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"Narcosis commences when any chemically indifferent substance has attained a certain molar concentration in the lipoids of the cell"

H.Meyer, 1899; E. Overton, 1901

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To Beverly, whose confidence sustained me and whose love endured the struggle.

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ABSTRACT

The fate and persistence of the pesticide, fenitrothion (0,0-dimethyl-0-(3-methyl-4-nitrophenyl) phosphorothioate) was monitored under both field and laboratory conditions. Rapid disapearance of the pesticide (half-life less than 2 days) in river and lake water was due to degradation of the parent compound via hydrolysis, photolysis and biolysis as well as to its sequestration by aquatic plants and unicellular algae. The high levels (9.99 ppt) attained in the green algal flagellate, Chlamydomonas reinhardii, together with observed inhibition of cell motility by the pesticide formulation, suggested that fenitrothion and/or its formulation additives (Aerotex 3470 and Atlox 3409F) were algicidal.

Further, in vitro laboratory studies with C. reinhardii demonstrated that Aerotex (1-10 ppm): 1. inhibited cell motility, population growth, ATP synthesis and CO_2 fixation; 2. enhanced staining with methylene blue, reduction of DCIP and leakage of C^{14}-photosynthate; and 3. induced ultrastructural changes in membrane configuration. An algal fluorometric screening procedure was developed to determine the relative algicidal activity of several aromatic hydrocarbon constituents of Aerotex as well as of several pesticides and formulation additives. A positive correlation was obtained between the algicidal activity (ICF_{100}) of the test chemicals and their lipophilicity (K_{ow}), ($\log ICF_{100} = 5.78 - 0.98 \log K_{ow}$ ($r = -0.92$)).

The relevance of the obtained toxicity data to the field situation was discussed.

RÉSUMÉ

On a fait une étude du devenir et de la rémanence du fénitrothion (0,0-dimethyl-0-(3-methyl-4-nitrophenyl)-phosphorothioate) sous des conditions de laboratoire et sur le terrain. La disparition rapide du pesticide (période de moins de deux jours) d'eau de lac et de riviere était due à la dégradation du composé par hydrolyse, par photolyse et par biolyse ainsi qu'a sa séquestre par des plantes aquatiques et des algues unicellulaires. Les niveaux élevés (9.99 ppt) obtenus chez l'algue verte flagellée Chlamydomonas reinhardii, ainsi que l'observation d'une inhibition du mouvement cellulaire causée par la formule du pesticide amena la suggestion que le fénitrothion et/ou une autre composante de la formule (Aerotex 3470 et Atlox 3409F) étaient algicides.

Egalement, des études de laboratoire in vitro avec C. reinhardii ont démontrées que l'Aerotex (1-10 ppm): 1. inhibait le mouvement cellulaire, la croissance de population, la synthèse de l'ATP et la fixation du CO_2; 2. augmentait la réduction du DCIP et la perte de produits de photosynthèse marqués par le C^{14}; et 3. causait des changements ultrastructuraux aux membranes. On a développé une méthode fluorométrique pour algues servant à determiner les propriétés algicides relatives aux hydrocarbures aromatiques constituants de l'Aerotex ainsi que de plusieurs pesticides et autres composés ajoutés aux formules. Une corrélation positive a été obtenue entre l'effet algicide (ICF_{100}) des composés étudiés et leur lipohilie (K_{ow}) ($\log ICF_{100} = 5.78 - 0.98 \log K_{ow}$ ($r = - 0.92$)).

On a finallement discuté l'application de ces données toxicologiques à la situation sur le terrain.

I - INTRODUCTION

I - INTRODUCTION

Contamination of the aquatic environment by synthetic pesticides is recognized as a major potential ecological problem. Recently, increasing concern has been expressed about the effects of pesticides on the unicellular phytoplankton (algae). This concern stems from a consideration of the ecological implications of pesticide-induced inhibition of algal photosynthesis (Bowes and Gee, 1971; Bowes, 1972).

The effects of pesticides on algae have been reviewed by Butler (1977) and also by Brown (1978). Although quite extensive, most of the reviewed literature has been concerned with pesticide effects in marine habitats and little consideration has been given to the effects of pesticides on freshwater algae. A case in point is the potentially injurious (algicidal) effects of aerially applied insecticide formulations on the unicellular algae inhabiting Canadian inland waters.

Fenitrothion[1] (0,0-dimethyl-0- (3-methyl-4-nitrophenyl)- phosphorothioate) has been used since 1965 to replace DDT for operational control of lepidopterous defoliators in Canadian forests (Fettes, 1968). By 1975, over 21 million hectares (ha) of forest land[2] had been sprayed with the pesticide at an average rate of 280 gm/ha, in an attempt to control the spruce budworm, Choristoneura fumiferana (Clemens)(N.R.C.C. No. 14104, 1975).

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[1] Fenitrothion was introduced in 1959 as an experimental insecticide by the Sumitomo Chemical Co., Japan, under the trade name Sumithion and independently by Bayer Leverbusen (Folithion) and by the American Cyanamid Co. (Accothion). It is synthesized by the reaction of 0,0-dimethyl phosphorochloridothioate with an alkali salt of 3-methyl-4-nitrophenol (Nishizawa et al., 1961).

[2] This is roughly three times the total area of New Brunswick (7.26 x 10^6 ha) (Colliers, 1946).

The initial purpose of this study was to examine the possibility that fenitrothion contaminated aquatic habitats. Before discussing the evolution of the project from this rather simple origin to its ultimate consideration of pesticide toxicity in algae, it is necessary to outline the relevant details of pesticide use in Canadian forests.

A. Insecticide Use in Canadian Forests

The spray history of the insecticides used to control insect infestations in Canadian forests has been reviewed by Nigam (1975) and also by Symons (1977). It will suffice, here, to list the types of insecticides used, and to give a general indication of the large amounts of insecticides that have been sprayed as well as the vast regions of forest involved.

The first aerial spray operations against Canadian forest insect infestations were conducted in the mid-1920's with calcium arsenate dust. After their termination in 1930, approximately 3 ha of forest had been sprayed with a total of approximatively 93 metric tons of arsenate[1].

The spray program was not renewed until 1944 when extensive spraying with the chlorinated hydrocarbon, DDT, was initiated. The extensive use of DDT was terminated in 1969 since environmental studies had demonstrated that this compound was extremely persistent. In fact, Yule (1970) reported that the half-life of DDT in forest soil was approximately 10 years. This observation came rather late , however, since by 1969, approximately 13,000 ha of forest land had been sprayed with over

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[1] The values reported in this section were originally obtained from Nigam (1975) and were subsequently summed and converted to metric units.

7,000 metric tons of DDT.

Nigam (1975) reported that out of 27 insecticides tested in the environment for replacing DDT, five insecticides have been used operationally in Canada including the organophosphates, phosphamidon and fenitrothion, and the carbamates, aminocarb, mexacarbate and carbaryl[1].

As evidenced by its extensive use, fenitrothion has been the major replacement for DDT. From 1965 to 1973, approximately 15,000 ha of forest land had been sprayed with over 4,500 metric tons of fenitrothion. More recent reports (Symons, 1977) have estimated that the total amount of fenitrothion deposited in Canada by 1977 had more than doubled (10,000 metric tons) since the previous estimate, and that since 1969, an average of 680 metric tons of the chemical had been applied on an annual basis to New Brunswick forests alone.

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[1] Chemical names of insecticides cited:

DDT: 1,1,1-trichloro-2,2-bis (p-chlorophenyl) ethane.

mirex: dodecachloro-octahydro-1,3,4-metheno-2H-cyclobutapentalene.

fenitrothion: 0,0-dimethyl-0-(3-methyl-4-nitrophenyl) phosphorothioate.

parathion: 0,0-diethyl 0-p-nitrophenyl phosphorothioate.

phosphamidon: 2-chloro-N,N-diethyl-3-hydroxycrotonamide dimethyl phosphate.

aminocarb (matacil): 4-dimethylamino-m-tolyl methylcarbamate.

mexacarbate (zectran): 4-dimethylamino-3,5-xylyl methylcarbamate.

carbaryl (sevin): 1-naphthyl methylcarbamate.

Since 1974, the amount of fenitrothion sprayed annually in Quebec has exceeded that in New Brunswick . Spray applications against spruce budworm and other forest pests have also been conducted in Newfoundland, Ontario, Manitoba and British Columbia (Symons, 1977). Since 1978, several spray programs in New Brunswick have employed matacil (aminocarb) in place of fenitrothion (Varty, 1980).

The main criteria used for selecting candidate insecticides for use in forest insect control in Canada are high toxicity to the target insect and low toxicity to fish, birds and mammals (Nigam, 1975). The impact then, of Canadian forest spray programs on algae is usually not a matter of consideration. Before discussing the algicidal potential of the spray formulations, it is necessary to consider the extent of contamination of aquatic habitats.

B. Contamination of Aquatic Habitats

1. Contamination of water

The literature concerned with the contamination of Canadian inland waters is relatively scarce considering the vast spray regions and the large insecticide deposits involved. Although the present project was concerned with the effects of fenitrothion dispersal in Canada, it should be noted that the use of this insecticide has been world-wide. Fenitrothion has been used throughout Europe, East Pakistan, Africa, the United Arab Republic, Japan, the Republic of China, New Zealand and Brazil (N.R.C.C. No. 14104, 1975).

In Canada, fenitrothion is registered for forest use only against spruce budworm, hemlock looper, and sawfly species (Symons, 1977). Infestations of the spruce budworm are the major concern of Canadian forest spray programs (Varty, 1980). By 1976, the total area of severe budworm infestations in Canada exceeded 30 million ha, of which about 5 million ha were in New Brunswick, nearly 12 million ha were in Quebec and over 13 million ha were in Ontario. Smaller infestations (less than 1 million ha) occurred in Nova Scotia, Prince Edward Island and Newfoundland (Symons, 1977).

The vast area involved has necessitated the use of aircraft for insecticide dispersal. Direct contamination of aquatic habitats situated within the spray region has been reported following aerial deposition of fenitrothion, while indirect contamination may result from spray drift or from surface run-off following rainfall (Eidt, 1975; Eidt and Sundaram, 1975).

Fenitrothion is applied either in a water emulsion or as an oil solution. Dispersal rates range from 138 to 413 gm of active ingredient (a.i.) per hectare (2-6 oz (a.i.)/acre) (N.R.C.C. No. 14104, 1975). Theoretically then (see Appendix (ii)), at these rates of spray a maximum concentration of 4.13 ppm (mg/l) of fenitrothion could be obtained in the top 1 cm layer of a water body situated within the spray region. This value could be doubled in cases where spray overlap occurs. Cases of double swathing have been reported, notably over rivers and lakes which have been used as boundary markers for spray blocks (Symons, 1977; Varty, 1980).

The actual maximum (peak) value for fenitrothion concentrations *in situ*, however, is reportedly much lower than 4.13 ppm. Post spray fenitrothion levels in river water peaked at only 75.5 ppb (ug/l) in one case (Lockhart *et al*., 1973), while even lower peak levels (less than 1 ppb) have been reported elsewhere (Eidt, 1975; Eidt and Sundaram, 1975).

The reasons usually given to explain the low levels of fenitrothion observed in water are spray drift, dilution (see Appendix (ii)), protection by the forest canopy, volatilization of the spray droplets (during their descent through the atmosphere), and degradation of the parent compound. Of these, only the first has been amply demonstrated in field studies. According to Armstrong and Randall (1969), the degree of temperature inversion in the zone above the trees is the major factor affecting the amount of insecticide reaching the target (forest canopy). The drift of the spray cloud from the target area is affected by the strength of the temperature inversion present at the time, and also by the amount of cross-wind.

Field observations during aerial spray applications have demonstrated that anywhere from 15-75% of the pesticide emitted from the aircraft reaches the forest canopy when applied during a temperature inversion condition, and in some cases under unstable conditions, "on-target" deposit was less than 2% of the total emitted material. Obviously, the portion of the spray deposit that does not land "on target" (i.e. on conifer foliage) may, as discussed previously, be transported to aquatic habitats.

After entering the aquatic habitat, fenitrothion may undergo rapid degradation to a variety of products[1] (Zitko and Cunningham, 1974; Greenhalgh et al., 1980). A brief discussion of fenitrothion degradation will be included here, since the rate of degradation of fenitrothion will determine the persistence of the compound in water, and hence its duration of exposure and potential toxicity to algae.

Unlike DDT, which is known to persist for up to two years in aquatic habitats (Yule and Tomlin, 1971), fenitrothion is not detectable by 40 days post-spray (N.R.C.C. No. 14104, 1975). The rapid degradation of fenitrothion in water is thought to involve a combination of hydrolytic, biolytic and photolytic mechanisms.

Laboratory studies have demonstrated that the hydrolysis of fenitrothion, with the subsequent formation of 3-methyl-4-nitrophenol, is highly dependent on the pH and temperature of the water (Kovacikova et al., 1973; Zitko and Cunningham, 1974). Hydrolysis, however, is not expected to occur appreciably in natural waters where pH ranges from 6.3-9.0 (McKee and Wolf, 1963). For example, Zitko and Cunningham (1974) reported that fenitrothion was stable for 45 days in tap water (pH 7), while Truchlick et al. (1972) reported that 50% of the fenitrothion remained in water (pH 9.2, 20-21°C) after 150 days.

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[1] Refer to Appendix (i).

Zitko and Cunningham (1974) reported that the rate of degradation of fenitrothion was greater in river water than in tap water and suggested that this was due to the formation of amino-fenitrothion by microbial degradation. In this connection, Yasuno et al. (1965) reported that the bacterium, Bacillus subtilis, rapidly degraded fenitrothion to amino-fenitrothion.

Photodegradation is also thought to play an important role in determining persistence of fenitrothion in natural waters. Lockhart et al. (1973) reported that fenitrothion was rapidly converted to two unidentified products when an aqueous solution of the pesticide was held in glass vessels and exposed to sunlight. They also reported that fenitrothion had a half-life of less than one day in sunlight but was stable in the dark.

Several "photo-products" of fenitrothion (including carboxy-fenitrothion, carboxy-fenitro-oxon, formyl-fenitrothion, fenitro-oxon, S-methyl-fenitrothion, 3-methyl-4-nitrophenol and 3-carboxy-4-nitrophenol)[1], have been identified in in vitro laboratory studies employing UV radiation[2] (Ohkawa et al., 1974; Greenhalgh, 1976). It is notable, that at the outset of the present project, these photo-products had not been identified in aquatic field studies.

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[1] Refer to Appendix (i).

[2] Germicidal lamp (wavelength mainly 253.7 nm).

The absorption of fenitrothion by suspended particulate matter (sediment, algae, organic debris, etc.) is another possible mechanism for explaining the rapid "dissapearance" of the compound in natural waters (N.R.C.C. No. 14104, 1975). The importance of fenitrothion absorption in relation to its potential toxicity in algae is presented in the following section.

2. Contamination of algae

Algae are expected to be efficient concentrators of pesticides, since their small size and consequently high surface-to-volume ratio permit rapid and thorough absorption. Further, since algal cells generally contain relatively large amounts of fats and oils (lipids) (Stewart, 1974), they should sequester lipophilic pesticides (e.g. DDT, fenitrothion) by partitioning[1].

In this connection, several algal species have been shown to possess a marked capacity to concentrate DDT from the ambient medium. Cox (1970) reported concentration factors[2] ranging from 25,000 to 80,000 for three species of marine algae exposed to 1 to 3 pptr (ng/l) concentrations of DDT. Rice and Sikka (1973) reported concentration factors ranging from 4,000 to 58,000 for six species of marine algae at a DDT concentration of 0.7 ppb. Sodergren (1968) reported that uptake of DDT by Chlorella was rapid ($\leq$ 15 sec) and permanent, since DDT was held without being

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[1] The theory of partitioning is discussed later.

[2] Concentration factor $= \frac{\text{(pesticide in algae)}}{\text{(pesticide in water)}}$

desorbed. Uptake was thought to be passive since killed cells absorbed DDT as efficiently as living cells. Hollister et al. (1975) reported concentration factors ranging from 3,200 to 7,300 for four species of algae exposed to 0.05 ppb of mirex, an organochlorine pesticide. They also reported that, depending on the algal species employed, the algae could absorb from 55% to 88% of the mirex present in the medium. This result is notable in connection with the previous discussion concerning the rapid "dissapearance" of pesticide residues in water samples.

In contrast to the literature concerning algal sequestration of organochlorine pesticides, reports dealing with bioconcentration of organophosphorus pesticides are relatively few. Gregory et al. (1969) reported concentration factors ranging from 50 to 116 for three species of algae and two protozoan species exposed to 1 ppm of parathion, while factors of 99 to 964 were obtained for DDT. Recently, Lakshminarayana and Bourque (1980) reported that preliminary studies indicated that freshwater algae may accumulate fenitrothion, and that up to 5.45% of the fenitrothion deposit was found associated with the algae taken from Peabody Lake, New Brunswick. Although in the latter study the toxicity of fenitrothion to algae was not studied, these results and the foregoing discussion are suggestive that initially low ambient concentrations of lipophilic pesticides could increase by partitioning in the algal population, and reach levels high enough to induce various acute or chronic algicidal effects.

This theory provides the basis for the hypothesis of the present study. Before formally presenting this hypothesis, it is necessary to consider the toxicity of pesticide formulations to algae.

C. The Algicidal Potential of Pesticide Formulations

1. Algicidal effects of pesticides

Reported algicidal effects of pesticides have been reviewed by Butler (1977) and also by Brown (1978). Most of these reports have dealt with the effects of organochlorine residues on algae. For example, the toxicity of DDT to several algal species has been well documented (Sodergren, 1968; Wurster, 1968; Christie, 1969; Rice and Sikka, 1973).

Sodergren (1968) reported that less than 0.3 ppb of DDT inhibited population growth of a freshwater species of Chlorella. Mechanisms for such inhibition have not been unequivocally demonstrated, however several reports have indicated that photosynthesis is affected. Wurster (1968) reported that DDT reduced the rate of photosynthesis in four species of marine algae. In vitro chloroplast preparations from both marine and freshwater algae have shown marked inhibition of photosynthetic electron transport by DDT (Bowes and Gee, 1971; Bowes, 1972). Batterson et al. (1972) suggested that DDT inhibits a membrane-bound ATP-ase in blue-green algae.

In general, organophosphorus pesticides are considered to be less toxic to algae than organochlorine pesticides (Birmingham and Colman, 1977). Christie (1969) reported a temporary inhibitory effect of malathion[1] at 100 ppm on the total population size of a mixed algal community. Clegg and Koevenig (1974) showed that 100 ppm of diazinon[2], a phosphorothioate pesticide, reduced ATP levels in three species of freshwater algae. Growth

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[1] malathion: 0,0-dimethyl S-1,2-di (ethoxycarbonyl) ethyl phosphorothioate.

[2] diazinon: 0,0-diethyl 0-(2-isopropyl-4-methyl-6-pyrimidinyl) phosphorothioate.

stimulation of algae exposed to organophosphate pesticides has also been reported (Cook and Connors, 1963; Birmingham and Colman, 1977).

2. Algicidal effects of spray additives

The literature concerned with the effects of pesticides on algae is often contradictory, and little attention has been given to proper standardization of the methodologies employed. One factor, that is commonly overlooked, is the presence of chemical additives in commercially prepared pesticide formulations used in field spray programs. These additives (emulsifiers, carriers, diluents, adjuvents, co-solvents, etc.) are used to provide a suitable formulation (emulsion, dilution, viscosity, etc.) of the pesticide for aerial dispersal. These additives are commonly regarded as "inert" ingredients of the pesticide formulation, and their potential toxicity to algae has not been well documented.

Recently, aqueous formulations of fenitrothion containing 10% fenitrothion, 1% Aerotex 3470, 1% Atlox 3409F and 88% water (v/v) have replaced the costlier oil-based formulations used previously in Canadian forest spray programs (Symons, 1977; Safe et al., 1977). The co-solvent[1] Aerotex 3470 is a high boiling point, fractional distillate of petroleum. The potential algicidal action of petroleum hydrocarbons in marine habitats exposed to crude oil spills has been reported previously (O'Brien and Dixon, 1976; Vandermeulen and Ahern, 1976).

Naphthalene and alkylated naphthalenes are among the major constituents of Aerotex and are also major components of the "water-soluble" fractions of crude oil (Anderson et al., 1974; Boylan and Tripp, 1971). Naphthalene has been shown to be highly interactive with marine life (Lee et al., 1978), and to inhibit the growth, photosynthesis and ATP

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[1] Aerotex is added to dissolve fenitrothion. Atlox, a detergent , is added to emulsify the Aerotex-fenitrothion solution in water.

synthesis of algae (Vandermeulen and Ahern, 1976). Recent reports by Soto *et al.* (1975 a,b; 1979 a,b) have indicated that the water soluble fraction of crude oil affects the motility, population growth, photosynthesis and cell ultrastructure of the fresh-water alga *Chlamydomonas angulosa*, and that similar, more marked effects were caused by naphthalene.

The relatively high naphthenic content of Aerotex (Safe *et al.*, 1977), and the wide-spread use of this and other oil distillates (e.g. fuel oils No. 2 and No. 4) for pesticide dispersal (Symons, 1977), prompted the present investigation. As mentioned previously, lipophilic (fat-soluble) compounds, including many pesticides (e.g. fenitrothion), as well as many petroleum constituents (e.g. naphthalene), would be expected to partition preferentially into algal lipids. This concept, therefore, can be applied to explain both pesticide and petroleum induced toxicity in algae.

For the purpose of the present study, it was considered necessary to evaluate the algicidal activity of the complete spray formulation (fenitrothion, Aerotex and Atlox) before assessing the environmental hazard of pesticide use.

D. The Theory of Partitioning

Before presenting the hypothesis of the present thesis , it is necessary to digress temporarily to explain the theory of partitioning. To comprehend this concept in its present context, consider a simple biphasic system, for example a layer of olive oil floating on top of a layer of water, both phases being contained in a separatory funnel. If a lipophilic substance such as fenitrothion was added to the system, and allowed to equilibrate, it would partition preferentially into the oil layer. Obviously, if the funnel had been shaken to provide a water suspension of oil droplets, the time taken to reach equilibrium would have been greatly reduced. This system, then, is analogous to a water suspension of algal cells exposed to fenitrothion, and one would similarly expect rapid uptake and sequestration of the pesticide in the algal lipids.

The lipophilicity of a chemical can be expressed quantitatively by determining its partition coefficient in a biphasic solvent system. By definition, the partition coefficient expresses the equilibrium concentration ratio of an organic chemical partitioned between an organic liquid (e.g. n-octanol)[1] and water (Chiou et al., 1977). Note that the concentration factor, used previously to express pesticide uptake in algae, is equivalent to an algae-water partition coefficient for solute concentrations recorded at equilibrium.

The partition coefficient is said to be volume independent, i.e. the ratio of the concentrations of solute distributed between two immiscible solvents is a constant and does not depend on the relative volumes of solvents used (Leo et al., 1971). However, it is notable that the concentration of the solute in either solvent is volume dependent. For example[2], if a solute (e.g. pesticide) has an oil-water partition coefficient ($K_{oil/w}$) of 200, and 20 mg were partitioned between equal 1 litre volumes of oil and water, the oil would contain about 19.9 mg. Hence, the oil concentration of the solute would be 19.9 mg/l or 19.9 ppm. If, however, 1 litre of water but only 1 ml of oil were used, the oil would contain 3.3 mg/ml or 3,300 ppm.

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[1] Octanol-water partition coefficient = $K_{ow} = \frac{\text{(solute in octanol)}}{\text{(solute in water)}}$

[2] The example is based on calculations given in Leo et al. (1971) for determing solvent extraction efficiencies.

Similarly, if the ratio of the oil/water volumes was reduced further, the oil concentration of the chemical would increase. It follows, then, that algae should concentrate lipophilic chemicals since the ratio of algal/water volumes (and hence algal lipids/water volumes) in situ is usually extremely small[1]. Although partition coefficients obtained with several organic solvents (e.g. oil, ethanol, ether, hexane, benzene) have been used as a quantitative measure of lipophilicity, n-octanol-water partition coefficients (K_{OW}) were used in the present study since they have been recorded for a greater variety of organic chemicals, and have been used more extensively in bioaccumulation studies, probably since octanol is thought to best imitate the fatty structure of membrane lipids (Leo et al., 1971; Seeman, 1972).

E. Experimental Design

The initial hypothesis of the present thesis was simply that fenitrothion contaminates aquatic habitats situated within forest spray regions. To investigate the fate, persistence and degradation of fenitrothion in water, a field study was conducted near Winnipeg, Manitoba following an operational spray program. Water and aquatic plant samples were taken from a stream located within the spray region, and were analyzed for fenitrothion and its degradation products.

A subsequent field study was conducted at Lac Bourgeois in the Gatineau Park, Quebec. In order to provide more rigorous control of environmental parameters, fabricated model field systems (1 m^3) were situated in the lake and were employed to determine the accumulation and persistence of fenitrothion in three species of unicellular algae:

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[1] Natural population densities rarely exceed 10^4 cells/ml (Stewart, 1974). This corresponds to a total cell volume of about 42 ul/l of water for a spherical cell of radius 10 um.

Chlamydomonas reinhardii, Chlorella pyrenoidosa, and Euglena gracilis. These algal species were selected for study because they are known to be ubiquitous inhabitants of freshwater aquatic environments and they have been used extensively in environmental pollution and plant physiology research.

Pesticide accumulation in Chlamydomonas was also studied in the laboratory with ^{14}C-labelled fenitrothion. During these studies, light microscope observations revealed that the pesticide formulation caused deflagellation and loss of motility in Chlamydomonas. After establishing that Aerotex was the toxic constituent of the formulation, a series of bioassays was conducted to determine the toxicity of Aerotex and the relative sensitivity of the algal species employed. The bioassay tests involved population growth studies in agar and liquid culture media, inhibition of ATP synthesis and CO_2 fixation, photoreduction of the Hill reagent DCIP (2,6-dichlorophenol indophenol), and electron microscope examination of cell ultrastructure.

Synthesis and evaluation of the bioassay results led to the development of the hypothesis of the present thesis. Simply expressed, it asserts that lipophilic chemicals, including petroleum hydrocarbons (Aerotex) as well as many pesticides (fenitrothion), are algicidal by virtue of their ability to partition preferentially within the lipid matrix of algal cell membranes. Further, their ability to cause membrane damage will depend on their ability to concentrate in membrane lipids and hence should positively correlate with their lipophilicity.

During the course of study, Hutchinson et al. (1979, 1980) reported that the octanol-water partition coefficient of several petroleum hydrocarbons (K_{OW}) could be positively correlated with the concentration of the hydrocarbon required to inhibit CO_2 fixation in algae.

To test this correlation, a method employing a plant fluorometer was developed in the present study for assessing the relative toxicity of several hydrocarbon constituents of Aerotex in Chlamydomonas. This method was also used to determine the algicidal activity of two insecticides (fenitrothion and matacil) and several formulation additives (Aerotex 3470, Atlox 3409F, Dowanol, Diluent-585 and Nonyl Phenol)[1] currently employed in New Brunswick spray programs (Varty, 1980).

The present fluorometric method took advantage of a miniaturized (solid-state) plant fluorometer that was recently developed at Simon Fraser University for in situ recording of fluorescence induction in higher plants (Schreiber et al., 1975). The apparatus was equipped with an external probe that was used in the present case to illuminate (670 nm) algae supported on filter pads while monitoring changes in chlorophyll "a" fluorescence intensity. Rapid changes in chlorophyll fluorescence that occur following illumination of dark-adapted photosynthetic tissue are commonly referred to as the "Kautsky effect" (Kautsky, 1943; for review see Papageorgiou, 1975). Fluorescence transients (changes in fluorescence intensity over time) are considered to be sensitive indicators of photosynthesis and have been extremely important in the elucidation of photosynthetic mechanisms (Schreiber et al., 1975). Although fluorometric analysis of algal photosynthesis has been well documented (Mohanty et al., 1971; Papageorgiou, 1975), the application of the method for assessing the relative algicidal activity of environmental pollutants is considered to be an original contribution of the present study.

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[1] Chemical description of the additives is given in Table 1. The additives (e.g. Aerotex) will be capitalized to differentiate these from the pesticides (e.g. fenitrothion) referred to in the text.

TABLE I

Test Chemical, Description and Source

Chemical	Description	Source
fenitrothion	O,O-dimethyl-O-(3-methyl-4-nitrophenyl) phosphorothioate	Sumitomo Chemical Co.
Aerotex 3470	petroleum distillate	Texaco Canada Ltd.
Atlox 3409F	detergent mixture	Atlas Chemical Co.
Dowanol	dipropylene glycol methyl ether	Dow Chemical Co.
matacil	4-dimethylamino-m-tolyl methyl carbamate	Chemagro Ltd.
matacil 1.8-D[1]	commercially prepared field formulation containing matacil, Nonyl Phenol and Diluent-585 (1.00:2.54:1.52 (v/v))	Chemagro Ltd.
Nonyl Phenol	mixture of monoalkyl phenols (predominantly para substituted)	Chemagro Ltd.
Diluent-585	petroleum distillate	Chemagro Ltd.

[1] Final treatment preparation followed the field dilution recipe (6) provided by Chemagro Ltd. (matacil 1.8-D: Diluent-585 (1:1.88)).

II - MATERIALS AND METHODS

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A. General Methodology

1. Chemicals

Purified samples of fenitrothion (greater than 99.5%) and its degradation products, fenitro-oxon, S-methyl-fenitrothion and desmethyl-fenitrothion were obtained from Agriculture Canada. Detailed methods for purification of fenitrothion and synthesis of these degradation products are described by Hallet _et al._ (1975). Purity was confirmed by gas chromatography (GC) and thin layer chromatography (TLC). ^{14}C-ring labelled fenitrothion (S.A., 5.54 mC/mM) was obtained from the Sumitomo Chemical Co., Japan. Samples of Aerotex 3470 and Atlox 3409F were gifts from Texaco Canada Ltd. and Atlas Chemical Co., respectively. The organic solvents used for extraction were glass distilled, pesticide grade (Caledon).

2. Analytical Methodology

(a) Gas liquid chromatography (GLC):

Fenitrothion and its degradation products were analysed on a Pye (model 104) gas chromatograph (GC), equipped with an alkali flame ionization detector (AFID). The glass column (1.8 m X 4 mm (i.d.)) contained 4% SE-30 Ultraphase/6% QF-1 chromosorb W (H.P.); 80-100 mesh. A second column packed with 3% SE-30 Ultraphase on Chromosorb W was used for crosschecking. Column temperature was 200°C, nitrogen 40 ml/min, air flow 500 ml/min, and hydrogen flow 35 ml/min. To quantitate residue levels, peak areas of duplicate sample injections were compared with intermittent duplicate standard injections. The residue level in organic matter (aquatic plants and algae) was calculated on a dry weight basis. Dry weight determinations were conducted separately for each species and were conducted in tared aluminum dishes at 80°C, to constant weight (ca. 24 hrs).

A second Pye GC equipped with an electron capture detector (EC) was used to analyze fenitrothion degradation products not detectable by the AFID. Methylation of samples to detect desmethyl products was conducted employing diazomethane as described by Hallet et al. (1975). Aerotex 3470 was analysed on a Hewlett-Packard GC as described by Safe et al. (1977). A 4% SE-30/6% QF-1 packed glass column (0.6m X 2mm (i.d.)) was used with an oven temperature of 90°C.

(b) Thin layer chromatography (TLC):

The enzyme inhibition technique described by Mendoza et al. (1968) using steer liver homogenate as the spray reagent, was used to visualize silica gel TLC plates (Fisher Rediplates) developed in a solvent system of 1:3 (v/v) ethyl acetate to cyclohexane. The ^{14}C-sample extracts were chromatographed as above, and the TLC plates were exposed to Kodak NO-Screen X-ray film for 2 weeks at -4°C.

(c) Liquid scintillation counting (LSC):

All sample extracts from radiolabel studies were analyzed with a Beckman LS-100C scintillation counter. Fifty ul aliquots of each extract were dispensed into Beckman plastic scintillation vials, each holding 10 ml of Fisher Scintiverse cocktail. Sample counts (cpm) were corrected for background, quenching and counting efficiency was determined by the external standard ratio method.

3. Algae and Culture Techniques

Algae were obtained axenic from the Culture Collection at Indiana University and were maintained in liquid culture in a Hotpack growth chamber (5 Klux[1], 16/8 hour photoperiod provided by cool white fluorescent lamps, 23°C). The cultures were maintained in 2800 ml Fernbach flasks held on a shaker at 78 oscillations/min. These growth conditions were standard for all treatments.

Chlamydomonas reinhardii (+ strain), *Chlorella pyrenoidosa* and *Scenedesmus obtusiusculus* were grown in autoclaved Bold's Basal Medium (BBM) (pH 6.7± 0.1), (Bold, 1949). *Euglena gracilis* was grown in Polytomella medium (Stein, 1973). Cell counts were made with an improved Neubauer hemacytometer. Exponential phase (9-12 day old) cultures were used in all bioassays and the algae were resuspended in fresh autoclaved BBM prior to use.

All glassware was of Pyrex^R (Corning Glass Works, Corning, N.Y.) glass. The following procedure was used to ensure cleanliness of glassware: (1) Soak in detergent overnight, (2) brush lightly, (3) rinse thoroughly with tap water, (4) rinse three times with pesticide-grade acetone, (5) rinse three times with distilled water, (6) store in a relatively dust-free cabinet, and (7) rinse six times with deionized distilled water just prior to use. Glassware employed for algal culture was kept separate from that used for pesticide treatment.

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[1] A light intensity of of 5 Klux (5×10^3 lumen/m^2) was standard for all culture and treatment procedures.

B. Field Procedures

1. Pine Creek Field Study

(a) Sample site

Two adjacent sections of Pine Creek (near Winnipeg, Manitoba) were selected that had relatively little coverage from the forest canopy. Current flow rates were measured with a Teledyne-Gurley (model 1622) flow meter. One section (Area 1) was virtually stagnant (0.02 m/sec, pH 7.5) while the other (Area 2) was fast flowing (0.18 m/sec, pH 8.2). Aquatic samples included surface-dwelling Lemna minor (duckweed) and submergent Ceratophyllum demersum (hornwort), both from Area 1, and submergent Butomus umbellatus (flowering rush) from Area 2.

(b) Sampling procedure

At eight selected post spray time periods (1-97/hr), water samples were collected in 1 litre amber, Nalgene^{R} bottles. Surface samples were collected by holding the bottle neck just beneath the water surface. Subsurface samples were taken at a depth ranging from 0.3-0.5m. Larger subsurface samples (23 l) were also collected for analysis of trace degradation products. Aquatic plant samples were collected in polyethylene bags from both areas for up to 192 hours post spray. All samples were kept on ice and transported to the laboratory in styrofoam coolers. Water and plant samples were kept in the dark at 4°C and -20°C respectively.

(c) Extraction procedure:

Water samples: Initially water extraction was carried out following the method of Lockhart et al (1973). 500 ml of each sample were filtered through Celite-545 and extracted three times with petroleum ether (100, 50, 50 ml) after addition of 2 gm of NaCl to prevent emulsification. The combined ether extract was passed through anhydrous sodium sulphate, concentrated to near dryness in a rotary evaporator, and brought up to 5 ml acetone for GLC analysis. All ether extractions were completed within 10 days post spray. Subsequent chloroform extractions of water spiked with fenitrothion, fenitro-oxon, and S-methyl-fenitrothion gave greater recoveries[1]. For this reason, the large water samples were extracted with chloroform. The samples were first filtered under vacuum through Whatman No. 43 filter paper. Three litres of water (filtrate) were extracted twice with chloroform (600, 300 ml). This was repeated 4 times until a total of 12 litres of each sample had been extracted.

The chloroform extract was passsed through anhydrous sodium sulphate, concentrated to near dryness and brought up to 10 ml acetone for GLC analysis. The remaining extracted water was freeze-dried and brought up in 5 ml methanol and was kept for determination of any polar products not extracted previously by chloroform partitioning. The residue obtained from the filtration procedure was extracted twice with 200 ml of ethyl acetate in a polytron sonicator, filtered through pre-rinsed Celite-545, concentrated and brought up to 10 ml acetone for GLC analysis.

Plant samples: Each sample (ca. 100 gm) of hornwort (Ceratophyllum demersum) or rush (Butomas umbellatus) was thawed at 37°C and the excess surface water was discarded. The sample was extracted three times (300, 200, 100 ml) with ethyl acetate in a Waring blender. The combined

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[1]

	Concentration (mg/l)	petroleum ether	chloroform
fenitrothion	1.0	97%	102%
fenitro-oxon	2.5	17%	104%
S-methyl fenitrothion	9.0	53%	97%

extracts were passed through anhydrous sodium sulphate, filtered through Celite-545 and concentrated to 25 ml. The concentrate was placed in a glass column (i.d. 22 mm) that had been dry packed successively with 3 cm Celite-545, 7.0 gm of a charcoal (Nuchar C-190N) and Celite mixture, and 3 cm of anhydrous sodium sulphate (Moody et al, 1977a). The extract was eluted successively with 100 ml of 25% ethyl acetate in benzene and 100 ml benzene. The eluant was concentrated to near dryness and brought up to 10 ml acetone for GLC analysis.

The procedure for duckweed (Lemna minor) extraction differed in that the homogenized sample was first separated into solid and aqueous components by centrifugation (10 min at 2°C, 5,900 X g). The residue was extracted and passed through charcoal as detailed above for the other species. The aqueous supernatant was decanted and extracted three times (100, 50, 50 ml) with chloroform and passed through anhydrous sodium sulphate. This extract was concentrated and brought up to 10 ml acetone for GLC analysis.

2. Lac Bougeois Field Study

(a) Sample site

Lac Bourgeois (Gatineau Park, Quebec) is considered to be typical of small lakes and large ponds in W. Quebec and E. Ontario (Dickmann et al., 1975). In situ measurements of the chemical characteristics of the lake water (pH 7.5, 22°C) were taken twice weekly for the duration of the study employing a Hack kit (No. Dr-EL) (Weinberger et al., 1981).

(b) Field stations

The field stations constituted open topped cubes (1 m^3) constructed from polyethylene (Canus Plastics Ltd., Ottawa). These were positioned approximately 1 meter apart, parallel to and approximately 11 metres from the south shore of Lac Bourgeois. Two containers with removable fitted top covers were made from opaque plastic to exclude light[1], another four were translucent and uncovered. The containers were filled with lake water 6 days prior to the scheduled spray date. To permit equilibration, a port located on one submerged side of each bag was left open until the spray operation.

(c) Additions of algae to field stations:

Late log phase cultures of Chlamydomonas, Chlorella and Euglena were grown as discussed previously. These were diluted with their respective algal medium to a population density of 1×10^4 cells/ml. Dialysis tubing (Fisherbrand 8-6670, 31 mm flat width, mw, cutoff 12,000) was used to hold 25 ml of algal culture. The dialysis bags, tied at both ends, were suspended 30 cm below the water surface 2 days before the fenitrothion application. Aquatic plants (Elodea canadensis) and lake sediment were also present in the field systems[2].

(d) Fenitrothion application:

Formulated fenitrothion (fenitrothion, 10%; Aerotex 3470, 1%; Atlox 3409F, 1%; and water 88% (v/v)) was applied with a hand-pump sprayer. At each of four stations, 100 ml of the formulation were applied to provide a fenitrothion concentration of 10 ppm (ul/l). Two stations were not sprayed and served as controls

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[1] Light meter readings demonstrated that less than 0.1% of the total amount of mid-day illumination reached the interior of the opaque stations

[2] This field study was conducted co-operatively with Mr. Bruce Boulton (M.Sc., 1980) who was responsible for the GLC analyses of aquatic plant and sediment samples. All water and algae GLC analyses were performed independently by the present author.

(e) Sampling procedure:

Water samples were collected twice daily for the first 2 days post-treatment and once on days 3,4,6,14 and 28. At each sampling time one litre of water was taken from the upper (10-35 cm subsurface) and lower (36-60 cm) water column of each station. One sample of each algal species was collected from each station at 1 hr post-spray and at 1,6,10 and 14 days post-treatment. The algae were transported on ice to the laboratory and kept at -20°C until extraction.

(f) Extraction procedure:

Water: The large number of water samples taken within a relatively short time period necessitated in situ extraction of the water samples on a rapid basis. For this purpose, a portable field laboratory was implemented that could be assembled within a half hour after arrival at the field site. The final system, manned by four technicians could simultaneously extract four water samples (the upper and lower water samples from each of two replicate stations) within an hour of collection. Briefly, each water sample was initially filtered to remove the suspended sediment. The water and suspended sediment were extracted separately and the extracted water and sediment were retained for further analysis. A detailed outline follows.

Four glass columns (40 x 2.2 cm) with sintered glass discs, were clamped to a metal rack attached to a small bench. Four, 500 ml Erlenmeyer flasks with side-arms were connected via rubber stoppers to each of the columns, and rubber vacuum hose connected the flasks by a series of plastic "T" joints. A vacuum pump was connected to this system and was run by a gas-powered electric generator. The columns were wet-packed in distilled water with 2-3 cm of Celite-545 prior to each experimental run. Four, 1 litre separatory funnels were held on ring clamps secured to a second bench.

For each sample, 1 litre of lake water was initially filtered under vacuum through a Celite-packed column. The Celite was rinsed with 10 ml of distilled water. Five hundred ml of the filtrate were then partitioned three times with chloroform (100 ml each time). The sample extracts were held in 12 oz amber glass bottles, each holding 3 gm of anhydrous sodium sulphate. Sample extracts were transported to a freezer for storage at a temperature of -10°C. The chloroform extracts had levels of fenitrothion that were sufficiently high to permit analysis without concentration of the total extract. One to 5 ml aliquots of each of the samples were brought up in 5 ml of acetone after the chloroform had been blown off under an air stream. The water samples that had been pre-extracted with chloroform were lyophilized and brought up in 5 ml methanol for analysis.

Algae: Each dialysis bag was initially thawed at room temperature. The contents of each bag were then filtered under vacuum through a glass column (30.7 x 1.1 cm) that had previously been wet-packed with 3-4 cm of Celite retained by a sintered glass disc. Three columns (with stopcocks) were run simultaneously (one for each algal species). The residue was rinsed under vacuum with 5 ml distilled water and the filtrates were discarded. The residue was then extracted with 50 ml of ethyl acetate. This was accomplished by pouring 10 ml of ethyl acetate into the column with the stopcock closed. After grinding (30 sec) the Celite inside the column with a glass pestle, the stopcock was opened to allow the solvent extract to flow out into a 125 ml Erlenmeyer flask. This was repeated until a total of 50 ml of solvent had been used in each of the three columns. Each extract was concentrated to near dryness in a flash evaporator at 40°C and then brought up to two ml of acetone for analysis.

C. Laboratory ^{14}C-Fenitrothion Partition Study

Algal uptake of fenitrothion was also studied in the laboratory. A late (12 day old) log phase culture of Chlamydomonas was centrifuged at 6000 X g for 10 minutes and resuspended in fresh BBM. Dialysis tubing was used to hold 10 ml of the culture (1.5 x 10^7 cells/ml). The top of the tube was left open for sampling purposes. For treatment, 200 ul of acetone containing 26.8 ug (1.19 x 10^6 dpm) of ^{14}C-ring labelled fenitrothion (SA: 5.54 mC/mM) were dispensed into a 100 ml breaker. The acetone was blown off under an air stream and 90 ml of BBM containing 1.0 mg of "cold" fenitrothion, 0.1 mg of Aerotex 3470 and 0.1 mg of Atlox 3409F were added to the beaker. In the 100 ml (90 ml BBM + 10 ml algae) system, there was a 10.3 ppm (0.3 ppm ^{14}C-fenitrothion) concentration of fenitrothion, formulated as in the Lac Bourgeois field study. Each dialysis bag was suspended in the treated BBM and supported with aluminum foil. A magnetic stirrer was used to keep the algae suspended in the bag and also to stir the treatment solution. Three systems were used with algae. For controls (without algae), an additional three systems were used with dialysis bags, each holding 10 ml of BBM. The systems were held under continuous illumination (5 klx) at 23°C in a growth chamber. At each sampling time (0, 0.5, 1, 1.5, 2, 3, 6, 24 and 48 hr), 200 ul of BBM were taken from both the outside and inside of each bag. The samples were dispensed into Scintiverse cocktail and analyzed by LSC, as described previously.

After 48 hr, 1 ml of algae from each dialysis bag was filtered under vacuum through a glass fibre filter (Whatman GF/C) and the filtrate was discarded. The algal residue was rinsed three times with 10 ml aliquots of BBM. Each rinse was allowed to remain on the filter for 5 min. prior to filtration. The three rinses were lyophilized and analyzed by LSC. The algal residue on the filter[1] was immersed in Scintiverse cocktail and analysed by LSC. The 48 hr counts taken for the algae at the termination of the dialysis study were used to calculate the amount of ^{14}C-activity present prior to rinsing. For background correction, filter pads were analyzed after filtration and successive rinsing of 1 ml of the BBM external to the dialysis bags.

D. Aerotex Phytotoxicity Studies

During the dialysis study, light microscope observations demonstrated that the algae became non-motile 2 days after treatment and cytolysis was apparent. Further motility tests with all combinations of fenitrothion, Aerotex and Atlox demonstrated that the Aerotex constituent was responsible for the observed impairment of motility. For this reason, several bioassays were conducted with several naphthalene constituents of Aerotex. These included naphthalene and 1-methyl-, 2-methyl-, 2,3-dimethyl-, and 2,6-dimethyl-naphthalene, and were obtained from Aldrich Chemicals Co.

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[1] The glass filters became transparent in Scintiverse cocktail and did not cause significant quenching. Millipore filters (plastic) were avoided since they absorbed significant amounts of ^{14}C-fenitrothion.

1. Bioassay Procedures

Unless indicated otherwise, emulsions of the toxicants in nutrient medium (BBM) were used for treatment. Stock emulsions of Aerotex or 1-methyl-naphthalene (1% (v/v in BBM)) were prepared immediately[1] prior to use by 1 minute sonication in a Polytron sonicator (No. 4369) at setting No. 5. Glass-distilled, deionized water was used in all cases for medium and treatment preparations.

(a) Motility studies:

Standard techniques employing a flagellar strain (Bradshaw, 1973), were used to determine the % deflagellation of Chlamydomonas treated with Aerotex (1-100 ppm (ul/l)). Suitable aliquots of Aerotex were added to the algal culture and motility was observed with a Zeiss light microscope. Non-motile treated cells were centrifuged and washed (three times) to determine the reversibility of the treatment. This study was repeated with stock acetone solutions of Aerotex and the naphthalene compounds. Acetone at the concentration employed (50 ul/100 ml BBM) did not affect cell motility.

Vital staining of Chlamydomonas with methylene blue was also conducted. Briefly, 1 ml of methylene blue (0.1%) was added to 1 ml of Chlamydomonas (2 x 10^6 cells/ml) pre-treated with Aerotex (1-100 ppm). The % of cells stained after 1, 5, 10, 15, 30 and 60 minutes was determined by counting with a hemacytometer.

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[1] Stock emulsions of Aerotex left overnight at room temperature were less effective in inhibiting cell motility than when freshly prepared.

(b) Agar bioassays:

An algal lawn technique, (Pulich et al, 1974; Wright, 1975), was used to test the effect of Aerotex concentration on Chlamydomonas, Chlorella, and Scenedesmus. Nine day old, exponentially growing cells (final concentration 0.5-1.0 x 10^6 cells/ml) were added to autoclaved agarized medium (1% Bacto-Agar), Difco No. 0140, in BBM that had cooled to 38°C. The agar was immediately poured into plastic petri dishes. Filter paper discs (Whatman No. 1, 55 mm) were soaked for 1 minute in a range of Aerotex concentrations (Table 5) and were placed equidistantly, 3 per plate, on the agar surface. The plates were incubated upside-down in a growth chamber (5 Klux, 16/8 hr photoperiod, 23°C) for 6-7 days. Following this, the diameter of each inhibition zone was measured.Agar bioassays were also conducted with the naphthalene compounds listed previously. These were dissolved in acetone for treatment of the filter discs. The acetone was allowed to evaporate for 30 sec prior to plating of the discs. Acetone controls did not inhibit growth.

(c) Liquid culture bioassays:

Axenic cultures of Chlamydomonas, Chlorella, and Scenedesmus were also used for liquid culture bioassays. Sterile, screw-cap tubes (Pyrex No. 9825, 22 x 150 mm) were used to hold 10 ml aliquots of autoclaved BBM. A suitable aliquot of a 1% stock preparation of Aerotex in cold sterile BBM was aseptically added to each tube to give final concentrations of 0, 1, 2, 5, 7.5, 10 and 100 ppm of Aerotex. This procedure was chosen since autoclaving or filter sterilization of oil products in aqueous emulsion may change their composition (Vandermeulen and Ahern, 1976). Identical aliquots (100 ul) of 9 day old algal cultures (4-6 x 10^6 cells/ml) in late log phase were dispensed into each tube after addition of the Aerotex. The tubes were loosely capped and placed on their sides on an Eberach Shaker (78 oscillations per minute) held in a growth chamber, and maintained under the conditions specified previously. Duplicate tubes were used for each Aerotex concentration. The use of culture tubes permitted a large number of replicated studies to be conducted simultaneously in one growth chamber. The large surface area to volume ratio inherent in this growth procedure permitted the use of a shaker for maintaining optimum aeration of cultures and for ensuring homogenous treatment conditions.

The change in population density was determined daily by reading the transmission of each culture tube in a Coleman (6/28) spectrophotometer at 540 nm. The transmission values were converted to $\log_2$ optical density (O.D.) and the initial value obtained for each culture was subtracted from each subsequent reading, (Stein, 1973). The Δ $\log_2$ O.D. values determined in this manner were plotted versus time to give a growth curve for each of the algal species, at each Aerotex concentration. Following the method of Stein (1973), standard curves were constructed for the three algal species to ensure a linear relationship between Δ $\log_2$ O.D. and cell density (Appendix (vi)). The use of culture tubes permitted the direct reading of O.D. without subsampling. The O.D. of tubes containing BBM alone did not change for the duration of the study.

Viable cell counts were conducted following standard techniques (Bradshaw, 1973). Briefly, Chlamydomonas was treated with Aerotex (10 ppm) as described above. After 1 hr treatment, aliquots of treated culture were diluted with sufficient BBM to permit assessment of cell density via colony counts after plating on BBM-supplemented agar. Colony counts of treated and control cultures were recorded after about 2 weeks growth under the conditions used for the agar bioassays.

(d) ATP study:

Nine day old cultures (100 ml) of Chlamydomonas, Chlorella, and Scenedesmus in late log phase were resuspended in autoclaved BBM and were dispensed into 250 ml screw-cap Erlenmeyer flasks. After 1 hr equilibration in the light, Aerotex was added to provide a concentration range of 0-100 ppm for each species. Triplicate flasks were used for each concentration. The flasks were held on a shaker and exposed to continuous illumination in the growth chamber at 23°C. Triplicate, 10 ml aliquots of each species at each treatment concentration were sampled after one hour. The procedure of Holms-Hansen and Booth (1966) as modified by Patterson et al (1970) was used for extraction and analysis of ATP. Briefly, the algae were centrifuged at 5,000 X g for 10 minutes and the pellet was extracted twice (5 min each time) with a total of 5 ml of boiling Tris buffer (0.02M, pH 7.75). The luminescence of the extracts was determined by LSC following addition of firefly luciferin-luciferase complex (Sigma Chemicals Ltd.). ATP levels were quantitated employing standard curves (Patterson et al, 1970). This study was repeated with Chlamydomonas as described above except the algae were maintained under either light or dark conditions for 1 hr both prior to and after treatment. It is noted that future studies should incorporate a P^{32} tracer direct measurement of phosphorylation.

E. Photosynthesis Assays

1. Carbon Dioxide Fixation Studies

Photosynthetic fixation of CO_2 by Chlamydomonas was determined by the method of Soto et al (1975a). Briefly, an exponential phase (9 day old) culture of Chlamydomonas was centrifuged at 500 X g for 10 minutes and resuspended in autoclaved BBM containing 1 mM $NaHCO_3$. The final cell population was 3.1×10^4 cells/ml.

Aerotex treatment was conducted in 250 ml Erlenmeyer flasks as outlined previously for the ATP study. At each sample time, 10 ml of the culture were dispensed into a Millipore glass filter apparatus holding two glass fibre filters (Whatman GF/C). After addition of 100 ul of solution containing 5 ul of $NaH^{14}CO_3$ (SA, 200 mC/mM), the culture was exposed to fluorescent light (5 klx) for exactly 3 minutes. The culture was then filtered under vacuum (filtration time: 15 sec) and rinsed with three 10 ml aliquots of BBM. The filter pads were analzed by LSC as described in the dialysis study. The counts obtained for the lower filter pads were used for background correction. "Dark" controls were conducted routinely. CO_2 fixation in the dark was less than 1% of that determined in the light.

Since the above procedure necessitated opening of the treatment flask at each sample time, the Aerotex was subject to volatilization. To negate this possibility, treatment was also conducted in screw-cap tubes filled with algal culture (headspace ca. 1 cm), with one tube being used at each sample time.

The effect of Aerotex (10 ppm) on cell leakage of ^{14}C-photosynthate was also conducted following the method of Hutchinson et al. (1979) except that the algae (Chlamydomonas) were held in dialysis tubing as described in the ^{14}C-partition study (II, C) and ^{14}C-activity in the dialysate was measured directly by LSC. Excess 1N HCl was added to the dialysate 10 min prior to counting, to remove un-fixed $NaH^{14}CO_3$. Both 10 days old and ca. 2 month old algal cultures were employed to examine the effect of cell age on leakage.

2. Photosynthetic Reduction of DCIP

The method of Hallier and Park (1969) was used to measure the photoreduction of 2,6-dichlorophenol indophenol (DCIP) by *Chlamydomonas*. The algae were held in screw-cap tubes and treated with Aerotex as described previously in the liquid culture bioassays. After 30 minutes, sufficient DCIP was added to the culture tube to provide a 1 uM concentration and the optical density (610 nm) was measured. The tube was returned to the shaker in the growth chamber (5 klx, 23°C) and O.D. readings were taken on a time basis thereafter. Triplicate tubes were used for each treatment concentration. The laboratory was dimly lit during these studies to prevent photoreduction during the O.D. measurements. For "dark"controls, the tubes were wrapped in aluminum foil and held in the growth chamber without illumination.

3. Fluorometric Analysis of Photosynthesis

(a) Algal treatment:

The pesticides and adjuvants selected for screening and their source of origin are listed in Table 1. The benzene and naphthalene test compounds were obtained from Aldrich Chemical Co. The test formulations selected for evaluation (Table 10) have been used extensively in Canada for spruce budworm control (Symons, 1977; Varty, 1980). Stock preparations of each test compound (100 mg/100 ml) were made immediately prior to use by one of two methods:

1. Stock solutions of non-volatile, acetone-soluble compounds were prepared with acetone. Suitable aliquots of stock solution were dispensed into 50 ml screw-cap Erlenmeyer flasks. The acetone was blown-off under an air stream and 45 ml of BBM containing

sufficient $NaHCO_3$ (CO_2 source) to provide a 1 mM concentration in 50 ml were immediately added to each flask. An identical aliquot (5.0 ml) of a 9-12 day old culture of Chlamydomonas in late log phase was then added to each flask to provide a final cell concentration of 2.6 x 10^4 cells/ml. The flasks were capped tightly, shaken, and placed on a shaker in the dark at 23°C. Dark conditions were used to negate the possibility of photodegradation of the toxicant.

2. Water soluble or volatile test compounds were suspended in BBM by mixing in a Polytron sonicator (No. 4369) at setting No. 5. Subsequent treatment followed the procedures outlined above for the acetone stock solutions, except that the total volume was corrected for the addition of algal medium.

A wide range of treatment concentrations (0.1, 1, 10, and 100 ppm) was initially tested to determine the proper range for subsequent studies. Triplicate flasks were used for each treatment concentration.

(b) Fluorometric analysis:

A Plant Productivity Fluorometer (model SF-10) was obtained from Richard Brancker Research Ltd., Ottawa, Canada. The fluorometer was used in conjunction with a Fisher Recordall^R Series 5000 chart recorder (signal input 10 vdc, chart speed 13 cm/min). The fluorometer was set at a light (680 nm) exposure of 10 sec and at maximum intensity (10^4 ergs/cm^2/sec).

After a 1 hour treatment each 50 ml algal sample was filtered under vacuum through a Whatman GF/C glass fibre filter. The glass filter was then placed on top of two Whatman No. 1 filter papers pre-moistened with BBM and the fluorometer probe was centered on top. During the filtration step and positioning of the probe, the algae were inevitably exposed to light. This reduced the sensitivity of the fluorometer response and it was necessary to readapt the cells to the dark. This was accomplished by leaving the probe on top of the algae for 30 seconds. An exact time period was required to obtain reproducible results. In this connection, previous studies have shown that the size of the initial fluorescence peak is directly proportional to the length of the preceeding dark period (Krause, 1973). A period of 30 sec dark adaptation was found to give sufficient sensitivity of response to permit accurate quantitation of the fluorescence transients in the present study. Duplicate analyses were made for each sample by repositioning the probe on the glass filter. Finally, the glass filter was washed under vacuum with 25 ml of BBM and re-analyzed to determine the reversibility of the treatment effect.

Following the above procedure, duplicate analyses of triplicate samples could be performed in less than 10 minutes. A maximum of 2 hours was required to determine the minimum concentration required for any compound to totally suppress the fluorescence response.

F. Transmission Electron Microscopy (TEM)

An exponential phase (9 day old) culture of Chlamydomonas was resuspended in fresh BBM (9.6 x 10^5 cells/ml) and treated for 1 hour in the dark with Aerotex (5 ppm). The treatment procedure followed that outlined previously in the ATP study.Algal treatment was also conducted with 1-methyl-naphthalene (5 ppm).

After treatment, the cells were pelleted by gentle centrifugation (setting No. 6 on IEC clinical centrifuge) and fixed according to a modified simultaneous glutaraldehyde-osmium tetroxide fixation procedure based on Franke et al. (1969). Equal parts of ice-cold 2% glutaraldehyde and 1% osmium tetroxide both buffered with 0.05M phosphate (pH 7), were mixed prior to use. Fixation for 1 hour was carried out at 0°C. The cells were then washed three times (10 min/wash), and postfixed for 1 hour in cold buffered 1% osmium tetroxide. After two subsequent washes in buffer, the cells were dehydrated in a graded acetone series and infiltrated with Spurr's hard resin mixture (Spurr, 1969), which was then polymerized at 70°C for 8 hours. Sections were cut with a DuPont diamond knife on a Sorval Porter-Blum MT-2B ultramicrotome, mounted on acetone treated copper grids without support and stained for 4 minutes in uranyl acetate (5% in 50% ethanol) and for 2 minutes in lead citrate (Reynolds, 1969). Observations and photographs were made with a Philips 201 electron microscope.

This study was repeated following the method described above except a glass knife was used for sectioning, while observations and photographs were made with an AEI-EMM6B electron miscroscope.

III - RESULTS

III RESULTS

A. Field Studies

1. Pine Creek Field Study

(a) Water samples:

The results of the GLC analyses for fenitrothion are given in Table 2, expressed as ppb (ug/l) of water. Values are given for both the surface and subsurface water samples taken from the stagnant and flowing sections of the stream.

Fenitrothion levels decreased from an initial value of 701 ppb recorded 1 hr post-spray (surface, Area 1), to less than 1 ppb, 84 hr post-spray. Relatively high levels of fenitrothion were detected in the suspended sediment (890 ppb, 26 hr post-spray, and 370 ppb, 72 hr post-spray) taken from Area 1. Trace levels of amino-fenitrothion were detected in the stream water (Table 2). Since this degradation product was not detected in the original spray formulation, it must have been formed in situ. Traces of desmethyl-amino-fenitrothion were detected in the lyophilized water samples taken from Area 1. Traces of S-methyl-fenitrothion were only detected in the surface water of Area 1 at 1 hr (4.4 ppb) and 10 hr (trace) post-spray. Analysis of the original spray formulation demonstrated the presence of 2.47% S-methyl-fenitrothion, a value consistent with those reported for other technical formulations (Greenhalgh et al., 1975).

(b) Aquatic plant and suspended sediment samples:

Table 3 presents the data obtained from the aquatic plant analyses. The levels of fenitrothion present in the surrounding water are given for comparison and demonstrate the accumulation of the pesticide in the plants from Area 1.

TABLE 2

Fenitrothion levels (ppb) in water taken from Pine Creek near Winnipeg, Manitoba following an operational aerial spray program.

PPB FENITROTHION (F) & AMINO-FENITROTHION (AF)								
	AREA 1 (STAGNANT)				AREA 2 (FLOWING)			
TIME	SURFACE		SUBSURFACE		SURFACE		SUBSURFACE	
(hr)	F	AF	F	AF	F	AF	F	AF
1.0	701	NA	9.46	0.05	1.32	TR	1.11	TR
10.0	44.1	0.35	9.03	0.08	0.57	TR	0.52	ND
23.0	14.1	0.11	7.45	0.08	0.28	ND	0.22	ND
50.5	3.57	0.08	2.73	0.09	0.14	ND	0.42	ND
70.0	1.77	0.07	1.15	0.08	0.24	ND	0.35	ND
84.3	0.84	0.04	0.61	0.03	0.23	ND	0.21	ND
97.0	0.67	0.02	0.57	0.02	0.23	ND	0.21	ND

NA = not analyzed
ND = not detectable (less than 0.01 ppb)
TR = trace amount

TABLE 3

Fenitrothion levels (ppm) in aquatic plants taken from Pine Creek.
The last column gives the levels in the surrounding water for comparison.

			SAMPLE MATERIAL			
SAMPLE SITE	SPECIES	TIME (Hr)	PPM RESIDUE	PPM SUPERNATANT	NET PPM	WATER PPB
Stagnant (Area 1)	L. minor (duckweed)	1	1.44	0.26	1.70	701
		10	4.00	0.19	4.19	44
		23	0.10	0.03	0.13	14
		192	0.03	0.002	0.032	(0.67: 97 hr)
Stagnant (Area 1)	C. demersum (hornwort)	23			0.12	7.45
		70			0.15	1.15
		192			0.14	(0.57: 97 hr)
Flowing (Area 2)	B. umbellatus (rush)	23			ND	0.22
		70			ND	0.35
		192			ND	ND

ND = Not detectable (less than 0.01 ppm plant or 0.01 ppb water)

The surface dwelling duckweed would have received the initial fenitrothion deposit directly, however, the data also shows rapid uptake of the pesticide by this species during the initial 10 hours post-spray. Fenitrothion was shown to be predominantly associated with the solid plant material (residue, Table 3). For example, less than 5% was associated with the aqueous component (supernatant, Table 3) of duckweed, 10 hr post-spray. Residues persisted at relatively high levels for at least 192 hours in the submergent hornwort.

2. Lac Bourgeois Field Study

(a) Water samples:

Figure 1 demonstrates the rapid diminution in fenitrothion levels in water taken from the field stations. There was no significant difference between the rates of dissapearance of the pesticide in the light (Fig. 1A) and dark (Fig. 1B) conditions. By two days post-spray, the fenitrothion levels in the upper and lower sections of the water column coincided, indicating that complete mixing had occurred.

The levels of fenitrothion degradation products detected in the water samples taken from both light and dark conditions are given in Table 4. The water samples were lyophilized and methylated with diazomethane prior to GLC analysis. Note that carboxy-, carboxy-amino-, and desmethyl-fenitrothion were only present in the water sampled from field stations exposed to light, while dimethyl-thiophosphate (DMTP) was detected in both light and dark-held samples. These products were identified by GLC and TLC and were further authenticated by GLC-mass spectrophotometry (Weinberger et al., 1981). A gas chromatogram of a methylated, lyophilized water sample taken from an illuminated field station is shown in Appendix (iii). After 2 days in the light, 33.2% of the deposit was still detectable in the water as fenitrothion, and 10.7% was present as degradation products.

FIGURE 1A

Fenitrothion levels (ppm) in water taken from Lac Bourgeois field stations exposed to sunlight. The average values of duplicate samples are shown together with standard range bars.

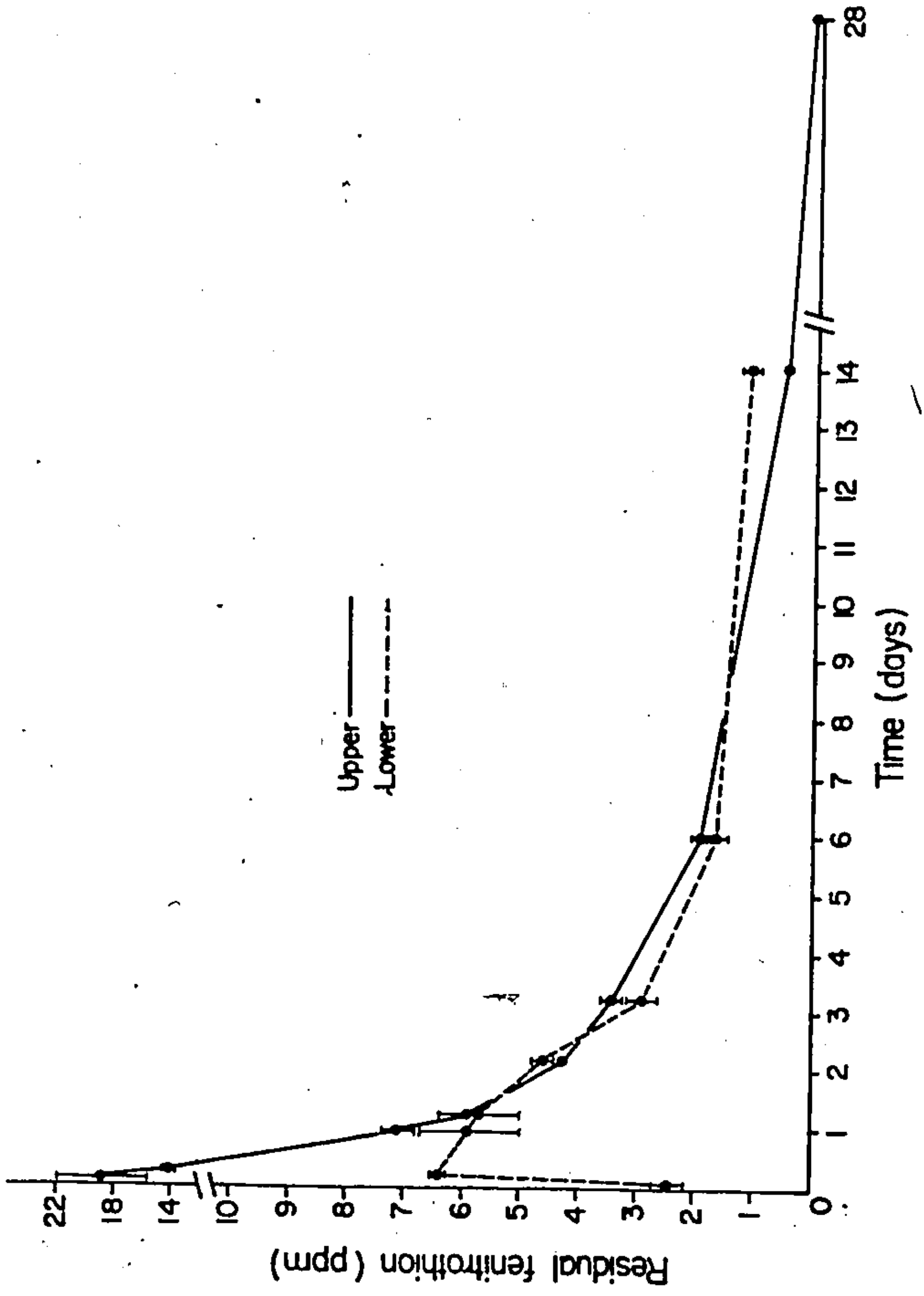


FIGURE 1B

Fenitrothion levels (ppm) in water taken from Lac Bourgeois field stations held in dark. The average values of duplicate samples are shown together with standard range bars.

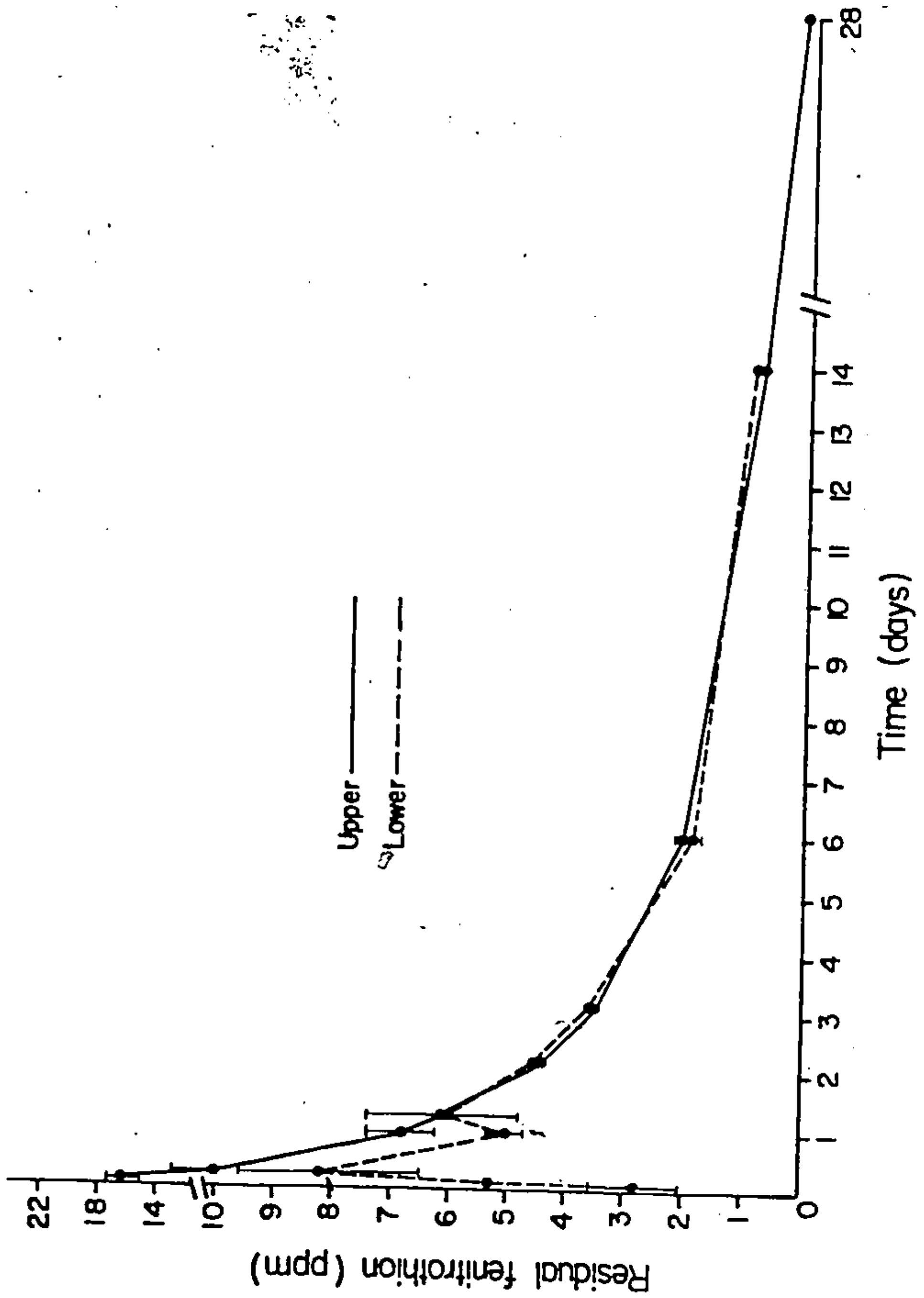


TABLE 4

Levels (ppm) of fenitrothion degradation products detected in lyophilized water samples taken from Lac Bourgeois.

The average values of duplicate samples are reported ± standard range.

	Fenitrothion (ft) Degradation Product (ppm)							
	carboxy ft		desmethyl ft		carboxy-amino-ft		dimethyl thiophosphate	
Time (Days)	L	D	L	D	L	D	L	D
0.2	0.06 (0.02)	TR	TR	ND	TR	ND	0.27 (0.01)	ND
1.2	0.18 (0.01)	ND	0.02 (0.01)	ND	0.12 (0.01)	ND	0.62 (0.05)	0.35[1]
6.0	0.23 (0.07)	ND	0.04 (0.02)	ND	0.20 (0.08)	ND	0.27 (0.05)	0.34[1]
14.0	0.04 (0.02)		TR		TR		0.09 (0.09)	
28.0	TR		ND		TR			

[1] Only one sample analyzed.

ft = fenitrothion
L = Light
D = Dark
TR = Trace amount
ND = Not detectable (less than 0.01 ppm)

( ) = Standard range

(b) Algae samples:

Figure 2 demonstrates rapid uptake and accumulation of fenitrothion in algae. Initially, fenitrothion levels were approximately two times greater in the light (Fig. 2A) than in the dark (Fig. 2B) for Chlamydomonas and Chlorella. After 2 days in the light, Chlamydomonas had accumulated 9.66 ppt (parts per thousand, ug/mg) of fenitrothion, representing a concentration factor of 2,196 times the ambient water concentration (4.4 ppm, Fig. 1A) present at that time. Degradation products of fenitrothion were not detected by GLC or TLC in any of the algal extracts. The total maximal algal absorption of fenitrothion accounted for only 0.004% of the initial deposit. Aquatic plant and sediment absorption, however, accounted for 0.5% and 38.7%, respectively (2 day sample time) (Weinberger et al., 1981).

B. Laboratory ^{14}C-Fenitrothion Partition Study

Figure 3 demonstrates rapid uptake of the ^{14}C-radiolabel by Chlamydomonas held in dialysis tubi[illegible]aboratory. The data, expressed as a ratio of the internal and external [illegible]ntrations of the ^{14}C-radiolabel, indicates that equilibrium was attained after about 6 hours. Note, however, that algal uptake of the ^{14}C-label increased slightly between 6 and 48 hr (Fig. 3).

At equilibrium (6 hr), the internal and external radiolabel concentrations of the controls (BBM alone) were approximately equal (ratio 1.0); however, the concentration in the dialysis bags holding the algae was 2.5 ± 0.25 times the external concentration. The concentration of radiolabel in the algae was calculated (see Appendix iv) from the observed ^{14}C-activity (dpm) in the algal culture after subtracting the ^{14}C-activity in an equal volume of BBM sampled

FIGURE 2A

Fenitrothion levels (ppt) in algae held in Lac Bourgeois field stations exposed to sunlight. The average values of duplicate samples are shown together with standard range bars.

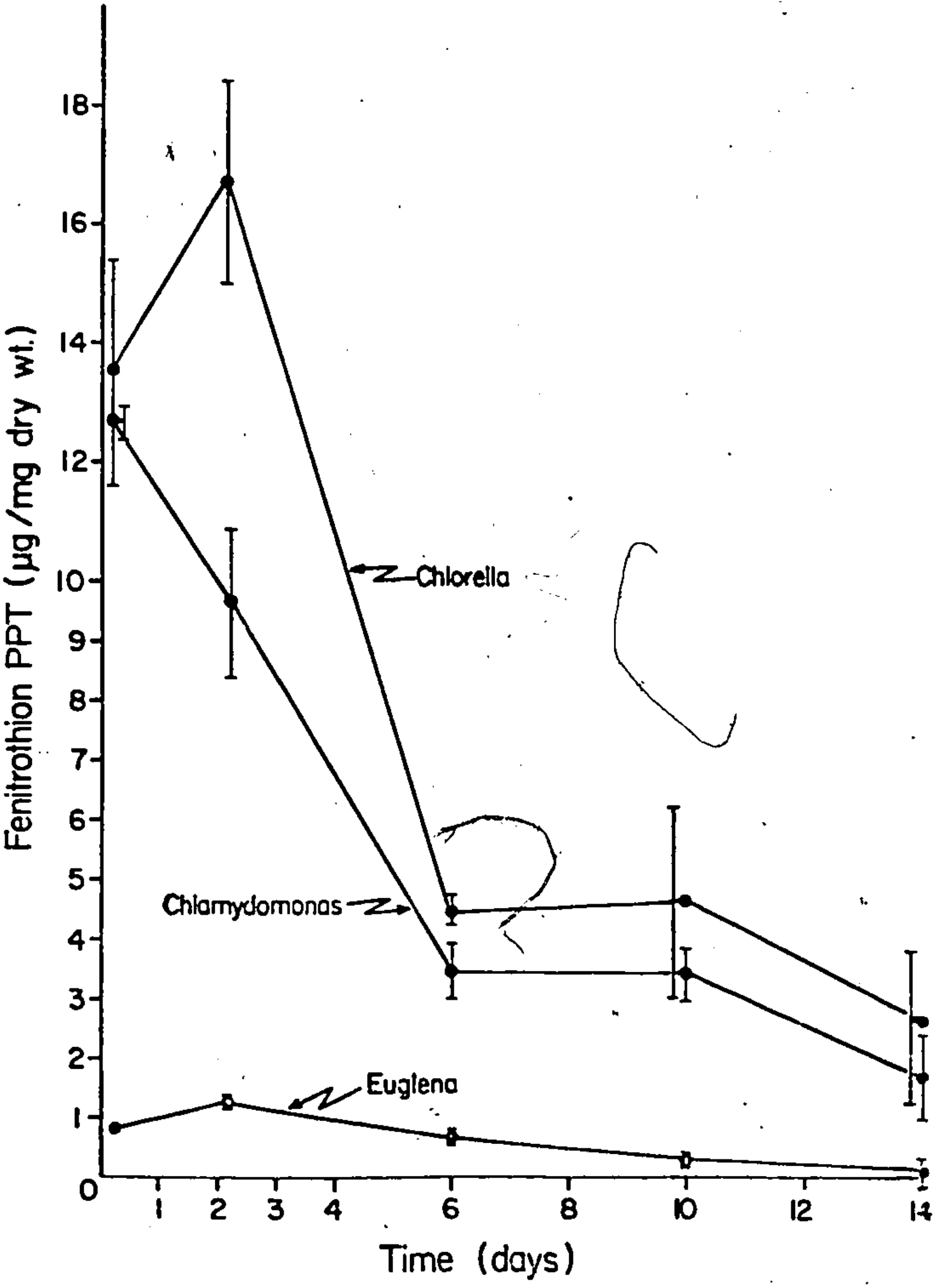


FIGURE 2B

Fenitrothion levels (ppt) in algae held in Lac Bourgeois field stations held in dark. The average values of duplicate samples are shown together with standard range bars.

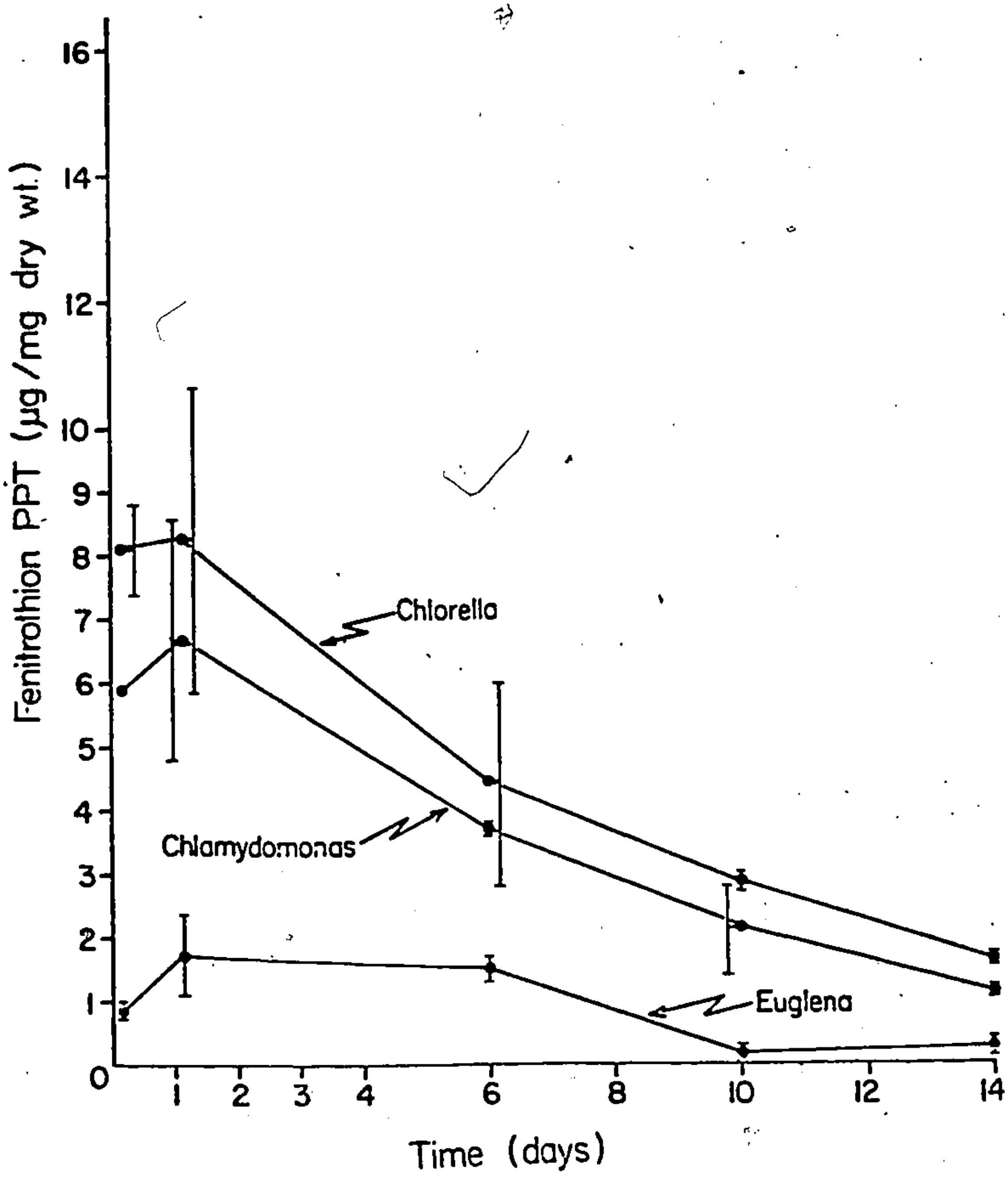
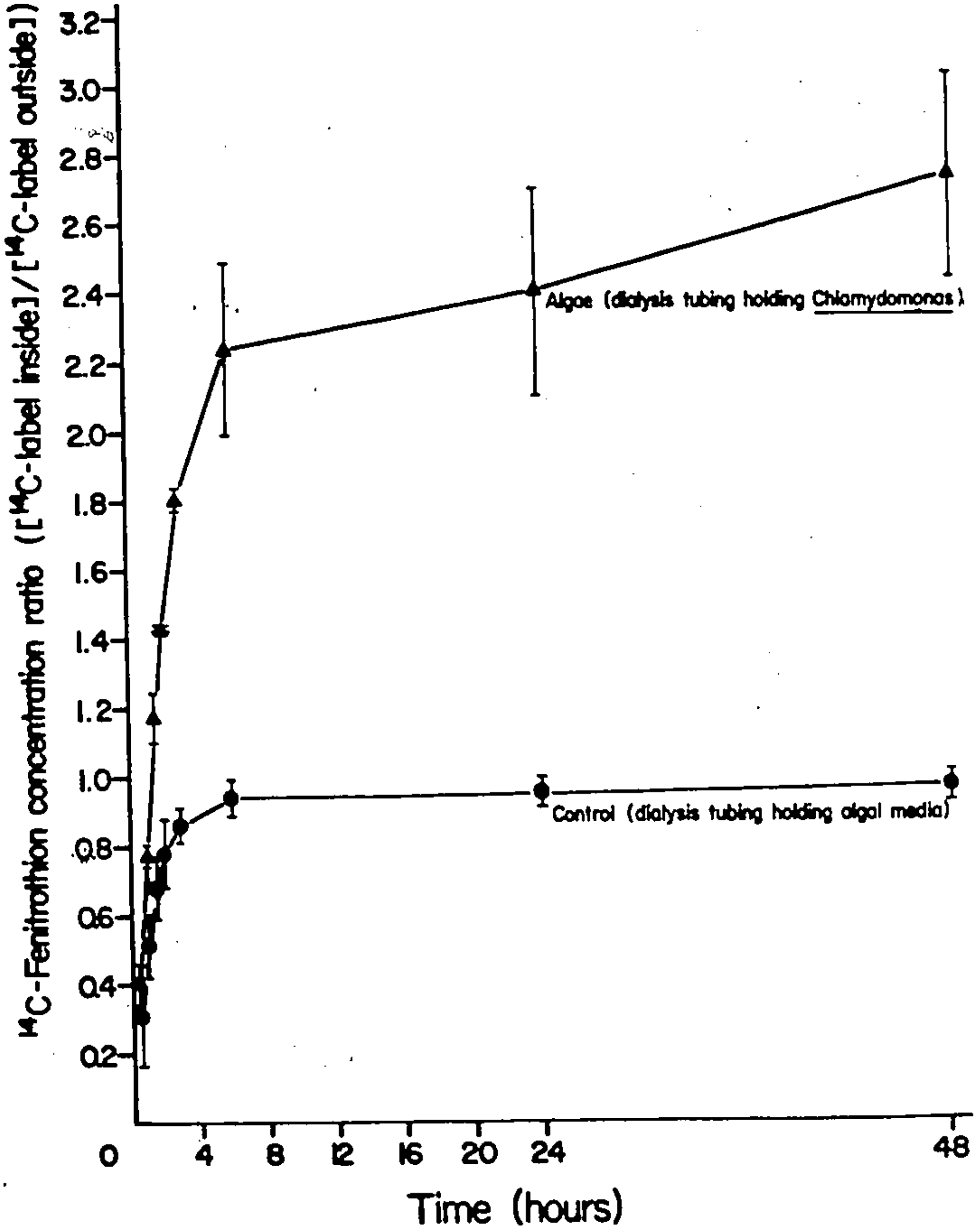


FIGURE 3

^{14}C-fenitrothion dialysis study: Ratio of internal-to-external ^{14}C-activity (dpm) versus time for dialysis bags holding algal media (BBM) or algae (Chlamydomonas). Mean values of triplicate samples are shown together standard deviation bars.



external to the bag[1]. The ^{14}C-activity in the algae was used to calculate the concentration of fenitrothion in the algae. After 6 hr, Chlamydomonas contained 8.92 ± 1.81 ppt (ug/mg) of fenitrothion[2]. This represents a concentration factor of 853 times the ambient water concentration (10.46 ppm) at 6hr. Assuming that 39% of the algal dry weight was lipid (Appendix iv), then the fenitrothion concentration in the lipid was 22.87 ppt representing a concentration factor of 2,186. The log of 2,185 = 3.34 which represents the log of the partition coefficient for fenitrothion[3] between the algal lipid and the external water (or log K_{lw}).

The results of the study in which Chlamydomonas (2 day treatment duration) was rinsed successively with a total of 30 ml of BBM demonstrated that a total of only 1.37 ± 0.26% of the ^{14}C-activity was leached out of the algae.

C. **Aerotex Phytotoxicity Studies**

1. Bioassay Procedures

(a) Analysis of Aerotex:

GLC analysis of Aerotex gave the following composition: naphthalene, 9.8%; 1-methyl-naphthalene, 12.9%; 2-methyl-naphthalene, 8.5%; 2,6-dimethyl-naphthalene, 4.2%; and 2,3-dimethyl-naphthalene, less than 0.5%. The remainder was mainly composed of benzene and alkylated benzenes (Safe et al., 1977). A gas chromatogram of Aerotex is included in Appendix v.

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[1] There was no significant difference between the ^{14}C-activity determined for the external BBM and the activity of an equal volume of internal BBM after removal of the algae by filtration.

[2] This is comparable to the concentration of fenitrothion determined for Chlamydomonas in the previous field study (12.78 ± 0.25) ppt, 5 hr).

[3] Note that this is comparable to 3.38, the literature value for log K_{ow} of fenitrothion (Chiou et al., 1977).

(b) Motility study and light microscopy:

Deflagellation and loss of motility of Chlamydomonas were evident (within 10 min) at concentrations as low as 5-10 ppm of Aerotex. Cell motility was not evident 24 hrs after treatment and cytolysis was apparent. A small fraction (less than 1%) of the cell population regained motility (within 30 min) if treated cultures were resuspended in fresh media within 1 hour of treatment. Concentrations of naphthalene ≤ 20 ppm did not impair motility while concentrations as low as 10 ppm of the methyl-naphthalenes caused loss of motility for at least 8 hours. By 24 hours, however, a fraction of the cell population treated with the methyl-naphthalenes (10 ppm) had regained its motility. Cellular vacuolation of Chlamydomonas was observed (light microscope x 400) following 30 min exposure to Aerotex (5 ppm). The vacuoles which were not evident in control cells (Fig. 11A), appeared green in the light microscope and were birefringent under phase constrast (Fig. 11B).

After addition of methylene blue, Chlamydomonas stained dark blue when the cells had been previously treated with Aerotex. The degree of staining (% of cell population stained) was directly related to both the duration of the Aerotex treatment and the concentration employed. For example, over 99% of the cells stained within 1 min of treatment with Aerotex at 100 ppm, however, at 20 ppm, 60 min were required to obtain over 99% staining[1]. After 60 min exposure to 10 ppm, 46 ± 5% staining was observed. At 5 ppm, 15-25% staining was observed 60 min after treatment while at 1 ppm, less than 1% staining was observed after 60 min.

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[1] The time refers to the duration of exposure to Aerotex and is exclusive of the staining procedure. After Aerotex treatment, the algae were left in the stain for 5 min prior to completion of cell counting (see "Materials and Methods", D.1(a)).

It was also observed that untreated algae yielded over 99% staining after heating for 10 min at 80°C followed by cooling to room temperature prior to addition of the stain. Untreated algae kept frozen at -60°C for 10 min and then thawed at room temperature exhibited less than 1% staining.

(c) Agar bioassays:

The size of the inhibition zone (Table 5) was proportional to the applied Aerotex concentration. *Chlorella* and *Chlamydomonas* were more sensitive to Aerotex than *Scenedesmus*. The algae did not grow on the inhibition zone for the duration (3 weeks) of the experiment. Of the naphthenic compounds tested, i.e. naphthalene, 1-methyl-, 2,3-dimethyl-, and 2,6-dimethyl-naphthalene, only 1-methyl-naphthalene inhibited growth of *Chlamydomonas* on agar and this compound was equally as inhibitory as Aerotex.

(d) Liquid culture bioassays:

The results of the liquid culture bioassays (Fig. 4A, B, C) demonstrated that the duration of the lag phase increased with increasing amounts of Aerotex until a concentration was reached at which no growth occurred. The average values of paired data were plotted for each growth curve when the standard range (indicated by vertical bars, Fig. 4A, B, C) was small. Otherwise, the data were plotted separately with two growth curves being plotted for one treatment concentration. The duration of the lag phase was determined for each growth curve by linear regression of the exponential portion of the curve followed by determination of the intercept of this line with the time axis. The lag phase extension (Δ L, Table 6) was determined by subtracting the L value of the appropriate control from the L value obtained for each treatment concentration. The specific growth rate (K, Table 6) was equivalent to the slope of the regression line obtained for each growth curve.

TABLE 5

Growth of algae on agar plates as affected by Aerotex concentration. The mean diameter of the inhibition zone (mm) is indicated together with the standard deviation.

Species	Aerotex Concentration (%)				
	0.01	0.05	0.10	0.50	1.00
Scenedesmus	0	0	0	8.8 (1.0)	20.3 (2.3)
Chlamydomonas	0	0	8.0 (0.7)	11.7 (0.8)	20.7 (1.6)
Chlorella	0	0	10.7 (1.1)	24.3 (2.8)	TI

TI = Total inhibition (plate diameter = 100 mm)

Note : Aerotex prepared in aqueous emulsion.

( ) = Standard deviation

TABLE 6

Growth of algae in liquid culture as affected by Aerotex concentration. The average values of lag phase extension (ΔL) in days are indicated and were calculated from the data in Fig. 6 as described in the text. The ΔL values of duplicate samples are indicated separately when replicates failed to agree.

	Aerotex (ppm)							
Species	K	0	1	2	5	7.5	10	100
Scenedesmus	0.89 (0.45)	0	0.73	1.25	1.28	2.12	2.71	NG
Chlamydomonas	0.68 (0.28)	0	0.71	6.25	6.11/ 9.49	11.70/ NG	NG	NG
Chlorella	0.67 (0.30)	0	4.02	5.17/ 8.56	8.27	12.41/ 20.92	8.67/ 20.99	NG

NG = No growth for duration of study.

Note : K is the specific growth rate (div/day).

( ) = Standard deviation

FIG. 4A

Growth curves of Scenedesmus for different Aerotex concentrations (ppm). The standard (I) of duplicate samples is plotted for each Aerotex concentration (0 ppm,○;1 ppm,●;2 ppm,△;5 ppm,▲;7.5 ppm,□; 10 ppm,■;100 ppm,χ). The symbol (x) superimposed on the x (time) axis refers to the concentration at which no growth occurred for the duration of the study.

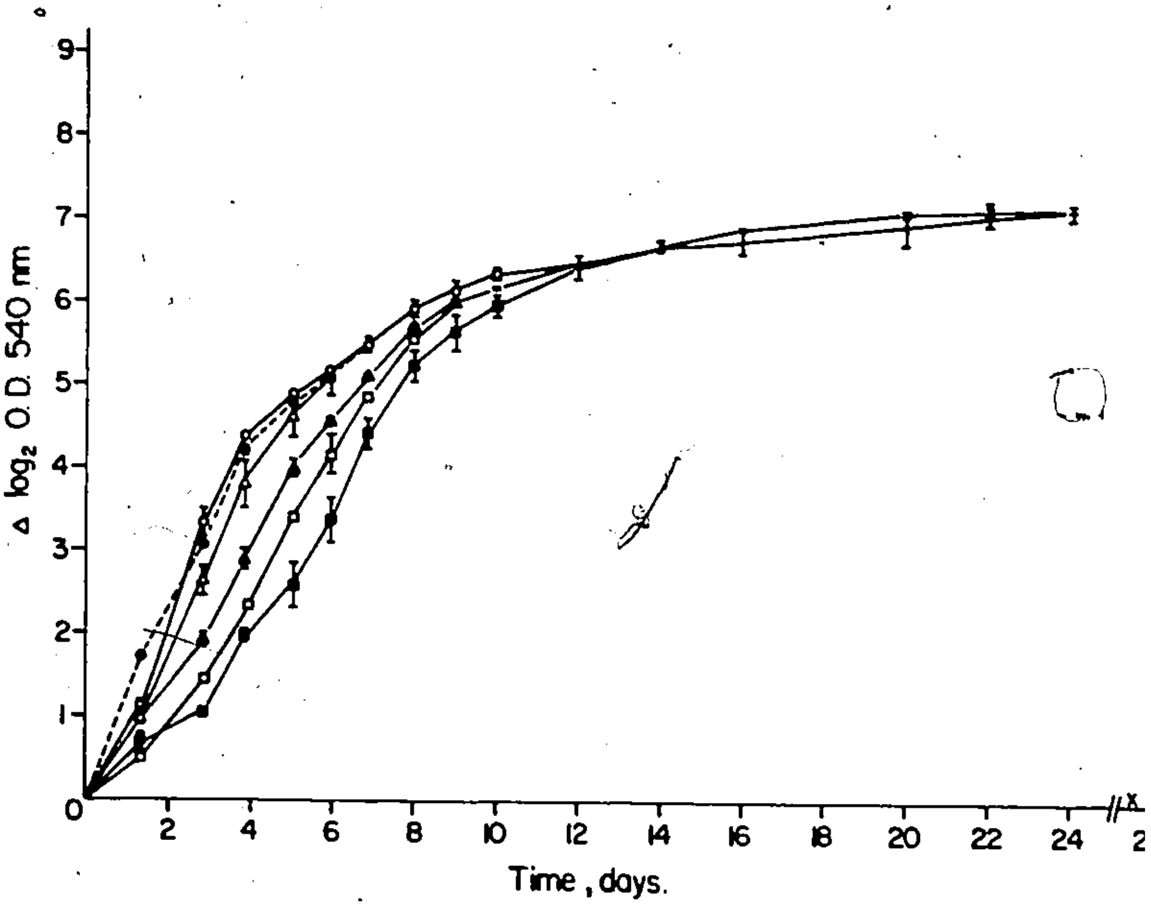


FIG. 4B

Growth curves of Chlamydomonas for different Aerotex concentrations (ppm). The standard range (I) of duplicate samples is plotted for each Aerotex concentration (0 ppm,○;1 ppm,●;2 ppm,△;5 ppm,▲;7.5 ppm,□;10 ppm,■;100 ppm,X). Replicates that did not agree are plotted separately . The symbols (□, ■, X) superimposed on the x (time) axis refer to concentrations at which no growth occurred for the duration of the study.

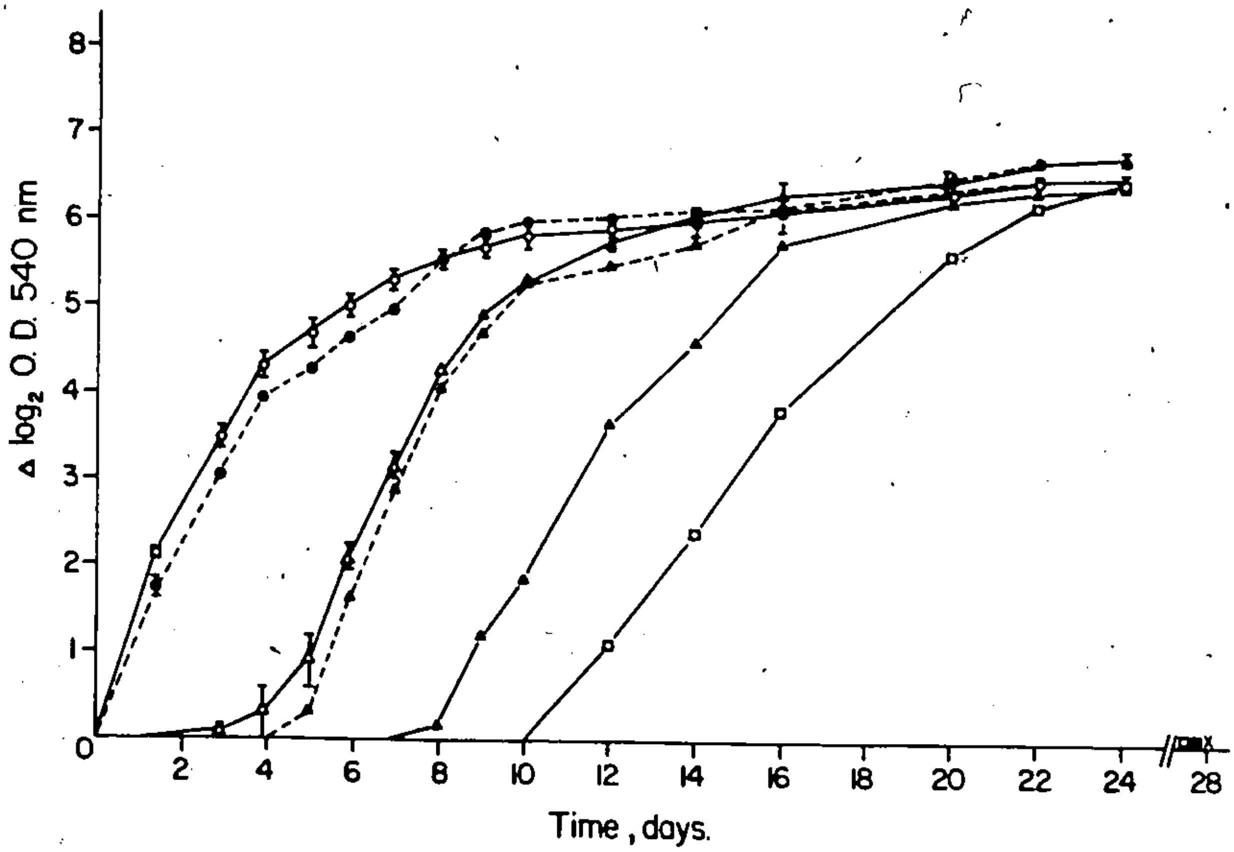
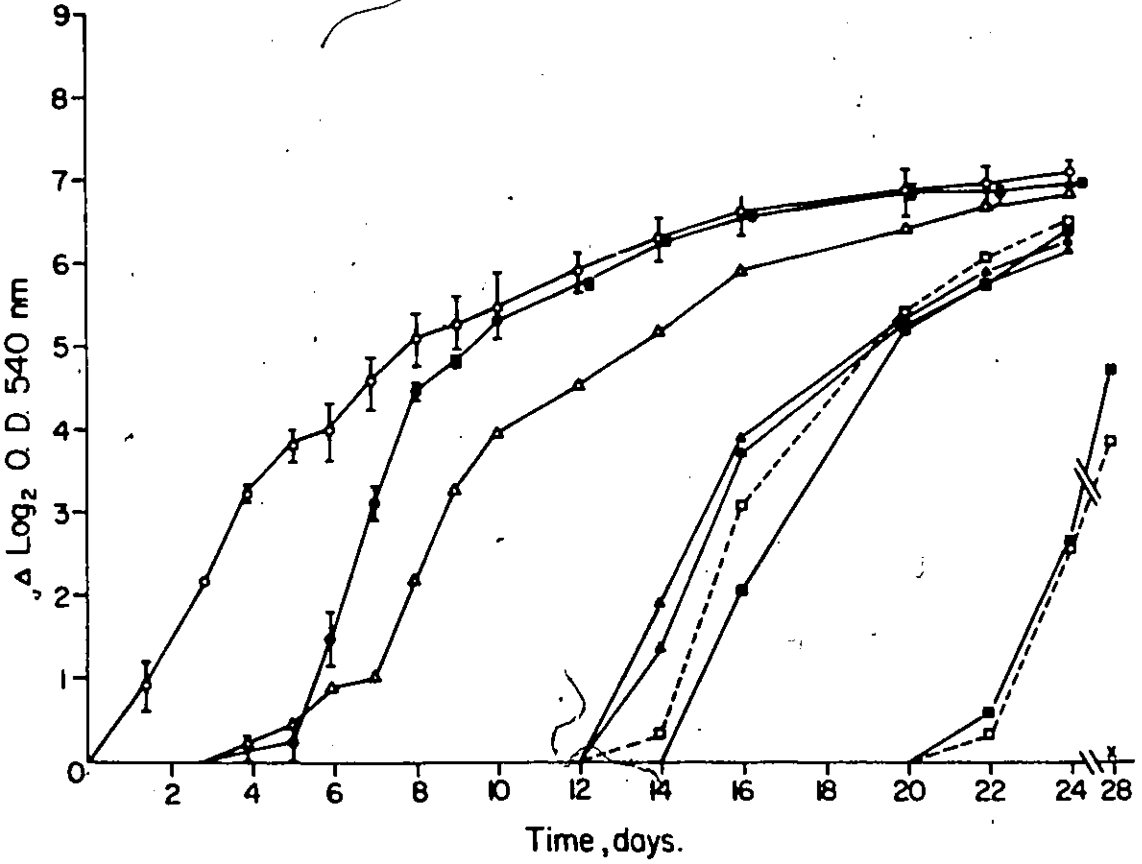


FIG. 4C

Growth curves for Chlorella for different Aerotex concentrations (ppm). The standard range (I) of duplicate samples is plotted for each Aerotex concentration (0 ppm,○;1 ppm,●;2 ppm,△;5 ppm,▲;7.5 ppm,□;10 ppm,■; 100 ppm,X). Replicates that did not agree are plotted separately. The symbol X superimposed on the x (time) axix refers to the concentration at which no growth occurred for the duration of the study.



The K values obtained for treated cultures and controls did not differ significantly ($P > 0.03$, student t-test 95% significance level) for any one species. There was no significant ($P > 0.01$) difference between the viable cell counts of control and Aerotex-treated (10 ppm, 1 hr) Chlamydomonas.

(e) ATP study:

A brief (1 hr) exposure of Aerotex to established cultures held in the light was sufficient to reduce the ATP levels in the sensitive species Chlamydomonas and Chlorella (Table 7). There was no significant difference ($P > 0.01$, student t-test, 95% significance level) between the levels of ATP determined for light and dark-held cultures of Chlamydomonas before treatment. Aerotex reduced ATP levels under both light and dark conditions (Table 8). Aerotex (50 ppm), alone did not alter the luminescence of ATP standards and algal extracts did not cause quenching.

D. Photosynthesis Assays

1. Carbon Dioxide Fixation Studies

Figure 5 demonstrates that there was a slight stimulation in CO_2 fixation by Chlamydomonas at low Aerotex concentrations (0.02-0.1 ppm). At higher concentrations ($\geq$ 2 ppm), CO_2 fixation was inhibited, with $\leq$ 10% of control fixation being observed between 5-10 ppm, Aerotex. Figure 6A demonstrates a rapid effect of Aerotex, with 71.5% inhibition of CO_2 fixation being recorded 5 min after treatment (5 ppm). After 1 hr, however, there was a gradual recovery of CO_2 fixation and 63% of the control fixation was observed after 24 hours. The data (Fig. 6A) was obtained from analysis of algae sampled successively from 250 ml flasks (see Materials and Methods, E.1.) which were opened at each sample time. There was no significant recovery ($\leq$ 10% of control) of CO_2 fixation for algae held for 24 hr in screw-cap tubes.

TABLE 7

Effect of Aerotex concentration on algal ATP levels. The values for % reduction of control ATP levels 1 hour after treatment are indicated together with standard deviation

Aerotex (ppm)	Scenedesmus	P	Chlamydomonas	P	Chlorella	P
1.0	3.0 (1.0)	⦣ .40	37.9 (10.5)	⦣ .50	19.5 (7.6)	∠ .10
2.0	3.3 (2.8)	⦣ .40	41.7 (3.0)	∠ .03	31.0 (8.7)	∠ .03
5.0	18.9 (3.0)	∠ .01	48.2 (0.9)	∠ .03	32.6 (6.4)	∠ .03
10.0	17.7 (8.0)	∠ .05	71.6 (0.4)	∠ .03		

Note: P determined by student t-test of data from treated cultures versus controls.

( ) = Standard deviation

TABLE 8

Effect of Aerotex on ATP levels in Chlamydomonas under light and dark conditions. The values for % reduction of control ATP levels 1 hour after treatment are indicated together with the standard deviation

Aerotex (ppm)	Light	Dark
1.0	6.4 (6.9)	16.9 (5.0)
2.0	7.7 (4.3)	10.6 (4.9)
5.0	21.8 (4.0)	17.2 (0.5)
10.0	94.5 (1.0)	93.2 (0.9)

( ) = Standard deviation

FIGURE 5

Inhibition of control $^{14}CO_2$ fixation in Chlamydomonas versus concentration (ppm) of Aerotex. Treatment duration was 1 hour. Mean values of triplicate samples are shown together with standard deviation bars.

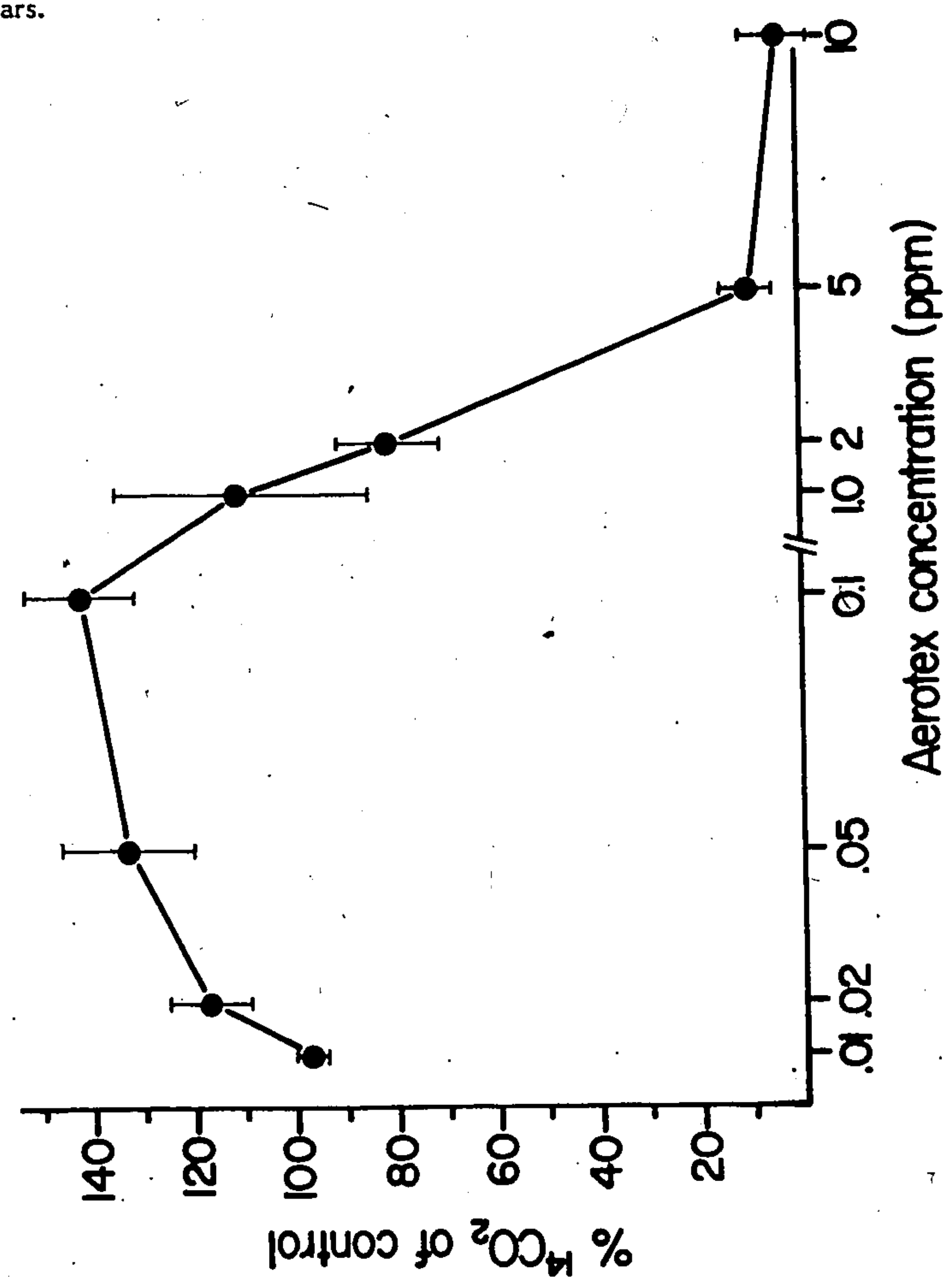


FIGURE 6A

Percent inhibition of control $^{14}CO_2$ fixation in Chlamydomonas versus duration of treatment with Aerotex (5 ppm). Mean values of triplicate samples are shown with standard deviation bars.

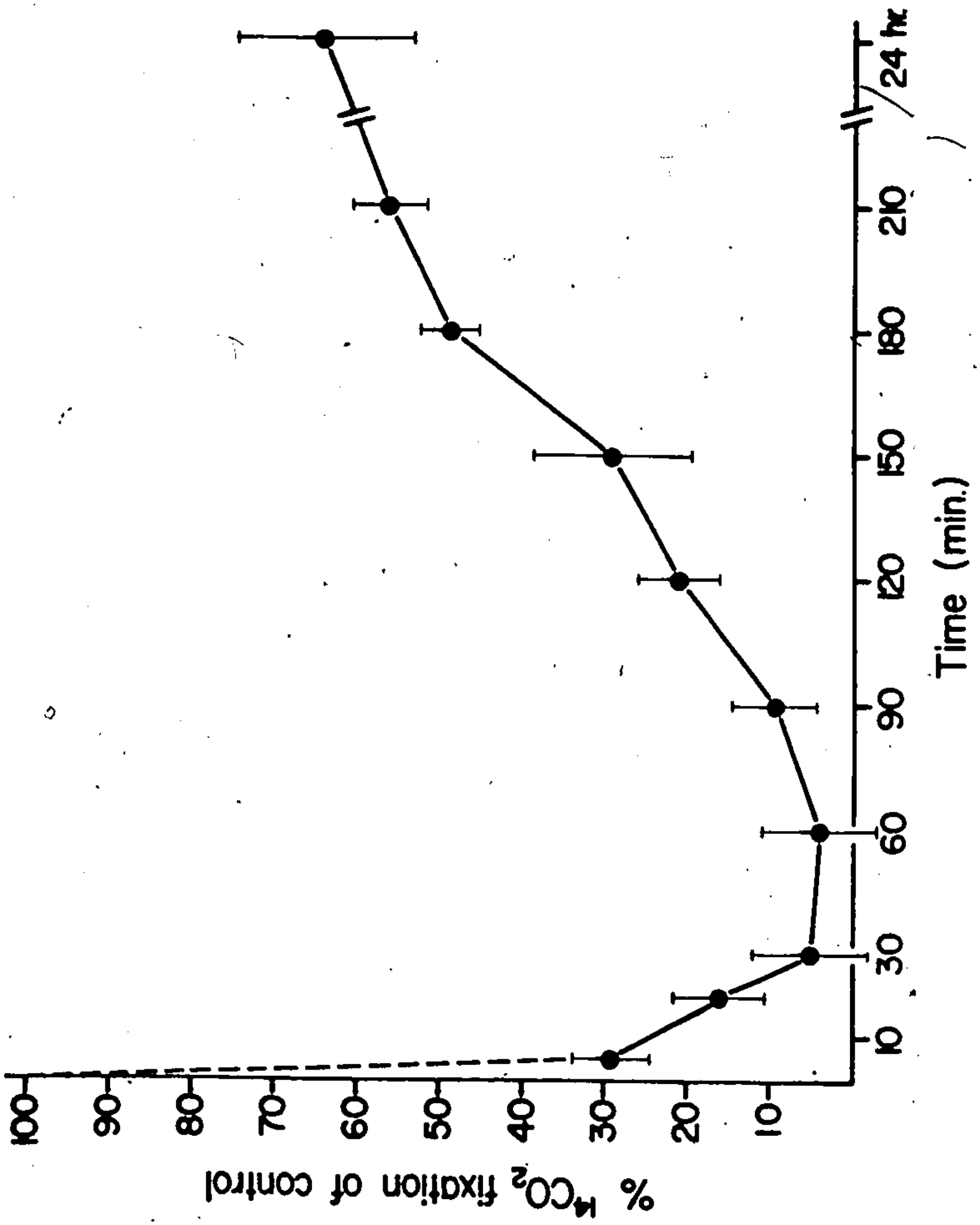


FIGURE 6B

Percent leakage of ^{14}C-photosynthate from control and Aerotex-treated (10 ppm) Chlamydomonas. Mean values of four replicate samples are shown together with standard deviation bars.

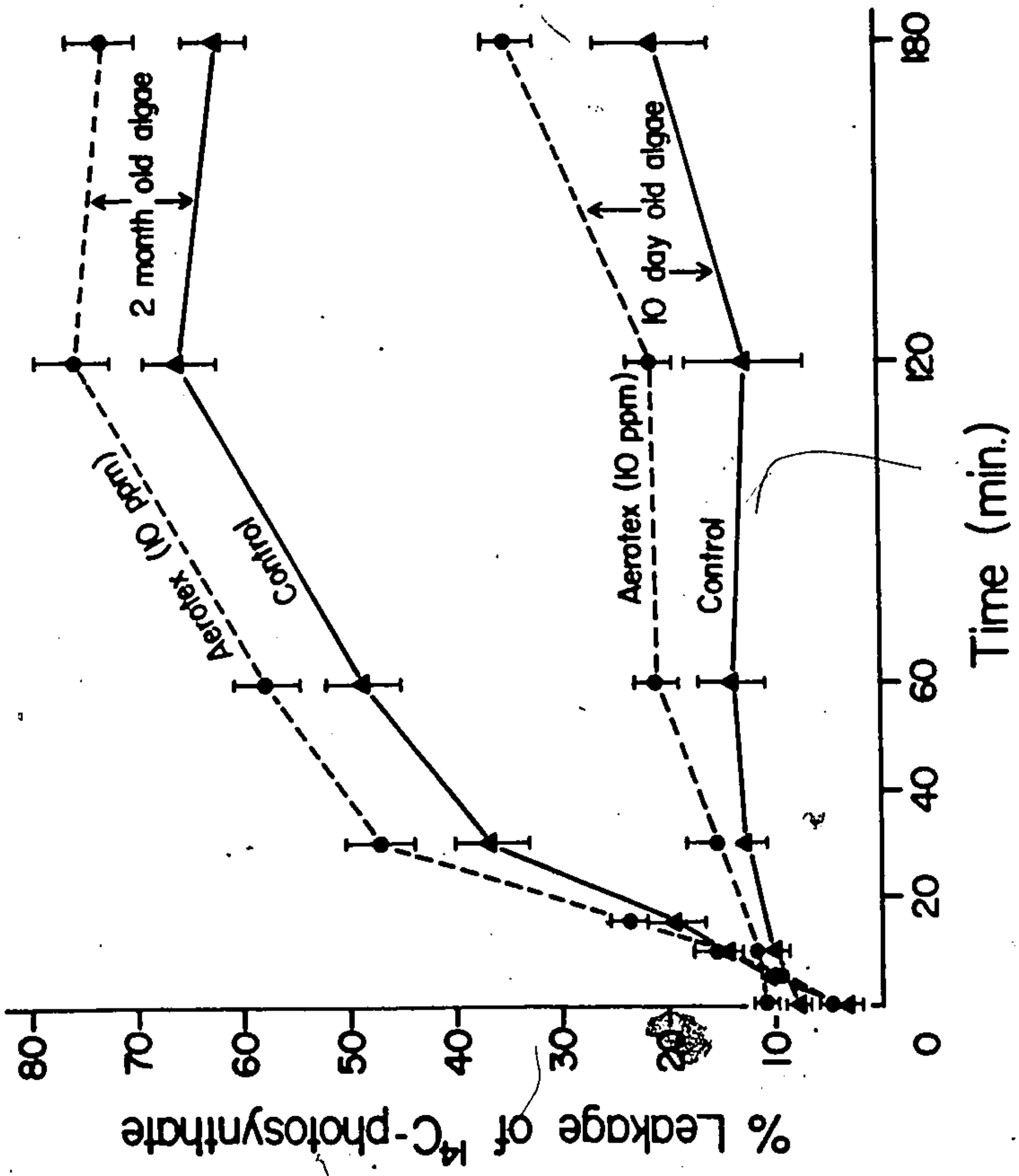


FIGURE 7A

Rate of reduction of DCIP by control and Aerotex-treated (10 ppm) Chlamydomonas exposed to light (5 Klx). Mean values of triplicate samples are shown together with standard deviation bars.

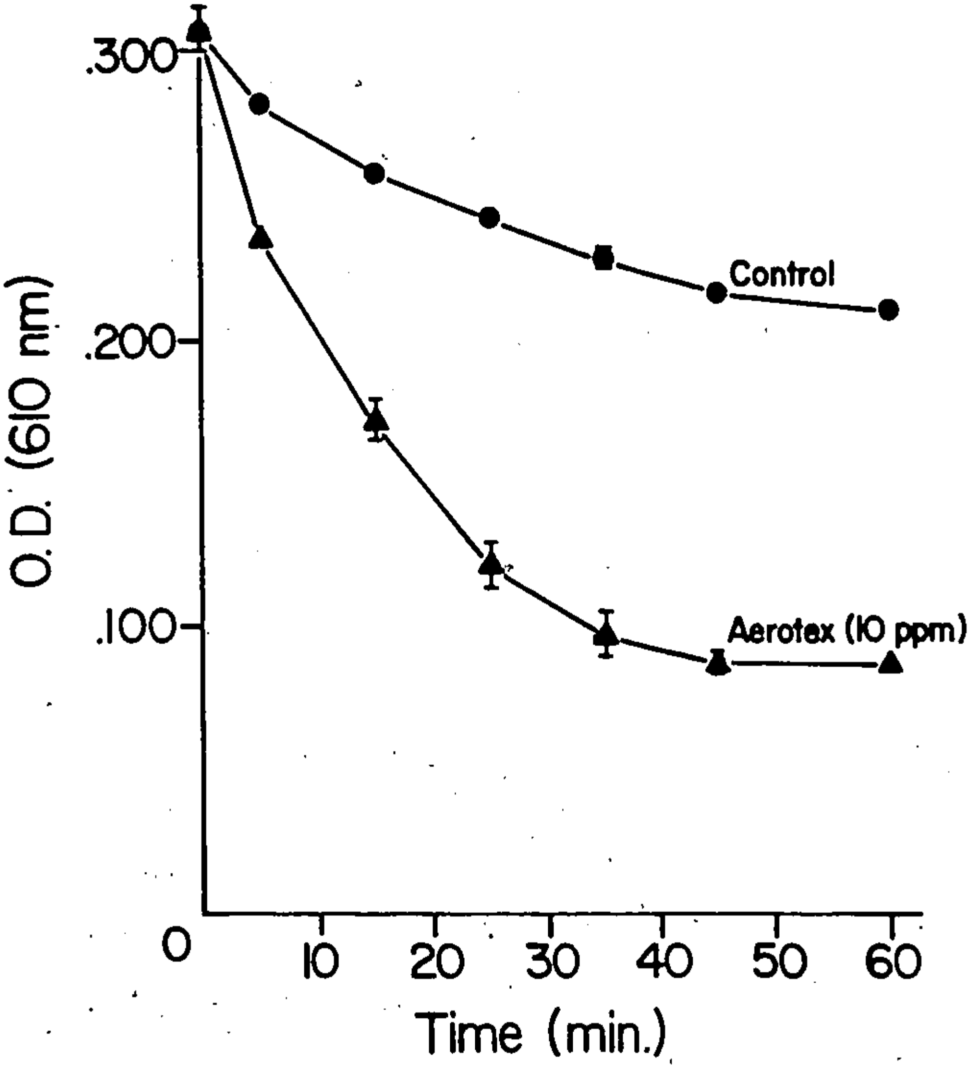
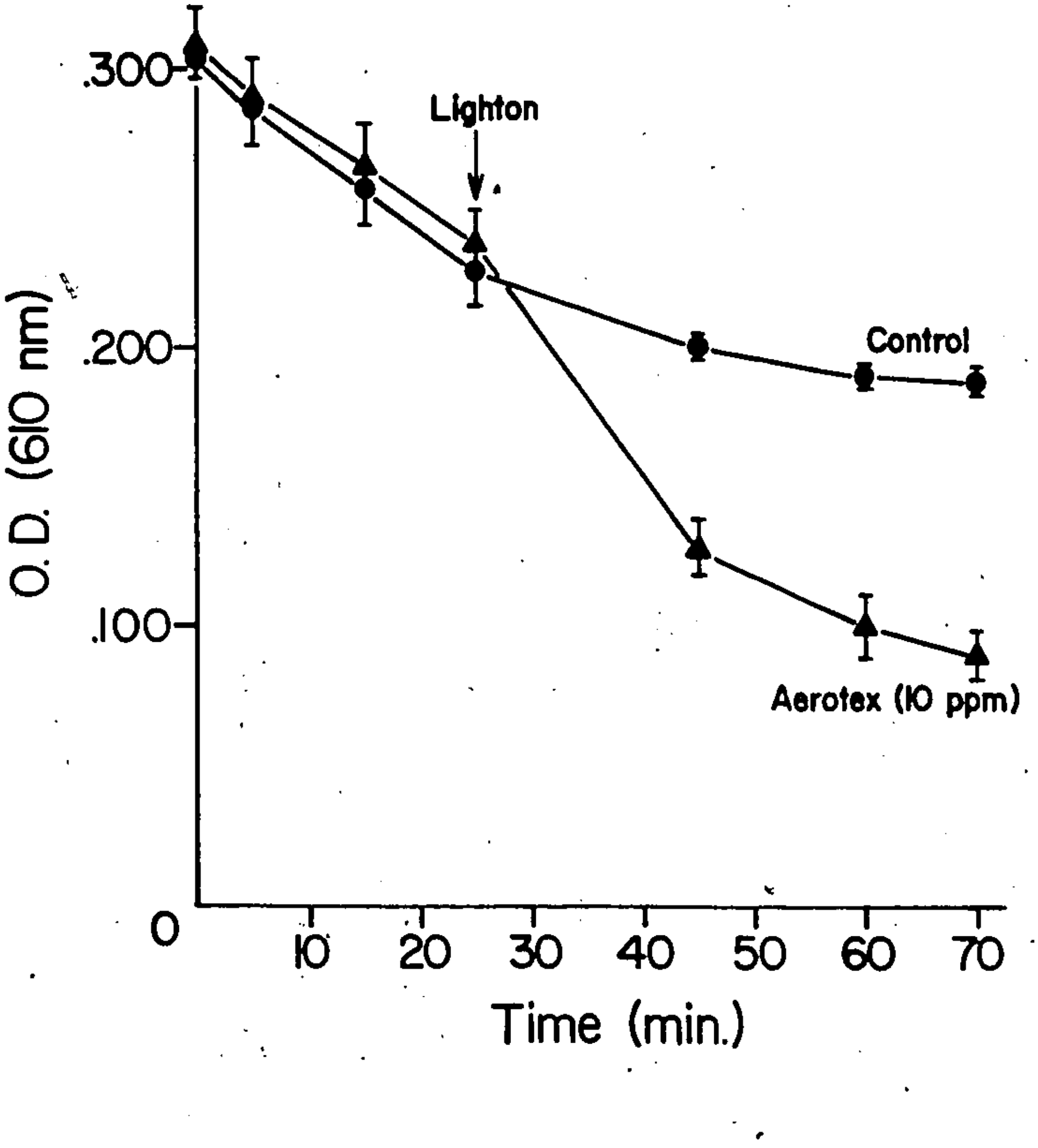


FIGURE 7B

Rate of reduction of DCIP by control and Aerotex-treated (10 ppm) Chlamydomonas held in dark for 25 minutes and then exposed to light (5 Klx). Mean values of triplicate samples are shown together with standard deviation bars.



The CO_2 fixation leakage study (Fig. 6B) demonstrated enhanced leakage of ^{14}C-material in Chlamydomonas treated with Aerotex (10 ppm) than control. ^{14}C-leakage was greater in the 2 month old culture for both the control and the treated algae, than in the 10 day old culture. Treatment of the dialysate with excess 1N HCl did not significantly reduce the unacidified sample counts while acidification of the ^{14}C-bicarbonate standards yielded $\geq$98% loss of activity.

2. Photosynthetic Reduction of DCIP

Aerotex (10 ppm) greatly increased the rate of reduction of DCIP by Chlamydomonas in the light (Fig. 7A) but not in the dark (Fig. 7B). DCIP was not reduced under light or dark conditions with algae treated with 100 ppm Aerotex. Further, DCIP was not reduced in the light when present with Aerotex (10 ppm) alone (i.e. without algae), or with heat-killed (80°C, 10 min) or freeze-killed (-20°C, 1 hr) algae. The addition of NH_4Cl (10mM) had no effect on the rate of DCIP reduction by Chlamydomonas either before or after treatment with Aerotex (10 ppm).

3. Fluorometric Analysis of Photosynthesis

Fig (i) shows a typical fluorescence transient obtained for untreated (control) cultures of Chlamydomonas showing the peak (P) characteristic of photosynthetically active cells which was absent for treated cultures. O, P, M and S divide the fluorescence transient into segments that are indicative of several photosynthetic partial reactions (Papageorgiou, 1975; Schreiber et al., 1978). The letter "T" on Fig (i) designates the termination of peak "P". The length of the P-T transient was used to quantitate photosynthetic activity.

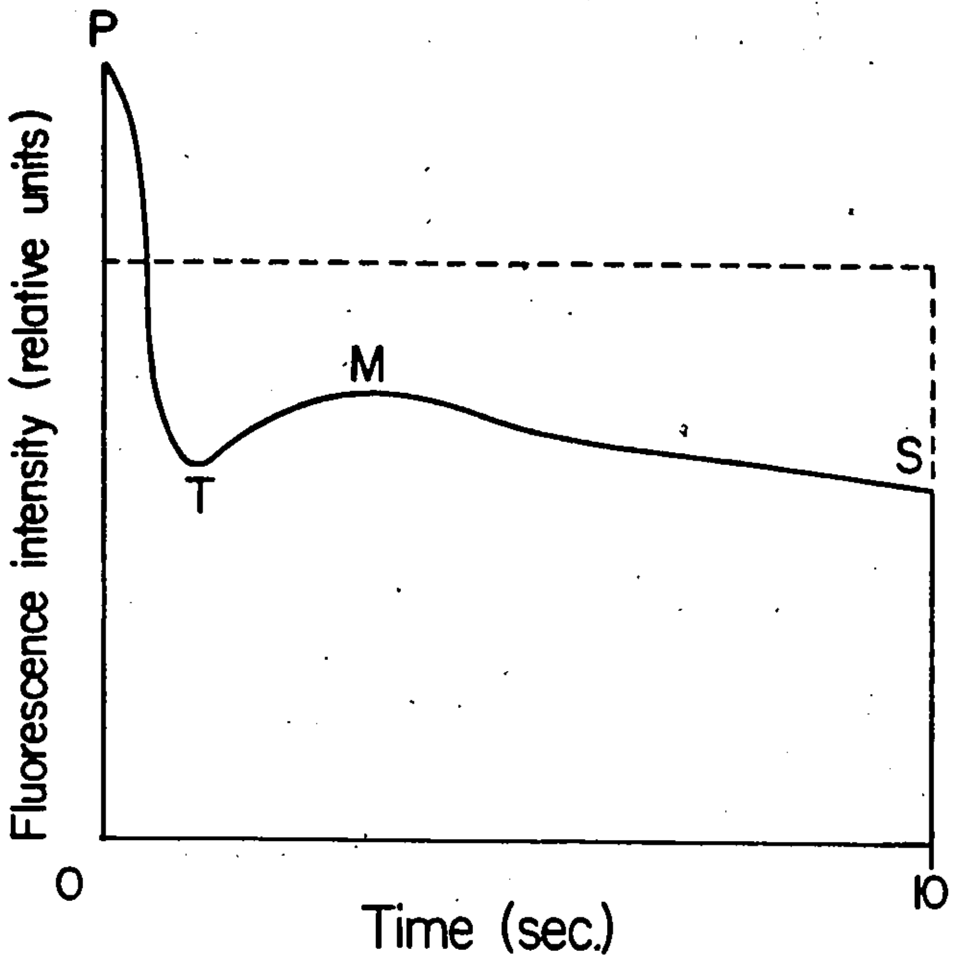


FIGURE (i)

The fluorescence transient in Chlamydomonas reinhardii: 670 nm; incident intensity 10^4 ergs/cm^2/sec. The solid line depicts a typical transient for untreated cells showing the points O, P, T, M, and S. The hatched line depicts a typical transient for cultures inhibited by chemical treatment.

A progressive decrease in the length of the P-T transient was observed as the treatment concentration of a test compound neared the level at which the fluorescence transient became flat as in Fig. (i). A flat fluorescence transient was also obtained for heat-treated (80°C), frozen (-60°C) or dessicated algae in the present study, and is generally held to be indicative of the absence of photosynthetic activity (Schreiber et al., 1978). The results obtained were quantitated by measuring the length of the P-T transient and expressing this as a percentage reduction (% inhibition, Table 9) of the control value. An analysis of variance was performed initially on the 6 replicate P-T values (3 replicate flasks x 2 sample analyses) obtained for each treatment concentration. In all cases, $F_{2,3}$ was less than 9.55 (p less than 0.95), permitting reporting data as a pooled mean ± standard deviation (Table 9).

The data obtained for the constituents of the fenitrothion and matacil formulations are presented in Table 9. The source and chemical description of the test compounds are given in Table 1. In some cases, when a pesticide formulation was used, the toxicity results were reported for the concentration present for one of the formulation constituents. For example, the toxicity data recorded from the fluorometer studies for matacil 1.8 D (matacil : Nonyl Phenol : Diluent-585 (1.00:2.54:1.52 (v/v)) was reported together with the concentration of Nonyl Phenol present after treatment (Table 9, footnote 3).

The second column (TR) in Table 9 indicates the treatment procedure used. The test compound was applied either in acetone which was then volatilized, or solubilized directly in BBM (as discussed in "Material and Methods", Section E.3). It is imperative to note the importance of adopting a correct treatment procedure. For example, Aerotex at 10 ppm did not inhibit the fluorometer response for Chlamydomonas if Aerotex was disolved in acetone and then

TABLE 9

Inhibition (%) of photosynthesis as determined by fluorometric analysis of C. reinhardii treated for 1 hour.

Chemical	TR[1]	Treatment Concentration (ppm)											
		0	0.1	0.2	0.5	0.75	1.0	5.0	10	20	30	40	100
fenitro[illegible]	D	0 (13.6)					-11.4[4] (31.1)	40.9 (19.7)	68.2 (7.6)	100 (0)			
Dowanol	W	O (5.1)					-3.2 (10.2)	-1.9 (7.0)	6.4 (12.7)				2.5 (21.7)
Atlox	W	0 (13.6)					-11.7 (17.4)	-1.9 (13.2)	5.2 (11.7)	60.1 (19.3)	100 (0)	100 (0)	
Aerotex	W	0 (6.1)					52.8 (9.8)	100 (0)	100 (0)	100 (0)			
fenitrothion:Aerotex:Atlox[2] (10:1:1)	W	0 (4.8)			-16.8 (11.1)		-9.6 (10.6)	25.5 (13.9)	41.3 (9.1)	100 (0)			
matacil	D	0 (14.9)					6.9 (4.6)	10.3 (9.1)	10.3 (5.7)	15.4 (4.6)			51.4 (8.0)
Diluant-585	W	0 (14.7)					-8.0 (10.7)	8.0 (8.0)	3.3 (4.0)	71.3 (5.3)	100 (0)	100 (0)	100 (0)
Nonyl Phenol	D	0 (7.8)	-17.2 (14.1)	0 (11.7)	54.7 (16.4)	100 (0)	100 (0)						
matacil 1.8-D[3]	D	0 (16.8)	-3.2 (16.1)		80.6 (12.3)	100 (0)	100 (0)						

[1] D = Dry treatment (in acetone blown off); W = Wet treatment (in BBM)

[2] Results are reported for the fenitrothion concentration (ppm) of the formulation

[3] Results are reported for the Nonyl Phenol concentration (ppm) of formulation

[4] A negative value was obtained when the P-T transient was greater than the control

exposed to an air stream for 5 min prior to treatment. If appplied in aqueous emulsion, however, Aerotex at 5 ppm was sufficient to totally supress the fluorometer (P-T transient) response (Table 9). In this connection, GLC analysis of Aerotex standards prepared in acetone demonstrated that ca. 50% of each of the hydrocarbon constituents of Aerotex had volatilized after 10 min exposure to an air stream. Similar GLC analyses of fenitrothion and matacil standards did not demonstrate significant volatilization after 10 min exposure to an air stream[1]. Nonyl Phenol was applied by the acetone procedure since a stable emulsion could not be prepared with the Polytron sonicator (the suspension started separating immediately after preparation). Contrary to the results obtained with Aerotex, the fluorometrically-determined toxicity of Nonyl Phenol was not reduced by 10 min exposure to an air stream.

Generally, the results (Table 9) demonstrated that a slight increase in the length of the P-T transient was obtained at low concentrations of the test compounds. This is consistent with the enhanced CO_2 fixation reported previously for low Aerotex concentrations. Higher treatment concentrations were associated with a decrease in the length of the P-T transient which is reported (Table 9) as an increase in the % inhibition of photosynthesis. The length of the P-T transients before and after washing with BBM did not differ significantly, except for matacil at 100 ppm, which before washing gave 51.4 ± 8.0% inhibition compared to 21.7 ± 6.6% after washing.

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[1] A maximum of 15-20 sec exposure to the air stream was sufficient to volatilize the acetone in the toxicity studies.

The lowest concentration of a test compound required to totally suppress the fluorometer response (P-T = 0) will be designated here as the ICF_{100} value. This value is reported as a concentration range for each test compound in Table 10. For example, 1 ppm of Aerotex was shown to cause 52.8% inhibition of photosynthesis while 100% inhibition was recorded for ≥ 5 ppm (Table 9). Hence, the ICF_{100} value for Aerotex is reported as 1-5 ppm (Table 10).

The following points should be considered when interpreting the results given in Table 10. The ICF_{100} values obtained for technical grade and purified samples of fenitrothion (see "Materials and Methods", section A.1.) were both between 10-20 ppm. Further, this value remained unchanged for fenitrothion formulated with either Aerotex and Atlox, or Dowanol and Atlox. Similarily, the ICF_{100} value for Nonyl Phenol was from 0.50-0.75 ppm either alone, or when present in the matacil 1.8-D formulation. The ICF_{100} values reported in Table 10 were obtained for a 1 hr treatment duration. Figure 8 plots the % inhibition of photosynthesis (decrease of P-T transient) versus duration of treatment with 5 ppm (upper limit of ICF_{100}, Table 10) of Aerotex. The time required to cause 50% inhibition was less than 8 minutes.

The ICF_{100} values determined for several hydrocarbon constituents of Aerotex as well as for Aerotex and ~~two~~ crude oils are listed in Table 11. The herbicide 2,4-D (2,4-dichloro-phenoxyacetic acid), was included for comparison. The ICF_{100} values were expressed as uM/l and the logarithm of each value is reported together with the respective log K_{OW} value (Hutchinson et al., 1980). When log K_{OW} values could not be obtained from the literature, they were calculated by the method of Leo et al. (1971). For example, the log K_{OW} value

TABLE 10

ICF$_{100}$ values - concentration (mg/l) required to totally inhibit photosynthesis in Chlamydomonas reinhardii within 1 hr of treatment determined by fluorometric analysis

Chemical	ICF$_{100}$ (ppm)
fenitrothion (pure)	10-20
fenitrothion (technical)	10-20
Aerotex 3470	1-5
Atlox 3409F	20-30
Dowanol	over 100
fenitrothion:Aerotex:Atlox (10:1:1)	10-20 (fenitrothion)
fenitrothion:Dowanol:Atlox (10:1:1)	10-20 (fenitrothion)
matacil	over 100
matacil 1.8-D	0.5-0.75 (Nonyl Phenol)[1]
Nonyl Phenol	0.5-0.75
Diluent-585	20-30

[1] The respective concentration of matacil was 0.16-0.24 ppm.

FIGURE 8

Percent inhibition of fluorometer (P-T) transient versus duration of treatment with Aerotex (5 ppm). Mean values of triplicate samples are shown together with standard deviation bars.

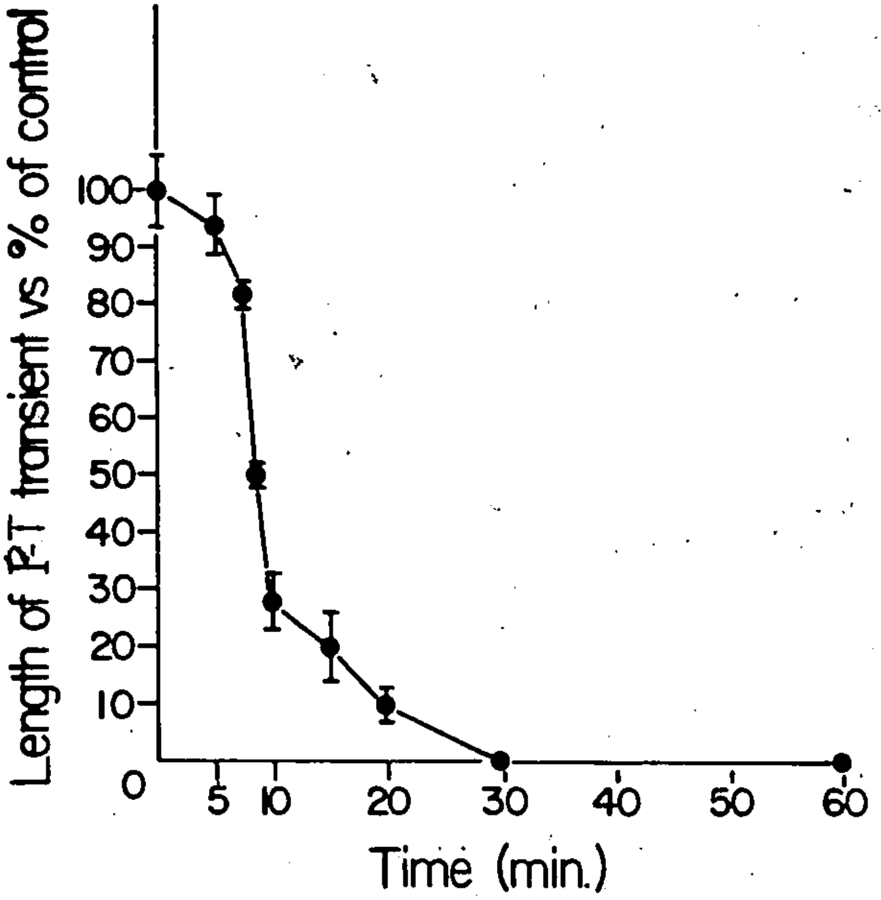

TABLE 11

ICF_{100} values (concentration required to totally inhibit photosynthesis in <u>C. reinhardii</u> within 1 hr of treatment) for benzene and naphthalene constituents of Aerotex. Values for several pesticides and petroleum derivatives are included for comparison. The log K_{ow} for each compound is also included.

COMPOUND	MW (gm)	ICF_{100} (mg/l)	ICF_{100} (uM/l)	Log ICF_{100} (log uM/l)	Log $K_{ow}{}^{3}$	Log S^{4} (log uM/l)
benzene	78.1	500-750	6402-9603	3.81-3.98	2.13 (a)	4.36 (a)
toluene	92.1	150-250	1629-2714	3.21-3.43	2.69 (a)	3.75 (a)
p-xylene	106.1	75-100	707-943	2.85-2.97	3.08 (a)	3.24 (a)
1,2,3-trimethyl-benzene	120.1	30-75	250-625	2.40-2.80	3.66 (c)	
1,2,4,5-tetramethyl-benzene	134.1	>100	> 746	> 2.87[5]	4.11 (c)	1.85 (a)
naphthalene	128.2	25-30	195-234	2.29-2.37	3.55 (a)	2.39 (a)
1-methyl-naphthalene	142.2	5-10	35.2-70.3	1.55-1.85	3.86 (a)	2.30 (a)
2-methyl-naphthalene	142.2	5-10	35.2-70.3	1.55-1.85	3.86 (a)	2.25 (a)
2,6-dimethyl-naphthalene	156.2	>5	>32.0	>1.51[5]	4.24 (c)	1.11 (d)
2,3,5-trimethyl-naphthalene	170.2	5-10	29.4-58.8	1.47-1.77	4.76 (c)	
fenitrothion	277	10-20	36.1-72.2	1.56-1.86	3.38 (b)	1.86 (c)
Nonyl Phenol	215	0.5-0.75	2.33-3.49	0.37-0.54	5.47 (c)	-0.70 (b)
2,4-dichloro-phenoxyacetic acid	221	300-500	1358-2262	3.13-3.35	2.81 (b)	3.60 (b)
Aerotex 3470	128.2[2]	1-3	7.8-23.4	0.89-1.37		
Crude oil 1[1]	128.2[2]	50-100	390-780	2.59-2.89		
Crude oil 2[1]	128.2[2]	50-100	390-780	2.59-2.89		

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[1] Crude oil 1 and 2 were Venezualan crude and Norman Wells Crude oils, respectively.

[2] The mw of naphthalene was used for calculations involving the petroleum products.

[3] The octanol-water partition coefficient (log K_{ow}) values were either obtained from the literature (a = Hutchinson <u>et al.</u>, 1980; b = Chiou <u>et al.</u>, 1977) or were calculated (c) by the method of Leo <u>et al.</u>, 1971 (see text).

[4] The log water solubility (Log S) values were obtained from the literature (a = Hutchinson <u>et al.</u>, 1980; b = Chiou <u>et al.</u>, 1977; c = N.R.C.C. No. 14104, 1975; d = Mackay and Schiu, 1977). The log S for Nonyl Phenol was calculated from the equation of Chiou <u>et al.</u>, 1977 (see text).

[5] Higher concentrations were not tested since they would be above the water solubility (S) of the compounds indicated by the log S value.

for p-Nonyl Phenol was calculated as follows:

$$
\begin{aligned}
\log K_{ow} \text{ (p-Nonyl Phenol)} &= \pi(\text{benzene}) + 9\,\pi(CH_2) + \pi(OH) \\
&= 2.13 + 9\,(0.5) + (-1.16) \\
&= 5.47 \text{ (Table 11)}
\end{aligned}
$$

A definition of the π substituent constants by means of Hammett sigma constants and a list of π constants for several substituent groups may be found in Leo et al. (1971).

The water solubilities (S, uM/l) of the test compounds are also reported in Table 11 and were obtained from the literature as indicated. The water solubility of Nonyl Phenol was calculated from the equation given by Chiou et al. (1977) as follows:

$$\log S = \frac{5.00 - \log Kow}{0.67}$$

$$\log S \text{ (p-Nonyl Phenol)} = \frac{5.00 - 5.47}{0.67} = -0.70 \text{ (Table 11)}$$

The following points should be considered when evaluating the results in Table 11. The treatment method differed from that used previously, in that all test compounds were applied in acetone, directly to the algal culture. This method was necessary for the solid volatile hydrocarbons, and was used throughout the series to ensure consistency. The acetone concentration (50 ul/50 ml algae) was kept constant for all tests and acetone alone did not affect the fluorescence transient. Further, the effect of acetone was considered to be negligible since the ICF_{100} values obtained previously (Table 10) without acetone, for many of the same compounds, are in agreement with those listed in Table 11[1]. When considering the reversibility of the treatment effect, it is

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[1] The ICF_{100} for Aerotex in Table 10 (aqueous Aerotex emulsion) was 1-5 ppm while 1-3 ppm is reported in Table 11 (Aerotex in acetone). This is because further concentrations (e.g. 3 ppm) were tested in the latter case.

important to note that there was no significant difference between the results obtained before and after washing with BBM. Further, a flat fluorescence transient was still obtained 24 hr after treatment at concentrations greater or equal to the ICF_{100} for all test compounds.

Figure 9 demonstrates an inverse correlation between the log K_{ow} values and log ICF_{100} values reported in Table 11. Linear regression of the data gave the following equation (correlation coefficient r = -0.92) which was used to plot the hatched line in Figure 9:

$$\text{Log } ICF_{100} = 5.78 - 0.98 \text{ Log } K_{ow}$$

The ICF_{100} values for Aerotex and the crude oils are included to the left of the plot for comparison. Note that the log K_{ow} values of these compounds are not known since these compounds are complex mixtures of hydrocarbons. On the average, Aerotex was 50 times more toxic than the two crude oils tested.

Figure 10 demonstrates a positive correlation between log S values and log ICF_{100} values reported in Table 11. Linear regression of the data gave the following equation (r = 0.94) and was used to plot the hatched line in Figure 10:

$$\text{Log } ICF_{100} = 0.56 + 0.68 \text{ Log } S$$

To test the combined (synergistic) effect of Aerotex constituents on Chlamydomonas, a toluene-based "synthetic" Aerotex was prepared with a naphthalene composition equivalent to that determined by the GLC analysis of Aerotex (see "Results" C.1.a.). The synthetic Aerotex contained 9.8% naphthalene, 12.9% 1-methyl-naphthalene, 8.5% 2-methyl-naphthalene, 4.2% 2,6-dimethyl-naphthalene and 64.6% toluene (w/v). The preparation was applied in acetone as described previously. Fluorometer measurements demonstrated that

FIGURE 9

Plot of concentration (log ICF_{100} (uM/l)) of test chemical required to supress the fluorometer (P-T) transient for chlamydomonas versus octanol-water partition coefficient (log Kow). Log ICF_{100} and log Kow values are from Table 11 the hatched line corresponds to the equation in the text.

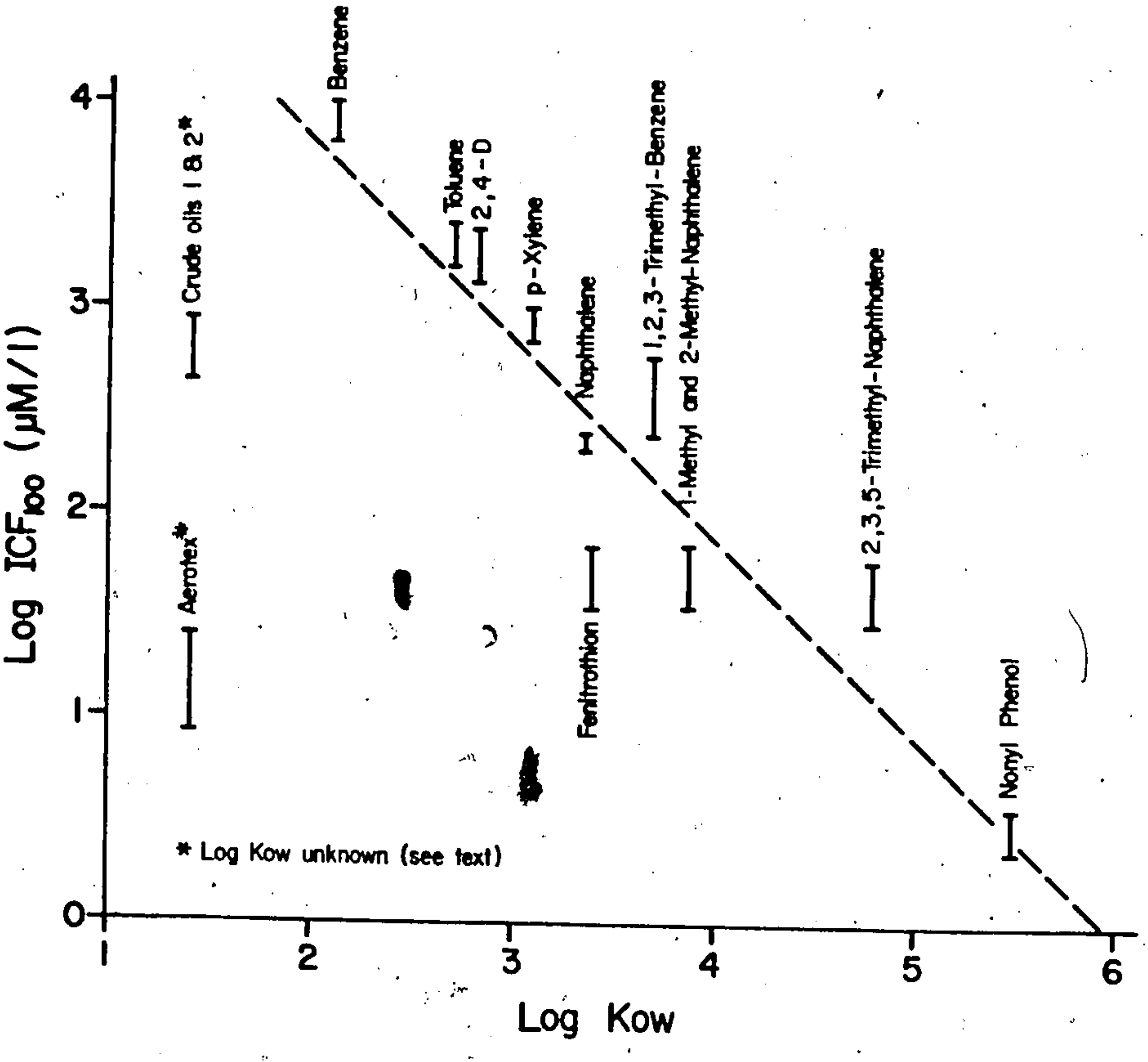


FIGURE 10

Plot of concentration (log ICF_{100} (uM/l)) of test chemical required to supress fluorometer (P-T) transient for Chlamydomonas versus water solubility (log S (uM/l)). Log ICF_{100} and log S values are from Table 11. The hatched line corresponds to the equation in the text.

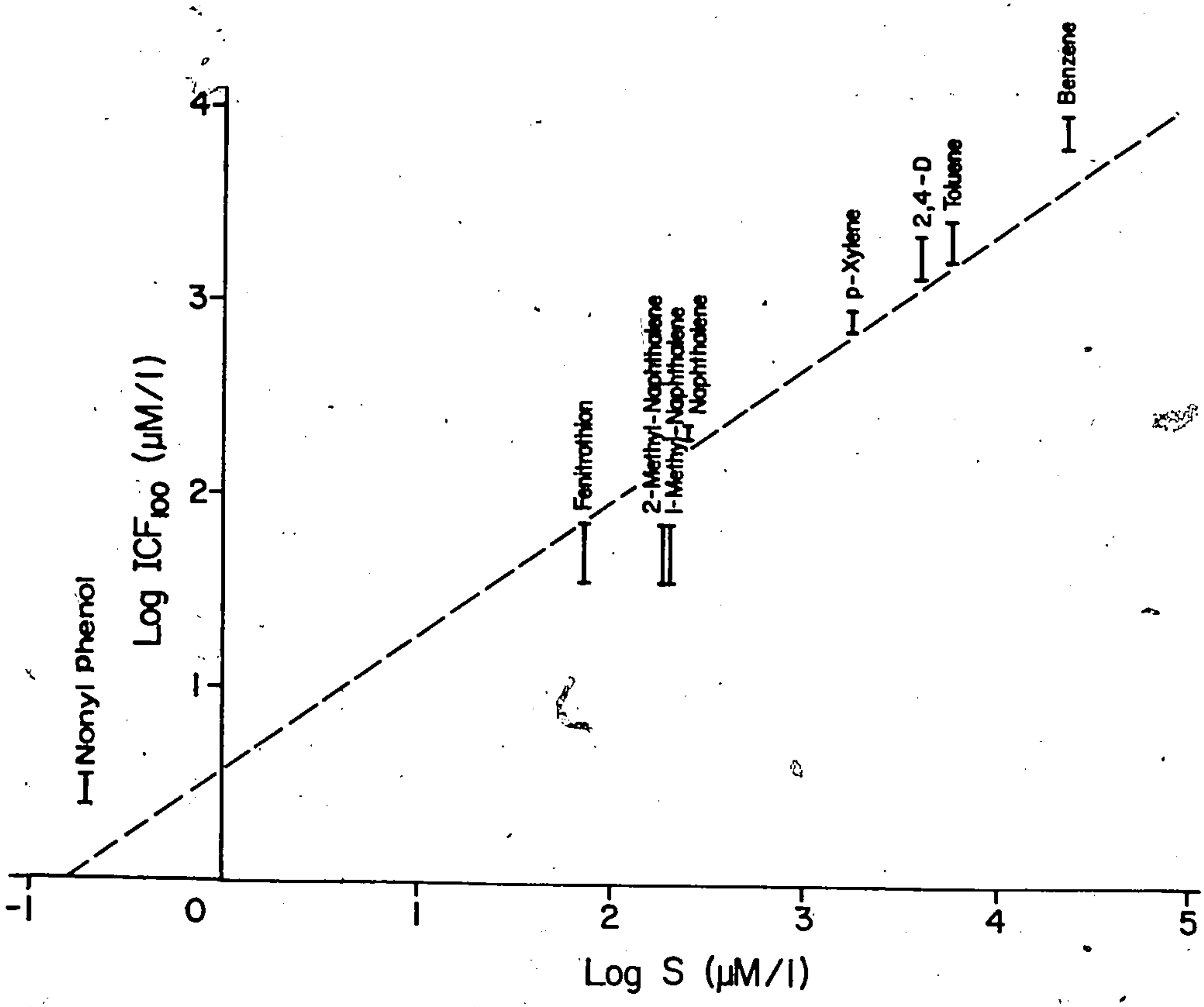
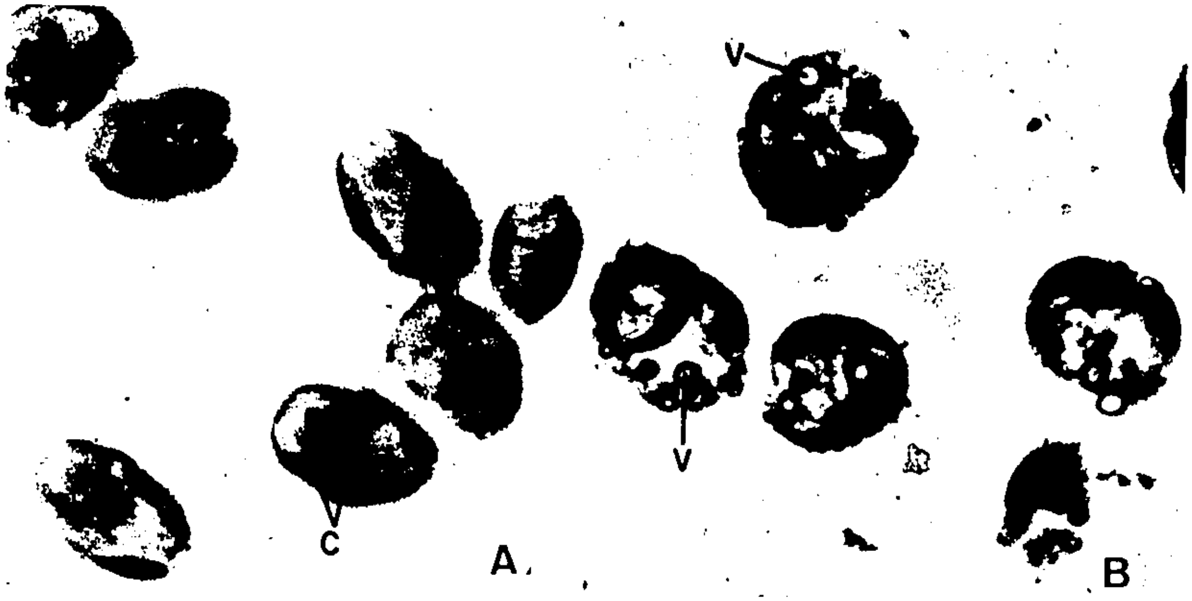


FIGURE 11A & B

Light micrographs of Chlamydomonas reinhardii X3, 750. A. cup-shaped chloroplast (c), pyrenoid (p) and nucleus (n) in untreated cells. B. Birefringent vacuoles (v) in phase contrast 1 hr after treatment with Aerotex (5 ppm)



the synthetic Aerotex was toxic between 10 ppm and 30 ppm. From the above composition, it can be calculated that the synthetic Aerotex contained 2.94 ppm naphthalene, 3.87 ppm 1-methyl-naphthalene, 2.55 ppm 2-methyl-naphthalene, 1.26 ppm 2,6-dimethyl-naphthalene, and 19.3 ppm toluene. These ppm values are less than the lowest toxic concentrations given in Table 11 for the respective hydrocarbons when tested individually.

E. Transmission Electron Microscopy (TEM)

Gross ultrastructural changes were observed in Chlamydomonas treated for 1 hr with Aerotex (5 ppm) (Figure 12C, D). Greatly swollen mitochondria were characteristic of Aerotex-treated cells as were negatively staining chloroplasts with wavy, diffuse thylakoids (Moody et al., 1981). In many cells (Fig. 12F), the number of thylakoids was greatly reduced and the remainder were stacked into grana-like structures.

In most treated cells (>99%), the inner mitochondrial membrane that normally constitutes the cristae (Fig. 12B) had unfolded and the dense matrix was not apparent (Figure 12D). However, Fig. 12E shows one mitochondrion with a clearly resolved double membrane, still containing some of the inner matrix.

The cell membrane was convoluted in treated cells and a fibrillar substance was observed adhering to the cells that appeared to be dissociated from the cell walls (Fig. 12D). The flagella were still attached to the control cells but deflagellation was evident in treated specimens. A large proportion of the cells treated with 1-methyl-naphthalene appeared similar to those treated with Aerotex; However, many cells were less affected with the only obvious difference from controls being a slightly plasmolyzed appearance (Fig. 12G).

FIGURE 12A & B

Electron micrographs of longitudinal sections of *Chlamydomonas reinhardii*. A. Control cell x 15,000. B. Control cell showing tubular cristae in mitochondria x 45,000. Figure legend: cell wall (cw), cell membrane (cm), chloroplast envelope (ce), thylakoids (t), mitochondrion (m), cristae (cr), pyrenoid (p), starch plate (sp), nucleus (n), site of attachment of flagella (sf).

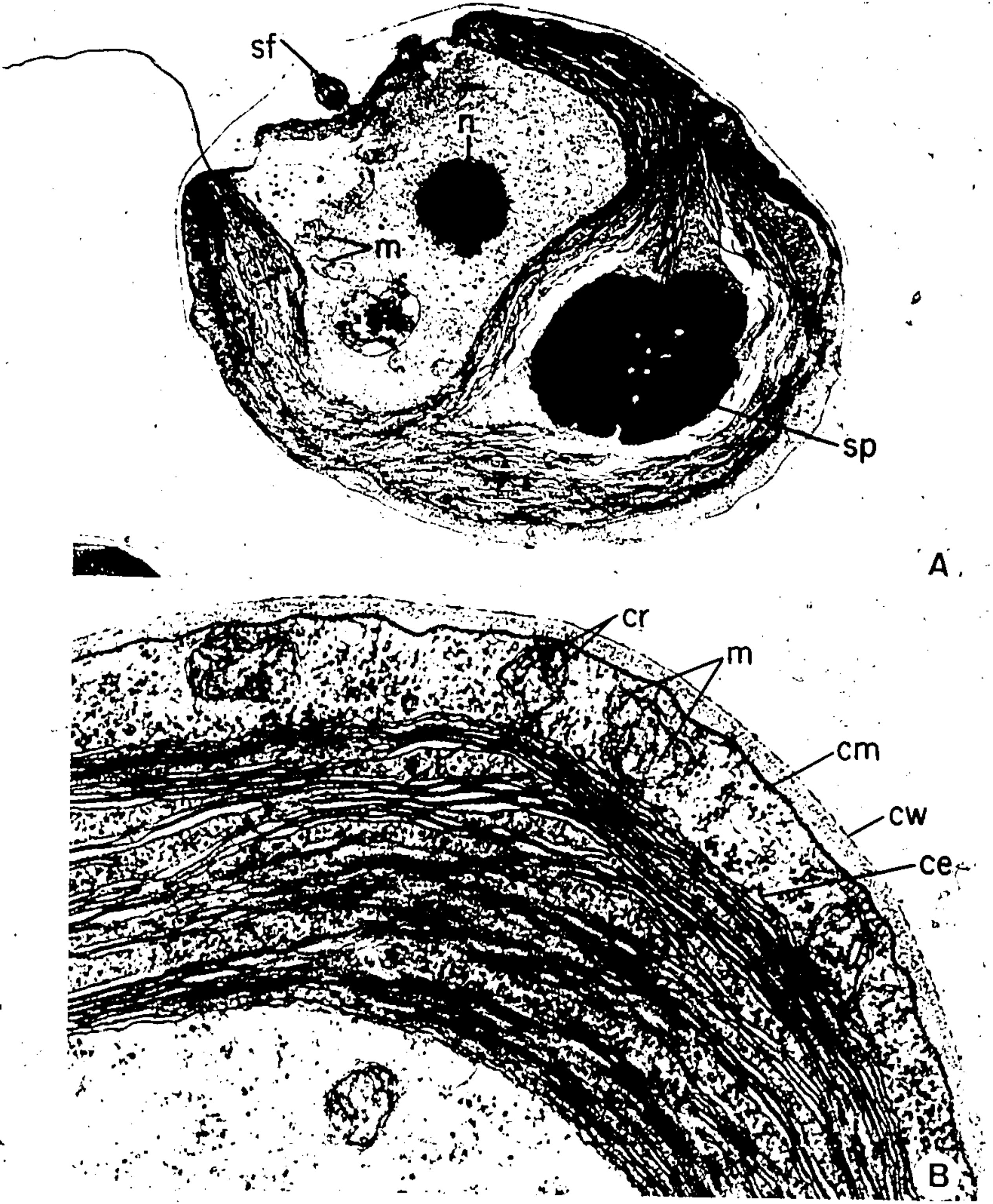

sf
n
m
sp
A
cr
m
cm
cw
ce
B

FIGURE 12C & D

Electron micrograph of longitudinal sections of *Chlamydomonas reinhardii*. C. Cell treated for 1 hr with Aerotex (5 ppm) x 15,000. D. Swollen mitochondria in cell treated for 1 hr with Aerotex (5 ppm) x 40,000. Figure legend: cell membrane (cm), pyrenoid (p), nucleus (n), mitochondrion (m), swollen mitochondrion (sm), thylakoids (t), fibrillar substance (f).

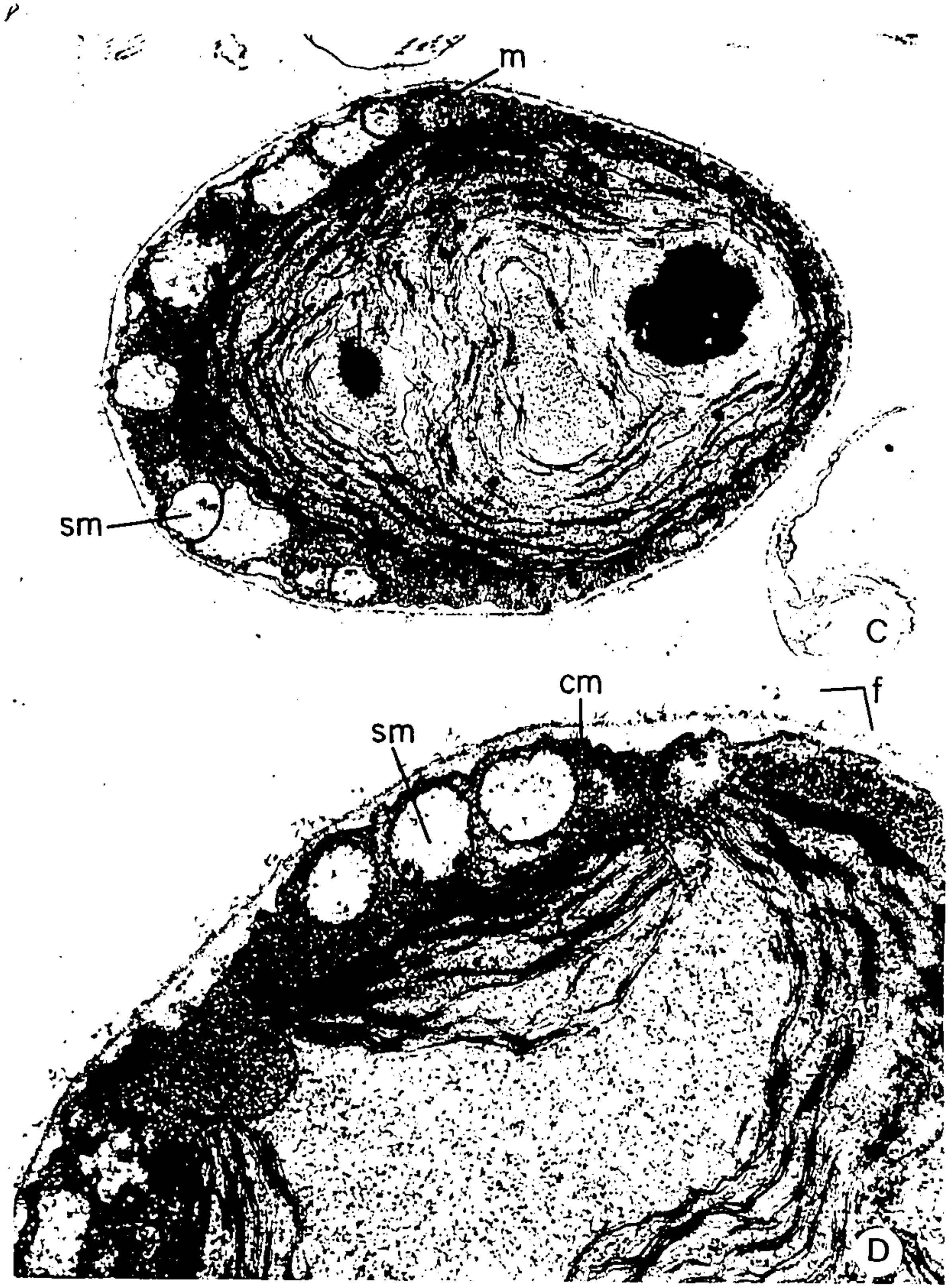
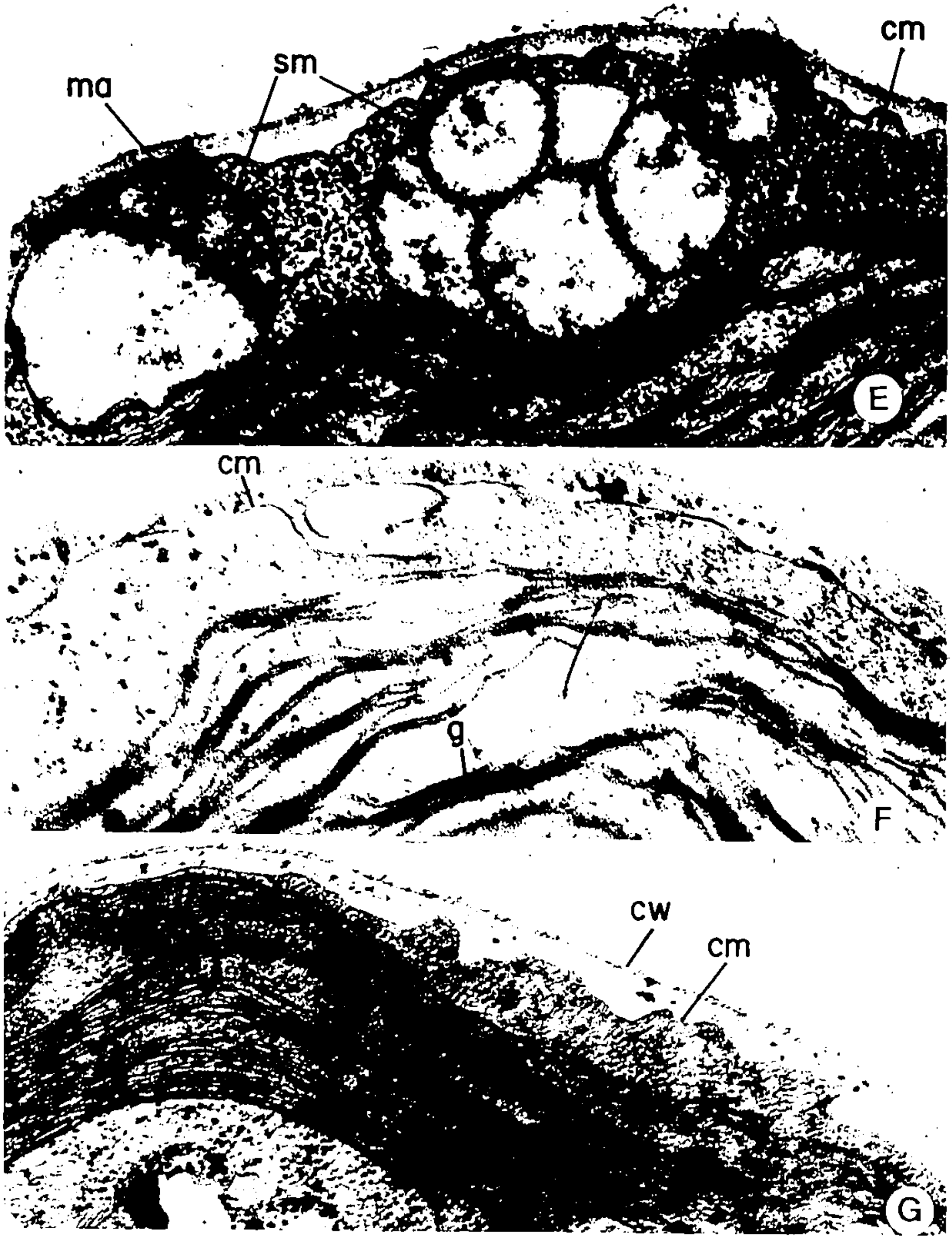

m
sm
C
cm
f
sm
D

FIGURE 12E,F & G

Electron micrographs of longitudinal sections of Chlamydomonas reinhardii. E. Swollen mitochondria with partial loss of matrix in cell treated for 1 hr with Aerotex (5 ppm) x 60,000. F. Grana-like stacking in cell treated for 1 hr with Aerotex (5 ppm) x 60,000. G. Plasmolyzed appearance of cell treated for 1 hr with 1-methyl-napthalene x 60,000. Figure legend: cell wall (cw), cell membrane (cm), granum (g), swollen mitochondria (sm), mitochondrial matrix (ma).


ma
sm
cm
E
cm
g
F
cw
cm
G

IV - DISCUSSION

IV - DISCUSSION

The course taken during the development of the present thesis necessitated the crossing of several borders of scientific endeavour. During this transition, certain basic principles were adopted from the literature, that, although encompassing a variety of fields, were consistent in inferring a relation between toxicity and lipophilicity. These principles, coupled with a basic understanding of partitioning theory, led to the development of the hypothesis of the present dissertation. Starting from a simple consideration of pesticide contamination of aquatic habitats, the derived experimental data led to a consideration of pesticide accumulation via partitioning in unicellular algae, which in turn led to a consideration of the relation between lipophilicity and toxicity of aromatic hydrocarbons. It remains to be shown that the experimental observations were relevant to certain principles held to be basic to an understanding of the anesthetic effect of lipophilic molecules in cell membranes.

Briefly reasserted, the hypothesis of the present study is that lipohilic chemicals, including petroleum hydrocarbons (Aerotex) as well as many pesticides (fenitrothion), are algicidal by virtue of their ability to partition preferentially within the lipid matrix of algal cell membranes. Further, their ability to cause membrane damage will depend on their ability to concentrate in membrane lipids and hence should positively correlate with their lipophilicity.

Before discussing the implications of this hypothesis, it will be instructive to consider the experimental studies that led to its development, and the validity of the derived observations in light of those reported elsewhere. This will proceed chronologically from the initial field studies, through the laboratory assessment of Aerotex-induced phytotoxicity and will conclude with the fluorometrically determined relation between lipophilicity and toxicity in algae. Following this, the theory of partitioning will be developed to show the consistency of the experimental results with the basic theory concerning the effects of anesthetic molecules in cell membranes. Final consideration will be given to the relevance of the present results to the field situation.

A. Field Studies

1. Pine Creek Field Study

(a) Water samples:

The observed rapid diminution in fenitrothion levels (half-life less than 24 hrs) in the water taken from Pine Creek was consistent with previous aquatic field studies (Lockhart et al., 1973; Eidt and Sundaram, 1975; Lakshminarayana and Bourque, 1980). This was expected in the flowing section (Area 2) of the stream where rapid mixing (and dilution within the water column) and downstream transport of the pesticide deposit would occur. Heavy rainfall at 21, 38, 65 and 84 hr post-spray could account for the slight fluctuation in levels recorded for Area 2 (Symons, 1977).

According to Eidt and Sundaram (1975) and Symons (1977), at the rate of pesticide dispersal used in the present study, a maximum fenitrothion water concentration of only 25 ppb should be expected for a water depth of 15 cm (assuming 40% "on-target" deposit and 50% filtration by forest canopy). The observation of 701 ppb at 1 hr in the surface layer of Area 1 (stagnant) illustrates the importance of water depth on the in situ dilution of pesticide residues (discussed previously in "Introduction" and in Appendix (ii)). This observation suggests that the neuston (surface-film inhabitants) may receive a brief exposure to relatively high pesticide concentrations.

The rapid diminution of fenitrothion levels in the stagnant water may also be due to microbial degradation. The in situ formation of amino- and desmethyl-amino-fenitrothion reported here is consistent with the in vitro studies of Yasuno et al. (1965) that demonstrated reduction of fenitrothion to the amino product by B. subtilis. The identification of trace levels of desmethyl-amino-fenitrothion was a notable achievement of the present methodology which employed lyophilization of water samples to recover polar (water-soluble) degradation products. This product has not been reported previously in aquatic field samples, although, it has been detected in in vitro laboratory studies (Zitko and Cunningham, 1974). The detection of trace levels of S-methyl-fenitrothion in the surface water may be significant since its in vitro anticholinesterase activity is 2 to 3 orders of magnitude greater than that of fenitrothion (Kovacicova et al., 1973).

(b) Plant samples:

The accumulation of fenitrothion in aquatic macrophytes has not been reported previously. Fenitrothion persisted ~~for~~ up to 192 hr in the submergent species (hornwort) but dissipated rapidly from the surface-dwelling species (duckweed). Fenitrothion dissipation in the latter case may be due to volatilization of the pesticide from the exposed leaf surfaces as documented previously for conifer tissue (Moody et al., 1977b). The sequestration of fenitrothion by the suspended particulate matter (containing algae) coupled with its accumulation in macrophytes, suggested that the pesticide could accumulate in aquatic microphytes such as the unicellular algae. For this reason, algae were included in the subsequent field study.

2. Lac Bourgeois Field Study

(a) Water samples:

The results of the Lac Bourgeois water analyses were consistent with the previous field study in showing rapid diminution of fenitrothion levels (half-life less than 2 days) and equilibration (less than 2 days) of the pesticide within the water column. On initial consideration, the observation of equally rapid "disappearance" of fenitrothion under dark and light conditions would seem to contradict the literature concerning the import of photodegradation of fenitrothion. There has been some criticism concerning the field relevance of in vitro studies such as those of Ohkawa et al. (1974) and Greenhalgh et al. (1976) that employ mercury lamp UV sources for irradiation. This criticism stems from the observation that the terrestrial solar spectrum is effectively depleted below approximately 2950 A, hence the UV radiation from a mercury lamp (2537 A) bears little relation to that expected from solar radiation in nature (Kenaga, 1972). The shorter wavelengths are filtered out by ozone in the upper atmosphere (Caldwell, 1971).

Lockhart et al., (1973), however, reported that fenitrothion was rapidly converted to two unidentified "photoproducts" in aqueous solutions exposed to sunlight but was stable in the dark. This is in agreement with the observation in the present study that three degradation products (desmethyl-, carboxy-, and carboxy-amino-fenitrothion) were only detected in the field stations exposed to light (Table 4). A fourth product (DMTP) was detected in both light and dark conditions, this being consistent with its non-photodegradative formation via hydrolysis (Zitko and Cunningham, 1974; Greenhalgh et al., 1980). It is notable that the carboxy-amino product has not been reported previously in either field or laboratory studies.

The identification of the photoproducts in the present case was aided by lyophilization of large water samples and also by diazomethane treatment of the recovered products. Lyophilization was shown to be necessary for recovering the polar (water-soluble) products since these products were detected in lyophilized water that had been pre-extracted with chloroform but were not detected in the chloroform extracts. It was also shown that the photoproducts had to be methylated by diazomethane treatment prior to their detection by GLC. These procedures, then, were an improvement on previously reported field extraction methods that rely solely on organic solvent partitioning and do not practice derivatization of sample extracts to ascertain the presence of "non-detectables". It has been previously asserted that fenitrothion poses no serious environmental hazard in aquatic habitats due to its rapid "dissapearance" in situ (Symons et al., 1977). This view will have to be re-evaluated in light of the present observations.

Mass balance calculations based on the data in Table 4 demonstrated that photodegradation accounted for up to 3% of the fenitrothion lost after 2 days in the light. This was negligible compared to the large amounts (e.g. 38.7% after 2 days; Weinberger et al., 1981) of pesticide sequestered by lake sediment, and explains the difficulty in observing a difference between the diminution rate of fenitrothion levels in the light and dark conditions. Further discussion of mass balance calculations is not considered relevant to the development of the present thesis and have been outlined in detail elsewhere (Weinberger et al., 1981). It should be mentioned, however, that although 80-95% of the fenitrothion was accounted for in early samples (up to 2 days), accountability was reduced at later sample times (60-70%, 6 days). Further in vitro studies in our laboratory have suggested that the plastic (polyethylene) used for constructing the field stations absorbed fenitrothion and could explain the reduced accountability at later sample times. Another explanation would be that further decomposition of the degradation products to non-detectable forms had occurred. In this connection, Daughton et al. (1976) reported that aquatic bacteria could utilize DMTP as a phosphorous source, while other reports have suggested that algal blooms following aerial application of the organophosphate pesticide, parathion, were due to phosphate-induced growth stimulation (Cook and Conners, 1963).

(b) Algae samples:

The rapid uptake and accumulation of fenitrothion by the unicellular algae in the present study were consistent with previous reports of algal concentration of lipophilic pesticides (Butler, 1977). The extremely rapid uptake of the pesticide and the high concentrating capacity of the algae (e.g. C.F. = 2,196 for C. reinhardii, light, 2 days) were consistent with the hypothesis that lipophilic pesticides accumulate in algae by partitioning into the cell lipids. In fact, the algal pesticide concentrations attained (e.g. 9.66 ppt or 9,660 ppm, C. reinhardii, light, 2 days), even when considered on a wet weight (w.w.) basis (9,660 ppm d.w. = 1,584 ppm w.w. for C. reinhardii), were far above the water solubility (20 ppm) of fenitrothion. The parallel diminution in algal-fenitrothion and lake water-fenitrothion levels observed at the later sample times is explicable simply on the basis of desorption. The observation that the polar degradation products were not detected in the algae was also consistent with the lipid partitioning theory since, as discussed previously, these compounds were relatively water-soluble.

The greater initial uptake of fenitrothion by C. reinhardii and C. pyrenoidosa in the light may be explicable on the basis of light-induced alteration of lipid composition (Nichols, 1968) and will be discussed later. An alternate explanation could be proposed invoking some form of light-dependent active uptake of fenitrothion. A mechanism of active uptake, however, seems unlikely considering the rapid uptake involved as well as the high intracellular concentrations attained. Several in vitro studies in our laboratory have not demonstrated significantly lower fenitrothion concentrations in freeze-killed C. pyrenoidosa versus controls. Similarly, Sodergren (1968) did not observe significantly different accumulation of DDT between controls and heat-killed C. pyrenoidosa and concluded that uptake was passive.

B. Laboratory ^{14}C-Fenitrothion Partition Study

The results of the laboratory ^{14}C-fenitrothion partition study were consistent with the previous field study in demonstrating rapid uptake and accumulation of fenitrothion in C. reinhardii. Since equilibrium was attained by 6 hr in both the algae and control systems, it is assumed that the dialysis tubing was the major rate-limiting factor involved, hence equilibrium should be attained in under 6 hr for algae suspended directly in medium. In fact, in vitro studies in our laboratory have demonstrated that maximal uptake occurs by 1 hr for C. reinhardii suspended directly in fenitrothion-treated medium. This is consistent with the extremely rapid uptake of DDT reported for C. pyrenoidosa (Sodergren, 1968), where maximal uptake (equilibrium) was established within 15 sec.

In the previous field study, fenitrothion levels in C. reinhardii held in the light were decreasing by day 2 (Fig. 2A). That this was not observed in the present case can be explained by the stability of fenitrothion under the present laboratory conditions. Several persistence tests conducted in our laboratory with both fenitrothion and ^{14}C-fenitrothion have demonstrated that significant degradation does not occur for at least 2 days (the duration of the present study) under identical laboratory conditions to those here. This is thought to be due to the relatively small UV light emmission from laboratory fluorescent lighting as compared to solar radiation. Further, the sediment which was the major "sink" for fenitrothion in the field study was omitted in the present case, and the study was conducted in glass (plastic stir bars were wrapped in aluminum foil and the dialysis tubing did not sequester fenitrothion).

The apparent contradiction that only 1.33% of the ^{14}C-fenitrothion leached from the algae after rinsing with medium, while desorption was proposed to explain the eventual decline in the algal-fenitrothion field levels, was probably due to the comparatively large water volumes present for back-partitioning (desorption) in the field situation. In fact, that rinsing with relatively small (10 ml) volumes of medium did not cause extensive leaching, is further evidence of partitioning <u>into</u> cell material (absorption) since surface-bound (adsorbed) molecules are expected to leach more readily (Sodergren, 1968).

As noted previously, the algal-fenitrothion levels in the present study continued to increase slowly from 6 to 48 hr. This can be explained on the basis that after initial rapid equilibration of the pesticide with the external cell membranes, the chemical would have to make many partitionings while crossing several cytoplasmic aqueous phases and internal membrane structures (e.g. through the thylakoid lamellae). this process would be much slower than the initial "surface" partitioning since many absorption-desorption steps would have to occur at the internal membrane surfaces.

The dialysis method improvised for the present study permitted immediate analysis of ^{14}-C-activity in the algae and circumvented the problems associated with sample storage and extraction. The importance of the algal partition coefficient referred to in the "Results" (Section III, B) will be discussed in a later section dealing with partition theory. After the termination of the study (48 hr), light microscope examination of the algae demonstrated a loss of cell motility. Since the algae were treated with formulated fenitrothion containing the adjuvants Aerotex and Atlox, further studies were conducted to determine whether the pesticide or adjuvants were toxic either alone or in combination. Results of these preliminary studies (involving cell motility studies and growth in agar and liquid culture media) indicated that Aerotex was the toxic constituent of the formulation and a series of bioassay tests was conducted to establish the mechanism of toxicity involved.

C. Aerotex Phytotoxicity Studies

The phytotoxicity of petroleum hydrocarbons to vascular plants has been documented previously as a result of the early usage of crude oil for fungicidal, insecticidal, and herbicidal purposes as well as for its more recent use as a pesticide carrier (Van Overbeek and Blondeau, 1954; Baker, 1970). It is generally agreed that the aromatic (ring-bearing) hydrocarbon (AH) constituents of petroleum are particularly toxic to higher plants (Baker, 1970) and a growing body of evidence, stemming from studies on the effects of oceanic crude oil spills, suggests a similarly high toxicity for AH compounds in algae (Kauss and Hutchinson, 1975; Vandermeulen and Ahern, 1976; O'Brien and Dixon, 1976; Hutchinson et al., 1980).

1. Aerotex Composition

The gas chromatographic analysis of Aerotex in the present study corroborated the report of Safe et al. (1977) that Aerotex contains relatively large amounts of mono- and di-methyl-naphthalenes. Anderson et al. (1974) reported that refined fuel oils were more toxic to marine organisms than crude oil and suggested that this was due to the higher concentrations of di- and triaromatic hydrocarbons in fuel oil. It has also been suggested that the degree of methylation of an aromatic hydrocarbon is directly related to toxicity in algae (Pulich et al., 1974; Kauss and Hutchinson, 1975). It was expected then, that the methyl-naphthalene constituents of Aerotex would enhance the toxicity of this fuel oil distillate. This is consistent with the hypothesis of the present thesis, since increased numbers of aromatic rings and/or methyl substituents of a molecule are held to be associated with increased lipophilicity (K_{OW}) (Leo et al., 1971).

2. Cell Motility

The loss of motility and deflagellation of C. reinhardii treated with Aerotex or its naphthalene constituents corroborated the observations of Soto et al. (1975b) on Chlamydomonas angulosa treated with water extracts of crude oil or naphthalene. Although loss of motility is not necessarily specific for a certain mode of toxicity (deflagellation of Chlamydomonas can result from osmostic or temperature stress (Salle, 1961)), the method was found to be useful for initial screening purposes. Aerotex at 100 ppm also induced deflagellation in Euglena gracilis but was not effective at 50 ppm. E. gracilis treated with Aerotex (100 ppm) initially underwent rapid morphological contortions, and by 1 hr, over 98% of the cells had become non-motile spheroids. This "rounding-up" and ultimate spheroid appearance of E. gracilis mimics the miscrospherocytosis of human erythrocytes that has been observed in cases of accidental naphthalene ingestion (Zuelzer and Apt, 1949). In the latter study, this effect was suggested to be due to a direct effect of napthalene on the cell membrane.

3. Vital Staining

The noted enhanced methylene blue staining of Aerotex-treated C. reinhardii, as well as providing a novel bioassay technique, may serve to indicate the mode of toxicity involved. Vital staining techniques have been reported for several plant and animal tissues. For example, Seepersad and Crippen (1978) employed aniline blue staining to distinguish between living and heat-killed zooplankton. It has been suggested that vital staining relies upon the loss of semi-permeability of cell membranes, in that after exposure of an organism to toxic conditions, intracellular uptake of normally non-permeant compounds occurs (i.e. dead cells stain) (Gaff and O'Kong'O-Ogala, 1971).

Methylene blue has been used as a vital stain in yeast cells (Fink and Kuhles, 1933 _in_ Gurr, 1960) and has also been used in histochemical studies to determine the presence of oxido-reductase activity (Roskin and Struve, 1947). Aerotex-induced inhibition of oxido-reductase activity in _C. reinhardii_ could be proposed to explain the differential staining noted in the present case. However, other equally credible explanations could be proposed (e.g. active exclusion of the dye molecules or Aerotex-induced changes in intracellular pH[1]) and the actual mechanism may not be as simple as that asserted by Gaff and O'Kong'O-Ogala (1971). Aerotex-induced loss of semi-permeability of algal cell membranes is consistent, however, with the cell "leakage" studies of Hutchinson _et al._ (1979) that will be discussed in a later section.

4. Agar and Liquid Culture Growth Studies

The agar bioassays provided a rapid means for assessing the relative sensitivity of algal species to Aerotex. The results (Table 5) demonstrated that

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[1] Methylene blue has a basic (cationic) chromophore and intensity of staining with cell proteins increases at higher pH (Gurr, 1960).

the algae listed in order of increasing sensitivity to Aerotex were S. obtusiusculus, C. reinhardii, and C. pyrenoidosa. The observation that 1-methylnaphthalene was more toxic than naphthalene in both the motility and agar studies is consistent with the theory that methyl groups enhance the toxicity of aromatic ring compounds. In contrast with the results of Pulich et al. (1974), the dimethyl-naphthalenes did not inhibit growth on agar, possibly because their greater hydrophobicity (Mackay and Shiu, 1977) retarded diffusion in the agar medium.

The results of the liquid culture bioassays (Table 6) indicated the relatively high toxicity of Aerotex. For example, Soto et al. (1975b) reported a lag phase extension (ΔL) of less than 1 day for C. angulosa exposed to the aqueous extract of two types of crude oil, while a saturated aqueous solution of naphthalene (34.4 ppm) yielded a Δ L of 2 days. In comparison, Aerotex at 2 ppm was sufficient to give a ΔL of about 6 days for C. reinhardii while 10 ppm totally inhibited growth (Table 6). Since the turbidimetric method used in the present study did not differentiate between living and dead cells, the lag phase extension may indicate a fractional kill of the inoculum or a temporary inhibition of cell division.

Kumar (1963) also reported that lag phase duration depended on treatment concentration when blue-green algae were exposed to antibiotics. In this case, the extended lag phase was thought to be indicative of a fractional kill of the inoculum, with only the resistant cells surviving treatment. The presence of resistant cells by chance in some of the treated cultures could explain the observed growth differences between replicate cultures at the higher treatment concentrations in the present study (Table 6). This explanation seems unlikely, however, since an extended lag phase was also observed when algae that survived the highest treatment concentrations were re-exposed to Aerotex. The alternate explanation that cell division was temporarily inhibited can be proposed on the basis that Aerotex volatilized from the culture media.

As noted in the "Results" section, "aged" aqueous emulsions of Aerotex were less effective than fresh preparations in inducing deflagellation in C. reinhardii. Further, gas chromatographic analysis of "neat" Aerotex standards exposed to an air stream demonstrated that the naphthalene constituents were extremely volatile. Similarly, Kauss et al. (1973) demonstrated that growth inhibition of Chlorella vulgaris by crude oil decreased proportionally to the length of time the oil was allowed to stand before algal treatment. Soto et al. (1975b) demonstrated that the lag phase of C. angulosa exposed to naphthalene was longer in closed than open culture flasks and this was consistent with their report (1975a) that by 1 day, over 90% of the ^{14}C-naphthalene had volatilized from algal medium (BBM) held in open systems. In fact, this effect has been well documented, since several reports have demonstrated that the reduced toxicity of aerated or "weathered" crude and fuel oils to marine organisms in general, is due to the volatilization of their AH constituents (Anderson et al., 1974; Dunstan et al., 1975; O'Brien and Dixon, 1976; Lee et al., 1978).

Soto et al. (1975b) suggested that the extension of the lag phase was due, at least partially, to a fractional kill of the inoculum. However, in the present case, viable cell counts did not demonstrate that a 1 hr treatment with Aerotex (10 ppm) caused mortality. This apparent contradiction can be resolved after considering the short treatment duration in the present case, as well as the fact that the treated algae had to be diluted with fresh medium to permit counting of separate colonies. This, in effect, was identical to the procedure used for determining the reversibility of treatment in the cell motility tests where some recovery of motility was observed if the algae were resuspended in fresh medium within 1 hr of treatment.

It is most likely, then, that both a fractional kill of the inoculum and inhibition of cell division of the surviving fraction were responsible for the observed lag phase extension in the present case. The positive correlation (Table 6) between the lag phase duration and Aerotex concentration further suggests a higher fractional kill and/or a greater time requirement for detoxification (volatilization) and/or a greater time requirement for cellular repair at the higher treatment concentrations.

5. ATP, DCIP and Carbon Dioxide Fixation Studies

A further indication of the mechanism of Aerotex-induced toxicity in algae can be gained from the collated results of the physiology studies. Hence the ATP, DCIP and CO_2 fixation results will be considered together.

(a) ATP studies:

The importance of adenosine-5'-triphosphate (ATP) as a "keystone" for providing the metabolic energy requirements of living cells has been reviewed by Patterson *et al.* (1970) in relation to the application of the firefly luciferin-luciferase assay system for determining algal ATP levels. Although other nucleoside triphosphates (e.g. CTP (cytidine-5'-triphosphate) and ITP (inosine-5'-triphosphate)) may cause light emission in the luciferin assay system (Holms-Hansen and Booth, 1966), the test is considered sufficiently specific for ATP determinations since the major proportion of light emission by algal extracts is attributable to ATP (Clegg and Koevenig, 1974). Employing this method, Clegg and Koevenig (1974) reported that several insecticides (DDT, aldrin, chlordane, diazinon and dieldrin) significantly reduced ATP levels in freshwater algae (*Chlamydomonas* sp., *Chlorella ellipsoidea* and *Euglena elastica*).

The present results (Table 7) parallel the preceding bioassay results in that C. reinhardii and C. pyrenoidosa were more sensitive than S. obtusiusculus and that the % reduction in ATP levels was directly related to Aerotex concentration. The results do not establish whether the lag phase extension noted in the liquid culture assays was due to initial mortality of the inoculum or to a temporary inhibition of cell division since they could be indicative of cell mortality and/or a sub-lethal reduction in the size of the ATP pool.

The reduction of ATP levels in C. reinhardii under both light and dark conditions suggested that Aerotex inhibited both photophosphorylation and oxidative phosphorylation. Naphthalene has previously been shown to reduce algal ATP levels under both light and dark conditions (Vandermeulen and Ahern, 1976).

(b) DCIP studies:

The blue dye, 2,6-dichlorophenol indophenol (DCIP) is a Hill reagent that has been used extensively in both higher plant and algal physiology studies to examine photosynthetic electron transport (Hallier and Park, 1969). Upon reduction, DCIP becomes colorless, hence the light-dependent, Aerotex-induced decrease in O.D. (Fig. 7) indicated that photosynthetic electron transport was stimulated. This effect has been well documented and is generally held to be due to uncoupling of photophosphorylation (ibid). The previously discussed reduction in algal ATP levels in the light is consistent with this explanation (Krogmann et al., 1959; Carafoli and Rossi, 1969).

Avron and co-workers (Krogmann, Jagendorf and Avron, 1959) discovered that NH_4Cl (1 mM) was a potent uncoupler of photophosphorylation in isolated spinach chloroplasts. The present observation that 10 mM NH_4Cl did not further enhance the Aerotex-induced stimulation of DCIP reduction could be proposed as positive evidence for uncoupling. However, NH_4Cl did not stimulate control DCIP reduction, probably because intact algal cells were used instead of isolated chloroplasts. In this connection, it is important to consider the study of Hallier and Park (1969) that demonstrated enhanced DCIP reduction after treatment of intact algal cells (e.g. *C. pyrenoidosa*) with formaldehyde (FA). They proposed their method as ~~an~~ improved technique for photosynthesis assays, since it circumvented problems associated with chloroplast isolation. They concluded that FA increased the permeability of algal cells, allowing DCIP to reach the intracellular chloroplast reducing sites. Aerotex, then, may similarly enhance cell permeability to DCIP. This is also consistent with the previously discussed differential staining obtained with methylene blue. A similar comparison can be made with the study of Howel and Walker (1972) in which toluene was used to render *C. reinhardii* permeable to nucleoside triphosphates for DNA synthesis studies. Toluene is a major AH constituent of petroleum (Anderson *et al.*, 1974) and is thought to induce "pore" formation in algal cell membranes (Lerner *et al.*, 1978).

The enhanced rate of DCIP reduction observed in the present study was explicable on the basis that Aerotex impaired the semi-permeability of algal cells and/or that uncoupling of photophosphorylation was involved. The two explanations are not necessarily mutually exclusive, however, since Aerotex could impair thylakoid membrane semi-permeability thereby abolishing transmembrane proton gradients required for ATP synthesis. This mechanism has been proposed previously to explain uncoupling effects (Carafoli and Rossi, 1969).

(c) Carbon dioxide fixation studies:

The rapid nature of Aerotex-induced inhibition of CO_2 fixation (71.5% inhibition in 5 min, Fig. 6A) was consistent with a biophysical effect of Aerotex on the cell membrane. The enhanced CO_2 fixation observed at low Aerotex concentrations (Fig. 5) has been reported previously for algae exposed to low (ppb) concentrations of crude oil (Gordon and Prouse, 1973). Stimulation of CO_2 fixation was reported in both laboratory and field studies for algae exposed to low concentrations of benzene, xylene, toluene or a No. 2 fuel oil (Parsons et al., 1976). Population growth stimulation has also been reported for algae exposed to "weathered" crude oil (Kauss and Hutchinson, 1975) and to low concentrations of benzene, toluene or xylene (Dunstan et al., 1975). It has been suggested that stimulation of both algal photosynthesis and growth are due to the presence of growth-regulating compounds and/or trace metals (acting as micronutrients) in crude oils (Baker, 1970; Gordon and Prouse, 1973). Dunstan et al. (1975), however, employed pure compounds and inferred that growth stimulation was related to an effect of hydrocarbons on membrane permeability.

The Aerotex-induced inhibition and subsequent recovery (Fig. 5) of CO_2 fixation has been reported previously for Chlamydomonas sp. and Monochrysis lutheri exposed to naphthalene (Kauss and Hutchinson, 1975; Vandermeulen and Ahern, 1976). Kusk et al. (1978) demonstrated full recovery (within 2 hr) of benzene-induced inhibition of photosynthetic oxygen evolution in Nitzschia palia, when treated cultures in glass-stoppered flasks were transferred to open containers. Similarly, Soto et al. (1975a), using open and closed culture flasks, demonstrated that recovery from crude oil- or naphthalene-induced inhibition of CO_2 fixation in C. angulosa was explicable on the basis of volatilization. This is consistent with the present observation that CO_2 fixation did not recover in the "closed" condition ("Results", D.1.) and also with the prior discussion concerning the relation between volatilization and lag phase duration in the growth studies.

The enhanced ^{14}C-leakage from Aerotex-treated C. reinhardii (Fig. 6B) was due to the efflux of ^{14}C-fixed material since acidification did not reduce sample ^{14}C-activity (Stein, 1973). The observation that greater overall leakage occurred with older algae tentatively suggests that cell permeability (leakiness) increases with aging, however, further replication with synchronized cultures is warranted.

A more in-depth study by Sikka et al. (1973) demonstrated that quinone pesticides (dichlone, O6K-quinone and chloranil) enhanced leakage of ^{14} C-photosynthate from C. pyrenoidosa. Both the rate of leakage and the total amount of leaked ^{14}C-material were directly dependent on pesticide concentration. These authors also determined that ^{14}C-labelled sucrose, amino acids and organic acids leaked from the cells but that ^{14}C-labelled lipids (possibly due to their size and non-polar nature) did not. They also suggested that quinone pesticides increased the permeability of the algae possibly by changing membrane structure. This mechanism was also proposed by Lee and Wilkinson (1973) to explain the leakage of plant (pea, corn, beet) cell contents resulting from treatment with an organophosphorous insecticide (chlorphenvinphos).

Membrane damage was also suggested by the study of Kauss and Hutchinson (1978) that demonstrated extensive leakage of potassium and manganese after treatment of Ankistrodesmus facultatus with benzene. Leakage of potassium and manganese also occurred after treatment of C. angulosa with naphthalene or crude oil (Hutchinson et al., 1981). Other extensive studies by Hutchinson et al. (1979) demonstrated that leakage (determined both by potassium and ^{14}C-photosynthate leakage studies) from C. angulosa and Chlorella vulgaris was enhanced by twenty chemically non-related petroleum hydrocarbons (including long-chain aliphatic and polynuclear aromatics). Further, they were able to demonstrate a positive correlation between the lipophilicity (K_{OW}) of hydrocarbons and their ability to induce leakage in algae.

D. Transmission Electron Microscopy

The ultrastructure of C. reinhardii has been examined intensively by numerous authors. The electron micrographs of control cells (Fig. 12A, B) were in agreement with those already published showing cup-shaped chloroplasts with dense staining thylakoids, and elongated mitochondria with tubular cristae (Ohad et al., 1967). The present observations were also consistent with the report of Shotz et al. (1972), that the mitochondria (10-15/cell) in C. reinhardii occupied about 3% of the cell volume, were usually close to the chloroplast surface, were articulated by constrictions, and frequently exhibited branching.

Soto et al. recently published electron micrographs of C. angulosa treated with aqueous crude oil extracts (1979b) and with naphthalene (1979a). While many similarities can be seen, notably the diffuse appearance of the thylakoids, the formation of grana-like structures and the diffuse nature of the cell wall, their observation that the mitochondria were not affected by treatment is in direct contrast to our own observations. Aerotex-treated cells typically had greatly swollen mitochondria. Their identification here was probably aided by the short (1 hr) treatment time used, as opposed to that (sampled after 1 day) used by Soto et al. (1979a,b). In a few cells some mitochondria with limited cristae were observed (Fig. 12C,D). However, in most cells, the inner mitochondrial membrane had unfolded completely giving the appearance of vacuoles enclosed by double membranes (Fig. 12E). The vacuoles apparent in the light microscope 1 hr after treatment (Fig. 11B) may have a mitochondrial origin. We suggest that the green coloration of these vacuoles may have been due to the accumulation of chlorophyll that had been displaced from the thylakoids. Greening of intercellular oils following crude oil application to higher plants has been reported previously (Baker, 1970). The presence of lipids was indicated by the birefringence of these vacuoles under phase contrast (Fig. 11B) and is consistent with the report of Soto et al. (1979a, b) that vacuoles present in treated C. angulosa stain with Sudan black B.

The mechanism of mitochondrial swelling in animal cells has been examined intensively (Blondin and Green, 1967). This phenomenon is less well documented for algal cells. However, the herbicide, 2,4-D has been reported to cause mitochondrial swelling in C. pyrenoidosa (Bertagnolli and Nadakavukaren, 1970). Swollen mitochondria have also been reported as a treatment effect of: the insecticide , methomyl on corn (Zea mays)(Koeppe et al., 1978); the polychlorinated biphenyl (PCB), Aroclor 1254 on shrimp hepatopancreas (Penaeus duorarum) (Couch and Nimmo, 1974); and the insecticide, DDT on fish (Tilapea aurea) (Coleman et al., 1977).

It has been suggested that mitochondria swollen to the extent observed in the present study provide conclusive evidence of acute toxicity (mortality) (York et al., 1980). Examination of ultrastructural damage of mitochondria in the protozoan, Amoeba proteus has recently been advocated as a model system for toxicological studies, especially for those dealing with uncoupling effects (Smith, 1980). York et al. (1980) demonstrated mycotoxin-induced swelling of corn mitochondria and reported that the inner mitochondrial membrane was severely affected. Uncouplers of oxidative phosphorylation (e.g. dinitrophenol (DNP)) are known to affect the permeability of the inner mitochondrial membrane (Carafoli and Rossi, 1969). It is tentatively suggested that the lipophilic hydrocarbon constituents of Aerotex may impair the normal impermeability of this membrane to ions (such as H^+) causing similar uncoupling effects. This would be consistent with the ATP data and also with the prior discussion concerning membrane leakage. Further in this connection, uncoupling of oxidative phosphorylation was proposed by Kusk (1978) to explain enhanced respiration (oxygen uptake) in the diatom, N. palea exposed to benzene and has also been proposed by Sinclair et al. (1977) to explain similar observations for C. vulgaris exposed either to the uncoupler, DNP, or to the PCB, Aroclor 1221. As well as a possible association between mitochondrial swelling and uncoupling, the initial cause of swelling may also be due to osmotic stress resulting from Aerotex-induced membrane leakage (isolated mitochondria swell in hypotonic media (Blondin and Green, 1967)). In this connection, freshwater algal cells are thought to have an internal (cytoplasmic) osmotic pressure of 5-6 atmospheres and this is considerably higher than that of the external medium (ca. 0.4 atmospheres for BBM) (Kauss and Hutchinson, 1978).

The negative staining property and wavy diffuse appearance of the chloroplast thylakoids in Aerotex-treated cells (Fig. 12C, D) were consistent with the previous studies (ATP, DCIP and CO_2 fixation) that demonstrated impairment of photosynthesis. The increased granal stacking observed with Aerotex was also reported for C. angulosa exposed to naphthalene (Soto et al., 1979a) and could have been due to changes in intracellular salt concentrations resulting from membrane leakage (Staehelin and Arntzen, 1979). The less severe ultrastructural damage induced by 1-methyl-naphthalene (5 ppm) as compared with Aerotex (5 pmm) was consistent with the fluorometer results (to be discussed).

It has been suggested that the forest spraying of formulated fenitrothion may be related to the relatively high incidence of Reye's Syndrome (RS - a viral-associated, often fatal condition in children) in New Brunswick (for review see Crocker, 1978). Crocker et al. (1974) reported that a normally sub-lethal dose of EMC virus was lethal to mice that had been exposed to the fenitrothion formulation. Fenitrothion alone, however, was not effective in enhancing viral-induced mortality. Partin et al. (1971) reported that liver mitochondria of RS patients were swollen and that the appearance of the organelles (matrix rarefaction) was peculiar to the RS condition and was the best means of diagnosis (Crocker, 1978). As well as Aerotex, other environmental contaminants such as paint thinners and household solvents have also been suggested as possible triggering agents or even that an intrinsic (endogenous) chemical factor was involved (blood sera from RS patients caused swelling of isolated liver mitochondria) (Crocker, 1978). Obviously, it is not contended that Aerotex-treated algae develop RS. One merely wishes to point out the similarities between the EM observations, with the hope that this may lead to a better understanding of the mechanism(s) involved.

E. Algal Fluorometry

The present study was undertaken to establish a rapid reproducible means for assessing the relative phytotoxicity of a wide array of environmental pollutants. The results indicated that algal fluorometry could provide a suitable screening procedure for further laboratory and field studies. The method is extremely rapid (maximum of 2 hr required to determine ICF_{100} for any compound), and sensitivity depends only on the algal species selected for screening. A wide variety of species could be employed in future studies to establish an order of species tolerance that could be used to relate the species composition of endemic algal populations to the water quality of a particular habitat. Such a table has been composed previously (Palmer, 1969) by arbitrarily conferring extra tolerance to those algal species reported most frequently inhabiting polluted water. It is notable that C. reinhardii was among the most tolerant species listed.

The observation that 1-5 ppm of Aerotex was sufficient to totally suppress the fluorometer response (ICF_{100} = 1-5 ppm) in C. reinhardii was consistent with the previous bioassays which demonstrated that Aerotex (1-10 ppm):

1. inhibited cell motility, population growth, ATP synthesis and CO_2 fixation;
2. enhanced staining with methylene blue, reduction of DCIP and leakage of ^{14}C-photosynthate;
3. induced ultrastructural changes in membrane configuration.

The enhanced photosynthesis suggested by the fluoremeter results (Table 9) for low concentrations of several test chemicals was consistent with the enhanced CO_2 fixation (Fig. 5) discussed previously for low Aerotex concentrations. The rapid Aerotex-induced inhibition of the fluorometer response (Fig. 8) was also consistent with the CO_2 fixation results (Fig. 6A). The observed lack of recovery of the fluorometer response after 24 hr treatment or after rinsing of treated (1 hr) algae with fresh media may appear to contradict the recovery of CO_2 fixation discussed previously for Aerotex-treated algae (Fig. 6A). However, as mentioned previously, the fluorometer treatments were conducted in the closed condition (screw-cap flasks) to curtail volatilization. Further, as discussed previously for elution of ^{14}C-fenitrothion in the dialysis study, the rapid rinsing and relatively small volume of BBM employed here was probably insufficient to achieve extensive back-partitioning (desorption).

The basic theory concerning the fluorometric analysis of photosynthesis was introduced previously. The time-course of fluorescence transient measurements are commonly arbitrarily categorized as being either "fast" or "slow" (Mohanty et al., 1971). The fluorescence transients recorded in the "fast" region are held to be due primarily to the redox level of the quencher, Q - the primary electron acceptor of photosystem II (ibid.). The P-T transient used for quantification of photosynthesis in the present study is considered to be part of the slow region (ibid.; Schreiber et al., 1978). Several hypotheses have been proposed to explain the slow fluorescence changes in intact green algae (Mohanty et al., 1971). Although the basic mechanism governing slow fluorescence induction is not fully understood, it is generally held that the P-T transient corresponds to trans-membrane ion flux-induced conformational changes of the thylakoid membranes (ibid.; Papageorgiou, 1975). Krause (1973) demonstrated with isolated spinach chloroplasts, that the slow fluorescence changes paralleled optical density (absorbance) changes that were thought to be due to photophosphorylation-associated shrinkage and expansion of the thylakoids. Further, uncouplers of photophosphorylation (e.g. cyclo-hexyl-ammonium chloride) are known to abolish the P-T decline and it has been suggested that this effect is due to an effect of uncouplers on trans-membrane proton gradients (Krause, 1973; Mohanty et al., 1971). The present observation, then, that several lipophilic chemicals (lipophiles) suppressed the P-T transient was further evidence that an impairment of membrane semi-permeability was involved.

The positive correlation attained between toxicity[1] and lipophilicity for several chemically non-related compounds (several AH constituents of Aerotex; the insecticide, fenitrothion; the adjuvant, Nonyl Phenol; and the herbicide, 2,4-D) suggested a common mode of action of lipophiles within the lipid matrix of algal cell membranes.

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[1] Toxicity will be used henceforth in place of algicidal activity.

The regression equation (correlation coefficient r = - 0.92) reported here for C. reinhardii,

$$(1)\quad \log ICF_{100} = 5.78 - 0.98 \log K_{OW}$$

was in close agreement with that reported by Hutchinson et al. (1979, 1980) for C. angulosa, (r = - 0.94),

$$(2)\quad \log x = 5.17 - 0.93 \log K_{OW}$$

on the basis of CO_2 fixation assays (x = concentration (uM/l) of hydrocarbon required to cause 50% inhibition). The present study also corroborated the report of Hutchinson et al. (1980) that the toxicity of hydrocarbons could be inversely correlated with their water-solubility. Note that this follows logically since less water-soluble (hydrophilic) chemicals are more lipophilic (hydrophobic) and hence should reach higher concentrations within algal lipids. This is consistent with the report of Korte et al. (1978) that the water solubility of 10 lipophilic chemicals could be inversely correlated with their degree of bioconcentration in Chlorella fusca. Further, in this connection, Chiou et al. (1977) demonstrated empirically that the water-solubility of 34 organic chemicals (including organochlorine and organophosphate pesticides, PCB's and petroleum hydrocarbons) was inversely related to their lipophilicity (K_{OW}), and explained this simply on the basis that the water-solubility of a lipophilic compound is a measure of the degree of partitioning of the chemical between itself and water[1].

Since the value of the K_{OW} of an AH compound is directly dependent on both the number of aromatic rings and the number of methyl-group ring-substituents (these having "additive-substituent properties" - Leo et al., 1971), the observation (Fig. 9) that toxicity increased along the series, benzene (B),

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[1] See Appendix (x) for the correlation between log K_{OW} and log S obtained with the present data.

toluene (mono-methyl-B), xylene (di-,methyl-B), naphthalene (bicyclic), 1- or 2-methyl naphthalene, was predictable in light of the present understanding that increased lipophilicity (K_{OW}) is associated with greater toxicity. This also explains the previous observation that 1-methyl naphthalene was more toxic than naphthalene in the cell motility and agar bioassay tests, and corroborates our previous assertion that the enhanced toxicity of Aerotex (as compared to crude oils) was due to its relatively high naphthenic content. In this connection, Aerotex was roughly 50X more toxic than the two crude oils tested (Fig. 9) and GLC analysis of the crude oils, conducted as described previously for Aerotex, has confirmed the higher naphthenic content of Aerotex (see Appendix v).

The observation that the naphthalene constituents of Aerotex were more toxic in combination (in "synthetic" Aerotex) than when tested alone has been reported previously for petroleum hydrocarbons (Pulich et al., 1974; Lee and Nicol, 1978). An additive effect of the AH constituents of Aerotex is also consistent with the observation that Aerotex (5 ppm) caused greater ultrastructural damage than 1-methyl-naphthalene (5 ppm). Assuming a common mode of action for AH compounds in general, an additive effect of their combined action would be expected.

Before closing this section, it is important to note that there are limits to the theory that increased lipophilicity will be necessarily associated with increased toxicity. For example, in the present study, 2,6-dimethyl-naphthalene and 1,2,4,5-tetramethyl-benzene were not toxic (Table 11). This so-called "cut-off" effect has been demonstrated in studies concerned with the anesthetic activity of lipophiles and has been explained on the basis that the water-solubility (and anesthetic activity) of AH compounds falls off dramatically within a homologous series (e.g. alkanes) beyond a certain maximum value of K_{OW} (Seeman, 1972).

F. The Theory of Partitioning and Anesthesia

The classical work of Meyer (e.g. 1899) and Overton (e.g. 1901) established that the potency of anesthethics was directly proportional to their olive oil/water partition coefficient and led to the Meyer-Overton theory of anesthesia: "Narcosis commences when any chemically indifferent substance has attained a certain molar concentration in the lipoids of the cell" (Seeman, 1972). However, pharmacological interest in this theory gradually declined since it did not lead to any really useful generalizations concerning the mechanism of narcotic activity (Leo et al., 1971).

Some renewed interest in the theory followed the work of Collander (e.g. 1954), that demonstrated that the rate of movement (permeating ability) of organic compounds in the Characean alga, Nitella, could be positively correlated with partition coefficients (Diamond and Wright, 1969). Recently, however, partition theory has been adopted by environmental toxicologists to relate the toxicity of lipophilic pollutants to their bioconcentration and toxicity in aquatic biota. For example, as well as the work of Hutchinson et al. (1980) concerning the toxicity of petroleum hydrocarbons in algae, partition coefficients have been related to the toxicity and bioaccumulation of PCB's in algae (Kleppel and McLaughlin, 1980; Biggs et al., 1980); bioaccumulation of AH compounds in Daphnia (Southworth et al., 1978), rate of absorption and toxicity of insecticides in fish (rainbow trout) (Yang and Sun, 1977), as well as to the toxicity of a variety of organic chemicals in mosquito larvae, bacteria, houseflies and rodents (Hansch and Fujita, 1964). Chiou et al. (1977) also demonstrated that the water-solubility of several organic compounds (including insecticides and PCB's) was inversely related to their bioconcentration in fish (rainbow trout).

Seeman (1972) has reviewed the membrane action of anesthetics within the context of lipophilicity. It is generally assumed that anesthetics have a common mode of action involving a primary effect on the cell (axon) membrane, however, some studies have indicated a primary effect on intracellular organelles such as mitochondria. It is also generally held that the mechanism of action of anesthethic molecules is structurally non-specific, since anesthetic activity is not dependent on the presence or absence of particular chemical groups in the molecule. Chemicals that exert an "in-common", structurally non-specific biological effect are classified as "biological depressants" and include anesthetics as well as hydrocarbons (aliphatic, aromatic and chlorinated) and insecticides (Kauss and Hutchinson, 1975). In fact, several petroleum hydrocarbons are known to be anesthetic (Goldacre, 1968) and naphthalene has been shown to induce narcosis in marine larval invertebrates (Sanborn and Malins, 1977). Insecticidal effects in insect neural membranes have also been compared to anesthetic effects and lipophilicity is generally held to be one of the basic requirements for insecticidal activity (Matsumura, 1975).

It is interesting to compare the results of recent toxicity studies concerning the effects of pesticides and aromatic hydrocarbons, while considering the current theories concerning the mode of action of anesthetics. The initial effect of anesthetics on cell membranes is thought to be due to an alteration of membrane structure with associated membrane "expansion" and "fluidization" effects (Seeman, 1972; Swenson and Narahashi, 1980). Domenech et al. (1977) first reported that pesticides (e.g. parathion) affected membrane fluidity. Micro-spectrofluorometric analysis of artificially prepared phospholipid vesicles (liposomes) has recently been proposed as a method for measuring uptake rates, intra-membrane diffusion, and membrane partition coefficients of organochlorine pesticides (Ohmann and Lakowicz, 1977). Membrane partition coefficients have been obtained for anesthetics in liposomes and have been shown to relate directly with K_{OW} values (Seeman, 1972).

Extensive studies with erythrocytes have demonstrated that low concentrations of anesthetics protect membranes from osmotic hemolysis but higher concentrations were lytic. At concentrations between those causing membrane-protection and membrane-lysis, a so-called "pre-lytic" stage is observed wherein the membrane becomes very permeable to ions but not to proteins (Seeman, 1972). This effect is comparable to the hydrocarbon-induced leakage in algae, discussed previously, and it is interesting that Van Overbeek and Blondeau (1954) proposed that the herbicidal activity of petroleum hydrocarbons was due to their ability to dissolve in cell membranes and to increase their permeability by displacing membrane lipids. The permeability of liposomes has also been shown to be increased by organophosphorous and organochlorine pesticides (Artunes-Madeira and Madeira, 1979).

There is an extensive literature on both the protective (stabilizing) and lytic (destabilizing) effects of anesthetics on lysosomal membranes (Seeman, 1972). Moore (1979) has recently suggested that polycyclic aromatic hydrocarbons (PAH) (e.g. methyl-naphthalenes) cause destabilization of lysosomal membrane structure and subsequent release of hydrolytic enzymes which induce autocytolysis of digestive cells in mussels (*Mytilus edulis*). In fact, the measurement of lysosomal destabilization has been used as a measure of toxicity in rat-liver lysosomes exposed to Aerotex 3470 (Crocker, 1978). "Lysosomal-like" organelles have been reported in aged cultures of *Euglena* (Palisano and Walne, 1972).

The hypothesis of the present thesis asserts that lipophilic chemicals, including petroleum hydrocarbons and pesticides, are algicidal by virtue of their ability to partition within the lipid matrix of algal cell membranes. Further, their ability to cause membrane damage will depend on their ability to concentrate in membrane lipids and hence should positively correlate with their lipophilicity (K_{OW}). In light of the present discussion, it is apparent that this hypothesis is simply a modern adoption of the Meyer-Overton theory of anesthesia. To further test this hypothesis, it is essential to determine the actual membrane concentration of lipophiles and to relate these <u>via</u> partition coefficients to their algicidal activity. These values are presently unavailable in the literature, however, a theoretical equation has been developed by the present author in order to calculate the algal lipid concentration of lipophiles, on the basis of partition theory. This equation (see Appendix viii) was obtained by simple mathematical rearrangement of the Hertz (1909) partition equation. A simple mathematical explanation for obtaining a maximum value of (0) (the concentration of lipophile in the organic or lipid phase) below a certain value of L (the volume of the organic phase) is given in Appendix (ix). As demonstrated in Appendix (viii), the data of Rice and Sikka (1973) for algal uptake of DDT could be predicted solely on the basis of partition theory.

Further, the present theory predicts that higher concentrations in algal lipids will be attained by more lipophilic compounds. Assuming that toxicity results once a certain concentration (or "molar occupation"; Seeman, 1972) of the algal membrane is attained, the present observation that lipophilicity directly relates to toxicity becomes understandable on the basis of partition theory. This is due to the fact that lower water concentrations (e.g. in BBM) of highly lipophilic compounds would be required to obtain a concentration in the membrane surpassing the tolerance limit. Further calculations given in Appendix (viii) suggest that for any lipohile, the log of the minimum concentration required in the algal lipids to cause toxicity will be a constant (i.e. the log of the toxic concentration in the lipid (membrane) is the same for "chemically indifferent" substances). In this connection, Meyer calculated that for gaseous substances, the molar concentration required to produce narcosis was a fixed value of 0.06M/l in brain tissue (Seeman, 1972).

A further consequence of the present theory is that the membrane lipid composition, itself, should affect the membrane partition coefficient of a particular lipophile. This is asserted since partition coefficients are known to be highly dependent on the organic phase (octanol, hexane, benzene, etc.) used as a reference system (Leo et al., 1971). It follows, then, that membrane lipid compositions (e.g. degree of unsaturation) that tend to promote partitioning would be more sensitive to lipophilic environmental pollutants.

As shown in Appendix (viii), higher algal lipid concentrations of lipophiles will result at lower algal population densities for compounds with a $K_{ow} \geq 10^6$ (e.g. DDT and PCB's). From the previous discussion, it follows that such chemicals would be more toxic at lower population densities. It is well documented that established (high population density) algal cultures are much less sensitive to pesticides than recently inoculated (low population density) cultures (Stein, 1973). Further, DDT and PCB have been shown to be more toxic at lower algal population densities (Butler, 1977; Kleppel and McLaughlin, 1980). This theory, then, has important consequences when considering the field relevance of in vitro toxicity data obtained with artificially high algal cell densities.

G. Relevance to the Field Situation

The initial field studies have indicated that the concentration of Aerotex reached in river water would never reach the lowest level (1 ppm) shown to affect algal viability in the bioassay studies. Further, much of the volatile toxic constituents of Aerotex may be lost during the descent of the spray droplets through the atmosphere (Howse et al., 1978). It can be calculated from the field data that the highest concentration of Aerotex that could be reached would be roughly 0.1 ppm. The oil concentration, however, would increase substantially if oil-based formulations (as opposed to the water-based formulations that contain ca. 1% Aerotex) were used. Of even greater consequence was the observation that the ICF_{100} of Nonyl Phenol for C. reinhardii was between 0.5 and 0.75 ppm, either alone, or when present in the Matacil 1.8-D field formulation recently sprayed in New Brunswick, where levels as high as 0.1 ppm of Nonyl Phenol have been reported in standing water following experimental application, albeit under "worst case" conditions (Varty, 1980). Holmes and Kingsbury (1980), however, reported levels of nonyl phenol up to 1.1 ppm in water taken from a stagnant stream, 4 hrs post-spray. This is consistent with the higher levels of fenitrothion detected in the stagnant section of Pine Creek (Table 2).

As discussed previously, when assessing field reports of pesticide concentrations in water, it is essential to consider the effect of dilution within the water column . Initial concentrations in the surface layer may be much higher than expected, and the present results indicate that even a brief (1 hr) exposure of the algal neuston (surface film inhabitants) inhabiting this layer would suffice to inhibit in situ photosynthesis.

It is difficult to relate in vitro unialgal bioassay results to the field situation. The in situ toxicity of lipophiles to algae may depend on environmental conditions which affect partition equilibria (e.g. salinity, temperature, pH (Leo et al., 1971)). Further, species-related differences in algal sensitivity were demonstrated in the present study and are well documented in the literature (Butler, 1977; O'Brien and Dixon, 1976). Algal sensitivity to lipophiles may depend on species-related differences in lipid composition which in turn may depend on such factors as light intensity, salinity, temperature, pH, nutrient supply and cell age (Kates, 1970; Stewart, 1974; Soto et al., 1977). In this connection, the greater uptake of fenitrothion by Chlamydomonas and Chlorella exposed to light in the field stations could be due to an effect of light on algal lipid composition (Nichols, 1965).

Many of the reports concerning pesticide and petroleum toxicity in algae are contradictory (Butler, 1977; O'Brien and Dixon, 1976) and it is suggested that this may be due to poor standardization of inter-laboratory treatment, algal culture and bioassay procedures. Unlike the work of Rice and Sikka (1973) for example, most reports do not consider the effect of population density on bioconcentration . As suggested previously, this may invalidate environmental lethal thresholds predicted from in vitro tests with established algal cultures. Identical intra-test population densities were used in the toxicity studies in present case and also in the studies of Hutchinson et al., (1979, 1980). Further, cell densities in the order of 10^4 cells/ml were used both in the present liquid culture bioassays and fluorometric studies, as well as in the CO_2 fixation assays used for assessing toxicity by Hutchinson et al. (1980), and are considered to be within the upper limit (10^4 cells/ml) of endemic algal populations (Stewa , 1974).

Several highly lipophilic chemicals (including DDT, PCB's and PAH's) are considered to be ubiquitous contaminants of aquatic habitats (Sodergren, 1968; Glooshenko and Glooshenko, 1975; Malins, 1977). It follows, then, that algae in situ will contain trace levels of several lipophilic environmental contaminants. In fact, "background" levels of many PAH compounds have been detected in algae sampled in marine habitats that were considered to be relatively free from industrial pollution (Malins, 1977). This "background contamination" is important when considering the previously asserted additive-effect of lipophiles in general. Since DDT, PCB's and PAH's are highly lipohilic, even trace levels in water would be concentrated to relatively high levels in algal lipids. Further exposure of algae to intermittent spraying with fenitrothion and Aerotex for example, could elevate the total lipophile concentration in the algae beyond the lethal threshold. Hence, algae in nature may be more susceptible to environmental pollution than predicted on the basis of in vitro toxicity tests with laboratory cultures.

There is some evidence to suggest that the algal species used in the present study are relatively resistant to organic pollution (Palmer, 1969). In fact, it has been reported that Chlamydomonas, Scenedesmus, Chlorella and Euglena "thrived" in river water following an accidental spill of fuel oil, and it was thought that this resulted from reduced competition after elimination of the more sensitive algal species (O'Brien and Dixon, 1976). It has also been reported that, on the basis of both field and laboratory studies, that flagellate species (e.g. Chlamydomonas) were more resistant to crude oil pollution than diatoms (Parsons et al., 1976; Thomas et al., 1977). Further, Hsiao (1978) reported that Chlamydomonas pulsatilla was more resistant than diatoms to crude oil spills in the Artic and suggested that this could lead to a dominant population of flagellate species in Artic water where diatoms normally constitute the base of the food chain.

It is generally held that the ecological importance of algae stems from their photosynthetic production of atmospheric oxygen, and their comprisal of the first link in the aquatic food chain. As well as direct lethal effects of environmental pollutants on the species composition of phytoplankton communities (e.g. the artificial selection of resistant species) indirect effects on higher trophic levels have been suggested on the basis that lipophilic contaminants sequestered by algae, are passed on through the food chain (Sodergren, 1973; Malins, 1977). Considering the wide-spread use of pesticides in forest spray programs (Symons, 1977), the fact that from 10 to 20 million gallons of petroleum by-products are used annually as pesticide co-solvents in the United States alone (Kay, 1974), and the estimation that the annual influx of petroleum into the ocean is roughly 3 million tons (Mallins, 1977) , it is essential that further studies be undertaken to assess the interaction of these chemicals with algae.

V - SUMMARY AND CONCLUSIONS

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The results of a field study conducted near Winnipeg, Manitoba demonstrated that fenitrothion contaminated river water following an operational spray program. The rapid dissipation (half-life less than 1 day) of the pesticide was considered to be due to its dilution within the water column, conversion to amino- and desmethyl-amino-fenitrothion (possibly via microbial degradation) and to its sequestration by aquatic macrophytes and suspended sediment. A subsequent field study conducted at Lac Bourgeois, Gatineau Park, Québec, confirmed the rapid dissipation of the pesticide (half-life less than 2 days) and demonstrated photodegradation of the pesticide to desmethyl-, carboxy-, and carboxy-amino-fenitrothion as well as hydrolysis to dimethyl thiophosphate. Rapid sequestration of fenitrothion by three species of unicellular algae (Chlamydomonas reinhardii, Chlorella pyrenoidosa and Euglena gracilis) was observed in the Lac Bourgeois field study and was confirmed by in vitro laboratory studies conducted with ^{14}C-fenitrothion and C. reinhardii. The high levels attained in C. reinhardii (9.66 ppt and 9.92 ppt in the Lac Bourgeois and laboratory studies, respectively) together with the observation that the pesticide formulation inhibited cell motility suggested that fenitrothion and/or its formulation additives (Aerotex 3470 and Atlox 3409F) were algicidal.

The results of preliminary in vitro bioassays demonstrated that Aerotex (1-10 ppm) inhibited algal growth in agar and liquid culture media, and decreased ATP levels. Further, Scenedesmus obtusiusculus was less sensitive to Aerotex than C. reihardii or C. pyrenoidosa. More extensive bioassays with C. reinhardii demonstrated that Aerotex (1-10 ppm):

1. inhibited cell motility, population growth, ATP synthesis and CO_2

fixation;

2. enhanced staining with methylene blue, reduction of DCIP and leakage of ^{14}C-photosynthate;
3. induced ultrastructural changes in membrane configuration.

These results suggested that Aerotex impaired the semi-permeability of algal cell membranes causing enhanced cell leakage, disruption of trans-membrane salt gradients and associated uncoupling effects. Although none of the studies, alone, unequivocally demonstrated Aerotex-induced membrane damage, the collated results provided strong circumstancial evidence that this was the case.

As a modern adaption of the "Meyer-Overton" theory of anesthesia, a general hypothesis was proposed that asserted that "lipophilic chemicals, including petroleum hydrocarbons as well as many pesticides, are algicidal by virtue of their ability to partition within the lipid matrix of algal cell membranes. Further, their ability to cause membrane damage will depend on their ability to concentrate in membrane lipids and hence should positively correlate with their lipophilicity". To test this hypothesis, an algal fluorometric screening procedure was developed to determine the relative algicidal activity for several aromatic hydrocarbon constituents of Aerotex as well as for several pesticides and formulation additives. These studies demonstrated that the algicidal activity (ICF_{100}) of the test chemicals could be positively correlated with their lipophilicity (K_{OW}) or inversely correlated with their water solubility, i.e.

$$(1)\quad \log ICF_{100} = 5.78 - 0.98 \log K_{OW} \qquad (r = -0.92)$$

$$(2)\quad \log ICF_{100} = 0.56 + 0.68 \log S \qquad (r = 0.94)$$

The experimental results, then, were consistent with the proposed hypothesis, however, further testing with a variety of lipophiles (insecticides, herbicides, PAH's, PCB's, etc.) would be warranted before concluding as to the comprehensive validity or predictive capacity of the obtained correlation.

During the course of study, several bioassay procedures were developed that could be implemented in future screening studies. The fluorometric method was considered to be an innovative procedure for assessing the relative algicidal activity of environmental pollutants. Although the determined ICF_{100} values for Aerotex (1-3 ppm) and Nonyl Phenol (0.5-0.75 ppm) were considered to be above the maximum in situ concentration of these additives, it was difficult to relate the results of these in vitro unialgal studies to the field situation. In this connection, the algal species used in the present study may be relatively insensitive and the in situ toxicity of lipophiles to algae may depend on such factors as salinity, temperature, pH, light intensity, nutrient supply, cell age, membrane lipid composition and algal population density. Further, the results suggested a potential additive effect of lipophilic pollutants in situ.

Since lipophilic chemicals are ubiquitous contaminants of aquatic habitats, it was considered essential that further studies be undertaken to assess the interaction of these chemicals with algae.

VI - REFERENCES

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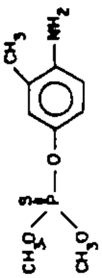
VII - APPENDICES

APPENDIX (i)

Fenitrothion degradation products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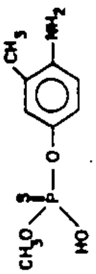
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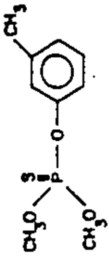
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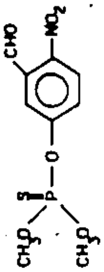
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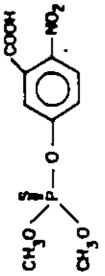
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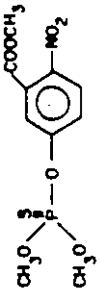
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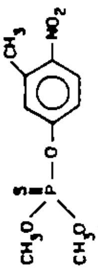
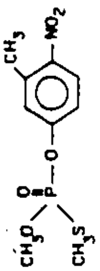

fenitrothion (F)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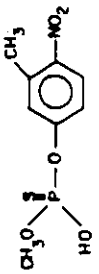
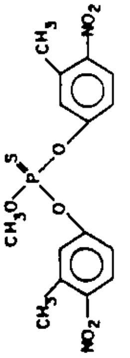
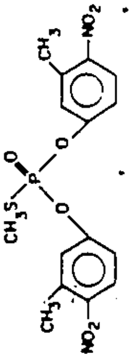

demethyl fenitrothion
(deMeF)


bis fenitrothion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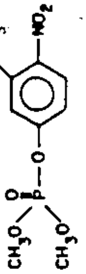
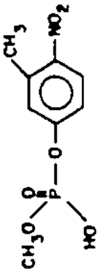

fenitrooxon(FO)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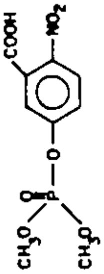
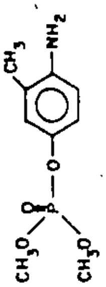
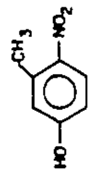
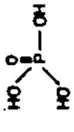
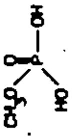
(deMeFO)


carboxy fenitrooxon

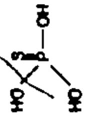

amino fenitrooxon

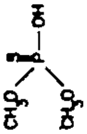

3-methyl-4-nitrophenol
(nitrocresol)

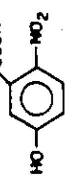

phosphoric acid
(PA)


methyl phosphoric acid


dimethyl phosphoric acid
(DMPA)


phosphorothioic acid
(PTA)


dimethyl phosphorothioic acid
(DMPTA)


3-carboxy-4-nitrophenol

APPENDIX (ii)

Calculation of maximum water concentration of fenitrothion

Assuming that fenitrothion is deposited on a water body at the upper rate (see text) of 413 gm/ha and the water depth = 1 cm, the water concentration of fenitrothion can be calculated as follows:

Since area of water = 1 ha = 1.00×10^8 cm^2(1),

the water volume for a depth of 1 cm = 1.00×10^8 cm^2 x 1 cm

= 1.00×10^8 cc

= 1.00×10^8 ml

= 1.00×10^5 l

Therefore, the water concentration

of fenitrothion = 413 gm/10^5 l

= 413 mg/100 l

= 4.13 mg/l

= 4.13 ppm

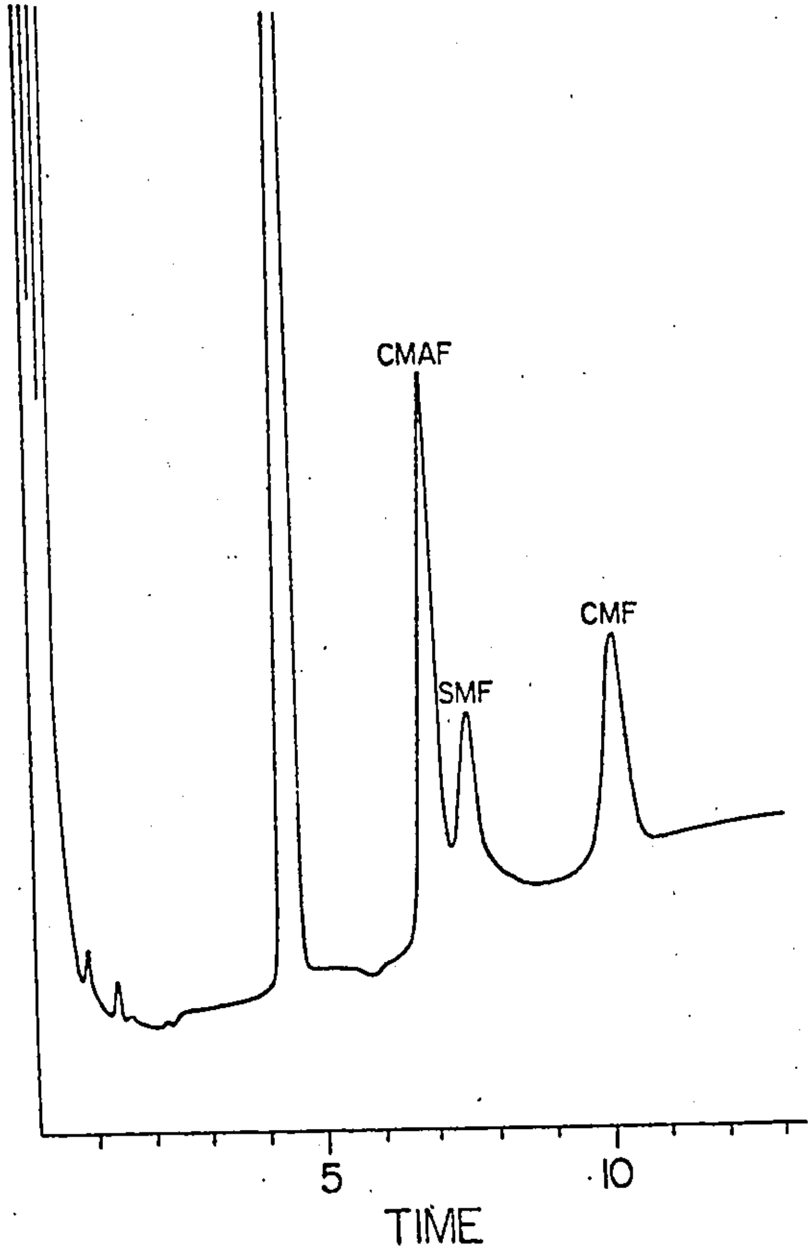
From the above, it is apparent that each 10 fold increase in water depth will result in a 10 fold decrease in concentration (e.g. for a 10 cm depth, concentration of fenitrothion = 0.41 ppm).

---

(1) 1 ha = 2.47 acres
1 acre = 4840 square yards
1 square yard = 8361 cm^2
Therefore, 1 ha = 2.47 x 4840 x 8361 = 1.00×10^8 cm^2.

APPENDIX (iii)

A gas chromatogram of the polar fraction of lake water from field study after lyophilization and methylation. The derivatives are carboxy-methyl-fenitrothion (CMF), carboxy-methyl-amino-fenitrothion (CMAF) and S-methyl-fenitrothion (SMF).



APPENDIX (iv)

Calculation of algal lipid/water partition coefficient from data obtained in the laboratory dialysis study.

1. Calculation of fenitrothion concentration in algae (6 hr sample time)

Replicate I

^{14}C-activity/ml (corrected for background and quenching) in dpm:

$$\begin{array}{ll} \text{Algae} & 28{,}617 \\ \text{Water} & \underline{-12{,}535} \\ & 16{,}082 \text{ dpm} \end{array} \qquad (1)$$

Since the SA of ^{14}C-fenitrothion = 5.54 mC/mM and the MW of ^{14}C-fenitrothion is 279 gm, then 1.23×10^{10} dpm = 279 mg. Therefore, from (1),

$$\begin{aligned} 16{,}082 \text{ dpm} &= \frac{16.082}{1.23} \times 10^{-10} \times 279 \text{ mg} \\ &= 3.65 \times 10^{-4} \text{ mg} \\ &= 365 \text{ ng/ml algae} \end{aligned}$$

From dry weight determinations,

1.23×10^{7} cells = 1.235 mg

In the present study, 1 ml of BBM contained 1.5×10^{7} cells, therefore:

1.5×10^{7} cells = 1.51 mg.

and therefore the algae had 365 ng ^{14}C-fenitrothion/1.51 mg or 242 ng/mg or 0.242 ppt (ug/mg).

Since there was initially 37.31 times as much cold fenitrothion (10 ppm) as ^{14}C-fenitrothion (0.268 ppm), then the total fenitrothion concentration in algae was 0.242 x 37.31 = 9.03 ppt.

2. Calculation of fenitrothion concentration in water

At 6 hr, the water (BBM) contained 12,535 dpm/ml. Using the SA for ^{14}C-fenitrothion as conducted previously yields 284 ng/ml ^{14}C-fenitrothion or 0.284 ppm. Correcting for the cold fenitrothion yields a total of 0.284 x 37.31 = 10.60 ppm.

3. Calculation of algae/water partition coefficient of fenitrothion (Kow)

$$K_{ow} = \frac{\text{(fenitrothion in algae)}}{\text{(fenitrothion in water)}}$$

$$= \frac{9.03 \text{ ppt}}{10.60 \text{ ppm}}$$

$$= 852$$

If the assumption is made that the algae contained 39% of their dry weight as lipid (Nichols, 1975) then, the algal lipid/water partition coefficient of fenitrothion (K_{lw}) is:

$$K_{lw} = 100/39 \times K_{ow}$$

$$= 100/39 \times 852$$

$$= 2,185$$

and log K_{lw} = 3.34.

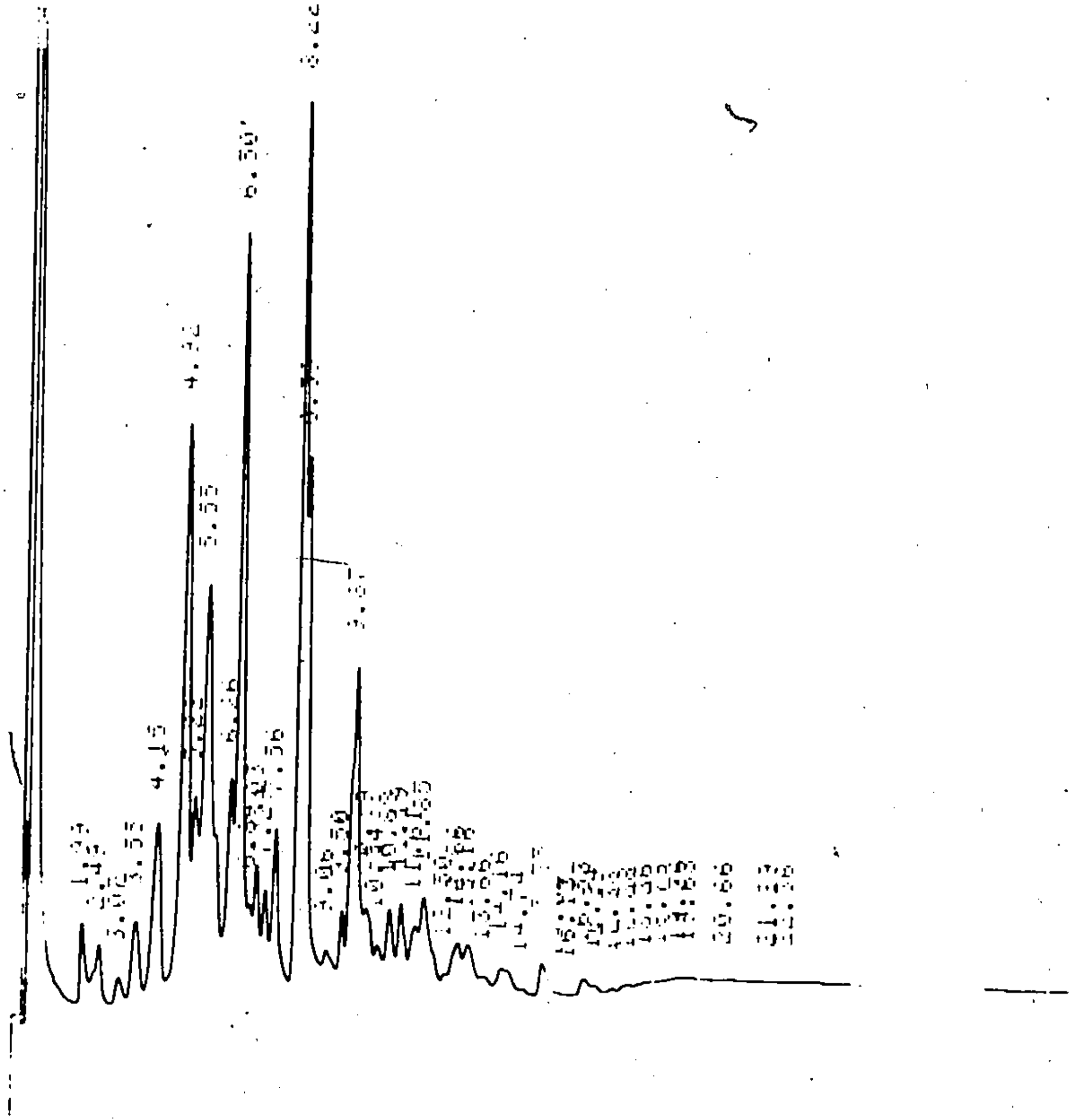
Similar calculations for the other two replicates samples gave algal fenitrothion concentrations of 7.05 and 10.67 ppt, and log K_{lw} values of 3.22 and 3.41, respectively.

Therefore, the average value at 6 hr for the concentration of algal fenitrothion was 8.92 ± 1.81 ppt and the average log K_{lw} was 3.32 ± 0.10.

APPENDIX (v)

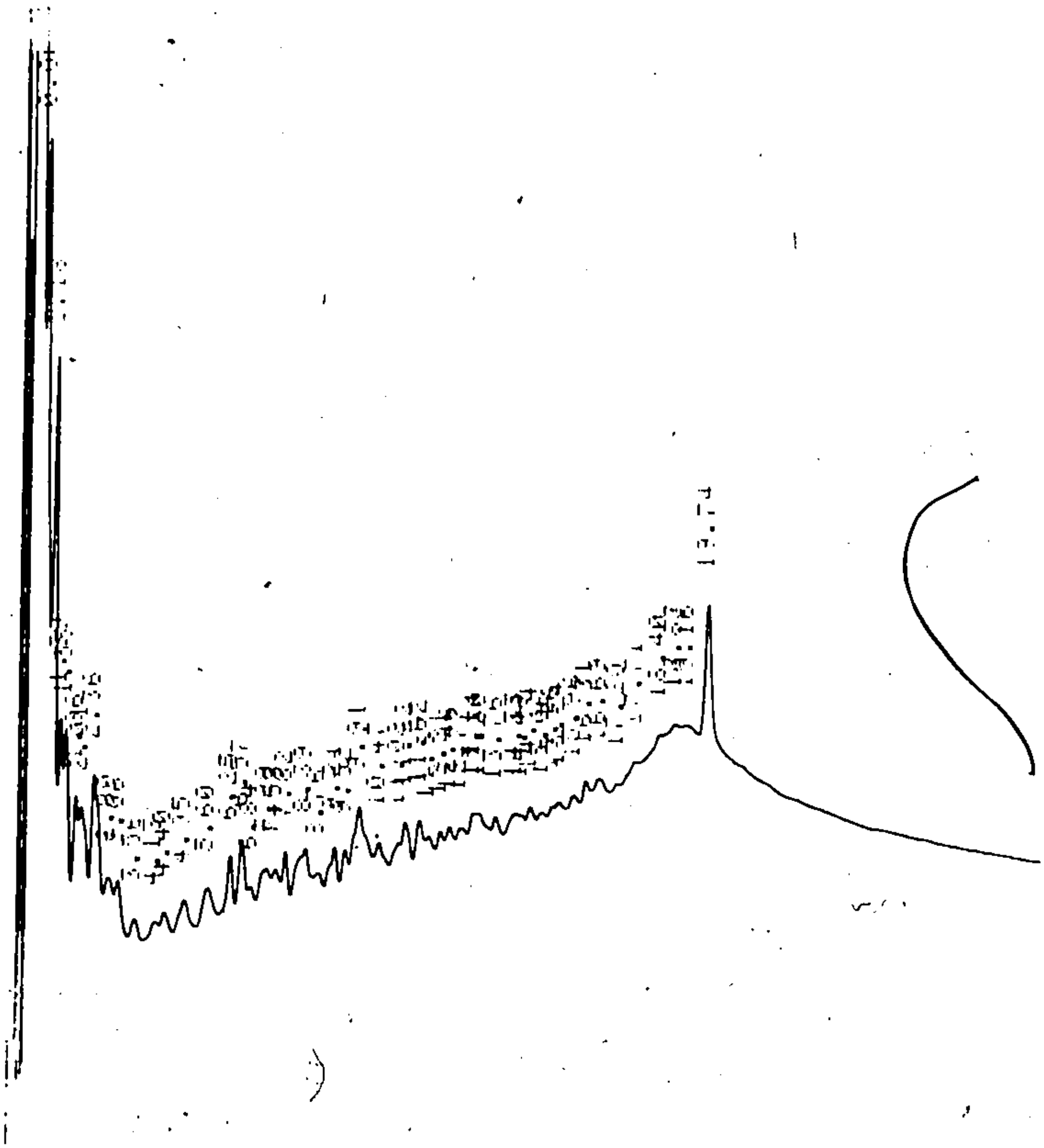
Gas chromatogram of Aerotex 3470

(1 ug; attenuation 2^8)



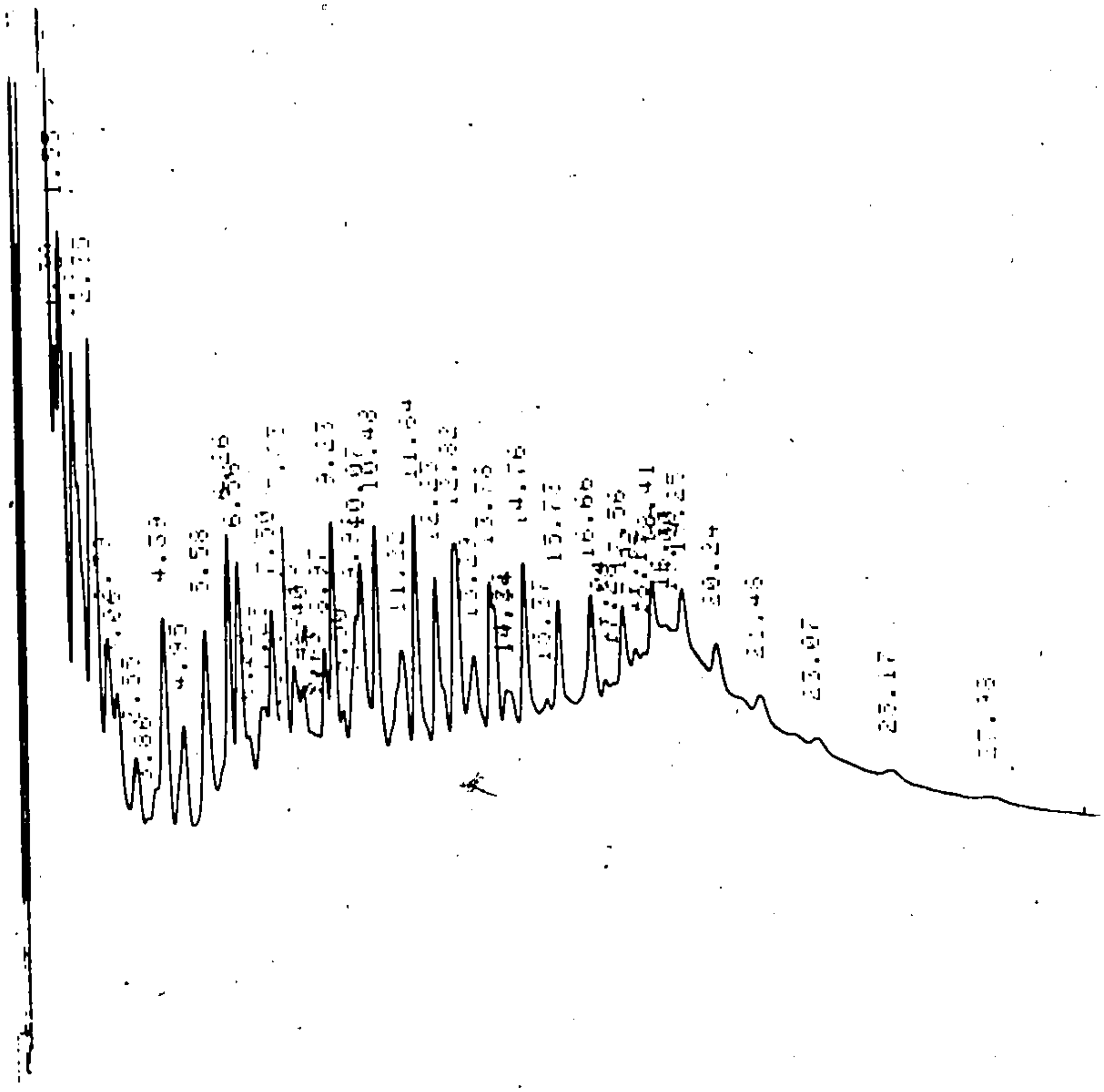
Gas chromatogram of Venezuelan crude oil

(1 ug; attenuation 2^6)



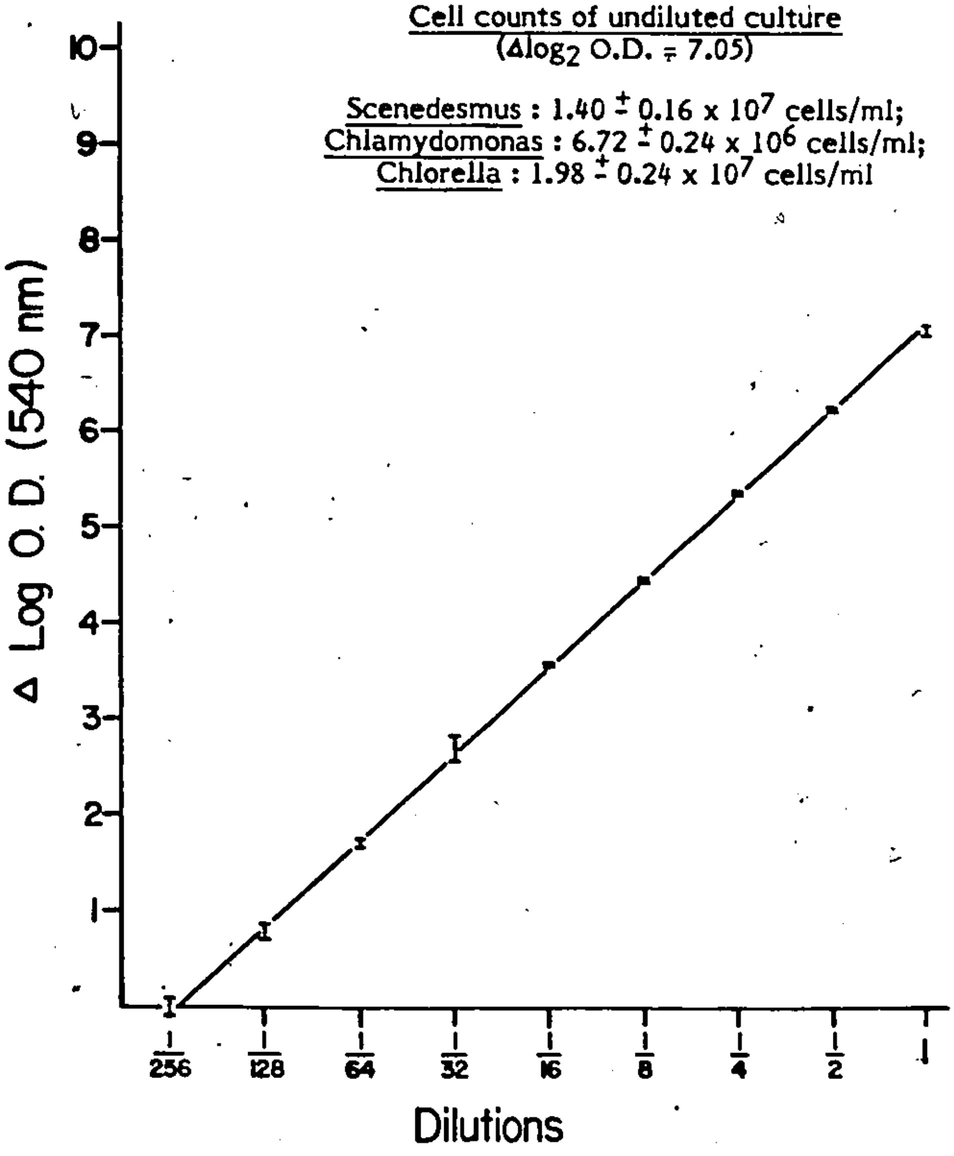
Gas chromatogram of Norman Wells crude oil

(1 ug; attenuation 2^6)



APPENDIX (vi)

Dilution curve for Scenedesmus, Chlamydomonas, and Chlorella
($\log_2$ O.D. readings for each species averaged together
with 3 replicate readings for each species)

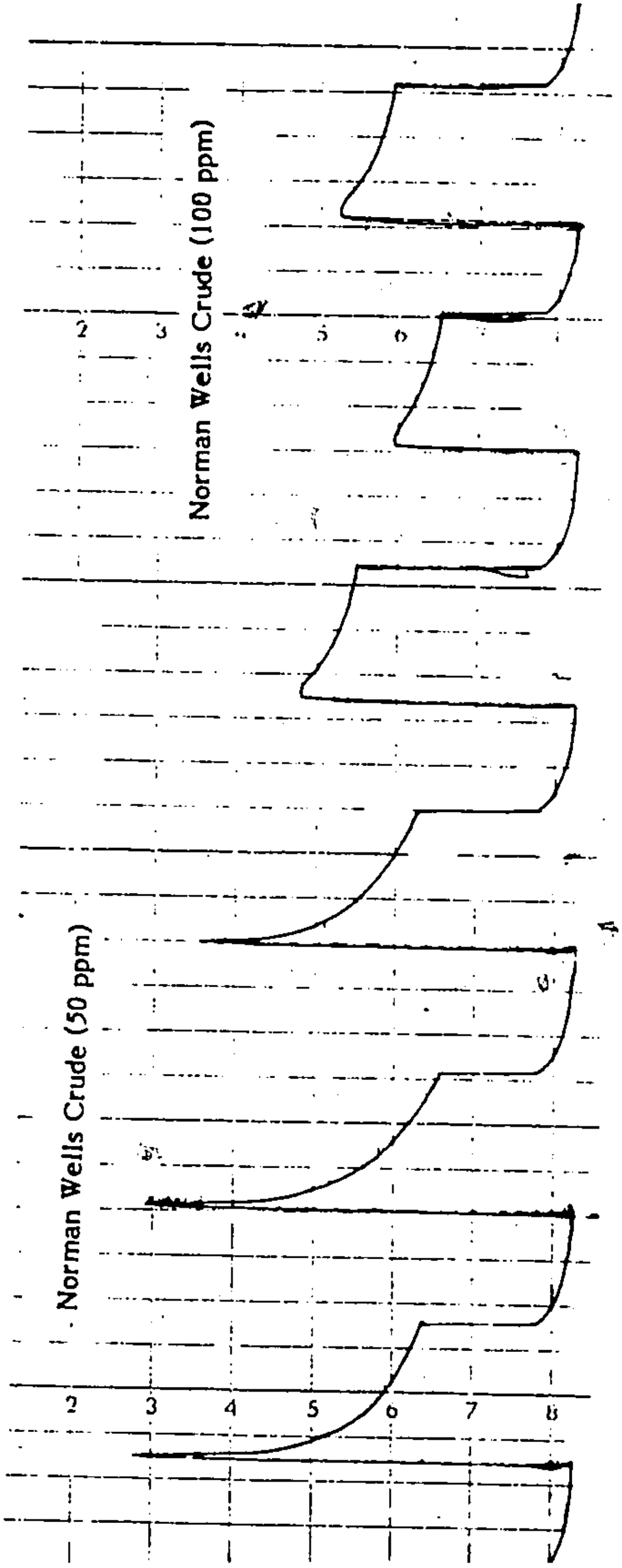


Cell counts of undiluted culture
($\Delta\log_2$ O.D. $\mp$ 7.05)

Scenedesmus : 1.40 $\pm$ 0.16 x 10^7 cells/ml;
Chlamydomonas : 6.72 $\pm$ 0.24 x 10^6 cells/ml;
Chlorella : 1.98 $\pm$ 0.24 x 10^7 cells/ml

APPENDIX (vii)

Fluorometer transients for C. reinhardii treated for 1 hr with Norman Wells crude oil



APPENDIX (viii)

The relation of partition theory to the uptake of lipophilic chemicals in algae and the algal lipid volume : Higher concentration at lower population density.

The Hertz (1909) formula relates the partition coefficient (K) to the number of extractions necessary to remove a given weight of solute from solution (Leo *et al.*, 1971). This formula, with symbols changed to conform to present usage, can be developed as follows:

If W ml of aqueous solution contains x_0 mg of solute extracted with L ml of an organic solvent (e.g. n-octanol), and x_1 mg of solute are extracted by the organic solvent, then x_1/L = concentration of solute in the organic phase and $(x_0 - x_1)/W$ = concentration in the water (mg solute remaining in water).

Since

$$K = \frac{[\text{solute in organic phase}]}{[\text{solute in water}]}$$

$$= \frac{x_1/L}{x_0 - x_1/W} \qquad (1)^1$$

Solving for x_1 (mg solute in organic phase), Equation (1) can be re-arranged as follows:

$$x_1 = \frac{K\,x_0}{(\frac{W}{L} + K)} \qquad (2)$$

---

[1] Leo *et al.* (1971) express the partition coefficient as P which = 1/K.

Since the concentration of solute in organic phase $[O] = x_1/L$, then from Equation (2);

$$[O] = \frac{K \; x_0}{L(\frac{W}{L} + K)}$$

$$= \frac{x_0}{\frac{W}{K} + L} \qquad (3)$$

Table 1 lists the calculated (Equation (3)) values for the concentration of a lipophilic pesticide that would result in the organic (lipid) phase following the establishment of a partition equilibrium with known values of x_0,L,W and K. These $[O]$ values are listed for decreasing volumes of the organic phase (L). Note that columns 1 to 3 list $[O]$ values for 10 mg/l (10 ppm) aqueous systems, for 3 different pesticides having K values in the order of 10^2 (e.g. 2,4-D), 10^3 (e.g. fenitrothion) and 10^6 (e.g. DDT). Columns 4 and 5 describe 0.1 ppm and 0.01 ppm systems respectively for $K = 10^6$.

It is apparent from Table 1 that:

1. There is a positive correlation between the partition coefficient (K) and the concentration $[O]$ of the pesticide in the organic phase.
2. Pesticides with relatively small K values ($\leq 10^3$) do not give significantly increased $[O]$ values when L is smaller than 10^{-4} l (i.e. a maximum value of $[O]$ is reached when L is smaller than 10^{-4} l). However, a pesticide with $K = 10^6$ does not reach a maximum $[O]$ until L 10^{-7} l.

TABLE 1

	1	2	3	4	5
x_o	10 mg	10 mg	10 mg	0.1 mg	0.01 mg
W	1000 ml	1000 ml	1000 ml	1000 ml	1000 ml
K	10^2	10^3	10^6	10^6	10^6
L (l)	[O] (mg/l)	[O] (mg/l)	[O] (mg/l)	[O] (mg/l)	[O] (mg/l)
10^{-1}	91	99	100	1	0.1
10^{-2}	500	909	1,000	10	1
10^{-3}	909	5,000	9.990	100	10
10^{-4}	990	9,090	99,000	990	99
10^{-5}	999	9,901	909,000	9,090	990
10^{-6}	1,000	9,990	5×10^6	5×10^4	5×10^3
10^{-7}	1,000	9,999	9.0×10^6	9.1×10^4	9.1×10^3
10^{-8}	1,000	10,000	9.9×10^6	9.9×10^4	9.9×10^3

3. Since DDT (K in the order of 10^6) has a solubility of 10% in olive oil (Sodergren, 1968), saturation of DDT in olive oil would occur (column 3) when L is $\leqq 10^{-4}$ l (0.1 ml) of olive oil (10% $\cong$ 100,000 ppm). Hence, experimental determination of $[O]$ would not show an increase in for L smaller than 10^{-4}. However, if smaller aqueous concentrations of DDT were used (columns 4,5) the effect would be observable.
4. $[O]$ is directly proportional to K. Hence if x_o = 10 ug (instead of 10 mg), the $[O]$ values listed in Table 1's columns 1-3 would be lower by a factor of 10^3 (i.e. they would be ppb instead of ppm).

This system, as well as being representative for bulk phase organic solvents[1] (olive oil), should also be representative of partitioning of lipophilic pesticides in algal fats and oils (lipids). The small lipid volumes per litre of water required to produce this "concentrating effect" are within the range of algal populations present in nature. Spherical unicells of 5u diameter with a population density of 10^8cells/l, would have a total volume of 6.6 ul (and a smaller lipid volume).

To test the ability of the derived formula to predict the bio-accumulation of DDT in algae, the concentration of DDT in algae was calculated using the data of Rice and Sikka, 1973. In their studies, 10 ml (W) of algae were exposed to 7 ng (x_o) of DDT (0.7 ppb). The concentrations of DDT in 6 algal species, $[A]$ were reported for algal population densities ranging from 0.1 to 1.0 mg cell dry

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[1] This phenomenon is taken advantage of in bulk phase partitioning studies. Small volumes of organic solvent (relative to the water volume) yield higher pesticide concentrations in the organic solvent for analysis (Leo <u>et al.</u>, 1971).

weight/10 ml culture. Assuming that 39% of the cell dry weight was lipid (Nichols, 1965), the cell dry weight/10 ml culture is readily expressed as lipid volume (L) as follows:

$$0.1 \text{ mg dry wt} = 0.1 \times 0.39 = 0.039 \text{ mg lipid}$$

Assuming that lipid volume is approximately equal to water volume,

$$0.039 \text{ mg} = 0.039 \text{ ul or } 0.039 \times 10^{-3} \text{ ml}^{1}$$

A value for K of 134,516 was calculated from the data given for the alga, Cyclotella nana. Since from the derived Equation (3) the DDT concentration in algal lipids is:

$$\begin{aligned}[A_1] &= \frac{x_0}{\frac{W}{K} + L} \\ &= \frac{7 \text{ ng}}{\frac{10 \text{ ml}}{134{,}516} + 0.039 \times 10^{-3} \text{ ml}} \\ &= 6.18 \times 10^{4} \text{ ng/ml lipid} \\ &= \underline{61.8 \text{ ng/mg}} \text{ lipid}\end{aligned}$$

and converting lipid weight back to cell dry weight, we obtain:

$$\begin{aligned}[A] &= 61.8 \times 0.39 \\ &= \underline{24.1 \text{ ng/mg}} \text{ cell dry wt}\end{aligned}$$

---

[1] This and the following underlined values are present in the second row of Table 2.

Following the above calculations, [A] values were calculated for other cell dry weights used by Rice and Sikka and are given in Table 2. Equation (3) is used where x_0= 7 ng, K = 134,516, and W = 10 ml.

Similar calculations performed for another species, Tetraselmis chui, (K = 19, 149) gave the [A] values listed in Table 3. Equation 3 was again used with x_0 = 7 ng, K = 19,149 and W = 10 ml.

These values for [A] are plotted versus cell dry weight in Figure 2. Note that this mathematically obtained plot is consistent with the experimentally determined data of Rice and Sikka (Figure 1). Note also that the algae with the smaller K value (T. chui) did not show the "concentrating effect" of small lipid volume.

TABLE 2

Calculated [A] values for C. nana

Cell dry Weight	L (ml)	[A] ng/mg lipid	[A] ng/mg cell dry wt
0.05	0.020×10^{-3}	74.2	30.0
0.10	0.039×10^{-3}	61.8	24.1
0.20	0.078×10^{-3}	46.0	18.0
0.40	0.156×10^{-3}	30.4	11.9
0.60	0.234×10^{-3}	22.7	8.9
0.80	0.312×10^{-3}	18.1	7.1
1.00	0.390×10^{-3}	15.1	5.9
1.20	0.468×10^{-3}	12.9	5.0

TABLE 3

Calculated [A] values for *T. chui*.

Cell dry Weight (mg)	L (ml)	[A] ng/mg lipid	[A] ng/mg cell dry wt
0.05	0.020×10^{-3}	12.9	5.0
0.10	0.039×10^{-3}	12.5	4.9
0.20	0.078×10^{-3}	11.7	4.6
0.40	0.156×10^{-3}	10.3	4.0
0.60	0.234×10^{-3}	9.3	3.6
0.80	0.312×10^{-3}	8.4	3.3
1.00	0.390×10^{-3}	7.7	3.0
1.20	0.468×10^{-3}	7.1	2.8

FIGURE 1

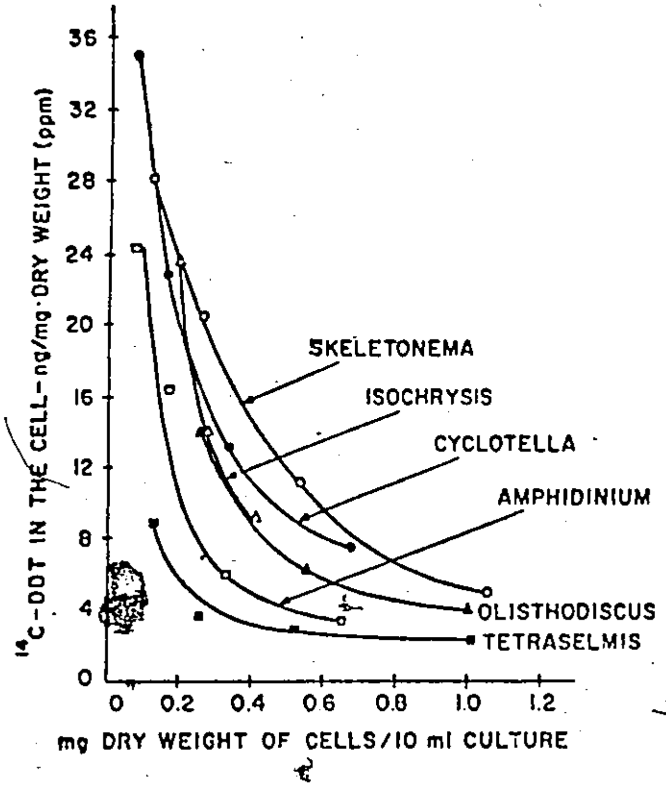
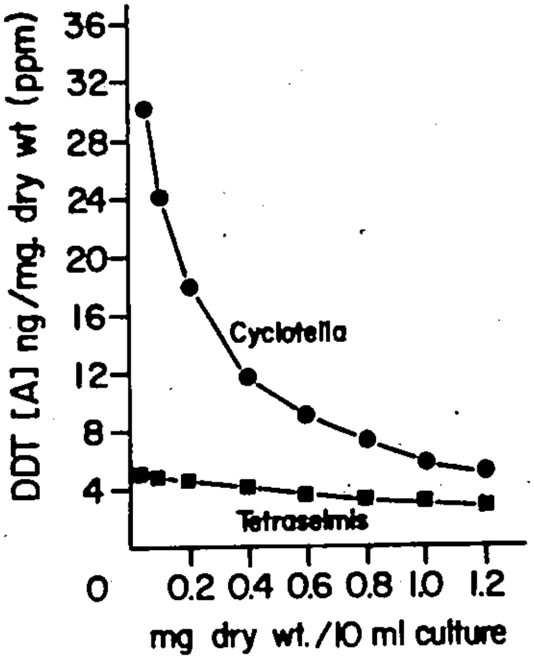


FIGURE 2



APPENDIX (ix)

A simple explanation for a maximum I O I below a critical L value

$$[\,0\,] = \frac{x_0}{\frac{W}{K} + L}$$

$$= \frac{x_0}{K^{-1} + L} \qquad \text{(if W = 1 litre)} \qquad (1)$$

When L is much smaller than K^{-1}, i.e. $L \ll K^{-1}$, then $K^{-1} + L = K^{-1}$

$$[\,0\,] = \frac{x_0}{K^{-1}}$$

$$= x_0\,K$$

This relation is evident from the data in Table 1 of Appendix (vii):

When $x_0 = 10$,

(and $K = 10^2$) $[\,0\,]_{max} = 10^3 = 10 \times K = x_0 K$ (Column 1)

Similarly,

(and $K = 10^3$) $[\,0\,]_{max} = 10^4 = x_0 K$ (Column 2)

and

(and $K = 10^6$) $[\,0\,]_{max} = 10^7 = x_0 K$ (Column 3)

When $x_0 = 0.1$

(and $K = 10^6$) $[\,0\,]_{max} = 10^5 = x_0 K$ (Column 4)

When $x_0 = 0.01$

(and $K = 10^6$) $[\,0\,]_{max} = 10^4 = x_0 K$ (Column 5)

Further, since

$$[\,0\,] = x_0\,K$$

$$\log\,[\,0\,] = \log\,(x_0 K)$$

$$\text{or:}\quad \log\,[\,0\,] = \log x_0 + \log K \qquad (2)$$

Since in a 1 litre system, x_0 mg = x_0 mg/l (initial water concentration) and the toxic concentration $ICF_{100} = x_0$ and $\log ICF_{100} = \log x_0$ (from (2)), then:

$$\log [0] = \log ICF_{100} + \log K \qquad (3)$$

where [0] is the concentration in lipids required to cause toxicity.

Using Equation (3) to sum the log ICF_{100} and log Kow values listed in Table 11 in the "Results" section of the thesis that were used in Figure 9 gives the following average value for log $[A_1]$, the concentration of the chemical in the algal lipids ($[A_1] \equiv [0]$ in Equation (3)):

$$\text{Average } \log [A_1] = 5.88 \pm 0.36 \text{ uM/l}$$

Similarly, calculations using Hutchinson's (1980) data gives:

$$\text{Average } \log [A_1] = 5.32 \pm 0.44 \text{ uM/l}$$

Hence, it is shown that the log of the concentration of any lipophile in the algal lipids to cause toxicity is a constant for the present data and also for the data of Hutchinson et al. (1980) which employed 38 chemically non-related compounds.

APPENDIX (x)

The correlation between log Kow and log S

The present studies demonstrated that:

$$(1)\quad \log ICF_{100} = 5.78 - 0.98 \log Kow$$

and

$$(2)\quad \log ICF_{100} = 0.56 + 0.68 \log S$$

Therefore:

$$5.78 - 0.98 \log Kow = 0.56 + 0.68 \log S$$

$$(3)\quad \log Kow = 5.33 - 0.69 \log S$$

This corroborates the empirical equation of Chiou et al. (1977) where:

$$(4)\quad \log Kow = 5.00 - 0.67 \log S$$

APPENDIX (xi)

Original ATP data

Algal ATP levels vs Aerotex concentration

- 1 hr. treatment in liquid culture.
- average values of three replicates.

Aerotex (ppm)	ATP ng/mg (d.w.) S	M	E	ng/cell x 10^{-4} S	M	E
0.0	328	338	3,422	0.28	1.22	4.40
0.1			2,744			3.53
1.0	318	210	2,755	0.27	0.76	3.54
2.0	317	197	2,361	0.27	0.71	3.04
5.0	266	175	2,306	0.23	0.63	2.96
10.0	270	96		0.23	0.35	

S = Scenedesmus obtusiusculus

M = Chlamydomonas reinhardii

E = Chlorella pyrenoidosa

APPENDIX (xii)

The ecological consequences of algae/pesticide interactions

Before closing this manuscript , it is desired to re-emphasize the ecological consequences of algae-pesticide interactions. The algae constitute the basic link in aquatic food chains and hence their contact with pesticides may lead to biomagnification of pesticide residues up the food chain, as well as to detrimental effects on primary consumers which have species-specific food requirements (Biggs et al, 1970; Birmingham and Colman, 1977; O'Brien and Dixon, 1976). It should be noted that even the sublethal effect reported here concerning the lag phase extension may suffice in situ to alter algal species composition in vernal bloom populations, at a time coincidentally concurrent with pesticide deposition for budworm control in Canadian forests (Armstrong and Randall, 1969).

The algae selected for study are ubiquitous in both freshwater and marine habitats. For example, several thermophilic, psychrophilic, halophilic and even terrestrial species of Chlamydomonas have been reported (Stewart, 1974). These algae (Chlamydomonas, Chlorella and Scenedesmus) have been refered to as "weedy" species and thrive in areas of high organic pollution (O'Brien and Dixon, 1976). Given their reported tolerance to pollutants in general (Palmer, 1969), the findings of the present thesis that relatively low concentrations of pesticides (or their formulants) are algicidal, strongly warrants that further studies be conducted to encompass other more susceptible species.

APPENDIX (iv)

Calculation of algal lipid/water partition coefficient from data obtained in the laboratory dialysis study.

1. Calculation of fenitrothion concentration in algae (6 hr sample time)

Replicate I

^{14}C-activity/ml (corrected for background and quenching) in dpm:

Algae 28,617
Water -12,535
16,082 dpm (1)

Since the SA of ^{14}C-fenitrothion = 5.54 mC/mM and the MW of ^{14}C-fenitrothion is 279 gm, then 1.23×10^{10} dpm = 279 mg. Therefore, from (1),

$$16{,}082 \text{ dpm} = \frac{16.082}{1.23} \times 10^{-10} \times 279 \text{ mg}$$

$$= 3.65 \times 10^{-4} \text{ mg}$$

$$= 365 \text{ ng/ml algae}$$

From dry weight determinations,

1.23×10^{7} cells = 1.235 mg

In the present study, 1 ml of BBM contained 1.5×10^{7} cells, therefore:

1.5×10^{7} cells = 1.51 mg

and therefore the algae had 365 ng ^{14}C-fenitrothion/1.51 mg or 242 ng/mg or 0.242 ppt (ug/mg).

Since there was initially 37.31 times as much cold fenitrothion (10 ppm) as ^{14}C-fenitrothion (0.268 ppm), then the total fenitrothion concentration in algae was 0.242 x 37.31 = 9.03 ppt.

2. Calculation of fenitrothion concentration in water

At 6 hr, the water (BBM) contained 12,535 dpm/ml. Using the SA for ^{14}C-fenitrothion as conducted previously yields 284 ng/ml ^{14}C-fenitrothion or 0.284 ppm. Correcting for the cold fenitrothion yields a total of 0.284 x 37.31 = 10.60 ppm.

APPENDIX (vii)

Fluorometer transients for C. reinhardii treated for 1 hr with Norman Wells crude oil