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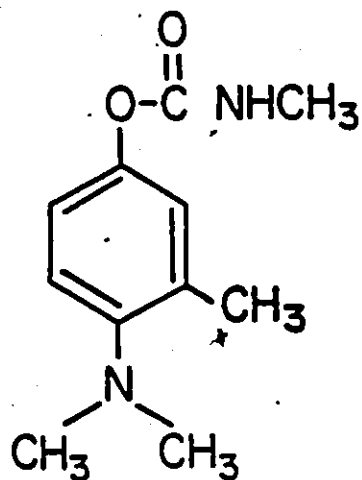
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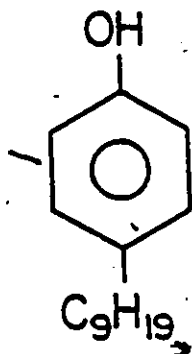
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AMINOCARB

(4-DIMETHYL AMINO 3 METHYL PHENYL - N METHYL CARBAMATE)

NONYLPHENOL



DILUENT OIL 585

- N - DECANE
- N - UNDECANE
- 1,2,4,5 TETRAMETHYL BENZENE
- N - DODECANE
- NAPHTHALENE
- N - TRIDECANE
- N - TETRADECANE
- 2,6 DIMETHYL NAPHTHALENE
- N - PENTADECANE
- N - HEXADECANE

- i -

ACKNOWLEDGEMENTS

The author would like to extend his sincere appreciation to Dr. P. Weinberger, Thesis Supervisor, for her interest, time, guidance and financial assistance throughout this research project.

I would particularly like to thank Dr. D.C. Mortimer, Dr. C. Nozzolillo and Dr. I. Arnason for many helpful discussions. Supervisory committees don't come any better!

Special thanks are extended to M. Ouellette and Don Champagne for many fruitful discussions and abundant help in the early stages of this project.

Fellow graduate students in Lab 212 are thanked for their interest, advice and encouragement towards the project and in providing a suitable atmosphere for research.

I sincerely thank Jacques Helie for the help with the drafting of figures and George Ben Ichavtchavadze for the plates. Jocelyne Rochon is thanked for her skillful typing of the thesis.

Gratitude is also expressed to all others, including the Support Staff of the Department of Biology who have contributed indirectly towards this thesis.

Finally, I would like to dedicate this thesis to Dr. R. Srinivasan without whose help pursuing graduate studies in Canada may have been just a dream.

ABSTRACT

Axenicly cultured fronds of Lemna minor L. were exposed to a range of ecologically relevant concentrations of the constituents of the carbamate pesticide formulation used in the Canadian spruce budworm program. The treatments included the active ingredient, aminocarb (4-dimethyl amino 3-methyl phenyl N methyl carbamate), nonylphenol (a non ionic surfactant), diluent 585 oil, (a 585 fuel oil distillate), or an aminocarb formulation, Matacil 1.8D which contained the three constituents. Exposure to aminocarb (0.10-2.50 µg/mL) or diluent oil 585 (0.15-3.75 µg/mL), did not affect biomass, frond development or metabolism. However, nonylphenol presented alone, or as Matacil 1.8D containing the same range of concentrations of these constituents, together with nonylphenol (2.50-6.25 µg/mL), significantly decreased frond growth, fresh and dry weights, total chlorophyll content, photosynthesis, chlorophyll fluorescence and total ATP content. However cell membrane permeability was not significantly affected. Phytotoxic symptoms of nonylphenol or Matacil 1.8D injury also included bleaching of fronds and detachment of roots. The degree of inhibition was directly attributable to the nonylphenol in the Matacil 1.8D mix. Nonylphenol considered to be the "inert" component of the Matacil formulation for registration purposes was the toxic agent, aminocarb, the "active" ingredient, was biologically "inert", over the range of concentrations used. The rapid biochemical effects of nonylphenol on many parameters made it difficult to ascertain whether the effects were primary or secondary, and its highly nonspecific toxicity suggested that

this surfactant may have multiple sites of action.

The uptake of aminocarb and nonylphenol was also examined. The bioaccumulation ratio was 15 - fold higher for nonylphenol than for aminocarb. The predicted values of bioaccumulation obtained from equations derived from the literature for plants was found to be reasonably close to the experimental values. The higher bioconcentration factor of nonylphenol was correlated to the greater K_{ow} of this compound. The possible use of Lemna minor as an indicator of pollution in aquatic areas is also discussed.

RESUME

Des frondes de Lemna minor L., cultivées en milieu stérile, furent exposées, à des niveaux écologiquement appropriés, aux constituants de la formulation insecticide de carbamate, utilisés dans les forêts canadiennes pour contrôler les infestations de tordeuses des bourgeons de l'épinette. Les traitements comprenaient l'ingrédient actif, l'aminocarb (4- diméthyle aminé 3- méthyle phénol N méthyle carbamate), le nonylphénole (un surfactant non-ionique), l'huile diluante #585 (un produit pétrolier), ou la formulation Matacil 1.8D, qui contient chacun des trois constituants. Ni l'aminocarb (0.1-2.5 µg/ml), ni l'huile diluante (0.15-3.5 µg/ml) n'affectèrent la croissance ou le métabolisme des frondes. Cependant le nonylphénole, seul (2.5-6.5 µg/ml) ou en formulation Matacil 1.8D (2.5-6.5 µg/ml de nonylphénole) causa une diminution considérable de la croissance des frondes, du poids sec, du poids frais, du contenu de chlorophylle, de la photosynthèse, de la fluorescence chlorophyllienne, et de l'ATP total. Le nonylphénole n'altéra pas de façon significative la perméabilité des membranes. Les symptômes de toxicité causés par le nonylphénole ou par le Matacil 1.8D comprennent également la décoloration des frondes et le détachement des racines. Le degré d'inhibition dû au Matacil 1.8D était directement imputable au composant nonylphénole. Ironiquement, le nonylphénole, composant "inerte" de la formulation Matacil 1.8D, fut l'agent le plus toxique alors que l'aminocarb, l'agent "actif", fut sans action aux doses employées dans cette étude. Puisque les effets biochimiques du nonylphénole, sur tant de paramètres, furent très rapides, il devint

difficile d'établir avec certitude, si les effets étaient primaires ou secondaires, et la toxicité non-spécifique de cet agent suggère qu'il agit à différents niveaux.

L'assimilation d'aminocarb et de nonylphénole fut aussi étudiée. La capacité d'assimilation fut 15 fois plus grande pour le nonylphénole que pour l'aminocarb. Ces valeurs expérimentales sont semblables à celles prédites par les équations rapportées dans la littérature pour les plantes. La haute capacité d'assimilation pour le nonylphénole correspond à la plus grande valeur de K_{ow} de cet agent. L'emploi possible de Lemna minor comme indicateur de pollution en milieu aquatique est aussi discuté.

TABLE OF CONTENTS

	Page
ACKNOWLEDGEMENTS	i
ABSTRACT	ii
RESUME	iv
INTRODUCTION	1
1.1 General Introduction	1
1.2 The spruce budworm program	2
1.3 <u>Lemna</u>	5
1.4 Environmental Hazards of the Spray Program	6
1.5 Rationale for present study	9
MATERIALS AND METHODS	11
2.1 Chemicals	11
Labelled Compounds	11
Luciferin Luciferase	12
ATP Standards	12
2.2 Plant material and conditions of culture	12
2.3 Bioassays	13
Growth studies: Frond number, wet and dry weights	13
Chlorophyll Content	14
Photosynthesis ¹⁴ CO ₂ incorporation	15
Fluorometric analysis	15
ATP Assay	16
Conductivity	17
2.4 ¹⁴ C Aminocarb Uptake	17
Nonylphenol uptake	18
2.5 Persistence of Aminocarb, Nonylphenol and Diluent Oil 585	19
2.6 Partition Co-efficient: Nonylphenol	20
2.7 Statistical Analysis of Data	20

	Page
EXPERIMENTAL RESULTS	22
3.1 Frond number and 96 hour IG ₅₀	22
3.2 Visual Observations	27
3.3 Fresh and Dry Weights	28
3.4 Total Chlorophyll Content	35
3.5 Photosynthesis	38
3.6 Fluorometry	38
3.7 Conductivity Measurements	41
3.8 ATP (Adenosine Triphosphate)	41
3.9 ¹⁴ C Aminocarb Uptake	43
3.10 Accumulation of Nonylphenol	47
3.11 Persistence of Aminocarb, Nonylphenol and Diluent oil in the Media	49
DISCUSSION	52
4.1 Aminocarb	54
4.2 Diluent Oil 585	54
4.3 Nonylphenol and Matacil 1.8D	55
4.4 Uptake and bioaccumulation	65
4.5 Relevance to Field Conditions	67
4.6 <u>Lemna minor</u> as an Indicator Species	69
4.7 Suggestions for Future Research	71
SUMMARY AND CONCLUSION	72
LITERATURE CITED	74

	Page
APPENDICES	80
Appendix (i)	80
Appendix (ii)	83
Appendix (iii)	86

LIST OF TABLES

TABLE	Page,
1. Lists the various concentrations of the compounds used in the study	14
2. Conditions for Gas Chromatography of Aminocarb and Nonylphenol and of Diluent Oil 585	21
3. 7 day Post-treatment Group Mean of Number of Fronds Following Treatment with Aminocarb, Diluent Oil, Nonylphenol and Matacil 1.8D	24
4. Effect of Aminocarb, Diluent oil, Nonylphenol and Matacil 1.8D on ATP levels	44
5 Uptake of Aminocarb by <u>Lemna minor</u> 1-7 days Post Treatment	45
6 Uptake of Nonylphenol by <u>Lemna minor</u> 1-7 days Post Treatment ..	48
7. Persistence of Aminocarb, Nonylphenol and Diluent Oil in Hillman M media (pH. 5.6) over a Seven Day Period	51

LIST OF FIGURES

FIGURES	Page
1. 1-7 days: Effect of Nonylphenol (0.25-6.25 $\mu\text{g}/\text{mL}$) on Frond Number (% of Control \pm SD)	25
2. 1-7 days: Effect of Matacil 1.8D (0.1-2.5 $\mu\text{g}/\text{mL}$ aminocarb) on Frond Number (% of Control \pm SD)	26
3. a and b. Comparative Determination of 96 hour IG_{50} of Nonylphenol and Matacil 1.8D respectively	29
4. 1 day Post treatment. Plants of <u>Lemna minor</u> Exposed to Nonylphenol Concentrations of 0, (Control), 0.25, 2.5 and 6.25 $\mu\text{g}/\text{mL}$ (ppm)	31
5. 1 day Post treatment. Plants of <u>L. minor</u> Exposed to Matacil 1.8D Concentration of 0 (Control), 0.1, 1.0 and 2.5 $\mu\text{g}/\text{mL}$ of aminocarb in the formulation mix	32
6. a and b. 7 day Post treatment: Fresh (0) and Dry (0) weights of <u>L. minor</u> Treated with Aminocarb (0-2.5 $\mu\text{g}/\text{mL}$) and Diluent Oil (0-3.75 $\mu\text{g}/\text{mL}$)	34
7. a and b. 7 day Post treatment: Fresh (0) and Dry (0) weights of <u>L. minor</u> Treated with Nonylphenol (0-6.25 $\mu\text{g}/\text{mL}$) and Matacil 1.8D (0-2.5 $\mu\text{g}/\text{mL}$ aminocarb content)	35
8. Chlorophyll Content (% of Control) 7 days Post treatment, Effects of a Range of Concentrations of Aminocarb, Nonylphenol, Diluent oil and Matacil 1.8D	36
9. Chlorophyll Content (% control) 1 day Post treatment: Effects of Matacil 1.8D mix. (2.5, 3.75 and 6.25 $\mu\text{g}/\text{mL}$ of aminocarb, diluent oil and nonylphenol respectively)	37
10. $^{14}\text{CO}_2$ Fixation 12 hours Post Treatment. Effects of Aminocarb (1.00 $\mu\text{g}/\text{mL}$), Diluent oil (1.50 $\mu\text{g}/\text{mL}$), Nonylphenol (2.50 $\mu\text{g}/\text{mL}$) and Matacil 1.8D (1.00 $\mu\text{g}/\text{mL}$ aminocarb content) ..	39
11. Effects of Nonylphenol (2.5, 5.00 and 6.25 $\mu\text{g}/\text{mL}$) on Chlorophyll Fluorescence Expressed as Percent of Control	40
12. a, b and c. Conductivity Measurements of the Media Bathing <u>Lemna minor</u> fronds. Effect of Treatment with 2.50, and 5.00 $\mu\text{g}/\text{mL}$ Nonylphenol.	42

13. Accumulation of Aminocarb in <u>L. minor</u> plants Exposed to 2.60 µg/mL, 1-7 days Post Treatment	47
14. Accumulation of Nonylphenol in <u>L. minor</u> Plants Exposed to 5 µg/mL 1-7 days Post Treatment	49

I - INTRODUCTION

1.1 General Introduction:

Rapid advances in modern technology has resulted in a dramatic increase in the production and use of chemicals. An array of chemicals have been used extensively to control agricultural and household pests. It has become evident, that in the course of this use, these pesticides have become widely dispersed in aquatic and terrestrial environments. This has been well documented in the literature in the past decade. (see reviews, Mulla et al 1981, Ware 1980). Recent public concerns have been expressed as to whether the potential environmental hazards resulting from the spray of millions of kilograms of pesticide in the forest environment outweighs the potential benefits obtained from curtailing insect predation. Many pesticides such as the widely used fenitrothion or aminocarb and their formulation products are not only restricted to the target organisms but may impinge on non target organisms as well. Registration and testing of new insecticide is an ongoing process both in Canada and elsewhere. New emphasis is being given to the role of adjuvants in formulations although there is presently no specific requirements for chemical and toxicological data on these products. It is hoped that the present study will contribute to our understanding of the biologically active nature of one particular formulation adjuvant (nonylphenol) that has been considered an "inert". Further, it is hoped that this study will act as a catalyst for other researchers and

legislators to question the current dogma which designates an automatic description of "inert" to all formulation additives.

1.2 The spruce budworm spray program.

Forests account for 1.24 million square miles of Canada's land area, the forest product industry being the dominant industry in many regions. Increasing demands on the forest resources for industrial raw material and recreational purposes have increased the need to protect the forest trees from insect damage (Prebble 1976).

In Canada, the spruce budworm, Choristoneura fumiferana (Clem), has become a major pest of the white spruce (Picea glauca), balsam fir (Abies balsamea), forest ecosystem. The budworm in its larval stage damages the spruce and fir trees by feeding on their needles in the month of May and June. Outbreaks last for six to ten years during which time large areas of mature forest may be killed. In 1976, about 60×10^6 ha of spruce-fir forests in the United States and Canada were attacked by the budworm. (Anonymous 1976, cited in McCarthy et al 1983). Some individual outbreaks in Ontario and Quebec have affected susceptible stands within areas as large as 181×10^5 to 207×10^5 ha. Estimated timber losses have ranged from 4.25×10^7 to 2.8×10^8 cu meters, depending on the size of the affected areas (Prebble 1976).

Because of the extensive area of coverage required to cope with the serious insect problem, aerial application of chemical insecticides was initiated in the mid 1940's. DDT became the insecticide of choice for budworm control. Initially, application rates were approximately 183g.active ingredient/ha, and this reduced the moth population by about

95%. However by the mid 1950's concern over non target effects on salmon fry in the Miramichi river caused application rates to be reduced to below 92g active ingredient/ha and a lower efficiency of control was accepted. Finally in 1968 the use of DDT was restricted in Canada, following recognition of its persistence and biomagnification through food webs and subsequent adverse effects on the ecosystem. This initiated a search for an appropriate replacement for DDT. In 1962, the organophosphate phosphomidon was sprayed experimentally, but its high cost and significant toxicity to birds made it unsuitable for treating large areas (Kingsbury 1976). In 1969, fenitrothion was introduced to replace DDT for operational control of lepidopterous defoliators, however, its use has been criticized as there appeared to be a possible link between the adjuvant present in the fenitrothion spray formulation and the potentiation of Herpes B virus resulting in Reyes syndrome in humans (Crocker et al 1976).

The carbamate insecticide aminocarb (4- dimethyl amino 3-methylphenyl N methyl carbamate), a non systemic broad spectrum insecticide was introduced for experimental use in 1970 and is presently one of the insecticides routinely used in Canadian forests. In 1973 a temporary registration for forest use was issued by Agriculture Canada and full registration followed in 1977 (NRC report on aminocarb 1982). For field use, aminocarb is formulated with a carrier and a primary solvent. Diluent 585 oil the fraction of No 2 fuel oil which distills at or below 585°F (308°C) is used as a carrier. It is composed of alkylated benzenes and naphthalenes. Nonylphenol, a mixture of monoalkyl phenols predominantly para-substituted, with about 3% of the ortho isomer and 4% 2-4 dinonylphenol, is added to all registered

formulations as a primary solvent to ensure uniform spreading of aminocarb on spruce foliage. Aminocarb mixed with nonylphenol and diluent oil 585 in the ratio 2:5:3 w/w/w has been registered as Matacil 1.80 oil soluble concentrate and is applied at the rate of 52 - 70 g active ingredient (a.i.) / ha. In Canada, formulated aminocarb was first used in Manitoba in 1965 against the fall cankerworm Alsophila pometaria (Nigam 1975). It was first field tested against the spruce budworm in New Brunswick in 1970 and has been used on an operational scale since 1975.

An appreciation of the magnitude of the potential problem posed by the ever increasing predation by the spruce budworm may be gained from a consideration of the immense extent of the areas sprayed. In New Brunswick in 1976, 24×10^3 ha were required to be sprayed, by 1977, this had increased to 120×10^3 ha. In Quebec alone in 1976 and 1977, 2.9×10^6 hectares were sprayed (Canadian Forestry Service 1977, 1978). In Newfoundland 243×10^3 hectares of forest land was sprayed with aminocarb in 1981. Spray operation planned for 1982 included coverage of 100×10^3 ha for Newfoundland and 1.2×10^6 ha for Quebec (FPMI Newsletter 1982). The total amount of aminocarb used in the provinces of Newfoundland, New Brunswick, Quebec and Ontario in the past decade has been about one million kgs (Nigam 1980). In Canada, as elsewhere, for registration purposes the pesticide is accepted as the active ingredient and the adjuvants as "inerts". Registration of aminocarb, the active ingredient under present investigation, was preceded by extensive invertebrate and mammalian toxicity testing. However, registration did not require testing on phytobiota. The present study was undertaken as it was thought advisable to monitor and evaluate whether aminocarb or its

adjuvants had any toxicological effects on non target plants in the ecosystem.

1.3 Lemna

Lemna minor L. (Tracheophyta, Lemnaceae, Arales), commonly called duckweed, a ready target for drift and airborne spray, was chosen as the test species. The plant is a free floating aquatic angiosperm, common in aquatic ecosystems. It is composed of 2 to 3 fronds and a long root with a conspicuous cap that hangs into the water from the underside of each plant. Vegetative reproduction is accomplished when the fronds of the plant produce similar fronds from the pocket in either side of the basal region. As the lateral fronds increase in size, any movement of the water surface can detach them from the base of the parent.

In the field, mats of Lemna, harbour a varied fauna of small invertebrates. A number of insects are obligate or facultative associates of L. minor. Obligates include the ephyrid fly and the rynchophorous beetle. The eggs are laid on the fronds which serve as food for the larvae and adults. Collembolans, Thysanura and spiders are facultative, on the duckweed. Lemna is also used as a food source by a number of other animals including ducks, waterbirds, herbivorous fish particularly Carpus lymprius, and snails, Physa spp. (Hicks 1937, Jacobs 1947 cited in Hillman 1961a).

In the laboratory the plant can be grown under axenic conditions, excluding the possibility of bacterial or algal contamination contributing to observed results. Rapid growth of uniform clonal

cultures makes it possible to monitor changes in biomass fairly easily. Since reproduction is normally vegetative, homozygous material can be obtained by starting with a single plant and maintaining a stock culture for as long as the investigation demands. Moreover, Lemna spp have been widely employed as an investigative tool in ecological studies as the plants are very sensitive to metals and other pollutants, (Blackman et al 1954, Hutchinson and Halina 1975). In this context it has also been considered as an indicator of pesticide and water pollution (Walker 1980, Nasu & Kugimoto 1981).

1.4 Environmental Hazards of the Spray Program

The forested areas which have been the sites of aminocarb spray operation include many rivers, lakes and streams. As a result of aerial application of aminocarb, a proportion of the spray released will inevitably drift to non target sites. Hindin et al (1966) reported that less than 35% of the DDT sprayed from an aircraft reached the target, presumably most of the remainder was carried in the atmosphere. A 50% loss of sprayed insecticide to a crop has also been indicated (Edwards 1976, Woodwell 1967).

Initial field investigations on aminocarb showed rapid dissipation of deposited carbamate into different components of the forest ecosystem (Sundaram et al 1976). Residues found in pond and stream water samples collected from Larose forest Que., after a single spray operation were 2.1 and 1.9 ppb respectively after a 0.6 day period and decreased to traces after 32 days. Studies on persistence have shown

that the $t_{\frac{1}{2}}$ of aminocarb in a pond is 4.4 days and 8.7 days in stream water (Sundaram et al 1976). It was suggested that the high value for the stream was due to high concentrations of insecticide carried into the stream from surrounding land by runoff water and washings from foliage. Tests carried out in our laboratory have shown a $t_{\frac{1}{2}}$ of 9.3 days for aminocarb in Matacil 1.8D (Weinberger et al 1982).

Studies on spruce showed that following a field spray the foliage contained 0.7 ppm of aminocarb at 0.6 days, this increased up to a maximum of 2.2 ppm by 4 days and by 64 days it had disappeared completely. The half life on foliage was 5.6 days. Forest soil did not contain any detectable levels of aminocarb on, or after, 0.6 days. (Sundaram et al 1976).

Aminocarb was first tested in 1970, (two applications of 85g a.i./ha), for side effects on aquatic organisms. No significant effects on juvenile salmon or aquatic insects were reported (Penniey 1971). An application of 103 g a.i./ha, however, caused a significant reduction in stonefly nymph populations. In another study, in eastern Canada, during the years 1971-1974, aminocarb was sprayed at doses ranging from 52 g a.i./ha to 105 g a.i./ha. There was a minimal effect on songbirds. Bees were subjected to adult forage knockdown, small mammals and amphibians were not affected. Minimal disturbances to aquatic organisms were noted. Stonefly nymphs were the only group identified suffering significant impact (Buckner, McLeod and Kingsbury 1975). Holmes and Kingsbury (1980), working with nonylphenol, found no effect on aquatic or terrestrial invertebrates or fish, at a dosage rate of 0.47 l/ha.

The acute toxicity of aminocarb, its adjuvants and its formulations, have been examined in a number of laboratory studies using a variety of organisms.

In studies with technical grade aminocarb, Couture et al (1979) reported a 14 day LC₅₀ of 0.1 mg/L and a 24 hour LC₅₀ of 0.8 mg/L for the algae Selenastrum capricornutum and Chlamydomonas variabilis respectively. The 48 hour LC₅₀ was shown to be 19 µg/L for the water flea, Daphnia magna, Nelson (1978). The 96 hour LC₅₀ for stonefly, Pteronarcella badia, amphipods Gammarus pseudolimnaeus and G. lacustris were 28 µg/L, 2.2 mg/L and 12 µg/L respectively, (Woodward & Mauck 1980, Sanders 1969). McLeese et al (1980) in their study showed the nominal 96 hour lethal threshold (LC₅₀) to be 0.2 mg/L for marine shrimp (Crangon septemspinosus) and concentrations of up to 5 mg/L causing no kill in the fresh water clam (Anodonta cataractae). For the fresh water crayfish, (Orconetes limosus), a 96 hour LC₅₀ of 33 mg/L has been reported, (Sundaram & Szeto 1979) Tests conducted on Atlantic salmon (Salmon salar) have been inconsistent. Nigam (1975) reported a 48 hour LC₅₀ of 1.10 mg/L whereas McLeese et al (1980) showed a 96 hour LC₅₀ of 8.7 mg/L. Toxicity tests conducted on mammals indicate an oral and dermal LD₅₀ of 30 & 275 mg/kg body weight for rats. (Mobay report 1979).

A limited number of laboratory tests on acute toxicity of the adjuvants on non-target aquatic fauna have been reported. For nonylphenol, Ernst (1981) showed a 360 hour LC₅₀ of 1.00 mg/L to soft shelled clam (Mya arenaria) and a 144 hour LC₅₀ of 5.00 mg/L when the clams were exposed to diluent oil 585. McLeese et al (1980) reported

the 96 hour LC₅₀ to marine shrimp (Crangon septempinnosa) and lobster (Homarus americanus) to be 0.4 and 0.2 mg/mL nonylphenol respectively. In the same study a 144 hour LC₅₀ of 5.00 mg/mL and > 5.00 mg/mL to freshwater clam (Anodonta cataractae) for nonylphenol and diluent oil respectively has been shown. In addition a 96 hour LC₅₀ of 5 mg/mL for marine annelid (Neris virens) and 5.00 mg/L to Crangon sp exposed to diluent oil has been reported.

Lethality tests with formulated aminocarb showed a 14 day LC₅₀ of 0.1 mg a.i./L and a 24 hour LC₅₀ of 0.6 mg a.i./L for the algae Selenastrum capricornutum and Chlamydomonas variabilis respectively (Couture et al 1979). The 96 hour LC₅₀ for the marine shrimp (Crangon septempinnosa) for clam (Anodonta sp) and the Atlantic salmon (Salmon salar) were 0.50, 1.0 and 3.5 mg/L respectively (McLeese et al 1980).

1.5 Rationale for present study

From an examination of the literature published one can state that previous research has largely investigated the effects of the insecticide aminocarb and the adjuvants on animals of the forest ecosystem. There is a dearth of literature on the effects of aminocarb and its adjuvants on aquatic plants which constitute an integral part of the fresh water food web. Studies on phytobiota would be worthwhile as the total energy budget of an ecosystem is extremely sensitive to any changes in primary productivity. Consequently, such changes in primary productivity may affect organisms at other levels in the food web and even diversity within a community. This latter perturbation has been

demonstrated by Mosser et al (1972) and Taub (1976) who have shown altered species composition after pesticide treatment.

The aim of the present research was to establish the relative phytotoxicity of aminocarb, diluent oil 585 and nonylphenol, presented alone or together in the Matacil 1.80 formulation. L. minor provided an ideal nontarget test organism. Additionally accumulation of nonylphenol and aminocarb was studied. To meet these objectives the following studies were undertaken.

(a) Effects on the whole plant were monitored by measuring frond number, fresh and dry weights and comparing control ((untreated sets) with plants exposed to the pesticide, its adjuvants, and the formulation mix.

(b) Effects at the cellular level were followed by determining total chlorophyll, photosynthesis ($^{14}\text{CO}_2$ uptake), chlorophyll fluorescence, total ATP. Cell membrane permeability changes were monitored by determining changes in conductivity.

(c) An Octanol-water partition coefficient for nonylphenol was obtained and uptake and bioaccumulation of aminocarb and nonylphenol followed.

The validity of Lemna minor as a biological indicator to monitor pollution of aquatic areas will be discussed on the basis of the data obtained.

II MATERIALS AND METHODS

2.1 Chemicals

Aminocarb (p-dimethylamino-m-tolyl N-methyl carbamate), nonyl-phenol, diluent oil 585, Matacil 1.8D OSC (2:5:3 w/w/w aminocarb: nonyl-phenol: diluent oil) were gifts from Mobay Co. Ltd. Mississauga Ont. Stock solution of each were prepared by dissolving the chemicals in glass distilled methanol. The solutions were then stored in a refrigerator (5°C). All solvents used for extraction of the xenobiotics were glass distilled, pesticide grade and purchased from Caledon Laboratories, Toronto. For scintillation counting, Oxiprep, Carbosorb and Permafluor were purchased from Packard Instrument Company Inc. Ill. U.S.A.

2.1.1 Labelled compounds

Sodium bicarbonate¹⁴C, specific activity 7.9 mCi/m mole was purchased in crystalline form from New England Nuclear, Boston, Mass, U.S.A. On delivery it was immediately dissolved in deionised, sterile water (pH 7.2) and stored at 5°C. ¹⁴C ring labelled aminocarb (S.A. 5.01 mCi/m mole) was purchased from New England Nuclear Co. U.S.A.

2.1.2 Luceferin Luciferase

Freeze dried extracts of firefly lanterns were obtained commercially (Sigma Chemical Co., Stock FLE-50) and stored at -20°C until used. Each vial contained the extract from 50mg of firefly lanterns in magnesium arsenate buffer. Each vial was rehydrated with 37.5 mL of deionised distilled water. After standing at room temperature for 1 hour the suspension was filtered (Whatman No. 4) and the filtrate incubated in an ice water bath for 24 hours. (Patterson et al. 1970).

2.1.3 ATP Standards

Adenosine 5' triphosphate (FF-ATP) was obtained from Sigma Chemical Company and stored at -20°C . Each vial contained 1 mg ATP and 40 mg MgSO_4 . To 1 mg of ATP was added 100 mL of deionised distilled water, to give a stock solution of 10 $\mu\text{g}/\text{mL}$ ATP. This stock solution was then stored at -20°C . Before the ATP analysis, standards ranging in concentration between 50-1000 ppb were prepared and kept in an ice bath. (Patterson et al. 1970).

2.2 Plant material and conditions of culture

Lemna minor plants were purchased from Carolina Biological Supplies U.S.A. Prior to culturing, the fronds were sterilized by immersion in 0.5% hypochlorite (30 seconds), followed by three distilled water

washings. Following this, they were transferred to sterilized Hillman M medium (1961b) fortified with 30 μ M EDTA, (Cleland and Briggs. 1967). The pH was adjusted to 5.6. After the hypochlorite treatment the fronds were partially bleached but subsequently healthy daughter fronds were produced. About 30 such healthy fronds were selected and transferred aseptically under a sterile hood to 500 mL Erlenmeyer flasks containing the 200 mL Hillman M medium. The flasks were maintained in a growth chamber (Sherer Controlled Environment Lab Model CEL 255-6), at 25°C \pm 1°C in continuous light provided by cool white fluorescent tubes (10 klux).

2.3 Bioassays

2.3.1 Growth studies: Frond number, wet and dry weights

Three plants of Lemna minor with a total of 10 fronds were selected randomly from a week old culture, and used as the initial inoculum for each test series (Table 1). The growth of the plants was daily determined for seven days by counting the number of fronds. Any degree of frond growth was counted as a whole frond. At the end of seven days wet and dry weights were determined for all clonal cultures. The wet weights were obtained after surface drying the plants on absorbent paper. The fronds were then freeze dried at -50°C for 1 hour, with a Virtis Condenser connected to a Welch Duo Vacuum Pump Model No. 1402 and the dry weights determined on a Mettler H54AR model

balance. All experiments were repeated at least twice at different times.

Table 1. Lists the various concentrations of the compounds used in the study.

Chemical	Final Concentration
Aminocarb	0, 0.10, 0.50, 1.00, 1.50, 2.00 or 2.50 µg/mL
Diluent Oil #585	0, 0.15, 0.75, 1.50, 2.25, 3.00 or 3.75 µg/mL
Nonylphenol	0, 0.25, 1.25, 2.50, 3.75, 5.00 or 6.25 µg/mL
Matacil 1.80 (aminocarb content)	0, 0.10, 0.50, 1.00, 1.50, 2.00 or 2.50 µg/mL

The final concentration of aminocarb in the media was cross-checked by extracting with ethylacetate and quantifying the amount of aminocarb by gas chromatography. (For details see Table 2).

2.3.2 Chlorophyll Content

In parallel experiments 7 day old fronds were analysed for total chlorophyll content. The plants were homogenized with a glass homogeniser in 4 mL of 80% acetone and centrifuged for 5 minutes. Total chlorophyll was determined according to the method of Arnon (1949).

In another set of experiments, Lemna minor plants were treated with Matacil 1.80 (2.5 µg/mL aminocarb). Fronds were harvested at 0, 4, 8, 16, 20, and 24 hours and total chlorophyll content determined as described above.

2.3.3 Photosynthesis $^{14}\text{CO}_2$ incorporation

Lemna minor plants were treated with aminocarb (1 $\mu\text{g}/\text{mL}$) diluent oil #585 (1.5 $\mu\text{g}/\text{mL}$), nonylphenol (2.5 $\mu\text{g}/\text{mL}$) or Matacil 1.80 (1 $\mu\text{g}/\text{mL}$ aminocarb). Control plants were grown in media without added xenobiotic. Each flask was inoculated with thirty fronds (3-4 fronds/plant), randomly selected from a one week old culture. As the experiment was of short duration more fronds were used than for the previous studies to ensure enough plant material for accurate analysis. Plants were harvested at the end of 12 hours and transferred to a 250 mL Erylenmeyer flask each containing 50 mL of HEPES - NaOH buffer (pH 7.2, 20mM). The reaction was started by adding unlabelled and labelled sodium bicarbonate to give a final concentration of 2mM bicarbonate. Plants were harvested every 30 minutes for 3 hours, washed twice with distilled water, to remove adsorbed $\text{NaH}^{14}\text{CO}_3$, lyophilized, weighed and then burnt in a sample oxidicer (Packard Tricarb Model #306). The $^{14}\text{CO}_2$ fixed in the plant was counted for 20 minutes using a liquid scintillation counter (Beckmann L.S. 3133P). Counts were corrected for quench.

2.3.4 Fluorometric analysis

For this study Lemna minor fronds were treated with 0, 2.5, 5, and 6.25 $\mu\text{g}/\text{mL}$ nonylphenol. Each flask was inoculated with 30 fronds randomly selected from a one week old culture. After 12, 24, 48, 72, 96 and 168 hours fluorometry studies were conducted for all treatments

sets. A plant productivity fluorometer Model SF-20 (Richard Branker Research Ltd., Ottawa, Canada) was used to measure the induction of fluorescence in the fronds. Four to six fronds with their roots excised were dark adapted by placing the fronds for 5 minutes on a plastic sheet with a fluorometer probe centered directly on top of them. Following this, the initial (I), peak (P), and terminal (T) readings were immediately recorded. These readings indicate the degree of integrity of the electron flow between PSII and PSI in the light reaction of photosynthesis. The gain was set at 0.8 the exposure time was 50 seconds and the wavelength of light was 670 nm. (Moody 1981).

2.3.5 ATP Assay

Lemna minor plants were treated with aminocarb (1 $\mu\text{g}/\text{mL}$ and 2.5 $\mu\text{g}/\text{mL}$), diluent oil 585 (1.5 $\mu\text{g}/\text{mL}$ and 3.75 $\mu\text{g}/\text{mL}$), nonylphenol (2.5 $\mu\text{g}/\text{mL}$ and 6.25 $\mu\text{g}/\text{mL}$) or Matacil 1.8D (1 $\mu\text{g}/\text{mL}$ and 2.5 $\mu\text{g}/\text{mL}$ of aminocarb). Each flask was inoculated with thirty fronds, (3-4 fronds/plant), randomly selected from a one-week-old culture. Fronds were harvested at the end of 12 hours and 24 hours, frozen in ethanol at dry ice temperatures of -40°C and stored overnight at -20°C . The ethanol was removed with a pipette and the plants were lyophilized to remove residual ethanol, following this, they were exhaustively extracted in Tris buffer at 100°C to a final volume of 5 mL. (Gower 1981). The extract, if not immediately used, was kept frozen.

For the ATP assays 0.5 mL of the extracted samples was mixed with 1.5 mL of firefly extract for 30 seconds and the ATP content was

determined as the measurement of light emission during the oxidation of luciferase (1 minute). A Beckmann L.S. 3133P liquid scintillation counter was used to measure light emission. Gain was set at 52 with an open window of 0-1000. Background light emission from the luciferin-luciferase preparation was measured prior to running standards and samples.

2.3.6 Conductivity

One hundred fronds of Lemna minor were floated in Hillman M media containing 0, 2.5, and 5 $\mu\text{g}/\text{mL}$ nonylphenol. After 12, 24, and 48 hours all plants, treated and control, were harvested and washed thoroughly in deionized distilled water. The plants were then incubated in 10 mL of deionized distilled water and gently shaken during incubation. At 0.5, 1.5, 3.0, and 18 hours the specific conductance of the ambient solution was measured using a SCM 2e conductivity meter. Leakage of electrolytes as measured by changes in electrical conductance was taken as an indicator of changes in cell membrane permeability during the treatment periods.

2.4.1 ^{14}C Aminocarb Uptake

Thirty fronds of L. minor were exposed to 2.64 $\mu\text{g}/\text{mL}$ ring labelled ^{14}C aminocarb (S. a. 5.01 mCi/m mole). Fronds were harvested at 3, 6, 12, 24, 48, 96, 120, and 168 hours, washed thoroughly with deionized distilled water to remove absorbed aminocarb. The fronds were then

freeze dried and the dry weights taken, following which the plants were burned in a Packard sample oxidizer and the ^{14}C recovered and counted on a LSC counter. The ^{14}C aminocarb in the media for each given time period was determined by taking 100 μl aliquots of the media into 10 mL of Scintiverse Scintillation Cocktail in counting vials and the ^{14}C radioactivity measured on a Beckman counter. Quench corrections were made using quench curves derived from external standards.

2.4.2 Nonylphenol uptake

In separate, parallel experiments, a further one hundred fronds of Lemna minor grown in 200 mL of Hillman M media were exposed to 5.0 $\mu\text{g}/\text{mL}$ nonylphenol to determine nonylphenol uptake with time. Plants were harvested after 6, 18, 24, 96 and 168 hours, washed in distilled water, dried with blotting paper and the fresh weights taken. The plants were then placed in 4 mL of methanol and stored at -20°C for 24 hours, following which the plants were homogenised and sonicated on a Model 300 Fisher Sonic Dismembrator. The methanol fraction was transferred to graduated centrifuge tubes and evaporated to a volume of 0.5 mL. A small volume (1-4 μl) of the sample was gas chromatographed on a HP Model 5880 gas chromatograph (Table 2) and the nonylphenol quantified using an authentic standard.

2.5 Persistence of Aminocarb, Nonylphenol and Diluent Oil 585

From an analytical grade (99.3%) aminocarb stock in methanol, an appropriate amount was transferred to 500 mL Erlenmeyer flasks. The methanol was blown off in a stream of nitrogen and 200 mL of Hillman M media (pH 5.6) added to give a final concentration of 1.12 ± 0.20 $\mu\text{g/mL}$ aminocarb (T=0). Three flasks were taken on days 1, 2, 4, and 7. The aminocarb was extracted in ethyl acetate (2 X 100 mL). Flash evaporated to 5 mL and then quantified in a GC model HP 5810 using external standards.

Similarly an appropriate aliquot of nonylphenol was transferred from a stock solution in methanol to 500 mL Erlenmeyer flasks. The methanol was blown off and 200 mL of media added to give a final concentration of 5.07 ± 0.37 $\mu\text{g/mL}$ (T = 0). Three flasks were taken on days 1, 2, 4, and 7. The nonylphenol was extracted in hexane (2 X 200 mL), flash evaporated to 3 mL and the nonylphenol quantified on a GC Model HP 5880 using external standards.

For studies with diluent oil 585, 50 μL of oil in acetone was transferred to 200 mL of Hillman M media to give a final concentration of 24.20 $\mu\text{g/mL}$ (T = 0). Three flasks were taken at 3, 6, 12, 24, 48, 72, and 168 hours. The diluent oil was extracted in hexane (2 X 200 mL) flask evaporated to 1 mL. A pinch of magnesium sulphate anhydrous was added to all extracts to remove residual water. A GC model HP 5880 was used for quantification. External standards were injected after every 3 injections. The GC conditions for aminocarb, nonylphenol and diluent oil are detailed in Table 2.

2.6 Partition Co-efficient: Nonylphenol

Two millilitres of nonylphenol from a standard prepared in octanol was introduced into a separatory funnel containing 250 mL of n-octanol saturated deionized water. The contents were gently shaken (to avoid formation of emulsions), about 100 times. The octanol fraction was then gas chromatographed directly. The nonylphenol in the water fraction was extracted with hexane (2 X 200 mL), flash evaporated to a 2 mL volume and transferred to a 10 mL centrifuge tube. The volume was further reduced to 0.5 mL using a stream of nitrogen. The nonylphenol present was quantified by gas chromatography using external standards.

2.7 Statistical Analysis of Data

Data collected from experiments were subjected to an analysis of variance. Biomedical Computer Program BMDP 2V developed by Department of biomathematics U.C.L.A. and the Statistical Package for Social Sciences from SPSS Incorporated were used for the analysis of variance. Significance was determined by Tukey's test at the 0.05 level. (Tukey 1949).

TABLE 2

Conditions for Gas Chromatography of Aminocarb and Nonylphenol.

	Aminocarb	Nonylphenol
Detector	nitrogen-phosphorous	flame ionization
Oven temperature	170°C	160°C
Injection port temp.	170°C	150°C
Detector temp.	250°C	300°C
Carrier	helium (30 mL/min.)	Nitrogen (30 mL/min.)
Air	(60 mL/min.)	(400 mL/min.)
Hydrogen	(3-5 mL/min.)	(30 mL/min.)
Column	4 feet, 2 mm inside diameter	4 feet, 2mm inside diameter
Column packing	3% SP. 2100 on ultra bond 20 M 80/100 mesh.	6% QF-1 plus 4% SE-30 on Chromosorb, high performance, 80/100 mesh.

Conditions for Gas Chromatography of Diluent Oil 585.

Detector	Flame ionization	
Oven temperature (programmed)	Oven temp. initial value 70°C Oven temp. initial time 3 min. Oven temp. programme rate 10°C Oven temp. final value 150°C Oven temp. final time in 10 min.	
Injection port temp.	150°C	
Detector temp.	300°C	
Gas carrier	Helium	flow (1 mL/min.)
Auxiliary	Helium	flow (30 mL/min.)
Air		flow (400 mL/min.)
Hydrogen		flow (30 mL/min.)
Column	25 meter capillary column methyl silicone cross linked 19091 A #102.	
Split Ratio	1:50	

III RESULTS

3.1 Frond number and 96 hour IG₅₀.

The toxic effects of aminocarb (0.1 µg/mL-2.5 µg/mL), diluent oil (0.15-3.75 µg/mL), nonylphenol (0.25-6.25 µg/mL) or Matacil 1.8D (0.1-2.5 µg/mL aminocarb) were obtained by daily counts of frond number, over a seven day period.

Table 3 lists the group mean of the number of fronds for the different treatments and their respective concentrations. The group mean was calculated by adding the number of fronds observed on each day for seven days. A Tukey's test, (Tukey 1949), performed on the data and assessed at the 0.05 level indicated a variability of response dependent on treatment. Aminocarb and diluent oil at none of the concentrations tested significantly affected frond multiplication. However, nonylphenol at concentrations as low as 1.25 µg/mL and Matacil 1.8D with nonylphenol concentrations of 1.25 µg/mL significantly decreased the rate of frond emergence.

In figures 1 and 2 the effects of treatment on frond number, expressed as percentage of control is plotted over a 7 day period. Increasing concentrations of nonylphenol or Matacil 1.8D were accompanied by a decreasing number of fronds. For nonylphenol, (1.25-5 µg/mL), and Matacil 1.8D, (0.5-2.5 µg/mL aminocarb), a significant reduction in frond number was evidenced after three days of exposure. For example, on the seventh day, frond multiplication was inhibited by 20, 27, 44 and

55% with 1.25, 2.5, 3.75, and 5 $\mu\text{g}/\text{mL}$ nonylphenol respectively. At the highest concentration of nonylphenol, (6.25 $\mu\text{g}/\text{mL}$), and Matacil 1.8D (containing 6.25 $\mu\text{g}/\text{mL}$ nonylphenol), a significant depression in frond emergence was noted after one day of treatment, and a 76% inhibition recorded on the seventh day.

Table 3. 7 day Post-treatment Group Mean of Number of Fronds Following Treatment with Aminocarb, Diluent Oil, Nonylphenol and Matacil 1:80.

	Treatment	Concentration µg/mL	Group mean Number of Fronds
A	Control	0	489a
	Aminocarb	2.5	488a
	Diluent oil 585	3.75	493a
B	Control	0	447a
	Nonylphenol	0.25	468a
		1.25	384b
		2.50	364b
		3.75	283c
		5.00	244d
		6.25	164e
	Matacil 1.80 (Concentration of nonylphenol)	0.25	442a
		1.25	355b
		2.50	340b
3.75		271c	
	5.00	240d	
	6.25	139e	

Values followed by the same letter indicate no significant difference at the 0.05 level (Tukey's test).

Figure 1 1-7 days: Effect of Nonylphenol (0.25-6.25 $\mu\text{g}/\text{mL}$) on Frond Number (% of Control \pm 1 SD)

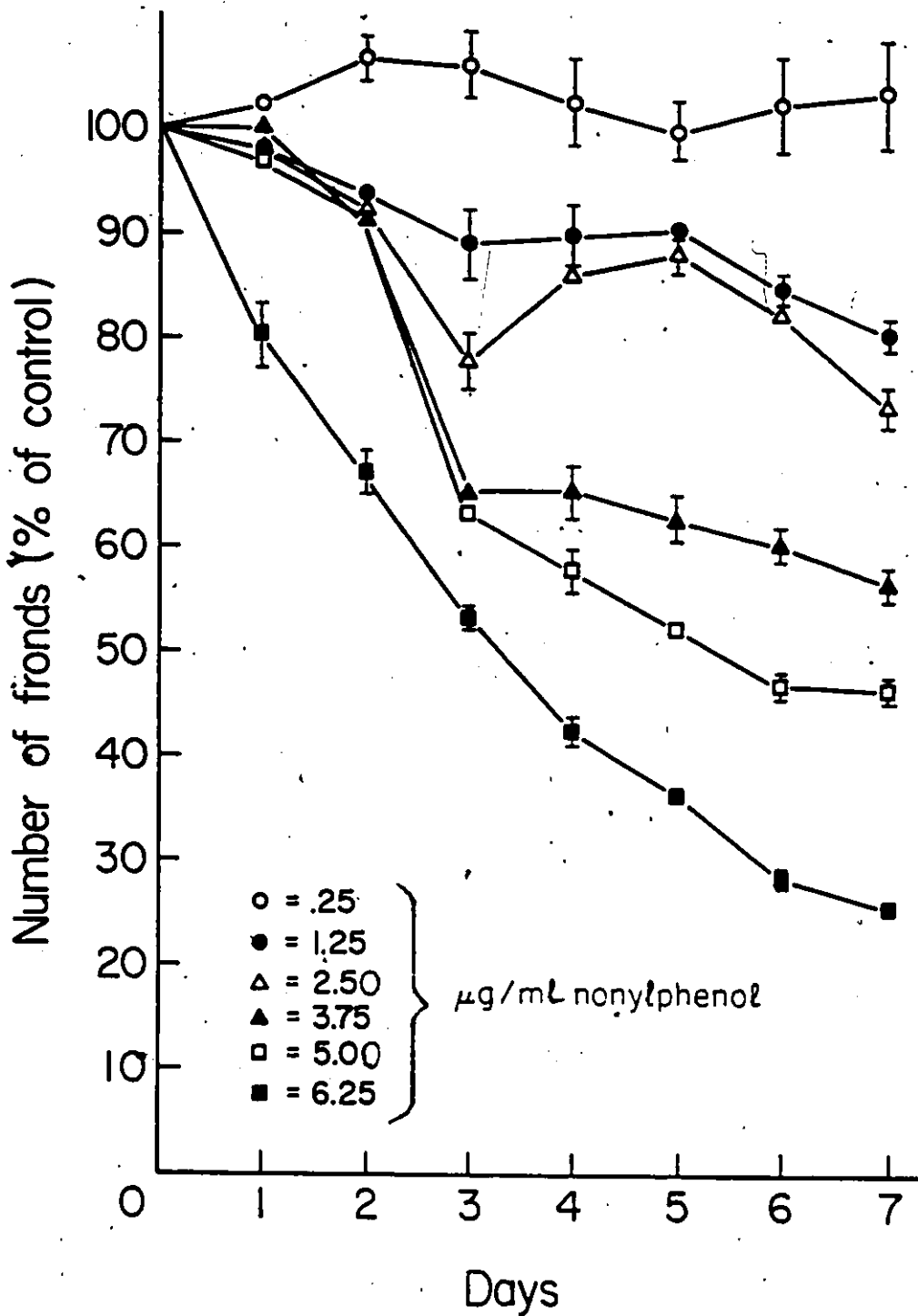




Figure 2 1-7 days: Effect of Matacil 1.8D (0.1-2.5 $\mu\text{g}/\text{mL}$ aminocarb) on Frond Number (% of Control \pm 1 SD)

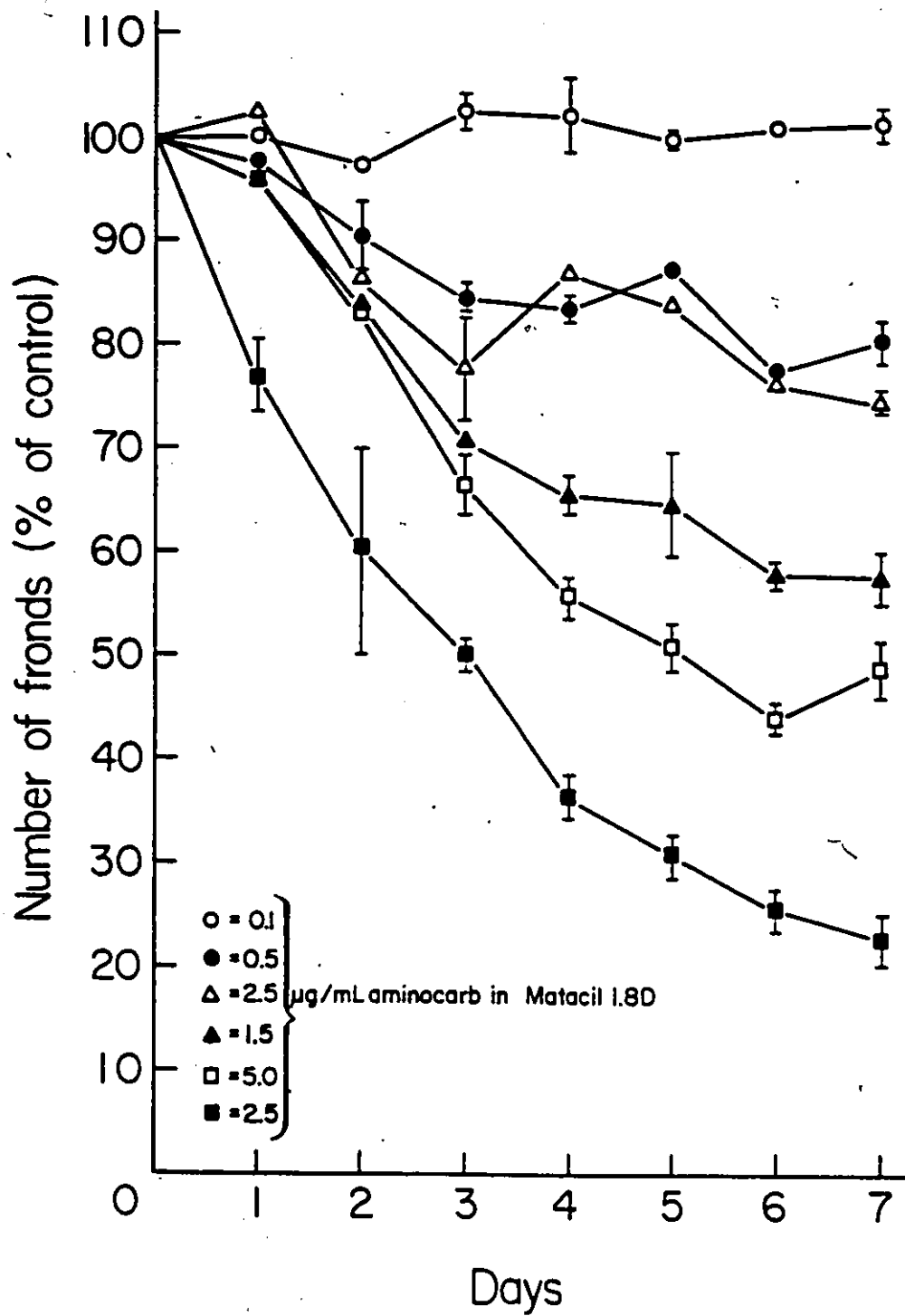


Figure 3(a) and 3(b) demonstrates that on the fourth day there is an apparent inverse relationship between the number of fronds produced and the concentration of xenobiotic to which they were exposed.

The straight line of best-fit was drawn through the points of concentration of nonylphenol, and Matacil 1.8D and the number of fronds (% of control) obtained. Correlation co-efficients of 0.98 and 0.96 respectively were obtained from the regression. A 96 hour IG₅₀ (inhibition of growth) traditionally used to measure short term toxicity was found to be 5.5 µg/mL both for nonylphenol and Matacil 1.8D (2.0 µg/mL aminocarb) containing 5 µg/mL nonylphenol.

3.2 Visual Observations.

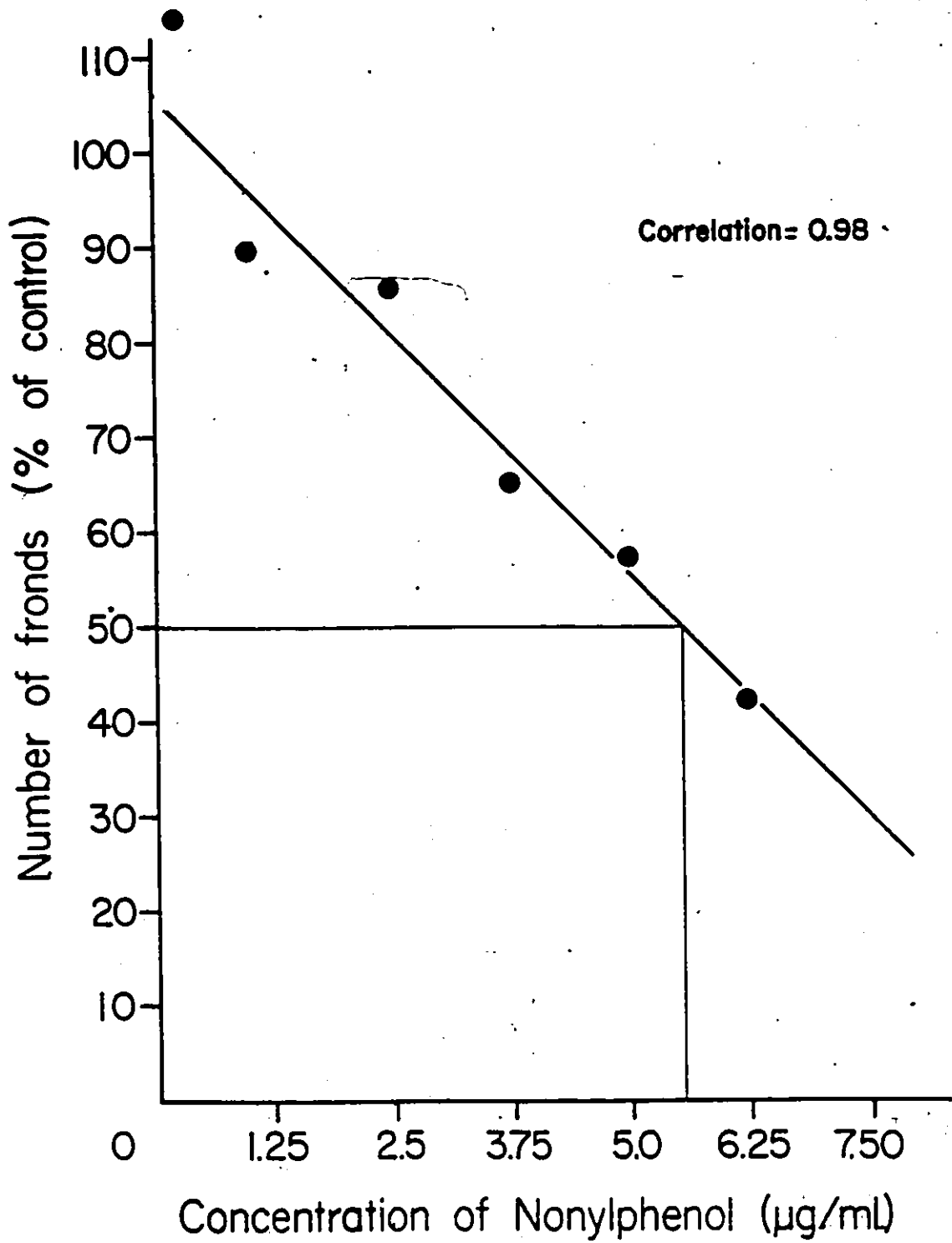
Monitoring of L. minor plants treated with aminocarb or diluent oil indicated that no visual change occurred in frond color or root growth, over the concentration range tested. However, plants exposed to nonylphenol concentrations of 2.50, 3.75 or 5.00 µg/mL showed partial bleaching of some fronds after one day of treatment although daughter fronds continued to be formed. Even at the highest concentration of nonylphenol, (6.25 µg/mL), which caused the initial 10 fronds to turn completely white, daughter fronds emerged on mother fronds. Some of the daughter fronds were partially bleached (Fig. 4). At concentrations above 3.75 µg/mL nonylphenol, detached roots were observed. Comparable results were obtained when fronds of L. minor were treated with Matacil 1.8D in the concentration range of 0.5-2.5 µg/mL aminocarb. For example at 6.25 µg/mL nonylphenol in the Matacil 1.8D formulation (2.5 µg/mL

aminocarb), the initial 10 fronds were completely bleached. Partially bleached daughter fronds were produced over time. The roots of these fronds easily detached (Fig. 5).

3.3 Fresh and Dry Weights.

To further delineate the effects of aminocarb, diluent oil, nonylphenol and Matacil 1.8D on L. minor, fresh and dry weights were measured after seven days of treatment. In the case of aminocarb and diluent oil no significant decrease in fresh and dry weights were observed at the highest concentrations used. (2.5 µg/mL aminocarb, 3.75 µg/mL diluent oil, Fig. 6a and 6b). Cultures exposed to nonylphenol and Matacil 1.8D in the concentration range of 2.5-6.25 µg/mL nonylphenol content exhibited a significant reduction in both fresh and dry weights. A linear relationship was obtained when concentration of pollutant was plotted against fresh and dry weights. (Fig. 7a and 7b). Nonylphenol significantly reduced fresh and dry weights in L. minor fronds above a concentration of 1.25 µg/mL and 2.5 µg/mL respectively after seven days of treatment. At the highest concentration of 6.25 µg/mL the fresh weight was reduced by approximately 50% and the dry weight by 45%. Comparable results were obtained with the formulation Matacil 1.8D where the nonylphenol content reached this concentration.

Figure 3a and 3b. Comparative Determination of 96 hour IG_{50} of Nonylphenol and Matacil 1.80 respectively.



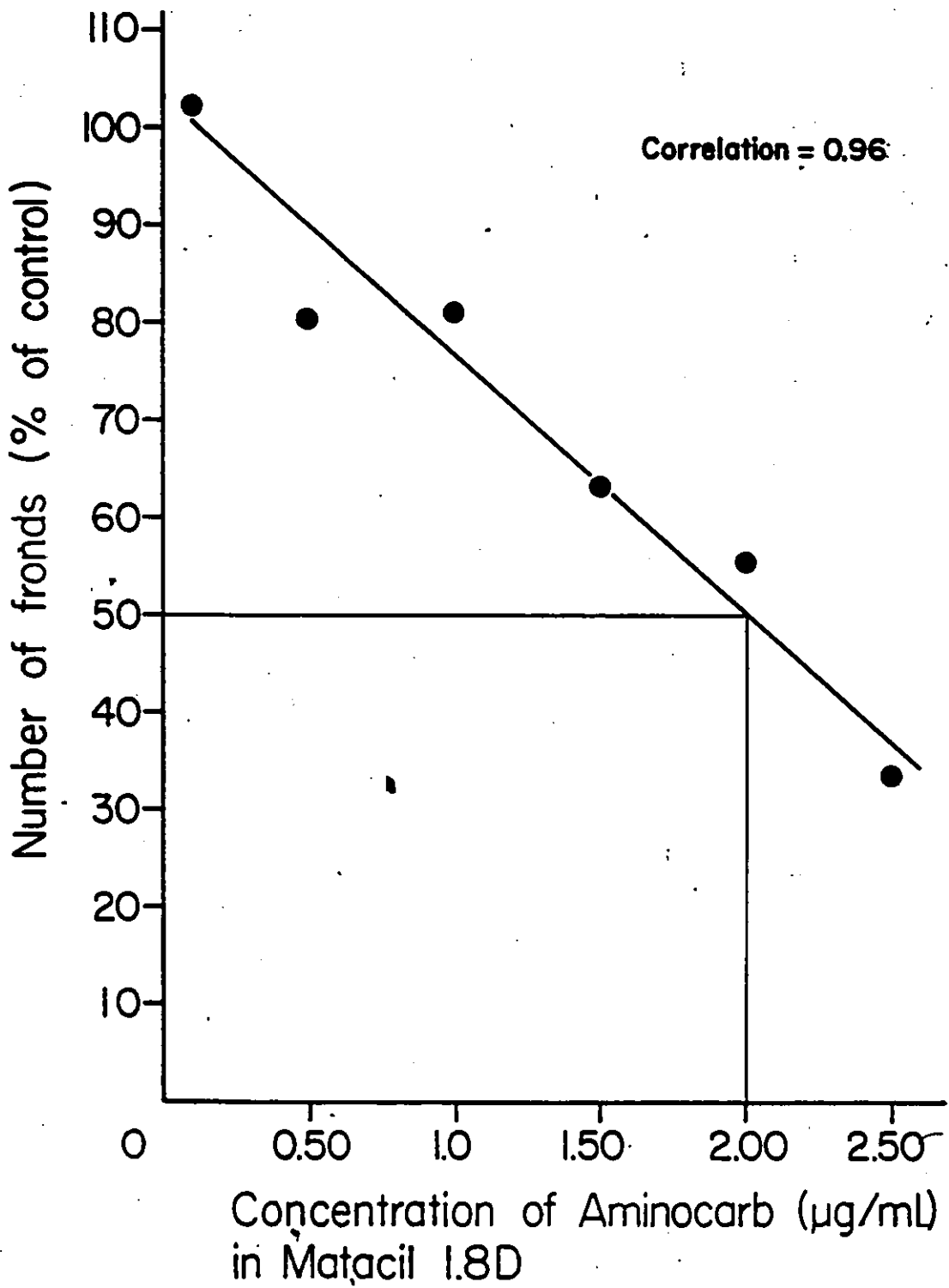
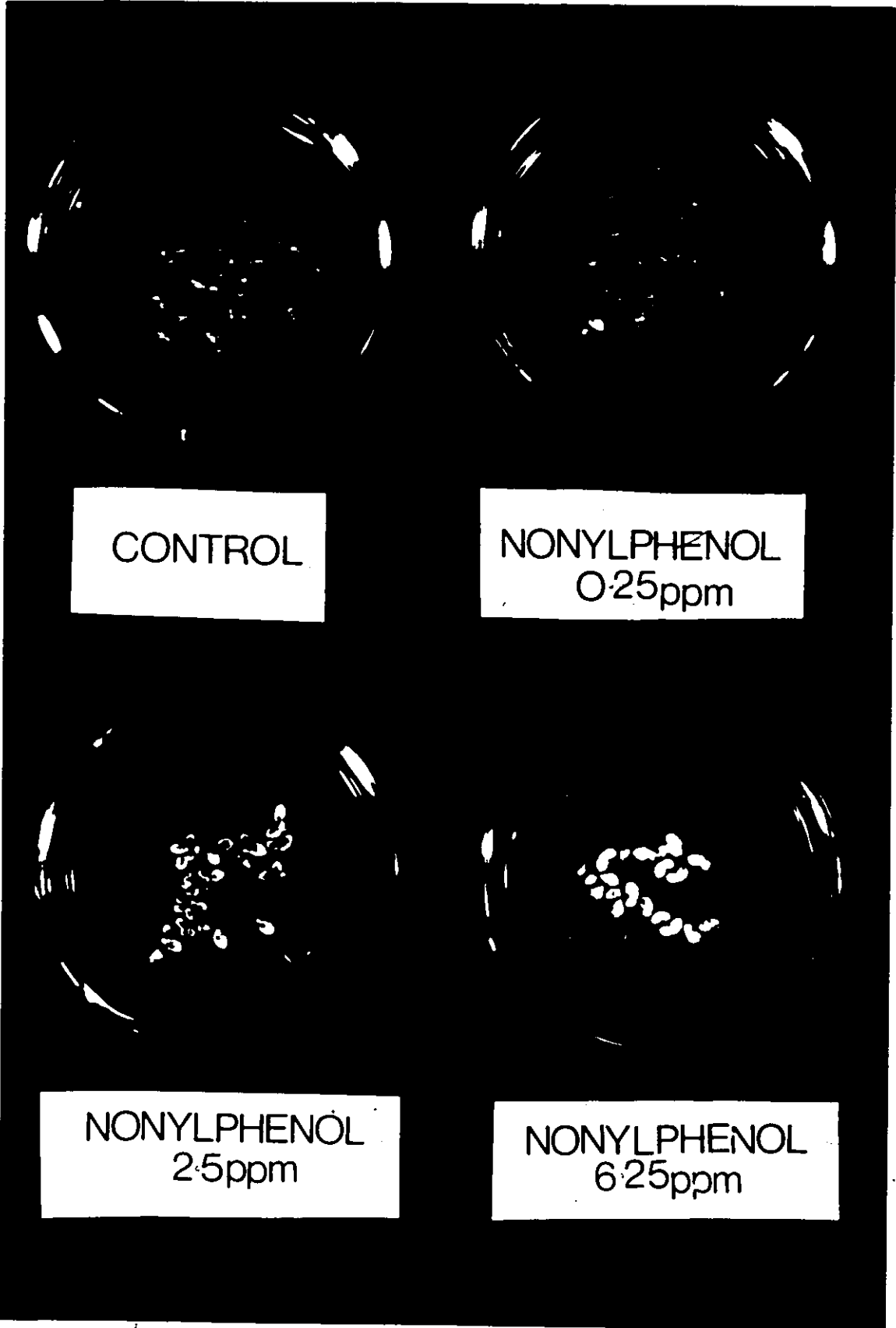


Figure 4 1 day Post treatment. Plants of Lemna minor Exposed to Nonylphenol Concentrations of 0, (Control), 0.25, 2.5 and 6.25 $\mu\text{g}/\text{mL}$ (ppm).



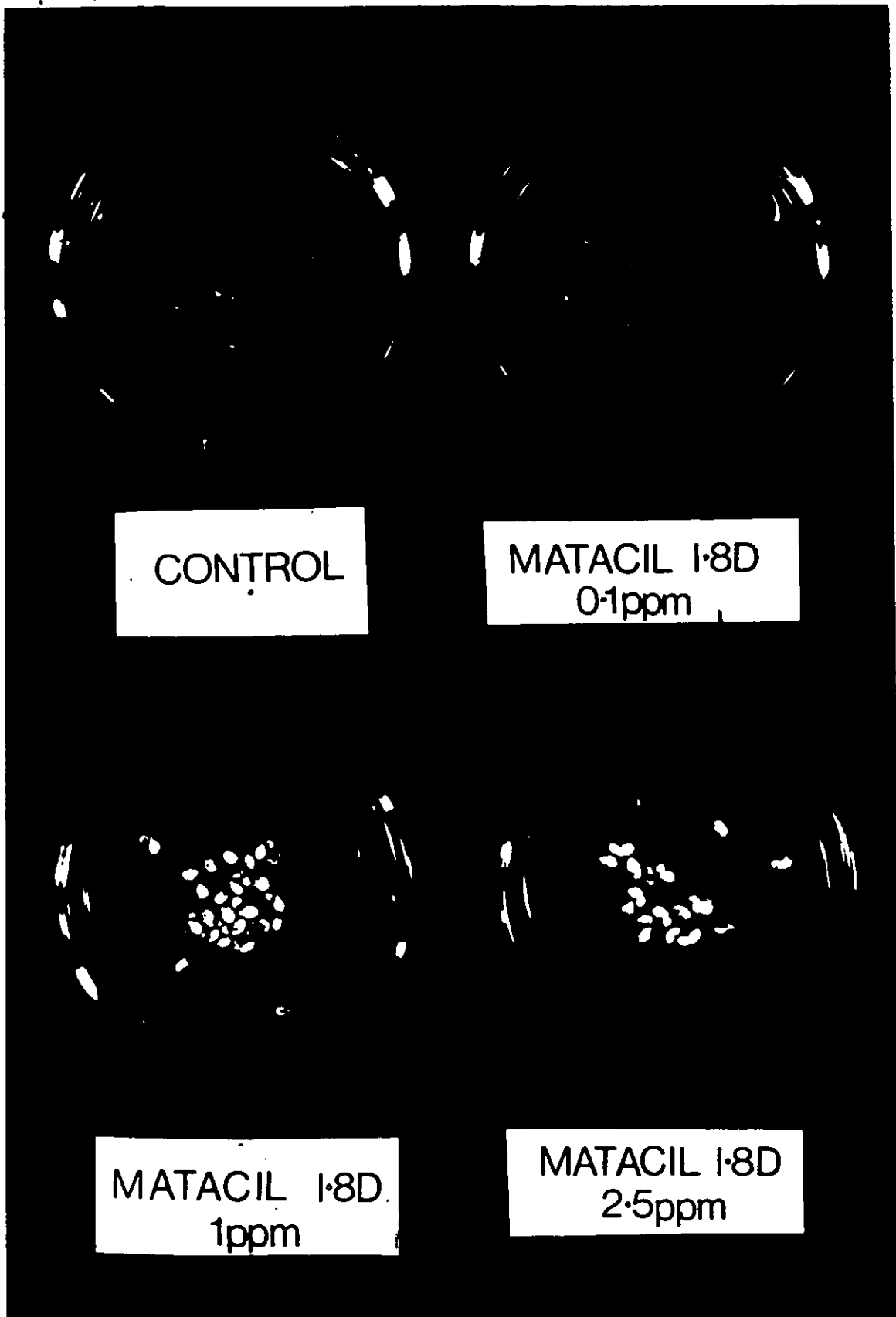
CONTROL

NONYLPHENOL
0.25ppm

NONYLPHENOL
2.5ppm

NONYLPHENOL
6.25ppm

Figure 5. 1 day Post treatment. Plants of L. minor Exposed to Matacil 1.8D Concentration of 0 (Control), 0.1, 1.0 and 2.5 $\mu\text{g}/\text{mL}$ of aminocarb in the formulation mix.



CONTROL

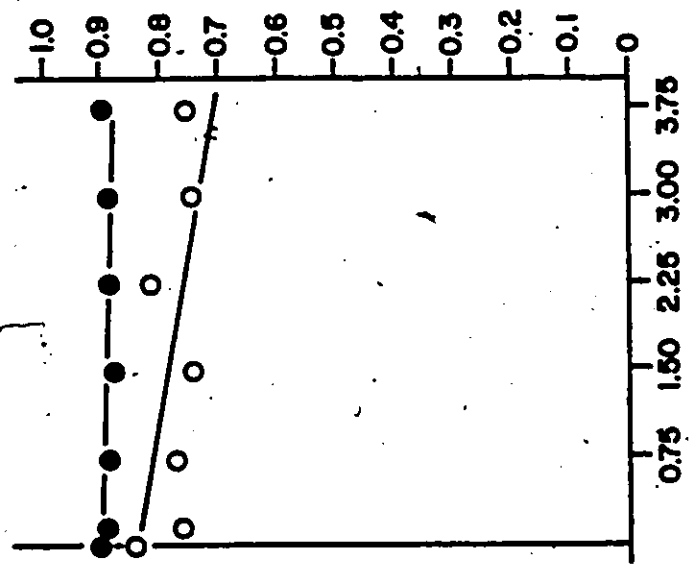
MATACIL 1:8D
0.1ppm

MATACIL 1:8D
1ppm

MATACIL 1:8D
2.5ppm

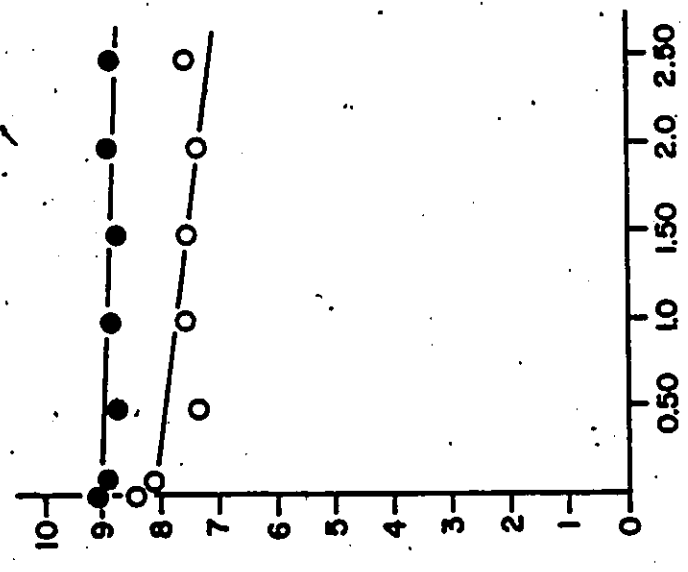
Figure 6a and 6b. .7 day Post treatment: Fresh (●) and Dry (○) weights of L. minor Treated with Aminocarb (0-2.5 µg/mL) and Diluent Oil (0-3.75 µg/mL).

Dry weight / 10 fronds (mgs)



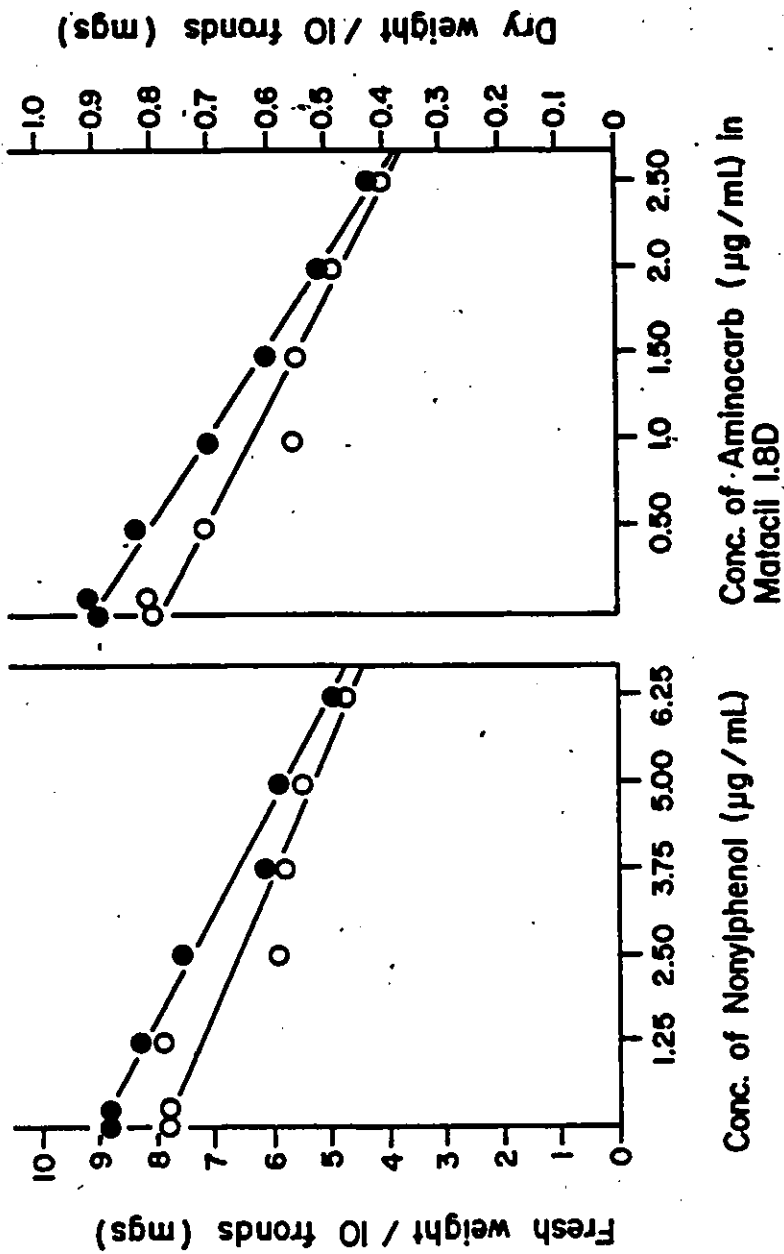
Conc. of diluent oil (µg/mL)

Fresh weight / 10 fronds (mgs)



Conc. of Aminocarb (µg/mL)

Figure 7a and 7b. 7 day Post treatment: Fresh (●) and Dry (○)
Weights of L. minor Treated with Nonylphenol (0-6.25 µg/mL) and Matabil
-1.60 (0-2.5 µg/mL aminocarb content).



3.4 Total Chlorophyll Content

The total chlorophyll content measured on the seventh day of growth indicated that aminocarb and diluent oil did not significantly inhibit total chlorophyll content in the concentration range of 0.1-2.5 µg/mL aminocarb or 0.15-3.75 µg/mL diluent oil ($P < 0.05$). However, for the xenobiotics nonylphenol and Matacil 1.80, the total chlorophyll content was a sensitive measure of toxicity and lead to an apparent linear relationship (Fig. 8). A concentration of nonylphenol of 1.25 µg/mL significantly depressed total chlorophyll content. A 50% reduction was obtained when fronds of L. minor were exposed to nonylphenol between 2.50 and 3.75 µg/mL. Similar results were obtained for Matacil 1.80 containing 1.25-6.25 µg/mL nonylphenol.

In a parallel experiment when the acute toxicity of Matacil 1.80 containing 2.5 µg/mL aminocarb, 3.75 µg/mL diluent oil and 6.25 µg/mL nonylphenol was examined, 50% of the damage was notable within approximately 18 hours post treatment (Fig. 9), and the damage could be totally ascribed to the nonylphenol in the 1.80 formulation.

Figure 8 Chlorophyll Content (% of Control) 7 days Post treatment, Effects of a Range of Concentrations of Aminocarb, Nonylphenol, Diluent oil and Matacil 1.8D. Control = 0.77 ± 0.07 mg/g fresh weight.

- (1) Concentration of Aminocarb, or Aminocarb in Matacil 1.8D.
- (2) Concentration of Nonylphenol.
- (3) Concentration of Diluent oil 585.

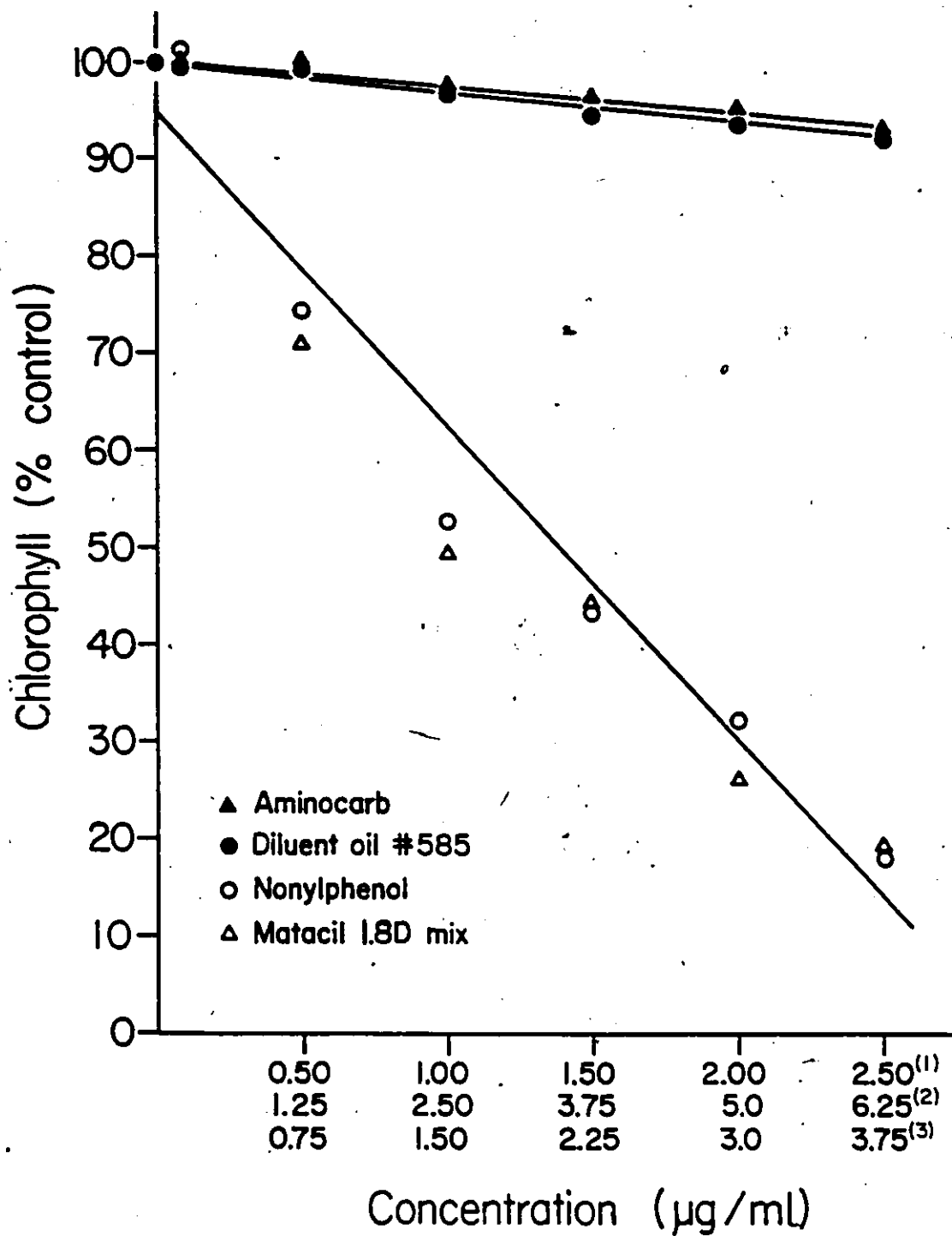
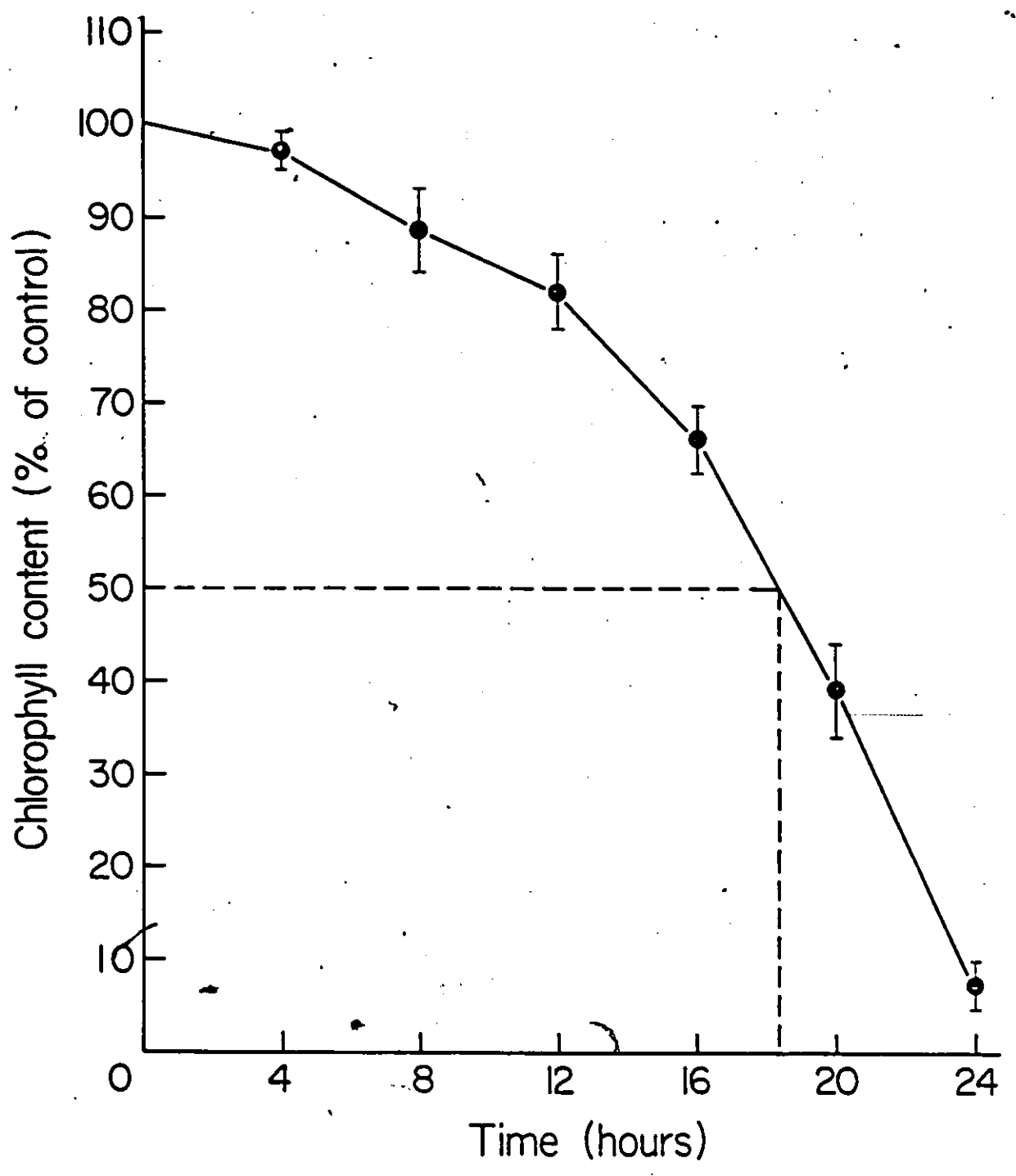


Figure 9. Chlorophyll Content (% control) 1 day Post treatment:
Effects of Matacil 1.8D mix. (2.5, 3.75 and 6.25 $\mu\text{g}/\text{mL}$ of aminocarb,
diluent oil and nonylphenol respectively). Control = 0.78 ± 0.06 mg/g
fresh weight.



3.5 Photosynthesis

In order to test whether the pollutants had an affect on photosynthesis, fronds exposed to the xenobiotics were transferred to HEPES NaOH buffer containing radio-labelled $\text{NaH}^{14}\text{CO}_3$ and the $^{14}\text{CO}_2$ assimilated was monitored in treated and control sets. Fronds exposed for 12 hours to aminocarb (1 $\mu\text{g}/\text{mL}$) or diluent oil (1.5 $\mu\text{g}/\text{mL}$), fixed approximately the same amount of $^{14}\text{CO}_2$ as the control plants over a three hour test period. In contrast, $^{14}\text{CO}_2$ fixation in L. minor fronds was severely affected by exposure to a concentration of 2.5 $\mu\text{g}/\text{mL}$ nonylphenol presented alone or as a constituent of the Matacil 1.8D mix. In these treatments sets, $^{14}\text{CO}_2$ fixation was reduced by 45% almost constantly over 3 hours as compared to untreated sets (Fig. 10).

3.6 Fluorometry

The inhibition of photosynthetic activity was further studied by measuring chlorophyll fluorescence indicated by P-I transients. Fronds exposed to 2.5 $\mu\text{g}/\text{mL}$ nonylphenol showed a 21% and 32% reduction of fluorescent activity after 12 and 24 hours respectively. Acclimation to this concentration of nonylphenol was later observed and after six days complete recovery was noted. Treatment with 5.0 $\mu\text{g}/\text{mL}$ nonylphenol was sufficient to reduce the P-I transient by 60% and 80% after 12 and 24 hours respectively. Recovery to control levels were observed on day 7. At the highest concentration of nonylphenol (6.25 $\mu\text{g}/\text{mL}$) a 99%

Figure 10. $^{14}\text{CO}_2$ Fixation 12 hours Post Treatment. Effects of Aminocarb (1.00 $\mu\text{g}/\text{mL}$), Diluent oil (1.50 $\mu\text{g}/\text{mL}$), Nonylphenol (2.50 $\mu\text{g}/\text{mL}$) and Matacil 1.80 (1.00 $\mu\text{g}/\text{mL}$ aminocarb content).

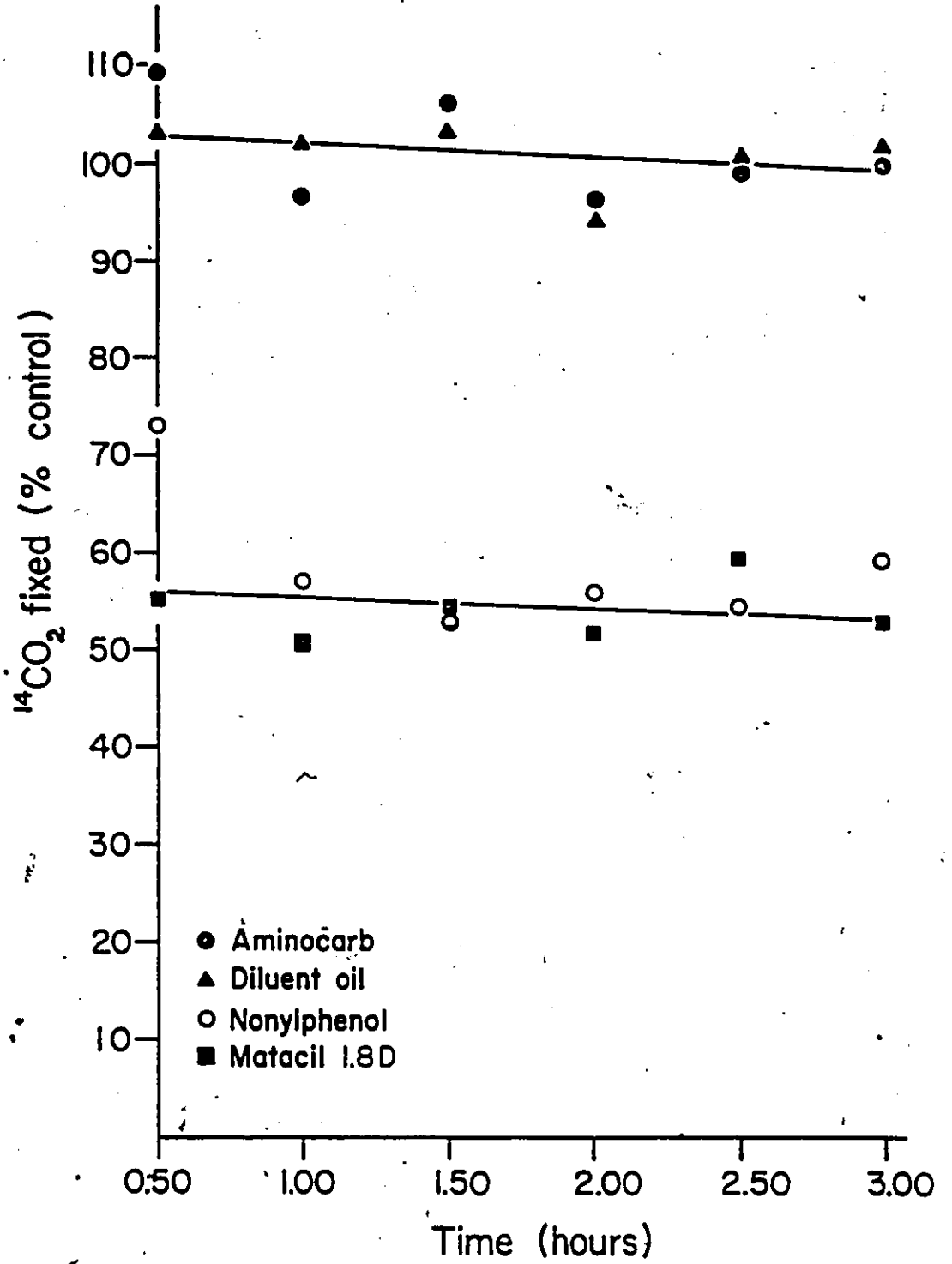
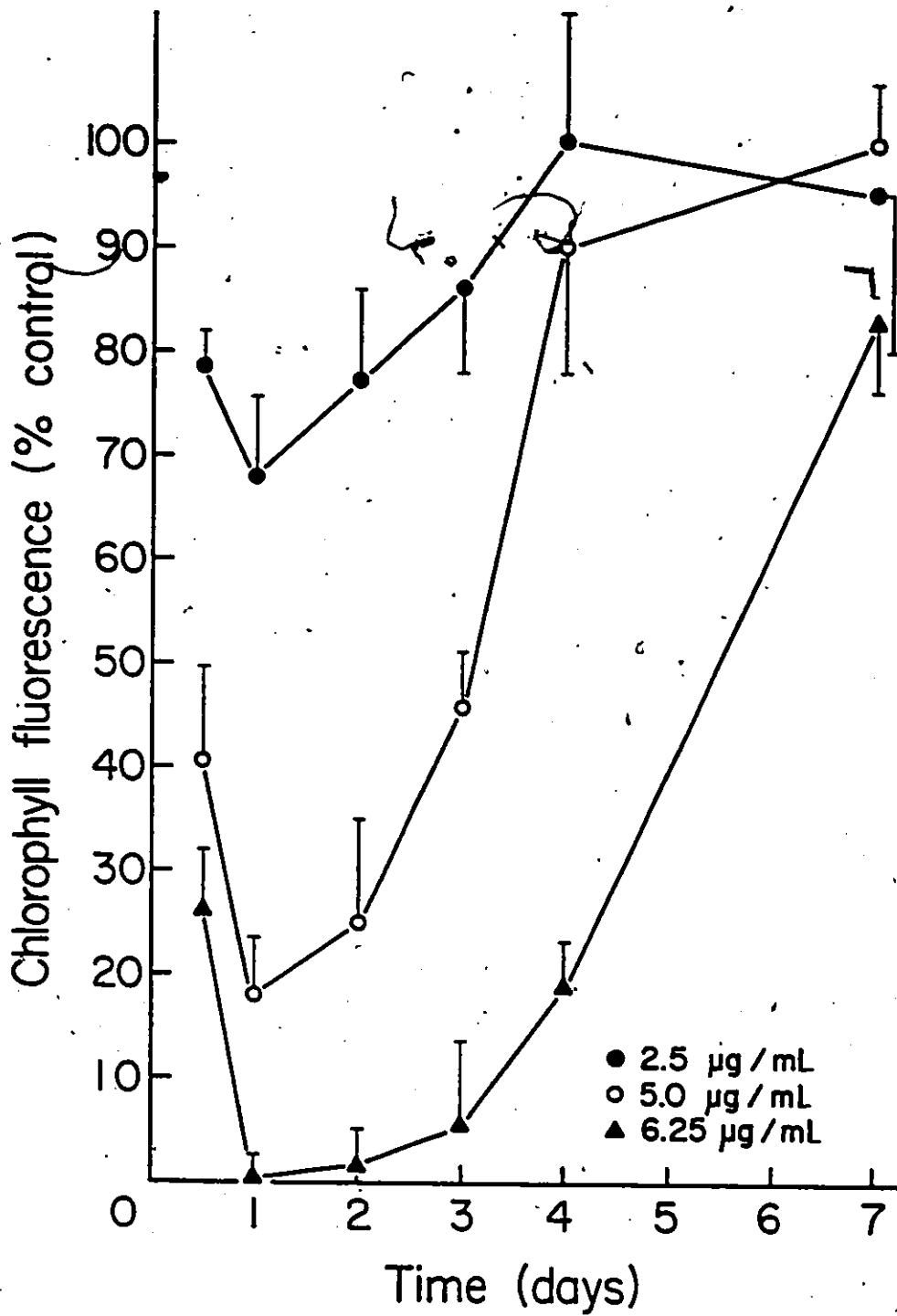


Figure 11. Effects of Nonylphenol (2.5, 5.00 and 6.25 $\mu\text{g}/\text{mL}$) on Chlorophyll Fluorescence Expressed as P.T. transient (% of Control).



inhibition of chlorophyll fluorescence was evidenced after 24 hours. This was not unexpected as the fronds were completely bleached. However, a gradual recovery was evidenced and by the seventh day only 17% inhibition was obtained. (Fig. 11).

3.7 Conductivity Measurements.

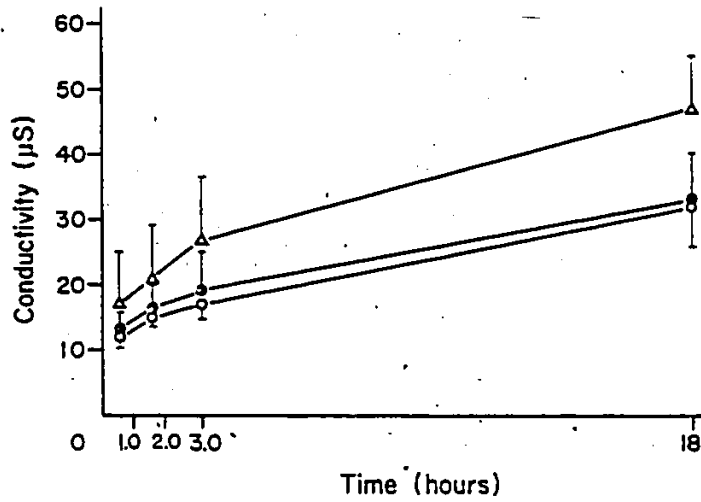
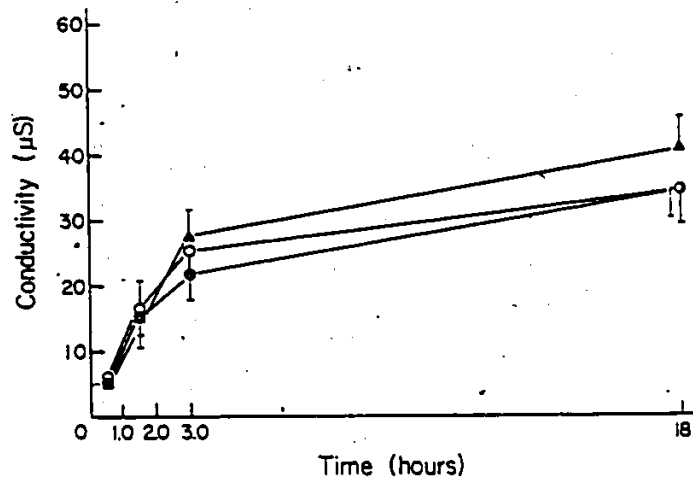
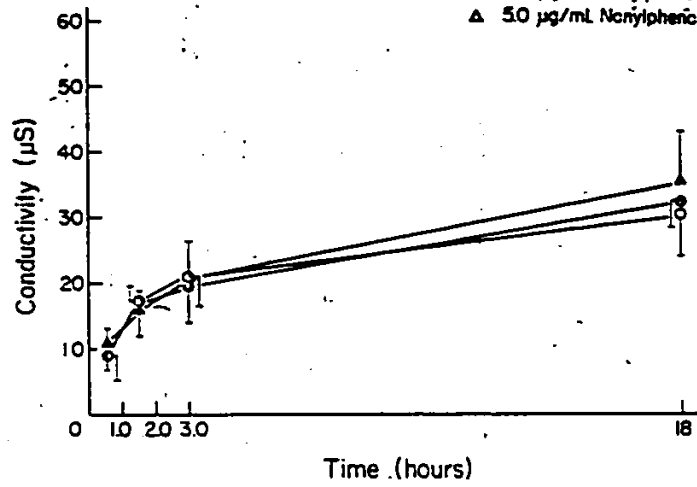
Leakage of solutes from the fronds into the ambient solution was followed for 18 hours to determine the effects of nonylphenol on cell membrane integrity. Changes in electrical conductivity of the solutions were taken as an indicator of leakage of electrolytes from the fronds. The values obtained from treated sets were compared with controls. The values indicated for 12 and 24 hours were very similar to those of controls (Fig. 12a and 12b). By contrast, there was an increasing trend in the conductivity of the media when the plants had been treated with 5 µg/mL nonylphenol for 48 hours (Fig. 12c).

3.8 ATP (Adenosine Triphosphate)

The total ATP content in the fronds was determined on control and treated sets by the well established luciferin-luciferase method. Table 4 lists the ATP content as % of control for the different treatments following 12 and 24 hours of exposure. After 12 or 24 hours neither aminocarb nor diluent oil reduced ATP levels significantly. However, treatment sets of L. minor exposed for 12 hours to nonylphenol (6.25 µg/mL) or Matacil 1.80 containing 6.25 µg/mL nonylphenol suffered a

Figure 12a, b and c. Conductivity Measurements of the Media
Bathing Lemna minor fronds. Effect of Treatment with 2.50, and 5.00
 $\mu\text{g/ml}$ Nonylphenol.

- Control
- 2.5 µg/mL Nonylphenol
- △ 5.0 µg/mL Nonylphenol



significant reduction. After 24 hours of treatment a 20% and 75% reduction in total ATP levels was indicated following exposure to 2.5 and 6.25 $\mu\text{g}/\text{mL}$ nonylphenol respectively. These reductions were obtained whether the nonylphenol was presented alone or as a part of the Matacil 1.80 formulation. Nonylphenol and Matacil 1.80 did not alter the luminescence of ATP standards at the concentrations used.

3.9 ^{14}C Aminocarb Uptake.

L. minor sequestered a maximum of 7.5% of the total ^{14}C activity from a 2.64 $\mu\text{g}/\text{mL}$ aminocarb solution reaching a peak concentration of 557 $\mu\text{g}/\text{mL}$ aminocarb equivalents at 96 hours. Following this, a gradual decline to 397 $\mu\text{g}/\text{g}$ at 7 days was obtained. This apparent dilution in the concentration of aminocarb in the plant was probably due to an increase in weight of plant tissue as the actual amount of aminocarb uptake by the plant reached a maximum by the seventh day. (Table 5).

The bioaccumulation ratio of aminocarb in L. minor fronds against time is shown in Figure 13. Bioaccumulation ratios are defined as the concentration of aminocarb in the plant divided by the concentration of the pesticide in the surrounding water for a given time period. The bioaccumulation gradually increased over time and an accumulation maximum of 227 was noted at 96 hours, followed by a decline to 170 on the seventh day. A mass balance was calculated and indicated a loss of aminocarb in the range of 0.75-4% in the course of the study.

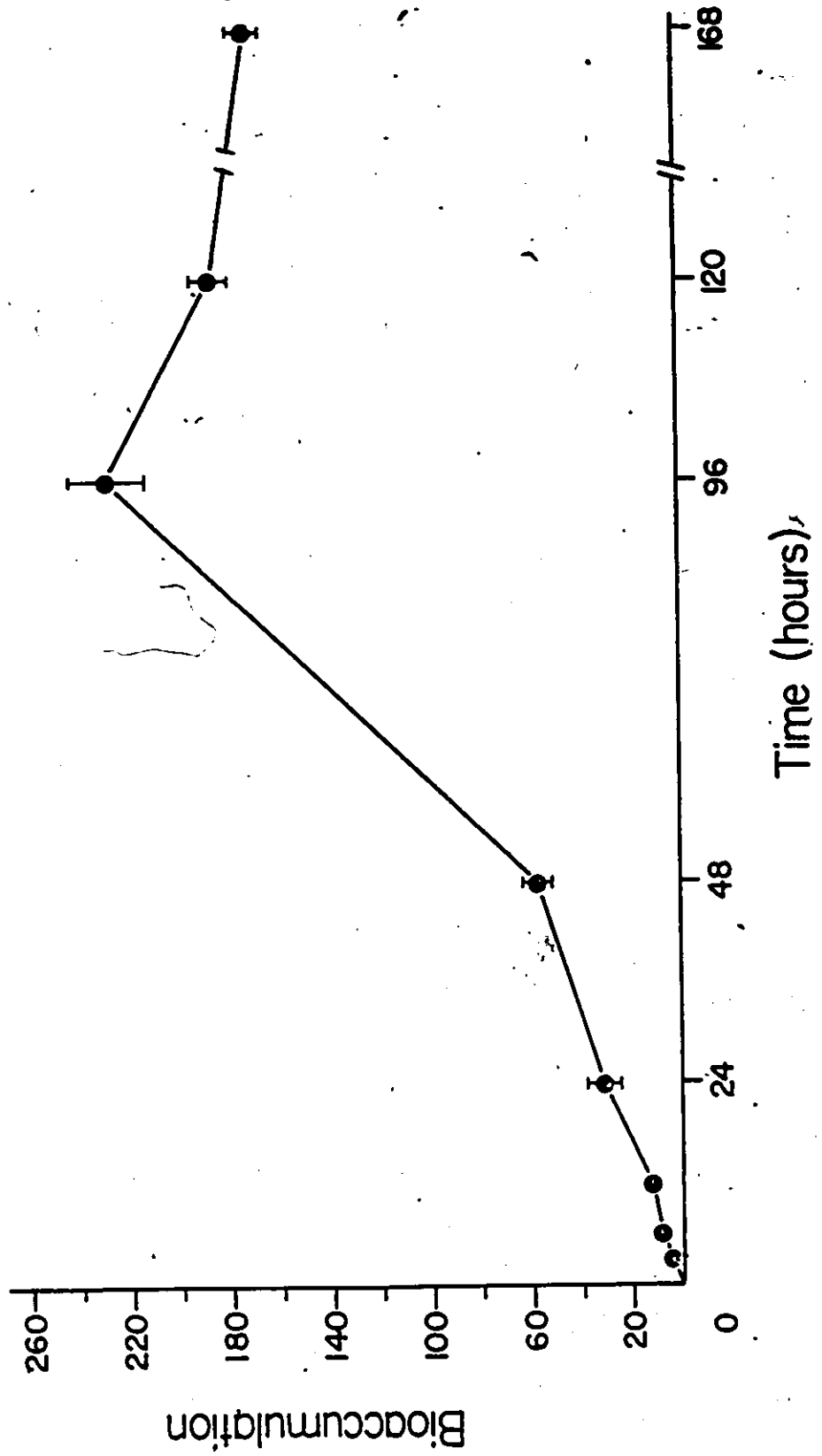
Table 4. Effect of Aminocarb, Diluent oil, Nonylphenol and Matacil 1.80 on ATP levels. The values as % of control after 12 and 24 hours post treatment are indicated together with their respective standard deviation. (Control at 12 hours = 1538 ± 209 $\mu\text{g/g}$ dry weight at 24 hours, 1122 ± 32 $\mu\text{g/g}$ dry weight).

Treatment	Concentration of xenobiotic $\mu\text{g/ml}$	ATP % of Control	
		12 hours	24 hours
Aminocarb	1.00 - 2.50	103.7 ± 8.5	104.2 ± 7.7
Diluent oil	1.50 - 3.75	92.2 ± 6.4	108.0 ± 7.4
Nonylphenol	2.50	90.3 ± 3.0	80.7 ± 10.8
	6.25	78.5 ± 5.5	25.3 ± 3.0
Matacil 1.80	1.00 2.50 1.50	83.7 ± 9.6	81.7 ± 2.3
	2.50 6.25 3.75	80.3 ± 6.0	24.3 ± 4.9

Table 5 Uptake of Aminocarb by Lemna minor 1-7 days Post Treatment

Time in (Hours)	Dry Weight (mg)	Total aminocarb in plant (µg)	Conc of aminocarb in plant (µg/gm dw)	Conc in media (µg/ml)
3	2.91	0.03	11.6 ± 0.5	2.60
6	2.90	0.05	18.6 ± 1	2.56
12	2.80	0.09	30.2 ± 0.4	2.55
24	4.60	0.35	76.3 ± 13	2.51
48	5.50	0.79	143.5 ± 9	2.46
96	15.40	8.5	557.0 ± 33	2.45
120	20.70	9.2	445.2 ± 13	2.40
168	25.20	9.9	396.9 ± 13	2.30

Figure 13. Accumulation of Aminocarb in L. minor plants Exposed to 2.60 $\mu\text{g}/\text{mL}$, 1-7 days Post Treatment.



3.10 Accumulation of Nonylphenol

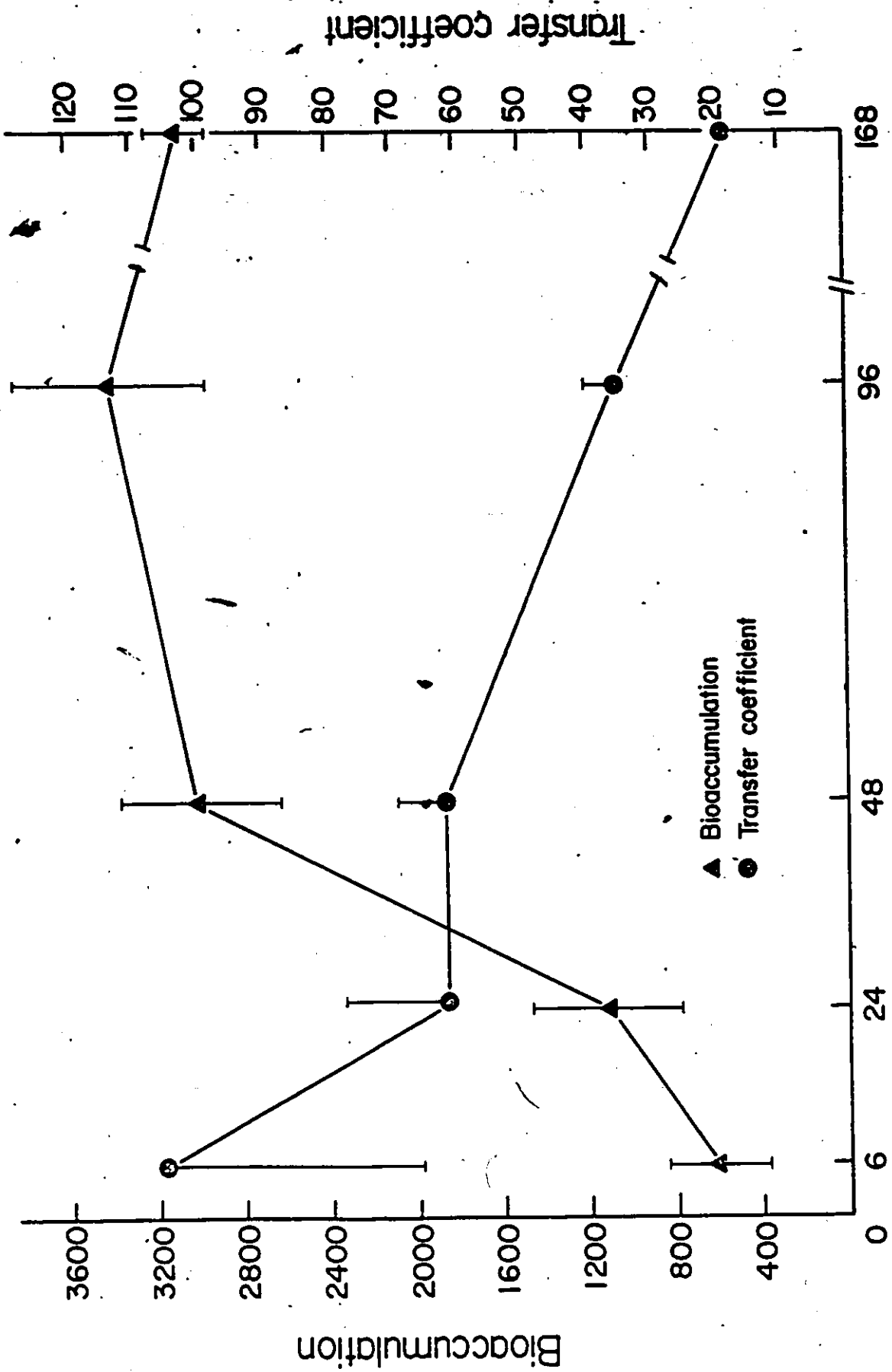
The uptake of nonylphenol from the media was determined over a seven day period following exposure of L. minor to 5 µg/mL nonylphenol. Initially, a gradual increase in the nonylphenol concentration from 3050 µg/g at 6 hours to 5240 µg/g at 24 hours was observed. A maximum concentration of 14,380 µg/g nonylphenol at 96 hours suggested that 15.4% of the total nonylphenol present in the media was taken up by the plants. Following this, after seven days, there was a slight decrease in the concentration to 12,960 µg/g of nonylphenol in the fronds (Table 6).

The bioaccumulation ratio of nonylphenol as a function of time is shown in figure 14. For the bioaccumulation calculations the amount of nonylphenol in the media was not experimentally determined but obtained theoretically by the difference of the total amount of nonylphenol initially present, to the amount in the plant at a given time. This was done as poor phase separation made it impossible to quantify the actual amount of nonylphenol. Bioaccumulation ratios increased with time initially, but after 48 hours, remained almost steady. The ratio ranged from 630 at 6 hours to 3,080 at 7 days. The transfer co-efficient (TC) is defined as the amount of water (g or ml) cleared of its nonylphenol content by one gram (dry weight) of L. minor in one hour. The TC was observed to be 100 at 6 hours and 18 at 7 days. This indicated a rapid uptake of nonylphenol between 0-6 hours followed by a slower intake of nonylphenol into the plant tissue.

Table 6 Uptake of Nonylphenol by Lemna minor 1-7 days Post Treatment

Time in (Hours)	Dry Weight (mg)	Total nonylphenol in plant (µg)	Conc of nonylphenol in plant (µg/gm dw)	Conc in media (µg/mL)
6	10.1 ± 2	32 ± 17	3050 ± 1140	4.8
24	9.7 ± 4	51 ± 19	5240 ± 1770	4.7
48	10.2 ± 5	133 ± 19	12990 ± 1280	4.3
96	10.7 ± 6	154 ± 17	14380 ± 1680	4.2
168	12.3 ± 4	160 ± 10	12960 ± 380	4.2

Figure 14. Accumulation of Nonylphenol in L. minor Plants Exposed to 5 µg/mL 1-7 days Post Treatment.



Time (hours)

Bioaccumulation

Transfer coefficient

▲ Bioaccumulation
● Transfer coefficient

3.11 Persistence of Aminocarb, Nonylphenol and Diluent oil in the

Media:

Table 7 shows the amounts of aminocarb, nonylphenol or diluent oil present in Hillman M. media (pH 5.6, 25°C), over a 0-7 day post injection. The concentrations were determined by gas chromatography. Aminocarb appeared to be fairly stable in the medium. A 32% loss was observed on the seventh day indicating a $t_{1/2}$ of > 7 days in the medium. No derivatives were detected.

Nonylphenol losses from the media were 11% at day 2 and 56% at day 7. The $t_{1/2}$ was roughly between 5-6 days. No transformed product were detected.

The diluent oil rapidly volitalised from the medium. A 36% loss was observed after 24 hours with non detectable concentrations reached after 72 hours. The $t_{1/2}$ was approximately between 24 and 36 hours indicating a relatively short persistence time..

Table 7. Persistence of Aminocarb, Nonylphenol and Diluent Oil in Hillman M media (pH. 5.6) over a Seven Day Period.

Time in days	0	1	2	4	7					
Concentration of Aminocarb in media (µg/mL)	1.1	1.0	0.9	0.8	0.8					
Concentration of Nonylphenol in media (µg/mL)	5.0	4.8	4.5	3.1	2.3					
Time in hours	0	3	6	12	18	24	36	48	72	168
Concentration of Diluent oil in media (µg/mL)	24.2	22.6	21.8	20.1	18.3	15.4	5.9	Traces	ND	ND

ND Not detectable.

DISCUSSION

To be commercially effective and environmentally safe modern pesticides require a high degree of specificity so that the pesticide will affect only specific organ systems in the target species. In reality, no synthetic pesticide approaches this specificity and consequently a variety of effects may be seen on nontarget organisms. In addition, pesticides are not the only material in a spray formulation. Because of their poor solubility it is frequently necessary to use substances that promote their solubility and enhance their performance characteristics. Chemicals such as surfactants, diluents and dispersing agents are used in formulating pesticides. These adjuvants may be phytocidal.

Concern about the impact of pesticides on non-target organisms expressed by biologists and environmentalists has formed the basis for legislation aimed at controlling or prohibiting the use of specific pesticides. As a result, extensive toxicological data on target and non-target organisms is required before the active ingredient in the spray mix is registered for use. Rarely are tests required on the individual components of the formulation mix. This could be attributed to the fact that registration of surfactants or other products offered as adjuvants in pesticide application is not required under the U.S. Federal law, although a few states do require registration of such products (Swisher 1982). For registration purposes, in Canada and elsewhere, these chemicals are considered to be "inerts". However with spread of their use in experimental work in physiology it has been

recognised that surfactants are not "inert" compounds but are highly active, capable of causing various biochemical and physiological effects (Parr and Norman 1965). The present investigation with nonylphenol, served to emphasize that this compound which is considered to be an "inert" in the Matacil 1.8D mix may be potentially phytotoxic.

For testing the toxicity of chemicals to nontarget aquatic plants there are innumerable parameters that could be measured as many possible sites and mechanisms of phytotoxic action have been found for a wide variety of chemicals (EPA report 1975). The response measured could be simple, such as production of a foliar lesion, to more complicated bioassays involving measurement of photosynthetic activity and/or determining the uptake of the chemical, to ensure proper environmental assessment of the test chemical. For example, a chemical may not alter the growth or reproduction of a plant but absorption and bioaccumulation of the chemical could be toxic to phytophagous organisms feeding on the plant.

In the present study a number of bioassays were adopted to test the potential toxicity of the chemicals in the Matacil 1.8D OSC formulation. In order to isolate the phytotoxicity of the components of Matacil 1.8D it was necessary to study the toxicity of the individual components namely aminocarb, nonylphenol and diluent oil as well as the Matacil 1.8D spray mix. The bioassays adopted were fast and reliable and unlike the LC₅₀ did not require that the test organism die before the chemical be assessed phytotoxic.

4.1 Aminocarb

Aminocarb the "active" ingredient in the Matacil 1.8D mix is a structurally specific poison which inhibits acetylcholine esterase resulting in neural disfunction and death of most animal species, the lethal dose varying between species. (Corbett 1974). N-methyl carbamates have been used not only as insecticides but are well known herbicides the primary mode of action in plants involving the inhibition of mitosis and meiosis (Amer and Farah 1975, 1976, Amer 1965). Studies on the effect of aminocarb on nontarget plants have indicated inhibition of cell division in algae at concentrations of 5 µg/mL (Chacin 1983) and a 50% reduction in sprout length in white birch seedlings accompanied by a significant reduction in the germination value at 50 µg/mL (Weinberger and Vladut 1981). However, in the present study, aminocarb showed no significant effect on frond multiplication in L. minor. This could possibly be explained on the basis that very low concentrations (0.1-2.5 µg/mL) of aminocarb were used in these studies to simulate concentrations which may be found in the environment. Furthermore, aminocarb did not reduce total chlorophyll, ATP content, or photosynthesis in the fronds.

4.2 Diluent Oil 585

Oils have been used extensively in insecticidal sprays as selective and general herbicides and as carriers for soluble agricultural chemicals. Research undertaken on phytotoxic properties of oils have indicated that toxicity increases along the series: paraffins,

naphthalenes olefins and aromatics (Baker 1970, Van Overbeek and Blondeau 1954). Further, Bruns et al (1955) found that aromatic solvents with a boiling point of 117-216°C and an aromatic content of not less than 85% were the most effective for controlling aquatic weeds. Currier and Peoples (1954) showed aromatic hydrocarbons rapidly killed aquatic plants at a concentration of 1% w/v. By contrast, diluent oil 585 (a mixture of aromatics) did not affect the growth of Lemna minor in this study. Weinberger and Rea (1981) also found the oil to have no effect on the growth and development of Chlorella pyrinoidosa Chick, or Chlamydomonas reinhardtii Dang alga. One possible suggestion for the lack of inhibitory effects observed may be due to the fact that concentrations used in the present study were below the threshold level required to cause a response. Furthermore the toxic components may have volatilized as the persistence studies showed a rapid loss of diluent oil from the media (Table 7). Similarly, Soto et al (1975) demonstrated that highly volatile naphthalenes exerted reduced toxic effects in open systems, suggesting that the reduced toxicity was due to rapid volatilization of the naphthalenes.

4.3 Nonylphenol and Matacil 1.8D

Before proceeding with the discussion it is notable that the effects observed on the different parameters tested with nonylphenol and Matacil 1.8D were not significantly different. As a result emphasis on the effects of nonylphenol will be discussed.

The frond multiplication studies partially indicated that nonylphenol was phytotoxic to L. minor. Although these studies did not necessarily specify a certain mode of toxicity, the method was useful for initial screening purposes. As the concentration of nonylphenol was increased, a proportional inhibition was observed. The inhibiting effect was not only dependant on concentration of the xenobiotic but also on the time of exposure. For example, significant effects at concentration between 1.25 and 5.00 µg/ml were only evident on the third day suggesting that a certain accumulation of nonylphenol was required within the plant to bring about a response.

The trends obtained in these studies partly followed the basic concept of dose to response relationship in which a sigmoid curve is obtained plotting response (% of control) against concentration. Only the linear portion of the curve was clearly demonstrated, presumably because very low or high concentrations were not used. The concentration range over which phytotoxicity occurred was quite narrow. This has also been shown for other surfactants (Parr 1982). In plotting 96 hour dose response curves probit plots were not used as mortality of fronds was not observed. Instead an index reporting the concentration which caused 50% reduction in growth was adopted (96 hour IG₅₀). This was similar to EC₅₀ values traditionally used in chemical toxicity tests with animals. Blackman et al (1954) used LD₅₀ values in his report for comparing the relative toxicity of different phenols in L. minor. Fronds which were colorless over more than half the surface were counted senescent. Thus the concentration which caused senescence in half the fronds i.e. median lethal dose (LD₅₀) was adopted as standard. This

method has serious limitations since the present study showed fully bleached fronds capable of producing daughter fronds. The 96 hr, IG₅₀ for which all fronds were counted whether green or bleached seemed more appropriate and served usefully in comparing the relative toxicity of nonylphenol and Matacil 1.80. An important inference drawn from these plots suggested nonylphenol to be the toxic component in the Matacil 1.80 mix.

Chlorophyll destruction appeared to be a major symptom of nonylphenol injury, with small increments of nonylphenol concentrations (2.5 to 5.00 to 6.25 µg/mL) a transition from partially bleached to completely bleached fronds was noted after 24 hours of treatment. This supports the suggestion by Blackman et al (1954) that the degree of chlorosis is very sensitive to small changes in concentration. The total chlorophyll quantified on day 7 indicated a proportional decrease with sequential increase in concentrations of the nonylphenol. Although this parameter was not determined on a daily basis, it is tempting to suggest from the visual observation studies and the short term study with Matacil 1.80 (fig. 9), that chlorophyll was severely affected in the initial inculcum (10 fronds) and that this initial traumatic effect was followed by slow recovery. Rapid diminution of chlorophyll suggested the attractive possibility that the primary site of nonylphenol action may be the chloroplast. Furthermore, nonylphenol, a highly lipophilic molecule (K_{ow} 15,000 McLeese et al 1981, 5,000 the present study), may preferentially solubilize in the lipid phase of internal membranes as the lipid content of the chloroplast is notably high (Granick 1949). Surfactants have been shown to solubilise membrane

lipids (Helinus & Simons 1975), due to their emulsifying effects on cytomembranes and other lipid containing structures.

Apparently, there have been no published attempts made to understand the mode of action of surfactant induced bleaching. However this is a well recognized phenomenon for herbicides. The mechanism of herbicidal bleaching has been discussed in great detail by Sandmann and Boger (1983). These authors have basically suggested two modes of action. One possibility involves the inhibition of carotene biosynthesis. It is well documented that carotenes play a protective role, preventing photooxidation of chlorophyll moieties. Decrease in carotenes leads to subsequent photodestruction of chlorophyll, peroxidation of other membrane components and decay of electron transport. The second possibility involves peroxidation of polyunsaturated fatty acids concurrently with the breakdown of carotenes, chlorophylls and decay of photosynthetic electron transport. Peroxidative bleaching may also occur if the harvested light energy is not channeled into chemical work. Besides the bleaching caused by either carotene loss or peroxidation, more targets for bleaching are conceivable such as the inhibition of chlorophyll biosynthesis itself. More studies are clearly needed with nonylphenol to clarify the specific mode of action involved.

A further indication of the mechanism of nonylphenol induced toxicity in L. minor was established from the present studies on photosynthesis, membrane permeability and total ATP. These parameters were followed because it was hypothesised that effects on these processes may precede more specific toxicological symptoms.

Photosynthesis consists of light and dark reactions. ATP and NADPH₂ are produced in the light reaction and O₂ is released. In the "dark", reaction CO₂ is reduced and incorporated into various organic compounds using ATP and NADPH₂. Toxic agents may affect photosynthesis either by altering the chloroplast activity for CO₂ fixation and/or by altering the diffusion of CO₂ to photosynthesising cells (Boyer and Younis 1980). The effect of nonylphenol on photosynthesis in L. minor was assessed by measurement of the rate of ¹⁴CO₂ fixation. The observation that photosynthesis was inhibited by 45% within 12 hours of treatment of L. minor fronds with 2.5 µg/mL toxicant strongly suggests that this was the most sensitive parameter tested during the studies. These findings paralleled closely the study of Elner et al (1982), who reported a 50% to 100% decrease in carbon assimilation by natural algal communities exposed to Matacil 1.8D which contained 0.98-1.5 µg/mL aminocarb respectively. They attributed the observed reduction in CO₂ fixation solely to the pesticide (aminocarb) in the formulation. The authors did not attempt to differentiate between the effects of the pesticide and its formulation adjunct one of which was nonylphenol.

In the present case a possible explanation for the inhibition of ¹⁴CO₂ incorporation could be related to the fact that nonylphenol being a lipophilic molecule may have changed the chloroplast activity possibly by intercalating in the thylakoid membranes thereby perturbing the membrane function. Tawne et al (1978) showed by electron micrographs that nonionic surfactants cause the grana to enlarge laterally and become an ordered lamellar system sometimes appearing as

abnormally large grana. It was proposed that surfactants loosen the bonding material between the grana membranes and with this disruption water flows into the intermembrane area. Moreover surfactants were observed to reduce the photosynthetic carbon dioxide fixation of isolated soybean cells, possibly by disrupting thylakoid membranes and thus sites of ATP and NADPH₂ formation. Subsequently a reduction in ATP may cause inhibition of photosynthesis, since ATP is needed to operate the Calvin cycle. Dodge (1983) showed that when carbon dioxide fixation is prevented, excess excitation energy leads to the formation of triplet chlorophyll, which may generate singlet oxygen leading to lipid peroxidation or directly induce proton abstraction from unsaturated fatty acids. These reactions instigate cellular disorganisation and finally death. Further support for the inhibition of photosynthetic activity were derived from the fluorometry data obtained in the present studies. Schreiber et al (1978) indicated that chlorophyll fluorescence was a sensitive measure of the photosynthetic energy conversion and is useful in the elucidation of the various reactions of the photosynthetic mechanism. The observation that the chlorophyll fluorescence measured as the P-T transients was inhibited by 20% with nonylphenol (2.5 ug/mL, 12 hours) was consistent with the previous studies which demonstrated that nonylphenol (2.5 µg/mL) inhibited ¹⁴CO₂ uptake and ATP formation. Subsequent recovery of fluorescence suggested that the daughter fronds produced at successive generations were less affected by nonylphenol, as compared to the initial inoculum. Similar trends may have been obtained for ¹⁴CO₂ fixation had the experiment been followed for a longer time period.

There is good agreement between damage estimated by the fluorescence assay and the visual symptoms with exception of the results obtained after 12 hours of exposure, in which the chlorophyll fluorescence characteristics were significantly affected before any visual symptom could be detected. This indicates that the fluorescence assay is capable of detecting injury before visible lesions are noticed. Total inhibition of chlorophyll fluorescence at 6.25 $\mu\text{g}/\text{mL}$ nonylphenol could be related to complete bleaching of the fronds after 24 hours of exposure, and was in agreement with Moody (1982) who demonstrated a ICF₁₀₀ at 0.75 $\mu\text{g}/\text{mL}$ nonylphenol in Chlamydomonas reinhardtii.

The mechanisms involved in the changes in chlorophyll fluorescence induction have been attributed to a number of factors (1) destruction of chlorophyll (2) damage to PS II donor site (H₂O splitting enzyme system), (3) inhibition of electron transport from PS II to PS I. (Schreiber et al 1978). Furthermore Krause (1973) and Mohanty et al (1971) found uncouplers of photophosphorylation to abolish the P-I transients suggesting the action of uncouplers on trans-membrane proton gradients. Papageorgiou (1975) also indicated that the P-I transients correspond to trans-membrane ion flux and induced conformational changes of the thylakoid membrane. This would be consistent with the prior discussion concerning membrane destruction with nonylphenol.

The fluorometry studies proved to be a rapid and sensitive method for studying nonylphenol effects on whole plants. This method could be conveniently used in screening of pollutants in the laboratory and the field, as the instrument is portable.

Furthermore, an interruption of photosynthesis may eventually lead

to a reduction in food reserves. This has been shown in the present study as there was a significant decrease in both fresh and dry weights at the end of seven days following exposure to 2.5 µg/mL nonylphenol. Other researchers have also reported a decrease in fresh and dry weights of higher plants exposed to nonionic surfactants. Horowitz and Givelberg (1979) showed Agral at 10 µl/L (ppb) 8 days post treatment reduced the fresh weight of the roots and shoots in Sorghum bicolor by 48% and 30%. Endo et al. (1969) observed Agro Gro¹ and Soil Penetrant¹ at 330 ug/mL to decrease shoot dry weight by 88% and 71% respectively at day 5.

It was interesting to note that, the cell membrane permeability measured by overall loss of intracellular material, was not affected. This does not seem unreasonable as nonylphenol being a nonionic surfactant, (i.e. there are no ionizable polar end groups on the molecule), may enter cells with greater ease concentrating in lipid rich organelles such as the chloroplast in preference to the plasmalemma. This proposition corresponds to the findings of earlier studies by St. John et al. (1974). These researchers took the approach of labelling isolated onion or soybean cells with ¹⁴C through fixation of ¹⁴CO₂ and then measuring subsequent surfactant effects on the loss of this label from the cells. Tween 20 (polyoxyethylene sorbitan monolaurate) and Daxad 21 (monocalcium salt of polymerized aryl alkyl sulphonic acids) at concentration of 0.1% w/v reduced photosynthesis (measured by ¹⁴CO₂ incorporation) by more than 50%, yet, they had no effect on

1 Chemical identity of Agro Gro and Soil Penetrant were not revealed.

leakage of radiolabel ^{14}C from the cells. The authors suggested that this was indicative of effects on intracellular membranes rather than the outer cell membrane.

Healey et al (1971) published electron micrographs of plant cells exposed to the anionic surfactant LAS (sodium (linear) dodecyl benzene sulphonate). After 4 hours of treatment swelling of thylakoids was evidenced but no apparent morphological changes in membrane systems other than the chloroplast was shown. When the treatment time increased to 48 hours the plasmalemma was intact although often pulled away from the cell wall, swelling of mitochondrial cristae were observed and severe changes in chloroplast morphology noted.

Thus it may be tentatively suggested that surfactant action on chloroplast membrane precedes destruction of the cell membrane.

Adenosine 5' triphosphate has been known to be the most common energy donating molecule in biochemical reactions. The luciferin-luciferase method for quantifying the amount of ATP in plants has been widely used. It should be noted that although other compounds with high phosphate transfer potentials are found in cells, it is unlikely that reduced luminescence found in extracts of treated fronds was due to these compounds, since ATP is more abundant in cells and has been demonstrated to give the greatest luminescence when added to firely extracts (Clegg and Koevenig 1974).

In the present studies it is difficult to explain why a 20% reduction in ATP levels (24 hour, 2.5 $\mu\text{g}/\text{mL}$) did not affect frond multiplication till day 3. Perhaps nonylphenol did not reduce the ATP

levels sufficiently within 24 hours to become a limiting factor for frond growth. This is in agreement with the findings of an earlier study with algae in which population densities were shown to be initially unaffected by decreased ATP levels (Clegg and Koevenig 1974). Moreover, the results noted in the present study that photosynthesis was reduced by 45% with minimal decrease in the level of levels could probably be a consequence of either increased respiration or decreased output in work (eg. action transport and biosynthesis). However, nonylphenol (6.25 µg/mL) did severely reduce ATP levels within 24 hours, suggesting effects on photophosphorylation and oxidative phosphorylation. Vernon (1965) demonstrated the non-ionic surfactant Triton X-100, commonly used in physiological studies, to separate membrane lipids from proteins and to uncouple photophosphorylation in spinach chloroplast at a concentration of 0.007%. Changes in permeability of grana and intergrana lamellae were suggested as the cause of the changes. Respiratory phosphorylation has also been reported to be uncoupled by Triton X-100. This was related to swelling of the mitochondrial membranes. Electron microscopic observations mentioned previously (Healy et al 1971) support the suggestion by these authors.

In conclusion the results obtained in the present study could all probably be explained on the basis of changed membrane integrity in the chloroplasts and mitochondria following exposure to nonylphenol whether presented alone, or as a component of the Matacil 1.8D formulation.

4.4 Uptake and bioaccumulation

The present studies which describe the uptake and bioaccumulation of aminocarb and nonylphenol have shown that nonylphenol tends to bioaccumulate in L. minor several times more than does aminocarb. This was to be expected, as a number of studies with organic pollutants and a variety of organisms have established a general trend for increase in pollutant accumulation with increasing octanol-water partition coefficients. Correlation between K_{ow} , water solubility and the bioconcentration factor have appeared in the literature. The K_{ow} is reflected in the solubility of the compound and plays a central role in predicting the values for transfer rate constants between compartments. It could be considered to be a quantitative measure of hydrophobicity of a compound. In this respect it has been used to predict the bioaccumulation of organic chemicals in animals (Neely et al 1974; Chiou et al 1977; Kenaga & Goring 1980; Veith et al 1979 and Ellegehausen et al 1980). Furthermore, the water solubility of organic compounds has been demonstrated to be inversely related to bioaccumulation and this relationship has proven useful in predicting the bioaccumulation in organisms (Chiou et al 1977; Körte et al 1978; Kenaga & Goring 1980). Aminocarb has a K_{ow} of 30 at pH 6.0 (Weinberger and Greenhalgh 1981), and nonylphenol a K_{ow} of 5,000 as calculated in the present study or 15,000 (McLeese et al 1981). The discrepancy in the values for

nonylphenol may have been a result of poor phase separation of nonylphenol between the octanol-water phase. Calculated values for the bioconcentration factor for aminocarb, and nonylphenol using regression equations was surprisingly low as compared to observed values. (Appendix ii). The apparent contradiction may have been due to a number of factors: (1) The equations cited in literature were calculated using fish as the model organism. The excretory system in animals is a well defined system, kinetic rate constants describing the rate of uptake and clearance of chemicals from fish are estimated and the BCF calculated when a steady state is reached. As a result these equations may not hold good for plant systems where there is no specific excretory system. (2) The biological half life ($t_{1/2}$) of an organic chemical has been shown to be higher in plants as compared to animals. For example the $t_{1/2}$ of aminocarb in trout is 32 hours whereas it is 600 hours in L. minor (Lockhart et al 1983). (3) The published equations do not contain a time factor making it difficult to interpret at what time a steady state was reached and the BCF calculated.

Having considered the drawbacks involved in predicting BCF of organic pollutants in plants with animal models, some interest in deriving equations for plants has been initiated. Korte et al (1978) demonstrated that BCF in the alga Chlorella fusca could be correlated with the water solubilities of 10 lipophilic chemicals. Ellgehausen et al (1980) derived a regression equation relating K_{ow} with BCF in algae. (Appendix ii). Briggs et al (1982) studied the uptake of chemicals by roots of barley and found that BCF was greater the more lipophilic the chemical.

During the course of the present study Lockhart et al (1983) working with a number of organic pollutants including aminocarb, studied uptake and depuration trends in L. minor. The measured BCF calculated by taking the ratio of uptake rate constants to depuration rate constants was 56 on a fresh weight basis, and 890 on a dry weight basis. This value was reasonably close to that found in the present studies (96 hour bioaccumulation was 227). As the K_{OW} of nonylphenol and fluorene are about the same, (K_{OW} of fluorene 4.18), it would be logical to compare the BCF of these compounds. Lockhart et al (1983) reported a BCF of 105 for fluorene which would convert to 1,678 on a dry weight basis. These results agree reasonably well with the measured bioaccumulation of 3,420 for nonylphenol (96 hours) obtained in the present studies. Bioaccumulation ratios of 2,000 for carbaryl in L. minor, and 1,280 for dieldrin in Elodea have been previously reported. (Kanazawa et al 1975, Sanborn & Ching Chieh Yu 1973), suggesting that aquatic plants could be a significant macro-reservoir of organic chemicals in aquatic systems, exposing herbivores to higher concentrations of a pollutant than present in the ambient environment.

4.5 Relevance to Field Conditions

One of the most important but also most difficult tasks following studies of this kind is to try to relate the data to field conditions. Much more field work must be carried out before reliable correlations can be made, but laboratory investigations serve as a basis to direct this further work. One shortcoming of field work, without preliminary

laboratory work, is the difficulty of relating observed effects to known effects of individual xenobiotics. For example, although some field studies on the toxicity of Matacil 1.8D showed effects on stonefly nymphs (Penney 1971), bees and songbirds (Buckner et al 1975), it is not clear whether the effects were caused by aminocarb or the adjuvants. Thus, preliminary laboratory studies could serve to answer such questions. The data obtained in the present studies clearly indicated that nonylphenol is the toxic component, to phytobiota, in the Matacil 1.8D spray formulation.

There is a dearth of literature on persistence and residues of nonylphenol in lake water under field conditions. However, studies on persistence in stream water samples have been reported. Sundaram et al (1980) showed 9.1 ppb of nonylphenol in stream water one hour post spraying decreasing to trace levels of 2.00 ppb by six hours. In the same study, up to 1.10 ppm of nonylphenol was found in water taken from a stagnant part of the same stream 4 hours post-treatment. This study indicated the possibility of nonylphenol concentrations being higher in lake water than in moving stream waters.

As nonylphenol and diluent oil 585 are sparingly soluble in water they would preferentially accumulate at the air water interface where free floating fronds of L. minor reside. As a consequence, concentrations of up to 6.25 µg/mL or higher of nonylphenol could impinge on the fronds leading one to hypothesise that there might well be some bleaching of fronds and a short or longer term reduction in frond number. Even in the best case situation, these perturbances could lead to a reduced vigour of affected Lemna population.

An extrapolation of results from this study to conditions which are found in contaminated streams and lakes is tenuous, however, the results will hopefully stimulate further investigations accompanied by field studies.

4.6 Lemna minor as an Indicator Species

Often in impact evaluation studies we seek methods of minimizing the number of variables to be measured in order to indicate the conditions of, or nature of, the environment we are studying. Indicator species have been used to evaluate the prevailing conditions in a variety of situations. For example, Eliassen (1952) conducted a long term study and summarized the changes in the species of fish, larger invertebrates and plankton in some streams as they change from slight to severe pollution and back to the unpolluted conditions. Thus, finding the appropriate set of species is crucial to the assessment of pollution in an aquatic system. To find an appropriate organism or organisms Butler et al 1971 suggested the following attributes for an organism to serve as a biological indicator. The organism should (1) accumulate the pollutant without being killed by the levels encountered in the environment. (2) be of reasonable size, giving adequate tissue for analysis. (3) be abundant throughout the study area. (4) be easy to sample, and hardy enough to survive in the laboratory. (5) be sufficiently long lived to allow the sampling of more than one year class if desired and (6) be sedentary in order to be representative of the study area.

In the present study L. minor proved to be a very sensitive species to nonylphenol pollution. The organism fulfilled most of the criteria stated above. For example the fronds were not killed even at the highest concentration used which was comparable to that expected in the field. The plants also accumulated reasonable amounts of nonylphenol.

An additional attribute for an indicator organism not cited by Butler et al (1971) could well be changes in appearance of plants or animals in the presence of pollutants. Foliar lesions or chlorosis of fronds could be a rapid tool for indication of nonylphenol pollution the degree of chlorosis of fronds allowing conclusions to be drawn as to the level of pollution.

Although a species or a related group of species can be used as indicators it would be more efficient and reliable to use more general characteristics such as chlorophyll, photosynthesis, and ATP content previously cited in literature. A rapid diminution of total chlorophyll and disruption of electron flow through PS11 and PS1 as indicated by the fluorometry studies were important symptoms of nonylphenol toxicity evidenced in the present research. As a result it is strongly suggested that these parameters be used on other phytobiota as an indicator of pollution in aquatic systems.

4.7 Suggestions for Future Research

- (a) It would be worthwhile to conduct electron microscopy studies to verify the suggestion that chloroplast membrane disruption precedes the effects on cell membranes. These experiments might also prove useful in elucidating the effects of nonylphenol on grana and stroma structures in chloroplasts.
- (b) It is important that an attempt be made to demonstrate preferential accumulation of nonylphenol in chloroplasts as opposed to the plasmalemma. Autoradiography studies with labelled nonylphenol should answer this question.
- (c) It could be interesting to determine the effects of nonylphenol on carotenoid and chlorophyll biosynthesis to further study the involvement of these processes in inducing bleaching.
- (d) The effect of nonylphenol on mixed cultures, for example natural assemblage of algae and L. minor would provide meaningful data to enable more accurate predictions to be made on the effects of nonylphenol under field situations.
- (e) Field studies after a spray operation would be worthwhile to follow. Harvesting L. minor fronds before and after a spray operation and conducting bioassays, may give an indication of the extent of pollution.

SUMMARY AND CONCLUSION

The phytotoxicity of Matacil 1.80 and its components aminocarb, nonylphenol and diluent oil 585, were investigated. The results demonstrated that at field relevant concentrations aminocarb the active ingredient, and diluent oil the carrier in the formulation mix, did not significantly effect the growth and metabolism of Lemna minor. This was probably due to the very low concentrations used (to simulate field concentrations) and the rapid volatilisation of diluent oil from the media.

The results with the nonylphenol and the Matacil 1.80 suggested the degree of inhibition to be directly attributable to the nonylphenol content in the formulation mix as there was no significant difference in the results obtained when Lemna minor plants were treated with nonylphenol or Matacil 1.80. The results indicated that nonylphenol perturbed the growth and development and this was evidenced by the fact that it, (1) inhibited frond emergence above a concentration of 1.25 $\mu\text{g}/\text{mL}$ and the 96 h IG₅₀ (Inhibition of growth) was calculated to be 5.50 $\mu\text{g}/\text{mL}$ nonylphenol. (2) The fresh and dry weights were significantly depressed at treatment levels of 1.25 $\mu\text{g}/\text{mL}$ and 2.50 $\mu\text{g}/\text{mL}$ respectively. (3) Bleaching of the fronds was observed 24 h post treatment following exposure of Lemna minor fronds to 2.5 $\mu\text{g}/\text{mL}$ nonylphenol and the total chlorophyll measured on day seven was inhibited by about 50% at a concentration of 3.75 $\mu\text{g}/\text{mL}$. (4) Exposure to 2.5 $\mu\text{g}/\text{mL}$ nonylphenol led to a 32% inhibition of photosynthesis as

indicated by chlorophyll fluorescence (P-I transients) and 45% as indicated by $^{14}\text{CO}_2$ incorporation 24 h and 12 h post treatment respectively. At 5.00 ug/mL an 80% inhibition of P-I transients was observed. (5) The total ATP levels in the fronds was inhibited by 20% and 75% at 2.50 and 6.25 ug/mL nonylphenol respectively 24 h post treatment. (5) Cell membrane permeability was not affected by any of the concentrations tested (2.50 and 5.00 ug/mL).

The results suggested that nonylphenol has a rapid biochemical effect on many parameters. As a result it was difficult to establish whether the effects were primary or secondary. However, it would be safe to suggest that the chloroplast membrane may be the primary target site leading to the array of effects observed.

The results of the bioaccumulation study demonstrated that the octanol water partition coefficient contributes directly to the uptake and bioaccumulation of a compound. Nonylphenol bioaccumulated roughly 15X greater than aminocarb in the fronds of Lemna minor. Calculated BCF values for aminocarb and nonylphenol using equations derived for animal models in the literature were much lower than the experimental values obtained in the present study. However, using equations derived for plant models, the experimental values were reasonably close to that derived theoretically. The use of K_{ow} as a preliminary screening test for lipophilic chemicals, may indicate the potential of the compound to bioaccumulate in living systems.

Finally from the data available it seems logical to suggest that Lemna minor is an ideal non target organism that should be routinely used as an indicator of pollution of aquatic habitats.

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Appendix (i)

Justification for adoption of 2.5 µg/mL figure as a measure of field application:

$$\text{Conversion factor} = 0.000702 \mu\text{l/cm}^2 = \text{Imp oz/acre}$$

$$\text{Rational for factor 1 acre} = 4.047 \times 10^7 \text{ sq cm}$$

$$\begin{aligned} 28.35 \text{ gm} &= 1 \text{ oz} \\ \text{Therefore 1 oz/acre} &= \frac{28.35}{4.047 \times 10^7} \\ &= 7 \times 10^{-7} \text{ gm/sq cm} \\ &= 0.7 \mu\text{g/sq cm} \end{aligned}$$

$$\begin{aligned} \text{Assuming density 1.0} &= 0.7 \times 10^{-6} \text{ ml/cm}^2 \\ &\text{or } 0.000702 \mu\text{l/cm}^2 \end{aligned}$$

Therefore the amount of aminocarb that should be placed in the petri dish (i.e. the amount which will be found following a 1 Imp oz/acre spraying) is

$$\begin{aligned} &(7 \times 10^{-7} \text{ mL/cm}^2) \times (63.5 \text{ cm}^2) \\ &= 441 \times 10^{-7} \text{ mL} \\ &= 4.41 \times 10^{-5} \text{ mL} \end{aligned}$$

The minimum amount of water that we need to completely cover the surface of a 9 cm petri dish is 15 mL. Then the concentration in the petri dish is,

$$\begin{aligned} &= \frac{4.41 \times 10^{-5} \text{ mL}}{15 \text{ mL}} \\ &= 2.93 \times 10^{-6} \\ &= 2.93 \text{ ppm (Since } 10^{-6} = 1 \text{ ppm)} \end{aligned}$$

Therefore 2.93 ppm should theoretically be found in a petri dish containing 15 mL of surface water following 1 Imp oz/acre spraying.

Aminocarb is generally sprayed at either

52 g a.i./ha	or	70 g a.i./ha	(2.5ac = 1 ha)
or - 2.08 g a.i./acre		28 g a.i./acre	
= 0.728 oz/acre		0.98oz/acre	(0.035oz = 1gm)
= 2.10 ppm		2.9 ppm	(1 Imp oz = 2.93 ppm)

These figures average to 2.5 ppm utilized in the present studies.

Appendix (ii)

Several papers have presented equations relating the solubility(S) of a compound to its water-octanol partition co-efficient (K_{ow}). This idea has been taken a step further to correlate the K_{ow} or S of a compound with its potential to bioaccumulate (BCF) in living organisms. In ii Appendix the BCF of aminocarb and nonylphenol has been calculated from a number of these previously reported equations.

Log K_{ow} aminocarb = 1.48 (Weinberger & Greenhalgh 1981)
Log K_{ow} nonylphenol = 3.69 (Present Study) or 4.17
(McLeese et al 1981)

Solubility of aminocarb. 2000 ug/mL (Mobay report 1979)

Solubility of nonylphenol derived theoretically from Chiou et al (1974) equation

K_{ow}	S (ug/mL)
3.69	19.80
4.19	3.46

Calculated Bioconcentration factors from regression equations formulated
for animals

Aminocarb

Equations used	Bioconcentration factor
A	8.43
B	0.77
C	3.61
D	0.32

- A $\text{Log BCF} = 0.542 \text{ Log } K_{ow} + 0.124$ (Neely et al 1974)
- B $\text{Log BCF} = 0.935 \text{ Log } K_{ow} - 1.495$ (Kenaga & Goring 1980)
- C $\text{Log BCF} = 0.850 \text{ Log } K_{ow} - 0.70$ (Veith et al 1979)
- D $\text{Log BCF} = 0.83 \text{ Log } K_{ow} - 1.71$ (Ellgehausen et al 1980)

Nonylphenol

Equation used	Bioconcentration factor	
	$K_{ow} = 3.69$	$K_{ow} = 4.17$
A	133	242
B	90	253
C	273	699
D	23	56

Calculated BCF factors from regression equations derived for algae:

Aminocarb

Equation used	BCF
E	5.97
F	724.00

E $\text{Log BCF} = 0.70 \text{ Log } K_{ow} - 0.26$ (Ellgehausen et al 1980)

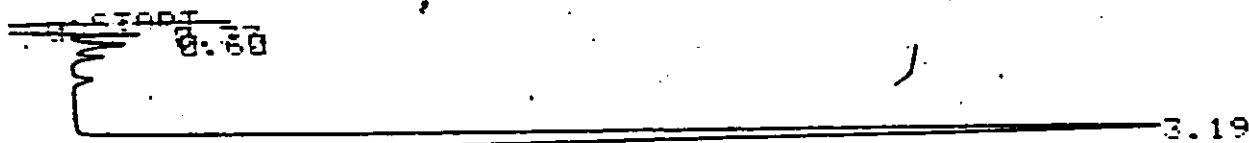
F $\text{Log BCF} = 4.94 - 0.33 \text{ Log } S$ (Korte et al 1978)

Nonylphenol

<u>Equation used</u>	S	K _{ow}	Source	Calculated BCF
E	-	3.69	This study	210
	-	4.17	McLeese(1981)	456
F	19.8	-	This study	3311
	3.46	-	McLeese(1981)	6025

Appendix (iii)

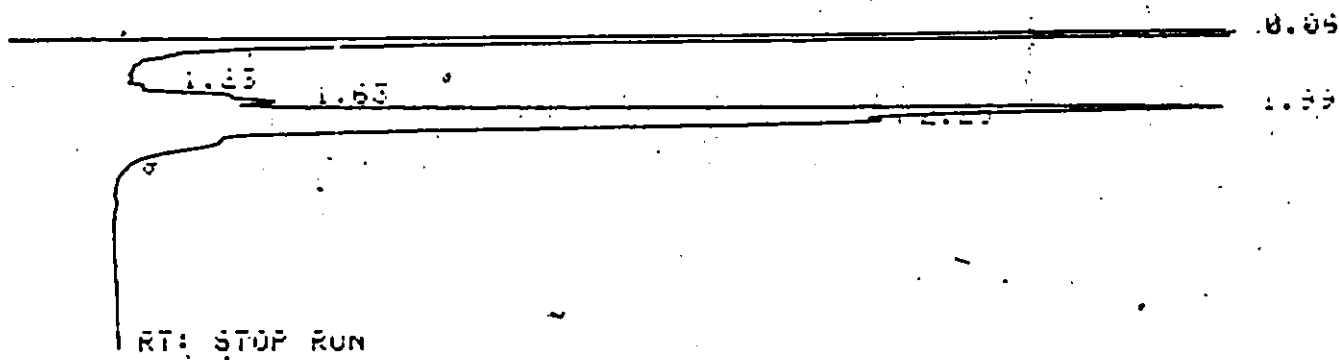
Gas chromatograph of aminocarb, nonylphenol and diluent oil 585, respectively.



ST
No 5830A
AREA %

RT	AREA	AREA %
0.16	1331	3.481
0.37	4670	12.213
0.60	207	0.541
3.19	32030	83.765

XF: 1.0000 E+ 0



RT: STOP RUN

DATA 5850A MANUAL INJECTION @ 16:59 NOV 4, 1982
AREA %

RT	AREA	TYPE	AREA %
0.04	211432.00	SV	13.916
0.06	1235450.00	VV	64.606
1.25	23.68	PP	0.002
1.65	296.11	SV	0.019
1.69	14158.50	VV	0.932

