Final Report

Study Title

Anaerobic Biodegradability Testing of the Perfluoroalkyl Substances PFOS, PFBS, PFOA, PFBA, MeFBSE, EtFOSE, 6:2-FTS, 6:2 FTOH and 8:2 FTOH, and also 246-Trichlorophenol and Pentachlorophenol

Data Requirement

Exploratory/Method Development

Author

Cleston C. Lange, Ph.D.

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Performing Laboratory

3M Environment, Health, Safety and Sustainability
Environmental Laboratory
3M Center, Bldg. 260-05-N-17
St. Paul, MN 55144

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101

SUMMARY

This report summarizes the results of anaerobic biodegradation experiments conducted from August 2005 to August 2006 under 3M Project E05-0544. The goal of the project was to develop effective and safe equipment procedures and test methods which would enable researchers of the 3M Environmental Laboratory to conduct anaerobic biodegradation testing of fluorinated surfactants and fluorinated polymers. This testing ability was needed to meet conditional requirements of U.S. EPA consent order agreements for the manufacture and sale of fluorinated materials in the U.S. To accomplish this goal, municipal anaerobic digester sludge was acquired from a local Twin Cities (MN) municipal anaerobic digester (Empire, MN) and the sludge was used to establish several sets of anaerobic test cultures. Those cultures were then dosed with a variety of perfluoroalkylated substances (PFAS). It is noteworthy that since the time that this work was performed, a number of research laboratories have published results which have confirmed many of the findings from this study (e.g. Zhang *et al.* 2013). Those findings and their relevance to this study's results are discussed herein.

In total nine PFAS test substances were chosen for the evaluation. They were chosen for a variety of reasons, including the following: First, several of them are synthetic building blocks for the manufacture of specialty fluorinated surfactants and fluorinated polymer protectant products and are expected environmental degradants of those fluorinated polymers (Schultz *et al.* 2003). Secondly, some of them are perfluorooctyl based (i.e. C8) synthons historically used in the manufacture of legacy fluorinated polymers and fluorinated surfactants which have been phased out in 2000-2001. Thirdly, some of the PFAS test substances are perfluorohexyl (C6) or perfluorobutyl (C4) based and were planned to be C8 replacements because of their anticipated more favorable environmental and toxicological profiles. Fourthly, some of the PFAS test substances were identified as the penultimate environmental degradation endpoints of PFAS-derived fluorinated polymers and fluorinated surfactants. Finally, some of the PFAS test substances were derived from electrochemical fluorination (ECF) process and others were derived from a telomerization (TM) process, and represent the two primary industrial processes used for making PFAS materials. The differences between ECF and TM have been discussed previously (Saez *et al.* 2008), amongst them one important difference is that ECF results in PFAS that are a mixture of linear and branched isomers, and also odd and even carbon perfluoroalkyl chain length, because of isomerization reactions that occur during the ECF process (Ignat'ev *et al.* 2003). The TM process results in predominantly even number perfluoroalkyl carbon chains and only linear isomer PFAS because of sequential step wise tetrafluoroethylene additions at the end of an elongating perfluoroalkyl chain during that process.

Most of the PFAS test substances were evaluated at a nominal concentration 1 μg/mL (1 ppm). The ECF-derived chemistries were perfluorooctanesulfonate (PFOS), perfluorobutanesulfonate (PFBS), perfluorooctanoate (PFOA), perfluorobutanoate (PFBA), 2-[(*N*-ethyl)perfluorooctanesulfonamido]ethanol (EtFOSE), and 2-[(*N*-methyl)perfluorobutane-sulfonamido]ethanol (MeFBSE). The TM-derived PFAS were 1,1,2,2-tetrahydro-

perfluorooctanesulfonate (a.k.a. 6:2 fluorotelomer sulfonate; 6:2 FTS), 1,1,2,2-tetrahydro-perfluorooctanol (a.k.a. 6:2 fluorotelomer alcohol; 6:2 FTOH) and 1,1,2,2-tetrahydro-perfluorodecanol (a.k.a. 8:2 fluorotelomer alcohol; 8:2 FTOH). In addition to the nine PFASs tested, 2,4,6-trichlorophenol (246-TCP) and pentachlorophenol (PCP) were also evaluated as potential positive controls for anaerobic biodegradation testing because they had previously been shown to undergo reductive dehalogenation to lesser halogenated phenol end products under methanogenic conditions (Mohn & Kennedy, 1992). The 246-TCP and PCP were dosed at nominal 1 μg/mL and 2 μg/mL, respectively. Some exceptions to the dosed concentrations listed above are noted in the individual test substance results sections.

Cultures were prepared as sets of replicate cultures, some contained sterilized sludge and acted as killed controls and others contained bioactive sludge with live microorganisms. Each culture consisted of 5 mL of anaerobic sludge culture in sealed nominal 42.5 mL amber-glass test vessels and were prepared in a nitrogen filled glove box devoid of oxygen (O_2). Low level addition of yeast extract solution to each provided necessary nutrients to sustain microorganisms in the sludge. The incubations were carried out in a glove box for up to 108 days. Many of the sets also were prepared in parallel with blank water cultures, blank sterile cultures and blank bioactive cultures for evaluating background from sludge and nutrients which were added to the cultures; blanks however were not dosed with equivalent 5-10 μL of methanol carrier solvent that the test cultures received. Each culture set was prepared to allow for at least duplicate or triplicate culture collections at each time point, with some exceptions as noted in the report. At select time points, cultures were collected and evaluated for biogas production. Select culture headspace gases were monitored by gas-phase FTIR analysis and by semi-quantitative full scan GC/MS analysis for gas composition and for potential volatile fluorocarbon products. The cultures were then extracted with acetone and analyzed for the test substance by LC/MS/MS or direct-inject GC/MS and for anticipated soluble biotransformation products by LC/MS/MS.

Formation of biogas occurred in all bioactive cultures and was determined by a volumetric displacement method. The composition of the biogas was verified as methane (CH_4) and carbon dioxide (CO_2) by gas-phase FTIR. Biogas did not form in sterilized control cultures and demonstrated that methanogenic conditions were successfully established during the study. The reductive dehalogenation of 246-TCP and PCP were observed in bioactive cultures based on measured loss of test substance and concomitant formation of lesser-chlorinated phenols in the culture medium. The incubation with 246-TCP resulted in stoichiometric formation of 4-chlorophenol (4-CP) and occurred without a significant lag period for its degradation to occur. PCP biodegradation occurred with an apparent 10 day lag phase, but ultimately formed several tetrachlorinated and trichlorinated phenols, but which was not consistent amongst replicates by the last time point. The degradation of 246-TCP and PCP did not occur in the sterile control cultures. The reductive dehalogenation of 246-TCP and PCP confirmed the establishment of healthy anaerobic methanogenic consortia inclusive of halorespiring microorganisms (i.e. microbes which use halogenated organics as terminal electron acceptor for anaerobic respiration). While 246-TCP was found to be a reliable positive control substance for future anaerobic biodegradation testing, PCP was determined to be a poor positive control substance

due to inconsistent results; inconsistency was likely due to toxicity of PCP and the tetrachlorophenol biotransformation product(s).

Of the nine PFAS that were tested, five were concluded to be recalcitrant to anaerobic biodegradation during the evaluations. This was not surprising given that all five are commonly detected in environmental matrices and four of the five are considered benign to normal environmental degradation mechanisms; 6:2 FTS has been shown to biodegrade aerobically. The five anaerobically stable substances were PFOS, PFBS, PFOA, PFBA and 6:2 FTS.

Four of the nine PFAS that were tested did undergo biodegradation anaerobically and all were PFAS alcohol substances. Two PFAS alcohols were derived from ECF synthesis, a C8 PFAS as EtFOSE and a C4 PFAS as MeFBSE. The other PFAS alcohols were TM-derived and were a C8 PFAS as 8:2 FTOH and a C6 PFAS as 6:2 FTOH.

Each of the PFAS alcohols underwent biotransformation reactions at the non-fluorinated portion of the molecule to generate a carboxylic or sulfinic acid end product. EtFOSE was biodegraded with the slowest rate and with only approximately 2.66 mole percent (mole%) biodegraded. The anaerobic biodegradation of EtFOSE resulted in the formation of 2[(*N*-ethyl) perfluorooctanesulfonamido]acetate (EtFOSAA) and perfluorooctane sulfinate (PFOSi). EtFOSE anaerobic biodegradation did not form (*N*-ethyl)perfluorooctane sulfonamide (EtFOSA), perfluorooctane sulfonamide (FOSA), PFOS or PFOA, each of which were previously observed as transient and/or terminal biotransformation products in aerobic municipal sludge cultures (Lange 2000, 2001a, Rhoads 2005). The low level of EtFOSE biodegraded and the slow degradation rate could not be determined by direct measure of EtFOSE and was only determined via the measurement of the accumulated biotransformation products in the culture medium.

MeFBSE biodegraded with the fastest apparent rate and approximately 75 mole% of MeFBSE was biodegraded to 2[(*N*-methyl)-perfluorobutanesulfonamido]acetate (MeFBSAA) and perfluorobutane sulfinate (PFBSi). Other anticipated PFAS products such as (*N*-methyl)-perfluorobutane sulfonamide (MeFBSA), perfluorobutane sulfonamide (FBSA), PFBA and PFBS were not formed.

Both of the fluorotelomer alcohol (FTOH) test substances biodegraded anaerobically. The FTOHs were removed from the bioactive and sterile cultures, however, biotransformation products were only observed in bioactive cultures. Approximately 8 mole% of the dosed 8:2 FTOH was biotransformed to 8:2 fluorotelomer carboxylate (8:2-FTCA) and 8:2 fluorotelomer unsaturated carboxylate (8:2 FTUCA). Similarly, 11 mole% of the dosed 6:2 FTOH biotransformed in analogous fashion to give 6:2 FTCA and 6:2 FTUCA. Neither FTOH test substance yielded perfluorinated carboxylates (PFCAs) which have been observed in other studies using aerobic sludge (Lange 2001b, Dinglasen *et al.* 2004, Wang *et al.* 2005). The reason for loss of FTOHs in sterile sludge controls was not determined, but could be due to irreversible binding of FTOHs to the sludge or the PTFE liner on the test vessel cap. The loss of FTOHS in sterile sludge controls has been observed in other studies (Saez *et al.*, 2008, Wang *et*

al. 2005). Additionally, anaerobic biotransformation products of FTOHs discovered since 2005-2006 were not measured for (e.g. 5:3 acid from 6:2 FTOH and 7:3 acid from 8:2 FTOH, Zhang *et al.* 2013), therefore it is likely that the estimated biodegradation rates for the FTOHs are biased low and this is discussed later in the report.

Overall, several conclusions were drawn from the results of this study. First, PFAS with a non-fluorinated alcohol moiety biotransform anaerobically in a predictable fashion and the biotransformation products were identified and quantified. Secondly, the rates of biodegradation appeared to be dependent on molecular weight, with the lower molecular weight substances biotransformed faster than the higher molecular weight substances (i.e. MeFBSE > 6:2 FTOH > 8:2 FTOH > EtFOSE); the faster and more abundant degradation of lower MW substances is likely related to an increased water solubility which in turn increases bioavailability. Thirdly, anaerobic biotransformation of the PFAS alcohols does not result in penultimate environmental endpoint perfluorinated sulfonates (PFSAs) or perfluorinated carboxylates (PFCAs). Accumulation of PFAS sulfinate end products from EtFOSE and MeFBSE suggests there is a requirement for O_2 and/or O_2-requiring enzymes/microbes for the next stage biotransformation reactions to generate perfluorinated sulfonates. Fourthly, the results of this study showed that 6:2 FTS, PFBS, PFOS, PFBA and PFOA are stable and do not undergo biological attack under anaerobic conditions. Fifth, degradation products identified in this study can be expected to sequester in low oxygen environments such as groundwater, sediment and wastewater treatment effluents and those are likely environmental sinks for precursors to perfluorinated sulfonates and carboxylates. Finally, this study resulted in development of a reliable analytical method for quantifying the target analyte PFASs in sludge matrices and established general method criteria results of batch QC elements. In general, it can be expected that matrix-matched calibration can be achieved with accuracy of within 100 $\pm$ 25% ($\pm$30% LLOQ) using the sample preparation and LC/MS/MS methods described, and QC results can be expected to be within 100 $\pm$ 30%. Calibration can be achieved over the range of approximately 5 ng/mL to 1000 ng/mL with quadratic fit and 1/x weighting to aid in low end quantitative accuracy, but LLOQs as low as 1.00 ng/mL were achieved for some analytes. Both internal standard and external standard methods of quantitation suffice, and it is analyte and IS dependent in terms of which quantitation method performs best.

While ultimately this work was performed to establish methods suitable for testing anaerobic biodegradability of fluorinated polymer and fluorinated surfactants for studies conducted under good laboratory practice (GLP) standards, two additional non-fluorinated substances 246-TCP and PCP were evaluate for their potential use as positive controls in such testing. The 246-TCP biodegraded reproducibly to a single end product and without any lag phase or toxicity effects. Degradation of 246-TCP was not observed in sterile controls. These observations indicate 246-TCP is a good positive control candidate. In contrast, pentachlorophenol (PCP) showed a long lag period and results on day-74 for 2 of 3 culture replicates showed loss of PCP with concomitant formation 2345- and 2346-tetrachlorophenol, and some 345-trichlorophenol, while the third replicate did not. Due to the observed lag, the inconsistent results and possible toxicity effects, PCP was not a good positive control substance candidate.

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1 INTRODUCTION

Anaerobic biodegradation is defined as the microbiologically catalyzed degradation of a substance in the absence of molecular oxygen (O_2). While typically under aerobic conditions O_2 serves as a final electron acceptor for metabolic processes, but in the absence of O_2 microorganisms find alternative electron acceptors to complete the biochemical redox reactions needed for cell growth and maintenance. Anaerobic microorganisms capable of such reactions are found in natural environments such as bogs, hot springs, subsurface water (i.e. groundwater), marine and freshwater sediments and submerged soils (i.e. paddy soil), and in manmade environments such anaerobic digesters, septic tanks and landfills, and in the gastrointestinal tract (colon) of animals and humans and specialized gut of ruminants.

During anaerobic digestion the microbiological breakdown of organic polymers involves four stages as shown in **Figure 1**. These stages are: 1) hydrolysis stage, in which organic polymers are broken down to form simple sugars, amino acids and hydroxylated fatty acids; 2) acidogenesis, or fermentation stage, is where further breakdown of products of the hydrolysis stage forms small organic acids; 3) acetogenesis stage is where the organic acids from acidogenesis are further broken down to form acetic acid, carbon dioxide and hydrogen; and 4) the methanogenesis stage, where conversion of acetate to methane by acetotrophic methanogens and/or conversion of hydrogen and carbon dioxide into methane by hydrogenotrophic methanogens occurs. In traversing from stages 1 to 4 of the anaerobic process, the redox potential decreases, with methanogenesis occurring under the more negative redox conditions. Some anaerobic environments can attain redox values as low as -530 mV (Mah & Sussman, 1967). For biogenic methane production to occur from a complex organic substrates such as a polymer, one can assume that the necessary microbial consortia for stages 1 through 4 are functioning and that redox is below 0 mV.

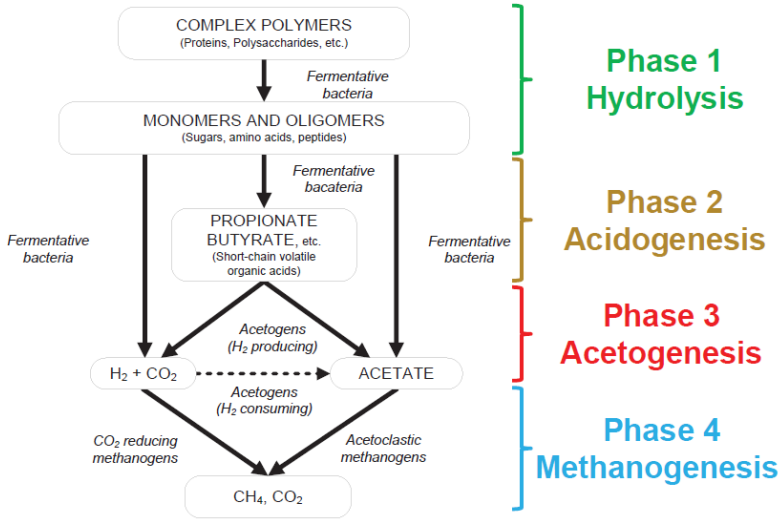

Figure 1. The four step anaerobic digestion Process (Source: BEEMS Module B7)

The work described in this report was initiated to develop test methods which could be used for reproducible establishment of anaerobic cultures for conducting GLP anaerobic biodegradability testing fluoropolymers; and conducted in support of consent order agreements between 3M Company and the U.S. EPA. While a draft protocol was written with the intent of conducting a more defined anaerobic test initially, it was determined early on that there was not enough information available regarding anaerobic culture preparations, proper dosing of fluorinated test substances into anaerobic cultures, identity of degradation products, nor recovery of test substance and degradation products from digester sludge matrix. Therefore the draft protocol was mostly ignored during the method development process. While the unsigned, draft protocol is included in the raw data package, it was not approved and was not a method development protocol and is only included to offer some insight to the mindset of the author during the refinement of the anaerobic biodegradation test method developed.

During this method development project, the establishment of methanogenic anaerobic cultures was accomplished using municipal anaerobic digester sludge as microbial inoculum. Cultures were maintained as biogas-producing cultures, with analytical confirmation of methane and carbon dioxide formation in the bioactive culture headspaces. The anaerobic cultures were dosed with several perfluoroalkyl substances (PFAS) and were incubated for up to 108 days. The test substances evaluated were inclusive of electrochemical fluorination (ECF) derived and telomerization (TM) derived PFAS. Several of the tested substances had been previously evaluated under aerobic biodegradation conditions during 3M Environmental Laboratory testing and the same biotransformation products identified in those studies served as the basis for the target biotransformation products during this study. Those study IDs were E00-2252 (EtFOSE), E01-0415 (EtFOSE, EtFOSAA, EtFOSA, FOSA, PFOSi , PFOS and PFOA), E01-0444 (PFOS), E01-0684 (6:2 FTOH and 8:2 FTOH) and E02-1325 (MeFBSE).

In addition to the PFASs, 2,4,6-trichlorophenol (246-TCP) and pentachlorophenol (PCP) were evaluated as potential positive control substances for future anaerobic biodegradation testing studies. Both biotransform via halorespiring microorganism under anaerobic conditions (i.e. reductive dehalogenation) and form stable, predictable end products (Woods *et al.* 1989, Miksell & Boyd 1985, and Mohn & Kennedy 1992). From the results obtained during this study, 246-TCP was determined to be a good candidate as a positive control substance for anaerobic biodegradation testing, while PCP was determined to not be a good positive control.

The description of the anaerobic test system developed and the results of incubations with the aforementioned test substances and control substances are described herein. The report is divided into sections based on each test substance evaluated during this study. Several of the PFAS test substances did undergo anaerobic biodegradation and biotransformation products were identified and quantified. Based on rates of formation for total products formed, estimated rates of biodegradation were determined. The implications of these findings to potential anaerobic environmental fate of those tested PFASs are also discussed.

2 TEST, CONTROL AND REFERENCE (TCR) SUBSTANCES

Table 1. Test Substances Evaluated During this Study (E05-0544)

Test Substances	Molecular Formula	3M TCR No.	Purity (%)	Stock Sol. ID	Stock Conc. in Methanol (µg/mL; ppm)
ECF-derived					
MeFBSE	$C_4F_9SO_2N[(CH_3)(CH_2CH_2OH)]$	TCR-631	95.55	05007-92	976
PFBS	$C_4F_9SO_3K$	TCR-282	97.3	05007-102	901
EtFOSE	$C_8F_{17}SO_2N[(CH_2CH_3)(CH_2CH_2OH)]$	SE-031	97.7	05007-91	1010
PFOS	$C_8F_{17}SO_3K$	TCR-741	99.9	05007-101	1060
PFOA	$C_7F_{15}CO_2H$	TCR-617	99.51	05007-99	1010
PFBA	$C_3F_7CO_2H$	TCR-757	99.2	05007-145	1030
Fluorotelomerization-Derived					
6:2 FTS	$C_6F_{13}CH_2CH_2SO_3K$	TCR-343	93.5	05007-100	954
6:2 FTOH	$C_6F_{13}CH_2CH_2OH$	TCR-1056	97	05017-66	1230
8:2 FTOH	$C_8F_{17}CH_2CH_2OH$	TCR-1058	97	05017-68	1300
Chlorinated Phenols					
PCP	C_6HCl_5O	TCR-1053	98.2	05017-62	1040
246-TCP	$C_6H_3Cl_3O$	TCR-937	99.6	05007-146	1220
A figures are shown to 3 significant figures, however more significant figure values were used in data calculations					

All substances were soluble in methanol at the stock solution concentrations described in **Table 1**. References substances used for quantitation were chosen based on predicted biodegradation products as shown in **Table 2**. The identity of the reference substances, molecular formula, abbreviations and purities are given in **Table 3.**

Table 2. Selection of Target Analytes Based on Test Substance Dosed

Target Analytes to Measure (i.e. Anticipated Biodegradation Products)	**Test Substance Dosed**								
	EtFOSE	PFOS	PFOA	MeFBSE	PFBS	PFBA	6:2 FTS	6:2 FTOH	8:2 FTOH
EtFOSE	*								
EtFOSAA	*								
EtFOSA	*								
FOSA	*	*							
PFOSi	*	*							
PFOS	*	*							
MeFBSE				*					
MeFBSAA				*					
MeFBSA				*					
FBSA				*	*				
PFBSi				*	*				
PFBS				*	*				
6:2 FTS							*		
6:2 FTOH							* [a]	*	
6:2 FTCA							*	*	
6:2 FTUCA							*	*	
8:2 FTOH									*
8:2 FTCA									*
8:2 FTUCA									*
PFBA				*	*	*			
PFHxA							*	*	
PFHpA								*	*
PFOA	*	*	*						*
PFNA									*

[a] 6:2 FTOH was intended to be measured in the 6:2 FTS cultures, but was not. However the 6:2 FTS culture did not demonstrate any loss of test substance based on direct measure of 6:2 FTS.

Table 3. Reference Substance (Study E05-0544)

Analyte	Abbreviation	Molecular Formula	Reference Substance ID	Purity
Perfluorobutanesulfonyl-Related Analytes				
2[(*N*-methyl)perfluorobutanesulfonamido]ethanol [a]	MeFBSE	$C_4F_9SO_2N[(CH_3)(CH_2CH_2OH)]$	TCR-631	95.55%
2[(*N*-methyl)perfluorobutanesulfonamido]acetate	MeFBSAA	$C_4F_9SO_2N$ $[(CH_3)(CH_2CO_2^-]$	TCR-707	50.1%
N-Methyl Perfluorobutane sulfonamide	MeFBSA	$C_4F_9SO_2NH(CH_3)$	TCR-632	97.25%
Perfluorobutane sulfonamide	FBSA	$C_4F_9SO_2NH_2$	TCR-314	99.3%
Perfluorobutane sulfinate	PFBSi	$C_4F_9SO_2^-$	TCR-723	51.2% [b]
Perfluorobutane sulfonate [a]	PFBS	$C_4F_9SO_3^-$	TCR-282	97.3%
Perfluorooctanesulfonyl-Related Analytes				
2[(*N*-ethyl)perfluorooctanesulfonamido]ethanol [a]	EtFOSE	$C_8F_{17}SO_2N[(CH_2CH_3)(CH_2CH_2OH)]$	TCR-078, SE-031	97.7%
2[(*N*-ethyl)perfluorooctanesulfonamido]acetate	EtFOSAA	$C_8F_{17}SO_2N$ $[(CH_2CH_3)(CH_2CO_2^-]$	TCR-881	98.63%
N-Ethyl Perfluorooctane sulfonamide	EtFOSA	$C_8F_{17}SO_2NH(CH_2CH_3)$	TCR-871	100% [c]
Perfluorooctane sulfonamide	FOSA	$C_8F_{17}SO_2NH_2$	TCR-280	98.94%
Perfluorooctane sulfinate	PFOSi	$C_8F_{17}SO_2^-$	TCR-7 (SD-007)	97.2%
Perfluorooctane sulfonate [a]	PFOS	$C_8F_{17}SO_3^-$	TCR-741	99.9%
Fluorotelomer-Related Analyte				
6:2 Fluorotelomer Sulfonate [a]	6:2 FTS (aka THPFOS)	$C_6F_{13}CH_2CH_2SO_3^-$	TCR-343	93.5%
6:2 Fluorotelomer Alcohol [a]	6:2 FTOH	$C_6F_{13}CH_2CH_2OH$	TCR-1056	97%
8:2 Fluorotelomer Alcohol [a]	8:2 FTOH	$C_8F_{17}CH_2CH_2OH$	TCR-1058	97%
6:2 Fluorotelomer acid	6:2 FTCA	$C_6F_{13}CH_2CO_2^-$	TCR-679	97%
6:2 Fluorotelomer unsaturated carboxylic acid	6:2 FTUCA	$C_5F_{11}CFCHCO_2^-$	TCR-681	97%
8:2 Fluorotelomer carboxylic	8:2 FTCA	$C_8F_{17}CH_2CO_2^-$	TCR-1156	95%
8:2 Fluorotelomer unsaturated carboxylic acid	8:2 FTUCA	$C_7F_{15}CFCHCO_2^-$	TCR-1089	98%
Perfluorinated Carboxylic Acid Analytes				
Perfluorononanoate	PFNA	$C_8F_{17}CO_2^-$	TCR-618	98.02%
Perfluorooctanoate [a]	PFOA	$C_7F_{15}CO_2^-$	TCR-617	99.51%
Perfluoroheptanoate	PFHpA	$C_6F_{13}CO_2^-$	TCR-267	98.2%

Table 3. Reference Substance (Study E05-0544)

Analyte	Abbreviation	Molecular Formula	Reference Substance ID	Purity
Perfluorohexanoate	PFHxA	$C_5F_{11}CO_2^-$	TCR-673	97.7%
Perfluorobutanoate [a]	PFBA	$C_3F_7CO_2^-$	TCR-757	99.2%
Chlorinated Phenol Analytes				
Pentachlorophenol [a]	PCP	C_6HCl_5O	TCR-1053	98.2%
2,3,5,6-Tetrachlorophenol	2356-TeCP	$C_6H_2Cl_4O$	TCR-1050	99.96%
2,4,6-Trichlorophenol [a]	246-TCP	$C_6H_3Cl_3O$	TCR-937	99.6%
2,6-Dichlorophenol	2,6-DCP	$C_6H_4Cl_2O$	TCR-1051	99.9%
2,4-Dichlorophenol	24-DCP	$C_6H_4Cl_2O$	TCR-1052	99.2%
2-Chlorophenol	2-CP	C_6H_5ClO	TCR 1055	99%
4-Chlorophenol	4-CP	C_6H_5ClO	TCR-1054	99.01%
Internal Standards				
Trimethylsilylpropane sulfonate (IS)	TMSPS	$Si(CH_3)_3(C_3H_6SO_3^-)$	TCR-938	100%
Perfluorohexane sulfonate	PFHS	$C_6F_{13}SO_3^-$	TCR-890	99.98%

[a] indicates a reference which was also a test substance in this study
[b] Purity was determined for the same lot of PFBSi material (3M lot 41-2601-2249-8) as TCR-1099 at 51.2%, but was determined after use of the substance in this study as TCR-723 (3M lot 41-2601-2249-8) with purity designated at 50% based on average of MSDS 45-55% value and was only value available at time of use.
[c] Purity not determined, assumed 100%

3 METHODS

The biodegradation of each test substance was inferred by loss of test substance, and/or formation of predicted polyfluorinated degradation products (see Table 3), and all determinations were compared to the results of killed (sterile sludge) and abiotic (no-sludge) controls which were prepared in parallel with the bioactive (live sludge) test cultures. None of the cultures were pre-acclimated to the test substances and as such the estimated initial rates are reflective of native ability of the digester sludge microorganisms to biodegrade the test substances and not reflective of adapted ability which could possibly have occurred if given more time to acclimate to the test substances. Most of the culture preparation and collection data and calibration and QC preparations for this project were documented in 3M Technical Notebooks No. 139716 (Cleston Lange) and No. 140568 (Peter Radford). This work was method development/exploratory in nature and therefore culture and standard preparations and extractions were not always recorded in accordance with the laboratory SOPs for documenting sample and calibration standard preparations. Also, there were no analytical criteria or analytical procedures specified by any signature-approved protocol or general project outline (GPO), albeit a draft protocol was written apriori and which did aid in defining the scope and general goals for this method development work. All of the reported results were evaluated for their merit based on the acquired analytical results with matrix-matched calibration standards and continuing calibration verification (CCV) checks, and select quality control (QC) samples prepared for select culture sets, and all results from bioactive cultures were compared results from sterile control cultures as confirmation of biodegradation when test substance loss or product formation was observed.

The operation and parameters of the test equipment, as described herein, were in many instances developed during this study in 2005-2006 with the intention of defining Standard Operating Procedures (SOPs) to support planned GLP studies at that time. Additional references that aided in this method development project were Tiedje and Shelton (1984), the U.S.EPA Fate, Transport and Transformation Test Guidance OPPTS 835.3400 and the OECD Guideline for the testing of Chemicals No. 308 and No. 311. However, many aspects of those guidance's were not followed or were considered irrelevant to this work and several changes were adopted during this development project to allow for ease of culture preparations in the glove box, as well as to allow for replicate cultures to be prepared, and to accommodate the sensitive analyte-specific analyses by liquid chromatography with tandem mass spectrometric detection (LC/MS/MS) and gas chromatography with mass spectrometric detection (GC/MS). As a result of the method development work described herein, and previously conducted aerobic biodegradation studies up to that time, a final equipment procedure for operation and maintenance of the anaerobic glove

box (ETS-9-028) and an analytical method for the extraction of biodegradation cultures for LC/MS analysis (ETS-8-001) were finalized in 2006 and added to the 3M Environmental Laboratory quality system. However, it is noted that not all results reported herein were consistent with the final parameters or criteria established in those SOPs since most of this work preceded those SOPs, nor did the results of this study always meet the acceptance criteria established in the final analytical method ETS-8-001. The SOPs developed were more importantly put in place during later stages of this study with the intended purpose to support the GLP studies for fluoropolymer anaerobic biodegradation conducted in 2006 (i.e. 3M anaerobic biodegradation studies E05-0627 and E05-0630).

In some instances the reanalysis of all time points for a test substance was conducted in a single analytical batch after completion of the incubations, and any previous intermittent analysis results were disregarded for determining the final biodegradation conclusions (i.e. MeFBSE). Calibration curves were typically constructed from analyzing sterilized sludge matrix extracted calibration standards. In some instances, QCs were prepared by fortifying target analytes into the equivalent of sterilized sludge cultures and were extracted the same as samples and analyzed. Analyses were typically, but not always, performed by internal standard (IS) method using trimethylsilylpropane sulfonate (TMSPS) as IS in extraction solutions and used to aid in baselining analytical results for test substance loss and/or product formation. In some instances, one or more anchor points [i.e. standards below the batch-defined lower level of quantitation (LLOQ)], were used to improve low end calibration accuracy. Also, in some instances, the LLOQ peak area response was not always 2-times the response of equivalent prepared method blanks. However, in all instances those data were evaluated more closely for merit and potential effects on final results and conclusions were considered, and in some instances were not used for final reporting. The use of laboratory control spikes (LCSs) and laboratory matrix spikes (LMSs) was not included during this study and some test, control and reference substances were not fully characterized at the time of use. However, as a result of findings during this study several were later more thoroughly characterized for use in GLP biodegradation studies once it was established they were anticipated degradation products of later tested fluoropolymers.

3.1 Collection, Storage and Handling of Anaerobic Digester Sludge

The anaerobic digester sludge for this study was obtained from the municipal anaerobic digester located in Empire, Minnesota and was collected and delivered to the 3M Environmental laboratory by Pace Analytical Services personnel. At the time of sludge collection the Empire wastewater treatment plant (WWTP) constituted one component of the larger Twin Cities metropolitan wastewater treatment system comprised of eight large interconnected WWTPs servicing over 300 million gallons of wastewater daily at that time. The Empire plant was the only Twin Cities Metro WWTP that had an on-sight anaerobic digester. For this study, the anaerobic digester sludge was delivered to 3M Environmental Laboratory on four occasions for testing purposes, as listed in **Table 4.**

Anaerobic sludge was typically received at ambient temperature in polypropylene 1-L bottles sealed tightly with white non-transparent lids. Once received at 3M each was labeled with unique IDs, described in **Table 4**, and then stored refrigerated until use. The bottles of anaerobic sludge used for the earlier phases of this study were originally logged into the 3M Environmental Laboratories LIM system under project E05-0544 as samples and were given unique sample IDs starting with E05-0544-xxx. Later, these bottles were reassigned with traceability (TN-A-) numbers and treated thereafter as reagents for preparation of cultures. Sludges were always stored with the lids tightly sealed and refrigerated prior to use. The anaerobic sludge was always used within 4 weeks of collection for preparation of test cultures. Anaerobic sludge has been reported to be viable for up to four weeks for anaerobic biodegradation evaluations (Shelton & Tiedje, 1984) and this storage period is more than the two weeks refrigerated storage suggested by U.S. EPA and OECD guidance. Sludge was typically transferred from the refrigerator to the glove box the day of or the day before culture preparations.

The sludge was always received as a dark-black semi-solid liquid with a texture of fine mud that maintained itself as a non-partitioning single phase. Pressure within sealed containers increased when stored at ambient temperature and was easily recognizable by bulging lids on the container. This was presumed to be due to very rapid anaerobic microbial gas production. The sludge had a distinct sulfide smell. Storage of the sludge at 4°C alleviated excessive pressure during storage, presumably due to the anticipated decrease in microbial activity at cold temperatures. Evaluation of the content or chemical makeup of the sludge was not typically performed, but some evaluations were performed on certain sludge collections and used to generalize the makeup of the sludge used throughout the study since it was anticipated that the anaerobic digester is maintained as a semi-continuous yet generally homeostatic system over time.

Sterilized sludge was prepared by autoclaving 500 mL of the anaerobic digester sludge for 30-45 minutes in a tempered-glass Nalgene™ bottle with autoclavable orange polypropylene lid and This study report and data were partially audited by the 3M Environmental Laboratory Quality Assurance Unit (QAU).

Table 4. Digester Sludge Collection Information

Sludge Source	Date Collected	3M Env. Lab LIMS ID	Volume (L)	Total Suspended Solids (g/L)*	3M Env. Lab Chain of Custody No.
Empire WWTP (MN)	10-August-2005	E05-0544-87193	2	ND	08429
Empire WWTP (MN)	29-August-2005	E05-0544-87854/87855	2	14.3	09251
Empire WWTP (MN)	18-October-2005	E05-0544-90220	2	14.3	09599
Empire WWTP (MN)	14-February-2006**	TNA-07526**	2	11.1; 18.1***	09435

*; Total suspended solids (TSS) determined by a modification of EPA method 160.2 and determined by weight of filtered sludge that was dried at between 85-110 °C.
ND; not determined.
**; sludge was not used in this study, but was characterized for content and reported to provide general characterization data for sludge obtained from the Empire (MN) WWTP anaerobic digester.
***; TSS was determined at both the 3M Environmental Laboratory; and Pace Analytical Services and different values reported (1st value is 3M result).

Sludge from the Empire, MN wastewater treatment plant anaerobic digester was collected on 2/14/2006 for another study, and was characterized by Pace Analytical Services (Pace project no.1027866, Report dated March 2006). The general sludge characterization results are shown below in **Table 5** to provide some insight to the sludge used in 2005 from the same Empire digester; however, the sludge evaluated was not used in this study.

Table 5. Empire Plant Digester Sludge Characterization Data

Parameter	Result	Units	Analysis Method
Total Suspended Solids (dry)	18,100	mg/L	EPA 160.2
Total Solids (dry)	20,600	mg/L	EPA 160.3
Nitrogen (Ammonia)	722	mg/L	EPA 350.1
Nitrogen (Total Kjeldahl)	984	mg/L	EPA 351.2
Phosphorous (Total)	454	mg/L	EPA 365.2
Ortho-Phosphate	47.8	mg/L	EPA 365.2
Sulfate	< 2.5	mg/L	EPA 375.4
Chemical Oxygen Demand (COD)	47,800	mg/L	EPA 410.4
Non-Purged Organic Carbon (TOC-NPOC)	1,100	mg/L	EPA 415.2

[a] these data determined for digester sludge that was not used in this study, but is included to provide general characterization data for digester sludge obtained from the Empire (MN) WWTP anaerobic digester around the time frame that sludge was collected for this study. These results are for Empire plant digester sludge obtained on February 2, 2006.

3.2 Culture Preparations

Anaerobic test cultures were prepared as one of five independently prepared sets of cultures over a period of approximately three months.

The first set of cultures was prepared as a very small exploratory set of test cultures and blank cultures and was primarily used for optimizing the methods of culture preparation and analysis and for some preliminary evaluation of potential anaerobic product formation from EtFOSE and MeFBSE. This first set of cultures (IDs E05-0544-001 to E05-0544-024) were prepared on 8/17/2005 using anaerobic sludge provided from the digester on 8/10/2005. This first set of cultures was dosed with and without EtFOSE or MeFBSE. Preparation of that set of cultures is referenced in 3M Technical notebook # 139716, pages 10-12 and most of the data associated with this set is excluded from this report due to the exploratory nature of that set.

The second preparation of culture sets (IDs E05-0544-025 thru E05-0544-129) occurred on 8/29/2005 using anaerobic sludge provided from the digester on 8/29/2005. Those cultures were dosed with either EtFOSE or MeFBSE at nominal 1 μg/mL (1 ppm) of each, and set up for time dependent collection and analysis. The preparation of that set of cultures is referenced in 3M technical notebook # 139716, pages 25-26.

The third preparation of culture sets (IDs E05-0544-130 thru E05-0544-219) occurred on 9/1/2005 using anaerobic sludge provided from the digester on 8/29/2005. That set of cultures was prepared with either 6:2 FTS, PFOS or PFBS for time dependent sample collection and analysis. Preparation of the third set of cultures is referenced in 3M technical notebook # 139716, pages 31-32.

The fourth preparation of culture sets (IDs E05-0544-220 thru E05-0544-240) occurred on 9/22/2005 using sludge collected on 8/29/2005. Those cultures were prepared with 246-TCP at nominal 1 ppm of each for time dependent collection and analysis. The preparation of those cultures is referenced in 3M Technical notebook (TNB) 139716, page 59.

Finally, a fifth preparation of cultures (IDs E05-0544-500 thru E05-0544-694) occurred on 11/11/2005 using sludge collected from the digester on 10/18/2005. Those sets of cultures were prepared for time dependent collection and analysis, and were dosed with PCP, PFOA, PFBA, 6:2 FTOH or 8:2 FTOH. Preparation of those cultures is referenced in 3M technical notebook # 139716, pages 79-84. With the exception of the first set of cultures and the fourth set of cultures, all of the culture sets were prepared with ample cultures to harvest cultures five times in triplicate per time point per culture type.

Note that for the apparent missing cultures with IDs E05-0544-241 to E05-0544-499 there were no culture prepared and those IDs were skipped.

The culture sets 2 through 5 included the following: active-sludge (live) cultures, sterilized-sludge (killed) control cultures, water control (abiotic) blanks, active-sludge (live) blanks, and sterilized-sludge (killed) blanks. The final prepared active cultures contained 4 mL of active-sludge plus 1 mL of sterile 10 mg/ml yeast extract (YE solution) and test substance. Sterile control cultures contained 4 mL of autoclave-sterilized sludge plus 1 mL of sterile 10 mg/mL YE solution and test substance. Active and sterile blank cultures were prepared identically, except they were not dosed with test substance. Abiotic control blanks received 4 mL of autoclave-sterilized Milli-Q™ water and 1 mL of sterile YE solution, and no test substance.

All cultures were prepared in nominal 42.5 mL amber-glass I-Chem™ vials with screw-caps containing PTFE-lined silicon-rubber septa. To each test vessel vial was added 1 mL of sterile 10 mg/mL YE solution under a normal atmosphere of air and then followed by the transfer of those vials to the glove box while keeping caps loose during transfer in the ante-chamber to displace air with N_2 gas. Once equilibrated to a low-oxygen environment in the glove box, 4 mL of anaerobic digester sludge was added. After adding sludge, the caps were sealed and test substance was then added to the appropriate cultures through the septa using a 25 µL or 50 µL Hamilton gas-tight syringe and methanol as carrier solvent. All test substances were added as stock solutions prepared in methanol and are listed in **Table 1** along with the other reference substances used in this study listed in **Table 3**. All fluorochemical (FC) test substances were dosed into cultures to nominal concentration of 1 µg/mL (1 ppm) by adding 5 µL of a nominal 1000 µg/mL stock solution into a 5 mL culture using a glass Hamilton gas-tight syringe. The 246-TCP cultures were tested at approximately 1.2 ppm of 246-TCP by adding 5 µL of a 1,220 ppm stock solution to a 5 mL cultures, except the last time point cultures which were dosed 2X at 2.44 ppm to evaluate higher level 246-TCP effects on the cultures. The pentachlorophenol (PCP) cultures were tested at approximately 2.0 ppm of PCP by adding 10 µL of a nominal 1000 ppm stock solution to a 5 mL culture. In many instances, blank cultures were not fortified with equivalent doses of blank methanol. This could have influenced the levels of methane/CO_2 gas productions in test cultures versus blanks by providing the additional methanol which would be expected to biodegrade, and therefore is acknowledged as a potential positive effect on rates and gas production in those cultures dosed with methanolic solutions. However, sterile controls and abiotic controls did receive the same quantity of test substance as bioactive test cultures and no significant difference was noted in gas production of blanks for culture sets prepared.

Once the test substance was added to the appropriate cultures, the cultures were mixed gently and incubated static and inverted (cap down) in the glove box to minimize losses of volatiles through the cap.

Note: Resazurin is a recommended colorimetric indicator of O_2 by some guidance documents to ensure anaerobic conditions, but was excluded from these study cultures. The conditions of the glove box (i.e. O_2 levels) during preparation and incubation from the oxygen sensor in the glove box served as the indicator for anaerobic conditions, as did positive formation of methane in headspace of bioactive cultures which is inhibited by O_2 and also was not formed in sterile cultures. Cultures were prepared and stored under strictly anaerobic conditions in the glove box which was maintained as described. Cultures were typically incubated static, but were periodically (approximately once every week or two) gently mixed to maintain culture homogeneity during the incubation period.

3.3 Anaerobic Glove Box "Incubation" Conditions

The anaerobic glove box (instrument name: Durga) used for this study was a Vacuum Atmospheres Inc. Nexus Model 100043 (S/N # 00420) controlled atmosphere glove box. The glove box was maintained at between 20-26°C during the incubation periods. The glovebox atmosphere was maintained with ultra-pure nitrogen (N_2) provided from a pressurized tank (Oxygen Service Company, MN). Trace oxygen and water in the glove box atmosphere were continuously removed using a built in circulating blower and regenerative O_2 and water removal system. Transfer of materials into and out of the glove box was performed using one of two vacuum antechambers. The levels of O_2 (in ppm or percent) within the chamber were monitored using a calibrated solid state oxygen sensor (Advanced Micro Instruments, Inc.) installed on the glove box. Moisture content was monitored by a Panametrix AMX 1 dew point meter model 705-844 installed on the glove box. . All anaerobic cultures were prepared, sealed tightly, and then incubated within the glove box to ensure maintenance of anaerobic conditions. During culture incubation periods, the glove box was maintained with an atmosphere of ultra-pure nitrogen and was typically $\leq$ 1 ppm of O_2. On occasion, the O_2 levels spiked to levels greater than 10 ppm due to transfer of materials in/out of the glove box. The transient oxygen spikes returned to 1 ppm, or less, soon thereafter. The recorded oxygen and temperature readings during the incubation time frames are shown in **Figure 2** and original records can be found in 3M Technical Notebook No. 139716 (Cleston Lange) or in the instrument logbook which was put into place for the glove box

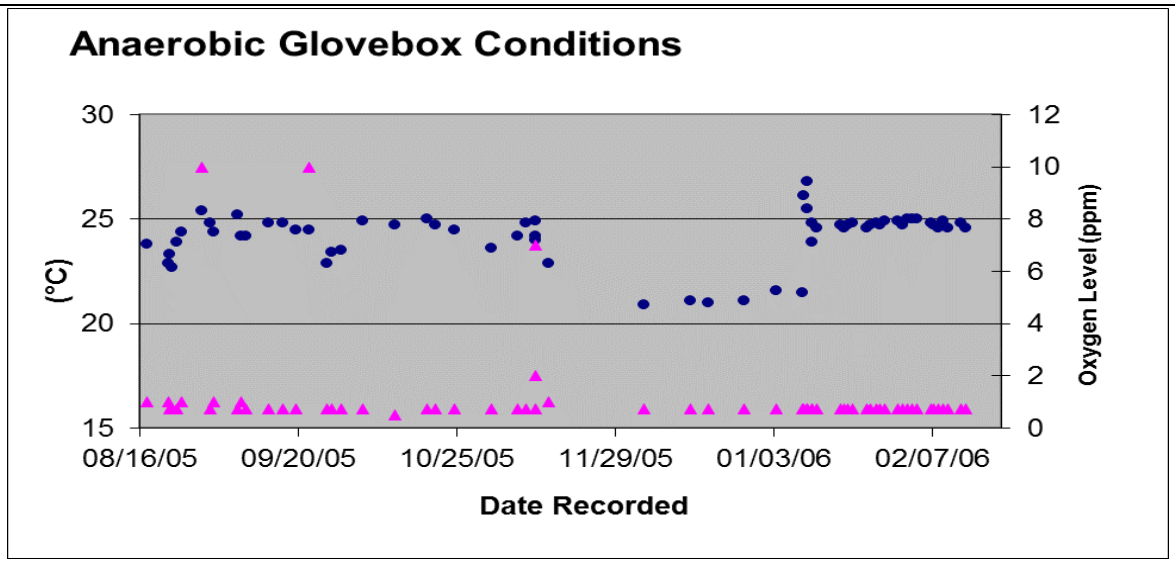

Figure 2. Glovebox temperature (●) and oxygen levels (▲) during study E05-0544.

3.4 Culture Collections

At each time point, select culture vessels were collected (harvested) from the glove box and were evaluated for production of gases (based on volumetric displacement method of a syringe barrel in a syringe). The gases were pushed back into the culture and were then frozen until such time that they could be extracted for analysis by LC/MS/MS and direct inject GC/MS. Cultures were typically harvested in duplicate or triplicate for each culture type at each collection time, except for the single culture collected for days 66 (MeFBSE) & 72 (EtFOSE0 for culture set 2, as described below.

Five sets of cultures were prepared during the course of this project. Due to the preliminary and highly developmental nature of culture set #1 prepared on 8/17/2005, and the lack of sufficient replicates and splitting the cultures out for several different analytical techniques, discussion about the 1st set of cultures is largely excluded from this report. The preparation dates, test substances dosed and incubation dates for the other culture sets were as follows: Set # 2 cultures were prepared on 8/29/2005 with MeFBSE or EtFOSE. The MeFBSE cultures were harvested on days 0, 10, 21, 29, 66 (70) and 108 and those dosed with EtFOSE were harvested on days 0, 10, 21, 29, 72 and 108. Cultures for day 70 (MeFBSE) sat on the bench for ~4 days after headspace analysis but prior to being frozen and extracted for analysis by LC/MS/MS, hence, the parenthetic value of 70 days which indicate LC/MS/MS results were for ~day 70. Headspace result for those MeFBSE cultures were for day 66.

Set # 3 cultures were dosed with PFOS, PFBS and 6:2 FTS (a.k.a. THPFOS) on 9/1/2005 and harvested on days 0, 7, 15, 26, 69 and 105. Set # 4 cultures were dosed with 246-TCP on 9/22/2005 and harvested on days 0, 5, 7, 15 and 47. Set # 5 cultures were prepared on 11/11/2005 and dosed with PFOA, PFBA, 6:2 FTOH, 8:2 FTOH or PCP and those cultures harvested on days 0, 10, 20, 74 and 94; the PCP cultures were harvested on different days 0, 3, 10, 20 and 74.

Special collection time points other than those listed above were as follows: on day 66 for culture set # 2 (MeFBSE), one active and one sterile culture for MeFBSE were removed from the glove box to perform headspace GC/MS analysis for potential volatile products, and those two samples sat on the lab bench until day 70 when they were frozen for later extraction and LC/MS/MS analysis (IDs E05-0544-112 and -129). Likewise, on day 72 for set # 2, one active and one sterile culture dosed with EtFOSE (IDs E05-0544-083 and -099) were removed from the glove box for headspace GC/MS analysis, along with cultures representing a day-72 active blank culture (E05-0544-054), a day-72 sterile blank culture (E05-0544-066) and a day-72 abiotic blank culture (E05-0544-033). All of those day-72 cultures were frozen immediately after headspace GC/MS analysis on that same day. On day 69 for culture set # 3, and active and sterile culture representing each for PFOS (E05-0544-204 and -219), PFBS (E05-0544-174 and -189) and 6:2 FTS (E05-0544-144 and -159) were removed from the glove box for headspace GC/MS analysis and then frozen immediately following the analysis on the same day.

3.5 Analytical Determinations

3.5.1 Gas Production Measurement

General gas production in the headspace of cultures was determined using a syringe barrel displacement method, which briefly was a 10 mL lure-lock polypropylene syringe with rubber tipped plunger coupled to a 21 Gauge 1 ½ inch beveled needle. The syringe plunger was pushed all the way into the syringe barrel to the zero mark and then the needle pierced thru the septa of a culture into the headspace, which promptly resulted in the plunger traveling up the barrel of the syringe, if gas pressure was present. Gas production was measured as the change in gas volume by reading the graduations on the syringe barrel after the plunger travel had ceased (measured to $\pm$ 0.1 mL). The same plunger was used for all cultures at the same sample collection day with replacement of the needle between samples and purging the syringe with air between different sets to avoid cross contamination of gases in the headspace. Gas volumes were recorded in 3M Technical notebook TNB 139716. All syringes and needles were used as provided by the manufacturer (Becton-Dickinson) and were not treated with silicon grease or other lubricants.

3.5.2 Gas Phase FTIR Analysis

Gas phase Fourier transform infrared (FTIR) spectroscopy analysis was conducted using a Midac Model I2001 FTIR with a nominal 10 cm (~ 100 mL) gas cell and using a 2001 ppm ethylene calibration standard (balanced with N_2) For sampling, 1 mL of static headspace from a culture was taken by gas tight syringe and introduced to the FTIR cell and analyzed. Nitrogen was used as a makeup gas for all measurements. The gas phase FTIR analysis was conducted primarily to quantify methane and carbon dioxide in culture headspaces following 3M Environmental Laboratory method ETS-8-031 and to qualitatively evaluate the gases for any potential high level volatile FC products. Only methane, carbon dioxide and water were observed by gas phase FTIR of culture headspaces; volatile fluorocarbon (C-F stretch) absorbance was not observed.

3.5.3 Headspace-GC/MS Analysis

Semi-quantitative/qualitative analysis of culture headspace gases for volatile fluorocarbon analytes was performed by gas chromatography with mass spectrometric detection (GC/MS) and was performed on cultures collected on day 66 (MeFBSE cultures only) and day 72 for cultures sets #2 and #3. The method used was slightly modified from that described in method ETS-8-016 and was modified for means of sample introduction via a syringe (ETS-8-016 is essentially EPA method TO15). A volatile surrogate standard was added to the culture headspace prior to sampling and then approximately 20 mL of headspace was withdrawn from each culture into a glass 50 mL Hamilton syringe fitted with a gas tight-valve. This was then injected onto a cryogenically cooled solid-phase Tenax™ trap to focus the volatiles for heated injection onto the GC/MS column. See data for analytical runs conducted on instrument ID “Zeke” in October-November 2005. Analytical batch Z051031.s was analysis of calibration standards. Batch Z051103.s included headspace analysis for cultures E05-0544-112 (bioactive MeFBSE, day 66) and -129 (Sterile MeFBSE, day 66). Batch Z051109.s included headspace analysis for cultures E05-0544-054 (bioactive blank, day 72), -144 (bioactive 6:2 FTS, day 69), -083 (bioactive EtFOSE, day 72), -174 (bioactive PFBS, day 69) and -204 (bioactive PFOS, day 69). Volatile fluorocarbon analytes were not detected above background for any of the analyzed culture headspaces, indicating they were not formed for EtFOSE, MeFBSE, 6:2 FTS, PFBS and PFOS. Culture sets #4 and #5 which were dosed with PFOA, PFBA, 6:2 FTOH, 8:2 FTOH, 246-TCP and PCP were not analyzed by headspace GC/MS.

3.5.4 Analytical Sample Preparation

Following headspace gas sampling/analysis, cultures were either frozen until later extraction with acetone, or were extracted immediately with acetone.

All 5 mL cultures were extracted by addition of 15 mL of acetone containing nominal 250 ng/mL of trimethylsilylpropane sulfonate (TMSPS) and 250 ng/mL of perfluorohexane sulfonate (PFHS) as internal standards (ISs). After addition of acetone extraction solution, each was briefly shaken to mix and then centrifuged at 1500 rpm to pellet the solids. Aliquots of supernatant were transferred to autovials for analyses. Analyses were performed against matrix matched calibration standards which were prepared by fortifying known quantities of target analytes into 4 mL of sterilized sludge plus 1 mL yeast extract solution followed up with extraction similar manner as samples using acetone extraction solution. All preparations associated with analytical samples were documented in 3M Technical notebook #139716 (Cleston Lange) and #140568 (Peter Radford).

3.5.5 Direct Inject GC/MS Analysis

The GC/MS analysis of acetone extracts for test substances 6:2 FTOH and 8:2 FTOH was performed using a Hewlett Packard 6890A gas chromatograph equipped with a 5973 mass-selective detector (MSD); shown as analytical run #24 (R060517a.S) in **Table 8**. The associated blank cultures prepared with the FTOH cultures were accidentally excluded from the analysis by the analyst, hence no background levels were established for the blank sludge cultures. The GC parameters included a split-less injection of 5.0 µL of acetone sample extract onto a 30 m x 0.250 mm ID x 0.001 mm film DB-1MS column was performed. A 15.5 minute temperature program was used with the following parameters: initial temp at 50°C for 1.00 minute; 20°C/minute to 300°C; hold 2 minutes at 300°C. The mass spectrometer was operated using electron impact (EI) source at 69.9 eV and 230°C, the MS quadrapole was at 150°C, GC to MS transfer line was at 250°C. Selected ion monitoring (SIM) mode was used to collect data for mass-to-charge ratio (*m/z*) positively charged ions with *m/z* 131, 169, 395, 405, 414 and 444.

Calibration standards for 8:2 FTOH were standards 140568-039D1 to -039D8 and for 6:2 FTOH were 140568-039C1 to -039C8. For GC/MS analysis of the 8:2 FTOH cultures, two QCs were analyzed in the analytical batch R060517.S approximately every 15 samples as CCVs and were identified as 140568-039D9 and 140568-039D10, described on page 41 of 3M TNB 140568. For GC/MS analysis of the 6:2 FTOH cultures, two QCs were analyzed in the analytical batch R060517a.S approximately every 15 samples as CCVs and were identified as 140568-039C9 and 140568-039C10. Those QC were prepared at 40 ng/mL and 400 ng/mL, respectively. Samples were analyzed for 6:2 FTOH and 8:2 FTOH with calibration standards identified as 140568-039C1 to -039C8 and described on page 41 also.

Quantitation was performed by external standard method using Analyst® software version 1.6.2 after conversion of Chemstation *.d files to common data format *.cdf file extension using Vx Capture™ and then to Analyst readable *.wiff extension using the Analyst Translat.exe program. Integration of the respective FTOH peaks was performed from the total ion chromatogram (TIC)

created by summing SIM ions *m/z* 169, 295, 305 and 314 for 6:2 FTOH (Peak Rt. 4.68 min.) and SIM ions *m/z* 169, 395, 405, and 414 for 8:2 FTOH (Peak Rt. 5.70 min.). Specific instrument parameters can be found with the raw data and the analytic run log associated with analytical run R060517a.S.

3.5.6 LC/MS/MS Analysis

After establishing the presence of and identities of biodegradation products by qualitative analysis (data not shown, see TNB 139716, pages 17-23), the quantitative LC/MS/MS analyses for degradation products was conducted for all test culture extracts. The LC/MS/MS method utilized an Agilent 1100 series high-pressure liquid chromatograph (HPLC: degasser, binary pump, autosampler and thermostat controlled column compartment) coupled to a triple quadrapole mass spectrometer with electrospray interface. Ions monitored for the target analytes are listed in **Table 6**. Chromatographic separations of fluorochemical analytes was performed at a flow rate of 1 mL/minute by 2-D chromatography which used an NG1 anion exchange (4 x 35 mm, 5µ; Dionex) guard column in-line with a Betasil C8 reverse-phase (4.6 x 150 mm, 5µ; Keystone) analytical column. Injection of sample extracts varied for different batches and ranged between 5 µL and 75 µL. The HPLC gradient was as described in **Table 7.** Quantitative LC/MS/MS analysis of chlorinated phenols was performed using the C8 column only (non NG1 column), but similar mobile phase conditions and gradient as described in **Table 7**. Analysis of the 6:2 FTS culture extracts was performed either by the NG1-C8 configuration above or using a different column configuration [Betasil C8 (4.6 x 150mm, 5µ) and Rocket C18 (3 x 37 mm, 3µ)] in tandem, with slightly modified aqueous/methanol gradient profile (see raw data). The specific HPLC and mass spectrometer operating parameters used for each analytical run are recorded within the raw data.

Quantitative analysis was typically performed using an 8 point calibration curve, with some exceptions as noted for EtFOSE which had 11 calibration standards analyzed. Curves were constructed using results from analysis of matrix-matched calibration standards typically ranging at nominal concentrations of 10.0, 25.0, 100, 250, 500, 750, 1000 and 2000 ng/mL of target analytes (5.00, 12.5, 50.0, 125, 250, 375 and 1000 ng/mL for PFBSi). For quantitation of the EtFOSE cultures at analyte concentrations below 10.0 ng/mL, three additional standards (total of 11 standards) were included with the additional three prepared at 1.00, 4.00 and 7.50 ng/mL.

Quality control samples (QCs) were prepared at 40 ng/mL and 400 ng/mL and analyzed as continuing calibration verification (CCVs) in all LC/MS/MS analytical batches used to analyze culture extracts for dosed substances MeFBSE, EtFOSE, PFOS, PFBS, PFOA and PFBA, except run #26 in which the CCVs were reinjection of the 250 ng/mL standard. Similarly, QCs were prepared at 40 ng/mL and 400 ng/mL and analyzed in direct inject GC/MS analytical batch for analysis of 6:2 FTOH and 8:2 FTOH.

The cultures dosed with TM derived substances (i.e. 6:2 FTOH, 8:2 FTOH and 6:2 FTS) were analyzed by LC/MS/MS for PFCAs, FTCAs, 6:2 FTS and FTUCAs. The CCVs in those analytical batches were the re-injection of a low-level (25.0 ng/mL) and mid-level (750 ng/mL) calibration standard throughout the run. CCVs were injected approximately every 10-15 samples and at the end of the run and were within 100 $\pm$ 30% accuracy.

The cultures dosed with 246-TCP were analyzed by LC/MS/MS in analytical run #12 and the CCVs in that run were the 800 ng/mL calibration standard. The PCP cultures were analyzed by LC/MS/MS in analytical run #27 and the CCVs in that run were reinjection of the 600 ng/mL calibration standard.

The lower limit of quantitation was analyte and batch specific and was defined as the lowest standard that gave good accuracy with the fitted calibration curve (100$\pm$ 30%) and that gave a peak area response at least 2-times the response of the associated blank analyzed just prior to the 1st calibration standard injection. LLOQs were $\leq$ 25.0 ng/mL for all analytes reported, and commonly were $\leq$ 10.0 ng/mL.

Exceptions to the normal rules for assigning LLOQs were in analytical run #21 where the LLOQ for PFOA was assigned as 4.00 ng/mL, but the curve included the 1.00 ng/mL standard (as an anchor point); but the 1.00 ng/mL standard was not 2-times the blank response. Also, the LLOQ in run #21 for PFOS was assigned as 7.5 ng/mL, however that calibration standard was not included in the calibration curve, but was 109% accurate and was more than 2-times the response of the blanks. The actual analyte LLOQs are shown in the individual results sections for each test substance.

All calibration standards included in calibration curves were within 100 $\pm$ 25% of theoretical and CCVs/QC results were within 100 $\pm$30%, with the following exceptions: **1)** one QC analyzed as a CCV for PFBSi in analytical run #5 recovered at 145% but did not affect the reported PFBSi results since all samples flanked by that QC were below the LLOQ (BLOQ); **2)** five CCVs in the latter half of the run #8 for 6:2 FTS were at 152%, 131%, 144%, 154% and 151%, data was reported based on most results flanked by those CCVs were BLOQ and the 6:2 FTS cultures analyzed gave results consistent with the dose levels; **3)** two CCVs in the latter half of analytical run #8 for 6:2 FTCA were 147% and 143%, but flanked samples were all BLOQ; **4)** two CCVs for 6:2 FTS in analytical run #8 were 152% and 131% and data flanked by those CCVs was reported and those data are flagged in the appropriate tables, and three other CCVs in that run for 6:2 FTS were 144%, 154% and 151% but flanked samples which were all < LLOQ and did not affect the reported results; **5)** one calibration standard for 24-DCP in analytical run #12 was 126%; **6)** one CCV in analytical run #26 was 69% for MeFBSE.

Table 6. Analytes and MS ions for LC/MS/MS and Direct Inject GC/MS Analyses (Study E05-0544)

LC/MS/MS Analiysis	Reference Material	Abbreviation [a]	Molecular Formula (anion shown)	Parent Ion (*m/z*)	Daughter Ion (*m/z*)
ECF-Derived	2[(*N*-Methyl)perfluorobutanesulfonamido]ethanol; acetate adduct	MeFBSE	$C_4F_9SO_2N[(CH_3)(CH_2CH_2OH)] \bullet CH_3CO_2$ -	416	59
	2[(*N*-methyl)perfluorobutanesulfonamido]acetate	MeFBSAA	$C_4F_9SO_2N[(CH_3)(CH_2CO_2$ -)]	370	219
	N-Methyl perfluorobutanesulfonamide	MeFBSA	$C_4F_9SO_2N(CH_3)$ -	312	69
	Perfluorobutanesulfonamide	FBSA	$C_4F_9SO_2NH$ -	298	78
	Perfluorobutane sulfinate	PFBSi	$C_4F_9SO_2$ -	283	219
	Perfluorobutane sulfonate	PFBS	$C_4F_9SO_3$ -	299	80
	2[(*N*-Ethyl)perfluorooctanesulfonamido]ethanol; acetate adduct	EtFOSE	$C_8F_{17}SO_2N[(CH_2CH_3)(CH_2CH_2OH)] \bullet CH_3CO_2$ -	630	59
	2[(*N*-Ethyl)perfluorooctanesulfonamido]acetate	EtFOSAA	$C_8F_{17}SO_2N[(CH_2CH_3)(CH_2CO_2$ -)]	584	169
	N-Ethyl perfluorooctanesulfonamide	EtFOSA	$C_8F_{17}SO_2N(CH_2CH_3)$ -	526	65
	Perfluorooctanesulfonamide	FOSA	$C_8F_{17}SO_2NH$ -	498	78
	Perfluorooctane sulfinate	PFOSi	$C_8F_{17}SO_2$ -	483	219
	Perfluorooctane sulfonate	PFOS	$C_8F_{17}SO_3$ -	499	80
Perfluorinated Carboxylates (can be ECF derived or TM derived)	Perfluorobutanoate	PFBA	$C_3F_7CO_2$ -	213	169 or 119
	Perfluorohexanoate	PFHxA	$C_5F_{11}CO_2$ -	313	269
	Perfluoroheptanoate	PFHpA	$C_6F_{13}CO_2$ -	363	319
	Perfluorooctanoate	PFOA	$C_7F_{15}CO_2$ -	413	369, 319 or 169
	Perfluorononanoate	PFNA	$C_8F_{17}CO_2$ -	463	419
Internal Standards (ISs)	Trimethylsilylpropane sulfonate (IS)	TMSPS	$Si(CH_3)_3(C_3H_6SO_3$ -)	195	80
	Perfluorohexanesulfonate (IS)	PFHS	$C_6F_{13}SO_3$ -	399	80

Table 6. Analytes and MS ions for LC/MS/MS and Direct Inject GC/MS Analyses (Study E05-0544)					
LC/MS/MS Analiysis	**Reference Material**	**Abbreviation [a]**	**Molecular Formula (anion shown)**	**Parent Ion (*m/z*)**	**Daughter Ion (*m/z*)**
Chlorinated Phenols	Pentachlorophenol	PCP	C_6Cl_5O -	263, 261	35
	2356-Tetrachlorophenol	2356-TeCP	C_6HCl_4O -	229,227	35
	246-Trichlorophenol	246-TCP	$C_6H_2Cl_3O$ -	195, 197	35
	24-Dichlorophenol	24-DCP	$C_6H_3Cl_2O$ -	161, 163	35
	4-Chlorophenol	4-CP	C_6H_4ClO -	127, 129	35
TM Derived	6:2 Fluorotelomer sulfonate	6:2 FTS (a.k.a THPFOS)	$C_6F_{13}CH_2CH_2SO_3$-	427	80
	6:2-fluorotelomer carboxylate	6:2-FTCA	$C_6F_{13}CH_2CO_2$ -	377	293
	Unsaturated 6:2 Fluorotelomer carboxylate	6:2 FTUCA	$C_5F_{11}CFCHCO_2$ -	357	293
	8:2-fluorotelomer carboxylate	8:2-FTCA	$C_8F_{17}CH_2CO_2$ -	477	393
	Unsaturated 8:2 Fluorotelomer carboxylate	8:2 FTUCA	$C_7F_{15}CFCHCO_2$ -	457	393
Direct inject GC/MS Analysis	**Analyte**	**Abbreviation**	**Molecular Formula (anion)**	**Quant Ion (*m/z*)**	
TM Derived	6:2 Fluorotelomer Alcohol	6:2 FTOH	$C_6F_{13}C_2H_4OH$	169, 295, 305, 314 (sum)	
	8:2 Fluorotelomer Alcohol	8:2 FTOH	$C_8F_{17}C_2H_4OH$	169, 395, 405, 414 (sum)	
[a] Naming of fluorinated compounds in this report is consistent with the terminology and classification set forth by Buck *et al*. 2011.					

Table 7. Typical HPLC Mobile Phase Gradient for NG1-C8 method (E05-0544)

Time (minutes)	% A	% B
0.00	3.0	97.0
0.50	3.0	97.0
5.00	95.0	5.0
9.00	95.0	5.0
9.01	3.0	97.0
12.00	3.0	97.0

Mobile Phase A equals 2 mM aqueous Ammonium Acetate Solution
Mobile Phase B equals HPLC grade Methanol
Flow rate was 1.00 mL/ minute
Note: Holding times between 5-9 minutes and 9.01 and 12.00 minutes may have varied to accommodate later eluting peaks during analysis of some analytes on alternative column setups, but gradients always remained the same.

The analytical batches used to report or confirm results for this study are described in **Table 8**.

Table 8. Study Analytical Batch Summary (Study E05-0544)

Run #	Purpose for Analysis	Date of Analysis	Analytical Run/Batch ID	Instrument Name/Type	Data Used for Reporting (Y/N)	Analytes Reported
1	Qualitative LC/MS & LC/MS/MS of cultures from set # 1	25-Aug-2005	Stan Data files\Projects\Anaerobic Biodeg\Anaerobic Screening 08-24-2005\Data Anaerobic Set 08-25-2005 Set #1.wiff	Stan (API 4000)	NO	None
2	Qualitative direct inject GC/MS of cultures from set #1	06-Sept-2005	Rufus 2\MSDChem\1\Data\Anaerobic Headspace06-sept-2005	Rufus 2 (Agilent GC- MSD)	NO	None
3	Qualitative LC/MS/MS of cultures from set #1	08-Sept-2005	Stan Data files\Projects\Anaerobic Biodeg\Anaerobic Screening 08-24-2005\08-Sept-200508-Sept-2005 (C4s MRM) and (C8s MRM)	Stan (API 4000)	NO	None
4	Quantitative. LC/MS/MS EtFOSE cultures (set 2) days 0 to 29	06-Oct-2005	Ollie data\Projects\E05-0544\2005_10_06\ E05-0544 EtFOSE Cultures & Blanks--06-OCT-2005	Ollie (API 4000 Q-Trap)	NO	None
5	Quant LC/MS/MS MeFBSE cultures (set 2) days 0 to 29	11-Oct-2005	Ollie data\Projects\E05-0544\2005_10_06\ E05-0544 MeFBSE Cultures & Blanks--11-OCT-2005	Ollie (API 4000 Q-Trap)	YES	MeFBSE, MeFBSAA, MeFBSA, FBSA, PFBSi, PFBS & PFBA

Table 8. Study Analytical Batch Summary (Study E05-0544)

Run #	Purpose for Analysis	Date of Analysis	Analytical Run/Batch ID	Instrument Name/Type	Data Used for Reporting (Y/N)	Analytes Reported
6	Quant LC/MS/MS PFBS cultures days 0, 7, 18 & 26	19-Oct-2005	a051019a.spl	Amelia (Quattro II)	YES	PFBS & PFBA
7	Quant LC/MS/MS, PFOS cultures days 0, 7, 18 & 26	20-Oct-2005	a051020b.spl	Amelia (Quattro II)	YES	PFOS & PFOA
8	Quant LC/MS/MS, 6:2 FTS cultures days 0,7,18 & 26	25-Oct-2005	a051025a.spl	Amelia (Quattro II)	YES	6:2 FTS, 6:2 FTUCA & PFHxA
9	Quant LC/MS/MS, PFBS cultures day 26	28-Oct-2005	a051028a.spl	Amelia (Quattro II)	NO	None, confirmatory results only
10	Quant LC/MS/MS, PFOS cultures day 26	31-Oct-2005	a051031a.spl	Amelia (Quattro II)	YES	FOSA & PFOSi
11	Quant LC/MS/MS, PFOS cultures day 0	01-Nov-2005	a051101a.spl	Amelia (Quattro II)	YES	FOSA & PFOSi
12	Quant LC/MS/MS, TCP cultures days 0, 5, 7, 15 & 47	08-Nov-2005	a051108a.spl	Amelia (Quattro II)	YES	246-TCP, 24-DCP & 4-CP
13	Quant LC/MS/MS for PFOS, PFBS and 6:2 FTS cultures (day 69), MeFBSE (day 70), and EtFOSE (day 72)	22-Nov-2005	a051122a.spl	Amelia (Quattro II)	NO	None, data not used to simplify reporting
14	Quant LC/MS/MS, MeFBSE cultures (set 2) day 70	23-Nov-2005	a051123a.spl	Amelia (Quattro II)	NO	None, confirmatory results only

Table 8. Study Analytical Batch Summary (Study E05-0544)

Run #	Purpose for Analysis	Date of Analysis	Analytical Run/Batch ID	Instrument Name/Type	Data Used for Reporting (Y/N)	Analytes Reported
15	Quant LC/MS/MS, PFBS culture day 69	30-Nov-2005	a051130a.spl	Amelia (Quattro II)	YES	PFBS, PFBSi, FBSA, PFBA
16	Quant LC/MS/MS, PCP cultures day 0, 3, 10 & 20 and TCP cultures day 0, 5, 7, 15 and 47	06-Dec-2005	a051206a.spl	Amelia (Quattro II)	NO	None, confirmatory results only
17	Quant LC/MS/MS, 6:2 FTOH and 8:2 FTOH cultures days 0, 10 & 20	14-Dec-2005	a051214a.spl	Amelia (Quattro II)	NO	None; Data was accidentally deleted and lost-results reported from a051215a
18	Quant LC/MS/MS, 6:2 FTOH and 8:2 FTOH cultures days 0, 10 & 20	15-Dec-2005	051215a.spl	Amelia (Quattro II)	YES	PFHxA, PFHpA, 6:2 FTCA & 6:2 FTUCA
19	Quant LC/MS/MS, PFOA and PFBA cultures days 0, 10 & 20	19-Dec-2005	a051219a.spl	Amelia (Quattro II)	YES	PFBA, PFHpA, PFOA, PFNA
20	Quant LC/MS/MS, PFBS cultures (day 105) and MeFBSE cultures (days 70 and 108); and EtFOSE cultures (days 0,10,21,29,72 and 108)	27-Dec-2005	a051227a.spl	Amelia (Quattro II)	YES	MeFBSE, MeFBSAA, MeFBSA, FBSA, PFBSi, PFBS & PFBA; EtFOSE, EtFOSAA, EtFOSA, FOSA, PFOSi, PFOS & PFOA
21	Quant LC/MS/MS, PFOS cultures (day 69) and 6:2 FTS cultures (days 69 and 105)	29-Dec-2005	a051229a.spl	Amelia (Quattro II)	YES	PFOS, PFOSi, FOSA, PFOA; 6:2 FTS, 6:2 FTUCA, 6:2 FTCA, PFHxA

Table 8. Study Analytical Batch Summary (Study E05-0544)

Run #	Purpose for Analysis	Date of Analysis	Analytical Run/Batch ID	Instrument Name/Type	Data Used for Reporting (Y/N)	Analytes Reported
22	Quant LC/MS/MS, 6:2 FTOH cultures for days 74 and 94	07-March-2006	A060307a.spl	Amelia (Quattro II)	YES	6:2 FTCA, 6:2 FTUCA, PFHxA and PFHpa
23	Quant LC/MS/MS, 8:2 FTOH and PFOA cultures for days 74 and 94	08-March-2006	A060308a.spl	Amelia (Quattro II)	YES	PFOA & PFNA
24	Quantitative direct inject GC/MS for 6:2 FTOH and 8:2 FTOH for days, 0, 10, 20, 74 and 94	17-May-2006	R060517a.S	Rufus (HP GC/MS)	YES	6:2 FTOH & 8:2 FTOH
25	Quant LC/MS/MS, PFOS cultures for day 105	14-July-2006	A060714a.spl	Amelia (Quattro II)	YES	PFOS, PFOSi, FOSA, PFOA, EtFOSE, EtFOSAA, EtFOSA
26	Quant LC/MS/MS, PFBA cultures for day 74 and 94	01-August-2006	A060801b.spl	Amelia (Quattro II)	YES	PFBA, PFBSi, PFBS and FBSA [Also MeFBSE, MeFBSAA, MeFBSA were analyzed, data not used]
27	Quant LC/MS/MS of PCP cultures for days 0, 3, 10, 20 & 74	16-August-2006	A060816b.spl	Amelia (Quattro II)	YES	PCP, 2356-TeCP, 246-TCP, 24-DCP and 4-CP
28	Quantitative LC/MS/MS of 8:2 FTOH cultures (day 0, 10, 20, 74 & 94) and Qualitative analysis of 6:2 FTOH Cultures (day 74 and 94)	18-August-2006	A060818a.spl	Amelia (Quattro II)	YES	PFHxA, PFHpA, PFOA, PFNA, 8:2 FTCA, 8:2 FTUCA
29	Semi quantitative/qualitative Headspace GC/MS of day 66 MeFBSE Cultures	03-Nov.-2005	Z051103.s	Zeke (Agilent GC- MSD)	YES	Volatiles

Table 8. Study Analytical Batch Summary (Study E05-0544)

Run #	Purpose for Analysis	Date of Analysis	Analytical Run/Batch ID	Instrument Name/Type	Data Used for Reporting (Y/N)	Analytes Reported
30	Semi quantitative/qualitative Headspace GC/MS of day 69 PFBS, PFOS and 6:2 FTS cultures; and day 72 bioactive blank and EtFOSE culture	03-Nov.-2005	Z051109.s	Zeke (Agilent GC- MSD)	YES	Volatiles

Note: This table is a comprehensive list of all analyses conducted for this study. However, not all data was used because of either its qualitative nature or redundancy with other runs for the same samples where better data quality was achieved. Data quality objectives were not defined for this study, hence data was never rejected because of failure to attain specific data quality objectives and the quality of the data reported are specified in each analytical data set.

4 RESULTS & DISCUSSION

The results reported herein were compiled from laboratory work conducted as part of a method development project for anaerobic methods and procedures needed to conduct GLP anaerobic biodegradability testing studies. The results of this discovery study provided early insight to the potential anaerobic biodegradability of several fluorochemical substrates (test substances), or the lack thereof for some. The cultures that were prepared utilized a rich anaerobic microbial inoculum from a municipal anaerobic digester sludge (Empire MN). The intent of this study was neither for regulatory submission nor to extrapolate potential rates of degradation in natural anaerobic environments, nor anaerobic wastewater treatment systems. However, these data do provide some insight into the potential fate of these substances in anaerobic environments in general.

Degradation curve fits were performed manually using ACD Labs Chemsketch 2012 software (freeware version 14.01). Chemical structures shown in degradation pathway figures were also generated using ACD Labs Chemsketch™ 2012.

4.1 Anaerobic Culture Test Parameters

Increases in culture gas volumes were determined after removal of the culture from the glove box on collection days and equilibration to ambient temperature. Changes in gas volume (ΔV) in the headspace was determined by a syringe barrel displacement method described in section 3.5.1. The change in gas volume are plotted in **Figures 3 and 4**. It was apparent within the first week of incubation that gas volume had increased in all of the bioactive-sludge cultures resulting in syringe barrel displacement values of approximately 5 mL to 7 mL, while no significant increase was realized in the sterile-sludge controls (not shown). Changes in gas volumes continued to rise throughout the experiment for the first three to four weeks in the bioactive cultures, after which the gas pressures appeared to plateau and then decreased during the remainder of the incubations. Increased gas volume was observed for bioactive cultures dosed with test substance versus bioactive blanks, and was assumed to be formed predominantly from the 5 μL of methanol solvent used to deliver test substances and which would have been converted to additional methane and carbon dioxide; methanol was not added to bioactive blanks and therefore only reflect methane and carbon dioxide formed from organic carbon in sludge and dilute yeast extract solution.

Semi-quantitative analysis by extractive gas-phase FTIR of headspace gases from day-26 and day-29 bioactive-sludge and sterile-sludge cultures for culture sets #3 and #2 (see section

3.2), respectively, showed methane and carbon dioxide were the primary gases in the headspaces of those bioactive cultures. Methane and carbon dioxide were not appreciably produced in sterile-sludge cultures. Additionally, the ratio of methane-to-carbon dioxide (approximately 4:1) was in good agreement with the ratio of methane and carbon dioxide expected for biogas (https://en.wikipedia.org). The gas-phase FTIR results for methane and carbon dioxide concentrations are presented in **Table 9.** No volatile fluorochemicals were observed in headspaces by gas-phase FTIR analysis.

Additional analyses for volatile fluorochemicals at for day 66, 69 and day 72 for cultures from culture sets #2 and #3 by a sensitive headspace GC/MS method showed that no volatile FCs were formed from any of the fluorochemical test substances. However, the GC/MS data did show the presence of volatile hydrogen sulfide and alkylated sulfides in the headspace of active cultures, but not in sterile cultures (data not shown), and suggests the cultures likely contained sulfate-reducing microorganisms.

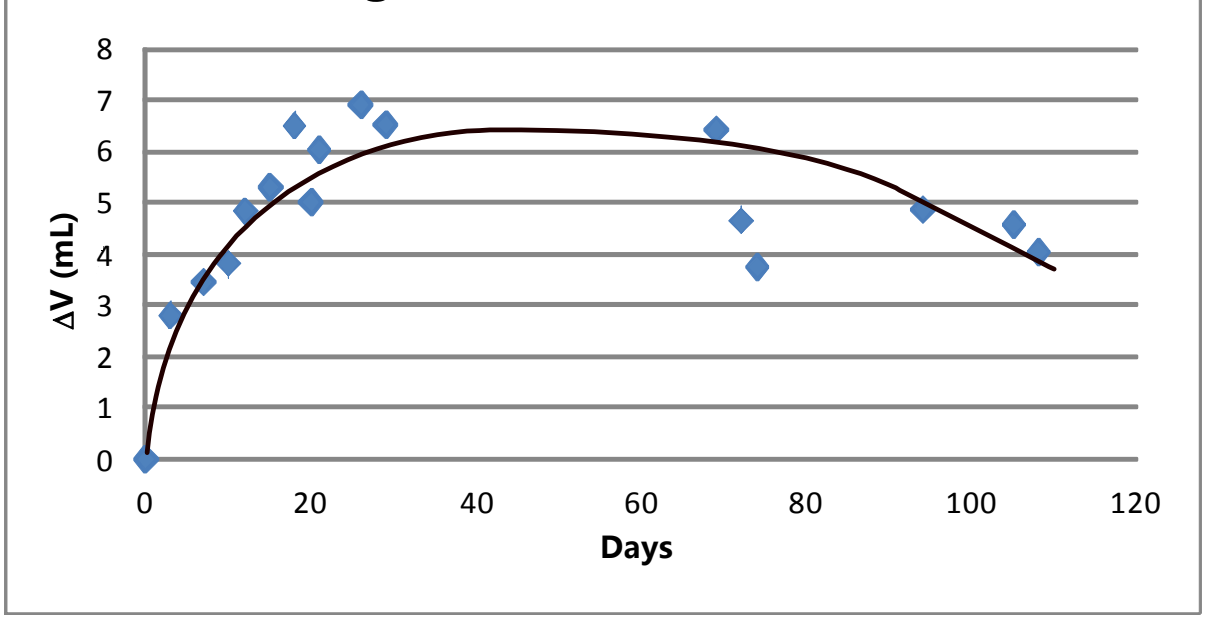

Figure 3. The average change in gas volumes measured for all bioactive cultures (blank cultures and dosed cultures) from culture sets #2, #3, #4, and #5. (Data manually fit for appearance)

Table 9. Methane & Carbon Dioxide Concentrations by Gas-Phase FTIR

Sample ID	Description	Methane (ppm)	Carbon Dioxide (ppm)	Gas Volume (ΔV; mL)
E05-0544-0028	Water Blank, Day 29	< MDL	1240	0.0
E05-0544-0061	Sterile-sludge Blank, Day 29	< MDL	542	0.0
E05-0544-0049	Active-sludge Blank, Day 29	99,600	28,300	5.0
E05-0544-0079	EtFOSE, Day 29, Active-sludge	131,00	34,700	7.7
E05-0544-0094	EtFOSE, Day 29, Sterile-sludge	1,860	681	0
E05-0544-0109	MeFBSE, Day 29, Active-sludge	131,000	25,100	7.2
E05-0544-0124	MeFBSE, Day 29, Sterile-sludge	1,830	< MDL	< MDL
E05-0544-0139	6:2 FTS, Day 26; Active-sludge	118,000	36,300	7.5
E05-0544-0154	6:2 FTS, Day 26; Sterile-sludge	< MDL	1660	< MDL
E05-0544-0199	PFOS, Day 26; Active-sludge	119,000	35,300	6.9
E05-0544-0214	PFOS, Day 26; Sterile-sludge	1,810	2,720	< MDL
E05-0544-0169	PFBS, Day 26; Active-sludge	124,000	41,100	7.4
E05-0544-0184	PFBS, Day 26; Sterile-sludge	786	1,660	< MDL

Note: The minimum detection limit (MDL) for CO_2 was 418 ppm, and the MDL for CH_4 was 780 ppm by extractive gas phase FTIR. All values in the table are corrected for dilution factors applied during analysis and are shown to 3 significant figures only.

4.2 Anaerobic Biodegradation Testing Results

4.2.1 EtFOSE Results

The test substance [2-(*N*-ethyl)-perfluorooctanesulfonamido]ethyl alcohol (EtFOSE) was dosed into anaerobic cultures at 1010 ng/mL (1.01 ppm) and incubated anaerobically for up to 108 days.

Quantitative LC/MS/MS analysis of processed culture extracts was conducted for EtFOSE and the anticipated biodegradation products EtFOSAA, EtFOSA, FOSA, PFOSi, PFOS and PFOA. Data was collected from analytical run #20 (A051227a-2.spl) as described in **Table 10**. While internal standard PFHS peaks were integrated and included in raw data results, quantitation was performed by external standard method for all target analyte and the IS was not used for quantitation. Calibration curves contained six or more calibration standards. Curve fits were quadratic with either 1/x or 1/x^2 weighting. The coefficient of determination (R) values for all calibration curves were $\geq$ 0.990. The lower limit of quantitation (LLOQ) for each analyte were as follows: PFOA (7.50 ng/mL), PFOSi (1.00 ng/mL), PFOS (7.50 ng/mL), FOSA (7.50 ng/mL), EtFOSA (7.50 ng/mL), EtFOSAA (4.00 ng/mL) and EtFOSE (1.00 ng/mL). Quantitative accuracy for all analytes was within 100$\pm$ 25% for calibration standards and QCs (used as analytical run CCVs). While data from analytical run #4 for analysis of EtFOSE day 0 to day 29 cultures was technically usable, it was not used for reporting and only served as confirmation of results for the entire culture set day 0 to day 108 determined in the single analytical run #20.

The LC/MS/MS analysis showed that EtFOSAA and PFOSi were produced at measurable levels in bioactive cultures, but not in sterile cultures. Both products steadily increased in concentration over the course of the 108 days of incubation. The maximum concentration of EtFOSAA reached 21.9 ng/mL at day 72, and PFOSi maximum value was reached at 4.58 ng/mL on day 72. The EtFOSE concentration in active cultures was not significantly reduced, suggesting the observed biotransformation was very slow and was likely the only reactions taking place. Furthermore, analysis of active and sterile culture headspaces at day-72 of incubations by a sensitive GC/MS assay showed that volatile fluorocarbon products were not generated.

At day-72, the time point with the maximal measured total product, there was 235 pmole of total product (EtFOSAA+ PFOSi), from a theoretical 8,844 pmole of EtFOSE dosed on day 0. This accounts for a maximum biotransformation of 2.66 mole% of the dosed EtFOSE. The average concentration of products at day-108 appeared to be less than at

day 72 and was likely an artifact of the analysis. Anticipated products EtFOSA, FOSA, PFOS and PFOA were not formed from anaerobic biodegradation of EtFOSE.

The semi-quantitative analysis of culture headspace for the day 72 EtFOSE-dosed bioactive culture E05-0544-083 was performed using a sensitive GC/MS assay in analytical run Z051109.s and showed that volatile fluorocarbons were not formed from the anaerobic biodegradation of EtFOSE.

Plots showing the measured concentrations for EtFOSE and the anaerobic transformation products *N*-EtFOSAA and PFOSi during 108 days of incubation are shown in **Figure 4**. Analysis of sterile culture extracts showed that EtFOSAA and PFOSi were not formed in sterile cultures (not shown). The measured concentrations of EtFOSE and anticipated products for active and sterile cultures are presented in **Table 10**.

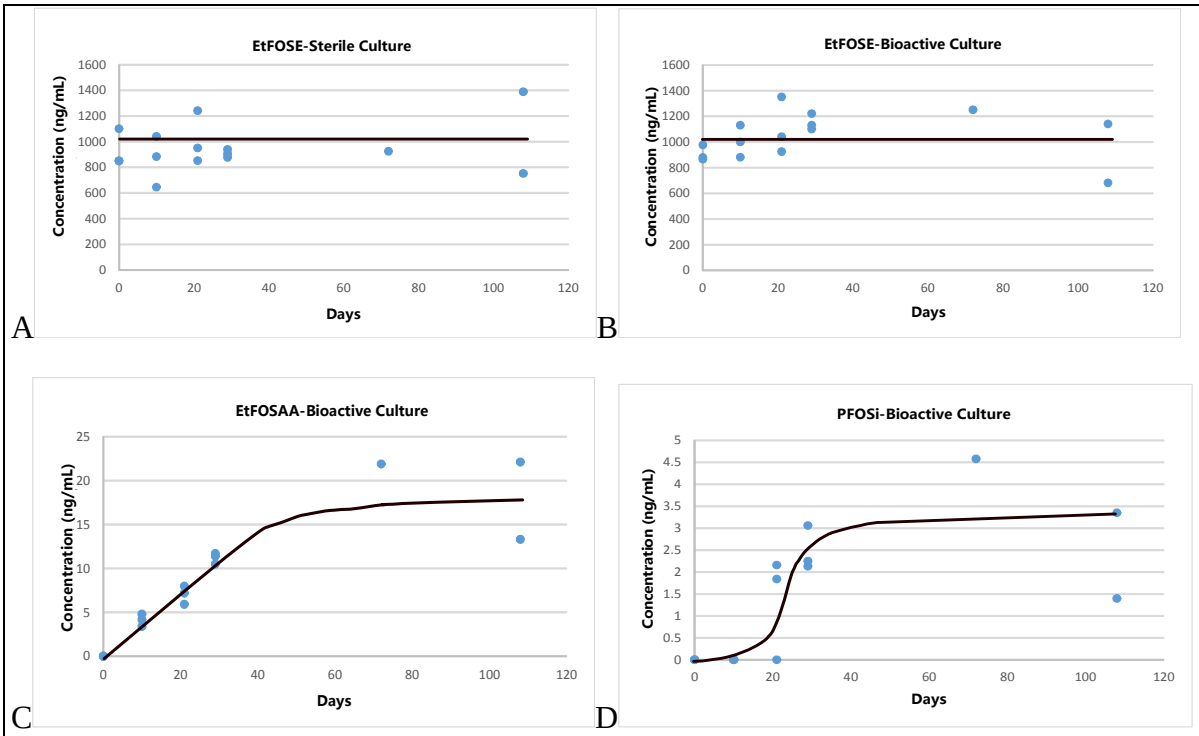

Figure 4. Measured EtFOSE, EtFOSAA and PFOSi concentrations in sterile cultures (A) and bioactive cultures (B, C &D), each dosed with EtFOSE. Data manually fit for appearance of trend.

EtFOSE biotransformed anaerobically at a very slow rate and could not be discerned by measurement of EtFOSE concentrations. However, it was discernible from formation of measureable levels of anticipated products EtFOSAA and PFOSi. The rate of product formation (summed for both products) appeared to be linear over the first 29 days and based on assumed zero-order rate gave an initial degradation rate of 4.11 pmole/day (**Figure 5**). An initial sludge-dependent biotransformation rate was calculated at 0.0718 pmole/day/mg sludge (dw). Based on the determined initial zero-order degradation rate, a zero-order half-life ($t_{1/2}$) of 1077 days was determined for EtFOSE; the $t_{1/2}$ does not take into consideration the fact that degradation was pseudo-first order over the length of the incubation, thus the half-life is likely underestimated.

Values used for converting ng/mL quantities of analytes to pmole quantities and determination of rates and mass balance were as describe in **Appendix A**.

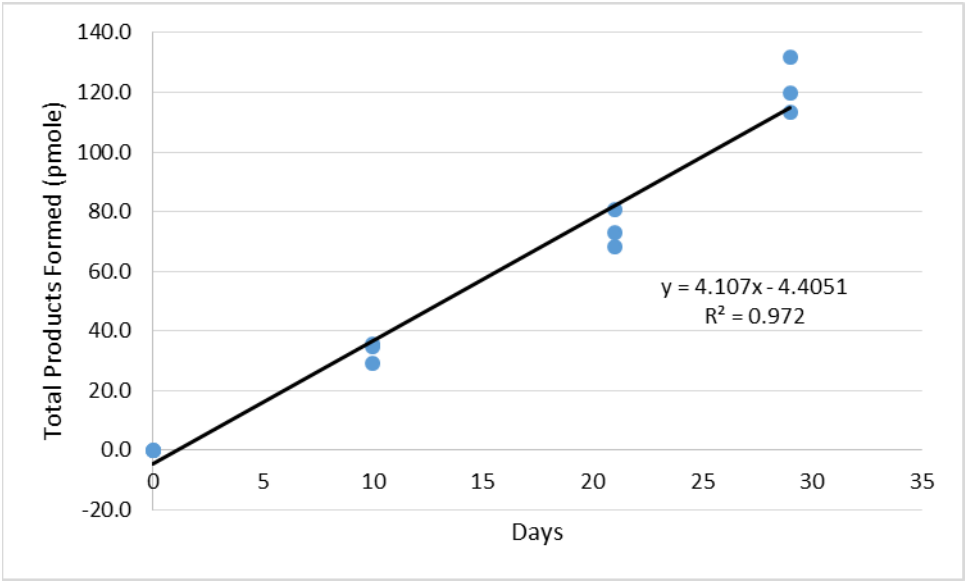

Figure 5. Linear plot of total products formed (pmole) from EtFOSE for the first 29 days.

Table 10. Analyte concentrations measured in bioactive and sterile anaerobic cultures incubated with EtFOSE.

Culture ID	Culture Type	Incubation Time (days)	Concentration (ng/mL)						
			EtFOSE	EtFOSAA	EtFOSA	FOSA	PFOSi	PFOS	PFOA
E05-0544-070	Bioactive Sludge w/ EtFOSE	0	977	<4.00	<7.50	<7.50	<1.00	<7.50	<7.50
E05-0544-071	Bioactive Sludge w/ EtFOSE	0	865	<4.00	<7.50	<7.50	<1.00	<7.50	<7.50
E05-0544-072	Bioactive Sludge w/ EtFOSE	0	879	<4.00	<7.50	<7.50	<1.00	<7.50	<7.50
E05-0544-073	Bioactive Sludge w/ EtFOSE	10	881	3.40 [a]	<7.50	<7.50	<1.00	<7.50	<7.50
E05-0544-074	Bioactive Sludge w/ EtFOSE	10	1130	4.08	<7.50	<7.50	<1.00	<7.50	<7.50
E05-0544-075	Bioactive Sludge w/ EtFOSE	10	999	4.17	<7.50	<7.50	<1.00	<7.50	<7.50
E05-0544-076	Bioactive Sludge w/ EtFOSE	21	1040	7.98	<7.50	<7.50	<1.00	<7.50	<7.50
E05-0544-077	Bioactive Sludge w/ EtFOSE	21	1350	7.19	<7.50	<7.50	1.84	<7.50	<7.50
E05-0544-078	Bioactive Sludge w/ EtFOSE	21	925	5.92	<7.50	<7.50	2.16	<7.50	<7.50
E05-0544-079	Bioactive Sludge w/ EtFOSE	29	1220	11.7	<7.50	<7.50	3.06	<7.50	<7.50
E05-0544-080	Bioactive Sludge w/ EtFOSE	29	1130	11.4	<7.50	<7.50	2.13	<7.50	<7.50
E05-0544-081	Bioactive Sludge w/ EtFOSE	29	1100	10.5	<7.50	<7.50	2.25	<7.50	<7.50
E05-0544-083	Bioactive Sludge w/ EtFOSE	72	1250	21.9	<7.50	<7.50	4.58	<7.50	<7.50
E05-0544-082	Bioactive Sludge w/ EtFOSE	108	1140	22.1	<7.50	<7.50	3.35	<7.50	<7.50
E05-0544-084	Bioactive Sludge w/ EtFOSE	108	681	13.3	<7.50	<7.50	1.40	<7.50	<7.50
E05-0544-085	Sterile Sludge w/ EtFOSE	0	1100	<4.00	<7.50	<7.50	<1.00	<7.50	<7.50
E05-0544-086	Sterile Sludge w/ EtFOSE	0	849	<4.00	<7.50	<7.50	<1.00	<7.50	<7.50
E05-0544-087	Sterile Sludge w/ EtFOSE	0	851	<4.00	<7.50	<7.50	<1.00	<7.50	<7.50
E05-0544-088	Sterile Sludge w/ EtFOSE	10	1040	<4.00	<7.50	<7.50	<1.00	<7.50	<7.50
E05-0544-089	Sterile Sludge w/ EtFOSE	10	645	<4.00	<7.50	<7.50	<1.00	<7.50	<7.50
E05-0544-090	Sterile Sludge w/ EtFOSE	10	883	<4.00	<7.50	<7.50	<1.00	<7.50	<7.50

Table 10. Analyte concentrations measured in bioactive and sterile anaerobic cultures incubated with EtFOSE.

Culture ID	Culture Type	Incubation Time (days)	Concentration (ng/mL)						
			EtFOSE	EtFOSAA	EtFOSA	FOSA	PFOSi	PFOS	PFOA
E05-0544-091	Sterile Sludge w/ EtFOSE	21	950	<4.00	<7.50	<7.50	<1.00	<7.50	<7.50
E05-0544-092	Sterile Sludge w/ EtFOSE	21	1240	<4.00	<7.50	<7.50	<1.00	<7.50	<7.50
E05-0544-093	Sterile Sludge w/ EtFOSE	21	852	<4.00	<7.50	<7.50	<1.00	<7.50	<7.50
E05-0544-094	Sterile Sludge w/ EtFOSE	29	939	<4.00	<7.50	<7.50	<1.00	<7.50	<7.50
E05-0544-095	Sterile Sludge w/ EtFOSE	29	877	<4.00	<7.50	<7.50	<1.00	<7.50	<7.50
E05-0544-096	Sterile Sludge w/ EtFOSE	29	905	<4.00	<7.50	<7.50	<1.00	<7.50	<7.50
E05-0544-099	Sterile Sludge w/ EtFOSE	72	924	<4.00	<7.50	<7.50	<1.00	<7.50	<7.50
E05-0544-097	Sterile Sludge w/ EtFOSE	108	752	<4.00	<7.50	<7.50	<1.00	<7.50	<7.50
E05-0544-098	Sterile Sludge w/ EtFOSE	108	1390	<4.00	<7.50	<7.50	<1.00	<7.50	<7.50

Notes: Results for the EtFOSE-dosed samples were reported from analytical run #20 of **Table 8.** Analysis included a set of matrix matched calibration standards (140568-045A01 to -045A11). Quantitation was by external standard method. Curve fits were quadratic with 1/x weighting, except for PFOS which was weighted $1/x^2$. R^2 values for all curves were $\geq$ 0.99. A low-level and mid-level QC at 40.0 ng/mL and 400 ng/mL (140568-45A12 and -45A13), respectively, were injected approximately every 15 samples as CCVs during the analysis. Quantitative accuracy for all analytes was within 100$\pm$ 25% for standards ($\pm$ 30% at LLOQ) and within 100 $\pm$ 30% for QCs (treated as CCVs throughout the run.
All values in **Table 10** are reported to three significant figures but higher precision data used in calculations.
Associated bioactive and sterile sludge blanks analyzed with the samples were all below the LLOQ for all analytes
[a] Value was determined by extrapolation of the curve slightly below the LLOQ of 4.00 ng/mL but within 75% of LLOQ acceptance range.

4.2.2 MeFBSE Results

The test substance [2-(*N*-methyl)-perfluorobutanesulfonamido]ethyl alcohol (MeFBSE) was dosed into anaerobic cultures at 976 ng/mL (2.73 μM) and then incubated anaerobically for up to 108 days.

Cultures were extracted for days 0, 10, 21, 29, 70)and 108. Quantitative LC/MS/MS analysis of processed culture extracts was conducted for MeFBSE, MeFBSAA, MeFBSA, FBSA, PFBSi, PFBS and PFBA. Data was collected from two analytical runs #5 and #20 as described in **Table 8**. Quantitation was by internal standard method for all analytes. Calibration curves contained six or more calibration standards, except PFBA in run #20 which had only 5 active points in the curve as footnoted in **Table 8**. The coefficient of determination (R) values for all calibration curves were $\geq$ 0.990. The lower limit of quantitation (LLOQ) for each analyte were always $\leq$ 25.0 ng/mL. Calibration standards included in the calibration curve all were within 100 $\pm$ 25% and QCs/CCVs were all within 100 $\pm$ 30%, except one QC analyzed as a CCV for PFBSi in analytical run #5 at 145%--but did not affect the reported PFBSi results since all samples flanked by that QC were below the LLOQ.

The LC/MS/MS results showed that MeFBSE was biodegraded with approximately 75% removed by day-72 (**Figure 6**) with concomitant formation of MeFBSAA and PFBSi as shown in **Figure 7**. Other anticipated products as MeFBSA, FBSA, PFBS and PFBA were not detected above the LLOQ during the 108 days of incubation (**Table 11**). MeFBSE biodegradation occurred with near equimolar production of MeFBSAA and PFBSi (summed), with mass balance calculated at 103%, 91.3%, 93.7%, 99.0%, 91.4% and 120% for cultures collected on days 0, 10, 21, 29, 70 and 108, respectively. The MeFBSE was not degraded in sterile controls, nor were any products formed.

The semi-quantitative analysis of the headspace gas for the MeFBSE-dosed bioactive and sterile cultures (E05-0544-112 and -129, respectively) occurred on day 66, and was performed using a sensitive GC/MS assay in analytical run Z051103.s. Results of headspace analysis showed that volatile fluorocarbons were not formed from the anaerobic biodegradation of MeFBSE. After the headspace analysis, those cultures sat on the bench top for ~3 days prior to being extracted for LC/MS/MS analysis and likely continued to degrade while stored on the lab bench, and is the reason why LC/MS/MS results indicate those culture times as day 70 rather than day 66.

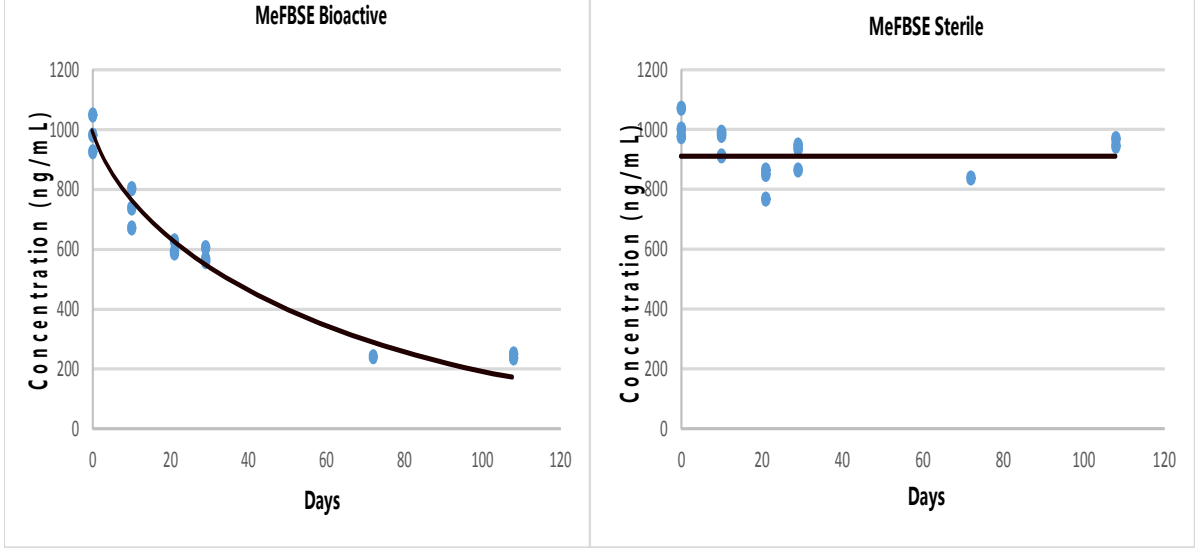

Figure 6. The MeFBSE (a.k.a. N-MeFBSE Alcohol) concentrations in active cultures (left) and sterile cultures (right). Data manually fit for appearance.

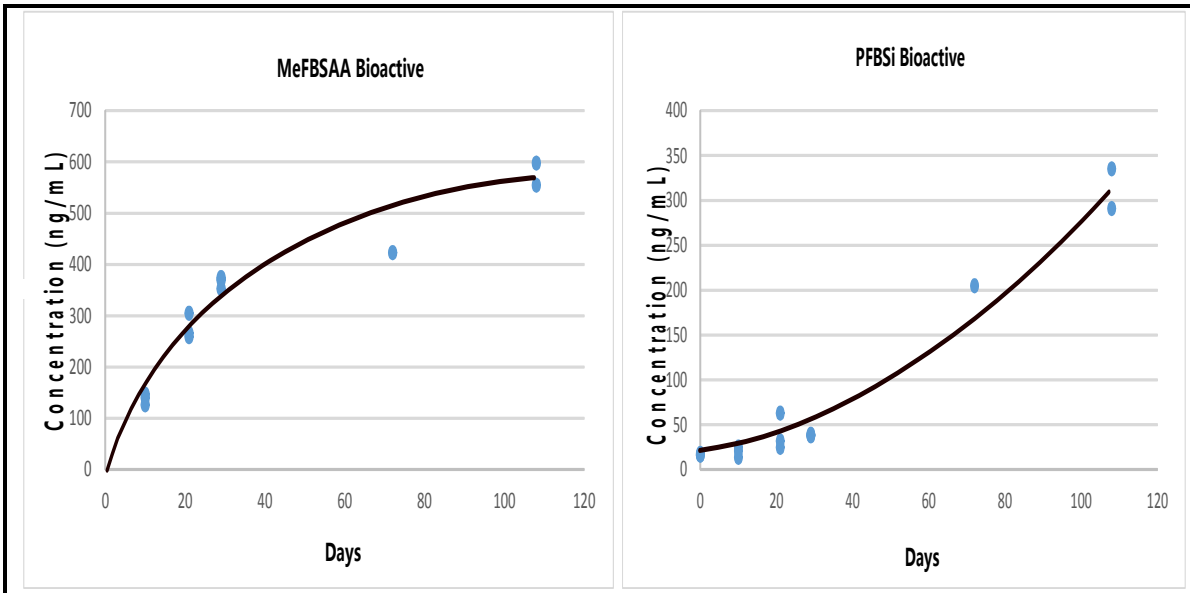

Figure 7. The MeFBSAA and PFBSi concentrations measured in active cultures dosed with MeFBSE (red circles). Data manually fit for appearance.

The formation of total products in pmole over the first 29 days (days 0, 10, 21 and 29) of incubation was linear and the slope of the fitted line was used to estimate a zero-order rate of degradation at 186 pmole/day (**Figure 8**). Based on that rate, a sludge-dependent

biodegradation rate for MeFBSE was determined at 3.26 pmole/day/mg sludge (dw) for MeFBSE. Based on the determined initial zero-order degradation rate, a zero-order half-life ($t_{1/2}$) of 36.7 days was determined for EtFOSE; the $t_{1/2}$ does not take into consideration the fact that degradation was pseudo-first order over the length of the incubation, thus the half-life value is likely underestimated.

Values used for converting ng/mL quantities of analytes to pmole quantities and determination of rates and mass balance were as describe in **Appendix A**.

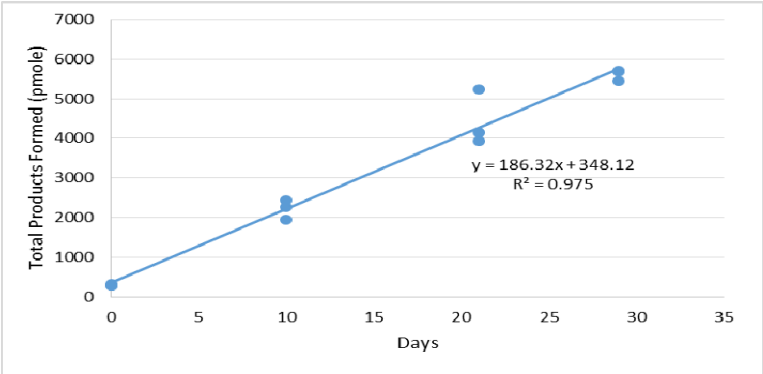

Figure 8. Plot of MeFBSE total products formation over first 29 days.

Note (MeFBSE Results): measured concentrations of PFBSi were determined using standards prepared from a reference substance which was assumed at 50% (w/v) in water at the time of preparation, but later this was shown by NMR analysis to be actually 51.2% (w/v) in water; however, measured values were not corrected for the 1.2% difference. Therefore, PFBSi values could be biased slightly low as a result, hence rates may be biased slightly low also.

Table 11. Analyte concentrations measured in active and sterile cultures incubated with MeFBSE

Culture ID	Culture Type	Incubation Time (days)	Concentration (ng/mL)						
			MeFBSE	MeFBSAA	MeFBSA	FBSA [s]	PFBSi [b]	PFBS	PFBA [c]
E05-0544-100	Active Sludge w/ MeFBSE	0	926	<10.0	<10.0	<10.0	16.3	<10.0	<10.0
E05-0544-101	Active Sludge w/ MeFBSE	0	982	<10.0	<10.0	<10.0	16.3	<10.0	<10.0
E05-0544-102	Active Sludge w/ MeFBSE	0	1050	<10.0	<10.0	<10.0	18.0	<10.0	<10.0
E05-0544-103	Active Sludge w/ MeFBSE	10	738	140	<10.0	<10.0	20.6	<10.0	<10.0
E05-0544-104	Active Sludge w/ MeFBSE	10	672	126	<10.0	<10.0	13.8	<10.0	<10.0
E05-0544-105	Active Sludge w/ MeFBSE	10	803	147	<10.0	<10.0	25.2	<10.0	<10.0
E05-0544-106	Active Sludge w/ MeFBSE	21	593	266	<10.0	<10.0	31.8	<10.0	<10.0
E05-0544-107	Active Sludge w/ MeFBSE	21	588	259	<10.0	<10.0	24.7	<10.0	<10.0
E05-0544-108	Active Sludge w/ MeFBSE	21	629	305	<10.0	<10.0	63.0	<10.0	<10.0
E05-0544-109	Active Sludge w/ MeFBSE	29	606	374	<10.0	<10.0	37.5	<10.0	<10.0
E05-0544-110	Active Sludge w/ MeFBSE	29	569	353	<10.0	<10.0	38.4	<10.0	<10.0
E05-0544-111	Active Sludge w/ MeFBSE	29	559	370	<10.0	<10.0	39.3	<10.0	<10.0
E05-0544-112	Active Sludge w/ MeFBSE	70 [e]	241	423	<25.0	<25.0	205	<25.0	<25.0
E05-0544-113	Active Sludge w/ MeFBSE	108	251	555	<25.0	<25.0	291	<25.0	<25.0
E05-0544-114	Active Sludge w/ MeFBSE	108	237	598	<25.0	<25.0	335	<25.0	<25.0
E05-0544-115	Sterile Sludge w/ MeFBSE	0	975	<10.0	<10.0	<10.0	<5.00	<10.0	<10.0
E05-0544-116	Sterile Sludge w/ MeFBSE	0	1070	<10.0	<10.0	<10.0	<5.00	<10.0	<10.0
E05-0544-117	Sterile Sludge w/ MeFBSE	0	1000	<10.0	<10.0	<10.0	<5.00	<10.0	<10.0
E05-0544-118	Sterile Sludge w/ MeFBSE	10	990	<10.0	<10.0	<10.0	<5.00	<10.0	<10.0
E05-0544-119	Sterile Sludge w/ MeFBSE	10	979	<10.0	<10.0	<10.0	<5.00	<10.0	<10.0
E05-0544-120	Sterile Sludge w/ MeFBSE	10	910	<10.0	<10.0	<10.0	<5.00	<10.0	<10.0
E05-0544-121	Sterile Sludge w/ MeFBSE	21	766	<10.0	<10.0	<10.0	<5.00	<10.0	<10.0
E05-0544-122	Sterile Sludge w/ MeFBSE	21	848	<10.0	<10.0	<10.0	<5.00	<10.0	<10.0
E05-0544-123	Sterile Sludge w/ MeFBSE	21	863	<10.0	<10.0	<10.0	<5.00	<10.0	<10.0
E05-0544-124	Sterile Sludge w/ MeFBSE	29	934	<10.0	<10.0	<10.0	<5.00	<10.0	<10.0

Table 11. Analyte concentrations measured in active and sterile cultures incubated with MeFBSE

Culture ID	Culture Type	Incubation Time (days)	Concentration (ng/mL)						
			MeFBSE	MeFBSAA	MeFBSA	FBSA [s]	PFBSi [b]	PFBS	PFBA [c]
E05-0544-125	Sterile Sludge w/ MeFBSE	29	863	<10.0	<10.0	<10.0	<5.00	<10.0	<10.0
E05-0544-126	Sterile Sludge w/ MeFBSE	29	947	<10.0	<10.0	<10.0	<5.00	11.9 [d]	<10.0
E05-0544-129	Sterile Sludge w/ MeFBSE	70 [e]	837	<25.0	<25.0	<25.0	<12.5	<25.0	<25.0
E05-0544-127	Sterile Sludge w/ MeFBSE	108	944	<25.0	<25.0	<25.0	<12.5	<25.0	<25.0
E05-0544-128	Sterile Sludge w/ MeFBSE	108	968	<25.0	<25.0	<25.0	<12.5	<25.0	<25.0

Notes: Results for the MeFBSE-dosed samples were reported from analytical runs #5 and #20 in **Table 8**. Culture extracts were analyzed against a set of matrix matched calibration standards. All curves contained minimally 6 data points and were quadratic fit with 1/x weighting, except for PFBS in run #20 which was weighted $1/x^2$.
Quantitation was by internal standard method. All calibration standards in calibration curves were within 100±25% (± 30% at LLOQ) of their theoretical value and CCVs (low and mid-level QCs at 40.0 ng/mL and 400 ng/mL) were injected every 10 to 15 samples and were within 100±30% of their theoretical values for all analytes, except one QC analyzed as a CCV for PFBSi in analytical run #5 which recovered at 145% but did not affect the reported PFBSi results since all samples flanked by that QC were below the LLOQ.
All values in table are reported to 3 significant figures while more precise data was used in data calculations.
Associated bioactive and sterile sludge blanks analyzed with the samples were all below the LLOQ for all analytes
[a] Purity of FBSA was assumed 100% at the time of use, but determined later at 97.3% and was not corrected for.
[b] Purity of PFBSi was assumed 50% at the time of use, but later determined to by 51.2% and was not corrected for.
[c] PFBA quantification in analytical run #20 included only 5 calibration standards in the curve with mid-points dropped. Data not affected since all results were < LLOQ.
[d] An aberrant PFBS result in the day 70 sterile culture was slightly above the LLOQ of 10 ng/mL but ignored for interpretation of results since PFBS was not observed in bioactive cultures; possibly derived from the sludge as background.
[e] Culture was collected on day 66 for headspace analysis, but sat out on bench for additional ~4 days prior to LC/MS/MS analysis, hence was designated as a day 70 culture.

4.2.3 PFOS Results

The test substance perfluorooctanesulfonate (PFOS) was dosed into anaerobic cultures at 1060 ng/mL (2.12 μM) and cultures incubated anaerobically for up to 105 days. Cultures were collected on days 0, 7, 18, 26, 69 and 105. Quantitative LC/MS/MS analysis of culture extracts was conducted for PFOS and the anticipated products PFOSi, FOSA and PFOA. Quantitative LC/MS/MS analysis of processed culture extracts was conducted for PFOS, FOSA, PFOSi and PFOA. Data for reporting results of PFOS cultures were collected in analytical runs #7, #10, #11, #21 and #25 in **Table 8**. Results are shown in **Table 12**. Cultures for day-7 and day-18 were not determined for PFOSi or FOSA.

The lower limits of quantitation (LLOQ) varied by batch for each analyte, but were always $\leq$ 25.0 ng/mL; LLOQs are indicated in **Table 12**. Calibration standards included in the calibration curve were determined at within 100 $\pm$ 25%, QCs analyzed as CCVs in the run were recovered within 100 $\pm$ 30%.

The results showed that PFOS did not biodegrade under anaerobic methanogenic sludge conditions for up to 105 days. This was demonstrated by the lack of any appreciable loss of PFOS (**Figure 9**) and the lack of formation for any fluorinated products PFOA, PFOSi or FOSA.

The semi-quantitative analysis of culture headspace for the day 69 PFOS-dosed bioactive culture E05-0544-204 was performed using a sensitive GC/MS assay in analytical run #30 of **Table 8** and showed that volatile fluorocarbons were not formed from anaerobic sludge incubations with PFOS.

In addition to not degrading, PFOS did not appear to inhibit methanogenisis since gas production seemed comparable in bioactive blanks as in bioactive PFOS cultures (data not shown).

The results of this study showed PFOS does not biodegrade under methanogenic conditions. These results corroborate the findings of Hollingsworth *et al.* (2005) who similarly found that anaerobic biodegradation of PFOS did not occur under methanogenic conditions during a 45 day trial. Those authors also concluded that inhibition of methanogenesis did not occur at up to 600 μg/mL of PFOS.

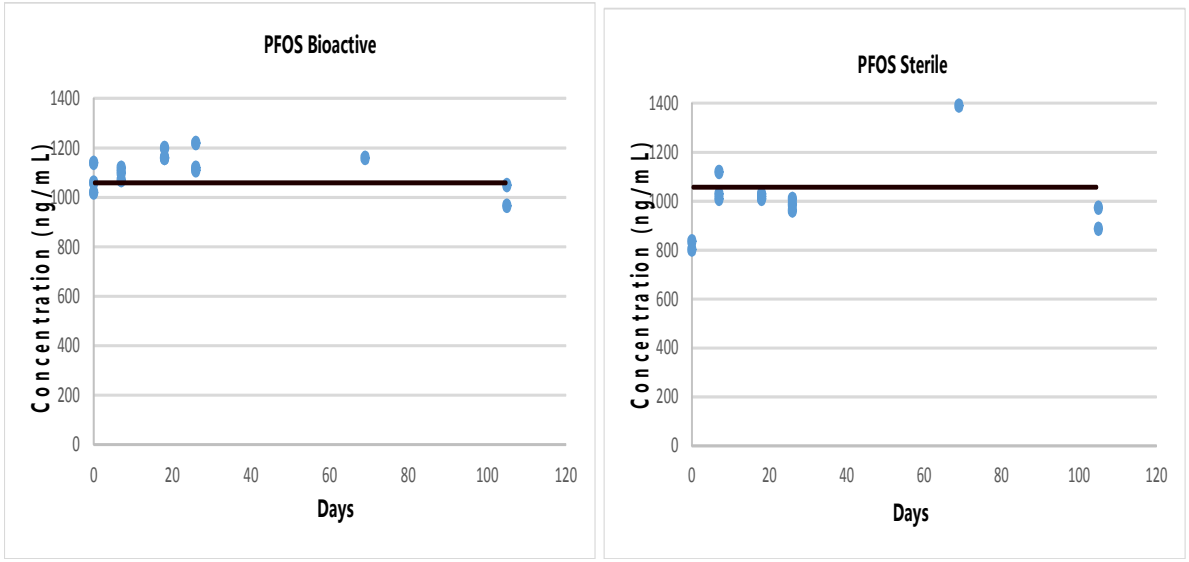

Figure 9. The PFOS concentrations measured in active cultures (left) and sterile cultures (right) dosed with PFOS. One apparent double spiked sterile culture was omitted (see **Table 12).** The line represents the dosed concentration of 1060 ng/mL

Table 12. Analyte concentrations measured in active and sterile anaerobic cultures dosed with PFOS.

Culture ID	Culture Type	Incubation Time (days)	Concentration (ng/mL)			
			FOSA	PFOS	PFOSi	PFOA
E05-0544-190	Active Sludge with PFOS	0	< 10.0	1140	< 10.0	< 25.0
E05-0544-191	Active Sludge with PFOS	0	< 10.0	1020	< 10.0	< 25.0
E05-0544-192	Active Sludge with PFOS	0	< 10.0	1060	< 10.0	< 25.0
E05-0544-193	Active Sludge with PFOS	7	Not Determined	1120	Not Determined	< 25.0
E05-0544-194	Active Sludge with PFOS	7	Not Determined	1070	Not Determined	< 25.0
E05-0544-195	Active Sludge with PFOS	7	Not Determined	1100	Not Determined	< 25.0
E05-0544-196	Active Sludge with PFOS	18	Not Determined	1160	Not Determined	< 25.0
E05-0544-197	Active Sludge with PFOS	18	Not Determined	1200	Not Determined	< 25.0
E05-0544-198	Active Sludge with PFOS	18	Not Determined	1160	Not Determined	< 25.0
E05-0544-199	Active Sludge with PFOS	26	< 10.0	1220	< 10.0	< 25.0
E05-0544-200	Active Sludge with PFOS	26	< 10.0	1110	< 10.0	< 25.0
E05-0544-201	Active Sludge with PFOS	26	< 10.0	1120	< 10.0	< 25.0
E05-0544-202	Active Sludge with PFOS [a]	105	< 10.0	1160	< 10.0	< 10.0
E05-0544-203	Active Sludge with PFOS [a]	105	< 10.0	966	< 10.0	< 10.0
E05-0544-204	Active Sludge with PFOS	69	< 4.00	1050	< 10.0	< 4.00
E05-0544-205	Sterile Sludge with PFOS	0	< 10.0	802	< 10.0	< 25.0
E05-0544-206	Sterile Sludge with PFOS	0	< 10.0	1770 [double spiked]	< 10.0	< 25.0
E05-0544-207	Sterile Sludge with PFOS	0	< 10.0	836	< 10.0	< 25.0
E05-0544-208	Sterile Sludge with PFOS	7	Not Determined	1120	Not Determined	< 25.0
E05-0544-209	Sterile Sludge with PFOS	7	Not Determined	1030	Not Determined	< 25.0
E05-0544-210	Sterile Sludge with PFOS	7	Not Determined	1010	Not Determined	< 25.0
E05-0544-211	Sterile Sludge with PFOS	18	Not Determined	1030	Not Determined	< 25.0
E05-0544-212	Sterile Sludge with PFOS	18	Not Determined	1010	Not Determined	< 25.0
E05-0544-213	Sterile Sludge with PFOS	18	Not Determined	1020	Not Determined	< 25.0
E05-0544-214	Sterile Sludge with PFOS	26	< 10.0	1010	< 10.0	< 25.0
E05-0544-215	Sterile Sludge with PFOS	26	< 10.0	987	< 10.0	< 25.0
E05-0544-216	Sterile Sludge with PFOS	26	< 10.0	962	< 10.0	< 25.0
E05-0544-219	Sterile Sludge with PFOS	69	< 4.00	1390	< 1.00	< 4.00
E05-0544-217	Sterile Sludge with PFOS [a]	105	< 10.0	973	< 10.0	< 10.0
E05-0544-218	Sterile Sludge with PFOS [a]	105	< 10.0	887	< 10.0	< 10.0

Note: Results for the PFOS-dosed samples were reported from analytical runs # 7, #10, #11, #21 and #25 in **Table 8**. Culture extracts were analyzed against a set of matrix matched calibration standards. Quantitation was by external standard method, except PFOA and PFOS in run # 7 which were quantified by internal standard method. Calibration curves contained minimally six data points with quadratic fit and 1/x weighting, except PFOS in run #21 and #25 which was weighted $1/x^2$. Calibration accuracy for standards included in the curve fit were within 100 $\pm$ 25% ($\pm$ 30% at LLOQ). CCVs (low and mid-level QCs at 40.0 ng/mL and 400 ng/mL, respectively) were injected approximately every 15 samples and were within 100$\pm$30% of their theoretical values for all analytes. LLOQs were always $\leq$ 25.0 ng/mL.
All values in the table are reported to 3 significant figures while more precise data was used in data calculations.
Associated bioactive and sterile sludge blanks analyzed with the samples were all below the LLOQ for all analytes
[a] Culture extracts were also analyzed in run #25 for EtFOSE, EtFOSAA and EtFOSA, but results excluded from table for brevity since those analytes were not expected products of PFOS degradation and were < 10.0 ng/mL in all samples.

4.2.4 PFBS Results

The test substance perfluorobutanesulfonate (PFBS) was dosed into cultures at 990 ng/mL and incubated anaerobically for up to 105 days.

Quantitative LC/MS/MS analysis of processed culture extracts was conducted for PFBS and anticipated biodegradation products FBSA, PFBSi and PFBA. Cultures analyzed were collected on days 0, 7, 18, 26, 69 and 105. Cultures for day-0, day-7, day-18 and day-26 were not measured for PFBSi nor FBSA. The LLOQ for all analytes were always $\leq$ 25.0 ng/mL. Data is reported from three analytical runs as described at the bottom of **Table 13**. PFBS concentrations measured in active cultures and sterile cultures are plotted in **Figure 10**. Concentrations of PFBS for day-0 sterile control cultures appeared low, but returned to near expected levels by day 7.

The semi-quantitative analysis of culture headspace for the day 69 PFBS-dosed bioactive culture E05-0544-174 in analytical run #30 of **Table 8** and showed that volatile fluorocarbons were not significantly formed from the anaerobic biodegradation of PFBS.

Overall, the results showed that PFBS did not biodegrade anaerobically over 105 days under methanogenic conditions. No anticipated products were measured above the LLOQ in any cultures and PFBS was recovered at near 100% at all time points. Furthermore, analysis of active and sterile culture headspaces at day 69 of incubations by a sensitive GC/MS assay showed no generation of volatile fluorocarbons, but production of sulfide compounds was observed in active cultures. The PFBS concentrations determined by LC/MS/MS in active and sterile cultures are plotted in **Figure 10**.

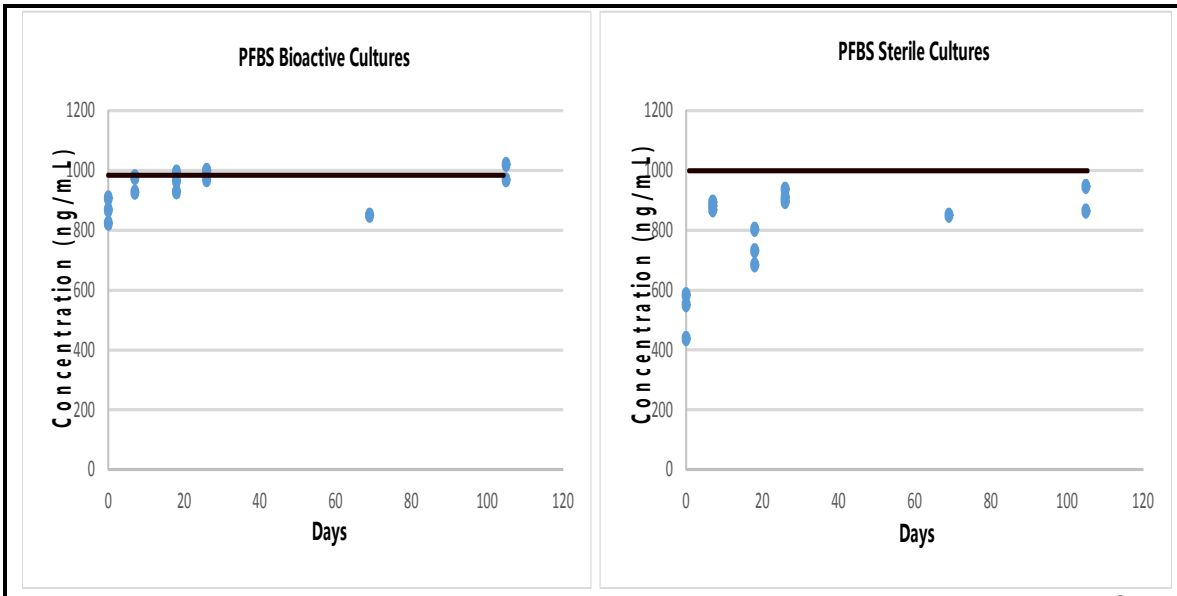

Figure 10. The PFBS concentrations measured in PFBS-dosed bioactive cultures (left) and sterile cultures (right). The line represents the dosed concentration of 990 ng/mL.

Table 13. Analyte concentrations measured in active and sterile anaerobic cultures dosed with PFBS.

Culture ID	Culture Type	Incubation Time (days)	Concentration (ng/mL)			
			FBSA [a]	PFBS	PFBSi [b]	PFBA
E05-0544-160	Active Sludge with PFBS	0	Not Determined	908	Not Determined	< 10.0
E05-0544-161	Active Sludge with PFBS	0	Not Determined	823	Not Determined	< 10.0
E05-0544-162	Active Sludge with PFBS	0	Not Determined	868	Not Determined	< 10.0
E05-0544-163	Active Sludge with PFBS	7	Not Determined	927	Not Determined	< 10.0
E05-0544-164	Active Sludge with PFBS	7	Not Determined	977	Not Determined	< 10.0
E05-0544-165	Active Sludge with PFBS	7	Not Determined	979	Not Determined	< 10.0
E05-0544-166	Active Sludge with PFBS	18	Not Determined	966	Not Determined	< 10.0
E05-0544-167	Active Sludge with PFBS	18	Not Determined	928	Not Determined	< 10.0
E05-0544-168	Active Sludge with PFBS	18	Not Determined	995	Not Determined	< 10.0
E05-0544-169	Active Sludge with PFBS	26	Not Determined	969	Not Determined	< 10.0
E05-0544-170	Active Sludge with PFBS	26	Not Determined	998	Not Determined	< 10.0
E05-0544-171	Active Sludge with PFBS	26	Not Determined	1000	Not Determined	< 10.0

Table 13. Analyte concentrations measured in active and sterile anaerobic cultures dosed with PFBS.

Culture ID	Culture Type	Incubation Time (days)	Concentration (ng/mL)			
			FBSA [a]	PFBS	PFBSi [b]	PFBA
E05-0544-174	Active Sludge with PFBS	69	< 10.0	850	< 5.00	< 10.0
E05-0544-172	Active Sludge with PFBS	105	< 25.0	969	< 12.5	< 25.0
E05-0544-173	Active Sludge with PFBS	105	< 25.0	1020	< 12.5	< 25.0
E05-0544-175	Sterile Sludge with PFBS	0	Not Determined	585	Not Determined	< 10.0
E05-0544-176	Sterile Sludge with PFBS	0	Not Determined	438	Not Determined	< 10.0
E05-0544-177	Sterile Sludge with PFBS	0	Not Determined	551	Not Determined	< 10.0
E05-0544-178	Sterile Sludge with PFBS	7	Not Determined	882	Not Determined	< 10.0
E05-0544-179	Sterile Sludge with PFBS	7	Not Determined	868	Not Determined	< 10.0
E05-0544-180	Sterile Sludge with PFBS	7	Not Determined	894	Not Determined	< 10.0
E05-0544-181	Sterile Sludge with PFBS	18	Not Determined	803	Not Determined	< 10.0
E05-0544-182	Sterile Sludge with PFBS	18	Not Determined	731	Not Determined	< 10.0
E05-0544-183	Sterile Sludge with PFBS	18	Not Determined	684	Not Determined	< 10.0
E05-0544-184	Sterile Sludge with PFBS	26	< 10.0	910	< 10.0	< 10.0
E05-0544-185	Sterile Sludge with PFBS	26	< 10.0	937	< 10.0	< 10.0
E05-0544-186	Sterile Sludge with PFBS	26	< 10.0	896	< 10.0	< 10.0
E05-0544-189	Sterile Sludge with PFBS	69	< 10.0	850	< 5.00	< 10.0
E05-0544-187	Sterile Sludge with PFBS	105	< 25.0	946	< 12.5	< 25.0
E05-0544-188	Sterile Sludge with PFBS	105	< 25.0	864	< 12.5	< 25.0

Note: Results for the PFBS-dosed samples were reported from analytical runs # 6, #15 and #20 in **Table 8**. Culture extracts were analyzed against a set of matrix matched calibration standards. Quantitation was by internal standard method for analytical runs #6 and #20, and external standard method for analytical run #15. Calibration curves contained minimally six data points. Curves in analytical run #6 were quadratic fit and weighted $1/x^2$, curves in analytical runs #6 and #20 were quadratic fit with 1/x weighting, except FBSA in run #15 which was $1/x^2$. Calibration accuracy for standards included in the curve fit were within 100 $\pm$ 25% ($\pm$ 30% at LLOQ). CCVs (low and mid-level QCs at 40.0 ng/mL and 400 ng/mL, respectively) were injected approximately every 15 samples and were within 100$\pm$30% of their theoretical values for all analytes. LLOQs were always $\leq$ 25.0 ng/mL.

All values in the table are reported to 3 significant figures while more precise data was used in data calculations.
Associated bioactive and sterile sludge blanks analyzed with the samples were all below the LLOQ for all analytes.
[a] Purity of FBSA was assumed 100% at the time of use, but determined later at 97.3% and was not corrected for.
[b] Purity of PFBSi was assumed 50% at the time of use, but later determined to by 51.2% and was not corrected for.

4.2.5 PFOA Results

The test substance perfluorooctanoate (PFOA) was dosed into cultures at 1010 ng/mL and incubated anaerobically for up to 94 days. Cultures analyzed were collected on days 0, 10, 20, 74 and 94.

Quantitative LC/MS/MS analysis of processed culture extracts was conducted for PFOA, perfluoroheptanoate (PFHpA), perfluorononanoate (PFNA) and PFBA on days 0, 10 and 20; and only PFNA and PFOA were measured at all other time points. Additionally, qualitative analysis for 8:2 fluorotelomer acid (8:2 FTCA) and 8:2 unsaturated fluorotelomer acid (8:2 FTUCA) was also conducted at all time points. Data was collected from two analytical runs #19 and #23 of **Table 8.** The PFOA, PFNA, PFHpA and PFBA concentrations measured in active and sterile cultures are shown in **Table 14** and plotted in **Figure 11**.

The analysis results showed that PFOA did not biodegrade anaerobically over 94 days under methanogenic conditions. There was no discernible difference between PFOA in bioactive cultures versus sterile cultures. Variation in the results can be attributed to the analytical method precision and accuracy. Additionally, lower molecular weight PFCAs as PFBA and PFHpA did not form in the first ~3 weeks of incubation, and the higher molecular weight PFNA did not form over the incubation period. Qualitative analysis also showed no anticipated fatty acid synthesis products 8:2 FTCA and 8:2 FTUCA (data not shown).

Note: Smaller perfluorinated acids could be anticipated degradation products of PFOA, but only PFBA and PFHpA were measured at days 0, 10 and 20. While the PFOA did not measurably biodegrade in active cultures versus the levels measured in sterile cultures, future studies might include analysis at later time points for low molecular weight perfluorinated acids in addition to those measured here, such as trifluoroacetate (TFA), perfluoropropanoate (PFPA), PFPeA and PFHxA, to ensure that none of those potential fluorinated acid products formed at low levels.

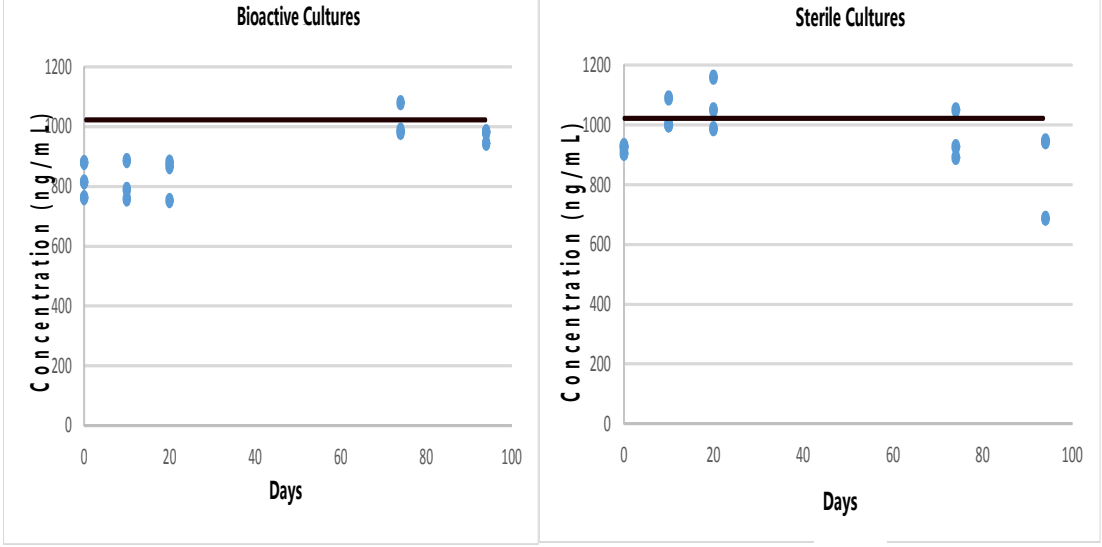

Figure 11. The PFOA concentrations measured in PFOA-dosed bioactive cultures (left) and sterile cultures (right). The line represents the dosed concentration of 1010 ng/mL

Table 14. Analyte concentrations measured in active and sterile anaerobic cultures dosed with PFOA.

Culture ID	Culture Type	Incubation Time (days)	Concentration (ng/mL)			
			PFOA	PFNA	PFHpA	PFBA [a]
E05-0544-635	Active Sludge with PFOA	0	880	< 10.0	< 10.0	< 10.0
E05-0544-636	Active Sludge with PFOA	0	762	< 10.0	< 10.0	< 10.0
E05-0544-637	Active Sludge with PFOA	0	815	< 10.0	< 10.0	< 10.0
E05-0544-638	Active Sludge with PFOA	10	887	< 10.0	< 10.0	< 10.0
E05-0544-639	Active Sludge with PFOA	10	790	< 10.0	< 10.0	< 10.0
E05-0544-640	Active Sludge with PFOA	10	758	< 10.0	< 10.0	< 10.0
E05-0544-641	Active Sludge with PFOA	20	866	< 10.0	< 10.0	< 10.0
E05-0544-642	Active Sludge with PFOA	20	753	< 10.0	< 10.0	< 10.0
E05-0544-643	Active Sludge with PFOA	20	881	< 10.0	< 10.0	< 10.0
E05-0544-644	Active Sludge with PFOA	74	1080	< 10.0	[b]	[c]
E05-0544-645	Active Sludge with PFOA	74	981	< 10.0	[b]	[c]
E05-0544-646	Active Sludge with PFOA	74	988	< 10.0	[b]	[c]
E05-0544-647	Active Sludge with PFOA	94	944	< 10.0	[b]	[c]
E05-0544-648	Active Sludge with PFOA	94	983	< 10.0	[b]	[c]
E05-0544-649	Active Sludge with PFOA	94	982	< 10.0	[b]	[c]

Table 14. Analyte concentrations measured in active and sterile anaerobic cultures dosed with PFOA.

Culture ID	Culture Type	Incubation Time (days)	Concentration (ng/mL)			
			PFOA	PFNA	PFHpA	PFBA [a]
E05-0544-650	Sterile Sludge with PFOA	0	925	< 10.0	< 10.0	< 10.0
E05-0544-651	Sterile Sludge with PFOA	0	905	< 10.0	< 10.0	< 10.0
E05-0544-652	Sterile Sludge with PFOA	0	931	< 10.0	< 10.0	< 10.0
E05-0544-653	Sterile Sludge with PFOA	10	1000	< 10.0	< 10.0	< 10.0
E05-0544-654	Sterile Sludge with PFOA	10	1000	< 10.0	< 10.0	< 10.0
E05-0544-655	Sterile Sludge with PFOA	10	1090	< 10.0	< 10.0	< 10.0
E05-0544-656	Sterile Sludge with PFOA	20	1160	< 10.0	< 10.0	< 10.0
E05-0544-657	Sterile Sludge with PFOA	20	1050	< 10.0	< 10.0	< 10.0
E05-0544-658	Sterile Sludge with PFOA	20	987	< 10.0	< 10.0	< 10.0
E05-0544-659	Sterile Sludge with PFOA	74	927	< 10.0	[b]	[c]
E05-0544-660	Sterile Sludge with PFOA	74	1050	< 10.0	[b]	[c]
E05-0544-661	Sterile Sludge with PFOA	74	891	< 10.0	[b]	[c]
E05-0544-662	Sterile Sludge with PFOA	94	943	< 10.0	[b]	[c]
E05-0544-663	Sterile Sludge with PFOA	94	947	< 10.0	[b]	[c]
E05-0544-664	Sterile Sludge with PFOA	94	688	< 10.0	[b]	[c]

Note: Data for PFOA-dosed samples were reported from analytical runs # 19 and #23 in **Table 8**. Culture extracts were analyzed against a set of matrix matched calibration standards. Quantitation was by internal standard method. Calibration curves contained minimally six data points. Curves in analytical run #19 and #23 were quadratic fit and weighted 1/x. Calibration accuracy for standards included in the curve fit were within 100 $\pm$ 25% ($\pm$ 30% at LLOQ). CCVs (low and mid-level QCs at 40.0 ng/mL and 400 ng/mL, respectively) were injected approximately every 15 samples in analytical runs #19 and #23, and all CCVs/QCs were within 100$\pm$30% of their theoretical values for all analytes. LLOQs for all data in **Table 14** were 10.0 ng/mL

All values in the table are reported to 3 significant figures while more precise data was used in data calculations.
Associated bioactive and sterile sludge blanks analyzed with the samples were all below the LLOQ for all analytes.
[a] Results for PFBA were reported from analysis of PFOA cultures in run #19, however, CCVs for PFBA were not included as bracketing QC for those samples.
[b] PFHpA data was collected for day74 and day 94 samples, but PFHpA is not reported. The PFHpA data was errantly not worked up at the time of analysis in 2006. Electronic data for run #23 was not retrievable in 2016 and only possession of original printed chromatograms and results, therefore PFHpA could not be determined.
[c] PFBA was not a target analyte during analysis of day 74 and 94 samples.

4.2.6 PFBA Results

The test substance perfluorobutanoate (PFBA) was dosed into cultures at 1030 ng/mL and incubated anaerobically for up to 94 days. Cultures analyzed were collected on days 0, 10, 20, 74 and 94.

Quantitative LC/MS/MS analysis results are reported for PFBA only. Data was collected from two analytical runs as described at the bottom of **Table 15**. PFBA concentrations measured in active and sterile cultures are plotted in **Figure 12** and showed no discernible loss of PFBA in either culture type. These results showed that PFBA did not biodegrade anaerobically over 94 days under methanogenic conditions in the active cultures, nor in the sterile cultures.

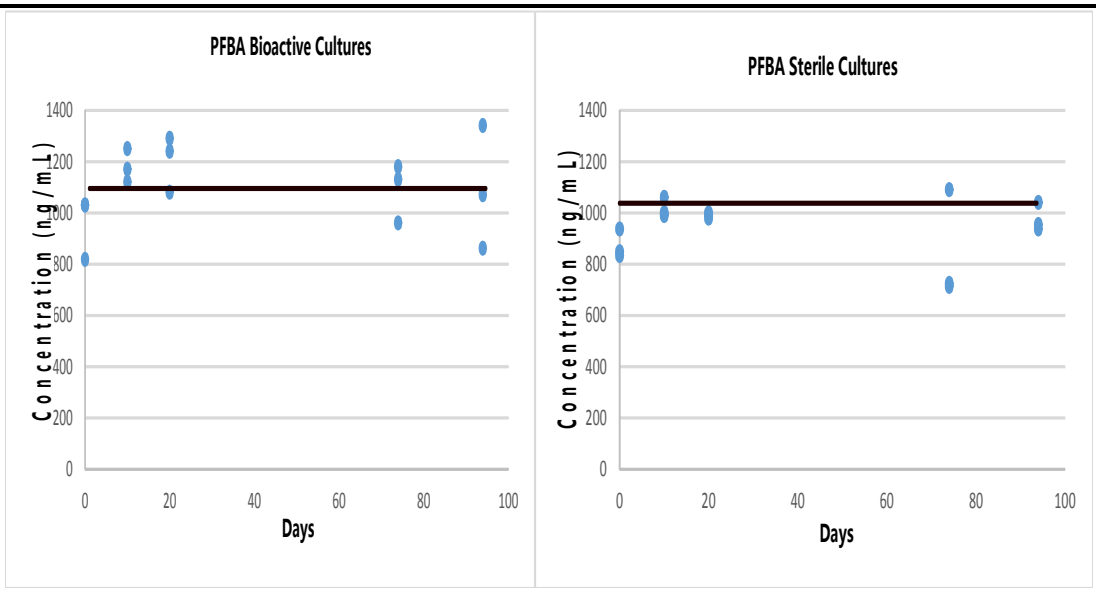

Figure 12. The PFBA concentrations measured in PFBA-dose bioactive cultures (left) and sterile cultures (right) dosed with PFBA. The line represents the dosed concentration of 1030 ng/mL.

Note: It may be expected that lower molecular weight perfluorinated acids could be generated as biodegradation products of PFBA, but none were analytically determined as part of this study. Future studies might include analysis for perfluorinated acids that are lower molecular weight than PFBA such as perfluoroacetate (i.e. trifluoroacetic acid, TFA) or perfluoropropanoate (PFPA).

Table 15. Analyte concentrations measured in active and sterile anaerobic cultures dosed with PFBA.

Culture ID	Culture Type	Incubation Time (days)	Concentration (ng/mL)
			PFBA
E05-0544-605	Active Sludge with PFBA	0	1030
E05-0544-606	Active Sludge with PFBA	0	818
E05-0544-607	Active Sludge with PFBA	0	1030
E05-0544-608	Active Sludge with PFBA	10	1170
E05-0544-609	Active Sludge with PFBA	10	1120
E05-0544-610	Active Sludge with PFBA	10	1250
E05-0544-611	Active Sludge with PFBA	20	1240
E05-0544-612	Active Sludge with PFBA	20	1080
E05-0544-613	Active Sludge with PFBA	20	1290
E05-0544-614	Active Sludge with PFBA	74	1180
E05-0544-615	Active Sludge with PFBA	74	960
E05-0544-616	Active Sludge with PFBA	74	1130
E05-0544-617	Active Sludge with PFBA	94	861
E05-0544-618	Active Sludge with PFBA	94	1340
E05-0544-619	Active Sludge with PFBA	94	1070
E05-0544-620	Sterile Sludge with PFBA	0	848
E05-0544-621	Sterile Sludge with PFBA	0	936
E05-0544-622	Sterile Sludge with PFBA	0	832
E05-0544-623	Sterile Sludge with PFBA	10	988
E05-0544-624	Sterile Sludge with PFBA	10	1060
E05-0544-625	Sterile Sludge with PFBA	10	1000
E05-0544-626	Sterile Sludge with PFBA	20	999
E05-0544-627	Sterile Sludge with PFBA	20	979
E05-0544-628	Sterile Sludge with PFBA	20	991

Table 15. Analyte concentrations measured in active and sterile anaerobic cultures dosed with PFBA.

Culture ID	Culture Type	Incubation Time (days)	Concentration (ng/mL) PFBA
E05-0544-629	Sterile Sludge with PFBA	74	725
E05-0544-630	Sterile Sludge with PFBA	74	1090
E05-0544-631	Sterile Sludge with PFBA	74	713
E05-0544-632	Sterile Sludge with PFBA	94	953
E05-0544-633	Sterile Sludge with PFBA	94	1040
E05-0544-634	Sterile Sludge with PFBA	94	936

Note: Results for the PFBA-dosed samples were reported from analytical runs # 19 and #26 in **Table 8**. Culture extracts were analyzed against a set of matrix matched calibration standards. Quantitation was by internal standard method for both runs. Calibration curves contained minimally six data points. Curves in analytical runs #19 and #26 were quadratic fit with 1/x weighting for PFBA. Calibration accuracy for standards included in the curve fit were within 100 ± 25% (± 30% at LLOQ). CCVs (low and mid-level QCs at 40.0 ng/mL and 400 ng/mL, respectively) were injected approximately every 15 samples in analytical run #19, and mid-level calibration standard (250 ng/mL) was re-injected as CCVs in analytical run #26, and all CCVs/QCs were within 100±30% of their theoretical values for all analytes. LLOQs for PFBA were 10 ng/mL in both runs.
All values in the table are reported to 3 significant figures while more precise data was used in data calculations.
Associated bioactive and sterile sludge blanks analyzed with the samples were all below the LLOQ for all analytes.
[a] PFBA cultures for days 74 and 94 were additionally analyzed for PFBS, PFBSi, FBSA, MeFBSA, MeFBSAA and MeFBSE in analytical run #26, but those results excluded from the table because they are not anticipated degradation products of PFBA.

4.2.7 6:2 FTS Results

The test substance 1,1,2,2-tetrahydro-tetradecafluorooctane sulfonate (6:2 fluorotelomer sulfonate, 6:2 FTS, a.k.a. THPFOS in data) was dosed into anaerobic cultures at a nominal concentration of 954 ng/mL and incubated anaerobically for up to 105 days. The results of the quantitative LC/MS/MS analysis for 6:2 FTS in the bioactive culture extracts versus sterile control culture extracts showed no discernible loss of 6:2 FTS over 105 days (**Figure 13**). In addition, the anticipated products perfluorohexanoate (PFHxA) and 6:2 unsaturated fluorotelomer acid (6:2 FTUCA) were not observed as products. The 6:2 FTS concentrations determined by LC/MS/MS in active and sterile cultures are shown in **Table 16**.

Matrix-interference appeared to cause signal enhancement for 6:2 FTS which was observed for mid-study time points, possibly due to transformation of sludge-related compounds in the cultures which facilitated signal enhancement for 6:2 FTS. In the day 69 and day-105 culture extracts a peak corresponding to the 6:2 unsaturated fluorotelomer carboxylate (6:2 FTUCA) was detected below the LLOQ in the sterile cultures, but was not present in the active cultures. However, coincidentally a peak was detected for 6:2 fluorotelomer carboxylate (6:2 FTCA) in the day-69 and day-105 active cultures that was not present in the equivalent sterile cultures. This could imply that a low concentration of 6:2 FTUCA may have been a contaminant of the 6:2 FTS and transformed anaerobically to 6:2 FTCA.

Semi-quantitative analysis of bioactive culture E05-0544-144 headspace at day-69 by a sensitive GC/MS assay showed no detectable volatile fluorocarbons (analytical run # 30 of **Table 8**).

The net result established from this data was that 6:2 FTS does not appear to biodegrade anaerobically at any appreciable rate over 105 days under methanogenic conditions. In the future, the anaerobic biodegradability of 6:2 FTS might be further evaluated by including the analysis of 6:2 fluorotelomer alcohol, a likely biotransformation product of 6:2 FTS that was not measured for the 6:2 FTS cultures. Additionally, analysis for smaller molecular weight perfluorinated acids such as TFA, PFPA, PFBA and PFPeA should also be included in future analyses of 6:2 FTS anaerobic cultures.

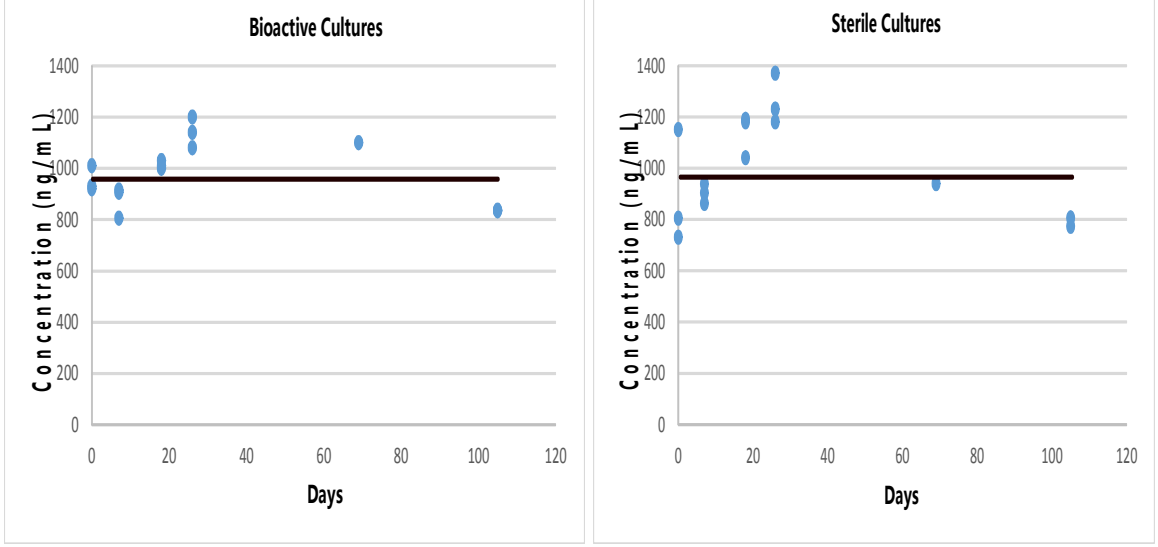

Figure 13. The 6:2 FTS concentrations measured in active cultures (left) and sterile cultures (right) dosed with 6:2 FTS. The line represents the dosed concentration of 954 ng/mL.

Table 16. Analyte concentrations measured in active and sterile anaerobic cultures dosed with 6:2 FTS

Culture ID	Culture Type	Incubation Time (days)	Concentration (ng/mL)			
			6:2 FTS	6:2 FTCA	6:2 FTUCA [b]	PFHxA
E05-0544-130	Active Sludge with 6:2 FTS	0	920	Not Determined	< 10.0	< 10.0
E05-0544-131	Active Sludge with 6:2 FTS	0	930	Not Determined	< 10.0	< 10.0
E05-0544-132	Active Sludge with 6:2 FTS	0	1010	Not Determined	< 10.0	< 10.0
E05-0544-133	Active Sludge with 6:2 FTS	7	907	Not Determined	< 10.0	< 10.0
E05-0544-134	Active Sludge with 6:2 FTS	7	915	Not Determined	< 10.0	< 10.0
E05-0544-135	Active Sludge with 6:2 FTS	7	805	Not Determined	< 10.0	< 10.0
E05-0544-136	Active Sludge with 6:2 FTS	18	1030	Not Determined	< 10.0	< 10.0
E05-0544-137	Active Sludge with 6:2 FTS	18	1000	Not Determined	< 10.0	< 10.0
E05-0544-138	Active Sludge with 6:2 FTS	18	1010	Not Determined	< 10.0	< 10.0
E05-0544-139	Active Sludge with 6:2 FTS	26	1080	Not Determined	< 10.0	< 10.0
E05-0544-140	Active Sludge with 6:2 FTS	26	1200	Not Determined	< 10.0	< 10.0
E05-0544-141	Active Sludge with 6:2 FTS	26	1140	Not Determined	< 10.0	< 10.0
E05-0544-144	Active Sludge with 6:2 FTS	69	1100	< 10.0	< 10.0	< 10.0
E05-0544-142	Active Sludge with 6:2 FTS	105	837	< 10.0	< 10.0	< 10.0
E05-0544-143	Active Sludge with 6:2 FTS	105	834	< 10.0	< 10.0	< 10.0
E05-0544-145	Sterile Sludge with 6:2 FTS	0	804 [a]	Not Determined	< 10.0	< 10.0
E05-0544-146	Sterile Sludge with 6:2 FTS	0	730 [a]	Not Determined	< 10.0	< 10.0
E05-0544-147	Sterile Sludge with 6:2 FTS	0	1150 [a]	Not Determined	< 10.0	< 10.0
E05-0544-148	Sterile Sludge with 6:2 FTS	7	903 [a]	Not Determined	< 10.0	< 10.0
E05-0544-149	Sterile Sludge with 6:2 FTS	7	862 [a]	Not Determined	< 10.0	< 10.0
E05-0544-150	Sterile Sludge with 6:2 FTS	7	938 [a]	Not Determined	< 10.0	< 10.0
E05-0544-151	Sterile Sludge with 6:2 FTS	18	1190 [a]	Not Determined	< 10.0	< 10.0
E05-0544-152	Sterile Sludge with 6:2 FTS	18	1040 [a]	Not Determined	< 10.0	< 10.0
E05-0544-153	Sterile Sludge with 6:2 FTS	18	1180 [a]	Not Determined	< 10.0	< 10.0

Table 16. Analyte concentrations measured in active and sterile anaerobic cultures dosed with 6:2 FTS

Culture ID	Culture Type	Incubation Time (days)	Concentration (ng/mL)			
			6:2 FTS	6:2 FTCA	6:2 FTUCA [b]	PFHxA
E05-0544-154	Sterile Sludge with 6:2 FTS	26	1180 [a]	Not Determined	< 10.0	< 10.0
E05-0544-155	Sterile Sludge with 6:2 FTS	26	1370 [a]	Not Determined	< 10.0	< 10.0
E05-0544-156	Sterile Sludge with 6:2 FTS	26	1230 [a]	Not Determined	< 10.0	< 10.0
E05-0544-159	Sterile Sludge with 6:2 FTS	69	939	< 10.0	< 10.0	< 10.0
E05-0544-157	Sterile Sludge with 6:2 FTS	105	805	< 10.0	< 10.0	< 10.0
E05-0544-158	Sterile Sludge with 6:2 FTS	105	773	< 10.0	< 10.0	< 10.0

Note: Results for the 6:2 FTS-dosed samples were reported from analytical runs # 8 and #21 in **Table 8**. Culture extracts were analyzed against a set of matrix matched calibration standards. Quantitation was by internal standard method for both runs. Calibration curves contained minimally six data points. Quantitation was by internal standard method and calibration curves were quadratic fit with 1/x weighting, except 6:2 FTS in run #21 which was quantified by external standard method. Calibration accuracy for standards included in the curve fit were within 100 ± 25% (± 30% at LLOQ). CCVs were re-injection of the 25.0 and 750 ng/mL calibration standard, and were injected approximately every 15 sample. CCVs were within 100±30% of their theoretical values for all analytes, with exceptions of 6:2 FTS in run #8 which had five CCVs between 131% and 154% and 6:2 FTUCA in run #8 which had two CCVs at 143% and 147%.
LLOQs were 25.0 ng/mL for 6:2 FTS (however 10 ng/mL standard included in curve fit and 10 ng/mL for PFHxA and 6:2 FTUCA in run #8. LLOQs were 10.0 ng/mL for 6:2 FTS, PFHxA, 6:2 FTCA and 6:2 FTUCA in analytical run #21.

Results shown are rounded to 3 significant figures while more precise values were used in data calculations.
Associated bioactive and sterile sludge blanks analyzed with the samples were all below the LLOQ for all analytes.
[a] Results were reported from analytical run #8 and the CCVs analyzed immediately after the 6:2-FTS dosed culture extracts were 152% and 131%, CCVs prior to those samples were 121% and 113%. Data was reported despite the high CCV results since there were not prerequisite pass/fail criteria.
[b] 6:2 FTUCA in run #8 had CCV results of 147% and 143% but those CCVs did not flank results for 6:2 FTS-dosed culture extracts and only flanked blank controls.

4.2.8 6:2 FTOH Results

The test substance 1,1,2,2-tetrahydro-tridecafluorooctan-1-ol (6:2 FTOH) was dosed into cultures at a nominal concentration of 1,230 ng/mL and were incubated anaerobically for up to 94 days. Extracted samples were analyzed by direct inject GC/MS for 6:2 FTOH and by LC/MS/MS for potential soluble degradation products 6:2 FTCA, 6:2 FTUCA, PFHpA and PFHxA. The results for 6:2 FTOH showed that there was considerable loss of 6:2 FTOH over the 94 days in bioactive cultures and in sterile cultures (**Figure 14**). The loss of 6:2 FTOH in sterile cultures was possibly due to irreversible adsorption to sludge or to the test vessel cap (i.e. PTFE liner). Irreversible adsorptive loss of FTOHs has been previously observed to PTFE liners of test vessels (Wang *et al.* 2005). The loss of 6:2 FTOH was more pronounced in bioactive cultures and was likely the result of the additive effect of 6:2 FTOH being biodegraded and lost to adsorption.

The biotransformation product 6:2 FTCA was formed in bioactive cultures at levels exceeding the LLOQ (**Figure 15**). Based on the average measured value at day 94, 10.9 mole% of the initial dosed 6:2 FTOH was accounted for as 6:2 FTCA (**Table 17**). The 6:2 FTCA product was not observed in sterile cultures, confirming its formation was due to microbiological activity. In addition, the anticipated product 6:2 FTUCA was observed qualitatively as a low level peak in chromatograms, and that peak response increased over time, but remained below the LLOQ of 10.0 ng/mL and thus was not quantifiable; the 6:2 FTUCA peak was not observed in sterile culture extracts. The anticipated products PFHxA and PFHpA were measured for but not observed as anaerobic degradation products.

During this study the 6:2 FTOH biotransformation product 5:3 acid and an aldehyde product 6:2 FTAL (Wang *et al.* 2005) were not measured. The 5:3 acid was recently identified as an anaerobic biodegradation product by Zhang *et al.* (2013a). However, the 6:2 FTAL is considered minor and short lived and is not a concern that it was not measured. However 5:3 acid is expected at significant levels anaerobically. The quantitative GC/MS results showed that approximately 300 to 500 ng/mL of additional 6:2 FTOH was lost in bioactive cultures versus the levels lost in sterile cultures, the maximum concentration of 6:2 FTCA was only 150 ng/mL and gave poor mass balance of approximately 40 mole% relative to the lost 6:2 FTOH. Therefore it is speculated that approximately 60 mole% of the lost 6:2 FTOH may have been biotransformed to 5:3 acid. The relevance of this is discussed later in this report.

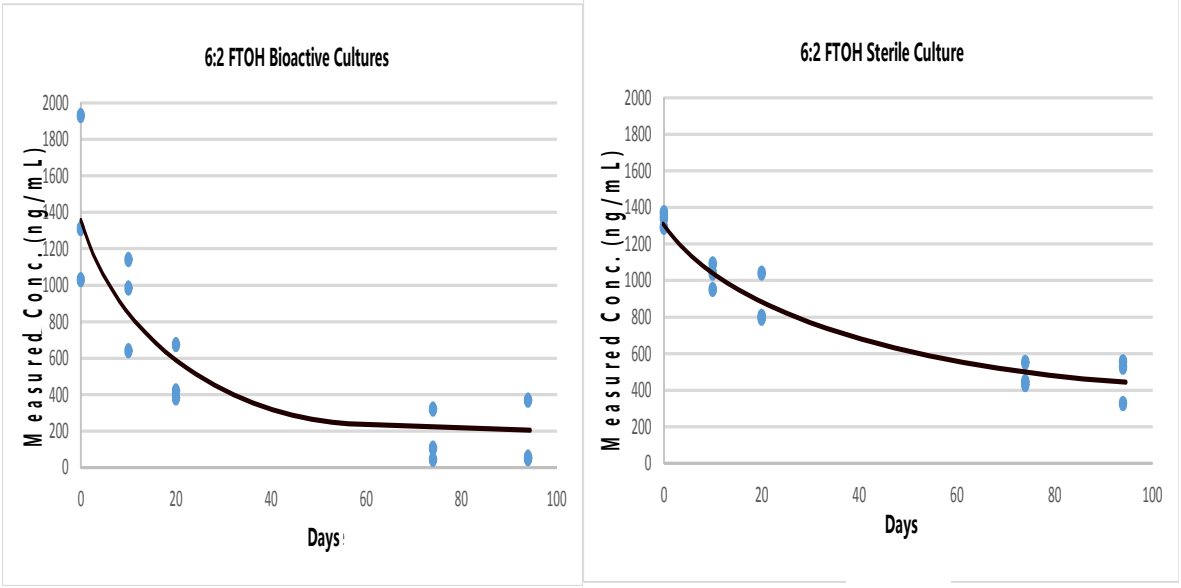

Figure 14. The measured 6:2 FTOH concentrations by GC/MS in bioactive cultures (left) and sterile cultures (right); dosed with nominal 1230 ng/mL 6:2 FTOH. Data manually fitted for appearance.

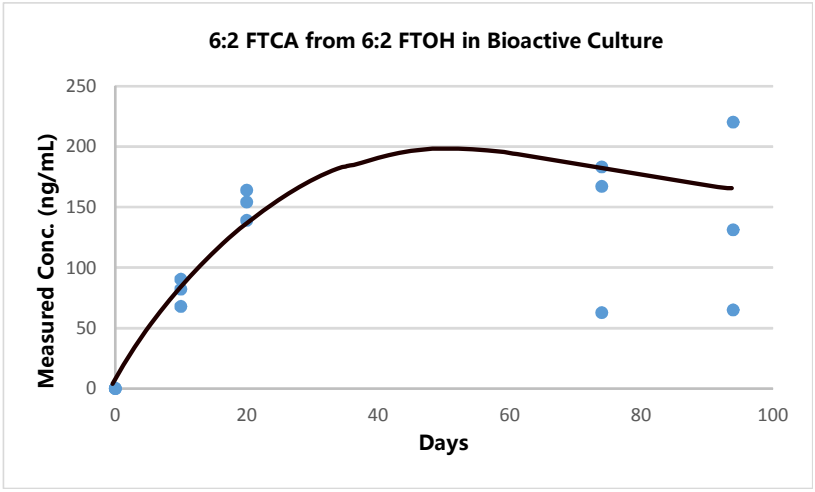

Figure 15. The formation of 6:2-FTCA from 6:2 FTOH in bioactive cultures. Data manually fitted for appearance.

The formation of 6:2 FTCA in the active cultures over the first 3 time points (i.e. first 20 days of incubation) was linear and those data were used to estimate an initial

biotransformation reaction rate, based on the 6:2 FTOH conversion to 6:2 FTCA only (**Figure 16**). The initial rate was calculated to be 102 pmole of 6:2 FTOH biotransformed to 6:2 FTCA per day under these test conditions. However, 5:3 acid was not measured and possibly accounted for ~60% of the degraded FTOH, therefore the rate is likely an underestimate and may be ~2 times faster than estimated. The sludge-dependent estimated biotransformation rate of 6:2 FTOH to 6:2 FTCA was calculated at 1.76 pmole per day per mg sludge (dry weight). An estimated $t_{1/2}$ was estimated at 83.8 days based on the initial rate and dose level (see **Table 21**). Rate calculations were performed as described in **Appendix A**.

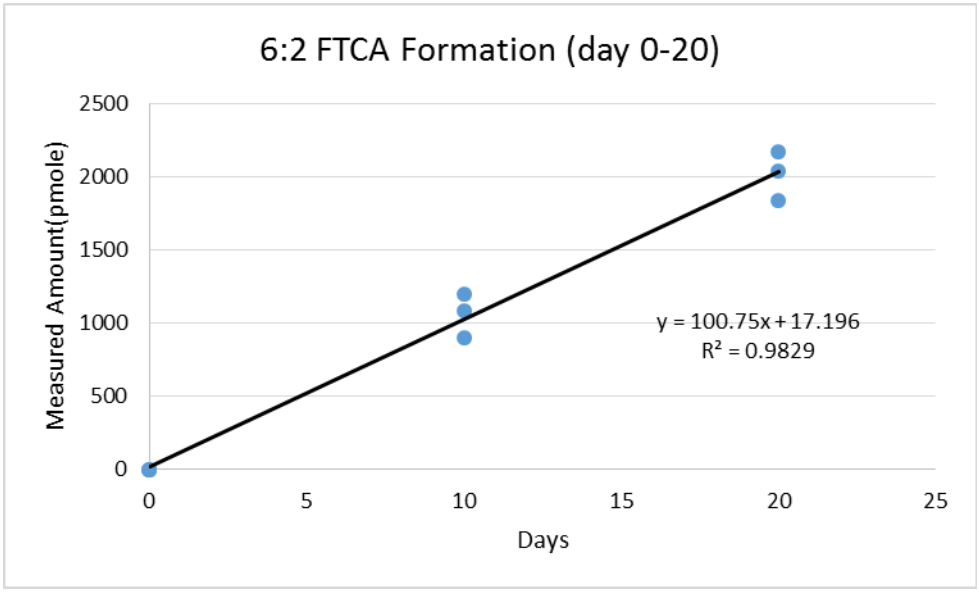

Figure 16. The linear curve fit of 6:2-FTCA (pmole) formed in cultures from 6:2 FTOH.

Table 17. Analyte concentrations measured in active and sterile anaerobic cultures dosed with 6:2 FTOH

Culture ID	Culture Type	Incubation Time (days)	Concentration (ng/mL)				
			6:2 FTOH (GC/MS)	6:2 FTCA	6:2 FTUCA	PFHpA	PFHxA
E05-0544-575	Active Sludge with 6:2 FTOH	0	1030	0	< 10.0	< 10.0	< 10.0
E05-0544-576	Active Sludge with 6:2 FTOH	0	1930	0	< 10.0	< 10.0	< 10.0
E05-0544-577	Active Sludge with 6:2 FTOH	0	1310	0	< 10.0	< 10.0	< 10.0
E05-0544-578	Active Sludge with 6:2 FTOH	10	983	82.1	< 10.0	< 10.0	< 10.0
E05-0544-579	Active Sludge with 6:2 FTOH	10	1140	90.3	< 10.0	< 10.0	< 10.0
E05-0544-580	Active Sludge with 6:2 FTOH	10	640	67.8	< 10.0	< 10.0	< 10.0
E05-0544-581	Active Sludge with 6:2 FTOH	20	674	164	< 10.0	< 10.0	< 10.0
E05-0544-582	Active Sludge with 6:2 FTOH	20	380	154	< 10.0	< 10.0	< 10.0
E05-0544-583	Active Sludge with 6:2 FTOH	20	419	139	< 10.0	< 10.0	< 10.0
E05-0544-584	Active Sludge with 6:2 FTOH	74	319	183	< 10.0	< 10.0	< 10.0
E05-0544-585	Active Sludge with 6:2 FTOH	74	106	167	< 10.0	< 10.0	< 10.0
E05-0544-586	Active Sludge with 6:2 FTOH	74	44.7	62.7	< 10.0	< 10.0	< 10.0
E05-0544-587	Active Sludge with 6:2 FTOH	94	48.4	131	< 10.0	< 10.0	< 10.0
E05-0544-588	Active Sludge with 6:2 FTOH	94	368	220	< 10.0	< 10.0	< 10.0
E05-0544-589	Active Sludge with 6:2 FTOH	94	56.4	64.8	< 10.0	< 10.0	< 10.0
E05-0544-590	Sterile Sludge with 6:2 FTOH	0	1290	< 10.0	< 10.0	< 10.0	< 10.0
E05-0544-591	Sterile Sludge with 6:2 FTOH	0	1340	< 10.0	< 10.0	< 10.0	< 10.0
E05-0544-592	Sterile Sludge with 6:2 FTOH	0	1370	< 10.0	< 10.0	< 10.0	< 10.0
E05-0544-593	Sterile Sludge with 6:2 FTOH	10	950	< 10.0	< 10.0	< 10.0	< 10.0
E05-0544-594	Sterile Sludge with 6:2 FTOH	10	1090	< 10.0	< 10.0	< 10.0	< 10.0
E05-0544-595	Sterile Sludge with 6:2 FTOH	10	1040	< 10.0	< 10.0	< 10.0	< 10.0
E05-0544-596	Sterile Sludge with 6:2 FTOH	20	1040	< 10.0	< 10.0	< 10.0	< 10.0
E05-0544-597	Sterile Sludge with 6:2 FTOH	20	803	< 10.0	< 10.0	< 10.0	< 10.0
E05-0544-598	Sterile Sludge with 6:2 FTOH	20	793	< 10.0	< 10.0	< 10.0	< 10.0
E05-0544-599	Sterile Sludge with 6:2 FTOH	74	429	< 10.0	< 10.0	< 10.0	< 10.0

Table 17. Analyte concentrations measured in active and sterile anaerobic cultures dosed with 6:2 FTOH

Culture ID	Culture Type	Incubation Time (days)	Concentration (ng/mL)				
			6:2 FTOH (GC/MS)	6:2 FTCA	6:2 FTUCA	PFHpA	PFHxA
E05-0544-600	Sterile Sludge with 6:2 FTOH	74	445	< 10.0	< 10.0	< 10.0	< 10.0
E05-0544-601	Sterile Sludge with 6:2 FTOH	74	551	< 10.0	< 10.0	< 10.0	< 10.0
E05-0544-602	Sterile Sludge with 6:2 FTOH	94	553	< 10.0	< 10.0	< 10.0	< 10.0
E05-0544-603	Sterile Sludge with 6:2 FTOH	94	524	< 10.0	< 10.0	< 10.0	< 10.0
E05-0544-604	Sterile Sludge with 6:2 FTOH	94	326	< 10.0	< 10.0	< 10.0	< 10.0

Note: Concentrations of 6:2 FTOH in dosed cultures were determined in GC/MS analytical run #24 in **Table 8**. Soluble biotransformation products were quantified in LC/MS/MS analytical runs #18 and #22 in **Table 8.** Culture extracts were analyzed against a set of matrix matched calibration standards. Calibration curves contained minimally six data points. Quantitation was by external standard method and calibration curve fit was quadratic with 1/x weighting for 6:2 FTOH. Calibration accuracy for standards included in the curve fit were within 100 $\pm$ 25% ($\pm$ 30% at LLOQ). The analytes 6:2 FTCA, 6:2 FTUCA, PFHpA and PFHxA were quantified by internal standard method and calibration curves were quadratic fit with 1/x weighting. Calibration accuracy for standards included in the curve fit were within 100 $\pm$ 25% ($\pm$ 30% at LLOQ). CCVs were a low and mid-level QCs (40.0 ng/mL and 400 ng/mL) and were injected approximately every 15 samples in analytical run #24 and results were within 100 $\pm$ 30%.

Results shown are rounded to 3 significant figures while more precise values were used in data calculations.
Associated bioactive and sterile sludge blanks analyzed with the samples were all below the LLOQ for all analytes.
LLOQs were 100 ng/mL for 6:2 FTOH, and 10 ng/mL for 6:2 FTCA, 6:2 FTUCA, PFHpA and PFHxA.

4.2.9 8:2 FTOH Results

The test substance 8:2 fluorotelomer alcohol (8:2 FTOH) was dosed into anaerobic cultures at a nominal concentration of 1210 ng/mL and incubated for up to 94 days. Culture extracts were analyzed by GC/MS and LC/MS/MS for 8:2 FTOH and anticipated PFAS products, respectively. The results showed that 8:2 FTOH was lost in both bioactive and sterile cultures (**Figure 17**). Loss of 8:2 FTOH in sterile cultures was presumed due to adsorptive loss to the sludge and/or test vessel, while loss in the bioactive cultures was due to an additive effect of adsorption and biodegradation. The LC/MS/MS results showed that 8:2 FTOH biotransformed to give 8:2-fluorotelomer acid (8:2 FTCA) and 8:2-fluorotelomer unsaturated acid (8:2 FTUCA), plotted in **Figure 18.** Anaerobic biodegradation of 8:2 FTOH did not result in formation of anticipated perfluorinated carboxylate products PFHxA, PFHpA, PFOA or PFNA. Based on the average measured concentrations for 8:2-FTCA and 8:2 FTUCA (**Table 18**), roughly 8.8 mole% of the 8:2 FTOH biotransformed to those two products over 94 days of incubation, with 8:2 FTCA being produced at about 5-times the level of 8:2 FTUCA.

Associated water blanks (no sludge) and sterile sludge and bioactive sludge blanks were less than LLOQ for all analytes.

The formation of 8:2 FTCA and 8:2 FTUCA in the active cultures over the first 3 time points (i.e. first 20 days of incubation) was linear and those data were used to estimate an initial biotransformation reaction rate for 8:2 FTOH (**Figure 19**). The initial rate was calculated to be 23.6 pmole of 8:2 FTOH biotransformed to 8:2 FTCA and 8:2 FTUCA per day under these test conditions. The sludge-dependent estimated biotransformation rate of 8:2 FTOH to 6:2 FTCA was calculated at 0.413 pmole per day per mg sludge (dry weight). A $t_{1/2}$ was estimated at 275 days based on the initial rate of product formation and dose level of 8:2 FTOH (see **Table 21**). Rate calculations were performed as described in **Appendix A**.

Note: The 7:3 acid was recently identified as an anaerobic biodegradation product of 8:2 FTOH under anaerobic conditions (Zhang *et al.* 2013a). However, the 7:3 acid product of 8:2 FTOH was not measured during this study and possibly accounted for a significant portion of the degraded FTOH, therefore the rate is likely an underestimate and may be significantly higher than estimated here.

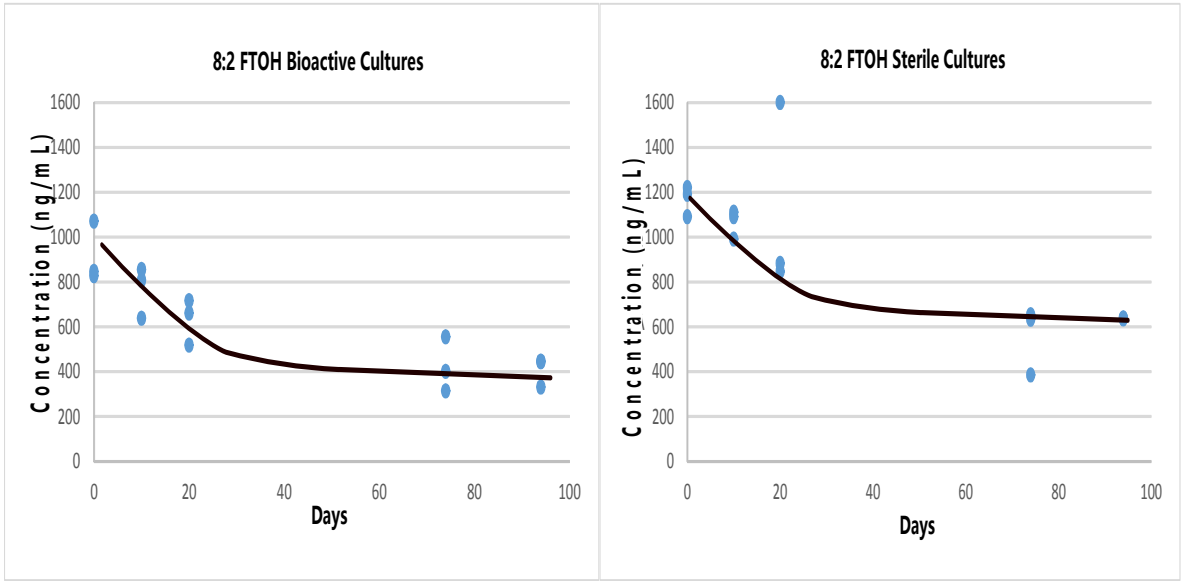

Figure 17. The 8:2 FTOH concentrations measured by GC/MS in bioactive cultures (left) and sterile cultures (right). Data manually fitted to demonstrated data trend.

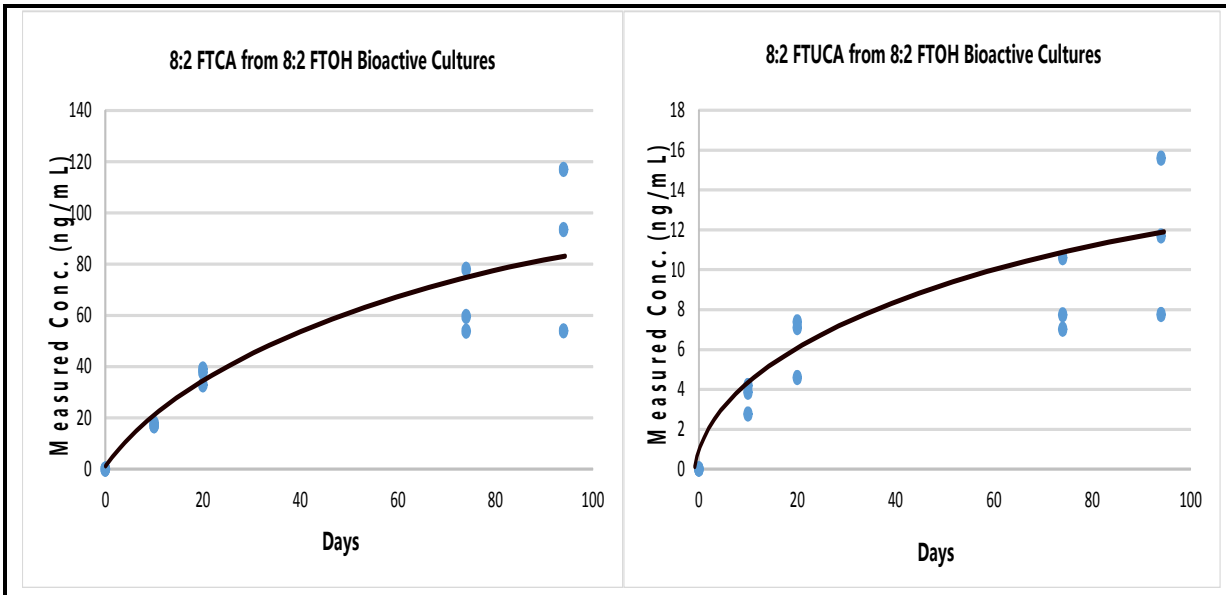

Figure 18. The 8:2 FTCA and 8:2 FTUCA formed in bioactive cultures dosed with 8:2 FTOH . Data manually fitted for appearance.

The initial biotransformation rate for 8:2 FTOH was calculated based on the cumulative formation of 8:2 FTCA and 8:2 FTUCA which only formed in the bioactive cultures. The summed values (pmole) were plotted versus incubation time (days) and fit with a line over the first 20 days (**Figure 19**). The slope of the fitted line was used to assign the rate of degradation. That initial rate was calculated at 23.6 pmole per day and equated to 0.413 pmole/day per mg sludge (dw). A $t_{1/2}$ value was estimated at 275 days based on initial rate. Calculations were performed as shown in **Appendix A**.

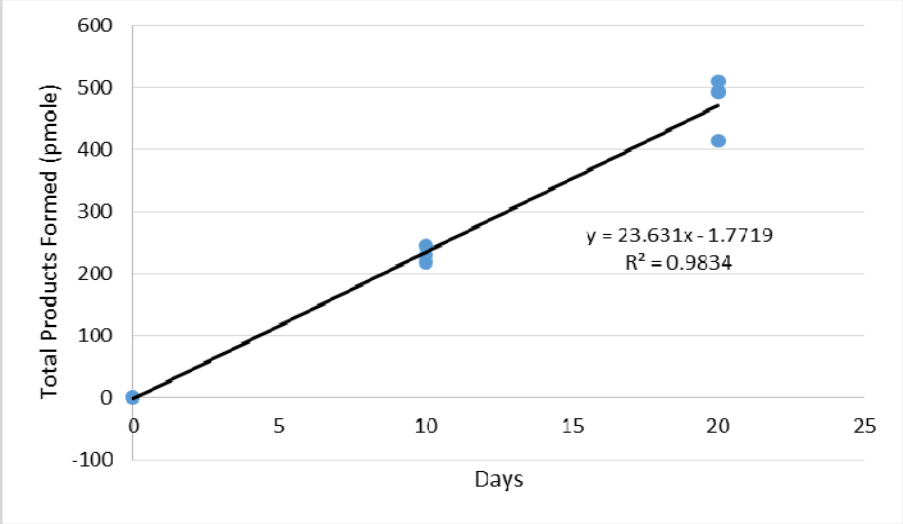

Figure 19. The linear curve fit of total product formed as 8:2 FTCA plus 8:2 FTUCA (pmole) formed in cultures from 8:2 FTOH.

Table 18. Analyte concentrations measured in active and sterile cultures dosed with 8:2 FTOH

Culture ID	Culture Type	Incubation Time (days)	Concentration (ng/mL)							
			8:2 FTOH (GC/MS)	8:2 FTOH (LC/MS)	8:2 FTCA	8:2 FTUCA	PFHxA	PFHpA	PFOA	PFNA
E05-0544-545	Active Sludge with 8:2 FTOH	0	1050	1030 [b]	< 5.00	< 1.00	< 1.00	< 1.00	< 25.0	< 1.00
E05-0544-546	Active Sludge with 8:2 FTOH	0	873	857	< 5.00	< 1.00	< 1.00	1.76 [a]	< 25.0	< 1.00
E05-0544-547	Active Sludge with 8:2 FTOH	0	1100	1010 [b]	< 5.00	< 1.00	< 1.00	1.20 [a]	< 25.0	< 1.00
E05-0544-548	Active Sludge with 8:2 FTOH	10	838	820	16.9	3.86	< 1.00	< 1.00	< 25.0	< 1.00
E05-0544-549	Active Sludge with 8:2 FTOH	10	785	760	18.1	4.19	< 1.00	< 1.00	< 25.0	< 1.00
E05-0544-550	Active Sludge with 8:2 FTOH	10	674	551	17.1	2.76	< 1.00	1.18	< 25.0	< 1.00
E05-0544-551	Active Sludge with 8:2 FTOH	20	743	711	39.2	7.39	< 1.00	< 1.00	< 25.0	< 1.00
E05-0544-552	Active Sludge with 8:2 FTOH	20	769	734	37.8	7.10	< 1.00	< 1.00	< 25.0	< 1.00
E05-0544-553	Active Sludge with 8:2 FTOH	20	547	572	32.9	4.59	< 1.00	< 1.00	< 25.0	< 1.00
E05-0544-554	Active Sludge with 8:2 FTOH	74	519	549	78.0	10.6	< 1.00	< 1.00	< 25.0	< 1.00
E05-0544-555	Active Sludge with 8:2 FTOH	74	345	305	53.9	7.02	< 1.00	< 1.00	< 25.0	< 1.00
E05-0544-556	Active Sludge with 8:2 FTOH	74	385	442	59.6	7.75	< 1.00	< 1.00	< 25.0	< 1.00
E05-0544-557	Active Sludge with 8:2 FTOH	94	439	532	93.5	11.7	< 1.00	< 1.00	< 25.0	< 1.00
E05-0544-558	Active Sludge with 8:2 FTOH	94	443	586	117	15.6	< 1.00	< 1.00	< 25.0	< 1.00
E05-0544-559	Active Sludge with 8:2 FTOH	94	342	308	54.0	7.76	< 1.00	< 1.00	< 25.0	< 1.00
E05-0544-560	Sterile Sludge with 8:2 FTOH	0	1110	1040 [b]	< 5.00	< 1.00	< 1.00	< 1.00	< 25.0	< 1.00
E05-0544-561	Sterile Sludge with 8:2 FTOH	0	1200	1340 [b]	< 5.00	< 1.00	< 1.00	< 1.00	< 25.0	< 1.00
E05-0544-562	Sterile Sludge with 8:2 FTOH	0	1170	1290 [b]	< 5.00	< 1.00	< 1.00	< 1.00	< 25.0	< 1.00
E05-0544-563	Sterile Sludge with 8:2 FTOH	10	1090	1090 [b]	< 5.00	< 1.00	< 1.00	< 1.00	< 25.0	< 1.00
E05-0544-564	Sterile Sludge with 8:2 FTOH	10	984	1030 [b]	< 5.00	< 1.00	< 1.00	< 1.00	< 25.0	< 1.00
E05-0544-565	Sterile Sludge with 8:2 FTOH	10	1030	1110 [b]	< 5.00	< 1.00	< 1.00	< 1.00	< 25.0	< 1.00
E05-0544-566	Sterile Sludge with 8:2 FTOH	20	1550	1240 [b]	< 5.00	< 1.00	< 1.00	< 1.00	< 25.0	< 1.00
E05-0544-567	Sterile Sludge with 8:2 FTOH	20	819	1100 [b]	< 5.00	< 1.00	< 1.00	< 1.00	< 25.0	< 1.00
E05-0544-568	Sterile Sludge with 8:2 FTOH	20	855	1020 [b]	< 5.00	< 1.00	< 1.00	< 1.00	< 25.0	< 1.00

Table 18. Analyte concentrations measured in active and sterile cultures dosed with 8:2 FTOH

Culture ID	Culture Type	Incubation Time (days)	Concentration (ng/mL)							
			8:2 FTOH (GC/MS)	8:2 FTOH (LC/MS)	8:2 FTCA	8:2 FTUCA	PFHxA	PFHpA	PFOA	PFNA
E05-0544-569	Sterile Sludge with 8:2 FTOH	74	639	848	< 5.00	< 1.00	< 1.00	< 1.00	< 25.0	< 1.00
E05-0544-570	Sterile Sludge with 8:2 FTOH	74	595	866	< 5.00	< 1.00	< 1.00	< 1.00	< 25.0	< 1.00
E05-0544-571	Sterile Sludge with 8:2 FTOH	74	382	455	< 5.00	< 1.00	< 1.00	< 1.00	< 25.0	< 1.00
E05-0544-572	Sterile Sludge with 8:2 FTOH	94	627	875	< 5.00	< 1.00	< 1.00	< 1.00	< 25.0	< 1.00
E05-0544-573	Sterile Sludge with 8:2 FTOH	94	588	863	< 5.00	< 1.00	< 1.00	< 1.00	< 25.0	< 1.00
E05-0544-574	Sterile Sludge with 8:2 FTOH	94	605	798	< 5.00	< 1.00	< 1.00	< 1.00	< 25.0	< 1.00

Note: Concentrations of 8:2 FTOH in dosed cultures were determined in GC/MS analytical run #24 in **Table 8**. Later, a LC/MS/MS method was developed for 8:2 FTOH and was determined in LC/MS/MS analytical run #28 of **Table 8**, but that data only used as confirmation of GC/MS results. Anticipated soluble biotransformation products were quantified and reported from LC/MS/MS analytical runs #18 and #28 of **Table 8**. Culture extracts were analyzed against a set of matrix matched calibration standards. Calibration curves contained minimally six data points. Quantitation of 8:2 FTOH by GC/MS was done by external standard method and calibration curve fit was quadratic with 1/x weighting. Soluble analytes determined by LC/MS/MS were quantified by internal standard method and curve fits were quadratic with 1/x weighting. Calibration accuracy for standards included in the curve fit were within 100 $\pm$ 25% ($\pm$ 30% at LLOQ). For analytical runs #18 and #24, CCVs were a low and mid-level QCs (40.0 ng/mL and 400 ng/mL) and were injected approximately every 15 samples, and results were within 100 $\pm$ 30%. For analytical run #28, CCVs were the re-injection of the 250 ng/mL calibration standards approximately every 15 samples, and result were within 100 $\pm$ 30%.

Results shown are rounded to 3 significant figures while more precise values were used in data calculations.
Associated bioactive and sterile sludge blanks analyzed with the samples were all below the LLOQ for all analytes.
LLOQs were 100 ng/mL for 8:2 FTOH, and 5.00 ng/mL for 8:2 FTCA, 1.00 ng/mL for 8:2 FTUCA, 1.00 ng/mL for PFNA, PFHxA and PFHpA, and 25.0 ng/mL for PFOA.
8:2 FTOH culture extracts for day 0, 10 and 20 were additionally analyzed for 6:2 FTCA and 6:2 FTUCA in analytical run #18, but those data not reported in the table for brevity, those results were < 10 ng/mL.
[a] PFHpA was detected slightly above the LLOQ of 1.00 ng/mL on day 0.
[b] Values were determined by extrapolation of the curve beyond the upper limit of quantitation (ULOQ) of 1000 ng/mL (used only as confirmatory results).

4.2.10 246-TCP Results

The 2,4,6-trichlorophenol (246-TCP) was tested because chlorophenols are amenable to LC/MS/MS quantitation by electrospray ionization and negative ion mode, similar to PFASs, and the anaerobic biotransformation of 246-TCP had been described as only occurring under methanogenic conditions using municipal anaerobic digester sludge granules (Mohn & Kennedy 1992.). Also, the biotransformation results in formation of 4-chlorophenol (4-CP) as a stable end product which can also be quantified by LC/MS/MS. It was expected that 246-TCP would undergo sequential reductive dehalogenations in the *ortho* positions of the molecule to form 4-chlorophenol if the cultures were methanogenic.

In this study, the 246-TCP was dosed into active-sludge and sterilized-sludge cultures at 1220 ng/mL and were then incubated anaerobically for up to 15 days. Duplicate cultures were established per culture type and were then incubated and collected on days 0, 5, 7, and 15. Additionally, two active and two sterile cultures were prepared with a dose of 246-TCP that was at 2X-concentrated as the other cultures (2440 ng/mL) and those were incubated for 47-days.

Quantitative LC/MS/MS analysis for 246-TCP, and the anticipated *ortho*-dechlorination products 2,4-dichlorophenol (24-DCP) and 4-CP, showed complete loss of the 246-TCP from active cultures by day-5, the first time point, transient formation of 24-DCP, and ultimately stoichiometric formation of 4-CP (**Table 19**). The 246-TCP did not degrade in the sterile-sludge controls and 4-CP was not formed. The anticipated intermediate product 24-DCP was transiently observed in active cultures, but was below the LLOQ of 100 ng/mL. The 2X-concentrated (2440 ng/mL) cultures collected on day-47 showed loss of 246-TCP in bioactive cultures and resulted in formation of 2X levels of 4-CP (avg. 2310 ng/mL). The 246-TCP was not biodegraded in sterile control cultures.

The 4-CP product accumulated in the anaerobic bioactive cultures dosed with 246-TCP and corroborates the findings of Mohn & Kennedy who showed that 4-CP was the primary product from 246-TCP incubation with acclimated sludge granules and that the 4-CP product was recalcitrant to further anaerobic biotransformation. However, in contrast to their study, the anaerobic digester sludge used here did not appear to require acclimation to 246-TCP to be able to degrade it. The lack of an acclimation period may be due to the additional inclusion of yeast extract nutrients to the cultures or may reflect the different sources of anaerobic sludge used or could be due to the lower, possibly less toxic dose evaluated here

versus the study by Mohn & Kennedy. The 246-TCP is less toxic than PCP (Vallecillo *et al.*, 1999), and has a determined IC_{50} of 7900 and 16000 ng/mL for anaerobic *Nitrosomonas* sp. and methanogens, respectively (Blum and Speece, 1991). This likely explains why 246-TCP was a better positive control substance in this study than PCP.

Mass balance determined for 4-CP on days 5, 7 and 15 ranged from 130 mole% to 165 mole% (relative to dose level of 1220 ng/mL 246-TCP). These high mass balance values could be due to enhanced signal response for 4-CP due to matrix effects in bioactive sludge versus calibration standards which were prepared in sterilized sludge.

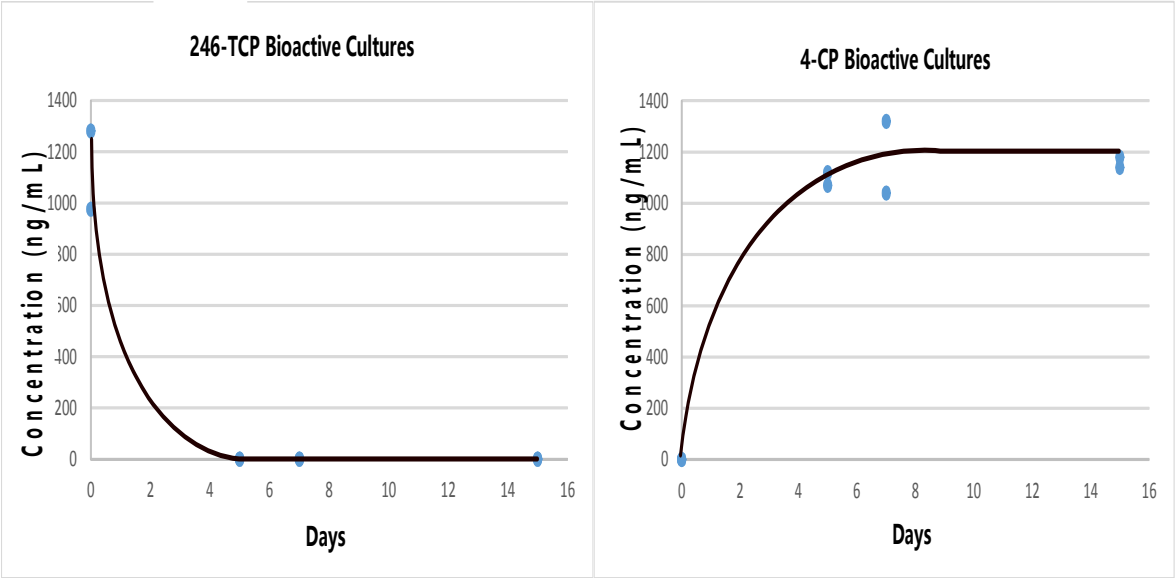

Figure 20. The loss of 246-TCP in bioactive cultures (left) and formation of 4-CP (right) for cultures dosed at 1220 ng/mL 246-TCP. Data manually fitted for appearance.

Table 19. Analyte concentrations measured in active and sterile cultures dosed with 246-TCP

Culture ID	Culture Type	Incubation Time (days)	Concentration (ng/mL)		
			246-TCP	24-DCP	4-CP
E05-0544-221	Active Sludge with 246-TCP	0	1280	< 100	< 100
E05-0544-222	Active Sludge with 246-TCP	0	975	< 100	< 100
E05-0544-223	Active Sludge with 246-TCP	5	< 100	< 100	1070
E05-0544-224	Active Sludge with 246-TCP	5	< 100	< 100	1120
E05-0544-225	Active Sludge with 246-TCP	7	< 100	< 100	1040
E05-0544-226	Active Sludge with 246-TCP	7	< 100	< 100	1320
E05-0544-227	Active Sludge with 246-TCP	15	< 100	< 100	1140
E05-0544-228	Active Sludge with 246-TCP	15	< 100	< 100	1180
E05-0544-229	Active Sludge with 246-TCP	47 [a]	< 100	< 100	2250
E05-0544-230	Active Sludge with 246-TCP	47 [a]	< 100	< 100	2370
E05-0544-233	Sterile Sludge with 246-TCP	0	1170	< 100	< 100
E05-0544-234	Sterile Sludge with 246-TCP	5	1220	< 100	< 100
E05-0544-235	Sterile Sludge with 246-TCP	5	1140	< 100	< 100
E05-0544-236	Sterile Sludge with 246-TCP	7	1190	< 100	< 100
E05-0544-237	Sterile Sludge with 246-TCP	7	975	< 100	< 100
E05-0544-238	Sterile Sludge with 246-TCP	15	1120	< 100	< 100
E05-0544-239	Sterile Sludge with 246-TCP	15	1140	< 100	< 100
E05-0544-240	Sterile Sludge with 246-TCP	47 [a]	3060	< 100	< 100
E05-0544-241	Sterile Sludge with 246-TCP	47 [a]	2850	< 100	< 100

Note: Concentrations of 246-TCP, 24-DCP and 4-CP determined by LC/MS/MS in analytical run #12 of **Table 8**. Culture extracts were analyzed against a set of matrix matched calibration standards. Calibration curves contained minimally six data points. Quantitation was by external standard method and calibration curve fit was quadratic with 1/x weighting. Calibration accuracy for standards included in the curve fit were within 100 $\pm$ 25% ($\pm$ 30% at LLOQ). CCVs were the re-injection of the 800 ng/mL calibration standards approximately every 15 samples, and result were within 100 $\pm$ 30%.

Results shown are rounded to 3 significant figures while more precise values were used in data calculations.
Associated bioactive and sterile sludge blanks analyzed with the samples were all below the LLOQ for all analytes.
LLOQs were 100 ng/mL for 246-TCP, 24-DCP and 4-CP

[a] Indicates the cultures were dosed at 2440 ng/mL (2x the normal dose concentration of the other cultures).

4.2.11 PCP Results

Pentachlorophenol (PCP) was dosed into cultures at 2070 ng/mL and cultures incubated anaerobically for up to 74 days (collected on days 0, 3, 10, 20 and 74). Quantitative LC/MS/MS analysis with chromatographic separation of chlorinated phenol analytes on a C8 reversed-phase column was achieved. Calibration standards were prepared with reference substances PCP, 2,3,5,6-tetrachlorophenol (2356-TeCP), 246-TCP, 24-DCP and 4-CP.

PCP appeared to require an acclimation period of approximately 10 days before significant biodegradation occurred. The PCP did not biodegrade in the sterile-sludge controls. As early as day 10, a major TeCP with peak retention times of 7.2 minutes and minor TeCP peak at 6.6 minutes were observed to form. The TeCP peaks were different retention time than the 2356-TeCP reference substance (6.5 min) and were tentatively assigned as 2,3,4,5-tetrachlorophenol (2345-TeCP) as major product and 2,3,4,6-tetrachlorophenol (2346-TeCP) as a minor product. In addition, a trichlorophenol peak was observed at 7.2 minutes, but did not share the same retention time with 246-TCP reference (7.0 min). This peak was tentatively identified as 3,4,5-trichlorophenol (345-TCP). Identities of the chlorinated phenols were assigned based on previous identification of PCP metabolites in methanogenic cultures by Mohn & Kennedy (1992) and Woods *et al.* (1989).

The biodegradation of PCP in the bioactive cultures by day 74 was apparent, but was not consistent amongst the replicates. For example, one culture showed near complete loss of PCP with formation of 2345-TeCP and 345-TCP, while another showed about 50% loss of the PCP, and the third replicate showed very little degradation of PCP.

The semi-quantitative results for 2345-TeCP and 345-TCP, determined using surrogate calibration with 2356-TeCP and 246-TCP, respectively, gave results higher than the PCP dose concentration (i.e. > 100% mass balance), indicating the 2345-TeCP and 345-TCP give MS/MS signal responses greater than the 2356-TeCP and 246-TCP reference substances, respectively.

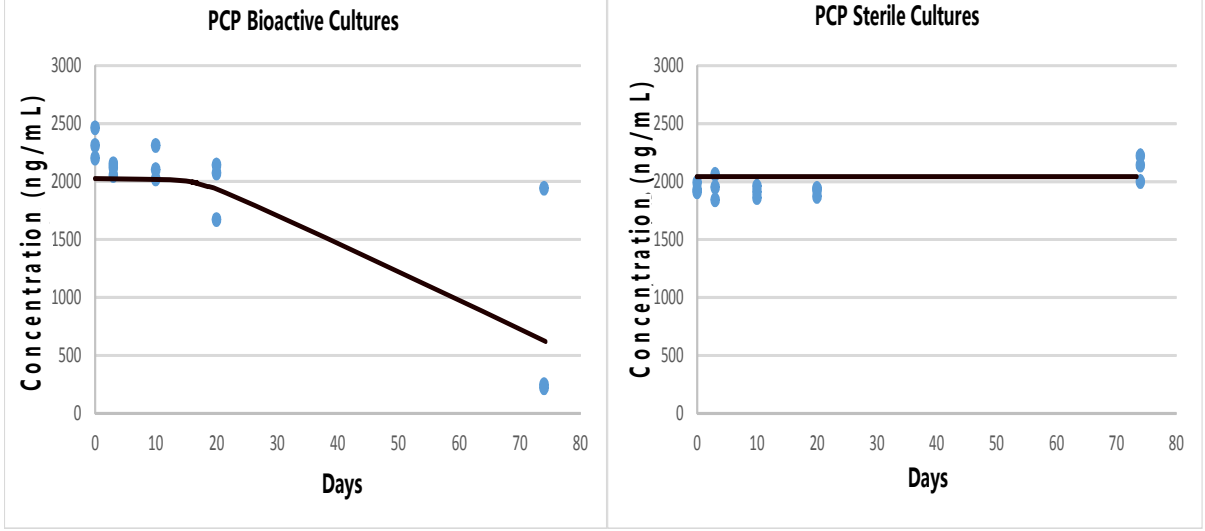

Figure 21. The measured PCP concentrations in bioactive cultures (left) and sterile cultures (right). Data manually fitted for appearance.

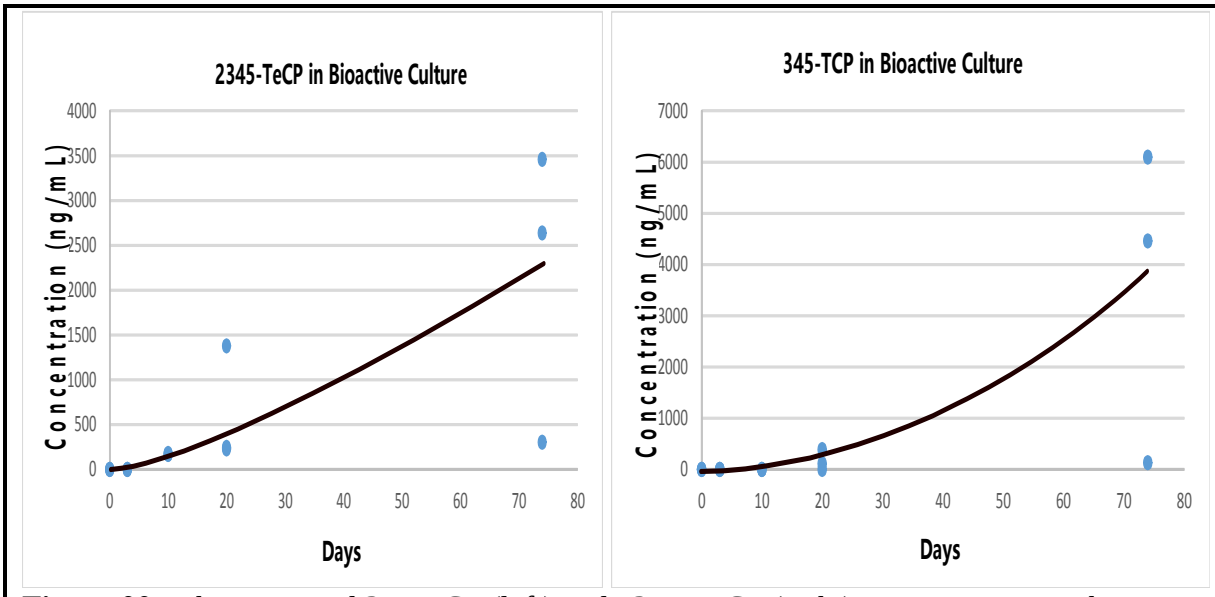

Figure 22. The measured 345-TCP (left) and 2345-TeCP (right) concentrations in bioactive PCP cultures; quantified from surrogate calibration using 246-TCP and 2356-TeCP calibration. Data manually fitted for appearance.

Table 20. Analyte concentrations measured in active and sterile cultures incubated with PCP

Culture ID	Culture Type	Incubation Time (days)	Concentration (ng/mL)							
			PCP [a]	2356-TeCP [a]	2345-TeCP [b]	2346-TeCP [b]	246-TCP [a]	345-TCP [c]	24-DCP [a]	4-CP [a]
E05-0544-665	Active Sludge with PCP	0	2460	< 100	< 100	< 100	<100	< 100	<100	<100
E05-0544-666	Active Sludge with PCP	0	2200	< 100	< 100	< 100	<100	< 100	<100	<100
E05-0544-667	Active Sludge with PCP	0	2310	< 100	< 100	< 100	<100	< 100	<100	<100
E05-0544-668	Active Sludge with PCP	3	2120	< 100	< 100	< 100	<100	< 100	<100	<100
E05-0544-669	Active Sludge with PCP	3	2050	< 100	< 100	< 100	<100	< 100	<100	<100
E05-0544-670	Active Sludge with PCP	3	2150	< 100	< 100	< 100	<100	< 100	<100	<100
E05-0544-671	Active Sludge with PCP	10	2020	< 100	168	< 100	<100	< 100	<100	<100
E05-0544-672	Active Sludge with PCP	10	2100	< 100	168	< 100	<100	< 100	<100	<100
E05-0544-673	Active Sludge with PCP	10	2310	< 100	181	< 100	<100	< 100	<100	<100
E05-0544-674	Active Sludge with PCP	20	2140	< 100	231	< 100	<100	< 100	<100	<100
E05-0544-675	Active Sludge with PCP	20	2070	< 100	249	< 100	<100	114	<100	<100
E05-0544-676	Active Sludge with PCP	20	1670	< 100	1380	< 100	<100	388	<100	<100
E05-0544-677	Active Sludge with PCP	74	1940	< 100	305	< 100	<100	136	<100	<100
E05-0544-678	Active Sludge with PCP	74	219	< 100	3460	< 100	<100	4460	<100	<100
E05-0544-679	Active Sludge with PCP	74	246	< 100	2640	< 100	<100	6100	<100	<100
E05-0544-680	Sterile Sludge with PCP	0	1930	< 100	< 100	< 100	<100	< 100	<100	<100
E05-0544-681	Sterile Sludge with PCP	0	1990	< 100	< 100	< 100	<100	< 100	<100	<100
E05-0544-682	Sterile Sludge with PCP	0	1910	< 100	< 100	< 100	<100	< 100	<100	<100
E05-0544-683	Sterile Sludge with PCP	3	1950	< 100	< 100	< 100	<100	< 100	<100	<100
E05-0544-684	Sterile Sludge with PCP	3	1840	< 100	< 100	< 100	<100	< 100	<100	<100
E05-0544-685	Sterile Sludge with PCP	3	2060	< 100	< 100	< 100	<100	< 100	<100	<100
E05-0544-686	Sterile Sludge with PCP	10	1860	< 100	< 100	< 100	<100	< 100	<100	<100
E05-0544-687	Sterile Sludge with PCP	10	1960	< 100	< 100	< 100	<100	< 100	<100	<100
E05-0544-688	Sterile Sludge with PCP	10	1910	< 100	< 100	< 100	<100	< 100	<100	<100
E05-0544-689	Sterile Sludge with PCP	20	1930	< 100	< 100	< 100	<100	< 100	<100	<100

Table 20. Analyte concentrations measured in active and sterile cultures incubated with PCP

Culture ID	Culture Type	Incubation Time (days)	Concentration (ng/mL)							
			PCP [a]	2356-TeCP [a]	2345-TeCP [b]	2346-TeCP [b]	246-TCP [a]	345-TCP [c]	24-DCP [a]	4-CP [a]
E05-0544-690	Sterile Sludge with PCP	20	1940	< 100	< 100	< 100	<100	< 100	<100	<100
E05-0544-691	Sterile Sludge with PCP	20	1870	< 100	< 100	< 100	<100	< 100	<100	<100
E05-0544-692	Sterile Sludge with PCP	74	2000	< 100	< 100	< 100	<100	< 100	<100	<100
E05-0544-693	Sterile Sludge with PCP	74	2220	< 100	< 100	< 100	<100	< 100	<100	<100
E05-0544-694	Sterile Sludge with PCP	74	2140	< 100	< 100	< 100	<100	< 100	<100	<100

Note: Concentrations of phenolic target analyzed were determined by LC/MS/MS in analytical run # 27 of **Table 8**. Extracts for days 0, 3, 10 and 20 were also analyzed in analytical run #16 but that data not reported due to unusually high PCP results and because the entire set of cultures were analyzed together in run #27 making the data more cohesive. Culture extracts in run #27 were analyzed against a set of matrix matched calibration standards. Calibration curves contained minimally six data points. Quantitation was by internal standard method using PFHS as IS, and calibration curve fit was quadratic with 1/x weighting. Calibration accuracy for standards included in the curve fit were within 100 $\pm$ 25% ($\pm$ 30% at LLOQ). CCVs were the re-injection of the 600 ng/mL calibration standard approximately every 15 samples, and result were within 100 $\pm$ 30%.

Results shown are rounded to 3 significant figures while more precise values were used in data calculations.
Associated bioactive and sterile sludge blanks analyzed with the samples were all below the LLOQ for all analytes.
[a] indicates the substance was a commercial reference substance included in the calibration standards
[b] Indicates the substance was quantified from surrogate calibration using response of 2356-TeCP reference (i.e. semi-quantitative results).
[c] Indicates the substance was quantified from surrogate calibration using response of 246-TCP reference (i.e. semi-quantitative results).

LLOQs were 100 ng/mL for PCP, 2356-TeCP, 246-TCP, 24-DCP and 4-CP.

5 RESULTS SYNOPSIS

Anaerobic methanogenic test conditions were successfully established as demonstrated by changes in gas pressure (measured by change in gas volume) in sealed cultures over the course of the experiment. Additionally, the composition of the gas was confirmed as primarily methane and CO_2 by gas phase FTIR analysis. Gas formation appeared to be stimulated by the presence of methanol, which was coincidentally added as the test substance solvent, but was not similarly stimulated in cultures that did not receive test substance. This increased gas production likely resulted from degradation of the methanol which likely was converted to form methane and/or carbon dioxide. Gas production in cultures did not appear to be inhibited by any of the tested fluorochemical substances at the nominal concentration of 1 mg/L. Cultures appeared to additionally contain a sulfate-reducing microbial community based on the observation of reduced hydrogen sulfide and alkyl-sulfide formation in the headspace of active cultures by GC/MS analysis. The existence of a sulfate-reducing microbial community might explain the loss of gas pressure in later time points as a result of possible methane oxidation by anaerobic methanotrophic bacteria that typically cohabitate with sulfate reducing consortia in anaerobic environments (Hanson and Hanson, 1996).

The substances 246-TCP and PCP were tested as potential positive control substrates for anaerobic biodegradation testing. The levels of PCP and 246-TCP, and their anticipated biotransformation products, were monitored by electrospray LC/MS/MS in negative ion mode. The 246-TCP biotransformed rapidly and was completely removed within 5 days, without any need for acclimation. The loss of 246-TCP occurred with transient formation of 24-DCP on day 15, and ultimately formation of only 4-CP on day 47. The identification of 24-DCP (transient) and 4-CP (final product) are consistent with the findings of previous work (Mohn & Kennedy, 1992). However, in that previous work, the authors showed that 246-TCP anaerobic biodegradation required acclimation of the sludge to 246-TCP before degradation occurred, whereas in this study acclimation of the sludge did not appear to be required. This could be due to the fact that in the previous study the authors tested 246-TCP at between 0.050 mM (9800 ng/ml) to 1.75 mM (346,000 ng/mL), levels that exceed the toxicity of 246-TCP to anaerobic bacteria; the IC_{50} for 246-TCP is 7900 ng/mL for methanogens and is 16000 ng/mL for anaerobic *Nitrosomonas* sp. The toxicity of the lesser halogenated phenols 24-DCP and 4-CP is significantly less than 246-TCP (Valecillo *et al.* 1999). The dose levels tested in this study were well below the IC_{50} concentrations, therefore the acclimation requirement in that pervious work could have been due to a toxicity effect which did not occur in this study.

The incubations with PCP showed that it was successfully biodegraded over 74 days, and did not biodegrade in the sterile-sludge controls. However, there was an apparent lag phase of ~10 days before PCP degradation occurred. Biotransformation reactions appeared to be

consistent with the reactions for 246-TCP, with reductive dehalogenation occurring at *ortho* positions to the hydroxyl group to yield 2345-TeCP and 345-TCP as tentatively identified major products; while dehalogenation in the *meta* and *para* position did not occur significantly occur. In contrast to 246-TCP results, the biodegradation of PCP in active cultures was not consistent for all culture replicates on day 74. For example, one culture showed near complete loss of PCP with formation of 2345-TeCP and 345-TCP, while another showed about 50% loss of the PCP, and the third replicate showed very little degradation of PCP. These results indicate the need for acclimated sludge to efficiently degrade PCP and that PCP may not serve as a good positive control substance for that reason. While it was speculated that PCP degradation proceeded by sequential *ortho*-dehalogenation to 2345-TeCP and then 345-TCP, as previously described by Woods *et al.* (1989) and Miksell & Boyd (1992), the identities of the products were not verified due to the lack of authentic reference substances for those two compounds. The lack of 2345-TeCP and 345-TCP references resulted in quantitation using surrogate calibration with 2356-TeCP and 246-TCP references, and which resulted in artificially high concentration values for 2345-TeCP and 345-TCP, and shown in **Table 20**.

It had been previously shown that PCP reductive dehalogenation only occurs under highly reducing environments (i.e. low redox conditions) where methanogenic or sulfate-reducing conditions exist (Hendriksen, *et al.* 1992). This work corroborates the work of other researchers and verifies that methanogenic cultures were successfully established in this study since PCP was reductively dechlorinated as expected. However, based on the results of this study, PCP was a poor positive control substances for these types of tests due to the need for acclimation, the formation of more than one product, and the inconsistent degradation results amongst replicates on day 74. These results were likely due to toxicity effects of PCP at the dosed concentration, as well as the concentrations of biotransformation products which formed in the cultures. Previous studies have shown that PCP is toxic to anaerobic *Nitrosomonas* sp. and methanogens at relative low levels with IC_{50} values of 60 ng/mL and 40 ng/mL, respectively (Blum and Speece, 1991). Also, the TeCPs are toxic at relative low levels, with IC_{50} values for 2356-TeCP at 1300 ng/mL and 130 ng/mL for anaerobic *Nitrosomonas* sp. and methanogens, respectively.

Both 246-TCP and PCP were biotransformed during this study via reductive dehalogenation reactions. Based on the tentative identification of the metabolites, the degradation pathways for 246-TCP and PCP are shown in **Figure 23**.

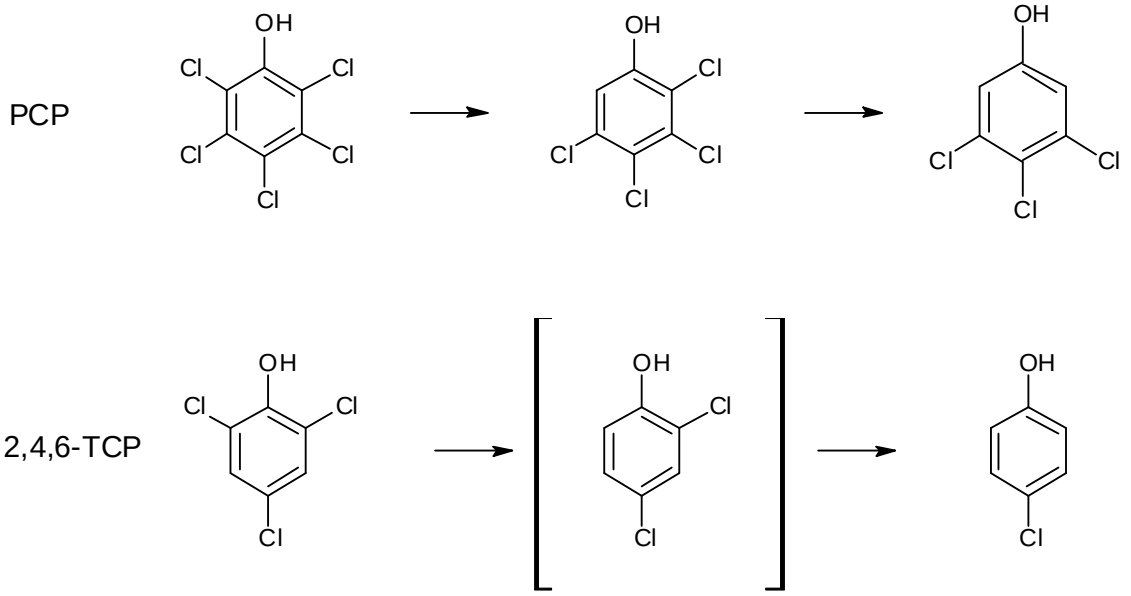

Figure 23. The anaerobic biotransformation pathways of PCP (top) and 246-TCP (bottom), as observed in this study (E05-0544).

Four of the nine tested PFASs in this study showed evidence of biodegradation, and all four were PFAS alcohols used as synthetic building blocks for fluorinated surfactants and fluorinated polymer protectant products. These included EtFOSE, MeFBSE, 6:2-FTOH and 8:2 FTOH. The five that did not biodegraded were PFOS, PFBS, PFOA, PFBA and 6:2 FTS.

EtFOSE was biotransformed with the slowest rate of the four alcohols which degraded during this study, and approximately 2.66 mole% biotransformed to 2[(*N*-ethyl)perfluorooctane-sulfonamido]acetate (EtFOSAA) and perfluorooctane sulfinate (PFOSi) after 108 days. The anaerobic degradation pathway for EtFOSE, as discerned from this study, is shown in **Figure 24**. The results are interesting because the EtFOSE terminal biotransformation products derived from anaerobic incubations are considered transient intermediate products during aerobic biodegradation of EtFOSE, and which ultimately degrades to PFOS under aerobic conditions (Lange 2000, 2001a, Rhoads 2008, Boulanger *et al.* 2005). Rhoads *et al.* (2008) concluded that EtFOSE does not biodegrade under anaerobic conditions, however, the findings from this study show differently and demonstrated that EtFOSE does biotransform anaerobically, albeit very slowly. The difference in results could be due to the very slow rate EtFOSE degradation, which could have been missed due to analytical limits or possibly differences from using sludge from other sources. The findings of this study do help explain the detection of EtFOSAA and PFOSi in environmental samples expected to have low oxygen such as wastewater treatment effluents (Boulanger *et al.* 2005, Higgins *et al.* 2007), marine sediments (Higgins *e.t. al.*2005, Benskin *et al.* 2013), biosolids (Sepulvado *et al.* 2011), and landfill leachates (Allred *et al.* 2015). Based on chemical similarity, 2-[(*N*-methyl)perfluorooctanesulfonamido]ethanol (MeFOSE) is expected to biodegrade anaerobically via the same reactions to form PFOSi and the analogous 2-[(*N*-methyl)perfluorooctanesulfonamido]acetate (MeFOSAA).

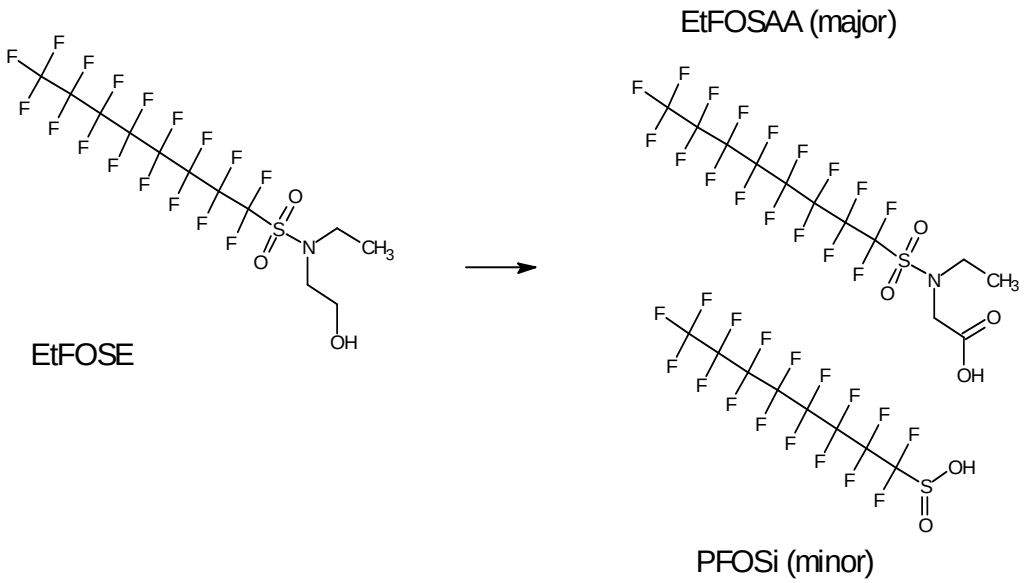

Figure 24. The anaerobic biotransformation pathway of EtFOSE observed (study E05-0544)

MeFBSE biodegraded with the fastest rate of the four PFAS alcohols that biodegraded during this study. After 108 days of incubation, approximately 75 mole% of the dosed MeFBSE was lost and with concomitant formation of two products, MeFBSAA and PFBSi. The degradation rate, determined as the rate of total product formed (pmole) per day per mg sludge (dw), was approximately 45-times faster than the rate determined for the slowest degraded PFAS alcohol, EtFOSE (**Table 21**). Anaerobic degradation of MeFBSE did not form any other products such as MeFBSA, FBSA, PFBS or PFBA, several of which do form during aerobic biodegradation of MeFBSE (Ellefson 2002b). The anaerobic degradation pathway of MeFBSE is shown in **Figure 25**.

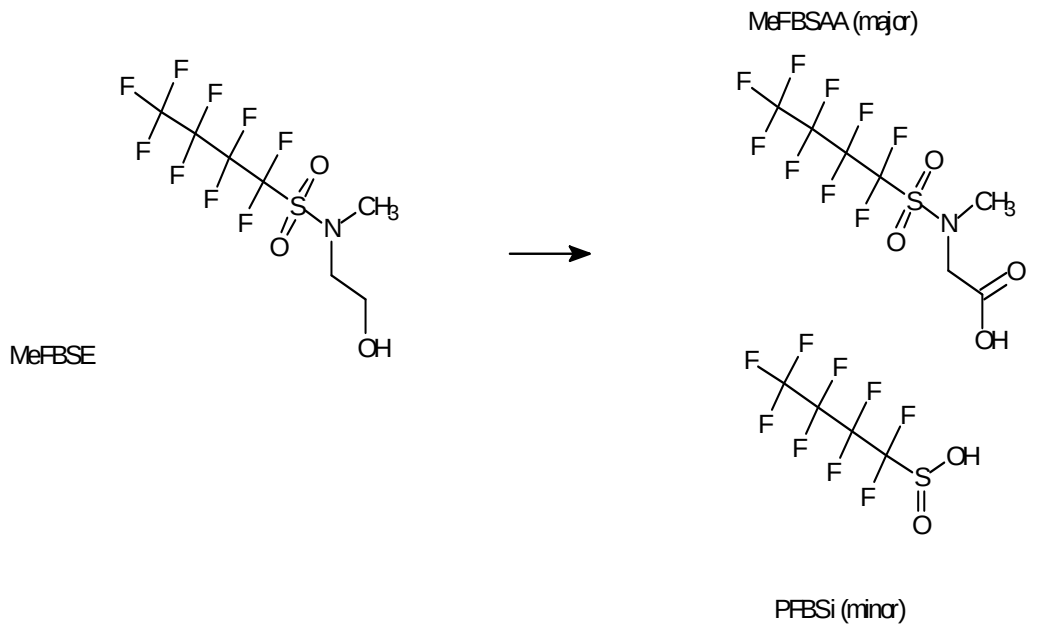

Figure 25. The anaerobic biotransformation pathway for MeFBSE determined in this study (E05-0544).

The 8:2 FTOH biodegraded with the second slowest rate, but was approximately 6-times the rate determined for EtFOSE **(Table 21**). The anaerobic biotransformation products identified from 8:2 FTOH in this study were 8:2 FTCA (major) and 8:2 FTUCA (minor), as shown in **Figure 26**. Those two products, in addition to a new product 7:3 acid, were verified in 2013 by Zhang *et al*., also shown in **Figure 26** for comparison. The lack of formation of perfluorinated carboxylates under anaerobic conditions, but previously identified as significant products from aerobic biodegradation (Lange. 2001 and Dinglasan *et al.* 2004), suggests that the formation of perfluorinated carboxylates from FTOHs may be the strictly confined to aerobic environments and likely catalyzed by microbial O_2-requiring enzymes.

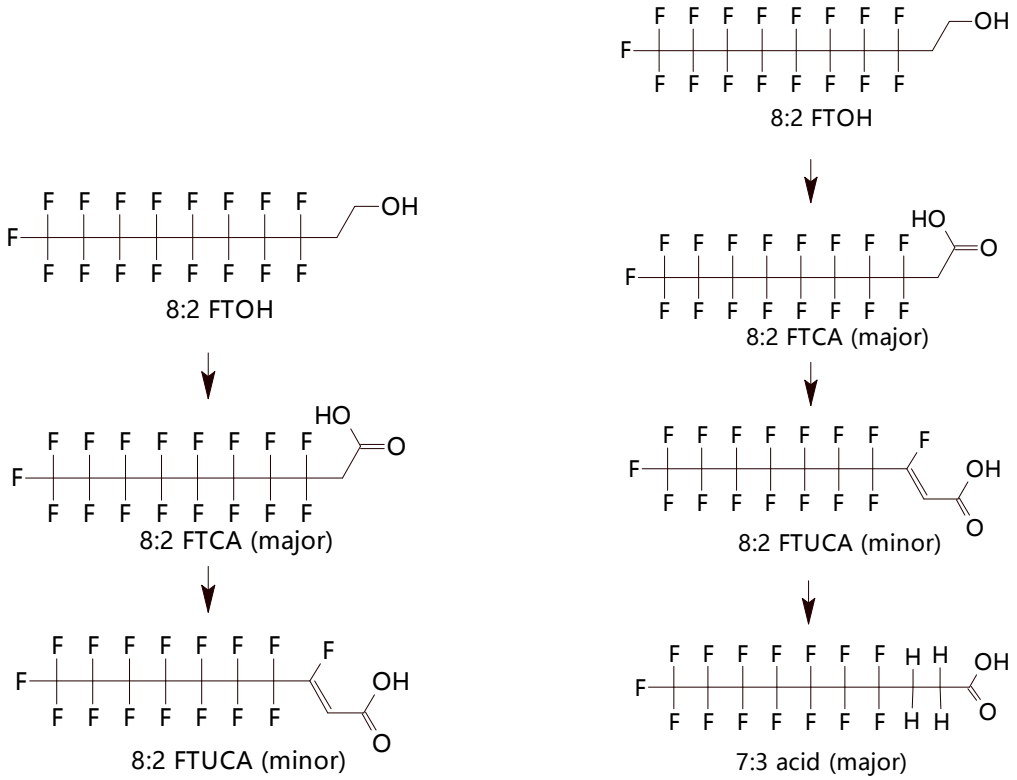

Figure 26. The anaerobic biotransformation pathway for 8:2 FTOH determined in this study shown on the left (E05-0544), and primary products determined by Zhang *et al.* (2013) shown on right.

The 6:2 FTOH degraded at about 25-times the rate determined for EtFOSE (**Table 21**), but approximately ½ the rate of MeFBSE in this study. Anaerobic biodegradation of 6:2 FTOH formed products analogous to those observed for 8:2 FTOH (**Figure 27**), however, while a 6:2 FTUCA product peak was observed it was not measurable above the LLOQ of 10 ng/mL. The only measurable product was the 6:2 FTCA. These products, in addition to 5:3 acid product, were verified in 2013 by Zhang *et al*., also shown in **Figure 27** for comparison. As with 8:2 FTOH, perfluorinated carboxylates did not form from 6:2 FTOH incubations under anaerobic conditions.

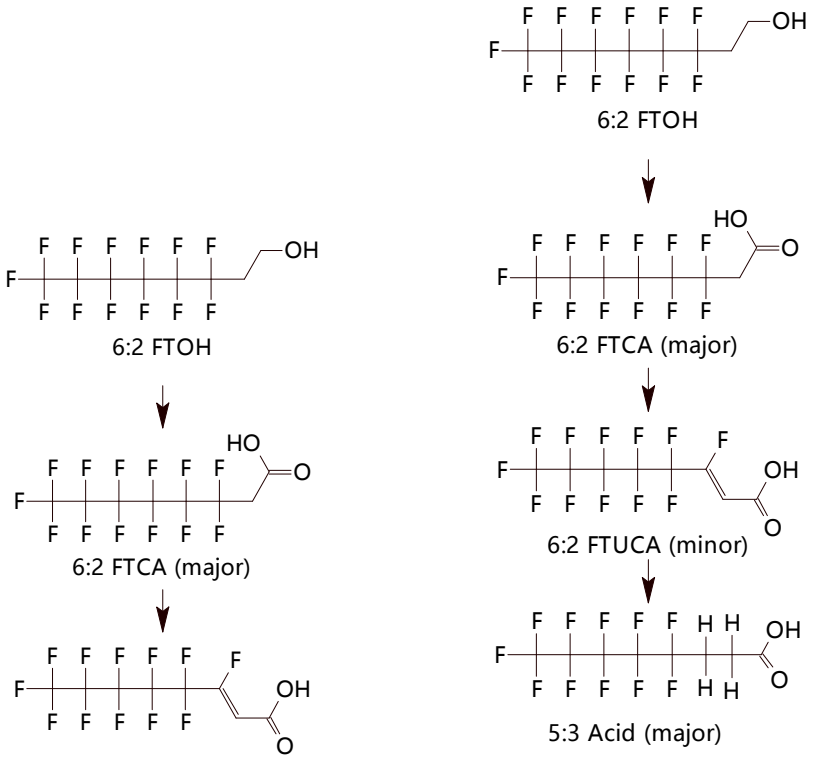

Figure 27. The anaerobic biotransformation pathway for 6:2 FTOH determined in this study shown on the left (E05-0544), and primary products determined by Zhang *et al.* (2013) shown on right.

6 CONCLUSION/DISCUSSION

Nine PFAS test substances were evaluated in this study, most of which had not been thoroughly evaluated under anaerobic conditions prior to this study in 2005-2006. Six were derived from ECF synthesis and were EtFOSE, MeFBSE, PFOS, PFBS, PFOA and PFBA and three were derived from TM synthesis (8:2 FTOH, 6:2 FTOH and 6:2 FTS).

The four PFAS alcohols are common precursors for synthesis of fluorinated polymers used as protectant products and specialty fluorinated surfactants. All four of the PFAS alcohols biodegraded under anaerobic sludge conditions, but with different rates of degradation that appeared to be related to water solubility and/or molecular weight (see **Table 21**). In contrast, the PFAS sulfonates (PFOS, PFBS and 6:2 FTS) and PFAS carboxylates (PFOA and PFBA) were all recalcitrant to anaerobic biodegradation during this study.

The formation of PFOSi and EtFOSAA anaerobically from EtFOSE, and likewise PFBSi and MeFBSAA from MeFBSE, is of importance because it shows that the sulfonamido alcohol precursors do not biotransform further to perfluorinated sulfonates (PFSAs) anaerobically, as has been observed under aerobic conditions (Lange 2001a, Ellefson 2002b). Therefore, analysis of environmental samples obtained from low oxygen environments (i.e. sediment, groundwater, sludge, etc.) may contain more sulfinate and sulfonamido acetate products than perfluorinated sulfonates and perfluorinated carboxylates. The formation and sequestration of the perfluoroalkyl sulfinates under anaerobic conditions also suggests that the biological mechanism underlying oxidation of perfluorinated sulfinates likely involves an oxidative step via O_2-requiring microbes/enzymes, and may not be the result of a hydrolysis reaction. Perhalofluoroalkyl sulfinates undergo oxidation to perfluorinated carboxylates with one-electron oxidants or by photooxidation reactions, but they can also undergo H_2SO_4 catalyzed acid hydrolysis to a mixture PFCAs and PFSAs (Hu *et al*. 1990).

Fluorotelomer carboxylates (FTCAs) and unsaturated carboxylates (FTUCAs) were identified as biotransformation products of 6:2 FTOH and 8:2 FTOH anaerobic biodegradation. The same products were observed as transient intermediates during aerobic biodegradation of FTOHs (Lange 2001b, Dinglasan *et al.* 2004, Wang *et al.* 2005), but not considered end products. The more recent work of Zhang *et al*. (2013) has confirmed that FTCAs and FTUCAs are products from FTOH anaerobic sludge incubations, but also identified a 7:2 acid and 5:3 acid as additional significant products from 8:2 FTOH and 6:2 FTOH, respectively. In that study the authors showed that 6:2 FTCA, 6:2-FTUCA and 5:3 acid were formed from 6:2 FTOH at a ratio of approximately 8:1:4, and that 8:2 FTCA, 8:2 FTUCA and 7:3 acid formed from 8:2 FTOH with a ratio of approximately 4:1:5. Because 5:3 acid and 7:3 acid were not known at the time this study was performed in 2005-2006, they were not quantified. However, based on the determinate ratios by Zhang *et al*.

(2013), the anaerobic biodegradation rates of 6:2 FTOH and 8:2 FTOH reported herein could be up to ~ 2-times faster than reported in **Table 21**. Therefore, the calculated half-life for 6:2 FTOH and 8:2 FTOH in this study could be ~ one-half of the value in Table 21. Rates in this study were calculated for domestic anaerobic sludge from Empire, MN and half-lives for 6:2 FTOH and 8:2 FTOH were determined at 83.8 days and 275 days, respectively, and were based on a lack of measurement for 5:3 acid and 7:3 acid. Therefore, the half-lives could be as short as ~42 days and ~140 days, respectively. Those half-life values are in good agreement with the determined half-lives by Zhang *et al.* for Wilmington, DE domestic anaerobic sludge which were determined at 30 days for 6:2 FTOH and 145 days for 8:2 FTOH.

The formation of FTCAs and FTUCAs as anaerobic end products is an important finding because both are known to be more toxic to aquatic organisms than perfluorinated carboxylic acids (Phillips *et al.* 2007) which are the anticipated penultimate end products of FTOH aerobic biodegradation. The loss of FTOHs in sterile sludge controls was observed in this study and hampered the ability to determine degradation rates based on loss of parent FTOH. However, irreversible binding of 8:2 FTOH to soils has been observed and increases with time and with organic carbon content (Liu & Lee, 2005). This phenomena has also been observed in other sludge biodegradation studies conducted since 2005-2006 (Saez *et al.*, 2008). Wang *et al.* 2005 suggested loss to PTFE containers as well, and in this study the caps of the culture vessels had a PTFE liner, and therefore that may also have contributed to loss of FTOHs in the controls. Similar types of losses were not observed for any of the other tested substances in this study: EtFOSE, MeFBSE, PFOS, PFBS, PFOA, PFBA or 6:2 FTS.

Prior to this study, the stability of 6:2 FTS, PFOS, PFBS, PFBA and PFOA under anaerobic conditions was not entirely clear. Although it had been speculated that they were stable, some laboratory results indicated they might not be. Unlike the PFAS alcohol test substances, the PFAS sulfonates (PFBS, PFOS and 6:2 FTS) and PFAS carboxylates (PFBA, PFOA) did not show evidence of anaerobic biodegradation under methanogenic conditions of this study. These results confirm the conclusions of Remde *et al*. (1996) who showed that PFOS does not biodegradable under anaerobic conditions in a modified ECETOC test over 28 days. Furthermore, a study by Hollingsworth *et al.* (2005) showed that PFOS did not biodegrade under methanogenic conditions or sulfate-reducing conditions for up to 45 days and 30 days of incubation, respectively, and that PFOS also did not appear to inhibit methanogenesis at up to 600 mg/L. In contrast it was reported by Schröder (2003) that PFOS was removed anaerobically within two days in anaerobic bioreactors using sewage sludge immobilized onto glass-foam beads. Additionally, they reported that PFOA was removed in 25 days in the same reactors once PFOS was removed. However, the authors did not detect any volatile fluorochemical products in the gases from the reactor by GC-ECD, nor were they able to measure any fluoride production by fluoride-selective electrode or fluorinated metabolites. Hence, biodegradation was not conclusively shown. Based on the relative

inertness of PFOS and PFOA to degradative attack in general, and lack of degradation in this study and by others, it is more likely that the loss of PFOS and PFOA observed by Schröder was due to adsorption to the test vessel or the sludge immobilized on glass-foam beads, or the glass foam beads themselves, and loss of PFOS and PFOA was likely not due to biodegradation.

The anaerobic stability of 6:2 FTS was somewhat surprising given that it is biodegraded under aerobic conditions (Key *et al.* 1996, Wang *et al.* 2011). 6:2 FTS is of environmental relevance because it has been identified as a component of some brands of aqueous firefighting foams (AFFF) and is a biodegradation product of 6:2 fluorotelomer thioether amido sulfonate (FtTAoS) which is also present in some AFFF formulations (Harding-Marjanovic *et al.* 2015) . Also, 6:2 FTS has been detected in ground water (Schultz and Barofsky, 2004) and municipal wastewater treatment plant effluents (Schultz *et al.* 2006).

The initial rates of degradation were determined from linear plots of the concentration data for total products formed the first few weeks of incubation (i.e. during the fastest rates of degradation); with the assumption of constant saturation of enzymes/cells with test substance above the C_{max} concentration needed to maintain a constant maximal rate during that period. The optimization of test substance concentration was not performed during this study and was outside the original scope, therefore all of the PFAS degradation rates were determined for a nominal test concentration of 1 μg/mL. It is noteworthy that this test concentration is higher than the reported water solubility concentrations for EtFOSE and 8:2 FTOH (Flaherty 2000, and Liu & Lee 2005). Nonetheless, the measured degradation rates appeared to increase as molecular weight of the PFAS test substance decreased (**Table 21),** and this likely is reflective of better bioavailability of the lower molecular weight substances due to greater water solubility. Future studies could be performed to evaluate effects of varying test concentration on rates of biotransformation and biotransformation product yields, as well as to test other PFAS compounds such as perfluoroalkyl sulfonamides.

Rates were based on assumption of quantifying all degradation products formed, and was an assumption which might have resulted in biased low rate estimates for 6:2 FTOH and 8:2 FTOH by a factor of ~2. Additionally, the incubation temperature may have been a factor for some data sets since modest variations in ambient temperature were observed. For example, the mean temperature for the first 4 weeks of MeFBSE and EtFOSE incubations was ~ 24°C, but for 6:2 FTOH and 8:2 FTOH, the mean temperature for the first four weeks of incubation was ~ 22°C due to ambient seasonal temperature fluctuations in the laboratory. The effects of temperature variations could have had an impact on the observed rates. Additionally, adsorption to sludge likely occurred in many cases and could have affected bioavailability and biodegradation rates. This was likely a factor for the 6:2 FTOH and 8:2 FTOH where apparent irreversible binding/loss of test substance in sterile controls was observed.

Table 21. Comparison of anaerobic biodegradation rates determined

Test Substance	MW	Water Solubility (ng/mL)	Sludge Biodeg. Rate (K_0) [pmole/day/$mg_{(dw)}$]	$t_{1/2}$ [a]	Rate Normalized to EtFOSE Rate
EtFOSE	571	151 [c]	0.0718	1077 days (2.95 yrs.)	1.00
8:2 FTOH	464	194 [d]	0.413 [b]	275 days (0.753 yrs.) [b]	5.75
6:2 FTOH	364	18,800 [e]	1.76 [b]	83.8 days (.230 yrs.) [b]	24.5
MeFBSE	357	141,000 [f]	3.26	36.7 days (0.101 yrs.)	45.4

Note: Estimated rates are specific to the conditions of the test described herein and are only presented for comparing the four biotransformed PFAS alcohol test substances to each other. The biodegradation of PFAS is very dependent on the physiological conditions of the test and is influenced by presence of enzyme inducers, microbial strain types present, and level of reducing energy present (Kim *et al.* 2013). PFOS, PFBS, 6:2 FTS, PFOA and PFBA were stable under anaerobic conditions and are therefore excluded from the table.
[a] The half-life estimates were determined based on initial rates and as described in Appendix A.
[b] Degradation rates may be biased low by a factor of ~ 2 due to not measuring 5:3 acid and 7:3 acid from 6:2 FTOH and 8:2 FTOH, respectively. That product(s) were described as additional major products of anaerobic degradation of FTOHs by Zhang *et al.* 2013; this also may result in overestimate of half-life ($t_{1/2}$) for each by a factor of ~ 2.
[c] Flaherty 2000
[d] Liu & Lee, 2005.
[e] Liu & Lee, 2007
[f] Ellefson 2002

Based on the results of this work it can be expected that the anaerobic end-products identified in this study may be dominant PFAS substances present in anaerobic environmental samples and that anaerobic environments may be environmental sinks for partially transformed fluorochemical precursors that are potential sources of perfluorinated sulfonates and carboxylates. It is comprehensible that if these environments are disturbed or oxygenated, that these substances may become aerobically degraded to generate perfluorinated carboxylates and perfluorinated sulfonates. Environments one might consider as anaerobic sinks for such precursors may include landfills and their associated leachates, wetlands, freshwater and marine sediments, groundwater and biosolids from WWTPs, and these findings could suggest that the limited analysis for PFOS and PFOA in environmental samples of such types may be skewed in terms of total PFOS/PFOA equivalents that are actually present. These conclusions are supported by the measurement of EtFOSAA and PFOSi as dominant PFAS in marine sediment collected from both the east and west coasts of the U.S. (Higgins *et al.* 2005) and also their presence in wastewater treatment plant (WWTP) sludge samples (Rhoads *et al.* 2005). Likewise, MeFBSAA, PFBSi, 8:2 FTCA and 8:2 FTUCA have been shown to be formed in leachate collected from anaerobic landfill

simulation chambers loaded with domestic waste solids (Allred *et al.* 2015). It was also shown by Sinclair and Kannan (2006) that 8:2 FTCA and 8:2 FTUCA were significant in the effluent waters of a wastewater treatment plant (WWTP) from New York State that received domestic, commercial and industrial waste.

Future environmental monitoring studies testing anaerobic environmental samples should consider monitoring for anaerobic degradation products identified in this study because they may be sequestered as potential sources of PFOA and PFOS if they are transported or oxygenated waters where they will biotransform to perfluorinated carboxylates and perfluorinated sulfonates, and will be a long-term continued source of such compounds into the environment.

7 LITERATURE CITED

3M Technical Notebook ID 139716, Cleston C. Lange, issued 2005.

3M Technical Notebook ID 140568. Peter M. Radford, issued 2005.

Allred, B.M., J.R. Lang, M.A Barlz, J.A Field. 2015. "Physical and biological release of poly- and perfluoroalkyl substance s(PFASs) from municipal solid waste in anaerobic model landfill reactors' Environ. Sci. Technol. 49:7648-7656.

BEEMS Module B7-Anaerobic Digestion; http://www.oardc.ohio-state.edu/beems/images/BEEMS_B7_Anaerobic_digestion.pdf

Benskin, J.., M.G. Ikonomou, F.A.P.C. Gobas, T.H. Begley, M.B. Woudneh, J.R. Cosgrove. 2013. "Biodegradation of N-ethyl perfluorooctane sulfonamido ethanol (EtFOSE) and EtFOSE-based diester (SAmPAP Diester) in marine sediments". *Environ. Sci. Technol.* 47:1381-1389.

Blum, D.J.W and R.E. Speece. 1991. "A database of chemical toxicity to environmental bacterial and its use in interspecies comparison and correlations". *J. Water Pollut. Control Fed.* 63(3):198-207.

Boulanger, B. J.D. Vargo, J.L. Schnoor, K.C. Hornbuckle. 2005. "Evaluation of perfluorooctane surfactant in a wastewater treatment system and in a commercial surface protection product". *Environ. Sci. Technol.* 39:5524-5530/

Buck, R.C., J. Franklin, U. Berger, J.M. Conder, I.T. Cousins, P. deVoogt, A.A. Jensen, K. Kannan, S.A. Mabury, S.P.J. van Leeuwen. 2011. "Perfluoroalkyl and polyfluoroalkyl

substances in the environment: terminology, classification, and origins." *Integrated Environmental Assessment and Management-SETAC.* **7**(4):513-541.

Dinglasan, J.J.A., Y. ye, E.A. Edwards, and S. A. Mabury. 2004. "Fluorotelomer alcohol biodegradation yields poly and perfluorinated acids". *Environ. Sci. Technol*. 38:2857-2864

Ellefson, M.E. 2002a. "Solubility of N-MeFBSE-OH in water". *3M Environmental Laboratory Report E02-0094.*

Ellefson, M.E 2002b. "Aerobic biodegradation of MeFBSE-OH". *3M Environmental Laboratory Report E02-1325.*

Flaherty, J. 2000. "Characterization of EtFOSE-OH. Phase: solubility of EtFOSE-OH in water, methanol and acetone". *3M Environmental Laboratory Report E00-1715*

Hanson, R.S. and T.E. Hanson. 1996. "Methanotrophic Bacteria". *Microbiol. Rev.* 60:439-471

Harding-Marjanovic, K.C., E.F. Houtz, S.Yi, J.A. Field, D.L. Sedlak, L. Alvarez-Cohen. 2015. "Aerobic biotransformation of fluorotelomer thioether amido sulfonate (Lodyne) in AFFF-amended microcosms". Environ. Sci. Technol. 49(13):766-7674.

Hekster, F.M., R.W.P.M. Laane, and P. de Voogt. 2003. "Environmental and toxicity effects of perfluoroalkylated substances". *Rev. Environ. Contam. Toxicol.* 179:99-121

Higgins, C.P., Field, J.A., Criddle, C.S., and R.G. Luthy. 2005. Quantitative determination of perfluorochemicals in sediments and domestic sludge". *Environ. Sci. Technol.* 39(11): 3946-3956.

Hollingsworth, J., R. Sierra-Alvarez, M. Zhou, K. L. Ogden and J. A. Field. 2005. "Anaerobic biodegradability and methanogenic toxicity of key constituents in copper chemical mechanical planarization effluents of the semiconductor industry". *Chemosphere* 59: 1219-1228.

Hu, C., Z. Xu, W. Huang. 1990. "Reaction of perhalofluoroalkyl sufinates with one-electron transfer oxidants. A facile method for the synthesis of perhalofluorocarboxylic acids." *J. Fluorine Chem*. 49:433-437.

Ignat'ev, N.V., U. Welz-Biermann, U.Heider., A. Kucheryna, S. vonAhsen, W. Habel, P. Sartori, H. Willner. 2003. "Carbon-chain isomerization during the electrochemical fluorination in anhydrous hydrogen fluoride-a mechanistic study". *J. Fluorine Chem.* 124:21-37.

Lange, C.C. 2000. "The aerobic biodegradation of *N*-EtFOSE alcohol by the microbial activity present in municipal wastewater treatment sludge". *3M Environmental Laboratory Report E00-2252.*

Lange, C.C. 2001a. "The 18-day aerobic biodegradation study of perfluorooctanesulfonyl-based chemistries". *3M Environmental Laboratory Report E01-0415*

Lange, C.C. 2001b. "Biodegradation screen study for DuPont Zonyl BA-type telomer alcohol". *3M Environmental Laboratory Report E01-0684*

Liu, J and L.S. Lee. 2005. "Solubility and sorption by soils of 8:2 fluorotelomer alcohol in water and cosolvent systems". *Environ. Sci. Technol.* 39(19):7535-7540

Liu, J and L.S. Lee. 2005. "Effect of fluorotelomer alcohol chain length on aqueous solubility and sorption by soils". *Environ. Sci. Technol.* 41(15):5357-5362

Liu, J. N. Wang, B. Szostek, R.C. Buck, P.K. Panciroli, P.W Folsom, L.M. Sulecki, C.A. Bellin. 2010. "6-2 Fluorotelomer alcohol aerobic biodegradation in soil and mixed bacterial culture". *Chemosphere* 78(4):437-444.

Mah, R.A. and C. Sussman. 1967. "Microbiology of anaerobic sludge fermentation: enumeration of the non-methanogenic anaerobic bacteria". *Appl. Microbiol.* 16:358-361

Miksell, M.D. and S.A. Boyd. 1985. "Reductive dechlorination of the pesticides 24-D, 24,5-T and pentachlorophenol in anaerobic Sludges". *J. Environ. Qual.* 14:337-340

Mohn, W.W. and K.J. Kennedy. 1992. "Limited degradation of chlorophenols by anaerobic sludge granules". *Appl. Env. Microbiol.* 58: 2131-2136.

Kim, H.M., N. Wang, K.H. Chu. 2013. "6:2 Flurotelomer alcohol (6:2 FTOH) biodegradation by multiple microbial species under different physiological conditions". *Appl. Microbiol. Biotechnol.* 98(4):1831-1840

Organization for Economic Cooperation and Development (OECD). 2002. "OECD Guideline for the Testing of Chemicals: Aerobic and Anaerobic Transformation in Aquatic Sediment Systems". OECD No. 308.

Phillips, M.M., M.J.A. Dinglasan-Panlilio, S.A. Mabury, K.R. Solomon, P.K. Sibley. 2007. "Fluorotelomer acids are more toxic than perfluorinated acids". *Environ. Sci. Technol.* 41:7159-7163.

Saez, M., P. deVoogt, J.R. Parsons. 2008. "Persistence of perfluoroalkylated substances in closed bottle tests with municipal sewage sludge". *Environ. Sci. Pollut. Res.* 15:472-477

Schultz, M.M., D.F. Barofsky, J.A. Field. 2003. "Fluorinated Alkyl Surfactants" Env. Eng. Sci. 20:487-501

Schultz, M.M. and D.F. Barofsky. 2004. "Quantitative determination of fluorotelomer sulfonates in groundwater by LC MS/MS". *Environ. Sci. Technol.* 38(6):1828-1835.

Schultz, M.M. and D.F. Barofsky. 2006. "Quantitative determination of fluorinated alkyl substances by large-volume-injection liquid chromatography tandem mass spectrometry characterization of municipal wastewater". *Environ. Sci. Technol.* 40(1):289-295.

Sepulvado, J.G., Blaine, A.C., Hundal, L.S. and C.P. Higgins. 2011. "Occurrence and fate of perfluorochemicals in soil following the land application of municipal Biosolids". *Environ. Sci. Technol.* 45(19):8106-8112.

Schröder, H.F. 2003. "Determination of fluorinated surfactants and their metabolites in sewage sludge samples by liquid chromatography with mass spectrometry and tandem mass spectrometry after pressurized liquid extraction and separation on fluorine-modified reversed-phase sorbents". *J. Chromatography A,* 1020: 131-151

Shelton, D.R. and J.M. Tiedje. 1984. "General method for determining anaerobic biodegradation potential". *Appl. Env. Microbiol.* 47: 850-857

Sinclair, E. and K. Kannan. 2006. "Mass loading and fate of perfluoroalkyl surfactants in wastewater treatment plants". *Environ. Sci. Technol.* 40:1408-1414.

United States Environmental Protection Agency (U.S.E.P.A.) 1998. Fate, Transport and Transformation Test Guidelines. OPPTS 835.3400. Anaerobic Biodegradability of Organic Chemicals. EPA 712-C-98-090

Vallecillo A. , P.A. Garcia-Encina, M. Pena. 1999. "Anaerobic biodegradability and toxicity of chlorophenols". *Wat. Sci. Tech.* 49(8):161-168.

Wang, N., B. Szostek, P. W. Folsom, L.M. Sulecki, V. Capka, R.C. Buck, W. R. Berti, and J.T. Gannon. 2005. Aerobic Biotransformation of ^{14}C-Labeled 8-2 Telomer B Alcohol by Activated Sludge from a Domestic Sewage Treatment Plant. *Environ. Sci. Technol* 39:531-538

Wang, N., J. Liu, R.C. Buck, S. H. Korzeniowski, B.W. Wolstenholme, P.W. Fosom, L.M. Sulecki. 2011. "6:2 Fluorotelomer sulfonate aerobic biotransformation in activated sludge of waste water treatment plants". *Chemosphere* 82(6):853-858.

Wang, Z., I.T. Cousins, M. Scheringer, K. Hungerbuhler. 2013. “Fluorinated alternatives to long-chain perfluoroalkyl carboxylic acids (PFCAs), perfluoroalkane sulfonic acids (PFSA) and their potential precursors”. *Environ. Int. J.* 60:242-248.

Woods, S. L., J.F. Ferguson and M.M. Benjamin. 1989. “Characterization of chlorophenol and chloromethoxybenzene biodegradation during anaerobic treatment”. *Environ. Sci. Technol.* **23**:62-68

Zhang, S. B. Szostek, P.K. McCausland, B.W. Wolstenhom, X. Lu, N. Wang, R.C. Buck. 2013a. “6:2 and 8:2 Fluorotelomer alcohol anaerobic biotransformation in digester sludge from a WWTP under methanogenic conditions”. *Env. Sci. Technol.* 47:4227-4235

Zhang,S. P. W. Folsom, B.W Wolstenhome, H. Sun, N. Wang, R.C. Buck. 2013b “6:2 Fluorotelomer alcohol biotransformation in an aerobic river sediment system”. *Chemosphere* 90(2):203-209

8 REPORT APPROVAL

Digitally signed by Cleston C. Lange
DN: c=US, st=MN, l=St. Paul, ou=3M Environmental Laboratory - authenticated by LRA, email=clange@mmm.com, o=3M, cn=Cleston C. Lange
Reason: I am the author of this document
Date: 2016.05.12 10:05:36 -05'00'

Cleston C. Lange, Ph.D., Project Lead and Report Author Date

Digitally signed by William K. Reagen
DN: c=US, st=MN, l=St. Paul, ou=Laboratory Director, ou=3M Environmental Laboratory - authenticated by LRA, email=wkreagen@mmm.com, o=3M, cn=William K. Reagen
Reason: I am approving this document
Date: 2016.05.12 12:26:39 -05'00'

William K. Reagen, Ph.D., Technical Director Date

This study report and data were audited by the 3M Environmental Laboratory Quality Assurance Unit (QAU).

Digitally signed by Kent R. Lindstrom
DN: c=US, st=MN, l=St. Paul, ou=3M Environmental Laboratory - authenticated by LRA, email=krlindstrom@mmm.com, o=3M, cn=Kent R. Lindstrom
Reason: I have reviewed this document
Date: 2016.05.12 10:17:46 -05'00'

QAU Representative Date

APPENDIX A: Data Calculations

Recovery Calculations:

Recovery = [measured concentration ÷ known concentration] x 100%

Calculation of picomole (pmole):

Example: If MeFBSE was dosed at 976 ng/mL in a 5 mL total volume culture, and MeFBSE is 357 ng/nmole, then the picomole amount dosed is calculated as follows:

[976 ng/mL] x [1 nmole/571 ng] x [5 mL] = 13.7 nmole
13.7 nmole X (1000 pmole/nmole) = 13700 pmole

Hence, there is nominal 13700 pmole of MeFBSE dosed into the 5 mL culture.

APPENDIX A (Continued): Data Calculations

Molecular weight values used for pmole calculations were as follows:

EtFOSE (571 ng/nmole)
EtFOSAA (584 ng/nmole); anion MW used
PFOSi (483 ng/nmole); anion MW used
MeFBSE (357 ng/nmole)
MeFBSAA (371 ng/nmole)
PFBSi (283 ng/nmole); anion MW used
6:2 FTOH (364 ng/nmole)
6:2 FTCA (378 ng/nmole); anion MW used
6:2 FTUCA (358 ng/nmole); anion MW used
8:2 FTOH (464 ng/nmole)
8:2 FTCA (478 ng/nmole); anion MW used
8:2 FTUCA (458 ng/nmole); anion MW used
PFOS (499 ng/nmole); anion MW used
PFBS (299 ng/nmole); anion MW used
PFOA (413 ng/nmole); anion MW used
PFBA (213 ng/nmole); anion MW used
6:2 FTS (425 ng/nmole) anion MW used
PCP (266 ng/nmole)
246-TCP (197 ng/nmole)
24-DCP (163 ng/nmole)
4-CP (129 ng/nmole)

APPENDIX A (Continued): Data Calculations

Calculation of Mass Balance and Percent Degradation:

Example: If there were 13700 pmole of MeFBSE dosed into a 5 mL total liquid volume culture and on day-72 there was 3380 pmole of MeFBSE remaining and 5510 pmole of MeFBSAA and 3610 pmole of PFBSi had formed, and no other analytes were detected, then the sum of total substances measured on day-72 would equal nominal 12500 pmole. The sum of total products formed would be 9120 pmole.

Overall mass balance would be [12500/13700] x 100% = **91.4 mole%**
Percent degradation of dose would be [(9120)/13700] x 100% = **66.7 mole%**

Calculation of Biodegradation/Biotransformation Rates and Half-lives:.

Using Excel 2007™, plot the measured pmole amount of test substance degraded, or pmole of total products formed, versus incubation time (days). The pmole should be on the y-axis and incubation time on x-axis. Only plot the first 3 or 4 time points to create a best fit with linear equation, and exclude later time points because of quadratic response as degradation slows down. Do not force the data through the origin, use the actual day-0 values measured. In the plot, include the linear equation of the fitted line and the R^2 value. The equation should be in the general format of $y = mx + b$. From the equation of the line, the slope of the line is the x-value in the equation and is calculated in pmole/day. Divide that rate value by the mass of sludge (dry wt.) initially added in each culture to get a sludge-dependent degradation rate in (pmole/day/mg sludge (dw). Rates were normalized to sludge content to account for the different mass of sludge (dry weight) in different culture sets and enabling comparison of rates for the different test substances that degraded.

All calculations in this study were based on nominal 5 mL culture volumes containing 4 mL of sludge; the sludge total suspended solids (TSS) value used for all experiments was 14.3 mg sludge (dw) per milliliter of digester sludge. This calculates to 57.2 mg sludge (dw) added per culture.

For half-life estimation (days): divide the dose at time zero (pmole) by the determined rate pmole/day, then divide that value by 2.