Study Title

Microbial Metabolism (Biodegradation)
Studies of Perfluorooctane Sulfonate (PFOS)
III. Anaerobic Sludge Biodegradation

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Study Completed On

31 October 2000

Submitted To

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Laboratory Project ID

Springborn Study No.: 290.6120

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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 for PFOS 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 anaerobic sludge 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 perfluorooctance 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 50.0-mL aliquot of the PFOA solution and a 30.0-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 2.50 mL of the 500 mg/L PFOA solution containing 300 mg/L THPFOS solution in a 50.0-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 aqueous stock solution was used to dose the PFOS anaerobic sludge system.

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 anaerobic biodegradation test sample a 10.0-mL aliquot was removed from the vessel containing the sample and centrifuged at 1200 x g (rotating radius of 12.5 cm, from RCF Nomagraph, IEC (International Equipment Company), 300 Second Ave., Needham Heights, Massachusetts, 02194) for 30 minutes with a Beckman Model 65-6R centrifuge. The procedures used for the resultant aqueous and biomass samples are detailed below.

3.1 Preparation of the Aqueous Test Samples

A 1:0-mL aliquot of the centrifuged supernatant was added to a volumetric flask and diluted to a volume of 25 mL with methanol. The samples were then filtered through a 0.2-µm filter (Titan, nylon membrane) prior to analysis. A 2-mL aliquot was removed from each sample and 25 µL of the 25.0 mg/L PFOA/15.0 mg/L THPFOS internal standard solution was added to the sample aliquot.

3.2 Preparation of the Biomass Samples

Each solid sample was extracted once with 40 mL of methanol by vortexing for 30 seconds, sonicating for 15 minutes, and shaking on a shaker table at 200 rpm for 30 minutes. The samples were then centrifuged at 1200 x g (rotating radius of 12.5 cm, from RCF Nomagraph, IEC (International Equipment Company), 300 Second Ave., Needham Heights, Massachusetts, 02194) for 30 minutes and the supernatant was decanted. The supernatant was then filtered through a 0.2-µm filter (Titan, nylon membrane) prior to analysis. If necessary, the sample extract (supernatant) was then diluted as appropriate in methanol. A 2-mL aliquot was removed from each sample extract and 25 µL of the 25 mg/L PFOA/15 mg/L THPFOS internal standard solution was added.

3.3 Quality Control Sample Preparation

Preparation of the aqueous and solid quality control (QC) samples is summarized in the following table. When necessary and prior to QC fortification, the media were centrifuged at 1200 x g for 30 minutes to separate the solid and aqueous portions. All QC samples were prepared by fortifying the appropriate control matrix (aqueous medium, solid medium) at the concentrations listed below. The QC samples were then treated following the same procedures as the test samples (as described above).

QC Sample Type	PFOS Stock Concentration (mg/mL)	Volume of Stock Solution Used (mL)	Control Matrix	Volume of Control Matrix (mL)	QC Sample Concentration (mg/L)
Aqueous	1.00	0.0250	1	5.00	5.00
	1.00	0.0500	1	5.00	10.0
	1.00	0.100	1	5.00	20.0
Solids	1.00	0.0250	2	10.0	2.50
	1.00	0.0250	2	10.0	2.50
	1.00	0.0500	2	10.0	5.00

1 = Aqueous portion from centrifuged anaerobic blank
2 = Solid portion from centrifuged anaerobic blank (10 mL)

3.4 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 TurbolonSpray® (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-alcohol)

* Based on 3M analytical method No. ETS-8-11.0

State file information:

Source parameters:

Polarity:	Negative
TurboIonSpray® 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

Twenty 160-mL serum bottles with crimped butyl rubber tops were established for this test, ten PFOS bottles and ten blank bottles. On test day 0, two 3.0-L batches of OECD mineral media were prepared and purged with nitrogen. The batches of mineral media were enriched and inoculated as follows: A 323-mL aliquot was removed from each mineral media batch to make space for the following, which were added to each batch while stirring; 6.00 mL of a 0.500 mg/mL resazurin stock solution to produce a 1.00 mg/L concentration in each mineral media batch, 16.5 mL of dried sludge extract to produce approximately 100 mg C/L concentration in each batch, and 300 mL of anaerobic digester sludge. The dried sludge extract was prepared on 4/29/99 as follows: dry sludge was collected from the rotating biological contacter (RBC) wastewater treatment, Bridgewater, Massachusetts. A 200-g aliquot was added to 800 mL of purified reagent grade water, and the sludge mixture was autoclaved for 30 minutes at 121 °C. After autoclaving, the sludge mixture was centrifuged and then filtered twice; the first time through a Whatman #41paper filter and the second time through a Whatman glass fiber filter. The resultant sludge extract was

refrigerated at 4 °C until used and was determined to contain approximately 18,400 mg C/L via analysis on a Dohrmann DC-80 carbon analyzer. The anaerobic sludge was collected from an anaerobic digestor at the Rockland, Massachusetts wastewater treatment plant, and was maintained so as to minimize exposure of sludge to oxygen.

A 100-mL aliquot of the enriched and inoculated mineral media solution was added to each serum bottle. The PFOS bottles were then dosed with 2.0 mL of a 1.06 mg/mL PFOS stock solution to produce a PFOS concentration of 20.8 mg/L. Nitrogen was used to purge the headspace in all the PFOS and blank bottles and the bottles were crimped closed. The bottles were maintained in the dark at 35 °C and were not fed with additional nutrients during the study.

On test Days 7, 14, 21, 28, 35, 42, 49, and 56, one inoculum blank bottle and one PFOS bottle were removed from incubation and stored at 4 °C prior to analysis. Day zero samples were not obtained. One blank tube was analyzed on days 7 and 56.

4.2 Results and Discussion

Table 1 presents the anaerobic sludge analytical data. Mass balances remained in the 101% to 108% range and indicated no apparent PFOS biodegradation over the 56-day period.

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.

Table 1. Results for test samples from the anaerobic biodegradation test.

Sample No./Type[a]	Nominal (mg/L)	PFOS Concentration					
		Measured (mg/L)			Measured (%)		
		Biomass	Medium	Total	Biomass	Medium	Total (Mass Balance)
Test Samples							
AN599-01/ Day 7	20.8	7.36	13.6	21.0	35.4	65.5	101
AN599-02/ Day 14	20.8	7.13	15.3	22.5	34.3	73.7	108
AN599-03/ Day 21	20.8	7.23	15.1	22.3	34.8	72.5	107
AN599-04/ Day 28	20.8	6.09	15.8	21.9	29.3	75.9	105
AN599-05/ Day 35	20.8	6.28	14.8	21.1	30.2	71.3	102
AN599-06/ Day 42	20.8	6.93	15.2	22.2	33.3	73.3	107
AN599-07/ Day 49	20.8	6.09	15.3	21.4	29.3	73.4	103
AN599-08/ Day 56	20.8	6.41	15.9	22.3	30.8	76.4	107
Blank Samples							
AN599-11	0.000	< 0.0100[b]	< 0.0100	< 0.0100	NA[c]	NA	NA
AN599-18	0.000	< 0.0100	< 0.0100	< 0.0100	NA	NA	NA
QC Samples[d]							
C799-191[e]	5.00	NA	5.05	NA	NA	101	NA
C799-192[e]	10.0	NA	10.2	NA	NA	102	NA
C799-193[e]	20.0	NA	19.4	NA	NA	96.9	NA
C799-194[f]	2.50	2.27	NA	NA	90.9	NA	NA
C799-195[f]	2.50	2.62	NA	NA	105	NA	NA
C799-196[f]	5.00	5.00	NA	NA	100	NA	NA

[a] Day 0 samples were not taken.
[b] Values expressed as < 0.0100 were below the limit of quantitation
[c] NA = not applicable
[d] QC = quality control sample.
[e] QC sample fortified in control medium.
[f] QC sample fortified in blank biomass.

Figure 1. Flow chart of extraction procedures.