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THE EFFECTS OF AMINOCARB AND ITS FORMULATION MATACIL 1.8D ON *CHLORELLA PYRENOIDOSA* CHICK AND *CHLAMYDOMONAS SEGNIS* ETTL

by

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A thesis submitted to the School of Graduate Studies in partial fulfillment of the requirements for the degree of Master of Science

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ABSTRACT

The effects of the carbamate pesticide aminocarb on two species of algae Chlorella pyrenoidosa and Chlamydomonas segnis were investigated. Aminocarb used to control spruce budworm infestations is formulated with nonylphenol and diluent oil 585 for field spraying. The formulated pesticide (Matacil 1.8D) inhibited population growth of Chlorella at high concentrations, but the pesticide alone actually stimulated population growth at the same concentrations. Nonylphenol was the toxic component in the formulation. In uptake studies employing C^{14}-aminocarb populations of Chlorella exposed to the aminocarb formulation accumulated a greater amount of the labelled pesticide over time than populations exposed to aminocarb alone. It was suggested that nonylphenol in the pesticide formulation affected membrane permeability thus increasing uptake of C^{14}-aminocarb.

Not only was the population growth of Chlamydomonas unaffected by the non-formulated pesticide, but the alga was also more resistant to the formulated pesticide than Chlorella. To better understand the effects of the formulated pesticide on algal growth synchronous populations of Chlamydomonas were treated at various times with Matacil 1.8D. A critical time for inhibition of doubling was identified indicating that DNA synthesis was affected.

RÉSUMÉ

Les effets de l'aminocarb, un pesticide carabamate sur les algues Chlorella pyrenoidosa et Chlamydomonas segnis ont été étudiés. On se sert de l'aminocarb pour contrôler les infestations de la tordeuse des bourgeons de l'épinette. Avant l'arrosage l'aminocarb est mélangé au nonylphénole et à l'huile diluente #585. Ce mélange, nommé Matacil 1.8D, est mortel pour Chlorella à forte concentration, mais le pesticide seul stimule la croissance de l'algue à la même concentration. L'additif nonylphénole est le constituent toxique du mélange. Dans des études d'assimilation avec le C^{14}-aminocarb, on a trouvé qu'une plus grande quantité d'aminocarb est assimilée par Chlorelle quand le pesticide est mélangé comme Matacil 1.8D que quand le pesticide est seul. Il a été suggéré que l'additif nonylphénole nuit à la perméabilité des membranes, augmentant ainsi la quantité d'aminocarb assimilée par l'algue.

L'accroissement des populations de Chlamydamonas n'a pas été affecté par le pesticide seul. En plus, l'algue Chlamydamonas est plus réfractaire au Matacil 1.8D que l'algue Chlorella. Pour mieux comprendre les effets du mélange d'insecticide sur les algues, des populations synchrones de Chlamydamonas ont été traitées à différents moments avec le Matacil 1.8D. Une période critique pour l'inhibition du dédoublement cellulaire a été observé. Cela indique que la synthèse de l'ADN est affectée.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Page

Page

INTRODUCTION

Forests cover more than one-third of the land area of North America. In the United States 750 million acres are forested and in Canada 550 million acres (McEwen and Stephenson, 1979). Forest pests are many. Destruction by insect pests such as several species of sawfly, looper, and weevil are not new to Canadian forests. In the past this destruction was not considered significant but today it is recognized that timber reserves are not infinite and this is particularly true of those species which are of economic use. These considerations together with a citizenry more sensitive to the consequences of ecological perturbation have led to a change in attitudes making the preservation of forests of paramount importance. In New Brunswick, for example, infestations by spruce budworm (Choristoneura fumiferana Clemens) caused a loss of 10-33% of the merchantable standing crop in 1975 (McEwen and Stephenson, 1979). Such losses are significant in New Brunswick where forestry comprises 20% of the economy. Not only are forests an important economic resource but they also serve as a refuge for wildlife and as a recreational area for people. The diverse order of Lepidoptera to which the budworm belongs contains the most damaging defoliating insects and the spruce budworm has caused extensive damage to the forests in North America.

Several species of spruce budworm live in geographically isolated regions of North America. The eastern spruce budworm is dominant in the forests of eastern Canada and Maine. The insect larvae over

winter among the needles of the host conifer. After completion of diapause in early spring the larvae begin to feed on vegetation. There are six larval instars and the last is the most destructive. The insect pupates for about ten days and emerges as a small brown moth. These moths usually mate the first day after emergence. Females deposit the eggs near their site of emergence thus starting another generation of spruce budworms.

Since 1700 spruce budworm outbreaks in eastern Canada and Maine have occurred at least six times previous to the present outbreak (Baskerville, 1975). A cycle has developed between the spruce budworm and the fir-spruce-birch forests. The pattern of budworm infestations in Green River, New Brunswick, was documented by Baskerville (1975). An outbreak occurred in 1877 killing most of the balsam fir in mature forests and leaving immature stands. These young dense stands dominated by fir matured until favorable weather brought about another budworm infestation from 1913 to 1919. The cycle began again with another budworm outbreak occurring in 1949. This outbreak had a different ending since experiments in Ontario indicated that DDT could control the budworm. Aerial application of DDT gained widespread use in spruce budworm control in northeastern United States and Canada from 1944 to the mid-1960's.

DDT was applied to New Brunswick forests at the rate of 1 lb active ingredient (AI) per acre from 1952 to the early 1960's. In spite of high larval mortality the outbreak of 1949 did not stop. This fact along with the observation of high fish mortality (Kerswill, 1959)

and reduced populations of aquatic invertebrates (Kingsbury, 1976) after spraying brought about a reduction in the DDT dosage to .25 lb/acre and the use of phosphamidon on forested areas near major lakes and streams. In 1968 DDT was banned in Canada for use in operational control programs against forest insects because research indicated that DDT was persistent in the environment (Kenaga, 1972; Bevenue, 1976) and its breakdown products were also toxic to living organisms (Brooks, 1974).

In 1969 fenitrothion was substituted for DDT and applied at the rate of 3 oz active ingredient/acre. Between 1952 and 1973 15 million pounds of DDT and more than 3 million pounds of fenitrothion were sprayed on 50 million acres of New Brunswick forests for spruce budworm control (Miller and Kettela, 1975). Spruce budworm infestation is not limited to New Brunswick forests. 25 million acres are affected in Ontario, 85 million acres in Quebec, and severe defoliation has occurred in Manitoba, Nova Scotia, Prince Edward Island, and Newfoundland.

In the 1960's concern over the use of pesticides lead to widespread testing in the laboratory and field of new chemical pesticides. Carbamate pesticides grew in popularity due to their low mammalian toxicity and nonpersistence (Kuhr and Dorough, 1976). Since 1972 the carbamate pesticide aminocarb, 4-dimethylamino 3-methylphenyl N-methyl carbamate (Appendix A), has been used experimentally and operationally to control spruce budworm infestation.

Aminocarb or Matacil® was first synthesized by Bayer Leverkasen in 1963 and has been researched in the United States and Canada by the

Chemagro Division of the Baychem Corporation. Aminocarb inhibits cholinesterase in the central nervous system (Kuhr and Dorough, 1976). Briefly, a nerve impulse causes the release of acetylcholine at the presynaptic membrane. Acetylcholine diffuses across the synapse to the postsynaptic membrane where it binds to receptor sites causing the cell to pass the impulse. The enzyme acetylcholinesterase hydrolyzes the acetylcholine so that stimulation of the receptor ceases. Aminocarb complexes with acetylcholinesterase thus preventing hydrolysis of acetylcholine. The accumulation of acetylcholine at the synapse allows continuous transmission of nerve impulses and nervous coordination deteriorates.

For operational use aminocarb is formulated with nonylphenol and a fuel oil distillate, 585 diluent oil, and this mix is registered as Matacil 1.8D OSC (Appendix A). The surfactant nonylphenol (a mixture of parasubstituted monoalkyl phenols) together with the fuel oil distillate (a mixture of low molecular weight alkanes and naphthalenes) maintain the lipophilic aminocarb in solution. In Matacil 1.8D the nonylphenol, diluent oil, and aminocarb are mixed in the ratio of 5:3:2 w/w/w, respectively. Matacil 1.8D is mixed with diluent oil for field spraying (common field dilutions of Matacil 1.8D : diluent oil range from 1:1.88 to 1:2.84) and the spray formulation is applied at the rate of .603 oz/ hectare (ha) (2-5 µg/ml aminocarb). For purposes of registration the adjuvants are regarded as biologically inert; the pesticide alone is rated as the active ingredient.

Initially investigations in pesticide research were undertaken on

the toxicity and mode of action of aminocarb in laboratory organisms. The metabolism of aminocarb by houseflies and rats and identification of the metabolites has been studied (Metcalf et al., 1967; Tsukamoto and Casida, 1967; Oonnithan and Casida, 1968). Research has also been conducted on the metabolism of aminocarb by bean plants (Abdel-Wahab et al., 1966; Kuhr and Casida, 1967). Several studies compared aminocarb metabolism in plants and animals (Lykken and Casida, 1969; Kuhr, 1970). An overview of the metabolic and degradative pathways of aminocarb is presented by Schlagbauer and Schlagbauer (1972).

Aerial applications of pesticides provided an efficient means for mass treatments of forests. But with this efficiency came the possibility of affecting a larger ecological area. Thus, investigations have shifted to the effects of the aminocarb spray formulation of nontarget organisms of the forest ecosystem. The effects of spraying on terrestrial flora have been studied. After trunk injection of aminocarb into spruce trees the young needles concentrated the pesticide up to 2.28 μg/ml after 21 days (Sundaram and Hopewell, 1977); after 89 days no aminocarb was detected in the foliage and little aminocarb was detected in the roots. No depression of fecundity or seed set of native forest plants was noted after aminocarb spray treatment (Thaler and Plowright, 1980). In fact, two plant species had significantly higher fecundity than the same species in the untreated control area.

More extensive work has been conducted on the effects of budworm spraying on terrestrial fauna. Certain hymenopterous pollinators are

affected by aerial treatment with the aminocarb spray formulation. Damage to adult foragers of domestic bees was observed at spray dosage of 52 g AI/ha (Buckner et al., 1975). The number of worker bees returned to normal levels after three days. No affect was noted on queens, brood, or newly emerged bees. Aminocarb spray formulation applied at 70 g AI/ha caused no increased mortality in bumble bees but a significant knockdown of solitary bees was observed (Plowright and Rodd, 1980).

Field investigations of forest bird populations (finches, sparrows, thrushes, and warblers) after aminocarb spray formulation application have revealed no ecological impact. Although some songbirds (yellow throats, black warblers, and white warblers) showed a slight reduction in numbers after spray applicaton at 52 g AI/ha, no adverse effects were noted at 70 g AI/ha (Buckner et al., 1975). Moreover, three applications of the spray formulation at 70 g AI/ha with an interval of six days between applications or two applications at 87 g AI/ha with five days between applications produced no increased mortality or interruption of activity such as singing and foraging in forest dwelling birds (Buckner and McLeod, 1977a). Birds also displayed no symptoms of pesticide stress such as erratic flight or bill wiping. Breeding territories remained intact after three aerial applications at 52 g AI/ha (Kingsbury and McLeod, 1980).

Small mammals such as mice, shrews, and voles appear also to be unaffected by the aminocarb spray formulation. No interruption of breeding was observed after application of 105 g AI/ha (Buckner et al.,

1973) or two applications of 52 g AI/ha (Buckner and Sarrazin, 1975). The effects of forest spraying on large mammals has not been extensively investigated although deer populations were reportedly unaffected after two applications of the spray formulation at 52 g AI/ha (Buckner and McLeod, 1977b).

Numerous studies have been undertaken on the effects of aminocarb on aquatic life since streams and lakes form an integral part of forest ecosystems. Laboratory investigations revealed a 48-hour LC_{50} (concentration of pesticide which killed 50% of the test species) of 1.1 µg/ml aminocarb for Atlantic salmon (Nigam, 1975) and a 24-hour LC_{50} of 39 µg/ml for a freshwater amphipod (Sanders, 1969). At low concentrations of aminocarb of 10-25 µg/ml bubble-making and unidirectional swimming were reduced in tadpoles. 24-hour LC_{50}'s for small and large tadpoles were 247 and 234 µg/ml, respectively (Lyons et al., 1976).

Aquatic ecosystems have been monitored after aminocarb formulation spraying for possible pesticide impact. Aquatic invertebrates are generally unaffected but certain species are more sensitive than others. For example, spray application at 52 g AI/ha reduced stonefly populations (Buckner et al., 1975) and increased blackfly larval drift (Holmes, 1979). No damage to adult or larval amphibians has been observed after applications of 70 g AI/ha (Buckner et al., 1975) or 105 g AI/ha (Rick and Gruchy, 1971). Fish populations were not affected by either one application (Holmes, 1979) or two applications (Buckner and McLeod, 1977b) of 52 g AI/ha of the spray formulation.

Field investigations of spray programs involving more than one

pesticide have been conducted. For example, the ecological impact of treatment with formulated fenitrothion at 140 g AI/ha followed by the aminocarb spray formulation at 52 g AI/ha ten to fourteen days later was studied (Buckner et al., 1974). The combined treatment had no effect on small mammals or birds. Another program employed two applications of the fenitrothion formulation at 280 g AI/ha followed by one application of the aminocarb spray formulation at 70g AI/ha (Kingsbury, 1978). After the aminocarb spray formulation no changes in bottom fauna, benthic fauna, or fish were observed, although short-lived increases in invertebrate (mayfly and stonefly nymphs or blackfly and midge larvae) drift were noted.

Most of the research in pesticide toxicology has emphasized the effects of the pesticide, not the formulation adjuvants on selected organisms. There have been, however, a few investigations comparing the effects of aminocarb, aminocarb formulations, or the formulation adjuvants on nontarget organisms. An aminocarb formulation with 17% pesticide was 300X more toxic to fish than technical grade aminocarb (Woodward and Mauk, 1980). Mussels exhibited low accumulation coefficients for the uptake of aminocarb or nonylphenol but a relatively high coefficient for diluent oil 585 (McLeese et al., 1980). Lethality of the aminocarb formulation to Atlantic salmon was attributed almost entirely to nonylphenol whereas, nonylphenol was only slightly more toxic than aminocarb to a marine crustacean (McLeese et al., 1980). In both organisms diluent oil 585 contributed little to the toxicity of the aminocarb formulation. The major toxicity of

Matacil 1.8D to cultured cells was demonstrated to be due to nonylphenol (Li et al., 1981).

Field investigations comparing aminocarb formulation sprayed at seasonal maximum dosage (175 g AI/ha) and nonylphenol sprayed to give an equivalent amount in this formulation were reported (Holmes and Kingsbury, 1980). Aminocarb residues of .024 µg/ml were detected in streams after .5 hr. and nonylphenol residues in flowing water peaked at .009 µg/ml after 1 hr. and in stagnant water levels as high as 1.1 µg/ml nonylphenol were detected after 4 hr. Whereas nonylphenol did not affect aquatic or terrestrial invertebrates, the formulation produced decreased bottom fauna populations with accompanying increased drift. The formulation also caused a significant knockdown of terrestrial invertebrates especially Diptera, Homoptera, and Hymenoptera. This effect was still evident after three days.

Research on the effects of the spruce budworm spray program on aquatic plants especially algae inhabiting lakes and streams in treated forests has been neglected. Algae perform a substantial part of the total photosynthesis in freshwater ecosystems (Talling, 1975 a, b) and form the base of extensive food webs. Research in algal pesticide toxicology is important since any perturbance of the primary productivity would affect the total energy budget of the system, and this could ultimately affect other organisms in the Eltonian food chain and even diversity within a community. This latter perturbation has been demonstrated by Mosser et al. (1972), Edwards (1972), Fisher et al. (1974), and Taub (1976) who found altered species composition after

pesticide treatments. Probably the first suggestion that pesticides affected algae was noted by Shane (1948) who observed algal blooms following pesticide application. Demonstration of pesticide toxicity to marine phytoplankton by Ukeles in 1962 emphasized the need for further investigations into the impact of pesticides on algae. Knowledge of the widespread contamination of waters by DDT led to extensive research into its effects on algae. The totality of the investigations on DDT represent the most extensive research effort conducted on any single pesticide (Butler, 1977).

The purpose of the present research was to widen the boundaries of our knowledge in relation to the relatively new pesticide, aminocarb, and its formulation mix Matacil 1.8D using the population dynamics of freshwater algae as a gauge of potential perturbation of freshwater systems within the forest ecosystem. While a great variety of algae live in aquatic environments two species of green algae were chosen for this investigation, namely, Chlorella pyrenoidosa Chick and Chlamydomonas segnis Ettl. Chlorella are found in freshwaters of northeastern Canada (NSERC Report, 1977) where aminocarb spraying occurs. Chlorella pyrenoidosa is also used extensively in laboratory studies. Chlamydomonas segnis is found in marshes of Manitoba (Badour et al., 1977) and could also be affected by spruce budworm treatment programs. Although its distribution is unknown and it is not a common laboratory species, it was used because it could be easily synchronized.

The main hypothesis of this thesis was that the aminocarb formulation Matacil 1.8D which contains nonylphenol, a nonionic

surfactant, and diluent oil, a mixture of petroleum hydrocarbons, would have a greater impact on algae than aminocarb alone. This simple hypothesis was founded on many studies which reported the toxic effects of both surfactants and petroleum hydrocarbons on plants and animals. For example, surfactants have been shown to enhance the toxicity of herbicides. A mixture of pichloram and the surfactant Atlox 210 was as effective as four times the amount of pichloram alone in reducing the numbers of saplings (Corns and Dai, 1967). Paraquat toxicity was also reportedly enhanced by several nonionic surfactants (Smith and Foy, 1967). Many surfactants including nonionics have been shown to inhibit the growth of crop plants such as oats, flax, and peas (Luzzati, 1974) and wild oats and radish (Merritt, 1976). Moreover, the toxicity of surfactants to aquatic organisms has been demonstrated. Lethal effects of surfactants have been reported for Atlantic salmon (Wildish, 1974), salmon fry (Kuroda, 1976), and barnacles (Wright, 1976). The growth of marine phytoplankton was inhibited in varying degrees by surfactants (Ukeles, 1965) and green algae actually sorbed and degraded certain surfactants (Davis and Gloyne, 1969).

The effects of phenolics especially the widespread pollutant polychlorinated biphenyl (PCB) on algae have been investigated. PCB reduced the growth rate and photosynthesis of marine diatoms (Powers et al., 1977) but did not affect a marine green alga or two species of freshwater algae (Mosser et al., 1972). On the other hand, PCB was highly toxic to Chlorella pyrenoidosa (Cole and Plapp, 1974). Low

concentrations of many phenolics have been shown to stimulate growth, photosynthesis, and respiration in algae whereas high concentrations were algicidal (Buikema, 1979).

The effects of petroleum hydrocarbons on algae have also been reported (Kauss and Hutchinson, 1975; Soto et al., 1975; Soto et al., 1977; Moody et al., 1981). These studies have shown that naphthalene, a major constituent of crude oils and a component of diluent oil, affected population growth, photosynthesis, and cell ultrastructure in freshwater algae.

These studies then would seem to substantiate the hypothesis that the aminocarb formulation Matacil 1.8D has the potential for greater effects on the two algal species than the aminocarb alone.

To elucidate any toxicity of aminocarb, Matacil 1.8D, or the formulation adjuvants, the effects of these chemicals on cell density and growth rate of both algal species were examined. The effects on cell size, chlorophyll content, and uptake in Chlorella pyrenoidosa and cell cycle in Chlamydomonas segnis were also assayed.

MATERIALS AND METHODS

2.1 Chemicals

Technical grade aminocarb, Matacil 1.8D, (aminocarb dissolved in nonylphenol and #585 oil, 2:5:3, w/w/w), nonylphenol, and diluent oil #585 were gifts of Mobay Co. Ltd., Mississauga, Ontario, Canada. Ring labelled C^{14}-aminocarb (5.01 mCi/nmole, 98% purity) was purchased form New England Nuclear (USA). The organic solvents used for preparation of standard concentrations of aminocarb, Matacil 1.8D, and adjuvants and for chlorophyll extraction were glass distilled, pesticide grade (Caledon). Chemicals used for preparation of algal culture media and Scintiverse used for liquid scintillation counting were purchased from Fisher Scientific Co. Difco Bacto-Agar was used to solidify culture media.

2.2 Algal Cultures

Cultures were periodically checked for viability and contamination by agar plating. Cell counts of initial inocula were made with an improved Neubauer hemacytometer.

2.2.1 Chlorella

Cultures of Chlorella pyrenoidosa Chick were obtained from the

Culture Collection at Indiana University.

(a) Growth studies and C^{14}-aminocarb studies

Liquid cultures were maintained in Bold's Basal Medium (BBM) pH 6.8 (Bold, 1942) in 20 x 150 mm Pyrex® screw-cap culture tubes and placed on a shaker (78 oscillations/min.).

To measure dead cell uptake in the C^{14}-aminocarb studies cells were frozen with liquid nitrogen and allowed to thaw at room temperature. Gross microscopic examination revealed intact cells and agar plating verified 100% mortality.

(b) Chlorophyll studies

Liquid cultures were maintained in BBM in 250 ml Pyrex® screw-cap Erlenmeyer flasks and placed on a shaker.

(c) TC_{50} determination of nonylphenol

Cells were cultured on solidified BBM in petri plates. BBM was solidified by adding agar at a concentration of 1.0% (Nichols, 1973).

Liquid cultures and agar plates were incubated in a Hotpack growth chamber (5 klux, 16hr light: 8hr dark photoperiod provided by Sylvania cool white fluorescent lamps, 23°C).

2.2.2 Chlamydomonas

Cultures of Chlamydomonas segnis Ettl were a gift from Dr. D.C. Mortimer, National Research Council, Ottawa, Ontario. For all studies algal cultures were maintained in the nutrient medium of Kuhl and Lorenzen (1964) as modified by Badour and Waygood (1971).

(a) Growth studies

Cells were grown in 20 X 150 mm Pyrex® screw-cap culture tubes, placed on a shaker, and incubated in a Hotpack growth chamber (5 klux, 12 hr light: 12 hr dark photoperiod provided by Sylvania cool white fluorescent lamps, 25°C).

(b) Cell cycle

Cells were grown in 500 ml Pyrex® filter flasks. The cultures were bubbled with air (.03% CO_2) and constantly mixed with a magnetic stirrer. The incubation conditions were the same as in the growth studies except the light was increased to 7 klux.

2.3 Growth Studies

The change in population density of the algal cultures was determined daily by reading the transmission of each culture tube in a Coleman (6/28) spectrophotometer at 540 nm. The transmission values were converted to optical density using the equation:

$$OD = \text{Log}_{10} \left(\frac{1}{T}\right)$$

where

OD = optical density

T = transmission

$= I_t / I_i$

and

I_t = transmitted light

I_i = incident light

The initial value obtained for each culture was subtracted from each subsequent reading and the ΔOD values determined in this manner were plotted versus time to give a growth curve for each treatment (Sorokin, 1973) (Appendix B). A straight line was fitted to each curve to permit calculation of growth rate (slope) and determination of the length of the lag phase (intercept of this line with the time axis). The use of culture tubes permitted the direct reading of OD without subsampling. The OD of tubes containing nutrient medium alone did not change for the duration of the studies.

The growth rate of the algal cultures was calculated using the equation:

$$\text{Growth rate} = \frac{X_2 - X_1}{t_2 - t_1}$$

where

X_1, X_2 = the ΔOD at the beginning and end of the period during which growth was measured

t_1, t_2 = corresponding times in days at which X_1 and X_2 were determined

The growth rate was determined for the first six days of growth in the Chlorella studies and the first five days of growth in the Chlamydomonas studies.

2.3.1 Chlorella

In parallel studies, aminocarb, Matacil 1.8D, nonylphenol and #585 oil were used at a range of concentrations above and below those used operationally in the budworm abatement program. Concentrations of .01 to 10.0 µg/ml of pesticide were used to simulate field levels or watershed contamination. In each case, the xenobiotics were added to triplicate tubes containing 4 X 10^6 cells to give final concentrations of .01, .1, 1.0, 3.0, and 10.0 µg/ml aminocarb or aminocarb as Matacil 1.8D, or .025, .25, 2.5, 7.5 or 25.0 µg/ml nonylphenol, or .015, .15, 1.5, 4.5 or 15.0 µg/ml diluent 585 oil. The concentrations of nonylphenol and 585 oil are the relative amounts found in the Matacil 1.8D series of .01 to 10.0 µg/ml aminocarb.

A 24hr TC_{50} for Chlorella cultured with nonylphenol concentrations of 0, 2.5, 5.0, and 7.5 µg/ml was determined. After treatment, the algal samples were washed with fresh medium and plated on nutrient enriched agar. After 2 weeks of growth in the controlled environmental chamber, the numbers of colonies per plate were counted.

2.3.2 Chlamydomonas

In parallel studies aminocarb or Matacil 1.8D were added to triplicate tubes containing 4 X 10^6 cells to give final concentrations of 1.0, 3.0, and 10.0 µg/ml aminocarb.

2.4 Cell Size Distribution

Following the 19 day treatment period Chlorella populations growing in the five concentrations of aminocarb or Matacil 1.8D were sampled for cell sizing. Cell numbers were counted and cell diameters were measured using an improved Newbauer chamber and an eyepiece linear micrometer. The proportion of cells in three size categories (0-3.7 μm, 3.75-7.4 μm, 7.45 - μm) was determined.

2.5 Chlorophyll Study

Chlorella cultures (1×10^6 cells/ml) were added to triplicate flasks containing 3.0 μg/ml aminocarb. Algal samples taken over 6 days were centrifuged and extracted with 100% methanol for 24 hrs. in the dark (Parsons, 1966). The samples were homogenized with a tissue grinder. The extract was measured in a Unicam SP 1800 Ultraviolet spectrophotometer at 650, 665, and 750 nm in a 1 cm cell. The optical clarity of the solution was checked with readings at 750 nm. Total chlorophyll and chlorophyll a and b were calculated using the equations derived by MacKinney:

$$Chl_T = 4\ A_{665} + 25.5\ A_{650}$$

$$Chl_a = 16.5\ A_{665} - 8.3\ A_{650}$$

$$Chl_b = 33.8\ A_{665} - 12.5\ A_{650}$$

(Holden, 1976).

2.6 Uptake of C^{14}-aminocarb

Chlorella cultures were added to triplicate tubes containing either aminocarb in Matacil 1.8D plus C^{14}-aminocarb or aminocarb plus C^{14}-aminocarb. In all cases, C^{14}-aminocarb represented about 20% of the total aminocarb. Sampling was conducted over 7 days except for the dead cell study which was sampled over 5 days.

Samples were suction filtered through Whatman GF/C glass fiber filters and washed with distilled water. The filter with algae and the filtrate were counted in Fisher Scintiverse using the C^{14}- and H^3-channels of the Beckman Scintillation Counter Model LS 233. The counts were corrected for quench using the External Standard-Channel Ratio (ESCR) Method (Beckman Instructions, 1976). Quench curves were prepared by plotting counting efficiency against the ESCR number (ratio of two counts of the external standard minus the sample) for each quenched standard. Population density and cell number were determined spectrophotometrically as discussed previously and the disintegrations per minute (dpm)/ 10^6 cells or the dpm/ml was calculated.

2.6.1 Effects of the formulation

Chlorella cultures (3×10^6 cells/ml) were treated with 3.0 µg/ml aminocarb or an equivalent amount of aminocarb formulated as Matacil 1.8D.

2.6.2 Dead cells

Freeze-killed algal cultures (3 x 10^6 cells/ml) were maintained in 3.0 µg/ml aminocarb.

2.6.3 Effects of cell concentration

Three different initial population densities of Chlorella (1 x 10^6, 2 x 10^6, and 3 x 10^6 cells/ml) were grown in 3.0 µg/ml aminocarb.

2.6.4 Binding of C^{14}-aminocarb

Chlorella cultures (2 x 10^6 cells/ml) were grown in 3.0 µg/ml aminocarb. The uptake of unwashed cells was compared with the uptake of cells receiving 3X the amount of wash or the normal amount of wash before scintillation counting.

2.7 Depuration of C^{14}-Aminocarb

Three day old Chlorella cultures growing in 3.0 µg/ml aminocarb enriched with C^{14}-ring labelled aminocarb were harvested by centrifugation and resuspended in fresh nutrient medium to give 3 x 10^6 cells/ml. Sampling and calculations were as described for the uptake studies.

2.8 Cell Cycle

Populations of Chlamydomonas were synchronized by the induction method of Badour and Waygood (1971). Briefly this required populations (1×10^6 cells/ml) to be subjected to alternating periods of 12 hrs light : 12 hrs dark. At the end of the dark period the algal suspension was diluted back to the original cell number. After 4 days the populations were synchronized: most cells (95%) divided at the same time and at the same rate.

Cultures were treated with 1.0 µg/ml aminocarb in Matacil 1.8D at the 10th (-2 hr darkness), 12th (0 hr darkness), or 14th (+2 hr darkness) hour of the cell cycle. The percent doubling of the control and treated cultures was compared.

2.9 Statistical Evaluation

The data was evaluated statistically using a BMDP Biomedical Computer Program, University of California, Berkeley, Calif. and a Tukey multiple range test (Neter and Wasserman, 1974).

RESULTS

3.1 Growth Studies

3.1.1 Chlorella

The population growth of Chlorella was not significantly affected by low concentrations (.01 or .1 µg/ml) of aminocarb or aminocarb in Matacil 1.8D (Figs 1 & 2). When the pesticide concentration was increased to 1.0 µg/ml, the algae in the non-formulated pesticide sets grew as well as those in the control sets but the growth of the algae in the formulated sets was adversely affected. This was evidenced by a 4 to 5 day delay in the initiation of population growth. Following this initial lag, the growth rate was significantly greater than control sets and by 19 days post-treatment the size of the populations was equal to those of the untreated controls (Fig. 3). Exposed to 3.0 µg/ml aminocarb the algae treated with the non-formulated pesticide consistently gave rise to populations with 15% greater OD ($\Delta OD = .13$) than the untreated controls, while the sets exposed to the formulated pesticide evidenced a prolonged 10-day lag in population growth. The growth rates of both sets were similar and relatively greater ($p>.05$) than control sets. Because of the initial lag, the final populations of the sets exposed to the formulated pesticide were less than 50% of the control sets after 19 days post-treatment (Fig. 4). At concentrations approximating a mild spill (10.0 µg/ml aminocarb)

Chlorella cells exposed to the non-formulated pesticide gave rise to populations 30% greater than untreated control sets. The growth rates of these populations were also significantly ($p<.05$) greater than the control populations. Formulated aminocarb at the same 10.0 µg/ml concentration of "active ingredient" pesticide was completely inhibitory (Fig. 5).

The mean OD over the 19 day study of populations treated with the non-formulated pesticide increased with increasing concentration of aminocarb (Fig. 6). Populations exposed to the two highest concentrations of aminocarb had significantly ($p<.05$) greater densities than control populations. Moreover, the growth rate of aminocarb treated populations increased with increasing concentration of pesticide, although only the growth rate of cultures treated with 10.0 µg/ml aminocarb was significantly ($p<.05$) greater (48%) than control cultures (Fig. 7).

In the formulated pesticide sets the growth rates (Fig. 7) of the populations treated with the two lowest concentrations of aminocarb were relatively lower than control populations resulting in somewhat lower mean ODs after 19 days (Fig. 6). Populations treated with 1.0 or 3.0 µg/ml aminocarb in Matacil 1.8D exhibited a lag phase before the initiation of growth resulting in lower mean ODs than control populations. However, their subsequent growth rates were higher than controls. This delay in growth increased with increasing concentration of formulated pesticide until complete inhibition of growth was evident at the highest concentration of 10.0 µg/ml aminocarb in Matacil 1.8D.

When populations of Chlorella were exposed to nonylphenol or diluent oil at concentrations comparable to those found in .01 or .1 µg/ml aminocarb formulated as Matacil 1.8D, the final cell populations were unaffected (Figs. 8 & 9). At concentrations of adjuvants equivalent to those in 1.0 µg/ml aminocarb formulated as Matacil 1.8D, the populations treated with diluent oil (1.5 µg/ml) were unaffected whereas the populations treated with nonylphenol (2.5 µg/ml) exhibited a 3-day delay in growth. However, by 25 days post-treatment the final population size was equal to untreated controls (Fig. 10). Exposed to concentrations of adjuvants found in the 3.0 µg/ml aminocarb formulated Matacil mix, again, only the sets treated with nonylphenol (7.5 µg/ml) were adversely affected. Initiation of population growth was delayed by 9 days and the final populations were about 30% less than control populations (Fig. 11). Treated with concentrations of adjuvants comparable to those obtained in the 10.0 µg/ml aminocarb formulation mix the diluent oil (15.0 µg/ml) did not affect population growth; whereas, the nonylphenol (25.0 µg/ml) completely inhibited the growth of Chlorella (Fig. 12). A 24 hr TC_{50} (concentration of pesticide which was toxic to 50% of the test organisms) was obtained with 1.5 µg/ml nonylphenol (Fig. 13).

The three highest concentrations of nonylphenol delayed the population growth of Chlorella resulting in decreased population densities after 25 days post-treatment. As the nonylphenol concentration increased the lag phase before growth also increased producing a decreasing mean OD for these populations (Fig. 14). The

growth rate of populations increased with increasing concentrations of nonylphenol until complete inhibition of growth was evident at the highest concentration (Fig. 15). Relatively higher growth rates of sets treated with 2.5 µg/ml nonylphenol resulted in a mean OD not significantly different from controls even after a 3-day lag in growth. Although the growth rate of populations exposed to 7.5 µg/ml nonylphenol was more than twice ($p<.05$) the rate of control populations, the mean OD after 25 days post-treatment was significantly less than controls because of the initial delay in growth.

The growth rate of populations exposed to diluent oil increased with increasing concentration to a maximum 25% greater than controls at 1.5 µg/ml oil. At higher concentrations of oil (4.5 or 15.0 µg/ml) the growth rates of treated populations were similar to the untreated controls (Fig. 15). This growth rate pattern associated with increasing concentration of diluent oil was reflected in a similar pattern in the mean ODs obtained in treated populations (Fig. 14). However, at any concentration neither the growth rate nor the mean OD of treated populations was significantly different from control populations.

3.1.2 Chlamydomonas

The population growth of Chlamydomonas was not significantly affected by any concentration of aminocarb (Fig. 16). Furthermore,

the growth rates of none of the treated populations were significantly different from the untreated controls (Fig. 17). Increasing concentration of aminocarb gave rise to final populations equal to those of control sets and hence mean ODs were not significantly different from controls (Fig. 18).

When the Chlamydomonas populations were exposed to the same concentrations of aminocarb formulated as Matacil 1.8D, population growth was depressed below the untreated controls and aminocarb treated populations (Fig. 19). 16 days post-treatment, the populations of sets exposed to 1.0 and 3.0 µg/ml aminocarb in Matacil 1.8D were 80% of the control populations and the sets exposed to 10.0 µg/ml aminocarb in Matacil 1.8D gave rise to final populations only 70% of control populations.

The growth rates of populations treated with the formulated pesticide decreased with increasing concentration of aminocarb in Matacil 1.8D (Fig. 17). However, at any given concentration of aminocarb, the growth rate of populations exposed to the formulated pesticide was consistently lower than populations exposed to the non-formulated pesticide. Populations exposed to 1.0 µg/ml aminocarb in the formulation had a growth rate only 13% greater than control populations. The growth rates of Chlamydomonas population exposed to 3.0 or 10.0 µg/ml aminocarb in the formulation were 72% and 52%, respectively, of the untreated controls, although only the effect of the highest concentration was significant. The main effects of

chemical and concentration were significant ($p < .05$) and the interaction was not. Over time these effects of the formulated pesticide on growth rates resulted in mean ODs lower than controls. As concentration of formulated pesticide increased, the mean OD over time decreased (Fig. 18).

Figure 1. Growth curves of Chlorella populations exposed to .01 µg/ml aminocarb or aminocarb in Matacil 1.8D versus untreated (control) populations. The average values of triplicate samples are shown together with standard deviation bars for days 8, 13, and 18.

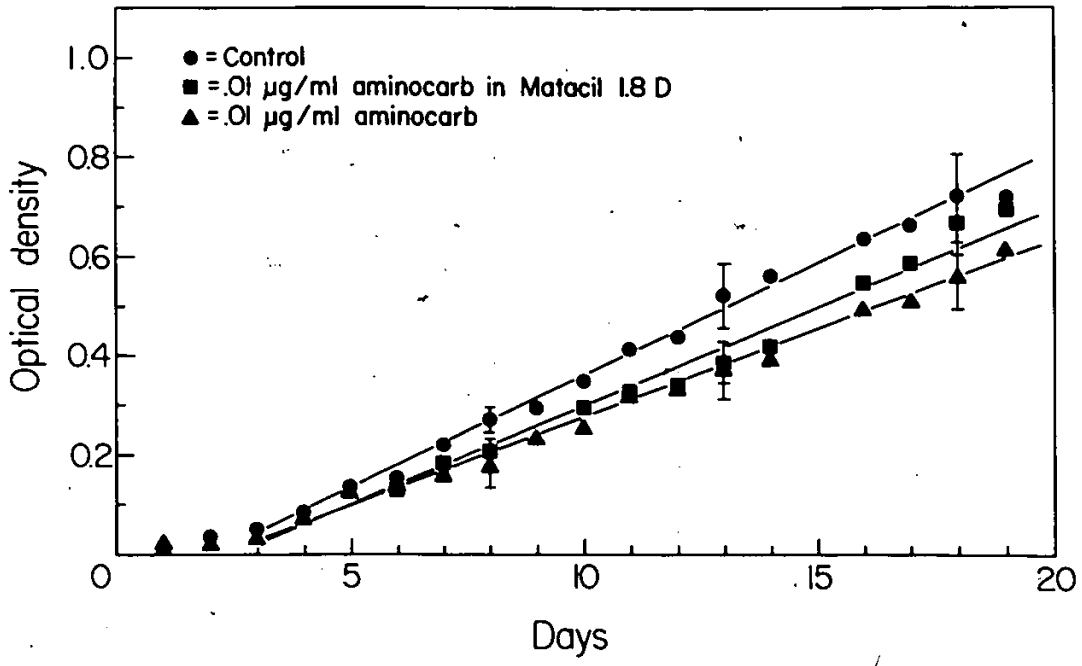

● = Control
■ = .01 µg/ml aminocarb in Matacil 1.8 D
▲ = .01 µg/ml aminocarb
Optical density
1.0
0.8
0.6
0.4
0.2
0
5
10
15
20
Days

Figure 2. Growth curves of Chlorella populations exposed to .1 µg/ml aminocarb or aminocarb in Matacil 1.8D versus untreated (control) populations. The average values of triplicate samples are shown together with standard deviation bars for days 8, 13, and 18.

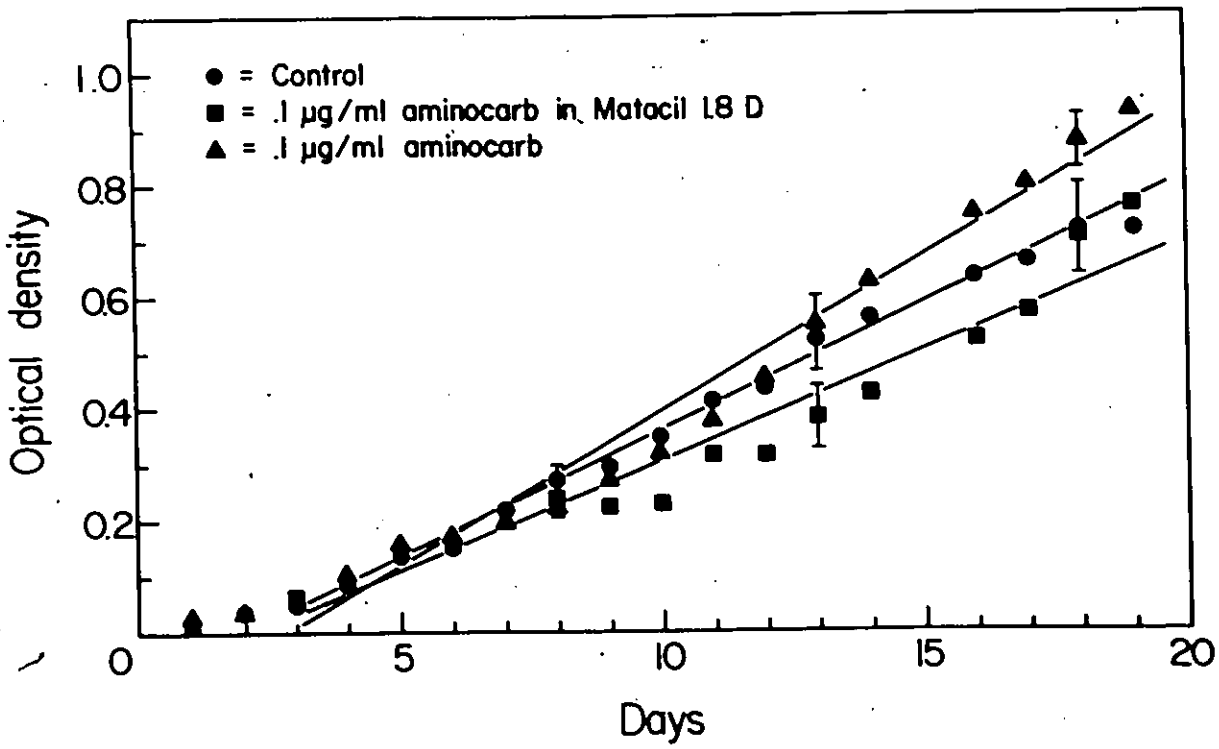

● = Control
■ = .1 µg/ml aminocarb in Matacil 1.8 D
▲ = .1 µg/ml aminocarb
Optical density
1.0
0.8
0.6
0.4
0.2
0
5
10
15
20
Days

Figure 3. Growth curves of Chlorella populations exposed to 1.0 µg/ml aminocarb or aminocarb in Matacil 1.8D versus untreated (control) populations. The average values of triplicate samples are shown together with standard deviation bars for days 8, 13, and 18.

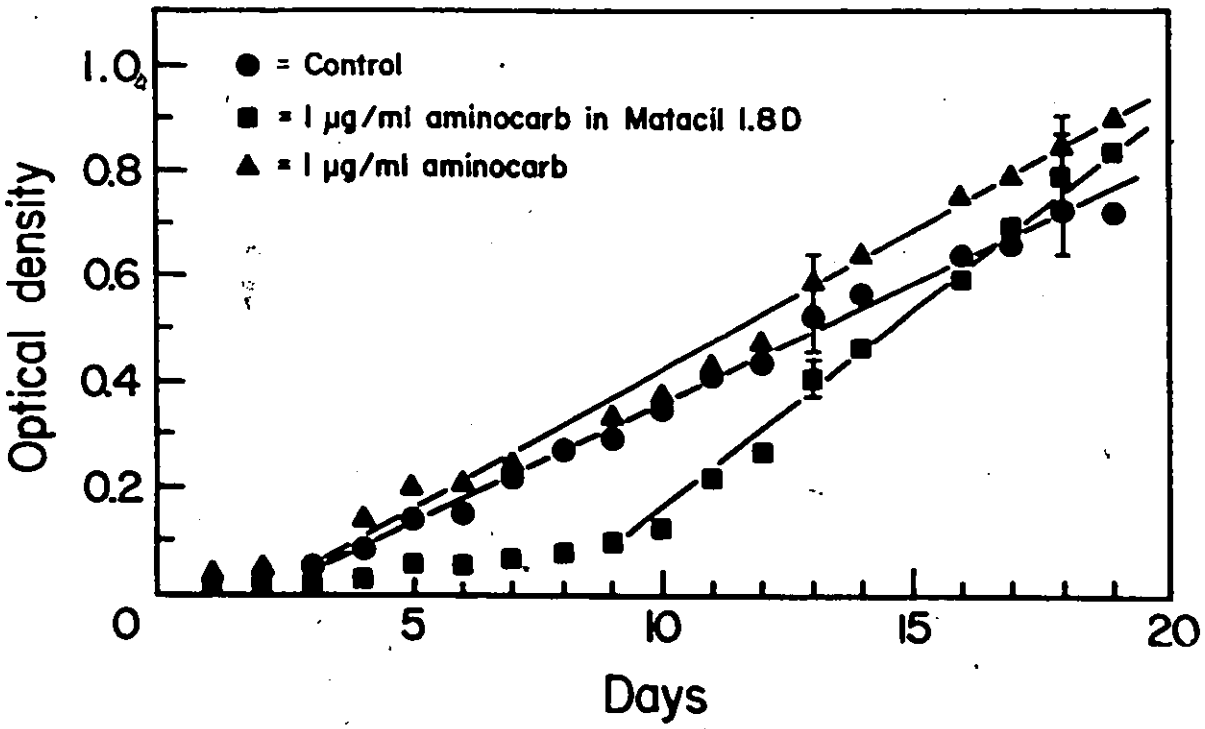

● = Control
■ = 1 µg/ml aminocarb in Matacil 1.8D
▲ = 1 µg/ml aminocarb
Optical density
1.0
0.8
0.6
0.4
0.2
0
5
10
15
20
Days

Figure 4. Growth curves of Chlorella populations exposed to 3.0 µg/ml aminocarb or aminocarb in Matacil 1.8D versus untreated (control) populations. The average values of triplicate samples are shown together with standard deviation bars for days 8, 13, and 18.

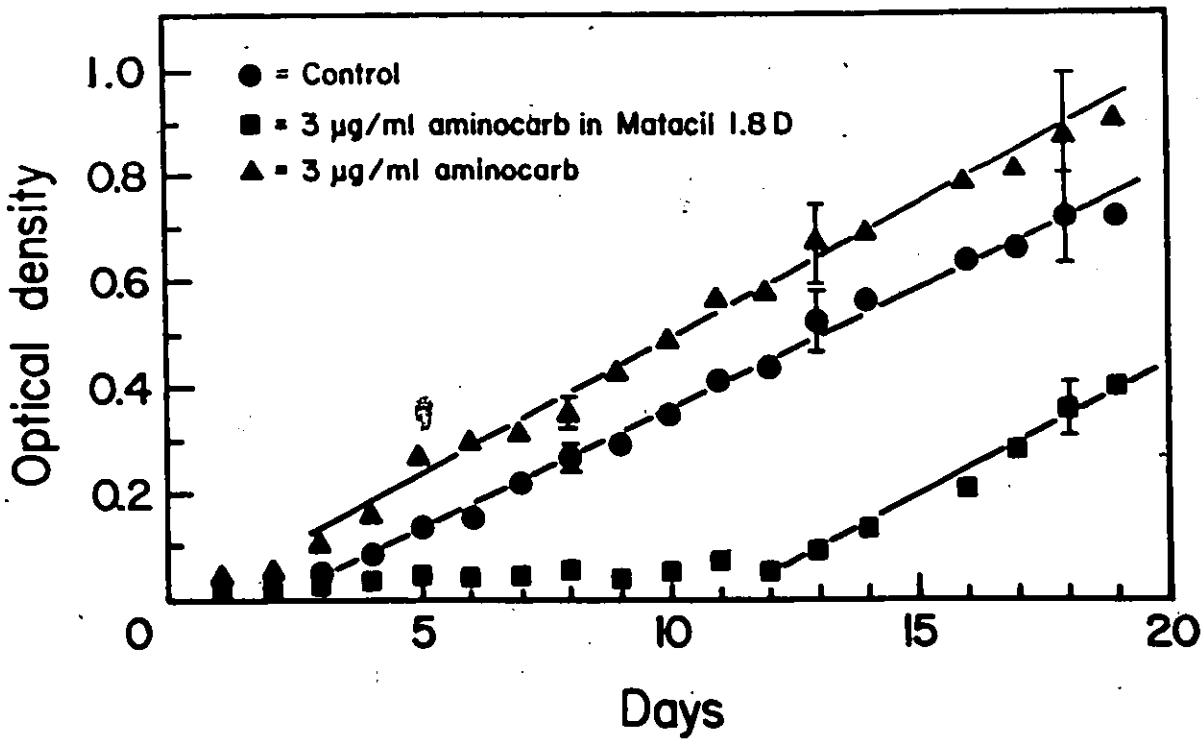

● = Control
■ = 3 µg/ml aminocarb in Matacil 1.8 D
▲ = 3 µg/ml aminocarb
Optical density
1.0
0.8
0.6
0.4
0.2
0
5
10
15
20
Days

Figure 5. Growth curves of Chlorella populations exposed to 10.0 µg/ml aminocarb or aminocarb in Matacil 1.8D versus untreated (control) populations. The average values of triplicate samples are shown together with standard deviation bars for days 8, 13, and 18.

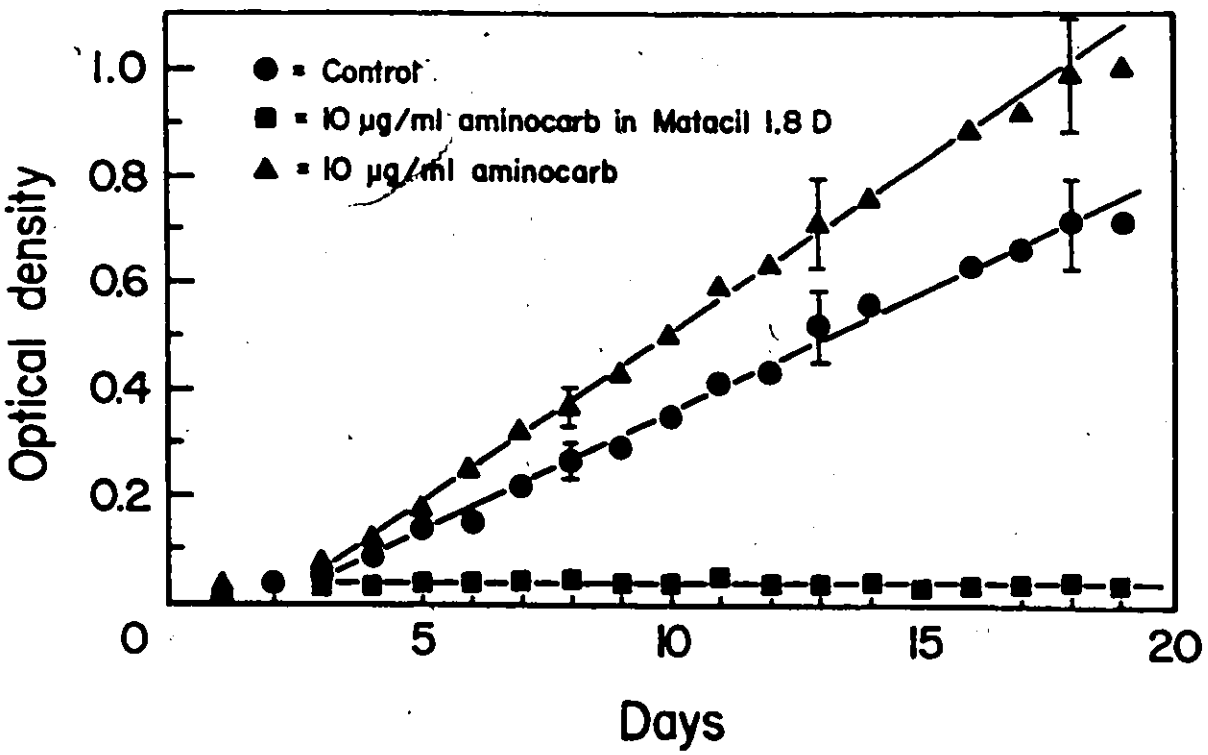

● = Control
■ = 10 µg/ml aminocarb in Matacil 1.8 D
▲ = 10 µg/ml aminocarb
Optical density
1.0
0.8
0.6
0.4
0.2
0
5
10
15
20
Days

Figure 6. The mean optical density ($\bar{X}$ OD) of Chlorella populations exposed to .01, .1, 1.0, 3.0, or 10.0 µg/ml aminocarb or aminocarb in Matacil 1.8D. The average values of triplicate samples over 19 days (N=57) are shown together with standard error bars. The $\bar{X}$ OD of control populations is represented by the horizontal line.

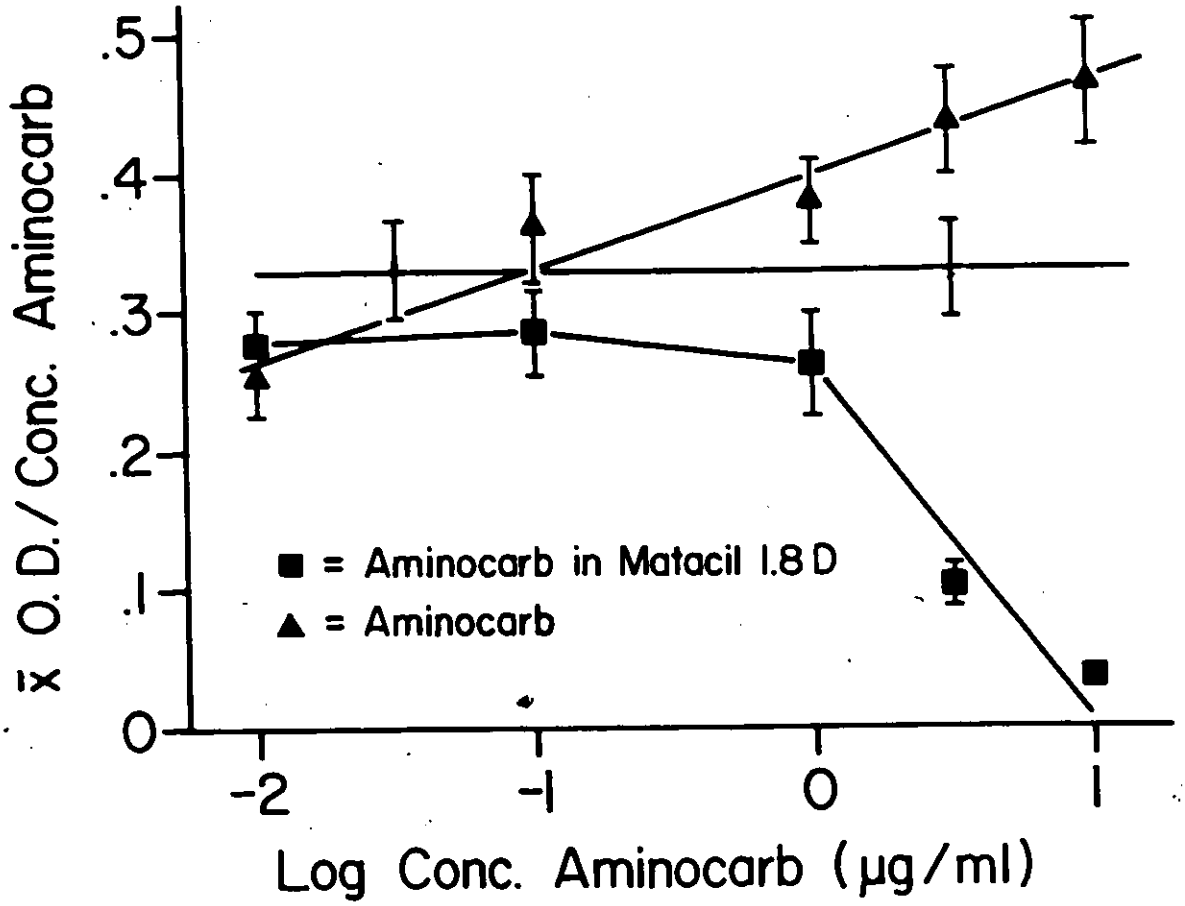

x O.D./Conc. Aminocarb
.5
.4
.3
.2
.1
0
-2
-1
0
1
■ = Aminocarb in Matacil 1.8 D
▲ = Aminocarb
Log Conc. Aminocarb (µg/ml)

Figure 7. Growth rates of Chlorella populations exposed to .01, .1, 1.0, 3.0, or 10.0 µg/ml aminocarb or aminocarb in Matacil 1.8D. the average values for the first six days of growth of triplicate samples are shown together with standard deviation bars. The growth rate of control populations is represented by the horizontal line.

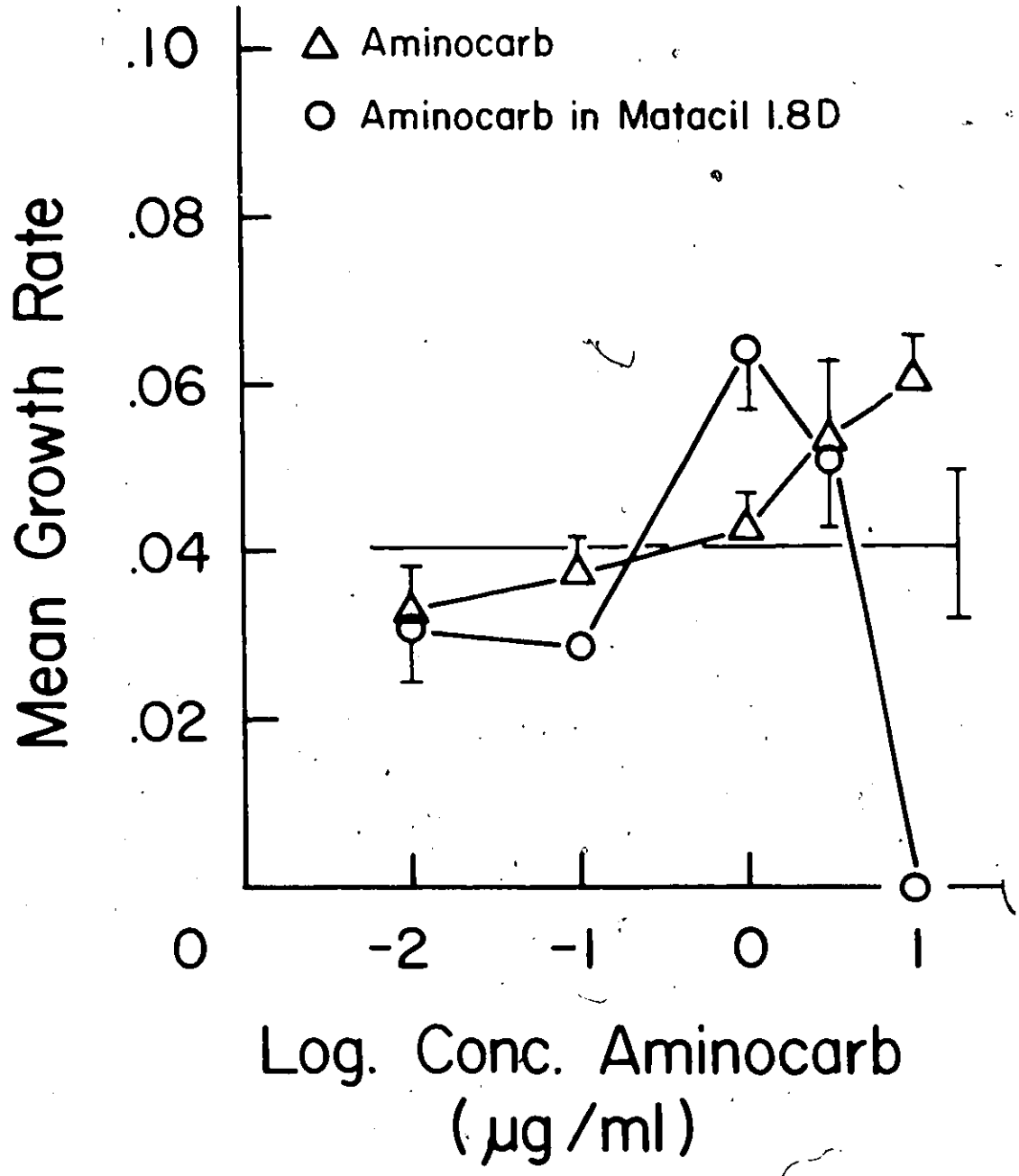

Δ Aminocarb
O Aminocarb in Matacil 1.8D
Mean Growth Rate
.10
.08
.06
.04
.02
0
-2
-1
0
1
Log. Conc. Aminocarb
(µg/ml)

Figure 8. Growth curves of Chlorella populations exposed to .015 µg/ml diluent oil or .025 µg/ml nonylphenol versus untreated (control) populations. The average values of triplicate samples are shown together with standard deviation bars for days 14, 20, and 25.

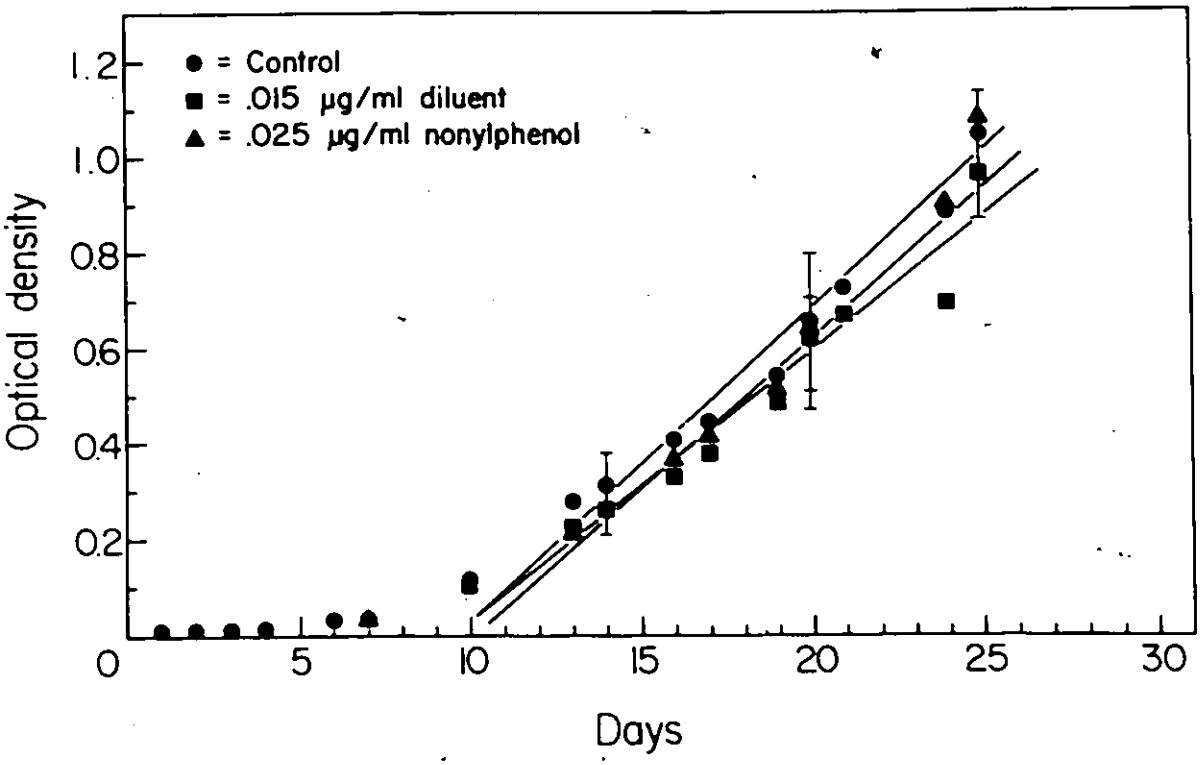

● = Control
■ = .015 µg/ml diluent
▲ = .025 µg/ml nonylphenol
Optical density
1.2
1.0
0.8
0.6
0.4
0.2
0
5
10
15
20
25
30
Days

Figure 9. Growth curves of Chlorella populations exposed to .15 µg/ml diluent oil or .25 µg/ml nonylphenol versus untreated (control) populations. The average values of triplicate samples are shown together with standard deviation bars for days 14, 20, and 25.

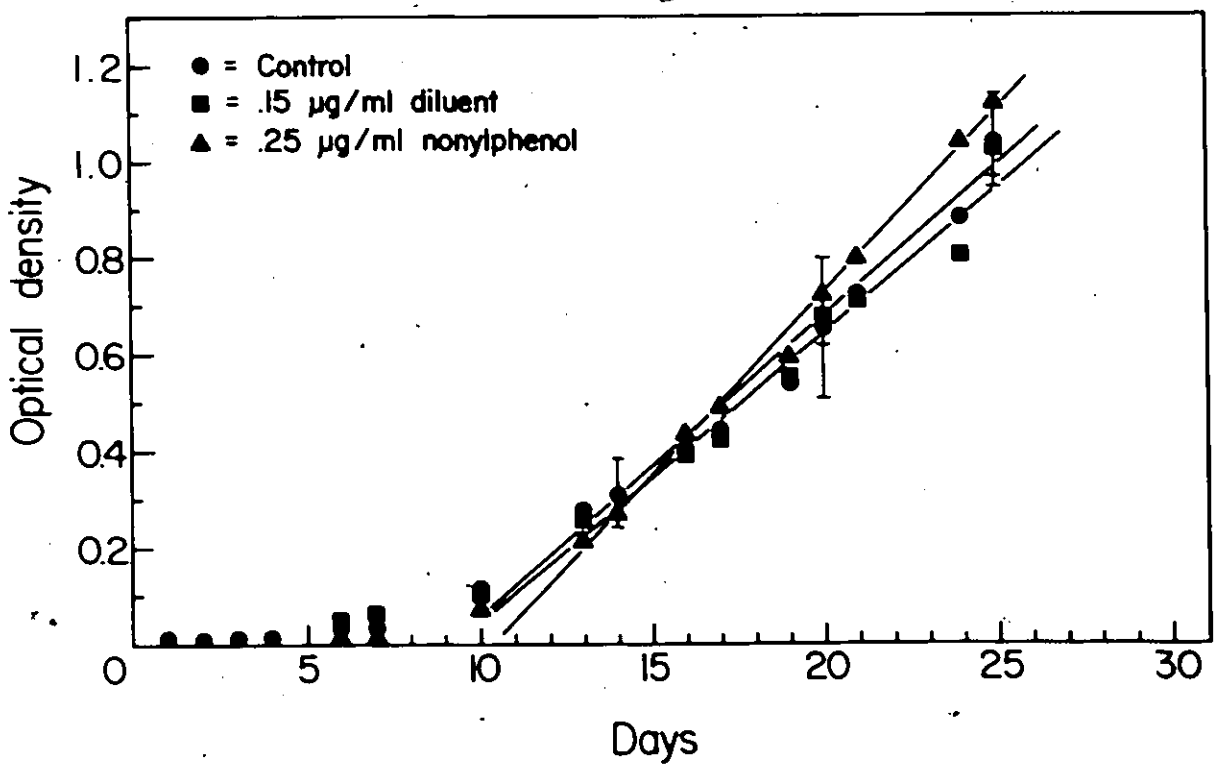

● = Control
■ = .15 µg/ml diluent
▲ = .25 µg/ml nonylphenol
Optical density
1.2
1.0
0.8
0.6
0.4
0.2
0
5
10
15
20
25
30
Days

Figure 10. Growth curves of Chlorella populations exposed to 1.5 µg/ml diluent oil or 2.5 µg/ml nonylphenol versus untreated (control) populations. The average values of triplicate samples are shown together with standard deviation bars for days 14, 20, and 25.

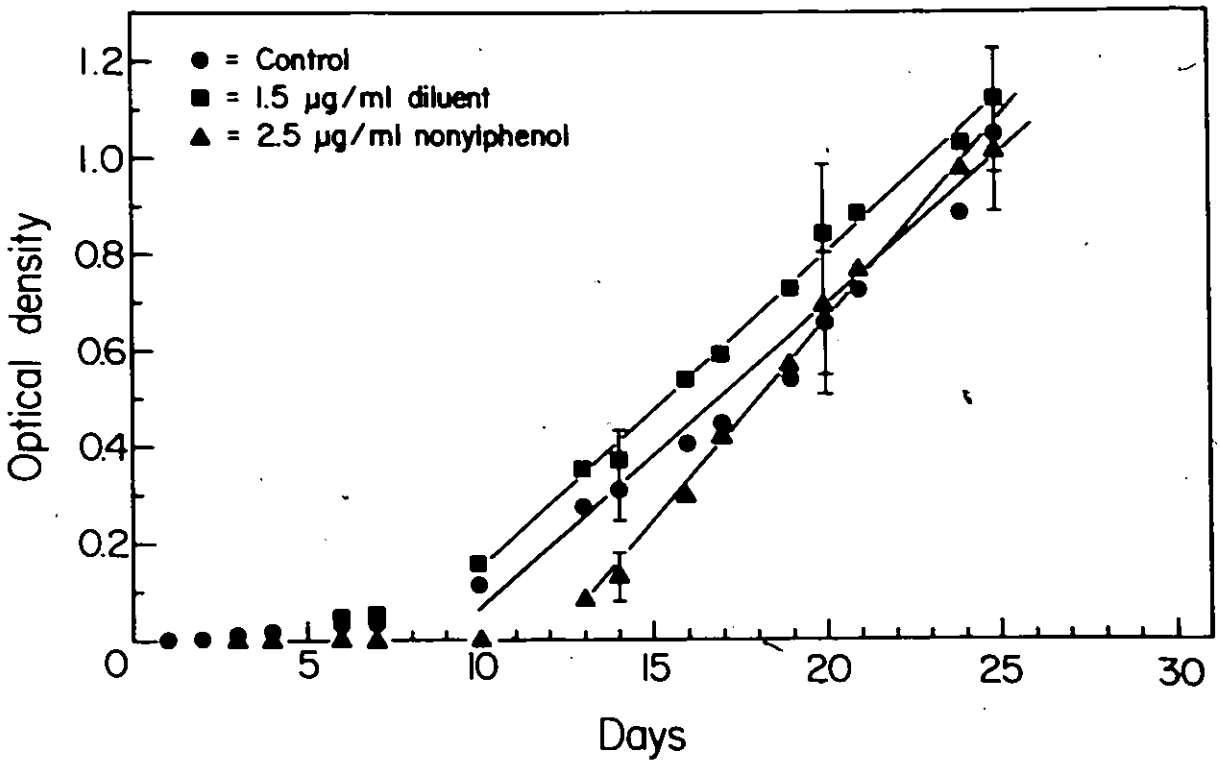

● = Control
■ = 1.5 µg/ml diluent
▲ = 2.5 µg/ml nonylphenol
Optical density
1.2
1.0
0.8
0.6
0.4
0.2
0
5
10
15
20
25
30
Days

Figure 11. Growth curves of Chlorella populations exposed to 4.5 µg/ml diluent oil or 7.5 µg/ml nonylphenol versus untreated (control) populations. The average values of triplicate samples are shown together with standard deviation bars for days 14, 20, and 25.

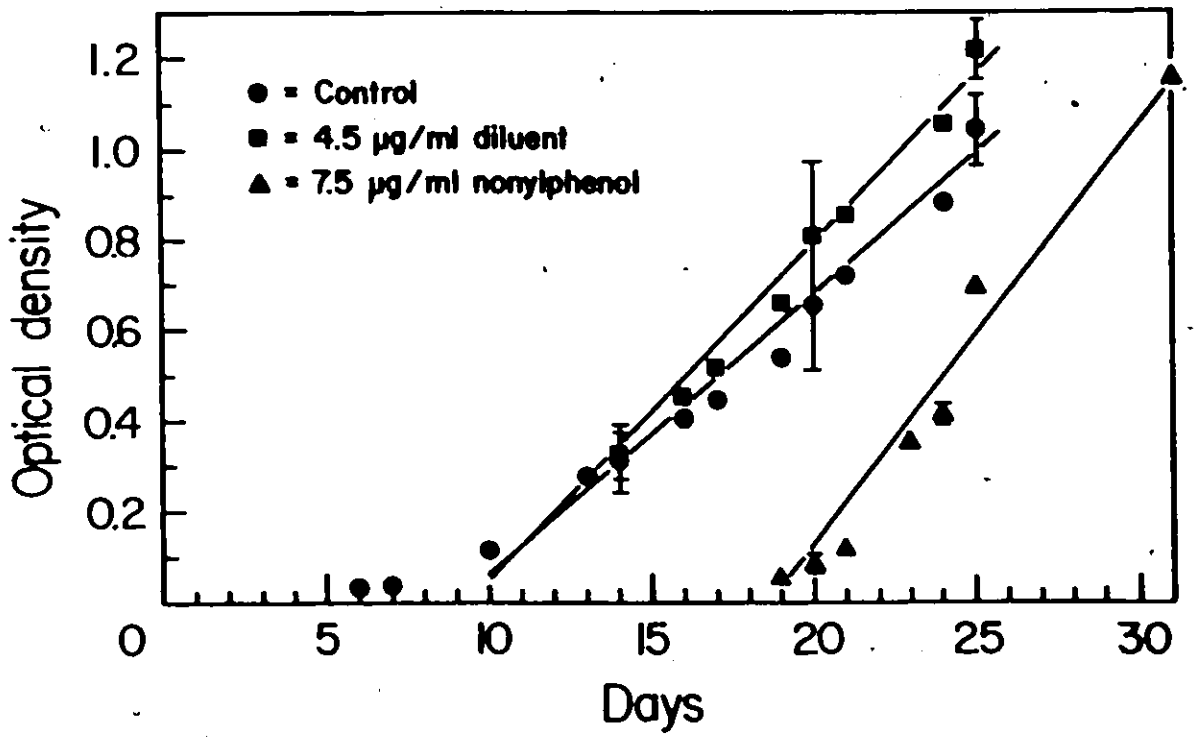

● = Control
■ = 4.5 µg/ml diluent
▲ = 7.5 µg/ml nonylphenol
Optical density
1.2
1.0
0.8
0.6
0.4
0.2
0
5
10
15
20
25
30
Days

Figure 12. Growth curves of Chlorella populations exposed to 15.0 µg/ml diluent oil or 25.0 µg/ml nonylphenol versus untreated (control) populations. The average values of triplicate samples are shown together with standard deviation bars for days 14, 20, and 25.

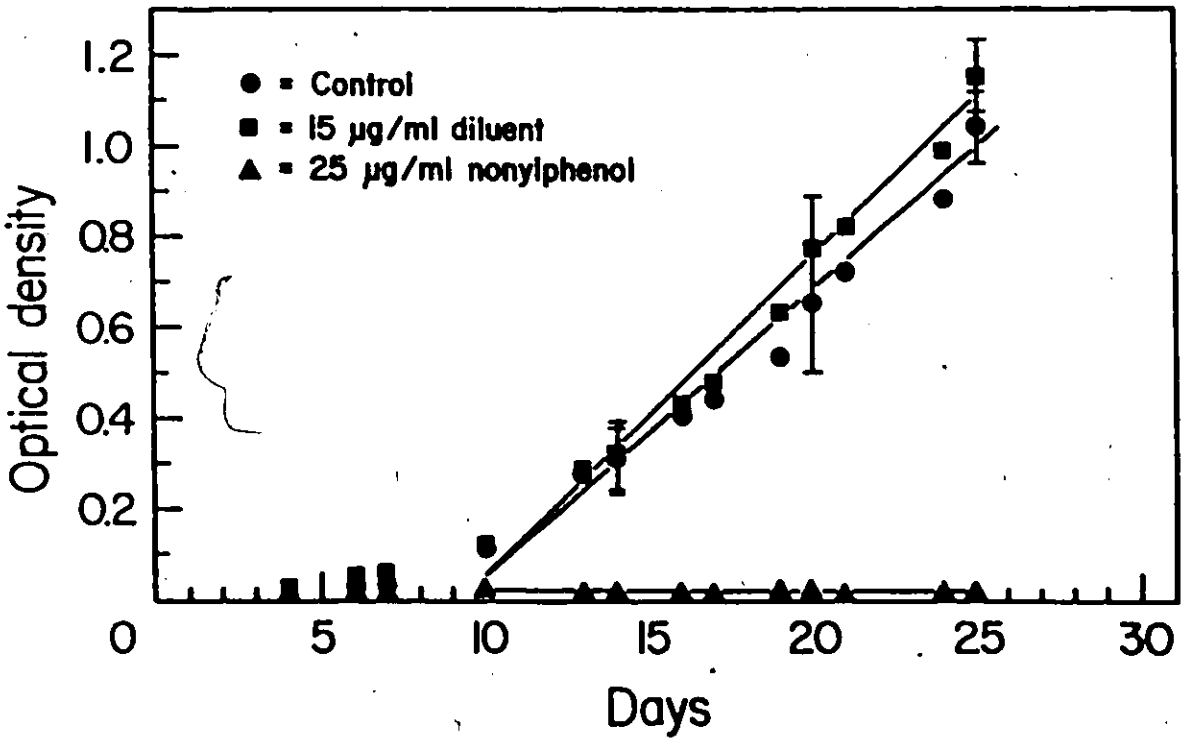

● = Control
■ = 15 µg/ml diluent
▲ = 25 µg/ml nonylphenol
Optical density
1.2
1.0
0.8
0.6
0.4
0.2
0
5
10
15
20
25
30
Days

Figure 13. Determination of 24 hr TC_{50} of Chlorella populations exposed to 0.0, 2.5, 5.0, or 7.5 µg/ml nonylphenol. The average values of triplicate samples are shown.

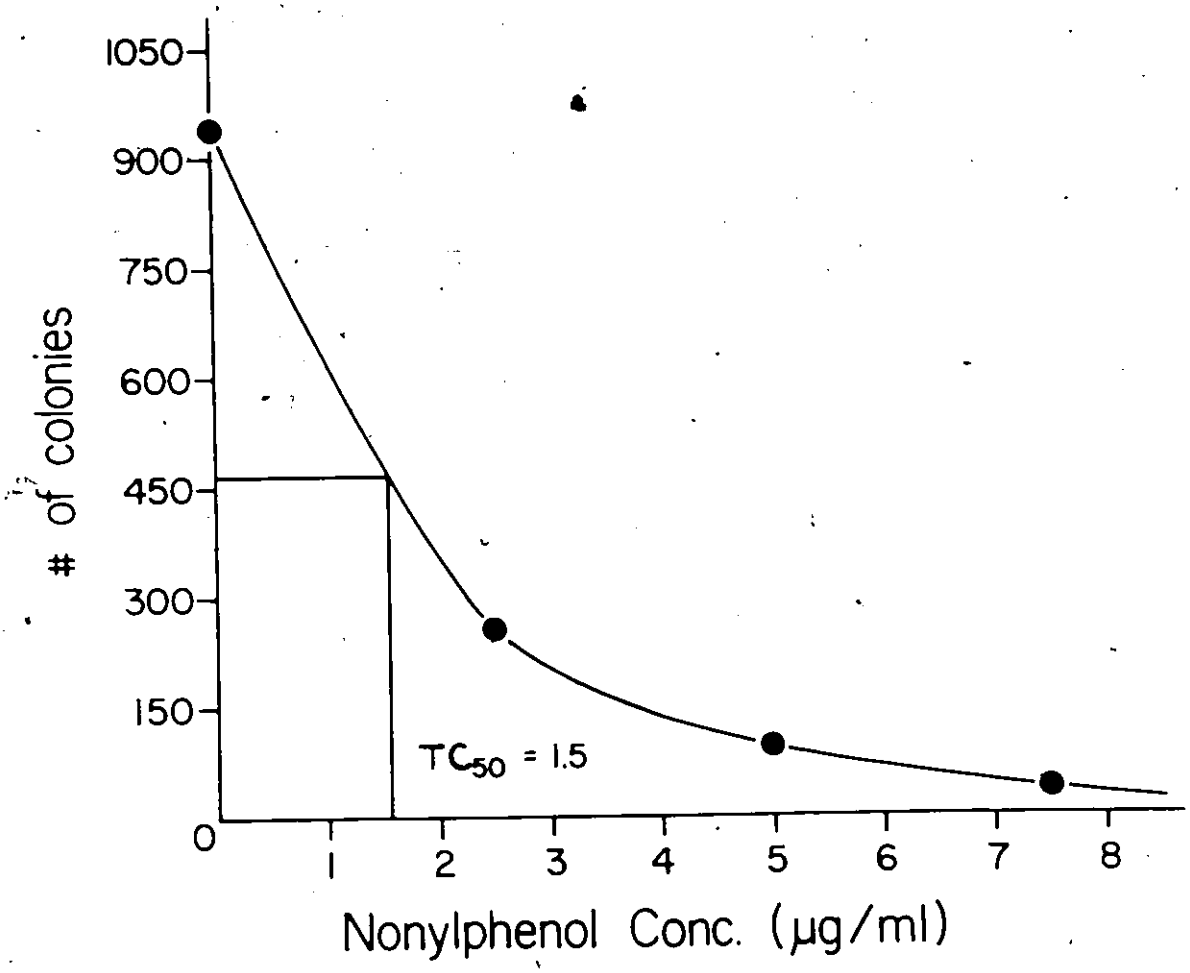

1050
900
750
600
450
300
150
0
# of colonies
TC50 = 1.5
1
2
3
4
5
6
7
8
Nonylphenol Conc. (µg/ml)

Figure 14. The mean optical density ($\bar{X}$ OD) of Chlorella populations exposed to concentations of diluent oil or nonylphenol equivalent to those found in .01, .1, 1.0, 3.0, or 10.0 µg/ml aminocarb in Matacil 1.8D. The concentrations of diluent oil or nonylphenol are indicated above or below the data points, respectively. The average values of triplicate samples over 25 days (N=75) are shown together with standard error bars. The $\bar{X}$ OD of control populations is represented by the horizontal line.

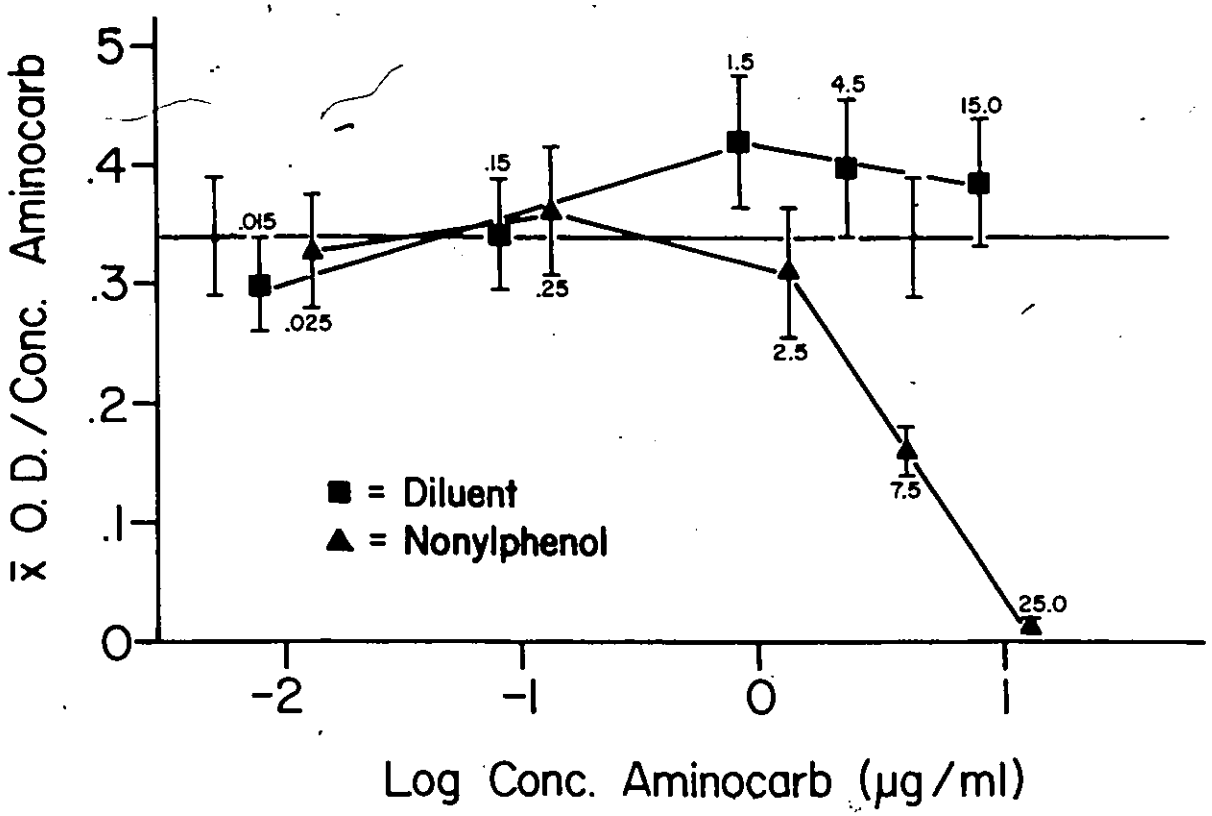

x O.D./Conc. Aminocarb
5
.4
.3
.2
.1
0
-2
-1
0
1
Log Conc. Aminocarb (µg/ml)
■ = Diluent
▲ = Nonylphenol
.015
.025
.15
.25
1.5
2.5
4.5
7.5
15.0
25.0

Figure 15. Growth rates of Chlorella populations exposed to concentrations of diluent oil or nonylphenol equivalent to those found in .01, .1, 1.0, 3.0, or 10.0 µg/ml aminocarb in Matacil 1.8D. The concentrations of nonylphenol or diluent oil are indicated above or below the data points, respectively. The average values for the first six days of growth of triplicate samples are shown together with standard deviation bars. The growth rate of control populations is represented by the horizontal line.

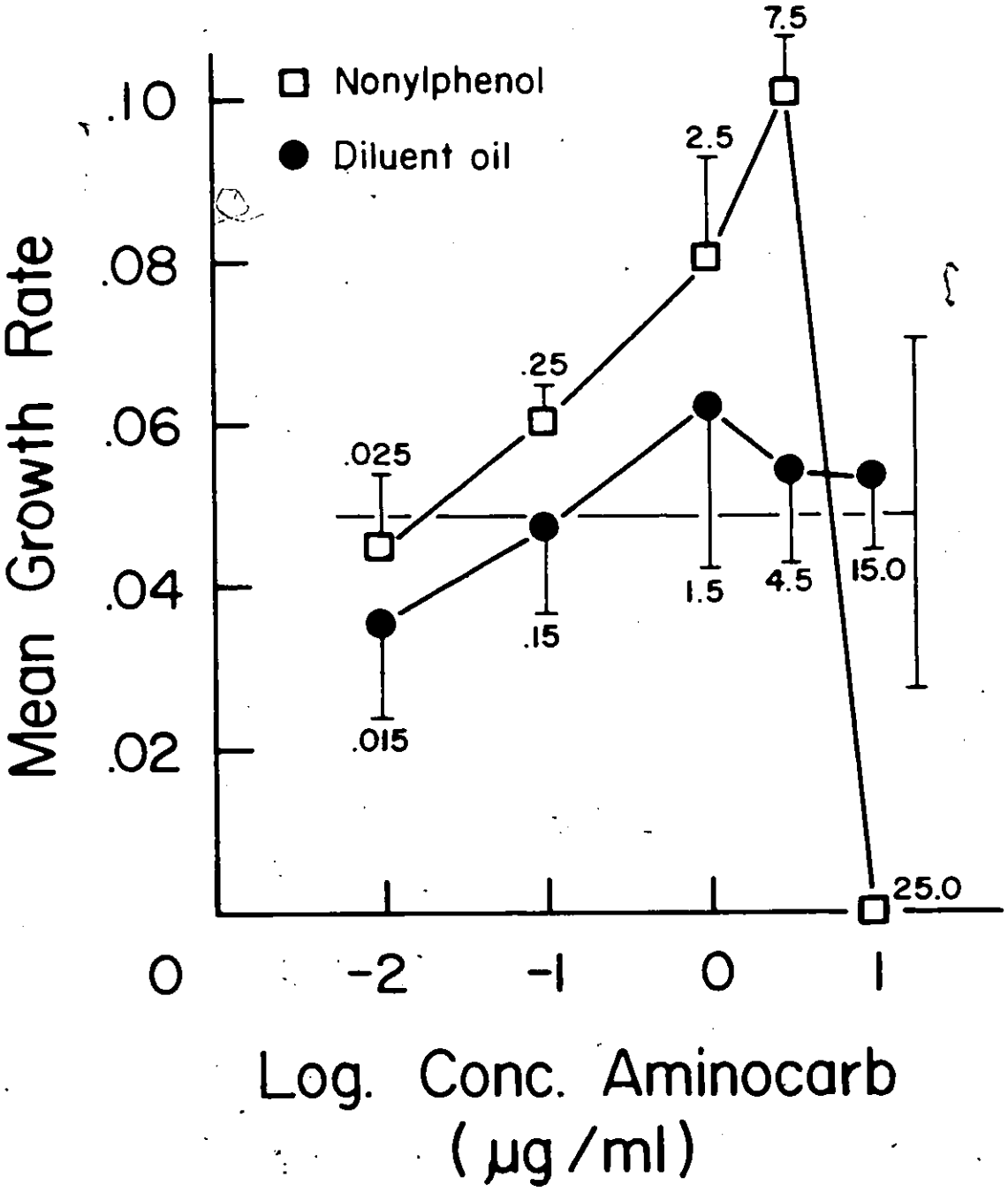

Nonylphenol
Diluent oil
Mean Growth Rate
.10
.08
.06
.04
.02
0
-2
-1
0
1
Log. Conc. Aminocarb
(µg/ml)
7.5
2.5
.25
.025
.015
.15
1.5
4.5
15.0
25.0

Figure 16. Growth curves of Chlamydomonas populations exposed to 1.0 µg/ml (a), 3.0 µg/ml (b), or 10.0 µg/ml (c) aminocarb versus untreated (control) populations. The average values of triplicate samples are shown together with standard deviation bars for days 7 and days 12 or 14.

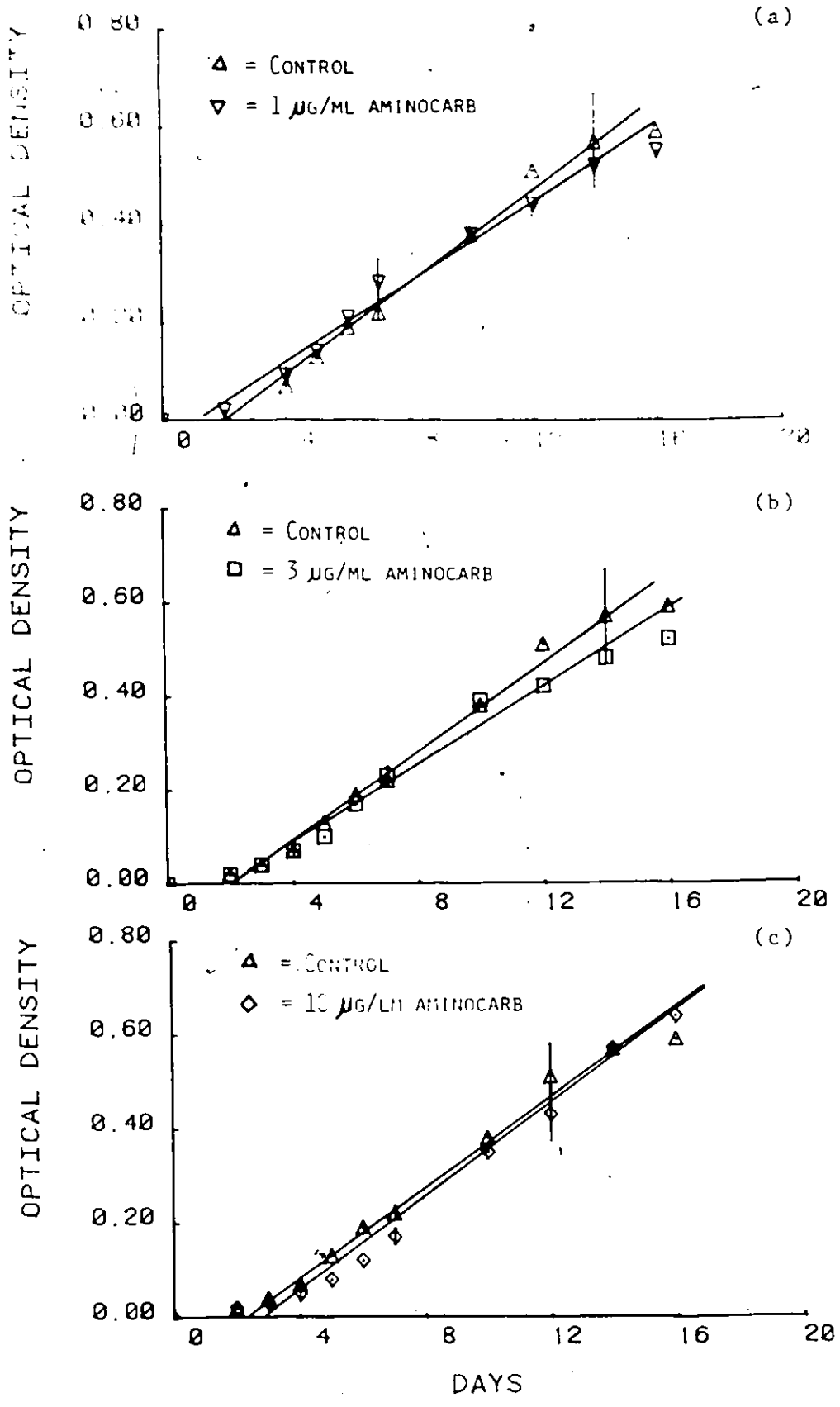

(a)
Δ = CONTROL
▽ = 1 µG/ML AMINOCARB
(b)
Δ = CONTROL
□ = 3 µG/ML AMINOCARB
(c)
Δ = CONTROL
◇ = 10 µG/ML AMINOCARB
OPTICAL DENSITY
0.80
0.60
0.40
0.20
0.00
0
4
8
12
16
20
DAYS

Figure 17. Growth rates of Chlamydomonas populations exposed to 1.0, 3.0, or 10.0 µg/ml aminocarb or aminocarb in Matacil 1.8D. The average values for the first five days of growth of triplicate samples are shown together with standard deviation bars. The growth rate of control populations is represented by the horizontal line.

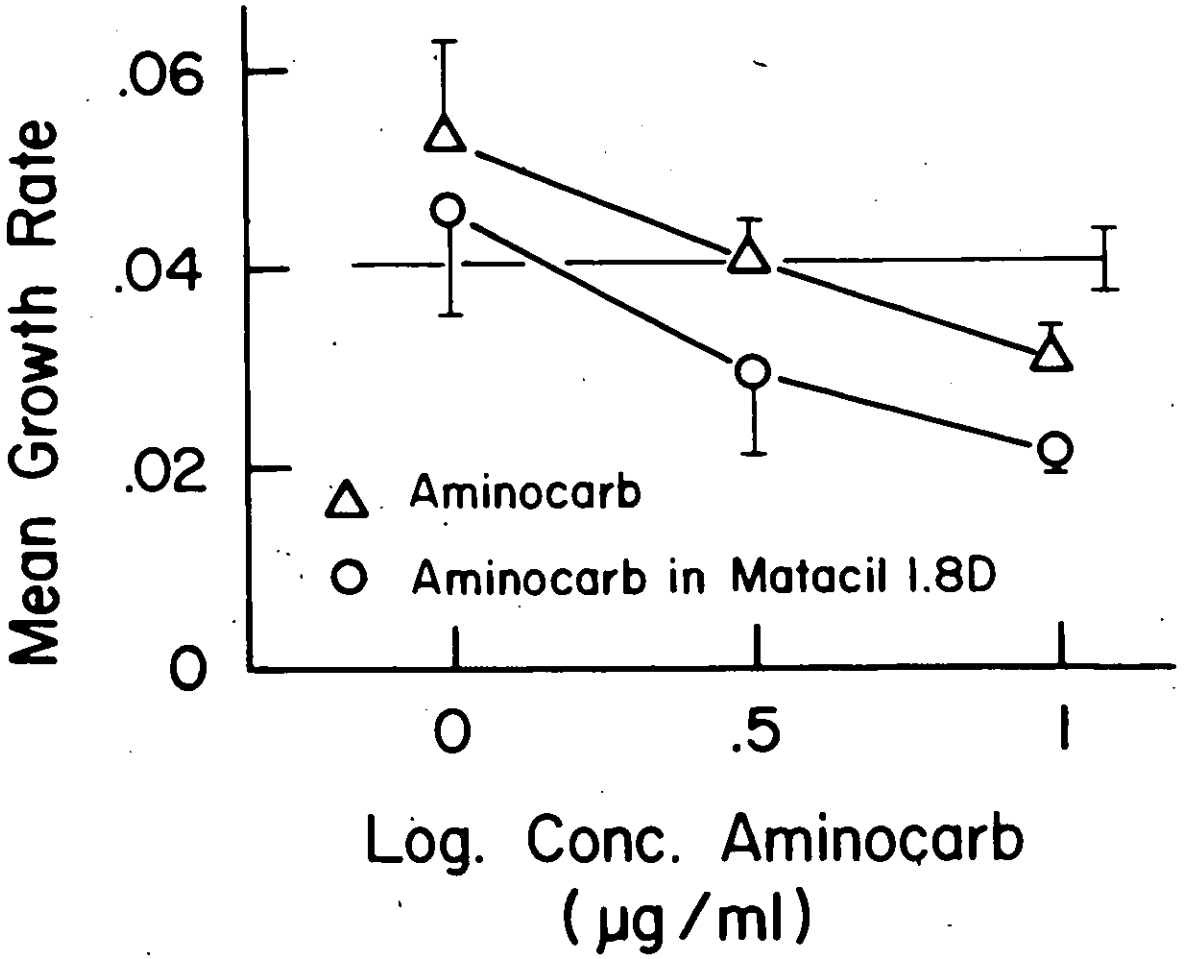

Mean Growth Rate
.06
.04
.02
0
Aminocarb
Aminocarb in Matacil 1.8D
0
.5
1
Log. Conc. Aminocarb
(µg/ml)

Figure 18. The mean optical density ($\bar{X}$ OD) of Chlamydomonas populations exposed to 1.0, 3.0, or 10.0 µg/ml aminocarb or aminocarb in Matacil 1.8D. The average values of triplicate samples over 16 days (N=48) are shown together with standard error bars. The $\bar{X}$ OD of control populations is represented by the horizontal line.

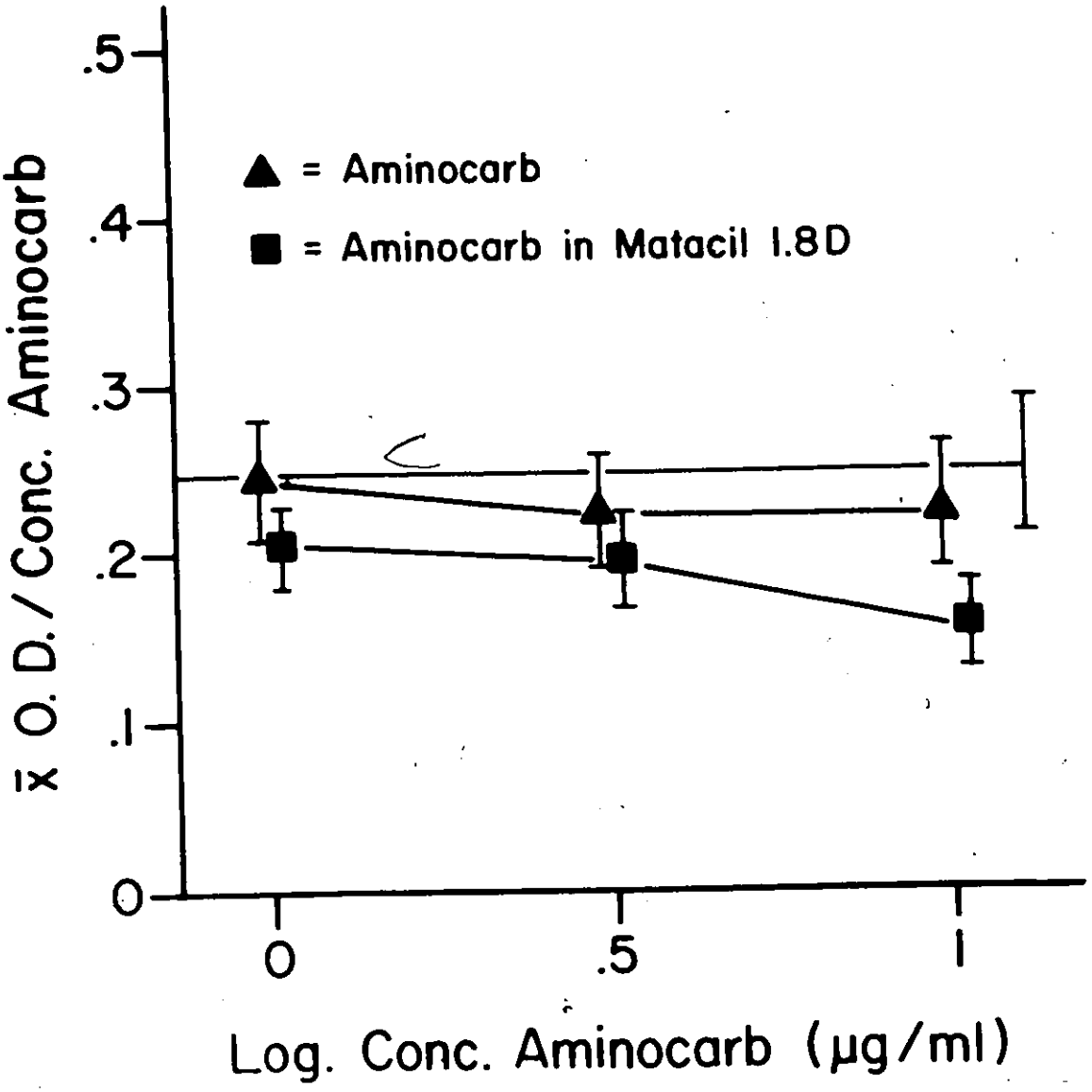
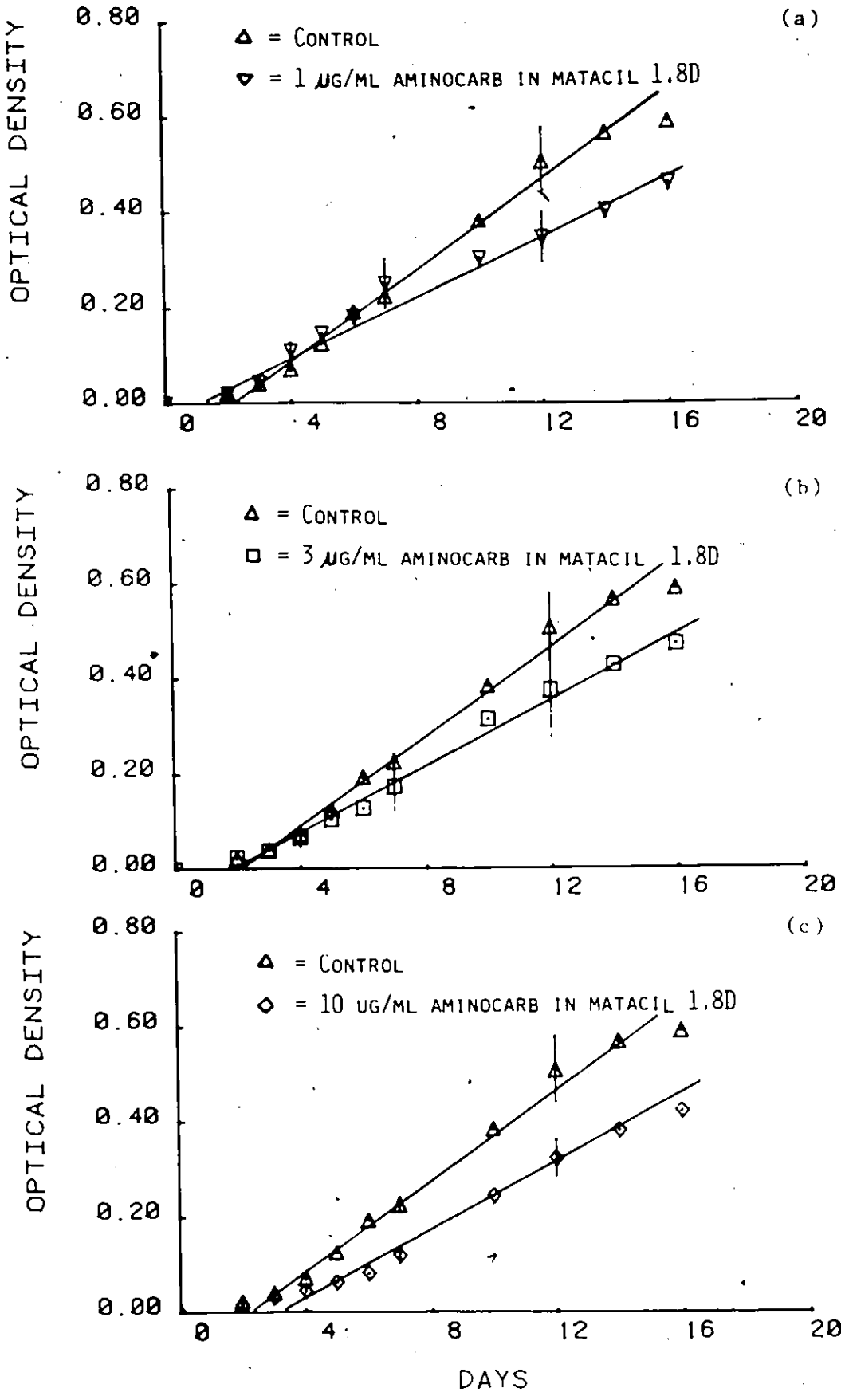

.5
.4
.3
.2
.1
0
x̄ O.D./Conc. Aminocarb
▲ = Aminocarb
■ = Aminocarb in Matacil 1.8D
0
.5
1
Log. Conc. Aminocarb (µg/ml)

Figure 19. Growth curves of Chlamydomonas populations exposed to 1.0 µg/ml (a), 3.0 µg/ml (b), or 10.0 µg/ml (c) aminocarb in Matacil 1.8D versus untreated (control) populations. The average values of triplicate samples are shown together with standard deviation bars for days 7 and 12.


(a)
OPTICAL DENSITY
0.80
0.60
0.40
0.20
0.00
△ = CONTROL
▽ = 1 µG/ML AMINOCARB IN MATACIL 1.8D
0
4
8
12
16
20
(b)
OPTICAL DENSITY
0.80
0.60
0.40
0.20
0.00
△ = CONTROL
□ = 3 µG/ML AMINOCARB IN MATACIL 1.8D
0
4
8
12
16
20
(c)
OPTICAL DENSITY
0.80
0.60
0.40
0.20
0.00
△ = CONTROL
◇ = 10 UG/ML AMINOCARB IN MATACIL 1.8D
0
4
8
12
16
20
DAYS

3.2 Cell Size Distribution

19 days post-treatment all populations of Chlorella growing in any of the range of concentrations of aminocarb tested (.01-10.0 µg/ml) contained a greater percentage of small cells than control populations (Table 1). The concentration of pesticide within this range had no effect, such that increasing amounts of aminocarb did not significantly alter the distribution of cell sizes, although populations exposed to 10.0 µg/ml aminocarb contained a greater percentage of cells in the largest size range than any other treated populations or control populations.

The size range distribution of populations exposed to .01 and.1 µg/ml aminocarb in Matacil 1.8D were similar to the aminocarb treated populations in that they also contained a greater percentage of small cells compared to the control sets. However, the distribution of cell sizes in populations treated with 1.0 and 3.0 µg/ml aminocarb in the formulation mix was similar to the control populations.

TABLE 1

Cell size distribution of Chlorella populations exposed to .01, .1, 1.0, 3.0, or 10.0 µg/ml aminocarb or aminocarb in Matacil 1.8D. The average values of four chamber counts of the Levy-Neubauer hemacytometer for triplicate samples (N=12) are reported.

	% Total Cells			
	0-3.7 µm[a]	3.75-7.4 µm[a]	7.45 µm[a]	N[b]
Control	11.7	88.3	0	213
Aminocarb				
.01 µg/ml	27.5	72.5	0	284
.1	19.3	80.4	.3	327
1.0	24.4	75.1	.5	422
3.0	23.8	76.2	0	450
10.0	22.3	75.3	2.4	336
Aminocarb in Matacil 1.8D				
.01 (.025)[c]	22.3	77.2	.5	224
.1 (.25)[c]	25.5	74.5	0	286
1.0(2.5)[c]	15.2	84.8	0	448
3.0(7.5)[c]	16.5	83.3	.2	557
10.0(25)[c]	0	0	0	0

[a] Diameter of Chlorella cells
[b] Total number of cells measured in all samples
[c] Concentration (µg/ml) of nonylphenol in Matacil 1.8D

3.3 Chlorophyll (Chl) Content

The total chlorophyll content of Chlorella treated with 3.0 µg/ml aminocarb was not significantly affected over the six day period (Table 2). On day 4, however, treated cells did contain significantly more chlorophyll. This difference was only temporary because the chlorophyll content of the treated populations was again similar to the control populations by day 6.

The amount of Chl_a fluctuated over the treatment period for both populations with no differences between control and treated sets except, again, on day 4 (Fig. 20). The average amount of Chl_a per day was similar for treated cells and untreated controls (Table 3). The amount of Chl_b remained fairly constant throughout the six days for treated and control populations with no differences between the daily average of both populations.

TABLE 2

Total chlorophyll (mg/l x 10^7 cells) of Chlorella populations exposed to 3.0 µg/ml aminocarb versus untreated (control) populations. The average values of triplicate samples are reported ± the standard deviation.

Day	Control	Treated	% Control
0	1.42 ± .032	1.36 ± .026	95
1	1.18 ± .009	1.24 ± .056	105
2	1.12 ± .043	1.20 ± .004	108
3	1.02 ± .010	.94 ± .152	93
4	1.03 ± .103	1.34 ± .355	130
6	1.33 ± .031	1.27 ± .059	96

Figure 20. Chl_a and Chl_b content of Chlorella populations exposed to 3.0 µg/ml aminocarb versus untreated (control) populations. The average values of triplicate samples are shown together with standard deviation bars.

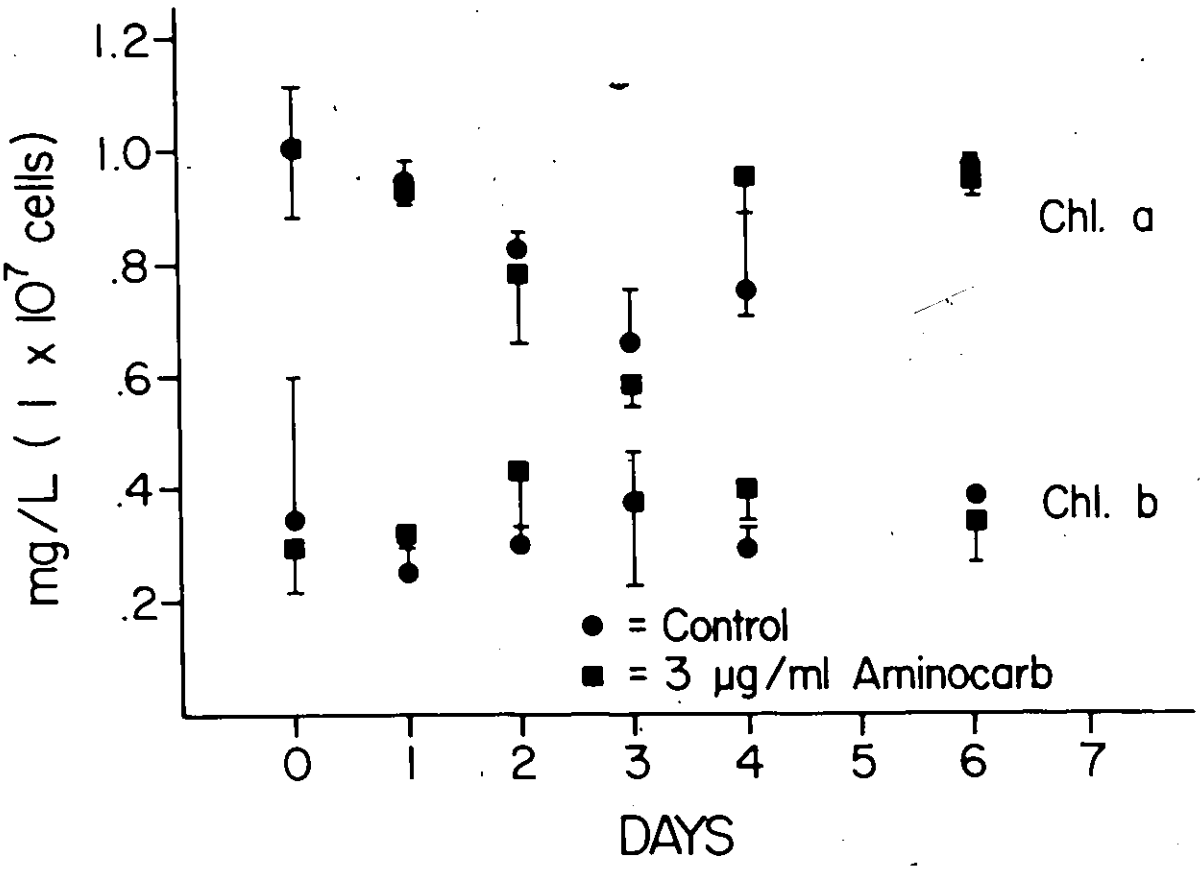

1.2
1.0
.8
.6
.4
.2
mg/L (1 x 10⁷ cells)
Chl. a
Chl. b
● = Control
■ = 3 µg/ml Aminocarb
0
1
2
3
4
5
6
7
DAYS

TABLE 3

Chl_a or Chl_b (mg/l x 10^7 cells) of Chlorella populations exposed to 3.0 µg/ml aminocarb versus untreated (control) populations. The average values of triplicate samples over 6 days (N=18) are reported ± standard deviations.

	Control	Treated
Chl_a	.866 ± .172	.872 ± .194
Chl_b	.319 ± .096	.353 ± .082

3.4 Uptake of C^{14}-Aminocarb

3.4.1 Effects of formulation

Uptake of the pesticide into the Chlorella cells was determined by direct C^{14} counts of the water and algae (corrected for their respective quench). Since the populations were growing throughout the experiment an increasing dpm over time may not solely be due to accumulation of C^{14}-aminocarb by the cells but may also be due to uptake by newly formed cells. Thus, if cell number doubled, then the dpm would double. Both populations exposed to the formulated pesticide or the non-formulated pesticide accumulated C^{14}-aminocarb over the seven day treatment period because the dpm increased by a greater factor, 20X and 12X, respectively, than population growth (Table 4). Moreover, the C^{14}-aminocarb accumulation over time was linear for the populations treated with aminocarb (r=.9861) and for the populations treated with the aminocarb formulated as Matacil 1.8D (r=.9912) (Fig. 21).

The uptake of C^{14}-aminocarb by populations exposed to the formulated pesticide was significantly greater than the uptake by populations exposed to the nonformulated pesticide even though the populations treated with Matacil 1.8D had lower cell numbers per day than the aminocarb treated populations (Fig. 21). After seven days post-treatment 1 x 10^6 Chlorella cells accumulated 0.10% of the added C^{14}-aminocarb in the non-formulated pesticide and 0.18% in the formulated pesticide. Thus, 1 x 10^6 cells had the capacity to

accumulate 7.0×10^{-2} µg of the total aminocarb in the non-formulated pesticide and 1.2×10^{-1} µg in the formulated pesticide.

3.4.2 Dead Cells

Uptake of C^{14}-aminocarb by freeze-killed Chlorella cells maintained in 3.0 µg/ml aminocarb was maximized by day 3 and remained fairly constant throughout the following two days (Fig. 21). On day 1 the uptake by the dead populations was similar to the uptake by populations exposed to the same concentration or pesticide presented as aminocarb or aminocarb in Matacil 1.8D. However, the accumulation of C^{14}-aminocarb by cells treated with the formulated pesticide or the non-formulated pesticide exceeded uptake by the dead cells on day 2 and day 3, respectively.

3.4.3 Effects of cell concentration

The density of cells had no effect on the accumulation of C^{14}-aminocarb by Chlorella populations growing in 3.0 µg/ml aminocarb (Table 5). Growth rate was inversely related to population density such that by Day 7 the ratio of initial cell densities (1:2:3) was no longer maintained. Increasing the initial cell concentration two or three fold produced no significant difference ($p > .05$) in the per cell uptake of the labelled pesticide by these populations over seven days

such that within the range of cell populations 1 x 10^6 to 5.73 x 10^6 uptake was not a function of cell number.

3.4.4 Binding of C^{14}-aminocarb

Tripling the amount of distilled water used to wash the algal samples before scintillation counting produced no significant difference in the dpm per day count for Chlorella populations growing in 3.0 µg/ml aminocarb (Fig. 22). The amount of C^{14}-aminocarb associated with the unwashed cell samples was significantly greater ($p<.05$) than washed samples over seven days. However, the pattern of C^{14}-aminocarb accumulation by the unwashed samples was similar to the uptake pattern of the washed samples. The differences between the dpm per day count of washed samples and unwashed samples decreased over the seven day period. The mean dpm per day of the washed samples increased from 6% of the unwashed samples at day 0 to 66% by day 7 (Table 6). In other words, 94% of the total dpm associated with the cell samples could be removed by washing on day 0, whereas, only 34% of the total dpm could be removed by washing on day 7.

TABLE 4

Uptake of C^{14}-aminocarb versus cell number of Chlorella populations exposed to 3.0 µg/ml aminocarb or aminocarb in Matacil 1.8D. The average values of triplicate samples are reported.

	Aminocarb		Aminocarb in Matacil 1.8D	
Day	$\bar{X}$ dpm/ml	$\bar{X}$ cell #/ml[a]	$\bar{X}$ dpm/ml	$\bar{X}$ cell #/ml[a]
0	347	3.00	337	3.00
1	636	3.24	1110	3.00
2	1939	4.30	3009	3.15
3	4549	4.93	—	—
4	—	—	6412	3.50
5	5955	5.03	—	—
7	8740	5.73	13,721	4.99

[a] All values x 10^6.

Figure 21. Uptake of C^{14}-aminocarb by Chlorella populations exposed to 3.0 µg/ml aminocarb or aminocarb in Matacil 1.8D and uptake by freeze-killed cells exposed to 3.0 µg/ml aminocarb. The average values of triplicate samples are shown together with standard deviation bars.

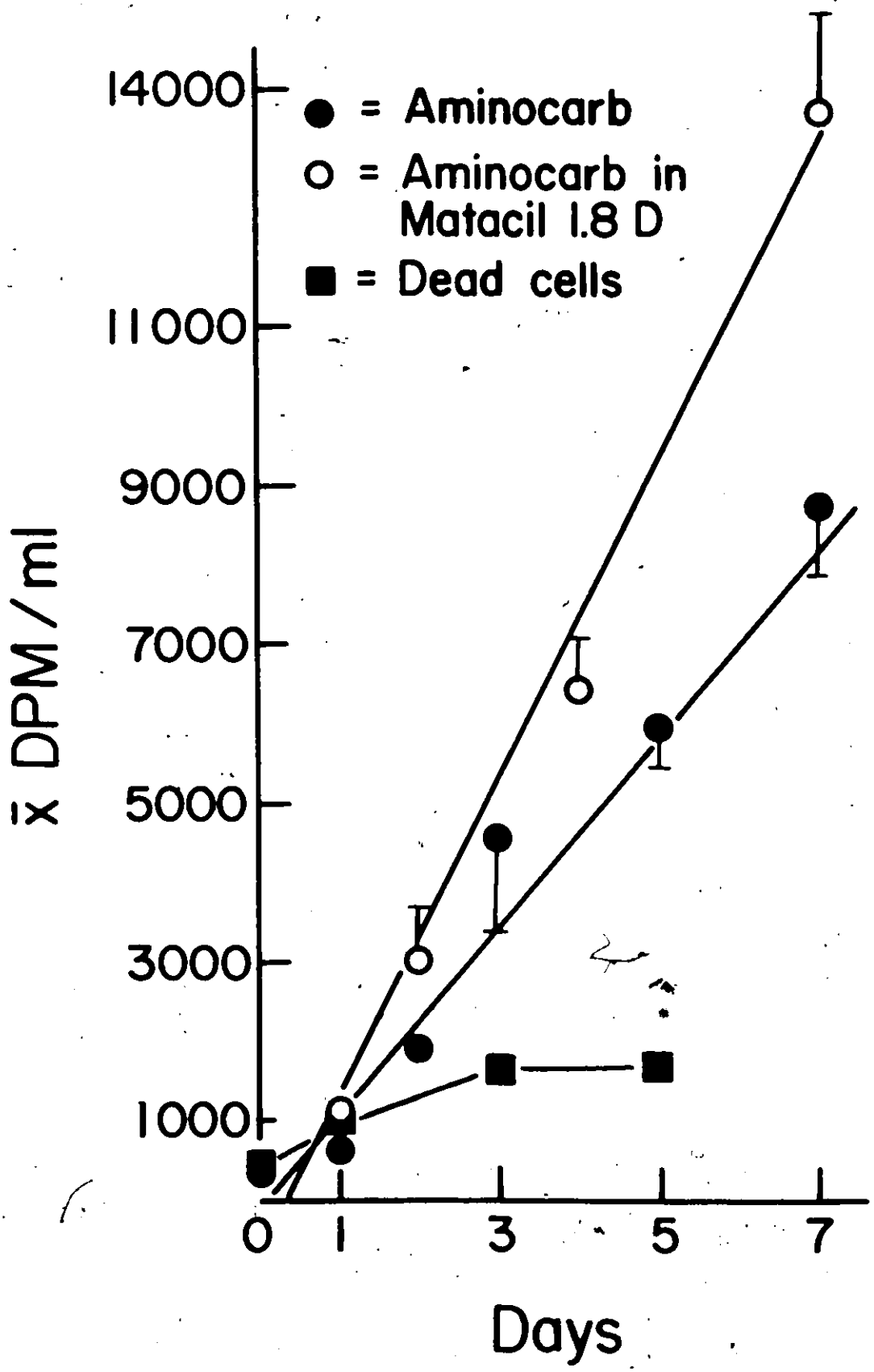

14000
11000
9000
7000
5000
3000
1000
x̄ DPM/ml
● = Aminocarb
○ = Aminocarb in Matacil 1.8 D
■ = Dead cells
0
1
3
5
7
Days

TABLE 5

Uptake of C^{14}-aminocarb ($\bar{X}$ dpm/10^6 cells) by different concentrations of Chlorella populations exposed to 3.0 µg/ml aminocarb. The average values of triplicate samples are reported ± standard deviations.

Day	1×10^6a	2×10^6a	3×10^6a
0	246 ± 89	186 ± 30	115 ± 7
1	273 ± 70	184 ± 36	197 ± 6
2	363 ± 54	272 ± 34	451 ± 12
3	583 ± 56	581 ± 57	923 ± 239
5	1073 ± 105	1213 ± 82	1184 ± 95
7	1403 ± 103	1710 ± 207	1525 ± 150
	3.23×10^6b	5.23×10^6b	5.73×10^6b

Note: $P > .05$ for all values as determined by ANOVA and Tukey multiple range test.

a initial cell concentration in cells/ml
b final cell concentration in cells/ml

Figure 22. Binding of C^{14}-aminocarb by Chlorella populations exposed to 3.0 µg/ml aminocarb. The average values of triplicate samples are shown together with standard deviation bars.

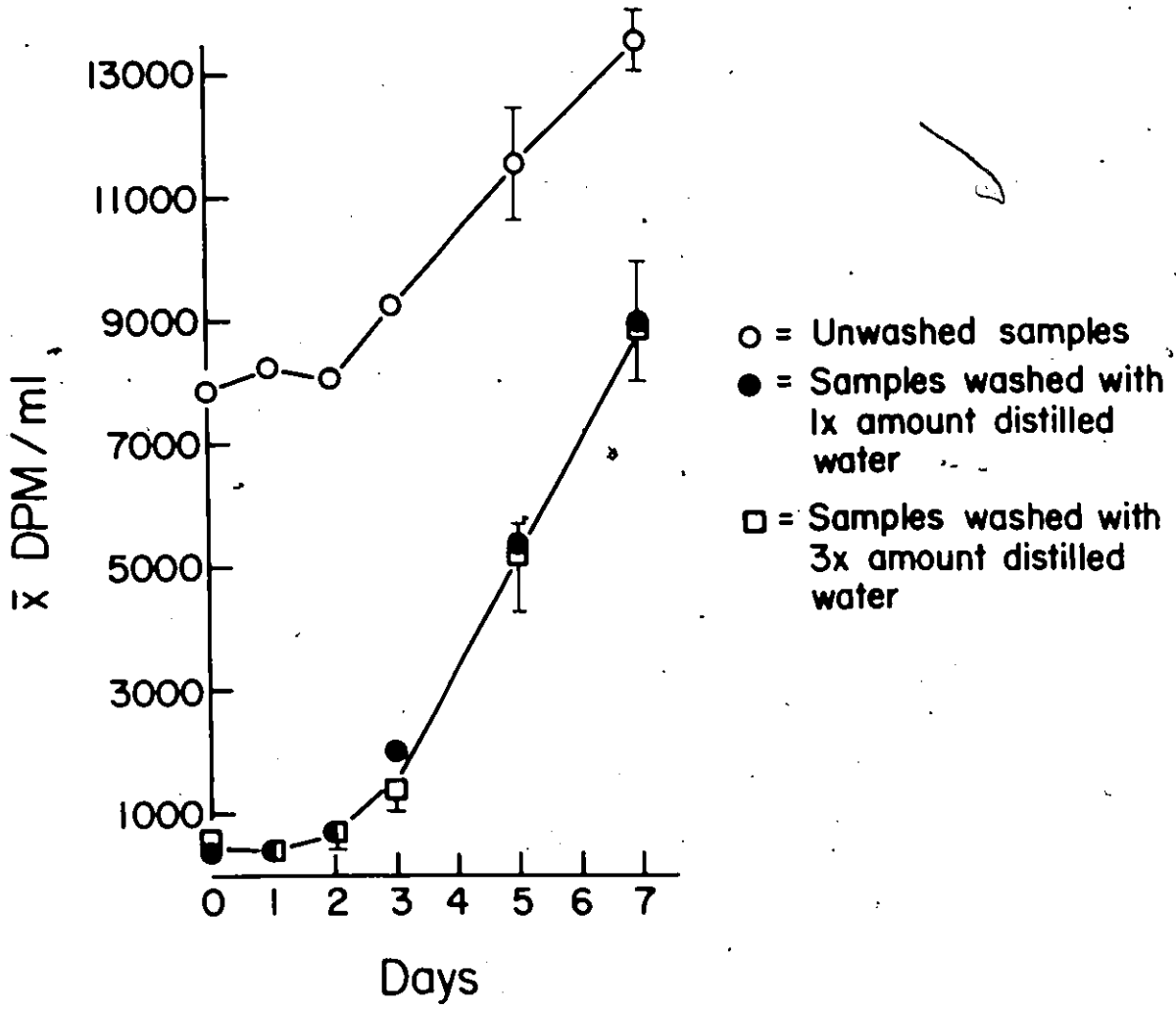

13000
11000
9000
7000
5000
3000
1000
1x DPM/ml
0 1 2 3 4 5 6 7
Days
○ = Unwashed samples
● = Samples washed with 1x amount distilled water
□ = Samples washed with 3x amount distilled water

TABLE 6

Binding of C^{14}-aminocarb by *Chlorella* populations exposed to 3.0 µg/ml aminocarb. The average values ($\overline{X}$ dpm/ml) of all washed samples (N=6) or unwashed samples (N=3) are reported ± standard deviations.

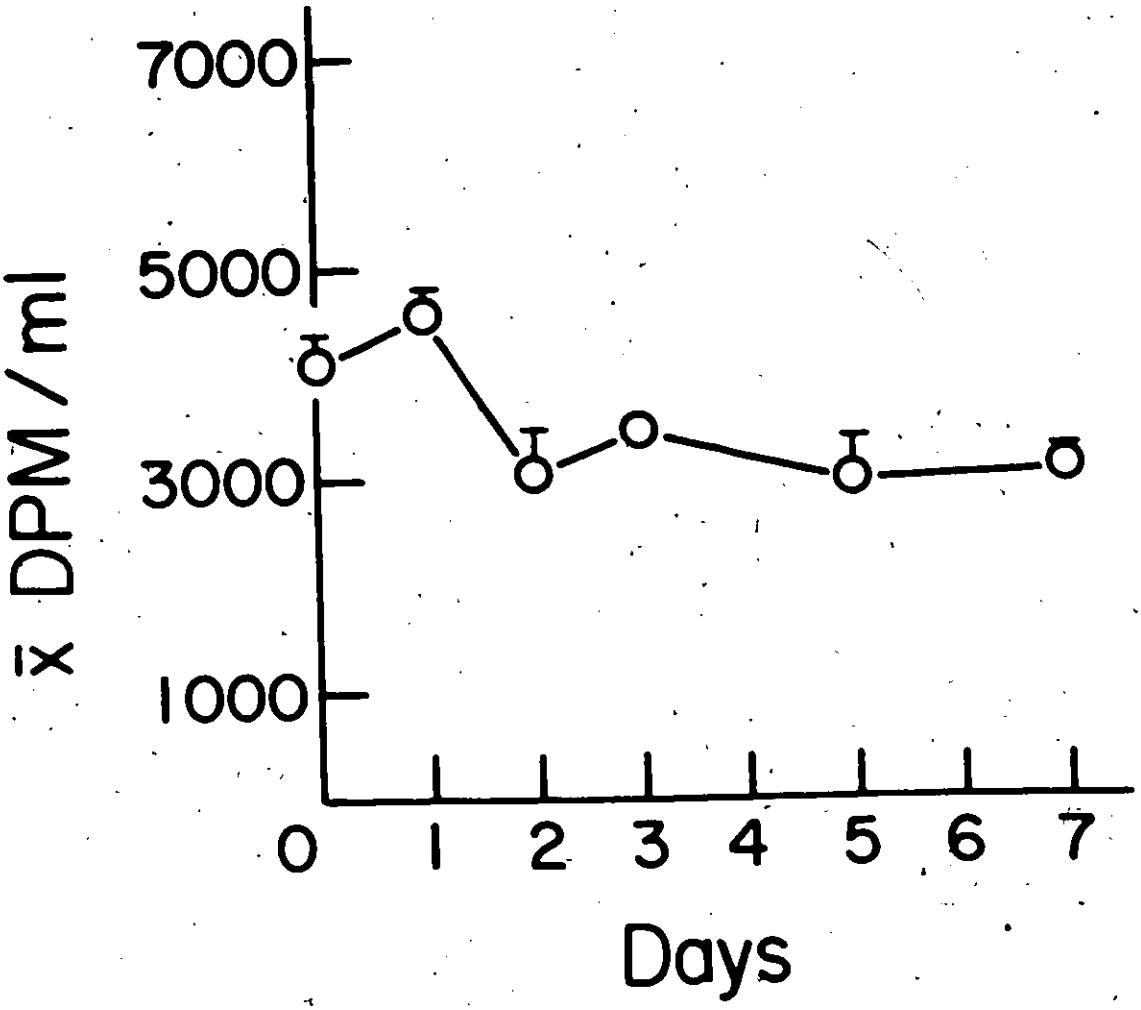
Day	$\overline{X}$ cell #/ml[a]	Unwashed	Washed	% Unwashed
0	2.00	7858 ± 390	486 ± 130	6
1	2.30	8267 ± 125	414 ± 58	5
2	2.78	8071 ± 21	713 ± 147	9
3	3.43	9277 ± 109	1704 ± 395	18
5	4.65	11,586 ± 949	5240 ± 1113	45
7	5.76	13,536 ± 588	8883 ± 859	66

[a] All values X 10^6

3.5 Depuration of C^{14}-Aminocarb

The depuration (loss of contaminant from the test organism) of C^{14}-aminocarb by Chlorella maintained in pesticide-free nutrient medium occurred very slowly throughout the seven days (Fig. 23). Although populations were growing, the dpm per ml per day decreased. On day 7 each ml of algae still contained 76% of the original C^{14} counts present on day 0.

Figure 23. Depuration of C^{14}-aminocarb by Chlorella populations exposed to 3.0 µg/ml aminocarb. The average values of triplicate samples are shown together with standard deviation bars.


7000
5000
3000
1000
x̄ DPM / ml
0
1
2
3
4
5
6
7
Days

3.6 Cell Cycle

Cell division in synchronized populations of Chlamydomonas was completed by the 16th hour of the cell cycle or the 4th hour of darkness (+4hr darkness). Cell division occurred between the 2nd and 4th hours of darkness with most cells (95%) forming two zoospores. The remaining cells did not divide or formed four zoospores.

Synchronized populations were treated with 1.0 µg/ml aminocarb in Matacil 1.8D at -2hr, 0hr, or +2hr darkness. The treatment times were chosen by extrapolating from the studies of Badour, et al. (1977) on DNA synthesis in the same algal species. These researchers found DNA synthesis began about four hours before cell division. At all treatment times of the synchronized populations most of the dividing cells (~95%) still formed two zoospores. However, cell division time was increased two hours in populations treated at -2hr darkness (Fig. 24) or at 0hr darkness (Fig. 25). These populations completed cell division at +6hr darkness. Populations treated at +2hr darkness were not affected with cell division occurring between the 2nd and 4th hours of darkness, this was comparable to the untreated populations (Fig. 26).

The formulated pesticide also inhibited doubling of the cells (Fig. 27). At the 4th hour of darkness about 95% of the cells in control populations had doubled. Populations treated at -2hr darkness were affected most with 59% less division than control populations at the 4th hour of darkness, while treatment at +2hr darkness had the

least effect with only 10% inhibition of doubling at the 4th hour of darkness. Populations treated at 0hr darkness exhibited 30% less cell division than control populations at the 4th hour of darkness. As mentioned previously treatment at -2hr or 0hr darkness extended division time two more hours such that division was completed at +6hr darkness. At this time populations treated at -2hr darkness still exhibited 38% less division than control populations and populations treated at 0hr darkness exhibited 17% less division.

Figure 24. The cell cycle of Chlamydomonas populations exposed to 1.0 µg/ml amino carb in Matacil 1.8D at -2hr darkness. The average values of triplicate samples for the initial experiment (a) and the replicate (b) are shown. Arrows indicate addition of the pesticide formulation.

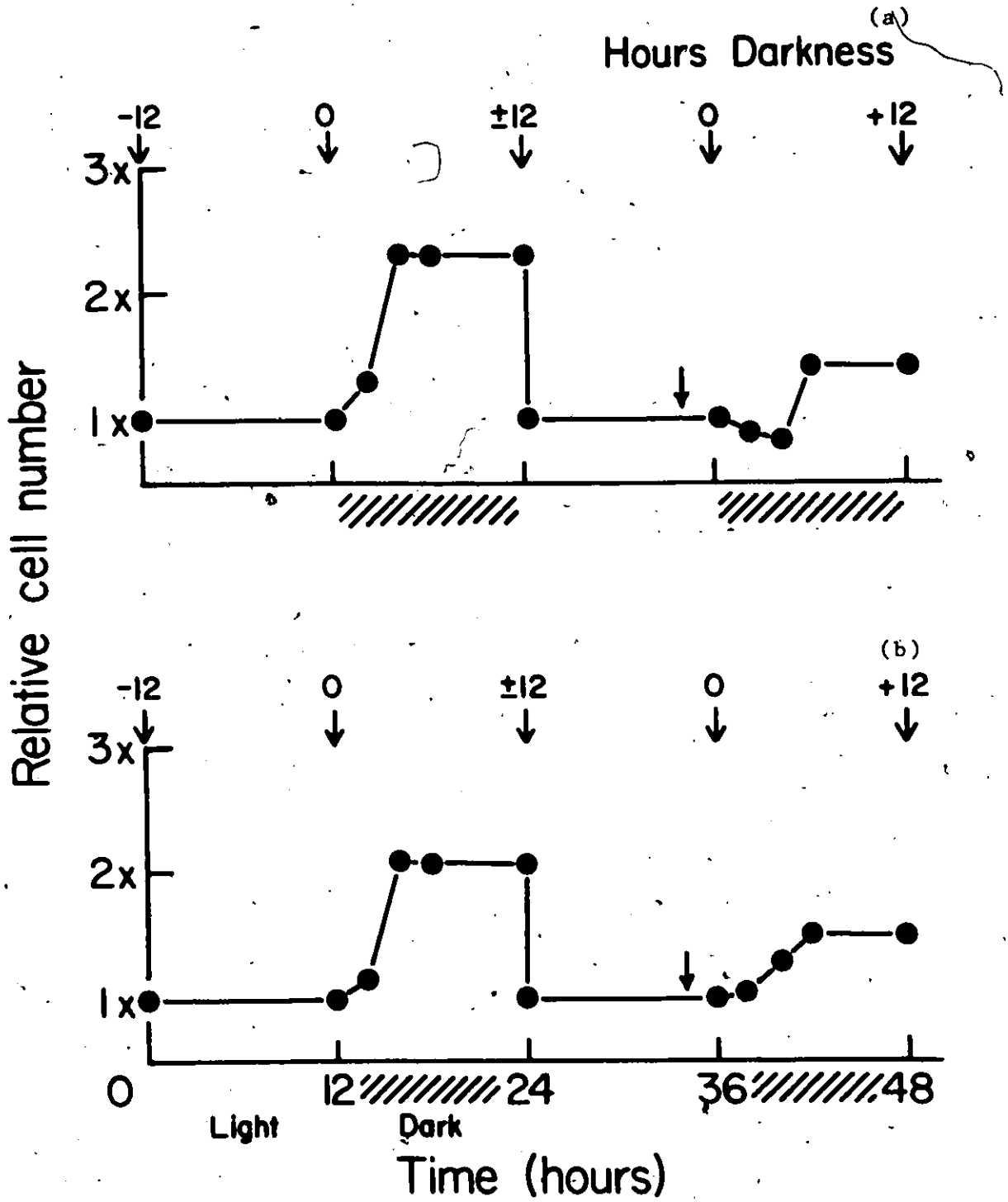

(a)
Hours Darkness
-12
0
±12
0
+12
3x
2x
1x
(b)
-12
0
±12
0
+12
3x
2x
1x
Relative cell number
0
12
24
36
48
Light
Dark
Time (hours)

Figure 25. The cell cycle of Chlamydomonas populations exposed to 1.0 µg/ml aminocarb in Matacil 1.8D at 0hr darkness. The average values of triplicate samples for the initial experiment (a) and the replicate (b) are shown. Arrows indicate addition of the pesticide formulation.

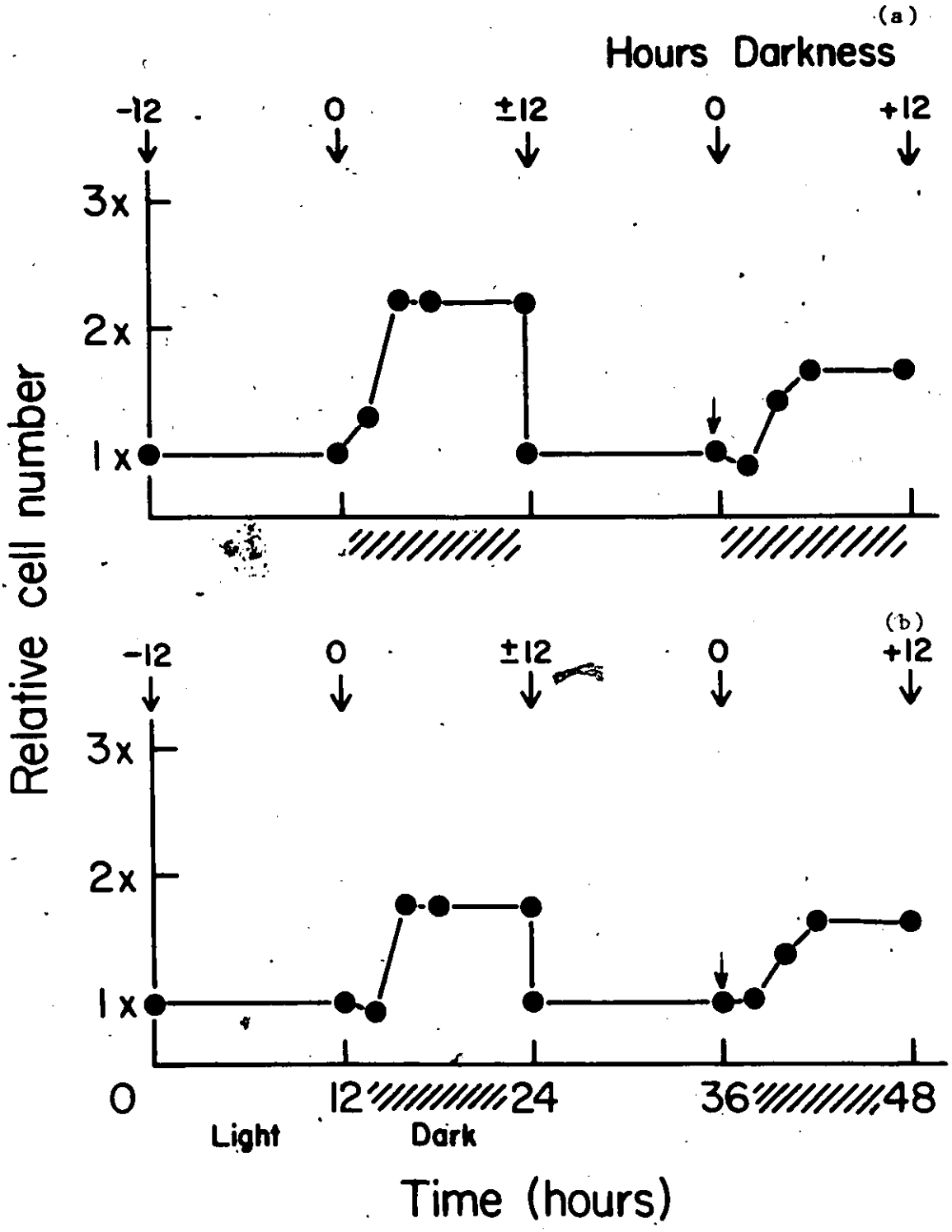

(a)
Hours Darkness
-12
0
±12
0
+12
3x
2x
1x
Relative cell number
(b)
-12
0
±12
0
+12
3x
2x
1x
0
12
24
36
48
Light
Dark
Time (hours)

Figure 26. The cell cycle of Chlamydomonas populations exposed to 1.0 µg/ml aminocarb in Matacil 1.8D at +2hr darkness. The average values of triplicate samples for the initial experiment (a) and the replicate (b) are shown. Arrows indicate addition of the pesticide formulation.

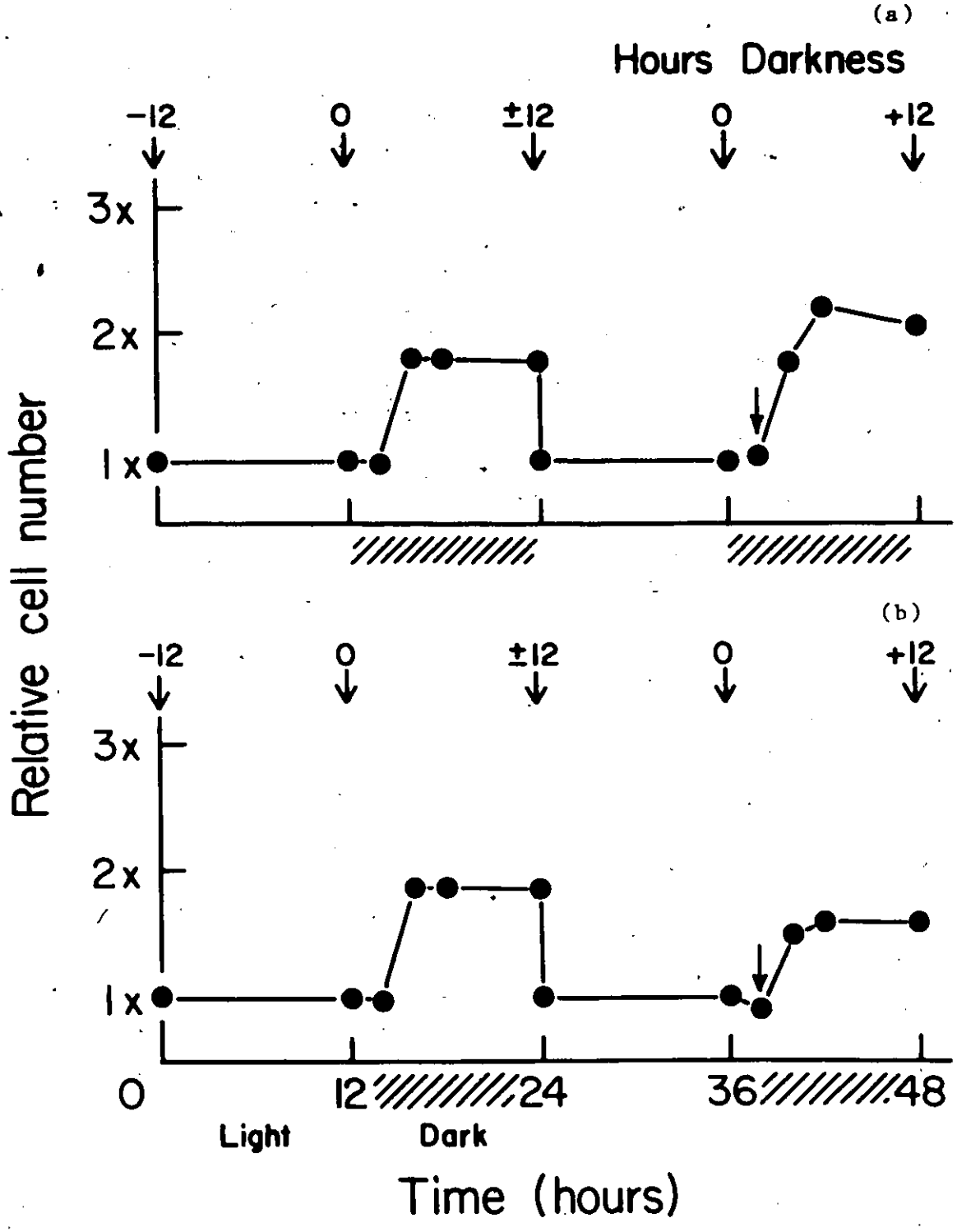
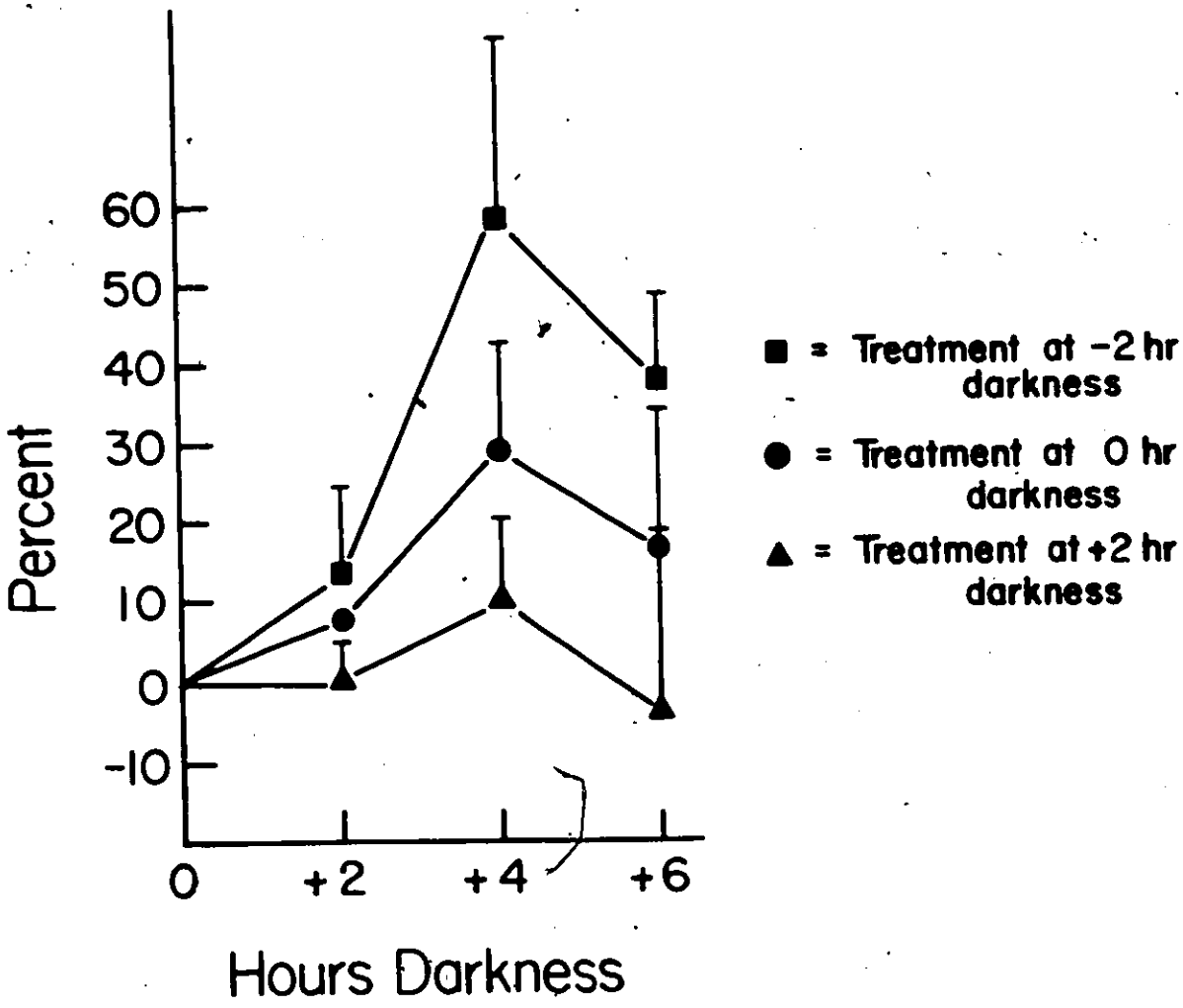

(a)
Hours Darkness
-12
0
±12
0
+12
3x
2x
1x
Relative cell number
(b)
-12
0
±12
0
+12
3x
2x
1x
0
12
24
36
48
Light
Dark
Time (hours)

Figure 27. Percent inhibition of doubling of Chlamydomonas populations exposed to 1.0 µg/ml aminocarb in Matacil 1.8D at different treatment times. The average values of triplicate samples for two experiments (N = 6) are shown together with standard deviation bars.


Percent
60
50
40
30
20
10
0
-10
0
+2
+4
+6
Hours Darkness
■ = Treatment at −2 hr darkness
● = Treatment at 0 hr darkness
▲ = Treatment at +2 hr darkness

DISCUSSION

The carbamate pesticide aminocarb is the active anticholinesterase chemical in Matacil 1.8D OSC which is used to control the spruce budworm in eastern Canada and Maine. Sprayed on tens of thousands of hectares of mixed forests the aminocarb formulation impinges upon a vast array of nontarget organisms in the forest ecosystem. Although spraying is discontinued over major lakes and streams (Kingsbury, 1977), pesticide contamination of waters could still occur through wind drift and surface runoff. Autotrophic microphytes in aquatic systems are the base of food webs of organisms in these habitats. Chlorella pyrenoidosa Chick and Chlamydomonas segnis Ettl are unicellular green algae which live in freshwater systems of forest ecosystems. Any effects that externally applied chemicals have on these organisms may well signal a cause for concern in terms of systems sensitivity.

No studies to date have reported the effects of the aminocarb formulation on algae living in lakes and ponds of forest ecosystems. The effects of other carbamate pesticides on algal growth have been reported. Growth of marine phytoplankton was suppressed in varying degrees by 0.1 and 1.0 µg/ml of the fungicide Nabam (disodium ethylene bis-dithio-carbamate hexahydrate) or the insecticide Sevin (1-naphthyl-N-methyl carbamate), while 10.0 µg/ml of either carbamate completely inhibited growth (Ukeles, 1962). Sevin was also toxic to Chlorella pyrenoidosa at concentrations of 0.1 µg/ml and above (Christie, 1969). After seven days 100 µg/ml of Sevin reduced Chlorella populations up to

30% of the controls. In long-term growth studies four algal species exhibited a threshold of toxicity between 1.0 and 10.0 µg/ml of formulated Zectran (4-dimethylamino-3,5-xylyl methylcarbamate) an insecticide used to control western spruce budworm (Snyder and Sheridan, 1974).

Chlorella and Chlamydomonas exhibited differential growth responses to the aminocarb and to the aminocarb formulation. This was not surprising because other studies have reported species related responses to other toxicants. For example, marine phytoplankton were inhibited in varying degrees by chlorinated hydrocarbons (Menzel et al., 1970) and a range of concentrations of polychlorinated biphenyls (PCBs) were needed to affect growth rates of marine and freshwater phytoplankters (Mosser et al., 1972). In the present set of experiments the non-formulated aminocarb in the range of 3.0 - 10.0 µg/ml stimulated the population growth of Chlorella but had no affects on the population growth of Chlamydomonas. Chlorella populations exposed to these concentrations of aminocarb had growth rates greater than controls thus resulting in greater cell numbers at the completion of the experiment. The growth rates of the Chlamydomonas populations were relatively unaffected by aminocarb and hence, the final population densities were similar to the controls.

Stimulation of population growth by pesticides is in itself an interesting phenomenon even though comparable effects on population growth of algae have been found by other investigators. Poorman (1973) exposed cultures of Euglena gracilis to a variety of insecticides and

herbicides and most of the chemicals in the range of 1.0 - 10.0 µg/ml stimulated population growth after a 24-hour exposure. However, after a 7-day exposure at 10.0 - 100.0 µg/ml the herbicides reduced algal growth while the insecticides stimulated growth. Klotz and Duysen (1972) also reported that respiration and growth of Chlorella pyrenoidosa were stimulated by low concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D). In particular, Stadnyk et al. (1971) showed that the carbamate insecticide carbaryl at 0.1 and 1.0 µg/ml stimulated population growth and carbon fixation of the green alga Scenedesmus quadricaudata.

The exact mechanism of growth stimulation by pesticides is unknown. In the present study increased pigment production was not concomitant with growth stimulation such that 3.0 µg/ml aminocarb did not affect total chlorophyll or chlorophyll a or b. Stadnyk et al. (1971) suggested that algal growth stimulation by carbaryl was due to degradation of the pesticide to NH_3 and formic acid. This would have provided an increased nitrogen source for growth. Pesticides could also provide a source of carbon for assimilation by algae. Eleven species of green algae including Chlorella yielded increased dry weights after myxotrophic growth on hydrocarbon substrates (Masters and Zajic, 1971). In the present study the stimulation of population growth observed in Chlorella cultures treated with aminocarb may have also been due to degradation of the insecticide to products utilized for growth (Alexander, 1980). Moreover, growth stimulation by aminocarb could also be due to its structural similarity to

indoleacetic acid and other auxins. Some researchers have proposed that auxin activity is determined by a charge distance of 5.5 Å between the carbon of a carboxyl group and a positive charge in the nucleus (Thimann, 1963). This charge-distance relationship is found in aminocarb (Greenhalgh, personal communication). Of course, the role of aminocarb in stimulating population growth of Chlorella warrants further investigation.

Stimulated growth can itself perturb the structure within a phytoplankton community when some algal populations are differentially affected (Kricher and Bayer, 1977). This could result in the substitution of a new dominant species in an algal assemblage. Stimulation of algal growth in aquatic ecosystems would have varying effects depending on the physical and chemical nature of the waters. In clear unpolluted lakes algal stimulation would cause temporary explosive bursts in primary production but in eutrophic lakes stimulation might be less noticeable in waters which already have long periods of high rates of primary productivity (Mathiesen, 1971). In terms of the effects on the ecological balance, stimulated growth of primary producers could increase productivity at all trophic levels in a food chain. It is interesting to note that after aminocarb formulation spray treatment at 70 Kg AI/ha Kingsbury (1978) observed a substantial increase in the numbers of zooplankton (copepods and rotifers) which he attributed to an increase in phytoplankton. Kingsbury stated that the increase in phytoplankton numbers was due to normal population fluctuations and not to growth stimulation by spray

treatment. The degree of stimulation indicated in this research (15-30%) may not evoke long-term perturbations in aquatic ecosystems as the half-life of aminocarb at pH 7.0-8.5 is only a matter of days (Weinberger and Greenhalgh, 1983). However, it could be important in terms of reducing feeding pressures on young fry.

As proposed in the Introduction, the aminocarb formulation had a greater impact on the population growth of the two algal species than aminocarb alone, although the responses were again species specific. Chlorella populations treated with 1.0 and 3.0 µg/ml aminocarb in Matacil 1.8D exhibited delays before growth and 10.0 µg/ml aminocarb in formulation was completely inhibitory. On the other hand, Chlamydomonas populations treated with 1.0 - 10.0 µg/ml aminocarb in Matacil 1.8D did not display any delay before initiation of growth. Although population densities of these cultures were 20 - 30% less than control populations after post-treatment, the highest concentration was not completely inhibitory to Chlamydomonas. That Chlamydomonas was more resistant to the aminocarb formulation than Chlorella was not unexpected, because Chlamydomonas was ranked above Chlorella in tolerating organic pollution in a compilation of reports on pollution-tolerant genera of algae (Palmer, 1969).

Delays in population growth have also been observed after treatment with other toxicants. For example, DDT caused lag phases lasting from 1-9 days in marine phytoplankton (Bowes, 1972) and certain chlorinated hydrocarbons caused lag periods before growth in blue-green algae (Batterton et al., 1971). Batterton et al. (1971) suggested that

adaptation and selection of insecticide-resistant cells occurred during lag periods. In fact, Lazaroff and Moore (1966) isolated sensitive and resistant clones from algal cultures which developed after a lag phase following pesticide treatment. Bowes (1972) also attempted to isolate "DDT-resistant" cells which developed after a lag phase but when resuspended into fresh medium containing the same concentration of DDT these cells still exhibited the same lag period.

Because cell densities were measured spectrophotometrically in this study, no differentiation could be made between living and senescent cells. Lag phases of Chlorella populations may have been due to a fractional kill or to a suppression of growth rate. The duration of the lag phase increased with increasing concentration of the aminocarb formulation indicating a fractional kill. But a concentration dependent lag phase may also suggest that cells needed time for morphological or physiological adaptations before growth could be resumed (Soto et al., 1975). Hence, a greater concentration of toxicant would cause greater cellular damage and more time would be needed for repairs. Furthermore, higher concentrations may have required more time to detoxify either by physical means such as volatilization or photodegradation or by the algal cells themselves. The former effect was demonstrated by Kauss and Hutchinson (1975) who found that the inhibition of growth of Chlorella vulgaris decreased with increasing "age" of crude oil extracts. "Aging" detoxified the oils by evaporation of the aromatic components. Onset of growth in Chlorella populations in the present study may have coincided with the loss of toxic components from the aminocarb formulation.

Chlorella populations were exposed to diluent oil and nonylphenol at concentrations comparable to those found in the range of aminocarb formulated as Matacil 1.8D employed in the initial growth studies (.01 - 10.0 µg/ml aminocarb). Many algae have reportedly exhibited lag phases before growth after treatment with crude oils and their components. This phenomenon was shown in certain marine algae (Pulich et al., 1974) and in the freshwater algae Chlorella vulgaris (Kauss and Hutchinson, 1975), Chlamydomonas angulosa (Soto et al., 1975) and Chlamydomonas reinhardtii (Moody et al., 1981). However, in the present study populations treated with diluent oil were unaffected by any concentration (.015 - 15.0 µg/ml oil); while the populations treated with the three highest concentrations of nonylphenol (2.5, 7.5, and 25.0 µg/ml) exhibited delays in growth. As the nonylphenol concentration increased the duration of the lag phase also increased until complete inhibition of growth was evident at the highest concentration. These results were similar to the results obtained with the aminocarb formulation. Nonylphenol in the Matacil 1.8D, not the diluent oil, produced the lag phases in Chlorella populations.

Again the question arose as to the factors involved in causing the lag phase. The results of the agar bioassays strongly suggested that nonylphenol caused a fractional kill of Chlorella thus producing a lag phase. If the nonylphenol temporarily depressed growth, then removal of the toxicant should have eventually produced growth, since in the agar bioassays cells exposed to nonylphenol were washed and plated on nonylphenol-free agar. Nonylphenol may have selected for "resistant"

or hardier cells as previously discussed, because their subsequent growth rates after the lag phase were higher than the controls. In fact, the growth rates of treated populations increased with increasing concentration of nonylphenol. Most studies have reported that when algal growth commences after a lag phase the doubling time of treated populations was similar to control populations (Bowes, 1972; Kauss and Hutchinson, 1975: Moody et al., 1981). However, increased cell division rates after lag periods were observed in Chlamydomonas angulosa after exposure to napthalene (Soto et al., 1975). These researchers suggested that the growth rate was inversely related to the initial cell density. This may be a plausible explanation for the results of the present study. In the agar bioassays the percent mortality of the algae increased with increasing concentration of nonylphenol. Thus, in the liquid bioassays the positive correlation of growth rates with nonylphenol concentration may have been related to decreasing initial cell densities caused by an increasing fractional kill. However, the positive correlation of growth rate with nonylphenol concentration was not evident in Chlorella populations exposed to the aminocarb formulation even though the range of concentrations of nonylphenol (.025 - 25.0 µg/ml) in the Matacil 1.8D was equivalent to the range used in the nonylphenol studies. In fact, populations exposed to 1.0 µg/ml nonylphenol had growth rates higher than populations treated with 3.0 µg/ml aminocarb in Matacil 1.8D (7.5 µg/ml nonylphenol). The lack of any clear relationship between

nonylphenol concentration in the aminocarb formulation and growth rate of Chlorella may have been due to the presence of the other components of the formulation, namely aminocarb and diluent oil.

It is known that carbamate herbicides such as propham and chloropropham are mitotic poisons which eliminate noxious plants by inhibiting cell division in roots (Ashton and Crafts, 1973). Isopropyl n-phenyl carbamate (IPC) which decreases root, shoot, and leaf growth in higher plants has been shown to inhibit cell division and growth in the green algae Oedogonium (Coss and Pickett-Heaps, 1974) and Chlamydomonas (Flavin and Slaughter, 1974). Chlorpropham (isopropyl 3-chlorocarbanilate) suppressed the growth of Chlorella populations at concentrations of 2.4 μM or above (Sumida et al., 1977). In synchronously growing populations of these alga an increase in cell number was completely inhibited by 14 μM chlorpropham but the average DNA per cell increased 3.0 fold after one turn of the cycle. This indicated that cell division was affected. It was conjectured that perhaps aminocarb would act similarly on cell division of the two algal species although the concentration range employed in this study (0.1-10.0 μg/ml aminocarb) did not reduce growth or cause lag periods. However, Chlorella populations exposed to the highest concentration of aminocarb (10.0 μg/ml) contained more cells in the largest size range (cell diameter > 7.45 μm) than control populations. These "giant" cells which represented only 2.4% of the total cell number may have resulted from arrested cell division. Perhaps, then, concentrations of aminocarb greater than those employed in this research might

significantly inhibit cell division and this warrants further investigation.

The results of the growth studies with Chlamydomonas suggested that the aminocarb formulation may have affected cell division rates. The growth rates of populations exposed to Matacil 1.8D were below the control populations. Synchronized populations of Chlamydomonas were employed to test the idea that cell division was indeed affected. Batch cultures of algae in exponential growth as used in the growth studies contained cells in all stages of development. In contrast all life cycles of cells in synchronous cultures are more or less coordinated with some processes developing gradually, for example, growth and protein or RNA syntheses, and some developing stepwise such as DNA synthesis (Lorenzen and Hesse, 1974). Synchronized populations provided a large amount of cellular material in similar physiological and morphological condition enabling treatment effects to be extrapolated to the cellular level.

The effects of the aminocarb formulation on synchronous populations of Chlamydomonas were related to the time of treatment. Treatment at the beginning of cell division (+2 hr darkness) had the least effect and treatment four hours before cell division (-2hr darkness) had the greatest effect. Treatment effects at two hours before cell division (0hr darkness) were intermediate between the other two treatment times. A time related effect was also exhibited by synchronized populations of Chlorella pyrenoidosa treated with chloramphenicol (CAP), an antibiotic which inhibits protein synthesis in bacteria. If CAP was added before the fifth hour of light, cell division was inhibited 80% but adding CAP at intervals

after the fifth hour caused a decrease in inhibition to zero by the end of the light period (Moberg et al., 1968).

The aminocarb formulation not only inhibited cell division but also increased the duration of cell division. Zoospore formation, however, was not affected, in that dividing cells still formed two zoospores. It was thought that pesticide stress might cause Chlamydomonas cells to form four zoospores as observed when the same species was subjected to air containing 5% carbon dioxide (Badour et al., 1977). Because treatment at the start of cell division had negligible effects and zoospore formation remained the same at all treatment times, it is proposed that the aminocarb formulation was not acting majorly to affect microtubule assembly (Ashton and Crafts, 1973).

Whereas division was completed at the fourth hour of darkness in control populations, division at this time in treated populations was still below that of controls. Division in treated populations was completed by the 6th hour of darkness. At end of the cell cycle 38% inhibition of growth (measured by cell counts) occurred at treatment four hours before cell division, 17% at treatment two hours before cell division, and no inhibition of growth occurred at treatment at the start of cell division. These results suggested that the aminocarb formulation may be affecting DNA synthesis. Badour et al. (1977) reported that DNA synthesis in the same algal species began about four hours before cell division. Treatment time at four hours before division began produced the greatest inhibition of cell division, thus, suggesting that DNA synthesis was affected. Of course,

other macromolecular syntheses (RNA, protein, and/or polysaccharides) might also have been affected by the aminocarb formulation and, hence, affected the completion of zoospore formation and release. Jeanne (1979), for example, found that synthesis of RNA or protein in synchronous cultures of Dunaliella was moderately inhibited by concentrations of lindane which strongly inhibited DNA synthesis. Inhibition of DNA synthesis by the aminocarb formulation could be a secondary effect with the primary effect being on all energy related reactions. Morris (1966a) found that chloramphenicol inhibited protein, polysaccharide, and nucleic acid syntheses in Chlorella which seemed to be caused by a more general effect on decreased ATP utilization by the treated cells (Morris, 1966b). More research is needed on the effects of the aminocarb formulation on macromolecules in synchronized populations.

The fact that synchronous populations of Chlamydomonas were most affected at four hours before cell division may also indicate that these algal cells were the most sensitive at this time in the cell cycle and were, therefore, killed by the treatment. It might be hypothesized that inhibition of doubling may only have represented a percent mortaility. However, this seems unlikely in the present case because Matacil 1.8D at concentrations ranging from 1.0 - 10.0 µg/ml aminocarb did not produce lag phases before growth in the Chlamydomonas populations. In future research treated synchronous populations could be plated on agar at the end of the cell cycle to test this possibility.

It is well known that algae accumulate pesticides (Butler, 1977). As the base of many food webs in aquatic ecosystems algae have the potential to transport pesticides to higher trophic levels. Södergren (1973) demonstrated that certain chlorinated hydrocarbons were taken up by Chlorella pyrenoidosa and transported to first-order consumers. Moreover, these consumers mainly accumulated the pesticides through the food chain rather than by uptake from the water. In the present study Chlorella populations accumulated C^{14}-aminocarb when exposed to either the non-formulated pesticide or the formulated pesticide. Södergren (1968) suggested that the rate of uptake of pesticides by dead algal cells was equivalent to the pesticides' rates of diffusion in water. Because freeze-killed Chlorella cells in the present study had a lower rate of uptake of C^{14}-aminocarb than the living cells, the mechanism of accumulation in the living cells could have been active. On a per cell basis the accumulation of C^{14}-aminocarb by freeze-killed cells was 36% of the amount accumulated by living cells exposed to aminocarb and only 20% of the amount accumulated by living cells exposed to Matacil 1.8D. Moreover, most uptake by dead cells occurred in the first day of treatment while living cells continued to accumulate C^{14}-aminocarb over a seven day period.

Chlorella populations exposed to the aminocarb formulation accumulated a greater amount of C^{14}-aminocarb over the seven days than populations exposed to aminocarb alone. By day seven, populations treated with Matacil 1.8D accumulated almost twice the amount of C^{14}-aminocarb as populations treated with aminocarb. The aminocarb

formulation reduced the population growth of Chlorella resulting in lower cell densities per day than populations growing in aminocarb. In the present research, cell concentration did not affect accumulation of C^{14}-aminocarb by Chlorella populations, although some other studies have reported a negative correlation between pesticide uptake by algal populations and cell density (Neudorf and Khan, 1975). Thus, the formulation adjuvants are probably responsible for the increased accumulation of C^{14}-aminocarb by populations exposed to Matacil 1.8D. Because nonylphenol, not the diluent oil, produced lag phases in population growth of Chlorella, it is proposed that nonylphenol influenced C^{14}-aminocarb uptake. It is further proposed that nonylphenol may have altered cellular membranes thus increasing the permeability of C^{14}-aminocarb. Although Chlorella possess both cell walls and cell membranes, the cell walls were probably not affected since results of other studies have indicated that phenols and surfactants only affect membranes. For example, PCB inhibited two algal species equally despite the fact that one alga had a cell wall and the other had none (Luard, 1973). The suggestion that surfactants like nonylphenol may affect membranes is not new. The dissemination of herbicides into plants and of pesticides through the cuticle of invertebrates is often improved by surfactants (Brian and Bland, 1965). Cytological observation of plants exposed to certain nonionic surfactants indicated cell membranes were affected (Endo et al., 1969). In particular, Chlamydomonas reinhardii exposed to 1.0 µg/ml nonylphenol for one hour showed evidence of membrane damage (Weinberger and Rea, 1981).

The exact mechanism by which surfactants affect membranes is unclear but some researchers have found a correlation between lethal concentrations of surfactants and their surface tension indicating disruption of cell membranes (Bode et al., 1978). The hypothesis that nonylphenol acts on membranes does not contradict the more widely held idea that phenolics are uncoupling agents of oxidative phosphorylation (Buikema et al., 1979). Uncouplers may penetrate membranes of organelles in which phosphorylation occurs thus destroying the selective permeability of these membranes to necessary ions (Ashton and Crafts, 1973). Larsson and Tillberg (1975) observed reduced phosphate uptake in Scenedesmus treated with PCB. They suggested that these effects were due to PCB action on plasmalemma not phosphorylation because oxygen uptake and evolution were not affected. As mentioned previously Morris (1966b) found that CAP affected ATP utilization in synchronized Chlorella populations. This could also indicate that membranes were affected by CAP and it it proposed that perhaps the inhibition of doubling in the synchronized Chlamydomonas populations by Matacil 1.8D may have been due to the effects of nonylphenol on cellular membranes.

The depuration of C^{14}-aminocarb by Chlorella cells occurred slowly over seven days. Moreover, it appeared that C^{14}-aminocarb became more tightly bound to the cells over time. Only 6% of the pesticide remained associated with the algae after washing on the first day compared to 66% on the seventh day. It is also possible that the C^{14}-

aminocarb became incorporated into cell organelles or products. Wheeler (1970) found the extraction of labelled dieldrin from Chlorella became more difficult over time indicating movement of the insecticide into "bound compartments" of the cell. The slow depuration of C^{14}-aminocarb and the increasing difficulty in removing the labelled pesticide over time strongly suggest that aminocarb was absorbed by the Chlorella. Essentially then first-order consumers could potentially ingest aminocarb for at least seven days whereas from purely chemical considerations it has been shown that aminocarb would have degraded long before this as the half-life of aminocarb at pH 6.8 is of the order of 36-48 hours (Weinberger and Greenhalgh, 1983).

Carbamate herbicides as mitotic poisons often produced increased cell size in treated plants (Ashton and Crafts, 1973). It was thought that aminocarb may act as a mitotic poison and produce larger cells in treated populations of Chlorella. Thus, cell sizes were measured after post treatment with aminocarb and aminocarb formulation. Populations exposed to all concentrations of aminocarb (.01 - 10.0 µg/ml) and to the two lowest concentrations of aminocarb in Matacil 1.8D (0.1 and 0.1 µg/ml aminocarb) contained a higher percentage of small cells than control populations. A greater percentage of small cells might indicate that these populations had higher growth rates. However, only the populations exposed to 3.0 and 10.0 µg/ml aminocarb had growth rates greater than controls. Furthermore, increasing growth rate

associated with increasing aminocarb concentration did not produce increasing numbers of smaller cells.

The cell size distribution in Chlorella populations exposed to 1.0 and 3.0 µg/ml aminocarb in Matacil 1.8D were similar to controls. These populations contained more large cells than other treated populations. Other studies have shown that chemicals related to nonylphenol affect cell size distribution. For example, PCB inhibited the growth of estuarine phytoplankton larger than 8 µm (Biggs et al., 1978) and coumarin, a naturally occurring phenolic, produced cells larger than controls in populations of Chlorella vulgaris (Ron and Mayer, 1959). Perhaps, then, the nonylphenol in the aminocarb formulation produced larger cells by acting as a mitotic poison or by affecting zoospore formation and/or membrane permeability as suggested by the present uptake studies. A loss of membrane selectivity in freshwater algae could result in increased diffusion of water into algal cells. Of course, this suggestion is highly speculative and the effects of aminocarb and the formulation adjuvants on algal cell size needs further investigation.

Changing the cell size distribution of primary producers could affect first-order consumers. Research has indicated that food size selection does, in fact, affect grazer populations (Wilson, 1973; Edmondson, 1965). These effects of altering algal cell size on feeding behavior may only be temporary. However, Powers et al. (1977) found that altered cell size distribution in marine alga caused by dieldrin

persisted through four generations even after removal of the pesticide. A more lasting change in cell size distribution of algae could have a greater impact on other organisms in the food chain.

SUMMARY AND CONCLUSIONS

The hypothesis proposed at the beginning of the research that the aminocarb formulation would have a greater impact on algal populations than aminocarb alone, was supported by these studies. Exposure to the aminocarb formulation led to an increased lag phase period before the onset of growth in populations of Chlorella and increased the uptake of C^{14}-aminocarb in these cells. The formulation also depressed growth of Chlamydomonas populations and more specifically inhibited doubling of synchronized cells. It was suggested that these effects of the aminocarb formulation were primarily due to nonylphenol in Matacil 1.8D. It was also hypothesized that the toxicity of nonylphenol was attributed to its effect on cellular membranes. That nonylphenol affected membrane permeability has also been supported by results of other studies employing surfactants and phenols and especially by cytological observations of Chlamydomonas cells exposed to nonylphenol. Nonylphenol could also have increased the toxicity of aminocarb by facilitating the uptake of the pesticide. However, aminocarb did not significantly affect population growth in either algal species. Exposure to nonylphenol alone led to increased lag phases before growth in Chlorella populations and this was probably due to a fractional kill of the algal cells as indicated by the agar bioassays. Aminocarb also had an impact on the algae but not to the same extent as the aminocarb formulation. Aminocarb stimulated population growth in Chlorella and also affected cell size distribution in these cells.

Pesticides enter aquatic ecosystems in many ways. Of course, contamination of waters by insecticides is usually unintentional either by atmospheric fallout on rain or dust during application, surface runoff from treated terrestrial areas, industrial effluent, sewage, or spills during processing, storage, and transportation. The persistence of pesticides in water depends on the physiochemical properties of the pesticides, such as water solubility and rate of hydrolysis and photolysis, the nature of the water, including abiotic and biotic factors, and sediments. Studies have reported that aminocarb or its formulation adjuvants are not very persistent in aquatic systems. Aminocarb in formulation exhibited a half-life in pond water of 4.4 days after spray application at 70 g AI/ha (Sundaram et al., 1976) and nonylphenol had a half-life of 2.5 days after spray application of a mixture of nonylphenol and diluent oil 585 at .47L/ha (Sundaram et al., 1980). The half-life of diluent oil 585 after spraying has not been reported. Although aminocarb and nonylphenol seem to dissipate rapidly, spraying Matacil 1.8D in the late spring to reduce the larval stages of the spruce budworm could perturb an aquatic environment at a time when its biota are barely recovering from thermal stresses of winter. The nonylphenol especially has the potential to be an added stressor to aquatic environments at a time when algae are already experiencing a shock period of higher light regime and a reduction of nutrients (Round, 1971). A reduction in algal populations may result in feeding stress for some aquatic biota. As a potential algicide nonylphenol could not only affect the intensity of feeding by

first-order consumers (Monakov, 1972) but could also cause grazer starvation (Taub, 1976). Moreover, nonylphenol could affect population numbers of zooplankton feeding on algae, since research has suggested a positive correlation between abundance of food and rate of reproduction (Edmondson, 1965).

The unicellular algae used in this study are the base of many food chains leading directly to zooplankton and indirectly to a wide interweaving web of organisms at higher trophic levels. Nonylphenol is not only used as a surfactant and solvent in the aminocarb formulation but it also has a wide range of industrial uses from germicides to oil and grease additives, detergents, dyestuffs and surface active and wetting agents. Nonylphenol has been detected in industrial waste waters from textile finishing plants and specialty chemical manufacturers (Buikema et al., 1979). The results of this research seem to dispute the conclusions of field trials and laboratory bioassays of some researchers. For example, Holmes and Kingsbury (1980) stated that nonylphenol sprayed at the maximal seasonal dosage gave no cause for concern for aquatic invertebrates or fish. The fact that nonylphenol adversely affected Chlorella and Chlamydomonas does not mean it may be toxic to other algal species or to higher organisms for that matter. However, it should signal the fact that it should not a priori be regarded as biologically inert.

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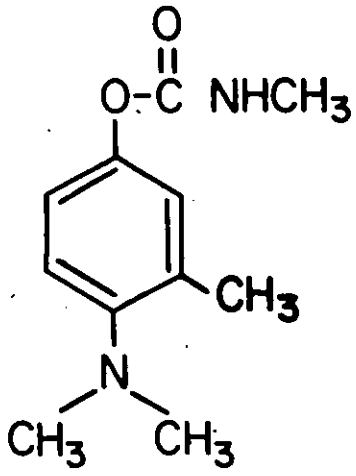
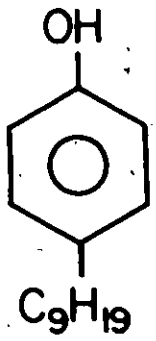
APPENDIX A



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

APPENDIX B

Standard curves were constructed to ensure a linear relationship between OD and cell density.