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ENVIRONMENTAL QUALITY RESEARCH

Shimshon Lerman, et al

California University

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ENVIRONMENTAL QUALITY RESEARCH

FIRST ANNUAL REPORT

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UNIVERSITY OF CALIFORNIA

FEBRUARY 1975

AEROSPACE MEDICAL RESEARCH LABORATORY
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This report for the period from February 1 to June 15, 1974 contains the results of research efforts of 4 projects concerned with defining the environmental effects of potential environmental contamination resulting from the use of certain Air Force materials. The 4 projects use different organisms as means of assessing effects. Project I uses the higher plants, the gymnosperms and angiosperms. Project II uses fish and aufwuchs. Project III utilizes unicellular algae and Project IV uses the eggs, embryo and larvae of the leopard frog. Materials being evaluated include hydrogen

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PRICES SUBJECT TO CHANGE

20. ABSTRACT(CONT'D)

chloride, hydrogen fluoride, RJ 4, RJ 5, JP 4, N phenyl-α-naphthylamine, p,p' dioctyldiphenylamine, octyl-phenyl-α-napthylamine.

Techniques for exposing organisms to these substances are discussed and the results of such exposures are presented.

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FOR THE COMMANDER

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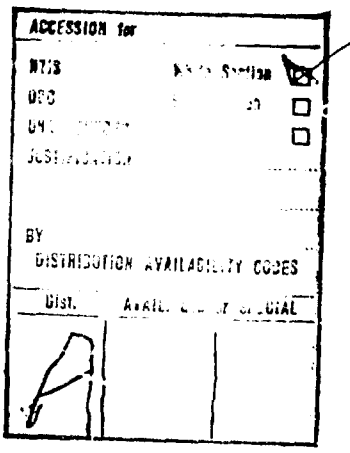

PREFACE

This is the First Annual Report of work performed under the Environmental Research Addendum P0009 of Air Force Contract AF33615-73-C-4059. Work under this portion of the contract covers the period February 1, to June 15, 1974 and encompasses projects I thru IV.

Project I is entitled, "Gymnosperm-Angiosperm Studies." Project II is entitled, "Fish and Aufwuchs Bioassay." Project III is entitled, "Use of Unicellular Algae and Evaluation of Potential Aquatic Contaminants." Project IV is entitled, "Effects of Pollutants on Eggs, Embryo and Larvae of the Leopard Frog, Rana pipiens."

Project Director for Project I is Dr. Shimshon Lerman, Project Director for Project II is Dr. Robert Cooper. Project Director for Project III is Dr. Jan Scherfig. Project Director for Project IV is Dr. Gerald Greenhouse.

Technical Progress for each of these projects is reported separately in the present document.

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PROJECT I
GYMNOSPERM, ANGIOSPERM STUDIES

GENERAL

This is the first annual technical report to be submitted in partial fulfillment of the contract.

RESEARCH PROGRAM

Screening For Plants Which Exhibit A Range Of Phytotoxic Responses To HCl Gas

Introduction

In comparison to other air pollutants, hydrogen chloride gas has only been considered to be of major concern in isolated cases. Haselhoff and Lindau(1) reported severe injury to vegetation in the vicinity of soda factories in England and Germany. They demonstrated a wide range of phytotoxic responses of various plant species to HCl gas. Seedlings of Viburnum and larch were killed after less than 2 days of exposure to 5-20 ppm of HCl. On the other hand, HCl concentrations as high as 1,000 ppm for 1 hour were required to produce bleached lesions on the leaves of fir, beech, and oak, and 2,000 ppm HCl 1 hour daily for 80 days to cause necrosis on the margins of maple, birch and pear leaves. Lacasse(2) observed HCl-type injury symptoms on vegetation within a half mile from a location on which incineration of polyvinyl chlorides took place. Shriner and Lacasse(3) exposed 28-day-old tomato plants to 5 ppm HCl gas for 2 hours. The test plants developed interveinal bronzing followed by necrosis within 72 hours after exposure. The relative sensitivity of 12 tree species to HCl gas was studied by Means and Lacasse(4). Coniferous and broadleaf seedlings, 2 to 5 years old, were exposed to concentrations of 3 to 43 ppm of HCl gas for 4 hours. The most sensitive of the broadleaf species was Liriodendron tulipifera, showing visible injury at 3 ppm. Pinus strobus was the most sensitive of the coniferous species, showing visible injury at 8 ppm. Thuja occidentalis was not injured at 43 ppm. Lind and London(5) exposed mature flowering marigold plants to high concentrations of HCl gas for 5-minute periods. Groups of plants which were exposed to 95 ppm showed little or no visible damage. Temporary wilting and bleached leaf spots were the responses of plants exposed to 300 ppm. Exposure to 2071 ppm of HCl gas resulted in severe wilting, marginal and interveinal leaf necrosis, stem collapse, and death of plants.

Selection Of Ornamental Plants

A group of 24 species and varieties of ornamental plants (table 1) were selected for the initial screening. The selection was based on:

a. Literature review.

b. Contractor's Experience.

c. Information obtained from the University of California Agricultural Extension Service, Santa Barbara, and two major companies which produce flower seeds in the vicinity of Vandenberg AFB.

d. Observations in the field by the contractor.

Production Of Ornamental Plants

Seeds of 11 out of the 24 plant species (marked* in table 1) were obtained from a retail seed supply house. The seeds were planted in peat moss-sand mixture. After germination, the young seedlings were transplanted into 4-inch plastic pots. U.C.-Type II soil mix (table 2) was selected as growing medium for the plants. The plants were fertilized weekly with standard Hoagland solution, starting from the second week after transplanting. Day temperatures were regulated by evaporative coolers equipped with activated charcoal filters. Daily temperature maxima were between 80-90 F. Occasionally, periods of extreme heat caused temperatures to exceed this range. Night temperatures ranged between 60 and 70 F.

Exposure Chambers

Two Plexiglass exposure chambers were modified to accommodate the exposure of plants to both gaseous and particulate pollutants. Each chamber measures 0.75 m sq by 1.0 m high. The basic design of the air-handling system is similar to that described by Heck et al.(6) Activated charcoal filtered air enters the chamber through 1.5-inch PVC pipe (figure 1). A blower on the exhaust side maintains a negative pressure of 0.4 inch of water in the exposure chamber at an airflow of approximately 40 cfm (two changes of chamber air every 1-minute). The rate of chamber equilibration after starting HCl flow into the chamber is presented in figure 2.

Equipment For Generating And Dispensing HCl Gas

A series of tests were completed to evaluate two methods for generating and dispensing HCl gas:

a. Gaseous HCl (99.5%) is diluted to a 10% level and then metered into the airstream entering the plant exposure chamber.

b. HCl gas is generated by the method described by Hill et al.(7) for generating HF gas. Air saturated with water vapor is bubbled through an aqueous solution of HCl. The desired concentrations of HCl gas at the exhaust tube was obtained by controlling the air flow and the temperature of the HCl solution.

Method b was found to be more reliable than a, and was selected for future study.

Equipment For Monitoring HCl Gas

A series of tests were conducted to evaluate three methods for monitoring HCl gas in the plant exposure chambers:

a. Continuous monitoring of chamber air: Electrochemical monitor. A Mast Microcoulomb instrument of the type commonly used to monitor total oxidants was adapted to measure HCl gas as described by Miller et al.(8) The system was found to be useful for monitoring HCl levels not greater than 3 mg/m^3 (2 ppm).

b. Periodic sampling of chamber air.
A given amount of air containing HCl gas was bubbled through a dilute solution of nitric acid. The Chloride was titrated with standard silver nitrate, using the potential difference between glass electrode and a silver electrode as an indication of the end point.

c. Continuous monitoring: Microcoulometer chloride analyzer. A research group from USAFSAM/VNL, directed by Dr. Robert J. Reyes, adapted a coulometric instrument for continuous HCl analysis. Tests were conducted to evaluate this method along with the periodic sampling. The two methods agreed within 10% at 10 ppm level of HCl gas in chamber air.

d. An Analytical Automatic Chloride Titrator, designed for the determination of chlorides in solution is being modified for continuous monitoring of HCl gas in air.

Exposure Of Plants To HCl Gas

Groups of 5 to 10 plants of various age levels from each species (marked** in table 1) were exposed to HCl gas at concentrations ranging from 1 to 25 ppm for a period of 20 minutes. An equal number of control plants were used for each experiment. Temperatures and relative humidity in the chambers at the time of exposure ranged from 75 to 95 F and 47 to 61%, respectively. The range of phytotoxic responses is presented in figures 3 through 12, and in table 3.

Screening For Plants Which Exhibit A Range Of Phytotoxic Responses To HF Gas

Equipment For Generating And Dispensing HF Gas

a. A generator for HF gas was constructed as described by Hill et al.(7)

Equipment And Methods For Monitoring HF Gas

a. Periodic sampling of chamber air. A given amount of air containing HF gas was bubbled through aqueous solution. The fluoride ions were determined with a specific ion electrode.

TABLE 1

LIST OF ORNAMENTAL PLANTS FOR THE INITIAL SCREENING
*Plants Currently Under Investigation
**Plants Exposed To HCl Gas

1. Alyssum (Alyssum, sp.)
**2. Aster (Callistephus chinensis)
3. Azalea (Rhododendron, sp.)
*4. Begonia (Begonia semperflorens)
**5. Calendula (Calendula officinalis)
6. Celosia (Celosia cristata)
**7. Cornflower (Centaurea cyanus)
**8. Cosmos (Cosmos, sp.)
9. Daisies (Rudbeckis hirata)
10. Geranium (Pelargonium, sp.)
11. Hollyhock (Althea rosea)
12. Lilac (Syringa, sp.)
13. Lobelia (Lobelia erinus)
**14. Marigold, American (Tagetes erecta)
**15. Marigold, French (Tagetes patula)
**16. Nasturtium (Tropaeolum, sp.)
**17. Petunia (Petunia, sp.)
18. Pine, ponderosa (Pinus ponderosa)
19. Salvia (Salvia splendens)
**20. Snapdragon (Antirrhinum majus)
21. Sweet Pea (Lathyrus odoratus)
22. Verbena (Verbena, sp.)
*23. Zinnia (Zinnia augustifolia)
24. Zinnia (Zinnia elegans)

TABLE 2

U.C.-TYPE II SOIL MIX

Ingredients per cubic yard of mix:

Soil	14 cu. ft
Canadian Peat Moss	7 " "
Redwood Shavings	7 " "
Single Super Phosphate	2-1/2 lbs
KNO_3	4 oz
K_2SO_4	4 oz
Dolomite Limestone	3-3/4 lbs
Oyster Shell Lime	1-1/2 lbs
Micronutrients	
Cu	30 ppm (dry basis)
Zn	10 "
Mn	15 "
Fe	15 "

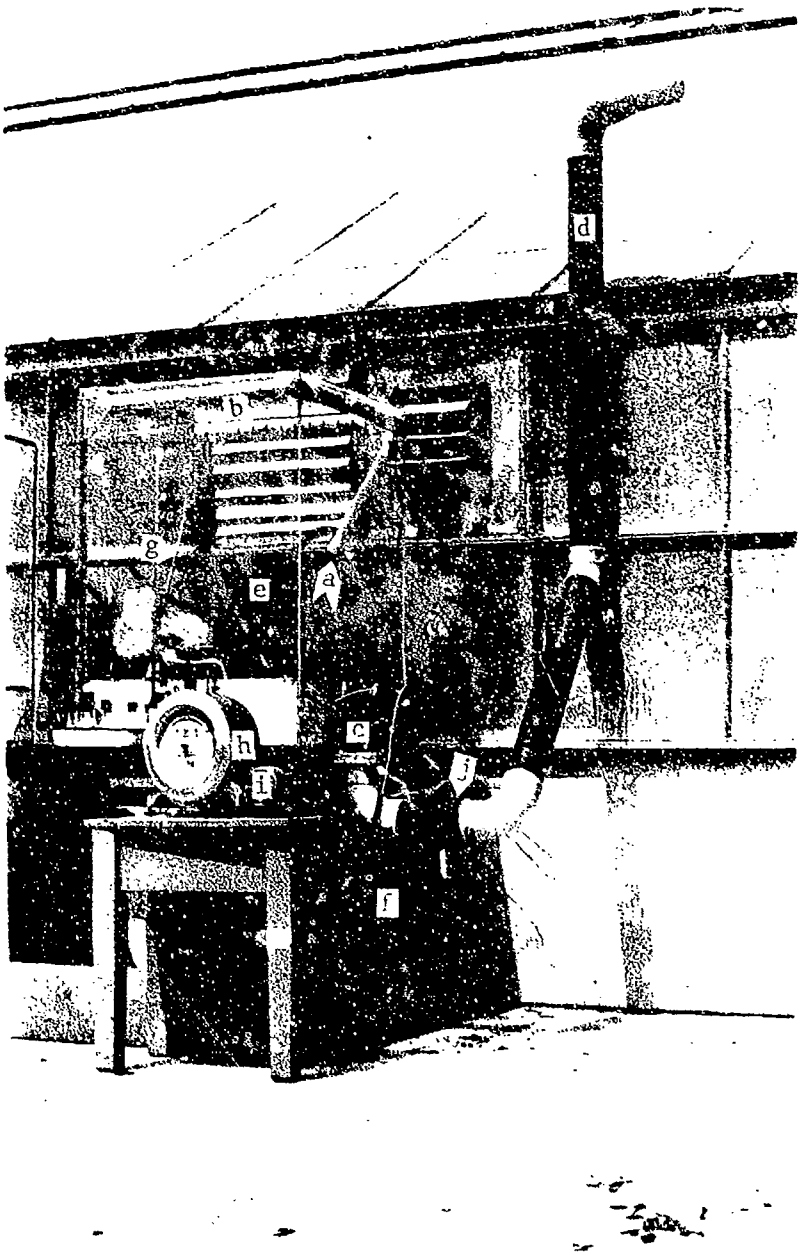

Figure 1. Exposure Chamber. a. Air inlet. b. Distribution tube (1.5 inch PVC tube). c. Blower. d. Exhaust duct (4-inch PVC). e. Toxicant dispensing tube (heated Teflon tubing from HCL/HF generators). f. Constant temperature bath (houses the toxicant generators). g. Sampling tube. h. Wet test meter. i. Sampling pump j. Wet and dry bulb thermocouples.

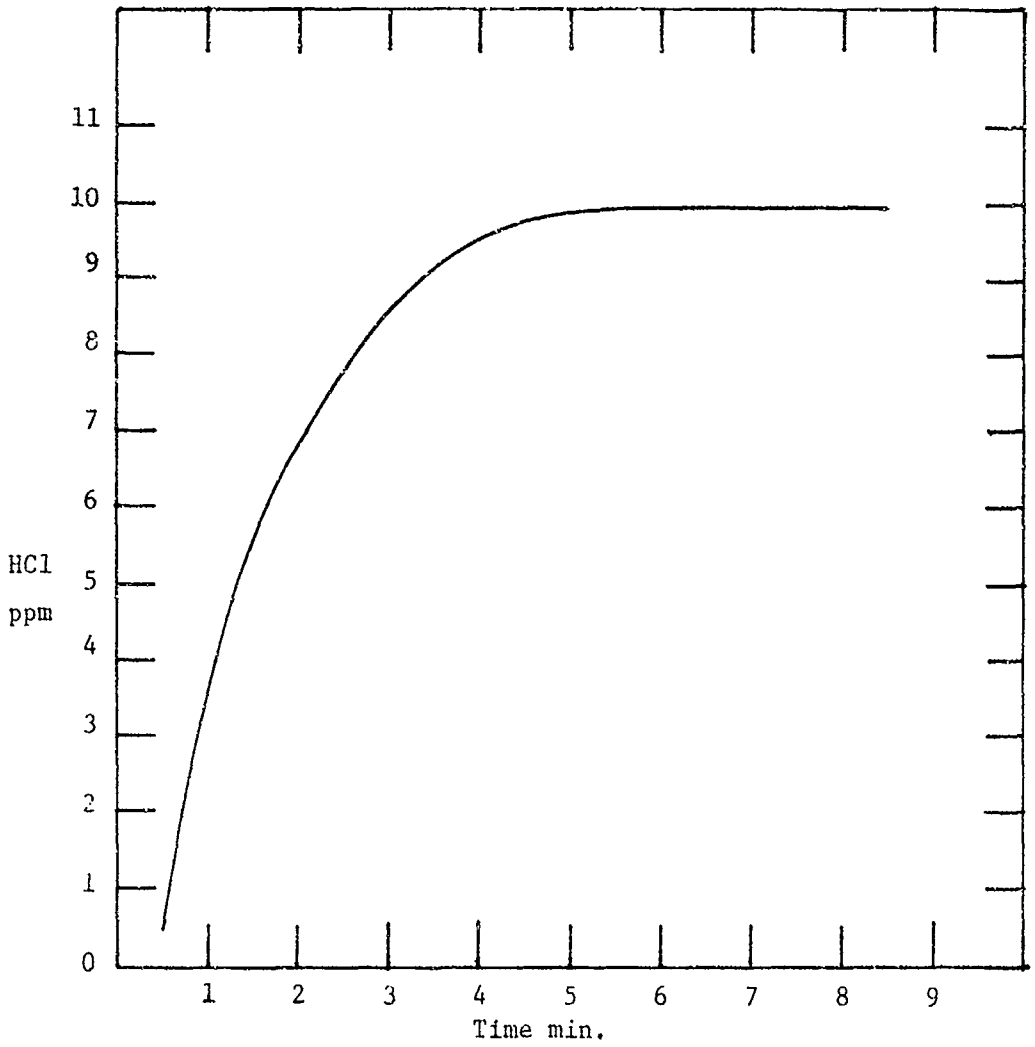

Figure 2. Rate of Equilibration of HCl Gas in the Exposure Chamber.

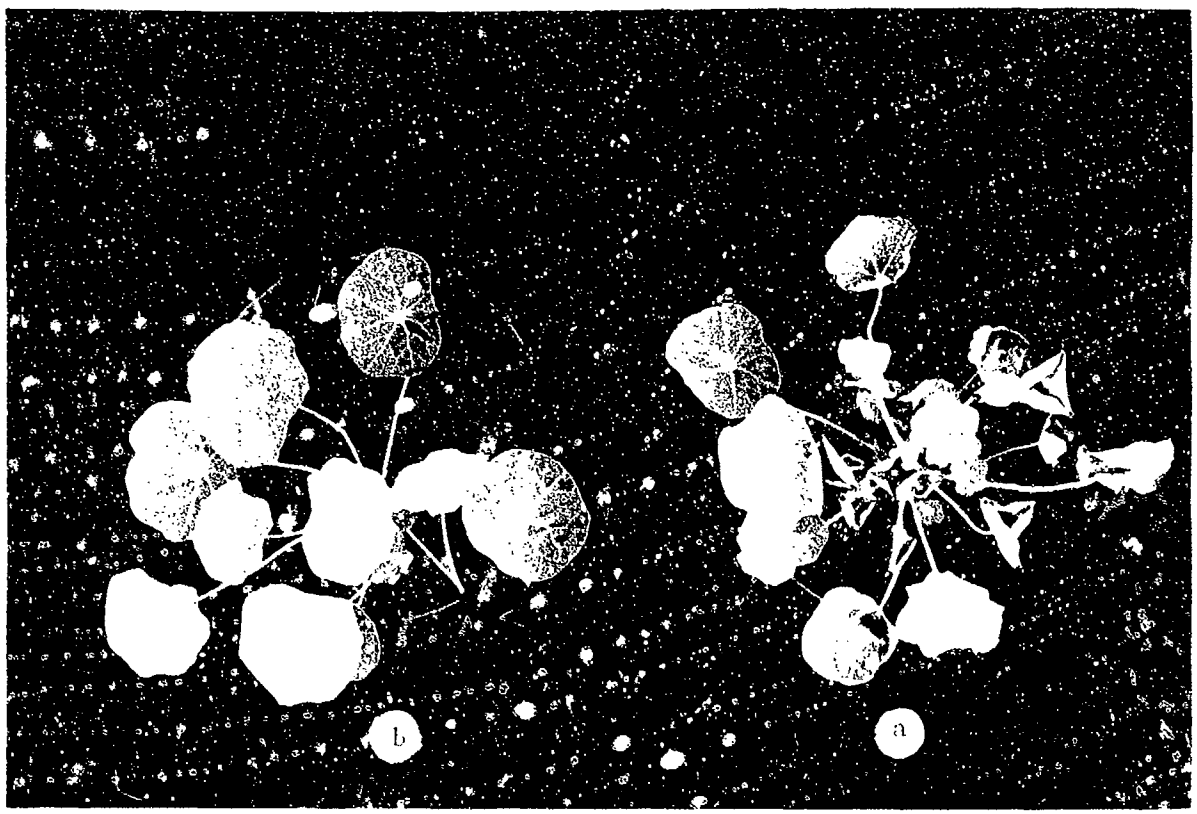

Figure 3. The Effects of HCl Gas on 29-Day-Old Nasturtium Plants. a. Plant exposed to 8.2ppm HCl for 20 minutes. b. Control plant.

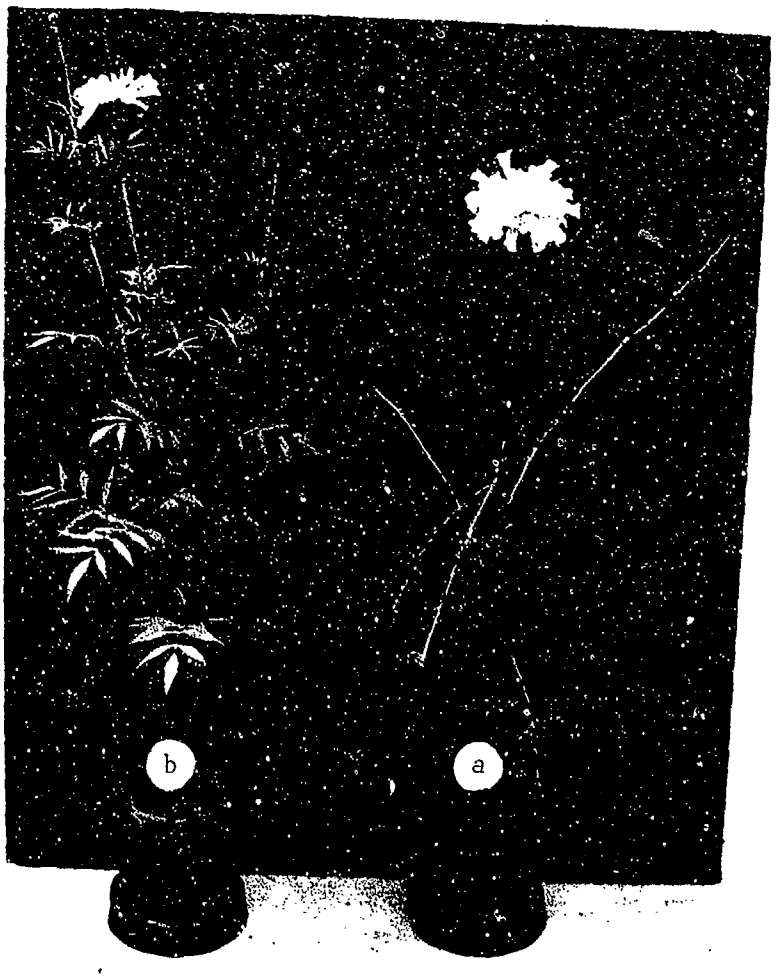

Figure 4. The Effects of HCl Gas on 91-Day-Old Marigold Plants. a. Plant exposed to 6.4ppm HCl for 20 minutes. b. Control plant.

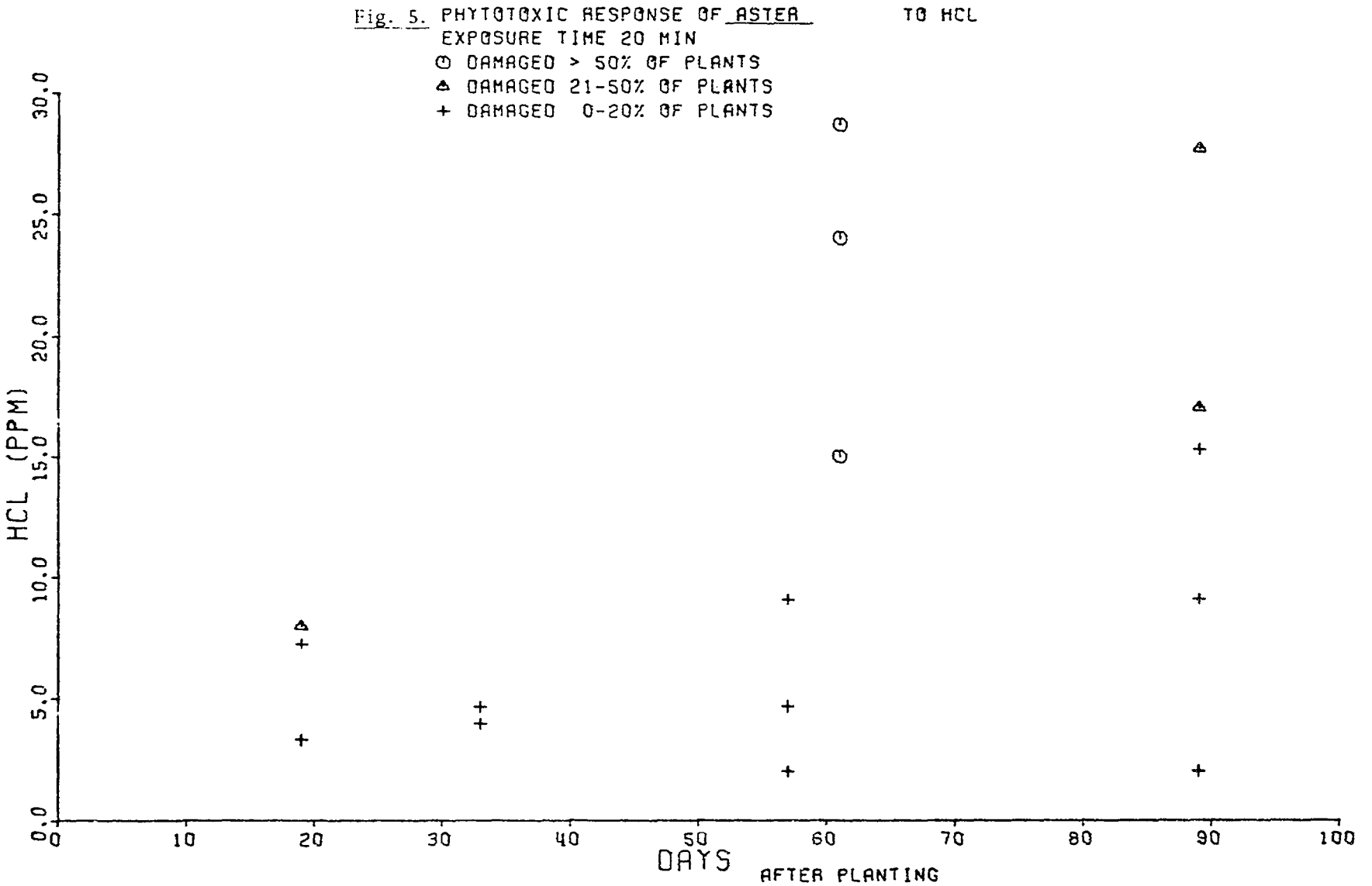
Fig. 5. PHYTOTOXIC RESPONSE OF ASTER TO HCL
EXPOSURE TIME 20 MIN
⊙ DAMAGED > 50% OF PLANTS
△ DAMAGED 21-50% OF PLANTS
+ DAMAGED 0-20% OF PLANTS
HCL (PPM)
0.0
5.0
10.0
15.0
20.0
25.0
30.0
0
10
20
30
40
50
60
70
80
90
100
DAYS AFTER PLANTING

Fig. 6. PHYTOTOXIC RESPONSE OF CALENDULA TO HCL
EXPOSURE TIME 20 MIN
⊙ DAMAGED > 50% OF PLANTS
△ DAMAGED 21-50% OF PLANTS
\+ DAMAGED 0-20% OF PLANTS

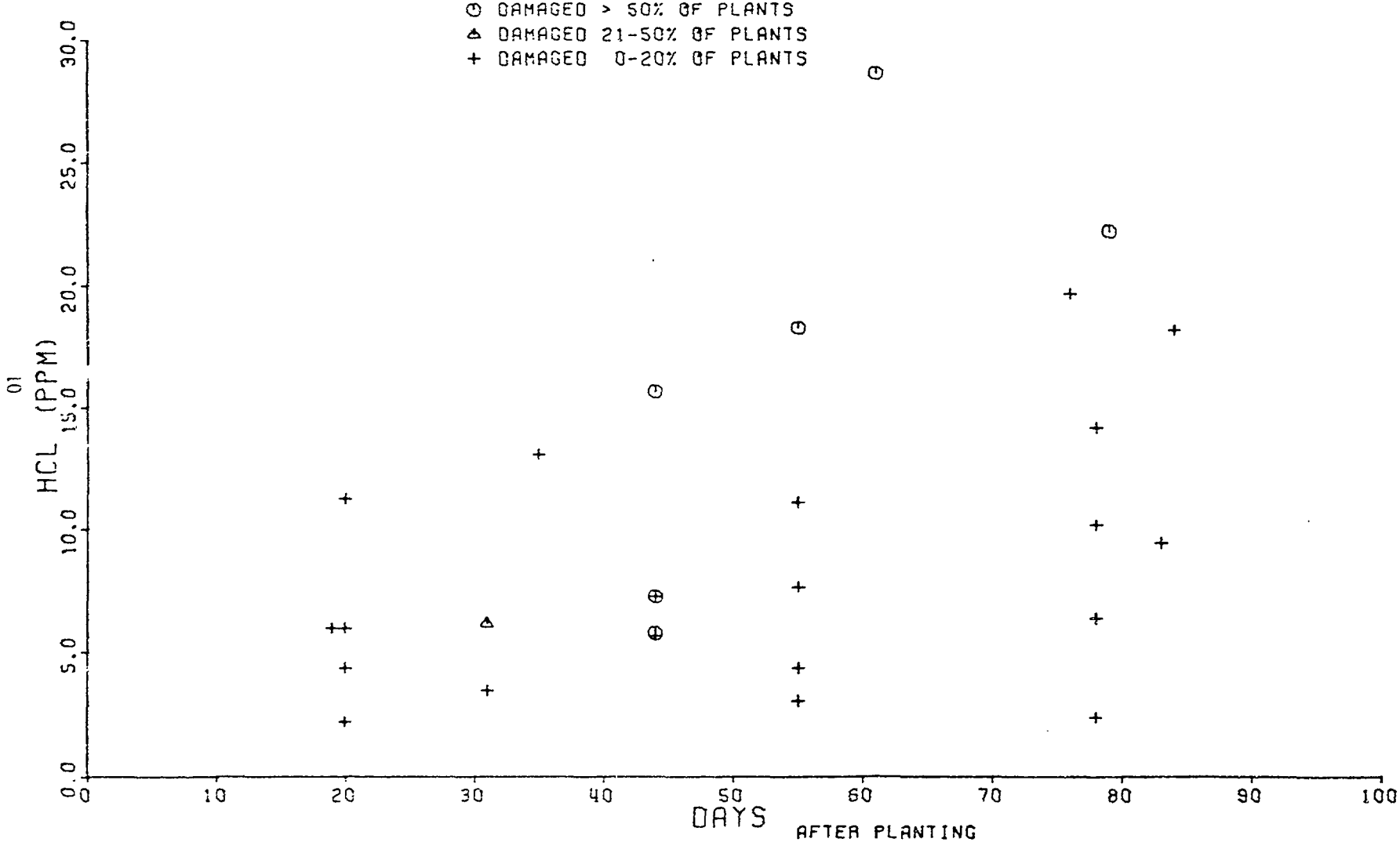

Fig. 7. PHYTOTOXIC RESPONSE OF CENTAUREA TO HCL
EXPOSURE TIME 20 MIN
⊙ DAMAGED > 50% OF PLANTS
△ DAMAGED 21-50% OF PLANTS
+ DAMAGED 0-20% OF PLANTS

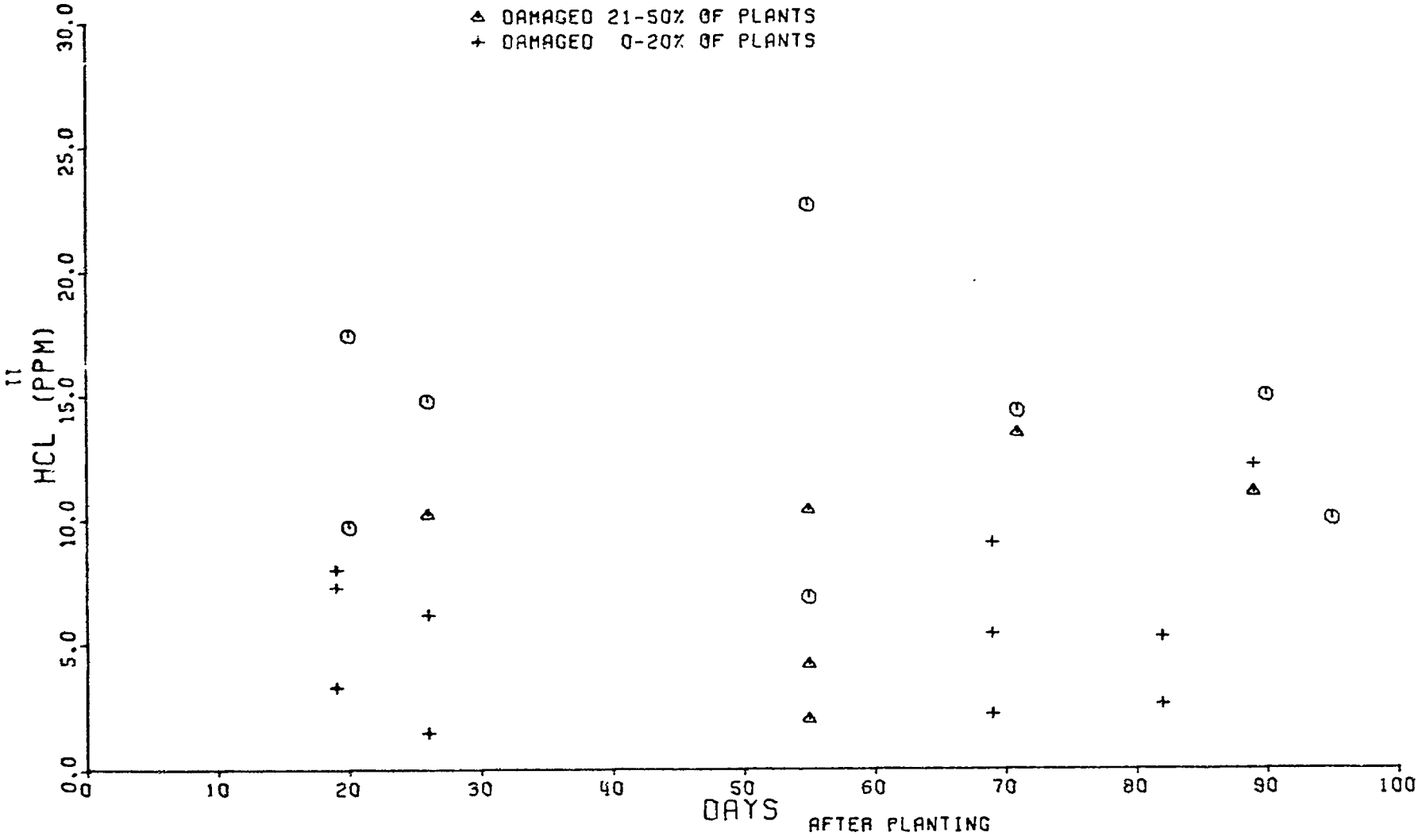

Fig. 8. PHYTOTOXIC RESPONSE OF COSMOS TO HCL
EXPOSURE TIME 20 MIN
⊙ DAMAGED > 50% OF PLANTS
△ DAMAGED 21-50% OF PLANTS
\+ DAMAGED 0-20% OF PLANTS

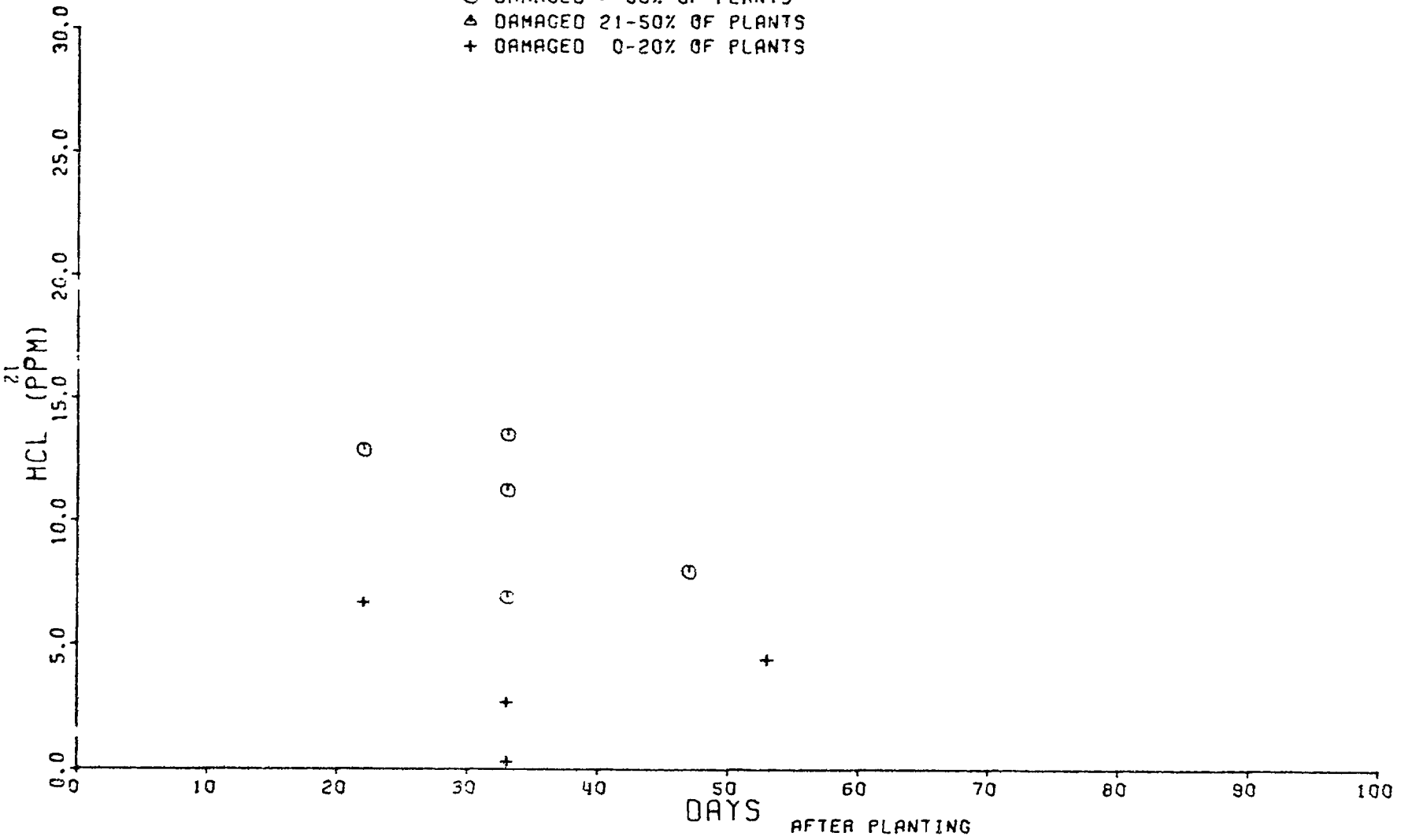

Fig. 5. PHYTOTOXIC RESPONSE OF MARIGOLD (AM) TO HCL
EXPOSURE TIME 10 MIN
⊖ DAMAGED > 50% OF PLANTS
△ DAMAGED 21-50% OF PLANTS
\+ DAMAGED 0-20% OF PLANTS

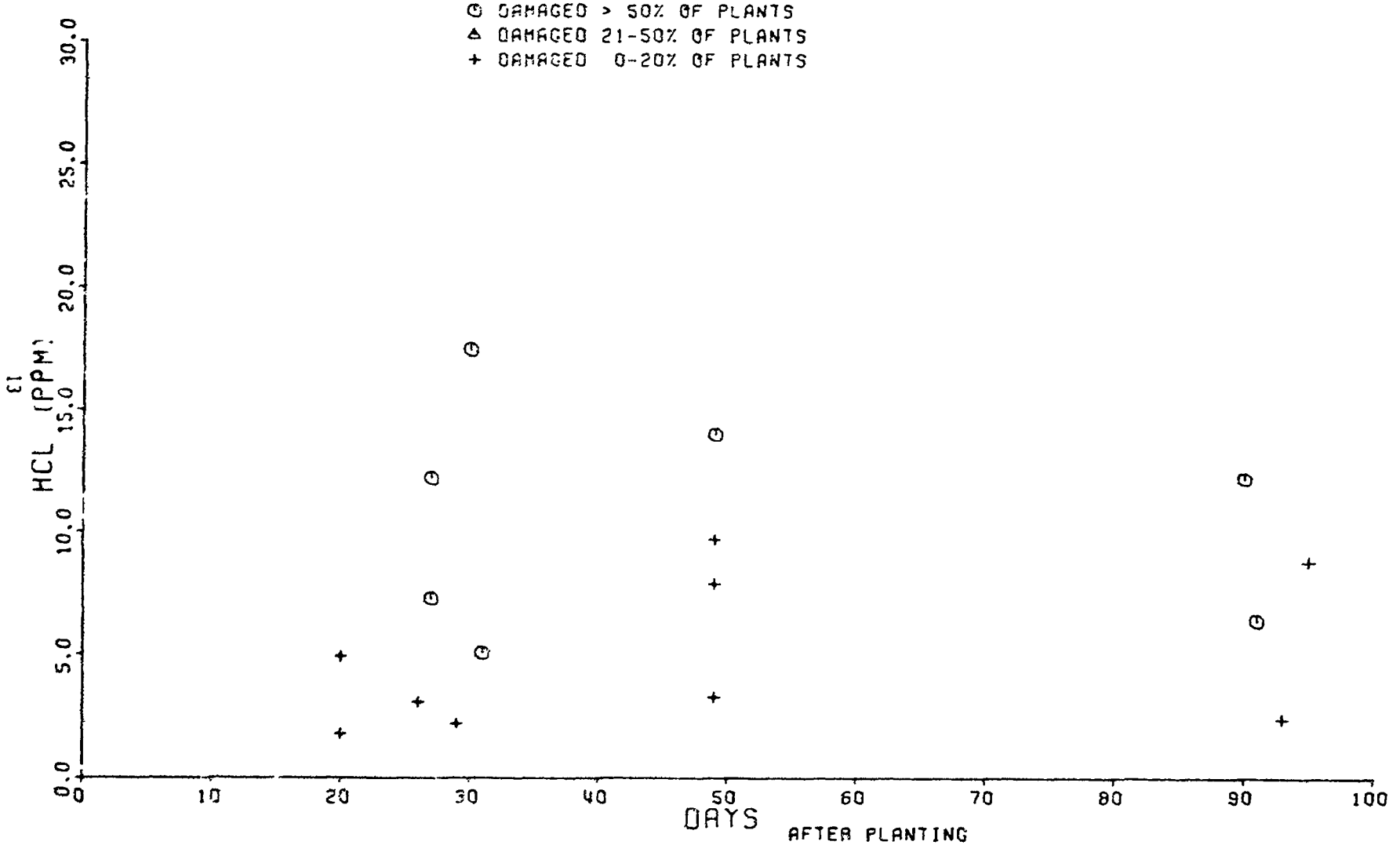

FIG. 16. PHYTOTOXIC RESPONSE OF MARIGOLD (FR) TO HCL
EXPOSURE TIME 20 MIN
⊙ DAMAGED > 50% OF PLANTS
△ DAMAGED 21-50% OF PLANTS
\+ DAMAGED 0-20% OF PLANTS

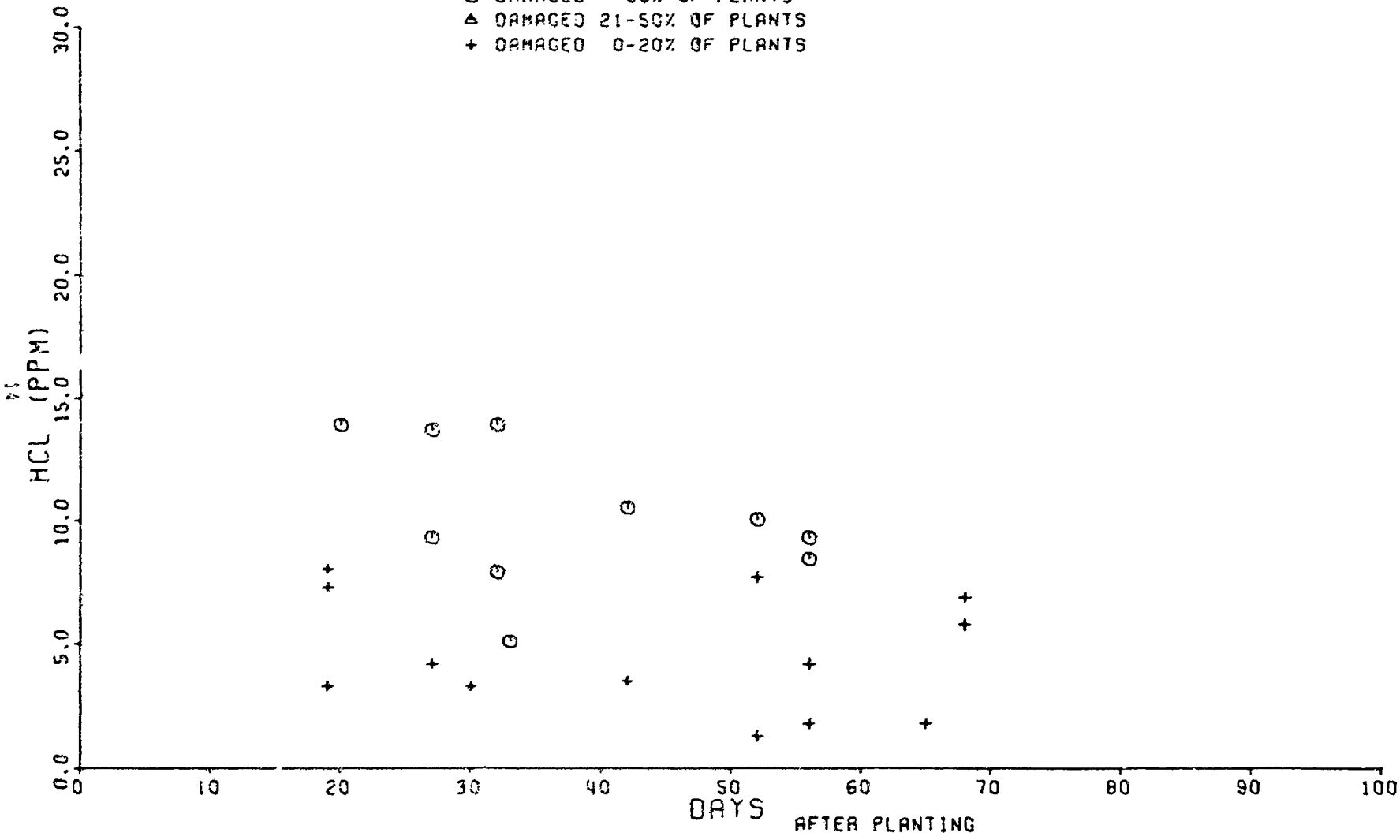

Fig. 11. PHYTOTOXIC RESPONSE OF NASTURIUM TO HCL
EXPOSURE TIME 20 MIN
⊙ DAMAGED > 50% OF PLANTS
△ DAMAGED 21-50% OF PLANTS
+ DAMAGED 0-20% OF PLANTS

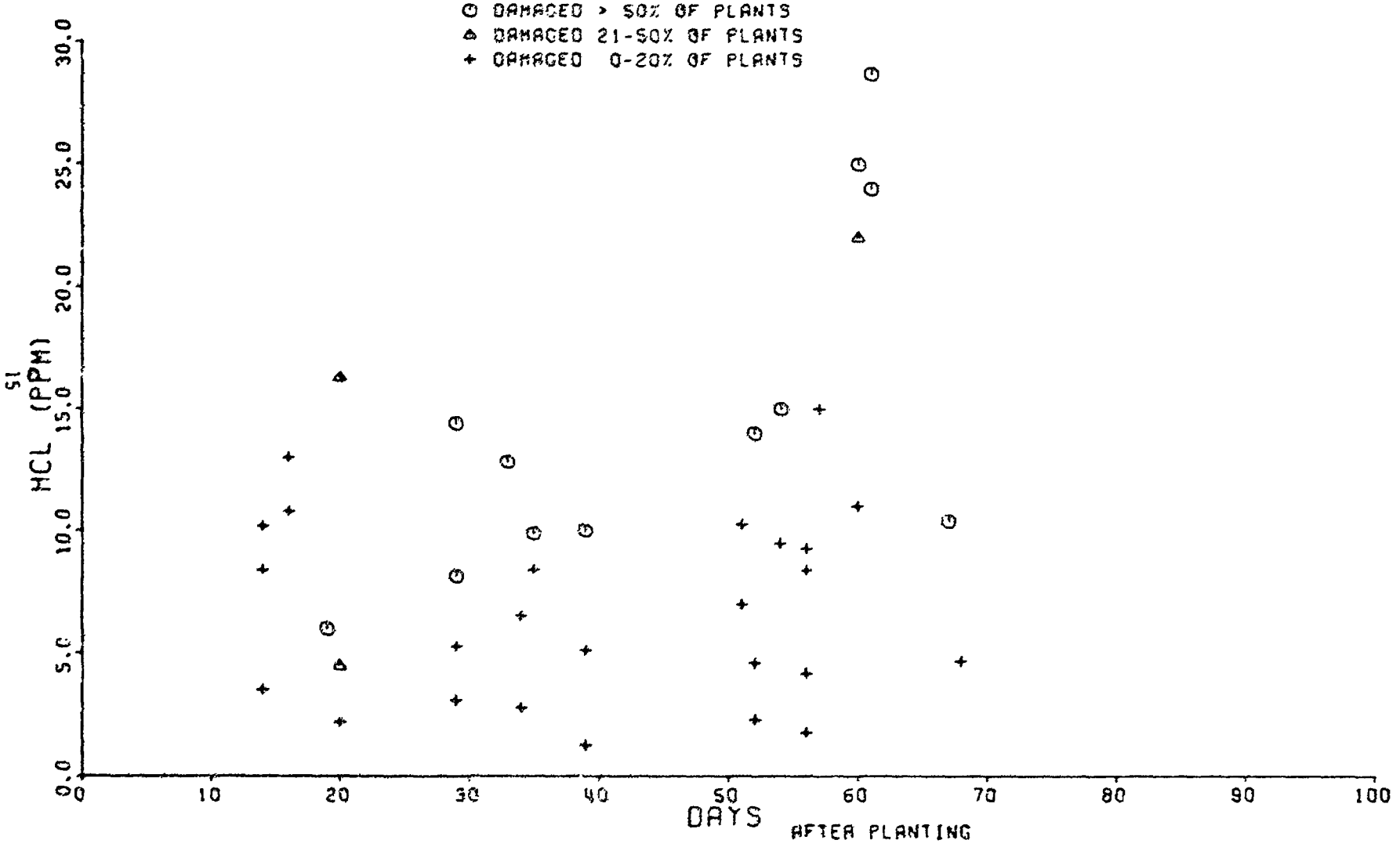

FIG. 12. PHYTOTOXIC RESPONSE OF ZINNIA TO HCL
EXPOSURE TIME 20 MIN
⊙ DAMAGED > 50% OF PLANTS
△ DAMAGED 21-50% OF PLANTS
+ DAMAGED 0-20% OF PLANTS

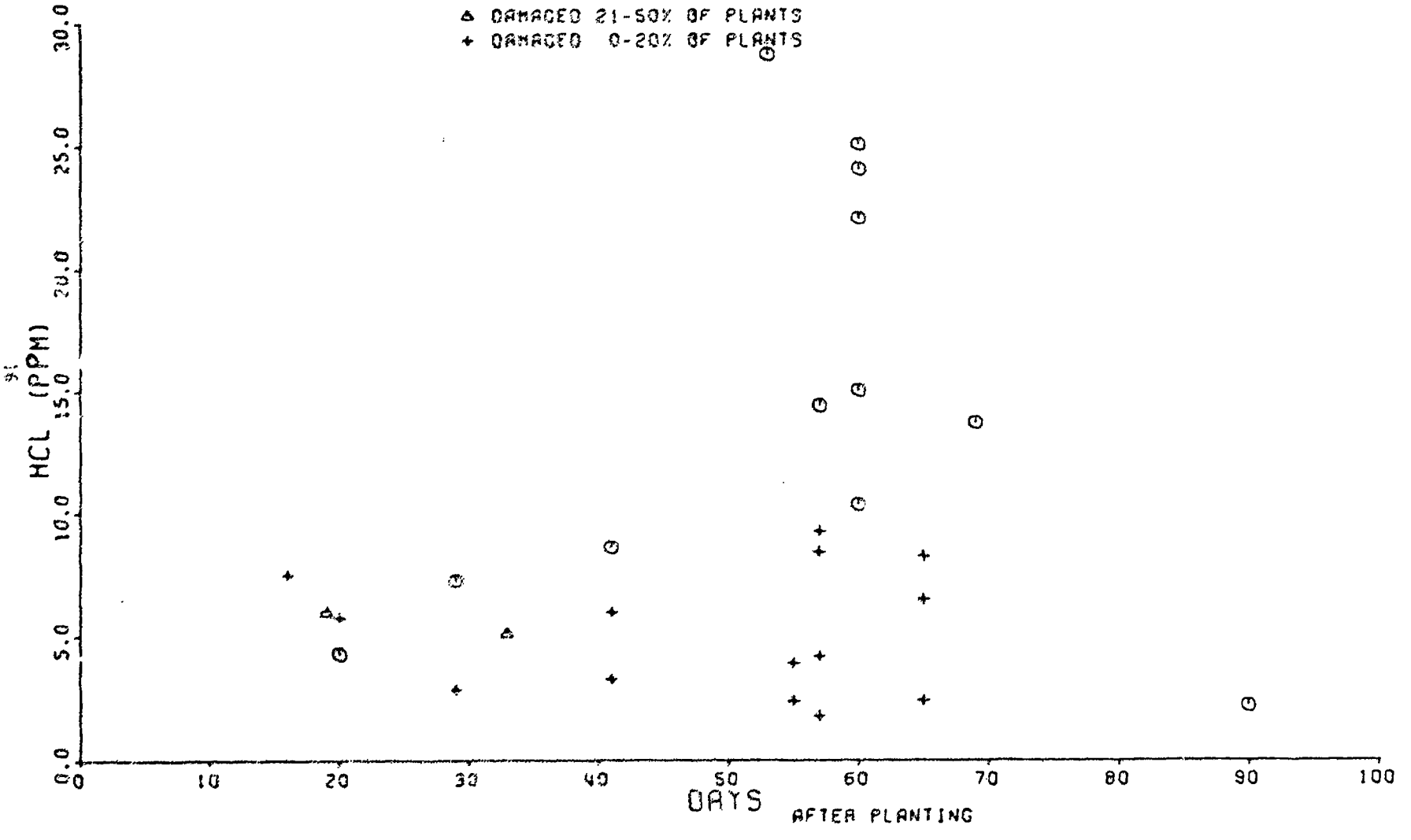

TABLE 3

EXPRESSION OF INJURY SYMPTOMS ON EIGHT PLANT SPECIES EXPOSED TO HCl GAS AT CONCENTRATIONS RANGING FROM 1-25 PPM FOR 20 MINUTES. Plants Were Evaluated 24 Hours After Exposure.

HCl Concentration	Aster	Calendula	Centaurea	Cosmos
15-25 ppm	Temporary wilting, extensive interveinal bronzing on lower leaf surface, necrosis of young tissue.	Temporary wilting, lower surface bronzing, discoloration, necrosis. The younger the leaf, the more distal the damage.	Extensive necrosis, rolling, speckling, temporary wilting, discoloration.	Extensive necrosis, extensive rolling, flower discoloration, tipburn of sepals.
7-14 ppm	Interveinal bronzing on lower surface, trace of necrosis.	Bronzing of lower leaf surface, interveinal necrosis, marginal discoloration.	Discoloration along the leaf margins, rolling.	Tipburn, tip rolling.
1-6 ppm	Trace of necrotic spots on young leaves.	Traces of lower surface bronzing.		Tipburn
	Marigold, Dwarf	Marigold, Sen. Birksen	Nasturtium	Zinnia
15-25 ppm	Severe necrosis of almost all leaves, rolling.	Severe necrosis, extensive rolling, tipburn of sepals on flowers.	Interveinal bleached lesions, on younger leaves in addition, marginal bleaching and rolling.	Bronzing on basal leaf portions, extensive necrosis and rolling on rest of leaf. Occasional petal necrotic spots.
7-14 ppm	Discoloration, necrosis of mid-aged leaves, some rolling.	Interveinal discoloration of mid-aged leaves, some rolling.	Discoloration, necrotic speckling, rolling.	Speckling, interveinal bronzing.
1-6 ppm	Traces of necrosis or discoloration.	Traces of necrosis or discoloration.	Traces of discoloration.	Trace of lower surface bronzing.

PROJECT II
FISH AND AUFWUCHS BIOASSAY

GENERAL

This is the first Annual Report to be submitted in partial fulfillment of the contract.

METHODS

Unless otherwise specified all determinations are performed in accordance with Standard Methods(9) as follows:

1. D.O. - Dissolved oxygen is measured using the azide modification of the the Winkler method.

2. BOD - Biological oxygen demand is measured by means of the dilution and seeding method.

3. COD - Chemical oxygen demand is measured by a modification of Standard Methods in which $HgSO_4$, $K_2Cr_2O_7$ and H_2SO_4 containing $AgSO_4$ are mixed together and cooled in a water bath prior to addition of sample.

4. 96-hour TL_{50} - Median tolerance limits are determined by Standard Methods utilizing semilog coordinate paper with concentration plotted on logarithmic axis and percent survival on arithmetic axis.

5. Aufwuchs Bioassay

   a. Aufwuchs are developed on roughened tygon tubing in growth unit racks according to the procedure of Krock and Mason(10).

   b. Biomass - Dry and ash-free weights are measured for periphyton according to Standard Methods.

   c. Chlorophyll a content measured using acetone extraction technique in Standard Methods.

   d. Metabolic measurements - Growth units are exposed to light and dark conditions according to the method of Krock and Mason(10).

Instrumentation Used For Specific Determinations Is As Follows:

1. pH - Radiometer, pH Meter 225 with a combination electrode.

2. Conductivity - Beckman conductivity bridge, Model RC-19.

3. Turbidity - Hach Turbidimeter, Model 2100, standardized with a formazin suspension.

4. TOC - Beckman Total Organic Carbon Analyzer, Model 915 with an Infrared Analyzer Model 215 A.

5. Chlorophyll - Beckman Spectrophotometer, Model DU.

COMPOUND PREPARATION

Development of a suitable method of solubilization of the fuels RJ 4, RJ 5 and JP 4 was the goal at the outset of experimentation.

Acetone was first investigated as a possible solvent for the fuels in static fish bioassays. At concentrations of up to 0.1% RJ 4 in 1 g/ℓ acetone there was 100% fish survival for eight days in both aerated and nonaerated series of uncovered 1 gal jars (3 fish/3ℓ). This indicated no acute effect of RJ 4, but a film on the surface demonstrated that acetone was not effective in solubilizing the fuel.

Vigorous shaking in a separatory funnel also failed to produce a stable saturated condition, and attention was then directed to use of a stainless steel Waring Blender. A reasonably stable emulsion was produced by the blender although some separation was noted. For this reason a standardized procedure was adopted in which 10 mℓ of fuel diluted to 1 ℓ with distilled water (1% by volume concentration) is blended for one hour and placed in a separatory funnel. After one hour residence to allow for separation, the lower portion or approximately 200 mℓ is drawn off and discarded. Then the middle zone is drawn off and retained for use.

Although accurate determination of the actual fuel concentrations in the emulsions must await quantitative measurement by gas chromatography, a rough estimate was made by comparison of the theoretical versus the measured TOC concentration of the emulsions with the results presented in table 1. Aliquots of 0.18mℓ of 1% by

TABLE 1

FUEL CONCENTRATION

Fuel	Theoretical			Measured		
	Volume Conc. %	Specific Gravity g/mℓ	Conc. mg/ℓ	TOC mg/ℓ	C %	Conc. mg/ℓ
RJ 4	0.00018	0.925	1.66	1.2	87.0	1.38
RJ 5	0.00018	1.0813	1.95	2.4	89.2	2.69
JP 4	0.00018	0.746	1.34	0.7	87.0	0.80

volume fuel emulsions were diluted to 1 ℓ giving theoretical volume concentrations of 0.00018%. Conversion to weight concentrations yielded theoretical values of 1.66 mg/ℓ for RJ 4, 1.95 mg/ℓ for RJ 5, and 1.34 mg/ℓ for JP 4. Based on total organic carbon (TOC) analyses the measured fuel concentrations were 1.38 mg/ℓ for RJ 4, 2.69 mg/ℓ for RJ 5, and 0.80 mg/ℓ for JP 4. The lower density fuels, RJ 4 and JP 4 tend to separate to the top resulting in a lower concentration in the portion drawn from the bottom, and the reverse phenomena was indicated by the high density RJ 5.

Another fairly crude estimate of the fuel concentration in the emulsions was performed by COD determinations. Theoretical COD's were computed for

RJ 4 and RJ 5 from their molecular formulas and compared to measured COD concentrations as follows:

RJ 4

Molecular formula = $C_{12}H_{20}$

$$24H_2O + C_{12}H_{20} \rightarrow 12CO_2 + 68H^+ + 68e^-$$

$$68e^- + 68H^+ + 17O_2 \rightarrow 34H_2O$$

$$C_{12}H_{20} + 17O_2 \rightarrow 12CO_2 + 10H_2O$$

$$17O_2 = 544 \text{ g}$$

M.W. RJ 4 = 164 g at a density of 0.925 g/mℓ

$$0.925 \left(\frac{544}{164}\right) = 3.068 \text{ g/mℓ}$$

Theoretical COD = 3.068×10^6 mg/mℓ

Measured COD = 2.1×10^6 mg/mℓ

The difference between measured and theoretical COD indicates about 70% solubilization which is in good agreement with the 80% solubilization indicated by TOC results.

RJ 5

Molecular formula = $C_{14}H_{20}$

$$28H_2O + C_{14}H_{20} \rightarrow 14CO_2 + 76H^+ + 76e^-$$

$$76H^+ + 76e^- + 19O_2 \rightarrow 38H_2O$$

$$C_{14}H_{20} + 19O_2 \rightarrow 14CO_2 + 10H_2O$$

$$19O_2 = 188 \text{ g}$$

M.W. RJ 5 = 188 at a density of 1.0813 g/mℓ

$$1.0813 \left(\frac{608}{188}\right) = 3.5 \text{ g/mℓ}$$

Theoretical COD = 3.5×10^6 mg/ℓ

Measured COD = 3.0×10^6 mg/ℓ

The difference of measured to theoretical COD indicates 86% solubilization of RJ 5 or much less than the TOC results which indicated 135% solubilization.

JP 4

Since JP 4 contains a mixture of C_6 to C_{13} compounds, it was not possible to compute the theoretical COD_5 without specific information on the exact composition. The measured COD was 4×10^5 mg/ℓ.

STATIC FISH BIOASSAYS

Nonemulsified Fuels

Two fish bioassays have been conducted in RFS tap water to determine the toxicity to golden shiners of RJ 4, RJ 5, and JP 4 in the nonemulsified form. In preliminary range-finding studies there were erratic fish-death rates noted under these conditions, leading to speculation that this may be due to chance encounters of fish with globules of fuel. The aim of full-scale studies was to investigate this phenomenon more fully and to determine whether meaningful TL_{50}'s can be established for the fuels when added in the pure form. The two studies are reported independently, and the results are then combined to yield TL_{50}'s based on 20 fish per concentration. The Richmond tap water used was of medium hardness with a measured concentration of 125 ppm and the alkalinity measured 100 mg/ℓ.

Study 1

The procedure was to provide the following toxicant volumes per 10 ℓ of dilution water: 1, 5, 10, 50 and 100 mℓ. Preceding the test, approximately 170 fish were placed in an acclimation tank for three days exposure to the 25 C temperature of the study. Sixteen jars of 20 ℓ capacity each received 10 ℓ of RFS water and were allowed to dechlorinate by aeration and come to temperature over the 3-day period.

Before introduction of the fish, each jar was aerated vigorously for 45 min, and then the experimental condition of minimal aeration was applied using non-dispersed air pumped slowly by means of Buchler polystaltic pumps. The initial DO was measured as 8.02 mg/ℓ and initial pH at 8.24.

Ten fish were then added to each jar and the toxicant was added in a manner to preclude contact with the fish. The feeding behavior of the fish is such that to discourage attempts to eat the toxicant as it sinks to the bottom, RJ 5 was introduced well below the water surface.

Table 2 shows that difficulty was experienced in maintaining the minimal required DO level of 4 mg/ℓ. The number of air bubbles per minute was increased on day 1 to the maximum capacity of the pump. The bubbles were not of constant size and where less than 100 bubbles per minute were measured, their size was larger than the norm. The two fuels which remained on the water surface RJ 4 and JP 4, caused a diminution of the DO at the higher concentrations indicating interference with oxygen transfer. In the RJ 4 series, the 50 mℓ/10ℓ conc. (Jar No. 5), the surface film almost covered the entire surface, and for the 100 mℓ/10 ℓ conc. (Jar No. 6) the entire surface was covered. At lower concentrations the surface action of the fuel formed circular globules. JP 4 formed a film instead of globules demonstrating a greater spreading capacity. The surfaces of Jars 15 and 16 were completely covered by films, but at lower concentrations there was exposed surface present.

TABLE 2

STUDY 1 — DISSOLVED OXYGEN CONCENTRATIONS
mg/ℓ

Jar No.	Toxicant	Toxicant Conc. mℓ/ℓ	Time in Hours					Aeration Rate Bubbles per Minute	pH	
			0	24	48	72	96		Initial	Final
1	control	0	7.94				4.84	180	8.25	7.42
2	RJ 4	0.1			4.75	5.48	4.74	120		7.50
3	RJ 4	0.5				4.93	5.69	130		7.52
4	RJ 4	1.0				4.68	4.64	164		7.45
5	RJ 4	5.0	8.05		4.24	4.95	5.59	62	8.25	7.41
6	RJ 4	10.0		3.64	3.15	3.35	2.59	116		7.20
7	RJ 5	0.1					4.59	126		7.20
8	RJ 5	0.5					4.81	156		7.44
9	RJ 5	1.0	8.05				5.14	140	8.25	7.53
10	RJ 5	5.0		4.04	3.92	3.15	3.13	70		7.39
11	RJ 5	10.0		3.72	3.48	5.00	5.02	58		7.52
12	JP 4	0.1					4.79	110		7.47
13	JP 4	0.5			4.75	4.00	4.94	132		7.45
14	JP 4	1.0		4.59	5.14	5.50	3.81	162		7.35
15	JP 4	5.0		4.12	3.32			168		
16	JP 4	10.0	8.05	4.13					8.20	

Table 3 shows that the fish survival pattern is erratic for RJ 4 and therefore only a range of concentrations can be estimated for the TL_{50}. With this limitation the 48 hr TL_{50} is 9250 mg/ℓ, the 96 hr TL_{50} approaches 100 mg/ℓ although there is 70% survival at the much higher concentration of 1000 mg/ℓ.

For the other two fuels the survival pattern is consistent. The toxicant concentration was corrected for density (specific gravity of RJ 5 = 1.0813), and the following TL_{50} measurements were computed.

	mg/ℓ
24-hour	-
48-hour	10,800
72-hour	5,410
96-hour	2,980

For JP 4 (specific gravity = 0.746) the following TL_{50} results were obtained:

	mg/ℓ
24-hour	1,045
48-hour	285
72-hour	285
96-hour	285

TABLE 3

FISH SURVIVAL IN STUDY 1

Jar No.	Toxicant	Toxicant Conc. mℓ/ℓ	Fish Survival Time in Hours			
			24	48	72	96
1	control	0	10	10	10	10
2	RJ 4	0.1	9	8	4	3
3	RJ 4	0.5	10	10	5	3
4	RJ 4	1.0	10	10	9	7
5	RJ 4	5.0	10	5	3	1
6	RJ 4	10.0	9	5	3	1
7	RJ 5	0.1	10	10	10	10
8	RJ 5	0.5	10	10	10	10
9	RJ 5	1.0	10	10	10	10
10	RJ 5	5.0	9	8	5	2
11	RJ 5	10.0	9	5	4	3
12	JP 4	0.1	10	10	10	10
13	JP 4	0.5	10	4	4	4
14	JP 4	1.0	6	3	3	3
15	JP 4	5.0	1	0	0	0
16	JP 4	10.0	0	0	0	0

Despite the obvious limitations of establishing a TL_{50} for insoluble materials, the range of acute toxicity can be estimated for fuel spills. Because of the initial difficulties with maintaining DO and to gain a greater measure of statistical validity, the study was repeated.

Study 2

In this study the problem with maintaining DO was combatted by providing 1.5 ℓ of water per fish, instead of 1 ℓ of water per fish. The only other difference from Study 1 was that the range of concentrations used for RJ 5 and JP 4 was narrowed.

Experimental conditions with respect to fuel concentrations, DO, pH, and TOC are reported in table 4. In this study the DO was at a satisfactory level for all jars except the three highest concentrations of JP 4. In these instances, the fish were badly stressed by the fuel, causing them to increase their respiration rate markedly. There were many fish deaths during the first 24 hours in these jars (as discussed in detail later), and stress probably initiated the decreased DO rather than vice versa. The aeration rate could not be increased due to the problem of stripping the fuel. The use of the higher ratio of 1.5ℓ of water per fish than previously had the desired effect of maintaining the DO in other jars that had decreased below 4 mg/ℓ in the first study.

There was no problem with pH encountered, and the TOC measurements indicated the limited solubility of the fuels. Only JP 4 demonstrated substantial initial

TABLE 4

EXPERIMENTAL CONDITIONS IN STUDY 2

Jar No.	Toxicant	Toxicant Conc. mℓ/ℓ	Dissolved Oxygen mg/ℓ				pH		TOC, mg/ℓ	
			Time in Hours							
			0	24	48	96	Initial	Final	Initial	Final
1	control	0	8.1			5.8	8.3	7.7	6.4	7.1
2	RJ 4	0.1	7.9			6.2		7.7	5.2	9.5
3	RJ 4	0.5				5.8		7.7	6.4	8.9
4	RJ 4	1.0			5.7	6.6		7.7	5.8	7.7
5	RJ 4	5.0			4.6	5.2		7.7	17.2	11.2
6	RJ 4	10.0	7.9		5.4	4.6	8.4	7.6	6.4	14.1
7	RJ 5	0.5	7.9			6.2	8.3	7.7	6.4	10.1
8	RJ 5	1.0			5.9	6.0		7.7	6.4	8.9
9	RJ 5	3.0				5.7		7.6	5.2	6.6
10	RJ 5	5.0				5.1		7.6	6.4	7.1
11	RJ 5	10.0	7.9		4.7	5.3		7.6	6.4	7.7
12	JP 4	0.1				5.0	8.3	7.6	7.6	7.7
13	JP 4	0.3	8.0			4.5		7.6	10.0	12.4
14	JP 4	0.5			2.0	2.2		7.4	14.8	17.0
15	JP 4	1.0		5.6	0.4	2.3		7.4	12.4	15.3
16	JP 4	3.0	8.0	5.5				7.4	10.6	22.3

solubilization (except for Jar 5 - the 5 mℓ/ℓ conc. of RJ 4). The pattern of solubility, which indicates a steady increase from the 0.1 mℓ/ℓ concentration up to a maximum of 0.5 followed by a steady decrease at the higher volumetric concentrations, is thought to be related to the fuel behavior when it is added to water in the nonemulsified form. At the 0.1 mℓ/ℓ concentration there is a globule of fuel about 2 inches in diameter with a few droplets of fuel dispersed on the surrounding surface. At the 0.3 mℓ/ℓ concentration a larger globule of fuel (about 4 inches in diameter) is present with a larger number of dispersed droplets scattered. The 0.5 mℓ/ℓ concentration has an elliptical shaped globule about 4 inches by 5-1/2 inches in size and still more dispersed droplets. However, at the higher concentration of 1.0 mℓ/ℓ there is an even film covering most of the water surface and a reduction in dispersed droplets. The 3.0 mℓ/ℓ concentration produces a film that covers the entire surface. It appears that the dispersed droplets are responsible for the solubilization that occurs and that little, if any, molecular exchange occurs between water and the globules.

Fish survival data is presented in table 5 and from this the following TL_m's, corrected for fuel density, were determined:

RJ 5, mg/ℓ

24 hours	
48 hours	9700
72 hours	950
96 hours	850

RJ 4, mg/ℓ

24 hours	
48 hours	630
72 hours	460
96 hours	

JP 4, mg/ℓ

24 hours	1300
48 hours	500
72 hours	420
96 hours	420

TABLE 5

FISH SURVIVAL IN STUDY 2

Jar No.	Toxicant	Toxicant Conc. mℓ/ℓ	Fish Survival Time in Hours			
			24	48	72	96
1	control	0	10	10	10	10
2	RJ 4	0.1	10	10	7	2
3		0.5	10	10	6	5
4		1.0	10	8	3	1
5		5.0	10	7	3	2
6		10.0	10	6	5	2
7	RJ 5	0.5	10	10	10	9
8		1.0	10	6	4	3
9		3.0	10	10	10	10
10		5.0	10	10	8	8
11		10.0	10	4	3	3
12	JP 4	0.1	10	10	10	10
13		0.3	10	10	10	10
14		0.5	10	8	6	6
15		1.0	8	1	1	1
16		3.0	2	0	0	

The RJ 5 data is erratic in this study with more deaths noted at a concentration of 1 mℓ/ℓ than at either 3 mℓ/ℓ or 5 mℓ/ℓ. However, when this data is combined with that of Study 1 and survivals are based on 20 fish, the results follow a uniform pattern with respect to time and concentration. The combined results also strengthen the data for JP 4.

A summary of the results of Study 1 and Study 2 and the TL_{50}'s from a plot of the combined results is as follows:

Time Hours	JP 4 TL_{50}, mg/ℓ		
	Study 1	Study 2	Combined
24	1045	1300	1170
48	285	500	450
72	285	420	380
96	285	420	380

Time Hours	JP 5 TL_{50}, mg/ℓ		
	Study 1	Study 2	Combined
24	-	-	-
48	10,800	9700	10,000
72	5,410	950	7,700
96	2,980	850	5,400

The acute toxicity of RJ 5 becomes meaningful when the data from the two studies are combined, and that this toxicity is quite low in comparison to that of JP 4. This comparison indicates the relative insolubility of RJ 5 compared to JP 4. The erratic nature of RJ 5 results may be due to chance encounters of fish with the pool of fuel on the bottom of the jars.

This chance encounter phenomenon may serve to explain the results for RJ 4 which present an unusual pattern of toxicity. Figure 1 shows that fish survival is dependent on time and relatively independent of concentration, as there is only a slight decrease in fish survival as concentration increases; the overwhelming factor is time of exposure. As length of exposure progresses for each 24-hour period after the first, there is a significant reduction in survival. These results suggest either that toxicity is a function of both time and concentration or that chance encounters of the fish with the fuel film on the surface are responsible. Results of the flume study (reported in a subsequent section) suggest the latter. The fish were placed in a separate container from the flume which simulated a running stream of water with an excess of fuel. Fish were continuously exposed to the stream of water which recirculated through the container, but there was no possibility of direct contact with the fuel globules. Under these circumstances fish survival with respect to RJ 4 was equivalent to the control, i.e. more than 90% over a period of 35 days. The flume study was

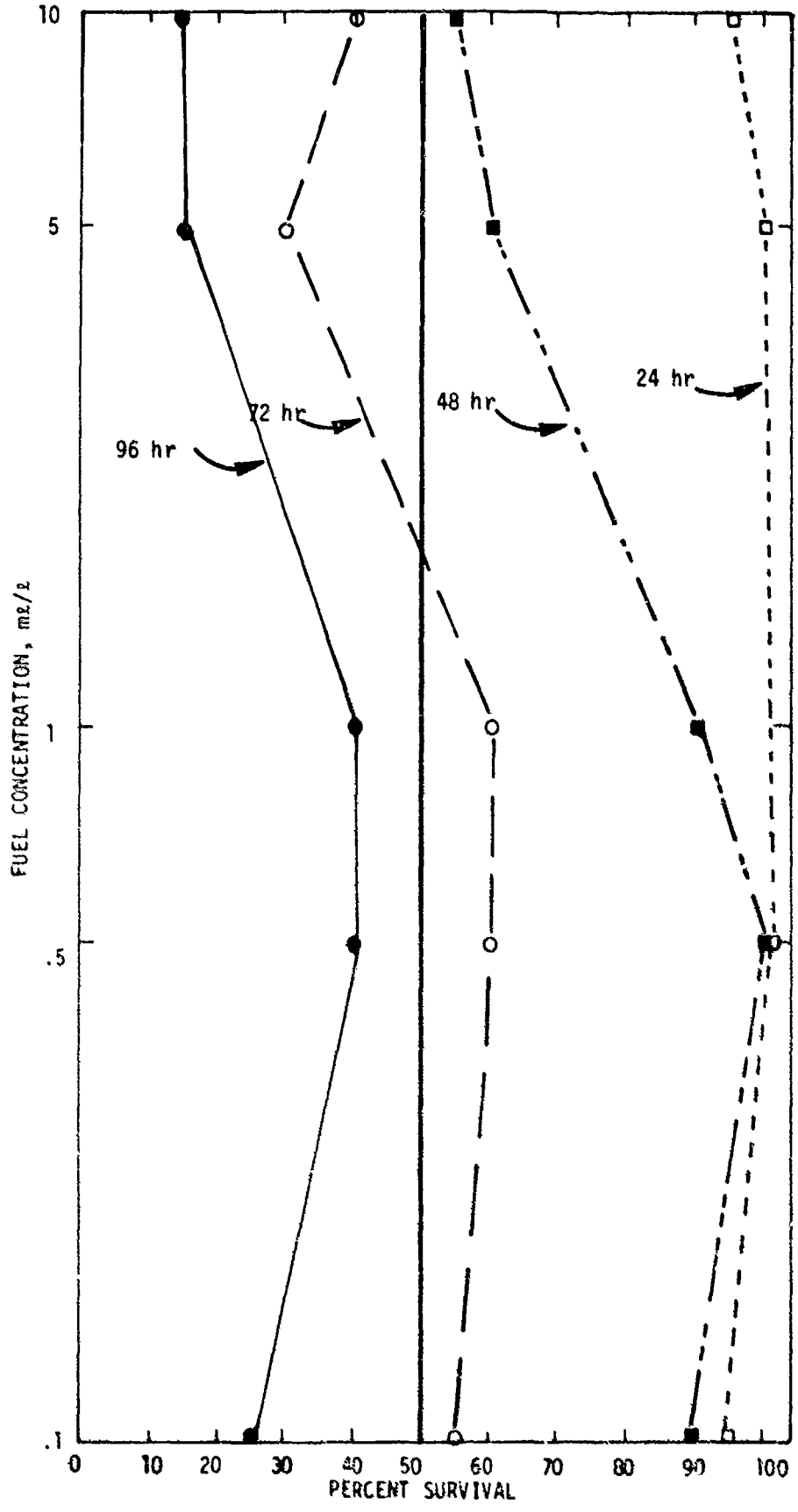

FIGURE 1. EFFECT OF RJ 4 ON FISH SURVIVAL

The flume study was terminated, and it was concluded that there is no toxicity due to solubilization of RJ 4 under normal stream conditions.

Assuming that encounters of fish with globules of fuel are responsible for deaths, then the impact of fuel concentration is a physical phenomenon and relates to percentage of surface area covered by the globule. the size of globules relative to fuel concentration was discussed previously, and the minor role played by concentration is due to the slightly greater probability of fish encounters with larger globules.

Emulsified Fuels

Emulsification of fuels represents the opposite extreme from the pure fuel situation in terms of boundary conditions that might exist if a spill occurs. Studies with emulsified fuels have all been preliminary to date with insufficient fish to provide statistical validity. Nevertheless, these range-finding studies have furnished good indications of the high degree of toxicity that might be expected. Due to the preliminary nature of these studies, only brief summaries will be presented. The procedure for emulsification of fuels was outlined previously.

Study 1

The toxicity of RJ 4 was investigated by exposing golden shiners to the following series of decimal dilutions: control, 0.01%, 0.005%, 0.001%, 0.0005% and 0.0001%. Five fish were placed in 10 ℓ of dechlorinated San Pablo Reservoir water (hardness = 108 mg/ℓ, Alkalinity = 81 mg/ℓ). The fish had an average weight of 1.34 g, an average length of 4.7 cm, and the water temperature was 23 C.

Results, corrected for fuel density, indicate the following TL_{50}'s:

	mg/ℓ
24-hour . .	51
48-hour . .	14
96-hour . .	6.5

Study 2

The toxicity of RJ 5 and JP 4 was studied in Richmond tap water using three fish per 3 ℓ at a temperature of 24 C. Decimal concentrations of 0.0001%, 0.001%, 0.01%, and 0.1% of each fuel were prepared.

Correcting for fuel density the 96-hour TL_{50} for RJ 5 was 0.7 mg/ℓ and for JP 4 was 12 mg/ℓ.

The results of these experiments indicate the high acute toxicity of the fuels in the emulsified form. JP 4 demonstrates relatively less toxicity than RJ 4 or RJ 5, which is the reverse of nonemulsified results. RJ 5 indicates the greatest toxicity in the emulsified form and by far the least acute toxicity in the nonemulsified form.

More bioassays are currently underway in order to fully investigate the acute toxicity of emulsified fuels.

AUFWUCHS

Lafayette Reservoir

A static periphyton bioassay was conducted to determine the photosynthesis and respiration response to the three fuels, RJ 4, RJ 5, and JP 4. It was originally planned to utilize a series of concentrations of each fuel, but limited growth restricted this test to a single concentration which was estimated to be of minimal toxicity based on results of fish bioassays. This concentration was 18×10^{-5}% by volume prepared by taking 0.18 mℓ of a 1% emulsion of each toxicant diluted to 1 ℓ.

The procedure for aufwuchs growth was to place two growth unit racks in the Lafayette Reservoir. Each rack consisted of 30 individual growth units of roughened tygon tubing, approximately 5 cm long and 1.2 cm in diameter. The racks were suspended 50 cm below the surface of the water by attachment to a wooden buoy anchored to the water intake tower of the reservoir.

The Lafayette Reservoir receives fresh water from the Sierra Nevada mountain range via the Mokelumne aqueduct. Considerable surface runoff is also contained in the reservoir making it one of the more eutrophic reservoirs in the East Bay Municipal Utility District system. For this reason the District frequently adds copper sulfate to retard algal growth. These treatments occurred two times during the seven week period of aufwuchs development. Immediately before each treatment the growth racks were removed from the reservoir, stored in a container of reservoir water for a period of 72 hours, and then returned.

The period of development was from March 27 to May 15, 1974, or 47 days. This lengthy period was required to obtain even a minimal growth deemed suitable for conducting the metabolic response test.

Utilization of the periphyton growth was as follows:

1. Preservation in formaldehyde (2 units)
    a. Scraped
    b. Intact
2. Biomass accumulation
    a. Volatile
    b. Ash component
3. Chlorophyll *a* accumulation
4. Metabolic characteristics
    a. Photosynthesis
    b. Respiration

Results, presented in table 6, represent the averages of four growth units per fuel with two units subjected to the light response and two units subjected to the dark response. Then all four units were analyzed for weight of biomass and chlorophyll *a* content.

TABLE 6

LAFAYETTE RESERVOIR AUFWUCHS STATIC BIOASSAY

Fuel	Standing Crop					Photosynthesis and Respiration				
	Weights			Chlorophyll *a*		mg O_2/Auf/Hr			PI(wt) mg O_2/g Org. Wt./Hr	PI(chl) mg O_2/mg Chl. *a*/Hr
	Dry mg/Auf	Organic mg/Auf	% Organic	mg/Auf	Organic Weight mg/g	Light (Net Photosyn.)	Dark (Resp.)	Gross Photosyn. (Light-Dark)		
RJ 4	36.0	27.5	78.5	0.0075	0.255	0.020	-0.025	0.045	1.6	6.0
RJ 5	46.0	35.5	77.5	0.0100	0.275	-0.005	-0.045	0.040	1.1	4.0
JP 4	47.0	36.5	77.0	0.0090	0.245	0	-0.050	0.050	1.4	5.5
Control	41.5	32.0	77.0	0.0011	0.340	0.040	-0.055	0.095	3.0	8.6

From the results it is apparent that there was a toxic response. The gross photosynthesis of the control was approximately twice (0.095) that of the three fuels, which at 0.045, 0.040 and 0.050 may be considered equal and average 47.5% of the control.

The photosynthetic index (PI) with respect to organic weight indicates the same order of magnitude of toxic response (45.5% of the control). The PI with respect to chlorophyll a yields an average for the three fuels of 5.17 or 60% of the 8.6 value of the control.

Mokelumne River

To augment the Lafayette Reservoir study, on May 23, 1974 four growth racks were placed in the Mokelumne River at a point approximately 1 mile above its confluence with the San Joaquin River in the Delta Region of Northern California. The buoy supporting the growth racks was anchored to a dock at the Willow Berm Laboratory operated by the U.S. Bureau of Reclamation and the California Department of Water Resources.

It is anticipated that a period of two to three weeks may be required for an adequate growth to develop.

CONTINUOUS FLOW BIOASSAY

Construction of Apparatus

Plans have been completed and construction is underway on 20 stainless steel tanks to be used for continuous flow bioassays. With this number of tanks it will be possible to examine four fuels at four concentrations each with one control for each group. The aim of these studies will be primarily to investigate the breeding success of fish and aufwuchs response in the presence of test fuels either in the emulsified or nonemulsified form.

Tank dimensions are 4 ft by 1 ft by 1 ft. Each tank will be fitted with removable perforated stainless steel dividers which can be used to provide separate chambers as needed for breeding fish, containing egg cups, developing fry, and growing aufwuchs. Proportional diluters will be used to provide the desired concentrations of fuels. Tanks will be operated at a liquid depth of 8 inches, a capacity of 75 l, and influent flow rates of 225 l per day per tank yielding theoretical residence times of 8 hours. To minimize concentration gradients that might occur due to degradation, recirculation will be provided by 10 l per minute submersible recirculation pumps.

The test specie of fish selected for these studies is Jordanella florida commonly known as flagfish. This selection was based on a number of advantages including a 6 to 8 week reproductive cycle, hardiness, ease of mating, and ease of sexing.

In preparation for the continuous flow studies, breeding success experimentation and development of a population of flagfish has begun. Six glass jugs are in use, each containing one male and three females which is the recommended ratio for breeding success. Small glass containers of coarse sand have been placed within the jugs to serve as egg cups. The ideal temperature

range for flagfish is 25-27°C, and the bioassay room is therefore now maintained in that range. It is also desirable to have 16 hours of light per day, and the room lights have been accordingly wired to a timer.

Flume Study

To simulate conditions that may exist in a stream if a fuel spill occurs, fish survival and the fate of fuels were studied in flumes located in the SERL pilot plant.

Four flumes, each with dimensions of 4 ft in length by 1 ft in width and 8 inches deep, were filled with dechlorinated RFS water. The volume of each flume was 64 ℓ and at the end of each flume a fish tank was provided containing 20 golden shiners in approximately 83 ℓ of water. Added to each flume was 1200 mℓ of fuel. The effluent port from each flume was 2 inches beneath the water surface to avoid removing the surface film of fuel (or bottom layer of fuel). Recirculating pumps were provided for continuous recirculation of the water from the container to the influent end of the flume. The input hose delivered recirculated water beneath the surface, but the effluent stream was allowed to fall into the fish container. A summary of the experimental conditions is as follows:

	Test Container			
	1	2	3	4
Fuel	JP 4	control	RJ 4	RJ 5
Flow, ℓ/min	10.7	10.9	9.1	8.2
Fuel quantity, mℓ	1200	0	1200	1200
Fuel appearance	covers surface	-	covers surface	thick globule covers about one-quarter of bottom area
Number of fish	20	20	20	20

The dilution water was Richmond Field Station tap water recirculated for a period of 3 days before fish were introduced in order to achieve dechlorination. During this period water was added as needed to maintain the specified volume. The study proceeded from April 29 to June 3, 1974. Other test conditions included the following: the average standard length of the golden shiners was 4.8 cm and the average net weight was 1.3 g; average maximum air temperature was 20.5 C (ranging from 16 to 27 C) and average minimum temperature was 10.5 C (ranging from 6.5 to 13 C).

Water characteristics during the course of the study are summarized in Table 7. The DO remained at a satisfactory level throughout, but the pH rose to a high level in the control and JP 4 flumes. TOC results indicated that JP 4 solubilized to an appreciable extent initially, and then diminished either by volatilization or degradation. Neither RJ 4 nor RJ 5 appeared significantly different from the control with respect to TOC.

TABLE 7

WATER CHARACTERISTICS DURING FLUME STUDY

Test	Time	Flume			
		Control	JP 4	RJ 4	RJ 5
TOC, mg/ℓ	2 hr	8.5	18.1	7.3	8.5
	96 hr	10.5	14.6	11.1	10.0
	11 days	7.1	9.4	6.9	7.5
DO, mg/ℓ	1 day	9.1	7.9	8.6	8.4
	32 days	8.1	7.7	7.6	7.9
pH	1 day	8.4	8.4	8.4	8.4
	32 days	9.2	9.5	8.7	8.3
Turbidity JTU	1 day	1.0	3.9	3.0	0.9

Fish survival results, presented in table 8, show there was 100% survival in the control through day 11 and 90% survival after 25 days. At this point the control was terminated because of a leak in the flume. The fish in the JP 4 container showed signs of stress within an hour after addition of the fuel and stress symptoms persisted for 4 days, but thereafter their appearance was normal. These symptoms correlate with the TOC results which indicated that JP 4 solubilizes. This resulted in a toxic effect initially, but as volatile components dissipated the fish were able to recover. The fish in the RJ 4 container had 100% survival until day 10. On the 11th day and until the end of the experiment, 95% of the fish survived. The fish in the RJ 5 container had 100% survival until the 17th day, 80% survival from the 21st to 25th day, 70% survival from the 28th to 31st day and 65% survival by the 32nd to 35th days. It is evident then that with RJ 5 the toxic effects are slow to develop compared to JP 4, but the cumulative effects are more pronounced.

The fate of the fuels was evaluated by odor and appearance. Throughout the experiment there was a fuel odor apparent in all the fish containers except the control. Initially fuel covered the entire surface area of the JP 4 and RJ 4 flumes, and one-fourth the bottom of the RJ 5 flume. At the termination of the experiment there was only a thin film which was not fuel covering approximately one-half the surface area of the JP 4 flumes, and the other half of the surface had a light brownish-yellow scum. The RJ 4 flume was still covered with fuel, but the film was thinner with many insects trapped between the fuel layer and the water surface. The surface of the RJ 5 flume was normal, but the layer of fuel on the bottom surface had an algae growth developing. All the flumes had an algal growth attached to their sides. The turbidity present in the flumes (reported in Table 7) was attributed to algae growth and bacteria buildup.

TABLE 8

FISH SURVIVAL IN FLUME STUDY

Day	Control	JP 4	RJ 4	RJ 5
0	20	20	20	20
1	20	20	20	20
2	20	20	20	20
3	20	19	20	20
4	20	19	20	20
7	20	18	20	20
8	20	18	20	20
9	20	18	20	20
10	20	18	20	20
11	20	18	19	20
14	19	18	19	20
18	18	18	19	20
21	18	18	19	16
22	18	18	19	16
23	18	18	19	16
24	18	18	19	16
25	18	18	19	16
28		18	19	14
29		18	19	14
30		18	19	14
31		18	19	14
32		17	19	13
35		17	19	13

PROJECT III

USE OF UNICELLULAR ALGAE FOR EVALUATION OF POTENTIAL AQUATIC CONTAMINENTS

GENERAL

During the first four months of the research, the efforts have been concentrated in the following areas:

1. Literature survey
2. Development of equipment and procedures
3. Experimental work

TECHNICAL PROGRAM

Literature Survey

The literature survey on the effects of oils and petrochemicals on algal growth has been completed.

A special effort was made to obtain specific references relating to the effects of the test compounds on algae. However, no such specific references were found.

The results of the general literature survey which resulted in about 40 recent and relevant publications can be summarized as follows:

1. Influences on photosynthesis are the principal effects noted for the algae, possibly because of their relative ease of measurement, but work on separation of physical stresses from internal physiological effects is needed(11) in order to more fully understand the precise disruptive properties of oils on biological systems.

2. Many of the precise physiological mechanisms of oil interference with terrestrial plant tissues are still speculative (12,13) and remain to be elucidated. The same comment is even more cogent for the algae.

3. Differences in cell wall and cuticular characteristics between algae and the higher plants could have significant bearing on the ability of oils to penetrate plant tissue. The relative abilities of the large variety of photosynthetic pigments in algae to resist destruction by oil is a complicating factor of some importance. Disturbance of the precursor pigments to the chlorophylls in the light energy transfer system in algal cells could cause severe metabolic problems even if the chlorophylls were not damaged.

4. Other needed work concerns the possible seasonal effects of oil on various algal species according to Boney(14). Consideration should always be given to the possible variable sensitivities of different stages in life histories of algae to the effects of pollutants. Care should also be taken when attempting to

correlate results for marine and fresh water situations because of the possible variable influences of environmental factors on the effects of oil on plant tissues. Baker (15) discussed these issues in some detail, especially the impact of temperature on oil toxicity. Because of their use commercially, various algae in zones affected by oil pollution, particularly in areas where it is somewhat chronic, should be examined for the possible accumulation of toxic residues. Various heavy metal compounds and carcinogens as discussed by Nelson-Smith (16) are found in oil and the possibility of their incorporation in algal tissues must be considered.

Development of Equipment and Procedures

Test Organisms

Three species of algae have been selected as the bioassay organisms and are maintained in pure culture. The three test organisms are:

Selenestrum capricornutum
Chlorella (not Axenic)
Chlorella (Axenic)

Selenestrum c. was selected as one of the assay organisms because it is one of the organisms prescribed by the U. S. Environmental Protection Agency (17) for algal assay investigations. The two cultures of *Chlorella* were selected on the basis of their suitability for the fundamental research investigations of the specific effects of the test compounds on the metabolism of algae. The first algal assay organism is being used in the batch screening experiments to determine the general effects of the test compounds. The possible general effects of the test compounds may for example be either growth stimulating, growth inhibitive, or combinations of the two. This algal assay organism will also be used in the continuous culture investigations of the effect of the test compounds on the biokinetic characteristics of the assay organism.

Bioassay Equipment

The standard batch assay equipment and procedures recommended by U. S. Environmental Protection Agency (17) are being used in this research work.

The only substantial modification from the standard procedure is related to the insolubility of the test compounds. Instead of having a uniform concentration of the test compound in the aqueous phase as it is envisioned in the EPA procedure (17), a specified amound of material is being added to the assay flasks. Only a portion of the material goes into solution while the remainder remains as a solid phase.

The continuous culture system is being modified to permit the handling of the relatively insoluble test compounds. It is intended to use only one concentration of each test compound in the feed solution to the continuous cultures. The concentration chosen is the saturation concentration for each test compound. It will be maintained by keeping the feed solution in contact with excess amounts of each test compound before the feed solution enter the individual chemostat. A separate screening and settling fixture will be developed to assure that none of undissolved test compound gets into the individual chemostats.

Analytical Procedures

The routine batch assay procedures (17) including the methods of determining cell growth, etc. are well established and are being used without modifications.

The main efforts in the area of analytical procedures are oriented towards the determination of the concentration of the test compounds in the 0.1 mg/ℓ to 100 mg/ℓ range. Two different procedures are being tried. The first of these is based on the determination of total organic carbon. This technique appears promising as a rapid test of the concentrations of the test compounds before any growth of algae takes place. However, it cannot be used after the bioassay because the metabolic by-products will interfere with the test. The second method used is gas chromatography. The procedures are being established and the intitial testing has been completed but with limited success. Because of the limited solubility of the test compounds, expecially RJ 5, an extraction step using isopentane will be evaluated.

Experimental Work

Batch assay testing has been intiated to determine the effects of the various test compounds on the growth to the test alga Selenastrum capricornutum.

First Batch Assay

Chemicals tested in the first main batch experiment were N phenyl-α-naphthylamine and p,p' dioctyldiphenylamine (Vanlube 81). Each chemical was tested at concentrations of 1mg/ℓ and 100 mg/ℓ. Six replicates of each concentration of the chemicals were tested in a base medium at nutrient levels of 1% and 33% (see table 1) of the referrnce medium NAAM as shown in table 2 (17). These two nutrient levels were selected as representative of the range of nutrients in natural lakes and streams.

TABLE 1

EXPERIMENTAL DESIGN FOR FIRST BATCH ASSAY

TEST COMPOUND	NUTRIENT CONCENTRATION a)	
	1 Percent	33 Percent
N phenyl-α-naphthylamine	2 Flasks 0 mg/ℓ	2 Flasks 0 mg/ℓ
	6 Flasks 1 mg/ℓ	6 Flasks 1 mg/ℓ
	6 Flasks 100 mg/ℓ	6 Flasks 100 mg/ℓ
p,p'dioctyldiphenylamine	2 Flasks 0 mg/ℓ	2 Flasks 0 mg/ℓ
	6 Flasks 1 mg/ℓ	6 Flasks 1 mg/ℓ
	6 Flasks 100 mg/ℓ	6 Flasks 100 mg/ℓ

The testing was conducted over a 22-day period and both algae cell numbers and total algae cell volume were measured as indication of the biological response to the test compound.

The results of the first batch assay are presented in the tables 3 through 6. The results indicate the following:

TABLE 2

REFERENCE MEDIUM COMPOSITION

Macronutrients - The following salts, Biological or Reagent grade, in milligrams per liter of glass-distilled water.

Compound	Concentration (mg/ℓ)	Element	Concentration (mg/ℓ)
$NaNO_3$	25.500	N	4.200
K_2HPO_4	1.044	P	0.186
$MgCl_2$	5.700	Mg	2.904
$MgSO_4 \cdot 7H_2O$	14.700	S	1.911
$CaCl_2 \cdot 2H_2O$	4.410	C	2.143
$NaHCO_3$	15.000	Ca	1.202
		Na	11.001
		K	0.469

Micronutrients - The following salts, Biological or Reagent grade, in micrograms per liter of glass-distilled water.

Compound	Concentration (µg/ℓ)	Element	Concentration (µg/ℓ)
H_3BO_3	185.500	B	32.460
$MnCl_2$	264.264	Mn	115.374
$ZnCl_2$	32.709	Zn	15.691
$CoCl_2$	0.780	Co	0.354
$CuCl_2$	0.009	Cu	0.004
$Na_2MoO_4 \cdot 2H_2O$	7.260	Mo	2.878
$FeCl_3$	96.000	Fe	33.051
$Na_2EDTA \cdot 2H_2O$	300.000		

TABLE 3

GROWTH OF SELENASTRUM IN THE PRESENCE OF p,p' dioctyldiphenylamine

CONCENTRATION OF BASE MEDIUM: 33% Standard Algal Assay Medium

CONCENTRATION OF TEST COMPOUND

Day of Growth	0 mg/ℓ Reps	0 mg/ℓ Cell Number 10^3 cells/mℓ	0 mg/ℓ Volume 10^5 μm^3/mℓ	1 mg/ℓ Reps	1 mg/ℓ Cell Number 10^3 cells/mℓ	1 mg/ℓ Volume 10^5 μm^3/mℓ	100 mg/ℓ Reps	100 mg/ℓ Cell Number 10^3 cells/mℓ	100 mg/ℓ Volume 10^5 μm^3/mℓ
0	2	1.0	0.5	6	1.0	0.5	6	1.0	0.5
3	2	330 (20) 100%	196 (16) 100%	6	231 (35) 70%	141 (50) 72%	6	255 (24) 77%	147 (20) 75%
7	2	1885 (78) 100%	642 (29) 100%	6	1073 (38) 57%	365 (8) 57%	6	980 (26) 52%	361 (17) 56%
10	2	2170 (4) 100%	793 (30) 100%	6	2214 (34) 102%	817 (20) 103%	6	2200 (49) 101%	821 (23) 103%
16	2	2251 (22) 100%	926 (2) 100%	6	2224 (33) 99%	912 (18) 99%	6	2198 (46) 98%	934 (13) 101%
22	2	2216 (20) 100%	964 (0) 100%	6	2257 (37) 102%	970 (14) 101%	6	2227 (46) 100%	1010 (17) 105%

TABLE 4

GROWTH OF SELENASTRUM IN THE PRESENCE OF p.p' dioctyldiphenylamine

CONCENTRATION OF BASE MEDIUM: 1% Standard Algal Assay Medium

CONCENTRATION OF TEST COMPOUND

Day of Growth	0 mg/ℓ Reps	0 mg/ℓ Cell Number 10^3 cells/mℓ	0 mg/ℓ Volume 10^5 μm^3/mℓ	1 mg/ℓ Reps	1 mg/ℓ Cell Number 10^3 cells/mℓ	1 mg/ℓ Volume 10^5 μm^3/mℓ	100 mg/ℓ Reps	100 mg/ℓ Cell Number 10^3 cells/mℓ	100 mg/ℓ Volume 10^5 m^3/mℓ
0	2	1.0	0.5	6	1.0	0.5	6	1.0	0.5
3	2	33 (2) 100%	25 (2) 100%	6	22 (3) 66%	17 (5) 70%	6	28 (3) 84%	32 (5) 128%
7	2	416 (15) 100%	172 (12) 100%	6	370 (25) 89%	148 (10) 82%	6	399 (27) 96%	164 (10) 95%
10	2	557 (29) 100%	252 (8) 100%	6	648 (36) 116%	259 (5) 103%	6	628 (22) 113%	292 (11) 116%
16	2	590 (14) 100%	297 (4) 100%	6	672 (49) 114%	278 (12) 97%	6	746 (60) 126%	350 (37) 122%
22	2	592 (18) 100%	301 (5) 100%	6	699 (40) 118%	306 (6) 101%	6	699 (14) 118%	333 (10) 111%

TABLE 5

GROWTH OF SELENASTRUM IN THE PRESENCE OF N phenyl-α-naphthylamine

CONCENTRATION OF BASE MEDIUM: 33% Standard Algal Assay Medium

CONCENTRATION OF TEST COMPOUND

Day of Growth	0 mg/ℓ Reps	0 mg/ℓ Cell Number 10^3 cells/mℓ	0 mg/ℓ Volume 10^5 μm^3/mℓ	1 mg/ℓ Reps	1 mg/ℓ Cell Number 10^3 cells/mℓ	1 mg/ℓ Volume 10^5 μm^3/mℓ	100 mg/ℓ Reps	100 mg/ℓ Cell Number 10^3 cells/mℓ	100 mg/ℓ Volume 10^5 μm^3/mℓ
0	2	1.0	0.5	6	1.0	0.5	6	1.0	0.5
3	2	330 (20) 100%	196 (16) 100%	6	212 (18) 64%	116 (7) 59%	6	10 (1) 3%	11 (1) 5%
7	2	1885 (78) 100%	642 (29) 100%	5	978 (21) 52%	361 (6) 56%	6	285 (149) 15%	119 (58) 19%
10	2	2170 (4) 100%	793 (30) 100%	6	1985 (33) 91%	812 (15) 102%	6	774 (340) 36%	362 (153) 46%
16	2	2251 (22) 100%	926 (2) 100%	6	2036 (29) 90%	941 (10) 102%	6	851 (367) 38%	457 (196) 49%
22	2	2216 (20) 100%	964 (0) 100%	6	2013 (29) 91%	971 (15) 101%	6	868 (367) 39%	483 (205) 50%

TABLE 6

GROWTH OF SELENASTRUM IN THE PRESENCE OF N phenyl-α-naphthylamine

CONCENTRATION OF BASE MEDIUM: 1% Standard Algal Assay Medium

CONCENTRATION OF TEST COMPOUND

	0 mg/ℓ				1 mg/ℓ			100 mg/ℓ		
Day of Growth	Reps	Cell Number 10^3 cells/mℓ	Volume 10^5 μm^3/mℓ	Reps	Cell Number 10^3 cells/mℓ	Volume 10^5 μm^3/mℓ	Reps	Cell Number 10^3 cells/mℓ	Volume 10^5 μm^3/mℓ
0	2	1.0	0.5	6	1.0	0.5	6	1.0	0.5
3	2	33 (2) 100%	25 (2) 100%	6	18 (2) 54%	11 (0.7) 46%	6	6 (1) 19%	7 (2) 27%
7	2	416 (15) 100%	172 (12) 100%	6	370 (25) 89%	149 (11) 86%	6	16 (2) 4%	12 (2) 7%
10	2	557 (29) 100%	252 (8) 100%	6	633 (18) 114%	265 (5) 105%	5	38 (5) 7%	27 (3) 11%
16	2	590 (14) 100%	287 (3) 100%	6	646 (19) 110%	293 (7) 102%	5	27 (7) 5%	10 (3) 4%
22	2	592 (16) 100%	301 (4) 100%	6	673 (15) 114%	310 (5) 103%	6	39 (8) 7%	22 (5) 7%

p,p' dioctyldiphenylamine:

This compound showed an intial small bioinhibition which changed into a small biostimulation towards the end of the test period.

N phenyl-α-naphthylamine:

This test compound showed a different pattern at the low concentration (1 mg/ℓ) as compared to the high concentration (100 mg/ℓ).

At the low concentration (1mg/ℓ), the phenomenon of initial bioinhibition was followed by biostimulation. However, at the high concentration (100 mg/ℓ) the results showed a very significant bioinhibition throughout the experiment with the final algae concentrations less than 10 percent of the concentrations observed in the control flasks. Even the 10 percent may be high because microscopical examinations indicated that the material counted did not all consist of algae cells.

Second Batch Assay

The second batch assay was started in May. The experimental design was modified to improve the statistical analysis of the results. The modified design is shown in table 7.

TABLE 7

CONCENTRATION OF TEST COMPOUND
AND NUMBER OF TEST FLASKS

Test Compound	NUTRIENT CONCENTRATION a)			
	1 Percent		33 Percent	
RJ 4	5 Flasks	0 mg/ℓ	5 Flasks	0 mg/ℓ
	5 Flasks	1 mg/ℓ	5 Flasks	1 mg/ℓ
	5 Flasks	100 mg/ℓ	5 Flasks	100 mg/ℓ
RJ 5	5 Flasks	0 mg/ℓ	5 Flasks	0 mg/ℓ
	5 Flasks	1 mg/ℓ	5 Flasks	1 mg/ℓ
	5 Flasks	100 mg/ℓ	5 Flasks	100 mg/ℓ

a) Percent of the standard reference medium (NAMM)

The preliminary results are shown in tables 8 through 11 for the RJ 4 and RJ 5 test compounds. It should be noted that the growth in the 33 percent base median has been completed but that the growth in the one percent base median has not leveled off after 14 days. This experiment is therefore continuing.

The preliminary results indicate that neither RJ 4 or RJ 5 show significant effects at the 1mg/ℓ concentrations. However, at the 100 mg/ℓ there is some inhibition of growth of the assay organism at the low base median concentration. The low base median concentration resembles the conditions found in very clean oligotrophic lakes.

FUTURE PLANNED WORK

After completion of the second batch assay experiment, the major focus of this investigational effort will be on the continuous culture experiments. It is expected that the continuous culture experiments for the first two test compounds will be completed in November 1974. In parallel to the continuous culture experiments, the work will continue on the improvement of the analytical techniques and establishment of the illuminated Warburg procedures.

TABLE 8

GROWTH OF SELENASTRUM IN THE PRESENCE OF RJ-4

CONCENTRATION OF BASE MEDIUM: 33% Standard Algal Assay Medium

CONCENTRATION OF TEST COMPOUND

Day of Growth	0 mg/ℓ Reps	0 mg/ℓ Cell Number 10^3 cells/mℓ	0 mg/ℓ Volume 10^5 μm^3/mℓ	1 mg/ℓ Reps	1 mg/ℓ Cell Number 10^3 cells/mℓ	1 mg/ℓ Volume 10^5 μm^3/mℓ	100 mg/ℓ Reps	100 mg/ℓ Cell Number 10^3 cells/mℓ	100 mg/ℓ Volume 10^5 μm^3/mℓ
0	5	1.0	0.5	5	1.0	0.5	5	1.0	0.5
4	5	576 (19) 100%	233 (7) 100%	5	428 (94) 74%	213 (4) 91%	5	452 (41) 78%	194 (18) 83%
6	5	1654 (32) 100%	734 (14) 100%	5	1542 (21) 93%	711 (15) 97%	5	1520 (77) 92%	708 (16) 96%
10	5	1898 (59) 100%	905 (29) 100%	5	1894 (113) 100%	920 (46) 102%	5	1691 (48) 89%	872 (12) 96%
14	5	1957 (64) 100%	959 (36) 100%	5	1907 (102) 97%	951 (54) 99%	5	1701 (40) 87%	895 (7) 93%

TABLE 9

GROWTH OF SELENASTRUM IN THE PRESENCE OF RJ-4

CONCENTRATION OF BASE MEDIUM: 1% Standard Algal Assay Medium

CONCENTRATION OF TEST COMPOUND

Day of Growth	0 mg/ℓ Reps	0 mg/ℓ Cell Number 10^3 cells/mℓ	0 mg/ℓ Volume 10^5 μm^3/mℓ	1 mg/ℓ Reps	1 mg/ℓ Cell Number 10^3 cells/mℓ	1 mg/ℓ Volume 10^5 μm^3/mℓ	100 mg/ℓ Reps	100 mg/ℓ Cell Number 10^3 cells/mℓ	100 mg/ℓ Volume 10^5 μm^3/mℓ
0	5	1.0	0.5	5	1.0	0.5	5	1.0	0.5
4	5	6.3 (0.5) 100%	6 (1) 100%	5	5.5 (0.8) 87%	5 (2) 83%	5	5.3 (0.3) 84%	4 (0.2) 67%
6	5	7.3 (0.5) 100%	6 (1) 100%	5	9.8 (3) 135%	6 (1) 100%	5	6.9 (0.6) 95%	4 (0.7) 67%
10	5	369 (23) 100%	156 (11) 100%	5	256 (126) 69%	98 (39) 63%	5	204 (60) 55%	90 (24) 58%
14	5	563 (49) 100%	258 (19) 100%	5	540 (106) 96%	242 (16) 94%	5	418 (42) 74%	198 (26) 77%

TABLE 10

GROWTH OF SELENASTRUM IN THE PRESENCE OF RJ-5

CONCENTRATION OF BASE MEDIUM: 33% Standard Algal Assay Medium

CONCENTRATION OF TEST COMPOUND

Day of Growth	0 mg/ℓ Reps	0 mg/ℓ Cell Number 10^3 cells/mℓ	0 mg/ℓ Volume 10^5 μm^3/mℓ	1 mg/ℓ Reps	1 mg/ℓ Cell Number 10^3 cells/mℓ	1 mg/ℓ Volume 10^5 μm^3/mℓ	100 mg/ℓ Reps	100 mg/ℓ Cell Number 10^3 cells/mℓ	100 mg/ℓ Volume 10^5 μm^3/ℓ
0	5	1.0	0.5	5	1.0	0.5	5	1.0	0.5
4	5	576 (19) 100%	233 (7) 100%	5	546 (16) 95%	218 (6) 94%	5	116 (38) 20%	66 (20) 28%
6	5	1654 (32) 100%	134 (14) 100%	5	1542 (58) 93%	709 (22) 97%	5	717 (282) 43%	399 (148) 54%
10	5	1898 (59) 100%	905 (29) 100%	5	1628 (33) 86%	808 (24) 89%	5	1620 (249) 85%	765 (128) 84%
14	5	1957 (64) 100%	959 (36) 100%	5	1756 (66) 89%	902 (42) 94%	5	1887 (234) 96%	990 (81) 103%

TABLE 11

GROWTH OF SELENASTRUM IN THE PRESENCE OF RJ-5

CONCENTRATION OF BASE MEDIUM: 1% Standard Algal Assay Medium

CONCENTRATION OF TEST COMPOUND

Day of Growth	0 mg/ℓ Reps	0 mg/ℓ Cell Number 10^3 cells/mℓ	0 mg/ℓ Volume 10^5 μm^3/mℓ	1 mg/ℓ Reps	1 mg/ℓ Cell Number 10^3 cells/mℓ	1 mg/ℓ Volume 10^5 μm^3/mℓ	100 mg/ℓ Reps	100 mg/ℓ Cell Number 10^3 cells/mℓ	100 mg/ℓ Volume 10^5 μm^3/mℓ
0	5	1.0	0.5	5	1.0	0.5	5	1.0	0.5
4	5	6.3 (0.5) 100%	6 (1) 100%	5	6.5 (1) 103%	4.5 (0.4) 75%	5	6.6 (0.4) 104%	7.8 (2.4) 130%
6	5	7.3 0.5) 100%	6 (1) 100%	5	8.1 (1) 111%	5.6 (0.9) 93%	5	7.9 (0.5) 109%	5.2 (0.4) 87%
10	5	369 (23) 100%	156 (11) 100%	5	370 (27) 100%	166 (13) 106%	5	184 (62) 50%	68 (24) 44%
14	5	563 (49) 100%	258 (19) 100%	5	477 (19) 85%	241 (8) 93%	5	342 (75) 61%	169 (37) 66%

PROJECT IV

EFFECTS OF POLLUTANTS ON EGGS, EMBRYOS AND LARVAE OF THE LEOPARD FROG, RANA PIPIENS

INTRODUCTION

Our object was to study the effect of compounds of interest to the Air Force upon the development of eggs, embryos, and larvae of the Leopard Frog, Rana pipiens. The project was divided into two parts, an initial study to determine lethal concentrations and exposure times to the above compounds, and a follow-up study to determine if any of the compounds were teratogenic. During the first 5 months of our research program we have kept to this outline except that we have added a second test species to our study, the South African Clawed Toad, Xenopus leavis. These animals are easier to maintain than the Rana in the laboratory, they spawn all year round, and are commercially available.

The compounds assigned to us by the Air Force were octyl-phenyl-α-naphthylamine, p,p'dioctyldiphenylamine, and N phenyl-α-naphthylamine. Two technical difficulties have affected the progress and interpretation of our work. First, we found that viability of the tadpoles we used in our studies is affected by handling and transferring operations. A number of our experiments had to be discontinued due to mechanical injury to the larvae. These problems are now solved. A second difficulty is introduced by the low aqueous solubility of the three compounds. Our results differ somewhat depending upon the method by which we add the compounds to water. Thus blending the compound into 'solution' with a Virtis Tissue Homogenizer appears to yield a more uniform suspension than merely swirling. Toxicity of at least one compound (N phenyl- -naphthylamine) is increased by this procedure. Heating also aids in dispersing at least one of the compounds (octyl-phenyl-α-naphthylamine) in aqueous medium. We have not yet determined if there is a concomitant increase in toxicity.

Laboratory Operations

Studies To Determine Lethal Concentrations Of The Three Amines

a. p,p' Dioctyldiphenylamine is the least toxic of the three amines tested. When continuously exposed to concentrations of up to 1 gram per liter R. pipiens eggs develop normally and continue to show normal viability for at least 30 days. Higher concentrations or longer exposure times have not been tested. Similar results have been obtained with 30-day exposures of up to 200 milligrams per liter using X. laevis eggs.

   Later stage larvae of both species have also been exposed to 200 milligrams per liter p,p' dioctyldiphenylamine without effect.

b. Octyl-phenyl-α-naphthylamine also appears to be relatively free of toxic effects. Fertilized eggs developed normally for at least 30 days when exposed to 1 gram per liter (R. pipiens) or 200 milligrams per liter (X. laervis). Tadpoles showed normal viability. Later stages of both species tolerated 200 milligrams per liter for at least 7 days. Higher concentrations or longer exposure times were not tested.

   In one recent study, octyl-phenyl-α-naphthylamine, when blended into suspension with the Virtis Tissue Homogenizer, was lethal to a group of 10 stage 45 Xenopus larvae at a concentration of 200 milligrams per liter. This experiment has not been repeated with Xenopus and confirmed; however, similar procedures have not proven lethal to Rana larvae.

c. N phenyl-α-naphthylamine is the most toxic of the three amines we are testing. When blended into suspension for 4 minutes with a Viritis Tissue Homogenizer, it is lethal to late stage pipiens and laevis larvae at 5 milligrams per liter within 24 hours (table 1). When fertilized eggs are continuously exposed to this compound, up to 200 milligrams per liter, they develop normally and show normal viability until stage 18 (table 2). However, from stage 20 on (characterized by beating heart and blood circulation in external gills) as little as 5 milligrams per liter is toxic when a blender is used; otherwise, toxicity occurs from 20-50 milligrams per liter.

Studies To Determine Effects Of Short Exposure To Lethal Concentrations Of N phenyl-α-naphthylamine

a. For these experiments tadpoles were exposed to various concentrations of amine for from 1 to 3½ hours and then transferred to fresh water which did not contain any amine. Thus far, only preliminary data are available. These indicate that concentrations of N phenyl-α-naphthylamine up to 50 milligrams per liter are not toxic when exposure time is 3½ hours or less. Higher concentrations and longer exposure times are being tested.

Teratogenic Effects Of Amines

a. These studies could not commence until lethal and sublethal doses and exposure times were ascertained. However, some preliminary information can be extracted from the foregoing studies, namely that exposure to p,p' dioctyldiphenylamine octyl-phenyl-α-naphthylamine using the preceding regimens does not cause gross morphological abnormalities in developing Xenopus and Rana tadpoles. Furthermore, N phenyl-α-naphthylamine does have an effect on larval development. The lethal doses used thus far have also caused noticeable retardation in growth rate (figure 1).

Histological Studies

In the foregoing experiments, specimens were observed and analyzed at the macroscopic level. Any subtle microscopic effects not leading to death would remain unobserved. We are, therefore, carrying out histological examinations on selected specimens to ensure that we are not overlooking derangement of internal organs.

TABLE 1. EFFECT OF EXPOSURE TO AMINES ON VIABILITY OF FROG LARVAE

TREATMENT	Number of larvae exposed	Number of larvae surviving 24 hours	Number of larvae surviving 48 hours
Control	125	125	125
N-phenyl-α-naphthylamine 5mg/l or greater	125	125	0
Octyl-phenyl-α-naphthylamine 50mg/l	125	125	125
p,p' Dioctyldiphenylamine 100mg/l	125	125	0

Larvae of stage 20 (heart beat) or later were used for this experiment.

TABLE 2. EFFECT OF EXPOSURE OF FERTILIZED FROG EGGS TO AMINES ON VIABILITY

TREATMENT	NUMBER OF EMBRYOS EXPOSED	NUMBER OF EMBRYOS SURVIVING			
		Stage 10 (dorsal lip)	Stage 14 (neurula)	Stage 18 (muscular response)	Stage 20 (heart beat)
Control	100	100	100	97	90
N-phenyl-α-naphthylamine					
20mg/l	100	100	100	0	
200mg/l	100	100	100		
Octyl-phenyl-α-naphthylamine					
20mg/l	100	100	100	95	92
200mg/l	100	100	100	90	88
p,p' Dioctyldiphenylamine					
20mg/l	100	100	100	91	85
200mg/l	100	100	100	90	80

a.

Control

b.

Compound

Figure 1. Effect of exposure to N phenyl-α-naphthylamine on development of frog larvae.

a. Camera lucida drawing of control stage 24.

b. Camera lucida drawing of larvae exposed continuously to N phenyl-α-naphthylamine.

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