

**FINAL REPORT**

# **In Situ Remediation of Aqueous Film Forming Foams and Common Co-Contaminants with the Dual Approach of Chemical Oxidation and Bioremediation**

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| <b>14. ABSTRACT</b><br><p>The physical and chemical properties of per- and polyfluoroalkyl substances (PFAS) make them difficult to remove from groundwater using traditional treatment technologies. Designing treatment systems for AFFF-impacted sites is further complicated by the frequent presence of co-contaminants and the fact that PFAS composition can vary significantly according to AFFF manufacturer and production year. However, in situ chemical oxidation (ISCO) and bioremediation can provide effective, relatively inexpensive and less energy intensive alternatives, especially when used in combination. The objective of this study was to develop knowledge leading to an in situ groundwater remediation strategy for PFAS and common co-contaminants. This strategy aimed to integrate chemical oxidation for PFAS removal with bioremediation for organic mass degradation. The hypothesis of this study was that in situ remediation of AFFF with co-contaminants can be achieved by sequentially applying ISCO based on persulfate chemistry in combination with bioremediation strategies. We hypothesized that the initial treatment of PFAS and co-contaminants with heat activated persulfate ISCO could remove chemical inhibitors of aquifer microorganisms and that subsequent downstream microbial activity for in situ mixed contaminants degradation will be stimulated. In addition, bioremediation of organics in AFFF and potential co-contaminants prior to ISCO can decrease the amount of oxidants needed for contaminant removal. Overall, elucidating the significance of the possible effects of ISCO on aquifer bacteria as well as the effects of bioremediation on subsequent ISCO could substantially improve the performance of remedial scenarios that combine ISCO with bioremediation. We evaluated the transformation of source-zone AFFF and co-contaminants during persulfate-based ISCO in the presence of aquifer solids, determined the effects of ISCO treatment on co-contaminant degrading bacteria, and evaluated the feasibility of employing bioremediation prior to persulfate based ISCO treatment to increase its effectiveness and decrease reagent requirements. Briefly, we found that repeated doses of 50-300 mM persulfate transformed over 95% of PFAS mass in 1000 g/L contaminated aquifer solids/groundwater matrices. It was also identified that high persulfate doses could inhibit co-contaminating groundwater bacterial communities. Our studies also indicated that low concentrations of PFAS precursors could be biotransformed to produce perfluoroalkyl acids with different product yields with different microbial communities and higher concentrations of individual PFAS could inhibit the degradation of common co-contaminants. Finally, we also established that high salt concentrations can impact the bulk solution behavior of perfluorooctane sulfonate (PFOS), specifically by increasing aggregation and interfacial activity. We believe that these results will be used to effectively manage AFFF-impacted sites, predict possible biotransformation pathways, and guide the utilization of heat-activated persulfate oxidation for in situ PFAS destruction.</p> |                               |                                             |                                                  |                                       |                                                                    |  |
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## List of Acronyms

|                         |                                                              |
|-------------------------|--------------------------------------------------------------|
| AFB                     | Air Force Base                                               |
| AFFF                    | Aqueous film forming foam                                    |
| AmPr-FHxSA              | n-dimethylammoniopropyl perfluorohexane sulfonamide          |
| AMS                     | Ammonium mineral salts                                       |
| ATP                     | Adenosine triphosphate                                       |
| BTEX                    | Benzene, toluene, ethylbenzene, and xylene                   |
| CB1190                  | <i>Pseudonocardia dioxanivorans</i> strain CB1190            |
| CMC                     | Critical micelle concentration                               |
| cis-DCE                 | cis-Dichloroethene                                           |
| DGBE                    | Diethylene glycol butyl ether                                |
| Dhc195                  | Dehalococcoides mccartyi strain 195                          |
| Dioxane                 | 1,4-Dioxane                                                  |
| DOC                     | Dissolved organic carbon                                     |
| Dvh                     | Desulfovibrio vulgaris Hildenborough                         |
| ECF                     | Electrochemical fluorination                                 |
| FHxSA                   | Perfluorohexane sulfonamide                                  |
| FHxSA-PrA               | Perfluorohexanesulfonamido propanoic acid                    |
| FHxSAA                  | Perfluorohexane sulfonamido acetic acid                      |
| PFHxSi                  | Perfluorohexane sulfinic acid                                |
| FC                      | Fold changes                                                 |
| FOSA                    | Perfluorooctane sulfonamide                                  |
| FTCA                    | Fluorotelomer carboxylic acids                               |
| FtOH                    | Fluorotelomer alcohols                                       |
| FtS                     | Fluorotelomer sulfonate                                      |
| FtTAoS                  | Lodyne                                                       |
| FtUCA                   | Fluorotelomer unsaturated carboxylic acids                   |
| GC-FID                  | Gas chromatography flame ionization detector                 |
| GC-MS                   | Gas chromatography mass spectrometry                         |
| GC-TD                   | Gas chromatograph coupled to a thermal conductivity detector |
| HPLC                    | High performance liquid chromatography                       |
| HAPO                    | Heat-activated persulfate oxidation                          |
| $\text{HSO}_4^{\cdot-}$ | Protonated sulfate radical                                   |
| IC                      | Ion chromatography                                           |
| ICP-MS                  | Inductively coupled plasma mass spectrometry                 |
| ISCO                    | In situ chemical oxidation                                   |
| LC                      | Liquid chromatography                                        |
| MS                      | Mass spectrometry                                            |
| NAS                     | Naval Air Station                                            |
| NOM                     | Natural organic matter                                       |
| OAmPr                   | N-oxidedimethylammoniopropyl                                 |
| PFAA                    | Perfluoroalkyl acids                                         |
| PFAS                    | Per- and polyfluoroalkyl substances                          |
| PFCA                    | Perfluoroalkyl carboxylic acid                               |
| PFHxA                   | Perfluorohexanoate                                           |

|       |                                                                                     |
|-------|-------------------------------------------------------------------------------------|
| PFHxS | Perfluorohexane sulfonate                                                           |
| PFSA  | Perfluoroalkyl sulfonic acid                                                        |
| PFOA  | Perfluorooctanoate                                                                  |
| PFOS  | Perfluorooctane sulfonate                                                           |
| SERDP | U.S. Department of Defense's Strategic Environmental Research & Development Program |
| SPME  | Solid phase microextraction                                                         |
| TCE   | Trichloroethene                                                                     |
| TOC   | Total organic carbon                                                                |
| TOP   | Total oxidizable precursor                                                          |
| VC    | Vinyl chloride                                                                      |

### **Keywords**

PFAS, AFFF, ISCO, persulfate, sulfate radical, TOP assay, bioremediation, biotransformation, precursors, TCE, dioxane, BTEX, microorganisms

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## Abstract

The physical and chemical properties of per- and polyfluoroalkyl substances (PFAS) make them difficult to remove from groundwater using traditional treatment technologies. Designing treatment systems for AFFF-impacted sites is further complicated by the frequent presence of co-contaminants and the fact that PFAS composition can vary significantly according to AFFF manufacturer and production year. However, in situ chemical oxidation (ISCO) and bioremediation can provide effective, relatively inexpensive and less energy intensive alternatives, especially when used in combination. The objective of this study was to develop knowledge leading to an in situ groundwater remediation strategy for PFAS and common co-contaminants. This strategy aimed to integrate chemical oxidation for PFAS removal with bioremediation for organic mass degradation. The hypothesis of this study was that in situ remediation of AFFF with co-contaminants can be achieved by sequentially applying ISCO based on persulfate chemistry in combination with bioremediation strategies. We hypothesized that the initial treatment of PFAS and co-contaminants with heat activated persulfate ISCO could remove chemical inhibitors of aquifer microorganisms and that subsequent downstream microbial activity for in situ mixed contaminants degradation will be stimulated. In addition, bioremediation of organics in AFFF and potential co-contaminants prior to ISCO can decrease the amount of oxidants needed for contaminant removal. Overall, elucidating the significance of the possible effects of ISCO on aquifer bacteria as well as the effects of bioremediation on subsequent ISCO could substantially improve the performance of remedial scenarios that combine ISCO with bioremediation. We evaluated the transformation of source-zone AFFF and co-contaminants during persulfate-based ISCO in the presence of aquifer solids, determined the effects of ISCO treatment on co-contaminant degrading bacteria, and evaluated the feasibility of employing bioremediation prior to persulfate based ISCO treatment to increase its effectiveness and decrease reagent requirements. Briefly, we found that repeated doses of 50-300 mM persulfate transformed over 95% of PFAS mass in 1000 g/L contaminated aquifer solids/groundwater matrices. It was also identified that high persulfate doses could inhibit co-contaminating groundwater bacterial communities. Our studies also indicated that low concentrations of PFAS precursors could be biotransformed to produce perfluoroalkyl acids with different product yields with different microbial communities and higher concentrations of individual PFAS could inhibit the degradation of common co-contaminants. Finally, we also established that high salt concentrations can impact the bulk solution behavior of perfluorooctane sulfonate (PFOS), specifically by increasing aggregation and interfacial activity. We believe that these results will be used to effectively manage AFFF-impacted sites, predict possible biotransformation pathways, and guide the utilization of heat-activated persulfate oxidation for in situ PFAS destruction.

## Executive Summary

### Introduction

Per- and polyfluoroalkyl substances (PFAS) are an active ingredient in aqueous film forming foams (AFFFs), a type of firefighting foam used for decades on military bases and at airports. Because of repeated contamination events, a diverse suite of PFAS have migrated into groundwater aquifers around the country, endangering drinking water for millions of people. Remediation of PFAS has become a priority in much of the world, as several of the most common PFAS are toxic and bioaccumulative. The physical and chemical properties of PFAS make them difficult to remove from groundwater using traditional treatment technologies. Designing treatment systems for AFFF-impacted sites is further complicated by the frequent presence of co-contaminants and the fact that PFAS composition can vary significantly according to AFFF manufacturer and production year. However, in situ chemical oxidation (ISCO) and bioremediation can provide effective, relatively inexpensive and less energy intensive alternatives, especially when used in combination. The objective of this study was to develop knowledge leading to an in situ groundwater remediation strategy for PFAS and common co-contaminants. This strategy aimed to integrate chemical oxidation for PFAS removal with bioremediation for organic mass degradation.

### Objectives

This project responded to SON number ERSON-17-01 to develop a greater understanding of synergistic treatment technologies for the remediation of PFAS and co-contaminants, with the effort to improve remediation strategies that minimize cost and maximize efficiency. The overall scope was to develop effective in situ remediation strategies for treating PFAS from AFFF, along with trichloroethene (TCE), and 1,4-dioxane (dioxane), by combining chemical oxidation with bioremediation. Our scope was focused on persulfate oxidation and its effects on biological remediation processes, the biodegradation of TCE and dioxane and PFAS impacts on these processes and integrating persulfate oxidation with biostimulation and/or bioaugmentation for complete removal of these co-contaminants. We began this project with the following hypotheses to develop and improve the performance of remedial strategies for in situ PFAS treatment:

1. In situ remediation of AFFFs with co-contaminants can be achieved by sequentially applying persulfate ISCO and bioremediation.
2. The initial treatment of PFAS and co-contaminants with persulfate ISCO will remove chemical inhibitors of aquifer microorganisms so that subsequent downstream microbial activity for in situ degradation of mixed contaminants can be stimulated.
3. Bioremediation of organics in AFFF and co-contaminants prior to ISCO will decrease the amount of oxidant needed for subsequent PFAS destruction.

By investigating two configurations—ISCO before bioremediation and ISCO after bioremediation—we aimed to determine the efficacy of ISCO on treating the three co-contaminants, AFFF, TCE, and dioxane, as well as determining the effect of ISCO on down-gradient microbial communities and down-plume bioremediation possibilities. Specifically, we outlined three objectives:

1. Evaluate low temperature persulfate heat-activation for the transformation of AFFF and co-contaminants in the presence of aquifer solids.
2. Determine the effects of AFFF ISCO products on co-contaminant-degrading bacteria, and evaluate the feasibility of down-gradient plume control via bioremediation.

3. Evaluate the feasibility of employing bioremediation of co-contaminants and labile organics in AFFF prior to ISCO treatment to increase effectiveness of PFAS destruction by activated persulfate and decrease reagent requirements.

We first set out to determine under what groundwater conditions PFAS could be destroyed by heat-activated persulfate oxidation (HAPO), especially at low temperatures. We also investigated HAPO efficiency in the presence of common aquifer constituents. Previous HAPO experiments in our lab (Bruton and Sedlak 2017, 2018) conducted in a pure water matrix or in a soil-water slurry (20 g/L aquifer solids) exhibited success in destroying precursor compounds, as well as (perfluorocarboxylic acids (PFCAs) at 85°C). In Objective 1 of this project, we lowered the activation temperature of the persulfate treatment to make chemical oxidation more energy efficient in the field and more compatible with downstream biological treatment. We also investigated persulfate dosing in batch experiments under various conditions occurring in groundwater aquifers, including higher aquifer solids concentrations, co-contaminants, and soil buffering capacity due to common soil minerals like calcite. During Objective 1 experiments, we discovered that the high salt concentrations from persulfate oxidation affected the behavior of perfluorooctane sulfonic acid (PFOS) in aqueous solutions due to the repeated doses of sodium persulfate (100 mM and higher). Given the significant implications of PFOS behavior on site investigation and management, we added an objective to our project:

4. Additional findings: Interpret and investigate salt effects on the solution behavior and measurement of PFOS.

### **Technical Approach**

The hypothesis of this study was that in situ remediation of AFFF with co-contaminants can be achieved by sequentially applying ISCO based on persulfate chemistry in combination with bioremediation strategies. We hypothesized that the initial treatment of PFAS and co-contaminants with heat activated persulfate ISCO could remove chemical inhibitors of aquifer microorganisms and that subsequent downstream microbial activity for in situ mixed contaminants degradation will be stimulated. In addition, bioremediation of organics in AFFF and potential co-contaminants prior to ISCO can decrease the amount of oxidants needed for contaminant removal. Overall, elucidating the significance of the possible effects of ISCO on aquifer bacteria as well as the effects of bioremediation on subsequent ISCO could substantially improve the performance of remedial scenarios that combine ISCO with bioremediation. We:

1. Evaluated the transformation of source-zone AFFF and co-contaminants during persulfate-based ISCO in the presence of aquifer solids,
2. Determined the effects of ISCO treatment on co-contaminant degrading bacteria, and
3. Evaluated the feasibility of employing bioremediation prior to persulfate based ISCO treatment to increase its effectiveness and decrease reagent requirements.

### **Results and Discussion**

#### *Objective 1: ISCO on AFFF and co-contaminants*

Our first goal was to establish if precursor and PFCA destruction was possible at lower persulfate activation temperatures. This approach could potentially lower remediation cost and enhance

potential for downstream bioremediation processes, thereby broadening applications of persulfate ISCO.

Our results indicated that >90% removal of PFCAs and precursors was achieved within about eight hours without aquifer solids present at 85°C (see Figure ES.1). The PFSA's were not oxidized by HAPSO (data not shown). At an activation temperature of 65°C, precursors and PFCAs were removed at an efficiency >95% in under 60 hours. With both conditions, the pH decreased below 3 rapidly due to the persulfate degradation (details in the report). Importantly, the 40°C test still resulted in >90% PFCA transformation, but over the course of about 10 days.

After establishing that HAPSO at 40°C was possible in the presence of aquifer solids we tested the PFCA inhibition in real PFAS-contaminated aquifer solids and groundwater from naval air station (NAS) Jacksonville, Florida through our collaborators on ESTCP ER-201729. We tested a range of persulfate concentrations, including 50, 100, 200, and 300 mM, added weekly as shown in Figure ES.2. Our results show over 97% of PFAS destruction under both low persulfate doses, though a longer reaction time was required. For all treatments, the DOC decreased by about 50% after 63 days, suggesting that some recalcitrant organics were resistant to persulfate oxidation and could have contributed to sulfate radical scavenging (more details in the report).

The success of low doses of persulfate during 40°C HAPSO is encouraging and likely has a milder impact to the aquifer environment. It would also result in lower chemical costs because less persulfate and neutralizing agent (e.g., carbonate or NaOH) after treatment would be required. However, the cost savings of many lower dose events would have to be compared with having fewer, high concentration oxidant delivery events. In ISCO field studies, it is common to have 1-2 oxidant delivery events with a median duration of 4-6 days each (Krembs 2008). HAPSO for PFAS destruction, on the other hand, may require 4+ delivery events depending on the chosen persulfate concentration.

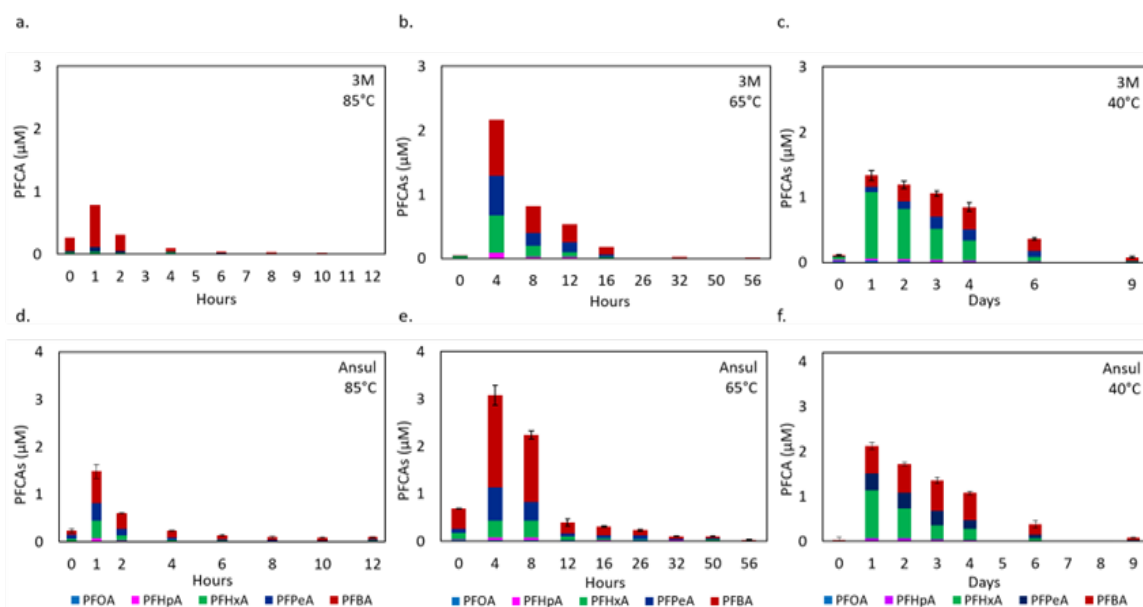


Figure ES. 1 3M and Ansul AFFF PFCA and precursor destruction by HAPSO at 85, 65, and 40°C. 3M tests shown in a, b, and c; Ansul tests shown in d, e, and f. Note the x-axis units; 85 and 65°C are shown in hours, while the 40°C HAPSO test took days. The initial increase in PFCAs between the first and second time points resulted from precursors transforming into PFCAs after persulfate addition.

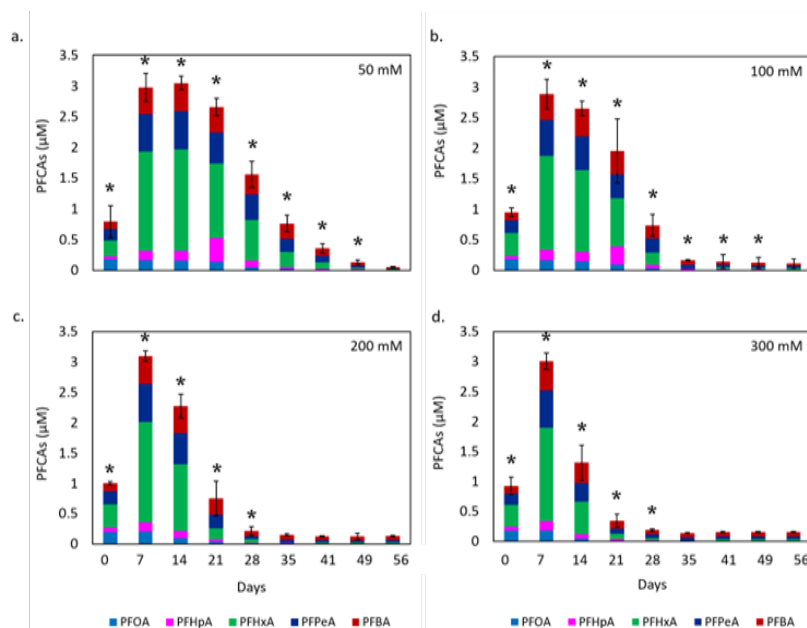


Figure ES. 2 HAPO at 40°C of contaminated groundwater and aquifer solids (1000 g/L), with a) 50 mM, b) 100 mM, c) 200 mM, and d) 300 mM sodium persulfate doses added weekly, shown by the asterisks. Error bars represent triplicate reactors.

### Objective 2: HAPO impacts on microorganisms

After HAPO treatment and subsequent pH neutralization, high levels of sulfate will remain in the aquifer, which will likely affect down-gradient microbial communities and bioremediation efforts. To examine this, we studied the effects of HAPO on enrichment cultures from AFFF-contaminated sites. We first exposed an enrichment culture, which had been enriched to degrade BTEX components, to increasing concentrations of sodium sulfate, as this would be a primary salt remaining after HAPO had been conducted. With all concentrations of sulfate (10, 50, 100, and 200 mM), BTEX was degraded similarly to the no-salt control (see Figure 29 in the main report).

Although the BTEX degradation was not impacted, we also extracted the DNA in each of the salt conditions to compare the phyla and genera in each of the triplicates (see Figures 30 and 31 in the main report). Proteobacteria relative abundance appeared to decrease as sulfate concentration increased. The relative abundance of Firmicutes remained relatively constant, between ~0.10-.25 with increasing salt concentration. Actinobacteria had an increase in relative abundance with increasing salt concentration (see Figure 30 in main report).

Additionally, *Rhodococcus*, known to degrade BTEX compounds, appeared to maintain a relative abundance of ~0.75 at sulfate concentrations 50, 100, and 200 mM, indicating that this genus can withstand relatively high concentrations of salts. This result indicates that *Rhodococcus* would be able to degrade BTEX compounds post-HAPO.

Similarly, we observed that most concentrations of the HAPO solution did not impact BTEX degradation (see Figures 29 and 30 in main report). Only the highest concentration, ~500 mM sulfate in HAPO solution, inhibited the degradation of benzene and toluene (Figure ES.3).

Interestingly, the ethylbenzene and ortho-xylene were degraded at the same rate across the salt conditions; the highest HAPO solution condition did not inhibit these two substrates' degradation.

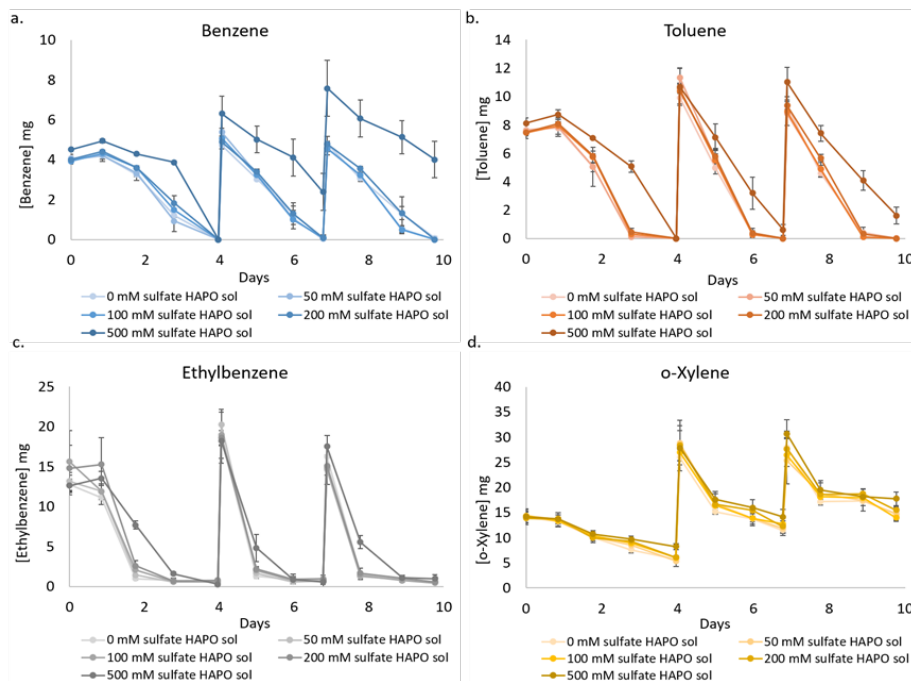


Figure ES. 3 BTEX degradation by an enrichment culture in the presence of increasing concentrations of HAPO solution (“HAPO sol”). The HAPO solution was a concentrated sodium persulfate solution that was heat-activated so that the persulfate was converted to sulfate. Additionally, the HAPO solution was neutralized before adding to the microcosms.

### Objective 3: Bioremediation of AFFF and co-contaminants before ISCO

The purpose of this task was to identify microcosms capable of consuming organics in 3M AFFFs. We compared the consumption of DOC in ECF-based AFFFs and fluorotelomer-based AFFFs. Generally in our tests, organics in fluorotelomer AFFFs degraded much more readily than in 3M AFFF (Figure 35). In one such test, ~250 mg/L of DOC in National Foam AFFF was consumed within 20 days compared to ~50 mg/L in 3M AFFF over 40 days (see Figure 35 in the main report). We hypothesized that the high concentrations of PFOS in 3M AFFFs were responsible for microbial inhibition of DOC degradation, and thus we found few conditions that resulted in rapid or effective DOC degradation for 3M AFFFs. During these tests, we started investigating the possible biotransformation of the prevalent 3M precursor AmPr-FHxSA.

We first were investigating the biotransformation of AmPr-FHxSA present in the 3M AFFF. In these AFFF experiments, consumption of organic carbon was slower in the enrichments containing the ECF-based formulation than those containing the fluorotelomer-based formulation (see Figure ES.4) despite adequate oxygen in both systems, similarly to the 3M AFFF tested in Figure ES.4. This suggests that some component(s) in the ECF-based AFFF can inhibit the microbial community, such as PFOS, which has been shown to be toxic to microorganisms (Fitzgerald et al

2019, Sheng et al 2021, Weathers et al 2016) and was present at concentrations three times that of AmPr-FHxSA. After 85 days, live and autoclaved controls showed a similar AmPr-FHxSA concentration decrease. Though not statistically significant compared to controls when averaged, two of the three replicates showed twice the concentration of FHxSA compared to the autoclaved controls (Figure ES.4). However, the multiple possible precursors within the formulation confounded the analysis. While we performed HRMS on multiple timepoints throughout the experiment, many of the results were not statistically significant compared to the initial timepoint (more details in Figure 37 of main report), making it challenging to draw conclusions on the fate of AmPr-FHxSA. There was no indication of biological activity in the autoclaved controls based on ATP (data not shown), which suggests any decreases of AmPr-FHxSA in the autoclaved controls were because of abiotic processes.

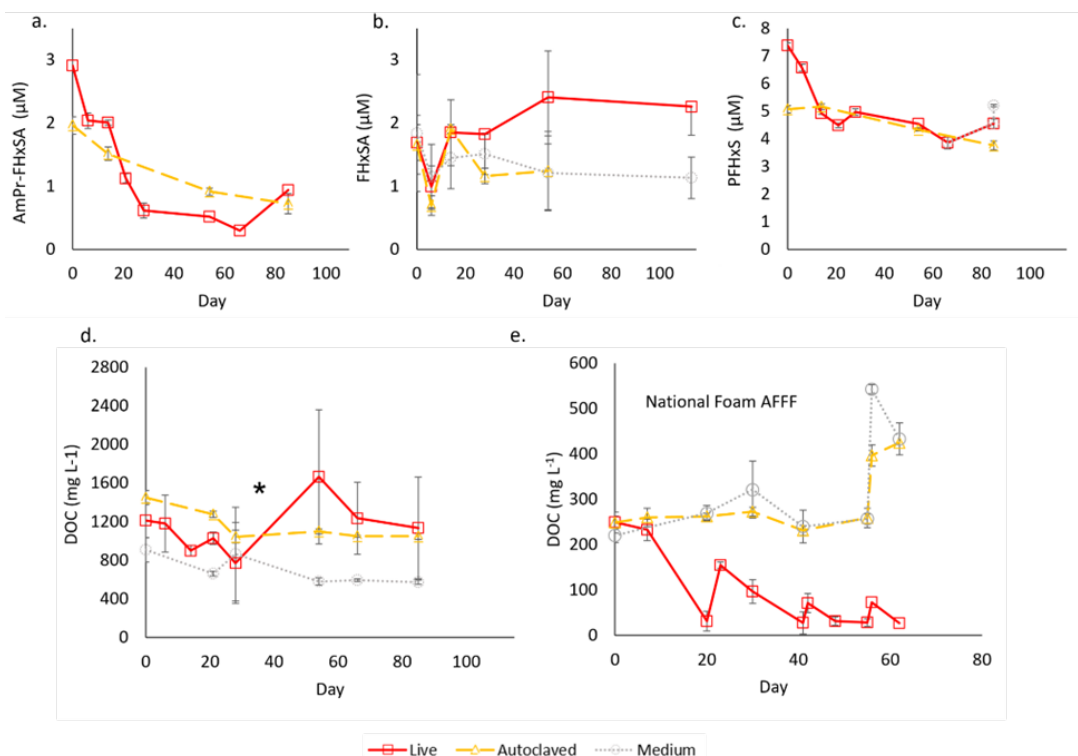


Figure ES. 4 ECF-based AFFF biotransformation study amended with DGBE. Concentrations of a) AmPr-FHxSA, b) FHxSA, c) PFHxS, and d) DOC for the ECF-based AFFF biotransformation test. Compared to the e) DOC degradation during a fluorotelomer-based AFFF biotransformation test, there was little to no DOC degradation in the ECF AFFF test, indicating recalcitrant non-fluorinated surfactants. Asterisk denotes respike with DGBE as additional carbon. 1:1,000 dilution AFFF.

To isolate possible aerobic biotransformation pathways of AmPr-FHxSA in the absence of PFOS or other inhibitors in AFFF, we performed biotransformation experiments by adding only AmPr-FHxSA with either methane, acetate, DGBE, or BTEX as a carbon source. We hypothesized that enrichments developed from these four conditions would result in functionally divergent microbial communities with different transformation products and yields. After 70 days of incubation, the concentration of AmPr-FHxSA decreased under all conditions but had the most significant

disappearance (83% decrease) in the acetate-oxidizing cultures, followed by the methane-oxidizing cultures (Figure ES.5). The live methane cultures resulted in the highest concentrations of generated targeted transformation products (Figure ES.5)—about 70 nM and 9 nM of FHxSA and PFHxS, respectively, or approximately 40% and 6.7% of the loss of the parent.

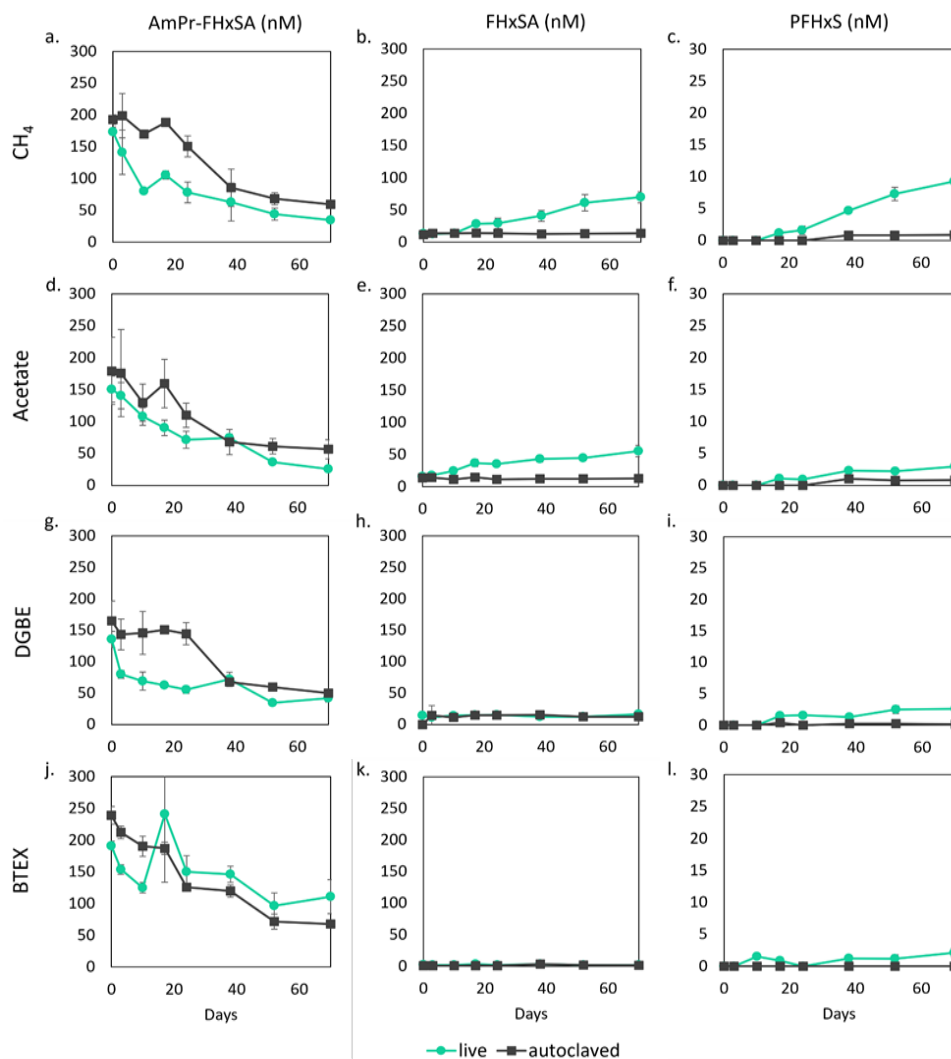


Figure ES. 5 From left to right, AmPr-FHxSA depletion and FHxSA and PFHxS production (nM) over 70 days under aerobic conditions with four carbon sources: a-c) methane (CH<sub>4</sub>), d-f) acetate, g-i) DGBE, and j-l) BTEX. Error bars represent standard deviation of triplicate reactors. Green circles represent the live cultures, while gray squares represent autoclaved controls.

Similar to the AFFF biotransformation experiment, AmPr-FHxSA decreased in the medium-only but without detectable FHxSA or PFHxS. Some AmPr-FHxSA loss could be explained by trace amounts of soil or biofilms not captured in sampling, as the autoclaved controls had less AmPr-FHxSA loss compared to the medium-only controls. However, because of the medium-only controls, we believe that the majority of the AmPr-FHxSA decrease is primarily due to sorption to the container. Low recovery could also have occurred from sorption to the needle syringe used for sampling or from aggregation or accumulation at interfaces, including air-water (Steffens et al

2021). Studies have shown that AmPr-FHxSA has a propensity to sorb to surfaces (Nickerson et al 2020), can be unstable from two oxidizable nitrogens and a lone pair of electrons (Liu et al 2021), and perhaps has temperature-dependent instability like other sulfonamides (Woudneh et al 2019). Despite the decrease in AmPr-FHxSA, autoclaved and medium-only controls did not form the stable intermediate FHxSA or terminal product PFHxS (Figure ES.5 and Appendix A). Because of the challenges with AmPr-FHxSA, we prioritized analyzing the differences in transformation products between the biotic cultures and abiotic controls.

In the methane, acetate, and DGBE cultures, approximately 12-15 nM FHxSA was present at the start of the experiment due to the AFFF-impacted inoculum source; this background was not in the BTEX culture, presumably because its inoculum source was older and had been amended with fresh media several times, diluting FHxSA below the limit of detection. Although the DGBE and BTEX cultures did not produce FHxSA, low concentrations (2.6 and 2.1 nM, respectively) of PFHxS were produced. This lack of FHxSA suggests that AmPr-FHxSA might be able to transform to PFHxS without accumulation of FHxSA, or that other intermediates not targeted in the LC-MS/MS method can be hydrolyzed directly to PFHxS. Given the stability of FHxSA in the environment (D'Agostino et al 2017), it is unlikely that this compound was formed as an intermediate that was quickly transformed to PFHxS.

Next, we measured fold changes in metabolite abundances to study mechanistic impacts of AmPr-FHxSA, its transformation products, AFFF, and the fluorine-free surfactant SDS on biological TCE and BTEX degradation pathways (Figures ES.6). In the TCE co-culture, two amino acids, arginine and alanine had the largest increase in FC with respect to the control (more details in Figure 56 of main report). Higher abundance of alanine occurred in all PFAS and also the fluorine-free SDS, implying this amino acid was not a PFAS-specific biomarker.

Conversely FCs for almost all amino acids in the BTEX enrichment were negative (see Figure 56). The amino acids with the most negative FC were L-citrulline and arginine during AmPr-FHxSA and FHxSA exposure. In addition to amino acids, the BTEX enrichment had increases of unsaturated fatty acids (stearic and palmitic acids). Finally, we identified transformation products of BTEX biodegradation, o-toluic (oxidation product of o-xylene), malonic and methylmalonic acids (formed from ring cleavage of BTEX compounds). The positive FCs in all of these transformation products signify incomplete biodegradation, but are difficult to interpret further.

We also evaluated increasing dilutions of AFFF in both biological systems (details in Figure 56). An AFFF concentration dependent decrease of Log 2 fold change of all metabolites can be seen in both BTEX and TCE systems, suggesting dose-response changes in the metabolite abundances.

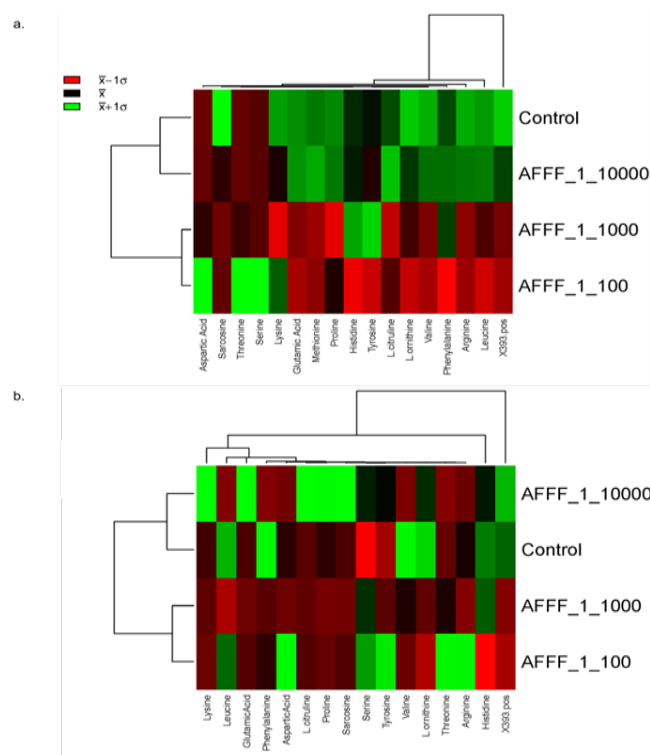


Figure ES. 6 . Heatmaps of Log 2 fold changes in metabolite abundance measured in the a) anaerobic TCE co-culture and b) aerobic BTEX enrichment culture with the three dilutions of AFFF. A concentration dependence is seen in the Euclidean distance with respect to the AFFF-free control (AFFF is 1:10000 most diluted, AFFF 1:100 is most concentrated).

#### Objective 4: Additional Findings

While effects of high ionic strength, specifically high sodium concentrations, are relevant to ISCO treated water matrices, we were interested in evaluating the effects in a high salinity, natural water matrix. To this end, we measured a range of detected PFOS concentrations in two high salinity matrices: a 200 mM Na<sub>2</sub>SO<sub>4</sub> solution and seawater collected from the San Francisco Bay. We observed a significant loss of PFOS in both matrices at PFOS concentrations above 5 mg/L, the loss ranging from 2-38 mg/L, that was dependent on the initial concentration of PFOS in Milli-Q water (see Figure 62 in main report). At PFOS concentrations < 5 mg/L, the concentration decrease was in the range of ~0.2-0.6 mg/L, likely primarily due to container-water interfacial sorption. The concentration loss in seawater was generally higher than in the Na<sub>2</sub>SO<sub>4</sub> solutions, which may be attributed to the presence of Ca<sup>2+</sup> leading to enhanced surface activity and aggregation of PFOS. These data suggest that high concentrations of monovalent salts may be of higher concern for accurate quantification of PFOS in sampling saline waters, while high concentrations of divalent salts may have a greater effect on PFOS interfacial behavior and sorption to soil in the vadose zone.

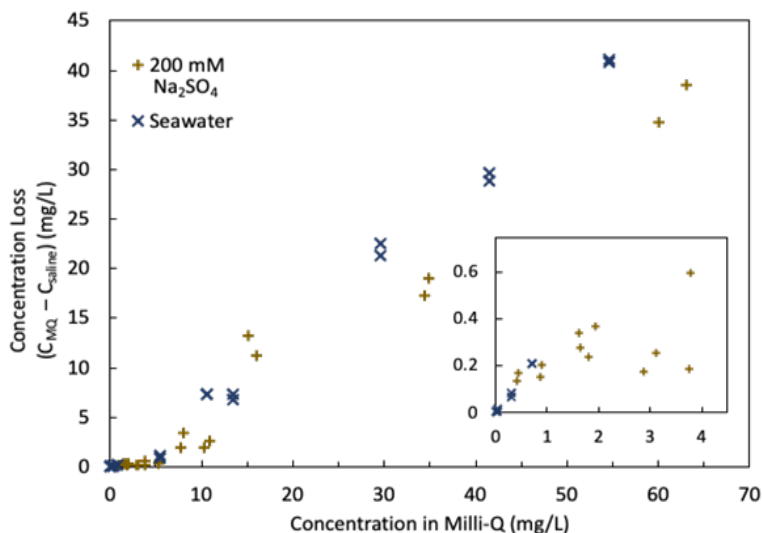


Figure ES. 7 PFOS concentration loss in saline bulk solution compared to PFOS concentration detected in Milli-Q. For the Na<sub>2</sub>SO<sub>4</sub> data, samples were taken from the same solution bottle before (CMQ) and after (C<sub>saline</sub>) salt addition. For the seawater data, PFOS was added to seawater (C<sub>saline</sub>) and Milli-Q water solutions (CMQ) in parallel and compared. Results from duplicate conditions are shown.

### Implications for Future Research and Benefits

Low temperature (40°C) HAPO can be an effective PFAS remediation strategy at AFFF source zones, though with some key limitations. Repeated injections of persulfate are likely needed for best transformation (50-300 mM, over several weeks) depending on the aquifer conditions. We also found that different microbial communities impacted by available carbon sources will affect AmPr-FHxSA biotransformation yields and product composition. Specifically, with methane and acetate as carbon sources, the resulting microbial communities had higher yields of FHxSA and PFHxS compared to DGBE and BTEX, and additionally contained a greater diversity of intermediate products.

At sites with multiple contaminants, high concentrations of AFFF may inhibit anaerobic chlorinated solvent bioremediation, while AFFF and potentially FHxSA and AmPr-FHxSA can negatively impact aerobic BTEX degradation. In source zones where AFFF concentrations are the highest, transformation of co-contaminants by microbial communities may be inhibited, while zones with lower AFFF concentrations or individual PFAS species will be less likely to interfere with bioremediation processes.

For the additional findings on salt effects on PFOS behavior in aqueous solution, our results indicate that PFASs were not transformed, destroyed, or removed during HAPO, though high salt concentrations can affect PFSA behavior in aqueous solutions. The observed effects of salt addition suggest that salt causes PFOS to 'salt out' or form aggregates in solution, causing a decrease in detected PFOS concentrations. Further investigation is needed to determine how salts affect PFAS under the complex conditions encountered at contaminated sites.

## Objectives

This project responded to SON number ERSON-17-01 to develop a greater understanding of synergistic treatment technologies for the remediation of PFAS and co-contaminants, with the effort to improve remediation strategies that minimize cost and maximize efficiency. The overall scope was to develop effective in situ remediation strategies for treating PFAS from AFFF, along with TCE, and dioxane, by combining chemical oxidation with bioremediation. Our scope was focused on persulfate oxidation and its effects on biological remediation processes, the biodegradation of TCE and dioxane and PFAS impacts on these processes, and integrating persulfate oxidation with biostimulation and/or bioaugmentation for complete removal of these co-contaminants.

Few PFAS destruction technologies exist, and fewer still can be applied as an in situ remediation strategy. ISCO has been used in practice for decades to treat a variety of organic pollutants with oxidants such as persulfate, hydrogen peroxide, and permanganate. PFAS, however, are some of the most stable and persistent organic pollutants. The only known oxidant that can destroy most PFAS is the sulfate radical produced from activated persulfate; however, PFASs cannot be destroyed by this method. We began this project with the following **hypotheses** to develop and improve the performance of remedial strategies for in situ PFAS treatment:

1. In situ remediation of AFFFs with co-contaminants can be achieved by sequentially applying persulfate ISCO and bioremediation.
2. The initial treatment of PFAS and co-contaminants with persulfate ISCO will remove chemical inhibitors of aquifer microorganisms so that subsequent downstream microbial activity for in situ degradation of mixed contaminants can be stimulated.
3. Bioremediation of organics in AFFF and co-contaminants prior to ISCO will decrease the amount of oxidant needed for subsequent PFAS destruction.

By investigating two configurations—ISCO before bioremediation and ISCO after bioremediation—we aimed to determine the efficacy of ISCO on treating the three co-contaminants, AFFF, TCE, and dioxane, as well as determining the effect of ISCO on down-gradient microbial communities and down-plume bioremediation possibilities. Specifically, we outlined three **objectives**:

1. Evaluate low temperature persulfate heat-activation for the transformation of AFFF and co-contaminants in the presence of aquifer solids.
2. Determine the effects of AFFF ISCO products on co-contaminant-degrading bacteria, and evaluate the feasibility of down-gradient plume control via bioremediation.
3. Evaluate the feasibility of employing bioremediation of co-contaminants and labile organics in AFFF prior to ISCO treatment to increase effectiveness of PFAS destruction by activated persulfate and decrease reagent requirements.

We first set out to determine under what groundwater conditions PFAS could be destroyed by HAPO, especially at low temperatures. We also investigated HAPO efficiency in the presence of common aquifer constituents. Previous HAPO experiments in our lab (Bruton and Sedlak 2017, 2018) conducted in a pure water matrix or in a soil-water slurry (20 g/L aquifer solids) exhibited success in destroying precursor compounds, as well as (PFCAs at 85°C. In Objective 1 of this

project, we lowered the activation temperature of the persulfate treatment to make chemical oxidation more energy efficient in the field and more compatible with downstream biological treatment. We also investigated persulfate dosing in batch experiments under various conditions occurring in groundwater aquifers, including higher aquifer solids concentrations, co-contaminants, and soil buffering capacity due to common soil minerals like calcite. During Objective 1 experiments, we discovered that the high salt concentrations from persulfate oxidation affected the behavior of PFOS in aqueous solutions due to the repeated doses of sodium persulfate (100 mM and higher). Given the significant implications of PFOS behavior on site investigation and management, we added an objective to our project:

**4. Additional findings: Interpret and investigate salt effects on the solution behavior and measurement of PFOS.**

This project combines the expertise of bioremediation and microbial processes from Dr. Lisa Alvarez-Cohen with Dr. David Sedlak's expertise in chemical oxidation and advanced oxidation processes for treatment of organic pollutants in contaminated waters.

## Background

PFAS are key components in AFFFs, complex chemical mixtures typically containing fluorinated and hydrocarbon surfactants and one or more glycol ether-based solvents, that have been widely used since the 1960s by the military and municipalities to extinguish hydrocarbon-fuel fires and to prevent re-ignition (Moody, 2002; Tuve, 1966). PFAS compounds, such as PFOA and PFOS, have been designated emerging contaminants due to concerns over environmental and human health effects (EPA, 2012). More specifically, PFAS are environmentally persistent, exhibit toxicity in primates and microbiota, and can bioaccumulate (Moody, 2002; Higgins, 2007; Blaine, 2014; Seacat, 2002). For these reasons, the use of PFOA, PFOS, and other “C8” perfluorinated chemicals has been discontinued. Repeated AFFF application at training facilities where fire-fighting exercises were conducted in unlined pits have led to elevated levels of PFAS in groundwater at sites that are often contaminated with chlorinated solvents, such as TCE and its toxic daughter products, 1,4-dioxane, a common stabilizer of chlorinated solvents, and fuel components comprising BTEX (McGuire, 2014; Guelfo, 2013; McKenzie, 2015). Although a number of studies have been conducted to develop remediation strategies for PFAS present in AFFF, few have been successful in identifying effective in situ processes that can remediate these compounds, and few have evaluated the effects of AFFF remediation strategies on the common co-contaminants.

The physical and chemical properties of PFAS make them difficult to remove from groundwater using traditional treatment technologies (Place, 2012). Designing treatment systems for AFFF-impacted sites is further complicated by the frequent presence of co-contaminants and the fact that PFAS composition can vary significantly according to AFFF manufacturer and production year. However, ISCO and bioremediation can provide effective, relatively inexpensive and less energy-intensive alternatives, especially when used in combination (Sutton, 2011).

### Chemical Oxidation of PFAS

ISCO is a common groundwater remediation strategy that involves injecting an aqueous oxidant into an aquifer for contaminant transformation (Siegrist, 2011). There are many types of oxidants (e.g., peroxide, permanganate, ozone, persulfate) that can transform a wide range of organic contaminants (e.g., chlorinated solvents, polycyclic aromatic hydrocarbons, pesticides, fuel hydrocarbons). Polyfluoroalkyl substances can be transformed into PFAAs by chemical or biological oxidation. Using hydroxyl radicals via the TOP assay, many precursors can be transformed to PFCAs (Houtz and Sedlak, 2012), but the hydroxyl radical cannot transform the PFAAs further. Sulfate radicals created during HAPO, in contrast, can mineralize PFCAs at an acidic pH, though are ineffective at transforming PFSAs (Bruton and Sedlak, 2017).

The TOP assay was developed as a way to quantify the mass of unknown precursor PFAS in a given aqueous matrix (Houtz and Sedlak, 2012). Precursor PFAS are so diverse in structure—and continue to be discovered—that many of the compounds cannot be quantified by current mass spectrometry methods. PFAAs, however, have commercially available mass labeled standards that ensure accurate quantification in many matrices. Therefore, the TOP assay works by converting precursor PFAS into PFAAs that can be measured by liquid chromatography mass spectrometry (LC-MS). By measuring the PFAAs before and after the TOP assay reaction, one can estimate the mass of unmeasurable precursor PFAS in a given sample.

The TOP assay is useful and accessible for estimating unknown PFAS mass. The reaction works by adding sodium hydroxide and persulfate (at a ratio of ~2.5 to 1) to an aqueous or slurry sample and letting the mixture react for 6-8 hours at 85°C. The heat activates the persulfate, creating sulfate radicals that instantaneously react with the HO<sup>-</sup>, creating hydroxyl radicals (Houtz and Sedlak, 2012). It has been shown that ECF-based precursors exposed to the TOP assay transform into the PFCA of equivalent carbon number (e.g., an eight carbon FOSA transforms to the eight carbon PFOA), while fluorotelomers transform into a mixture of shorter chain PFCAs (e.g., 8:2 FtS transforms to PFOA, PFHpA, PFPeA, PFBA and a small percentage to PFNA) (Houtz and Sedlak, 2012).

The TOP assay is particularly useful as a measurement tool, but some researchers have also experimented with the hydroxyl radical for in situ remediation projects. Precursors, with charged non-fluorinated head groups, are often more sorbtive to soil matrices than PFAAs. Therefore, some have proposed using hydroxyl radicals in situ to transform a mixed PFAS plume into PFAAs to be more easily pumped out of the aquifer for further ex situ treatment processes (Crimi, SERDP; Shojaei, 2021).

Previous research in our lab established the efficacy of HAPO to transform PFCAs at high temperatures (85°C) and an acidic pH (<3) (Bruton and Sedlak 2017 & 2018). Persulfate can be activated by several mechanisms (e.g., heat, high pH, transition metals) to create one or two sulfate radicals (SO<sub>4</sub><sup>•-</sup>), which are strong, non-selective oxidants that can transform organic pollutants. Specifically, Bruton & Sedlak showed that HAPO can completely transform PFOA and some precursor compounds into shorter-chain PFCA products, ultimately leading to mineralization into F<sup>-</sup> and CO<sub>2</sub> (Bruton and Sedlak 2017 & 2018). Precursor PFAS are first transformed into PFCAs, then those produced PFCAs undergo further transformation (Figure 1).

A mechanism of PFCA transformation by sulfate radical proposed by Kutsuna and Hori (2007) and summarized by Vecitis (2009) describes a stepwise “unzipping” of PFOA to create shorter chain PFCAs (Figure 1), which begins with a direct electron transfer from the carboxylate headgroup to the sulfate radical. Interestingly, it has been shown that PFCA destruction by sulfate radical can only occur at a pH <3. To understand this pH dependence, a group modeled different scenarios of sulfate radical reacting with PFCAs, and determined that deprotonated PFOA reacting with an HSO<sub>4</sub><sup>•-</sup> was the most favorable reaction, which would happen at pH values less than about 3 (Zhang, 2019). A more recent study disagreed with the protonated sulfate radical theory, showing that the radical does not protonate when accounting for water molecules in their calculations (Carre, 2021). Rather, they suggested that the pH dependence of PFCA transformation was determined by the acid catalysis of heat-activated persulfate, coupled with the decrease in sulfate radical scavengers at these acidic pH values (Carre, 2021). Regardless of the mechanism, as persulfate degrades to sulfate radical and eventually to sulfate, two moles of protons are produced per mole of persulfate, naturally driving down the solution pH to the required acidity for PFCA mineralization (Equation 1).



This pH requirement, however, could prove to be a challenging requirement for successful transformation of PFCAs. This study evaluated HAPO of PFAS at low temperatures (40°C) in the presence of co-contaminants and aquifer solids as a way to determine the ability of this treatment to work as an in situ treatment strategy that could help address the military's need to achieve site closure by means of timely and cost-effective remediation.

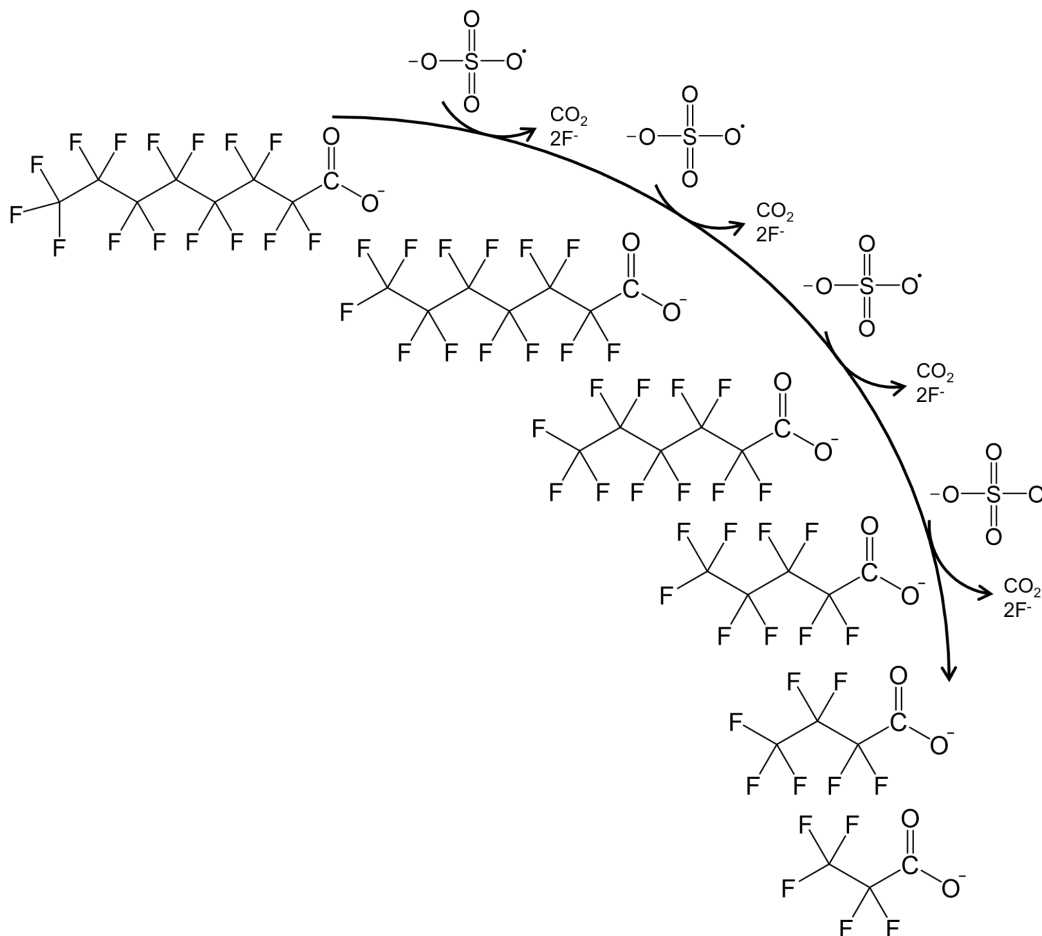


Figure 1. Representation of the stepwise unzipping of PFCAs by the sulfate radical.

## **Biotransformation of PFAS**

At the time of writing the proposal for this research, there were few biotransformation studies for PFAS precursors, and they mostly investigated fluorotelomers. For example, FTOHs and FtTAoS were investigated for pathways and products; while they can ultimately transform to PFCAs, both 8:2 and 6:2 FTOH form intermediate products including the fluorotelomer with a carboxylic acid or ketone headgroup replacing the alcohol, which can then transform to PFCAs of equal or fewer carbon number (Liu et al., 2013). Incubation times range from 1-7 months and PFCA yield ranges from 1-40% (Liu et al., 2013).

8:2, 6:2, and 4:2 FtTAoS were investigated in our lab for aerobic biotransformation using an enrichment culture seeded by soil from an AFFF-contaminated air force base (Harding-Marjanovic et al., 2015). Each of these three compounds disappeared from the cultures within 20 days of incubation, producing transformation products such as FtS, FTCA, FtUCA, and PFCAs.

Only in the past few years has the study of ECF-based precursor biotransformation increased and become more prominent in literature. As a result, part of our Objective 3 includes our own study on biotransformation of ECF-based precursor AmPr-FHxSA, a common precursor found in 3M AFFFs.

## Materials and Methods

PFAS analytical standards (C4–C10 PFCAs; 4:2, 6:2, and 8:2 FtS; 5:3, 7:3, 6:2, 8:2 FtCA; 6:2, 8:2 FtUCA, AmPr-FHxSA, and FHxSA) and mass labeled standards were purchased from Wellington Laboratories (Guelph, ON, Canada). AmPr-FHxSA used for biotransformation experiments was purchased from Chemieliva Pharmaceutical Co., Ltd (China, 97%) and FHxSA from Synquest Laboratories Inc. (USA, 95%). DGBE was purchased from TCI chemicals (>99%). BTEX compounds (>98%), methane (99%), and sodium acetate, were purchased from Sigma Aldrich. All other chemicals used in media or analyses were purchased from Sigma Aldrich or Fisher Scientific at the highest purity available.

Generally, for PFAS analyses, samples stored in methanol were vortexed, centrifuged (10 minutes at 15,000g), and transferred into LC-MS/MS vials with mass-labeled internal standard mixture (2 µg/L [<sup>13</sup>C]-PFAS). Samples were analyzed with an Agilent 1260 Infinity Binary HPLC System coupled to an Agilent 6460 triple quadrupole mass spectrometer. Chromatographic separation was achieved using a Zinc-Diol (12.5×4.6 mm, 6 µm) and Zorbax C18 XDB guard column (12.5×4.6 mm, 5 µm) before a Zorbax C18 XDB analytical column (50×4.6 mm, 1.8 µm). The LC-MS/MS configuration was modified to decrease PFAS contamination from the system. The mobile phase was a water/methanol gradient, each with 10 mM ammonium acetate at 0.4 mL/min. Samples were prepared such that PFAS concentrations fell above the lowest standard, 0.2 µg/L.

### Objective 1: ISCO on AFFF and co-contaminants

Previous experiments in our lab demonstrated the efficacy of HAPO of some precursor compounds and PFCAs at 85°C in the presence of low concentrations of aquifer solids (Bruton and Sedlak, 2017; 2018). Our first goal was to establish the efficacy of precursor and PFCA destruction at lower temperatures. Lowering the temperature could lower remediation cost and enhance potential for complementary bioremediation processes, thereby broadening applications of persulfate ISCO.

HAPO experiments were conducted with 50 mM sodium persulfate (Na<sub>2</sub>S<sub>2</sub>O<sub>8</sub>), in the presence or absence of aquifer solids (20 g/L) at 85, 65, and 40°C using either Ansulite (Ansul) or 3M AFFF formulations that were diluted 1:10,000 (1-3 µM PFAS in final concentrations). Experiments at 65 and 85°C were conducted in a temperature-controlled water bath, while 40°C experiments were in an incubator. Aquifer solids from two locations—Tinker AFB (Oklahoma, US) and Davis-Monthan AFB (Arizona, US)—were sent for chemical analysis to the University of California, Davis to determine total and organic carbon and total iron for use in the following experiments (Appendix A). All solids used were dried at 100°C, homogenized, and passed through a 600 µm sieve before use in experiments. Concentrated Ansul and 3M AFFFs were provided by Prof. Jennifer Field at Oregon State University from the AFFF archive in the Field laboratory. During time course experiments, routine PFAS analyses were performed with LC-MS/MS as well as the total oxidizable precursor (TOP) assay. Persulfate was measured using a colorimetric assay (Liang et al., 2008) and dissolved organic carbon (DOC) was measured using a total organic carbon (TOC) analyzer.

After we observed that the presence of Tinker and Davis-Monthan aquifer solids could inhibit PFCA transformation at 40°C and that the pH remained above 3, we hypothesized that those

aquifer solids contained buffering minerals such as calcite that had prevented the pH from descending below 3 that is necessary for PFCA transformation during HAPO. To test this, we performed HAPO experiments with 50 mM persulfate, Milli-Q water, 1:10,000 diluted 3M and Ansul AFFF, and 0-50 mM of calcium carbonate (calcite) without aquifer solids at 40°C. We then performed HAPO on both types of AFFF in slurries of Milli-Q water and aquifer solids from two locations (i.e., Tinker AFB and Davis-Monthan AFB) at 20 g/L after a pre-treatment step with 1 N sulfuric acid (H<sub>2</sub>SO<sub>4</sub>). We reasoned that pre-acidifying a buffered aquifer would improve the efficacy of PFCA transformation during persulfate ISCO. This treatment acidified the slurry to a pH of 2 before persulfate amendment for PFAS transformation. The bottles were kept in the 40°C incubator and sampled regularly for PFAS analysis.

In addition to examining the effects of aquifer solid buffering, we investigated the effect of co-contaminants on HAPO treatment of PFAS. Experiments with 3M and Ansul AFFFs (diluted 1:10,000) were performed with 7.6 and 76 µM (1 and 10 mg/L) TCE in 160 mL sealed glass serum bottles. Each set contained 20 g/L of Tinker AFB aquifer solids. Before persulfate addition, the slurries were pre-acidified to pH 2 with sulfuric acid. After 50 mM sodium persulfate was added to the glass bottles, they were maintained in a 40°C incubator and sampled periodically. PFAS were measured by LC-MS/MS and chloride and chlorate were measured by Dionex ICS 1100 ion chromatography (IC).

Preliminary experiments with AFFF and dioxane were conducted without solids present to determine if dioxane oxidation slowed PFCA or precursor transformation due to competition for sulfate radicals. These experiments were conducted in glass bottles with 100 mL final volume, 1:10,000 diluted 3M and Ansul AFFF, and 11.3 µM (1 mg/L) dioxane. Based on prior tests demonstrating rapid dioxane oxidation, bottles were first kept in a 40°C water bath for eight hours for high-frequency dioxane sampling, then were transferred to a 40°C incubator for longer term PFAS sampling. Dioxane was measured using SPME GC-MS after 1:1,000 in 10 mL of Milli-Q water with three grams of NaCl (Shirey and Linton, 2006).

To mimic aquifer conditions and further investigate potential persulfate ISCO inhibitors of PFCA transformation at low activation temperatures, we performed HAPO experiments in slurries with a ratio of one-to-one aquifer solids to groundwater (e.g., 50 grams solids, 50 mL water) at 40°C. Experiments began in collaboration with ESTCP project ER-201729 and used contaminated aquifer solids and groundwater from NAS Jacksonville, Florida. Before and after HAPO treatment, TOP assay was performed on the groundwater and the aquifer solids. After TOP, the aquifer solids were extracted using basic methanol (Houtz et al., 2013) and analyzed by LC-MS/MS for PFCAs/PFSAs. The NAS site in Jacksonville, Florida was identified as an ideal site for PFAS ISCO treatment due to the low buffering capacity and metal leachability potential in soil and low alkalinity in the groundwater. Dissolved metals in pH 7 and pH 3 adjusted with sulfuric acid and aqua regia digestion of Jacksonville aquifer solids were analyzed with liquid chromatography inductively coupled plasma mass spectrometry (LC-ICP-MS) (Grebel et al., 2016) to estimate the maximum solubilized metals released during HAPO.

Bench-scale single and weekly persulfate dose experiments (50-500 mM persulfate) were conducted to optimize PFAS destruction. In experiments with weekly oxidant doses, persulfate

was measured using a different colorimetric assay (Huang et al., 2002) than previously mentioned due to the higher concentrations.

Some of the 1:1 groundwater/aquifer solids experiments were performed with a pre-treatment step with hydrogen peroxide and citrate-chelated iron (II), which was considered in ER-201729; the exothermic peroxide decomposition would provide the heat required for HAPO and also oxidize labile organics in groundwater, which might have otherwise scavenged sulfate radicals. A slurry of contaminated groundwater and an equal mass of aquifer solids contained 12% hydrogen peroxide (i.e., 3.46 M peroxide), 5 mM  $\text{Fe}_2(\text{SO}_4)_3$ , and 10 mM  $\text{Na}_2\text{C}_6\text{H}_5\text{O}_7$ . After five days of reaction, the peroxide was completely degraded and persulfate was added to begin HAPO at 40°C.

Before and after HAPO treatment with Jacksonville slurry (1:1 groundwater/solids), we performed dissolved metal analysis using ICP-MS on 0.45-micron membrane filtered samples (Grebel et al., 2016). Post-treatment pH neutralization experiments with  $\text{CaCO}_3$  and NaOH will be performed to determine reprecipitation of metals that were mobilized during HAPO.

## **Objective 2: ISCO effects down-gradient**

After HAPO treatment and subsequent pH neutralization, high levels of sulfate will remain in the aquifer, which will likely affect down-gradient microbial communities and bioremediation efforts. Previous research in our laboratory investigated the effect of sulfate on a TCE dechlorinating enrichment and demonstrated that 5 mM sulfate does not inhibit TCE dechlorination (Mao et al. 2017). However, sulfide (~2 mM) generated by sulfate-reducing bacteria co-existing within the enrichment does inhibit dechlorination activity. This subtask aims to build on this research, as substantial knowledge gaps still remain. Specifically, no equivalent research has yet been done for dioxane biodegradation using CB1190. In addition to high sulfate concentrations, solutions from HAPO of AFFF will likely contain unreacted PFSA's and persulfate, which could affect the biodegradation of co-contaminants.

1,4-dioxane-degrading CB1190 was grown on AMS mineral salts medium and fed 5 mM 1,4-dioxane as its sole carbon source. To these triplicate reactors, increasing concentrations of PFOS were added to examine what concentration of PFOS leftover from HAPO treatment could inhibit co-contaminant degradation. These microcosms were kept in a 30°C shaking (150 rpm) incubator and sampled every 2-4 days for 1,4-dioxane concentrations.

An aerobic toluene degrading enrichment culture was similarly exposed to increasing concentrations of PFOS. This culture was maintained by a mineral salts medium, and fed toluene in multiple doses. Headspace samples were taken every 1-2 days; 100  $\mu\text{L}$  were injected into a GC-FID for toluene quantification, as described in the next section.

A BTEX-degrading enrichment culture was maintained in our lab for months, kept in a 37°C incubator, and regularly fed fresh media and BTEX compounds. This culture was used as inoculum for several experiments, including exposing this culture to increasing concentrations of sodium sulfate and increasing concentrations of a HAPO solution to determine if co-contaminants can be transformed after performing HAPO.

### **Objective 3: Bioremediation of AFFF and co-contaminants before ISCO**

AFFF microcosms were performed in triplicate using a mineral salts medium, a 5% soil inoculum from a separate Air Force base in South Dakota, and a 1:1000 dilution of neat AFFF. The inoculum contained trace amounts of soil. Autoclaved and medium-only controls were also included in triplicate. 50 mL of liquid slurry was added to 250 mL bottles to allow for ample oxygen exchange into the liquid. The bottles were capped with Mininert valve caps, and incubated in the dark (130 rpm, 30°C). 500-700 µL were taken about once a week (then less frequently the latter half of the experiment). About 400 µL of the sample was frozen aqueous, and 100 µL were diluted with 300 µL of basic methanol (2% NH<sub>4</sub>OH) for PFAS analysis; all were stored in -20°C until analysis. Oxygen and CO<sub>2</sub> were monitored in the headspace using Hewlett Packard 5890 Series II GC-TCD held isocratically at 35°C and DOC was measured using a Total Organic Carbon Analyzer (Shimadzu TOC-VCSH) as an indication of substrate consumption in the AFFF. DGBE was amended once. To compare microbial consumption of non-fluorinated carbon between 3M AFFF and fluorotelomer-based AFFF, additional microcosms under similar conditions described above were amended with either 1:1000 diluted ECF-based AFFF or the fluorotelomer-based National Foam AFFF. Upon depletion of DOC, we amended the microcosms with 1.5 mM of DGBE. Heat-killed and medium-only controls were also included for this experiment.

For the four carbon source experiments, AmPr-FHxSA was added to each condition. Each enrichment consisted of triplicate live treatments and autoclaved controls. One set of triplicate medium-only controls were conducted without any of the four carbon sources added. All controls were amended with 0.5 mL of an antibiotics mixture (1 mg/mL in MilliQ water of kanamycin, chloramphenicol, and streptomycin) to suppress biological activity. All bottles contained 19.5 mL of AMS media, 0.5 mL of phosphate buffer, and 5 mL of inoculum that contained trace amounts of soil (5 mL water added to medium-only controls) in 160 mL glass serum bottles. Methane, acetate, DBGE, and BTEX were added to achieve a target concentration of ~16 mM carbon. AmPr-FHxSA was added from a 10 mM stock solution (in 1:1 isopropyl alcohol: methanol) to a final concentration of ~0.2 µM. Background PFAS concentrations from the AFFF-impacted soil-derived inocula were 1-2 orders of magnitude lower than the concentration of AmPr-FHxSA.

The microcosms were placed horizontally in a 30°C shaking incubator (130 rpm) and sampled (500-700 µL) weekly for the first half of the experiment, then every 14 days. Aqueous samples were stored at -20°C and were measured within a week. Samples for PFAS analysis were immediately diluted in basic methanol (2% NH<sub>4</sub>OH; 25% sample in final mixture) and stored at -20°C until LC-MS/MS analysis. Oxygen was monitored weekly and supplemented when below ~5% of the headspace. Carbon sources were measured weekly and amended when needed (weekly for DGBE, acetate, and BTEX, once for methane). DOC was measured as a proxy for DGBE and acetate. BTEX and methane were measured directly with a GC-FID. ATP was also monitored as a proxy for biological activity.

BTEX and methane were measured on a GC-FID (Agilent 7890A) with a GS-GasPro column, injecting 100 µL of headspace air. For BTEX, the oven was set at 50°C for two minutes, then ramped 50°C/min until it reached 260°C. It was held at 260°C for two minutes. With this method, the retention times were 3.9, 4.8, 5.6, and 5.8 for benzene, toluene, ethylbenzene, and ortho-xylene. Methane was measured using a different method throughout which the oven remained at 70°C

with the retention time being about 1.1 minutes. In both methods, the injector port and detector were kept at 250°C.

Oxygen and carbon dioxide were measured on a Hewlett Packard 5890 Series II GC-TCD with an inner stainless steel column held at 350°C. The retention times for CO<sub>2</sub> and O<sub>2</sub> were ~0.8 and 1.2 minutes.

To confirm microbial activity, ATP was measured each week for all reactors—live, autoclaved, and medium-only using a luminescence-based assay adapted from Hammes et al. Briefly, a reagent was made using a BacTiter-Glo™ Buffer solution and a lyophilized BacTiter-Glo™ Substrate (Promega). Reagent and MilliQ water were separately heated in a heating block for three minutes at 38°C, then combined (50 µL Reagent, 450 µL diluted sample), vortexed, and incubated at 38°C for 20 seconds. The sample was then transferred to a Luminometer (GloMax® 20/20 Single Tube Luminometer Model#E6080) to be measured as an integral in relative light units over 10 seconds. These units were converted to ATP concentrations using a standard curve using a rATP stock (Promega; Cat. #P1132). All live reactors maintained ATP concentrations above ~50 pM for most of the experiment (much more for the DGBE and BTEX enrichments), while autoclaved and medium controls remained at 0 pM. Aqueous samples for ATP measurements were analyzed within 6 hours of sample collection.

Samples from the AFFF-based biotransformation study, as well as day 0 and day 70 timepoints from the four carbon source biotransformation study were sent to Colorado School of Mines for HRMS analysis. These samples were run on an ultra-high pressure liquid chromatography system (uHPLC, SCIEX Nexera LC) equipped with Sciex X500R Quadrupole Time-of-Flight Mass Spectrometer (QToF-MS) system. A delay column (Luna C18, 100Å, 30×3 mm, 5 µm) was installed for removal of potential contamination from the LC system. The analytical column was two Agilent Zorbax Diol guard columns (12.5×4.6 mm, 6 µm), a Phenomenex C18 guard column (2×4 mm), and Phenomenex Gemini C18 column (110Å, 100×3 mm, 5 µm), and column temperature was maintained at 40°C. The injection volume was 1000 µL. The mobile phase was a water/methanol gradient, each with 20 mM ammonium acetate at 0.6 mL/min (Table S4). Data acquisition on SWATH® in Data-Independent Acquisition mode allowed full scan of the infused sample and the data set was processed using Sciex OS software for both target analysis using analytical standard chemicals and nontarget screening with a list of suspected PFAS as previously described. Hits were identified based on mass error (<5 ppm), isotope ratio difference (<10%), and reasonable retention time based on known retention times of target compounds of similar mass. Samples were only run with negative electrospray ionization.

Genomic DNA was isolated for 16S rRNA gene amplicon sequencing at the end of the carbon source experiment. Biomass slurry samples (2 mL) were taken from each live treatment on day 70 and centrifuged at 10,000g for 10 minutes. After decanting the supernatant, DNA was extracted from pellets using a Dneasy PowerSoil Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. DNA quantity and quality was assessed using a NanoDrop Spectrometer (Thermo Fisher Scientific, Wilmington, DE, USA). The extracted DNA was stored in sterile, parafilm 1.5 mL centrifuge tubes at -80°C and sent to Novogene Corporation Inc. (Sacramento, CA, USA) for bacterial 16S rRNA gene amplification and sequencing.

### **Additional Findings — Salt Effects on PFOS Detection and Interfacial Behavior**

In our HAPO experiments we observed that levels of PFASs in the supernatant decreased with each high weekly dose of sodium persulfate (i.e., 200 and 500 mM doses), though previously in our lab and elsewhere (Bruton and Sedlak, 2018, 2017; Park et al., 2016), HAPO treatment did not transform PFASs. Focusing first on PFOS, we identified pH and ionic strength as two potential variables affecting the observed decrease in concentration in the solution. Additionally, to assess if PFOS adsorbed to solids, we evaluated the difference in PFOS concentrations in sand and sand-free experiments.

Solutions were prepared with Milli-Q water and Ottawa sand (50-70 mesh particle size) (1:1 v/w) in 150 mL polypropylene bottles. PFOS was added via a concentrated stock solution prepared from technical grade perfluorooctane sulfonate acid (PFOS) (40% in water) for all salt-effect experiments. Sulfuric acid was used to adjust the pH of Na<sub>2</sub>SO<sub>4</sub> treated reactions to match the pH and ionic strength of HAPO. Upon preparation, aqueous solutions were equilibrated at 40°C overnight to match the activation temperature for previous HAPO experiments. We added Na<sub>2</sub>SO<sub>4</sub> after the equilibration period. Solutions were sampled prior to salt addition and at various time intervals post-salt addition. Between sampling periods, solutions were stored at 40°C either in the incubator or in a temperature-controlled water bath. The standard sampling procedure for aquifer solid and water slurries was to take a 2 mL aliquot from the reaction bottle, centrifuge the sample at 10,000 g, 25°C for 10 minutes, then dilute a portion (<50% of the aqueous sample) in MeOH (1:1 v/v) and centrifuge again. Sample centrifugation was implemented in lieu of ultra-filtration prior to LC-MS analysis.

While the Na<sub>2</sub>S<sub>2</sub>O<sub>8</sub> and Na<sub>2</sub>SO<sub>4</sub> comparison experiments demonstrated the effects of salts on PFOS concentrations, we considered that the observed PFOS decrease may be an artifact of the sample preparation and sampling method, and not an accurate representation of the bulk solutions. The effects of subsampling versus a full bottle extraction were compared to determine if PFOS was adsorbing to the bottle upon salt addition. The effect of centrifugation on PFOS quantification was also tested.

To test the effect of subsampling, solutions containing 20 µM PFOS in 15 mL of Milli-Q water were prepared. After equilibration overnight at 40°C, sample aliquots were taken in triplicate and diluted in MeOH (1:1 v/v); then, the sample was centrifuged (10,000 g, 25°C, 10 minutes). The aqueous sample with no solids was not centrifuged as no particulates were present in any of the samples. Samples were taken in triplicate prior to salt addition and immediately diluted in MeOH. 1 M Na<sub>2</sub>SO<sub>4</sub> was added and the solutions were sampled again three hours post-salt addition. To replicate the standard sampling procedure in a full-bottle extraction, 12 mL of MeOH was added to the experiment bottle and shaken to recover remaining PFOS, then centrifuged. Samples were taken for LC-MS/MS quantification.

We also considered the effect of sample preparation on PFOS concentration. Two sample preparation methods were compared pre- and post-salt addition. In Method 1, an aqueous aliquot was first centrifuged, then diluted in MeOH (1:1 v/v) and centrifuged again prior to dilution for LC-MS/MS analysis; this was in accordance with the standard sample preparation procedure for aquifer solids and water slurry experiments. In Method 2, an aqueous aliquot was taken from the experiment bottle, diluted directly in MeOH, centrifuged, and diluted for LC-MS/MS analysis.

## Results and Discussion

### Objective 1: ISCO on AFFF and co-contaminants

The results described in this Objective are being drafted into a manuscript that will be submitted to a peer-reviewed journal.

### *Effect of Temperature, Buffering, and Co-Contaminants on PFAS Transformation*

Achieving an 85°C persulfate activation temperature for an in situ remediation strategy is possible but would be energy-intensive, challenging, time-consuming, and expensive. Our first goal was to establish if precursor and PFCA destruction was possible at lower persulfate activation temperatures. Lowering the temperature could lower remediation cost and enhance potential for downstream bioremediation processes, thereby broadening applications of persulfate ISCO.

HAPSO at 85°C was performed as a positive control for the other tests conducted at the lower activation temperatures, 65 and 40°C (Figure 2). Similar to previous 85°C experiments, >90% removal of PFCAs and precursors was achieved within about eight hours without aquifer solids present. The PFSAs were not oxidized by HAPSO (data not shown). At an activation temperature of 65°C, precursors and PFCAs were removed at an efficiency >95% in under 60 hours. With both conditions, the pH decreased below 3 rapidly due to the persulfate degradation (Figure 3a&b, Equation 1). The 40°C test still resulted in >90% PFCA transformation, but over the course of about 10 days. The pH decreased to below 3 within about five days, and there was still plenty of persulfate remaining by the end of the experiment (Figure 3c & d).

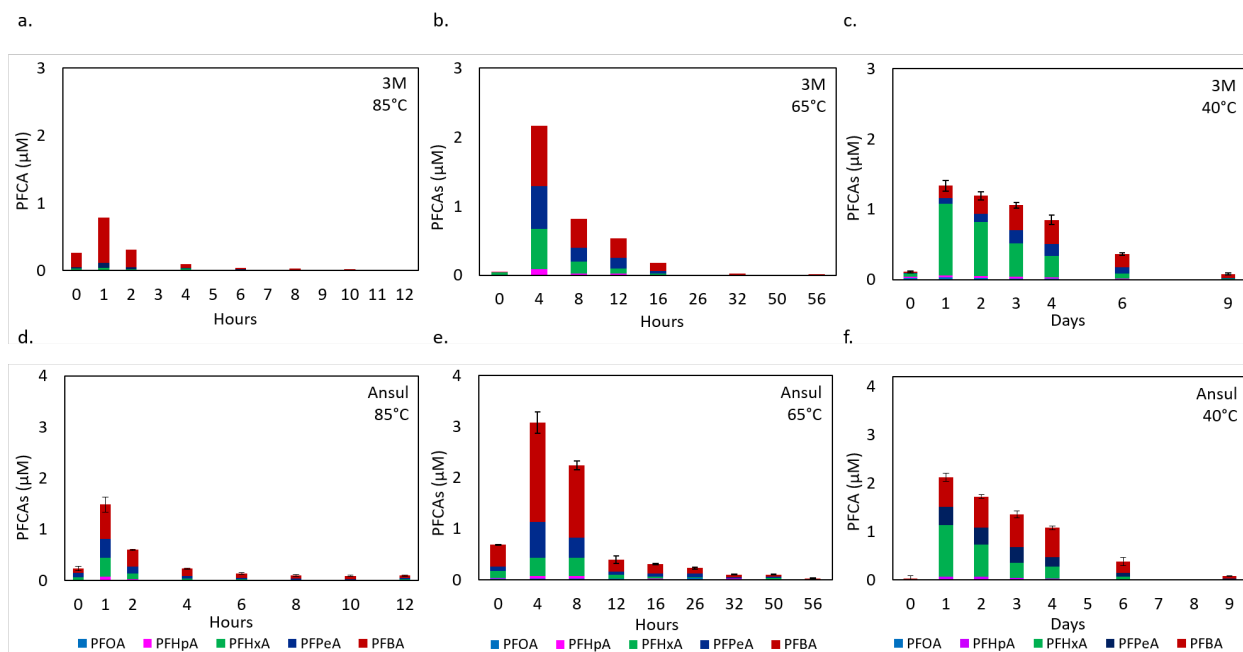


Figure 2. 3M and Ansul AFFF PFCA and precursor destruction by HAPSO at 85, 65, and 40°C. 3M tests shown in a, b, and c; Ansul tests shown in d, e, and f. Note the x-axis units; 85 and 65°C are shown in hours, while the 40°C HAPSO test took days. The initial increase in PFCAs between the first and second time points resulted from precursors transforming into PFCAs after persulfate addition.

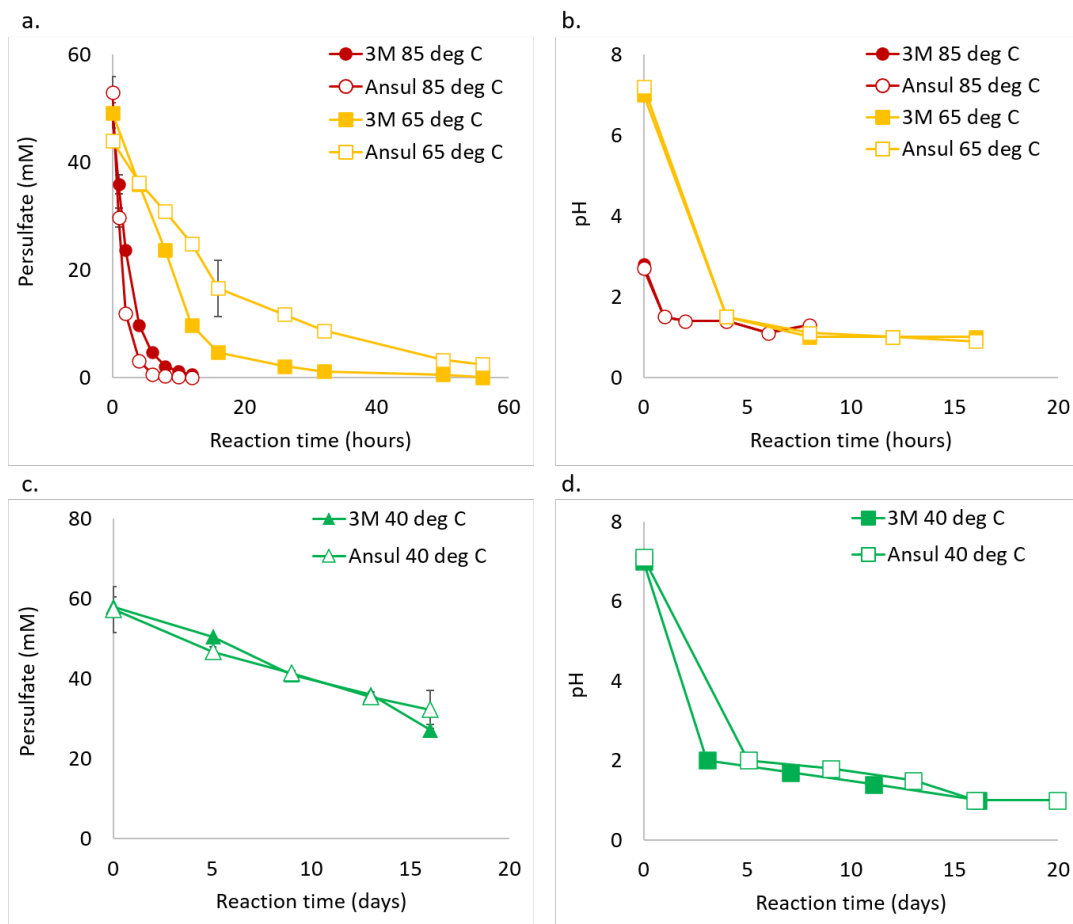


Figure 3. a and c) Persulfate concentrations and b and d) pH for the 85°C (red), 65°C (yellow), and 40°C HAP (green) test. Persulfate degrades on the order of days for the 40°C test, though the pH decreases to below three within the first 4 days.

We then repeat these experiments, but in the presence of 20 g/L of aquifer solids. Figure 4 compares the activation temperature results without and with solids for Ansul AFFF. PFCA transformation occurs, though slightly delayed and with more PFCAs remaining on the final timepoint. The 40°C test, however, resulted in complete inhibition of PFCA transformation. Precursors transformed into PFCAs between days 0 and 5, but no subsequent degradation occurred (Figure 4), due to a persistent circumneutral pH. After 27 days, about 30 mM of persulfate remained in solution (data not shown), indicating that a lack of persulfate activation was not limiting PFCA transformation but impeding the further PFCA destruction.

After observing the complete inhibition of PFCA transformation by aquifer solids at 40°C, we performed experiments to examine whether this effect was due to the buffering of the solids coupled with the lower rate of persulfate activation. We repeated the 40°C HAPO experiment with 3M and Ansul AFFFs, but with increasing concentrations of calcite instead of solids to mimic the buffering capacity of the aquifer solids. We added between 2-25 mM calcite to the AFFF-water mixture, then added persulfate. As shown in Figures 5 and 6, as the activated persulfate acidified the solution, PFCA transformation began and then continued to the end of our sampling period. Precursors transformed into PFCAs regardless of pH over the first day of the experiment. Higher concentrations of calcite resulted in longer initial lag times before PFCA transformation, but did not inhibit transformation once the buffering capacity was depleted.

As mentioned above, two recent studies have investigated the pH-dependence of activated persulfate oxidation of PFCAs (Zhang et al 2019, Carre et al 2021). One suggested that sulfate radical scavengers, especially hydroxide, were primarily responsible for the lack of PFCA transformation at pH values above 3 (Carre et al 2021). Therefore, it is integral to have an acidic solution before PFCA mineralization can occur. If employing HAPO at field sites, engineers should expect to see PFCA concentrations increase at pH > 3, while precursors transform, and only see PFCA concentrations decrease when the pH falls below 3.

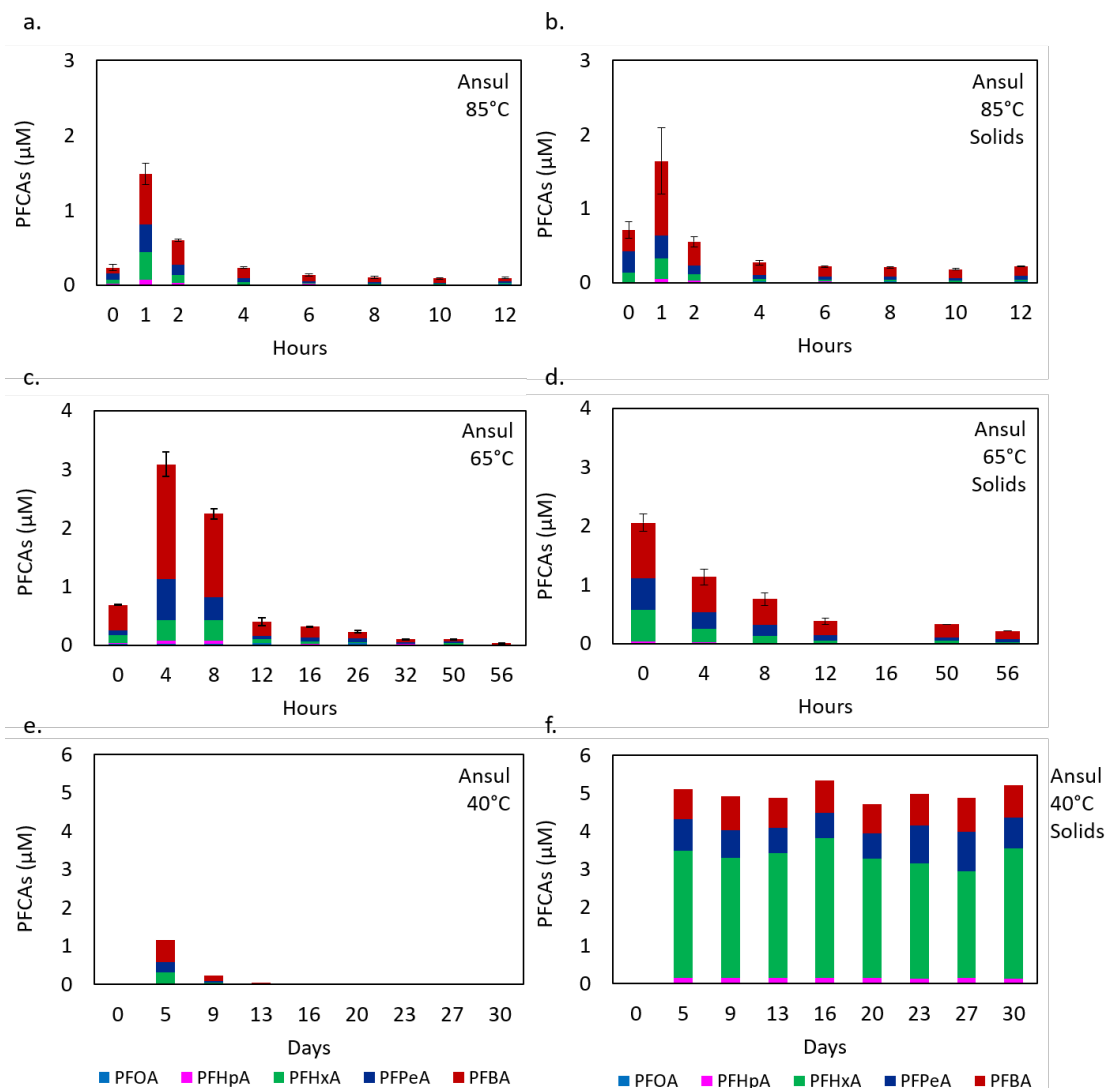


Figure 4. HAPO of Anul AFFF without (a, c, e) and with (b, d, f) aquifer solids at 85 (a, b), 65 (c, d), and 40°C (e, f) with 50 mM persulfate. As shown in f, precursors were transformed into PFCAs but no PFCA transformation occurred after a month.

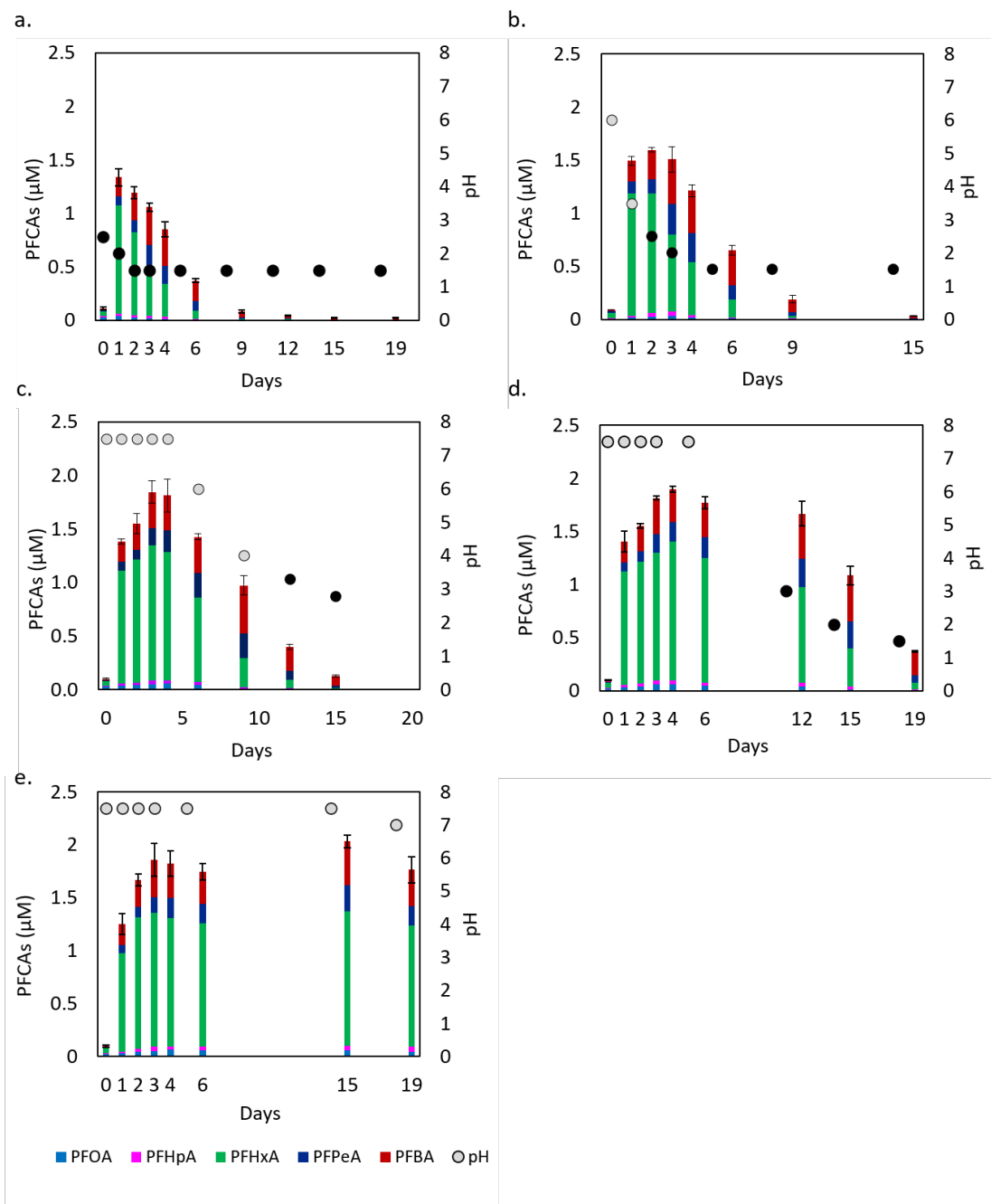


Figure 5. The effects of pH buffering on PFCA transformation by the addition of calcium carbonate (calcite) at 40°C activation temperature. a) 0 mM, b) 2 mM, c) 5 mM, d) 10 mM, and e) 25 mM calcite was added to duplicate test tubes. This test used 10,000x diluted 3M AFFF and 50 mM sodium persulfate. The dots indicate pH measurements and turn black once the pH is within the range for PFCA transformation.

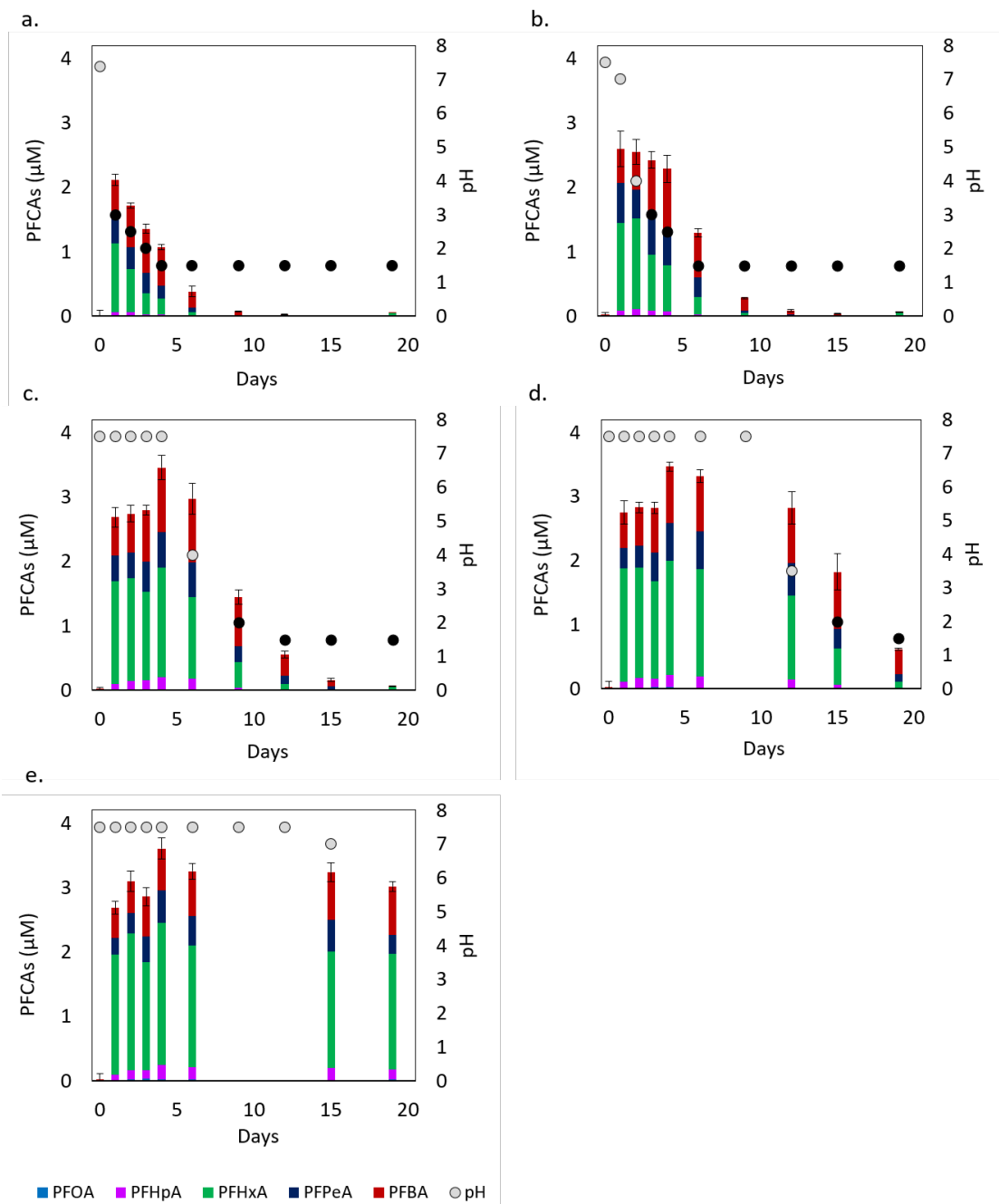


Figure 6. The effects of pH buffering on PFCA transformation by the addition of calcium carbonate (calcite) at 40°C activation temperature. a) 0 mM, b) 2 mM, c) 5 mM, d) 10 mM, and e) 25 mM calcite was added to duplicate test tubes. This test used 10,000x diluted Ansol AFFF and 50 mM sodium persulfate. The dots indicate pH measurements and turn black once the pH is within the range for PFCA transformation.

Greater concentrations of buffering minerals in an aquifer would mean a higher concentration of persulfate needed to acidify the groundwater, increasing the treatment time in the field. To overcome this buffering-induced lag time, we tested the acidification of water-soil slurries prior to HAPO as a practical pretreatment step to enhance PFCA transformation (Figures 7 and 8). We constructed a suite of batch tests using either Tinker or Davis-Monthan AFB aquifer solids (Table 1) that were amended with either 3M or Ansul AFFFs. We compared pre-acidified bottles (to pH 2 with 1 N sulfuric acid) to those without this step.

Over the course of about 20 days, the slurries with sulfuric acid addition showed significantly higher PFCA degradation than the slurries without the addition of acid, though the pH in the Davis-Monthan AFB slurries (Figure 7) did slowly decrease to below 3, possibly leading to PFCA degradation. Following pre-acidification, the Davis-Monthan AFB slurries showed quicker PFCA degradation than the Tinker AFB slurries, likely due to the higher carbonate concentrations in the Tinker AFB solids. After acidifying to pH 2, the pH of the Tinker AFB slurry increased over the first two days, which postponed the onset of PFCA destruction. It is likely that additional acid or persulfate would have enabled faster transformation and more complete removal of PFCAs during the timeframe of the experiment. The Davis-Monthan slurries, however, achieved a greater degree of PFCA destruction after about 20 days because the pH stayed below 3 after the initial acidification. These results indicate that buffering capacity of aquifer solids could halt PFCA transformation, even if the slurry is initially acidified, and that pH must be monitored throughout treatment for best PFAS removal.

The results from the calcite and pre-acidification experiments indicate that groundwater and aquifer solids buffering can be overcome by designing persulfate dose accordingly (activation of higher persulfate concentrations results in higher  $H^+$  concentrations) or pre-acidifying the groundwater. Needing the groundwater to be acidic for PFCA transformation could result in greater hazard and requires caution if implementing this strategy as an in situ PFAS source zone treatment.

Table 1. Soil characteristics of two aquifer samples used in the pre-acidification slurry experiments. Shown here are N, C, TOC, Zn, Mn, Fe, and Cu.

| Sample No. | Description    | N (Total) % | C (Total) % | TOC % | Zn (Total) ppm | Mn (Total) ppm | Fe (Total) ppm | Cu (Total) ppm |
|------------|----------------|-------------|-------------|-------|----------------|----------------|----------------|----------------|
| 1          | Davis-Monthan, | <0.020      | 0.73        | <0.02 | 57             | 338            | 16100          | 17             |
| 1 dup      | AZ solids      | <0.020      | 0.75        | <0.02 | 55             | 342            | 15400          | 17             |
| 2          | Tinker, OK AFB | <0.020      | 1.15        | <0.02 | 37             | 840            | 15300          | 24             |
| 2 dup      | solids         | <0.020      | 1.18        | <0.02 | 35             | 810            | 13300          | 22             |

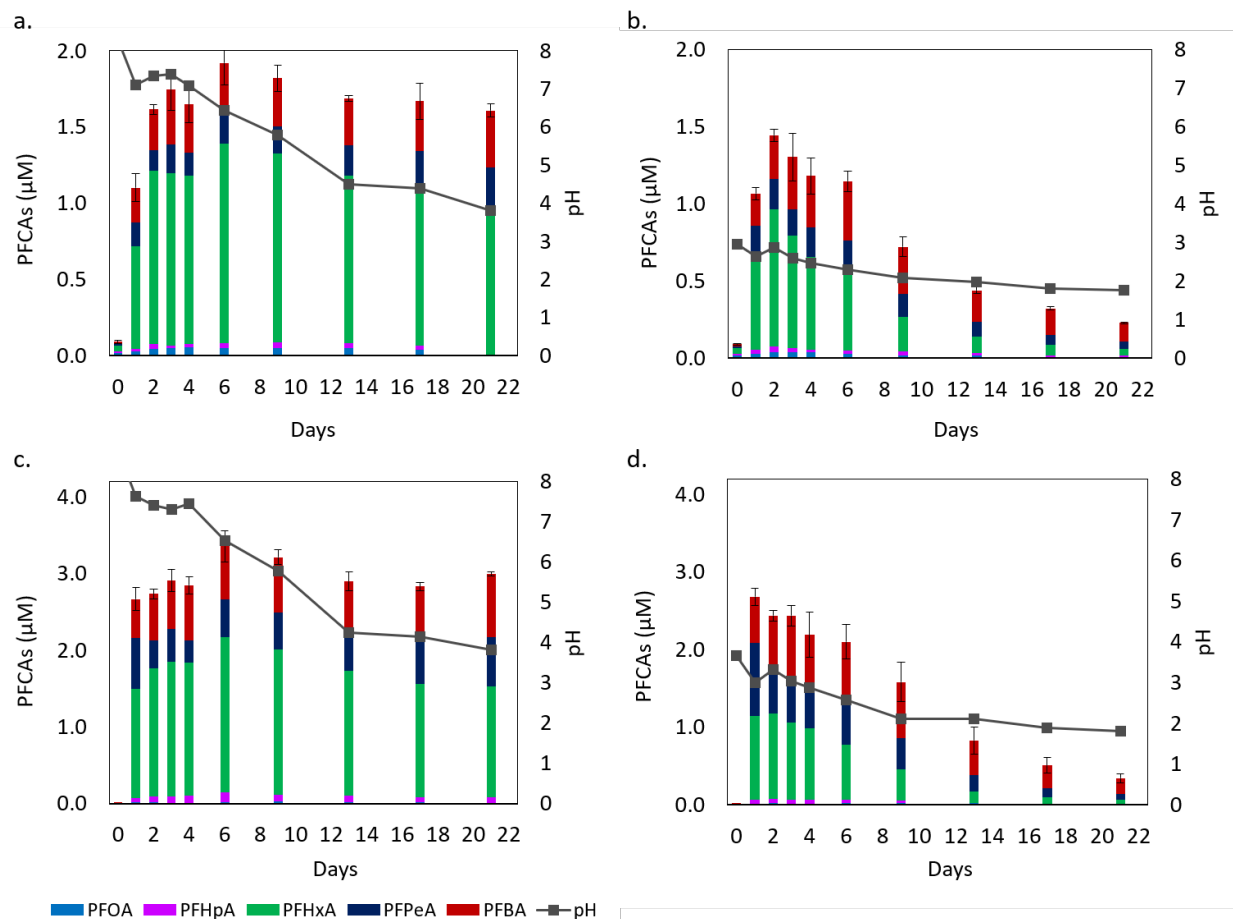


Figure 7. Pre-acidification increases PFCA transformation efficiency in the presence of Arizona-based Davis-Monahan aquifer solids. Duplicate test tubes were exposed to a) 3M AFFF without pre-acidifying, b) 3M AFFF with pre-acidifying, c) Ansul AFFF without pre-acidifying, and d) Ansul AFFF with pre-acidifying. All tests were exposed to 50 mM sodium persulfate at a heat-activation temperature of 40°C.

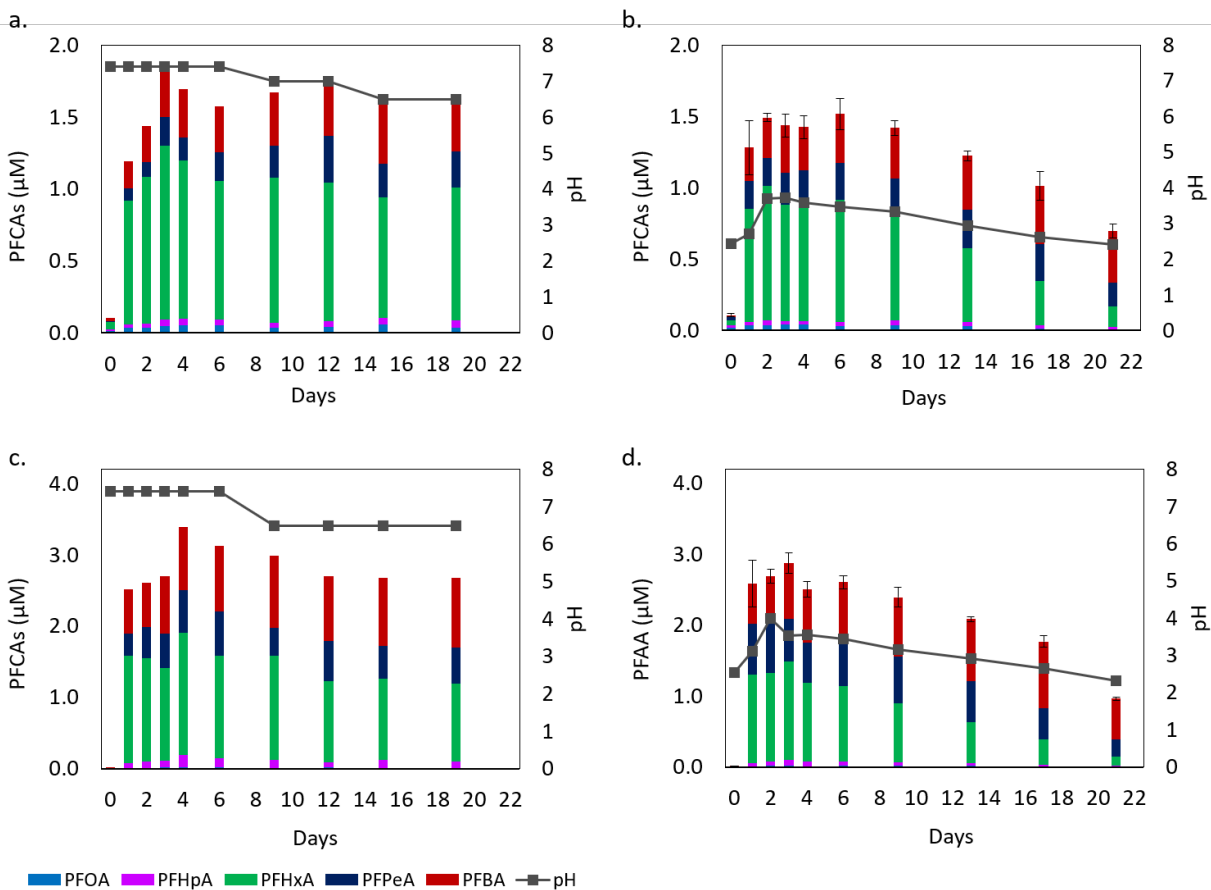


Figure 8. Pre-acidification increases PFCA transformation efficiency in the presence of Oklahoma-based Tinker AFB aquifer solids. Duplicate test tubes were exposed to a) 3M AFFF without pre-acidifying, b) 3M AFFF with pre-acidifying, c) Ansul AFFF without pre-acidifying, and d) Ansul AFFF with pre-acidifying. All tests were exposed to 50 mM sodium persulfate at a heat-activation temperature of 40°C.

After establishing that HAPO at 40°C was possible in the presence of aquifer solids—with the assistance of a pre-acidification step—environmentally-relevant concentrations of common co-contaminants TCE and dioxane were added to AFFF solutions to determine if these compounds inhibit precursor or PFCA transformation. Based on the previous pre-acidification results, TCE was added to a soil slurry that was pre-acidified with sulfuric acid before beginning HAPO at 40°C.

Samples taken hourly at the start of the experiment showed rapid transformation of TCE in the presence of both types of AFFF. Samples taken daily tracked PFAS and chloride concentrations. After an initial increase of chloride concentrations from the degradation of TCE, there was a steady decrease as chloride was oxidized to chlorate at an acidic pH (Figures 11). As the World Health Organization lists a maximum drinking water concentration of chlorate at 8.4 µM, chlorate formation is concerning and would need to be monitored carefully under HAPO conditions. Proper remediation strategies would also need to be implemented if the chlorate concentration is above the safety level, including TCE bioremediation prior to HAPO as proposed in Objective 3.

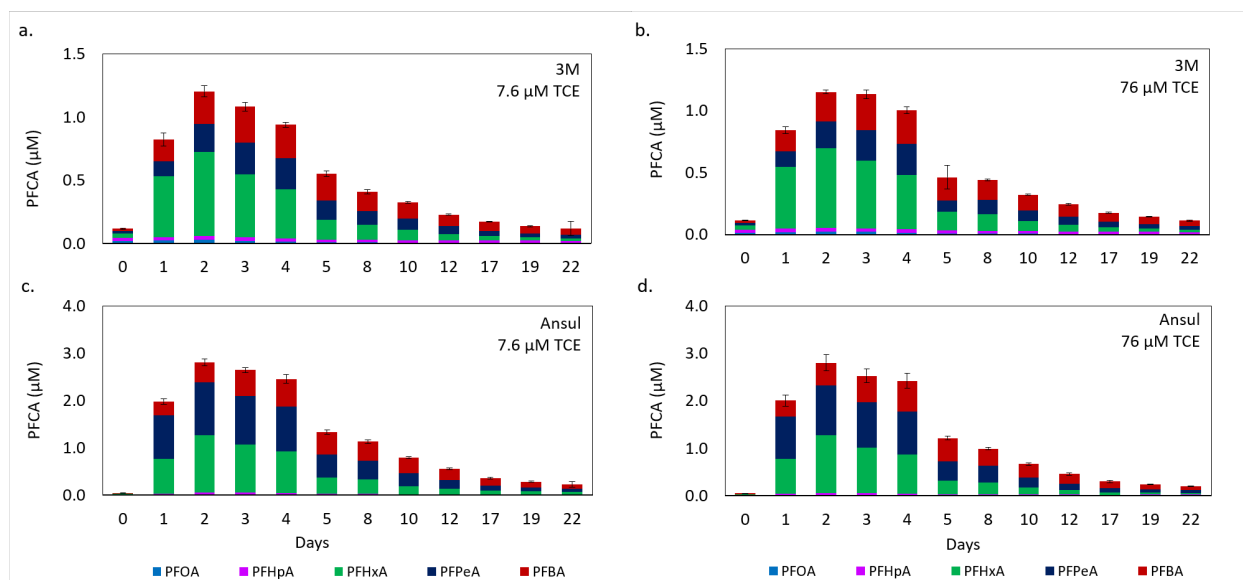


Figure 9. PFCA transformation in the presence of TCE for slurries with 20 g/L aquifer solids, Milli-Q water, and diluted AFFF. The slurries were pre-acidified to pH 2 with sulfuric acid to encourage PFCA transformation.

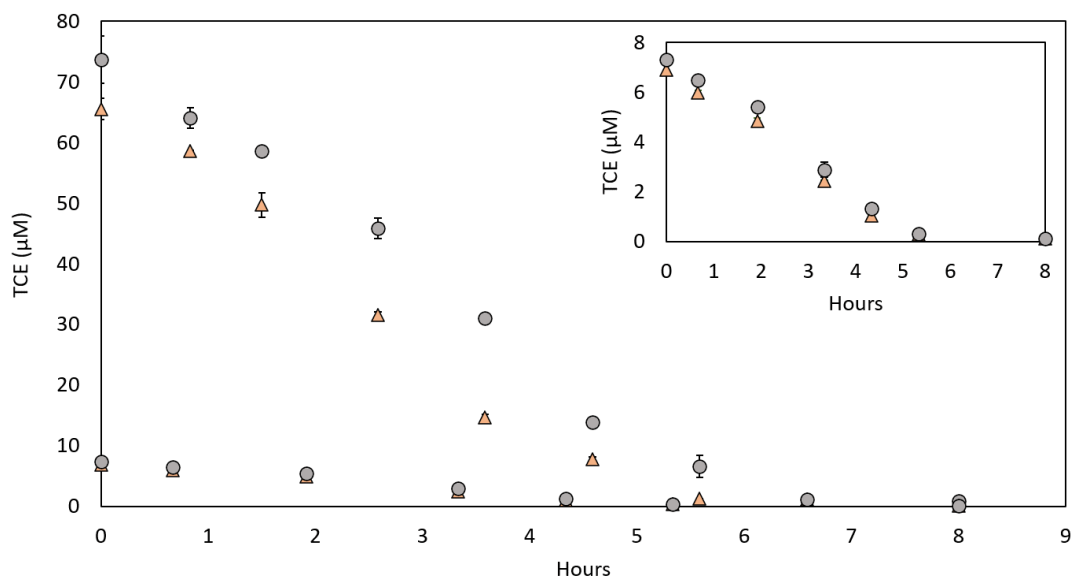


Figure 10. HAPO of AFFF and TCE mixtures. TCE was mineralized at a) 7.6  $\mu\text{M}$  (1 ppm) and b) 76  $\mu\text{M}$  (10 ppm) after 6-8 hours.

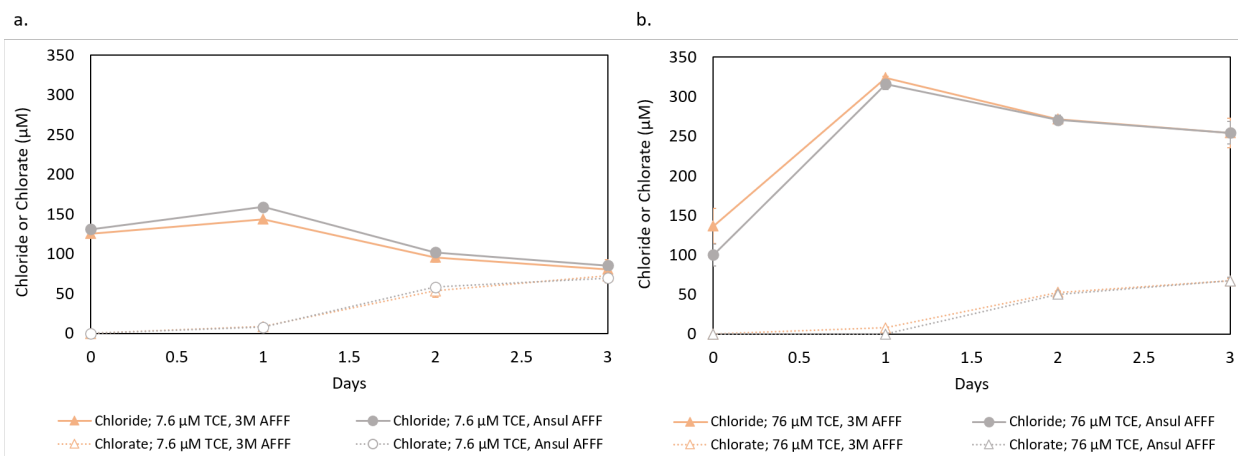


Figure 11. Chloride and chlorate concentrations produced from TCE HAPO. a) shows results from adding 7.6  $\mu\text{M}$  TCE and b) 76  $\mu\text{M}$  TCE. Chloride is shown in solid lines and circles; chlorate is shown in open circles and dotted lines. Pink represents the 3M AFFF test, and gray the Ansul AFFF test.

Preliminary HAPO experiments in the presence of dioxane did not stall the subsequent destruction of precursors and PFCAs after 14 days (Figure 12). Precursors and PFCAs were removed from solution by over 95% for both AFFF formulations. Additionally, dioxane itself was degraded within about 11 hours of persulfate addition and heat-activation at 40°C (Figure 13). 88% of the dissolved organic carbon was oxidized, independent of initial concentration that varied based on the AFFF present in solution.

PFCAs are much more recalcitrant than organic co-contaminants such as TCE and 1,4-dioxane. Even with a high ratio of oxidant to PFAS, it takes days for PFCAs to mineralize, versus only hours for the co-contaminants to mineralize at 40°C. These co-contaminants may be able to be oxidized by the persulfate anion ( $S_2O_8^{2-}$ ) without activation, while PFCAs can only be transformed by the sulfate radical at acidic pH values. Additionally, rate constants of sulfate radicals with longer-chain PFCAs are orders of magnitude lower than with natural organic matter (NOM), chloride,  $Fe^{2+}$ , hydroxide, carbonate, etc. (Appendix A). At an AFFF remediation site, engineers may see slower initial transformation of precursors into PFCAs if there are organic co-contaminants present, and may require higher doses of persulfate. Concentration ranges for persulfate dosing to overcome aquifer inhibitions are discussed in the next section.

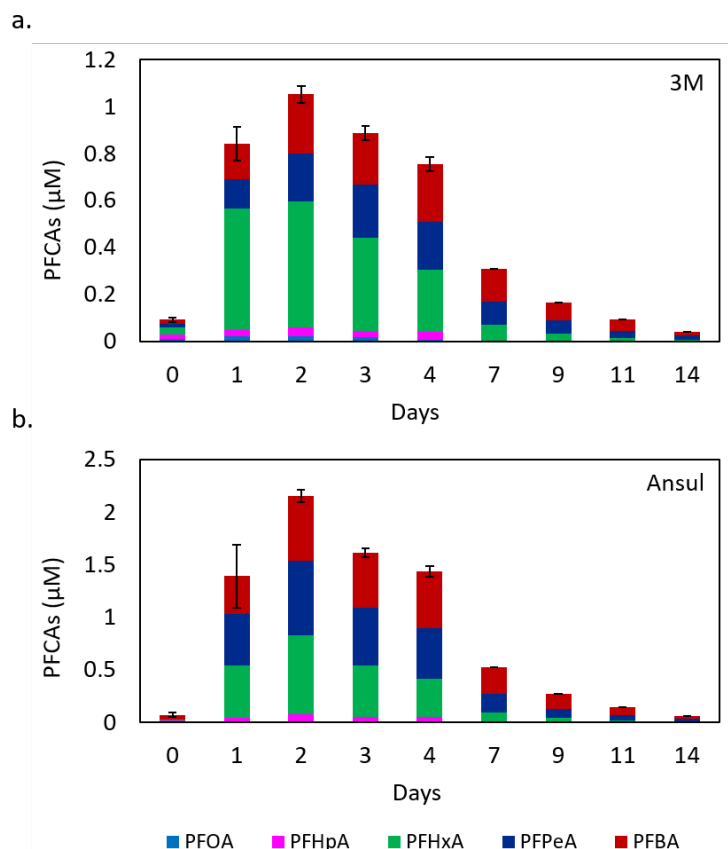


Figure 12. Precursor transformation and subsequent destruction of PFCAs in the presence of ~12  $\mu$ M (1 ppm) dioxane under 40°C HAPO conditions.

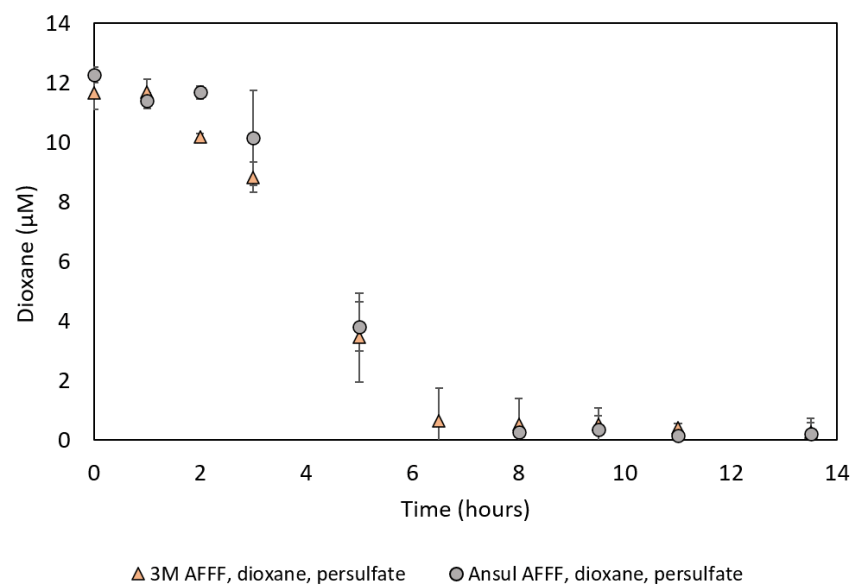


Figure 13. Dioxane degradation during a 40°C ISCO of 3M and Ansul AFFF co-contaminated with dioxane using 50 mM persulfate.

### *Applying and Improving HAPO Performance Under Realistic Site Conditions*

The aforementioned HAPO experiments were all conducted with 50 mM persulfate and, where solids were present, 20 g/L were added. To more closely simulate aquifer conditions, and in collaboration with ESTCP ER-201729, the following treatability studies consisted of a one-to-one ratio of groundwater to solids (i.e., 1000 g/L).

We received PFAS-contaminated aquifer solids and groundwater from NAS Jacksonville, Florida through our collaborators on ESTCP ER-201729. We performed PFAS and TOP analyses on groundwater and solids (5-35 feet below ground surface) and determined that the groundwater contained approximately 3.6  $\mu\text{M}$  PFOS, and a TOP PFCA concentration of 2.7  $\mu\text{M}$ , most of which was the six-carbon chain, PFHxA. The pH was 6 and the total alkalinity was 2.2 meq/L, or 1.1 mM (110 mg/L) as  $\text{CaCO}_3$ . The DOC of filtered groundwater was approximately 3.3 mM (40 mg/L). The aquifer solids did not show strong buffering capacity that would have hindered the efficiency of HAPO treatment for the Jacksonville site. We tested dissolved metals in the groundwater and solids acid digestion (sulfuric acid pH 3 and aqua regia) to quantify the maximum amount of metals that could be released upon acidification via HAPO (Figures 15, 16, 17).

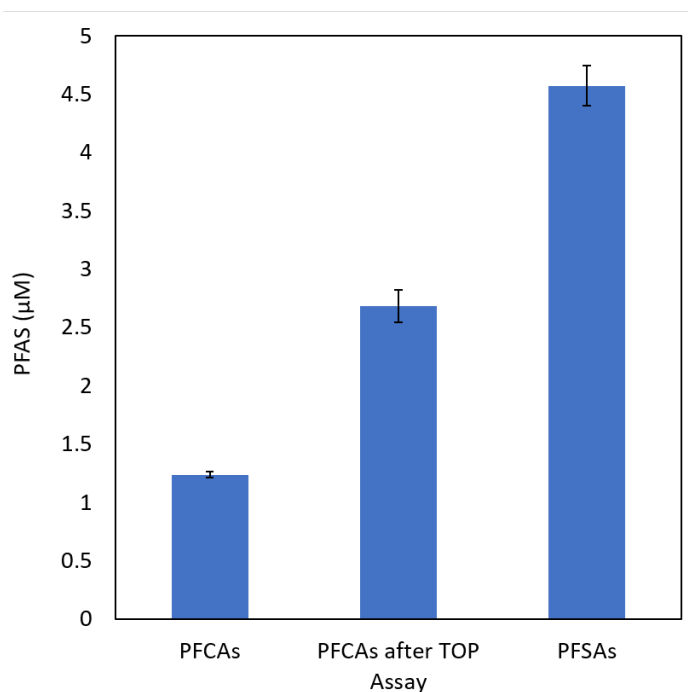


Figure 14. PFAS profile of the Jacksonville groundwater samples. The sum of PFCAs and precursors measured via the TOP assay was about 3  $\mu\text{M}$  whereas PFSAs were about 4.5  $\mu\text{M}$ .

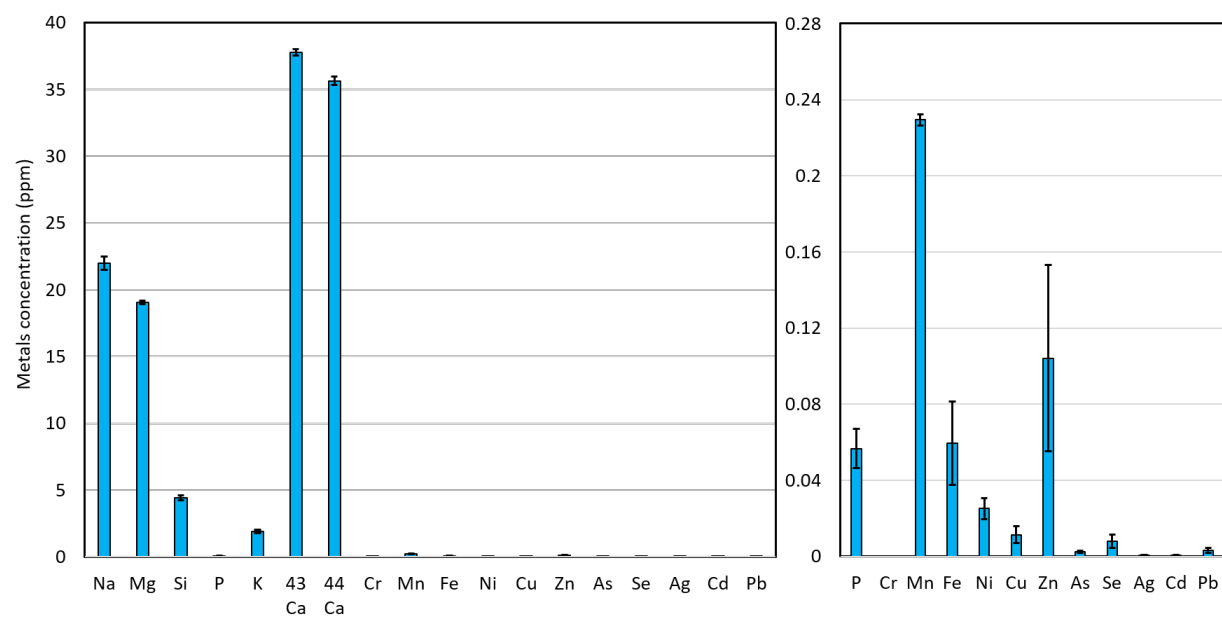


Figure 15. Dissolved metals (mg/L) on NAS Jacksonville groundwater contaminated with AFFF.

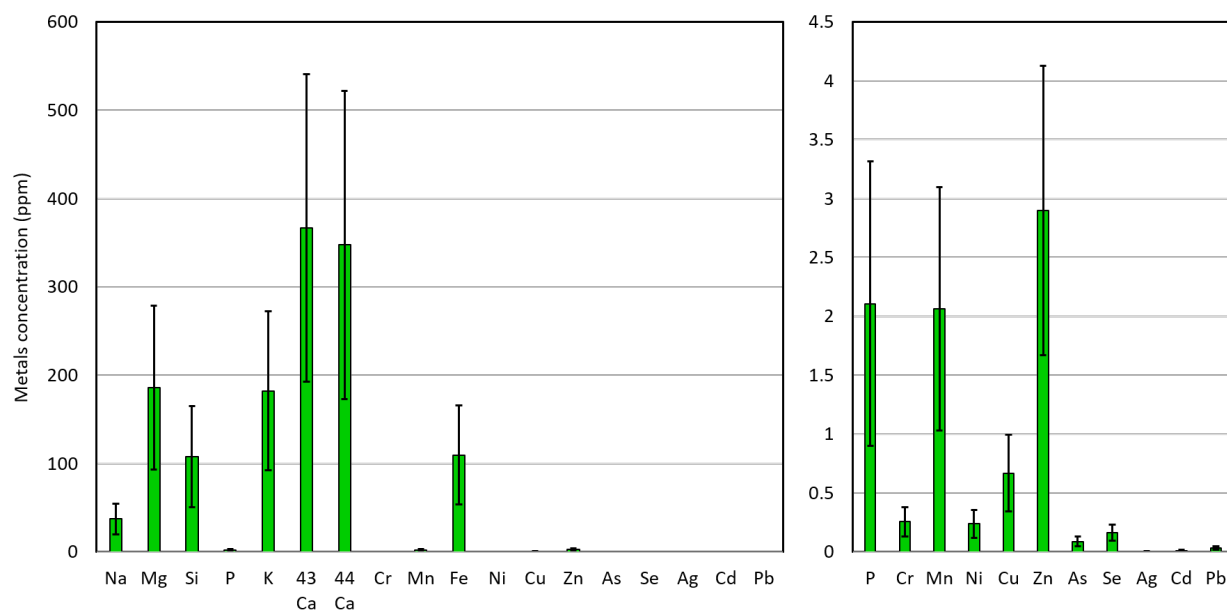


Figure 16. Dissolved metals (mg/L) after digestion of Jacksonville solids with sulfuric acid at pH 3.

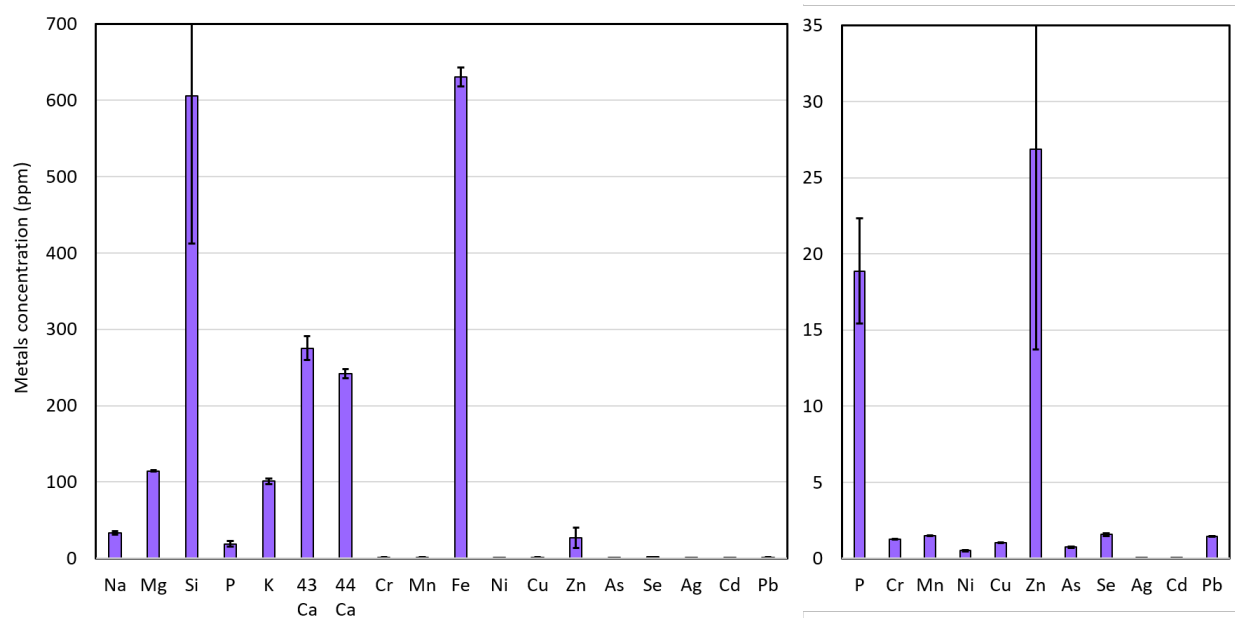


Figure 17. Dissolved metals (mg/L) digested with aqua regia in Jacksonville solids.

As the geochemical conditions at a given site can vary widely, the metals released during HAPO need to be included during the development of in situ treatment strategies. Metal reprecipitation by pH neutralization is a possible strategy to prevent unintentional release of heavy metals.

To optimize the persulfate dose for efficient PFAS transformation, preliminary tests with one-to-one ratio of solids and groundwater were conducted to explore the range of persulfate concentrations needed under these conditions. Tests with 50, 100, and 200 mM persulfate added on day 0 resulted in only precursor transformation into PFCAs, and no further PFCA degradation (data not shown). Another one-to-one test was conducted with a 500 mM persulfate dose added day 0, followed by a 100 mM persulfate addition after three weeks (Figure 18). This, too, resulted in PFCA production but then the stalling of PFCA transformation.

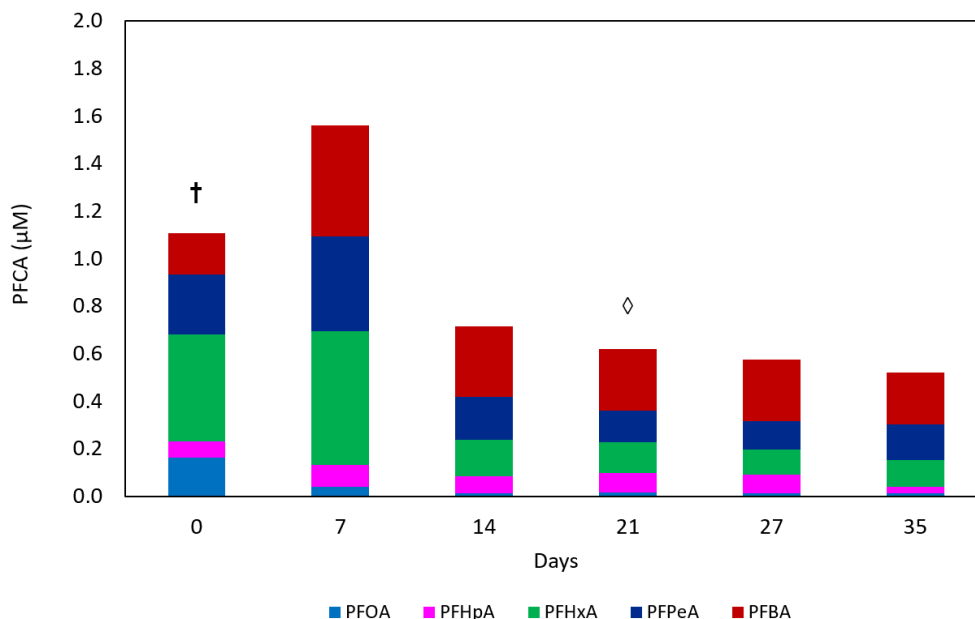


Figure 18. One 500 mM dose (†) of persulfate added Day 0 during 40°C HAPO was insufficient for PFCA transformation over the course of one month, despite an additional 100 mM dose (◇) after three weeks.

Informed by these negative results, we conducted a series of experiments with weekly additions of persulfate to encourage PFCA transformation to continue throughout the course of the approximately one-month experiment timelines. This experiment was conducted with a hydrogen peroxide pretreatment step (Figure 19). Working with our collaborators on ESTCP ER-201729, we established that an in situ heating mechanism for HAPO could be the addition of peroxide for its exothermic reaction. We tested adding peroxide,  $(\text{Fe}_2\text{SO}_4)_3$ , and sodium citrate to encourage Fenton-like chemistry, with controlled release of iron. The peroxide degraded by day 5; subsequently, persulfate was added on day 5 to begin the weekly doses. We tested four weekly doses of 200 or 500 mM persulfate, and 200 mM persulfate proved to be the most effective, resulting >97% PFCA and precursor destruction over 35 days (Figure 20). Interestingly, 500 mM persulfate doses were less effective (92% degradation of PFCAs and precursors). We hypothesize that the excess sulfate radicals might have caused a radical self-scavenging, reducing the efficiency of PFAS oxidation.

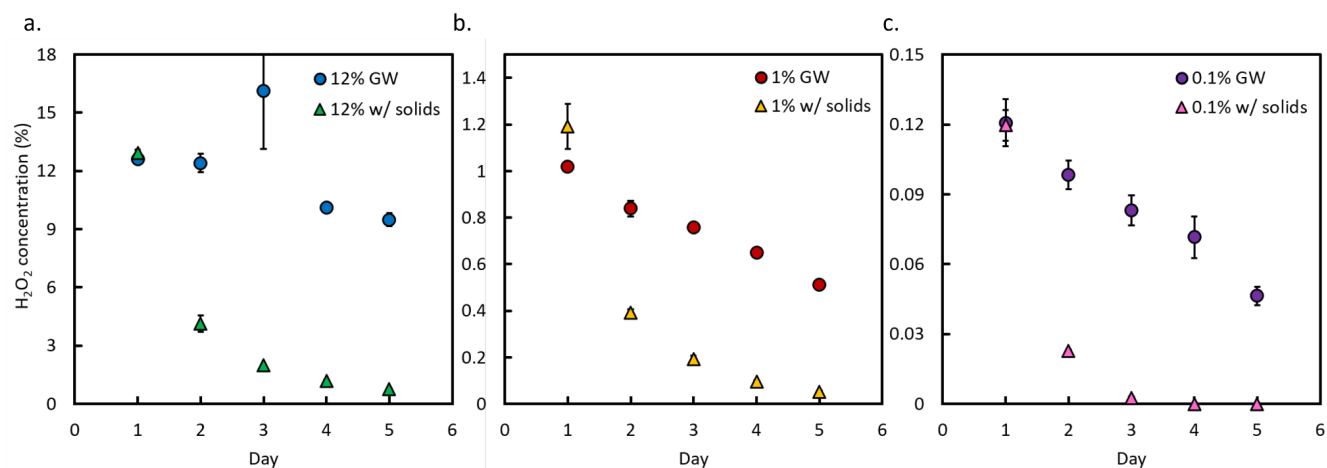


Figure 19. Hydrogen peroxide concentrations over 5 days added to just the groundwater or the groundwater-solid slurries at a) 12%, b) 1%, and 0.1% by volume. Peroxide depleted more rapidly in the presence of the aquifer solids.

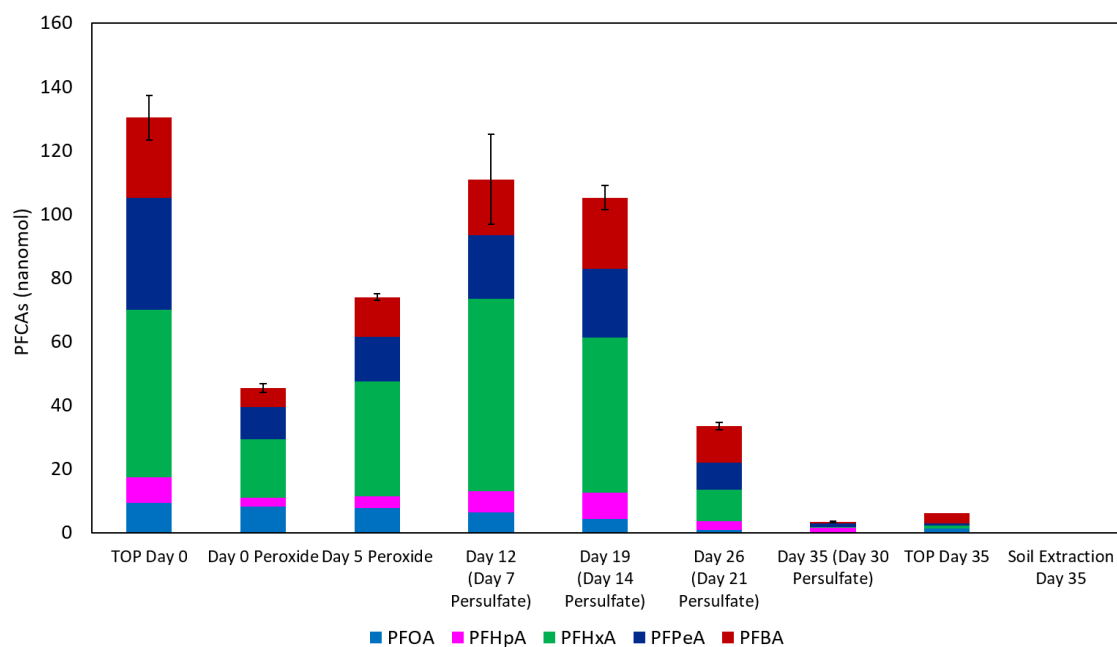


Figure 20. Peroxide was added Day 0 and depleted until Day 5 in this 1000 g/L groundwater-soil slurry. Then, 200 mM persulfate doses were added Day 5 and each week subsequent until Day 35. We compared these results with TOP results conducted on the groundwater-soil slurry before the start of the experiment, and at the end. A soil extraction test (Houtz et al 2013) was performed and all PFAS were below the limit of detection.

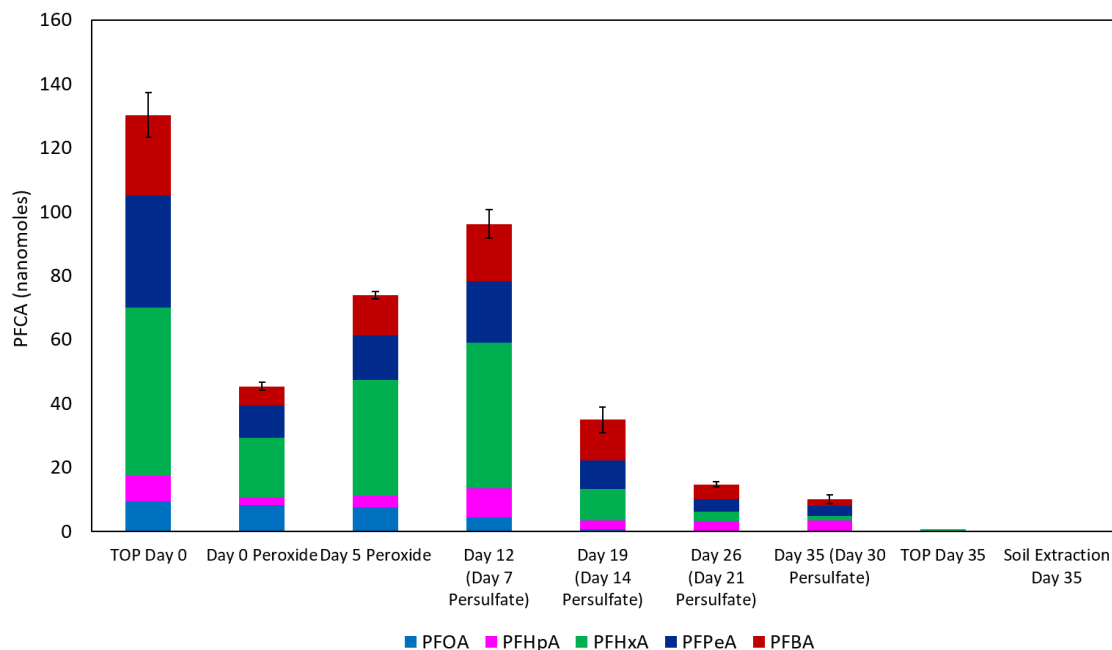


Figure 21. The same experiment as in Figure 20, but with 500 mM persulfate doses added on Day 5 and each week subsequent until Day 35.

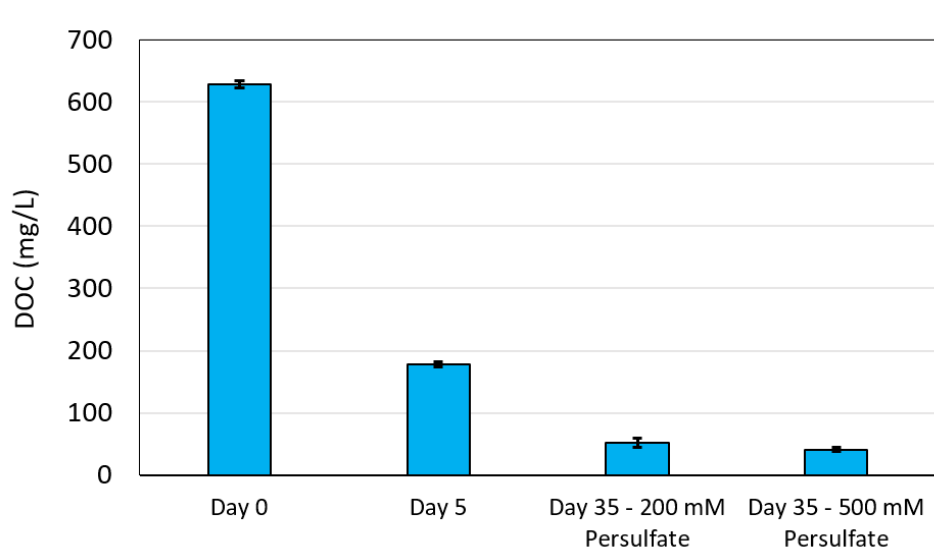


Figure 22. DOC concentrations starting on Day 0, after the peroxide addition on Day 5, and after the 200 mM or 500 mM weekly persulfate doses as shown in Figures 20 and 21.

Interestingly, instead of observing constant PFSA concentrations as we had come to anticipate from previous HAPO tests, we observed a steady decrease in PFSA especially of PFOS (Figure 23). This trend and possible explanations are discussed in detail in the Additional Findings sections. In short, we hypothesized that the ionic strength of the solutions affected the bulk solution behavior of PFOS, creating sampling artifacts.

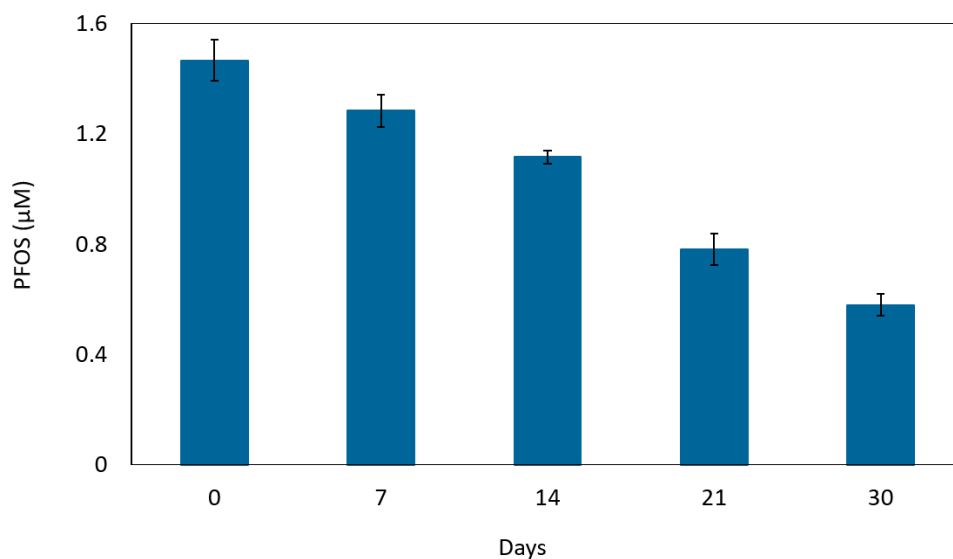


Figure 23. PFSA concentrations during 40°C HAPO of one-to-one slurry of Jacksonville aquifer solids and groundwater. Starting after the Day 5 measurement point following peroxide degradation, weekly doses of 200 mM sodium persulfate were added. PFOS concentrations decreased each week, which we investigated in follow-up experiments described in Additional Findings.

We repeated these weekly persulfate dosing experiments to verify these results without the peroxide pretreatment step. We tested a range of persulfate concentrations, including 50, 100, 200, and 300 mM, added weekly as shown in Figure 24. Our results show over 97% of PFAS destruction under both low persulfate doses, though a longer reaction time was required. For all treatments, the DOC decreased by about 50% after 63 days, suggesting that some recalcitrant organics were resistant to persulfate oxidation and could have contributed to sulfate radical scavenging (Figure 25).

The success of low doses of persulfate during 40°C HAPO is encouraging and likely has a milder impact to the aquifer environment. It would also result in lower chemical costs because less persulfate and neutralizing agent (e.g., carbonate or NaOH) after treatment would be required. However, the cost savings of many lower dose events would have to be compared with having fewer, high concentration oxidant delivery events. In ISCO field studies, it is common to have 1-2 oxidant delivery events with a median duration of 4-6 days each (Krembs 2008). HAPO for PFAS destruction, on the other hand, may require 4+ delivery events depending on the chosen persulfate concentration.

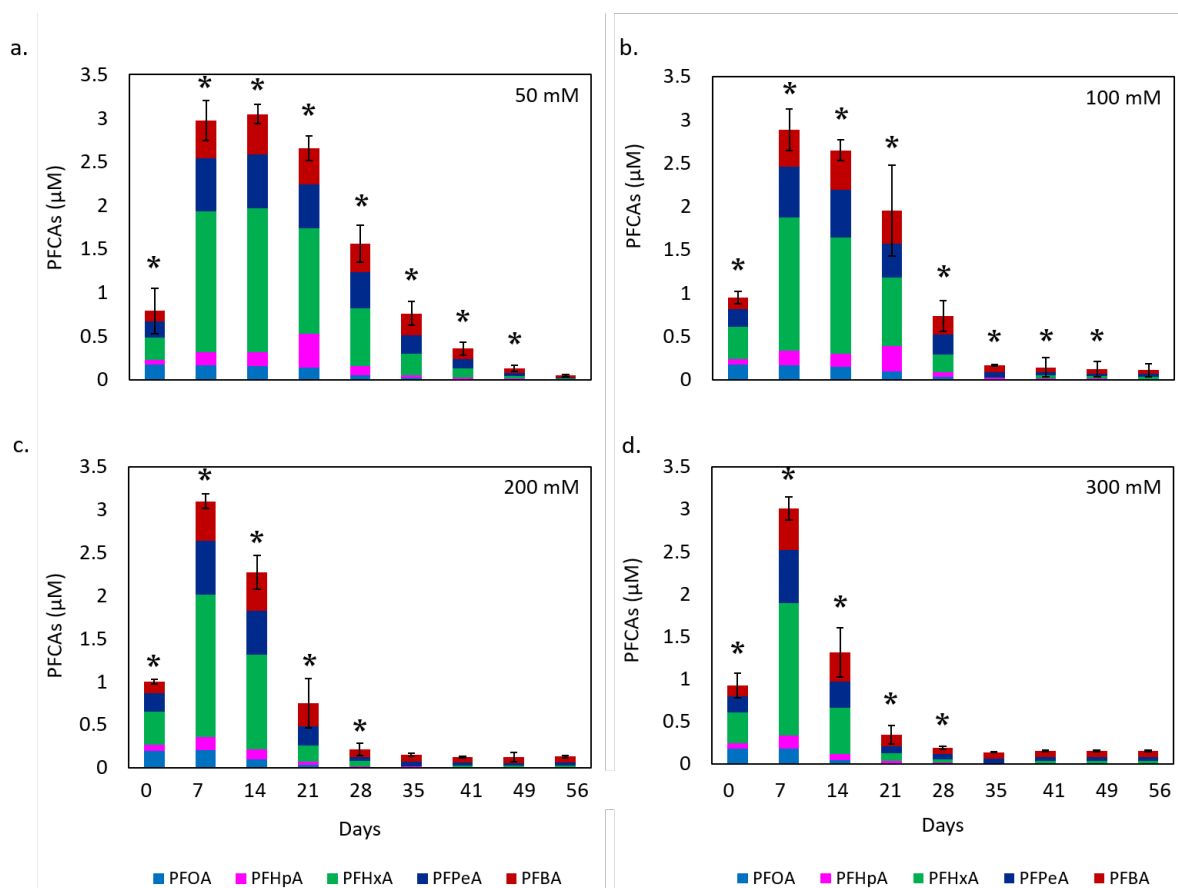


Figure 24. HAPO at 40°C of contaminated groundwater and aquifer solids (1000 g/L), with a) 50 mM, b) 100 mM, c) 200 mM, and d) 300 mM sodium persulfate doses added weekly, shown by the asterisks. Error bars represent triplicate reactors.

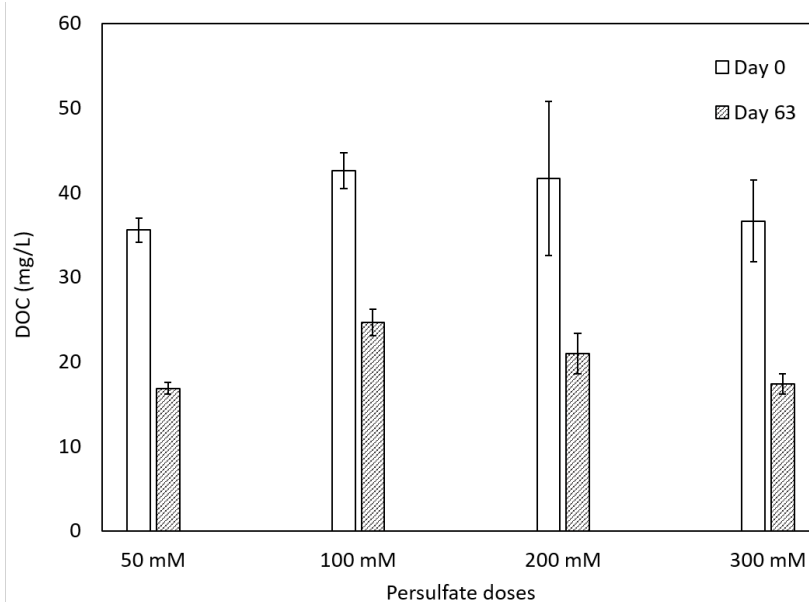


Figure 25. DOC concentrations at the beginning and end of the experiment shown in Figure 24. Despite the dosing, DOC decreased to ~18-25 mg/L, potentially indicating that there existed recalcitrant organic compounds that the sulfate radicals did not degrade.

Once HAPO treatment of an AFFF-contaminated site is completed, the groundwater would have to be neutralized. We investigated adding calcite or sodium hydroxide to neutralize the slurry tests from the HAPO test shown in Figure 4.6, and monitored how metals were affected. For the final time point of the experiment, increasing concentrations of persulfate (the weekly doses of 50, 100, 200, and 300 mM persulfate) added resulted in higher concentrations of dissolved metals measured (Figure 26). After neutralizing the slurries with either calcite (55 g/L for 50 mM persulfate dose condition, up to 135 g/L for 300 mM persulfate dose condition) or sodium hydroxide (27 g/L for 50 mM persulfate dose condition, up to 70 g/L for 300 mM persulfate dose condition), metals concentrations decreased significantly; both neutralization methods resulted in precipitation and 99% removal of metals from the aqueous phase (Figure 26).

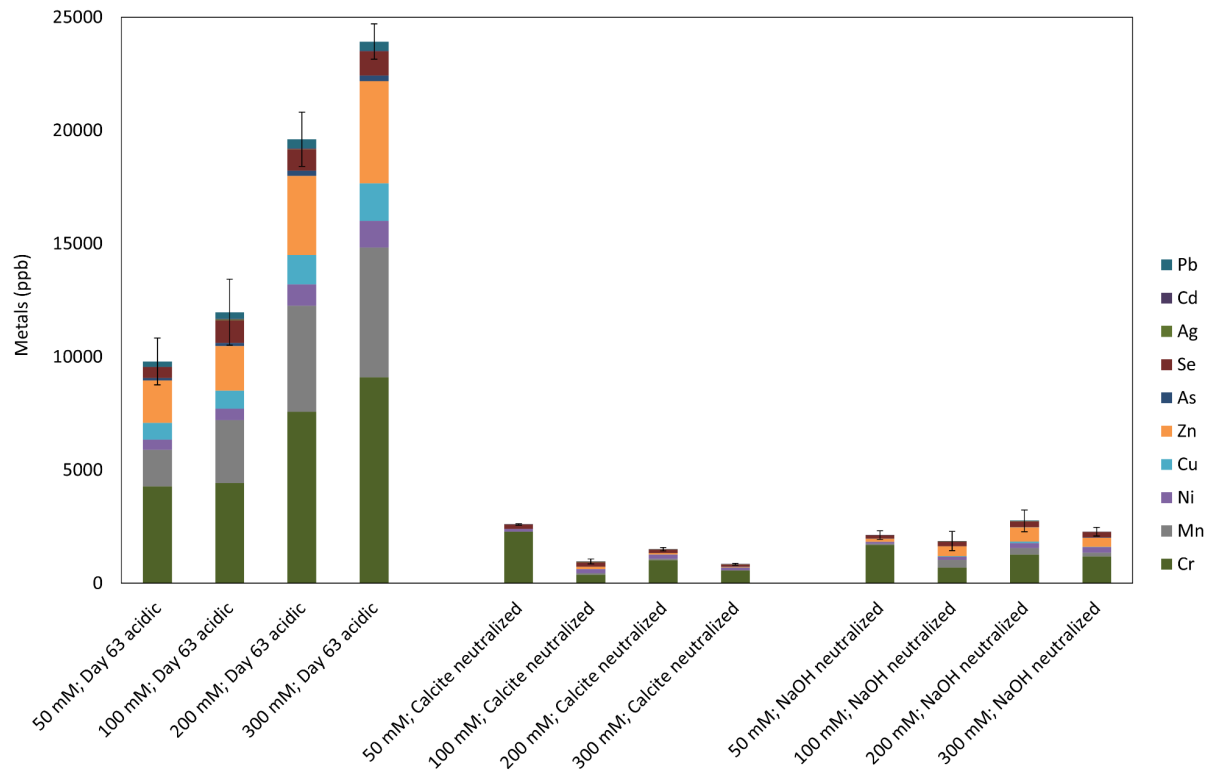


Figure 26. Dissolved metal concentrations ( $\mu\text{g/L}$ ) resulting from the various persulfate concentrations added during  $40^\circ\text{C}$  HAPO experiment with 1000 g/L aquifer solids. Results are shown immediately after the experiment (in the first four columns) and after calcite (second four columns) or NaOH neutralization (final four columns). Metals shown are chromium (Cr), manganese (Mn), nickel (Ni), copper (Cu), zinc (Zn), arsenic (As), selenium (Se), silver (Ag), cadmium (Cd), and lead (Pb).

## Objective 2: ISCO effects down gradient

### *PFAS impacts on microorganisms*

Since HAPO does not transform PFASs such as PFOS, we first tested what concentrations would be inhibitory to co-contaminant-degrading microbial communities as PFOS would remain after any applied HAPO treatment. We tested increasing concentrations of PFOS on aerobic pure culture CB1190, which degrades dioxane as its carbon source and electron donor (Figure 27), as well as on an enrichment culture seeded from an AFFF-contaminated site that degrades toluene (Figure 28).

CB1190 showed inhibition of dioxane degradation at 100  $\mu\text{M}$  PFOS, while 1  $\mu\text{M}$  and 10  $\mu\text{M}$  had no effect compared to the control (Figure 27). Alternatively, the toluene degrading enrichment culture experienced a delay in toluene degradation when exposed to 50  $\mu\text{M}$ , 100  $\mu\text{M}$ , and 175  $\mu\text{M}$  PFOS; interestingly, when a second toluene dose was added to the culture, in the 50  $\mu\text{M}$  and 100  $\mu\text{M}$  sets the toluene degradation occurred much more rapidly than the first dose. This result suggests that some enrichment cultures can overcome the negative impact of PFOS, whereas pure cultures such as CB1190 may be more irreversibly impacted.

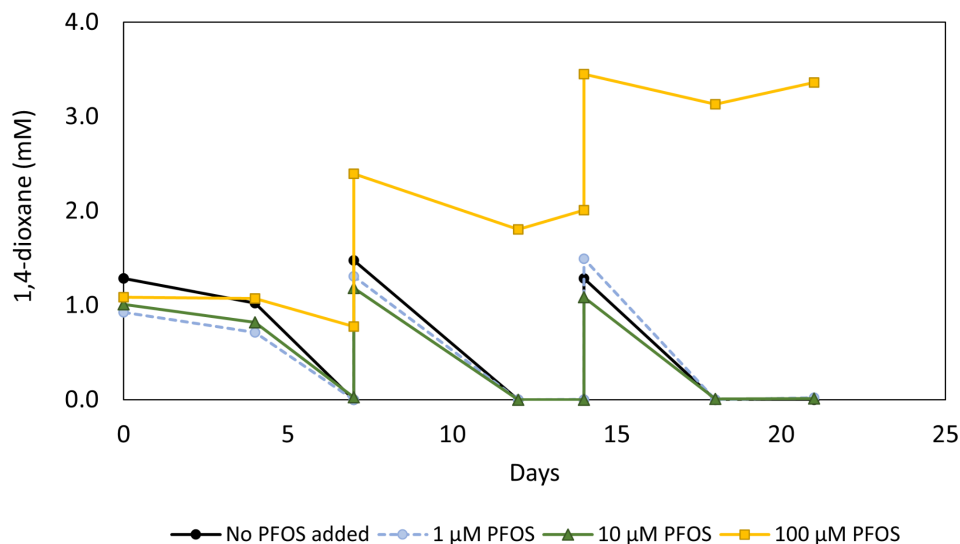


Figure 27. Effects of increasing PFOS concentrations on the pure culture *Pseudonocardia dioxanivorans* CB1190 using 1,4-dioxane as its substrate.

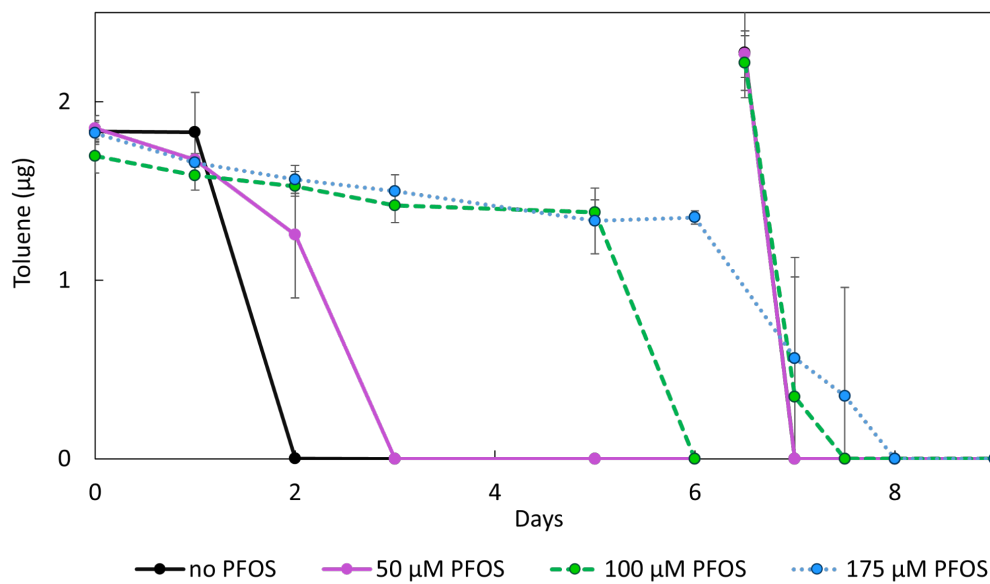


Figure 28. Toluene degradation by an aerobic enrichment culture from an AFFF contaminated AFB, exposed to increasing concentrations of PFOS. While the presence of PFOS delayed toluene degradation for the first toluene dose, a second dose was rapidly transformed in the presence of 50 and 100 µM PFOS.

### *HAPO impacts on microorganisms*

We then started the examination of the effects of HAPO on enrichment cultures from AFFF-contaminated sites. We first exposed an enrichment culture, which had been enriched to degrade BTEX components, to increasing concentrations of sodium sulfate, as this would be a primary salt remaining after HAPO had been conducted. With all concentrations of sulfate (10, 50, 100, and 200 mM), BTEX was degraded similarly to the no-salt control (Figure 29).

Although the BTEX degradation was not impacted, we also extracted the DNA in each of the salt conditions to compare the phyla and genera in each of the triplicates (Figures 30 and 31). Proteobacteria relative abundance appeared to decrease as sulfate concentration increased. The relative abundance of Firmicutes remained relatively constant, between ~0.10-0.25 with increasing salt concentration. Actinobacteria had an increase in relative abundance with increasing salt concentration (Figure 30).

Additionally, *Rhodococcus*, known to degrade BTEX compounds, appeared to maintain a relative abundance of ~0.75 at sulfate concentrations 50, 100, and 200 mM, indicating that this genus can withstand relatively high concentrations of salts. This result indicates that *Rhodococcus* would be able to degrade BTEX compounds post-HAPO in the resulting sulfate concentration after HAPO was no higher than 200 mM.

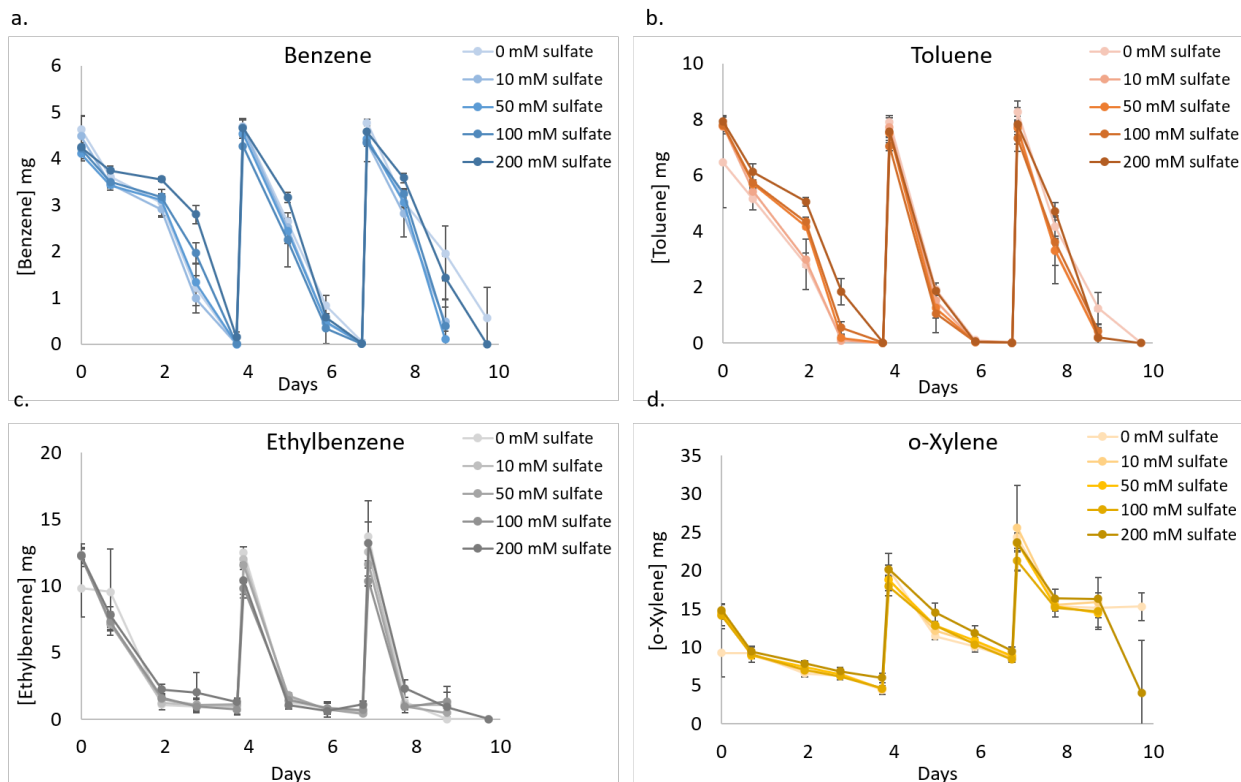


Figure 29. BTEX degradation by an enrichment culture in the presence of increasing concentrations of sodium sulfate. The enrichment culture was not impacted by the highest concentration of sulfate, indicating that microbial communities can be robust in the presence of salts.

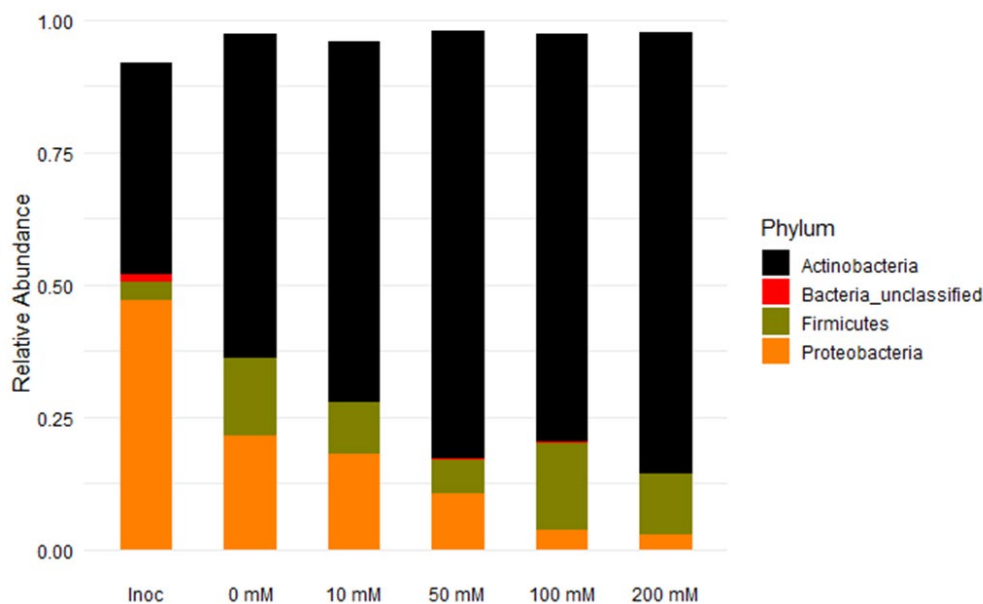


Figure 30. Phyla of the BTEX-degrading inoculum and for each of the sodium sulfate concentrations shown in Figure 29. While BTEX degradation was not impacted by salt

concentration, the present phyla were. Proteobacteria were the least abundant in the presence of 200 mM sodium sulfate.

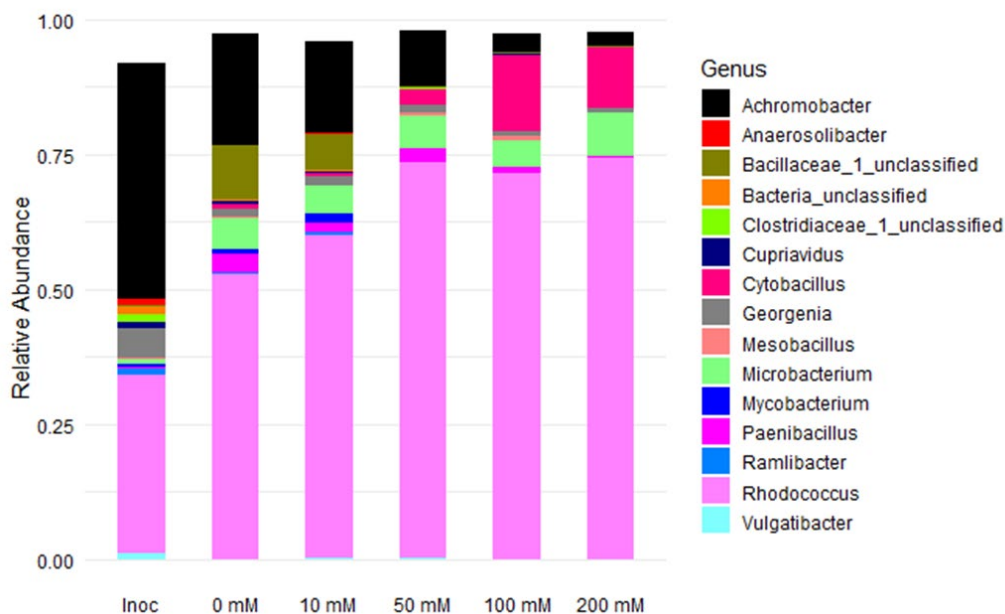


Figure 31. Genera of the BTEX-degrading inoculum and for each of the sodium sulfate concentrations shown in Figure 29. Rhodococcus, known to degrade BTEX, remained at high (~0.75) relative abundance at the three highest sodium sulfate concentrations, 50-200 mM.

We then repeated this salt effects experiment, but with an actual HAPO solution. The HAPO solution was made by heat-activating a concentrated solution of sodium persulfate at 85°C until the persulfate was converted to sulfate. We then neutralized the solution with NaOH so that the acidity of the solution did not impact the microbial enrichment cultures. We exposed the same BTEX-degrading community to increasing concentrations of the HAPO solution, which had approximate concentrations of sulfate of 0, 50, 100, 200, and 500 mM. We added the 500 mM condition after we found that 200 mM did not impact BTEX degradation from Figure 29.

Similarly to Figure 29, Figure 30 shows that most concentrations of the HAPO solution did not impact BTEX degradation. Only the highest concentration, ~500 mM sulfate in HAPO solution, inhibited the degradation of benzene and toluene (Figure 32). Interestingly, the ethylbenzene and ortho-xylene were degraded at the same rate across the salt conditions; the highest HAPO solution condition did not inhibit these two substrates' degradation.

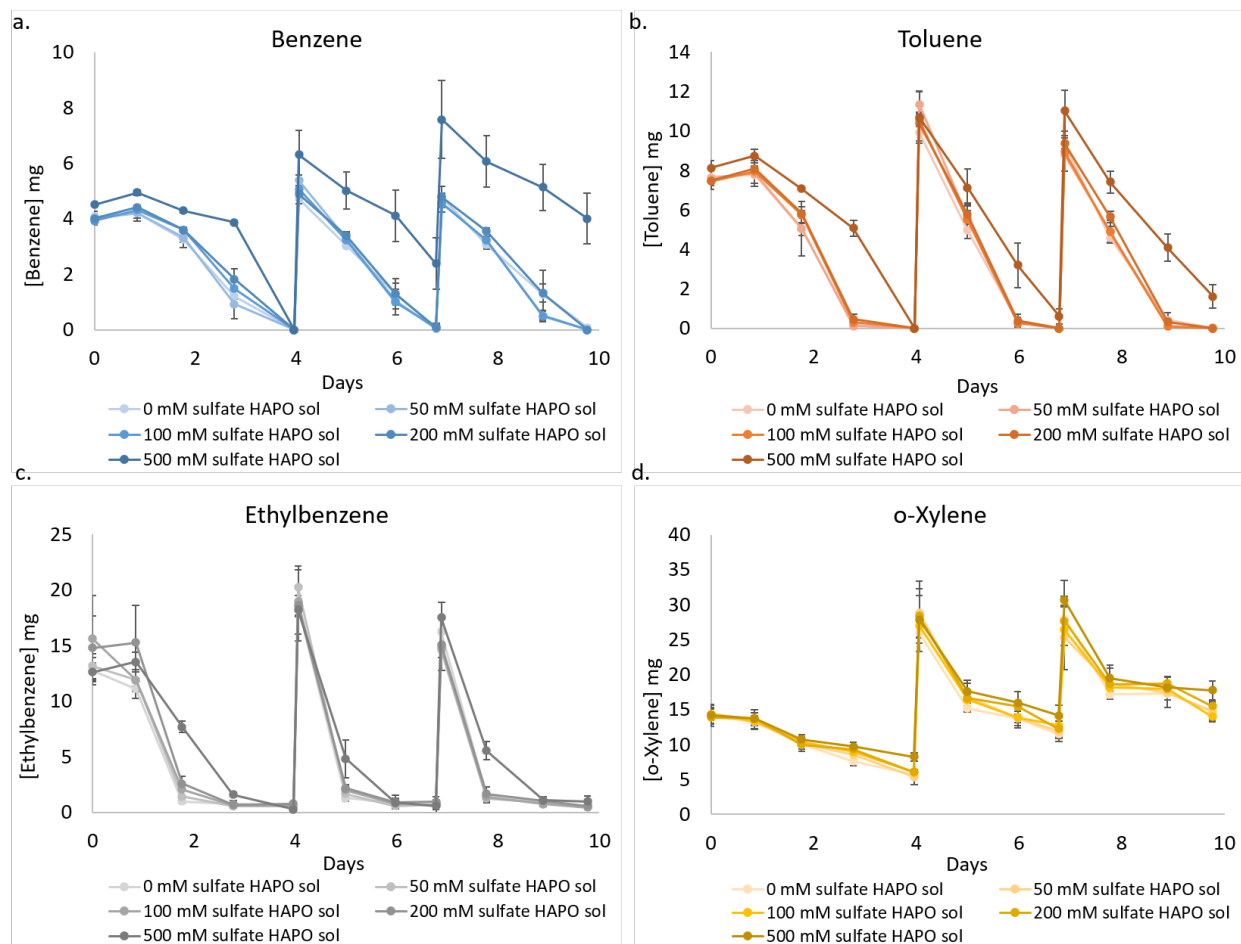


Figure 32. BTEX degradation by an enrichment culture in the presence of increasing concentrations of HAPO solution (“HAPO sol”). The HAPO solution was a concentrated sodium persulfate solution that was heat-activated so that the persulfate was converted to sulfate. Additionally, the HAPO solution was neutralized before adding to the microcosms.

The Actinobacteria initially increased in relative abundance with increasing salt, but then dropped in abundance in the presence of 500 mM sulfate as HAPO solution. In its place, Firmicutes had an increase in relative abundance at 500 mM sulfate as HAPO solution. Similarly, *Rhodococcus* was negatively impacted at the highest HAPO solution concentration, dropping in abundance from ~0.60 to ~0.45.

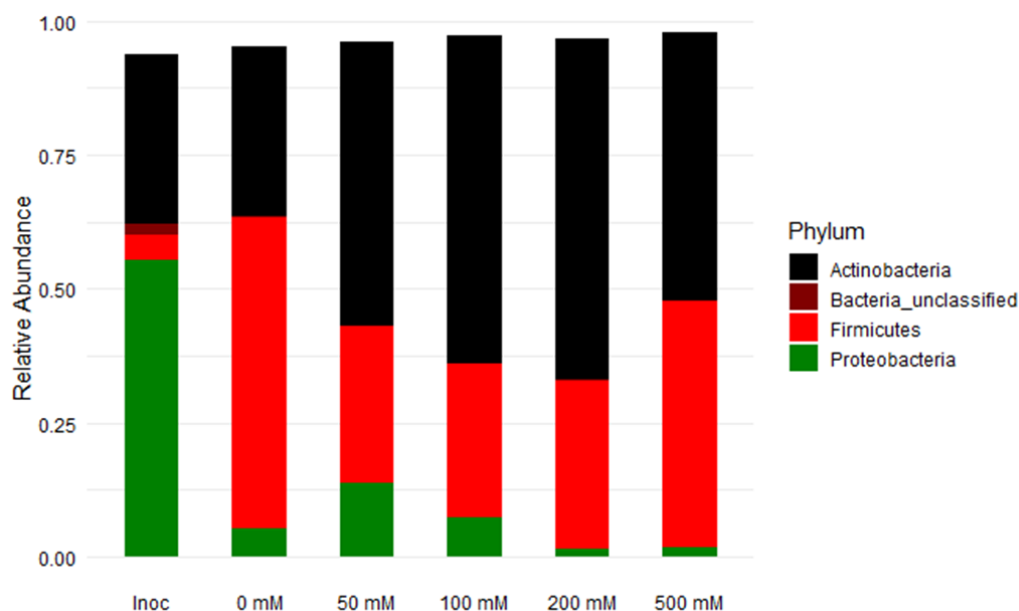


Figure 33. Phyla of the BTEX-degrading inoculum and for each of the HAPO solution concentrations shown in Figure 32. Actinobacteria relative abundance dropped between 200 mM and 500 mM concentration of the HAPO solution as sulfate.

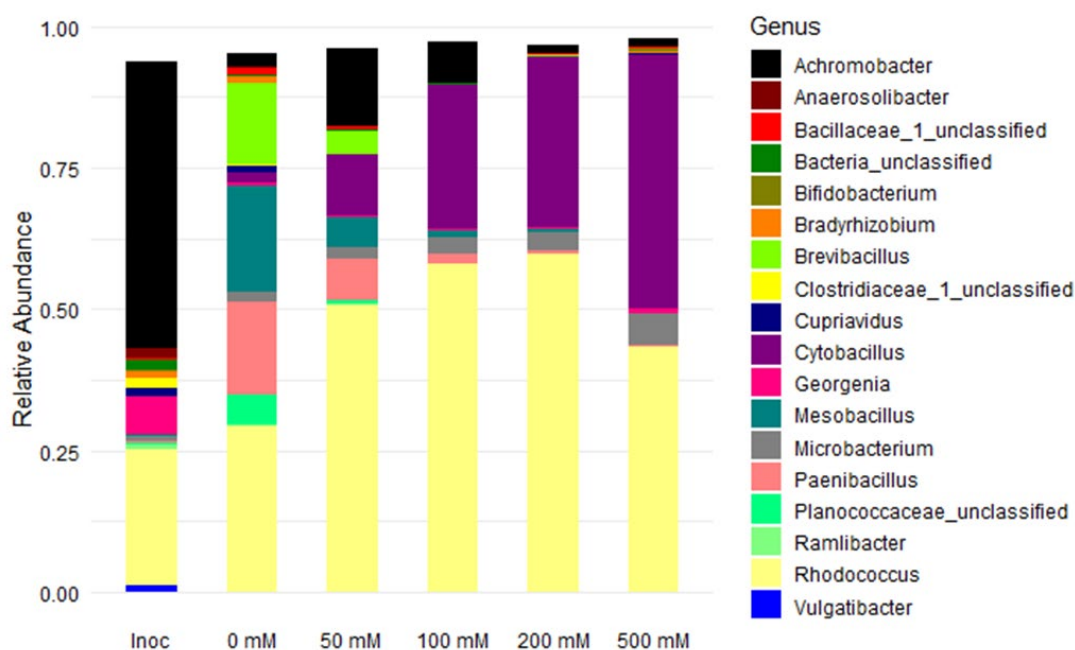


Figure 34. Genera of the BTEX-degrading inoculum and for each of the HAPO solution concentrations shown in Figure 32. Cytobacillus appeared to increase in relative abundance as the salt concentration increased. Rhodococcus relative abundance dropped between 200 mM and 500 mM concentration of the HAPO solution as sulfate.

### Objective 3: Bioremediation of AFFF and co-contaminants before ISCO

#### *Consumption of AFFF Organics and Biotransformation of ECF-Based Precursors*

The purpose of this task was to identify microcosms capable of consuming organics in 3M AFFFs. We compared the consumption of DOC in ECF-based AFFFs and fluorotelomer-based AFFFs. Generally in our tests, organics in fluorotelomer AFFFs degraded much more readily than in 3M AFFF (Figure 35). In one such test, ~250 mg/L of DOC in National Foam AFFF was consumed within 20 days compared to ~50 mg/L in 3M AFFF over 40 days (Figure 35). We hypothesized that the high concentrations of PFOS in 3M AFFFs were responsible for microbial inhibition of DOC degradation, and thus we found few conditions that resulted in rapid or effective DOC degradation for 3M AFFFs. During these tests, we started investigating the possible biotransformation of the prevalent 3M precursor AmPr-FHxSA.

The results from this Subtask have been accepted for publication in *Environmental Science & Technology* (Cook et al 2022; DOI: 10.1021/acs.est.2c00261)

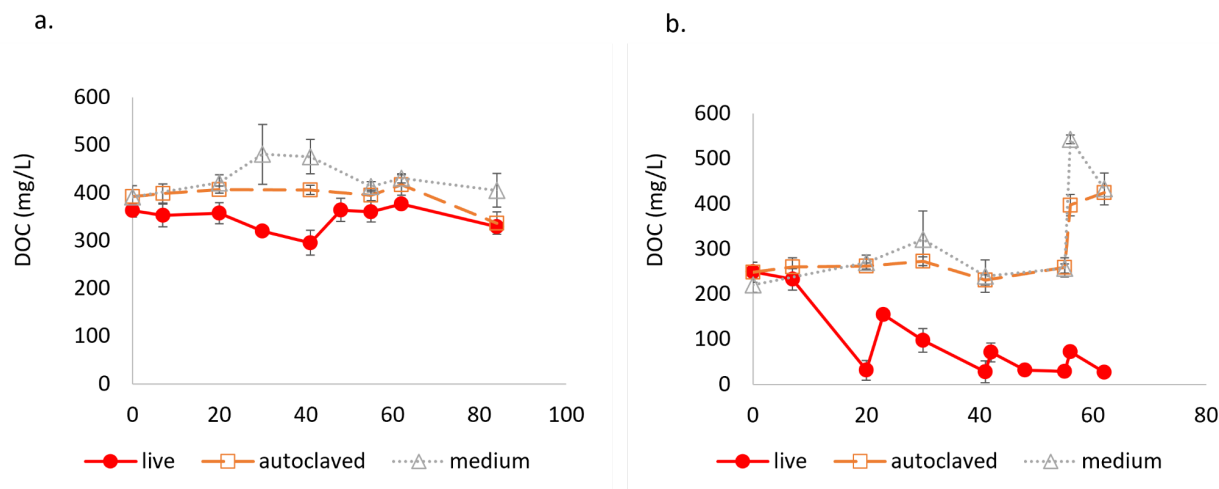


Figure 35. DOC in microcosm experiments with a) 3M AFFF and b) National Foam AFFF.

We first were investigating the biotransformation of AmPr-FHxSA present in the 3M AFFF. In these AFFF experiments, consumption of organic carbon was slower in the enrichments containing the ECF-based formulation than those containing the fluorotelomer-based formulation (Figure 36) despite adequate oxygen in both systems, similarly to the 3M AFFF tested in Figure 36. This suggests that some component(s) in the ECF-based AFFF can inhibit the microbial community, such as PFOS, which has been shown to be toxic to microorganisms (Fitzgerald et al 2019, Sheng et al 2021, Weathers et al 2016) and was present at concentrations three times that of AmPr-FHxSA. After 85 days, live and autoclaved controls showed a similar AmPr-FHxSA concentration decrease. Though not statistically significant compared to controls when averaged, two of the three replicates showed twice the concentration of FHxSA compared to the autoclaved controls (Figure 36). However, the multiple possible precursors within the formulation confounded the analysis. While we performed HRMS on multiple timepoints throughout the experiment, many of the results were not statistically significant compared to the initial timepoint (Figure 37), making it challenging to draw conclusions on the fate of AmPr-FHxSA. There was no indication of

biological activity in the autoclaved controls based on ATP (data not shown), which suggests any decreases of AmPr-FHxSA in the autoclaved controls were because of abiotic processes.

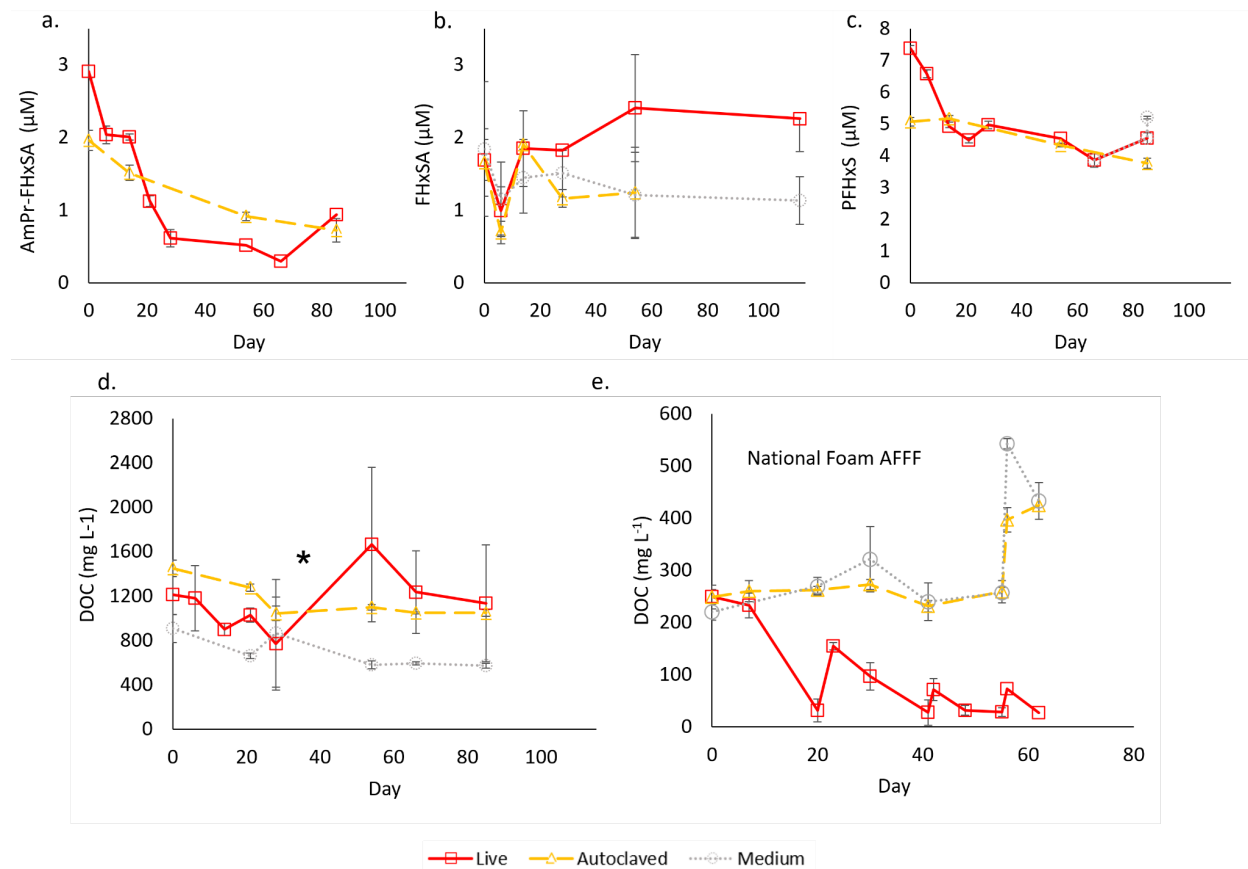
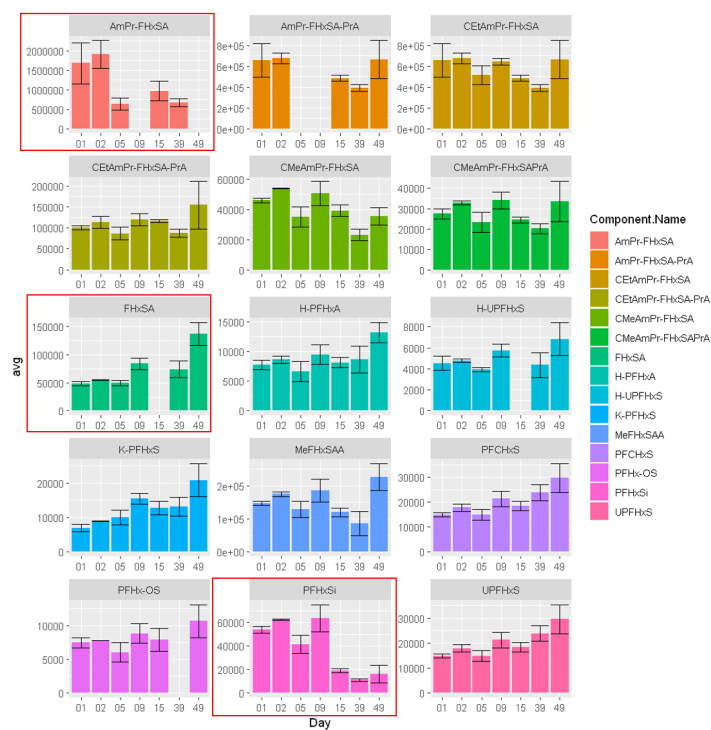
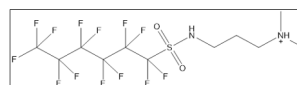


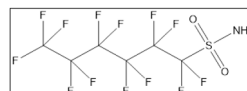
Figure 36. ECF-based AFFF biotransformation study amended with DGBE. Concentrations of a) AmPr-FHxSA, b) FHxSA, c) PFHxS, and d) DOC for the ECF-based AFFF biotransformation test. Compared to the e) DOC degradation during a fluorotelomer-based AFFF biotransformation test, there was little to no DOC degradation in the ECF AFFF test, indicating recalcitrant non-fluorinated surfactants. Asterisk denotes respike with DGBE as additional carbon. 1:1,000 dilution AFFF.



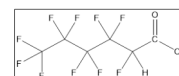
AmPr-FHxSA (parent compound)



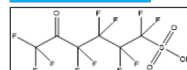
FHxSA



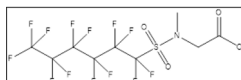
H-PFHxA



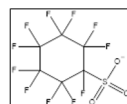
K-PFHxS



MeFHxSAA



PFCHxS



UPFHxS

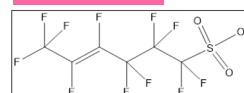


Figure 37. HRMS results, and relevant structures, from the ECF-based AFFF biotransformation experiment.

To isolate possible aerobic biotransformation pathways of AmPr-FHxSA in the absence of PFOS or other inhibitors in AFFF, we performed biotransformation experiments by adding only AmPr-FHxSA with either methane, acetate, DGBE, or BTEX as a carbon source. We hypothesized that enrichments developed from these four conditions would result in functionally divergent microbial communities with different transformation products and yields. After 70 days of incubation, the concentration of AmPr-FHxSA decreased under all conditions but had the most significant disappearance (83% decrease) in the acetate-oxidizing cultures, followed by the methane-oxidizing cultures (Figure 38). The live methane cultures resulted in the highest concentrations of generated targeted transformation products (Figure 38)—about 70 nM and 9 nM of FHxSA and PFHxS, respectively, or approximately 40% and 6.7% of the loss of the parent.

Similar to the AFFF biotransformation experiment, AmPr-FHxSA decreased in the medium-only but without detectable FHxSA or PFHxS. Some AmPr-FHxSA loss could be explained by trace amounts of soil or biofilms not captured in sampling, as the autoclaved controls had less AmPr-FHxSA loss compared to the medium-only controls. However, because of the medium-only controls, we believe that the majority of the AmPr-FHxSA decrease is primarily due to sorption to the container. Low recovery could also have occurred from sorption to the needle syringe used for sampling or from aggregation or accumulation at interfaces, including air-water (Steffens et al 2021). Studies have shown that AmPr-FHxSA has a propensity to sorb to surfaces (Nickerson et al 2020), can be unstable from two oxidizable nitrogens and a lone pair of electrons (Liu et al 2021), and perhaps has temperature-dependent instability like other sulfonamides (Woudneh et al 2019). Despite the decrease in AmPr-FHxSA, autoclaved and medium-only controls did not form the stable intermediate FHxSA or terminal product PFHxS (Figure 38 and Appendix A). Because of the challenges with AmPr-FHxSA, we prioritized analyzing the differences in transformation products between the biotic cultures and abiotic controls.

In the methane, acetate, and DGBE cultures, approximately 12-15 nM FHxSA was present at the start of the experiment due to the AFFF-impacted inoculum source; this background was not in the BTEX culture, presumably because its inoculum source was older and had been amended with fresh media several times, diluting FHxSA below the limit of detection. Although the DGBE and BTEX cultures did not produce FHxSA, low concentrations (2.6 and 2.1 nM, respectively) of PFHxS were produced. This lack of FHxSA suggests that AmPr-FHxSA might be able to transform to PFHxS without accumulation of FHxSA, or that other intermediates not targeted in the LC-MS/MS method can be hydrolyzed directly to PFHxS. Given the stability of FHxSA in the environment (D'Agostino et al 2017), it is unlikely that this compound was formed as an intermediate that was quickly transformed to PFHxS.

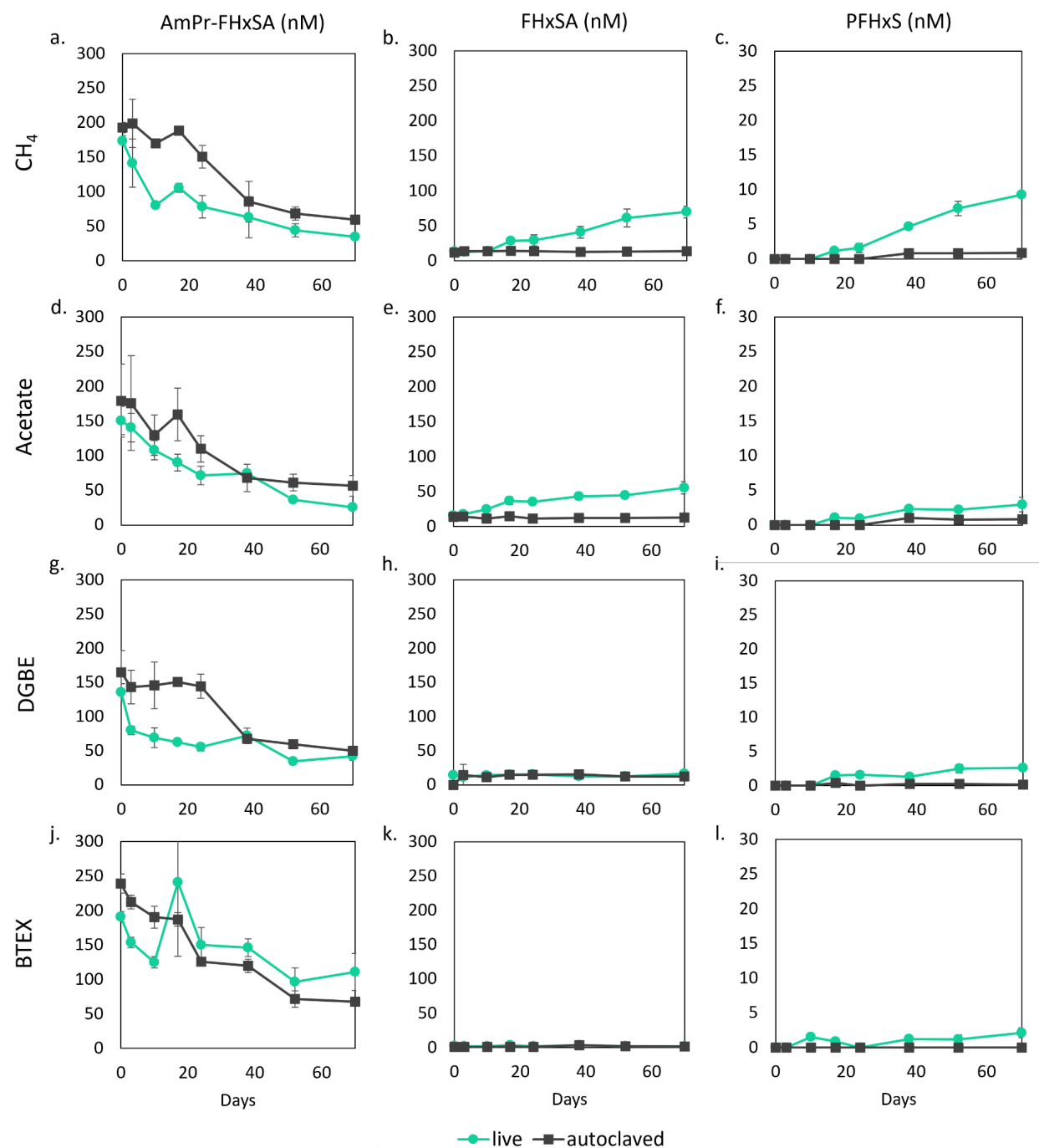


Figure 38. From left to right, AmPr-FHxSA depletion and FHxSA and PFHxS production (nM) over 70 days under aerobic conditions with four carbon sources: a-c) methane (CH<sub>4</sub>), d-f) acetate, g-i) DGBE, and j-l) BTEX. Error bars represent standard deviation of triplicate reactors. Green circles represent the live cultures, while gray squares represent autoclaved controls.

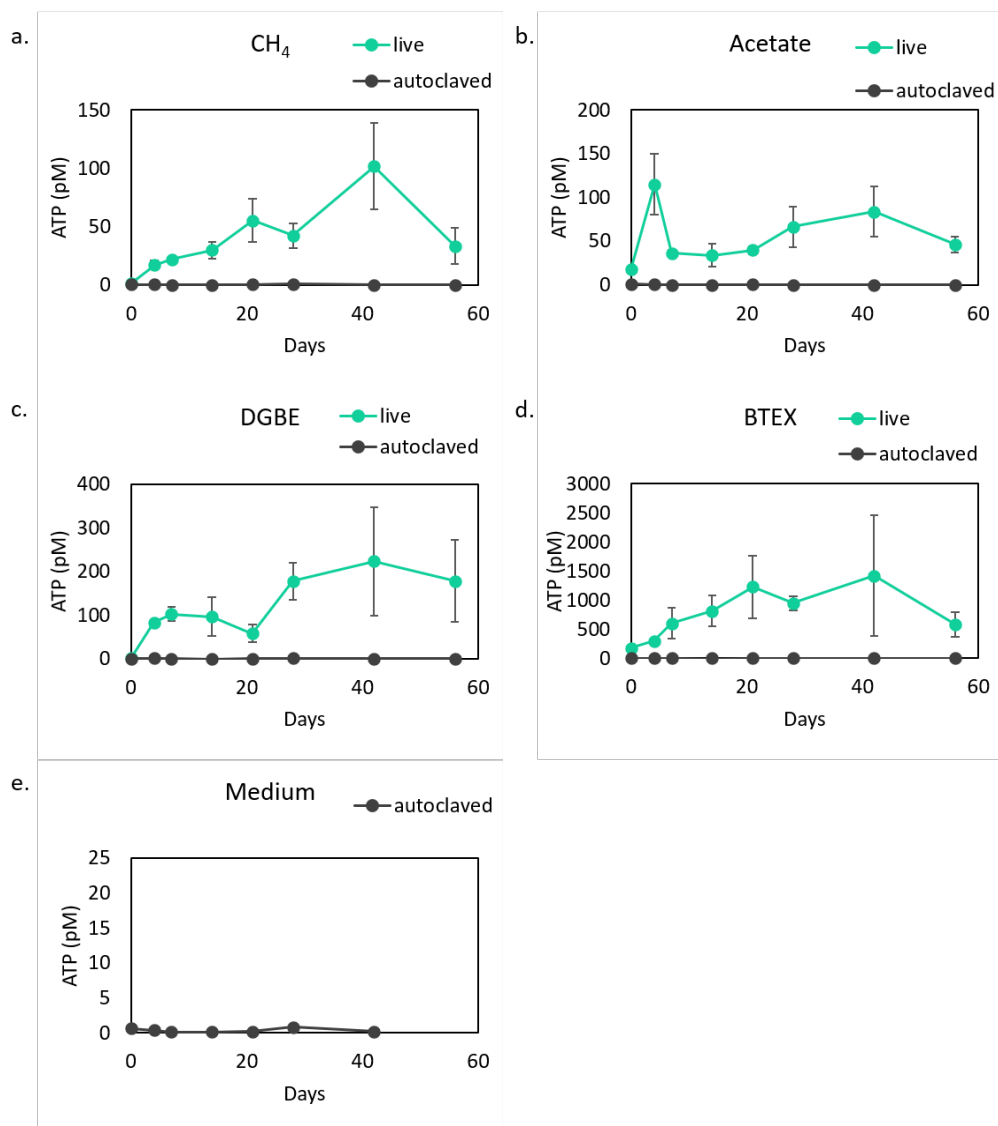


Figure 39. ATP measurements for the live and autoclaved controls for each of the four carbon sources, as well as the medium controls.

We compared the percentage of FHxSA and PFHxS production (based on initial parent concentration) in the live controls to the perfluorooctyl-based analogue in literature. In this study, we found FHxSA production to be approximately 40% and 36% in the methane and acetate cultures, respectively, after 70 days compared to Liu et al (2021) who reported approximately 7.5% FOSA production after 90 days. PFHxS production was 5%, 2%, 2%, and 1% for methane, acetate, DGBE, and BTEX cultures over 70 days compared to 2.6% PFOS production after 90 days. Although it is challenging to compare based on the different microbial communities and conditions, our results suggest that some aerobic microbial communities can result in much faster rates of FHxSA production compared to FOSA and slightly faster or similar rates, depending on the community, of PFHxS production compared to PFOS.

Additionally, because some literature suggests a difference in biotransformation rates of branched versus linear isomers of ECF-based precursors (Liu et al 2019), we examined the ratios of branched versus total (linear + branched) peak areas of AmPr-FHxSA and PFHxS on day 70 (Table 2). Linear AmPr-FHxSA precursors may be more readily transformed into PFHxS by soil microbial communities. All reactors had a higher percentage of branched compared to the AmPr-FHxSA stock (Table 2), suggesting that linear AmPr-FHxSA sorbed more than the branched isomer. Moreover, the methane and acetate live controls had higher percentages of branched AmPr-FHxSA on day 70 compared to the autoclaved controls. While the AmPr-FHxSA stock solution contained an average branched percentage of  $9.3 \pm 1.4\%$  (Table 2), the acetate live cultures resulted in 28% branched AmPr-FHxSA remaining on day 70 compared with 15.4% in the autoclaved reactors. This result indicates that, especially in the acetate cultures, linear AmPr-FHxSA was more likely to be biotransformed compared to its branched isomer. Additionally, the percentage of branched PFHxS produced, particularly in the methane treatments (2.8% branched), was lower than the percentage branched of the parent compound, further suggesting that linear PFHxS precursors may be more readily transformed into PFHxS by soil microbial communities.

To evaluate the mass balance, we performed the TOP assay on day 70 samples for the four carbon source treatments. FOSA and similar eight carbon ECF-based precursors have been shown to produce PFOA during the TOP assay (Houtz et al 2012, Martin et al 2019). Therefore, we expected the TOP assay to produce PFHxA in concentrations similar to the initial concentration of AmPr-FHxSA, minus the PFHxS produced biotically. Indeed, the only detected PFAAs after the TOP assay were PFHxA and PFHxS (Figure 40) but resulted in incomplete balances likely due to sorption of AmPr-FHxSA as mentioned. An appreciable fraction of the PFHxA produced likely came from the oxidation of FHxSA, which was a biotransformation product during the experiment. For example, 70 nM of FHxSA was produced by day 70 in the methane enrichments, which, if completely transformed to PFHxA during the TOP assay, would account for about 77% of the PFHxA produced during the TOP assay. For the acetate, DGBE, and BTEX cultures, this ratio was 45, 20, and 3.5%, respectively. The remaining PFHxA produced during the assay presumably came from other biotransformation products and/or from the remaining AmPr-FHxSA. Interestingly, the PFHxS concentrations after the assay were greater than the concentrations measured on day 70 of the biotransformation experiment. As will be discussed, this could point to some transformation of perfluoroalkyl sulfonamides to PFHxS during the TOP assay.

Table 2. Percent branched AmPr-FHxSA and PFHxS on Day 70. The stock solution of AmPr-FHxSA contained  $9.3 \pm 1.4\%$  branched AmPr-FHxSA as measured on the LC-MS/MS.

|                    | AmPr-FHxSA Day 70 |         |        |  | PFHxS Day 70 |         |        |
|--------------------|-------------------|---------|--------|--|--------------|---------|--------|
|                    | Average           | Std Dev | T test |  | Average      | Std Dev | T test |
| Methane Live       | 17.4              | 0.18    | 0.003  |  | 2.8          | 0.31    | 0.139  |
| Methane Autoclaved | 15.7              | 0.28    |        |  | 11.5         | 6.01    |        |
| Acetate Live       | 28.2              | 2.09    | 0.005  |  | 9.5          | 4.46    | 0.236  |
| Acetate Autoclaved | 15.4              | 0.69    |        |  | 22.3         | 9.73    |        |
| DGBE Live          | 15.2              | 1.39    | 0.642  |  | 8.6          | 6.48    | 0.471  |
| DGBE Autoclaved    | 15.8              | 0.36    |        |  | 14.8         | 5.68    |        |
| BTEX Live          | 10.5              | 2.11    | 0.064  |  | 11.8         | 5.07    | —      |
| BTEX Autoclaved    | 14.5              | 0.27    |        |  | —            | —       |        |
| Medium Control     | 17.8              | 3.08    | —      |  | —            | —       | —      |

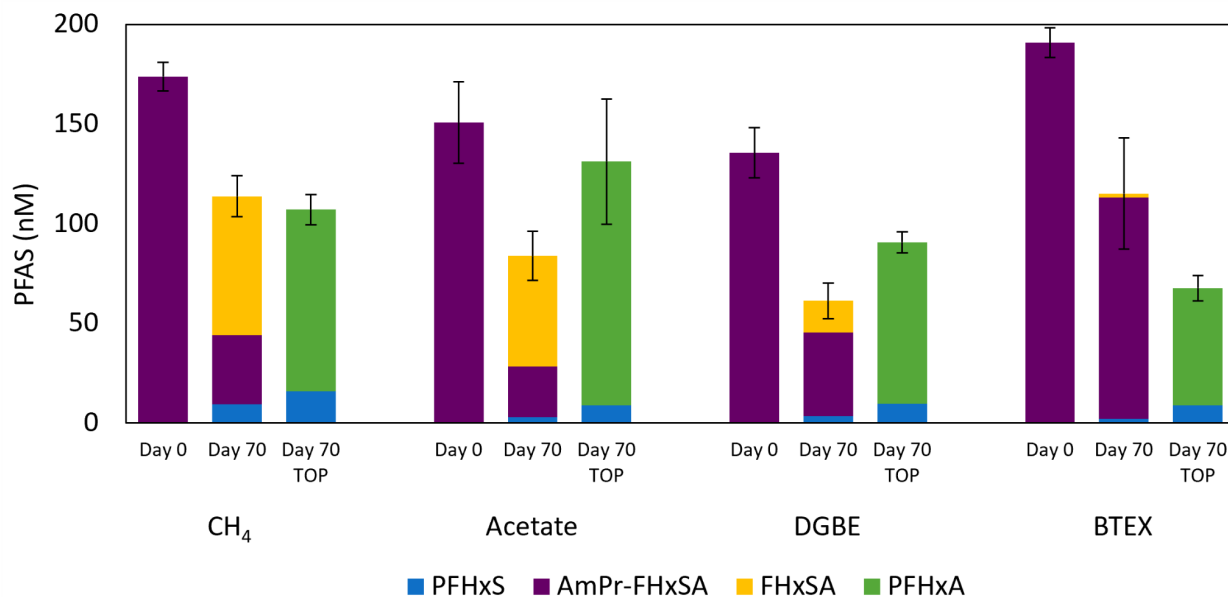


Figure 40. The concentrations of parent on day 0, parent and biotransformation products on day 70, and the TOP assay results conducted on day 70 samples.

We performed HRMS on samples from days 0 and 70 of the live treatments, and on day 70 of the autoclaved sets to explore non-targeted transformation products. Combining the results from HRMS and LC-MS/MS, in addition to some proposed transformation products in recent literature and in *enviPath*, a transformation pathway is proposed in Figure 41. Overall, the methane and acetate cultures had the greatest number of transformation products identified, both containing FHxSA-PrA, FHxSAA, FHxSA, PFHxSi, and PFHxS, and the acetate cultures additionally containing OAmPr-FHxSA. In the DGBE cultures FHxSAA, PFHxSi, and PFHxS were measured, and in the BTEX cultures only PFHxSi and PFHxS. Perfluoroalkyl sulfonamido acids such as FHxSA-PrA and FHxSAA have been detected in the environment, including in AFFF-impacted groundwater and landfill leachate (Barzen-Hanson et al 2017, Allred et al 2014, Nguyen et al 2011).

We detected FHxSA-PrA in the methane and acetate live cultures, as well as one DGBE replicate, though at about an order of magnitude lower peak area than the acetate and methane cultures. On the proposed pathway between AmPr-FHxSA and FHxSA-PrA, we include potential demethylated intermediates based on *enviPath* predictions and Liu et al's (2021) investigation of perfluorooctyl-based ECF-based precursor biotransformation. Also proposed on *enviPath* and in literature was an aldehyde intermediate (Figure 39) (Mejia et al 2016). FHxSA-PrA was additionally measured in one of the DGBE autoclaved controls with a similar peak area as the live replicate, opening the possibility of abiotic transformation to FHxSA-PrA in the DGBE condition.

FHxSAA was likely produced after FHxSA-PrA and was detected in the methane, acetate, and DGBE live cultures, though at an order of magnitude less than FHxSA-PrA. This acetic acid could then lead to FHxSA, which increased in the methane and acetate cultures as shown in Figure 38. We also suggest that FHxSA-PrA can lead directly to FHxSA, as proposed by Liu et al. for the eight-carbon homologs (Liu et al 2021). While FHxSAA could be an intermediate between FHxSA-PrA and FHxSA, we believe it would be a minor pathway based on *enviPath*'s predictions and literature. For example, Rhoads et al (2008) observed FOSA before detecting FOSAA in activated sludge biotransformation of Et-FOSE and thus suggested that FOSAA to FOSA was a minor pathway. Similarly, Yin et al (2018) proposed that direct dealkylation of Et-FOSA to FOSA was faster than biotransformation of FOSAA to FOSA, corroborating these hypotheses. Nevertheless, it is also possible that FHxSAA is a transient intermediate.

FHxSA could then be hydrolyzed to either PFHxSi or directly to PFHxS. Rhoads et al (2008) proposed perfluorooctane sulfinic acid (PFOSi) as a biotransformation intermediate between FOSA and PFOS, and PFOSi has been found to be highly correlated with FOSA in a marine mammal study (Ahrens et al 2009). The methane cultures had the highest peak area of PFHxSi, which correlates with high concentrations of FHxSA and PFHxS in this treatment. Conversely, FHxSA concentrations did not increase in the DGBE or BTEX cultures despite increasing concentrations of PFHxS (Figure 38), indicating that there was rapid transformation to PFHxSi or PFHxS, resulting in no accumulation of FHxSA. Another possibility is that AmPr-FHxSA, FHxSA-PrA, and/or FHxSAA could directly produce PFHxS via biotic hydrolysis. To inform this possibility, we can consider the perfluorooctyl equivalent, FOSA. FOSA has been shown to be quite stable biotically and is readily measured at AFFF-impacted sites and urban surface waters, as is FHxSA (McGuire et al 2014, D'Agostino et al 2017, McDonough et al 2021). The half-life of FOSA in aerobic soils has been predicted to be >700 days (Liu et al 2019). Many ECF-based

biotransformation studies that measured FOSA and PFOS as key transformation products did not observe sequential peak times over the course of their experiments that would have suggested FOSA transforming to PFOS (Liu et al 2021, Avendano et al 2015, Liu et al 2019, Mejia et al 2016, Zhang et al 2020). In a recent follow up FHxSA biotransformation experiment with acetate as a carbon source, we measured less than 2% PFHxS after 90 days of incubation (data not shown), compared to 2.4% PFHxS in the acetate cultures observed in this study after 70 days. Therefore, while FHxSA may be a key biotransformation product of ECF-based precursors, it could be a rate limiting bottleneck in the pathway shown in Figure 41. This hypothesis is supported by high frequency detection in surface waters impacted by AFFF (D'Agostino et al 2017). The recalcitrance of FHxSA underscores the significance of perfluoroalkyl sulfonamides and the need to include short-chain homologs in monitoring lists. More research should be done on their prevalence in the environment, potential toxicity, and human exposure.

While PFHxSi peak areas increased between days 0 and 70 for the methane, acetate, DGBE, and BTEX cultures, it was also detected in the methane, DGBE, and BTEX autoclaved controls, indicating that this compound could be produced abiotically. Finally, PFHxS was found in each of the four carbon source live cultures (and in none of the autoclaved controls) indicating that the production of PFHxS necessitates a biological step. Interestingly, when comparing the pathway step probabilities in enviPath, transformation of any of the precursors (i.e., AmPr-FHxSA, FHxSA-PrA, and the grayed-out theoretical intermediates) directly to PFHxS was more probable than the stepwise transformation shown on the left side of Figure 41. Additionally, Chen et al (2020) suggests that the eight-carbon equivalent to OAmPr-FHxSA could biologically transform directly to PFOS, meaning that OAmPr-FHxSA similarly could be an immediate precursor to PFHxS. Because there are potentially several direct biotransformation precursors to PFHxS (e.g., FHxSA-PrA to PFHxS, FHxSA to PFHxS, etc.), it is possible that several of these pathways can occur simultaneously. If FHxSA biotransformation to PFHxS is as slow as FOSA to PFOS, FHxSA could prove to be a long-term biotic source of PFHxS at AFFF-contaminated sites, even long after AmPr-FHxSA or other ECF-based precursors have transformed. Based on our results, this could be especially important where methane and acetate are present carbon sources.

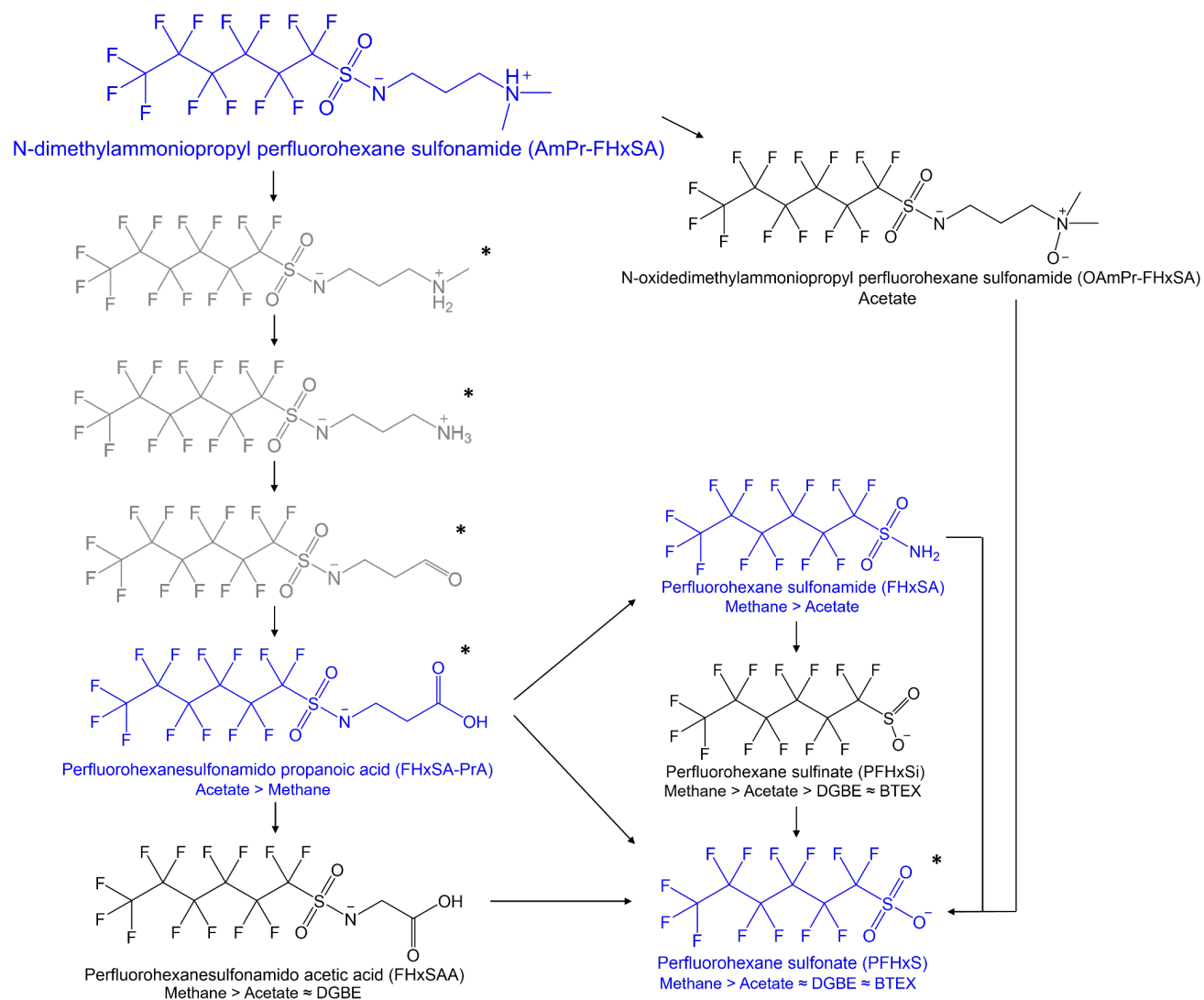


Figure 41. Proposed transformation pathway of AmPr-FHxSA under various carbon source treatments. The faded structures in gray were not measured in this study but proposed in literature (Liu et al 2021) for the eight-carbon analogues and/or on envPath. Asterisks denote products proposed via envPath. The blue structures were measured in higher abundances via HRMS.

We extracted the DNA for each of the live treatments on day 70 to compare the phyla, genera, and diversity of each of the four carbon source enrichment cultures (Figure 42). The BTEX, methane, and DBGE cultures were predominantly composed of Proteobacteria (57%, 77%, and 77% respectively), whereas the acetate culture was primarily made up of Actinobacteria (56%) with a smaller proportion of Proteobacteria (31%) (Figure 42). Actinobacteria made up an appreciable, though lesser proportion of the three Proteobacteria-dominant cultures (5-27%). These two phyla were similarly found to dominate a PFAS aerobic soil microcosm experiment when not provided an external source of organic carbon (Zhang et al 2020), suggesting that Proteobacteria and Actinobacteria are tolerant to PFAS contamination.

The methane culture, which contained the highest concentrations of transformation products, was also composed of an appreciable relative abundance of *Acidobacteria* (8%) compared to the other three treatments ( $\leq 2\%$ ), and a modest relative abundance of *Gemmatimonadetes* ( $>1\%$ ), which was not seen in any of the other three enrichments. These two phyla are less frequently discussed in the literature but may also be tolerant to PFAS (Bao et al 2018, Cai et al 2020, Stock et al 2013). The methane cultures were dominated by *Ramlibacter* and *Cupriavidus* genera and had a much larger fraction of low abundance taxa compared to the other three cultures (Figure 42), which likely contributed to the significantly ( $p < 0.05$ ) greater alpha diversity in this culture (Figure 42). Greater biodiversity entails a broader suite of soluble enzymes that could react with precursors, leading to the higher yield of FHxSA and PFHxS.

The acetate cultures were dominated by *Mycobacterium* (52%), which is known to transform alkanes, and has been shown to transform 6:2 fluorotelomer alcohol (VanInsberghe et al 2015). Given this and its high relative abundance, we hypothesize that *Mycobacterium* was in part responsible for biotransformation of PFAS in the acetate culture. However, we were unable to find experimental evidence in literature confirming this genus's ability to transform ECF-based precursors like AmPr-FHxSA.

*Cupriavidus*, in addition to having a high abundance in the methane cultures, was also found in high abundance in the DGBE cultures, which had much fewer transformation products. Their shared high abundance of *Cupriavidus* likely contributed to the close clustering of these two enrichments when the relative abundances were plotted in principal component analysis space (Appendix A), though did not correlate with biotransformation product yield between these two carbon sources.

The BTEX culture was the least diverse (Figure 42), though not significantly less so than the acetate and DGBE cultures. It was dominated by *Achromobacter* and *Rhodococcus*, both of which are known BTEX degraders (Nielsen et al 2006, Deeb et al 1999). Combined, these two organisms accounted for 72% of the relative abundance in this culture. It is possible that this extreme specialization is responsible for the slower rate of AmPr-FHxSA biotransformation. While the enzymes produced may have been efficient in degrading BTEX, they appear to be unable to transform AmPr-FHxSA in an appreciable amount. This result diverges from the findings on fluorotelomer biotransformation in our laboratory (Olivares et al 2022), which showed that adding BTEX as an additional carbon source resulted in more PFAA product formation.

All cultures contained *Rhodococcus*, which can participate in sulfur oxidation in 6:2 fluorotelomer sulfonamido alkyl betaine (Van Hamme et al 2004). It has been proposed in perfluorooctyl-based

PFAS biotransformation studies that PFOSi is a biotic precursor to PFOS, this step microbially mediated via sulfur oxidation (Liu et al 2021). If PFHxSi was indeed biotically transformed to PFHxS in our experiments, *Rhodococcus* could be responsible for this step, being able to oxidize the sulfinate head group to a sulfonate.

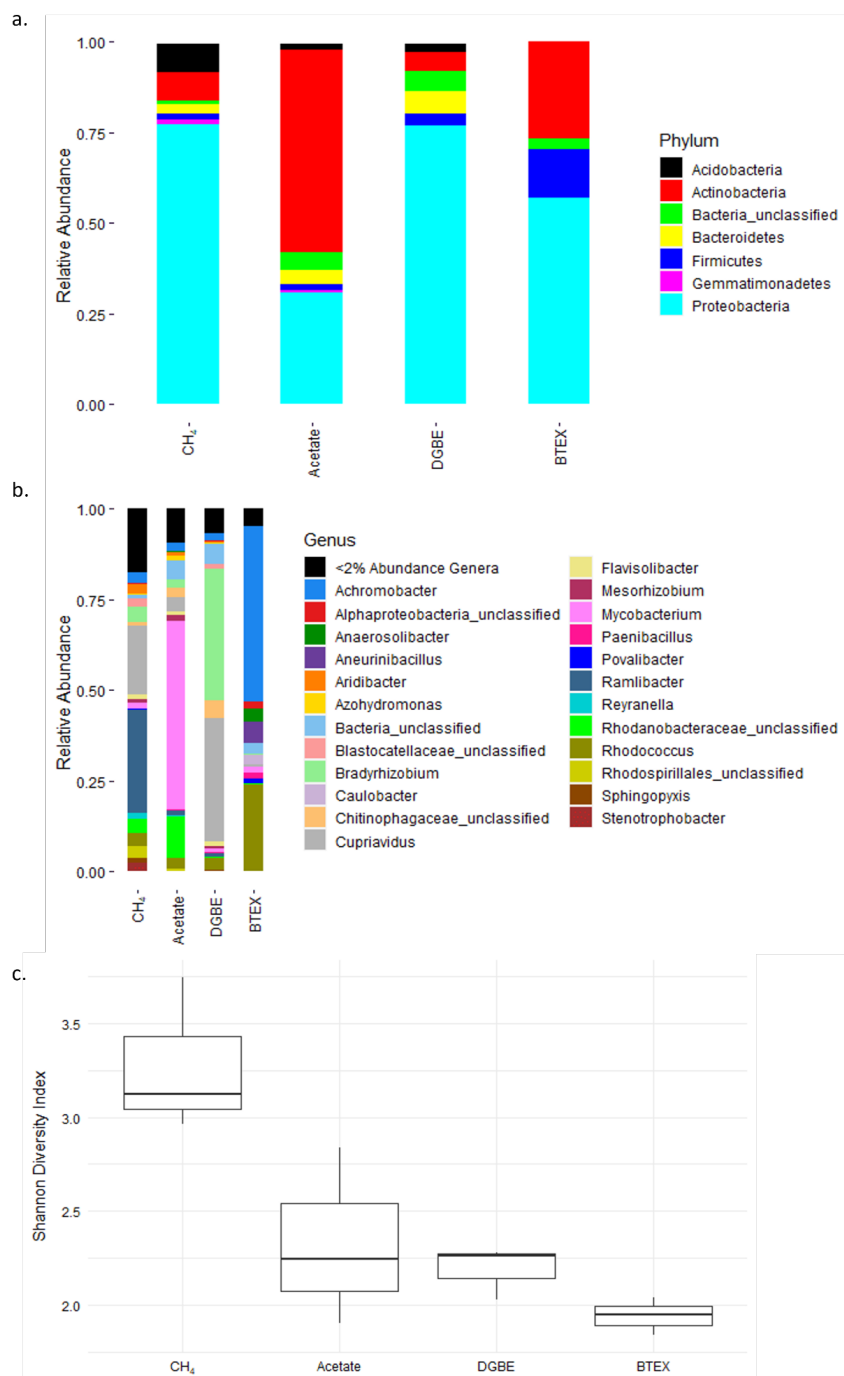


Figure 42. The a) phyla, b) genera, and c) Shannon Diversity Index from each of the four carbon source conditions. DNA was extracted from day 70; results are averaged between triplicate live samples.

While biotransformation of AmPr-FHxSA can lead to the production of PFHxS under a range of microbial communities, applying chemical oxidation such as sulfate radical oxidation as a remediation strategy could prevent the biotic production of PFSA from AFFF-impacted source zones. To explore different chemical oxidation processes, we first performed the TOP assay on AmPr-FHxSA and FHxSA individually (Figure 43). With both AmPr-FHxSA and FHxSA, most of the mass was converted to PFHxA after 12 hours. Unexpectedly, a small amount of PFHxS was produced, forming about 1% of the product in AmPr-FHxSA TOP assay and 4.3% in the FHxSA TOP assay. To quantify the PFHxS, we concentrated the samples for PFHxS to fall within the range of the calibration curve. No PFHxS was detected in the controls. Additionally, this experiment was repeated three times and each time produced 1-1.5% of PFHxS from AmPr-FHxSA and 4-5% PFHxS from FHxSA. To date, it has been widely accepted that PFSA are not produced during the TOP assay; while fluorotelomer-based precursors result in a suite of PFCAs in the assay, ECF-based precursors are reported to be converted to the corresponding chain-length PFCA (e.g., FOSA to PFOA) (Plumlee et al 2009, Houtz and Sedlak 2012, Martin et al 2019). Robel et al (2017) found PFOS production during reaction of textiles during the TOP assay but could not isolate which precursor might have been its oxidation precursor. Therefore, this could be the first example of specific PFAS producing a PFSA during the assay. Although the PFHxS yield is low, we believe that this result underscores the unique properties of sulfonamides and should be considered in TOP assay analyses with perfluoroalkyl sulfonamides as ignoring PFSA formation could cause underestimation of total PFAS at sites with high ECF-based precursor concentrations.

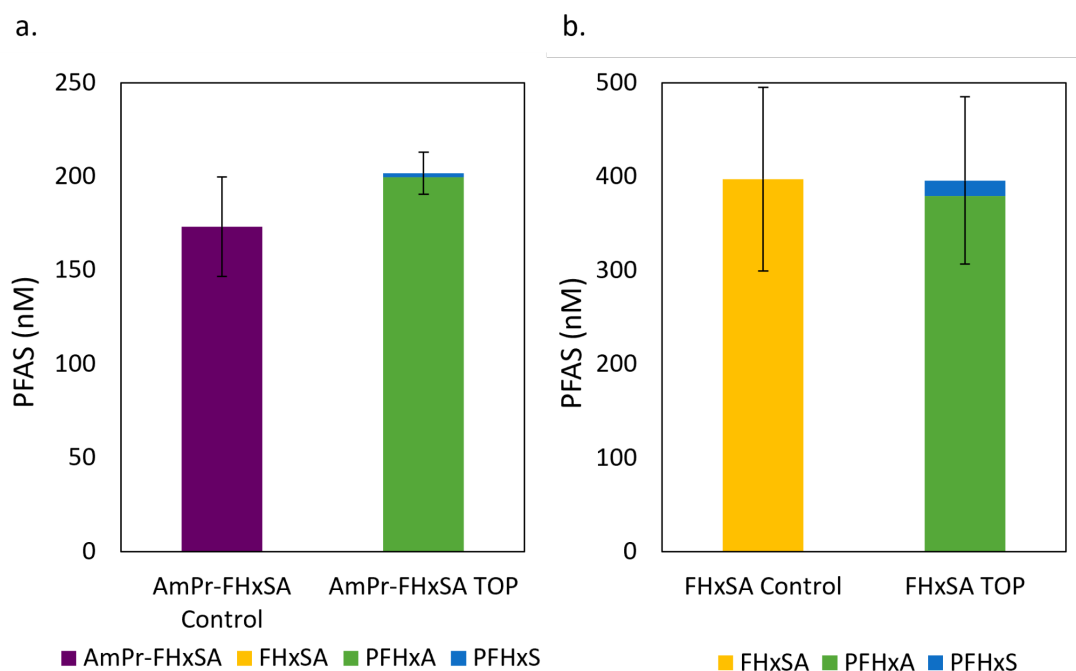


Figure 43. TOP assay results from reacting a) AmPr-FHxSA and b) FHxSA in Milli-Q water. Both precursors converted mostly to PFHxA via hydroxyl radical, but some PFHxS was detected. Error bars represent triplicates.

We then compared the TOP results with potential mineralization of AmPr-FHxSA and FHxSA using sulfate radicals during heat-activated persulfate oxidation, as activated persulfate oxidation

of PFAS has been proposed as a potential in situ remediation technique (Bruton and Sedlak 2017 and 2018, Carre et al 2021). Sulfate radicals from activated persulfate can transform precursor PFAS into PFCAs and at pH values below 3 these radicals can sequentially oxidize PFCAs, leading to mineralization (Bruton and Sedlak 2017 and 2018, Park et al 2016). Upon adding sodium persulfate and placing these reactors in an 85°C water bath, persulfate quickly converts to sulfate radicals and produces protons ( $\text{S}_2\text{O}_8^{2-} + \text{H}_2\text{O} \rightarrow 2\text{SO}_4^{\cdot-} + 2\text{H}^+ + \frac{1}{2}\text{O}_2$ ), acidifying the solution (Bruton and Sedlak 2017). AmPr-FHxSA and products were fully transformed in less than five hours, while FHxSA and products were fully transformed in about 24 hours (Figure 44), showing that these ECF-based precursors can be transformed under acidic conditions using activated persulfate. AmPr-FHxSA was first transformed to FHxSA and carboxylic acids, which underwent chain shortening. While FHxSA took longer to transform, it was no longer detected after five hours, suggesting that FHxSA is more recalcitrant than AmPr-FHxSA.

Similarly to the TOP assay, PFHxS was detected during HAPO of FHxSA (Figure 44). One of the triplicates contained PFHxS at the first sample point, which might indicate some rapid transformation of FHxSA to PFHxS since the controls did not contain PFHxS (Figure 44). Across the triplicates, the concentration of PFHxS appears to increase between 0 and 5 hours, and levels off after 5 hours; this timing corresponds to when FHxSA was no longer detected (between 3 and 5 hours).

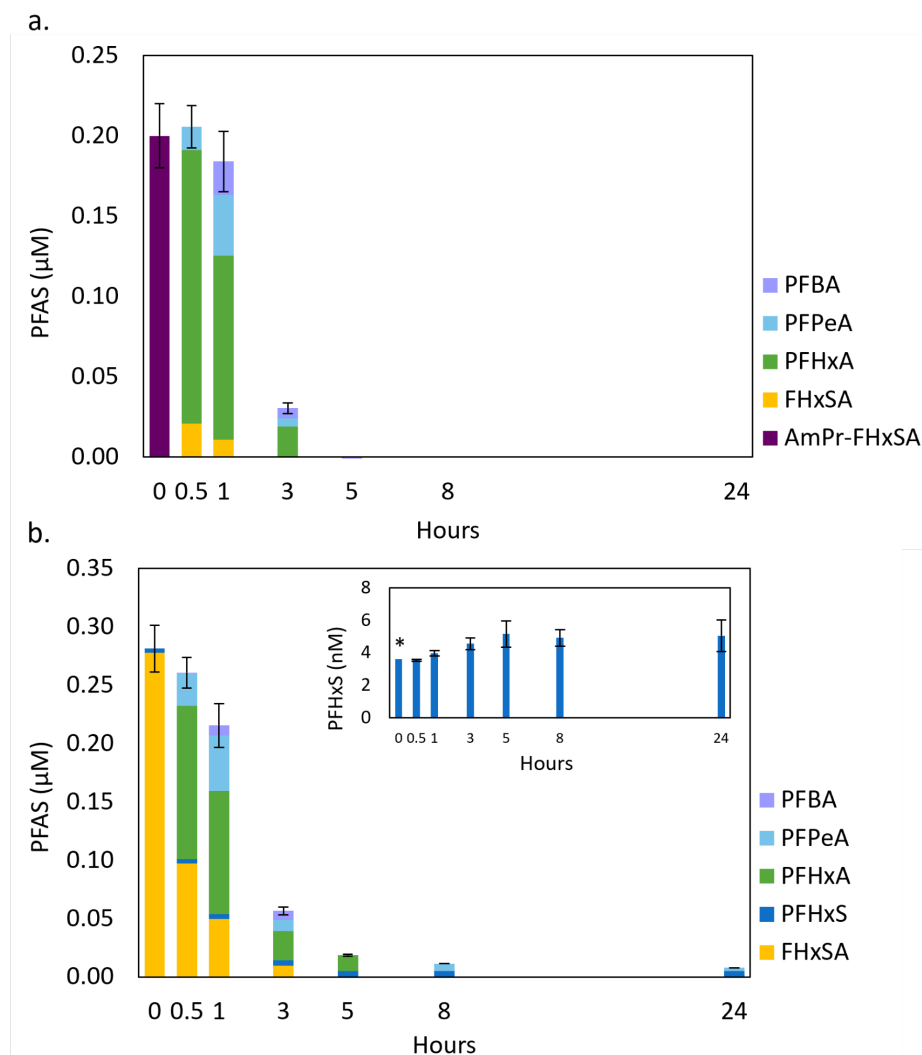


Figure 44. Heat-activated persulfate oxidation performed on a) AmPr-FHxSA and b) FHxSA diluted in MilliQ water with 50 mM sodium persulfate. The pH quickly dropped to below 3 within the first hour and remained acidic. Inset in b) is the concentration of PFHxS in nM, increasing within the first 5 hours of reaction time, corresponding to the time it takes FHxSA to transform. The asterisk (\*) indicates that PFHxS was only detected in one of the triplicate samples at hour 0. PFPeA = perfluoropentanoic acid; PFBA = perfluorobutanoic acid. Error bars represent triplicate reactors. Controls are shown in Figure 45.

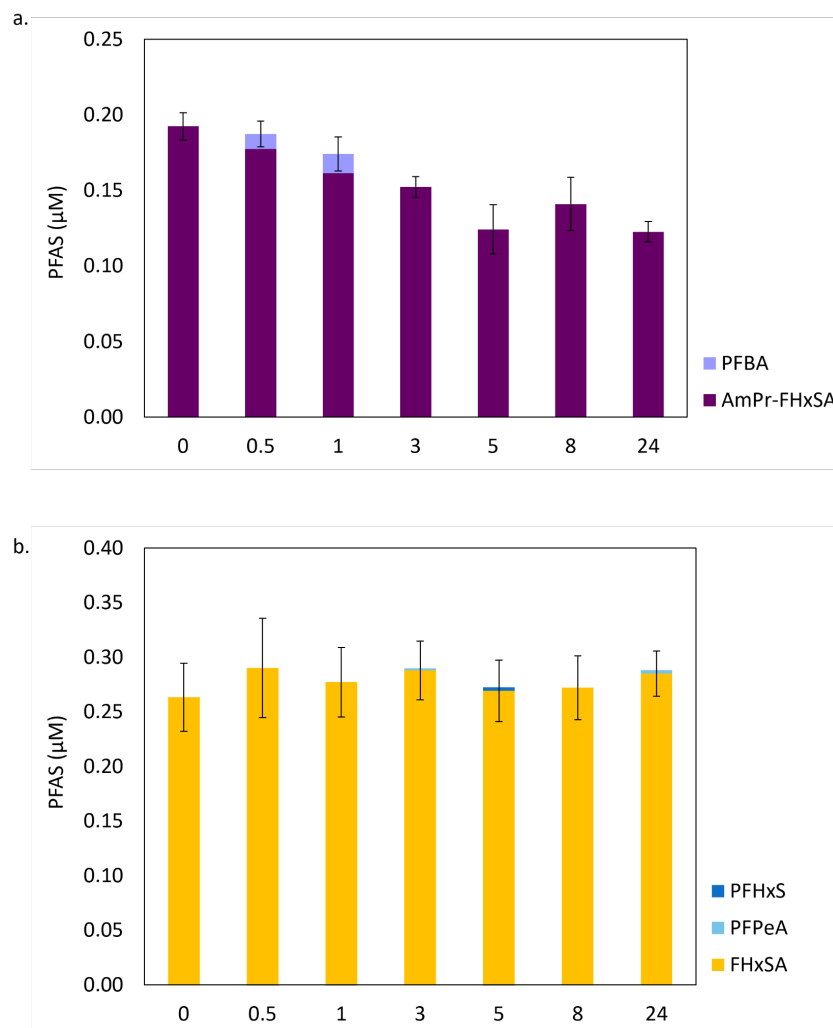


Figure 45. Controls for heat-activated persulfate oxidation of a) AmPr-FHxSA and b) FHxSA.

These controls were kept in a 4°C fridge to avoid heat-activation of the persulfate. The decrease in AmPr-FHxSA could be due to sorption to the reactor containers and/or from high ionic strength induced sampling artifacts, as has been shown for PFOS in literature (Steffens et al 2021).

### ***Effects of PFAS on Biodegradation of Co-Contaminants***

The results from this Subtask are being drafted into a manuscript for submission to a peer-reviewed journal.

Recently, researchers have shown the widespread prevalence of shorter-chained PFAS in AFFF-impacted groundwater, including PFHxS and AmPr-FHxSA, as well as FHxSA, a biotransformation product of perfluorohexyl-based ECF precursors including AmPr-FHxSA. Several groups have started reporting the biotransformation pathways and rates of ECF-based precursors, but no one has yet reported the potential toxicity of these compounds on remedial microbial communities.

We exposed an aerobic BTEX-degrading enrichment culture, an anaerobic toluene-degrading enrichment (both seeded from AFFF-contaminated sites), and an anaerobic TCE-degrading co-culture to multiple ECF-based PFAS and an ECF-based AFFF to determine possible inhibitory effects on these bacteria. We tested 1  $\mu\text{M}$  and 10  $\mu\text{M}$  (i.e., approximately 0.5 and 5 mg/L) of these compounds and investigated the effect of electron donor/acceptor consumption, adenosine triphosphate (ATP) production, amino acid production, and community changes to determine how bioremediation processes may be affected by the presence of PFAS. We chose these concentrations as they are representative of groundwater in AFFF-impacted source zones.

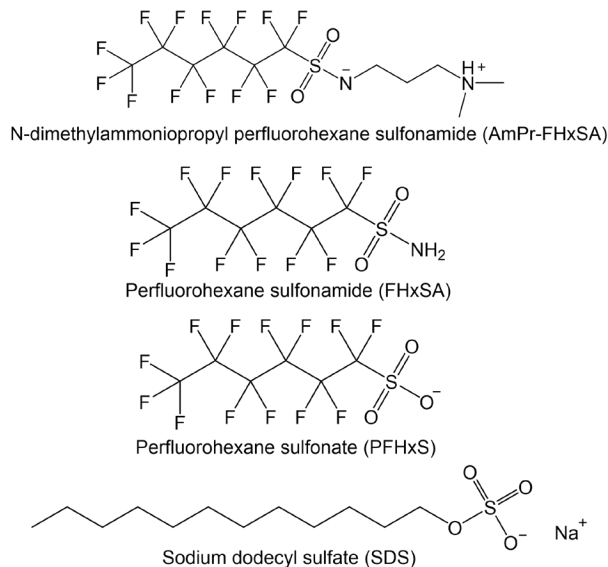


Figure 46. Surfactants tested in this study. All contained hexafluoroalkyl chains, except for the non-PFAS control, SDS.

Dhc195 and Dvh exist in a syntrophic relationship that results in more rapid and robust dechlorination of TCE to ethene, as previously described. Dhc195 uses chlorinated solvents as electron acceptors, transforming TCE to cis-DCE, VC, and finally to ethene with the support of sulfate-reducing Dvh. When we exposed this co-culture to 1 and 10  $\mu\text{M}$  individual PFAS and SDS, there was no effect on the rate of dehalogenation of TCE to ethene compared to the no-surfactant controls (Figure 47). We repeated this experiment with three dilutions of the AFFF: 100x, 1,000x, and 10,000x (Figure 48). The 10,000x diluted replicates performed similarly to the control, while the 1,000x diluted set resulted in a slowed, though complete degradation of TCE to ethene (Figure 48). In contrast, the microcosms that contained 100x diluted AFFF resulted in complete inhibition of TCE dechlorination, with no production of cis-DCE, VC, or ethene (Figure 48). Therefore, the combined effects of the PFAS in the 1,000x diluted AFFF delayed TCE degradation while the concentration range of 100x diluted AFFF—38  $\mu\text{M}$  PFHxS, 65  $\mu\text{M}$  AmPr-FHxSA, and 200  $\mu\text{M}$  of PFOS—inhibited the culture entirely. Since PFOS was present in the AFFF at an order of magnitude higher than the next highest PFAS, the concentration of PFOS could have controlled whether or not the AFFF dilution was inhibitory, but this could have also been due to other ingredients in the AFFF, including anticorrosives or non-fluorinated surfactants or solvents.

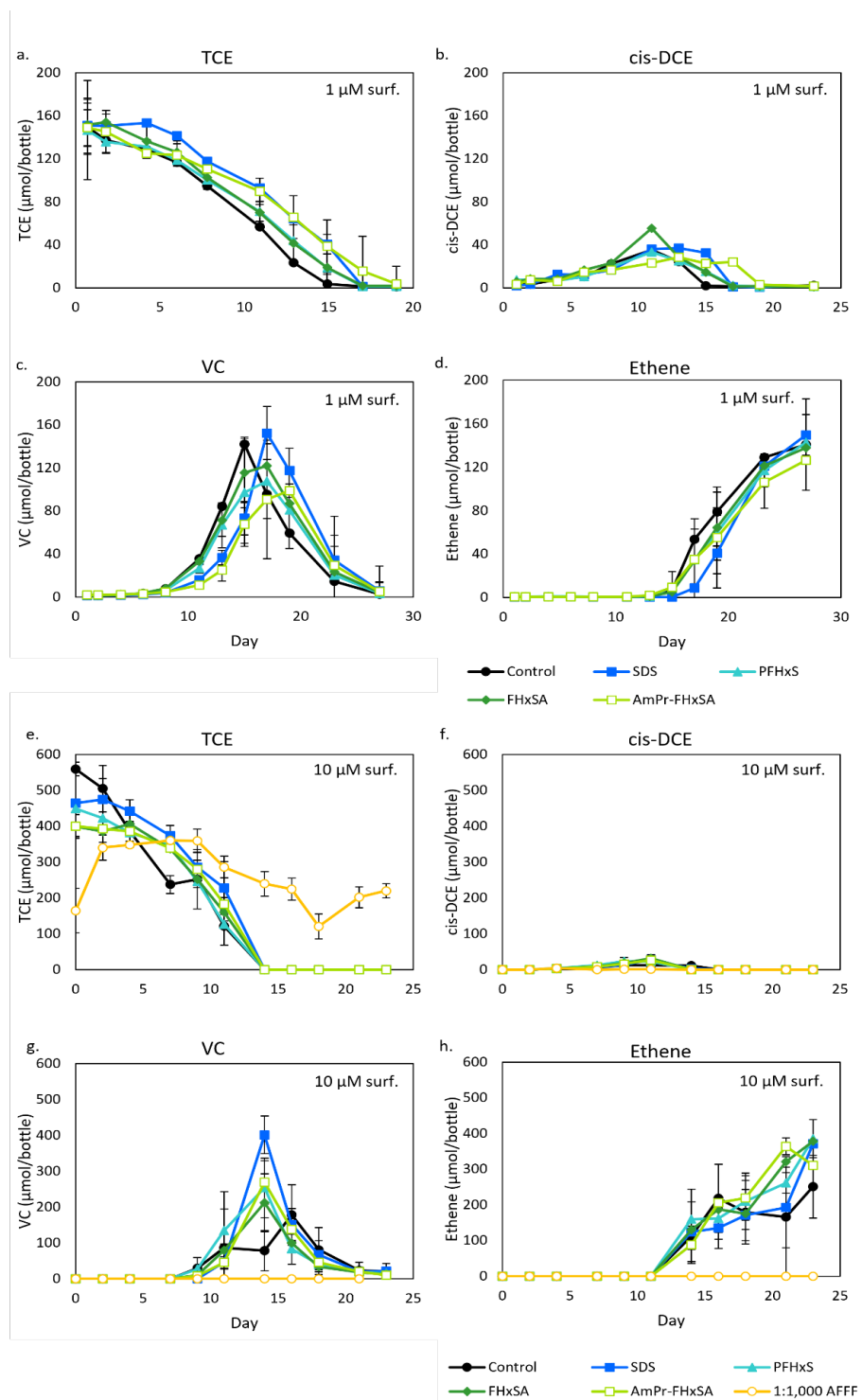


Figure 47. TCE (a,e), DCE (b,f), and VC (c,g) dehalogenation and ethene production (d,h) with the anaerobic culture exposed to 1  $\mu$ M of surfactants (a-d) or 10  $\mu$ M surfactants (e-g). Black circles are control, blue squares are non-fluorinated control SDS, light blue triangles PFHxS, dark green diamond FHxSA, and light green outlined white squares AmPr-FHxSA.

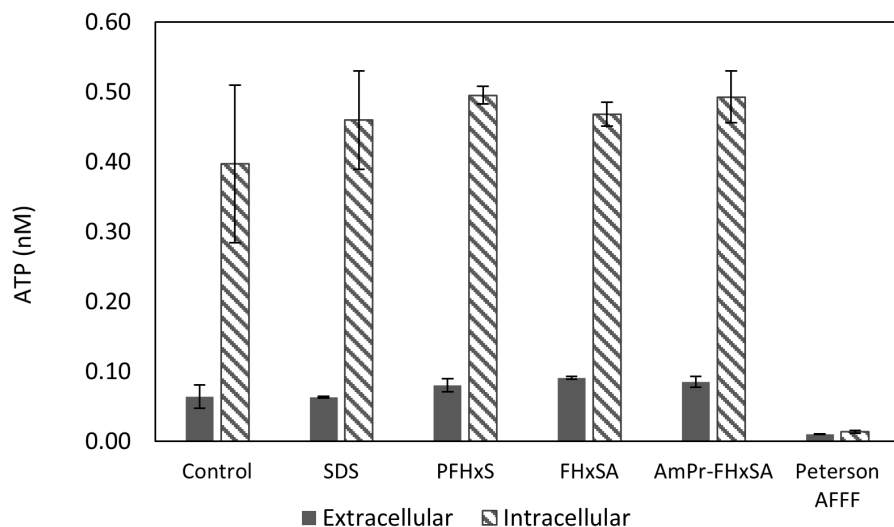


Figure 48. ATP concentrations in nM from the TCE co-culture on day 14 of the 10  $\mu$ M surfactant experiment, as shown in Figure 47. Extracellular, shown in solid gray, represents ATP measured after filtering the biomass samples. Intracellular, shown in striped gray, represents ATP measured in total without filtering with the extracellular concentration subtracted. Error bars represent standard deviation of triplicate cultures.

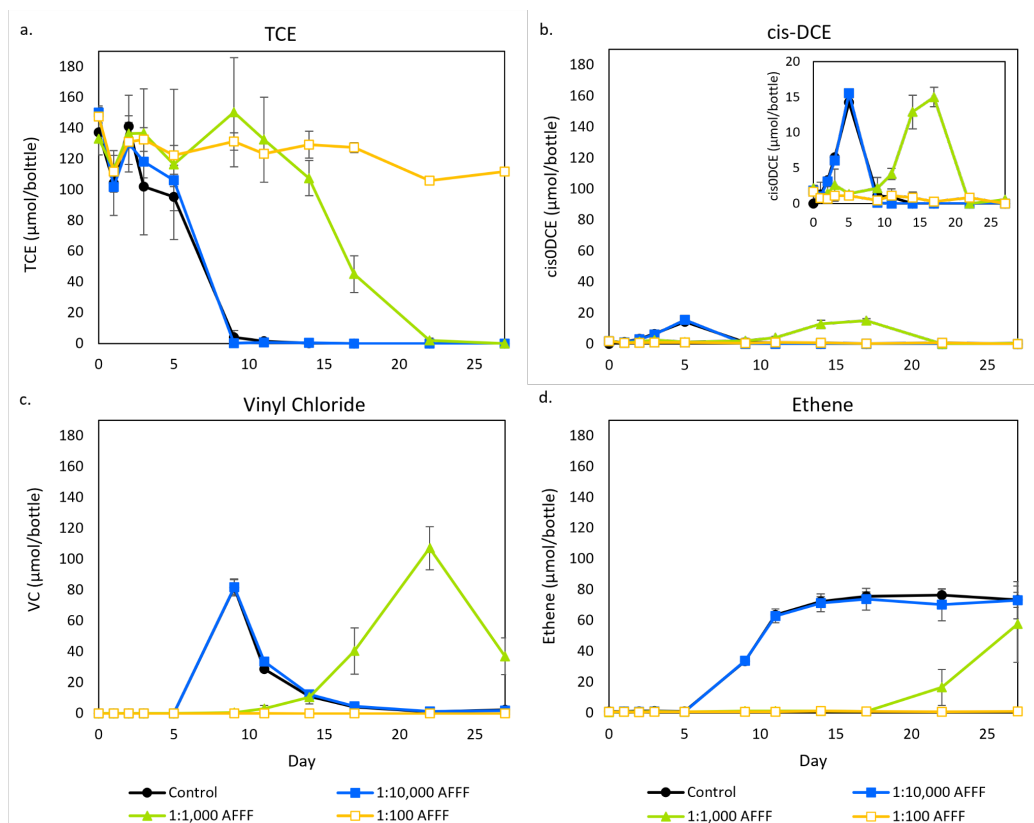


Figure 49. a) TCE dechlorination and subsequent production of b) cis-DCE, c) VC, and d) ethene by the anaerobic co-culture. This culture was exposed to increasing dilutions of an ECF-based

AFFF: black circles are no-AFFF control, blue squares 10,000 diluted AFFF, green triangles 1,000x diluted, and yellow/white squares 100x diluted. Error bars represent triplicate bottles.

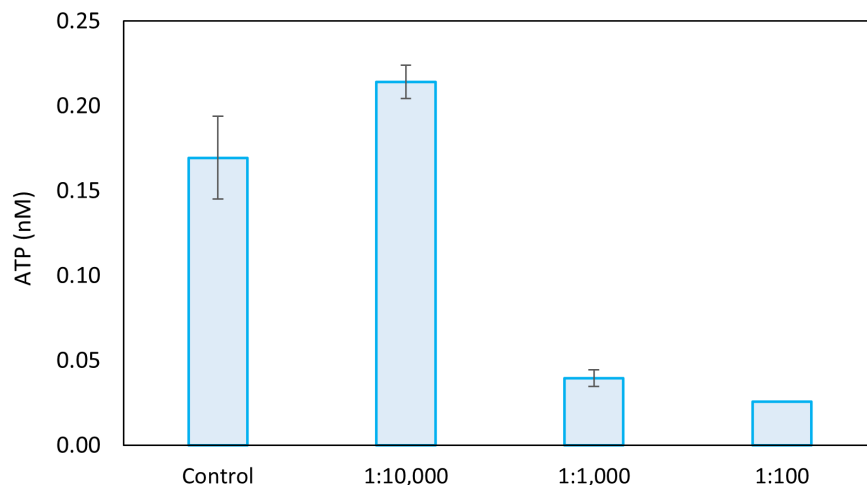


Figure 50. ATP concentrations in nM from the TCE co-culture on day 16 of the AFFF dilution experiment as shown in Figure 49.

In the TCE dehalogenation tests, 1  $\mu\text{M}$  and 10  $\mu\text{M}$  surfactant additions (i.e., SDS, PFHxS, FHxSA, and AmPr-FHxSA) did not impact anaerobic dehalogenation. However, AFFF-exposed treatments resulted in decreased dehalogenation. The cultures exposed to 1,000x diluted AFFF contained about 24% ATP compared to the control on day 16, while the 100x AFFF cultures had 18% of the ATP (Figure 50). The genus *Dehalococcoides* has been shown to have a unique cell wall; instead of peptidoglycan, the cell wall structure resembles the S-layer protein subunit walls that are typical of archaea. These S-layer type proteins could be the main contributor to the co-culture's resistance to PFAS, especially when the compounds are added individually. Indeed, it has been shown that *Dehalococcoides* species can be resistant to peptidoglycan biosynthesis associated antibiotics.

The aerobic BTEX-degrading enrichment culture, which uses BTEX as its carbon source and electron donor, did not exhibit any inhibitory effects when exposed to 1  $\mu$ M of PFAS and SDS. Exposure to 10  $\mu$ M FHxSA or AmPr-FHxSA, however, completely inhibited BTEX consumption compared to every other triplicate condition (Figure 51). All triplicate sets, except for those exposed to AmPr-FHxSA or FHxSA, rapidly consumed the BTEX within a day and were consequently amended with BTEX components on day 3 while the experiment was continued to determine if the AmPr-FHxSA and FHxSA triplicate sets would demonstrate any substrate degradation.

When repeated with dilutions of AFFF (Figure 52), BTEX degradation in the 100x and 1,000x AFFF sets was slowed compared to the control and 10,000x AFFF dilution. Because one of the organic components in AFFF with highest concentrations (26-420 g/L) is DGBE (commercially known as butyl carbitol), we tested its inhibition potential in additional toxicity tests but found no indication of inhibition to aerobic BTEX biodegrading activity compared to the BTEX-only live control. Other non-PFAS components in AFFF with biocide activity include hydrocarbon surfactants and recently found benzotriazole corrosion inhibitors present in AFFF at 120-360 mg/L, which may also account for some inhibitory impact, but were not tested individually in this study.

It is interesting that PFHxS in isolation did not inhibit BTEX degradation, while FHxSA and AmPr-FHxSA did. The most notable difference among these compounds is the headgroup charge (Figure 46). Negatively charged PFAAs have been shown to disrupt bacterial cell membranes, but the effects of neutral and zwitterionic PFAS on membrane integrity have not been reported to our knowledge. It is possible that the neutral FHxSA permeate cell membranes more effectively than negatively charged surfactants, and that the zwitterion AmPr-FHxSA could have interfered with the membrane and membrane-bound proteins such as ATP synthase.

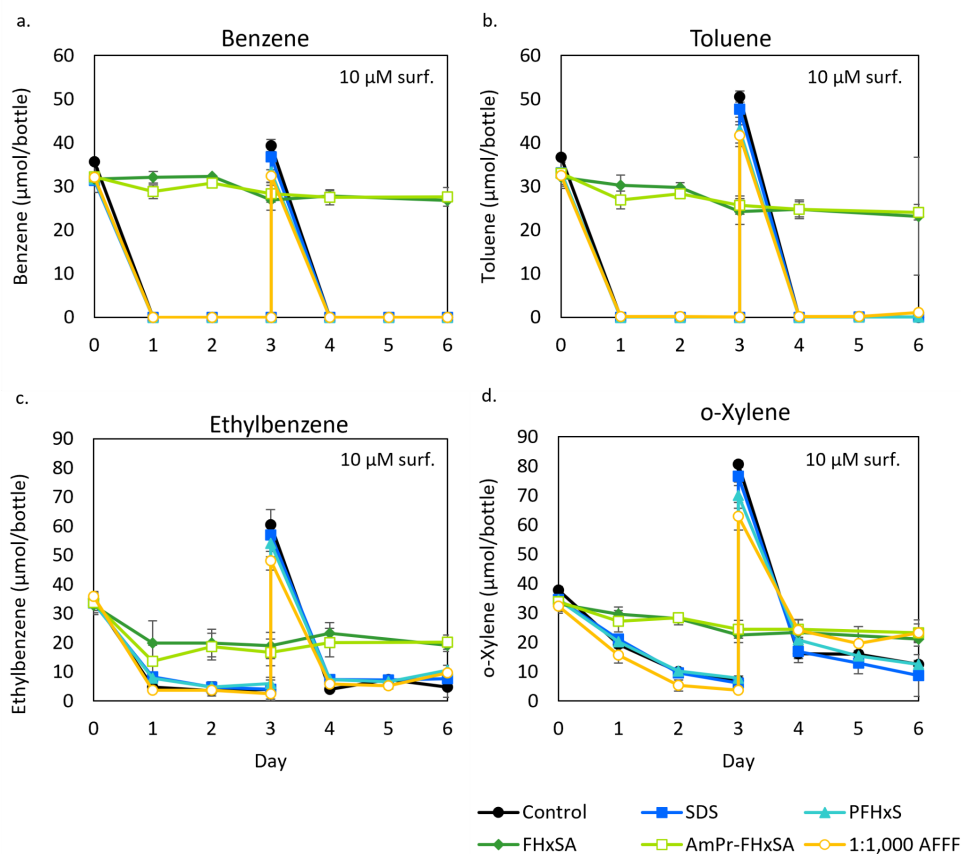


Figure 51. Aerobic degradation of a) benzene, b) toluene, c) ethylbenzene, and d) ortho-xylene by an AFFF-impacted enrichment culture. Black circles are no-PFAS control, blue squares SDS, turquoise triangle PFHxS, dark green diamond FHxSA, light green outlined white squares AmPr-FHxSA, and yellow open circles 1,000x diluted AFFF. Error bars represent triplicate bottles. Additional BTEX was added Day 3 to all reactors that had already consumed most of their substrate; additional BTEX was not added to the AmPr-FHxSA and FHxSA cultures because they had not demonstrated BTEX degradation.

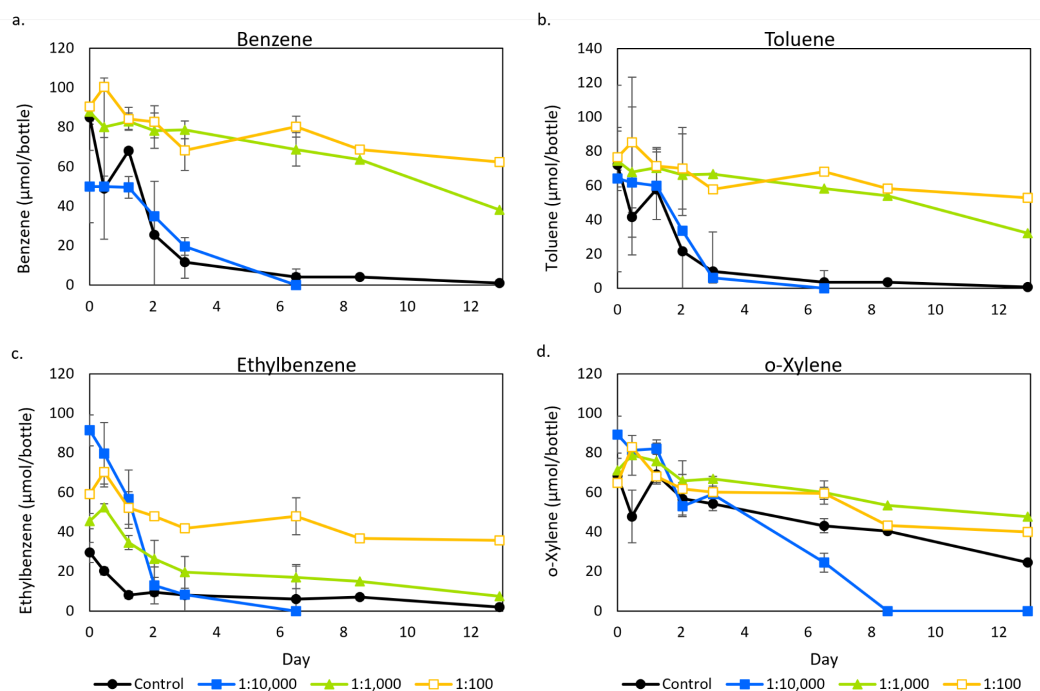


Figure 52. Aerobic BTEX degrading culture consuming a) benzene, b) toluene, c) ethylbenzene, and d) ortho-xylene in the presence of dilutions of AFFF. Black circles are the control, blue squares are 10,000x diluted AFFF, light green triangles 1,000x diluted AFFF, and yellow white circles 100x diluted AFFF.

To compare with an anaerobic culture, we dosed a toluene degrading culture under nitrate- and sulfate-reducing conditions with the three AFFF dilutions and AmPr-FHxSA. Neither AmPr-FHxSA (1 or 10  $\mu$ M), nor any of the three AFFF dilutions inhibited toluene degradation in these cultures (Figures 53 and 54).

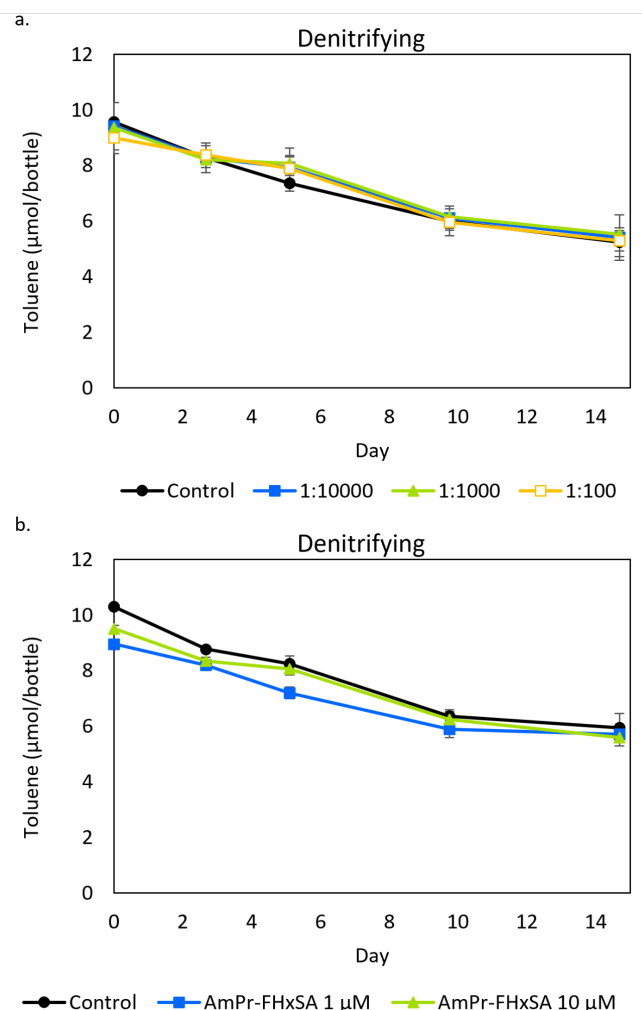


Figure 53. Anaerobic toluene degradation with the anaerobic enrichment culture under nitrate reducing conditions, exposed to a) dilutions of AFFF or b) two concentrations of AmPr-FHxSA.

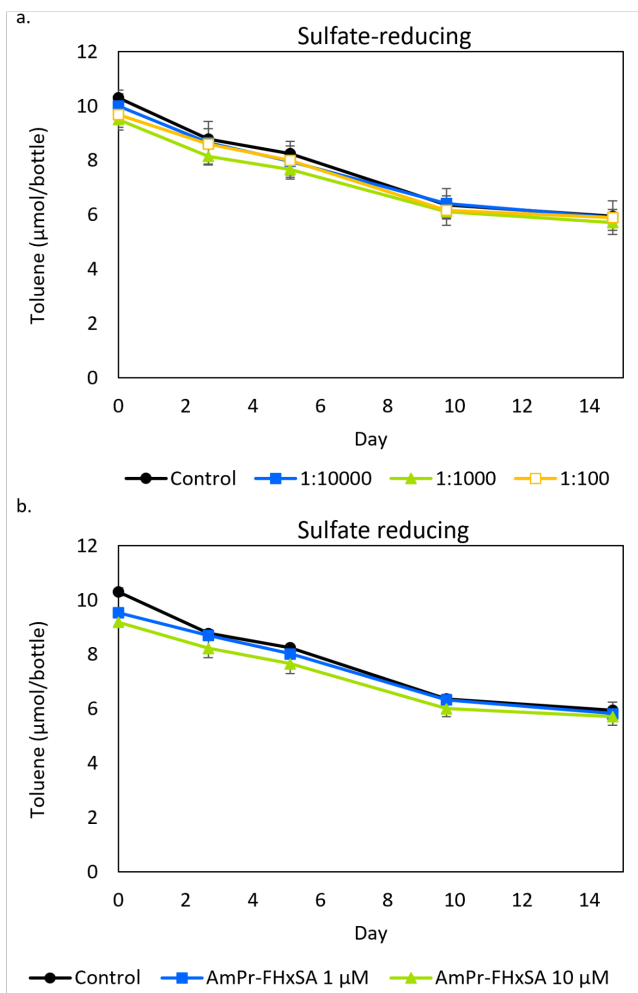


Figure 54. Anaerobic toluene degradation with the anaerobic enrichment culture under sulfate reducing conditions, exposed to a) dilutions of AFFF or b) two concentrations of AmPr-FHxSA.

On day 4 of the 10  $\mu$ M surfactant test with the aerobic BTEX enrichment cultures, we sampled biomass for DNA extraction and sequencing to compare differences between the cultures exposed to different surfactants. Unfortunately, potentially because of the high concentrations of surfactants used, several of the samples failed the quality control measures due to amplification failure. It is possible that the surfactants interfered with the DNA extraction procedure; in the future, when analyzing PFAS-impacted microbial communities, a PFAS extraction method may need to be first employed before DNA extraction to avoid downstream amplification errors. Therefore, the results that passed quality control, the inoculum source, FHxSA, and AmPr-FHxSA exposed cultures, are described here.

The inoculum was dominated by *Rhodococcus* and *Achromobacter* (Figure 55), two known BTEX degraders. Cultures exposed to FHxSA and AmPr-FHxSA, in addition to *Rhodococcus* and *Achromobacter*, also contained *Sediminibacterium* and unclassified *Alphaproteobacteria*. The inoculum and the FHxSA culture shared a small abundance of *Vulgatibacter*, while the AmPr-FHxSA cultures were unique in containing *Acinetobacter*.

*Acinetobacter* are gram-negative bacteria known for being antibiotic resistant bacteria, including to compounds such as penicillin and aminoglycoside. In addition to being antibiotic resistant, *Acinetobacter* are generally robust organisms, being able to persist in a range of temperatures and moisture contents. In studies investigating bioremediation of petroleum hydrocarbons, *Acinetobacter* has been demonstrated to be able to use fuels as their carbon source and electron donor. It is interesting that, based on its persistence, *Acinetobacter* was not detected with significant abundance in the FHxSA cultures. Its absence could be attributed to the ability of FHxSA to passively diffuse into cell membranes compared to AmPr-FHxSA, leading to inhibition of this genus.

There is not much literature on the *Vulgatibacter* genus, which was found in the inoculum and in the FHxSA cultures. This genus contains gram negative obligate heterotrophic aerobes.<sup>36</sup> *Sediminibacterium*, present in both FHxSA and AmPr-FHxSA cultures, is similarly an understudied genus with only seven species, which are also gram negative heterotrophic aerobes. Its higher abundance with the PFAS cultures compared to the control suggests that *Sediminibacterium* can withstand high PFAS concentrations. Interestingly, *Pseudomonas* was present in both the FHxSA and AmPr-FHxSA cultures, its higher abundance compared to the control suggesting that this genus could be more resistant to PFAS compared to *Rhodococcus*, which declined in abundance compared to the control (Figure 55). While *Rhodococcus* was sensitive to the presence of PFAS, *Achromobacter*, the other key BTEX-degrader, seems unaffected evidenced by its consistent relative abundance in all cultures.

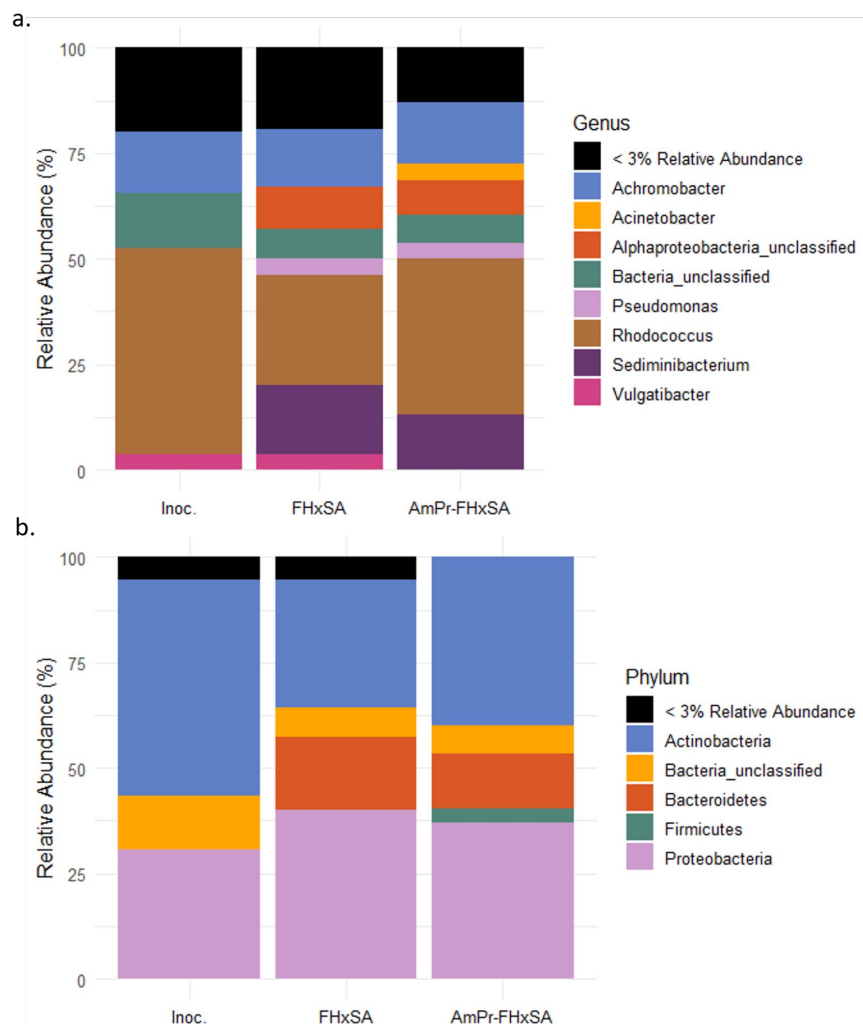


Figure 55. a) Genera and b) Phyla of the inoculum, 10  $\mu$ M FHxSA exposed cultures, and 10  $\mu$ M AmPr-FHxSA exposed cultures in the aerobic BTEX degrading enrichment culture.

We measured fold changes in metabolite abundances to study mechanistic impacts of AmPr-FHxSA, its transformation products, AFFF, and the fluorine-free surfactant SDS on biological TCE and BTEX degradation (Figures 56, 57) pathways. In the TCE co-culture, two amino acids, arginine and alanine had the largest increase in FC with respect to the control (Figure 56a). Higher abundance of alanine occurred in all PFAS and also the fluorine-free SDS, implying this amino acid was not a PFAS-specific biomarker.

Conversely FCs for almost all amino acids in the BTEX enrichment were negative (Figure 56b). The amino acids with the most negative FC were L-citrulline and arginine during AmPr-FHxSA and FHxSA exposure. Based on the KEGG *Rhodococcus* sp. pathways, glutamate is used in the synthesis of arginine, which in turn is reversibly converted into citrulline. From the amino acid FCs, proline seemed to be a FHxSA specific biomarker. Proline is formed from glutamate. In addition to amino acids, the BTEX enrichment had increases of unsaturated fatty acids (stearic and palmitic acids) (Figure 56b). Finally, we identified transformation products of BTEX biodegradation, o-toluic (oxidation product of o-xylene), malonic and methylmalonic acids (formed from ring cleavage of BTEX compounds). The positive FCs in all of these transformation products signify incomplete biodegradation, but are difficult to interpret further. This is because their concentration is a balance between formation and degradation of the transformation product.

In addition to testing individual PFAS, we also evaluated increasing dilutions of AFFF in both biological systems (Figure 56). An AFFF concentration dependent decrease of Log 2 fold change of all metabolites can be seen in both BTEX and TCE systems, suggesting dose-response changes in the metabolite abundances. The BTEX enrichment, however, had more varied results; as there were 3-5 key genera in these cultures (Figure 55), the culture likely has a network of materials exchange and co-dependence, leading to more varied metabolites concentrations as each species may react differently to the surfactant exposure. The TCE dechlorinating culture generally had clearer trends as shown on the heatmaps, potentially because there were only two species in the culture responding to the PFAS.

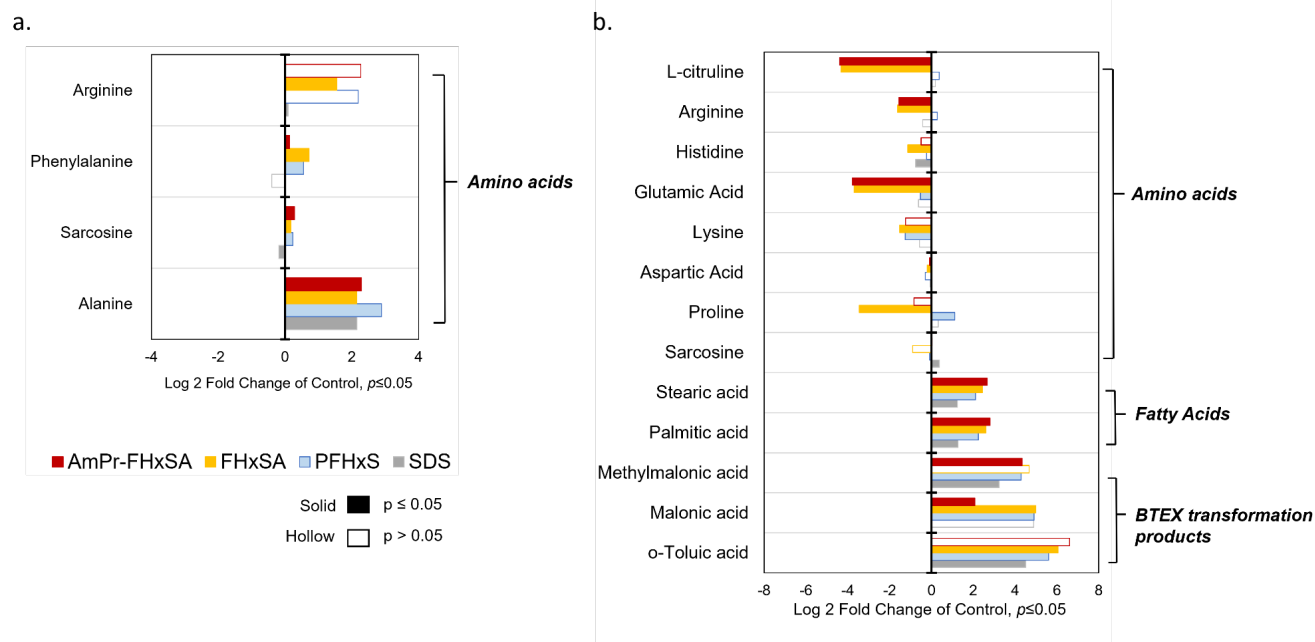


Figure 56. Log 2 fold changes of total (intra- and extracellular) metabolite abundance detected in the a) anaerobic TCE co-culture and b) aerobic BTEX enrichment cultures exposed to AmPr-FHxSA. Fold changes with  $p \leq 0.05$  are shown in solid bars, otherwise only the outline of the bar is shown (hollow).

For the TCE dechlorinating co-culture, the control sets clustered similarly to the 10,000x diluted AFFF. The 1,000x AFFF dilution resulted in higher relative concentrations of tyrosine and histidine compared to the control and 10,000x AFFF dilution. Tyrosine can be linked to polysaccharide production, while histidine residues are common in phosphoproteins. Interestingly, aspartic acid, serine, and threonine are also common in phosphoproteins; the phosphorylation of proteins is a key cellular mechanism to either activate or deactivate an enzyme based on it being phosphorylated or dephosphorylated or cellular signaling for a cell to respond to a change in its environment. This could indicate phosphorylation or dephosphorylation of these residues on particular proteins in response to the presence of the AFFF. Additionally, aspartic acid is used to synthesize both lysine and peptidoglycan. Since lysine decreases in concentration from the control to 10,000x and then 1,000x AFFF dilutions, this could point to negative interactions with peptidoglycan synthesis.

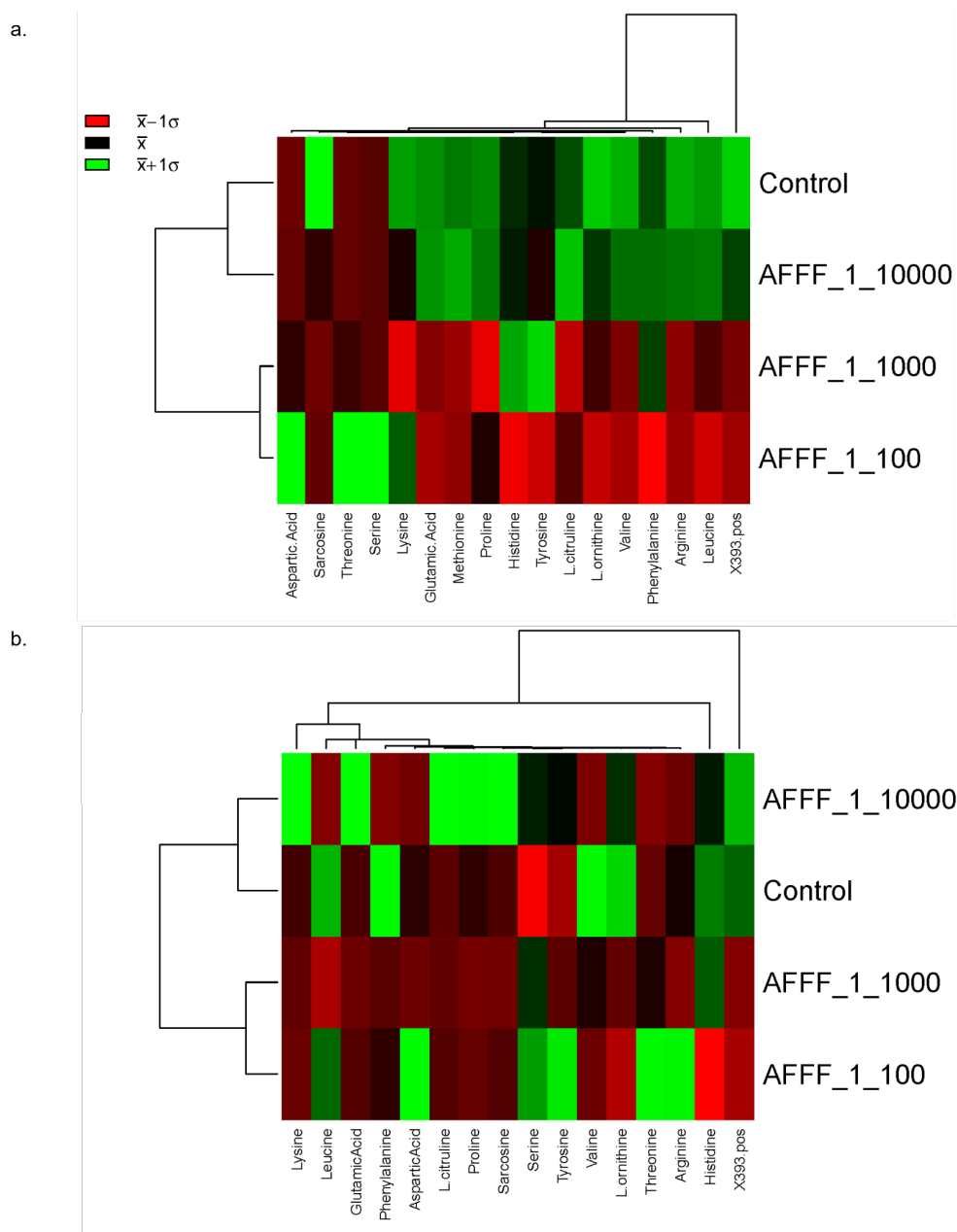


Figure 57. Heatmaps of Log 2 fold changes in metabolite abundance measured in the a) anaerobic TCE co-culture and b) aerobic BTEX enrichment culture with the three dilutions of AFFF. A concentration dependence is seen in the Euclidean distance with respect to the AFFF-free control (AFFF is 1:10000 most diluted, AFFF 1:100 is most concentrated).

## Additional Findings — Salt Effects on PFOS Solution Behavior

The following results have been published: Steffens, S.D., Cook, E.K., et al. (2021) Under-reporting Potential of Perfluorooctanesulfonic acid (PFOS) under High Ionic Strength Conditions. *Environmental Science & Technology Letters*.

Mentioned previously in Objective 1, experiments with high, weekly doses of persulfate resulted in decreasing levels of PFOS throughout the experiment, even though HAPO was proven to be ineffective against PFOS and other perfluoroalkyl sulfonic acids (Bruton and Sedlak 2017 and 2018, Park 2016). During the HAPO reaction, acid is produced upon persulfate activation at elevated temperatures ( $\geq 35^{\circ}\text{C}$ ) (Liu et al 2014).

Given the products of the HAPO reaction, we identified pH and ionic strength as two potential variables that could affect the observed PFOS decrease. In order to mimic HAPO conditions and determine if  $\text{Na}_2\text{S}_2\text{O}_8$  was responsible for the decrease in PFOS, we compared the effect of a 200 mM dose of  $\text{Na}_2\text{S}_2\text{O}_8$  to a 200 mM dose of  $\text{Na}_2\text{SO}_4$ . Sulfuric acid was used to adjust the pH of the  $\text{Na}_2\text{SO}_4$  amended solution to match the pH of the persulfate reaction. No chemical reaction was expected in the acidified  $\text{Na}_2\text{SO}_4$  negative control. As shown in Figure 58, the PFOS concentration decreased immediately after dosing with  $\text{Na}_2\text{S}_2\text{O}_8$  or  $\text{Na}_2\text{SO}_4$ , prior to any significant pH decrease in the solution. A similar PFOS concentration decrease was observed on Day 1 after salt addition in both conditions. After the initial decrease, the concentration remained relatively constant over the 5-day sampling period in both conditions.

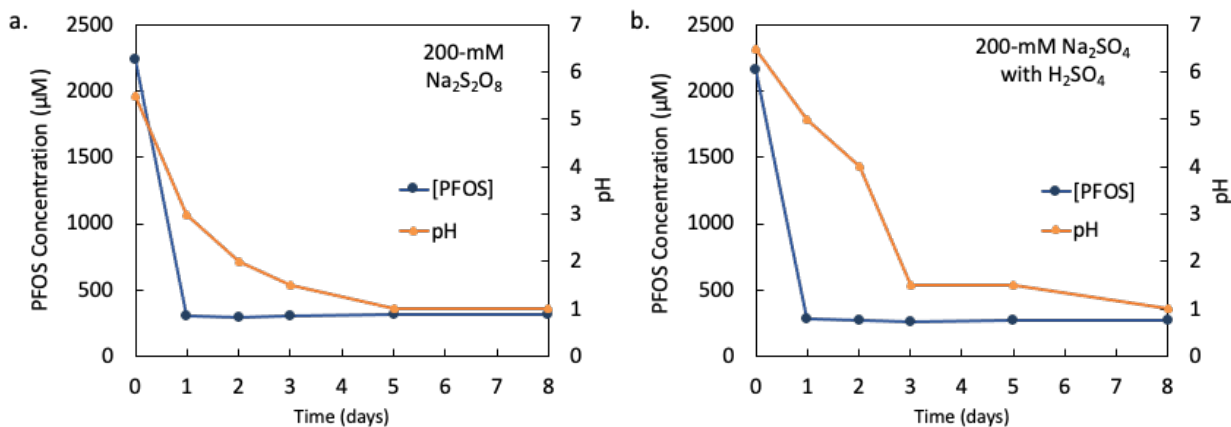


Figure 58. Change in PFOS concentration with pH decrease and an initial dose of (a) 200 mM  $\text{Na}_2\text{S}_2\text{O}_8$  and (b) 200 mM  $\text{Na}_2\text{SO}_4$ . Bottles contained Milli-Q water, sand (50% w/v), and  $\sim 2$  mM PFOS.  $\text{Na}_2\text{S}_2\text{O}_8$  and  $\text{Na}_2\text{SO}_4$  were added after day 0 sampling;  $\text{H}_2\text{SO}_4$  added after day 1 sampling to  $\text{Na}_2\text{SO}_4$  condition only. Lack of error bars indicates preliminary results from single samples.

The results of these experiments indicate that the PFOS concentration decrease was directly related to the salt addition and was not pH dependent. The insignificant effect of pH is likely related to the low  $\text{pK}_a$  of PFOS. The calculated  $\text{pK}_a$  of PFOS is -3.27, indicating that PFOS is a very strong acid (Goss 2008). Therefore, PFOS is expected to be fully ionized under the experimental

conditions tested, and an increase in solution acidity to pH 1 would have a negligible effect on the ionization state of PFOS.

To further assess the impact of salt on observed PFOS concentration, we tested a range of  $\text{Na}_2\text{SO}_4$  concentrations in aqueous solutions containing source zone relevant PFOS concentrations. The standard sample preparation procedure, with centrifugation of the aqueous sample, was used in all experiments going forward. The target concentrations of PFOS was 20  $\mu\text{M}$  and  $\text{Na}_2\text{SO}_4$  concentrations were varied from 250 to 500 mM. A decrease in PFOS concentration was observed after salt addition in the 500 mM and 1000 mM  $\text{Na}_2\text{SO}_4$  conditions, consistent with observations in the pH experiment. The results of increasing salt concentrations are summarized in Figure 59.

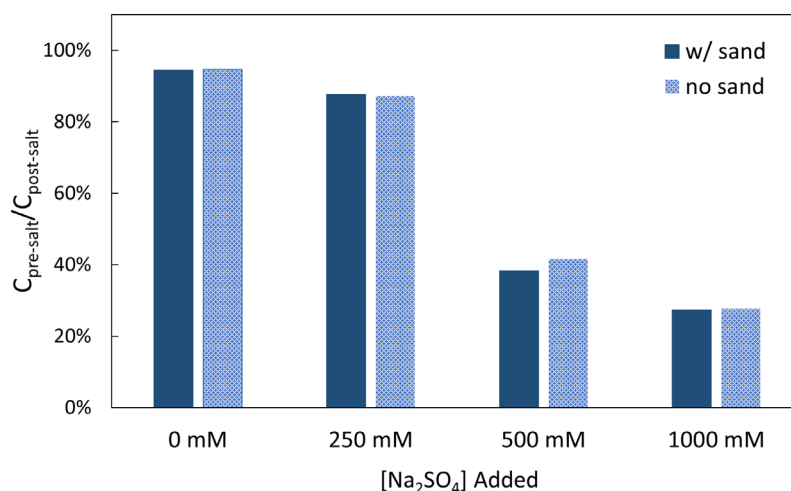


Figure 59. Detected PFOS concentration in bulk solution before and 1-hour after  $\text{Na}_2\text{SO}_4$  addition, in bottles with and without sand. Initial PFOS concentration was ca. 10 mg/L (20  $\mu\text{M}$ ), 250 to 1000 mM  $\text{Na}_2\text{SO}_4$  added as solid; averages are from duplicate samples. No significant difference was observed in bottles with sand suggesting PFOS adsorption to sand is not the mechanism for PFOS loss in bulk solution.

Given that PFOS concentrations decreased nearly equivalently for the sand and sand-free conditions, we concluded that the observed concentration decrease could not be attributed solely to adsorption. Additionally, the significance of the concentration drop is directly related to the amount of salt added, as evidenced by comparing the 250 mM  $\text{Na}_2\text{SO}_4$  dose to the 500 mM and 1000 mM  $\text{Na}_2\text{SO}_4$  dose. The concentration drop occurs almost immediately after salt addition.

To analyze the potential effect of subsampling from the experiment bottles, and evaluate if sorption to the container could be driving observed decreases in PFOS concentration in the bulk solution, we performed full bottle extractions with MeOH in parallel with the routine sample preparation of solution aliquots. Results from the subsampling versus full bottle extraction experiment are shown in Figure 60. Surprisingly, almost no difference in PFOS concentration was observed after salt addition in the subsample or the full bottle extraction 3-hours after salt addition, despite that previous results indicated a sharp decrease in PFOS under equivalent conditions. We hypothesized that centrifugation of the aqueous solution, which was eliminated in this experiment, might be impacting the PFOS concentration in bulk solution.

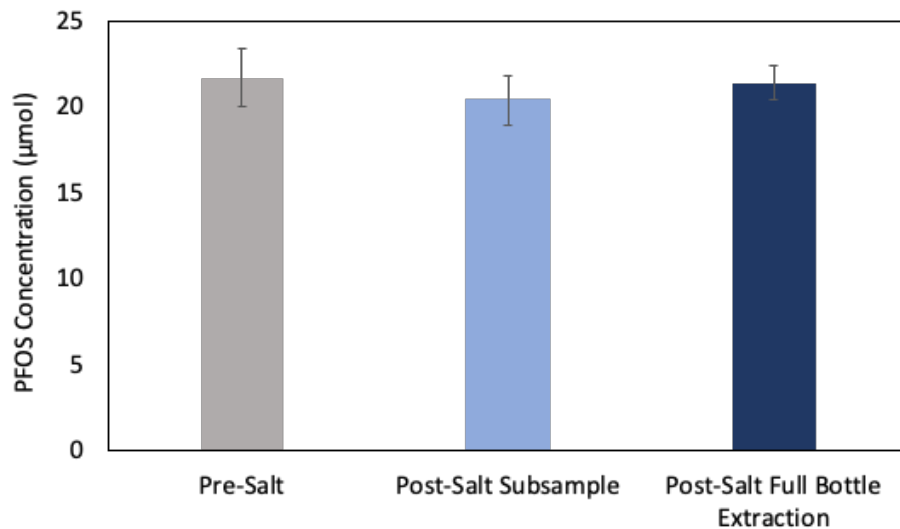


Figure 60. PFOS concentration detected pre-salt addition and post-salt addition, detected in an aliquot (subsample) and from a full bottle extraction. Reaction bottles were prepared in duplicate; samples were taken three hours after salt addition (1000 mM Na<sub>2</sub>SO<sub>4</sub>) in triplicate. Aqueous aliquots were removed and diluted in MeOH (2x dilution), then MeOH was added to the reaction bottle (2x dilution). Samples were centrifuged after dilution in MeOH. Values and error bars represent averages and standard deviations of six samples.

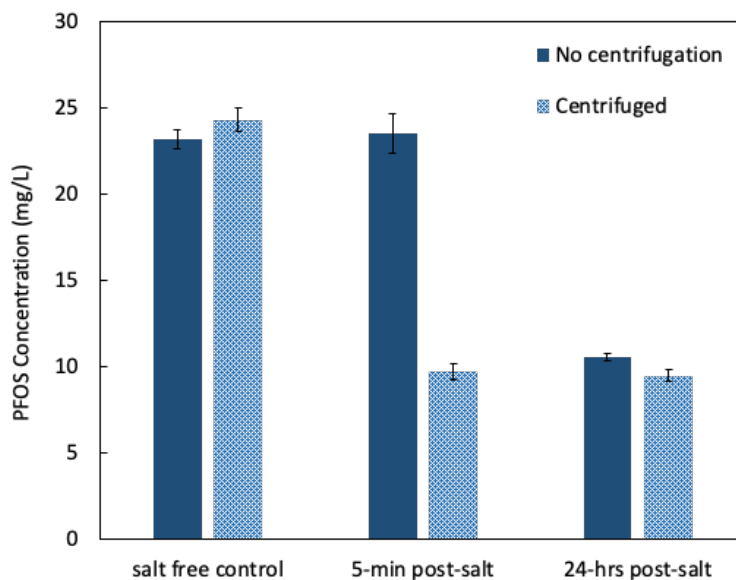


Figure 61. Effect of centrifugation of aqueous samples on PFOS detected in bulk solution. Initial PFOS concentration was ca. 25 mg/ L; 500 mM Na<sub>2</sub>SO<sub>4</sub> was added. Error bars represent the standard deviation of triplicate samples.

To evaluate this hypothesis, we compared PFOS concentrations detected in aqueous samples upon salt addition, with and without a centrifugation step. When a 25 mg/L PFOS solution was prepared in 500 mM Na<sub>2</sub>SO<sub>4</sub> solution, only 40% of the added PFOS was detected after centrifugation at 10,000 g, whereas a 101% recovery was obtained in the non-centrifuged solution (Figure 61). Storing the salt-containing PFOS solution on a benchtop for 24 hours prior to sampling the bulk solution yielded losses similar to those observed for the centrifuged solutions (Figure 61), suggesting that phase separation of aggregates or sorption to the container may occur over this time period.

While effects of high ionic strength, specifically high sodium concentrations, are relevant to ISCO treated water matrices, we were interested in evaluating the effects in a high salinity, natural water matrix. To this end, we measured a range of detected PFOS concentrations in two high salinity matrices: a 200 mM Na<sub>2</sub>SO<sub>4</sub> solution and seawater collected from the San Francisco Bay. We observed a significant loss of PFOS in both matrices at PFOS concentrations above 5 mg/L, the loss ranging from 2-38 mg/L, that was dependent on the initial concentration of PFOS in Milli-Q water (Figure 62). At PFOS concentrations < 5 mg/L, the concentration decrease was in the range of ~0.2-0.6 mg/L, likely primarily due to container-water interfacial sorption. The concentration loss in seawater was generally higher than in the Na<sub>2</sub>SO<sub>4</sub> solutions, which may be attributed to the presence of Ca<sup>2+</sup> leading to enhanced surface activity and aggregation of PFOS.

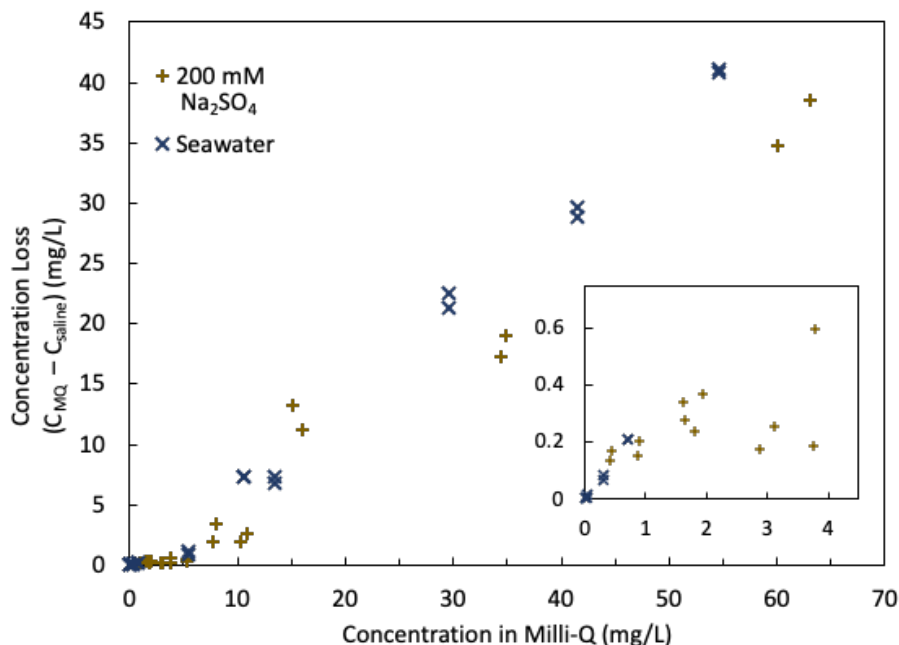


Figure 62. PFOS concentration loss in saline bulk solution compared to PFOS concentration detected in Milli-Q. For the Na<sub>2</sub>SO<sub>4</sub> data, samples were taken from the same solution bottle before ( $C_{MQ}$ ) and after ( $C_{saline}$ ) salt addition. For the seawater data, PFOS was added to seawater ( $C_{saline}$ ) and Milli-Q water solutions ( $C_{MQ}$ ) in parallel and compared. Results from duplicate conditions are shown.

We continued the investigation of the effects of salts on PFOS behavior, focusing on specific salt effects and quantification of air-water interfacial sorption through surface tension measurements.

Initially, we compared groundwater relevant mono- and divalent salts on PFOS detection in bulk solution. We found that the monovalent salts NaCl and KCl resulted in over 50% reduction in detected PFOS concentration in the bulk solution. The divalent salts MgCl<sub>2</sub> and CaCl<sub>2</sub> resulted in approximately 20% reduction in PFOS concentration (Figure 63). In contrast, surface tension experiments indicated decreased surface tension, and therefore, higher interfacial activity, in the presence of divalent salts compared to the monovalent salts (Figure 64). Together, these data suggest that high concentrations of monovalent salts may be of higher concern for accurate quantification of PFOS in sampling saline waters, while high concentrations of divalent salts may have a greater effect on PFOS interfacial behavior and sorption to soil in the vadose zone.

The results of the salt effects studies summarized in this Additional Findings section were published in Environmental Science and Technology Letters (Steffens et al 2021).

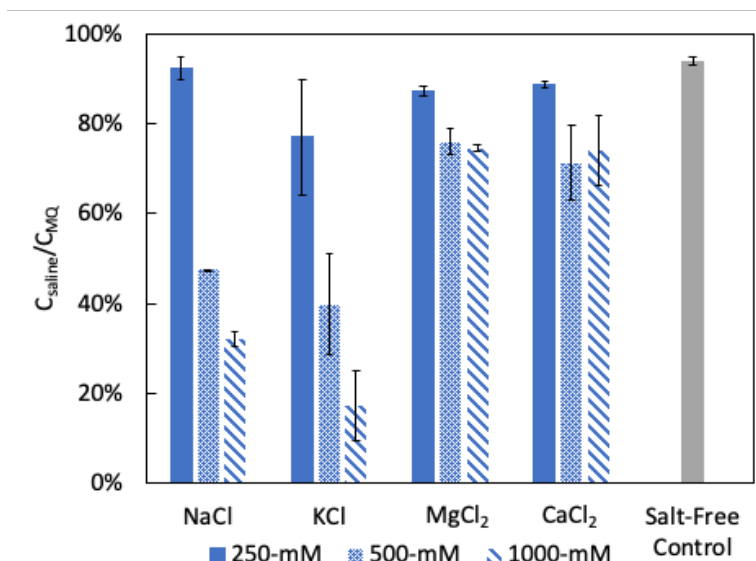


Figure 63. Effects of 250-1000 mM monovalent and divalent salts on decreased detection of PFOS in bulk solution. PFOS concentration was measured before ( $C_{MQ}$ ) and three hours after ( $C_{saline}$ ) salt addition; initial concentration was ca. 10 mg/L. Error bars represent the standard deviation of triplicate samples.

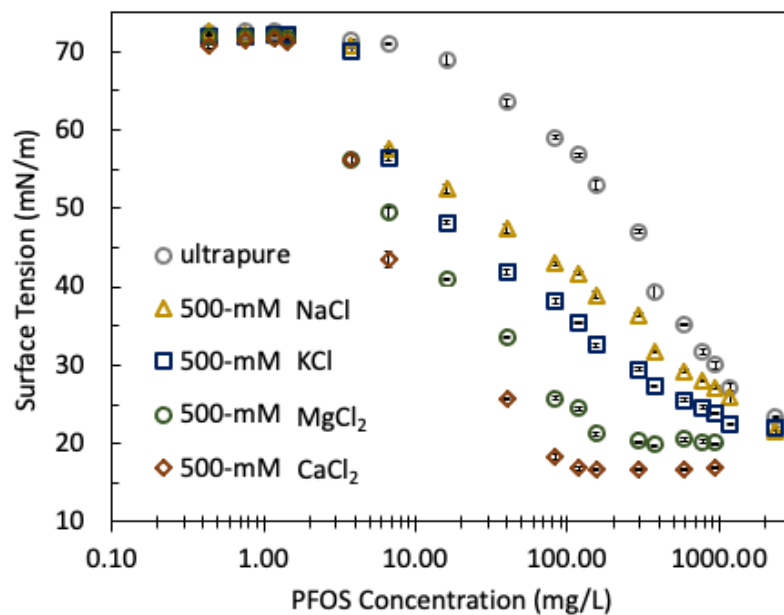


Figure 64. Surface tension isotherms for PFOS in 500 mM salt solutions at 22°C. Surface tension values and error bars are from the average and standard deviation of three drop measurements. The addition of monovalent ( $\text{Na}^+$ ,  $\text{K}^+$ ) and divalent cations ( $\text{Mg}^{2+}$ ,  $\text{Ca}^{2+}$ ) lowers the surface tension of the solutions below that of PFOS at equivalent concentrations in ultrapure water.

## Conclusions and Implications for Future Research/Implementation

### Objective 1

Low temperature (40°C) HAPO can be an effective PFAS remediation strategy at AFFF source zones, though with some key limitations. The aquifer should have low chloride concentrations, low buffering mineral content, low concentrations of toxic metals, and ideally be composed of precursor PFAS and/or PFCAs. Co-contaminants such as 1,4-dioxane and chlorinated solvents (and potentially many others) will be transformed under HAPO and much more quickly than the PFAS. Repeated injections of persulfate are likely needed for best transformation (50-300 mM, over several weeks) depending on the aquifer conditions. While some metals may be mobilized based on the acidic pH caused by HAPO, neutralization post-treatment can precipitate and immobilize the vast majority. Unintended byproducts such as chlorate and mobilized metals should be monitored post-HAPO and post-neutralization, as well as the pH of the aquifer.

ISCO projects have been steadily increasing since the 1990s for a range of organic contaminants, under a range of geological conditions, and to achieve different remediation goals (e.g., mass reduction, maximum concentration limits, alternative (risk-based) cleanup levels). Although in situ HAPO as an ISCO treatment for PFAS contaminants has its own set of challenges and inhibitions, it is one tool in the PFAS treatment toolbox that can mineralize PFAS in situ, an advantage over ex situ treatment options. Some industrial PFAS sites as well as landfill leachates with a higher concentration of PFCAs and precursors could be better suited for HAPO compared to some AFFF sites that have a high abundance of PFOS.

HAPO is a unique destruction method because it mineralizes two major classes of PFAS without creating a slew of unknown transformation products. Each site and PFAS-contaminated matrix is unique, and HAPO can, and should, be one of the many tools to help break the global, persistent PFAS cycle.

### Objective 2

In this objective, enrichment cultures exposed to high concentrations of salt that would occur in situ post-HAPO were shown to be very resistant to high salt conditions. A BTEX-enrichment culture from an AFFF-contaminated site was still able to degrade several doses of BTEX compounds in the presence of 500 mM sulfate as HAPO solution. These results show that at sites with AFFF co-contaminants such as fuels, naturally occurring microbial communities can continue to consume these substrates even after an in situ HAPO treatment for PFAS.

Additionally, we showed that microorganisms were able to withstand exposure to PFOS, only experiencing inhibition effects at PFOS concentrations at ~100 µM. CB1190 showed inhibition of dioxane degradation at 100 µM PFOS, while 1 µM and 10 µM had no effect compared to the control. A toluene degrading enrichment culture experienced a delay in toluene degradation when exposed to 50 µM, 100 µM, and 175 µM PFOS. Interestingly, when a second toluene dose was added to the culture, in the 50 µM and 100 µM sets, the toluene degradation occurred much more rapidly than the first dose. This result suggests that some enrichment cultures can overcome the negative impact of PFOS, whereas pure cultures such as CB1190 may be more irreversibly impacted.

### Objective 3

We found that different microbial communities impacted by available carbon sources will affect AmPr-FHxSA biotransformation yields and product composition. Specifically, with methane and acetate as carbon sources, the resulting microbial communities had higher yields of FHxSA and PFHxS compared to DGBE and BTEX, and additionally contained a greater diversity of intermediate products. The methane oxidizing community was the most diverse, which could point to a correlation between microbial diversity and increased biotransformation of ECF-based precursors. Lack of microbial diversity could especially be significant at sites where there has been a long history of BTEX contamination, or other commonly used solvents, that could result in a site's microbial community being more enriched or specialized for a particular substrate, resulting in less precursor biotransformation. Additionally, while FHxSA is a main biotransformation product with the microbial communities utilizing methane and acetate, this compound could be a bottleneck, resulting in slow, long-term PFHxS production. In our study, each of the microbial communities tested resulted in some PFHxS production; however, chemical oxidation of both AmPr-FHxSA and FHxSA primarily results in PFHxA under the TOP assay, which can be mineralized under heat-activated acidic persulfate oxidation. Therefore, persulfate oxidation could be used as a remediation tool for an AFFF-contaminated site to prevent long-term biotic production of FHxSA and PFHxS. However, a small fraction of FHxSA also transforms to PFHxS during chemical oxidation and appears to be more recalcitrant than AmPr-FHxSA during heat-activated persulfate oxidation. These latter two results point to the unique characteristics of perfluoroalkyl sulfonamides, which should be carefully monitored at AFFF-contaminated sites, especially FHxSA and other short-chain sulfonamides that are not usually included in monitoring lists.

At sites with multiple contaminants, high concentrations of AFFF may inhibit anaerobic chlorinated solvent bioremediation, while AFFF and potentially FHxSA and AmPr-FHxSA can negatively impact aerobic BTEX degradation. PFAS may act by interfering with cells' proton motive force, which drives ATP production thus reducing the cell's ability to create energy currency. Neutral PFAS such as FHxSA may be able to penetrate cell membranes more effectively than negatively charged PFAS, and zwitterionic PFAS may use electrostatic interactions to interfere with cell proteins. Additionally, changes in metabolite concentrations point to certain amino acids associated with cell signaling and protein activation being impacted by the presence of AFFF. In source zones where AFFF concentrations are the highest, transformation of co-contaminants by microbial communities may be inhibited, while zones with lower AFFF concentrations or individual PFAS species will be less likely to interfere with bioremediation processes.

### Additional Findings

For the additional findings on salt effects on PFOS behavior in aqueous solution, our results indicate that PFSA were not transformed, destroyed, or removed during HAPO, though high salt concentrations can affect PFSA behavior in aqueous solutions. That is, using standard quantification techniques, aqueous concentrations of PFOS appeared to decrease during ISCO treatment conditions, i.e. low pH and high ionic strength. The observed concentration decrease was directly related to the addition of salt, either in the form of  $\text{Na}_2\text{S}_2\text{O}_8$  or  $\text{Na}_2\text{SO}_4$ . The results of sand-free and sand-containing solution experiments indicate that PFOS, or its aggregates, were not adsorbing to sand in a significant manner. Both the concentration of salt added and the concentration of PFOS in solution affected the magnitude of the PFOS decrease; higher salt

concentrations produced the largest concentration decreases, and high PFOS concentrations were most affected by salt addition. The observed aqueous concentration decreased immediately after salt addition. The observed effects of salt addition suggest that salt causes PFOS to 'salt out' or form aggregates in solution, causing a decrease in detected PFOS concentrations. To accurately assess the PFSA concentrations, these results should be considered, especially during sample preparation and storage.

Upon investigating the salting-out mechanism further, we found that at sufficiently high PFOS concentrations (i.e., above 5 mg/L), the salting-out effect can lead to under-reporting of PFOS concentrations under ISCO-like and real seawater conditions. The effect of decreasing PFOS in bulk solution was strongest for monovalent salts, namely  $\text{Na}^+$  and  $\text{K}^+$ . Relative to divalent cations (i.e.,  $\text{Mg}^{2+}$  and  $\text{Ca}^{2+}$ ) the effect of the salt on increasing PFOS surface activity—a factor critical to fate and transport—was stronger than for monovalent cations. These results suggest that the presence of monovalent salts is more relevant for sampling artifacts in bulk solution. The presence of divalent salts may be more relevant for modeling partition coefficients and informing transport studies, particularly of PFOS in unsaturated zones.

Further investigation is needed to determine how salts affect PFAS under the complex conditions encountered at contaminated sites. For example, AFFF also contains hydrocarbon-based surfactants and solvents, such as diethylene glycol butyl ether (DGBE). Salts may also affect the behavior of shorter and longer chain PFAS (e.g., perfluorohexanesulfonic acid and perfluorodecanesulfonic acid). Finally, it may be possible to exploit salt induced aggregation behavior as a means of enhancing the removal of PFOS and other PFAS from hazardous waste and other matrices that contain high concentrations of contaminants.

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## Appendices

### Appendix A. Supporting Data

#### Modified BAV1 Media Recipe

One liter of BAV1 media was prepared by adding 10 mL of salts solution (1 g NaCl; 0.5 g  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ ; 0.2 g  $\text{KH}_2\text{PO}_4$ ; 0.3 g  $\text{NH}_4\text{Cl}$ ; 0.3 g KCl; 15 mg  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  per liter of Milli-Q water), 1 mL of trace elements solution (10 mL of 25% w/w HCl; 1.5 g  $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$ ; 0.19 g  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ ; 0.1 g  $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ ; 70 mg  $\text{ZnCl}_2$ ; 6 mg  $\text{H}_3\text{BO}_3$ ; 36 mg  $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ ; 24 mg  $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$ ; 2 mg  $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$  per liter of Milli-Q water), 1 mL of Se/Wo solution (6 mg  $\text{Na}_2\text{SeO}_3 \cdot \text{H}_2\text{O}$ ; 8 mg  $\text{NaWO}_4 \cdot 2\text{H}_2\text{O}$ ; 0.5 g NaOH per liter of Milli-Q water), and 2.52 g of  $\text{NaHCO}_3$ .

#### AMS Media Recipe

One liter of Ammonium Mineral Salts (AMS) media was prepared by adding salts (0.66 g  $(\text{NH}_4)_2\text{SO}_4$ ; 1.0 g  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ ; 0.015 g  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ ), 1 mL AMS trace elements solution (0.5 g  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ ; 0.4 g  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ ; 0.02 g  $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ ; 0.015 g  $\text{H}_3\text{BO}_3$ ; 0.01 g  $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$ ; 0.25 g EDTA; 0.05 g  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ ; 0.005 g  $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$  per liter of Milli-Q water), 1 mL Stock A (5.0 g Fe-Na EDTA; 2.0 g  $\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$  per liter of Milli-Q water), and 20 mL phosphate buffer (113 g  $\text{K}_2\text{HPO}_4$ ; 47.0 g  $\text{KH}_2\text{PO}_4$  per Liter of Milli-Q water). Specifically, the AMS recipe without the phosphate buffer was added to individual bottles, capped, and sterilized in an autoclave. The phosphate buffer solution was filter-sterilized separately and added to the autoclaved bottles under sterile conditions.

### Objective 1

Table A. 1 LC-MS/MS mobile phase gradient for negative mode in HAPO experiments.

| Time  | A % | B % |
|-------|-----|-----|
| 0     | 90  | 100 |
| 2     | 90  | 100 |
| 8     | 5   | 70  |
| 12    | 5   | 70  |
| 12.50 | 90  | 40  |
| 19    | 90  | 40  |

Table A. 2 Rate constants with sulfate radicals and PFAS and aquifer constituents.

| Reactants                                   |                                             | Products                                    |                                                            | Rate constant<br>(M <sup>-1</sup> s <sup>-1</sup> ) |  | Reference                |
|---------------------------------------------|---------------------------------------------|---------------------------------------------|------------------------------------------------------------|-----------------------------------------------------|--|--------------------------|
| Radical propagation                         |                                             |                                             |                                                            |                                                     |  |                          |
| SO <sub>4</sub> <sup>•-</sup>               | HO <sup>•</sup>                             | HSO <sub>5</sub> <sup>-</sup>               |                                                            | 1.00E+10                                            |  | Das, T.N. 2001           |
| SO <sub>4</sub> <sup>•-</sup>               | SO <sub>5</sub> <sup>•-</sup>               | S <sub>2</sub> O <sub>8</sub> <sup>2-</sup> | 0.5O <sub>2</sub>                                          | 8.96E+09                                            |  | Qian, Yajie, et al. 2015 |
| SO <sub>4</sub> <sup>•-</sup>               | SO <sub>4</sub> <sup>•-</sup>               | S <sub>2</sub> O <sub>8</sub> <sup>2-</sup> |                                                            | 8.20E+09                                            |  | Criquet & Leitner 2009   |
| HO <sup>•</sup>                             | HO <sub>2</sub> <sup>•</sup>                | H <sub>2</sub> O                            | O <sub>2</sub>                                             | 6.60E+09                                            |  | Sehested et al 1968      |
| HO <sup>•</sup>                             | HO <sup>•</sup>                             | H <sub>2</sub> O <sub>2</sub>               |                                                            | 5.00E+09                                            |  | Kamel et al 2009         |
| SO <sub>4</sub> <sup>•-</sup>               | HO <sub>2</sub> <sup>•</sup>                | HSO <sub>4</sub> <sup>-</sup>               | O <sub>2</sub>                                             | 3.50E+09                                            |  | Jiang et al. 1992        |
| SO <sub>5</sub> <sup>•-</sup>               | SO <sub>5</sub> <sup>•-</sup>               | S <sub>2</sub> O <sub>8</sub> <sup>2-</sup> | O <sub>2</sub>                                             | 2.20E+08                                            |  | Das, T.N. 2001           |
| SO <sub>5</sub> <sup>•-</sup>               | SO <sub>5</sub> <sup>•-</sup>               | SO <sub>4</sub> <sup>•-</sup>               | SO <sub>4</sub> <sup>•-</sup> O <sub>2</sub>               | 2.10E+08                                            |  | Das, T.N. 2001           |
| SO <sub>4</sub> <sup>•-</sup>               | OH-                                         | HO <sup>•</sup>                             | SO <sub>4</sub> <sup>2-</sup>                              | 8.30E+07                                            |  | Neta et al 1988          |
| HO <sup>•</sup>                             | H <sub>2</sub> O <sub>2</sub>               | H <sub>2</sub> O                            | HO <sub>2</sub> <sup>•</sup>                               | 2.70E+07                                            |  | Buxton et al 1988        |
| S <sub>2</sub> O <sub>8</sub> <sup>2-</sup> | HO <sup>•</sup>                             | HSO <sub>4</sub> <sup>-</sup>               | SO <sub>4</sub> <sup>•-</sup> 0.5O <sub>2</sub>            | 1.20E+07                                            |  | Peternel et al 2013      |
| SO <sub>4</sub> <sup>•-</sup>               | H <sub>2</sub> O <sub>2</sub>               | HSO <sub>4</sub> <sup>-</sup>               | HO <sub>2</sub> <sup>•</sup>                               | 1.20E+07                                            |  | Jiang et al. 1992        |
| SO <sub>4</sub> <sup>•-</sup>               | HSO <sub>5</sub> -                          | SO <sub>5</sub> <sup>•-</sup>               | HSO <sub>4</sub> <sup>-</sup>                              | 1.00E+06                                            |  | Das, T.N. 2001           |
| SO <sub>4</sub> <sup>•-</sup>               | S <sub>2</sub> O <sub>8</sub> <sup>•-</sup> | S <sub>2</sub> O <sub>8</sub> <sup>2-</sup> | SO <sub>2</sub> O <sub>2</sub>                             | 9.92E+05                                            |  | Qian, Yajie, et al. 2015 |
| HO <sub>2</sub> <sup>•</sup>                | HO <sub>2</sub> <sup>•</sup>                | H <sub>2</sub> O <sub>2</sub>               | O <sub>2</sub>                                             | 8.30E+05                                            |  | Bielski 1985             |
| HO <sup>•</sup>                             | HSO <sub>4</sub> <sup>-</sup>               | SO <sub>4</sub> <sup>•-</sup>               | H <sub>2</sub> O                                           | 3.74E+05                                            |  | Yu et al 2004            |
| SO <sub>4</sub> <sup>•-</sup>               | S <sub>2</sub> O <sub>8</sub> <sup>2-</sup> | S <sub>2</sub> O <sub>8</sub> <sup>•-</sup> | SO <sub>4</sub> <sup>2-</sup>                              | 1.80E+05                                            |  | Yu et al 2004            |
| SO <sub>4</sub> <sup>•-</sup>               | H <sub>2</sub> O                            | HO <sup>•</sup>                             | HSO <sub>4</sub> <sup>-</sup>                              | 1.82E+03                                            |  | Herrmann et al 1995      |
| S <sub>2</sub> O <sub>8</sub> <sup>•-</sup> | SO <sub>4</sub> <sup>2-</sup>               | S <sub>2</sub> O <sub>8</sub> <sup>2-</sup> | SO <sub>4</sub> <sup>•-</sup>                              | 9.50E-02                                            |  | Yu et al 2004            |
| S <sub>2</sub> O <sub>8</sub> <sup>•-</sup> | H <sub>2</sub> O                            | HO <sup>•</sup>                             | H <sup>+</sup> S <sub>2</sub> O <sub>8</sub> <sup>2-</sup> | 8.30E-04                                            |  | Qian, Yajie, et al. 2015 |
| PFCAs                                       |                                             |                                             |                                                            |                                                     |  |                          |
| SO <sub>4</sub> <sup>•-</sup>               | C <sub>2</sub> F <sub>5</sub> COOH          | CF <sub>3</sub> COOH                        | Byproducts                                                 | 9.31E+07                                            |  | Qian, Yajie, et al. 2015 |
| SO <sub>4</sub> <sup>•-</sup>               | C <sub>3</sub> F <sub>7</sub> COOH          | C <sub>2</sub> F <sub>5</sub> COOH          | Byproducts                                                 | 1.05E+07                                            |  | Qian, Yajie, et al. 2015 |
| SO <sub>4</sub> <sup>•-</sup>               | C <sub>4</sub> F <sub>9</sub> COOH          | C <sub>3</sub> F <sub>7</sub> COOH          | Byproducts                                                 | 1.26E+06                                            |  | Qian, Yajie, et al. 2015 |
| SO <sub>4</sub> <sup>•-</sup>               | C <sub>5</sub> F <sub>11</sub> COOH         | C <sub>4</sub> F <sub>9</sub> COOH          | Byproducts                                                 | 7.02E+05                                            |  | Qian, Yajie, et al. 2015 |
| SO <sub>4</sub> <sup>•-</sup>               | C <sub>6</sub> F <sub>13</sub> COOH         | C <sub>5</sub> F <sub>11</sub> COOH         | Byproducts                                                 | 2.68E+05                                            |  | Qian, Yajie, et al. 2015 |
| SO <sub>4</sub> <sup>•-</sup>               | C <sub>7</sub> F <sub>15</sub> COOH         | C <sub>6</sub> F <sub>13</sub> COOH         | Byproducts                                                 | 2.59E+05                                            |  | Qian, Yajie, et al. 2015 |
| Aquifer Constituents                        |                                             |                                             |                                                            |                                                     |  |                          |
| SO <sub>4</sub> <sup>•-</sup>               | Fe <sup>2+</sup>                            | SO <sub>4</sub> <sup>2-</sup>               | Fe <sup>3+</sup>                                           | 9.90E+08                                            |  | Neta et al 1988          |
| SO <sub>4</sub> <sup>•-</sup>               | NOM                                         | Byproducts                                  |                                                            | 2.35E+07                                            |  | Xie et al 2015           |
| SO <sub>4</sub> <sup>•-</sup>               | Mn <sup>2+</sup>                            | SO <sub>4</sub> <sup>2-</sup>               | Mn <sup>3+</sup>                                           | 2.00E+07                                            |  | Neta et al 1988          |
| SO <sub>4</sub> <sup>•-</sup>               | CO <sub>3</sub> <sup>2-</sup>               | CO <sub>3</sub> <sup>•-</sup>               | SO <sub>4</sub> <sup>2-</sup>                              | 6.50E+06                                            |  | Zuo et al 1999           |
| SO <sub>4</sub> <sup>•-</sup>               | HCO <sub>3</sub> <sup>-</sup>               | CO <sub>3</sub> <sup>•</sup>                | H <sup>+</sup> SO <sub>4</sub> <sup>2-</sup>               | 3.60E+06                                            |  | Chameides & Davis 1982   |
| SO <sub>4</sub> <sup>•-</sup>               | HPO <sub>4</sub> <sup>2-</sup>              | HPO <sub>4</sub> <sup>•-</sup>              |                                                            | 1.20E+06                                            |  | Neta et al 1988          |
| SO <sub>4</sub> <sup>•-</sup>               | NO <sub>3</sub> <sup>-</sup>                | NO <sub>3</sub> <sup>•</sup>                | SO <sub>4</sub> <sup>2-</sup>                              | 3.60E+05                                            |  | Neta et al 1988          |

Table A. 3 Rate constants with sulfate radicals and chloride species, and relevant pKa values.

| <i>Chloride</i>          |                                               |                          |                          |               |            |                          |
|--------------------------|-----------------------------------------------|--------------------------|--------------------------|---------------|------------|--------------------------|
| $\text{SO}_4^{\bullet-}$ | $\text{ClO}_2^{\bullet} + \text{H}_2\text{O}$ | $\text{ClO}_3^-$         | $\text{SO}_4^{2-}$       | $2\text{H}^+$ | 8.23E+09   | Qian, Yajie, et al. 2015 |
| $\text{Cl}^{\bullet}$    | $\text{Cl}^-$                                 | $\text{Cl}_2^{\bullet-}$ |                          |               | 8.00E+09   | Yu et al 2004            |
| $\text{Cl}_2^{\bullet-}$ | $\text{Cl}_2^{\bullet-}$                      | $\text{Cl}_2$            | $2\text{Cl}^-$           |               | 6.41E+09   | Neta et al 1988          |
| $\text{HO}^{\bullet}$    | $\text{Cl}^-$                                 | $\text{ClOH}^{\bullet-}$ |                          |               | 4.30E+09   | Jayson et al 1973        |
| $\text{HO}^{\bullet}$    | $\text{ClO}_2^{\bullet}$                      | $\text{ClO}_3^-$         | $\text{H}^+$             |               | 4.00E+09   | Neta et al 1988          |
| $\text{Cl}^{\bullet}$    | $\text{Cl}_2^{\bullet-}$                      | $\text{Cl}_2$            | $\text{Cl}^-$            |               | 2.10E+09   | Yu & Barker 2003         |
| $\text{HO}^{\bullet}$    | $\text{ClO}_2^-$                              | $\text{ClO}_2^{\bullet}$ | $\text{OH}^-$            |               | 1.00E+09   | Neta et al 1988          |
| $\text{Cl}^{\bullet}$    | $\text{CO}_3^{2-}$                            | $\text{Cl}^-$            | $\text{CO}_3^{\bullet-}$ |               | 5.00E+08   | Yang et al 2014          |
| $\text{SO}_4^{\bullet-}$ | $\text{Cl}^-$                                 | $\text{SO}_4^{2-}$       | $\text{Cl}^{\bullet}$    |               | 4.70E+08   | Yuan et al 2014          |
| $\text{Cl}^{\bullet}$    | $\text{SO}_4^{2-}$                            | $\text{SO}_4^{\bullet-}$ | $\text{Cl}^-$            |               | 2.50E+08   | Yu et al 2004            |
| $\text{Cl}^{\bullet}$    | $\text{HCO}_3^-$                              | $\text{Cl}^-$            | $\text{CO}_3^{\bullet-}$ | $\text{H}^+$  | 2.20E+08   | Yang et al 2014          |
| $\text{Cl}^{\bullet}$    | $\text{Cl}^{\bullet}$                         | $\text{Cl}_2$            |                          |               | 8.80E+07   | Wu et al 1980            |
| $\text{Cl}^{\bullet}$    | $\text{H}_2\text{O}$                          | $\text{ClOH}^{\bullet-}$ | $\text{H}^+$             |               | 1.30E+03   | Liang et al 2006         |
| <i>pH</i>                |                                               |                          |                          |               |            |                          |
| $\text{HSO}_4^{\bullet}$ |                                               | $\text{SO}_4^{\bullet-}$ | $\text{H}^+$             |               | pKa < 1.9  |                          |
| $\text{HSO}_4^-$         |                                               | $\text{SO}_4^{2-}$       | $\text{H}^+$             |               | pKa = 1.9  |                          |
| $\text{H}_2\text{CO}_3$  |                                               | $\text{HCO}_3^-$         | $\text{H}^+$             |               | pKa = 6.3  |                          |
| $\text{HCO}_3^-$         |                                               | $\text{CO}_3^{2-}$       | $\text{H}^+$             |               | pKa = 10.3 |                          |

### Objective 3

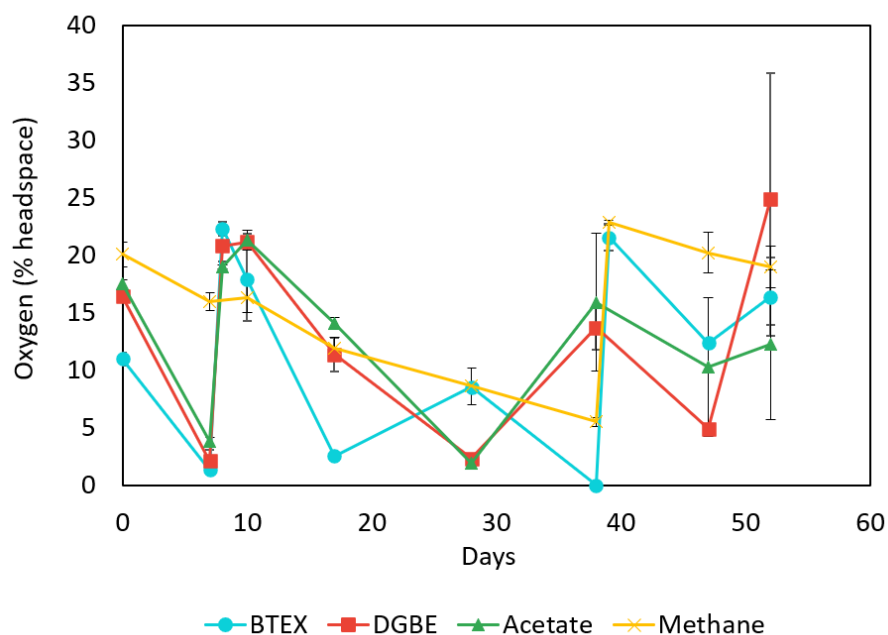


Figure A. 1 Oxygen consumption and replenishment for the four carbon source experiment.

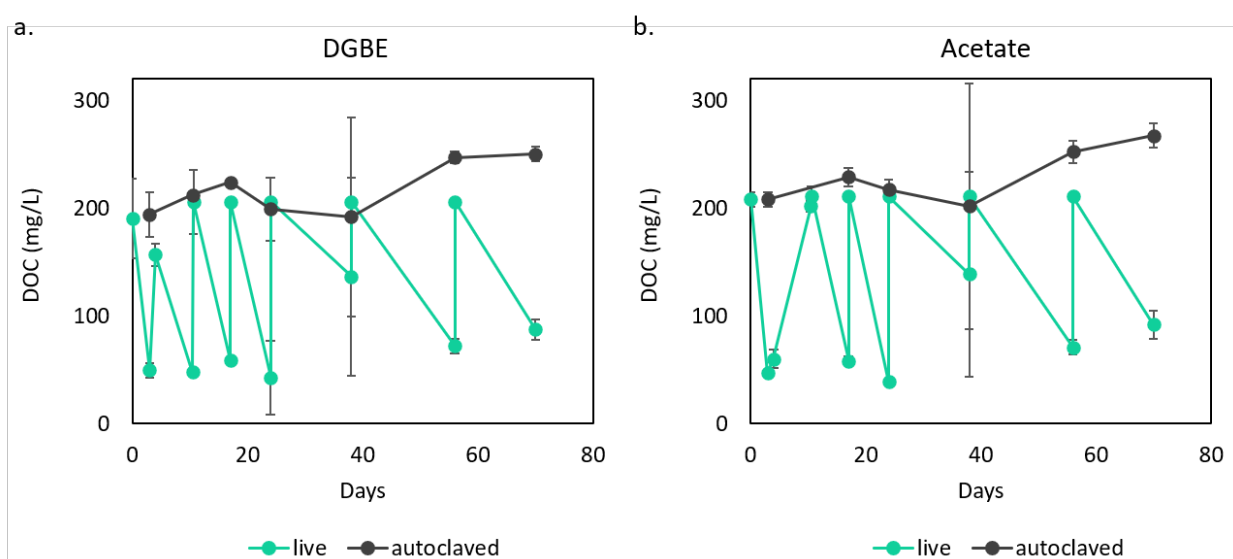


Figure A. 2 DOC degradation for the DGBE and acetate cultures for the four carbon source experiment.

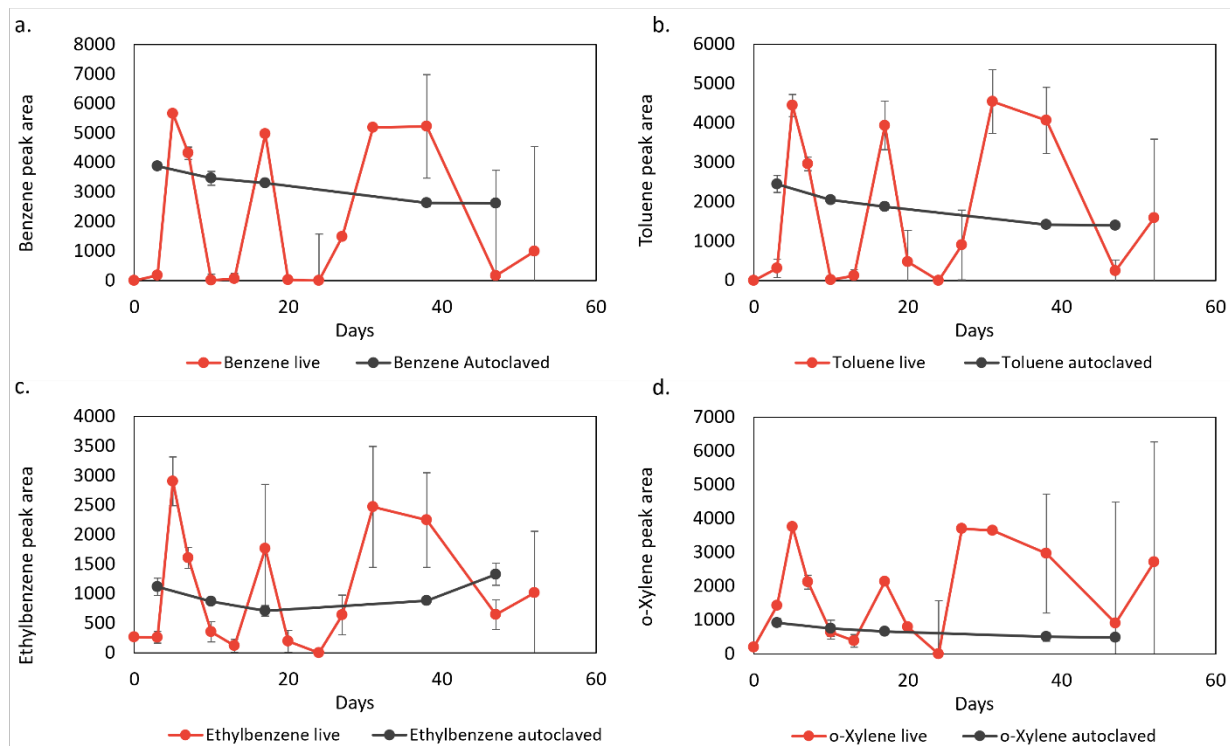


Figure A. 3 BTEX measurements for the live and autoclaved triplicate cultures for the four carbon source experiment.

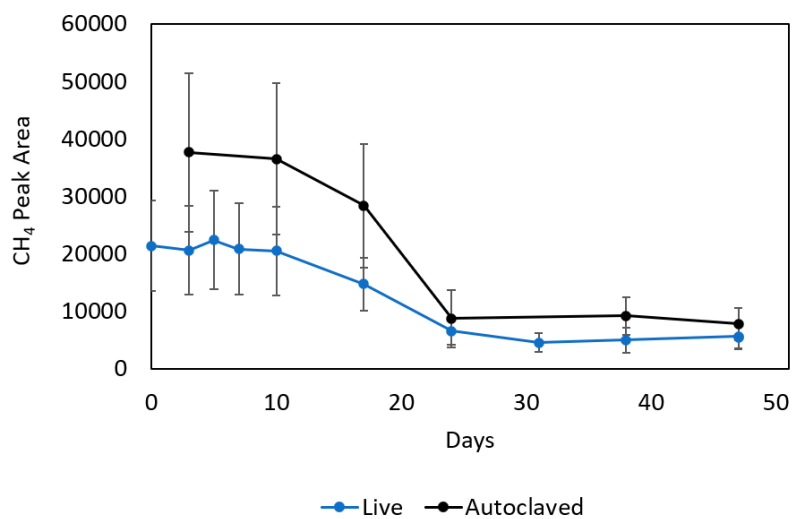


Figure A. 4 Methane measurements for live and autoclaved triplicate cultures for the four carbon source experiment.

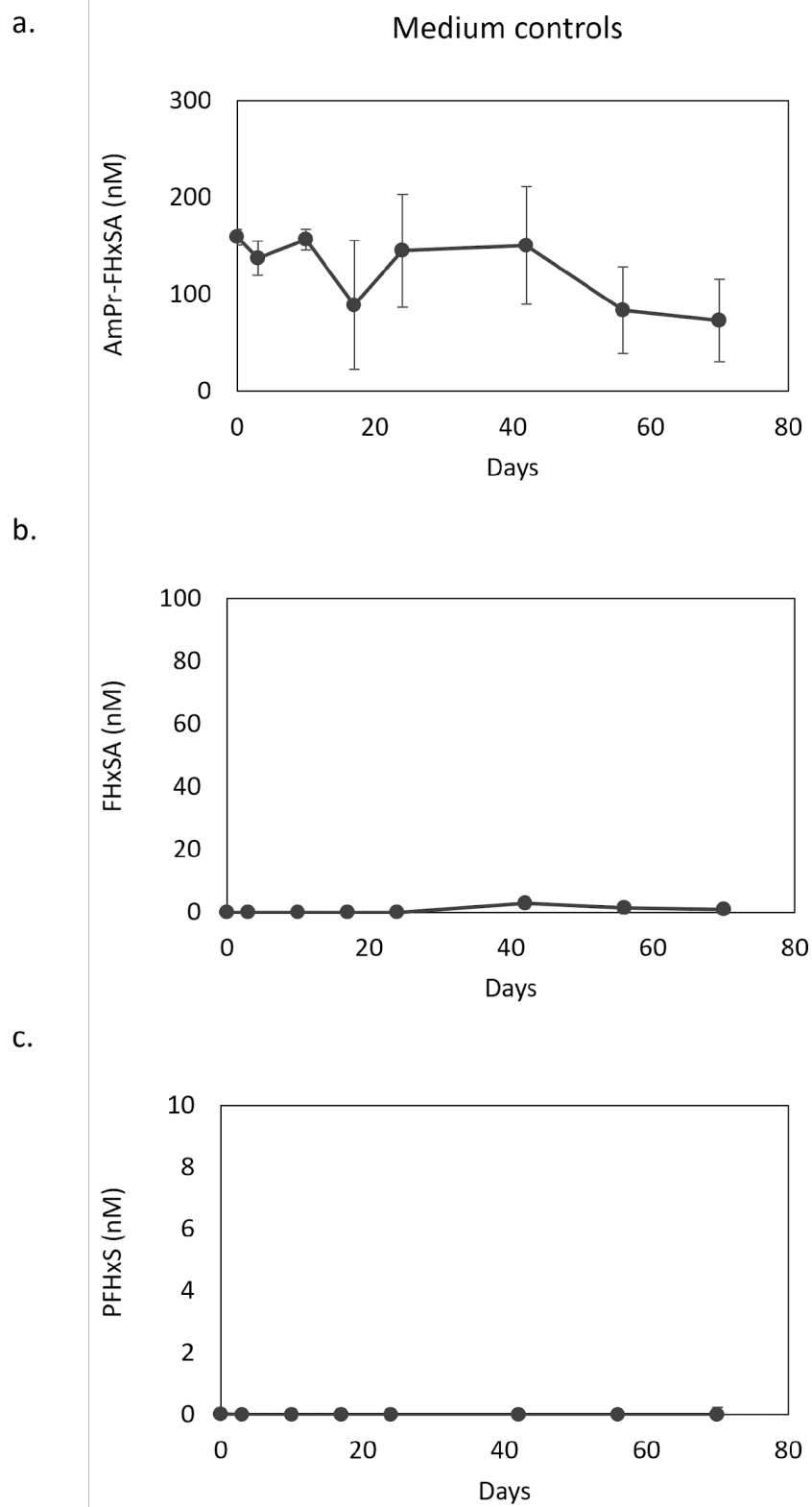


Figure A. 5 PFAS measurements for the medium controls of the four carbon source experiment using LC-MS/MS.

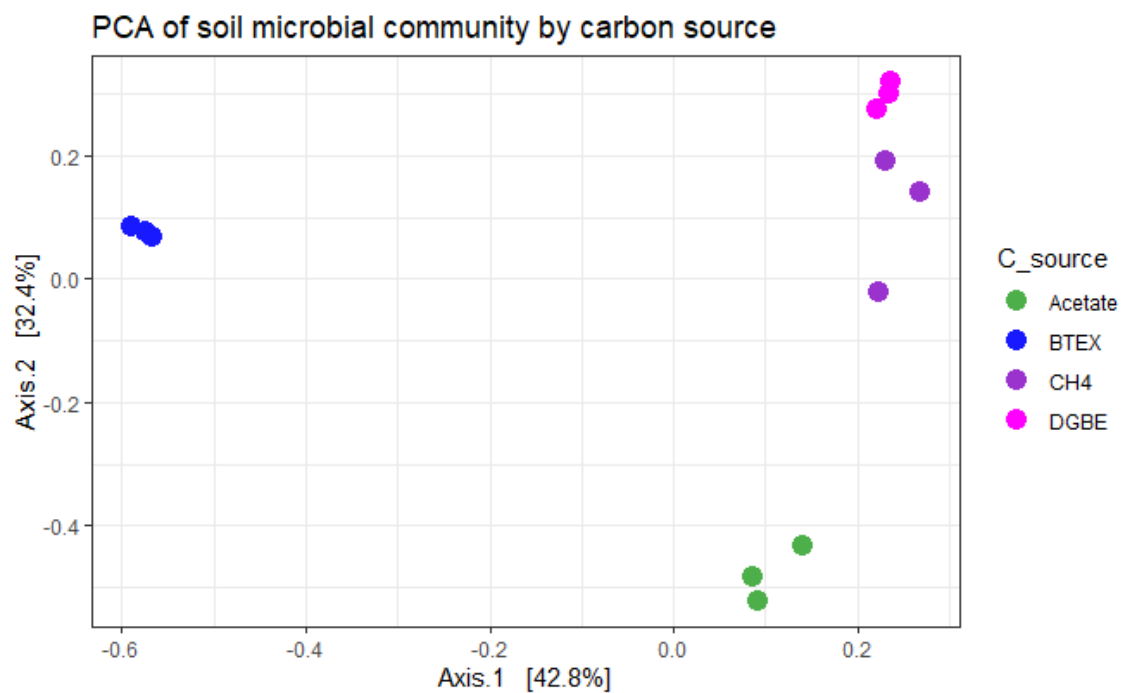


Figure A. 6 Principal component analysis space of each of the four carbon sources according to their detected genera.

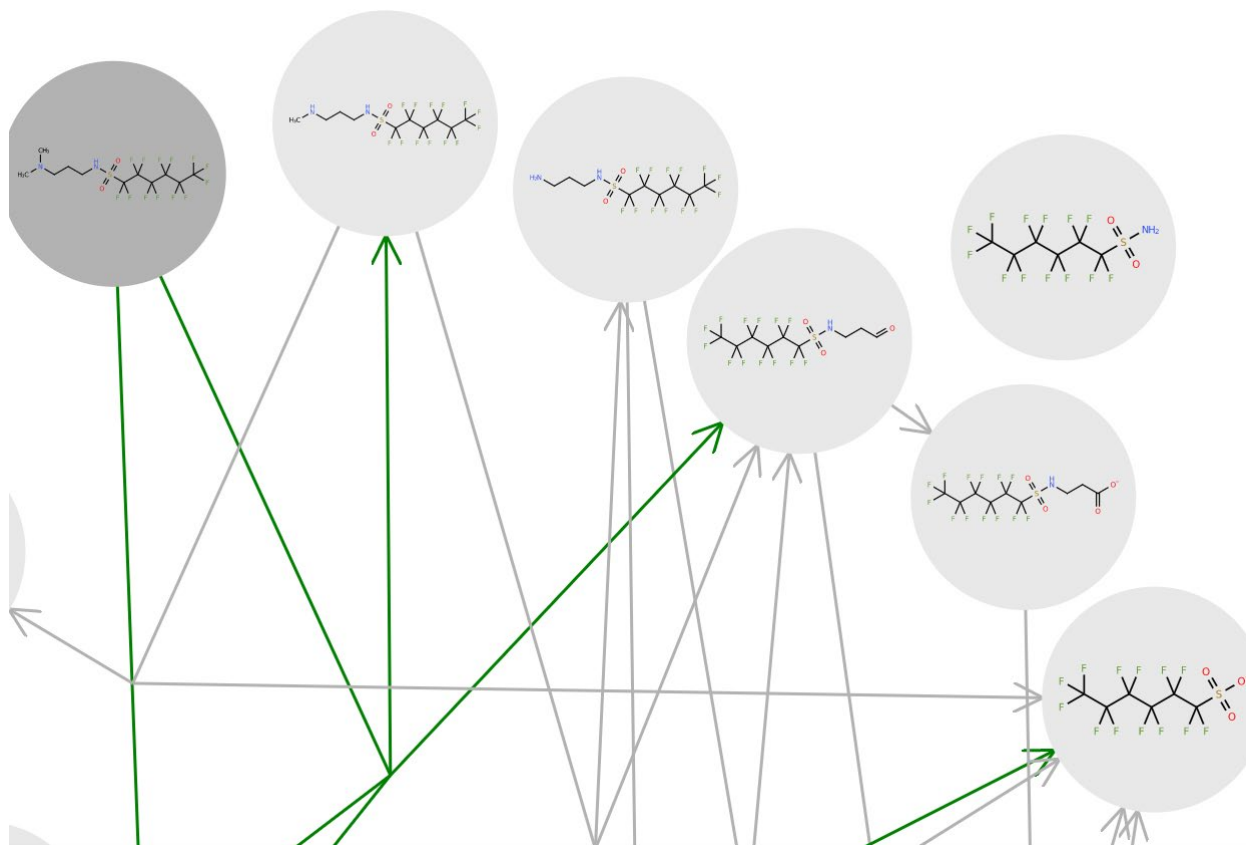


Figure A. 7 Proposed pathway zoomed in on fluorinated results (EnviPath).

SMILES ID used: FC(C(F)(F)C(F)(F)C(F)(F)C(F)(F)C(S(=O)(NCCCN(C)C)=O)(F)F)(F)F. FHxSA is shown here, but was manually added by us during analysis; it was not originally suggested as a transformation product of this parent.

Table A. 4 PFAS concentrations in ECF-based AFFF. Duplicate samples run on the LC-MS/MS of the diluted AFFF stock solution. Error is simply the difference between the two replicates.

| Conc ( $\mu$ M) | AmPr-FHxSA | PFBA | PFPeA | PFHxA | PFHpA | PFOA | PFBS | PFHxS | PFOS  |
|-----------------|------------|------|-------|-------|-------|------|------|-------|-------|
| Average         | 6573       | 359  | 279   | 956   | 270   | 937  | 1408 | 3825  | 20274 |
| Error           | 615        | 8    | 51    | 130   | 9     | 68   | 53   | 1193  | 756   |

Table A. 5 PFAS in targeted LC-MS/MS analyses.

| Compound   | Internal Standard | Precursor Ion | Product Ion | Fragmentor Voltage (V) | Collision Energy (V) | Polarity |
|------------|-------------------|---------------|-------------|------------------------|----------------------|----------|
| MPFBA      | -                 | 217           | 172         | 50                     | 5                    | Neg      |
| MPFPeA     | -                 | 266           | 222<br>221  | 60                     | 2                    | Neg      |
| MPFHxA     | -                 | 315           | 270         | 60                     | 5                    | Neg      |
| MPFOA      | -                 | 417           | 372         | 70                     | 2                    | Neg      |
| MPFNA      | -                 | 468           | 423         | 70                     | 5                    | Neg      |
| MPFHxS     | -                 | 403           | 103         | 150                    | 40                   | Neg      |
| MPFOS      | -                 | 503           | 80          | 190                    | 60                   | Neg      |
| PFBA       | MPFBA             | 213           | 169         | 50                     | 2                    | Neg      |
| PFPeA      | MPFPeA            | 263           | 219<br>68.9 | 60<br>92               | 2<br>8               | Neg      |
| PFHxA      | MPFHxA            | 313           | 269<br>119  | 80                     | 2<br>15              | Neg      |
| PFHpA      | MPFOA             | 363           | 319<br>169  | 80                     | 2                    | Neg      |
| PFOA       | MPFOA             | 413           | 369<br>169  | 80                     | 3<br>14              | Neg      |
| PFNA       | MPFNA             | 463           | 419<br>219  | 80                     | 2<br>15              | Neg      |
| PFBS       | MPFHxS            | 299           | 99<br>80    | 120                    | 30<br>70             | Neg      |
| PFHxS      | MPFHxS            | 399           | 99<br>80    | 125                    | 50<br>80             | Neg      |
| PFOS       | MPFOS             | 499           | 99<br>80    | 122                    | 50<br>80             | Neg      |
| FHxSA      | MPFOS             | 398           | 78          | 125                    | 36                   | Neg      |
| FOSA       | MPFOS             | 498           | 78          | 125                    | 36                   | Neg      |
| 6-2 FtS    | MPFOS             | 427           | 407<br>80   | 140                    | 25<br>35             | Neg      |
| AmPr-FHxSA | MPFOS             | 485           | 85          | 135                    | 30                   | Pos      |

Table A. 6 LC-MS/MS mobile phase gradient for four carbon source experiment. LC-MS/MS was run with water and methanol mobile phase, each with 10 mM ammonium acetate.

| Time | Water % | Methanol % |
|------|---------|------------|
| 0    | 75      | 25         |
| 5    | 50      | 50         |
| 13   | 5       | 95         |
| 16   | 5       | 95         |
| 16.5 | 75      | 25         |
| 23   | 75      | 25         |

Table A. 7 HRMS mobile phase gradient for four carbon source experiment. HRMS was run with water and methanol mobile phase, each with 10 mM ammonium acetate.

| Time | Water % | Methanol % |
|------|---------|------------|
| 0    | 90      | 10         |
| 0.5  | 50      | 50         |
| 8    | 1       | 99         |
| 13   | 1       | 99         |
| 13.5 | 90      | 10         |
| 20   | 90      | 10         |

Table A. 8 Average HRMS peak areas for suspect screening compounds related to AmPr-FHxSA biotransformation in live treatments (n=3) for day 0.

|         | OAmPr-FHxSA | FHxSA-PrA | FHxSAA | PFHxSi    |
|---------|-------------|-----------|--------|-----------|
| Acetate | 1199*       | ND        | ND     | 1567.5±65 |
| BTEX    | ND          | ND        | ND     | 997*      |
| DGBE    | ND          | ND        | ND     | 829*      |
| Methane | 3109*       | ND        | ND     | ND        |

ND means not detected. Asterisk(\*) denotes that only one replicate had a detectable peak. N-oxidedimethylammoniopropyl-perfluorohexanesulfonamide (OAmPr-FHxSA); Perfluorohexane sulfonamido propanoic acid (FHxSA-PrA); N-methylperfluorohexane sulfonamic acetic acid (FHxSAA); perfluorohexane sulfinic acid (PFHxSi)

Table A. 9 Background PFAS (µg/L; without dilution factor included) detected via LC-MS/MS on day 0 for four carbon source experiment.

| Sample                | N-TAmP-FHxSA | FHxSA | AmPr-FHxSA | 6-2 FtTAoS | 6-2 FtS | 6-2 FtSaB | PFBA  | PFPeA | PFHxA | PFHpA | PFOA  | PFNA | PFBS  | PFHxS | PFOS  |
|-----------------------|--------------|-------|------------|------------|---------|-----------|-------|-------|-------|-------|-------|------|-------|-------|-------|
| Name                  | ug/L         | ug/L  | ug/L       | ug/L       | ug/L    | ug/L      | ug/L  | ug/L  | ug/L  | ug/L  | ug/L  | ug/L | ug/L  | ug/L  | ug/L  |
| std00                 | 0.00         | 0.00  | 0.01       |            | 0.23    | 0.18      |       | 0.04  |       | 0.04  | 0.01  | 0.02 |       |       | 0.00  |
| std01                 | 0.19         | 0.10  | 0.19       | 0.06       | 0.26    | 0.26      | 0.14  | 0.28  | 0.30  | 0.26  | 0.23  | 0.21 | 0.22  |       | 0.16  |
| std02                 | 0.45         | 0.40  | 0.43       | 0.54       | 0.37    | 0.34      | 0.59  | 0.44  | 0.45  | 0.50  | 0.49  | 0.47 | 0.49  | 0.57  | 0.30  |
| std03                 | 0.99         | 0.86  | 0.96       | 1.37       | 0.97    | 1.15      | 1.08  | 0.92  | 0.99  | 1.02  | 1.04  | 1.00 | 0.90  | 0.78  | 0.97  |
| std04                 | 2.24         | 2.16  | 2.19       | 2.52       | 2.35    | 2.36      | 2.21  | 2.15  | 2.23  | 2.41  | 2.33  | 2.32 | 1.98  | 2.03  | 2.23  |
| std05                 | 4.99         | 4.69  | 4.85       | 4.99       | 5.32    | 4.75      | 5.05  | 5.25  | 4.85  | 5.08  | 5.13  | 5.20 | 5.54  | 5.60  | 4.82  |
| std06                 | 7.55         | 7.47  | 7.67       | 5.75       | 8.10    | 7.69      | 7.15  | 7.51  | 7.89  | 7.27  | 7.35  | 7.51 | 7.33  | 7.33  | 7.45  |
| std07                 | 10.08        | 10.18 | 10.14      | 10.92      | 9.50    | 10.07     | 10.35 | 10.06 | 10.16 | 10.15 | 10.20 | 9.94 | 10.02 | 10.03 | 10.18 |
| BTEX_d0_live_01       | 0.00         | 0.00  | 1.61       | 0.02       | 0.25    | 0.18      | 0.13  | 0.00  | 0.00  | 0.04  | 0.00  | 0.00 | 0.00  | 0.08  | 0.00  |
| BTEX_d0_live_02       | 0.00         | 0.00  | 2.21       | 0.00       | 0.26    | 0.18      | 0.13  | 0.02  | 0.00  | 0.04  | 0.00  | 0.00 | 0.00  | 0.05  | 0.00  |
| BTEX_d0_live_03       | 0.00         | 0.00  | 2.14       | 0.04       | 0.00    | 0.18      | 0.13  | 0.00  | 0.00  | 0.04  | 0.00  | 0.00 | 0.00  | 0.09  | 0.02  |
| BTEX_d0_autoclaved_01 | 0.00         | 0.00  | 2.91       | 0.00       | 0.23    | 0.19      | 0.13  | 0.00  | 0.00  | 0.04  | 0.00  | 0.00 | 0.00  | 0.08  | 0.05  |
| BTEX_d0_autoclaved_02 | 0.00         | 0.00  | 2.39       | 0.07       | 0.24    | 0.18      | 0.00  | 0.00  | 0.00  | 0.04  | 0.00  | 0.00 | 0.00  | 0.09  | 0.03  |
| BTEX_d0_autoclaved_03 | 0.00         | 0.00  | 2.31       | 0.06       | 0.23    | 0.18      | 0.13  | 0.00  | 0.00  | 0.05  | 0.00  | 0.00 | 0.00  | 0.07  | 0.00  |
| DGBE_d0_live_01       | 0.01         | 0.10  | 1.51       | 0.06       | 0.23    | 0.19      | 0.14  | 0.00  | 0.00  | 0.04  | 0.00  | 0.00 | 0.00  | 0.07  | 0.13  |
| DGBE_d0_live_02       | 0.00         | 0.08  | 1.71       | 0.02       | 0.27    | 0.19      | 0.14  | 0.00  | 0.00  | 0.04  | 0.00  | 0.00 | 0.00  | 0.07  | 0.00  |
| DGBE_d0_live_03       | 0.00         | 0.09  | 1.64       | 0.03       | 0.27    | 0.18      | 0.00  | 0.00  | 0.00  | 0.00  | 0.00  | 0.00 | 0.00  | 0.07  | 0.00  |
| DGBE_d0_autoclaved_01 | 0.00         | 0.48  | 1.46       | 0.02       | 0.24    | 0.19      | 0.00  | 0.00  | 0.00  | 0.04  | 0.00  | 0.00 | 0.00  | 0.09  | 0.06  |
| DGBE_d0_autoclaved_02 | 0.00         | 0.43  | 2.08       | 0.07       | 0.00    | 0.18      | 0.13  | 0.03  | 0.00  | 0.04  | 0.00  | 0.00 | 0.00  | 0.09  | 0.05  |
| DGBE_d0_autoclaved_03 | 0.00         | 0.73  | 2.27       | 0.00       | 0.24    | 0.18      | 0.16  | 0.00  | 0.00  | 0.05  | 0.00  | 0.00 | 0.00  | 0.09  | 0.04  |
| Acetate_d0_live_01    | 0.00         | 0.14  | 1.43       | 0.02       | 0.24    | 0.18      | 0.00  | 0.00  | 0.00  | 0.04  | 0.00  | 0.00 | 0.00  | 0.09  | 0.03  |
| Acetate_d0_live_02    | 0.00         | 0.14  | 1.31       | 0.00       | 0.25    | 0.00      | 0.13  | 0.00  | 0.00  | 0.04  | 0.00  | 0.00 | 0.00  | 0.09  | 0.01  |
| Acetate_d0_live_03    | 0.00         | 0.11  | 1.59       | 0.08       | 0.24    | 0.00      | 0.13  | 0.00  | 0.00  | 0.04  | 0.00  | 0.00 | 0.00  | 0.06  | 0.04  |

|                              |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
|------------------------------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|
| Acetate_d0_autoclave<br>d_01 | 0.00 | 0.06 | 1.15 | 0.00 | 0.26 | 0.00 | 0.13 | 0.00 | 0.00 | 0.04 | 0.00 | 0.00 | 0.00 | 0.09 | 0.05 |
| Acetate_d0_autoclave<br>d_02 | 0.00 | 0.13 | 1.41 | 0.00 | 0.23 | 0.18 | 0.00 | 0.01 | 0.00 | 0.04 | 0.00 | 0.00 | 0.00 | 0.08 | 0.06 |
| Acetate_d0_autoclave<br>d_03 | 0.00 | 0.06 | 2.01 | 0.03 | 0.24 | 0.18 | 0.13 | 0.00 | 0.00 | 0.04 | 0.00 | 0.00 | 0.00 | 0.10 | 0.07 |
| CH4_d0_live_01               | 0.00 | 0.02 | 1.50 | 0.00 | 0.24 | 0.19 | 0.13 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.10 | 0.05 |
| CH4_d0_live_02               | 0.00 | 0.09 | 1.65 | 0.00 | 0.28 | 0.18 | 0.13 | 0.00 | 0.00 | 0.04 | 0.00 | 0.00 | 0.00 | 0.07 | 0.04 |
| CH4_d0_live_03               | 0.00 | 0.04 | 1.63 | 0.00 | 0.28 | 0.19 | 0.13 | 0.00 | 0.00 | 0.04 | 0.00 | 0.00 | 0.00 | 0.08 | 0.00 |
| CH4_d0_autoclaved_<br>01     | 0.00 | 0.08 | 1.26 | 0.03 | 0.27 | 0.18 | 0.13 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.08 | 0.00 |
| CH4_d0_autoclaved_<br>02     | 0.00 | 0.07 | 1.76 | 0.00 | 0.26 |      | 0.14 | 0.00 | 0.00 | 0.04 | 0.00 | 0.00 | 0.00 | 0.07 | 0.00 |
| CH4_d0_autoclaved_<br>03     | 0.00 | 0.09 | 2.83 | 0.04 | 0.24 |      | 0.00 | 0.00 | 0.00 | 0.04 | 0.00 | 0.00 | 0.00 | 0.08 | 0.00 |
| Medium_d0_autoclav<br>ed_01  | 0.00 | 0.00 | 1.48 | 0.03 | 0.24 | 0.18 | 0.00 | 0.00 | 0.00 | 0.04 | 0.00 | 0.00 | 0.00 | 0.07 | 0.02 |
| Medium_d0_autoclav<br>ed_02  | 0.00 | 0.00 | 1.63 | 0.00 | 0.23 | 0.18 | 0.13 | 0.00 | 0.00 | 0.04 | 0.00 | 0.00 | 0.00 | 0.08 | 0.05 |
| Medium_d0_autoclav<br>ed_03  | 0.00 | 0.00 | 1.52 | 0.00 | 0.23 | 0.18 | 0.00 | 0.00 | 0.00 | 0.04 | 0.00 | 0.00 | 0.00 | 0.09 | 0.02 |

Table A. 10 Average HRMS peak areas for suspect screening compounds related to AmPr-FHxSA biotransformation in live and autoclaved treatments (n=3) for day 70 for four carbon source experiment.

|         |            | OAmPr-FHxSA | FHxSA-PrA  | FHxSAA    | PFHxSi    |
|---------|------------|-------------|------------|-----------|-----------|
| Acetate | Live       | 4885±4235   | 48238±3231 | 1159±190  | 1620±1439 |
|         | Autoclaved | ND          | ND         | ND        | ND        |
| BTEX    | Live       | ND          | ND         | ND        | 447±775   |
|         | Autoclaved | ND          | ND         | ND        | 1161±1015 |
| DGBE    | Live       | ND          | 1217±2108  | 341±591   | 1461±619  |
|         | Autoclaved | ND          | 1195±2069  | ND        | 3686±312  |
| Methane | Live       | ND          | 22895±6601 | 3084±2755 | 4077±1017 |
|         | Autoclaved | ND          | ND         | ND        | 2599±892  |

ND means not detected.

Table A. 11 Percentage of AmPr-FHxSA decrease between days 0 and 70, averaged between the triplicate reactors for four carbon source experiment.

|                    | % decrease of AmPr-FHxSA | Std Dev | T test     |
|--------------------|--------------------------|---------|------------|
| Methane live       | 80.1                     | 0.6     | 0.000<br>4 |
| Methane autoclaved | 69.2                     | 1.0     |            |
| Acetate live       | 83.0                     | 2.6     | 0.003<br>5 |
| Acetate autoclaved | 68.4                     | 1.4     |            |
| DGBE live          | 69.0                     | 5.4     | 0.938<br>7 |
| DGBE autoclaved    | 68.7                     | 6.8     |            |
| BTEX live          | 42.2                     | 11.7    | 0.143<br>3 |
| BTEX autoclaved    | 71.3                     | 0.9     |            |
| Medium control     | 54.1                     | 2.7     | --         |

The T test was performed in Excel to compare AmPr-FHxSA removal between the live and autoclaved reactors.

Table A. 12 Analysis of AmPr-FHxSA stock. Stock was diluted in MilliQ water (over several runs on the LC-MS/MS) to calculate the branched percentage.

| µg/L                 | Area                 | µg/L           | Area           | µg/L                  | Area                  |                       |
|----------------------|----------------------|----------------|----------------|-----------------------|-----------------------|-----------------------|
| Linear +<br>Branched | Linear +<br>Branched | Linear<br>only | Linear<br>only | Estimated<br>branched | Estimated<br>branched | %<br>branched<br>area |
| 2.1182               | 8135                 | 1.879          | 7170           | 0.2392                | 965                   | 11.9                  |
| 2.3826               | 8079                 | 2.1806         | 7363           | 0.202                 | 716                   | 8.9                   |
| 3.8943               | 11244                | 3.5108         | 10107          | 0.3835                | 1137                  | 10.1                  |
| 3.1713               | 42418                | 2.8017         | 37068          | 0.3696                | 5350                  | 12.6                  |
| 3.7806               | 48168                | 3.434          | 43453          | 0.3466                | 4715                  | 9.8                   |
| 3.4234               | 42826                | 3.1131         | 38651          | 0.3103                | 4175                  | 9.7                   |
| 9.2994               | 88439                | 8.5155         | 80787          | 0.7839                | 7652                  | 8.7                   |
| 8.1966               | 87744                | 7.51           | 80171          | 0.6866                | 7573                  | 8.6                   |
| 8.6443               | 89220                | 7.9596         | 81950          | 0.6847                | 7270                  | 8.1                   |
| 8.2864               | 85142                | 7.5954         | 77830          | 0.691                 | 7312                  | 8.6                   |
| 8.7451               | 89792                | 8.0631         | 82591          | 0.682                 | 7201                  | 8.0                   |
| 8.1137               | 85882                | 7.4892         | 79070          | 0.6245                | 6812                  | 7.9                   |
| 15.6915              | 151797               | 14.3283        | 138390         | 1.3632                | 13407                 | 8.8                   |
| 14.6414              | 140922               | 13.438         | 129133         | 1.2034                | 11789                 | 8.4                   |
| 11.574               | 113234               | 10.5942        | 103431         | 0.9798                | 9803                  | 8.7                   |
|                      |                      |                |                |                       | Average               | 9.3                   |
|                      |                      |                |                |                       | Std dev               | 1.4                   |

Table A. 13 Concentrations of PFHxS during persulfate oxidation of FHxSA in triplicate.

|                  | Raw PFHxS Conc ug/L |       |       |       |       |       |       |
|------------------|---------------------|-------|-------|-------|-------|-------|-------|
| Hour             | 0                   | 0.5   | 1     | 3     | 5     | 8     | 24    |
| FHxSA HAPO rep 1 | 0                   | 0.127 | 0.149 | 0.177 | 0.215 | 0.196 | 0.218 |
| FHxSA HAPO rep 2 | 0.129               | 0.130 | 0.139 | 0.154 | 0.163 | 0.165 | 0.159 |
| FHxSA HAPO rep 3 | 0                   | 0.126 | 0.141 | 0.160 | 0.179 | 0.169 | 0.167 |
|                  | PFHxS Conc nM       |       |       |       |       |       |       |
| Hour             | 0                   | 0.5   | 1     | 3     | 5     | 8     | 24    |
| FHxSA HAPO rep 1 | 0.00                | 3.52  | 4.14  | 4.91  | 5.97  | 5.45  | 6.06  |
| FHxSA HAPO rep 2 | 3.59                | 3.60  | 3.86  | 4.27  | 4.53  | 4.59  | 4.42  |
| FHxSA HAPO rep 3 | 0.00                | 3.49  | 3.91  | 4.45  | 4.96  | 4.69  | 4.63  |

Table A. 14 Eluent gradient for LC-MS/MS metabolites method.

| Time  | A % | B % |
|-------|-----|-----|
| 0     | 0   | 100 |
| 2     | 0   | 100 |
| 7     | 30  | 70  |
| 7.7   | 30  | 70  |
| 9     | 60  | 40  |
| 9.5   | 60  | 40  |
| 10.25 | 70  | 30  |
| 12.75 | 0   | 100 |
| 17    | 0   | 100 |

Table A. 15 Analytes for metabolite LC-MS/MS analysis.

| Compound                 | Precursor Ion | Product Ion | Fragmentor | Collision Energy | Polarity |
|--------------------------|---------------|-------------|------------|------------------|----------|
| Glutathione-s2           | 613.2         | 355         | 100        | 5                | +        |
| 393-pos                  | 393.3         | 57.2        | 80         | 40               | +        |
| Glutathion               | 308           | 308         | 92         | 0                | +        |
| Oxaloacetic acid-con     | 271           | 157         | 92         | 8                | -        |
| Oxaloacetic acid         | 271           | 113         | 92         | 12               | -        |
| Tyrosine                 | 182.1         | 136.1       | 25         | 12               | +        |
| L-citrulline             | 176           | 159.1       | 72         | 4                | +        |
| L-citrulline-conf        | 176           | 70.2        | 72         | 20               | +        |
| Arginine                 | 175.1         | 70.1        | 75         | 28               | +        |
| Aconitic acid            | 169           | 125.1       | 68         | 4                | -        |
| Phenylalanine            | 166.1         | 120.1       | 25         | 5                | +        |
| Hilidine                 | 156.1         | 110         | 25         | 12               | +        |
| Methionine               | 150           | 104         | 75         | 8                | +        |
| Glutamic acid            | 148.1         | 84.1        | 75         | 16               | +        |
| Lysine                   | 147.1         | 84.1        | 50         | 16               | +        |
| Aspartic acid            | 134           | 74          | 50         | 15               | +        |
| L-ornithine              | 133           | 116         | 72         | 4                | +        |
| L-ornithine              | 133           | 70.2        | 72         | 16               | +        |
| Leucine                  | 132.1         | 86.1        | 25         | 8                | +        |
| Isoleucine               | 132.1         | 86.1        | 25         | 8                | +        |
| Threonine                | 120           | 74.1        | 25         | 10               | +        |
| Valine                   | 118.1         | 72.1        | 25         | 8                | +        |
| Proline                  | 116.1         | 70.1        | 50         | 16               | +        |
| 2-methyl<br>oxobutanoate | 116           | 74          | 180        | 10               | -        |
| Maleic acid              | 115           | 71.2        | 46         | 8                | -        |
| Serine                   | 106.1         | 60.1        | 25         | 8                | +        |
| Sarcosine                | 90.1          | 44.2        | 46         | 12               | +        |
| Alanine                  | 90.1          | 44.1        | 25         | 8                | +        |
| Glycine                  | 76            | 30.1        | 25         | 5                | +        |

## **PFAS sample preparation and LC-MS/MS analysis**

### **Materials & Instrumentation**

Mass-labeled internal standard compounds were purchased from Wellington Laboratories. The mass-labeled compounds arrive in glass ampules ranging from 1.0-50 ug/L, and are diluted to make stock solutions for preparation of internal standard solution and standard working solutions for the preparation of a standard curve. PFAS are quantified by LC-MS/MS equipped with electrospray ionization in negative mode (Triple Quad 6460A, Agilent Technologies) multiple reaction monitoring (MRM).

### **Reproducibility & Quality Control**

To ensure accuracy and precision in measurements obtained from LC-MS/MS analysis, the method included quantification of a known sample supplied by the EPA, method blanks, and standard checks throughout the sample run. For example, the EPA sample of known PFOA, PFOS concentration was analyzed at the beginning of each LC-MS/MS run, as well as throughout the duration of the run, approximately every 30 samples. Along with running method blanks approximately every 10 samples, this allowed determination of any changes in concentration of target analytes due to instrumentation or analyte carry over. Selected standard curve samples were also checked throughout the run. A method blank sample (50/50 MeOH:H<sub>2</sub>O) was run at the beginning of an LC-MS run and approximately every 10 samples throughout the analytical run. An acceptable level of detection for any analytes in the method blank was expected to be <50% of the lowest calibration standard. A laboratory control sample prepared from an EPA standard solution was run at the beginning and end of each sample run, with the acceptable level of each analyte being between 80-120% of the nominal value. A calibration curve was run and analyzed at the beginning of each run, and acceptable with an  $R^2 \geq 0.99$ . For all analytes, the  $S/N \geq 10$  were considered significant peaks for quantification.

## List of Analytes

Table A. 16 PFAS compounds analyzed via LC-MS/MS.

| Compound   | Internal Standard | Precursor Ion | Product Ion | Fragmentor Voltage (V) | Collision Energy (V) | Polarity |
|------------|-------------------|---------------|-------------|------------------------|----------------------|----------|
| MPFBA      | -                 | 217           | 172         | 50                     | 5                    | Neg      |
| MPFPeA     | -                 | 266           | 222<br>221  | 60                     | 2                    | Neg      |
| MPFHxA     | -                 | 315           | 270         | 60                     | 5                    | Neg      |
| MPFOA      | -                 | 417           | 372         | 70                     | 2                    | Neg      |
| MPFNA      | -                 | 468           | 423         | 70                     | 5                    | Neg      |
| MPFHxS     | -                 | 403           | 103         | 150                    | 40                   | Neg      |
| MPFOS      | -                 | 503           | 80          | 190                    | 60                   | Neg      |
| PFBA       | MPFBA             | 213           | 169         | 50                     | 2                    | Neg      |
| PFPeA      | MPFPeA            | 263           | 219<br>68.9 | 60<br>92               | 2<br>8               | Neg      |
| PFHxA      | MPFHxA            | 313           | 269<br>119  | 80                     | 2<br>15              | Neg      |
| PFHpA      | MPFOA             | 363           | 319<br>169  | 80                     | 2                    | Neg      |
| PFOA       | MPFOA             | 413           | 369<br>169  | 80                     | 3<br>14              | Neg      |
| PFNA       | MPFNA             | 463           | 419<br>219  | 80                     | 2<br>15              | Neg      |
| PFBS       | MPFHxS            | 299           | 99<br>80    | 120                    | 30<br>70             | Neg      |
| PFHxS      | MPFHxS            | 399           | 99<br>80    | 125                    | 50<br>80             | Neg      |
| PFOS       | MPFOS             | 499           | 99<br>80    | 122                    | 50<br>80             | Neg      |
| FHxSA      | MPFOS             | 398           | 78          | 125                    | 36                   | Neg      |
| FOSA       | MPFOS             | 498           | 78          | 125                    | 36                   | Neg      |
| 6-2 FtS    | MPFOS             | 427           | 407<br>80   | 140                    | 25<br>35             | Neg      |
| AmPr-FHxSA | MPFOS             | 485           | 85          | 135                    | 30                   | Pos      |

### Preparation of Standards

Concentrated PFAS standard stocks and Standard Working Solutions for the standard curve were prepared according to the following protocol. All solutions should be made with LC-MS-grade methanol and stored at 4°C. A PFAA mix from Wellington Laboratories (Native Perfluorinated Acid/Sulfonate Mix 1.2 mL– PFAC-MXB) was used for preparation of standard curve solutions. For target analytes not in the PFAA mix, individual compounds also purchased from Wellington Laboratories were used (e.g., 4:2 FtS, 6:2 FtS). A 20-mL glass scintillation vial should be used for preparation of a standard mix. The scintillation vial should be rinsed with MeOH (3x) and allowed to air dry prior to stock solution preparation. PFAS concentrations of 250 ug/L for a concentration stock solution were targeted and volumes of MeOH and Wellington standard were added accordingly. A standard working solution, from which the standard curve was prepared, was prepared in a similar manner in a pre-rinsed 20-mL scintillation vial at a concentration of 25 ug/L. From the standard working solution, the standard curve samples were prepared according to the concentrations listed in the table below.

Table A. 17 Standard curve of the samples.

| Level | Final Conc (ppb) | H <sub>2</sub> O (μL) | IS (μL) | Stock (μL) | MeOH (μL) |
|-------|------------------|-----------------------|---------|------------|-----------|
| 7     | 10               | 125                   | 25      | 100        | 0         |
| 6     | 7.5              | 125                   | 25      | 75         | 25        |
| 5     | 5.0              | 125                   | 25      | 50         | 50        |
| 4     | 2.5              | 125                   | 25      | 25         | 75        |
| 3     | 1.0              | 125                   | 25      | 10         | 90        |
| 2     | 0.5              | 125                   | 25      | 5          | 95        |
| 1     | 0.2              | 125                   | 25      | 2          | 98        |
| 0     | 0                | 125                   | 25      | 0          | 100       |

### LC-MS/MS Parameters

PFAS analytes were quantified by LC-MS/MS equipped with electrospray ionization in negative mode (Triple Quad 6460A, Agilent Technologies) multiple reaction monitoring (MRM). Gas and Sheath Gas Heater temperatures were 325 and 350°C, respectively. Gas and sheath gas flows were 9 L/min. The nebulizer was kept at 25 psi and the capillary voltage was 3.5 kV. Samples were prepared for analysis by dilution in MeOH to a target concentration of 10 µg/L to ensure quantification within the calibration range (0.2-10 µg/L). The isotope dilution method was used to account for any potential matrix effects. Mass labeled [<sup>13</sup>C]-PFAAs (2-5 µg/L) were added to LC-MS/MS sample vials for each sample in the final stage of sample preparation. Analytes were separated using a Zinc-Diol guard column coupled to Zorbax C18 XDB guard and analytical columns (Agilent Technologies). The mobile phase (0.4 mL/min) was 5 mM ammonium acetate in water (A) and 5 mM ammonium acetate in MeOH (B) with a solvent gradient: hold 0-2 min 95% A, ramp to 10% A by 10 min, hold 10-11.5 min 10% A, ramp to 95% A by 12 min, hold 12-18 min 95% A. The LC-MS/MS setup includes delay C18 columns at the outlet of each of the pump heads to decrease contamination from PTFE components in the instrument. PFAS were quantified by the transitions and collision energies listed in Section 4.5, Table 1. Mobile phase blanks were run every 10 samples and select calibration samples were re-run every 20 samples to prevent contamination or carryover in the analysis.

## Additional Findings

Table A. 18 Critical micelle concentration of PFOS in 500 mM salt solutions calculated from surface tension isotherm data.

|                          | $\frac{\partial \gamma}{\partial \ln C}$ | y-intercept | IFT Minima |         | CMC<br>(mg/L) | R <sup>2</sup> |
|--------------------------|------------------------------------------|-------------|------------|---------|---------------|----------------|
|                          |                                          |             | (mN/m)     | ln(CMC) |               |                |
| ultrapure                | -12.592                                  | 115.7       | 23.36      | 7.333   | 1530          | 0.9941         |
| 500 mM NaCl              | -6.753                                   | 72.842      | 22         | 7.5281  | 1860          | 0.9922         |
| 500 mM KCl               | -6.0854                                  | 64.086      | 21.92      | 6.929   | 1021          | 0.9809         |
| 500 mM MgCl <sub>2</sub> | -8.8324                                  | 65.811      | 20.31      | 5.151   | 172           | 0.9953         |
| 500 mM CaCl <sub>2</sub> | -9.9881                                  | 62.621      | 16.84      | 4.583   | 98            | 0.9986         |

Table A. 19 Summary of fitted parameters from the Szyszkowski equation and the calculated maximum surface excess values ( $\Gamma_{\max}$ ).

|                          | $\gamma_0$<br>(mN/m) | Fit Range<br>(mg/L) | $a$    | $b$   | $\Gamma_{\max}$<br>(mg/m <sup>2</sup> ) | R <sup>2</sup> |
|--------------------------|----------------------|---------------------|--------|-------|-----------------------------------------|----------------|
| ultrapure                | 72.24                | 40-1200             | 0.2084 | 58.27 | 3.04                                    | 0.990          |
| 500 mM NaCl              | 72.29                | 6-1000              | 0.0887 | 0.827 | 1.295                                   | 0.991          |
| 500 mM KCl               | 71.9                 | 6-800               | 0.0908 | 0.473 | 1.350                                   | 0.988          |
| 500 mM MgCl <sub>2</sub> | 72.01                | 4-160               | 0.132  | 0.719 | 1.919                                   | 0.994          |
| 500 mM CaCl <sub>2</sub> | 71.81                | 4-120               | 0.158  | 0.778 | 2.286                                   | 0.968          |

Table A. 20 Air-water interfacial uptake ( $\Gamma$ ) and calculated air-water partition coefficient ( $k_{aw}$ ) for PFOS in ultrapure Milli-Q water and a 200 mM Na<sub>2</sub>SO<sub>4</sub> (I=0.6 M).

|                                        | $\Gamma$ (mg m <sup>-2</sup> ) | $k_{aw}$ (m)       |
|----------------------------------------|--------------------------------|--------------------|
| Ultrapure                              | 0.252 ± 0.051                  | 2.24E-02 ± 5.1E-03 |
| 200 mM Na <sub>2</sub> SO <sub>4</sub> | 0.536 ± 0.269                  | 7.94E-02 ± 4.2E-02 |

## **Appendix B. List of Scientific/ Technical Publications**

### **Papers**

Cook, E.K., et al. (In prep.) PFAS Impacts on the Bioremediation of Co-contaminants Trichloroethene and Combined Benzene, Toluene, Ethylbenzene, and Xylene (BTEX).

Cook, E.K., et al. (Accepted.) Biological and Chemical Transformation of the Six- Carbon PFAS N-dimethyl ammonio propyl perfluorohexane sulfonamide (AmPr-FHxSA). *Environmental Science & Technology*. 10.1021/acs.est.2c00261

Olivares, C.I., Yi, S., Cook, E.K., et al. (2022) BTEX degradation Increases Yield of Perfluoroalkyl Carboxylic Acids from Aerobic 6:2 FtTAoS Biotransformation. *Environmental Science: Processes & Impacts*, 24(3), 439-446.

Steffens, S.D., Cook, E.K., et al. (2021) Under-reporting Potential of Perfluorooctanesulfonic acid (PFOS) under High Ionic Strength Conditions. *Environmental Science & Technology Letters*.

Cook, E.K. (2019) Feasibility tests for in situ chemical oxidation of PFAS in a contaminated aquifer. *Geosyntec Graduate Student Paper Competition*.

### **Presentations**

Olivares, C.I., Cook, E.K. et al. Interactions between polyfluorinated compounds in aqueous film forming foams and bioremediation of BTEX and TCE. ACS Spring 2021 Meeting. Virtual. April 14, 2021.

Cook, E.K., et al. Field demonstration design of heat-activated persulfate in situ chemical oxidation (ISCO) of PFASs in Jacksonville, FL. 257th American Chemical Society Annual Spring meeting. Orlando, FL. March 30-April 4, 2019.

Olivares, C.I., Cook, E.K., et al. Combined persulfate chemical oxidation and bioremediation as in-situ strategies for AFFF and BTEX co-contaminant impacted sites. 257th American Chemical Society Annual Meeting. Orlando, FL. March 30-April 4, 2019.

Cook, E.K., et al. Heat-activated persulfate in situ chemical oxidation (ISCO) of co-contaminants 1,4-dioxane and perfluorocarboxylic acids (PFCAs). Groundwater Resources Association's Western Groundwater Congress. Sacramento, CA. September 25-27, 2018.

Cook, E.K. and Tom McKeag. Finding Nature-inspired Alternatives to PFAS in Durable Water Repellency (DWR): An Academic/ Industry Approach. ACS Green Chemistry & Engineering Conference. Portland, OR. 18 June 2018.

Cook, E.K., et al. Combining bioremediation and in situ chemical oxidation for treatment of aqueous film forming foams (AFFFs) in groundwater. 255th American Chemical Society National Meeting. New Orleans, LA. March 18-22, 2018.

### **Posters**

Olivares, C.I., Cook, E.K., Sedlak, D.L., Alvarez-Cohen. Metabolic markers of AFFF polyfluorinated compound transformation inhibition to microbial biodegradation of aromatic hydrocarbons and chlorinated solvents. Gordon Conference: Environmental Sciences Water. Holderness, NH. June 19-24, 2022.

Cook, E.K., Sun, Y., Olivares, C.I., Yi, S., Sedlak, D.L., Alvarez-Cohen, L. In situ remediation of aqueous film forming foams and common co-contaminants with the dual approach of chemical oxidation and bioremediation. SERDP and ESTCP Symposium & Workshop, Washington D.C., Nov 3-5, 2019.

Cook, E.K., Campbell, G., Olivares, C.I., Hawley, E., Marvin, B., Deeb, R., Kornuc, J., Sedlak, D.L., Alvarez-Cohen, L. Optimization of persulfate in situ chemical oxidation (ISCO) for per- and polyfluoroalkyl substance treatment in groundwater and aquifer solids at PFAS-impacted site in Jacksonville, FL. SERDP & ESTCP Symposium & Workshop, Washington D.C., Nov 3-5, 2019.

Cook, E.K., C.I. Olivares, Y. Sun, D.L. Sedlak, L. Alvarez-Cohen. Bench-scale studies on the efficacy of in situ chemical oxidation of per- and polyfluoroalkyl substances (PFASs) in a contaminated aquifer. REMTEC Summit. Denver, Colorado. February 26-28, 2019.

Cook, E.K., Sun, Y., Olivares, C.I., Yi, S., Bruton, T.A., Sedlak, D.L., Alvarez-Cohen, L. Soil Constituents to consider when selecting an aquifer to perform heat-activated persulfate in situ chemical oxidation (ISCO) of PFASs. SERDP & ESTCP Symposium & Workshop, Washington D.C. November 28, 2018.

Cook, E.K., Sun, Y., Olivares, C.I., Yi, S., Sedlak, D.L., Alvarez-Cohen, L. Optimization of persulfate in situ chemical oxidation (ISCO) for per- and polyfluoroalkyl substance treatment in groundwater and aquifer solids at PFAS-impacted site in Jacksonville, Florida. SERDP & ESTCP Symposium & Workshop, Washington D.C. November 28, 2018.

Olivares, C.I., Cook, E.K., Sun, Y., Yi, S., Sedlak, D.L., Alvarez-Cohen, L. Combined persulfate chemical oxidation and bioremediation as in-situ remediation strategies for AFFF components and co-contaminants. 2018 Gordon Research Conference on Environmental Sciences: Water, Holderness, NH. June 24-29, 2018.

Cook, E.K., Sun, Y., Olivares, C.I., Yi, S., Sedlak, D.L., Alvarez-Cohen, L. In situ remediation of aqueous film forming foams and common co-contaminants with the dual approach of chemical oxidation and bioremediation. SERDP & ESTCP Symposium & Workshop, Washington D.C. November 28-30, 2017.