

WHEN CHEMISTRY MEETS MICROBIOLOGY: BIOTRANSFORMATION OF
EMERGING ORGANIC CONTAMINANTS

BY

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DISSERTATION

Submitted in partial fulfillment of the requirements
for the degree of Doctor of Philosophy in Environmental Engineering in Civil Engineering
in the Graduate College of the
University of Illinois Urbana-Champaign, 2021

Urbana, Illinois

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ABSTRACT

In recent years, with the increasing use of organic chemicals such as pesticides, pharmaceuticals, personal care products, and many other types of anthropogenic chemicals, there have been emerging concerns about the potential risk of these compounds to natural environments and public health. The release of such emerging organic contaminants (EOCs) into the environment threatens the ecosystem's health and functioning. Microbial biotransformation shows its potential in alleviating the environmental detections of many EOCs. However, limited studies tackle the EOCs biotransformation mechanisms from both pathways' elucidation and responsible microorganisms' identification aspects. Thus, the primary focus of this study aims to build a framework to comprehensively study the fate of EOCs as well as their biotransformation patterns under the different environmental conditions, specifically focusing on investigating the transformation products, the biotransformation pathways, the responsible microorganisms, and ultimately the responsible genes and enzymes.

I started the project by investigating the EOCs biotransformation capabilities of nitrifiers (i.e., nitrite-oxidizing bacterium *Nitrobacter* sp. and ammonia-oxidizing bacterium *Nitrosomonas europaea*). The results showed that the experimental used nitrite-oxidizing bacteria could not biotransform any investigated EOCs, whereas *N. europaea* showed the biotransformation capabilities for several EOCs. Notably, the results present strong evidence favoring ammonia monooxygenase (AMO) responsible for EOCs biotransformation, which likely occurred via co-metabolism. Furthermore, for the first time, we demonstrated that abiotic EOCs transformation by hydroxylamine, the product of AMO-mediated ammonia oxidation, likely represents another transformation mechanism of EOCs by ammonia oxidizers.

I then compared the abilities of three phylogenetically distant ammonia oxidizers, *Nitrososphaera gargensis*, an ammonia-oxidizing archaeon; *Nitrosomomas nitrosa* Nm90, an ammonia-oxidizing bacterium; and *Nitrospira inopinata*, the only complete ammonia oxidizer available as a pure culture, to biotransform the selected EOCs. The results exhibited that among three ammonia oxidizers, only *N. inopinata* was able to biotransform carbendazim when ammonia was supplied as the energy source. The exclusive biotransformation of carbendazim by *N. inopinata* is likely enabled by an enhanced substrate promiscuity of its unique AMO and its much higher substrate (for ammonia) affinity compared with the other two ammonia oxidizers.

Among various EOCs, organofluorine compounds (e.g., per- and polyfluoroalkyl substances, PFAS) have caused increasing global concerns due to their persistence in the environment, bioaccumulation in organisms, and toxicity to human beings and ecosystems. Therefore, I further investigated the microbial biotransformation and biodefluorination of structurally different per- and polyfluorinated compounds. Surprisingly, under the anaerobic condition, the microbial cleavage of C–F bonds in PFAS was demonstrated for the first time by the release of F^- and the formation of the corresponding defluorination products. Moreover, I found that the α , β -unsaturation was crucial for fluorinated compounds biotransformation by the defluorinating enrichment culture. Furthermore, the defluorination activity was significantly enhanced along with the enrichment processes, indicating the growth of defluorinating microorganisms. Given the toxicity of fluoride ion (F^-), the enhanced defluorination activity also suggested that the responsible microorganisms must defend themselves against the intracellular F^- buildup. Taken together, the fluoride ion efflux transporter and the reductase responsible for α , β -unsaturated double bonds reduction were proposed as two biomarkers for selecting the responsible

defluorinating microorganisms. By 16S rRNA gene amplicon sequencing and metagenomic sequencing, the community dynamics of the defluorinating enrichment culture were elucidated.

One anaerobic bacterium isolate that met both selection criteria was then chosen as a representative for the biotransformation experiments. The results showed that the selected microorganism could biotransform the selected perfluorinated compound via the same biotransformation and biodefluorination pathways as observed in the enrichment community. Based on the differential gene expression, the electron transferring trajectory, the defluorination pattern, and the possible responsible genes/enzymes for fluorinated compound defluorination and hydrogenation were further elucidated.

Overall, this work advances the fundamental understanding of the fate of various EOCs in natural and engineered environments and opens up diverse venues for both fundamental science and engineering applications. Those include identifying and characterizing the responsible microorganisms/genes/enzymes for EOCs removal, establishing novel EOCs bioremediation strategies, and designing more readily degradable chemical structures.

ACKNOWLEDGEMENTS

Six years ago, I joined the Department of Civil and Environmental Engineering at the University of Illinois at Urbana-Champaign and started my *M.S. + Ph.D.* program. People say, “*I survived my Ph.D.*” when they graduate. I think only PhDs can understand this “inside joke.” Looking back now, it has been an incredible and memorable journey for me, and I feel grateful and encouraged by what has been achieved. In Chinese, there is a saying, “How far a person can go depends on who they travel with; how talented a person can become depends on who they have as their mentors; how successful a person can achieve depends on who they have as their companions.” Here, I would like to take this opportunity to express my sincere gratitude to the fantastic people I met during the last few years.

First of all, I would like to express my sincere gratitude and heartily thanks to my advisor Prof. Yujie Men for guiding me throughout my entire graduate study (*M.S. + Ph.D.*). I was trained with a *B.Eng.* in Hydrology, making it challenging for me to start my graduate study in Environmental Science. I still remember how nervous I was the first time I reached out to Dr. Men to inquire about the availability of a research assistant position in her lab working on environmental microbiology-relevant projects. Thank you for and seeing the little sparkle in me and offering me the opportunity to do science. The six years of learning from you and working with you are the most precious time of my life. I remember everything you mentioned to me about how to be a good researcher. Thank you for teaching me all the analytical chemistry, biochemistry, and molecular biology skills and encouraging me to learn new techniques in bioinformatics.

I truly appreciate the help and support from my Ph.D. committee members: Prof. Wen-Tso Liu (University of Illinois at Urbana-Champaign), Prof. Thanh H. (Helen) Nguyen (University of Illinois at Urbana-Champaign), and Prof. Jinyong Liu (University of California, Riverside). Thank

you for being my committee members in both Preliminary Exam and Final Defense, and thank you for guiding me through the most important period of my Ph.D. life. The course CEE540 (i.e., Remediation Design) taught by Dr. Ran Mei and Prof. Wen-Tso Liu led me through bioinformatics, which later became an essential tool for my research in Chapter 6 and Chapter 7. Thank you for helping me understand the most critical concepts in environmental microbiology and ecology. The first course I took at UIUC was CEE440 (i.e., Fate and Cleanup of Environmental Pollutants), taught by Prof. Thanh H. (Helen) Nguyen. Thank you for introducing me to the field of Environmental Science and Engineering. Prof. Jinyong Liu is a fantastic collaborator, a good mentor, and a helpful friend. Thank you for your help, guidance, insights, and suggestions for all these years, which I believe will become one of the most valuable treasures for my future independent academic career.

In addition, I would like to express my thanks to my collaborators Prof. Ping Han, Dr. Li-jun Zhou, Prof. Zhenyu Tian, Prof. Michael Wagner, and all the coauthors in my papers. I appreciate your support and help at the early stage of my research. Many thanks to Dr. Zhong (Lucas) Li from the Roy J. Carver Biotechnology Center (UIUC) for the help with HPLC-HRMS measurement and non-target analysis. I would like to thank Chris L. Wright and Hernandez Alvaro Gonzalo at the Roy J. Carver Biotechnology Center (UIUC) for the 16S rRNA gene amplicon sequencing and metagenomic and metatranscriptomic sequencing support.

My special thanks are given to my colleagues: Dr. Yue Xing, Shun Che, Kunyang Zhang, Jing Yuan, Zhaohui An, Bosen Jin, Dr. Huaqing Liu, and Chujing Zheng. Thank you all for the discussion of my research projects and for helping me with my Ph.D. defense rehearsal. Yue, we joined the group and graduated with our Ph.D. around the same time. I discussed my research and my personal life with you almost as much as with Dr. Men. Thank you for being there and listening

to me. Shun, you, Yue, and me, we three moved from UIUC all the way to UCR together. I cannot imagine how life would be without both of you. Thank you for helping me out for all these years. Kunyang, I learned a lot from you during the two and a half years when you were doing your master's degree at UIUC. I admire your courage in deciding to change your major for your future career. Jing and Zhaohui, thank you for helping me with my preliminary exam and defense rehearsal. Bosen, thank you for constantly reminding me to stay healthy and take care of myself. I like your optimistic attitude to life. Huaqing, thank you for bringing the pure culture isolation techniques into the group. I have always had an interest in learning new approaches in environmental microbiology. Chujing, you joined the group during the last stage of my Ph.D. study when I was super occupied by my lab works and experiments. We might have fewer overlaps as you are in a different research direction, but thank you for bringing joy and passion into the group.

I would like to thank some important people at the Department of Civil and Environmental Engineering of the University of Illinois and Urbana-Champaign. Dr. Shaoying Qi, thank you for helping me initiate all my research and designing the reactors from scratch. Prof. Vernon Snoeyink, thank you for organizing the Culture Awareness Speech Enhancement (CASE). I benefit a lot during the practice. Joan Etta Christian, thank you for helping me maintain my student status at UIUC and taking care of my student registration and graduation. You are the most helpful graduate student advisor I have ever met so far.

I would like to thank some people at the Department of Chemical and Environmental Engineering of the University of California, Riverside. Dr. Michael J. Bentel, you are one of the most important collaborators I have. Thank you for encouraging me when I was looking for a postdoc position. Dr. Changxu Ren, you are the first friend I made here at UCR. Thank you for helping me out with the moving and transition from UIUC to UCR. Zekun Liu, thank you for

sharing your life experiences with me and reminding me to stay happy and healthy. Jinyu Gao and Tianchi Liu, thank you for sharing your lab space with me when my lab was under renovation and thank you for helping me with my experiments. Tianchi, we met at Orlando during the ACS conference and became friends since then. Thank you for organizing the 2021 Thanksgiving vacation, I really had a great time hanging out with you!

Moreover, I would like to thank my seniors and my friends. Dr. Ran Mei, Dr. Yijue Diao, and Dr. Mengwei Han, you are the most outstanding and hardworking researchers I have ever met so far in my life. Thank you for being the role models and having me in your squad. My friends Minkai Wang and Shiyi Chen, thank you for having me in your lives, and thank you for being my companions whenever I needed you.

I would like to thank the financial support from UIUC CEE Department, UIUC Graduate College, UCR CEE Department, NSF, SERDP, ACS Division of Environmental Chemistry, CAPEES, and China Scholarship Council.

I also want to say thank you to my parents, my father Jinbo Yu, and my mother Li Wang. Thank you for loving me, supporting me, and keeping me out from the wind and rain in my life as much as you can. At this stage of life, I understand that you do not have too much to give, and you can do little to help. But I know you provide me with everything you have and help me whenever I needed. I never said “I love you” in front of you, but I love you more than anything in the world combined. You are my strength as I roamed the world alone. Friends of mine are either starting their families or starting their own business; friends of yours are becoming grandparents and enjoying family happiness. Yet, I am still on the way to pursuing my dream, and we all know that the path of my future is for sure not paved. You told me that I am not the smart type ever since I was a little kid, but I can still get to wherever I want as long as I devote myself to it. My

determination is never waved, but I was afraid you might see things differently and push me to the next chapter of my life. On the contrary, you told me there is no rush when people are doing something they love, and people will never be lonely and become wanderers as long as their hearts are beating and have purposes in mind. You raised me as a person who sees things positively and always loves the world. You taught me to accept who I am. You encouraged me to learn from past failure and use it in the future. You are my role models of how I see the world.

I dedicate this dissertation to my parents, Jinbo Yu and Li Wang, for loving me and supporting me all the way through. Thank you for raising me to be the person I am today.

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CHAPTER 1: INTRODUCTION

1.1 Emerging Organic Contaminants (EOCs)

Emerging organic contaminants (EOCs) include pesticides, pharmaceuticals, personal care products, and many other types of anthropogenic chemicals such as per- and polyfluoroalkyl substances (PFAS). The environmental detections of most EOCs are usually at low concentrations (a few ng/L to tens of µg/L),¹⁻³ thus, in many studies, EOCs are also referred as the micropollutants.⁴⁻⁶ In recent years, EOCs are getting emerging concerns as their potential adverse effects on the ecosystem and public health.^{3, 7-9} For example, there are many studies demonstrating that the detection of antibiotics in the environment could potentially lead to the increase of antibiotic resistance of microorganisms.^{10, 11} Moreover, industrial chemicals such as PFAS are found to be bioaccumulated in organisms.^{12, 13} Not only the directly released EOCs, some of the transformation products may share the same concerns. In a recently published paper, the researcher identified that a transformation product from the tire rubber particle leachate could induce acute mortality in coho salmon.¹⁴ Microbial biotransformation shows its potential in alleviating the environmental detections of many EOCs. However, limited studies tackle the EOCs biotransformation mechanisms from both pathways' elucidation and responsible microorganisms' identification aspects.

1.2 The Biotransformation of EOCs in WWTPs

Most of the EOCs enter the environment from industrial and commercial facilities, contamination sites (e.g., oil or other organic chemicals spilling sites), road runoff, sewage

systems, and wastewater treatment plants (WWTPs).^{3, 15} For instance, the detection of a wide range of EOCs WWTPs haven been frequently reported in many studies.¹⁶⁻²¹ It is very important to notice that municipal WWTPs acted as not only a source of many EOCs but also a potential barrier of EOCs entering the ecosystems. EOCs removal in WWTPs mainly occurs during the biological treatment processes, which can be attributed to (1) biological removal, including biotransformation^{18, 20, 22-25} and bioaccumulation²⁶ and (2) abiotic removal, including compounds volatilization, sorption² to the sludge, and abiotic degradation.²⁷ Among all these EOCs removal routes, the biotransformation is considered the major responsible mechanism.²⁸ A number of studies have shown the significant correlations between EOCs removal and the nitrification activities of the nitrifying activated sludge.^{18, 20, 29, 30} To be specific, it has been previously reported that the EOCs removal was significantly enhanced/decreased as the responses of nitrification activity was stimulated/inhibited.¹⁹ Based on these observations, the nitrifiers are hypothesized to be one of the essential responsible players during the EOCs biotransformation. However, in the nitrifying activated sludge studies, it is difficult to disentangle the roles of the co-existing heterotrophs from the nitrifiers. Therefore, pure nitrifiers biotransformation studies are needed to fully demonstrate the roles of nitrifiers played in EOCs removal.

Nitrifiers contain both ammonia-oxidizing microorganisms and nitrite-oxidizing microorganisms.³¹ Currently, ammonia-oxidizing microorganisms can be categorized into ammonia-oxidizing bacteria (AOB), ammonia-oxidizing archaea (AOA), and the recently

discovered complete ammonia-oxidizing bacteria (comammox bacteria). Functionally, all three types of ammonia oxidizers can obtain energy by oxidizing ammonia/ammonium to nitrite, yet physiologically all three types of ammonia oxidizers vary from each other. For AOB, ammonium is oxidized to hydroxylamine by a membrane bound enzyme (i.e., ammonia monooxygenase, AMO), and hydroxylamine is further oxidized to nitrite by enzyme nitrite oxidoreductase. In AOA, it is an unknown enzyme complex that carries the oxidation of hydroxylamine. For comammox bacteria, there is a unique enzyme (i.e., nitrite oxidoreductase), which can further oxidize the nitrite to nitrate.³² For nitrite-oxidizing microorganisms, inorganic nitrogen NO_2^- was oxidized to NO_3^- for energy conservation. Both ammonia-oxidizing microorganisms and nitrite-oxidizing microorganisms are chemolithotrophs that assimilate inorganic carbon (e.g., CO_2) as carbon sources for cell growth.³¹ A pure strain of the ammonia-oxidizing thaumarchaeote *Nitrososphaera gargensis* has been demonstrated to use cyanate as the energy source.³³ Cyanate was converted to ammonium and carbon dioxide in *N. gargensis* by an enzyme called cyanase.³³ Given the differences among nitrifiers, the biotransformation capabilities of the three types of ammonia oxidizers as well as the nitrite oxidizers remain elusive and need further investigation.

Theoretically, the biotransformation of EOCs could occur either metabolically or cometabolically. For metabolic biotransformation, the energy released for the corresponding reactions should be sufficient to support the growth of responsible microbes, and the carbons in the investigated EOCs could be assimilated into the biomass. Unlike the metabolic processes, for

cometabolic EOCs removal, the reactions only occurred when other primary substrates were also provided. Due to the nature of most EOCs (bio)transformation processes do not provide any building blocks for microbes (e.g., nitrifiers), it is hypothesized that the cometabolic biotransformation should play major role in EOCs removal, yet explicit tests of this hypothesis are needed. Regardless of the biotransformation mechanisms, the EOCs biotransformation efficiencies vary among compounds' properties and the treatment processes. As a result, the incomplete removed EOCs and their transformation products are discharged into the environment and threaten the entire ecosystem's health.²² Thus, investigating the biotransformation pathways, as well as the transformation products, are of particular importance to evaluate the environmental impacts of both the parent compounds and the transformation products.¹⁹

1.3 Per- and polyfluoroalkyl Substances (PFAS)

Among hundreds and thousands of EOCs, per- and polyfluoroalkyl substances (PFAS) are getting much more attention in recent years. PFAS comprise a class of anthropogenic organic chemicals which are widely used for industrial and commercial applications in recent years due to their unique properties like hydrophobicity, lipophobicity, and thermal stability.³⁴⁻³⁷ According to the latest report of the Organization for Economic Co-operation and Development (OECD), PFAS are defined as fluorinated chemicals that contain at least one fully fluorinated methyl group ($-\text{CF}_3$) or methylene group ($-\text{CF}_2-$) carbon atoms.³⁸ The persistence,³⁹ bioaccumulation,^{12, 40} and toxicity of PFAS⁴¹ to the ecosystem and public health⁴² have triggered global concerns of such

contaminants.^{43, 44} The highly stable Carbon-Fluorine (C–F) bonds⁴⁵ in PFAS make them very recalcitrant in the environment. Therefore, the ultimate goal for PFAS remediation is defluorination. So far, many breakthroughs have been achieved in PFAS physiochemical treatment technologies.^{37, 46-50} Yet, in the aspect of bioremediation, most studies focus on the biotransformation/biodefluorination of polyfluorinated structures.⁵¹⁻⁵⁵ The defluorination of the polyfluorinated compounds was initiated by HF elimination at the α and β position of fluorotelomer carboxylic acid⁵⁶⁻⁵⁸ or by desulfonation of fluorotelomer sulfonic acid.^{59, 60} For instance, the biotransformation of 6:2 fluorotelomer alcohol (6:2 FTOH, an important ingredient in aqueous film-forming foam) under aerobic conditions by various microbial communities and microbial species have been previously reported.^{53, 57-67} Compared to the biotransformation of polyfluorinated structures, convincing reports on microbial cleavage of C–F in perfluorinated structures are even fewer. Despite the strong C–F bonds in perfluorinated structures, the toxicity of the defluorination product, fluoride ion (F^-), to nearly all living beings also indicates that microbial defluorination is difficult and rare.^{68, 69} In order to achieve a sustained defluorination activity, the responsible microbes must defend the intracellular fluoride ion buildup by expelling the fluoride ion out of the cell. Thus, studies identifying the responsible defluorinating microorganisms/genes/enzymes and investigating the defluorination mechanisms (combining both defluorination transformation products identification and F^- measurement) are needed to fill this knowledge gap in the field of microbial defluorination.

1.4 Organizations of This Dissertation

The overall objective of this dissertation is to build a framework to comprehensively study the fate of EOCs as well as their biotransformation pattern under the different environmental conditions, specifically focusing on investigating the biotransformation pathways, transformation products, the responsible microorganisms, genes, and enzymes. Chapter 1 reviewed the current status of EOCs biotransformation and stated two major objectives this dissertation aimed to fulfill: (1) investigate the exact roles of nitrifiers (i.e., ammonia-oxidizing microorganisms and nitrite-oxidizing microorganisms) played in some EOCs biotransformation, (2) provide solid evidence (i.e., defluorination products identification, pathways elucidation, and fluoride ion measurement) for microbial cleavage of C–F bonds in PFAS. Chapter 2 investigated the specific roles of AOB and NOB played during selected EOCs biotransformation and provided concrete evidence for ammonia monooxygenase being the responsible enzyme. Chapter 3 compared the EOCs biotransformation capabilities of three types of ammonia oxidizers (i.e., AOB, AOA, and comammox) and demonstrated the distinct biotransformation pattern of the comammox bacterium. Chapter 4 focused on one specific type of EOCs (i.e., per- and polyfluoroalkyl substances, PFAS) and investigated the microbial cleavage of C–F bonds in two branched and unsaturated per- and polyfluorinated structures. Chapter 5 extended the investigation to a comprehensive set of unsaturated fluorinated compounds and demonstrated specific structures for anaerobic PFAS degradation. Chapter 6 continued the research at the microbial community level and revealed the

community dynamics of the possible responsible microorganisms and genes for the reductive defluorination reactions. Chapter 7 continued the investigation of reductive defluorination microorganisms by pure culture experiments. The defluorination pattern and possible responsible genes/enzymes were further elucidated by whole genome sequencing, RNA sequencing, and proteomics analysis. Chapter 8 summarized the findings and implications regarding the study and proposed a systematic workflow for EOCs biotransformation investigations.

CHAPTER 2: AMMONIA MONOOXYGENASE-MEDIATED COMETABOLIC BIOTRANSFORMATION AND HYDROXYLAMINE-MEDIATED ABIOTIC TRANSFORMATION OF MICROPOLLUTANTS IN AN AOB/NOB COCULTURE

2.1 Abstract

Biotransformation of various micropollutants (MPs) has been found to be positively correlated with nitrification in activated sludge communities. To further elucidate the roles played by ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB), we investigated the biotransformation capabilities of an NOB pure culture (*Nitrobacter* sp.), and an AOB (*Nitrosomonas europaea*)/NOB (*Nitrobacter* sp.) co-culture for fifteen MPs, whose biotransformation was reported previously to be associated with nitrification. The NOB pure culture did not biotransform any investigated MP, whereas the AOB/NOB co-culture was capable of biotransforming six MPs (i.e., asulam, bezafibrate, fenhexamid, furosemide, indomethacin, and rufinamide). Transformation products (TPs) were identified, and tentative structures were proposed. Inhibition studies with octyne, an ammonia monooxygenase (AMO) inhibitor, suggested that AMO was the responsible enzyme for MP transformation that occurred co-metabolically. For the first time, hydroxylamine, a key intermediate of all aerobic ammonia oxidizers, was found to react with several MPs at concentrations typically occurring in AOB batch cultures. All of these MPs were also biotransformed by the AOB/NOB co-culture. Moreover, the same asulam TPs were detected in both biotransformation and hydroxylamine-treated abiotic transformation experiments, whereas rufinamide TPs formed from biological transformation were not detected during hydroxylamine-mediated abiotic transformation, which was consistent with

the inability of rufinamide abiotic transformation by hydroxylamine. Thus, in addition to cometabolism likely carried out by AMO, an abiotic transformation route indirectly mediated by AMO might also contribute to MP biotransformation by AOB.

2.2 Introduction

In recent years, with an increasing use of organic chemicals such as pesticides, pharmaceuticals and personal care products, there are emerging concerns about the potential risk of these contaminants to natural environments and public health.^{3, 7-9} These compounds are typically detected at very low concentrations in aquatic systems (i.e., ng/L ~ µg/L levels),^{1, 2} thus are also referred to as micropollutants (MPs). Conventional wastewater treatment plants (WWTPs) are one major sink of MPs.⁷⁰ Biotransformation is an essential mitigation route of MPs in WWTPs, but most of these systems are not designed specifically for MP removal. Thus, MP removal in WWTPs is generally incomplete and its efficiency is highly compound specific.^{24, 71, 72} Remaining MPs and their transformation products (TPs) are discharged into aquatic systems, which may cause adverse effects.⁷³⁻⁷⁵ Hence, further studies of MP biotransformation mechanisms and identification of their TPs are necessary and will in the long run help us to better understand the environmental fate of MPs, design removal or monitoring strategies, as well as establish precaution guidelines.^{1,}

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Nitrification, the aerobic oxidation of ammonia via nitrite to nitrate, is an essential step for the removal of nitrogen compounds in WWTPs. In many WWTPs, this process is catalyzed by the

concerted activity of ammonia-oxidizing bacteria (AOB) converting ammonia to nitrite, and nitrite-oxidizing bacteria (NOB) converting nitrite to nitrate, although ammonia-oxidizing archaea (AOA)⁷⁷⁻⁸⁰ the archaeal counterpart of AOB and the recently discovered complete ammonia oxidizers (comammox) converting ammonia to nitrate⁸¹⁻⁸³ have also been detected in some WWTPs. Previous studies have shown correlations between nitrification and biotransformation of some MPs in nitrifying activated sludge communities.^{17, 71, 73, 84, 85} For example, MP biotransformation was enhanced when nitrification activity was stimulated.³⁰ In addition, MP biotransformation in activated sludge communities decreased when nitrification was completely inhibited by ammonia monooxygenase (AMO) inhibitors.^{30, 74, 86, 87} In a recent study, the biotransformation of a number of MPs was inhibited by AMO inhibitors, and more than 50% inhibition was observed for four MPs (i.e., asulam, trimethoprim, monuron, and clomazone), providing strong indications that ammonia oxidizers (particularly AOB) were involved in the biotransformation of some MPs.⁷¹ However, the correlations observed in inhibition studies does not necessarily lead to causal relationships. In order to demonstrate the actual role played by AOB in MP biotransformation, and to identify the responsible enzymes and transformation pathways, complementary pure culture studies are required.

It has been hypothesized that AMO, a substrate-promiscuous key enzyme of all autotrophic ammonia oxidizers is responsible for nitrifier-mediated MP biotransformation.^{18, 23, 30} So far, whole-cell studies have been used to investigate AMO functionality due to the unavailability of

purified AMO with retained activity.⁸⁸ While it has been shown that AMO is able to oxidize some hydrocarbons and halogenated hydrocarbons co-metabolically,^{89, 90} direct evidence for MP transformation by this enzyme is largely lacking. Furthermore, the results of the inhibition studies could also suggest that the MP biotransformation was carried out by microorganisms whose growth is highly dependent on ammonia oxidizers. For example, the inhibition of ammonia oxidizers in nitrifying activated sludge will strongly reduce nitrite production, and thus also inhibit NOB. Therefore, there is an urgent need to investigate the MP biotransformation potential of nitrite oxidizers, too. Given that there is no nitrite accumulation in nitrifying activated sludge communities and the accumulated nitrite in AOB pure cultures may lead to abiotic nitration reactions of some MPs,^{91, 92} a co-culture system containing AOB and NOB species is more relevant to the actual nitrification condition, and will be used in this study to investigate the roles of nitrifiers.

The goal of this study was (i) to investigate biotransformation capabilities of AOB and NOB using the model AOB species *Nitrosomonas europaea* and NOB species *Nitrobacter* sp.. Fifteen MPs including pharmaceuticals and pesticides were selected, whose biotransformation has been associated with nitrification in activated sludge by an inhibition study,⁷¹ (ii) to identify responsible enzymes for the transformation of selected MPs, and (iii) to further understand the respective transformation mechanisms. Biotransformation experiments were conducted using the NOB pure culture and a nitrifying co-culture containing the AOB and the NOB species. Metabolic

and co-metabolic biotransformation mechanisms were tested by using the biotransformed MP as the sole energy source. AMO inhibition experiments were carried out to demonstrate the involvement of AMO in MP biotransformation. Abiotic transformation experiments using the key ammonia oxidation intermediates (e.g., hydroxylamine) were also conducted to further unravel mechanisms of MP biotransformation by ammonia oxidizers.

2.3 Materials and Methods

MP Selection

Based on a previous inhibition study of MP biotransformation in activated sludge, 15 MPs (Table S1) were selected, whose biotransformation was inhibited by more than 20% when adding the bacterial AMO inhibitor octyne.⁷¹ These MPs are commonly used pesticides and pharmaceuticals, including acetamiprid, asulam, bezafibrate, carbendazim, clomazone, fenhexamid, furosemide, indomethacin, irgarol, levetiracetam, monuron, rufinamide, tebufenozide, thiacloprid, and trimethoprim. In addition, two compounds (mianserin and ranitidine) that were previously shown to be biotransformed by ammonia-oxidizing pure cultures,¹ were included as positive controls for biomass activities (Table A.1). Reference compounds and methanol (HPLC grade) were purchased from Sigma-Aldrich (St. Louis, MO). Five internal standards including trimethoprim-d3, metoprolol acid-d5, carbendazim-d4, and furosemide-d5, were obtained from C/D/N isotopes (Quebec, Canada) and Toronto Research Chemicals (Toronto, Canada). The bacterial AMO inhibitor 1-octyne^{93, 94} was purchased from Fisher Scientific. Stock

solutions of MPs and internal standards were prepared individually in methanol at 1 g L⁻¹, except for carbendazim and its corresponding internal standard carbendazim-d4, which were prepared in methanol at 0.1 g L⁻¹. All stock solutions were stored at – 20 °C. A mixture of standards (100 mg L⁻¹ for each MP) was prepared via appropriate dilution of the stock solutions in methanol.

Cultivation of Nitrifying Pure and Co-cultures.

The AOB strain *Nitrosomonas europaea* Winogradsky (ATCC 19718) and the NOB strain *Nitrobacter* sp. (ATCC 25381) were obtained from the American Type Culture Collection (ATCC). They were maintained in 50 mL suspension culture flasks with filter screw caps (VWR, Radnor, PA) containing 20 mL of a modified basal medium with 4.0 g L⁻¹ CaCO₃ for buffering the pH around 8.³³ All cultures were incubated at 28 °C in a dark incubator and shaken at 120 rpm. Seven mM NH₄Cl or NaNO₂ was added as the growth substrate in the AOB or NOB cultures, which was re-amended upon depletion. The culture performance (ammonia or nitrite oxidation) was consistent and stable during each feeding cycle of the entire incubation period. The AOB/NOB co-culture was obtained by growing the two strains together, and maintained in the same basal medium with 2 mM NH₄Cl as the substrate, which was re-added upon depletion of both NH₄⁺ and NO₂⁻. All cultures were sub-cultured (10%, v/v) into autoclaved fresh medium after three doses of the respective substrate (NH₄Cl or NaNO₂). Additional pure AOB and AOA species (i.e., *Nitrosomonas nitrosa* Nm90 and *Nitrososphaera gargensis*) were maintained as described previously.¹

Biotransformation Experiments

The capability of MP biotransformation by the NOB pure culture and the AOB/NOB co-culture was investigated in batch experiments. Pre-grown biomass was harvested by centrifugation at $10,000 \times g$ (4 °C for 15 min). The biomass was re-suspended in autoclaved fresh medium and concentrated to reach a similar ammonia or nitrite oxidation rate (~ 2 mM per 8 hours). Individual MP or mixed MP stock solution was added to empty culture flasks. After the organic solvent methanol was evaporated entirely, 25 mL of thoroughly mixed concentrated NOB pure culture or AOB/NOB co-culture was inoculated. As the mixture of tested MPs inhibited the physiological activity of *N. europaea* at concentrations over 30 $\mu\text{g/L}$ (each), and did not show inhibition on *Nitrobacter* sp. at a concentration up to 100 $\mu\text{g/L}$ (each), an initial MP concentration of 100 $\mu\text{g/L}$ (each) was used for the NOB experiments, while 10 $\mu\text{g/L}$ (each) was applied for the AOB/NOB co-culture. The culture flasks were shaken at 150 rpm for 5 min to re-dissolve the MPs. Weight loss due to evaporation in each flask was measured and compensated by adding sterile nanopure water every 24 hours. Two mM NH_4Cl and 2 mM NaNO_2 were added to the AOB/NOB co-culture and the NOB culture at the beginning, respectively, and were re-added afterwards to keep the ammonia/nitrite oxidation activity. For the AOB/NOB co-culture, 2 mM NH_4Cl was re-added every 12 hours during the first two days, and every 24 hours afterwards. For the NOB pure culture, 2 mM NaNO_2 was re-added every 24 hours. Samples (1 mL) were taken immediately after the first addition of NH_4Cl or NaNO_2 , and centrifuged at $16,000 \times g$ (4 °C for 15 min). Supernatant was

collected and stored at 4 °C for LC-MS/MS and nitrogen species (i.e., NH_4^+ , NO_2^- , and NO_3^-) measurements. Cell pellets were stored at – 20 °C for quantitative PCR (qPCR) analysis. Samples were taken using the same procedure at subsequent time points during an incubation period of 168 hours. At the end of incubation, cells in 10 mL culture were collected by centrifugation, washed with sterile medium for three times, and stored at – 20 °C for intracellular MP and TP analysis. Biomass-free sterile fresh medium control and heat-inactivated biomass (autoclavation at 121 °C for 20 min) control were set up in triplicates in the same way as the biotransformation experiments described above. Briefly, in both medium and heat-inactivated controls, the same MPs were added, and ammonium (2 mM), nitrite (2 mM) and nitrate (14 mM) were added to mimic the levels of N-species present in the biotransformation experiments.

Asulam Inhibitory Effect Tests

Biotransformation experiments were performed as described above using the same pre-grown co-culture with different initial concentrations of asulam (0, 10, 20, 30, 40, 50, and 80 $\mu\text{g/L}$). Two mM ammonia was supplied every 24 hours. Supernatant samples in a time series were taken over a 72-h incubation period, and ammonia degradation and asulam biotransformation were determined as described above.

AMO Inhibition Experiments

To investigate the role of AMO in asulam biotransformation, the bacterial AMO inhibitor octyne ($\sim 10 \text{ mg/L}$)^{71, 95} was added to the AOB/NOB co-culture with 10 $\mu\text{g/L}$ asulam after a 72-h

incubation. We chose octyne as the AMO inhibitor instead of the commonly used allylthiourea (ATU) and acetylene because (i) ATU has been found not specific to AMO;⁷¹ (ii) acetylene is in gaseous phase, thus it is difficult to keep its inhibitory effect in aerobic reactors with fast air exchange; (iii) octyne is added as liquid, and was found to have more rapid inhibition than acetylene.⁹⁵ All inhibition experiments were performed in quadruplicates, three of which were sampled in a time series over a 168-h incubation period for LC-MS/MS analysis, cell growth, and N-species measurements. The remaining replicate was sampled every 24 hours to monitor the ammonia oxidation activity using Hach test tubes for NH_4^- and NO_3^- -N according to the manufacturer's instructions. Octyne was re-added as soon as recovery of ammonia oxidation activity was detected.

Role of Ammonia Oxidation Intermediates in MP Transformation

To investigate the effects of the ammonia oxidation intermediates, hydroxylamine (NH_2OH) and nitric oxide (NO) on MP biotransformation, MP transformation experiments after addition of either NH_2OH or NO were carried out in sterile fresh medium. Based on previously measured concentrations of extracellular NH_2OH produced by ammonia oxidizers in batch reactors and the observation that 5 μM NH_2OH was decomposed within 10 hours in the medium used,⁹⁶ 5 μM NH_2OH was added every 12 hours over a 168-h incubation period. As a comparison to AMO-mediated intermediate NH_2OH , MP transformation by NO, a non-AMO-mediated intermediate was also carried out. Excess NO was added in the form of 114.3 μM NONOate (Fisher Scientific,

Hampton, NH), which releases $\sim 200 \mu\text{M}$ NO during the 168-h incubation (first-order process with half-life of 56 hours at 25°C). A mixture of all 17 MPs were added at a concentration of $20 \mu\text{g/L}$ for each compound. Samples at 0, 12, and 168 h were taken for LC-HRMS analysis.

Analytical Method

MPs were analyzed by liquid chromatography coupled to a high-resolution quadrupole orbitrap mass spectrometer (LC-HRMS/MS) (Q Exactive, Thermo Fisher Scientific). For LC analysis, $50 \mu\text{L}$ sample was loaded onto a C_{18} Atlantis-T3 column (particle size $3 \mu\text{m}$, $3.0 \times 150 \text{ mm}$, Waters), and eluted at a flow rate of $350 \mu\text{L/min}$ with nanopure water (A) and acetonitrile (B) (both amended with 0.1% formic acid), at a gradient as follows: 5% B: 0 – 1 min, 5% – 100% B: 1 – 8min, 100% B: 8 – 20 min, and 5% B: 20 – 26 min. The compounds were measured in full scan mode on HRMS at a resolution of 70,000 at m/z 200 and a scan range of m/z 50 – 750 in a positive/negative switching mode. The limit of quantification (LOQ) for each MP is determined as the lowest concentration with a detection variation $< 20\%$, which was listed in [Table A.1](#).

Cell Extraction for Intracellular Concentration Measurement

A cell extraction procedure from a previous study⁹⁷ was used with slight modification. Briefly, internal standards were spiked in cell pellets collected from 10 mL culture (at a final concentration of $4 \mu\text{g/L}$ for each) followed by an addition of 2 mL lysis solvent containing methanol (0.5% formic acid): nanopure water (0.1% w/w EDTA), 50: 50 (v/v). The cells were disrupted by ultrasonication at 50°C for 15 min, centrifuged at $10,000 \times g$ for 10 min. The

supernatant was collected in a glass vial. This procedure was repeated twice for a better recovery. Finally, ~ 6 mL supernatant was evaporated to dryness under a gentle stream of dinitrogen gas at 40 °C. The analytes were re-dissolved in 0.5 mL filter-sterilized fresh medium without CaCO₃, which were then centrifuged at 10,000 × *g* at 4 °C for 10 min. The supernatant was collected for LC-HRMS measurement.

Transformation Product Identification

Both suspect screening and non-target screening were carried out to identify TPs. Suspect screening was done by TraceFinder 4.1 EFS software (Thermo Fisher Scientific). TP suspect lists were compiled using an automated metabolite mass prediction script, which considered a number of known redox and hydrolysis reactions, as well as several conjugation reactions at primary and secondary levels. Plausible TPs were identified according to the following criteria: i) mass accuracy tolerance < 5ppm; ii) isotopic pattern score > 70%; iii) peak area > 5 × 10⁶; iv) increasing trend over time, or first increase then followed by a decrease; v) absent in biological samples without MP addition or heat-inactivated controls. Sieve 2.2 software (Thermo Fisher Scientific) was used for non-target screening and the TP candidates were selected based on the same criteria. MS² fragment profiles of TP candidates were obtained using data-dependent MS/MS scan, to help elucidate TP structures. MarvinSketch (NET6.2.0, 2014) was used for drawing, displaying, and characterizing chemical structures, ChemAxon (<http://www.chemaxon.com>).

Ammonium, Nitrite, and Nitrate Measurements

Ammonia/ammonium was measured by a colorimetric method.⁹⁸ Nitrite was measured by photometry with the sulfanilamide N-(1-naphthyl) ethylenediamine dihydrochloride (NED) reagent method.⁹⁹ Nitrate was first reduced to nitrite by vanadium chloride, and then detected by the acidic Griess assay.¹⁰⁰ The detection limit for ammonium, nitrite, and nitrate was 10 μ M.

Quantitative PCR

Genomic DNA was extracted by DNeasy Blood & Tissue Kit (QIAGEN, Germantown, MD) according to manufacturer's instructions. Cell growth was measured by qPCR. Specific primers targeting the 16S rRNA genes of *N. europaea*, (forward: 5'-GCAGCAGAGGGGAGTGGAAAT-3', reverse: 5'-CGTGCATGAGCGTCAGTGTC-3'), and *Nitrobacter* sp. (forward: 5'-CCATGACCGGTTCGCAGAGAT-3', reverse: 5'-AACTAAGGACGGGGGTTGCG-3') were designed and validated using the web-based tool, Primer-BLAST (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>). FAST SYBR Green reagents (Thermo Fisher Scientific) were used for qPCR according to the manufacturer's instructions. In general, each 20- μ L reaction mixture contained 2.5 μ L of gDNA sample or serially diluted standard, 10 μ L 2 $\times$ Fast SYBR Green master mix, and 0.625 μ M of the forward and reverse primers. The PCR procedure included an initial deactivation at 95 $^{\circ}$ C for 20s, followed by 40 thermal cycles at 95 $^{\circ}$ C for 20s, then at 60 $^{\circ}$ C for 30s. Genomic DNA of *N. europaea* and *Nitrobacter* sp. were quantified by NanoDrop One (Thermo Fisher Scientific) and served as qPCR standards.

2.4 Results and Discussion

MP Biotransformation by the AOB/NOB Co-culture and the NOB Pure Culture

According to a previous study,⁷¹ the biotransformation of four selected compounds (i.e., asulam, monuron, clomazone, and trimethoprim) in a nitrifying activated sludge community was highly associated with nitrification (50 – 90% inhibition when nitrification was inhibited). To further demonstrate the causal relationship between nitrifiers and MP biotransformation, we used an AOB/NOB co-culture that mimics the complete nitrification system in WWTPs to investigate the biotransformation of the four selected MPs. This co-culture setup also reduces the potential for biologically mediated abiotic nitritation that has been observed for some MPs previously.^{91, 92} As AOB generally represent the dominant ammonia-oxidizers in municipal WWTPs,⁷⁷ the AOB model organism *N. europaea* was selected to construct the co-culture with the NOB model organism *Nitrobacter* sp.. *Nitrobacter* frequently occurs in nitrifying activated sludge.^{101, 102} However, it should be noticed that NOB of the genera *Nitrotoga*^{101, 103, 104} and *Nitrospira*¹⁰⁴⁻¹⁰⁶ often outnumber *Nitrobacter* in nitrifying activated sludge systems. As biomass production for biotransformation experiments is relatively slower with members of these genera, a *Nitrobacter* strain was selected for the experiments, but future studies should also include other NOB species. As expected, the co-culture converted all added ammonium to nitrate at a conversion rate of 2 mM per 8 hours with no nitrite accumulation. A relative removal of 20% of a MP was arbitrarily selected as a threshold for a positive biotransformation result to ensure that other factors like

measurement variations did not cause false-positive results. Among the four MPs, only asulam (as well as mianserin and ranitidine that were included as positive controls for MP biotransformation) were biotransformed (> 20% removal) by the co-culture during a 168-h incubation (Fig. 2.1A). Inconsistent with the activated sludge inhibition study,⁷¹ no significant transformation of monuron, clomazone, and trimethoprim was observed by the co-culture (Fig. A.1). Intracellular asulam concentrations of the co-culture in the biotransformation experiments were below the detection limit. This excludes active biosorption/uptake by living cells, a phenomenon observed for heavy metals.¹⁰⁷

To elucidate whether the NOB species *Nitrobacter* sp. contributed to the biotransformation in the co-culture, we tested the biotransformation of asulam, as well as all the other 16 investigated MPs by the *Nitrobacter* sp. pure culture. Although the nitrite oxidation of *Nitrobacter* sp. was not inhibited by the added MPs, there was no obvious biotransformation (> 20% removal) for any of the tested MPs over a 168-h incubation period (Fig. A.2). Therefore, although NOB was co-inhibited with AOB in previous inhibition studies due to the inhibited nitrite production,^{24, 30, 71, 108} they seem less likely contribute to the biotransformation of the selected MPs, which showed a correlation with nitrification activity. These results also indicate that the MP biotransformation in the co-culture was carried out by the AOB species *N. europaea*.

Although biotransformation of monuron, clomazone, and trimethoprim was strongly associated with nitrification in a previous study,⁷¹ these MPs were not biotransformed by the

ammonia oxidizers tested in this study. The inability of trimethoprim biotransformation by *N. europaea* is consistent with findings by Khunjar et al.²³ This discrepancy between pure culture studies and community inhibition studies is possibly because the nitrifying activated sludge in the inhibition study contained ammonia-oxidizers with different biotransformation capabilities. This seems less likely, given the same biotransformation compound specificity (i.e., of the four compounds, only asulam was biotransformed at a 144-h removal of 25% – 40%) observed for the two AOB species (*N. europaea* and *Nitrosomonas nitrosa* Nm90 isolated from activate sludge¹⁰⁹) and the very distantly related AOA species (*Nitrososphaera gargensis*)³³ tested in this study (Fig. A.3). Nevertheless, another recently discovered ammonia oxidizer, comammox was also detected in some WWTPs, which carries out complete ammonia oxidation to nitrate, and is physiologically distinct from the other ammonia oxidizers (i.e., AOB and AOA).¹¹⁰ Although beyond the scope of this study, it is worthwhile to investigate their roles in MP biotransformation in future studies. Another possible reason for the discrepancy between pure culture studies and community inhibition studies is that NOB species other than *Nitrobacter*, or heterotrophic microorganisms growing dependently on nitrifiers¹¹¹ in the nitrifying activated sludge were responsible for the biotransformation of the MPs that were not transformed by the tested ammonia oxidizers. For example, studies have indicated that trimethoprim can undergo various biotransformation pathways under both aerobic (nitrifying) and anoxic (denitrifying) conditions.¹¹² Therefore, it is highly likely that heterotrophic microorganisms physiologically interacting with nitrifiers carried

out the biotransformation of trimethoprim in nitrifying activated sludge communities. Although lack of previous bacterial biotransformation studies, the biotransformation of monuron and clomazone, similar with trimethoprim biotransformation was likely carried out by heterotrophs.

Transformation Product Identification

To identify TPs of asulam, suspect screening was first carried out. The self-compiled suspect list includes ~ 1000 compounds derived from asulam via various reactions, including conjugation at primary and secondary levels. By suspect screening, two asulam TPs were identified in the nitrifying co-culture, with exact masses of $[M-H]^-$ at 230.0129 (denoted “TP230”), and at 274.9979 (denoted “TP274”) (Fig. 2.1B). The formula of TP230 is $C_8H_9O_5NS$ ($-NH+O$ from asulam), and the formula of TP274 is $C_8H_8O_7N_2S$ ($-2H+3O$ from asulam). Both TPs were likely formed from oxidation reactions, and their hypothetical structures are proposed (Fig. A.4). TP230 was probably formed by the oxidation of the amine group ($-NH_2$) of asulam to a hydroxyl group ($-OH$), and TP274 likely contains a nitro group from oxidation of the amine group, as well as a hydroxyl group added on the aromatic ring (Fig. A.4). It is worth noting that the removal of asulam did not lead to a complete breakdown of the main structure into simpler molecules, as both identified TPs have similar or even larger masses than asulam. MS^2 analysis were further carried out to validate the proposed structures of the two TPs (Fig. A.4). However, the MS^2 fragments of the two TPs did not provide clear evidence for the position of the adding $-OH$ or nitro group. As the corresponding standard compounds with the proposed TP structures were unavailable, there

were no reference MS² fragmentation profiles to compare with, thus the exact TP structures remain unclear.

According to the peak areas, the two TPs account for ~ 40% of the removed asulam, resulting in an incomplete mass balance. Therefore, we further carried out non-target screening to identify TP candidates that might be missing from the suspect screening. However, except the above two TPs, no additional plausible TP was detected. We also analyzed possible TPs in the intracellular samples using suspect and non-target screening. According to the same screening criteria, no TP candidate was detected in the intracellular samples, which made it unlikely of active uptake of TPs into living cells. As asulam is a relatively small molecule (m/z 231.0434, $[M+H]^+$), possible reasons for the incomplete mass balance include: (1) formation of even smaller molecules that are not detectable by LC-MS; (2) asulam and the two TPs were detected in two ESI modes (positive for asulam, and negative for the TPs). The ionization efficiency under negative mode is usually lower than positive mode, resulting in lower response intensities for the same concentration of compounds.

The incomplete biotransformation of asulam and poor mass balance nicely illustrate that it is crucial to elucidate TP profiles during transformation processes to obtain a comprehensive understanding of the environmental fate of parent MPs. Future research is needed to better understand the ecological impact and putative health risks associated with TPs from incomplete MP transformation.

Co-metabolic Biotransformation of Asulam

To investigate the biotransformation mechanism of asulam by *N. europaea* in the nitrifying co-culture, limited ammonium (one-time addition of 0.05 mM NH₄Cl at the beginning of the experiment) was added to the co-culture only as the nitrogen source, and asulam was added as a putative energy source. Under these conditions, no asulam biotransformation was observed (Fig. 2.2A), with no ammonia oxidation (Fig. 2.2B) and little cell growth (Fig. 2.2C). In comparison, asulam was continuously biotransformed with active ammonium oxidation and cell growth in the control with ammonium as the energy and nitrogen source (Fig. 2.2). These results suggest that asulam biotransformation by *N. europaea* in the co-culture occurred via co-metabolism. Similarly, previous studies have shown that AOB can co-metabolically biotransform a number of other MPs, which has often been hypothesized to be carried out by AMO given its broad substrate spectrum.^{1, 73, 74, 90, 113-115} However, experimental support for this hypothesis is scarce.

Experimental Support for AMO as the Responsible Enzyme for Asulam Biotransformation.

To investigate whether AMO is the responsible enzyme for asulam biotransformation, inhibitory effects of asulam on ammonia oxidation was first examined. Compared to the no-asulam control, no inhibition of ammonia oxidation was observed for asulam at 10, 20, and 30 µg/L (Fig. A.5). The ammonia oxidation rate started to decrease with 40 µg/L asulam by 4.7%, and the inhibition increased up to 15.5% at a concentration of 80 µg/L (Fig. A.5). Interestingly, the

inhibitory effect of asulam on ammonia oxidation decreased gradually after re-feeding the co-culture with ammonium (Fig. A.5). This is possibly because, the ratio of ammonia to asulam increased after re-feeding with ammonium as asulam was removed over time which led to a decrease in potential substrate competition for AMO. Moreover, newly synthesized AMO during cell growth could also lead to the recovery of ammonia oxidation with a longer incubation. The inhibitory effect of asulam on ammonia oxidation suggested that asulam might be a competitive substrate for AMO, which was likely the responsible enzyme for asulam biotransformation. A substantial inhibitory effect of asulam was observed at 80 µg/L (c.a., 348 nM) (Fig. A.5), indicating that asulam possibly has an even higher affinity to AMO than ammonia (the ammonia affinity of the AMO of *N. europaea* is reported between 20 – 70 µM NH₃, c.a., ~ 330 – 1200 µM of total ammonium at pH 8).¹¹⁰

In addition, a specific mechanistic inhibitor of bacterial AMO, octyne,^{93, 94} was used to further examine whether AMO was the responsible enzyme for the cometabolic asulam biotransformation. Octyne was added after 72 hours during asulam biotransformation in the nitrifying co-culture. As the addition of octyne resulted in instantaneous inhibition of AMO activity, we hypothesized that, if AMO was the responsible enzyme, immediate inhibition of asulam biotransformation would occur after octyne addition, while if other enzymes carried out the biotransformation, a lagged inhibition would be expected as their activities likely persist for a while after AMO inhibition.⁸⁸ Ammonia oxidation and asulam transformation was determined 2

hours after the octyne addition. Compared to the no-octyne-treated control, in the octyne-treated co-culture, complete inhibition of ammonia oxidation was observed (Fig. 2.3A), and asulam biotransformation also completely ceased (Fig. 2.3B). Consistently, no growth of AOB and NOB occurred after octyne exposure (Fig. 2.3C&D). These results provide strong support for the assumption that AMO is the enzyme involved in asulam biotransformation by *N. europaea* in the co-culture.

Abiotic MP Transformation by NH₂OH, the Intermediate Formed by AMO

In addition to being converted by AMO as non-specific substrates, it is also possible that MPs abiotically react with hydroxylamine, the product of ammonia oxidation by AMO.⁹⁶ In order to test this, we conducted abiotic MP transformation experiments with the addition of NH₂OH at concentrations occurring in AOB batch cultures.⁹⁶ We also tested MP transformation by NO, a non-AMO-mediated ammonia oxidation intermediate of AOB. NO can be formed by nitrite reductase (NirK) in AOB.¹¹⁶⁻¹¹⁸ Moreover, it is produced as an obligate intermediate by hydroxylamine dehydrogenase (HAO) of AOB.^{119, 120} All 15 MPs and the two control MPs mianserin and ranitidine were exposed to hydroxylamine and NO, respectively. The abiotic transformation was compared to the biological transformation by the AOB/NOB co-culture (Fig. 2.4). Eight MPs were biotransformed by the AOB/NOB co-culture (Fig. 2.4, Fig. A.6), whereas no biotransformation was observed in the tested NOB pure culture for all MPs (Fig. A.2). Six out of the eight MPs were also transformed abiotically in NH₂OH-added fresh medium (with even

higher removals), and only rufinamide and bezafibrate did not exhibit transformation by NH_2OH (Fig. 2.4). In contrast, only three of the eight MPs, i.e., indomethacin, ranitidine and fenhexamid, were transformed by NO, taken into consideration the abiotic removal of mianserin and indomethacin (61% and 22%, respectively) in fresh medium with no NH_2OH or NO addition (Fig. 2.4). Transformation of those three compounds by NO were less pronounced than by NH_2OH (Fig. 2.4). Furthermore, one cannot exclude the possibility that the transformation of the three MPs was attributed to reactions with the NONOate residual rather than the released NO.

Further analyses on TPs focused on MPs transformed by NH_2OH . For asulam, TP230 and TP274 identified during biotransformation in the co-culture were also detected in the NH_2OH -treated fresh medium. The similar compound specificities and TP profiles between biotransformation and NH_2OH -mediated abiotic transformation suggest that abiotic MP transformation by NH_2OH might be an important transformation route indirectly mediated by AMO, which was not recognized previously. The abundance of TP230 in the NH_2OH -treated fresh medium after 168 hours was higher than that in the co-culture when adding the same concentration of asulam (20 $\mu\text{g/L}$) (Fig. 2.5). One should notice that more asulam was converted in the NH_2OH -treated fresh medium (> 80%) than in the co-culture (~ 60%) after 168 hours (Fig. 2.4), which might result in the higher formation of TP230 in the NH_2OH -treated fresh medium. In other words, if asulam removal in the co-culture reached the same level as that in the NH_2OH -treated fresh medium, the abundance of TP230 under both conditions would be similar. Under the assumption

that the abundance of TPs formed from non-enzymatic transformation by NH_2OH in the co-culture was similar as that observed in fresh medium with NH_2OH addition, this result suggests that the formation of TP230 was more likely via the NH_2OH -mediated abiotic transformation route. Nevertheless, the abundance of TP274 in the AOB/NOB co-culture was substantially higher than that in the NH_2OH -treated fresh medium (Fig. 2.5), which indicates the co-occurrence of non-enzymatic and enzymatic MP transformation pathways for the formation of TP274.

Among the other five MPs (i.e., mianserin, indomethacin, ranitidine, furosemide, and fenhexamid) transformed abiotically in NH_2OH -added fresh medium, no plausible TP could be detected for indomethacin, furosemide, and fenhexamid in both the AOB/NOB co-culture and the NH_2OH -added fresh medium. For mianserin and ranitidine, consistent with the previous study,¹ the same TPs: TP279 for mianserin, and TP273, TP289, TP330 for ranitidine were detected in the AOB/NOB co-culture, as well as in the NH_2OH -added fresh medium, but at lower concentrations compared to those in the AOB/NOB co-culture (Fig. A.7). One possible reason is that in the AOB/NOB co-culture the same TPs were formed via both AMO-mediated biotransformation and NH_2OH -mediated abiotic transformation, leading to a higher TP formation.

Moreover, by suspect screening, two major TPs of rufinamide with exact masses of $[\text{M}+\text{H}]^+$ at 240.0597 (TP240), and 312.0790 (TP312) (Fig. A.8) detected in the AOB/NOB co-culture were not detected in the NH_2OH -treated fresh medium, which was in agreement with no transformation of rufinamide by NH_2OH abiotically (Fig. 2.4).

With NH_2OH treatment, it is surprising that the identified TPs were in oxidized forms of the respective MPs, given that NH_2OH is nucleophilic and typically serves as a reducing agent. Moreover, no reduced forms of asulam TPs could be detected when targeting the corresponding TP suspects based on reported reductive reactions of NH_2OH with compounds that also have carboxylic ester functional groups as asulam.¹²¹ The oxidized forms of TPs were probably formed from reactions of the MPs with some oxidizing intermediates from the reaction of NH_2OH with dissolved O_2 . In this context, it is interesting to note that although the initial dose of NH_2OH (5 μM) was in excess compared to the MP concentrations ($\sim 0.06 - 0.12 \mu\text{M}$), the abiotic transformation of MPs by NH_2OH did not reach the highest level after 12 hours with the addition of a single dose of NH_2OH , but further increased upon reamendment of NH_2OH during a longer exposure (Fig. 2.4). This could be due to faster degradation of NH_2OH when reacting with dissolved O_2 than reacting with the MPs, or due to the possibility that the MPs reacted with intermediates from NH_2OH decomposition in water, which could be formed at lower concentrations. However, the exact pathways and mechanisms of NH_2OH -mediated abiotic MP transformation need to be further elucidated.

2.5 Conclusion

Nitrifiers are ubiquitously distributed in natural environments, as well as engineered systems such as WWTPs. Although positive correlations have been previously observed between MP biotransformation and nitrification activities,⁷¹ the responsible enzymes and involved

pathways are not well understood. Here, we demonstrate that the NOB *Nitrobacter* sp. did not contribute to the biotransformation of all tested MPs whose biotransformation was associated with nitrification activity according to nitrifying activated sludge inhibition studies.⁷¹ We also show that only 6 of 15 MPs, whose biotransformation positively correlated with the activity of nitrifiers in a nitrifying activated sludge community were transformed by the AOB species in an AOB/NOB (*N. europaea*/*Nitrobacter* sp.) co-culture. Similar results were observed in other studies,^{23, 73, 108} where more MPs were biotransformed by complex communities such as nitrifying activated sludge than by various pure ammonia-oxidizing cultures, including AOB and AOA. Besides potential contributions of other physiologically diverse ammonia oxidizers and NOB, including the recently discovered complete ammonia oxidizers^{82, 83} to MP biotransformation, it is highly likely that heterotrophic bacteria interactively co-existing with nitrifiers also make important contributions to MP transformation in nitrifying activated sludge. Thus, keeping the diversity and complexity in biological systems could be important to obtain a higher overall removal of MPs. Further studies are needed to investigate effects of microbial diversity on MP biotransformation, and to identify the key heterotrophs that co-exist with nitrifiers and contribute to MP biotransformation.

Importantly, we present evidence favoring that AMO of *N. europaea* is the responsible enzyme for asulam biotransformation, which likely occurred via co-metabolism. Furthermore, we report for the first time that abiotic MP transformation by hydroxylamine, the product of AMO-mediated ammonia oxidation, likely represents another transformation mechanism of MPs by

ammonia oxidizers. It expands our knowledge on MP transformation pathways and highlights that the reactive intermediates of different environmental microbial guilds might play an important yet understudied role in MP transformation. As an $\text{NH}_4\text{-N}$ concentration (2 mM, c.a. 28 mg/L as N) relevant to typical influent of municipal WWTPs was used in the batch reactors of this study, similar MP transformation pathways might be occurring in nitrifying activated sludge in continuous flow reactors in WWTPs. However, it needs to be further investigated whether the actual free NH_2OH available for abiotic MP transformation in WWTPs was similar with that in lab-scale batch reactors. Altogether, these findings give a better understanding of roles played by nitrifiers (i.e., AOB and NOB) in MP biotransformation, the responsible enzyme, and the underlying mechanisms, which will provide important insights into the fate of MPs in various nitrifying environments.

2.6 Special Acknowledgements of Chapter 2

I would like to acknowledge that this work has been published in *Environmental Science and Technology*¹²² and has been previously included in my *M.S. Thesis*.¹²³ It is one of the most important works during my *M.S. + Ph.D.* program, which was not only the first study investigating the responsible enzymes of ammonia-oxidizing bacteria in biotransformation micropollutants but also a transition chapter for the following chapters in studying the biotransformation mechanisms of various micropollutants by different types of microbial consortia. After discussing with my

advisor Prof. Yujie Men and consulting with UIUC Graduate College, I decided to include this work here as the second chapter of my Ph.D. dissertation.

2.7 Figures of Chapter 2

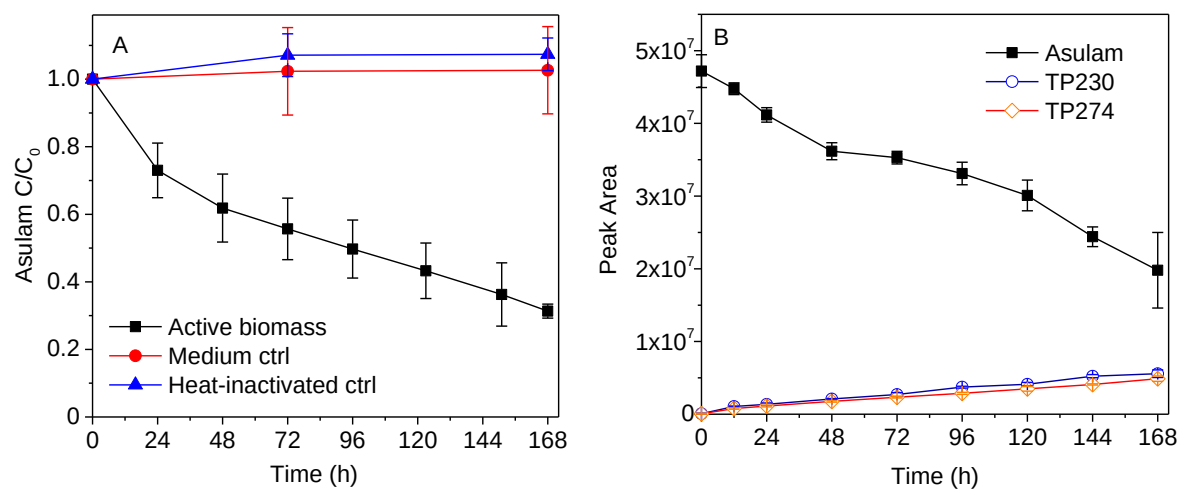


Figure 2.1 Biotransformation (A) and TP formation (B) of asulam (initial concentration: 10 µg/L) in the AOB/NOB co-culture (n=3; TP274 and TP230 were not detected in the medium or the heat-inactivated control).

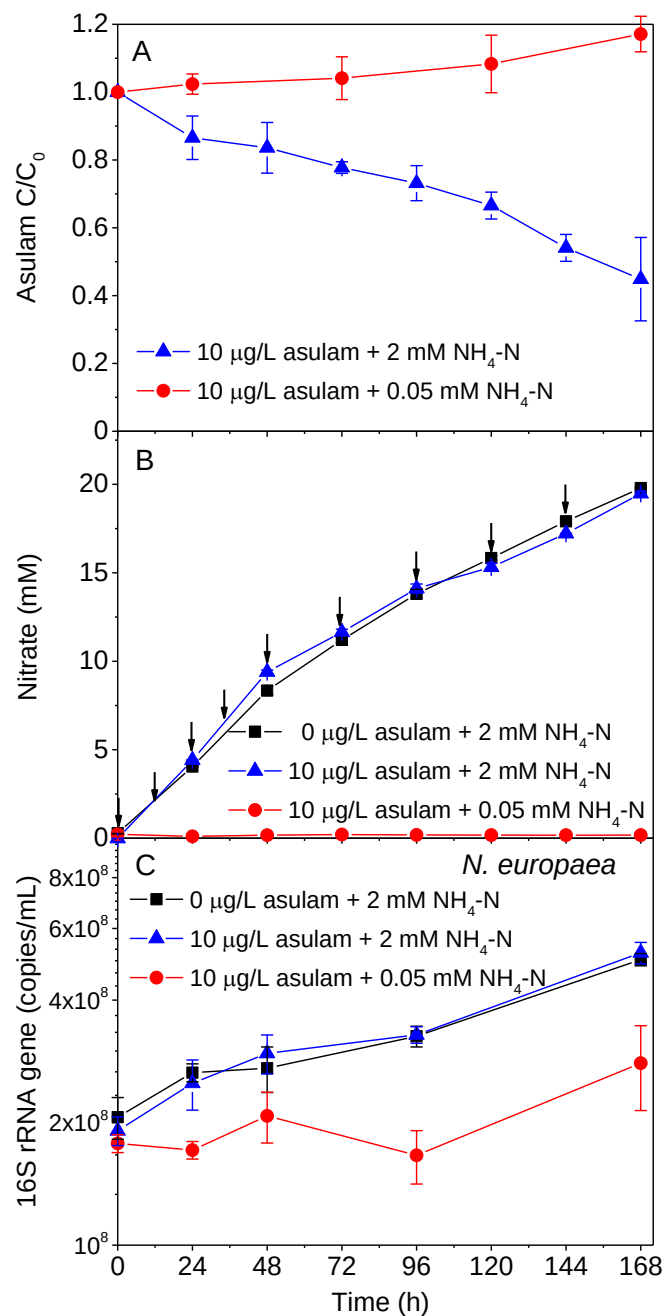


Figure 2.2 Asulam biotransformation by the AOB/NOB co-culture grown with reamended and limited NH₄-N (A: asulam removal; B: nitrate formation; C: cell growth of the AOB *N. europaea*; 2 mM ammonium was added repeatedly as indicated by arrows; 0.05 mM ammonium was only added at 0 h; n=3).

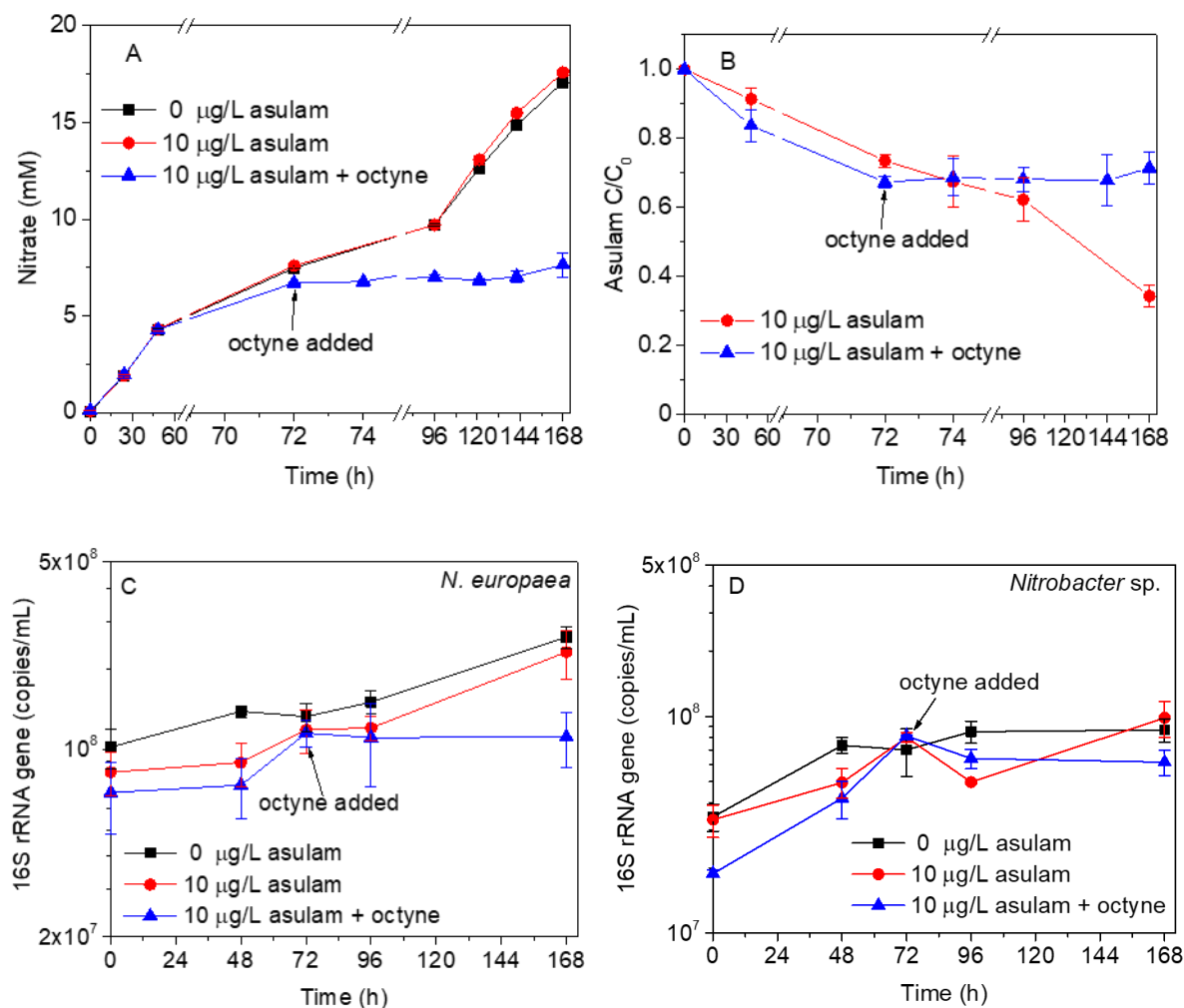


Figure 2.3 Nitrate formation from a total of 14 mM ammonium addition (nitrite was below detection limit under all conditions) (A), asulam removal (B), growth of *N. europaea* (C) and *Nitrobacter* sp. (D) in the AOB/NOB co-culture with and without the addition of octyne (72h results were measured before octyne addition, $n=3$).

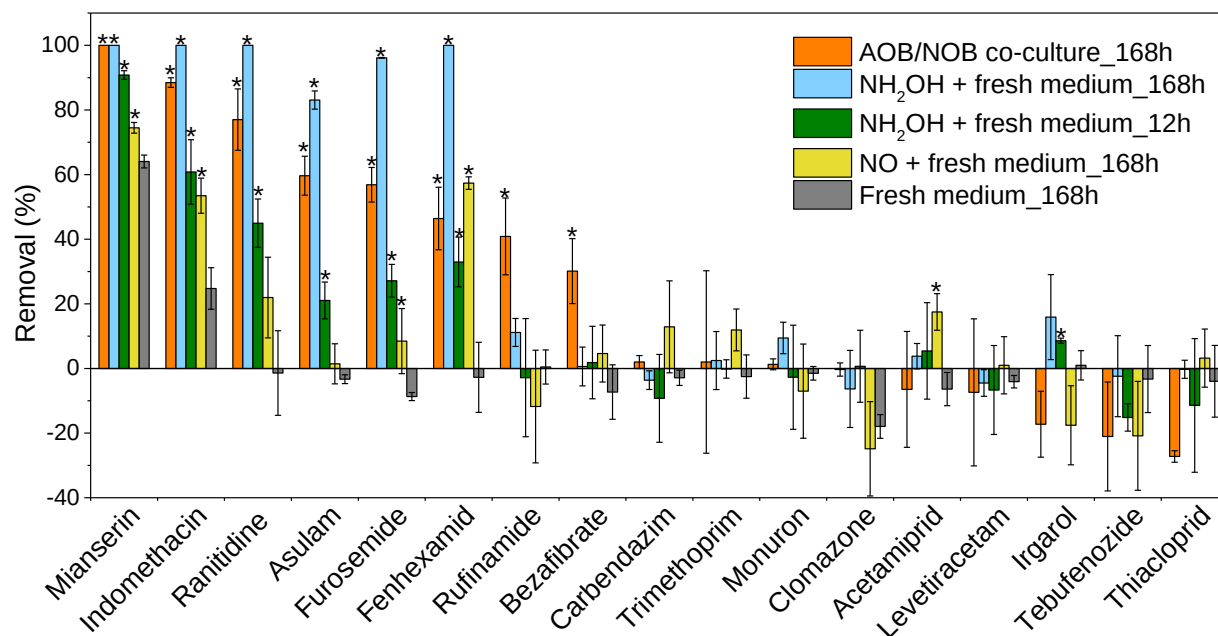


Figure 2.4 Comparison of MP transformation in NH₂OH- or NO-added sterile fresh medium and in the AOB/NOB co-culture (* indicates significant difference from the 168h removal in the fresh medium control, $p < 0.05$, $n = 3$).

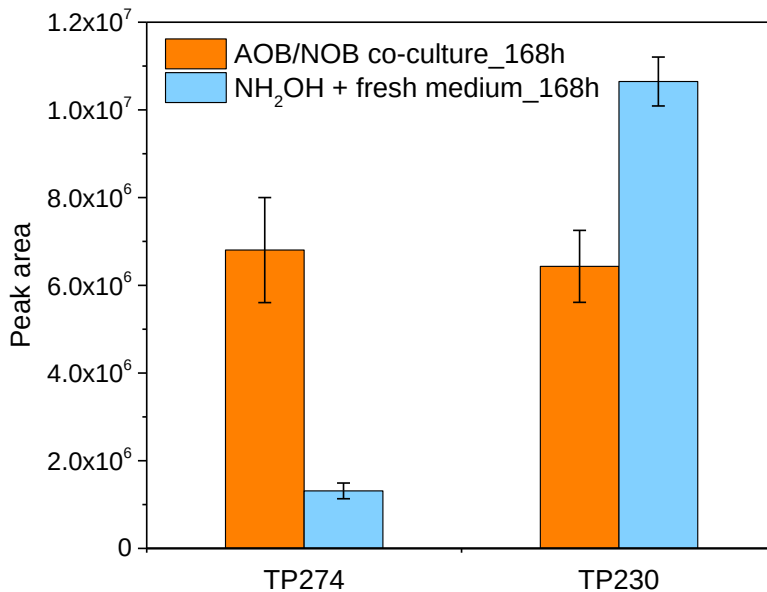


Figure 2.5 Formation of asulam TPs in the AOB/NOB co-culture and in the NH₂OH-treated fresh medium after a 168-h incubation with the same addition of asulam (neither of the two TPs was detected in the medium or heat-inactivated biomass control, $n=3$).

CHAPTER 3: SPECIFIC MICROPOLLUTANT BIOTRANSFORMATION PATTERN BY THE COMAMMOX BACTERIUM *NITROSPIRA INOPINATA*

3.1 Abstract

The recently discovered complete ammonia-oxidizing (comammox) bacteria are occurring in various environments, including wastewater treatment plants. To better understand their role in micropollutant biotransformation in comparison with ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA), we investigated the biotransformation capability of *Nitrospira inopinata* (the only comammox isolate) for 17 micropollutants. Asulam, fenhexamid, mianserin, and ranitidine, were biotransformed by *N. inopinata*, *Nitrososphaera gargensis* (AOA) and *Nitrosomonas nitrosa* Nm90 (AOB). More distinctively, carbendazim, a benzimidazole fungicide, was exclusively biotransformed by *N. inopinata*. The biotransformation of carbendazim only occurred when *N. inopinata* was supplied with ammonia but not nitrite as the energy source. The exclusive biotransformation of carbendazim by *N. inopinata* was likely enabled by an enhanced substrate promiscuity of its unique AMO and its much higher substrate (for ammonia) affinity compared with the other two ammonia oxidizers. One major plausible transformation product (TP) of carbendazim is a hydroxylated form at the aromatic ring, which is consistent with the function of AMO. This TP was further transformed, and smaller linear TP candidates were identified. These findings provide fundamental knowledge on the micropollutant degradation potential of a comammox bacterium, to better understand the fate of micropollutants in nitrifying environments.

3.2 Introduction

Micropollutants, including pesticides, pharmaceuticals, and personal care products, which occur at low levels (ng – µg/L) are a considerable environmental concern due to their potential adverse effects on ecosystems and human health.^{8, 70, 124, 125} Therefore, it is important to obtain an in-depth understanding of the environmental fate of these compounds. Biotransformation of

micropollutants plays an essential role in their removal, particularly in wastewater treatment plants (WWTPs) and has recently been extensively investigated.^{20, 70, 126} More specifically, biotransformation of various micropollutants is associated with ammonia oxidation activity of nitrifying activated sludge and biofilms in WWTPs.^{16, 17, 20, 21, 29, 71, 87, 127, 128} Consistently, biotransformation of some micropollutants is catalyzed by enriched or pure cultures of ammonia-oxidizing microbes.^{25, 129}

Ammonia-oxidizing bacteria (AOB) are typically the dominant ammonia oxidizers in municipal WWTPs.¹³⁰⁻¹³² Ammonia-oxidation is initiated by ammonia monooxygenases (AMO), a substrate promiscuous key enzyme^{90, 133-135} of all known ammonia oxidizers. Some AOB metabolically or co-metabolically catalyze the transformation of various compounds including polycyclic aromatic hydrocarbons and pharmaceuticals.^{108, 126, 128, 133, 134, 136} Besides AOB, ammonia-oxidizing archaea (AOA) have also been molecularly detected and/or cultured in nitrifying activated sludge of some industrial and municipal WWTPs.^{78, 132, 137-139} In 2015, complete ammonia oxidizers (comammox), which as single organisms oxidize ammonia all the way to nitrate via nitrite, were discovered.^{32, 82} Phylogenetic analyses revealed that the *amoA* genes of comammox bacteria differ from those of known AOB and AOA species, and represent a distinct lineage of AMO enzymes within this enzyme family⁸². All known comammox bacteria belong to the genus *Nitrospira* and have been detected in various environments including WWTPs.^{32, 82, 140-142} *Nitrospira inopinata* is the only comammox isolate available to date, and has a much higher affinity for ammonia than many AOA and all tested AOB.¹¹⁰ The distinct enzymatic repertoire of comammox bacteria as well as their high substrate affinity might enable them to perform important ecological services in natural and engineered environments, which have not yet been well understood.

A series of recent studies provided additional insights into the mechanisms behind micropollutant degradation by AOB and AOA.^{27, 129} Two AOB strains *Nitrosomonas nitrosa* Nm90, *Nitrosomonas* sp. Nm95 isolated from industrial sewage,¹⁴³ and the AOA strain *Nitrososphaera gargensis* isolated from a hot spring effluent mat,^{33, 144} showed biotransformation of two pharmaceuticals (mianserin and ranitidine) through co-metabolism likely mediated by AMO upon ammonia oxidation.¹²⁹ Consistently, biotransformation of 31 out of 79 structurally different micropollutants was inhibited after application of AMO inhibitors in a nitrifying activated sludge community.⁷¹ A follow-up study revealed that a portion of micropollutants whose biotransformation was associated with nitrification was biotransformed by the AOB *Nitrosomonas europaea* via both cometabolic biotransformation by AMO and abiotic transformation by hydroxylamine, an intermediate produced by AMO during ammonia oxidation.¹⁴⁵

Nothing is yet known about the role of comammox bacteria in the turnover of micropollutants. Differences in biotransformation activities of these microbes compared to AOB and AOA might be expected, as they possess unique AMO enzymes and the complete enzyme set for nitrification. As comammox bacteria are widespread in WWTPs, targeted studies exploring their potential contribution to micropollutant biotransformation associated with nitrification are needed, to better understand the fate of those micropollutants during wastewater treatment and in other nitrifying environments. The goal of this study was to expand the fundamental knowledge on micropollutant biotransformation potential of different ammonia oxidizers in isolation, with a focus on the newly discovered comammox bacteria. We selected 17 commonly used pharmaceuticals and pesticides whose biotransformation was inhibited by more than 20% when adding AMO inhibitors in a nitrifying activated sludge community.⁷¹ The biotransformation of these micropollutants by the comammox isolate *N. inopinata* was tested and compared with the

biotransformation by the AOA *N. gargensis* and the AOB *N. nitrosa* Nm90. Two pharmaceuticals (mianserin and ranitidine) that are biotransformed by *N. gargensis* and *N. nitrosa* Nm 90¹²⁹ were also included to study their degradability by *N. inopinata*. Major transformation products (TPs) were identified. The mechanism of MPs biotransformation by *N. inopinata* and the responsible enzyme(s) were further investigated.

3.3 Materials and Methods

Micropollutant Selection

The target micropollutants (i.e. acetamiprid, asulam, bezafibrate, carbendazim, clomazone, fenhexamid, furosemide, indomethacin, irgarol, levetiracetam, mianserin, monuron, ranitidine, rufinamide, tebufenozide, thiachloprid, and trimethoprim) were selected based on the following criteria: 1) their biotransformation was inhibited > 20% by AMO inhibitors in a previous activated sludge study;⁷¹ 2) the reference standards are commercially available. A complete list of the investigated micropollutants and their applications can be found in [Table B.1](#). The reference standards of selected micropollutants were purchased from Sigma-Aldrich (St. Louis, MO), and Toronto Research Chemicals (Toronto, Canada). Five internal standards including trimethoprim-d3, metoprolol acid-d5, carbendazim-d4, and furosemide-d5 were obtained from C/D/N Isotopes (Quebec, Canada) and Toronto Research Chemicals (Toronto, Canada). Stock solutions of all tested compounds were prepared in methanol with concentration of 1 g/L except carbendazim (0.25 g/L) and stored at – 20 °C until use. Given the potential inhibitory effects of these micropollutants on ammonia oxidizers,^{129, 145} we divided the 17 micropollutants into two groups (i.e., Group A and Group B) to reduce the total number of micropollutants and the total concentration in the mixtures. Micropollutants in each group were added as a mixture.

Cultivation of Microorganisms

All strains were maintained in the same modified basal medium¹¹⁰ with 4 g/L CaCO₃ for buffering the pH at ~ 8.0. *N. inopinata* was incubated at 42 °C in the dark without shaking, and 2 mM NH₄Cl was added as growth substrate periodically upon depletion (~ every 7 days). *N. gargensis* was incubated at 46 °C in the dark without shaking, and 2 mM NH₄Cl was added as growth substrate ~ every 6 days. *N. nitrosa* Nm90, was obtained from the AOB strain collection of the University of Hamburg (Germany),¹⁴³ and was incubated at 37 °C in the dark with shaking at 90 rpm, amended with 2 mM NH₄Cl every ~ 6 days. The nitrite-oxidizing bacterium *Nitrospira defluvii*¹⁴⁶ was incubated at 37 °C in the dark without shaking, and 1 mM NaNO₂ was added as growth substrate every ~ 7 days.

Micropollutant Biotransformation Screening

The micropollutant biotransformation capabilities of the comammox *N. inopinata*, AOA *N. gargensis* and AOB *N. nitrosa* Nm90 were investigated in batch experiments. Pre-grown biomass was harvested by centrifugation at $7,380 \times g$ for 30 min at 10 °C, the pellet was re-suspended in fresh medium to obtain 2-fold concentrated biomass. In our previous study, the concentration of MPs in WWTPs ranges from several ng/L to less than a hundred µg/L.¹⁶ Thus, the corresponding volume of mixed micropollutant stock solutions to reach an initial concentration of 20 µg/L (for each micropollutant) was first added into empty sterile bottles. After the organic solvent methanol was evaporated, 25 mL of thoroughly mixed concentrated culture with 2 mM NH₄Cl was inoculated into 100 mL glass bottles, respectively. NH₄Cl was re-added back to 2 mM when below 1 mM. In order to fundamentally understand MP biotransformation capabilities of each strain, cultures were incubated at their optimal growth temperatures for *N. inopinata* (42 °C), *N. gargensis* (46 °C) and *N. nitrosa* Nm90 (37 °C). First time point samples (0.5 mL) were taken immediately after biomass addition and then centrifuged at $16,240 \times g$ at 4 °C for 10 min, 0.3 mL

of supernatant was transferred into 2 mL amber glass vials (with 300 µl-inserts), capped and stored at 4 °C in dark until LC-HRMS/MS analysis (max. 30 days of storage until analysis), while 0.1 ml of supernatant was transferred into 1.5 mL Eppendorf tubes and stored at – 20 °C for further chemical measurements. The cell pellets were stored at – 20 °C for total protein measurement. Subsequent samples were taken in the same way for *N. inopinata* at 18, 24, 48, 72, 96, 144, 240, and 336 h, *N. gargensis* at 4, 24, 48, 72, 96, 144, and 240 h and *N. nitrosa* Nm90 at 16, 24, 48, 72, 94, 144, 240, 336, and 504 h. The incubation period is relevant to typical solid retention times (6 – 16 days) in suspended growth nitrifying reactors of WWTPs.¹⁴⁷

Abiotic (without added biomass, with 4 g/L CaCO₃) and heat-inactivated biomass (121°C and 103 kPa for 20 min) control experiments were set up in the same way as biotransformation experiments, to examine the sorption potential of the selected micropollutants to the CaCO₃ precipitates in fresh medium and to the biomass, respectively, as well as the abiotic transformation potential at 46 °C (the highest temperature for cultivating the tested strains). For abiotic and heat-inactivated biomass controls, 2 mM ammonium and 6 mM nitrite were added (for *N. inopinata* controls, 6 mM nitrate was also added) to mimic the highest N levels in the biological samples and investigate possible abiotic transformation in the presence of ammonium, nitrite and nitrate. Samples were taken at the same time points during the same incubation period as for the biotransformation experiments. In parallel, cultures amended with ammonium only (no micropollutant addition) were also set up as the positive control for ammonia oxidation activity. All experiments were performed in triplicates.

Biotransformation of Carbendazim by N. inopinata

To confirm the biotransformation of carbendazim by *N. inopinata*, the same biotransformation experiments were set up with an individual addition of carbendazim (40 µg/L)

and inoculated with 2 – 4 times concentrated biomass. To test whether the biotransformation of carbendazim by *N. inopinata* was associated with ammonia and/or nitrite oxidation, experiments were designed providing *N. inopinata* ammonium or nitrite as the sole energy source. Biotransformation experiments were set up using concentrated *N. inopinata* biomass under the following conditions: 1) 2 mM NH₄-N (with re-amendment upon depletion to a total of 10 mM), with and without carbendazim; 2) 0.5 mM NaNO₂ (with re-amendment upon depletion to a total of 2.5 mM), 0.05 mM NH₄-N, with and without carbendazim; 3) 0.5 mM NaNO₂ (with re-amendment upon depletion to a total of 2.5 mM), 0.05 mM NH₄-N and 10 µM allylthiourea (ATU, a common AMO inhibitor resulting in a complete inhibition of ammonia oxidation at 10 µM), with and without carbendazim, and 4) 1 mM NH₄-N and 10 µM ATU (for ammonia oxidation inhibition control). We used 0.5 mM NaNO₂ as the accumulation of nitrite during complete ammonia oxidation by *N. inopinata* under these conditions can reach this level. We used ATU to inhibit comammox AMO by copper depletion, rather than a mechanistic inhibitor specific to AOB such as octyne because the specificity of octyne to comammox AMO has not yet been investigated. Control experiments with heat-inactivated *N. inopinata* biomass were set up with the addition of 2 mM NH₄Cl, 0.5 mM NaNO₂, 6 mM NaNO₃, 10 µM ATU, and 40 µg/L carbendazim. Time series samples were taken over a 29-day incubation period. The two other ammonia oxidizers (*N. gargensis* and *N. nitrosa* Nm90), as well as the nitrite-oxidizing *N. defluvii* isolated from a WWTP,¹⁴⁸ were tested with individual carbendazim compound addition for comparison. Cultures were amended with 2 mM NH₄-N and 2 mM NO₂-N (with re-amendment back to 2 mM when below 1 mM) for ammonia-oxidizers and the nitrite-oxidizer, respectively. Samples were taken in time series over a 14-day incubation period.

Ammonium, Nitrite and Nitrate Measurements

NH_4^+ , NO_2^- and $\text{NO}_2^-/\text{NO}_3^-$ (NO_x) concentrations were all measured colorimetrically. $\text{NH}_4\text{-N}$ was measured by a colorimetric method.¹⁴⁹ Standards were prepared in the medium and ranged from 100 to 2000 μM NH_4Cl . Nitrite was measured using a sulfanilamide N- (1-naphthyl) ethylenediamine dihydrochloride (NED) reagent method.¹⁵⁰ Nitrate was reduced to nitrite by Vanadium chloride and measured as NO_x by the Griess assay.¹⁵¹ Standards were prepared in the medium and ranged from 100 to 2000 μM NO_x and from 100 to 2000 μM nitrite. All colorimetric analyses were performed using an Infinite 200 Pro spectrophotometer (Tecan Group AG, Männedorf, Switzerland).

Total Protein Measurement

Total protein was measured using a Pierce BCA Protein Assay Kit (Thermo Scientific, Regensburg, Germany) according to the manufacturer's instructions.

Estimation of Kinetic Parameters

To quantitatively compare biotransformation activities among biological samples, we estimated biotransformation rate constants (k_{bio}) normalized to total protein (Table B.2) by using a first-order model described previously,¹²⁹ which incorporates abiotic sorption and transformation, and biotransformation processes.^{152, 153} A Bayesian fitting procedure was used for parameter estimation as described elsewhere.¹⁵² The median value calculated from the fitting procedure was used as the estimated k_{bio} , with the 5% and 95% percentile values representing the estimation uncertainty. The fitting quality was evaluated by plotting measured data against model predictions including 90% credibility intervals and by the root-mean-square errors.

Analytical Method

Micropollutants as well as their transformation products (TPs) were analyzed by liquid chromatography coupled to a high-resolution quadrupole orbitrap mass spectrometer (LC-

HRMS/MS) (Q Exactive, Thermo Fisher Scientific) as described previously.¹⁴⁵ In general, 50 μ L sample was loaded onto a C₁₈ Atlantis- T3 column (particle size 3 μ m, 3.0 $\times$ 150 mm, Waters), and eluted at a flow rate of 350 μ L/min with nanopure water (A) and acetonitrile (B) (both amended with 0.1% formic acid), at a gradient as follows: 5% B: 0 – 1 min, 5% – 100% B: 1 – 8min, 100% B: 8 – 20 min, and 5% B: 20 – 26 min. Compounds in the eluate were measured in full scan mode on HRMS at a resolution of 70,000 at m/z 200 and a scan range of m/z 50 – 750 in a positive/negative switching mode. The data-dependent MS² using exact masses of identified transformation products (TPs) was triggered after each full scan with a resolution of 17,500 at 200 m/z at a normalized collision energy of 40. Dynamic exclusion technology was used to accurately capture MS² spectra of each TP.

Cell Extraction for Intracellular Micropollutant Concentration Measurement

A cell extraction procedure from a previous study⁹⁷ was adopted with slight modification. Briefly, internal standards were spiked in cell pellets collected from 10 mL culture (at a final concentration of 4 μ g/L for each) followed by an addition of 2 mL lysis solvent containing methanol (0.5% formic acid): nanopure water (0.1% w/w EDTA), 50: 50 (v/v). The cells were disrupted by ultrasonication at 50 °C for 15 min, centrifuged at 8,000 $\times g$ for 10 min. The supernatant was collected in a glass vial. This procedure was repeated for two more times for a better recovery. Finally, $\sim$ 6 mL supernatant was evaporated to dryness under a gentle steam of dinitrogen gas at 40 °C. The analytes were re-dissolved in 0.5 mL filter-sterilized fresh medium without CaCO₃, which were then centrifuged at 8,000 $\times g$ at 4 °C for 10 min. The supernatant was collected for LC-HRMS measurement.

Transformation Product (TP) Identification

As described in previous studies,^{129, 145, 154} both suspect screening and non-target screening were carried out to identify TPs. Generally, suspect screening was performed by TraceFinder 4.1 EFS software (Thermo Scientific). TP suspect lists were compiled using an automated metabolite mass prediction script, which considered a number of known redox and hydrolysis reactions, as well as several conjugation reactions at primary and secondary levels. Plausible TPs were identified according to the following criteria: i) isotopic pattern score > 70%; ii) peak area > 5×10^6 ; iii) increasing trend over time, or an increase followed by a decrease; iv) absent or at lower levels in biological samples without micropollutant addition and in heat-inactivated controls. Sieve 2.2 software (Thermos Scientific) was used for non-target screening and the TP candidates were selected based on the same criteria. MS² fragment profiles of TP candidates were obtained using data-dependent MS/MS scan, which were used to elucidate TP structures. MarvinSketch (NET6.2.0, 2014) was used for drawing, displaying and characterizing chemical structures (ChemAxon, <http://www.chemaxon.com>).

3.4 Results and Discussion

Micropollutant Biotransformation by N. inopinata

First, we tested for absorption of the selected micropollutants to CaCO₃ precipitates and for abiotic transformation in the commonly used basal medium³³ containing ammonium, nitrite and nitrate at the highest possible concentrations as those in the biological samples. MP abiotic transformation tests were performed at the highest incubation temperature (46 °C), and we assumed absence or lower abiotic transformation rates at lower temperatures. No more than 20% abiotic removal was observed for 15 of the 17 tested compounds (data not shown). Indomethacin was almost completely transformed abiotically after 120 h and was excluded from further analysis. Fenhexamid exhibited a 36% removal after 94 h and 86% after 504 h (Fig. B.1). Heat-inactivated

biomass was used for testing the absorption of micropollutants on inactivated cells. No obvious micropollutant removal (> 20%) was observed over a 94-h incubation. With a prolonged incubation of 504 h, fenhexamid in the heat-inactivated AOB culture showed a removal of 62% (Fig. B.1). These results indicate that except indomethacin and fenhexamid, all the other tested micropollutants did not adsorb on CaCO₃ precipitates or inactivated biomass, nor exhibit abiotic transformation after at least 240 h incubation (for selected data see Fig. B.1). The abiotic transformation of fenhexamid after 94 h was not through nitration observed for other micropollutants,⁹¹ as no corresponding transformation products were detected. Moreover, fenhexamid likely adsorbed on CaCO₃. The higher removal of fenhexamid in the medium control than in the heat-inactivated control containing the same amount of CaCO₃ precipitates (4 g/L) could indicate that the surface of the CaCO₃ particles was occupied by dead cells in the heat-inactivated control, resulting in a reduced absorption of fenhexamid. In addition, at a concentration of 20 µg/L, the tested micropollutant mixtures (Table B.1) exhibited no inhibitory effects on ammonia oxidation by the three ammonia oxidizers or nitrite oxidation by *N. inopinata* (Fig. B.2).

Five of the 17 tested micropollutants, i.e., asulam, carbendazim, fenhexamid, mianserin and ranitidine showed biotransformation by *N. inopinata*. Except for asulam, which was only slightly biotransformed by 16 %, the other four compounds exhibited 45 – 85 % removals during the 240-hour incubation period (Fig. 3.1A & B.1). For the tested AOA and AOB strains a similar compound specificity was observed. These strains biotransformed asulam, fenhexamid, mianserin, and ranitidine, although at various biomass-normalized rates (Table B.2, Fig. 3.1B). Among the tested micropollutants these four compounds were also previously reported by us to be biotransformed by another AOB strain (*N. europaea*). In addition, *N. europaea* also transformed rufinamide and benzafrate, but at relatively lower removals than the other four compounds.¹⁴⁵

For asulam, *N. gargensis* exhibited the highest biotransformation rate among the three ammonia oxidizers, about 5 times higher than that of *N. nitrosa* Nm 90, while for biotransformation of fenhexamid, *N. nitrosa* Nm90 was the most efficient strain with a 5 times higher biotransformation rate than those of *N. inopinata* and *N. gargensis* (Fig. 3.1B). The two pharmaceuticals, mianserin and ranitidine, which were demonstrated to be biotransformed by *N. gargensis* and *N. nitrosa* Nm 90,¹²⁹ were also biotransformed by *N. inopinata* with 240-h removals of 87 % and 73 %, respectively (Fig. 3.1A, Table B.2). Compared to AOA (*N. gargensis*) and AOB (*N. nitrosa* Nm90), respectively, the biotransformation rate constants of *N. inopinata* were 6 and 8 times higher for mianserin, and 8 and 22 times higher for ranitidine. In addition, the previously identified TPs of mianserin, ranitidine, and asulam during biotransformation by the AOA and AOB species^{129, 145} were also detected during biotransformation by *N. inopinata* (Fig. B.3). All these TPs were formed via oxidation reactions that might be carried out by AMO,¹²⁹ which indicates that *N. inopinata* also likely converted mianserin and ranitidine via the same mechanism by AMO.

Surprisingly, and different from the above four micropollutants, carbendazim was biotransformed only by *N. inopinata* with a 240-h removal of 48 % (Fig. 3.1A). The biotransformation rate of carbendazim by *N. inopinata* was 8 – 17 times lower than those of fenhexamid, mianserin and ranitidine. Moreover, according to our previous study, carbendazim cannot be transformed by either hydroxylamine or nitric oxide, which is different from the other four biotransformed compounds that underwent both co-metabolic biotransformation by bacterial AMO and abiotic biotransformation mediated by ammonia oxidation intermediates.¹⁴⁵ It was previously hypothesized that the higher affinity of the archaeal AMO for the co-metabolic substrate lead to a higher rate of micropollutant biotransformation by the AOA *N. gargensis* than by the AOB *N. nitrosa* Nm90.¹²⁹ Thus, the exclusive biotransformation of carbendazim by *N.*

inopinata and its higher biotransformation rates of mianserin and ranitidine are also likely attributed to its phylogenetically different AMO and the remarkably high affinity of this enzyme.¹¹⁰ In addition, *N. inopinata* excretes much more hydroxylamine than *N. gargensis* and *N. nitrosa* Nm90 during ammonia oxidation in batch culture with an initial ammonium concentration of 2 mM,⁹⁶ which will result in higher hydroxylamine-mediated abiotic transformation for the four compounds that can react with hydroxylamine (except for carbendazim).¹⁴⁵ The lower biotransformation rate of asulam by *N. inopinata* compared to the other tested ammonia-oxidizers suggests that AMO affinities vary among micropollutants. It is worth noting that carbendazim is structurally different from asulam and the other three biotransformed compounds as it contains a benzimidazole group. Thus, future studies should include more benzimidazole compounds to test whether compounds within this group are more generally biotransformed by *N. inopinata*.

Notably, besides AMO that is encoded by all known aerobic ammonia oxidizers, *N. inopinata* possesses a nitrite oxidoreductase (NXR), which enables its additional nitrite oxidation activity compared to AOB and AOA.⁸² Although a previous study has demonstrated that the nitrite-oxidizing bacterium (NOB) *Nitrobacter* sp. was not able to biotransform any of the micropollutants investigated in this study,¹⁴⁵ it remains to be demonstrated whether the NXR enzyme of *N. inopinata*, that is significantly different to *Nitrobacter* NOB^{82, 146} is contributing to the observed biotransformation reactions.

Mechanisms of Carbendazim Biotransformation by N. inopinata

We first confirmed the biotransformation of carbendazim in the absence of the other micropollutants by *N. inopinata* using an individual addition of carbendazim to the three investigated ammonia oxidizers. Consistent with the mixed micropollutant addition, among the three tested nitrifiers, only *N. inopinata* was able to biotransform carbendazim (Fig. 3.2, Fig. B.4).

To further confine which enzymes were involved in carbendazim biotransformation and elucidated the underlying mechanisms, we investigated carbendazim biotransformation activities by *N. inopinata* cells under various conditions: 1) ammonium as the energy source (2 mM with reamendment upon depletion to a total of ~10 mM), where both AMO-involved ammonia oxidation and NXR-mediated nitrite oxidation were active; 2) nitrite as the energy source (0.5 mM with reamendment upon depletion to a total of ~2.5 mM), where NXR was active while the activity of enzymes involved in ammonia oxidation were suspended due to the lack of ammonium; 3) nitrite as the energy source with same additions as in 2) but with an AMO inhibitor (ATU), where NXR was active and the activity of AMO was completely inhibited. When using nitrite as the energy source, we still added a small amount of ammonium (0.05 mM) as nitrogen source, as *N. inopinata* cannot assimilate nitrite.⁸² To eliminate potential AMO activity by oxidizing the added trace amount of ammonium for energy conversion, the commonly used AMO inhibitor ATU¹⁵⁵ was used. The inhibitory effect of ATU was confirmed as no nitrate formation was observed in *N. inopinata* grown on 1 mM ammonium in the presence of 10 μ M (ca. 1.16 mg/L) ATU (Fig. 3.2B). In all experiments, only the ammonium-amended *N. inopinata* culture biotransformed carbendazim with a 206-h removal of 57 %, whereas nitrite-amended *N. inopinata* did not show any carbendazim biotransformation (Fig. 3.2A).

All added ammonium/nitrite was completely converted by *N. inopinata* to nitrate (final product) in the absence of ATU. Ammonia oxidation was totally inhibited by the added ATU which did not affect nitrite oxidation. The maximum nitrate production rate from ammonium was slightly smaller with carbendazim addition than without (Fig. 3.2B). This difference was due to either a slight inhibitory effect of carbendazim, or the lower initial biomass of *N. inopinata* in the experiments with carbendazim addition (Fig. 3.2C). A sustained activity of NXR and an overall

metabolic activity of *N. inopinata* was indicated by the continuous oxidation of nitrite to nitrate in *N. inopinata* cells when nitrite was provided as the sole energy source with or without ATU addition. However, carbendazim biotransformation activity was lost in *N. inopinata* with nitrite (with or without ATU addition) (Fig. 3.2A), suggesting that the biotransformation of carbendazim by *N. inopinata* was associated with ammonia oxidation rather than NXR-mediated nitrite oxidation. Even in *N. inopinata* fed with ammonia, there was a lag phase of ~ 100 h before carbendazim was actively biotransformed (Fig. 3.2A & Fig. 3.3). This suggests that the biotransformation of carbendazim was via a co-metabolic mechanism by AMO, in which the conversion of carbendazim by AMO is mechanistically possible only when ammonia is present and ammonia oxidation is actively occurring. It is also worth noting that, no growth of *N. inopinata* on nitrite was observed no matter ATU was added or not, despite sustained catabolic activity and the presence of a small amount of ammonia (0.05 mM) for N-assimilation (Fig. 3.2C). The reason for the inability of *N. inopinata* to grow on nitrite in the presence of a suitable nitrogen source remained unknown, one possible reason was insufficient reducing power (ammonia) for carbon fixation with and without ATU, as *N. inopinata* lacks the pathway to generate reducing power from nitrite to quinone via the reverse electron transport for carbon fixation.⁸²

We also tested carbendazim biotransformation by a (non-ammonia-oxidizing) nitrite-oxidizing bacterium *N. defluvii*^{146, 148}, which is phylogenetically related to *N. inopinata*. Within a 14-d incubation, *N. defluvii* showed no carbendazim biotransformation activity (Fig. B.4). This corroborates that enzymes involved in the nitrite-oxidizing metabolic pathway did not contribute to the biotransformation of carbendazim.

Carbendazim Transformation Product (TP) Identification.

TPs of carbendazim biotransformation by *N. inopinata* were analyzed to better understand the biotransformation pathways and mechanisms. By suspect and non-target screening, four plausible carbendazim TPs (Fig. 3.3) with m/z values of 208.0713 ($[M+H]^+$, TP208), 148.0601 ($[M+H]^+$, TP148), 296.0273 ($[M-H]^-$, TP296), and 116.0492 ($[M-H]^-$, TP116) were identified according to the screening criteria. The confidence level of structure elucidation for each plausible TP was assigned based on the criteria set up by Schymanski *et al.*¹⁵⁶

Among the four TPs, TP208 and TP148 were the two dominant ones during carbendazim biotransformation by *N. inopinata* over ~ 29 d. TP208 was accumulated during the first 96 h, then was further converted, and followed by the formation and accumulation of TP148 (Fig. 3.3). Based on the predicted formula ($C_9H_9O_3N_3$) and the detection of three fragments from the MS² scan containing an intact aromatic ring with an oxygen element, TP208 was likely formed via hydroxylation of the aromatic ring of carbendazim (confidence level 2b) (Fig. 3.4). This indicates that aromatic hydroxylation of carbendazim was the first transformation reaction of carbendazim biotransformation, which is a typical reaction carried out by AMO.⁹⁰ Thus, consistent with the above result that carbendazim biotransformation only occurred in *N. inopinata* grown on ammonia, AMO is highly likely the responsible enzyme initiating carbendazim biotransformation.

MS² fragments of TP148 revealed the same MS² fragmentation profile as O-acetyl-L-serine ($C_5H_9O_4N$) deposited in MassBank MS² library (MassBank Accession: PR100272) (<https://massbank.eu>). We further obtained the MS² spectra of O-acetyl-L-serine using the authentic standard, as well as the MS² spectra of its isomer N-acetyl-L-serine, both of which matched with the MS² spectra of TP148 at the same retention times (RTs) (Fig. B.5). Thus, we identified TP148 as acetyl-L-serine (confidence level 1) in general without differentiating proportions of the two isomers. The sums of peak areas at the two RTs were used to analyze the

formation of TP148. O-acetyl-L-serine is an intermediate of cysteine biosynthesis in bacteria. In line with that, TP148 was detected in *N. inopinata* grown on ammonium without the addition of carbendazim. Moreover, TP148 was only detected in *N. inopinata* with ammonia addition but not with nitrite addition (nitrite does not support *N. inopinata* growth), nor in the medium of the other two AOA and AOB species grown on ammonia. This indicates that TP148 was exclusively excreted by actively growing *N. inopinata* cells. The efflux of metabolites of the cysteine biosynthesis pathway, including O- and N-acetyl-L-serine was observed in *Escherichia coli* by a facilitator protein.¹⁵⁷ *N. inopinata* might possess similar facilitator proteins for the excretion of acetyl-L-serine. Notably, TP148 (acetyl-L-serine) abundance was more than doubled with the addition of carbendazim in *N. inopinata* cultures (Fig. B.6A), suggesting two possibilities: (i) acetyl-L-serine was over excreted for unknown reasons by *N. inopinata* when exposed to carbendazim without being a transformation product; (ii) acetyl-L-serine was formed from carbendazim biotransformation by *N. inopinata*, which would be the first evidence for micropollutant biotransformation via ring cleavage reactions by comammox bacteria. However, the exact biotransformation pathways from carbendazim to TP148 still remain elusive.

The other two minor TPs (peak area < 10⁷), i.e., TP296 (C₉H₇O₇N₅) and TP116 only showed up after about 8 days (Fig. 3.3), suggesting that they were secondary TPs. TP296 was likely formed by the addition of two nitro groups and one –OH group to the aromatic ring (confidence level 3) (Fig. B.7), whereas the formula and the structure of TP116 remain elusive (confidence level 5) as the exact mass does not give a plausible formula within the mass tolerance threshold (5 ppm), and the MS² profile does not provide sufficient information for an unambiguous structure (Fig. B.8). Similar to TP148, TP116 was also detected in *N. inopinata* without carbendazim addition, but at lower levels than in carbendazim biotransformation samples (Fig.

B.6B). Thus, like TP148, TP116 was a metabolic intermediate of *N. inopinata*. The additional amount of TP116 in *N. inopinata* with carbendazim addition could be due to the stimulated excretion of the metabolic intermediate in the presence of carbendazim, or an additional formation as a true TP from carbendazim biotransformation. The benzimidazole functional group in carbendazim was altered or lost in all four TPs, likely resulting in less toxicity of the TPs.

To examine active uptake by living cells, we further analyzed the intracellular occurrence of carbendazim and the detected TPs in extracts of washed cells collected at the end of the incubation using suspect and non-target screening based on the same screening criteria. No carbendazim or TP was detected, suggesting no active uptake or accumulation of carbendazim or TPs in the living cells. As the reference compounds were not available for all TPs, we used peak areas to estimate the mass balance by assuming the parent compounds and the TPs have the same ionization efficiency on LC-HRMS.¹⁵⁴ We observed an incomplete mass balance (~ 45% recovery) of carbendazim. This could be due to the formation of smaller or nonionizable TPs undetectable by the current LC-HRMS method, or due to the inaccurate estimation of concentrations based on peak areas without taking into account the different ionization efficiencies in positive (for carbendazim, TP 208 and TP148) and negative (for TP296 and TP116) ionization modes. If ring-cleavage biotransformation was occurring, carbendazim could also be partially assimilated into the biomass or be mineralized to carbon dioxide.

3.5 Conclusion

Comammox represent a group of nitrifiers that have been previously overlooked. They possess physiological characters distinct from the other common ammonia and nitrite oxidizers and may play important yet understudied ecological roles in natural and engineered environments. Therefore, in this study we explored their contributions to the environmental fate of

micropollutants. The comammox isolate *N. inopinata* was the only one among the three tested ammonia oxidizers that can biotransform carbendazim, a benzimidazole fungicide widely used over the past decades and frequently detected in wastewater, groundwater and surface water.¹⁵⁸⁻¹⁶⁴ In the environment, carbendazim is very stable and relatively persistent.¹⁶⁵⁻¹⁶⁷ A few isolated bacteria are able to mineralize or degrade carbendazim as the sole nitrogen and carbon source.¹⁶⁸⁻¹⁷² The identified common pathway of carbendazim degradation by those bacteria starts with carbendazim hydrolysis, forming 2-aminobenzimidazole, which was further converted to 2-hydroxybenzimidazole, 1,2-diaminobenzene, catechol, and then to carbon dioxide.^{170, 172} Among the above reported biotransformation intermediates, only 1,2-diaminobenzene was detected in this study at low levels, but occurred in all experimental samples and controls. The peak areas in the heat-inactivated control were even higher than those in the biotransformation samples, indicating its formation was due to abiotic rather than biological degradation of carbendazim. Different from the reported metabolic pathways, *N. inopinata* employed co-metabolic pathways by oxidizing carbendazim into a hydroxylated TP first, which was further converted to other TPs different from the ones identified in carbendazim mineralization by other bacterial species. As comammox bacteria are found in various environments such as soil, sediments, freshwater, and WWTPs, they may make special contributions to carbendazim removal in those systems.

Mianserin is a widely used second-generation tetracyclic antidepressant¹⁷³ and ranitidine is a highly used gastrointestinal drug which inhibits stomach acid production.^{174, 175} Both are detectable at ng/L levels in various environments.^{174, 176} In this study, *N. inopinata* is able to biotransform these two compounds, like previously tested AOA and AOB species.¹²⁹ Asulam is one of the most effective systemic herbicides for controlling the growth of a variety of bracken by inhibiting germination.^{177, 178} Fenhexamid (a dicarboximide) is a widespread fungicide that inhibits

DNA and RNA synthesis and cell division in fungi.¹⁷⁹⁻¹⁸¹ Both asulam and fenhexamid are heavily used in agriculture. Here, we determined for the first time that all tested ammonia oxidizers have abilities to biotransform these two compounds, providing new clues for the fate of these two compounds in nitrifying agro-environments. To fundamentally understand the biotransformation capabilities of the different ammonia oxidizers, for each strain investigated the biotransformation rate constants were calculated and compared at their optimal growth temperature. Ideally, the biotransformation rate constants should be corrected by the temperature correction factor (Q_{10} for a 10 °C temperature difference) to a typical temperature found in systems important for micropollutant biotransformation. Q_{10} is reaction- and culture/enzyme-specific, and various Q_{10} values (1 – 17.6) have been determined for AOA and AOB cultures.^{182, 183} However, as Q_{10} values between 20 °C and the optimal temperature for the investigated ammonia oxidizers are unknown, the actual temperature effects of MP biotransformation are uncertain. Decreased activities may be expected at a lower temperature. Nonetheless, for all five compounds substantial removal (> 20%) was reported in a nitrifying activated sludge community at room temperature, where ammonia oxidizers showed significant contribution to the transformation of these micropollutants.⁷¹

In summary, the discovery of comammox *Nitrospira* has fundamentally changed our view of nitrification.^{32, 82} In this study, we demonstrate that the comammox organism *N. inopinata* (the only comammox isolate available) is capable of biotransforming five micropollutants (including a compound not transformed by the tested AOA and AOB) suggesting that comammox should be considered as a player for micropollutant biotransformation, too. The distinct ecological niche of comammox due to its remarkably high substrate affinity and yield in comparison to AOA and AOB might add weight to the role of comammox bacteria in micropollutant biotransformation, especially in oligotrophic ecosystems where comammox bacteria are the dominant ammonia

oxidizers.¹¹⁰ Different removal percentages and rate constants of the tested micropollutants may be expected for other comammox bacteria with different origins from *N. inopinata*. The findings collectively expand our knowledge of micropollutant biotransformation by ammonia oxidizers and provide important fundamental insights into the potential fate of certain micropollutants in nitrifying environments colonized by different ammonia oxidizers.

3.6 Special Acknowledgements of Chapter 3

I would like to acknowledge that the work presented in Chapter 3 has been published in *Environmental Science and Technology*.¹⁸⁴ It is the first study reporting the specific micropollutants biotransformation pattern of comammox bacterium among three types of ammonia oxidizers. Collaborating with researchers from the Chinese Academy of Sciences, East China Normal University, and University of Vienna, we have further investigated the biotransformation mechanisms of sulfonamides,²⁷ lincomycin and fluoroquinolone antibiotics¹⁸⁵ by three types of ammonia oxidizers. Two manuscripts have been published in *Water Research*. Both studies compared the parent compounds removal and reaction pathways and elucidated the roles of ammonia oxidizers played during the biotransformation reaction. In this dissertation, the results of the two collaborative studies will not be discussed.

3.7 Figures of Chapter 3

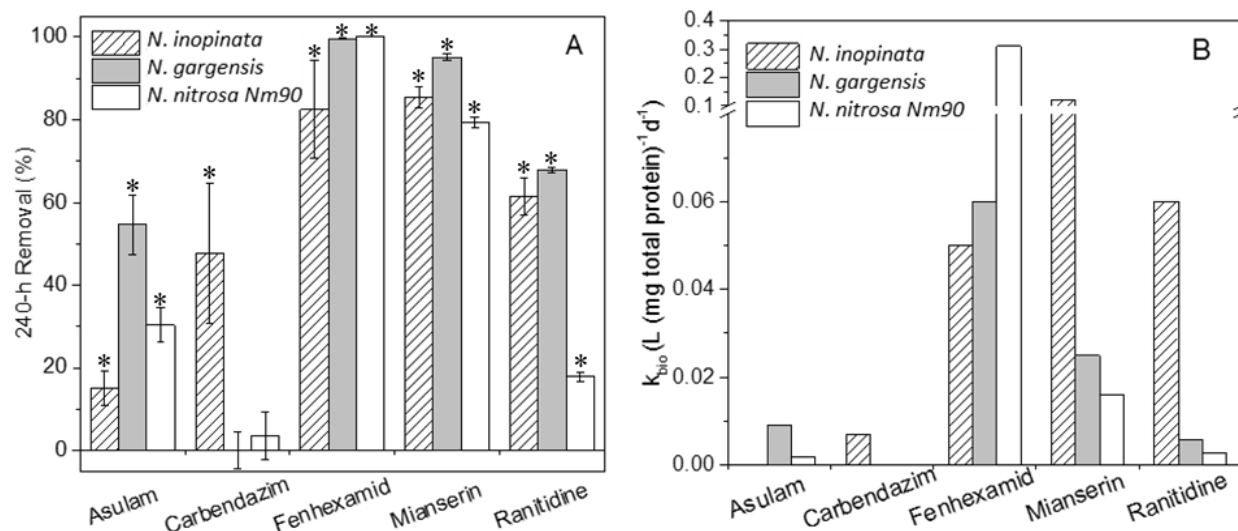


Figure 3.1 A: Micropollutants biotransformed by *Nitrososphaera gargensis* (AOA), *Nitrosomonas nitrosa* Nm90 (AOB), and *Nitrospira inopinata* (comammox) (n = 3, * indicates statistically significant difference from the abiotic and heat-inactivated biomass controls, p < 0.05); B: biomass-normalized biotransformation rate constants, k_{bio} (bars represent the median k_{bio} values calculated based on a first-order model and a Bayesian fitting procedure, taken into account the adsorption and abiotic transformation; see Table S2 for complete results of simulated rate constants; k_{bio} of mianserin and ranitidine biotransformation by *N. gargensis* and *N. nitrosa* Nm90 was adopted from Men *et al.*, 2016¹²⁹).

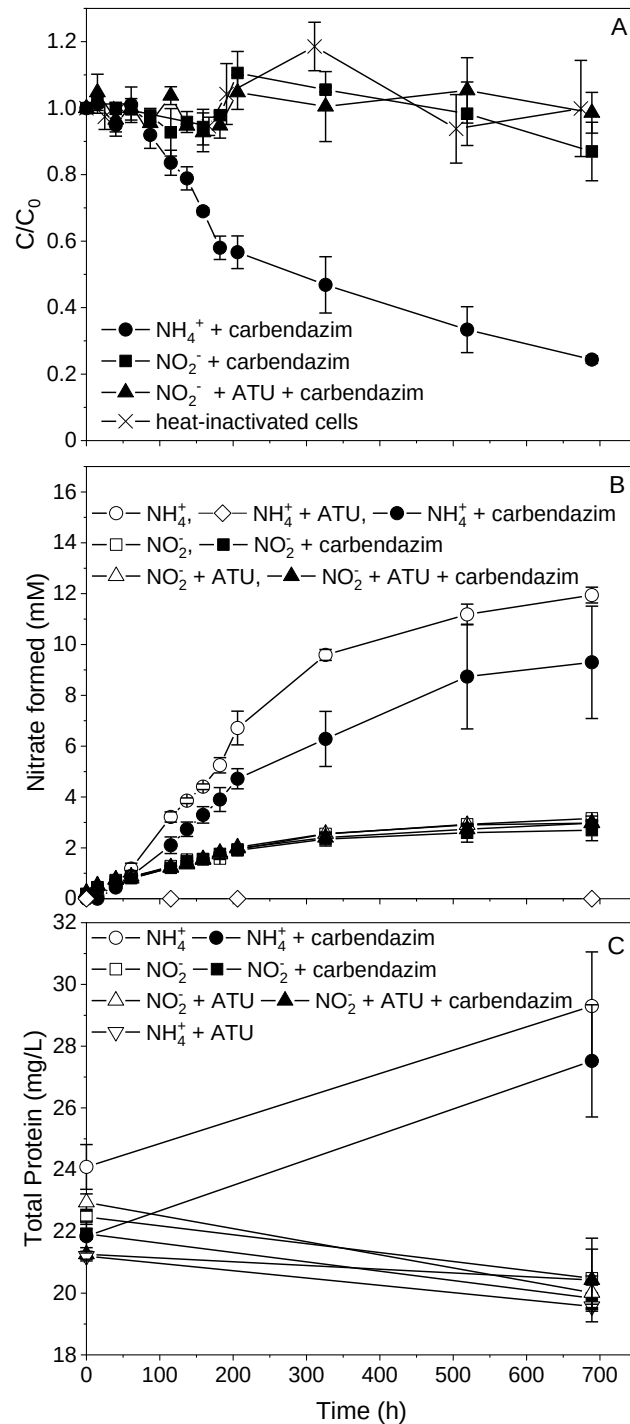


Figure 3.2 Carbendazim biotransformation (A), nitrate production (B), and cell growth measured as total protein (C) of *N. inopinata* under different growth conditions (n=3).

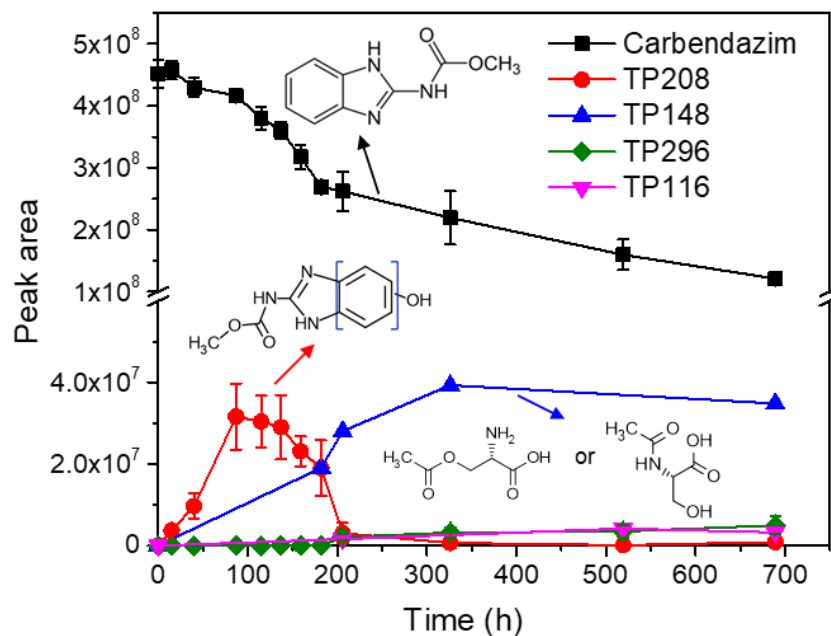
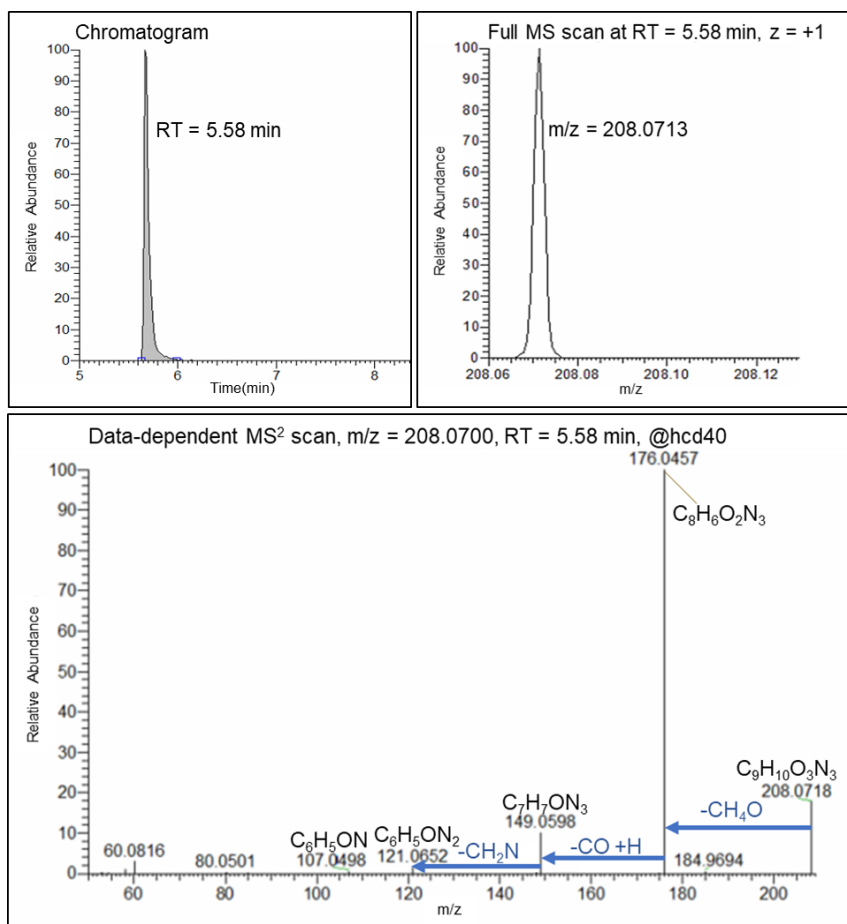


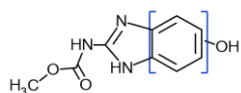
Figure 3.3 Carbendazim biotransformation and TP formation by *N. inopinata* (n=3; note: for TP148 and TP116, the data shown were the averaged peak areas in *N. inopinata* with carbendazim addition subtracted by the averaged peak areas in *N. inopinata* without carbendazim addition, representing the net formation of the two TPs).



Formula: C₉H₉O₃N₃

Atomic Modification: +O

Hypothetical Structure:



Confidence level: 2b

Figure 3.4 Carbendazim TP208 structure elucidation

CHAPTER 4: MICROBIAL CLEAVAGE OF C–F BONDS IN TWO C₆ PER- AND POLYFLUORINATED COMPOUNDS VIA REDUCTIVE DEFLUORINATION

4.1 Abstract

C–F bond is one of the strongest single bonds in nature. Although microbial reductive dehalogenation is well known for the other organohalides, no microbial reductive defluorination has been documented for perfluorinated compounds except for a single, non-reproducible study on trifluoroacetate. Here, we report on C–F bond cleavage in two C₆ per- and polyfluorinated compounds via reductive defluorination by an organohalide-respiring microbial community. The reductive defluorination was demonstrated by the release of F[–] and the formation of the corresponding product when lactate was the electron donor, and the fluorinated compound was the sole electron acceptor. The major dechlorinating species in the seed culture, *Dehalococcoides*, were not responsible for the defluorination, as no growth of *Dehalococcoides* or active expression of *Dehalococcoides* reductive dehalogenases was observed. It suggests that minor phylogenetic groups in the community might be responsible for the reductive defluorination. These findings expand our fundamental knowledge of microbial reductive dehalogenation and warrant further studies on the enrichment, identification, and isolation of responsible microorganisms and enzymes. Given the wide use and emerging concerns of fluorinated organics (e.g., per- and polyfluoroalkyl substances, PFAS), particularly the perfluorinated ones, the discovery of microbial defluorination under common anaerobic conditions may provide valuable insights into the environmental fate and potential bioremediation strategies of those notorious contaminants.

4.2 Introduction

Thousands of organofluorine compounds are on the market, used with diverse applications ranging from surfactants for fire-fighting and oil production, as well as oil/water-repelling materials in commercial goods¹⁸⁶ to refrigerants,¹⁸⁷ and reagents in catalysis.¹⁸⁸ Due to their wide applications, the organofluorine compounds, especially the per- and polyfluoroalkyl substances (PFAS), have caused increasing global concerns on their persistence in the environment, bioaccumulation in organisms, and toxicity to human beings and ecosystems.¹⁸⁶ Many of them have been frequently detected in various environments.^{35, 189, 190} Tremendous efforts have been and are being made to understand the environmental fate and transport of the structurally diverse fluorinated compounds and to develop effective removal strategies. Due to the strong Carbon-Fluorine (C–F) bond,⁴⁵ to date, the defluorination of fluorinated compounds relies on energy-intensive physiochemical approaches.^{37, 46-48, 191} Only sporadic studies on microbial defluorination have been reported since the 1960s when the hydrolytic dehalogenase catalyzing aerobic hydrolytic defluorination of monofluoroacetate was identified.¹⁹² Moreover, there has been only one single study showing the stepwise reductive defluorination of trifluoroacetate (TFA, CF_3COOH) to acetate in a methanogenic microbial community,¹⁹³ yet proved to be non-reproducible,^{194, 195} with no follow-up studies on the responsible microorganisms/enzymes. An isolate from the phylum *Synergistetes* in the absence of known fluoroacetate dehalogenase genes

has shown reductive defluorination activities on monofluoroacetate but not TFA when grown on the fermentation of amino acids.^{196, 197}

For $> C_2$ fluorinated compounds, microbial defluorination has been observed for polyfluorinated telomeric structures (i.e., fluorinated compounds containing CH_2 moieties) under both aerobic and anaerobic conditions,^{54, 57, 198, 199} yet no microbial defluorination of perfluorinated structures was reported until very recently that Huang and Jaffé observed F^- release from PFOA/PFOS in Feammox cultures using Fe^{3+} as the required electron acceptor.²⁰⁰ However, convincing evidence for microbial reductive defluorination, in terms of F^- release and formation of the reductive defluorination products, is still lacking from all previous studies.

According to the thermodynamic calculation, reductive defluorination of fluorinated compounds is biologically feasible.²⁰¹ Thus, one intriguing yet not well-addressed question is that can microbes carry out reductive defluorination of $> C_2$ perfluorinated structures? In this study, we aimed to fill this knowledge gap and investigate microbial reductive defluorination of a variety of per- and polyfluorinated structures. Recent studies reported abiotic reductive defluorination of branched and unsaturated perfluorinated compounds in a vitamin B_{12} -Ti(III) catalytic system at room temperature,^{44, 46} where Ti(III) recycles and reduces Co(III) in B_{12} into Co(I), the active species carrying the reductive defluorination.²⁰² It suggests that reductive defluorination of perfluorinated compounds with similar structures might also be carried out in an anaerobic microbial system, where an enzymatically catalytic system for reductive dehalogenation, such as

B₁₂-dependent reductive dehalogenases (RDase), is present.²⁰³⁻²⁰⁵ We herein used the subcultures of a well-studied dechlorinating enrichment culture and pure dechlorinating cultures to test the biotransformation and biodefluorination of five structurally different per- and polyfluorinated compounds. The biotransformation/biodefluorination products (TPs) were identified, the plausible pathways were further interpreted, and the possible microorganisms involved in the reductive defluorination were discussed. The discovery of microbial defluorination of perfluorinated compounds via the H–F exchange reductive pathway fills the knowledge gap of microbially mediated reductive dehalogenation of organofluorine compounds. The findings shed light on a better understanding of microbial processes that may affect the fate and transport of fluorinated compounds in the environment.

4.3 Materials and Methods

Chemicals

Standard compounds of perfluoro-n-octanoic acid (CAS number: 335-67-1, PFOA), perfluoro-3,7-dimethyloctanoic acid (CAS number: 172155-07-6, PFdiMeOA), (E)-perfluoro(4-methylpent-2-enoic acid) (CAS number: 103229-89-6, PFMeUPA), 4,5,5,5-tetrafluoro-4-(trifluoromethyl)-2-pentenoic acid (CAS number: 243139-64-2, FTMeUPA), and 4,5,5,5-tetrafluoro-4-(trifluoromethyl) pentanoic acid (CAS number: 243139-62-0, FTMePA) were purchased from SynQuest Laboratories (Alachua, FL) and used without further purification. For PFOA, PFdiMeOA, PFMeUPA, FTMeUPA, and FTMePA, 10 mM stock solutions of each

standard were prepared anaerobically in autoclaved Milli-Q water in 160 mL sealed serum bottles and stored at room temperature until use. Detailed compound structures information is listed in [Table C.1](#).

Cultures and Growth Conditions

The *Dehalococcoides*-containing TCE-dechlorinating enrichment (KB1) was generously provided by SiREM Lab (<https://www.siremlab.com/>). Pure *Dehalococcoides mccartyi* BAV1 (ATCC BAA-2100) and FL2 (ATCC BAA-2098) were purchased from American Type Culture Collection (ATCC). Pure *Dehalobacter restrictus* was purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) (DSM-9455). All cultures were maintained in 160-mL sealed serum bottles containing 90 mL sterile anaerobic basal medium with 100 µg/L vitamin B₁₂ as previously described,^{206, 207} and 60 mL Ar/CO₂ headspace. Vitamin B₁₂ was provided as an essential cofactor for the defluorinating pure cultures. For the maintenance of KB1 culture, 5 mM lactate and 2 µL neat TCE (ca. 220 µM) were added as the primary electron donor and electron acceptor, respectively, and were re-added periodically. The three dechlorinating pure cultures were maintained in the same basal medium with H₂/Ar/CO₂ headspace. For *Dehalococcoides mccartyi* BAV1, 5 mM acetate and 2 µL neat *cis*-DCE were supplied upon depletion. For *Dehalococcoides mccartyi* FL2 and *Dehalobacter restrictus*, 5 mM acetate, 2 µL neat TCE were supplied upon depletion. All cultures were incubated at 34 °C in a dark incubator without shaking.

Biodefluorination Experiments

Ten mL of the dechlorinating enrichment (KB1) or the pure dechlorinating culture was inoculated into 90 mL sterile basal medium amended with 5 mM lactate as described above. A sole electron acceptor was added in two scenarios: (1) 75 μ M individual organofluorine compound as the defluorination experimental group; (2) 220 μ M TCE/*cis*-DCE as the culture activity control. The electron donor (i.e., lactate for KB1 and H₂ for *Dehalococcoides* pure cultures) and the electron acceptors were re-added after depletion. The dechlorinating activity was examined by measuring TCE and the dechlorination products via gas chromatography coupled to a mass selective detector (GC-MSD) (Fig. C.1). For organofluorine-added cultures, aqueous samples were taken subsequently during the incubation period for the measurement of parent compounds and F⁻. Briefly, culture suspension was centrifuged at 16,000 $\times$ g (4 °C for 30 min) for F⁻ measurement (2 mL) and the LC-HRMS/MS measurement (2 mL). Cell pellets were stored properly for DNA (-20 °C) and RNA (-80 °C) extraction, respectively. Heat-inactivated biomass controls were set up by inoculating 10 mL autoclaved (at 121 °C for 20 min for two cycles) culture in the same basal medium amended with the same nutrients including the same concentrations of vitamin B₁₂, the electron donor (lactate for KB1 subcultures and H₂ for *Dehalococcoides* pure cultures), and the electron acceptor (organofluorines). All experimental groups and controls were set up in triplicates.

Fluoride Ion Measurement by Ion-Selective Electrode

Concentration of fluoride ion (F⁻) in the culture supernatant was determined by an ion-

selective electrode (ISE, HACH, Loveland, CO) connected with an HQ30D Portable Multi Meter (HACH). A 100 μg fluoride ionic strength adjustment powder (HACH) was added into 2 mL culture supernatant, and the F^- concentration was then measured with the ISE-Multi Meter system. The ISE was calibrated each time before sample measurement according to the manufacturer's instructions. The limit of quantification of ISE was 0.01 mg/L (ca. $\sim 0.5 \mu\text{M}$) F^- . In addition, ion chromatography (IC) was used to validate the fluoride measurement by ISE. The limit of quantification of IC was 1 μM F^- . For ISE method validation, six mock samples with F^- concentrations ranging from 0 to 100 μM were prepared in the medium matrix, as well as three samples prepared from a real supernatant sample (measured as 32.7 μM by ISE and 31.0 μM by IC) with the addition of 0, 9.5, and 49.8 μM F^- standard. F^- concentrations in all samples were measured by ISE and IC, and the results were compared. The two methods exhibited similar (10% or less difference) fluoride concentration measurement results (Fig. C.2).

Fluoride Ion Measurement by Ion Chromatography

Dionex ICS-5000 ion chromatography system equipped with a conductivity detector and a Dionex IonPac AS10 Analytical column (4 $\times$ 250mm) (Thermo Fisher Scientific, Waltham, MA) was used for the ISE validation. The high purity potassium hydroxide eluent was generated by Dionex ICS-6000 Eluent Generator with following gradient: 3 mM for 0 –20 min, 40 mM for 20 –25 min (slope = 7.4 mM/min), 40 mM for 25 –70 min: 3 mM (slope = 7.4 mM/min) for 70 –75 min, and 3 mM for 75 –95 min. The flow rate was set at 1 mL/min, and the suppressor

(AERS_4mm) current was set at 99 mA. The Chromeleon 7.1 (Thermo Fisher Scientific) was used for data acquisition and analysis. The retention time of F⁻ is 17.4 min, which is consistent throughout the analysis (Fig. C.3).

High-performance Liquid Chromatography Coupled to High-Resolution Tandem Mass Spectrometry (HPLC-HRMS/MS) Analysis

Concentrations of the parent compounds and transformation products were analyzed by high-performance liquid chromatography coupled to a high-resolution quadrupole orbitrap mass spectrometer (HPLC-HRMS/MS, Q Exactive, Thermo Fisher Scientific) in the Metabolomics Lab of Roy J. Carver Biotechnology Center at the University of Illinois at Urbana-Champaign. For HPLC analysis, a 50 µL sample was loaded onto a Zorbax SB-Aq column (particle size 5µm, 4.6×50 mm, Agilent), and eluted with 10 mM ammonia formate (A) and methanol (B) at a flow rate of 350 µL/min. The linear gradient for LC separation was 100% A for 0 – 1 min, 100% – 2% A for 2 – 15 min, 2% – 100 % A in 1 min, and 100% A for 16 – 21 min. For HRMS, mass spectra were acquired in full scan mode at a resolution of 70,000 at m/z 200 and a scan range of m/z 50 – 750 under negative/positive switch ionization (ESI) mode. The Xcalibur 4.0 and TraceFinder 4.1 EFS (Thermo Fisher Scientific) were used for data acquisition and analysis. The limit of quantification (LOQ) for each standard compound was determined as the lowest concentration of calibration standards with a detection variation < 20% (Table C.1).

Transformation Product (TP) Identification

Suspect screening and nontarget screening were conducted to identify transformation products as previously described^{122, 184, 208} with slight modification. Briefly, the suspect screening was done by TraceFinder 4.1 EFS and Xcalibur 4.0 software (Thermo Fisher Scientific). TP suspect lists were generated by an automated metabolite mass prediction script,¹²⁹ which was modified to specifically predict the defluorination products via different biological reaction pathways, including a number of known reduction reactions, hydrolysis reactions, and conjugation reactions at both primary and secondary levels. Plausible TPs were selected based on the following criteria: (i) mass accuracy tolerance < 5 ppm; (ii) isotopic pattern score > 90%; (iii) peak area > 10⁸; (iv) increasing trend along time or first increase then decrease; (v) no formation in heat-inactivated controls and absent in biological samples without any organofluorine addition; (vi) not detected as an in-source fragment of the parent compound or any other identified TPs (in-source fragments are peaks identified by MS full-scan, which are also MS² fragments and have the same retention time and formation pattern as the parent compound or the other TPs). For non-target screening, software Sieve 2.2 (Thermo Fisher Scientific) was used for data analysis. The potential TPs were selected based on the same criteria as suspect screening. For TPs with authentic standards (Table C.1), MS² fragment profiles of both standard compounds and TP candidates were acquired using a data-dependent MS² scan based on the exact mass of the precursor ion to elucidate the structures of TPs. MS² fragment profiles of TPs without available authentic standards (Table C.2) were compared with the predicted fragments by Competitive Fragmentation Modeling for

Metabolite Identification (CFM-ID, <http://cfmid.wishartlab.com/>).²⁰⁹ The confidence level of structure elucidation for each TP was assigned based on the criteria set up by Schymanski et al.¹⁵⁶ MarvinSketch (version 19.20.0) was used for drawing, displaying, and characterizing chemical structures, ChemAxon (<http://www.chemaxon.com>).

Gas Chromatography Coupled to Mass Selective Detector (GC-MSD) for TP Analysis in Gaseous Phase

Headspace in a culture bottle was pre-concentrated by solid phase-microextraction (SPME). SPME fibers (silica-fused) coated with polydimethylsiloxane-divinylbenzene (PDMS-DVB, 65 μ m) were purchased from Supelco (Bellefonte, PA). The PDMS-DVB coated SPME fiber has been reported to effectively extract headspace volatile organic compounds such as terpenes, aldehydes, ketones, and alcohols.^{210, 211} Before sampling, the SPME fiber carried by a needle was conditioned in the GC inlet at 260 °C for 15 min.²¹¹ During sampling, the SPME needle was injected into the culture bottle and exposed to the headspace for ~24 hours to extract the gas-phase analytes. Analytes retained on the fiber were assessed using a gas chromatography/electron ionization-mass spectrometry (GC/EI-MS) (Agilent 6890N GC and 5975 MSD, Agilent Technologies, Santa Clara, CA). Headspace samples from PFMeUPA-fed cultures were measured after PFMeUPA was gone. The headspace sample from the heat-inactivated control was also measured, serving as the matrix blank.

The collected SPME samples were manually injected into the GC inlet in splitless mode and desorbed for 3 min at 250 °C. Analytes desorbed from the SPME fiber were separated by a DB-5MS column (30 m × 0.25 mm i.d., 0.25 µm film) (Agilent Technologies). The flow rate of the carrier gas (i.e., helium) was 1 mL/min. The GC temperature was initially at 30 °C, held for 1 min, increased to 150 °C at 5 °C min⁻¹, then increased to 250 °C at 50 °C min⁻¹, and held for 4 min. The total run time was 31 min. EI was performed using electrons with a kinetic energy of 70 eV. Data acquisition was performed in a positive mode with a full scan (m/z 40 – 300). MS fragments of plausible transformation products were interpreted based on the following criteria: 1) no detection in the matrix blank; 2) the same retention time for all fragments of one product.

GC-MSD Analysis for Chlorinated Solvents

Chlorinated ethenes and ethene in all experimental samples fed with TCE/*cis*-DCE were regularly measured by injecting 500 µL headspace sample into a GC-MSD (6850 Network GC system, 5975C VL MSD) equipped with an Rtx-200 capillary column (30 m × 250 µm × 1 µm; Shimadzu, Columbia, MD). The oven temperature was programmed to hold at 45 °C for 1 minute, increased to 200 °C in 3.44 minutes, and held at 200 °C for 1 minute. The temperatures of the injector and the detector were maintained at 220 °C and 250 °C, respectively. Data acquisition was performed at selected ion monitoring mode.

Bond Dissociation Energy (BDE) Calculation

GAUSSIAN 09 quantum chemistry package was used to obtain the C–F BDEs for the

parent compound and TPs examined in Fig. 4.2. All molecular structures were optimized at the B3LYP/6-311+G(2d,2p) level of theory.²¹²⁻²¹⁵ Grimme's empirical dispersion correction with Becke-Johnson damping (GD3-BJ) was employed to approximate the dispersion interaction between molecules.²¹⁶ The SMD continuum solvation model was selected to simulate the solvent effect implicitly.²¹⁷ Frequency examinations of all optimized geometries were done to confirm that the local minima were reached instead of obtaining the first-order saddle point. The BDE for each C–F bond was calculated through a previously reported formula:

$$BDE = (H_{\text{radical}[PFAS\text{minus}F]}^* + H_{\text{radical}F}^*) - H_{\text{parent}PFAS}^*$$

where H^* represents the enthalpy of formation.⁴⁶

DNA Extraction and Quantitative Polymerase Chain Reaction (qPCR)

Biomass from 0.5 mL culture was sampled from each biological replicate. Microbial genomic DNA was extracted by DNeasy PowerSoil Kit (QIAGEN, Germantown, MD) according to the manufacturer's instructions. Cell growth was measured by qPCR using primers targeting universal bacterial 16S rRNA genes and 16S rRNA genes of *Dehalococcoides* spp., *Dehalobacter* spp., and *Geobacter* spp. (Table C.3). Genomic DNA of *Dehalococcoides mccartyi* FL2 and *Dehalobacter restrictus* were quantified by NanoDrop One (Thermo Fisher Scientific) and served as qPCR standards. The relative abundance of reductive dehalogenase genes was determined by qPCR using the primers listed in Table C.3. PowerUp SYBR Green reagents (Thermo Fisher Scientific) were used for qPCR according to the manufacturer's instructions. Briefly, every 20 μ L

reaction mixture contained 2.5 μL of gDNA sample or serially diluted standard, 10 μL of $2 \times$ PowerUp SYBR Green master mix solution, and 1.25 μL 10 μM of forward and reverse primers. The PCR procedure included an initial deactivation at 95 $^{\circ}\text{C}$ for 2 min, followed by 40 thermal cycles at 95 $^{\circ}\text{C}$ for 1 s, then at 60 $^{\circ}\text{C}$ for 30 s.

Reverse-Transcription qPCR (RT-qPCR)

RNA was extracted using acid-phenol: chloroform: isoamyl alcohol (25: 24: 1) and precipitated in ethanol at -20°C as previously described.²¹⁸ RNA was cleaned up using the RNeasy PowerClean Pro CleanUp Kit (QIAGEN) according to the manufacturer's instructions. Contaminating DNA in the RNA samples was removed by Turbo DNase Kit (Thermo Fisher Scientific) following the manufacturer's instructions. qPCR was carried out to verify the removal of genomic DNA contamination from the purified RNA. The quality of RNA was examined by agarose gel electrophoresis.

SuperScript III First-Strand Synthesis System (Thermo Fisher Scientific) was applied for complementary DNA (cDNA) synthesis according to the manufacturer's instructions. In general, the 10 μL cDNA synthesis mix containing 8 μL of RNA sample, 1 μL of primer (random hexamers) and 1 μL of 10 mM dNTP mix was incubated at 65 $^{\circ}\text{C}$ for 5 min, then placed on ice for 1 min. The cDNA synthesis mix was added to each RNA/primer mixture and incubated at 25 $^{\circ}\text{C}$ for 10 min then 50 $^{\circ}\text{C}$ for 50 min. The reaction was terminated at 85 $^{\circ}\text{C}$ for 5 min, and then the entire tube was chilled on ice. At last, 1 μL of RNase H was added to each tube and incubated at 37 $^{\circ}\text{C}$ for 20

min. The cDNA synthesis products were stored at $-20\text{ }^{\circ}\text{C}$ for qPCR measurement using primers listed in [Table C.3](#).

The relative gene expression of RDase genes was calculated using the double delta Ct method²¹⁹ (as shown in the following equation) with *Dehalococcoides* 16S rRNA gene as the reference gene.

Relative gene expression = $2^{-\Delta\Delta\text{Ct}}$, where

$$\Delta\Delta\text{Ct} = \Delta\text{Ct}_{\text{Organofluorine-added sample}} - \Delta\text{Ct}_{\text{TCE-added control}}, \quad \Delta\text{Ct} = \text{Ct}_{\text{RDase gene}} - \text{Ct}_{\text{16S rRNA gene}}$$

4.4 Results and Discussion

Microbial Reductive Defluorination of a C₆ Perfluorinated Compound

The reductive defluorination was conducted in subsequent transfers (10%, v/v) of a well-studied and commercially available dechlorinating enrichment culture KB-1[®] (SiREM, Ontario, Canada). We investigated three perfluorinated compounds that exhibited abiotic reductive defluorination activities in a Ti (III)-vitamin B₁₂ catalytic system,⁴⁶ as well as two polyfluorinated compounds structurally similar to the C₆ perfluorinated compound (see compound details in Materials and Methods, [Table C.1](#)). We obtained strong evidence for microbial reductive defluorination of the C₆ perfluorinated compound (i.e., PFMeUPA), as well as the polyfluorinated FTMeUPA in the following aspects: (i) complete parent compound biotransformation in biological samples but not in the heat-inactivated control ([Fig. 4.1A & B](#)), indicating microbially/enzymatically mediated biotransformation; (ii) significant F⁻ release ([Fig. 4.1A & B](#))

with the formation of the corresponding defluorination products (Fig. 4.2A – D), indicating reductive defluorination; and (iii) no defluorination activity when no electron donor was provided, thus excluding defluorinating fermentation (Fig. C.4). For the saturated perfluorinated structures (PFOA and PFdiMeOA) or the saturated polyfluorinated structure (FTMePA), no microbial defluorination was observed (Fig. C.5), suggesting that the double bond (C=C) was crucial for the microbial reductive defluorination of FTMeUPA.

Transformation Product (TP) Analysis Reveals the C–F Bond Cleavage Positions and The Biotransformation Pathways of PFMeUPA and FTMeUPA

We have identified the major transformation products (TPs) for PFMeUPA and FTMeUPA, according to the LC-High-Resolution MS/MS data (see details in the Materials and Methods). Authentic standards were used to confirm the structures of some TPs in Fig. 4.2C & D. For those without available authentic standards, the TP structures were inferred according to the MS² fragmentation patterns and the model-predicted MS² spectra²⁰⁹ (Materials and Methods, Table C1 & C2, Fig. 4.3, Fig. C.6 – C.11). Due to the difference in MS ionization efficiency among different organofluorine structures (Table C.4), the peak areas were only used to indicate the formation trend of individual TPs but not their relative abundance.

The TP profiles indicate two major biotransformation pathways: reductive defluorination and hydrogenation. The first-step reductive defluorination is the major reaction leading to the significant F[–] release from PFMeUPA, forming TP256 (–F+H) (Fig. 4.2A and Reaction 1 in Fig.

4.2C). TP256 has an MS² fragment of C₃F₇⁻, representing the intact branched tail in the structure (Fig. 4.3). It indicates that the first C–F bond cleavage was at the *sp*² carbons of the double bond rather than the *sp*³ ones. It seems that the *sp*² C–F bonds are more readily for reductive defluorination despite its higher BDE than the tertiary *sp*³ C–F bond in PFMeUPA (Fig. 4.2C). Similarly, the first C–F bond cleavage also occurred at the *sp*² carbon during the B₁₂-catalyzed abiotic reductive defluorination of another unsaturated compound 2,3,3,3-tetrafluoropropene.¹⁸⁷ The C=C bond provides a readily binding site for catalysts containing a transition metal, such as Co, Fe, Ni, and Mn, which can carry out reductive defluorination of unsaturated organofluorines in abiotic catalytic systems.^{46, 187, 220-222} Similarly, the C=C bond may facilitate the binding of the defluorinating enzymes, which likely possess a metal center, to initiate the reductive defluorination. All the above-mentioned metals are also biologically relevant and could be the catalytic center of defluorinating enzymes, which may or may not be B₁₂-dependent. Investigating an extended list of unsaturated/saturated and branched/linear organofluorines, identifying and isolating the responsible defluorinating enzymes/microorganisms are needed in future studies to fully demonstrate the actual roles of the double bond (C=C) and B₁₂ in the microbial reductive defluorination. In FTMeUPA, which is unsaturated but lacks *sp*² C–F bonds, the reductive defluorination occurred at one *sp*³ C–F bond, most likely the tertiary *sp*³ C–F bond that has a much lower BDE than the primary *sp*³ C–F bonds, forming the corresponding TP221 (Fig. 4.2B and Reaction 5 in Fig. 4.2D).

Besides reductive defluorination, the other major pathway for both PFMeUPA and FTMeUPA was hydrogenation, forming the corresponding saturated carboxylic acids (TP276 and TP241, Reaction 2 in Fig. 4.2C and Reaction 4 in Fig. 4.2D, respectively). The hydrogenation pathway occurred in parallel with the reductive defluorination pathway because the hydrogenation products of PFMeUPA and FTMeUPA (i.e., TPA276 and TP241) were accumulated to a plateau without a further decrease (Fig. 4.2A & B). It seems that the saturated products were more recalcitrant to reductive defluorination than the unsaturated parent compounds, again suggesting the important role of the C=C bond. The hydrogenation of unsaturated polyfluorinated carboxylic acids is not uncommon and has been observed in both aerobic and anaerobic sludges.^{57, 199} The hydrogenation of α , β -unsaturated carboxylic anions forming the corresponding saturated carboxylic acids can be catalyzed by enoate reductases with nicotinamide adenine dinucleotide (NADH) as the cofactor from anaerobic microorganisms such as *Clostridium kluyveri*.²²³ In addition, flavin-based ene-reductases from the “Old Yellow Enzyme” family possessed by a variety of microorganisms may also carry out the reduction of activated alkenes that contain a carboxyl as the electron-withdrawing group (EWG) and an additional EWG such as a halogen.²²⁴ However, it is still unclear whether microorganisms can obtain energy for growth from such hydrogenation reactions.

Microbial Preference Between the Two Major Biotransformation Pathways of PFMeUPA and FTMeUPA

It is worth noting that although FTMeUPA was biotransformed faster than PFMeUPA (~70 days vs. ~130 days for > 90% removal), the F⁻ released from FTMeUPA was less and slower (Fig. 4.1). The defluorination degree of the removed FTMeUPA during the entire incubation period was 4~5%, which is much lower than what would have been expected if all FTMeUPA was subject to the first-step reductive defluorination (i.e., one out of seven F, corresponding to 14%). In contrast, the defluorination degree of the removed PFMeUPA was about the same as if all PFMeUPA underwent the first-step reductive defluorination (i.e., one out of nine F, corresponding to 11%). This result indicates that the reductive defluorination at the *sp*² C–F bond was favored over the hydrogenation pathway for PFMeUPA, whereas the *sp*³ C–F bond cleavage was less favorable than the hydrogenation of the F-free *sp*² carbons for FTMeUPA. In addition, if the accumulation of TP276 (Fig. 4.2A) was in a significant amount, the total 11% defluorination of PFMeUPA would suggest a secondary defluorination. However, we were not able to determine the accurate portions of PFMeUPA that underwent reductive defluorination and hydrogenation due to the lack of reference standards of TP256 and TP276. According to the LC-HRMS/MS results, we observed a further transformation of TP256 after 90 days (Fig. 4.2A), but the only identified secondary TP (TP259) is a hydrogenation product of TP256 (Reaction 3 in Fig. 4.2C). Plausible secondary defluorination TPs, including FTMeUPA and its transformation products (i.e., TP221 and TP241), were not observed during the biotransformation of PFMeUPA. Thus, the secondary defluorination products of PFMeUPA, if any, could be smaller molecules that are not

detectable by LC-HRMS/MS. We further used GC-MS to identify possible secondary defluorination products from the headspace. According to GC-MS fragment interpretation, we identified two plausible secondary defluorination products (Fig. C.12A & B). They were two olefin isomers with the second sp^2 C–F bond or the tertiary sp^3 C–F bond cleaved. As we did not detect the corresponding carboxylic acids of those two olefins by LC-MS/MS, and the GC-MS data indicated the presence of the decarboxylation product of TP256 (Fig. C.12C), the second C–F bond cleavage likely occurred after the decarboxylation of TP256. Due to the low resolution of GC-MS and the lack of standards, the structures of the two secondary defluorination products could not be validated or quantified. The preferred position of the second C–F bond cleavage remains elusive. Collectively, the cleavage of the first C–F bond in a perfluorinated structure is more critical and of greater significance given the recalcitrance of perfluorinated structures to biodegradation. Our findings suggest that the presence of C=C bond in a perfluorinated structure plays a crucial role in initiating the first C–F bond cleavage, which can result in the formation of polyfluorinated structures with C–H bond(s) that are more vulnerable to attack by other environmental microorganisms under aerobic or anaerobic conditions.^{61, 199, 225}

Microbial Reductive Defluorination Capabilities of the Identified Dehalorespiring Microorganisms in the Community

The dominant dehalorespiring microorganism in the investigated KB-1 culture is *Dehalococcoides* spp.²²⁶ The versatility of B₁₂-dependent reductive dehalogenases of

Dehalococcoides spp. in metabolizing various organohalides makes them the most promising candidate capable of microbial reductive defluorination of the two fluorinated compounds.²²⁷ Thus, we first looked at the growth and activities of *Dehalococcoides* spp. in the PFMeUPA/FTMeUPA-fed culture, in terms of the 16S rRNA gene abundance and its transcriptional levels, as well as the transcriptional levels of 14 RDase genes²²⁸ identified in the community. Unexpectedly, no *Dehalococcoides* growth or RDase gene transcription was observed during the incubation period when PFMeUPA/FTMeUPA was provided as the sole electron acceptor (Fig. 4.4A & B, Fig. C.13, Fig. C.14). In comparison, cultures with TCE addition exhibited active growth of *Dehalococcoides* (Fig. 4.4A & B, Fig. C.13). Theoretically, the energy generated from the added PFMeUPA/FTMeUPA (~75 μ M) could sustain the growth of *Dehalococcoides* via reductive dehalogenation if it occurred. Therefore, the dominant chloroethene-respiring *Dehalococcoides* spp. in the community were not responsible for the reductive defluorination. It was corroborated by the two isolated *Dehalococcoides* strains, *D. mccartyi* FL2 and BAV1, not being able to cleave F from PFMeUPA but exhibiting reductive dechlorination activities (Fig. C.15).

We then looked at the growth of another two dechlorinators in this community: the second dominant TCE-dechlorinating species *Geobacter* spp.²²⁶ and the minor dechlorinating species *Dehalobacter* spp.²²⁹ Again, *Geobacter* spp. showed no growth in the PFMeUPA-fed culture, hence were not responsible for the reductive defluorination (Fig. C.16). Nevertheless, we observed

significantly higher growth and activities of *Dehalobacter* in the PFMeUPA/FTMeUPA-fed culture compared to the TCE-fed ones (Fig. 4.4C & D, Fig. C.17). One should note that the initial abundance of *Dehalobacter* in the seed culture fed with TCE was extremely low ($< 10^3/\text{mL}$). Such low *Dehalobacter* abundance was consistent with what was observed in the TCE-fed subcultures of the same microbial community.²²⁹ Several *Dehalobacter* strains have been demonstrated to biodegrade chlorinated compounds such as TCE, trichloroethane (TCA), and TCB.²²⁹⁻²³¹ A similar emergence of *Dehalobacter* population with a significant decrease of *Dehalococcoides* population was observed when the terminal electron acceptor was switched from TCE to 1,2,4-TCB.²²⁹ The abilities of *Dehalobacter* spp. to utilize diverse chlorinated compounds to alleviate substrate competition with other dehalorespiring microorganisms make them likely involved in the defluorination of PFMeUPA and FTMeUPA. Thus, we further examined the defluorination capabilities of the pure culture of *Dehalobacter restrictus* strain PER-K23, the closest strain to *Dehalobacter* spp. in the community.²²⁹ *Dehalobacter restrictus* strain PER-K23 is known as an obligate organohalide respiring bacterium, which is able to dechlorinate tetra- or trichloroethene with hydrogen as the sole electron donor.²³² However, we did not detect any F^- release from PFMeUPA and FTMeUPA (Fig. C.18). As *Dehalobacter* spp. in the community showed higher growth and activities when fed with PFMeUPA/FTMeUPA than TCE, one may still not exclude the possibility that the actual *Dehalobacter* spp. in the community had different physiological characteristics and were able to carry out the reductive defluorination of PFMeUPA/FTMeUPA.

Alternatively, they could carry out the biotransformation of primary intermediates, such as the first C–F bond cleavage product TP256 of PFMeUPA, thus dependent on the microorganisms involved in the primary biotransformation. Microorganisms responsible for the reductive defluorination may also be other members in the community, which are quite different from those for reductive dechlorination, and the responsible enzymes were different from the known B₁₂-dependent RDases.

4.5 Conclusion

According to this study, microbial reductive defluorination of > C₂ perfluorinated compounds does exist in nature. The unsaturated structures seem to be more bioavailable, and the first C–F bond cleavage at the *sp*² position is a crucial first step to destruct perfluorinated structures, which could lead to further defluorination of the formed polyfluorinated products by environmental microorganisms. As the dominant dechlorinating species in the community were not responsible for the reductive defluorination, the actual defluorinating microorganisms were likely those in low abundances. Thus, higher defluorinating activities and perhaps a wider fluorinated substrate range would be expected for enriched and/or isolated defluorinating microorganisms. Therefore, future studies are needed to enrich and/or isolate the microorganisms responsible for the reductive defluorination and characterize their physiological properties in the highly enriched cultures and/or isolates. While the enrichment, identification, and isolation are still ongoing due to the slow growth of the defluorinating microorganisms, unveiling the microbial

pathways of breaking the “hardest-ever” carbon–halogen bond via reductive dehalogenation has already initiated one significant step further toward a complete understanding of anaerobic microbial reductive defluorination.

In recent years, some short-chain ($\leq C_6$), branched, and unsaturated organofluorine compounds have already been used as alternatives to the legacy per- and polyfluoroalkyl substances (PFAS) (e.g., perfluorooctanoic acid, PFOA and perfluorooctanesulfonic acid, PFOS), and some have been detected in various environments as the original forms and the transformed forms from certain parent PFAS.^{233, 234} Given the increasing concerns of PFAS worldwide, the findings here will provide important implications and open up diverse venues for both fundamental science and engineering applications. Those include identifying and characterizing the defluorinating microorganisms/enzymes for potential bioremediation strategies, as well as assessing the environmental fate and biodegradability of PFAS, particularly for the unsaturated and/or branched perfluorinated structures. In addition, as PFAS cannot be completely phased out in the short term,²³⁵ the structural specificity of biodefluorination may provide implications to the molecular design of fluorochemicals with enhanced biodegradability, shorter half-life, hence less bioaccumulability.

4.6 Special Acknowledgements of Chapter 4

I would like to acknowledge that the work presented in Chapter 4 has been published in *Environmental Science and Technology*.²³⁶ I would like to thank the ACS Division of

Environmental Chemistry for selecting this paper as one recipient of the C. Ellen Gonter Environmental Chemistry Awards of 2019. I want to thank Chinese-American Professors in Environmental Engineering and Science (CAPEES) for awarding this paper as the 2021 CAPEES Founding President Best Paper Awards.

4.7 Figures of Chapter 4

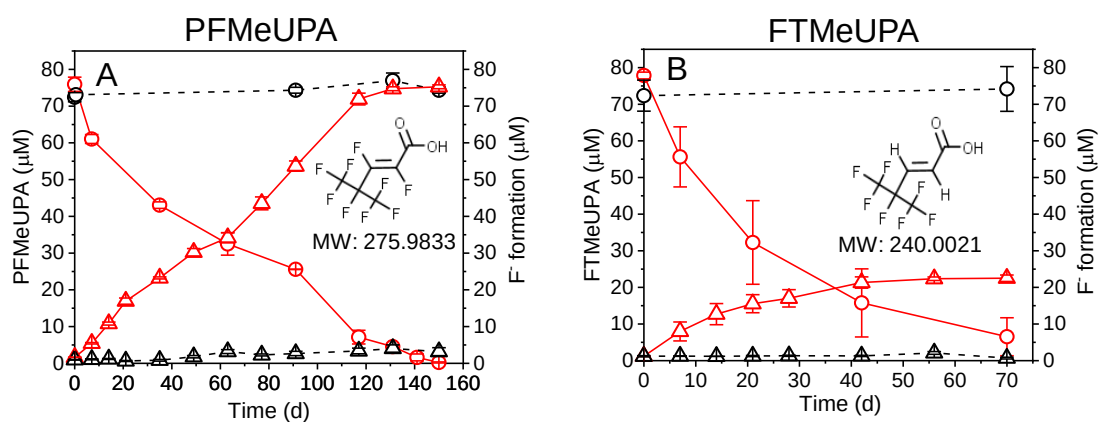


Figure. 4.1 Parent compound removal and fluoride ion release for PFMeUPA (A) and FTMeUPA (B) (red: biological samples; black: heat-inactivated controls; circles: parent compound; triangles: fluoride ion; n=3).

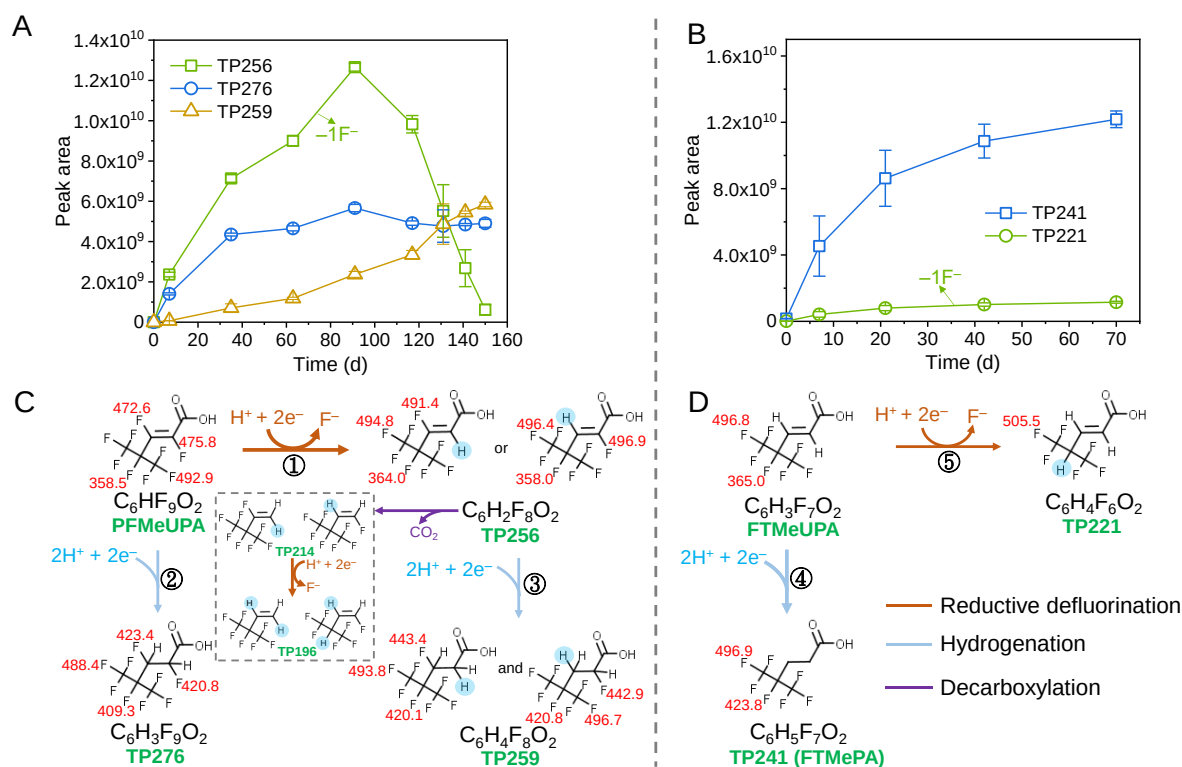
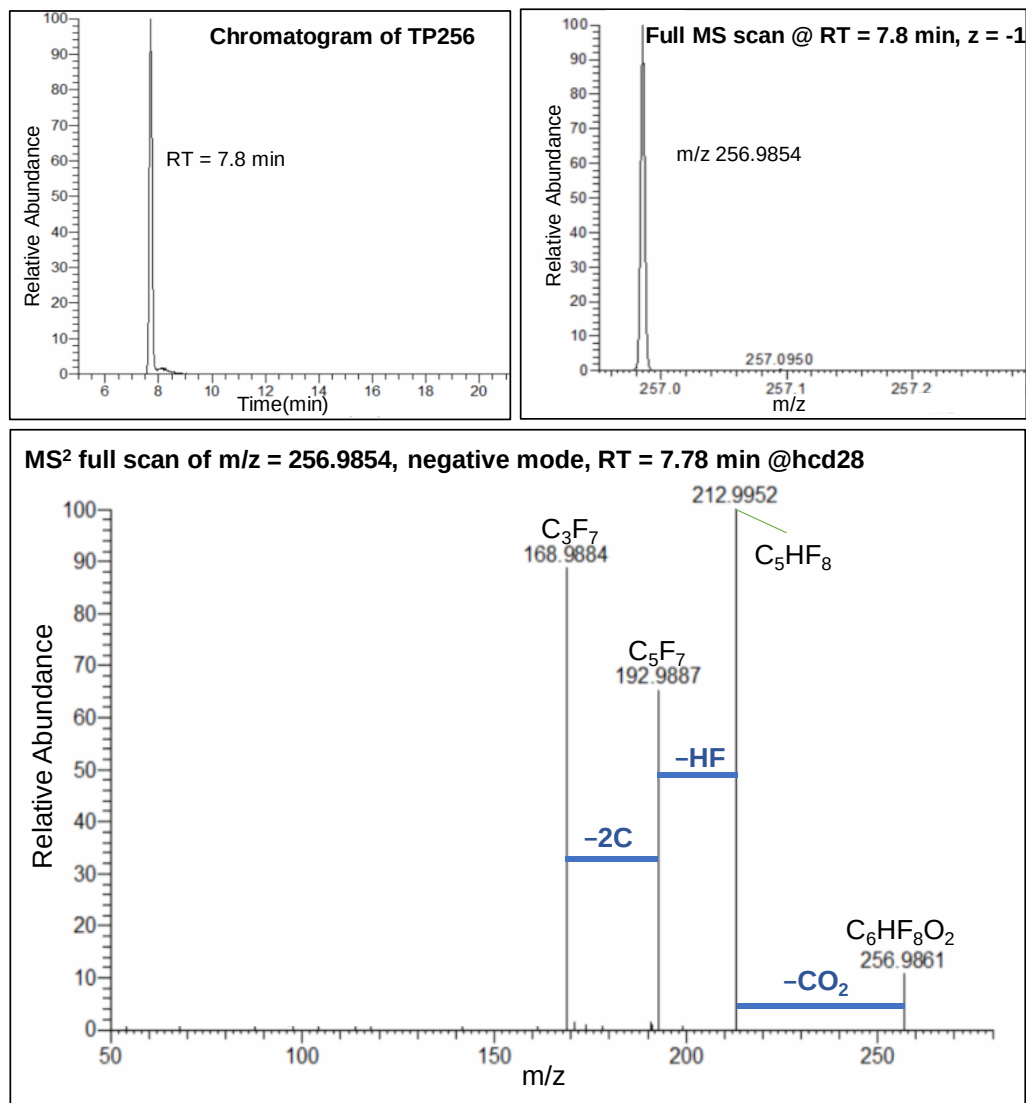


Figure. 4.2 The temporal trend of TPs identified by LC-HRMS/MS during PFMeUPA (A) and FTMeUPA (B) biotransformation (arrows indicate the TPs from reductive defluorination, $n=3$); proposed biotransformation pathways of PFMeUPA (C) and FTMeUPA (D) (the red number next to a C–F bond is the calculated bond dissociation energy in kJ/mol; TPs in the grey dashed box were identified in the headspace by GC-MS, whose formula and structures need further validation; the other TPs were identified in the aqueous phase by LC-HRMS/MS, the most plausible structure of which were shown according to the confirmed formula and MS² fragments).



Formula: $C_6H_2F_8O_2$

Atomic Modification: $-F + H$ of PFMeUPA

Hypothetical Structure:

Confidence level: 2b

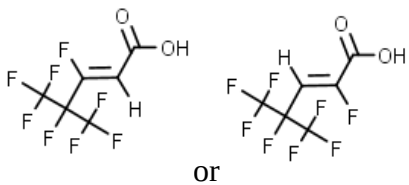


Figure. 4.3 Structure elucidation of the first defluorination product of PFMeUPA (TP256).

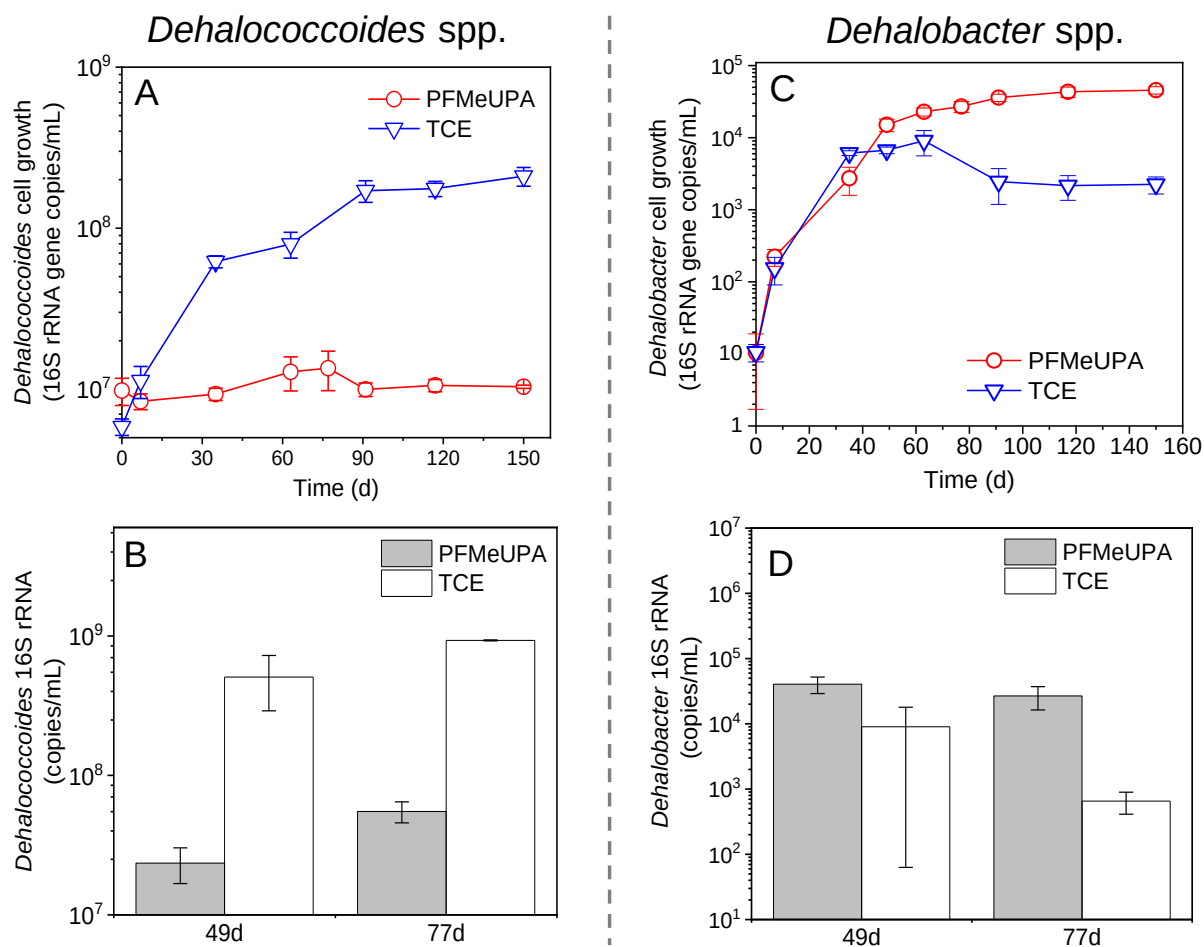


Figure 4.4 The growth (as 16S rRNA gene copy numbers) (A) and activities (as 16S rRNA copy numbers) (B) of *Dehalococcoides* spp., and the growth (C) and activities (D) of *Dehalobacter* spp. in KB-1 subcultures (10% v/v inoculation of KB-1) fed with ~ 75 μ M PFMeUPA compared to those grown on trichloroethene (TCE) (n=3).

CHAPTER 5: MICROBIAL DEFLUORINATION OF UNSATURATED PER- AND POLYFLUORINATED CARBOXYLIC ACIDS UNDER ANAEROBIC AND AEROBIC CONDITIONS: A STRUCTURE SPECIFICITY STUDY

5.1 Abstract

The recently discovered microbial reductive defluorination of two C₆ branched and unsaturated fluorinated carboxylic acids (FCAs) provided valuable insights into the environmental fate and potential bioremediation strategies of per- and polyfluoroalkyl substances (PFAS). However, a systematic investigation is needed to further demonstrate the role of C=C double bonds in the biodegradability of unsaturated PFAS in both anaerobic and aerobic environments. Here, we examined the structure-biodegradability relationships of thirteen FCAs, including nine commercially available unsaturated FCAs and four structurally similar saturated ones, by a reductively defluorinating enrichment and activated sludge communities. The transformation products were identified to elucidate the transformation/defluorination pathways. The results showed that under anaerobic conditions, the α , β -unsaturation is crucial for FCA biotransformation via reductive defluorination and/or hydrogenation pathways. With sp^2 C–F bonds being substituted by C–H bonds, the reductive defluorination became less favorable than hydrogenation. Moreover, for the first time, we reported enhanced degradability and defluorination capability of specific unsaturated FCA structures with trifluoromethyl (–CF₃) branches at the α/β -carbon. Such FCA structures can undergo anaerobic abiotic defluorination in the presence of reducing agents and significant aerobic microbial defluorination. Given the diverse applications and emerging concerns of fluorochemicals, this work not only advances the fundamental understanding of the

fate of unsaturated PFAS in natural and engineered environments but may also provide important insights into the design of readily degradable fluorinated alternatives to existing PFAS compounds.

5.2 Introduction

Organofluorines, particularly per- and polyfluoroalkyl substances (PFAS), have been widely used as surfactants and oil/water repelling materials³⁴⁻³⁷ due to their unique properties (e.g., hydrophobicity, lipophobicity, and thermal stability). The highly stable Carbon-Fluorine (C–F) bonds⁴⁵ in PFAS make them very recalcitrant in the environment. Because of their environmental persistence, bioaccumulation, and toxicity,¹⁸⁶ the frequent occurrence of PFAS in various environments²³⁷⁻²³⁹ has caused emerging concerns to public health and ecosystems. So far, tremendous efforts have been made on PFAS regulations and treatment approaches, yet most of the efforts are spent on the legacy PFAS, such as perfluorooctanoic acid (PFOA), perfluorooctanesulfonic acid (PFOS), and others in the Unregulated Contaminant Monitoring Rule (UCMR 3) listed by the U.S. EPA.^{43, 44, 240} Consequently, fluorochemical manufacturers have been phasing out those legacy PFAS and developing fluorinated alternatives.^{186, 241} However, it has been recently realized that some PFAS alternatives (e.g., perfluoroalkyl ether carboxylic acids or short/branched-chain PFAS) revealed unpredictable toxicities and even higher mobilities than the legacy PFAS.^{35, 47, 242, 243} Moreover, their concentrations and diversities in the environment have rapidly increased compared to the legacy PFAS.²⁴⁴⁻²⁴⁷ As PFAS have been widely used in consumer products and industrial applications, and some of their applications are essential and

currently without established alternatives,²⁴⁸ it is urgent to design novel fluorinated alternatives with enhanced degradability while maintaining similar functional properties.

The biodefluorination of fluorinated compounds (mostly the polyfluorinated structures) have been previously reported.^{51, 52, 54, 57, 68, 192, 196, 197, 249, 250} The defluorination of the polyfluorinated structures, was initiated by HF elimination at the α and β position of fluorotelomer carboxylic acid⁵⁶⁻⁵⁸ or by desulfonation of fluorotelomer sulfonic acid.^{59, 60} Compared to biotransformation of polyfluorinated compounds, convincing reports on microbial cleavage of C–F in perfluorinated structures are even fewer.^{200, 236} Based on fluoride formation and transformation product identification, our recent study has reported microbial reductive defluorination of two branched unsaturated per- and polyfluorinated carboxylic acids in an anaerobic enrichment.²³⁶ It revealed that the biotransformation and biodefluorination of fluorinated carboxylic acids (FCAs) may be structure-dependent, and the C=C double bond could play an essential role in the reductive defluorination of FCA. However, since only two unsaturated structures were investigated in the previous study,²³⁶ the structure-specificity of FCAs biotransformation by the anaerobic defluorinating enrichment has not been well demonstrated.

In this study, we filled this knowledge gap and aimed to investigate the transformation and defluorination capabilities of all commercially available unsaturated FCAs (linear and branched) as well as the structurally similar saturated ones by the anaerobic defluorinating enrichment. We identified the major transformation products (TPs), elucidated the transformation pathways, and

examined the microbial preference of defluorinating and non-defluorinating pathways. Based on the pathway preference under the anaerobic condition, we further investigated the aerobic biotransformation and biodefluorination of the unsaturated FCAs and compared to the anaerobic condition. We proposed specific structures that may have enhanced (bio)degradability in both anaerobic and aerobic conditions. These findings expand our knowledge of the fate and transport of PFAS in both natural and engineered environments, which could ultimately provide insights into the PFAS source tracking and the structural design of readily degradable fluorinated PFAS alternatives.

5.3 Materials and Methods

Chemicals

Sixteen FCA standards, which were used in this study as parent compounds in the biotransformation experiments or analytical reference compounds, were purchased from SynQuest Laboratories (Alachua, FL) and used without further purification ([Fig. 5.1](#)). For all standard compounds, 10 mM stock solutions were prepared anaerobically in autoclaved Milli-Q water in 160-mL sealed serum bottles and stored at room temperature until use. The limit of quantification (LOQ) for each standard compound was determined as the lowest concentration of calibration standards with a detection variation within $\pm 20\%$. The detailed compound information was provided in [Table D.1](#).

Maintenance of The Anaerobic Defluorinating Enrichment.

As described previously,²³⁶ the defluorinating enrichment was maintained in 160-mL sealed serum bottles containing 90 mL sterile anaerobic basal medium with 100 µg/L vitamin B₁₂ and the reducing agent, cysteine-sulfide (48mg/L cysteine and 96mg/L sodium sulfide). The headspace contains 60 mL Ar/CO₂ (75:25, v/v). For routine maintenance, 5 mM lactate and 75 µM **MeU-C6a** (PFMeUPA) were added as the primary electron donor and sole electron acceptor, respectively. Lactate was re-amended upon depletion. After the first dose of **MeU-C6a** being completely consumed, cultures were subsequently transferred (5%, v/v) into 90 mL fresh basal medium containing 5mM lactate and 75 µM **MeU-C6a**. The defluorination activity of the subcultures was verified by the continuous formation of fluoride and complete removal of the parent compound.

FCA Biotransformation by the Anaerobic Defluorinating Enrichment.

After the consumption of the first dose of **MeU-C6a**, 5 mL of the enrichment culture was inoculated into 90 mL sterile fresh medium. Approximately 75 µM of individual FCA was added into the subcultures as the sole electron acceptor with 5 mM lactate as the primary electron donor. For each batch of the experiment, an **MeU-C6a**-added positive control was set up to verify the defluorinating activity of the subcultures. Heat-inactivated biomass controls were set up by inoculating 5 mL autoclaved (at 121 °C for 20 min for two cycles) culture in the same basal medium with parent compound amended the same way as described above to determine the abiotic degradation and potential adsorption of each compound. Samples were taken subsequently during

the incubation period for the measurement of the parent compound, transformation products (TPs), and F^- . Briefly, at each sampling time, 3 mL aqueous suspension was centrifuged at $16,000 \times g$ for 30 min ($4^\circ C$). Two mL supernatant was used for F^- measurement and the remaining (~ 1 mL) was stored at $4^\circ C$ in dark for LC-HRMS/MS measurement. All experimental samples and controls were performed in triplicates. Sufficient incubation time was given, and the incubation was stopped when either all parent compound was depleted, or the fluoride/TP formation reached a plateau. The same for the aerobic biotransformation experiment.

Abiotic Defluorination of FCAs with Reducing Agents

To test the FCA reactivity with the reducing agent (i.e., cysteine-sulfide), individual FCAs ($\sim 75 \mu M$) that were degraded in the anaerobic defluorinating enrichment were added into 90 mL sterile anaerobic Milli-Q water with the addition of 48 mg/L cysteine and 96 mg/L sodium sulfide. Milli-Q water amended with only FCA and without the reducing agent was set up as the control. Experiments were performed in triplicates. Three mL supernatant was periodically taken via centrifugation for F^- measurement and LC-HRMS/MS analysis. To avoid any further abiotic degradation of the parent compound in the HPLC vials, we measured the samples on LC-HRMS/MS immediately after sampling.

FCA Biotransformation by Aerobic Activated Sludge

As described in our previous study,⁵⁵ activated sludge communities were taken from the aeration tank in a local municipal WWTP the same day of the experiment. Fifty mL of activated

sludge was inoculated into a 150 mL glass bottle. To maintain the activity of the activated sludge community during the incubation period, we added methanol (~ 47 mg/L) and $\text{NH}_4^+\text{-N}$ (~ 14 mg/L), which were re-amended every other day.²⁵¹⁻²⁵³ All reactor bottles were loosely capped and shaken at 150 rpm at room temperature, with the dissolved oxygen maintained at 6.5 –7.0 mg/L during the entire incubation period. For the biotransformation experiments, ~ 50 μM of individual FCA was added into the bottles inoculated with 50 mL activated sludge. Abiotic controls were set up using autoclaved sludge filtrate to determine the abiotic defluorination of the investigated FCAs. Briefly, activated sludge was filtered through a 0.22 μm filter (Durapore Membrane Filters), and the filtrate was then autoclaved at 121 $^\circ\text{C}$ for 40 min. The same amount of FCAs and nutrients (methanol and $\text{NH}_4^+\text{-N}$) were added to each abiotic control bottle. Biomass-only controls by inoculating the same amount of activated sludge and nutrient but without adding any FCAs were also set up to evaluate the defluorination of any potential F-containing chemicals in the filtrate, but no defluorination was observed when no FCA was amended ([Fig. D.1](#)). All experiments were set up in triplicates. After mixing for 5 min, 3 mL of aqueous suspension was taken as 0-day samples in the same way as described above. Subsequent samples were taken during the incubation period of 14 – 21 days, for the measurement of parent compounds, transformation products (TPs), and F^- . We weighed the reactor bottle every day. On average, the water loss due to evaporation was < 0.1 mL/d (i.e., a max. total water loss < 2.1 mL out of 50 mL), thus it was neglected for the measurements.

Fluoride Measurement.

F⁻ concentrations in the supernatant were measured by an ion-selective electrode (ISE, HACH, Loveland, CO) connected to an HQ30D Portable Multi Meter (HACH), as previously described.^{55, 236} Briefly, before each measurement, the ISE was calibrated according to the manufacturer's instructions. For the sample measurement, a 100 µg fluoride ionic strength adjustment powder (HACH) was dissolved into 2 mL supernatant. The F⁻ concentration was then determined by the ISE-Multi Meter system. The detection limit was 0.01 mg/L (ca. 0.5 µM). The ISE method has been previously cross-checked by an ion chromatography (IC) in the two matrices used in this study, the anaerobic medium and the activated sludge filtrate, where the two methods showed less than 10% differences.^{55, 236} The F⁻ released from FCA was determined by subtracting F⁻ detected in the biomass-only control from F⁻ detected in the experimental group. For FCAs that have impurities being abiotically defluorinated, the F⁻ released from FCA was calculated by subtracting F⁻ detected in the abiotic control from F⁻ detected in the experimental group. The transformation percentage and defluorination degree of FCAs were calculated as follows:

$$\text{Transformation percentage (\%)} = \frac{\text{Removed FCA (\mu M)}}{\text{Total added FCA (\mu M)}} \times 100\%$$

$$\text{Defluorination degree (\%)} = \frac{\text{F}^{-} \text{ released from FCA (\mu M)}}{\text{Removed FCA (\mu M)} \times \# \text{ of F in one molecule}} \times 100\%$$

Ultra-high-performance Liquid Chromatography Coupled to High-resolution Tandem Mass Spectrometry (UHPLC-HRMS/MS) Analysis.

The FCA parent compounds were analyzed by UHPLC-HRMS/MS (Q Exactive, Thermo Fisher Scientific) as described previously.^{49, 55} For UHPLC analysis, 2 μ L sample was loaded onto a Hypersil GOLD column (particle size 1.9 μ m, 100 $\times$ 2.1 mm, Thermo Fisher Scientific) and eluted with nano-pure water (A) and methanol (B) (both amended with 10 mM ammonium acetate) at a flow rate of 300 μ L/min, with a gradient as follows: 95% A: 0 – 1 min, 95% – 5% A: 1 – 6 min, 5% A: 6 – 8 min, and 95% A: 8 – 10 min. For HRMS, mass spectra were acquired in full scan mode at a resolution of 70,000 at m/z 200 and a scan range of m/z 50 – 750 in the negative mode of electrospray ionization (ESI). To elucidate the structures of transformation products, a data-dependent MS² scan was performed at a resolution of 17,500 at m/z 200. Xcalibur 4.0 (Thermo Fisher Scientific) was used for data acquisition and analysis.

Transformation product (TP) Identification

Both suspect and non-target screening were conducted to identify TPs as previously described^{122, 184, 208, 236} with slight modification. For suspect screening, the TP suspect lists were generated by a self-written automatic metabolite mass prediction script,¹²⁹ which was modified and used to specifically predict the potential TPs of the investigated FCA structures. Plausible TPs were identified using Compound Discoverer 3.1 (Thermo Fisher Scientific) based on the following criteria: (i) mass tolerance < 5 ppm; (ii) isotopic pattern score > 90%; (iii) peak area > 10⁵; (iv) peak area showing either an increasing trend or first increasing then decreasing trend over time; (v) no formation in the heat-inactivated control or the biomass-free fresh medium control; (vi) not

detected as an in-source fragment of the parent compound or other identified TPs. For non-target analysis, all detected features that met the above criteria were picked by Compound Discoverer. The formula of identified TPs was further validated by Xcalibur 4.0. MS² fragments were used for TP structure elucidation. The confidence level of the structure elucidation for each TP was assigned based on the criteria set up by Schymanski et al.¹⁵⁶ ChemDraw Professional 20.0 and MarvinSketch (version 19.20.0, ChemAxon, <http://www.chemaxon.com>) were used for drawing, displaying, and characterizing chemical structures.

5.4 Results and Discussion

α , β -unsaturation is Crucial for FCA Biotransformation in the Anaerobic Defluorinating Enrichment

To demonstrate the structure-specificity of FCA biodegradability and the role of C=C double bonds and branched structures, we used the same anaerobic defluorinating enrichment culture that was reported to reductively defluorinate two branched and unsaturated FCAs, i.e., **MeU-C6a** (PFMeUPA) and **MeU-C6b** (FTMeUPA).²³⁶ We investigated the biotransformation/defluorination feasibilities of a comprehensive set of commercially available unsaturated FCAs, together with the structurally similar saturated ones. The tested FCAs also have linear/branched structures with various chain lengths and fluorine substitution degrees. Among the nine unsaturated FCAs, the six structures with α , β -unsaturation ($-\text{C}=\text{C}-\text{COOH}$) (i.e., **U-C4b** (1:3 FTUCA), **U-C5a**, **MeU-C5e**, **MeU-C6a**, **MeU-C6b**, and **U-C8b** (6:2 FTUCA)) showed nearly

complete biological transformation/defluorination (no degradation in the heat-inactivated controls). In contrast, the structurally similar saturated FCAs of the latter three structures (i.e., **C5a**, **C5b**, and **Me-C6b**) revealed no biodegradability (Fig. 2). Moreover, the tertiary sp^3 C–F bond in the branched moiety of MeO-C6c (GenX) was not able to be microbially cleaved like the one in **MeU-C6b**,²³⁶ although the bond dissociation energy of the tertiary sp^3 C–F bond in GenX is low (ca. 432.2 kJ/mol⁴⁷). It was likely due to the saturated structure of GenX, which made it less bioavailable. **U-C5c**, which has a terminal C=C bond, was not biotransformed, either (Fig. 5.2). The other two unsaturated FCAs (**MeU-C4c** and **MeU-C5d**) showed abiotic transformation and defluorination, which will be discussed in the following section. The linear unsaturated perfluorinated FCA (i.e., **U-C5a**) exhibited a similar defluorination activity to the branched and unsaturated perfluorinated structure²³⁶ (i.e., **MeU-C6a**), indicating that it is the C=C double bond that determined the anaerobic microbial defluorination of those two structures. Collectively, the α , β -carbon unsaturation in FCA was crucial for the initiation of anaerobic microbial defluorination by the investigated enrichment, which seemed independent of fluorine substitution degrees and the linear/branched structures.

Biotransformation Pathway Elucidation and the Pathway Preference of The Biotransformed Unsaturated FCAs in the Anaerobic Defluorinating Enrichment

For the biotransformed FCAs, we elucidated the biotransformation and biodefluorination pathways based on the F^- formation and the identified TPs. Consistent with what was previously

reported for **MeU-C6a** (PFMeUPA) and **MeU-C6b** (FTMeUPA),²³⁶ the other biotransformed unsaturated FCAs also underwent two pathways: (i) reductive defluorination ($-F+H$) and (ii) hydrogenation ($+2H$) and exhibited different pathway preferences correlated with the number of fluorine substitutions on the unsaturated carbons. By multiplying the defluorination degree by the total number of F in the molecule, we were able to determine how many fluoride ions would have been released from one molecule of the parent compound if all had undergone defluorination. If the defluorination degree was less than one over the number of F in the molecule, the parent compound would also undergo non-defluorinating pathways. Thus, we used the defluorination degree to evaluate the preference of reductive defluorination over the non-defluorinating hydrogenation pathway for all biotransformed unsaturated FCAs. The two perfluorinated unsaturated structures (i.e., **U-C5a** and **MeU-C6a**) showed higher defluorination degrees. Similar to **MeU-C6a**,²³⁶ **U-C5a** was completely biotransformed, corresponding to at least 52 μM F^- release after taking the abiotic F^- formation into account (Fig. 5.3A, Fig. D.2). It is worth noting that the abiotic F^- formation in the heat-inactivated control was from the impurities of **U-C5a** standard as no removal of **U-C5a** or formation of any TPs was observed during the entire incubation period (Fig. 5.3A). We assumed that the maximum F^- released from the impurities in the biotransformation group was the same as detected in the heat-inactivated control. Then, we gave a conservative estimation of the F^- released from **U-C5a** by subtracting the F^- formed in the heat-inactivated control from that in the biotransformation group, resulting in 52 μM , which

corresponds to a defluorination degree of 14.3% (Fig. 5.3A), as if that all of the parent compounds exclusively underwent the first C–F cleavage via reductive defluorination (reaction 1 in Fig. 5.3C). The detection of the hydrogenation product of **U-C5a** (**U-C5a_TP226**, reaction 2 in Fig. 5.3C, Fig. 5.3B, and Fig. D.3) demonstrated that only a portion of **U-C5a** underwent reductive defluorination, forming the first C–F cleavage product (**U-C5a_TP206**, Fig. 5.3B and Fig. D.4). A second C–F cleavage should also occur, perhaps after the decarboxylation of the primary defluorination product as reported for **MeU-C6a**.²³⁶ However, the primary defluorination product (**U-C5a_TP206**) seemed to largely undergo hydrogenation, as its rapid decrease was corresponding to the increase of the hydrogenation product (**U-C5a_TP209**, Fig 5.3B and Fig. D.5). The TP formation trends, together with the overall defluorination degree, suggest that the second C–F bond cleavage was not as significant as the first one. In comparison, the defluorination degree of **MeU-C6b**, which has no F substitution on the unsaturated carbons, was only 4%,²³⁶ less than 14% (1 out of 7 F in the molecule), indicating that more than half of the parent compound underwent the hydrogenation pathway and the defluorination was at the tertiary sp^3 C – F bond. For **U-C4b** (1:3 U acid), which has neither fluorine substitutions on the unsaturated carbons nor the tertiary sp^3 C–F bond in a branched moiety as the one in **MeU-C6b**, it only underwent hydrogenation with the formation of the corresponding product (+2H of **U-C4b**, 1:3 FTCA) (Fig. D.6).

To further examine the influence of fluorine substitutions on the pathway preference between reductive defluorination and hydrogenation, we analyzed the biotransformation of **U-C8b** (6:2 FTUCA) (Fig. D.7), which is the only commercially available FCA structure with α , β -unsaturation and one fluorine substitution on the unsaturated carbon. It was rapidly biotransformed, together with the release of fluoride, which reached a plateau within the first week of incubation (Fig. 5.4A, Fig. D.8). The hydrogenation (reaction 2 in Fig. 5.4B) product (i.e., 3-fluoro 5:3 acid) was also formed (Fig. 5.4A and Fig. D.9), which seemed subject to very slow secondary biotransformation (reaction 4 then reaction 3 in Fig. 5.4B) corresponding to the slight formation of F^- and 5:3 FTCA after the first week (Fig. 5.4A and Fig. D.10). Thus, we inferred that the major defluorination pathway was via reductive defluorination of the sp^2 C–F bond in the parent compound (reaction 1 in Fig. 5.4B). However, the reductive defluorination product, 5:3 FTUCA, was not detected. It was likely due to its fast turnover to the 5:3 FTCA via hydrogenation (reaction 3 in Fig. 5.4B), which was also observed in anaerobic digester sludge.¹⁹⁹ Like **U-C5a**, due to the abiotic F^- formation from impurities of **U-C8b** standard (Fig. D.8), we alternatively used the formation of 5:3 FTCA, which can be accurately quantified using the reference compound, to determine the defluorination degree (Fig. 5.2) and the percentage of **U-C8b** that underwent defluorination (Fig. 5.4B). Since the 5:3 FTCA formed from 3-fluoro 5:3 acid was minor, we considered nearly all 5:3 FTCA formed (12 μ M) was from the reductive defluorination pathway, corresponding to 17.1% (12 μ M/70 μ M) of the parent compound that underwent

defluorination. The remaining 82.9% of the parent compound underwent the hydrogenation pathway (Fig. 5.4B), suggesting that this pathway was favored. The anaerobic biotransformation products of 6:2 FTUCA detected here were consistent with those in the anaerobic digester sludge reported by Zhang et al.¹⁹⁹ In that study, 6:2 FTUCA was also quickly removed within a week, forming the transient intermediate 5:3 FTUCA at a low-level, as well as another two semi-stable/stable products, 3-fluoro 5:3 acid and 5:3 FTCA. Different from the very slow transformation of 3-fluoro 5:3 acid observed in this study, Zhang et al. observed substantial decrease of 3-fluoro 5:3 acid, corresponding to a continuous increase in 5:3 FTCA with no detection of 5:3 FTUCA after 6:2 FTUCA was depleted. It indicates that the activity of microorganisms carrying out the HF elimination of 3-fluoro 5:3 acid in this study was much lower than that in the digester sludge community studied by Zhang et al. We re-analyzed the TP formation data reported in the Supporting Information by Zhang et al. and found that more than 50 mol% out of the 94 mol% total formation of 5:3 FTCA was formed after 6:2 FTUCA was consumed. As 5:3 FTUCA, once formed, could be quickly hydrogenated to 5:3 FTCA, any 5:3 FTCA formed after the depletion of the parent compound should be from the other intermediate, which is 3-fluoro 5:3 acid via reaction 4 and 3 in Fig. 4B. Thus, the preference of hydrogenation pathway over the reductive defluorination pathway for 6:2 FTUCA is also implied in the results from another study using a different anaerobic microbial community.¹⁹⁹ Collectively, the reductive defluorination of structures with one fluorine substitution on the unsaturated carbon was less favorable than the

perfluorinated unsaturated structures in the anaerobic defluorinating enrichment. Instead, the hydrogenation pathway became more favorable as more sp^2 C–F bonds being replaced with C–H bonds. Effective inhibition of the enzymes/microorganisms carrying out the hydrogenation pathway is desired to improve the overall defluorination degree of the unsaturated FCA structures. Even though the hydrogenation could be inhibited, the improvement of the overall defluorination could still be limited to the cleavage of the sp^2 C–F bonds and the tertiary sp^3 C–F bond (for branched unsaturated structures). Introducing branches, particularly more fluorinated moieties at the α , β -unsaturated carbons might enhance the overall defluorination degree.

Abiotic Transformation and Defluorination of Unsaturated FCAs with a Trifluoromethyl ($-CF_3$) Branch at the α/β Carbon in the Presence of Reducing Agents

Among the three commercially available α/β -branched unsaturated FCAs (i.e., **MeU-C4c**, **MeU-C5d**, and **MeU-C5e**), the two with a $-CF_3$ branch (i.e., **MeU-C4c** and **MeU-C5d**) exhibited abiotic transformation and defluorination in the presence of cysteine and sulfide, the commonly used reducing agents in anaerobic culture media. In comparison, no transformation or defluorination was observed when no reducing agent was provided (Fig. D.11). Notably, the $-CF_3$ branch in **MeU-C5d** significantly enhanced the degradability and defluorination (Fig. D.10 – D.15) compared to the $-CH_3$ branch at the same position in **MeU-C5e**, which only underwent slow microbial hydrogenation (Fig. D.16 – D.18). The similar defluorination degrees of **MeU-C5d** in both the active enrichment culture and the anaerobic (with N_2 headspace) biomass-free sterile

Milli-Q water indicate an abiotic defluorination process. Similarly, the $-\text{CF}_3$ branched structure **MeU-C4c** became more vulnerable to degradation and defluorination than its linear isomer **U-C4b** (Fig. D.10 and D.19). The 60 – 80% defluorination degree of **MeU-C5d** corresponds to a release of 4 – 5 F from the molecule if all **MeU-C5d** could have undergone defluorination. Because not all **MeU-C5d** underwent defluorination pathway according to the formation of the non-defluorinating cysteine conjugation product (Fig. D.12 and Fig. D.15), for the portion of **MeU-C5d** that underwent the abiotic defluorination, more F, perhaps all the six, could be cleaved. However, no defluorination intermediates were detected, perhaps due to the simultaneous cleavage of nearly all C–F bonds and the formation of small products that cannot be detected by LC-HRMS. The abiotic defluorination could be spontaneous when the redox potential was lowered below a threshold by the reducing agent or via reactions with either cysteine or sulfide. Further studies are needed to examine the abiotic defluorination mechanisms.

The previously mentioned abiotic defluorination of impurities in the heat-inactivated controls of **U-C5a** and **U-C8b** was also attributed to the presence of reducing agents, as no F^- release was detected when no reducing agents were added (Fig. D.11). Interestingly, the total F^- formation from **U-C8b** in the biological samples was even lower than the abiotic F^- formation from **U-C8b** impurities in the abiotic control (Fig. D.8). **U-C8b** was demonstrated to be biodefluorinated and transformed into 5:3 FTCA (Fig. 5.4). If the same amount of F^- was released from the impurities in the biological samples, the total F^- should be higher instead of lower. This

observation could be explained that the impurities might have unsaturated structures, which were more likely to be microbially hydrogenated than abiotically defluorinated in the biological samples. The hydrogenation pathway was demonstrated to be exclusively enzyme-mediated as no hydrogenation product was detected during the abiotic transformation of **MeU-C5d** (Fig. D.12C). It, again, indicates that the microbial hydrogenation of unsaturated structures (particularly those with no sp^2 C–F bonds) could compete with the (bio)defluorination pathways under anaerobic conditions.

Aerobic biotransformation and biodefluorination of the unsaturated FCAs.

Complementarily, the unsaturated FCAs that showed low removal and defluorination under the anaerobic condition exhibited higher biotransformation and biodefluorination aerobically. For example, **U-C5c** with a terminal C=C bond, which did not show any anaerobic biotransformation, exhibited 30% aerobic removal via a 2-F release pathway (~ 80% defluorination of the removed part) (Fig. 5.5).⁵⁵ On the other hand, all investigated perfluorinated structures (both unsaturated and saturated) and the saturated FCAs did not show aerobic defluorination by the activated sludge communities.

Moreover, **MeU-C4c**, which underwent complete abiotic transformation but only with ~10% defluorination in the anaerobic condition, exhibited a complete aerobic removal within a day and a complete defluorination within two weeks (Fig. 5.6A). The further defluorination after the depletion of **MeU-C4c** indicates the defluorination may occur to the biotransformation

intermediate of **MeU-C4c**. Since hydrogenation was demonstrated to be the major aerobic biotransformation pathway for the two unsaturated structures, **MeU-C5e** (Fig. D.20) and **MeU-C6b** (Fig. D.21), we inferred that the complete defluorination of **MeU-C4c** likely started from the hydrogenation step, forming 2-methyl-trifluoropropionate (Fig. 5.6B), which could undergo the same defluorination pathway as reported in our recent study (Fig. D.22).²⁵⁴ Interestingly, we observed a 4-day plateau of fluoride release after the first-day incubation (~43.8% defluorination) (Fig. 5.6A). It is likely due to the first step defluorination via HF elimination was relatively fast, but the next two steps of defluorination needed a longer acclimation for the microbes. However, no intermediates were identified by the LC-HRMS, probably because they were mainly in CoA forms in microbial cells, which were difficult to extract and detect, leaving relatively low levels of the acid forms in culture suspension. In the abiotic control, a low level (< 10 μ M) of F^- was released with no removal of **MeU-C4c** (Fig. 5.6A), indicating aerobic abiotic defluorination of **MeU-C4c** impurities. Since the F^- released from impurities was not as significant as that from the parent compound **MeU-C4c**, it did not affect the biodefluorination analysis. However, if the F^- released from impurities and the target compound were comparable, it may interfere with the assessment of biodefluorination of the target compound, which should be taken into consideration in future studies related to FCAs remediation.

Besides, **MeU-C5d**, which showed abiotic transformation (100% after 18 days) and defluorination (77.5%) in the anaerobic condition (Fig. 5.2), also underwent aerobic microbial

defluorination with an even higher defluorination degree (82%), although the removal was incomplete after 21 days (Fig. 5.5 and 5.7). Three defluorination intermediates were identified, including the two defluorinating TPs (**MeU-C5d_TP139** and **MeU-C5d_TP121**) and the hydrogenation product (**MeU-C5d_TP209**) (Fig. 5.7B and C). **MeU-C5d_TP139** was likely further biotransformed to **MeU-C5d_TP121** as it slightly decreased after 9 days with an increase of **MeU-C5d_TP121** (Fig. 5.7B). Given that the formation of **MeU-C5d_TP209** did not contribute to the defluorination, the actual defluorination degree could be even higher than 82%, and perhaps all six C–F bonds in the molecule were cleaved via the proposed pathway in Fig. 5.7C.

The linear unsaturated structure, **U-C8b** (6:2 FTUCA), also exhibited an enhanced defluorination in the aerobic condition compared to the anaerobic condition (10% vs. 1%) (Fig. 5.6 and Fig. 5.2). It is likely because more parent compound underwent the defluorination pathways in the aerobic activated sludge. The preferred hydrogenation pathway, forming 3-fluoro 5:3 acid, in the anaerobic defluorinating enrichment was not observed during the aerobic biotransformation (Fig. D.23C and D). 6:2 FTUCA is a key biotransformation intermediate of 6:2 fluorotelomer alcohol (6:2 FTOH, an important ingredient in aqueous film-forming foam, AFFF) under aerobic conditions by various microbial communities and microbial species.^{53, 57-67} Many of the previously reported TPs were also detected here (Fig. D.23B – D), and among them **C6a** (PFHxA) and **C5a** (PFPeA) seemed to be the two major ones. Besides, we also detected two new TPs (2H-PFHpA and 2H-PFHxA) (Fig. D.23D and E and Fig. D.24 & D25), which were not

reported previously for 6:2 FTOH,⁵⁴ while Wang et al. detected 2H-PFOA from the aerobic biotransformation of 8:2 FTOH.⁶⁷ We did not detect 5:2 ketone and 5:2 sFTOH, two important TPs of 6:2 FTUCA reported in previous studies.^{58, 67} 5:2 sFTOH was formed from 5:2 ketone, which was likely a decarboxylation product after β -hydroxylation of 6:2 FTUCA.⁶⁷ This pathway seemed not occur in the activated sludge community we investigated. Thus, we proposed two major pathways, similar to β - and α -oxidation, leading to all detected TPs of 6:2 FTUCA (Fig. D23E). Despite various defluorinating TPs, the low defluorination degree and the accumulation of TPs indicate that the majority of C–F bonds still stayed intact in the TPs, which remained microbially recalcitrant.

5.5 Conclusion

F[−] release has been used as a necessary indicator of defluorination activities. For biodefluorination assays, it is worth noting that some impurities in the purchased standard compound may be defluorinated, and the abiotic defluorination of impurities could be suppressed by other competing enzymatic pathways, such as biohydrogenation of unsaturated structures, in biological samples. If the F[−] released from impurities was at significant levels, it may interfere the F[−]-based assessment of the bioavailability of the target organofluorine molecule, resulting in either overestimation (for **U-C5a**) or underestimation (for **U-C8b**). Thus, besides F[−] measurements, comprehensive analyses of the target parent compound and the related TPs in the

biotransformation experimental groups and various abiotic control groups must be included to ensure an accurate assessment in biodefluorination studies.

A comprehensive understanding of the structure-specific biotransformation and biodefluorination of unsaturated FCAs is significant for not only the development of biological treatment technologies but also the source tracking and the environmental fate of PFAS.²⁵⁵ The structure-biodegradability relationships revealed in this study emphasize that not all FCA structures could be turned over by microbes. FCAs with the same signature structures or functional groups exhibited similar biotransformation performance in the investigated microbial communities. More specifically, the α , β -unsaturation rather than the $-\text{CF}_3$ branch is the required structure for anaerobic biotransformation by the investigated anaerobic defluorinating enrichment when the fluorinated compound was provided as the sole electron acceptor. With the α , β -unsaturation, the tertiary sp^3 C–F bond can also be anaerobically defluorinated. The fluorine substitution degree, the presence of branches, and the chain length in the α , β -unsaturated FCAs did not affect the bioavailability. However, the reaction preference (defluorination vs. hydrogenation) was affected mainly by the fluorine substitution degree. The more F to be substituted by H at the C=C double bonds, the more favorable the hydrogenation will be over the defluorination under the anaerobic condition. The finding implies that although perfluorinated structures were exclusively defluorinated anaerobically, anaerobic conditions may not be suitable for biodefluorination of polyfluorinated structures with fewer fluorine substitutions, which could

be better defluorinated aerobically. Thus, the combination of anaerobic and aerobic conditions may be implemented in PFAS bioremediation to degrade a broader range of PFAS structures and achieve deeper defluorination.

Moreover, we demonstrated that the introduction of one $-\text{CF}_3$ branch at either of the unsaturated C in the α , β -unsaturated FCAs could significantly enhance the degradability (anaerobic abiotic defluorination and aerobic biodefluorination). It provides important insights and opens new avenues for the design of novel fluorinated PFAS alternatives that are readily (bio)degradable. We acknowledge that due to the unavailability of some specific FCA structures, only a limited number of α , β -branched, unsaturated FCA structures were tested. The generality of the finding still needs to be further demonstrated when more desired structures are available. Collaborations between researchers from academia and fluorochemical industries may be needed to examine the structure-reactivity relationship using custom-synthesized structures and discover fluorinated PFAS alternatives with retained functionality, maximized degradability, and minimized adverse impact to the environment.

5.6 Special Acknowledgements of Chapter 5

I would like to acknowledge that the work presented in Chapter 5 has been invited for submission by *Environmental Science and Technology* under the Special Issue “Emerging Contaminants: Fluorinated Alternatives to Existing PFAS Compounds”. By the time I submitted

the dissertation, Chapter 5 is in revision. Thus, it is expected to have several modifications of this current version in the published journal.

5.7 Figures of Chapter 5

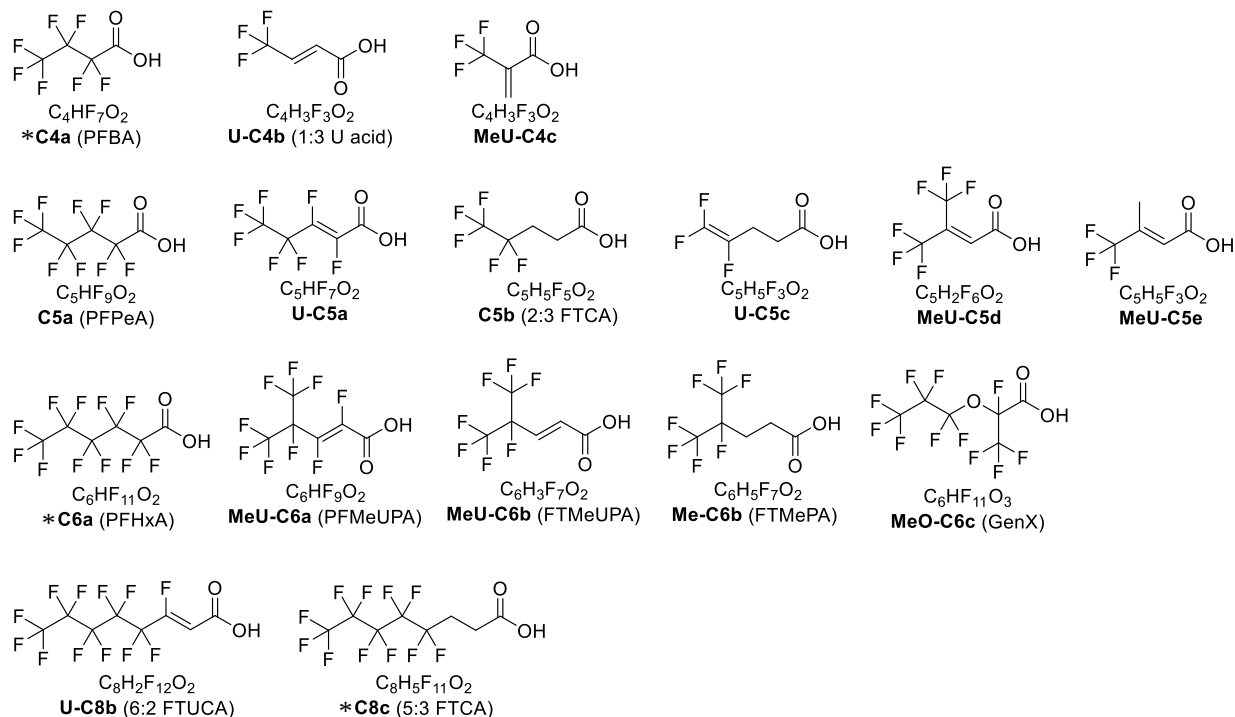


Figure 5.1 Structures of the 16 FCA standards used in this study, including the 13 FCAs used as parent compounds and three (*) used as the analytical standards of transformation products (“U” stands for unsaturated structures; “Me” stands for the structures with either $-CF_3$ or $-CH_3$ branches; “O” stands for the ether structure).

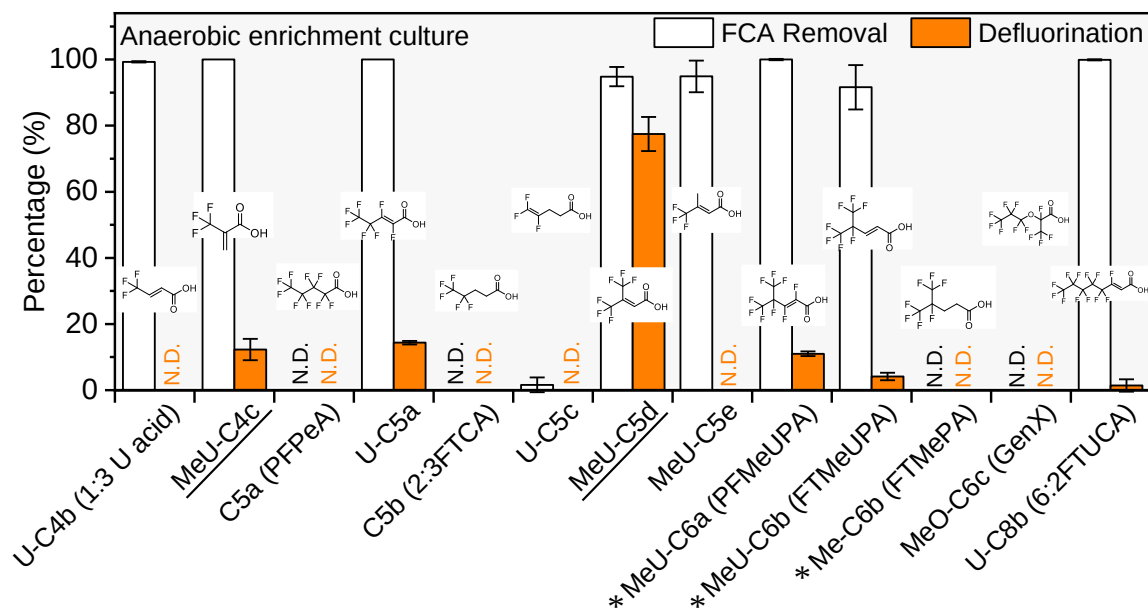


Figure 5.2 Parent compound removal and defluorination degree of thirteen FCAs that underwent biological transformation/defluorination in the anaerobic defluorinating enrichment (*: data acquired from our previous study²³⁶; __: abiotic transformation and defluorination; ; sufficient incubation times (18 – 150 days) were performed until all parent compound was depleted or the fluoride/TP formation reached a plateau, and the end time point data were shown in the figure; N.D.: not detectable; n=3).

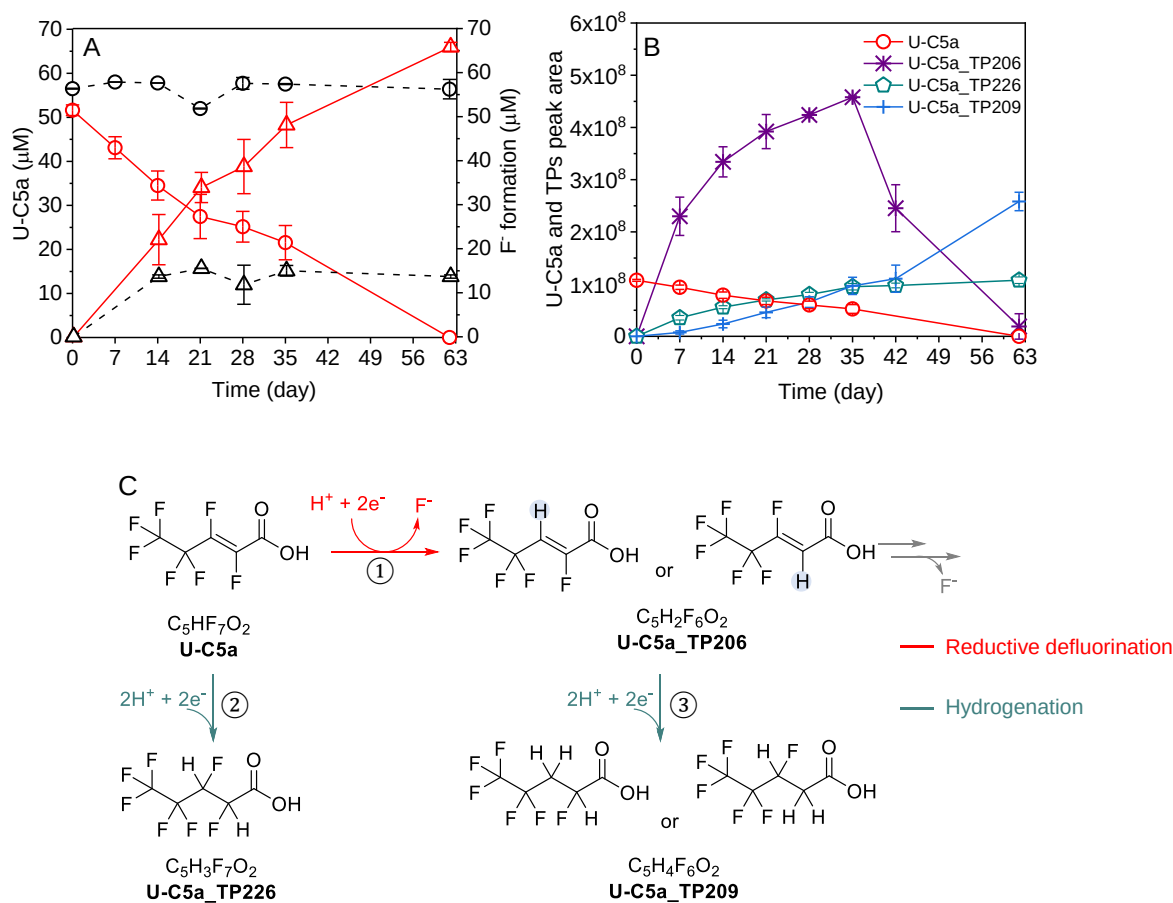


Figure 5.3 U-C5a removal and fluoride release in the anaerobic defluorinating enrichment (A) (red: biological samples; black: heat-inactivated controls; circles: parent compound; triangles: fluoride formation; $n=3$); temporal trends of the parent compound and the TPs during U-C5a biotransformation (B) (Note: the TPs of U-C5a identified in the biological samples were not detected in the heat-inactivated control; due to different ionization efficiencies, the peak area only indicates the degradation/formation trend of each compound but not their relative abundances); proposed biotransformation pathways of U-C5a (C).

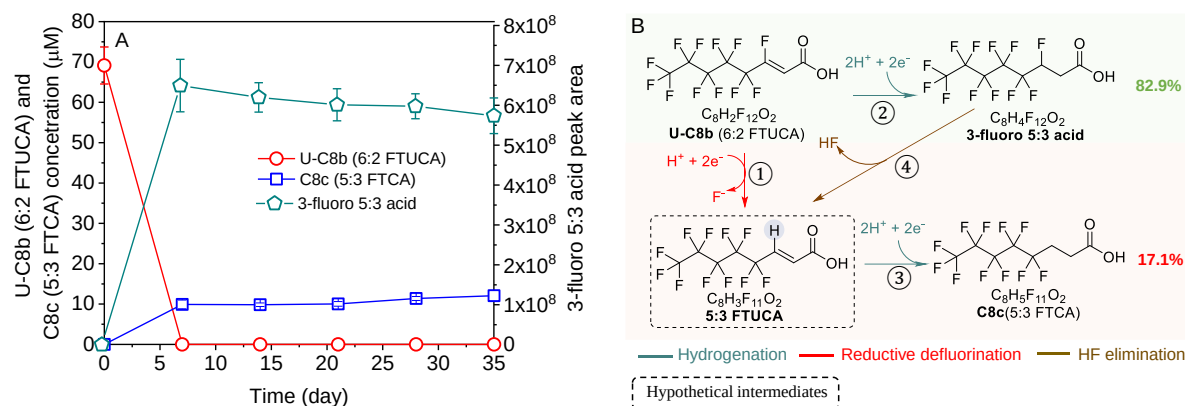


Figure 5.4 Temporal trend of the **U-C8b** and the TPs during the anaerobic biotransformation (A) (Note: in the heat-inactivated control, U-C8b did not show any removal, and the two TPs of **U-C8b** identified in biological samples were not detected); proposed biotransformation pathways of **U-C8b** (B) (the percentage indicates the molar ratio of parent compound subject to the specific route: 82.9% for hydrogenation and 17.1% for defluorination; the dashed box indicates hypothetical intermediates not detected).

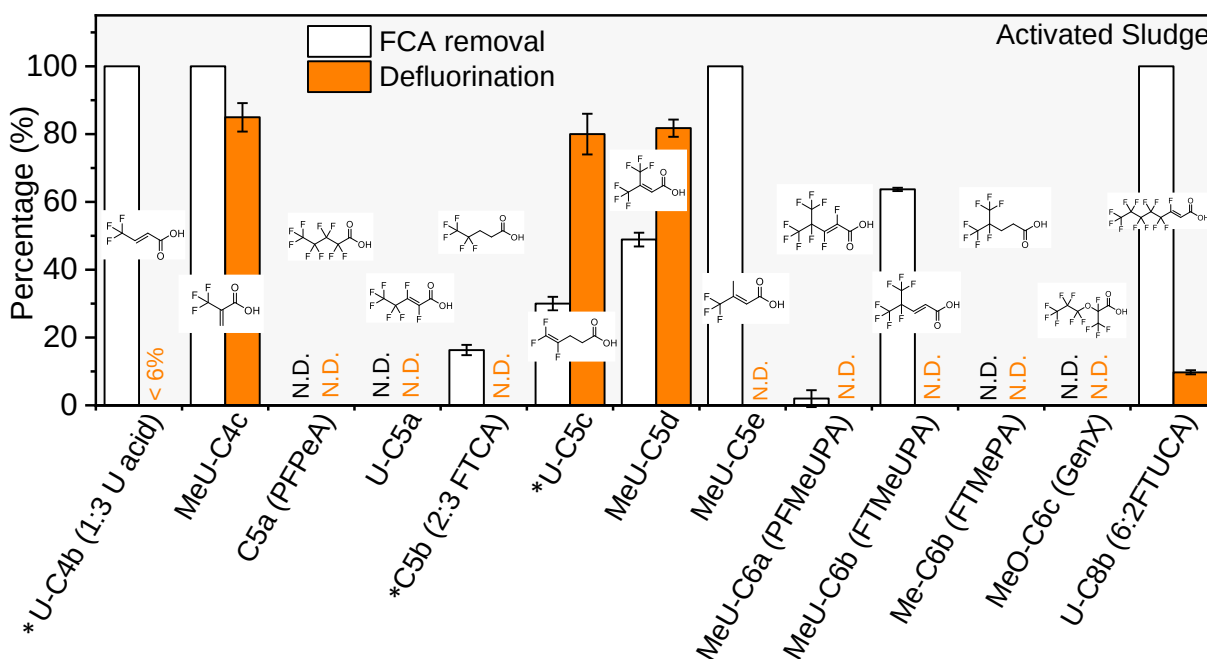


Figure 5.5 Parent compound removal and defluorination degree of all tested structures in activated sludge (*: data acquired from our previous study⁵⁵; all compounds were incubated for 14-day, except for U-C5c, which was incubated for 72 h as it reached the max. removal and defluorination after 24 h⁵⁵; N.D.: not detectable; n=3).

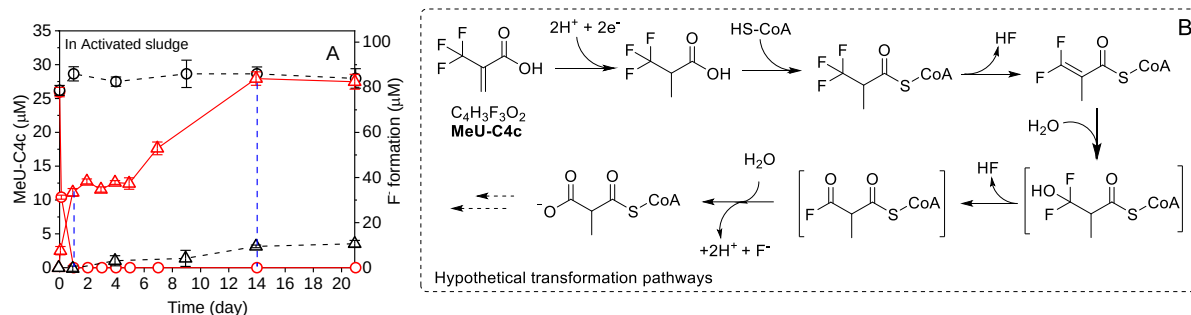


Figure 5.6 MeU-C4c removal and fluoride release in activated sludge (A) (red: biological samples; black: autoclaved sludge filtrate controls; circles: parent compound; triangles: fluoride; n=3); proposed hypothetical transformation pathways of MeU-C4c (B) (Note: in brackets are unstable intermediates; the dashed box indicates that none of the proposed intermediates was detected).

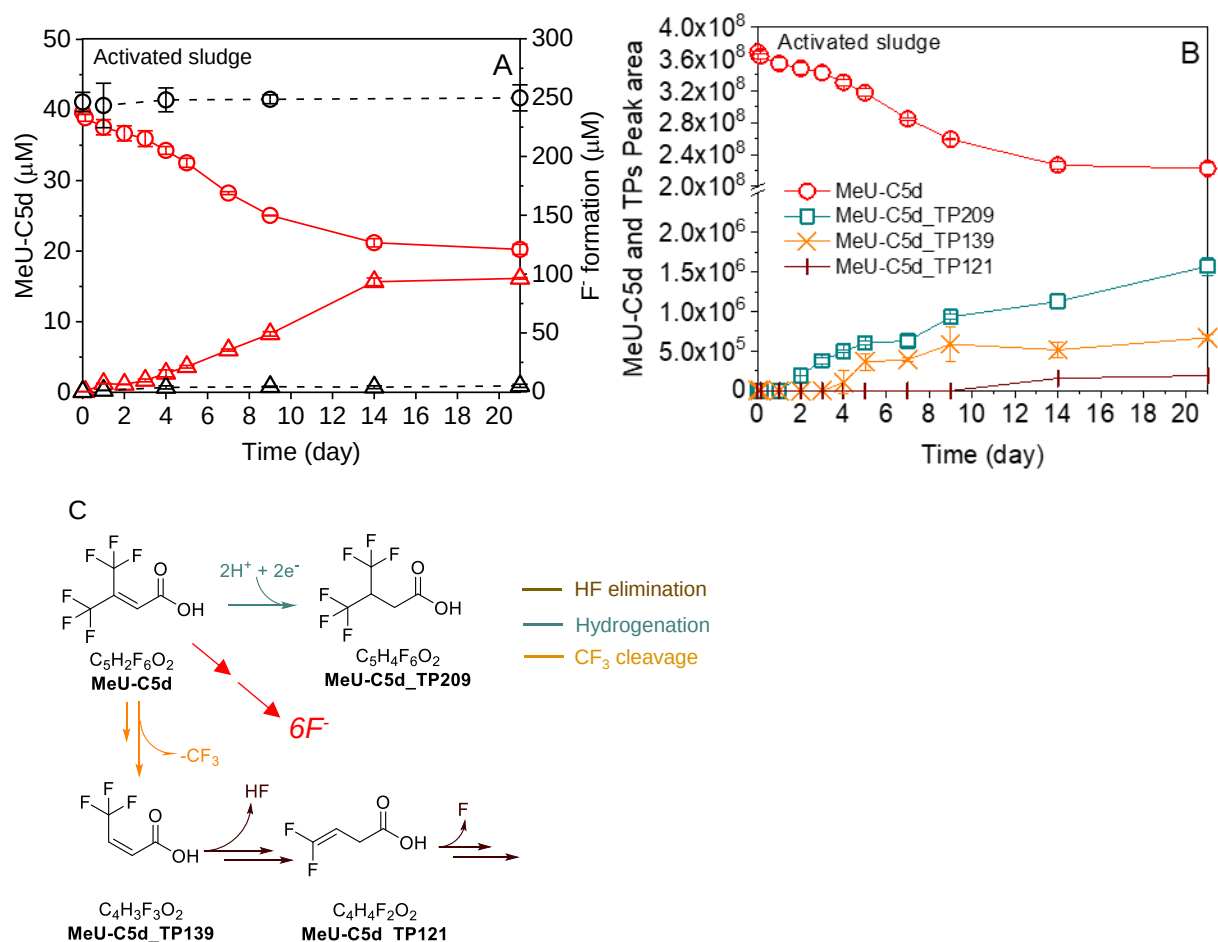


Figure 5.7 Aerobic removal of **MeU-C5d** and fluoride release in the activated sludge community (A) (red: biological samples; black: abiotic controls; circles: parent compound; triangles: fluoride; n=3); the temporal trend (peak areas) of parent compound and TPs during **MeU-C5d** biotransformation (B) (Note: Due to the differences in ionization efficiencies of TPs, the peak areas only indicate the formation trend of each TP but not their relative abundance); the proposed biotransformation pathways of **MeU-C5d** (C) (Note: **MeU-C5d_TP139** is an isomer of **U-C4b** as it has a different retention time (0.87 min) than **U-C4b** (2.36 min), although they share the same formula).

CHAPTER 6: COMMUNITY DYNAMICS AND IDENTIFICATION OF FUNCTIONAL GENES DURING THE ENRICHMENT OF AN ANAEROBIC DEFLUORINATING CULTURE

6.1 Abstract

The discovery of microbial reductive defluorination of unsaturated fluorinated compounds by an anaerobic enrichment culture could potentially lead to new biotechnologies in organofluorine compounds remediation. It was previously demonstrated that the well-known dechlorinators (e.g., *Dehalococcoides* spp., *Geobacter* spp.) in the enrichment were not involved in the reductive defluorination, suggesting the responsible defluorinating microorganisms might be other previous overlooked members in the community. In this presented study, we characterized the electron donor specificity of the enrichment culture and found that the responsible microbes are versatile in utilizing multiple organic substrates as well as H₂. The 16S rRNA gene amplicon sequencing indicated community dynamics during the enrichment process. The Scanning Electron Microscopy imaging of the microbial community likely suggested a strong syntrophic relationship of the major components. We further used metagenomic and metatranscriptomic sequencing to reconstruct the enriched genomes in the community and identify the possible microbes and genes responsible for the reductive defluorination of the specific perfluorinated structure. In total, 46 bin genomes were retrieved. Possible suspects were found to encode fluoride ion efflux related genes as well as the reductases that are known for the α , β -unsaturated carboxylic acid reduction. The findings expand our knowledge of the anaerobic microbial defluorination and provide significant

insights into the identification of biomarkers indicative of the potential of microbial reductive defluorination.

6.2 Introduction

Due to the unique properties of per- and polyfluoroalkyl substances (PFAS) (e.g., hydrophobicity, lipophobicity, and thermal stability), PFAS are becoming more and more pervasive. Heavily fluorinated compounds, especially the perfluorinated ones, have negative impacts on nearly all living organisms, resulting in increasing regulations being established to remediate such contaminants. However, due to the strong C–F bonds in PFAS, only a few studies on microbial defluorination, especially for perfluorinated structures,^{200, 236, 256} have been documented, making the environmental cleanup of such heavily fluorinated compounds by bioremediation approaches still in its infancy.

In both natural and engineering environments (e.g., wastewater treatment plants), microbial degradation and transformation play the most important roles in alleviating the concentrations of organic contaminants. In our previous studies (Chapter 5 and 6),²³⁶ α , β -unsaturated perfluorinated carboxylic acids were found to be reductively defluorinated by an anaerobic enrichment culture, which was originated from a commercial bioaugmentation culture (KB1). KB1 enrichment culture are known to dechlorinate organochlorine compounds such as TCE to ethene.²²⁸ The most dominant responsible microbes in KB1 during the reductive dechlorination have been reported as *Dehalococcoides* spp. However, according to our previous investigations, the *Dehalococcoides*

spp and the previously identified reductive dehalogenase genes in KB1 might not be responsible for the reductive defluorination.²³⁶ Therefore, the research questions that have been scientifically and practically intriguing but not yet addressed are: “Which types of microorganisms are responsible for the defluorination?” and “Which genes or enzymes are necessary for the microbial reductive defluorination?”

In this study, we filled these knowledge gaps by investigating the physiological characters of the defluorinating enrichment culture. Specifically, using previously reported PFMeUPA as the model compound, we examined its reductive defluorination feasibility coupled with different types of electron donors. The SEM imaging of the enrichment culture was also conducted to visualize the micro-interactions of the components in the enrichment culture. The microbial community succession from the original seed sludge to the defluorinating enrichment was tested by 16S rRNA gene sequencing. Furthermore, by metagenomic sequencing, we revealed the community structure after six subculture events. We proposed the possible responsible species and the functional genes/enzymes involved in the PFMeUPA biotransformation (reductive defluorination and hydrogenation). These findings, especially the criteria we proposed for screening the responsible defluorinating microbes and the involving genes we identified for the microbial reductive fluorination, provided significant insights into the future discovery, study, and engineer of microorganisms that can efficiently defluorinate perfluorinated compounds.

6.3 Methods and Material

Chemicals

Standard compounds of (E)-perfluoro(4-methylpent-2-enoic acid) (CAS number: 103229-89-6, PFMeUPA or MeU-C6a) were purchased from SynQuest Laboratories (Alachua, FL) and used without further purification. 10 mM stock solution of PFMeUPA was prepared anaerobically in autoclaved Milli-Q water in 160 mL sealed serum bottles and stored at room temperature until use.

Enrichment and Maintenance of PFMeUPA-defluorinating Microorganisms.

After the first dose of PFMeUPA was completely consumed, the KB-1 subculture (10% KB-1, v/v) (subculture 1) grown on lactate was subsequently transferred into a 90 mL fresh basal medium containing 5 mM lactate and 75 μ M PFMeUPA for subculture events (subculture 2 to 6). The defluorination of additional PFMeUPA doses was also tested in the precedent subcultures (subculture 1 to 5). Aqueous samples and cell pellets were taken in the same way as described previously to measure the concentration of F^- . Cells pellets were stored in $-20\text{ }^{\circ}\text{C}$ for DNA extraction and $-80\text{ }^{\circ}\text{C}$ for RNA extraction, respectively.

Biodefluorination of PFMeUPA with Different Electron Donors.

The enrichment was inoculated (10% v/v) into fifteen 160-mL serum bottles containing 90 mL fresh medium as described above and 75 μ M PFMeUPA. The fifteen bottles were maintained with five electron donor conditions: (1) H_2 (3 bottles, with H_2/CO_2 headspace and acetate as the

carbon source; (2) no electron donor control (3 bottles, with Ar/CO₂ headspace); (3) methanol (3 bottles with 5mM methanol and Ar/CO₂ headspace); (4) butyrate (3 bottles with 5mM butyrate and Ar/CO₂ headspace); (5) positive control (three bottles, with 5 mM lactate and Ar/CO₂ headspace). Aqueous samples were taken subsequently during the incubation period for the measurement of defluorination activity.

Fluoride Ion Measurement by Ion-Selective Electrode

As described in previous studies (Chapter 5 and Chapter 6),^{55, 236} the concentration of fluoride ion (F⁻) in the culture supernatant was measured by an ion-selective electrode (ISE, HACH, Loveland, CO) connected with an HQ30D Portable Multi Meter (HACH). Briefly, a 100 µg fluoride ionic strength adjustment powder (HACH) was added into 2 mL culture supernatant, and the F⁻ concentration was then measured with the ISE-Multi Meter system. Ion chromatography (IC) was used to validate the fluoride measurement by ISE, which exhibited similar (10% or less difference) fluoride concentration measurement results compared to the ISE method.

16S rRNA Gene Amplicon Sequencing and Sequence Analyses

Genomic DNA for 16S rRNA amplicon sequencing was extracted from 1.5 mL biomass samples at 0-day (before PFMeUPA was added) and 150-day (after the first dose of PFMeUPA was consumed) in subculture 1. Complementary DNA (cDNA) was synthesized from RNA that was extracted from 1.5 mL biomass samples taken at 77-day. The genomic DNA extraction, RNA extraction, and cDNA synthesis procedures are described above. The Illumina 16S Metagenomic

Sequencing Library Preparation protocol (15044223 B) was used for library preparation ((Illumina, San Diego, CA, USA). Briefly, the concentration of genomic DNA and cDNA were quantified by Qubit 4 Fluorometer (Thermo Fisher Scientific, Wilmington, DE), and diluted to 5ng/μl using 10 mM Tris pH 8.5 buffer solution. For the stage PCR amplification, a total of 25-μL reaction mixture contained 5 μL of DNA sample, 12.5 μL of 2x KAPA HiFi HotStart ReadyMix solution, and 5 μL 1μM of forward and reverse PCR primers. A universal bacterial primer set 341F (5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG)/805R (5' GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC) targeting the V3-V4 region of bacterial 16S rRNA gene was used for the PCR amplification. The PCR procedure included an initial deactivation at 95 °C for 3 min, followed by 25 thermal cycles at 95 °C for 30 s, 55 °C for 30 s, and then at 72 °C for 30 s, and final extension at 72 °C for 5 min. The PCR products were purified using the AMPure XP beads system before the sequencing library preparation. For the library preparation, Nextera XT Index Kit (Illumina) was used to attach the dual indices and Illumina sequencing adapters onto the PCR amplicons according to the instructions.

Library sequencing was performed using an Illumina MiSeq platform (2 × 300 bp) at the Roy J. Carver Biotechnology Center at the University of Illinois at Urbana-Champaign, IL, USA. An average coverage of 201,015 reads for each DNA sample was obtained. Sequences were analyzed on the microbiome bioinformatics platform QIIME 2 v2019.1 (<https://qiime2.org/>)²⁵⁷.

Raw sequences were first imported into QIIME 2 and subject to assembly, quality control (with a minimum quality score of 25), and feature table construction using DADA2²⁵⁸. The obtained amplicon sequencing variances were further clustered into operational taxonomic units (OTUs) with a minimum of 97% in similarity using Vsearch²⁵⁹. Chimeric sequences were then identified by Uchime²⁶⁰. The most abundant sequence was automatically selected as the representative sequence for each specific OTU. The closest taxonomy of each OTU was first assigned using q2-feature-classifier²⁶¹ customized for the primer sets used in this study with Silva SSU database release 132²⁶², and then corrected by BLAST using the reference sequences. 16S rRNA amplicon Sequencing data have been deposited in the NCBI Sequence Read Archive and are available under BioProject accession number PRJNA611458.

Quantitative PCR (qPCR) Measurement on Relative Abundances and Relative Growth of Selected OTUs

Ten of OTUs were selected for qPCR measurement. Except for *Dehalococcoides* spp. (i.e., OTU187), all the other OTUs were active members and enriched during the defluorination of PFMeUPA in the first lactate-fed subculture (Sub1) according to the 16S rRNA and 16S rRNA gene amplicon sequencing results. Specific primers (Table E1) targeting the selected OTUs were designed and validated using Primer-BLAST (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>), where the database was customized to all identified sequences from the 16S rRNA gene amplicon sequencing. Total bacterial abundance was determined using the primer set targeting universal

bacterial 16S rRNA genes listed in [Table E1](#). The relative abundance and the relative growth of each target OTU were calculated by the following equations with the assumption of 100% amplification efficiency for all primer sets.

Relative Abundance = $2^{-\Delta Ct} \times 100\%$, where $\Delta Ct = Ct_{OTU} - Ct_{Universal\ Bacteria}$

Relative Growth (fold change of copy numbers) = $2^{-\Delta Ct}$, where $\Delta Ct = Ct_{Day\ n} - Ct_{Day\ 0}$ (Day n is when the first dose of PMeUPA was consumed).

Metagenomic Sequencing

After the first dose of PFMeUPA was completely consumed in subculture 5 enrichments, cultures were transferred (5% v/v) to three serum bottles containing 95 fresh medium as previously described. The bottles were maintained under with lactate as primary electron donor (3 bottles, with 5 mM lactate and Ar/CO₂ headspace) and 75 μ M PFMeUPA as electron acceptor. Aqueous samples were taken subsequently during the incubation period for the measurement of PFMeUPA and F⁻. Six mL samples from each bottle were taken at 61-d and centrifuged at 16,000 g 4 °C for 20 min. Cell pellets were collected and stored in a – 20 °C refrigerator for DNA extraction. Sequencing was performed at the Roy J. Carver Biotechnology Center at University of Illinois at Urbana-Champaign using the Illumina NovaSeq 6000 platform S4 flowcell (paired-reads, 2 × 150bp length). The shotgun genomic libraries were prepared with the Hyper Library construction kit from Kapa Biosystems (Roche) for metagenomic sequences.

Metagenomic Assembly and Binning

The draft genome assembly and binning were performed on the Department of Energy Systems Biology Knowledgebase (KBase) platform (<https://www.kbase.us/>). For each sample, the metagenomic data were trimmed and quality-filtered using Trimmomatic v 0.36 with default parameters. The clean reads of triplicate samples were then merged into one library. The taxonomy abundance of individual sample compared to the merged library were compared using KAIJU. The merged clean reads were used for downstream analysis. Two assembling software, MEGAHIT v1.2.9 and IDBA-UD v1.1.3, were applied to assemble the clean reads into contiguous fragments (contigs) with minimum contig length setting at 1000. Two sets of contigs generated from two different assemblers were examined by running Compare Assembled Contig Distributions v1.1.2. As the assembly from running MEGAHIT generated the best quality contigs (high N50, low L50 and fewest contigs), therefore, it was used for the downstream analysis. Two binning programs, MetaBAT v1.7 and MaxBin v2.2.4 were applied to the metagenomic assembly and generated two independent sets of bins. DAS-Tool v1.2 was then used to compare and merge the results generated from MetaBAT and MaxBin. The quality (i.e., completeness and contamination) of the bins was assessed using CheckM v1.0.18. Bins with > 70% completeness and < 10% contamination were selected for downstream analysis. The metagenomic abundance of each bin genome in the community was calculated as described previously with slightly modifications.^{263, 264} Briefly, quality-filtered metagenomic sequence reads from the merged library were mapped to individual bin genome using Bowtie2 v2.3.2. The taxonomic assignments for each bin were assessed using

GTDB-TK v1.3.0, and the genome annotation and the maximum likelihood phylogenetic tree were generated by RASTtk v1.073 and Insert Set of Genomes Into Species Tree v2.2.0, respectively. The detailed metagenomic analysis workflow was shown in [Fig 6.1](#). Sequencing data can be found in Kbase under the corresponding workflow (will be open to public once the manuscript have been published).

Scanning Electron Microscopy (SEM) Imaging of the Enrichment Culture

The SEM imaging procedure was adopted from a previous study with slight modifications.²⁶⁵ Briefly, fifty mL subculture 6 enrichment were used for SEM imaging, which was performed with a SUPRAU 40VP field emission scanning electron microscope at California NanoSystems Institute (CNSI). Samples were prepared by overnight fixation with 2.5% glutaraldehyde added directly to the microbial culture at room temperature. After removing the glutaraldehyde with deionized water, the samples were repeatedly spun down and resuspended in deionized water, then dehydrated in increasing concentrations of ethanol (25%, 50%, 75%, 90%, 100%, and 100%, 10 min each). Lastly, the dehydrated biomass samples were treated with hexamethyldisilazane (HMDS) for 1 hour. The biomass in HMDS were then drop-casted on a silica wafer for overnight drying at room temperature. The silica wafer with biomass on was sputtered with ~3 nm gold layer before imaging.

6.4 Results and Discussion

Physiological Characteristics of the Reductive Defluorinating Community

We first examined different organic substrates (i.e., lactate, methanol, and butyrate) in PFMeUPA microbial reductive defluorination. The results showed that the enrichment culture was able to reductively defluorinate PFMeUPA regardless of the type of organic substrate (Fig. E1A). In the organic substrate fed dechlorinating enrichment culture, acetogens or fermenters usually convert the organic carbon into H_2 and acetate, which was used as the electron donor and carbon source by microorganisms like dechlorinators in the community.²⁶⁶ Therefore, we further tested whether H_2 could be used as a direct electron donor for the reductive defluorination of PFMeUPA. The results showed that H_2 could support the PFMeUPA defluorination with acetate being provided as the carbon source (Fig 6.2). As fermenters and fermentation processes were significantly inhibited when no fermentable organics were provided, the reductive defluorination observed in the H_2 -fed culture was less likely a co-metabolism carried out by fermenters. However, it was interesting to see that the defluorination activity was decreased in H_2 -fed subcultures (Fig 6.2B).

In contrast, the reductive defluorination activity was sustained in the lactate-fed controls after up to five subsequent transfers (Fig. 6.3). Furthermore, we observed an enhanced defluorination activity for subsequent PFMeUPA doses, which could be due to the growth of the defluorinating microorganisms in the community. Similar enhancement of reductive dehalogenation activity coupled to cell growth has been observed in a trichloroethene (TCE)-dehalorespiring pure culture.²⁶⁷ The slower defluorination in the H_2 -fed subculture than lactate-

fed ones might be due to the limitation of H₂ transfer (Fig. E1B). As the interspecies H₂ transfer between two species of microorganisms was more efficient than microorganisms directly uptake the dissolved H₂ from the gas phase, it was expected to observe an enhanced and sustained defluorination activity in the lactate-fed subculture than the H₂-fed ones (Fig. E1B). We acknowledge that without isolating the responsible microorganism(s), the actual physiological and biochemical traits, including the data on optimal growth temperature, pH value, preferred salt concentrations and nutrients, remained question marks. However, the sustained and enhanced defluorination activity in mixed cultures already suggested that the responsible microbes could cooperate with other co-existing microbes under anaerobic conditions. In line with the enhanced defluorinating results, the SEM imaging of the enrichment culture also suggested a syntrophic growing condition of the microbial community (Fig. 6.4).

16S rRNA Gene Amplicon Sequencing Revealed the Community Structure of the Enrichment Culture

We examined the community succession along the enrichment process by 10% v/v transfers into the basal medium with lactate as the primary electron donor and PFMeUPA as the sole electron acceptor. We identified the microbial groups, in terms of operational taxonomic units (OTUs), potentially involved in the reductive defluorination based on the following criteria: (1) the growth is active during defluorination and can be sustained in subcultures, i.e., an increase of relative abundance in subcultures compared to the seed culture (Fig. 6.5A); (2) growth with H₂ as

the electron donor, although the defluorination activity was not as sustained as that in lactate-fed culture (Fig. 6.5B). Consistent with the qPCR analysis results and pure culture biotransformation data illustrated in Chapter 4, the dominant dechlorinating species in the seed culture, *Dehalococcoides*²²⁶, did not contribute to the defluorination, as *Dehalococcoides* were diluted out without any growth rather than enriched (Fig. 6.5A). Among the OTUs enriched in the first PFMeUPA-fed subculture (Fig. 6.5A), there was only one (OTU68) closely related to species (i.e., *Dehalobacter* spp.) known for reductive dechlorination^{229, 268-270}. We observed significantly higher growth of this *Dehalobacter*-like OTU on lactate during the defluorination of PFMeUPA and FTMeUPA than that during the dechlorination of TCE (Fig. C17). It suggests the involvement of *Dehalobacter* in the reductive defluorination. The emergence of the *Dehalobacter* population has been previously observed by switching the terminal electron acceptor from TCE to the structurally different 1,2,4-trichlorobenzene²²⁹. However, in the H₂-fed culture, the growth of *Dehalobacter*-like OTU (OTU68) was not as significant as that in the lactate-fed ones with the same amount of PFMeUPA (~ 75 µM) being consumed (Fig. 3B). In addition, the isolate closest to *Dehalobacter* spp. (i.e., *Dehalobacter restrictus* strain PER-K23) in the community²²⁹ did not show F⁻ release from PFMeUPA or FTMeUPA when H₂ was provided as the electron donor (Fig. C18, Chapter 4). Thus, although *Dehalobacter* might still contribute, other microorganisms should play the major role in reductive defluorination.

According to the 16S rRNA gene amplicon sequencing analysis, all the identified archaea presented in the enrichment belong to methanogens (Fig. E2). The defluorination activities were not affected (Fig. E3) when we inhibited the methanogens (by adding the methanogen-inhibitor, 2-bromoethanesulfonate, BES, 1 mM). It indicated that methanogens in the enrichment culture, which were known to carry out co-metabolic dechlorination,^{271, 272} were not responsible for the reductive defluorination. In Chapter 7, we thoroughly studied the PFMeUPA biotransformation feasibility using one strain of pure methanogens. Thus, we consider that the major defluorinating microorganisms should belong to bacteria, which exhibited significant growth (relative growth)> 10, higher than the subculturing ratio 1:10) on H₂. Based on this criterion, we identified five OTUs (i.e., OTU169, 6, 138, 83, and 30) as potential defluorinating microorganisms or microorganisms supportive of the defluorination (Fig. 6.5). The taxonomic groups closest to those OTUs are commonly present in dechlorinating communities and/or general anaerobic environments. Among them, *Lactivibrio* (OTU30) belongs to the phylum *Synergistetes*, one strain (*Synergistetes* strain MFA1) from which has been reported to carry out reductive defluorination of monofluoroacetate but not trifluoroacetate with amino acids as the growth substrate^{196, 197}. Moreover, species from the genus *Mesotoga* (OTU83) have been found to carry reductive dehalogenase genes, indicating its potential role in reductive dehalogenation²⁷³. In addition, *Sedimentibacter* (OTU169) and *Petrimonas* (OTU6) have been detected in several dechlorinating enrichments as essential microorganisms supportive of the dechlorinators, *Dehaobacter* and *Dehalococcoides*, when H₂

was provided as the electron donor^{268-270, 274}. It is worth noting that these microbial groups were minor ones in the community (relative abundances < 3%). If they were responsible for the reductive defluorination, higher activities and perhaps a wider fluorinated substrate range would be expected after they are highly enriched or isolated. Moreover, all these suspected microorganisms were not only present in dehalorespiring communities but also in various anaerobic environments. These widely spread, phylogenetically diverse, and yet-to-be cultured microorganisms suggest that their new ecological roles in reductive defluorination may ubiquitously exist in nature. To further investigate the comprehensive genomic information as well as to elucidate the functional species and responsible enzymes during the defluorination processes, metagenomic sequencing of the enrichment culture was conducted.

Metagenomic Analysis Revealed the Structure Dynamic of the Enriched Defluorinating Culture

We first examined the taxonomy structures and abundance among three replicate samples. The results showed that three sets of reads libraries exhibited highly similarities (Fig. E.4). Therefore, we further combined triplicate reads' libraries into one merged library, which was used for downstream analysis similarities (Fig. E.4). In total, 46 bin genomes were retrieved, and their relative abundance and closet taxonomy were shown in Table 6.1. Consistent with the 16S rRNA gene amplicon sequencing analysis, functionally, the key components of the enrichment could be divided into two major categories: fermenters/acetogens, and methanogens. Starting from lactate, fermenting bacteria (e.g., the most abundant species in the enrichment, Bin 3) were able to degrade

lactate to H₂ and acetate through the acetyl-CoA pathway, and to propionate via either acryloyl-CoA pathway or methylmalonyl-CoA pathway (Fig. E.5). Under the methanogenic condition, propionate was oxidized by syntrophic consortia of proton-reducing acetogenic bacteria and methanogens. The acetogens oxidize propionate to acetate and H₂, according to the reaction: $\text{propionate}^- + 3\text{H}_2\text{O} \rightarrow \text{acetate}^- + \text{HCO}_3^- + \text{H}^+ + 3\text{H}_2$. It is worth noting that the Gibbs free energy of the above reaction was highly positive (calc. +76.1 kJ), which made the above reaction thermodynamically unfavorable. However, due to the rapid consumption of H₂ by methanogens, acetogens were then able to grow. Moreover, acetoclastic methanogenesis was also able to utilize acetate to form acetyl-CoA, which also dragged the reaction forward.²⁷⁵ Notably, one of the most abundant methanogens identified by metagenomic sequencing (Bin 7) was found to be *Methanothrix soehngenii*. *M. soehngenii* is a typical acetoclastic methanogen that used acetate as the sole energy source.²⁷⁶ The SEM image of *M. soehngenii* revealed a rod shape morphology. Cells are combined end to end, forming a bamboo shape for the filaments surrounded by a sheath-like structure.²⁷⁷ The same bamboo shape filaments were also observed in the defluorinating enrichment culture SEM imaging (Fig. 6.4F). Given the relatively high abundance of *M. soehngenii* in the enrichment culture, the observed bamboo shape filaments in Fig. 6.4F were highly likely *M. soehngenii*. Again, the special distribution and interactions between *M. soehngenii* and the surrounding microbes indicated a syntrophic growing relationship.

According to our previous study (Chapter 5 and Chapter 6),²³⁶ PFMeUPA primarily underwent reductive defluorination ($-F+H$) and hydrogenation reaction ($+2H$). During the entire reaction process, none of the six carbons in PFMeUPA was assimilated by the cells. It indicated that the responsible defluorinating microbes must be able to utilize either organic carbon or fix CO_2 during the cell synthesis while defluorinate PFMeUPA. It is also worth noting that F^- was generated and accumulated as one of the key products during the reductive defluorination. F^- has been known for their adverse effects on microorganisms' metabolism and growth.²⁷⁸ In some microorganisms, there are proteins (i.e., F^- transporters *crcB*²⁷⁹ and F^-/H^+ antiporters *eriC*^{F280}) expelling the high concentration of F^- out from the cells. The enhanced defluorination activity, together with the increased F^- concentrations (Fig. 6.3), indicated that the responsible defluorinating microorganisms must have the ability to execute similar mechanisms to defend themselves against the intracellular F^- buildup. The C–F bond cleavage will not be retained in populations without rapid and efficient export of the F^- . Thus, the ability to encode a fluoride ion expelling gene(s) is a constraint for selecting the responsible defluorinating microorganisms.⁶⁸

With multiple doses of PFMeUPA being added into the culture, the biotransformation rate of PFMeUPA was significantly increased (Fig. 6.3). In contrast, the total defluorination degree remained the same (in total, one F was cleaved from the structure). It suggested that the two major reactions of PFMeUPA (i.e., reductive defluorination and hydrogenation) were simultaneously proceeded and enhanced, indicating that both reactions might be carried out by the same

responsible microorganism(s) and even by the same enzymes. Otherwise, with fluoride concentration in the medium kept increasing and the subculture events kept proceeding, the hydrogenation responsible microbes should be diluted out or inhibited. Therefore, an enhanced defluorination degree PFMeUPA should be observed. In our previous study (Chapter 5), we have explicitly demonstrated that under the anaerobic condition, the α , β -unsaturation in the FCAs are essential for microbes to initiate the biotransformation reaction. Enzymes that reduced the C=C double bond in α , β -unsaturated carboxylic acid have been previously reported, which predominantly belong to the “Old Yellow Enzyme (OYE)” family of NAD(P)H/flavin-dependent oxidoreductase.²⁸¹ Other than the old yellow enzymes, there are other enzyme complex that are also known for α , β -unsaturated carboxylic acid reduction, such as the caffeyl-CoA reductase-Etf complex presented in many acetogens.^{282, 283} The low potential electron carrier ferredoxin also makes it possible for fluorinated compounds reductive defluorination.²⁸³ Besides the reduction of C=C double bonds, OYEs are also found to cleave the ester bonds in nitrate esters.²⁸⁴ Given that the structures of PFMeUPA (i.e., α , β -unsaturated double bond connected with three electron-withdrawing groups), it is highly likely that NAD(P)H/flavin-dependent oxidoreductase of the OYE family was able to reductively cleave the C–F in PFMeUPA while reducing the C=C double bond at the same time.

Taken together, to identify the plausible defluorinating microorganisms in the enrichment, we further investigated the genomic information of individual bin genomes. The responsible

candidates were selected based on the following criteria: (1) encodes either *crcB* or *eriC^F* genes on its genome, (2) encodes NAD(P)H/flavin-dependent oxidoreductase (Old Yellow Enzyme) related genes, and (3) encodes dehalo-relevant genes (e.g., reductive dehalogenase genes) or other reductases that are known for contaminants reduction (i.e., arsenate reductase, etc).

According to the results (Table 6.1), hydrolytic dehalogenase-related genes were detected in most bin genomes (Table 6.1). As no hydrolytic dehalogenation products ($-F+OH$) were detected during the PFMeUPA biotransformation, it was less likely the hydrolytic dehalogenases were the responsible enzymes for the reductive defluorination reaction. Among 46 retrieved bin genomes, nearly all of them (except for Bin 19) encoded arsenite extruding genes (i.e., *ARC3* or *arsA*). It has been previously investigated that the efflux permeases for arsenite detoxification are widely occurring in extant microbes.²⁸⁵ The missing annotation of *ARC3* or *arsA* in Bin 19 was likely due to the incomplete genome assembly (85.56% completeness). Thirty-six bin genomes encoded arsenate reductase. The reduction of As (V) to As (III) requires the compound uptake via phosphate-binding proteins,²⁸⁶ two equivalents electron transferred from electron donor to arsenate via arsenate reductase, and the transformation product As (III) was expelled out of the cell. Given the similarities between PFMeUPA reduction and arsenate reduction (e.g., compound uptake, two-electron transferring, product exporting, etc.), it is likely that the proteins that participated in arsenate reduction were also involved in PFMeUPA biotransformation. Future pure culture

biotransformation studies are needed to fully demonstrate the exact roles of arsenical-related encoding genes in fluorinated compounds biodefluorination.

In line with our hypotheses, fluoride ion exporting genes (i.e., *crcB* or *eriC^F*) were found in 17 assembled bin genomes (Table 6.1), suggesting these microbes were able to maintain a low and steady-state intracellular fluoride concentration. Among the 17 suspects, two bin genomes (i.e., Bin 51 *Dichloromethanomonas* and Bin 57 *Bryobacteraceae*) also encoded NADPH/flavin-dependent OYE, making them the most promising candidates for the biotransformation of PFMeUPA. It is also worth noting Bin 51 also encodes a tetrachloroethene reductive dehalogenase gene, which is known for PCE reductive dechlorination. In our previous study (Chapter 5),²³⁶ we demonstrated the *tceA* in *D. mccartyi* FL2, *bcrA* in *D. mccartyi* BAV1, and the other 14 previously reported reductive dehalogenase genes (including *vcrA*) in KB1 culture were not responsible for PFMeUPA defluorination. However, at the functional level, there are other genes encoding different types of reductive dehalogenases, which are involved in specific steps of dehalogenation.²⁸⁷ For instance, *pvcA*, *pteA*, *pcbA1*, *pcbA4*, *pcbA5* encoding reductive dehalogenases were able to cleave C–Cl bond from tetrachloroethene (PCE, $\text{CCl}_2=\text{CCl}_2$).²⁸⁸⁻²⁹¹ Given the structural similarities between PCE and PFMeUPA, it is still possible that the tetrachloroethene reductive dehalogenase in Bin 51 was able to catalyze the reductive defluorination of PFMeUPA. We further calculated the differential gene expressions of Bin 51 and Bin 57 between PFMeUPA added, and no PFMeUPA added control. Unexpectedly, there was no

significant differential gene expression being observed. Possible reasons for this phenomenon are: (1) the biotransformation of PFMeUPA are cometabolism with other energy-conserving reactions, with sufficient primary substrate being supplied, the addition of extra PFMeUPA would not alter the metabolism processes and (2) at the time when we sampled the RNA samples (Day 41 of the subculture 6), there were not sufficient fluoride ion or TPs being generated. Thus, the inhibition effects of fluoride ions and TPs on specific enzymes (e.g., pyrophosphatase and enolase)⁶⁸ might not be significant.

6.5 Conclusion

Meta-omics analysis of the enrichment culture comprehensively revealed the community dynamics and the functional species during the reductive defluorination. The inhibition of methanogens did not affect the overall reactivity of the PFMeUPA, indicating the minor role of methanogens played in PFMeUPA biotransformation. However, as methanogenesis is one of the driving forces in the enrichment that supports the growth of other components (e.g., acetogens), the activities of methanogens might be necessary for the long-term maintenance of the enrichment culture.

Halide ions are one essential product during all types of dehalogenation processes. Distinct from the other halide ions (e.g., Cl^-), F^- is highly toxic to nearly all prokaryotic and eukaryotic cells. The increased F^- in medium (or any other environment) can inhibit the activity of essential enzymes and affect the metabolism of microorganisms. The enhanced defluorination activity

observed in subcultures with multiple doses of PFMeUPA strongly suggested that the defluorinating responsible microbes must be able to fight against the intracellular F^- accumulation. Furthermore, the simultaneously enhanced hydrogenation reaction activity and the fixed overall defluorination degrees indicated the hydrogenation reaction and reductive defluorination of PFMeUPA might be carried out by the same microorganisms and even by the same enzymes. It explained why the stoichiometry of PFMeUPA underwent different reaction pathways was not affected during the enriching processes. Given that the α , β -unsaturation is necessary for microbes in the enrichment to initiate the biotransformation reaction, the low potential reductase was proposed as the most plausible enzymes that were responsible during the PFMeUPA biotransformation. Altogether, future studies with pure culture investigation systems are needed to demonstrate whether the fluoride ion expelling mechanisms can help the cells alleviate the negative effect caused by C–F bond cleavage and whether such low potential reductases are responsible for the reductive defluorination.

6.6. Special Acknowledgements of Chapter 6

Here I acknowledged that by the time I submitted the dissertation, I was doing additional isolation experiments. In total, 4 pure isolates and one co-culture are picked for sequencing analysis and defluorination activity test. Please refer this part of results in my future publications regarding this research topic.

6.7 Figures and Table of Chapter 6

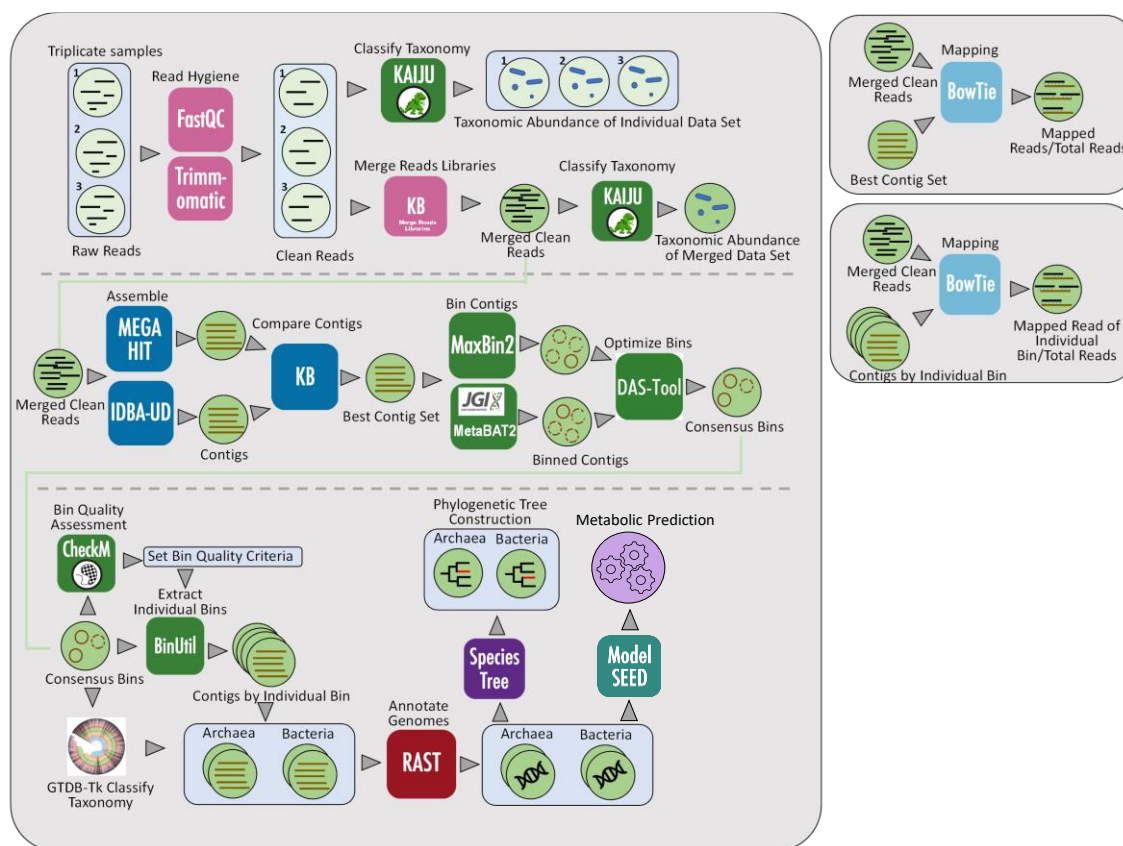


Figure 6.1. Metagenomic sequencing analysis workflow (Kbase platform).

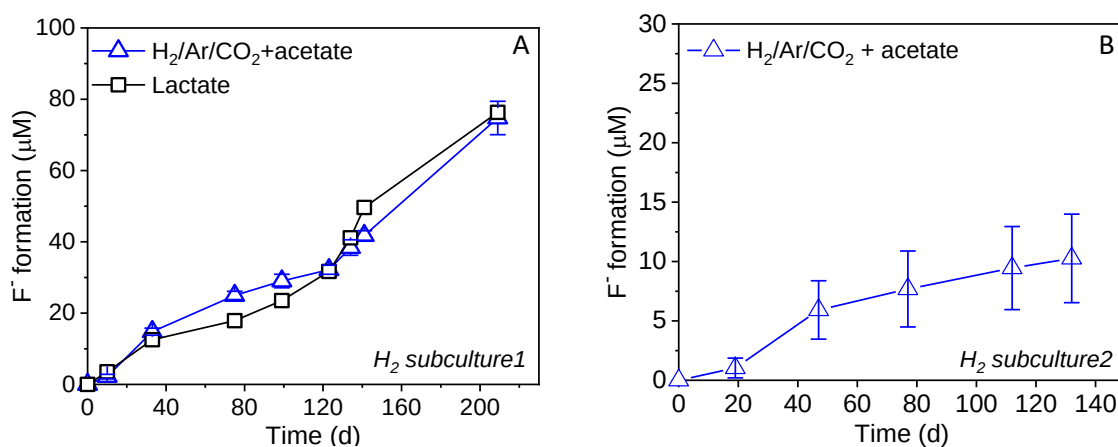


Figure 6.2 Fluoride ion formation of PFMeUPA with different electron donors (A), fluoride ion formation in H_2 -fed subculture 2 ($n=3$).

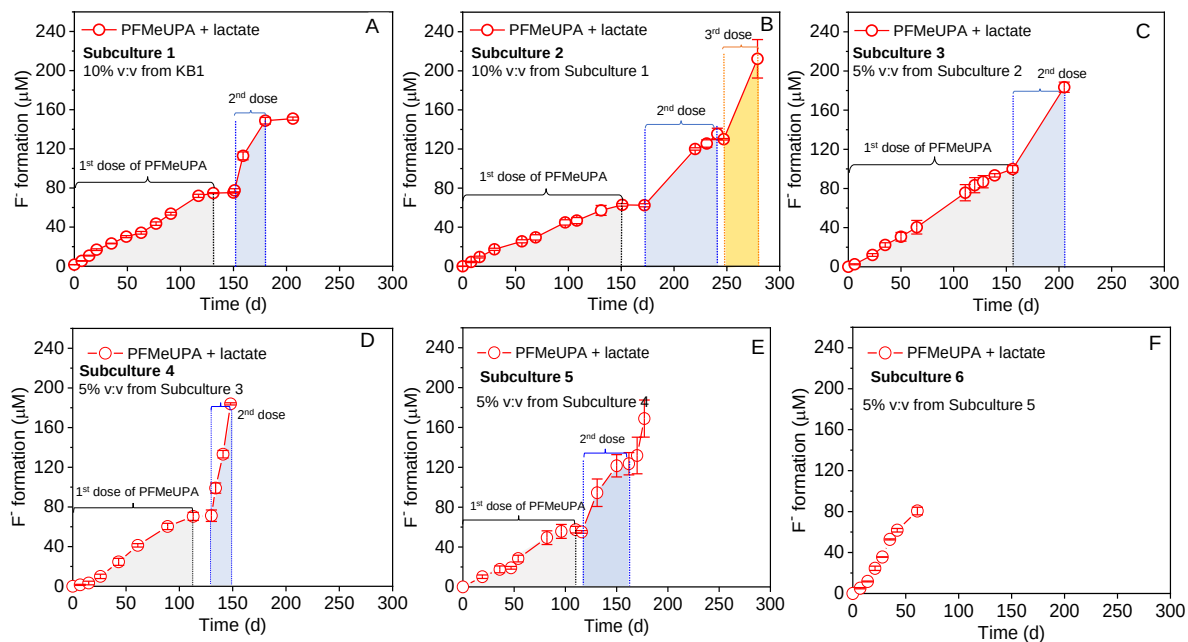


Figure 6.3 Fluoride ion formation in (A) Subculture 1 (inoculated with KB-1, 10% v/v), (B) Subculture 2 (inoculated with Subculture 1, 10% v/v), (C) Subculture 3 (inoculated with Subculture 2, 5% v/v), (D) Subculture 4 (inoculated with Subculture 3, 5% v/v), (E) Subculture 5 (inoculated with Subculture 4, 5% v/v), and (F) Subculture 6 (inoculated with Subculture 5, 5% v/v).

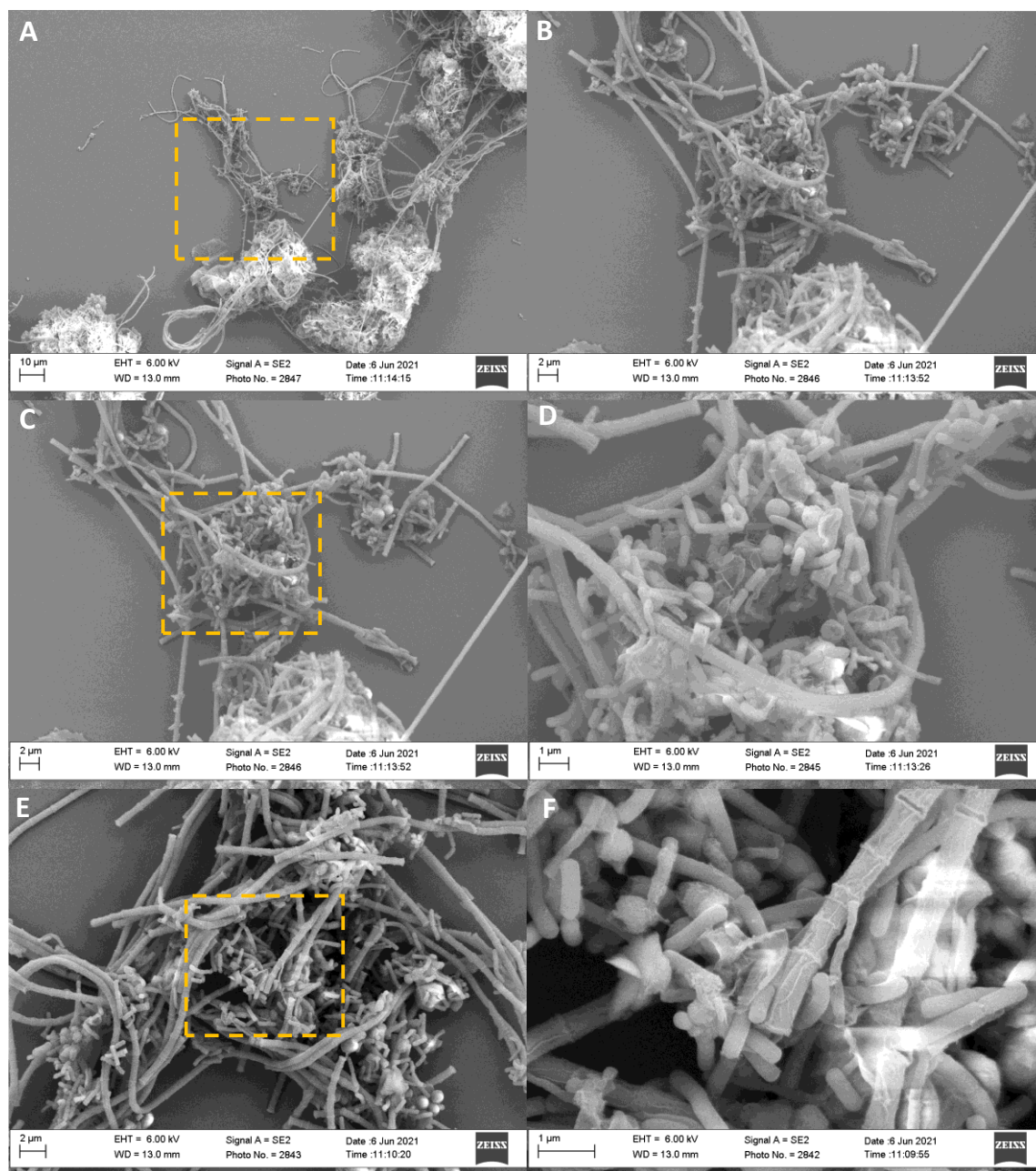
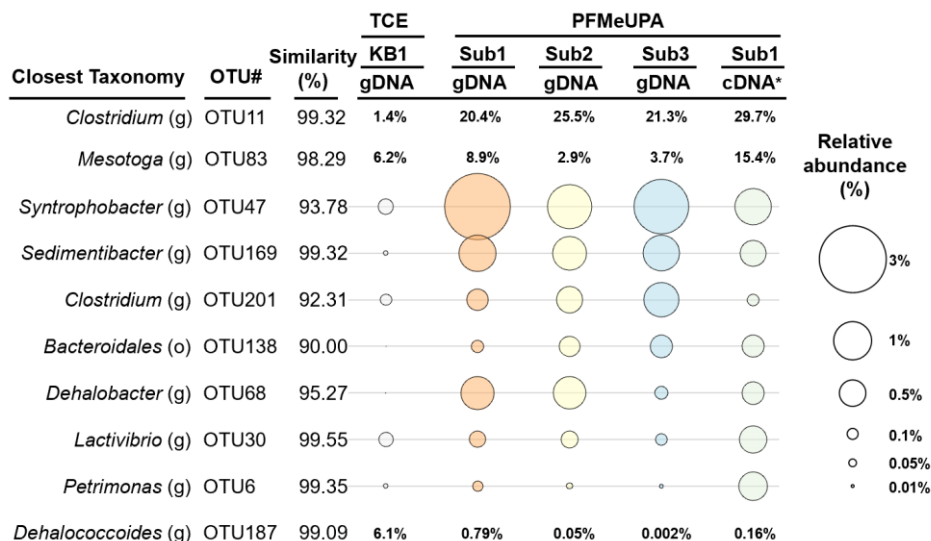


Figure 6.4 SEM of the microbial consortia on a silicon wafer (the right panel B, D, F and the zoomed-in area of right panel A, C, E). (Note: the culture samples went through the fixation, washing, and resuspension in DI water, and dehydration with ethanol solutions before imaging. Because the culture was not directly imaged, the special distributions among microbes in the enrichment culture might be interrupted with the treatment processes, yet the species micro-interaction in terms of the syntrophic growing traits should be retained and imaged)

A



B

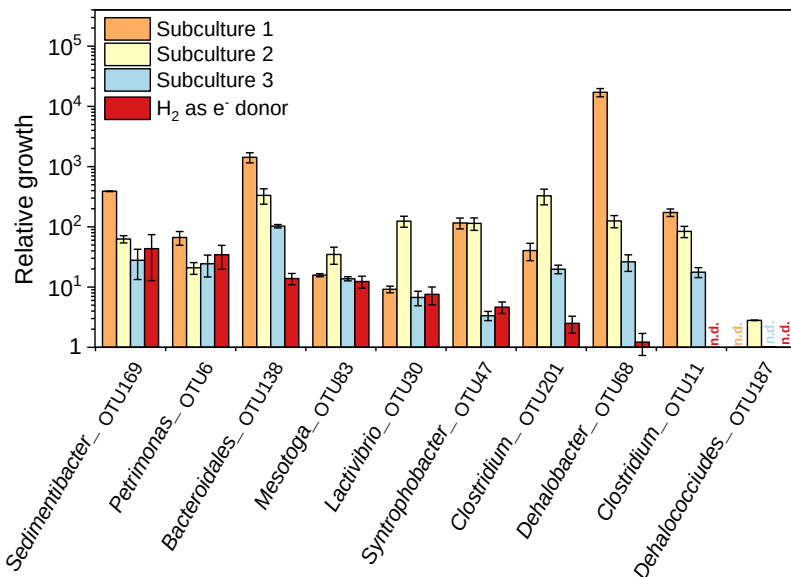


Figure 6.5 (A) Relative abundances of selected bacteria OTUs over the total bacteria OTUs in the seed culture (KB1 subculture on TCE) and the three lactate-fed PFMeUPA-defluorinating subcultures based on the qPCR results and their relative activities based on the 16S RNA sequencing results (the lowest taxonomic rank is assigned to each OTU: g, genus; o, order). (B) relative growth of the selected OTUs in the lactate- and H₂-fed PFMeUPA-defluorinating subcultures, measured by qPCR after the depletion of the first dose of PFMeUPA.

Table 6.1 Relative abundance, closest taxonomy, and detection of selected genes of 46 bin genomes.

Kingdom	Bin ID#	Relative Abundance	Closest Taxonomy	Expel fluoride		NAD(P)H/flavin oxidoreductases (Old Yellow Enzyme family)	Tetrachloroethene reductive dehalogenase	Ortho-chlorophenol reductive dehalogenase	Haloalkane dehalogenase-like protein	Haloacid dehalogenase-like hydrolase	2-haloacid dehalogenase	Arsenate reduction related			
				Fluoride Ion Transporter CrcB	EriC-type fluoride/proton exchange protein							Arsenate reductase	Arsenical-resistance protein ACR3	Arsenical resistance operon repressor	Arsenical pump-driving ATPase
Bacteria	Bin3	10.98%	<i>Syntrophobacter fumaroxidans</i> (s)	No	Yes	Yes	No	No	No	No	No	Yes	Yes	No	No
	Bin96	6.86%	<i>Clostridium H</i> (g)	No	No	No	No	No	No	No	No	Yes	Yes	Yes	Yes
	Bin21	5.50%	<i>Eremiobacterota</i> (p)	Yes (CrcB)	No	No	No	No	Yes	Yes	No	Yes	Yes	No	No
	Bin88	3.34%	<i>Mesotoga</i> (s)	No	No	No	No	No	No	Yes	No	No	Yes	No	No
	Bin5	2.38%	<i>Treponematales</i> (o)	No	No	No	No	No	No	No	No	No	Yes	No	Yes
	Bin17	1.24%	<i>Anaerolineaceae</i> (f)	No	No	No	No	No	No	No	No	Yes	No	No	No
	Bin30	1.08%	<i>Syntrophales</i> (o)	No	No	No	No	No	No	Yes	No	Yes	No	Yes	No
	Bin10	0.89%	<i>Armatimonadota</i> (p)	Yes (CrcB)	No	No	No	No	No	No	No	Yes	Yes	Yes	No
	Bin74	0.87%	<i>Bacteroidales</i> (o)	Yes (CrcB)	No	No	No	No	No	Yes	No	Yes	No	No	No
	Bin32	0.84%	<i>Desulfovibrionaceae</i> (f)	Yes (CrcB)	No	No	No	No	No	Yes	No	Yes	Yes	No	No
	Bin34	0.77%	<i>Synergistales</i> (o)	No	No	No	No	No	No	Yes	No	Yes	Yes	No	No
	Bin37	0.43%	<i>Sedimentibacter</i> (g)	Yes (EriC ^F)	No	No	No	Yes	No	No	No	Yes	Yes	Yes	No
	Bin18	0.41%	<i>Anaerolineae</i> (c)	No	No	No	No	No	No	Yes	No	Yes	No	No	No
	Bin43	0.33%	<i>Phycisphaerae</i> (c)	Yes (CrcB)	No	No	No	No	No	No	No	Yes	Yes	Yes	No
	Bin16	0.30%	<i>Cloacimonas</i> (g)	No	No	No	No	No	No	No	No	No	Yes	No	No
	Bin20	0.26%	<i>Anaerolineaceae</i> (f)	No	No	No	No	No	No	Yes	No	Yes	No	No	No
	Bin77	0.26%	<i>Mesotoga</i> (g)	No	No	No	No	No	No	Yes	Yes	No	Yes	No	No
	Bin44	0.24%	<i>Armatimonadota</i> (p)	No	No	No	No	No	No	No	No	Yes	Yes	No	No
	Bin45	0.22%	<i>Anaerolineaceae</i> (f)	Yes (CrcB)	No	No	No	No	No	No	No	Yes	No	No	No
	Bin41	0.20%	<i>Bacteroidetes vadinHA17</i> (f)	Yes (CrcB)	No	No	No	No	No	No	No	Yes	Yes	No	No
	Bin57	0.19%	<i>Bryobacteraceae</i> (f)	Yes (CrcB)	Yes	Yes	No	No	No	Yes	No	Yes	Yes	No	Yes

Notes: p: phylum; c: class; o: order; f: family; g: genus; s: species

Table 6.1 continued

Kingdom	Bin ID#	Relative Abundance	Closest Taxonomy	Expel fluoride		NAD(P)H/flavin oxidoreductases (Old Yellow Enzyme family)	Tetrachloroethene reductive dehalogenase	Ortho-chlorophenol reductive dehalogenase	Haloalkane dehalogenase-like protein	Haloacid dehalogenase-like hydrolase	2-haloacid dehalogenase	Arsenate reduction related			
				Fluoride Ion Transporter CrcB	Eric-type fluoride/proton exchange protein							Arsenate reductase	Arsenical-resistance protein ACR3	Arsenical resistance operon repressor	Arsenical pump-driving ATPase
Bacteria	Bin50	0.18%	<i>Anaerolineaceae</i> (f)	No	No	No	No	No	No	Yes	No	Yes	No	Yes	Yes
	Bin51	0.17%	<i>Dichloromethanomonas</i> (g)	Yes (CrcB)	Yes	Yes	No	No	No	No	No	Yes	Yes	Yes	No
	Bin35	0.17%	<i>Spirochaetota</i> (p)	No	No	No	No	No	No	No	No	Yes	Yes	No	No
	Bin85	0.16%	<i>Sphaerochaetaceae</i> (f)	No	No	No	No	No	No	Yes	No	Yes	Yes	Yes	No
	Bin63	0.09%	<i>Bacteroidales</i> (o)	No	No	No	No	No	No	No	No	Yes	Yes	Yes	No
	Bin72	0.07%	<i>Treponematales</i> (o)	No	No	No	No	No	No	Yes	No	Yes	Yes	No	No
	Bin23	0.07%	<i>Aminobacteriaceae</i> (f)	Yes (CrcB)	No	No	No	No	No	No	No	No	Yes	No	No
	Bin64	0.06%	<i>Syntrophomonadaceae</i> (f)	No	No	No	No	Yes	No	No	No	Yes	Yes	Yes	Yes
	Bin6	0.05%	<i>Bacteroidales</i> (o)	No	No	No	No	No	No	No	No	Yes	Yes	Yes	Yes
	Bin2	0.04%	<i>Peptostreptococcales</i> (o)	Yes (Eric ^F)	No	No	No	No	No	No	No	Yes	Yes	Yes	No
	Bin4	0.04%	<i>Caldatribacteriales</i> (o)	No	No	No	No	No	No	No	No	No	Yes	Yes	No
	Bin25	0.04%	<i>Proteiniphilum</i> (g)	Yes (CrcB)	No	No	No	No	No	No	Yes	Yes	Yes	Yes	Yes
	Bin69	0.04%	<i>Syntrophorhabdaceae</i> (f)	No	No	No	No	No	No	No	No	Yes	Yes	No	Yes
	Bin47	0.04%	<i>Anaerolineaceae</i> (f)	Yes (CrcB)	No	No	No	No	No	No	No	Yes	Yes	No	No
	Bin24	0.04%	<i>Anaerovoracaceae</i> (f)	No	No	No	No	No	No	No	No	Yes	Yes	No	No
	Bin62	0.03%	<i>Caldisericia</i> (c)	No	No	No	No	No	No	No	No	No	Yes	No	No
	Bin19	0.02%	<i>Levilinea</i> (g)	No	No	No	No	No	No	Yes	No	No	No	No	No
	Bin80	0.01%	<i>Anaerovoracaceae</i> (f)	Yes (CrcB)	No	No	No	No	No	No	No	Yes	Yes	Yes	No
	Bin52	0.01%	<i>Anaerolineae</i> (c)	No	No	No	No	No	No	Yes	No	Yes	Yes	No	No
	Bin12	0.01%	<i>Aminicenantales</i> (o)	No	No	No	No	No	No	No	No	No	Yes	No	No
Archaea	Bin1	13.58%	<i>Methanobacterium</i> (g)	No	No	No	No	No	No	No	No	Yes	No	No	No
	Bin7	9.85%	<i>Methanothrix soehngenii</i> (s)	No	No	No	No	No	No	No	No	No	No	Yes	No
	Bin39	0.55%	<i>Methanomassiliicoccales</i> (o)	No	No	No	No	No	No	Yes	No	Yes	Yes	No	Yes

Table 6.1 continued

Kingdom	Bin ID#	Relative Abundance	Closest Taxonomy	Expel fluoride		NAD(P)H/flavin oxidoreductases (Old Yellow Enzyme family)	Tetrachloroethene reductive dehalogenase	Ortho-chlorophenol reductive dehalogenase	Haloalkane dehalogenase-like protein	Haloacid dehalogenase-like hydrolase	2-haloacid dehalogenase	Arsenate reduction related			
				Fluoride Ion Transporter CrcB	EriC-type fluoride/proton exchange protein							Arsenate reductase	Arsenical-resistance protein ACR3	Arsenical resistance operon repressor	Arsenical pump-driving ATPase
Archaea	Bin53	0.07%	<i>Methanoregula</i> (g)	Yes (<i>CrcB</i>)		No	No	No	No	Yes	No	Yes	Yes	No	No
	Bin65	0.03%	<i>Methanomethylovorans hollandica</i> (s)	Yes (<i>CrcB</i>)		No	No	No	No	Yes	No	Yes	Yes	No	No

CHAPTER 7: MICROBIAL DEFLUORINATION OF UNSATURATED PER- AND POLYFLUORINATED CARBOXYLIC ACIDS BY ANAEROBIC BACTERIUM ISOLATES: COMBINING WHOLE-GENOME SEQUENCING, RNA-SEQ, AND PROTEOMICS ANALYSIS TO REVEAL THE REDUCTIVE DEFLUORINATION PATTERN

7.1 Abstract

This study investigated the defluorination capabilities of five strains of microorganisms (i.e., *Syntrophobacter fumaroxidans*, *Clostridium homopropionicum*, *Mesotoga primus*, *Acetobacterium bakii*, and *Methanosarcina barkeri*). Under the optimal growing conditions of individual strains, *S. fumaroxidans*, *C. homopropionicum*, *M. primus* could not biotransform any perfluorinated compound (i.e., PFMeUPA). In contrast, PFMeUPA was slightly biotransformed by *M. barkeri* (less than 10%), and the biotransformation was cometabolism with methanogenesis. Surprisingly, with glycolysis processes being activated, *A. bakii* showed sustained defluorination activity of PFMeUPA, and the biotransformation/defluorination rate was even higher than that of a previously reported defluorinating enrichment community. Transformation products analysis of PFMeUPA in *A. bakii* culture indicated the same biotransformation pathways as that had been observed in the enrichment culture, suggesting that the defluorinating mechanisms and the responsible enzymes might be identical in two cultures. We further conducted RNA sequencing analysis between the controls with and without PFMeUPA added. The results showed that the genes for fluoride ion expelling (*crcB*) were highly expressed, indicating a fluoride ion detoxification mechanism during the microbial reductive fluorination. Moreover, the intracellular CO₂ accumulation evidenced by the downregulation of carboxysomes related genes suggested that

the electron generated from the glycolysis processes were going into another electron acceptor (i.e., PFMeUPA). Such electron bifurcation phenomenon highly suggested that the caffeyl-CoA reductase was likely the responsible enzyme for PFMeUPA reduction. Additionally, the highly expressed genes for arsenite detoxification suggested that the exporting of fluorinated transformation products might share the same enzyme sets with the arsenite. Proteomics analyses between PFMeUPA added, and no PFMeUPA added controls revealed a significant change in the protein level between the two groups. It suggested that the reductive defluorination affected the total metabolism of the *A. bakii* at a protein level. This study, for the first time, demonstrated that the reductive defluorination capabilities of individual microorganisms. Given the widely occurrence of acetogens in both natural and engineered environments, the findings shed light on the global fluoride cycle carried out by microorganisms. Ultimately, the identification of the reductive defluorination and fluoride ion defending enzymes provide significant insights into the environmental defluorinating microorganisms screening and advanced the study of the fate of fluorinated compounds in nature.

7.2 Introduction

Microbial reductive defluorination of α , β -unsaturated perfluorinated carboxylic acids have been previously investigated using anaerobic enrichment originated from KB1 culture. The microbial community of the enriched culture has been studied according to the metagenomic sequencing results. Moreover, fluoride ion transportation genes and the low potential reductase

that can attack the α , β -unsaturated bonds have been proposed as the most promising biomarkers for selecting defluorinating microorganisms. However, based on the metagenomic sequencing analysis, the essential roles such enzymes cannot be fully demonstrated. Thus, to fulfill this knowledge gap, pure culture experiments are conducted in this study. For the pure culture with reductive defluorination capability, RNA sequencing was further conducted to study the responsible genes involved in the fluorinated compound biotransformation.

7.3 Methods and Materials

Chemicals

Fluorinated carboxylic acids (FCAs) that showed defluorination in the enrichment culture were selected (Chapter 5). Briefly, 4 FCA standards used in this study as parent compounds in the biotransformation experiments or analytical reference compounds were purchased from SynQuest Laboratories (Alachua, FL) and used without further purification ([Table F.1](#)). For all standard compounds, 10 mM stock solutions were prepared anaerobically in autoclaved Milli-Q water in 160-mL sealed serum bottles and stored at room temperature until use. The limit of quantification (LOQ) for each standard compound was determined as the lowest concentration of calibration standards with a detection variation within $\pm 20\%$.

Cultivation of Microorganisms

Syntrophobacter fumaroxidans (DSM 5847) and *Clostridium homopropionicum* (DSM 10017) were purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen

(DSMZ). *Mesotoga primus* (ATCC, BAA-2239), *Acetobacterium bakii* (DSM 8239), and *Methanosarcina barkeri* (DSM 800) were purchased from American Type Culture Collection (ATCC). To test the biotransformation capabilities of individual strains, all cultures were cultivated under their optimal growing conditions as instructed by DSMZ or ATCC.

Biotransformation of Fluorinated Compounds by Individual Strains.

After the growth of individual strain has been observed, each strain of microorganism has been transferred (5%, v/v) into six serum bottles with 95 mL fresh medium amended with all essential nutrients recommended by either ATCC or DSMZ. The six bottles were set up into two conditions: (1) growing positive control with no PFMeUPA addition and (2) biotransformation control with approximately 80 μ M of PFMeUPA being provided. Heat-inactivated biomass controls were set up by inoculating 5 mL autoclaved (at 121 °C for 20 min for two cycles) culture in the same medium. The parent compound was then amended the same way as described above to determine the abiotic degradation and potential adsorption of each compound. Samples were taken subsequently during the incubation period for the measurement of the parent compound, transformation products (TPs), and F⁻. Briefly, at each sampling time, 3 mL aqueous suspension was centrifuged at 16,000 $\times$ g for 30 min (4 °C). Two mL supernatant was used for F⁻ measurement, and the remaining (~ 1 mL) was stored at 4 °C in dark for LC-HRMS/MS measurement. The cells were stored in -20 °C for DNA extraction. All experimental samples and controls were performed in triplicates.

Methanogens Inhibition Biotransformation Experiment

PFMeUPA biotransformation by *M. bakeri* with methanogenesis activity being inhibited were tested. Briefly, *M. bakeri* were transferred (5% v/v) into six bottles containing 95 mL fresh medium. Three of them were amended with 1 mM of the methanogen-inhibitor, 2-bromoethanesulfonate (BES) with re-addition subsequently throughout the incubation period, to completely inhibit methanogenesis. The other three bottles that contained no BES were set as the positive control. The defluorination results as aqueous F^- concentrations were compared between two controls. Methane (CH_4) concentrations in the headspace were measured by Gas Chromatography Coupled with a Flame Ionization Detector (GC-FID) (Agilent, CA), which is used as the indicator for methanogenesis activity.

Toxicity examination of PFMeUPA in A. bakii

To test the toxicity of PFMeUPA in *A. bakii* culture, a titration experiment with PFMeUPA concentration ranging from 0 μ M up to ~ 1000 μ M were conducted. Briefly, different concentrations of PFMeUPA were added into *A. bakii* culture (5% inoculation, v/v). Three mL aqueous suspension was taken at each sampling time and centrifuged at $16,000 \times g$ for 30 min (4 °C). Two mL supernatant was used for F^- measurement, and the remaining was stored at 4 °C in dark for LC-HRMS/MS measurement. The cells were stored in -20 °C for DNA extraction. All experimental samples and controls were performed in duplicates.

Ultra-high-performance liquid chromatography coupled to high-resolution tandem mass spectrometry (UHPLC-HRMS/MS) analysis.

The FCA parent compounds were analyzed by UHPLC-HRMS/MS (Q Exactive, Thermo Fisher Scientific) as described previously^{49, 55} with slightly modifications. For UHPLC analysis, 2 μ L sample was loaded onto a Hypersil GOLD column (particle size 1.9 μ m, 100 $\times$ 2.1 mm, Thermo Fisher Scientific) connected with a Hypersil GOLD guard column (particle size 3.0 μ m, 10 $\times$ 2.1 mm, Thermo Fisher Scientific) and eluted with nano-pure water (A) and methanol (B) (both amended with 10 mM ammonium acetate) at a flow rate of 280 μ L/min, with a gradient as follows: 95% A: 0 – 1 min, 95% – 5% A: 1 – 6 min, 5% A: 6 – 8 min, and 95% A: 8 – 10 min. For HRMS, mass spectra were acquired in full scan mode at a resolution of 70,000 at m/z 200 and a scan range of m/z 50 – 750 in the negative mode of electrospray ionization (ESI). Xcalibur 4.0 (Thermo Fisher Scientific) was used for data acquisition and analysis. Transformation products (TPs) screening was conducted using Compound Discoverer 3.1 (Thermo Fisher Scientific) as previously described (Chapter 5). ChemDraw Professional 20.0 and MarvinSketch (version 19.20.0, ChemAxon, <http://www.chemaxon.com>) were used for drawing, displaying, and characterizing chemical structures.

Fluoride Ion Measurement by Ion-Selective Electrode

As described in previous studies,^{55, 236} the concentration of fluoride ion (F^-) in the culture supernatant was measured by an ion-selective electrode (ISE, HACH, Loveland, CO) connected

with an HQ30D Portable Multi Meter (HACH) and further validated using Ion Chromatography. For the ISE measurement, a 100 µg fluoride ionic strength adjustment powder (HACH) was added into 2 mL culture supernatant, the F⁻ concentration was then measured by the ISE-Multi Meter system.

Whole-Genome Sequencing Analysis

Biomass from 1.5 mL culture was sampled from each biological replicate. Microbial genomic DNA was extracted by DNeasy PowerSoil Kit (QIAGEN, Germantown, MD) according to the manufacturer's instructions. The concentration of genomic DNA was measured by the Qubit 4 Fluorometer (Thermo Fisher Scientific, Wilmington, DE). The DNA sequencing was performed by Microbial Genome Sequencing Center (MiGS). Briefly, genomic DNA samples were treated with Illumina DNA library preparation procedure and sequenced on the NextSeq 2000 platform (paired-end reads, 2 × 151 bp).

Differential Genes expression evidenced by RNA-seq

RNA was extracted using acid-phenol: chloroform: isoamyl alcohol (25: 24: 1) and precipitated in ethanol at – 20 °C as previously described.²¹⁸ RNA was cleaned up using the RNeasy PowerClean Pro CleanUp Kit (QIAGEN) according to the manufacturer's instructions. Contaminating DNA in the RNA samples was removed by Turbo DNase Kit (Thermo Fisher Scientific) following the manufacturer's instructions. The quality of RNA was examined by agarose gel electrophoresis. RNA samples were sent to Microbial Genome Sequencing Center

(MiGS) for sequencing analysis. Briefly, RNA samples were treated with RiboZero Plus for rRNA depletion followed by an Illumina Stranded RNA library preparation procedure, and sequenced on a NextSeq 2000 using 2x51bp reads.

Proteomics Analysis

The proteomics analyses were performed by the University of California, Los Angeles (UCLA) Proteome Research Center (PRC). Briefly, 6 mL of whole-cell samples of individual biological replicates were sent to UCLA-PRC on dry ice. Protein extraction and digestion, peptide analysis and protein identification were conducted by UCLA-PRC. Briefly, the proteomics analysis were conducted in the following sequences: sample preparation, digestion and desalting, LC-MS acquisition, and analysis. For sample preparation, bacteria pellets were lysed in lysis solution (8M Urea, 0.1M Tris-HCl pH 8.0, 1%SDS) containing AEBSF, pepstatin, leupeptin and benzonase. Samples were incubated with rotation for 30 min at temperature and then sonicated for 10 seconds in an ice-water bath using an ultrasonic cell homogenizer. Lysates were clarified by centrifugation at 16,100g for 15 min at 4°C. Protein amount in each Lysate were normalized by absorbance at 280 nm and aliquoted 50 µg for the following digestion. For digestion and desalting, 50 µg protein aliquots were reduced and alkylated via sequential 20 min incubations with 5 mM TCEP and 10 mM iodoacetamide at room temperature in the dark while being mixed at 1200 rpm in an Eppendorf thermomixer. 10 µl of carboxylate-modified magnetic beads (CMMB and also widely known as SP31) was added to each sample. Ethanol was added to a concentration of 50%

to induce protein binding to CMMB. CMMB were washed 3 times with 80% ethanol and then resuspended with 50µl 50mM TEAB. The protein was digested overnight with 0.1 µg LysC (Promega) and 0.8 µg trypsin (Pierce) at 37 °C. Following digestion, 1ml of 100% acetonitrile was added to each sample to increase the final acetonitrile concentration to over 95% to induce peptide binding to CMMB. CMMB were then washed 3 times with 100% acetonitrile and the peptide was eluted with 50 µl of 2% DMSO. Eluted peptide samples were dried by vacuum centrifugation and reconstituted in 5% formic acid before analysis by LC-MS/MS. For the LC-MS acquisition and analysis, peptide samples were separated on a 75µM ID, 25cm C₁₈ column packed with 1.9 µM C₁₈ particles (Dr. Maisch GmbH HPLC) using a 140-minute gradient of increasing acetonitrile concentration and injected into a Thermo Orbitrap-Fusion Lumos Tribrid mass spectrometer. MS/MS spectra were acquired using Data Dependent Acquisition (DDA) mode. MS/MS database searching was performed using MaxQuant (1.6.10.43) against the *Xanthobacter autotrophicus* reference proteome from Uniprot (UP000036873, *Acetobacterium bakii* DSM 8239, 3495 entries). Statistical analysis of MaxQuant label-free quantitation data was performed with the artMS Bioconductor package, which performs the relative quantification of protein abundance using the MSstats Bioconductor package (default parameters). Intensities were normalized across samples by median-centering the log₂-transformed MS¹ intensity distributions. The abundance of proteins missing from one condition but found in more than 2 biological replicates of the other condition for any given comparison were estimated by imputing intensity values from the lowest

observed MS1-intensity across samples and p-values were randomly assigned to those between 0.05 and 0.01 for illustration purposes.

7.4 Results and Discussions

Reductive Defluorination of PFMeUPA by Four Pure Cultures.

Besides the reductive defluorination, fermentation (carried out by fermenters), acetogenesis (carried out by acetogens), and methanogenesis (carried out by methanogens) are the other three key metabolic processes that occurred in the enrichment culture. According to our previous community analysis (Chapter 6), Bin 3 (i.e., *Syntrophobacter fumaroxidans*), Bin 96 (i.e., *Clostridium H*) and Bin 88 (*Mesotoga*) were three of the most abundant species detected in the enrichment culture with their closest relatives available. *S. fumaroxidans*, *C. homopropionicum*, *M. primus* were found to utilize multiple types of organic substrates, which is consistent with versatile substrate utilization characters observed for the enrichment culture. Thus, we first tested the PFMeUPA biotransformation capability of *S. fumaroxidans*, *C. homopropionicum*, *M. primus* under their optimal growing conditions, respectively. The results showed that none PFMeUPA can be biotransformed by the tested strains, and no fluoride ion formation was observed either, indicating that in the enrichment culture, these most abundant fermenting species might not be responsible for the PFMeUPA reductive defluorination. Notably, the whole genome re-sequencing of the three experimental used pure cultures revealed no fluoride ion transporting related genes on their genomes. In line with our proposed defluorinating microbes screening philosophy: a

sustained microbial defluorination must couple with an F^- detoxification process.

We then examined the reductive defluorination capabilities of acetogens and methanogens. As most of the components in the enrichment culture were yet uncultured ones with no closest relatives (at species level) being identified, we chose the acetogen *A. bakii* and the methanogen *M. barkeri* as two model strains for the defluorination examinations. Both *A. bakii*²⁸² and *M. barkeri*²⁹² are versatile in utilizing multiple organic substrates and H_2/CO_2 for energy conservation. Most importantly, the fluoride ion transporter (*crcB*) are found in both strains' genome (we did whole-genome sequencing for *A. bakii* and used the annotated *M. barkeri* genome from NCBI). Theoretically, if the reductive defluorination did occur, both strains should be able to export the F^- out of the cell and maintain a low and steady-state intracellular F^- concentration.

Firstly, we found that the selected methanogen *M. barkeri* could biotransform a small portion of added PFMeUPA with either methylotrophic and/or hydrogenotrophic methanogenesis being activated (Fig. 7.1). Consistent with the fluoride ion formation results, after the first week of incubation, the biotransformation of PFMeUPA slowed down until nearly ceased. The reductive defluorination did not resume when we switched the substrate from methanol and H_2/CO_2 to only H_2/CO_2 , indicating it is not the competition of primary organic substrate (i.e., methanol) inhibited the defluorination activity. The product analysis revealed the same reaction pathways of PFMeUPA in *M. barkeri* as in the enrichment culture, suggesting the responsible enzymes in *M. barkeri* might be the same as that in the enrichment culture, although methanogens were not the responsible

microorganisms. In Chapter 6, we did a methanogens inhibition experiment and found the defluorination was not affected when methanogens were inhibited. Thus, we then conducted a methanogenesis inhibition on the *M. barkeri* pure culture. The results showed that with methanogenesis activity being inhibited, the biotransformation of PFMeUPA was shut down as a response, suggesting a cometabolism of PFMeUPA biotransformation (Fig. 7.2).

Interestingly, we observed some formation of two TPs in the BES inhibited groups, suggesting trivial (bio)transformation (not distinguished by HRMS/MS) of PFMeUPA (Fig. 7.2D). During the methanogenesis, coenzyme M (CoM, 2-mercapto-ethane sulfonate) serves as a cofactor that is involved in the final step of methanogenesis by transferring a methyl group to methyl-CoM reductase. BES is a non-toxic structural analogue of coenzyme M (CoM). By irreversible combining with the methyl-CoM reductase, BES can efficiently inhibit the methanogenesis activity. The formation of TPs after BES inhibition suggested that the leftover ATPs in *M. barkeri* may still sustain some PFMeUPA biotransformation. With ATPs being consumed, the reaction ceased eventually. It also indicated that the reductive defluorination and the hydrogenation of PFMeUPA are not energy conservation processes but energy consumption reactions.

Surprisingly, for the first time we observed a sustained and complete biotransformation and reductive defluorination of perfluorinated compound (i.e., PFMeUPA) by one strain of pure culture *A. bakii*. *A. bakii* was able to completely defluorinate PFMeUPA with the acetogenesis being activated (Fig. 7.3). The product analysis revealed the TPs (i.e., TP256, TP276, and TP259) detected

in the enrichment culture were also detected in the *A. bakii*. The defluorination degree of PFMeUPA by *A. bakii* was calculated as 10.13%, which was very close to that have been observed in the enrichment culture (i.e., 11%), indicating the primary defluorination mechanisms (i.e., the first step reductive defluorination) between *A. bakii* and the enrichment culture should be identical (Fig. 7.3).

Differential Gene Expressions Reveal the Reductive Defluorination Processes

According to the PFMeUPA titration experiment, we found that even though the defluorination could occur with up to ~10 doses of PFMeUPA (1 dose of PFMeUPA equal to ~ 89 μ M), with the concertation of PFMeUPA being increased, the defluorination activity was inhibited (Fig. F.1). After 20-day incubation, PFMeUPA was completely consumed in 1 dose addition controls (Fig. 7.3 and Fig. F.1). Furthermore, we found that the defluorination activity was significantly higher in the first five controls (up to ~ 450 μ M PFMeUPA addition) than in the remaining high concentration controls (with 6 to 10 doses of PFMeUPA addition). To further elucidate which genes and enzymes might be involved in the reductive defluorination, we chose a ~ 400 μ M PFMeUPA testing concertation for the RNA sequencing, which will provide continuous reductive defluorination activity for a relatively long-term incubation period.

As *A. bakii* were able to utilize both organic substrates (e.g., fructose) and H_2/CO_2 for energy conservation, we then tested whether the different acetogenesis pathways (utilizing fructose only, H_2/CO_2 only, and fructose with H_2/CO_2) affected the defluorination activity. The results

showed that the defluorination of PFMeUPA can be observed in all three investigated conditions, whereas exhibited a slower activity in H₂/CO₂ only condition (Fig. F.2). One possible reason for this phenomenon was that under the H₂/CO₂ only condition, the growth of *A. bakii* was much slower than that with fructose provided (Fig. F.3). Less biomass production resulted in the slower defluorination activity.

Microbial Reductive Defluorination Pattern in A. bakii Evidenced By Differential Gene Expression Analysis and Proteomics Analysis

To further elucidate the reductive defluorination responsible genes and enzymes, we did RNA sequencing for both “fructose + PFMeUPA” condition and “fructose + H₂/CO₂ + PFMeUPA” condition. For each condition, there are two controls being set up (i.e., with PFMeUPA added and without PFMeUPA added). RNA samples were taken in the middle of reductive defluorination processes compared between two controls. Genes with significant up/down regulation (i.e., > 2-fold change, *p-value* < 0.05) were selected as suspected genes which regulations were likely related to the PFMeUPA biotransformation (Fig. 7.4). The detailed up/down gene regulation are included in Table F1—F2. As the defluorination activity is similar in both conditions, to simply the investigation system, we focus on the fructose-fed only condition for downstream analysis, where fructose was the only electron donor provided for the cell synthesis as well as for the reductive defluorination and hydrogenation of PFMeUPA.

We conducted both transcriptomic analysis and proteomics analysis. The results indicated

that with the addition of PFMeUPA, both RNA and protein compositions exhibit differences. However, based on the proteomics analysis, the responsible enzymes still could not be demonstrated. It is very important to notice that to map the up/down regulated genes to the corresponding proteins, RNA samples and protein samples were taken simultaneously. After the analyses, we found that not too much mapped data could be retrieved. Even though the observations were not expected for the purpose of this study, it was for sure not uncommon for comparative analyses of the transcriptome and the proteome of pure culture study. One possible reason for it is that the microorganisms' responded to the environmental changes (e.g., the addition of PFMeUPA) by rapid and radical reprogramming the transcriptome. Whereas for the proteome, it reflects the cumulative result of how microbes cooperate with the environment during the incubation period. To identify the responsible genes and enzymes involved in the microbial defluorination, the RNA sequencing data were adopted as it serves as a real-time “snapshot” of the changing environment during the PFMeUPA being continuously biotransformed and the environmental fluoride ion concentration increased.

According to the differential gene expression results ([Table F1—F4](#), [Fig. 7.5](#)), we first observed that a higher expression of gene *crcB* (fluoride ion transporter). *CrcB* is known as a fluoride riboswitch with a conserved RNA structure. It has been demonstrated that these RNAs (i.e., *crcB*) were able to sense the elevated intracellular fluoride ion concentration and help to mitigate the fluoride ion out of the cells. Our previous study (Chapter 6) proposed using “fluoride

ion efflux transporter” as a selection criterium for identifying the reductive defluorinating microorganisms. In this presented study, we, for the first time, demonstrated the importance of fluoride ion transporter in reductive defluorinating microorganisms. More importantly, the higher expression of *crcB* in *A. bakii* suggested the reductive defluorination occurred inside the cell, indicating a fluorinated compound uptake mechanism. The ATP synthase, pyrophosphatase, and the Na^+/H^+ antiporter-related genes were found to be downregulated, which were highly likely due to the inhibitory effects of fluoride ions (Table F.2 & F.3, Fig. 7.5). The inhibition of fluoride ions to ATPase and some essential cellular enzymes (e.g., pyrophosphatase and enolase) have been previously documented.⁶⁸ Most interestingly, we found that several genes on the *pdu* operon (e.g., *pduABK*) were down-regulated in the PFMeUPA added condition. The related protein of *pduA*, *pduB*, and *pduK* genes belong to carboxysomes, which are known as microcompartments that contain enzymes involved in carbon fixation. When the available CO_2 is limited, carboxysomes can concentrate CO_2 inside the shell and elevate the CO_2 concentrations. The downregulation of carboxysome-related genes indicated a high/accumulated intracellular CO_2 concentration. It has been previously reported that acetogens could reduce the carbon-carbon double bond in caffeate when fructose was provided as the electron donor. In the presence of both caffeate and CO_2 , the CO_2 utilizing pathways were inhibited as no acetate formation through the corresponding pathways can be observed. One possible reason for this is that compared to CO_2 , caffeate might be considered as a better electron acceptor by the microorganisms. During the caffeate reduction, the

NADH/ferredoxin-dependent caffeyl-CoA reductase was transferring two electrons to caffeate-CoA. Such carbon-carbon double bond reduction is similar to the Old Yellow Enzyme (OYE).

Similar to caffeate, PFMeUPA also has an α , β -unsaturated carbon bond and the same electron-withdrawing group (i.e., carboxylic acid group). It is highly likely that the biotransformation of PFMeUPA shared the same mechanisms, even the same enzyme sets as caffeate reduction. These genes were also found to be downregulated with fructose + H₂ condition when PFMeUPA was added (Table F.4 & F.5). When external H₂ was provided to the culture, the Wood–Ljungdahl pathways were more activated as the H₂, in this case, was not limited to the glycolysis activity. Thus, a faster CO₂ consumption was expected. Again, the downregulation of the carboxysomes related genes under H₂ provided condition suggested the intracellular accumulation of CO₂. It indicated that the electron flow into the PFMeUPA and the caffeyl-CoA reductase was the most promising responsible enzyme. Caffeate reduction processes involved an activation reaction of caffeate to caffeyl-CoA. With NADH serving as reductants (i.e., electron donor), the responsible enzyme caffeyl-CoA reductase must couple with the electron transfer flavoprotein to form a stable complex (a.k.a., caffeyl-CoA reductase-Etf complex) and then conduct the caffeyl-CoA reduction reactions. Given the complexity of enzyme complex purification and activity test, current findings are already significant for demonstrating the reductive defluorination capability of perfluorinated compounds by a single bacterium and proposing the possible biotransformation mechanisms, including the enzyme identification.

7.5 Conclusions

Pure culture studies including three strains of fermenters, one strain of acetogen, and one strain of methanogen were conducted for investigating the defluorination activity of one selected perfluorinated carboxylic acid (PFMeUPA). The results showed that none of the fermenters were able to biotransform PFMeUPA. In contrast, methanogens showed slightly biotransformation of the compound but significantly less than what had been observed in an anaerobic methanogenic defluorinating enrichment culture. And the biotransformation of PFMeUPA was found as co-metabolism with the methanogenesis activity. Surprisingly, we found that *A. bakii* was able to biotransform PFMeUPA and exhibited the same transformation pathways and defluorination degrees as observed in the enrichment community. Furthermore, by differential gene expression data analysis, the possible responsible genes and enzymes for reductive defluorination and hydrogenation were elucidated. The findings not only broadened the boundaries of what we have known for microbial defluorination but also provided significant insights into the development of future bioremediation approaches for PFAS.

7.6 Special Acknowledgements of Chapter 7

By the I submitted the dissertation, I am doing (1) protein extraction experiments to test enzyme activity to PFMeUPA biotransformation, (2) additional strains of pure acetogens biotransformation experiments to test if other acetogens can carry out such reductive defluorination (i.e., *Acetobacterium woodii* (DSM 1030), *Acetobacterium fimetarium* (DSM

8238), and *Acetobacterium malicum* (DSM 4132)), and (3) PFAS structure specificity test to study whether acetogens can biotransform more diverse of PFAS structures. Furthermore, I am also adding additional RNA sequencing experiments (400 μM PFMeUPA and 800 μM PFMeUPA) and tested (1) the short-term influence of the addition of PFMeUPA to the *A. bakii* (RNA samples were taken 90 min after the addition of PFMeUPA) and (2) the long-term high PFMeUPA (800 μM PFMeUPA) to the *A. bakii*. Because this part of results is confidential by nature and I am still refining the manuscript, I will not share these data (as well as the raw sequencing data) here in this chapter. I will be included the data in my future peer-reviewed papers. Please refer to the future publications regarding this topic for more information.

7.7 Figures in Chapter 7

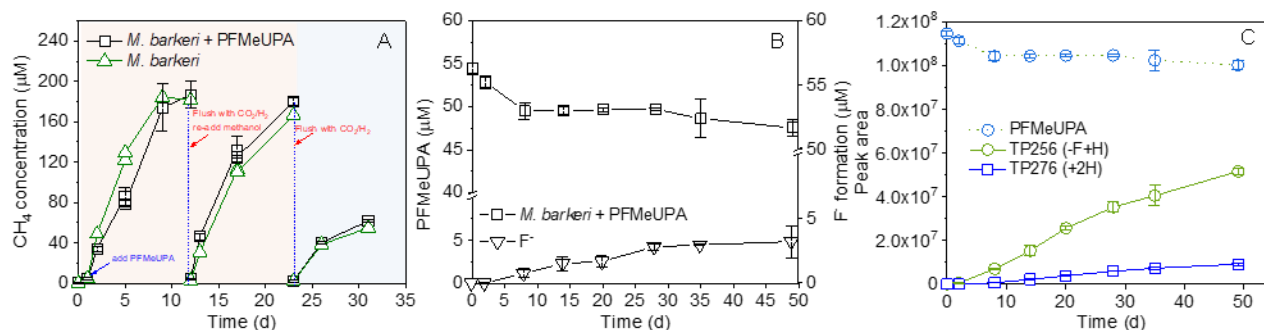


Figure 7.1 CH₄ formation in PFMeUPA added and no PFMeUPA added controls (A) (Note: the headspace was flushed with CO₂/H₂ at 12-day and 23-day, 0.8 mL methanol was added at 0-day and 12-day, PFMeUPA was added after the first day incubation once the methanogenesis activity has been observed), PFMeUPA biotransformation and fluoride ion formation (B), temporal trend of the PFMeUPA and the TPs during the biotransformation (C) (n=3).

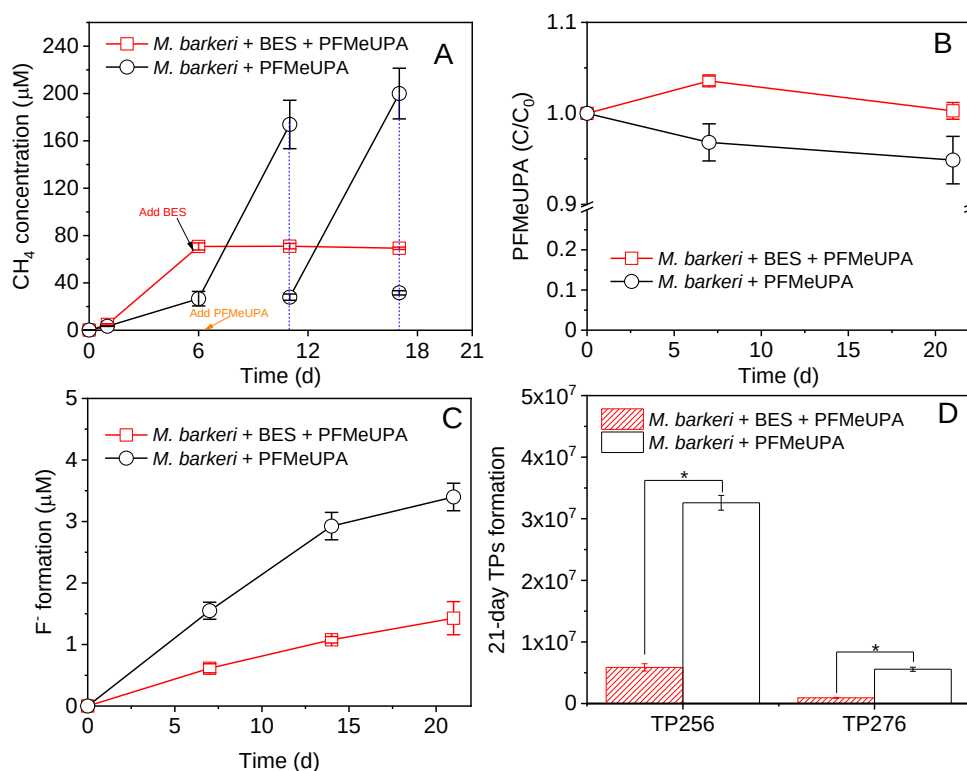


Figure 7.2 CH₄ formation in BES added and no BES added controls (A) (Note: BES and PFMeUPA were added at 6-day), PFMeUPA biotransformation in two controls (B), fluoride ion formation in two controls (C), TPs formation at 21-day in two controls (D) (Note: * indicated significant difference between two controls) (n=3).

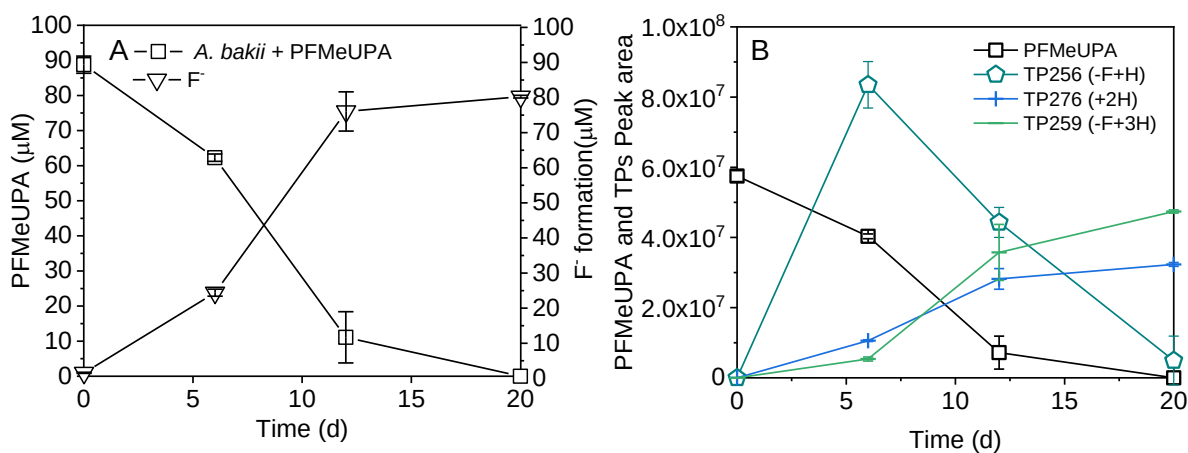


Figure 7.3 PFMeUPA removal and fluoride release in the *A. bakii* culture (A) (Note: no abiotic degradation of PFMeUPA was observed; temporal trends of the parent compound and the TPs during PFMeUPA biotransformation (B) (n=3).

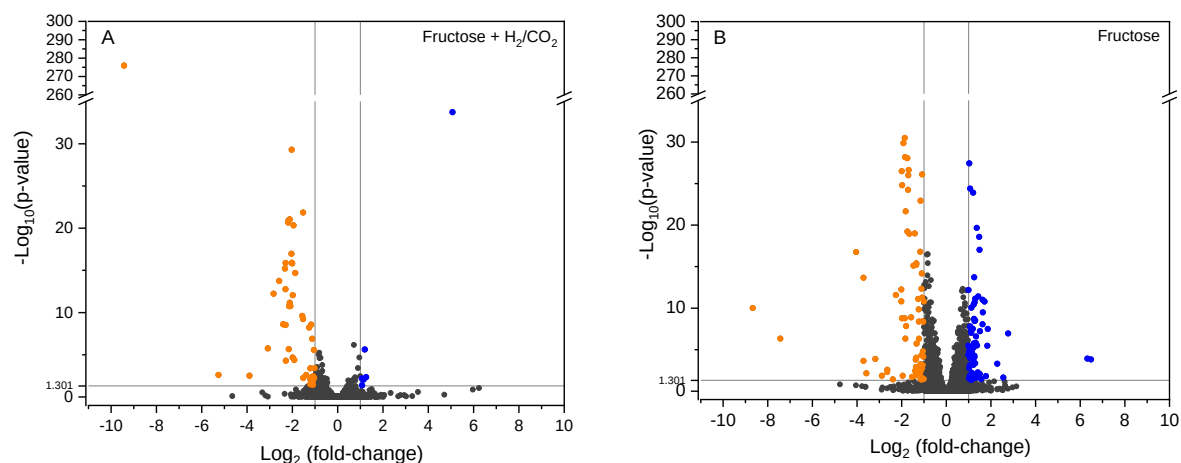


Figure 7.4 Differential gene expression with and without the addition of PFMeUPA in fructose with H_2/CO_2 acetogenesis (A) and fructose only acetogenesis (B) conditions. (Note: genes with p -value < 0.05 , fold-change > 2 were selected: orange dots represents genes with down regulation; blue dots represents genes with up-regulation) ($n=3$).

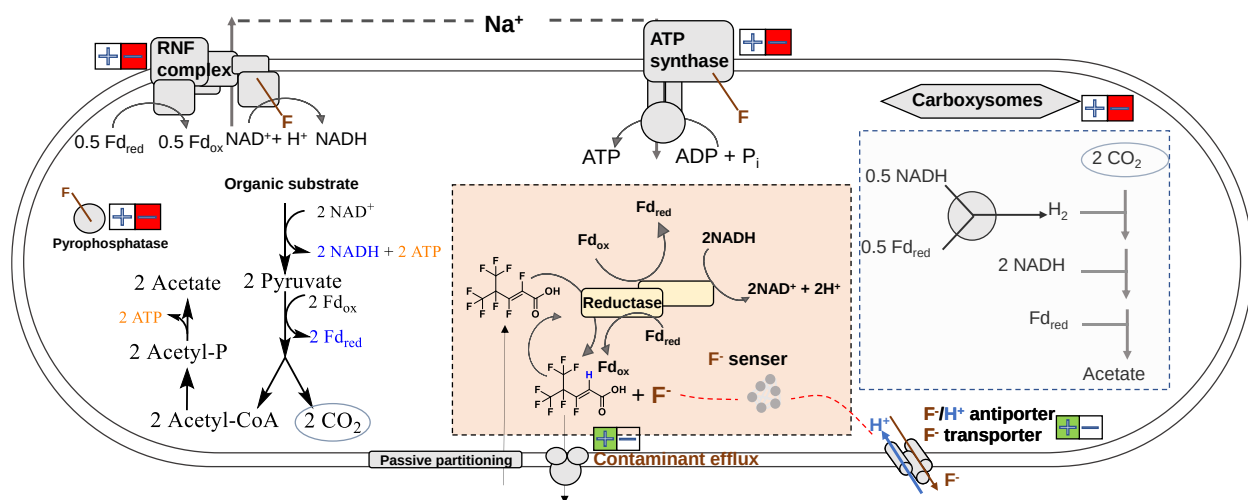


Figure 7.5 Proposed model of PFMeUPA biotransformation in *A. bakii*. (Note: green plus symbol indicates the up-regulation of corresponding genes; red minus symbol indicates the down regulation of related genes)

CHAPTER 8: CONCLUSION

8.1 Summary of the Findings of This Dissertation

Emerging organic contaminants (EOCs) can be defined as a general group of unregulated organic pollutants. Microbial biotransformation shows its potential in alleviating the environmental concentrations of many EOCs from wastewater and water. Thus, the major objective of this study aims to build a framework to comprehensively study the fate of EOCs as well as their biotransformation patterns under the different environmental conditions, specifically focusing on investigating the transformation products, the biotransformation pathways, the responsible microorganisms, and the responsible genes and enzymes. For transformation products analysis and reaction pathways elucidation, advanced analytical chemistry technology (i.e., high-resolution mass spectrometry) are used, and target and non-target data analysis approaches are conducted. For the responsible microorganisms/genes/enzymes' identification, meta-omics sequencing approaches (i.e., 16S rRNA gene sequencing analysis, metagenomic and metatranscriptomic sequencing analysis, whole-genome sequencing analysis, RNA sequencing analysis, and proteomics analysis) are adopted ([Fig. 8.1](#)).

- Chapter 1 reviewed the current status of EOCs biotransformation. The major objective of this dissertation is in two folds: (1) investigate the roles of nitrifiers played in EOCs removal and (2) study the feasibility of microbial cleavage of C–F bonds in PFAS.
- Chapter 2 disentangled contributions of AOB and NOB species to the transformation of

EOCs whose removal in nitrifying activated sludge was associated with nitrification activity, and further explored different transformation mechanisms. The findings showed that the NOB *Nitrobacter* sp. did not contribute to biotransformation of all tested EOCs and the AOB *Nitrosomonas europaea* in the *N. europaea*/*Nitrobacter* sp. co-culture was capable of biotransforming 8 out of 15 tested EOCs, whose biotransformation was previously found to be associated with nitrification. Strong evidence is obtained for enzymatic transformation of asulam by AMO via cometabolism. Furthermore, hydroxylamine (the AMO-mediated ammonia oxidation intermediate) was found, for the first time, to react with EOCs abiotically, with similar compound specificities and transformation product profiles as the biological transformation.

- Chapter 3 focused on roles of comammox bacteria in EOCs biotransformation. We found *N. inopinata* exhibited similar compound specificity for biotransformation as the other two investigated ammonia oxidizers (one AOB and one AOA species). More excitingly, we found that a benzimidazole fungicide carbendazim was exclusively biotransformed by the comammox bacterium *N. inopinata*. Like EOCs biotransformation by AOB that we reported in Chapter 2, carbendazim biotransformation by *N. inopinata* was also associated with ammonia monooxygenases, likely via co-metabolism.
- Chapter 4 investigated the defluorination activities of per- and polyfluorinated compounds by subcultures of a well-studied dechlorinating enrichment (KB-1). We demonstrated

microbial reductive defluorination of two C₆ branched and unsaturated structures, including one perfluorinated compound, as well as elucidated the defluorination/biotransformation pathways.

- Chapter 5 investigated defluorination activities of an anaerobic defluorinating enrichment and aerobic activated sludge community using a comprehensive list of unsaturated fluorinated carboxylic acids and their structure-similar saturated ones. We found that α , β -unsaturation in FCAs was crucial for initiating anaerobic microbial biotransformation and biodefluorination, which was independent of the fluorine substitution degrees (per- or poly-fluorinated) and the linear/branched structures. Reductive defluorination and hydrogenation are two primary biotransformation pathways for the unsaturated FCAs in the anaerobic defluorinating enrichment. With sp^2 C–F bonds being substituted by C–H bonds, the reductive defluorination became less favorable than hydrogenation. Furthermore, the tertiary sp^3 C–F bond in unsaturated FCAs could also be cleaved. The findings suggested that introducing multiple double bonds in the FCA structures or more fluorinated moieties at the α , β -unsaturated carbons may enhance the overall defluorination degree. Interestingly, unsaturated FCAs with trifluoromethyl (–CF₃) branch at the α/β carbon exhibited enhanced abiotic degradation and defluorination in the presence of reducing agents. Moreover, such structures also showed significant aerobic microbial defluorination in the activated sludge communities. Given the diverse applications and

emerging concerns of fluorochemicals, this work not only advances the fundamental understanding of the fate of unsaturated PFAS in natural and engineered environments but may also provide important insights into the design of readily degradable fluorinated PFAS alternatives.

- Chapter 6 applied meta-omics sequencing analysis approaches and revealed community structures of the defluorinating enrichment culture. Based on the defluorination activity results and metagenomic sequencing analysis data, we proposed that the fluoride ion expelling mechanisms might be necessary for the responsible microorganisms to defend the intracellular fluoride ion build-up. Furthermore, according to the results we obtained in Chapter 5, we concluded that the α , β -unsaturation in FCAs was crucial for initiating anaerobic microbial biotransformation and biodefluorination. Thus, it was highly likely that the responsible enzyme(s) for reductive defluorination and hydrogenation should be identical. The low potential reductase might be the responsible enzyme(s) for both reactions.
- Chapter 7 continued the investigating using pure cultures, by testing the biotransformation and biodefluorination capabilities of five selected pure isolates, we demonstrated that the acetogens *A. bakii* exhibited the same reaction mechanisms as what we observed for the enrichment culture and proposed the possible genes related to the biotransformation reaction.

8.2 Figure of Chapter 8

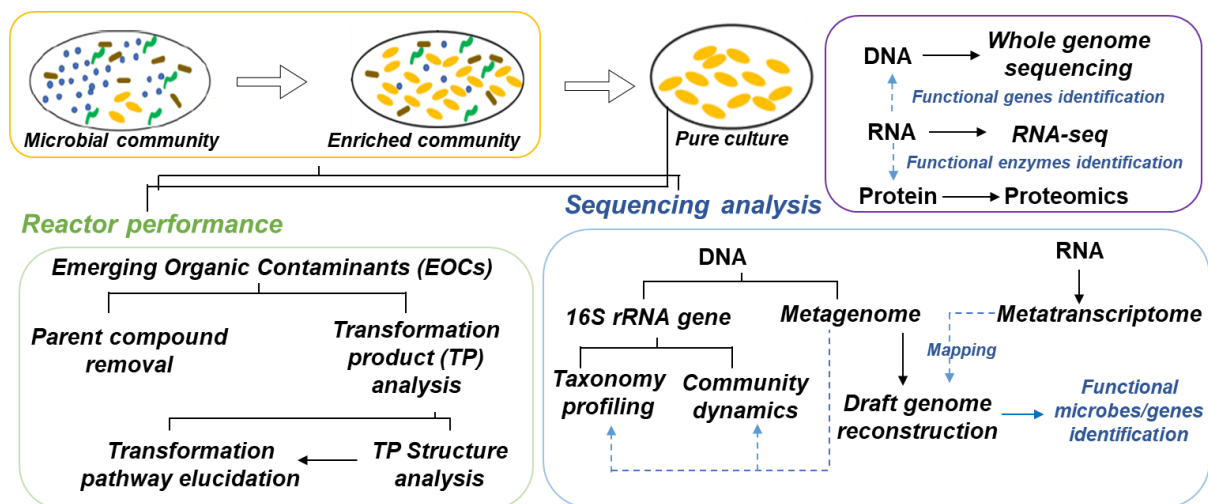


Figure 8.1 The proposed framework for studying EOCs biotransformation and identifying the responsible microorganisms, genes, and enzymes.

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APPENDIX A: SUPPLEMENTARY MATERIAL FOR CHAPTER 2

A.1 Supplementary Figures and Table

Table A.1 Characteristics of MPs investigated in Chapter 2.

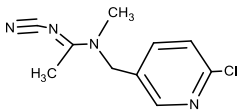
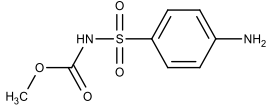
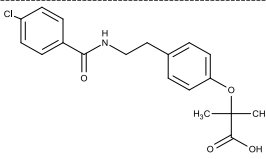
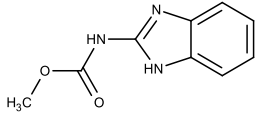
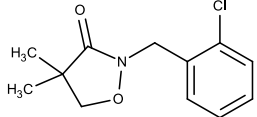
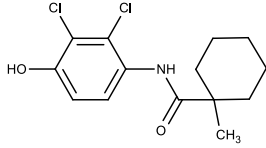
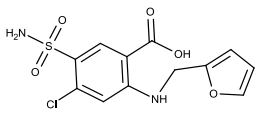
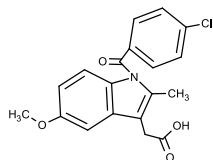
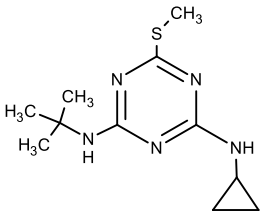
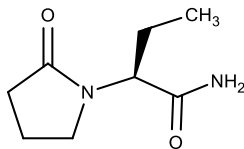
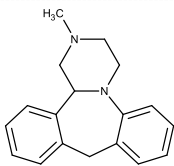
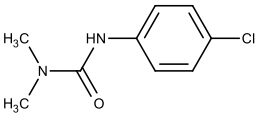
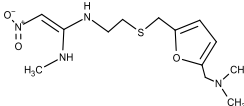
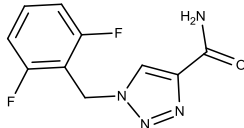
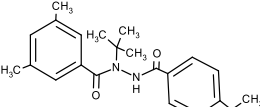
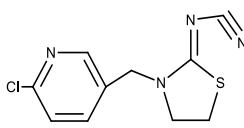
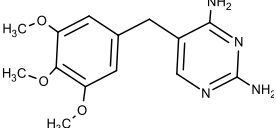
Name	Formula	Molecular weight	m/z	Structure	LOQ (µg/L)
Acetamiprid	C ₁₀ H ₁₁ ClN ₄	222.0672	223.0750		1
Asulam	C ₈ H ₁₀ O ₄ N ₂ S	230.0361	231.0439		1
Bezafibrate	C ₁₉ H ₂₀ ClNO ₄	361.1081	362.1159		0.5
Carbendazim	C ₉ H ₉ N ₃ O ₂	191.0694	192.0773		1
Clomazone	C ₁₂ H ₁₄ ClNO ₂	239.0713	240.0791		0.5
Fenhexamid	C ₁₄ H ₁₇ Cl ₂ NO ₂	301.0636	302.0715		1
Furosemide	C ₁₂ H ₁₁ ClN ₂ O ₅ S	330.0077	328.9999		1
Indomethacin	C ₁₉ H ₁₆ O ₄ NCl	358.0841	357.0768		5

Table A.1 Continued.

Name	Formula	Molecular weight	m/z	Structure	LOQ (µg/L)
Irgarol	C ₁₁ H ₁₉ N ₅ S	253.1361	254.1439		0.5
Levetiracetam	C ₈ H ₁₄ N ₂ O ₂	170.1055	171.1133		1
Mianserin	C ₁₈ H ₂₀ N ₂	264.1627	265.1699		1
Monuron	C ₉ H ₁₁ ClN ₂ O	198.0559	199.0638		1
Ranitidine	C ₁₃ H ₂₂ N ₄ O ₃ S	314.1413	315.1485		1
Rufinamide	C ₁₀ H ₈ F ₂ N ₄ O	238.0666	239.0744		0.5
Tebufenozide	C ₂₂ H ₂₈ N ₂ O ₂	352.2150	353.2229		0.5
Thiacloprid	C ₁₀ H ₉ ClN ₄ S	252.0236	253.0314		0.5
Trimethoprim	C ₁₄ H ₁₈ N ₄ O ₃	290.1379	291.1457		1

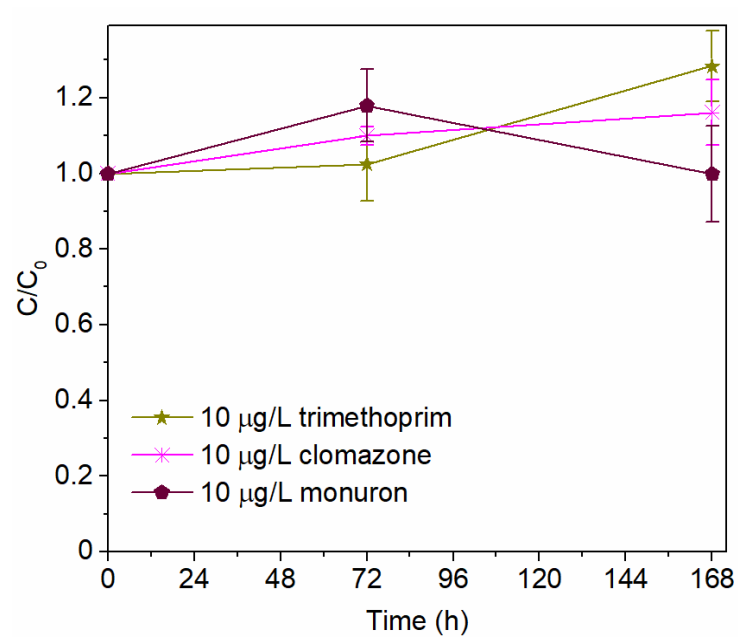


Figure A.1 Biotransformation of clomazone, monuron, and trimethoprim by the AOB/NOB co-culture (n=3).

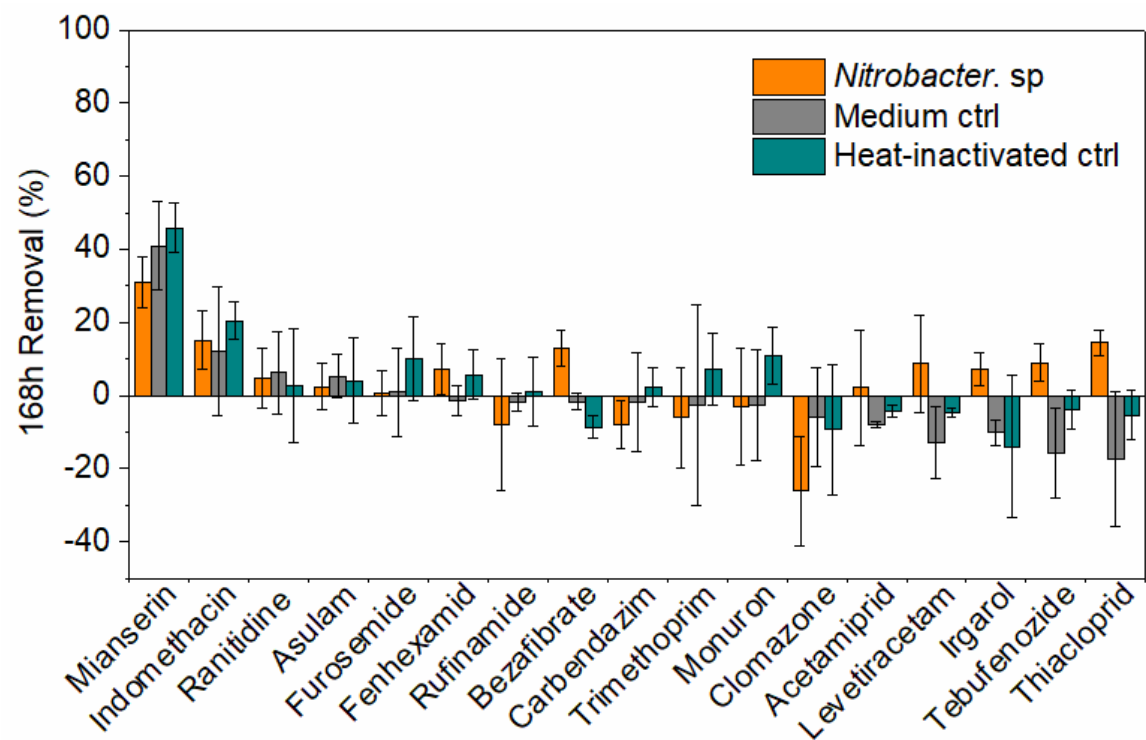


Figure A.2 Biotransformation of MPs by the NOB pure culture (n=3)

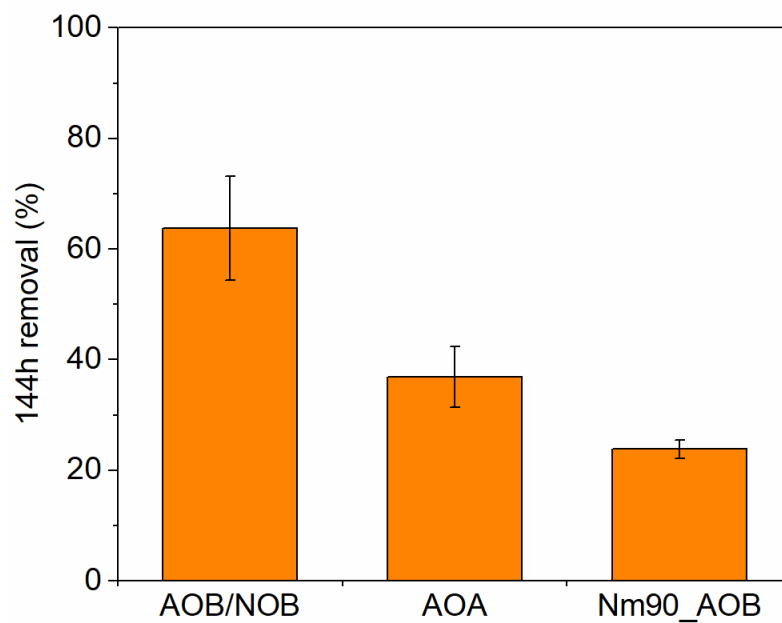
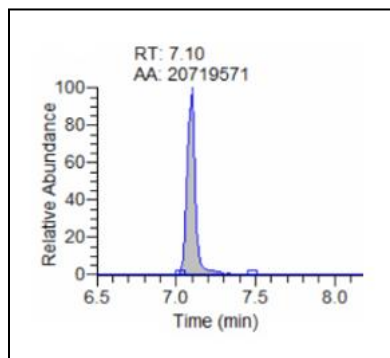


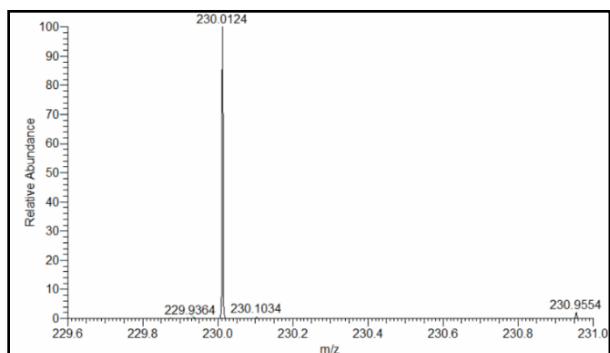
Figure A.3 144h-removal of asulam by the AOB/NOB co-culture, *Nitrososphaera gargensis* (AOA), and *Nitrosomonas* sp. Nm90 (AOB) at an ammonia oxidation rate of 1~2 mM/d (n=3).

(A)

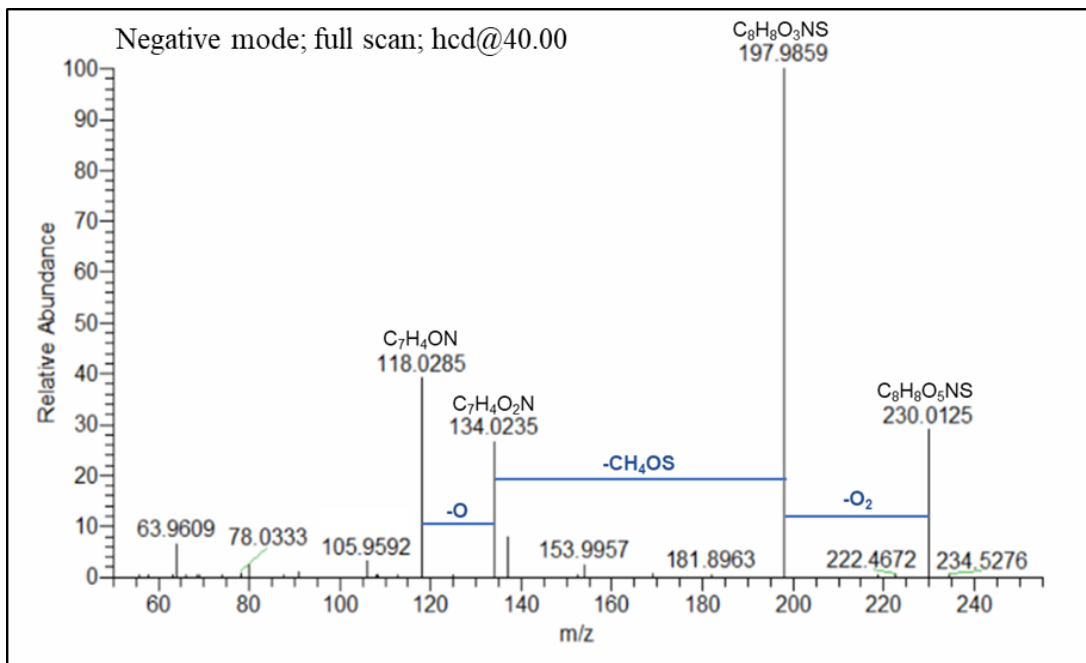
Chromatogram_TP230



MS Spectra_TP230



MS² Spectra_TP230



Formula

C₈H₉O₅NS

Atomic Modification

-NH +O

Hypothetical Structure

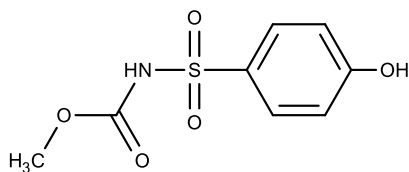
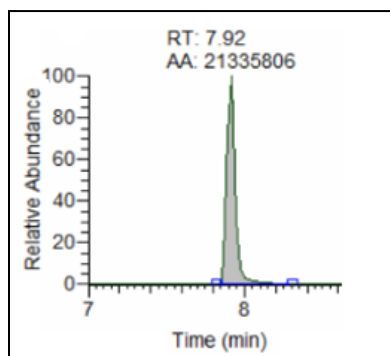


Figure A.4 Structure elucidation of the two asulam TPs (A: TP 230 and B: TP274).

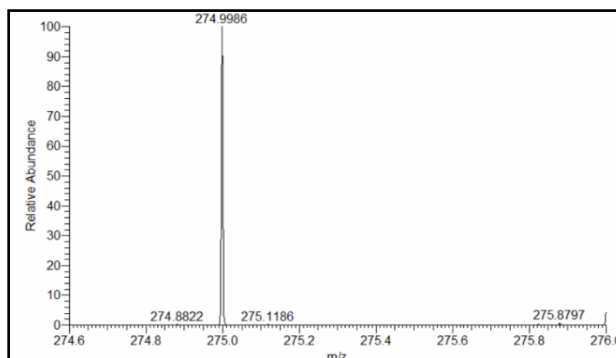
Figure A.4 Continued.

(B)

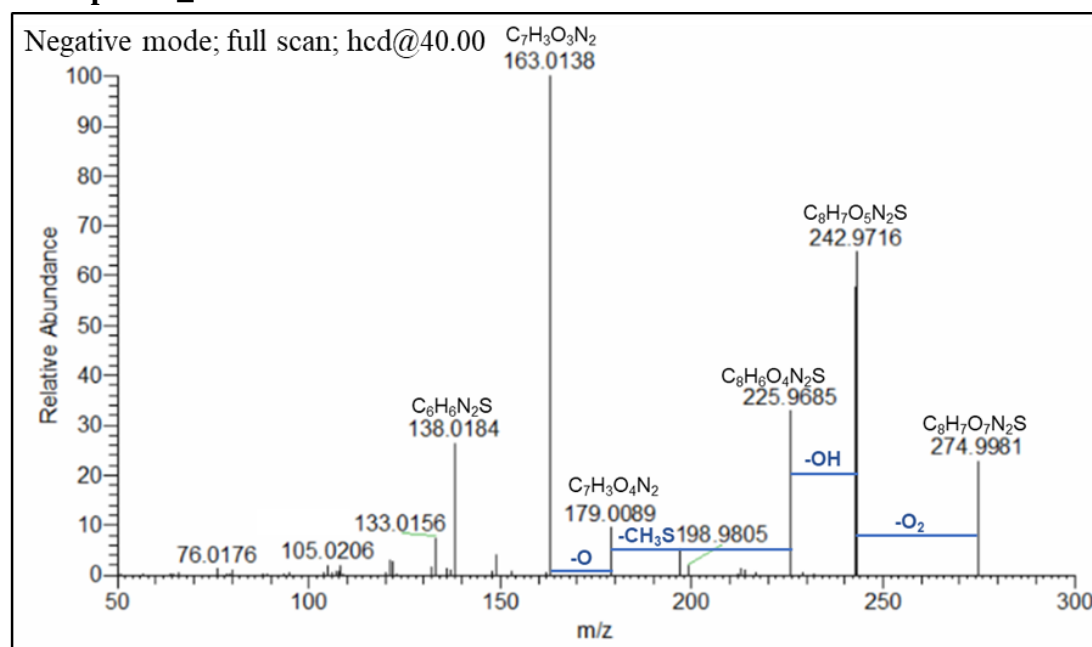
Chromatogram_TP274



MS Spectra_TP274



MS² Spectra_TP274



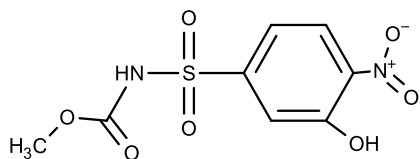
Formula

C₈H₈O₇N₂S

Atomic Modification

-2H +3O

Hypothetical Structure



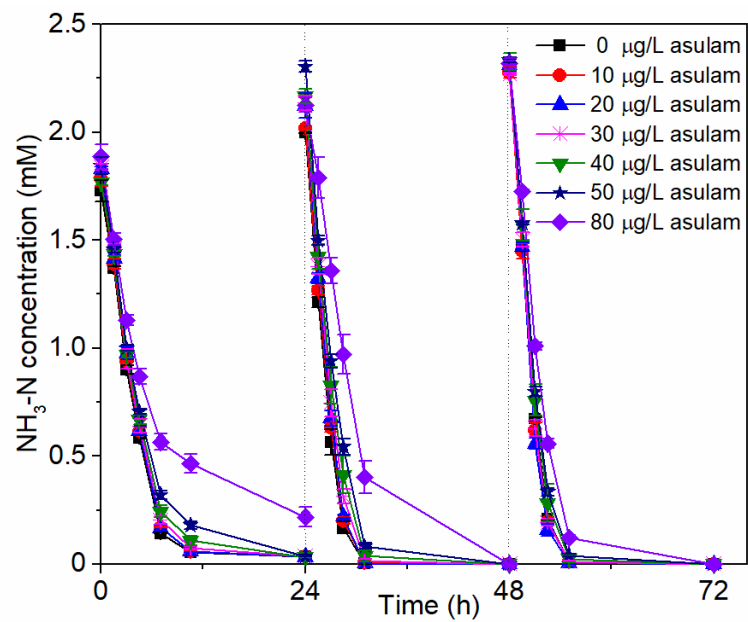


Figure A.5 Effects of different asulam concentrations on ammonia oxidation by the AOB/NOB co-culture (n=3)

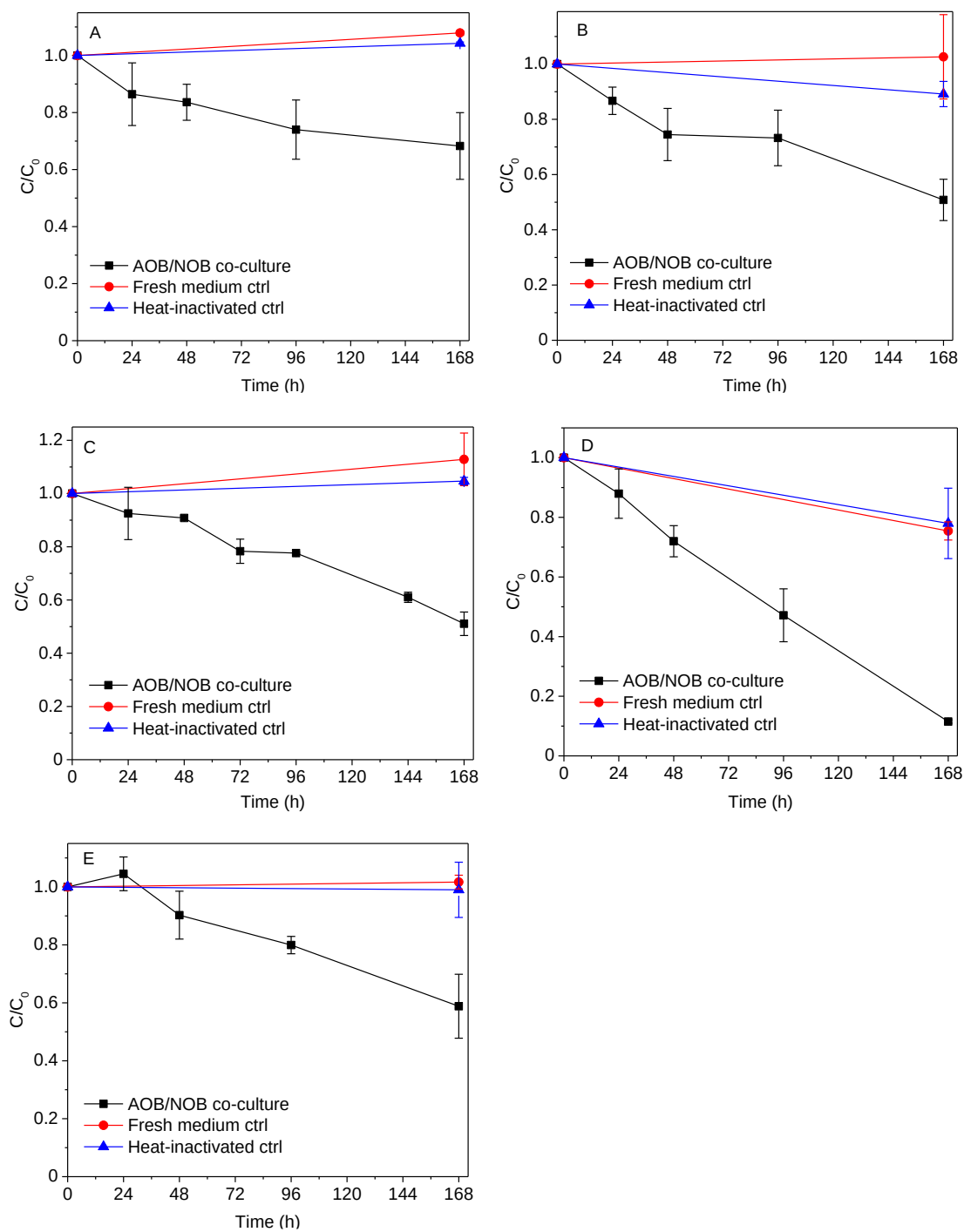


Figure A.6 Biotransformation of bezafibrate (A), fenhexamid (B), furosemide (C), indomethacin (D), rufinamide (E) (n=3).

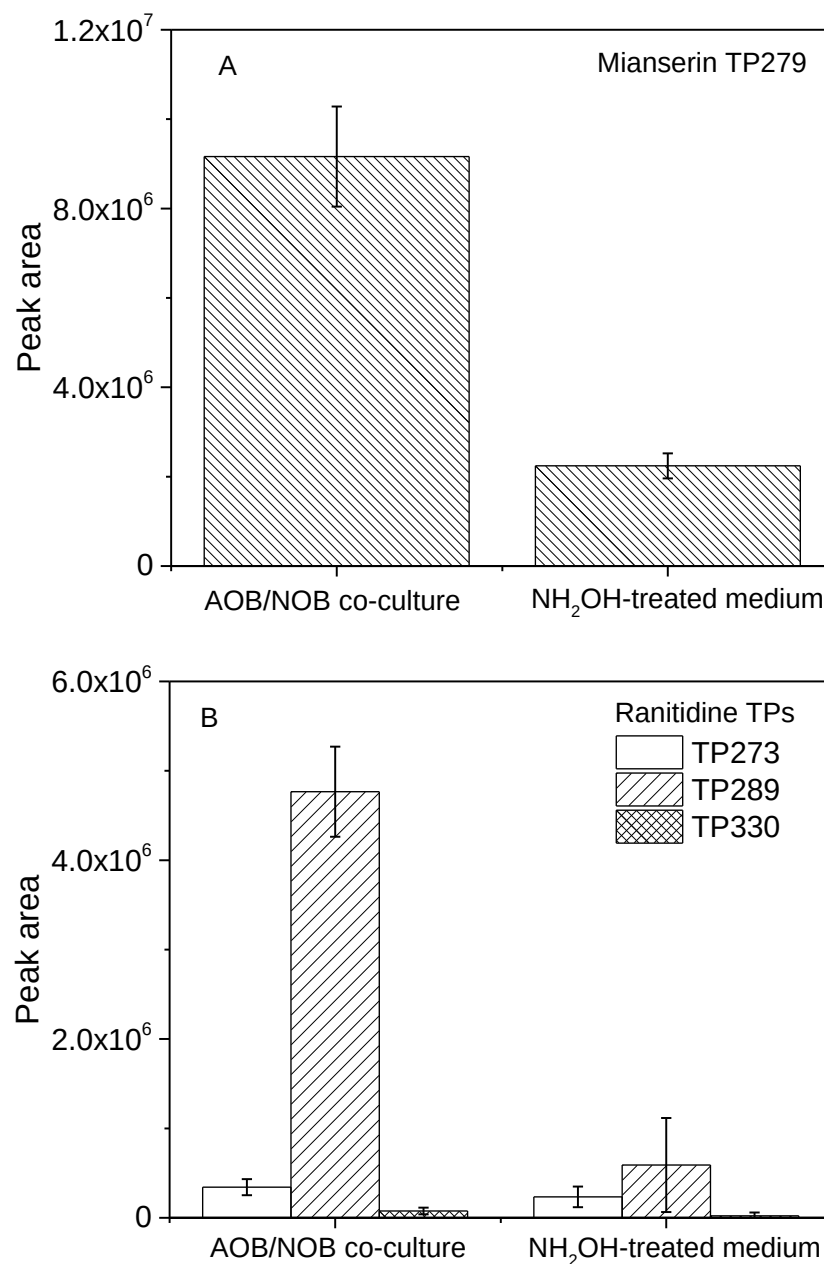


Figure A.7 Formation of mianserin TP (A) and ranitidine TPs (B) in the AOB/NOB co-culture and in the NH_2OH -treated fresh medium after a 168-h incubation with the same addition of parent compounds (none of the TPs was detected in the medium or heat-inactivated biomass control; $n=3$).

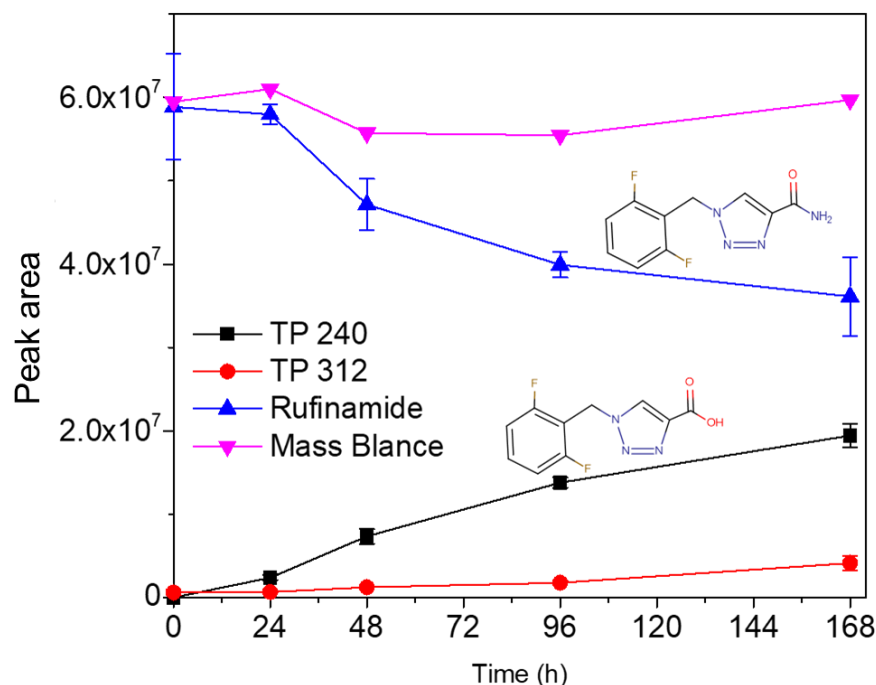


Figure A.8 Formation of rufinamide TPs in the AOB/NOB co-culture (n=3). The formula of TP240 was $C_{10}H_8O_2N_3F_2$ (-NH₂, +OH from rufinamide), and the formula of TP312 was $C_{13}H_{11}O_4N_3F_2$ (-NH₂, +OH, +O, +C₃H₄O from rufinamide). The mass balance line in magenta represents the sum of peak areas of TP240, TP312, and rufinamide, indicating a good mass balance regarding peak areas. Proposed structures of TP240 was also included. For TP230, a deamination, two hydroxylation, and a conjugation reaction (+C₃H₄O) were speculated to happen on the parent compound rufinamide. However, due to the lack of standard compounds, the structure of TPs cannot be fully elucidated.

APPENDIX B: SUPPLEMENTARY MATERIAL FOR CHAPTER 3

B.1 Supplementary Figures and Tables

Table B.1 List of micropollutants investigated and their detection on LC-HRMS/MS

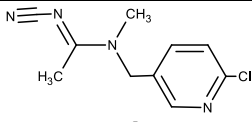
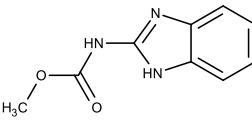
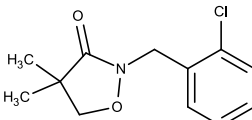
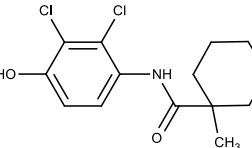
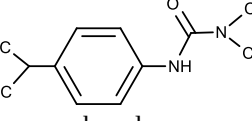
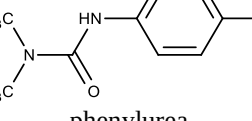
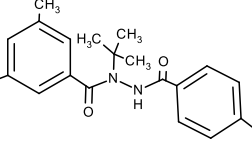
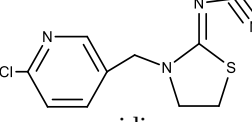
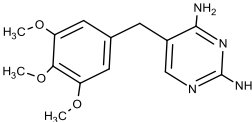
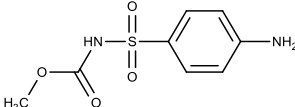
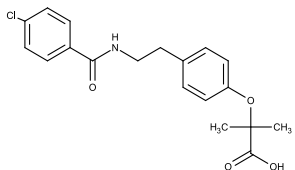
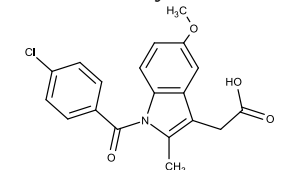
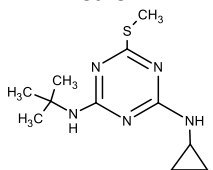
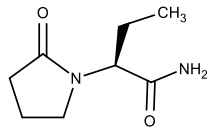
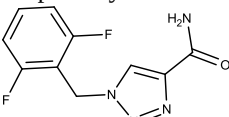
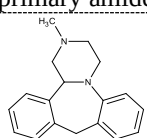
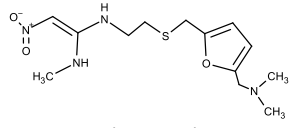
Group	Name	Formula	Structure	m/z	RT (min)	Category
	Acetamiprid	C ₁₀ H ₁₁ ClN ₄	 amidine	223.0745	7.62	Insecticide
	Carbendazim	C ₉ H ₉ N ₃ O ₂	 carbamate	192.0768	5.90	Fungicide
	Clomazone	C ₁₂ H ₁₄ ClNO ₂	 other	240.0786	9.55	Herbicide
	Fenhexamid	C ₁₄ H ₁₇ Cl ₂ NO ₂	 secondary amide	302.0709	10.00	Fungicide
A	Isoproturon	C ₁₂ H ₁₈ N ₂ O	 phenylurea	207.1491	9.02	Herbicide
	Monuron	C ₉ H ₁₁ ClN	 phenylurea	199.0633	8.38	Herbicide
	Tebufenozide	C ₂₂ H ₂₈ N ₂ O ₂	 secondary amide	353.2224	10.38	Insecticide
	Thiacloprid	C ₁₀ H ₉ ClN ₄ S	 amidine	253.0309	8.05	Insecticide
	Trimethoprim	C ₁₄ H ₁₈ N ₄ O ₃	 other	291.1452	6.03	Pharmaceutical

Table B.1 Continued.

Group	Name	Formula	Structure	m/z	RT (min)	Category
	Asulam	C ₈ H ₁₀ N ₂ O ₄ S	 other	231.0434	7.00	Herbicide
	Bezafibrate	C ₁₉ H ₂₀ ClNO ₄	 secondary amide	362.1154	9.09	Pharmaceutical
	Indomethacin	C ₁₉ H ₁₆ ClNO ₄	 other	358.0841	9.91	Pharmaceutical
B1	Irgarol	C ₁₁ H ₁₉ N ₅ S	 thioether	254.1434	8.85	Algaecide
	Levetiracetam	C ₈ H ₁₄ N ₂ O ₂	 primary amide	171.1128	6.07	Pharmaceutical
	Rufinamide	C ₁₀ H ₈ F ₂ N ₄ O	 primary amide	239.0739	7.41	Pharmaceutical
	Mianserin	C ₁₈ H ₂₀ N ₂	 tertiary amine	265.1699	6.84	Pharmaceutical
B2^a	Ranitidine	C ₁₃ H ₂₂ N ₄ O ₃ S	 tertiary amine	315.1485	5.54	Pharmaceutical

^aCompounds in B2 group were only included in *N. inopinata* biotransformation experiments.

Table B.2 Biomass-normalized first-order biotransformation rate constants (k_{bio}) of MPs by *N. gargensis*, *N. nitrosa* Nm90 and *N. inopinata*

(a) Biotransformation of asulam and fenhexamide by *N. gargensis*, *N. nitrosa* Nm90 and *N. inopinata*

Cultures	k_{bio}^a [L·(mg total protein) ⁻¹ ·d ⁻¹]		k_a (d ⁻¹)		k_d [L·(mg total protein) ⁻¹]	
	Asulam	Fenhexamide	Asulam	Fenhexamide	Asulam	Fenhexamide
<i>N. gargensis</i>	0.009 (0.006, 0.01) ^b	0.06 (0.03, 0.36)	0.003 (0.0002, 0.01)	0.004 (0.0004, 0.01)	0.01 (0.001, 0.07)	0.08 (0.005, 0.95)
<i>N. nitrosa</i> Nm90	0.002 (0.0001, 0.02)	0.31 (0.28, 0.36)	0.02 (0.001, 0.14)	0.004 (0.0003, 0.02)	0.05 (0.004, 0.29)	n.a. ^c
<i>N. inopinata</i>	n.a.	0.05 (0.02, 0.6)	n.a.	0.02 (0.008, 0.03)	n.a.	0.09 (0.004, 1.8)

(b) Biotransformation of mianserin, ranitidine and carbendazim by *N. inopinata*

k_{bio}^a [L·(mg total protein) ⁻¹ ·d ⁻¹]			k_a (d ⁻¹)			k_d [L·(mg total protein) ⁻¹]		
Mianserin ^d	Ranitidine ^d	Carbendazim	Mianserin	Ranitidine	Carbendazim	Mianserin	Ranitidine	Carbendazim
0.12 (0.07, 1.4)	0.06 (0.03, 0.13)	0.007 (0.005, 0.01)	0.009 (0.001, 0.02)	0.009 (0.001, 0.03)	0.005 (0.0004, 0.02)	0.015 (0.001, 0.09)	0.015 (0.001, 0.09)	0.008 (0.001, 0.04)

^a Normalized to total protein;

^b Values are reported as median (5%, 95% percentiles);

^c Not applicable.

^d The previously reported k_{bio} values for *N. gargensis* and *N. nitrosa* Nm90¹²⁹ were used to compare to the biotransformation kinetics of mianserin and ranitidine by *N. inopinata*, which is summarized in Figure 1B of the main text.

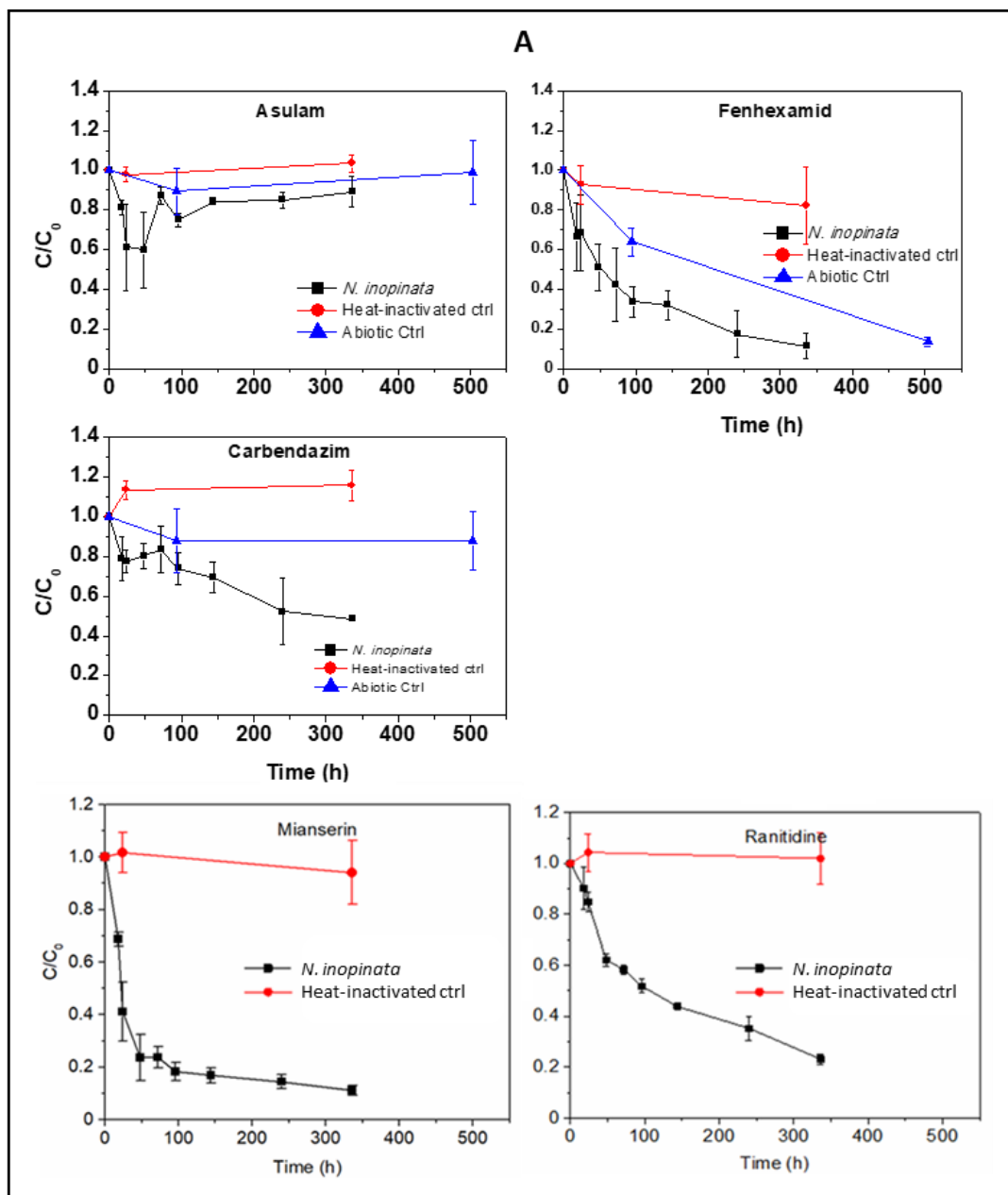
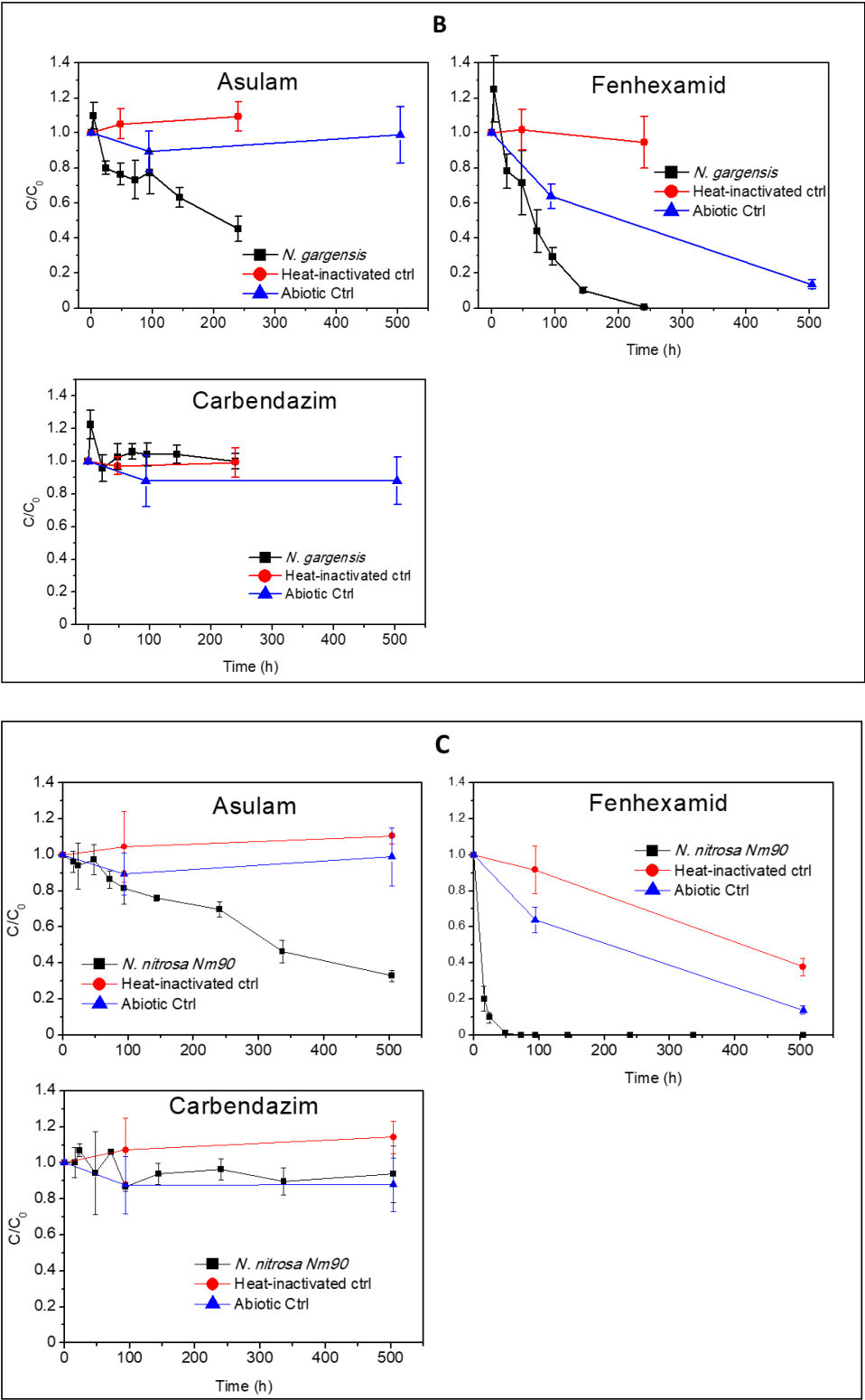


Figure B.1 Biotransformation curve of selected MPs by *N. inopinata* (A), *N. gargensis* (B) and *N. nitrosa* Nm90 (C)

Figure B.1 Continued.



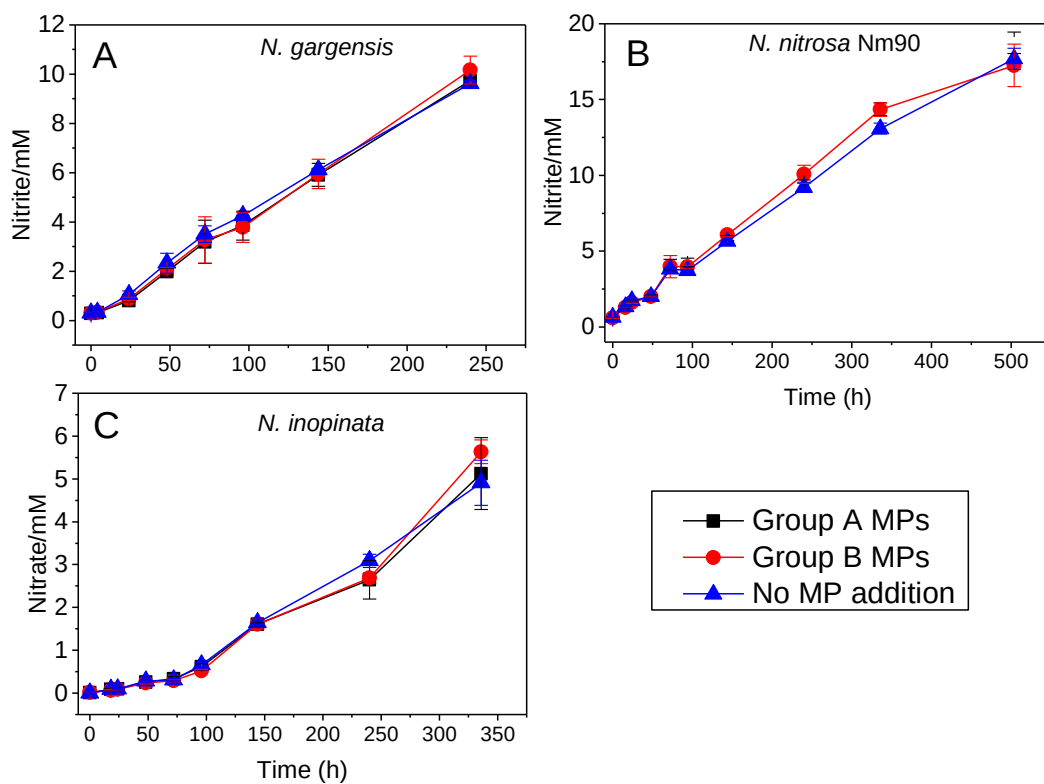


Figure B.2 Nitrite/nitrate production of *N. gargensis* (A), *N. nitrosa* Nm90 (B) and *N. inopinata* (C) with addition of groups of MPs (20 $\mu\text{g/L}$ each).

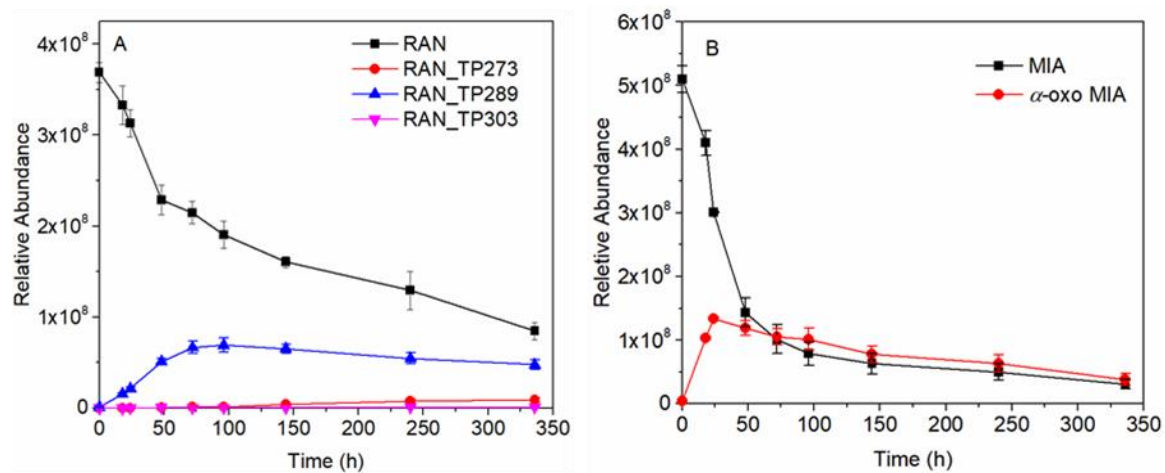


Figure B.3 TP formation of ranitidine (A) and mianserin (B) by *N. inopinata*

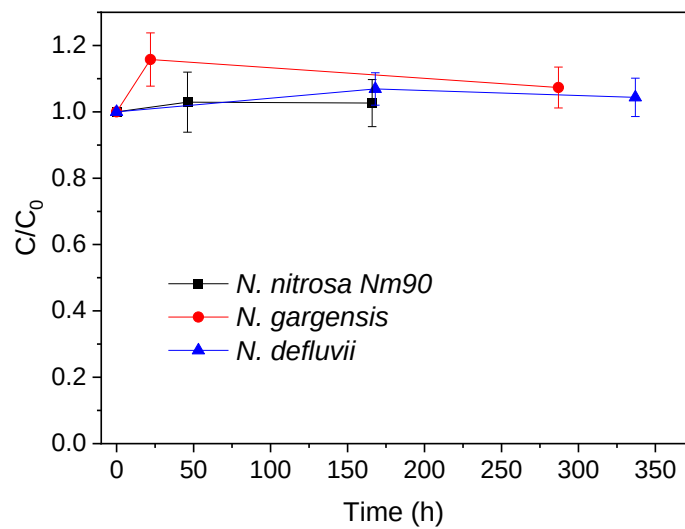


Figure B.4 Removal of carbendazim after individual addition (without the other micropollutants) by *N. gargensis*, *N. nitrosa* Nm90 and *N. defluvii* during ammonia/nitrite oxidation.

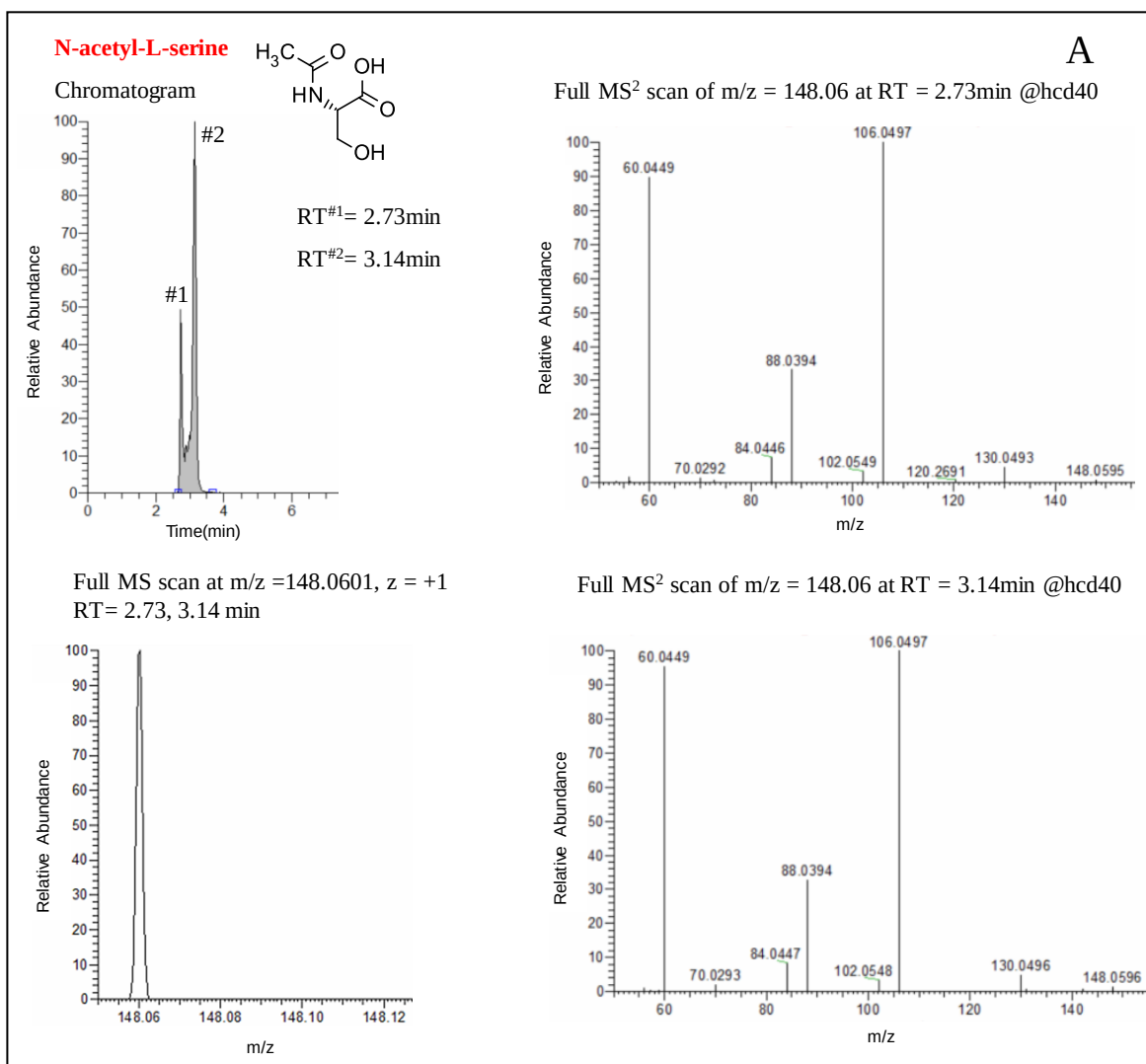


Figure B.5 Full MS scan and MS² fragmentation profiles of N-acetyl-L-serine (A), O-acetyl-L-serine (B), and carbendazim TP148 (C).

Figure B.5 Continued.

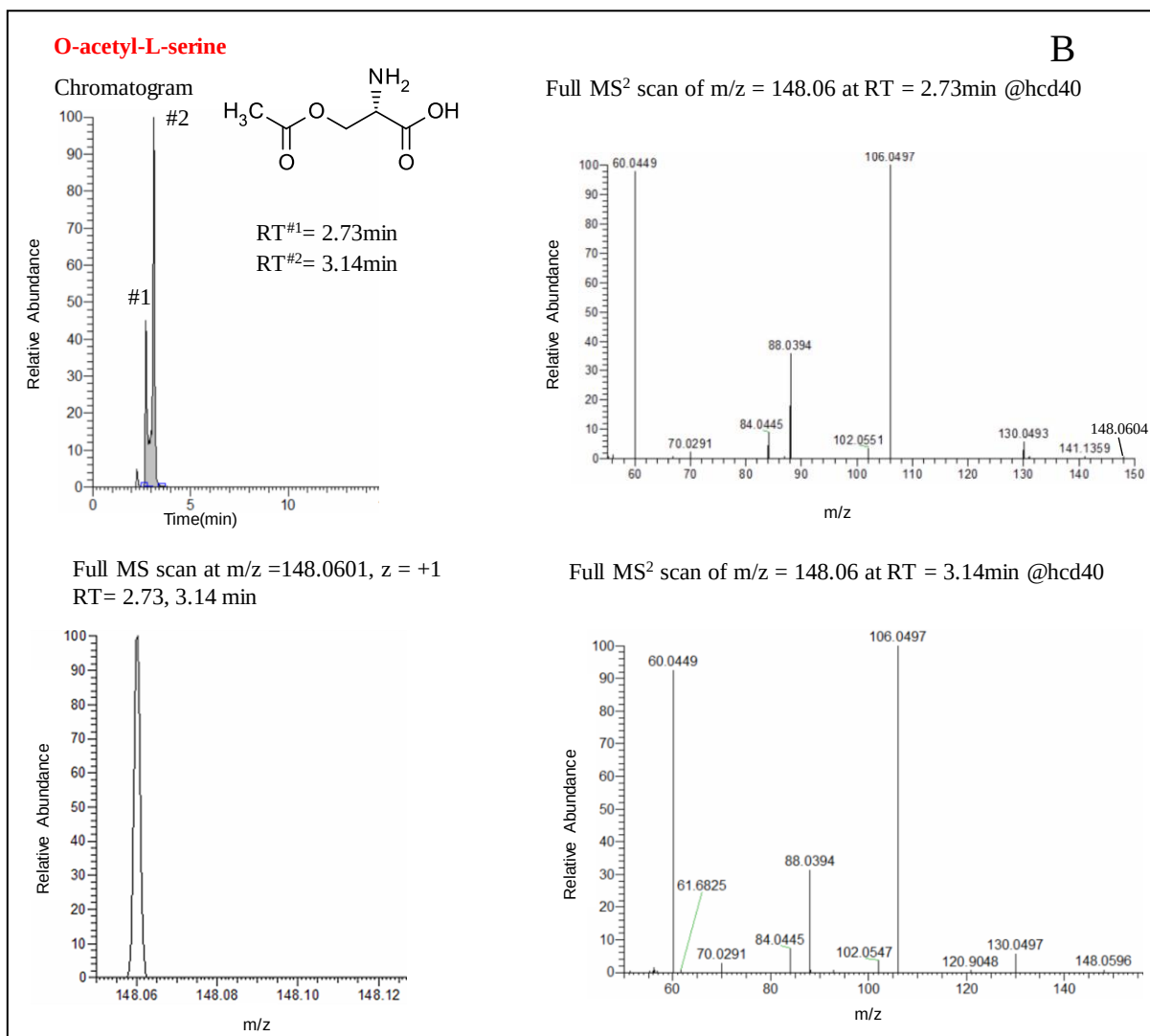
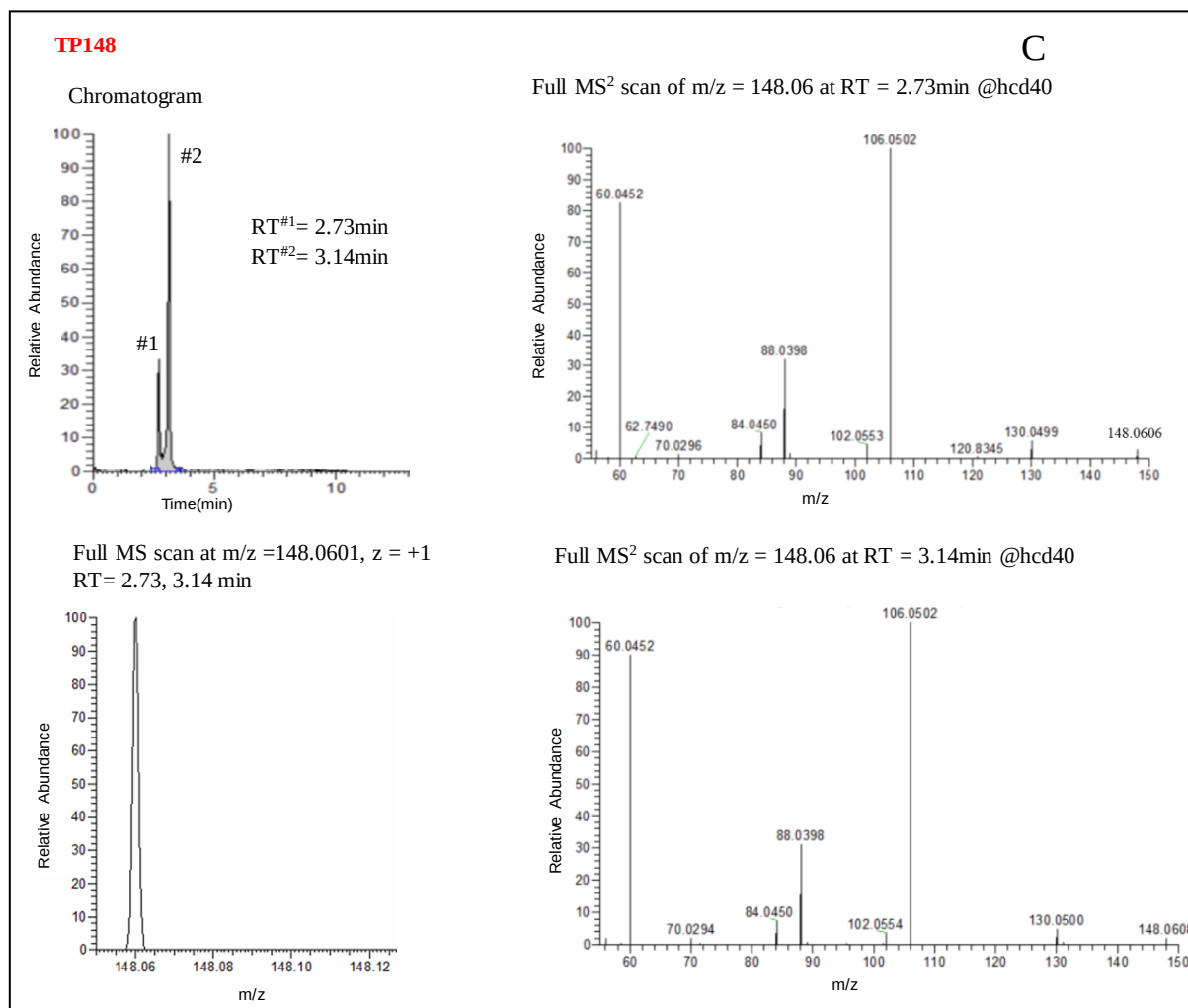


Figure B.5 Continued.



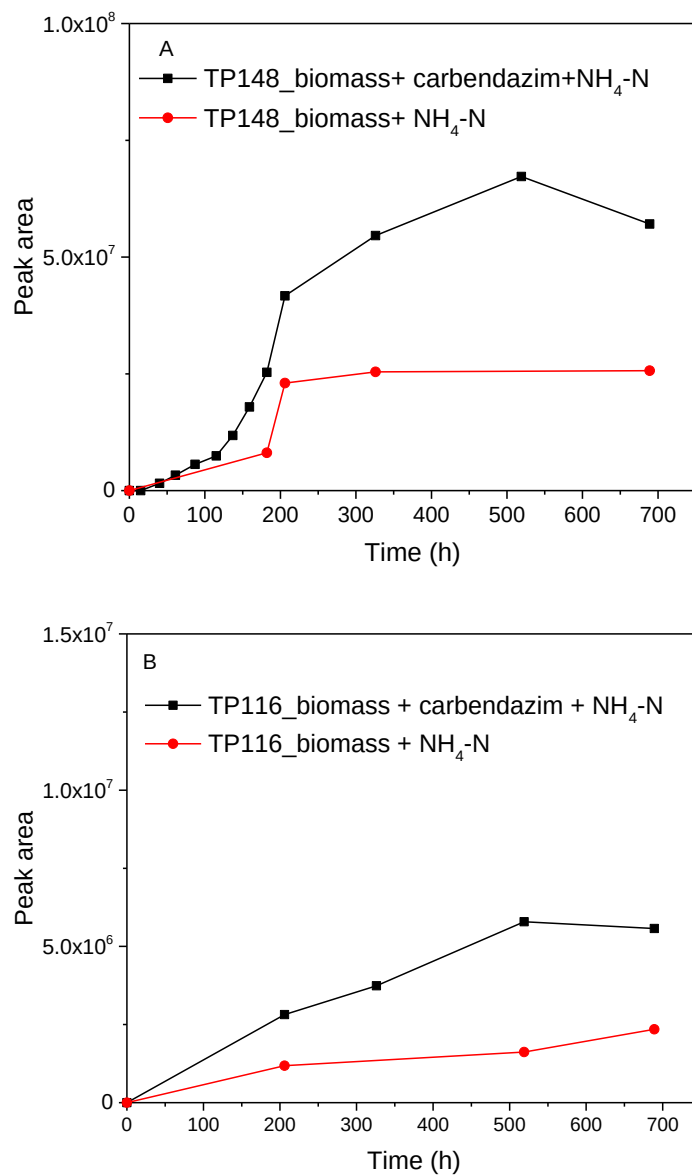
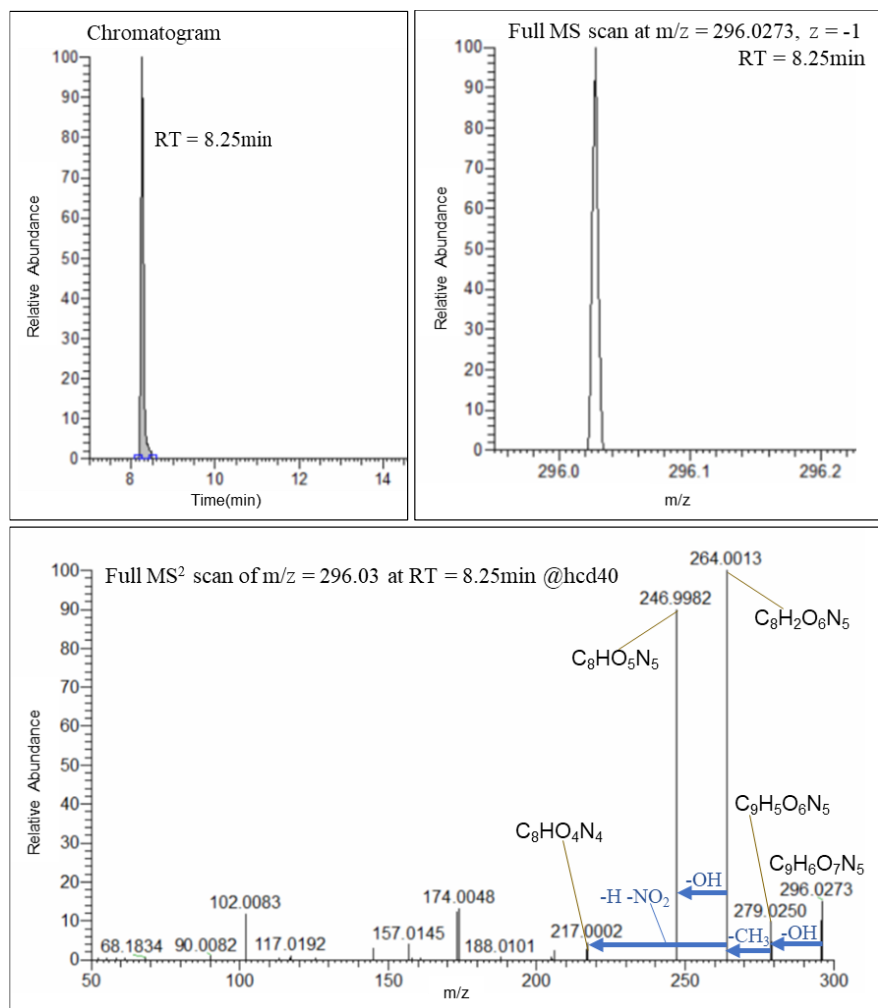


Figure B.6 The formation of TP148 (A) and TP116 (B) in *N. inopinata* with and without carbendazim.



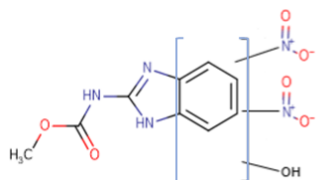
Formula

$C_9H_7O_7N_5$

Atomic Modification

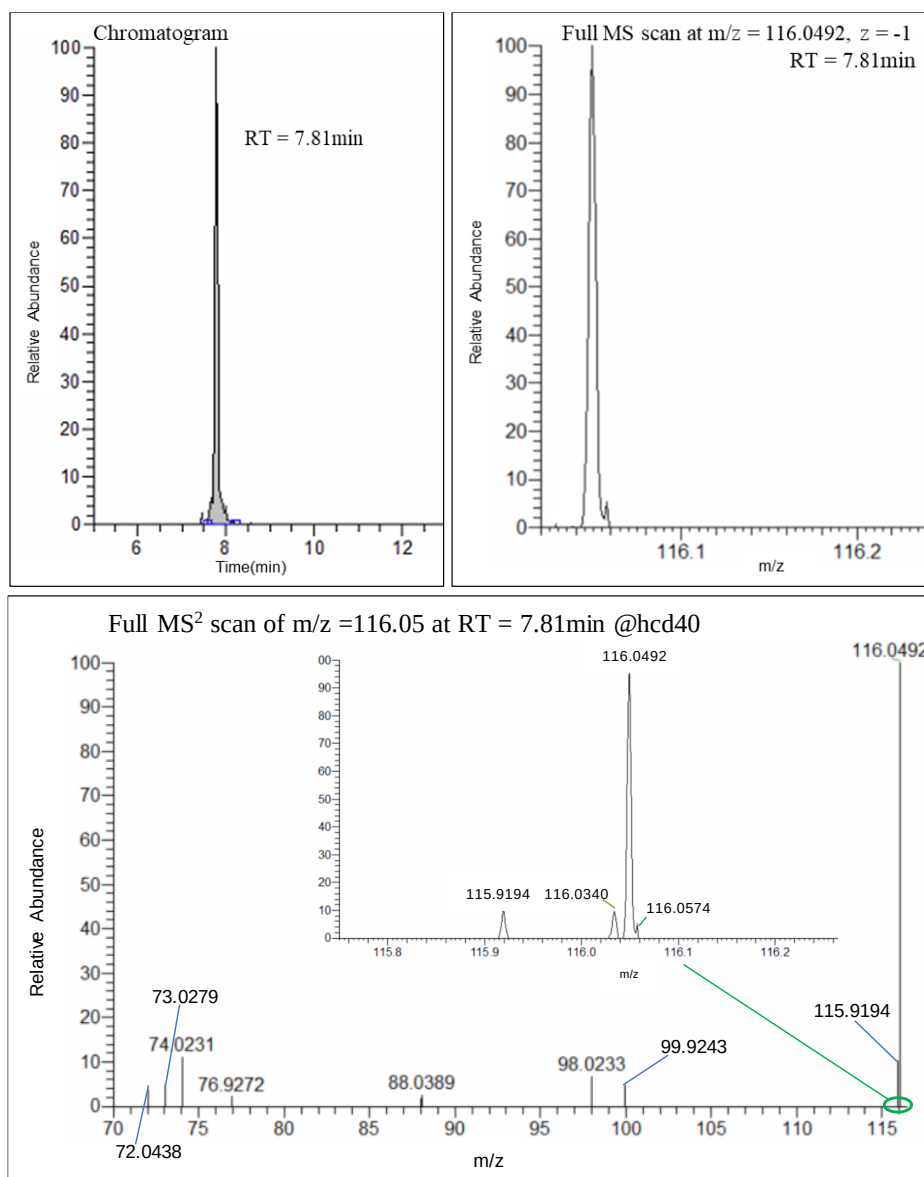
$-2H + 5O + 2N$

Hypothetical Structure



Confidence Level: 3

Figure B.7 Full MS scan and MS² fragmentation profiles of carbendazim TP296.



No plausible formula assigned

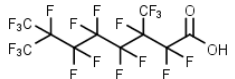
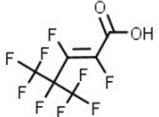
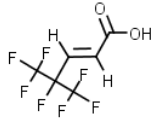
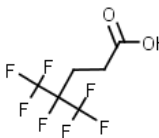
Confidence level: 5

Figure B.8 Full MS scan and MS² fragmentation profiles of carbendazim TP116.

APPENDIX C: SUPPLEMENTARY MATERIAL FOR CHAPTER 4

C.1 Supplementary Figures and Tables

Table C.1 Standard compound information.

Standards name	Formula	[M-H] ⁻	Observed Fragments	Predicted Fragments	Structure	LOQ (μM)
PFOA	C ₈ HF ₁₅ O ₂	412.9664	368.9765 ; 218.9858; 168.9883; 118.9914	368.9766 ; 268.9830		0.01
PFdiMeOA	C ₁₀ HF ₁₉ O ₂	512.9600	468.9706 ; 318.9804 ; 268.9833; 218.9854; 168.9883; 118.9912	468.9702 ; 318.9798 ; 68.9958		0.01
PFMeUPA	C ₆ HF ₉ O ₂	274.9760	230.9859 ; 180.9892; 68.9942	230.9862 ; 168.9894		0.01
FTMeUPA	C ₆ H ₃ F ₇ O ₂	238.9949	218.9879 ; 198.9815 ; 174.9977 ; 168.9883 ; 154.9913; 132.9895	220.9843; 218.9886 ; 198.9824 ; 195.0050; 192.9894; 174.9988 ; 168.9894 ; 148.9856; 125.0020		0.01
FTMePA	C ₆ H ₅ F ₇ O ₂	241.0105	221.0059 ; 200.9978 ; 180.9911; 177.0137	222.9999; 221.0043 ; 202.9934; 200.9980 ; 197.0203; 177.0144		0.01

Numbers in **bold** are the detected MS² fragments that match the predicted ones.

Table C.2 Transformation product information.

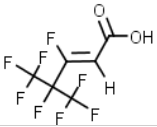
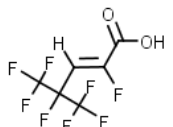
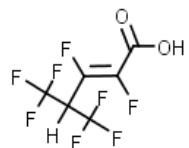
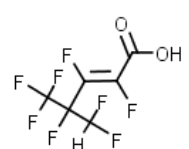
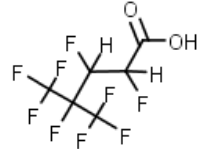
Compound ID	Formula	[M-H] ⁻	Observed Fragments	Predicted Fragments	Structure	LOQ (μM)
TP256	C ₆ H ₂ F ₈ O ₂	256.9854	212.9952; 192.9887; 168.9884	212.9956; 192.9894; 168.9894		n.a.
				236.9792; 212.9956; 192.9894; 186.9824; 168.9894; 142.9926		
				236.9792; 212.9956; 192.9894; 150.9988		
				236.9792; 212.9956; 192.9894; 150.9988; 106.9950		
TP276	C ₆ H ₃ F ₉ O ₂	276.9917	212.9949; 192.9886	258.9811; 256.9854; 233.0018; 230.9862; 212.9956; 206.9886; 168.9894; 162.9988		n.a.

Table C.2 Continued

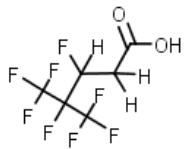
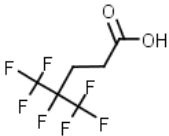
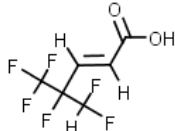
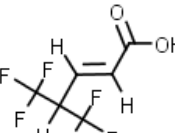
Compound ID	Formula	[M-H] ⁻	Observed Fragments	Predicted Fragments	Structure	LOQ (μM)
TP259	C ₆ H ₄ F ₈ O ₂	259.0011	238.9951; 195.0043; 174.9960; 168.9985; 154.9915	240.9905; 238.9949; 215.0112; 212.9956; 195.0050; 188.9980; 170.9875; 168.9918; 145.0082		n.a.
				240.9905; 238.9949; 215.0112; 212.9956; 195.0050; 188.9980; 170.9875; 168.9918; 145.0082		
TP241 (Confirmed as FTMePA)	C ₆ H ₅ F ₇ O ₂	241.0105	221.0059; 200.9978; 180.9911; 177.0137	222.9999; 221.0043; 202.9934; 200.9980; 197.0203; 177.0144		0.01

Table C.2 Continued

Compound ID	Formula	[M-H] ⁻	Observed Fragments	Predicted Fragments	Structure	LOQ (μM)
TP221	C ₆ H ₄ F ₆ O ₂	221.0043	200.9974; 180.9910; 152.9958; 132.9895; 130.9917; 108.9893; 104.9943	202.9937; 200.9980 ; 182.9875; 180.9918 ; 177.0144; 174.9988; 168.9918; 157.0082; 154.9926; 151.0012; 132.9907 ; 130.9926 ; 125.0020; 107.0114; 104.9958		n.a.
			200.9974; 180.9910; 152.9958; 132.9895; 130.9917; 108.9893; 104.9943	202.9937; 200.9980 ; 182.9875; 180.9918 ; 177.0144; 174.9988; 168.9918; 157.0082; 154.9926; 151.0012; 132.9907 ; 130.9926 ; 125.0020; 107.0114; 104.9958		
TP221	C ₆ H ₄ F ₆ O ₂	221.0043	200.9974; 180.9910; 152.9958; 132.9895; 130.9917; 108.9893; 104.9943	202.9937; 200.9980 ; 182.9875; 180.9918 ; 177.0144; 174.9988; 168.9918; 157.0082; 154.9926; 151.0012; 130.9926 ; 107.0114		n.a.
			200.9974; 180.9910; 152.9958; 132.9895; 130.9917; 108.9893; 104.9943	202.9937; 200.9980 ; 182.9875; 180.9918 ; 177.0144; 174.9988; 168.9918; 157.0082; 154.9926; 151.0012; 130.9926 ; 107.0114		

n.a.: not available; TP names in **bold** have structures either confirmed with the standard compounds (confidence level 1) or highly plausible (inferred from MS² fragments, confidence level 2b) structures; Fragments in **bold** are those matching the predicted ones.

Table C.3 Primer sets information

Gene target	Primer name	Sequence (5'-3')	References
Universal bacterial 16S rRNA gene	Unibac_341f	CCTACGGGAGGCAGCAG	293
	Unibac_518r	ATTACCGCGGCTGCTGG	
<i>Dehalococcoides</i> 16S rRNA gene	Dhc_f	GGTAATACGTAGGGAAGCAAGCG	207, 294, 295
	Dhc_r	CCGGTTAAGCCGGGAAATT	
<i>Dehalobacter</i> 16S rRNA gene	Dhb_447f	GATTGACGGTACCTAACGAGG	296
	Dhb_647r	TACAGTTTCCAATGCTTTACGG	
<i>Geobacter</i> _1 16S rRNA gene	Geo_f	CTTGCTCTTTTCATTTAGTGG	226
	Geo_r	AAGAAAACCGGGTATTAACC	
<i>rdhA1</i>	rdhA1_246f	ATCGGAGCTGCACAAGTAGG	228
	rdhA1_336r	TCTTGTGAGCGGTGTCTTTG	
<i>rdhA2</i>	rdhA2_720f	CAAAGGAGATGTTCCGGTGT	228
	rdhA2_985r	CAGGTGGAAGACCGGTTA	
<i>rdhA3</i>	rdhA3_1149f	CATTCTCCGGGAAGAAAACA	228
	rdhA3_1379r	CCAGGCTTCCTTGTCTTCAG	
<i>rdhA4</i>	rdhA4_754f	TTGTTATGCCCAATATGA	228
	rdhA4_925r	TCTATCCATTTGCCCCAGAC	
<i>rdhA5</i>	rdhA5_1017f	GATGCAGGCATTTACCGTTT	228
	rdhA5_1137r	GTCTCTTTGCCTTCGGTCAG	
<i>rdhA6</i>	rdhA6_318f	ATTTAGCGTGGGCAAAACAG	228
	rdhA6_555r	CCTTCCCACCTTGGGTATTT	
<i>rdhA7</i>	rdhA7_1391f	GCTAAAGAGCCGTCATCCTG	228
	rdhA7_1539r	GCAGTAACAACAGCCCCAAT	
<i>rdhA8</i>	rdhA8_845f	CCCAAGGTAGGTGTGCAGAT	228
	rdhA8_1016r	CCCGGTTAGTTACCCCGTAT	
<i>rdhA9</i>	rdhA9_251f	CTGACCTTGAAACCCCTGAA	228
	rdhA9_425r	TTGCCACCCATTTCCATATT	
<i>rdhA10</i>	rdhA10_710f	GCTGAAACACCCACCAAACT	228
	rdhA10_860r	CGACAAAGGGGAATCTTTGA	
<i>rdhA11</i>	rdhA11_429f	TAATGGCAACCGGAGGTAAG	228
	rdhA11_609r	TCTACCGGTATGGCCTGAAC	
<i>rdhA12</i>	rdhA12_864f	AGGAGTTCCTGTGGGGACTT	228
	rdhA12_994r	TTTGGGGGTCATAACTGCTC	
<i>rdhA13</i>	rdhA13_1356f	CAGGGTACCTGTCCCTTCAA	228
	rdhA13_1493r	AGGGTTCTTCCGTCCGTACT	
<i>rdhA14</i>	rdhA14_642f	GAAAGCTCAGCCGATGACTC	228
	rdhA14_846r	TGGTTGAGGTAGGGTGAAGG	

Table C.4 Comparison of selected PFASs ionization efficiency.

Compound	Ionization efficiency ratio
FTMeUPA: PFMeUPA	5.69
FTMePA: PFMeUPA	0.57
FTMeUPA: FTMePA	9.93

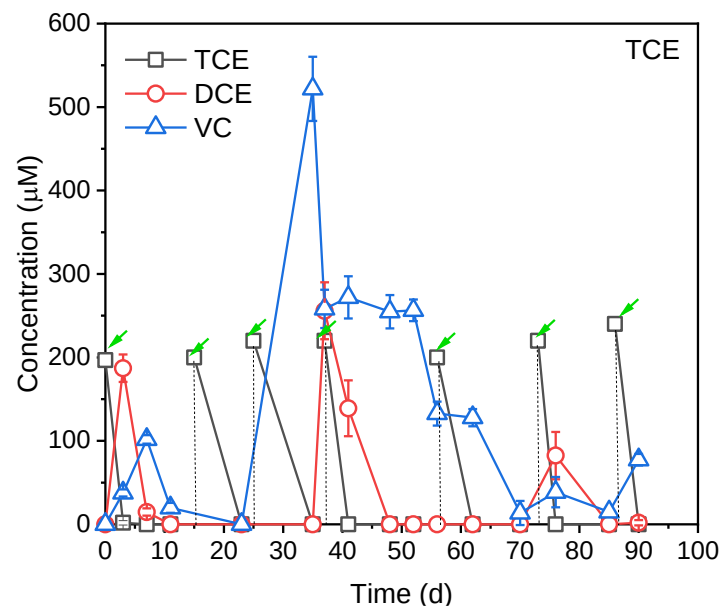


Figure C.1 TCE, *cis*-DCE, and VC in the culture with only TCE added (green arrows indicate the times when TCE was re-added).

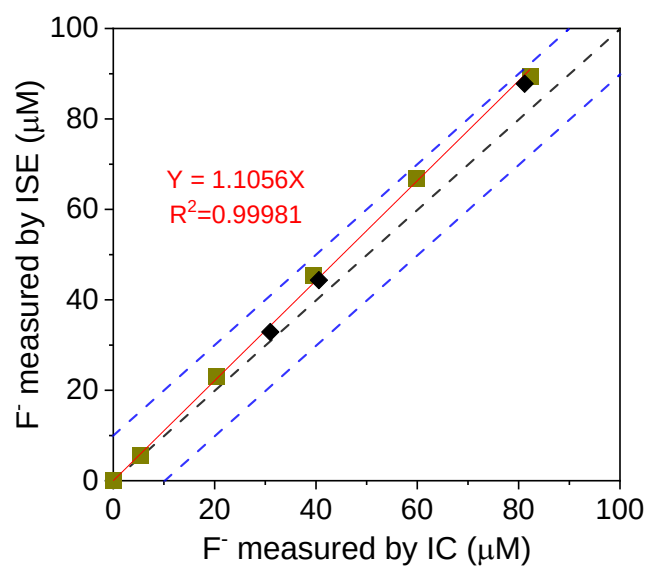


Figure C.2 Comparison of F⁻ measured by ISE and IC using mock samples (dark green) and real supernatant samples with standard addition (black). The black dash line shows the 1:1 line, the blue dash line indicates the $\pm 10\%$ variation compared to the 1:1 line, and the red line shows the linear regression (with an equation of $Y=1.1056X$) of the mock samples.

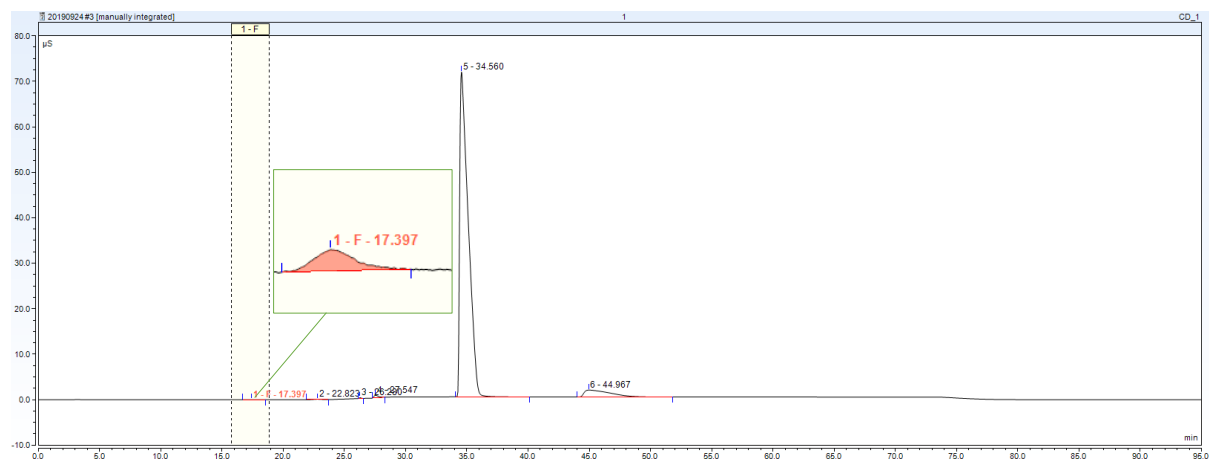


Figure C.3 A representative IC chromatogram showing all peaks detected in the culture medium matrix during the full elution period (95 min), and a zoomed-in area (in a green-box) containing the F⁻ peak at 17.4 min (the baseline used for integration was determined by connecting the start and the end points of the fluoride peak that were automatically recognized by the instrument based on the signal intensity, as indicated in red in the inserted graph).

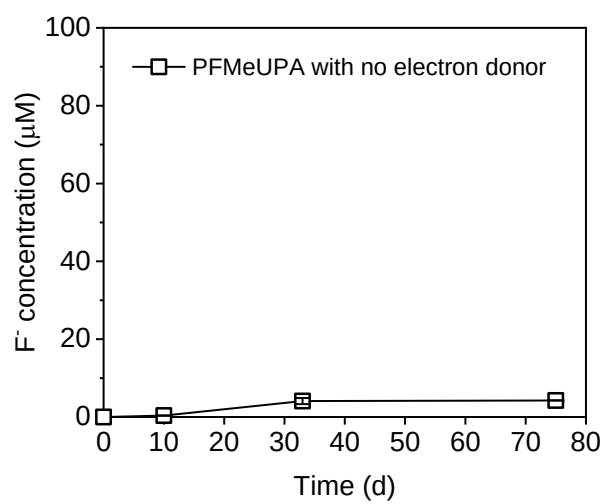


Figure C.4 Fluoride ion release from PFMeUPA without electron donor (n=3).

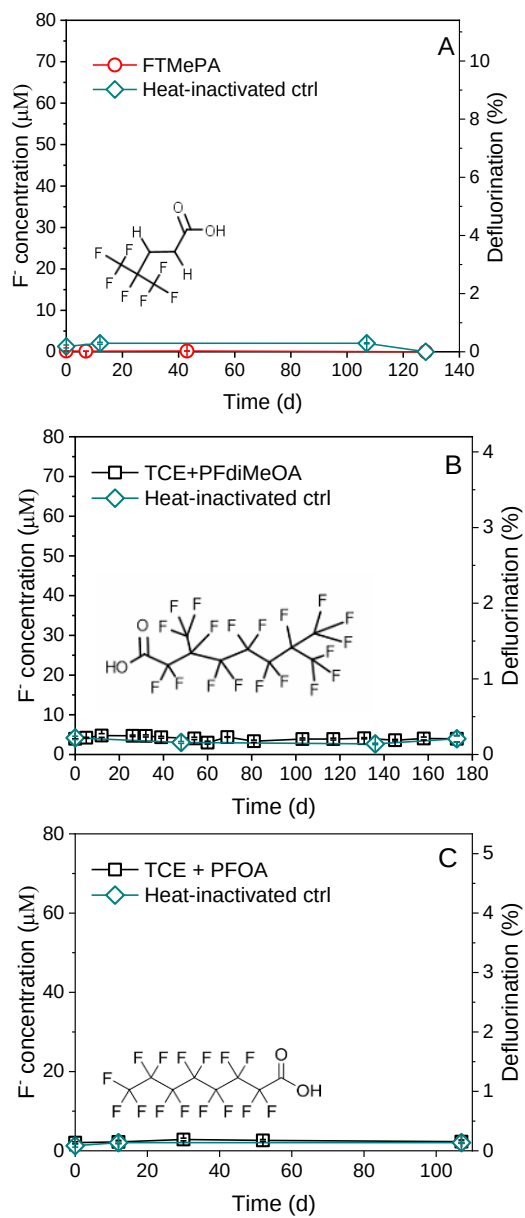
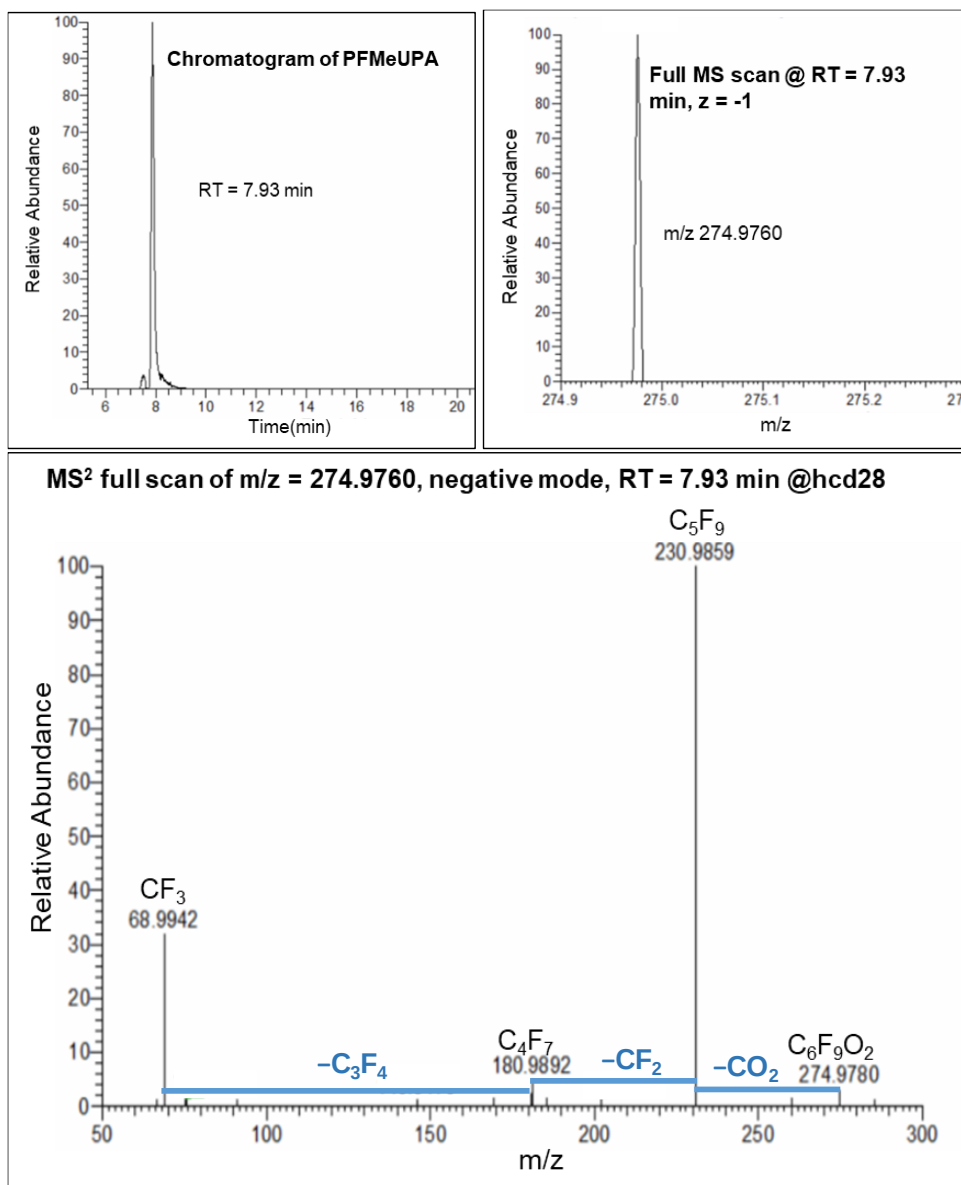


Figure C.5 Fluoride ion release from 100 μM of FTMePA (A), PFdiMeOA (B), and PFOA (C) in the dechlorinating microbial community (n=3).



Formula: C₆HF₉O₂

Structure:

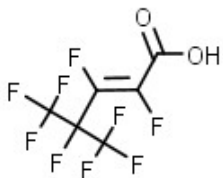
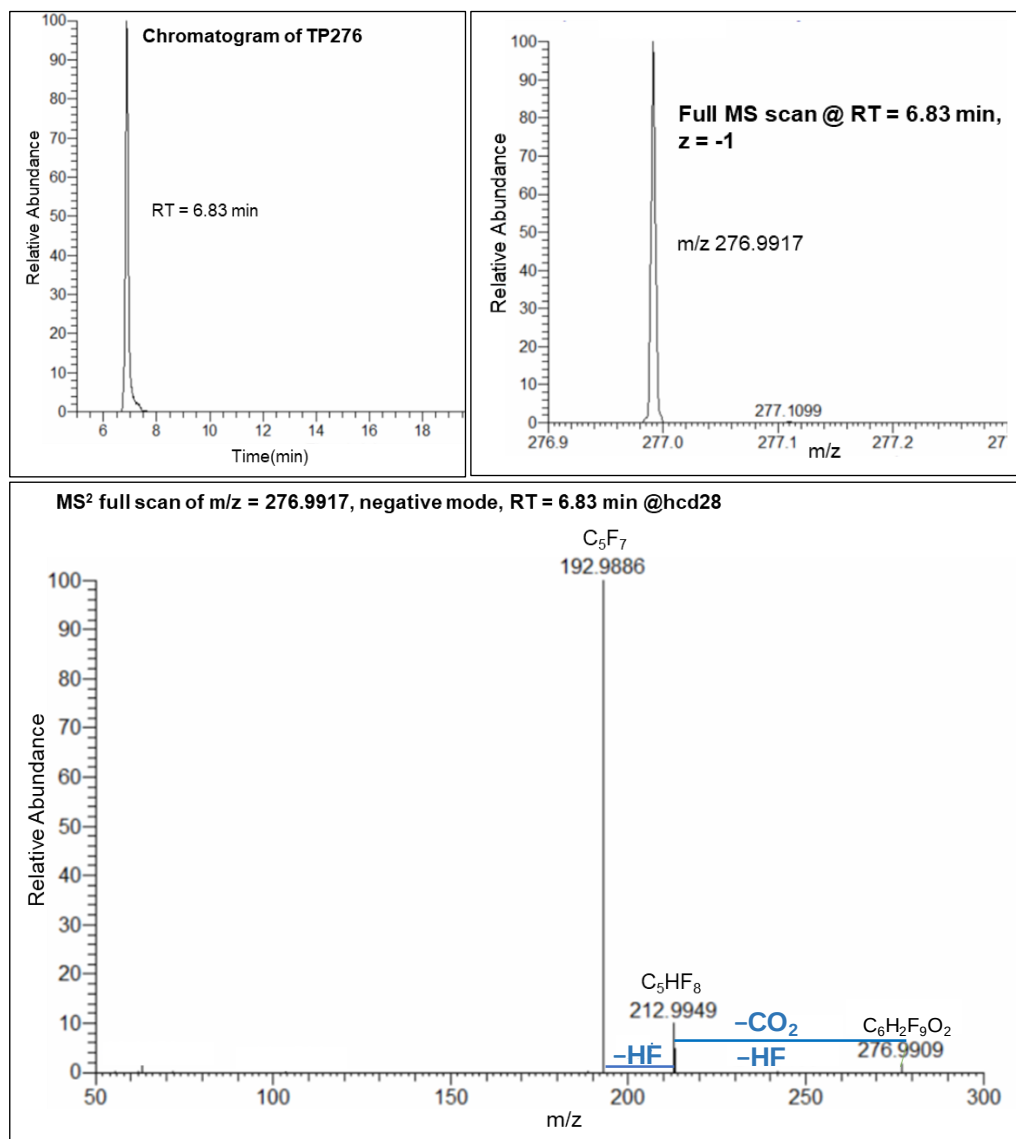


Figure C.6 MS² fragments of PFMeUPA



Formula: C₆H₃F₉O₂

Atomic Modification: +2H of PFMeUPA

Confidence level: 2b

Hypothetical Structure:

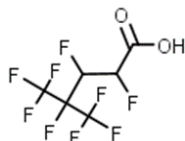
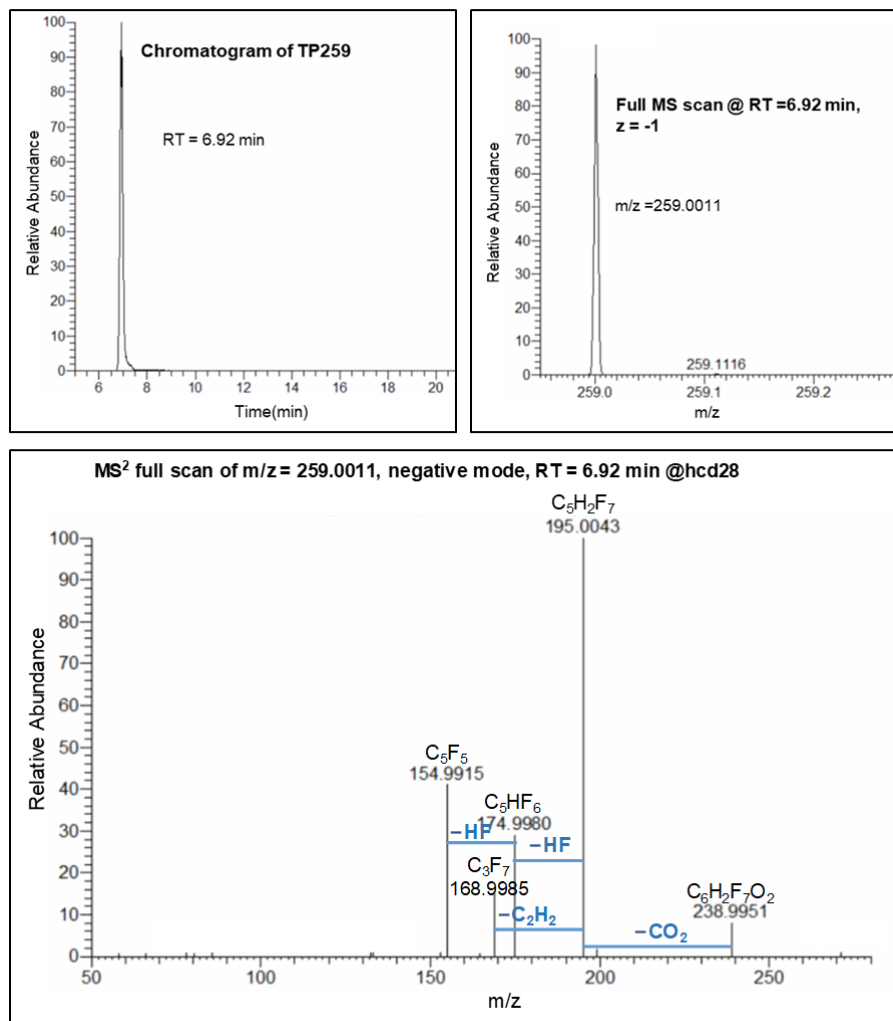


Figure C.7 TP276 structure elucidation



Formula: C₆H₄F₈O₂

Atomic Modification: -F + 3H of PFMeUPA

Confidence level: 2b

Hypothetical Structure:

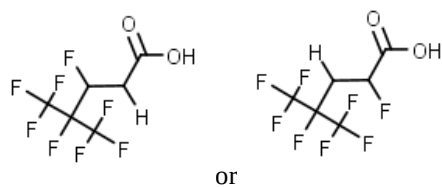
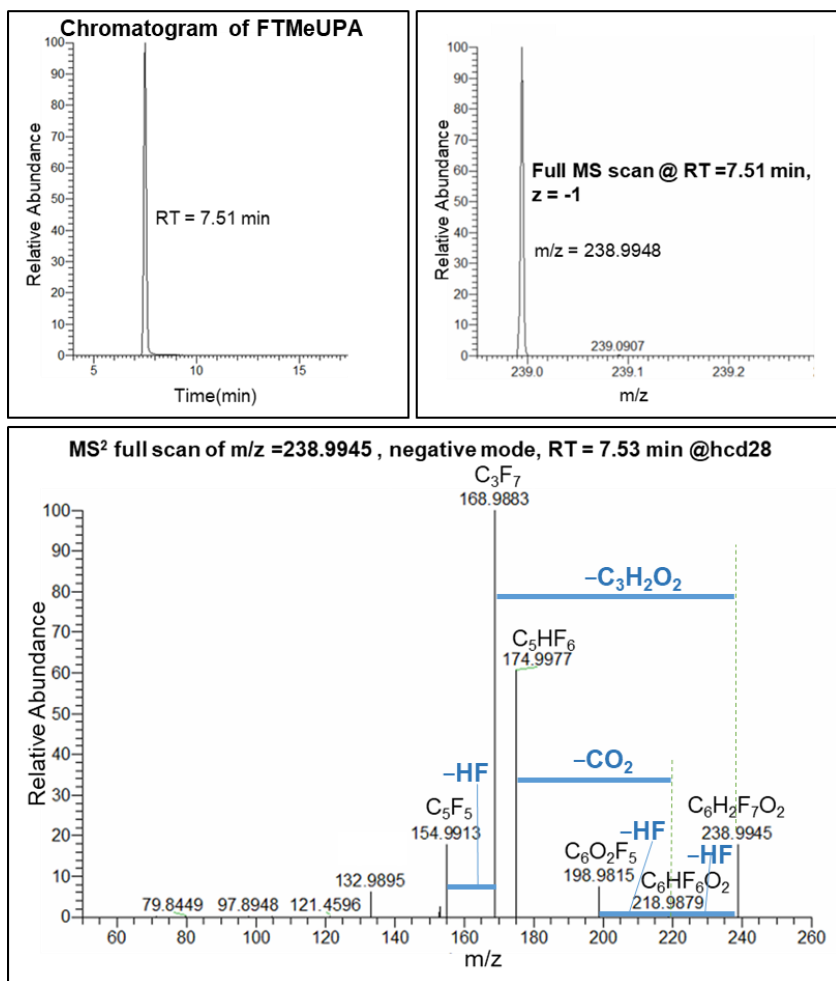


Figure C.8 TP259 structure elucidation



Formula: $C_6H_3F_7O_2$

Structure:

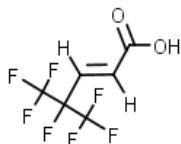
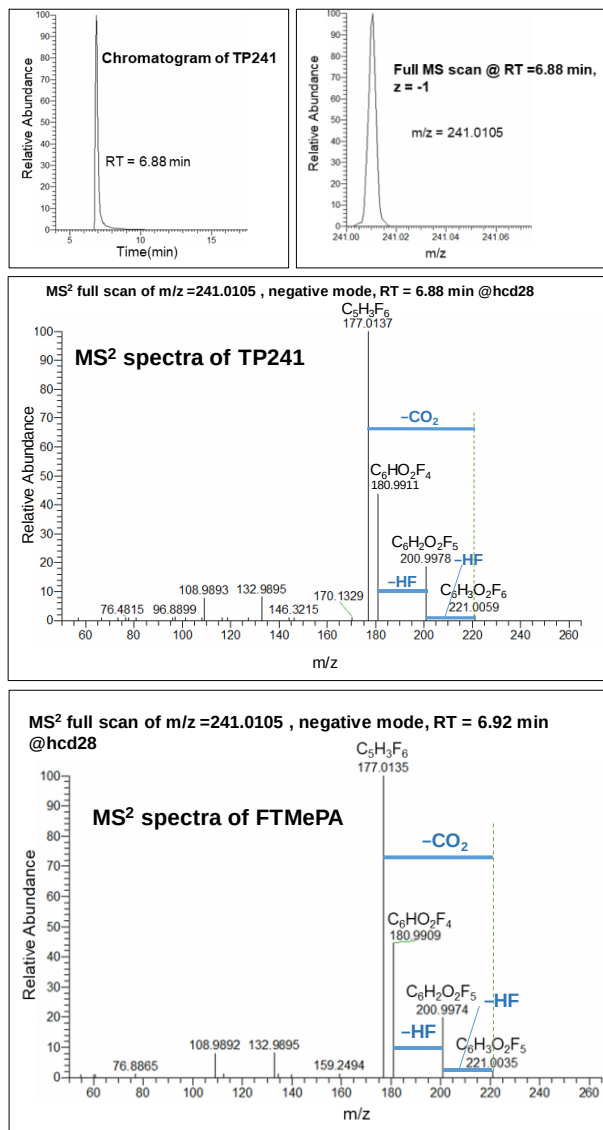


Figure C.9 MS² fragments of FTMeUPA



Formula: C₆H₅F₇O₂

Atomic Modification: +2H of FTMeUPA

Confidence level: 1

Confirmed Structure:

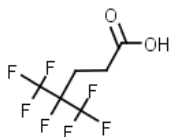
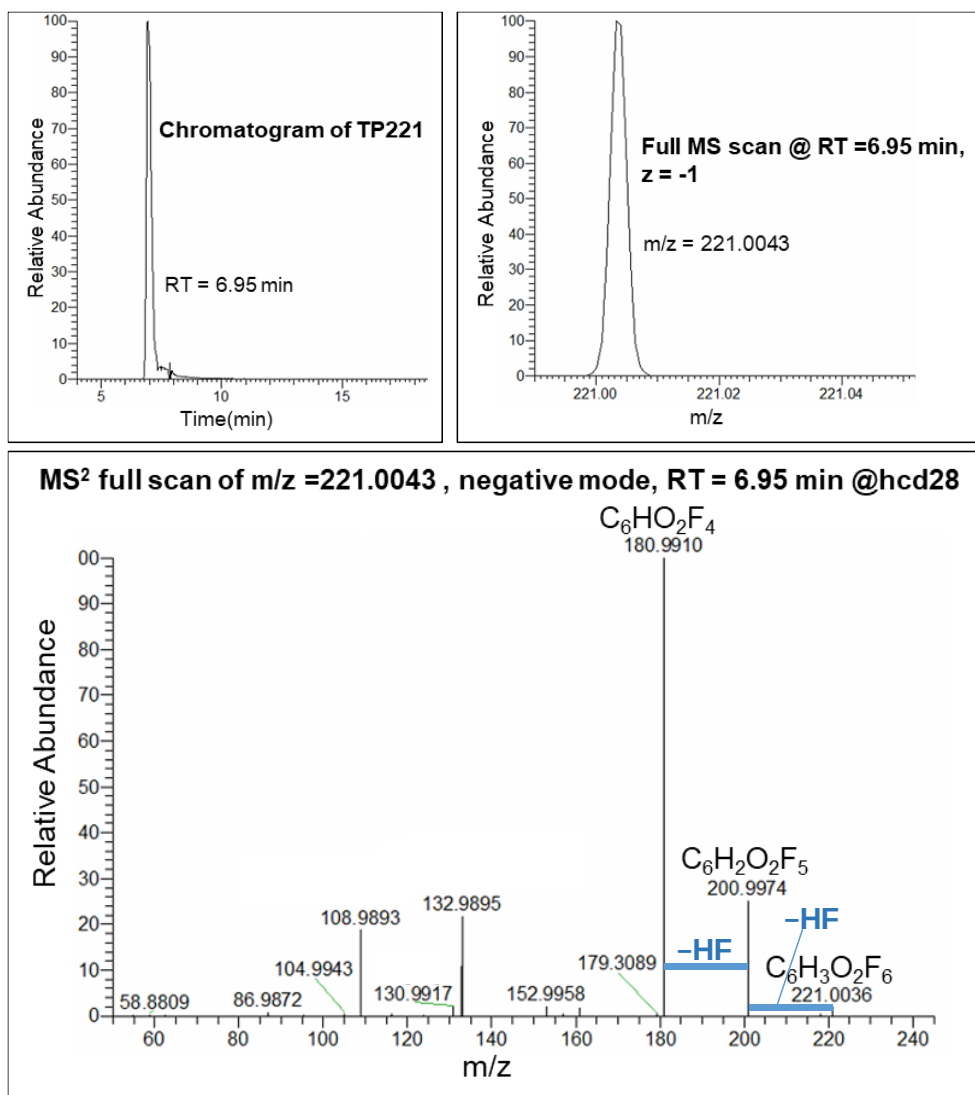


Figure C.10 TP241 structure elucidation



Formula: $C_6H_4F_6O_2$

Atomic Modification: -F +H of FTMeUPA

Confidence level: 2b

Hypothetical Structure:

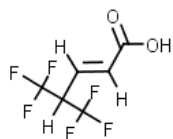


Figure C.11 TP221 structure elucidation

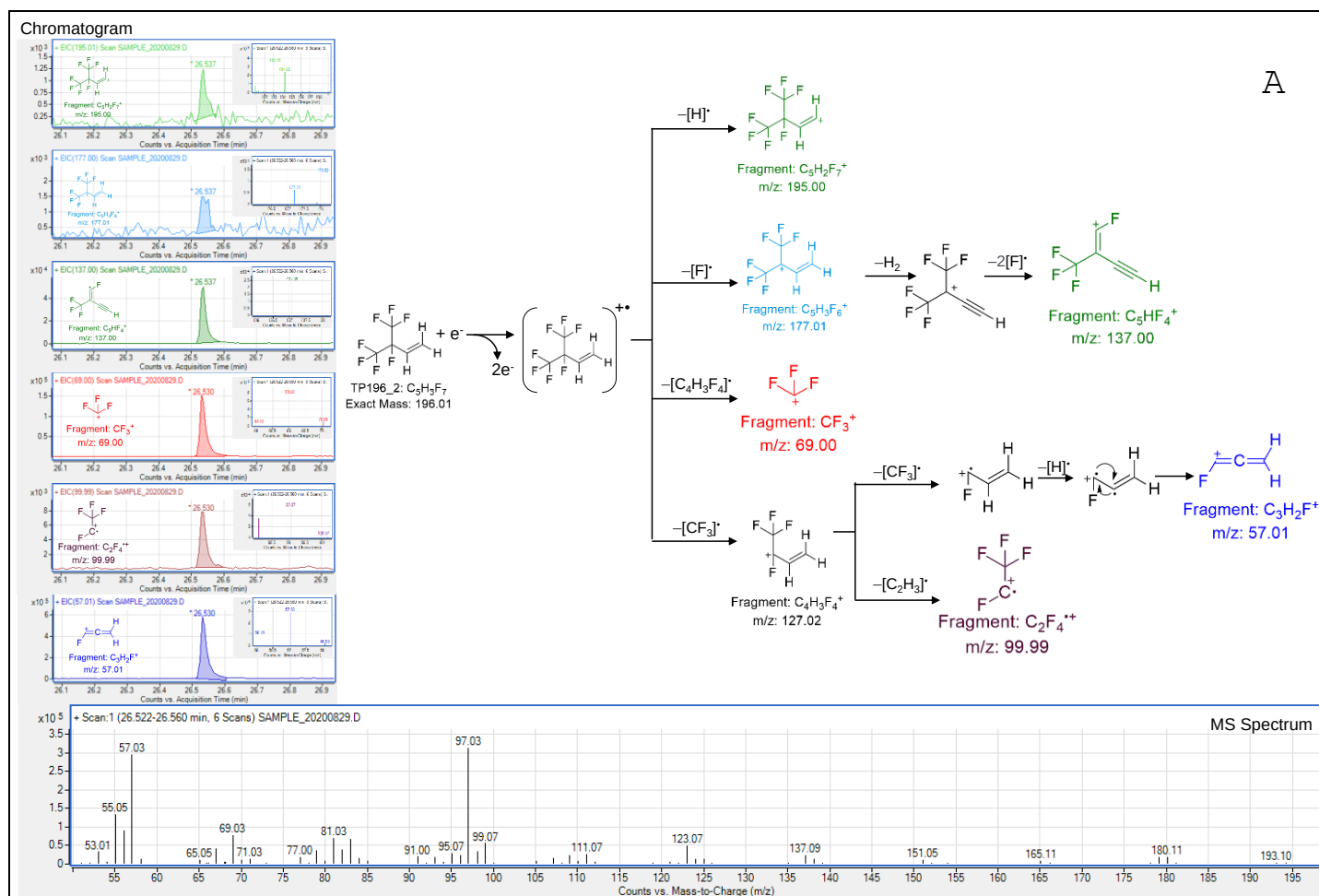


Figure C.12 Structure elucidation of gas phase transformation products (A: TP196 with the secondary C–F bond cleavage at the sp^2 carbon; B: TP196 with the secondary C–F bond cleavage at the tertiary carbon; C: TP214, the decarboxylation product of TP256). In each sub-figure, the left panel represents the extracted ion chromatograph for each detected fragment, with inserted graphs showing the corresponding mass spectra; the top-right panel shows the interpretation of fragmentation pathways; the bottom panel shows the full-scan mass spectrum. Carboxylic acids transformation products, i.e., TP256 and TP259 were also detected on GC-MS but at a different RT, which were not shown in this figure.

Figure C.12 Continued.

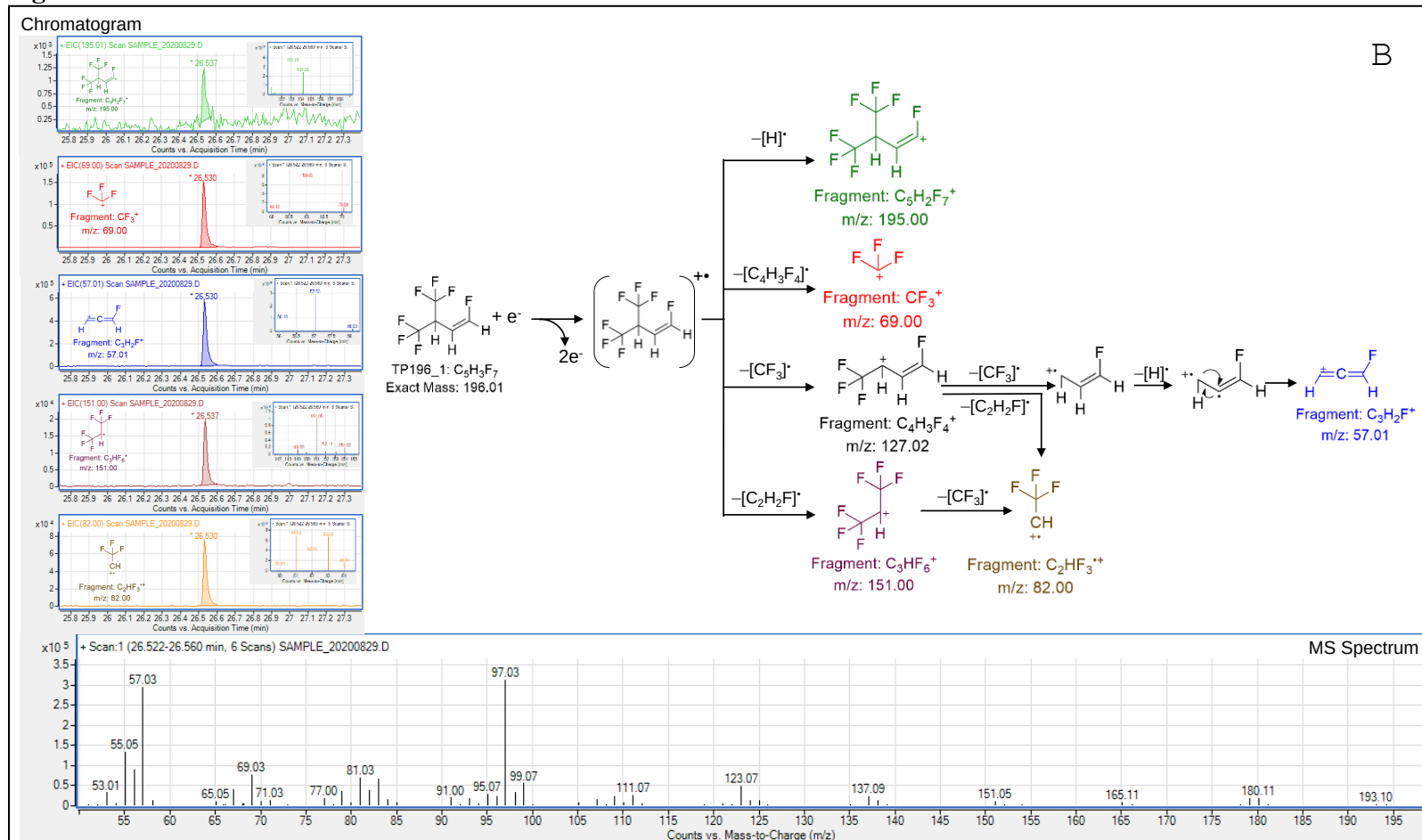
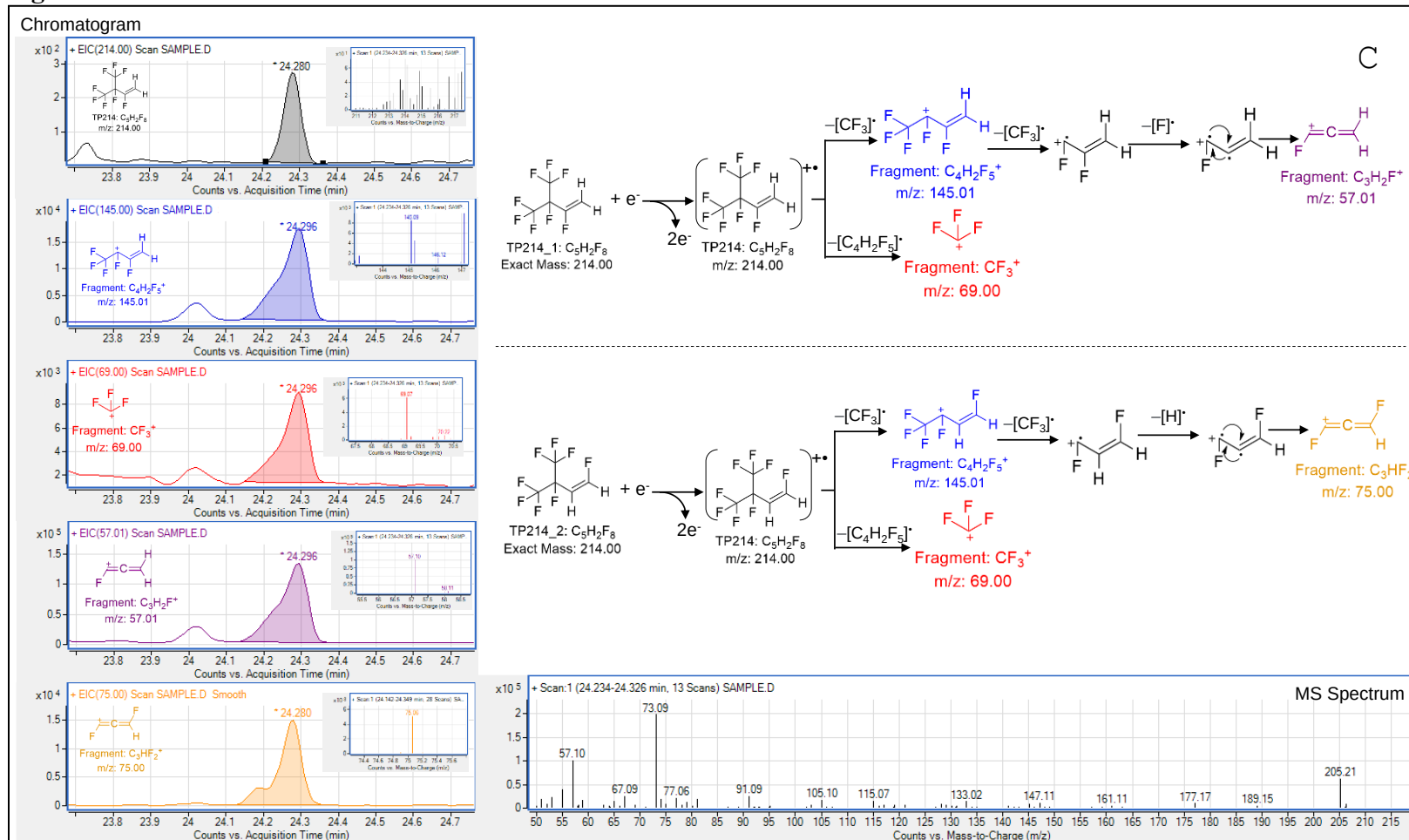


Figure C.12 Continued.



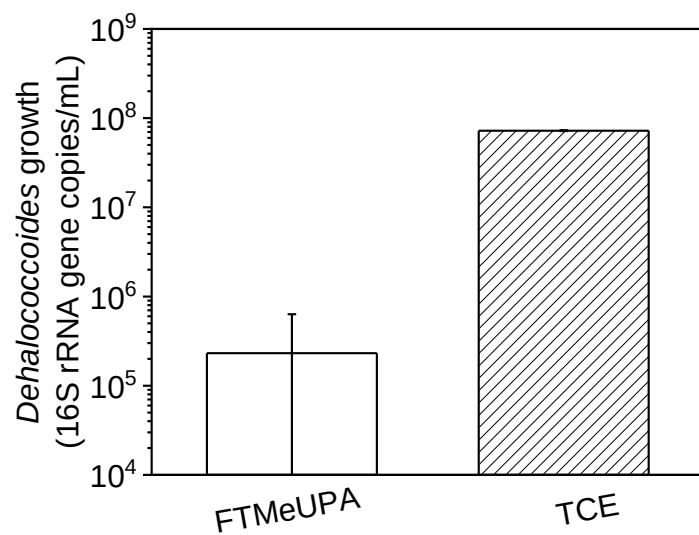


Figure C.13 The growth of *Dehalococcoides* spp. after 70 days in FTMeUPA-fed cultures compared to TCE-fed ones (n=3).

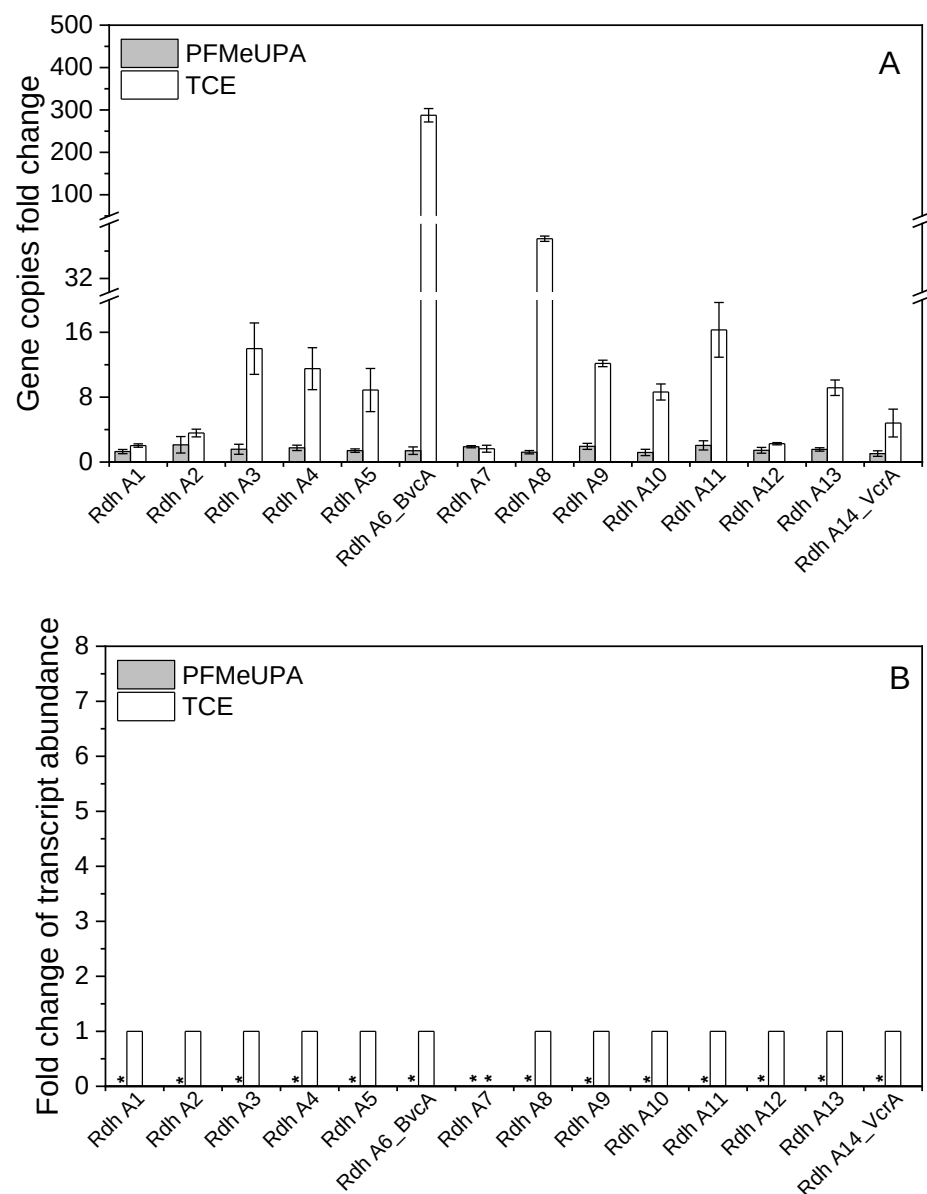


Figure C.14 Fold changes (91d/0d) of reductive dehalogenase gene (Rdh) copy numbers (A) and relative expression levels (gene transcripts) in PFMeUPA-added samples in comparison to those in TCE-added samples on 77d (B) (16S rRNA gene is the reference gene; n=3; *: no gene expression).

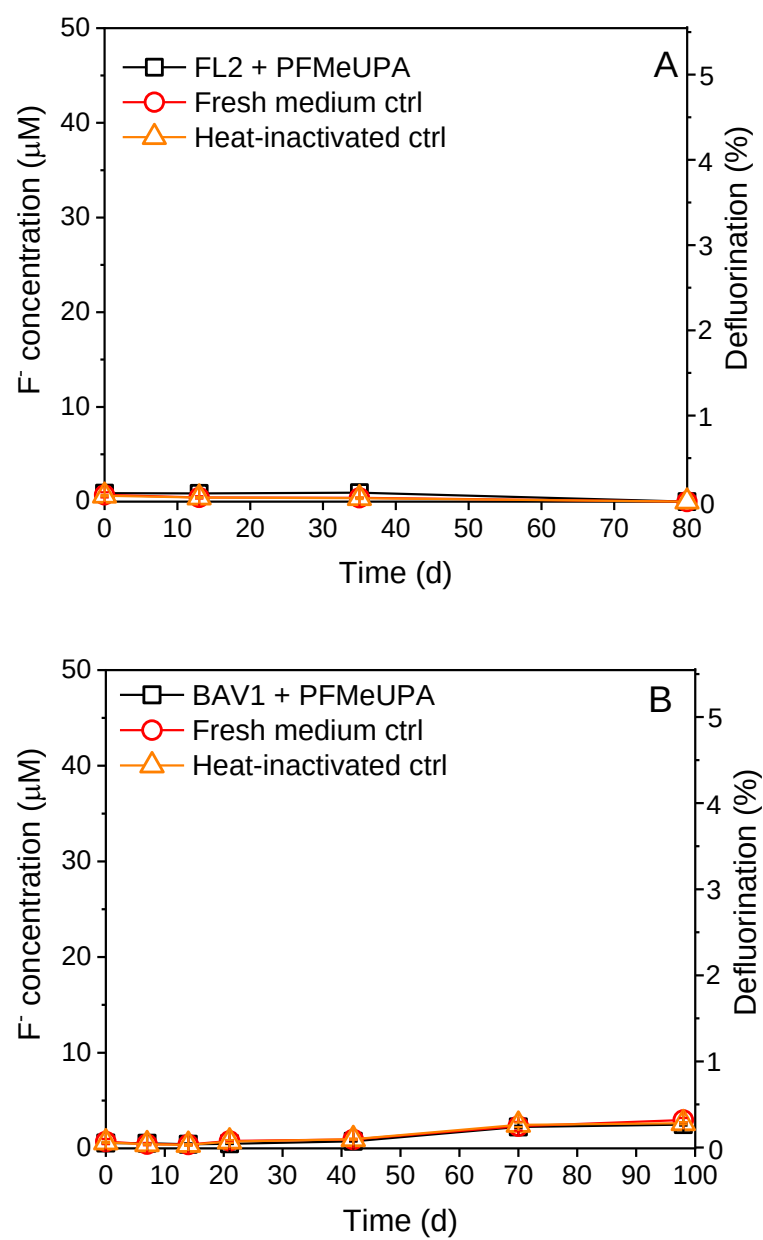


Figure C.15 Biotransformation of PFMeUPA by *D. mccartyi* FL2 (A) and BAV1 (B) (n=3).

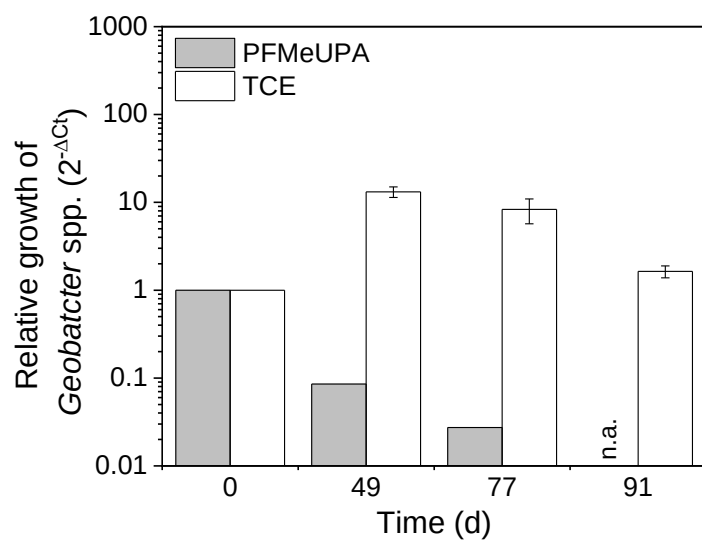


Figure C.16 Relative growth of *Geobacter* spp. in PFMeUPA-fed cultures and TCE-fed ones in terms of the fold-change of 16S rRNA gene copy numbers compared to day 0. (n.a.: no amplification).

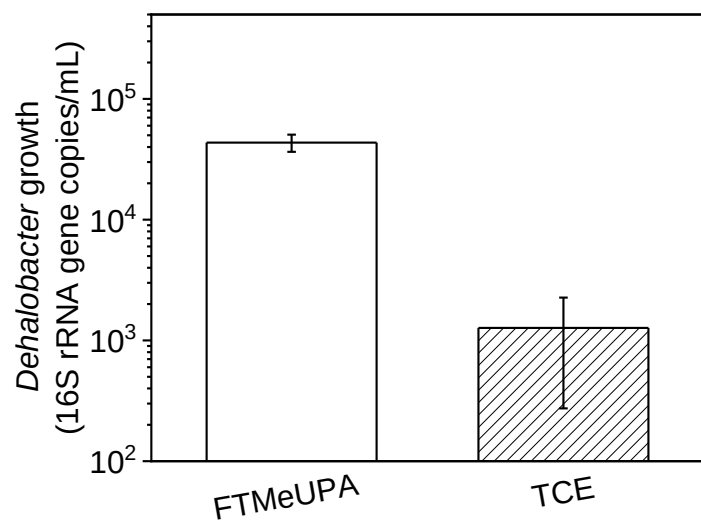


Figure C.17 The growth of *Dehalobacter* spp. after 70 days in FTMeUPA-fed cultures compared to TCE-fed ones (n=3).

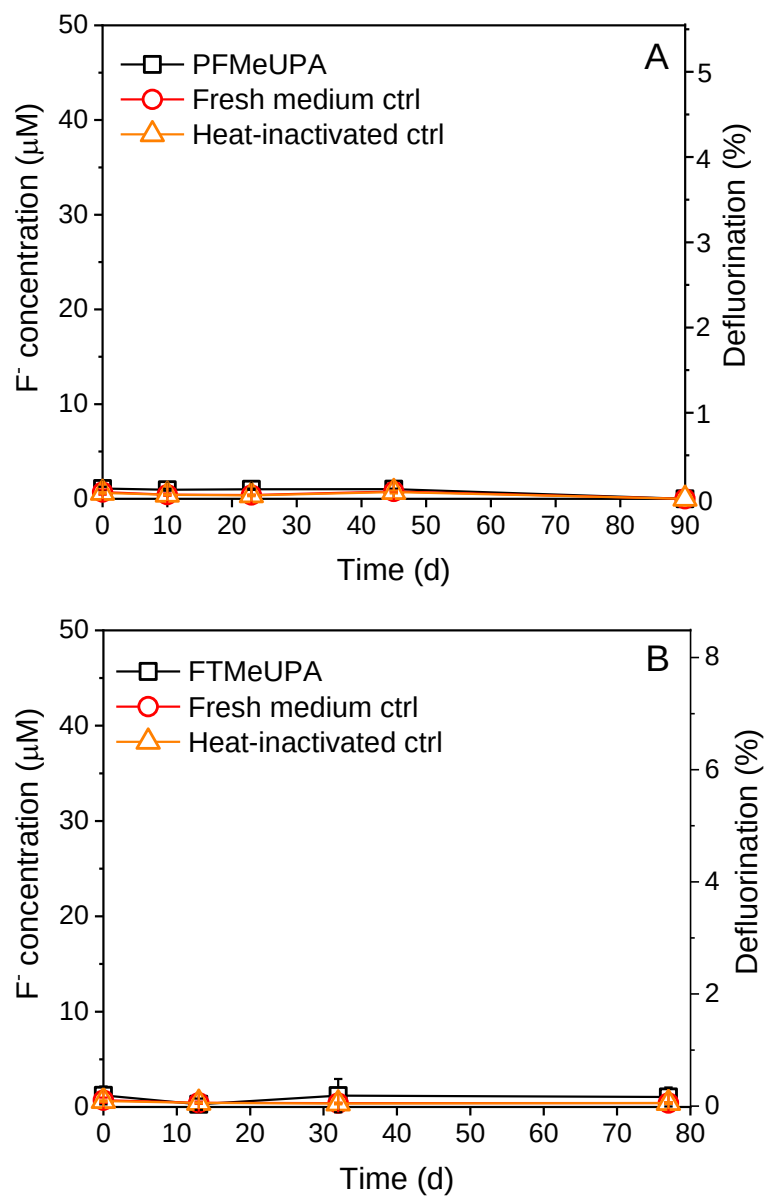


Figure C.18 Biotransformation of PFMeUPA (A) and FTMeUPA (B) by *Dehalobacter restrictus* (n=3).

APPENDIX D: SUPPLEMENTARY MATERIAL FOR CHAPTER 5

D.1 Supplementary Figures and Table

Table D.1 Standard compound information

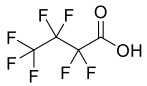
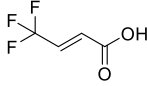
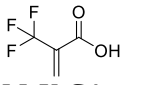
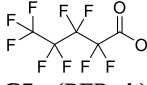
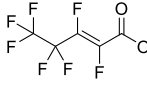
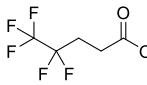
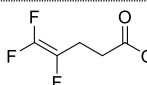
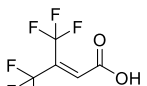
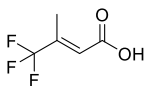
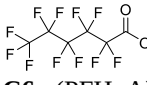
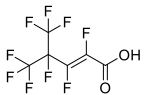
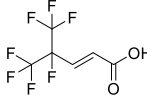
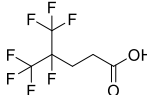
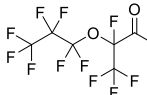
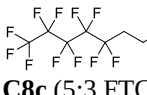
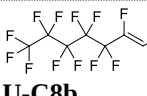
Structure (acronym)	PFAS Standard Information					LOQ (μ M)
	CAS	Formula	Purity	[M-H] ⁻	RT (min)	
 C4a (PFBA)	375-22-4	C ₄ HF ₇ O ₂	98%	212.9786	5.06	0.025
 U-C4b (1:3 U acid)	71027-02-6	C ₄ H ₃ F ₃ O ₂	97%	139.0006	2.36	0.05
 MeU-C4c	381-98-6	C ₄ H ₃ F ₃ O ₂	98%	139.0006	1.76	1
 C5a (PFPeA)	2706-90-3	C ₅ HF ₉ O ₂	98%	262.9754	6.17	0.05
 U-C5a	103229-91-0	C ₅ HF ₇ O ₂	97%	224.9786	5.73	0.1
 C5b (2:3 FTCA)	3637-31-8	C ₅ H ₅ F ₅ O ₂	95%	191.0131	4.95	0.2
 U-C5c	110003-22-0	C ₅ H ₅ F ₃ O ₂	97%	153.0163	2.60	5
 MeU-C5d	1763-28-6	C ₅ H ₂ F ₆ O ₂	97%	206.9880	4.86	0.5
 MeU-C5e	69056-67-3	C ₅ H ₅ F ₃ O ₂	97%	153.0163	3.54	0.2
 C6a (PFHxA)	307-24-4	C ₆ HF ₁₁ O ₂	97%	312.9722	6.58	0.025

Table D.1 Continued

Structure (acronym)	PFAS Standard Information					LOQ (μ M)
	CAS	Formula	Purity	[M-H] ⁻	RT (min)	
 MeU-C6a (PFMeUPA)	103229-89-6	C ₆ HF ₉ O ₂	97%	274.9754	6.37	0.5
 MeU-C6b (FTMeUPA)	243139-64-2	C ₆ H ₃ F ₇ O ₂	97%	238.9943	6.20	0.5
 Me-C6b (FTMePA)	243139-62-0	C ₆ H ₅ F ₇ O ₂	97%	241.0099	6.09	0.5
 MeO-C6c (GenX)	13252-13-6	C ₆ HF ₁₁ O ₃	97%	328.9671	6.73	1
 C8c (5:3 FTCA)	914637-49-3	C ₈ H ₅ F ₁₁ O ₂	97%	341.0035	7.04	0.2
 U-C8b (6:2 FTUCA)	70887-88-6	C ₈ H ₂ F ₁₂ O ₂	95%	356.9784	7.06	0.5

Note: U: unsaturated; Me: with branches (–CF₃ or –CH₃); **MeU-C6b** and **U-C5a** are the corresponding unsaturated structures of **Me-C6b** and **C5a**, respectively.

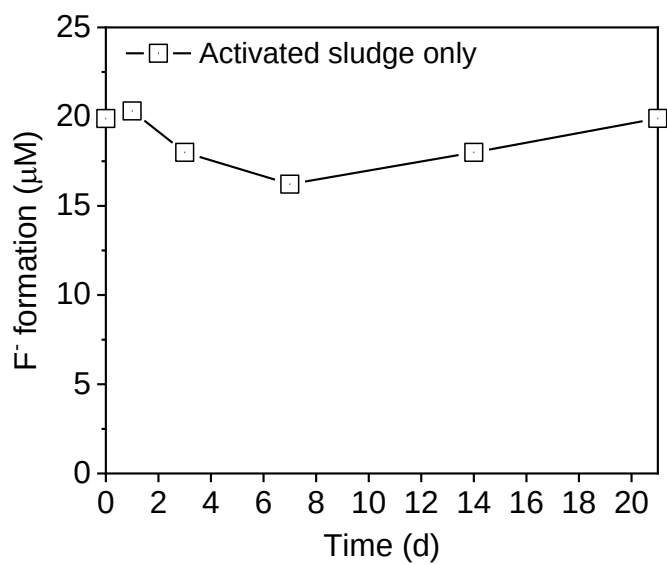
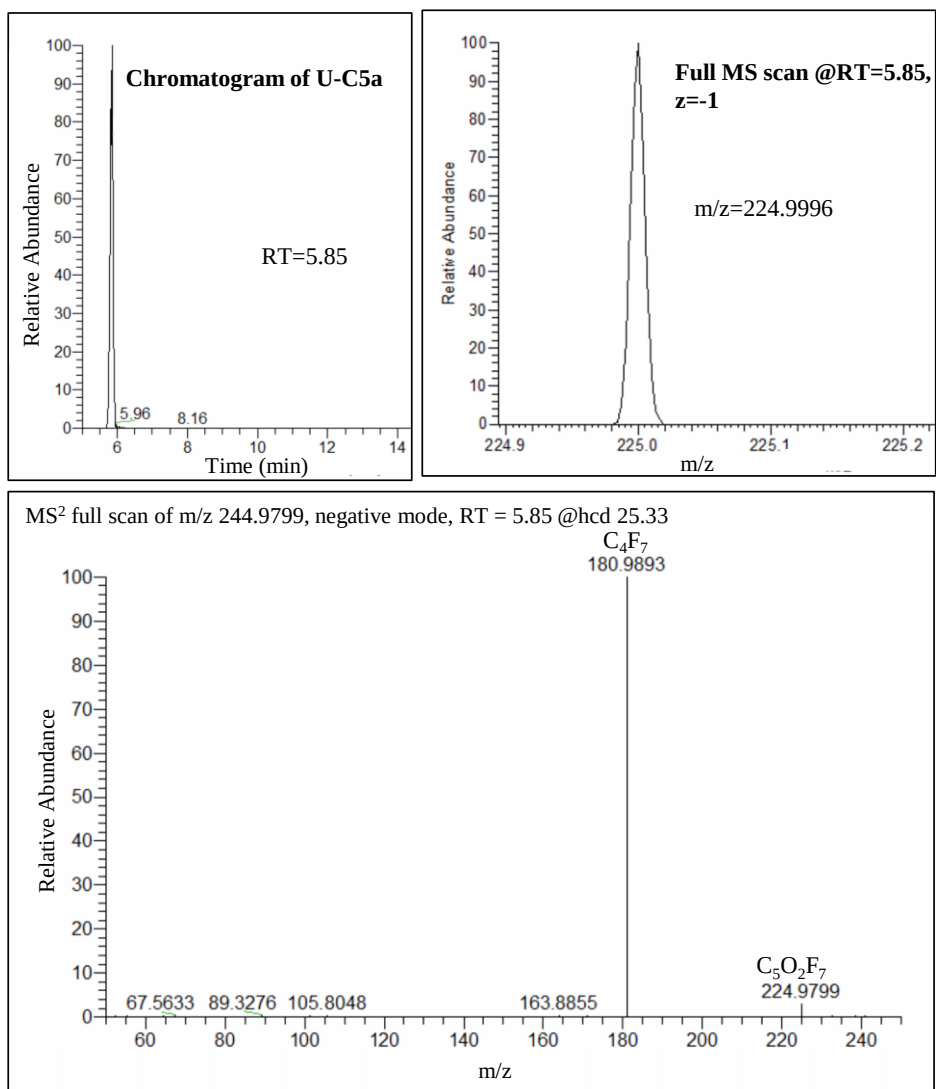


Figure D.1 fluoride release in activated sludge biomass-only control.



Formula: C₅HO₂F₇

Compound Structure:

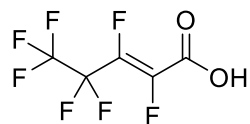
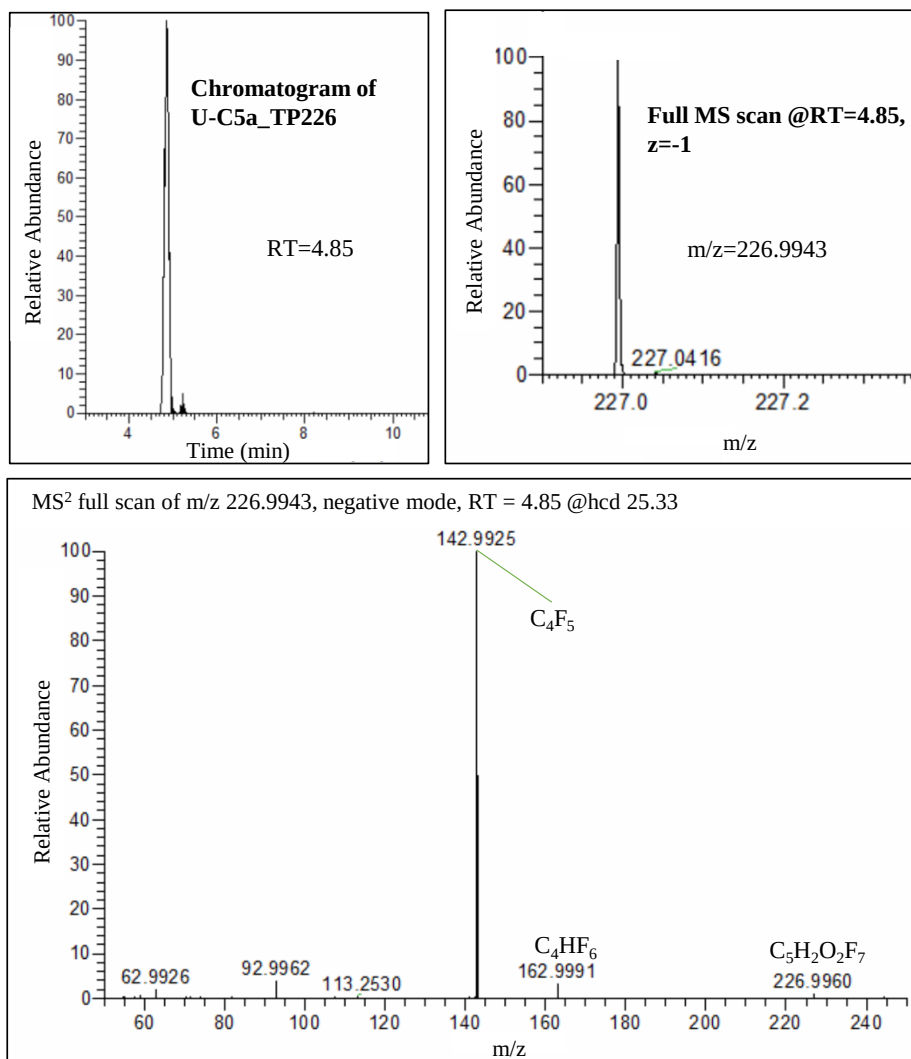


Figure D.2 MS² fragments of **U-C5a** standard.



Formula: C₅H₃O₂F₇

Atomic Modification: +2H of U-C5a

Confidence level: 2b

Hypothetical structure:

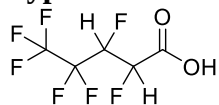
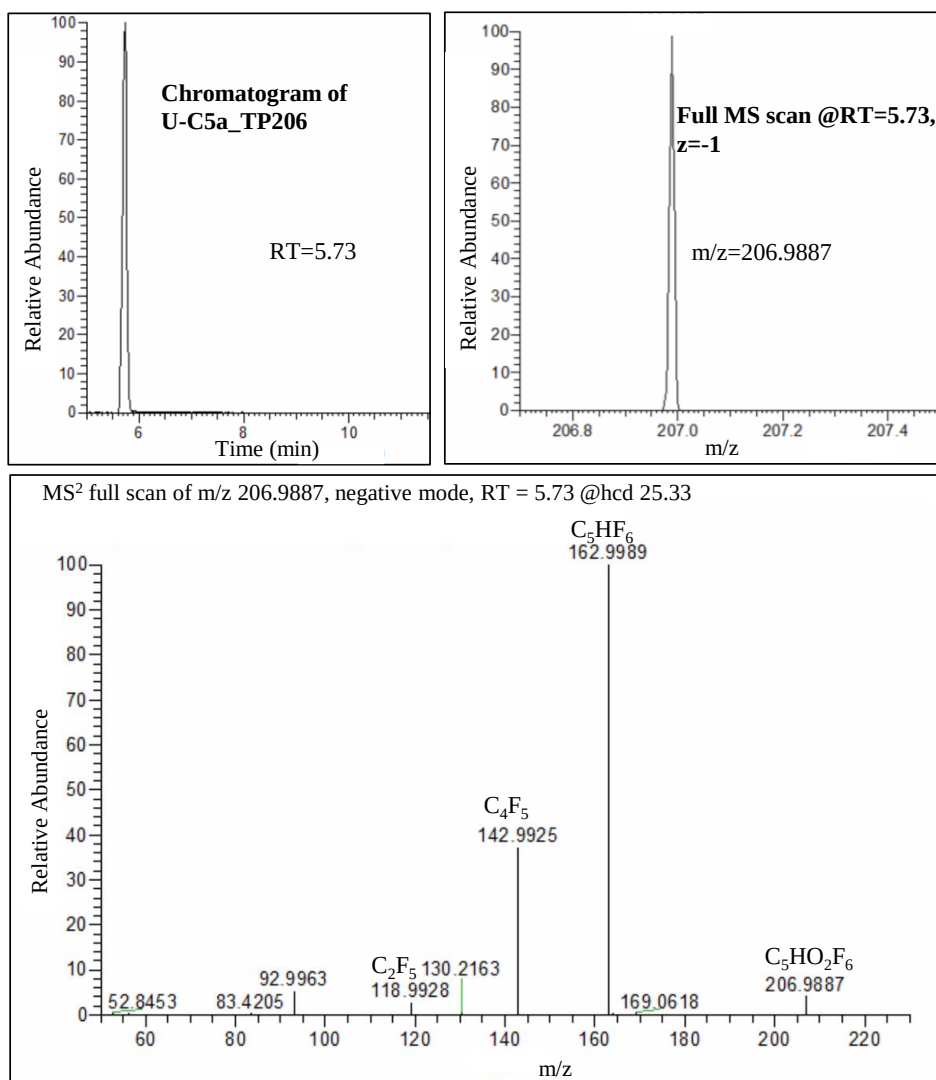


Figure D.3 U-C5a_TP226 structure elucidation.



Formula: C₅H₂O₂F₆

Atomic Modification: -F+H of U-C5a

Confidence level: 2b

Hypothetical structure:

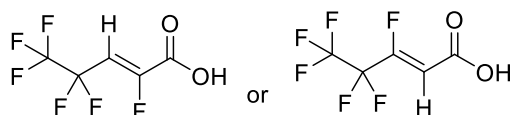
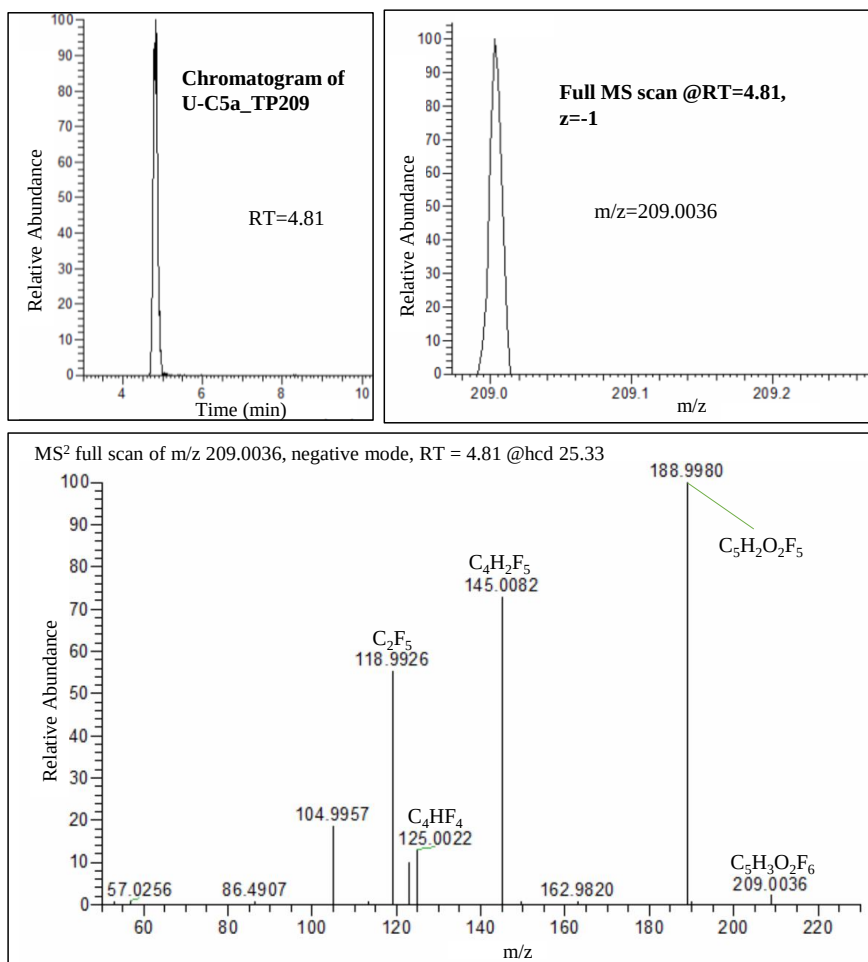


Figure D.4 U-C5a_TP206 structure elucidation.



Formula: C₅H₄O₂F₆

Atomic Modification: +2H of U-C5a_TP206

Confidence level: 2b

Hypothetical structure:

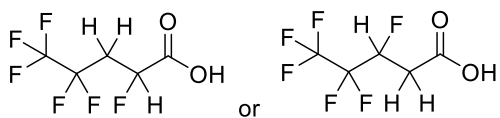
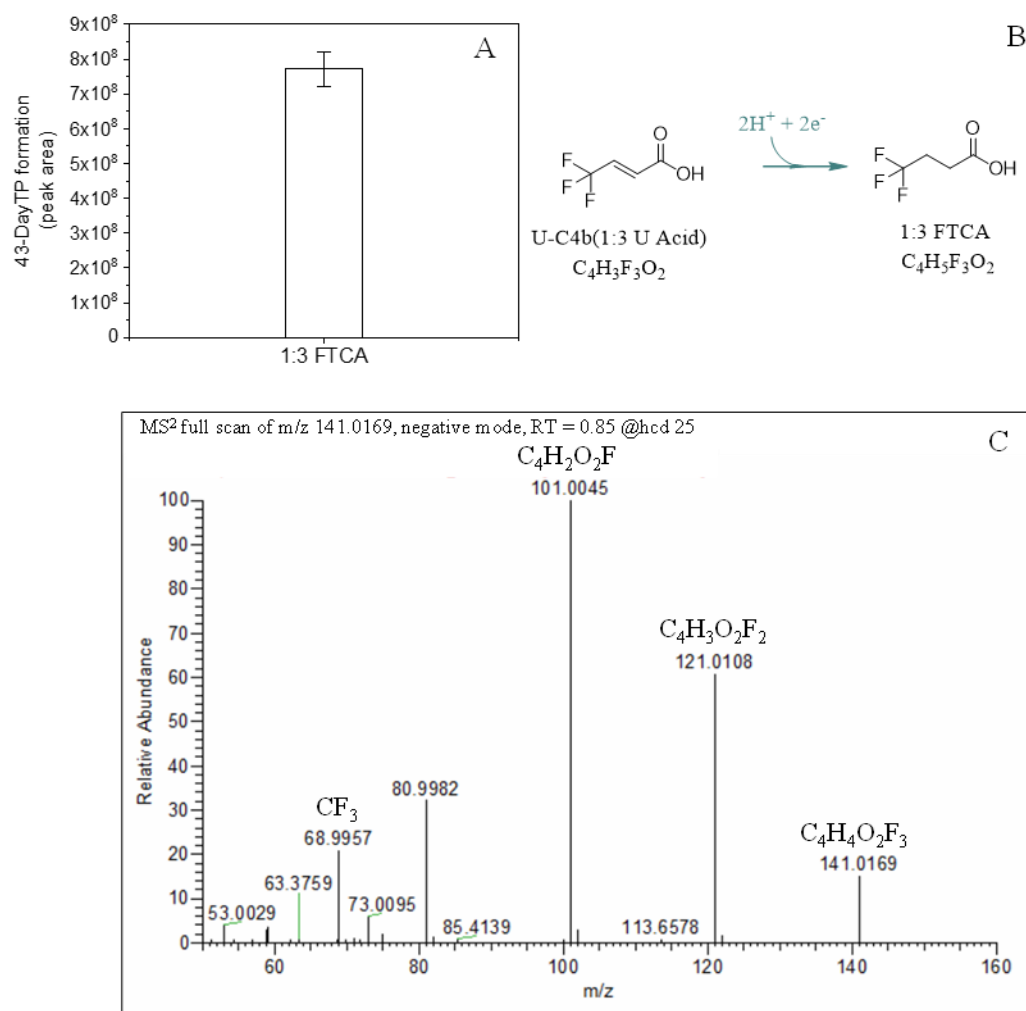


Figure D.5 U-C5a_TP209 structure elucidation.

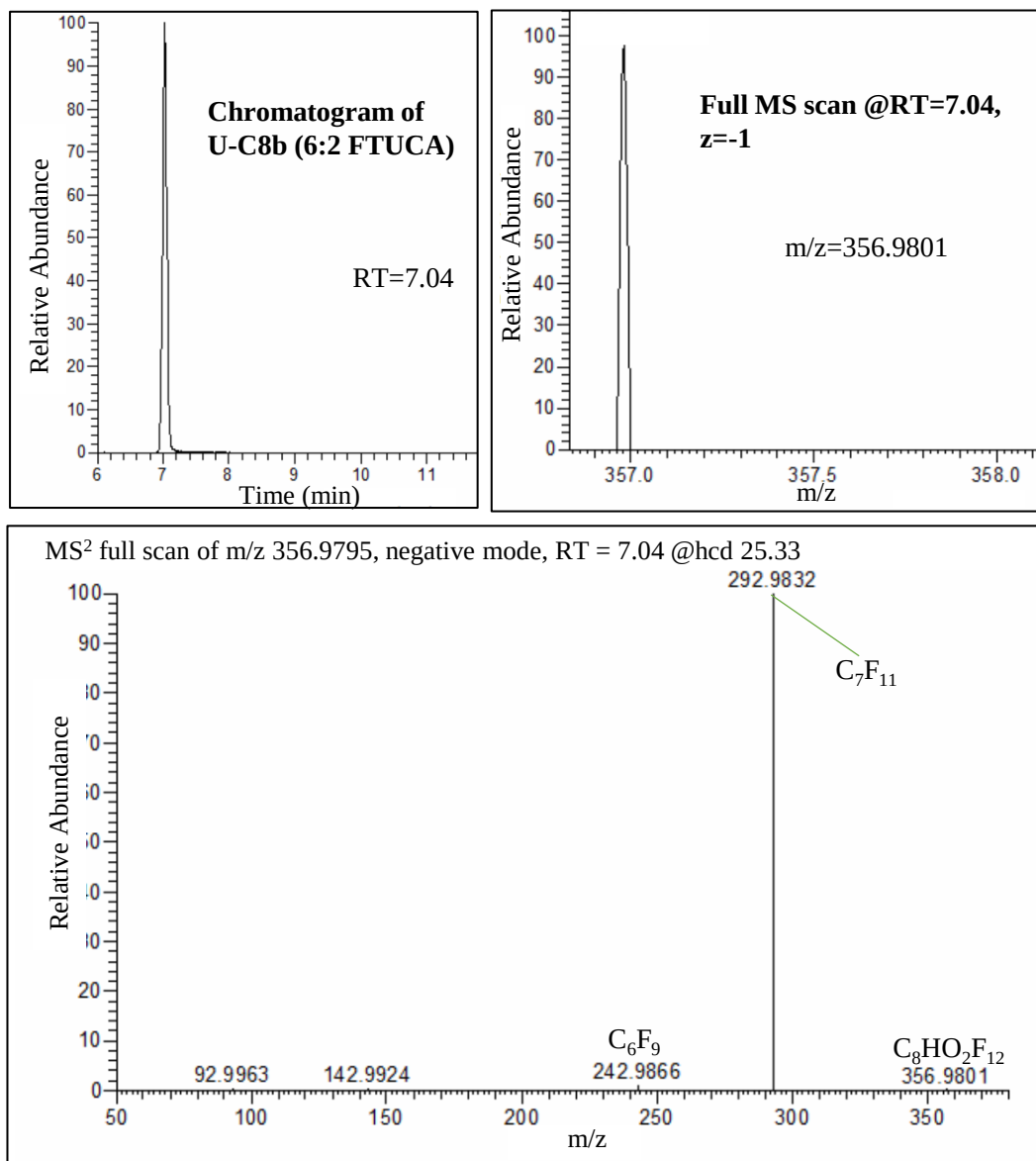


Formula: $C_4H_5O_2F_3$

Atomic Modification: +2H of U-C4b

Confidence level: 2b

Figure D.6 Formation of **U-C4b** (1:3 U acid) hydrogenation product (i.e., 1:3 TFCA) (A); proposed **U-C4b** biotransformation pathway (B); MS² fragments of the hydrogenation product 1:3 FTCA. (Note: **U-C4b** was 100% biotransformed into 1:3 FTCA, which was quantified using corresponding analytical standards)



Formula: C₈H₂O₂F₁₂

Compound Structure:

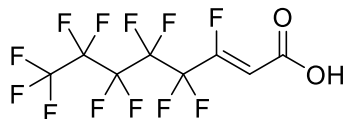


Figure D.7 MS² fragments of **U-C8b** standard.

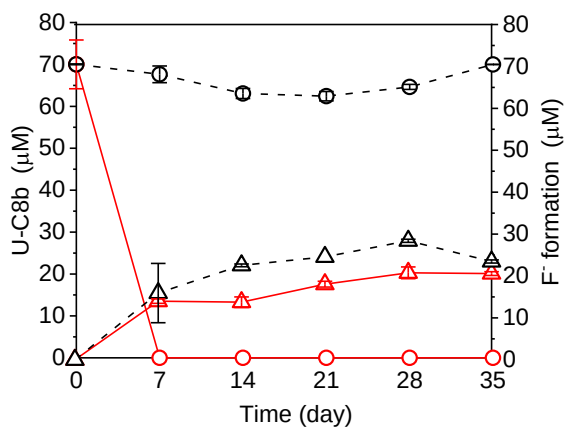
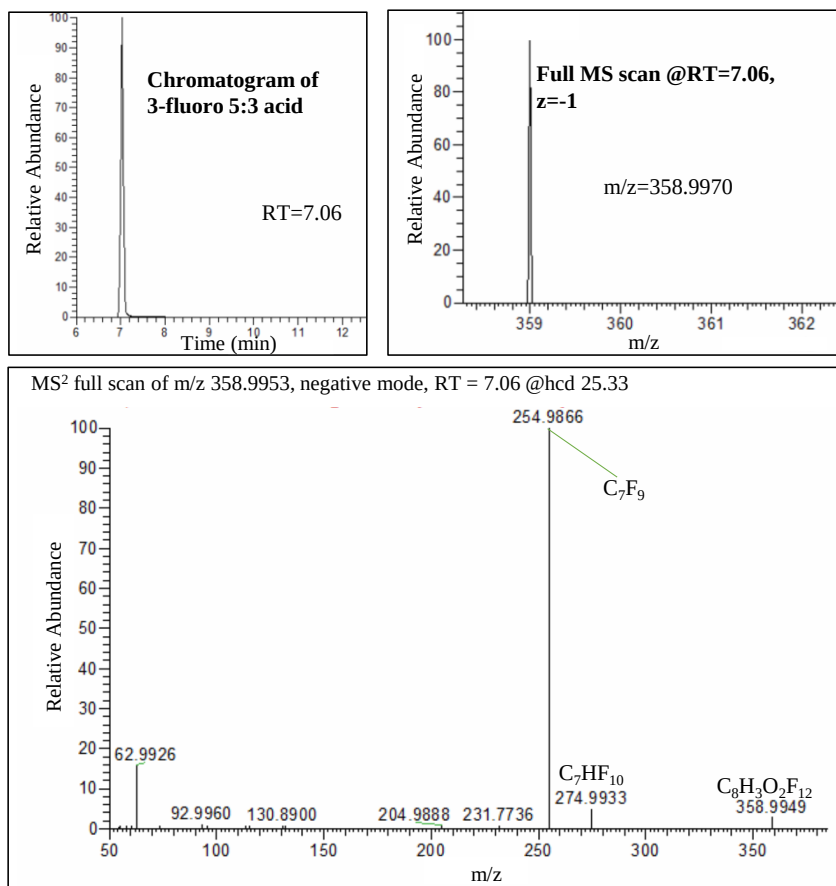


Figure D.8 U-C8b (6:2 FTUCA) removal and fluoride release in the anaerobic defluorinating enrichment (red: biological samples; black: heat-inactivated controls; circles: parent compound; triangles: fluoride formation; n=3) (Note: F⁻ detection in heat-inactivated controls was attributed to the impurities defluorination).



Formula: C₈H₄O₂F₁₂

Atomic Modification: +2H of U-C8b

Confidence level: 2b

Hypothetical structure:

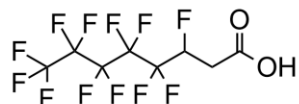


Figure D.9 3-fluoro 5:3 acid structure elucidation.

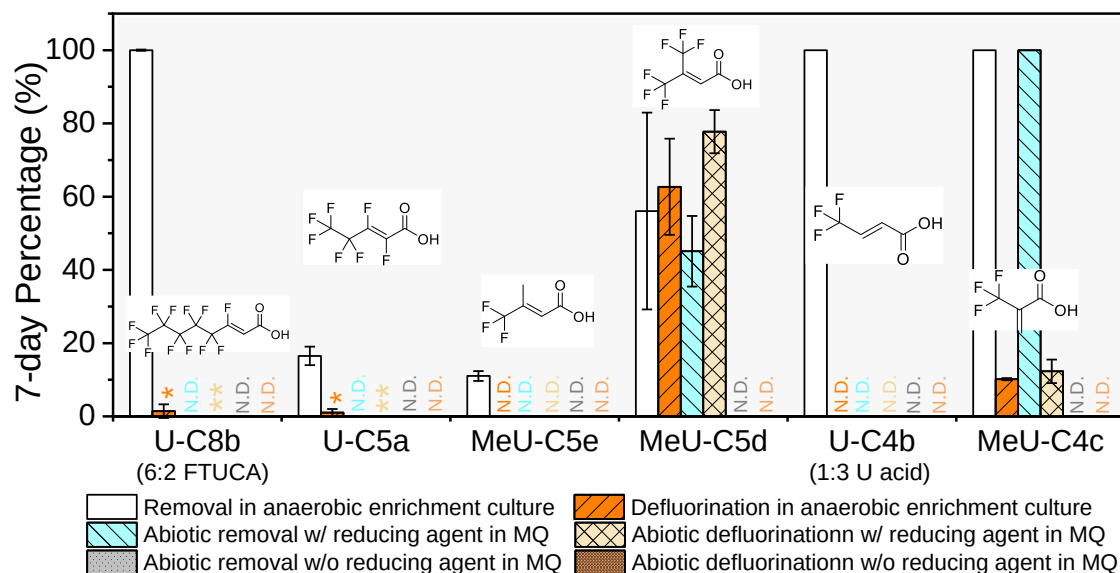


Figure D.11 The removal and defluorination degree of **MeU-C4c** and **MeU-C5d** compared to other selected unsaturated structures under different anaerobic conditions (n=3) (*: due to F^- release from impurities, the defluorination degrees of **U-C8b** and **U-C5a** were calculated according to the formation of the defluorination TP 5:3 FTCA and the F^- detected in the biological samples subtracted the F^- released in the heat-inactivated control, respectively; **: there was F^- release under this condition, which was attributed to the abiotic defluorination of impurities but not the target parent compound as the parent compound showed no removal).

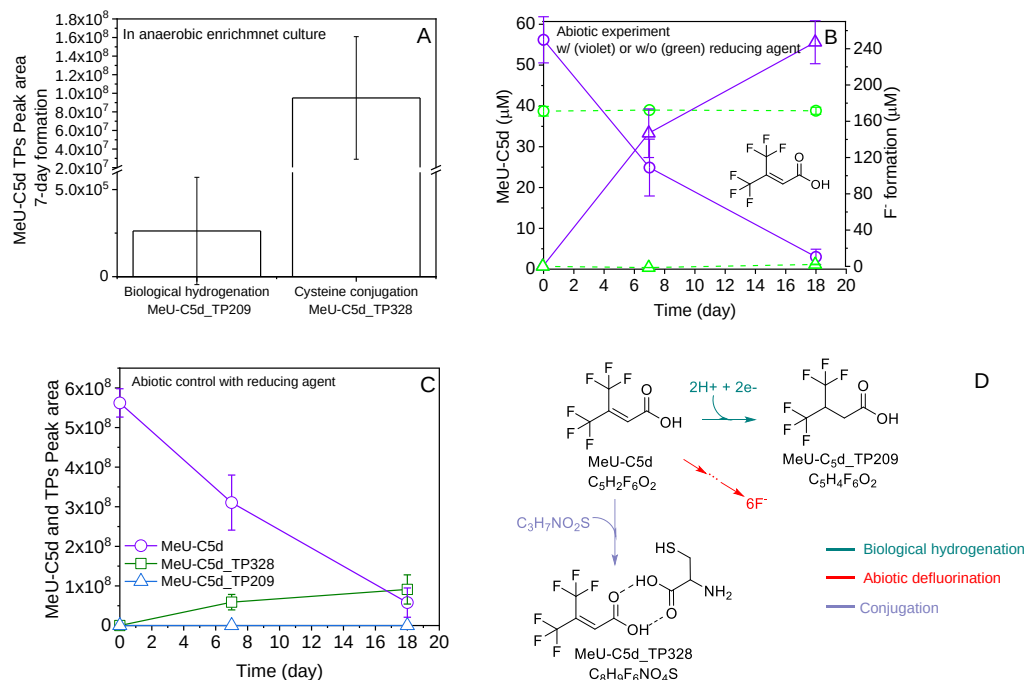
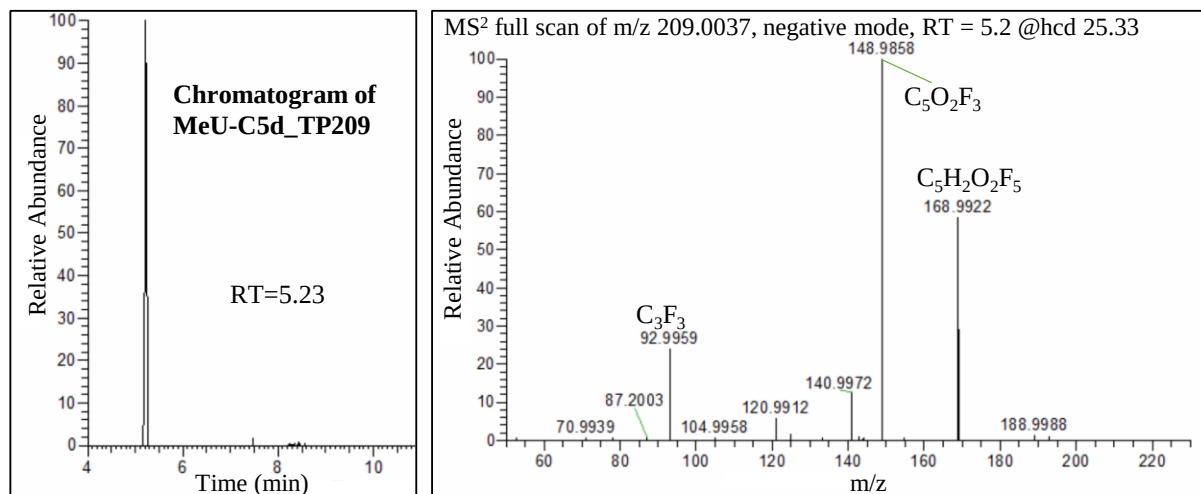


Figure D.12 7-day formation of **MeU-C5d** hydrogenation product and cysteine conjugation product in the anaerobic enrichment culture (A); **MeU-C5d** removal and fluoride release in biomass free Milli-Q water (B) (violet: abiotic samples with the reducing agent, cysteine and sulfide; green: abiotic samples without the reducing agent; circles: parent compound; triangles: fluoride); the temporal trend (peak areas) of the parent compound and the TPs during abiotic transformation of **MeU-C5d** (C); proposed transformation pathways of **MeU-C5d** (D). (Note: the hydrogenation product **MeU-C5d**_TP209 was only identified in the enrichment culture; n=3).



Formula: C₅H₄O₂F₆

Atomic Modification: +2H of MeU-C5d

Confidence level: 3

Hypothetical structure:

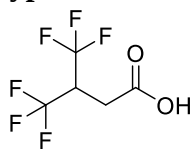


Figure D.14 MeU-C5d_TP209 structure elucidation. (Note: the mass spectrum of m/z=209.0037 did not show in the MS² fragmentation, likely due to the fragmentation in the MS full scan).

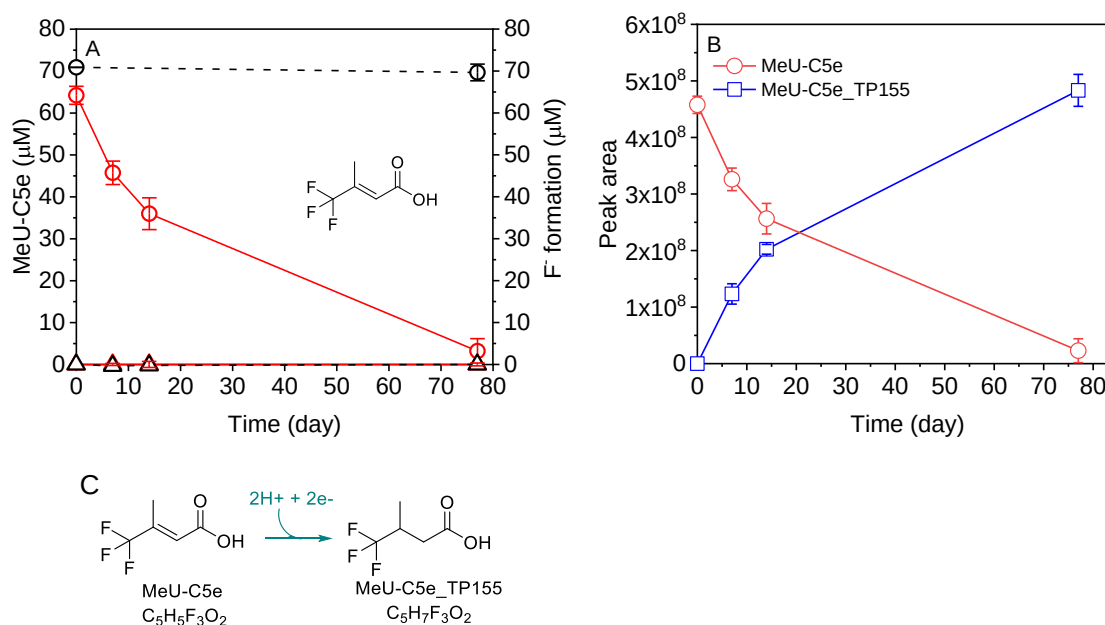
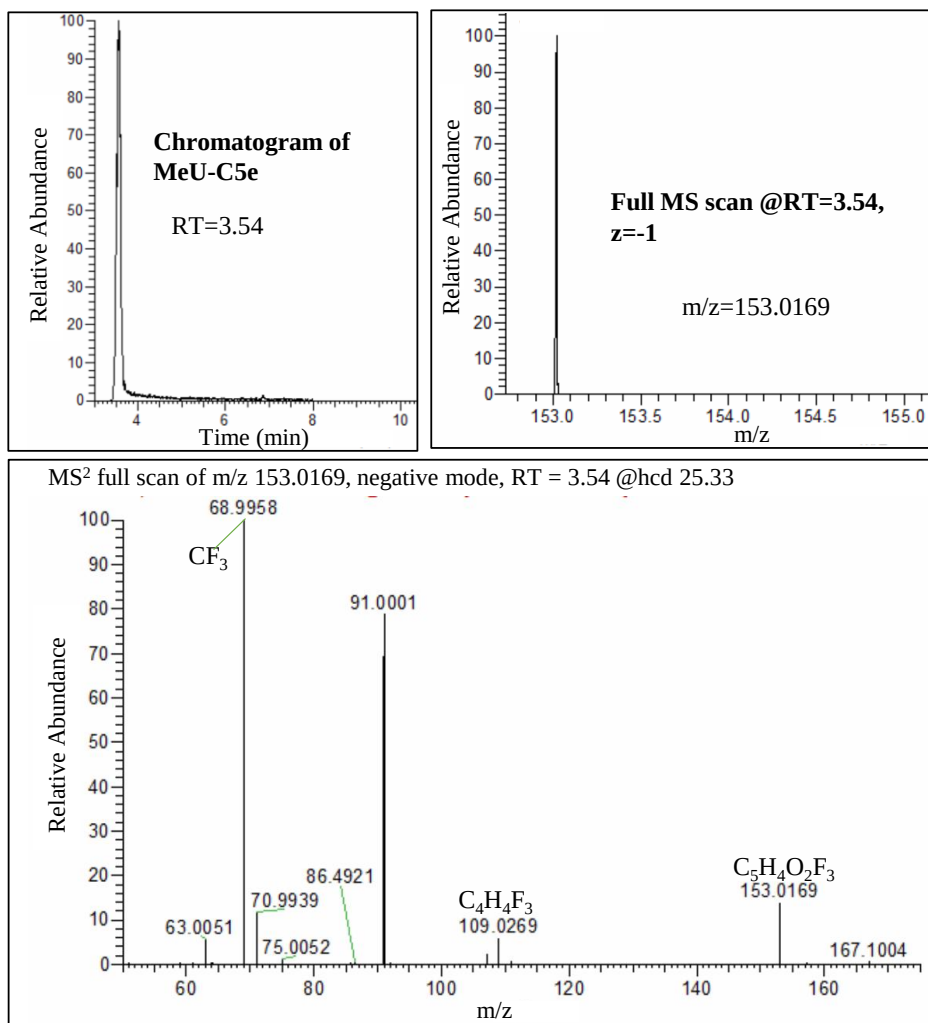


Figure D.16 MeU-C5e removal and fluoride release attributed to the parent compound degradation in the anaerobic defluorinating enrichment (A) (red: biological samples; black: autoclaved sludge filtrate controls; circles: parent compound; triangles: fluoride ion; n=3); the temporal trend of TPs identified by LC-HRMS/MS during MeU-C5e biotransformation (B); the proposed transformation pathway of MeU-C5e (C).



Formula: C₅H₅O₂F₃

Compound Structure:

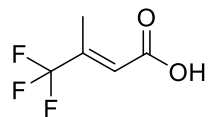
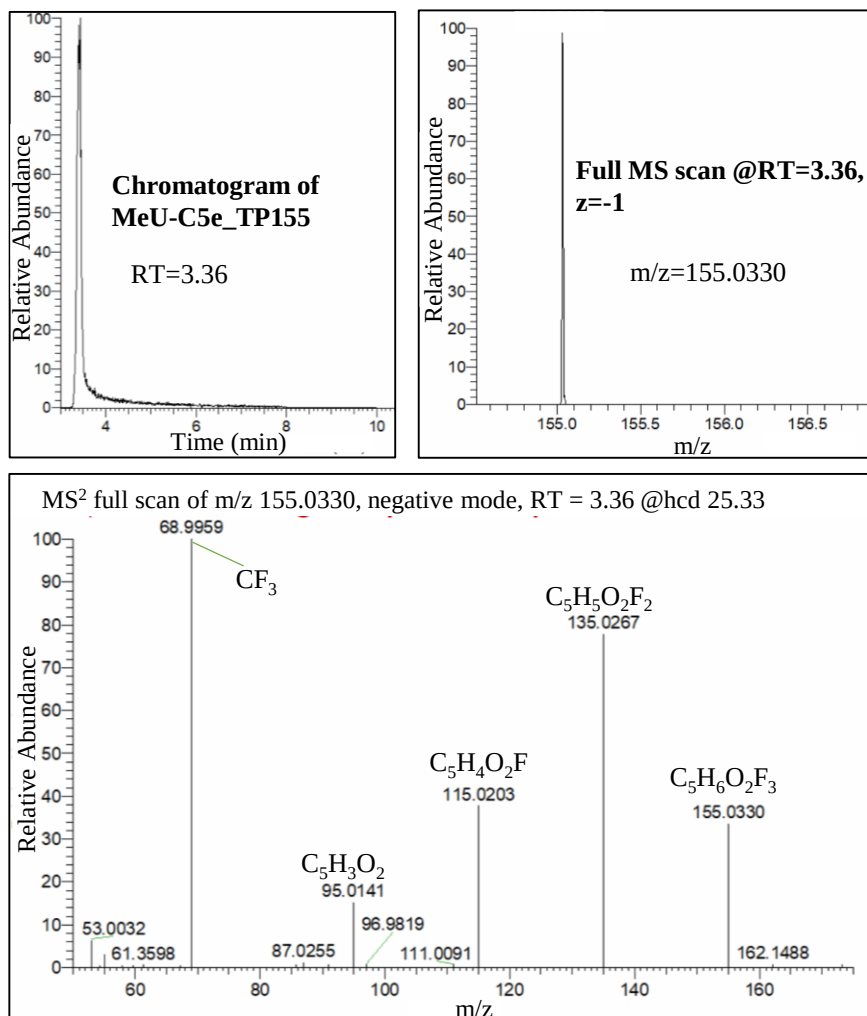


Figure D.17 MS² fragments of **MeU-C5e** standard.



Formula: C₅H₇O₂F₃

Atomic Modification: +2H of MeU-C5e

Confidence level: 2b

Hypothetical structure:

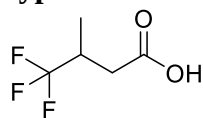


Figure D.18 MeU-C5e_TP155 structure elucidation.

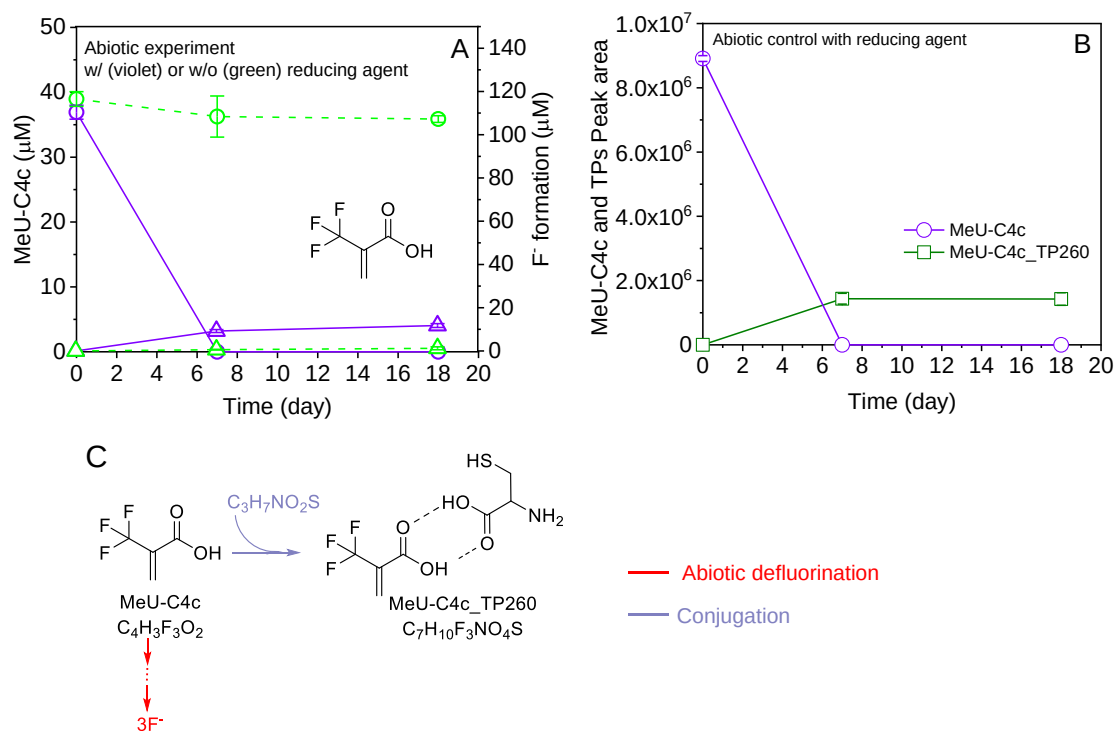


Figure D.19 MeU-C4c removal and fluoride release in the abiotic medium with and without the reducing agent (cysteine and sulfide) (A) (circles: parent compound; triangles: fluoride ion; n=3); the temporal trend of MeU-C4c and MeU-C4c_TP260 with the reducing agent (B); the proposed transformation pathways (C).

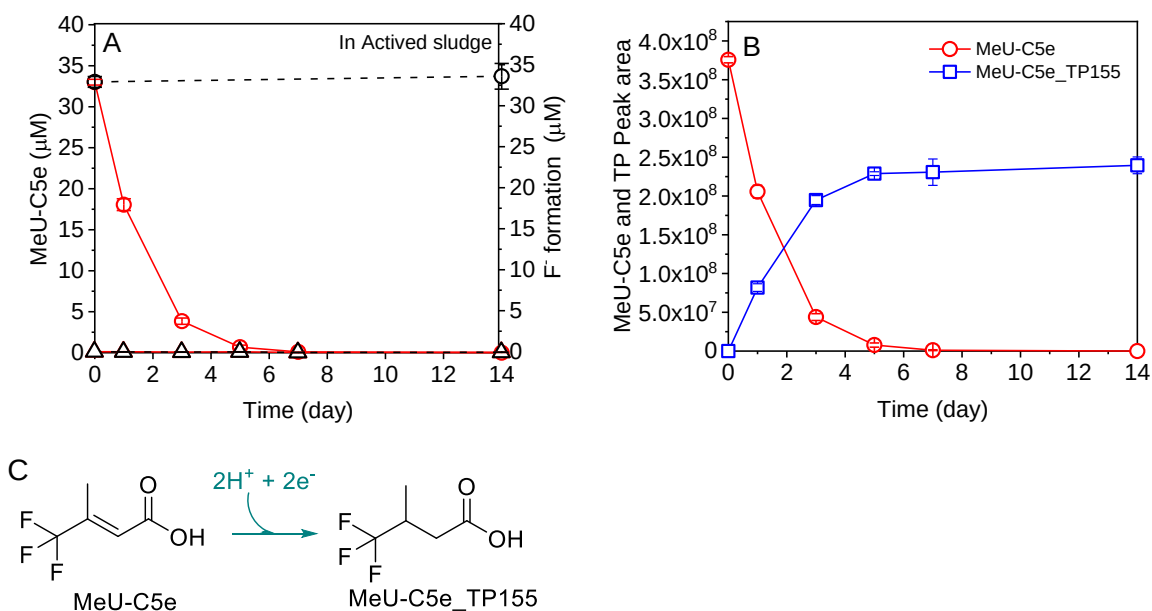


Figure D.20 **MeU-C5e** removal and fluoride release in the activated sludge communities (A) (red: biological samples; black: autoclaved sludge filtrate controls; circles: parent compound; triangles: fluoride ion; n=3); the temporal trend of TPs identified by LC-HRMS/MS during **MeU-C5e** biotransformation (B); the proposed transformation pathway of **MeU-C5e** (C).

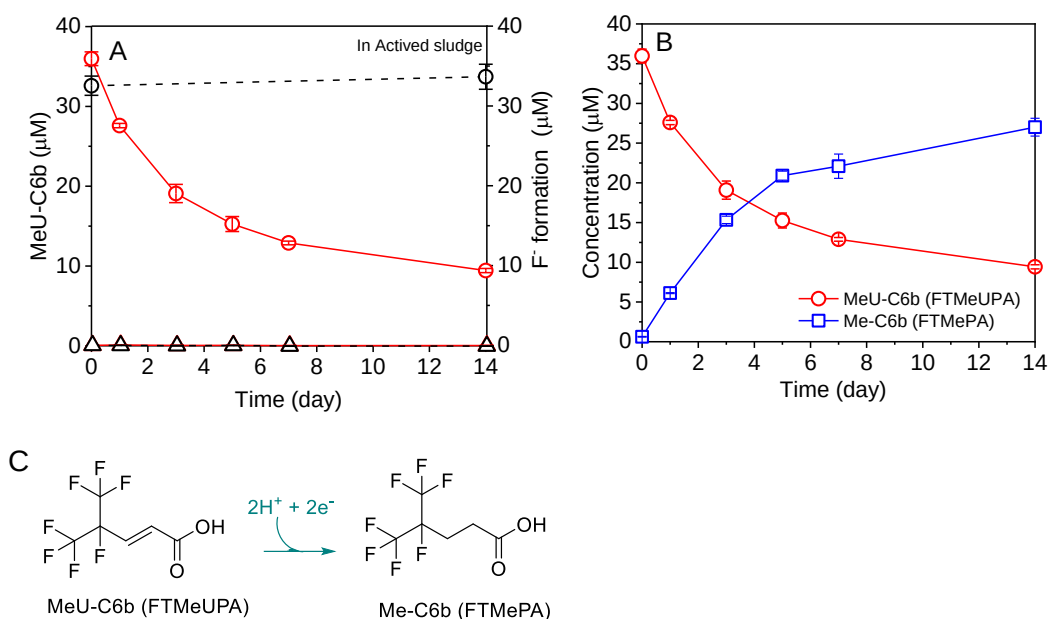


Figure D.21 **MeU-C6b** removal and fluoride release in the activated sludge communities (A) (red: biological samples; black: heat-inactivated controls; circles: parent compound; triangles: fluoride ion; n=3); the removal of **MeU-C6b** and the formation of the corresponding hydrogenation TP (i.e., **Me-C6b**) in biological samples (B); the proposed transformation pathway of **MeU-C6b** (C). (Note: All biotransformed **MeU-C6b** was converted to **Me-C6b** based on the LC-HRMS/MS quantification).

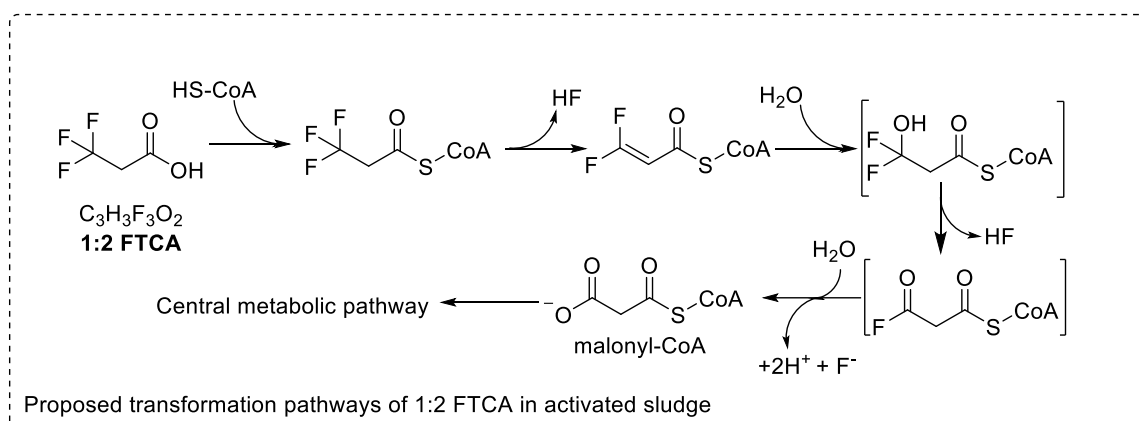


Figure D.22 The defluorination pathway of 1:2 FTCA proposed in a previous publication⁵⁵ (Note: in brackets are unstable intermediates; other intermediates were not detected, either, likely due to the fast turnover).

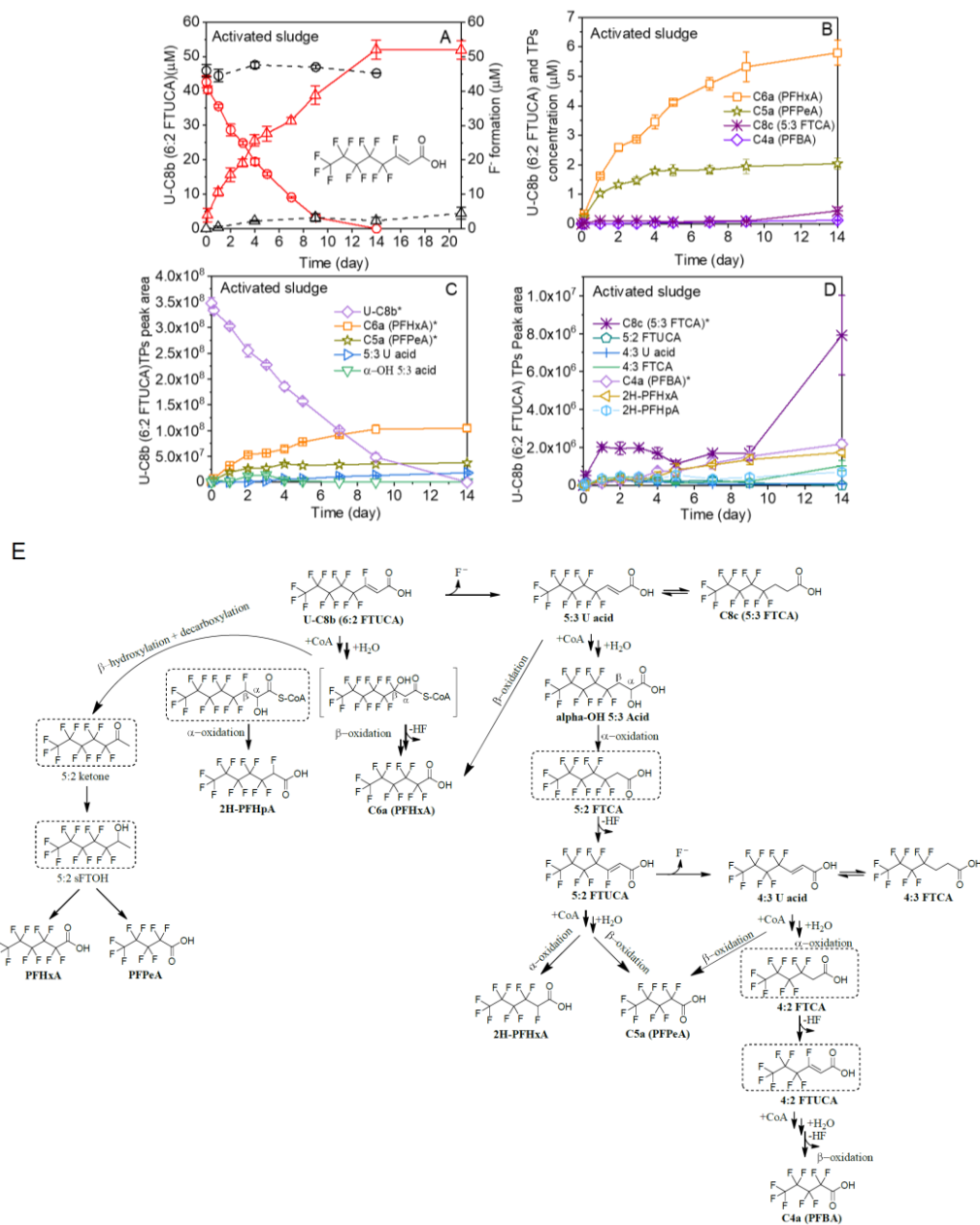
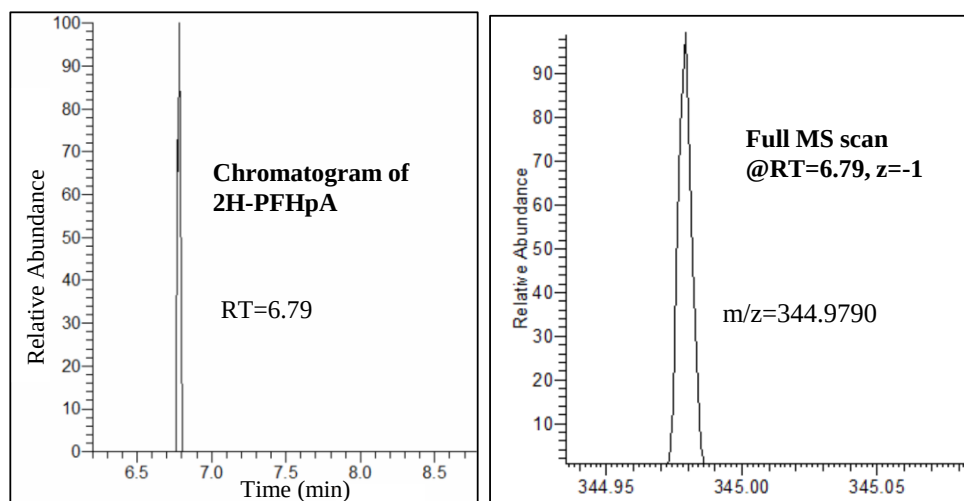


Figure D.23 Aerobic biotransformation of **U-C8b** (6:2 FTUCA) and fluoride release in the activated sludge community (A) (red: biological samples; black: abiotic control samples; circles: parent compound; triangles: fluoride; n=3); the quantification of some TPs during **U-C8b** biotransformation (B); temporal trends (peak areas) of **U-C8b** and TPs (C and D) (Note: C and D have different scales of y axis; *: TPs that can be quantified); the in the investigated activated sludge community via α- and β-oxidation-like reactions (E). (Note: pathways via the formation of 5:2 ketone and 5:2sFTOH reported in other studies,^{57, 58, 61, 62, 64, 65} were included on the left, which were not detected in this study; for other TPs, those in dashed boxes were hypothetical intermediates not detected; in brackets were unstable intermediates not detected; all others in bold were TPs detected in this study)



Formula: $C_7H_2O_2F_{12}$

Confidence level: 3

Hypothetical structure:

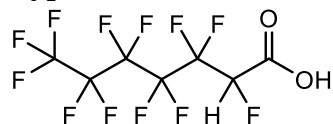
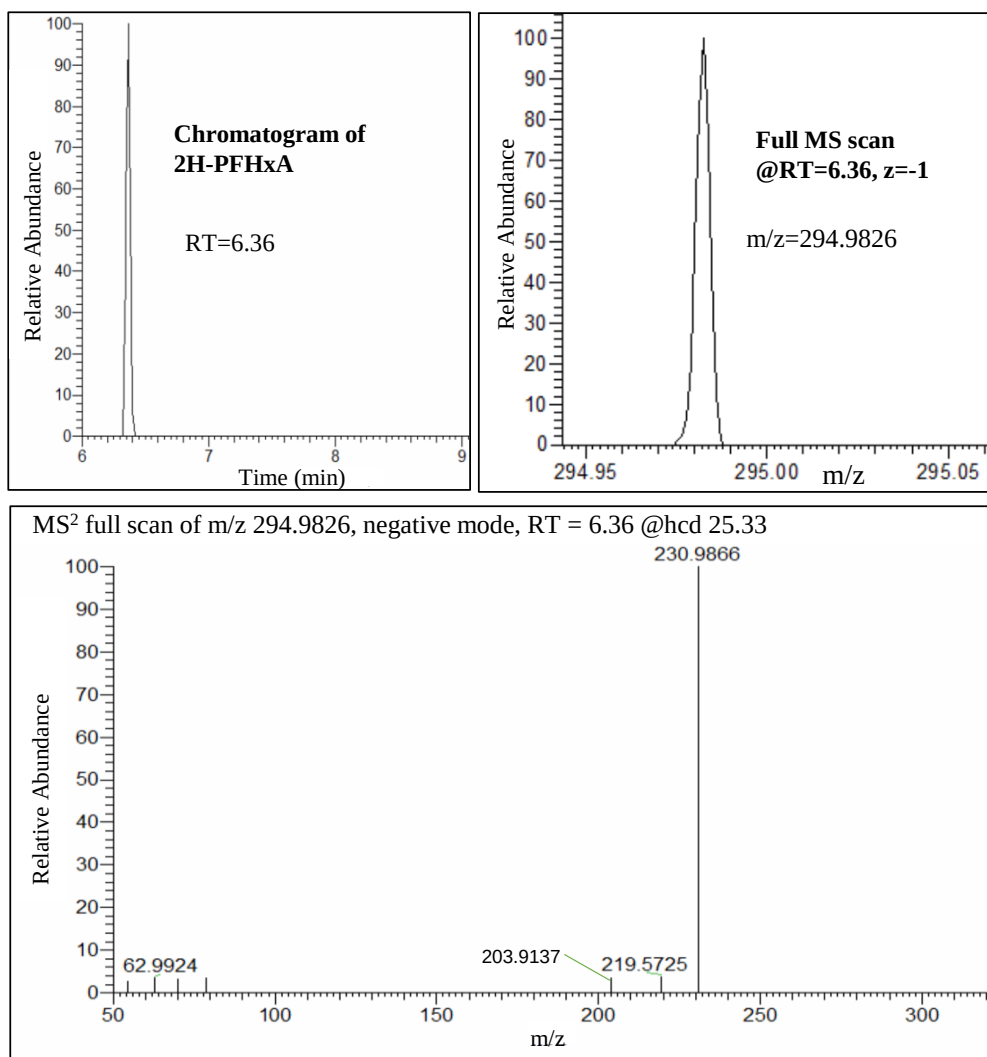


Figure D.24 2H-PFHpA structure elucidation (Note: due to the low intensity of 2H-PFHpA, the MS^2 fragments cannot be acquired even under the data-dependent mode).



Formula: C₆H₂O₂F₁₀

Confidence level: 2b

Hypothetical structure:

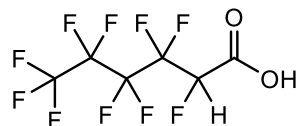


Figure D.25 2H-PFHxA structure elucidation.

APPENDIX E: SUPPLEMENTARY MATERIAL FOR CHAPTER 6

E.1 Supplementary Table and Figures

Table E.1 Primer sets information of selected OTUs.

Gene target	Primer name	Sequence (5'-3')
Universal bacteria 16S rRNA gene	Unibac_341f	CCTACGGGAGGCAGCAG
	Unibac_518r	ATTACCGCGGCTGCTGG
OTU47	OTU47_ Syntrophobacter_f	CCTGGGCTTAACCTGGGAAG
	OTU47_ Syntrophobacter_r	GCGTCAGTTACCGTCCAGAA
OTU169	OTU169_ Sedimentibacter_f	CGCGTGAGTGAAGAAGGTCT
	OTU169_ Sedimentibacter_r	CTTCCTCCTTGGCTACCGTC
OTU201	OTU201_ Armatimonadetes_f	GGTGATCAAGCCTGTGGTGA
	OTU201_ Armatimonadetes_r	TCTACGCATTCCACCGCTAC
OTU138	OTU138_ Rikenellaceae_f	TGTGCGGAATGTGTGGTGTA
	OTU138_ Rikenellaceae_r	ATTGCCCTTAGTGTGCTGCCT
OTU166	OTU166_ Spirochaetaceae_f	GTAAAGGGCATGTAGGCGGT
	OTU166_ Spirochaetaceae_r	ACTCAAGCCTCGCAGTTCTC
OTU68	OTU68_ Dehalobacter_f	CTGATGTGAAAGTGCGGTGC
	OTU68_ Dehalobacter_r	AGTCCAGAAAGTCGCCTTCG
OTU30	OTU30_ Lactivibrio_f	GCGGTAATACGTAGGGGACG
	OTU30_ Lactivibrio_r	ATTCCACTTGCCTCTCCAGC
OTU70	OTU70_ Syntrophorhabdus_f	CGGAAAGAACGACCCTGTGA
	OTU70_ Syntrophorhabdus_r	ATTCCGAACAACGCTTGAC
OTU68	OTU6_ Petrimona_f	TGGTGTAGCGGTGAAATGCT
	OTU6_ Petrimona_r	GCATGAGCGTCAGTAGTGGT

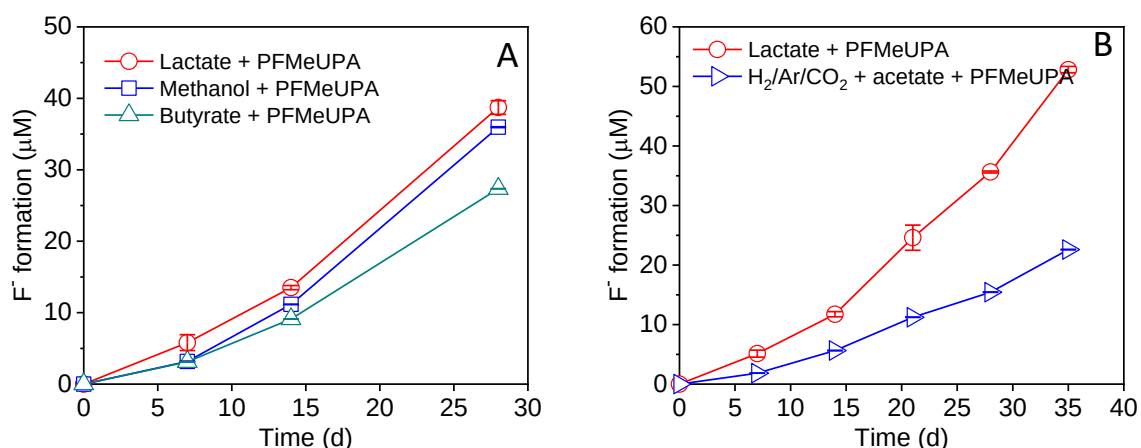


Figure E.1 Fluoride ion formation of PFMeUPA with different organic substrates (A), comparison of defluorination of PFMeUPA with lactate as primary electron donor and H_2 as directly electron donor (B) (n=3).

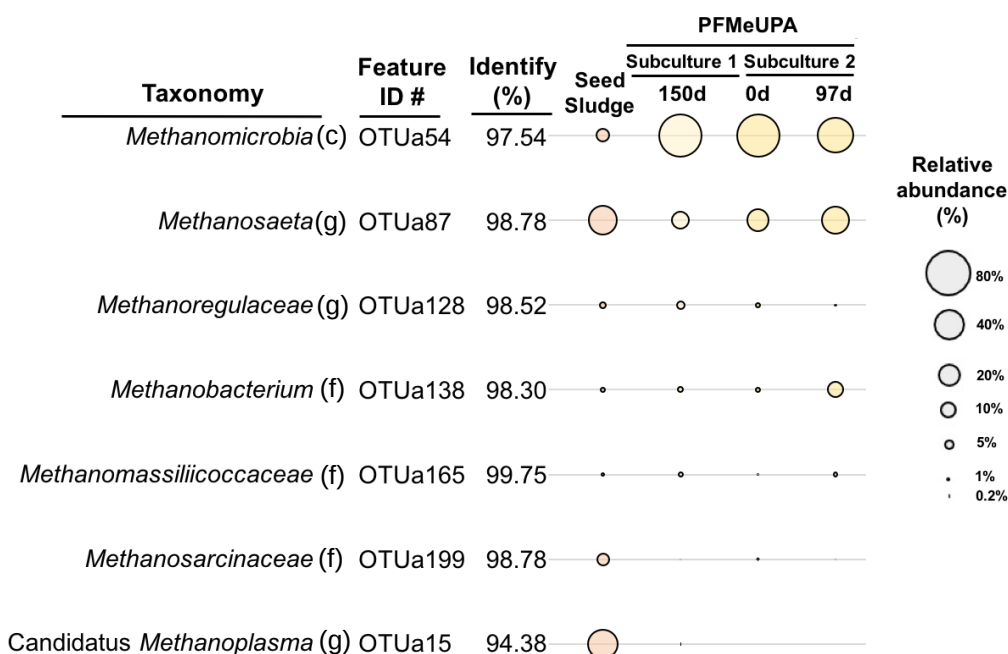


Figure E.2 Relative abundances of selected archaea OTUs over the total archaea OTUs detected in the seed culture (KB1 subculture on TCE) and the two lactate-fed PFMeUPA-defluorinating subcultures based on the 16S RNA sequencing results (the lowest taxonomic rank is assigned to each OTU: c, class; f, family g, genus).

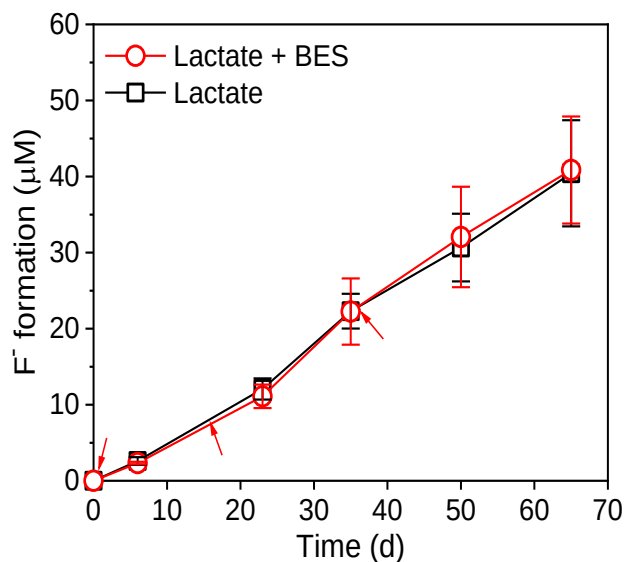


Figure E.3 Fluoride ion formation with and without the inhibition of methanogens by BES (inoculated with Subculture 2, 5% v/v; red arrows indicate the addition of 1 mM BES).

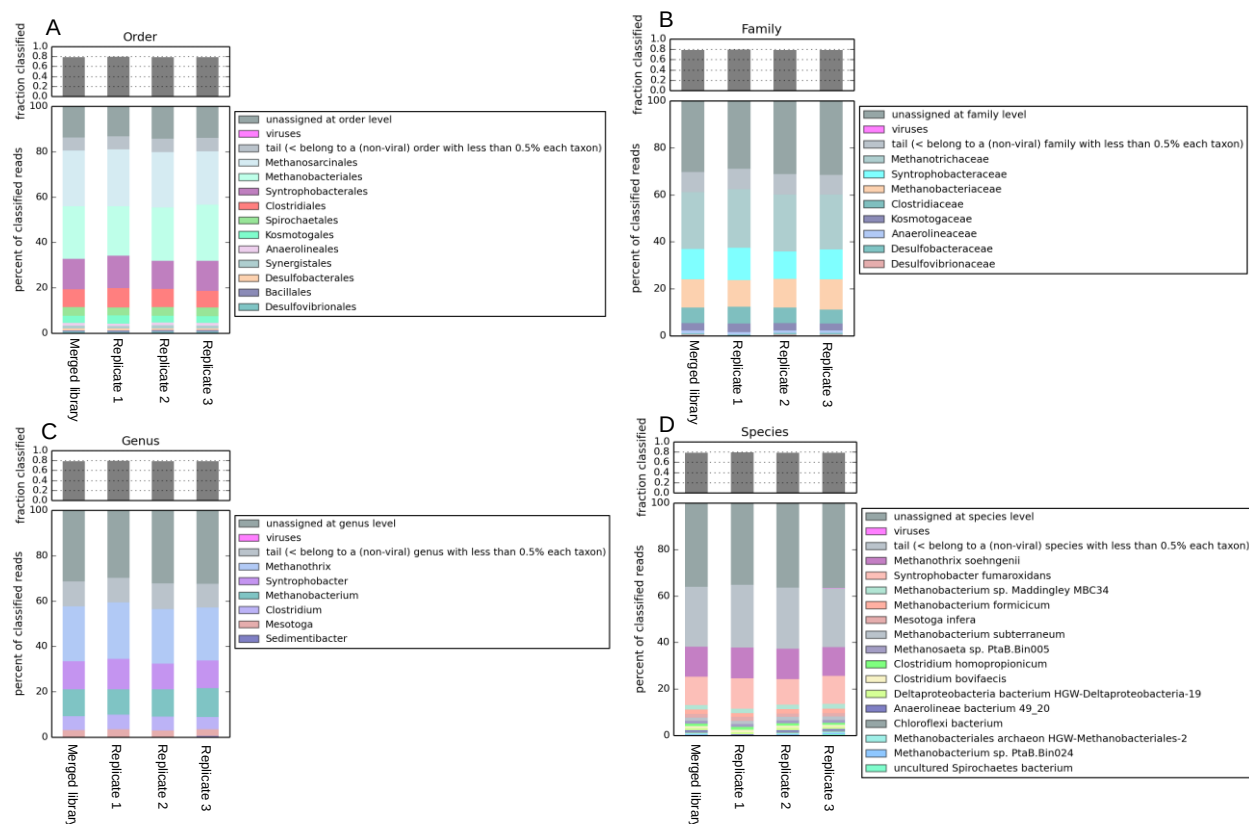
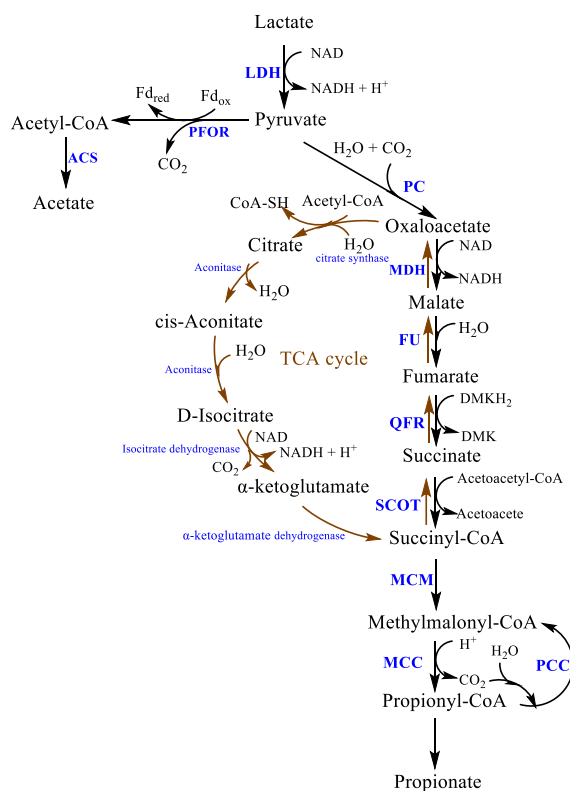


Figure E.4 Taxonomy comparison using KAIJU between merged reads library and individual reads library at order (A), family (B), genus (C), and species (D) level.



Abbreviations for enzymes:

PFOR: pyruvate ferredoxin oxidoreductase

LDH: Lactate dehydrogenase (Lactational oxidoreductase)

ACS: Acetyl-CoA synthetase or Acetate-CoA ligase

PC: Pyruvate carboxylase or pyruvate: carbon dioxide ligase (ADP-forming)

MDH: Malate dehydrogenase or Malate- NAD oxidoreductase

FU: fumarase

QFR: fumarate reductase

SCOT: succinyl-CoA: acetoacetate CoA-transferase

MCM: (R)-Methyl malonyl-CoA CoA-carboxylmutase

MCC: (S)-methyl malonyl-CoA carboxy-lyase (propanol-CoA-forming)

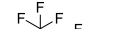
PCC: Propanoyl-CoA: carbon-dioxide ligase (ADP-forming)

Figure E.5 Major lactate fermentation pathways and TCA cycles of Bin 3 in the defluorinating enrichment culture.

APPENDIX F: SUPPLEMENTARY MATERIAL FOR CHAPTER 7

F.1 Supplementary Tables and Figures

Table F.1 Standard compound information

Structure (acronym)	PFAS Standard Information					RT (min)	LOQ (μM)
	CAS	Formula	Purity	[M-H] ⁻			
 MeU-C6a (PFMeUPA)	103229-89-6	C ₆ HF ₉ O ₂	97%	274.9754	6.37	0.5	

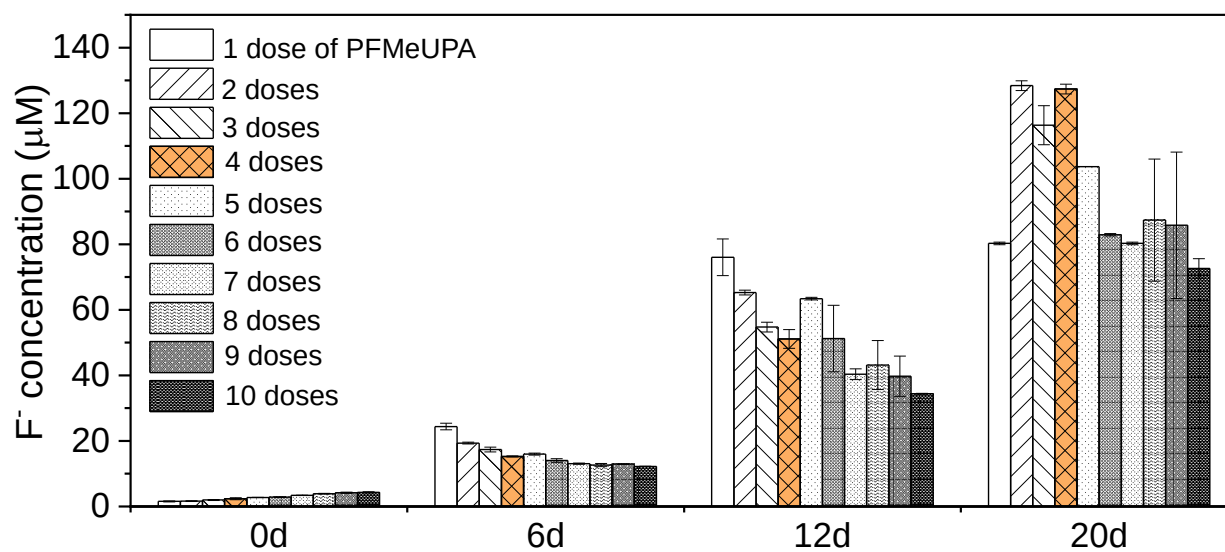


Figure F.1 Fluoride ion formation in *A. bakii* with different PFMeUPA addition. (Note: 1 does equals $\sim 89 \mu$ M PFMeUPA (quantified by HPLC-HRMS/MS); the orange color indicate the selected concentration for RNA-seq experiments).

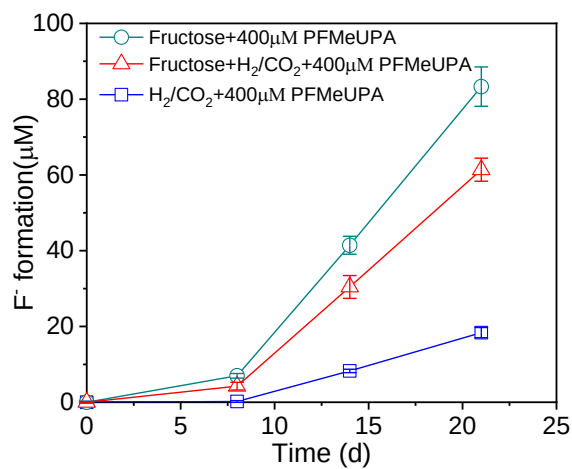


Figure F.2 Fluoride ion formation in *A. bakii* with different primary substrates.

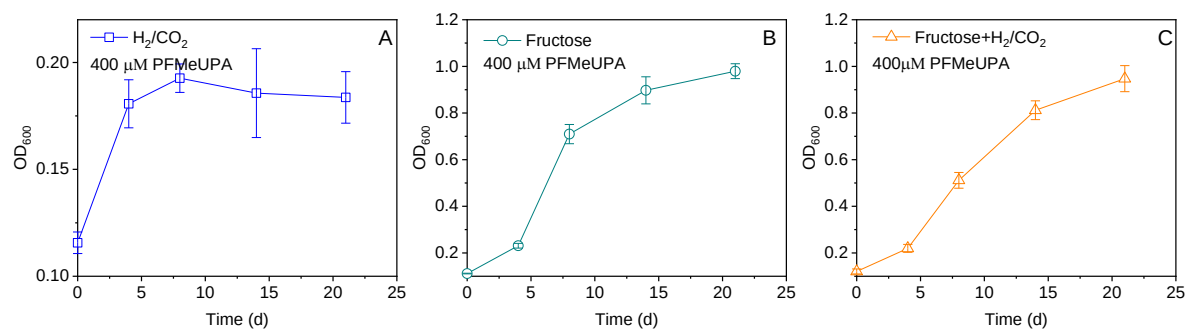


Figure F.3 OD₆₀₀ of *A. bakii* with different primary substrates with the addition of PFMeUPA.

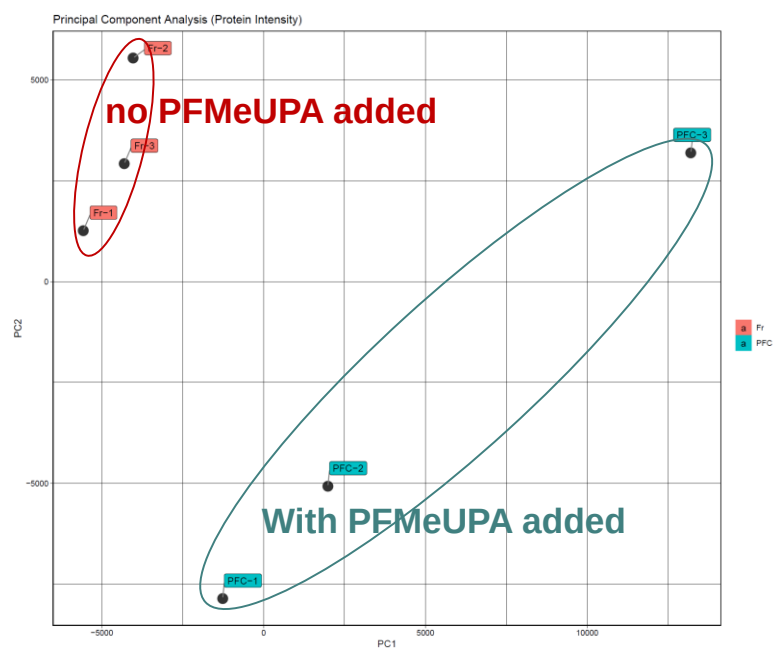


Figure F.4 PCA analysis of the protein composition in *A. bakii* with (green plots) and without (red plots) ~400 μ M PFMMeUPA added controls.

Table F.2 Up-regulated genes in fructose condition

Feature ID	Type	Function
CDS.1080	gene	Alcohol dehydrogenase (EC 1.1.1.1)
CDS.1123	gene	Transcriptional regulator, <i>PadR</i> family
CDS.1151	gene	Putative sulfite reductase, GSU1351 type
CDS.1161	gene	Integral membrane protein
CDS.1177	gene	Nicotinate dehydrogenase, FAD-subunit (EC 1.17.1.5)
CDS.1489	gene	Uncharacterized protein CPF_2956
CDS.1497	gene	Phosphate ABC transporter, substrate-binding protein <i>PstS</i> (TC 3.A.1.7.1)
CDS.1552	gene	Site-specific tyrosine recombinase <i>XerC</i>
CDS.1556	gene	Unknown function
CDS.1559	gene	hypothetical protein
CDS.156	gene	D-alanyl-D-alanine dipeptidase (EC 3.4.13.22)
CDS.1580	gene	Fluoride ion transporter <i>CrcB</i>
CDS.1585	gene	Alkyl hydroperoxide reductase subunit C-like protein
CDS.1621	gene	Ammonium transporter
CDS.1872	gene	Unknown function
CDS.1905	gene	Excinuclease ABC subunit C
CDS.1913	gene	Carboxyl-terminal protease (EC 3.4.21.102)
CDS.1998	gene	Type II/IV secretion system protein <i>TadC</i> , associated with Flp pilus assembly
CDS.2000	gene	Unknown function
CDS.2090	gene	L-aspartate oxidase (EC 1.4.3.16)
CDS.2091	gene	Quinolinate synthetase (EC 2.5.1.72)
CDS.2169	gene	Unknown function
CDS.2170	gene	arsenical resistance operon repressor
CDS.2171	gene	Asenic metallochaperone <i>ArsD</i> , transfers trivalent metalloids to <i>ArsAB</i> pump
CDS.2203	gene	Cold shock protein of CSP family
CDS.2265	gene	Acetyl-CoA synthase corrinoid activation protein
CDS.2362	gene	hypothetical protein
CDS.2363	gene	Unknown function
CDS.2365	gene	Unknown function
CDS.2396	gene	Unknown function
CDS.2736	gene	Efflux ABC transporter, ATP-binding protein
CDS.2755	gene	Unknown function
CDS.2765	gene	hypothetical protein
CDS.2766	gene	hypothetical protein
CDS.2852	gene	Thioredoxin
CDS.2853	gene	Thioredoxin
CDS.2854	gene	Unknown function
CDS.2858	gene	Thiol:disulfide oxidoreductase related to <i>ResA</i>
CDS.2859	gene	Cytochrome c-type biogenesis protein <i>CcdA</i> (<i>Dsbd</i> analog)
CDS.2935	gene	Unknown function
CDS.3002	gene	Unknown function
CDS.3003	gene	hypothetical protein
CDS.3062	gene	Predicted ATPase related to phosphate starvation-inducible protein <i>PhoH</i>
CDS.3152	gene	Orotate phosphoribosyltransferase (EC 2.4.2.10)
CDS.3315	gene	Efflux ABC transporter, ATP-binding protein
CDS.3389	gene	hypothetical protein
CDS.3390	gene	Protein with domain similar to <i>SecA</i>
CDS.3439	gene	Transporter, <i>EamA</i> family
CDS.3440	gene	Nuclease subunit of the excinuclease complex
CDS.3520	gene	Putative sulfite reductase, GSU1351 type
CDS.3548	gene	Ferredoxin 3 fused to uncharacterized domain
CDS.3549	gene	FIG00515007: hypothetical protein
CDS.3584	gene	Unknown function
CDS.3834	gene	Unknown function
CDS.3952	gene	hypothetical protein
CDS.3968	gene	Nitroreductase family protein
CDS.3970	gene	Thioredoxin
CDS.4009	gene	Uncharacterized membrane protein, <i>YraQ</i> family
CDS.4066	gene	Regulatory protein <i>ArsR</i>
CDS.463	gene	Arsenite/antimonite pump-driving ATPase <i>ArsA</i> (EC 3.6.3.16)
CDS.625	gene	Hydroxylamine reductase (EC 1.7.99.1)
CDS.654	gene	Transcriptional regulator, <i>MarR</i> family
CDS.725	gene	Phosphate ABC transporter, permease protein <i>PstC</i> (TC 3.A.1.7.1)
CDS.726	gene	Guanosine-3',5'-bis(diphosphate) 3'-pyrophosphohydrolase (EC 3.1.7.2) / GTP pyrophosphokinase (EC 2.7.6.5), (p)ppGpp synthetase II
CDS.795	gene	Two-component transcriptional response regulator, <i>AtoC</i> family
CDS.986	gene	Uncharacterized UPF0118 membrane protein

Table F.3 Down-regulated genes in fructose condition

Feature ID	Type	Function
CDS.1094	gene	Na ⁺ /H ⁺ antiporter
CDS.1286	gene	Lead, cadmium, zinc and mercury transporting ATPase (EC 3.6.3.3) (EC 3.6.3.5), Copper-translocating P-type ATPase (EC 3.6.3.4)
CDS.139	gene	hypothetical protein
CDS.1396	gene	Ethanolamine utilization protein similar to <i>PduV</i>
CDS.1443	gene	Proline/sodium symporter <i>PutP</i> (TC 2.A.21.2.1) @ Propionate/sodium symporter
CDS.1446	gene	diguanylate cyclase/phosphodiesterase (GGDEF & EAL domains) with PAS/PAC sensor(s)
CDS.1538	gene	ABC transporter, substrate-binding protein
CDS.1539	gene	ABC transporter, permease protein
CDS.1600	gene	Short-chain-fatty-acid--CoA ligase
CDS.1640	gene	hypothetical protein
CDS.1643	gene	Leader peptidase (Prepilin peptidase) (EC 3.4.23.43) / N-methyltransferase (EC 2.1.1.-)
CDS.1661	gene	ATP synthase F ₀ sector subunit c (EC 3.6.3.14)
CDS.1662	gene	ATP synthase F ₀ sector subunit c (EC 3.6.3.14)
CDS.1665	gene	ATP synthase delta chain (EC 3.6.3.14)
CDS.1666	gene	ATP synthase alpha chain (EC 3.6.3.14)
CDS.1668	gene	ATP synthase beta chain (EC 3.6.3.14)
CDS.1760	gene	Peptide-methionine (S)-S-oxide reductase <i>MsrA</i> (EC 1.8.4.11)
CDS.1810	gene	Uncharacterized corrinoid protein
CDS.1811	gene	hypothetical protein
CDS.2058	gene	Unknown function
CDS.2102	gene	Unknown function
CDS.2103	gene	Unknown function
CDS.2104	gene	Hydroxymethylpyrimidine ABC transporter, ATPase component
CDS.2105	gene	Hydroxymethylpyrimidine ABC transporter, substrate-binding component
CDS.2106	gene	Hydroxymethylpyrimidine ABC transporter, transmembrane component
CDS.2107	gene	Protein of unknown function DUF77
CDS.2140	gene	Hydroxymethylpyrimidine phosphate kinase <i>ThiD</i> (EC 2.7.4.7)
CDS.2141	gene	Unknown function
CDS.2142	gene	Thiamin-phosphate pyrophosphorylase (EC 2.5.1.3)
CDS.2143	gene	Hydroxyethylthiazole kinase (EC 2.7.1.50)
CDS.2242	gene	PTS system, glucitol/sorbitol-specific IIB component (EC 2.7.1.198) / PTS system, glucitol/sorbitol-specific IIC component 2
CDS.2243	gene	PTS system, glucitol/sorbitol specific IIC component
CDS.2244	gene	hypothetical protein
CDS.2274	gene	Oxidoreductase, short-chain dehydrogenase/reductase family
CDS.2289	gene	Unknown function
CDS.2354	gene	Transcriptional regulator, <i>LysR</i> family
CDS.2384	gene	hypothetical protein
CDS.2511	gene	Unknown function
CDS.2615	gene	hypothetical protein
CDS.2616	gene	Propanediol utilization polyhedral body protein <i>PduT</i>
CDS.2617	gene	hypothetical protein
CDS.2618	gene	Cob (III)alamin reductase @ Cob (II)alamin reductase
CDS.2619	gene	CoA-acylating propionaldehyde dehydrogenase
CDS.2620	gene	Hypothetical protein PA1329
CDS.2621	gene	ATP:Cob(II)alamin adenosyltransferase (EC 2.5.1.17) @ ATP:Cob(II)alamin adenosyltransferase (EC 2.5.1.17), propanediol utilization
CDS.2622	gene	Propanediol utilization polyhedral body protein <i>PduN</i>
CDS.2623	gene	hypothetical protein
CDS.2624	gene	Ethanolamine utilization protein <i>EutJ</i>
CDS.2625	gene	Phosphate propanoyltransferase (EC 2.3.1.222)
CDS.2626	gene	Propanediol utilization polyhedral body protein <i>PduK</i>
CDS.2627	gene	Propanediol dehydratase reactivation factor small subunit
CDS.2628	gene	Propanediol dehydratase reactivation factor large subunit
CDS.2629	gene	Propanediol dehydratase small subunit (EC 4.2.1.28)
CDS.2630	gene	Propanediol dehydratase medium subunit (EC 4.2.1.28)
CDS.2631	gene	Propanediol dehydratase large subunit (EC 4.2.1.28)
CDS.2632	gene	Propanediol utilization polyhedral body protein <i>PduB</i>
CDS.2633	gene	Propanediol utilization polyhedral body protein <i>PduA</i>
CDS.2719	gene	Unknown function
CDS.2737	gene	Efflux ABC transporter, permease protein
CDS.2816	gene	Thymidylate kinase (EC 2.7.4.9)
CDS.3341	gene	Cobalt ECF transporter, ATPase component of energizing module <i>CbiO</i>
CDS.3346	gene	Vitamin B ₁₂ ABC transporter, ATP-binding protein <i>BtuD</i>
CDS.3518	gene	ABC transporter, ATP-binding protein
CDS.3973	gene	Iron (III) ABC transporter, ATP-binding protein
CDS.3998	gene	Heterodimeric efflux ABC transporter, permease/ATP-binding subunit 2
CDS.4060	gene	Nitrite reductase probable [NAD(P)H] subunit (EC 1.7.1.4)
CDS.4061	gene	Nitrite reductase probable electron transfer 4Fe-S subunit (EC 1.7.1.4)
CDS.489	gene	hypothetical protein
CDS.626	gene	Phosphomethylpyrimidine synthase <i>ThiC</i> (EC 4.1.99.17)
CDS.627	gene	Substrate-specific component <i>ThiW</i> of predicted thiazole ECF transporter
CDS.786	gene	ABC transporter, permease protein (cluster 3, basic aa/glutamine/opines)
CDS.792	gene	Phosphoenolpyruvate-dihydroxyacetone phosphotransferase (EC 2.7.1.121), ADP-binding subunit <i>DhaL</i>

Table F.3 Continued

Feature ID	Type	Function
CDS.793	gene	Phosphoenolpyruvate-dihydroxyacetone phosphotransferase (EC 2.7.1.121), dihydroxyacetone binding subunit <i>DhaK</i>
CDS.916	gene	Unknown function
CDS.918	gene	Putative hydrolase

Table F.4 Up-regulated genes in fructose+H₂ condition

Feature ID	Type	Function
CDS.1579	gene	Fluoride ion transporter <i>CrcB</i>
CDS.1580	gene	Fluoride ion transporter <i>CrcB</i>
CDS.255	gene	Unknown function
CDS.3844	gene	Acetyltransferase, GNAT family
CDS.3845	gene	Formate-tetrahydrofolate ligase (EC 6.3.4.3)
CDS.488	gene	Unknown function

Table F.5 Down-regulated genes in fructose+H₂ condition

Feature ID	Type	Function
CDS.2111	gene	Acetoin catabolism protein X, possible NAD kinase
CDS.2113	gene	Acetoin dehydrogenase E1 component alpha-subunit (EC 2.3.1.190)
CDS.2112	gene	Acetoin dehydrogenase E1 component beta-subunit (EC 2.3.1.190)
CDS.2388	gene	Alcohol dehydrogenase (EC 1.1.1.1)
CDS.2546	gene	Alcohol dehydrogenase (EC 1.1.1.1)
CDS.2621	gene	Uncharacterized conserved protein <i>GlcG</i>
CDS.2843	gene	Cation-transporting ATPase, E1-E2 family
CDS.2619	gene	CoA-acylating propionaldehyde dehydrogenase
CDS.3727	gene	CoA-disulfide reductase (EC 1.8.1.14) / Disulfide bond regulator
CDS.2618	gene	Cob(III)alamin reductase @ Cob(II)alamin reductase
CDS.1959	gene	Deoxyribose-phosphate aldolase (EC 4.1.2.4)
CDS.1960	gene	Deoxyribose-phosphate aldolase (EC 4.1.2.4)
CDS.2110	gene	Dihydrolipoamide acetyltransferase component (E2) of acetoin dehydrogenase complex (EC 2.3.1.-)
CDS.2109	gene	Dihydrolipoamide dehydrogenase (EC 1.8.1.4)
CDS.2624	gene	Ethanolamine utilization protein <i>EutI</i>
CDS.1244	gene	Ethanolamine utilization protein similar to <i>PduU</i>
CDS.1964	gene	FIG00031715: Predicted metal-dependent phosphoesterases (PHP family)
CDS.1884	gene	Glycerol dehydrogenase (EC 1.1.1.6)
CDS.2108	gene	hypothetical protein
CDS.2615	gene	hypothetical protein
CDS.2617	gene	hypothetical protein
CDS.2623	gene	hypothetical protein
CDS.2620	gene	Hypothetical protein PA1329
CDS.2625	gene	Phosphate propanoyltransferase (EC 2.3.1.222)
CDS.626	gene	Phosphomethylpyrimidine synthase ThiC (EC 4.1.99.17)
CDS.1605	gene	Phytoene synthase (EC 2.5.1.32)
CDS.2417	gene	Prephenate and/or arogenate dehydrogenase (unknown specificity) (EC 1.3.1.12) (EC 1.3.1.43)
CDS.2631	gene	Propanediol dehydratase large subunit (EC 4.2.1.28)
CDS.2630	gene	Propanediol dehydratase medium subunit (EC 4.2.1.28)
CDS.2628	gene	Propanediol dehydratase reactivation factor large subunit
CDS.2627	gene	Propanediol dehydratase reactivation factor small subunit
CDS.2629	gene	Propanediol dehydratase small subunit (EC 4.2.1.28)
CDS.2633	gene	Propanediol utilization polyhedral body protein <i>PduA</i>
CDS.2632	gene	Propanediol utilization polyhedral body protein <i>PduB</i>
CDS.2626	gene	Propanediol utilization polyhedral body protein <i>PduK</i>
CDS.2622	gene	Propanediol utilization polyhedral body protein <i>PduN</i>
CDS.2616	gene	Propanediol utilization polyhedral body protein <i>PduT</i>
CDS.1932	gene	Tyrosine-protein kinase <i>EpsD</i> (EC 2.7.10.2)
CDS.3237	gene	Uncharacterized corrinoid protein
CDS.3356	gene	Uncharacterized protein MJ1441
CDS.3236	gene	Uncharacterized pterin binding enzyme DSY4972
CDS.3235	gene	Uroporphyrinogen-III decarboxylase
CDS.3346	gene	Vitamin B ₁₂ ABC transporter, ATP-binding protein <i>BtuD</i>
CDS.1957	gene	Xylulose kinase (EC 2.7.1.17)
CDS.1958	gene	Xylulose kinase (EC 2.7.1.17)
CDS.2290	gene	Xylulose kinase (EC 2.7.1.17)