Study Title

Microbial Metabolism (Biodegradation)
Studies of Perfluorooctane Sulfonate (PFOS)
II. Aerobic Soil Biodegradation

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1.0 INTRODUCTION

The biodegradation program for perfluorooctane sulfonate (PFOS) was designed to offer a wide range of conditions to maximize the chance for selection and enrichment of microbial populations that could metabolize this chemical as well as other unique fluorochemicals. In addition to enrichment, the program was also designed to optimize for co-metabolism of fluorochemicals. This was done by continual addition of fresh inoculum and complex natural nutrients. The overall goal of these studies, therefore, was to observe loss of parent material and formation of degradation products as quantitatively as possible within the limits of the study designs.

Three aerobic systems were examined: a sewage treatment based system to select for faster growing species (Zymogenous), a soil based system to select for slower growing species (Autochthonous), and a pure culture system for examining specific metabolic capabilities (Cytochrome P_{450} monooxygenase). One anaerobic system was studied: 10% anaerobic digester sludge.

The overall screening program was based on key factors to maximize the chance for enrichment of those organisms capable of metabolizing unique chemicals. Among these factors were:

- testing and enrichment under non-toxic conditions
- the use of natural ecosystems as the basis for enrichment
- the use of natural nutrients from those ecosystems with supplemental trace minerals, co-factors and vitamins
- the continual introduction of new microbes from different natural sources
- the periodic replenishment of natural nutrients without diluting out the species being enriched
- the provision for a realistic time frame for enrichment and acclimation
- the protection of microorganisms from toxic products or metabolites by use of low substrate concentrations, replenishment of nutrients, balanced medium (C:N:P, etc.), proper pH and provision of a protective surface for growth (vermiculite, sand, soil, activated C, diatomaceous earth, etc.)
- the enrichment in more concentrated (higher biomass and test substance concentration) systems and examination of biodegradation in more dilute systems
- the separation of systems selective for fast growing (zymogenous) and slow growing (autochthonous) species
- use of pure cultures containing the cytochrome P_{450} monooxygenase enzyme system known to metabolize complex molecules.

Throughout the experimental program the principles outlined above for enrichment were incorporated. The results of the studies are summarized in four separate reports and provide a

basis for the future direction of the program to better understand the environmental fate of fluorochemicals. This report summarizes the results of exposure of PFOS to an aerobic soil biodegradation system.

2.0 TEST SUBSTANCE, INTERNAL STANDARDS, AND SOLUTION PREPARATION

2.1 Test Substance and Internal Standards

The test substance, perfluorooctane sulfonate potassium salt (PFOS, lot no. TN-A-2130), an off-white powder, was received on 25 January 1999 (SLI No. 70-93) from 3M Environmental, St. Paul, Minnesota. Prior to study completion, analytical characterization of the PFOS test substance was not conducted. Therefore, all calculations in the report are based on PFOS purity of 100%. After study completion, a sample of a 1.06 mg/mL PFOS stock solution (SLI No. 70-93A), see Section 2.2.2) was sent to 3M Environmental Laboratory for evaluation of impurities using LC/MS-TOF. Compounds looked for were the C2 to C10 PFOS analogous materials, and many of them were observed in both the 1-µL and 10-µL injections (e.g., masses 249, 299, 349, 399, 449, 499, and 549 were observed as peaks). The C2 to C10 carboxylates related to PFOA were looked for, and some were found. For example, the masses 213, 263, 313, 363, 413, and 463 which correspond to the C4, C5, C6, C7, C8, and C9 perfluorinated carboxylates, respectively, were observed; however, perfluorinated carboxylates were estimated to be present at <0.2% of the total material. FOSA was looked for but not observed. The perfluorooctane sulfinate was also looked for at mass 483, but not found. The percentage of each component, based on signal intensity, is presented in the following table and is based on an assumption that the signal ratio for each is 1:1 with PFOS.

Perfluorinated Alkyl Sulfonates Observed

Analyte	Peak Response (Area)	Total (%)
Perfluorononane sulfonate (C9) response	0.990	0.400
Perfluorooctane sulfonate (C8) response	223.245	90.23
Perfluoroheptane sulfonate (C7) response	5.786	2.34
Perfluorohexane sulfonate (C6) response	4.262	1.72
Perfluoropentane sulfonate (C5) response	5.326	2.15
Perfluorobutane sulfonate (C4) response	5.190	2.10
Perfluoropropane sulfonate (C3) response	2.622	1.06
Total	**247.421**	**100**

The internal standard, perfluorooctanoic acid (PFOA, lot no. 07216AS), an off-white solid wax, was received on 1 April 1999 (SLI No. 71-94) from Aldrich, Milwaukee, Wisconsin. An additional internal standard, 1,1,2,2-tetrahydroperfluorooctane sulfonate (THPFOS), a brown crystal was received on 18 January 1999 (SLI No. 70-83) from 3M Environmental, St. Paul, Minnesota. Upon receipt at Springborn, the samples of test substance and internal standards were stored in their original containers at room temperature in a dark, ventilated cabinet.

2.2 Preparation of Stock Solutions and Reagents

2.2.1 Internal Standard Stock Solutions. A 25.0 mg/L PFOA stock solution containing 15.0 mg/L THPFOS internal standard was prepared in the following manner. A 1000 µg/mL PFOA solution was prepared by placing 0.1002 g of PFOA in a 100-mL volumetric flask and bringing to volume with methanol. A 1000 µg/mL THPFOS solution was prepared by placing 0.1002 g of THPFOS in a 100-mL volumetric flask and bringing to volume with methanol. A 50-mL aliquot of the PFOA solution and a 30-mL aliquot of the THPFOS solution were placed in a 100-mL volumetric flask and brought to volume with methanol which resulted in a 500 mg/L PFOA solution containing 300 mg/L THPFOS. The 25.0 mg/L PFOA/15.0 mg/L THPFOS internal standard solution was then prepared by placing 2.5 mL of the 500 mg/L PFOA solution containing 300 mg/L THPFOS solution in a 50-mL volumetric flask and bringing to volume with methanol. No visible signs of undissolved substances were observed in any of the methanol solutions.

2.2.2 PFOS Stock Solutions. Two PFOS stock solutions were prepared and used for dosing the activated sludge/sediment, closed vial and toxicity test systems. At the time of test initiation, the actual purity of the PFOS sample had not been determined and was assumed to be 100% for preparation of stock solutions. A 1.06 mg/mL PFOS stock solution (SLI No. 70-93A) was prepared by placing 0.1061 g of PFOS in a 100-mL volumetric flask and bringing to volume with purified reagent water. This stock solution was used to dose the toxicity assay and activated sludge/sediment acclimation flasks. A 1.01 mg/mL PFOS stock solution (SLI No. 70-93E) was prepared by placing 0.1011 g of PFOS in a 100-mL volumetric flask and bringing to volume with purified reagent water. This stock solution was used to dose the closed vial (headspace) aerobic biodegradation test system. These stock solutions were suspensions and were used after vigorous shaking and sonication to deliver homogeneous suspensions to the test systems. Homogeneity was confirmed by the LC/MS analysis of a 1-mL aliquot of an aqueous PFOS stock solution that

resulted in a 100.2% recovery (SLI No. F499-59). Note that the solubility of PFOS in purified reagent grade water is 0.567 mg/mL (SD = 52.8, CV = 9.31%, n = 6) (VanHoven and Nixon, 1999).

A 1.00 mg/mL PFOS primary stock solution (SLI No. 70-93C) was prepared by placing 0.1004 g of PFOS in a 100-mL volumetric flask and bringing to volume with methanol. This stock solution was used in the preparation of quality control samples.

A final PFOS primary stock solution with a concentration of 1.22 mg/mL (SLI No. 70-93D) was prepared by placing 0.1223 g of PFOS in a 100-mL volumetric flask and bringing to volume with methanol. Secondary stock solutions with concentrations of 1.22, 12.2, and 122 mg/L were prepared by placing the appropriate volume of the 1.22 mg/mL primary stock solution in a 50.0-mL volumetric flask and bringing to volume with methanol. The primary and secondary stock solutions were used to prepare calibration standards. Preparation of the calibration standards is detailed in the following table.

Concentration of Stock Solution	Fortification Volume (mL)	Final Volume (mL)	Diluent	Standard Concentration (mg/L)
1.22 mg/mL	0.100	50.0	Methanol	2.44
1.22 mg/mL	0.0500	50.0	Methanol	1.22
1.22 mg/mL	0.0250	50.0	Methanol	0.610
122 mg/L	0.0500	50.0	Methanol	0.122
12.2 mg/L	0.125	50.0	Methanol	0.0305
1.22 mg/L	0.410	50.0	Methanol	0.0100

The calibration standards were stored in amber bottles with Teflon®-lined crimp caps. Aliquots were removed as needed for each LC/MS analysis.

The internal standards were mixed in the same proportions and added (prior to LC/MS analysis)to the calibration standards in the same manner they were added to the test samples (i.e., 25 µL of 25 mg/L PFOA/15 mg/L THPFOS to 2.00 mL of standard). Only the 1.06 mg/mL PFOS (70-93A) stock was used to dose the soil for the soil biodegradation test.

2.2.3 Ammonium Acetate Stock Solution. Ammonium acetate solutions (2 mM) were prepared by adding 0.151 g of ammonium acetate to a 1000-mL volumetric flask and bringing to volume with purified reagent water.

3.0 PREPARATION OF SAMPLES AND ANALYTICAL METHODS

The methods used in the analysis of the aqueous and solid samples are summarized in the flow chart presented in Figure 1. For each test sample, the entire test sample was transferred to a 22-mL accelerated solvent extractor (ASE) cell. Any space remaining in the ASE cell was filled with Ottawa sand. The cells were capped, shaken, and then placed on the Dionex ASE™ for extraction. The extraction was performed following the program presented below. The program was performed twice; the extracts collected separately and analyzed.

3.1 Dionex ASE™ Method

Pressure:	1500 psi
Temperature:	100 °C
Preheat Time:	0 minutes
Purge During Preheat:	Off
Heat Time:	5 minutes
Static Time:	5 minutes
Flush Volume:	60%
Purge time:	60 seconds
Static Cycles:	1
Solvent :	100% methanol

Prior to analysis, the sample extracts were filtered through a 0.2-µm filter (Titan, nylon membrane) and diluted as appropriate in methanol. A 2-mL aliquot was removed from each sample extract and 25 µL of the 25.0 mg/L PFOA/15.0 mg/L THPFOS internal standard solution was added.

3.2 Quality Control Sample Preparation

Preparation of the quality control (QC) samples is summarized in the following table. The 10-g (dry weight) aliquots were removed from each QC sample, supplemented with PFOS, mixed using a spatula and placed into 22-mL ASE cells for extraction on the Dionex ASE™.

PFOS Stock Concentration (mg/mL)	Volume of Stock Solution Used (mL)	Amount of Control Matrix[a] (g)[b]	QC Sample Concentration (mg/kg)
1.00	0.250	10.0	25.0
1.00	0.250	10.0	25.0
1.00	0.250	10.0	25.0

[a] Soil from the study
[b] Dry weight

3.3 Instrumental Conditions

The following instrumental conditions were used during the analysis of the test samples.

Instrumental System: Hewlett-Packard Model 1050 quaternary pump, membrane degasser, autosampler, PE Sciex API 100 LC/MS, PE Sciex TurboIonSpray® (electrospray)
Column: Keystone Betasil C18, 5 µm, 100 Å, 150 x 2 mm column with a Betasil C18 guard column
Mobile phases: A: 2 mM ammonium acetate in purified reagent water
B: 100% Methanol
Flow Rate: 0.3 mL/min
Gradient program:

Time (min)	%A	%B
0	60	40
8.5	10	90
11	10	90
13	0	100
17	0	100
20	60	40

Run time: 20 min
Equilibration delay: 10 min
Injection volume: 10 µL

LC/MS parameters

Experiment information:

Scan type: Q1, SIM
Scan time: 2.01 sec
Peak Hopping: Disabled
Mass defect: 0 mmu/100 amu
Pause time: 2 msec
Dwell time: 400 msec
Masses scanned (amu)*: 413 (PFOA), 427 (THPFOS), 499 (PFOS), 616, 630 (N-EtFOSE-OH)
* Based on 3M analytical method No. ETS-8-11.0

State file information:

Source parameters:

Polarity:	Negative
Turbolon spray voltage:	-5000 volts
Temperature:	400 °C
Orifice Potential:	-20 volts
Nebulizer gas:	air (high purity)
Auxiliary gas:	nitrogen

4.0 TEST PROCEDURES, RESULTS, AND DISCUSSION

4.1 Test Procedures

Test vessels used during the aerobic soil study were 40-mL I-Chem glass vials with silicone/Teflon®-lined septum screw caps. Three soils and two sediments from diverse sources in Massachusetts were collected, air dried briefly by spreading out on aluminum foil on a laboratory bench to remove excess water, and sieved through a 2-mm screen. Soils were collected from a hardwood forest in Hanson, MA, a pine forest in Onset, MA and a river bank in Bridgewater, MA. Sediments were from brackish sites below the Wareham wastewater treatment plant outfall and from the Narrows area in Wareham, MA. The percent moisture was then determined for each soil and sediment and a 200-g (dry wt.) aliquot of each soil and sediment were thoroughly mixed using a spatula. The water holding capacity (WHC) of the soil/sediment mixture was determined by adding 10 g of the mixture to a glass column, pumping water up through the soil column until breakthrough was observed and recording the amount of water added. The volume of water added was divided by the amount of dry soil present and resulted in a measured value of 39.5% WHC.

Ten gram aliquots (based on dry weight) of the soil sediment/mixture were added to each of twenty test vessels (ten for the test substance, ten blanks). The soils were initially adjusted to 75% of the WHC by adding water and the nutrients described below. During the study, soil moisture was monitored each week by weighing individual vessels and adding reagent water, if required. Soil biomass was not measured prior to initiation of the study, but was determined on day 83 to confirm that a viable microbial population still existed.

Potting soil extract was prepared by mixing 400-g soil potting soil (from a local nursery) with one liter of water and autoclaving for 45 minutes. After cooling and settling, the potting soil extract was filtered and the TOC was determined to be 16,360 mg/L via analysis on a Dohrmann Model DC80

total organic carbon analyzer. Sample injections for each replicate were performed in duplicate to demonstrate reproducibility.

Soils in each of the twenty test vessels were dosed with, 10 µL of a 1/100th strength trace mineral solution (Trace Minerals Research, Roy, Utah), 100 µL of yeast extract (5 mg/mL stock, Acumedia, Baltimore, MD), 30 µL of potting soil extract and 100 µL reagent water. These additions brought the soil moistures to approximately 75% of WHC. A 200-µL aliquot of the 1.06 mg/mL PFOS stock solution (SLI No. 70-93A) was then added to the test substance test vials, resulting in an PFOS concentration of approximately 21.2 mg/kg soil. Blank control soils received 20-µL of methanol. Following dosing, the vials were incubated in the dark at 22 ± 3 °C for twenty weeks in an environmental chamber. Soil microbial biomass was determined at day 83 of the study by both fumigation/extraction and standard plate counts to evaluate the microbial population.

On test Days 7, 14, 21, 28, 35, 42, 49, 56, and 63, one blank tube and one PFOS tube were removed from incubation and stored at 4 °C prior to analysis.

4.2 Results and Discussion

The fumigation/extraction technique (SLI SOP No. 2.4.17) is a measure of the carbon from microbial sources and is determined as the difference in TOC between soils fumigated with chloroform and non-fumigated (Brookes, et. al., 1987). The biomass of the control soil at day 83 of the study was determined to be 17.4 mg C/100 g soil. Standard plate counts (SLI SOP No. 7.26) on nutrient agar determined the microbial population to be 6×10^5 cells/g. The biomass values are typical for an active microbial community.

Table 1 summarizes the fate of PFOS in the soil system over the 63-day test period. Mass balances ranged from 102% to 121% and indicated that essentially no PFOS metabolism occurred during the study. Analysis of the QC samples with each set of test system samples resulted in measured concentrations which were consistent with the recovery range determined during the method validation study. Based on these results, it was established that the appropriate quality control was maintained during the analyses of the test samples.

5.0 CONCLUSIONS AND FUTURE STUDIES

Acclimation to PFOS degradation, if it occurs, may take substantial time. It has been noted that anoxic settling ponds at the 3M plant in Decatur, Alabama contain materials tentatively identified as perfluorooctane sulfinate (reduced PFOS). Thus, it may be beneficial to sample both aerobic and anaerobic sites known to have been exposed to PFOS for a prolonged time period. Microbes in such a system may be able to use PFOS as an electron acceptor or possibly a source of sulfur. Time decay studies with these inocula may provide the best chance to demonstrate PFOS metabolism.

REFERENCES

VanHoven, Raymond L. and Willard B. Nixon. 1999. Determination of the Water Solubility of PFOS by The Shake Flask Method, Wildlife International LTD. Project Number 454C-107, OECD Guideline for the Testing of Chemicals, 105 Water Solubility, May 3, 1999.

Brooks, P.C, D.S. Jenkinson, and E.D. Vance. 1987. An extraction method for measurement of soil microbial biomass carbon. Soil Biol. Biochem. 19:703-707.

Table 1. Results for the test samples from the aerobic soil biodegradation study.

Sample No.[a]/ Type	Nominal (mg/kg)	PFOS Concentration	
		Measured (mg/kg)	Measured (%)
		Total	Total (Mass Balance)
Test Samples			
SO499-01/Day 7	21.2	22.6	107
SO499-02/Day 14	21.2	21.3	101
SO499-03/Day 21	21.2	20.4	96.1
SO499-04/Day 28	21.2	23.4	111
SO499-05/Day 35	21.2	22.7	107
SO499-06/Day 42	21.2	22.8	108
SO499-07/Day 49	21.2	21.8	103
SO499-08/Day 56	21.2	21.9	103
SO499-09/Day 63	21.2	24.1	114
Blank Samples			
SO499-11/Day 7	0.00	< 0.0100[b]	NA[c]
SO499-12/Day 14	0.00	< 0.0100	NA
SO499-19/Day 63	0.00	< 0.0100	NA
QC[d] Samples			
C999-75	25.0	26.2	105
C999-76	25.0	27.0	108
C999-77	25.0	27.1	108

[a] Day 0 samples were not taken.
[b] Values represented by < 0.0100 were below the limit of quantitation.
[c] NA = not applicable
[d] QC = quality control sample

Figure 1. Flow chart of extraction procedures.