PERFLUOROALKYL ACIDS AND MICROORGANISMS: IMPLICATIONS FOR
SUBSURFACE TRANSPORT AND MICROBIAL PROCESSES

by

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A thesis submitted to the Faculty and the Board of Trustees of the Colorado School of Mines in partial fulfillment of the requirements for the degree of Doctor of Philosophy (Hydrology).

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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>vii</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>ix</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>xi</td>
</tr>
<tr>
<td>ACKNOWLEDGMENTS</td>
<td>xii</td>
</tr>
<tr>
<td><strong>CHAPTER 1</strong> INTRODUCTORY REMARKS</td>
<td>1</td>
</tr>
<tr>
<td><strong>CHAPTER 2</strong> PERFLUOROALKYL ACIDS INHIBIT REDUCTIVE DECHLORINATION OF TRICHLOROETHENE BY REPRESSING DEHALOCOCCOIDES</td>
<td>7</td>
</tr>
<tr>
<td>2.1 Introduction</td>
<td>8</td>
</tr>
<tr>
<td>2.2 Materials and Methods</td>
<td>10</td>
</tr>
<tr>
<td>2.2.1 PFAA Preparation and Aqueous Analysis</td>
<td>10</td>
</tr>
<tr>
<td>2.2.3 Cellular preparation</td>
<td>11</td>
</tr>
<tr>
<td>2.2.4 Reductive dechlorination with the mixed culture</td>
<td>11</td>
</tr>
<tr>
<td>2.2.5 Phylogenetic sequencing</td>
<td>12</td>
</tr>
<tr>
<td>2.3 Results and Discussion</td>
<td>14</td>
</tr>
<tr>
<td>2.3.1 Effect of PFAAs on reductive dechlorination by a mixed culture</td>
<td>14</td>
</tr>
<tr>
<td>2.3.2 Effects of PFAA exposure on community structure</td>
<td>16</td>
</tr>
<tr>
<td>2.3.3 Reductive dechlorination of axenic Dehalococcoides</td>
<td>23</td>
</tr>
<tr>
<td>2.4 Environmental Implications</td>
<td>26</td>
</tr>
<tr>
<td><strong>CHAPTER 3</strong> ENHANCED BIOFILM PRODUCTION BY A TOLUENE DEGRADING RHODOCOCCUS OBSERVED AFTER EXPOSURE TO PERFLUOROALKYL ACIDS</td>
<td>29</td>
</tr>
<tr>
<td>3.1 Introduction</td>
<td>30</td>
</tr>
<tr>
<td>3.2 Materials and Methods</td>
<td>32</td>
</tr>
<tr>
<td>Section</td>
<td>Title</td>
</tr>
<tr>
<td>---------------------------------</td>
<td>----------------------------------------------------------------------</td>
</tr>
<tr>
<td>3.2.1</td>
<td>PFAA Preparation and Aqueous Analysis</td>
</tr>
<tr>
<td>3.2.2</td>
<td>Cellular Preparation, Harvest, and Protein Measurements</td>
</tr>
<tr>
<td>3.2.3</td>
<td>Toluene Biodegradation</td>
</tr>
<tr>
<td>3.2.4</td>
<td>Growth and EPS Production</td>
</tr>
<tr>
<td>3.2.5</td>
<td>Soil Enrichments</td>
</tr>
<tr>
<td>3.2.6</td>
<td>Quantification of Gene Expression</td>
</tr>
<tr>
<td>3.3</td>
<td>Results and Discussion</td>
</tr>
<tr>
<td>3.3.1</td>
<td>Effect of PFAAs on Toluene Degradation</td>
</tr>
<tr>
<td>3.3.2</td>
<td>Effect of PFAA Presence on Growth and Biofilm Formation</td>
</tr>
<tr>
<td>3.3.3</td>
<td>Expression of Stress Genes</td>
</tr>
<tr>
<td>3.4</td>
<td>Environmental Implications</td>
</tr>
<tr>
<td><strong>CHAPTER 4</strong></td>
<td>SORPTION EFFECTS OF PERFLUOROALKYL ACIDS EXPOSED TO MICROORGANISMS</td>
</tr>
<tr>
<td>4.1</td>
<td>Introduction</td>
</tr>
<tr>
<td>4.2</td>
<td>Materials and Methods</td>
</tr>
<tr>
<td>4.2.1</td>
<td>PFAA Preparation and Analysis</td>
</tr>
<tr>
<td>4.2.2</td>
<td>Solids-associated PFAA extraction</td>
</tr>
<tr>
<td>4.2.3</td>
<td>Cellular sorption</td>
</tr>
<tr>
<td>4.2.4</td>
<td>Sorption with soil and increasing biomass</td>
</tr>
<tr>
<td>4.2.5</td>
<td>Calculating Sorption Coefficients</td>
</tr>
<tr>
<td>4.3</td>
<td>Results and Discussion</td>
</tr>
<tr>
<td>4.3.1</td>
<td>Sorption of PFAAs to bacteria</td>
</tr>
<tr>
<td>4.3.2</td>
<td>Sorption of PFAAs with cells and soil</td>
</tr>
</tbody>
</table>
CHAPTER 5  FUTURE WORK: EVALUATING TRANSPORT AND ECOLOGY IN FLOW-THROUGH SYSTEMS ................................................................. 64

5.1  Introduction .............................................................................................................. 64

5.2  Experimental Design ............................................................................................... 65

5.3  Preliminary Results ................................................................................................. 67

5.3.1  Microbiological activity ...................................................................................... 67

5.3.2  Permeability ........................................................................................................ 68

5.3.3  Non-reactive tracer breakthrough ...................................................................... 68

5.3.4  PFAA breakthrough ........................................................................................... 69

5.4  Continuing Efforts ................................................................................................... 71

CHAPTER 6  CONCLUDING REMARKS ...................................................................... 73

REFERENCES CITED ................................................................................................................. 75

APPENDIX ........................................................................................................................................ 84

S.1  Supporting information for Chapter 2 ........................................................................ 84

S.1.1  Dual-Indexed Primer Specifications ...................................................................... 84

S.1.2  Amplification Program ......................................................................................... 85

S.1.3  Sequencing Post-Processing Scripts ..................................................................... 85

S.2  Supporting information for Chapter 3 ........................................................................ 92

S.2.1  Aqueous loss control ............................................................................................ 92

S.2.2  PFAA competition control .................................................................................... 92

S.2.3  RT-qPCR method details and QA/QC ................................................................ 94

S.2.4  RT-qPCR on LB grown samples ......................................................................... 97

S.2.5  Statistical significance of RT-qPCR results ......................................................... 99

S.2.6  Additional results .................................................................................................. 100
ABSTRACT

Perfluoroalkyl acids (PFAAs) are contaminants of emerging concern found throughout the environment. The interactions between subsurface microbiological process and PFAAs are largely unknown. Similarly, the effects of active microorganisms on PFAA transport are not well understood. This work explores these interactions by assessing co-contaminant biodegradation in the presence of PFAAs, shifts in microbial ecosystems, and stress-related effects on select microorganisms. Additionally, transport characteristics of PFAAs in the presence of pure cellular material and active microbiology are addressed.

PFAAs are often found in aqueous film-forming foams (AFFF) used for fire suppression and often co-occur in groundwater with chlorinated solvents and BTEX compounds (benzene, toluene, ethylbenzene, and xylene). Here we show that reductive dechlorination by a methanogenic, mixed culture was significantly inhibited when exposed to concentrations representative of PFAA source zones (>66 mg/L total of 11 PFAA analytes, 6 mg/L each). Significant repression (8-fold decrease in abundance) of the pivotal reductive dechlorinator *Dehalococcoides* corresponded to an enhancement of methane-generating Archaea within the community (9-fold increase). Growth and dechlorination by axenic cultures of *Dehalococcoides mccartyi* strain 195, which can completely dechlorinate TCE to non-toxic ethene, were similarly repressed under these conditions. These results suggest that enhanced reductive dechlorination of chlorinated solvents could be impeded in subsurface environments.

This work also addresses the effects of PFAAs on biodegradation of toluene. No effect on toluene degradation rate or induction time was observed when active cells of *Rhodococcus jostii* strain RHA1 were exposed to toluene and a mixture of PFAAs at concentrations of 110 mg/L total PFAAs. However, exposure to aqueous PFAA concentrations above 2 mg/L each was
associated with enhanced aggregation of bacterial cells and extracellular polymeric substance production. This behavior was accompanied by two- to three-fold upregulation of stress-associated genes, *sigF3* and *prmA*, during growth of this *Rhodococcus* in the presence of PFAAs. These results suggest that biological responses, such as microbial stress and biofilm formation, could be more prominent than suppression of BTEX biodegradation in subsurface locations where PFAAs occur with hydrocarbon fuels.

To address the impacts of microbiological presence of PFAA transport, this dissertation evaluated aqueous sorption coefficients for PFAAs onto cellular material. Calculated logarithmic distribution coefficients (logK$_d$) generally increase with increasing carbon chain length within the range of 2.3 to 4.7, exceeding the published sorption coefficients for sorption to cellular organic matter by nearly an order of magnitude. Microcosms containing soil amended with inactivated bacterial cells at quantities representative of subsurface growth in a biostimulated zone revealed changes in organic carbon normalized distribution coefficients as a function of biomass and analyte. These results demonstrate that PFAAs preferentially sorb to intact bacterial cells over soil-associated organic carbon and that traditional normalization techniques to bulk organic carbon may not accurately predict sorption of all PFAAs in microbially active zones. This phenomenon is a function of carbon chain length: … This information may have significant effects on our ability to predict subsurface fate and transport of PFAAs. Future research is proposed that focuses on upscaling sorption behavior and community ecology by assessing controlled flow-through column scenarios. These systems will evaluate changes in PFAA sorption and desorption as a function of biomass growth, coupled with the monitoring of biofilm production and corresponding advective shifts as a function of PFAA concentration.
LIST OF FIGURES

Figure 1.1  Summary graphic of Chapter Two……………………………………………………………3
Figure 1.2  Summary graphic of Chapter Three……………………………………………………………4
Figure 1.3  Summary graphic of Chapter Four………………………………………………………………5
Figure 2.1  Reductive dechlorination by the mixed culture…………………………………………………………15
Figure 2.2  Changes in abundance based on PFAA exposure do not correlate with phylogeny……………………………………………………………………………………………………………………………16
Figure 2.3  Principle component analysis shows distinct clustering……………………………………………………………18
Figure 2.4  Abundances of Dehalococcoides and Methanobacterium…………………………………………………20
Figure 2.5  Dechlorination is inhibited by PFAA presence in cultures of D. mccartyi 195….25
Figure 3.1  Toluene concentrations over time………………………………………………………………………40
Figure 3.2  Optical density, protein for cells grown in the presence and absence of PFAAs..41
Figure 3.3  Flocculation response at different PFAA concentrations…………………………………………………42
Figure 3.4  Photographs and microscopy of RHA1 grown with and without PFAAs….……45
Figure 4.1  Organic carbon normalized sorption coefficients are dependent on carbon chain length and biomass…………………………………………………………………………………………………………60
Figure 5.1  Columns were divided into five sets……………………………………………………………………65
Figure 5.2  Microbial activity as measured by ATP…………………………………………………………68
Figure 5.3  Permeability decreases upon microbial stimulation……………………………………………………69
Figure 5.4  Dispersivity of the non-reactive tracer increases over time with biostimulation..70
Figure 5.5  Fitted breakthrough of PFOA and PFHxS…………………………………………………………71
Figure S.2.1 Aqueous loss controls remain quantifiable……………………………………………………………94
Figure S.2.2 Toluene degradation is not affected by a PFAA mixture, PFOS alone, or PFOA alone………………………………………………………………………………………………………………………………96
Figure S.2.3  Melt curve analysis.................................................................98
Figure S.2.4  Standard curves for sigF3.........................................................99
Figure S.2.5  Long-term growth curves for RHA1.........................................101
Figure S.2.6  EPS measurements for two different extraction methods..............101
Figure S.2.7  More flocs are observed in soil-derived communities grown in the presence of 110 mg/L PFAAs.................................................................102
Figure S.3.1  EPS content is minimal in each sorption system except –Soil...........107
LIST OF TABLES

Table 1.1  Characteristics of PFAAs used in this study……………………………………... 2
Table 2.1  Reductive dechlorination rates by the mixed culture………………………….. 15
Table 2.2  Dechlorination and production rates by axenic *Dehalococcoides*……………….. 24
Table 3.1  Increased transcription of stress-associated genes……………………………… 47
Table 4.1  Organic carbon normalized distribution coefficients…………………………….. 58
Table 5.1  Modeled parameters for PFOA and PFHxS………………………………………… 71
Table S.1.1  Observed taxa……………………………………………………………… 89
Table S.1.2  Relative abundance……………………………………………………………… 90
Table S.1.3  Fold changes and statistical significance……………………………………….. 91
Table S.2.1  RT-qPCR primer specifications……………………………………………….. 94
Table S.2.2  Errors, efficiencies, slopes, and intercepts for each gene and media……………… 96
Table S.2.3  CP cycles for negative RT-qPCR controls……………………………………… 98
Table S.2.4  Initial RNA concentrations and CP for *prmA* analyses……………………… 103
Table S.2.5  Initial RNA concentrations and CP for *sigF3* and *gyrB* analyses…………… 104
Table S.2.6  Fold change and statistical significance……………………………………….. 105
Table S.3.1  Low organic carbon loamy sand properties……………………………………… 107
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Members of the GEM Lab have taught me what a lab is, and have given me the chance to teach newcomers the same lessons. Dr. Stephanie Carr, Dr. Lisa Gallagher, Dr. Halley Eydal, Dr. Rob Almstrand, Dr. Kristin Mikkelsen, Zack Jones, Dina Drennan, Chelsea Bokman, Chris Trivedi: we have struck the ultimate balance of keeping each other sane while driving each other crazy. Heartfelt thanks to Dr. A. Blaine, Dr. J. Guelfo, Dr. K. Christiansen, and Dr. S. Roberts for showing me the ropes of analytical chemistry. I wouldn’t have had this opportunity without funding from the Strategic Environmental Research and Development Program (ER-2126) and the National Science Foundation (CBET-1055396).

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DEDICATION

This work is dedicated to the memory of two strong women: Inez Pavon and Ethel Weathers.

Without the curiosity, independence, and passion you instilled upon your children,
grandchildren, and great-grandchildren, these words would not exist
CHAPTER 1
INTRODUCTORY REMARKS

Perfluoroalkyl acids (PFAAs) are contaminants of emerging concern detected throughout the environment. PFAAs are a subset of poly- and perfluoroalkyl substances (PFASs), and can also be the end products of fluorotelomer alcohol degradation.\(^1,2\) They have unique chemical properties: they are fully fluorinated carbon chains of varying lengths,\(^3\) they have hydrophobic and oleophobic tails, and possess the ability to form micelles.\(^4\) These characteristics lend themselves to many industrial applications including non-stick coatings, fire retardants, and pesticides.\(^5-7\) Some PFAAs, along with PFASs, are critical components of aqueous film-forming foam (AFFF) used for fire suppression.\(^3,7-9\) The pervasive use and environmental longevity of PFAAs, coupled with improvements in quantification, have contributed to the far-reaching environmental detection of these compounds.\(^10-13\) PFAAs have been identified in soil, surface water, groundwater, water and wastewater treatment effluent, and have been observed in humans, crops, and wildlife.\(^3,5,6,14,15\) These compounds are environmentally recalcitrant, are known to bioaccumulate, have demonstrated toxicity effects in primates and rats,\(^10,16-19\) and have been detected in human kidneys, livers, and blood serum.\(^5,6,15,20\)

The U.S. Environmental Protection Agency recognizes two common PFAAs as emerging contaminants, perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA), and has established provisional drinking water health advisories and soil-screening values for these compounds.\(^6\) These were the two most manufactured PFASs in 2009,\(^6,21\) however, current industry trends are shifting towards increased usage of short-chain PFAAs.\(^10,21\) PFAAs fall into two primary classes: carboxylates (including PFOA) and sulfonates (such as PFOS).\(^3\) To better understand the behavior of these compounds, the work presented herein uses an eleven-
component mixture of PFAAs containing perfluorobutanoate (PFBA), perfluoropentanoate (PFPeA), perfluorohexanoate (PFHxA), perfluoroheptanoate (PFHpA), PFOA, perfluorononanoate (PFNA), perfluorodecanoate (PFDA), perfluoroundecanoate (PFUnA), perfluorobutanesulfonate (PFBS), perfluorohexanesulfonate (PFHxS), and PFOS unless otherwise noted. Properties of these compounds are shown in Table 1.1.

Table 1.1. PFAA analytes used in this study and corresponding characteristics

<table>
<thead>
<tr>
<th>Compound</th>
<th>Abbreviation</th>
<th>Structure</th>
<th>Purity</th>
</tr>
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<tbody>
<tr>
<td>Perfluorobutanoic acid</td>
<td>PFBA</td>
<td>n = 2</td>
<td>98%</td>
</tr>
<tr>
<td>Perfluoropentanoic acid</td>
<td>PFPeA</td>
<td>n = 3</td>
<td>97%</td>
</tr>
<tr>
<td>Perfluorohexanoic acid</td>
<td>PFHxA</td>
<td>n = 4</td>
<td>97%</td>
</tr>
<tr>
<td>Perfluoroheptanoic acid</td>
<td>PFHpA</td>
<td>n = 5</td>
<td>100%</td>
</tr>
<tr>
<td>Perfluoroctanoic acid</td>
<td>PFOA</td>
<td>n = 6</td>
<td>96%</td>
</tr>
<tr>
<td>Perfluorononanoic acid</td>
<td>PFNA</td>
<td>n = 7</td>
<td>97%</td>
</tr>
<tr>
<td>Perfluorodecanoic acid</td>
<td>PFDA</td>
<td>n = 8</td>
<td>98%</td>
</tr>
<tr>
<td>Perfluoroundecanoic acid</td>
<td>PFUnA</td>
<td>n = 9</td>
<td>95%</td>
</tr>
<tr>
<td>Perfluorobutane sulfonate</td>
<td>PFBS</td>
<td>m = 3</td>
<td>98%</td>
</tr>
<tr>
<td>Perfluorohexane sulfonate</td>
<td>PFHxS</td>
<td>m = 5</td>
<td>98%</td>
</tr>
<tr>
<td>Perfluoroctane sulfonate</td>
<td>PFOS</td>
<td>m = 7</td>
<td>98%</td>
</tr>
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The ubiquitous use of PFAAs, particularly as AFFF for military firefighter training, has led to the significant introduction of PFAAs into groundwater in sites that are co-contaminated with other chemicals, such as chlorinated solvents or hydrocarbons. The biodegradation of these co-contaminants commonly found at AFFF-impacted sites (i.e., BTEX: benzene, toluene, ethylbenzene, and xylene) can provide a long-term mechanism for attenuation. Chlorinated solvents such as trichloroethene (TCE) and its toxic daughter products cis-dichloroethene (cDCE) and vinyl chloride (VC) can also be biologically attenuated through a process known
as enhanced reductive dechlorination (ERD). This is a bioremediation strategy for chlorinated solvents in which an electron donor, such as lactate (biostimulation), or potentially a known microbial consortium containing *Dehalococcoides* (bioaugmentation), is supplied.\textsuperscript{25} The impacts of PFAA presence on continued biodegradation of these common subsurface contaminants has not previously been addressed. Similarly, the effects of PFAAs on community structure and microbial processes have not been widely explored.

The second chapter of this dissertation discusses the inhibitory effects of PFAAs on reductive dechlorination of TCE by a *Dehalococcoides* containing methanogenic mixed culture and has been submitted to peer review. This paper has been recommended for acceptance to *Environmental Science and Technology*. Figure 1.1 is a summary graphic of the primary conclusions of Chapter 2: PFAA presence at source-zone levels of contamination completely inhibit TCE dechlorination by directly repressing *Dehalococcoides*, the only known genus to completely degrade TCE to non-toxic ethene.\textsuperscript{26–28} Analysis of the community structure reveals decreases in relative abundances of select microorganisms and increases in others that are either excelling in the presence of PFAAs or filling the putative niche left over from the restricted taxa.

![Figure 1.1. Summary graphic of Chapter 2 describes the inhibition of TCE dechlorination by direct repression of *Dehalococcoides*.](image-url)
For example, methanogenic Archaea, a direct competitor of *Dehalococcoides*, increases in abundance upon PFAA exposure while *Dehalococcoides* is inhibited. Work addressing similar objectives was performed in collaboration with Katie Harding-Marjanovic and Lisa Alvarez-Cohen at University of California, Berkeley and is currently in review.\(^{29}\) This work focuses on inhibitory behavior that has been observed in the presence of select AFFF formulations containing a range of PFASs, however systems with equivalent concentrations of only perfluoroalkyl sulfonates did not affect dechlorination.

Chapter three discusses whether the bioremediation of hydrocarbons is inhibited. It focuses on degradation of toluene by a common, and well-studied soil microbe: *Rhodococcus jostii* strain RHA1 in order to enable mechanistic inquiries. The primary conclusions of this work, published in *Environmental Science and Technology*,\(^{30}\) can be graphically interpreted in Figure 1.2. Unlike reductive dechlorination systems, there is no observed inhibition of toluene degradation. However, an increase in stress-related gene transcription, coupled with enhanced extracellular polymeric substance (EPS) production was documented as a biological effect of PFAA presence, rather than a chemical interaction.

Figure 1.2. Summary graphic of Chapter 3 demonstrates the continued degradation of toluene, even as RHA1 undergoes a stress response and enhanced extracellular polysaccharide formation. Reprinted with permission from *Environmental Science and Technology*. 

4
An increase in EPS production *in situ* may result in changes in PFAA transport. Chapter 4, in preparation for submission as a short letter communication, evaluates PFAA sorption to microbial surfaces in contrast to sediments. Figure 1.3 graphically summarizes some of the primary conclusions of Chapter 4, particularly that PFAAs sorb preferentially to cellular organic carbon over organic carbon associated with sediments. The degree of preferential cellular sorption appears to be dependent on PFAA carbon chain-length. Enhanced EPS production may provide additional cellular sorption sites or may coat soil surfaces, creating a system in which PFAA transport is retarded. However, constriction of pore spaces as a result of biofilm formation may change the advective transport characteristics of the fluid. A flow-through system is discussed in Chapter 5 as future work that could be approached to quantify PFAA retardation as a result of biostimulation in a loamy sand with comparatively low concentrations of organic carbon (0.17%). Additionally, this system will be used to understand effects of long-term exposure of PFAAs on microbial community structure that can be compared to the trends noted in Chapter 2 for the reductive dechlorinating consortium.

![Figure 1.3. Summary graphic of Chapter 4 illustrates increasing PFAA sorption as a function of increasing biomass.](image-url)
The appendix of this dissertation includes supplementary information for Chapters 2, 3, and 4. This section includes additional experimental data, results from experimental controls, quality assurance and quality control measures, sequence post-processing scripts, and copyright permissions for Chapter 3.

As a whole, this dissertation addresses both direct effects on growth and metabolism as well as indirect interactions between PFAAs and microbiology such as preferential association or sorption. Insights gleaned by addressing the impacts of PFAA presence on microbial processes in this series of laboratory inquiries, specifically the continued bioremediation of co-contaminants, shifts in community structure, expression of microbial stress, and production of EPS, can be used to better predict and understand the effects of PFAAs that are currently, and will continue to be, occurring in the subsurface. By accounting for the impacts of microbiology on PFAA transport in a laboratory system, we are able to understand the potential feedbacks between active microbiota and PFAA behavior. This work provides a better understanding of the interactions between microorganisms and PFAAs ranging from impacts on microbial processes such as bioremediation, growth, and biofilm production, to changes in advective and sorptive properties of PFAAs.
CHAPTER 2
PERFLUOROALKYL ACIDS INHIBIT REDUCTIVE DECHLORINATION OF TRICHLOROETHENE BY REPRESSING DEHALOCOCCOIDES

A paper recommended for acceptance to Environmental Science and Technology

Tess S. Weathers*,†, Katie Harding-Marjanovic†, Christopher P. Higgins†,‡, Lisa Alvarez-Cohen§, Jonathan O. Sharp†,‡

Abstract

The subsurface recalcitrance of perfluoroalkyl acids (PFAAs) used in aqueous film-forming foams could have adverse impacts on microbiological processes used for the bioremediation of comingled chlorinated solvents such as trichloroethene (TCE). Here we show that reductive dechlorination by a methanogenic, mixed culture was significantly inhibited when exposed to concentrations representative of PFAA source zones (>66 mg/L total of 11 PFAA analytes, 6 mg/L each). TCE dechlorination, cis-dichloroethene, and vinyl chloride production and dechlorination, and ethene generation were all inhibited at these concentrations of PFAAs. Phylogenetic analysis revealed that the abundances of 65% of the operational taxonomic units (OTUs) changed significantly when grown in the presence of PFAAs, although repression and/or enhancement resulting from PFAA exposure did not correlate with putative function or

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*Tess Weathers was the primary author in the writing of this manuscript.
phylogeny. Notably, there was significant repression of *Dehalococcoides* (8-fold decrease in abundance) coupled with a corresponding enhancement of methane generating Archaea (a 9-fold increase). Growth and dechlorination by axenic cultures of *Dehalococcoides mccartyi* strain 195 were similarly repressed under these conditions, confirming an inhibitory response of this pivotal genus to PFAA presence. These results suggest that chlorinated solvent bioattenuation rates could be impeded in subsurface environments near PFAA source zones.

2.1 Introduction

Poly- and perfluoroalkyl substances (PFASs) are contaminants of emerging concern found throughout the environment.\(^5\) PFASs are used in a diverse range of industrial, consumer, and commercial applications including pesticides, non-stick coatings, and in fire-fighting foams.\(^5\)–\(^7\) These compounds are environmentally recalcitrant, exhibit toxicity effects in primates and microbiota, and can bioaccumulate.\(^10,16–18,30\) The prevalent use and environmental longevity, coupled with improvements in quantification, have led to widespread environmental detection in groundwater, surface water, soil, and air, as well as detection in human serum, wildlife, and food crops.\(^10–13\) The U.S. Environmental Protection Agency has set provisional drinking water health advisories for two common PFASs: 0.4 μg/L for perfluorooctanoate (PFOA) and 0.2 μg/L for perfluorooctanesulfonate (PFOS).\(^6\)

Perfluoroalkyl acids (PFAAs), a subset of PFASs, are present in and can arise from components of aqueous film-forming foams (AFFF) used for fuel fire suppression.\(^5\) Use of AFFF for military firefighter training has led to the introduction of PFAAs into groundwater in sites that are often contaminated with chlorinated solvents\(^7,22,31,32\) such as trichloroethene (TCE) and its toxic daughter products cis-dichloroethene (cDCE) and vinyl chloride (VC).\(^25\) Enhanced reductive dechlorination (ERD) is a bioremediation process for chlorinated solvents in which an
electron donor, such as lactate (biostimulation), or potentially a known microbial consortium containing Dehalococcoides (bioaugmentation) is supplied. Members of the genus Dehalococcoides are known to completely degrade tetrachloroethene (PCE) and TCE to ethene, thus limiting the potential for toxic accumulation of vinyl chloride. Dehalococcoides have been incorporated into ERD consortia in laboratory settings and are often found in aquifers wherein complete PCE and TCE dechlorination has been observed. It is yet unknown how PFAAs may impact microbial communities relevant to chlorinated solvent bioremediation. While biodegradation of PFAAs is not expected, there are concerns regarding potential adverse effects from PFAA exposure on subsurface microbial communities and co-contaminant degradability. Biodegradation of another commonly co-located contaminant, toluene, was not impacted in pure culture studies, however the presence of PFAAs did correlate to increased formation of extracellular polysaccharides and enhanced transcription of stress-related genes. Consequently, unanticipated impacts on PFAA or chlorinated solvent fate and transport in groundwater sources are possible, ranging from effects on co-contaminant degradation and microbial processes, to changes in sorptive properties of PFAAs as a result of biostimulation or bioaugmentation.

The objective of this study was to assess potential impacts of these nontraditional contaminants, PFAAs, on reductive dechlorination of TCE. This was accomplished by querying for suppression of reductive dechlorination activity by a Dehalococcoides-containing methanogenic mixed community cultivated in the presence of varied concentrations of PFAAs. In parallel, we contrasted the community structure and putative functionality of these microcosms to understand ecological shifts and metabolic redundancy within this consortium that could be attributed to PFAA presence. Insights from the ecological profiles were then used
to challenge an axenic culture of Dehalococcoides with PFAAs to understand the direct impact on this important bacterial genus. Results revealed suppression of Dehalococcoides growth and reductive dechlorination rates in both pure culture and mixed assemblages.

2.2 Materials and Methods

2.2.1 PFAA Preparation and Aqueous Analysis

Purity corrected stock solutions of an eleven-analyte mixture were prepared as described elsewhere. PFAA salts (Sigma Aldrich) were suspended in a 70/30 (v/v) methanol/water solution. Water used in this study was generated with a Milli-Q system (Millipore). Unless otherwise specified, each mixture contained perfluorobutanoate, perfluoropentanoate, perfluorohexanoate, perfluoroheptanoate, PFOA, perfluorononanoate, perfluorodecanoate, perfluoroundecanoate, perfluorobutanesulfonate, perfluorohexanesulfonate, and PFOS. These compounds represent a range of carbon chain lengths and are commonly found in AFFF. Empty culture bottles were spiked with the PFAA mixture while in an anaerobic chamber (90% nitrogen, 5% hydrogen, 5% carbon dioxide) and the methanol was evaporated as described elsewhere. Final PFAA concentrations were verified using liquid chromatography tandem mass spectroscopy with stable-isotope surrogate standards (Wellington Laboratories). Aqueous samples were centrifuged to remove particulates, sampled, and diluted as appropriate. A SCIEX 3200 mass spectrometer (MDS Sciex) was utilized with MultiQuant for quantitation to verify PFAA recovery.

2.2.2 Dechlorination measurements

Chloroethenes, ethene, and methane were measured by injecting 100 µL of culture headspace into an Agilent 7890A gas chromatograph equipped with a flame ionization detector and 30 m x 0.32 mm J&W capillary column (Agilent Technologies). Hydrogen concentrations
were measured by injecting diluted headspace samples into a gas chromatograph fitted with a reductive gas detector (Trace Analytical). Between 50 and 300 μL of culture headspace was withdrawn for each hydrogen measurement and diluted in 17 mL glass vials purged with N₂ to generate concentrations within the linear calibration range of the instrument. The total volume of extracted headspace was tracked throughout the incubation to ensure that the same approximate volume was removed from all bottles (1.5 to 1.9 mL). Initial TCE dechlorination rates were determined by obtaining the slope of a time-course regression through the linear portion of the degradation curve (days 1 through 4) after accounting for loss as a function of time generated from the abiotic control. Production rates for cDCE, VC, and ethene were also tabulated during the linear portion of the generation curve for each sample.

2.2.3 Cellular preparation

Anaerobic experiments were inoculated with a *Dehalococcoides*-containing methanogenic mixed culture that was maintained in a stock bottle amended with exogenous cobalamin co-factors, such as vitamin B₁₂, to enable TCE dechlorination. This stable and robust culture maintained in Berkeley, CA was derived from TCE-contaminated groundwater. The *Dehalococcoides* strains in this culture are most similar to *Dehalococcoides mccartyi* strain 195. The described culture ferments lactate to produce hydrogen and acetate, the necessary electron donor and carbon source for *Dehalococcoides*. Pure culture experiments using *Dehalococcoides mccartyi* strain 195 were maintained under the same conditions as the mixed culture.

2.2.4 Reductive dechlorination with the mixed culture

Batch systems containing TCE, lactate, and a PFAA mixture were designed to evaluate microbial degradation rates. ERD experiments contained triplicate sets of 22, 66, or 110 mg/L
total PFAAs added to sterile 60 mL serum bottles as well as 0 mg/L controls spiked with non-PFAA containing 70/30 v/v methanol/water. These concentrations were chosen to reflect what may be observed near a PFAA source zone\(^7\) (110 mg/L total at 10 mg/L each of 11 analytes) and to determine if there is a PFAA concentration threshold wherein biological effects are not observed. Following PFAA addition and methanol evaporation described in the prior section, bottles were sealed with butyl rubber stoppers. Next, 48.5 mL of an autoclaved mineral salts medium\(^37\) containing 20 mM lactate as electron donor and 100 μg/L vitamin B\(_{12}\) was added to stoppered bottles with a sterile syringe. During media addition, an exhaust needle was inserted into the stopper to avoid bottle pressurization, while low flow rates were used to minimize PFAA volatilization and flushing. After media addition, the headspace was gently flushed with N\(_2\)/CO\(_2\) (90:10) to remove residual hydrogen and oxygen. Each bottle was then amended with approximately 20 μmoles of TCE (Sigma Aldrich, 99.9%) and allowed to sit for at least 24 hours to facilitate TCE and PFAA equilibration. At time zero, the bottles were inoculated with 3% (v/v) of the previously-grown methanogenic mixed community stock culture and incubated in the dark at 34ºC for the duration of the experiment. All bottles were inverted several times at each sampling point to promote PFAA mixing. Abiotic controls devoid of microorganisms were prepared for the parallel pure-culture experiment. After TCE dechlorination profiles were generated, the bottles were sampled for phylogenetic sequencing and verification of PFAA concentrations.

2.2.5 Phylogenetic sequencing

Every sample containing the methanogenic mixed culture was extracted after dechlorination profiles were complete (~8 days). DNA was extracted with the PowerSoil DNA Isolation Kit (MO BIO Laboratories, Inc.). After extraction, DNA was initially quantified with a
Qubit 2.0 Fluorometer and dsDNA HS Assay Kit (Life Technologies). Each sample was amplified using a TC-412 Thermocycler (TECHNE) with 2 μL DNA template, 2 μL each forward and reverse primers at 10 μM each, 6.5 μL PCR grade water, and 12 μL Phusion 2× MasterMix (New England BioLabs, Inc.). Dual-indexed primers (515F and 805R) target the V4 region of the 16s rRNA gene (Integrated DNA Technologies) and are described in the SI along with the amplification protocol. Select samples were run via gel electrophoresis to confirm amplicons of a desired length of roughly 300 base pairs. Samples were purified and normalized with the SequalPrep Normalization Kit (Invitrogen) according to the manufacturer’s protocol and pooled into a 2 mL RNase and DNase free sterile microcentrifuge tube. Pooled samples were concentrated with ultra-.05 30K Centrifugal Filter Devices (Amicon) according to the provided protocol. Pooled samples were quantified using the Qubit Fluorometer as described above and diluted and re-quantified if necessary to normalize concentrations. Half of the final pooled sample volume was archived at -80°C with the balance sent for MiSeq sequencing using the V2 250 Cycle Paired-End kit at BioFrontiers Institute (Boulder, CO).

Sequence post-processing was performed with MacQIIME version 1.9.038 with OTU picking and chimera screening using Usearch6139,40 and alignment with PyNAST.41,42 Taxonomy was assigned with Greengenes reference database version 12_1043 using RDP Classifier 2.2.44 FastTree 2.1.3 was used to generate the phylogenetic tree45 and was further manipulated in FigTree.46 Weighted, non-rarefied beta diversity was visualized with EMPeror,47 and adonis and ANOSIM test statistics were calculated using compare categories.48 Samples were filtered so the minimum total observed fraction per OTU was 0.001. Taxa were summarized such that the OTU identifier with the greatest total abundance was retained: if the lowest classification for multiple OTU identifiers resulted in the same taxonomic assignment, the specific OTU identifier with the
greatest total abundance was retained. The abundance of the resulting merged OTU reflects the
sum of each OTU with the same taxonomic lineage. Differential abundances were calculated
with the DeSeq2 package; the adjusted p values are reported herein.\textsuperscript{49} The relative abundances
represent the averages ± standard deviation between the three experimental replicates per
condition. Reporting of results and analysis within this work reflects the lowest identified
taxonomic level of each OTU. Functionality was assigned based on the class level information
for the non-dechlorinating members of the consortium according to the comparative
metagenomics study performed by Hug et al.\textsuperscript{28} The methanogenic mixed culture used herein is
similar to the ANAS culture maintained in Berkeley, California in Hug et al.\textsuperscript{26,28} which also uses
lactate as an electron donor.\textsuperscript{26,50} Sequence data have been submitted to the National Center for
Biotechnology Information Sequence Read Archive database (BioProject PRJNA302232). Post-
processing scripts can be found in the appendix.

2.3 Results and Discussion

2.3.1 Effect of PFAAs on reductive dechlorination by a mixed culture

Batch microbial incubations across a range of PFAA concentrations revealed that TCE
dehlorination was severely inhibited when the methanogenic mixed culture was grown in the
presence of 110 mg/L PFAA (Figure 2.1). This corresponded with reduced cDCE production as
well as limited VC and ethene production. Inhibition effects on TCE dechlorination were also
observed at 66 mg/L manifested by a decrease in dechlorination rates of TCE as compared to the
case without PFAAs (p<0.01), along with a decrease in cDCE, VC, and ethene production rates
(Table 2.1). Dechlorination of TCE, production and dechlorination of cDCE and VC, and
production of ethene occurred similarly between cases with no PFAAs and those with 22 mg/L.
Analogous inhibitory behavior has also been observed in the presence of select AFFF
formulations containing a range of PFASs. The eleven-component PFAA mixture appeared to exert this selective pressure; systems with equivalent concentrations of only perfluoroalkyl sulfonates were not affected.

Table 2.1. Dechlorination (-) and production (+) rates by the mixed culture

<table>
<thead>
<tr>
<th></th>
<th>TCE</th>
<th>cDCE</th>
<th>VC</th>
<th>Ethene</th>
</tr>
</thead>
<tbody>
<tr>
<td>No PFAAs</td>
<td>-5.8 ± 0.2</td>
<td>+1.4 ± 0.0</td>
<td>+5.5 ± 0.2</td>
<td>+0.3 ± 0.3</td>
</tr>
<tr>
<td>22 mg/L</td>
<td>-4.3 ± 2.9</td>
<td>+1.5 ± 0.7</td>
<td>+3.5 ± 2.7</td>
<td>+0.3 ± 0.4</td>
</tr>
<tr>
<td>66 mg/L</td>
<td>-3.3 ± 1.0</td>
<td>+1.0 ± 0.2</td>
<td>+4.5 ± 1.3</td>
<td>+0.0 ± 0.1</td>
</tr>
<tr>
<td>110 mg/L</td>
<td>-0.5 ± 0.1</td>
<td>+0.1 ± 0.1</td>
<td>+0.1 ± 0.1</td>
<td>0.0 ± 0.0</td>
</tr>
</tbody>
</table>

*Values represent triplicate averages ± standard deviation

Figure 2.1. Reductive dechlorination by the mixed culture is inhibited by 110 mg/L total PFAAs (□) with slower rates of dechlorination for 66 mg/L total PFAAs (○), while 22 mg/L total PFAAs (△) is not significantly different from no PFAAs (×) (p > 0.2 for each sampling time and compound). TCE profiles depicted in (A). Similar trends are reflected for downstream dechlorination products cDCE (B) and VC (C) as well as ethene (D). Error bars represent the standard error of triplicate incubations.
2.3.2 Effects of PFAA exposure on community structure.

To understand how the eleven-component PFAA mixture herein affected the dechlorination process, relative abundance, community dynamics, and putative functionalities were explored via next-generation sequencing. Phylogenetic sequencing of the methanogenic assemblage resulted in 17 total operational taxonomic units (OTUs) after filtering to 0.1% as described in Methods and Materials (Figure 2.2). This core microbiome was observed regardless of PFAA concentration; however, the relative abundance of select taxonomic units varied with increasing PFAA exposure.

Figure 2.2. Changes in abundance based on PFAA exposure do not correlate with phylogeny. Arrows represent the log$_2$ fold change in abundance between no PFAAs and 110 mg/L total PFAAs with arrow size correlating to magnitude of log$_2$ fold change. An upward pointing arrow represents an increase in abundance when grown with PFAAs, downward represents a decrease in abundance when grown with PFAAs. Putative functions within the community are annotated as methionine synthesis (■), fermenters (▲), corrinoid synthesis (○), and oxygen scavenging (✘). Nodes represent FastTree local bootstrap values: values between 80-100% (●), values between 50-80% (○), and values less than 50% (○).
The functions and taxonomy described herein reflect what is typically observed in dechlorinating methanogenic mixed-cultures\textsuperscript{28,36} and are thus applied to this particular system. These functions are hypothetical, as data are being compared to a similar culture that is cultivated in semi-batch (verses batch) and likely contains a different strain of *Dehalococcoides* than the methanogenic mixed-culture used herein.\textsuperscript{26,36} This comparison is appropriate for putative functionality considering the overall taxonomic similarities between the two cultures.

This mixed-culture is fermentative and methanogenic. The general pathway of reductive dechlorination within this community begins with conversion of lactate, the electron donor, to hydrogen by a set of fermenters.\textsuperscript{51} In this study, fermentation has been putatively assigned to eleven OTUs including members from the classes of Bacteroidia\textsuperscript{52} and Clostridia and single members from Spirochaetes and Synergistia (Figure 2.2).\textsuperscript{28,53} The hydrogen can then be used by *Methanobacterium bryantii* to produce methane or to complex with chlorine as it is removed from the electron acceptors (TCE, cDCE, or VC), by *Dehalococcoides*, eventually producing ethene.\textsuperscript{28,54} Most of the remaining OTUs in this culture, along with those found within Clostridia and Spirochaetes, can be functionally assigned to a combination of methionine or corrinoid synthesizers (two functions *Dehalococcoides* lacks), or can fulfill the niche of oxygen scavengers.\textsuperscript{28} These hypothesized interactions only address functionality as it relates to *Dehalococcoides* vitality; it is likely that additional processes are occurring within the system, however they are not included in this analysis. This general community composition is similar to what has been described elsewhere for reductive dechlorinating cultures sustained on lactate, which includes orders such as Clostridiales, Bacteroidales, and Spirochaetales, along with δ-proteobacteria, *Dehalococcoides*, and Archaeal methanogens.\textsuperscript{28,50,51}
The presence of PFAAs at varied concentrations tended to shift the relative abundances of many of these organisms. Analysis of beta diversity depicted two distinct groups: the samples with 110 mg/L total PFAAs clustered independently from the 0, 22, and 66 mg/L samples (Figure 2.3).

![Figure 2.3. Principle component analysis of weighted non-rarefied taxon abundance shows distinct clustering of cultures grown in the presence of 110 mg/L PFAAs (□) on an axis that explains 60.6% of the variability. The rest of the cultures, grown either in the presence of 22 mg/L (▲), 66 mg/L (○), or without PFAAs (◆) do not exhibit any significant clustering between samples.](image)

This is also observed statistically; both adonis and ANOSIM metrics exhibit statistical significance (\(p=0.013\) and \(p=0.011\), respectively) when the samples are grouped by concentration. Although the diversity of samples grown with 66 mg/L PFAAs is not significantly different from 22 mg/L or those without PFAAs, there is still a reduction in dechlorination rate as discussed previously. TCE dechlorination in both the 22 and 66 mg/L samples is well
correlated to the relative abundance of *Dehalococcoides* in each sample, with Pearson correlation coefficients of -0.72 and -0.91 respectively, even though the community diversity between samples does not cluster at 22 mg/L or 66 mg/L. This correlation further supports that *Dehalococcoides* abundance is a driving factor for both differences in community structure and inhibition of TCE dechlorination upon PFAA exposure.

The most extreme shift in community structure occurred when comparing samples without PFAAs and those grown in the presence of 110 mg/L total PFAAs. Samples that effectively dechlorinated TCE without PFAAs had an average abundance of 4.5% ± 0.2% of *Dehalococcoides*, compared to an average abundance of 0.5% ± 0.2% for samples grown in the presence of 110 mg/L total PFAAs. This is an 8.4 fold change decrease in abundance (p<0.01) for communities grown in the presence of 110 mg/L PFAAs. An inverse shift in the relative abundance of methanogenic Archaea was also observed: without PFAAs the *Methanobacterium bryantii* abundance was 0.5% ± 0.3%, and increased to 3.8% ± 0.9% with 110 mg/L PFAAs resulting in an 8.5 fold change increase (p<0.01) (Figure 2.2). This relationship is expected; *Dehalococcoides* and methanogens actively compete for the available hydrogen to either produce HCl through the dechlorination reaction or to produce methane, respectively.28,54 The relative abundances of *Dehalococcoides* and *Methanobacterium bryantii* correlate well with TCE dechlorination rates (R² = 0.68 for both comparisons) as seen in Figure 2.4.

Although the inverse correlation between *Dehalococcoides* and Archaea is anticipated in light of the interplay between TCE reduction and methane consumption,54 our understanding of shifts and corresponding relationships between non-dechlorinating members of the community is limited. A consistent trend was observed in all members of δ-proteobacteria: no significant change in relative abundance was observed upon exposure to PFAAs. Members of this class
Figure 2.4. Abundances of *Dehalococcoides* (□) and *Methanobacterium bryantii* (△) exhibit correlations with PFAA concentration (X) (panel A). TCE dechlorination rates also correlate with *Dehalococcoides* abundance (panel B) ($R^2 = 0.68$) and with Archaea abundance (panel C) ($R^2 = 0.68$).
(such as an unspecified *Desulfovibrionaceae* and *Desulfovibrio* sp.) are hypothesized to scavenge oxygen and synthesize corrinoid cofactors necessary for *Dehalococcoides*. Members of δ-proteobacteria also encode hydrogenases necessary for the conversion of hydrogen cations to H₂ along with the ability to produce exogenous methionine. Reductive dechlorination by *Dehalococcoides* irreversibly loses functionality upon exposure to oxygen, thus *Dehalococcoides* may rely on oxygen scavenging organisms, such as δ-proteobacteria, within the mixed community. Another OTU that was not affected by PFAA presence was *Treponema*, a genus known for corrinoid and methionine synthesis, oxygen scavenging, and the ability to ferment lactate to produce H₂ (Figure 2.2). As these organisms are not significantly impacted by PFAA presence, they are likely not contributing to the inhibition of *Dehalococcoides* vitality and activity.

Conversely, eleven OTUs exhibited statistically different abundances (p<0.05) when grown in the presence of 110 mg/L PFAAs. To understand the potential effects of PFAAs on the putative functions within the methanogenic mixed culture, we will focus on the scenario wherein the largest shifts in relative abundance occurred: between samples without PFAAs and those with 110 mg/L PFAAs, although similar trends were observed when comparing between 22 mg/L, 66 mg/L, and 110 mg/L (Table S.1.3). Taxa that may be responsible for corrinoid synthesis and oxygen scavenging exhibited varied responses to PFAA presence, thus the inhibition of *Dehalococcoides* is not definitively connected to repression of organisms capable of corrinoid or methionine synthesis, or to oxygen scavenging within the community. For example, we hypothesize that members of the order Clostridiales are fermenters and can synthesize methionine within this system. Many of these OTUs were enhanced in the presence of 110 mg/L PFAAs: an unspecified Clostridiales increased by a fold-change of 4.7, *Coprococcus*
increased by 2.4-fold, *Oscillospira* was enhanced 3.4-fold, and Veillonellaceae increased with a fold-change of 1.5. Others capable of fulfilling these niches were inhibited at 110 mg/L PFAAs: *Sedimentibacter* decreased 1.5-fold, and an unspecified Ruminococcaceae decreased 1.7-fold. Christensenellaceae, another Clostridiales, was not significantly impacted by PFAA presence at all. The reason some members of Clostridiales are enhanced while others are repressed or unaffected is yet to be understood.

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members of Clostridiales are enhanced while others are repressed or unaffected is yet to be understood.

Similarly, two OTUs in the order Bacteroidales, which contribute to fermentation and potentially methionine synthesis, were observed: one unidentified Bacteroidales was repressed in the presence of 110 mg/L PFAAs by a fold-change of 7.1, while Porphyromonadaceae was enhanced 1.8-fold. Also repressed at 110 mg/L was WCHB1-05 in the family Anaerolinaceae, which decreased by a fold change of 4.8. This OTU was the most closely related to *Dehalococcoides* as a member of the phylum Chloroflexi. Fold changes and statistical significance for each OTU and concentration comparison can be viewed in the appendix, Table S.1.3. This exercise demonstrates that the selective pressures as a result of PFAA presence are highly variable; in general, enhancement and repression do not trend with putative functionality or with phylogeny.

**2.3.3 Reductive dechlorination of axenic *Dehalococcoides***

As the previously described experiments were performed with mixed cultures, it is impossible to determine whether *Dehalococcoides* was directly affected by PFAAs, or if another subset of the community upon which *Dehalococcoides* is dependent was affected. For example, it is impossible to determine whether *Methanobacterium* excels in the presence of PFAAs or if *Methanobacterium* fills the niche resulting from *Dehalococcoides* inhibition. To understand whether *Dehalococcoides* is directly affected by PFAA exposure, the TCE dechlorination experiment was repeated with *Dehalococcoides* in pure culture. With *Dehalococcoides mccartyi* strain 195 maintained axenically, the inhibitory effects of PFAAs on TCE dechlorination behavior was similar to that of the more complex methanogenic community (Figure 2.5). The rates of production and dechlorination of each solvent with *Dehalococcoides* in pure culture
further illustrate increasing degrees of inhibition with increasing PFAA content (Table 2.2).

Pronounced inhibition was observed at 110 mg/L with a significant reduction in TCE
dechlorination rates for this concentration as compared to dechlorination rates devoid of PFAAs
(p<0.01) (Table 2.2, Figure 2.5). Correspondingly, minimal cDCE and VC production occurred
with 110 mg/L. Ethene generation was not observed above the background from inoculation
carry-over in any of the treatments, which may be explained by the inefficiency of
*Dehalococcoides* in pure culture,37 coupled with TCE presence across these incubations. The
dechlorination of TCE is more energetically favorable than that of VC, therefore significant
generation and subsequent dechlorination of VC is not expected to occur if TCE remains in the
system.37 TCE dechlorination rates with 66 mg/L PFAAs were also significantly inhibited when
contrasted with systems without PFAAs (p<0.01). A delayed rate of cDCE and VC production
was observed at 66 mg/L, although rates of VC production were similar between 66 mg/L and
the case without PFAAs. At 22 mg/L total PFAAs, the average TCE dechlorination rate was
lower than the system lacking PFAAs, yet the inhibition of the rate was not statistically
significant (Figure 2.5). Correspondingly, both cDCE and VC production rates were slightly
retarded at 22 mg/L, although the difference did not maintain statistical significance.

| Table 2.2. Dechlorination (-) and production (+) rates of axenic *Dehalococcoides* |
|---------------------------------|---------------------|-------------------|-------------------|-------------------|
|                                  | TCE (day 3-6)       | cDCE (day 1-6)    | VC (day 5-10)     | Ethene (day 0-10) |
| No PFAAs                        | -2.5 ± 0.2          | +1.3 ± 0.1        | +0.8 ± 0.1        | 0.0 ± 0.0         |
| 22 mg/L                         | -2.1 ± 0.6          | +1.1 ± 0.1        | +0.7 ± 0.1        | 0.0 ± 0.0         |
| 66 mg/L                         | -1.2 ± 0.4          | +0.5 ± 0.1        | +0.6 ± 0.3        | 0.0 ± 0.0         |
| 110 mg/L                        | -0.6 ± 0.2          | 0.0 ± 0.0         | 0.0 ± 0.0         | 0.0 ± 0.0         |
| Abiotic                         | -0.4 ± 0.1          | 0.0 ± 0.0         | 0.0 ± 0.0         | 0.0 ± 0.0         |

*Values represent triplicate averages ± standard deviation
Figure 2.5. Dechlorination is also inhibited by PFAA presence in cultures of D. mccartyi strain 195. TCE dechlorination (A, dotted) is inhibited by 110 mg/L total PFAAs (□) and behaves similarly to the abiotic control (†). Slower rates of dechlorination were observed for 66 mg/L total PFAAs (○) and mild inhibition at 22 mg/L total PFAAs (△) compared to no PFAAs (×). Cis-DCE content (A, dashed), VC content (B, dotted) and ethene content (B, dashed) reflect the same trends of inhibition at each PFAA concentration. Error bars represent the standard error of triplicate incubations.
2.4 Environmental Implications

Understanding reductive dechlorination in the presence of PFAAs is of environmental concern due to the common co-location of PFAAs and chlorinated solvents, and the reliance on microbiological processes for chlorinated solvent attenuation. *In situ* concentrations up to 7 mg/L for a single PFAA analyte in groundwater have been measured; a choice of 10 mg/L of each analyte as investigated here may be representative of a source zone or subsurface worst-case scenario, especially considering increases in environmental detection and measurement sensitivity of a variety of PFASs. Additionally, increased PFAA sorption in the presence of non-aqueous phase liquids (NAPLs) has been observed, particularly at higher PFAA concentrations. This may result in an accumulation of PFAAs where TCE is present, even outside of source zones. It is also known that PFAA precursor compounds found in AFFF may generate additional PFAAs in groundwater over time. As the depth of knowledge regarding the concentration and distribution of PFAA analytes is continually growing, this is a current approximation of what may be observed near a source zone *in situ*. Based on the results from this study, it is clear that at these high concentrations reflective of PFAA source zones or areas of NAPL co-contamination, dechlorination of TCE is significantly inhibited both within the dechlorinating mixed community and with *Dehalococcoides* in pure culture. Phylogenetic investigations revealed that the presence of PFAAs at high concentrations directly impacts *Dehalococcoides* relative abundance and has a varied effect on the composition of the methanogenic mixed community.

It has been observed elsewhere that dechlorinating communities provided with acetate, lactate, or methanol as electron donors appear to be phylogenetically distinct from one another. These different communities, however, still exhibit conserved metabolic pathways, particularly
for methionine and corrinoid synthesis, fermentation, and oxygen scavenging. This implies that although community shifts may occur in the presence of PFAAs, the machinery required to facilitate *Dehalococcoides* growth and dechlorination may still be provided, suggesting that there exists a degree of functional redundancy within the methanogenic mixed culture. Exploring the putative functions of the mixed methanogenic communities may not necessarily provide the single key to the mechanisms behind the repression of *Dehalococcoides*, however, this is an important step in understanding the effects of PFAAs on environmentally relevant systems. To understand whether particular processes or functions are affected by PFAA presence, additional exploration such as proteomics or functional metagenomics may be utilized to understand the impacts of PFAA exposure.

The parallel observations of impacts on reductive dechlorination by *Dehalococcoides* both axenically and in community incubations confirm that source-level concentrations of PFAAs will have a direct impact on *Dehalococcoides*. However, due to the variation in community responses, it is difficult to ascertain the mechanisms affecting the repression of *Dehalococcoides*. This repression could potentially be attributed to a stress response or inhibition of vitamin B$_{12}$ uptake. For example, vitamin B$_{12}$, in the form of cyanocobalamin, is a required enzymatic cofactor for reductive dehalogenases and contains both a cyano group and 5’6’-dimethylbenzimidazole as the lower α-ligand. *Dehalococcoides* growth can be hindered by changing the type of lower ligand: we can hypothesize that if PFAAs chemically interact with this lower ligand, it may be converted to a form that is unusable for *Dehalococcoides*. Additionally, while we do not expect or observe any defluorination, there is evidence of potential physicochemical interactions between PFAAs and vitamin B$_{12}$. Binding of PFAAs onto B$_{12}$ is not well understood, but may be observed considering the molar excess.
of PFAAs used in this study: at 110 mg/L PFAAs, there are four orders of magnitude more PFAAs present (3x10^{-4} \text{ mol/L}) than vitamin B_{12} amendments (7x10^{-8} \text{ mol/L}). This interaction may result in a deficit for use by Dehalococcoides; a minor change in vitamin B_{12} availability may dramatically affect Dehalococcoides’ ability to reductively dechlorinate.\textsuperscript{37,59} Future work to examine the effects of increasing vitamin B_{12} dosage in the presence of high concentrations of PFAAs or the quantification of cobalamin lower ligand moieties may help elucidate these hypotheses.

In summary, this study addresses the impacts of PFAAs on the enhanced reductive dechlorination of TCE and a community responsible for that process. PFAAs and TCE are environmental pollutants, the former is recalcitrant and not easily remediated \textit{in situ}; the latter is often treated with bioremediation via reductive dechlorination. Interestingly, high concentrations, similar to what may be found near a PFAA source zone, significantly inhibit TCE dechlorination by directly repressing Dehalococcoides. Because of Dehalococcoides’ role in reductive dechlorination, suppression of Dehalococcoides growth may result in an accumulation of VC, especially if other dechlorinators that are not capable of attenuating this carcinogen, such as Geobacter, are active and unaffected.\textsuperscript{28} In sites that are co-contaminated with both chlorinated solvents and PFAAs, biostimulation or bioaugmentation to enhance reductive dechlorination processes may not be the most suitable option for solvent remediation. Additionally, if ERD is the current cleanup strategy for chlorinated solvents and is observed to be ineffective, the presence of coexisting PFAAs may be the cause of inhibition. These results underscore the need for complete site investigation, monitoring, and exploration to ensure continued chlorinated solvent bioremediation in locations that may contain PFAAs.
CHAPTER 3

ENHANCED BIOFILM PRODUCTION BY A TOLUENE DEGRADING RHODOCOCCUS
OBSERVED AFTER EXPOSURE TO PERFLUOROALKYL ACIDS

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Abstract

This study focuses on interactions between aerobic soil-derived hydrocarbon degrading bacteria and a suite of perfluorocarboxylic acids and perfluoroalkylsulfonates that are components of aqueous film-forming foams used for fire suppression. No effect on toluene degradation rate or induction time was observed when active cells of *Rhodococcus jostii* strain RHA1 were exposed to toluene and a mixture of perfluoroalkyl acids (PFAAs) including perfluorooctanoic acid (PFOA) and perfluorooctane sulfonate (PFOS) at concentrations near the upper bounds of groundwater relevance (11 PFAAs at 10 mg/L each). However, exposure to aqueous PFAA concentrations above 2 mg/L each was associated with enhanced aggregation of bacterial cells and significant increases in extracellular polymeric substance production. Flocculation was only observed during exponential growth and not elicited when PFAAs were added to resting incubations; analogous flocculation was also observed in soil enrichments.


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Aggregation was accompanied by two- to three-fold upregulation of stress-associated genes, $\text{sigF3}$ and $\text{prmA}$, during growth of this $\text{Rhodococcus}$ in the presence of PFAAs. These results suggest that biological responses, such as microbial stress and biofilm formation, could be more prominent than suppression of co-contaminant biodegradation in subsurface locations where poly and perfluoroalkyl substances occur with hydrocarbon fuels.

3.1 Introduction

Poly and perfluoroalkyl substances (PFASs) are emerging contaminants that are widespread in many facets of the environment: surface water, soil, air, and groundwater.\textsuperscript{14} Advances in detection and quantification have led to growing concerns of exposure. Within the broader class of PFASs, a subset of perfluoroalkyl acids (PFAAs) are environmentally persistent, toxic, and can bioaccumulate.\textsuperscript{10,15–17} These molecules have been detected in human kidneys, livers, and blood serum.\textsuperscript{5,6,15,20} To mitigate exposure, the U.S. Environmental Protection Agency has set provisional drinking water health advisories for two common PFAAs: 0.4 μg/L for perfluorooctanoate (PFOA) and 0.2 μg/L for perfluorooctanesulfonate (PFOS).\textsuperscript{6}

PFAs are fully fluorinated carbon chains of varying lengths and two primary classes: carboxylates and sulfonates.\textsuperscript{3} These chemicals possess distinctive properties: they have hydrophobic and oleophobic tails, and tend to form micelles.\textsuperscript{4} Due to these unique aspects, PFASs are used in many industrial applications, such as non-stick coatings, fire retardants, and pesticides.\textsuperscript{5,6} Some PFASs, as well as their precursors, are critical components of aqueous film-forming foam (AFFF) used for fire suppression.\textsuperscript{3,7–9} Extensive use of AFFF for military firefighter training has lead to the significant introduction of PFAAs into groundwater in sites that are often contaminated with other chemicals, such as chlorinated solvents or hydrocarbons.\textsuperscript{7,8}
The biodegradation of recalcitrant hydrocarbons commonly found at AFFF-impacted sites (i.e., BTEX: benzene, toluene, ethylbenzene, and xylene), has been extensively documented and provides a long-term mechanism for attenuation. Bacterial strains of Rhodococcus, a genus of common soil microbes, have been particularly noted for their resistance to toxicity and ability to degrade a range of organic compounds with bioremediation and industrial applications. They are also noted for their utility for mechanistic exploration in laboratory investigations due to genetic annotation and a diversity of biodegradation pathways. To date, it remains unclear to what extent PFAAs may impact microorganisms relevant to hydrocarbon bioremediation. While biodegradation of PFAAs is not expected, in part due to increased rigidity of the fluorinated chain that may enable resistance to enzymatic interactions, concerns regarding potential adverse effects from PFAA exposure on microbial populations and subsequent co-contaminant degradability have been expressed and could complicate remediation efforts in contaminated subsurface systems.

The objective of this study was to examine the interactions between a suite of 11 PFAAs and microbes as they relate to contaminant metabolism and cellular growth in aqueous batch systems. First, we evaluated the effects of PFAA concentrations on toluene degradation and induction time by a well-characterized soil bacterium, Rhodococcus jostii strain RHA1. Next, we followed the growth of this microorganism during PFAA exposure and analyzed the subsequent flocculation and production of extracellular polymeric substances (EPS), a component of biofilms, which was also observed during growth of soil-derived communities. Finally, reverse transcriptase quantitative polymerase chain reactions (RT-qPCR) were utilized to quantify the upregulation of stress-associated genes for cells grown in the presence of PFAAs. Collectively, this study assessed possible changes in subsurface bioremediation potential of co-
contaminants, as well as impacts on microbial behavior including biofilm production and stress responses that may be elicited by exposure to PFAAs.

3.2 Materials and Methods

3.2.1 PFAA Preparation and Aqueous Analysis

Purity-corrected stock solutions of an eleven-analyte mixture were made using salts from Sigma-Aldrich in a 70/30 v/v methanol/aqueous solution. The components in this mixture were: perfluorobutanoate (PFBA), perfluoropentanoate (PFPeA), perfluorohexanoate (PFHxA), perfluoroheptanoate (PFHpA), PFOA, perfluorononanoate (PFNA), perfluorodecanoate (PFDA), perfluoroundecanoate (PFUnA), perfluorobutanesulfonate (PFBS), perfluorohexanesulfonate (PFHxS), and PFOS (Table 1.1). This mixture was used in the following experiments unless otherwise noted. The appropriate volume of the PFAA mixture in 70/30 v/v methanol/aqueous solution was added to experimental vessels to achieve target aqueous concentrations and left overnight or until the methanol from the spiking solution had evaporated to minimize residual methanol that could be utilized as a carbon source for microbial respiration. Initial control experiments indicated insignificant losses of PFAAs from this evaporation step (Figure S.2.1). To verify PFAA concentrations in aqueous suspensions, samples were centrifuged to remove particulates, sampled, and diluted as appropriate according to published methods, followed by measurement via liquid chromatography tandem mass spectroscopy (LC-MS/MS) utilizing stable-isotope surrogate standards purchased from Wellington Laboratories. An Applied Biosystems 3200 mass spectrometer (MDS Sciex) was utilized with Analyst for quantitation. Control experiments were performed to ensure that PFAA losses due to experimental setup (i.e. vessel type, stopper type, sampling procedure) were quantifiable and repeatable (Figure S.2.1), and competition effects between different PFAA analytes were negligible (Figure S.2.2).
3.2.2 Cellular Preparation, Harvest, and Protein Measurements

Bacterial cultures of *Rhodococcus jostii* RHA1 were archived at -80°C and maintained axenically using Luria-Bertani (LB) agar plates. Liquid LB (Difco) cultures were grown in either 500 mL or 1 L glass flasks using a volume to liquid ratio of 5:1. Subsequent generations were propagated with 1 or 10% liquid inoculum. For cells grown on toluene (Pharmco-Aaper, HPLC UV-grade, 99.7%), 500 mL capped pyrex bottles with 100 mL of minimal salts media were inoculated directly from the plate. Toluene (20 μL) was supplied to a glass test tube resting in the pyrex bottle and allowed to mix via headspace. For induction and degradation experiments, cells were harvested and washed using 0.1 M potassium-phosphate buffer (pH ~7.0). Harvested cells were then added to experimental vessels at target specific protein concentrations after PFAA addition, methanol evaporation, and media equilibration. All growth and assay experiments occurred at 30°C and 150 rpm unless otherwise described. To quantify cellular protein, aliquots of 0.5 or 1 mL were centrifuged at 15,000 x g for 3 minutes, decanted, and stored at -80°C until analysis. Protein extraction included NaOH digestion, physical disruption, and heated incubations at 100°C as described elsewhere. Cellular protein was quantified using the colorimetric Coomassie Plus Assay Reagent (Bradford) with bovine serum albumin (BSA) as the standard (Thermo Scientific).

3.2.3 Toluene Biodegradation

Batch systems containing toluene and a PFAA mixture (no PFAAs in the control) were designed to assess the degradation rates of this model substrate. Degradation experiments were performed in 60 mL amber glass bottles and sealed with non-PTFE containing butyl rubber stoppers. Experiments were performed in triplicate in 0.1 M potassium-phosphate buffer (pH 7; 50 mL total) for toluene degradation. This buffer was chosen to reduce interference during LC-
MS/MS analysis of PFAAs. PFAAs were added as described above to achieve a target final concentration of 10 mg/L for each analyte (110 mg/L total PFAAs), followed by the addition of potassium-phosphate buffer and neat toluene through the butyl rubber stopper with a 5 μL high precision glass syringe (Hamilton). Toluene partitioned into the aqueous phase via headspace, resulting in an initial aqueous concentration of 34.6 mg toluene per L. This mixture was allowed to equilibrate for at least 12 hours prior to the addition of toluene-grown, washed, and harvested cells of *Rhodococcus jostii* (an initial quantity of 22 mg/L protein approximated from optical density measurements and OD-protein correlations established from growth experiments discussed herein). Headspace samples of 50 μL were removed approximately every hour until at least 50% toluene degradation was observed. Gas phase toluene concentrations were measured using a Hewlett Packard 5890 Series II Plus gas chromatograph flame ionization detector (GC-FID) with a 0.25 μm Rxi-5ms fused silica column (Restek). A standard curve was developed with known aqueous toluene standards that were created, stored, and sampled in the same manner as the reaction vessels. Concentration in the headspace was extrapolated to the liquid phase using a dimensionless Henry’s Law constant of 0.337 for a 30°C system.

To evaluate the potential lag in degradation for cells newly introduced to toluene as the only carbon source, a similar procedure was used as described above. Cells were added from a washed culture grown in LB broth with an initial protein content of 55 mg/L approximated as discussed above. Headspace was immediately sampled after inoculation, and was sampled approximately every 4 hours until at least 50% toluene degradation was observed. Induction times were calculated by evaluating the slope by linear regression of the degradation period (between hours 4 and 8) and calculating the intersection of this slope with the average toluene concentration of early linear data points (hours 0 to 4) to avoid bias based on sampling times.
The calculated intersection represents the time to induction, or the approximate time at which significant toluene degradation began.

### 3.2.4 Growth and EPS Production

Growth experiments were performed in one liter sealed polypropylene bottles in triplicate containing PFAAs, LB media, and a liquid inoculum of *R. jostii* RHA1 (~0.2 mg/L initial protein concentration) with a 1:10 liquid to headspace ratio. To evaluate the effects of varied PFAA concentrations, this experiment was also performed at 0.1, 0.2, 0.5, 1, 2, 5, and 10 mg/L of each PFAA analyte wherein 50 μL of inoculum was transferred to 4.95 mL LB media in 50 mL polypropylene tubes sealed with parafilm and placed at an angle of 10°. A single sample was taken after three days of growth for this entire range of concentrations and analyzed for optical density.

Optical density was measured using a Jenway 6505 UV/Vis Spectrophotometer. EPS production was quantified by using a modified anthrone reagent (Fisher Scientific) total sugar and polysaccharide assay with glucose (Sigma Aldrich) as a standard. Prior to polysaccharide analysis, samples were centrifuged at 15,000 x g for 3 minutes. The supernatant was decanted and stored at 4°C. EPS was harvested by adapting two methods that account for loosely and tightly bound EPS. Both resulted in agreement of trends, however, the method using NaCl produced greater EPS yields and is presented herein (protocol and results from a method using EDTA are found in Figure S.2.6). This extraction includes a room temperature incubation to release loosely bound EPS, followed by a heated incubation with 20 g/L NaCl to release tightly bound EPS. The following modifications were implemented: initially, a cellular aliquot was weighed and transferred to a 15 mL polypropylene conical tube with 5 mL of MilliQ™ water per 1.5 g cells, and only NaCl was used for the chemical extraction.
Light microscopy images were generated with 20 μL of liquid sample on glass slides performed with an Olympus CX41 light microscope. To prepare the cells for scanning electron microscopy (SEM) with a Hitachi TM3000 SEM, 1 mL aliquots were sampled after 4 days of growth when the differences in degree of flocculation were largely apparent. These aliquots were stored at 4°C until dehydration and fixing; a visualization protocol was adapted and is described in the appendix.

### 3.2.5 Soil Enrichments

Cultures derived from circumneutral pH soil were harvested from below turf grass at a depth of 7.5 to 10 cm in Golden, Colorado. Following sample collection, 5 g soil was placed into 100 mL LB media at a volume to liquid ratio of 5:1. Every 12 hours, 0.5 mL of liquid inoculum was moved to fresh LB media with a 10:1 volume to liquid ratio and incubated as described above. After 4 generations, 50 μL of inoculum was transferred to 4.95 mL LB media in 50 mL polypropylene tubes containing 10 mg/L of each PFAA (110 mg/L total) or no PFAAs prepared as described previously. Tubes were prepared in triplicate, sealed with parafilm, and placed at an angle of 10° within the incubation conditions. Flocs from each replicate were measured and counted using an Olympus CX41 light microscope with a Bright-Line Hemacytometer counting chamber and Infinity Analyze software.

### 3.2.6 Quantification of Gene Expression

To evaluate genetic upregulation, 50 μL of strain RHA1 grown in minimal media with 40 mM sodium pyruvate (Sigma Aldrich) was transferred to 50 mL polypropylene tubes containing either 10 mg/L of each PFAA (110 mg/L total) or no PFAAs and 4.95 mL fresh media in triplicate. A parallel set grown with LB was also performed; preparation and results are included in the appendix. Tubes were sealed with parafilm and placed at an angle of 10° in a
30°C incubator at 150 rpm. Samples were aerated on day 2, 3, and 5 by opening the tube and gently pipetting in order to ensure ample oxygen transfer into the medium. When flocculation was most apparent (day 6) samples were centrifuged, decanted, resuspended in 2 mL RNALater (Ambion), and stored at 4°C until RNA extraction. RNA from 1.5 mL well-mixed aliquots was extracted using the MoBio PowerBiofilm RNA Isolation Kit (VWR). The manufacturer protocol was followed with a final elution of 100 μL prior to DNAse treatment and removal using the Turbo DNA-free Kit (Ambion). DNAse treated RNA was concentrated to a final volume of 40 μL as described in the MoBio PowerBiofilm RNA Isolation Kit procedure. RNA was quantified using a Qubit 2.0 Fluorometer with a Qubit RNA HS Assay Kit. All purified RNA was stored at -80°C until use.

Two primer sets for stress-related genes were utilized: *sigF3*<sup>63</sup> and *prmA*.<sup>61</sup> *GyrB*<sup>78,79</sup> was used as a reference gene with primers as described for *Rhodococcus opacus* and adjusted for RHA1 specificity using GenBank Primer-BLAST.<sup>80</sup> *SigF3* and *prmA* primers were designed using Geneious version 6.1,<sup>81</sup> tested for specificity using GenBank Primer-BLAST, and synthesized by Integrated DNA Technologies (Table S.2.2). Amplification was performed in triplicate for each biological replicate in 96-well plates using a Roche LightCycler 480II. Wells were prepped with qScript One-Step SYBR Green qRT-PCR Kit (Quanta Biosciences). Each 25 μL reaction contained 2 μL of extracted RNA, 0.4 μM each of a forward and reverse primer, 8 μL nuclease free water, 12.5 μL SYBR Green Master Mix, and 0.5 μL qScript Reverse Transcriptase. Quantification protocols were adapted from Sharp et al.:<sup>61</sup> reverse transcription occurred for 10 minutes at 50°C, followed by initial denaturation for 5 minutes at 95°C. All temperature ramping occurred at 4.4°C/s unless otherwise specified. Amplification followed with 40 cycles of a 95°C hold for 10 seconds, 30 seconds at 55°C (2.2°C/s ramp rate), and
quantification after 1 minute at 72°C. The absolute quantification/second derivative maximum method was used for quantification within the Roche LightCycler 480II version 1.5 software. To test for DNA contamination, each sample was also amplified in duplicate without the reverse transcriptase enzyme in addition to a triplicate negative control with nuclease free water (Figure S.2.4).

To calculate statistical significance, samples were compared to a set of gene specific DNA standard curves and normalized to total RNA concentration as recommended elsewhere. A description of standard curve formulation can be found in the SI. Student’s t-tests for samples with unequal variance were performed on analytical triplicates to encompass both biological and technical sources of error as done elsewhere. Upregulation-fold change calculations were performed by utilizing a relative expression model that includes a correction for variations in real-time efficiencies. An additional evaluation for significance utilizing randomization tests within the Relative Expression Software Tool (REST) was also performed. RT-qPCR details following the Guidelines for Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) such as efficiencies, melt curves, and raw data can be found in the appendix.

3.3 Results and Discussion

3.3.1 Effect of PFAAs on Toluene Degradation

If the presence of PFAAs affects microbial processes, it may be manifested as changes in toluene degradation rates or variations in induction time needed for the synthesis of toluene-degrading enzymes. Toluene was selected as a representative recalcitrant hydrocarbon, as similar metabolic machinery is utilized for the aerobic biodegradation of all BTEX compounds by oxygenase-type enzymes. The genes responsible for toluene degradation are hypothesized to be
part of the ethylbenzene dioxygenase pathway,\textsuperscript{69,78} and are found in two of the linear plasmids within the RHA1 genome.\textsuperscript{66} In our experiments, near-complete toluene degradation by strain RHA1 was observed regardless of the presence of PFAAs at collective concentrations as high as 110 mg/L. Cultures that were grown on toluene as the sole electron acceptor prior to harvest exhibited a rapid onset of aerobic toluene biodegradation with no visible lag in batch assays. Just 5.7±0.3\% of the initial toluene remained after 6.5 hours for the systems with PFAAs versus 3.3±2.3\% for the systems without PFAAs (Figure 3.1). The protein normalized rate of toluene degradation for the system with PFAAs was 0.183±0.009 mg toluene / mg protein per hour; without PFAAs the degradation rate was 0.200±0.007 mg toluene / mg protein per hour. These values are not significantly different (p > 0.05), strongly suggesting that the presence of high concentrations of PFAAs does not affect enzymatic affinity for toluene degradation nor does it exhibit any clear short-term toxicological effect on cells acclimated to the presence of toluene.

An analogous experiment was then conducted to understand whether the presence of PFAAs inhibits the synthesis of enzymes involved in aerobic toluene degradation. Strain RHA1 grown in LB media and then introduced to toluene as the sole carbon source after harvesting did not exhibit significant differences in rates or induction times with PFAA exposure (Figure 3.1). After about 13 hours, 7.9±3.8\% of the toluene remained in the systems with PFAAs, and 11.5±2.3\% of the toluene remained in systems without PFAAs. Induction times, which represent a transcription / translation response to the presence of toluene as a target substrate, were not significantly different: 3.65±0.33 hours for systems with PFAAs versus 3.65±0.17 hours for systems without PFAAs. After the induction time had passed, the rates of toluene degradation were similar in both systems regardless of PFAA presence: the rate for PFAA systems was 0.051±0.026 mg toluene / mg protein per hour while the rate for systems without PFAAs was
0.056±0.003 mg toluene / mg protein per hour. These rates are slower than those observed for the system grown on the target substrate for both cases with and without PFAAs, which can be expected if *R. jostii* is still in the process of acclimatizing to toluene as the sole carbon source. This suggests that the rate of degradation should increase over time, however that degree of resolution was not observed as a result of experimental confines.

As there were no significant alterations in degradation rate or induction time at 10 mg/L of each PFAA (110 mg/L total), effects at lower concentrations are unlikely. Field concentrations of PFAAs in groundwater have been measured up to 7 mg/L for a single analyte; a choice of 10 mg/L of each analyte may be representative of an environmental worst-case scenario. The

![Figure 3.1. Toluene concentration over time for toluene grown systems (open symbols, initial protein ~22 mg/L) and induction time and concentrations for those grown on Luria-Bertani broth (closed symbols, initial protein ~55 mg/L) are not affected by the presence of PFAAs. Points represent the triplicate average toluene concentration in the liquid phase (C_w) calculated from headspace measurements using Henry’s Law constant of 0.337 for systems with PFAAs (□ ■) and without (△ ▲), error bars represent the standard deviation. Abiotic controls shown with circles (○ ●).](image)
similarities in toluene degradation rates and induction times further support the inference that strain RHA1 could continue to degrade toluene when exposed to high concentrations of individual components or mixtures of PFAAs.

3.3.2 Effect of PFAA Presence on Growth and Biofilm Formation

While a metabolic impact was not observed for cells grown and then subsequently exposed to PFAAs and toluene, significant differences in optical density trends and values were observed for *R. jostii* strain RHA1 during the exponential phase of growth in the presence of similarly high concentrations of PFAAs (Figure 3.2A). This anomaly was attributed to cellular

![Figure 3.2 Visible cellular flocculation results](image)

Figure 3.2 Visible cellular flocculation results in lower optical density readings for cells grown in the presence of PFAAs (panel A, □) than for those grown in the absence of PFAAs (panel A, △). However, protein content trends remain similar between cells grown in the presence of PFAAs (panel B, □) and those grown in the absence of PFAAs (panel B, △).

3.3.2 Effect of PFAA Presence on Growth and Biofilm Formation

While a metabolic impact was not observed for cells grown and then subsequently exposed to PFAAs and toluene, significant differences in optical density trends and values were observed for *R. jostii* strain RHA1 during the exponential phase of growth in the presence of similarly high concentrations of PFAAs (Figure 3.2A). This anomaly was attributed to cellular
flocculation associated with the presence of PFAAs, which was visibly observed in all replicates. Optical density is a common proxy for measuring microbial growth in liquid media but one that loses quantitative meaning when refractive particle sizes differ. For an alternative representation of biomass accumulation, the protein contents at various points throughout the growth curves were analyzed (Figure 3.2B). In contrast to optical density, quantified protein extracted from biomass grown in the absence and presence PFAAs were not significantly different, thus the actual quantity of cellular biomass was not affected by the presence of the PFAAs. When exploring the effects of varied PFAA concentrations, growth after three days exhibited a decrease in optical density relating to flocculation interference at total PFAA concentrations in excess of 22 mg/L (Figure 3.3).

![Figure 3.3. Flocculation response at different PFAA concentrations (1 – 110 mg/L total) as represented by OD. A drop in OD correlating with flocculation occurs above 22 mg/L total PFAAs.](image)

This flocculation was not observed when PFAAs were added to harvested cells during toluene degradation experiments described in the previous section. Additionally, the flocs that formed during exponential growth broke apart after approximately 7 days in a more stationary phase (Figure S.2.5) without any change in PFAA concentration. These results suggest that
flocculation was associated with a biological response rather than surfactant-derived chemical interactions with PFAAs. The presence of PFAAs, at least at these very high concentrations, impacts the manner of *R. jostii*’s growth with enhanced potential for biofilm formation and cellular aggregation.

To test whether the flocculation phenomena was a result of enhanced EPS production, a combination of microscopy and quantitation by carbohydrate analysis was employed. Photographs of liquid samples (Figure 3.4A) and light micrographs of *R. jostii* depict homogenously distributed cells in solution without PFAAs (Figure 3.4C). The system with PFAAs, however, exhibits large and dense cellular clumps with little to no free-floating cells (Figure 3.4B, 3.4D). Scanning electron microscopy (SEM) also captures differences between the systems grown with PFAAs and those without. When grown in the absence of PFAAs, cells were more uniform in size with an average cellular length of approximately 1.8±0.82 μm (Figure 3.4E). In contrast, when grown in the presence of PFAAs, the average cellular length doubled to approximately 3.4±1.6 μm (Figure 3.4F). This lengthening is potentially end-to-end adhesion as a result of EPS production or an artifact of incomplete cell division, as has been observed in resource limited systems and linked to a stress response. SEM images of strain RHA1 grown in the presence of PFAAs also capture collapsed EPS (Figure 3.4F). The EPS is typically a highly hydrated substance, but after the dehydration steps necessary for SEM visualization, EPS can be observed as a collapsed structure.

Extraction of EPS from the cellular matrix yielded statistically significant (p < 0.05) increases in carbohydrates for the systems grown with PFAAs. In the case without PFAAs, 0.052±0.012 g carbohydrates / g cellular dry weight was recovered, versus 0.139±0.050 g carbohydrates / g cellular dry weight for the case with 10 mg/L of each PFAA. This near tripling
of carbohydrate content coupled to micro and macroscopic visualization of cells grown with PFAAs provides strong evidence for enhanced EPS production, which may lead to increased biofilm formation in subsurface systems.

To further extrapolate the relevance of this axenic bacterial response, increased flocculation was also observed in soil-derived communities enriched in LB media and exposed to PFAAs. Increased floc counts, as well as larger floc areas, were observed under these conditions as compared to control communities grown without PFAAs (Figure S.2.7). The capability of microorganisms to produce EPS is widely observed. The presence of PFAAs and the associated toxicological effects on microbial populations may encourage increased EPS formation as a stress response or through environmental changes. Microbial flocculation in the presence of surfactant-like molecules, such as PFAAs, may be counterintuitive if the flocculation is merely a physical process, however EPS production and subsequent flocculation may be protecting and isolating microcolonies so that they may continue to grow and reproduce.

3.3.3 Expression of Stress Genes

Species of *Rhodococcus* have been shown to produce EPS as a potentially protective stress response under a variety of conditions including exposure to toxins and desiccation, carbon and nitrogen starvation, as well as to facilitate adhesion to polyethylene and subsequent three-dimensional microcolony formation. Specifically, the production of EPS can act as a barrier between the cells and the dry environment under desiccation stress, or as a protective layer between the cells and toxins in solution including antibacterial agents and surfactants. External stressors, such as changes in ionic strength, have even been applied to cultures in order to induce EPS production. Alternatively, the unique chemical properties of PFAAs, particularly
Figure 3.4. Large flocs are visible when *R. jostii* RHA1 is grown in the presence of PFAAs (right column); without PFAAs, the microbes are evenly distributed in solution (left column). A. Photograph of the bottom of a representative 1 L bottle used for growth without PFAAs and (B) with PFAAs. C. Light microscopy images of a representative sample without PFAAs (n=25) and (D) with PFAAs (n=32). E. SEM images capture cells as lighter, similarly sized, rod-shaped articles without PFAAs (n=15). F. More dehydrated EPS (gray background slurry) is captured via SEM in images of systems with PFAAs (n=28).
the ability to form micelles, may result in microbial aggregation independent of bacterial processes.

To test whether EPS production occurs in tandem with a stress response in the presence of PFAAs, the utilization of strain RHA1 with its large metabolic potential and annotated genome enables us to target for specific genes that correlate to cellular stress responses and regulation in order to better mechanistically explore this phenomenon. This method does not necessarily prove that EPS production is a result of stress, or even that a stress response occurs as a function of cellular flocculation, instead we are assessing the variety of cellular responses that may occur upon exposure to PFAAs and are further exploring this interaction. To achieve this, quantification of stress-related genes within RHA1 using RT-qPCR was performed.

The \textit{prmA} gene is a part of the propane monooxygenase operon, and has been observed to be upregulated during solvent exposure and starvation conditions, potentially as a part of a general stress response. Similarly, \textit{sigF3} is a gene encoding a sigma factor, and has been linked to desiccation, heat, and osmotic stress and is potentially part of a regulatory stress network. Both genes are found in the chromosome of the RHA1 genome. Upregulation of either or both of these genes would imply activation of a stress response to an environmental variable. When RHA1 was grown with 110 mg/L total PFAAs (+PFAA), there was a two- to three-fold upregulation of both genes of interest (p≤0.05) over cells grown without PFAAs (-PFAA) (Table 3.1). Similar upregulation trends of statistical significance were also observed for cells grown in nutrient rich media (LB broth) and can be seen in the appendix.

Although increased expression of these genes can be correlated to a stress response, the particular stress trigger as a result of PFAA exposure (such as nutrient limitation, environmental toxicity, or even flocculation behavior itself) is yet unknown. Whether stress conditions occur in
a mixed culture or even *in situ* has not been evaluated, however considering the observation of increased flocculation in soil-derived cultures as previously discussed, microbial stress effects in PFAA source zones may be likely.

<table>
<thead>
<tr>
<th>Table 3.1. Increased transcription of stress-associated genes</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gene</strong></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td><em>sigF3</em></td>
</tr>
<tr>
<td><em>prmA</em></td>
</tr>
</tbody>
</table>

### 3.4 Environmental Implications

Ideally, subsurface zones contaminated with hydrocarbons will undergo some degree of biodegradation even in the absence of active engineering practices.$^{23,24}$ Due to the co-occurrence of hydrocarbons and PFAAs *in situ*, the continued hydrocarbon bioremediation in the presence of PFAAs is a widespread concern.$^{7-9}$ The effects of PFAAs on microorganisms has not yet been studied, as compared to the effects on macroorganisms. For example, an average of 50 ng/mL of PFOA (one of the 11 analytes tested in our work) has been detected in human populations exposed to fluorinated surfactants.$^{94}$ Rats have been shown to break down precursor compounds into PFOS,$^{19}$ bioaccumulation factors for PFAS uptake into radish, celery, tomato, and sugar snap pea crops have been calculated,$^{10}$ and primates express decreased body weight and increased liver weight upon exposure to PFOS.$^{18}$ The body of knowledge regarding human and environmental interactions with previously undetected poly and perfluoroalkyl substances is continually growing,$^{12}$ and may eventually provide more accurate field and biologically-associated levels of exposure. If microorganisms are detrimentally impacted by PFAA exposure, we may see environmental effects of concern that could include inhibition of co-contaminant
degradation, shifts in carbon cycling, or changes in subsurface chemical properties such as pH or metal mobility.\textsuperscript{90,95}

Aqueous batch experiments assessing the interactions between PFAAs and a soil microbe capable of hydrocarbon degradation highlight potential impacts on subsurface systems. Although these laboratory based systems are not necessarily representative of every subsurface environment, the comparison of PFAA-containing cultures to those without can provide us with insights regarding the effects of PFAAs while controlling and monitoring variables such as temperature, microbial community effects, and contaminant mass balances. The laboratory work described herein could complement and support future research investigating anaerobic systems, mixed communities, and/or dynamic systems.

While the presence of PFAAs did not appear to alter metabolic traits relating to toluene bioremediation potential or net biomass growth of \textit{R. jostii} strain RHA1, cells grown in the presence of high concentrations of PFAAs (≥22 mg/L total PFAAs) exhibited enhanced EPS formation and subsequent flocculation. Stress-related genes are also upregulated 2- to 3-fold over the cells grown without PFAAs. Multiple lines of evidence support that aggregation of RHA1 results from a biological response rather than only physicochemical interactions: 1.) flocculation was not observed when harvested cells were exposed to media containing PFAAs as in the toluene biodegradation experiments; 2.) observed flocs eventually break apart during stationary phase without a change in PFAA concentration; 3.) flocculation is coupled to an increase in EPS production; and 4.) the upregulation of stress-related genes implies that a biological response is occurring.

The ability to enhance flocculation is most often used in wastewater treatment trains as a part of the activated sludge process.\textsuperscript{96} Flocculation has also been implemented in the biofuels
industry, and can be seen in natural processes in both oceanic and freshwater systems. Flocculation can be encouraged by exposure to surfactants, such as sodium dodecyl sulfate (SDS), which can change the surface hydrophobicity of the cells. Although PFAAs exhibit surfactant-like properties and can often be co-located with non-fluorinated surfactants, the effects of PFAAs on cellular flocculation and subsequent EPS production had not previously been evaluated.

Increased EPS production in situ may have a variety of impacts, including biofouling or clogging, which may result in the ensuing formation of preferential subsurface pathways, accelerating the transport of both co-contaminants and PFAAs. Alternatively, decreases in permeability due to biofilm formation may result in retarded transport of PFAAs or co-contaminants, especially considering the preferential PFAA sorption to organic carbon. Regarding the sorptive properties of PFAAs, this enhanced biofilm formation may increase cellular exposure to aqueous PFAAs or co-contaminants, or it may decrease PFAA contact with soil particles.

Continued research assessing additional microorganisms of interest as well as increasingly environmentally relevant assemblages can be conducted to verify in situ impacts of PFAAs. Collectively, these results suggest that the effects of PFAA exposure may be of less concern for co-contaminant biodegradation than for other processes in environmental systems. In particular, enhanced biofilm formation and increased microbial stress responses could impact physical subsurface transport, contaminant sorption, and perhaps even microbial community structure.
CHAPTER 4

SORPTION EFFECTS OF PERFLUOROALKYL ACIDS EXPOSED TO MICROORGANISMS

A paper prepared for submission as a short communication.

Abstract

Perfluoroalkyl acids (PFAAs) often co-occur with hydrocarbons or chlorinated solvents. The bioremediation of these co-contaminants via bioaugmentation or biostimulation is common practice, however, an accurate understanding of PFAA transport within microbiological systems is lacking. This study evaluates single point aqueous sorption coefficients for a suite of perfluoroalkyl acids including perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA), onto microbial biomass. Organic carbon normalized sorption coefficients (logK_{oc}) derived by exposing PFAAs to inactive cellular material result in an increase in sorption by over an order of magnitude compared to soil organic carbon sorption coefficients found in literature. For example, sorption coefficients for PFOS are 4.05±0.07 L/kg for cellular organic carbon and 2.80±0.08 L/kg for soil organic carbon. Cellular sorption coefficients follow the same trends for those observed in soils: increasing sorption with increasing carbon chain length for 8 carbons and up, and increasing sorption with decreasing carbon chain length for 4-7 carbons. Assessment of microcosms containing soils amended with inactive cells also depicted changes in organic carbon normalized distribution coefficients as a function of biomass and analyte. The results of this study demonstrate that normalization to soil organic carbon may not accurately predict sorption of all PFAA compounds in environmental settings with low organic carbon soils, and the degree of preferential PFAA sorption to cells is dependent on carbon chain length. This information
may have significant effects on subsurface fate and transport assessments of perfluoroalkyl acids particularly in biostimulated environments.

4.1 Introduction

Poly- and perfluoroalkyl substances are considered contaminants of emerging concern. Often utilized in aqueous film-forming foams (AFFF), poly- and perfluoroalkyl substances have been detected throughout the environment in soil, surface water, groundwater, and wastewater treatment effluent. They have been detected in humans and wildlife, and tend to bioaccumulate in plants and animals. Perfluoroalkyl acids (PFAAs), a subset of poly- and perfluoroalkyl substances, are components of AFFF along with PFAA precursors. The US Environmental Protection Agency has established provisional health advisories for the two most manufactured perfluoroalkyl substances in 2009, perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA). Current industry trends are now shifting towards the increased manufacture and usage of short-chain PFAAs. The use of AFFF for fire training activities has resulted in the co-location of PFAAs and other common groundwater contaminants: hydrocarbons and chlorinated solvents. The biodegradation of these co-contaminants has been extensively documented, whether through techniques such as natural attenuation or enhanced reductive dechlorination (ERD). ERD generally occurs via biostimulation with the addition of an electron donor such as lactate, or by bioaugmentation, where a known microbial consortium containing Dehalococcoides is added to the subsurface.

Equilibrium partitioning coefficients for a range of PFAAs have previously been quantified for sorption to soils of various properties; these can be used to predict retardation in the subsurface. Traditionally, these values are evaluated in laboratory systems with clean, well classified, microbially inactive soils, yet have shown increased solid-phase
partitioning with increasing quantities of organic carbon.\textsuperscript{105,107} Considering the high quantities of organic carbon in cellular material,\textsuperscript{108} the tendency for PFAA bioaccumulation,\textsuperscript{11,17} coupled with the potential for enhanced extracellular polymeric substance production in the presence of PFAAs,\textsuperscript{30} the failure to account for microbiological material as an organic carbon source within normalized distribution coefficients may lead to a bias in estimating sorption coefficients. Sorption to microbial surfaces, and thus potential transport in stimulated biological settings such as those observed in subsurface bioremediation scenarios, has yet to be addressed.

The objective of this study was to examine sorption behavior of a suite of PFAAs to cellular material. This was accomplished by establishing the distribution coefficients for sorption to Gram-positive bacterial cells, and by evaluating the observed sorption effects in mixed microcosms containing both cellular amendments and soil. Organic carbon normalized sorption coefficients were calculated using two different models. We conclude that the typical method of normalizing distribution coefficients to organic carbon is not sufficient to describe PFAA sorption in systems where cellular matter is present.

4.2 Materials and Methods

4.2.1 PFAA Preparation and Analysis

An eleven-analyte mixture of purity corrected stock solutions (Sigma-Aldrich) was prepared as described previously.\textsuperscript{30,109} The components were suspended in 70/30 v/v methanol aqueous solution and include: perfluorobutanoate (PFBA), perfluoropentanoate (PFPeA), perfluorohexanoate (PFHxA), perfluoroheptanoate (PFHpA), PFOA, perfluorononanoate (PFNA), perfluorodecanoate (PFDA), perfluoroundecanoate (PFUnA), perfluorobutanesulfonate (PFBS), perfluorohexanesulfonate (PFHxS), and PFOS. Each experimental vessel was spiked with the appropriate volume of this mixture and evaporated as described elsewhere.\textsuperscript{30,109} Liquid
chromatography tandem mass spectroscopy (LC-MS/MS) using an Applied Biosystems 3200 mass spectrometer (MDS Sciex) with stable isotope surrogate standards (Wellington Laboratories) and Analyst and MultiQuant for quantitation was utilized according to previously published methods.22

4.2.2 Solids-associated PFAA extraction

The PFAA solid phase extraction procedure has been described elsewhere with a few modifications.22 Two variations of the extraction method were performed depending on the sorption experiment: one for pure cells testing the inactivation procedure, one for the combined cells and soil. Samples containing combined soil and/or cells were separated into aliquots in 50 mL polypropylene conical tubes with 2 ng of PFAA mass-labeled surrogate. For sorption to cells only, the cellular material was transferred to 50 mL polypropylene conical tubes after the addition of 99/1 v/v methanol and ammonium hydroxide through vortexing, mixing, and stirring with a methanol rinsed spatula; this was repeated twice more to the 50 mL tube to ensure adequate transfer of the cellular material, resulting in 30 mL of extraction solvent and cells. After shaking (2 hours) and centrifugation (20 minutes at 2700 rpm), the supernatant was then transferred to a new polypropylene conical tube and placed under a stream of nitrogen (Organomation Associates Inc. N-EVAP 112) until near-dryness. After three rounds of extraction, 7 mL of 99/1 v/v methanol and acetic acid was added to each tube and incubated at room temperature to reconstitute and ensure PFAA removal from the tube surface. For extractions from soil and cells together, ~20 mg of Supelclean ENVI-Carb carbon (Supelco Inc.) was added to each sample, centrifuged, and the supernatant was used for LC-MS/MS analysis. For sorption to cells only, the PFAA mass-labeled surrogate solution was added prior to clean-up (80 uL of 10 000 ng/L surrogate solution) along with 1.4 mL of the reconstituted extract. This
was then sent through Supelclean ENVI-Carb Clean-up Columns (Supelco Inc.) with about 200 mg of ENVI-Carb in each column. An aliquot of 350 uL cleaned solution was transferred to a 2 mL microcentrifuge tube and diluted as necessary to fit within the linear calibration range as described previously.22

4.2.3 Cellular sorption

Bacterial cultures of *Rhodococcus jostii* strain RHA1 were maintained axenically as described elsewhere.30,61 This is a well-characterized soil bacterium recognized for its ability to degrade a wide-range of environmental organic contaminants.66,68 *R. jostii* RHA1 growth occurred at 30°C and 150 rpm. Cellular sorption experiments were performed in 15 mL polypropylene conical tubes spiked with 50 µg/L of each PFAA and a harvested concentrate of strain RHA1 for final protein concentrations of ~860 µg/mL in 13.5 mL of 0.1 M potassium-phosphate buffer. Incubations and abiotic controls were conducted in triplicate. Three different chemical and physical approaches to minimize cellular activity were assessed to minimize the effects of microbial activity and growth on the interpretation of PFAA sorption: incubation at 4°C, pasteurization at 80°C prior to the experiment, and the addition of sodium azide. Chemical deactivation with sodium azide produced the best PFAA recovery and was thus the chosen inactivation method for the following analyses; considering mass loss to the vial as a function of abiotic controls and the reported aqueous masses, total recovery of all PFAAs in the sodium azide system ranged between the acceptable 70% to 130% for every analyte except for PFOS, for which the total mass balance was 62%. The lower total recovery for PFOS likely does not significantly impact the resultant $K_d$ value as the distribution coefficients were determined from directly measured solid phase and solution phase concentrations.22 After inoculation, 0.01% by
weight sodium azide was added to each tube, inverted until visibly mixed, and placed in sideways in the 4°C incubator at 150 rpm.\textsuperscript{71}

After 7 days, the samples were centrifuged at 8 000 x g for 2 minutes, 500 µL of the liquid was sampled for an interim PFAA analysis, and the samples were then rehomogenized with light vortexing and inversion to verify equilibrium conditions. To avoid loss of solution, 500 µL of 0.1M potassium-phosphate buffer (pH 7.0) was added subsequent to this sampling event. A control set of samples were not sampled at this interim point, but were otherwise treated equally to verify that no changes in PFAA sorption occurs as a result of interim sampling. At day 14, all tubes were centrifuged at 12 000 x g for 5 minutes. The supernatant was poured off after sampling for PFAA and protein analyses to verify minimal cellular lysis.

The cellular material was stored at 4°C prior to PFAA extraction. To quantify cellular protein, aliquots of 0.5 or 1 mL were centrifuged at 15 000 x g for 3 minutes, decanted, and stored at -80°C until analysis. Cellular protein was extracted and quantified using the colorimetric Coomassie Plus Assay Reagent (Bradford) with bovine serum albumin (BSA) as the standard (Thermo Scientific) as described previously.\textsuperscript{61}

4.2.4 Sorption with soil and increasing biomass

Five sets of triplicate microcosms were prepared to assess the effect of increasing soil mass on sorption. Axenic RHA1 was grown and pasteurized as described above. A loamy sand with low organic carbon content was chosen for these experiments, soil properties can be viewed in the appendix. Each microcosm with soil contained loamy sand at a ratio of soil mass to water volume (rsw) of 0.1 g/mL. The first set contained only soil and no amended biomass (-Bio). The second set contained soil and was amended with a small quantity of pasteurized RHA1, resulting in a protein content of 0.03 g/L suspended in potassium-phosphate buffer at pH 7 (BioLow). The
third set contained soil and a moderate quantity of pasteurized RHA1 with a protein content of 0.74 g/L in potassium-phosphate buffer (pH 7; BioMid). The fourth set contained soil and a large quantity of cellular material with a protein content of 3.4 g/L in potassium phosphate buffer (pH 7; BioHigh). The fifth set (-Soil) contained this same high protein content of cellular material and no soil. The solid-phase PFAA extraction for this final set was performed in the same manner as those containing soil to eliminate extraction method biases. A control set without cells or soil was also created to quantify losses to the system. Mass balance was within 70-130% for every sample and analyte with the exception of over-recovery for the –Soil case (PFHxS at 145% and PFOS at 178%) and the BioHigh case (PFOS only at 140%). Again, direct estimation of sorption coefficients resulting from LC-MS/MS analysis of both phases should not be significantly impacted by mass recovery.\(^{22}\) Each vial was vortexed, sealed with parafilm, and placed at an angle of 10° in a 4°C incubator at 150 rpm. After six days, 1 mL of mixed slurry was sampled from each microcosm and stored for protein analysis. The remaining sample was spun at 5000 x g for ten minutes. The supernatant was sampled for liquid PFAA analysis; the remainder was archived at -20°C. The solid portion was archived at -80°C for extracellular polymeric substance (EPS) extraction, and 4°C for PFAA solid-phase extraction. EPS extraction was performed using an NaCl method according to previously adapted methods.\(^{30,75}\)

4.2.5 Calculating Sorption Coefficients

The distribution coefficient associated with the solid phase, \(K_d\), was calculated using Equation 4.1.

\[
K_d = \frac{C_s}{C_w}
\]

(EQ 4.1)
where both quantities are determined by LC-MS/MS. \( C_s \) is the concentration associated with the solid phase, and \( C_w \) is the aqueous concentration. The organic carbon normalized sorption coefficient (\( K_{oc} \)) is calculated as in Equation 4.2.

\[
K_{oc} = \frac{K_d}{f_{oc}}
\]

(EQ 4.2)

where \( f_{oc} \) is the mass fraction of organic carbon in the system. Traditionally, this value is based on soil organic carbon approximations only: for the loamy sand used herein the \( f_{oc} \) is equal to 0.0017. This organic carbon fraction can be modified as in Equation 4.3 to reflect the addition of organic carbon in the form of cellular material (\( f_{ocmix} \)).

\[
f_{ocmix} = \frac{M_{occell} + M_{ocs\text{oil}}}{M_{tot}}
\]

(EQ 4.3)

where \( M_{occell} \) is the mass of organic carbon derived from the cellular portion, \( M_{ocs\text{oil}} \) is the mass of organic carbon resulting from soil, and \( M_{tot} \) is the total mass of solids in the system. \( M_{occell} \) is derived by assuming 53% of the protein content as measured from the slurry after incubation is organic carbon.\(^\text{108}\) \( M_{ocs\text{oil}} \) is the organic content of the loamy sand (0.0017) multiplied by the total mass of soil added to the system. \( M_{tot} \) was observed by applying the sample-specific water content to the final measured mass of cells and solid added to the system. This \( f_{ocmix} \) can also be used to normalize \( K_d \) values according to Equation 4.4:

\[
K_{ocmix} = \frac{K_d}{f_{ocmix}}
\]

(EQ 4.4)

where \( K_{ocmix} \) is the sorption coefficient normalized to an organic carbon fraction containing both cells and soil.
4.3 Results and Discussion

4.3.1 Sorption of PFAAs to bacteria

To assess the transport potential of PFAAs in active subsurface biostimulation or bioaugmentation scenarios the magnitude and contribution of PFAA sorption to cellular surfaces should be established. Single point distribution coefficients ($K_d$ values) were calculated by exposing chemically deactivated cellular material to PFAAs and measuring both aqueous and sorbed PFAA fractions. Organic carbon normalized distribution coefficients, normalized by organic carbon in protein and by organic carbon in volatile suspended solids for each analyte follow consistent patterns regarding chain length (Table 4.1).

<table>
<thead>
<tr>
<th></th>
<th>Cellular Log$K_{oc}$ (Protein, L/kg)</th>
<th>Cellular Log$K_{oc}$ (Dry Wts, L/kg)</th>
<th>Soil Log$K_{oc}$ (L/kg)$^a$</th>
<th>Soil Log$K_{oc}$ (L/kg)$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>PFBA</td>
<td>2.82 ± 0.00</td>
<td>2.41 ± 0.00</td>
<td>--</td>
<td>1.88 ± 0.11</td>
</tr>
<tr>
<td>PFPeA</td>
<td>2.70 ± 0.04</td>
<td>2.29 ± 0.04</td>
<td>--</td>
<td>1.37 ± 0.46</td>
</tr>
<tr>
<td>PFHxA</td>
<td>2.68 ± 0.02</td>
<td>2.26 ± 0.02</td>
<td>--</td>
<td>1.31 ± 0.29</td>
</tr>
<tr>
<td>PFHpA</td>
<td>3.04 ± 0.02</td>
<td>2.62 ± 0.02</td>
<td>--</td>
<td>1.63 ± 0.15</td>
</tr>
<tr>
<td>PFOA</td>
<td>3.50 ± 0.04</td>
<td>3.09 ± 0.04</td>
<td>2.06</td>
<td>1.89 ± 0.02</td>
</tr>
<tr>
<td>PFNA</td>
<td>3.85 ± 0.06</td>
<td>3.43 ± 0.06</td>
<td>2.39 ± 0.09</td>
<td>2.36 ± 0.04</td>
</tr>
<tr>
<td>PFDA</td>
<td>4.20 ± 0.20</td>
<td>3.78 ± 0.20</td>
<td>2.76 ± 0.11</td>
<td>2.96 ± 0.15</td>
</tr>
<tr>
<td>PFUnA</td>
<td>4.34 ± 0.22</td>
<td>3.93 ± 0.22</td>
<td>3.30 ± 0.11</td>
<td>3.56</td>
</tr>
<tr>
<td>PFBS</td>
<td>2.93 ± 0.02</td>
<td>2.52 ± 0.02</td>
<td>--</td>
<td>1.79 ± 0.10</td>
</tr>
<tr>
<td>PFHxS</td>
<td>3.42 ± 0.01</td>
<td>3.00 ± 0.01</td>
<td>--</td>
<td>2.05 ± 0.08</td>
</tr>
<tr>
<td>PFOS</td>
<td>4.05 ± 0.07</td>
<td>3.63 ± 0.07</td>
<td>2.57 ± 0.13</td>
<td>2.80 ± 0.08</td>
</tr>
</tbody>
</table>

$^a$ Higgins^{105}$; $^b$ Guelfo^{22}$

Previously, it was hypothesized that distribution coefficients would increase with increasing chain length. For example, Higgins and Lathy^{13} described an increase of 0.5 to 0.6 log
units per CF$_2$ moiety. Due to increases in detection abilities, more information can now be obtained for the shorter carbon chained sulfonates and acids. For these smaller chains, $\log K_{oc}$ values actually decreased with increasing chain length for the shorter PFBA, PFPeA, and PFHxA. This trend has also been previously observed$^{22,110}$ the positively correlated trend begins with PFHxA through PFUnA. For cellular sorption, there is an average increase of 0.33±0.12 log units per CF$_2$ moiety for the acids PFHxA through PFUnA (Figure 4.1). The sulfonates also increase with increasing chain length at an average of 0.56±0.10 per CF$_2$ moiety. Although the trends correlate well with previously derived $K_{oc}$ values, the increase in sorption as a function of cellular presence is not well captured.

4.3.2 Sorption of PFAAs with cells and soil.

To verify whether the observed increase in sorption is quantifiable in the presence of soil, and to establish a more appropriate normalization technique, microcosms including a loamy sand and cellular amendments were evaluated. A range of organic carbon derived from biomass was targeted: for the BioLow systems, the organic carbon fraction averaged 51 ± 1.0 μg C per gram dry weight, the BioMid systems contained 600 ± 12 μg C per gram dry weight, and the BioHigh samples contained 5340 ± 200 μg C per gram dry soil. Delta $\log K_{OC}$ values derived from single-point sorption coefficients (Figure 4.1) demonstrate that there is an increase in sorption in cases with moderate to high cellular biomass, represented with the BioMid and BioHigh samples. This quantity is calculated by subtracting the $\log K_{OC}$ values for the –Bio control from the $\log K_{OC}$ values of each sample containing cells. Biomass organic carbon quantities between 150-500 μg per gram of dry soil have been observed in biochar amended soils,$^{33}$ while cellular organic carbon fractions up to 670 μg C per gram of dry soil have been observed in grassland and woodland soils.$^{34}$ Furthermore, biomass has been observed at over 1000 μg C per gram of dry
sampled from woodland and agricultural soils. Therefore, any effects observed herein for the BioMid system may be observed upon in situ analyses.

Figure 4.1. The changes in organic carbon normalized sorption coefficients ($\Delta \log K_{OC}$) are dependent on carbon chain length and on biomass presence. Normalization to organic carbon in the soil only, A, depicts increasing sorption corresponding to increasing biomass content, particularly for longer chain PFAAs: BioHigh samples (x) sorb more strongly than –Soil samples ( ), which sorb more strongly than BioMid samples (○) for longer chain sulfonates and acids. All sorb more strongly than both BioLow samples (□). Delta values are calculated by $\log K_{OC}$(with cells) – $\log K_{OC}$(-Bio). Normalization to a mixture of biomass and soil organic carbon (B) reduces the $\Delta \log K_{OC}$ values.
Organic carbon normalized distribution coefficients, $K_{oc}$, for each amendment and PFAA analyte can be seen in Figure 4.1A. The $f_{oc}$ from soil only represents a typical approximation that does not account for cellular mass. An adaptation employing $f_{ocmix}$ from Equation 4.3 was used to account for both the organic carbon component of the soil along with the organic carbon fraction of the cells (Figure 4.1B). Typically, agreement within 0.3 log units for a particular compound is considered acceptable;\textsuperscript{111} this agreement was observed for comparison between BioLow samples and those devoid of bacterial additions regardless of $f_{oc}$ normalization technique. However, the sorption coefficients in BioMid and BioHigh amended samples for select analytes are outside of this acceptable range for both organic carbon fraction calculations. When using the organic carbon of the soil only ($f_{oc}$), BioMid samples have $K_{oc}$ values above those of –Bio for PFDA, PFUdA, and PFOS. $K_{oc}$ values are larger for BioHigh samples with PFAAs longer than PFHxA, along with PFHxS and PFOS. This indicates that if traditional $K_{oc}$ values derived from soil are applied to systems with average to high biomass, sorption and subsequent retardation will be under-predicted for longer chain-length PFAAs.

Use of $f_{ocmix}$ to account for both biological and soil organic carbon provides better agreement for longer chain PFAAs, but tends to over-predict sorption for shorter PFAAs. For the environmentally relevant BioMid samples, $K_{ocmix}$ values as calculated from equation 4 are still too high for PFUdA and PFOS. For BioHigh samples, the $K_{ocmix}$ values for the shorter-chain acids PFBA, PFPeA, PFHxA, and PFBS are less than those for the –Bio system. Although normalizing to $f_{ocmix}$ provides a better picture of sorption in the presence of biomass than normalization to pure soil carbon, use of this constant to understand in situ behavior may not be representative of environmental conditions. Of particular concern is the resulting over-prediction of short-chain sorption in scenarios representative of average biomass content.
Collectively, we draw three main conclusions from this study: (1) PFAA sorption to cellular material is stronger than sorption to natural organic matter associated with soil; (2) normalization to the organic carbon fraction of the soil may be inappropriate \textit{in situ} due to the presence of microbial growth, particularly in areas were bioremediation of co-contaminants may be stimulated. Instead, an organic carbon fraction approximating the contribution from biomass should be implemented; and (3) increased sorption to cells versus soil is dependent on carbon chain-length, in particular, longer chain acids tend to exhibit increased preference for cellular sorption than do the shorter chain analytes. Considering the increased awareness and quantification of PFAAs in the environment, understanding of PFAA fate and transport \textit{in situ} is a burgeoning field of study. Inappropriate application of abiotic, laboratory-derived equilibrium sorption coefficients may result in insufficient predictions of retardation; depending on the type of organic carbon normalization, retardation may either be under- or over-predicted as a function of carbon chain-length. The increase in cellular sorption may also be augmented due to the potential for enhanced biofilm formation in the presence of source-zone quantities of PFAAs.\textsuperscript{22} This biofilm or EPS production may change the sorptive properties of the soil by providing additional cellular sorptive sites, such as protein, by coating cellular surfaces and inhibiting contact with soil particles, or by decreasing permeability in subsurface settings. In this study, no significant EPS production was observed (Figure S.3.1) or expected due to chemical inactivation prior to the addition of PFAAs. Addressing sorption between a soil microbe and PFAAs in batch systems provides significant environmental insight, though behavior in natural systems is notably more complicated. Consideration of sorption characteristics within an active mixed culture is a logical progression, as is a more mechanistic understanding behind the preferential sorption. For example, pulsed-field gradient nuclear magnetic resonance spectroscopy may be used to
understand sorption to organic matter at a molecular level. If the mechanics of sorption are well understood, a more appropriate sorption normalization technique can be developed that not only reflects differences between PFAA analytes, but can also account for the presence of ubiquitous biological material.
CHAPTER 5
FUTURE WORK: EVALUATING TRANSPORT AND ECOLOGY IN FLOW-THROUGH SYSTEMS

This chapter represents a summary of experimental data representing both ecological shifts in the presence of PFAAs along with PFAA transport in biostimulated flow-through environments. The laboratory column experiments and analysis described herein are being explored and synthesized to determine their promise for future work and dissemination.

5.1 Introduction

The fate and transport of perfluoroalkyl acids (PFAAs) in the presence of active microbial communities has not been widely investigated. This study explores the transport of a suite of perfluorocarboxylic acids and perfluoroalkylsulfonates, including perfluorooctanoic acid (PFOA) and perfluorooctane sulfonate (PFOS), in microbially active settings. It has been demonstrated that organic carbon normalized sorption coefficients derived by exposing inactive cellular material to PFAAs result in more than an order of magnitude increase in sorption compared to soil organic carbon sorption coefficients found in literature (e.g. sorption coefficients for PFOS are 4.05±0.07 L/kg for cellular organic carbon and 2.80±0.08 L/kg for soil organic carbon). This increase in sorption, coupled with enhanced extracellular polymeric substance produced as a biological response to PFAA exposure at source-level concentrations may result in PFAA retardation in situ and/or changes in subsurface flow parameters.

To address the upscaling of this phenomenon, fourteen flow-through columns packed with low-organic carbon sediment were biostimulated with 10 mg/L glucose and exposed to PFAA concentrations of 11, 55, or 110 mg/L total of 11 distinct analytes for approximately 200 pore volumes at 6 cm³/hr. Each concentration of PFAA was assigned to columns in triplicate,
including a control devoid of PFAA amendment. Two columns inactivated with 1 g/L sodium azide\textsuperscript{22} served as further controls for microbial growth. Breakthrough and tailing of each analyte was measured and subsequently modeled with Hydrus-1D\textsuperscript{113} to explore sorption coefficients over time for microbially-active columns and to establish the effects of biostimulation on PFAA transport. Phylogenetic 16S rRNA gene sequencing was also performed at two points during the experiment in order to query whether or not long-term exposure of PFAAs influenced microbial community structure.

### 5.2 Experimental Design

Fourteen 2.8cm x 8cm (diameter x length) polypropylene and glass columns (Kimble-Chase) were wet-packed and tamped with low organic carbon loamy-sand (soil properties discussed in Table S.3.1) in autoclaved artificial groundwater (AGW).\textsuperscript{22} For the duration of the experiment (85 days), flow was maintained at approximately 6 mL/hr. Columns were initially flushed for at least 3 pore volumes before introduction of a bromide tracer to establish baseline measurements of pore velocity. Figure 5.1 depicts the five different experimental systems: A) biostimulated triplicate columns without PFAAs, termed “-PFAA”; B) duplicate inactivated

![Figure 5.1](image)

Figure 5.1. Columns were divided into 5 sets: A. one biostimulated set did not have PFAAs; B. one set without biology that was inactivated with sodium azide and was flushed with 110 mg/L total PFAAs; C. one biostimulated set with 110 mg/L total PFAAs; D. a biostimulated set with 55 mg/L total PFAAs; and E. a biostimulated set with 11 mg/L total PFAAs.
columns with sodium azide as described elsewhere\textsuperscript{22} and 110 mg/L total PFAAs (10 mg/L of 11 different PFAA analytes) termed “-Bio”; C) biostimulated columns in triplicate with 110 mg/L total PFAAs, termed “110”; D) biostimulated triplicate columns with 55 mg/L total PFAAs (5 mg/L each of 11 analytes), titled “55”; E) triplicate biostimulated columns with 11 mg/L total PFAAs (1 mg/L each of 11 analytes), defined as “11”. The upper limit of PFAA concentrations were chosen based on source-zone concentrations observed \textit{in situ}.\textsuperscript{7} This is also where microbial effects on EPS formation\textsuperscript{30} (see also Chapter 3) and community succession within laboratory batch systems\textsuperscript{109} (Chapter 2) have been documented. The lower level, 11 mg/L total, is the highest concentration at which EPS production was not consistently observed as discussed in Chapter 3.\textsuperscript{30}

Upon completion of the initial 90 g/L bromide tracer, biostimulated columns were amended with roughly 10 pore volumes of local, turf-grass derived Luria-Bertani microbial enrichments resuspended in 1:4 v:v Luria-Bertani broth to AGW. After this inoculation period, AGW was amended with 10 mg/L glucose to continue biostimulation. Glucose amendments were calculated stoichiometrically such that if all glucose was utilized, residual oxygen would remain in the system, ideally maintaining an aerobic community. PFAAs at the desired concentration were also added to the AGW after evaporation.\textsuperscript{29,30,109} Aqueous PFAA samples were sampled from each column upon introduction of PFAAs to capture the contaminant front. After approximately 80 pore volumes (~27 days), another bromide tracer test was implemented to represent changes in advective transport that could result from microbial colonization or other experimental variables. To accompany the non-reactive breakthrough, falling-head permeability was also measured around this time. After tailing of the bromide tracers (~37 days), one column from each of the 110, 55, 11, and –PFAA sets was removed for solid-phase PFAA extraction,\textsuperscript{22}
protein, EPS, and ATP extraction and quantification\textsuperscript{,109} and next-generation sequencing\textsuperscript{,29,109}. This dataset represents the microbial characteristics that may occur upon uninterrupted PFAA exposure in the subsurface. At this time, the PFAAs were no longer added to the influent.

Aqueous PFAA samples archived at this time describe the tailing behavior of the PFAA pulse. Flushing with AGW continued for approximately 90 pore volumes (around day 70), until a third and final bromide tracer test was conducted. Each column was then removed and archived as described above.

### 5.3 Preliminary Results

Samples for aqueous and solid-associated PFAAs have been archived and quantified with LC-MS/MS according to previously published methods\textsuperscript{22}. ATP was also quantified immediately after extraction\textsuperscript{114}. Samples for next-generation sequencing were prepped and sequenced as performed in Chapter 2. Post-processing has not yet been completed. Protein and EPS samples have also been archived, but have not been evaluated.

#### 5.3.1 Microbiological activity

Respiration as surrogate for microbial activity in the inlet, middle, and effluent end of the columns was approximated by ATP concentration immediately after the columns were taken offline. Figure 5.2 shows that the activity in biostimulated columns was greater than the inactivated columns, and that activity is greatest at the inlet. This is expected considering the increased availability of nutrients upon entrance to the column. This data was developed from duplicate measurements for each extraction, with two samples extracted for ATP per column location. Unfortunately, ATP was only quantified for one –Bio column upon contamination of the alternate –Bio column. There are no significant activity differences as a function of PFAA
exposure, with the exception of the middle portion of the 11 column where activity appears to be stimulated.

![Graph showing ATP levels at different positions](image)

Figure 5.2. Microbial activity as measured by ATP is greater for the biostimulated columns than for the inactivated system (□). Activity is greatest at the inlet for all biostimulated conditions and least at the outlet. There is no significant difference in activity between 110 mg/L total PFAAs (○), 55 mg/L total PFAAs (●), 11 mg/L total PFAAs (▲), and for the system without PFAAs (▲).

### 5.3.2 Permeability

Permeability was quantified via falling-head tests\(^{115}\) at the point of longest PFAA exposure (~37 days). Only permeability from 110, -PFAA, and –Bio has been analyzed. Results are shown in Figure 5.3, and demonstrate that there is a reduction in permeability as a result of stimulated microbiology. Additionally, the reduction in permeability is greatest for the systems amended with 110 mg/L total PFAAs vs. the system without PFAAs (no overlap in average ± standard deviations), which may be a result of biofilm formation.

### 5.3.3 Non-reactive tracer breakthrough

Modeling of bromide tracers using Hydrus-1D were utilized to verify the calculated porosity from packing and to estimate the change in modeled longitudinal dispersivity ($\alpha_L$).
Dispersivity, as fit by Hydrus-1D assuming constant porosity, can be used to reflect spreading as a potential result from microbial colonization of the pore spaces. Figure 5.4 shows an increase in this fitted parameter as a function of time for biostimulated systems regardless of PFAA exposure. Average modeled dispersivities for each column are represented in Figure 5.4; error bars represent the standard deviation. Only one breakthrough for the first non-reactive –Bio tracer has been analyzed.

\[ \text{Figure 5.3. Permeability decreases upon microbial stimulation (110, -PFAA) compared to –Bio, and is further reduced in the system with 110 mg/L total PFAAs.} \]

### 5.3.4 PFAA breakthrough

All applicable PFAA influent, effluent, and solid-associated samples have been analyzed by LC-MS/MS. The front of the PFOA and PFHxS breakthroughs have been modeled by Hydrus-1D for the 110 mg/L and -Bio columns only. The model input includes PFAA data from each column exposed under that particular condition. The modeled fits are shown in Figure 5.5.
For these particular analytes, it is evident that the best fit for the case with PFAAs includes a larger dispersivity. A distribution coefficient, \( K_d \), is also modeled by Hydrus-1D.

From \( K_d \), the retardation factor, \( R \), can be calculated using equation 5.1:

\[
R = 1 + \frac{\rho_b}{n} K_d
\]

(EQ 5.1)

where \( \rho_b \) is the bulk density of the soil (initially estimated at 1.85 g/cm\(^3\)), and \( n \) is the porosity as fit by the initial non-reactive breakthrough. A retardation factor of 1 implies no retardation at all; logically, increases in sorption will increase the retardation factor. Table 5.1 summarizes the porosity, distribution coefficient, retardation factor, and dispersivity for PFOA and PFHxS in the –Bio and 110 mg/L columns. Modeled fits agree with the findings from the non-reactive tracers, namely that the fit parameter of longitudinal dispersivity is greater in after exposure to biostimulation and 110 mg/L PFAAs than for the case without active biomass for both PFOA and PFHxS. The retardation factor is also greater in the presence of active biomass.
An assessment of the preliminary results shows the potential for novel findings for in situ applications of PFAA transport. Biomass appears to be affecting PFAA transport by increasing spreading behavior and enhancing retardation, at least for PFOA and PFHxS at concentrations relevant to PFAA source-zones.

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<th>PFHxS</th>
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<tr>
<td></td>
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<td>K_d</td>
</tr>
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<td>-Bio</td>
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<td>0.5E-3</td>
</tr>
<tr>
<td>110 mg/L</td>
<td>0.3</td>
<td>0.056</td>
</tr>
</tbody>
</table>

An assessment of the preliminary results shows the potential for novel findings for in situ applications of PFAA transport. Biomass appears to be affecting PFAA transport by increasing spreading behavior and enhancing retardation, at least for PFOA and PFHxS at concentrations relevant to PFAA source-zones.

**Figure 5.5.** Fitted breakthrough (solid line) of PFOA for –Bio systems (A) reflects a steeper front, and thus less dispersivity than for PFOA in the 110 mg/L system (B). Similar trends are observed for PFHxS in the –Bio system (C) and 110 mg/L (D).

**5.4 Continuing Efforts**

There are additional analyses required to complete this study. In particular, a significant modeling effort must be initiated to model dispersivity, retardation coefficients, and desorption...
parameters for each analyte within each column. Breakthrough and tailing data for each system and each analyte is yet to be completed. To support this, non-reactive tracer data for the remaining systems also needs to be modeled. In order to affirm the hypothesis that there is increased biofilm formation at elevated levels of PFAA exposure, EPS and protein will be extracted from the inlet, middle, and outlet of each column. To address effects of PFAAs on community ecology, post-processing from next-generation sequencing results must also be analyzed. The completion of this data set should result in a novel assessment of the impacts of PFAA presence on ecological properties and general microbiological attributes that could influence hydrologic properties as well as PFAA transport in flow-through systems.
CHAPTER 6
CONCLUDING REMARKS

This work represents the growing understanding of interactions between perfluoroalkyl acids and microbiology. Laboratory-based exploration of environmental problems provides a well-defined avenue to predict and unravel mechanistic explanations of true in situ behavior. By increasing complexity in laboratory systems, namely by including microorganisms and assessing microbiological impacts, we are one step closer to comprehending the nature of these emerging contaminants in the subsurface. The assessment of perfluoroalkyl acid impacts on bioremediation is of critical importance considering the frequent co-location of PFAAs and other contaminants that are known to undergo bioremediation such as chlorinated solvents and hydrocarbons.

Although the potential for inhibited biodegradation of chlorinated solvents near PFAA source zones is alarming, continued bioremediation of hydrocarbons, specifically BTEX compounds, is likely to continue. This underscores the diverse impacts PFAAs may have on differing microbial communities. Similarly, the impacts of PFAA presence on the vitality, and thus putative functionality, are also diverse. The inhibition of Dehalococcoides growth can be directly linked to the lack of observed reductive dechlorination, however other microorganisms such as Archaea or members of δ-proteobacteria are enhanced or not significantly impacted by PFAA presence.

Perfluoroalkyl acids also have the potential to affect microbes at a cellular level: stress responses in R. jostii RHA1 accompanied with increased EPS production, are observed at PFAA concentrations relevant to source zones.

Increasing EPS production may have several implications for PFAA transport. It is shown in Chapter 4 that PFAAs sorb more strongly to cellular organic carbon, potentially resulting in retardation of PFAAs in situ. The increased production of EPS observed in batch
systems may also contribute cellular-derived organic carbon, or may even inhibit access of PFAAs to soil. While sorption may increase in these systems through these interactions, advective transport may also be affected. By increasing biofilm formation, the pore structure of the soil matrix may change. Constriction of pores reduces permeability, and may result in either complete clogging of the advective channels, or increases in Darcy velocity. Ideally, this relationship will be better understood upon completion of the future work discussed in Chapter 5.

While the bench-scale studies incorporating perfluoroalkyl acids and microbiology are critical, it is always pertinent to understand the big picture. To do so, one must understand the limitations of a laboratory environment: the assumptions that are employed and the deviations between laboratory and environmental forcings must all be understood to accurately apply the new knowledge to an environmental system. Feedback between bench-scale experiments, model parameterization and predictions, and environmental data and observations is required to perpetuate knowledge and comprehension of each individual facet. Each type of research can build upon and support the others. Ideally, the studies herein can help inform modeling studies and \textit{in situ} remediation, and can provide an understanding of changes in subsurface ecology, including the potential ramifications of these shifts on desired subsurface microbial processes. Results from modeled predictions and environmental responses can further inform the direction and interpretation of these subsequent laboratory pursuits.
REFERENCES CITED


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APPENDIX

The appendix is grouped by chapter, with the first section, S.1, relating to Chapter 2. Section S.2 is the supplemental information for Chapter 3, and section S.3 corresponds to Chapter 4. Copyright permissions for Chapter 3 are included at the end of the appendix.

S.1 Supporting information for Chapter 2

The supporting information for Chapter 2 contains the sequencing primer specifications and amplification program, the sequencing post-processing scripts, a summary table of the observed taxa, relative abundances, and a table of the fold changes and statistical significance.

S.1.1 Dual-Indexed Primer Specifications

501-508 PCR primer design – forward primer:

<illumina adapter><i5 index>< primer pad><linker><v4 16Sf>

Illumina adapter: AATGATACGGCGACCACCGAGATCTACAC

i5 index: NNNNNNNN

Primer pad: TATGGTAATT

Linker: GT

V4 16Sf: GTGCCAGCMGCCGCGGTAA

701-712 PCR primer design – reverse primer:

<illumina adapter><i7 index>< primer pad><linker><v4 16Sr>

Illumina adapter: CAAGCAGAAGACGGCATACGAGAT

i7 index: NNNNNNNN

Primer pad: AGTCAGTCAG

Linker: CC

V4 16Sr: GGACTACHVGGGTWTCTAAT
S.1.2 Amplification Program

The amplification program began with initial denaturation for two minutes at 95°C, followed by the amplification cycles that included 95°C for 20 seconds, 55°C for 15 seconds, and 72°C for 5 minutes. The final extension occurred at 72°C for 10 minutes and ended with a final hold at 4°C.

S.1.3 Sequencing Post-Processing Scripts

#Join paired ends
multiple_split_libraries_fastq.py -i /joined_renamed_tw
   -o /split_out_tw --demultiplexing_method sampleid_by_file --include_input_dir_path --remove_filepath_in_name --read_indicator .join.

#Pick open reference OTUs
pick_open_reference_otus.py -i seqs.fna
   -r /macqiime/greengenes/gg_13_8_otus/rep_set/97_otus.fasta
   -o /open_ref_otus
   -p /pick_open_ref_otus_parameters.txt -m usearch61

#OTU table summary
biom summarize-table
   -i /open_ref_otus/otu_table_mc2_w_tax.biom
   -o /open_ref_otus/otu_table_summary

#Divide samples for different projects and summarize table
filter_samples_from_otu_table.py
   -i /open_ref_otus/otu_table_mc2_w_tax.biom
   -o /open_ref_otu/otu_table_tw_erd.biom
   --sample_id_fp /erd_sample_ids.txt

biom summarize-table
   -i /otu_table_tw_erd.biom
   -o /otu_table_summary_tw_erd

#Rarify OUT table to lowest count
single_rarefaction.py
   -i /otu_table_tw_erd.biom
   -o /otu_table_tw_erd_21761.biom
   -d 21761

#Summarize rarefied and non-rarefied OTU tables
summarize_taxa_through_plots.py
   -i /otu_table_tw_erd_21761.biom
   -o /wf_taxa_summary_tw_erd_21761
   -m /150107_illumina_mapping_tsw.txt
summarize_taxa_through_plots.py
   -i /otu_table_tw_erd.biom
   -o /wf_taxa_summary_tw_erd_norare
   -m /150107_illumina_mapping_tsw.txt

#Make .tre file
make_phylogeny.py -i /rep_set_aligned_pfiltered.fasta
   -o /rep_st_tw.tre

#Set alpha diversity parameters
echo "alpha_diversity:metrics shannon,PD_whole_tree,chao1,observed_species" > alpha_params.txt

#Calculate alpha diversity on rarefied and non-rarefied
alpha_rarefaction.py
   -i /otu_table_tw_erd_21761.biom
   -m /150107_illumina_mapping_tsw_erd.txt
   -o /wf_arare_tw_erd/
   -p alpha_params.txt
   -t /rep_st_tw.tre

alpha_rarefaction.py
   -i /otu_table_tw_erd.biom
   -m /150107_illumina_mapping_tsw_erd.txt
   -o /wf_arare_tw_erd_norare/
   -p alpha_params.txt
   -t /rep_st_tw.tre

#Calculate beta diversity on rarefied and non-rarefied
beta_diversity_through_plots.py
   -i /otu_table_tw_erd_21761.biom
   -m /150107_illumina_mapping_tsw_erd.txt
   -o /wf_bdiv_tw_erd_21761
   -t /rep_st_tw.tre

beta_diversity_through_plots.py
   -i /otu_table_tw_erd.biom
   -m /150107_illumina_mapping_tsw_erd.txt
   -o /wf_bdiv_tw_erd_norare
   -t /rep_st_tw.tre

#Filter samples for comparison
filter_samples_from_otu_table.py
   -i /otu_table_mc2_w_tax.biom
   -o /otu_table_tw_erd_AllMix.biom
   --sample_id_fp /erd_sample_ids_AllMix.txt

   # This is to further separate files (e.g. AllMix = all PFAAs and 0, PFSA = sulfonates and 0, 22ppm means only
   # samples at 22 (sulfonates and allmix)
   filter_samples_from_otu_table.py
   -i /otu_table_mc2_w_tax.biom
   -o /otu_table_tw_erd_PFSA.biom
   --sample_id_fp /erd_sample_ids_PFSA.txt
filter_samples_from_otu_table.py
-i /otu_table_mc2_w_tax.biom
-o /otu_table_tw_erd_22ppm.biom
--sample_id_fp /erd_sample_ids_22ppm.txt

filter_samples_from_otu_table.py
-i /otu_table_mc2_w_tax.biom
-o /otu_table_tw_erd_66ppm.biom
--sample_id_fp /erd_sample_ids_66ppm.txt

filter_samples_from_otu_table.py
-i /otu_table_mc2_w_tax.biom
-o /otu_table_tw_erd_110ppm.biom
--sample_id_fp /erd_sample_ids_110ppm.txt

# Filter samples to >0.001 abundance, repeated for each OTU table
filter_otus_from_otu_table.py
-i /otu_table_tw_erd_All_no_10.biom
-o /otu_table_tw_erd_All_no_10_minfrac_001.biom
--min_count_fraction 0.001

# Summarize by taxa and convert to integers, repeated for each OTU table
summarize_taxa.py
-i /otu_table_tw_erd_All_no_10_minfrac_001.biom
-o otu_table_All_no_10_taxa_abs_minfrac_001.biom
-a

# Convert to json file format, repeated for each OTU table
biom convert -i /otu_table_tw_erd_All_no_10_minfrac_001_L6.biom
-o /otu_table_tw_erd_All_no_10_minfrac_001_L6_minfrac_001_L6.biom
--table-type="OTU table"
--to-json

# Differential abundance using DeSeq, repeated for each comparison
differential_abundance.py
-i /otu_table_tw_erd_All_no_10_minfrac_001_L6_minfrac_001_DESeq.txt
-m /150107_illumina_mapping_tsw_erd.txt -c PFAA -x 10ppm -y NoPFAA
-a DESeq2_nbinom –d

# Make new .tre file to use with FIGtree
make_phylogeny.py
-i /rep_set_aligned_pfiltered.fasta
-o /rep_st_tw_fig.tre

# Prune tree file
filter_tree.py
-i /rep_st_tw_fig.tre
-o erd_minfrac001_pruned.tre
-t /erd_minfrac001_tips_to_keep.txt

# Create distance matrices (then manually change their names)
beta_diversity.py -i /otu_table_tw_erd_AllMix_minfrac_001.biom
    -m weighted_unifrac -o /beta_div/ -t /rep_st_tw.tre

# Adonis statistical test
compare_categories.py --method adonis
    -i /weighted_unifrac_otu_table_tw_erd_AllMix_minfrac_001.txt
    -m 150107_illumina_mapping_tsw_erd_AllMix.txt -c PFAA -o /adonis/ -n 999

# ANOSIM statistical test
compare_categories.py --method anosim
    -i /weighted_unifrac_otu_table_tw_erd_AllMix_minfrac_001.txt
    -m /150107_illumina_mapping_tsw_erd_AllMix.txt -c PFAA -o /anosim/ -n 999

# Redo adonis and ANOSIM without 110
# Remove 110
filter_samples_from_otu_table.py
    -i /otu_table_tw_erd_AllMix_minfrac_001.biom
    -o /otu_table_erd_AllMix_minfrac_001_no110.biom
    -m /150107_illumina_mapping_tsw_erd_AllMix_stats.txt
    -s 'Location:Dechlor'

# Redo beta div without 110, manually rename file
beta_diversity.py -i /otu_tab_erd_AllMix_minfrac_001_no110.biom
    -m weighted_unifrac -o /beta_div/ -t /rep_st_tw.tre

# Run tests without 110
compare_categories.py --method adonis
    -i /weighted_unifrac_otu_tab_erd_AllMix_minfrac_001_no110.txt
    -m /150107_illumina_mapping_tsw_erd_AllMix_stats_no110.txt -c PFAA
    -o /adonis/ -n 999

compare_categories.py --method anosim
    -i /weighted_unifrac_otu_tab_erd_AllMix_minfrac_001_no110.txt
    -m /150107_illumina_mapping_tsw_erd_AllMix_stats_no110.txt -c PFAA
    -o /anosim/ -n 999
Table S.1.1 Observed taxa

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<th>Class</th>
<th>Order</th>
<th>Family</th>
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<th>Species</th>
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<td>Spirochaetaceae</td>
<td>Treponema</td>
<td>4325657</td>
</tr>
<tr>
<td>Bacteria</td>
<td>Synergistetes</td>
<td>Synergistia</td>
<td>Synergistales</td>
<td>Synergistaceae</td>
<td>vadinCA02</td>
<td>70446</td>
</tr>
<tr>
<td>Unassigned</td>
<td>Other</td>
<td>Other</td>
<td>Other</td>
<td>Other</td>
<td>Other</td>
<td>N/A</td>
</tr>
</tbody>
</table>
Table S.1.2 Relative abundance

<table>
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<tr>
<th>OTU</th>
<th>No PFAAs</th>
<th>22 mg/L</th>
<th>66 mg/L</th>
<th>110 mg/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanobacterium bryantii</td>
<td>0.51 ± 0.29</td>
<td>1.14 ± 0.63</td>
<td>0.81 ± 0.25</td>
<td>3.80 ± 0.92</td>
</tr>
<tr>
<td>Bacteroidales</td>
<td>5.17 ± 4.23</td>
<td>2.81 ± 2.10</td>
<td>0.43 ± 0.14</td>
<td>0.57 ± 0.10</td>
</tr>
<tr>
<td>Porphyromonadaceae</td>
<td>6.64 ± 2.10</td>
<td>7.83 ± 2.61</td>
<td>9.08 ± 2.18</td>
<td>9.58 ± 0.21</td>
</tr>
<tr>
<td>WCHB1-05</td>
<td>0.27 ± 0.02</td>
<td>0.17 ± 0.07</td>
<td>0.22 ± 0.10</td>
<td>0.05 ± 0.03</td>
</tr>
<tr>
<td>Dehalococcoides</td>
<td>4.52 ± 0.24</td>
<td>4.80 ± 2.54</td>
<td>5.74 ± 1.19</td>
<td>0.49 ± 0.17</td>
</tr>
<tr>
<td>Clostridiales</td>
<td>2.95 ± 1.54</td>
<td>8.30 ± 8.31</td>
<td>3.36 ± 3.17</td>
<td>12.92 ± 0.71</td>
</tr>
<tr>
<td>Sedimentibacter</td>
<td>17.0 ± 2.05</td>
<td>15.7 ± 3.04</td>
<td>15.6 ± 0.93</td>
<td>10.1 ± 0.71</td>
</tr>
<tr>
<td>Christensenellaceae</td>
<td>1.75 ± 3.00</td>
<td>2.64 ± 4.53</td>
<td>3.21 ± 3.98</td>
<td>0.01 ± 0.01</td>
</tr>
<tr>
<td>Coprococcus</td>
<td>0.10 ± 0.06</td>
<td>0.01 ± 0.02</td>
<td>0.23 ± 0.10</td>
<td>0.21 ± 0.04</td>
</tr>
<tr>
<td>Ruminococcaceae</td>
<td>0.51 ± 0.15</td>
<td>0.56 ± 0.22</td>
<td>0.79 ± 0.29</td>
<td>0.26 ± 0.08</td>
</tr>
<tr>
<td>Oscillospira</td>
<td>0.21 ± 0.00</td>
<td>0.46 ± 0.28</td>
<td>0.48 ± 0.28</td>
<td>0.62 ± 0.13</td>
</tr>
<tr>
<td>Veillonellaceae</td>
<td>24.3 ± 1.48</td>
<td>20.9 ± 1.15</td>
<td>25.0 ± 4.53</td>
<td>30.7 ± 2.85</td>
</tr>
<tr>
<td>Desulfovibronaceae</td>
<td>20.3 ± 3.63</td>
<td>19.8 ± 2.17</td>
<td>20.3 ± 0.25</td>
<td>16.8 ± 0.32</td>
</tr>
<tr>
<td>Desulfovibrio putealis</td>
<td>0.63 ± 0.22</td>
<td>0.56 ± 0.37</td>
<td>0.70 ± 0.11</td>
<td>0.80 ± 0.18</td>
</tr>
<tr>
<td>Desulfovibronaceae - other</td>
<td>0.40 ± 0.32</td>
<td>0.50 ± 0.56</td>
<td>0.34 ± 0.05</td>
<td>0.20 ± 0.27</td>
</tr>
<tr>
<td>Treponema</td>
<td>13.6 ± 1.42</td>
<td>12.9 ± 1.53</td>
<td>13.2 ± 1.70</td>
<td>12.0 ± 0.96</td>
</tr>
<tr>
<td>vadinCA02</td>
<td>0.91 ± 0.53</td>
<td>0.64 ± 0.81</td>
<td>1.20 ± 0.81</td>
<td>0.83 ± 0.50</td>
</tr>
<tr>
<td>Unassigned</td>
<td>0.23 ± 0.39</td>
<td>0.36 ± 0.62</td>
<td>0.21 ± 0.34</td>
<td>0.00 ± 0.00</td>
</tr>
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</table>
Table S.1.3. Fold changes and statistical significance (highlighted values p<0.05)

<table>
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<tr>
<th>OTU</th>
<th>No PFAAs vs 110 mg/L</th>
<th>No PFAAs vs 22 mg/L</th>
<th>No PFAAs vs 66 mg/L</th>
<th>22 mg/L vs 66 mg/L</th>
<th>22 mg/L vs 110 mg/L</th>
<th>66 mg/L vs 110 mg/L</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>log2 Fold-Change</td>
<td>p (adj)</td>
<td>log2 Fold-Change</td>
<td>p (adj)</td>
<td>log2 Fold-Change</td>
<td>p (adj)</td>
</tr>
<tr>
<td>Methanobacterium bryantii</td>
<td>3.1  0.000</td>
<td>0.8  0.572</td>
<td>0.5  0.574</td>
<td>-0.6  0.896</td>
<td>1.7  0.019</td>
<td>2.4  0.000</td>
</tr>
<tr>
<td>Bacteroidales</td>
<td>-2.8  0.009</td>
<td>-0.2  0.942</td>
<td>-1.9  NA</td>
<td>-2.4  0.022</td>
<td>-2.3  0.026</td>
<td>0.6  0.351</td>
</tr>
<tr>
<td>Porphyromonadaceae</td>
<td>0.7  0.004</td>
<td>0.3  0.812</td>
<td>0.3  0.574</td>
<td>0.1  0.896</td>
<td>0.3  0.291</td>
<td>0.3  0.457</td>
</tr>
<tr>
<td>WCHB1-05</td>
<td>-2.3  0.000</td>
<td>-0.5  0.677</td>
<td>-0.3  0.713</td>
<td>0.2  0.896</td>
<td>0.2  0.896</td>
<td>-1.8  0.046</td>
</tr>
<tr>
<td>Dehalococcoides</td>
<td>-3.1  0.000</td>
<td>0.1  0.942</td>
<td>0.1  0.761</td>
<td>0.1  0.896</td>
<td>0.3  0.896</td>
<td>-3.3  0.000</td>
</tr>
<tr>
<td>Clostridiales</td>
<td>2.2  0.000</td>
<td>0.8  0.572</td>
<td>0.0  0.996</td>
<td>-1.2  0.850</td>
<td>0.5  0.545</td>
<td>2.1  0.001</td>
</tr>
<tr>
<td>Sedimentibacter</td>
<td>-0.6  0.009</td>
<td>-0.1  0.942</td>
<td>-0.3  0.574</td>
<td>-0.1  0.896</td>
<td>-0.6  0.023</td>
<td>-0.4  0.285</td>
</tr>
<tr>
<td>Christensenellaceae</td>
<td>-5.2  NA</td>
<td>0.1  NA</td>
<td>0.1  NA</td>
<td>-0.2  NA</td>
<td>-6.4  NA</td>
<td>-4.3  NA</td>
</tr>
<tr>
<td>Coproccocus</td>
<td>1.3  0.026</td>
<td></td>
<td>0.5  0.703</td>
<td>2.6  0.046</td>
<td>3.6  0.006</td>
<td>0.1  0.872</td>
</tr>
<tr>
<td>Ruminococcaceae</td>
<td>-0.7  0.044</td>
<td>0.3  0.715</td>
<td>0.4  0.574</td>
<td>0.3  0.896</td>
<td>-1.0  0.082</td>
<td>-1.3  0.018</td>
</tr>
<tr>
<td>Oscillospira</td>
<td>1.8  0.000</td>
<td>0.8  0.572</td>
<td>0.8  0.392</td>
<td>-0.1  0.896</td>
<td>0.5  0.538</td>
<td>0.6  0.441</td>
</tr>
<tr>
<td>Veillonellaceae</td>
<td>0.5  0.006</td>
<td>-0.2  0.677</td>
<td>-0.1  0.761</td>
<td>0.1  0.896</td>
<td>0.6  0.006</td>
<td>0.5  0.175</td>
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<td>Desulfovibrioaceae</td>
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<td>0.0  0.942</td>
<td>-0.2  0.703</td>
<td>-0.1  0.896</td>
<td>-0.2  0.082</td>
<td>0.0  0.872</td>
</tr>
<tr>
<td>Desulfovibrio putealis</td>
<td>0.5  0.169</td>
<td>-0.1  0.942</td>
<td>0.0  0.996</td>
<td>0.2  0.896</td>
<td>0.5  0.538</td>
<td>0.4  0.457</td>
</tr>
<tr>
<td>Desulfovibrioaceae - other</td>
<td>-0.7  0.679</td>
<td>0.1  0.942</td>
<td>-0.3  0.761</td>
<td>-0.5  0.896</td>
<td>-1.1  0.538</td>
<td>-0.5  0.700</td>
</tr>
<tr>
<td>Treponema</td>
<td>0.0  0.729</td>
<td>0.0  0.942</td>
<td>-0.2  0.574</td>
<td>-0.1  0.896</td>
<td>-0.1  0.538</td>
<td>0.1  0.849</td>
</tr>
<tr>
<td>vadinCA02</td>
<td>0.0  0.935</td>
<td>-0.2  0.942</td>
<td>0.2  0.846</td>
<td>0.6  0.896</td>
<td>0.4  0.731</td>
<td>-0.3  0.704</td>
</tr>
<tr>
<td>Unassigned</td>
<td>-4.3  NA</td>
<td>0.1  NA</td>
<td>0.0  NA</td>
<td>-0.5  NA</td>
<td>-5.3  NA</td>
<td>NA</td>
</tr>
</tbody>
</table>
S.2 Supporting information for Chapter 3

The supporting information for Chapter 3 contains a discussion and data regarding aqueous loss controls and competition controls. RT-qPCR details and QA/QC are also specified according to the MIQE guidelines.86

S.2.1 Aqueous loss control

This control experiment was performed under the same conditions as the toluene degradation experiments (discussed within the main text, Materials and Methods: Toluene biodegradation) but without cellular material and toluene. Recovery after evaporation actually increased for shorter chain acids, and slightly decreased for most sulfonates. Because the final PFAA concentrations remain quantifiable, we are able to utilize the evaporation step in future experiments (Figure S.2.1). Additionally, though we may get some loss to the air-water interface when using glass bottles, the final aqueous concentration is still quantifiable, even at concentrations lower than those utilized for the 10 mg/L experiments.

S.2.2 PFAA competition control

Competition may be manifested in preferential sorption to cellular material or preferential losses to the air-water interface.105,106 Three sets of toluene degradation control experiments were performed including 0.1M potassium-phosphate buffer (pH ~ 7) and either PFOA, PFOS, or the entire 11 analyte mix at 10mg/L each. Another set included Rhodococcus sp., toluene, and either PFAA, PFOS, or the entire 11 analyte mix at 10 mg/L each. The final set included only Rhodococcus sp. and either PFAA, PFOS, or the entire 11 analyte mix at 10 mg/L each. These experiments were set up as described for the toluene degradation experiment. Aqueous PFOS and PFOA independently yielded similar results to the PFOS and PFOA within the mix, implying competitive effects are not a concern for these systems (Figure S.2.2).
Figure S.2.1. Final PFAA concentrations remain quantifiable, even after an evaporation step (cross hatches, white) for two different concentrations: 0.1 mg/L of each analyte (cross hatches, gray) and 1 mg/L (white, black) in glass bottles sealed with butyl rubber stoppers.

Figure S.2.2. Toluene degradation is not affected by an 11-component PFAA mixture (□), by PFOS alone (○), or by PFOA alone (x) as compared to a control without PFAAs (○). The average degradation rate is 1.02±0.07 mg/L toluene / hr.
S.2.3 RT-qPCR method details and QA/QC

RNA extractions were performed on 1.5 mL aliquots of 5 mL sample resuspended in 2 mL RNAlater for samples grown in minimal media. All samples were tested for inhibition by running dilutions, no inhibition effects were observed. Primers were ordered from Integrated DNA Technologies prepared as Lab Ready with standard desalting. Primer details can be found in Table S.2.1.

Table S.2.1. RT-qPCR primer specifications

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Gene ID</th>
<th>Amplicon Length</th>
<th>Sequence</th>
<th>GC Content (%)</th>
<th>Tm (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>sigF3 119F</td>
<td>ro04728</td>
<td>323</td>
<td>5’TGG GTC ATG TTG CCG AAG AA3’</td>
<td>50</td>
<td>57.0</td>
</tr>
<tr>
<td>sigF3 441R</td>
<td></td>
<td></td>
<td>5’GAA CTG CAC CTC TCC CTC AC3’</td>
<td>60</td>
<td>57.6</td>
</tr>
<tr>
<td>prmA 394F</td>
<td>ro00441</td>
<td>210</td>
<td>5’ACG ATC CAG ATG AAC CTC AAG AA3’</td>
<td>43.5</td>
<td>55.8</td>
</tr>
<tr>
<td>prmA 603R</td>
<td></td>
<td></td>
<td>5’TAC GAA CAG GGT GTT GGT GAA C3’</td>
<td>50</td>
<td>57.2</td>
</tr>
<tr>
<td>gyrB 607F</td>
<td>ro008268</td>
<td>121</td>
<td>5’GTC TAC AGC TTC GAG ACG3’</td>
<td>55.6</td>
<td>54.7</td>
</tr>
<tr>
<td>gyrB 727R</td>
<td></td>
<td></td>
<td>5’TGA CGA CCT CTT CCG TG3’</td>
<td>58.8</td>
<td>55.7</td>
</tr>
</tbody>
</table>

PCR product was analyzed via gel electrophoresis to verify primer specificity in tandem with melt-curve genotyping. All positive samples amplified above the limits of detection. Melt curves were generated with a 5 minute hold at 95°C, followed by one minute at 65°C (2.2°C/s ramp rate), and finally quantification every 5°C during a 1.1°C /s ramp up to 98°C. The program ended with 10 seconds of cooling to 37°C at 2.2 °C /s. Melt curves are shown in Figure S.2.3 and depict uniform peaks at the appropriate temperature for the product length for both samples and standards. Earlier peaks correspond to negative controls. LightCycler® 480 version 1.5.0 (Roche) was used to program thermal cycling and to calculate errors and efficiencies with the Absolute Quantification/Second Derivative Maximum algorithm. Errors, efficiencies, standard
Figure S.2.3. Melt curve analysis for (A) LB grown *sigF3* (B) LB grown *prmA*, (C) sodium pyruvate grown *sigF3*, (D) sodium pyruvate grown *prmA*, and (E) *gyrB* for both types of samples depict uniform melting and amplicon lengths. The early outlier peaks in all cases are negative controls.
curve slopes and y-intercepts are shown in Table S.2.2. DNA was quantified using a NanoDrop Light Spectrophotometer (Thermo Scientific) using the dsDNA protocol. Yield was negligible and purity (A260/A280) was within the limits as recommended within the MoBio Biofilm RNA Extraction protocol for samples diluted with nuclease free water.

Table S.2.2. Errors, efficiencies, slopes, and intercepts for each gene and media.

<table>
<thead>
<tr>
<th>Growth Media</th>
<th>Gene</th>
<th>Error</th>
<th>Efficiency</th>
<th>Slope</th>
<th>Y-Intercept</th>
</tr>
</thead>
<tbody>
<tr>
<td>Both</td>
<td>gyrB</td>
<td>0.014</td>
<td>2.018</td>
<td>-3.279</td>
<td>30.93</td>
</tr>
<tr>
<td>LB</td>
<td>sigF3</td>
<td>0.0104</td>
<td>2.062</td>
<td>-3.182</td>
<td>37.28</td>
</tr>
<tr>
<td></td>
<td>prmA</td>
<td>0.0236</td>
<td>1.917</td>
<td>-3.538</td>
<td>35.95</td>
</tr>
<tr>
<td>Minimal</td>
<td>sigF3</td>
<td>0.0129</td>
<td>2.052</td>
<td>-3.204</td>
<td>29.66</td>
</tr>
<tr>
<td></td>
<td>prmA</td>
<td>0.0149</td>
<td>1.948</td>
<td>-3.453</td>
<td>31.52</td>
</tr>
</tbody>
</table>

Standard curves for *prmA*, *gyrB*, and *sigF3* were developed by bead purifying the PCR product amplified from experimental template using Agencourt AMPure XE (Beckman Coulter). Purity was verified using gel electrophoresis to obtain a single band of the target length. Purified DNA was then quantified and diluted as appropriate for each standard curve, with the exception of the *sigF3* standard curve used for LB grown samples (see “RT-qPCR on LB Grown Samples” in the appendix). Standards and PCR products were stored at -20°C. Testing for DNA contamination was performed by performing controls on each sample in duplicate without the reverse transcription enzyme. For the LB grown samples, each RT control was below detection or amplified with high uncertainty as shown in Figure S.2.4 with the exception of a single well for LB grown No-PFAA *sigF*, sample 7. Because no DNA contamination was observed in the corresponding replicate sample, we assumed the DNA contamination only occurred in that specific well and that the corresponding sample template was not compromised. For the samples grown on minimal media, all RT controls were below detection, measured with high uncertainty, or amplified after the negative controls, with the exception of No-PFAA *sigF* samples 7, 8, and
9. Because every replicate of this negative control amplified above the limit of detection, this entire sample has been removed from the analysis.

**S.2.4 RT-qPCR on LB grown samples**

RNA from 1 mL well-mixed aliquots from the samples grown in LB was extracted using the Qiagen RNEasy RNA Extraction kit. Protocols involving enzymatic lysis, proteinase K digestion, mechanical disruption, and purification of total RNA from bacterial lysate were performed prior to DNase treatment and removal using the Turbo DNA-free Kit (Ambion). RNA was quantified as described in the main text. A set of standard curves for *sigF3* (used only for cells grown on LB) were developed from extracted genomic DNA from RHA1 using the PowerSoil DNA Isolation Kit (MoBio). The target gene was amplified from the genomic DNA using the amplification protocol described herein, followed by gel electrophoresis on the PCR product. The target band was extracted from the gel using E.Z.N.A. Gel Extraction Kit (Omega bio-tek), quantified using Qubit dsDNA HS Assay Kit, and diluted as necessary. Standards for *sigF3* derived through extraction and purification of the gel band were utilized for the LB grown cells instead of the bead-purified set due to the reproducibility at the lower bounds of detection, as can be seen in Figure S.2.4. For measurement of *prmA*, samples were diluted as necessary to fall between a range of 1 – 2 ng/μL RNA.
Table S.2.3. CP cycles for negative RT-qPCR controls.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Replicate</th>
<th>prmA Cq or CP (cycles)</th>
<th>sigF3 Cq or CP (cycles)</th>
<th>gyrB Cq or CP (cycles)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LB No PFAA -RT Control</td>
<td>1</td>
<td>ND</td>
<td>35&lt;sup&gt;a&lt;/sup&gt;</td>
<td>35&lt;sup&gt;a&lt;/sup&gt;</td>
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<td></td>
<td>2</td>
<td>ND</td>
<td>35&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>ND</td>
<td>ND</td>
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<tr>
<td></td>
<td>5</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>ND</td>
<td>22.13&lt;sup&gt;a&lt;/sup&gt;</td>
<td>35&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td></td>
<td>8</td>
<td>ND</td>
<td>23.70&lt;sup&gt;a&lt;/sup&gt;</td>
<td>ND</td>
</tr>
<tr>
<td>LB 110 mg/L PFAA -RT Control</td>
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<td>ND</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td></td>
<td>7</td>
<td>ND</td>
<td>ND</td>
<td>35&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>ND</td>
<td>ND</td>
<td>35&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
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</tr>
<tr>
<td></td>
<td>2</td>
<td>35&lt;sup&gt;a&lt;/sup&gt;</td>
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</tr>
<tr>
<td>Minimal Media No PFAA -RT Control</td>
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<td>ND</td>
<td>35&lt;sup&gt;a&lt;/sup&gt;</td>
<td>35&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
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<sup>a</sup>High uncertainty

<sup>b</sup>Nonspecific binding (multiple melt curve peaks)

<sup>c</sup>Amplification after negative control
Figure S.2.4. Standard curves for *sigF3* were more reproducible for LB grown cells, especially as dilution increased, when the gel band was extracted and purified (A) than when the PCR product was bead purified (B). Dilution factors are shown within the graphs overlaid on the corresponding amplification curves.

### S.2.5 Statistical significance of RT-qPCR results

Multiple tests were performed to verify the statistical significance of the RT-qPCR results. The upregulation ratio, $R$, was calculated according to Equation S.2.1:\cite{82}

$$R = \frac{E_{\text{targ}}^{C_{q_{\text{targ,control}}} - C_{q_{\text{targ,sample}}}}}{E_{\text{ref}}^{C_{q_{\text{ref,control}}} - C_{q_{\text{ref,sample}}}}}$$

(EQ S.2.1)

where $E$ is the efficiency of the target or reference gene, $C_q$ is the average quantification cycle for the control replicates (in this case no PFAAs) or the sample replicates (110 mg/L PFAAs). A
randomization approach using the Relative Expression Software Tool (REST)\textsuperscript{83} was used to calculate statistical significance. In this approach, Cq values are randomly and repeatedly reallocated between control and sample with a new R value calculated at each step. After the specified number of iterations (2000 – 10,000), the probability that the control and sample groups are significantly different, or if the target gene is upregulated or downregulated, is provided.

The student’s t-test for samples with unequal variance was also performed on this dataset. For this evaluation, copy numbers per sample were normalized to the mass of RNA present in the sample prior to amplification, as shown in Tables S5 and S6. The student’s t-test was performed on 9 replicates (three biological replicates with three technical replicates) to encompass the variability inherent in both facets of the experiment, as executed in Sharp et al.\textsuperscript{61}

Normalization to the reference gene and statistical analysis using REST was not performed on the \textit{prmA} gene on samples grown in LB due to minimum sample volume requirements. Statistical significance on RNA normalized expression using student’s t-test was performed.

\textbf{S.2.6 Additional results}

\textbf{S.2.6.1 Complete RHA1 growth curves}

RHA1 growth curves extended beyond the 2 days depicted in Figure 3.2. The data for the duration of the experiment is shown below in Figure S.2.5.

\textbf{S.2.6.2 EPS extraction and measurement}

EPS extraction using the EDTA method was used as described elsewhere\textsuperscript{76} with the use of 10 mL of 10mM EDTA per sample.
Figure S.2.5. Long-term growth curves of RHA1 grown with PFAAs (square) and without PFAAs (circle) show an eventual leveling off of growth. The late increase of growth in the presence of PFAAs to within a standard deviation of the case without PFAAs is caused by an eventual breakup of the cellular aggregates.

Figure S.2.6. EPS as measured by a carbohydrate assay with glucose as a standard is more abundant in the presence of PFAAs (gray) than without PFAAs (white) for two types of extraction methods.
S.2.6.3 Soil Derived Community Floc Assessment

Communities derived from local soil and sustained in Luria-Bertani media also flocculated in the presence of PFAAs, as seen in Figure S.2.7.

Figure S.2.7. More flocs are observed in soil-derived communities grown in the presence of 110 mg/L total PFAAs (gray) than in those grown in the absence of PFAAs (white). The largest observed flocs also occurred when soil-derived communities were grown in the presence of 110 mg/L total PFAAs.


### S.2.6.4 RT-qPCR CP Values and Upregulation Data for LB Grown Systems

Table S.2.4. Initial RNA concentrations and Cq (CP) for all *prmA* analyses

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<thead>
<tr>
<th>Media</th>
<th>Sample</th>
<th>Replicate</th>
<th>RNA Concentration (ng/μL)</th>
<th><em>prmA</em> Cq or CP (cycles)</th>
</tr>
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<tbody>
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Table S.2.5. Initial RNA concentrations and Cq (CP) for \textit{sigF3} and \textit{gyrB} analyses

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Table S.2.6. Fold change and statistical significance for LB grown and minimal media grown cells

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<th>Fold Change</th>
<th>Student's t-test</th>
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S.3 Supporting information for Chapter 4

The supporting information for Chapter 4 contains a discussion on the derivation of sorption coefficients, the EPS content measured during sorption experiments, and properties of the loamy sand.

S.3.1 Calculating sorption coefficients

Organic carbon sorption coefficients were calculated after quantifying both the aqueous and solid phase concentrations of PFAAs. First, a sorption coefficient accounting for losses to the vial ($K_v$) were calculated from aqueous phase controls using Equation S.3.1. If losses to the system were less than 10%, $K_v$ was assumed to be 0.

$$K_v = \frac{1}{C_w/C_{add}} - 1$$

(EQ S.3.1)

where $C_w$ is the concentration in the aqueous phase (mg/L) as measured with LC-MS/MS and $C_{add}$ is the concentration initially added to the system (mg/L). The distribution coefficient associated with the solid phase, $Kd$, was calculated using Equation S.3.2.

$$Kd = \frac{Cs}{Cw} = \frac{Ms}{C_w \times rsw \times V}$$

(EQ S.3.2)
where $M_s$ is the mass associated with the solid phase as quantified with LC-MS/MS, $r_{sw}$ is the ratio of solid to water, and $V$ is the volume of the reactor. For the cellular system, $r_{sw}$ was calculated from optical density assuming an OD$_{600}$ of 2.27 = 8159 mg/L wet weight of cells. For the soil system, $r_{sw}$ was 10%. Conversion to organic carbon normalized sorption coefficients was calculated as discussed elsewhere. For the cellular system, conversions to organic carbon normalized distribution coefficients were performed in two ways: by converting cellular mass to protein, and by measuring volatile suspended solids. The protein approach assumes a correlation with optical density of 0.01 mg/L of protein for an optical density of 1, and an organic carbon fraction within protein of 53%. The volatile suspended solids approach assumes 50% of the volatile suspended solids is organic carbon. Mass balance checks were performed by calculating a sorption coefficient accounting for loss to the vial ($K_v$) in controls without a solid phase. The fraction of analyte lost to the vial ($f_v$) was calculated using Equation S.3.3.

$$f_v = \frac{K_v}{K_v + r_{sw} Kd + 1}$$  

(EQ S.3.3)

The summation of the mass in the aqueous phase with the masses sorbed to the solid phase and the vial should ideally fall within 70 – 130% of the total mass added to the system as defined by the limits expected from LC-MS/MS quality control.
Figure S.3.1. EPS content is minimal in each sorption system except –Soil. Error bars represent the standard deviation of triplicate systems.

Table S.3.1 Low organic content loamy sand properties

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</tr>
<tr>
<td>Percent Clay</td>
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