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ABSTRACT

The toxicological effects of the pesticide aminocarb and its adjuvants in the Matacil 1.8D formulation were monitored using Chlamydomonas segnis as the test organism. The parameters followed included number of cells, biomass changes in terms of total dry weight, protein, carbohydrate and nucleic acids (DNA and RNA). The null hypothesis, that the physiological and developmental status of the organism at the time of stressor application would differentially affect the response, was tested. To this end, separately, and mixed as a formulation, the pesticide and its adjuncts were added to populations of synchronized cells at 4 different phases of their life cycle, namely, at -4 and -2 h before the dark period (corresponding to the end of G₁ and S-phase), at the initial switch over to the dark period (the end of S phase and the beginning of G₂) and 2 h into the dark cycle (when cytokinesis was observed).

Aminocarb applied at a concentration of 50 $\mu\text{g ml}^{-1}$ during the S-phase (following macromolecular synthesis) was algicidal and algistatic. The synthesized macromolecules were unaffected. As a consequence these molecules accumulated in the cells and DNA synthesis was stimulated. Treatment at -4 h was algistatic but not algicidal. At 0 h, the inhibition of cell division was related to the pesticide concentration. However, treatment at +2 h into the dark period did not significantly affect cell number. Low concentrations, up to 1 $\mu\text{g ml}^{-1}$ of aminocarb, affected neither cell division nor the macromolecular content of the cells.

Nonylphenol (2.5 $\mu\text{g ml}^{-1}$) inhibited cell division at all the time periods tested. Cells treated in the S-phase, -2 h before the dark cycle were the most sensitive with respect to the perturbation of cell division

following treatment; algistatic and algicidal effects were observed. Treatment at -4 h and 0 h resulted in algistasis alone. When the cells were in cytokinesis (+2 h). Nonylphenol application resulted in a 75% inhibition of this process. Accumulation of macromolecules was observed as a result of the inhibition of cell division and these effects related to the phase of the cell cycle at the time of the treatment.

In the light, the chlorophyll content was reduced following exposure to (2.5 µg ml⁻¹) Nonylphenol. Treatment at -4 h was the most sensitive period with respect to chlorophyll a and b content. Chlorophyll a was reduced by 70% and chlorophyll b by 30%. Treatment at -2 h resulted in a 33% reduction in chlorophyll a content, chlorophyll b was unaffected. No significant effects were observed following treatment at 0 h and +2 h darkness.

Diluent oil (750 µg ml⁻¹) did not affect either cell division or macromolecular synthesis at any of the time periods tested. The exception was obtained when the cells were treated at -2 h (S-phase). A 20% reduction in cell number was observed.

The formulated pesticide (Matacil 1.8D containing 1 µg ml⁻¹ aminocarb, 2.5 µg ml⁻¹ Nonylphenol and 1.5 µg ml⁻¹ diluent oil) was both algicidal and algistatic when applied at -2 h (S-phase). The concentrations of aminocarb and oil in the formulation were at the NOE (no effect) level. The toxicological responses were generally similar to those evoked by Nonylphenol alone, apart from the RNA response, where augmentation was obtained above that evoked by the application of Nonylphenol alone. Treatment with Matacil during the light periods (-4 and -2 h) evoked identical responses to those obtained with Nonylphenol.

Possible mechanisms for these toxicological responses are discussed.

RESUME

Les effets toxicologiques du pesticide, aminocarb et de ses composants dans la formulation Matacil 1.8D furent détectés en utilisant Chlamydomonas segnis comme organisme-test. Les paramètres utilisés, comprennent le nombre de cellules et les changements de biomasse en fonction de la totalité du poids sec, des protéines, des carbohydrates et des acides nucléiques (ADN et ARN). L'hypothèse nulle, que le statut physiologique et développemental de l'organisme, au temps où l'agent fut appliqué, affecterait différemment la réponse, fut testée.

A cette fin, le pesticide et ses composants, seuls ou mélangés tout comme la formulation, furent ajoutés à des populations de cellules synchronisées à 4 phases différentes de leur cycle vital, nommément, à -4 et -2 h. avant la période obscure (ce qui correspond à la fin de la phase G₁ et S), au début de la phase obscure (fin de S et commencement de G₂) et 2 h. après le début du cycle obscur (lorsque la cytotinèse est observée).

Aminocarb, lorsqu'appliqué à une concentration de 50 $\mu\text{g ml}^{-1}$ durant la phase S (après la synthèse des macromolécules) fut algicide et algistatique. Les macromolécules synthétisées ne furent pas affectées. Conséquemment, ces molécules s'accumulèrent dans les cellules et la synthèse de l'ADN fut stimulée.

Le traitement à -4 h fut algistatique, mais non algicide. A 0 h., l'inhibition de la division cellulaire fut reliée à la concentration du pesticide. Cependant, le traitement à +2 h. dans la période obscure n'affecta pas de façon significative le nombre de cellules. De basses concentrations, jusqu'à 1 $\mu\text{g ml}^{-1}$ d'aminocarb n'affectent ni la division cellulaire, ni le contenu macromoléculaire des cellules.

Le Nonylphénol ($2.5 \mu\text{g ml}^{-1}$) inhiba la division cellulaire à toutes les périodes testées. Les cellules traitées durant la phase S, -2 h. avant le cycle obscur furent les plus sensibles quant à la perturbation de la division cellulaire à la suite du traitement; des effets algistatiques et algicides furent observés. Les traitements à -4 h et 0 h. résultèrent en algistasis seulement. L'application du Nonylphénol durant la cytokinèse (+2 h.) mena à une inhibition de 75% du processus. L'accumulation de macromolécules fut observée à la suite de l'inhibition de la division cellulaire et cet effet dépend de la phase dans laquelle était la cellule à l'instant du traitement.

En présence de lumière, le contenu de chlorophylle fut réduit après exposition au Nonylphénol ($2.5 \mu\text{g ml}^{-1}$). Le traitement à -4 h. fut la période la plus sensible en ce qui concerne le contenu de chlorophylle a et b. La chlorophylle a fut réduite de 70% et la chlorophylle b de 30%. A -2 h., la chlorophylle a fut réduite de 33%, mais la chlorophylle b ne fut pas influencée. Aucun effet significatif ne fut observé à 0 h. et +2 h à l'obscurité.

L'huile diluante ($750 \mu\text{g ml}^{-1}$) n'affecta pas la division cellulaire ou la synthèse macromoléculaire à aucune des périodes testées. L'exception se présente lorsque les cellules furent traitées à -2 h. (phase S). Une réduction de 20% du nombre de cellules (par rapport aux contrôles), fut observée.

La formulation du pesticide (Matacil 1.8D contenant $1 \mu\text{g ml}^{-1}$ d'aminocarb, $2.5 \mu\text{g ml}^{-1}$ de Nonylphénol et $1.5 \mu\text{g ml}^{-1}$ d'huile diluante) fut algicide et algistatique lorsqu'appliquée à -2 h. (phase S). Les concentrations d'aminocarb et d'huile dans la formulation étaient au niveau NOE (sans effets). Les réponses toxicologiques furent généralement simi-

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Un mécanisme possible pour ces réponses toxicologiques est discuté.

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INTRODUCTION

The increasing contamination of aquatic environments by chemical pesticides is a pressing and worrisome concern since it may lead to a range of problems. Two major ecological concerns relate to the possibility that species composition may change when aquatic communities are exposed to these stressors and thus the stability of an ecosystem may be disrupted. Furthermore, some toxic residues may accumulate in organisms of lower trophic levels. Such residues may not only act as additional stressors, but also pose hazards for further accumulation at higher levels of the food chain. Algae, the important primary producers of aquatic environments have received relatively little attention with respect to possible destabilizing effects of pesticides or their formulation adjuncts. Two reviews cover the major aspects of this research and they deal principally with pesticide effects on marine algae and the accumulation of organochlorine compounds. (Butler, 1977; Brown, 1978). However, millions of hectares (ha) of Canadian mixed forest areas are annually sprayed with insecticides and during the course of the aerial application extensive fresh water systems in these habitats are contaminated. It is, therefore, important to know the effects of pesticide formulations on the aquatic phytobiota indigenous to these fresh water habitats.

Aminocarb (4-(dimethylamino)-3-(methylphenol methylcarbamate) has been used since 1971 in Eastern Canada to control the spruce budworm, Choristoneura fumiferana, Clemens. In Canada, its usage has been approved at the rate of 52 or 70 g of "active" ingredient per ha. The "inert" materials in the registered formulation include Nonylphenol, an

non ionic detergent consisting mainly of p-substituted nonylphenols and 585 insecticide fuel oil, a no. 2 fuel oil distillate. The registered formulation mix (Matacil[™] 1.8D OSC) consists of aminocarb: Nonylphenol: 585 diluent oil in the ratio of 20:50:30 W/W/W.

The aim of the present research was to study the effects of the pesticide aminocarb and its formulation adjuvants on an algal species indigenous to fresh water habitats in Canadian forests.

The alga selected was Chlamydomonas segnis. This alga readily responds to synchronization and was therefore an ideal organism to use in order to obtain insights into the effects of the formulants and formulation on the normal cell cycle and on some key macromolecules.

1.1 History of Insecticide Use in Canadian Forests

Canada's land area is 9.7 million km², of which 3.14 million km² are classed as "forest land". The productive portion of these forests is estimated to be 2 million km². (Reed, 1980). Forest land is one of the most valuable natural resources of Canada. The importance is without question both economic and social, as the timber provides jobs directly and indirectly for 1.3 million Canadians and is the major contributor to the Canadian gross national product. The growth of the forest industry has helped to build the prosperity of the provinces with timber resources by permitting fiscal expansion, encouraging increased transport facilities, supply and service industries and increasing foreign exchange.

It is important to note that the forests also provide a natural resource for recreational activities as well as encouraging wildlife habitat conservation. The aesthetic quality derived from the beauty and

serene peace of these areas is such that it is impossible to assess a monetary value. Public awareness of the fragility and economic value of this resource is increasing daily; for example, presently, scientific interest is being generated to use the forest ecosystems to improve the quality and quantity of water yields (Anderson and Smith, 1976; Wildish, 1980).

The over-riding economic significance of the dependence of Canada on the forest industry intensifies, considerably the necessity to provide protection for the trees of the forests.

Over the past two decades the damage by insects to Canadian forests has resulted in an estimated annual reduction in harvest of the order of 88 million m³. In the last decade, the most serious loss has been due to spruce budworm defoliation of fir and spruce stands (Reed, 1980; Blais, 1983). In order to keep predation at its present level, without permitting an increase, and therefore protect the Canadian forest land from incremental losses, aerial application of pesticides is necessary in those areas slated for lumber use within the next decade. The active ingredients of the sprays are chemical compounds which are toxic to the spruce budworm larvae.

Although aerial dusting against forest defoliators was first tried in the 1920's using mainly calcium arsenate dust (Nigam, 1975), present aerial application techniques were developed when DDT came into widespread use. In the 1950's, the first spray operation was undertaken when 75×10^3 ha of New Brunswick forests were sprayed with DDT at 1.120g ha⁻¹. In 1953 the application was halved but the area sprayed was increased to 73×10^4 ha. During the following five years DDT was applied at 280g/ha. In 1957, the area sprayed increased to 23×10^3 ha. (Symons,

1977). DDT began to be withdrawn in 1968 when some of its negative environmental effects were publicized. Such effects included persistence, killing of non-target organisms and accumulation in food chain organisms (Bevenue, 1976). In 1961, phosphamidon, an organophosphorous insecticide, was introduced as a substitute for DDT. However, due to its toxicity to birds it was not considered environmentally safe, (Nigam, 1975). Since 1969, fenitrothion, an organosphosphorous insecticide, has been used in Canadian chemical control programs (Prebble, 1975). In 1970, the area sprayed in New Brunswick increased from 17×10^5 to 40×10^5 ha. Notwithstanding these spray programs, by 1976 the total spruce budworm infestation distributed between New Brunswick, Quebec, Ontario, Maine and British Columbia exceeded 30×10^6 ha, (Symons, 1977).

Since 1977, the spray program in New Brunswick has employed aminocarb in addition to fenitrothion. The carbamate pesticide aminocarb is distributed in Canada by the Chemagro Division of the Baychem Corporation of Germany. It is the active ingredient in Matacil 1.8D. Technical aminocarb has been granted registration as an acceptable pesticide in the United States, Canada, Germany and Australia (Taylor, 1978). In Canada it is used to control spruce budworm. The pesticide is marketed as a formulation. The adjuvants added aid in the flow characteristics of pesticide sprays and enhance persistence of the pesticide. The essential function of aminocarb is to inhibit cholinesterase activity in the central nervous systems of the spruce budworm larvae and other agricultural pests (Kuhr and Dorough, 1976). The basic carbamate structure (Appendix A) is similar to acetylcholine. It complexes with acetylcholinesterase in the nerve synapse thus preventing hydrolysis of acetylcholine. The accumulation of acetylcholine causes continuous transmission of nerve

impulses thus impairing nervous coordination. In insects, this is manifested in hyper-activity, tremors-convulsions, paralysis and death. The rate of mortality is enhanced by concomitant dehydration of the central nervous system and subsequent histological degeneration (Gerolt, 1983). By 1978 and 1979, spray operations were based either on fenitrothion or aminocarb (Matacil) formulations, (Varty, 1980). In Quebec, operational sprays involved treatments with formulated fenitrothion and/or aminocarb. In Newfoundland, aminocarb was used in 3.76×10^5 ha and in Ontario, the pesticide of preference was aminocarb. In 1980-81 aerial applications of insecticides were conducted in New Brunswick, Quebec, Newfoundland, and Maine and on a smaller scale in Ontario and Nova Scotia. The total area damaged by budworm predation was 3.7×10^6 ha on the Eastern seaboard of North America. The main control chemicals used were fenitrothion and aminocarb at 210g and 52 or 70g of active ingredient per ha. respectively. By 1982, 1.7×10^6 ha were sprayed in New Brunswick of which 4×10^5 ha. of forest were sprayed mainly with fenitrothion and aminocarb formulations.

The aerial applications are made early in the morning (6-8 a.m.) or before dusk (19-21 h) to avoid air turbulence and to minimize dissipation of the spray cloud drift above the trees. All the spraying is conducted between the end of May and end of June, (Ketella, 1977). Aminocarb is applied in a formulation containing 20% aminocarb, 50% Nonylphenol and 30% of diluent oil and at 70g or 52g AI ha⁻¹. Two sprays about 5.7 days apart are applied at the time that the budworm is at the stage between the third and fifth instar larvae (Prebble, 1975).

1.2 Contamination of Aquatic Habitats

Over the past decade, millions of tonnes of pesticides have been added to the environment. Most pesticides reach the aquatic environment by direct application, drift, or leaching to ground waters. Although direct applications of aminocarb to large water bodies is generally avoided in Canada, (Kingsbury, 1978), all bodies of water in and around the sprayed areas may be contaminated either by direct or indirect spray, (Varty, 1980; McEwen and Stephenson, 1979).

The maximum concentration of Matacil in pond and stream water has been recorded at $2.6 \mu\text{g ml}^{-1}$, 4 days post-spray at an application rate of 78 g/ ha^{-1} , (Sundaran et al. 1980). Coady (1978), reported that after an aerial spray of $90 \text{ g of AI ha}^{-1}$ of aminocarb the peak concentration declined to below detectable limits within a day. Holmes (1979), recorded a wide range of aminocarb concentrations from $1 \mu\text{g L}^{-1}$ to $33 \mu\text{g L}^{-1}$ in one stream 4.4 h after one spray operation and $5.7 \mu\text{g L}^{-1}$ in a lake after two sprays. Elner and Wildish (1981), measured aminocarb residues in a small pond sprayed with the Matacil (aminocarb) 1.8D formulation. The aminocarb concentration in a pond ranged from $53 \mu\text{g L}^{-1}$, 13 h after spraying to $7 \mu\text{g L}^{-1}$, 12 h later. In a Newfoundland pond a concentration of $81 \mu\text{g L}^{-1}$ was found 36 hours post-spray at $70 \text{ g of A.I. ha}^{-1}$ (Osborne, 1979). Nonylphenol residues of $9.1 \mu\text{g L}^{-1}$ were reported present in a stream water 1 h after a Matacil application (Sundaran et al. 1980). This concentration declined to $4.5 \mu\text{g L}^{-1}$ 3 h post-spray. After five days only a trace amount was detected. In stagnant water, 4 h after spraying, the concentration of Nonylphenol was $1.1 \mu\text{g ml}^{-1}$. The residues declined to $0.1 \mu\text{g ml}^{-1}$ within 6 h post-spray and were not detectable

after 3 days. The rapid dissipation of Nonylphenol in stream water could be due mainly to the dilution by water flow and other physical factors such as surface evaporation and co-distillation.

Nonylphenol, one of the adjuvants in the Matacil 1.8D formulation, is a surfactant and has a wide range of industrial uses, from germicides, to oil and grease additives, detergents, shampoos and dyestuffs. Through its use, in these and other products, millions of pounds enter the environment. Concentrations of $1.5 \mu\text{g ml}^{-1}$ have recently been recorded in sewage water (Buikema et al. 1979).

Some surfactants have been found to be toxic to plants. For example, Newman and Jagendorf (1965), working with isolated spinach chloroplast found that Triton X-100 at low concentrations stimulated the Hill reaction and at high concentrations inhibited the reaction. Inhibition of mitosis in pea (Pisum sativum) seedlings by surfactants was reported by Nethery (1967). He hypothesized that inhibition may have resulted simultaneously at several stages of the mitotic cycle.

Horowitz and Givelgerg (1979) exposed plant roots to surfactant solutions and found a leakage of ions and amino acids into the bathing media. The amino acids that they detected were possibly dissociation products of membrane lipoproteins as a result of the binding of the surface active compounds to the cell membrane. Surface binding could affect membrane enzymatic activity and denaturing of protein membrane constituents.

Detergents affect biological membranes because they bind to the functional sites of the membrane proteins, removing cofactors necessary for enzyme activity (Helenious and Simons, 1975). A number of membrane enzymes have been found to depend on specific lipids for activity. When these

lipids are removed the enzymes are inactivated. In addition, the formation of micelles of detergent and phospholipid bring about membrane modification and depletion, or reduction, of intracellular sulfate.

Phenol compounds and their effects on aquatic environments have been reviewed by Buikema et al. (1979) the toxic effects of these compounds range from algistatic effects at low concentrations, to algicidal effects at high concentrations. The possible mode of action of some phenols on algae may be due to the uncoupling of oxidative phosphorylation and to the phenolic binding of the acid thiol groups. For example, Stom and Beym (1976), studied the effects of phenolic compounds on Nitella and they observed that the phenolics were able to modify the physico-chemical properties of the cytoplasm with complete inhibition of cyclosis. Jayaweera et al. (1982), found that pentachlorophenol (PCP) induced a decrease of membrane electrical potential. The toxic effects of pentachlorophenol are associated with the absorption of PCP on cell and sub-cellular membranes.

Algal-pesticide effects at the cellular and macromolecular level have been investigated by Sumida et al. (1977). They found that the growth of Chlorella was suppressed by 2.4 μM or higher concentrations of chlorphropham, a carbamate herbicide, 50% inhibition of cell division was observed with 1.3 μM , and protein and cell wall polysaccharide synthesis was inhibited, although lipid synthesis was stimulated.

The toxic effects of Lindane, which contains 99% of hexachlorocyclohexane were studied by Hansen (1979) on two green algae (Chlorella sp and C. pyrenoidosa). Toxicity was shown to be concentration dependant, 511 $\mu\text{g L}^{-1}$ Lindane was lethal to both algae. Between 511 and 200 $\mu\text{g L}^{-1}$, others cultures were not capable of reproduction. Between 100 and 10 μg

L^{-1} , 50% cell reduction was observed. In the range of 5-10 $\mu g\ ml^{-1}$, Lindane effects on cell division, cell cycle and some biosynthetic processes of two other unicellular algal, (Dunaliella bioculata and Amphidium carterae) were also noted (Jeanne, 1979).


Adjuvants, the spray additives, are always regarded as "inert" constituents of pesticide formulations, and their potential toxic effects to algae have not been considered. Recently, Moody et al. (1981) noted the algicidal properties of Aerotex 3470 and one of its components (methyl-naphthalene). They pointed out that ATP levels decreased in treated algae and that these xenobiotics produced gross ultrastructural changes in the cell. Further, Weinberger and Rea (1980) working with Chlorella pyrenoidosa found that the exponential growth rate of these algae was depressed by Nonylphenol and an LC_{50} (concentration which is lethal to 50% of a test population) was obtained with 1.5 $\mu g\ ml^{-1}$. Weinberger and Vladut (1981) observed a synergistic toxic effect between aminocarb and its adjuvants. Augmented toxicity towards jackpine and paper birch seedlings was observed when pesticide and adjuvants were presented as a formulation as compared with single chemical effects.

1.3 Rationale for Project

Apart from the work of Elner et al. (1982) and Weinberger and Rea (1980) little information has been published on the effects of aminocarb, Nonylphenol and Matacil 1.8D on algal cells. The present study was concerned with the effects of aminocarb and its adjuvants, namely, Nonylphenol and diluent oil 585 on cell division and macromolecular synthesis during the cell cycle of the motile alga Chlamydomonas segnis in synchro-

nized cultures. This alga was selected for study because it is known to be an inhabitant of Canadian aquatic environments where operational spray programs occur (Badour et al. 1972).

As in all eukaryotic cells, the cell cycle of Chlamydomonas segnis is divided, into the G_1 , S, G_2 , and mitotic (M) phases. The G_1 phase is the period that corresponds to the general growth and biosynthetic processes of the cell and accounts for 70% of the cell cycle. The S-phase is the period where synthesis of thymidine kinase, histones, and DNA synthesis take place. During G_2 phase the synthesis of microtubular protein is completed and M phase is the period that corresponds to cell division. These latter three periods occupy only 30% of the cell cycle (Fig. I). Also, previous work has produced a large amount of information concerning its cell cycle and physiological characteristics (Badour et al. 1972, 1977). It is an organism which is easy to synchronize, therefore the cell populations are homogenous and reproducibly cultured. Since Chlamydomonas segnis doubles its mass and cell number during one 24 h generation, effects on cell division and biochemical parameters were easily detected.



SECTION II

MATERIALS AND METHODS

2.1 Chemicals

Technical grade aminocarb (97.3% pure), Nonylphenol, diluent oil N^o 585 and Matacil 1.8D were all gifts from Mobay Co. Ltd. Mississauga, Ontario, Canada.

Bovine serum albumin (BSA), glucose and E. coli DNA standards for the macromolecular determination of protein, carbohydrate and DNA, respectively, were purchased from Sigma Chemical Co. USA. Perchloric acid (PCA), sulfuric acid, amyacetate, phenol and 2N Folin Ciocalteau phenol reagent were all reagent grades from Fisher Scientific Co. All organic solvents were glass distilled pesticide grade (Caledon). Chemicals used for the preparation of the algal culture media and reagents were purchased from Fisher Scientific Co.

2.2 Materials

An improved Neubauer-Levy haemocytometer was routinely used to determine cell counts using a light microscope (Wild Heerbrugg, Switzerland). A refrigerated Sorval Superspeed RC2-B centrifuge having a rotational capacity of 20,000 rpm was used to separate the algal cells from the media. A clinical centrifuge having a rotational capacity of 5,000 rpm and conical glass centrifuge tubes were used to sediment the residues after extraction of cell materials with various solvents. A filtration

unit and millipore filter (H.A. 0.45 μm) from Millipore Corporation were used for the chlorophyll extractions and glass microfibre filters (Whatman GF/A) for dry weight determinations. A Fisher isotemp oven (Fisher Scientific Co., USA) was used to dry the cells. A Hotpack programmed environmental incubator was used for cell culture. A 21 UVD Bausch & Lomb spectrophotometer with a wavelength range of 200-1000 nm was used in conjunction with analytical procedures to quantify the macromolecular determinations.

2.3 Algal and Culture Techniques

Chlamydomonas segnis was obtained from Dr. M. Czuba and Dr. D. Mortimer of the National Research Council, Ottawa, Ont., Canada. The alga were cultivated in Kuhl's liquid medium, (Kuhl and Lorenzen, 1964) which was auto-claved prior to use (final pH 6.8). The algal cells were induced to divide synchronously when they received a light intensity of 5.5 Klux obtained from Sylvania cool white fluorescent lamps and incubated in a Hotpack growth chamber (25°C). A photoperiod of 12 hr. light: 12 hr. dark was used. The culture was stirred constantly and bubbled with air (0.03% CO₂). Dilution of the culture to a standard cell density (2 x 10⁵ cells/ml) was routinely performed at the end of each dark period. Population densities were estimated by using a haemocytometer grid.

Prior to exposing Chlamydomonas segnis to the range of concentrations of the pesticide and adjuvants used in the present studies, the cellular content of the biochemical parameters to be followed were determined on control, nontreated sets of cells. Data was obtained on cell number, dry weight, total cellular protein, insoluble carbohydrate, RNA

and DNA at each of the experimental periods corresponding to -4 and -2 h before the dark period, at 0 h, and +2 h into the dark phase. These results provided the basis for comparison with the treatment sets. Table II.

2.4 Pesticide Application and Sampling

Aminocarb, Nonylphenol, diluent oil or Matacil 1.8D OSC were added to Kuhl's liquid medium after being dissolved in acetone. The final concentration of acetone was 0.2% in all the cases. The algal cells were then added to give a population density of 2×10^5 cells/ml. A control without solvent and a solvent control were also prepared.

The pesticide aminocarb, and/or the adjuvants and formulation, were applied at four experimental periods: four hours before the dark period corresponding to the end of G_2 , here designated as -4 h; two hours before the dark period corresponding, to the S-phase (-2 h); at the beginning of the dark period, that is the end of S-phase and the beginning of G_2 (0 h) and two hours into the dark period, corresponding to cytokinesis (+2 h). The concentrations of the xenobiotics used in parallel sets of experiments are given in Table 1.

The experimental design required that synchronized algal cultures be stressed with the test chemicals (aminocarb, Nonylphenol and diluent oil) at predetermined times of the cell cycle, and that cell number, dry weight, protein, carbohydrate, DNA and RNA content of the cells be assayed following completion of the cell cycle. In the Nonylphenol and Matacil treatment sets, chlorophyll determinations were also made.

Table 1. - Xenobiotics and the concentrations ($\mu\text{g ml}^{-1}$) used as test solutions.

Xenobiotic	Concentration ($\mu\text{g ml}^{-1}$)
Aminocarb	0.05, 0.5, 1, 5, 10 and 50
Diluent oil 585	5, 10 and 50
Nonylphenol	2.5
Matacil 1.8D	5 (1:2.5:1.5 $\mu\text{g ml}^{-1}$ of aminocarb: Nonylphenol: diluent oil respectively)

All experimental sets were set up in triplicate and repeated at least twice at different time periods

2.5 ANALYTICAL METHODOLOGY

2.5.1 Dry Weight

Three 50 ml aliquots of the algal suspension were taken at the end of the dark period. Cells were separated from the culture medium by filtration on a previously weighed glass microfibre filter (Whatman GF/C). The algal suspensions were washed with distilled water and then oven-dried for 4 hrs. at 100^U C. The filters with dry algae were cooled to room temperature in a dessicator for 15 mins. and then weighed. The dry weight was determined by difference between dry preweighed filter and oven-dried filter and alga. (Sorokin, 1973).

2.5.2 Chlorophyll Extraction and Determination

Chlorophyll determinations were made on the Nonylphenol and Matacil treatment sets.

Three 50 ml aliquots of the algal cultures were taken at the end of the dark period (0 h) and the cells were separated from the medium by filtration on a millipore filter (H.A. 0.45 μ m). The filter with the alga was placed into centrifuge tubes containing 10 ml of 90% acetone. These extracts were kept in the dark at 16^U C for 24 h after which the samples were centrifuged (5,000 rpm for 10'). Blanks with 90% of acetone and paper filter were prepared. The absorbance (A) of the extracted chlorophyll was read at 665, 645 and 630 nm in a Spectronic 21 UVD Bausch & Lomb spectrophotometer.

Quantitative chlorophyll values were obtained from the following equations:

$$Ca = 11.6 A_{665} - 1.31 A_{645} - 0.14 A_{630}$$

$$Cb = 20.7 A_{645} - 4.34 A_{665} - 4.42 A_{630}$$

(Hansmann, 1973)

2.5.3 Protein Extraction

Three 50 ml aliquots of the Chlamydomonas culture were collected by centrifugation for 10 min. at 5,000 r.p.m. The precipitate was extracted once with 90% acetone and twice with ethanol - ether (1:1) at 40^U C for 10 mins. to remove pigments and lipids. The decolorized residue was then collected by centrifugation (5,000 rpm for 10 min.), chilled with ice, and washed with 5 ml ice-cold 1N perchloric acid (PCA). The residue was then washed with 5 ml of cold ethanol, followed by ether and then dried with air. The dried material was solubilized with 2 ml of 1N NaOH and placed in a water bath for 18 hrs. at 37^U C. (Iwamura et al. 1970). Protein was determined by the method of Layne (1957) in which 0.2 ml of the extract was diluted to 0.30 ml with 1N NaOH. To this, 3 ml of Reagent (50 ml of 2% Na₂CO₃ and 1 ml of 5% CuSO₄ · H₂O in 1% sodium tartarate) was added. After 10 mins., 0.30 ml of 1N Folin Ciocalteu Reagent was added with a syringe. The mixture was allowed to stand at room temperature (22^U C) for 30 mins. absorbance at 720 nm were determined using a Bausch & Lomb spectrophotometer and protein was quantified from a calibration curve using BSA standards (10 - 150 μg ml⁻¹).

2.5.4 Carbohydrate Determination

0.20 ml of the hydrolyzate obtained in the above protein extraction was used for carbohydrate determinations. The aliquots taken for this purpose were adjusted to 2.0 ml by adding 1N NaOH. Then 0.5 μ l of phenol reagent (10 ml of H₂O - 90 ml of 90% phenol) was added and mixed thoroughly. Finally, 5 ml of concentrated H₂SO₄ was rapidly added and the mixture allowed to stand for 30 mins. before reading the absorbance at 485 nm. A calibration curve was prepared using a standard solution (10-150 μ g/ml glucose). (Kochert, 1978).

2.5.5 RNA Extraction

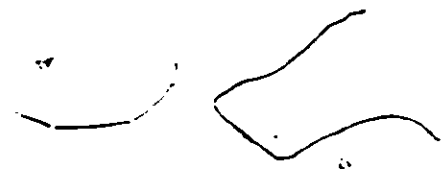
1.5 ml of the hydrolyzate obtained in the protein extraction was chilled and then neutralized with 0.16 ml of ice chilled 60% PCA. Then 0.10 ml of MgCl₂ (1M sol.) and 0.10 ml of BSA (0.5%) were added. The mixture was stirred and left standing for several minutes in an ice bath, then acidified with 0.2 ml of ice-chilled 60% PCA. To this, a further 1.94 ml of PCA was added. After centrifugation, the optical density of the supernatant was measured at 260 and 320 nm. The concentration of RNA was calculated by the equation:

$$\text{RNA } (\mu\text{g ml}^{-1}) = 29.5 \times (A_{260} - A_{320}) \times 4$$

A blank was prepared containing 1N PCA in 1.50 ml of 1N NaOH. (Iwamura et al., 1970).

2.5.6 DNA Extraction

The residue obtained in the above RNA extraction was washed with 5 ml of cold ethanol, then with ether and dried with air. The DNA extract was diluted with 2 ml of 0.5 N PCA and mixed with 1 ml of HCl 2.5 N and 1 ml of 0.06% indole solution. The color was developed following incubation of the mixture in a boiling water bath for 10 mins. This was then extracted with 4 ml of amylacetate. The absorbance of the extracted solution was read at 490 nm against the blank and compared with the values obtained with the standard DNA (prepared by dissolving in 0.5 N PCA). From this stock solution, working standards were prepared in the same way as the unknown samples to obtain data for delineating a calibration curve. (Keck, 1956).



SECTION III

RESULTS3.1 Controls

It was observed that the dry weight of the untreated controls increased 2.5 - fold, from 33 to 83 pg/cell throughout the light period (Table II). During the first part of the dark period (+2 h or 14 h) the dry weight of Chlamydomonas cells was similar to the dry weight of the cells at the beginning of light period (designated as 0 h in Table II).

The protein content of the untreated controls was 12 pg/cell at the beginning of the light period and it increased to 27 pg/cell over the light period, thereby doubling the initial content.

Total carbohydrates increased about 4 - fold from 9 to 34 pg/cell over the light period. The carbohydrate content began to decrease just before cellular division (14 h or +2 h), corresponding to the decrease obtained in dry weight.

The cellular RNA content doubled from 3 to 6 pg/cell over the light period and DNA doubled from 0.4 ± 0.1 to 1.0 ± 0.1 pg/cell during the synthesis phase.

Dry weight, protein, carbohydrate and RNA increased in a linear manner throughout the light period. DNA remained constant until two hours before the dark period when it doubled. As cell division occurred, the macromolecular content of these parameters returned to their initial values showed in Table II at the beginning of the light period.

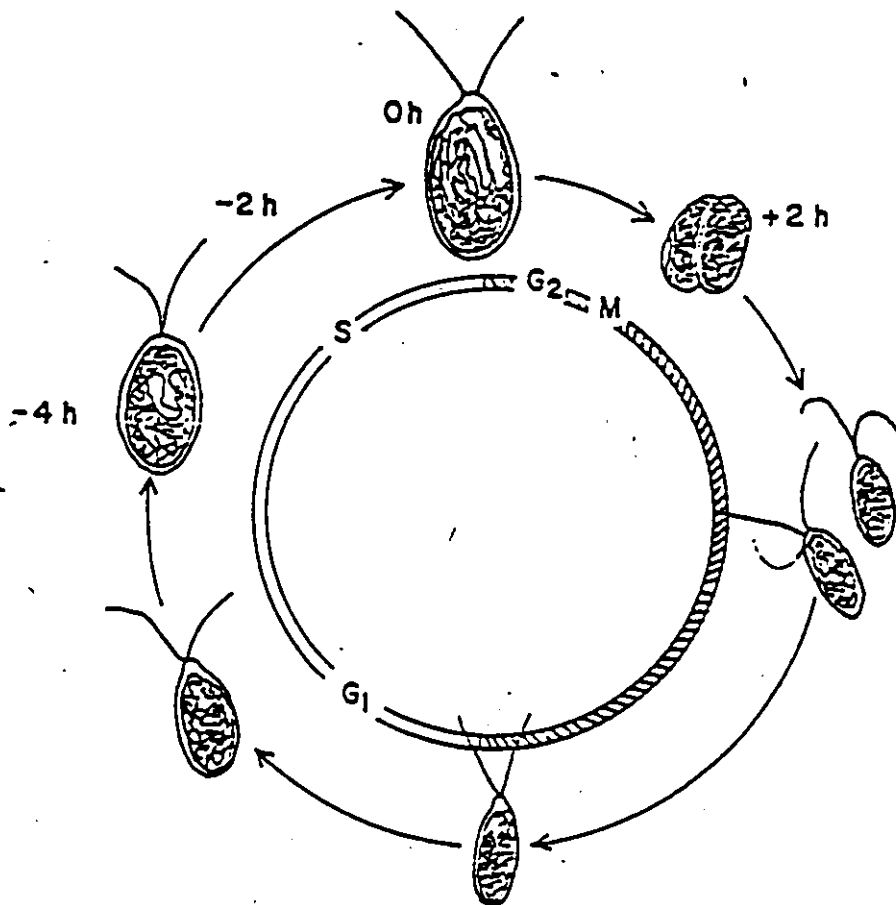


Fig. I. Diagrammatic representation of the asexual cycle of *C. segnis* in synchronous culture supplied with modified air. (Badour et al. 1972).

Table II. Metabolic parameters of control, untreated cells of Chlamydomonas
seguis at designated time periods \pm standard deviation.

Parameter	pg per cell				
Corresponding times h	0	8	10	12	14
in cell cycle, h.		-4	-2	0	+2
Dry weight	33 \pm 4	42 \pm 2	61 \pm 2	83 \pm 1	70 \pm 1
Protein	12 \pm 3	18 \pm 3	24 \pm 3	27 \pm 4	29 \pm 8
Carbohydrate	9 \pm 2	23 \pm 6	31 \pm 6	34 \pm 9	18 \pm 6
RNA	3 \pm 0.4	4 \pm 0.6	5 \pm 1	6 \pm 2	6 \pm 2
DNA	0.4 \pm 0.1	0.4 \pm 0.3	0.6 \pm 0.3	1 \pm 0.1	0.8 \pm 0.1
Time corresponds to	12h light period			dark period	

3.2 Aminocarb

Aminocarb at the lowest concentrations (0.05, 0.5 and 1 $\mu\text{g ml}^{-1}$) did not significantly affect any of the parameters examined following treatment at the -4, -2, 0, and +2 h experimental periods. Perturbations were observed at the three highest concentrations of aminocarb tested, namely, 5, 10 and 50 $\mu\text{g ml}^{-1}$ (Table III, IV. Fig. 2a). The effects on cell number and each biochemical parameter at each treatment level are given as pg/cell and percent of the control.

3.2.1 Cell Number

The effects of treatment with aminocarb on cell population growth at each of the experimental periods were followed and are summarized below and detailed in Fig. 2b and Table III.

-4 h: Exposure of Chlamydomonas cells to aminocarb, 50 and 10 $\mu\text{g/ml}$; arrested cell division whereas 5 $\mu\text{g ml}^{-1}$ inhibited the cell division process by 25%.

-2 h: At this time period, 50 $\mu\text{g ml}^{-1}$ of aminocarb not only completely inhibited cell division but was also algicidal. The initial cell number was decreased by 75%.

Following treatment with 10 $\mu\text{g ml}^{-1}$ the cell number remained constant throughout the experiment periods, whereas following exposure to 5 $\mu\text{g/ml}$ cell division was reduced by 25% compared to the control.

0 h: Exposure to 50 and 10 $\mu\text{g ml}^{-1}$ significantly reduced cell division by 40 and 20% respectively whereas the sets treated with 5 $\mu\text{g/ml}$ showed no significant differences from control.

+2 h: None of the algal cells exposed at this time period were affected by any of the aminocarb concentrations.

The most sensitive period relative to progression through the cell cycle was -2 hr. At 0 h, $10 \mu\text{g ml}^{-1}$ provided the threshold level for toxicity.

3.2.2 Dry Weight

Dry weight changes signalling effects on overall metabolic synthetic activity and biotic vigor are shown in Fig. 3 and Table (IV). Summaries of these effects are given below.

-4 h: A 1.5 to 2- fold increase in dry weight from $33 \pm 4.4 \text{ pg/cell}$ to 52, 61 and 51 pg/cell respectively was observed following exposure to 50, 10 or $5 \mu\text{g ml}^{-1}$ respectively (Tables II and IV). Treatment at -4 h evoked the greatest response. The 1.5, 2- fold increase in dry weight varied proportionally with the reduction in cell division (3.2.1).

-2 h: At this time period, $50 \mu\text{g ml}^{-1}$ of pesticide produced a 2- fold augmentation in dry weight. No effects on this parameter were obtained in the 10 or $5 \mu\text{g ml}^{-1}$ aminocarb treatment sets.

0 h: At this time period, 50 and $10 \mu\text{g ml}^{-1}$ of aminocarb significantly increased the cellular dry weight, a 1.4 to 1.7- fold increase in dry weight was observed, while $5 \mu\text{g ml}^{-1}$ of aminocarb did not significantly alter cellular dry weight when treatment and control sets were compared.

+2 h: At this time period, none of the treatments significantly altered the cellular dry weight.

3.2.3 Total protein per cell

The effects of aminocarb on total protein are given in Fig. 2 and Table IV and detailed in Fig. 4 and a summary of the data is given below.

-4 h: Exposure to 50 and 10 $\mu\text{g ml}^{-1}$ led to a 2-fold increase in protein content over the control at both treatment levels. Similarly, the % of protein as a function of dry weight almost doubled. Exposure to 5 $\mu\text{g ml}^{-1}$ pesticide did not significantly affect the protein content.

-2 h: Exposure to 50 $\mu\text{g ml}^{-1}$ pesticide significantly increased the protein content. A 2.5-fold augmentation was observed compared to the control. 10 or 50 $\mu\text{g ml}^{-1}$ treatments did not significantly affect the protein content.

0 h: Exposure of the cells to 50 $\mu\text{g ml}^{-1}$ did not significantly affect the protein content per cell, although the cell number was reduced by 40% by this treatment. (refer to 3.2.1).

Treatment with 10 $\mu\text{g ml}^{-1}$ led to a 2-fold increase in protein as compared with the untreated controls even though some 20% algiastasis was observed (refer to 3.2.1), that is, progression through the cell cycle was delayed but protein synthesis not only continued, but was actually stimulated. 5 $\mu\text{g ml}^{-1}$ did not significantly affect total cellular protein.

+2 h: None of the treatments affected the total cellular protein content.

3.2.4 Insoluble carbohydrate per cell

Perturbation effects on the total insoluble carbohydrate content of

C. segnis cells are shown in Fig. 5. Summaries of these effects follow.

- 4 h: Exposure to 50 and 10 $\mu\text{g ml}^{-1}$ pesticide led to a 1.6 to 1.7-fold increase, respectively, in insoluble carbohydrate. 10 $\mu\text{g ml}^{-1}$ was the threshold value for perturbation as no effects followed treatment at the 5 $\mu\text{g ml}^{-1}$ level.
- 2 h: Exposure to 50 $\mu\text{g ml}^{-1}$ evoked the same response as noted at -4 h, namely, a 1.6-fold increase in insoluble carbohydrate. No effects were observed following exposure to 10 or 5 $\mu\text{g ml}^{-1}$ indicating that this was a less sensitive phase of the cell cycle.
- 0 h: Exposure to 50 or 10 $\mu\text{g ml}^{-1}$ evoked a 2-fold augmentation in carbohydrate content levels in terms of pg/cell as well as on a dry weight basis. This period was slightly more sensitive than the -4 h treatment period.
- +2 h: None of the treatments (50, 10 or 5 $\mu\text{g ml}^{-1}$) led to significant changes in the insoluble carbohydrate content of the cells, again supporting the dry weight data.

3.2.5 Total RNA per cell

In Fig. 6 the effects of aminocarb-treatment on total cellular RNA are graphically displayed. The highlights of the observed changes are detailed below.

- 4 h: Exposure of the cells to 50 $\mu\text{g ml}^{-1}$ caused a 2-fold accumulation in RNA levels compared with the control. This period was the most sensitive period with respect to RNA content. 10 and 5 $\mu\text{g ml}^{-1}$ pesticide did not cause significant effects.
- 2 h: Exposure of the cells to 50, 10 and 5 $\mu\text{g ml}^{-1}$ pesticide did not produce significant effects on RNA content.
- 0 h: Exposure to 10 $\mu\text{g ml}^{-1}$ pesticide significantly increased cel-

lular RNA in treated cells by 1.8- fold.

Fifty and 5 $\mu\text{g ml}^{-1}$ did not affect RNA content of the cells. The lack of effect at the 50 $\mu\text{g ml}^{-1}$ treatment level was unexpected.

+2 h: None of the treatments led to a significant change in the total cellular level of RNA.

3.2.6 DNA

Total cellular DNA fluctuations with treatment level and time of exposure are shown in Fig. 7. Summaries are given below.

-4 h: A 2.5, 3 and 1.6- fold augmentation in DNA content was observed followed exposure to 50, 10 and 5 $\mu\text{g ml}^{-1}$ pesticide respectively. Evidently, the 50 and 10 $\mu\text{g ml}^{-1}$ treatments had similar effects. A dose response effect was observed at the 5 $\mu\text{g ml}^{-1}$ level. There was a 50% reduction in this treatment whereas cell number was decreased by 25%.

-2 h: Exposure of the cells to 50 $\mu\text{g ml}^{-1}$ led to a 2- fold accumulation in DNA. In section 3.1.1 it was noted that this level of treatment was potentially algicidal. In these cells then, DNA accumulated. No effects on DNA content were obtained in the 10 or 5 $\mu\text{g ml}^{-1}$ aminocarb treatment sets.

0 h: At this time period, exposure of the cells to 50 and 10 $\mu\text{g ml}^{-1}$ of pesticide produced a 2- fold increase in DNA. As cell number was lowered by 40 and 20% respectively, supra-stimulated synthesis of DNA was evident following these treatments.

+2 h: None of the treatments led to significant changes in DNA content of the cells.

Table III. Effects of aminocarb (50, 10, 5 $\mu\text{g ml}^{-1}$) on cell number of Chlamydomonas segnis ($\times 10^3$) as observed at the end of the dark period. \pm standard deviation.

Time of Application	Aminocarb ($\mu\text{g ml}^{-1}$)		
	50	10	5
- 4hr	1.9 \pm 0.1d	2 \pm 0.4d	2.8 \pm 0.6b
- 2hr	0.5 \pm 0.1d	2 \pm 0.3d	3 \pm 0.3c
0hr	2.4 \pm 0.1d	3 \pm 0.2c	3.7 \pm 0.8a
+ 2hr	3.7 \pm 0.2a	3.8 \pm 0.4a	3.9 \pm 0.4a
Control	4 \pm 0.2		
Initial No. of cells	2 \pm 0.2		

a = Indicates no significant difference

b, c, d = Indicates significant difference from the controls at the 0.05, 0.01 and 0.005 level respectively (t-test).

Figure 2a. Effects of different concentrations of aminocarb (50, 10 and 5 $\mu\text{g ml}^{-1}$) at the end of the dark period on dry weight and macromolecular content of Chlamydomonas segnis applied at different times of the cell cycle.

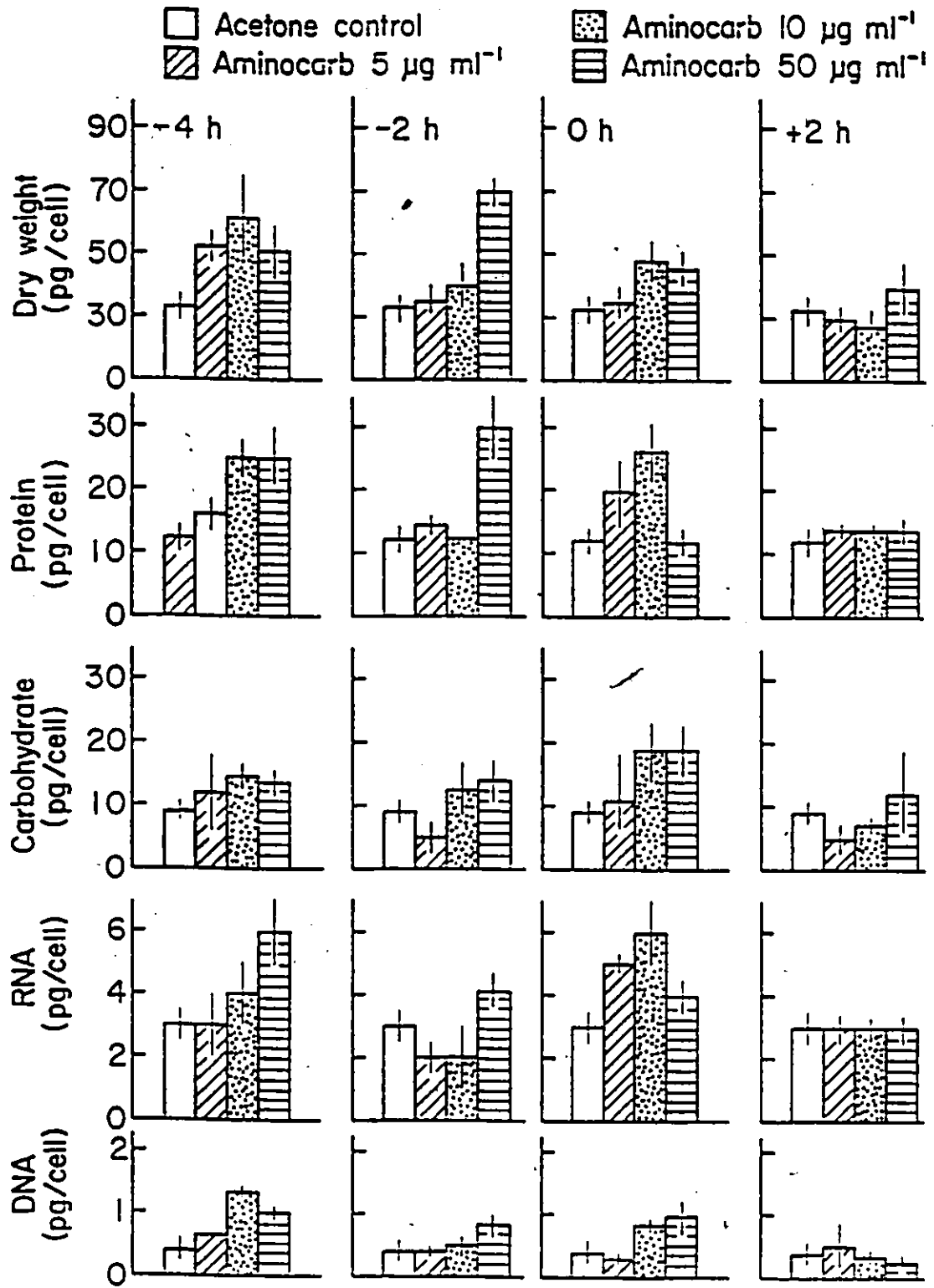


Table IV.- Effects of different concentrations of aminocarb (50, 10, and 5 $\mu\text{g} \cdot \text{ml}^{-1}$) at the end of the dark period on dry weights and macromolecular content of *Chlamydomonas reinhardtii* applied at different times of the cell cycle

Time of treatment - 4h									
Parameter	50 $\mu\text{g} \cdot \text{ml}^{-1}$			10 $\mu\text{g} \cdot \text{ml}^{-1}$			5 $\mu\text{g} \cdot \text{ml}^{-1}$		
	pg/cell	% of control	% of increase as % dry wt.	pg/cell	% of control	% of increase as % dry wt.	pg/cell	% of control	% of increase as % dry wt.
Dry weight	52 _d	150		61 _b	180		51 _b	150	
Protein	25 _d	200	75	25 _d	200	75	16 _a	130	47
Carbohydrate	14 _d	160	41	15 _c	170	44	12 _c	140	36
RNA	6 _d	190	18	4 _a	130	12	3 _a	100	10
DNA	1 _d	250	3	13 _d	300	4	06 _d	160	2

Time of treatment -2h									
Parameter	50 $\mu\text{g} \cdot \text{ml}^{-1}$			10 $\mu\text{g} \cdot \text{ml}^{-1}$			5 $\mu\text{g} \cdot \text{ml}^{-1}$		
	pg/cell	% of control	% of increase as % dry wt.	pg/cell	% of control	% of increase as % dry wt.	pg/cell	% of control	% of increase as % dry wt.
Dry weight	70 _d	212		40 _a	120		35 _a	106	
Protein	30 _b	250	91	12 _a	100	36	14 _a	116	42
Carbohydrate	14 _d	160	42	13 _d	150	40	5 _a	58	15
RNA	4 _a	130	12	2 _b	66	5	2 _a	66	5
DNA	08 _d	200	2	05 _a	120	15	04 _a	100	1

Time of treatment 0h									
Parameter	50 $\mu\text{g} \cdot \text{ml}^{-1}$			10 $\mu\text{g} \cdot \text{ml}^{-1}$			5 $\mu\text{g} \cdot \text{ml}^{-1}$		
	pg/cell	% of control	% of increase as % dry wt.	pg/cell	% of control	% of increase as % dry wt.	pg/cell	% of control	% of increase as % dry wt.
Dry weight	46 _c	140		48 _d	168		35 _d	106	
Protein	12 _a	100	38	27 _d	200	81	20 _a	160	60
Carbohydrate	18 _d	200	56	18 _d	200	55	11 _a	130	34
RNA	4 _a	120	11	6 _c	180	16	5 _a	160	14
DNA	1 _c	220	3	08 _d	200	3	03 _a	75	1

Time of treatment +2h									
Parameter	50 $\mu\text{g} \cdot \text{ml}^{-1}$			10 $\mu\text{g} \cdot \text{ml}^{-1}$			5 $\mu\text{g} \cdot \text{ml}^{-1}$		
	pg/cell	% of control	% of increase as % dry wt.	pg/cell	% of control	% of increase as % dry wt.	pg/cell	% of control	% of increase as % dry wt.
Dry weight	40 _a	120		28 _a	84		30 _a	87	
Protein	14 _a	119	41	14 _a	110	41	14 _a	110	42
Carbohydrate	12 _c	140	37	7 _c	82	20	5 _a	61	16
RNA	3 _a	100	10	3 _a	100	10	3 _a	100	10
DNA	03 _a	75	1	03 _a	88	1	05 _a	110	1

a.- Indicate no significant difference.

Figure 2b. Effects of different concentrations of aminocarb (50, 10, and 5 $\mu\text{g ml}^{-1}$) on cell number of Chlamydomonas segnis when the pesticide was applied at different times of the cell cycle. The average values of triplicate samples are shown together with standard deviation bars.

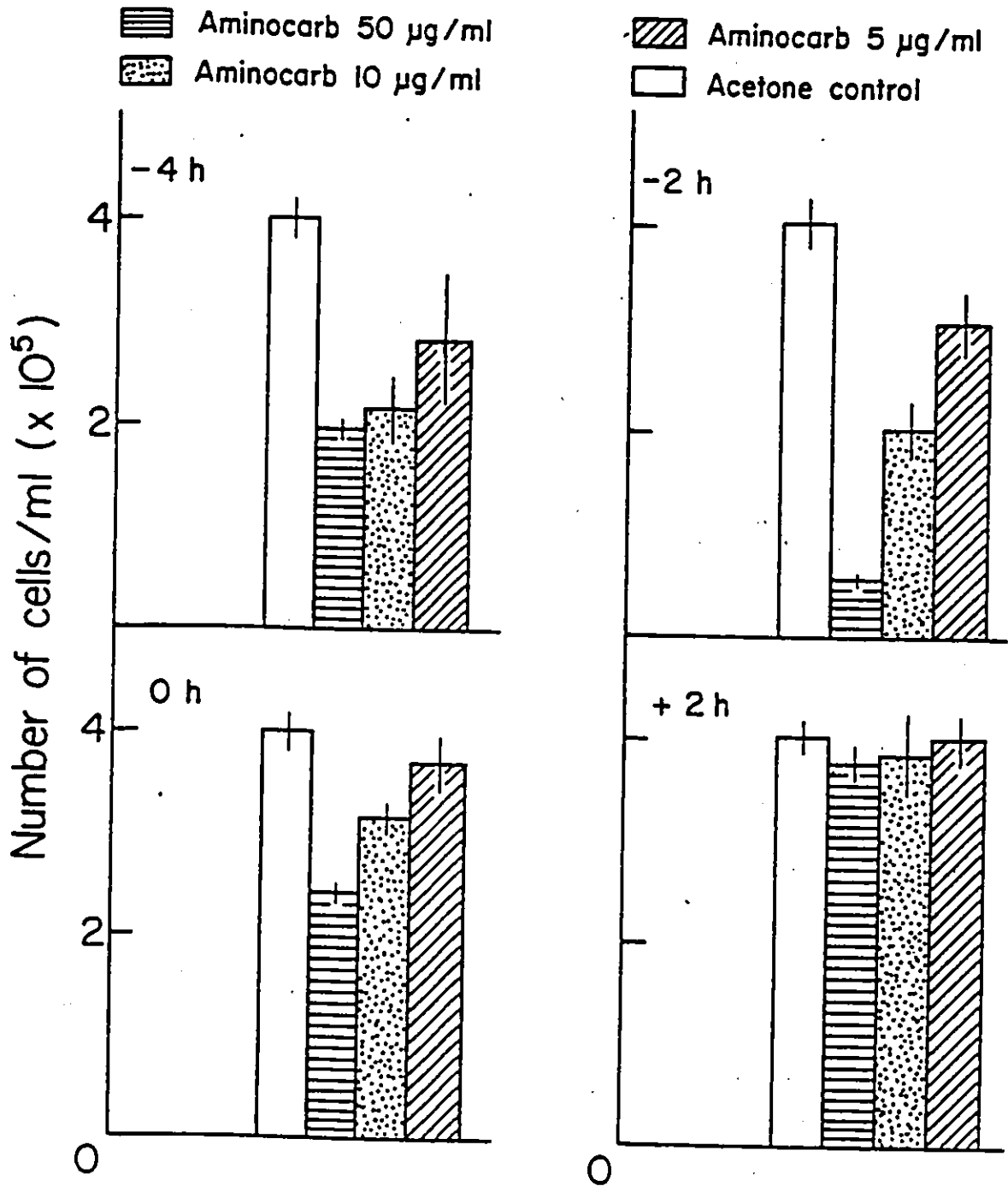




Figure 3. Effects of different concentrations of aminocarb (50, 10, and 5 $\mu\text{g ml}^{-1}$) on the dry weight content of Chlamydomonas segnis when the pesticide was applied at different times of the cell cycle. The average values of triplicate samples are shown together with standard deviation bars. Arrows indicate addition of the pesticide.  Light,  Dark periods.

(c.f. Tables II and IV)

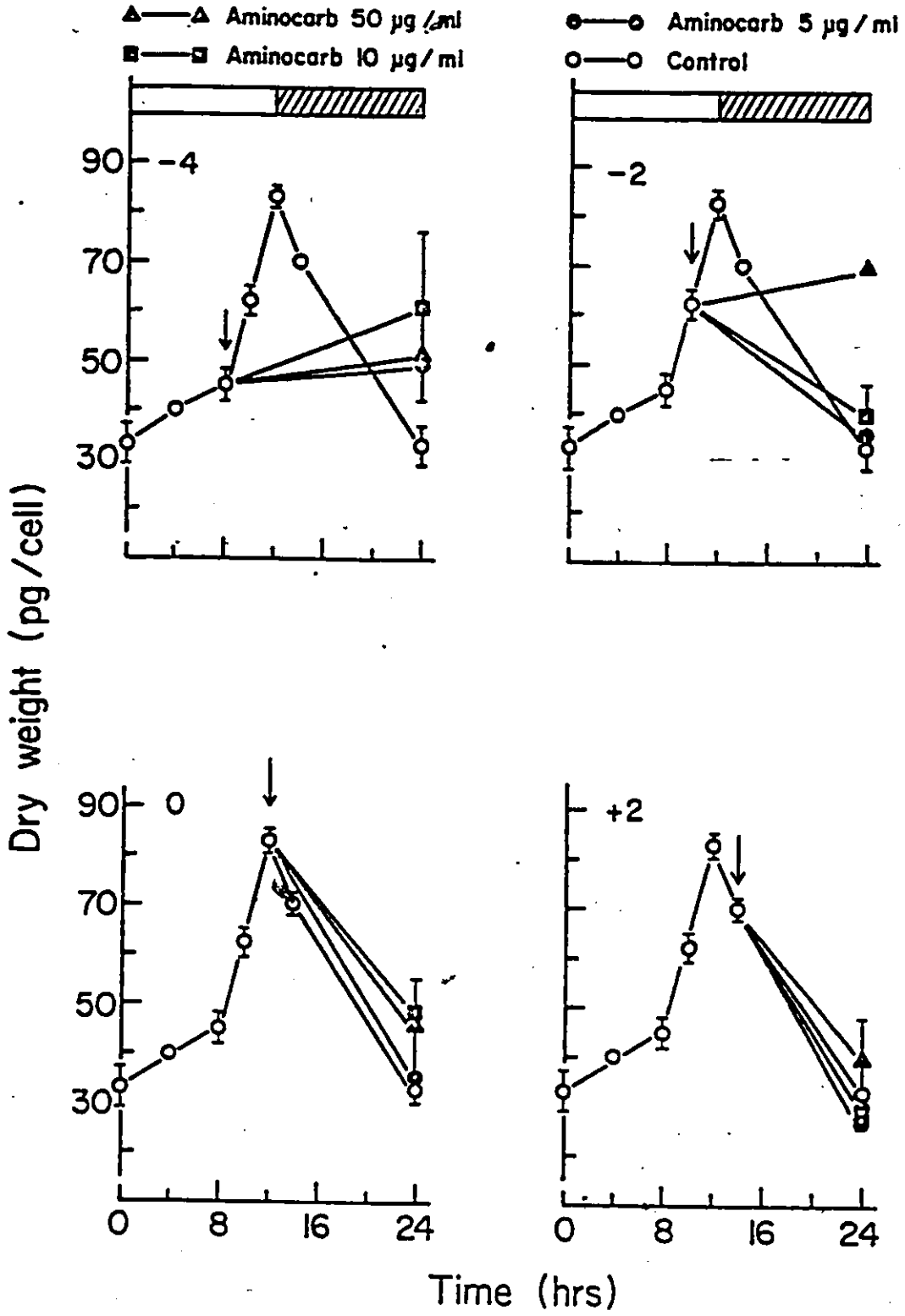
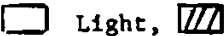



Figure 4. Effects of different concentrations of aminocarb (50, 10, and 5 $\mu\text{g ml}^{-1}$) on the protein content of Chlamydomonas segnis when the pesticide was applied at different times of the cell cycle. The average values of triplicate samples are shown together with standard deviation bars. Arrows indicate addition of the pesticide.  Light,  Dark periods.
(c.f. Fig. 2a and Table IV)

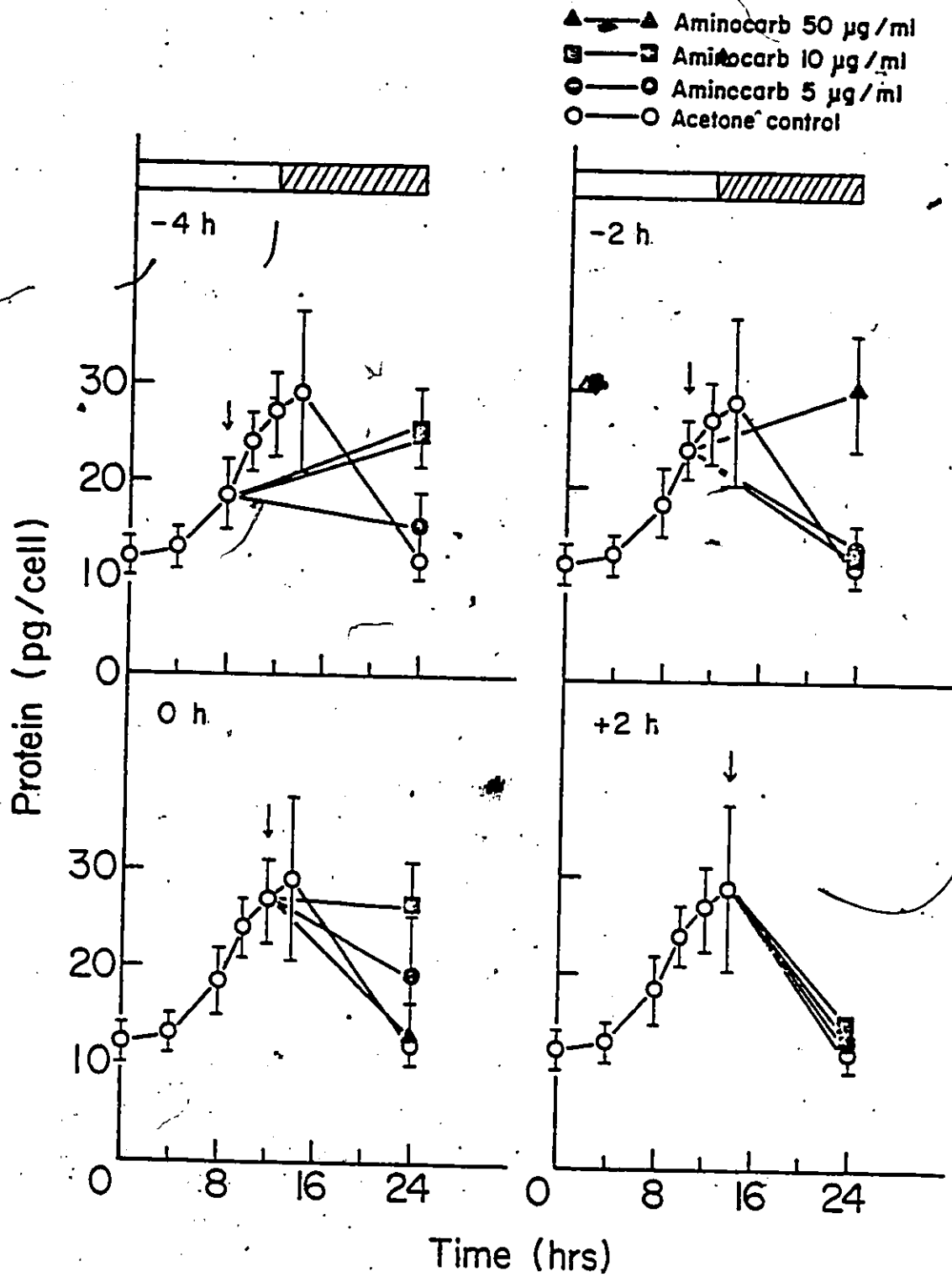




Figure 5. Effects of different concentrations of aminocarb (50, 10, and 5 $\mu\text{g ml}^{-1}$) on carbohydrate content of Chlamydomonas segnis when the pesticide was applied at different times of the cell cycle. The average values of triplicate samples are shown together with standard deviation bars. Arrows indicate addition of the pesticide.  Light,  Dark periods.

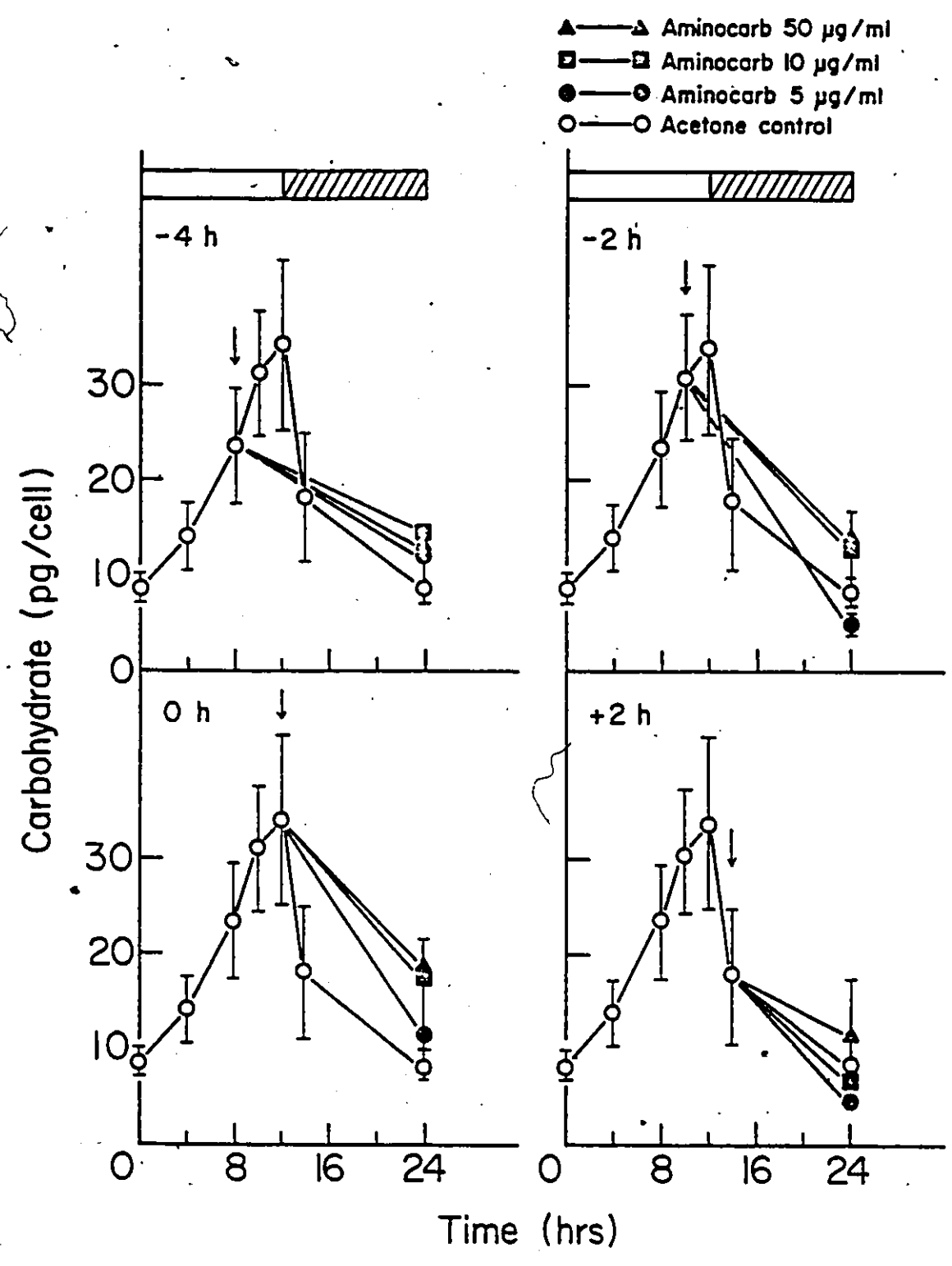


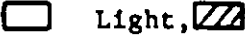


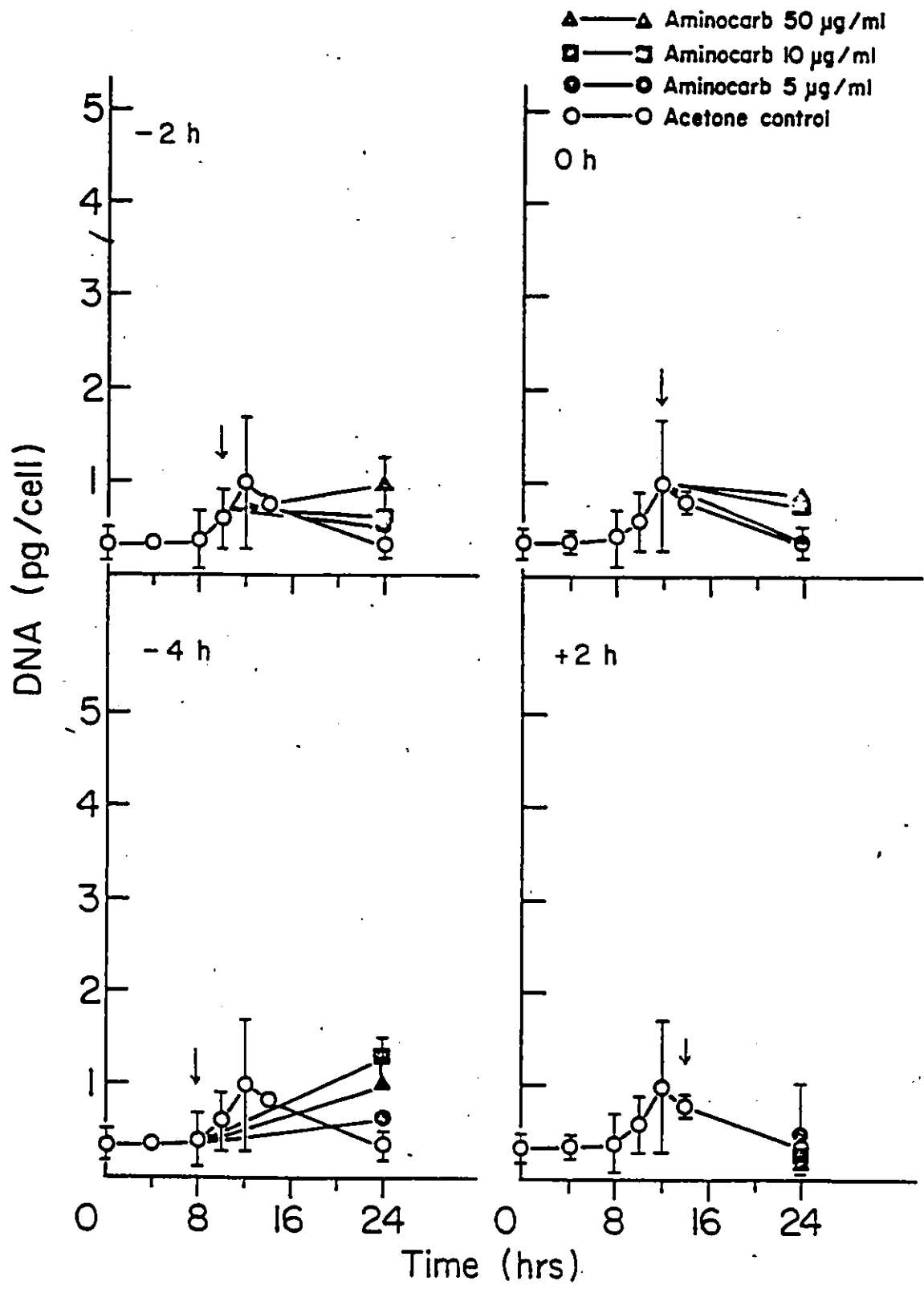
Figure 6. Effects of different concentrations of aminocarb (50, 10, and 5 $\mu\text{g ml}^{-1}$) on RNA content of Chlamydomonas segnis when the pesticide was applied at different times of the cell cycle. The average values of triplicate samples are shown together with standard deviation bars. Arrows indicate addition of the pesticide.  Light,  Dark periods.

Figure 7. Effects of different concentrations of aminocarb (50, 10, and 5 $\mu\text{g ml}^{-1}$) on DNA content of Chlamydomonas segnis when the pesticide was applied at different times of the cell cycle. The average values of triplicate samples are shown together with standard deviation bars. Arrows indicate addition of the pesticide.





3.3 Diluent oil

3.3.1 Cell number

Treatment with 50, 10 and 5 $\mu\text{g ml}^{-1}$ of diluent oil at -4, 0 and 2 h did not significantly affect cell number whereas treatment at -2 h with 50 $\mu\text{g ml}^{-1}$ significantly decreased the cell number by 20%. However, treatment at this time with the lower concentrations of oil, namely, 10 and 5 $\mu\text{g ml}^{-1}$ did not significantly affect overall cell number. (Fig. 8).

3.3.2 Protein, carbohydrate, RNA and DNA

None of these parameters were affected by any of the tested concentrations of oil at any of the treatment times. (Figs. 9, 10, 11, 12).

Figure 8. Effects of different concentrations of diluent oil (50, 10, 5 $\mu\text{g ml}^{-1}$) on cell number of Chlamydomonas segnis when the xenobiotic was applied at different times of the cell cycle. The average values of triplicate samples are shown together with standard deviation bars.

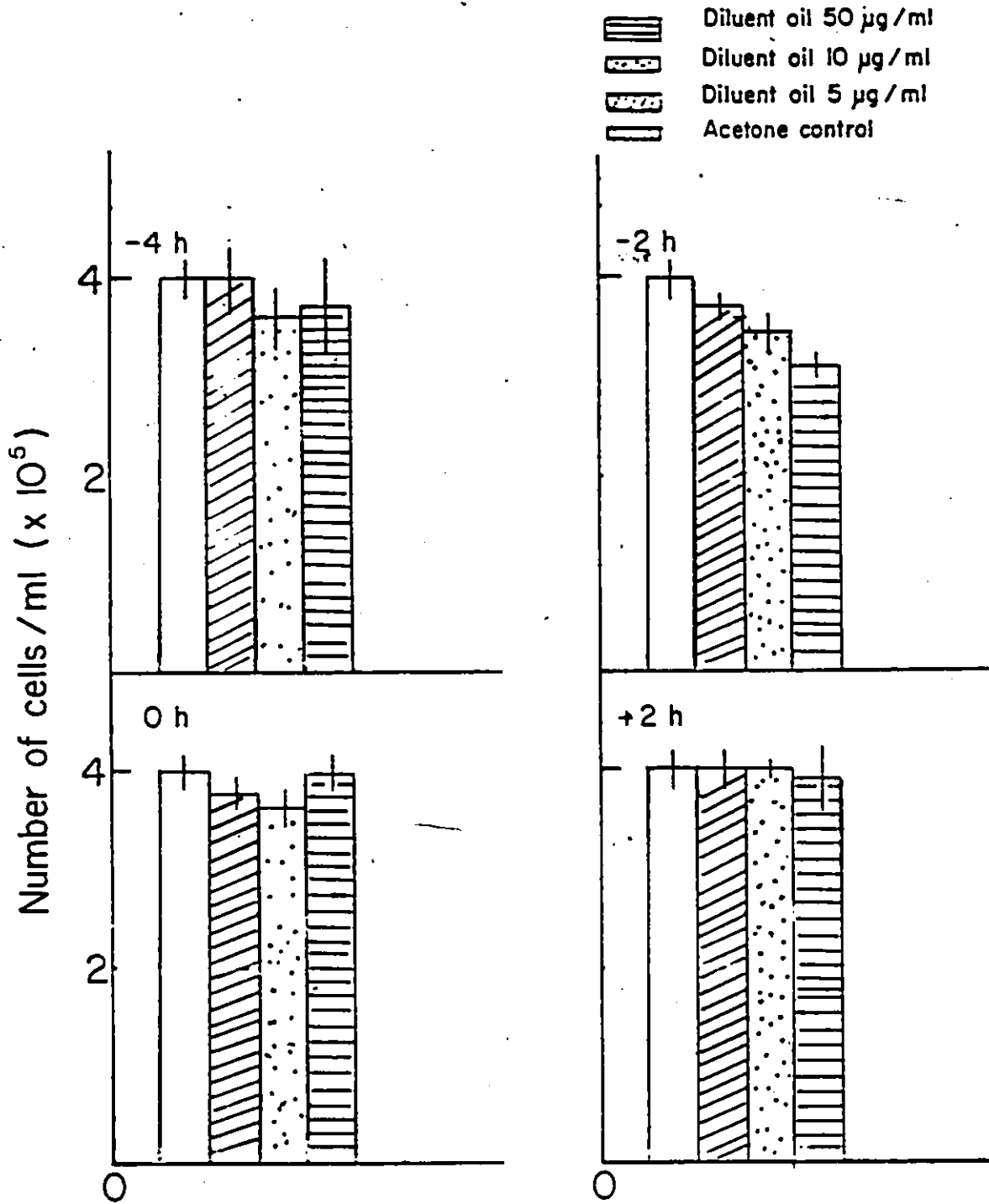




Figure 9. Effects of different concentrations of diluent oil (50, 10, 5 $\mu\text{g ml}^{-1}$) on protein content of Chlamydomonas segnis when the xenobiotic was applied at different times of the cell cycle. The average values of triplicate samples are shown together with standard deviation bars. Arrows indicate addition of diluent oil.  Light,  Dark periods.

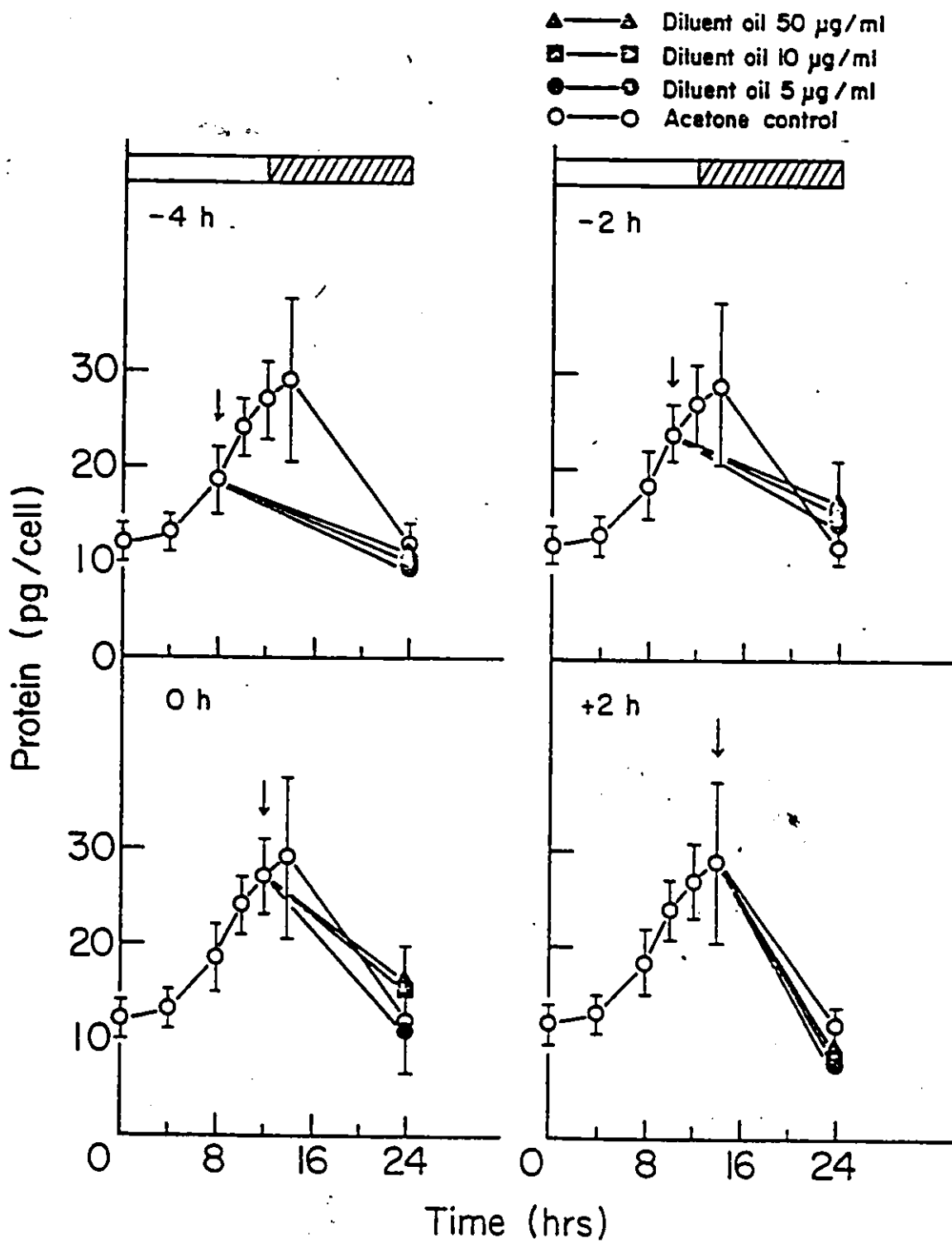
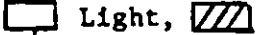



Figure 10. Effects of different concentrations of diluent oil (50, 10, 5 $\mu\text{g ml}^{-1}$) on carbohydrate content Chlamydomonas segnis when the xenobiotic was applied at different times of the cell cycle. The average values of triplicate samples are shown together with standard deviation bars. Arrows indicate addition of diluent oil.  Light,  Dark periods.

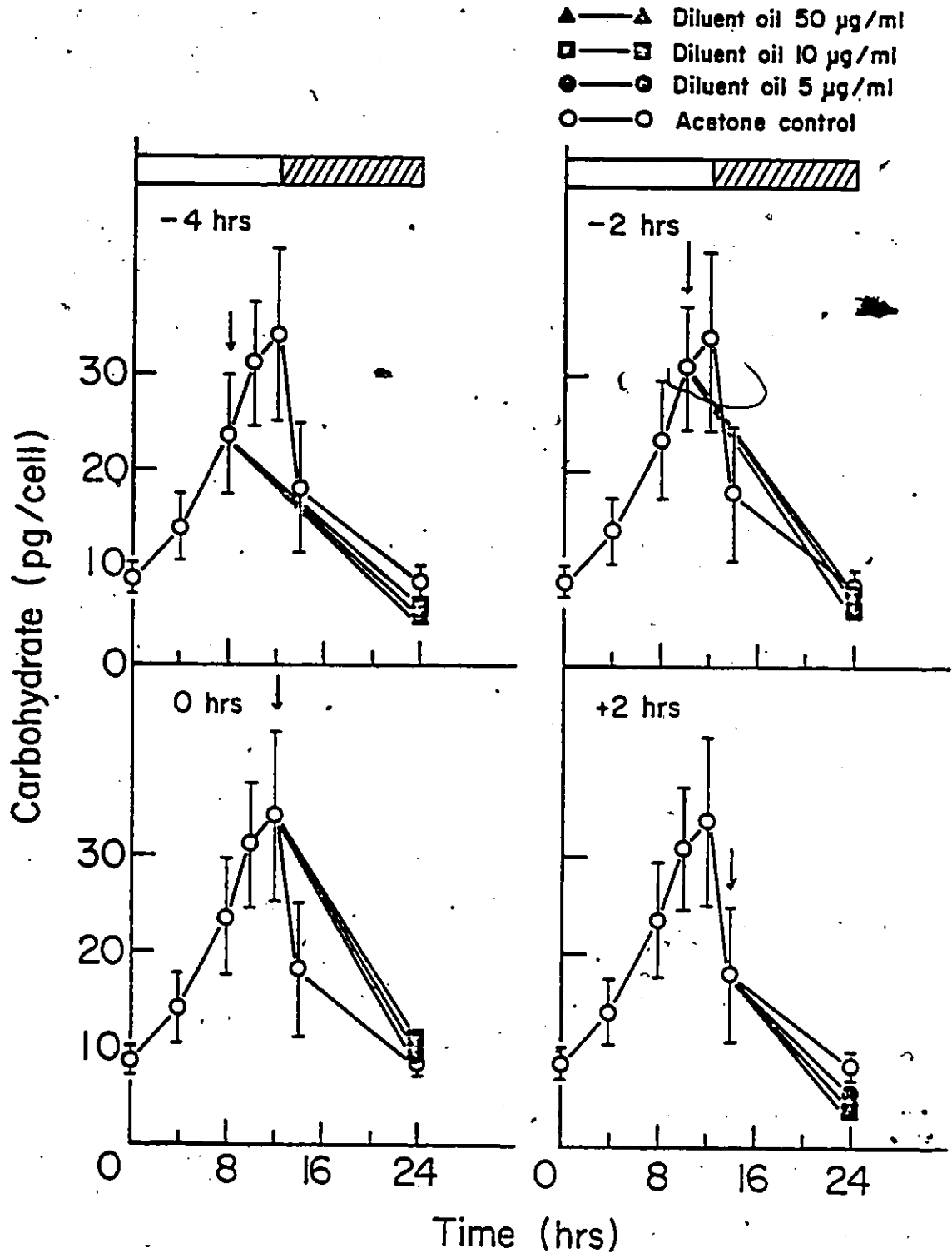
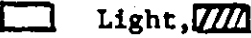

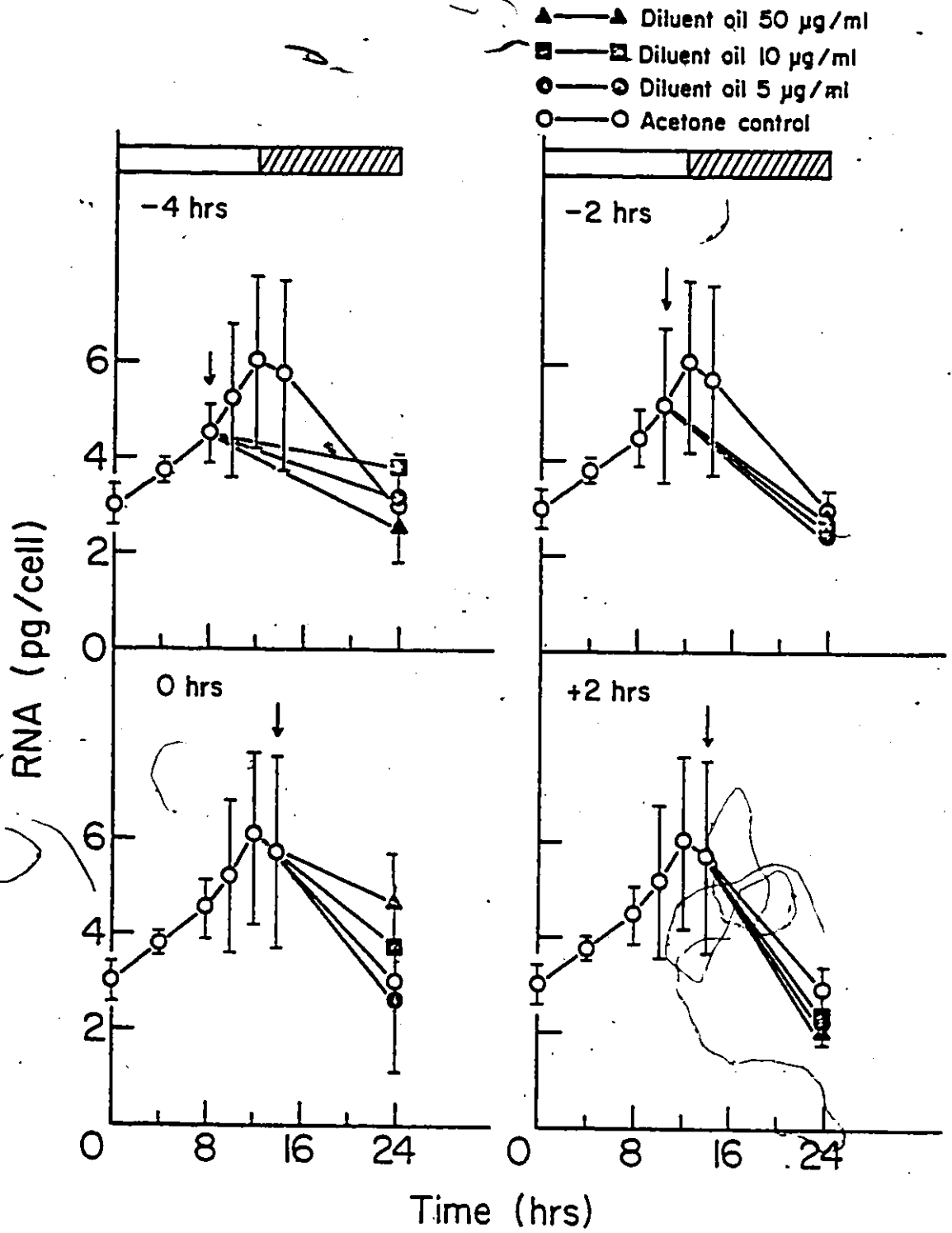


Figure 11. Effects of different concentrations of diluent oil (50, 10, 5 $\mu\text{g ml}^{-1}$) on RNA content of Chlamydomonas seguis when the xenobiotic was applied at different times of the cell cycle. The average values of triplicate samples are shown together with standard deviation bars. Arrows indicate addition of diluent oil.  Light,  Dark periods.



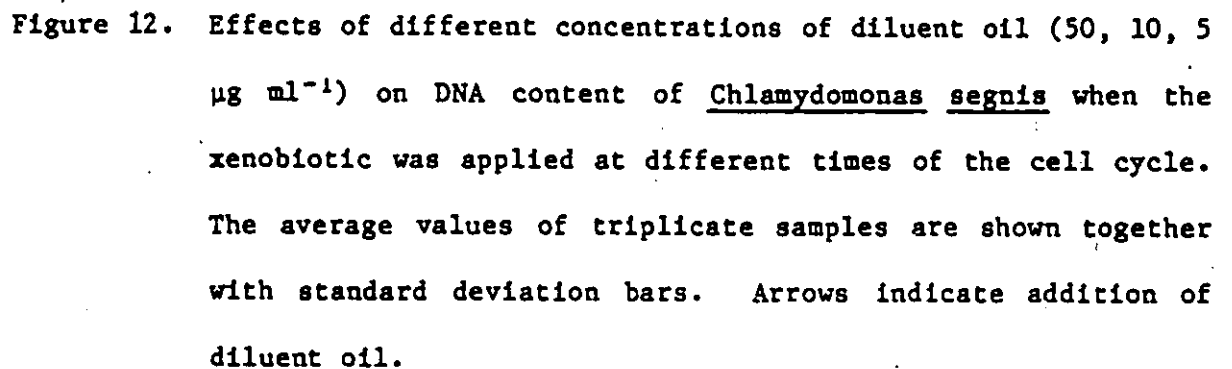


Figure 12. Effects of different concentrations of diluent oil (50, 10, 5 $\mu\text{g ml}^{-1}$) on DNA content of Chlamydomonas segnis when the xenobiotic was applied at different times of the cell cycle. The average values of triplicate samples are shown together with standard deviation bars. Arrows indicate addition of diluent oil.

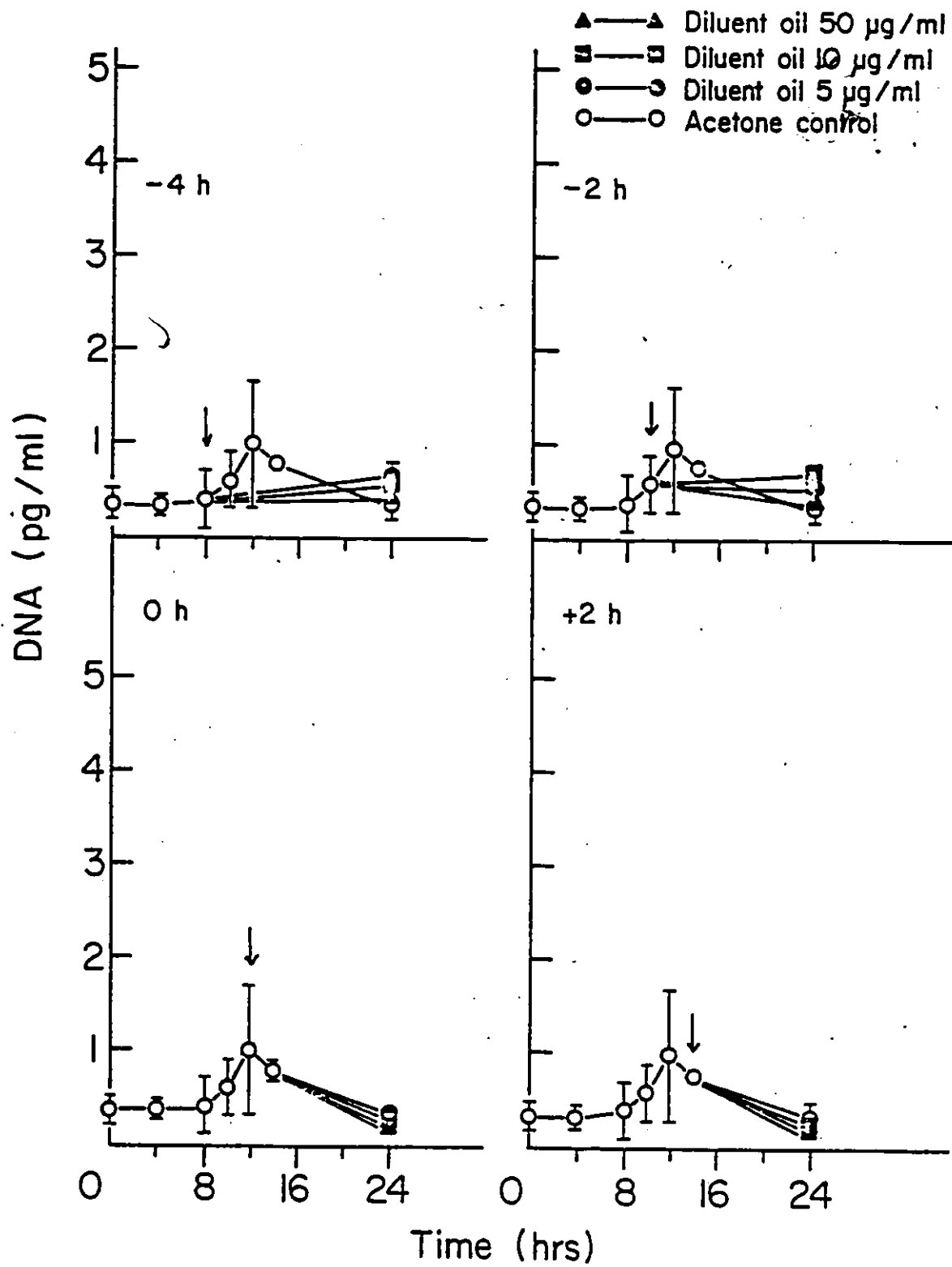


Table V. Effects of diluent oil (50, 10, 5 $\mu\text{g ml}^{-1}$) on cell number of Chlamydomonas segnis ($\times 10^3$) as observed at the end of the dark period.
 \pm standard deviation.

Time of Treatment	Diluent oil ($\mu\text{g ml}^{-1}$)		
	50	10	5
-4hr	3.7 \pm 0.5	3.6 \pm 0.4	4 \pm 0.5
-2hr	3.2 \pm 0.1	3.5 \pm 0.4	3.7 \pm 0.3
0hr	4 \pm 0.2	3.7 \pm 0.2	3.9 \pm 0.2
-2hr	3.9 \pm 0.4	4 \pm 0.1	4 \pm 0.3
Control	4 \pm 0.2		
Initial	No. of cells 2 \pm 0.2		

3.4 Nonylphenol (2.5 $\mu\text{g ml}^{-1}$)

The effects of Nonylphenol on the cell cycle events and macromolecular synthetic capacity of C. segnis cells were followed and are separately described below.

3.4.1 Cell Number

In general, 2.5 $\mu\text{g ml}^{-1}$ of nonylphenol was algistatic or algicidal at all the time periods tested (Table VI, Fig. 13).

-4 h: Exposure of the cells to 2.5 $\mu\text{g ml}^{-1}$ of Nonylphenol completely inhibited cell division, there was no change in the cell number from the initial value over the course of the experiment.

-2 h: Exposure of the cells to Nonylphenol at this time caused a 30% reduction in the initial cell number. Cell division was inhibited and an algicidal effect was seen. This time period was the most sensitive period with respect to effects on cell number.

0 h: Results were similar to those obtained at -4 h, that is, exposure of the cells to Nonylphenol led to a complete inhibition of cell division. The effects were 100% algistatic but not algicidal.

+2 h: Exposure of the cells to Nonylphenol at the time that the cells were undergoing cytokinesis was algistatic to 75% of the population. Mitosis was completed in all the cells but the autospores were released in only 25% of the population.

Because of this, final cell number increased by only 25% relative to the initial cell number.

Nonylphenol appears to affect the cell cycle at three different points depending upon the time of application relative to the cell cycle. Applied at -4 hr, -2 hr and 0 hr, the cells were held in G₂ and further, at -2 hr, some 30% of the cells were killed. Whereas applied at +2 hr, when mitosis was actually taking place, the mitotic cycle (prophase through telophase and cytokinesis) was unaffected, but zoospore release was inhibited in 75% of the treated cells.

3.4.2 Total Cellular Dry Weight

In the present study, it was observed that in Chlamydomonas cultures growing synchronously, dry weight increased continuously until the end of the light period, then cell dry weight decreased thereafter with the release of 2 autospores in the dark period, leading to a doubling in the population. This response was altered when the culture was subjected to Nonylphenol treatment, the pattern of change being dependent on the time of the cycle at which the Nonylphenol treatment was initiated. (Fig. 14, Table VII).

-4 h: When cells were exposed to Nonylphenol at this time period the subsequent increase in dry weight, in the light period, was moderate. (Fig. 14). Additionally, cellular dry weight did not decrease toward the end of the dark cycle as it did in the control sets, thus resulting in a 1.6 augmentation in cell dry weight by the end of the dark period as compared with the control sets. The cellular increase in dry weight is likely related to the absence of cell division.

- 2 h: Exposure of the cells to Nonylphenol caused a 2- fold increase in cell dry weight compared with the controls at the end of the dark period.
- 0 h: Results were similar to those obtained at -2 h exposure, that is, there was a 2- fold accumulation in dry weight per cell as a result of inhibition of cell division. However, taking into consideration the level of cell dry weight at the time of treatment, some decrease was observed which suggests that some hydrolytic enzyme activity could be involved in this decrease.
- +2 h: Exposure to Nonylphenol at this time period caused a 1.5- fold accumulation in dry weight of the treated cells compared with the control cells. This period was the least sensitive period with respect to cell dry weight content. In the control, carbohydrate content normally decreased following the dark period. In the first 2 hours of the dark period, cells usually mobilize and utilize carbohydrates in preparation for cell division. This leads to a decrease in carbohydrate content in control cells which continues as the cells divide and release the 2 autospores. In the treated sets, however, cell division occurred in only 25% of the cells, and as noted above, cell dry weight decrease was proportionate to this indicating the close relationship between cell dry weight and auto-spore formation and release.

3.4.3 Total protein content

The total protein content of the Chlamydomonas cells was also sensitive to treatment concentration and cell cycle phase at time of applica-

tion, (Fig. 15).

- 4 h: At this time of the treatment application the cellular protein content was 1.6- fold more than the initial amount of protein present in each cell at the beginning of the light period. At the end of the dark period, the protein content in treated cells remained at more or less the same level as when the pesticide was applied (1.7- fold) whereas in the control sets protein per cell was halved. This indicates that protein synthesis was inhibited after treatment in the light period and that protein per cell was accumulated in the dark period as a result of cell division inhibition.
- 2 h: Exposure of the cells to Nonylphenol led a 2- fold protein accumulation. Similarly, the % of protein on a dry weight basis also doubled. (Table VII).
- 0 h: Exposure of the cells to Nonylphenol caused a 1.8- fold accumulation in cellular protein in treated cells, compared to the control cells. Following treatment, accumulation at this time period (0 hr) was less than at -2 hr, this could indicate that 20% of the total protein may have been lost.
- +2 h: Exposure of the cells to Nonylphenol caused a 3- fold increase in cellular protein levels in treated cells compared to the controls. This increase may be correlated with the 75% inhibition in cell division noted under 3.3.1 or perhaps supra-stimulation of protein synthesis after treatment.

3.4.4 Insoluble carbohydrate content

At all treatment periods (-4, -2, 0, and +2 h), the normal decrease in carbohydrate content during the dark phase was partially inhibited. As a result, the carbohydrate content per cell was significantly higher in treated cells than in the control cells. A 1.6, 2.0, 1.7 and 1.6-fold accumulation relative to control was observed at -4, -2, 0, and +2 h respectively. (Fig. 16).

These results could not be completely related to the inhibition of cell division observed at -4, -2, and 0 and the 75% inhibition observed at +2 h. This indicates that other factors beside cell division are involved in carbohydrate degradation in the dark period and these were not affected by the treatment.

3.4.5 Total RNA content

Sensitivity of RNA to Nonylphenol treatment was clearly apparent and is shown in Fig. 17 and noted below for each treatment period.

-4 h: Exposure of the cells to Nonylphenol treatment led to a partial decrease (by 24%) in RNA content per cell. However, the decrease was smaller than that observed in the control, (24% compared to 50% in control sets) and the RNA content was therefore, somewhat higher at the end of the cycle. A 1.5-fold accumulation per cell of RNA was observed compared with the control.

-2 h: Exposure to Nonylphenol evoked a 2-fold augmentation in RNA due to inhibition of cell division in the dark period.

- 0 h: Exposure of the cells to Nonylphenol did not cause any significant change in RNA content compared with the controls. This result was unexpected because cell division was arrested. However, the decrease of RNA could be due to degradation of RNA and also related to the observed 20% decrease in cellular protein.
- +2 h: Exposure of the cells to Nonylphenol led to a 1.7- fold accumulation of RNA. This accumulation can be related to the 75% inhibition of cell division.

3.4.6 Total cellular DNA content

Effects of Nonylphenol treatment on DNA content were not unexpected but the lack of effect at +2 h was not predicted, Fig. 18, Table VII.

- 4 h: Exposure to Nonylphenol caused a 2.6- fold increase in the cellular DNA content compared with control cells. This indicates that besides accumulation an actual 20% stimulation of DNA synthesis occurred.
- 2 h: Exposure of the cells to Nonylphenol tripled their DNA content from 0.4 to 1.3 pg/cell. At this time period, DNA synthesis was stimulated 3- fold above that observed for non-treated control cells.
- 0 h: Exposure to Nonylphenol led to a 3.7- fold increase in DNA content.
- +2 h: Exposure to Nonylphenol at this time did not show any significant change in DNA content. This result was unexpected because 75% of cell inhibition was observed. Perhaps, depolymerization of the DNA molecule occurred.

3.4.7 Chlorophyll (a) and (b)

Differences in sensitivity of chlorophyll a and chlorophyll b with respect to treatment and cell phase were the most interesting feature of this series.

- 4 h: Exposure of the cells to Nonylphenol led to a 62% decrease in chlorophyll (a), from 1.3 $\mu\text{g}/\text{ml}$ to 0.5 $\mu\text{g}/\text{ml}^{-1}$ (Table IX). A decrease by 33% in chlorophyll (b) was also observed (Table X). This time period proved to be the most sensitive with respect to chlorophyll (a) and (b).
- 2 h: Exposure of the cells to Nonylphenol caused a decrease in chlorophyll (a) content, from 1.5 to 1.1 $\mu\text{g}/\text{ml}^{-1}$. No significant decrease in chlorophyll (b) was observed indicating differences in chlorophyll (a) and chlorophyll (b) sensitivity to treatment.
- 0 h: Exposure of the cells to Nonylphenol did not show any significant effect on the cellular content of chlorophylls (a) or (b).
- +2 h: Similarly to the treatment applied at 0 h no significant effect on chlorophyll (a) or (b) content was observed at this time period.

Table VI. Effect of exposure to Nonylphenol ($2.5 \mu\text{g ml}^{-1}$) on the number of cells of a population of Chlamydomonas segnis ($\times 10^3$) treated at 4 time periods in the life cycle as observed at the end of the dark period. \pm standard deviation.

Nonylphenol ($2.5 \mu\text{g/ml}$)	
-4hr	1.9 ± 0.1
-2hr	1.4 ± 0.1
0hr	2.2 ± 0.3
+2hr	2.5 ± 0.4
Control	4 ± 0.2
Initial	
No. of cells	2 ± 0.3

Table VII. Effect of Nonylphenol (2.5 ug/ml) on dry weight and macromolecular content of Chlamydomonas segnis applied at different times of the cell cycle.

Parameter	-4 h			-2 h		
	pg/cell	% control	% of increase as as % of dry w.	pg/cell	% control	% of increase as % of dry w.
Dry weight	54 c	160		63 c	200	
Protein	21 d	170	63	28 c	220	81
Carbohydrate	14 d	160	42	18 d	200	53
RNA	5 d	150	14	6 b	200	18
DNA	1 d	260	3	1.3 c	300	4

Parameter	0 h			+2 h		
	pg/cell	% control	% of increase as as % of dry w.	pg/cell	% control	% of increase as % of dry w.
Dry weight	66 c	200		51 c	150	
Protein	22 b	180	67	36 d	300	107
Carbohydrate	15 d	170	45	14 d	160	42
RNA	4 a	120	12	5 d	170	16
DNA	1.5 d	370	45	0.4 a	100	1.2

a.- Indicates no significant difference.

b, c, d, Indicates significant difference at the 0.05, 0.01 and 0.005 level respectively. (T-test).

Figure 13. Effect of $2.5 \mu\text{g ml}^{-1}$ of Nonylphenol on cell number of Chlamydomonas segnis when Nonylphenol was applied at different times of the cell cycle. The average values of triplicate samples are shown together with standard deviation bars.

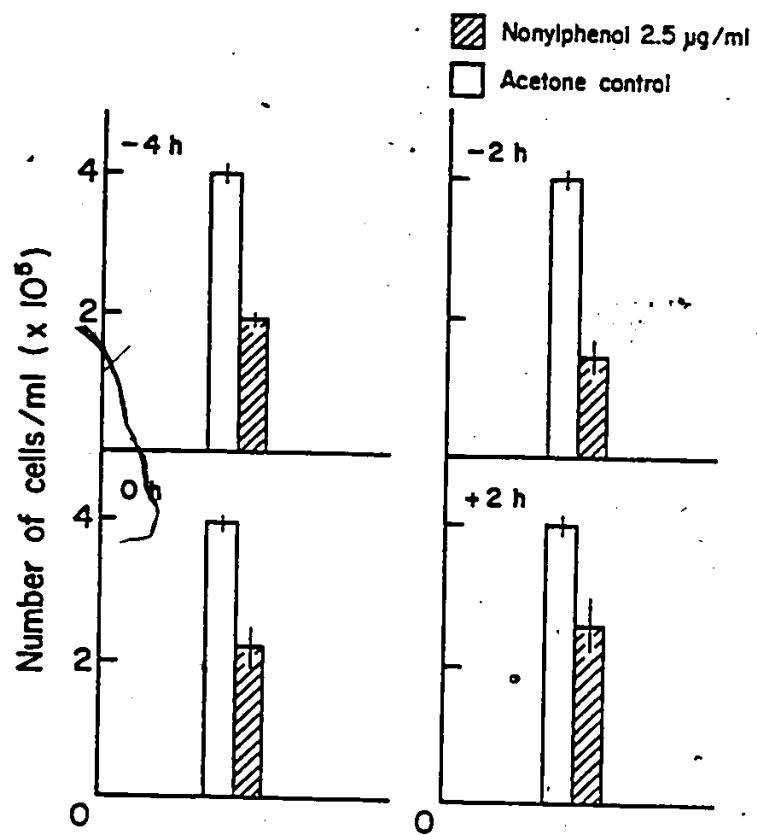


Figure 14. Effect of $2.5 \mu\text{g ml}^{-1}$ of Nonylphenol on dry weight content of Chlamydomonas segnis when Nonylphenol was applied at different times of the cell cycle. The average values of triplicate samples are shown together with standard deviation bars. Arrows indicate addition of the xenobiotic.

□ Light, ▨ Dark periods.

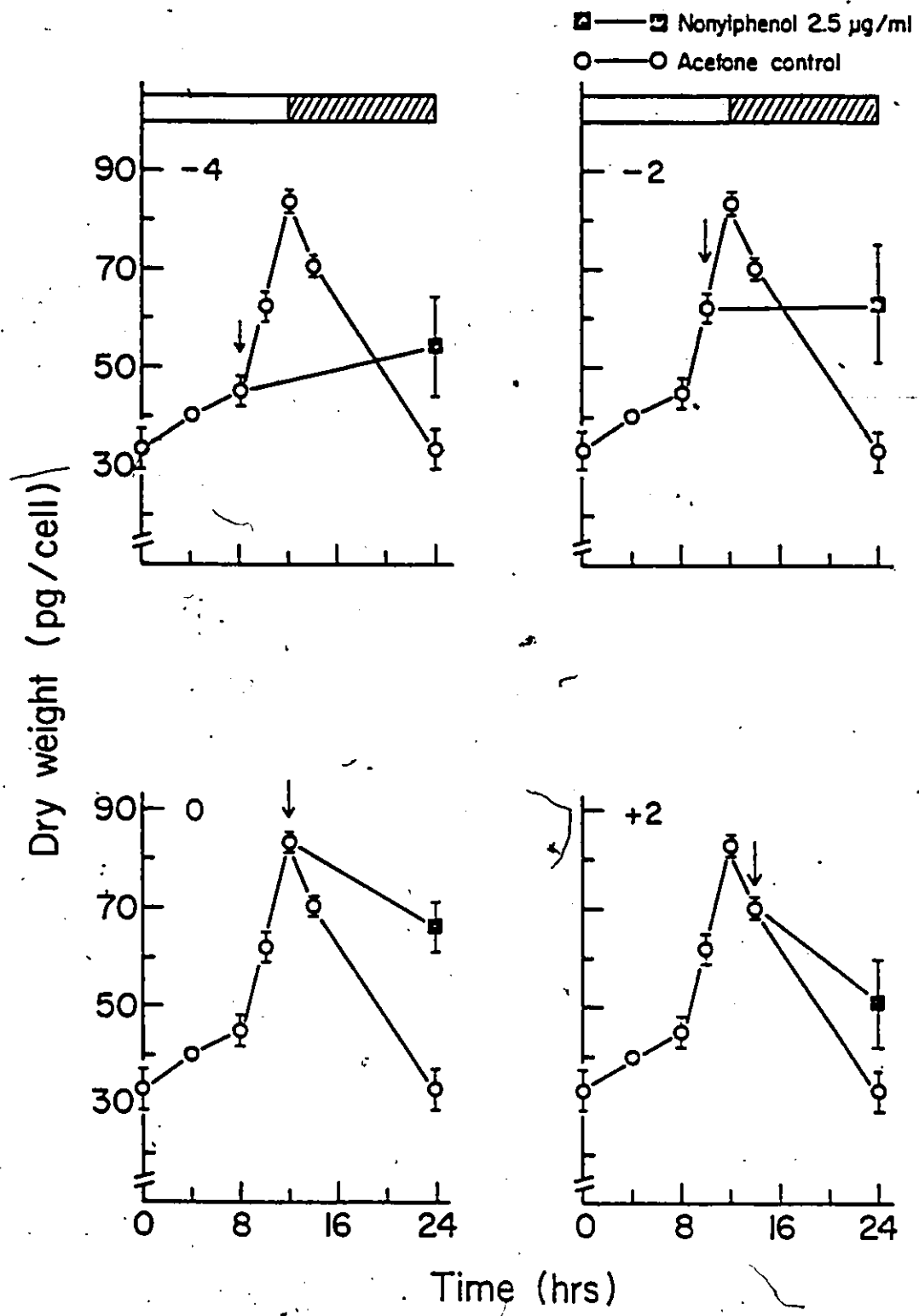


Figure 15. . Effect of $2.5 \mu\text{g ml}^{-1}$ of Nonylphenol on protein content of Chlamydomonas segnis when Nonylphenol was applied at different times of the cell cycle. The average values of triplicate samples are shown together with standard deviation bars. Arrows indicate addition of the xenobiotic.

□ Light, ▨ Dark periods.

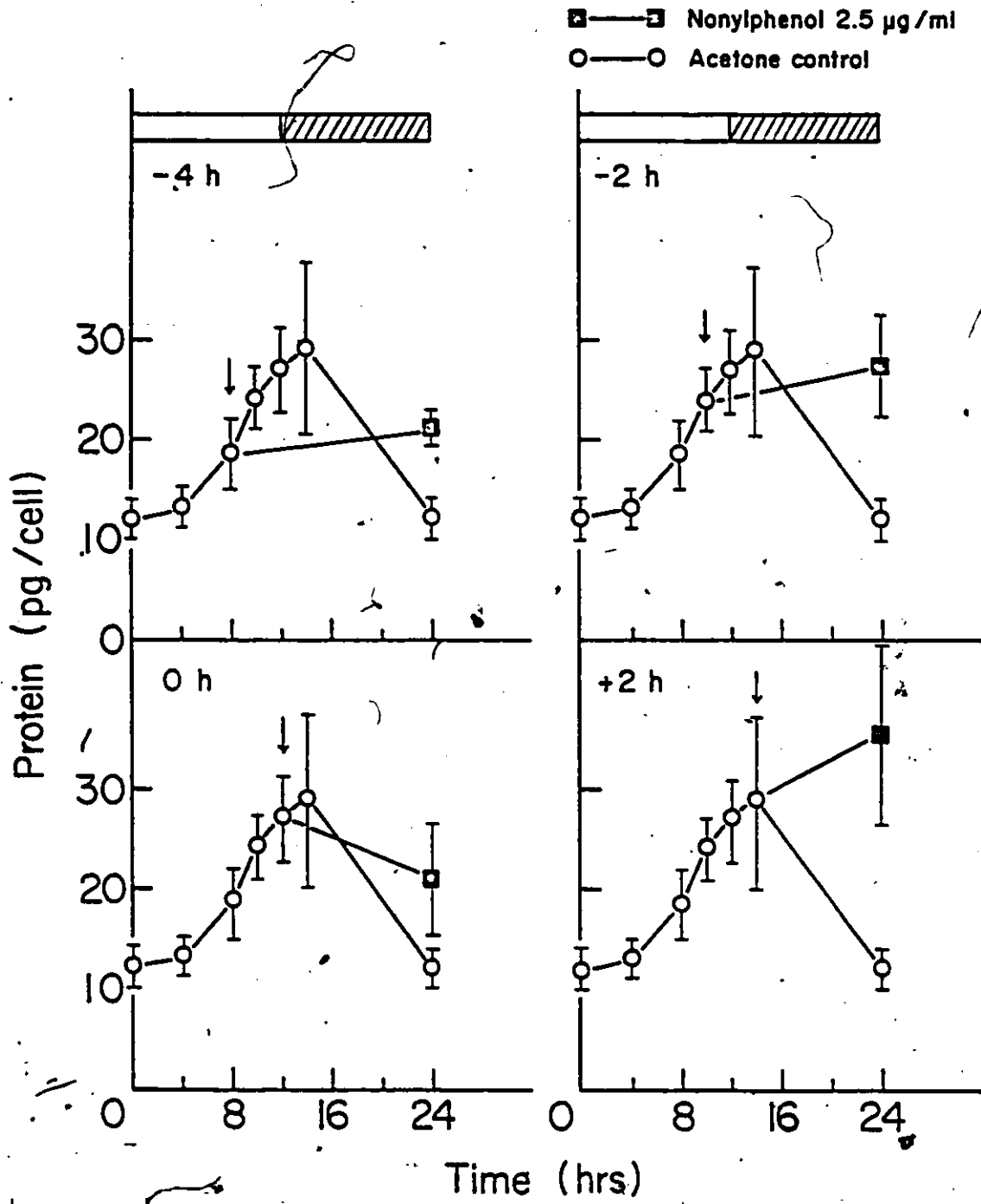


Figure 16. Effect of 2.5 ug ml^{-1} of Nonylphenol on carbohydrate content of Chlamydomonas segnis when Nonylphenol was applied at different times of the cell cycle. The average values of triplicate samples are shown together with standard deviation bars. Arrows indicate addition of the xenobiotic.

□ Light, ▨ Dark periods.

Figure 17. Effect of $2.5 \mu\text{g ml}^{-1}$ of Nonylphenol on RNA content of Chlamydomonas segnis when Nonylphenol was applied at different times of the cell cycle. The average values of triplicate samples are shown together with standard deviation bars. Arrows indicate addition of the xenobiotic.

□ Light, ▨ Dark periods.

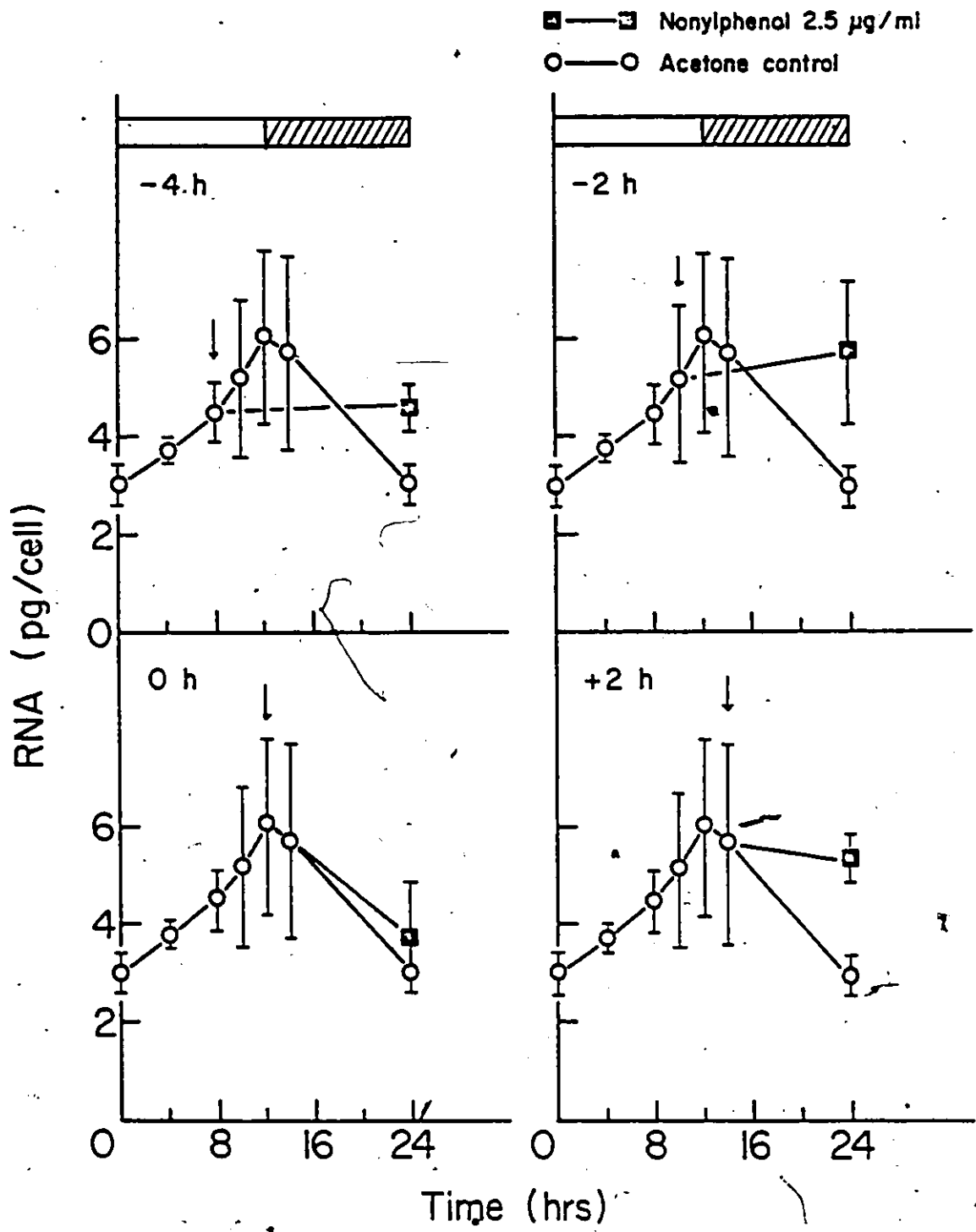
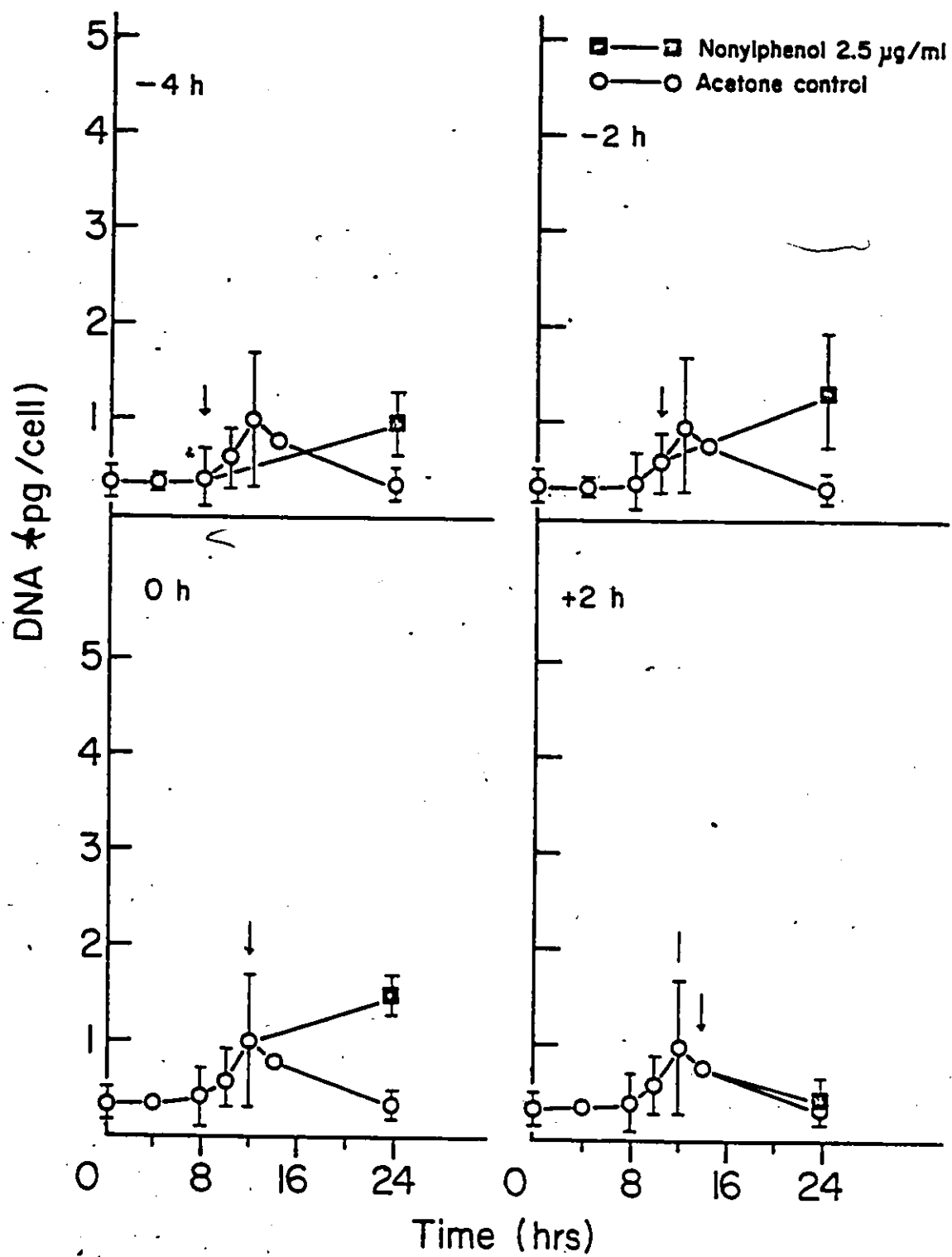


Figure 18. Effect of $2.5 \mu\text{g ml}^{-1}$ of Nonylphenol on DNA content of Chlamydomonas segnis when Nonylphenol was applied at different times of the cell cycle. The average values of triplicate samples are shown together with standard deviation bars. Arrows indicate addition of the xenobiotic.



3.5 Matacil 1.8D

One treatment ($5 \mu\text{g ml}^{-1}$ Matacil) and one exposure period alone (-2 h) were features of this experimental set. However, chlorophyll content was observed following treatment at all times (-4, -2, 0 or +2 h darkness). The $5 \mu\text{g ml}^{-1}$ of the formulation Matacil 1.8D corresponds to $1 \mu\text{g ml}^{-1}$: $2.5 \mu\text{g ml}^{-1}$: $1.5 \mu\text{g ml}^{-1}$ of aminocarb, Nonylphenol and diluent oil respectively in the mix. (Tables VIII, IX and X, and Fig. 25).

3.5.1 Cell number

Treatment of Chlamydomonas cells at -2 h darkness with Matacil 1.8D resulted in a 25% decrease of the initial cell number and total inhibition of cell division, Fig. 19. This treatment was both algistatic and algicidal.

These results were similar to those obtained with the Nonylphenol treatment at this time (-2 h) period. No significant differences were observed between the two sets of data (Fig. 26). On the other hand, treatment of the cells with $1 \mu\text{g ml}^{-1}$ aminocarb alone did not show any significant effects on cell number. This indicates that Nonylphenol may be the primary toxic factor in the aminocarb formulation. (Fig. 19).

3.5.2 Dry weight (Fig. 20, Table VIII)

At the time of the treatment application, the cellular dry weight content was 2-fold more than the initial amount. At the end of the dark period, the dry weight in treated cells remained at the same level as

when the pesticide was applied (2-fold), whereas in the control sets the dry weight decreased to the initial level with autospore release. This indicates that the already synthesized dry weight was retained throughout the dark period as cell division did not occur. (Fig. 20, Table VIII).

This result was also observed following the Nonylphenol treatment. No significant differences were observed in the effects evoked by Nonylphenol and Matacil (Fig. 26); again supporting the fact that the toxic effects in Matacil 1.8D were due to Nonylphenol.

3.5.3 Protein content

The protein content of the cells exposed to Matacil 1.8D did not decrease at the end of the dark period as it did in the control cells. A 1.7-fold augmentation in protein content was found in treated cells as a consequence of complete inhibition of cell division. (Fig. 21, Table VII).

This result was not significantly different from that obtained following Nonylphenol treatment (Fig. 26). In both cases, the reduction from a 2-fold increase in protein to 1.7-fold level indicates that some proteolysis may have taken place.

3.5.4 Insoluble carbohydrate content

At the end of the dark period, the normal % decrease in carbohydrate content in the cells exposed to Matacil 1.8D was not observed. Treated cells showed only a 54% decrease in carbohydrate whereas in control cells the decrease was 75%, as a consequence, the carbohydrate content was

higher in treated cells compared with controls. As cell division was completely arrested at this time period the observed decrease in carbohydrate may be due to increased starch hydrolysis. This result is similar to the effect evoked by $2.5 \mu\text{g ml}^{-1}$ of nonylphenol at this time period (-2 h) (Fig. 26).

Aminocarb treatment ($1 \mu\text{g ml}^{-1}$) did not cause any significant effect on total carbohydrate. The 2-fold decrease in biomass observed in Section 3.5.2 provides a summation of the perturbant effects on both carbohydrate and protein metabolism.

3.5.5 Total RNA content

The RNA content of the cells exposed to $5 \mu\text{g ml}^{-1}$ of Matacil 1.8D did not decrease in the dark period as evidenced in the control cells (Table II). Because of this, the treated cells had more than double the RNA content of the control cells. These results indicate that induction of RNA synthesis may have occurred after treatment. As cell division did not occur in the treated cells, RNA was accumulated. (Fig. 23).

3.5.6 DNA content

Treatment caused an increase in DNA content. At the end of the dark period treated cells showed three-fold more DNA than untreated controls. This result indicates that a stimulation of DNA synthesis may have occurred as a result of pesticide treatment at this time period (S-phase). No significant differences were observed between the Nonylphenol effects and Matacil effects which indicate, once again, that the toxicity of Matacil

was due to the Nonylphenol content in the formulation, (no significant effects were observed in the aminocarb treatment). (Fig. 24).

3.5.7 Chlorophyll content

-4 h: Exposure of the cells to Matacil 1.8D ($5 \mu\text{g ml}^{-1}$) led to a 70% reduction in chlorophyll a (Chl_a) from $1.3 \mu\text{g ml}^{-1}$ to $0.4 \mu\text{g ml}^{-1}$. (Table IX).

The cellular content of chlorophyll b (Chl_b) was also significantly decreased from $0.3 \mu\text{g/ml}$ to $0.2 \mu\text{g/ml}$. (Table X). The effects on Chl_a were highly significant. (Table IX).

-2 h: Exposure of the cells to Matacil caused a 53% decrease in Chl_a , from 1.5 to $0.8 \mu\text{g ml}^{-1}$ (-2h). The content of Chl_b was not affected at this time period. These results suggest that Chl_a was more sensitive to Matacil during the -4 h treatment than the -2 h, whereas Chl_b was sensitive only at -4 h.

0 & +2 h: Exposure of the cells to Matacil 1.8D did not affect Chl_a or Chl_b content at either of these treatment periods.

Visual observation of Chlamydomonas after treatment with Nonylphenol ($2.5 \mu\text{g ml}^{-1}$) or Matacil 1.8D ($2.5 \mu\text{g ml}^{-1}$ of Nonylphenol) showed chlorosis of the cells when the treatment was applied at -4 h and to a lesser extent at -2 h (Fig. 25). This agrees with the quantitative results obtained for chlorophyll content during the same treatment periods.

Table VIII. Effect of Matacil 1.8D (1:2.5:1.5 $\mu\text{g ml}^{-1}$ of aminocarb, nonylphenol and diluent oil respectively) on the macromolecular content (pg/cell and % of control) of Chlamydomonas segnis when the pesticide was applied at 2h before the dark period (-2 h).

	pg/cell	% of control	% of increase as % of dry wt.
Dry weight	64 a	190	
Protein	21 b	170	62
Carbohydrate	20 b	200	59
RNA	8 b	200	23
DNA	1.5 b	300	4.5

a.- Significant difference obtained at the 0.01 level.

b.- Significant difference obtained at the 0.005 level. (t-test).

Figure 19. Effect of $5 \mu\text{g ml}^{-1}$ of Matacil 1.8D (1:2.5:1.5 $\mu\text{g ml}^{-1}$ of aminocarb, Nonylphenol, and diluent oil respectively), and Nonylphenol alone on the cell number of Chlamydomonas cells when the pesticide was applied at -2 h before the dark period. The average values of triplicate samples are shown together with standard deviation bars.

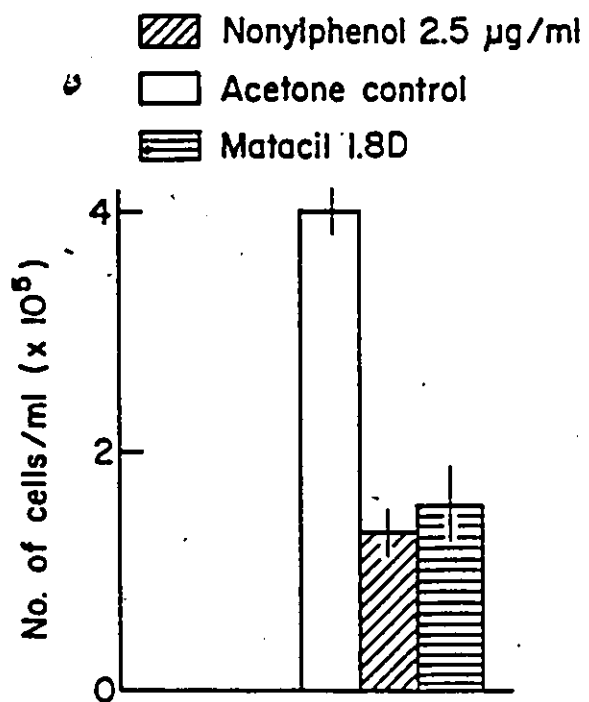


Figure 20. Effect of $5 \mu\text{g ml}^{-1}$ of Matacil 1.8D ($1:2.5:1.5 \mu\text{g ml}^{-1}$ of aminocarb, Nonylphenol, and diluent oil respectively) and Nonylphenol alone on the dry weight content of Chlamydomonas cells when the pesticide was applied at -2 h before the dark period. The average values of triplicate samples are shown together with standard deviation bars. Arrows indicate addition of the pesticide formulation.

□ Light, ▨ Dark periods.

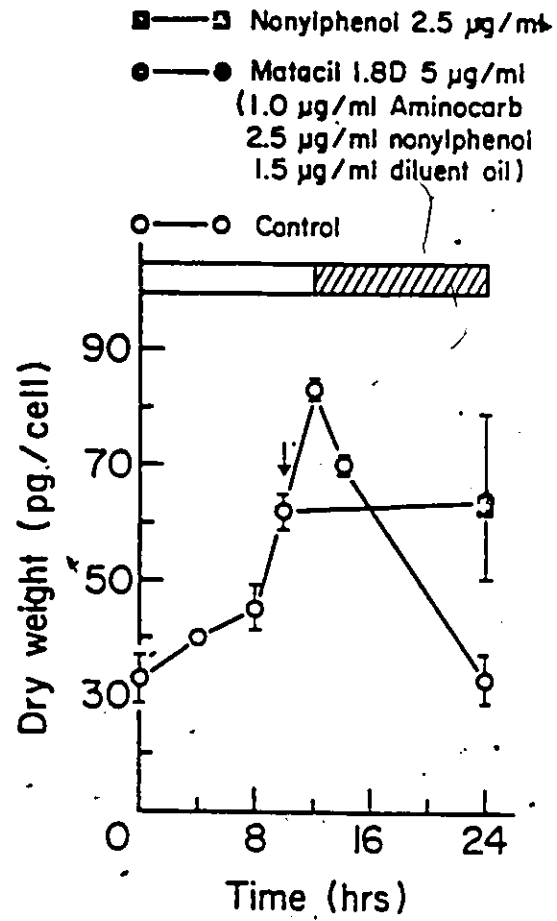


Figure 21. Effect of $5 \mu\text{g ml}^{-1}$ of Matacil 1.8D ($1:2.5:1.5 \mu\text{g ml}^{-1}$ of aminocarb, Nonylphenol, and diluent oil respectively) and Nonylphenol ($2.5 \mu\text{g ml}^{-1}$) alone on protein content of Chlamydomonas cells when the pesticide was applied at -2 h before the dark period. The average values of triplicate samples are shown together with standard deviation bars. Arrows indicate addition of the pesticide formulation.

□ Light, ▨ Dark periods.

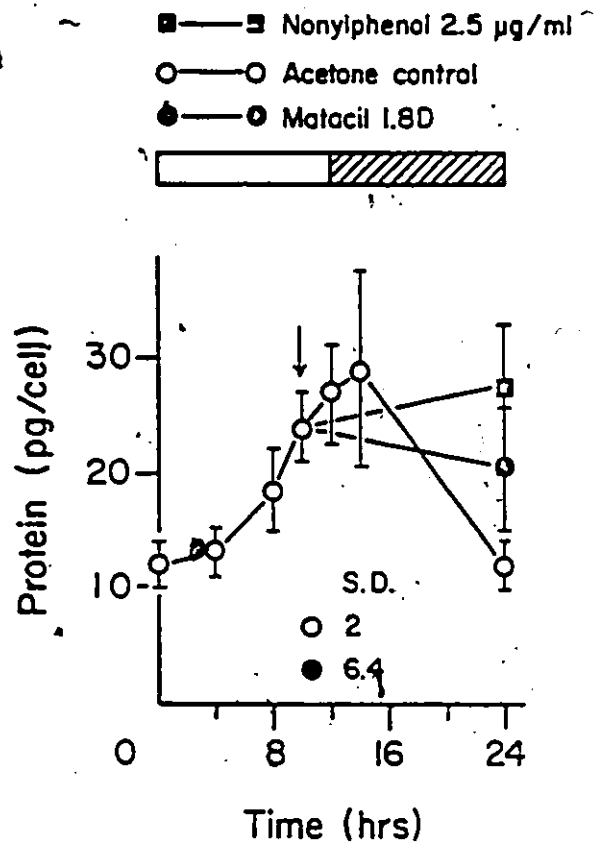


Figure 22. Effect of $5 \mu\text{g ml}^{-1}$ of Matacil 1.8D ($1:2.5:1.5 \mu\text{g ml}^{-1}$ of aminocarb, Nonylphenol, and diluent oil respectively) and Nonylphenol ($2.5 \mu\text{g ml}^{-1}$) alone on the carbohydrate content of Chlamydomonas cells when the pesticide was applied at -2 h before the dark period. The average values of triplicate samples are shown together with standard deviation bars. Arrows indicate addition of the pesticide formulation.

□ Light, ▨ Dark periods.

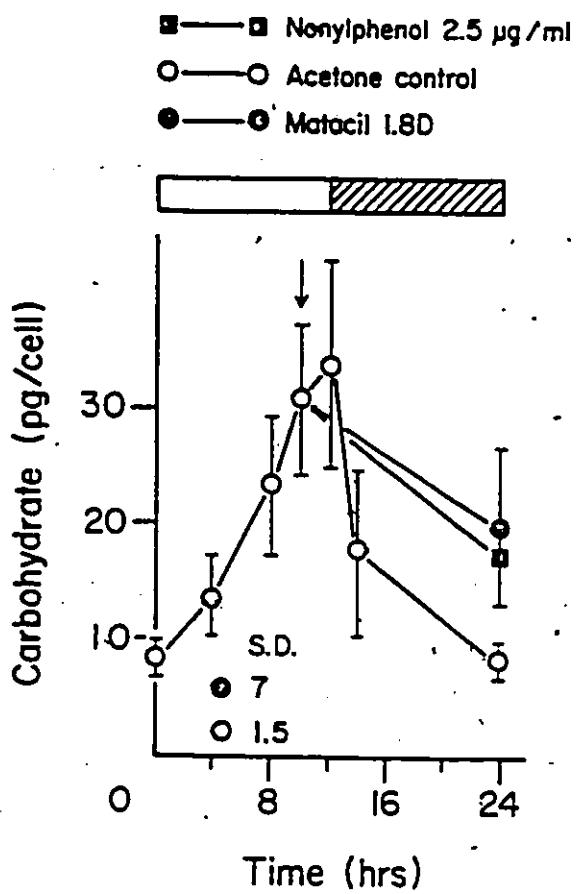


Figure 23. Effect of $5 \mu\text{g ml}^{-1}$ of Matacil 1.8D ($1:2.5:1.5 \mu\text{g ml}^{-1}$ of aminocarb, Nonylphenol, and diluent oil respectively) and Nonylphenol ($2.5 \mu\text{g ml}^{-1}$) alone on RNA content of Chlamydomonas cells when the pesticide was applied at -2 h before the dark period. The average values of triplicate samples are shown together with standard deviation bars. Arrows indicate addition of the pesticide formulation.

□ Light, ▨ Dark periods.

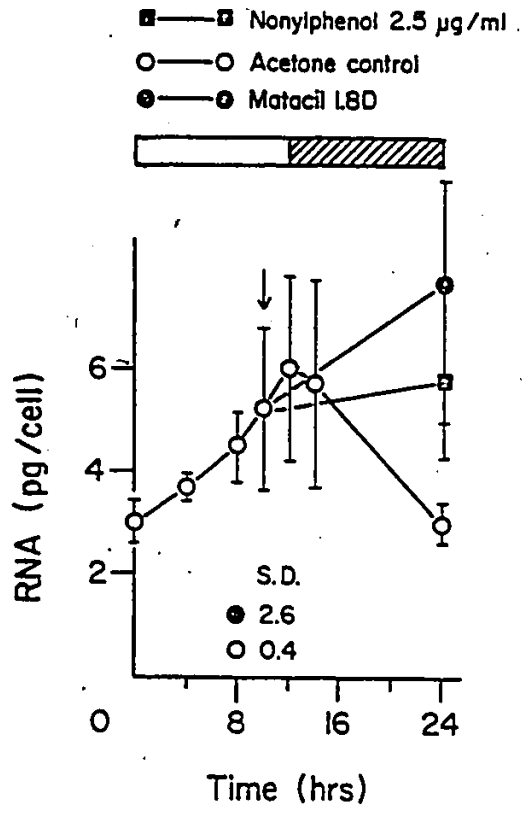


Figure 24. Effect of $5 \mu\text{g ml}^{-1}$ of Matacil 1.8D (1:2.5:1.5 $\mu\text{g ml}^{-1}$ of aminocarb, Nonylphenol, and diluent oil respectively) and Nonylphenol ($2.5 \mu\text{g ml}^{-1}$) alone on DNA content of Chlamydomonas cells when the pesticide was applied at -2 h before the dark period. The average values of triplicate samples are shown together with standard deviation bars. Arrows indicate addition of the pesticide formulation.

□ Light, ▨ Dark periods.

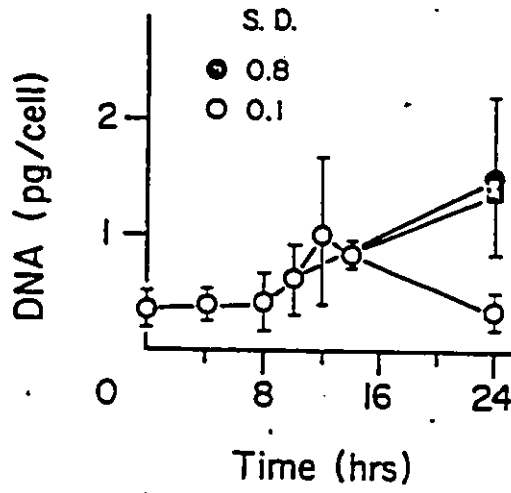
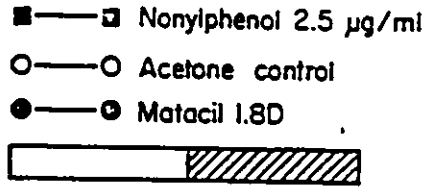
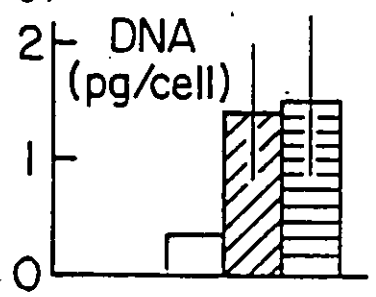
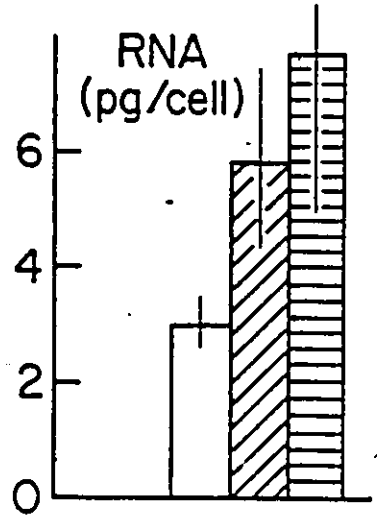
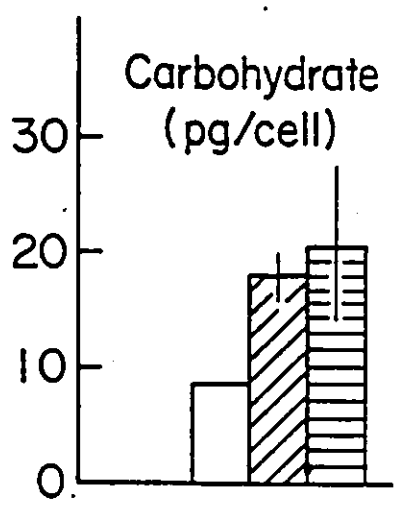
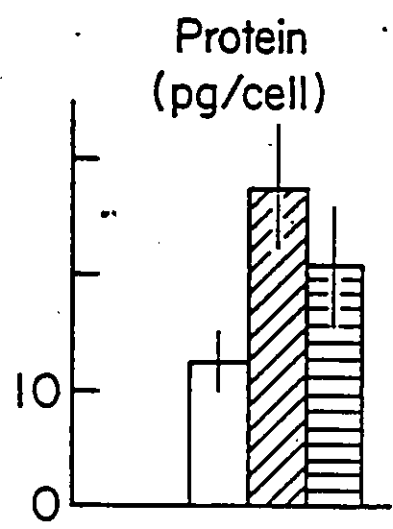
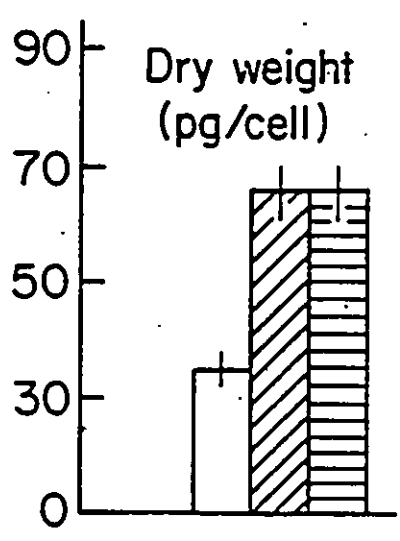


Figure 26. Effect of $5 \mu\text{g ml}^{-1}$ of Matacil 1.8D (1:2.5:1.5 $\mu\text{g ml}^{-1}$ of aminocarb, Nonylphenol, and diluent oil respectively) and Nonylphenol alone on the dry weight and macromolecular content of Chlamydomonas cells when applied at -2 h before the dark period. The average values of triplicate samples are shown together with standard deviation bars.






-  Nonylphenol 2.5 µg/ml
-  Matacil 1.8D containing 2.5 µg/ml NP
-  Acetone control

TABLE IX Chlorophyll a content ($\mu\text{g ml}^{-1}$) of Chlamydomonas population exposed to $2.5 \mu\text{g ml}^{-1}$ of Nonylphenol or $5 \mu\text{g ml}^{-1}$ of Matacil at different times of the cell cycle versus untreated control populations. The average values of triplicate samples are reported \pm the standard deviation.

	+2 hr	0 hr	-2 hr	-4 hr
Control	1.3 ± 0.2	1.5 ± 0.04	1.5 ± 0.0	1.3 ± 0.02
Nonylphenol	1.6 ± 0.1 a	1.4 ± 0.05 a	1.1 ± 0.1 b	0.5 ± 0.1 d
Matacil	1.6 ± 0.2 a	1.4 ± 0.2 a	0.8 ± 0.12 c	0.4 ± 0.1 d

a.- Indicates no significant difference.

b,c,d.- Indicates significant difference at the 0.05, 0.01 and 0.005 level respectively. (t-test).

TABLE X Chlorophyll b content ($\mu\text{g ml}^{-1}$) of Chlamydomonas population exposed to $2.5 \mu\text{g ml}^{-1}$ of Nonylphenol or $5 \mu\text{g ml}^{-1}$ of Matacil at different times of the cell cycle versus untreated populations. The average values of triplicate samples are reported \pm the standard deviation.

	+2 hr	0 hr	-2 hr	-4 hr
Control	0.4 ± 0.03	0.4 ± 0.03	0.33 ± 0.01	0.3 ± 0.00
Nonylphenol	0.5 ± 0.05 a	0.5 ± 0.1 a	0.3 ± 0.05 a	0.2 ± 0.02 b
Matacil	0.6 ± 0.05 a	0.5 ± 0.05 a	0.3 ± 0.04 a	0.2 ± 0.02 b

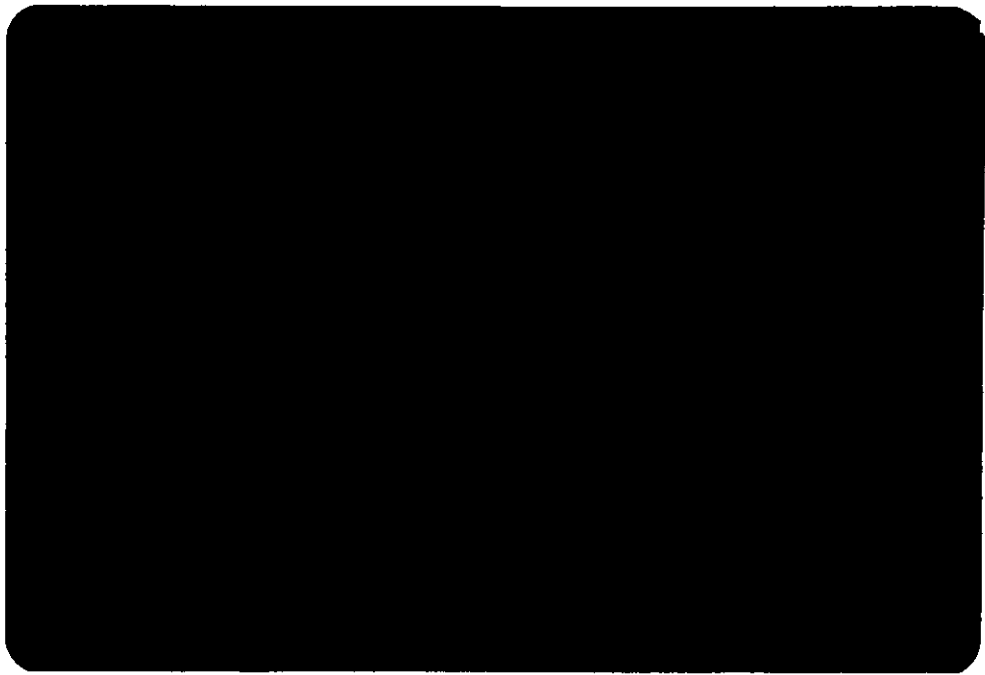
a.- Indicates no significant difference.

b.- Indicates significant difference at the 0.05 level. (t-test).

Figure 25. Chlamydomonas cells treated with $5 \mu\text{g ml}^{-1}$ of Matacil 1.8D or $2.5 \mu\text{g ml}^{-1}$ of Nonylphenol at different times of the cell cycle (-4, -2, 0, and +2 h darkness)

A.-Control cells. B.-Cells treated with Matacil.

C.-Cells treated with Nonylphenol.



SECTION IV

DISCUSSION

The present results indicate that high concentrations of aminocarb, (5, 10, and 50 $\mu\text{g ml}^{-1}$) dramatically affected the cell cycle events in the alga Chlamydomonas segnis. Daughter cell release and mitosis were prevented and accumulation of macromolecular products was observed as a consequence of cell division arrest.

In plants and especially in algae little is known about the mechanism of action of aminocarb. However, some carbamate pesticides have been shown to inhibit algal growth. For example, the insecticide Carbaryl (1-naphthyl-N-methyl carbamate) at concentration as low as 0.1 $\mu\text{g/ml}$ was toxic to Chlorella pyrenoidosa (Christie, 1969). Similarly, Carbaryl inhibited growth of Amphiprora, Amphora, Nitzschia and Chlorococcum at concentrations of 10, 2, 1, and 0.5 $\mu\text{g ml}^{-1}$. All four genera were inhibited by Carbaryl at 2 $\mu\text{g ml}^{-1}$ in single species cultures (Maly and Ruber, 1983). Also the growth of Synechococcus lividus, Oscillatoria terebriformis, Scenedesmus quadricauda and Navicula pelliculosa was arrested by Zectran (4-dimethylamino-3, 5-xylylmethylcarbamate) at a concentration of 10⁴ $\mu\text{g/l}$ (ppb). Zectran affected both respiration and photosynthesis in the cyanophytes at concentrations of 0.5 and 12.5 $\mu\text{g ml}^{-1}$. However, S. quadricauda and N. pelliculosa were unaffected by these concentrations (Snyder and Sheredan, 1974).

The results of application of aminocarb at different times in the cell cycle suggest that the inhibition of cell division might be caused by interference by the pesticide with some of the events that occur to

initiate the cell division process. These would include microtubule formation and changes in metabolic status. Also, the pesticide treatment proved to have quite a different consequence depending on the stage of development at which the synchronous cells had been treated. Presumably, other internal chemical factors such as specific enzymes may also be influenced, which change their activity at specific time of the cell cycle (Kates and Jones, 1967).

It was found that the inhibitory effects of aminocarb in Chlamydomonas cells were most pronounced when $50 \mu\text{g} \cdot \text{ml}^{-1}$ or $10 \mu\text{g} \cdot \text{ml}^{-1}$ of the pesticide was applied at -2 hr (S-phase). At this time, cell lysis occurred and inhibition of cell division was observed. This treatment time corresponds to the period of DNA synthesis, however, DNA synthesis was not inhibited. Perhaps, Chlamydomonas cells were more sensitive, at this point in the cell cycle, to the events leading to cytokinesis and zoospore release, than to DNA synthesis. At this time (-2 hr, S-phase) all the requirements necessary for DNA synthesis are already completed. Once DNA synthesis has started in a cell which has an adequate supply of nutrients, it continues until it is completed. (Mazia, 1961; Wheatley, 1982).

On the other hand, it may be supposed that transient structural changes in the membrane and also in the protoplasm were responsible for the observed decrease of the initial cell number. Possibly, lysosomal destabilization by the pesticide treatment may have occurred that led to an increase of lytic enzymes and consequently lysis of the cells. Lysosome-like organelles have been reported in cells of Euglena granulate (Palisano and Walne, 1972). In addition, Swanson and Floy (1979) working with Asteromonas gracilis, reported the localization of acid phosphatase in vesicles which are the primary lytic compartments of these cells.

Effects following treatment at 0 h (beginning of G_2) were intermediate between -4 hr (end of G_1) and +2 hr (cytokinesis). Treatment when the cells were undergoing cytokinesis (+2 hr) had the least effect on cell number. This may be due to the fact that at this time period microtubule assembly and macromolecular synthesis for the "event" period were already completed, so that no radical destabilization followed pesticide exposure.

Other carbamate pesticides have been shown to inhibit cell division. For instance, N-phenyl carbamate (Propham) at $40 \mu\text{g}\cdot\text{ml}^{-1}$ was found to interfere with cell division in the green alga Oedogonium cardiacum (Coss and Pickett-Heaps, 1974). Also, growth of Chlorella was inhibited by 50% by a number of carbamate herbicides including Propham $14 \mu\text{g ml}^{-1}$, Chlorpropham $2.7 \mu\text{g ml}^{-1}$, Fermuron $0.5 \mu\text{g}\cdot\text{ml}^{-1}$, Propanil $0.18 \mu\text{g ml}^{-1}$ and Asulam $6 \mu\text{g}\cdot\text{ml}^{-1}$. (Wright, 1972). When Chlorella cells were treated with $14.1 \mu\text{g ml}^{-1}$ Chlorpropham the DNA content of the cells increased but the cells did not divide, indicating that there was only inhibition of cell division (Sumida et al., 1977). In addition, 50% growth reduction in Chlamydomonas reinhardtii was caused by $2.7 - 3 \mu\text{g}\cdot\text{ml}^{-1}$ Chlorpropham (Maule and Wright, 1983). Propham has been shown to act primarily on the microtubule organizing centers of Euglena gracilis. In this case a high proportion of the cells were in the G_2 phase of the cell cycle. Polymorphic bodies, plastid and nuclear abnormalities in the cytoplasm following treatment were observed (Vannini et al., 1982).

In the present study, the macromolecular contents probably accumulated as a consequence of the inhibition of cell division. The most sensitive period of treatment to affect protein content occurred when the pesticide ($50 \mu\text{g}\cdot\text{ml}^{-1}$) was applied at -2 h darkness (S-phase). RNA also

accumulated as a result of inhibition of cell division. It is known that proteins rich in leucine and thiol groups, as well as the RNA associated with these principal proteins, are reorganized prior to the onset of cell division and that they provide the macromolecular requirement necessary for cell division to occur, as well as probably triggering the mechanism which sends a cell into mitosis (Mazia, 1961). Probably, alterations at this level may have happened but this work did not differentiate specific proteins or RNA.

The sensitivity of the carbohydrate synthesizing and hydrolytic capacities of the cells was greatest at 0 h, at the onset of the dark period (G_2 phase). Sensitivity of carbohydrate synthesis at this phase of the cell cycle may be explained by the observed arrest of cell division coupled with the probable inhibition of phosphorylation processes. It is interesting to note that on a comparative basis, carbohydrate content increase coincided with cell cycle arrest, and of the molecules monitored this was the macromolecular compound which was least accumulated. This may be explained by the fact that an increase of the activity of two starch degrading enzymes, namely, polyglucan phosphorylase and α -amylase occur at the onset of the dark period (Wanka et al., 1970; Hirokawa et al., 1982). If these enzymes were not adversely affected by the pesticide then hydrolytic degradation of accumulated carbohydrate would occur during mitotic arrest.

Cell division and macromolecular content of Chlamydomonas populations were unaffected by any of the concentrations of diluent oil tested at any of the time periods, apart from -2 h (S-phase). At this time, $50 \mu\text{g ml}^{-1}$ of oil led to a decrease in the cell number.

Diluent oil 585, a $n^u 2$ fuel oil distillate is a mixture of low

boiling point oils and contains a range of naphthalene compounds. Naphthalene has been found to be very toxic to algae. For example, inhibition of growth was observed in the fresh water alga Chlorella vulgaris (Kauss and Hutchinson, 1978) and Chlamydomonas angulosa (Soto et al., 1975). Moody et al. (1981) found that Aerotex which contains naphthalene inhibited the growth of Chlamydomonas reinhardtii, Chlorella pyrenoidosa and Scenedesmus obtusiusculus. In addition, a n^o 2 fuel oil extract depressed the photosynthetic ¹⁴C incorporations in Vaucheria (Bott and Rogenmuser, 1978). However, the inability of diluent oil to affect the parameters studied here may be due to the fact that lower boiling point oils usually have lower toxicities (Van Overbeek and Blondeu, 1954). Furthermore, naphthalene, which is normally highly toxic to algae, may have evaporated from the media because the algae were continuously bubbled with air and low molecular weight volatile compounds will not persist for long under these conditions (Soto et al., 1975; Vandermeulen and Ahern, 1976).

With Nonylphenol (2.5 $\mu\text{g ml}^{-1}$) the greatest effect was observed at -2 h (S-phase) and the least effect at +2 h (cytokinesis). These results have shown that Nonylphenol has algicidal properties. Following treatment at -2 h the initial number of cells decreased throughout the experiment.

The toxicity of phenol compounds is well documented (Buikema et al., 1979) and in particular Weinberger and Rea (1980) reported a 24 h LC₅₀ of 1.5 $\mu\text{g ml}^{-1}$ nonylphenol for Chlorella pyrenoidosa, Chick.

The mechanism by which Nonylphenol prevented the cell from dividing has not been elucidated. It has been reported that solutions of polyphenols bring about depletion of the content of SH groups in Nitella (Stom

and Beym, 1976). This might inactivate or prevent key mitotic SH-protein synthesis. It is suggested that inhibition of cell division may be caused by the interference of Nonylphenol with some of these proteins. However, their inhibition would probably not be detected by simply measuring the gross protein content. Mihara and Hase (1975) reported that a certain "protein factor" is released and activated in the cell at a specific time to initiate cell division. Perhaps, then, Nonylphenol binds to this factor, alters its configuration, changes or denatures it and so prevents the initiation of cell division.

Recently, it has been pointed out that phenol compounds such as dinoseb at a concentration as low as 10^{-4} M inhibited the uptake of Ca^{2+} by the zucchini plant mitochondria consequently, Ca^{2+} accumulated in the cytoplasm (Hertel and Marme, 1983). As Ca^{2+} balance is important to cell division (Wheatly, 1982), as well as ATPase activity (Marme, 1982), alteration of Ca^{2+} might produce a dramatic cellular decrease in ATP depriving the cell of the necessary energy for cell division to occur.

Other phenolic compounds have reportedly affected membranes. For example, pentachlorophenol has been shown to act primarily on the membrane function of the alga Selenastrum capricornutum (Jayawera et al., 1982). Recently, four algal species exposed to $1 \mu\text{g ml}^{-1}$ of polychlorinated biphenyls showed disruption of their cell organelles (mitochondria and chloroplasts) and loss of cleavage of the cytoplasm following nuclear division (Mahanty et al., 1983).

In the present study when the Chlamydomonas cells were treated with Nonylphenol or Matacil 1.8D at different times of the cell cycle their chlorophyll was unaffected at 0 and +2 h. An abrupt loss of chlorophyll was, however, observed following treatment at -4 h (end of G_1) and at -2

h (S-phase). At these periods the cells were in the light phase of their cycle. Bleaching of the cells was observed which may have been due to the direct effect of Nonylphenol on biomembranes. Disruption of the electron transport systems in the thylakoid membrane was probably a primary cause of bleaching. Surfactants have been shown to affect electron flow during light exposure periods (Sandmann and Boger, 1982). This could account for bleaching effects although this does not rule out the probability of deactivation of some enzymes necessary for maintaining the functional and structural integrity of the thylakoid chloroplast membranes. The specific mechanisms by which Nonylphenol produced chlorosis is not known. Phenol compounds generally have been shown to be inhibitory uncouplers which produce pigment breakdown and lipid peroxidation reflected by a decrease in chlorophyll content. The degradation of chlorophyll is considered to result from photooxidation induced by the inability of chlorophyll to dissipate its absorbed excitation energy when electron transport is inhibited. The phytotoxic symptom of chlorosis is thus produced by the electron transport inhibitors only in the light and the effects are proportional to light intensity (Moreland, 1980) and in the present study, to light time exposure.

At -4 h, both chlorophyll pigments Chl_a and Chl_b were degraded whereas at -2 h, only Chl_a was affected. No effect on Chl_a and Chl_b content was observed when the treatment was applied at 0 h and -2 h darkness, again emphasizing the light-mediated inhibition.

It has also been shown that apart from the adjuvants, pesticide treatment alone, in some cases, interferes with peroxisomal enzyme activities under light conditions. For example, Feierabend and Kemmerich (1983) found that the herbicides amino triazole and haloxydine interfer-

red with the appearance of peroxisomal enzyme activities. The inactivation of these enzymes was the result of a photooxidative process.

There were no significant differences between the sets treated with Nonylphenol and those treated with Matacil 1.8D and it was concluded that the toxic effects of Matacil 1.8D on chlorophyll content were due to the presence of Nonylphenol in the formulation. The results obtained when Chlamydomonas populations were exposed to field relevant concentrations of Matacil 1.8D suggest that at the exposure levels Nonylphenol was the toxic component in the formulation as no effects were observed in algal populations treated with either aminocarb ($1 \mu\text{g ml}^{-1}$) alone, or diluent oil alone at the concentrations present in the Matacil 1.8D formulation.

These results agree with the result of Li et al. (1981) who reported that cell populations of cultured cells of fish were dramatically reduced within the first 24 h following exposure to $5 \mu\text{g ml}^{-1}$ of formulated aminocarb. They also indicated that cytological effects such as surface lesions on the treated cells had occurred. In addition, McLeese et al. (1980) pointed out that the lethality of the aminocarb formulation to salmon was due to Nonylphenol. Moreover, Elner et al. (1982) studied the effects of formulated aminocarb on algal communities, dominated by flagellated Chlorophyta, and found a 50% reduction in carbon assimilation (EC 50) at $0.98 \mu\text{g ml}^{-1}$. Carbon uptake was not detectable at $1.5 \mu\text{g ml}^{-1}$ of aminocarb in the formulation.

The accumulation in the macromolecular content of the compounds presently studied followed the same trend in the cells treated with Nonylphenol as those treated with Matacil 1.8D. The major exception was RNA content in which a possible inductive effect was observed. The stress induced by all 3 xenobiotics may have induced augmentation of RNA synthesis and was indicative of the perturbation evoked by exposure to both the pesticide and the Nonylphenol adjuvant.

GENERAL COMMENTS

Prediction of the ecological effects that would follow a pesticide spray by extrapolating the results presented here are complicated by a number of factors such as the simplicity of unialgal assays and the deficiency of community interaction that can only be studied by directly looking at changes in specific communities and ecosystems. However, populations, communities and ecosystems, and the biological and chemical mechanisms by which they interact are of a complexity such that is almost impossible to understand and assign significance of the effects at those levels of organization. Furthermore, chemicals tend to behave in a variety of ways depending upon the complexity and potential sinks of the environment, such as volatilization, adsorption, bioaccumulation and chemical transformations which frequently confound the significance of the results. Actually, the LC_{50} (that concentration which is lethal to 50% of a test population) represents a drastic possible means of obtaining a preliminary evaluation of the possible environmental hazard posed by chemicals. In addition to looking at LC_{50} 's, the life cycle of the affected organisms should also be studied. The organisms response to the pesticide may be expressed as a change in reproductive fitness because the organism is a part of a community in its ecosystem, any effects on that organism will probably have implication at the population and community level. Sub-lethal effects as well as acute-level tests are important in predicting the potential ecological effects of the toxicant in the ecosystems. Sub-lethal tests should be taken into account. The stage of development at which the test organism is the most sensitive to the toxicants should be determined. In the present study, Chlamydomonas

segnis showed great sensitivity to Nonylphenol ($2.5 \mu\text{g ml}^{-1}$) at -2 h. This study also highlights the fact that the time of pesticide application is also an important factor when considering the effects of a pesticide on non-target as well as target organism. It is possible then, that a complete population might be altered or destroyed at some sensitive stage of its development. Therefore, there is a need for regulations to be put in place regarding the use of potential toxicants and for the need for the assessment of life cycle hazards based on sublethal tests using non-target and target organisms.

SUMMARY AND CONCLUSION

Some developmental and physiological effects of aminocarb, its formulation adjuncts in the Matacil 1.8D formulation and the pesticide formulation itself were observed in Chlamydomonas segnis. The hypothesis that these xenobiotics would affect the physiological and developmental status of the cells, at the time of stressor application, was supported by these studies.

Exposure to a high concentration of aminocarb ($50 \mu\text{g ml}^{-1}$) led to algistatic and algicidal effects following treatment at -2 h treatment (S-phase). Cells in this phase of growth were also most sensitive to treatment with ($2.5 \mu\text{g ml}^{-1}$) Nonylphenol and Matacil 1.8D.

Nonylphenol alone reduced chlorophyll a and b content when the cells were at the end of G_1 phase (-4 h), only chlorophyll a content was reduced when the cells were treated in S-phase (-2 h). No effects were observed at 0 h and +2 h darkness. Effects on the content of chlorophyll a and b comparable to these were produced by Matacil 1.8D treatments.

These studies indicate that the toxic effects of the aminocarb formulation were primarily due to the Nonylphenol in the Matacil 1.8D formulation. It is probable that one of the sites of primary toxicity is the cellular membranes.

It is important to notice that the high concentrations of aminocarb used in this study ($50 \mu\text{g ml}^{-1}$) would not occur under ordinary field conditions. This concentration could only occur following an accidental spill; therefore, it can be reasonably argued that aminocarb will have minimal effects on flagellated algae at the levels found in the operational program ($<80 \mu\text{g ml}^{-1}$). On the other hand, exposure to ($2.5 \mu\text{g ml}^{-1}$)

Nonylphenol reveals the potential toxicity of this surfactant, at the actual concentration in the Matacil formulation. The most serious effects were observed at the critical stage of cell division. The concentration evoking these responses has been reported present in natural waters subject to domestic and industrial pollution, or simply by runoff from agricultural lands and forest areas.

The general and over-riding conclusions from this study support the original null hypothesis. Further, they indicate that a fundamental change must take place in the way in which adjuvants are viewed. They can no longer be regarded, a priori, as "inerts", their activity must be fully assessed before registration is granted for their use.

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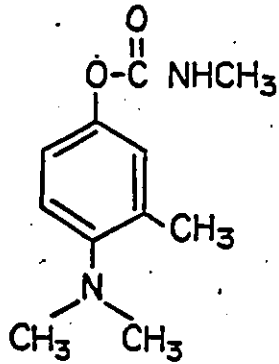
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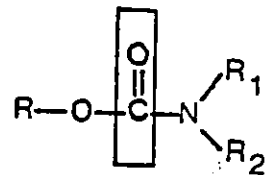
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APPENDIX A



AMINOCARB

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



BASIC CARBAMATE CHEMICAL

STRUCTURE

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

NONYLPHENOL

