

Biodegradation Studies of Fluorocarbons (8/12/76) report
and Biodegradation Studies of Fluorocarbons - III
(7/19/78) report. (Note: both reports summarized with
one robust summary)

BIODEGRADATION

TEST SUBSTANCE

Identity: Perfluorooctanesulfonate; may also be referred to as PFOS or FC-95. (1-Octanesulfonic acid, 1,1,2,2,3,3,4,4,5,5,6,6,7,7,8,8,8-heptadecafluoro-, potassium salt, CAS # 2795-39-3)

Remarks field: The test substance is a white powder of uncharacterized purity.

The following is a reduced overview of previously completed biodegradation studies. While these studies have concluded that 1-Octanesulfonic acid, 1,1,2,2,3,3,4,4,5,5,6,6,7,7,8,8,8-heptadecafluoro-, potassium salt is persistent, new testing is underway to determine if a mechanism exists for the biological degradation of this substance.

METHOD

Methods: Warburg Determination¹; Shake Culture study modeled after the Soap and Detergent Association's presumptive test for the determination of ABS/LAS biodegradability².

Type: Aerobic

GLP: No

Year completed: 1976¹, 1978²

Contact time (units): 3 hours¹, 2.5 months²

Innoculum: Activated Sludge

RESULTS

No biodegradation was observed in either the Warburg study or the 2.5 month shake culture biodegradation study.

DATA QUALITY

Reliability: Klimisch ranking = 2¹. This study meets all criteria for quality testing, but analytical methodology is questionable.

Klimisch ranking = 2². This study meets criteria for quality testing, but analytical methodology is questionable.

No reference compounds or abiotic vessels were included in either of the studies.

Footnotes:

¹Warburg Determination

²Shake Culture study modeled after the Soap and Detergent Association's presumptive test for the determination of ABS/LAS biodegradability

REFERENCES

1. Fate of Fluorochemicals in the Environment, Project number 75-6398-29, E.A. Reiner, August 12, 1976, 3M Company, Environmental Laboratory.
2. Fate of Fluorochemicals in the Environment, Project number 9970612613, E.A. Reiner, July 19, 1978, 3M Company, Environmental Laboratory.

OTHER

Submitter: 3M Company, Environmental Laboratory, P.O. Box 33331, St. Paul, Minnesota, 55133

Last changed: 5/3/00



TECHNICAL REPORT SUMMARY

DATE: August 12, 1976

TO: Technical Communications
Center, 201-25

Environmental LABORATORY, DEPT. NUMBER 0222

MICROFORM COPIES:

Title Biodegradation Studies of Fluorocarbons	
Project: Fate of Fluorochemicals in the Environmental	Project Number: 75-6398-29 Report No. (3 digits)
To: R. L. Bohon	
By: E. A. Reiner	Employee Number: 47816
Objective: To determine the biodegradability of selected 3M fluorocarbon compounds.	Notebook Reference: 40671 Pgs. 29-37, 41-50
	No. of pages including covers: 12

SECURITY

Company Confidential(Open)

Special Authorization(Closed)

IF SUMMARY REPORT

Has information in this report been covered by other reports submitted to TCC?

- ☒ No
☐ Partially
☐ Completely

Please keyword information not included in other reports and give page numbers of new material:

3M CHEMICAL REGISTRY

New chemicals reported?

- ☒ No ☐ Yes

KEYWORDS

Select general, specific, and 3M product terms from 3M Thesaurus. Enclose suggested terms in parentheses.

EE & PC - Div.
Environ - Assess
Biology
Bacteria
Bioscreening
Fluorochemical
Biodegradable**ABSTRACT** and Conclusions. (System can accommodate 200-250 words)

Biodegradation studies using a Warburg respirometer were conducted on FC-95, FM 3422, FC-128, and hydrogen analogs of FC-95 and FM 3422. No biodegradability was observed on FC-95, although an approximate hydrogen analog of FC-95 was readily degradable. FM 3422 and FC-128 both were demonstrated to undergo some biodegradation. Attempts to isolate degradation products of FM 3422, from the Warburg studies, and from a subsequent activated sludge study were unsuccessful.

SPECIFIC PROBLEMS remaining to reach objective.

Continued attempts will be made to isolate and identify the biodegradation products of FM 3422 and other 3M fluorocarbons. Work with radioactivity labeled FM 3422 is being considered.

BIODEGRADATION STUDIES OF FLUOROCARBONS

SUMMARY AND RECOMMENDATION

No biodegradation was observed in Warburg studies on FC 95. Biodegradation of FC 95 is improbable because it is completely fluorinated. The resistance of this compound to biodegradation by an acclimated microbial culture, however, has not yet been demonstrated.

Warburg studies on FM 3422 and FC 128 both indicated that some biodegradation occurred. The products of this biodegradation are not known. Semicontinuous activated sludge studies on FM 3422 did not confirm or disprove the Warburg findings.

Future investigation of the biodegradability of the fluorocarbon compounds would be greatly facilitated by the development of an analytical procedure for FC 95. Warburg studies using purified FC 128 should be made to confirm the present findings.

Studies on the biodegradability of FM 3422 were hindered by its low water solubility. This problem could be overcome using FM 3422 radioactivity labeled on its hydrocarbon portion provided this material had a high specific activity (>5 mci/m mole) and purity. Biodegradation of a saturated solution of the labeled compound could be measured by detecting $^{14}\text{CO}_2$ evolution.

INTRODUCTION

The susceptibility to microbial modification is an important parameter in the study of the environmental fate of any class of compounds. It is the most important form of degradation for organic compounds.

A vast array of organic compounds can be completely degraded by microorganisms. So vast in fact that it was once believed by some that given enough time and the proper conditions, microorganisms could degrade any organic material. This doctrine of microbial infallibility is still a common misconception(1).

Perfluorinated compounds are extremely resistant to biodegradation (2). Although compounds with single fluorines have been shown to release fluoride ions as a result of biodegradation, perfluorinated compounds have rarely or never been shown to undergo natural degradation. For this reason, no modification of the perfluoro components of compounds in this study was anticipated. However, modification of its hydrocarbon components seemed possible.

An understanding of the partial degradation products is important since the environment will be exposed to these products in addition to the undegraded materials.

METHODS AND MATERIALS

Chemicals

The chemicals used in these experiments are shown in Table I.

TABLE I. CHEMICALS USED IN BIODEGRADATION EXPERIMENTS

FM 3422	$\text{C}_8\text{F}_{17}\text{SO}_2\overset{\text{C}_2\text{H}_5}{\underset{ }{\text{N}}}\text{C}_2\text{H}_4\text{OH}$
Hydrogen Analog of FM 3422	$\text{C}_8\text{H}_{17}\text{SO}_2\overset{\text{C}_2\text{H}_5}{\underset{ }{\text{N}}}\text{C}_2\text{H}_4\text{OH}$
FC 95	$\text{C}_8\text{F}_{17}\text{SO}_3\text{K}$
Sipex-ols	$\text{C}_8\text{H}_{17}\text{OSO}_3\text{Na}$
FC 128	$\text{C}_8\text{F}_{17}\text{SO}_2\overset{\text{C}_2\text{H}_5}{\underset{ }{\text{N}}}\text{CH}_2\text{COOK}$

They were obtained from Don Ricker of the Commercial Chemical Division in September, 1975. FM 3422 (N-et Fose alcohol) was identified as 788 CC 745-2. The FC.95 used was from lot 583. Lot numbers were not given for the FM 3422 hydrogen analog or the sipex-ols (RM 26442).

These chemicals were selected for a number of reasons. FC 95 is essentially the fluorocarbon constituent of a large number of 3M fluorocarbon compounds. FM 3422 is an intermediate in the production of 3M fluorocarbons, and FC 128 is a finish fluorocarbon product. Sipex-ols and the Hydrogen Analog of FM 3422 were selected for comparison to the fluorocarbons. Sipex-ols is an approximate hydrogen analog of FC 95. These hydrogen analogs were tested because biologically labile fluorocarbons have frequently been found to be gratuitously defluorinated by enzymes which normally remove a hydrogen. Thus, it seemed probable that microbial growth on hydrogen analogs could select populations of organisms which could more completely degrade fluorocarbons.

WARBURG DETERMINATION

Warburg studies were conducted according to the attached standard procedures. (Attachment) Microorganisms were collected from the mixed liquor of the Pigs Eye treatment system, washed, and suspended in a basal salts medium and used at a concentration of 2000 mg of biological solids per liter.

Water insoluble substrates were emulsified in water prior to addition to the Warburg flasks. Emulsions were made using a Blackstone model EP-2 ultrasonic probe, base 1/2 inch, at 100% power. Logarithmic dilutions in water were made of the test substrates, and 1/2 ml was placed in the first side arm of the Warburg flasks. Controls contained 1/2 ml of 10 g/l glucose solution or deionized water in this side arm. The second side arm contained either glucose or deionized water.

Oxygen uptake was first observed in each flash for a period up to 1.5 hrs. with readings at 10-15 min. intervals to establish the endogenous activity. This was followed by addition of the first side arm and continued oxygen monitoring for approximately 2 hrs. Addition of the second side arm containing glucose, a readily degradable material, allowed a further evaluation of the toxicity of the previously added material.

Semicontinuous Activated Sludge Studies

*check this
it was 1.5 L **
A week-long semicontinuous activated sludge (SCAS) study was conducted on FM 3422. The microorganisms used were obtained, as before, from Pigs Eye Treatment Plant. One Hundred Fifty ml of activated sludge was added to 3 SCAS reactors and tap water as a control to a fourth. FM 3422 was added to 3 reactors below the water surface in 1/2 ml of absolute alcohol. Each addition increased the FM 3422 concentration by 33 mg/l. Pure ethanol was added to one sludge-containing reactor as a control. The operation of the semicontinuous reactors is shown in Figure 1.

The SCAS reactors were aerated for 23 hrs. with 500 ml/min. of air while the contents of each reactor were stirred with a magnetic stirrer to prevent settling. After the aeration period, the sludge was settled for an hour and one liter of supernatant was replaced with primary effluent from the Pigs Eye Plant.

FM 3422 was added at the beginning of the Test Cycles 1, 2, and 4. Samples were taken at the start and end of each test cycles and from the supernatant after settling.

The aeration chambers used in the SCAS studies were plexiglass cylinders 13" high with a 4" internal diameter. A side arm allowed drainage of the supernatant leaving the 500 ml with the settled sludge undisturbed.

Analytical

Samples taken at the termination of the first Warburg study on FM 3422 were evaluated by thin layer chromatography (Central Research analytical work req. No. A59412). The samples were extracted into dichloromethane, dried to a small volume, and separated on Woelm silica plates. The developing solvent system was 10:90 ethanol, chloroform (V:V). The developed plates were visualized by the iodine starch technique and compared to known standards with a detection limit of one μ g of FM 3422.

Samples for the SCAS study were extracted into n-octanol and separated by gas chromatography with an electron capture detector. Extractions were performed in capped 50 ml polypropylene centrifuge tubes and phases separated by centrifuging at 26,700XG for 10 minutes.

RESULTS AND DISCUSSION

Warburg - FM 3422

Results from the Warburg study on FM 3422 are summarized in Figure 2. This experiment was performed by first sonicating FM 3422 and its analog in water to make emulsions of approx. 24,000 mg/l of the FM 3422 and 11,000 mg/l of the FM 3422 analog. Since FM 3422 and its hydrogen analog are not very soluble in water, it was felt that forming an emulsion would put more of these compounds in contact with the microorganisms in the Warburg study.

STEP 1: Add test compound,
media, and
microorganisms

STEP 2: Aerate and mix
for 23 hours

STEP 5: Re-add
test compound
and media.
Repeat
cycle.

magnetic
stirring bar

air sparger

supernatant

drain

sludge

STEP 4: Drain supernatant.

STEP 3: Stop aeration and mixing.
Let sludge settle.

FIGURE 1: Test cycle for semicontinuous activated sludge reactor.

FM 3422

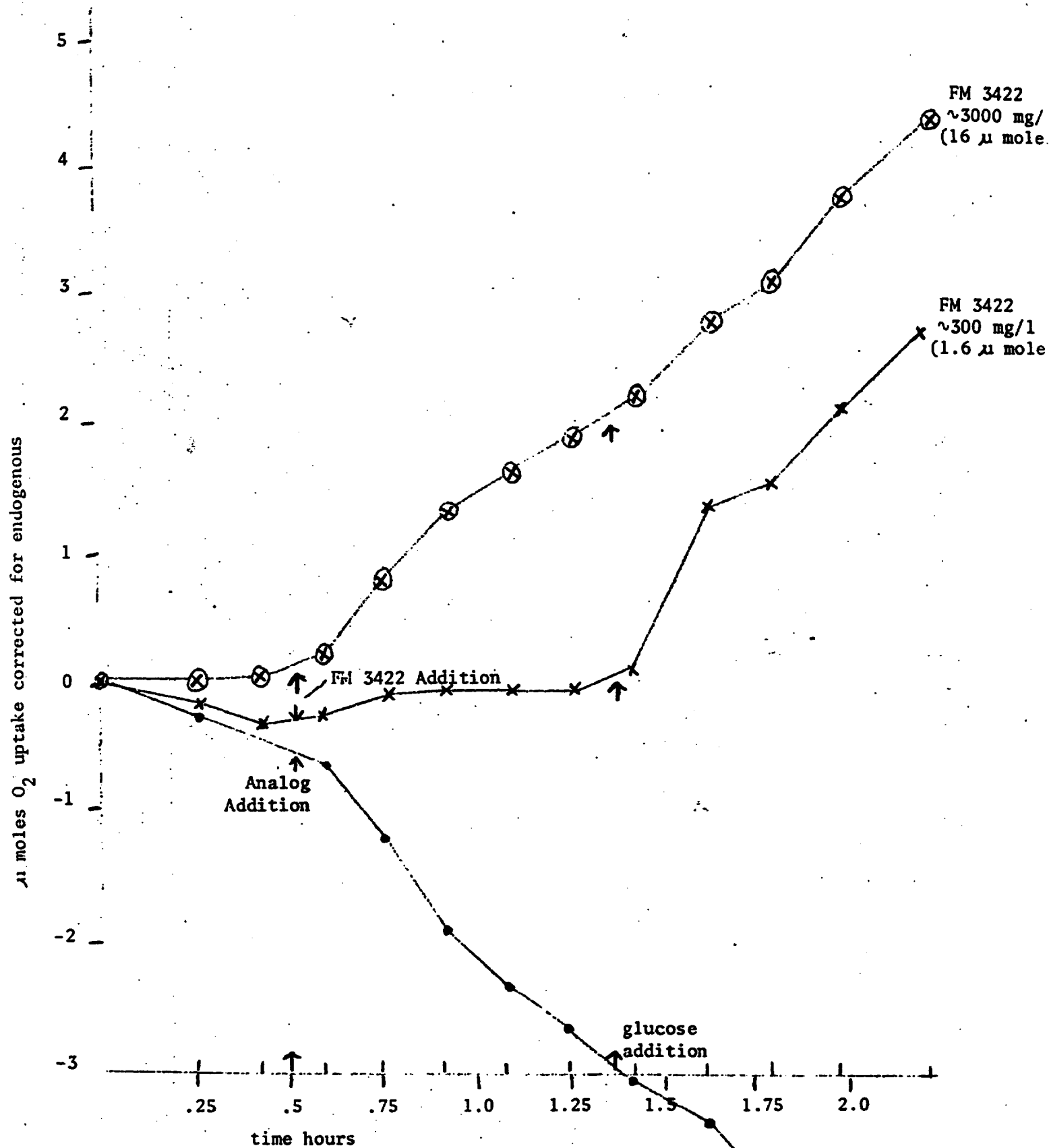
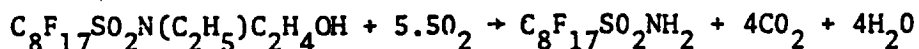


FIGURE 2: Warburg study of FM 3422 and its hydrogen analog.

Hydrogen Analog
~1800 mg/l
(21 μ mole)

While the FM 3422 analog was relatively easily emulsified and stable once emulsified, the FM 3422 was not. Approximately one hour was required to put 75% of the FM 3422 into emulsion, and this material proceeded to slowly come back out of emulsion. In about two to three hours, excess FM 3422 emulsion, which had not been used in the experiment, turned into a semi-solid gel.

Complete chemical oxidation of the hydrocarbon component of the FM 3422 at the highest concentration (~16 μ moles) would require 87 μ moles of O_2 based on the following equation:



Microbial oxidation rarely exceeds 60% of the chemical oxidation. In this experiment, only 2-3 micro moles of oxygen uptake was observed. However, oxygen uptake was continuing at the end of this experiment. Addition of glucose to the FM 3422 culture also produced increased oxygen uptake, confirming that the FM 3422 emulsion was not inhibitory to the microbial culture.

On the other hand, the hydrogen analog of FM 3422 showed significant toxicity. Upon addition of the most concentrated emulsion of the analog, endogenous oxygen uptake ceased and was not restored even after the addition of glucose to the culture. The negative slope of the hydrogen analog's oxygen uptake curve (Figure 2) is due to the endogenous correction and not oxygen evolution. Similar results were obtained in a second Warburg experiment with FM 3422 and its hydrogen analog.

How? Analysis of FM 3422 has shown it to be quite pure. The oxygen uptake observed was greater than would be expected from impurities in the compound. It is conceivable that sonication produced degradation products that were biodegradable, but not detectable by thin layer chromatography. It is also possible that some of the hydrocarbon components of FM 3422 molecule were degraded. However, using thin layer chromatography we were unable to detect any materials formed as a result of the biodegradation of FM 3422. It is not known if the hydrogen analog of FM 3422 itself is toxic. Thin layer and gas chromatography showed this material to be impure. Gas chromatograph* showed the analog to be 90% pure with two major contaminants. The contaminants may have been the cause of the observed toxicity.

SCAS - FM 3422

The semicontinuous activate sludge (SCAS) study was a second attempt to isolate the hypothesized degradation products of FM 3422. This study was conducted over a period of 1 week with samples taken at the initiation and end of each 24-hr. cycle. The FM 3422 samples added in an ethanol solution rapidly separated from the liquid phase, and as a result may have had too small a surface area to allow significant microbial degradation.

n-octanol extracts of the samples were analyzed by gas chromatography. No new leaks were formed as a result of exposure of the FM 3422 to the microorganisms. If some of the FM 3422 had been degraded to the sulfonic acid, it would not have been detected. The sulfonic acid is not sufficiently volatile to pass through the gas chromatography column.

* performed by Commercial Chemicals Division

The n-octanol extracts could not be separated by thin layer chromatography because of the low volatility of this solvent. Frozen nonextracted samples still exist at this date and could be extracted into a more volatile solvent for thin layer analysis.

Three additions of FM 3422 in 33 ppm increments were made during the SCAS experiment. The FM 3422 settled with the solids and for the most part remained in the reactor when the supernatant was withdrawn. The final concentration (although not in solution) was approximately 100 mg/l. This material was not homogeneously distributed and accumulated on the sides of the reactors.

WARBURG FC-95

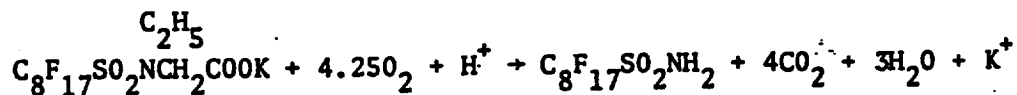
The results of Warburg studies with FC-95 are graphed in Figure 3.

No oxygen uptake was observed as a result of the addition of FC-95. This material also caused no toxic effects. Sipel-ol, an approximate hydrogen analog of FC-95, was shown to be readily biodegradable and to have no toxic effects.

The Sipel-ols was soluble at all concentrations tested (as high as 1700 mg/l). FC-95 was incompletely soluble at 4000 mg/l, but was completely in solution at 400 mg/l. The lack of degradation with FC-95 was expected since perfluorinate compounds are characteristically nonbiodegradable.

WARBURG FC-128

Oxygen uptake curves from Warburg studies on FC-128 as shown in Figure 4. These results indicate that FC-128 is readily biodegradable. Assuming biodegradation occurs as is shown below, approximately 70% of the theoretical maximum oxygen uptake occurred within the 7-hr. experimental period. This oxygen uptake is greater than expected and appeared to be continuing at the end of the experiment. These results are somewhat in question since this FC-128 is known to be an impure chemical.



μ moles O_2 uptake corrected for endogenous.

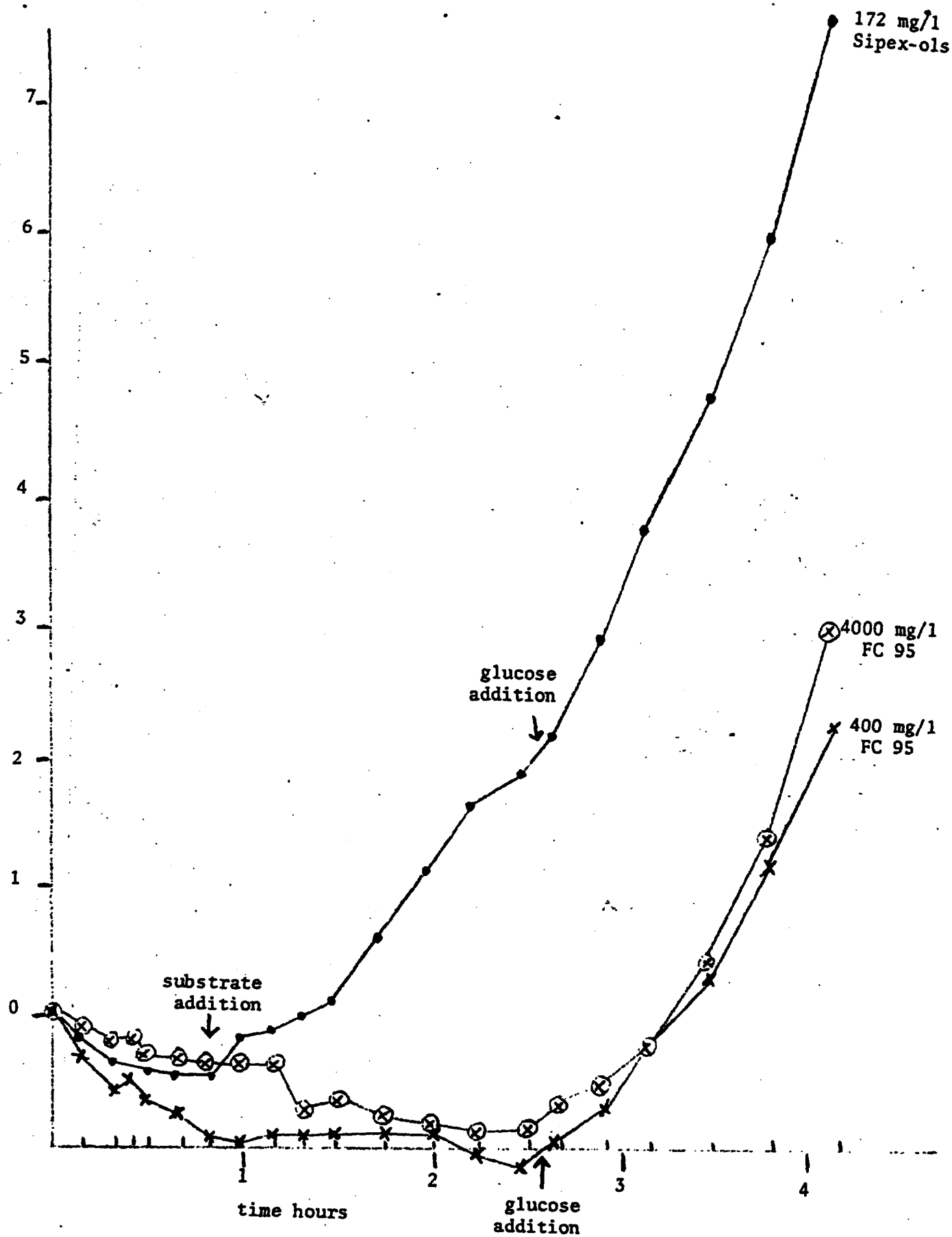


FIGURE 3: Warburg study of FC 95 and Sipex-ols.

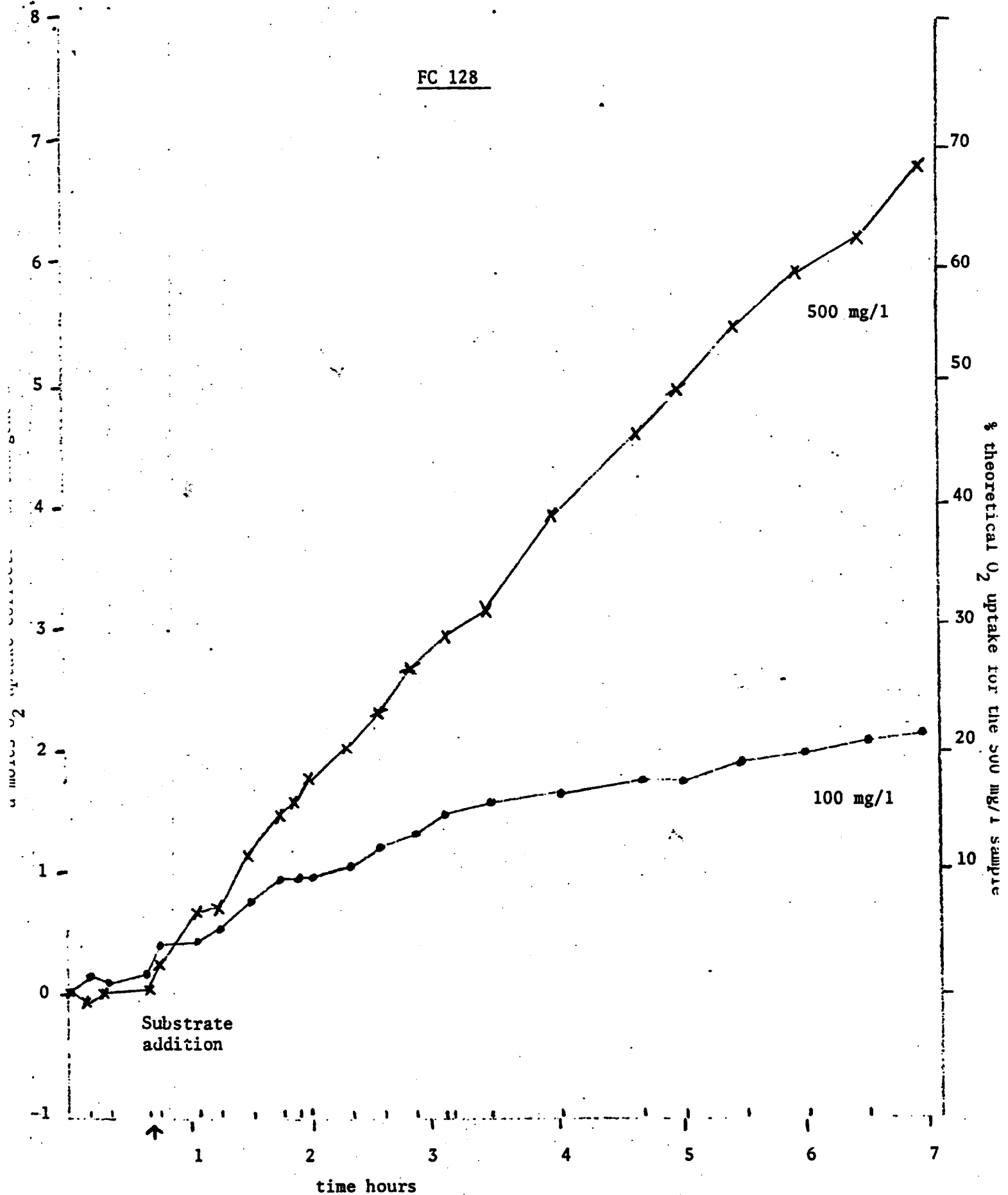


FIGURE 4: Warburg study of FC 128.

REFERENCES:

- (1) Alexander, M.; Biodegradation: Problems of Molecular Recalcitrance and Microbial Fallibility. Adv. Appl. Microbial 7: 35-80, 1965.
- (2) Chapman, P. J.; Department of Biochemistry, University of Minnesota, St. Paul, Minnesota, Personal Communications 2/24/76.

ATTACHMENT I

STANDARD PROCEDURE FOR WARBURG DETERMINATIONS

1. Design experiment and calculate concentrations of materials to add.
2. Fill water bath (DI water if left in bath).
3. Adjust temp. of bath (several hrs. or overnight).
4. Place manometers in desired order.
5. Prepare thermobarometer. Add about 3ml of H₂O to 1 flask.
6. Set out glassware in desired order (to match the manometer with which they were calibrated).
7. Lightly grease center-well top with stopcock grease that can be removed with solvent. Add 0.2 ml 10% KOH.
8. Prepare samples in DI water (or according to request) to add to side arms. Keep refrigerated until used.
9. Prepare cells (keep cells cold at all times but avoid freezing).
 - A. Centrifuge 0° C.
 - B. Wash with cold BSM - centrifuge.
 - C. Resuspend in cold BSM.
 - D. Determine concentration of an aliquot with the spectronic 20 at 600 nm. Adjust remainder to desired conc. C basic salts medium. Refrigerate until use.
 - E. Take sample of final adjusted sludge for standard MLSS analysis.
10. Add samples to side arms (usually 1 ml if one side arm, ½ ml to each side arm if 2 side arms).
11. Add 2 ml of washed cells to flasks.
12. Add filter paper strip to alkali in center cup.
13. Attach flasks to the correct manometer.
14. Retighten flasks after about 5 min. shaking in bath.
15. Leave stopcock open to atmosphere, and let temp. adjust for an additional 10 min.
16. Adjust level in manometer to 150 with stopcock open (close stopcock).
17. Begin readings (always adjust closed arm of manometer to 150 mm before reading).
18. Add contents of side arms according to experiment design requirements.

19. Take readings periodically (on open arms) throughout course of experiment.

20. Disconnect and clean flasks.

- A. Rinse with water.
- B. Wash off grease with acetone.
- C. Acid wash.
- D. Rinse with DI Water.



Form 6747-11 A

TECHNICAL REPORT SUMMARY

Date
7/19/78

TO: TECHNICAL COMMUNICATIONS CENTER - 201-2CN

(Important - If report is printed on both sides of paper, send two copies to TCC.)

Division Environmental Laboratory (EE & PC)		Dept. Number 0535
Project Fate of Fluorochemicals in the Environment		Project Number 9970612613
Report Title Biodegradation Studies of Fluorocarbons - III		Report Number 5
To D. L. Bacon		
Author(s) E. A. Reiner		Employee Number(s) 47816
Notebook Reference 44703, p. 6-14, 21, 25-27, 29, 35, 39-43, 46; 45727, p. 32-35; 49400, p. 11-12.		No. of Pages Including Coversheet 17
SECURITY ► ☐ Open (Company Confidential)	☐ Closed (Special Authorization)	3M CHEMICAL REGISTRY ► ☐ New Chemicals Reported ☒ Yes ☐ No

KEYWORDS:
(Select terms from 3M
Thesaurus. Suggest other
applicable terms.)

(Biodegradation)
EE & PC-Div.
Envir. Assess.
Fluorochemical
Degradation

CURRENT OBJECTIVE:

To evaluate the susceptibilities of FC-95 and FC-143
to microbial decomposition.

REPORT ABSTRACT: (200-250 words) This abstract information is distributed by the Technical Communications Center to alert 3M'ers to Company R&D.

A biodegradation study is described which allows the evaluation of the susceptibility of FC-95 and FC-143 to aerobic microbial degradation. The culturing procedures used in this study are modeled after the Soap and Detergent Association's presumptive (shake culture) test for the determination of ABS/LAS biodegradability. Microbial inocula were obtained from activated sludge collected at Chemolite, Decatur and Metro waste treatment plants. Analytical procedures included GLC, TLC, ¹⁴C-scintillation counting and analysis for released fluoride. Degradation of reference compounds demonstrated the suitability of the biodegradation test conditions.

Information Liaison
Initials: SEW

SUMMARY

Fluorochemicals FC-95 and FC-143 were shown to be completely resistant to biodegradation in a 2½-month shake culture biodegradation study. The mixed microbial test cultures used in this study were derived from activated sludge inocula obtained from three waste treatment systems (Chemolite, Decatur, & the Twin Cities Metro plant). The cultures were maintained in dilute yeast extract-basal salts media supplemented with the hydrogen analog of the respective fluorochemicals. Test cultures also contained FC-95 or FC-143. Phenol and 1-dodecene-derived linear alkyl sulfonate (LAS) were used as reference compounds. Their degradation demonstrated that biodegradation could occur under the test conditions. All cultures were transferred 15 times over the 2½-month period, and temperature was controlled at 25° C. during the latter half of the experiment.

In the final growth period, degradation products of ¹⁴C-labeled fluorochemicals were assayed for by thin-layer chromatography (TLC) and gas liquid chromatography (GLC). Chemicals separated by TLC were visualized by TLC-autoradiograph. Methylated and nonmethylated culture extracts separated by GLC were detected by electron capture. No degradation products were detected. Scintillation counting showed that all radioactivity associated with the labeled fluorochemicals remained in the culture medium.

In all but the final growth period, fluorocarbon biodegradation was monitored simply by measuring the initial and final fluoride concentration in the media. No increase in fluoride concentration was observed indicating that if biodegradation did occur, it did not result in the release of fluoride. Control cultures supplemented with fluoride showed that fluoride is not lost from the media under the experimental conditions used.

While this study cannot rule out the possibility that conditions could be found that would allow the biodegradation of these compounds, the results of this study suggest that these chemicals are likely to persist in the environment for extended periods unaltered by microbial catabolism.

INTRODUCTION

The fluorochemicals selected for this study, FC-143 and FC-95, have perfluorinated carbon chains and are chemically stable. The perfluorinated portion of fluorocarbons have not been found to be susceptible to biological degradation (1). Therefore, biodegradation studies were conducted on these compounds primarily for the sake of completeness. Without such testing, it could not be said with certainty that these compounds would resist microbial modification.

Since biodegradation was unlikely, the best feasible test conditions for biodegradation were selected. Inocula were obtained from areas considered likely to contain acclimated microorganisms. Long acclimation periods were used in an attempt to select and develop populations of microbes capable of degrading these compounds, and hydrogen analogs of the fluorocarbons were added to try to select organisms that might gratuitously "cometabolize" the fluorocarbons.

METHODS AND MATERIALS

Chemicals

FC-95, FC-143, the hydrogen analog of FC-95, ammonium octanoate (the hydrogen analog of FC-143), carbon-14 labeled FC-143, and carbon-14 labeled FC-95 were obtained from Commercial Chemicals Division. These chemicals were used as received unless designated otherwise (Arthur Mendel-Report in Progress).

Standard linear alkylate sulfonate prepared for use as a reference compound for biodegradation studies was obtained from the US/EPA Laboratory in Cincinnati, Ohio. Except where noted, all other compounds were reagent grade.

Culture Media

The control medium used in these studies had the composition shown in TABLE 1.

TABLE 1

CONTROL MEDIUM COMPOSITION

- 1) Basal salts solutions:
 - 1.0 g/l - NH_4Cl
 - 2.0 g/l - K_2HPO_4
 - 0.25 g/l - $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$
 - 0.002 g/l - $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$
- 2) Well water - 25 ml/l
- 3) Yeast extract - 0.3 g/l
- 4) Hydrogen analogs of either FC-95
or FC-143 - 20 mg/l

Media were prepared from stock solutions which were combined and brought to volume just prior to each culture transfer. A fresh solution of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ was prepared and dry yeast extract was used in media preparation at each transfer. The pH of all media was adjusted to 7.5 with 1.0 N HCl and if overshoot adjusted back with 1.0 N NaOH. The well water was added to insure an adequate supply of trace elements. Analyses of the well water made during the 12-month period prior to the initiation of this study showed its calcium hardness to range from 92 to 144 mg/l expressed CaCO_3 . Any precipitate resulting from the addition of well water was removed by filtration through a #54 Whatman filter.

The purified hydrogen analogs of FC-95 and FC-143 were used in biodegradation test media and controls. These compounds were included in an attempt to select a microbial population likely to degrade the fluorocarbons. Enzymes capable of catalyzing defluorination reactions are frequently identical to enzymes involved in carbon-hydrogen bond cleavage (1). Additional components of other specific media are listed in TABLE 2.

TABLE 2
GROWTH MEDIA FORMULATIONS

<u>Media</u>	<u>Components</u>
FC-95	FC-95 Control Medium + 50 mg/l FC-95
FC-143 Test	FC-143 Control Medium + 50 mg/l FC-143
Phenol Controls	FC-95 or FC-143 Control Medium + 30 mg/l Phenol
LAS Controls	FC-95 or FC-143 Control Medium + 30 mg/l Standard Linear Alkyl- benzenesulfonate (LAS)
Fluoride Controls	FC-95 or FC-143 Control Medium + 33.2 mg/l NaF (15.0 mg/l F ⁻)
¹⁴ C-FC-95 Test	FC-95 Control Medium + 50 mg/l ¹⁴ C-FC-95
¹⁴ C-FC-143 Test	FC-143 Control Medium + 50 mg/l ¹⁴ C-FC-143
FC-95 + LAS	FC-95 Control Medium + 30 mg/l LAS + 50 mg/l FC-95
FC-143 + LAS	FC-143 Control Medium + 30 mg/l LAS + 50 mg/l FC-143

Culturing Procedures

The initial growth period was started by inoculating 49 ml of each medium with 1 ml of activated sludge supernatant. The activated sludge used was a mixture of two sludges collected on the day of inoculation. The sludge was obtained from the Metropolitan Waste Control Commission's Metro plant in Saint Paul, Minnesota, and the Chemolite Waste Treatment Plant in Cottage Grove, Minnesota.

Following inoculation, the cultures in polypropylene Erlenmeyer flasks were shaken at 200 rpm on rotary shakers at room temperature (4).

At the end of each growth period, each culture was transferred to identical fresh media using a 1% inoculum from the preceding culture (i.e., 0.5 ml of existing culture to 49.5 ml of identical new medium).

The growth period between transfers varied as is noted in TABLE 3. A 10 ml sample was taken from each culture at 10 minutes after inoculation or culture transfer and at the end of each growth period. Samples were centrifuged for 10 min. at 17,000 x g prior to analysis of the centrifugate. Deviations from this culturing procedure are noted in TABLE 3.

The final growth period differed from preceding periods. Media were prepared with Carbon-14 labeled FC-95 and FC-143. One hundred ml cultures were grown in flasks on a rotary shaker in a growth chamber controlled at $25^{\circ}\text{C} \pm 1$. Twenty ml samples were taken at 10 min., 2 days and at 7 days.

Chemical Analysis

Fluoride ion concentrations were measured using a fluoride ion electrode (Orion ion analyzer fluoride electrode model 96-09), and a standard curve drawn from the results of measurements of accurately prepared fluoride standards. The concentrations of these fluoride standards bracketed the concentrations present in the experimental samples. Fluoride curves were set up at each sampling period, except for transfer 1. For the analyses following this transfer, a 1.0 ppm fluoride standard was used to calibrate the instrument with the assumption that the slope of the previous fluoride curve remained constant.

Phenol analysis was done according to Standard Methods for the Examination of Water and Wastewater, 14th Edition, 1975. Linear alkylbenzenesulfonate (LAS) was analyzed for by the methylene blue, chloroform extraction method described in the 14th edition of Standard Methods (3), except in transfers 8-14, LAS was analyzed by a modification of this method. In this modified method, the samples was diluted to 100 ml in a separatory funnel. Also added to the separatory funnel were 25 ml of Standard Methods methylene blue solution and 100 ml of chloroform. This mixture was shaken for 30 seconds, allowed to settle, swirled, and the chloroform drawn off through glass wool into a 2.5 cm diameter, spec 20 curvette. Percent transmittance was read at 652 nm and compared to a standard curve prepared with surfactant samples of known concentration treated in the same manner.

TABLE 3

SUMMARY OF CULTURING PROCEDURES USED
IN THE SHAKE FLASK BIODEGRADATION STUDY OF
FC-95 AND FC-143

<u>Transfer #</u>	<u>Culture Growth Period (days)</u>	<u>Notes</u>
0	3	Used activated sludge inoculum from Metro and Chemolite.
1	3	FC-143-hydrogen analog added to 143 cultures and controls.
2	4	
3	3	At the time of culture transfer, 1 ml of Decatur sludge supernatant added to cultures.
4	3	LAS replaced phenol as a reference compound. LAS media was inoculated with a mixture of control culture and Chemolite and Decatur sludge supernatant.
5	3	
6	3	The use of fluoride control was discontinued.
7	6	Shaker was inadvertently turned off, possibly for 5 days, during this growth period.
8	3	1/2 ml of Metro sludge supernatant was added to all cultures.
9	6	
10	4	
11	4	In this and subsequent growth periods, cultures were grown in a reciprocating shaker-water bath at 100 strokes per min. and 25° C.
12	6	
13	6	
14	8	
-	+6 (2)	
15	<u>7</u>	

78 days = Total Enrichment Period

Carbon 14 Counting Techniques

Scintillation counting was performed on 1 ml samples of culture centrifugate added to Aquasol^(R), and counted with an internal standard quench correction. The radioactivity of these samples was compared to known weight samples of ^{14}C -FC-95 or ^{14}C -FC-143 added directly to Aquasol.

Solid samples were collected directly onto millipore HA 0.45 μm filters composed of cellulose acetate and cellulose nitrate. The filters were then washed with deionized^(R) water and placed into paper combustion cones^(R), wet with Combustaid^(R), and combusted in Agrichem's Packard^(R) combustion equipment. The $^{14}\text{CO}_2$ resulting from combustion was trapped in a scintillation fluid containing an organic amine and counted in Agrichem's Packard scintillation counter. Samples were recounted with an internal standard for quench correction.

Thin-Layer Chromatography (TLC)

Thin-layer chromatography was performed to detect radioactive metabolites of ^{14}C -FC-95 and ^{14}C -FC-143. Ten ml culture samples were collected and immediately frozen. These samples were stored frozen for about 1 month. The samples were extracted immediately after thawing with 10 ml of ethyl acetate. The samples were then centrifuged at 17,000 x g to ensure the separation of the ethyl acetate, water, and solids phases. The water phase and portions of the ethyl acetate phase were evaporated to dryness under N_2 . The dried samples were resuspended in a 9:1 hexane:ethyl ether mixture. (Some samples which evaporated to dryness in air before spotting were resuspended in methanol.) The resuspended samples were spotted on E. Merck silica gel GF₂₅₄. Small spots of solids residue were also applied directly to these plates. All samples were referenced against a mixture of ^{14}C -FC-143 and ^{14}C -FC-95. The plates were developed with 10% ethanol in ethyl acetate and visualized by exposing Kodak no-screen x-ray film on the plates for one week.

TLC was repeated on the remaining portion of the solvent samples. The solvent was allowed to evaporate to dryness in air, and the residue resuspended in methanol. These plates were spotted more heavily, developed as before, and visualized with x-ray film for 2 weeks.

Gas-Liquid Chromatography (GLC)

Ethyl acetate extracts were prepared as described in the thin-layer chromatography methods. Control solutions were made by dissolving ^{14}C -FC-95 and ^{14}C -FC-143 in ethyl acetate. Portions of the ethyl acetate extract samples and the ethyl acetate control solutions were also methylated. Aliquots of the methylated and nonmethylated ethyl acetate extracts and controls were injected onto the 5713 Hewlett Packard gas chromatograph with electron capture detector. Methylated samples were injected within 3 hrs. of their methylation. The chromatographic column was 12 ft. x 1/8" O.D. stainless steel packed with 20% DC 200 (12,500 CS) on 10% Bentone 34 and 70% 80/90 mesh Anakrom P.A. The injection port temperature was 250° C., and the detector temperature 300° C. The column temperature was programmed to hold for 4 min. at 75° C., to rise to 180° C. at 8° C. per min., and to hold at 180° C. The flow rate was adjusted to 35 ml/min. of Argon/methane, 95/5.

Methylations were performed by adding a 20 μl aliquot of a 1 $\mu\text{g/ml}$ $\text{C}_9\text{F}_{19}\text{COOH}$ solution, as a reference compound to each sample. Diazomethane was then added until a yellow color persisted. The samples were then loosely capped, swirled and allowed to stand for 15 minutes. Nitrogen was blown over the samples until the yellow color disappeared, and the sample was returned to its original volume with ethyl acetate.

RESULTS AND DISCUSSION

Fluoride Release

In all but the final growth period, degradation of FC-95 and FC-143 was monitored only by analysis of fluoride concentration at the beginning and end of each culture period. It was assumed that if the fluorochemical portions of these molecules were degraded, fluoride ion would accumulate in the media. To ensure that fluoride was not lost from the culture by absorption, precipitation or volatilization, control cultures were grown with 15 mg/l of fluoride. This fluoride concentration is approximately what would result if FC-95 or FC-143 underwent degradation with 50 percent fluoride release. The results of the fluoride analyses conducted on different days showed considerable variation. This was due to the variable and very sluggish response of the fluoride electrode. TABLE 4a shows the results obtained at each transfer. TABLE 4b shows the results obtained when the same samples, which had been stored in polyethylene containers, were analyzed together after the termination of the experiment. Despite the variability due to the analytical technique, the results indicate that fluoride, if released to the media through biodegradation, would not be lost from the media.

The results of the fluoride analysis on fluorocarbon-containing cultures and controls are shown in TABLE 5. The results show that no biodegradation with fluoride release occurred.

TABLE 4a

INITIAL AND FINAL FLUORIDE CONCENTRATION
(mg/l) OF FLUORIDE SUPPLEMENTED CONTROLS
MEASURED BY SPECIFIC ION ELECTRODE AT
THE TIME OF TRANSFER

<u>Transfer #</u>	FC-95		FC-143	
	<u>Fluoride Control</u> <u>Initial</u>	<u>Final</u>	<u>Fluoride Control</u> <u>Initial</u>	<u>Final</u>
0	21	23	20	21
1	23	22	21	20
2	20	22	18	21
3	26	17.5	25	16.5
4	16.5	19.2	16	17.3

TABLE 4b

INITIAL AND FINAL FLUORIDE CONCENTRATION
(mg/l) OF FLUORIDE SUPPLEMENTED CONTROLS
MEASURED BY SPECIFIC ION ELECTRODE
MEASURED COLLECTIVELY AT END OF STUDY

<u>Transfer #</u>	FC-95		FC-143	
	<u>Fluoride Control</u> <u>Initial</u>	<u>Final</u>	<u>Fluoride Control</u> <u>Initial</u>	<u>Final</u>
0	16.4	16.2	15.7	17.0
1	15.6	16.2	15.7	15.0
2	15.6	16.2	14.5	16.4
3	16.2	16.2	16.4	15.6
4	15.6	15.7	15.7	17.0
5	19.3	17.0	15.0	16.4

TABLE 5

INITIAL AND FINAL FLUORIDE CONCENTRATION
(mg/l) OF FC-143 AND FC-95-CONTAINING
CULTURES AND OF NONSUPPLEMENTED 95 AND
143 CONTROL CULTURES

<u>Transfer #</u>	<u>FC-95 Test</u>		<u>95 Control</u>		<u>FC-143 Test</u>		<u>143 Control</u>	
	<u>Init.</u>	<u>Final</u>	<u>Init.</u>	<u>Final</u>	<u>Init.</u>	<u>Final</u>	<u>Init.</u>	<u>Final</u>
0	0.46	0.51	0.31	0.33	<0.1	<0.1	<0.1	<0.1
1	0.50	0.46	0.36	0.36	<0.1	<0.1	<0.1	<0.1
2	0.42	0.66	0.34	0.56	<0.1	<0.1	<0.1	<0.1
3 (5)	1.75	1.6	1.75	1.5	.83	1	.81	1
4	0.73	0.71	0.68	0.60	<0.1	<0.1	<0.1	<0.1
5	0.72	0.78	0.61	0.68	<0.1	<0.1	<0.1	0.56
6	0.73	0.8	0.63	0.70	<0.1	<0.1	<0.1	<0.1
7	0.14	0.17	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
8	0.90	0.84	0.66	0.66	<0.1	<0.1	<0.1	<0.1
9	0.84	0.73	0.72	0.60	<0.1	<0.1	<0.1	<0.1
10	0.72	0.81	0.60	0.68	<0.1	<0.1	<0.1	<0.1
11	0.81	0.80	0.69	0.62	<0.1	<0.1	<0.1	<0.1
12	0.74	0.73	0.66	0.62	<0.1	<0.1	<0.1	<0.1
13	0.73	0.84	0.64	0.66	<0.1	<0.1	<0.1	<0.1
14	0.81	0.78	0.66	0.64	<0.1	<0.1	<0.1	<0.1

Reference Compounds

Reference compounds were used to demonstrate that the biodegradation test conditions used were suitable to degrade compounds known to be somewhat resistant to degradation.

In the first four growth periods, 30 mg/l phenol was added to two cultures which were identical to the test cultures, except that they lacked fluorocarbons. Analytical problems prevented the measurement of phenol concentration during the first three growth periods. In the fourth growth period, phenol was found to degrade to less than 1.3 mg/l, the limit of sensitivity of the method as applied. This demonstrated that the test conditions were suitable for the biodegradation of phenol.

In the fifth through final growth periods, reference linear alkyl sulfonate (LAS) was used as the reference compound. This compound is a standard reference material used in the Soap and Detergent Association's biodegradation test method for anionic surfactants(6). This material is considered to be relatively easily degraded. In the Soap and Detergent Association's shake flask biodegradation test, the results are considered invalid if the removal of 1-dodecene-derived LAS is not nearly complete.

The data showing the extent of degradation of LAS in surfactant supplemented controls are depicted in TABLE 6. The data showing the equivalent amount of methylene blue active substances in the controls not supplemented with LAS are depicted in TABLE 7. Little LAS degradation occurred during the first few adaptive transfers. Three transfers were required before the majority of the LAS began to degrade in the surfactant supplemented control for FC-95. Five transfers were required for LAS degradation in the 143 control. Therefore, it appeared that organisms capable of degrading 1-dodecene-derived LAS were not initially present in sufficient numbers for LAS degradation. The test condition allowed for enrichment of these organisms, but enrichment occurred at a slower rate than had been anticipated. Consequently, changes were made in the procedure to increase the rate and likelihood of acclimating organisms capable of degrading the fluorochemicals. Growth periods were extended from 3 to 4-6 days, and temperature was raised from room temperature (<20° to 22°C) to a constant temperature of 25°C. Results of LAS degradation in the final growth period are shown in TABLE 8.

In the growth periods following transfers 11 and 12, an experiment was done to determine if 50 mg/l of FC-95 or FC-143 inhibited the degradation of LAS. These results are shown in TABLE 9. FC-95 appears to have an inhibiting effect on the microbial degradation of LAS. However, its presence was not completely inhibitory. Comparison with TABLES 8 and 6 shows that the presence of 50 mg/l of FC-95 inhibited LAS degradation by 18% and 23% during these two test periods. On the other hand, within the limits of the precision of our method, FC-143 did not appear to have a significant effect on LAS degradation.

In the final growth period, 50 mg/l of carbon 14-labeled FC-95 and FC-143 were used as test substrates in place of the nonlabeled fluorochemicals. Both FC-95 and FC-143 cultures were prepared in triplicate. The concentrations of the radioactive fluorocarbons present in the aqueous phase as determined by scintillation counting are shown in TABLE 10. The initial FC-95 concentration is much lower than expected. This low value could have resulted from a systematic error in the collection of the initial FC-95 samples. It is also possible that FC-95 had not completely dissolved in the cultures when the first sample was taken, but this seems unlikely, since the initial values for FC-95 concentration from all 3 parallel cultures were almost identical (30.3, 29.8 and 30.4 mg/l). Nevertheless, the remaining data show that the radioactivity associated with FC-95 and FC-143 remained in solution during the entire 7-day degradation test period. Analysis of the biological solids showed some binding of radioactive material, but the vast majority remained in the liquid phase.

TABLE 6

CONCENTRATION OF LAS (mg/l) IN SUPPLEMENTED
CONTROLS AND % LAS REMOVED

Transfer #	95 - Surfactant Control			143 - Surfactant Control		
	LAS			LAS		
	Init.	Final	% Removal(7)	Init.	Final	% Removal
4	31.5	26.8	18.4	35.5	29.5	19.4
5	28.3	27.5	0.1	32.8	25.8	21.2
6	29.8	25.5	15.5	27.0	24.0	7.0
7	25.0	12.0	91.1	25.0	30.6	-2.0
8	31.2	3.75	89.8	37.0	35.8	11.1
9	33.0	3.17	95.1	38.9	13.7	94.6
10	32.7	2.33	95.9	42.7	12.8	89.7
11	31.0	2.0	95.0	39	13.7	93.8
12	31.3	2.33	96.5	41.3	19.7	77.9
13	31.7	2.5	93.5	41.3	18.0	88.3
14	31.3	3.0	92.8	40.3	13.7	90.7

TABLE 7

CONCENTRATION OF METHYLENE BLUE ACTIVE SUBSTANCE
(mg/l) IN NONSUPPLEMENTED CONTROLS

<u>Transfer #</u>	<u>95 - Control</u>		<u>143 - Control</u>	
	<u>Init.</u>	<u>Final</u>	<u>Init.</u>	<u>Final</u>
4	1.0	1.9	4.5	4.5
5	1.15	.38	4.5	3.5
6	0.50	.75	7.1	5.50
7	5.25	10.2	5.0	10.2
8	3.0	0.88	9.0	10.9
9	5.75	1.83	10.9	12.2
10	4.17	1.17	12.3	9.67
11	4.33	0.67	11.5	12.0
12	3.0	1.33	12.3	13.3
13	3.67	0.67	11.3	14.5
14	3.67	1.0	14.5	11.3

TABLE 8

CONCENTRATION OF MBAS (mg/l) IN SURFACTANT
SUPPLEMENTED AND NONSUPPLEMENTED CONTROLS
DURING FINAL GROWTH PERIOD

<u>Time</u>	<u>FC-95 Controls</u>			<u>FC-143 Controls</u>		
	<u>#1 LAS Suppl.</u>	<u>#2 LAS Suppl.</u>	<u>Non- Suppl.</u>	<u>#1 LAS Suppl.</u>	<u>#2 LAS Suppl.</u>	<u>Non- Suppl.</u>
Initial	28.7	29.3	1.0	34.0	36.7	6.5
Day 2	13.0	26.0	1.0	8.0	22.3	5.3
Day 7	1.67	2.0	.7	6.3	6.3	4.0
% LAS Removal (7)	96.5	95.4	-	91.6	92.3	-

TABLE 9

EFFECT OF FC-95 AND FC-143 ON THE
BIODEGRADATION OF LAS ANALYZED
FOR AS MBAS

<u>Transfer #</u>	<u>FC-95 + LAS Culture</u>			<u>FC-143 + LAS Culture</u>		
	<u>Init.</u>	<u>Final</u>	<u>% LAS Removal(8)</u>	<u>Init.</u>	<u>Final</u>	<u>% LAS Removal(8)</u>
11	58.0	34.7	73.6	53.7	27.3	97.8
12	64.3	40.3	78.9	53.7	34.0	71.4

TABLE 10

CONCENTRATION OF ^{14}C -FC-95 OR ^{14}C -FC-143 IN
THE CENTRIFUGATE OF TEST CULTURE DURING
THE FINAL GROWTH PERIOD

	<u>^{14}C-FC-95 Cultures</u>		<u>^{14}C-FC-143 Cultures</u>	
	<u>Concentration</u>	<u>Standard Deviation</u>	<u>Concentration</u>	<u>Standard Deviation</u>
Init.	30.1 mg/l	0.3 mg/l	46.2 mg/l	0.9 mg/l
Day 2	52.8	0.5	48.0	0.3
Day 7	53.5	3.1	49.7	0.4

Thin-layer chromatography did not reveal the presence of radioactive metabolic products of either FC-143 or FC-95. Likewise, gas liquid chromatography of the same culture extracts, both before and after methylation, showed no products that were not initially present or not also present in controls. From the combination of these results, it can be concluded that no biodegradation of these fluorochemicals occurred.

REFERENCES AND FOOTNOTES

- (1) Goldman, Peter, Enzymology of Carbon-Halogen Bonds. Degradation of Synthetic Organic Molecules in the Biosphere, Nat. Acad. of Sci., Washington, DC (1972).
- (2) There was a six-day period before the onset of the final growth period during which the test cultures were shaken at 25° C. in the presence of FC-95 or FC-143.
- (3) Standard Methods for the Examination of Water and Wastewater, 14th Edition, American Public Health Association (1975).
- (4) Daytime temperatures were observed to range between 20 and 22° F. Night temperatures were not measured during that part of the study in which cultures were shaken at ambient temperature (see TABLE 3). However, measurement made near the termination of this 2½-month study, in January, showed that nighttime temperature frequently drops to 17° C.
- (5) At this transfer, Decatur sludge was added which contained a high fluoride concentration.
- (6) Subcommittee on Biodegradation Test Methods of the Soap and Detergent Association, A Procedure and Standards for the Determination of the Biodegradability of Alkyl Benzene Sulfonate and Linear Alkylate Sulfonate. J. of the American Oil Chemists' Society, 42:986 (1966).
- (7) Percent LAS removal was calculated as:

$$\% \text{ Removal} = \frac{(\text{MBAS}_{\text{SI}} - \text{MBAS}_{\text{CI}}) - (\text{MBAS}_{\text{SF}} - \text{MBAS}_{\text{CF}})}{\text{MBAS}_{\text{SI}} - \text{MBAS}_{\text{CI}}} \times 100$$

Where:

MBAS_{SI} = The initial methylene blue active substances (MBAS) concentration of the surfactant supplemented culture.

MBAS_{CI} = The initial MBAS concentration of the nonsupplemented control (TABLE 7).

MBAS_{SF} = The final MBAS concentration of the surfactant supplemented culture.

MBAS_{CI} = The final MBAS concentration of the nonsupplemented control (TABLE 7).

(7) The percent LAS Removal was calculated as:

$$\% \text{ Removal} = \frac{(\text{MBAS}_{\text{STI}} - \text{MBAS}_{\text{CI}}) - (\text{MBAS}_{\text{STF}} - \text{MBAS}_{\text{CF}})}{(\text{MBAS}_{\text{SI}} - \text{MBAS}_{\text{CI}})} \times 100$$

Where:

MBAS_{STI} = The initial methylene blue active substances (MBAS) concentration of the culture supplemented by both LAS surfactant and either FC-95 or FC-143.

MBAS_{CI} = The initial MBAS concentration of the nonsupplemental control (TABLE 7).

MBAS_{STF} = The final MBAS concentration of cultures supplemented with surfactant and fluorocarbon.

MBAS_{CF} = The final MBAS concentration of the nonsupplemental control (TABLE 7).

MBAS_{SI} = The initial MBAS concentration of the surfactant supplemental culture (TABLE 6).

It was assumed that MBAS concentration due to FC-95 or FC-143 was not reduced by the biodegradation or other loss of these compounds.

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