

Study Plan for Biodegradation This includes studies on:
aerobic acclimated closed bottle biodegradation, aerobic
soil/sediment biodegradation, pure culture aerobic, and
fluorochemical decomposition process.

Study Designs – 3M Microbial Metabolism Program

Goal: To optimize conditions that permit enrichment of microbial populations capable of metabolizing fluorochemicals.

A. Introduction

The program and test systems described below offer a wide range of conditions designed to enrich for microbial populations that can metabolize fluorochemicals. Even though these systems are fairly complex and require significant maintenance, they are designed primarily as "screening" systems to assess if microbial metabolism of individual fluorochemicals can occur. At the screening phase, all possible control systems, multiple replicates, etc. can not (and should not) be included because of the number of analytical samples generated and the effort required to service each test system. Because of the complexity of many fluorochemicals, metabolism may be limited or not occur in most of the systems. If metabolism is seen in screening systems, they will serve to guide more detailed metabolism studies under a wider range of test conditions.

The key to a successful microbial selection or enrichment program (i.e. one that maximizes the chance for enrichment of those organisms capable of metabolizing fluorochemicals) is often based on providing the following:

- Use of natural ecosystems as the basis for enrichment
- Use of natural nutrients from those ecosystems with supplemental trace minerals, co-factors and vitamins
- Continual introduction of new microbes from different natural sources
- Periodic replenishment of natural nutrients without diluting-out the species being enriched.
- Providing a realistic time frame to permit enrichment and acclimation
- 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 (vermiculate, sand, soil, activated C, diatomaceous earth, etc.)
- Enrichment in more concentrated (higher biomass & test material conc.) systems and examination of biodegradation in more dilute systems.
- Separation of systems selective for fast growing (zymogenous) and slow growing (autochthonous) species.

Note that throughout the scheme outlined below, we have attempted to incorporate the principles outlined above for enrichment. Most of what is described has been gained from our background in microbial ecology and practical experience with isolation and culture of unique microorganisms.

B. Aerobic Enrichment Systems – General Discussion

Three aerobic systems are proposed: a sewage treatment based system to select for faster growing species (Zymogenous), a soil based system to select for slower growing species (Autothonous) and a pure culture system for examining specific metabolic capabilities (Cytochrome P-450 monooxygenase).

1. Sewage treatment based system

Secondary aerobic sewage treatment systems include primarily activated sludge (AS), rotating biological contactors (RBC) and trickling filter (TF) wastewater treatment plants. We also include as part of sewage treatment systems the immediate receiving water and top-most floc (sediment) on the banks of the receiving stream or pond (tertiary treatment!). Two sources of activated sludge will be used, Wareham and New Bedford, and one RBC plant, Bridgewater, will be sampled. Wareham and Bridgewater are primarily domestic wastewater treatment plants (WTP) of about 1MGPD flow. New Bedford is a new 30 MGPD WTP which receives light industry and domestic waste (approximately 20:80). The New Bedford plant has been shown to have greater metabolic capabilities for certain classes of chemicals. The receiving water system, site for sediment collection, will be downstream from the Wareham STP outfall, since New Bedford discharges far out into the Bay. There are no TF plants in the nearby area, but microbial growth from the Bridgewater RBC plant should mimic attached growth from a TF plant. Fresh raw sewage from the Wareham plant will be collected on a weekly basis and used as the primary growth medium. DOC from each of these plants is typically below 40 mg/L, so supplementation with activated sludge extract to achieve levels of ~100 mg C/L will be done. AS extract is prepared from dried Bridgewater AS by mixing one part dried sludge with 4 parts water and autoclaving for 30 min. After cooling the mixture is settled and filtered through a fiberglass filter. TOC of this extract is in the range of 3500 mg/L.

Test vessels for acclimation will be baffled 300-mL Erlenmeyer (DeLong neck) flasks containing ~100 mL of enrichment medium and sealed with a polyurethane plug. Natural sewage will be supplemented with OECD mineral salts at the concentrations specified for the OECD 301B test, trace minerals (Trace Minerals Corp. 0.01 mL/100 mL) and yeast extract (5 mg/100 mL). A sandy soil will also be added (1 g/100 mL) to provide a surface for microbial growth as well as an additional source of microorganisms. This soil was collected by American Ag Services, Inc. on 10/28/98 and is classified as a loamy sand with 77% sand, 21% silt and 2% clay. It contains 2.5% organic matter and has a pH of 4.9. Activated sludge, RBC sludge and receiving water sediment (1 g/L each d. wt. basis) will be the primary source of microorganisms. Test material will be added at a concentration of 20 mg a.i./L. A blank control flask containing inoculum but no test substance will also be included. Once per week, media from acclimation systems will be centrifuged and two-thirds of the supernatant and 10% of the biomass will be removed. Fresh medium and test material will be added. Additional fresh biomass (activated sludge, RBC sludge and sediment at a

concentration of 10% of the original concentration) will be added and the pH adjusted to ~7.0, if necessary. Weekly addition of ~100 mg C/L from natural sewage and AS extract should allow the microbial population to increase on a weekly basis (perhaps by 50 mg cells/L based on a 50% growth yield from C).

On a weekly basis, test medium and 10% of the centrifuged biomass will be extracted and analyzed for DOC, parent material and potential metabolites. Depending on the test substance, foam stoppers may also be extracted and analyzed. The acclimation process will continue until evidence of test material degradation has occurred or for a maximum of 8-10 weeks. If microbial metabolism is seen, a more definitive assay will be conducted. Assay for rates of degradation and metabolite formation will be conducted in sealed 20 or 40-mL serum bottles containing two-thirds of the volume as nutrient medium, inoculum and test material (biomass concentrations in the 10-50 mg/L range). Bottle headspace will periodically be assayed for carbon dioxide and volatile organics with a Thermoglas 1200 TOC analyzer. Medium will also be assayed for parent material and metabolites via appropriate LC-MS technology.

Prior to initiation of testing, each fluorocarbon will be examined for its microbial toxicity. The assay system will consist of 125-mL Erlenmeyer flasks to which 50-mL OECD mineral salts medium containing 20 mg/L test substance and 10 mg/L ^{14}C -labelled sodium benzoate will be added. Blank controls will be included and contain all ingredients except test substance. The flasks will have a reservoir above the test medium that contains 750 μL 0.1N KOH. The assay will be initiated by adding 1.0 mL of medium from the blank control flask, described above, and sealing the vessel with a silicone stopper. Assay for $^{14}\text{CO}_2$ will be conducted at 4-6 hours and for several days (e.g. at days 1, 2 and 4) by removing 100 μL from the KOH reservoir and counting on the LSC. Toxicity will be assessed by comparing the rate of $^{14}\text{CO}_2$ evolved from the test substance and control systems.

2. Soil/sediment based system

Soil/sediment studies will be conducted in 50-ml test vessels containing 20 g of the soil/sediment mixture. Individual test vessels will be sacrificed at each time interval. Three soils and two sediments, freshly collected, will be used as the source of microorganisms. Soils will be from several diverse sources (hardwood forest, pine forest and a river bank soil/sediment from Bridgewater), mixed and screened through a 2-mm mesh screen. The two sediments will be collected from the Agawam River from areas below the Wareham WTP and at low tide from a brackish site behind the town of Wareham. These sediments will be combined in equal volumes based on dry weight. Equal amounts of each soil and the one sediment mixture will be combined (dry wt. basis) supplemented with trace minerals (1.0 mL/kg, yeast extract (50 mg/kg) and soil extract (100 mg C/kg). Soil extract is prepared from a rich organic soil or humus by mixing one part with four parts water and autoclaving for 30 minutes. After cooling and filtration through a glass fiber filter, TOC is in the range of 500-800 mg C/L. Test

material will be added at 20 mg/kg. Blank control soils/sediment systems will also be set up and will contain all ingredients except test substance.

On a weekly basis, individual test vessels will be sacrificed and analyzed for the particular fluorocarbon. Soil extract and yeast extract will be added as fresh nutrients and to adjust soil moisture.

3. Pure culture system

Springborn maintains a bank of bacteria, actinomycetes and fungi that have been demonstrated to metabolize complex molecules in a manner very similar to mammalian (microsomes) and plant species. These species have been isolated from natural ecosystems (soils primarily) and contain the cytochrome P-450 monooxygenase enzyme system. Mammalian systems (liver hepatocytes) are known to rapidly metabolize Et-FOSE-OH and there is potential for these microorganisms to do the same.

We have reviewed a portion of our data base consisting of nineteen species and ten complex molecules and have selected three fungi and one actinomycete based on their ability to metabolize the greatest range of molecules. These species are: *Cunninghamella echinulata*, *Mucor circinelloides*, *Phanerochaete chrysosporium* and *Streptomyces griseus*. These cultures will be grown in complex media in 250-mL shake flasks in the presence of the chemical of interest for seven days. Following growth, cells will be separated from broth and both will be extracted and examined for the parent material and metabolites. Also, since certain cultures may not readily metabolize difficult to degrade substances, resting cell suspensions (washed to remove complex organics growth nutrients) may be examined for their ability to transform the test substance. If certain cultures are observed to metabolize the chemical of interest, definitive studies will be conducted to establish kinetics of degradation and to identify metabolites.

C. Aerobic Enrichment Systems – Test System Summaries

1. Sewage Treatment Acclimation System (*Zymogenous sp.*)

a. Test vessels – 300-mL baffled Erlenmeyer flasks, polyurethane & s.s. stoppers.

b. Test medium – Natural sewage from one A.S. plant + OECD mineral salts + trace minerals & yeast extract (50 mg/L). Supplementation will be done with A.S. extract to achieve ~100 mg/L C. Also, an appropriate surface for microbial growth (sandy soil). Total volume 100 mL/flask; incubation at 22°C.

c. Inoculum – A.S from Wareham & New Bedford, attached microbial growth (periphyton) from Bridgewater RBC STP, receiving water sediment (floc) from Wareham STP (each @1000 mg/L d.wt. solids).

d. Test Material – Test system will be dosed at 20 a.i. mg/L. Blank controls with no test material will also be included and flasks will be somewhat larger (500-1000 mL) to accommodate analytical needs for five test substances. Additional flasks, identical in every way except that they contain no biomass will also be included as analytical controls. Potential toxicity of test material will be established via respirometry (3-4 day study in a miniaturized test system using ^{14}C -benzoate). Sterile controls may be added based on analytical requirements.

e. Enrichment Conditions – Once per week, test medium will be centrifuged and $2/3^{\text{rd}}$ of the supernatant and 10% of the biomass will be removed. Medium removed will be replaced with fresh medium and test material. An additional amount of A.S., RBC microbes and sediment equivalent to 10% of the original amount will also be added weekly. Medium pH will be adjusted to ~7.0 with 0.1N HCl or NaOH if necessary. Enrichment will continue for at least 8-10 wks.

f. Analysis – On a weekly basis, test medium and 10% of the centrifuged biomass will be extracted (if appropriate) and analyzed for parent & metabolites. Samples not assayed immediately will be refrigerated. Carbon analysis will be conducted on supernatants. Analysis of foam plugs may be included based on the test material. Analytical results will trigger need for definitive testing as indicated below.

g. Test Material Requirements – Enrichment systems will require 16.0 mg of active ingredient (a.i.) [2.0 mg day 0, 1.5 mg weekly for 9 weeks, and 1.0 mg for toxicity assay]. Definitive assays will require about 10 mg [set up 16 vials with 20 mL medium].

h. Definitive Assays – For enrichment systems showing positive signs of biodegradation, metabolism profiles will be developed in 20 or 40-mL serum vials. Vials will contain $2/3^{\text{rd}}$ volume as nutrient medium and inoculum at a concentration from the acclimated enrichment medium (10-50 mg solids/L) plus test material. Headspace will be assayed on days 0, 1, 3, 7, 14, 21 and 28 for CO_2 , volatile organics and perhaps O_2 . Medium will be assayed for parent and metabolites on days 0, 7, 14, 21 & 28.

2. Soil Based System (*Autochthonous sp.*)

a. Test vessels – 50-mL culture tubes with polyurethane stoppers.

b. Inoculum – Three soils and two sediments collected from various sites in the Cape Cod area will be used. Soils will be sieved through a 2 mm screen and mixed. Sediments will be collected at low tide and mixed on an equal d.wt. basis with the soils. Ten grams of the soil mixture will be added to each culture tube.

c. Test Material – Test material will be added at 20 mg/kg. Blank controls with no test material will also be included. Need for abiotic controls will be dependant

on test material. Potential toxicity of test material would not need to be re-established via respirometry since it will be done in the sewage system.

d. Enrichment Conditions – Soils will be adjusted to approximately 75% moisture holding capacity (MHC) with soil extract prepared from a rich organic soil or peat. MHC is determined on dried soil samples placed in a column and passing water through the column until break-through. Additional soil extract, trace minerals and yeast extract will be added on a weekly basis. The enrichment will continue for at least 8-10 wks.

e. Analysis – On a weekly basis, blank tubes and tubes containing test material will be sacrificed, extracted and analyzed for parent & metabolites. Based on analytical results, fresh samples of the same soil/sediment mixture will be re-dosed with test material and kinetics of degradation will be monitored over time.

f. Test Material Requirements – Enrichment systems will require 6 mg [0.4 mg for each tube times enough tubes for 10 weeks, and 2 mg for toxicity assay]. Definitive assays will require about another 6 mg.

3. Pure Culture Systems (Cytochrome P-450 Enzyme System)

a. Test vessels – 250-mL Erlenmeyer flasks, polyurethane & ss stoppers.

b. Test medium – Growth will be conducted in complex microbiological media (soybean grits-glucose) for all species. Total volume ~60 mL /flask; incubation at 26°C.

c. Microorganisms – Four species [*Cunninghamella echinulata*, *Mucor circinelloides*, *Phanerochaete chrysosporium* and *Streptomyces griseus*] will be evaluated for their ability to metabolize test materials.

d. Test Material – Test material will be added at 20 mg/L. Blank controls with no test material will also be included.

e. Growth Conditions – Actively growing cultures will be transferred from agar slants to fresh medium (Stage 1). Stage 1 cultures will be grown for 3-6 days and 6.0 mL will be transferred to 60-mL of fresh medium containing test material (Stage 2). Stage 2 cultures will be grown for seven days until the early to mid-stationary phase of growth. Cells will be separated from broth and each examined analytically. Based on results from growing cell suspensions, cells may also be grown in complex medium without test material to the early stationary phase, centrifuged and washed. Washed cells would then be resuspended in mineral salts medium containing trace minerals, vitamins and test material (resting cell suspensions). After 72 hr, cells and broth will be separated for analysis.

f. Analysis – Cells and broth are extracted (at times zero and after seven days) and analyzed for parent & metabolites. Foam stoppers may be analyzed based on potential volatility of test materials or degradation products.

g. Test Material Requirements – Assays will require 9.0 mg for growing cells and an additional 9.0 mg if resting cells are studied..

h. Definitive Assays – Establish kinetics of degradation and metabolite identification.

D. Test matrix

Tables 1 and 2 summarize the proposed test sampling matrix. Table 1 displays the samples under study and those to be assayed on a weekly basis. Five test substances (Group 1) will be examined first. Additional test substances (Group 2) as well as test systems will be added over time. Table 2 presents more detail of the actual numbers of samples for each test substance on a weekly basis. A "0" in Table 2 indicates the day of initiation for that particular test matrix and that no sample was taken. Pure culture studies are sampled on day "0".

E. Additional test matrices

Additional test matrices will include anaerobic sludges and sediments as well as soil and sewage sources provided by 3M. These will be added to this text in the near future. Also, the need to incorporate sterile control matrices will be evaluated as analytical results are received.

Anaerobic Enrichment Systems

A. Sewage Treatment Acclimation and Assay Systems

Test vessels – 160-mL serum bottles with crimped butyl rubber or Teflon® stoppers. Assay based on OPPTS 835-3400 and subsequent modifications by Birch *et al.* At least three bottles will be set up for each test material.

Test medium – OECD mineral salts + trace minerals & vitamins and 0.0002% resazurin will be added to A.S. extract. Total volume ~100 mL/bottle after inoculum addition.

Inoculum – Mixture of anaerobic digester sludge from Wareham & New Bedford STP's will be mixed equally under conditions to maintain anaerobicity and added to the test medium (10% v/v). Incubation will be at 35°C.

Test Material – Addition in the 30-50 mg/L (carbon basis) range. Blank controls with no test material also included.

Enrichment Conditions – On a weekly basis, add additional activated sludge extract through the septum while maintaining anaerobic conditions. Continue enrichment for at least 8 wks.

Analysis – On a weekly basis, remove 5 mL of mixed liquor from the test vessels, centrifuge test medium, extract and analyze for parent & metabolites. Methane and carbon dioxide will also be followed in the headspace via GC.

Test Material Requirements – Enrichment systems will require 22.5 mg [50 mg/L C – assume 75 mg/L test material, 300 mL medium = 7.5 mg per vessel].

Definitive Assays – Definitive test systems will be used to evaluate metabolic rates in more detail and will be designed based on results from the initial test bottles. Definitive assays will probably contain less biomass to facilitate analytical measurements.

B. Soil/Sediment Acclimation and Assay Systems

Test vessels – 160-mL serum bottles with crimped butyl rubber or Teflon® stoppers. Assay based on OPPTS 835-3400 and work of Suffita *et al.* At least three bottles will be set up for each test material.

Test medium – OECD mineral salts + trace minerals & vitamins and 0.0002% resazurin will be added to an aquifer sediment extract. Total volume ~100 mL/bottle after inoculum addition.

Inoculum – Inoculum will be obtained from an anoxic receiving water or aquifer. Water and sediment will be collected under conditions to maintain anaerobicity and added to the test medium (~5 g d.wt. basis). Incubation will be at 22°C.

Test Material – Addition in the 30-50 mg/L (carbon basis) range. Blank controls with no test material also included.

Enrichment Conditions – On a weekly basis, add additional aquifer extract (5 mL) through the septum while maintaining anaerobic conditions. Continue enrichment for at least 8 wks.

Analysis – On a weekly basis, remove 5 mL of mixed liquor from the test vessels (anaerobically), centrifuge test medium, extract and analyze for parent & metabolites. Methane and carbon dioxide will also be followed in the headspace via GC.

Test Material Requirements – Enrichment systems will require 22.5 mg [50 mg/L C – assume 75 mg/L test material, 300 mL medium = 7.5 mg per vessel].

Definitive Assays – Definitive test systems will be used to evaluate metabolic rates in more detail and will be designed based on results from the initial test

bottles. Definitive assays will probably contain less biomass to facilitate analytical measurements.

Table 1. 3M Test Matrix *

Product	Clean System (M) **								Sewage (M+B)								Soil (S)							
	Week #								Week #								Week #							
	0	1	2	3	4	6	8	10	0	1	2	3	4	6	8	10	0	1	2	3	4	6	8	10
	Date								Date								Date							
	4/5	4/12	4/19	4/26	5/3	5/17	5/24	5/31	4/5	4/12	4/19	4/26	5/3	5/17	5/31	6/14	4/26	5/3	5/10	5/17	5/24	6/7	6/21	7/5

Blank Control	0	3	5	5	5	6	7	8
FC-1395	0	0	1	1	1	1	1	1
AO Foamer	0	1	1	1	1	1	1	1
54F	0	0	1	1	1	1	1	1
Et. PFOS alc.	0	1	1	1	1	1	1	1
PFOS	0	1	1	1	1	1	1	1
27C					0	1	1	1
FX-3539						0	1	1
PFOSA							0	1
34E								
Total	0	6	10	10	10	12	14	16

0	6	10	10	10	12	14	16
0	0	2	2	2	2	2	2
0	2	2	2	2	2	2	2
0	0	2	2	2	2	2	2
0	2	2	2	2	2	2	2
0	2	2	2	2	2	2	2
				0	2	2	2
					0	2	2
						0	2
0	12	20	20	20	24	28	32

0	3	5	5	6	7	8	8
	0	1	1	1	1	1	1
0	1	1	1	1	1	1	1
	0	1	1	1	1	1	1
0	1	1	1	1	1	1	1
0	1	1	1	1	1	1	1
			0	1	1	1	1
				0	1	1	1
					0	1	1
0	6	10	10	12	14	16	16

Product	Pure Culture (M+B)				Anaerobic Digester (B+M)								Anaerobic Sediment (B+M)							
	G				Week #								Week #							
	Days #0 & 7				0	1	2	4	6	8	10		0	1	2	4	6	8	10	
	4/12	4/19	4/26	5/3	5/3	5/10	5/17	5/31	6/14	6/28	7/12		5/10	5/17	5/24	6/7	6/21	7/5	7/19	

Blank Control		16	32	32	16
FC-1395		8	8		
AO Foamer		0	8	8	
54F		0	0	8	8
Et. PFOS alc.		0	8	8	
PFOS		8	8		
27C				8	8
FX-3539					
PFOSA					
34E					
Total		32	64	64	32

0	6	10	10	12	14	14
	0	2	2	2	2	2
0	2	2	2	2	2	2
	0	2	2	2	2	2
0	2	2	2	2	2	2
0	2	2	2	2	2	2
			0	2	2	2
				0	2	2
					0	
0	12	20	20	24	28	28

0	4	10	10	12	14	14
	0	2	2	2	2	2
0	2	2	2	2	2	2
	0	2	2	2	2	2
0	2	2	2	2	2	2
0	0	2	2	2	2	2
			0	2	2	2
				0	2	2
0	8	20	20	24	28	28

* Sterile controls may be included based on analytical results.

** Legend : M = medium; P = foam plug (optional); B = biomass/sludge; S = soil; G = growing cells; R = resting cells

** Other High Priority Products - Existing (FX-3539, FC-807A, FC-1395, FC-228, FC-845); New (L15655, L15656, L15660) Standards (PFOSAA, PFOSA, Me.PFOS Alc.)

[illegible]

Table 2 (cont.) 3M weekly samples

			Week of: (# samples)																																
Test Matrix	Product		4/5					4/12					4/19					4/26					5/3					5/10							
			W	Th	F			M	T	W	Th	F			M	T	W	Th	F			M	T	W	Th	F			M	T	W	Th	F		
Pure Culture	Blank	1						8		8					8	8	8		8							8									
	54F	1																								8									
	FC-1395	1						8							8											8									
	AO Foamer	1																																	
	Et.PFOS Alc.	1																																	
	PFOS	1																																	
	Blank	2																																	
	27C	2																																	
	FX-3539	2																																	
PFOSA	2																																		
35E	2																																		
Anaerobic Sludge	Blank	1																																	
	54F	1																																	
	FC-1395	1																																	
	AO Foamer	1																																	
	Et.PFOS Alc.	1																																	
	PFOS	1																																	
	Blank	2																																	
	27C	2																																	
	FX-3539	2																																	
PFOSA	2																																		
35E	2																																		
Anaerobic Sediment	Blank	1																																	
	54F	1																																	
	FC-1395	1																																	
	AO Foamer	1																																	
	Et.PFOS Alc.	1																																	
	PFOS	1																																	
	Blank	2																																	
	27C	2																																	
	FX-3539	2																																	
PFOSA	2																																		
35E	2																																		
Total	Group 1		0	0	0		16	0	16	0	0		16	16	16	0	16		0	16	0	16	16		0	0	20	16	0		###	###	###	###	###
Group 1 samples- Mon.= FC-1395, Tues.= 54F, Wed.= PFOS, Thur.= Et.PFOS Alc., Fri.= AO Foamer																																			
Total	Group 2		0	0	0		0	0	0	0	0		0	0	0	0	0		0	0	16	0	0		0	0	16	0	0		16	0	0	16	0
Group 2 samples- Mon.= , Tues.=, Wed.= 27C, Thur.= FX-3539, Fri.= PFOSA																																			