BIODEGRADATION POTENTIAL OF PERFLUOROOCTANOATE AND PERFLUOROOCTANE SULFONATE

A Thesis

by

PARVATHY THELAKKAT KOCHUNARAYANAN

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

August 2011

Major Subject: Civil Engineering

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Approved by:

Chair of Committee,	Kung-Hui Chu
Committee Members,	Bill Batchelor
	Thomas Wood
Head of Department,	John Niedzwecki

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ABSTRACT

Biodegradation Potential of Perfluorooctanoate and Perfluorooctane Sulfonate.

(August 2011)

Parvathy Thelakkat Kochunarayanan, B.Tech., University of Kerala, India

Chair of Advisory Committee: Dr. Kung-Hui Chu

Perfluorooctanoate (PFOA) and Perfluorooctane sulfonate **(**PFOS) are two environmentally persistent perfluorinated compounds widely used for many industrial and consumer products due to their high thermal, oxidative resistance and surface repellence to water and oil. Their reproductive and developmental toxicity in lab animals and their persistence in environment have raised a serious concern for humans and animals. Trace amounts of these compounds have been found in water bodies, human blood, and wildlife samples. PFOA and PFOS are currently listed in Environmental Protection Agency's drinking water Contaminant Candidate List and in the list of Persistent Organic Pollutants in the Stockholm Convention.

The strong covalent bond between carbon and fluorine present in PFOA and PFOS makes them stable and resistant to conventional treatment processes. Several advanced chemical processes can degrade PFOA and PFOS under high temperatures and pressures or other extreme conditions. However, the potential of biodegradation as a treatment technology for these compounds hasn't been developed successfully. This thesis focuses on evaluating the biodegradation potential of PFOA and PFOS.

Fluoroacetate dehalogenase is an enzyme capable of defluorinating fluorinated aliphatic compounds. In this study, the potential of fluoroacetate dehalogenase-expressing microorganisms to biodegrade PFOA and PFOS is examined. Two known fluoroacetate dehalogenase-expressing strains and fluoroacetate-degrading mixed cultures were used. The effect of ammonia in the enzyme activity was extended to study its effect on the biodegradation of PFOA and PFOS. Fluoride ions released during the mineralization of the PFOA and PFOS was used as a proof of biodegradation. The experiments with fluoroacetate dehalogenase-expressing strains and mixed culture consortia enriched from soil showed an increase in fluoride concentration in the solution thus indicating the possibility of successful biodegradation of PFOA and PFOS. Based on the fluoride ion content, it was also concluded that ammonia inhibits the enzyme activity in one of the two pure strains.

DEDICATION

I dedicate this thesis to my husband Kiran & my brother Rajeev

ACKNOWLEDGEMENTS

I am extremely grateful to my advisor, Dr. Kung-Hui Chu, for her guidance, encouragement and constant support throughout the course of this research. I would also like to thank Dr. Bill Batchelor and Dr. Thomas Wood, for serving on my thesis committee.

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I have no words to express my gratitude to my family, especially my husband and brother for their immense support and care. Without their love and support, I would not have made it through.

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CHAPTER I

INTRODUCTION

Perfluorinated compounds are a group of organofluorine compounds where fluorine atoms replace all hydrogen atoms. Under this group, perfluorooctanoate (PFOA) and perfluorooctane sulfonate (PFOS) are the most widely used compounds in industrial and consumer products due to their high thermal, oxidative resistance and surface repellence to water and oil (*1*). For example, PFOA is used in non-stick cookwares, water and stain proof coatings, protective coatings, and high temperature lubricants (*1*). PFOS is applied as a surfactant in fire fighting foams, as protective coatings for clothes, in insecticides etc. Exposures to these compounds have found to induce reproductive and developmental toxicity and liver toxicity in lab animals (*2*). In humans, the mean serum elimination half-lives for PFOA and PFOS have been observed to be 3.8 and 5.4 years respectively (*3, 4*). These compounds are currently listed in Environmental Protection Agency's drinking water Contaminant Candidate List (CCL 3) (*5*).

Both PFOA and PFOS are environmentally persistent compounds and their widespread use has resulted in global contamination. Trace amounts of these compounds have been found in the environment (*6, 7*) and in human blood and wildlife sample (*8, 9*). Drinking water contamination of tap and surface water samples in many countries (*6*) and trace level detection in ocean waters (*7*) have been reported for PFOA

This thesis follows the style of *Environmental Science & Technology*.

and PFOS. In Australia, drinking water is reported to have 9.7 ng/l of PFOA and 16 ng/l of PFOS (*10*). The lake water in China was reported to have around 4.2 ng/l of PFOS and 5.4 ng/l of PFOA (*6*). Undoubtedly these compounds have posed emerging threat to water quality.

The carbon- fluorine bond in PFOA and PFOS is the strongest covalent bond known (*11*), making them difficult to be removed by conventional water and wastewater treatment processes (*12*). While these compounds can be removed by chemical oxidation under extreme conditions (*13, 14*), biological degradation is a desirable option, since biological processes have a potential to degrade these compounds under environmentally relevant conditions.

The possibility of biodegradation has been examined based on thermodynamic data and calculations (*15*). However, the lack of experimental research in this area is notable. No bacteria or enzymes capable of degrading PFOA and PFOS have been identified. Among numerous known dehalogenases, an enzyme called fluoroacetate dehalogenase is known for its ability to cleave carbon-fluorine bond. However, no studies have examined whether PFOA and PFOS can be biodegraded by fluoroacetate dehalogenase. This research focuses on studying the biodegradation of PFOA and PFOS using fluoroacetate or similar dehalogenases.

The hypothesis of this study is that aerobic fluoroacetate dehalogenase-expressing microorganisms can degrade PFOA and PFOS. The biodegradation potential of PFOA and PFOS were examined through the following three specific objectives:

- Evaluate the biodegradation potential of PFOA and PFOS using known fluoroacetate dehalogenase-expressing strains.
- Examine the biodegradation potential of PFOA and PFOS using fluoroacetate-degrading mixed cultures.
- Investigate the effects of ammonia on the biodegradation of PFOA and PFOS.

The rationale behind selecting the fluoroacetate degraders for this research is the specificity of the enzyme for fluorinated aliphatics. The idea of this project is derived from the facts that i) defluorination is a thermodynamically possible reaction and ii) the cleavage of C-F bond is reported for shorter chain fluorinated aromatic and aliphatic compounds (*15*). Accordingly, it can be extended to longer chain compounds like PFOA and PFOS. It is also found that bacteria can obtain energy for growth from this defluorination process (*15*). Two fluoroacetate degraders, *Pseudomonas fluorescens* DSM8341 and *Burkholderia* sp. FA1 were chosen as model strains. These strains degrade sodium fluoroacetate by producing the enzyme fluoroacetate dehalogenase (*16, 17*). The fluoroacetate is hydrolytically defluorinated to produce glycolate ion and fluoride ion. *Burkholderia* sp. FA1 was isolated from Garden soil in Japan (*16*). *Pseudomonas fluorescens* DSM8341 was isolated from Western Australian soil (*18*). In the current study we tested our hypothesis that these strains could also be successful in degrading the organofluorine compounds under consideration, which are PFOA and PFOS. Since the model strains used for this study capable of mineralizing organofluorine compounds are both isolated from soil, we assumed that the mixed

culture from soil may have some fluoroacetate degraders present in it. Hence soil inoculum was selected to enrich fluoroacetate degraders.

Experiments were also conducted to examine biodegradation of PFOA and PFOS using mixed cultures originally enriched with sodium fluoroacetate and PFOA. As ammonia has been reported to affect the function of fluoroacetate dehalogenase (*19*) , it is expected that ammonia would also affect the biodegradation of PFOA and PFOS, if any. Thus, the third part of experiments was designed to examine this aspect.

There are five chapters in this thesis. The literature review for this research is covered in Chapter II. Detailed experimental methods and materials used in this study are described in Chapter III. Results and discussion are covered in Chapter IV. The final chapter, Chapter V summarizes future work based on the results of current research.

CHAPTER II

LITERATURE REVIEW

SOURCES, PROPERTIES, AND FATE OF PFOA AND PFOS

Among perfluorinated compounds, PFOA and PFOS are the two most studied compounds. They are widely used in non-stick, water and stain proof coatings, protective coatings, high temperature lubricants, and as surfactants (*1*). The commercial applications vary from water and stain resistant coatings in clothes and carpets to coatings for paper, aqueous film forming foams (AFFF), food wrappers, and many others (*20*). PFOA and PFOS were first manufactured by 3M company adopting Simons Electro-Chemical Fluorination (ECF) process (*20*). In this process, a selected organic compound is reacted with hydrogen fluoride in the presence of electric current to replace all the hydrogen atoms in the organic compound with fluorine atoms producing the perfluorinated equivalent. Currently, DuPont is the only PFOA manufacturer in the United States. These compounds can be indirectly produced from the biodegradation of precursors such as fluorotelomer alcohols (*21*).

PFOA has eight carbon atoms and fifteen fluorine atoms with a carboxylic acid group at the end. PFOS has seventeen fluorine atoms attached to the eight carbon chain with a sulfonic acid group at the end. Figure 1 and Figure 2 show the structures of PFOA and PFOS respectively.

PFOA and PFOS exhibit peculiar and unique properties which can be attributed to the unique fluorine chemistry. Fluorine is the most electronegative element, resulting in the formation of a very strong and polar C-F bond with a very high dissociation

energy of 110 kCal/mol (*22*). This imparts high thermal, chemical, electrical, oxidative stabilities to these compounds. The three non-bonding electrons in the fluorine atom and the protective shield provided by the carbon atom in the carbon-fluorine bond provides significant protection from attack (*23*).

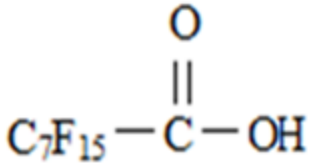


Figure 1 Structure of PFOA

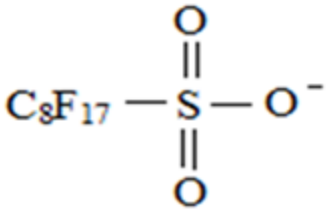


Figure 2 Structure of PFOS

These compounds are both hydrophobic and oleophobic. The carboxylic acid and sulfonic acid groups when attached to the pefluorinated chains make them hydrophilic and hence PFOA and PFOS have surfactant properties. Due to the surface

active properties of PFOS, the octanol-water partitioning coefficients could not be calculated (*24, 25*). Hence the related factors such as sediment adsorption factor, bioconcentration factor are not predicted. The solubility of PFOS is 550 mg/l in water at 24-25^{o}C (*26*).

PFOA has a log $K_{air/water}$ value of −2.4 and a log K_{ow} of 4.3 suggesting that sorption and volatilization are two main transport processes (*27*). Since it is both hydrophobic and oleophobic, it finds its application in fire fighting industry and helps to extinguish the fire and also to prevent the subsequent inflaming. Due to the low acid dissociation constant of PFOS, it occurs in the environment in complete dissociated form (*23*).

TOXICITY OF PFOA AND PFOS

PFOA and PFOS are found to induce reproductive and developmental toxicity in humans and animals (*2*). These compounds get absorbed quickly and are circulated in liver and serum in humans and animals (*3*). In a hazard assessment study conducted by OECD, it was found that the PFOS binds to the protein and not to the lipid fraction (*24*). An EPA review suggests that a short term exposure to PFOS can lead to weight loss, liver toxicity, and decrease in the thyroid hormones level (*28*). In animals, in addition to reproductive and developmental toxicity, liver toxicity has also been reported.

In rats, high concentration of PFOA was observed in blood samples within 2 hours of exposure showing the rapid absorption of the compound (*29*). In humans, exposure to PFOA has been found to affect organ growth (*30*). In addition to the reproductive toxicity, exposures to PFOA and PFOS affect the fecundity of both males

and females (*31*). PFOA and PFOS are found to cause peroxisome proliferation, fatty acid metabolism in animals, especially rats (*32*). Acute dermal and inhalation toxicities were reported for rats and death was reported after 3-7 days after dermal exposure (*33*). In humans, the mean serum elimination half-lives have been observed to be 3.8 years for PFOA and 5.4 years for PFOS (*3*). In rats, the elimination of PFOA differs greatly with gender. For female rats, the average time for excretion is about 13 hours while for the males it is around 7 days (*34*). A similar sex-related variation is not found in humans (*3*). It has been found that rats take about 90 days to discard PFOS from the body (*35*). The long elimination time may have contributed to the presence of these compounds in living organisms.

ENVIRONMENTAL PERSISTENCE OF PFOA AND PFOS

PFOA and PFOS have been found in human blood and wildlife samples (*8, 9*). A study reveals that among the blood samples collected from about 645 adult donors, the PFOS concentrations ranged from about 4 ppb to 1656 ppb (*8*). In wild life, PFOS has been found to accumulate in various species like bald eagles, and marine mammals (*36, 37*). PFOA and PFOS have been detected in endangered species like Red Panda and Giant Panda in China (*38*). Thus the distribution of these compounds is widespread and covers all over the globe. Due to their toxicity and persistence, these compounds pose serious threat to human and ecological health. The PFOA and PFOS are currently listed in Environmental Protection Agency's drinking water Contaminant Candidate List (CCL 3) (*5*). PFOS has been added to the list of Persistent Organic Pollutants in the Stockholm Convention (*39*). Tap and surface water samples were contaminated with

PFOA and PFOS in many countries (*6, 10*). Trace level of these chemicals were also detected in ocean waters (*7*). In Australia the drinking water is reported to have 9.7 ng/l PFOA and 16 ng/l PFOS (*10*). The lake water in China was reported to have around 4.2 ng/l of PFOS and 5.4 ng/l of PFOA (*6*). Undoubtedly these compounds have posed emerging threat to water quality.

CURRENT TREATMENT TECHNOLOGIES

Physical and Chemical Treatment

The fact that the carbon-fluorine bond present in PFOA and PFOS is the strongest covalent bond known make it difficult to remove them by conventional water and wastewater treatment processes. PFOA and PFOS were found to be resistant towards biodegradation under aerobic conditions (*40-42*). Advanced oxidation methods using different oxidizing agents like ozone, Fenton's reagent, combination of ozone and hydrogen peroxide, ozone and UV light were inefficient in removing PFOA and PFOS (*43*). However, sonochemical degradation can effectively degrade both the compounds to produce CO, CO_2, fluoride ion, and sulfate ion in aqueous media (*44*). In this process, ultrasound waves were applied under very high temperature (600-1000 K) (*13*) and pressures to form hydroxyl radicals. For organic pollutants, hydroxyl radicals thus formed causes degradation while for hydrophobic compounds, high temperature causes direct combustion in addition to the radical formation. Another study reported a half life period as low as 43 minutes for PFOS and 22 minutes for PFOA by sonochemical method (*45*). The study concluded that that the PFOA and PFOS molecules undergo

pyrolysis at the surface between the cavitation bubbles and solution and reported sonochemical degradation of PFOS in the presence of tert-butyl alcohol to form PFOA (*45*).

Photodegradation is a possible treatment method for both PFOA and PFOS (*46*). The degradation of PFOA by photochemical method in the presence of a photocatalyst (tungstic heteropolyacid), oxygen, water, and ultraviolet-visible light irradiation was reported to produce shorter chain perfluoroalkanes with 4-6 carbon atoms. This method proves to be very efficient, removing about 90% PFOA after 72 hours of irradiation (*14*). Another study reported the photochemical degradation of PFOA using persulfate ion (*47*). The reaction was initiated by the attack of high energy sulfate radical. This method can be used for PFOA degradation in wastewater (*48*). A similar photodecomposition method has been reported for PFOS in water using UV irradiation, and alkaline 2-propanol (*49*). Hori et al. (*47*) suggested another method using zerovalent iron in subcritical water ("hot water with pressure sufficient to maintain the liquid state") as a degradation method for PFOS. No bioaccumulative perfluorinated compound is formed as product in this method.

Biological Treatment Technologies

Only a few studies explored the biodegradation potential of PFOA and PFOS (*40-42, 50*). Neither decrease in PFOA and PFOS concentrations nor release of fluoride ion by activated sludge over 18 day under aerobic conditions was reported (*41*). A similar study using activated sludge found that PFOS concentration reduced in 2 days and PFOA decreased in 25 days under anaerobic conditions (*42*). Liou et al. (*50*)

conducted an anaerobic biodegradation study of PFOA using different electron acceptors. But the study couldn't prove the degradation of PFOA. Key et al. (*40*) reported the biodegradation of organosulfur compounds with at least one hydrogen atom when tested under aerobic, sulfur limiting conditions. However, no biodegradation was observed for PFOS (*40*). Despite the possibility of biodegradation based on thermodynamic data and calculations (*15*), bacterium capable of degrading PFOA and PFOS has not been identified. More studies focusing on the biodegradation potential of PFOA and PFOS are needed.

BIODEFLUORINATION OF ALIPHATIC COMPOUNDS

Dehalogenation is an important step in the biodegradation of organohalogen compounds and different dehalogenases are known for their ability to dehalogenate aliphatic and aromatic compounds. Haloalkane dehalogenase,haloacid dehalogenases, and halohydrin dehalogenases. are some of them (*51*). This section summarizes the biological defluorination of selected aliphatic compounds.

Organofluorine Sulfonates

Key et al. (*40*) studied the biodegradation of the fluorinated sulfonates namely perfluorooctane sulfonate (PFOS), difluoromethane sulfonate (DFMS), trifluoromethane sulfonate (TFMS), 2,2,2-trifluoroethane sulfonate (TES) and 1H,1H,2H,2H-perfluorooctane sulfonate (H-PFOS) by *Pseudomonas* sp. strain D2. Under aerobic and sulfur limited conditions, PFOS and TFMS were persistent and did not show any

degradation. However for the organofluorine sulfonates with hydrogen, partial or complete defluorination was observed (*40*).

Trifluoroacetate (TFA)

Visscher et al. (52) reported the degradation of trifluoroacetate in oxic and anoxic sediments. The result also showed that the degradation under oxic condition will lead to the formation of fluoroform. A 90-week study conducted by Kim et al. (*53*) showed that TFA can undergo cometabolism under anaerobic conditions.

Trifluoroacetoacetate

The degradation of ethyl 4,4,4-trifluoro acetoacetate was observed to be occurring at the C-4 position (*54*). The usual reductive dehalogenation reactions of β-keto esters have been reported to be at α-carbon atom (*55*). The defluorination of trifluoroacetoacetate is shown in Figure 3.

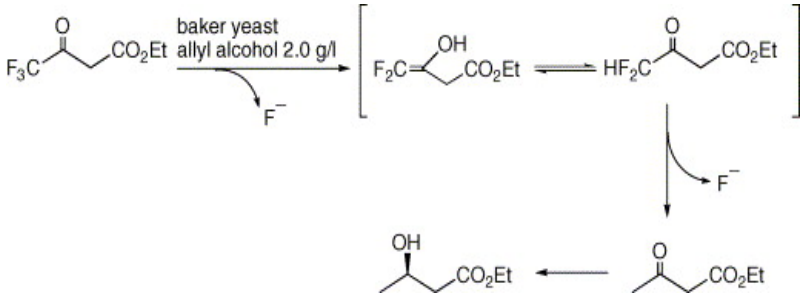


Figure 3 Defluorination of Trifluoroacetoacetate (*56*)

Fluoroacetate

Fluoroacetate is a toxic chemical and is used as a rodenticide. Goldman (*57*) reported the microbial defluorination of fluoroacetate by fluoroacetate dehalogenase in a pseudomonad isolated from soil. This study reported the degradation of the compound to give glycolate ion and fluoride ion. Figure 4 explains the defluorination mechanism of fluoroacetate dehalogenase. The enzyme fluoroacetate dehalogenase has been isolated from *Moraxella* sp. B, a soil *Pseudomonas* sp. and *Fusarium solani, Burkholderia* sp. FA1, *Pseudomonas fluorescens* DSM8341 (*16, 18, 58, 59*).

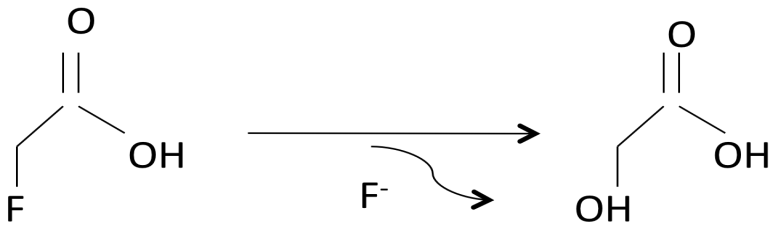


Figure 4 Defluorination via Fluoroacetate Dehalogenase (*56*)

Thus from the review, fluoroacetate dehalogenase is likely the only enzyme that catalyzes the dehalogenation reaction of an aliphatic organofluorine compound. The enzyme fluoroacetate dehalogenase is known for its ability to cleave the highly stable carbon-fluorine bond (*17*). Other dehalogenases capable of dehalogenating aliphatics are not found to be successful in the case of fluorinated aliphatics. For example, L-2-haloacid dehalogenase which is capable of catalyzing hydrolytic dehalogenation of

various haloaliphatics and haloacetates is unable to defluorinate fluorinated compounds (*60*).

EFFECT OF AMMONIA IN THE ACTIVITY OF FLUOROACETATE DEHALOGENASE ENZYME

Ammonia has been reported to inhibit the activity of the enzyme fluoroacetate dehalogenase (*19*). The reaction mechanism of fluoroacetate dehalogenase based on an X-ray crystallography study (*61*) is given in Figure 5. Ichiyama et al. (*19*) studied the catalysis linked inactivation of the enzyme. Catalysis linked inactivation typically involves a chemical reaction between the complex formed by enzyme-substrate action and a reagent not linked to the complex. This reaction leads to a permanent modification for the catalytic residue. The study was focused on whether a nucleophilic reagent like ammonia can cause this inactivation (*19, 62*). It was found that during defluorination of fluoroacetate in the presence of ammonia, the ester formed intermediately was attacked by ammonia and the enzyme activity was inhibited. Figure 6 shows the inhibition of the enzyme activity produced by the attack of ammonia. The experimental result suggests that the fluoroacetate dehalogenase's activity was completely inhibited by the action of ammonia. In this research, we try to see whether ammonia has a similar effect on the degradation of PFOA and PFOS by the two model strains.

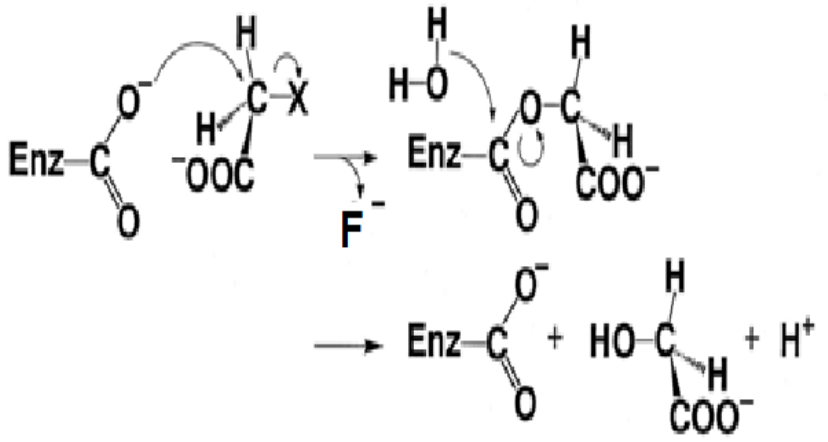
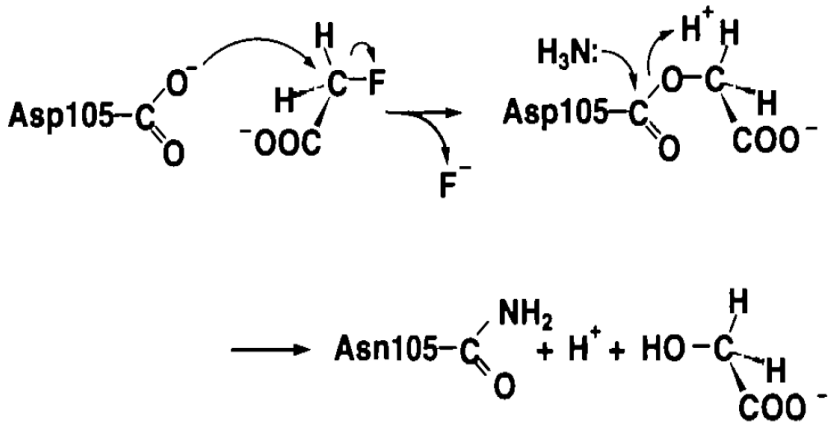


Figure 5 Reaction Mechanism of Fluoroacetate Dehalogenase (*58*)



Scheme 2. **Catalysis-linked inactivation of FAc-DEX by ammonia.**

Figure 6 Ammonia Inhibition of the Enzyme Activity (*19*)

CHAPTER III

MATERIALS AND METHODS

MATERIALS

Chemicals

Perfluorooctanoic acid (PFOA, 96%) and potassium salt of perfluorooctanesulfonic acid (PFOS-K salt, >98%) were obtained from Tokyo Kasei Kogyo Co. ltd. The stock solutions (400 mg/l) were prepared in filtered DI water and stored in glass bottles. Sodium fluoroacetate (95%) was purchased from Pfaltz and Bauer. The stock solution (10 g/l) was prepared by dissolving sodium fluoroacetate in filtered DI water.

Pure Strains and Growth Conditions

Burkholderia sp.FA1 (16) was generously provided by Dr. Kurihara (Institute for Chemical Research, Kyoto University, Japan). The cells were maintained in agar plates (1% polypeptone, 0.5% yeast extract, and 0.5% NaCl). The strain was grown aerobically in Brunner medium supplemented with 2 g/l sodium fluoroacetate and 0.01% yeast. The incubation was done at 30^{o}C in the dark with shaking at 170 rpm.

Pseudomonas fluorescens DSM8341 (18) was purchased from the German Collection of Microorganisms and Cell cultures (DSMZ) as lyophilized cells. The freeze dried culture was rehydrated and reactivated in 5 ml of nutrient broth (DSMZ Medium 1). After this reactivation, the strain was cultivated in Brunner medium (DSMZ Medium 457) supplemented with 1 g/l sodium fluoroacetate and 0.01% yeast. Brunner medium

was used for growing Pseudomonas fluorescens DSM8341 and Burkholderia sp.FA1 unless otherwise noted. Both strains were incubated at 30^{o}C in the dark with shaking at 170 rpm.

Enrichment of PFOA/PFOS-degrading Consortia

Garden soil collected from Texas A&M University was used as the inoculum. The enrichment of fluoroacetate degraders from soil mixed culture was performed following the method adopted for isolating *Burkholderia* sp.FA1 (*16*). 2ml of soil suspension (10% w/v) was added to 10 ml of the specific growth medium used for isolation of Burkholderia strain. The medium prepared was supplemented with 2 mg/l PFOA. After 10 days of incubation, 100 µl of cells were transferred to four separate vials having the following contents: 1) fresh medium spiked with 2 mg/l PFOA 2) fresh medium spiked with 2 mg/l PFOA and 100 µl of R2A medium 3) fresh medium spiked with 2 mg/l PFOS 4) fresh medium spiked with 2 mg/l PFOS and 100 µl of R2A medium. The mixed culture sets are labeled as M1-1, M1-2, M2-1, and M2-2 respectively. Figure 7 shows details of enrichment and maintenance of the mixed culture.

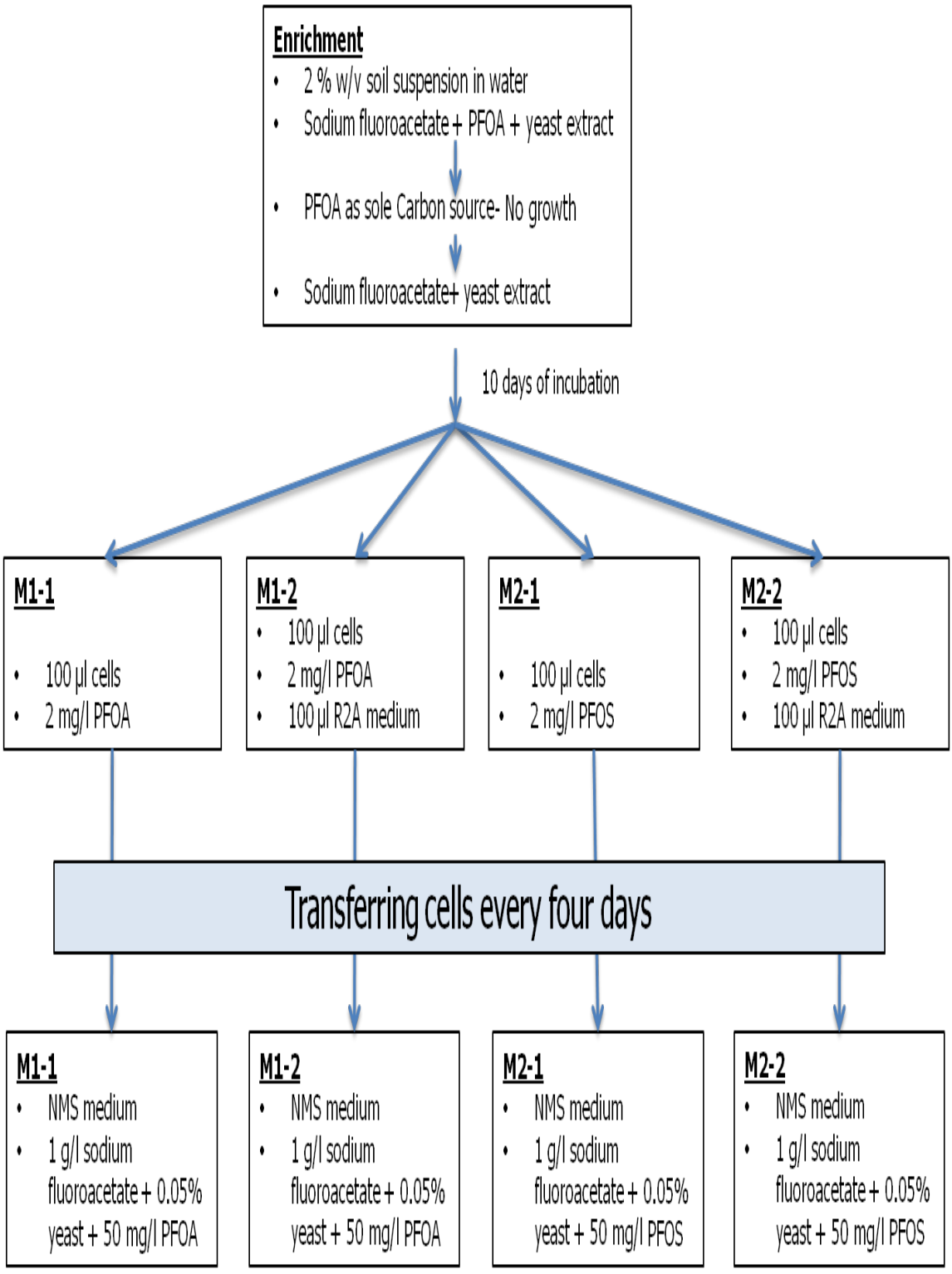


Figure 7 Enrichment of Fluoroacetate Degrading Mixed Cultures

Growth Media

Nitrate Mineral Salt (NMS) and Ammonia Mineral Salt (AMS) Medium

The NMS medium was prepared as described by Chu and Alvarez (*63*). The constituent chemicals and their concentrations in g/l are: 100 ml of $NaNO_3$ solution (9.995 g/l), 100 ml of Na_2HPO_4 (8.660 g/l) solution, 100 ml of solution containing K_2SO_4 (1.708 g/l), $MgSO_4$ $7H_2O$ (0.370 g/l), $CaSO_4$ 2 H_2O (0.121 g/l), $FeSO_4$ $7H_2O$ (0.222 g/l), 10 ml of solution containing KI (0.020 g/l), $ZnSO_4$ $7H_2O$ (0.060 g/l), $MnSO_4$ (0.030 g/l), H_3BO_3 (0.010 g/l), $CoSO_4$ (0.110 g/l). The pH was adjusted to 7.5 and the total volume was made to 1 liter. The medium was sterilized by autoclaving. For AMS medium, the $NaNO_3$ was replaced by $(NH_4)_2SO_4$ (15.539 g/l) keeping the rest of the recipe the same.

Nutrient Broth Medium (DSMZ Medium 1)

The medium was prepared by dissolving peptone (5.0 g), and meat extract (3.0 g) in 1 liter of distilled water as developed by DSMZ. The solution pH was adjusted to 7.0.

Brunner Medium (DSMZ Medium 457)

The recipe developed by DSMZ was followed for preparing Brunner medium with the following constituents: Na_2HPO_4 (2.44 g), KH_2PO_4 (1.52 g), $(NH_4)_2SO_4$ (0.50 g), $MgSO_4$ $7H_2O$ (0.20 g), $CaCl_2$ 2 H_2O (0.05 g), 10 ml of trace element SL-4 solution and distilled water 1000ml. The pH was adjusted to 6.9 and the medium was sterilized by autoclaving. The SL-4 solution can be prepared by EDTA (0.50 g), $FeSO_4$ $7H_2O$ (0.20 g), trace element SL-6 solution 100 ml and distilled water 900ml. The SL-6

solution can be prepared by dissolving $ZnSO_4$ x 7 H_2O (0.10 g), $MnCl_2$ $4H_2O$ (0.03 g), H_3BO_3 (0.30 g), $CoCl_2$ $6H_2O$ (0.20 g), $CuCl_2$ $2H_2O$ (0.01 g), $NiCl_2$ $6H_2O$ (0.02 g), Na_2MoO_4 $2H_2O$ (0.03 g).

Biomass Preparation for Degradation Experiments

Resting Cells of Burkholderia sp.FA1

100 ml of sterilized Brunner medium was spiked with 2 g/l sodium fluoroacetate and 0.01% yeast in a 250 ml glass flask. 1 ml of culture pre-grown in the Brunner medium under the same conditions was added to the 100 ml medium keeping the cell: medium ration as 1:100. The solution was incubated in dark at 30^oC by horizontal shaking at 170 rpm for 1.5 days till it reaches an OD of around 0.9. The cells were harvested by centrifugation at 10,000x g rpm for 4 minutes, resuspended in the NMS or AMS medium for degradation experimental use.

Resting Cells of Pseudomonas Fluorescens DSM8341

100 ml of sterilized Brunner medium was spiked with 1 g/l sodium fluoroacetate and 0.01% yeast in a 250 ml glass flask. 1 ml of culture pre-grown in the Brunner medium under the same conditions was added to the 100 ml medium keeping the cell: medium ration as 1:100. The solution was incubated in dark at 30^oC by horizontal shaking at 170 rpm for 2 days till it reaches an OD of around 0.8. The cells were harvested by centrifugation at 10,000x g rpm for 4 minutes, resuspended in the NMS or AMS medium for degradation experimental use.

Resting Cells of Mixed Cultures M1-1 and M1-2

100 ml of sterilized NMS medium was spiked with 1 g/l sodium fluoroacetate, 50 mg/l PFOA and 0.05% yeast in a 250 ml glass flask. 1 ml of culture pre-grown in the NMS medium under the same conditions was added to the 100 ml medium keeping the cells:medium ratio as 1:100. The solution was incubated in dark at 30^{o}C by horizontal shaking at 170 rpm for 1.5 days till it reaches an OD of around 1.0. The cells were harvested by centrifugation at 10,000x g rpm for 4 minutes, resuspended in the NMS medium for degradation experimental use.

Resting Cells of Mixed Cultures M2-1 and M2-2

100 ml of sterilized NMS medium was spiked with 1 g/l sodium fluoroacetate, 50 mg/l PFOS and 0.05% yeast in a 250 ml glass flask. 1 ml of culture pre-grown in the NMS medium under the same conditions was added to the 100 ml medium keeping the cell: medium ration as 1:100. The solution was incubated in dark at 30^{o}C by horizontal shaking at 170 rpm for 1.5 days till it reaches an OD of around 1.0. The cells were harvested by centrifugation at 10,000x g rpm for 4 minutes, resuspended in the NMS medium for degradation experimental use.

METHODS

Chemical Analysis

The mineralization of these fluorinated compounds should lead to increased concentration of fluoride ion in solution. Fluoride release will be measured to quantify the amount of degradation. The fluoride ion release was measured using the fluoride ion

selective electrode (Orion 9609BNWP) following manufacturer's instructions. The sensitivity of fluoride electrode was 0.02 mg/l. While using fluoride electrode to measure fluoride release in samples with NMS growth medium, standard solutions with different fluoride concentrations were prepared using NMS medium and 100 mg/l sodium fluoride stock solution. Similarly for experiments with AMS, standard solutions with different fluoride concentrations were prepared by serial dilution using AMS medium. In the case of pure strains, the spent growth medium obtained by growing the cells in NMS medium with sodium acetate and yeast was used for preparing standard curve. While measuring fluoride release in samples, the standard curves were plotted every 3 hours to maintain accuracy. All types of controls and samples were filtered using 0.22 um nylon membrane filter to remove bacteria before being analyzed for fluoride.

Experimental Design

The aerobic biodegradation potential of PFOA and PFOS were examined through three specific tasks as described below:

Task 1. *Examination of degradation ability of PFOA and PFOS by microorganisms capable of cleaving C-F bond.*

Pseudomonas fluorescens DSM8341 and *Burkholderia* sp. FA1 were used as the model strains for this study because they are known for their ability to cleave C-F bond via fluoroacetate dehalogenase (*16, 17*). The degradation tests of PFOA and PFOS were conducted using resting cells. Production of fluoride ion was monitored using a fluoride probe.

Task 2. Examination of degradation ability of PFOA and PFOS using mixed culture.

A parallel experiment was conducted using PFOA/PFOS enrichment mixed cultures. A series of microcosm was constructed using soil as inoculum. Degradation tests were conducted using resting cells. Similarly, liquids samples were collected and analyzed for the production of fluoride ions.

Task 3. Effect of ammonia on the biodegradation of PFOA and PFOS.

The effect of ammonia on the biodegradation of PFOA and PFOS was studied using pure strains. As ammonia has inhibiting effects on the activity of fluoroacetate dehalogenase (*19*), degradation tests were performed using resting cells in medium containing one of the two different nitrogen sources, ammonia or nitrate. The results from degradation tests with two different nitrogen sources will be compared to see if there is any inhibiting effect for ammonia on enzyme activity.

Detailed Experimental Approach

Task 1a: Biodegradation of PFOA/PFOS using resting cells of Burkholderia sp. FA1

The strain was pre-grown in Brunner medium with 2 g/l sodium fluoroacetate and 0.01% yeast extract in dark at 30^{o}C by horizontal shaking at 170 rpm for 1.5 days. The cells were harvested by centrifugation at 10,000xg rpm for 4 minutes. The supernatant was discarded and the pellets were resuspended in NMS medium for experimental use. The starting optical density (measured @600nm) of the resting cell suspension was 2.07. The experiments were conducted in 43-ml glass vials containing 10 ml of the cell suspension. To start the experiment, the vial was spiked with 250 µl of PFOA stock solution to achieve the final PFOA concentration at 10 mg/l. Blanks were

prepared similarly, except without the resting cells. Killed controls were used that contained formaldehyde-killed cells and PFOA. Controls were prepared using resting cells suspension of a non-PFOA/PFOS degrading bacterium *E. coli*. At each sampling time, vials were sacrificed for fluoride measurement. The liquid samples were first filtered with a 0.22 μm nylon filter to remove bacteria before being analyzed for fluoride. All controls and samples were in duplicates. Biodegradation of PFOS experiments were conducted similarly, expect that PFOS was used. The initial PFOS concentration was 10 mg/l.

Task 1b: Biodegradation of PFOA/PFOS using resting cells of Pseudomonas fluorescens DSM8341

Biodegradation with PFOA/PFOS were also conducted using resting cells of *Pseudomonas fluorescens* DSM8341. The experimental procedure was similar as described in Task 1a, except that the starting optical density (measured @600nm) of the resting cells was 1.63 and the experimental duration was 2.5 days.

Task 2: Biodegradation of PFOA/PFOS using mixed culture Resting Cells

The biodegradation tests for PFOA were performed similarly as described in Task 1a, except that the resting cells of mixed cultures M1-1 and M1-2, were used. The starting optical densities (measured @600nm) for M1-1and M1-2 were 1.35 and 1.47 respectively. For PFOS biodegradation tests, the starting optical densities (measured @600nm) for resting cells of M2-1 and M2-2 were 1.41 and 1.68, respectively. The same experimental procedure was followed as described in Task 1a.

Task 3. Effect of Ammonia in the degradation of PFOA and PFOS using pure strains

To examine the effects of ammonia on the biodegradation of PFOA and PFOS by the pure strains, experiments were conducted following the same procedure, except that the resting cells were resuspended in AMS medium. The incubation period was 2 days for *Burkholderia* sp. FA1 and 2.5 days for *P. fluorescens* DSM8341.

CHAPTER IV

RESULTS AND DISCUSSION

The biodegradation potentials for PFOA and PFOS by both pure culture and mixed culture were determined based on defluorination of these compounds. The maximum theoretical fluoride concentration that would be measured was 6.9 mg/l for PFOA and 6 mg/l for PFOS if both compound were completely defluorinated. The results of these experiments are described and discussed below.

DEGRADATION TESTS WITH PURE CULTURE

Biodegradation Potential of PFOA and PFOS by *Burkholderia* sp. FA1

Figure 8 shows the release of fluoride ion in the samples at the end of degradation tests using resting cells of *Burkholderia* sp. FA1 in NMS and AMS medium. Killed controls showed high readings for fluoride ion contents, suggesting the presence of unknown compounds that interfere with the reading of the fluoride ion probe. The average of the difference in fluoride releases of sample and killed control was used for determining the fluoride release. Table 1and Table 2 present the detailed results of fluoride measurement for degradation tests using *Burkholderia* sp. FA1 with NMS and AMS.

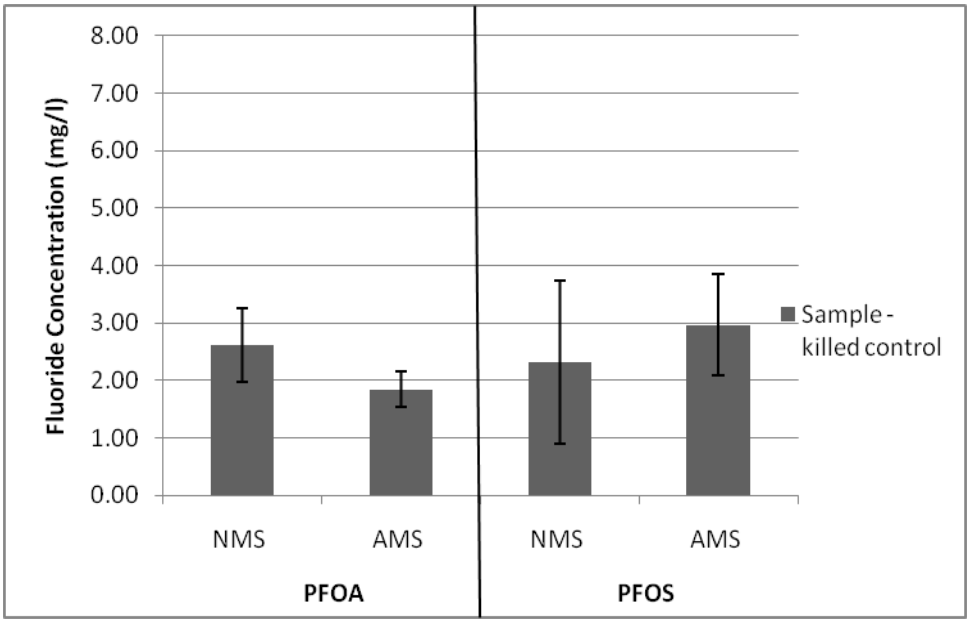


Figure 8 Fluoride Release from PFOA/PFOS by *Burkholderia* sp. FA1

Table 1: Fluoride Results for Degradation Tests using Resting Cells of *Burkholderia* sp.FA1 in NMS

Type	Bacterium	Medium	Duration of incubation (day)	PFOA (mg/l)	PFOS (mg/l)	Average Measured Fluoride concentration (mg/l)	Range (+/-)
Blank 1	No cells	NMS	0	10	0	-0.12	0.00
Blank 2	No cells	NMS	2	10	0	-0.03	0.00
Sample 1	*Burkholderia* sp.FA1	NMS	0	10	0	6.94	0.01
Sample 2	*Burkholderia* sp.FA1	NMS	2	10	0	7.85	0.12
Killed Control 1	*Burkholderia* sp.FA1	NMS	0	10	0	2.43	0.00
Killed Control 2	*Burkholderia* sp.FA1	NMS	2	10	0	5.23	0.52
Live control 1	*Burkholderia* sp.FA1	NMS	0	0	0	5.52	0.02
Live control 2	*Burkholderia* sp.FA1	NMS	2	0	0	5.63	0.04
Control 1	*E. coli*	NMS	0	10	0	-0.07	0.00
Control 2	*E. coli*	NMS	2	10	0	0.45	0.04
Blank 1	No cells	NMS	0	0	10	-0.05	0.05
Blank 2	No cells	NMS	2	0	10	-0.15	0.17
Sample 3	*Burkholderia* sp.FA1	NMS	0	0	10	4.98	0.14
Sample 4	*Burkholderia* sp.FA1	NMS	2	0	10	8.07	1.42
Killed Control 3	*Burkholderia* sp.FA1	NMS	0	0	10	2.50	0.05
Killed Control 4	*Burkholderia* sp.FA1	NMS	2	0	10	5.75	0.00
Live control 1	*Burkholderia* sp.FA1	NMS	0	0	0	5.52	0.02
Live control 2	*Burkholderia* sp.FA1	NMS	2	0	0	5.63	0.04
Control 3	*E. coli*	NMS	0	0	10	0.08	0.06
Control 4	*E. coli*	NMS	2	0	10	0.36	0.08

Table 2: Fluoride Results for Degradation Test using Resting Cells of *Burkholderia* sp.FA1 in AMS

Type	Bacterium	Medium	Duration of incubation (day)	PFOA (mg/l)	PFOS (mg/l)	Average Measured Fluoride concentration (mg/l)	Range (+/-)
Blank 1	No cells	AMS	0	10	0	0.08	0.00
Blank 2	No cells	AMS	2	10	0	0.33	0.05
Sample 1	*Burkholderia* sp.FA1	AMS	0	10	0	5.33	0.24
Sample 2	*Burkholderia* sp.FA1	AMS	2	10	0	4.95	0.13
Killed Control 1	*Burkholderia* sp.FA1	AMS	0	10	0	0.86	0.21
Killed Control 2	*Burkholderia* sp.FA1	AMS	2	10	0	3.11	0.18
Live control 1	*Burkholderia* sp.FA1	AMS	0	0	0	1.40	0.04
Live control 2	*Burkholderia* sp.FA1	AMS	2	0	0	1.52	0.06
Control 1	*E. coli*	AMS	0	10	0	0.71	0.04
Control 2	*E. coli*	AMS	2	10	0	-0.48	0.65
Blank 1	No cells	AMS	0	0	10	0.59	0.24
Blank 2	No cells	AMS	2	0	10	0.31	0.09
Sample 3	*Burkholderia* sp.FA1	AMS	0	0	10	5.23	0.00
Sample 4	*Burkholderia* sp.FA1	AMS	2	0	10	6.12	0.71
Killed Control 3	*Burkholderia* sp.FA1	AMS	0	0	10	0.89	0.19
Killed Control 4	*Burkholderia* sp.FA1	AMS	2	0	10	3.15	0.19
Live control 1	*Burkholderia* sp.FA1	AMS	0	0	0	1.40	0.04
Live control 2	*Burkholderia* sp.FA1	AMS	2	0	0	1.52	0.06
Control 3	*E. coli*	AMS	0	0	10	1.07	0.05
Control 4	*E. coli*	AMS	2	0	10	0.65	0.00

Biodegradation Potential of PFOA and PFOS by *P. fluorescens* DSM8341

Figure 9 shows the release of fluoride ions in the samples at the end of degradation tests (t=2.5 days) using resting cells of *Pseudomonas fluorescens* DSM8341. The average of the difference in fluoride releases of sample and killed control was used for determining the fluoride release. Table 3 and Table 4 present the detailed results of

fluoride measurement for degradation tests using *P. fluorescens* DSM8341 in NMS and AMS.

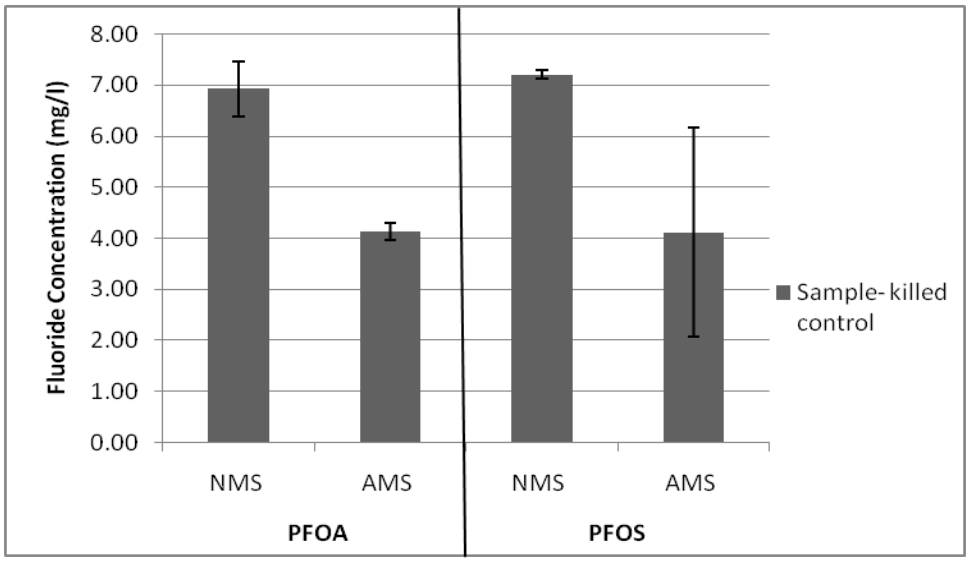


Figure 9 Fluoride Release from PFOA/PFOS by *P. fluorescens* DSM8341

Table 3: Fluoride Results for Degradation Tests using Resting Cells of *P. fluorescens* DSM8341 in NMS

Type	Bacterium	Medium	Duration of incubation (day)	PFOA (mg/l)	PFOS (mg/l)	Average Measured Fluoride concentration (mg/l)	Range (+/-)
Blank 1	No cells	NMS	0	10	0	-0.12	0.20
Blank 2	No cells	NMS	2.5	10	0	-0.03	0.03
Sample 1	*P. fluorescens* DSM8341	NMS	0	10	0	4.61	2.61
Sample 2	P. fluorescens DSM8341	NMS	2.5	10	0	7.57	0.53
Killed Control 1	P. *fluorescens* DSM8341	NMS	0	10	0	0.54	0.08
Killed Control 2	P. *fluorescens* DSM8341	NMS	2.5	10	0	0.65	0.02
Live control 1	P. *fluorescens* DSM8341	NMS	0	0	0	-0.30	0.01
Live control 2	P. *fluorescens* DSM8341	NMS	2.5	0	0	-0.30	0.05
Control 1	*E. coli*	NMS	0	10	0	-0.07	0.00
Control 2	*E. coli*	NMS	2.5	10	0	0.47	0.04
Blank 1	No cells	NMS	0	0	10	-0.05	0.06
Blank 2	No cells	NMS	2.5	0	10	0.00	0.08
Sample 3	*P. fluorescens* DSM8341	NMS	0	0	10	4.87	2.79
Sample 4	P. fluorescens DSM8341	NMS	2.5	0	10	7.87	0.08
Killed Control 3	P. *fluorescens* DSM8341	NMS	0	0	10	0.54	0.06
Killed Control 4	P. *fluorescens* DSM8341	NMS	2.5	0	10	0.67	0.01
Live control 3	P. *fluorescens* DSM8341	NMS	0	0	0	-0.60	0.03
Live control 4	P. *fluorescens* DSM8341	NMS	2.5	0	0	-0.50	0.04
Control 3	*E. coli*	NMS	0	0	10	0.08	0.06
Control 4	*E. coli*	NMS	2.5	0	10	0.67	0.02

Table 4: Fluoride Results for Degradation Test using Resting Cells of *P. fluorescens* DSM8341 in AMS

Type	Bacterium	Medium	Duration of incubation (day)	PFOA (mg/l)	PFOS (mg/l)	Average Measured Fluoride concentration (mg/l)	Range (+/-)
Blank 1	No cells	AMS	0	10	0	0.08	0.10
Blank 2	No cells	AMS	2.5	10	0	0.19	0.12
Sample 1	*P. fluorescens* DSM8341	AMS	0	10	0	4.11	0.47
Sample 2	P. fluorescens DSM8341	AMS	2.5	10	0	4.53	0.14
Killed Control 1	P. *fluorescens* DSM8341	AMS	0	10	0	0.25	0.02
Killed Control 2	P. *fluorescens* DSM8341	AMS	2.5	10	0	0.40	0.03
Live control 1	P. *fluorescens* DSM8341	AMS	0	0	0	3.88	0.05
Live control 2	P. *fluorescens* DSM8341	AMS	2.5	0	0	4.18	0.08
Control 1	*E. coli*	AMS	0	10	0	0.71	0.04
Control 2	*E. coli*	AMS	2.5	10	0	0.73	0.07
Blank 1	No cells	AMS	0	0	10	0.59	0.24
Blank 2	No cells	AMS	2.5	0	10	0.14	0.30
Sample 3	*P. fluorescens* DSM8341	AMS	0	0	10	3.94	0.36
Sample 4	P. fluorescens DSM8341	AMS	2.5	0	10	4.47	2.03
Killed Control 3	P. *fluorescens* DSM8341	AMS	0	0	10	0.29	0.03
Killed Control 4	P. *fluorescens* DSM8341	AMS	2.5	0	10	0.36	0.03
Live control 3	P. *fluorescens* DSM8341	AMS	0	0	0	3.98	0.20
Live control 4	P. *fluorescens* DSM8341	AMS	2.5	0	0	4.30	0.28
Control 3	*E. coli*	AMS	0	0	10	1.07	0.05
Control 4	*E. coli*	AMS	2.5	0	10	0.82	0.02

Discussion of Results

The results of fluoride ion measurement from the degradation tests, suggest that biodegradation of PFOA and PFOS may be occurring. The blanks did not show any release of fluoride. The fluoride data in Table 1 shows that the killed controls of *Burkholderia* sp. FA1 have relatively high values of fluoride release. This behavior was unexpected and a possible reason for the high value may be the interference from

unknown compounds present in the solution. However, the fluoride contents measured from *P. fluorescens* DSM8341 were negligible for both PFOA and PFOS.

Comparing Figure 8 and Figure 9, it can be concluded that experiments with NMS medium and *P. fluorescens* DSM 8341 show a higher amount of fluoride release than experiments with *Burkholderia* sp. FA1. The fluoride release in experiments with PFOS follows the same pattern, with *P. fluorescens* DSM 8341 showing higher fluoride release than *Burkholderia* sp. FA1. Similarly, fluoride release from PFOS in NMS medium was greater with *P. fluorescens* DSM 8341. Between PFOA and PFOS, fluoride release is greater for PFOS. However, since there is lack of additional data to quantitatively prove degradation of PFOS, further study is needed to confirm this.

DEGRADATION TESTS WITH MIXED CULTURE

Figure 10 and Figure 11 show the release of fluoride ion in the samples at the end of degradation tests using resting cells of mixed culture. As mentioned earlier, M1-1 and M1-2 are the potential PFOA degrading consortia, and M2-1 and M2-2 are potential PFOS degrading consortia. The average of the difference in fluoride releases of sample and killed control was used for determining the fluoride release. Table 5 presents the detailed results of fluoride measurement for degradation tests using mixed culture.

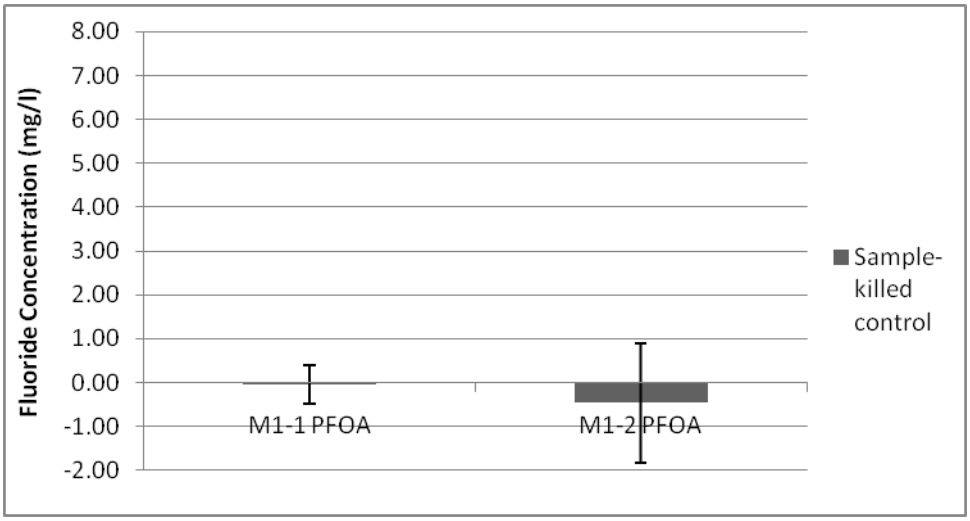


Figure 10 Fluoride Release from PFOA using Mixed Culture

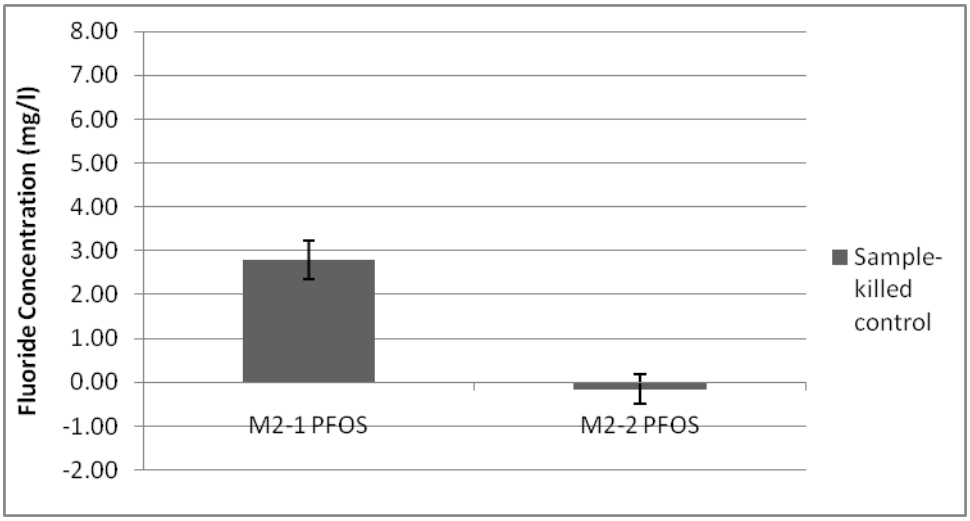


Figure 11 Fluoride Release from PFOS using Mixed Culture

Table 5: Fluoride Results for Degradation Tests using Mixed Culture

Type	Bacterium	Medium	Duration of incubation (day)	PFOA (mg/l)	PFOS (mg/l)	Average Measured Fluoride concentration (mg/l)	Range (+/-)
Blank 1	No cells	NMS	0	10	0	-0.12	0.40
Blank 2	No cells	NMS	2	10	0	-0.03	0.20
Sample 1	M1-1	NMS	0	10	0	0.56	0.27
Sample 2	M1-1	NMS	2	10	0	1.26	0.29
Killed Control 1	M1-1	NMS	0	10	0	0.55	0.08
Killed Control 2	M1-1	NMS	2	10	0	1.30	0.14
Blank 3	No cells	NMS	0	10	0	-0.12	0.50
Blank 4	No cells	NMS	2	10	0	-0.03	0.10
Sample 3	M1-2	NMS	0	10	0	0.22	0.14
Sample 4	M1-2	NMS	2	10	0	0.59	1.20
Killed Control 3	M1-2	NMS	0	10	0	0.51	0.17
Killed Control 4	M1-2	NMS	2	10	0	1.06	0.17
Blank 5	No cells	NMS	0	0	10	-0.05	0.05
Blank 6	No cells	NMS	2	0	10	-0.15	0.17
Sample 5	M2-1	NMS	0	0	10	1.36	0.09
Sample 6	M2-1	NMS	2	0	10	1.78	0.29
Killed Control 5	M2-1	NMS	0	0	10	-0.90	0.00
Killed Control 6	M2-1	NMS	2	0	10	-1.01	0.14
Blank 7	No cells	NMS	0	0	10	-0.05	0.08
Blank 8	No cells	NMS	2	0	10	-0.15	0.20
Sample 7	M2-2	NMS	0	0	10	-0.19	-0.24
Sample 8	M2-2	NMS	2	0	10	0.75	0.20
Killed Control 7	M2-2	NMS	0	0	10	-0.58	0.06
Killed Control 8	M2-2	NMS	2	0	10	0.91	0.14

Discussion of Results

The fluoride release data suggests the possible biodegradation of PFOA and PFOS using mixed culture. Figure 10 shows that fluoride releases from PFOA are

comparable for M1-1 and M1-2. The killed controls for M1-1 and M1-2 show significant fluoride ion content after 2 days of incubation. Fluoride releases from blanks are less. Thus it can be concluded that PFOA may be biodegradable by the PFOA-degrading mixed culture consortia that was enriched from soil.

Figure 11 shows the ability of M2-1 and M2-2 to degrade PFOS. Killed controls and blanks do not show any increase in fluoride concentration at the end of the experiments. From the amount of fluoride released, M2-1 can be considered to degrade PFOS better than M2-2. Thus based on fluoride data, it can be concluded that there is a microbial activity occurring in the samples that produce fluoride ion from PFOS. The fluoride measurement data for the four mixed culture consortia corroborates our earlier observation that PFOS may be more biodegradable than PFOA.

EFFECT OF AMMONIA ON THE BIODEGRADATION OF PFOA AND PFOS

Discussion of Results

Figure 8 and Figure 9 show the fluoride measurement data from degradation tests using pure cultures in AMS and NMS media. The results suggest that there is a possible inhibition effect of ammonia on the biodegradation of PFOA and PFOS by *P.fluorescens* DSM8341 and is well pronounced in experiments with *P. fluorescens* DSM8341. However, for *Burkholderia* sp.FA1 such an inhibition effect is evident for PFOA only. For experiments with PFOS the fluoride content was higher than the corresponding release in NMS. The decrease in the fluoride release from the samples can be attributed to the inhibition effect of ammonia on the enzyme activity.

CHAPTER V

CONCLUSIONS AND FUTURE DIRECTIONS

The main question addressed by this study is whether PFOA and PFOS are biodegradable. The study using two pure fluoroacetate degrading strains suggest that they may be able to biodegrade PFOA and PFOS. Future studies are needed to further confirm the observation of this study.

As a part of this study, we enriched potential PFOA/PFOS degrading consortia from soil. The enrichment cultures are found to produce an increased concentration of fluoride compared to killed controls. Since such a release is absent in blanks, the probabilities that PFOA and PFOS are being biodegraded are high.

The biodegradation potential of PFOA and PFOS were assessed based on the increased fluoride concentration in solution. However, the lack of metabolite data and quantitative proof corroborating the biodegradation of PFOA and PFOS need to be addressed. Future study should also isolate PFOA/PFOS-degrading strains from the mixed culture consortia developed in this study.

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