

BIODEGRADATION

TEST SUBSTANCE

Identity: N-ethylperfluorooctane sulfonamidoethanol; may also be referred to as N-EtFOSE Alcohol or FM-3422. (1-Octanesulfonamide, N-ethyl-1,1,2,2,3,3,4,4,5,5,6,6,7,7,8,8,8-heptadecafluoro-N-(2-hydroxyethyl)-, CAS # 1691-99-2)

Remarks: Material is an off-white, waxy solid of uncharacterized purity. The following summary includes a history of studies.

METHOD:

Methods followed: Modification of OECD 302A – Inherent Biodegradability: Modified SCAS Test.; Warburg Determination (Standard Procedure included with study); COD analysis; Shake-Flask Die Away Study (Procedure described in study)

Test Type: Aerobic

GLP: No

Years studies were performed: 1976 - 1978

Contact time: 7 days to 6 months

Inoculum: Activated sludge collected from an aeration basin at the municipal waste treatment plant in St. Paul, Minnesota was primarily used. Alternate inocula taken from the Decatur, Alabama manufacturing facility were also utilized with the understanding that these media were the most likely to have developed naturally enriched cultures capable of degrading fluorochemicals.

Remarks: The initial study in 1976 was a Warburg oxygen uptake experiment. This testing resulted in oxygen uptake equivalent to approximately 3% of the theoretical oxygen demand for the hydrocarbon moiety. However, no degradation products were detected.

Further experiments included testing on non-stabilized and surfactant-stabilized N-EtFOSE alcohol. In the initial semicontinuous activated sludge (SCAS) study, N-EtFOSE Alcohol was simply added to the media in an ethanol solution. The N-EtFOSE Alcohol rapidly congealed and separated from the water phase. It was postulated that biodegradation may not have been observed because enzymes could not "attack" the coagulated N-EtFOSE Alcohol molecules. Since emulsions of water-insoluble molecules have been shown to be more susceptible to enzyme attack (Dickson, L. S. Liu, M.Sc., Ph.D.), a second SCAS degradation study of N-EtFOSE Alcohol was conducted in 1978 using emulsified N-EtFOSE Alcohol.

RESULTS

None of the testing conducted could definitely prove loss of N-EtFOSE alcohol through biodegradation process. Analytical capability at the time could not verify the presence of degradation products or give explanations for why the test substance became non-extractable during some of the experiments.

CONCLUSIONS

It appears that N-EtFOSE Alcohol does not degrade, at least to detectable levels or by verifiable processes, under the conditions of these tests including with high cell densities and extended contact periods. These studies cannot rule out the possibility that conditions could be found that would allow for biodegradation, nor could they exclude the possibility that a microorganism could eventually be enriched that could metabolically alter this chemical. Testing did indicate that N-EtFOSE alcohol was not inhibitory to bacteria.

DATA QUALITY

Reliability: Klimisch ranking = 3. The analytical methodology is questionable. Sample purity was not sufficiently characterized. Procedural problems were present in several of the studies. Analytical methodology did not exist at this time to detect possible degradation products.

REFERENCES

3M Technical Report Summary, "*Biodegradation Studies of Fluorocarbons*", E. A. Reiner, 8/12/76

Request for Laboratory Work, Lab Request No. 3844, COD, Dale Bacon, July 1977.

3M Interoffice Correspondence, "*Chronological Review of Biodegradation Studies on FM 3422*", E. A. Reiner, August 12, 1977

3M Technical Report Summary, "*Biodegradation Studies of Fluorocarbons – II*", Report number 4, E. A. Reiner, 1/9/78

OTHER

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

Last changed: 5/18/00



TECHNICAL REPORT SUMMARY

TO:
Technical Communications
Center, 201-25

DATE: August 12, 1976

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

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

How 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.

006324

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

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.

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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.

006328

FM 3422

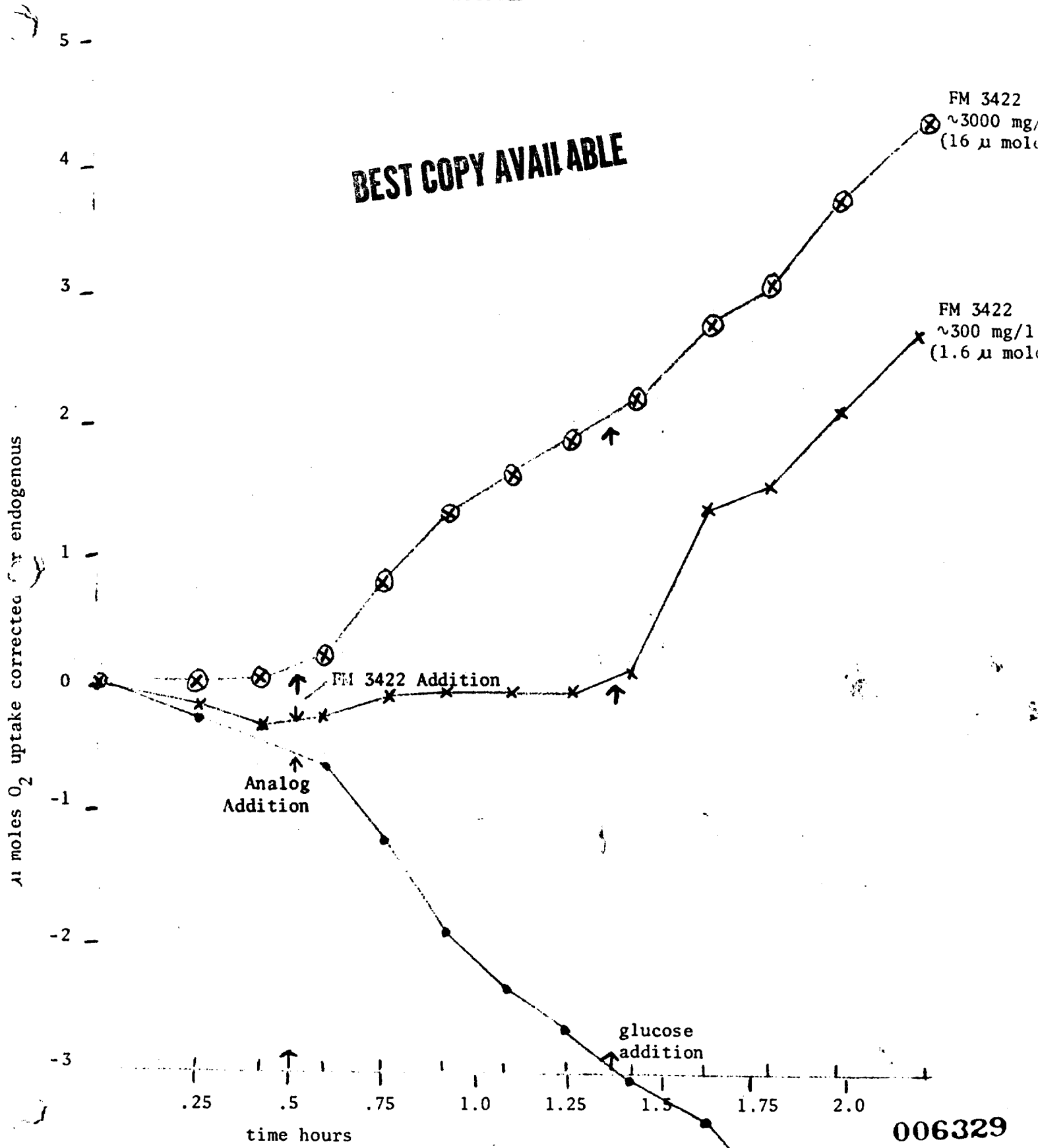
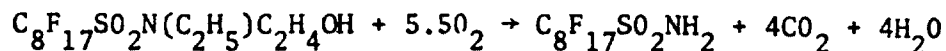


FIGURE 2: Warburg study of FM 3422 and its hydrogen analog.

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 ($\sim 16 \mu$ moles) would require 87μ 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.

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

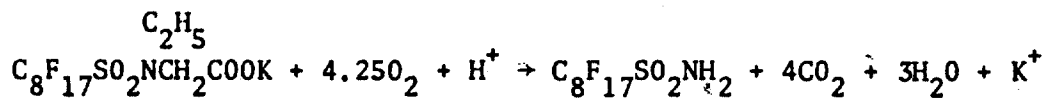
The results of Warburg studies with FC-95 are graphed in Figure 3.

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.



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μ moles O_2 uptake corrected for endogenous.

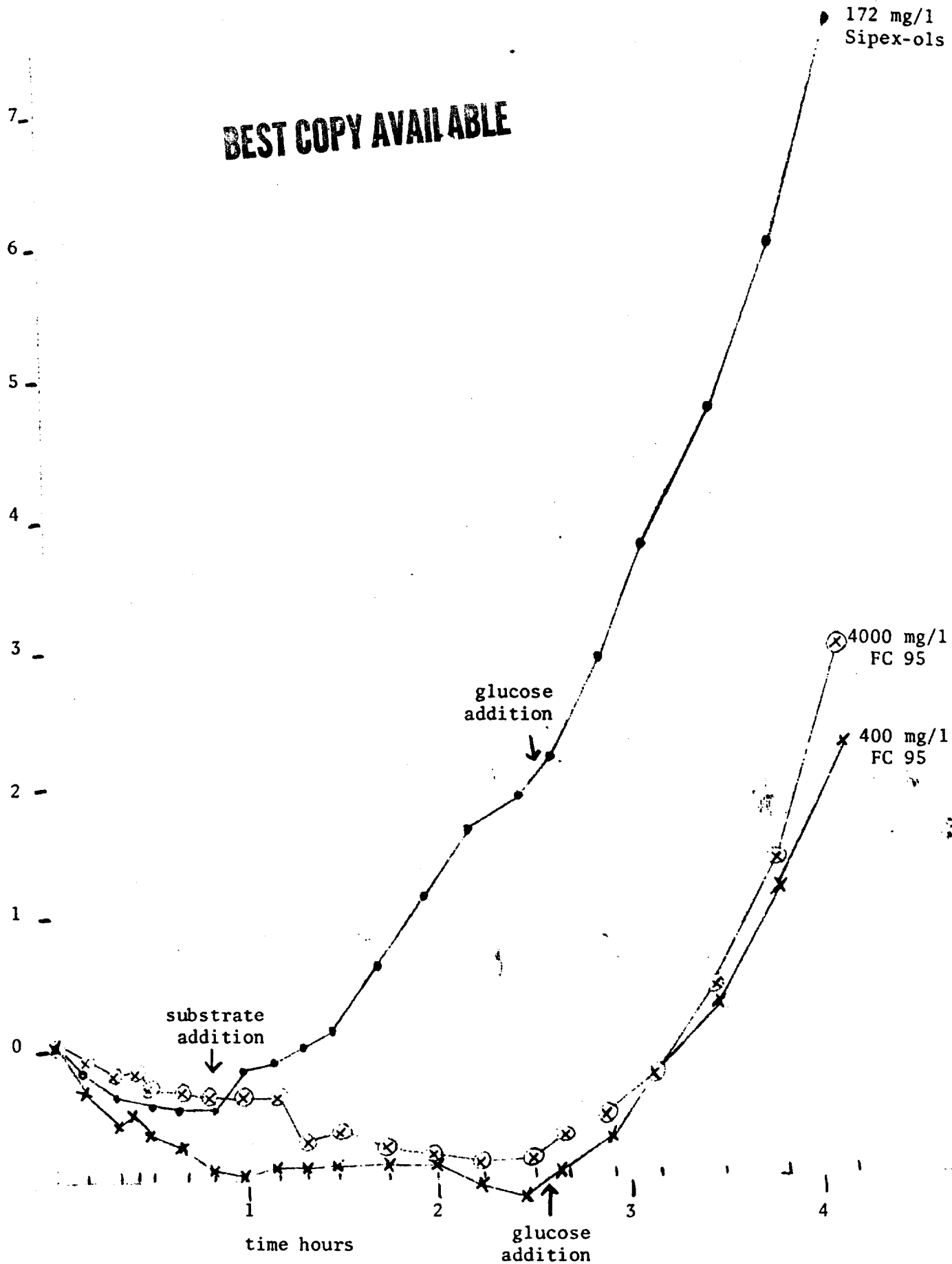


FIGURE 3: Warburg study of FC 95 and Sipex-ols.

006332

FC 128

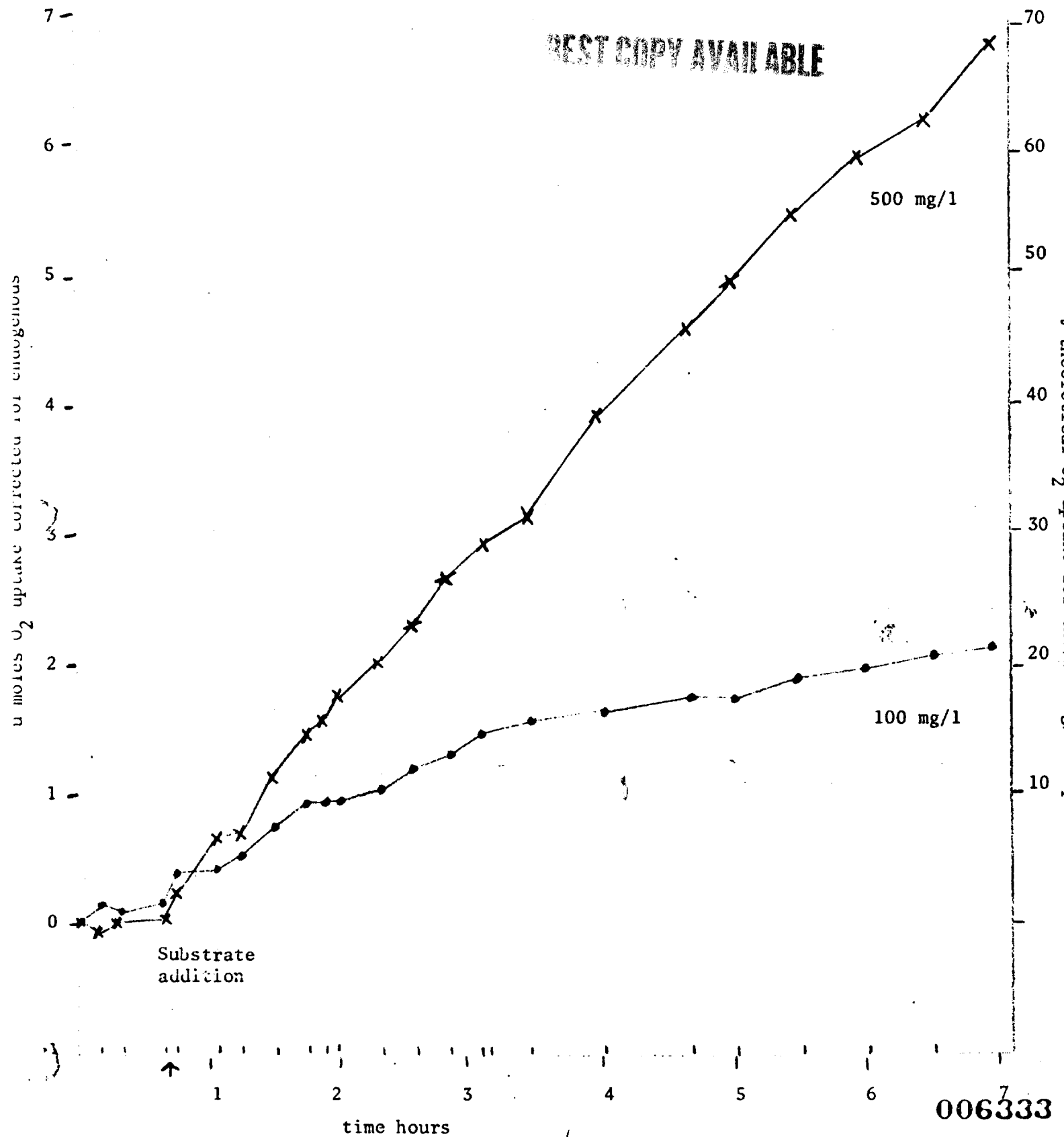


FIGURE 4: Warburg study of FC 128.

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 H_2O 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 $0^{\circ} C$.
 - 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, $\frac{1}{2}$ 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.

See P28 Notebook 46671

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File # M-3422

* leaked + tightened at 12 min

* Glucose high in side arm flask. Glucose could be flowing into the flask

⊙ Chemicals added at 27 min. - 33 min.

+ ~~Chlorine~~ Water went into other arm.

add ~~250~~ 250g glucose for side am
start 80 min - 83 min

110. 150

006337

PROJECT NO. _____

3M TECHNICAL NOTEBOOK NO.

40671 28

Subject:

Warburg determination of toxicity of
FM-3422 and its hydrogen analog

Date: 9-22-75

Project: To determine toxicity of these compounds to Microorganisms
at that concentration levels can be selected for~~Reference~~ Semicontinuous activated sludge biodegradation
studies. (Regardless of results low concentration still should be
used because degradation products may be toxic)(The following procedure will be followed on on)
(9-24-75)Procedure:

- ① Follow outlined procedure for Warburg Determination*
(EAR 7/10/75) and for making BSM (EAR 7/10/75).
- ② Collect cells from Pipe Eye activated sludge
tank on day prior to Warburg operation.
When collecting sludge let it drain from line
for a few minutes to ensure that fresh
active sludge is obtained.
- ③ Wash sludge 2x with Cold BSM as soon as
possible after returning to the lab. ~~and~~
Adjust MLSS to 3000 mg/l using standard
curve developed on Spectronic-20 for
Chenobite sludge**. Determine MLSS on
adjusted sludge using Good ~~and~~ crucible and
glass fiber filter gravimetric procedure
for comparison.
- ④ Prepare Warburg flasks as outlined
on Next Page.

* These procedures are in Warburg Data Book

** This curve is in Warburg Data Book.

Signature _____

006338

Date: _____

Read and Understood _____

Date: _____

PROJECT NO. _____

3M TECHNICAL NOTEBOOK NO.

40671 29

Subject:

FM-3422 + H analog warburg study contract.

Date: _____

Object:

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Reference:

flask #	flask code Name	flask Contents	Vent + Side arm #1	Stopper Side arm #2	Center Well
①	Thermobarometer	~3 ml DI H ₂ O	—	—	—
②	(Endogenous control)	(2 ml of 3000 mg/g Washed Sludge)	(1/2 ml DE water)	(1/2 ml DI water)	(.2 ml 10% KOH)
③	(glucose only control #1)	??	(1/2 ml 1/2 ml 0.5 g/50 ml glucose solution)	??	??
④	(glucose control #2)	??	(1/2 ml - 0.5 g/50 ml glucose solution)	??	??
⑤	(0.4 mg/l FM 3422)	??	??	(1/2 ml 2.4 mg/l FM 3422 aqueous solution or emulsion*)	??
⑥	(4.0 mg/l FM 3422)	??	??	1/2 ml 24 mg/l FM 3422	??
⑦	40 mg/l FM 3422	??	??	1/2 ml 240 mg/l FM 3422	??
⑧	400 mg/l FM 3422	??	??	1/2 ml 2400 mg/l FM 3422	??
⑨	4000 mg/l FM 3422	??	??	1/2 ml 24,000 mg/l FM 3422 aqueous emulsion	??

Continued Next page

* Preparation of FM-3422 or H analog solutions or emulsions see ~~next~~ page 31.

Signature _____

006339

Date: _____

Read and Understood _____

Date: _____

PROJECT NO. _____

3M TECHNICAL NOTEBOOK NO.

40671 30

Subject: FM - 3422 + H analog Working Study Cat

Date: 9-22-75

Object:

Reference:

Flask #	Flask Name	flask Contents	Vent Side arm # 1	Stopper Side arm # 2	Center well
(10)	(0.186 myl/l Hydrogen analog of FM 3422*)	(2 ml of 3000 myl/l Washed Sludge)	(1/2 ml 0.5g/sol ml) glucose Solution	1/2 ml 1.12 myl/l Aqueous solution or emulsion of the hydrogen analog of FM 3422**	0.2 myl/l of 10% KOH
(11)	(1.86 myl/l H analog of FM 3422)	>>	>>	1/2 ml 11.2 myl/l Analog solution or emulsion	>>
(12)	18.6 myl/l H analog	>>	>>	1/2 ml 112 myl/l analog emulsion	>>
(13)	186 myl/l H analog	>>	>>	1/2 ml 1120 myl/l analog emulsion	>>
(14)	1860 myl/l H analog	>>	>>	1/2 ml 11200 myl/l analog emulsion	>>

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* This is equimolar with the 0.4 myl/l FM 3422 solution.

MW FM 3422 ($C_{12}H_{10}F_{17}S_1N_1O_3$) = 571.29MW Hydrogen analog ($C_{12}H_{27}S_1N_1O_3$) = 265.46

$$0.4 \left(\frac{265.46}{571.29} \right) = 0.186$$

** See next page for prep of solution or emulsion.

Signature _____

006340

Date: _____

Read and Understood _____

Date: _____

Subject: FM 3422 + H analog Warburg study. Cat and
Prep of Emulsion or Solution of FM 3422 and
Object: Its Hydrogen analogs.

Date: 9-22-75

Reference:

Since FM 3422 and its hydrogen analog are not very soluble emulsions may have to be prepared in order to put them in contact with the microorganisms in the warburg studies.

Bring ~~about~~ 1.2 g of FM 3422 To 50 ml ~~the~~ with H₂O (DI) Heat to melt FM 3422 and Sonicate to form an emulsion. As soon as possible, to avoid separation of emulsion, take 5 ml of this 24,000 mg/l solution, and transfer to 45 ml of DI water to form a 2,400 mg/l solution. Mix, and repeat ~~the~~ 5 ml to 45 ml dilutions to form 240 mg/l, 24 mg/l liter, and 2.4 mg/l emulsions or solutions.

Do the same with the hydrogen analog of FM 3422, except that 0.558 g of the analog should be added to 50 ml of H₂O. This will make 11,200 mg/l emulsion or solution. Also make 1120, 112, 11.2, and 1.12 mg/l emulsion solutions.

If emulsion can't be made by the above procedure try making emulsions in a blender. If this also doesn't work see if a better sonicator is available.

Signature _____

Date: _____

Read and Understood _____

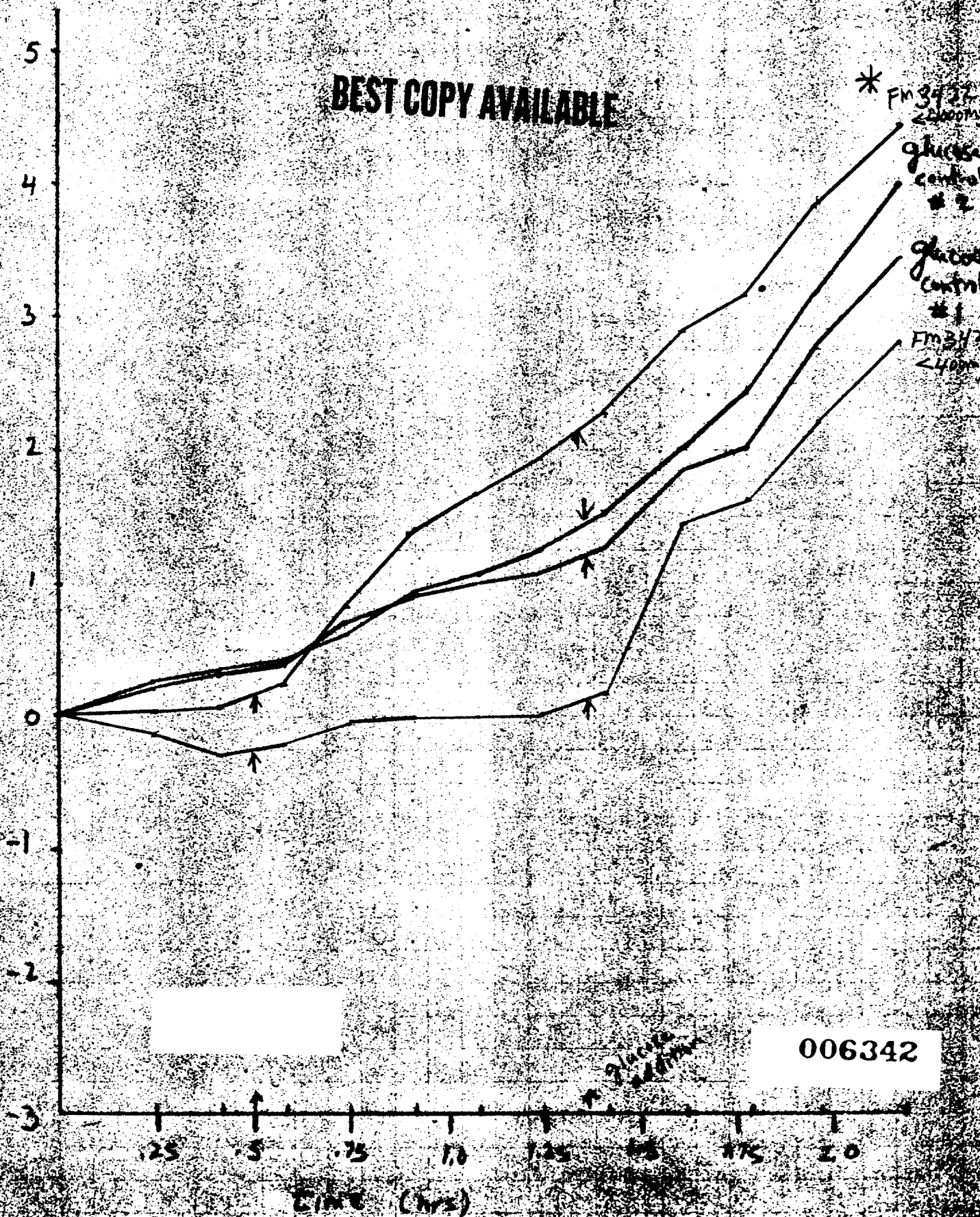
006341

Date: _____

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FM 3422 7/25/55 Run 4

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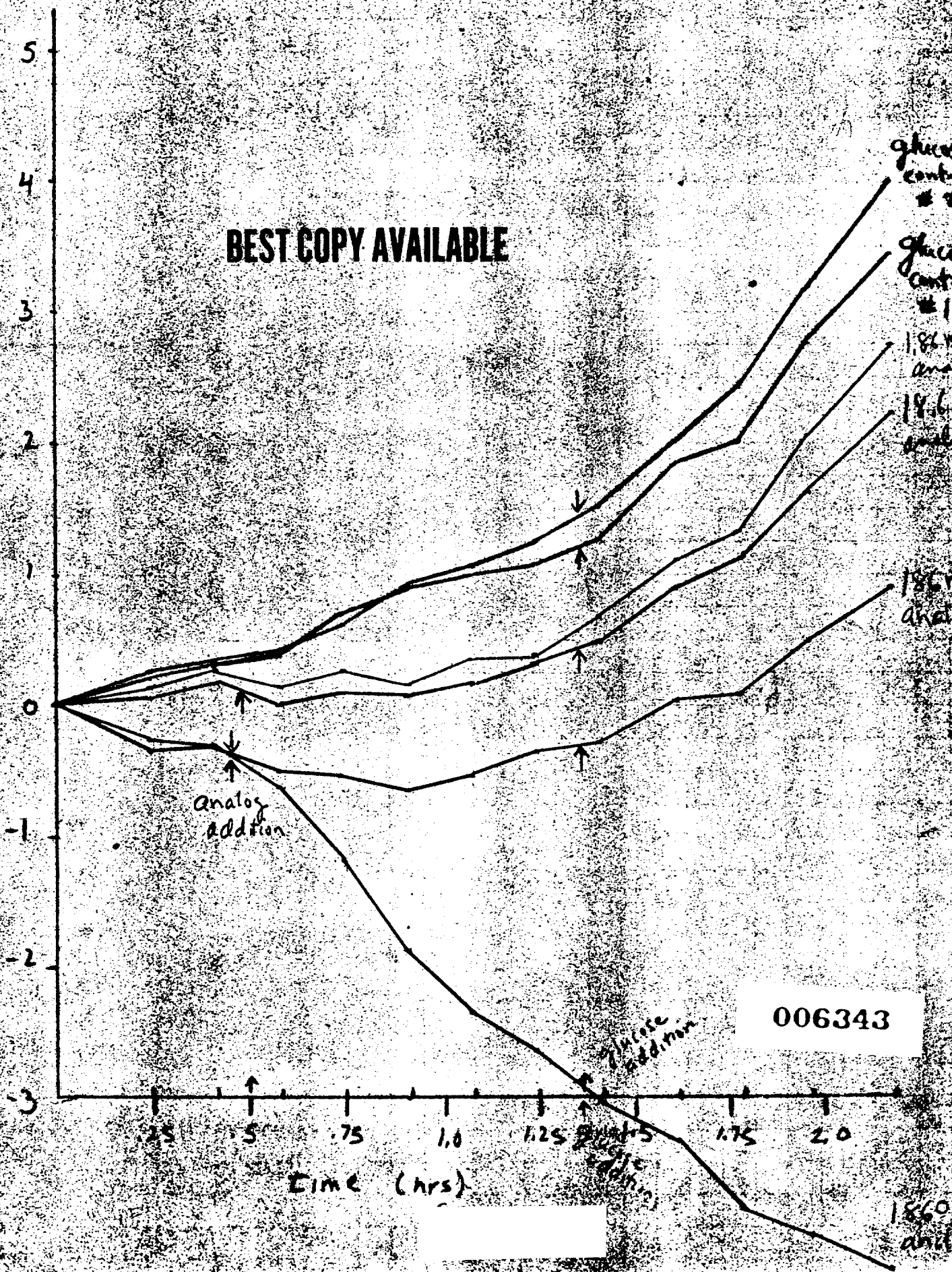


* not all went into emulsion. Till case probably cleared to 3000 mg/l

FM 3422 exp

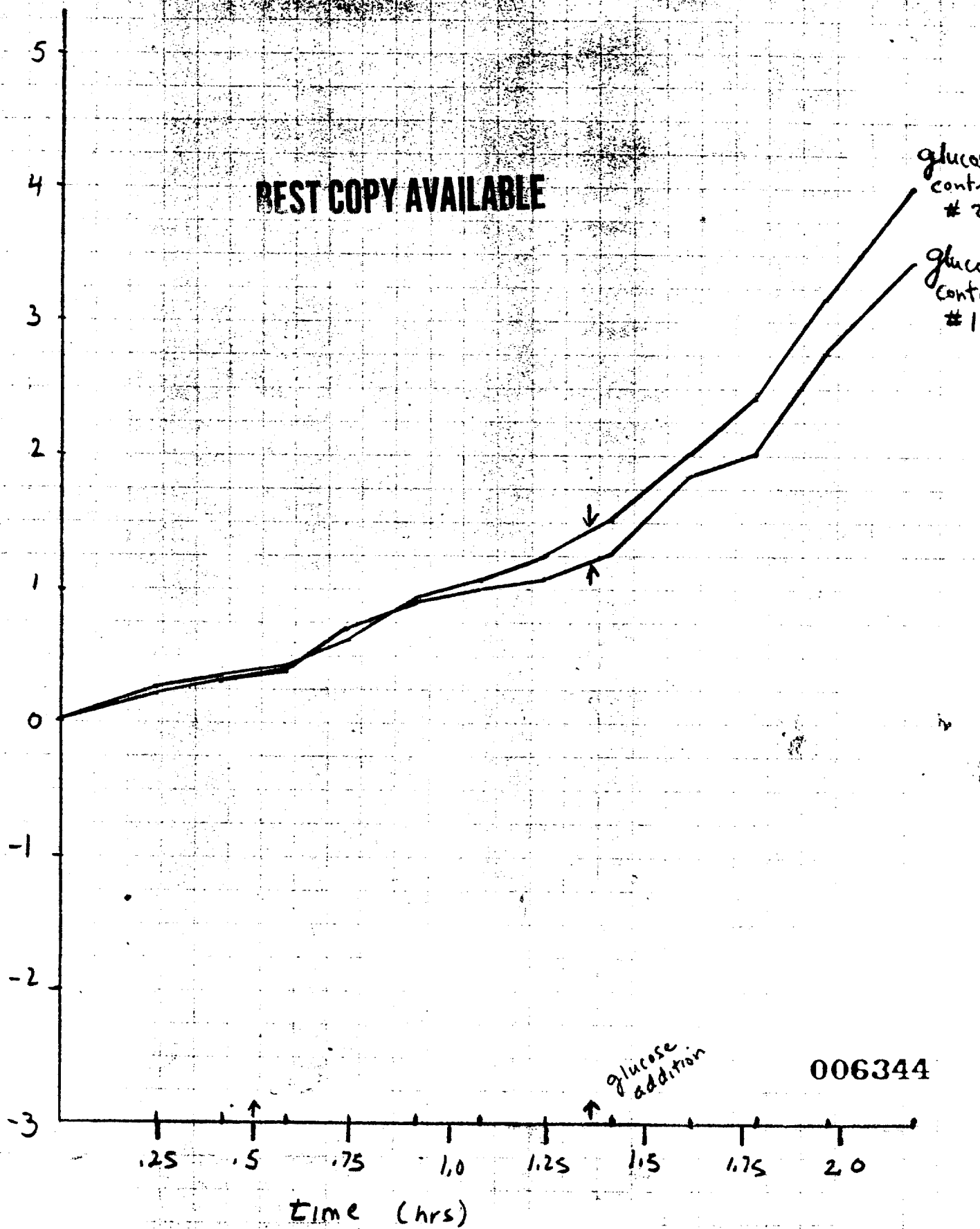
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μ mole O_2 uptake converted to endogenous

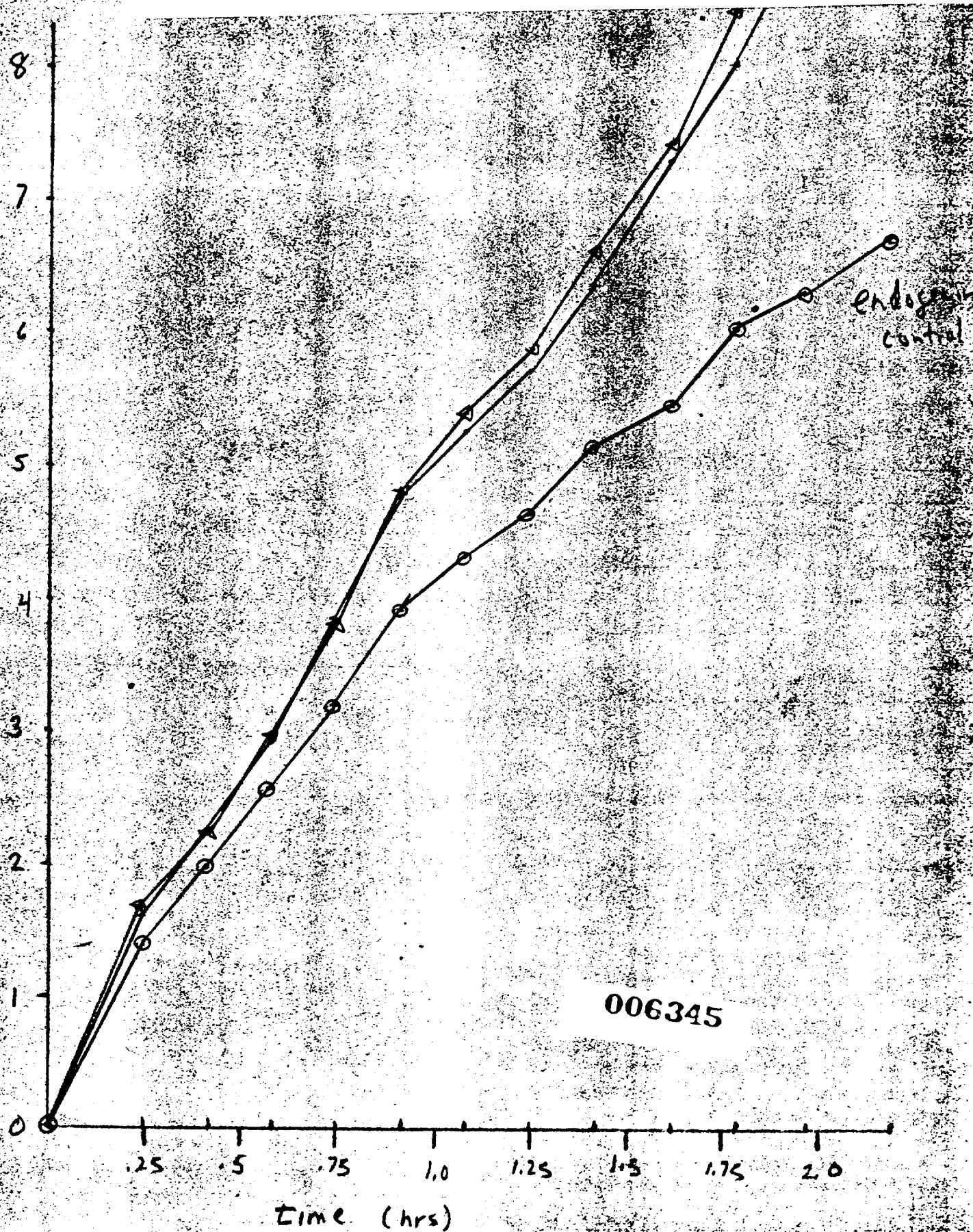


μ mole O_2 uptake corrected for endogenous

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$$FC = 0.08310$$

$\frac{1}{2}$ ml H_2O both side arms

[illegible]

Footnotes over.

H_2O + glucosyl side arms

Flask Contents: glucose control #

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08315

H₂O is 15% from glycerol seed

006348

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Footnotes over

08443

006349

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Footnotes over:

08467

g/14 com
in second
side am

006350

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Footnotes over

08447

006351

Footnotes over.

08543

WARBURG O₂ UPTAKE CALCULATION SHEET

006352

Footnotes over.

05359

WARBURG O₂ UPTAKE CALCULATION SHEET

glenn

Footnotes over.

* adjusted back to 150 after last reading

glen

006354

Footnotes over. * returned to 150 after last reading

Flask Contents:

Title: Warburg Determination of Toxicity of FM-3422 + analysis

Date: 9-25-75

Flask No.: 13

Flask Contents:

006355

Footnotes over.

06277

Flask Contents: 1860 mg/l.e. analog

006356

Footnotes over.

PRODUCT ECOLOGICAL ASSESSMENT
REQUEST FOR LABORATORY WORK

Ref: ENVIR. ASSESS. INQ. # _____
DIVISION _____

LAB REQUEST NO. 3844

PRODUCT: _____

REQUESTER

Name: Dale Baun Phone No. _____
Request Date: 7/77
Project No.: 9900012600 Prob. No. _____
Code No.: _____
Sample Date: _____ Date Needed: _____
Description: _____
Purpose of Tests: _____

LABORATORY

Date Received Sample: _____
Analyst: _____
Date Completed: _____
Hours Spent: _____
Y _____ B _____ P _____
Please Call Requester If Delays Are Encountered.

PHYSICAL & CHEMICAL PROPERTIES

Heat of Combustion Cal/G (BTU/Lb.)

Density

Ash

Other

FC98 Lt 35

FC93 Lt 253

FC95
Lt 583

FC92 Lt 714

WATER POLLUTION

pH

COD

BOD₅

BOD, Ultimate

TOC

GC

Oxygen Uptake

Dehydrogenase Activity (TTC)

Other

✓ 5.000

✓ 180

✓ < 4,000

✓ 250,000

nitrogen
run
at 100
ml/min

BIOASSAY

Specify Organism and Method:

Fat head 96 h

✓

✓

LEACHATE FROM LEACHING TEST

pH

COD

BOD₅

BOD, Ultimate

TOC

GC

Bioassay

Other

OTHER

Bioaccumulation

Persistence

Degradation Products

006357

Subject: Chronological Review of
Biodegradation Studies
on FM 3422

August 12, 1977

TO: D. L. BACON
FROM: E. A. REINER

The following reviews the progress that has been made in the biodegradation study of FM 3422 over the course of the Fate of Fluorochemicals Project.

The first step in our biodegradation study of FM 3422 was a Warburg oxygen uptake experiment. The Warburg work was conducted using an unstabilized FM 3422 emulsion in water produced by sonication. When FM 3422 was exposed to activated sludge as the sole carbon source, O_2 uptake equivalent to about 3% of the theoretical O_2 demand for the hydrocarbon portion was observed within a few hours. No degradation products were detected by thin-layer chromatography (TLC). However, these results were confirmed by a second Warburg study.

This work was followed by attempts to collect degradation products in quantities sufficient for identification. A semicontinuous activated sludge study using nonemulsified FM 3422 was conducted for a one-week period with daily readdition of FM 3422. No degradation products were detectable by gas liquid chromatography (GLC) in n-octanol extracts or by TLC in ethyl acetate extracts. The FM 3422 added in ethanol rapidly congealed and separated from aqueous suspension. As a result, too little surface area may have been available for detectable microbial degradation.

A method was then developed to make nontoxic, stable FM 3422 emulsions in order to increase the likelihood of microbial modification of FM 3422. Liu has shown this approach to be effective with polychlorinated biphenyls (PCB) (1). Stabilized PCB emulsions are biodegradable; nonemulsified PCB's are not. However, the incubation of emulsified FM 3422 in SCAS reactors again did not yield ethyl acetate extractable material that could be identified as biodegradation products of FM 3422 by either TLC or GLC. It also was impossible to quantify the loss of FM 3422 by this approach since FM 3422 did not remain homogeneously distributed throughout the system.

Die-away experiments were conducted to quantitatively measure depletion of FM 3422 by biological degradation. Measured quantities of FM 3422 were shaken with activated sludge in Erlenmeyer flasks. Flasks were sacrificed at 0, 1, 2, 4, and 8 days, acidified, extracted and analyzed for FM 3422 by GLC. No consistent reduction in GLC peak height was observed in this 8-day experiment. A new GLC peak was observed that was not present in control cultures.

747 *Better way*

006358

August 15, 1977

Successive seven-day adaptive transfers of FM 3422 acclimated cultures were then made into nutrient media containing emulsified FM 3422. Unexpectedly, the final concentration of ethyl acetate extractable FM 3422 was found to decrease (see attached bar graphs). The newly observed unidentified GLC peak was present both in initial and final FM 3422 containing samples. This suggested that the material causing this peak was formed chemically (not biologically) from FM 3422 as a result of the extraction and/or analytical technique. This material was found only in samples acidified to pH 1 prior to extraction. Attempts to identify this material by TLC in our lab and by Central Research failed. The acidification step in the extraction procedure was found unnecessary and dropped.

Modifications in the methods of making FM 3422 emulsions and in the extraction technique reduced the variability in results. Our ability to obtain repeatable results over the same incubation period is demonstrated in the attached bar graphs. Alternate inocula, in addition to Pig's Eye sludge, were also tried as sources of microorganisms. A year-old Decatur soil sample gave no loss of extractable FM 3422 during the first seven-day growth period. However, loss was observed after the second period and increased in subsequent adaptive transfers (see graph 3). New Decatur soil and Decatur activated sludge were found capable of decreasing the amount of extractable FM 3422 during the first seven-day growth period as well as in subsequent growth periods. However, it must be noted that sterile technique was not used in all of these experiments. Thus, it is possible that all of these cultures became contaminated by the already developed acclimated culture present in the laboratory.

No metabolites have yet been identified. An attempt is now being made to identify a possible (but I feel improbable) metabolite separated by TLC.* Very distinct spots of this material were present in two 135 ppm FM 3422 containing cultures, but a spot of similar intensity was also observed in a control culture. The control culture contained only 2.7 ppm of FM 3422 that was present in its inoculum. Both TLC and GLC confirmed this low FM 3422 level in the control.

A number of experiments are now underway in the laboratory and others are being written with the objective of determining what causes FM 3422 to become non-extractable. An attempt is being made to determine if FM 3422 has been conjugated, polymerized, or modified to a form that can be converted back to unmodified FM 3422 by acid or base hydrolysis. Although it is unlikely that FM 3422 is degraded to the point of fluoride ion release to the media, this possibility is being investigated. An attempt will be made using sterile technique to clarify whether viable acclimated cultures are necessary to effect the change in FM 3422 which makes it nonbiodegradable. A materials balance experiment with ^{14}C labeled FM 3422 will be conducted to determine if the nondetectable portion or product of FM 3422 is located in the ethyl acetate phase, the water phase, or in the microbial solids.

Other future objectives are to look for biodegradation products of FC-128. This compound is chemically very similar to FM 3422. FC-128 has a carboxyl salt in place of a hydroxyl substituted carbon, and FC-128 is a possible degradation product of FM 3422. FC-128 was found to be readily degradable in Warburg studies.

* Infrared, GLC and TLC analysis of the extract now suggest that this material is a complexed form of FM 3422 (8/17/77).

August 15, 1977

Identification of the biodegradation product of FM 3422 has proven to be a more elusive goal than originally expected. However, because of FM 3422's central position in the fluorocarbon line, it is important that we determine what is happening to this molecule. It is likely that the noncostly experiments we have planned will give us the answers we need.

Eric G. Reiner

EAR/cel

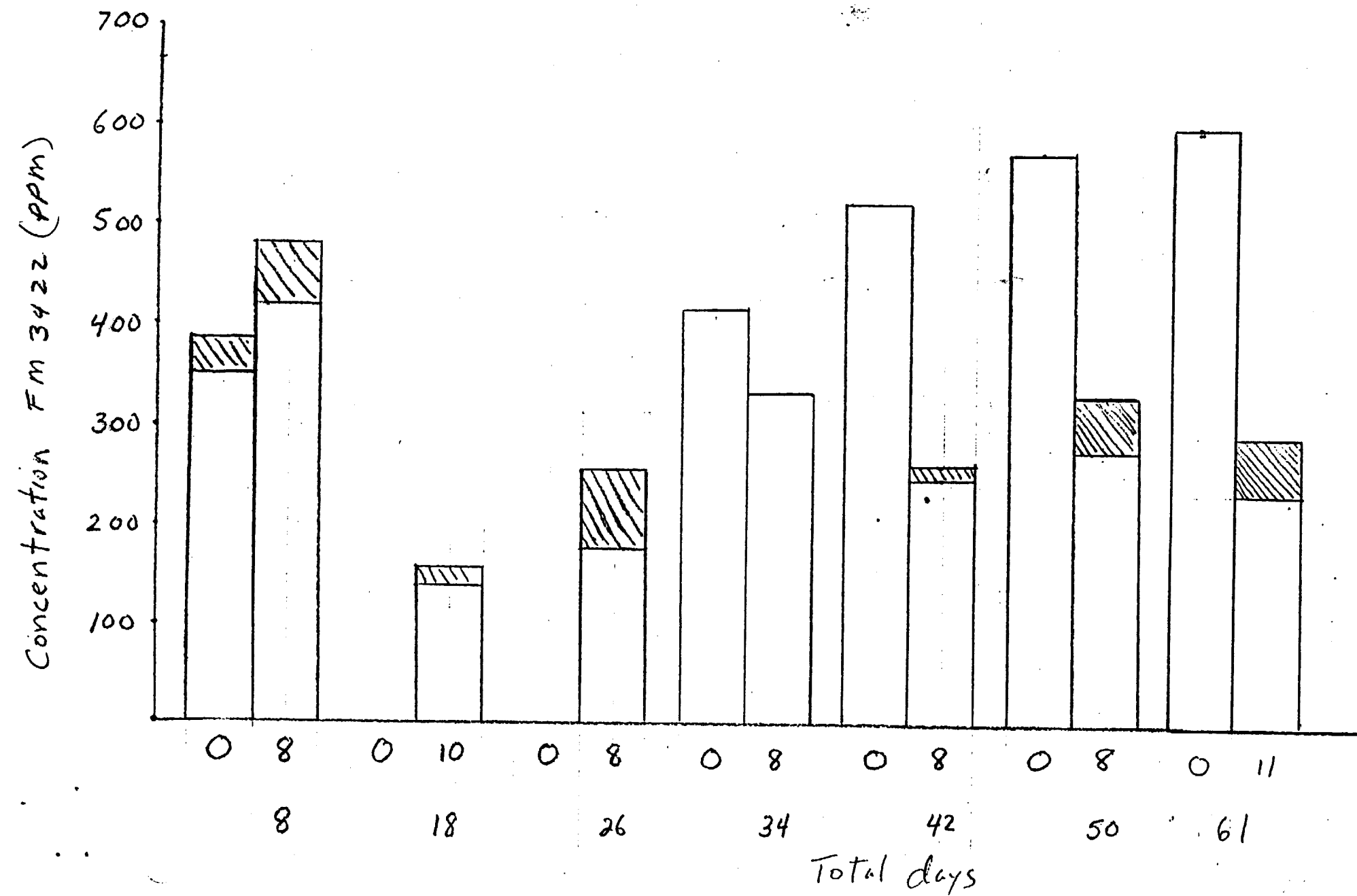
Reference:

- (1) Dickson L. S. Liu, M.Sc., Ph.D. (British Columbia), "Biodegradation, An Environmental Solution to Some Toxic Organic Compounds," Environmental Conservation, Vol. 3, No. 2, Summer 1976.

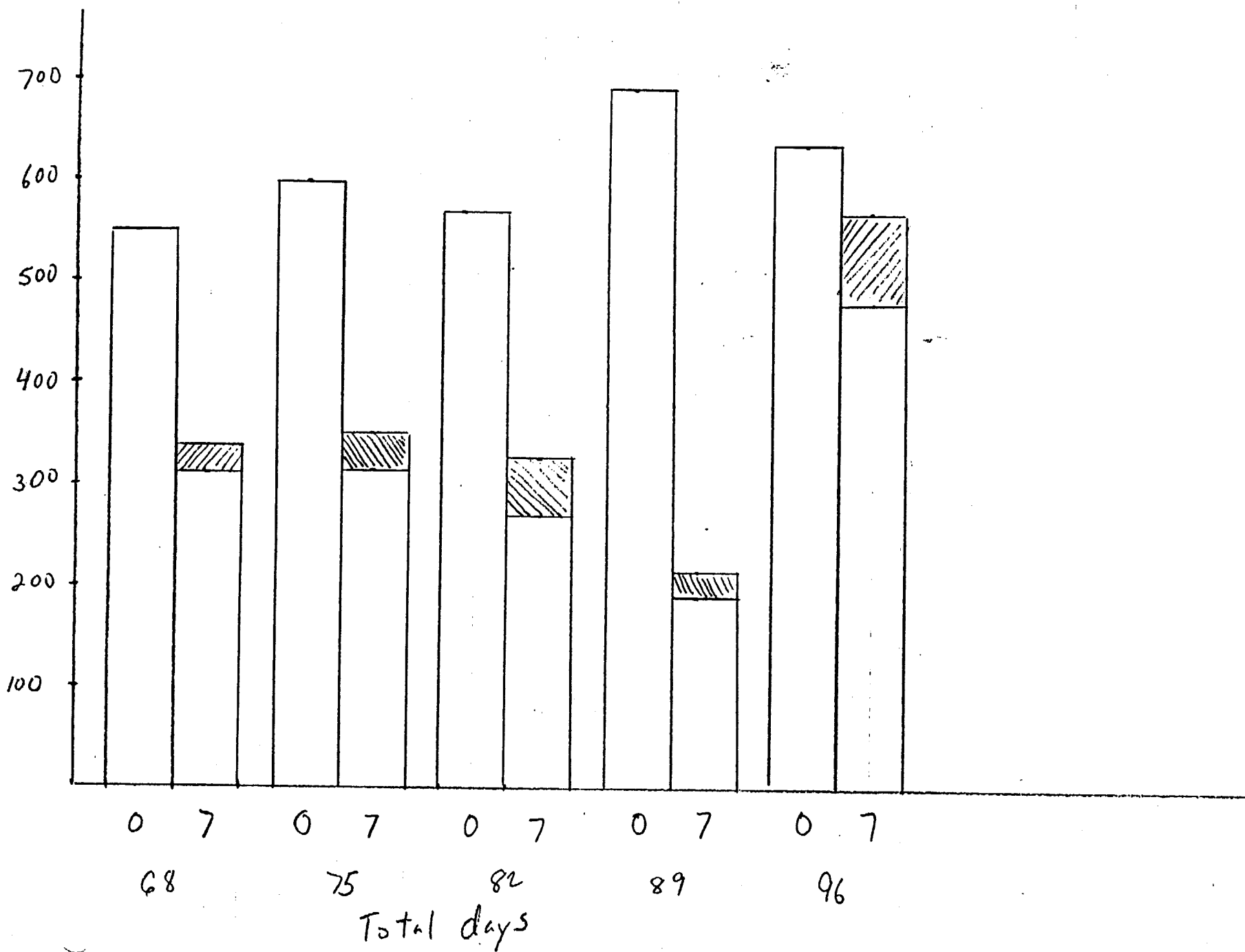
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Adaptive Transfers

006361

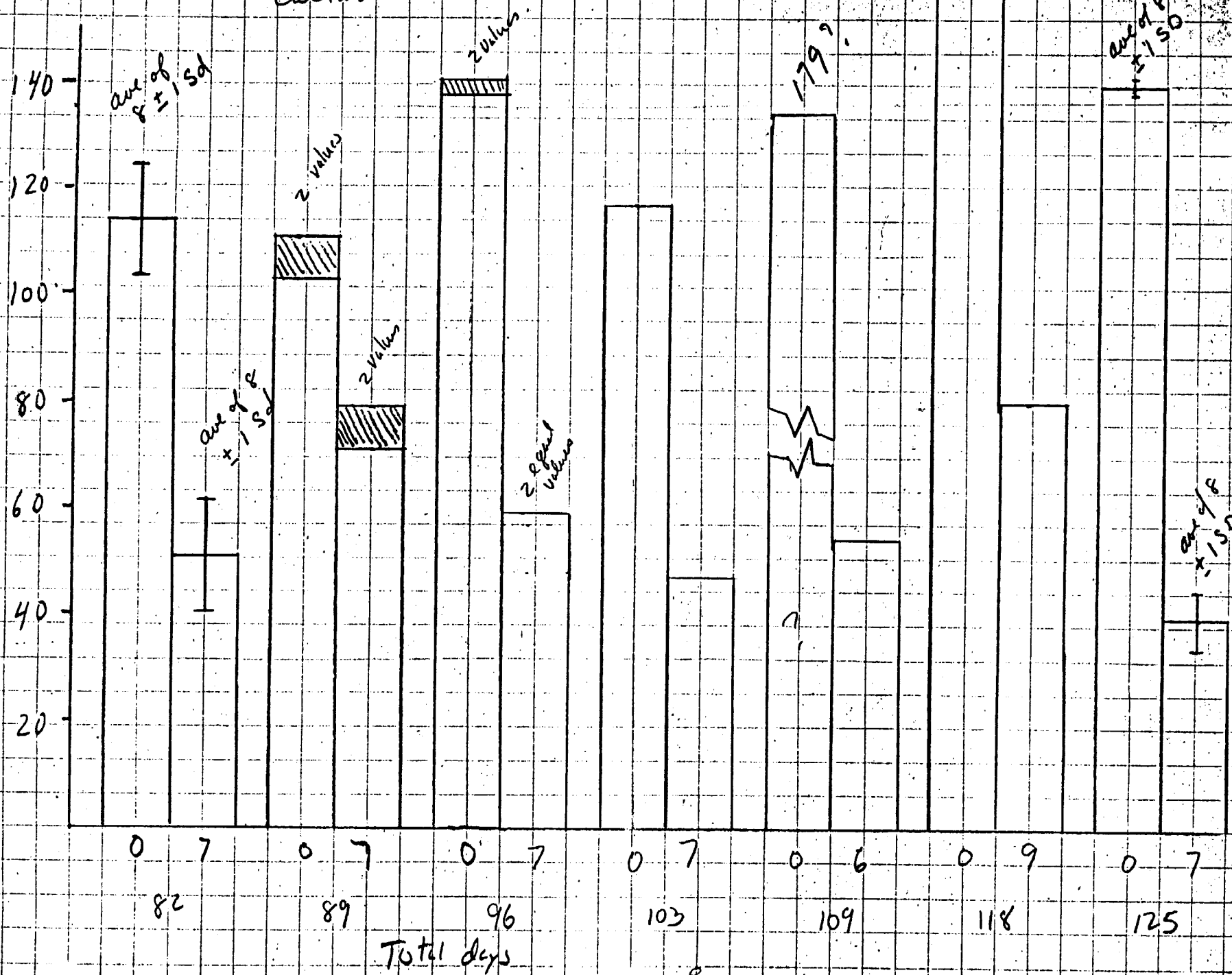


Concentration Fm 3422 (ppm)



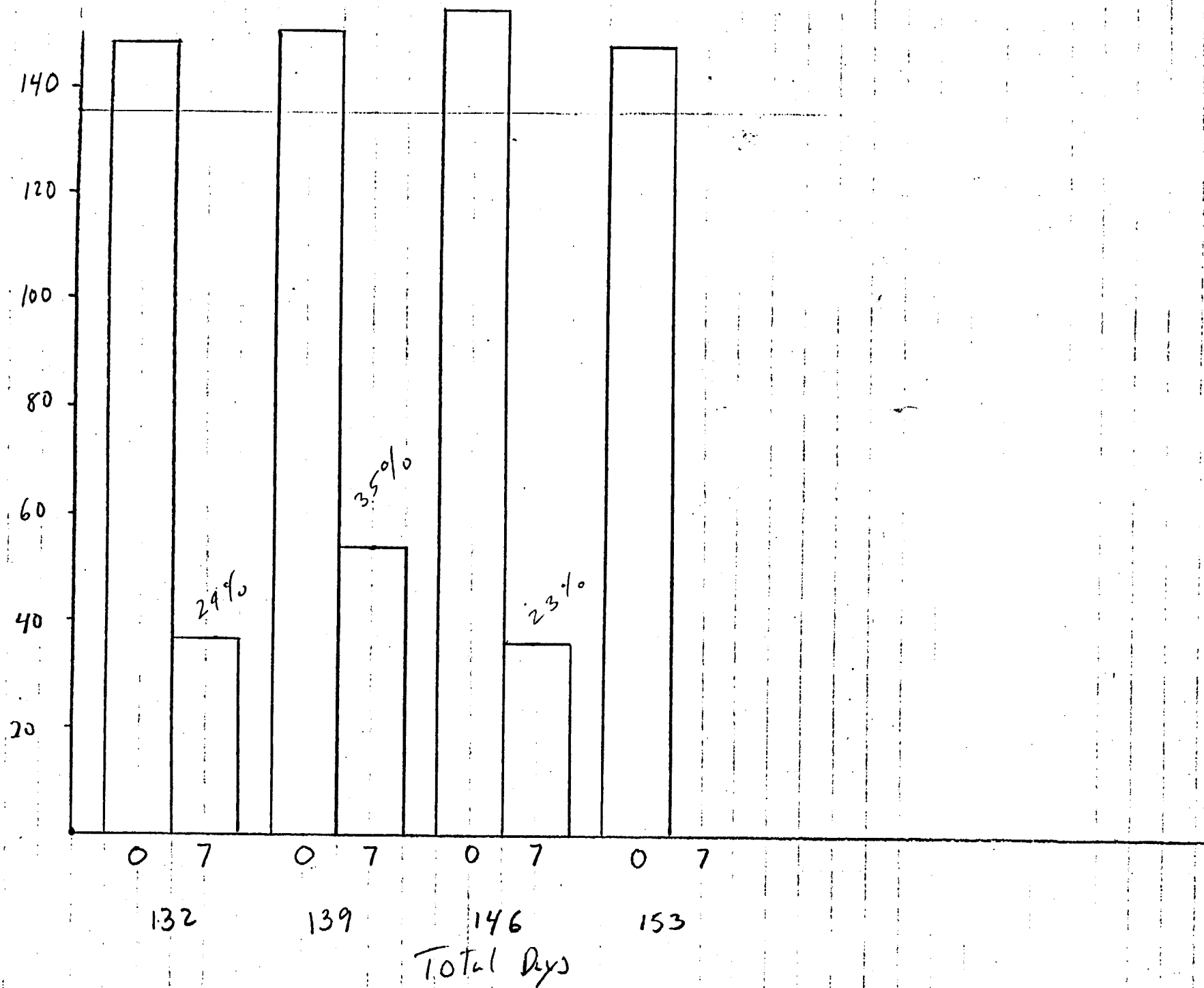
Adoptive Transfers in Centrifuge Tube Expt

Concentration FM 3422 (PPM)



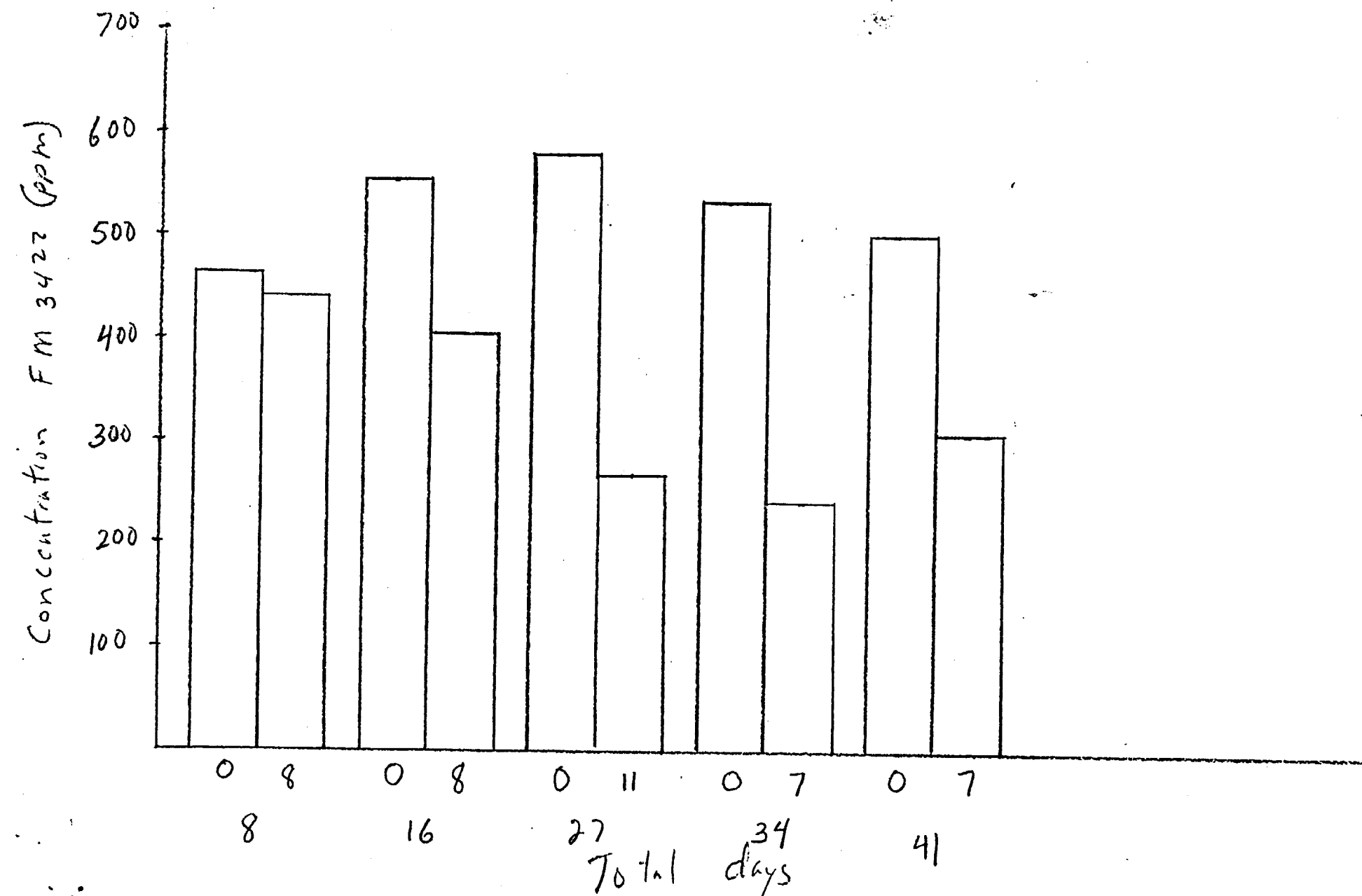
006363

Concentration Fm 3422 (ppm)

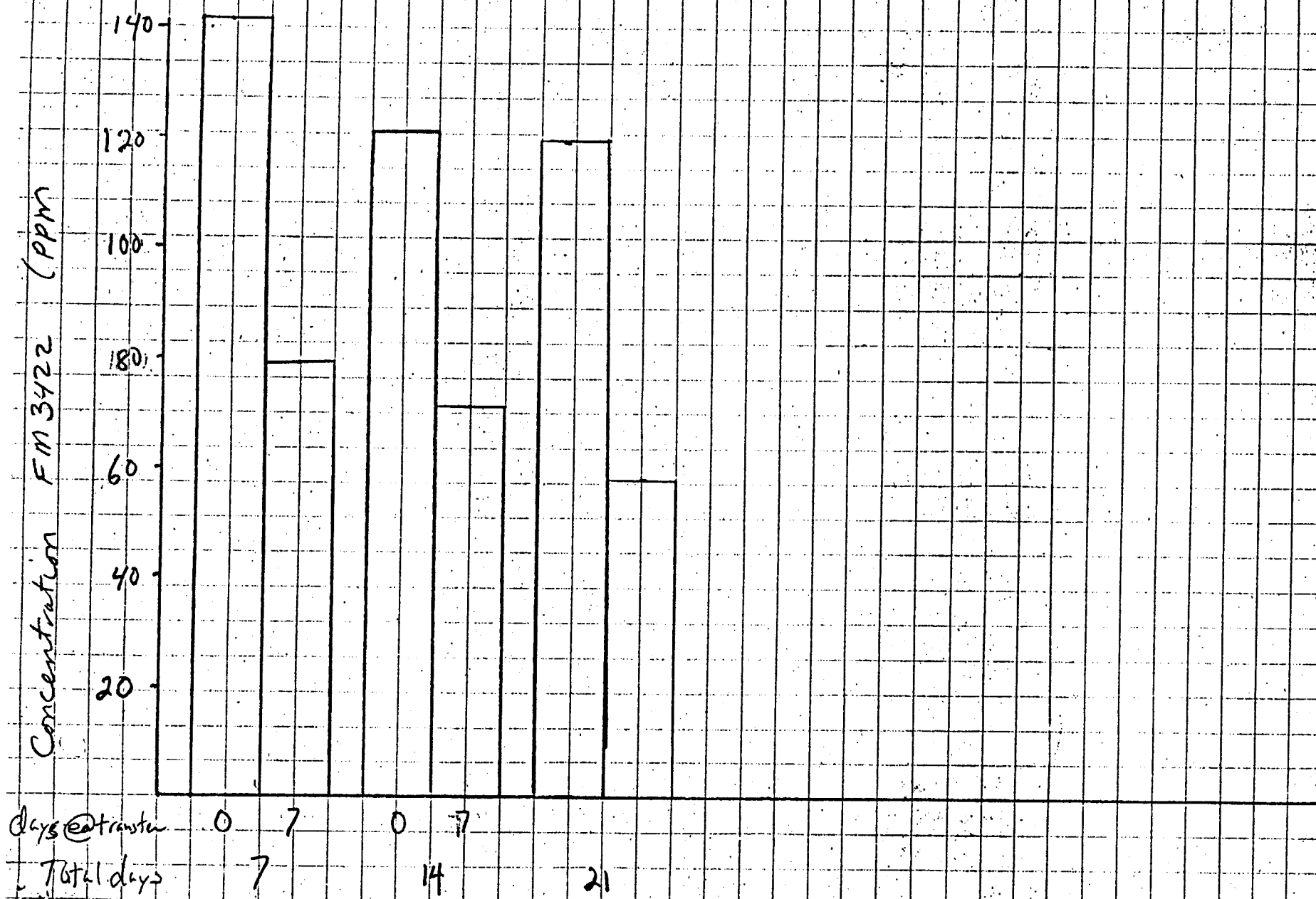


Decatur Soil Adaptive Transfers (year old soil)

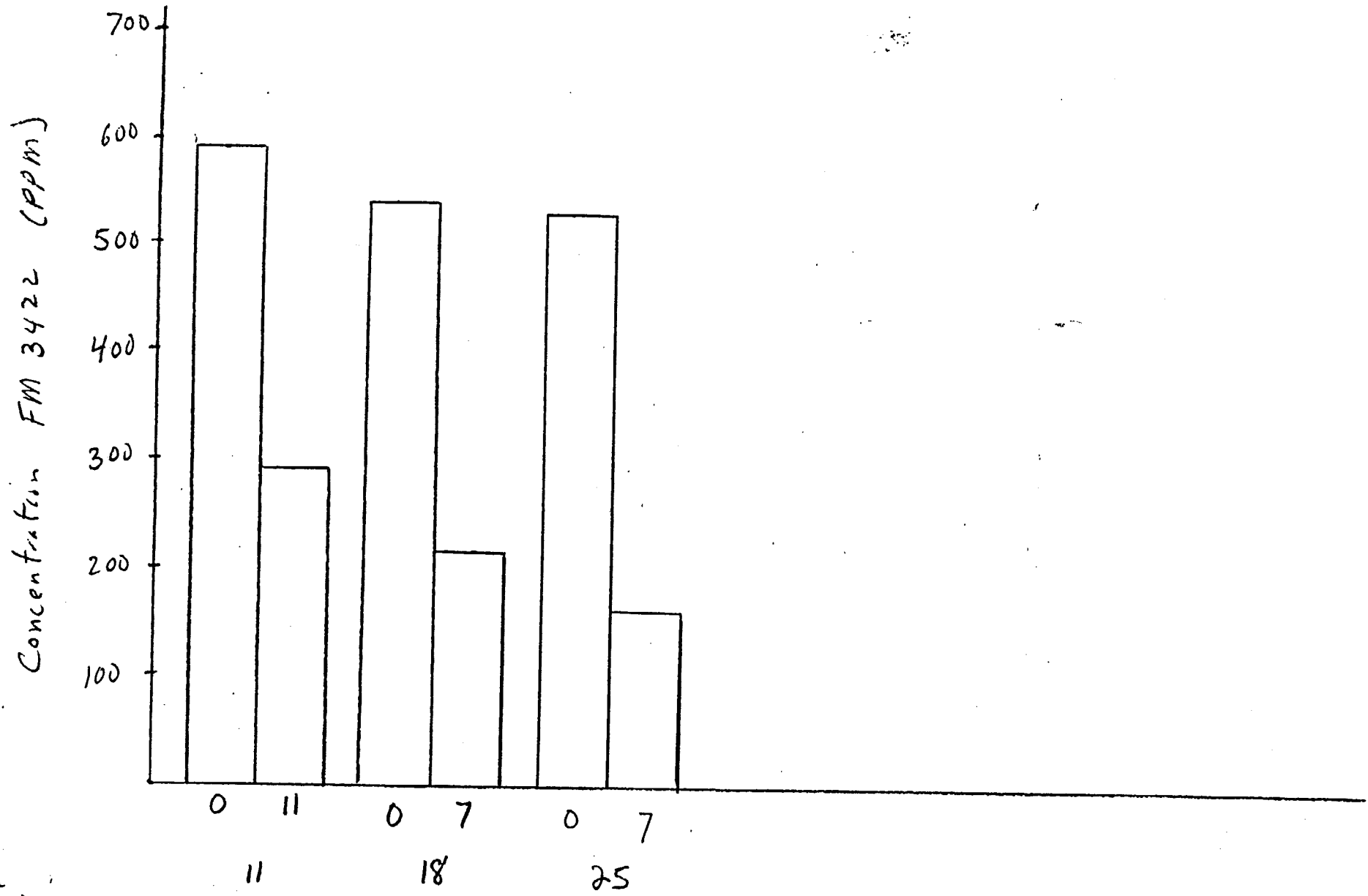
006365



new Soil from UEC star adaptation transfer



006366



TECHNICAL REPORT SUMMARY

Date
1/9/78

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	0222
Project	Fate of Fluorochemicals in the Environment	Project Number	9970612613
Report Title	Biodegradation Studies of Fluorocarbons - II	Report Number	4
To	D. L. Bacon		
Author(s)	E. A. Reiner	Employee Number(s)	47816
Notebook Reference	40671, p. 54-56; 45727, p. 1-30; 46269, p. 7, 14, 15, 18, 21, 25, 29; 41947, p. 50, 51, 55; 44191, p. 27-32	No. of Pages Including Coversheet	20
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 susceptibility of FM 3422 to biological decomposition.

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

Biodegradation studies are described which allow the evaluation of the susceptibility of FM 3422 to aerobic microbial degradation. Biodegradation test procedures used are modifications of common biodegradation test methods. They include semicontinuous activated sludge and shake flask die-away studies with sequential adaptive transfers. Microbial inocula were obtained from several environmental sources. Some sources were selected for their likelihood of containing microbial populations enriched in the capability of degrading FM 3422 or similar fluorochemicals. Analytical procedures used included GLC, TLC, ¹⁴C-scintillation counting and analyses for released fluoride. Interpretation of biodegradation results from these studies was hindered by the low solubility of FM 3422, its volatility, and its affinity for suspended organic material. This binding made it difficult to quantitatively extract FM 3422 from the biological solids of the test cultures. Nontoxic detergent stabilized emulsions were used to circumvent problems caused by low water solubility.

SUMMARY AND CONCLUSIONS

FM 3422 was found to be completely resistant to biodegradation under the conditions described in this report. These conditions are considered to be optimum for biological degradation. FM 3422 was also found to have a strong affinity for organic solids.

In all experiments described herein, FM 3422 was exposed as a surfactant stabilized emulsion, to heterogeneous microbial cultures, under aerobic conditions, and near neutral pH (7.1-7.5). Concentrations of FM 3422 ranged from 100-500 mg/l. Microorganisms were obtained or developed from soil or waste treatment sludges. Some microbial sources were selected because of their previous or continuous exposure to fluorochemicals. It was felt that these sources were likely to have developed naturally enriched cultures capable of degrading fluorochemicals. FM 3422 was exposed to both high (~2000 mg/l) and low (~100 mg/l) cell densities. Weekly transfers of the low cell density cultures allowed acclimation to FM 3422 to proceed for six months.

This study cannot rule out the possibility that conditions could be found that would allow biodegradation of this compound, nor can it exclude the possibility that a microorganism could eventually be enriched that could metabolically alter this chemical under the conditions described in this report. Nevertheless, the results of this quite extensive study strongly suggest that FM 3422 is likely to persist in the environment for extended periods unaltered by metabolic attack. Its observed affinity for organics suggests that FM 3422 released to the environment is likely to sorb to organics such as those present in soil or in the sediments of aquatic ecosystems. Table 1 summarizes the experiments conducted in the study of FM 3422 biodegradation.

TABLE 1

SUMMARY OF EXPERIMENTS PERFORMED IN
FM 3422 BIODEGRADATION STUDY

<u>Experiment</u>	<u>Findings</u>
1) 10-day semicontinuous activated sludge biodegradation study of surfactant stabilized FM 3422.	No FM 3422 biodegradation products were detected in ethyl acetate extracts by TLC or GLC.
2) 6-month shake flask die-away study with weekly transfers.	Again no degradation products were detected in ethyl acetate extracts. However, >50% and sometimes >75% of the initially ethyl acetate extractable FM 3422 was missing from extracts from 7-day-old cultures.
3) Materials balance experiment.	An attempt to measure the fractions of ^{14}C -FM 3422 present in the water, ethyl acetate, and solids phases of 7-day-old cultures failed. This apparently was due to FM 3422 volatilization during solids drying. When mixed with grown cells, FM 3422 became nonextractable too rapidly to be explained by a metabolic phenomenon.
4) Fluoride release	Fluoride concentration did not increase during die-away studies with cultures acclimated to FM 3422. This suggests no modifications occur to the perfluoro portion of FM 3422.
5) Attempts to recover nonethyl acetate extractable FM 3422 from cell cultures:	
A) Hydrolysis	Neither acid or base hydrolysis freed non-extractable FM 3422.
B) Binding to Containers	Irreversible binding to culture container did not explain nonextractable FM 3422.
C) Volatilization from Cells	Nonextractable FM 3422 was 1/3 recovered by volatilizing at 100°C. from culture solids and condensing.
D) Hot ethyl acetate extracts	Ethyl acetate extracts at 60°C. did not recover additional FM 3422.
E) Reflux with Dioxane	Refluxing culture solids with 1,4-dioxane recovered all nonextractable FM 3422 from the solids of 7-day-old cultures. Complete recovery of FM 3422 shows that no biodegradation occurred.

006370

INTRODUCTION

Our early work evaluating the susceptibility of FM 3422 to microbial degradation has been described in a previous report (1). This work included two separate Warburg respirometric experiments which, convincingly, showed increased oxygen utilization by unacclimated biological sludge immediately following addition of unstablized emulsions of unpurified FM 3422. In these two experiments, with FM 3422 as the sole exogenous carbon source, oxygen uptake above the endogenous level accounted for 3% of the oxygen required to completely oxidize the hydrocarbon portion of the molecule. The reason for this remains unexplained. One possibility is that biodegradable fragments of FM 3422 may have been formed as a result of the emulsion formation by sonication.

Attempts to isolate FM 3422 degradation products from the Warburg flasks following the observed oxygen uptake were unsuccessful, as were those from a subsequent semicontinuous activated sludge (SCAS) biodegradation study. In the SCAS study solutions of FM 3422 in ethanol were added directly to the biological reactors. Added in this manner, the FM 3422 rapidly congealed and separated from the water phase accumulating on the reactor walls and with the biological solids. This separation from the water phase may have made FM 3422 unavailable to react with degradative enzymes.

In the present report, further attempts to characterize the susceptibility of FM 3422 to biodegradation are described.

METHODS AND MATERIALS

Most methods are described in the experimental section. The following standard methods and materials were used repeatedly throughout the experiment.

Extraction Procedure

10.0 ml samples were extracted in polypropylene centrifuge tubes by adding 10.0 ml of ethyl acetate. The samples were then capped with polypropylene caps and inverted 50 times. Phase separation was ensured by centrifuging for 10 minutes at about 12,000 x gravity. A correction has to be made for the decreased volume of the solvent phase and the increased volume of the water phase following extraction. After shaking the volume of the aqueous phase increases from 10.0 to 11.0 ml and the volume of the ethyl acetate solvent phase decreases from 10.0 to 9.0 ml. Therefore, a determination of the number of ppm of FM 3422 originally present in the water phase which is extracted into ethyl acetate is made by multiplying the concentration found in the ethyl acetate phase by 0.90.

Media

The basal salts media used in the semicontinuous activated sludge experiment consisted of:

- 1.0 g/l NH_4Cl
- 2.0 g/l K_2HPO_4
- 0.25 g/l $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$
- Adjusted to pH 7.2 with strong acid

The standard media used in die-away and subsequent studies contained:

133 ppm of FM 3422 (emulsified)

2 g/l K_2HPO_4

2.4 g/l YM Broth

830 mg/l Alconox

~6 g/l Ethyl acetate

pH ~7.2

It was prepared by emulsifying 0.2 g of FM 3422 dissolved in water-saturated ethyl acetate in 100 ml of 12.5 g/l Alconox solution that had been prepared using ethyl acetate-saturated water. Emulsification was accomplished by mixing for 30 seconds with a model SDT Tissumizer. The resulting emulsion was then diluted in a K_2HPO_4 solution which in turn was diluted in a weak YM broth media.

The surfactant containing media were demonstrated to be nontoxic to microorganisms by inoculating these media and comparing the dissolved oxygen concentration depletion rates from these cultures with those of nonsurfactant-containing cultures. These comparisons were made initially, after one hour, and after one day of exposure to the surfactant-containing media.

Chemical Analyses

TLC and GLC analyses are described in the Progress Report by A. Mendel (2).

Chemicals

The following chemicals were used in this study:

- 1) Sublimated FM 3422 (see report of Art Mendel - (2))
- 2) Radioactive FM 3422 (see report of Art Mendel - (2))
- 3) Ethyl acetate - Mallenckrodt, A.R.
- 4) YM Broth - DIFCO - Bacto Dehydrated
Bacto Yeast Extract - 14.3%
DIFCO Malt Extract - 14.3%
Bacto-Peptone - 23.8%
Bacto-Dextrose - 47.6%
- 5) Ether - Anhydrous, J. T. Baker, "Baker Analyzed."
- 6) Siponate DS-10 - A purified alkylaryl - sulfonate supplied by Alcolac, Incorporated.
- 7) Alconox - A mixture of alkylarylsulfonates, lauryl alcohol sulfates, phosphates, carbonates and synergistic agents - supplied by Alconox, Incorporated.
- 8) 1,4-Dioxane - Reagent grade - repackaged, supplier unknown.
- 9) Aquasol - Xylene-based scintillation solution - supplied by New England Nuclear.

006372

EXPERIMENTAL

Semicontinuous Activated Sludge Study

In an earlier study, no degradation products of FM 3422 were detected by TLC or GLC in a semicontinuous activated sludge (SCAS) study in which FM 3422 was simply added to the media as an ethanol solution, and FM 3422 rapidly congealed and separated from the water phase (1). It was postulated that biodegradation may not have been observed because enzymes could not "attack" the coagulated FM 3422 molecules. Since emulsions of water-insoluble molecules have been shown to be more susceptible to enzyme attack (3), a second SCAS degradation study of FM 3422 was conducted using emulsified FM 3422.

Prior to the initiation of this study, several surfactants were tested for their ability to stabilize FM 3422 emulsions. The two most satisfactory were Alconox and Siponate DS-10. These were both used in the SCAS study.

The emulsions for this study were made by dissolving 3 g of FM 3422 in 3 ml of ethyl acetate. Portions of this solution were then added to 5 g/l solutions of Alconox or Siponate DS-10, and emulsified by mixing for 30 seconds with a tissumizer. The concentrations of chemicals in the emulsions thus formed were 5 g/l Alconox or DS-10, 2.7 g/l ethyl acetate, and 3 g/l FM 3422. Emulsions with only ethyl acetate and one of the two surfactants were used as controls.

Six semicontinuous activated sludge units were set up by adding 2 liters of fresh activated sludge to each unit. This sludge was collected from an aeration basin at the municipal waste treatment plant in Pig's Eye, Minnesota. The sludge was settled and the bottom 500 ml, containing all of the settled cells, was retained. One liter of medium was then added to the settled sludge in each reactor, and identical medium readded at the beginning of each test cycle. These media were made by diluting portions of the above-described emulsions in the basal salts medium described in the methods and materials section.

Table 2 shows the concentrations of ethyl acetate, Alconox, Siponate DS-10, and FM 3422 present in 1.5 l contents of each reactor at the beginning of the first test cycle.

TABLE 2
CONCENTRATION OF ORGANIC COMPOUNDS IN
SEMICONTINUOUS ACTIVATED SLUDGE REACTORS AT THE
BEGINNING OF THE FIRST TEST CYCLE

SCAS Reactor	Initial Concentration (ppm)			
	Ethyl Acetate	Alconox	DS-10	FM 3422
1	560	830	0	0
2	560	0	830	0
3	460	830	0	510
4	460	0	830	510
5	90	170	0	100
6	90	0	170	100

006373

The semicontinuous activated sludge procedure was described in a previous report (1), and is briefly outlined in Figure 1.

This experiment was conducted for 10 days with samples taken at the beginning and end of the first and last test cycle. After the samples were taken, they were centrifuged. The solids and centrifugate were extracted separately. The centrifugate was extracted two times, first at the pH of the sample ($\sim$ pH7) and again after adjusting to pH 1 with concentrated sulfuric acid. The cells were extracted only once at pH 7. The ethyl acetate extracts were then evaporated, resuspended in methanol and separated by TLC.

Problems with foaming developed in all reactors during the first test cycle. This foaming caused the loss of some suspended solids in reactors 1, 2, 3, and 4. Little or no solids were lost from reactor 5, and most of the suspended solids were lost from reactor 6. In all subsequent test cycles, foaming and loss of solids were controlled by blowing a stream of air on the water surface of each reactor. This also increased the rate of evaporation, and reactors were brought back to their original volume with deionized water prior to draining or sampling.

There were no new spots that developed on TLC plates that would indicate that a degradation product of FM 3422 was accumulating. This was true both in the extracts of the suspended solids and in the extracts of the centrifugates.

There were indications that biodegradation of the surfactant took place during the course of the final test cycle. Spots which could be attributed to Alconox or DS-10 were present in the extracts of the initial samples. These spots were replaced by new spots from later samples and these new spots were completely gone (except in reactor 4) at 24 hours.

It appears that FM 3422 had not degraded, at least to a detectable level, under these conditions with high cell densities and a 10-day acclimation period. These are considered to be optimum conditions for biodegradation.

Shake Flask Die-Away Studies with Adaptive Transfers

Growth of a heterogeneous cell population in nutrient media containing emulsified FM 3422 with sequential adaptive transfers was initiated for two reasons. First, it was very difficult to quantitatively measure the removal of FM 3422 from SCAS studies. This was due to FM 3422's very low water solubility and its affinity for cellular material. On the other hand, the entire contents of die-away cultures could easily be extracted eliminating this problem of nonhomogeneous sampling. The second reason for starting shake flask studies is that this adaptive transfer procedure is an accepted method of enriching organisms from nature that are capable of growth on xenobiotics or rarely-occurring organic materials.

In an attempt to obtain organisms capable of growth on FM 3422, inocula were obtained from a number of sources. These sources included three soil samples from 3M's Decatur, Alabama plant, taken from areas that were likely to have been exposed to fluorochemicals, biological sludge from the Decatur plant, and sludge from the Metropolitan Waste Treatment Plant at Pig's Eye, Minnesota. Separate shake flask cultures were started from these various inocula, but they were eventually combined into a single master culture.

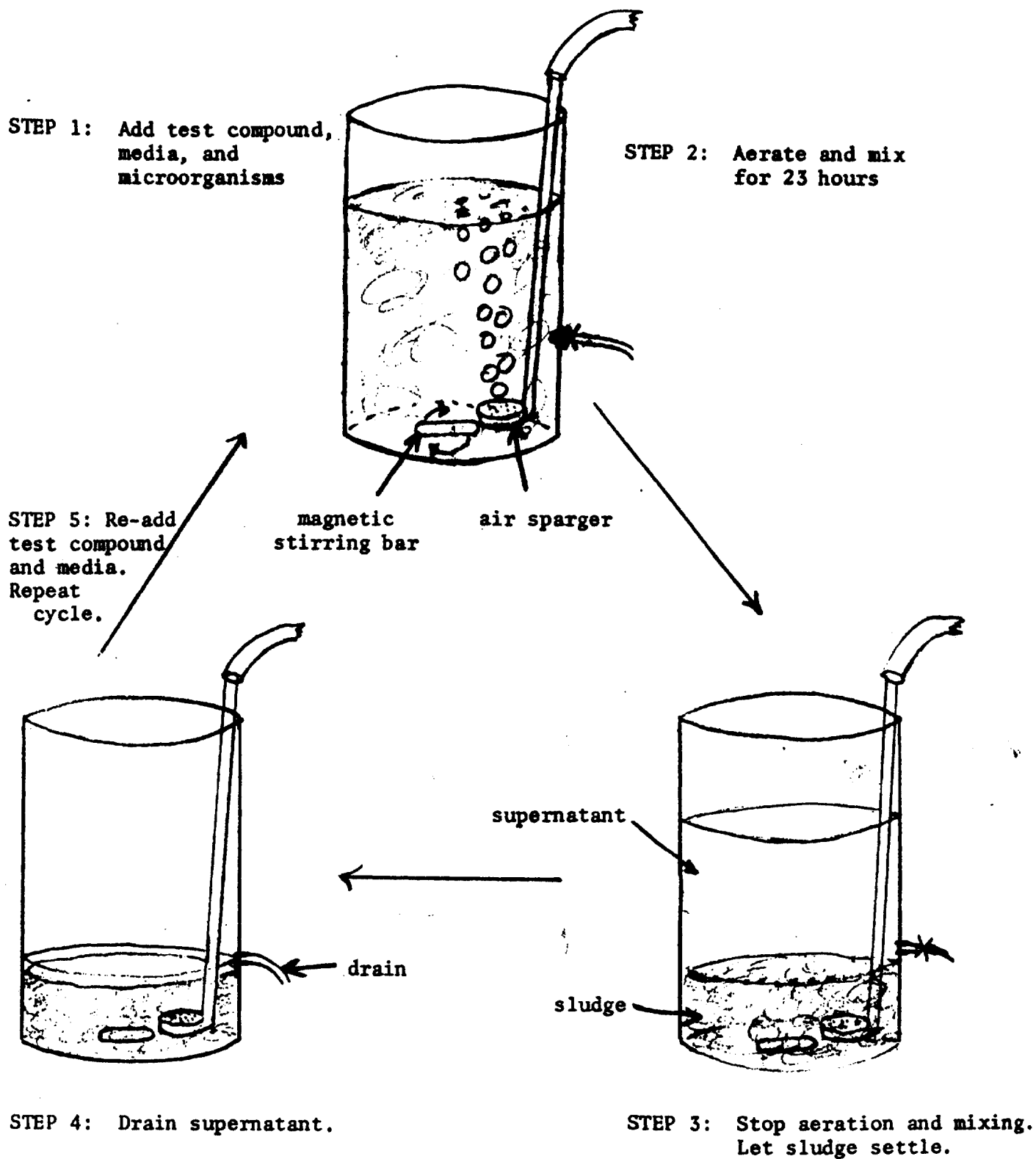


FIGURE 1. Test cycle for semicontinuous activated sludge reactor.

006375

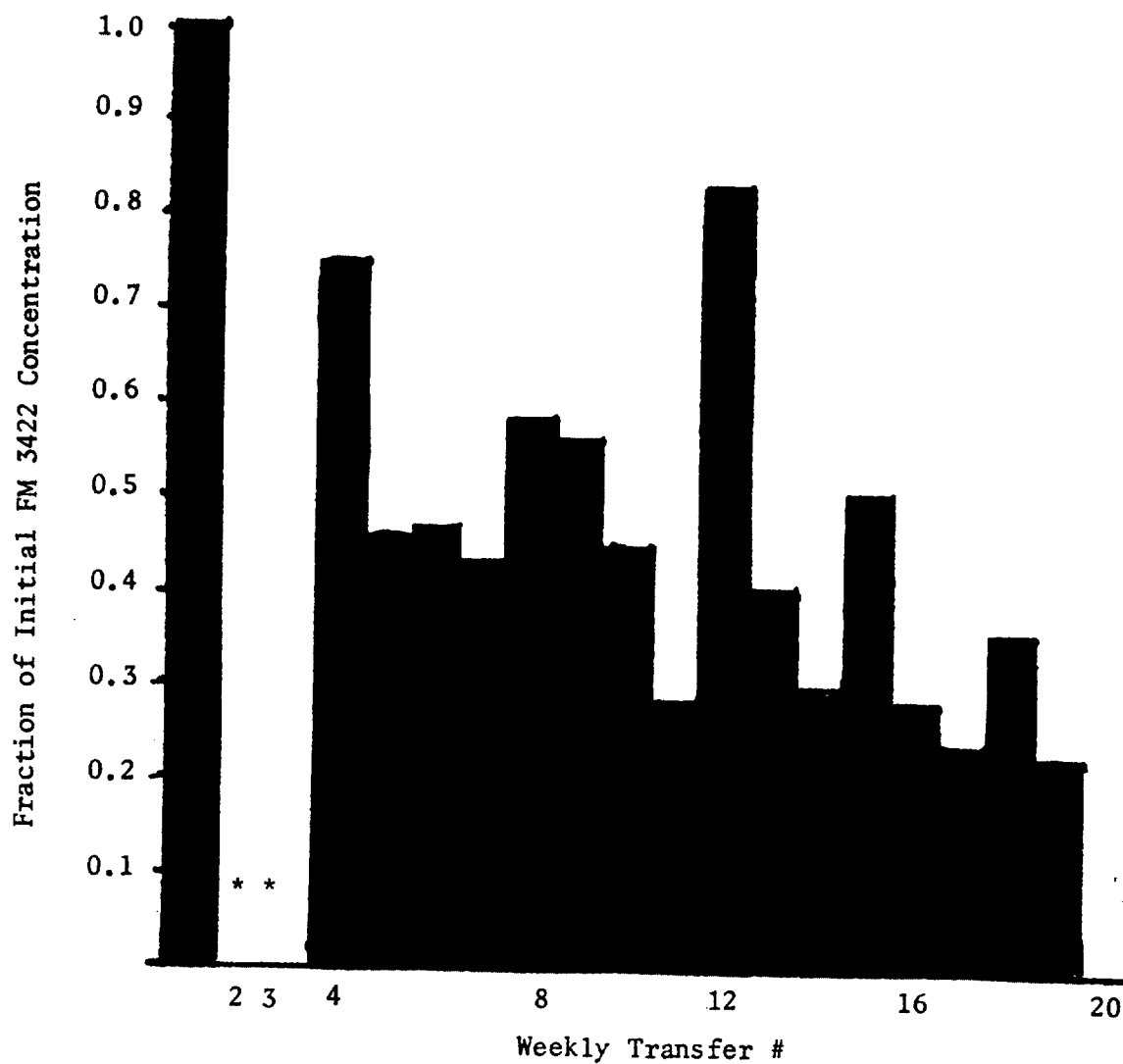
Initially, the adaptive transfers were conducted in glass Erlenmeyer flasks containing 50 ml of culture medium. The total contents of the flask were acidified with sulfuric acid to pH 1, supplemented with a saturated NaCl solution, extracted two times, and the inside of the flask washed with solvent two times. Total phase separation was accomplished by centrifugation. Extracts and washings were combined prior to GLC analysis. Acidification and salt addition were later eliminated from the extraction procedure. Salt addition proved to be of no benefit and sulfuric acid addition caused the formation of a new unidentified peak in the GLC analysis. Acid addition was also of no value in improving the extractability of FM 3422. This newly-formed peak apparently was a chemical modification of FM 3422, and it was converted back to FM 3422 on thin-layer plates. Since it was present both in extracts taken initially and at the end of the growth period, it was obviously not a metabolic product.

The preparation of the media used in early shake flask cultures differed from the later improved and standard procedure described in the methods and materials section only in that water-saturated ethyl acetate, and ethyl acetate-saturated water were not used in its preparation. As a result, however, the FM 3422 was not as completely emulsified in this media. The nonemulsified FM 3422 was intentionally separated and not included in the culture media. Thus, the initial FM 3422 concentration was variable and its concentration known only to be something less than 133 ppm, which was the concentration that would have been present had all the FM 3422 been emulsified. As with the standard media, this initially-used media was demonstrated to be nontoxic to microorganisms by the fact that cell respiration occurred normally in its presence.

Initially, cultures were shaken at room temperature (20-25°C.) and transfers made every 8 days. Transfer times were later changed to 7 days for convenience. Cultures were extracted both at the beginning and end of each culture growth period.

The results were quite variable from transfer to transfer. This can be seen in Figure 2. In most cases, the amount of FM 3422 that could be extracted with ethyl acetate at the end of the incubation period was less than that which was extractable immediately following culture inoculation. Usually, 50% or more of the initially-extracted material was not recoverable from the week-old grown cultures. No degradation products were discovered by either TLC or GLC of the ethyl acetate culture extracts. At this point, it was not known whether the unrecovered FM 3422 were being completely degraded, metabolically modified to materials which could not be extracted, or whether it or its metabolites were bound to the biomass of the culture container.

A number of experiments were subsequently conducted to determine what caused a portion of the FM 3422 to become nonextractable by ethyl acetate.



*Initial concentrations were not measured in the second and third weekly adaptive transfer periods.

FIGURE 2. Ratio of final to initial FM 3422 concentration in equal volume ethyl acetate extracts of week-old shake flask cultures from adaptive transfer experiment.

TLC and GLC Analysis of Extracts from Shake Flask Studies

In one TLC experiment, ethyl acetate extracts of 3 cultures were found to contain a material which produced very distinct spots with a smaller R_f than FM 3422. The TLC visualizing procedure suggested that the new spots contained fluorocarbons. Two of the cultures producing these spots contained 135 ppm of FM 3422 prior to extraction.

The third culture contained less than 3 ppm of FM 3422 (introduced with the FM 3422 acclimated inocula). Despite this great difference in initial concentration, the "new" spots from the extracts of the three cultures proved to be of approximately equal intensity, but FM 3422 was detected by TLC only in the extract of the two 135 ppm cultures.

The extracts of these three cultures were combined. The material causing this new spot isolated by preparative TLC and again repurified by thin-layer chromatography. Attempts were made to characterize this material. GLC of this material in our lab only yielded a material with GLC characteristics identical to FM 3422. Measurements were not made of the amount of material injected. IR spectra of this material showed it to have a carbonyl absorption at $\sim 6\mu\text{m}$ and an absorption fairly characteristic of a fluorocarbon compound between 8 and $9\mu\text{m}$. Bands similar to FM 3422, but not overlapping with FM 3422, were also present. This suggested a complex with a material like urea. Mass spectra obtained following direct insertion of the unknown into the mass spectrophotometer showed ammonia, CO_2 , glycerin, and FM 3422. The relative concentration of FM 3422 to other materials² could not be determined. The presence of urea was not noted. FM 3422 appeared in the mass spectra work to volatilize at a higher temperature than pure FM 3422; this may have been due to its being complexed. GC-mass spec of the unknown material yielded mainly glycerin as identified by the mass spec. (In fact, more than one peak was identified by mass spec as glycerin.) Unlike the work in our lab, FM 3422 was not detected, although the same liquid phase, Carbowax 20M, was used at a higher temperature. This product has not been further characterized. It does not appear to be a major degradation product.

Fluoride Release Determinations

Measurements of F^- concentrations, taken initially and after 7 days, have shown no change in either control cultures or cultures that showed $\sim 50\%$ decreases in ethyl acetate extractable FM 3422 concentration.

In this experiment, nine cultures were prepared and inoculated with a small amount of a heterogeneous culture that had been maintained for 6-8 transfers on FM 3422-containing media. Three of these cultures were grown in the absence of FM 3422 and extracted, three were grown in the presence of emulsified FM 3422 and extracted, and three were extracted prior to cell growth and immediately after FM 3422 addition. The ethyl acetate phase was removed from all cultures. Fifty-one, sixty-one, and fifty-six percent of the initially extractable FM 3422 could not be extracted from the three cultures that had been grown in the presence of FM 3422. Less than 0.1 ppm of F^- was present in any of the nine cultures. Had all of the nonextractable FM 3422 been degraded in the culture grown with FM 3422 with complete F^- ion release to the media, ~ 40 ppm would have been found. These results indicate that, as expected, the perfluorinated portion of the FM 3422 molecules is not metabolically attacked.

Attempts to Free Nonextractable FM 3422 by Hydrolysis

A possible explanation for the observation that 50% or more of the initially-extractable FM 3422 frequently cannot be extracted into ethyl acetate after incubation with acclimated microbial cultures is that it has been conjugated or polymerized forming a nonextractable material. The objective of this experiment was to determine if acid or base hydrolysis could convert FM 3422 back to an extractable form.

In this experiment, cultures which had been grown in the presence of emulsified FM 3422 on the standard media were extracted with ethyl acetate. The concentration of FM 3422 in the ethyl acetate extract was determined. The cultures were then extracted again after hydrolysis at 80°C. and at pH 1 or pH 13 for various lengths of time. Concentrated H₃PO₄ was used for acidification and 20 molar NaOH was used to increase the pH. Following hydrolysis, all samples were neutralized and adjusted to equal volumes before extraction at room temperature.

The results of this experiment are shown in Table 3. An average of 28.4% of the FM 3422 was extractable before cellular growth. Very little of the remaining FM 3422 could be extracted even after hydrolysis. In fact, it appears that longer exposure to these hydrolysis conditions decreased the amount of FM 3422 that could be recovered in a second extraction.

TABLE 3

RECOVERY OF "NONEXTRACTABLE" FM 3422
FOLLOWING ACID OR BASE HYDROLYSIS AT 80°C

<u>pH</u>	<u>Time at 80°C</u>	<u>% of "Nonextractable" FM 3422 Recovered</u>
7	0	2.7
1	0	12
1	0.25 hr.	3.9
1	2.5 hr.	1.5
1	25 hr.	0.8
13	0 hr.	8.2
13	2.5 hr.	3.2
13	25 hr.	0.4

Materials Balance Experiments

Biodegradation studies were conducted using ^{14}C -FM 3422. The primary objective of these experiments was to determine the relative portions of FM 3422 present in the aqueous, solvent, and solids phases of extracted cultures after growth in the presence of emulsified ^{14}C -FM 3422.

In this experiment, the standards media was prepared both with and without ^{14}C -FM 3422 (100 ppm). This media was inoculated with a heterogeneous culture that had been maintained for approximately 20 transfers at weekly intervals on the standard FM 3422 media. Three of the new cultures containing ^{14}C -FM 3422 were extracted immediately after inoculation, and three identical cultures were extracted after one week of growth. Three cultures not containing FM 3422 were also grown for one week. At this point, ^{14}C -FM 3422 emulsion was added to these cultures, bringing their concentration to 100 ppm. The cultures were then shaken for an additional fifteen minutes and extracted. Three one-ml samples from the aqueous and ethyl acetate phases were taken from each of the nine extracted cultures, added to Aquasol^(R) in scintillation vials, and counted. The solids from the grown cultures (essentially no solids were present initially) were transferred to combustion cups, dried in an oven at 103°C . for two hours and combusted using Agrichem's Packard combustion equipment. The $^{14}\text{CO}_2$ from combustion was trapped in a scintillation fluid containing an organic amine and counted. Samples were counted with an internal standard, quench correction, and their radioactivity compared to known volume samples of ^{14}C -FM 3422 emulsion added directly to Aquasol.

This experiment was conducted two times. In the first attempt, the ^{14}C -FM 3422 emulsion was inadvertently not stabilized with Alconox, and it broke rapidly. Also, ^{14}C -FM 3422 emulsion was not added to the cultures that had been grown before FM 3422 addition because the week-old unstabilized emulsion had already broken.

The results of the first attempt at this experiment are shown in Tables 4 and 5.

	<u>Culture Extracted Initially</u>	<u>Culture Extracted After 1 Week</u>
Ethyl acetate	103%±11%	102%±6%
Water	0.04%±0.008%	0.02%±0.02%
Solids	--	<u>0.3%±.4%</u>
Total Recovered	103%±11%	102%±6.4

Table 4. Percent ± 1 Standard Deviation of initially added ^{14}C -FM 3422 recovered in first of 2 Materials Balance experiments.

Method of Determining FM 3422 Concentration			
		<u>Electron Capture GLC</u>	<u>Scintillation Counting</u>
Culture Extracted Initially	Extract Concentration % of Added Material Accounted For.	91 ppm $\pm$ 4.2 ppm 82%	115 ppm $\pm$ 12 ppm 103%
Culture Extracted After 1 Week	Extract Concentration % of Added Material Accounted For.	83 ppm $\pm$ 5.0 ppm 75%	113 ppm $\pm$ 7 ppm 102%

Table 5. Concentration of FM 3422 in ethyl acetate extracts $\pm$ 1 standards deviation from the first Materials Balance experiment as determined by scintillation counting and by electron capture GLC.

The results of the second run of the Materials Balance experiment are shown in Tables 6 and 7.

	<u>Culture Extracted Initially</u>	<u>Culture Extracted After 1 Week</u>	<u>Grown Cultures Extracted After Fifteen Minutes</u>
Ethyl acetate	89.7 $\pm$ 3.6	39.9 $\pm$ 9.8	33.6 $\pm$ 3.0
Water	4.0 $\pm$ 1.3	0.4 $\pm$ 0.3	1.0 $\pm$ 0.4
Solids	--	3.9 $\pm$ 6.6	0.1 $\pm$ 0.05
Total Recovered	93.7 $\pm$ 4.9	44.2 $\pm$ 16.7	34.7 $\pm$ 3.4

Table 6. Percent $\pm$ one standard deviation of initially added ¹⁴C-FM 3422 recovered in second of two Materials Balance experiments.

	<u>Method of Determining FM 3422 Concentration</u>	
	<u>Electron Capture GLC</u>	<u>Scintillation Counting</u>
Culture Extracted Initially	99.8 ppm±0.7 ppm	99.6 ppm±4.0 ppm
Culture Extracted After 1 Wk.	44.0 ppm±8.7 ppm	44.3 ppm±10.8 ppm
Grown Culture Extracted After 15 Minutes	31.1 ppm±2.7 ppm	37.3 ppm±3.3 ppm

Table 7. Concentration of FM 3422 in ethyl acetate extracts from the second Materials Balance experiment as determined by scintillation counting and by electron capture GLC.

The results of these experiments were valuable despite the fact that complete recoveries of FM 3422 were not obtained. In the first attempt, it was found that when FM 3422 was added as an unstabilized emulsion, the ability to extract this material was not lost after 7 days of shaking. We have observed that unstabilized emulsions break within a few hours on sitting with the precipitation of coagulated clumps of FM 3422. This presumably happened in this experiment as well, and in this form, it remains extractable despite contact with a viable biological culture. It is possible that surfactant facilitates the uptake of FM 3422. However, it is more likely that emulsification increases the number of FM 3422 molecules contacting and, subsequently, binding with microbial solids.

As noted above, all of the ^{14}C -FM 3422 was not accounted for in the second attempt at this experiment. As is seen in Table 6, most of this material could be recovered initially, but it was not recovered after significant biological solids had accumulated. The most probable explanation for this loss of radioactive FM 3422 is that it was absorbed by the cellular material and bound so strongly that it could not be extracted by ethyl acetate. However, it apparently was not so strongly bound that it could not be volatilized from the cellular material by heating to 103°C . Loss to volatilization is the most probable explanation for the low recoveries.

This experiment also suggests that absorption is a physical phenomenon. FM 3422 was also not extractable from the pregrown cultures that were shaken with emulsified FM 3422 for only 15 minutes. This rate of removal is too rapid to be explained by a metabolic phenomenon, particularly since the organisms had not been acclimated to FM 3422. It can't be determined from this experiment whether or not the FM 3422 which is presumably bound to the cells is eventually metabolized to another chemical form. This would require recovery of the bound FM 3422 and confirmation by a method such as GLC that the recovered material is still FM 3422.

Adsorption on Culture Containers

A possible explanation for the decrease in extractable FM 3422 in grown cultures is that the material was being strongly bound to the polypropylene test culture containers. This possibility was considered remote since similar results were obtained in both glass and polypropylene containers. Nevertheless, it was investigated in the following procedures:

A media was prepared that differed from the standard 133 mg/l FM 3422 emulsion media only in that it did not contain the nutrient YM broth necessary as a nitrogen source for cell growth. Ten ml of this media was added to each of nine tubes. Three were extracted immediately, three were capped with polypropylene caps, and three were plugged with foam stoppers. The capped and stoppered tubes were shaken for one week and extracted and analyzed by GLC using the standard procedures.

<u>Sample</u>	<u>Cap</u>	<u>Time in Polypropylene Tube Prior to Extraction</u>	<u>% Initially Added FM 3422 Recovered</u>
I #1	Polypropylene	15 min.	79
I #2	Polypropylene	15 min.	79
I #3	Polypropylene	15 min.	78
P #1	Polypropylene	7 Days	75
P #2	Polypropylene	7 Days	78
P #3	Polypropylene	7 Days	84
F #1	Foam	7 Days	92
F #2	Foam	7 Days	93
F #3	Foam	7 Days	92

Table 8. Percent of initially added FM 3422 extracted from polypropylene tubes.

The results of this experiment are shown in Table 8.

The reasons for the low FM 3422 recoveries obtained in this experiment are not known. There also is no explanation for the greater recoveries obtained with the tubes that were shaken with foam stoppers. In the week-long shaking procedure, the contents of the tubes never came in contact with either the polypropylene cap or the foam stopper. Also, the foam stoppers had not been used before in any experiments.

Despite the incomplete recovery of FM 3422, these results do not explain the phenomenon repeatedly seen in earlier experiments. In early experiments, high recoveries of FM 3422 were obtained initially before the cell culture had developed and low recoveries of FM 3422 were obtained in ethyl acetate extracts done after the culture had grown for seven days. In this experiment with negligible microbial populations, the initial FM 3422 recoveries were not significantly different from the recoveries after 7 days. It, therefore, appears that the increasing loss of ability to extract FM 3422 from the culture with time requires the presence of biological solids.

Recovery of FM 3422 From Cells By Volatilization and Condensation

As previously noted, the most probable explanation for the loss of FM 3422 in the Materials Balance experiment is that it volatilized from the solids phase during the two hours drying period. For this reason, it was believed that it might be possible to quantitatively recover sorbed FM 3422 by volatilizing it from cells and collecting it by condensation on a "cold finger."

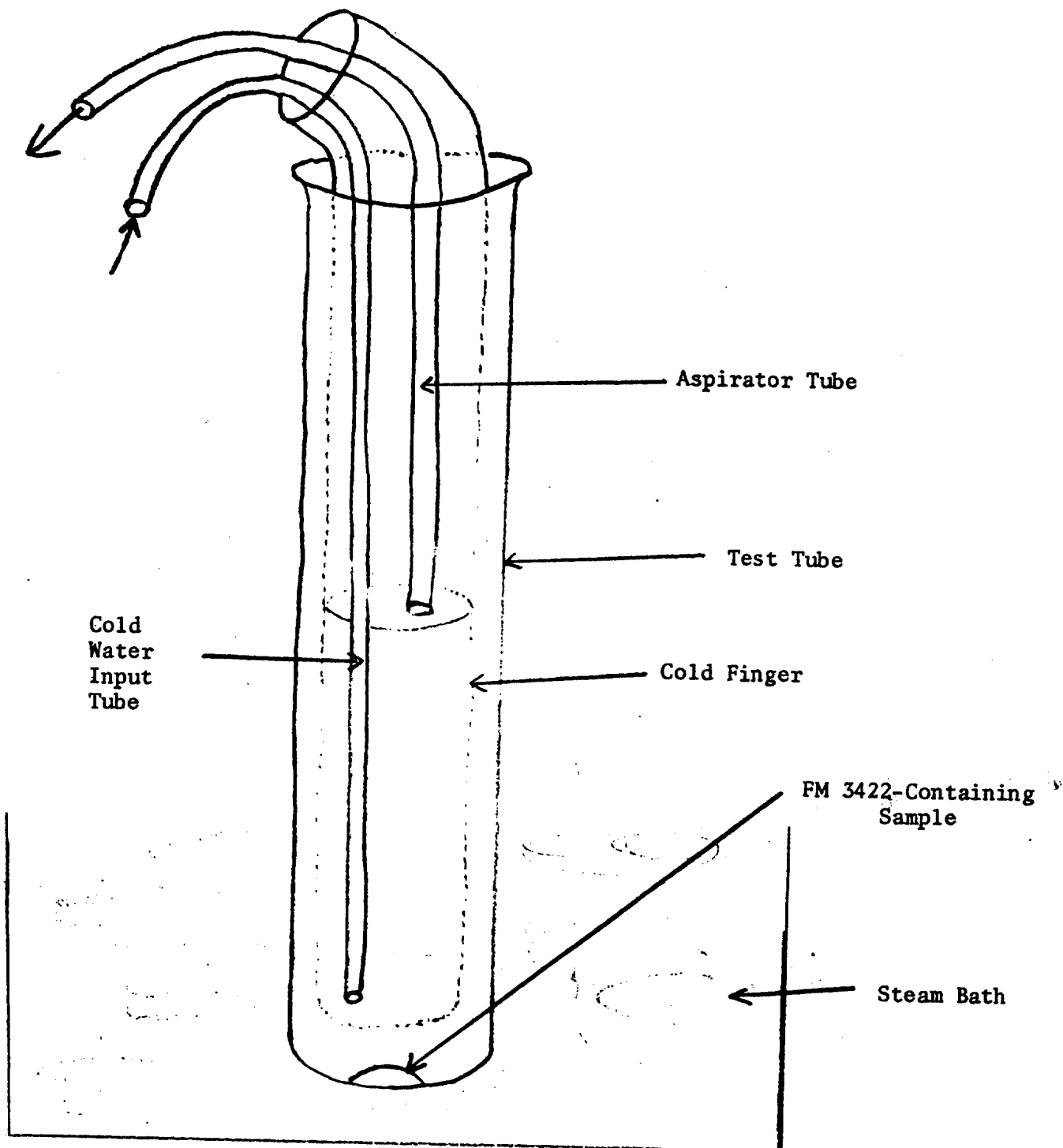


Figure 3. Apparatus used to recover FM 3422 from cells by volatilization and condensation.

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The feasibility of this approach was evaluated by the following experiment which was run in duplicate. FM 3422 (1 mg) was placed in the base of a large test tube. A second smaller test tube was placed inside the first, and was effectively made into a cold finger by continuously running water into its base and aspirating it from the top. The base of this apparatus was then placed in a steam bath (Figure 3). After 15 minutes, the cold finger was removed and the sublimated FM 3422 washed from its surface into a known volume of ethyl acetate. GLC analysis indicated that 32% and 42% of the originally added FM 3422 was sublimated and collected in two trials.

The above sublimation procedure was then repeated in duplicate using week-old 10 ml cell cultures in the standard media. The small inoculum for these cultures came from a heterogeneous FM 3422 acclimated culture. These cultures were extracted with equal volumes of ethyl acetate (10 ml), and the liquid phases removed. The undried biomass was transferred to the outer tube of the above-described apparatus and exposed to the steam bath for 45 minutes. The ethyl acetate washings from the cold finger were analyzed by GLC, but very little of the nonextracted FM 3422 was recovered. The cells were dried under nitrogen and again exposed in the steam bath for an additional two hours.

The results of this experiment are shown in Table 9. The FM 3422 that was not extractable into ethyl acetate and presumably bound to the cells, was not quantitatively recovered by sublimation and condensation. Perhaps complete recovery would have been possible had the cells been exposed to the steam bath for a longer period. Although this experiment did not rule out the possibility that some of the bound FM 3422 may have been chemically modified by metabolism, it did indicate that a large fraction of the bound FM 3422 was unchanged.

	<u>Culture #1</u>	<u>Culture #2</u>
Extracted into Ethyl Acetate	15.6%	21.0%
Volatilized from "Wet" Cells	0.5%	0.3%
Volatilized from N ₂ Dried Cells	<u>25.6%</u>	<u>21.8%</u>
Total FM 3422 Recovered	41.7%	43.1%

Table 9. Percent of initially added emulsified FM 3422 recovered from cells by ethyl acetate extraction followed by volatilization and condensation of remaining FM 3422.

Hot Ethyl Acetate Extracts

In an attempt to increase the recovery of the FM 3422 sorbed to the culture biomass, extractions were done using hot ethyl acetate. This procedure involved addition of 2.5 ml of FM 3422 emulsion to week-old, 10 ml cultures that had been grown on nutrient media. FM 3422 emulsion was also added to two, 10 ml deionized water samples. The resulting 12.5 ml samples, which contained 133 ppm FM 3422, were shaken for 15 minutes on a rotary shaker to allow sorption of the FM 3422 by the biomass, and extracted with equal volumes of ethyl acetate at room temperature or at 40° C. or 60° C. Phase separation was insured by centrifugation at 12,000 rpm and the concentration of FM 3422 in the ethyl acetate phase was determined by gas chromatography.

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The results of this extraction procedure are shown in Table 10.

<u>Sample</u>	<u>Aqueous Phase Composition</u>	<u>Extraction Temperature (°C)</u>	<u>ppm FM 3422 in Ethyl Acetate Extract</u>
1	Deionized Water	22	115
2	Deionized Water	22	133
3	Microbial Culture	22	52
4	" "	22	55
5	" "	40	35
6	" "	40	31
7	" "	60	51
8	" "	60	56

Table 10. Hot ethyl acetate extracts of water or biological cultures after 15 minutes of shaking with emulsified FM 3422 (133 ppm).

These results indicate that hot ethyl acetate extraction does not improve the extractability of FM 3422 from the biomass. The amount of FM 3422 extracted at 60°C. is no higher than that extracted at 22°C. The reason for the lower concentration of FM 3422 in the 40°C. extract is not known. The results also confirm the previous observation that emulsified FM 3422 is absorbed by cells within 15 minutes. This rapid loss from a nonacclimated culture suggests a physical/chemical absorption process rather than biological degradation.

Dioxane Extraction of FM 3422 from Culture Solids

In a preliminary experiment, it was found that all of the FM 3422 not extracted by ethyl acetate could be recovered by refluxing the culture solids with 1,4-dioxane. Attempts to repeat this observation were initially hindered by problems with the electron capture detector of our gas chromatograph. These problems were apparently caused by the presence of dioxane. Central Research overcame this analytical problem and the experiment was repeated. The results (Table 11) demonstrate quite conclusively that FM 3422 is not metabolically altered in seven days by shake flask cultures that have been maintained with weekly transfers on FM 3422-containing media for over six months.

<u>Culture Extracted with Ethyl Acetate at:</u>	<u>% of FM 3422 Extracted By Ethyl Acetate</u>	<u>Procedure for Extracting Culture Solids</u>	<u>% FM 3422 Recovered From Solids</u>	<u>Total % Recovery of FM 3422</u>
15 min.	99.3	No solids present	--	99.3
15 min.	99.3	No solids present	--	99.3
15 min.	101.3	No solids present	--	101.3
7 days	25.8	(Refluxed in Dioxane for 2 hrs.)	77.9	103.7
7 days	27.1	" "	80.1	107.2
7 days	31.1	(Dried solids with N ₂ & refluxed in Dioxane for 2 hrs.)	75.0	106.1
7 days	30.4	(Refluxed in Dioxane for 1 min.)	73.5	103.9
7 days	29.6	(Extracted with Dioxane at Room Temperature)	68	97.6

Table 11. Recovery of FM 3422 from culture solids by extracting or refluxing with Dioxane.

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