls (Figure A2.5). No discernable trend was observed for 7:3 FtCA or 6:2 FtCA concentrations in live microcosms compared to autoclaved controls (Figures A2.5 and A2.6), while no 8:2 FtCA was detected in live or autoclaved microcosms (Figure A2.6). Production of several PFCAs was also observed in live microcosms. PFHxA, PFPeA, and PFBA (Figure 2.2C) accounted for the majority of PFCA products detected (48%, 40%, and 10% of total PFCAs quantified on day 60, respectively), while lesser amounts of PFHpA and PFOA were detected (Figure A2.4). PFCA products accounted for approximately 1.5% of the total FtTAoS biotransformed by day 60, while all produced FtS, FtCA, and PFCA compounds together accounted for just over 10% of total FtTAoS transformed. Biotransformation products were not detected in medium controls.

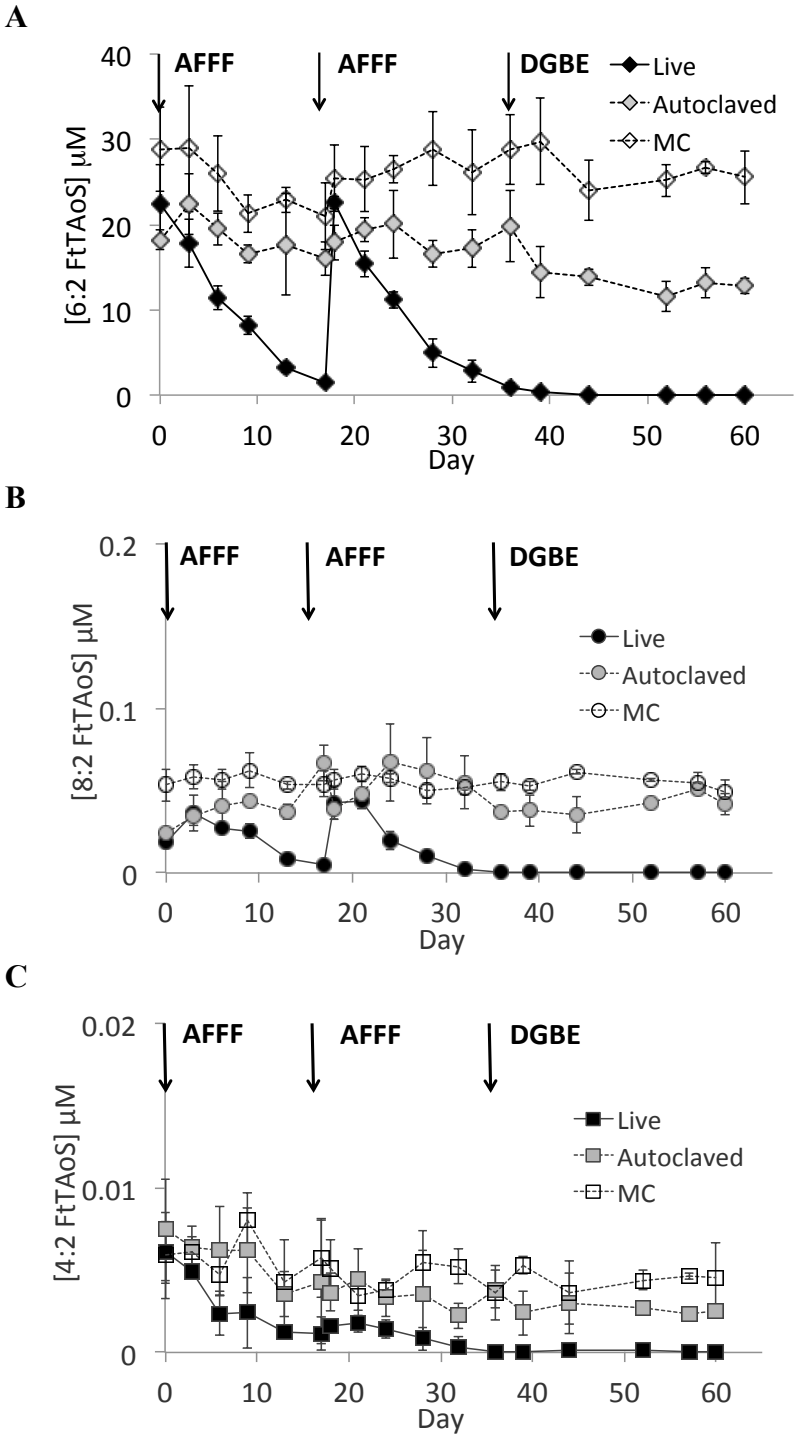


Figure 2.1. Biotransformation of 6:2 FtTAoS (A), 8:2 FtTAoS (B), and 4:2 FtTAoS (C) in live microcosms with two AFFF amendments. Note the y-axis scales in the B and C plots are significantly less than the scale in the A plot. The second AFFF dose and DGBE amendment were made to live microcosms only and not autoclaved or medium-control (MC) experiments. Error bars represent the standard deviation of triplicate experimental bottles.

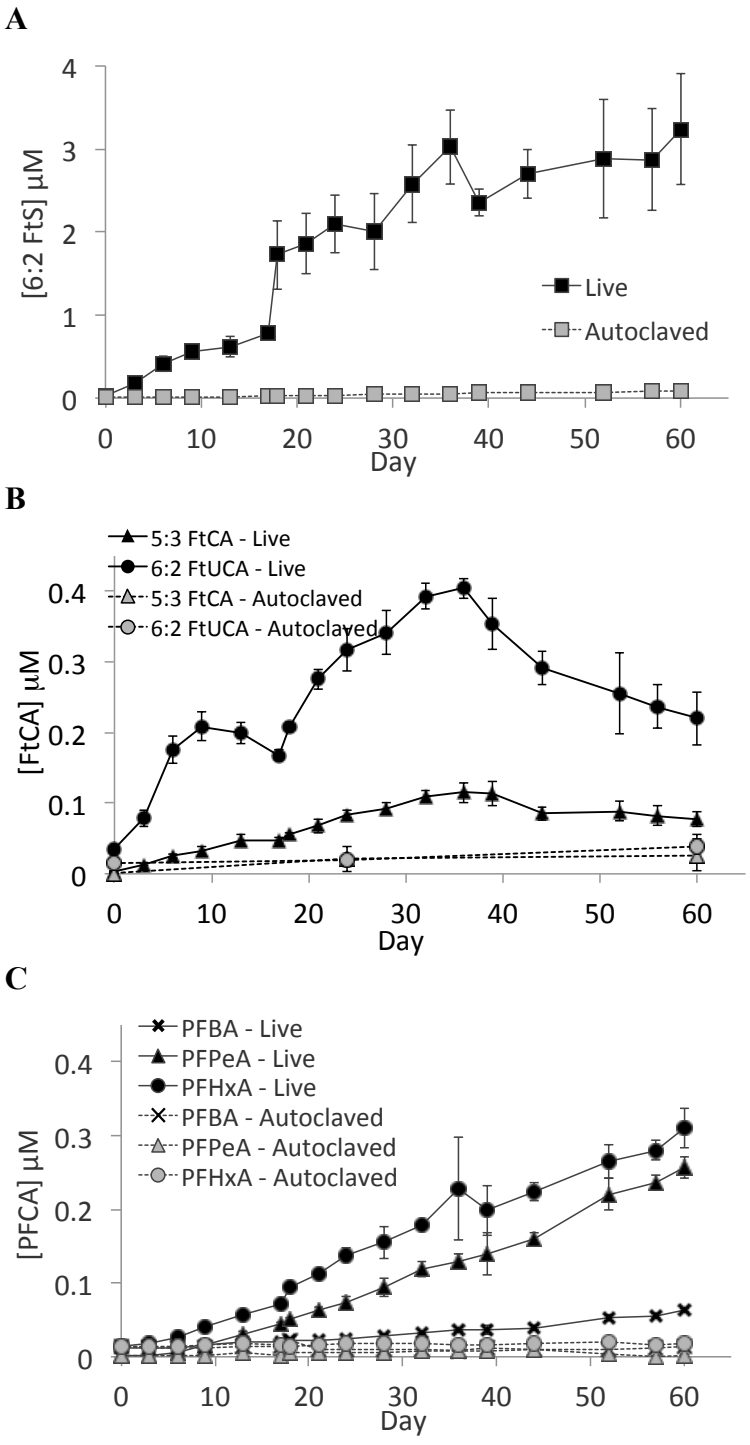


Figure 2.2. Production of major transformation intermediates identified by direct LC/MS-MS measurement: 6:2 FtS (A), 6:2 FtUCA and 5:3 FtCA (B), and PFBA, PFPeA, PFHxA (C). Note the y-axis scales in the B and C plots are one tenth of the scale in the A plot.

2.3.2 Intermediate Product Identification

LC/MS-MS molecular ion scans indicated the presence of four polyfluorinated compounds in the microcosms for which no analytical standards were available: molecular ions 602, 618, 702, and 718. LC/MS-MS retention times and chromatograms for the ions are shown in Table A2.5 and Figures A2.11 and A2.12, respectively. Molecular ion 602 was observed in live, autoclaved, and medium-control microcosms (Figure 2.3A), indicating that it was present in the AFFF formulation. In contrast, molecular ions 618, 702, and 718 were observed only in live microcosms (Figures 2.3B, A2.9, and A2.10, respectively). The analyte response of ion 602 appeared to increase slightly in live microcosms over time and then decrease, consistent with a compound that is both produced and consumed. The response of the ion also increased slightly in autoclaved microcosms but not medium controls, suggesting that some of its production could have occurred through an abiotic reaction involving the solid phase. The instrumental analyte response of m/z 586 (6:2 FtTAoS) and m/z 686 (8:2 FtTAoS) obtained during the same MS analyses are plotted for comparison in Figures A2.7 and A2.8, respectively. If identical instrumental responses are assumed for 586, 602 and 618, approximately 50% of the loss of 6:2 FtTAoS can be explained by the production of 602 and 618. If the same assumption is made for ions 686, 702, and 718, approximately 70% of the loss of 8:2 FtTAoS can be explained by the production of 702 and 718.

The exact masses of 602 and 618 were determined from HRMS and indicated that the molecules had the same atomic composition as 6:2 FtTAoS plus the addition of one (602) or two (618) oxygen atoms (Table A2.5). Key structural features were then proposed based on an LC/MS-MS mass spectrum generated for each molecular ion under a selected ion mode (Figure A2.13). The product ion scans of both 602 and 618 showed fragments corresponding to the entire non-fluorinated part of 6:2 FtTAoS adjacent to the thioether moiety (m/z 205) as well as the terminal sulfonate moiety (m/z 135) contained within m/z 205 (Figure A2.13). Since these two fragments were also observed in the mass spectrum of 6:2 FtTAoS, it is likely that the oxygen additions occurred on the part of the molecule containing the fluorotelomer thioether group. Thus, it is confirmed that the proposed structures for 602 and 618 possess one or two oxygens (respectively) on the thioether functional group of 6:2 FtTAoS. These compounds are designated 6:2 fluorotelomer sulfoxide amido sulfonate (6:2 FtSOAoS) and 6:2 fluorotelomer sulfone amido sulfonate (6:2 $FtSO_2AoS$), and their structures are shown in Figure 2.4. Although exact mass measurements for molecular ions 702 and 718 were not obtained due to their low relative abundance in live microcosm samples, the mass spectra generated from MS product ion scans on 686, 702, and 718 produced mass fragments m/z 205 and 135, suggesting oxygen addition on the 8:2 fluorotelomer thioether moiety. Molecular ions 702 and 718 were designated 8:2 FtSOAoS and 8:2 $FtSO_2AoS$, respectively (Figures A2.9 and A2.10). Molecular ions 502 and 518, which would correspond to the singly and doubly oxygenated species of 4:2 FtTAoS (4:2 FtSOAoS and 4:2 $FtSO_2AoS$, respectively), were not detected in these microcosms with either LC/MS-MS molecular ion scans or HRMS. This is likely due to the low initial abundance of 4:2 FtTAoS in the AFFF.

Although Weiner *et al.* 2013 proposed the occurrence of a sulfoxide and sulfone species as potential biotransformation products of 6:2 FtTAoS, it was not definitely determined whether 6:2 FtSOAoS was a biotransformation product as no production of the compound was observed in live microcosms. 6:2 $FtSO_2AoS$ was also not detected in any culture. Here, their exact molecular masses were determined, evidence supporting their proposed molecular structures is provided, and time course trends confirming their role as FtTAoS biotransformation products were constructed. The identification of these species describes the mechanism through which multiple congeners of FtTAoS may aerobically biotransform to FtS compounds.

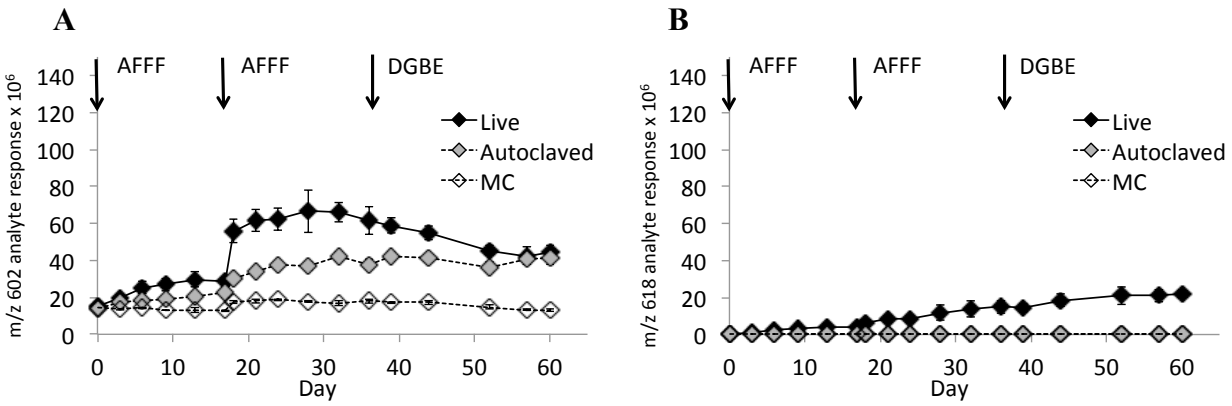


Figure 2.3. The average LC/MS-MS analyte response of molecular ions 602 (A) and 618 (B) normalized to the mass labeled-6:2 FtS internal standard in live, autoclaved, and medium control (MC) microcosms. The second AFFF and DGBE amendment were given to live microcosms only. Error bars represent the standard deviation of triplicate microcosms.

2.3.3 FtTAoS Biotransformation Pathways

The proposed biotransformation pathways for 4:2, 6:2, and 8:2 FtTAoS (Figure 2.4) were based on quantification of 4:2, 6:2, and 8:2 FtS, 5:3 FtCA, 6:2 and 8:2 FtUCA, and C_4-C_8 PFCAs with certified analytical standards, as well as the detection of intermediate compounds identified by HRMS and molecular fragmentation patterns. Because more 6:2 FtSOAoS was produced in live microcosms than in autoclaved controls in the 20 days following the first AFFF amendment, it is likely that this reaction was attributable to both microbial activity and an abiotic reaction. Thus, the first two steps in the biotransformation reactions of each FtTAoS compound are confirmed to be sequential oxygen additions on the thioether group to form FtSOAoS and $FtSO_2AoS$, which are then followed by the formation of FtS through a third oxygen addition and cleavage of a carbon-carbon bond, resulting in a fluorotelomer sulfonate and an alkyl amidosulfonate group. The oxidation of 6:2 FtS to 6:2 FtUCA, 5:3 FtCA, PFHxA and PFPeA in aerobic microcosms inoculated with activated sludge has been reported previously [Wang *et al.* 2011, Weiner *et al.* 2013]. In this study, the production of PFHpA, PFOA, and 8:2 FtUCA (Figures A2.4 and A2.5), which were the likely products of 8:2 FtS biotransformation (Figure A2.3), were consistent with those observations and formed an analogous oxidation pathway for

8:2 FtTAoS (Figure A2.15). Production of PFBA and some 4:2 FtS was also observed in live microcosms (Figures 2.1D and A2.3, respectively). Although PFBA is expected to be a terminal oxidation product of 4:2 FtS, its concentration in live microcosms at the end of the 60 day incubation period (0.5 μM) was much greater than the total estimated 4:2 FtTAoS biotransformed (<0.01 μM), suggesting that it also was a biotransformation product of 6:2 FtS produced from 6:2 FtTAoS.

Cleavage of the carbon-carbon bonds during the oxidation of fluorotelomer sulfonates and alcohols has been observed in previous studies. For example, Wang *et al.* 2011 detected PFBA formation (accounting for approximately 0.14% mol/mol) when 6:2 FtS was aerobically oxidized, while production of PFBA was also observed when 6:2 FtOH was biotransformed under aerobic conditions [Liu *et al.* 2010, Kim *et al.* 2012, Kim *et al.* 2014]. In this study, PFBA accounted for approximately 0.1% (mol/mol) of the total amended 6:2 FtTAoS at the end of the incubation. It is possible that 8:2 FtS biotransformation also produced the C_{n-2} PFCA product, PFHxA; aerobic 8:2 FtOH biotransformation to PFHxA has been previously observed [Kim *et al.* 2012]. Although approximately 0.3 μM PFHxA was formed, it was likely that the majority of it was produced from 6:2 FtS oxidation as the concentration of 8:2 FtTAoS in the AFFF in this study is much lower than that of 6:2 FtTAoS. For both 6:2 and 8:2 FtTAoS biotransformation in these microcosms, the terminal C_n and C_{n-1} PFCA products (PFHxA/PFPeA and PFOA/PFHpA, respectively) were produced in approximately equimolar quantities. Similar findings were reported for the biotransformation of 6:2 FtS and 6:2 FtOH [Liu *et al.* 2010, Wang *et al.* 2011].

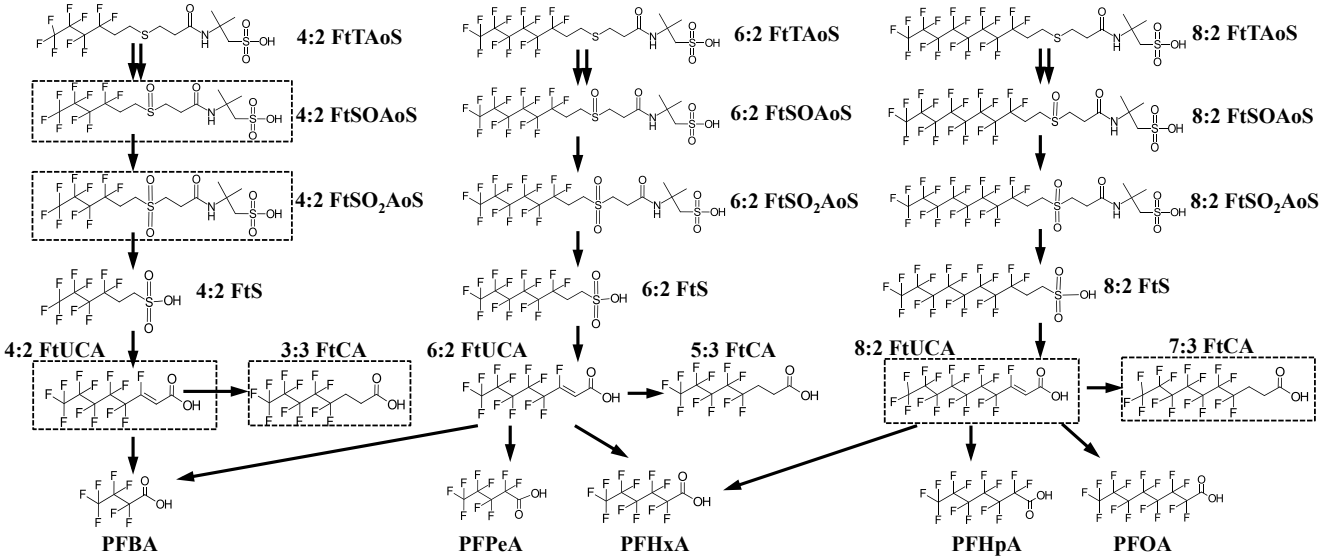


Figure 2.4. Proposed biotransformation pathways of 4:2, 6:2, and 8:2 FtTAoS by aerobic soil microcosms. Compounds in dashed boxes are proposed biotransformation intermediates and were not directly detected in microcosms. The double arrow indicates that the reaction occurs both biologically and abiotically.

Generally, the rate of FtTAoS disappearance observed in this study was faster than the rate of transformation of intermediate compounds, indicating that the rate-limiting step in PFCA production likely occurs after FtSOAoS production. It is unknown whether the observed FtTAoS biotransformation occurs metabolically or cometabolically by the soil microbial community in these microcosms. Three aliquots each of over 100 mg/L of organic carbon were supplied to the microcosms in the two AFFF amendments and the one DGBE amendment. The labile organic compounds were each mineralized within three days, as evidenced by the low organic carbon concentration measured in the live microcosms (Figure A2.1). Biotransformation of FtTAoS and production of FtS and PFCA products continued to occur after the labile organic carbon compounds disappeared, and no significant differences in PFAS transformation rates were observed in the presence of higher concentrations of labile organic carbon. Even after all of the FtTAoS had been biotransformed on day 40, when DGBE was added to the live microcosms, no significant change in the production rate of FtS or PFCA products was observed (Figure 2.1). Because the presence or absence of labile carbon did not appear to have a significant impact on PFCA production, it is likely some factor other than labile organic carbon controlled the rate of FtTAoS biotransformation.

2.3.4 Precursor Oxidation Assay and Mass Balance

The FtS and PFCA biotransformation products that could be quantified directly by LC/MS-MS (4:2, 6:2, 8:2 FtS, 6:2 FtUCA, 5:3 FtCA, and C_4-C_8 PFCAs) accounted for approximately 10% of the total FtTAoS transformed. The PFCA products alone accounted for approximately 1.5% of the FtTAoS transformed, a recovery within the range of those observed in other aerobic FtS and FtOH biotransformation studies (0.3-10% mol/mol) [Dinglasan *et al.* 2004, Wang *et al.* 2005, Liu *et al.* 2010, Wang *et al.* 2011, Kim *et al.* 2012, Kim *et al.* 2014].

Because a total mass recovery of FtTAoS was not observed, the total oxidizable precursor assay was employed on microcosm samples to check the material balance. Seven samples from live microcosms that were taken before, during, and after biotransformation of the two aliquots of FtTAoS were subjected to the assay. Of the 22 μM of 6:2 FtTAoS that disappeared during the first 20 days of incubation in live microcosms, the precursor assay recovered approximately 90% of the compound as C_4-C_8 PFCA products (Figure 2.5). After the second AFFF addition, the recovery ranged from 80-100%. Use of the precursor assay performed on samples from autoclaved and medium control microcosms yielded similar results (Figures A2.16 and A2.17). As described by Houtz *et al.* 2012, the medium control samples (Figure A2.17) also confirm that approximately 75% of a fluorotelomer PFAA precursor is recoverable in PFCA products using this assay. The incomplete recovery of the precursor in some samples may be due to the production of PFCAs with fewer than four carbons that are not quantified by this LC/MS-MS method [Houtz *et al.* 2012, Houtz *et al.* 2013]. Additionally, the concentration distribution of PFCA congeners (C_4:C_5:C_6) produced from the precursor assay is consistent with products of C_6 fluorotelomer precursor oxidation [Houtz *et al.* 2012], suggesting that 6:2 FtTAoS and its related C_6 transformation products were the primary oxidizable PFASs in these microcosms. Although the generation of PFNA can occur from the oxidation of an 8:2 fluorotelomer, it was not detected in this study, likely due to the low initial concentrations of 8:2 FtTAoS.

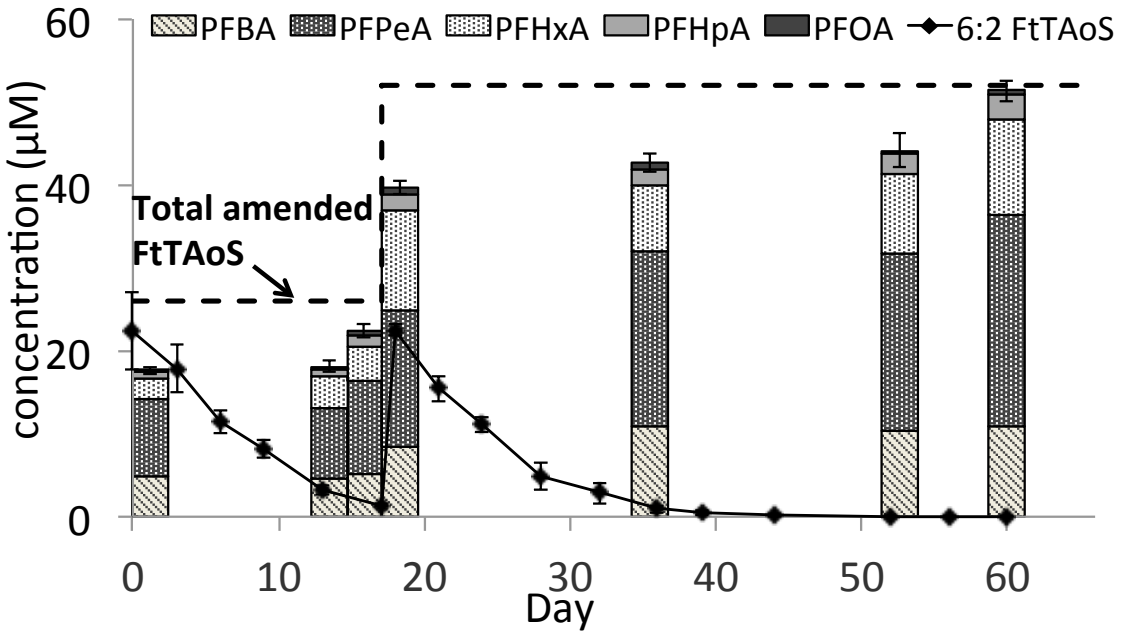


Figure 2.5. Concentrations of 6:2 FtTAoS and PFCA products generated in live microcosm samples after oxidation in the PFAA precursor assay. The dotted line represents the total amended FtTAoS and was obtained by summing the 4:2, 6:2, and 8:2 average FtTAoS concentrations measured in the medium controls.

Although the majority of the total amended FtTAoS was not accounted for as intermediates and terminal products, the precursor assay provided insight into the PFAS mass balance. The nearly complete recovery of amended FtTAoS as oxidized PFAA precursors before, during, and after biotransformation confirmed that all unidentified transformation compounds remained in the soil-media slurry. If cleavage of the fluoroalkyl tail to less than a C_4 perfluorinated chain or complete defluorination had occurred, the assay would have yielded lower recoveries. Although Weiner *et al.* 2013 identified 6:2 FtOH as a potential transformation product of 6:2 FtTAoS, the nearly complete recovery of PFAA precursors suggested that this pathway was not significant in these microcosms.

2.4 Environmental Implications

The results of this study may help to explain the occurrence of FtS and PFCA compounds in groundwater and soil at sites where AFFF was released [Moody *et al.* 2002, Awad *et al.* 2011, Moody *et al.* 2003, Schultz *et al.* 2004, Houtz *et al.* 2013, Backe *et al.* 2013]. As FtTAoS is present in AFFF from several manufacturers, its biotransformation to FtS and PFCAs under conditions representative of soil, groundwater, and surface water is important for understanding the present occurrence and potential PFCA generation in the environment. Although FtTAoS is biotransformed over a period of weeks, its conversion to PFCAs appears to be slow. Other intermediate biotransformation products (*e.g.* FtS and FtUCA) may constitute a significant source of PFASs in FtTAoS-contaminated environments. Furthermore, measuring only PFCAs and FtS in environmental samples may not completely characterize the potential for long-term release of PFCAs. The total oxidizable precursor assay can be used to obtain a more precise estimate of the concentration of intermediate products that are not quantifiable by direct analysis. Two of the identified intermediate compounds, 6:2 FtSOAoS and 6:2 $FtSO_2AoS$, appear to have slow transformation rates relative to 6:2 FtTAoS, indicating they may also occur in subsurface environments.

CHAPTER 3: Effects of perfluoroalkyl acids (PFAAs) on TCE dechlorination by a *Dehalococcoides* enrichment culture

*A modified version of this text is part of a prepared manuscript: Weathers, T.S.; Harding, K.C.; Alvarez-Cohen, L.; Higgins, C.P.; Sharp, J.O. "Perfluoroalkyl acids and soil microbes: potential impacts on biofilm formation and co-contaminant transformation" *August 2014.*

3.1 Introduction

Poly and perfluoroalkyl substances (PFASs) are emerging contaminants that are widespread in many facets of the environment: surface waters, soil, air, and groundwaters [Hansen *et al.* 2002]. Advances in detection and quantification have led to growing concerns of exposure. Within the broader class of PFASs, a subset of perfluoroalkyl acids (PFAAs) are environmentally persistent, toxic, and can bioaccumulate [Van de Vijver *et al.* 2003, Moody *et al.* 2002, Higgins *et al.* 2007, Blaine *et al.* 2014]. The compounds have been detected in human kidneys, livers, and blood serum [Van de Vijver *et al.* 2003, Key *et al.* 1997, Kuklenyik *et al.* 2004]. To mitigate exposure, the U.S. Environmental Protection Agency (EPA) has set provisional drinking water health advisories for two common PFAAs: 0.4 µg/L for perfluorooctanoate (PFOA) and 0.2 µg/L for perfluorooctanesulfonate (PFOS) [U.S. EPA 2012].

PFAAs have fully fluorinated carbon chains of varying lengths; molecules with a four carbon chain up to a thirteen carbon chain have been manufactured in two common classes: acids and sulfonates [Prevedouros *et al.* 2006]. These chemicals possess distinctive properties; they have hydrophobic and oleophobic tails, and tend to form micelles [La Mesa *et al.* 1987]. Due to these unique aspects, PFAAs are used in many industrial applications, such as non-stick coatings, fire retardants, and pesticides [Prevedouros *et al.* 2006]. They have also been a component of legacy aqueous film-forming foams (AFFF) used for fire suppression [Prevedouros *et al.* 2006, Moody and Field 1999, Moody and Field 2000, Moody *et al.* 2003]. Extensive use of AFFF for military firefighter training has lead to the significant introduction of PFAAs into groundwater in sites that are often contaminated with other chemicals, such as hydrocarbons or chlorinated solvents [Moody and Field 1999, Moody and Field 2000].

The biodegradation of hydrocarbons commonly found at AFFF-impacted sites (*i.e.*, BTEX: benzene, toluene, ethylbenzene, and xylene), has been extensively documented [Deeb *et al.* 2002, Patterson *et al.* 1993]. Chlorinated solvents, such as trichloroethene (TCE) and its toxic daughter products *cis*-dichloroethene (cDCE) and vinyl chloride (VC), are also common at AFFF-impacted sites [Moody and Field 1999, Moody and Field 2000, Ballapragada *et al.* 1997]. Biological reductive dechlorination (also known as enhanced reductive dechlorination, or ERD) is a bioremediation process in which an electron donor, such as lactate, is applied to generate a reducing environment and stimulate the activities of reductive dechlorinating microbial communites. Microorganisms of the genus *Dehalococcoides* (Dhc) are known to completely degrade TCE to ethene, and are therefore targeted members of these dechlorinating communities [Holmes *et al.* 2006, Maymó-Gatell *et al.* 1997, Cupples *et al.* 2003]. Dechlorinating enrichment cultures containing strains of *Dehalococcoides mccartyi* and capable of dechlorinating TCE to ethene have been developed as bioaugmentation cultures for addition to sites with insufficient indigenous *Dehalococcoides* strains [Ballapragada *et al.* 1997, Freedman *et al.* 1989, Men *et al.* 2013].

To date, it remains unclear to what extent PFAAs may impact microbes relevant to hydrocarbon and chlorinated solvent bioremediation. While biotransformation of PFAAs is not expected [Key *et al.* 1997], concerns regarding potential adverse effects from PFAA exposure on subsurface microbial populations and subsequent co-contaminant degradability have been expressed [Moody and Field 1999]. Unexplored microbe-PFAA interactions may lead to unintended impacts on PFAA, hydrocarbon, or chlorinated solvent levels in water sources. This study sought to examine the effects of PFAAs on *Dehalococcoides* organisms with and without the presence of soil.

3.2 Materials and Methods

3.2.1 PFAA Preparation and Aqueous Analysis

Purity-corrected stock solutions of an eleven-analyte mixture were made using salts from Sigma-Aldrich in a 70/30 v/v methanol/aqueous solution as discussed in Sepulvado and Higgins 2013. The components in this mixture were: perfluorobutanoate (PFBA), perfluoropentanoate (PFPeA), perfluorohexanoate (PFHxA), perfluoroheptanoate (PFHpA), PFOA, perfluorononanoate (PFNA), perfluorodecanoate (PFDA), perfluoroundecanoate (PFUnA), perfluorobutanesulfonate (PFBS), perfluorohexanesulfonate (PFHxS), and PFOS (Table S1). This mixture was used in the following experiments unless otherwise noted. To spike each experiment with the PFAA mixture, the appropriate volume of PFAAs in 70/30 (v/v) methanol/aqueous solution was added to every experimental vessel to achieve the target aqueous concentrations. The vessels were left unsealed overnight or until the methanol from the spiking solution had evaporated to limit residual methanol that could be utilized as a carbon source for the microbial populations. Initial control experiments indicated insignificant losses of PFAAs from this evaporation step, even at 1 and 0.1 mg/L (Figure A3.1). For PFAA analysis of aqueous suspensions, samples were centrifuged, sampled, and diluted as appropriate following the methods in Sepulvado and Higgins 2013 and measured via liquid chromatography tandem mass spectroscopy (LC/MS-MS) utilizing stable-isotope surrogate standards purchased from Wellington Laboratories. An Applied Biosystems 3200 mass spectrometer (MDS Sciex) was utilized with Analyst for quantitation. Control experiments were performed to ensure that PFAA losses due to experimental setup (*i.e.* evaporation procedure) were quantifiable and repeatable (Figure A3.1).

3.2.2 Reductive Dechlorination in the Presence of Soil

By simplifying batch systems to contain only one dechlorinating species, PFAA effects on metabolism (such as degradation, enzymatic induction, growth) can be clearly attributed to a single type of organism without concern for competition or mixed population dynamics. To extrapolate to increasingly environmentally relevant, anaerobic systems, reductive dechlorination by a mixed community in the presence of soil was assessed for deviations in chlorinated solvent degradation upon exposure to PFAAs. Batch systems containing TCE, lactate, soil, and a PFAA mixture were designed to evaluate the degradation rates of a *Dehalococcoides*-containing methanogenic enrichment culture. Reductive dechlorination experiments contained quadruplicate sets of 50 μg/L of each PFAA (11 analytes) added as described above to sterile 60 mL serum bottles. Following PFAA addition and methanol evaporation, 5 g of a classified air-dried loam (f_{oc} = 4.5% described in Guelfo and Higgins 2013) was added to each bottle for a soil to liquid

ratio of 0.1 g/mL. The PFAA and soil preparation was performed in an anaerobic chamber (90% nitrogen, 5% hydrogen, 5% carbon dioxide) to reduce losses of PFAAs and ensure anaerobic conditions. Next, 48.5 mL of an autoclaved mineral salts medium [Men *et al.* 2013] containing 20 mM lactate as electron donor and 100 µg/L vitamin B_{12} was added to stoppered bottles with a sterile syringe. During media addition, an exhaust needle was inserted into the stopper to avoid bottle pressurization. After media addition, the headspace was gently flushed with N_2/CO_2 (90:10) to remove residual hydrogen and oxygen. Each bottle was then amended with approximately 17 µmoles of TCE (Sigma Aldrich, 99.9%) and allowed to sit for at least 24 hours to facilitate chloroethene and PFAA equilibration. At time zero, the bottles were inoculated with 3% (v/v) of the previously-grown methanogenic mixed community stock culture [Men *et al.* 2013] and incubated in the dark at 34ºC for the duration of the experiment. All bottles were inverted several times at each sampling point to promote PFAA mixing. Two control sets were also prepared using the method above: one set was not amended with the PFAA stock solution (-PFAA) and one was not amended with TCE (-TCE). Additionally, control bottles were established by pasteurizing un-inoculated bottles containing growth media without lactate or vitamin B_{12} in a heated water bath until the soil slurry reached approximately 80ºC. The water bath pasteurization was conducted prior to TCE amendment.

3.2.3 Reductive Dechlorination without Soil

To evaluate growth effects of high concentrations of PFAAs on the dechlorinating community, batch bottles containing 1 mg/L, 10 mg/L of each PFAA (11 analytes), and a non-PFAA containing control were performed in triplicate without soil. Samples were prepared and inoculated as described previously for the dechlorinating microbial community. TCE, cDCE, VC, ethene, methane, and H_2 were measured as before as a proxy for growth. All bottles were incubated for approximately 1 week at 34ºC with periodic mixing. Once near-complete TCE degradation occurred in the control and 1 mg/L PFAA conditions, a second dose of 17 µmoles of TCE was added. An additional 0.2 mM lactate was amended to the control, 1 mg/L and 10 mg/L PFAA bottles. The TCE and lactate were added to promote further biomass production. The cultures were incubated for several hours before being shipped overnight from UC Berkeley to Colorado School of Mines in an insulated container. Upon receipt, samples were incubated at room temperature for one week to maximize growth. To determine whether flocculation may have occurred at a microscopic scale, each bottle was destructively sampled for EPS analysis after a final optical density and ATP measurement. TCE dechlorination rates were determined by obtaining the slope of a time-course linear regression through days four through six.

3.2.4 Chloroethene, Hydrogen, and Methane Measurement

Chloroethenes, ethene, and methane were measured by injecting 100 µL of culture headspace into an Agilent 7890A gas chromatograph equipped with a flame ionization detector (GC-FID) and 30 m x 0.32 mm J&W capillary column (Agilent Technologies). Hydrogen concentrations were measured by injecting diluted headspace samples into a gas chromatograph fitted with a reductive gas detector (RGD) (Trace Analytical) [Men *et al.* 2013]. Between 50 and 300 µL of culture headspace was withdrawn for each hydrogen measurement and diluted in 17 mL glass vials purged with N_2 to generate concentrations within the linear calibration range of the instrument. The total volume of extracted headspace was tracked throughout the incubation to ensure that the same approximate volume was removed from all bottles (1.5 to 1.9 mL).

To normalize dechlorination rates, Dhc cell numbers were quantified by 16S rRNA genes from extracted DNA. Cells were collected by centrifuging 1.5 mL of culture slurry at 15,000 x g for 10 minutes. Total DNA was extracted using the PowerSoil DNA Isolation Kit (MO BIO Laboratories) according to the manufacturer's instructions. Dhc 16S rRNA genes were quantified using qPCR with the protocol, primers, and standards described in Men *et al.* 2013, and it was assumed that each Dhc cell carries one 16S rRNA gene. Dhc cell counts were quantified in both the stock inocula culture at time zero and duplicate samples extracted from one culture bottle in each experimental set on day 12.

3.2.5 Cellular Preparation, Harvest, and Protein Measurements

To quantify cellular protein, aliquots of 0.5 or 1 mL were centrifuged at 15,000 x g for 3 minutes, decanted, and stored at -80°C until analysis. Cellular protein was quantified using the colorimetric Coomassie Plus Assay Reagent (Bradford) with bovine serum albumin (BSA) as the standard (Thermo Scientific) as described previously [Sharp *et al.* 2007]. Measurement of adenosine triphosphate (ATP) as an indicator for microbial activity was performed [as in Eydal and Pedersen 2007] using ATP biomass kit HS for total ATP in living cells (Biothema AB). Samples were diluted up to ten-fold during extraction to ensure measurement within the standard range.

Optical density was measured using a Jenway 6505 UV/Vis Spectrophotometer. EPS production was quantified by using a modified anthrone reagent (Fisher Scientific) total sugar and polysaccharide assay with glucose (Sigma Aldrich) as a standard [Morris 1948]. Prior to polysaccharide analysis, samples were centrifuged at 15,000 x g for 3 minutes. The supernatant was decanted and stored at 4°C. The EPS was harvested by adapting two methods that both account for loosely and tightly bound EPS: a NaCl Method [Aguilera and Souza-Egipsy 2008] and an EDTA Method [Zhang *et al.* 1999]. Both methods resulted in an agreement of trends, however the NaCl Method will be discussed herein. The NaCl Method largely follows Aguilera and Souza-Egipsy 2008, with the following exceptions: initially, a cellular aliquot was weighed and transferred to a 15 mL polypropylene conical tube with 5 mL of MilliQ^{TM} water per 1.5 g cells, and only NaCl was used for the chemical extraction.

3.3 Results and Discussion

3.3.1 Effect of PFAAs on Reductive Dechlorination in the Presence of Soil.

Batch bottles containing a loamy soil, TCE, and 50 µg/L of each PFAA analyte (550 µg/L total) were amended with lactate and a dechlorinating culture to determine the effects of low concentration (*i.e.* environmentally relevant) PFAAs on chlorinated solvent degradation rates. Soil was added to experimental bottles to validate the ability of the enrichment culture to degrade TCE in the presence of PFAAs in a simulated aquifer system. The soil also provided sorption sites for PFAAs, resulting in slightly lower aqueous PFAA concentrations than the 50 µg/L target amendment concentration. Concentration-specific soil/aqueous distribution coefficients (K_D) were calculated for each PFAA in the bottles and then normalized to the soil's f_{oc} (4.5% as described in Guelfo and Higgins 2013) to obtain a K_{oc} (Figure 3.1). The plotted K_{oc} values represent the average obtained from biological quadruplicates. The mass balance-approximated K_{oc} values for target PFAAs are within the range of those reported previously [Higgins and Luthy 2006, Guelfo and Higgins 2013], including the anomalous higher sorption tendencies of the shorter-chained perfluorocarboxylic acids (C_4-C_7 PFCAs and C_4 PFSA) which do not follow the chain-length dependent trend of the longer-chained PFAAs. PFAA sorption does not appear to be significantly impacted by either the presence of TCE or the inoculated microbial community. Although Guelfo and Higgins 2013 found that the presence of NAPL generally increased the sorption of C_4-C_6 PFCAs to soil, the aqueous concentrations of TCE in the these experiments (approximately 340 µM) was well below solubility, and therefore may not have been high enough to significantly impact PFAA partitioning.

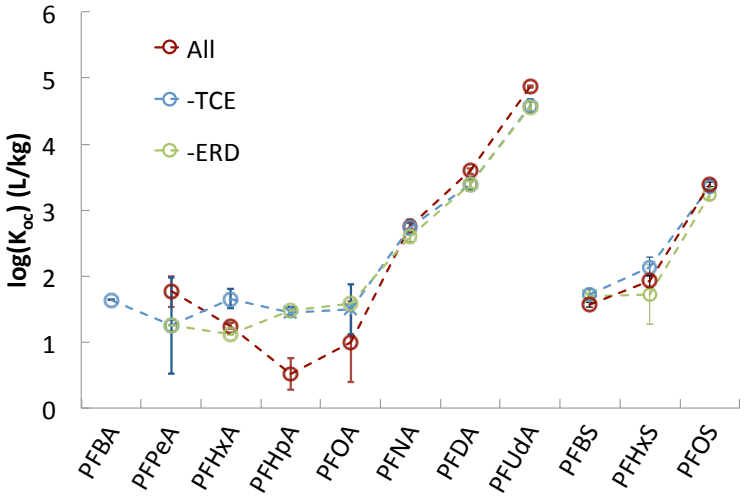


Figure 3.1. Mass balance approximated K_{oc} values for each PFAA (50 µg/L of each) in the cultures. "All" indicates experimental bottles that contain soil, media, TCE, and the microbial community, while "-TCE" and "-ERD" indicate conditions without TCE and the microbial community ("enhanced reductive dechlorination" community), respectively.

In the experimental bottles constructed with soil, biodegradation of TCE was observed in all biotic systems, while no significant dechlorination occurred in uninoculated bottles containing pasteurized soil (Figure 3.2). No significant difference in TCE degradation was observed between cultures with and without PFAAs (Figure 3.2). The initial TCE dechlorination rates were 17.2+-1.6 and 17.4+-1.7 μM TCE/day/10^9 Dhc cells for bottles with PFAAs and without, respectively. The presence of 50 μg/L of each PFAA did not significantly affect TCE degradation, or cDCE and vinyl chloride production (Figures 3.3A and 3.3B, respectively). Complete dechlorination of TCE occurred in both live cultures by day seven. No significant ethene production was observed in these cultures by day seven, as expected. The Dhc strain in this community harbors the *tce*A RDase gene and only slowly, co-metabolically dechlorinates VC to ethene [Men *et al.* 2013].

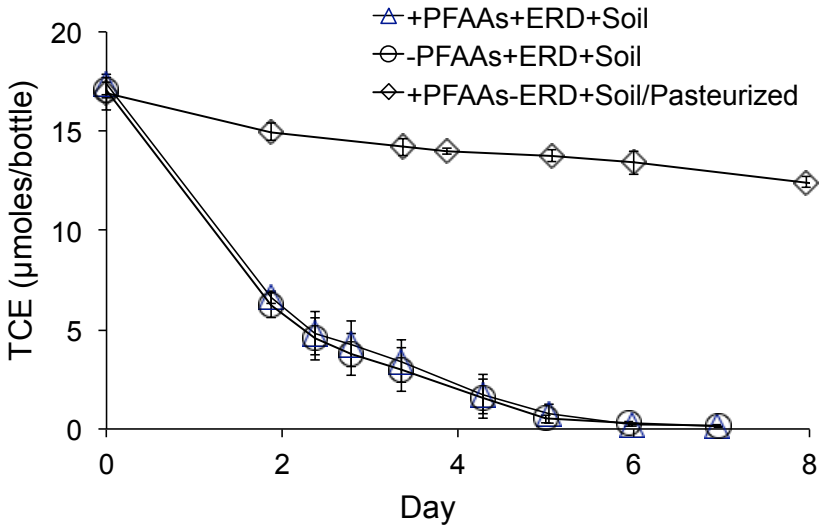


Figure 3.2. TCE concentrations in soil-containing bottles with and without PFAAs (50 μg/L each).

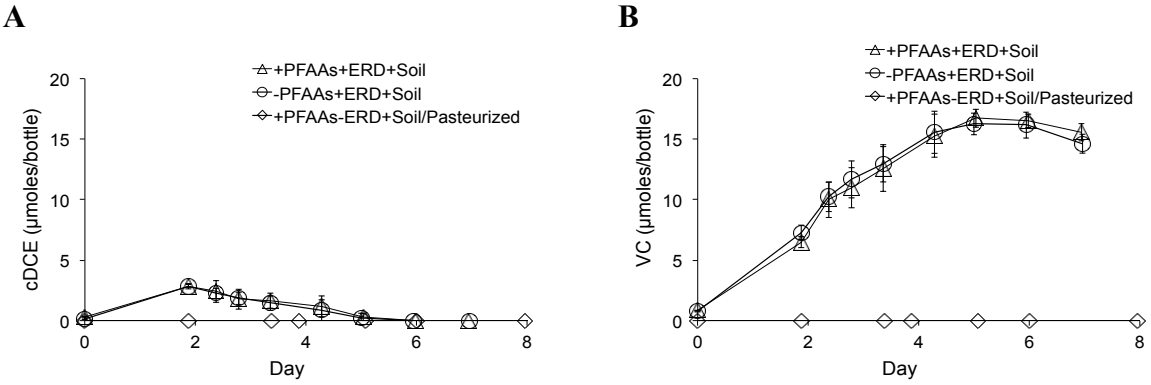


Figure 3.3. Concentrations of cDCE (A) and vinyl chloride (B) in soil-containing cultures with and without PFAAs (50 μg/L each).

3.3.2 Effect of PFAAs on Reductive Dechlorination in Soil-Free Cultures.

In soil-free cultures amended with higher concentrations of PFAAs (1 and 10 mg/L of each PFAA), TCE dechlorination did not occur in the presence of 10 mg/L each PFAA; however it was unaffected by the presence of 1 mg/L of each PFAA (Figure 3.4). No cDCE or VC was generated in the 10 mg/L condition, while no difference in the production of these products was observed in the control and 1 mg/L condition (Figure 3.5 A and B). Similarly to the soil-containing cultures, no ethene production was observed by day 8 in any of the cultures.

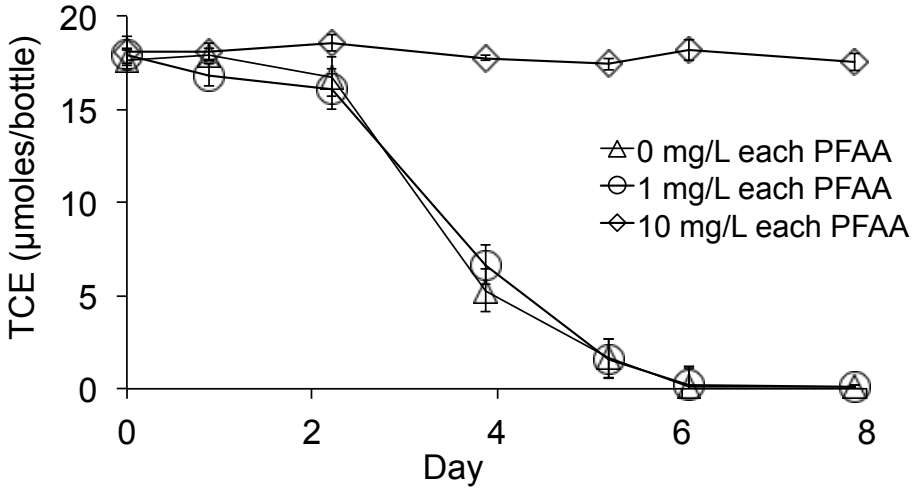


Figure 3.4. TCE concentrations in soil-free dechlorinating cultures with 1 and 10 mg/L of each PFAA as well as a 0 mg/L PFAA control culture.

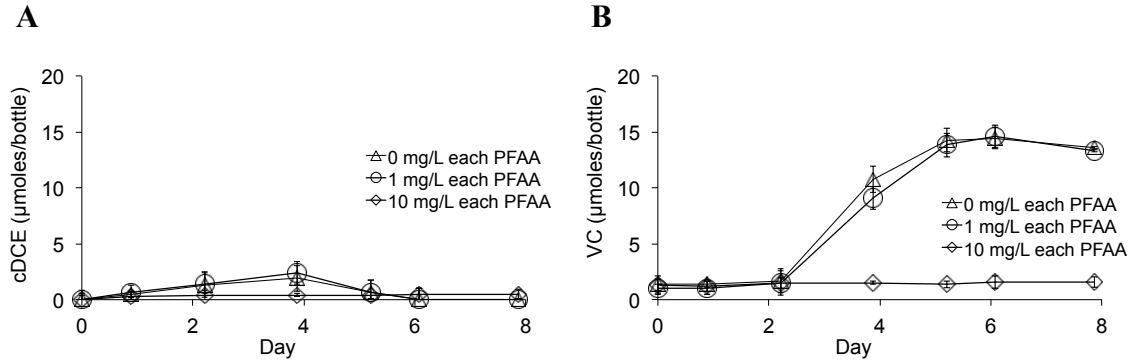


Figure 3.5. Concentrations of cDCE (A) and vinyl chloride (B) in soil-free dechlorinating cultures with 1 and 10 mg/L of each PFAA as well as a 0 mg/L PFAA control culture.

3.3.3 Effect of PFAAs on Enrichment Culture Growth.

Optical density (OD_{600}) measurements taken in the soil-free dechlorinating community at the conclusion of the experiment indicate that overall microbial growth occurs in the presence of high concentrations of PFAAs (10 mg/L each) relative to the non-amended control (Figure 3.6), even though dechlorination did not occur and presumably Dhc growth was inhibited. This finding is confirmed by slight increases in methane production observed in the 10 mg/L-amended condition (Figure A3.2). It is possible that Dhc inhibition allowed methanogenic organisms to compete for and utilize the available hydrogen, and that their growth was reflected in the increased OD measurements in the 10mg/L condition (Figure 3.6). It is unclear why the ATP appears to decrease in the bottles that show greater OD measurements (Figure 3.6). ATP can generally be used as an indicator of the active microbial biomass as it is usually not conserved in non-viable cells. Hence, it is expected that increasing ATP concentrations would be correlated to increasing biomass. It is possible that the presence of higher concentrations of PFAAs selected for the growth of organisms that produce lower ATP concentrations per cells. It could also be possible that while more rapid microbial growth occurred initially in the 10 mg/L condition, the higher concentration of PFAAs caused more members of the community to enter into a late-exponential or stationary growth phase by the time the ATP was extracted at the end of the experiment.

EPS measurements obtained from the soil-free cultures (Figure 3.7) indicate that no significant differences in polymeric substance production were observed according to the PFAA concentration. This suggests that the cultures had no bias towards flocculation or biofilm production in the presence of PFAAs. This is in contrast to results previously obtained from an aerobic hydrocarbon-degrading pure culture. The cellular growth of *Rhodococcus jostii* RHA1 changed in the presence of higher concentrations of PFAAs (10 mg/L each of 11 analytes), with increased EPS production facilitating the formation of large microbial flocs during the degradation of toluene, but resulting in no difference in toluene degradation rates (data not shown). It is hypothesized that the flocculation might have offered protection to RHA1 in the presence of the PFAAs, facilitating the continued degradation of toluene.

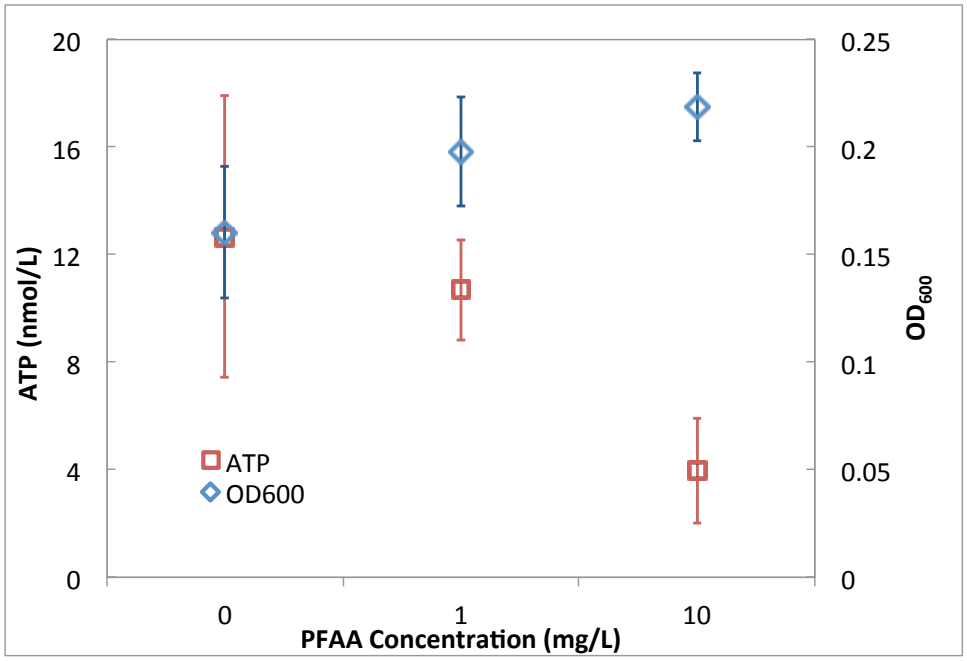


Figure 3.6. Optical density and ATP measurements in the soil-free dechlorinating cultures amended with 0, 1, and 10 mg/L of each PFAA.

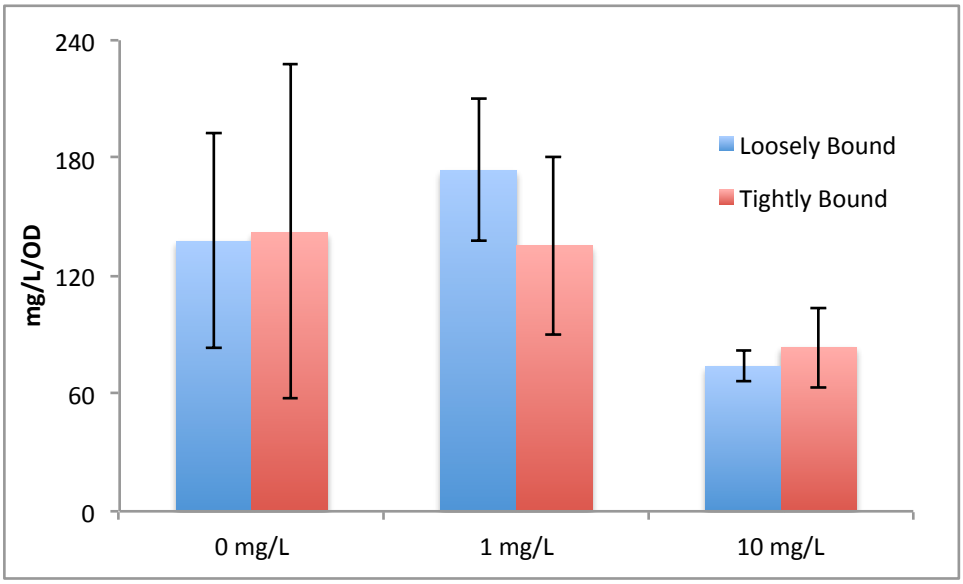


Figure 3.7. EPS concentrations in the soil-free dechlorinating cultures amended with 0, 1, and 10 mg/L of each PFAA.

3.4 Environmental Implications

TCE dechlorination by an anaerobic microbial community was inhibited by high concentrations of PFAAs (10 mg/L each of 11 PFAAs). Changes in growth characteristics or indicators of flocculation were not observed within the community regardless of PFAA presence. It is possible that species within the community do not have protective mechanisms such as the ability to produce EPS that would ensure continued degradation of TCE. These findings may have impacts on co-contaminant degradability and PFAA transport, particularly in the source zones of PFAA contamination where their concentrations are high. Not only may reductive dechlorination be less effective than predicted, but the continued presence of chlorinated solvents, particularly as NAPLs, may provide additional sorption sites for PFAAs, thus retarding PFAA transport in the subsurface [Guelfo and Higgins 2013].

This study shows that there was no significant impact of low concentrations of PFAAs on TCE dechlorination in a Dhc-containing microbial community, both in the presence and absence of soil. However, Dhc was inhibited in soil-free cultures in the presence of high concentrations of PFAAs. It is unknown whether dechlorination would still be inhibited at high PFAA concentrations in the presence of soil. This study also indicates that the calculated organic-carbon distribution coefficients (K_{oc}) for the soil-containing dechlorinating communities were not significantly impacted by the presence of TCE (< 340 μM) or the inoculated Dhc culture. This result lends more confidence to the use of these coefficients for predicting PFAA groundwater transport in NAPL-free subsurface areas where TCE reductive dechlorination is occurring by the native microbial community.

CHAPTER 4: Effects of aqueous film-forming foam (AFFF) on TCE dechlorination by a *Dehalococcoides* enrichment culture

4.1 Introduction

Aqueous-film forming foams (AFFFs) are used for the suppression of liquid-fueled fires from jet fuel or flammable solvents [Moody and Field 2000, Tuve and Jablonski 1966]. These water-based formulations typically consist of various fluorinated and hydrocarbon surfactants and one or more glycol ether-based solvents [Moody and Field 2000]. Most AFFFs are at least 8 to 20 percent (w/w) diethylene glycol butyl ether (DGBE). AFFF has been optimized for rapid fire suppression through the addition of poly- and perfluoroalkyl substances (PFAS), key constituents that allow for a reduction in surface tension that facilitates foam spreading and fire smothering [Moody and Field 2000]. Studies that have characterized the PFASs present in various AFFF formulations indicate that PFAS composition varies significantly according to AFFF manufacturer and year of production [Place *et al.* 2012, D'Agostino and Mabury 2014]. AFFF produced by National Foam, Ansul, Chemguard, and Buckeye generally contain fluorotelomer-based PFASs, which are polyfluorinated compounds consisting of a perfluorinated chain adjacent to two to three nonfluorinated carbons connected to an ionic functional group of varying length and structure [Place *et al.* 2012]. AFFF produced by the 3M Company typically contains perfluorinated sulfonates (PFSAs) and a suite of perfluoroalkyl sulfonamide amino carboxylates (PFSaAmA) and perfluoroalkyl sulfonamido amines (PFSaAm) [Place *et al.* 2012].

Repeated use of AFFF at military, industrial, and municipal sites has led to widespread groundwater and soil contamination by multiple classes of PFASs [Moody *et al.* 2003, Weiss *et al.* 2012, Schultz *et al.* 2004, Houtz *et al.* 2013, Backe *et al.* 2013, McGuire *et al.* 2014]. Firefighter training exercises conducted regularly at Air Force bases historically released untreated AFFF wastewater and fuels into the environment [Moody and Field 2000, Houtz *et al.* 2013, Backe *et al.* 2013]. Because chlorinated ethenes were frequently ignited as the flammable component in the training exercises, the infiltrated wastewater often consisted of AFFF and solvents such as trichloroethene (TCE) [Moody and Field 2000, Moody and Field 1999, McGuire et al. 2014]. This has led to a number of waste sites containing both TCE and PFASs.

Reductive dechlorination of trichloroethene (TCE) by *Dehalococcoides mccartyi* (Dhc) is a frequently employed *in situ* remediation strategy at many TCE-contaminated sites [Stroo and Ward 2010, McCarty 1997]. The hydrogen, acetate, and vitamin B_{12} required for Dhc growth and dechlorination are often generated by fermentation of labile reduced organics injected into the subsurface to stimulate the activity of microbial populations co-existing with Dhc in complex subsurface communities [Duhamel and Edwards 2006, He *et al.* 2007, Men *et al.* 2013, Lee *et al.* 2008]. Historic and repeated infiltration of reduced carbon sources in AFFF have the potential to create a reducing environment conducive to TCE bioremediation by Dhc. However, the effects of AFFF dechlorination by Dhc and the metabolism of microorganisms fermenting AFFF organics are not well described. Because AFFF is a complex mixture of chemicals, one or more of its components could adverseley impact dechlorination. Remediation at TCE and AFFF contaminated sites has so far focused largely on chlorinated solvent contamination with little regard to the effects of PFAS presence and concentration may have on performance outcomes. The recent development of advanced analytical techniques to detect and quantify more PFAS compounds specific to AFFF has also allowed for the enumeration of an increased number of environmentally-relevant PFASs [Place *et al.* 2012, Houtz *et al.* 2012]. Better characterization of co-contaminated aquifers coupled with an improved understanding of the effects of AFFF on *in situ* microbial communities will lead to more effective TCE bioremediation.

This study sought to determine the effects of various AFFF formulations on TCE dechlorination by exposing an enriched anaerobic microbial community containing a *Dehalococcoides mccartyi* strain to three different formulations: 3M Light Water (3%), National Foam Aer-O-Water 3EM (3%), and Ansul Ansulite (3%). These formulations represent AFFF products that were widely applied during fire-training exercises at various U.S. military bases beginning as early as 1976 [Place *et al.* 2012]. The goals of this study were to determine whether dechlorination can occur in the presence of high concentrations of AFFF and whether the microbial communities are capable of utilizing AFFF carbon solvents to produce the hydrogen and acetate necessary to drive TCE dechlorination by Dhc. The AFFF concentrations used in this study represent conditions relevant to the repeated exposure of some subsurface microbial communities to the foams and the cumulative impacts of certain persistent AFFF components, such as perfluorocarboxylates (PFCAs) and perfluorosulfonates (PFSAs), on the activity of Dhc. The effects of individual AFFF components on the communities, such as reduced organics and certain PFAS compounds, were then examined in an effort to explain differences in dechlorination observed among the amended AFFF formulations.

4.2 Materials and Methods

4.2.1 Chemicals

AFFF 3% formulations manufactured by Ansul (Ansulite), 3M (Light Water), and National Foam (Aer-O-Water 3EM) with estimated manufacture dates of 2008, 1998, and 2008, respectively, were obtained from a U.S. military base as previously described [Place *et al.* 2012, Houtz *et al.* 2013]. Each AFFF was stored in a sealed polyethylene tube at room temperature in the dark and the same stocks were used throughout the study to ensure consistency in PFAS and solvent composition. Commercial source materials containing fluorotelomer thioamido sulfonate (FtTAoS), fluorotelomer sulfonamido betaine (FtSaB), fluorotelomer sulfonamido amine (FtSaAm), perfluoroalkyl sulfonamido amine (PFSaAm), and perfluoroalkyl sulfonamide amino carboxylate (PFSaAmA) were obtained from the Fire Fighting Coalition, and the concentrations of 6:2 FtTAoS, 6:2 FtSaB, and 6:2 FtSaAm in various stock solutions were previously determined by Backe *et al.* 2013. All PFCAs, PFSAs, fluorotelomer sulfonates (FtSs) and isotopically-labeled standards used for PFAS quantification by LC/MS-MS were purchased from Wellington Laboratories (Guelph, Ontario, CA). TCE and diethylene glycol butyl ether (DGBE) were purchased from Sigma Aldrich (St. Louis, MO), while all other chemicals were purchased from either Fisher Scientific (Waltham, MA) or Sigma Aldrich at the highest purity possible.

4.2.2 Culture Growth Conditions

All experiments were conducted in either 60 or 160 mL glass serum bottles containing 50 to 100 mL of a reduced mineral salts medium [Lee *et al.* 2006], 10 to 60 mL of a N_2/CO_2 (90:10) headspace, 100 µg/L vitamin $B_{12,}$ and 18 to 25 µmoles of neat TCE that had been equilibrated 24 hours prior to culture inoculation. Approximately 3 to 5% (vol/vol) of a previously-grown Dhc-containing mixed culture was inoculated at day zero into medium representing each of the tested growth conditions and then incubated at 34ºC in the dark without shaking for the duration of the experiment. The Dhc enrichment culture used for this study has been maintained in batch culture in our laboratory for over five years as previously described [Men *et al.* 2013] and propagated through sequential subculture during this time. The culture is typically provided lactate as an electron donor, amended with low initial concentrations of TCE (0.2 mM) to promote methanogenic activity, and given 100 µg/L vitamin B_{12} as an exogenously-supplied co-factor necessary for reductive dehalogenase (RDase) activity [Men *et al.* 2013]. The culture contains a *Dehalococcoides mccartyi* strain similar to Dhc strain 195 in genomic content and function [Men *et al.* 2013]. The strain employs a *tce*A RDase for the metabolic dechlorination of TCE to cis-dichloroethene (cDCE) and vinyl chloride (VC) and co-metabolic dechlorination of VC to ethene [Men *et al.* 2013].

AFFF amendment experiments. In experiments constructed to examine the effects of AFFF on TCE dechlorination and to test the ability of AFFF to support dechlorination as the sole carbon and energy source, 300 µL of Ansul, 3M, and National Foam AFFF were separately amended to 100 mL of growth medium containing 25 µmoles of TCE and 100 µg/L vitamin B_{12}. The AFFF was added with a sterile polypropylene syringe and the growth medium, TCE, and AFFF were equilibrated for 24 hours before culture inoculation. 5 mL of Dhc enrichment culture was then inoculated into the medium, inverted several times to mix, and incubated at 34ºC for 5 hours before the first sample was taken. A set of sterile controls was prepared by inoculating previously-autoclaved culture into the medium containing AFFF and TCE, while a set of culture-free medium controls was prepared by substituting 5 mL of sterile medium for inoculation culture. The autoclaved and medium controls were constructed to quantify any abiotic reactions occurring in the cultures as well as potential PFAS losses due to irreversible binding to biomass or bottle walls. In 3M AFFF-amended cultures, approximately 2.5 mM DGBE was amended to live bottles on day 13, while two additional 300-µL doses of 3M AFFF were amended to live bottles on days 18 and 40. Ansul and National Foam AFFF-amended live bottles both received a 300-µL dose of 3M AFFF on day 33. All bottles were inverted periodically throughout the incubation to promote PFAS mixing. At each sampling time point, between 1 and 1.5 mL of well-mixed culture was removed with a sterile syringe and stored in a 2-mL polypropylene centrifuge tube. Approximately 200 µL of the culture was immediately diluted in an equal volume of HPLC-grade methanol and reserved for LC/MS-MS analysis, while the remaining culture was centrifuged at 15,000 x g for 10 minutes and stored at -20ºC for future analyses. All experiments were performed in triplicate.

DGBE amendment experiments. To determine the ability of DGBE to support TCE dechlorination as a fermentable carbon and energy source, 5 mL of Dhc enrichment culture was inoculated into triplicate experimental bottles containing 100 mL growth medium, 100 µg/L vitamin B_{12}, 2.5 mM DGBE, and 25 µmoles TCE. The DGBE concentration was chosen to approximate the concentration that would be expected with a 300-µL AFFF amendment that is approximately 20% (w/w) DGBE, according to reported MSDS information on AFFF foams [FC-203CF, MSDS No CKQCB, Ansulite 3% AFFF AFC-5-A, MSDS, Aer-O-Water 3EM, MSDS No. MNS210]. DGBE was amended as a sterile stock solution previously prepared in the same anaerobic growth medium. Two additional carbon amendment conditions were tested to determine the potential toxicity of DGBE to TCE dechlorination in the cultures: triplicate bottles containing only 20 mM lactate, and triplicate bottles containing 20 mM lactate and 2.5 mM DGBE. Bottles initially amended with DGBE-only received four additional 2.5 mM doses of DGBE on days 28, 36, 92, and 118, while bottles initially amended with lactate + DGBE received one additional 2.5 mM dose of DGBE on day 92. All bottles were incubated at 34ºC for 200 days and re-amended with 25 µmoles of TCE on day 92. All DGBE experiments were sampled periodically by extracting 1 to 1.5 mL of well-mixed culture into a polypropylene centrifuge tube, centrifuging at 15,000 x g for 10 minutes, and storing at -20ºC for future use. All experiments were performed in triplicate.

PFAS and AFFF component amendment experiments. To determine the specific impacts of certain AFFF components, including PFASs, on TCE dechlorination by Dhc, triplicate cultures were prepared with anaerobic growth medium containing 20 mM lactate, 25 µmoles TCE, and either 18 mg/L ethylene glycol, 12 mg/L 1-propanol, 45 mg/L FtTAoS, 16 mg/L 6:2 FtSaB, or 32 mg/L 6:2 FtSaB. Lactate-only controls containing no additional AFFF compounds was also constructed in parallel. The concentration of the various added components was intended to match the expected concentration of each component in the AFFF-amended experiments to facilitate comparison of observed TCE dechlorination effects among the experiments. The concentration of the various constituents in AFFF-amended experiments was determined by either measuring concentrations in the foams directly (6:2 FtTAoS and 6:2 FtSaB) or estimating concentrations from the closest published MSDS information for each foam type (ethylene glycol, 1-propanol), which typically provides a specific gravity of the AFFF and percent by weight of each foam constituent [FC-203CF, MSDS No CKQCB, Ansulite 3% AFFF AFC-5-A, MSDS, Aer-O-Water 3EM, MSDS No. MNS210]. All bottles were incubated for five days at 34ºC and inverted periodically to promote mixing.

To further assess the impact of PFSAs and PFCAs on TCE dechlorination (collectively termed perfluoroalkyl acids or PFAAs), six different PFAA-amended conditions were tested with enrichment cultures amended with 20 mM lactate and 22 µmoles TCE. Three concentrations of a suite of 11 PFAAs were tested: 2, 6, and 10 mg/L of each PFAA. The suite of PFAAs consisted of PFBA, PFPeA, PFHxA, PFHpA, PFOA, PFNA, PFDA, PFUnA, PFBS, PFHxS, and PFOS. This amendment yielded three tested conditions with total PFAA concentrations of 22, 66, and 110 mg/L, respectively. Additionally, three concentrations of PFSAs were tested: 7.3, 22, and 36.7 mg/L of PFBS, PFHxS, and PFOS each, yielding three tested conditions with total PFSA concentrations of 22, 66, and 110 mg/L, respectively. A PFAA or PFSA methanol stock solution containing each respective set of tested compounds was first evaporated in triplicate 60 mL

serum bottles in an anaerobic chamber and then stoppered and amended with 48.5 mL anaerobic growth medium, 20 mM lactate, and 22 µmoles TCE. The medium was equilibrated with the lactate, PFAAs or PFSAs, and TCE for 48 hours before inoculating with 1.5 mL Dhc enrichment culture and incubating for seven days at 34ºC. Bottles were inverted once a day for several times to promote mixing. Triplicate bottles containing no PFAAs were also constructed in parallel to serve as positive controls.

4.2.3 Analytical Methods

Chloroethene, methane, and hydrogen measurement. TCE, cDCE, VC, ethene, and methane were regularly measured in all experiments by injecting 100 µL of culture headspace into an Agilent 7890A GC-FID equipped with a GS-GasPro capillary column (30 m x 0.32 mm; Agilent Technologies, Inc., Santa Clara, USA). The oven temperature was ramped up from 45 to 200°C in 4 minutes, with a 1 minute hold at 200°C. The injector and detector temperatures were maintained at 220 and 250ºC, respectively. Hydrogen concentrations were measured by injecting diluted headspace samples into a GC fitted with a reductive gas dectector (Trace Analytical, Menlo Park, CA, USA) as previously described [Men *et al.* 2013, Freeborn *et al.* 2005]. Between 50 and 300 µL of culture headspace was withdrawn for each hydrogen measurement and diluted in 17 mL glass vials purged with nitrogen to generate concentrations within the linear calibration range of the instrument.

PFAS measurement. PFAS compounds were quantified on an Agilent 6410 LC/MS-MS operating in both positive and negative electrospray ionization modes using the MS parameters, ion transitions, and internal standards as previously described [Houtz *et al.* 2013]. Diluted culture samples reserved for LC/MS-MS quantification were first vortexed for 30 minutes at room temperature and then centrifuged at 15,000 x g for 10 minutes. The supernatant was diluted with HPLC-grade methanol and water and then amended with 50 µL of an internal standard stock containing 20 to 40 µg/L of various internal standards [Houtz *et al.* 2013]. PFAS analytes for which a commercial analytical standard or quantified source material was available were directly quantified using isotope dilution. For the PFAS compounds in the 3M AFFF formulation used in this study: C4, C5, C6, and C8 perfluoroalkyl sulfonamide amino carboxylates (PFSaAmA) and C4, C5, C6, and C8 perfluoroalkyl sulfonamido amines (PFSaAm), the concentrations in the cultures were estimated according to the method described in the supporting information of Houtz *et al.* 2013, which first normalized the LC/MS-MS analyte responses of the sulfonamide compounds to the PFHxS internal standard response and then quantifies a concentration utilizing an empirically-generated relative response coefficient for each PFSaAmA and PFSaAm perfluorinated chain length.

Organic acid quantification. Acetate, lactate, propionate, and butyrate were quantified with on an HPLC equipped with an Aminex HPX-87H ion exclusion column (300 x 7.8mm, Bio-Rad, Hercules, CA) and a photodiode array detector (PDA) set at 210 nm (Waters, Milford, MA). Solvent consisted of 5 mM H_2SO_4 and was provided at a constant flow rate of 0.6 mL/min. 200 µL of centrifuged culture sample was acidified with H_2SO_4 in a 1 mL amber glass vial, and all organic acids were quantified using external calibration curves as previously described [He *et al.* 2007].

Dhc 16S rDNA quantification. Dhc cell numbers were determined by quantifying Dhc 16S rRNA gene copies in the AFFF-amended cultures. Cells were collected from 1.5 mL of centrifuged culture sample (15,000 x g for 10 minutes) taken from AFFF amended incubations on days 0, 12, 20, 31, 41, and 53. DNA was extracted from the cell pellets using the PowerSoil DNA Isolation Kit (MO BIO Laboratories, Inc, Carlsbad, CA, USA) according to the manufacturer's instructions. Dhc 16S rRNA gene copies were quantified with quantitative polymerase chain reaction (qPCR) using the protocol, primers, and standards described previously [Men *et al.* 2013]. DNA extraction and 16S qPCR enumeration conducted on non-AFFF controls (data not shown) indicates that the AFFF surfactants had no significant effect on extraction and qPCR efficiency. It is assumed the Dhc strain in this enrichment culture contains one 16S rRNA gene copy per cell [Men *et al.* 2013].

4.3 Results

4.3.1 AFFF Amendment Experiments

When 300 µL AFFF was amended to TCE dechlorinating communities as the sole carbon and energy source, active dechlorination was only observed with the 3M AFFF and was completely inhibited with the Ansul and National Foam formulations (Figure 4.1A-C). Aqueous hydrogen concentrations in the active 3M and inactive Ansul cultures were maintained below 0.12 µM, while the inactive National Foam-AFFF amended culture exhibited H_2 concentrations of 2 µM after 10 days and remained approximately constant for the duration of the incubation (Figure 4.2A). To determine whether the supplemental carbon in the 3M AFFF provided additional fermentable growth substrates to facilitate dechlorination, DGBE and/or 3M AFFF were amended to the cultures. For the cultures originally fed 3M AFFF, DGBE and 3M AFFF were added when dechlorination stalled, on days 12, 18, and 40 (Figure 4.1A). Approximately 10 µmoles TCE were dechlorinated from days 12 to 30, while no further dechlorination was observed after day 30 (Figure 4.1A), yielding a total of 25 µmoles TCE that were dechlorinated to cDCE and vinyl chloride. Small amounts of aqueous hydrogen production were observed after each 3M AFFF amendment, but not following DGBE amendment (Figure A4.2). Increases in methane production similarly corresponded to AFFF amendment, yielding a total of 16 µmoles methane production during the 60-day incubation, while no increase in methane concentration was observed in the 5 days following DGBE amendment (Figure 4.1D). For the National Foam- and Ansul-amended cultures, no dechlorination was observed even after the amendment of 3M AFFF, and no significant methane production was detected (Figure A4.1). No TCE dechlorination, methane, or hydrogen was detected in autoclaved cultures or medium control bottles under any AFFF amendment condition.

Acetate production was greatest in National Foam cultures (~2 mM), followed by 3M cultures (~1 mM) and Ansul cultures (~0.5 mM) (Figure 4.2B), while approximately 1 mM acetate was measured at time 0 in both live and autoclaved cultures due to carryover from the inoculated culture stock (Figure 4.2B). Lactate and butyrate were not detected in any of the live, autoclaved, or medium control cultures, while approximately 1 mM propionate was measured at time 0 in both live and autoclaved cultures due to organic acid carryover from the inoculated stock culture. The propionate concentration remained unchanged in all cultures throughout the incubation. No significant Dhc growth, as measured by 16S rRNA gene copy numbers, was detected in any of the AFFF amendment cultures (Figure A4.3).

The concentrations of all measured PFAS compounds remained unchanged during the incubation of each AFFF formulation, indicating that the compounds in the three tested foams wre persistent in these anaerobic cultures and likely did not transform abiotically or through microbial biotransformation (Figures A4.4-A4.7). PFOS was the most abundant PFAS in 3M amended cultures, while PFBS, PFHxS, PFHpS, C_4-C_6 PFSaAm, and C_4-C_6 PFSaAmA were also detected (Figure A4.4B). Each PFAS compound approximately doubled and then tripled following the second and third respective 3M AFFF doses to these cultures (Figure A4.4A-B). PFOS was present in 3M amended cultures at 25 mg/L after the first amendment and then increased to 40 and 60 mg/L following successive amendments (Figure A4.4A).

6:2 FtSaB, 6:2 FtSaAm, 6:2 FtS, and 8:2 FtS were detected in National Foam amended cultures and together yielded a total PFAS concentration of approximately 30 mg/L (Figure A4.6A). The total PFAS concentration increased to 70-80 mg/L after day 33 following 3M amendment to the National Foam-containing cultures, reflecting a 40 mg/L total PFAS contribution from the 3M dose (Figure A4.6A). 6:2 FtTAoS was the only PFAS detected in Ansul-containing cultures and was present at approximately 70 mg/L. This represented the total PFAS concentration in the cultures until 3M was amended on day 33, after which the PFAS concentration increased to 110-120 mg/L (Figure A4.7A). In each of the three AFFF conditions tested, the total PFAS concentrations in autoclaved and medium controls remained relatively constant throughout the incubation, indicating that no significant abiotic reactions or physical losses occurred in any of the experiments (Figure A4.5, A4.6B, A4.7B).

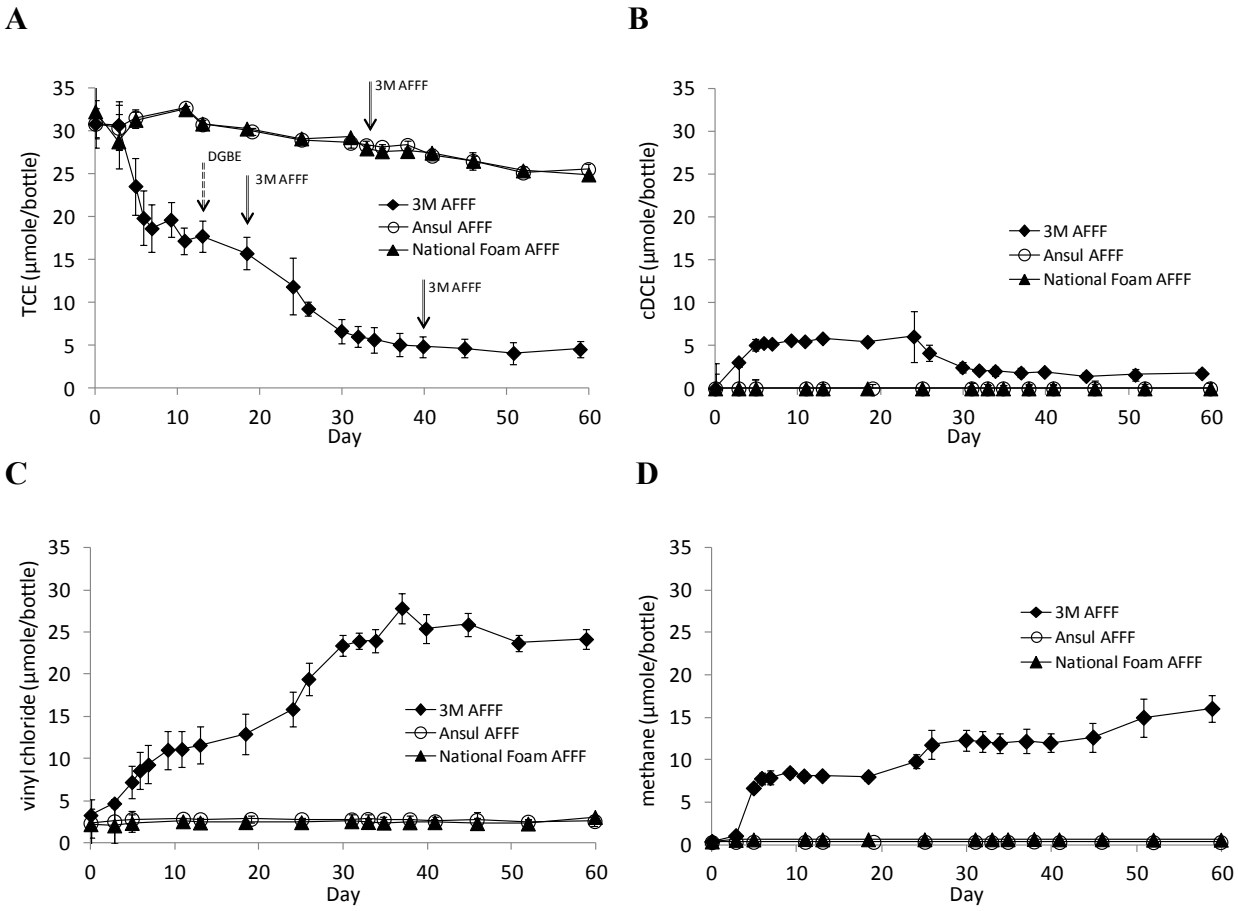


Figure 4.1. Concentrations of TCE (A) cDCE (B), vinyl chloride (C), and methane in cultures amended with 3M, National Foam or Ansul AFFF. Legend indicates the AFFF that was initially amended to the cultures at time 0. Arrows on A denote additional 3M AFFF (solid) and DGBE (dotted) amendments to cultures. Error bars represent the standard deviation of triplicate biological bottles.

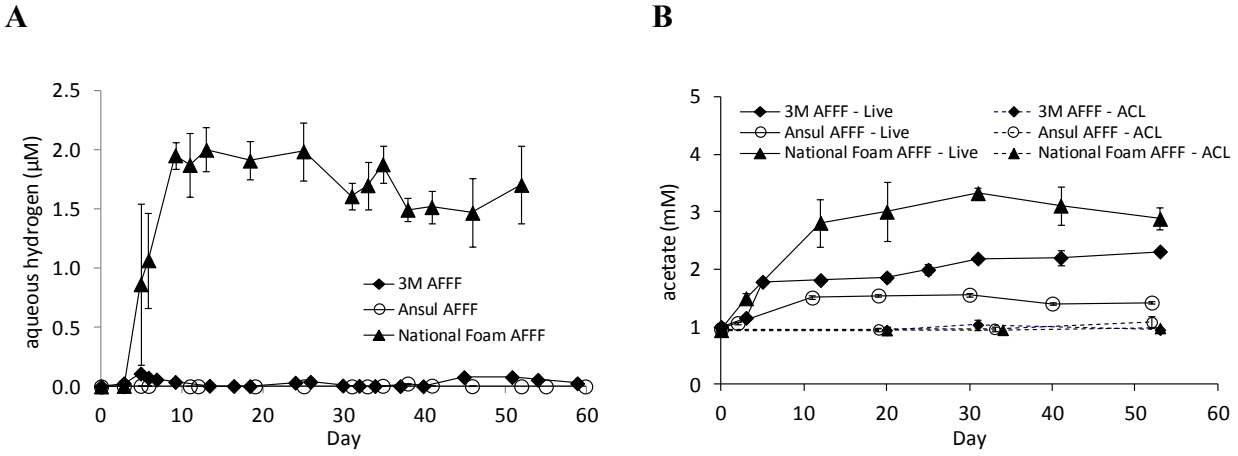


Figure 4.2. Hydrogen production (A) and acetate production (B) in 3M-, Ansul- and National Foam-AFFF amended TCE-dechlorinating cultures.

4.3.2 DGBE Amendment Experiments

To confirm that DGBE can serve as a fermentable organic capable of driving TCE dechlorination, multiple amendments of 2.5 mM DGBE were made to Dhc enrichment cultures as sole organic carbon and energy source (amendments made on days 0, 28, 36, 92 and 118) (Figure 4.3A-D). Two doses of 25 μmoles TCE were dechlorinated to vinyl chloride and ethene with dechlorination rates that were slower than those for lactate-containing cultures. Interestingly, no difference was observed between cultures amended with lactate + DGBE and those amended with lactate only (Figure 4.3A), suggesting that lactate is more labile for fermentation. All conditions received a second dose of TCE on day 90, while only the lactate + DGBE and DGBE-only conditions received additional carbon at that time (2.5 mM DGBE). TCE dechlorination stalled in both the lactate + DGBE and lactate conditions after approximately 125 days (Figure 4.3A).

Approximately 15-20 μmoles methane were produced in the lactate and lactate + DGBE conditions during the first 25 days (Figure 4.4A), while no methane was initially produced in the DGBE-only condition. Methane was produced (2 μmoles) after day 60 in the DGBE only condition and again after day 150 when the TCE was completely removed (Figure 4.4A). Aqueous hydrogen concentrations in the lactate and lactate + DGBE conditions increased rapidly to 0.15 μM in the first two days (Figure 4.4B), after which the hydrogen was subsequently consumed, leaving a residual concentrations of 0.05 μM until the second TCE amendment when it decreased to nearly 0.0005 μM (Figure 4.4B and A4.8). In the DGBE-only condition, hydrogen remained just under 0.001 μM throughout most of the incubation (Figure A4.8), with slight increases after days 60 and 150 when the TCE was completely removed (Figure A4.8). No significant acetate production was measured in the DGBE-only condition, while approximately 7 mM acetate was produced in the lactate and lactate + DGBE conditions, as expected with the standard growth of this culture on lactate (Figure 4.4C). All conditions contained approximately 1 mM acetate and 1 mM proprionate at time 0 as the organic acids were present in the culture inoculum stock. Lactate, butyrate, and propionate production were not observed in DGBE-only amended cultures.

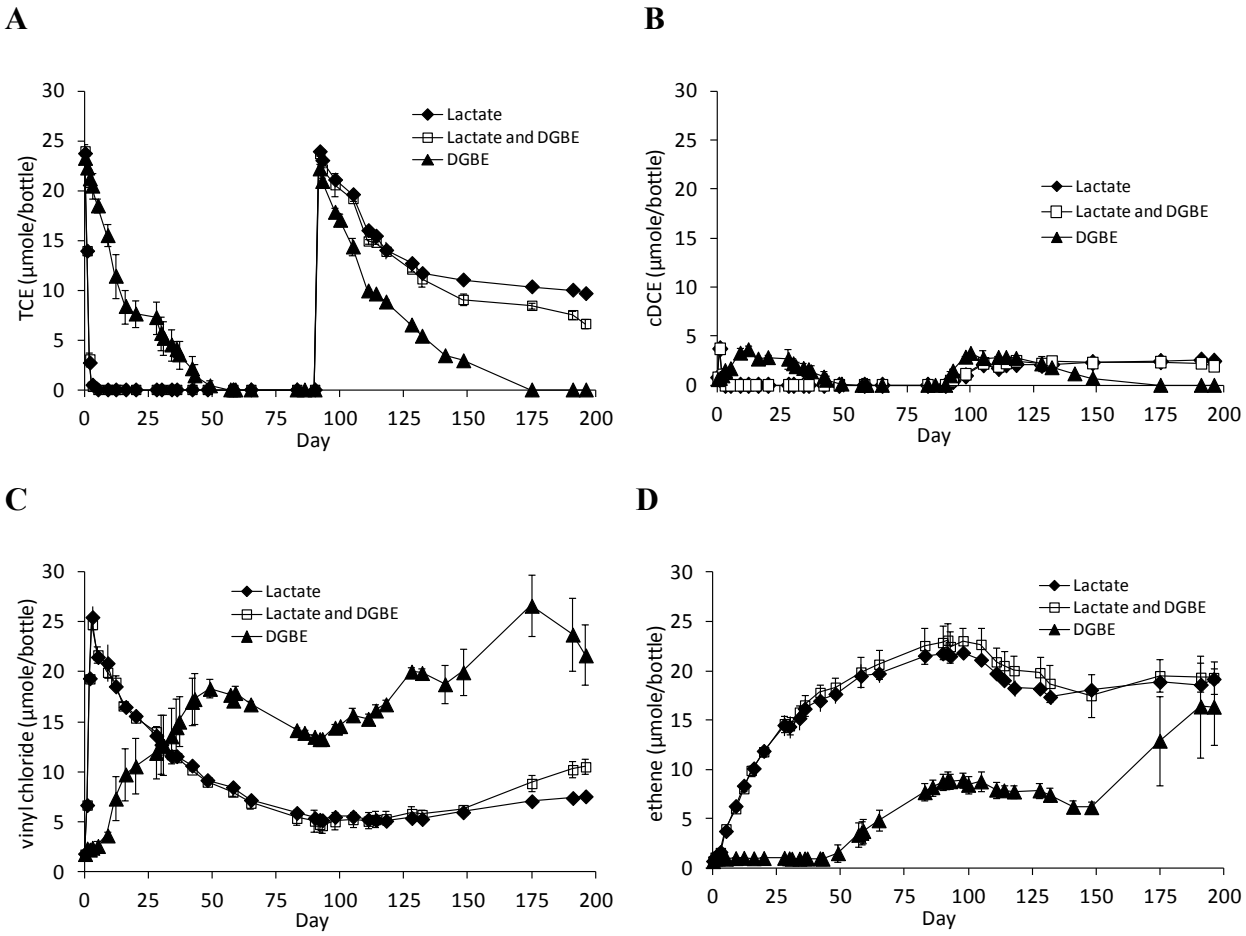


Figure 4.3. Concentrations of TCE (A), cDCE (B), VC (C), and ethene (D) cultures amended with lactate, lactate + DGBE, or DGBE-only as sole carbon and energy sources.

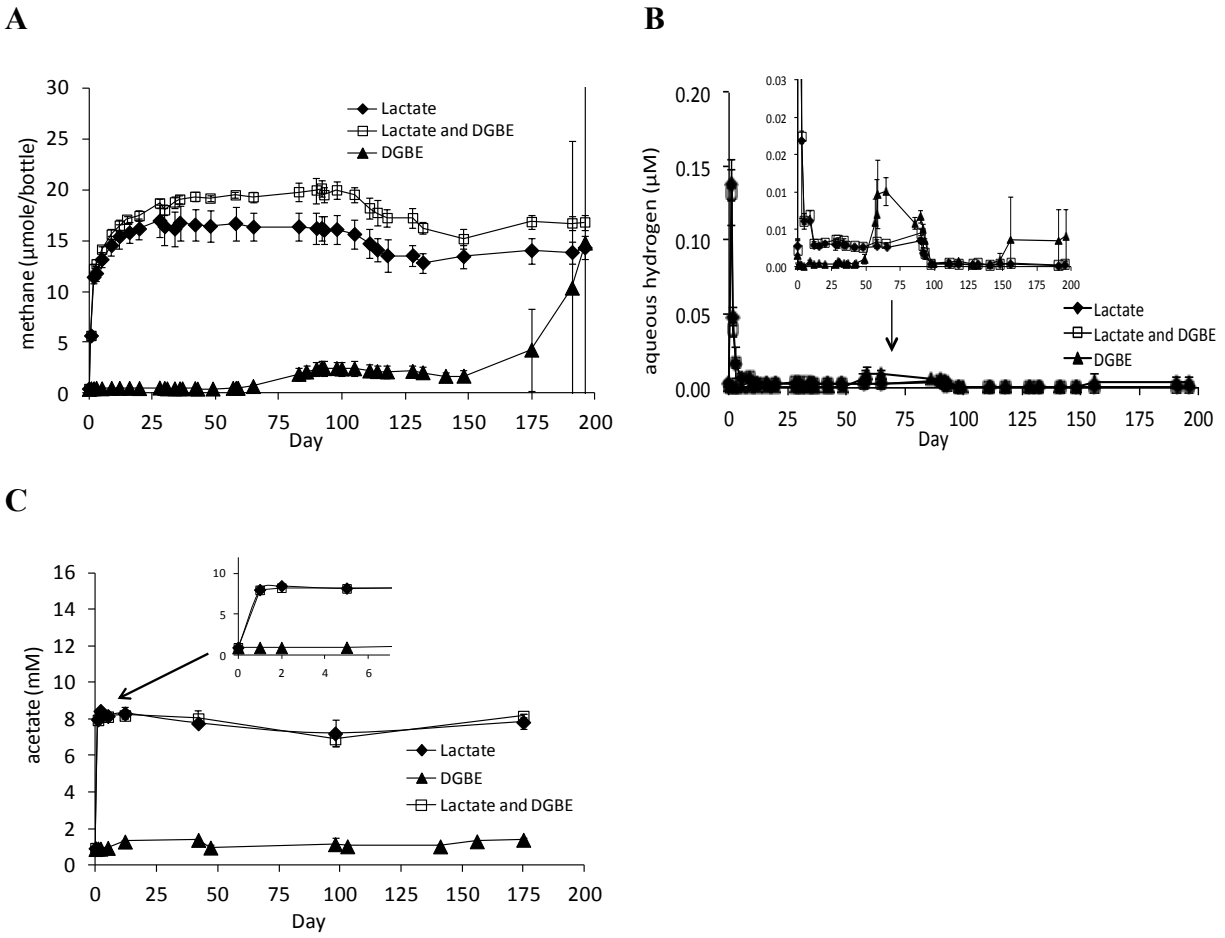


Figure 4.4. Concentrations of methane (A), aqueous hydrogen (B) and acetate (C) in cultures amended with either lactate, lactate + DGBE, or DGBE-only as a carbon and energy source. Inset on B shows reduced y-axis scale to show hydrogen concentrations.

4.3.3 PFAS and AFFF Component Amendment Experiments

To provide insight into which substances in National Foam and Ansul AFFFs may have inhibited TCE dechlorination, the principal fluorinated surfactants, 6:2 FtSaB and 6:2 FtTAoS, as well as two solvents, ethylene glycol and 1-propanol, that are reported to comprise the National Foam and Ansul AFFF formulations at 6 to 8% and 0.4%, respectively [Ansulite 3% AFFF AFC-5-A MSDS, Aer-O-Water 3EM MSDS No. MNS210], were amended to lactate-fermenting cultures. The tested concentrations of each of the compounds were intended to reproduce concentrations present in AFFF-amended experiments. TCE dechlorination was not affected by the presence of 18 mg/L ethylene glycol, 12 mg/L 1-propanol, or 45 mg/L 6:2 FtTAoS when compared to a non-amended control; however, dechlorination was slowed by the presence of 16 mg/L 6:2 FtSaB and stalled completely in the presence of 32 mg/L FtSaB (Figure 4.5). Production of dechlorination daughter products was not observed in the 32 mg/L FtSaB condition, while cDCE dechlorination to vinyl chloride was significantly slower in the 16 mg/L FtSaB condition compared to the non-amended control (Figure A4.9A-B). No difference in

cDCE, vinyl chloride, and ethene production was observed between the control culture and the cultures amended with ethylene glycol, propanol, or FtTAoS (Figure A4.9A-C). Hydrogen production and consumption rates did not vary significantly among the conditions tested, although consumption after day 1 may have been slightly faster in the non-amended control (Figure A4.10B). The concentration of both FtSaB and FtTAoS were stable over the incubation period indicating the PFASs did not biotransform or bind to biomass.

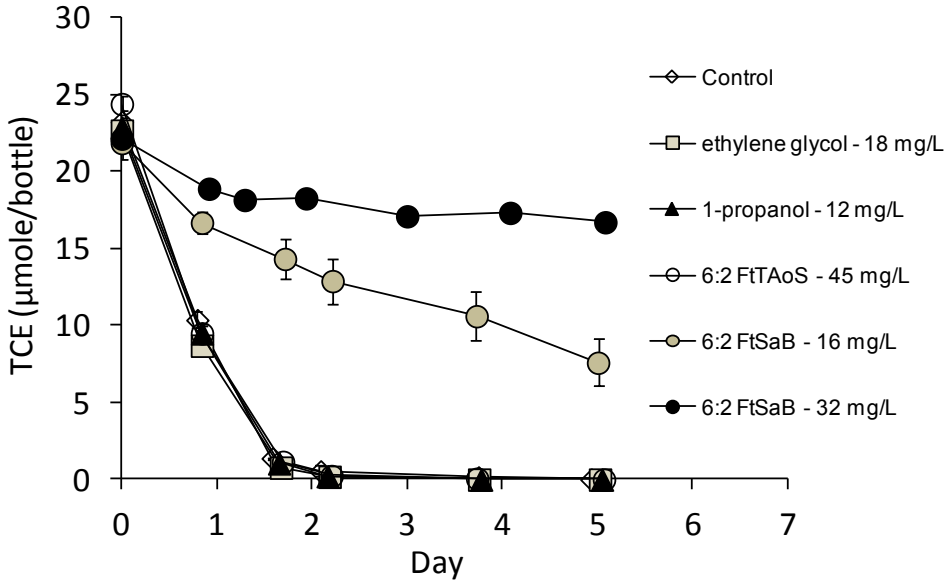


Figure 4.5. TCE dechlorination in the presence of 16 and 32 mg/L 6:2 FtSaB, 18 mg/L ethylene glycol, 12 mg/L 1-propanol, and 45 mg/L 6:2 FtTAoS.

No TCE dechlorination occurred in the presence of 110 mg/L total PFAAs, while dechlorination rates were not significantly affected by the presence of 22, 66, and 110 mg/L total PFSAs and 22 mg/L total PFAAs (Figure 4.6). TCE dechlorination may have been slightly inhibited in the presence of 66 mg/L total PFAAs, however, there were larger standard deviations for the TCE concentrations of the triplicate culture bottles in this condition (Figure 4.6). No cDCE, vinyl chloride, or ethene was produced in cultures amended with 110 mg/L PFAAs, while the compounds' production in all other conditions remains identical to the no-PFAA control (Figure A4.11A-C). Slightly more methane and hydrogen are produced in the 110 mg/L PFAA-amendment condition than the no-PFAA control (Figure A4.12A-B), although very little methane is produced overall in all of the conditions (<5 μmoles), including the control (Figure A4.12A).

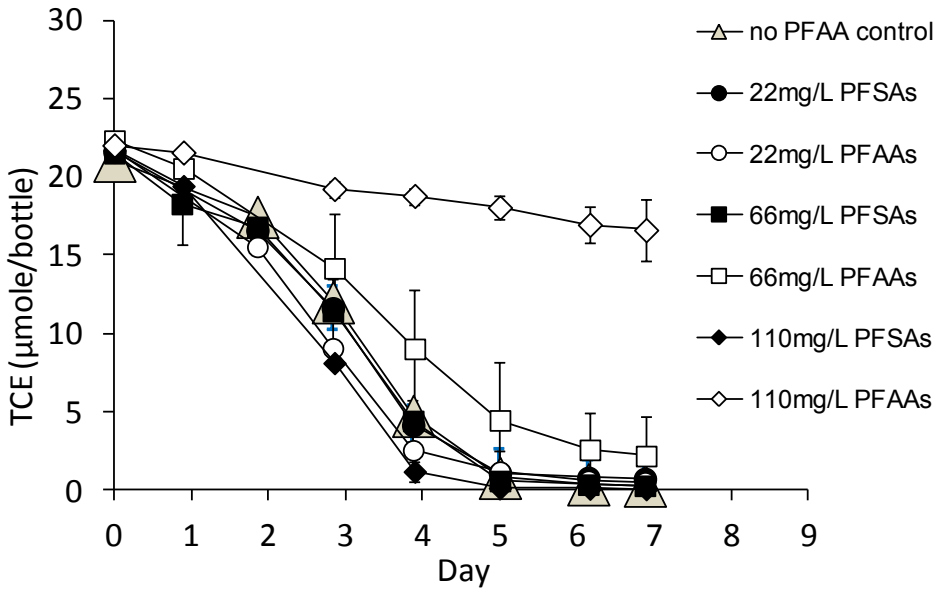


Figure 4.6. TCE dechlorination in the presence of various concentrations of PFAAs (PFCAs + PFSAs) or PFSAs.

4.4 Discussion

The carbon solvents in all three AFFF formulations were fermentable by the anaerobic microbial community used in this study, despite the fact that only one of the three (3M) was capable of supporting dechlorination. The varying concentrations of hydrogen and acetate produced from each AFFF likely reflect the foams' differing initial solvent compositions, the culture's ability to ferment the solvents, and any effects other foam constituents may have had on fermenting microbial populations.

Reported MSDS information indicates that although total carbon solvents typically constitute ~20% (w/w) of an AFFF formulation, foams contain varying concentrations of DGBE along with other solvents. National Foam AFFF typically contains 8-12% (w/w) ethylene glycol, a know microbial carbon and energy source that could be responsible for the higher concentrations of acetate and hydrogen observed in National Foam-amended cultures (Figure 4.2A-B). The anaerobic degradation of ethylene glycol to acetate, hydrogen, and ethanol by a variety of microorganisms has been observed previously [Gaston *et al.* 1962, Dwyer and Tiedje 1983, Dwyer and Tiedje 1986, Strab and Schink 1986], including its fermentation by a *Desulfovibrio* strain [Dwyer and Tiedje 1986], a genus commonly found in dechlorinating microbial communities and represented in the culture used in this study [Men *et al.* 2013]. Although the addition of 18 mg/L ethylene glycol to cultures fermenting lactate did not produce more hydrogen than cultures given only lactate in this study (Figure A4.10B), it is likely the microorganisms preferentially consumed the lactate over the ethylene glycol, as the cultures are regularly maintained in lactate-containing growth medium.

Although to our knowledge anaerobic microbial degradation of DGBE has not yet been reported, a variety of polyethylene glycol compounds (PEGs), including diethylene glycol (DEG) and triethylene glycol (TEG), have been shown to biodegrade under anaerobic conditions to acetate and hydrogen, and even to methane when degraded by methanogenic consortia [Dwyer and Tiedje 1983, Dwyer and Tiedje 1986]. However, in this study, the observed dechlorination coupled with the low hydrogen concentrations and absence of acetate accumulation observed in cultures provided DGBE as the sole carbon and energy source (Figure 4.4B-C) indicated that the fermentation of DGBE in these cultures was stoichiometrically able to support reductive dechlorination. Interestingly, the methane and hydrogen concentrations began to increase in these cultures immediately following the disappearance of each TCE dose – on day 50 and after day 150, suggesting that TCE may inhibit the complete fermentation of DGBE to hydrogen, methane, and acetate. This may also have been demonstrated in cultures initially amended with 3M AFFF as the methane concentrations appeared to inversely correlate with TCE concentrations. However, the inhibition appears to be unidirectional as 2.5 mM DGBE did not inhibit TCE dechlorination in the presence of 20 mM lactate (Figure 4.3A). Although DGBE fermentation appears to at least partially support TCE dechlorination by producing small amounts of hydrogen electron donor (Figures 4.3 and 4.4), all AFFF-amended experiments produced greater concentrations of both hydrogen and acetate, even though the initial supplied mass of organic carbon to DGBE-amended experiments was approximately the same. This suggests that lower concentrations of the other carbon sources in AFFF could be responsible for the hydrogen, acetate, and methane production in the AFFF-amended experiments (Figures 4.1 and 4.2). These organics may include ethylene glycol in the National Foam formulation, 1-propanol and hexylene glycol in Ansul AFFF or hydrocarbon surfactants that are reported to be

present in all three AFFFs at 1-11% (w/w). However, due to the proprietary nature of AFFF foams, specific components are not well described in type and concentration.

TCE dechlorination occurred in the presence of 0.3% (v/v) 3M AFFF concentrate in water, but was inhibited in the presence of the same dilution of National Foam and Ansul AFFF formulations. The dechlorination in 3M-amended cultures appears to have been supported primarily by the fermentation of 3M AFFF carbon sources to hydrogen and acetate that promoted dechlorination activity of Dhc, although it is unclear why TCE stalled after 30 days. Although sufficient hydrogen and acetate were also produced in the Ansul and National Foam-amended cultures to support dechlorination (aqueous hydrogen concentration above the minimum threshold range for Dhc (1 - 2 nM) [Yang and McCarty 1998, Löffler *et al.* 2013]) no TCE degradation was observed, suggesting that chemical(s) unique to Ansul and National Foam formulations specifically inhibit Dhc dechlorination but not microbial fermention. As methane production was also not observed in Ansul and National Foam-amended cultures, the same chemicals inhibitory to TCE dechlorination might also suppress methanogenic activity. The lack of methane production following the amendment of 3M AFFF to Ansul- and National Foam-containing cultures confirms the presence of methanogenic inhibitory compounds in those formulations.

TCE dechlorination inhibition in National Foam amended cultures may have been due to the presence of its principal PFAS compound, 6:2 FtSaB. Interestingly, higher concentrations of the principle PFAS in Ansul AFFF, 6:2 FtTAoS had no effect on TCE dechlorination (Figure 4.5). Although the tested concentration of 6:2 FtTAoS in the PFAS-amended experiment was slightly lower (45 mg/L) than the measured concentration in the Ansul-amended experiment (60 mg/L), if dechlorination inhibition in the presence of Ansul AFFF was due primarily to the presence of this PFAS, it might be expected that at least some effect on TCE dechlorination would be observed at 45 mg/L 6:2 FtTAoS. It thus remains unclear why no TCE dechlorination was observed in Ansul AFFF-amended experiments. The total initial concentration of PFASs in cultures with 3M AFFF was 33% higher (by mass) than the concentration in cultures with National Foam AFFF (40 and 30 mg/L, Figures A4.4A and A4.6A, respectively), however, TCE dechlorination occurred even after two doses of 3M AFFF had been amended (Figure 4.1A). This suggests that dechlorination inhibition may not only be dependent on PFAS concentration, but on type and structure. Furthermore, 6:2 FtTAoS and 6:2 FtSaB both possess 6 perfluorinated carbons and have similar molecular weights (587 and 570 grams/mole for 6:2 FtTAoS and 6:2 FtSaB, respectively). If dechlorination inhibition was primarily due to the effects of the fluoroalkyl chains, it would be expected that 45 mg/L 6:2 FtTAoS would instead show greater inhibitory effects on TCE degradation than 32 mg/L 6:2 FtSaB due to the increased quantity of $-CF_2$ groups in the FtTAoS-amended cultures. This may indicate that dechlorination inhibition in these experiments is attributable to the effects of the PFASs' non-fluorinated functional groups.

The simplest PFAS functional group structures are represented in the PFSA and PFAA amendment experiments (Figure 4.6), where the effects of having a sulfonate or carboxylate group adjacent to the perfluoroalkyl chain are differentiated. Because the concentration of each PFSA in the 110 mg/L total PFSA condition is greater than the concentration of each PFSA in the 110 mg/L total PFAA condition (Figure 4.6), then either the PFCAs are singularly responsible for TCE dechlorination inhibition in this experiment, or the combination of PFCAs and PFSAs together cause inhibition. Yet, dechlorination activity still occurs with higher

concentrations of PFAAs than 6:2 FtSaB, suggesting that the inhibition in FtSaB-amended experiments is likely attributable to the PFAS's non-fluorinated functional group. FtSaB was the only tested PFAS in this study that contains a non-fluorinated betaine functional group, which could potentially aid in the binding of the compound to negatively charged biomolecules, such as proteins and nucleic acids. Such binding might interfere with normal cellular metabolic and replication processes.

Although the mode of dechlorination inhibition of Dhc is not clear, it is possible that the PFASs are directly interacting with Dhc cells to prevent dechlorination, *e.g.* by interacting specifically with RDases to prevent dechlorination or binding to Dhc's proteinaceous S-layer and preventing proper transport functions. A previous study found that the hydrocarbon surfactant Tween-80 specifically inhibited dechlorination by Dhc organisms, but not other gram-positive and gram-negative dechlorinating isolates [Amos *et al.* 2007]. Surfactant sorption or partial diffusion through Dhc's S-layer might prevent the cells' proper metabolism and replication, and could potentially explain the absence of observable Dhc growth in the 3M AFFF-amended experiments (Figure A4.3).

4.5 Environmental Implications

The results of this study suggest that there is a complex interplay between AFFF and TCE bioremediation communities dependent on the AFFF formulation and composition. The carbon solvents in the three tested AFFFs in this study were fermentable under anaerobic conditions and produced the hydrogen and acetate necessary to support dechlorination by *Dehalococcoides*. Although the biodegradation of DGBE appears to produce lower concentrations of hydrogen than other carbon constituents in the foams, such as ethylene glycol, organics in each of the AFFF formulations were capable of providing the hydrogen and acetate required for dechlorination by Dhc.

However, the type and quantity of PFAS compounds present may determine whether dechlorination will occur in the presence of AFFF, and the total mass concentration of PFAS compounds may be insufficient for predicting TCE degradation. Although the PFAS concentrations examined in this study are up to an order of magnitude higher than those found in the limited number of field studies that quantified known compounds in soil and groundwater at AFFF-impacted field sites, it has also been revealed that major fractions of PFASs in the field are currently unidentified and may constitute up to 30% of the total PFASs detected in the samples [Houtz *et al.* 2013, McGuire *et al.* 2014]. Additionally, there is increasing evidence that site remediation activities and *in situ* biotransformation may be responsible for the conversion of AFFF PFAS compounds, such as FtTAoS, into more water-soluble PFCAs [McGuire *et al.* 2014, results of Chapter 2], potentially increasing the load of PFAAs microbial communities are exposed to.

AFFF application at firefighter training sites was often heterogeneous with respect to time and formulation use. Foams from different manufacturers and production years were often used at the same site, leading to groundwater and soil contaminated with multiple classes of carbon solvents and PFASs. Additional research is needed to describe the effects of a range of AFFF concentrations on microbial communities in the presence of soil and the potential combinatorial effects of multiple formulations types. These experiments should be coupled to fundamental studies with Dhc isolates that examine the mechanisms of PFAS inhibition and seek to determine whether the inhibition is reversible. A holistic understanding of the effects of AFFF on TCE-dechlorinating microbial communities will lead to better sequential treatment operations at AFFF and chloroethene-contaminated sites.

CHAPTER 5: Effects of varying growth conditions on TCE stable carbon isotope fractionation by *Dehalococcoides* cultures

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5.1 Introduction

The chlorinated hydrocarbon and industrial solvent trichloroethene (TCE) is one of the most common groundwater contaminants in the United States [Westrick *et al.* 1984, NRC 1994, US DHHS 2005]. Past disposal and storage methods that resulted in the widespread contamination of groundwater aquifers pose a significant threat to human health due to the potential for transport to potable water and irrigation supplies. As aquifer depletion and overdrafting accelerate in the United States [Zekster *et al.* 2005], this threat continues to grow. The anaerobic dechlorination of TCE to non-toxic ethene by microorganisms of the widely-occurring soil bacterium *Dehalococcoides mccartyi* (Dhc) makes *in situ* bioremediation with indigenous or augmented strains a cost-effective and less invasive remediation strategy at many contaminated sites [Lovley 2003, Lemming *et al.* 2010]. Confirming the transformation of chlorinated solvents in field environments that have complex stratigraphy and are often ill-characterized remains, however, a challenging task. Physical attenuation processes such as dilution, dispersion and sorption may dominate concentration profiles, and co-contamination or off-site sources of compounds resembling biological daughter products of TCE reduction, such as *cis*- and *trans*-dichloroethene (cDCE and tDCE) or vinyl chloride (VC), can complicate site degradation assessments.

Measuring a compound's stable isotope ratio has been used increasingly in recent years by practitioners aiming to confirm and quantify *in situ* contaminant transformation [Elsner *et al.* 2005, Bombach *et al.* 2010]. Because a detectable and unique shift in a compound's isotope composition accompanies an irreversible chemical or biological transformation, quantifying the kinetic isotope fractionation with compound-specific isotope analysis (CSIA) can provide evidence for the contaminant's degradation and elucidate potential transformation pathways [Meckenstock *et al.* 2004, Bloom *et al.* 2000]. This analysis relies on the slightly faster enzymatic reaction of lighter isotopes (*e.g.* ^{12}C) over heavier isotopes (^{13}C), leaving a dynamic enrichment of heavy isotopes in the residual substrate and an accumulation of lighter isotopes in the product. The kinetic isotope fractionation of chlorinated hydrocarbons follows a distribution described by the Rayleigh equation and is quantified in the laboratory for a given compound and reaction pathway by reporting an enrichment factor, e [Lollar *et al.* 1999, Lee *et al.* 2007]. Because biological and chemical transformations of chlorinated ethenes cause isotope shifts that are one to two orders of magnitude greater than those produced by physical processes, various co-occurring transport or phase-transfer processes have only a minor impact on the overall isotopic fractionation [Huang *et al.* 1999, Poulson and Drever 1999]. Coupling CSIA with measured geochemical parameters, contaminant site concentrations, and hydrogeological data may further inform site remediation decisions by confirming the occurrence and activity of reductive dechlorination.

Currently, quantifying the *in situ* bioremediation of organic compounds with CSIA requires the extrapolation of laboratory-acquired enrichment factors to field isotope data [Hunkeler *et al.* 2004, Morrill *et al.* 2005, Chartrand *et al.* 2005]. The application of a non-representative enrichment factor can lead to significant errors in estimated degradation rates [Nijenhuis *et al.* 2005]. Previous studies that have measured this shift during the reductive dechlorination of chlorinated ethenes showed that enrichment factors can be highly variable from site-to-site and culture-to-culture, and are not only a function of the degrading microorganisms and enzymes, but also vary according to independent experiments conducted by different

laboratories [Bloom *et al.* 2000, Lee *et al.* 2007, Fletcher *et al.* 2011, Cichocka *et al.* 2008, Slater *et al.* 2001, Duhamel *et al.* 2002, Liang *et al.* 2007]. Enrichment factors currently reported for TCE dechlorination range between -2.5 and -16‰ for pure cultures and enriched communities [Bloom *et al.* 2000, Lee *et al.* 2007, Fletcher *et al.* 2011, Cichocka *et al.* 2008, Liang *et al.* 2007] Dhc strains possessing the *tce*A reductive dehalogenase (RDase) gene were reported by different laboratories to have enrichment factors of -9.6±0.4 ‰ and -13.7±1.8 ‰ for strain 195 [Lee *et al.* 2007, Cichocka *et al.* 2008], and -8.0±0.4 ‰ for strain FL2 [Fletcher *et al.* 2011]. Significantly different enrichment factors (-8.4±0.3 ‰ and -5.1±0.3‰) were also reported by two laboratories for the reductive dechlorination of 1,1-DCE by the *bvc*A RDase in Dhc strain BAV1 [Lee *et al.* 2007, Fletcher *et al.* 2011]. This suggests that the same enzyme may produce different isotopic fractionations, and that there is not always reproducibility between laboratories for a given organism, enzyme, or compound. This raises a question about whether specific growth and enrichment conditions or culture maintenance protocols could contribute to differences in the observed fractionation patterns. Additionally, the enrichment factors for most of these organisms were quantified under conditions designed to maximize cellular growth and dechlorination rates.

As *in situ* environmental conditions often differ significantly from those established for Dhc laboratory growth, it is important to explore the potential impacts of growth conditions on isotopic fractionation. This study sought to determine the effects of variable growth conditions on the stable carbon isotope fractionation of TCE by examining the fractionation produced by a single RDase (*tce*A) present in a variety of cultures. The growth conditions of *tce*A-containing Dhc were varied while the organisms were grown in pure culture, co-culture, and in enriched microbial communities, and their respective enrichment factors were quantified. Tested growth conditions were chosen on the basis of relevancy to field environments, potential differences in applied enrichment techniques, and achievability in the laboratory given Dhc's strict growth and nutritional requirements. Some tested conditions, such as limited vitamin B_{12} in the growth medium and induced nitrogen fixation, are known to produce changes in Dhc195's physiological and dechlorination activity [Lee *et al.* 2009, He *et al.* 2007, Johnson *et al.* 2009, Lee *et al.* 2012]. Elevating aqueous vinyl chloride concentrations above 1.6 mM was previously shown in our laboratory to slow TCE dechlorination by Dhc195 (unpublished data). A reduced incubation temperature causes long dechlorination lag periods and slower TCE transformation rates to cDCE. Dhc growth in co-cultures and enriched mixed communities is generally more robust with greater cell yields and faster cell-normalized dechlorination rates [Duhamel *et al.* 2002, He *et al.* 2007, Men *et al.* 2011]. Here it is hypothesized that different physiological states which produce wide variations in enzymatic dechlorination rates may lead to changes in TCE isotope fractionation by altering the suite of cellular kinetic processes that control the observed fractionation, such as membrane transport and enzyme-substrate binding. Similarly, the growth of Dhc in varying enrichment environments which have different community structures and activities could produce changes in enzyme activity and associated kinetic processes, leading to changes in fractionation. To isolate the observed differences in fractionation produced by different RDases dechlorinating different chloroethenes, the isotope fractionation of one chloroethene (TCE) was quantified by one RDase (*tce*A) present in multiple cultures during reductive dechlorination to cDCE.

5.2 Materials and Methods

5.2.1 Chemicals

All chemicals used in the following experiments were obtained from Fisher Scientific (Houston, TX), or Sigma Aldrich (St. Louis, MO). The TCE amended to each culture (Sigma Aldrich, highest purity possible) was taken from the same stock bottle in order to minimize variation in the starting isotope composition.

5.2.2 Culture Growth Conditions

All experiments were conducted in 160 mL glass serum bottles containing 100 mL of liquid media and 50 to 80 μmoles of neat TCE that had been equilibrated 24 hours prior to inoculation using materials previously described [He *et al.* 2003]. Cultures were transferred at 3 to 10% (v/v) from maintenance bottles to fresh anaerobic medium representing their respective nutritional and growth conditions. Each experiment was constructed with triplicate bottles and autoclaved controls, except the standard growth control which was constructed using six biological replicates. For the standard growth control experiment, Dhc195 was grown in pure culture under conditions previously described [He *et al.* 2003]. This includes growth at 34°C in 100 mL defined mineral medium with 5mM acetate as a carbon source, 60 mL H_2/CO_2 (80:20) headspace for electron donor, 5.6 mM NH_4Cl, 100 μg/L vitamin B_{12} and approximately 78 μmoles of TCE. For the B_{12} and VC stress experiments, a standard-grown Dhc195 culture was sub-cultured (3% v/v) in biological triplicates into medium containing no B_{12} or medium equilibrated with 300 μmoles of gaseous VC, with 78 μmoles of TCE in each condition. This provided experimental growth conditions with effective B_{12} concentrations less than 3 μg/L, a concentration similar to what was shown to limit TCE dechlorination and cellular growth rates in pure Dhc cultures [He *et al.* 2007], and with an elevated aqueous VC concentration of approximately 1.6 mM. This concentration of VC is nearly three times higher than the initial aqueous concentration of TCE and approximately four times higher than the aqueous VC concentration that results from the complete reduction of 78 μmoles TCE. Previous experiments in our laboratory indicated that amending media with 1.6 mM aqueous VC slowed dechlorination of TCE by Dhc195 relative to control cultures (unpublished data). Because the functional RDase of Dhc195 (*tce*A) can only co-metabolically catalyze VC reduction to ethene, VC accumulation could potentially occur in environments lacking organisms that possess *vcr*A, *bvc*A, or other functionally equivalent enzymes. A culture of Dhc195 capable of growth and dechlorination at the suboptimal temperature of 22°C was maintained over the course of a year and sub-cultured at 10% v/v multiple times in order to test decreased temperature affects on dechlorination rates. For isotope experiments, stock bottles were sub-cultured at 10% v/v into biological triplicates containing 78 μmoles of TCE and incubated at 22°C for the duration of the experiment. A serially-diluted culture of Dhc195 actively fixing atmospheric dinitrogen was also maintained in our laboratory for over 3.5 years and was sub-cultured and grown in a modified medium under a pressurized nitrogen headspace ($8.3 \cdot 10^4$ N/m^2) for these experiments, as previously described [Lee *et al.* 2009].

Co-cultures of Dhc195 and *Desulfovibrio vulgaris* Hildenborough (DVH) were maintained with 5 mM lactate as a carbon source as previously described [Men *et al.* 2011]. Stable co-cultures of Dhc195 and *Syntrophomonas wolfei* had also been maintained in the laboratory for over 2 years by sub-culturing 5% v/v into 100 mL medium with either 20 mM crotonate and 55 µmoles TCE or 5 mM lactate and 78 µmoles TCE. All co-cultures were maintained in the same mineral salts medium with a N_2/CO_2 headspace (90:10) and 100 µg/L amended B_{12} at 34°C.

The carbon isotope fractionation of TCE was also determined for a Dhc-containing mixed community before and after a two-year enrichment period under three defined growth conditions. The development of these communities is reported elsewhere [Men *et al.* 2013] and includes the following 3 enrichments that were all derived from the same parent culture, a methanogenic community amended with low initial TCE (55 µmoles) and 100 µg/L B_{12} (LoTCEB12/Parent). The subsequent 3 enrichments included methanogen-inhibited cultures amended with high initial TCE (78 µmoles) and (1) no B_{12} (HiTCE) or (2) 100 µg/L B_{12} (HiTCEB12), and (3) a methanogenic culture amended with low TCE (55 µmoles) and 100 µg/L B_{12} (LoTCEB12). The effects of carbon source limitation on TCE fractionation were also explored using the HiTCEB12 culture that was starved of lactate after 4 days (HiTCEB12/LowLac).

5.2.3 TCE Degradation Rate Calculation

An initial TCE degradation rate was quantified for each culture by assuming an insignificant cell growth rate and calculating the slope of a time-course linear regression through the first 3 to 5 measured TCE concentration points for each biological replicate. This typically represented the first 2 to 5 days of TCE degradation for Dhc pure and co-cultures, and 1 to 2 days for Dhc enrichment cultures. All numbers were normalized to the number of inoculated Dhc cells, determined from quantifying 16S rDNA copy numbers [Men *et al.* 2011, Men *et al.* 2013, Lee *et al.* 2012] and subsequently adjusting for the appropriate dilution. Average TCE degradation rates are reported along with their standard deviations, which represent all biological replicates quantified.

5.2.4 Experimental Methods and Isotopic Quantification

The concentrations of TCE and its transformation products cDCE, VC, and ethene were measured regularly by injecting 100 µL of culture headspace into a GS-GasPro capillary column (30 m x 0.32 mm; Agilent Technologies, Inc., Santa Clara, USA) on an Agilent 7890A gas chromatograph equipped with a flame ionization detector (GC-FID). The applied GC temperature program ramped the oven from 45 to 200°C in 4 minutes, with a 1 minute hold at 200°C. Samples for isotope analysis were prepared by injecting 7 mL of liquid culture into a previously-evacuated and stoppered 10 mL glass vial containing NaCl and 1 M NaOH to facilitate chloroethene volatilization and cell death, respectively. Headspace samples (200-1000 µL) were then taken from each 10 mL vial and injected into a cryogenic stainless steel loop mounted on a 6-port valve (Valco Instruments Co. Inc., Houston, USA). Samples were injected into the gas chromatograph by switching the 6-port valve and heating the loop with a heat gun. Gases were separated chromatographically on a DB-VRX fused silica capillary column (30 m x

0.25 mm Agilent Technologies, Inc., Santa Clara, USA). After GC separation, the chloroethenes were combusted to CO_2, water was removed, and the carbon isotope ratio was measured in a Micromass JA Series Isoprime Isotope ratio mass spectrometer (Micromass, Manchester, UK). Stable carbon isotope ratios are expressed in the conventional **δ**-notation in parts per mil (‰) relative to the Vienna-Peedee Belemnite (V-PDB) standard. Analytical replicates of samples were periodically conducted when two to three sampling points in each experiment were subjected to duplicate analysis. Repeated analysis of laboratory standards indicates that the uncertainty associated with each stable carbon isotope measurement is approximately ±0.5‰. A time zero sample was collected shortly after culture inoculation to obtain a carbon isotope ratio representative of undegraded TCE, and subsequent samples were collected at regular time intervals thereafter to capture dynamic isotopic shifts over degradation time courses covering 60 to 95% of total initial TCE supplied, with 7 to 21 measured time points per fractionation curve. Because 7 mL of liquid culture was withdrawn for each time point, 49 to 147 mL was withdrawn from the triplicate set in total. A similar number of samples were taken from each bottle so that liquid volumes remained approximately equal between replicate cultures. Sample removal constituted 16 to 49% of initial culture volume, and was accounted for in all chloroethene concentration calculations.

5.2.5 Enrichment Factor Calculation

The TCE isotope fractionation for all experiments was quantified with the Rayleigh model (Equation 5.1) and described as an enrichment factor, ε, obtained from a linear regression of a rearranged form of the equation (Equation 5.2) [Mariotti *et al.* 1981].

$$\frac{R}{R_0} = f_R^{\varepsilon/1000} \qquad \textit{Equation 5.1}$$

$$10^3 ln \frac{10^{-3}\delta^{13}C+1}{10^{-3}\delta^{13}C_0+1} = \varepsilon\, ln(f_R) \qquad \textit{Equation 5.2}$$

R_0 and R are the carbon isotope ratios of the initial and remaining reactant, respectively, $\delta^{13}C_0$ and $\delta^{13}C$ are the isotope ratios in their corresponding delta notation, and f_R is the molar fraction of substrate remaining, determined for each time point relative to the total mass measured at time zero. The calculated f_R used at every time point was corrected for cumulative sample removal. Each experiment showed a minimal spread in fractionation factors between duplicate and triplicate sets. Thus, the data was combined to produce an un-weighted average fractionation, which was reported as a single enrichment factor for each growth condition examined. The linear regression coefficient (r^2) and 95% confidence interval (CI) calculated for each enrichment factor are also reported.

5.3 Results and Discussion

For all experiments in this study, the Rayleigh equation produced a good fit to the carbon isotope ratios collected in each tested condition (r^2>0.87). The quantified enrichment factors, r^2 values, and 95% CIs for the tested culture conditions are reported in Table 1, and these newly reported enrichment factors are plotted along with the previously reported minimum and maximum enrichment factors for the reductive dechlorination of TCE by Dhc in Figures 5.1 and 5.2 (dotted lines indicate TCE enrichment factors -2.5 and -16.0‰ previously reported for enrichment cultures KB-1 [Bloom *et al.* 2000] and ANAS [Lee *et al.* 2007], respectively). The individual Rayleigh plots and linear regressions for each experiment in this study are provided in the Appendix (Figures A5.1-A5.13), as well as time-course plots for measured TCE, cDCE, and VC (Figures A5.14-A5.16 for Dhc195 pure and co-cultures, and Figures A5.17-A5.19 for Dhc enrichment cultures). The initial TCE dechlorination rates for most tested culture conditions in this study are also listed in Table 5.1, normalized to the initial number of Dhc cells. In all cultures in this study, the sequential dechlorination of TCE to cDCE and then to VC was observed and most likely occurred via the *tce*A reductive dehalogenase enzyme. The step-wise dehalogenation of TCE to VC through a cDCE intermediate occurs metabolically in *tce*A-containing organisms, while the reduction of VC to ethene occurs through a slower co-metabolic reaction [Maymó-Gatell *et al.* 1997]. In these experiments, both cDCE and VC were produced by Dhc195 in pure and co-cultures as well as the Dhc in the enrichment communities (Figures A5.14-A5.16 and A5.17-A5.19). However, because the experiments ended immediately after TCE transformation, little to no ethene generation had yet been observed.

Table 5.1. Carbon isotope Rayleigh enrichment factors (ε) and initial reaction rates for the reductive dechlorination of TCE by *tce*A-containing Dhc under a variety of growth and enrichment conditions measured in this study or previously reported.

Culture[a]	Growth Condition	TCE ε (‰)	95% CI	r^2	Initial TCE degradation rate (µmoles TCE / day / 10^9cells)	Culture[b]	TCE ε (‰)	95% CI	r^2
Dhc195 pure cultures						***Previously Reported***			
Dhc195	Standard growth	-13.3	±0.7	0.98	3.7 ± 2.8	Dhc195[c]	-9.6	±0.4	0.99
Dhc195	Decreased Vitamin B_{12} (3 µg/L)	-12.7	±1.0	0.97	1.9 ± 0.6	Dhc195[d]	-13.7	±1.8	0.95
Dhc195	Elevated vinyl chloride (1.6 mM aq)	-12.5	±0.4	0.99	0.9 ± 0.5	Dhc FL2[e]	-8.0	±0.4	0.98
Dhc195	Nitrogen fixing	-14.4	±0.8	0.99	6.0 ± 2.9	ANAS/Dhc enrichment[c]	-16.0	±0.6	0.99
Dhc195	22° C incubation	-13.3	±0.9	0.96	*NA	KB-1/Dhc enrichment[f]	-2.5	n/a	0.91
Dhc195 co-cultures						KB-1/Dhc enrichment[f]	-13.8	n/a	0.98
Dhc195/ *D. vulgaris* Hildenborough	5 mM lactate-fed co-culture	-13.0	±2.0	0.87	8.7 ± 0.4	KB-1/Dhc enrichment[f]	-6.6	n/a	1.0
Dhc195/*S. wolfei*	5 mM lactate-fed co-culture	-10.4	±1.2	0.93	11.7 ± 2.4	BDI/Dhc enrichment[g]	-15.3	±0.6	n/a
Dhc195/*S. wolfei*	20 mM crotonate-fed co-culture	-13.3	±1.0	0.98	8.6 ± 1.1	Abiotic with cyanocobalamin[h]	-16.6	n/a	0.99
Dhc enrichment cultures									
Dhc enrichment	LoTCEB12/Parent	-8.9	±0.4	0.98	7.9 ± 0.4				
Dhc enrichment	LoTCEB12	-6.8	±0.8	0.93	6.8 ± 0.5				
Dhc enrichment	HiTCEB12	-8.7	±1.3	0.90	4.0 ± 0.5				
Dhc enrichment	HiTCE	-9.4	±0.7	0.97	7.3 ± 1.2				
Dhc enrichment	HiTCEB12/LowLac	-7.2	±0.3	0.98	8.9 ± 0.7				

[a]Data from this study. [b]Data previously reported. [c]Data from Lee *et al*. 2007. [d]Data from Cichocka *et al*. 2008. [e]Data from Fletcher *et al*. 2011. [f]Data from Bloom *et al*. 2000. [g]Data from Liang *et al*. 2007. [h]Data from Slater *et al*. 2001. *Dhc cell growth at 22ºC was not determined for this study.

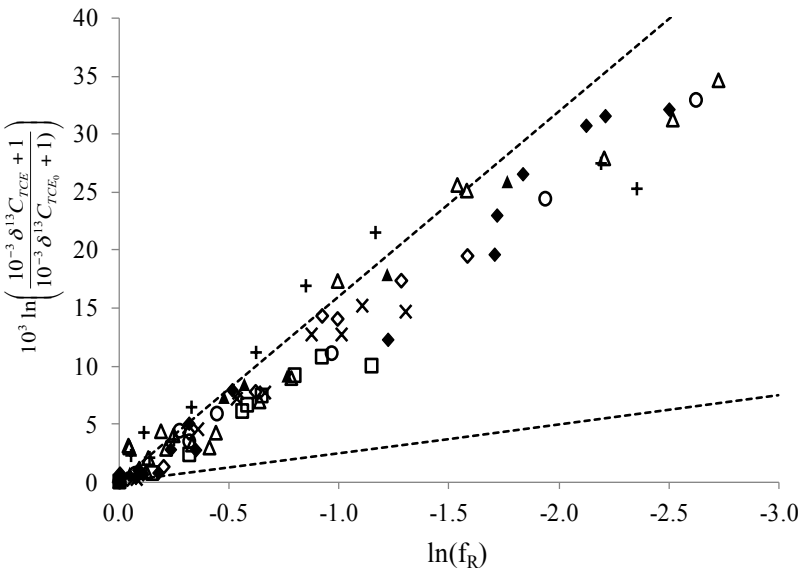

Figure 5.1. The Rayleigh equation linear regression plots for the carbon isotope fractionation of TCE during reductive dechlorination by strain 195 grown in pure and co-culture under a variety of growth conditions. Dotted lines represent the previously reported minimum and maximum TCE isotope fractionations by Dhc. Legend: (◆) Dhc195, standard growth; (▲) Dhc195, nitrogen fixing; (×) Dhc195, decreased B_{12}; (△) Dhc195, 22°C incubation; (○) Dhc195, elevated VC; (+) Dhc195/DVH; (◇) Dhc195/*S. wolfei* w/ 20mM crotonate; (□) Dhc195/*S. wolfei* w/ 5mM lactate.

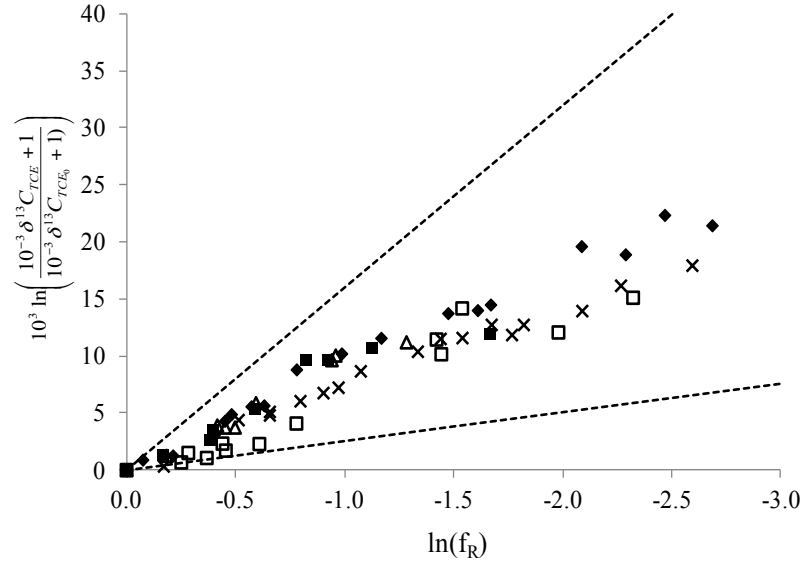

Figure 5.2. The Rayleigh equation linear regression plots for the carbon isotope fractionation of TCE during reductive dechlorination by Dhc-containing enrichments under a variety of growth conditions. Dotted lines represent the previously reported minimum and maximum TCE isotope fractionations by Dhc. Legend: (◆) LoTCEB12/Parent; (■) HiTCEB12; (△) HiTCE; (×) HiTCEB12/LowLac; (□) LoTCEB12.

5.3.1 Effects of Growth Conditions on TCE Fractionation by Dhc195

To investigate the effects of growth conditions on the stable carbon isotope fractionation of TCE, experiments were performed to compare quantified fractionation factors during reductive dechlorination by Dhc195 grown under standard conditions to those subjected to four different growth conditions. These included: 1) trace amended B_{12} concentration (reduced from 100 µg/L to 3 µg/L), 2) forced dinitrogen fixation, 3) reduced incubation temperature (from 34°C to 22°C), and 4) exposure to 1.6 mM VC (a product of cDCE dechlorination that Dhc195 cannot metabolize for energy).

TCE degradation rates for each tested culture varied according to the applied growth condition (Table 5.1). Reducing the amended B_{12} concentration from 100 µg/L to less than 3 µg/L slowed the TCE degradation rate. Vitamin B_{12} is known to play an important role in the metabolism of Dhc as it serves as a corrinoid co-factor necessary for activity of the reductive dehalogenases, and must be obtained from the environment as Dhc cannot synthesize it *de novo* [Yi *et al.* 2012]. It was previously shown that Dhc195 cellular growth and TCE dechlorination rates significantly increased when the supplied B_{12} concentration was increased from 1 µg/L to 100 µg/L [He *et al.* 2007]. Because groundwater is often oligotrophic with low levels of nutrients, corrinoid co-factors may be growth-limiting substances in some subsurface environments.

Vinyl chloride exposure (1.6 mM) also slowed initial TCE degradation rates (Table 5.1) with 78 µmoles of TCE degraded in 24 days in the high VC condition versus 19 days in the standard condition. Due to the slower co-metabolic reduction of VC to ethene by the *tce*A RDase in strain 195, VC accumulation may occur in environments where contaminant transformation is catalyzed primarily by that enzyme and vinyl chloride reductive dehalogenases, such as *vcr*A or *bvc*A, are not present or active.

When forced to fix nitrogen, Dhc195 was previously shown to have significantly different metabolic and growth activities compared to ammonium-amended cultures, as observed by a decreased cell yield and VC production rate, earlier growth transition into stationary phase, and increased expression of the nitrogenase-encoding gene *nif*D [Lee *et al.* 2009]. Indeed, this is consistent with the known physiological stresses often induced by the energy-intensive nitrogen-fixation process, which potentially could occur in groundwater environments with limited aqueous fixed nitrogen. Finally, TCE dechlorination at 22ºC was much slower than the 34ºC standard condition, exhibiting a lag phase of approximately 16 days before degradation began and requiring over 40 days to transform 78 µmoles of TCE to VC and ethene. Because chloroethene-impacted groundwater in the United States is typically 10-20ºC and may have substantial variations depending on depth and season, examining the impact of temperature variation on the kinetic isotope fractionation, particularly at a reduced growth temperature, is important for interpreting *in situ* isotope data.

The standard growth experiment generated an enrichment factor of -13.3±0.7‰, while the four tested growth conditions: decreased B_{12}, nitrogen fixation, 22°C incubation, and elevated VC produced enrichment factors of -12.7±1.0‰, -14.4±0.8‰, -13.3±0.9‰, and -12.5±0.4‰, respectively. All tested growth conditions of the Dhc195 culture yielded enrichment factors within the uppermost and lowermost bounds of the 95% CI of the standard condition,

indicating that the altered growth conditions produced little to no significant variation in the quantified isotope fractionation. Interestingly, however, the TCE enrichment factor for the Dhc195 standard condition in this study (-13.3±0.7‰) differed significantly from that quantified by our lab and reported in Lee *et al.* 2007 (-9.6±0.4‰). These values represent two different fractionations observed in the same culture in which the same growth conditions were applied for both experiments and the same analytical protocols and equipment were used. This raises the possibility that the isotope fractionation could reflect physiological and genetic changes occurring in the organism as it adapts to sequentially sub-cultured growth in the laboratory over time. Unknown factors attributed to sample handling by different experimenters also cannot be ruled out. After isolation Dhc195 was initially grown with tetrachloroethene (PCE) in an undefined medium containing filtered sludge extract [Maymó-Gatell *et al.* 1997]. The isolate was being grown in a defined mineral medium under TCE amendment when the Lee *et al.* 2007 study was conducted and further propagated under those conditions to the time of this study. Given this change in cultivation conditions during prolonged isolated growth, physiological adaptation over time that might result in slightly increased TCE transport rates or faster enzyme kinetic steps preceding bond cleavage is possible. The reported abiotic carbon isotope fractionation of TCE by cyanocobalamin is -16.6‰ [Slater *et al.* 2003], which represents the intrinsic isotope fractionation produced by the cleavage of a carbon-chlorine bond. Corrinoid-catalyzed biological TCE fractionations that are closest in magnitude to this abiotic fractionation, such as those observed in the enrichment culture ANAS (-16.0±0.6‰) and for *Sulfurospirillum multivorans* (-16.4±1.5‰) [Lee *et al.* 2007], suggest that the rate-limiting step in the enzyme-catalyzed degradation pathways of these organisms is likely the high-energy cleavage of the carbon-chlorine bond. Smaller fractionations, such as those observed for Dhc195, may represent a masking of the intrinsic fractionation by one or more lower-energy kinetic steps that precede bond breakage. The increase in Dhc195 TCE fractionation from the 2007 study (Lee *et al.* 2007) to this one may indicate that rate changes have occurred in the degradation pathway whereby preceding kinetic steps have become faster relative to bond-breakage, shifting the rate-limitation onto the carbon-chlorine bond cleavage, and further revealing the intrinsic fractionation effect. Conformational movements in enzymes are often essential to their functions and can be slow relative to bond cleavage. It is possible these enzymatic motions have become faster in the *tce*A enzyme in Dhc195 relative to carbon-chlorine bond breakage, which would decrease their role in rate-limited catalytic throughput, and thus increase the observed isotopic fractionation. Increased rates of chloroethene transport through membrane structures and to catalytic sites might similarly increase the observed fractionation.

As temperature is known to affect a wide array of cellular processes and rate constants, enzymatic and other, it is somewhat surprising that the fractionation remained unchanged during dechlorination at a 12°C-lower (34°C versus 22°C) incubation temperature. A reduced incubation temperature could slow the rate of preceding kinetic processes relative to carbon-chlorine bond breakage, increasing the masking effect and yielding a lower overall fractionation (less negative enrichment factor). However, given the uniformity of the results between the two temperatures, masking effects do not appear to be significantly affected by this temperature reduction. Although this is the first report of a Dhc TCE fractionation factor measured at 22ºC, Schmidt *et al.* 2010 found that the isotopic fractionation during aerobic cDCE degradation in groundwater microcosms were also consistent over two tested temperature incubations (23 and 13ºC).

Limiting B_{12} in the growth medium of Dhc195 to 1 μg/L was previously shown to cause reductions in the TCE dechlorination rate and cell densities, and results suggested that the rate reduction was caused by decreased cell density rather than the weakening of individual cells [He *et al.* 2007]. However, microarray analyses showed that limited B_{12} caused differential regulation of a number of genes in Dhc195, including those encoding corrinoid transport and synthesis-related proteins, as well as putative cobalamin riboswitches [Johnson *et al.* 2009]. Differences in co-factor transport and up-take could lead to changes in RDase activity rates, leading to differences in isotopic fractionation. However, quantified isotope fractionations were the same between high and low B_{12} concentrations, suggesting that differences in corrinoid salvaging efforts by Dhc195 do not cause enzymatic changes that lead to significant differences in the bulk isotopic fractionation.

Previous transcriptomic and proteomic analyses confirm that a wide array of strain 195's membrane-associated transporters and electron carriers are differentially regulated during nitrogen-fixation [Lee *et al.* 2012]. However, nitrogen fixation did not produce a change in the TCE isotope fractionation of Dhc195, indicating that the regulatory changes caused by N_2-fixation did not impact TCE fractionation. Finally, the TCE fractionation factor was also not affected by exposure to high VC concentrations, despite the significantly decreased TCE degradation rate observed in this study.

5.3.2 Effects of Co-Culture Growth on TCE Fractionation by Dhc195

TCE fractionation was also quantified during dechlorination by two syntrophic co-cultures in order to understand potential impacts of interspecies substrate transfer on isotope fractionation when Dhc195 receives its hydrogen and carbon source from fermenting organisms. Initial dechlorination rates in the tested co-cultures were generally greater than that observed for the Dhc195 isolate in standard condition, consistent with previous observations that Dhc growth with fermenting organisms is more robust than growth alone [Men *et al.* 2011]. In this experiment, initial dechlorination rates for the Dhc195/*S. wolfei* and Dhc195/DVH co-cultures grown on 5mM lactate and the Dhc195/*S. wolfei* co-culture fed crotonate were higher than the Dhc195 isolate (Table 5.1).

TCE fractionation by Dhc195/DVH with 5 mM lactate generated an enrichment factor of -12.6±2.0‰, while Dhc195/*S. wolfei* produced enrichment factors of -10.4±1.2‰ and -13.5±1.0‰, when provided 5 mM lactate or 20 mM crotonate, respectively. Only the Dhc/*S. wolfei* co-culture with 5 mM lactate produced an enrichment factor (-10.4±1.2‰) slightly outside the range of the standard growth control (-13.3±0.7‰), however the difference is similar to the magnitude of the confidence interval for many tested cultures, suggesting that the presence of syntrophic organisms had little effect on the quantified fractionation in these experiments.

5.3.3 Effects of Growth Condition on TCE Fractionation by Enrichment Cultures

TCE fractionation was quantified in dechlorinating microbial communities before and after a two-year enrichment period performed with 3 different growth conditions: high TCE amendment (HiTCEB12), low TCE amendment (LoTCEB12), and no exogenous B_{12} amendment (HiTCE). Determining whether fractionation values are affected by disparate growth conditions is important for the application of laboratory-generated values to field environments where varying conditions occur. In the Dhc-containing communities, the high and low TCE amendments were provided to inhibit or promote methanogenic activity, respectively [Men *et al.* 2013]. Accordingly, the abundance of total Archaea was two orders of magnitude lower in LoTCEB12 than HiTCE and HiTCEB12, and the abundance of microorganisms of the genera *Pelosinus*, *Dendrosporobacter*, and *Sporotalea* was greater in the LoTCEB12 condition [Men *et al.* 2013]. Microarray analysis and 16S rRNA sequencing of these cultures have confirmed the presence of a single dechlorinating Dhc strain which is closely related to Dhc195 in genetic composition and physiology [Men *et al.* 2013]. The cultures were shown to possess only one of the functionally-confirmed RDase genes, *tce*A, and its copy numbers correlate closely to Dhc 16S rDNA copy numbers in each enrichment [Men *et al.* 2013]. A previously-performed physiological characterization of these enrichments indicated that similar Dhc cell yields were achieved among all cultures. Although varying initial TCE dechlorination rates were observed for the Dhc enrichments in this study, they were similar to the rates observed in the co-cultures and generally greater than those of the isolates (Table 5.1). In a subsequent experiment, non-methanogenic culture HiTCE was starved of lactate for approximately 20 days, generating HiTCELowLac, a culture with an initial TCE degradation rate similar to the other Dhc enrichments, but whose rate slowed significantly after approximately 4 days, and stalled at 90% TCE degradation after 20 days (Figure A5.17 in the Appendix), suggesting the dechlorination reaction was limited by the available hydrogen.

The isotope fractionations were -8.9±0.4‰, -8.7±1.3‰, -9.4±0.7‰, and -6.8±0.8‰ for the LoTCEB12/Parent, HiTCEB12, HiTCE, and LoTCEB12 conditions, respectively (Table 5.1, Figure 5.2). The fractionation factors for all enrichment community conditions are lower than those generated by the Dhc195 isolate and co-cultures. The enrichment factors of the LoTCEB12 and LoTCEB12/Parent cultures (-6.8±0.8‰ and -8.9±0.4‰) are outside of each other's CIs; however the difference in values (2.1‰) approaches the magnitude of the confidence interval found for some Dhc cultures (Table 5.1). The TCE fractionation also remained consistent despite the post-enrichment growth change applied to the non-methanogenic, B_{12}-amended culture (HiTCEB12/LowLac, -7.2±0.3‰). This suggests that even limiting Dhc-growth substrates enough to slow degradation does not produce differences in TCE fractionation. The largest difference between fractionation factors in the Dhc enrichments is a relatively small 2.4‰ (HiTCEB12/LowLac and HiTCE), indicating that both a post-enrichment applied growth stress and a long-term enrichment of this community under different culturing pressures only minimally affect the isotope fractionation. Enrichment factors remained consistent over a two year enrichment period despite active or inhibited methanogenesis and the absence of exogenously supplied vitamin B_{12}. Although fractionation differences were observed over time for the Dhc195 pure culture (this study and Lee *et al.* 2007), it is possible the adaptation response of Dhc in a community may be more muted than that in a pure culture due to the stabilization and optimization of its growth environment by supportive micoorganisms.

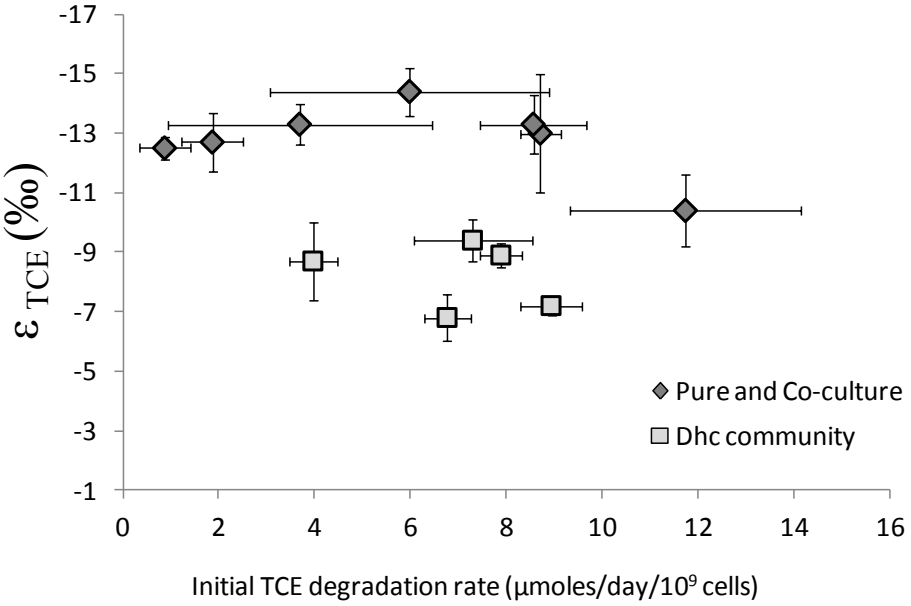

Figure 5.3. TCE enrichment factors plotted against the cell-normalized initial TCE degradation rate for each growth condition according to culture type (22ºC condition not plotted). In this study there is no significant correlation between degradation rate and the TCE enrichment factor. Vertical error bars represent a 95% confidence interval, while horizontal error bars represent the degradation rate's standard deviation for biological triplicates.

Although TCE degradation rates varied significantly among the tested cultures (Table 5.1), the magnitude of the generated enrichment factors did not correlate with the magnitude of the dechlorination rates (Figure 5.3). This indicates that in the growth conditions tested, the quantified isotopic fractionation likely did not reflect a mass-transfer limited masking process. Although there are no significant differences in fractionation among the tested growth conditions of the isolate, co-cultures, or the enriched communities, there is a difference between the fractionation factors of Dhc195 and the enrichment communities. In fact, most Dhc195 and enriched culture enrichment factors were well outside of each other's 95% CIs, with differences of up to 7.6‰, suggesting that the fractionation is strain-specific. This is especially interesting given that both the Dhc195 and the enrichment cultures utilize *tce*A for dechlorination. In fact, the Dhc enrichment strain is closely related to Dhc195 in behavior and genomic content and shares over 80% percent of its genes with the isolate, including most genes related to corrinoid uptake and remodeling [Men *et al.* 2013]. Furthermore, cobalamin has been shown to be the preferred corrinoid form for TCE dechlorination by the Dhc enrichment strain. Excess cobalamin (3-4 µg/L) has been detected in cultures without exogenous B_{12} amendment (HiTCE) [Men *et al.* 2013], indicating that it is produced by other microorganisms in the community and utilized by Dhc for growth. Although Dhc195 can utilize alternative corrinoid forms [Yi *et al.* 2012], cobalamin appears to be the favored co-factor as cell yields and dechlorination rates are greatest relative to six other corrinoids when they are each provided separately in culture growth medium [Yi *et al.* 2012]. Yet, the difference between the strains' observed enrichment factors is sufficient to result in substantial variations in the calculated extent of biotransformation (overestimation by

up to 30% for an isotopic shift of 10‰) [Elsner *et al.* 2005]. This supports the notion that a compound's enrichment factor is difficult to predict for a given organism and enzyme based on genetic or physiological similarity to another organism [Lee *et al.* 2007, Fletcher *et al.* 2011, Cichocka *et al.* 2008]. Because the fractionation was shown to be consistent under a variety of environmental conditions, TCE degradation rates, and culture compositions, the variation in ε values between Dhc strains is likely caused by one or more unique structural features of the specific strains. These fractionation differences could represent small variations in cell membrane structure and composition which could lead to varying rates of TCE uptake and transport to enzymatic sites, or slight differences in RDase folding and structure which might lead to differences in enzyme-substrate binding configurations and access to catalytic sites. Variations in slower kinetic processes that precede carbon-chlorine bond cleavage could serve to decrease the overall extent of fractionation. The results of this study show that closely related organisms may possess cellular kinetic processes that are sufficiently different to produce distinct isotope fractionations.

5.4 Environmental Implications

In summary, the carbon isotope fractionation of TCE during reductive dechlorination by Dhc195 remained unchanged despite a variety of different growth conditions in both pure and co-cultures. The fractionation was also similar and reproducible for numerous tested growth conditions of a highly enriched Dhc-containing community. This study is the first to systematically investigate the effects of growth condition on the TCE isotope fractionation by Dhc and demonstrates the relative stability of enrichment factors under temperature variation, co-factor limitation, nitrogen fixation, elevated VC concentrations, and various enrichment strategies applied to mixed communities in the laboratory. These results suggest that although enrichment factors may depend on a site-specific Dhc strain, once the fractionation for that strain has been established for a given site it is unlikely to change under varying environmental conditions, such as nutrient depletion, product accumulation, or seasonal temperature fluctuations. This study allows for increased confidence in the coupling of isotope data to reactive transport models that may be applied at a given site under a variety of environmental conditions with a single enrichment factor. An enrichment factor that remains unchanged despite a variety of growth conditions leads to better predictive modeling under various potential environments and makes the application of these models to estimate field biodegradation from *in situ* isotope data more robust.

However, this study provides evidence that TCE fractionation can be strain-dependent and difficult to predict from knowledge of operating enzymes and physiologic or genetic similarity to other organisms. Dhc strains harboring the same functional dehalogenase may produce significantly different fractionations in closely related organisms. This study suggests that microcosm experiments constructed from site-specific soil or groundwater would be the most effective approach for predicting TCE dechlorination enrichment factors. Alternatively, the best state of practice may be in choosing an enrichment factor within the range of those published for a given chloroethene that most conservatively estimates the extent of *in situ* biotransformation.

CHAPTER 6: Summary and Conclusions

6.1 Summary

The research described in this dissertation investigated the fate and biotransformation of trichloroethene (TCE) and aqueous film-forming foams (AFFFs) under the aerobic and anaerobic conditions encountered at contaminated firefighter training sites. The results of these studies suggest that complex microbial interactions occur at AFFF-impacted sites and that the dynamic biotransformation of TCE and polyfluoroalkyl substances (PFASs) may be closely linked to not only the redox condition but also to the type and amound of AFFF. In Chapter 2 it was shown that 4:2, 6:2, and 8:2 fluorotelomer thioamido sulfonate (FtTAoS), present in several widely used AFFF formulations, aerobically biotransform to persistent perfluorinated carboxylic acids. The rate of perfluorocarboxylate (PFCA) formation was generally slow relative to FtTAoS disappearance, indicating that the accumulation and slow transformation of intermediate compounds occurred. In Chapters 3 and 4, the effects of various PFASs and AFFF formulations were tested with an anaerobic TCE-dechlorinating microbial community. In Chapter 3 it was shown that low concentrations of PFAAs had no impact on the TCE dechlorination rate in the presence of soil. However, in soil-free cultures, dechlorination was inhibited in the presence of high concentrations of PFAAs. In Chapter 4 it was shown that although the microbial communities were capable of fermenting AFFF carbon solvents to hydrogen and acetate to support Dhc, TCE dechlorination was inhibited in the presence of two of the three formulations. The effects of various PFASs and perfluroalkyl acids (PFAAs) on TCE dechlorination were further investigated in Chapter 4. Generally, dechlorination occurred in the presence of higher concentrations of PFCAs and perfluorosulfonic acids (PFSAs), while it was inhibited by lower concentrations of an AFFF-derived PFAS, 6:2 FtSaB. Finally, in Chapter 5, the stable carbon isotope fractionation of TCE was investigated under varying growth conditions of *Dehalococcoides* (Dhc). Although the fractionation did not change according to growth condition, the study provided evidence that the fractionation is Dhc-strain specific.

6.2 Biotransformation of Fluorotelomer Thioamido Sulfonate in AFFF

In Chapter 2, the aerobic biotransformation pathways of 4:2, 6:2, and 8:2 FtTAoS were characterized by determining the fate of the compounds in soil microcosms amended with an AFFF solution. FtTAoS is the principle PFAS in AFFF formulations made by at least three different manufacturers and was historically applied at firefighter-training areas on various U.S. military bases. The biotransformation of FtTAoS occurred in live culture microcosms and produced 4:2, 6:2, and 8:2 FtS, 6:2 FtUCA, 5:3 FtCA, and C_4 to C_8 PFCAs. Two additional biotransformation products corresponding to singly and doubly oxygenated forms of 6:2 FtTAoS, were also identified through mass spectra analysis and high resolution MS. An oxidative assay was used to quantify precursors of perfluoroalkyl acids in order to check the material balance by indirectly quantifying the total concentration of polyfluorinated compounds. The assay produced nearly complete mass recovery after FtTAoS biotransformation, with 10% (mol/mol) of the amended FtTAoS accounted for in FtS, FtCA, and PFCA products. The transformation rates of identified products appear to be slow relative to FtTAoS, indicating that some intermediates may persist in the environment. The results of this study help to explain the occurrence of FtS and PFCA compounds in groundwater and soil at AFFF-impacted sites, and suggest the potential for significant quantities of intermediate compounds that are not routinely analyzed to form during biotransformation of PFASs in AFFF.

6.3 Effects of AFFF and PFASs on TCE dechlorination

The application of AFFF to extinguish chlorinated solvent-fueled fires has led to the widespread co-contamination of PFASs and TCE in groundwater and soil. Reductive dechlorination of TCE to ethene by Dhc is a frequently used *in situ* remediation strategy at many contaminated sites; however, the effects of AFFF and PFASs on TCE dechlorination by anaerobic microbial communities have not been examined.

In the study presented in Chapter 3, varying concentrations of PFAAs were tested on a Dhc-containing enrichment community. In the presence of soil, 50 μg/L each of a suite of 11 PFAAs had no impact on TCE dechlorination compared to a control culture containing no PFAAs. The cell-normalized TCE degradation rates were identical, and chloroethene product formation remained unchanged between the two culture conditions. In a soil-free planktonic culture, 1 mg/L each of the same suite of 11 PFAAs had no effect on TCE dechlorination, while 10 mg/L of each PFAA completely inhibited dechlorination. This study also indicated that the calculated organic-carbon distribution coefficients (K_{oc}) for the soil-containing dechlorination communities were not significantly impacted by the presence of 45 mg/L TCE or the inoculated Dhc culture. These results indicate that TCE dechlorination may only be impacted in highly-contaminated groundwater and soil sites, where the total concentration of PFAAs exceeds 100 mg/L. This also suggests that documented PFAA K_{oc} values can be used to predict PFAA groundwater transport in systems containing non-NAPL TCE undergoing bioremediation.

In the study presented in Chapter 4, three AFFF formulations were amended to the growth medium of a Dhc-containing enrichment community to determine their impacts on TCE dechlorination. The communities were capable of fermenting carbon solvents in all AFFFs to hydrogen and acetate when the foams were amended as the sole source of carbon and energy, but the product concentrations varied significantly according to formulation. The fermentation of diethylene glycol butyl ether (DGBE), the primary glycol ether solvent in most AFFFs, when amended alone produced less hydrogen and acetate than AFFF-amended experiments, suggesting that smaller quantities of other organic carbon substances in the foams may be more easily fermentable. TCE was dechlorinated in the presence of one AFFF formulation, while no dechlorination occurred in the presence of two other formulations. In PFAS-amended experiments, 16 mg/L 6:2 fluorotelomer sulfonamido betaine (6:2 FtSaB) slowed TCE dechlorination while 32 mg/L FtSaB completely inhibited dechlorination, suggesting dechlorination did not occur in one AFFF-amended experiment due to the presence of its most abundant PFAS. In cultures amended with PFAAs, 110 mg/L total PFSAs did not inhibit TCE dechlorination, while 110 mg/L total PFAAs did, suggesting that inhibition is dependent on PFAS structure as well as concentration. This study revealed a dynamic interplay between AFFF and TCE bioremediation communities that may be highly dependent on the AFFF formulation and composition.

6.4 Impact of Varying Growth Conditions on TCE Isotope Fractionation

To quantify *in situ* bioremediation using compound specific isotope analysis (CSIA), isotope fractionation data obtained from the field is interpreted according to laboratory-derived enrichment factors. Although previous studies that have quantified dynamic isotopic shifts during the reductive dechlorination of TCE indicate that fractionation factors can be highly variable from culture-to-culture and site-to-site, the effects of growth condition on the isotope fractionation during reductive dechlorination have not been previously examined. Here, carbon isotope fractionation by *Dehalococcoides mccartyi* 195 (Dhc195) maintained under a variety of growth conditions was examined. Enrichment factors quantified when Dhc195 was subjected to four sub-optimal growth conditions, including decreased temperature (-13.3±0.9‰), trace vitamin B_{12} availability (-12.7±1.0‰), limited fixed nitrogen (-14.4±0.8‰), and elevated vinyl chloride exposure (-12.5±0.4‰), indicate that the fractionation is similar across a range of tested conditions. The TCE enrichment factors for two syntrophic co-cultures, Dhc195 with Desulfovibrio vulgaris Hildenborough (-13.0±2.0‰) and Dhc195 with Syntrophomonas wolfei (-10.4±1.2‰ and -13.3±1.0‰), were also similar to a control experiment. In order to test the stability of enrichment factors in microbial communities, the isotope fractionation was quantified for Dhc-containing groundwater communities before and after two-year enrichment periods under different growth conditions. Although these enrichment factors (-8.9±0.4‰, -6.8±0.8‰, -8.7±1.3‰, -9.4±0.7‰, and -7.2±0.3‰) were predominantly outside the range of values quantified for the isolate and co-cultures, all tested enrichment conditions within the communities produced nearly similar fractionations. Enrichment factors were not significantly affected by changes in any of the tested growth conditions for the pure cultures, co-cultures or the mixed communities, indicating that despite a variety of temperature, nutrient, and co-factor-limiting conditions, stable carbon isotope fractionations remain consistent for given *Dehalococcoides* cultures.

6.5 Future Research Recommendations

Thus far, relatively few studies have examined the biotransformation of PFASs in AFFF. With the exception of 6:2 FtSaB biotransformation observed in aquatic organisms [Moe *et al.* 2003] and the FtTAoS biotransformation characterized in microbial cultures in this study and briefly in Weiner *et al.* 2013, no other biotransformation pathways for the AFFF PFASs shown in Figure 1.5 have been reported. Elucidating the transformation pathways for the perfluorosulfonamide compounds in 3M AFFF is particularly important as 3M AFFF accounts for up to 75% of the AFFF stored on U.S. military bases, and there are currently no restrictions on the future use of stockpiled AFFF [Place *et al.* 2012, Darwin 2004]. The low concentrations of perfluorsulfonamides compounds detected in contaminated groundwater and sediments where the historical application of 3M AFFF occurred [Houtz *et al.* 2013, Backe *et al.* 2013] suggest that these compounds may undergo *in situ* abiotic or biological transformation.

The environmental and growth factors that control the rate of PFAS biotransformation must also be investigated in order to develop reactive transport models that can predict *in situ* PFCA production rates. Although Kim *et al.* [2012 and 2013] found that certain FtOH pathways in pure cultures are preferred depending on the presence of an enzyme-inducing substrate or reducing agent, similar conditions should also be tested on mixed cultures to simulate the synergistic activities among organisms in a community. Preliminary data obtained from

microcosms amended separately with FtTAoS, DGBE, and AFFF suggest that while a labile carbon source enhances the biotransformation rate of FtTAoS, transformation still occurs in the absence of an exogenous carbon source in the microbial community. This result is consistent with the study presented in Chapter 2 where FtTAoS transformation continued to occur for more than 15 days after all AFFF-carbon sources had been depleted. However, if complete FtTAoS biotransformation to PFCAs represents the diverse activities of multiple groups of microorganisms in a soil community, certain carbon and nutritional requirements may be needed by microorganisms executing one or more steps in the transformation pathway. Thus, the factors controlling individual FtTAoS transformation steps could be determined by adding certain intermediates, such as 6:2 FtS and 6:2 FtUCA, as the initial amended substrate to soil communities cultured under varying growth conditions.

Finally, the search for an organism or culture that can metabolically detoxify perfluoroalkyl compounds through reductive defluorination should be continued and expanded. Prior to the 1980s, PCE and TCE were largely considered to be recalcitrant in the environment and toxic to microorganisms, especially in anoxic environments; however, following the discovery and characterization of chloroethene-degrading cultures, including those that respire the compounds and link the degradation to their growth, PCE and TCE are now considered to be readily biodegradable in groundwater given the appropriate redox conditions. As pointed out in Parsons *et al*. 2008, thermodynamic estimations suggest that energy yield following reductive defluorination of a perfluorinated compound is achievable, and thus theoretically possible. The enrichment of aquifer sediments and groundwater from contaminated sites possessing a long history of AFFF application likely would yield the best chances for obtaining a culture capable of PFAS reductive defluorination. The development of a robust bioaugmentation culture capable of PFOS or PFOA degradation would be a significant advance for the remediation of fluorochemicals in contaminated groundwater.

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Appendix: Chapter 2

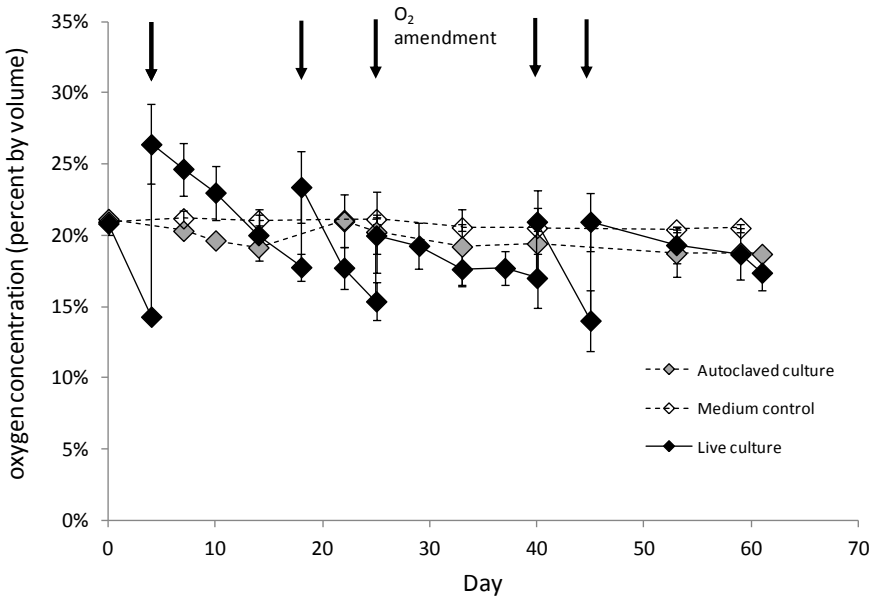


Figure A2.1. Oxygen concentrations in microcosm bottles. Oxygen amendments denoted by the arrows occurred in live microcosms only. Error bars represent the standard deviation of triplicate microcosm bottles.

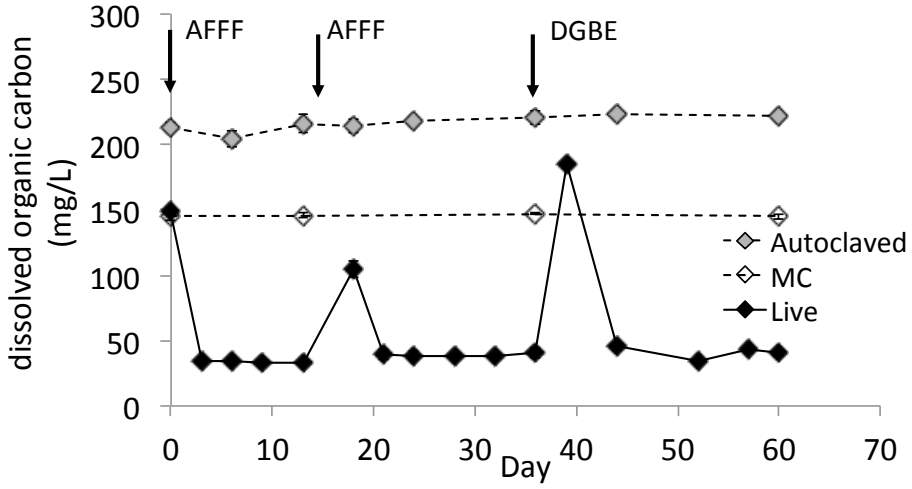


Figure A2.2. Dissolved organic carbon concentrations in microcosms. AFFF and DGBE amendments denoted by the arrows occurred in live microcosms only. Error bars represent the standard deviation of triplicate microcosm bottles. MC = medium control.

Table A2.1. Monitored ion transitions, MS conditions, and internal standard for 4:2 FtS on Agilent LC-MS/MS.

Compound	Internal Standard	Molecular Ion	Fragmentor Voltage (V)	Quantifier Ion (m/z)	Collision Energy (V)	Qualifier Ion (m/z)	Collision Energy (V)	Polarity
4:2 FtS	[$^{13}C_2$] 6:2 FtS	327	135	307	20	81	20	Negative

Table A2.2. Monitored ion transitions, MS conditions, and internal standards for Shimadzu Nexera X2 UHPLC / ABSciEX 5500 Triple Quad MS system.

Compound	Internal Standard	Molecular Ion	Decluster Potential (V)	Quantifier Ion (m/z)	Collision Energy (V)	Collision Cell Exit Potential (V)	Qualifier Ion (m/z)	Collision Energy (V)	Collision Cell Exit Potential (V)	Polarity
5:3 FtCA (FPePA)	[$^{13}C_2$] 6:2 FtS	341	50	237	20	15				Negative
7:3 FtCA (FHpPA)	[$^{13}C_4$] PFOA	441	55	337	30	25	317	18	13	Negative
6:2 FtCA (FHEA)	[$^{13}C_2$] 6:2 FtS	377	35	293	22	25	137	12	15	Negative
8:2 FtCA (FOEA)	[$^{13}C_5$] PFNA	477	60	393	20	15	217	28	15	Negative
6:2 FtUCA (FHUEA)	[$^{13}C_2$] 6:2 FtS	357	40	293	16	11	121	50	13	Negative
8:2 FtUCA (FOUEA)	[$^{13}C_5$] PFNA	457	40	393	20	15	343	52	31	Negative

Table A2.3. Solvent gradient program used for FtCA quantification on Simadzu Nexera X2 UHPLC / ABSciEX 5500 Triple Quad MS system.

Time (min)	% MeOH
0.1	30
1	50
3.5	80
4	90
5	90
6	30
7	30

Quantification of 4:2 and 8:2 FtTAoS for which authentic standards were not available: For the quantification of 6:2 FtTAoS, a commercial source material was available. To quantify 4:2 and 8:2 FtTAoS, the raw instrument responses for the compounds were first normalized to the instrument response of the mass labeled-6:2 FtS internal standard. This response ratio was then applied to the calibration curve obtained for 6:2 FtTAoS. This is delineated in Table S4.

Table A2.4. Calibration parameters and quantification of FtTAoS on Agilent LC-MS/MS.

Analyte	Calibration Range μg/L	Number of calibration point	R^2	Internal Standard
4:2 FtTAoS	*calculated using 6:2 FtTAoS calibration curve*			$[^{13}C_2]$ 6:2 FtS
6:2 FtTAoS	0.5 - 10	5	>0.95	$[^{13}C_2]$ 6:2 FtS
8:2 FtTAoS	*calculated using 6:2 FtTAoS calibration curve*			$[^{13}C_2]$ 6:2 FtS

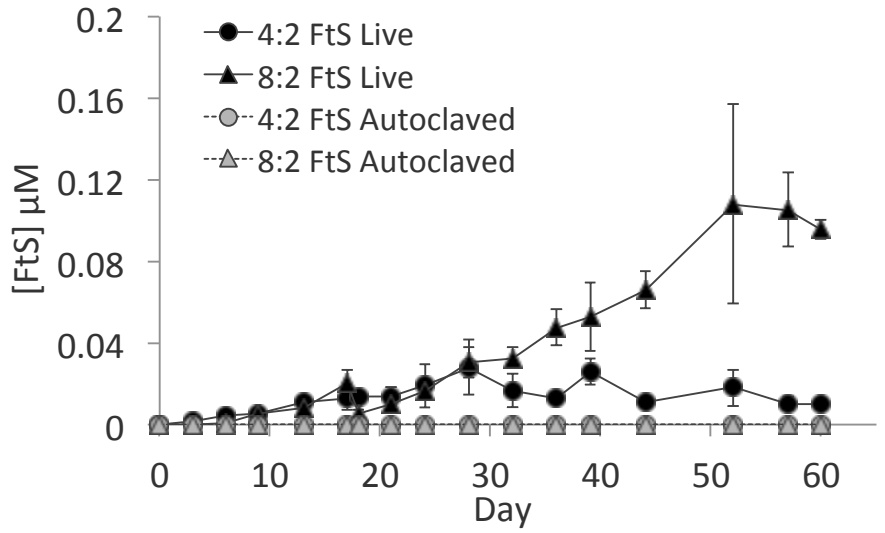


Figure A2.3. 4:2 and 8:2 FtS concentrations in microcosms. Error bars represent the standard deviation of triplicate microcosm bottles.

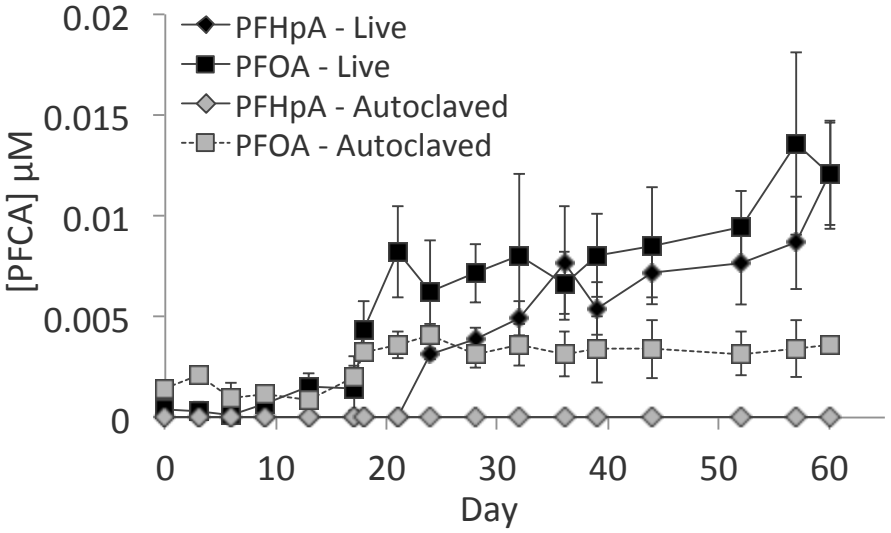


Figure A2.4. PFHpA and PFOA concentrations in microcosms. Note y-axis scale on this plot is one-tenth that of Figure S3. Error bars represent the standard deviation of triplicate microcosm bottles.

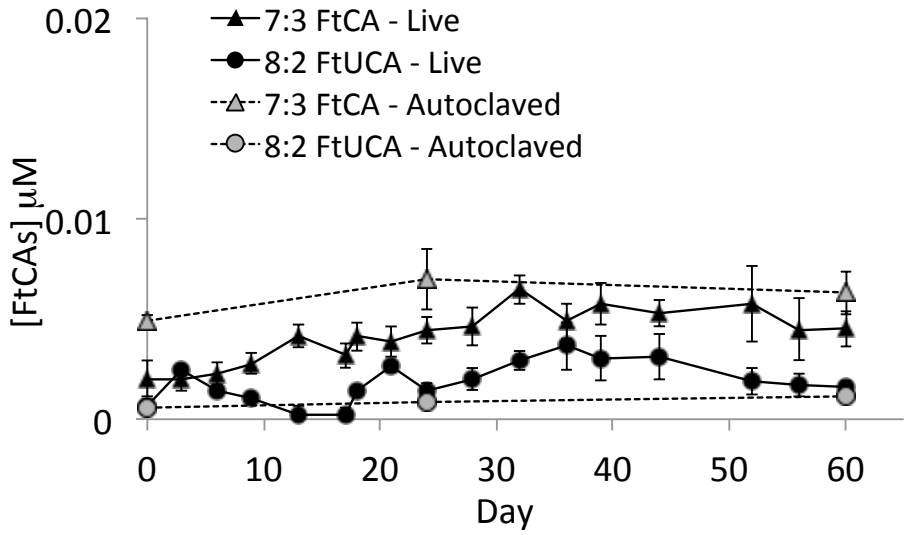


Figure A2.5. Concentrations of 7:3 FtCA and 8:2 FtUCA in microcosms. Error bars represent the standard deviation of triplicate microcosm bottles.

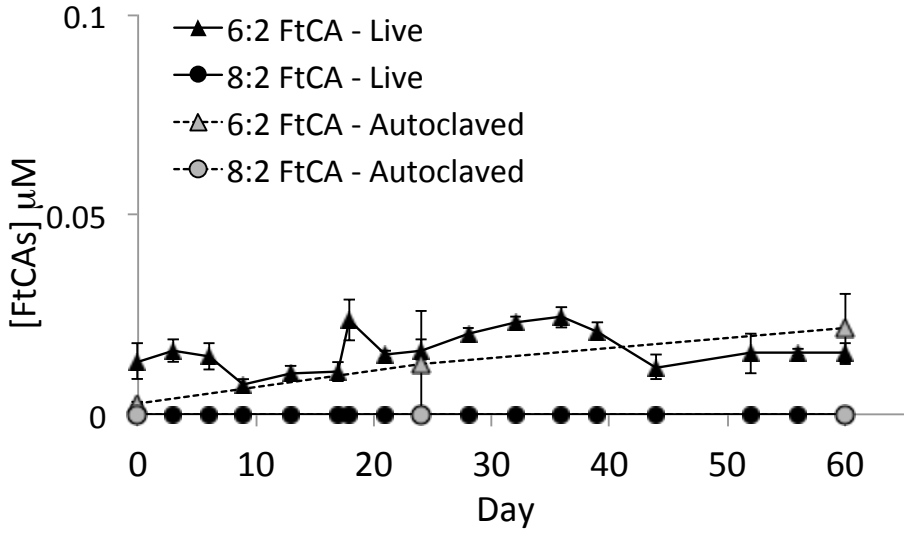


Figure A2.6. Concentrations of 6:2 FtCA and 8:2 FtCA in microcosms. . Error bars represent the standard deviation of triplicate microcosm bottles.

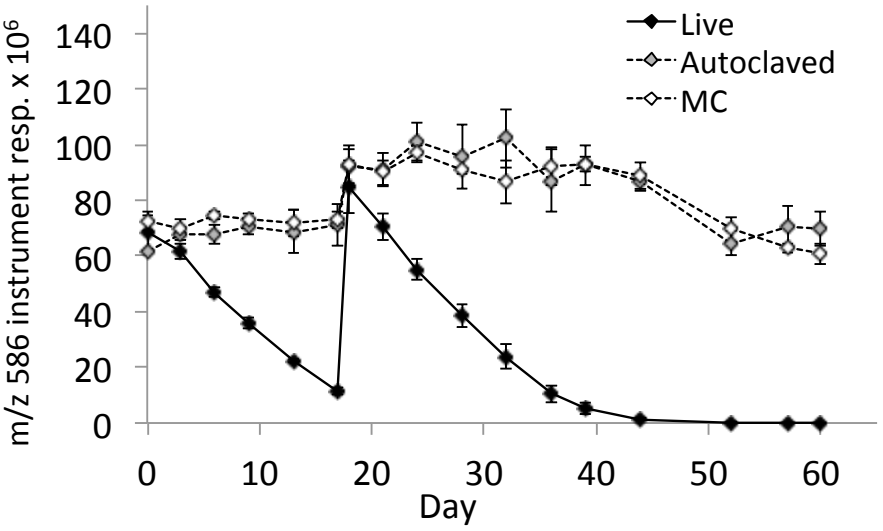


Figure A2.7. Average LC/MS-MS response of molecular ion 586 (6:2 FtTAoS) at a retention time of 8.0 minutes, normalized to the response for the mass labeled-6:2 FtS internal standard. Error bars represent the standard deviation of triplicate bottles. MC is medium control.

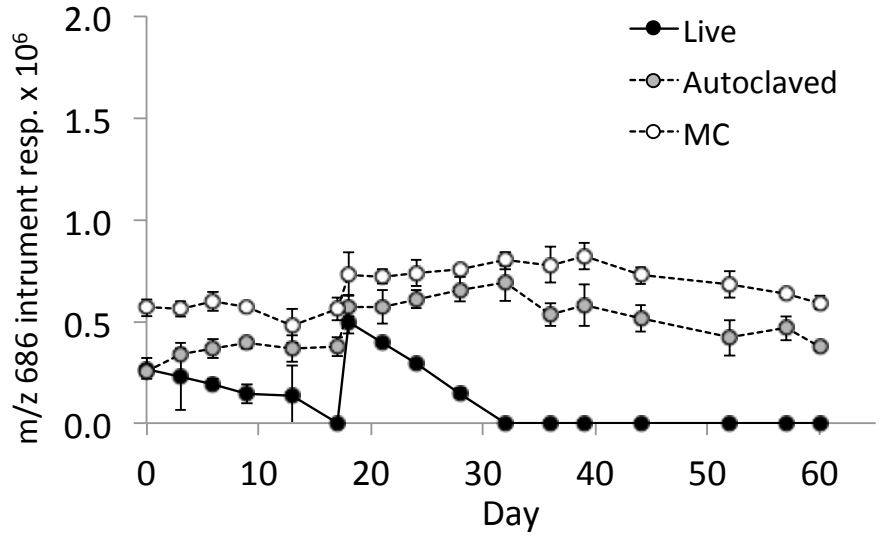


Figure A2.8. Average LC/MS-MS response of molecular ion 686 (8:2 FtTAoS) at a retention time of 8.1 minutes, normalized to the response of the mass labeled-6:2 FtS internal standard. Error bars represent the standard deviation of triplicate bottles. MC is medium control.

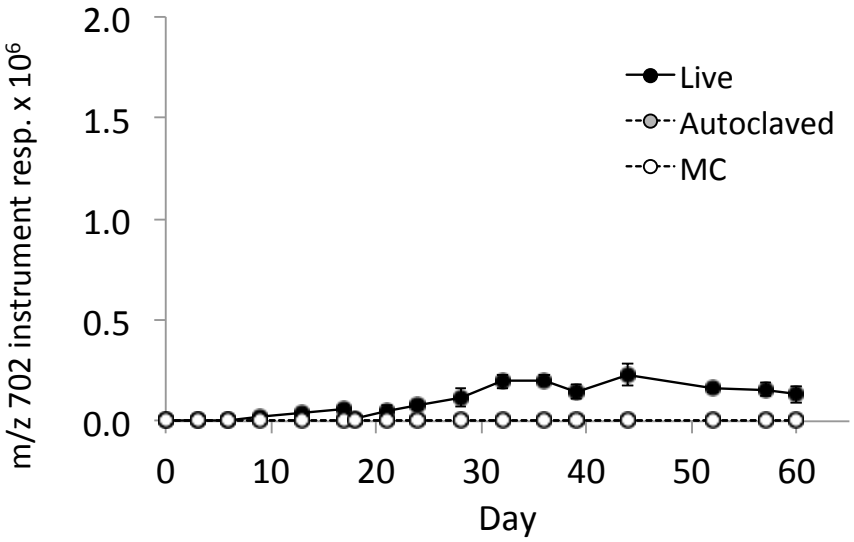


Figure A2.9. Average LC/MS-MS analyte response of molecular ion 702 at a retention time of 8.5 minutes, normalized to the response of the mass labeled-6:2 FtS internal standard. Error bars represent the standard deviation of triplicate bottles. MC is medium control.

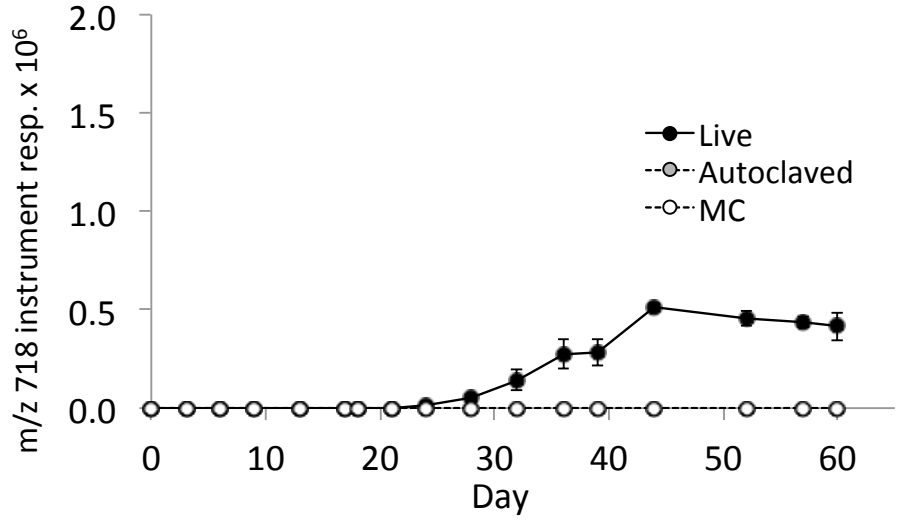


Figure A2.10. Average LC/MS-MS response of molecular ion 718 at a retention time of 8.6 minutes, normalized to the response of the mass labeled-6:2 FtS internal standard. Error bars represent the standard deviation of triplicate bottles. MC is medium control.

Table A2.5. Measured mass, theoretical mass, and mass accuracy of intermediate products identified by high resolution mass spectrometry. Retention time of compounds refers to LC/MS-MS elution time.

Compound	m/z	Retention Time, minutes	Composition	Measured Mass	Theoretical Mass	Mass Accuracy, ppm
6:2 $FtSO_2AoS$	618	8.1	$C_{15}H_{17}O_6NF_{13}S_2$	618.0276	618.0295	-3.11
6:2 FtSOAoS	602	8.0	$C_{15}H_{17}O_5NF_{13}S_2$	602.0329	602.0346	-2.84
8:2 $FtSO_2AoS$	718	8.6	*NA*			
8:2 FtSOAoS	702	8.5	*NA*			

NA: Masses could not be confirmed with HRMS.

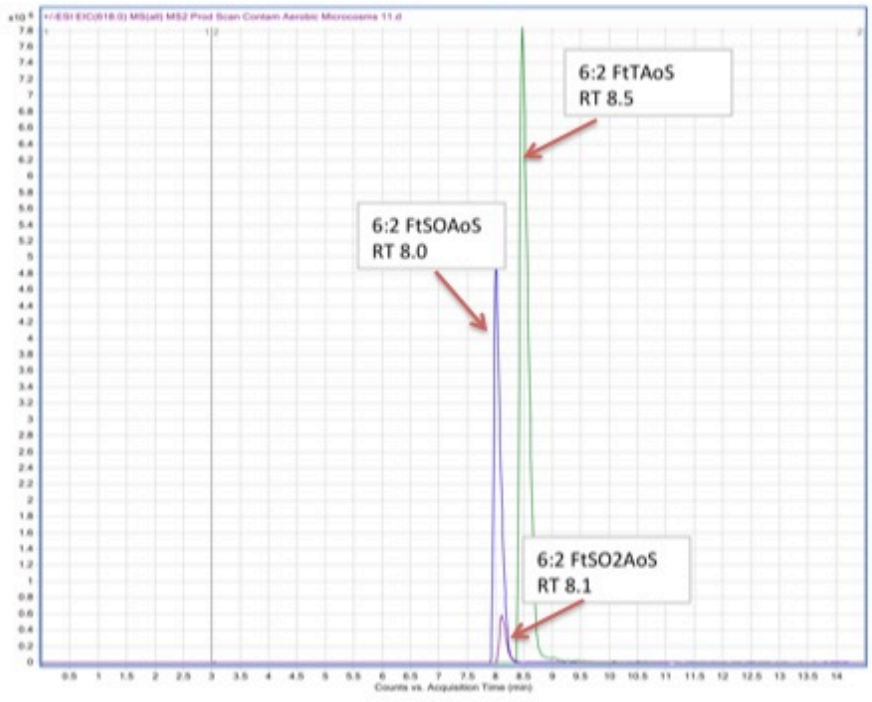


Figure A2.11. Chromatogram of 6:2 FtTAoS (m/z 586), 6:2 FtSOAoS (m/z 602), and 6:2 $FtSO_2AoS$ (m/z 618) molecular ions in a live microcosm on day 29 of incubation.

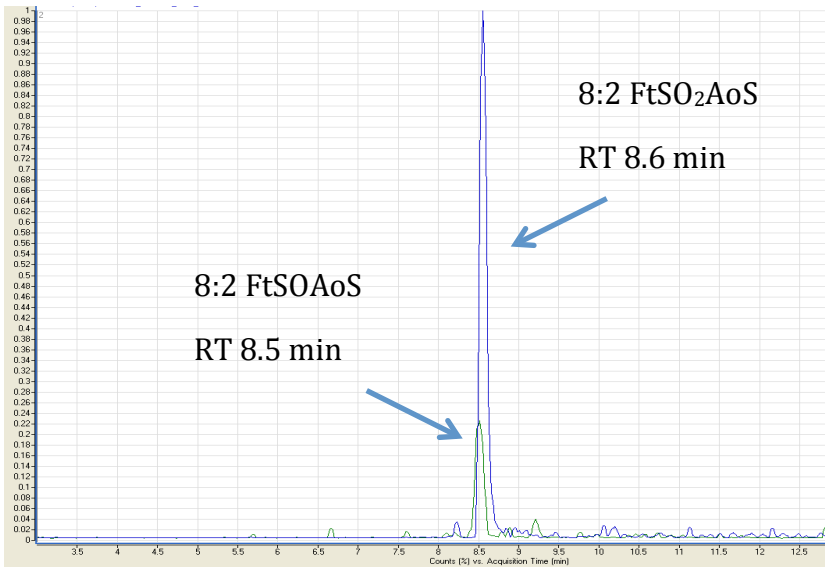


Figure A2.12. Chromatogram of 8:2 FtSOAoS (m/z 702), and 8:2 $FtSO_2AoS$ (m/z 718) molecular ions in a live microcosm on day 60 (8:2 FtTAoS, m/z 686, was not detected at the end of the incubation).

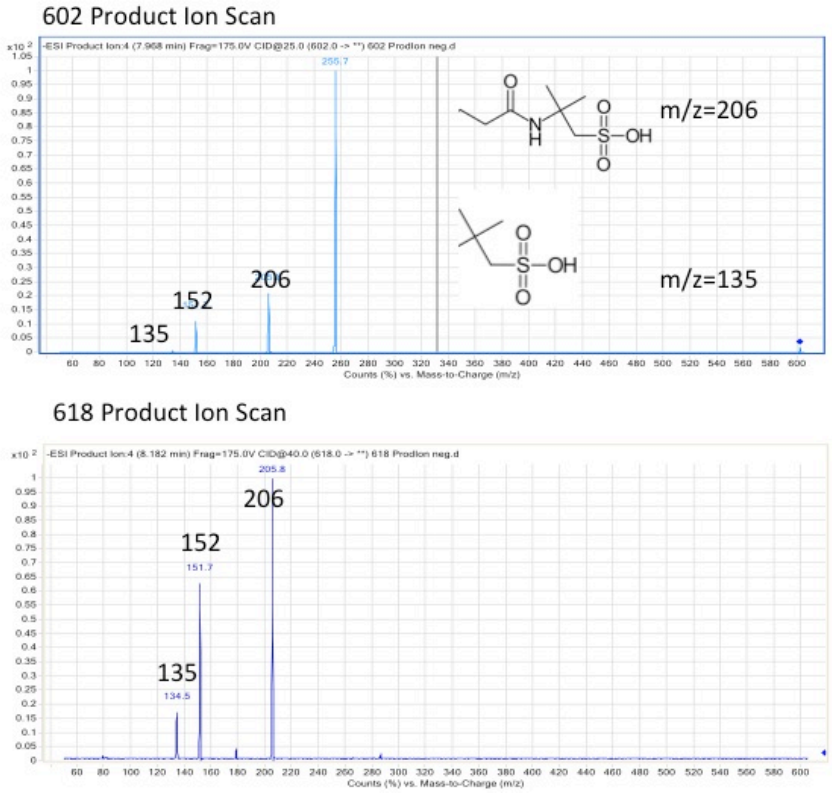


Figure A2.13. Product ion scans of m/z 602 (6:2 FtSOAoS) and 618 (6:2 $FtSO_2AoS$) and proposed structures of daughter ions.

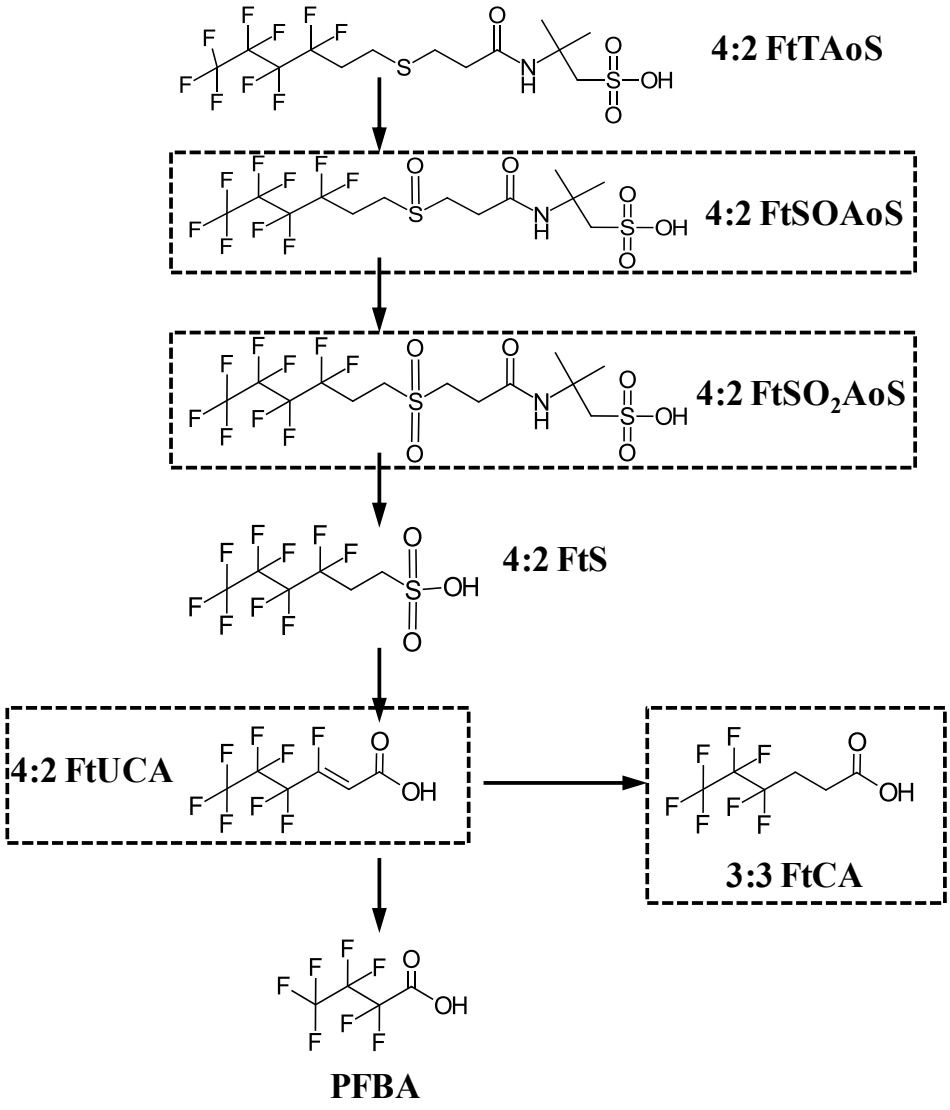


Figure A2.14. Proposed 4:2 FtTAoS biotransformation pathway. Compounds in dotted boxes are proposed biotransformation products that were not detected in microcosms.

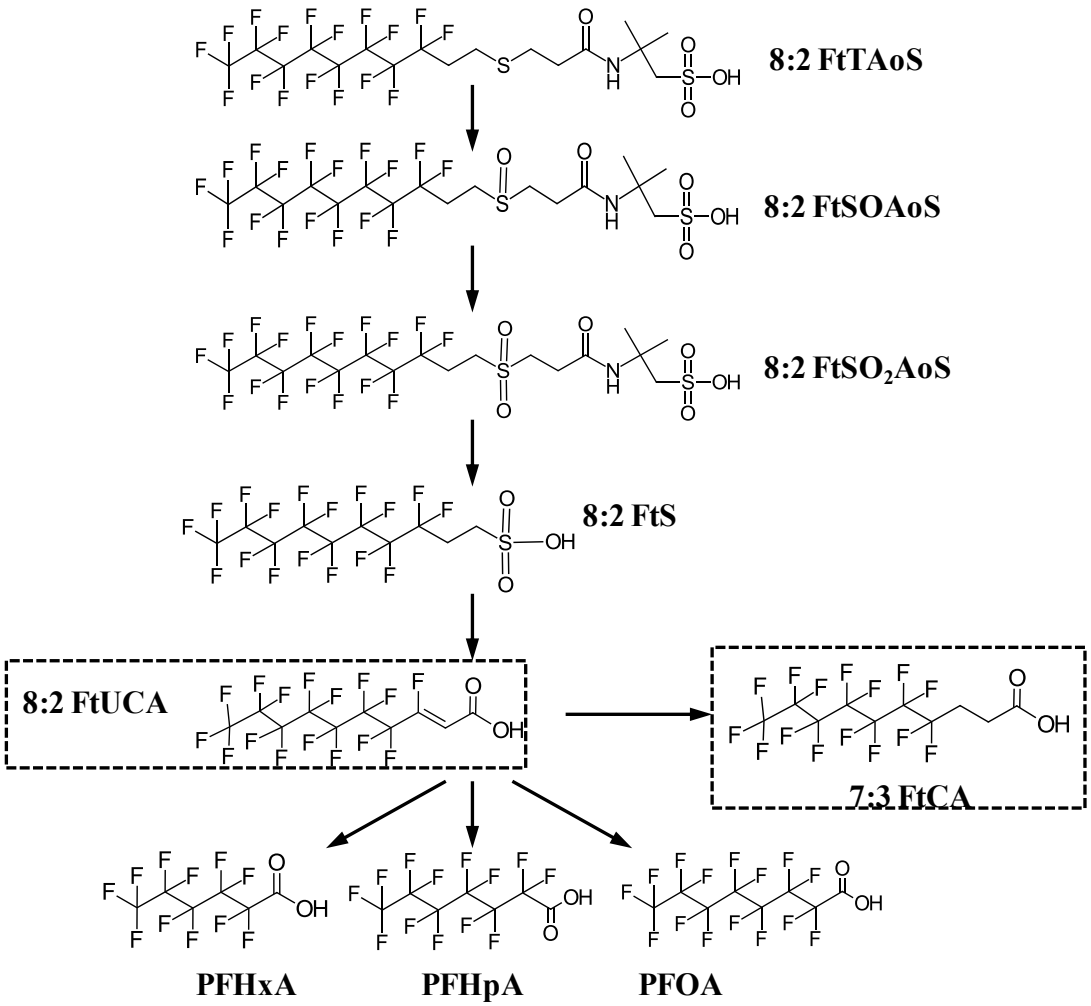


Figure A2.15. Proposed 8:2 FtTAoS biotransformation pathway. Compounds in dotted boxes are proposed biotransformation products that were not detected in microcosms.

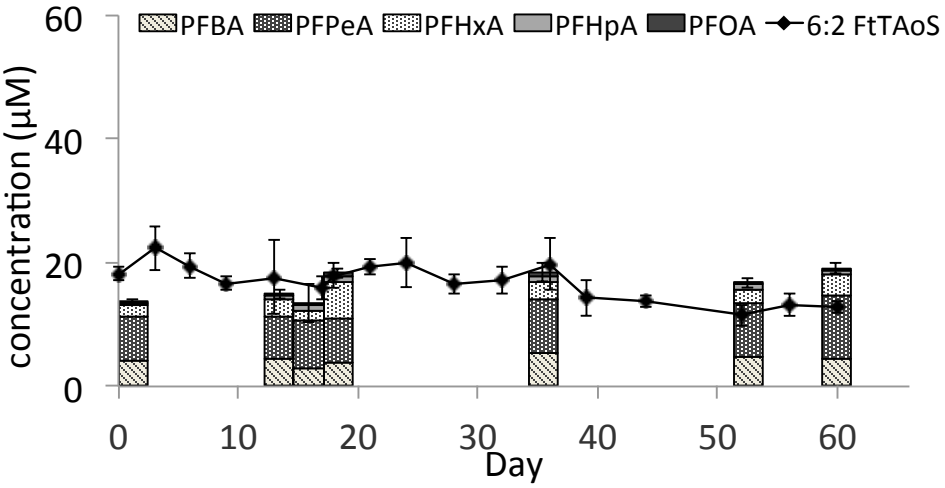


Figure A2.16. Concentration of 6:2 FtTAoS measured in the autoclaved microcosms and concentration of PFCA products measured in autoclaved microcosm samples after they were subjected to the total oxidizable precursor assay.

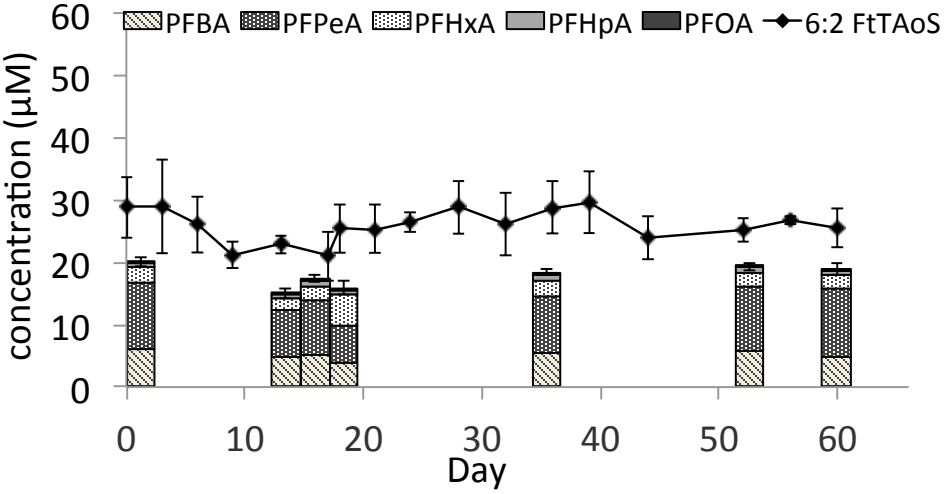
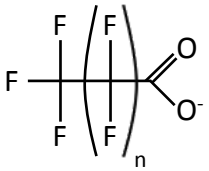
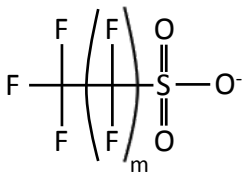


Figure A2.17. Concentration of 6:2 FtTAoS measured in the medium controls and concentration of PFCA products measured in medium control samples after they were subjected to the total oxidizable precursor assay.

Appendix: Chapter 3

Table A3.1. PFAA analytes used in this study and corresponding characteristics

Compound	Abbreviation	Structure[1]		Purity
Perfluorobutanoic acid	PFBA		n = 2	98%
Perfluoropentanoic acid	PFPeA		n = 3	97%
Perfluorohexanoic acid	PFHxA		n = 4	97%
Perfluoroheptanoic acid	PFHpA		n = 5	100%
Perfluorooctanoic acid	PFOA		n = 6	96%
Perfluorononanoic acid	PFNA		n = 7	97%
Perfluorodecanoic acid	PFDA		n = 8	98%
Perfluoroundecanoic acid	PFUnA		n = 9	95%
Perfluorobutane sulfonate	PFBS		m = 3	98%
Perfluorohexane sulfonate	PFHxS		m = 5	98%
Perfluorooctane sulfonate	PFOS		m = 7	98%

PFAA preparation: Purity-corrected stock solutions of an eleven-analyte mixture were made using salts from Sigma-Aldrich in a 70/30 v/v methanol/aqueous solution as discussed previously [Sepulvado and Higgins 2013]. To achieve a stock solution of 50, 30, or 20 g/L of pure analytes. The components in this mixture were: perfluorobutanoate (PFBA), perfluoropentanoate (PFPeA), perfluorohexanoate (PFHxA), perfluoroheptanoate (PFHpA), PFOA, perfluorononanoate (PFNA), perfluorodecanoate (PFDA), perfluoroundecanoate (PFUnA), perfluorobutanesulfonate (PFBS), perfluorohexanesulfonate (PFHxS), and PFOS (Table S1). These stock solutions were then combined at the appropriate volumes and diluted with additional 70/30 v/v methanol/MilliQ™ water to create spiking solutions.

Experimental aqueous loss control. To assess whether there are aqueous PFAA losses due to evaporation of methanol during PFAA amendment, the concentrations of each PFAA were measured before and after evaporation. Two different target concentrations were assessed (0.1 mg/L and 1 mg/L of each PFAA) to determine if losses may be concentration dependent (Figure S1).

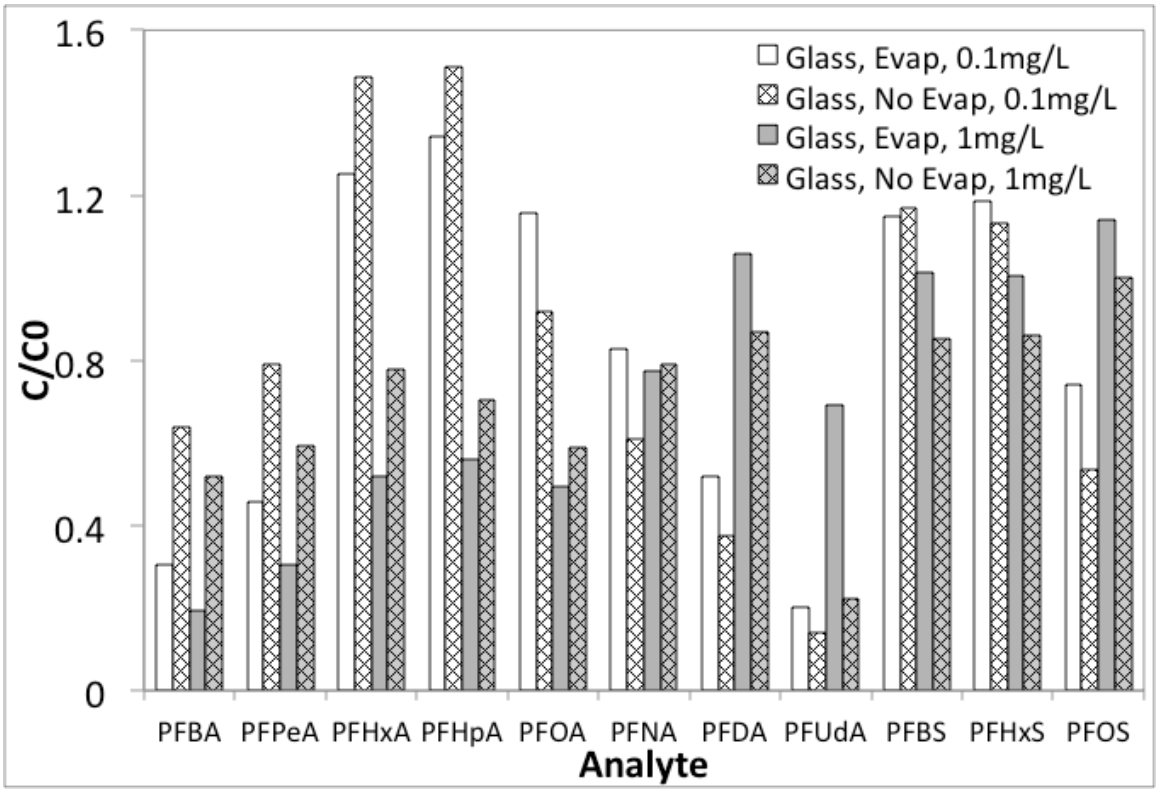


Figure A3.1. PFAA concentrations before and after evaporation step (cross-hatches) for two different concentrations: 0.1 mg/L (white bars) and 1 mg/L (grey bars) in glass bottles sealed with butyl rubber stoppers.

Recovery after evaporation increased for shorter chain acids, and slightly decreased for most sulfonates. Because the final PFAA concentrations remain quantifiable, we are able to utilize the evaporation step in future experiments. Additionally, though we may get some loss to the air-water interface when using glass bottles, the final aqueous concentration is still quantifiable, even at concentrations lower than those utilized for the 10 mg/L-each PFAA experiments.

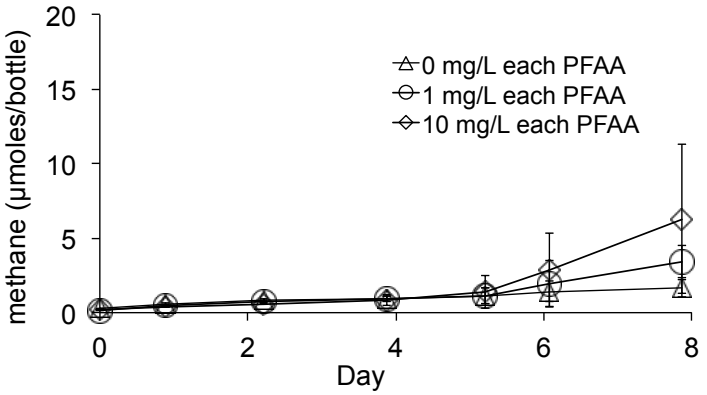


Figure A3.2. Methane concentrations in dechlorinating microbial communities amended with 0, 1, or 10 mg/L of each PFAA (11 analytes total).

Appendix: Chapter 4

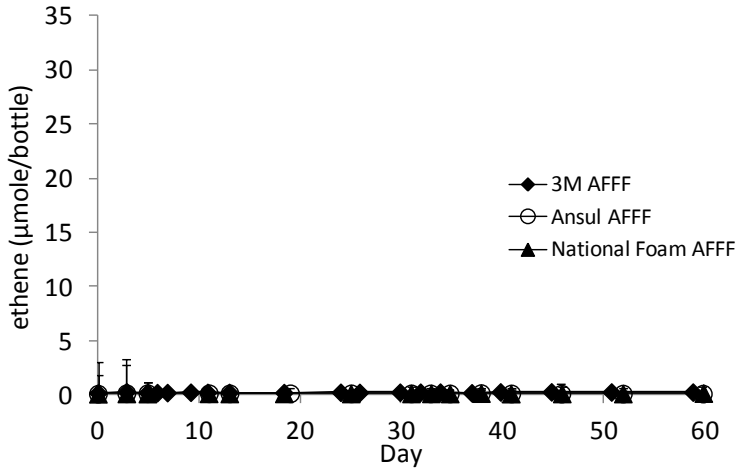


Figure A4.1. No ethene production occurs when cultures are amended with a 3M, Ansul or National Foam AFFF after 60 days. Error bars represent the standard deviation of triplicate biological bottles.

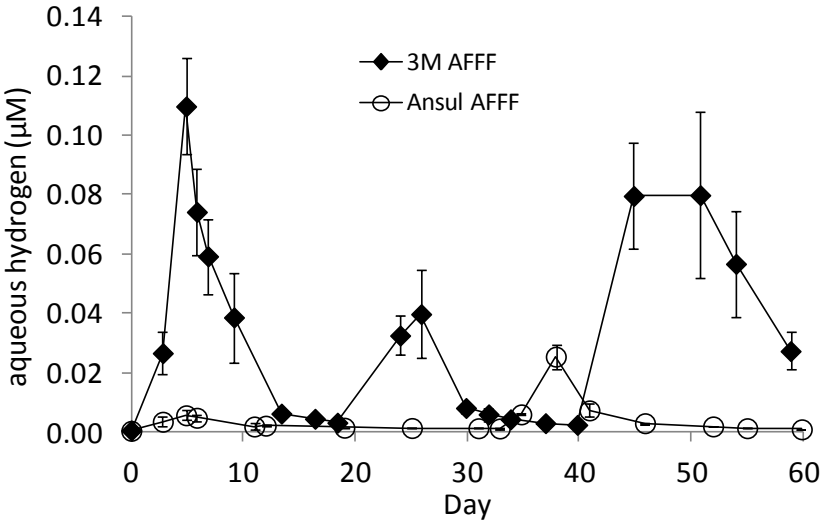


Figure A4.2. Hydrogen production and consumption in 3M and Ansul AFFF-amended cultures. This figure is related to text Figure 2A: the y-axis scale has been decreased in order to zoom in on the measured hydrogen concentrations in these conditions.

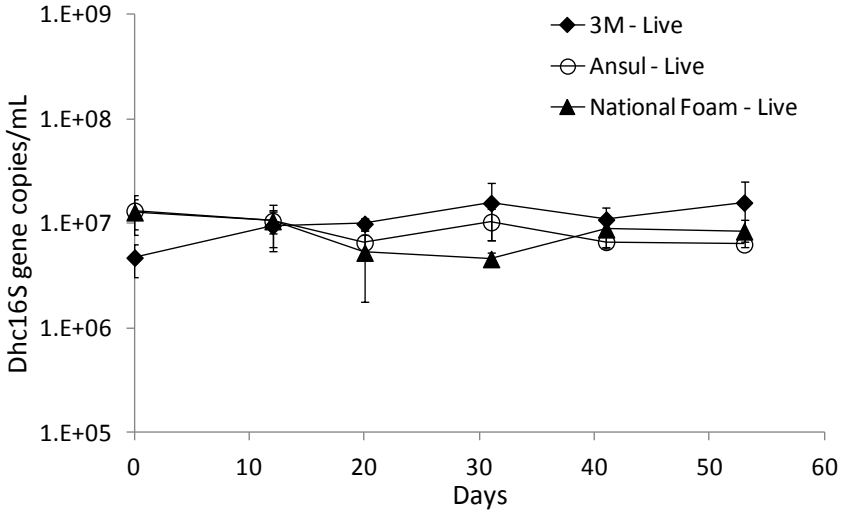


Figure A4.3. *Dehalococcoides* 16S rDNA copies in 3M, Ansul, and National Foam-amended TCE-dechlorinating cultures. No significant Dhc growth is detected in any of the AFFF amendment conditions. Error bars represent the standard deviation of triplicate biological bottles.

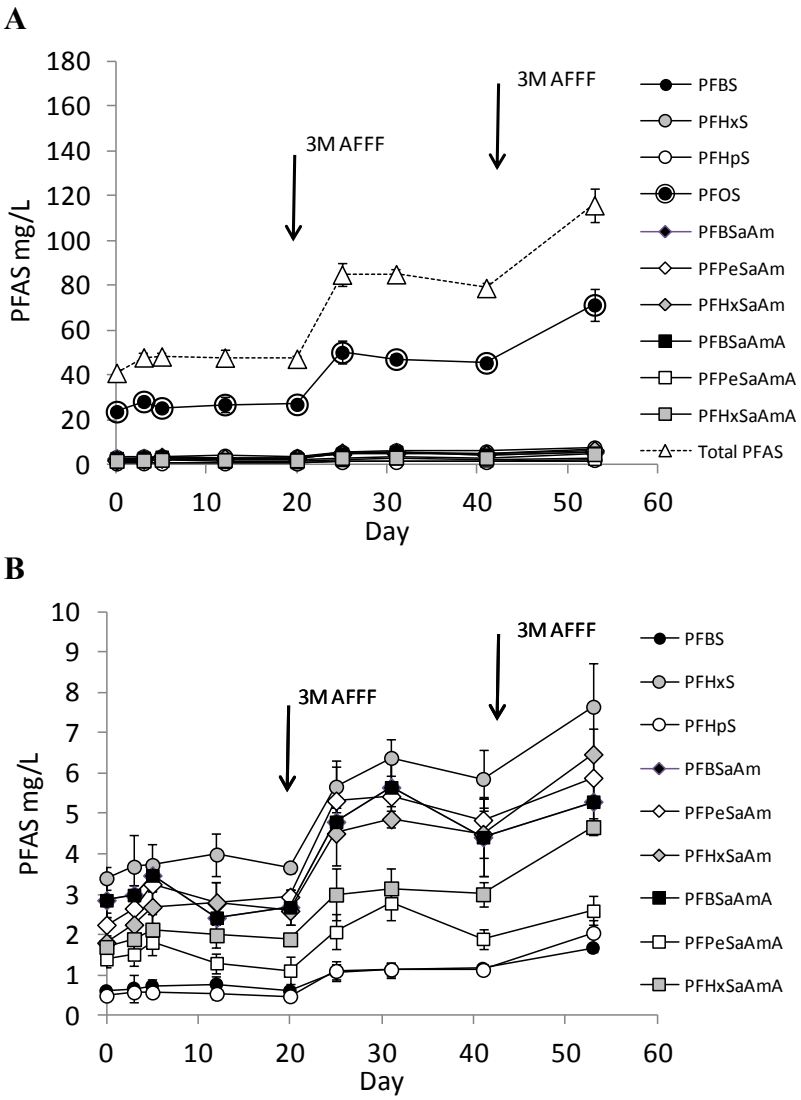


Figure A4.4. Concentrations of all measured PFAS compounds in live TCE-dechlorinating cultures initially amended with 3M AFFF (A, upper). The arrows denote two additional 3M AFFF amendments that were made to the live cultures. The "Total PFAS" concentration (A) represents the sum of all PFAS compounds plotted in A and B. Plot B (lower) is related to plot A: the y-axis scale has been decreased to zoom in on measured PFAS compounds. Error bars represent the standard deviation of triplicate biological bottles.

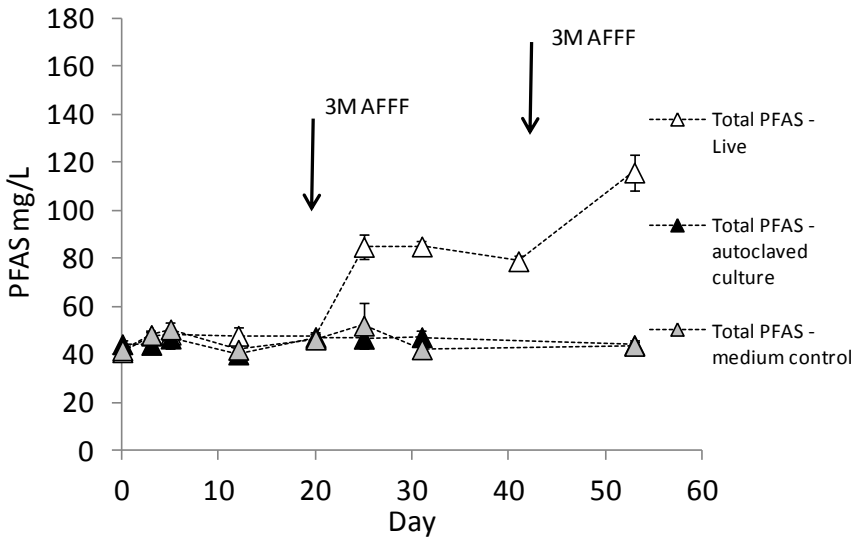


Figure A4.5. The total PFAS concentrations measured in live cultures, autoclaved cultures, and medium controls initially amended with 3M AFFF. The two additional 3M AFFF amendments were made to the live cultures only.

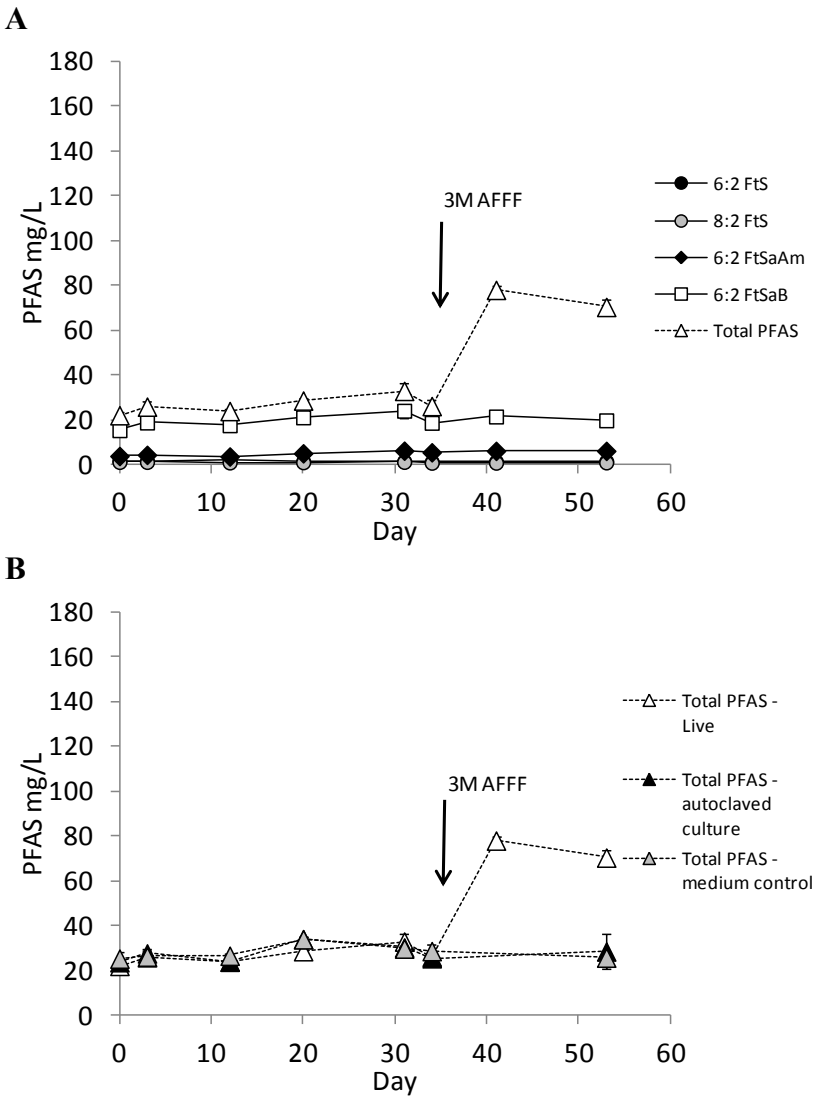


Figure A4.6. Concentrations of all measured PFAS compounds in live cultures initially amended with National Foam AFFF (A, upper). The arrow denotes a 3M AFFF amendment that was made to live cultures only. The "Total PFAS" concentration in A represents the sum of all PFAS compounds measured in the live cultures, while plot B (lower) shows the total PFAS concentration measured in the autoclaved cultures and medium control.

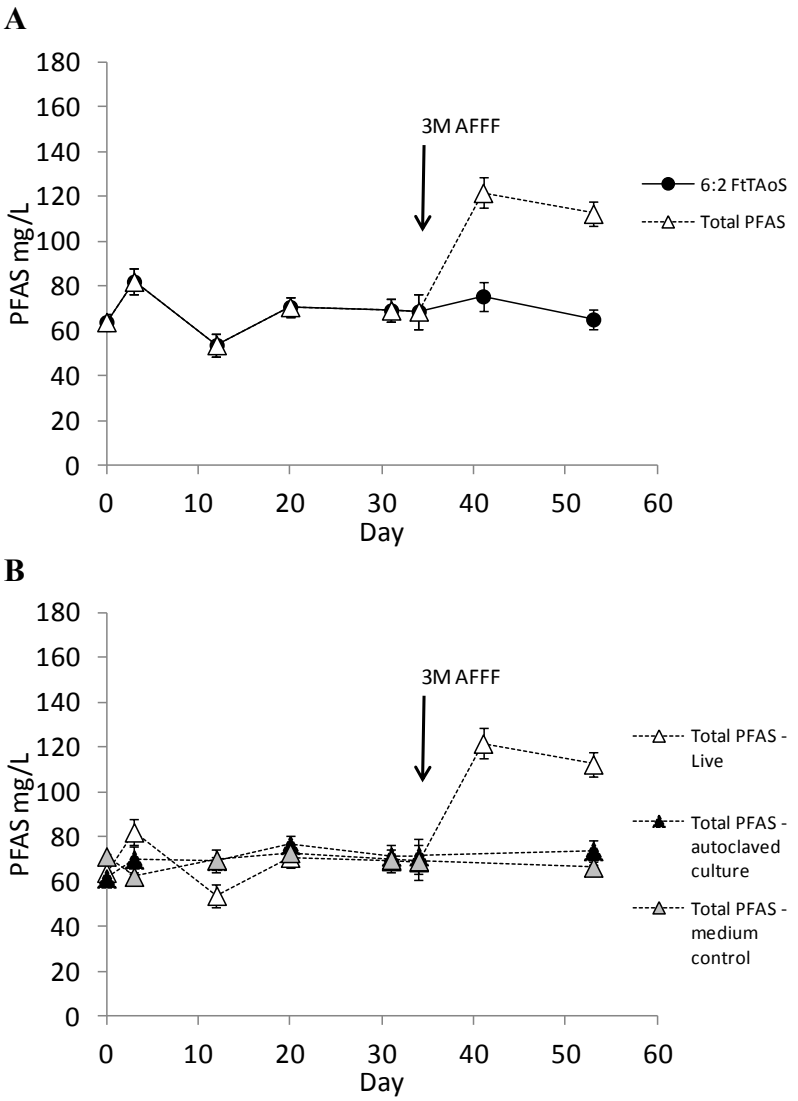


Figure A4.7. Concentrations of all measured PFAS compounds in live cultures initially amended with Ansul AFFF (A, upper). The arrow denotes a 3M AFFF amendment that was made to live cultures only. The "Total PFAS" concentration in A represents the sum of all PFAS compounds measured in the live cultures, while plot B (lower) shows the total PFAS concentration measured in the autoclaved cultures and medium control.

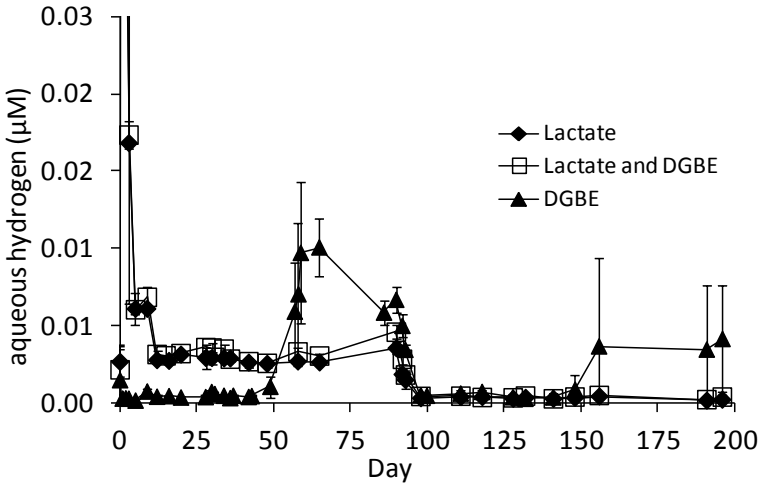


Figure A4.8. Hydrogen production and consumption in lactate, lactate + DGBE, and DGBE-only amended conditions. This figure is related to text Figure 4B: the y-axis scale has been decreased in order to zoom in on the measured hydrogen concentrations in these experiments.

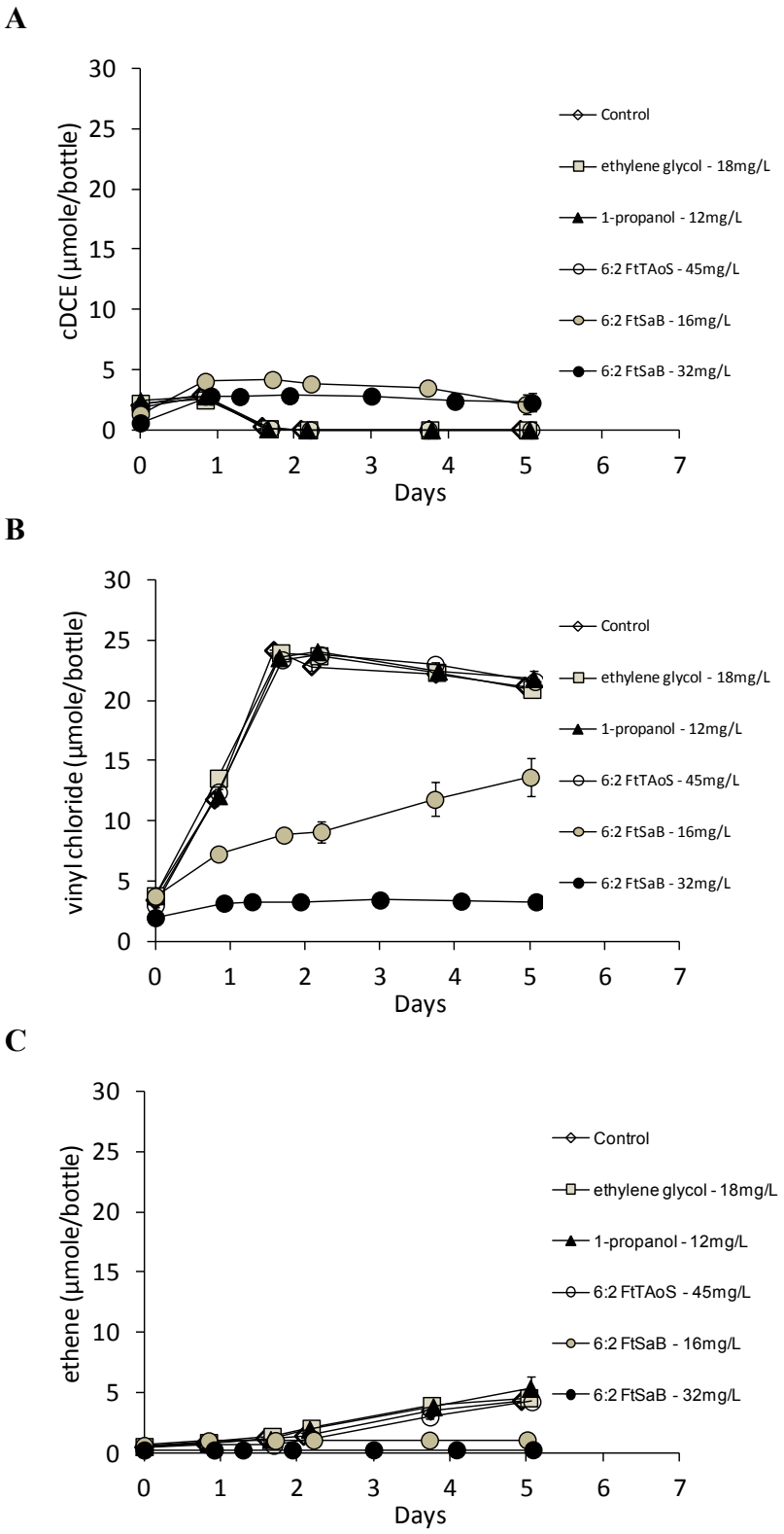


Figure A4.9. Production of cDCE (A), vinyl chloride (B), and ethene (C) following TCE dechlorination in a *Dehalococcoides* enrichment culture amended with various concentrations of AFFF constituents.

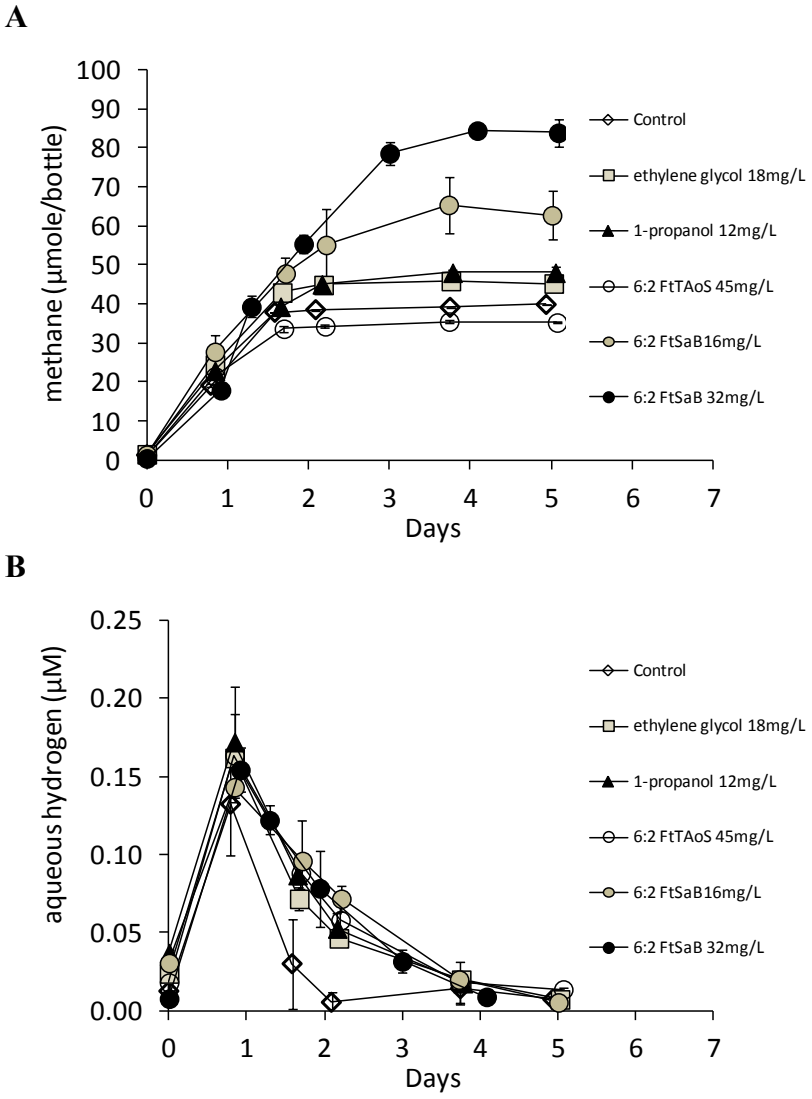


Figure A4.10 Methane production (A) and hydrogen production and consumption (B) during TCE dechlorination in a *Dehalococcoides* enrichment culture amended with various concentrations of AFFF constituents.

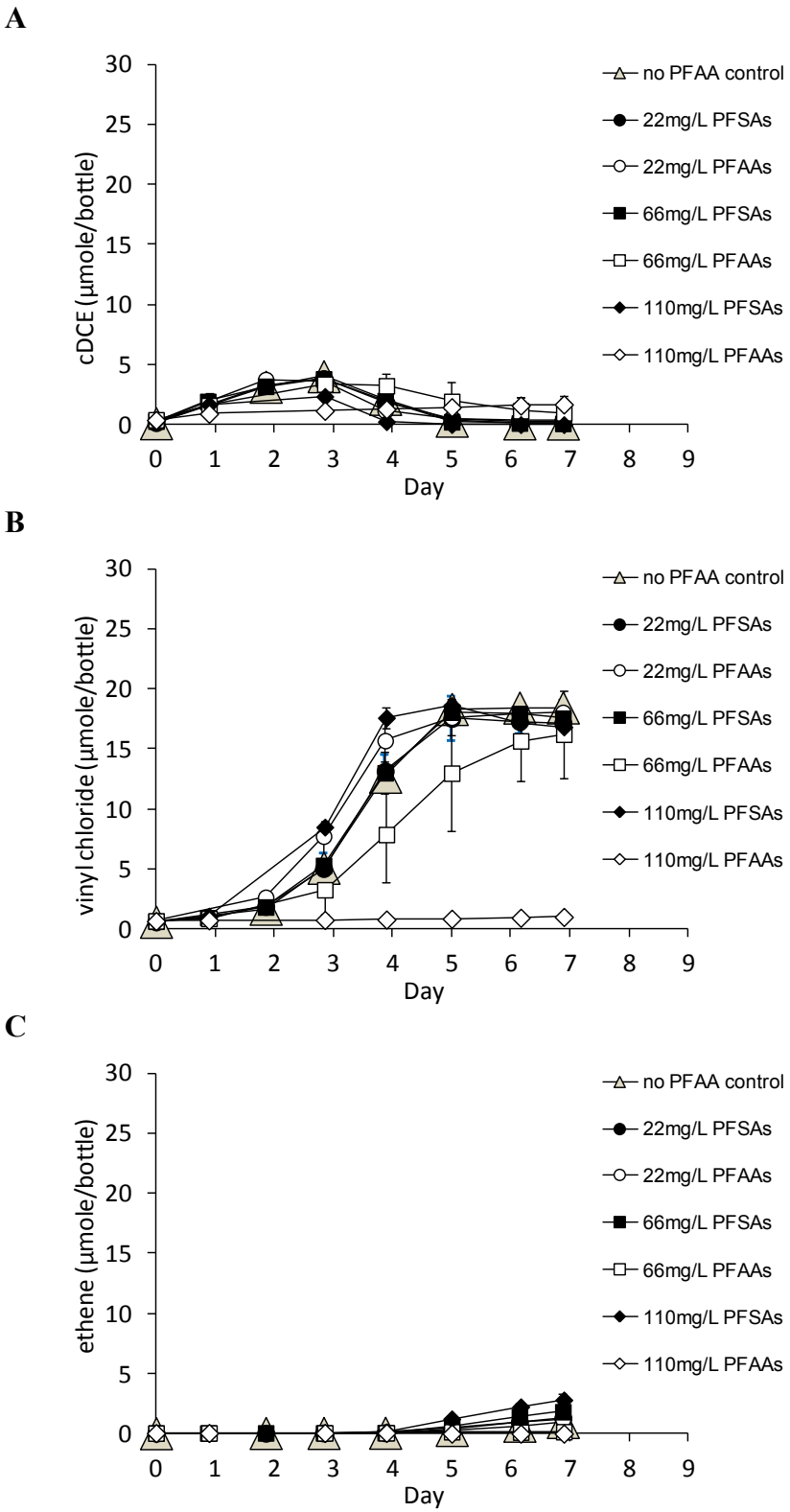


Figure A4.11. Production of cDCE (A), vinyl chloride (B), and ethene (C) following TCE dechlorination in a *Dehalococcoides* enrichment culture amended with various concentrations of PFAAs (PFSAs and PFCAs) and PFSA

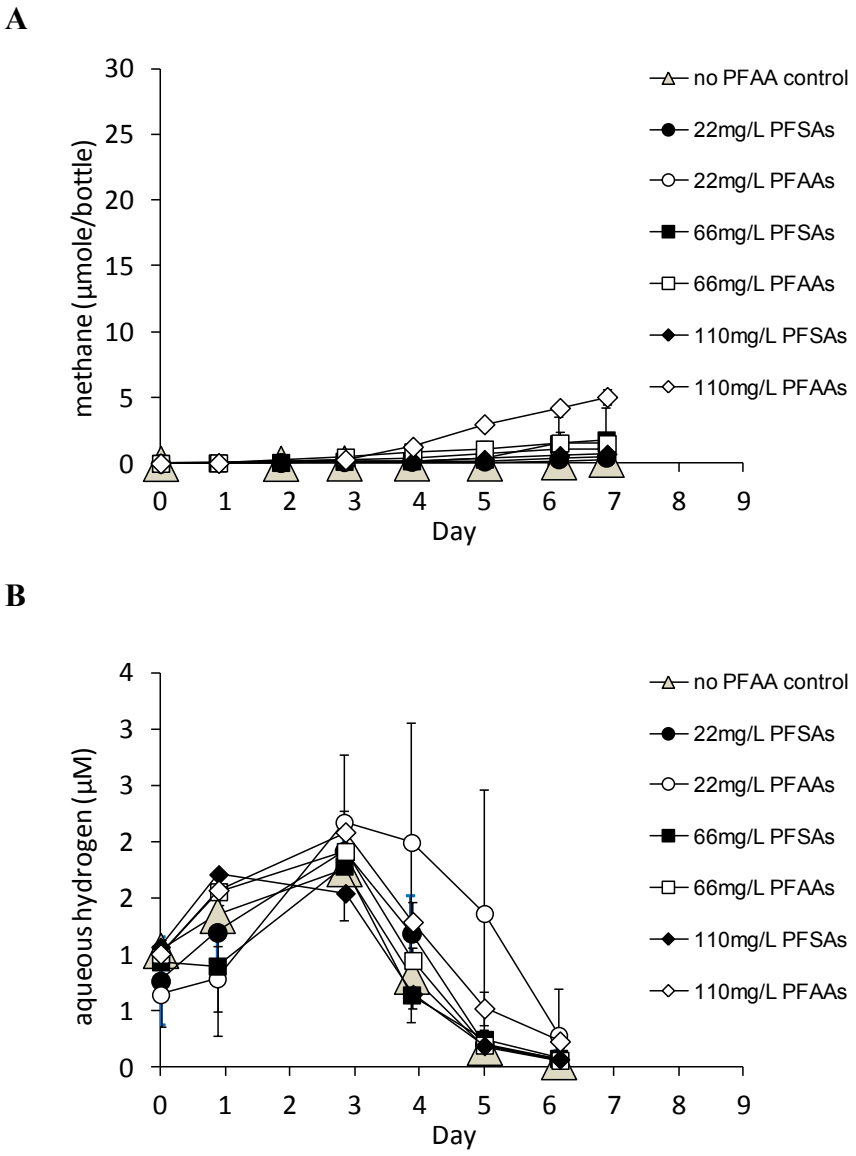


Figure A4.12. Methane production (A) and hydrogen production and consumption (B) during TCE dechlorination in a *Dehalococcoides* enrichment culture amended with various concentrations of PFAAs (PFSAs and PFCAs) and PFSAs.

Appendix: Chapter 5

Figures A5.1-A5.13. Stable carbon isotope fractionation of TCE during different culture growth conditions (A), and the linear regression plot of the Rayleigh equation used to calculate the enrichment factor, ε (B). Symbols collectively represent measurements from triplicate bottles.

Fig. A5.1. Dhc strain 195 – standard growth

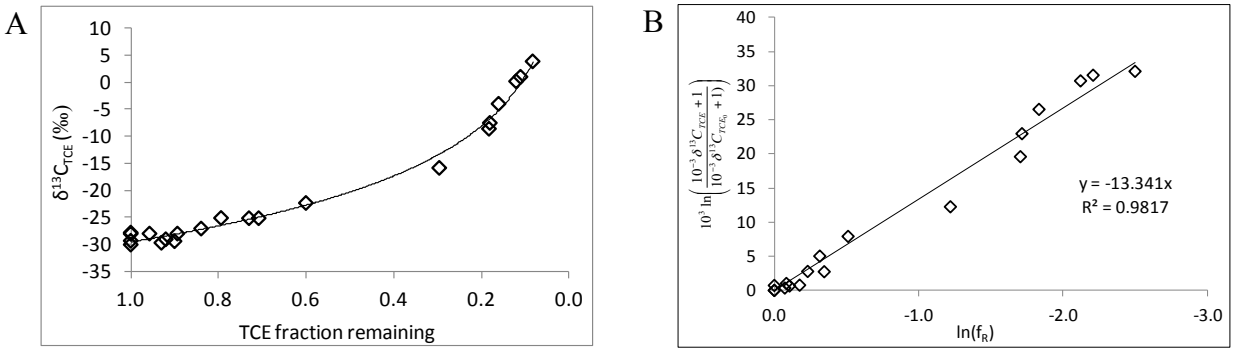


Fig. A5.2. Dhc strain 195 – nitrogen fixing

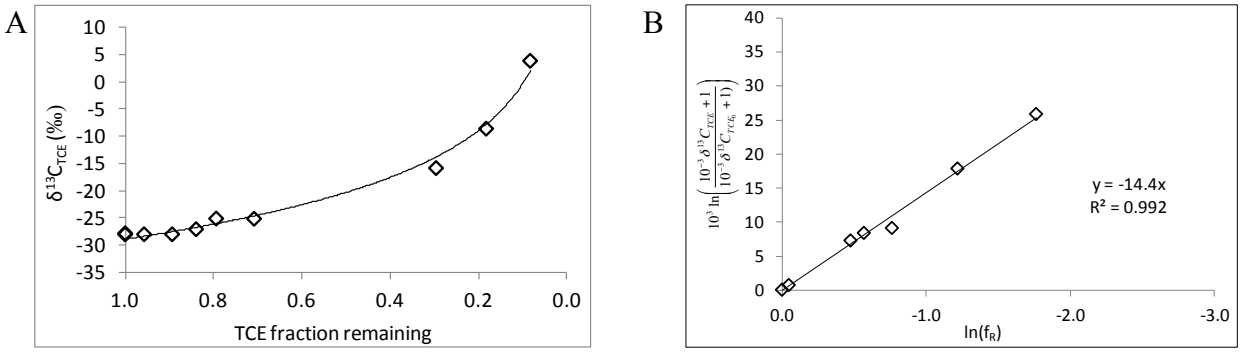


Fig. A5.3. Dhc strain 195 – 3 µg/L vitamin B_{12}

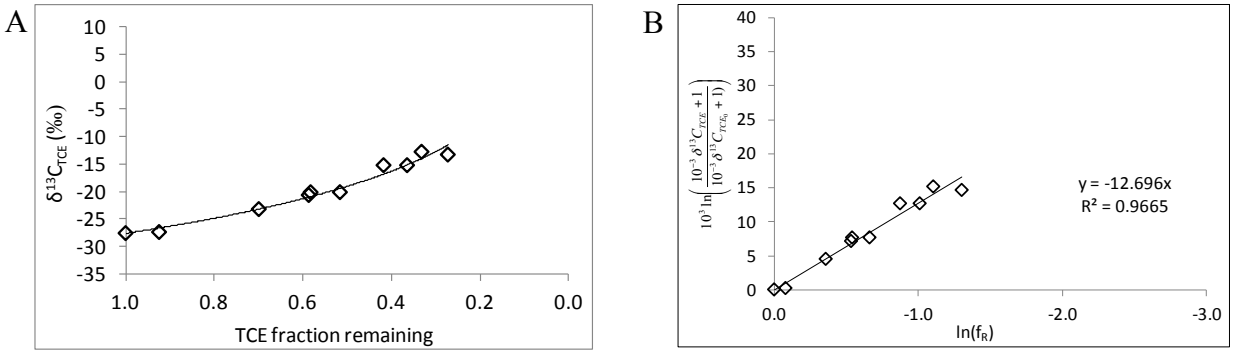


Fig. A5.4. Dhc strain 195 – 22ºC incubation

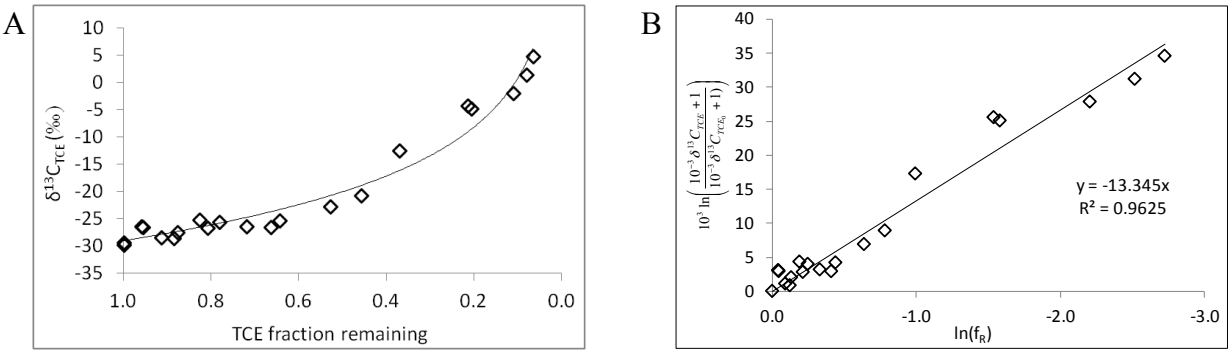


Fig. A5.5. Dhc strain 195 – 1.6 mM aqueous vinyl chloride

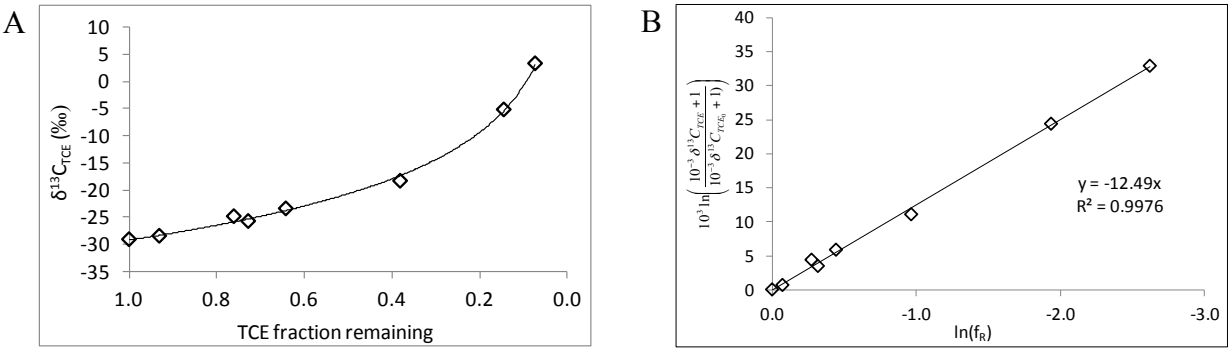


Fig. A5.6. Dhc strain 195 + *Desulfovibrio vulgaris* Hildenborough – 5mM lactate

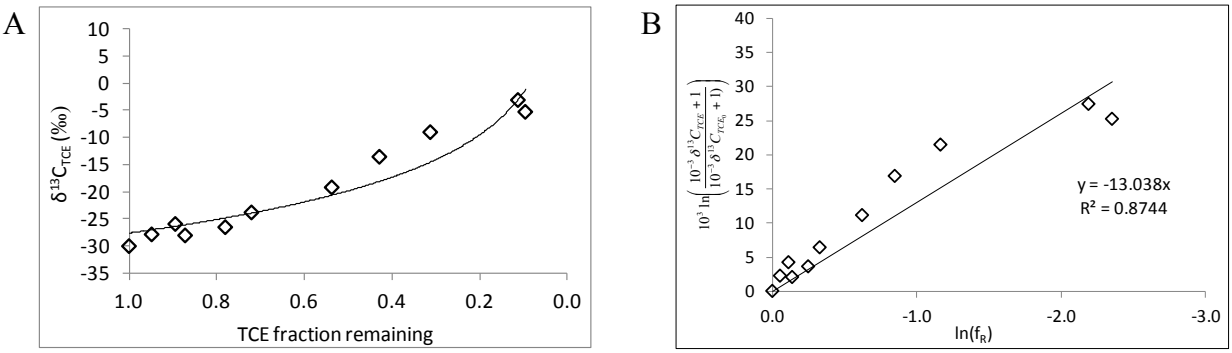


Fig. A5.7. Dhc strain 195 + *Syntrophomonas wolfei* – 5 mM lactate

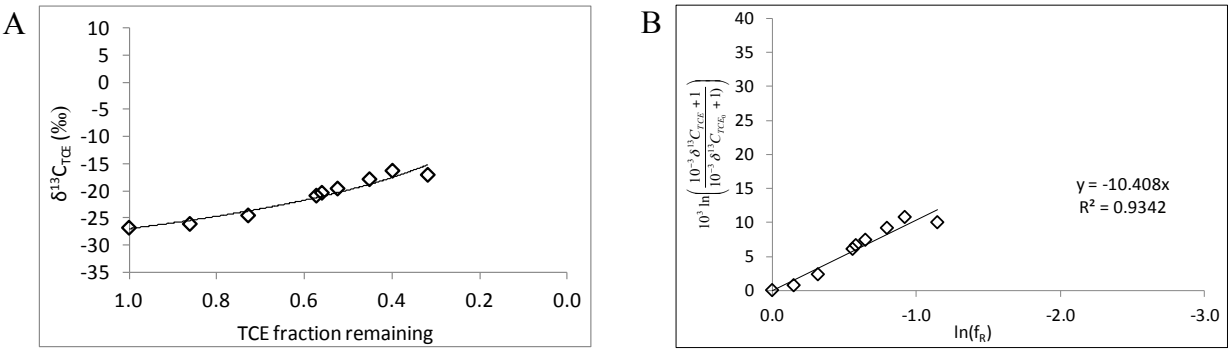


Fig. A5.8. Dhc strain 195 + *Syntrophomonas wolfei* – 20 mM crotonate

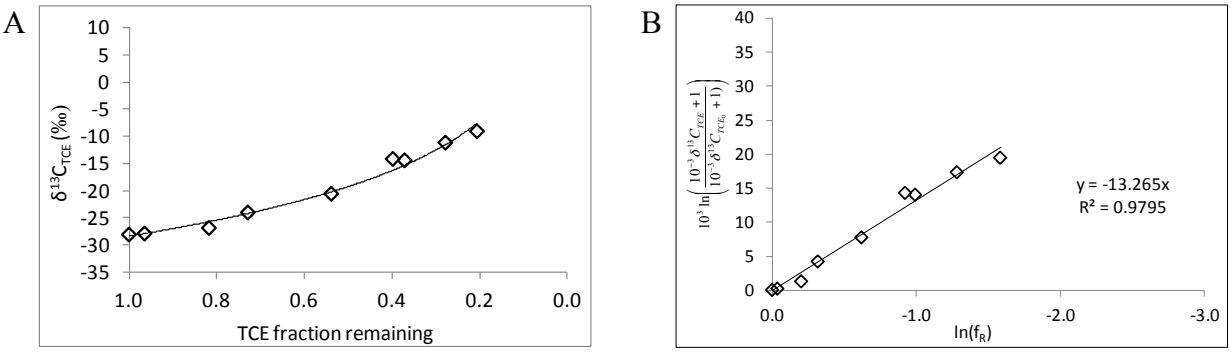


Fig. A5.9. Dhc enrichment – LoTCEB12/Parent

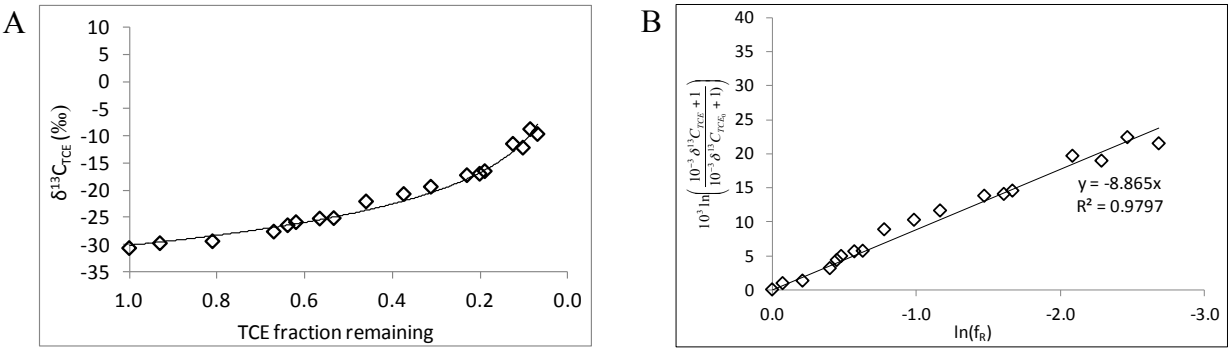


Fig. A5.10. Dhc enrichment – LoTCEB12

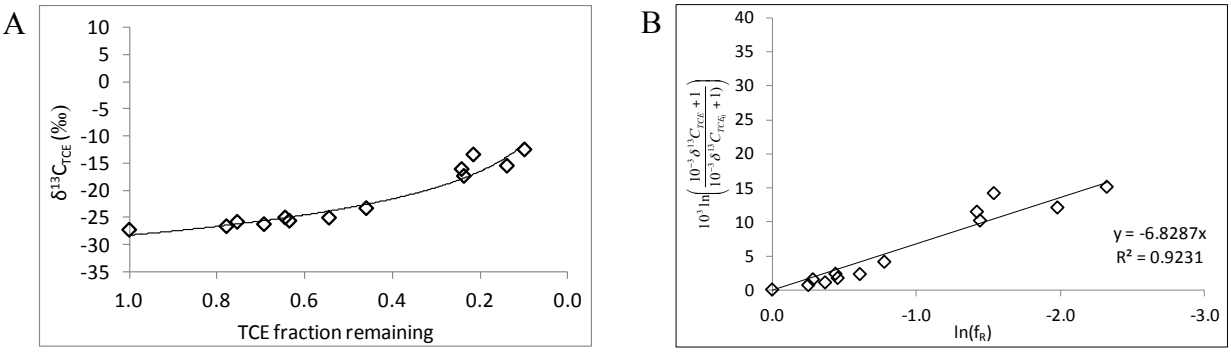


Fig. A5.11. Dhc enrichment – HiTCEB12

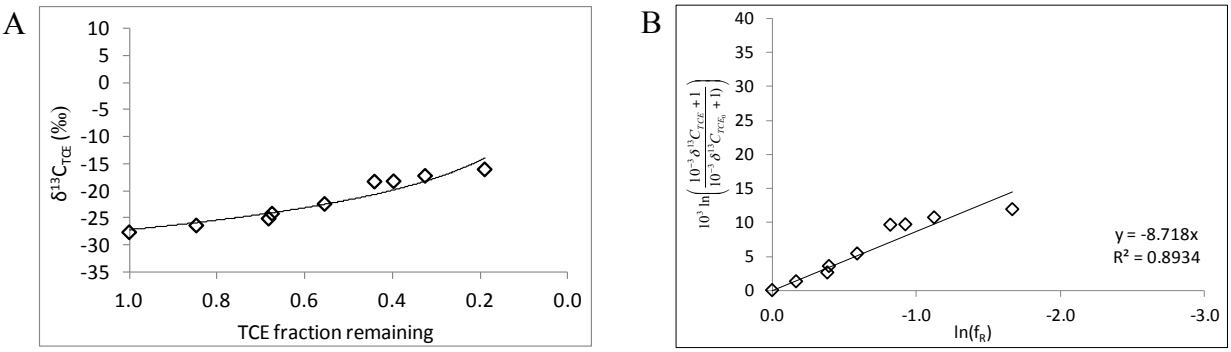


Fig. A5.12. Dhc enrichment - HiTCE

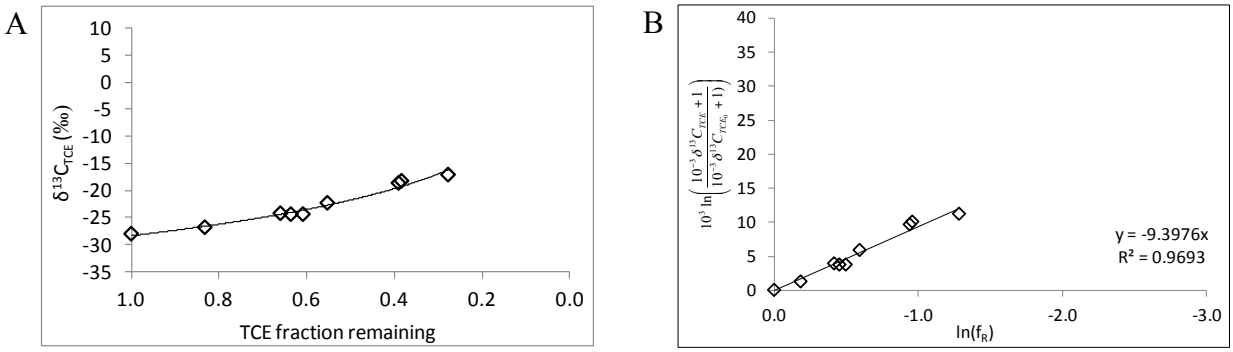
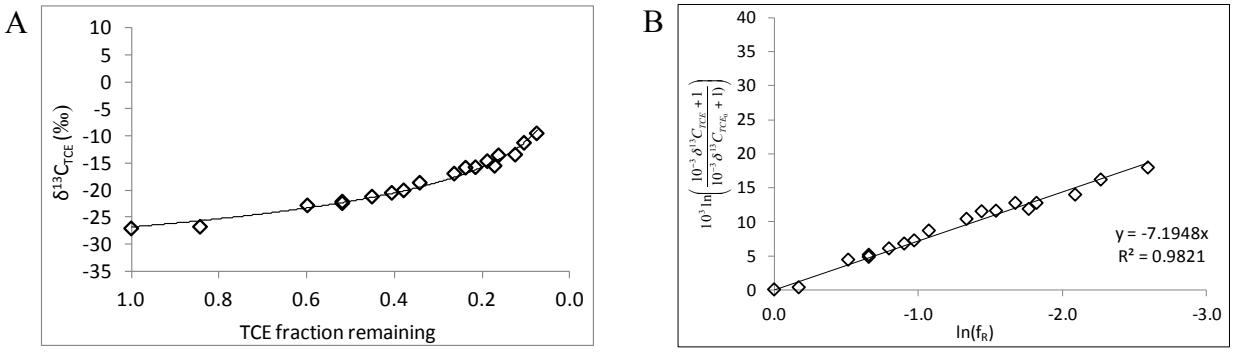


Fig. A5.13. Dhc enrichment – HiTCE/LowLac



Figures A5.14-A5.16. Time course plots for TCE degradation (A5.14), cDCE production (A5.15) and VC production (A5.16) in Dhc195 pure and co-cultures. Error bars, where shown, represent the standard deviation for biological triplicates. The calculated fraction of each chloroethene was corrected for sample removal. The vinyl chloride fraction plotted for "Dhc195, elevated VC" includes biologically produced VC only.

Fig. A5.14. TCE degradation in Dhc195 pure and co-cultures.

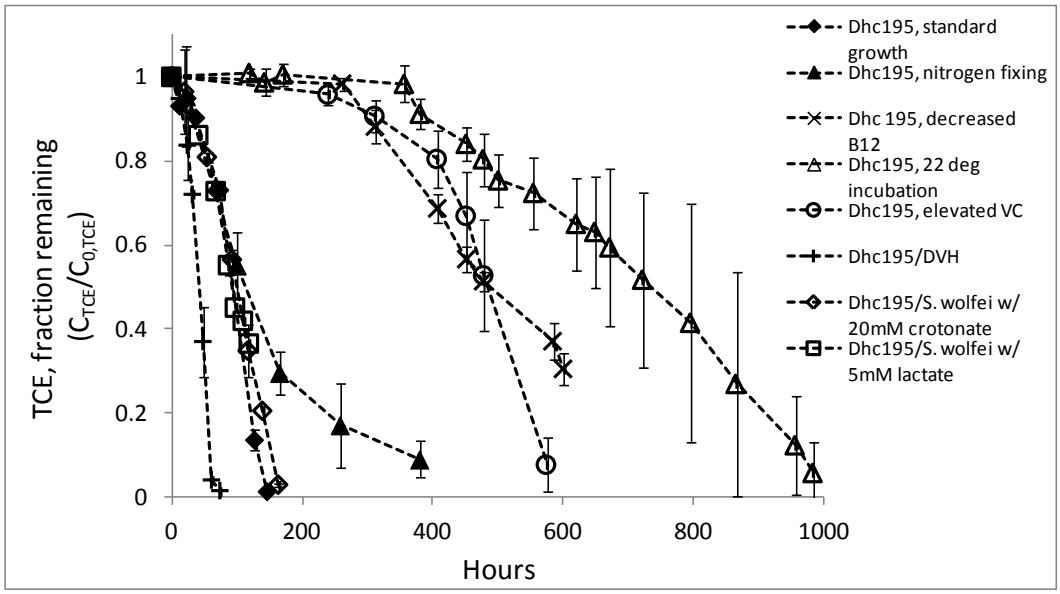


Fig. A5.15. cDCE production in Dhc195 pure and co-cultures.

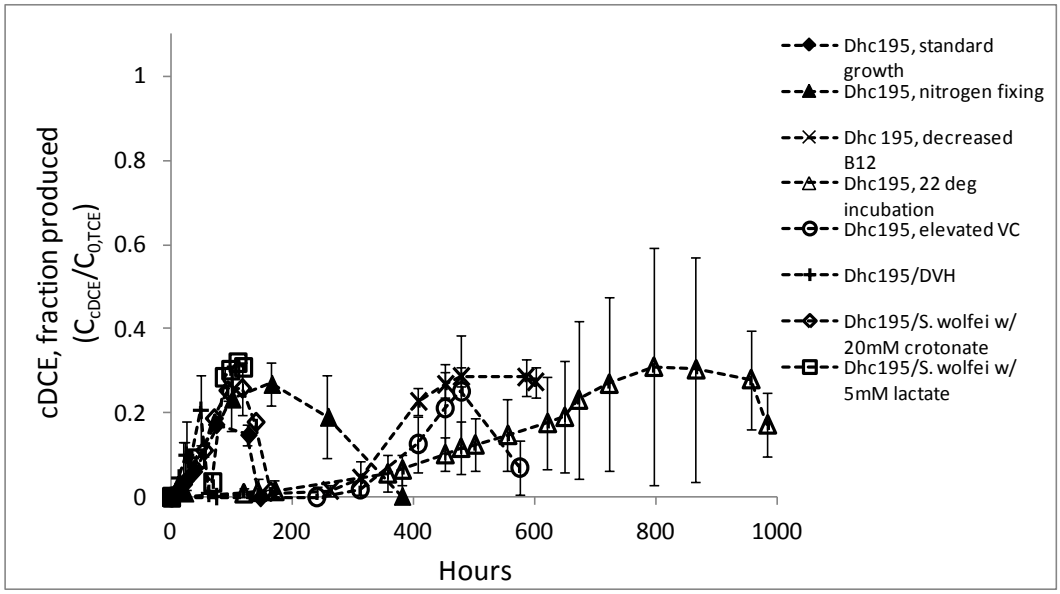
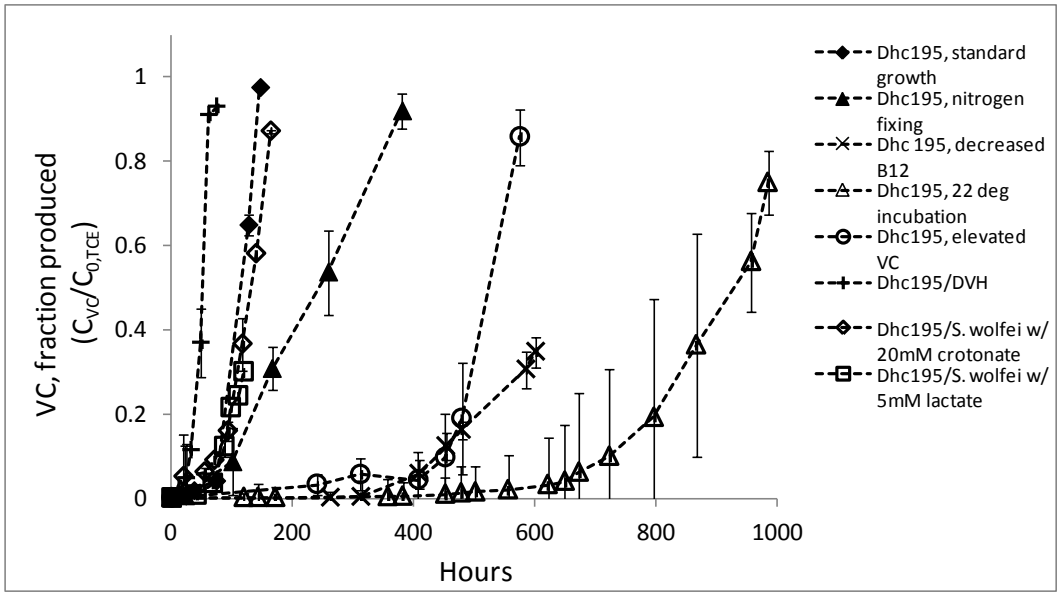


Fig. A5.16. VC production in Dhc195 pure and co-cultures.



Figures A5.17- A5.19. Time course plots for TCE degradation (A5.17), cDCE production (A5.18) and VC production (A5.19) in Dhc enrichment cultures. Error bars, where shown, represent the standard deviation for biological triplicates. The calculated fraction of each chloroethene was corrected for sample removal.

Fig. A5.17. TCE degradation in Dhc enrichment cultures

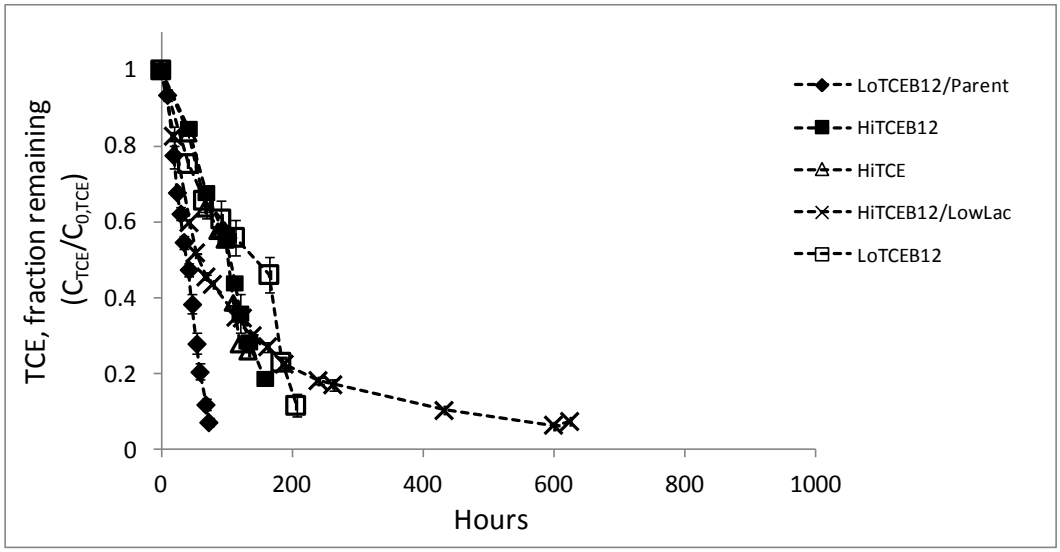


Fig. A5.18. cDCE production in Dhc enrichment cultures.

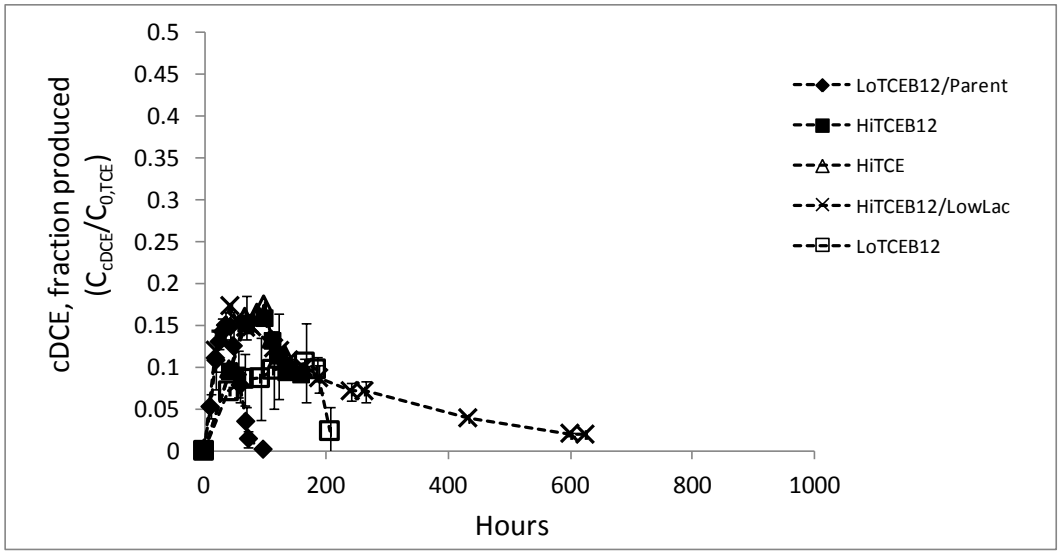


Fig. A5.19. VC production in Dhc enrichment cultures.

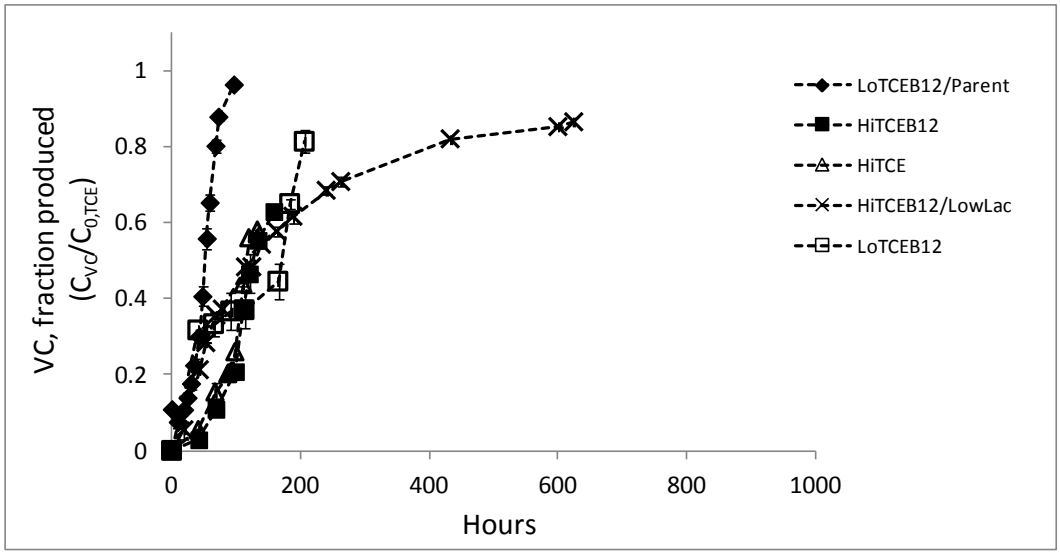


Figure A5.20. Product isotope ratios during reductive dechlorination of TCE to cDCE and VC by Dhc enrichment culture LoTCEB12/Parent. The dotted line indicates the isotope mass balance calculated at each time point by summing the product of each chloroethene molar ratio (corrected for sample removal) and its associated isotope ratio.