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BIODEGRADATION OF THE ORGANOPHOSPHORUS INSECTICIDE FENITROTHION BY THE ALGA CHLAMYDOMONAS REINHARDTII: THE ROLE OF CYTOCHROME P450 MONOOXYGENASE

By Mary Francis Ladouceur

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

July 1992

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To my parents I express loving gratitude for their moral support and understanding throughout these years of study.

This thesis is dedicated to Dr. Pearl Weinberger.

ABSTRACT

Cultures of Chlamydomonas reinhardtii (1 x 10^6 cells/mL) were incubated with 5.0 μg/mL of the insecticide fenitrothion, (FEN), [O,O-dimethyl-O-(3-methyl-4-nitrophenyl)phosphorothioate]. The rate of abiotic fenitrothion degradation in Gorman and Levign growth medium (pH=6.8, T=21 °C,Vita Lite^R 40 w/m^2), was enhanced 9-fold in the light relative to the dark under abiotic conditions ($T_{1/2}$ values of 145 h (light) and 54 days (dark)). C. reinhardtii cultures had fenitrothion half life values of 53.1 h and 143 h in the light and dark respectively. This represented a 3-fold difference in degradation rate in the light relative to abiotic degradation.

Endogenous metabolism by C. reinhardtii produced significant amounts of 3-methyl-4-nitrophenol (nitrocresol), demethyl fenitrothion, formyl fenitrothion, hydroxymethyl fenitrothion, and carboxyfenitrothion within the cells. This biodegradation was slower in the dark than in the light. Elimination of carboxy fenitrothion in the light and 3-methyl-4-nitrophenol and formyl fenitrothion in the dark, from the intracellular fractions, contributed to the existing levels of these metabolites produced by extracellular abiotic reactions (hydrolysis, oxidation, photolysis and photooxidation).

Studies of algal biodegradation of fenitrothion in the presence of 5.0 μg/mL fenitrothion plus 23.2 μg/mL phenobarbital,(a cytochrome P_{450} monooxygenase inducer), demonstrated a 10 fold increase in the levels of intracellular hydroxymethyl fenitrothion relative to control algal cultures. This metabolite of cytochrome

P_{450} monooxygenase was also inhibited in algal cultures incubated with piperonyl butoxide, an inhibitor of the enzyme's activity. Piperonyl butoxide also produced a 1.7 fold increase in fenitrothion half life, due to inhibition of PSMO.

It was concluded from this study that degradation of fenitrothion by metabolic activity of cytochrome P_{450} monooxygenase may be a significant determinant in fenitrothion degradation in C. reinhardtii cultures. Hydroxymethyl fenitrothion and demethyl fenitrothion metabolites were produced in this alga at significant levels by this enzyme complex. Subsequent hydrolysis and oxidation reactions by other endogenous phase II enzymes, produced 3-methyl-4-nitrophenol, fenitrooxon, formyl fenitrothion, carboxy fenitrothion, and carboxy fenitrooxon. The amounts of formyl fenitrothion, carboxy fenitrothion and, carboxyfentrooxon were demonstrated to depend on the availability of intracellular hydroxymethyl fenitrothion pool produced by algal PSMO activity, while NC and DSM levels were attributable to other cellular enzymes.

The phenomenon of fenitrothion degradation by endogenous metabolism in C. reinhardtii may be a significant biotic factor in common with other non-target aquatic organisms, which impacts on the chemical fate of xenobiotic residues in the environment.

Abbreviations

CFO	carboxy fenitrooxon
CFT	carboxy fenitrothion
DSM	demethyl fenitrothion
DMPA	di-methylphosphoric acid
DMPTA	di-methylphosphorothioic acid
FEN	fenitrothion
FO	fenitrooxon
FF	formyl fenitrothion
MPA	methylphosphoric acid
MPTA	methylphosphorothioic acid
NC	3-methyl-4-nitrophenol
OHMEF	hydroxymethyl fenitrothion
PA	phosphoric acid
PSMO	polysubstrate monooxygenases
RER	rough endoplasmic reticulum
SER	smooth endoplasmic reticulum
SMF	s-methylfenitrothion

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CHAPTER 1

1.0 INTRODUCTION

Humans live and thrive in their environments with many other organisms. All organisms require food and shelter as essential elements for successful reproduction and survival. There are numerous competitive interactions between animal species for food. In addition, the human population is rapidly approaching 6 billion people (Buchel, 1983), which places a tremendous stress on the global habitat and resources as we attempt to maintain public health against *specific bacterial and viral agents.*

Humans have classified these competing animals and microorganisms as pests. Biocidal compounds which kill these pests have been developed ranging from organometallic compounds to organochlorine, organophosphate, carbamate, and urea compounds. A given pest can be controlled by more than one group of chemical compounds. Pesticides eradicate or control populations of pest organisms using acute lethal doses as low as parts per billion.

1.1 Pesticide Effectiveness

An effective pesticide is one that has a unique biochemical target in the pest organism, such that it does not intoxicate non-target organisms. Non-target organisms are those organisms which are not intended to be controlled nor eradicated by the pesticide under consideration. Efficient pesticides have immediate toxic action. Immediate eradication of a pest population is necessary, because financial losses through pest damage can not be sustained.

Effective pesticides must be degraded quickly after pest intoxication has occurred to avoid the intoxication of non-target organisms. Physical, chemical and biological factors in the soil, air and aquatic environments contribute to this process and determine the residual pesticide toxicity to non-target organisms.

1.2 Cost/Benefit Analysis for Pesticides

An alternative assessment of pesticide efficiency is based on the cost/benefit ratio (Coulston, 1973). Cost and benefit of pesticides can be evaluated from both economic and ecological viewpoints.

1.2.1 Economic Factors

Cost is evaluated economically in terms of profit obtained from the sale of a pesticide product, relative to the expenses incurred in the research, development, (R & D), and manufacture of a pesticide. Expenses in R & D arise due to the necessity for biological screening of a pesticide for its toxicity, its metabolic and abiotic degradation, and its persistent residue behaviour and ecology. Field studies which test pesticide activity, determine formulation requirements, application rates, and patent protection are also costly (Niessen, 1975).

The financial benefit from the investment in pesticide manufacture is the monetary return from intensive use of pesticide products. Labour costs for mechanical weeding and harvesting are alleviated. The high demand for pesticides in domestic,

agricultural, and forestry applications sustains the industry and supports national economies (Buchel and Bayer, 1983).

1.2.2 Ecological Factors

The ecological benefits of pesticide use are two-fold. Human health is maintained by reducing insect borne disease such and malaria and yellow fever, and by the successful control of pest populations in forests, agricultural crops and in our homes. Prey populations may be protected by controlling the pest predator, as in the case of protection of forests from the variety of lepidopteran predator species. These benefits have been evidenced in third world countries where the use of pesticides reduce food product losses, and maintain food supply, addressing the problem of unequal global food distribution. A common opinion in third world countries are that these benefits from using pesticides, is worth the cost of risk to human health that may result from their misuse (Buchel, 1983).

The ecological damage of pesticide use results from abuse and increased dependency on pesticides. The many years of monoculture and intensive pesticide use has been accompanied with an increase in the resistance of the pest population to the pesticide applied. It has also resulted in cross-resistance to related pesticides. This requires that increased levels of pesticide be applied to the susceptible crops to control the pests.

1.3 Ecological Risk Identification and Control

Organisms which the pesticide is not used against (non-target organisms) are often exposed to pesticides or to their toxic metabolites. The hazards associated with this exposure, if severe enough, will cause physiological damage, or impact on population dynamics. Ecological interactions in aquatic and terrestrial environments may be impacted upon and in these respects the true ecological cost of pesticide use is not predictable (McEwen and Stephenson, 1979).

The problems of environmental impact are addressed in studies during biological screening of new pesticides prior to registration. If a marketed pesticide demonstrated severe health risks, from epidemiology studies for example, these pesticides have been banned by national legislative bodies and similar regulatory agencies. These products include lindane and heptachlor (1970), chlordane (1978), and alachlor (1985) (IASA, 1986).

Much research and development for biological screening is necessary to satisfy the regulations for environmental safety and yet still produce an effective product. Current pesticide use and manufacture is controlled by regulatory bodies. Previously registered pesticides, are also reevaluated in this context, specifically in the area of the impact of toxic persistent residues on non-target organisms (IASA, 1986).

Some alternative measures to control pests without pesticides include hormone mimics, bacterial and viral toxins, parasites, attractants, and chemosterilants. Alternative herbicidal agents

include plant hormones and growth regulators, natural plant chemical defenses, and biotechnology developments in breeding resistance into plants. These alternative pest control measures are useful where some damage can be tolerated, and can be integrated into a multifaceted pest management program.

1.4 Environmental Fate of Pesticides

The environmental fate of a pesticide depends on its rate of degradation. The rate of degradation will determine the pesticides availability, while the degradation products will determine the persistence of pesticide toxicity. Non-target organisms can contribute to pesticide degradation, but other environmental factors will determine the significance of the non-target organisms' influence.

1.4.1 Environmental Distribution

The exposure of pesticide residues to degradative factors depends on the micro-environmental compartment in which the residues persists. In aquatic environments, these micro-environmental compartments include the water column, sediments, suspended solids (humic substances), and biological sinks where bioaccumulation can occur such as bacteria, plants, invertebrates and vertebrates (Lockhart et al., 1982, Travis et al., 1988).

1.4.2 Degradation

The degradation rates of pesticides depend on the type of compound, and this property is unique to each pesticide. Two external factors that affect pesticide degradation include exposure to biotic and abiotic conditions.

1.4.2.1 Abiotic Degradation

Abiotic degradation processes are non-enzymatic chemical reactions. These include hydrolysis, photolysis, and adsorption to humic substances (Miller and Hebert, 1987). Hydrolysis is the breaking of a bond by the addition of water. Hydrolysis can be either base catalysed by the nucleophilic hydroxyl ion or catalysed by low pH. Photolysis reactions are hydrolysis reactions where the addition of light energy provides energy for breaking bonds. Interaction of ionic bonds and reactive groups of humic substances may adsorb pesticides and light adsorption by the humic substance will photosensitize the pesticide and facilitate hydrolysis.

1.4.2.2 Biotic Degradation

Microorganisms, algae, plants, and invertebrates can degrade xenobiotic residues in soil or water. Pesticide residues may also be degraded after accumulation into vertebrates (Miyamoto et al., 1979). This potential biotic degradation is due to endogenous metabolism of these organisms.

Knowledge of the pesticide's chemical structure permits the prediction of their bioaccumulation, persistence and biochemical

fate. (Freed et al., 1977). Accumulation and storage of pesticide residues is related to and depends on the compound's solubility in water and its octanol/water partition coefficient. These properties are directly correlated with lipid membrane solubility and biomagnification potential (Lu and Metcalf, 1975).

Partitioning of pesticides into cytosolic or lipid cellular fractions determines which enzymes are available for their degradation. Non-polar xenobiotics tend to be sequestered and metabolized in lipid fractions while the more polar xenobiotics are localized in the cytoplasm and conjugated into excretable forms. Xenobiotic degradation is mediated by mixed function oxidase in the lipid fractions followed by conjugation in the cytosolic fraction.

1.5 Enzyme Function

Some definitions for chemical reactions and enzyme function are needed to describe the degradative capabilities of enzyme systems in non-target organisms.

1.5.1 Chemical Reactions

Detoxification of pesticides in non-target organisms is mediated by biological enzymes. All reactions have a requirement for activation energy (ΔG°) (Laidler,1978).

1.5.1.1 Activation Energy

Activation energy is the energy required to form the transition species of the reaction possessing the most energy. This energy barrier ΔG° must be overcome in order for a reaction to proceed. Activation energy has two components; (1) entropy, which is temperature dependant (S), and (2) enthalpy, (H), which depends on bond formation and cleavage.

Enthalpy is a measure of the bond strength of the reacting bonds which are broken formed during a reaction (Laidler, 1978). The energy required to break a bond represents an input of enthalpy and is positive. Bond formation releases enthalpy which is negative.

Entropy (S) is a measure of the energy of randomness of a system (Laidler, 1978). Entropy is lost when bonds are formed, and is gained when bonds are broken.

The activation energy ΔG° must be added to the reaction system in order for the reaction to proceed, by activating the reactants to the transition state complex, which has the highest probability of forming the products through the breaking of bonds.

1.5.1.2 Rates of Reactions

In biological systems, metabolism occurs through spontaneous reactions in cycles of energy exchange. The rates of these reactions however, are slow unless promoted by enzyme catalysis. Catalysts combine with reactants to lower activation energy of transition states. The uncatalysed rate can be predicted from the

difference in thermodynamic stability of the products compared to that of the reagents which is known as the overall energy of reaction ΔG° (Lehninger, 1975). This general rule is Hammond's Postulate and is stated quantitatively as the "Linear Free Energy Relationship" in which the activation energy is a function of the Gibbs free energy of the reaction:

$$\Delta G^{\ddagger} = f(\Delta G^{\circ})$$

$$= f(\Delta H^{\circ} - T\Delta S^{\circ}) = (H^{\ddagger} - T\Delta S^{\ddagger})$$

Here, the free energy barrier to reaction, $\Delta G^{\ddagger}$ increases linearly as the free energy difference between the reagent and product, ΔG°. Hammonds's Postulate relates the geometry of the transition state to the geometry of the reactants and the products and suggests that the free energy of the transition state (that is the $\Delta G^{\ddagger}$ will approximate the free energy of the side (reactants or products) which it most resembles in geometry.

$\Delta G^{\ddagger}$ is also related to the rate of reaction. Hammonds Postulate can be interpreted in thig context when considering transition state diagrams in which the location of the transition state complex is located early along the reaction coordinate in fast reactions and later along the coordinate in slow reactions. Transition complexes formed early in the reaction generally require less activation energy while those formed later in the reaction sequence require more activation energy $\Delta G^{\ddagger}$.

Experimentally, the rate of a reaction is:

$$\text{Rate} = k\,[A]^{a}$$

in which k is the rate constant at one temperature and pressure and

$[A]^a$ are the concentrations of reagents to a power a, depending on the order of the reaction. This applies to a reaction such as:

A -------->B

where the equilibrium constant is K = [B]/[A], and the rate of reaction is equal to the disappearance of A or $-d[A]/dt = k[A]^a$ (March, 1971). The rate of reaction is related to equilibrium constant K, and under first order conditions, when a=1, such that

$$G^\cdot = -RT \ln K^\cdot$$

where $K^\cdot$ is related to the appearance of the activated complex of A ($A^\cdot$). By substitution for $\blacktriangle G^\cdot$, $f(\blacktriangle G^\circ) = -RT \ln k$

Thus the rates of reactions are expected, in most cases, to be predictable from knowledge of the free energies of formation of reagents and products, which are values available in standard tables and are calculated according to Hess' law. Hess' law of constant heat summation states that the total heat effect in a chemical reaction is independent of the number or order of steps, but depends only on the starting and final states of the system under consideration (Mortimer, 1975).

Since this is a general model of chemical reactivity, it applies not only to endogenous biochemicals but also to xenobiotics, including pesticides. Most pesticides are small molecules and the products can be predicted and often experimentally characterized. The $\blacktriangle G^\circ$ calculations can then be used to predict $\blacktriangle G^\cdot$ and hence reaction rates, k, for degradation processes. In most situations these rates are very slow at biological temperatures. Thus degradation is only possible at

significant rates if promoted by enzymes.

1.5.2 Enzyme Catalysis

Enzyme inhibition by pesticides disrupts the optimum reaction conditions. Enzymes enhance optimal reaction conditions of a reaction by lowering the free energy of activation ($\blacktriangle G^{\bullet}$) of a reaction (Laidler, 1978). This is due to enzyme structure and interactions of enzymes with substrates.

Biological reactions requiring enzymes include condensation of amino acids to form proteins and hydrolysis reactions of glycerides. The process of breaking the water molecule to form hydroxide and protons is common to all biological reactions, and has the highest enthalpy of activation requirement of any biological reaction. As a result, the difference between biological substrates in their activation energy requirements therefore depends on the net change of entropy of reaction rather than enthalpy. Therefore enzymes function in biological reactions by lowering the entropy activation energy requirement.

1.6 Microsomal Monooxygenase

Polysubstrate Monooxygenase (PSMO) is an important lipid soluble mixed function oxidase enzyme system, involved in metabolism of xenobiotics (Ortiz de Montellano, 1986). The system is present in bacteria, fungi, plants, invertebrates and vertebrates. PSMO has been most extensively studied in mammals namely, mice, rats and rabbits. It has also been studied in humans

where its endogenous content is much lower than that induced in laboratory animals (Wang et al., 1980). It is located in microsomal fractions of SER and RER (smooth endoplasmic reticulum and rough endoplasmic reticulum) of hepatic tissue.

PSMO is also found in lung, kidney, skin and gastro-intestinal tract. In plants, cytochrome P_{450} monooxygenase has a significantly lower enzyme activity and exists in fewer forms than does the animal monooxygenase. It has been detected in the microsomes of rhizomes, tubers or roots, seed endosperm, hypocotyl, radicle or cotyledons and in photosynthetic tissues such as stems, leaves, apical buds, inflorescence or fruit (Hendry, 1986). Cytochrome P_{450} monooxygenase is also found in microorganisms (Pseudomonas putida) and in fungi (Fusarium oxosporum)(Shoun et al., 1989).

1.6.1 Components of Microsomal Monooxygenase

The essential functional components of the PSMO complex includes the active site cytochrome P_{450} (EC 1.14.14.1), in a globin protein embedded in lipid. This is accompanied by cofactors, NADPH cytochrome P_{450} reductase (EC 1.6.2.4), NADPH reducing power, cytochrome b_5 and its reductase (Figure 1).

The structure of cytochrome P_{450} protein includes a haem moiety and an apoprotein. The haem moiety is synthesized in the mitochondria, while the apoprotein is transcribed on ribosomes and is inserted into microsomal membranes. The haem is later incorporated into the apoprotein molecule. The resulting cytochrome protein has four active sites for substrate hydroxylation.

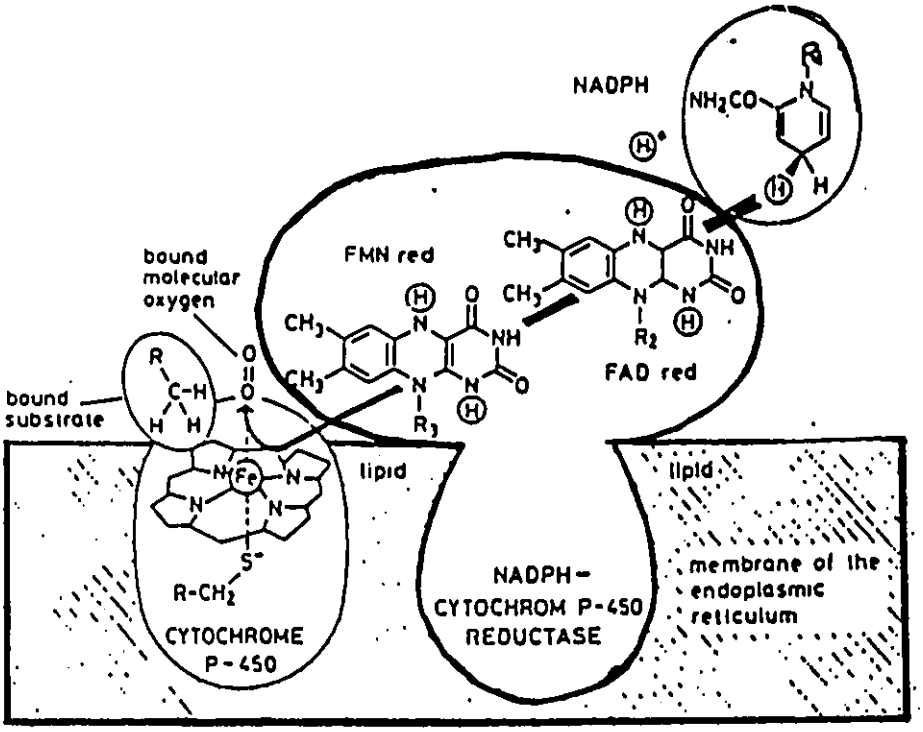


FIGURE 1

Schematical drawing of the constituents of the mammalian liver microsomal cytochrome P_{450} monooxygenase enzyme complex (Ortiz de Montellano, 1986).

Different isozymes of cytochrome P_{450} exist, based on different substrate specificity, molecular weight, enzyme activity, species and response to inducing agents. Phenobarbital induced cytochrome P_{450} of rabbits is a 55,464 dalton protein (Heinemann and Ozols, 1983), while 3-methylcholanthrene-induced rats have a 53,000 dalton cytochrome protein.

NADPH dependant-cytochrome P_{450} reductase is a flavoprotein consisting of one molecule each of FMN (flavin mononucleotide) FAD (flavin adenine dinucleotide). It can exist in any of four electron reduced species which are produced during oxidation of a monooxygenase substrate (Coon et al.,1982). It is located outside the endoplasmic reticulum membrane bound by a 70 amino acid residue. It is distributed homogenously in the rough and smooth endoplasmic reticulum. This enzyme forms a 1:1 complex with the cytochrome P_{450} protein.

The cytochrome b_5 reduction system with its NADPH reductase flavoprotein is also located in the rough endoplasmic reticulum with lateral heterogeneity and is a component of the cytochrome P_{450} complex. Cytochrome b_5 is involved in the donation of electrons into P_{450} through NADPH. It does not form a complex with the cytochrome P_{450} protein nor with cytochrome P450 reductase.

Phospholipids are an essential component in which the cytochrome P_{450} monooxygenase is imbedded. The key lipid is dilauroyl phosphatidylcholine (Janig et al., 1977). The lipid interacts with the PSMO complex and determines the overall monooxygenase activity by regulating the rotation of the enzyme system within the

membrane.

1.6.2 PSMO Function

PSMO activity converts C-H groups into C-OH The C-H bond strength determines the oxidizing power required to hydroxylate the substrate. All PSMO substrates have the same requirement for activated oxygen, and hence the same energy requirement for activation of the oxygen. This activated oxygen must be inserted into the substrate C-H bond and each different substrate requires different oxidizing powers. The substrates induce de novo synthesis of PSMO with the required oxidizing power as determined by the substrate C-H bond strength (Brownlee and Hollebone, 1986). Substituents on the substrate molecule that change the electronegativity of C-H bonds will influence the oxidizing power required for hydroxylation, and contribute to the specificity of the induced PSMO for the inducing substrates.

PSMO differs from other biological enzymes because it does not interact with substrate shapes to lower entropy of activation (ΔS^*), but instead lower the activation enthalpy of reaction (ΔH^*) through electronic effects. This is because cytochrome P_{450} acts to create an oxygen centre in the substrate by the addition of electrons. This process depends on the availability of free electrons and on the oxidizing power necessary to hydroxylate the C-H bond. From the viewpoint of entropy, all reactions are equally likely, however enthalpy (ΔH^*) will be very important in determining G^* of hydroxylation of substrates by PSMO (Hollebone, 1986).

PSMO activity involves several steps which have been described in several models. An early example by Estabrook and Werringloer (1976), diagrammed in Figure 2, postulates that the PSMO active site exists as a 6-coordinate ferric (Fe^{3+}) complex. After the substrate binds, the active site is reduced to a ferrous (Fe^{2+}) complex by the addition of the first electron by NADPH dependant cytochrome P_{450} reductase (containing the FAD/FMN flavoprotein). Myoglobin of the cytosol provides molecular oxygen for cytochrome P_{450} from the cytoplasm. An intra-molecular electron shift occurs from the ferrous complex to localize the electron in the molecular oxygen and produce the ferric form of the complex. The second electron is added to the oxygen-substrate-haem-protein complex by the cytochrome b_5 reductase.

Diprotonation of an oxygen molecule produces water and the resulting O-O bond cleavage results in insertion of O^- into the lipid soluble substrate which is bound to the hydrophobic binding site on P450. The product, which is relatively more polar than the substrate, induces the rotation of the protein as the product is released, leaving the cytochrome P_{450} protein binding site free to repeat the cycle (Figure 2).

1.6.3 Alternative Models

Alternative modifications to this hypothesized model of PSMO function have been proposed which differ in several aspects including: (1) the order of binding of oxygen and substrate and their coordination with enzyme rotation events, (2) the timing and

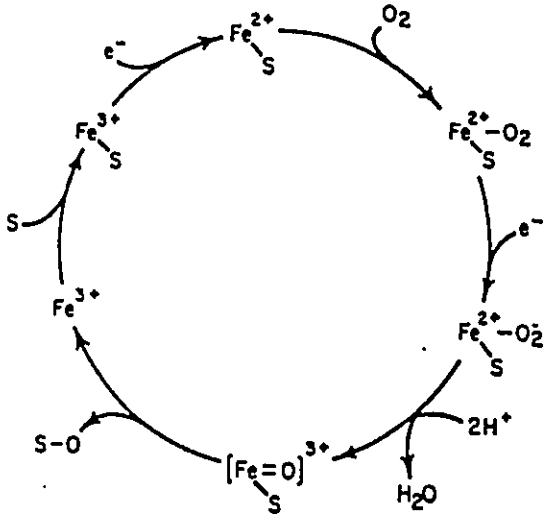


FIGURE 2

The proposed mechanism for the mammalian cytochrome P_{450} monooxygenatic cycle during hydroxylation of a membrane soluble substrate R-H. (Ortiz de Montellano, 1986)

number of added electrons, and (3) the existence of a stable Fe^{3+}-O^{2-}-substrate complex. One such alternative mechanism is shown in Figures 2(a) and 2(b) which differs from the previous model in the three aspects mentioned.

Hollebone (1986), proposes an alternative model based on more extensive evidence (Figure 3). In order for the substrate to be hydroxylated the xenobiotic substrate must be embedded in the lipid, the oxygen is supplied by the cytosol, the active site must rotate from the cytosol into the lipid to come into contact with the substrate. As a result the hydroxylation of substrates by PSMO should proceed in the following order : (1) oxygen binding and loss of an OH^- group, (2) rotation of active site bearing oxygen into the internal membrane region (3), binding of this active site with the substrate, and (4) production and the release of the hydroxylated substrate.

The loss of an hydroxyl group before rotation of the globin produces an $S\text{-}Fe^{2+}\text{-}O^+$ complex. Upon rotation into the membrane, a front side electrophilic attack by the activated oxygen occurs at the C-H bond of the substrate (Mansuy et al., 1984). Addition of electrons is required only during the substrate hydroxylation step, and are provided by FAD, $2e^-$ at a time. In this way extra reaction steps such as adding single electrons during stabilization of iron complexes or O_2^- are not be necessary as were seen in Figure 2.

Figure 3 shows the model of microsomal monooxygenase function as proposed by Hollebone, (1986). Based on extensive experimental evidence and chemical energy requirements, it provides a

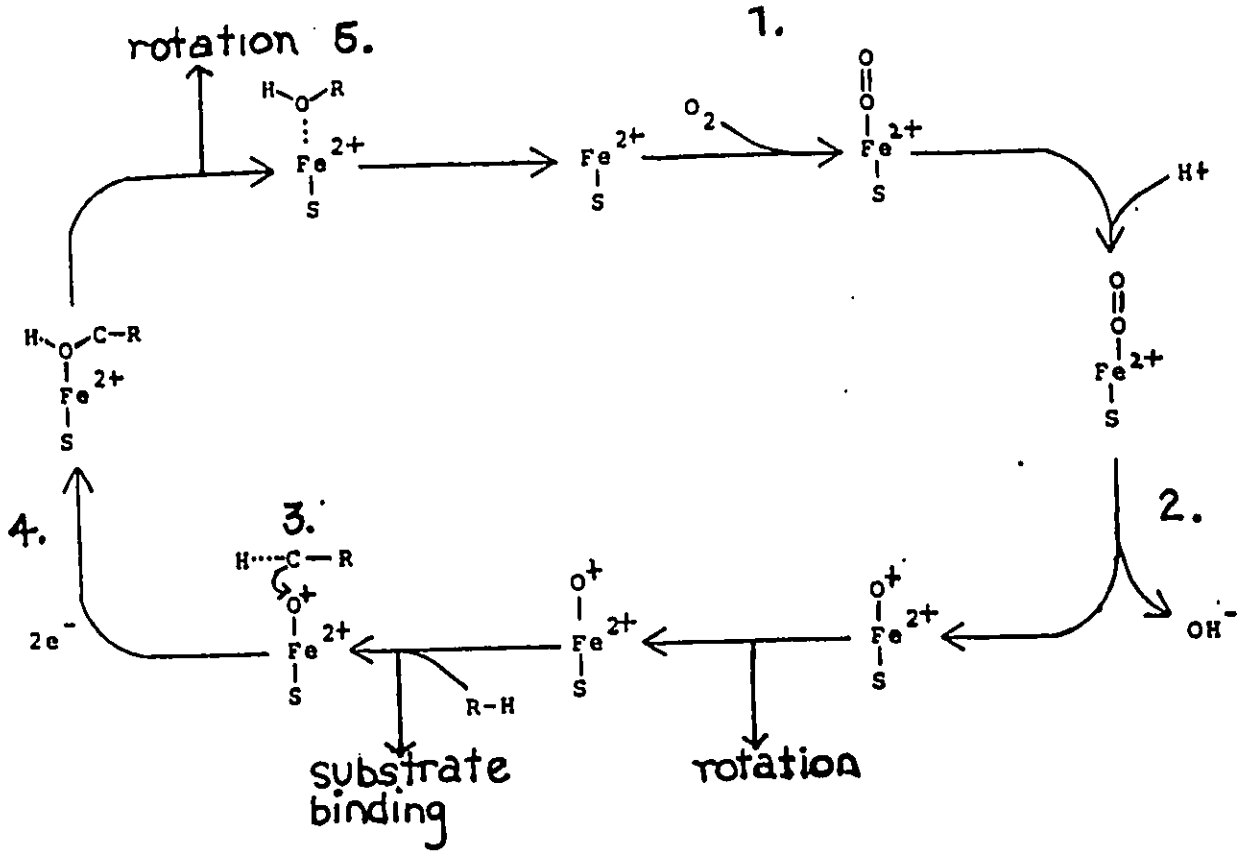


FIGURE 3 Hydroxylation of lipid-soluble substrates by cytochrome P_{450} monooxygenases

(1) Oxygen is bound to a P-450-Fe^{2+} site facing the cytosol.(2) After heterolytic fixation of the oxygen, the cytochrome P450 protein can rotate in the lipid. (3) Substrate hydroxylation occurs, with the addition of two electrons from FAD (4). The hydroxylated product can force rotation of the P450 protein, permitting release of the product to the cytosol (5), which re-initiates the cycle (Hollebone, 1986).

scientifically sound mechanism for the function of PSMO.

1.6.4 Endogenous Reactions of Microsomal Monooxygenase

PSMO is involved in the metabolism of cholesterol and prostaglandin (Murakami et al., 1982, Powell, 1978). These substrates have their own specific PSMO isozymes, although other inducible forms of PSMO are involved in the metabolism of xenobiotics.

In plants, cytochrome PSMO is also involved in endogenous metabolism by several well defined reactions. One endogenous form of plant cytochrome PSMO is involved in the synthesis of p-hydroxy-cinnamic acid from trans-cinnamic acid by trans cinnamate 4-monooxygenase. This form of PSMO is wide spread among plants (Karasaki, 1983), and causes p-coumaric acid conversion to quinones and anthocyanin.

Other isozymes exist with specificity for other plant substrates. These include monooxygenases for lauric acid (for waxes and cutins), alkaloids, phytoalexins, gibberellin, abscisic acid and kaurene monooxygenase for synthesis of the diterpene 7b-hydroxykaurenoic acid in developing seeds,(Hendry et al., 1986, Salaun et al., 1984, Hasson and West, 1976). Reactions of endogenous cytochrome P_{450} involve hydroxylation transformations, N-demethylation of monuron, N-methyl-p-chloroaniline, and benzephetamine (Mathews et al., 1982, Karasaki et al. 1983). This supports the possibility that detoxification of xenobiotics occurs by plant cytochrome P_{450} monooxygenase as it does in animals.

1.6.5 Induction and Inhibition of PSMO

Induction of substrate specific PSMO by endogenous or xenobiotic substrates results in de novo synthesis of PSMO protein and support systems. The new active sites on this protein will act to hydroxylate the inducing chemical stressors in order to promote cytosolic elimination and to maintain homeostasis in the cell.

Xenobiotics are detected by detector compounds in the cell which sense the xenobiotic's presence in the cell in the same way that antibodies detect antigens (Cook et al., 1983). It is thought that migration of the detector-xenobiotic substrate to the nucleus induces a multigene family to be expressed through transcription and translation of ribonucleic acid (RNA) (Nakamura et al., 1983). This enhanced genetic expression results in protein synthesis and increases levels in cytochrome P_{450} protein. Nucleotide sequences which are expressed upon induction by 3-methylcholanthrene and phenobarbital have been determined (Gonzales and Kasper, 1982).

Induction occurs to compensate for a decreased efficiency of the pre-induced levels of PSMO activity by synthesizing more of the enzyme. This corresponds with the findings that the newly induced cytochrome PSMO has a greater catalytic activity than the endogenous forms (Nebert, 1979a).

Chemically unstable intermediates of parent compounds, which are too lipophilic for elimination, can normally also be detoxified as long as the dose of the chemical does not overload or damage enzymes in chemical defense systems. However, overloading of the detoxification mechanisms can result in temporary or irreversible

inhibition of monooxygenase activity. The unstable metabolite intermediates can then bind irreversibly to the haem iron of the active site of the PSMO. This can result in inhibition of enzyme activity, degradative conversion of cytochrome P_{450} to cytochrome P_{420}, lipid peroxidation, and denaturation of other proteins in the monooxygenase complex.

Safrole is a hepatocarcinogen that dehydrates to form a carbene intermediate. This in turn forms a ligand complex with PSMO haem and degrades PSMO to the P_{450} form. Likewise, other methylene dioxyaryl compounds such as piperonyl butoxide, amphetamines, lipophilic amines, hydroxyl amines, carbanions and organic thiols (P=O bonds) behave similarly (Franklin, 1977).

1.6.6 Suicidal Substrates of PSMO

PSMO activity may result in the production of epoxides and free radicals are that are strong electrophiles. The metabolites may destroy PSMO activity by denaturation of PSMO enzyme components. Substrates which are metabolised by PSMO in this manner and inhibit subsequent PSMO activity are suicidal substrates. Epoxides and free radicals also react with nucleophilic proteins and nucleic acids, forming covalent bonds and changing their conformational structure. Lipid peroxidation, denaturation of P_{450} enzyme components and other enzymes, irreversible haem oxidation, cytotoxicity, mutagenicity, and carcinogenicity are effects of metabolism of suicide substrates (Gelboin et al, 1976).

Some suicidal substrates include chloroform, carbon

tetrachloride, and PAH's. Chloroform metabolism produces phosgene, and carbon tetrachloride metabolism produces carbene radicals ($\cdot CCl_3$)(Gelboin et al., 1976). The generation of these cytotoxic metabolites induces production of additional PSMO with an even higher oxidizing power necessary to handle secondary chemical stress. Each new PSMO isozyme may sustain the process of suicidal reactions until the damage is severe enough that homeostasis cannot be maintained and the cell dies.

1.6.7 Synergistic Xenobiotic Mixtures: Toxicity

Metabolism of xenobiotics can convert a toxic parent compound to toxic or non-toxic metabolites, which are readily excreted via the aqueous phases of the body. It can also produce activated metabolic intermediates which remain lipid soluble. When mixtures of xenobiotics are encountered, induction of PSMO may lead to synergistic effects.

Potentiation of a xenobiotic occurs in a synergistic mixture when the LD_{50} (or EC_{50}) of one xenobiotic is decreased by the activity of the another xenobiotic (Owais, 1978). This is due to the induction of PSMO activity by one of the xenobiotics, followed by the production of toxic metabolites of the second xenobiotic. These toxic metabolites from the second xenobiotic would not normally be produced with the pure xenobiotic alone.

Inhibition occurs in cases where the first xenobiotic induces PSMO. The PSMO then metabolizes this inducing xenobiotic, in a suicidal manner, destroying the PSMO. There is therefore no PSMO

available to metabolize any subsequent xenobiotic. In this way, the metabolism of the second pesticide is inhibited (Putnam and Penner, 1974).

1.6.8 Xenobiotic Metabolism

PSMO induced during xenobiotic metabolism has a lower substrate specificity than does endogenous PSMO, therefore, efficient transformation of many xenobiotic stressors is possible. PSMO is involved in the hydroxylation, oxidation, reduction, hydrolysis, N and O dealkylation, desulfuration, dehydrogenation, and dehalogenation of xenobiotics to polar compounds. These are known as phase I reactions (Gillette et al., 1972, Mandel, 1971; Parke, 1976; Karashima et al., 1977; Siest et al., 1978; Ulrich et al., 1978).

Other enzymes are embedded in the endoplasmic reticulum and are associated functionally with the monooxygenase. These include O_2 and NADPH dependant amine oxidase involved in N oxidation, and epoxide hydratase (EC 4.2.1.63) (Patton et al., 1980). This latter enzyme is important in the metabolism of xenobiotics with aromatic and aliphatic double bonds. Epoxides and arene oxides can result from oxidation of these bonds and epoxide hydratase converts these into diols (Lu and Metcalf, 1975).

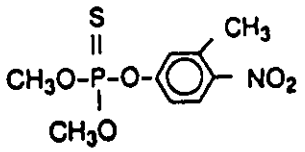
Phase II reactions consist of coupling the xenobiotic intermediate compound to cytosolic compounds such as sulphate, fatty acids, glycine, glucuronic acid, glutathione, glucose, or glycogen (Jenner and Testa, 1978). UDP-glucuronosyltransferase is

found in animal liver cells, glutathione alkyl and aryl transferases are found in liver, kidney, spleen, lung and small intestine. Plants do not contain glucuronosyltransferase and therefore cannot produce glucuronosyl conjugates of xenobiotics. In plants, glycosyltransferases and glutathione transferases are present so that glycosylation and gluthathion conjugation of metabolites can occur (Sandermann et al., 1977). Other cytosolic phase II reactions include: oxidations by alcohol dehydrogenase and aldehyde dehydrogenase, monoamine and diamine oxidations, aldehyde reduction reactions, and ester or amide hydrolysis. These additional reactions may be necessary prior to phase II conjugation reactions (Price et al., 1975).

PSMO activity is also involved in the activation of pro-insecticide activity in order for them to be effective pesticides. In organophosphate insecticides, PSMO is a key enzyme because action of the pesticide in these cases actually depends on the desulfuration of P=S to P=O. For example thimet undergoes S-oxidation in cotton plants to become the active insecticide (Bowman and Casida, 1957). Within the midgut of insects desulfuration of the P=S group to P=O occurs to activate the insecticide. Pro-insecticides are designed with P=S groups which necessitate this desulfuration conversion, since P=O cannot pass through all membranes whereas P=S groups can.

1.7 Fenitrothion

Fenitrothion is an organophosphate insecticide which has been used in Canadian agriculture and forestry since 1969, against the spruce budworm Choristoneura fumiferana at doses of 275 g/ha on target forest areas (NRCC no. 14104, 1975).



FENITROTHION

0,0-DIMETHYL-0-(4-NITRO-m-TOLYL) PHOSPHOROTHIOATE

This pesticide was chosen as the non-persistant pesticide with which to study the degradative role of non-target organisms because its toxicity, degradation and behaviour in the environment has been extensively studied (NRCC No. 14104, 1975).

1.7.1 Fenitrothion Toxicity

Fenitrothion is acutely toxic to insects, however it is relatively less selective for mammals. This is based on steric interactions of the acetylcholinesterase anionic and esteratic sites, with the ring methyl and phosphate moieties respectively, on the activated fenitrooxon. The distance between these two moieties in fenitrooxon is 5.2-6.5 Å. Mammalian and insect cholinesterases differ in that the anionic-esteratic sites are separated by 4.3-4.7 A and 5.0-5.5 Å respectively. In addition, the CH_3 is more readily

oxidized by animals to detoxify the molecule and eliminate it (Hollingworth et al., 1967) . As a result, the probability for anticholinesterase activity is much higher in insects than mammals (Hollingworth et al., 1967a).

The mechanism of nervous system poisoning by fenitrothion involves anticholinesterase activity. The chemical structure of fenitrothion mimics the structure and reactivity of acetylcholine, the natural substrate of cholinesterases (Eto, 1976). Cholinesterases are inhibited by phosphorylation at their esteratic site by organophosphates (Bayer, 1983).

Table 1 lists the toxicity of fenitrothion to aquatic organisms. A comparison of lethal toxicity for aquatic organisms, with that of insects and humans, demonstrates that the lethal concentrations for aquatic non target organisms are less than that of insect target organisms.

The toxic manifestations of fenitrothion in mammals includes inhibition of growth (Misu et al., 1966), depression of cholinesterase levels in rats (Sumitomo, 1972a), teratogenicity affecting fetal weight in rats (Hazelton, 1974b), and potentiation of other pesticides by decreasing the LD_{50} of malathion and Surecide in rats (Hladka et al., 1974). No evidence exist of mutagenicity or neurotoxicity (Sumitomo, 1972d and Benes et al. 1973).

1.7.2 Levels of Environmental Residues of Fenitrothion

In Canada, fenitrothion was applied by aircraft over terrestrial forest ecosystems. The fenitrothion deposited can

Table 1 Fenitrothion Toxicity

Organism	Dose or Aquatic Concentration of Fenitrothion	Effect
Fish [1]		
Rainbow Trout	1.28 μg/mL 0.02 μg/mL	48 h LC_{50} 4 week LC_{50}
Blue gill	2.72 μg/mL	48 h LC_{50}
Carp	4.4 μg/mL	48 h LC_{50}
Invertebrate		
Daphnia[1]	0.0092 μg/mL	3 h LC_{50}
Housefly[2]	5.0 mg/kg	acute lethal
Mammals[2]		
Rats	250 mg/kg	acute oral LD_{50}
Mice	870 mg/kg	acute oral LD_{50}
Humans[3]	2.0 g/m^2, for 6-8 weeks sprayed on walls	depression of cholinesterase activity

[1] Miyamoto, 1969.

[2] NRCC No. 14104.

[3] Schrader, 1961.

contaminate other environmental compartments in which non-target organisms exist. Fenitrothion is lost by atmospheric drift, and also surface ground runoff and accidental spraying to end up in water bodies which are a sink (Sundaram, 1973 and Sundaram, 1974a). Terrestrial and aquatic environments are therefore unequally exposed to fenitrothion.

In general however, fenitrothion, like most organophosphates, has a short half life, being degraded to non-toxic polar compounds in organism and easily eliminated (Eto, 1976). Some common metabolites of fenitrothion found in aquatic ecosystems are shown in Figure 4.

Hydrolysis of fenitrothion to yield NC occurs at pH>8.0. At pH< 7.0 in natural waters NC and DSM are produced (Greenhalgh et al., 1980). Aminofenitrothion is an additional metabolite found in natural waters resulting from microbial degradation of fenitrothion. Photodegradation by natural light wavelengths produces FO, CFO, CFT, and carboxymethyl fenitrothion (Greenhalgh et al., 1980).

The potential for fenitrothion contamination in aquatic environments after an operational spray is thought to exist. For example, Wildish (1971) estimated that in a one foot deep stream, assuming no drift and complete mixing, an operational spray of 138 g/ha could produce levels of residual fenitrothion at 0.045 mg/L. However, due to volatilization, adsorption to suspended solids, and chemical reactions such as photolysis and hydrolysis, the highest levels ever detected in water bodies is between 1.0 and 75.5 µg/mL.

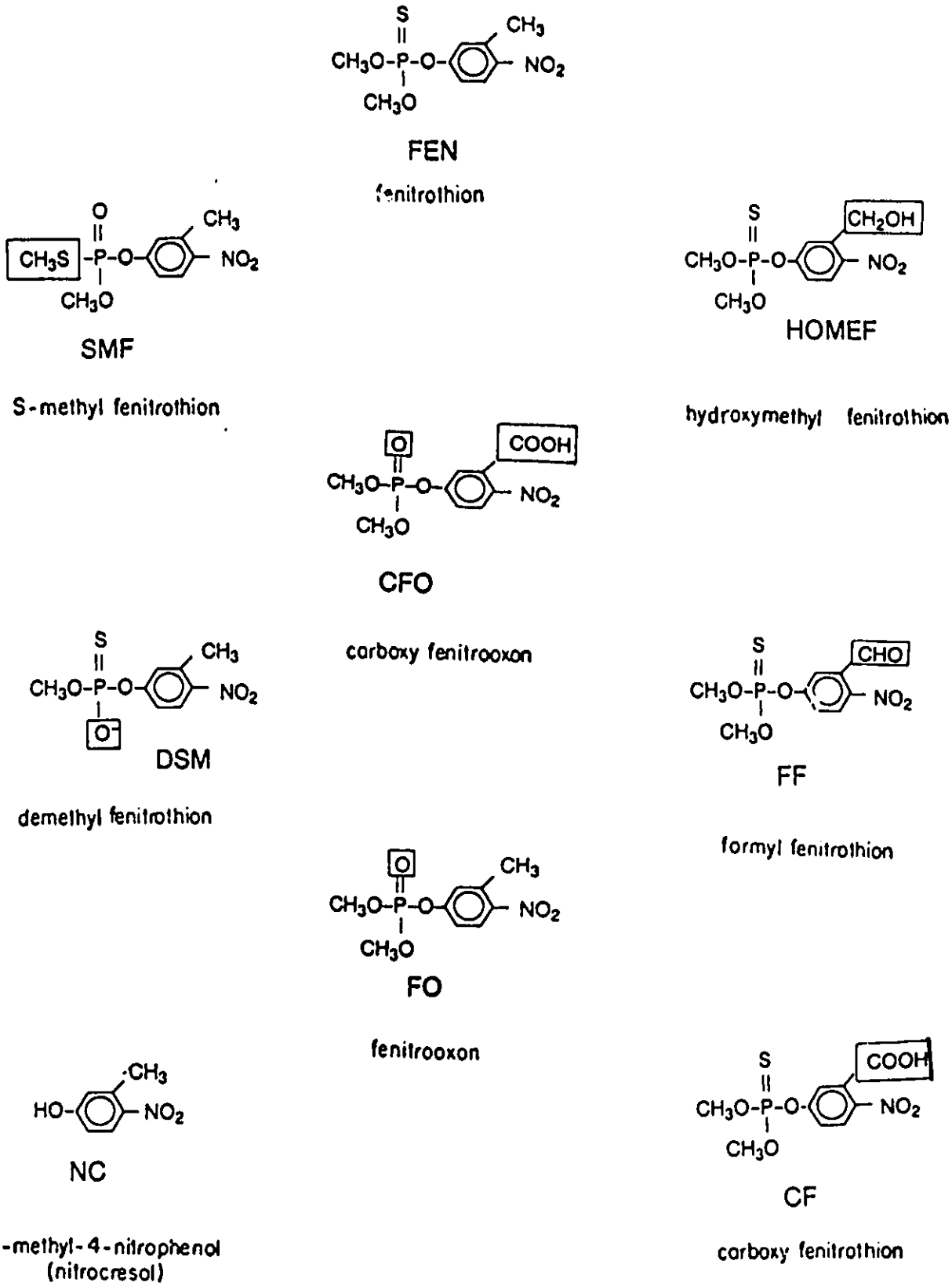


FIGURE 4 Chemical structure of fenitrothion and its metabolites

These levels are much lower than the concentrations that elicit toxic effects in non-target aquatic organisms. Residues usually disappear from aquatic environments on a time scale of hours to days after operational sprays with no fenitrothion remaining after 40 days, and no toxic residues of fenitrothion metabolites persist in the food chain (Eidt, 1975, Penny, 1971 and Flannagan, 1973).

Aquatic organisms do not have a high fenitrothion bioaccumulation ratio (Miyamoto et al., 1979). The metabolic residues are rapidly excreted from snail, daphnia, algal and fish species. The drug metabolizing ability of their cytochrome P_{450} mixed function oxidases, however, are significantly lower than that of mammals, with mouse> rat> rainbow trout> carp> snail> algae (Miyamoto et al., 1979).

1.7.3 Environmental Exposure, Metabolism and Toxicity of Fenitrothion in Non-Target Organisms

Fish

Stream caged rainbow trout, and salmon have been studied for the effects of fenitrothion exposure. After an exposure of 275 g/ha fenitrothion, rainbow trout accumulated an average of 0.5 mg/kg bw fenitrothion after 24 h with some measured values as high as 1.84 mg/kg. This bioaccumulation was temporary, however, with only 0.02 mg/kg remaining after 4 days (Lockhart et. al., 1973). The effect of fenitrothion on fish is similar to mammals with depression of acetylcholinesterase levels in adult brain and whole bodies of

young atlantic salmon (Zitko et al., 1970, Wildish et al., 1971).

Carp, sunfish, rainbow trout, and euryhaline fish (Oryzias latipes and Mugil cephalus) degrade fenitrothion to 3-methyl-4-nitrophenol and its B-glucuronide conjugate, fenitrooxon, demethyl fenitrothion and demethyl fenitrooxon (Benke et al., 1974 and Lech, 1973, Miyamoto et al., 1979, Takimoto et al., 1987).

Invertebrates

Crayfish survived the operational sprays yet accumulated 1.37 mg/kg bw fenitrothion, however no indication of the potential bioconcentration into its fish predators is known (Leonhard, 1974). Fenitrothion is detoxified in the hepatopancreas of blue crab Callinectes sapidus to 3-methyl-4-nitrophenol, and demethyl fenitrothion by glutathion aryl and alkyl transferase (Johnston and Corbett, 1986). Shrimp, Palaemon paucidens and the water flea, Daphnia pulex also produce NC conjugates with glucose or sulphates (Takimoto et al., 1987). Both the shrimp and the bluecrab produce FO as a toxic metabolic residue.

Lower in the aquatic food chain, populations of aquatic insects such as stone flies and caddis flies are diminished after operational sprays of 138 to 275 g/ha fenitrothion, but as with the crayfish, extrapolations of the effect on their salmonid predator population is not known (MacDonald and Penny 1969). Snails, Cipangopaludina japonica and Physa acuta degrade fenitrothion by oxidative dealkylation (DSM) and dearylation reactions (NC), by oxidative desulfuration (FO), by side-chain oxidation, and by reduction and sulphate or glucose conjugation (Miyamoto et al.,

1979).

Cotton leaf worm degrades fenitrothion by mixed function oxidases and the addition of piperonyl butoxide decreases the rate of fenitrothion degradation in Spodoptera littoralis (Dogheim and El Guindy, 1980). Spruce budworm degrades fenitrothion to the same metabolites as those produced in other invertebrate organisms previously discussed, as well as producing conjugates of NC with glucosides and amino acids (Sundaram, 1988).

Suspended Solids

Suspended solids readily absorb fenitrothion and they can serve as a storage site, from which fenitrothion or its degradation residues can be released (Choudhry, 1981). Microorganism and algal uptake of fenitrothion changes its bioavailability of and that of its toxic metabolites to the food chain and non-target organisms. (Zitko and Cunningham, 1975).

Aquatic Plants

Myriophyllum, Sagittaria, and Elodea were analysed during a study of fenitrothion uptake by aquatic plants, and all three genera retained significant levels of fenitrooxon, s-methyl fenitrothion and 3-methyl-4-nitrophenol after exposure to 10 ppm fenitrothion (Weinberger et al., 1982). Chlamydomonas segnis significantly decreased the abiotic half-life of fenitrothion in the light from 30 h to 15 h (Caunter and Weinberger, 1988).

Other studies with unicellular algae demonstrated that fenitrothion and the metabolites FO, DSM, SMF, and CFT were detected in Chlamydomonas and Chlorella in both the light and dark

(Weinberger et al., 1982). Lemna minor, Cerratophyllum demersum, and Butomus umbellatus accumulate fenitrothion, removing it from the aquatic environment such that after 10 h post spray (250 g/ha), no aquatic residues were detected (Moody et al., 1978). This study did not identify fenitrothion metabolites in the plant fractions.

1.7.4 Current Studies in Degradation of Aquatic Residues of Fenitrothion

Individual processes contributing to fenitrothion disappearance from water fractions of aquatic environments have been extensively studied in isolated systems. However, the relative importance of each of the degradation factors in the environment is less studied (Yule and Duffy, 1972, Kovacicova et al., 1973, Lockhart et al., 1973, Zitko and Cunningham, 1975, Okawa et al. 1974, Weinberger et al. 1982, Zepp and Schlotzhauer, 1983, Caunter and Weinberger 1988). Processes including hydrolysis, which is temperature and pH dependant, photolysis and oxidations, and photosensitization by humic substances, have been implicated as the major degradation routes which could produce the low aquatic half life observed for fenitrothion.

Rapid uptake by non-target plants, suspended solids, phytoplankton, and zooplankton invertebrates, followed by metabolism also contribute significantly to the low aquatic persistence of fenitrothion. Algae, plants, aquatic invertebrates, and their grazers potentially provide a sink for

fenitrothion uptake during its disappearance from the water fraction, however, work indicates that the sediment is the major sink for aquatic residues (Weinberger et al, 1982., Lekshminarayana and Bourque 1980, and Zepp and Schlotzhauer, 1983).

Uptake by phytobiota could account for the fenitrothion half life of 4 days in natural waters observed by Lockhart, (1973). This is likely because aquatic half-life is a measure of the amount of parent compound remaining in the water fraction, and does not account for the fenitrothion residue in other environmental compartments. Due to fenitrothion lipophilicity (log octanol/water partition coefficient = 3) and its low water solubility, (log μmoles/L = 2) algae and suspended solids are most likely to absorb fenitrothion (Chiou, et al., 1977). The mechanism and efficiency of this aquatic compartment will affect the risk of exposure to other non-target organisms.

There are a few studies that examine accumulation and degradation by aquatic plants. They examine chemical and physical degradation rather than biodegradation. These studies give little insight into the contribution of aquatic plant sinks to the fate of fenitrothion, and its toxicity hazards through food chain persistence (Miyamoto et al. 1979, Johnston and Corbett, 1986, and Levi et al., 1988). Therefore, although the aquatic persistence of fenitrothion may be known, the contribution of non-target organisms relative to abiotic factors has not been clearly elucidated.

1.8 Hypothesis

The hypothesis formulated for the aquatic model in this study was that Chlamydomonas reinhardtii has a significant role in degrading fenitrothion relative to abiotic degradation. It was hypothesized that C. reinhardtii can significantly decrease the half life of fenitrothion relative to its abiotic half life. If this is true under the experimental conditions of this study, it was further hypothesized that the endogenous metabolic reactions of C. reinhardtii would be involved in the degradation of fenitrothion. It was thought that the biodegradation of fenitrothion by C. reinhardtii would yield a different spectrum of fenitrothion metabolites (predominantly fenitrothion oxidation products: OHMEF, FF, CFT and CFO) from the spectrum produced by abiotic degradation (predominantly non-enzymatic oxidation/photooxidation and hydrolysis/photolysis products: DSM, NC, SMF, FO).

It was further hypothesized that cytochrome P_{450} monooxygenase is involved in algal biodegradation of fenitrothion. Based on the known activity of cytochrome P_{450} monooxygenase in hydrolysis, desulfuration, demethylation and oxidation reactions, it was hypothesized that cytochrome P_{450} monooxygenase is involved in fenitrothion degradation by: (A) side chain oxidation to produce OHMEF, FF, and CFT, (B) desulfuration to produce of FO, CFO, and (C) demethylation and enzyme catalyzed hydrolysis to produce DSM and NC..

1.8.1 Objectives

The objectives of this study which test the hypothesis are:

1) To determine if C. reinhardtii does significantly decrease the half life of fenitrothion relative to its abiotic half life,

2) To determine if there is significant uptake of fenitrothion by C. reinhardtii,

3) To determine and quantify the significant differences in the spectrum of fenitrothion biodegradation products in C. reinhardtii, from the abiotic fenitrothion degradation products,

4) To determine if cytochrome P_{450} activity is involved in C. reinhardtii biodegradation of fenitrothion.

1.9 Experimental Design

To set up an experiment which provides a valid test of the hypothesis, choices of the pesticide fenitrothion, the non-target organism Chlamydomonas reinhardtii, and the exposure conditions were made. The choices taken for this study are highly typical of environmental conditions in temperate zones and should be applicable to similar organisms when exposed to related pesticides.

1.9.1 Non Target Organism: Chlamydomonas reinhardtii

Chlamydomonas reinhardtii was the alga chosen to study fenitrothion degradation by a non-target organism. This alga is ubiquitous to freshwater ecosystems. Uptake of fenitrothion in Chlamydomonas, and other species of unicellular algae has been documented as a significant sink for fenitrothion in freshwater ecosystems (Lekishmyanara and Bourque, 1980).

Chlamydomonas is easy to handle, and can be quickly cultured for experimental use to achieve high cell densities of plant material. It is easy to maintain as a mitotically synchronous culture of cells produced from an isogenic strain. C. reinhardtii is a sensitive indicator species representative of evolutionary primitive plants. Being unicellular, this alga is easy to manipulate during extraction methodology and does not require extensive tissue disruption techniques.

1.9.2 Experimental Conditions of Algal Exposure to Fenitrothion

1.9.2.1 Fenitrothion Toxicity Experiments

This laboratory study was done under controlled conditions. The growth medium is buffered at pH=6.8 and the temperature is kept constant (in a temperature controlled growth chamber at 21± 0.5 °C) to optimize algal growth conditions. Natural waters differ in pH ranging from 6.1 to 7.2 (Lekshminarayana and Bourque, 1980). Vita Lite^{R} illumination (Dura Test, Ultra Hi Output) was used (intensity of 40 w/m^{2}) with high intensity u.v. light (290-310 nm) (Weinberger and Caunter, 1988). These wavelengths overlap with the peak

absorption of fenitrothion (Figures 5a and 5b).

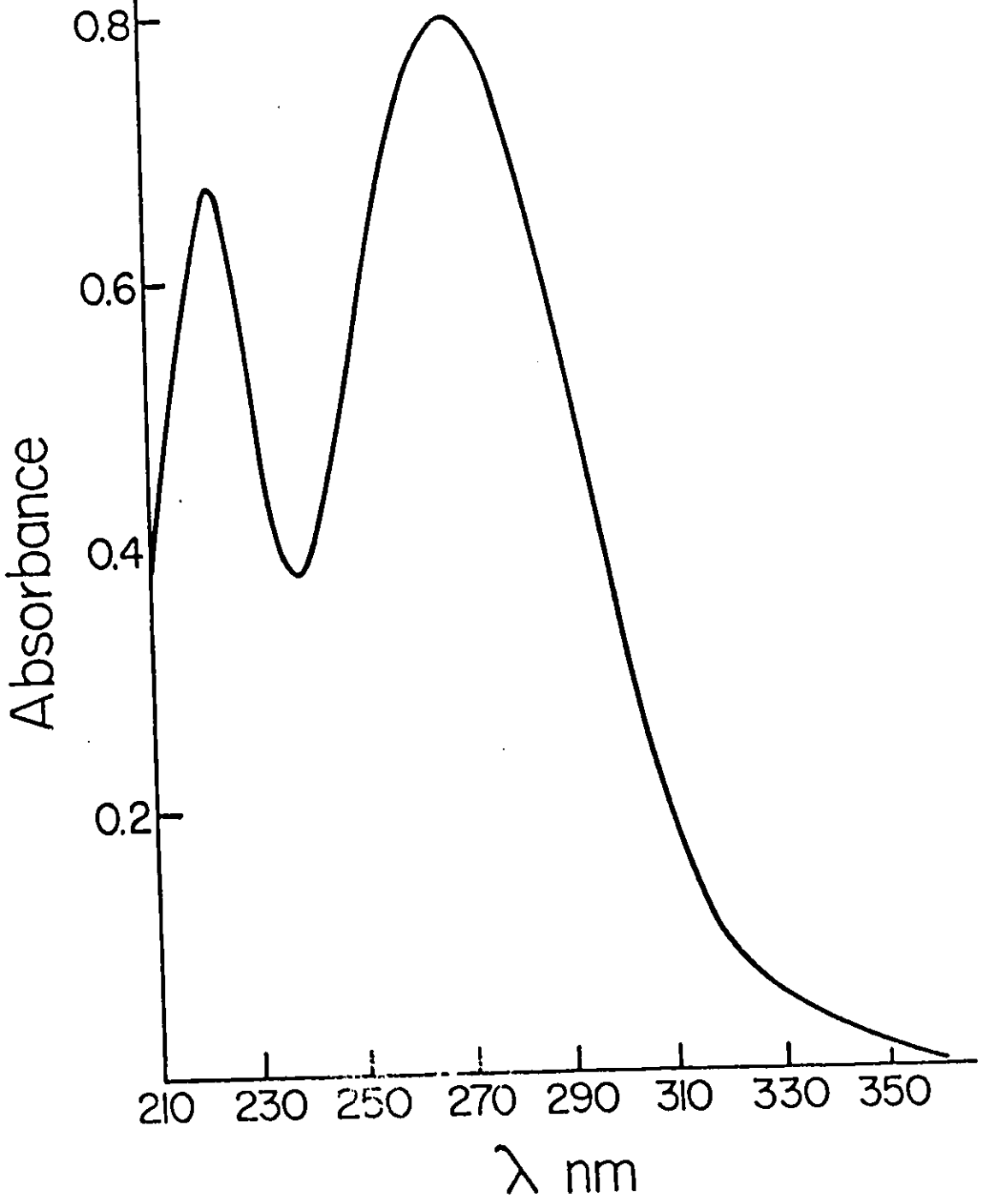
These conditions serve as control conditions for abiotic fenitrothion degradation without alga, in Gorman and Levign medium at neutral pH. By using both light and dark abiotic controls, hydrolysis, and photoenhanced degradation without algae can be elucidated and compared with biotic reactions. By controlling these conditions the growth of C. reinhardtii is optimized and the background factors can be minimized to avoid confounding these results with those of biodegradation.

In order to determine the role of C. reinhardtii in the biodegradation of fenitrothion, an exposure concentration of must first be determined. It is necessary that the physiological impact of the fenitrothion exposure on the algal cultures must be known. The concentration used must not be toxic to the alga, be of similar order of magnitude as environmental residues, and be present at a high enough concentration to be detected by the experimental methodology. By using a larger concentration than the residues in the environment, the limitations of an increased environmental pesticide load on the degradation mechanism can be determined.

The working level of fenitrothion to be used, is determined by exposing algal cultures to a range of fenitrothion concentrations, and then monitoring the subsequent effects on physiological parameters. The Ec_{50} values calculated for the measured parameters is used to quantify the toxicity of fenitrothion to the alga. (Ec_{50} is the exposure concentration of chemical which results in a 50% decrease in the measured parameter

FIGURE 5 (a)

UV absorbance spectrum of Fenitrothion in methanol.



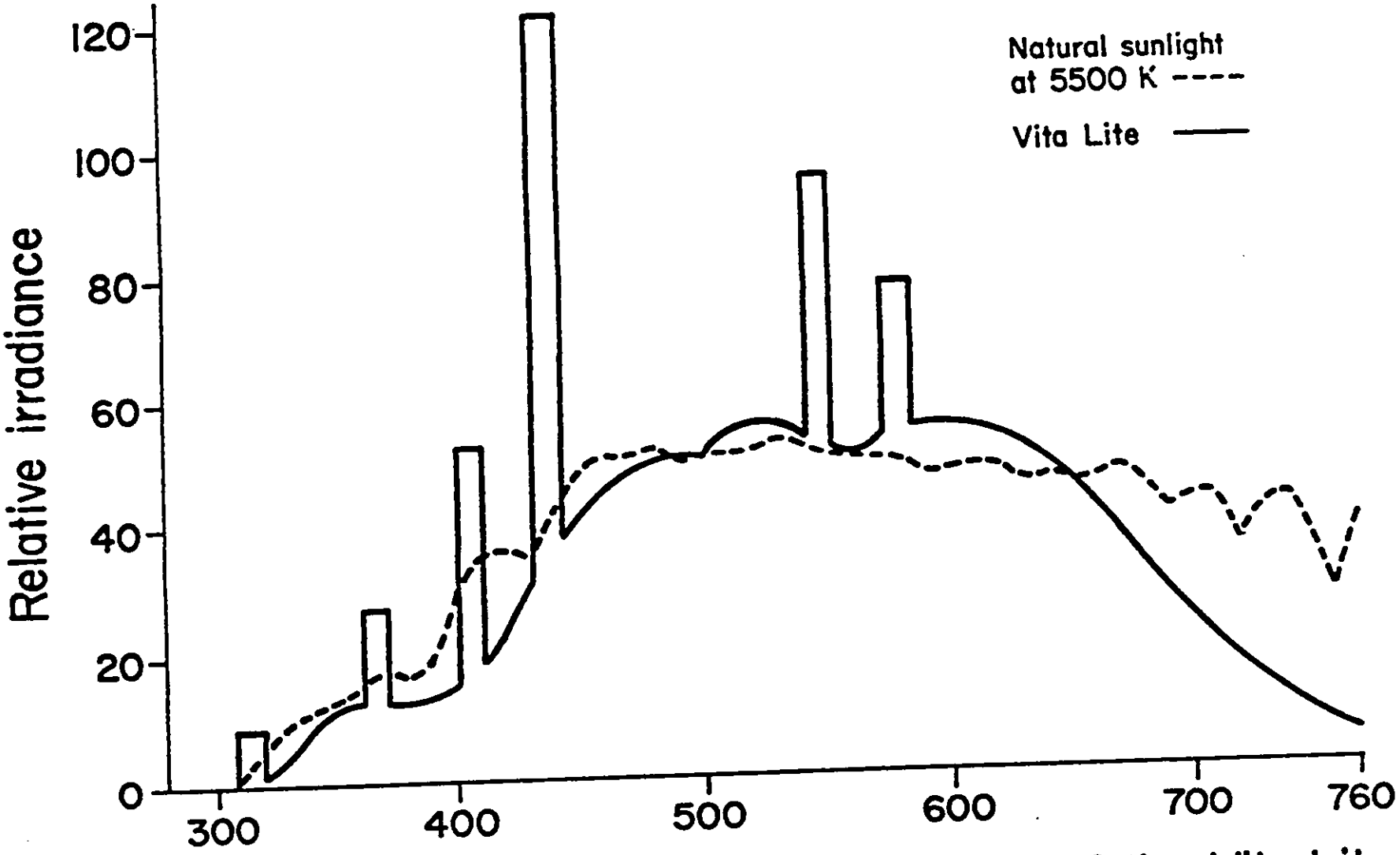


Figure 5b Relative spectral irradiance distribution of the Vita Lite fluorescent lamp compared with natural light at 5500 K (Mass et al, 1974)

relative to the control)

1.9.2.2 Abiotic Degradation

To determine the role of C. reinhardtii in the degradation of and formation of metabolites of fenitrothion, the metabolism in the absence of the alga must be elucidated. This must be determined under sterile conditions with controlled pH, temperature, and illumination conditions. The metabolites of fenitrothion formed in the dark represent degradation due to enzyme independent hydrolysis and oxidation reactions. Illumination of abiotic controls with fluorescent or Vita Lite^{R} will account for photolysis and photo-oxidation reactions. This establishes the background degradation in the absence of biotic factors for the light and dark exposure conditions.

1.9.2.3 Algal Biodegradation of Fenitrothion

Subsequent experiments will determine the metabolic rate of fenitrothion degradation in the growth medium in the presence of cultures of C. reinhardtii. The mass balance of ^{14}C-ring labelled fenitrothion will be used to monitor fenitrothion partitioning between the extracellular and cellular algal fractions. The metabolic residues of fenitrothion, in the intracellular and extracellular fractions of these cultures, will be extracted and quantified by GC an by comparison with fenitrothion metabolite standards.

Fenitrothion biodegradation by C. reinhardtii, in this study, may proceed by hydrolysis, demethylation, and oxidation reactions. This is based on the assumption that algae have the same enzymes as other aquatic and terrestrial plants, such as glutathion-S alkyl and aryl transferases, cytochrome P_{450} monooxygenase, and cytosolic oxidase. Fenitrooxon (FO), S-methyl fenitrothion (SMF), 3-methyl-4-nitrophenol (NC), desmethylfenitrothion (DSM), hydroxymethyl fenitrothion (OHMEF), formylfenitrothion (FF), carboxyfenitrothion (CFT) carboxyfenitrooxon (CFO), phosphates (PA, PTA, MPTA,DMPTA), desmethylfenitrooxon (DSMFO), and glucoside or glutathion conjugates of these derivatives are all possible algal metabolites of fenitrothion.

Elimination of these metabolites from the cellular fractions of the alga to the external cell medium, will enhance the existing amounts of these metabolites produced by photolytic and hydrolytic reactions in the external cell medium. In order to determine the effect that algal degradation has on metabolite residue bioavailability in the external medium, elimination of fenitrothion metabolites to external growth medium must be monitored. After incubation of fenitrothion with algal cultures for one week (at which time, some metabolites are produced), the cell pellet can then be separated from the pesticide containing medium, washed, and resuspended in growth medium without pesticide. Any pesticide found in the growth medium after further incubation in cold medium would represent metabolite residues produced in the cells and released to

the external medium due to elimination.

Whether the abiotic degradation of fenitrothion is enhanced more by the effects of light or by the biotic influence of C. reinhardtii can be determined. This is done by calculating the biotic: abiotic ratio of the levels of metabolites detected in the dark. Secondly the ratio of abiotic light:dark ratio for detected metabolites is determined. By comparing these ratios, the light enhancement of abiotic fenitrothion degradation relative and biotic enhancement of abiotic degradation can be compared.

1.9.2.4 PSMO Studies

To determine if cytochrome P_{450} monooxygenase is involved in the biodegradation of fenitrothion, phenobarbital, an inducer of this enzyme, is incubated with C. reinhardtii during the biodegradation of fenitrothion. One would expect that if this enzyme is involved in fenitrothion biodegradation, that the previously observed algal mediated fenitrothion degradation would be enhanced by phenobarbital treatment. This is consistent with the theory that induced monooxygenase synthesis in plants is evoked by phenobarbital and results in enhanced degradation of xenobiotic stressors (Reichhart et al., 1980). In particular, the metabolic reactions by algal cytochrome P_{450} monooxygenases which produce the OHMEF from a phase I reaction,while other oxidation products of fenitrothion (FF, CFT, CFO) would be produced from OHMEF by phase II reactions. These metabolites would be enhanced by phenobarbital relative to non-enzymatic oxidation and hydrolysis reactions which

produce NC, DSM, SMF, FO and phosphoric acids derivatives MPA, PA, DMPA.

Incubation of piperonyl butoxide, an inhibitor of monooxygenase activity, with fenitrothion treated C. reinhardtii cultures would produce a decrease in cytochrome P_{450} monooxygenase mediated metabolism of fenitrothion. The metabolite OHMEF, would be detectable intracellularly at levels significantly lower than that of biotic control cultures, treated with fenitrothion only. Intracellular hydrolysis and oxidation reactions producing FO, NC, SMF and DSM would be unaffected by this inhibition treatment. The amounts of CFT, CFO and FF produced by side chain oxidation will change in relative proportion with the availability of OHMEF to phase II enzymes. This would be indirectly related to induction and inhibition of cytochrome P_{450} monooxygenase.

The concentration of the inhibitor and the inducer compound to be used during algal metabolism of fenitrothion needs to be determined. This is done by monitoring the physiological response of the algae to different concentrations of phenobarbital or piperonyl butoxide, and by calculating the Ec_{50}. A ratio of inducer:pesticide or inhibitor:pesticide to be applied in this study must approximate a concentration which is not physiologically toxic to C. reinhardtii, and yet approximates the known ratios used in studies of the synergistic effects of phenobarbital or piperonyl butoxide on xenobiotic degradation. These values are one tenth the pesticide concentration for piperonyl butoxide (Iyengar, 1989) and 13.92 mg/g plant tissue for phenobarbital (Reichhart et al., 1980).

In addition, physiological parameters must be monitored in the presence of the test concentrations of inhibitor with fenitrothion, and in the case of fenitrothion with the inducer compound, to determine the synergistic stress on the algal cultures during the metabolism study. Controls without algae which monitor the degradation of fenitrothion in the growth medium with the inhibitor or inducer compound will also be studied to see if these chemicals alone can affect the half-life of the pesticide.

These studies will provide a useful test of the implications of the hypothesis that (1) algal biodegradation of fenitrothion has a significant effect on its half-life relative to abiotic degradation, (2) that PSMO activity has a role in algal biodegradation of fenitrothion, and (3) that non-target organisms contribute significantly to fenitrothion degradation under environmentally relevant conditions.

CHAPTER 2

MATERIALS AND METHODS

2.0 Chemicals and Organic Solvents

Analytical grade fenitrothion (> 99%) and ^{14}C-ring labelled fenitrothion (>98% 5.54 mCi/mM specific activity) were obtained from Sumitomo Chemical Company, Japan. Standards of fenitrothion derivatives were obtained from Agriculture Canada, Chemical and Biological Research Institute, Ottawa, Canada. These included fenitrooxon, demethyl fenitrothion, aminofenitrothion, 3-methyl,4-nitrophenol, s-methyl fenitrothion, hydroxymethyl fenitrothion, formyl fenitrothion carboxyfenitrothion, carboxy fenitrooxon, and phosphate moieties, PA, MPA, DMPA, MPTA, and DMPTA. The purity of these metabolites was determined using gas chromatography.

Piperonyl butoxide and diazald (N-methyl-N-nitroso-p-tolylsulphonamide) were obtained from Aldrich Chemicals, Milwaukee Wisconsin, U.S.A.. Phenobarbital was obtained from Allen and Hanbury, Toronto, Canada. The solvents used for extraction were pesticide grade and were obtained from BDH Chemicals, Toronto, Canada.

2.1 Algal Culture Techniques

2.1.1 Algae

Wild type Chlamydomonas reinhardtii was obtained from the University of Toronto Culture Collection, Department of Botany, University of Toronto, Toronto, Canada. Agar slants of Gorman and

Levign medium were used to store C. reinhardtii at 4°C. Axenic, mitotically synchronous cultures of C. reinhardtii were used in all metabolic studies and in all physiological studies. Synchronous cultures were obtained by plating wild type cells of C. reinhardtii onto 1.5% agar plates containing minimal medium supplemented with 0.4% vitamin-free casamino acids (Surzycki, 1971). Approximately one hundred colonies per plate were allowed to grow for 6 days at 25°C in an Hotpack programmed incubator under Sylvania Coolwhite light (4000 lux). The largest green colonies were selected and ten of these were mixed together to form a new isogenic strain with a genetic background such that the strain liberates its daughter cells almost immediately after cytokinesis at 16-17 h of the cell cycle.

2.1.2. Growth Medium

C. reinhardtii cultures were maintained in Gorman and Levign growth medium (Appendix A)(Gorman and Levign, 1965).

2.1.3 Inoculation of Cultures for Synchronous Growth

A seed culture of cells was grown to stationary phase in continuous light (3 days, 25°C). At 6-7 h before the dark phase, 3.0 litres of Gorman and Levign growth medium was inoculated with 1.5×10^8 cells so that the cell density was 5.0×10^4 cells/ml. The cultures were bubbled with 0.03% CO_2 in air in order to agitate the cells and to supply carbon dioxide for growth.

2.1.4 Synchronization of C. reinhardtii Cultures

Cells were synchronized using a 24h light-dark cycle at an intensity of 6000 lux (12 h light:12 h dark). The temperature was maintained at 21.00 ± 0.50 °C. After the fourth dark period the culture was diluted by half, and then again every 24 h thereafter so that the cell division remained synchronous. Cell counts were made every day to ensure that the cell number did not exceed $5x10^6$ cells per ml (Surzycki, 1971).

2.2 Fenitrothion Treatment

In all studies, synchronous C. reinhardtii cultures were treated in the S-phase of mitosis, the most sensitive phase of the growth cycle (2 h before the dark phase) (Weinberger and DeChacin, 1987). Fenitrothion was weighed by the method of difference of weights using a Metler analytical balance H54AR. Fenitrothion stock solutions were prepared by dissolving analytical grade(>99% purity) fenitrothion in acetone.

To treat algal cultures with fenitrothion, first the appropriate volume of fenitrothion stocks were added to the appropriate volume of growth medium. Radioactively labelled ^{14}C-fenitrothion (ring labelled) was added in the same way as cold fenitrothion. A 375 µL volume of a 12.0 µCi/ml stock of labelled fenitrothion was added to the final volume of 20 ml algal cultures. This was equivalent to 0.225 µCi/ml culture. Finally the appropriate volume of cell culture was added to the growth medium to achieve the desired cell density and desired concentration of

fenitrothion. Aliquots of culture were handled using sterile techniques. The mixtures were agitated on a horizontal Eberbach shaker to achieve aeration and equal exposure of the algal cells to the pesticide. Cultures were exposed to Vita Lite^{R} (Dura Test, 40 wm^{-2}, wavelength 290-700 nm, at 21°C ± 0.5). Dark treatments were incubated in foil covered tubes with the light exposed treatments.

2.3 Studies of Fenitrothion Toxicity to C. reinhardtii

Algal cultures (2.0 x 10^5 cells/ml) were treated with 0.0, 1.0, 5.0, 10.0, 20.0, and 100 µg/ml fenitrothion and were incubated in Gorman and Levign growth medium under Vita Lite^{R} and in the dark. After different time periods of incubation, physiological parameters were monitored as described below. Probit analysis of the data was done to determine the EC_{50} of fenitrothion for each parameter (Finney, 1952). The concentration of pesticide representative of environmental residues to be used in subsequent metabolism studies was determined. This was done to ensure that the test concentration of fenitrothion was physiologically non-toxic and did not impact on algal homeostasis.

2.3.1 Growth parameters: Cell Number and Optical Density

Cell numbers were measured by examining ten aliquots from each culture tube under a light microscope using an improved Neubauer haemocytometer grid. Optical density was measured on a Bausch and Lomb spectrophotometer at 540 nm (Sorokin, 1973). Standard growth curves of the change in logarithm of optical density (▲O.D.) versus

time were plotted following the method of Sorokin (1973). The values for intrinsic growth rate,(r), and maximum carrying capacity, (K), were obtained using an IBM compatible Stats Graphics software with non-linear regression capabilities. A logarithmic growth model (Sorokin, 1973) was incorporated into the software:

$$N_t/N_o=K/(1+ e^{a-rt})$$

where N_t is the cell number after generation t, K is the maximum carrying capacity of the C. reinhardtii culture, a is a constant, and r is the growth rate calculated for the exponential phase of the growth curve.

2.3.2 Dry Weight

Cell weight of C. reinhardtii cultures treated with the test concentrations of fenitrothion was determined at time zero and every 6 h afterwards up to 48 h. Three replicate samples (5.0 ml) of control and treatment sets were filtered on pre-dried and pre-weighed Whatman GF/C glass fibre filters (2.5 cm diameter, 0.45 μm pore size). The filters with algae were oven dried at 100°C in an Isotemperature oven for 4 h, cooled for 15 min to room temperature in a desiccator, and weighed. The dry weight was determined as the difference between dry pre-weighed filter and oven-dried filter with algae (Sorokin, 1973).

2.3.3 Pigment Content

Chlorophyll a and b pigments were extracted following the method of Hansmann (1973). Triplicate sets of 5.0 ml of fenitrothion treated cultures and control cultures were filtered on MSI Magna nylon 66 membrane filters (0.45 µm pore size, 2.5 cm diameter). The filters were extracted for 24h in 5.0 ml 90% acetone in the dark at 16°C after which the samples were centrifuged for 10 min at 5000 rpm in a Sorvall RC 2-B automatic refrigerated centrifuge. The absorbance of pigment samples was read at 665, 645, and 630 nm, on a Spectronic 21 UVD Bausch and Lomb spectrophotometer using Bausch and Lomb spectrophotometer tubes. These readings were zeroed using blanks containing 5.0 ml 90% acetone and a dissolved filter, treated as were the pigment extracts. Chlorophyll a and b concentrations of each replicate were obtained by using the equations:

Chl a= 11.6 (a 665)-1.31 (a 645)-0.14 (a 630)

Chl b= 20.7 (a 645)-4.34 (a 665)-4.42 (a 630)

(mg/L) (where a=absorbance)

The ratio of chlorophyll a to b was also calculated and compared with controls.

2.4 Fenitrothion Degradation

2.4.1 Abiotic Degradation

Triplicate sets of 20 ml Gorman and Levign growth medium were incubated with 5.0 μg/ml fenitrothion or fenitrothion plus ^{14}C-ring labelled fenitrothion, in 50 ml pyrex culture tubes capped with teflon lined screw caps. Three identical treatments were prepared and incubated at 21°C in either the dark, fluorescent Cool White light, or Vita Lite^{R}. Each of the three treatments were represented in triplicate and the experiment was repeated once to provide n=6. The light intensity was adjusted to 40 w/m^2 which was measured using a LI-COR quantum/radiometer/LI 185B.

The complete volume of each growth medium sample was analysed at each of the following time periods (0, 24, 48, 60, 96, 168, and 336 h). These abiotic controls without C. reinhardtii were extracted and analysed to identify the residual fenitrothion and fenitrothion metabolites. Mass balance and quantification of radiolabelled pesticide and pesticide metabolites were done using thin layer chromatography, and liquid scintillation counting. The rate of degradation and half-life of fenitrothion under these different abiotic light regimes were also calculated.

2.4.2 Biotic Degradation

C. reinhardtii cultures (1.0 x 10^6 cells/mL, 1.0 mg/mL algae) were incubated at 22°C with 5.0 μg fenitrothion /mL of Gorman and Levign medium (pH=6.8) for two weeks. Three replicate sets were incubated under Vita Lite^{R} and three parallel treatments were

incubated in the dark. In addition, control abiotic treatment sets of growth medium without alga were incubated in the light and dark.

This detailed set-up was repeated using radioactive labelled ^{14}C-ring fenitrothion incubated in both the algal treatments and the control treatments. At time periods of 0, 39, 60, 168, and 336 h, algal samples were separated in cell and medium fractions and analysed separately according to the extraction methodology. Metabolite residues were detected and quantified using liquid scintillation counting and gas chromatography, and were compared with the abiotic controls. Accumulation of ^{14}C-methoxy labelled fenitrothion into C. reinhardtii from cell medium was also monitored and compared with accumulation of ring ^{14}C-radio-labelled fenitrothion.

2.4.3 Elimination Studies

Elimination of fenitrothion metabolites from algal cells into external growth medium was monitored by incubating 4 sets of algal cultures in triplicate (n=3) with fenitrothion (5.0 µg/mL). Two sets of triplicate samples for each analysis time period were incubated in the dark and two sets were incubated under Vita Lite^{R}.

After one week one triplicate set from the light treatment and one from the dark treatment were separated into cell and medium fractions. The metabolites were extracted and quantified.

At one week, the remaining two treatments incubated in the light or dark were centrifuged and the cell pellet was removed from the supernatant cell medium. The cell pellets were washed in cell

medium without fenitrothion, and were resuspended in pesticide free medium to incubate for an additional week. After two weeks, cellular and extracellular fractions were analysed as were the one week samples to identify and quantify the metabolite residue.

Four triplicate sets of abiotic controls of 5.0 µg/mL fenitrothion in growth medium were incubated in the light (2 sets) or the dark (2 sets) as were the biotic algal treatments. Two sets of controls (one light and one dark treatment) were extracted into chloroform and aqueous soluble fractions at one week, and the remaining two sets were analysed at two weeks.

Cell medium fractions at one week and two weeks were compared with cell medium fractions of cultures which were resuspended in pure medium after one week. A comparison of these latter three fractions was done to determine if the algal mediated increase in metabolites in the external growth medium was due to elimination of metabolites produced in the cell fractions.

2.5 PSMO Studies

2.5.1 Physiological Studies

Cultures of algae were treated with phenobarbital (PSMO inducer compound) without fenitrothion to determine the concentration that was physiologically non-toxic to the algae. Cultures of C. reinhardtii (1×10^6 cells/mL, 1.0 mg algae/mL) were incubated with a range of treatment concentrations of phenobarbital (0.00232, 0.0232, 0.232, 2.32, 23.2 µg/mL) in Gorman and Levign growth medium (pH=6.8, 21.0 ± 0.5°C). Triplicate sets (20 mL

volume) for each concentration were incubated for two weeks in 50 mL culture tubes stoppered with teflon lined screw caps. The cultures were illuminated under Vita Lite^{R} and agitated on a shaker. Each day the transmittance of these cultures was measured, growth curves were constructed, and growth rates and maximum carrying capacity were determined as described previously.

Similarly the concentration of piperonyl butoxide which was physiologically non-toxic to C. reinhardtii was also determined. Cultures were treated with a range of concentrations of piperonyl butoxide (0.005, 0.05, 0.5, 5.0, and 50.0 µg/mL), and were incubated under Vita Lite^{R} in culture tubes. The effect of test concentrations of piperonyl butoxide on growth rate and biomass was determined. In addition, three sets of 150 mL of algal culture (1 x 10^{6} cells/mL) were incubated in 250 mL cotton stoppered erlenmeyer flasks with the treatment concentrations of piperonyl butoxide. These were incubated for 2.0 weeks under Vita Lite^{R} as were the culture tubes. Each day 1.0 mL aliquots were removed for cell weight determinations, and 4.0 mL aliquots were analysed for chlorophyll a and chlorophyll b content. Once the treatment concentrations of phenobarbital and piperonyl butoxide were determined, these same physiological studies were repeated with the test concentration of phenobarbital or piperonyl butoxide with 5.0 µg/mL fenitrothion. This served to monitor any effects on the physiological parameters during incubation of algae with fenitrothion and the cytochrome P_{450} monooxygenase inducer or inhibitor compounds.

2.5.2 Metabolism Studies

To detect the involvement of cytochrome P_{450} monooxygenase in the algal mediated degradation of fenitrothion, 23.3 µg/ml phenobarbital or 0.5 µg/mL piperonyl butoxide were added to C. reinhardtii cultures with 5.0 µg/mL fenitrothion. Triplicate sets of 20 mL cultures of treated alga were incubated for two weeks in Gorman and Levign growth medium on a shaker (pH=6.8, 21°C ± 0.5) in 50 mL culture tubes under Vita Lite® illumination. Triplicate sets with radiolabelled fenitrothion were also prepared for each of the analysis time periods. At 0, 39, 60, 168, 336 h, the algal cultures were extracted and analysed following the protocol for algae samples, using lsc and gc.

Induction of P450 monooxygenase activity was monitored by comparing half life of fenitrothion and metabolite production within phenobarbital treated algal fractions, with that of the control algal metabolism. Inhibition of cytochrome P_{450} monooxygenase was monitored by comparing these same parameters in the presence of piperonyl butoxide treatment with that of control algal metabolism. Any increase or decrease in any of these factors was analysed in relation to induction or inhibition of monooxygenase activity.

2.6 Extraction Methodology

2.6.1 Extraction of Growth Medium and Cell Medium Samples

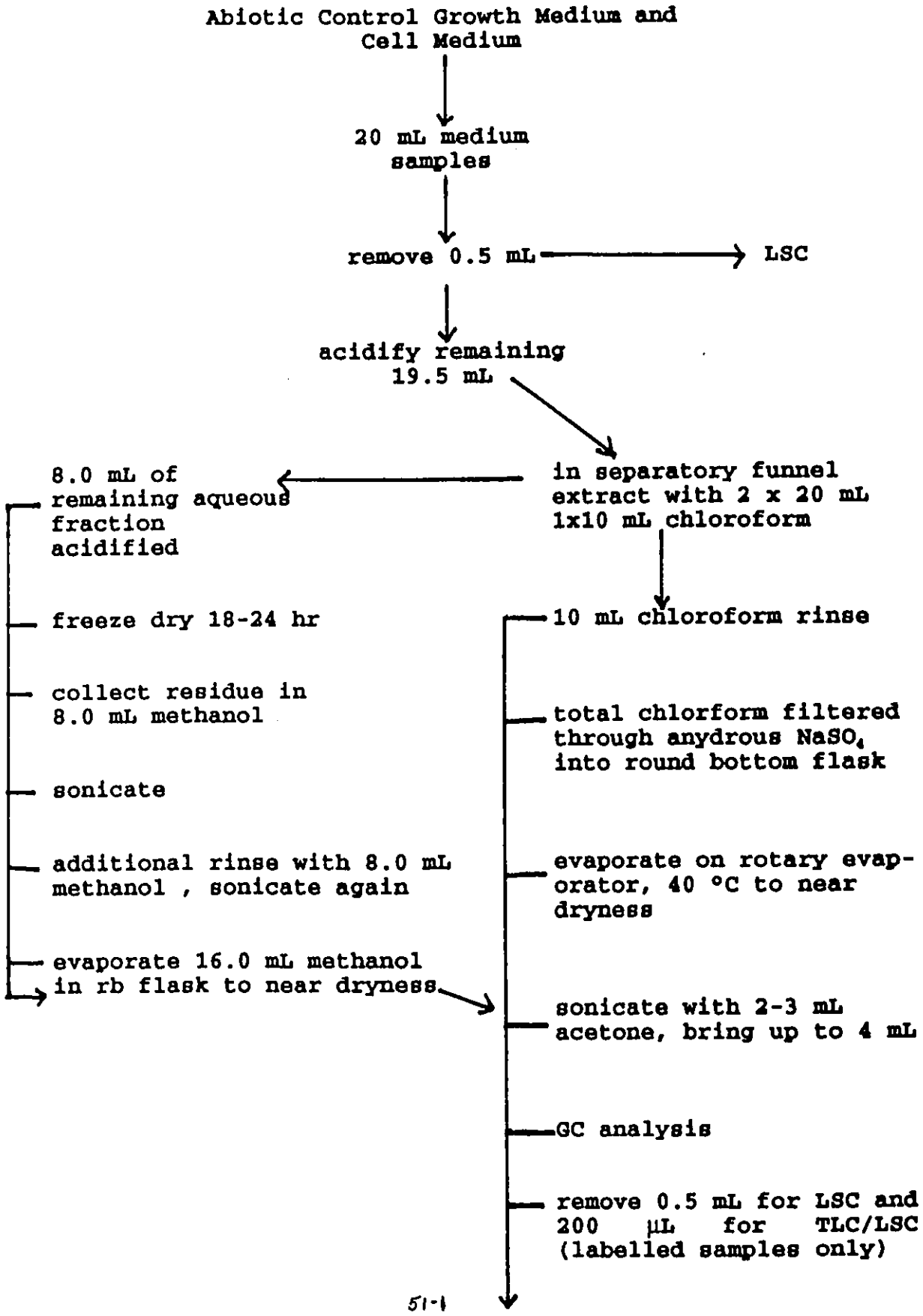
Medium samples were analysed at 0, 39, 60, 168, and 336 h (Figure 6). The 0.5 mL aliquots of the samples treated with radiolabelled pesticide were removed from the original 20 mL volume for mass balance analysis. Medium samples with or without radiolabelled pesticide were then acidified from pH 6.8 to pH 3.9-4.1 using 2-3 drops of acetic acid. pH was measured on an ionanalyzer/501 (Orion Research). The samples were extracted with 2 x 20 mL followed by 1 x 10 mL of chloroform in 125 mL separatory funnels. The chloroform fractions were separated from the aqueous medium and filtered through anhydrous sodium sulphate to remove traces of water. A rinse volume of 10 mL chloroform was added to the chloroform fraction to give a final volume of 60 mL in a 100 mL round bottom flasks.

The chloroform fractions were evaporated to near dryness, under vacuum with a Buchi rotary evaporator unit,(Brinkman) at 40°C. The remaining residue was sonicated in a Polytron sonicator (Tronic Corp) with 2-3 mL acetone, and brought up to 4.0 mL in acetone. 8.0 ml samples of the remaining aqueous fractions were freeze dried for 18-24 h in a Labconco freeze drier (Fisher).

The dried residue was rinsed with 8.0 mL of methanol and sonicated to loosen the residue. This was repeated with an additional 8.0 mL of methanol. The combined 16.0 mL from each sample was evaporated to near dryness in 20 mL round-bottomed pyrex flasks. After additional sonication with 2-3 mL acetone, the final

Figure 6

Extraction Methodology



volume was brought up to 4.0 mL acetone. These acetone samples containing chloroform and aqueous soluble extracts of fenitrothion residues were analyzed using gc and tlc.

Liquid scintillation counting and tlc/lsc were used for radiolabelled samples to quantify the amount of fenitrothion remaining and the amount of fenitrothion metabolites produced. A derivatization step was necessary for the acetone samples with aqueous soluble metabolite prior to gc analysis as is described later.

2.6.2 Algal Samples

A 0.5 mL aliquot (medium plus cells) was removed from each algal culture which was incubated with ring labelled fenitrothion, and was counted by lsc. Another 1.0 mL aliquot was removed from each algal culture sample, (both the non-labelled and labelled treatment sets) and filtered on a dry pre-weighed Whatman GF/C glass fibre filter. The filter was washed with 2 ml distilled water, dried at 100°C for 4 h, cooled to room temperature in a desiccator and weighed to determine the mass of algae/mL culture. The filters of radioactive samples were dissolved in Scintran-X scintillation counting fluid and counted by lsc to determine the algal accumulation of labelled pesticide and fenitrothion metabolites for the mass balance.

The remaining 19.0 mL of algal culture samples, (18.5 mL for labelled fenitrothion treatments), was centrifuged in 30 mL Corex tubes in a Sorvall centrifuge for 20 min at 5,000 rpm. A 0.5 mL

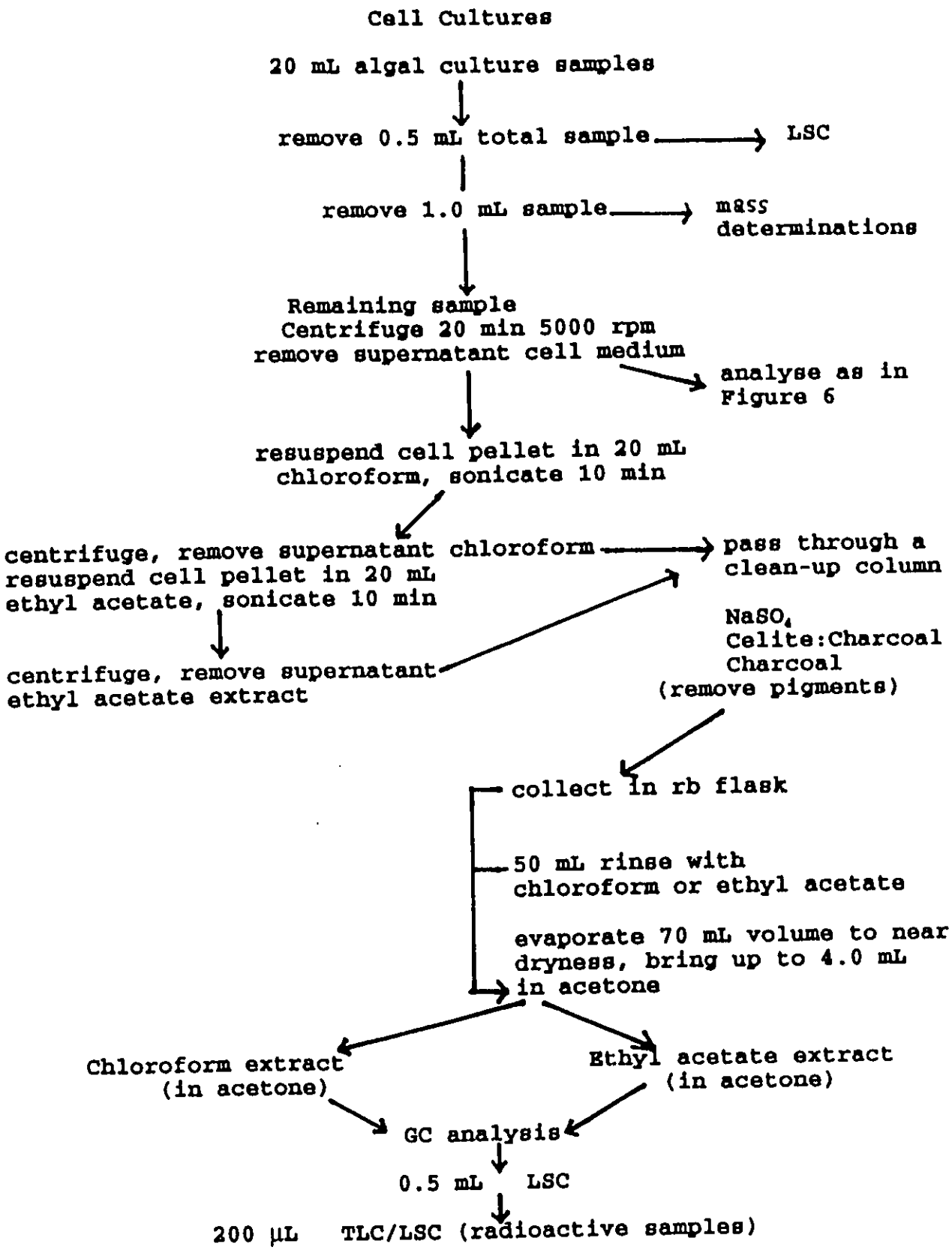
aliquot of the supernatant cell medium was counted by lsc and the remaining supernatant cell medium was stored at 4°C in the dark. These fractions were later analyzed by the method described for control growth medium.

The cell pellets were resuspended in 20 mL chloroform and sonicated for 10 min to remove residues of fenitrothion and fenitrothion metabolites from the cell fraction. After centrifuging the cell pellet from the supernatant chloroform fraction, the cells were resuspended in 20 mL ethyl acetate and sonicated for another 10 minutes. The chloroform fractions and ethyl acetate fractions were passed through a cleanup column to remove pigments which produce interfering peaks on gc chromatograms. The clean up column consisted of a 24 inch long 0.75 inch diameter column which was packed with 2-3 g of celite followed by 1:6 charcoal:celite mixture (7.0 g charcoal) and finally 2-3 g of anhydrous sulphate. The packed column was rinsed with 50 mL hexane and this fraction was discarded. 50 ml of ethyl acetate or chloroform was then used to for elution of columns for ethyl acetate and chloroform extracts respectively (Figure 7).

The total 70 mL volumes were evaporated to dryness in 125 mL round bottom flasks, and brought up to 4.0 mL acetone. These chloroform and ethyl acetate cells fractions containing metabolites residues were quantified by gc and lsc mass balance methodologies.

Figure 7

Extraction Methodology



2.7 Analytical Methodology

2.7.1 TLC

The developing solvent mixtures tlc were made immediately prior to spotting the tlc plates and were permitted to equilibrate for 30 minutes in the developing chamber prior to developing the tlc plates.

Extracted fractions of medium or cell fractions (200 μL) with non-labelled pesticide were plated against standard metabolites on pre-washed (pesticide grade methanol) and dried (30 min at 70°C) Fisher brand Redi/Plate™ silica plates (250 μm thickness) containing activated zinc sulphate fluorescent indicator. The plates were developed in two dimensions using chloroform:methanol (3:1) and cyclohexane:ethylacetate (3:1) to detect and determine the rf of the following fenitrothion metabolites: fenitrothion, fenitrooxon, formyl fenitrothion, hydroxymethyl fenitrothion, S-methyl fenitrothion, demethyl fenitrothion, and 3-methyl-4-nitrophenol.

Metabolites were visualized by U.V. fluorescence using a UVA lamp (245 nm, UVS-11 Mineralight®) and three different reagent sprays. Metabolites containing the 3-methyl-4-nitrophenol moiety were detected using a 10% alcoholic potassium hydroxide solution, (Hollingworth et al., 1967b), which gave yellow spots after heating the plate for 5.0 min at 80°C. A 0.50% solution of 2,6-dibromo-N-chloro-p-benzoquinoneimine dissolved in cyclohexane was used to detect metabolite residues containing the P=S moiety after heating for 7 min at 110°C (Menn et al., 1957). Spots appeared yellow to

brownish-red.

Phosphorus was visualized using perchloric acid ammonium molybdate reagent spray (March et al., 1954). 10 mL of 1.0N HCl, 25 mL 4% ammonium molybdate, 5.0 mL 72% perchloric acid, and 60 mL distilled water were combined and sprayed on the developed plates. This was followed by heating for 2 min at 80°C, and illumination for 15-30 min under u.v.(365 nm) light. Phosphomolybdenum appeared as blue spots. The metabolites were therefore identified both by their R_f values and their reaction to the reagent sprays.

Table 2 shows the limits of detection of fenitrothion metabolites using thin layer chromatography techniques combined with u.v. visualization or lsc techniques described earlier. The R_f value for each metabolite on the different types of silica, and cellulose plates is also listed in Table 2.

The limits of detection ranged from 10 ng to 700 ng by this analytical technique. Pesticide residue analysis requires that the most sensitive analytical techniques be used. For this reason TLC was used only for studies of partitioning of radiolabelled fenitrothion moieties, while GC was used for quantification of metabolic residues.

2.7.2 Radiolabelled Samples

200 μL of medium or cell fraction samples with ^{14}C-ring labelled pesticide were plated on Kodak Chromagram tlc paper (100 μm thickness) with fluorescent indicator and developed in chloroform:methanol (3:1) and cyclohexane:ethyl acetate (3:1)

Table 2 R_f Values and Limits of Detection for Fenitrothion Metabolites During TLC Separation

Metabolites	R_f Values		
	$MeOH/CHCL_3$	Cyclohex:Ea	NH_4OH/water/ propanol
fenitrothion (10 ng)	0.85	0.58	1.0
fenitrooxon (10 ng)	0.4	0.09	1.0
aminofenitrothion (180 ng)	0.0	0.0	0.9
formylfenitrothion (700 ng)	0.8	0.8	1.0
S-methylfenitrothion (500 ng)	0.6	0.15	1.0
carboxyfenitrothion (500 ng)	0.0	0.0	0.78
carboxyfenitrooxon (500 ng)	0.0	0.0	0.84
formylamino-fenitrothion (100 ng)	0.35	0.4	n/a
denitrofenitrothion (100 ng)	0.0	0.35	n/a
carboxyethyl-aminofenitrothion (20 ng)	0.75	0.37	0.98
Hydroxymethyl fenitrothion (200 ng)	0.4	0.25	0.81
Demethylfenitrothion (100 ng)	0.0	0.0	0.72
DMPTA	0.0	0.0	0.01
Nitrocresol (30 ng)	0.42	0.38	0.91
DMPA	0.0	0.0	0.53
MPA	0.0	0.0	0.16
PA	0.0	0.0	0.05

n/a not analysed by tlc with this solvent system

solvent systems. The metabolites were identified from their R_f value during illumination under UVA light. The paper plates were cut into 0.5 cm strips from the origin to the solvent front, placed in scintillation vials, and counted as described bellow.

2.7.2.1 Extraction Methodology

The extraction methodology resulted in some loss of the initial radiolabelled ring ^{14}C-fenitrothion. Table 3 shows the mass balance of recovery of % original label in each extracted fraction. The results from each fraction are discussed below.

2.7.2.2 Medium Samples

Loss of up to 10% of radiolabelled material was lost during the residue extraction process. Extraction from aqueous samples involves the processes of freeze drying and during roto-evaporation, during which some loss of fenitrothion residue could be lost. Loss of residue during roto-evaporation was minimized by not allowing the sample to evaporate to dryness, and by repeatedly rinsing the flask while collecting the residue concentrate.

2.7.2.3 Cellular Fraction Samples

About 5.0% of the ^{14}C-label was lost from the cell pellet upon work-up to obtain the chloroform and ethylacetate concentrates.

Table 3

Percent Recovery of ^{14}C-ring Labelled Fenitrothion After Extraction of Pesticide Residue from Cellular, Cell Medium, and Control Medium Fractions[2] after 24 h Incubation of *C. reinhardtii* Culture ($1x10^6$ cells/mL) in the Dark with 5.0 µg/mL Fenitrothion

	Fraction	Percent Recovery[1] of Original Label
A	Control Medium (Total)	100%
	Aqueous Soluble Fraction	20.15 ± 5.40
	Chloroform Soluble Fraction	78.70 ± 5.97
		Total = 98.85 ± 11.01
B	Cell Medium	65.13 ± 3.11
	Aqueous Soluble Fraction	16.80 ± 0.85
	Chloroform Soluble Fraction	50.26 ± 3.99
		Total = 67.06 (102.9%) ± 4.84
C	Cell Pellet	10.70 ± 0.45
	Chloroform Extract	6.15 ± 2.05
	EthylAcetate Extract	4.10 ± 2.68
		Total = 10.25 (96%) ± 4.73

1 Mean ± s.d., n=3
2 Extraction procedure is described in Figure 6 and Figure 7.

2.7.2.4 Cell Medium Samples

Up to 5% of the label was lost from cell medium samples. However, at least 15% was lost when combining recovery of all the fractions from the cell treatment. The control medium samples only lost 10% of original ^{14}C-label. Roto-evaporation could account for 5% and the freeze drying a further 5%, suggesting that the remaining 5% of loss is due to the centrifugation and cell washing procedures done to separate the cells from the cell medium. These processes are the only differences between the extraction procedures for the cell medium and control medium samples.

2.7.3 Mass Balance

0.5 mL of total extracts from growth medium fractions or cell fractions with radiolabelled fenitrothion were each counted by lsc to determine the percent label in each fraction relative to the total original pesticide added. In addition the tlc cuttings were counted so that the mass balance could account for the percent label in all metabolites.

The 0.5 ml samples of extracted medium, cell fractions, or tlc cuttings were dissolved in 8.0 mL Scintran-X liquid scintillation cocktail (BDH Chemicals) in 10 mL polyethylene scintillation vials (Canlab) sealed with foil lined screw-caps. All of the radioactive samples were counted on a Packard 2000 CA Tricarb liquid scintillation counter and were corrected for luminescence and quenching using a quench curve. A quench curve was constructed for each sample type: cell extracts (chloroform or ethyl acetate),

growth medium extracts (chloroform and aqueous), and tlc samples.

2.7.4 Gas Chromatography

2.7.4.1 Synthesis of Diazomethane

A 50 mL volume of diazomethane was prepared at one time using a Diazald Kit. 5.0 mL ethanol (95%) were added to 2.0 mL of 5.0 g of KOH in 8 mL of H_2O, in a 100 mL distillation flask fitted with a dropping funnel and an efficient condenser set downward for distillation. The condenser was connected to two receiving flasks in series. The second flask contained 20-30 mL ether. The inlet tube of the second receiver dipped below the surface of the ether. Both receivers were cooled to 0°C using crushed ice.

The flask containing the alkali solution (KOH) was heated in a water bath to 65°C (using a stirring hot plate). A solution of 4.3 g of Diazald in 40.0 mL of ethyl ether was added through the dropping funnel into the KOH solution at a rate of about 2 drops/sec (total time 25 min). The rate of addition approximately equalled the rate of distillation. When the dropping funnel was empty, another 8.0 mL of ether were added slowly until the distillate ether was colourless. The combined distillate contained approximately 3.0 g diazomethane. The diazomethane could be stored for one week at 20°C, in a container protected from light.

2.7.4.2 Derivatization

Immediately prior to GC analysis, samples containing aqueous soluble metabolites of fenitrothion were derivatized with diazomethane in a fume hood. 0.5 mL of diazomethane were added to 1.0 mL of concentrated samples of cell and medium fraction extracts. These bright yellow samples were allowed to stand for 3.0 min at 20°C. The samples were then evaporated under a stream of nitrogen to the original 1.0 mL volume (Shafik and Enos, 1969). The samples were immediately analysed by gas chromatography.

The derivatization reaction resulted in methylation of the acid moiety of fenitrothion metabolites forming esters. Consequently the metabolite residues were made less polar. This facilitated the GC analysis of polar metabolites.

2.7.4.3 GC Analysis

1 μL samples of the extracts in acetone were analysed using a Hewlett Packard model 5830A gas chromatograph equipped with an on column injection port and a flame ionization detector. Separation was achieved on a DB5 Megabore capillary column (id= 0.545 mm, length 8.5 m, 1.5 μm fused silica coating). The carrier gas was He (flow rate of 21 mL/min), the detector gas flow were air (300 mL/min) and hydrogen (30 mL/min). The column was temperature programmed as follows: initial temperature: 120°C, hold time: 1.5 min, ramp at 15°C/min to 245°C, final hold time: 1.0 min, inj temp: 200°C, det temp:250°C.

Known amounts of metabolic standards were injected between test samples under the same chromatographic conditions. The amount of each metabolite per sample was quantified by:

$$\frac{\text{peak height of injected sample (mm) x amount of standard (ng)}}{\text{peak height (mm) response of known standard}} \quad (1)$$

Table 4 shows the retention times and the limits of detection obtained for fenitrothion metabolites quantified by capillary gas chromatography. This was measured at an attenuation of 2^3 using metabolite standards of purities greater than 92% and analytical grade fenitrothion (>99.8%). SMF (6.5%) and NC (1.2%) accounted for the impurities of standards other than the analytical grade fenitrothion. These values of impurities were used to correct the conversion factor in (1) which is used to quantify the amount of metabolite residues. The standard curve relating concentration injected to response in peak height for GC analysis had an r value of 0.96.

2.8 Calculation of Fenitrothion Half Life

The quantified amount of pesticide measured by GC (µg/mL of growth medium) was expressed as percent original fenitrothion added. To test if the degradation is first order, i.e. independent of initial fenitrothion concentration, a plot of ln % fenitrothion remaining against time was constructed.

A first order reaction is represented by the equation $\ln[A] = \ln[A_o] - kt$ or $-kt = 2.303 \log c/c_o$. The ratio of amount of pesticide

Table 4

Retention Time and Limits of Detection of Fenitrothion Metabolites using Gas Chromatographic Analysis with an FID Detector

Metabolite	Retention Time (min)	Limit of Detection (ng) attenuation=2^3
Fenitrothion	7.31	0.260 ± 0.001
Fenitrooxon	7.41	0.668 ± 0.001
S-methylfenitrothion	8.49	0.567 ± 0.003
3-methyl-4-nitrophenol	2.79	0.950 ± 0.001
Desmethylfenitrothion	8.55	0.145 ± 0.0006
Formylfenitrothion	8.65	0.507 ± 0.002
Hydroxymethylfenitrothion	8.92	0.590 ± 0.005
Carboxyfenitrothion	9.26	0.456 ± 0.006
Carboxyfenitrooxon	9.39	0.493 ± 0.001

is unitless and therefore the rate is independent of concentration. The rate was calculated as the slope of the graph of ln % fenitrothion remaining versus time, k =-2.303 log 0.5/t, and the half life calculated using $t_{1/2}$= 0.693/k. The equation representing fenitrothion degradation was then determined for abiotic control and biotic degradation by substituting k into the exponential first order equation:

$$C_t = C_o\ e^{-kt}$$

(Frost et al., 1961)

2.9 Statistical Analysis

Quantified values of fenitrothion metabolite residues were analysed using Tukeyś analysis of variance (Tukey, 1949) using an SPSSX CMS mainframe program. Sigmaplot graphics software was used to construct the graphs and calculate the student T-test 95% confidence intervals. Probit analysis of raw data to determine Ec_{50} values for physiological parameters of fenitrothion treated cultures was done using an SPSSX SAS probit analysis program. RS1 was used to test that data was from a normal distribution, to test if variances were different, and the Student-Newman-Keuls multiple range test was done to determine significant differences between means of different treatments.

Chapter 3

Results

3.1 Fenitrothion Toxicity Studies

Probit analysis of the effect of fenitrothion on the physiological parameters monitored in C. reinhardtii is shown in Table 5. The EC_{50} concentrations are listed for growth rate, maximum carrying capacity, cell weight, chlorophyll a, chlorophyll b, and ratio of chlorophyll a:b. The concentration chosen for subsequent degradation studies of fenitrothion is 5.0 µg/ml fenitrothion.

3.1.1 Population Dynamics

There was no significant effect on the population dynamics with a 5.0 µg/ml fenitrothion treatment relative to control populations as measured by growth rate and maximum carrying capacity (Figure 8(a) and Figure 8(b)). Cell weight however, decreased over time while the population size increased (Figure 9). This suggests that at 5.0 µg/ml, fenitrothion induces a change in size of the individual cells of the C. reinhardtii population. This could be due to the density stresses of high cell numbers in a non-replenished culture medium.

3.1.2 Chlorophyll Concentration

Figures 10(a), 10(b), and 10(c) demonstrate the resilience of the treated algal population at 5.0 µg/ml fenitrothion with respect to chlorophyll a, b and the chlorophyll a:b ratio.

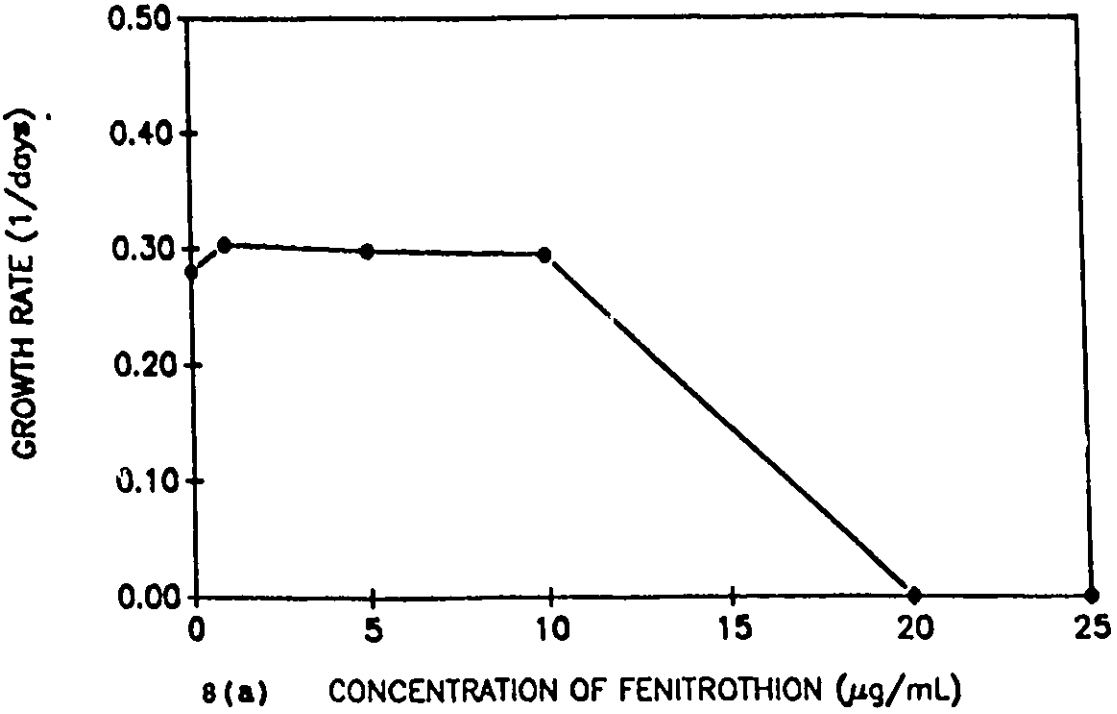
Table 5 Probit Analysis of Physiological Parameters of C. reinhardtii Cultures Treated with Fenitrothion

Parameter	EC_{50}*	Fiducial Limits -	+
Growth Rate	15.3	8.20	20.9
Carrying Capacity (Population Maximum)	15.4	9.52	22.6
Cell Weight	7.2	2.64	14.7
Chlorophyll A	7.6	5.16	1.91
Chlorophyll B	9.7	3.97	17.7
Ratio chlorophyll a:b	17.4	11.5	24.2

* Concentration in ug/mL at which there is 50, percent inhibition of the given physiological parameters. Calculated using SAS probit analysis

Table 6 Abiotic Degradation Rates and Half Life of Fenitrothion in Gorman and Levign Growth Medium Incubated at 21 ± 0.5 °C, pH=6.8, Under Different Light Regimes

Light Regime	Degradation Rate Constant(s^{-1}) (k)	Half-Life (h)
Dark	0.0026 ± 0.0001	270
Cool White Light	0.01 ± 0.0001	70
Vita Lite®	0.023 ± 0.00007	30



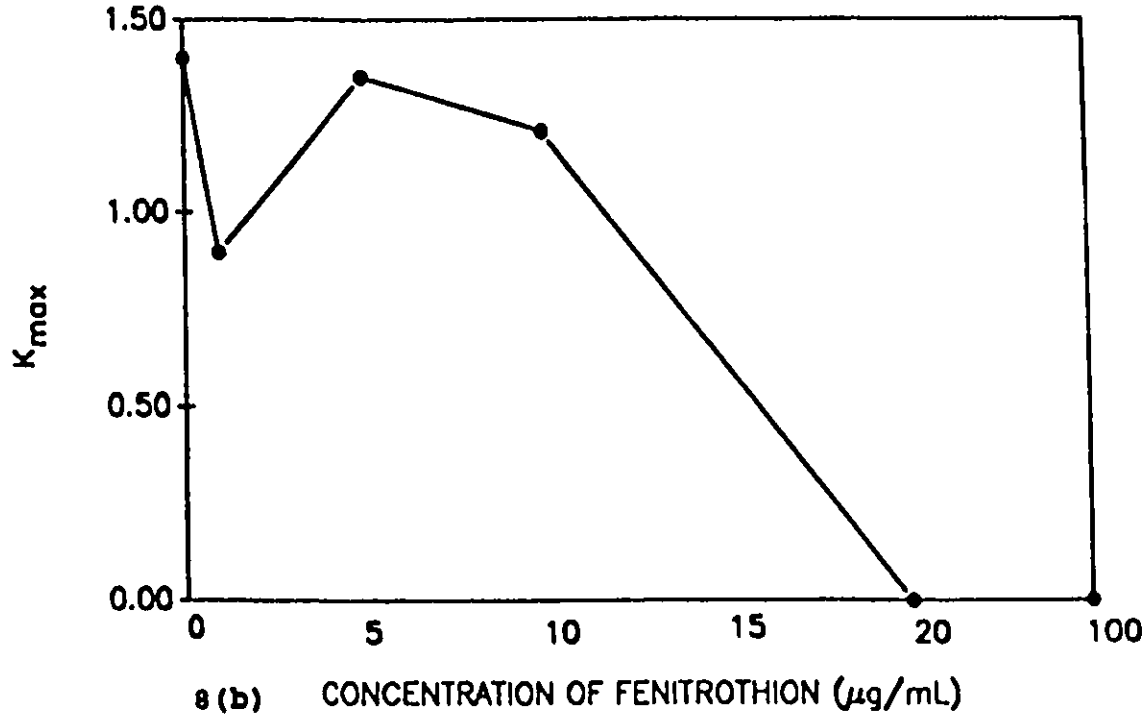


Figure 8

(a) Growth rate and (b) maximum carrying capacity of C. reinhardtii cultures treated with a range of concentrations fenitrothion.

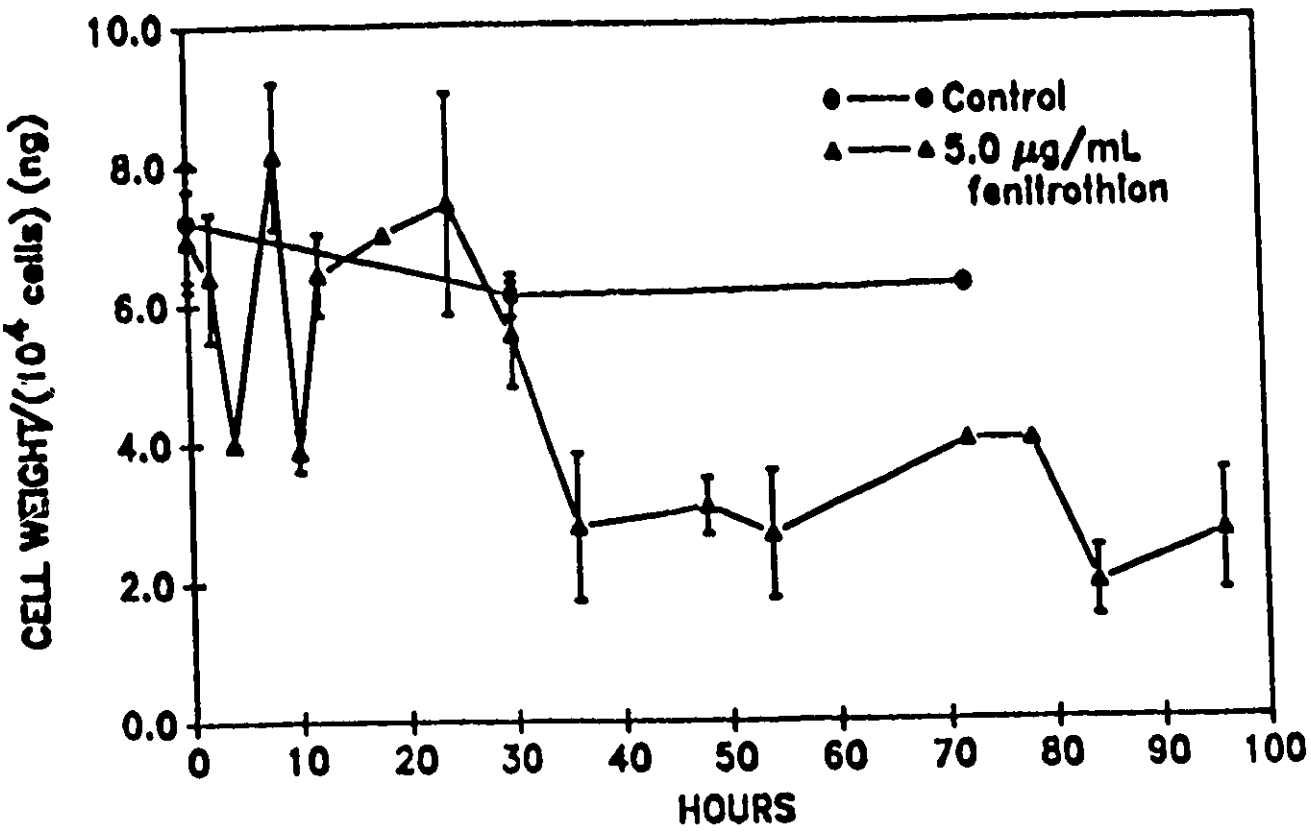


Figure 9

Cell weight of C. reinhardtii cultures treated with 5.0 ug/ml fenitrothion. n=3, p=0.05, mean±95% c.i..

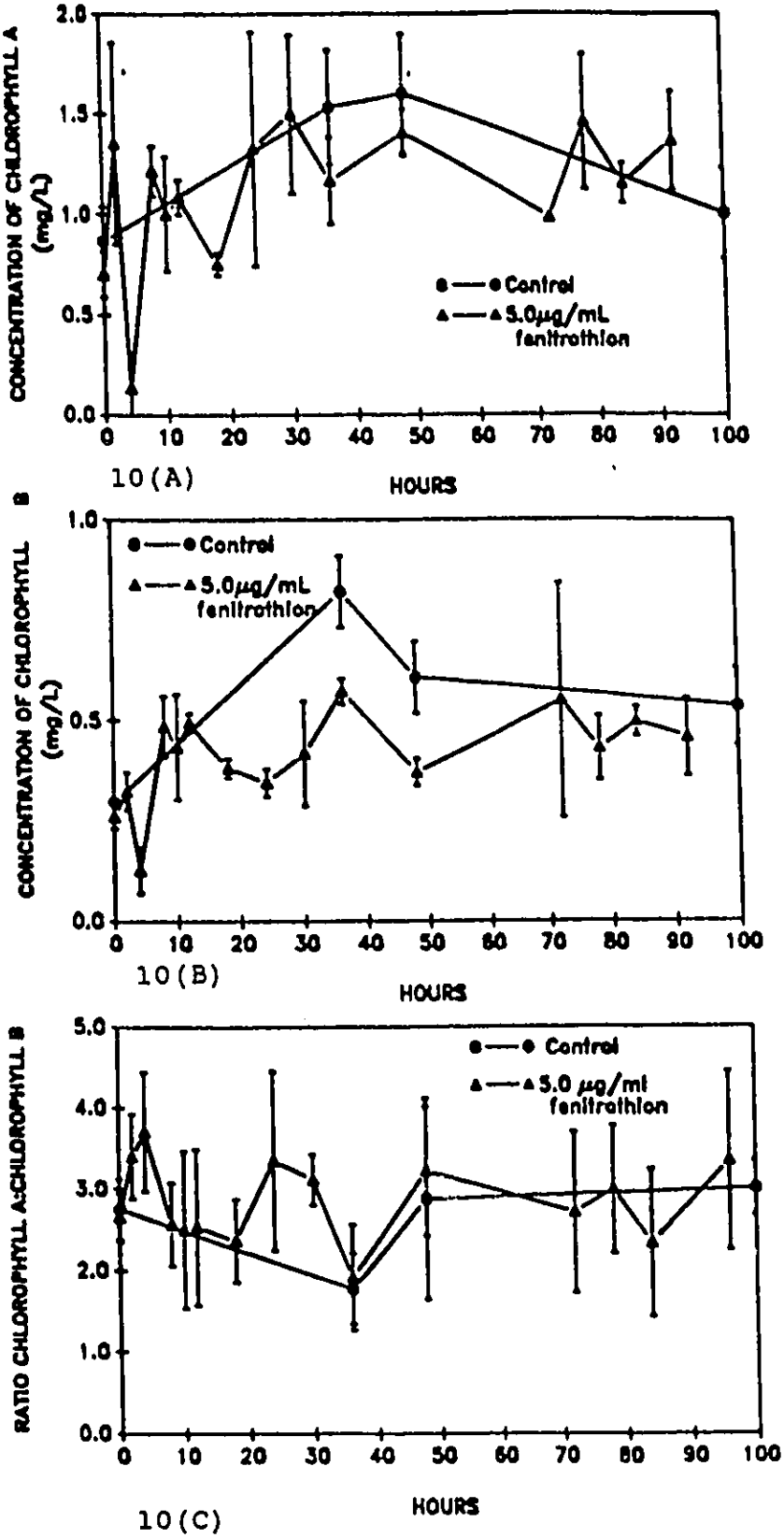


FIGURE 10

Concentration of (a) chlorophyll a (b) chlorophyll b and (c) ratio chlorophyll a:b of C. reinhardtii cultures treated with 5.0 µg/mL fenitrothion. n=3, p=0.05, mean ± 95% c.i..

Fluctuations in chlorophyll a concentration and a:b ratio in C. reinhardtii did not differ significantly from that of control populations. Chlorophyll b differed significantly at 30 h after which it did not differ from the controls up to 100 h. This induction response is a typical one resulting from stress by low concentrations of xenobiotics and represents an attempt at maintaining homeostasis (Stebbing, 1982).

3.2 Abiotic Degradation

Sterile growth medium provides an artificial microenvironment in which to study the abiotic degradation of fenitrothion in the absence of bacteria, algae or other organisms. Abiotic degradation in growth medium involves the effects of relevant physical and chemical factors such as pH, temperature, and electromagnetic radiation. These factors affected the degradation of fenitrothion in this study.

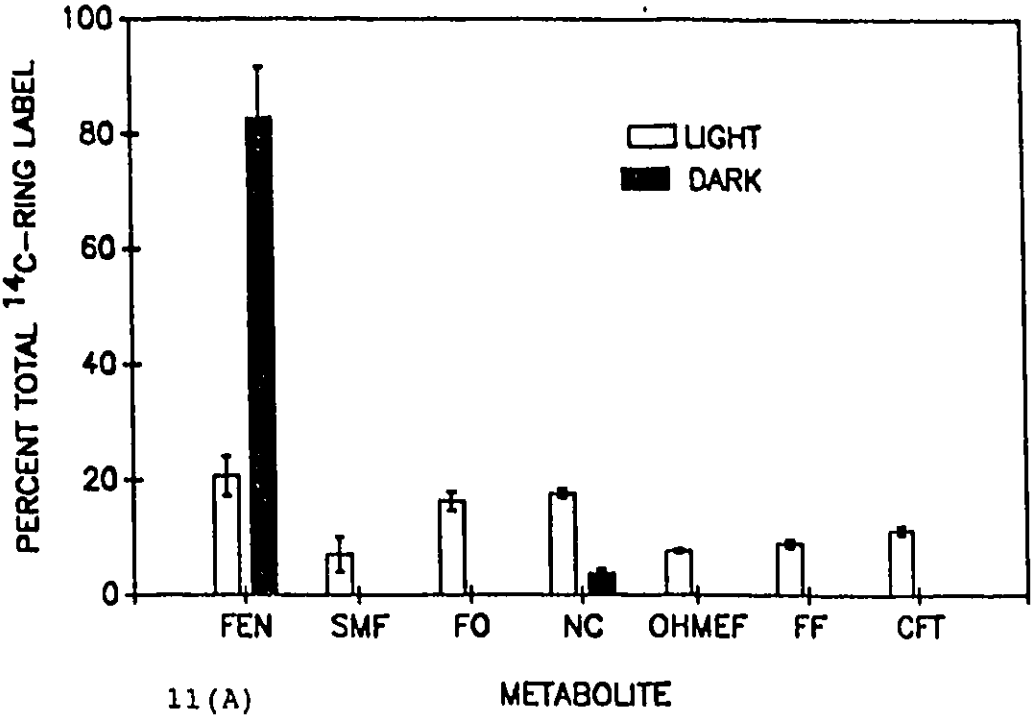
In the chemical environment under study, typical oxidation metabolites included FF, OHMEF, CFT, CFO, and FO. Phosphate ester metabolites DMPA, MPA and PA are also oxidation metabolites. Hydrolysis reaction produce DSM, NC and phosphate ester metabolites from fenitrothion by SN2 reactions and the splitting of water. This produces leaving groups from fenitrothion: methanol with DSM, and phosphate esters with NC. DSM and NC are therefore identified under abiotic conditions as hydrolysis metabolites. Their relative amounts depends on the pH of the growth medium, with high pH's producing predominantly NC and neutral pH producing predominantly

DSM. SMF is produced by the alkylation of the sulphur group of fenitrothion by another fenitrothion molecule, fenitrothion thus acting as an alkylating agent on itself.

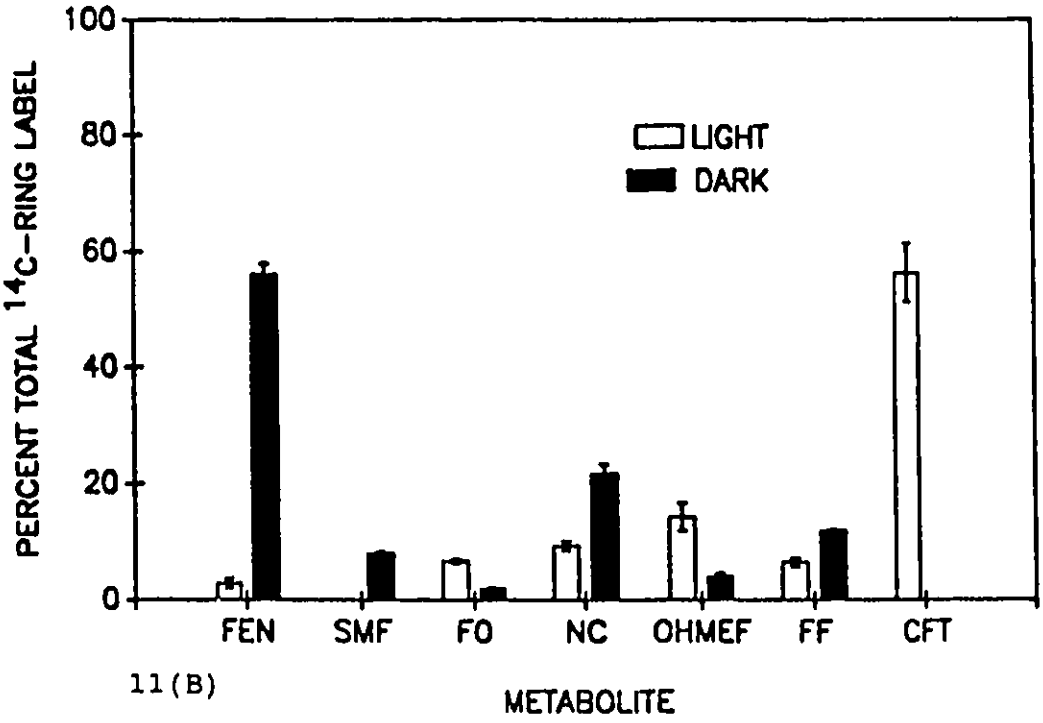
Abiotic degradation rates and relative amounts of metabolites are enhanced by temperature and light. Oxidation of fenitrothion can be enhanced by light, specifically the ultra violet wavelengths, and are therefore defined as photolysis and photooxidation reactions. Photolysis and photooxidation enhance the rate of fenitrothion degradation relative to that of oxidation and hydrolysis. This will affect the relative amounts of the metabolites at any given time during degradation, relative to oxidation and hydrolysis. This abiotic study established the metabolites produced under the controlled pH and temperature in the absence of biotic factors to provide a background with which to compare biotic fenitrothion metabolites.

3.2.1 Metabolites of Abiotic Fenitrothion Degradation

Figure 11(a) and Figure 11(b) illustrate the fenitrothion derivatives detected by thin layer chromatography and liquid scintillation counting of spots after 48 h and 1 week incubation in the dark and under Vita Lite^{R}. The dark incubated growth medium had 80% residual fenitrothion at 48 h with 5% NC (Figure 11(a)). In the light significantly less fenitrothion (20%) remained in the growth medium with the rest of the ring label distributed between the other metabolites at levels between 5 and 20%. Ninety-three to 97% of the recovered label was identified as fenitrothion derivatives



11(A)



11(B)

FIGURE 11

Abiotic fenitrothion metabolites detected in growth medium by tlc and lsc after (a) 48 hours and (b) after 1 week incubation of 5.0 µg/mL fenitrothion in growth medium. n=6,p=0.05, mean ± 95% c.i..

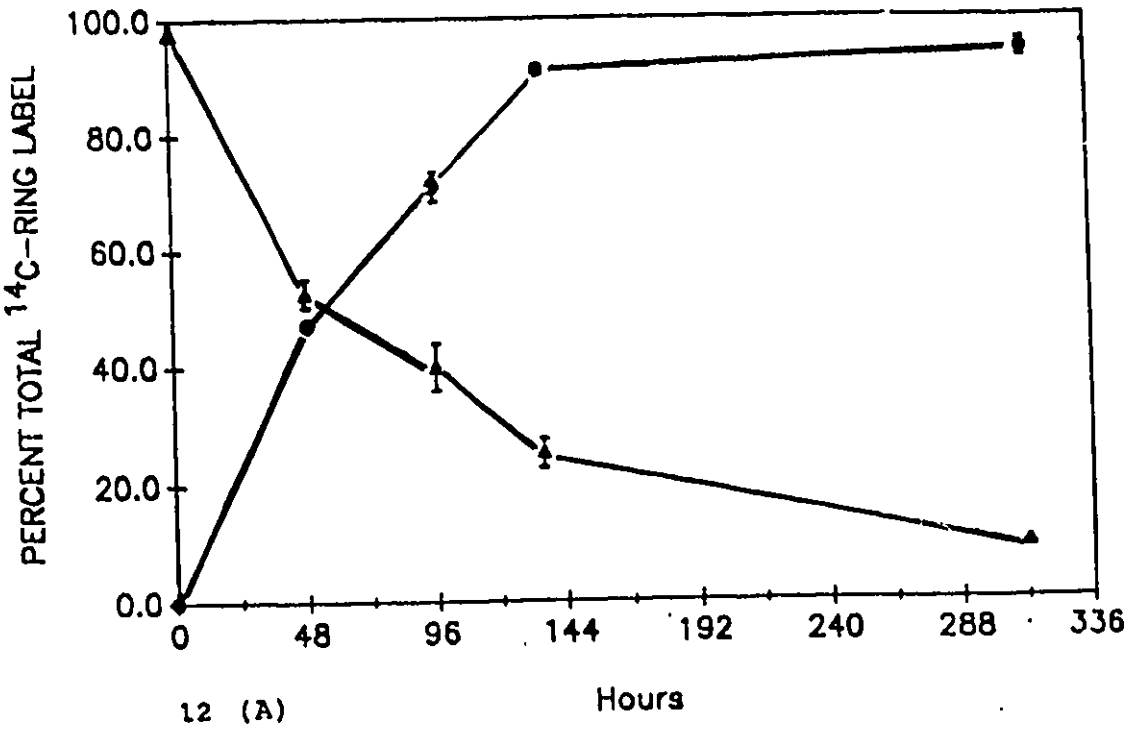
including SMF, FO, NC, OHMEF, FF, CFT, with the latter being the predominant metabolite.

Non-radioactive moieties were detected on cellulose plates using the reagent sprays. Thin layer chromatography with standards revealed that phosphoric acid (PA), methyl-phosphoric acid (MPA) and dimethyl-phosphorothioic acid (DMPTA) were produced in the dark and only PA was produced in the light treatments. Quantification of these moieties was not done. However, their total amounts would not exceed that of NC, the other moiety obtained when these phosphoric and phosphorothioic acids are produced.

After one week, 57% of the ^{14}C-ring labelled fenitrothion residue remained in the dark treated growth medium while only 3.0% remained in the light treatment. The remaining label in the dark was in NC (at a level significantly greater than that at 48 h), and in SMF, FO, OHMEF and FF which were not previously detected (Figure 11(b)). All of these derivatives were detected in the light at 48 h. At one week in the light, the remaining label residues were distributed among the same derivatives as were detected for these treatments at 48 h.

3.2.2 Mass Balance

Mass balance of the total ring labelled moieties of fenitrothion in the system was monitored for 12 days (Figure 12(a) and Figure 12(b)). 93% to 97% of the radiolabelled ring moiety was recovered and identified in the light and dark treatments as fenitrothion or its metabolites. The observed transfer of ^{14}C-ring



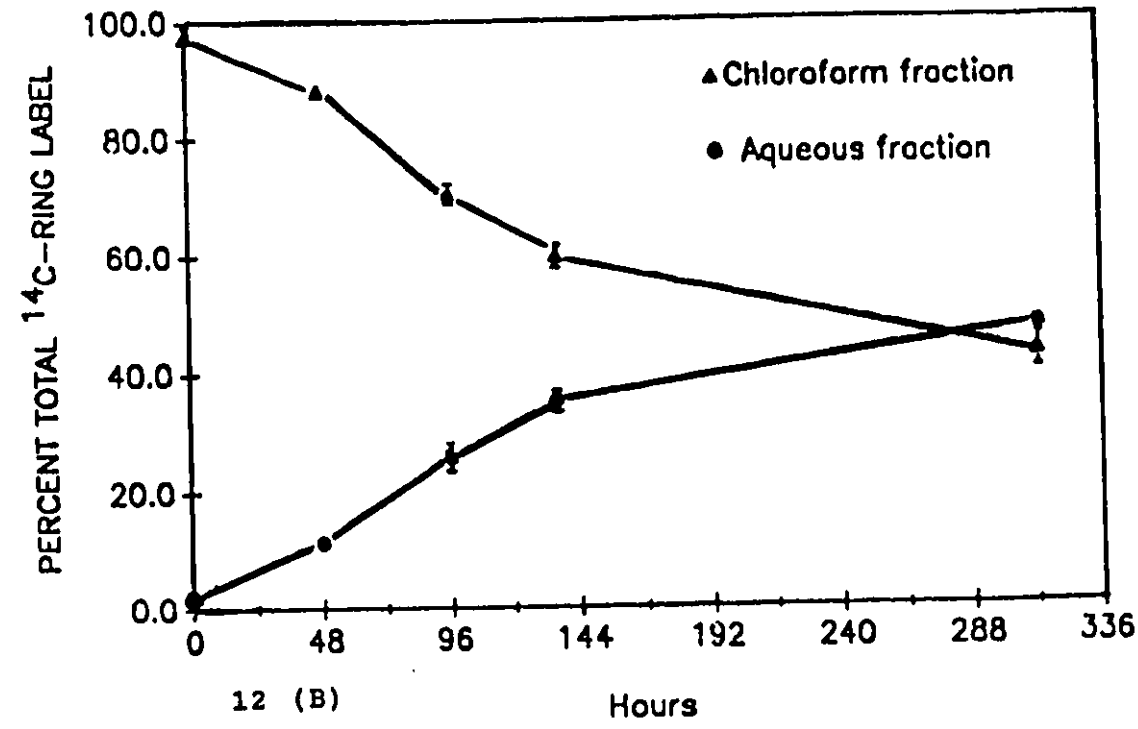


Figure 12

Mass balance of partitioning of ^{14}C-ring labelled fenitrothion moieties between polar and apolar fractions of growth medium during abiotic degradation of fenitrothion (a) in the light and (b) in the dark. n=6, p=0.05, mean ±95% c.i..

radiolabel from the chloroform fraction into the aqueous fraction of growth medium occurred in both the light and in the dark.

At all times greater than 48 h the amount of ring label in the chloroform fraction of medium and the amount of label in the aqueous fraction was significantly greater in the light than in the dark incubated treatments. These differences in half-life and fraction partitioning between light and dark treatments were also reflected in the amounts of fenitrothion derivatives detected by thin layer chromatography analysis.

3.2.3 Rate Constant of Degradation

Figure 13 shows the degradation of fenitrothion in Gorman and Levign growth medium as measured by gas chromatography. The half-lives under different illumination conditions are listed in Table 6 as was calculated from the growth rate from the log plot % fenitrothion remaining versus time using the equation:

$$T_{1/2} = 0.693/k$$

where k= slope is the temperature and concentration independent degradation rate constant. The half life in the dark was 3.9 times greater than under Cool White fluorescent light and 9 times greater than under Vita Lite^R. Vita Lite^R illumination produced the greatest rate of fenitrothion degradation. After two weeks there was 40% original fenitrothion remaining in the dark, 20% with fluorescent light and, and 2.0% with Vita Lite (Figure 13).

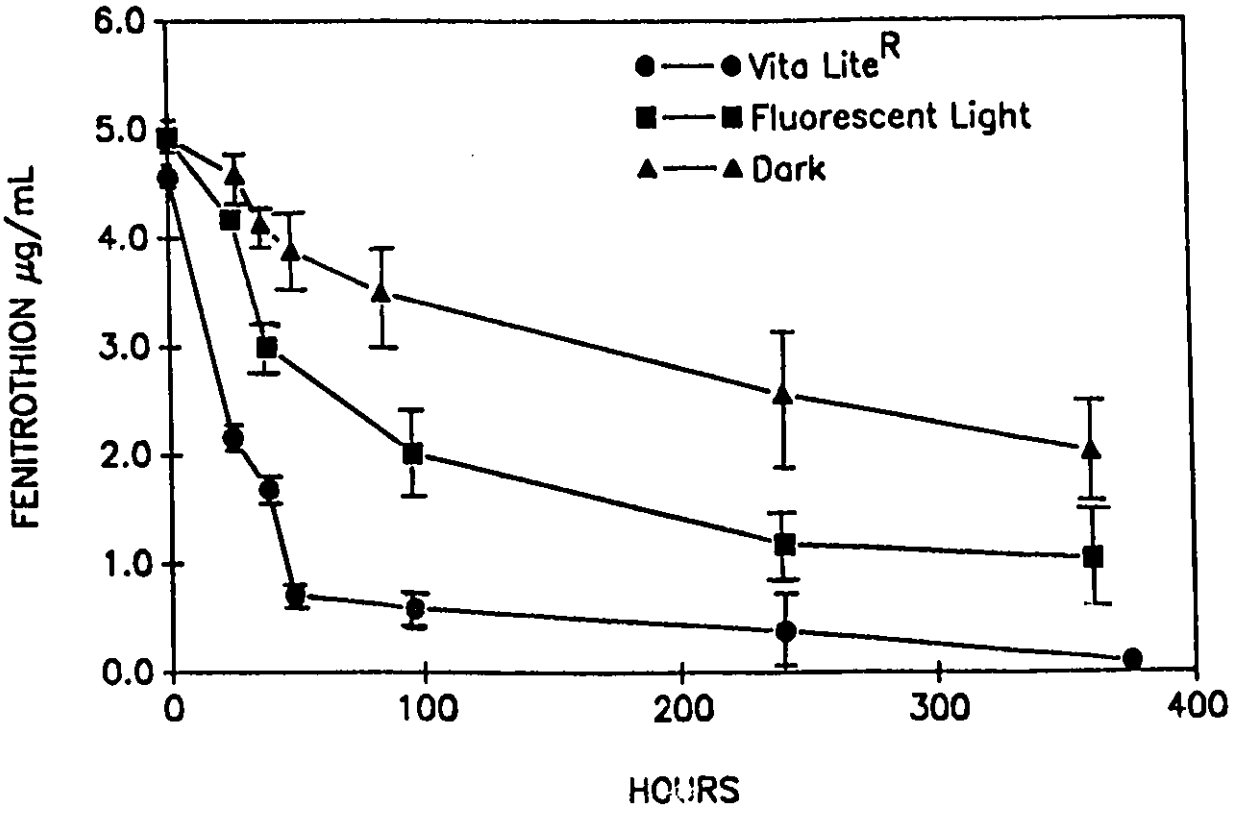


FIGURE 13

Abiotic degradation of fenitrothion from Gorman and Levign growth medium incubated with 5.0 µg/mL fenitrothion under different light regimes. n=6, p=0.05, mean ± 95% c.i..

3.3 Biotic Fenitrothion Metabolism

3.3.1 Biotic Fenitrothion Metabolism by C. reinhardtii

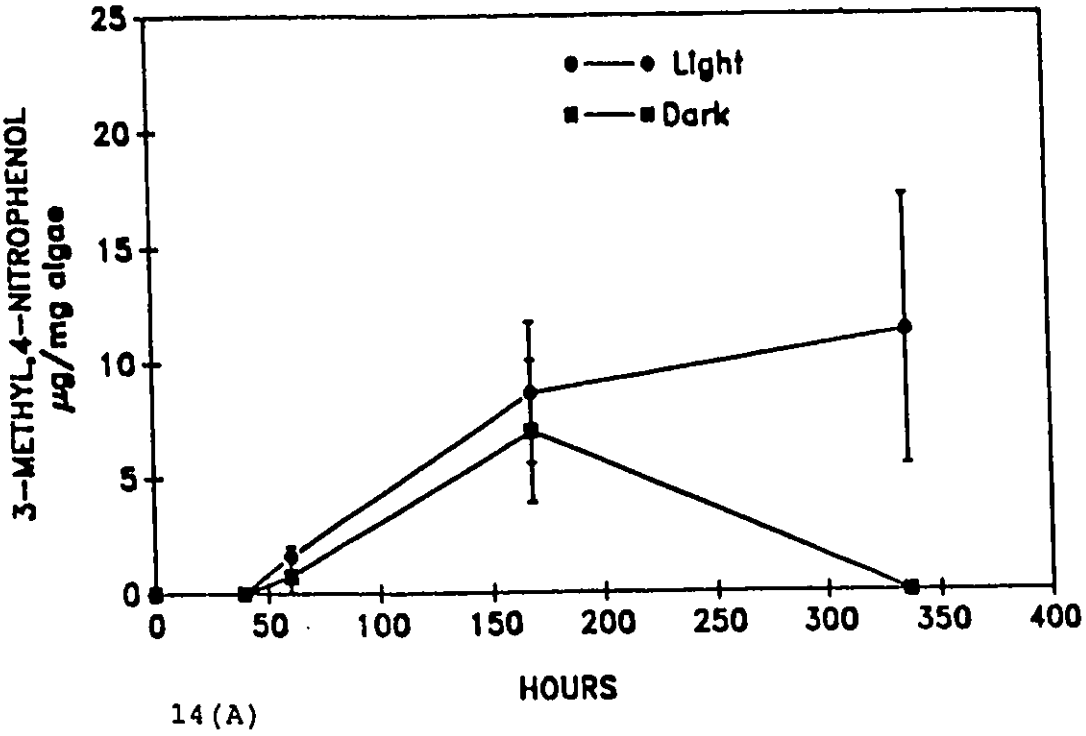
3.3.1.1 Biotic Intracellular Metabolites

Gas chromatographic analysis of the fenitrothion metabolites in C. reinhardtii detected significant amounts of NC, DSM, OHMEF, and CFT in both the light and dark biotic treatments (Figure 14(a), 14(b), 15(a) and 15(c)). FF was only detected in the dark treatment cell fraction (Figure 15(b). The ratio of the metabolites detected in the cell fractions in the light relative to incubation in the dark are shown in Figure 16. The ratios for DSM and OHMEF detected in the light relative to the dark are significantly different from 1.0 at one week and those of CFT and NC are significantly different from 1.0 at two weeks.

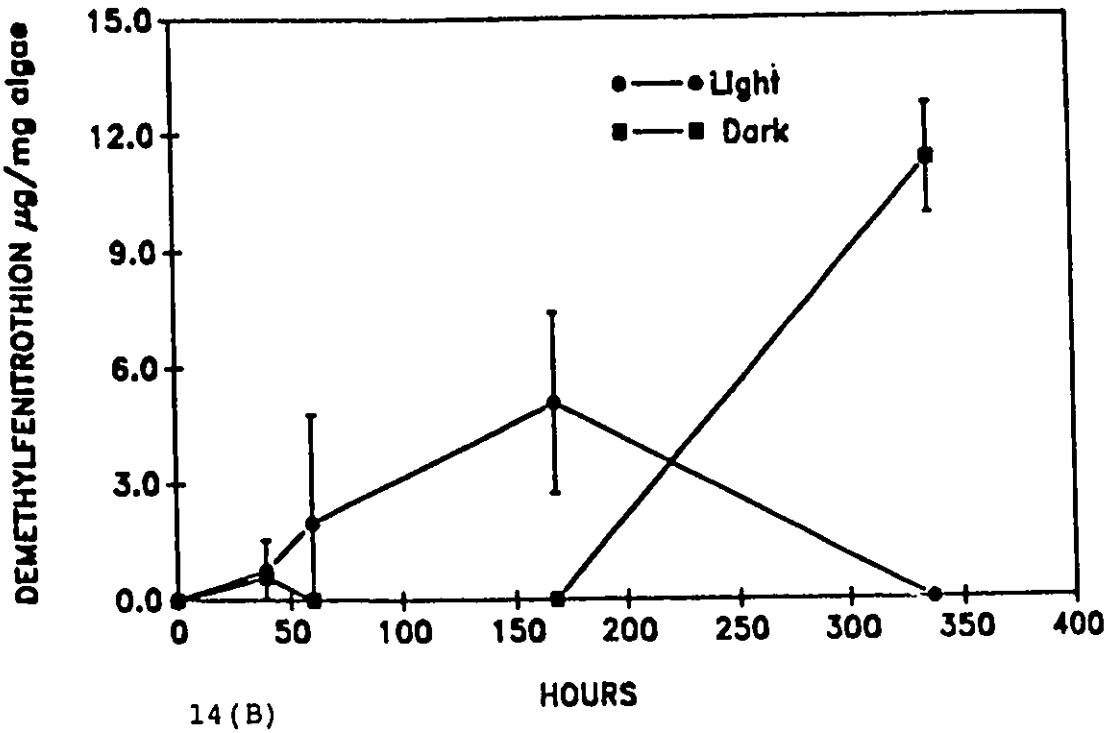
These results demonstrate that the amounts of fenitrothion metabolites detected in the light biotic treatments were dependant not only on the presence of C. reinhardtii and on light energy provided by Vita Lite^{R} (U.V.) but also on incubation time. This suggests that the biotic production of fenitrothion degradation in C. reinhardtii may be mediated by a time dependant mechanism.

3.3.1.2 Comparison of Biotic and Abiotic Degradation of Fenitrothion in Growth Medium

In the control medium, SMF, OHMEF, CFT, NC, and DSM were detected in the light and CFO ,NC and DSM were detected in the dark (Figures 17, 18, and 19). In the biotic treatments, the cell medium contained detectable amounts of NC, DSM, SMF, CFT and FF in both



14(A)



14(B)

FIGURE 14

Amount of (a) 3-methyl-4-nitrophenol (NC) and (b) desmethyl fenitrothion, (DSM), detected in cellular fractions of C. reinhardtii cultures incubated in the light and dark with 5.0 µg/mL fenitrothion. n=3, p=0.05, mean ±95& c.i., $1x10^6$ cells/mL.

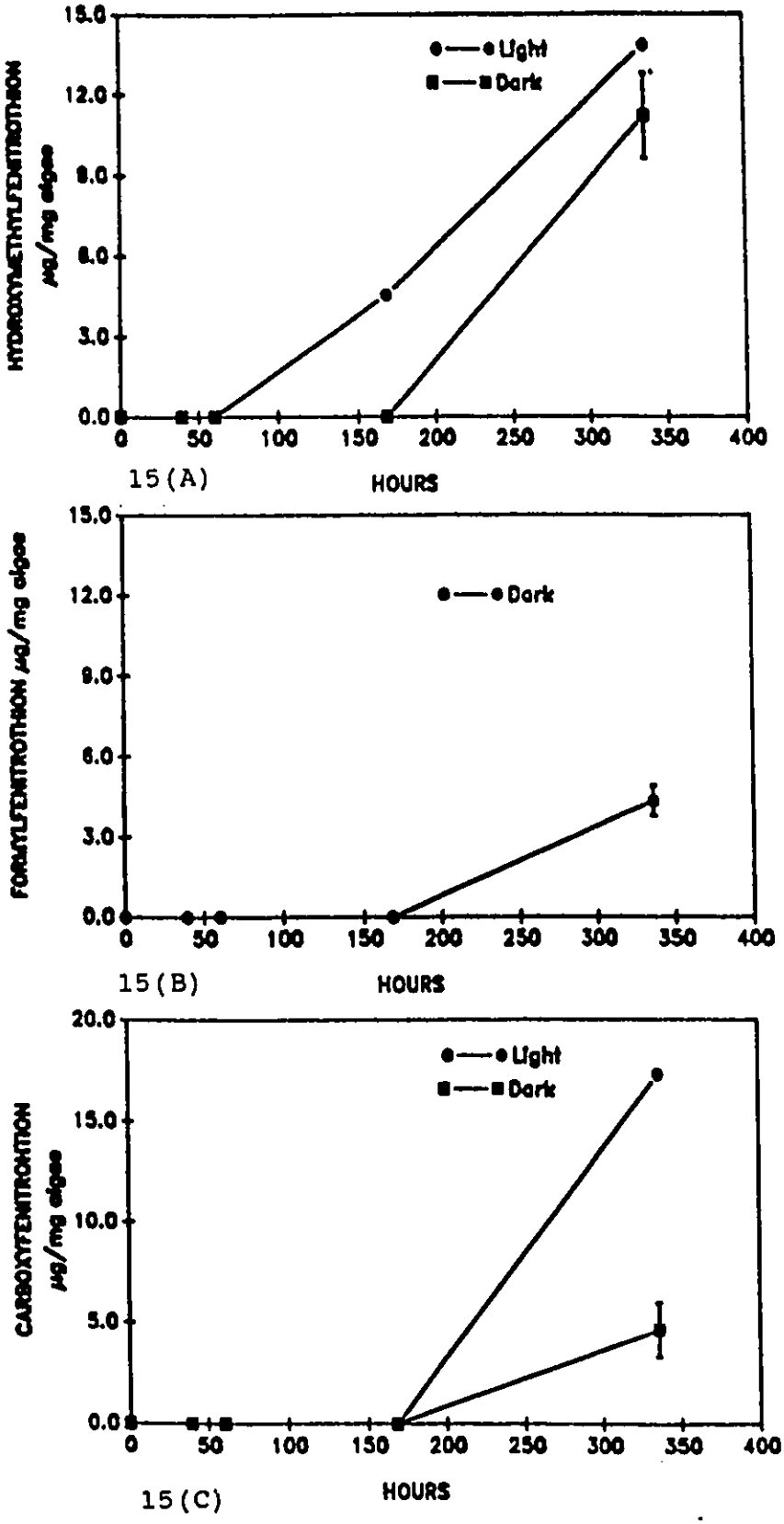


FIGURE 15

Amount of (a) hydroxymethylfenitrothion, (OHMEF), (b) formyl fenitrothion, (FF), and (c) carboxyfenitrothion, (CFT) detected in cellular fractions of C. reinhardtii cultures incubated in the light and dark with 5.0 µg/mL fenitrothion. n=3, p=0.05, mean ±95& c.i., $1x10^6$ cells/mL.

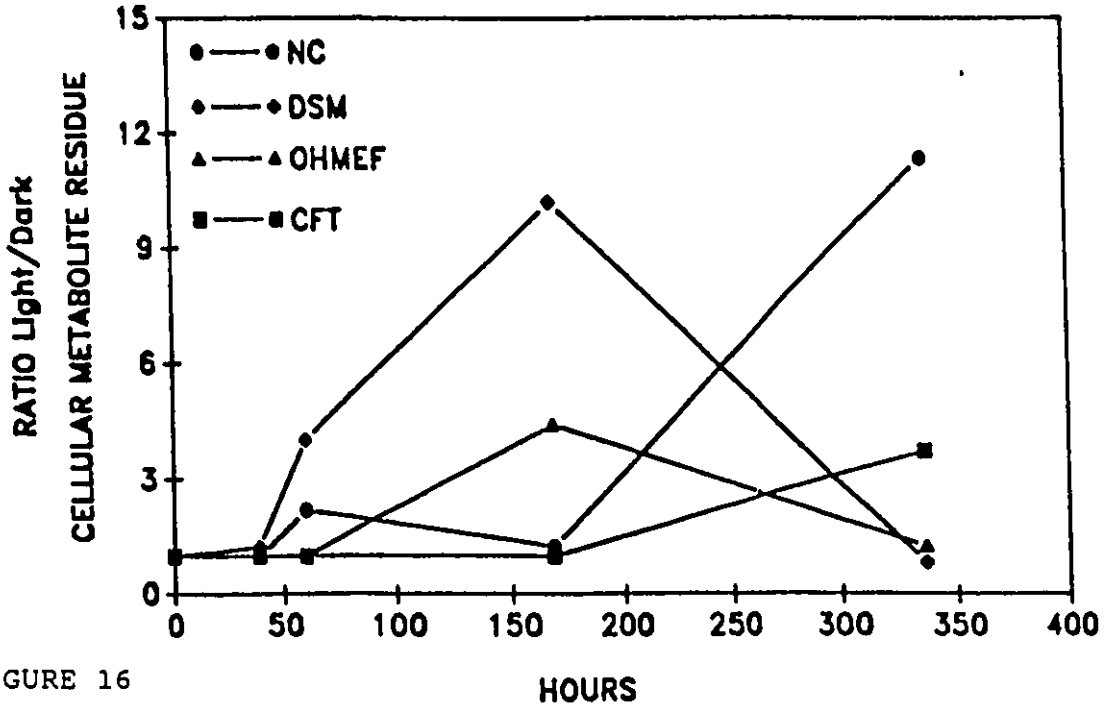


FIGURE 16

Light : Dark ratio of amount of fenitrothion metabolites detected in cellular fractions of C. reinhardtii cultures incubated with 5.0 µg/mL fenitrothion. n=3, p=0.05, mean ± 95% c.i., $1x10^6$ cells/mL.

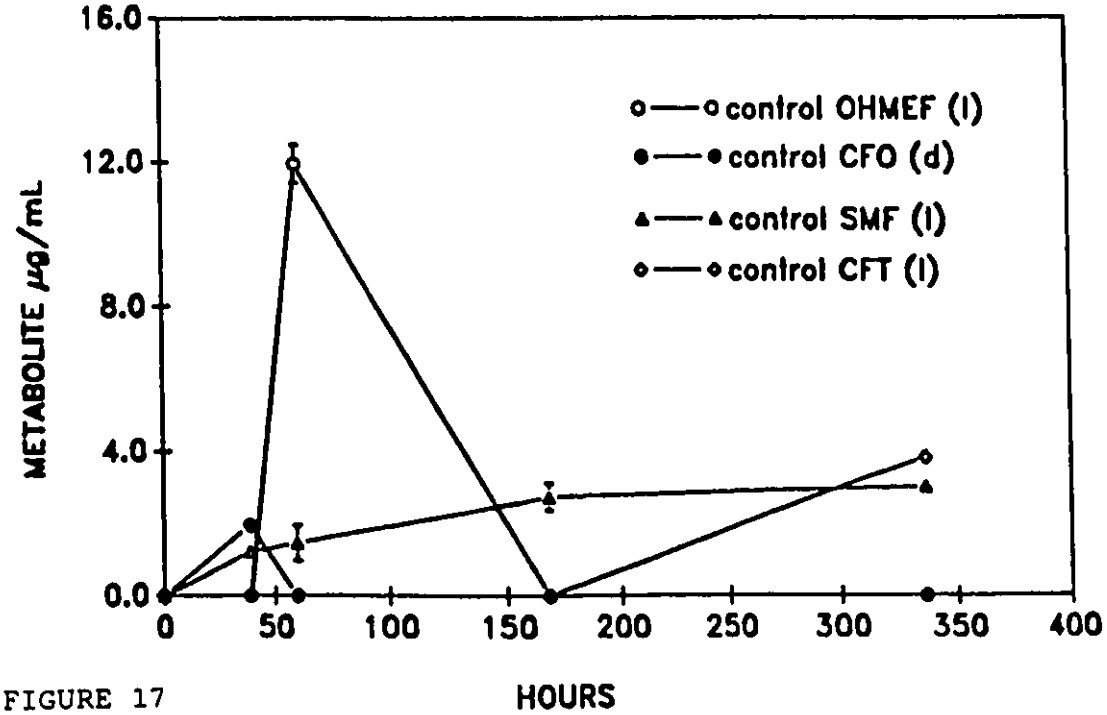


FIGURE 17

Amount of fenitrothion metabolites detected in control abiotic treatments of growth medium incubated in the light and dark with 5.0 µg/mL fenitrothion. n=3, p=0.05, mean ± 95% c.i..

the light and dark treatments (Figures 18, 19, 20(a) and 20(b). OHMEF was detected only in the light treatments and FO was detected only in the dark treatments of cell medium.

The biotic:abiotic ratio of the mean values of fenitrothion derivatives detected in the growth medium in the light and in the dark are shown in Table 7 and Table 8. NC and DSM ratios were significantly different from 1.0 in both the light and the dark. Likewise the biotic/abiotic ratios of OHMEF and SMF were significantly different from 1.0 for the light treatments. However these metabolites were detected at higher levels in the abiotic medium rather than the biotic treatments (Table 7).

The biotic/abiotic ratios of quantified metabolites differed from 1.0 not only in magnitude but also in a time dependant fashion. NC ratio different significantly from 1.0 at 2 weeks in the light and 48 h in the dark. DSM was also a significant metabolite relative to abiotic treatments at 48 h in the dark, and at one week in the light.

The only other metabolites detected in both control and treatment sets were OHMEF, SMF and CFT, and these were detected in the light only. In this instance the biotic/abiotic ratios demonstrated that SMF and OHMEF in the biotic treatments were not as significant at 60 h to two weeks as they were in the abiotic control. CFT, however was significantly greater in biotic than abiotic growth medium at two weeks (Table 7).

The metabolites which were detected in the light biotic treatments of cell medium, and which were the significant

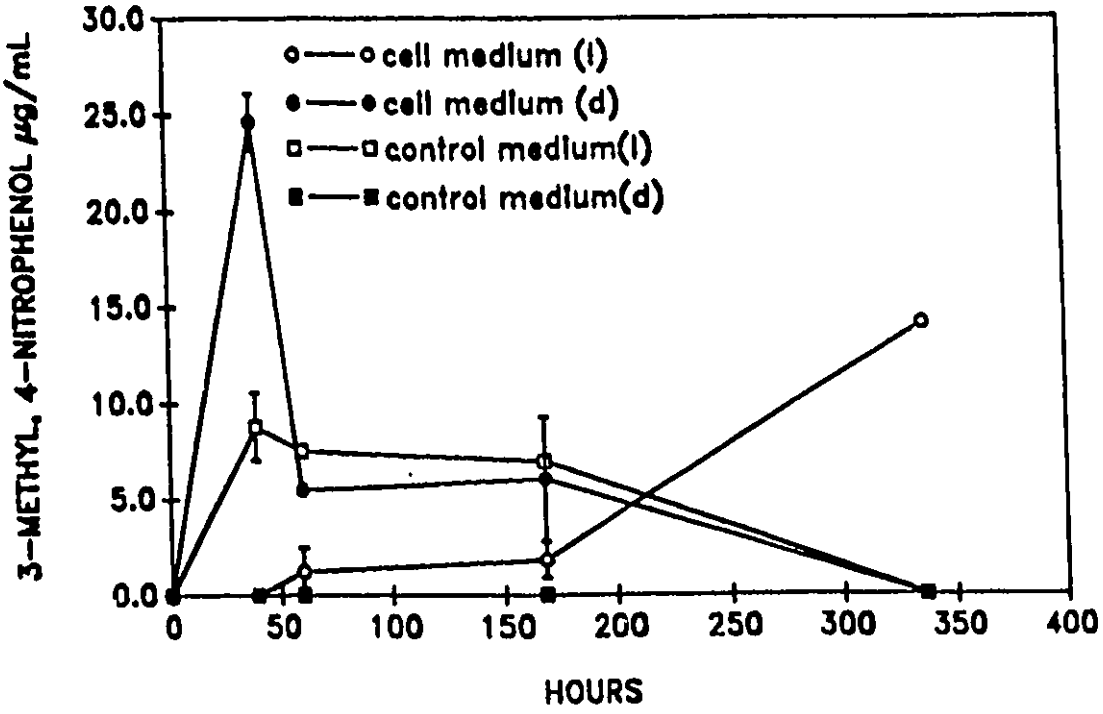


FIGURE 18

Amount of 3-methyl-4-nitrophenol detected in abiotic and biotic treatments incubated in the light and dark with 5.0 µg/mL fenitrothion. n=3, p=0.05, mean ± 95% c.i..

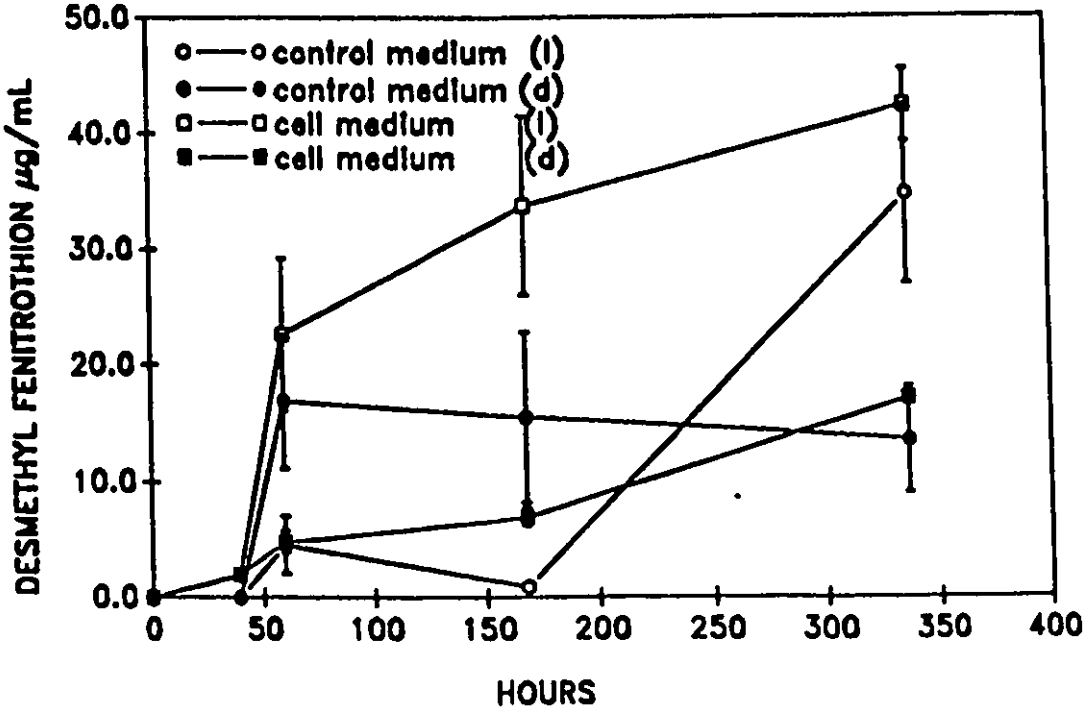


FIGURE 19

Amount of desmethylfenitrothion detected in abiotic and biotic treatments incubated in the light and dark with 5.0 µg/mL fenitrothion. n=3, p=0.05, mean ± 95% c.i..

Table 7

Biotic:Abiotic Ratios of Amounts of Fenitrothion Metabolites Detected in Growth Medium in the Light During Degradation of Fenitrothion

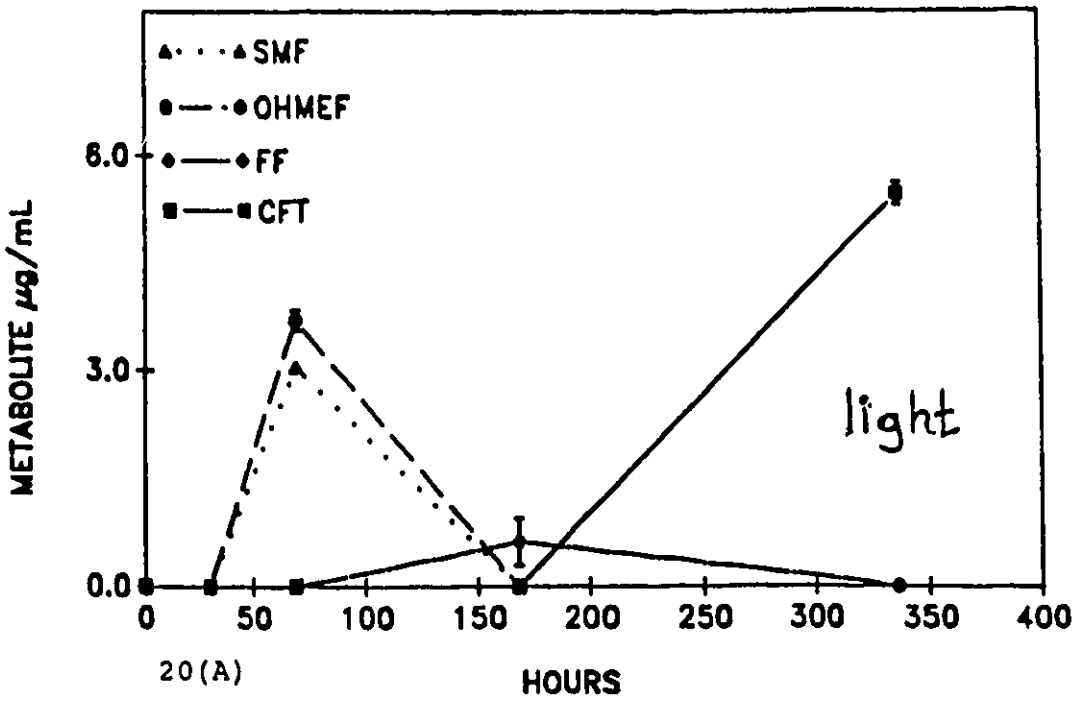
Metabolite	Time (h) 0	48	60	168	336
NC	1.0 ± 0.0	0.02* ±0.0	0.2 ±1.1	0.28 ±2.0	105* ±0.0
DSM	1.0 ±0.0	4.1* ±0.0	5.0 ±10	38.3* ±8.0	1.0 ±16.0
SMF	1.0 ±0.0	0.01* ±0.0	1.61* ±0.0	0.006* ±0.0	0.004* ±0.0
CFT	1.0 ± 0.0	1.0 ±0.0	1.0 ±0.0	1.0 ± 0.0	1.55* ±0.033
FF	1.0 ±0.0	1.0 ±0.0	1.0 ±0.0	23* ±0.001	1.0 ±0.0
OHMEF	1.0 ±0.0	1.0 ±0.0	0.322* ±0.2	1.0 ±0.0	1.0 ±0.0
CFO	1.0 ±0.0	1.0 ±0.0	1.0 ±0.0	1.0 ±0.0	1.0 ±0.0
FO	1.0 ±0.0	1.0 ±0.0	1.0 ±0.0	1.0 ±0.0	1.0 ±0.0

* denotes means that are significantly different, mean ± 95% c.i., n=3, p=0.05

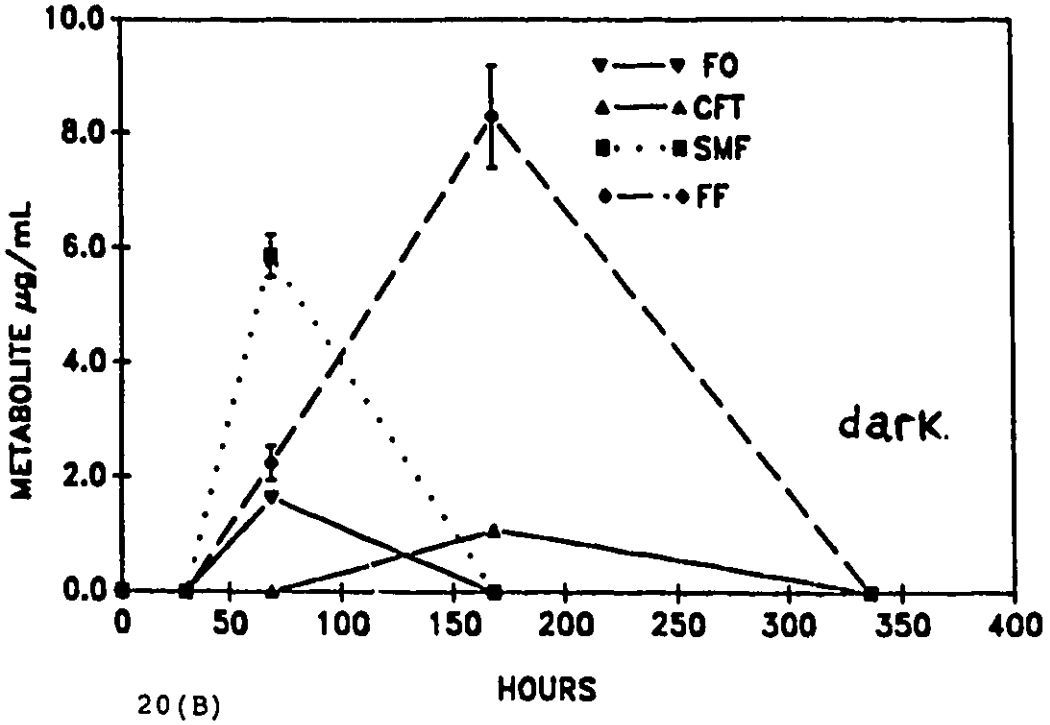
Table 8 Comparison of Ratios of Amounts of Fenitrothion Metabolites in Growth Medium During Biotic and Abiotic Degradation of Fenitrothion in the Light and Dark

Metabolite	Ratio	Time (h) 0	48	60	168	336
NC	Abiotic L/D	1.0 ±.02	58* ±.002	53.2* ±0.0	51.7* ±0.002	1.0 ±0.0
	Dark B/A	1.0 ±0.0	176* ±0.003	36.8* ±0.0	35.4* ±.004	1.0 ±0.0
DSM	Abiotic L/D	1.0 ±0.0	2.0* ±.01	0.23* ±.05	0.058* ±.04	2.18 ±35
	Dark B/A	1.0 ±.02	3.8* ±.2	0.27 ±2.0	0.7 ±.5	1.2 ±.012
SMF	Abiotic L/D	1.0 ±0.0	130* ±0.0	136* ±0.0	246* ±0.0	283* ±0.0
	Dark B/A	1.0 ±0.0	1.0 ±0.0	447* ±0.0	1.0 ±0.0	1.0 ±0.0
OHMEF	Abiotic L/D	1.0 ±0.0	1.0 ±0.0	24.6* ±0.0	1.0 ±0.0	1.0 ±0.0
	Dark B/A	1.0 ±0.0	1.0 ±0.0	1.0 ±0.0	1.0 ±0.0	1.0 ±0.0
FF	Abiotic L/D	1.0 ±0.0	1.0 ±0.0	1.0 ±0.0	1.0 ±0.0	1.0 ±0.0
	Dark B/A	1.0 ±0.0	1.0 ±0.0	80.76* ±0.0	308* ±0.002	1.0 ±0.0
CFT	Abiotic L/D	1.0 ±0.0	1.0 ±0.0	1.0 ±0.0	1.0 ±0.0	422* ±0.003
	Dark B/A	1.0 ±0.0	1.0 ±0.0	1.0 ±0.0	111* ±0.0	1.0 ±0.0
CFO	Abiotic L/D	1.0 ±0.0	0.01* ±0.0	1.0 ±0.0	1.0 ±0.0	1.0 ±0.0
	Dark B/A	1.0 ±0.0	0.01 * ±0.0	1.0 ±0.0	1.0 ±0.0	1.0 ±0.0

* Denotes ratios significantly different than 1.0, n=3, p=0.05, mean ± s.d. L=light, D=dark, A=abiotic, B=biotic.



20(A)



20(B)

FIGURE 20

Amount of fenitrothion metabolites detected in biotic cell medium of C. reinhardtii cultures incubated with 5.0 µg/mL fenitrothion (a) in the light, and (b) in the dark. n=3, p=0.05, mean ± 95% c.i., $1x10^6$ cells/mL.

Table 9

Light:Dark Biotic Ratio of Amount Fenitrothion Metabolites Detected in the Cell Medium Fraction of C. reinhardtii Cultures Incubated with 5.0 µg/mL Fenitrothion

Metabolite	Biotic Light:Dark Ratio Time (h)				
	0	48	60	168	336
NC	1.0 ±0.0	.005* ±0.00	0.325* ±0.0 0	0.32 ±9.0	105* ±0.0
DSM	1.0 ±0.0	1.0 ±0.02	4.4* ±16	5.3* ±0.3	2.20* ±0.08
OHMEF	1.0 ±0.0	1.0 ±0.0	6.78* ±0.01	1.00 ±0.32	1.0 ±0.73
FF	1.0 ±0.0	1.0 ±0.0	0.12* ±0.001	0.075* ±0.40	1.0 ±0.001
SMF	1.0 ±0.0	1.0 ±0.0	0.5* ±0.01	1.0 ±0.0	1.0 ±0.0
CFT	1.0 ±0.0	1.0 ±0.0	1.0 ±0.0	0.009* ±0.11	666* ±0.0
FO	0.02* ±0.0	0.02* ±0.00	.01 ±0.0	0.02* ±0.0	0.02* ±0.0

* Denotes ratios significantly different from 1.0, n=3, p=0.05, mean ± s.d..

predominating metabolites at two weeks included CFT, NC and DSM in the light and NC and DSM in the dark (Figure 18, 19, 20(a) and 20 (b). At 30 h the predominating metabolites were SMF, and OHMEF in the light and FF, SMF, and FO in the dark.

Table 8 represents the abiotic light:dark and the dark biotic:abiotic ratios of metabolites detected in growth medium incubated with and without C. reinhardtii. The first ratio represents the difference in fenitrothion degradation products in light treatment with that in the dark treatment. The second ratio represents the effect that the algal culture (or biotic factor) has in enhancing abiotic dark degradation of fenitrothion. In comparing these two ratios, it was observed that the C. reinhardtii alga enhanced fenitrothion metabolite production more than the light treatment did. This was observed for NC, SMF, FF, and CFT (Table 8).

When comparing the biotic:abiotic ratios of detected metabolites in growth medium in the light with that detected in the dark (Table 7 and Table 8), it was observed that DSM was the only metabolite where this ratio was more significant greater in the light than in the dark.

3.3.2 Mass Balance of Fenitrothion Residues

3.3.2.1 Distribution in Growth Medium

In the presence of C. reinhardtii cultures, the distribution of ring labelled moieties of ^{14}C-fenitrothion in the aqueous fraction of growth medium approached 60% in the light and 60% in

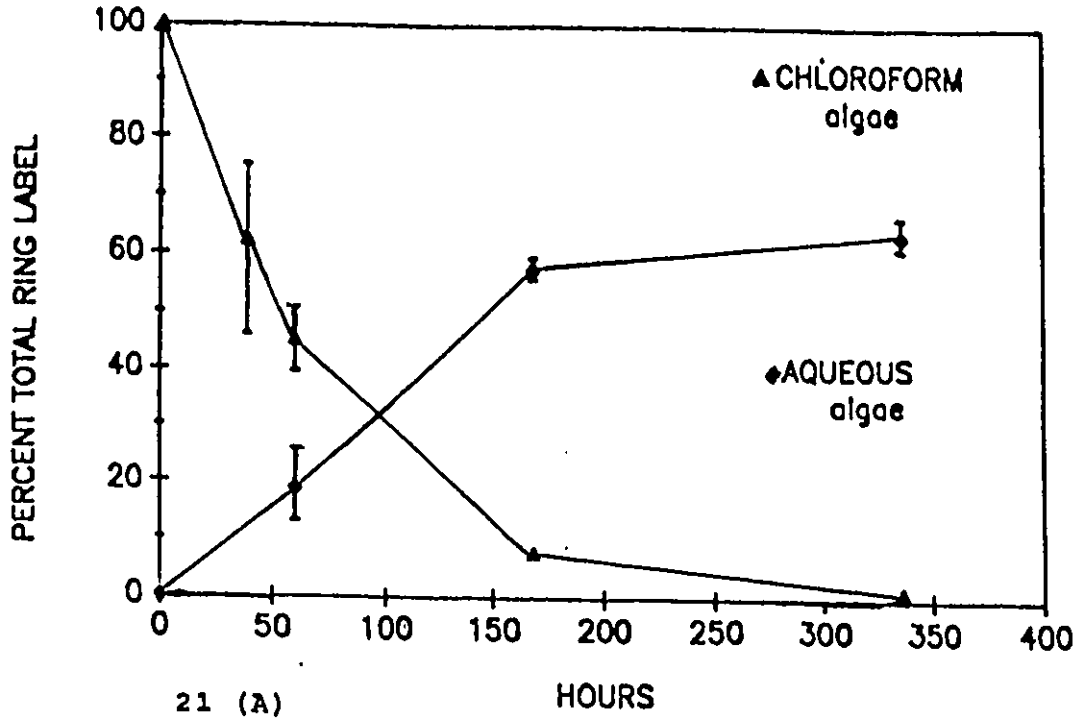
the dark at two weeks (Figure 21(a) and Figure 21(b). These values were significantly greater than that of the corresponding aqueous abiotic controls in the light and dark (Figure 21(c) and Figure 21(d).

In addition, the labelled ring moieties disappeared faster from the apolar fraction of growth medium incubated with C. reinhardtii than in the abiotic controls in both the light and in the dark. These results indicate that the partitioning of radiolabelled moieties from apolar to polar fractions of growth medium and the associated mechanisms for doing so, were significantly greater in the presence of C. reinhardtii, and were further enhance with this alga under Vita Lite^{R} illumination.

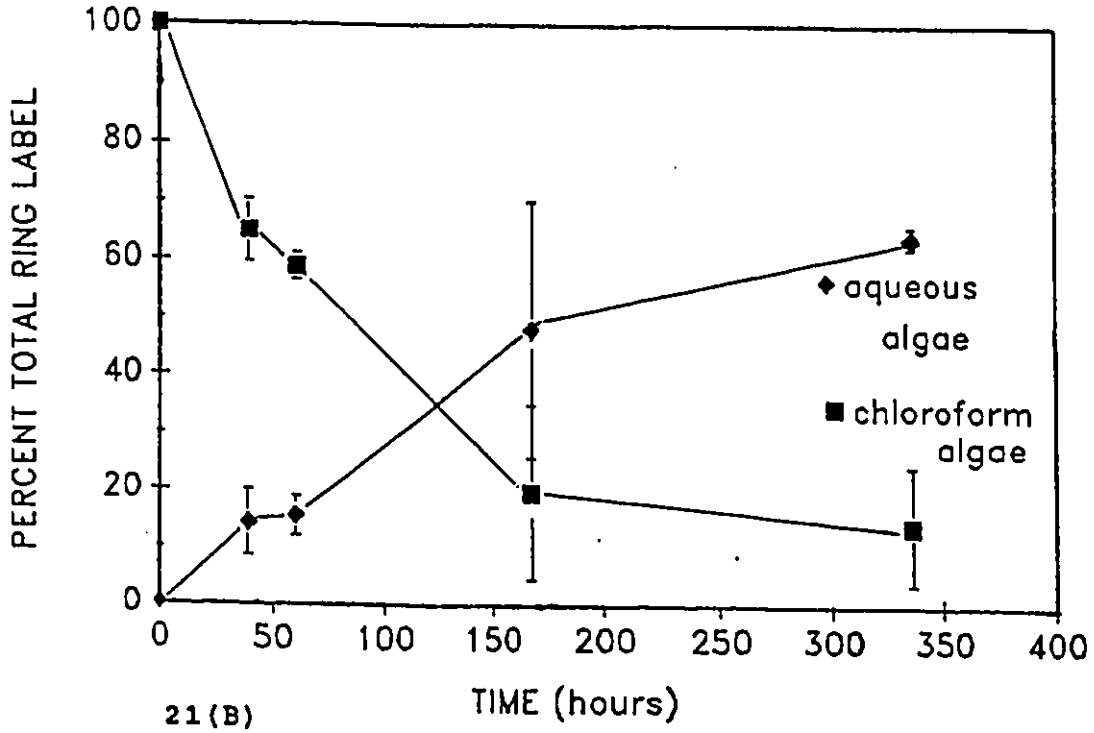
3.3.2.2 Distribution in Intracellular Fractions

Peak cellular accumulation of ^{14}C-ring labelled fenitrothion reached approximately 60% in the light at 60 h and 12% in the dark at one week and decreased there after (Figure 22(a) and Figure 22(b)). There was a lag in the accumulation of radiolabel into C. reinhardtii in the dark which indicated that a light dependant uptake process may have been involved. Of this total accumulated ^{14}C-ring label, net accumulation of fenitrothion by C. reinhardtii accounted for 4.0% in the dark and 1.0 % in the light (Figure 23(a)). This was equivalent to 0.8 mg/g algae in the dark and 0.2-0.3 mg/g algae in the light biotic treatments.

The ratio of mean values for uptake of fenitrothion in the dark relative to the light demonstrates that net accumulation of



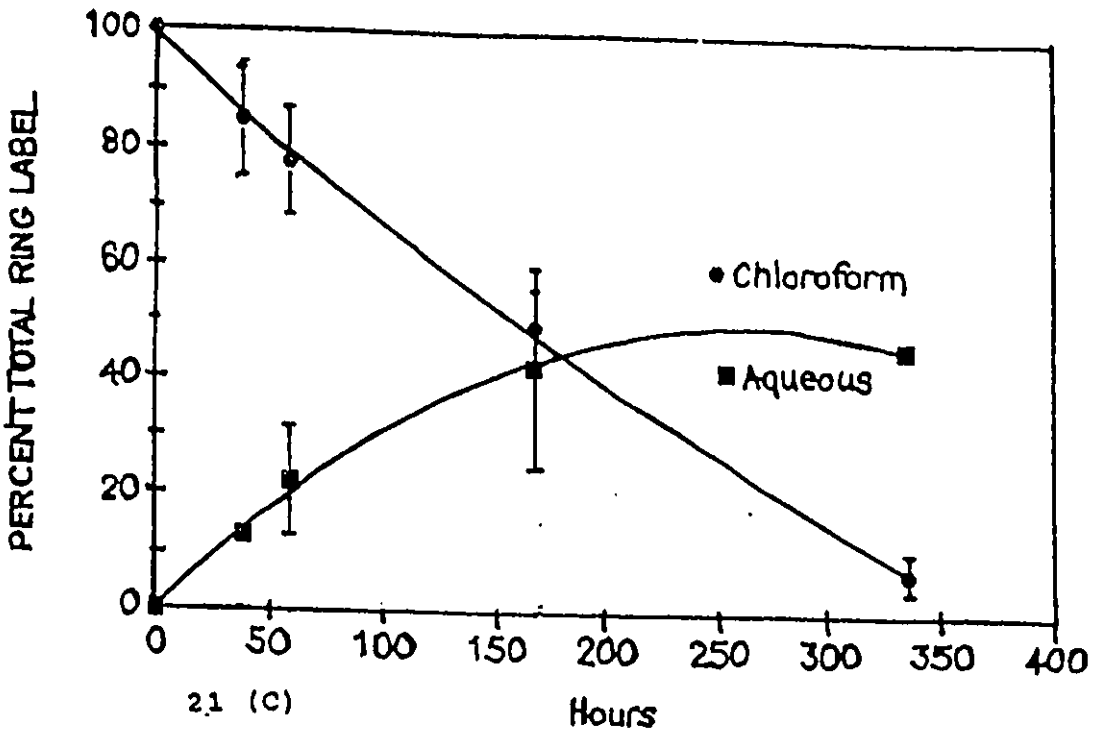
21 (A)



21(B)

Figure 21

Distribution of ^{14}C-ring labelled fenitrothion in growth medium in the presence of C. reinhardtii in (A) the light and (B) the dark.n=3, p=0.05, mean ± 95% c.i..



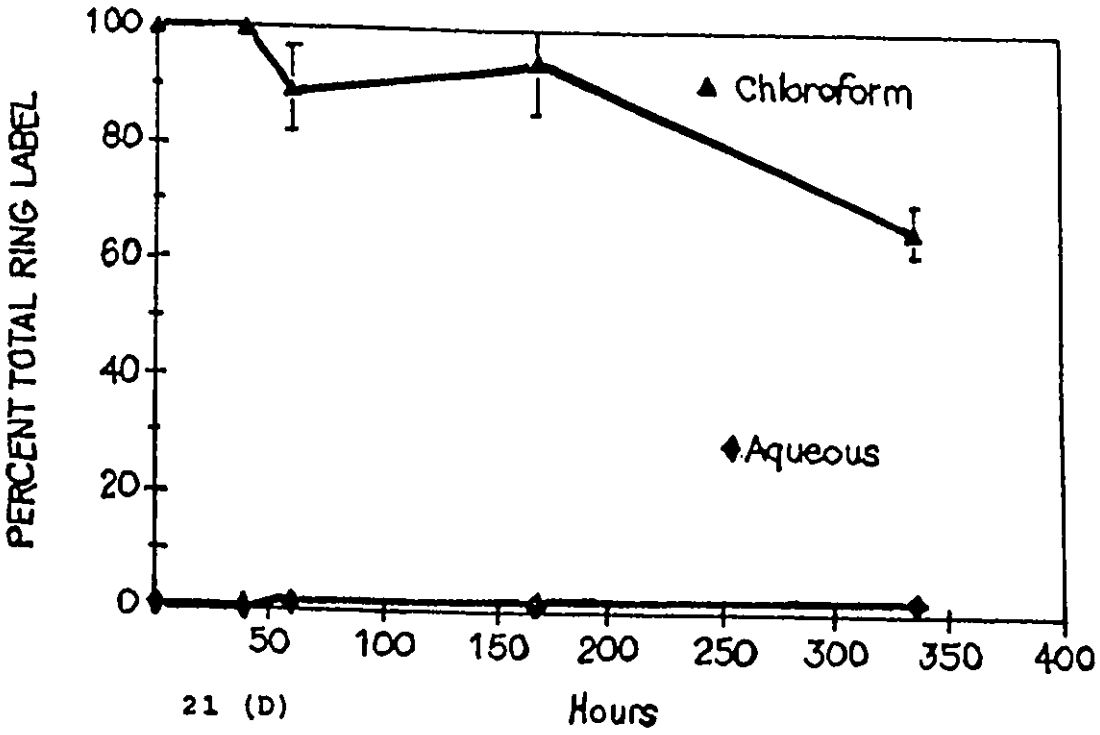
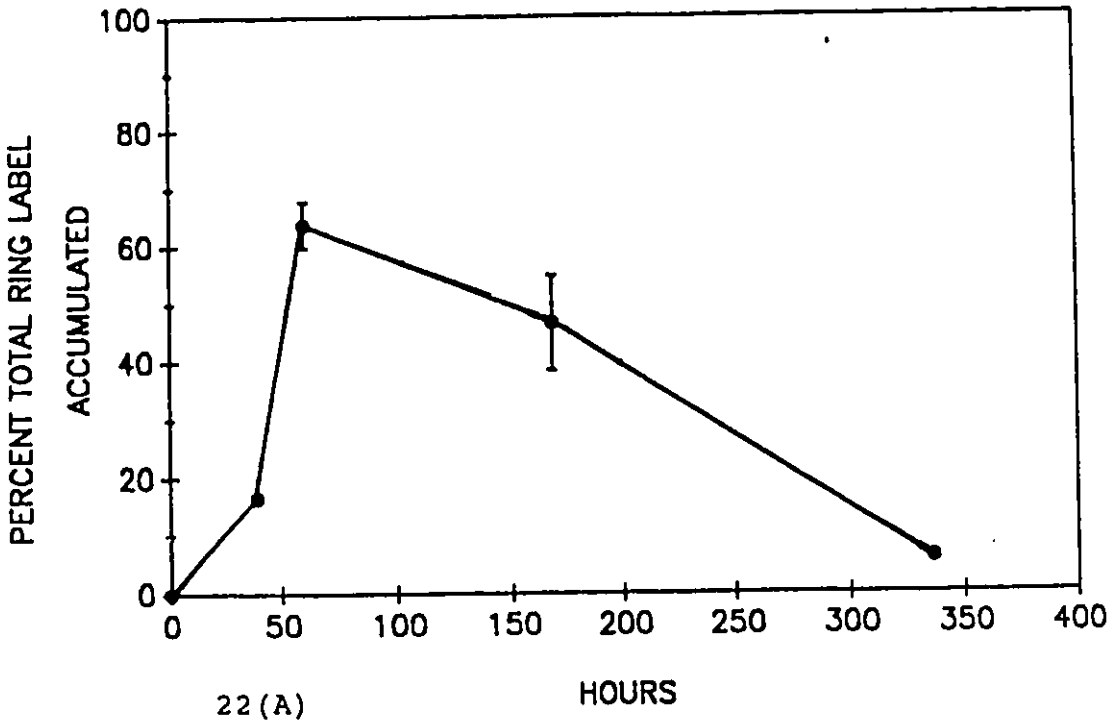
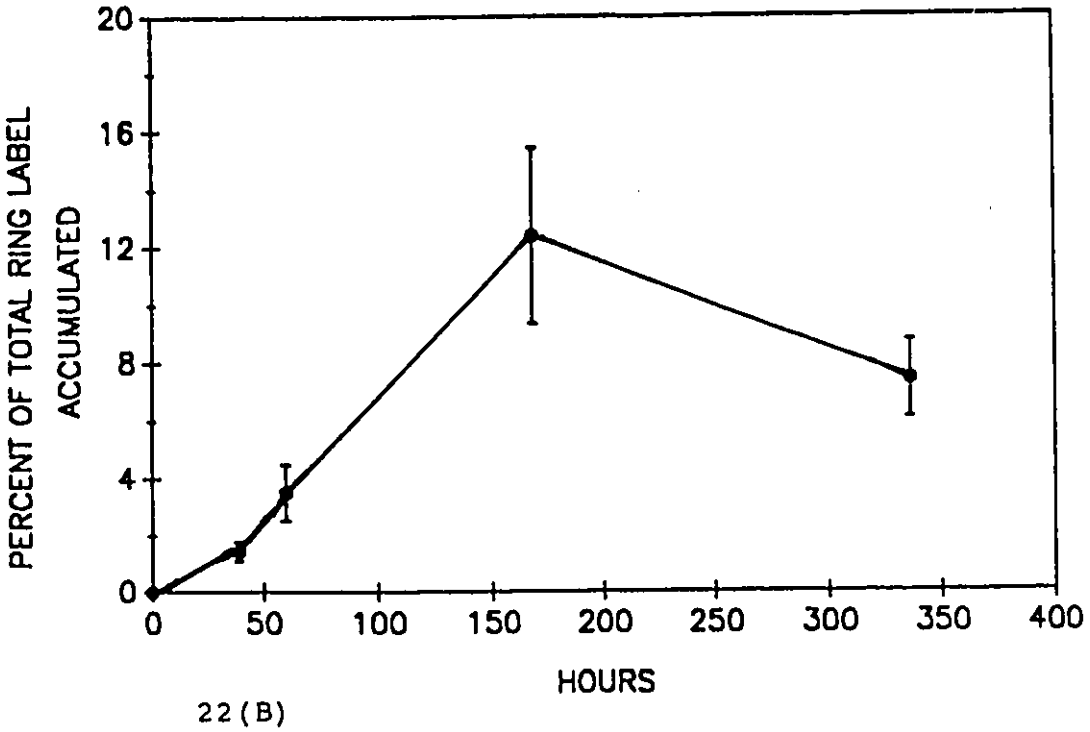


Figure 21

Distribution of ^{14}C-ring labelled fenitrothion in abiotic growth medium (C) in the light and (D) in the dark. n=3, p=0.05, mean ± 95% c.i..



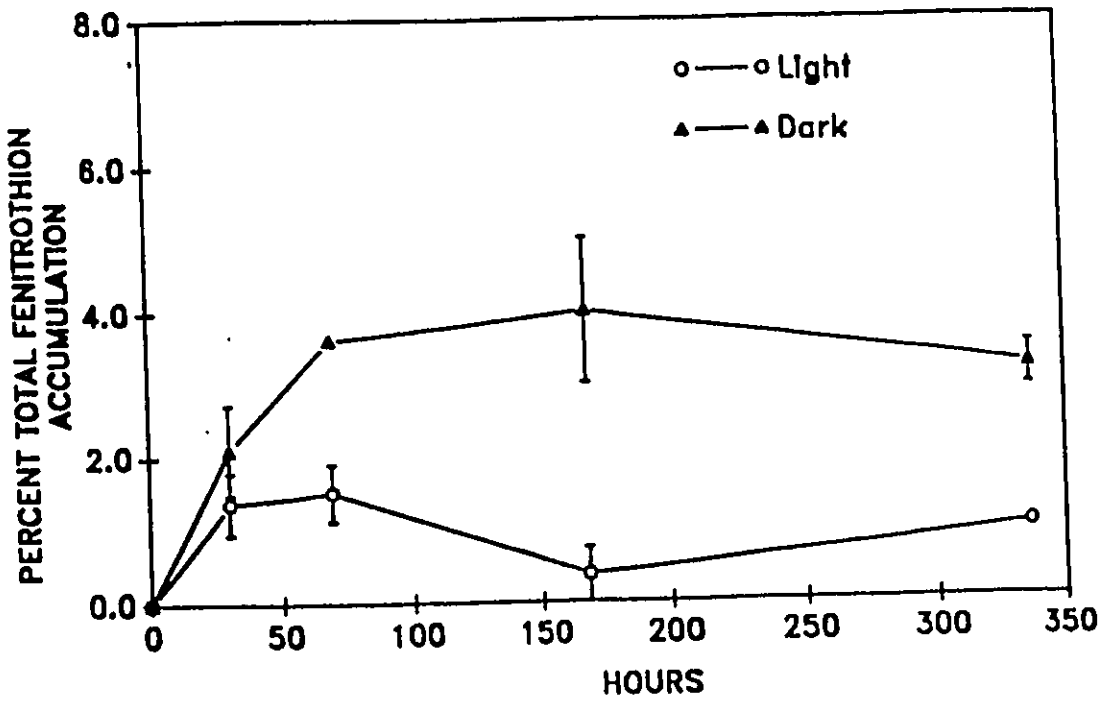
22(A)



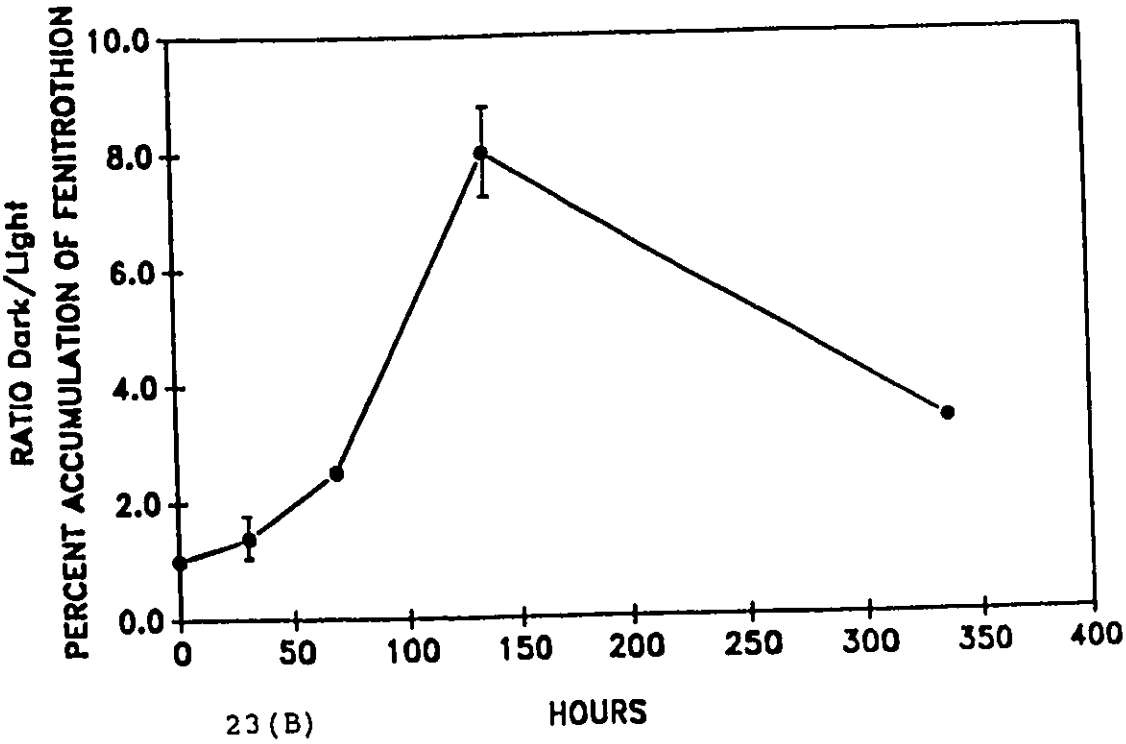
22(B)

FIGURE 22

Accumulation of fenitrothion ^{14}C-ring labelled moieties from growth medium by C. reinhardtii cultures incubated with 5.0 µg/mL fenitrothion (a) in the light and (b) in the dark. n=3, p=0.05, mean ± 95% c.i., 1x10^6 cells/mL.



23(A)



23(B)

FIGURE 23

(a) Accumulation of fenitrothion and (b) ratio percent fenitrothion accumulation in the dark relative to the light, from growth medium by C. reinhardtii cultures incubated with 5.0 µg/mL fenitrothion. n=3, p=0.05, mean ± 95% c.i., $1x10^6$ cells/mL.

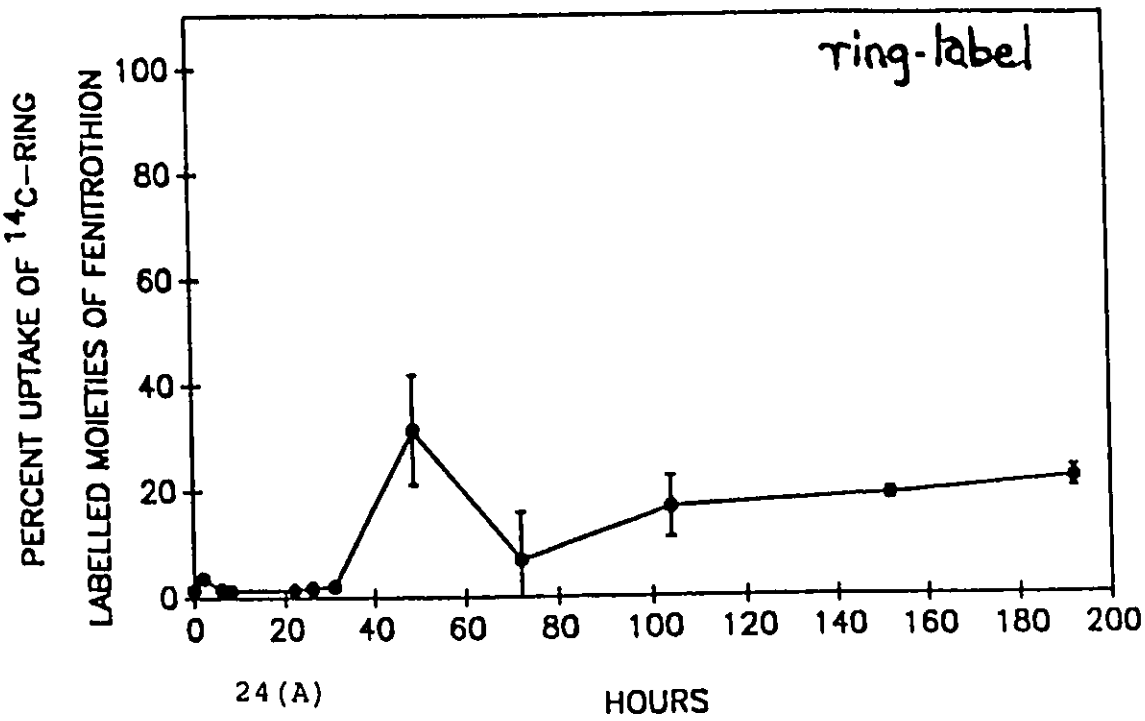
fenitrothion in the dark was more significant than in the light (Figure 23(b)). The rest of the label that was accumulated over the period of incubation was accounted for by the metabolites of fenitrothion.

3.3.2.3 Differential Accumulation of Fenitrothion Moieties

Accumulation of ^{14}C-fenitrothion labelled at the ring carbon and at the methoxy carbon is shown in Figures 24(a) and 24(b) respectively. The net accumulation of ring moieties and phosphate moieties of fenitrothion peaked at 48 h and decreased afterward to 20% of the total label for up to 200 h. Less ring labelled moieties (20-30%) were accumulated than the methoxy-labelled moieties (50-60%). The uptake and depuration processes account for the net accumulation observed and differences in net accumulation of the different labels may be due equally to differences in both processes.

3.3.3 Biotic Rate of Degradation

Gas chromatography analysis shows that fenitrothion in growth medium was significantly less in the presence of C. reinhardtii cultures as compared with that in abiotic growth medium (Figure 25(a)). This was true in both the light and in the dark treatments. Fenitrothion degradation in the dark biotic treatments was also significantly greater than that of the light abiotic controls. Figure 25(b) represents the log % fenitrothion remaining in growth medium calculated from Figure 25(a).



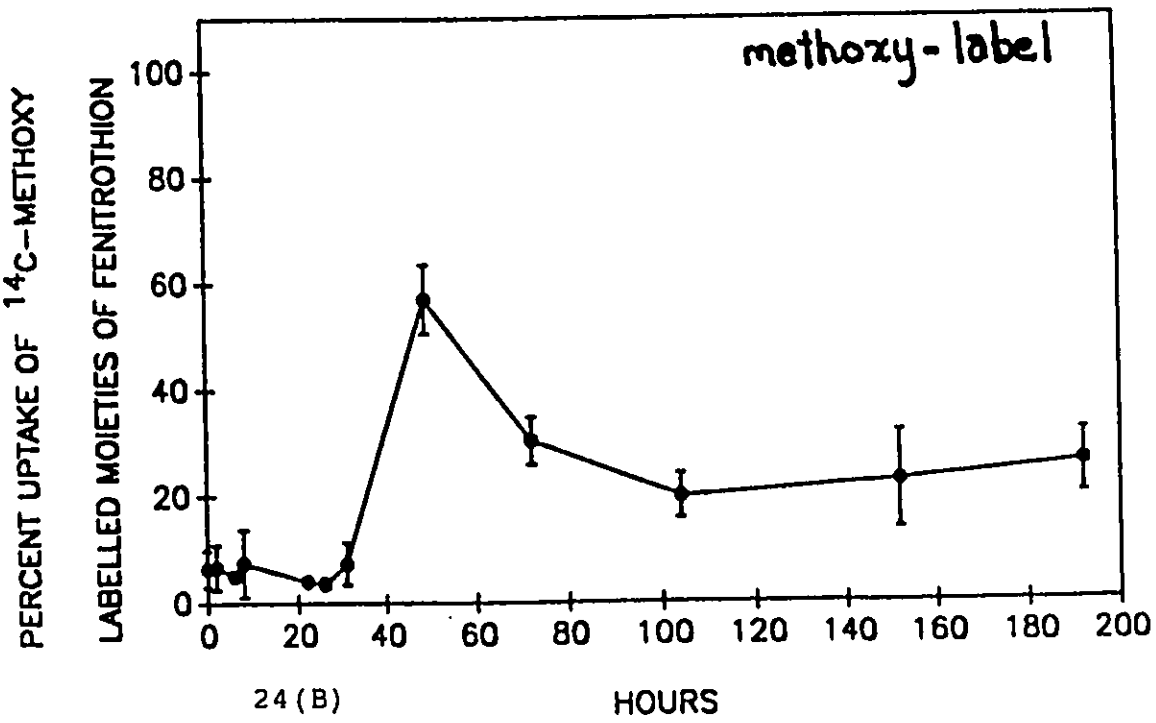
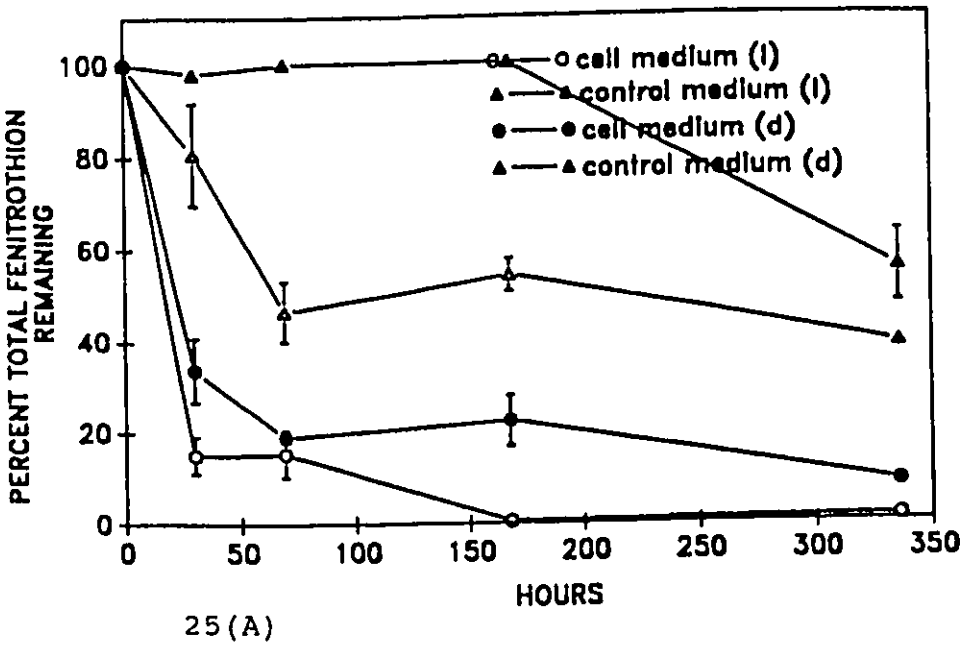
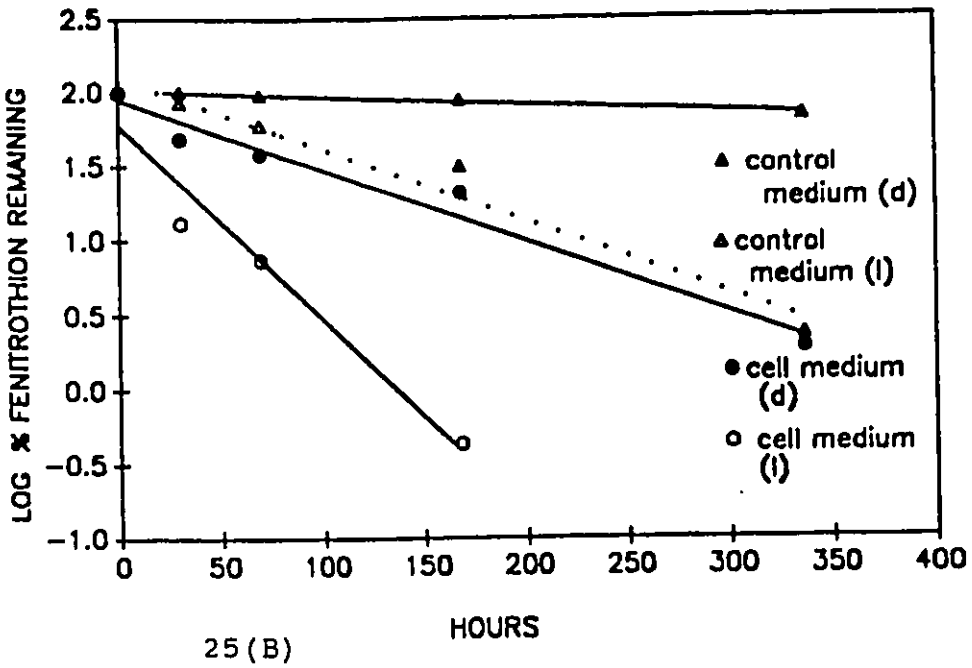


FIGURE 24

Cellular accumulation by C. reinhardtii cultures of (a) ring labelled, and (b) methoxy labelled ^{14}C-fenitrothion, from growth medium incubated in the light with 5.0 µg/mL fenitrothion. n=3, p=0.05, mean ± 95% c.i., 1x10^{6} cells/mL.



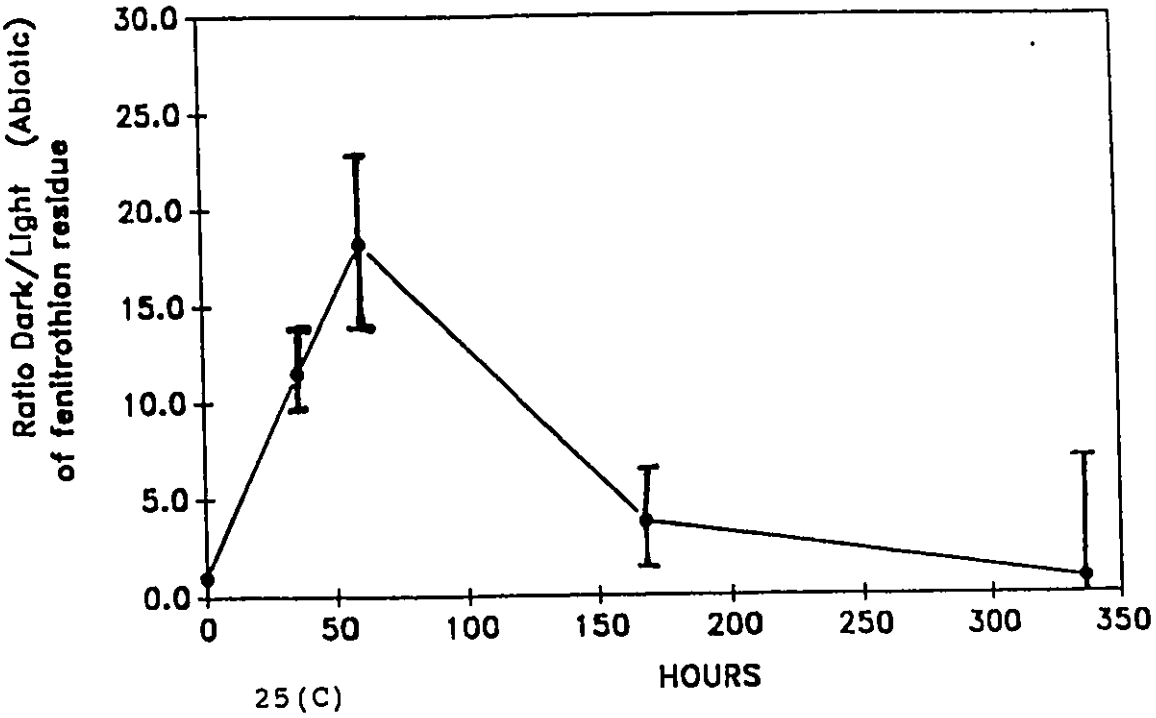
25(A)



25(B)

FIGURE 25

(a) Disappearance of fenitrothion from biotic and abiotic growth medium incubated in the light or dark with 5.0 µg/mL fenitrothion. (b) Log plot of percent original fenitrothion remaining from (a).



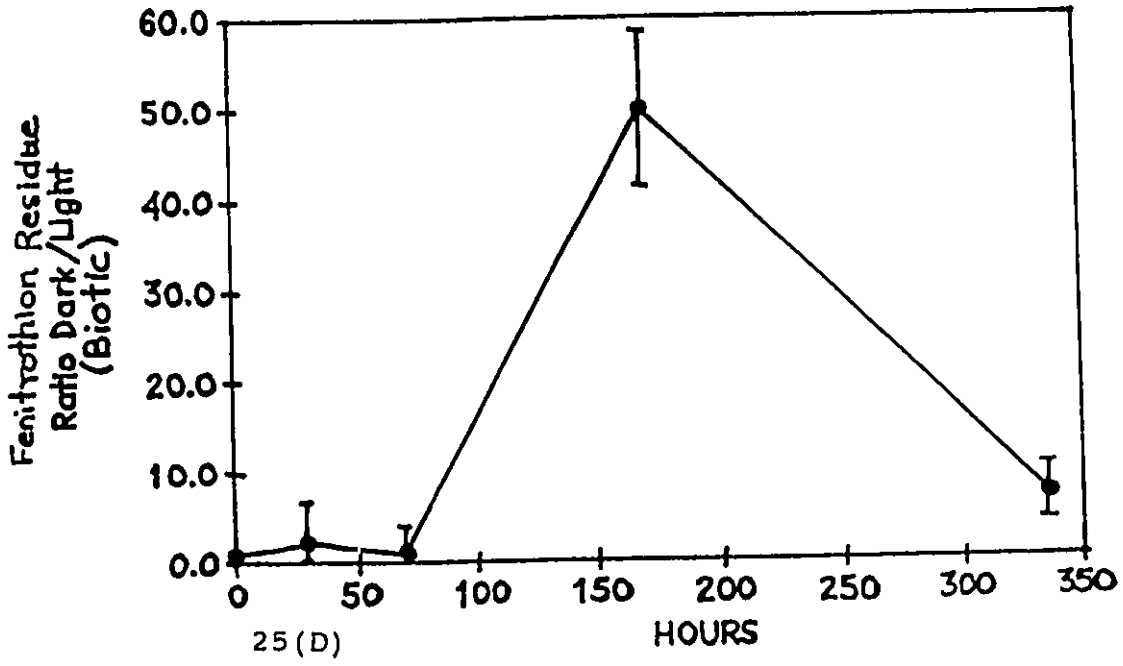
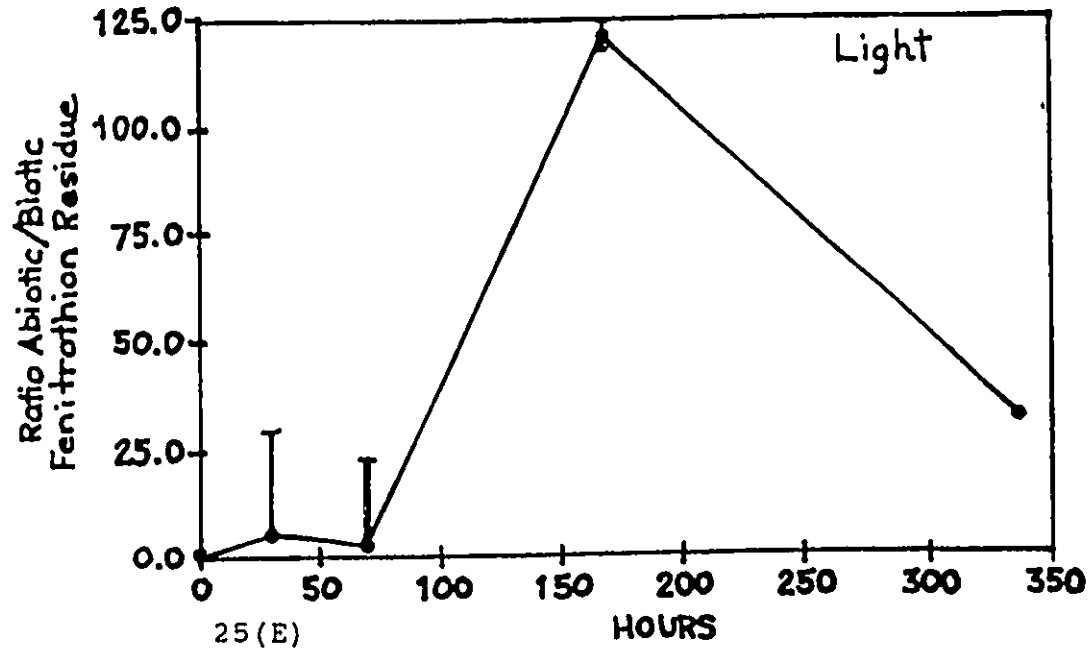


FIGURE 25

(c) Abiotic and (d) biotic dark:light ratios of values from 26 (a). n=3, p=0.05, mean ± 95% c.i..



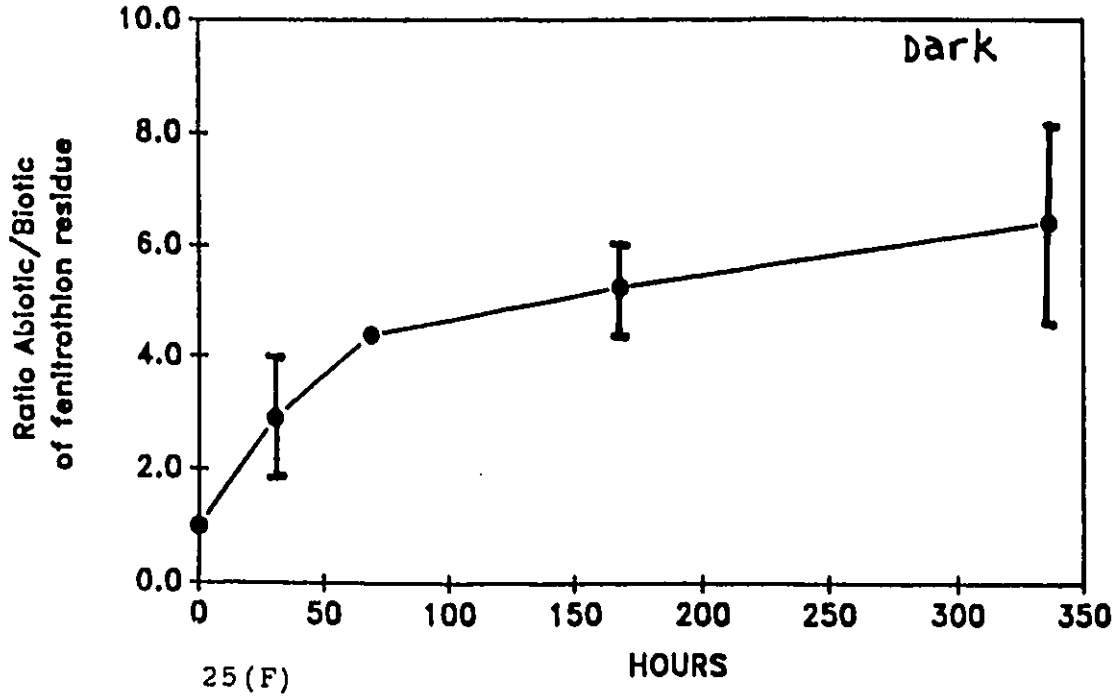


FIGURE 25

(e) Light and (f) dark abiotic:biotic ratios of values from (a). n=3, p=0.05, mean ± 95% c.i..

The slopes of the lines of Figure 25(b) represent the degradation rate constant (k) of fenitrothion in each case (days $^{-1}$) (Table 10). The k value in the light biotic treatment was 2.7 times greater than that of the light abiotic control. The value of k for dark biotic treatment was 9.8 times greater than that of the dark control (Table 10). Appendix C shows the statistical analysis for this data. The multiple range test analysis shows that the presence of C. reinhardtii enhances fenitrothion degradation relative to light abiotic treatment. This relationship existed in both the light and the dark. However, abiotic light and biotic dark treatments did not differ in their degradation rate constant.

Figure 25(c) and 25(d) show the ratio of the mean values from Figure 25(a) of the percent fenitrothion remaining in the dark relative to the light. Under abiotic conditions the ratio was significantly greater than 1.0 after 39 h until one week. This suggests that the influence of light on the degradation of fenitrothion in this laboratory model had its most significant effects at these times (Figure 25(c)). Under biotic conditions with C. reinhardtii, the difference in ratio of fenitrothion remaining in the dark relative to that of the light was significantly different from 1.0 at 69 h until two weeks (Figure 25(d)).

These results suggest that the effects of light on fenitrothion degradation were not as significant in this model in the presence of C. reinhardtii as they were in abiotic controls. This also suggests that there may be a metabolic role of alga as a

Table 10

Rate Constant of Degradation and Half Life of Fenitrothion in Gorman and Levign Growth Medium Under Abiotic and Biotic Conditions in the Dark and Under Vita Lite^{R} Calculated from the Log Transformation of Figure 25 (a). 40wm^{-2}, pH=6.8, T=21± 0.5°C[a].

Light Regime	R Value	Degradation Rate Constant	Half Life (days)
Dark abiotic	0.989	$-0.0005 \pm 6.0 \times 10^{-6}$	54
Light abiotic	0.985	$-0.0047 \pm 3.6 \times 10^{-5}$	6.04
Dark biotic	0.982	$-0.0049 \pm 9.1 \times 10^{-5}$	5.95
Light biotic	0.977	$-0.013 \pm 4.0 \times 10^{-4}$	2.2

[a] See equation in Methods and Materials for half life calculations obtained from log plot % fenitrothion remaining versus time.

See appendix C for details of statistic for this data

process responsible for the enhanced degradation of fenitrothion in the biotic treatments, other than just the effects of light in algal photosensitization.

In comparison, Figure 25(e) and 25(f) demonstrate that there was a significant difference in the abiotic/biotic ratio of means of percent fenitrothion remaining in both the light and in the dark. In the light there were one to two orders of magnitude of difference less of fenitrothion remaining in the biotic and abiotic treatments at one week and at two weeks (Figure 25(e)). In the dark at all time periods other than time zero, the ratio was significantly different than 1.0 signifying that the amount of fenitrothion remaining in the dark biotic treatment was significantly less than that of the abiotic controls (Figure 25(f)).

The significant biotic enhancement of fenitrothion degradation in the presence of C. reinhardtii occurs in both the light and dark. This suggests that not only is U.V. illumination important, but also the presence of alga is important for producing the low half-life of fenitrothion in this study.

3.3.4 Elimination of Fenitrothion Metabolites

Table 11 shows the amount of elimination (desorption) of fenitrothion metabolites from the cellular fractions of C. reinhardtii cultures during incubation with fenitrothion. Columns 1 and 2 show the amount of each metabolite detected in cell medium after one and two weeks of incubation of algal cultures with

Table 11

Amount of Elimination of Fenitrothion Metabolites From C. reinhardtii Cultures in Extracellular Medium after Two Weeks Incubation With 5.0 µg/mL Fenitrothion

Metabolite	Amount (µg/mL) in Growth Medium Control cultures[1] 1 week	2 weeks	Washed cultures[2] 2 weeks
CFO	ld[3]	ld	20.0 ±6.3
CFT	ld	6.8 ±3.1	29.0 ±4.7
FF	1.4 ±0.7	1.7 ±0.02	ld
OHMEF	ld	ld	15.0 ±3.8
SMF	ld	ld	ld
DSM	32.0 ±5.6	43.3 ±6.2	15.0 ±2.9
NC	ld	14.9 ±5.4	6.297 ±2.73

[1] Growth medium of cell cultures incubated with fenitrothion (5.0 µg/mL). This represents metabolites detected in cell medium due to abiotic production and due to cellular production and elimination.

[2] Growth medium of cell cultures whose cells were resuspended in non labelled medium without fenitrothion after 1 week incubation with labelled fenitrothion (5.0 µg/mL). This represents elimination of the metabolite from inside the cell.

[3] ld= amounts less than the limits of detection, see table 4

fenitrothion. These values represent abiotic and biotic degradation, and elimination processes.

Column three represents the amounts of metabolites detected in cell medium of cultures which were incubated in fenitrothion for one week, and subsequently washed and re-suspended in fresh pesticide-free growth medium. Any pesticide in the medium at two weeks would have come from the intracellular fraction, and would not be due to abiotic degradation. Significant elimination of CFO, CFT, OHMEF, NC and DSM occurred in the light. DSM and NC detected in extracellular growth medium at two weeks in the non washed treatments was greater than for the elimination treatment, suggesting that there was significantly more abiotic production of these metabolites than biotic production. No elimination of metabolites was detected under dark treatment conditions.

3.4 Assessment of PSMO Role in Fenitrothion Metabolism

The enhanced degradation of fenitrothion metabolism in the presence of C. reinhardtii relative to abiotic degradation occurred in the light and in the dark. It was hypothesized that the activity of cytochrome P_{450} monooxygenase was involved as the principle route of biotic degradation. These studies tested that hypothesis since most of the metabolites are oxidized fragments of the original molecule.

3.4.1 Determination of Control Levels of PSMO Inducer and Inhibitor Compounds

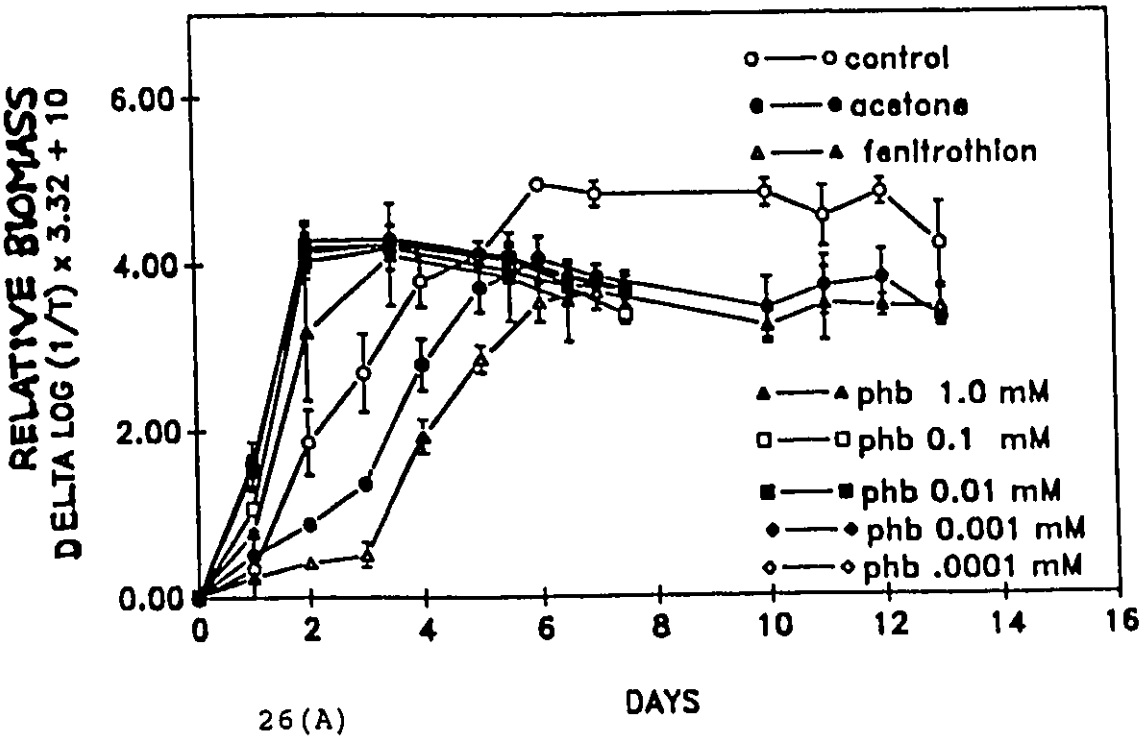
At low concentrations, phenobarbital and piperonyl butoxide can respectively induce and inhibit PSMO and may affect the rate of degradation and composition of the fenitrothion residues produced by C. reinhardtii. The concentration of these compounds which cause physiological and biochemical processes in alga is not known. In order to minimize these toxic effects, a low concentration of these inducer and inhibitor compounds must be determined which can be used in subsequent studies of the role of PSMO in C. reinhardtii.

3.4.1.1 Growth Studies

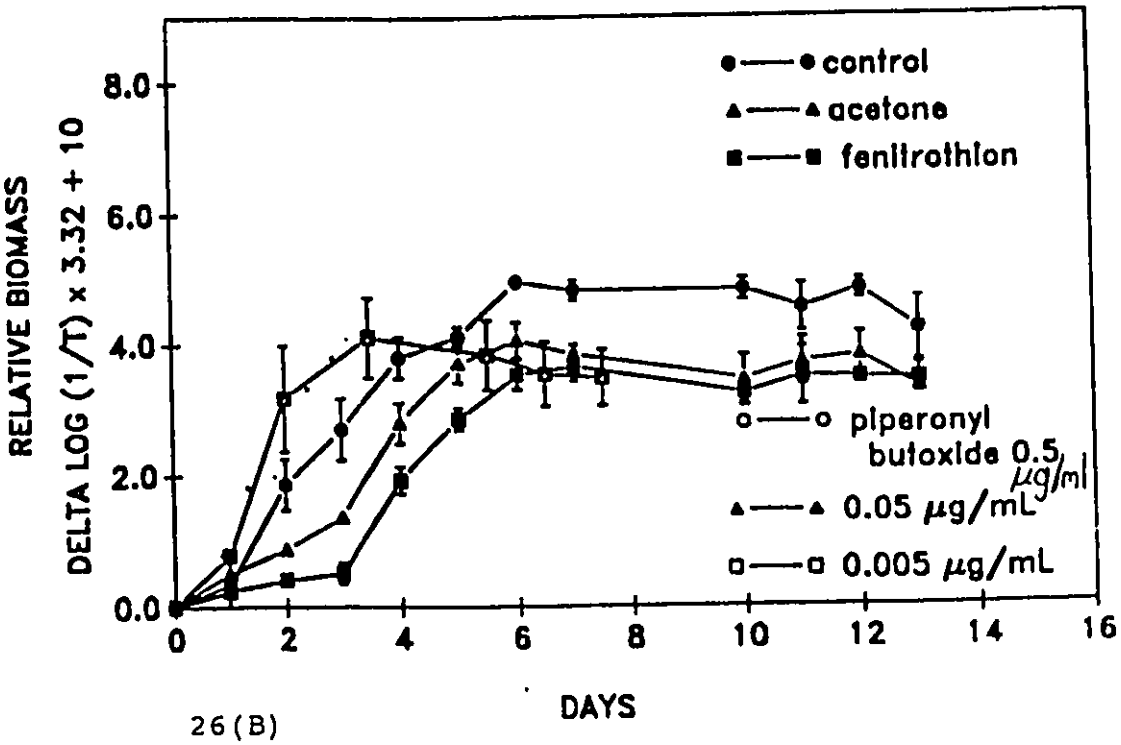
Figure 26(a) and (b) shows the growth curves for C. reinhardtii in the presence of 5.0 µg/mL fenitrothion, and two controls, one with alga only and one with acetone (equivalent to that used to dissolve the fenitrothion in the treatments). In addition, growth curves of cultures with phenobarbital or piperonyl butoxide are also shown.

The growth rates for the control, acetone and fenitrothion treatments were 0.186, 0.223, and 0.285 s^{-1} respectively (Table 12). The fenitrothion treatment was significantly different from the control. The maximum attainable biomass for the acetone control and fenitrothion treatment were significantly different from the control (Table 12).

After 12 h incubation of the algal cultures with 50.0 and 5.0 µg/mL piperonyl butoxide, the cells became chlorotic and were non-



26(A)



26(B)

FIGURE 26

Growth curve of C. reinhardtii cultures incubated in the light with (a) a range of concentrations of phenobarbital, and (b) a range of concentrations of piperonyl butoxide. n=3, p=0.05, mean ± 95% c.i..

Table 12

Growth Rate and Maximum Attainable Biomass of *C. reinhardtii* Cultures Incubated with Fenitrothion, Piperonyl Butoxide or Phenobarbital in the Light

Treatment	Growth Rate[a]	Maximum Attainable Biomass[a]
Control	0.186	4.2
Acetone	0.223*	3.8*
Fenitrothion 5.0 µg/mL	0.285*	3.49*
Piperonyl Butoxide 0.5 µg/mL	0.296*	4.1
0.05 µg/mL	0.283*	4.03
0.005 µg/mL	0.276*	3.98
Phenobarbital 23.2 µg/mL	0.298*	4.11
2.32 µg/mL	0.296*	4.19
0.232 µg/mL	0.312*	4.20
0.0232 µg/mL	0.331*	4.32
0.00232 µg/mL	0.314*	4.24

* Denotes values significantly different from the control cultures.

[a] relative units as calculated from ln (1/transmission). See methods for growth parameters.

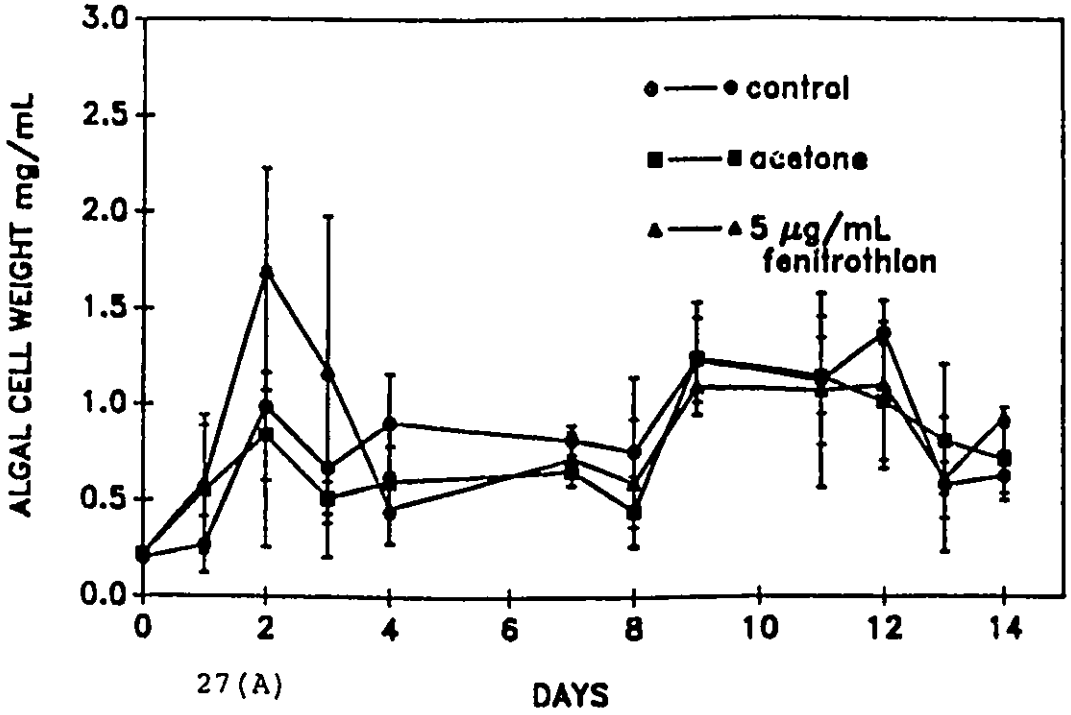
motile. Cultures of C. reinhardtii with 0.5, 0.05, and 0.005 µg/mL of this cytochrome P_{450} inhibitor were not affected in this way. The growth rates of these cultures at these latter concentrations were not significantly different from the fenitrothion treatment. In contrast, the maximum attainable biomass of piperonyl butoxide treated cultures were significantly greater than the fenitrothion treated cultures (Table 12).

Figure 26(a) shows the growth curves for C. reinhardtii cultures incubated with the cytochrome P_{450} inducer phenobarbital. These phenobarbital treated cultures had significantly higher growth rates than the fenitrothion and control treatments at all concentrations tested. The maximum attainable biomass at all phenobarbital treatments was not significantly different from that of the fenitrothion and acetone treatments nor the control algal cultures (Table 12).

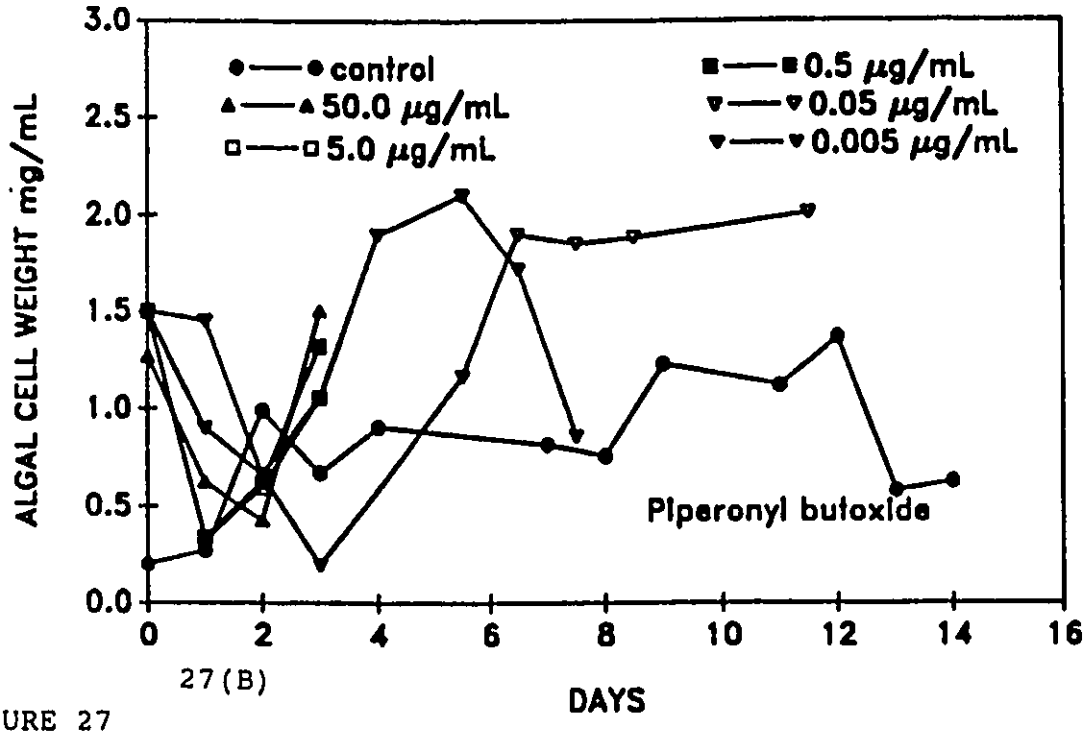
3.4.1.2 Cell Weight

Cell weight of C. reinhardtii cultures treated with fenitrothion, acetone or under control conditions did not differ significantly from each other over a 14 day period (Figure 27(a)). The cell weight of cultures incubated with treatment concentrations of piperonyl butoxide ranging from 50.0, 5.0, to 0.50 µg/ml was significantly greater than that of fenitrothion treated and control cultures after 2 days (Figure 27(b)).

After 4 days, 0.05, 0.005 µg/mL piperonyl butoxide treatments had a significantly greater cell weight relative to controls and



27(A)



27(B)

FIGURE 27

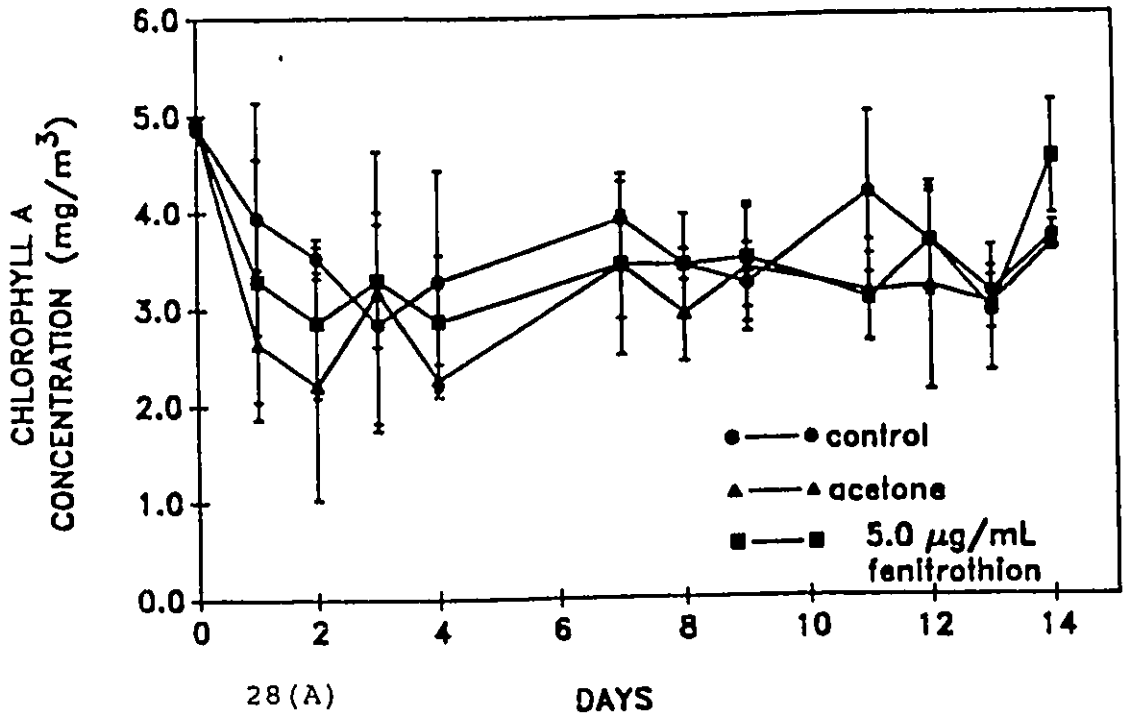
Cell weight of C. reinhardtii cultures incubated in the light with (a) 5.0 µg/mL fenitrothion or (b) a range of concentrations of piperonyl butoxide.n=3, p=0.05, mean ± 95% c.i., 2.0 x 10^5 cells/mL.

fenitrothion treated cultures. After 8-11 days cell weight at these lower treatment concentrations decreased to the level of the controls (Figure 27(b)). At the higher concentrations (50.0, 5.0 and 0.5 μg/mL piperonyl butoxide) cell weight continued to increase until the cultures became chlorotic and the cultures reached senescence.

3.4.1.3 Chlorophyll a and Chlorophyll b Concentration

Figure 28(a) shows that the chlorophyll a concentration of the acetone control or fenitrothion treated cultures of C. reinhardtii did not differ significantly from the control cultures. Chlorophyll b in acetone and fenitrothion treated cultures was significantly greater than that of the control set at day 3 as was observed in the earlier fenitrothion toxicity studies (Figure 28 (b)). At day 4 there was a significant decrease in chlorophyll b concentration relative to the control. Chlorophyll b concentration of fenitrothion treated cultures were also significantly different from the control sets at 11 and 14 days (Figure 28 (b)).

Chlorophyll a and chlorophyll b concentration of C. reinhardtii cultures treated with all the test concentrations of piperonyl butoxide differed significantly from that of the control cultures (Figures 29(a) and 29(b)). Chlorophyll a and b of cultures treated with 50.0 and 5.0 μg/mL piperonyl butoxide decreased significantly after 1 day and relatively no pigments were present as compared with the control set. This corresponds with the previous observation that cells became chlorotic and non motile at



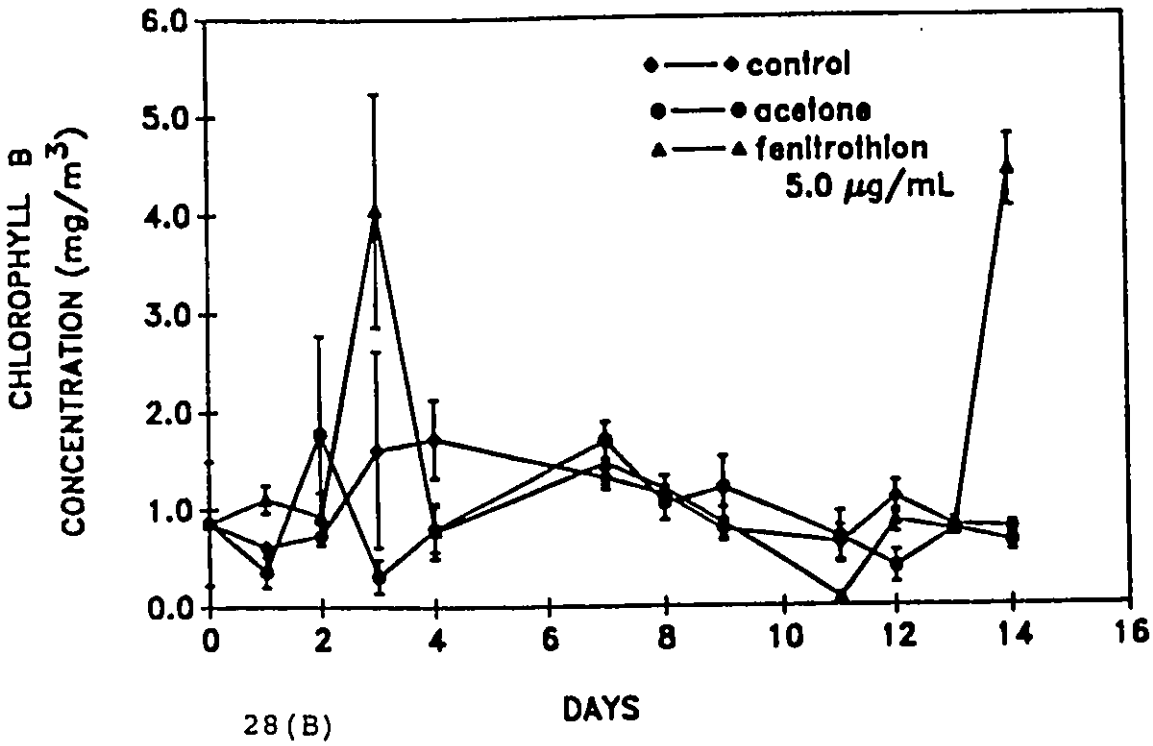
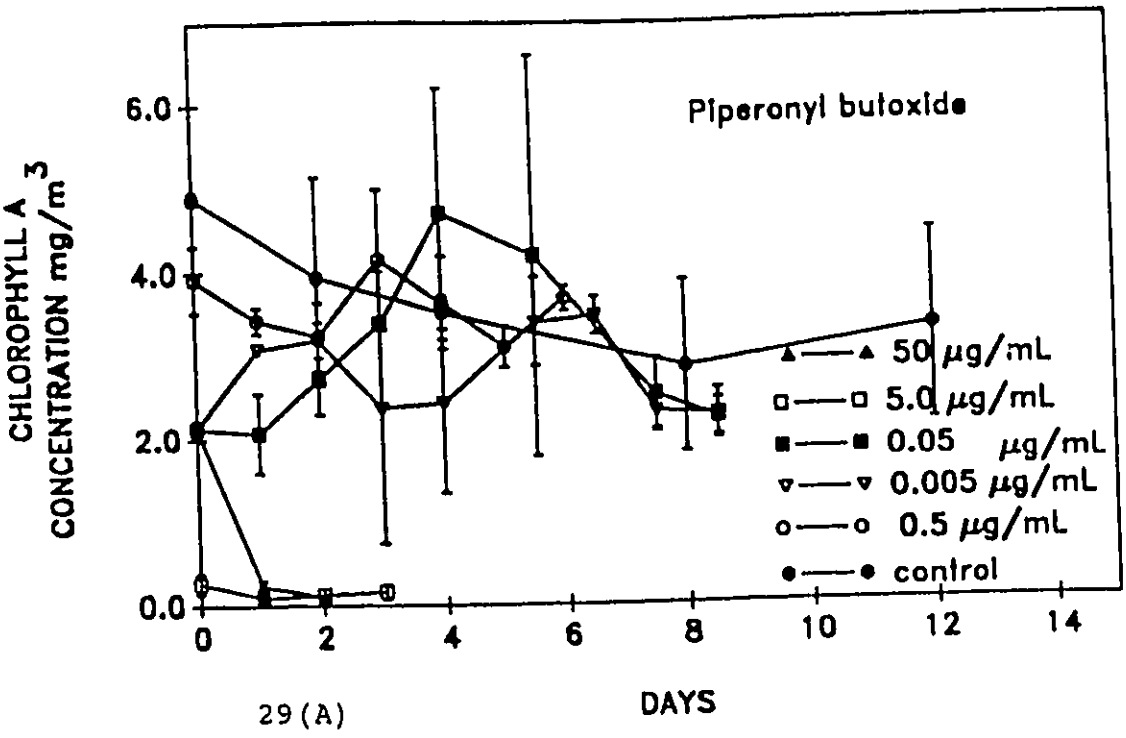
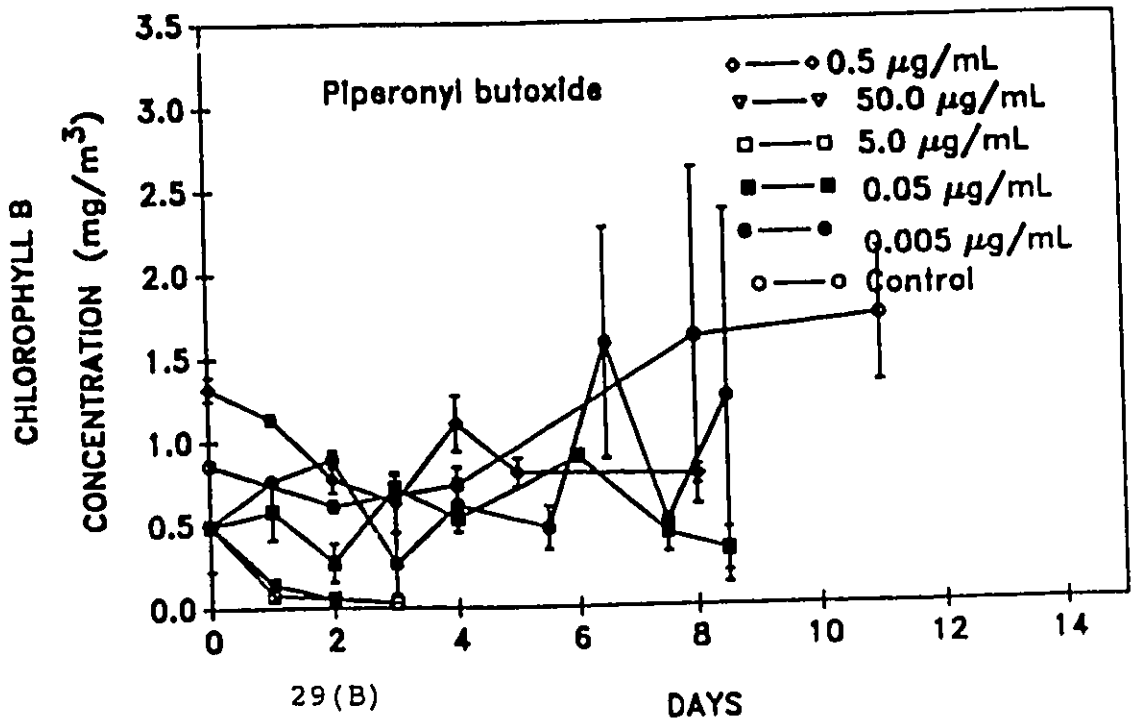


FIGURE 28

Concentration of (a) chlorophyll a and (b) chlorophyll b of C. reinhardtii cultures incubated in the light with 5.0 µg/mL fenitrothion. n=3, p=0.05, mean ± 95% c.i., 2.0 x 10^5 cells/mL.



29(A)



29(B)

FIGURE 29

Concentration of (a) chlorophyll a and (b) chlorophyll b of C. reinhardtii cultures incubated in the light with a range of concentrations of piperonyl butoxide. n=3, p=0.05, mean ± 95% c.i., 2.0 x 10^5 cells/mL.

these same treatment concentrations. The significant differences in pigment concentration for the 0.5, 0.05, and 0.005 μg/mL treatments occurred after 7 and 3 days for chlorophyll a and b respectively (Figure 29(a) and 29(b)). The sensitivity of C. reinhardtii cultures to piperonyl butoxide was greater for chlorophyll b than for chlorophyll a.

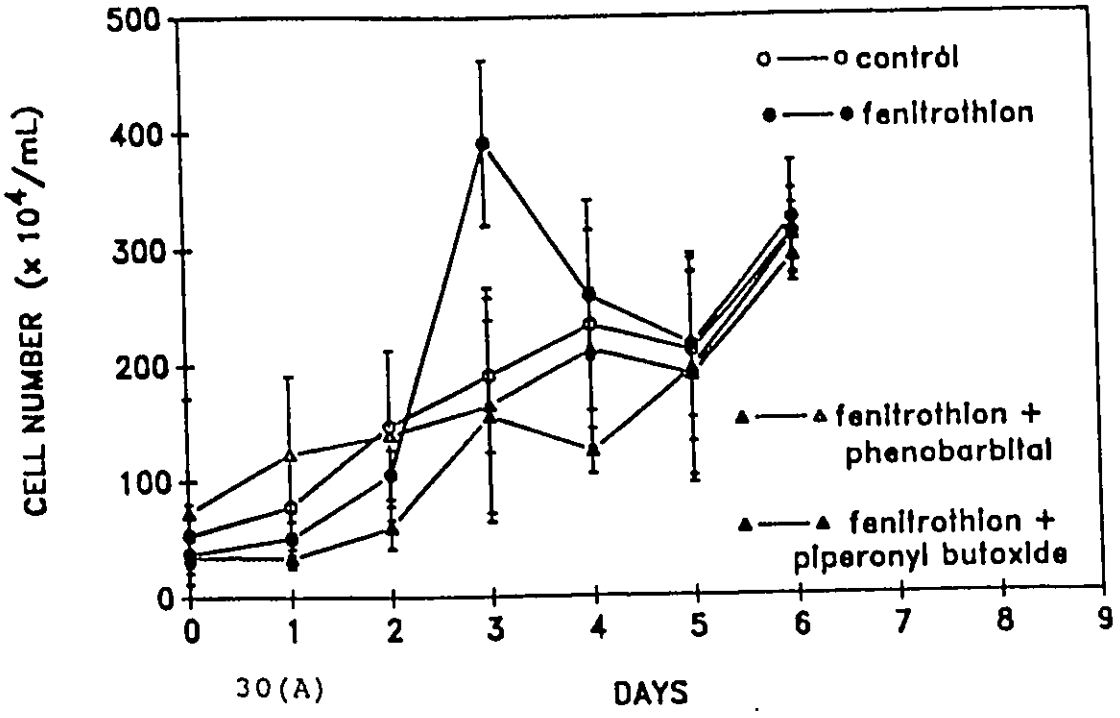
3.4.2 Toxicity of Fenitrothion with Piperonyl Butoxide or Phenobarbital to C. reinhardtii

These studies were done to determine if the mixtures of fenitrothion with phenobarbital or piperonyl butoxide were physiologically toxic to C. reinhardtii.

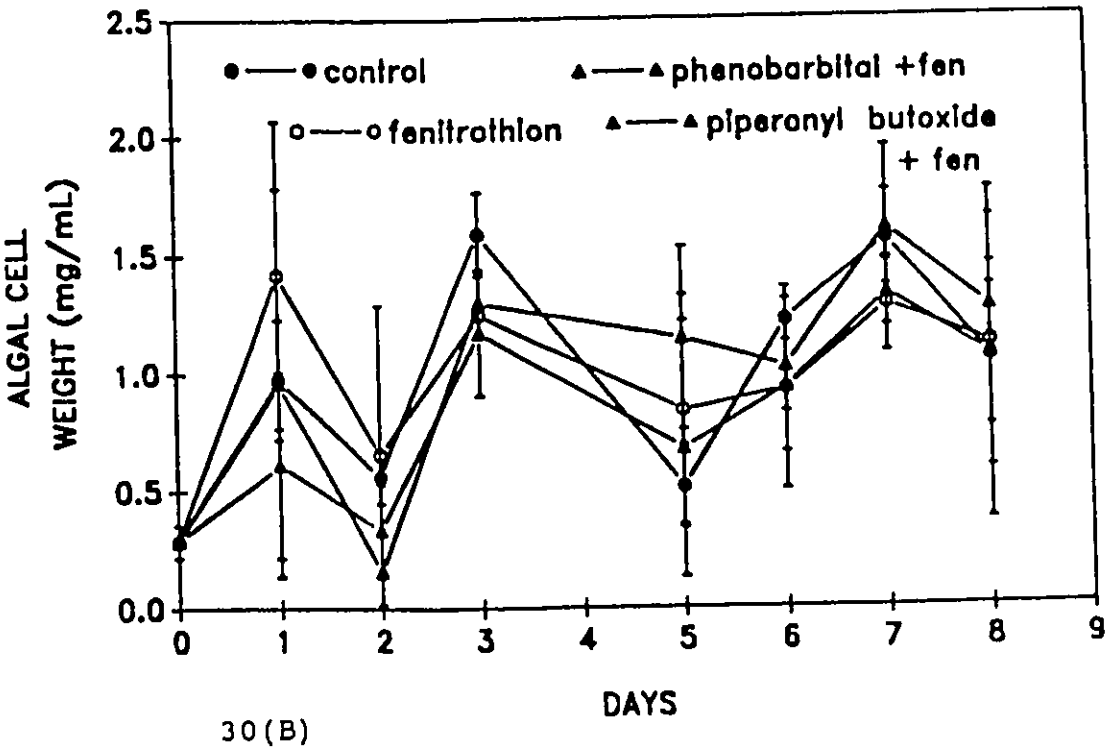
3.4.2.1 Growth Studies

Cell number of cultures of C. reinhardtii incubated with 5.0 μg fenitrothion/mL growth medium plus 23.2 μg/mL phenobarbital did not differ significantly from the control nor the fenitrothion treatments (Figure 30 (a)). The cultures treated with 0.5 μg/mL piperonyl butoxide and 5.0 μg/mL fenitrothion differed significantly from the control up to 3 days, but did not differ significantly from the fenitrothion treated cultures during this time (Figure 30 (a)).

At 4 days, cell number in this fenitrothion plus piperonyl butoxide treatment was significantly less than both the control and fenitrothion treatments. However, no significant difference existed between any of these treatments from 5 to 6 days. All of these



30(A)



30(B)

FIGURE 30

(a) Cell number and (b) cell weight of C. reinhardtii cultures incubated in the light with 5.0 µg/mL fenitrothion, 5.0 µg/mL fenitrothion plus 23.2 µg/mL phenobarbital, or 5.0 µg/mL fenitrothion plus 0.5 µg/mL piperonyl butoxide. n=3, p=0.05, mean ± 95% c.i., 2.0 x 10^5 cells/mL.

fluctuations in cell number were also found in the control cultures of C. reinhardtii and can be considered as natural background variation.

3.4.2.2 Cell Weight

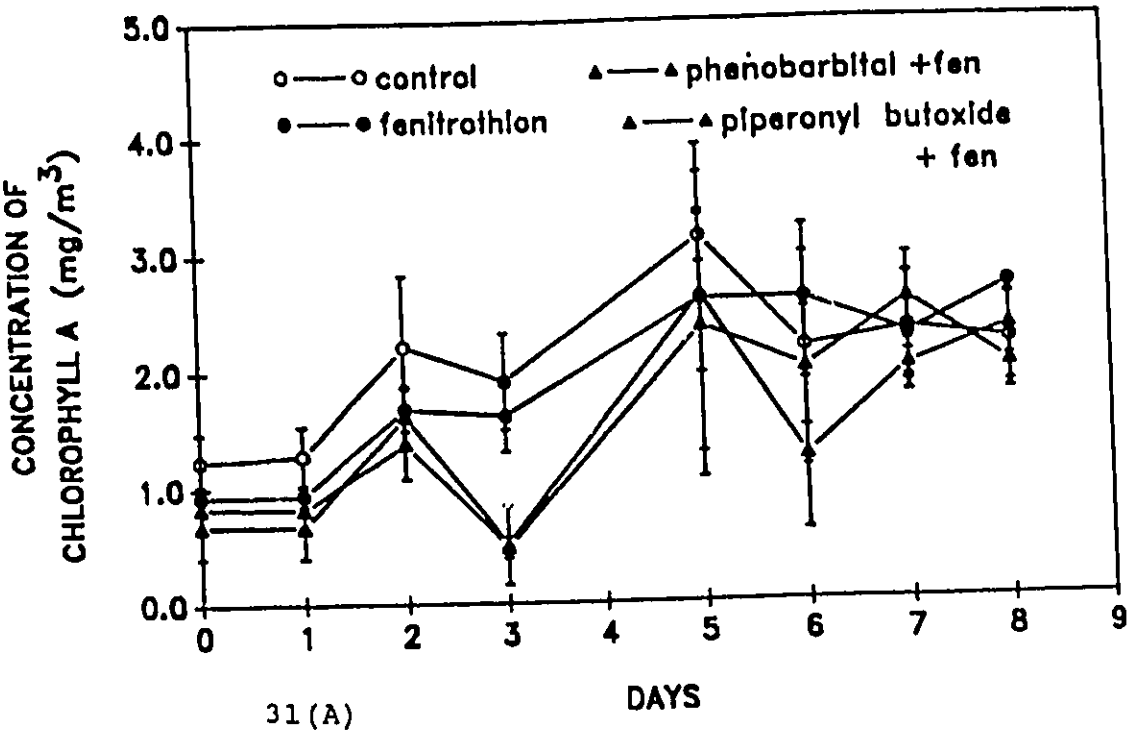
Similarly, there was no significant difference between cell weight of C. reinhardtii cultures incubated with fenitrothion and those incubated with fenitrothion plus phenobarbital or fenitrothion plus piperonyl butoxide (Figure 30(b)).

3.4.2.3 Chlorophyll Concentration

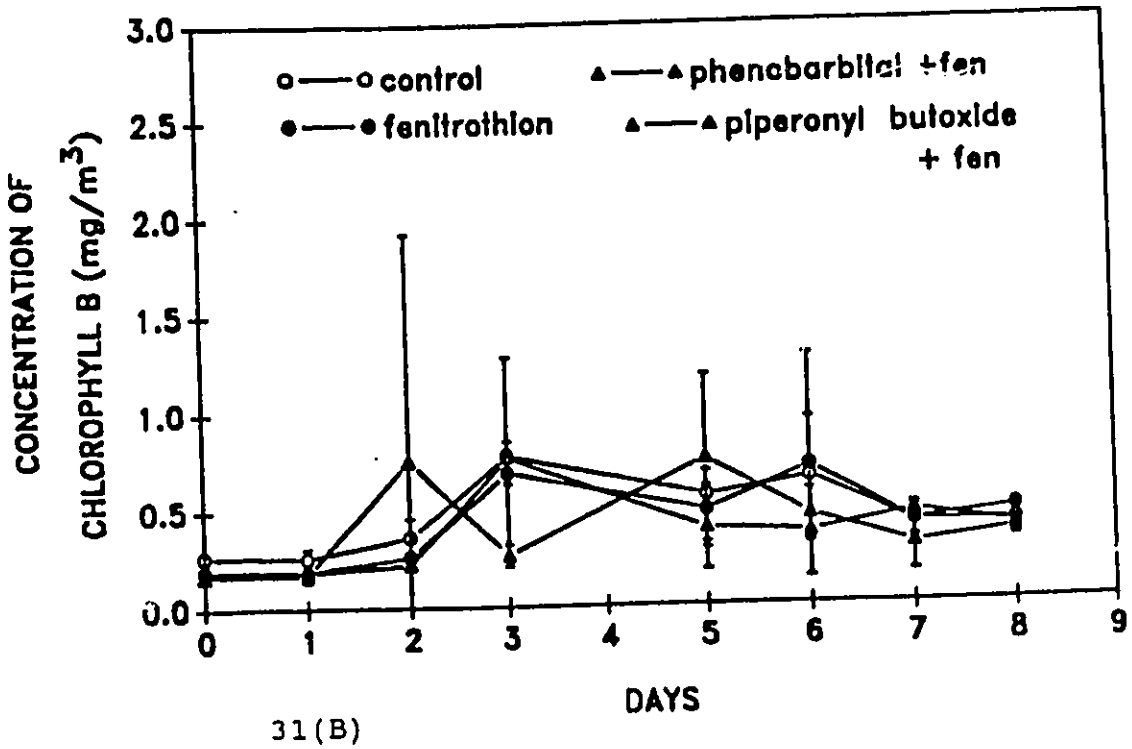
Chlorophyll a and chlorophyll b concentration was also not significantly affected by the treatments of fenitrothion with phenobarbital or piperonyl butoxide (Figures 31(a) and 31(b)). Both mixtures were significantly less than the control and fenitrothion treatment for chlorophyll a at 3 days, however this was the only significant difference.

These results demonstrated that the treatment concentration of piperonyl butoxide (0.5 μg/mL) do not significantly stress C. reinhardtii cultures when incubated with 5.0 μg/mL fenitrothion with respect to the physiological parameters. Any physiological stresses on the individual cells of the algal cultures during incubation with these treatment mixtures were not acutely toxic.

These concentrations of piperonyl butoxide and phenobarbital were used in subsequent studies of metabolism; whether due to homeostasis or due to detoxification mechanisms, the algal cultures



31(A)



31(B)

FIGURE 31

(a)Concentration of chlorophyll a and (b)chlorophyll b of C. reinhardtii cultures incubated in the light with 5.0 µg/mL fenitrothion, 5.0 µg/mL fenitrothion plus 23.2 µg/mL phenobarbital, or 5.0 µg/mL fenitrothion plus 0.5 µg/mL piperonyl butoxide.n=3, p=0.05, mean ± 95% c.i., 2.0 x 10^5 cells/mL.

were resilient to any physiological stress due to these synergistic mixtures.

3.4.3 Degradation and Biodegradation of Fenitrothion in the Presence of PSMO Inducer and Inhibitor

Cultures of C. reinhardtii were first studied to elucidate the fenitrothion metabolites produced under control biotic conditions with 5.0 μg/mL fenitrothion alone. The production of metabolites under these control conditions were then compared to the rate of degradation and amounts of metabolites in the induced and inhibited algal culture treatments. The expected result if PSMO activity has a principle role in algal metabolism of fenitrothion, is that the induced and the inhibited cultures will respectively show an increase and a decrease in the rate of fenitrothion degradation.

Figure 32(a) shows the disappearance of fenitrothion from growth medium incubated with 5.0 μg/mL fenitrothion plus 23.2 μg/mL phenobarbital or 0.5 μg/mL piperonyl butoxide. The rate of degradation of fenitrothion and half life values for the abiotic medium in these phenobarbital and piperonyl butoxide treatments did not differ significantly from the abiotic fenitrothion control (Table 13).

In the presence of C. reinhardtii cultures, the rate constant of degradation of fenitrothion from the piperonyl butoxide treatment was significantly lower relative to the biotic fenitrothion control (Table 13 and Figure 32(b)). This may be because the piperonyl butoxide inhibition affects the process of

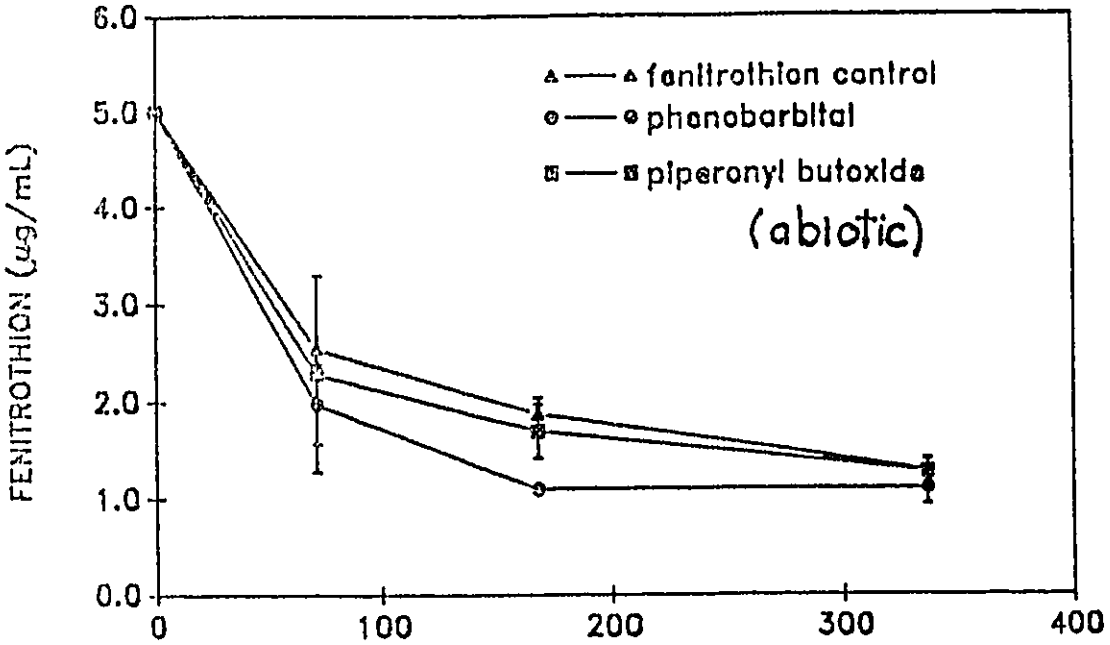
Table 13

Half Life of Fenitrothion in Growth Medium Under (A) Biotic Conditions with C.reinhardtii and (B) Abiotic Conditions Without Algae

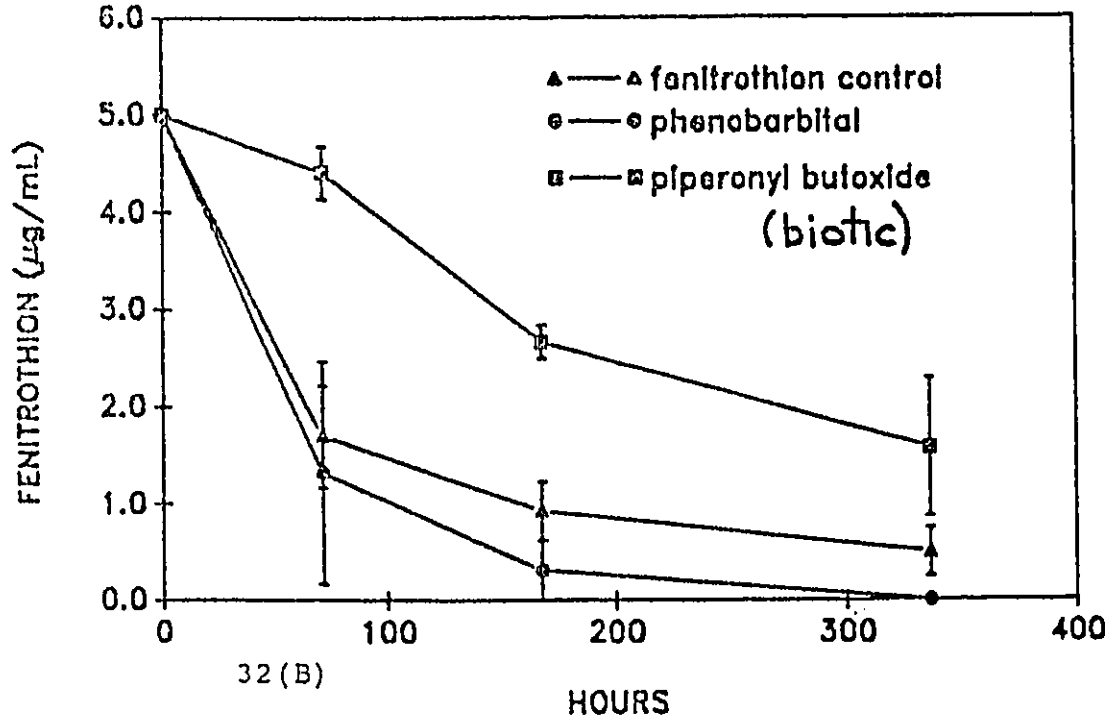
Treatment	Half Life (Days)[a] Biotic	Abiotic	Rate Constant Biotic ($days^{-1}$)	Abiotic
Phenobarbital plus Fenitrothion	7.2	15.8	0.096 ± 0.001	0.0438 ±0.0003
Fenitrothion Control	7.4	16.0	0.0936 ± 0.0007	0.0433 ±0.0001
Fenitrothion plus Piperonyl Butoxide	12.25	16.40	0.0566 ± 0.0003	0.0422 ± 0.006

[a] Calculated from the slope of the plot log %fenitrothion remaining versus time using the equation $t_{1/2} = \frac{0.693}{k}$

where k is the negative value of the slope



32(A)



32(B)

FIGURE 32

Disappearance of fenitrothion from (a)abiotic and (b) biotic growth medium incubated in the light with 5.0 µg/mL fenitrothion, 5.0 µg/mL fenitrothion plus 23.2 µg/mL phenobarbital, or 5.0 µg/mL fenitrothion plus 0.5 µg/mL piperonyl butoxide.n=3, p=0.05, mean ± 95% c.i., 1 x 10^6 cells/mL.

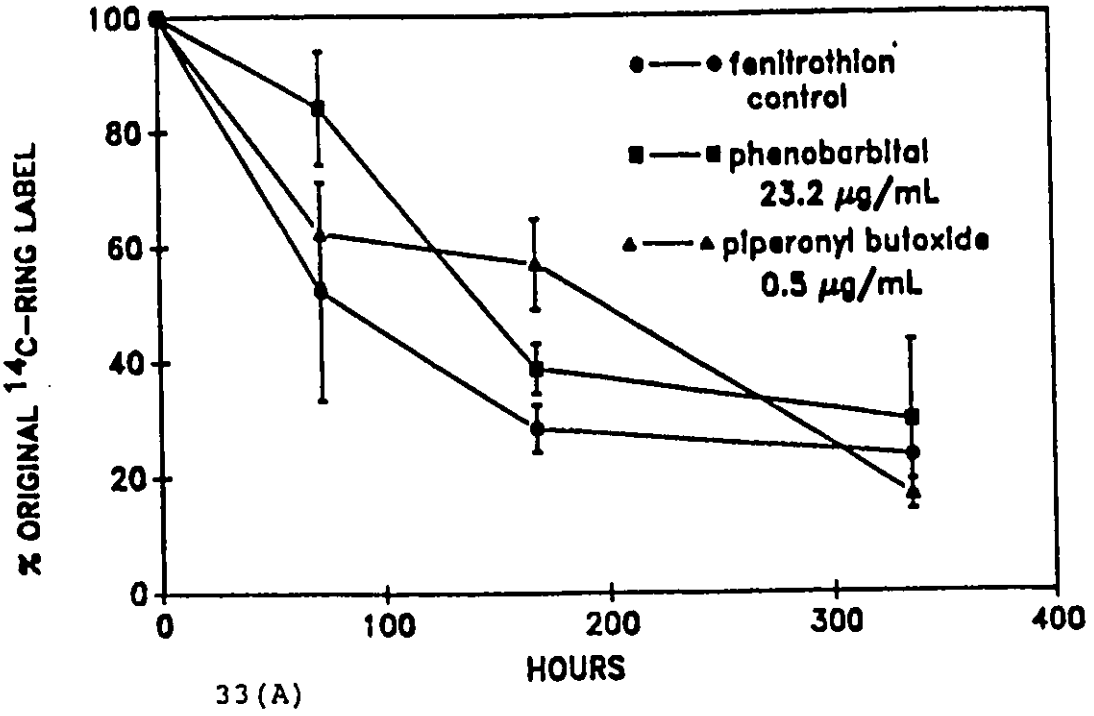
FEN conversion to fenitrothion metabolites in a significant amount, that it is reflected in the degradation rate. In contrast, the rate constant of fenitrothion biodegradation in the phenobarbital treatments was not significantly different from that in the fenitrothion algal controls (Figure 32(b)). All treatments with algae had degradation rates and half life values which were significantly less than their respective abiotic controls (Table 13).

3.4.3.1 Abiotic Degradation Control

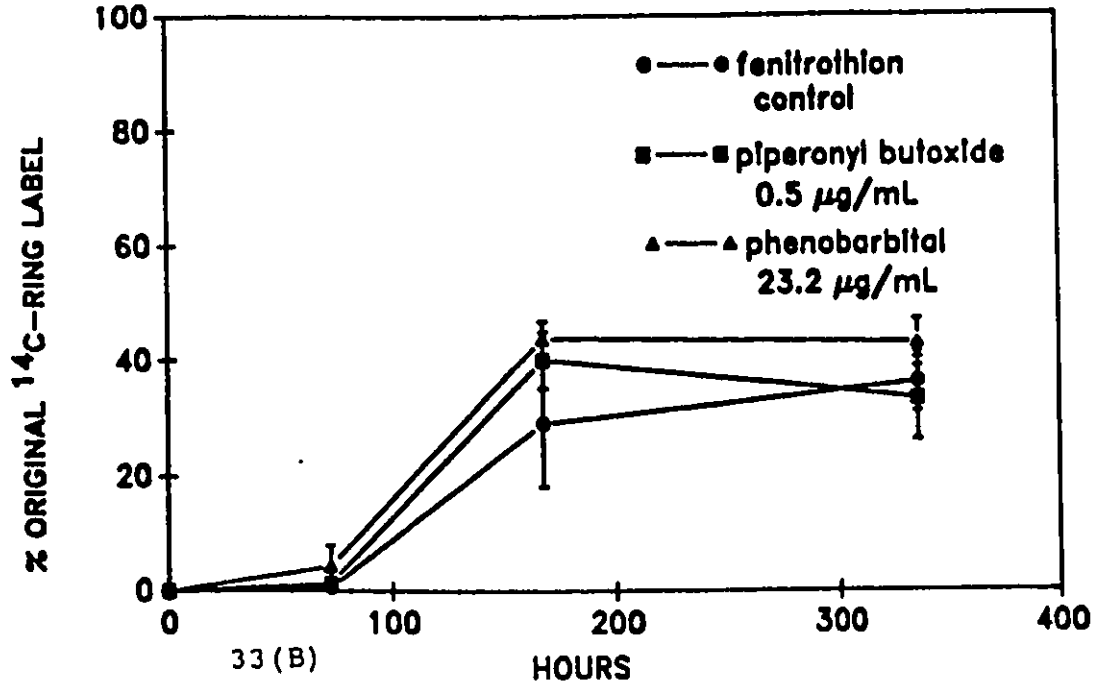
3.4.3.1.1 Differential Distribution of ^{14}C-ring Label

Figure 33(a) represents the disappearance of the total ring labelled fenitrothion and its derivatives from growth medium treated with fenitrothion, fenitrothion plus phenobarbital or fenitrothion plus piperonyl butoxide. In these treatments without algae, approximately 20-40% of the ^{14}C-ring labelled fenitrothion remained in the chloroform fraction of growth medium at 2 weeks (Figure 33(a)). There was no significant difference in the disappearance of radiolabel from the growth medium between the three treatment groups.

Figure 33(b) represents the distribution of ring labelled fenitrothion derivatives in the aqueous fractions of growth medium treated with fenitrothion, fenitrothion plus phenobarbital, or fenitrothion plus piperonyl butoxide. In these abiotic treatments, the amount of polar fenitrothion derivatives in the aqueous fraction were not significantly different between fenitrothion



33(A)



33(B)

FIGURE 33

Levels of ^{14}C-ring labelled fenitrothion moieties detected in (a)chloroform fraction and (b) aqueous fraction of abiotic growth medium incubated in the light with 5.0 µg/mL fenitrothion, fenitrothion plus phenobarbital, or fenitrothion plus piperonyl butoxide.n=3, p=0.05, mean ± 95% c.i..

control and the treatments with cytochrome P_{450} inhibitor or inducer (Figure 33(b)). This indicates that phenobarbital and piperonyl butoxide do not affect the distribution of metabolites between polar and non-polar fractions of the growth medium.

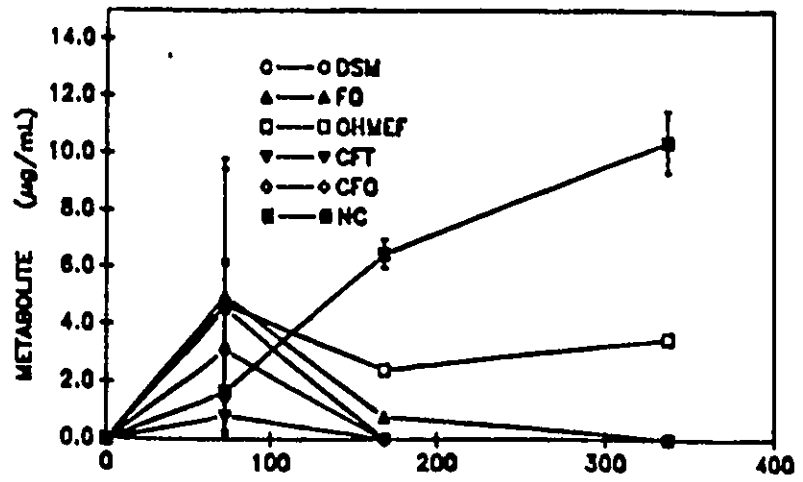
3.4.3.1.2 Abiotic Degradation Controls

Abiotic Fenitrothion Control

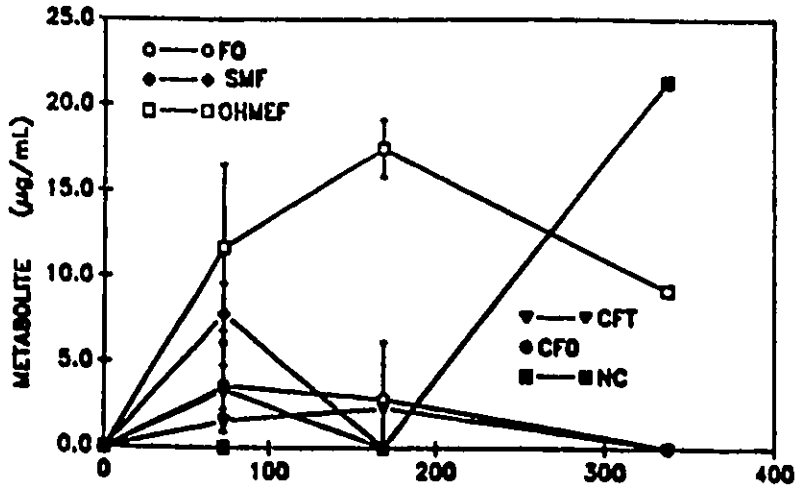
The fenitrothion degradation products in the abiotic medium incubated with fenitrothion alone are shown in Figure 34(a). All the products were detected after the first few hours. At 3 days, the amounts of all products except NC peaked with significantly less CFO and CFT than DSM, FO, and OHMEF. At two weeks OHMEF and NC were the predominant products in this control.

Abiotic Phenobarbital Control

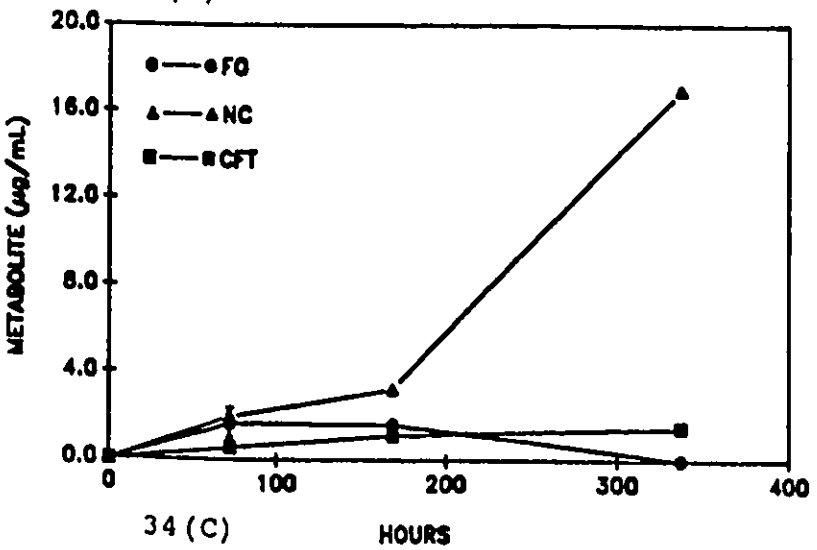
Figure 34(b) shows that the same products were detected in the abiotic phenobarbital controls as were detected in the medium of the abiotic fenitrothion controls (Figure 34(a)), except for DSM. In addition, significantly greater levels of SMF were produced in the presence of the cytochrome P_{450} inducer. Significantly more CFO, OHMEF and NC were detected in the phenobarbital abiotic controls, than in the abiotic fenitrothion controls (Figure 34(a)). This suggests that phenobarbital is a photosensitizer and is involved in producing the significantly higher abiotic levels of OHMEF and NC observed here relative to abiotic fenitrothion degradation. Less FO and CFT were produced in the abiotic phenobarbital controls, however the residues persisted until two weeks as compared to the



34 (A)



34 (B)



34 (C)

FIGURE 34

Amount of fenitrothion metabolites detected in the growth medium fractions of abiotic treatments incubated in the light with (a) 5.0 µg/mL fenitrothion, (b) fenitrothion plus 23.2 µg/mL phenobarbital, and (c) fenitrothion plus 0.5 µg/mL piperonyl butoxide. n=3, p±0.05, mean ± 95% c.i..

abiotic fenitrothion controls.

Abiotic Piperonyl Butoxide Control

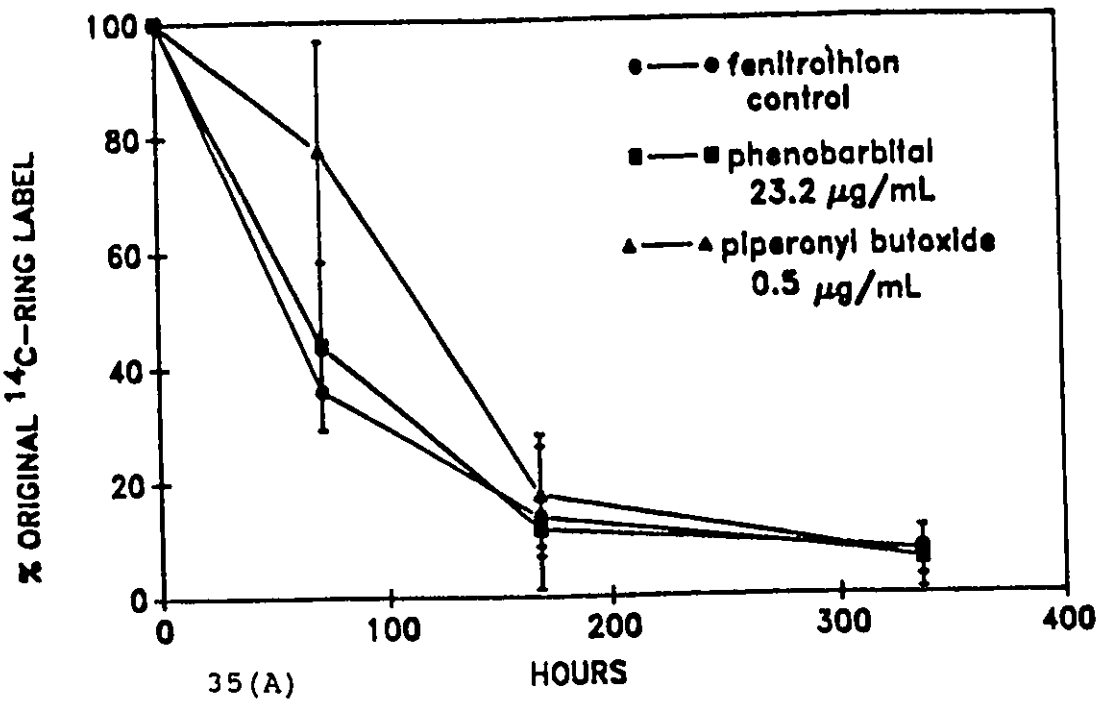
In the presence of piperonyl butoxide (Figure 34 (c)), FO levels were significantly less than in the medium of abiotic fenitrothion controls at all time periods (Figure 34 (a)), while NC levels were significantly greater at two weeks. CFT was detected at 3 days and persisted longer in the medium of piperonyl butoxide abiotic controls than it did in the abiotic fenitrothion controls (Figure 34(a) and 34(c)). All of the other products detected in the abiotic fenitrothion treatments were not seen in the abiotic controls with piperonyl butoxide. There were predominantly hydrolysis reactions occurring in these treatments with little oxidation metabolites.

3.4.3.2 Biotic Degradation Control

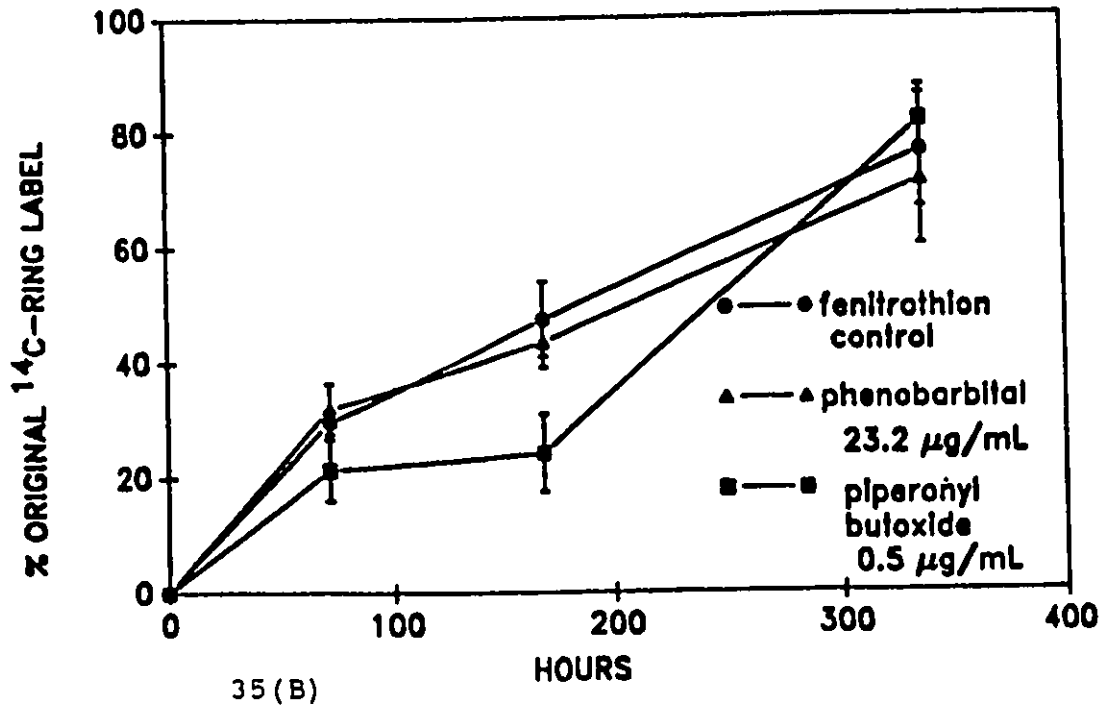
3.4.3.2.1 Differential Distribution of ^{14}C-ring Label

In the algal treatments, the amount of radiolabelled ring moieties of fenitrothion remaining in the chloroform fraction of cell medium at two weeks approached 10-20% (Figure 35(a)). While in the polar fraction of the growth medium the label approached 60-80% (Figure 35(b)).

Intracellular accumulation of ring labelled fenitrothion moieties in the biotic fenitrothion control approached 10% after two weeks (Figure 36(a)). This represented 10% of the original 5.0 μg/mL fenitrothion initially added (0.5 μg/mg algae)(Figure 36(b)).



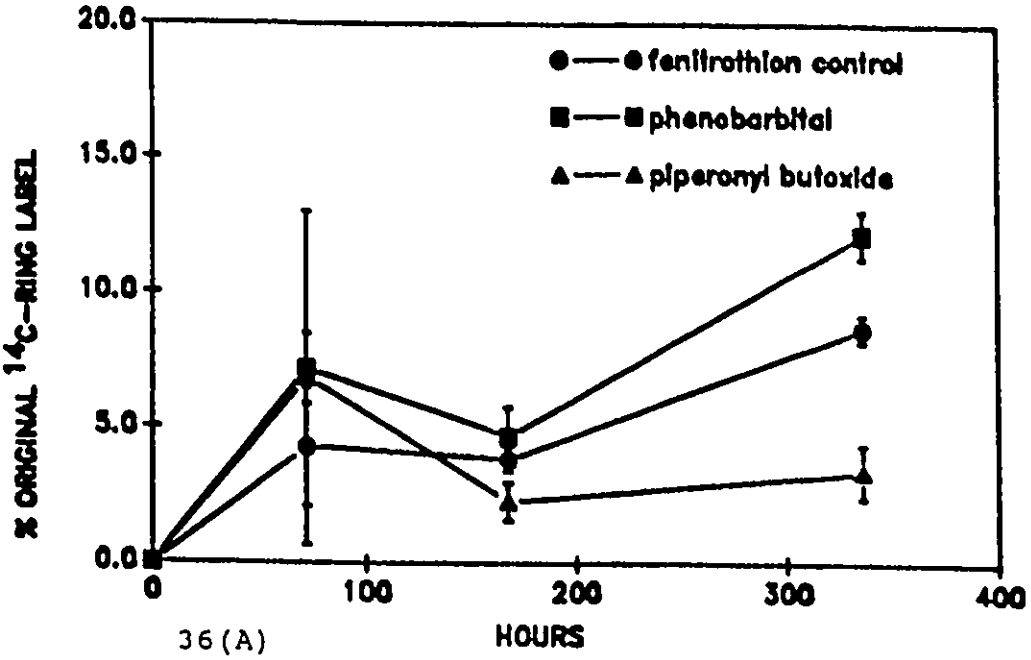
35(A)



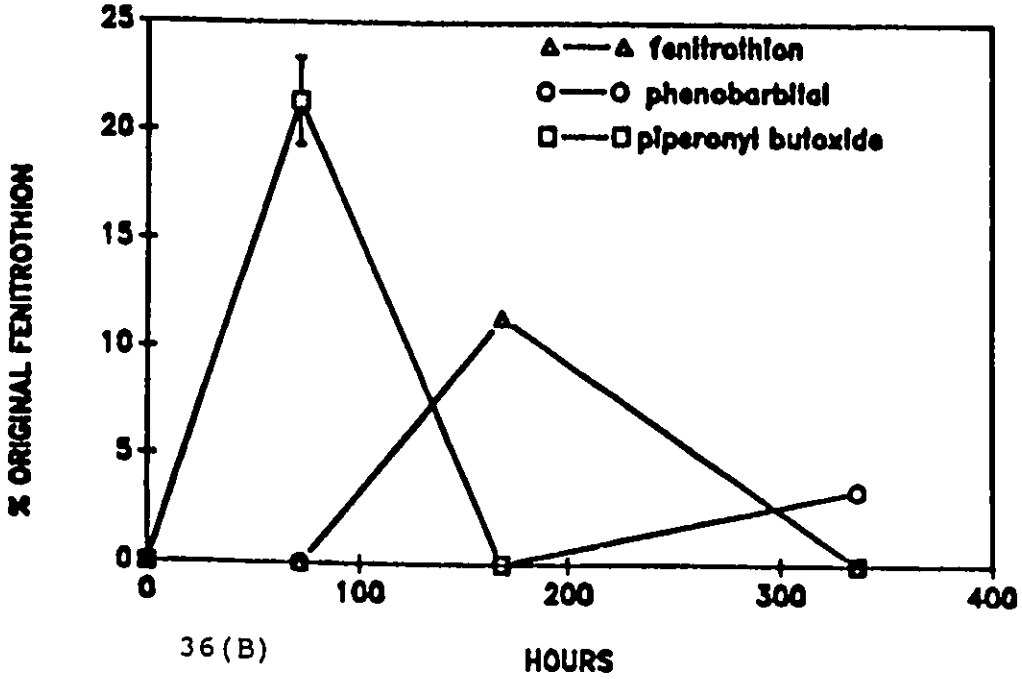
35(B)

FIGURE 35

Levels of ^{14}C-ring labelled fenitrothion moieties detected in (a) the chloroform and (b) the aqueous fraction of biotic growth medium incubated in the light with 5.0 µg/mL fenitrothion, fenitrothion plus phenobarbital, or fenitrothion plus piperonyl butoxide. n=3, p=0.05, mean ± 95% c.i., 1 x 10^6 cells/mL.



36(A)



36(B)

FIGURE 36

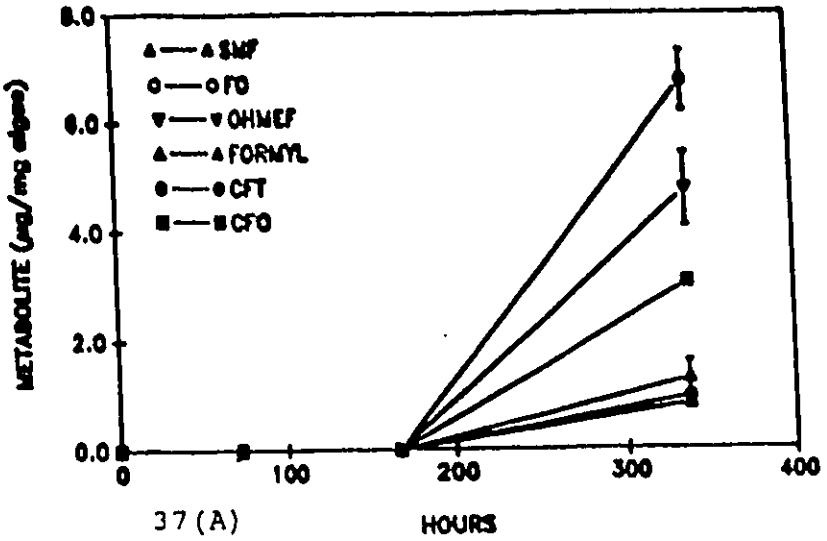
Percent accumulation of (a) ^{14}C-ring labelled fenitrothion moieties and (b) original fenitrothion in the cellular fraction of C. reinhardtii cultures incubated in the light with 5.0 µg/mL fenitrothion, fenitrothion plus phenobarbital, or fenitrothion plus piperonyl butoxide. n=3, p=0.05, mean ± 95% c.i., 1 x 10^6 cells/mL.

3.4.3.2.2 Metabolites:Fenitrothion Control

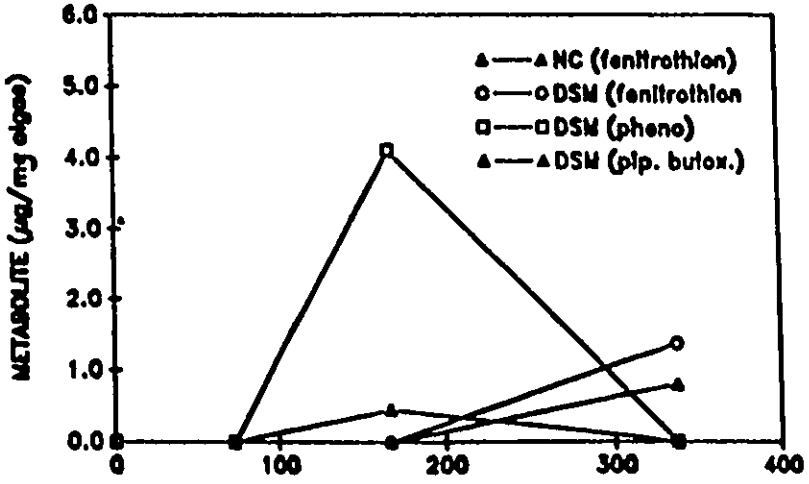
The possibility that the metabolic mechanism of fenitrothion degradation by C. reinhardtii involved cytochrome P_{450} monooxygenase was investigated by examining the extent of and time dependence of this process in the presence of the cytochrome P_{450} inhibitor and inducer. Figure 37(a) and 37(b) show the fenitrothion metabolites detected in cell fractions of C. reinhardtii cultures incubated with 5.0 µg/mL fenitrothion. The metabolites detected in the cell fractions of the control fenitrothion treatment included NC, DSM, SMF, FO, OHMEF, FF, CFT, and CFO. All of these metabolites were detected after one week and the amounts increased until after two weeks.

In the fenitrothion biotic control, hydrolysis metabolites NC and DSM, the fenitrothion isomer SMF, and the oxidation metabolites FO and FF were detected in significantly lesser amounts than that of CFT, CFO and OHMEF at two weeks. The relative amounts of these metabolites suggests that CFO and CFT are the final products of oxidation of the ring methyl group in fenitrothion. FF and OHMEF, however, are the intermediate metabolites in the oxidation process. The results suggest that the hydrolysis reactions and oxidative desulfuration reactions producing NC and DSM and FO are less significant processes in this treatment that the oxidation reactions producing OHMEF, CFT, CFO, and FF. It is also possible that DSM is being produced by glutathione-S-alkyl transferase.

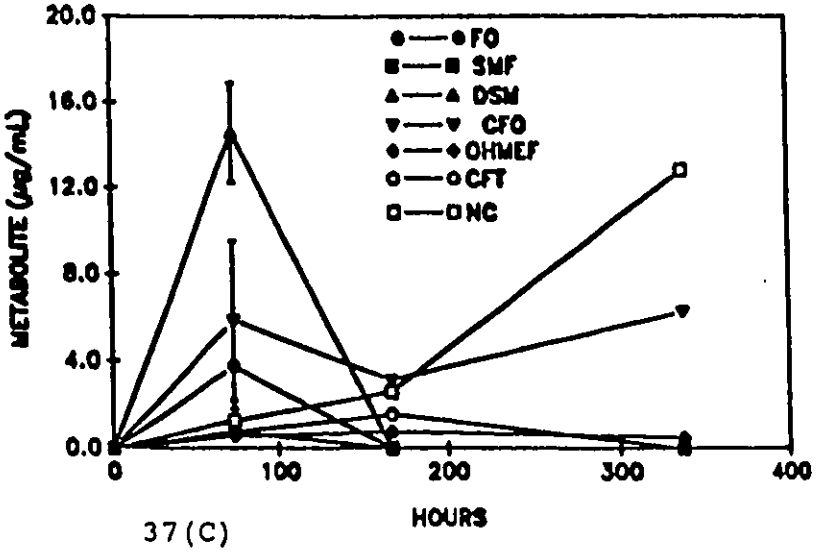
In the presence of C. reinhardtii cultures, fenitrothion was degraded in the biotic fenitrothion controls to produce aqueous



37 (A)



37 (B)



37 (C)

FIGURE 37

Fenitrothion metabolites detected in the intracellular fractions (a,b) and extracellular fractions (c) of C. reinhardtii cultures incubated in the light with 5.0 µg/mL fenitrothion. n=3, p=0.05, mean ± 95% c.i., 1 x 10^6.

soluble metabolites identical to those in the abiotic medium except for SMF which was not detected in the biotic medium of fenitrothion treatments (Figure 37(c)). Significantly greater amounts of SMF, CFO, and DSM and significantly less of FO, OHMEF, and NC were detected in the cell medium than in the control medium at 3 days (Figure 34(a)). The predominant metabolites in cell medium at 2 weeks were NC and CFO with residual amounts of OHMEF (Figure 37(c)), as opposed to NC and OHMEF as the predominant metabolites in abiotic medium at two weeks (Figure 34(a)).

3.4.3.3 Induced and Inhibited Biotic Degradation

3.4.3.3.1 Differential Distribution of ^{14}C-Ring Labelled Fenitrothion

The amount of ^{14}C-ring labelled fenitrothion metabolites remaining in the chloroform fraction of cell medium for both the fenitrothion plus phenobarbital and the piperonyl butoxide plus fenitrothion treatments is shown in (Figure 35(a)). This amount did not differ from the fenitrothion algal control cultures. After two weeks all biotic treatments were significantly lower than the corresponding abiotic controls in the amount of ring label remaining in the chloroform fraction of growth medium.

These results demonstrate that the presence of piperonyl butoxide or phenobarbital did not significantly affect the distribution of fenitrothion metabolites in algal cultures relative to the biotic fenitrothion control culture.

Algal cultures treated with fenitrothion plus phenobarbital or

piperonyl butoxide had significantly greater amounts of polar fenitrothion metabolites detected in the aqueous medium fractions after two weeks relative to their respective abiotic controls (Figure 35(b)). However, there were no significant differences in the levels of polar radiolabelled moieties in the growth medium of these biotic treatments at two weeks relative to the biotic fenitrothion control medium. Piperonyl butoxide had an early inhibitory effect on the detection of polar metabolites at one week.

Intracellular accumulation of ^{14}C-ring labelled fenitrothion metabolites in algal cultures approached 10% after two weeks for the phenobarbital plus fenitrothion treatment (Figure 36(a)). Initial accumulation at 72 h (5-6%) in this treatment did not differ significantly from the phenobarbital or fenitrothion control sets. Accumulation in piperonyl butoxide plus fenitrothion treated cultures was significantly less at two weeks (3.0 %).

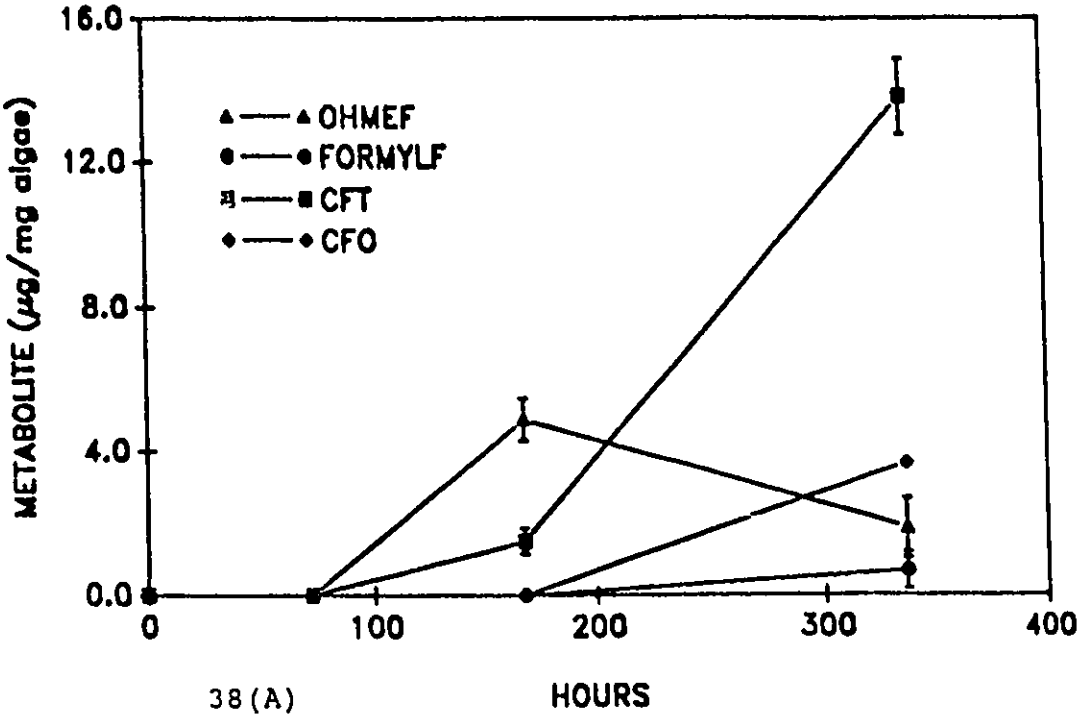
Figure 36 (b) illustrates the accumulation of fenitrothion as quantified by gas chromatography by C. reinhardtii during incubation with 5.0 µg/mL fenitrothion. The piperonyl butoxide treated cultures showed and accumulation of 25% of the original fenitrothion as early as 72 hours. This was significantly greater than the peak accumulation of fenitrothion observed in the fenitrothion control and the phenobarbital treatment at 1 and 2 weeks respectively. The amount of fenitrothion accumulated in these two latter treatments were also significantly different from each other.

Although these results indicate that the net accumulation of label is not significantly different between the treatments as is seen in Figure 36(a), cellular accumulation of fenitrothion is significantly different. There is a greater initial stimulation of fenitrothion uptake by the cells incubated in piperonyl butoxide as compared to the fenitrothion control and the phenobarbital treatment.

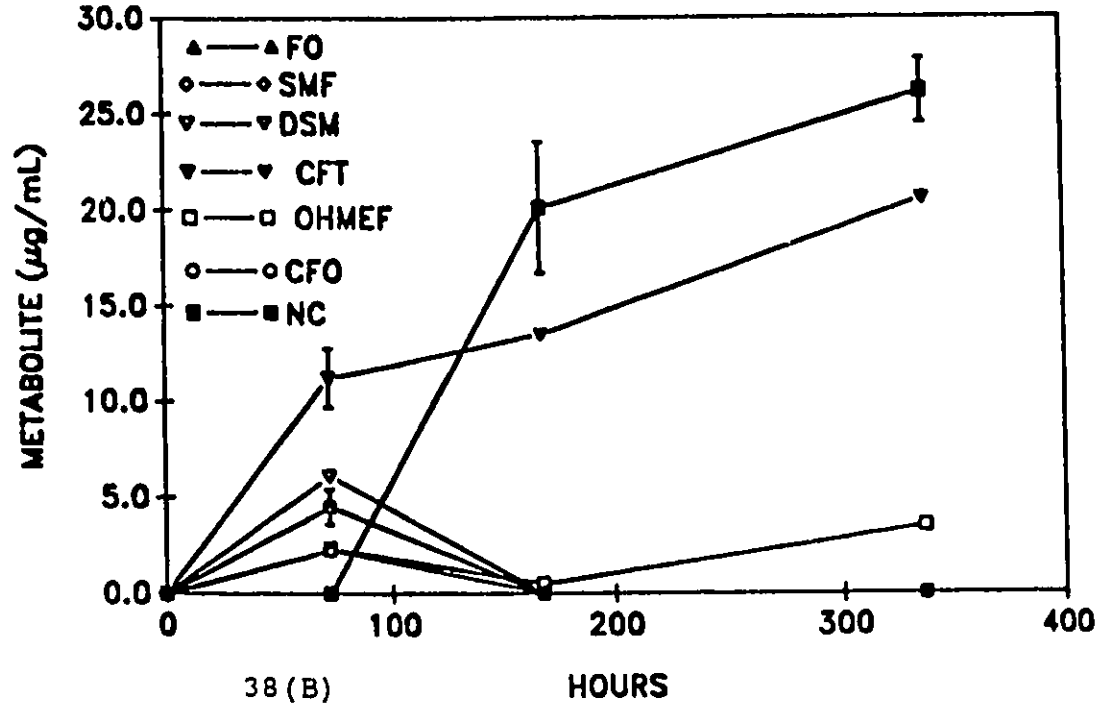
3.4.3.3.2 Induced Biotic Metabolism:Phenobarbital Treatment

The fenitrothion metabolites detected in the intracellular fractions of C. reinhardtii cultures incubated with 23.2 µg/mL phenobarbital included DSM, OHMEF, FF, CFT, and CFO (Figure 38(a) and 37(b)). DSM, OHMEF, and CFT levels detected after 3 days were significantly greater than the amounts of these metabolites in the fenitrothion control at one week. Significantly more CFO was detected at 2 weeks in the phenobarbital treatments than in the fenitrothion controls (Figure 38 (a) and 37(b)). The amount of FF detected in phenobarbital treatments did not differ significantly from the fenitrothion control.

Table 14(A) shows the ratios comparing the amount of fenitrothion metabolites detected in cellular fractions of fenitrothion controls with that of phenobarbital treated cultures. The ratios show that the amounts of OHMEF, CFT, and DSM detected in the cellular fractions were produced significantly earlier at one week in the phenobarbital treatment in amounts significantly greater than in the fenitrothion control.



38 (A)



38 (B)

FIGURE 38

Amount of fenitrothion metabolites detected in the (a) intracelluar and (b) biotic growth medium fractions of C. reinhardtii cultures incubated in the light with fenitrothion plus 23.2 µg/mL phenobarbital. n=3, p=0.05, mean ± 95% c.i., 1 x 10^6 cells/mL.

Table 14 Fenitrothion:Treatment Ratios of Amount of Intracellularly metabolites in C. reinhardtii Cultures Incubated with (a) Fenitrothion and Phenobarbital or (b) Fenitrothion and Piperonyl Butoxide.

Metabolite	Ratio at given time (h)			
	(A) Phenobarbital:Fenitrothion			
	0.0 h	60 h	138 h	336 h
NC	1.0	1.0	1.0	0.188
CFO	1.0	1.0	1.0	1.08
DSM	1.0	1.0	118	0.25
OHMEF	1.0	1.0	11.08 ± 0.003	0.627 ±0.22
SMF	1.0	1.0	1.0	0.022
FF	1.0	1.0	1.0	0.5±0.1
CFT	1.0	1.0	162.22	1.94 ±0.78
FO	1.0	1.0	1.0	0.022
	(B) Fenitrothion:Piperonyl Butoxide			
NC	1.0	1.0	1.0	5.319
CFO	1.0	1.0	1.0	185
DSM	1.0	1.0	0.11	39.39
OHMEF	1.0	1.0	1.0	10.67 ±0.005
SMF	1.0	1.0	1.0	97.0
FF	1.0	1.0	1.0	0.535 ± 0.02
CFT	1.0	1.0	1.0	5.78 ± 0.529
FO	1.0	1.0	1.0	45

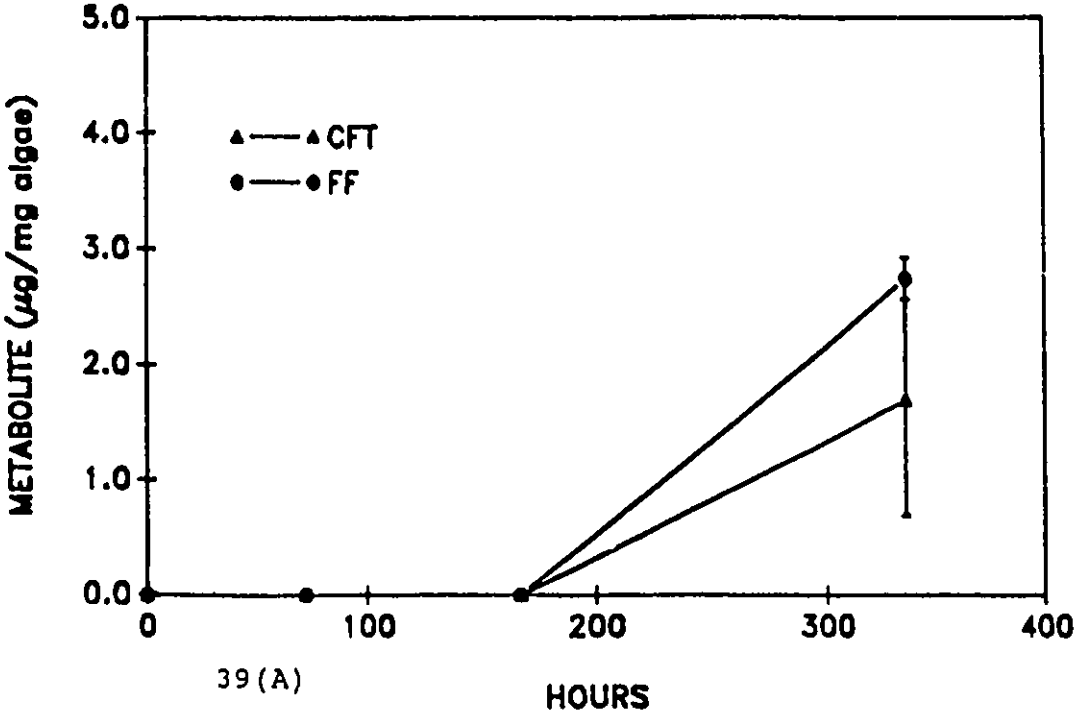
n=3, p=0.05, mean ± s.d., s.d = 0.0 unless indicated

Significantly more NC, DSM, CFT, and significantly less OHMEF were detected in the cell medium of phenobarbital treated algae, than were detected in the abiotic medium of the phenobarbital treatment (Figure 34(b) and 38(b)). There was no significant difference in the amounts of FO, CFO and SMF between these same treatments, however FO in abiotic medium of phenobarbital treatments persisted until after one week (Figure 34(b) and 38(b)).

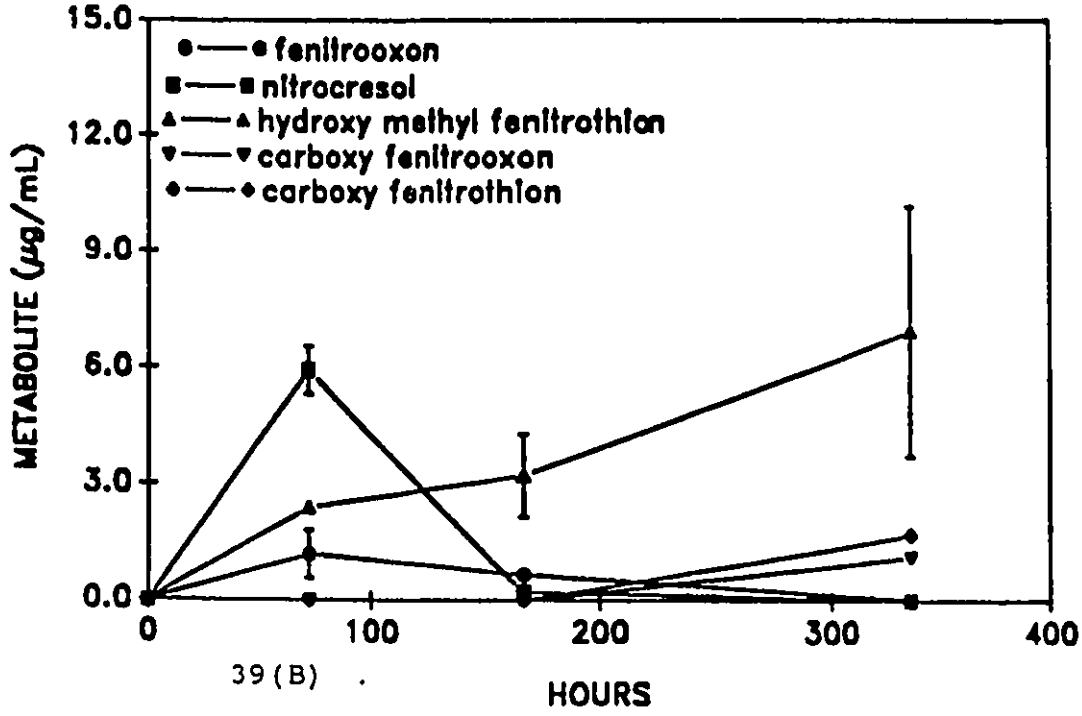
3.4.3.3.3 Inhibited Biotic Metabolism:Piperonyl Butoxide Treatment

Figure 39 (a) and 37(b) show the fenitrothion metabolites detected in the cell fractions of piperonyl butoxide treated cultures of C. reinhardtii. DSM detected after 3 days was significantly less than the amount of DSM detected in the control treatment at one week (Figure 37(b)). The levels of FF detected in the piperonyl butoxide treatments were not significantly different from those in the controls, whereas the CFT detected was significantly less than that of the controls (Figure 37(a)). No other metabolites were detected in the cell fractions of piperonyl butoxide treatments suggesting that cytochrome P_{450} monooxygenase activity is related to cellular metabolism of fenitrothion in Chlamydomonas reinhardtii.

Algal cultures incubated with piperonyl butoxide had significantly greater amounts of OHMEF in the cell medium than did the abiotic piperonyl butoxide treatment (Figure 39(b) and 34(c). This was the predominant metabolite at two weeks in the biotic



39(A)



39(B)

FIGURE 39

Amount of fenitrothion metabolites detected in the (a) intracellular and (b) biotic growth medium fractions of C. reinhardtii cultures incubated in the light with fenitrothion plus 0.5 µg/mL piperonyl butoxide. n=3, p=0.05, mean ± 95% c.i., 1 x 10^6 cells/mL.

piperonyl butoxide treatment as compared with NC in the abiotic treatment. There was no significant difference in the amount of CFT and FO, however, there were significantly greater amounts of NC in the biotic cell medium, at an earlier time period of 60 h, than in the abiotic medium of the piperonyl butoxide treatments (Figures 34(c) and 39(b)). CFO was detected in the cell medium of piperonyl butoxide treated cultures at two weeks which was not observed in the abiotic piperonyl butoxide treatment (Figure 34 (c)).

The ratio of amount of intracellular metabolites (fenitrothion control:piperonyl butoxide treatment are listed in Table 14(B). The ratios demonstrate that in the piperonyl butoxide treatment NC, DSM, SMF, FO, OHMEF, CFT and CFO detected in the cellular fractions of algae, were significantly lower than that of the control fenitrothion treated cultures. FF was the only metabolite in this treatment with greater amounts detected than the control treatment. The lack of significant levels of OHMEF in cellular fractions of the piperonyl butoxide treated algal cultures, relative to fenitrothion control cultures, suggests that the activity of cytochrome P_{450} monooxygenase, (inhibited in this treatment), is directly involved in the degradation of fenitrothion.

The results also suggest that other metabolites are produced by cytochrome P_{450} monooxygenase and are inhibited by inhibiting monooxygenase activity as has occurred in this study. These include NC, FO, DSM and the metabolites from subsequent ring carbon

oxidation of OHMEF (FF, CFO, and CFT). The results shown in Table 14(A) suggest that the production of intracellular OHMEF, (the first step in the production of polar ring carbon oxidation metabolites of fenitrothion), which was induced by phenobarbital, may be a reaction of cytochrome P-450 monooxygenase. FO, SMF and FF were not directly affected by either piperonyl butoxide nor by phenobarbital mixtures with fenitrothion, therefore their production may be due to alternative or additional cytoplasmic or lipid embedded enzymes.

3.4.3.4 Comparison of Control and Induced and Inhibited Biotic Fenitrothion Metabolism by C. reinhardtii

Table 15 shows the biotic:abiotic ratios of fenitrothion metabolites detected in the cell medium of the three different treatments. The effect of C. reinhardtii cultures in enhancing the levels metabolites in growth medium relative to that in abiotic medium, can be compared among treatments.

The algal enhancement of NC, OHMEF in piperonyl butoxide treatments was greater than in the fenitrothion control, while the enhancement of CFT and CFO was greater in the fenitrothion control. This enhancement may be due in part to differences in depuration of metabolites from the cellular fractions adding to the existing levels of these metabolites produced abiotically.

In the phenobarbital treatment, biotic enhancement of the levels of NC, DSM, FO, and CFT was greater than that of the

Table 15

Biotic:Abiotic Ratio of Amount of Fenitrothion Metabolites Detected in Growth Medium of Treatments Incubated with (a) Fenitrothion, (b) Piperonyl Butoxide plus Fenitrothion and, (c) Fenitrothion plus Phenobarbital

Metabolite	Ratio at given Time (h)			
	0 h	60 h	168h	336 h
	(A) Fenitrothion Biotic:Abiotic			
NC	1.0	0.93 ±0.02	0.38 ±0.06	0.245 ±0.778
DSM	1.0	3.56 ±0.146	1.0	1.0
SMF	1.0	63.13	1.0	1.0
FO	1.0	0.857 ±0.159	0.0173	1.0
OHMEF	1.0	0.075 ±0.026	0.275 ±0.028	0.256 ±0.023
FF	1.0	1.0	1.0	1.0
CFT	1.0	1.203 ±0.02	192	1.0
CFO	1.0	2.13 ±0.223	185	302
	(B) Piperonyl Butoxide Biotic:Abiotic			
	0 h	60 h	168h	336 h
NC	1.0	2.97 ±0.63	0.058 ±0.08	0.01
FO	1.0	0.93 ±0.11	0.31 ±0.031	1.0
OHMEF	1.0	5.57 ±0.001	6.57 ±0.003	12.79 ±0.01
CFT	1.0	0.57	0.018	1.06 ±0.01
CFO	1.0	1.0	1.0	74

Table 15 (continued)

(C) Phenobarbital Biotic:Abiotic

	0.0h	60h	168h	336 h
NC	1.0	1.0	149.6 ±0.003	1.08 ±0.36
DSM	1.0	148	1.0	1.0
SMF	1.0	0.448 ±0.060	1.0	1.0
FO	1.0	2.35 ±0.008	0.005	1.0
OHMEF	1.0	0.94 ±0.40	0.024 ±0.01	0.33 ±0.04
CFT	1.0	7.89 ±0.171	5.17 ±0.182	2222
CFO	1.0	0.623 ±0.180	1.0	1.0

n=3, p=0.05, mean ± s.d., s.d. = 0.0 unless indicated otherwise

fenitrothion control, while biotic enhancement of SMF and CFO was more significant in the medium of the fenitrothion control. These results suggest that phenobarbital induced production of cellular metabolites of fenitrothion increases the pool of polar metabolites and non intended fenitrothion metabolites of cytochrome P-450 monooxygenase available for depuration into the extracellular medium.

The observed effects of the cytochrome P_{450} monooxygenase inducer and inhibitor compounds on the biotic degradation of fenitrothion by C. reinhardtii suggest that this family of enzymes may be involved as a cellular detoxification mechanism in this alga.

Chapter 4
Discussion

4.1 Physiological Toxicity Studies

Fenitrothion toxicity studies on Chlamydomonas reinhardtii algal cultures demonstrated that 5.0 μg/mL of fenitrothion was the non-toxic exposure concentration for use in the studies of aquatic persistence and biotic degradation of this pesticide. The toxicity studies monitored the chronic effect of a range of concentrations of fenitrothion on some physiological parameters of the algal cultures.

4.1.1 Fenitrothion Toxicity Studies

The range of fenitrothion concentrations studied included sub-acute field relevant concentrations of 1.0 to 5.0 μg/mL to acute concentrations of 10-20 μg/mL of fenitrothion. The plant load under these conditions at a cell number of 1×10^6 cells/mL is equivalent to 5.0 μg/mg algae (fresh weight) algae in 1.0 mL of culture sample. Environmental pesticide load to a mixture of aquatic plants was 16.28 μg/mg algae in 1.0 mL volume of Lac Bourgeois water, which contained Chlorella, Euglena, and Chlamydomonas species (Moody et al., 1978).

The physiological parameters of pigment concentration, growth rates and maximum carrying capacity were not significantly affected at the exposure level of 5.0 μg/mL (Table 5, Figures 8,9, and 10). Algal culture cell weight was decreased significantly relative to control cultures after 30 h incubation with fenitrothion. This was

due to the cellular mechanisms of homeostasis. This was evidenced by the population dynamics of the algal cultures: growth rate and maximum carrying capacity of cultures treated with 5.0 µg/mL fenitrothion did not differ significantly from that of control cultures (Figures 8(a) and 8(b)).

The studies of Wong and Chang (1988) agree with this study, of the effect of fenitrothion on C. reinhardtii growth, photosynthesis and chlorophyll a concentration. Their study demonstrated that low concentrations of 1 to 5.0 µg/mL fenitrothion stimulated cell growth and chlorophyll a synthesis in C. reinhardtii cultures and inhibited these parameters only at concentrations greater than 10 µg/mL fenitrothion.

C. reinhardtii proves to be a sensitive indicator species in laboratory studies that is representative of algal responses to environmental pesticide residues. It is commonly used as an indicator species in environmental studies of organophosphorus insecticide toxicity to aquatic plants and algae (Irmer and Wachholz, 1986).

In forest applications, fenitrothion is applied early in the morning. In this study however, it was applied during the most sensitive phase of the growth cycle, 2h before the dark.
The environmental application at that time of day is to ensure a maximum knock down exposure to the target pest organism, the spruce budworm. Adjuvants are included in the environmental formulation, which are not used here. Acetone was used here as an ingredient of the stock solutions and it is known to produce autophotolysis.

Based on these important differences between environmental and laboratory exposure, it would be very likely to find different results in metabolic fate or toxicity to the algal population between these two situations. These facts together with the different exposure concentration itself, will only enable this study to yield an understanding of the types of physiological and metabolic responses and does not attempt to define absolutes of what occurs in the environment.

4.1.2 Piperonyl Butoxide and Phenobarbital Toxicity Studies

These studies have determined that 23.2 μg/mL phenobarbital does not affect the growth rate of C. reinhardtii populations tested. Likewise the concentrations of piperonyl butoxide ranging from 0.5 to 0.005 μg/mL did not significantly affect the growth parameters of C. reinhardtii cultures. These parameters included growth rate, maximum population biomass, cell weight, and concentration of photosynthetic pigments. Subsequent studies monitored the synergistic effect of fenitrothion plus phenobarbital and fenitrothion plus piperonyl butoxide on the Chlamydomonas reinhardtii algae (Figures 26 to 31).

None of the physiological parameters, as previously monitored, were affected by these mixtures of fenitrothion plus inhibitor or inducer, relative to control cultures treated with fenitrothion only. The algal cultures were resilient to any physiological stress by these mixtures, demonstrating that these chemical mixtures would not be acutely toxic to algae in the subsequent

metabolic studies. The treatment levels of 0.5 μg/mL piperonyl butoxide and 23.2 μg/mL phenobarbital were used in subsequent metabolic studies of the effects of these cytochrome P_{450} monooxygenase inhibitor and inducer compounds on the biotic degradation of fenitrothion by C. reinhardtii cultures.

4.2 Abiotic Fenitrothion Degradation

4.2.1 Fenitrothion Control

Abiotic degradation of fenitrothion in the absence of C. reinhardtii in Gorman and Levign growth medium yielded a half life in the light of 30-70 h and 11.5 days in the dark (pH=7.0, T=21°C, Table 6). These values are comparable to the results of laboratory studies by Greenhalgh et al., (1980), and Caunter and Weinberger, (1988). These two studies yielded half-life values of 1.5-2 days in the light and 49.5 days in the dark at pH = 7.5, (T=23°C), while at pH=6.8, the half-life value in the light was 30.3 h.

These studies demonstrated that the degradation of fenitrothion is temperature and pH dependant. Half life values of fenitrothion decrease with increasing temperature, with twice the rate of hydrolysis at 40°C than at 30°C (Nishizawa, 1961a). These are the properties of an SN_2 reaction mechanism.This suggests that hydrolysis may be the major abiotic degradation route and that hydrolysis products NC, phosphates, and DSM may be the predominant fenitrothion metabolites which account for the low abiotic persistence of fenitrothion.

As the pH increases from 3.0 to 8.0, the hydrolysis rate of

fenitrothion increases, producing NC, PA, MPA, DMPA, DMPTA, and DSM (Truchlik et al., 1972). At pH values greater than 8.0, base catalyzed hydrolysis predominates, producing NC as the major metabolite. As the pH increases beyond 8.0, the hydrolysis rate increases further with a hydrolysis rate 1000 times greater at pH=12.3 than at pH=9.2 (Truchlik et al., 1972).

In this study the metabolites of abiotic degradation in Gorman and Levign medium in the light at one week were due to oxidative desulfuration of P=S to P=O, side chain oxidation at the ring methyl group (OHMEF, FF, CFO, and CFT), and some hydrolysis to NC. At 48 h however, NC FO and SMF metabolites predominated whereas the metabolites OHMEF, FF, CFO, and CFT were less significant. These same metabolites, as well as amino fenitrothion (AF), were identified in lake water samples buffered at pH 7.5 and T= 23°C (Greenhalgh et al., 1980). AF was detected in natural water (or in laboratory samples of non sterilized lake water) due to microbial reduction metabolism on fenitrothion (Ohkawa et al., 1974).

The dark abiotic treatments yielded NC, FF and SMF as the major metabolites at one week with minor levels of FO, OHMEF, with no CFT or CFO detected. These identified metabolites accounted for 93-97% of the 14-C radio-labelled ring methyl moiety. Hydrolysis was a more significant reaction as opposed to oxidation. In the dark the phosphoric and phosphorothioic acid metabolites from fenitrothion hydrolysis were mainly methylated forms of PTA, rather than of PA as was observed in the light. The light illumination also enhanced oxidation of the ring methyl group relative to the

dark treatments with FF and CFT being detected at higher levels in the light than in the dark.

These differences between the quantitative composition of fenitrothion metabolites in the dark and light abiotic treatments are related to the light energy of the wavelengths used. The Vita Lite^{R} used in these studies mimics the intensities of natural light absorbed by fenitrothion (Figure 5(b)). The small peak at 310 nm corresponds with the peak of fenitrothion absorbance in the u.v. wavelength range 270-310 nm. These wavelengths of u.v. light accelerate the degradation of fenitrothion by transferring high energy electromagnetic energy to the pesticide and provide activation energy for hydrolysis and oxidation reactions.

In the dark this energy is not available and hydrolysis to NC and DSM therefore occurs more slowly. Other abiotic metabolites of fenitrothion due to isomerization (SMF) and non-enzymatic photooxidation (FO, CFT, FF, OHMEF, and CFO) may also be less favoured in the absence of the u.v. intensity of energy (Figures 11(a) and 11(b)).

Other laboratory and environmental studies using microcosms with organisms from multiple levels of the food chain have been used to study the chemical fate of fenitrothion, its distribution and half-life in the different biotic compartments (Weinberger et al., 1982, Miyamoto et al., 1979). However the mechanism of degradation in aquatic plants and algae has not been elucidated as has that of aquatic vertebrates and invertebrates. The relative contribution of algae as a metabolic sink in fenitrothion

degradation was further investigated in this study.

4.2.2 Abiotic Phenobarbital and Piperonyl Butoxide Controls

4.2.2.1 Degradation Rate Constant and Partitioning of ^{14}C-Fenitrothion

Results of the abiotic fenitrothion degradation study in growth medium with phenobarbital or piperonyl butoxide demonstrate that the half-life and degradation rate constant (k) of fenitrothion is not affected in these inducer or inhibitor mixtures with fenitrothion (Table 13). Abiotic fenitrothion degradation producing polar metabolites resulted in partitioning of polar products of fenitrothion with the ^{14}C-ring label into the aqueous fraction of growth medium. This was accompanied by the disappearance of label from the chloroform soluble fraction of growth medium. No significant difference in this partitioning process occurred between control cultures and cultures incubated with the cytochrome P_{450} inducer and inhibitor compounds (Figure 33(a) and 33 (b)).

4.2.2.2 Degradation Products of Fenitrothion

Piperonyl butoxide and phenobarbital affected abiotic fenitrothion degradation in Gorman and Levign medium by increasing the level of OHMEF in the phenobarbital treatments, by increasing the level of NC detected in abiotic piperonyl butoxide treatments, and by decreasing the amounts of DSM, OHMEF, and CFO in the piperonyl butoxide control (Figures 37(b), 38(c) and 39(b) and 34).

These significant differences in fenitrothion degradation are not correlated with changes to cytochrome P_{450} monooxygenase activity since there is no algae in these treatments.

The increased levels of OHMEF in the abiotic phenobarbital treatment may indicate that phenobarbital is enhancing oxidation in the absence of C. reinhardtii. When comparing Figure 35(b) with 35(a), hydrolysis and oxidation degradation of fenitrothion is enhanced by phenobarbital relative to fenitrothion abiotic control. After 200 h, oxidation metabolites predominate. This enhanced oxidation may be due to the keto groups on the phenobarbital molecule and may result in the photosensitization of fenitrothion. This photosensitization would produce desulfuration of P=S to P=O, enhancing hydrolysis to NC (accounting for less FO). In addition, side chain oxidation is enhanced by photosensitization relative to abiotic fenitrothion degradation with inducer.

Piperonyl butoxide treatment also enhanced abiotic hydrolysis of fenitrothion (Figure 34(c)). Here, CFT persisted longer than FO because was not as easily hydrolysed to NC. If first converted from CFT to CFO hydrolysis would be facilitated.

These variations in degradation product levels did not change the half life of fenitrothion in these different abiotic treatments. The difference in products are significant, however, in establishing background control levels to compare with the respective biotic treatments with fenitrothion and phenobarbital or fenitrothion and piperonyl butoxide.

The use of separate abiotic controls for each treatment are

necessary because the composition and quantities of fenitrothion degradation products varied significantly among the different abiotic treatments. In order to compare the results of biotic fenitrothion degradation between the three C. reinhardtii culture treatments the biotic:abiotic ratio of each metabolite residue is first determined for each treatment. The values of this biotic enhancement ratio can then be compared between treatments of phenobarbital, piperonyl butoxide and fenitrothion control cultures. The metabolites produced in the intracellular fractions of the cultures can be compared directly between the treatment and control culture groups.

4.3 Biotic Fenitrothion Degradation

The biotic influences causing the decreased fenitrothion persistence in this laboratory model included the possibilities of algal intracellular accumulation and subsequent metabolic degradation. (Weinberger et al., 1982,and Caunter and Weinberger, 1988). Other factors in the aquatic environments include humic substances which photosensitize pesticide residues, sediments which provide a storage sink for chemically active pesticide residues, and bacteria, which introduce a reductive degradation pathway to biodegrade pesticide residues. These latter three possibilities each contribute significantly to the degradation of environmental pesticide residues, but were controlled or absent in this study. Accumulation and endogenous metabolism will be discussed as the significant processes producing the low biotic half life observed

for fenitrothion residues in this aquatic study.

4.3.1 Rate Constant of Biodegradation

Degradation of fenitrothion in this laboratory model was enhanced in Gorman and Levign medium ir the presence of Chlamydomonas reinhardtii relative to abiotic degradation. Table 10 shows that the biotic half life of fenitrothion is significantly less in the light and dark biotic treatments by a factor of 2.7 and 9.06 respectively, as compared to the abiotic treatments.

Enhanced biotic degradation is also evidenced in Figures 25(e) and 25(f), where the abiotic/biotic ratios of fenitrothion remaining are significantly greater than 1.0 in the light and in the dark. Even in the dark treatments the presence of C. reinhardtii can significantly decrease the persistence of fenitrothion to a value less than in the light abiotic treatments. The environmental relevance of this result is uncertain, since it is difficult to isolate an environmental abiotic control, when there are always biotic influences in environmental studies of aquatic persistence of fenitrothion.

Results of degradation of fenitrothion by C. reinhardtii in the mixtures of 5.0 μg/mL fenitrothion with 0.5 μg/mL piperonyl butoxide or 23.2 μg/mL phenobarbital showed that all the biotic treatments enhanced the k value of fenitrothion relative to their abiotic controls (Table 13). The biotic:abiotic ratios of the fenitrothion degradation rates are 2.19, 2.16, and 1.34 s^{-1} in the phenobarbital, fenitrothion control, and piperonyl butoxide treated

cultures respectively. This demonstrates that one of the effects of piperonyl butoxide on algal degradation of fenitrothion is that it significantly decreased the rate constant of fenitrothion relative to biotic fenitrothion controls. Although it was hypothesized that phenobarbital would enhance algal degradation of fenitrothion, the effect of phenobarbital was not significantly reflected in degradation rate constant, but rather in the cellular accumulation of fenitrothion and in the metabolite production as is discussed below.

4.3.2 Accumulation of and Distribution of Fenitrothion and its Products in Biotic Fenitrothion Controls

Accumulation processes in C. reinhardtii occurred in both the light and the dark treatments (Figure 22(a) and 22(b)). During accumulation and subsequent metabolism, the radioactive carbon label in the phenyl ring of the original fenitrothion will be found in metabolites including all phenyl ring containing metabolites, while PA, PTA, and their methylated derivatives would be unlabelled. As a result these latter metabolites were not traced with the ring carbon label. The methoxy carbon labelled moieties, however would trace any metabolite containing a methylated phosphate moiety but would not trace PA, PTA, nor NC and 50% of the DSM.

The results of Figures 24(a) and 24(b) demonstrated that up to 40% and 60% of radioactively labelled ring and methoxy ^{14}C were accumulated respectively. Therefore up to 40% of the accumulated

species may have been in the form of the intact fenitrothion molecule, or DSM, FF, CFO, CFT, OHMEF if abiotic metabolism of fenitrothion began before cellular uptake occurred.

Either at least 15-20% of ^{14}C-methoxy label was accumulated separately from the ring moiety as methyl phosphates and phosphorothioates, or the depuration of the ring labelled moieties occurred more rapidly than that of the methoxy labelled moieties of fenitrothion. This is likely if glutathione alkyl transferase binds the ^{14}C-methoxy moieties, thus storing them intracellularly, while the resulting DSM and NC were more readily eliminated by depuration.

Figures 22(a) and 22(b) demonstrated that C. reinhardtii does not maintain the peak net accumulation of 40 to 60% of the label in the light and the dark treatment sets, and Figure 23(a) shows that 1.2% of the original fenitrothion concentration exposure is accumulated in the dark and that the rest of the label accumulated is in the form of metabolites as evidenced by the gc results discussed later. This is also the case in the light with 3.5% of the radiolabel accumulated as fenitrothion. These amounts are equivalent to 0.2-0.3 mg fenitrothion /g algae in the light and 0.8 mg fenitrothion /g algae in the dark.

The results of this study suggest that the ring moieties were being eliminated into the external growth medium after the period of peak accumulation due to elimination processes. This occurred more rapidly in the light than in the dark, possibly accounting for the low observed net accumulation of fenitrothion relative to the

total ^{14}C-ring label accumulation.

The greater net accumulation of fenitrothion by intracellular algal fractions in the dark relative to the light is demonstrated in Figure 23(b) where the ratio of dark to light of accumulated fenitrothion is significantly greater than 1.0 at later than 69 h. This suggests that either the uptake of fenitrothion in the light is slower, or that the degradation processes are delayed in the dark relative to light treatment sets. Both possibilities are feasible based on the results of Figure 21 and the metabolite residues detected in Figure 16.

The data in Figure 25(a) shows that at 0-48 h approximately 20% more fenitrothion remains in the dark than in the light. If fenitrothion is being accumulated from the extracellular medium, then the pool of FEN available for uptake is itself slightly less. At times later than this, there is no significant difference in the extracellular FEN, yet the amount accumulated does differ between the biotic light and dark. It seems more likely that cellular metabolism degrading fenitrothion is occurring more rapidly in the light than in the dark, accounting for the difference in intracellular FEN, rather than differences in rate of FEN uptake.

4.3.3 Accumulation and Distribution of Fenitrothion in Induced and Inhibited Biotic Treatments

In C. reinhardtii cultures, fenitrothion accumulation is also affected by the mixtures of fenitrothion with PSMO inducer or inhibitor. Phenobarbital treated cultures showed a greater net

accumulation and piperonyl butoxide treated cultures showed a decrease in net accumulation of label at two weeks relative to control fenitrothion cultures (Figure 36(a)). This decrease may be one of the reasons for the low biotic rate of fenitrothion degradation in the piperonyl butoxide treated cultures. Whether the accumulation process is energetically active or passive is not known (Weinberger et al., 1982).

It can also be suggested that there were differences in the timing and in the extent of biotic formation of polar intracellular metabolites which replaced fenitrothion. This would result in the same amount of label persisting in the cell, while the amount of fenitrothion decreased (Figure 36(b)).

Both the phenobarbital and piperonyl butoxide treated cultures showed enhanced levels of polar metabolites relative to their respective abiotic controls without algae (Figure 33(a), 33(b), 35(a) and (b)). However, piperonyl butoxide and phenobarbital treated algal cultures did not demonstrate significant differences in partitioning of radiolabelled fenitrothion moieties in comparisons with fenitrothion control cultures of C. reinhardtii.

This suggests that the most significant effects of piperonyl butoxide and phenobarbital on fenitrothion degradation were on the composition and quantity of intracellular fenitrothion metabolites produced without inhibiting the production of polar fenitrothion residues due to extracellular reactions. The inhibition of intended reactions of PSMO by piperonyl butoxide therefore could not have totally inhibited fenitrothion degradation by C. reinhardtii.

Other studies of environmental persistence of aquatic fenitrothion residues demonstrated that macrophytes and microphytes accumulated as much as 12-15 mg fenitrothion/kg dry weight (Weinberger et al., 1982, Moody et al., 1978). These plants included Myriophyllum, Elodea, Sagittaria, Chlamydomonas and Chlorella species, Lemna minor, and Ceratophyllum demersum demonstrated rapid environmental accumulation (4.19 and 0.15 ppm respectively), during a 10 h period post spray at an application rate of 4 oz/acre. The high levels of accumulation peaked by 10 h and persisted until 192 h in hornwort C. demersum. This occurred under stagnant conditions, but their study did not identify the metabolite residues (Moody et al., 1978).

Uptake of ring labelled ^{14}C-fenitrothion moieties by Chlorella in laboratory studies with 10 ppm pesticide (in atlox 3409, 1.0% aerotex 3470, and 88% H_2O v/v) peaked in an algal/water model at 4 h (Weinberger et al.,1982). This was followed by desorption and was greater in the light than in the dark by 2-5 fold. This is comparable to the 5 fold difference observed in this study (Figures 22(a) and 22(b)).

These studies support the idea that a light energized active uptake mechanism exists for xenobiotics. Ultimately, the lipid solubility of the pesticide may determine the passive mechanism of cellular accumulation, and light induced changes in algal lipid composition may change the extent of xenobiotic absorption by algae and aquatic plants (Nichols, 1965). Plant loads for pesticides in plant microcosms exposed to 10 ppm fenitrothion reached 74 and 24

in the light and dark for Elodea densa and 417 for Chlorella in this same study (22°C, 16:8 h photoperiod, 5000 lux),(Weinberger et al., 1982). In cases such as these, within organisms, metabolic degradation becomes significant.

Bioaccumulation, as studied in other aquatic organisms from a 0.1 ppm fenitrothion exposure in a dynamic flow system at 25°C, include 235 for fish, (Oryzias latipes), 53 and 18 for snails,(Physa acuta, and Cipangopaludina japonica respectively), and 71 and 6 for crustaceans (Daphnia pulex, and Palaemon paucidens), (Takimoto et al., 1987). Therefore through accumulation, aquatic algae and plants may contribute significantly to the degradation of fenitrothion under high pesticide loads, and therefore determine the chemical fate and true persistence of the aquatic fenitrothion residue.

Soil and aquatic sediments have chemical and physical properties that facilitate the binding of pesticide residues. In aquatic sediments and soil there is no photodegradation without light penetration. In these environmental compartments, biodegradation by bacteria and algae becomes an even more significant process than it is in the water column.

4.4 Fenitrothion Biotic Metabolites

No previous studies have monitored the fenitrothion metabolites produced in C. reinhardtii. This study monitored the fenitrothion metabolites detected simultaneously in both the external aqueous medium and in the intracellular algal fractions.

Cellular metabolites detected in C. reinhardtii included NC, DSM, OHMEF, and CFT in both the light and the dark.

Extracellular fractions of fenitrothion treated C. reinhardtii cultures contained NC, DSM, SMF, OHMEF, FF, CFT, and FO metabolites of fenitrothion. FO however, was only detected in the dark treatments and OHMEF was detected only in the light treatment (Figures 18 to 20). The corresponding abiotic controls produced NC, DSM, OHMEF, SMF, and CFT residues in the light and DSM, NC, and CFO metabolite residues in the dark.

4.4.1 Hydrolysis Metabolites

4.4.1.1 Biotic Fenitrothion Control

Enzymatic hydrolysis produced NC and DSM in the cellular fractions after only 48 h in the light (Figure 14 (a) and 14(b)), simultaneous to peak accumulation of ring carbon radiolabelled fenitrothion (Figure 22(a)). The cellular DSM residue thereafter decreased (Figure 14(a)), possibly being converted to NC or due to elimination to the extracellular medium (34.6 % of extracellular DSM residue) (Table 11). Elimination of NC in the dark accounted for 42% of the extracellular NC residue which appeared after one week (Table 11, Figure 18). NC in the extracellular fractions may also be due to hydrolysis of other cellular metabolites as OHMEF and CFT which also underwent elimination, but is not likely due to abiotic degradation since abiotic NC levels peaked earlier at 48 h.

In the dark, cellular residues of DSM appeared latter than the NC residues did, and NC residue levels decreased while the

intracellular DSM residue in the dark was detected after one week and two weeks (Figure 14). No elimination of NC nor of DSM to the dark extracellular fractions occurred and this process therefore had no effect on extracellular levels of NC relative to the abiotic dark treatments. The early dark peak levels of NC at 48 h may be due to cell surface interaction but is not due elimination nor photoenhanced degradation. Extracellular levels of DSM did not differ from abiotic levels in the dark, although they were significantly lower than the DSM residues in light biotic and abiotic medium samples (Figure 19).

These results for the hydrolysis reactions of fenitrothion in this algal culture are possible if the hydrolysis proceeds by producing predominantly NC in the light with DSM residues being latter converted to NC accounting for the indirect contribution of DSM to the NC intracellular levels. The Vita Lite^{R} energy may be significantly enhancing this process such that in the light, significantly more hydrolysis of DSM to NC and more elimination of NC and DSM occurs than in the dark.

In the dark however, the predominance of hydrolysis metabolite DSM over NC as the cellular residue from FEN may indicate that conjugation of NC to sugars and sulphates may be occurring. In addition glutathione-alkyl transferase activity would account for production of DSM, another conjugation type reaction. Therefore in the light, hydrolysis metabolites may be produced enzymatically and then are rapidly eliminated, whereas in the dark conjugation processes predominate.

Under both light and dark treatment conditions, NC and DSM are the initial metabolites produced from accumulated FEN as opposed to oxidation metabolites of fenitrothion. Elimination of intracellular hydrolysis metabolites significantly affects the levels of these metabolites in extracellular medium in the light treated cultures, but does not occur in the dark.

In the alga Oedegonium, Warwick-Fisher (1985) detected intracellular residues of the hydrolysis metabolite NC but there was no DSM detected. NC was detected in the algae at 0.013 ppm and in the extracellular medium at 0.198 ppm in the external medium. This study by Warwick-Fisher,(1985), was done in a microcosm with algae, snail, fish and midges. Although Oedogonium was not the only organism in the study, Fisher demonstrated that NC residues were found in the intracellular algal fractions.

4.4.1.2 Induced and Inhibited Biotic Treatments

DSM produced by PSMO activity, contributed to the detected levels of this intracellular metabolite in fenitrothion treated algal cultures. DSM levels were at a maximum in phenobarbital cultures relative to fenitrothion treated cultures, (Figure 37(b)) and minimal in the piperonyl butoxide treatment (Figure 37(b)). This suggests that DSM may be a metabolite from PSMO activity. DSM and NC were significantly enhanced in the extracellular medium of phenobarbital treated algal cultures relative to the phenobarbital abiotic treatments (Table 13 and Table 15). Elimination of greater amounts of DSM from the phenobarbital treatments than from the

biotic fenitrothion control may therefore account for the higher biotic extracellular levels of DSM in the phenobarbital treatments.

It was difficult to elucidate whether NC was a biodegradation product of cytochrome P_{450} monooxygenase activity. Although it was produced in the cell fractions of biotic fenitrothion controls, it was not detected at the given analysis times in either the phenobarbital nor the piperonyl butoxide treated cultures (Figure 37 (a) and (b)).

Biotic enhancement of NC levels in the extracellular medium were significantly greater in the phenobarbital treatment and significantly less in the piperonyl butoxide treatment, than in the biotic fenitrothion controls at one week. However, the intracellular levels of NC were greater for the biotic fenitrothion controls (Table 14). This suggests that NC elimination alone, would not account for the increased extracellular levels of this metabolite in the induction and inhibition treatments. Instead, it is likely that oxidation metabolites, especially oxon metabolites with a P=O group were eliminated extracellularly, and then hydrolysed abiotically to NC.

Therefore cytochrome P_{450} monooxygenase activity is not directly responsible for the enhanced levels of NC found in the extracellular fractions of biotic treatment. The NC level depends indirectly on PSMO because its activity affects the amount of oxidation metabolites available for elimination. The hydrolysis itself is occurring extracellularly and abiotically.

This process was more significant in the phenobarbital biotic

treatments than in the fenitrothion biotic controls. At two weeks, biotic enhancement of NC did not differ between the two treatment sets, (Figure 15) because the abiotic levels of NC were more predominant than the biotic source of these treatments.

NC and FO production has been demonstrated by (Levi, Hollingworth et al., 1988), to be a fenitrothion metabolite in mice due to microsomal cytochrome P_{450} monooxygenase activity. 1.0 mM of p-nitroanisole was used to inhibit cytochrome P_{450} monooxygenase activity during in vitro metabolism of 25 μM fenitrothion. 1.0 mg of phenobarbital/kg body weight was used to induce cytochrome P_{450} levels in the mice prior to isolation of the monooxygenase used in in vitro studies.

Their study demonstrated that FO and NC were the predominant metabolites of fenitrothion degradation by cytochrome P_{450} monooxygenase and that phenobarbital treatment induced FO to higher levels than NC. In addition their study showed that piperonyl butoxide inhibited fenitrothion metabolism to FO and the NC:FO ratio decreased. This demonstrated that the levels of NC were not significantly affected relative to FO, and suggests that FO may be produced directly from of cytochrome P_{450} monooxygenase activity where as NC is an indirect result of PSMO activity. Other metabolites of fenitrothion were not monitored and were not considered in their study: OHMEF, FF, CFT, CFO, and SMF. Nonetheless the results of Levi et al.,(1988), support the findings in this study; that enhanced levels of NC with phenobarbital treatment is due to enhanced hydrolysis of FO.

Their findings also support this study of fenitrothion degradation by C. reinhardtii. FO production from fenitrothion may depend on cytochrome P_{450} monooxygenase activity, although it may be of minor importance, relative to OHMEF (C-H bond hydroxylation), DSM (demethylation) and secondary oxidation metabolites (FF, CFT, CFO).

4.4.2 Oxidation Metabolites

4.4.2.1 Biotic Fenitrothion Control

Non hydrolysis products of fenitrothion due to successive oxidations of the 3-methyl carbon on the phenyl ring included OHMEF, FF, CFT, and oxon their oxon metabolites, OHMEFO, FFO and CFO. OHMEF appeared in cellular fractions of the light treatment sets after only 60 h incubation with fenitrothion, more than 100 hours earlier than it did in the dark (Figure 15). Subsequently after 1 week there were CFT residues detected in cellular fractions of light exposed cultures which were significantly greater than that in the dark (Figure 15 and Figure 16). FF was detected in the cellular fractions of dark treated algal cultures, but was not detected in the light treated cultures (Figure 15(b)). OHMEF, CFT and FF can each be further degraded by hydrolysis to NC.

Elimination of OHMEF was not significant in the light nor the dark extracellular medium, and C. reinhardtii did not enhance OHMEF levels relative to abiotic medium in the light nor the dark (Figure 19 and 20). No significant elimination of FF occurred in the light but rather rapid conversion of FF to CFT in the cellular fraction

occurred (Figure 15 (a), (b) and (c)). In the dark however, FF was a major oxidation metabolite in the extracellular medium in the dark (Figure 15 (b)).

Production of CFT from FF in the cellular fractions occurred, producing higher residue levels of CFT in the light than in the dark algal treatments (Figure 15(c)). CFT elimination significantly enhanced extracellular levels of this metabolite relative to abiotic medium in the light (Table 11, Figure 17, and Figure 20 (a)).

In the fenitrothion treated cultures of algae incubated in the dark (Figure 15 (a), 15 (b) and 15(c)), the conversion of fenitrothion to OHMEF, FF, and CFT was a significant process. FF and CFT were detected in the extracellular medium at levels greater than in the dark abiotic controls (Figure 17 and 20(b)). These results support the suggestion that elimination is the likely reason for enhanced extracellular levels of these metabolites.

None of the metabolites OHMEF, FF, CFT, and CFO were detected in algal fractions of other laboratory and field studies, however Warwick-Fisher, (1985) detected four compounds by tlc and scintillation counting whose chemical structures were unknown. These metabolites accounted for 62.86 % of the external cell residue and 23 % of the internal fenitrothion residues in oedogonium and FF, OHMEF, CFT and CFO may have been among these unidentified metabolites.

It is believed that the algae initially produced OHMEF by cytochrome P_{450} monooxygenase mediated oxidation of the lipid

incorporated fenitrothion. This intermediate metabolite was subsequently oxidized by cytoplasmic enzymes to produce FF, CFT and CFO in a time dependant manner with the most polar metabolites CFT and CFO appearing last. The overall observed distribution of fenitrothion metabolites during the two week incubation demonstrates that oxidized metabolites of increasing polarity were being produced and eliminated to the surrounding cell medium in the light and the dark.

Reactions of cytochrome P_{450} monooxygenase may have been responsible for the oxidation of fenitrothion to DSM, FO or NC, although hydroxylation is the more likely reaction of this enzyme (Hollebone, 1986). In addition, glutathione-S-alkyl transferase may have produced DSM, and SMF may be produced by alkylation of DSM by excess FEN (Hallet et al., 1977).

The mechanism of the observed light enhancement of this oxidation sequence may be related to the photosynthetic production of NADPH reducing equivalents required by cytochrome P_{450} monooxygenase (Benveniste et al., 1977), and by redox reactions such as are involved in fenitrothion degradation. Induction of cytochrome P_{450} monooxygenase activity and endogenous levels by light has been demonstrated in plants (Benveniste et al., 1978). Therefore in C. reinhardtii cultures studied here, Vita Lite^R may likely be inducing the production of OHMEF relative to the dark treatments, either

(a) indirectly due to enhanced rates of NADPH (monooxygenase cofactor) production;and/or

(b) directly by induction of endogenous algal cytochrome P_{450} levels or activity.

The subsequent differences observed in intracellular levels of FF, CFT and CFO detected during light exposure and the dark exposure, may therefore be due to the different degradation rates for FEN to OHMEF. This affects the amount of intracellular OHMEF available for further biodegradation to FF, CFT, CFO, and NC.

4.4.2.2 Induced and Inhibited Biotic Treatments

The most significant effect of phenobarbital on the production of oxidized fenitrothion metabolites involved DSM, OHMEF and CFT (Table 14 Figure 37(b) and Figure 38(a)). OHMEF and CFT production occurred after 72 h, and peaked at one week and two weeks respectively, at levels greater than in fenitrothion treated cultures. This was followed by the production of CFO as the subsequent oxidation metabolite.

It was hypothesized that phenobarbital would induce levels and activity of cytochrome P_{450} monooxygenase in C. reinhardtii cultures, and increase the amount of the metabolite OHMEF. The effect of phenobarbital in these results, however, showed a rapid production of intracellular levels of OHMEF followed by a decrease of this same metabolite, simultaneous to the appearance of CFT.

No OHMEF was detected in the piperonyl butoxide treatment, suggesting that this metabolite of cytochrome P_{450} monooxygenase was inhibited because the monooxygenase activity was inhibited (Figure 39(a)). Extracellular levels of OHMEF were not significantly

enhanced by C. reinhardtii in the phenobarbital treatments relative to fenitrothion biotic controls, however when comparing growth medium in abiotic treatments phenobarbital was found to enhance OHMEF levels, possibly by photosensitization. Although OHMEF appeared in the extracellular medium of this treatment, it was rapidly converted to CFT and the OHMEF levels did not persist (Figure 34 (b) and Table 15).

Biotic enhancement of OHMEF in growth medium occurred in the piperonyl butoxide biotic treatments relative to the abiotic piperonyl butoxide controls (Table 15). This enhancement was greater here than the biotic enhancement found in the fenitrothion treatment. The greater enhancement of OHMEF here is not likely due to elimination of this metabolite from the pool of intracellular metabolites, since it was not detected at the given analysis times (Figure 38 (c)). Non-enzymatic oxidation through photosensitization by the oxidizing agent piperonyl butoxide, or may likely account for the biotically enhanced levels of extracellular OHMEF in the piperonyl butoxide treatment.

Significantly greater levels of intracellular CFT were produced in the phenobarbital biotic treatment than in the control fenitrothion treated cultures. Extracellular medium of phenobarbital biotic treatments had significantly greater amounts of CFT than in the phenobarbital abiotic controls possibly due to depuration of the intracellular residue.

Piperonyl butoxide significantly decreased the levels of all metabolites produced in the cellular fractions of algal cultures,

such that the only oxidative metabolites produced were FF and CFT (Figure 39(a)). The residual levels of CFT and FF detected in piperonyl butoxide treated cultures were not produced from any significant amount of OHMEF precursor from monooxygenase activity, but by other cytoplasmic of lipid embedded oxidases. These two metabolites accounted for all of the identifiable pesticide residue, as opposed to the fenitrothion treated cultures which had six additional metabolites (Figure 37(a), 37(b)).

Extracellular residues of CFT and CFO in piperonyl butoxide biotic treatments were not significantly enhanced relative to the abiotic piperonyl butoxide controls. This is because the large ratio value of 74, for CFO for instance, does not represent any biological significance. This is because the absolute values of biotic CFO extracellular levels are not significantly greater than those of the fenitrothion treated cultures (Figure 38 (b) and 33 (a)).

Photosensitization by piperonyl butoxide in algal cultures may have accounted for the observed residues of extracellular CFO, as opposed to true biotic effects from cellular metabolism in the piperonyl butoxide treatment. Since abiotic hydrolysis and photosensitization reactions were not affected by piperonyl butoxide treatments photosensitizing reactions may therefore have produced CFO and hydrolysis reaction produced the NC detected in the extracellular fraction of algal cultures (Figure 39(b)).

4.4.3 Toxic Fenitrothion Metabolites

4.4.3.1 Biotic Fenitrothion Control

FO and SMF total residues produced over the two week period in the light were significantly less in the biotic medium than in the abiotic growth medium. SMF and FO may have subsequently been hydrolysed to NC (Figure 18). In the dark, however extracellular residues of FO and SMF were significantly greater than those residues in abiotic cell medium (Figure 17 and Figure 20(b)).

4.4.3.2 Induced and Inhibited Biotic Treatments

SMF was not detected in the piperonyl butoxide nor in the phenobarbital treated cultures of algae and it was not determined if this metabolite was produced by monooxygenase activity. It may have been produced non-enzymatically by transmethylation of DSM by excess FEN. Induction and inhibition treatments did not produce intracellular levels of FO, and although there were extracellular levels, this metabolite did not persist and was not significantly different from the levels in the fenitrothion control cultures.

Identification of accumulated fenitrothion residues in Sagittaria, Elodea, and Myriophyllum species included FO, SMF, and NC in the light up to 53.32, 16.0 and 31.4 μg/g dry weight respectively after exposure to 10 ppm fenitrothion (Weinberger et al., 1982). In the dark only NC and DMPTA were detected in these aquatic plants. Both FO and SMF are potentially toxic; FO is the actively toxic form of fenitrothion and SMF is an isomer of FEN.

SMF can be formed by alkylation on the S by enzymes. This suggests that algal accumulation can result in chemical changes in the activity of the parent pesticide fenitrothion.

In a study with the alga Oedogonium, NC, SMF, and FO as well as four unidentified products were detected in the intracellular and cell medium fractions at residue levels up to 60 ng/mL. The occurrence of these metabolites in the presence of Oedogonium was pH dependant with less metabolism at pH=8.3 than at a pH=6.8 (Warwick-Fisher, 1985), however this difference did not affect the toxicity of the resulting metabolite residues. At slightly acidic or basic pH, fenitrothion persist longer than just a few days in natural water, but at a more basic pH of 8.0, base catalysed hydrolysis of FEN, FO and SMF to NC is more rapid.

In the study of Hallet et al., (1977), FO and SMF residues were detected in yellow birch, white spruce and white pine seeds at peak levels of 10.0 and 8.0 ppm. These were identified as true intracellular metabolites of fenitrothion after only four days of incubation with 4×10^{-5} M fenitrothion which decreased to less than two ppm at 21 days. It was suggested by Hallet et al., (1977), that microsomal monooxygenases were involved in the production of FO in the seeds. However, their study concluded that it was glutathione transferase which produced SMF, that gave the seeds the ability to survive large doses of fenitrothion.

SMF and FO hydrolysis to NC and phosphorothioic or phosphoric acids, occurs more rapidly than hydrolysis of fenitrothion to NC. This is due to the enhanced positive charge on the phosphorus in

SMF and FO relative to FEN. This promotes base catalyzed hydrolysis by SN2 nucleophilic attack of hydroxyl groups. This potential route of rapid degradation may suggest that FO and SMF do not present a significant toxic hazard, however, FO residues are more toxic than FEN and SMF is an isomer of FEN.

Therefore, although the production of FO and SMF residues from fenitrothion in the presence of C. reinhardtii decreases the half life of fenitrothion, these degradation products represent a potential hazard to non target organisms if eliminated extracellularly into the environment. However, in this study all treatments demonstrated that biotic degradation of fenitrothion by C. reinhardtii does not produce persisting levels of these relatively toxic metabolite residues. Nonetheless it is important to not only monitor the half life of the parent compound xenobiotic but to also identify the chemical fate and toxicity of pesticide residues, to accurately assess the persistence of toxic activity.

4.5 Model of the Role of PSMO in Degradation of Fenitrothion by C. reinhardtii

Trends in the production of fenitrothion metabolites in C. reinhardtii cultures demonstrated that fenitrothion is metabolised to produce OHMEF, FF, CFT, CFO, NC, DSM, FO, SMF and phosphate moieties. It was hypothesized that the production of OHMEF, NC, DSM and FO would depend on cytochrome P_{450} monooxygenase activity. Subsequent side chain oxidation of OHMEF at the benzyl alcohol moiety would produce FF, CFT, and CFO metabolites would be produced

also depending on PSMO activity or cytoplasmic reactions. Elimination of these intracellular metabolites would increase the levels of these metabolites in the biotic extracellular fractions.

Cytochrome P_{450} monooxygenase function is controlled by electronic effects alone and does not depend on entropy of reaction as do other biological enzymes. The enthalpy change, however, of a reaction associated with insertion of an hydroxyl group into an available C-H bond of a substrate is significant in that it determines which C-H bond will be modified.

Fenitrothion is lipophilic, and when it is inserted in the endoplasmic reticulum, it presents a variety of sites to PSMO enzymes. The easiest C-H bond site is that of the CH_3 (methyl) group at the 3-C position on the ring moiety. It presents three possible hydroxylation sites which are equivalent. Each of these possible hydroxylation sites have equal likelihood of being involved in cytochrome P_{450} monooxygenase degradation reactions and OHMEF is the product formed.

Due to the availability of free paired electrons, in O,P and S groups, electrophilic centres, and resonating bonds, alternative sites are available for cytochrome P_{450} activity. In fenitrothion, the likelihood of these unintended reactions occurring depends on the electronegativity of the moieties surrounding a given pair of bonded or free electrons. P-O-aryl, P-O alkyl, and P=S bonds are possible alternative substrate sites for cytochrome P_{450} monooxygenases. Hydroxylation at these sites would produce the fenitrothion metabolites DSM, NC, FO, DMPTA, DMPA, MPA, PTA and

PA.

The polar metabolite, OHMEF, would be extruded from the endoplasmic reticulum and increase the cytoplasmic pool of this intermediate metabolite available for further metabolism by cytoplasmic enzymes such as transferase, base catalyzed intracellular hydrolysis or conjugation to other moieties. Thus, OHMEF would be oxidized to FF, CFT and CFO, (D)MP(T)A oxidized to PA, and the metabolites FO, DSM and SMF, may be further hydrolysed to NC. Fenitrothion may also be isomerized to SMF via DSM due to glutathione-s-transferase or transmethylation by excess FEN. Elimination of these secondary metabolites would add to the levels of the extracellular metabolites detected under biotic conditions.

Cytochrome P_{450} monooxygenase inhibitor (piperonyl butoxide) and inducer (phenobarbital), were used in this study to demonstrate the involvement of PSMO in the algal degradation of fenitrothion, producing OHMEF with a benzyl alcohol moiety. It was hypothesized that, the levels OHMEF would decrease in the presence of piperonyl butoxide and increase in the presence of phenobarbital relative fenitrothion treated control cultures. This would occur if OHMEF was a key metabolite in algal degradation of fenitrothion by cytochrome P_{450} monooxygenase. This is based on the assumption that the benzyl alcohol moiety is not produced in significant amounts by other cellular mechanisms.

It was hypothesized that NC and DSM would also be affected by adding these inducer and inhibitor compounds. The results showed, however, that alternative degradation mechanisms exist in algae

that degrade FEN to NC and DSM. However, significant variation of levels of any of the metabolites FF, CFT, and CFO in the presence of phenobarbital or piperonyl butoxide, would provide additional evidence of algal cytochrome P_{450} monooxygenase being involved in the side chain oxidation of fenitrothion.

This is because the production of OHMEF, FF, and CFT depends on the hydroxylation of each of the C-H bonds on the ring methyl substituent at the C-3 position. This produces COH--->CHO--->COOH. It is feasible that cytochrome P_{450} monooxygenase could be involved in such a reaction, but it is equally likely that OHMEF is extruded into the cytoplasm. If OHMEF is does not rapidly undergo desulfuration to the OHMEF oxon metabolite or rapidly hydrolysed to NC, then cytoplasmic oxidation reactions may be involved in FF and CFT production.

The results of the studies with phenobarbital and piperonyl butoxide suggested that DSM and OHMEF are likely the only metabolites of fenitrothion that depend on degradation by cytochrome P_{450} monooxygenase in C. reinhardtii. The NC produced in this study did not depend of the activity of PSMO, and although it was hypothesized to be a feasible metabolite of PSMO reactions, it was likely produced by other intracellular metabolism.

The levels of the toxic fenitrothion metabolites SMF and FO produced by C. reinhardtii do not persist nor are they significantly affected by changes in cytochrome P_{450} monooxygenase activity, due to their rapid conversion to NC, which also did not depend on PSMO activity. FO and DSM depuration from C. reinhardtii

metabolism did not significantly affect their extracellular levels and the algal culture in this laboratory model but rather accelerated the degradation and detoxification of fenitrothion at the level of 5.0 μg/mL. The results of the PSMO studies give support to the hypothesis that cytochrome P_{450} monooxygenase are involved in the metabolism of fenitrothion. This phenomenon may be involved in determining the chemical fate of aquatic residues of other structurally related compounds with lipophilic moieties and suitable C-H bond sites.

4.6 Future Research Considerations

Some problems encountered in this study could be addressed in additional experiments with improvements to the experimental design. The three major considerations for improvement include the use of additional controls, in vitro studies for fenitrothion degradation by reconstituted cytochrome P_{450} monooxygenase enzyme complex isolated from algae, and better reproducibility of results.

Additional controls are necessary to better distinguish the differences in metabolite composition and amounts between (a) the abiotic fenitrothion hydrolysis versus oxidation, (b) between photolysis and hydrolysis, and (c) between oxidation and photooxidation. The light abiotic controls used in this study for fenitrothion degradation, quantified the fenitrothion metabolites produced from non enzymatic hydrolysis, photolysis, oxidation and photooxidation combined. The dark abiotic controls used in this study for fenitrothion degradation, quantified the fenitrothion

metabolites produced from non-enzymatic hydrolysis and oxidation combined.

This created a previously unforseen problem because each metabolite observed in the light abiotic treatments could have been produced by one of at least two abiotic processes. For instance NC and DSM may be produced by hydrolysis or photolysis, and OHMEF, FF, CFT, FO, and CFO may have been produced abiotically by oxidation or photolysis (light enhanced hydrolysis). However the relative contributions of these abiotic degradation mechanisms could not be determined because an underlying control was missing.

Additional abiotic degradation controls using N_2 during degradation of fenitrothion in the dark and in the light should have monitored the metabolites produced in growth medium in an anaerobic atmosphere. These controls would have determined which of NC and DSM were produced by hydrolysis, and determine if anaerobic conditions affected the production of any of the metabolites FO, OHMEF, FF, CFT, CFO. It is known that abiotic oxidation of P=S to P=O depends on aerobic conditions, but it would not be produced in anaerobic conditions, whereas NC would be. If an anaerobic atmosphere was used for light abiotic degradation of fenitrothion photooxidation and oxidation metabolites would have been suppressed and therefore distinguished from the levels of metabolites produced from hydrolysis and oxidation within the light treatments.

In this way the composition and amounts of fenitrothion metabolites produced in both the light and dark by non-enzymatic abiotic reactions could have been measured (Table 16). These

Table 16 Suggested Improvements to Experimental Controls

Treatment	Control Condition for
(A) Fenitrothion in abiotic growth medium, dark illumination, nitrogen atmosphere.	Identification of hydrolysis metabolites in the dark anaerobic treatment.
(B) Fenitrothion in abiotic growth medium, dark illumination, oxygen atmosphere.	Identification of hydrolysis and oxidation metabolites in the dark aerobic treatment.
(C) Fenitrothion in abiotic growth medium, Vita Lite^{R} illumination, nitrogen atmosphere.	Identification of photolysis and hydrolysis metabolites in the light anaerobic treatment.
(D) Fenitrothion in abiotic growth medium, Vita Lite^{R} illumination, oxygen atmosphere.	Identification of photolysis, hydrolysis, oxidation and photo-oxidation metabolites produced in the light aerobic treatment.

controls would also enable better separation of extracellular fenitrothion residues produced by abiotic processes from those produced by in algal cultures and released from the intracellular fractions.

There is a very complex set of interactions occurring intracellularly between different reaction pathways. They are difficult to sort out chemically in the context of determining what enzyme reactions are responsible. Cytoplasmic reactions of hydrolysis, conjugation, and oxidation producing NC, DSM, and FO, FF and CFT, respectively, were not separated from metabolism by cytochrome P_{450} monooxygenase. This is because the metabolism studies were done in vivo rather than in vitro. This is why a reconstituted microsomal monooxygenase system from C. reinhardtii would be very useful and would be an essential step for subsequent follow-up studies. Likewise glutathione alkyl transferase enzyme systems and other cytoplasmic enzymes would have to be reconstituted as in vitro systems in order to effectively evaluate their relative contribution to cellular degradation of FEN.

This study did not attempt to isolate and purify algal cytochrome P_{450} monooxygenase in C. reinhardtii. In order to isolate this monooxygenase, which involves cell wall lysis, protoplast isolation, and cellular fractionation to obtain the sufficiently large quantities of microsomal fractions of this algae must be carried out (Klein et al., 1983, Schlosser et al.,1976). Microsomes must be isolated without disrupting the large central vacuole to avoid the contamination of the cellular fraction with

photosynthetic pigments. These pigments interfere with the assay of cytochrome P_{450} protein content.

Purification of the microsomal fraction and column chromatography must be done to obtain the fraction containing the most PSMO activity. Isolation of the cytochrome P_{450} monooxygenase protein has never been attempted in photosynthetic plant tissue, and although the column separation methodology has been developed for mammalian microsomes development of this methodology for algae would have been difficult.

Since algal cytochrome P_{450} monooxygenase was not purified and characterized for its essential functional components, an in vitro study of fenitrothion metabolism by this enzyme was not possible. This enzyme complex, if isolated and purified from the alga, could be reconstituted with its functionally essential components and characterized for substrate specificity toward other xenobiotics. The response in activity and levels of this protein to known cytochrome P_{450} inhibitor and inducer compounds could also have been studied. The toxic effect on algae would not have to be considered in in vitro studies and higher concentrations of inhibitor compounds could be used to monitor the kinetics of algal cytochrome P_{450} monooxygenase activity.

Problems occurred in the reproducibility of results due to the attempt to use isogenic strains of C. reinhardtii cultures. The method used by Surzycki, (1971), produces mitotically synchronous algal cultures from an isogenic colony of algal cells. Each study of biotic degradation of fenitrothion required that the algae be

obtained from an isogenic strain, but different experiments did not use algae from one strain isolation procedure. Although the cultures are mitotically synchronous, there could be biochemical variation between cultures grown from separate clone colonies. Therefore the first study of algal degradation of fenitrothion and the study of cytochrome P_{450} monooxygenases were not true replicate cultures of algae. As a result, the rates of biotic degradation of fenitrothion, and the amounts of the metabolites between these two studies were different.

Statistical analysis was used to compare the biotic:abiotic ratios of metabolite residues in different treatments to determine the variation between treatment groups. Statistical significance, however, may not represent biological significance of results. For example, in comparing the algal enhancement of metabolite residues in growth medium with abiotic medium of phenobarbital treated cultures, biotic enhancement of NC occurred (biotic:abiotic ratio = 150). This represented a difference in 20 μg/mL of NC residue, whereas the biotic enhancement ratio for DSM (148) in medium fractions represented a residue difference of only 5.0 μg/mL. This occurred because the limits of detection for each metabolite differed. As a result it could be concluded from biotic enhancement ratios alone that phenobarbital induced DSM and NC equally (148 and 150), however, absolute amounts show that NC was more significantly enhanced. Therefore the biological significance of the results must also be evaluated as well as the statistical significance.

The studies here were limited to an in vivo system, based on

the assumptions that (a) cytochrome P_{450} was present in Chlamydomonas, (b) that its activity was activated and inhibited respectively by phenobarbital and piperonyl butoxide, and (c) that this activity was involved in fenitrothion degradation by C. reinhardtii as in other plant, invertebrates and vertebrate isozymes.

A limited interpretation of algal degradation of fenitrothion resulted because the monooxygenase activity was not isolated from other endogenous cellular metabolism. DSM can be produced in cellular fractions by glutathione-alkyl transferase and may not be attributed solely to the activity of cytochrome P_{450} monooxygenase activity.

It was hypothesized that the production of intracellular residues of the metabolites FF, CFT, and CFO depended on the availability of OHMEF in the cellular fractions and therefore indirectly on the activity of cytochrome P_{450} monooxygenase. In an in vitro study, this process could be monitored in the absence of competing cytoplasmic oxidation reactions. In the present study it must be concluded that both enzyme groups could partially contribute to the production of these oxidation metabolites.

CHAPTER 6
SUMMARY AND CONCLUSIONS

To date there is no direct evidence through isolation and assay of cytochrome P_{450} protein, that C. reinhardtii or any other green algae contain cytochrome P_{450} monooxygenase activity. Nonetheless fungi, bacteria, plants, and aquatic and terrestrial vertebrates and invertebrates are known to contain these monooxygenase, which have the ability to degrade fenitrothion as well as other organophosphates. Many plants including rice, white pine, white spruce, and yellow birch seeds, cauliflower, duckweed, hornwort, rush, Chlorella and Chlamydomonas have been demonstrated in laboratory and field studies, to contain metabolite residues of fenitrothion.

In this study, fenitrothion was determined to be non-toxic to C. reinhardtii cultures at a 5.0 µg/mL level with respect to the measured parameters of population dynamics and physiological processes. Cellular accumulation of fenitrothion was rapid due to the lipophilic nature of fenitrothion (log K_{ow}= 3), approaching a maximum after 30 h in the light and 50 h in the dark.

The half life of fenitrothion in the presence of C. reinhardtii cultures was enhanced in the light and dark conditions relative to their abiotic controls yielding values of 53.1 h and 143 h, respectively. The use of dark controls of algal cultures incubated with fenitrothion has demonstrated that although ultra violet wavelengths of light optimized the biotic degradation of fenitrothion, algal accumulation and degradation occurred

independent of light energy. NC, DSM, FO, SMF, OHMEF, FF, CFT and CFO were produced in the intracellular fractions of the algal cultures.

The environmental relevance of intracellular metabolism by algae and other aquatic plants, in determining the persistence of aquatic fenitrothion residues was not emphasized in any of the previous studies by Caunter and Weinberger (1988), Weinberger, Greenhalgh et al.,(1982), Moody, Greenhalgh et al.,(1978), and Greenhalgh et al., (1980). Their research emphasized that photolysis, hydrolysis, microbial reductive degradation, photosensitization by algal pigments and sediment adsorption, volatilization, and plant accumulation of the resulting metabolites, were the most significant processes in fenitrothion degradation in aquatic ecosystems.

This study, however, simultaneously monitored the production of metabolites in the extracellular medium and in the intracellular fractions, was designed to eliminate microbial degradation, and minimized hydrolytic degradation by using pH and temperature controlled abiotic controls and cultures. Under these conditions this study demonstrated that an endogenous metabolic factor in C. reinhardtii is a fenitrothion degradation process of environmental significance.

Elimination of metabolites from intracellular fractions was demonstrated to be a light mediated process and it was suggested that the light treatment changes the algal lipid composition and cell membrane permeability to fenitrothion as was also proposed in

Chlorella vulgaris by Nichols,(1965).

CFT, CFO, OHMEF, NC, and DSM were shown to contribute significantly to the levels of extracellular metabolites of fenitrothion in the algal cultures. It was also suggested that the existing levels of fenitrothion metabolite residues in the extracellular medium which were not enhanced by biotic degradation and elimination, were produced by abiotic hydrolysis and photolysis (NC, DSM, and phosphates), isomerization (SMF), and non enzymatic oxidation and photooxidation (OHMEF, FF, CFT, and CFO).

This study therefore demonstrates that endogenous cellular metabolism of a non target aquatic algae has the potential to degrade fenitrothion. The study also provides an original contribution in that it demonstrated that cytochrome P_{450} monooxygenase (PSMO) are involved in algal degradation of fenitrothion in a predictable manner according to known cytochrome PSMO function. This predictable function can be extended to any organophosphate xenobiotic and to many other groups of aquatic xenobiotic residues.

The production of the metabolite OHMEF was associated with cytochrome P_{450} monooxygenase activity as was hypothesized. Production of OHMEF was inhibited by 0.5 μg/mL piperonyl butoxide treatment and was induced by 23.2 μg/mL phenobarbital treatment. These responses to monooxygenase inhibitor and inducer compounds emulated the response observed in other plants and animals (Dogheim et al., 1981, Fonne-Pfister, 1988). The production of DSM was also related to cytochrome P_{450} monooxygenase activity, but was also due

to glutathione-alkyl-transferase activity. It was further suggested that the production of FF, CFT, CFO and NC depended on the pool of intracellular OHMEF residues available for subsequent cytosolic oxidation, hydrolysis, and conjugation reactions.

In this way the levels of these secondary metabolites were indirectly induced and inhibited by phenobarbital and piperonyl butoxide treatments respectively. These polar metabolic residues were ultimately hydrolysed to NC either intracellularly or in the growth medium after elimination. SMF and FO residues produced by algal metabolism did not persist significantly in extracellular fractions, suggesting that these toxic metabolites in natural ecosystems would not create a serious hazard to non target organisms. It was also suggested that light treatment enhanced monooxygenase function due to possible dependence on NADPH reducing equivalents produced in the light dependant reactions of photosynthesis.

Future research considerations suggested an attempt to isolate and conduct an in vitro assay of the levels and activity of algal cytochrome P_{450} monooxygenase, to characterize the essential components of the protein complex and to elucidate the effect that light has on monooxygenase activity. This would provide the required evidence to support the results obtained in these studies, which demonstrated the involvement of cytochrome P_{450} monooxygenase in fenitrothion degradation.

Under certain environmental conditions, other chemical and physical factors may in fact be the predominating process in

degradation of fenitrothion in aquatic ecosystems. However this study recognizes the significance of endogenous metabolism by non target algal species as a contributing process in xenobiotic degradation. Cytochrome P_{450} monooxygenase activity should be considered as an environmentally relevant factor in determining the true chemical fate and environmental hazard of xenobiotic chemicals.

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Appendix A Gorman and Levign Growth Medium

The ingredients of the growth medium per litre volume are as follows:

Tris base	2.42 g	
glacial acetic acid	1.0 mL	
deionized water	975 mL	
TAP salts	25.0 mL	32.0 g NH_4Cl 8.0 g $MgSO_4.7H_2O$ 4.0 g $CaCl_2.2H_2O$ in deionized water to 2 litres
concentrated phosphate	0.375 mL	576 g anhydrous K_2HPO_4 288 g KH_2PO_4 in deionized water to 2 litres
Trace elements	1.0 mL	

Trace metals were prepared as follows:

	g salt	mL H_2O
EDTA, disodium salt	50	250
$ZnSO_4.7H_2O$	22	100
H_3BO_3	11.4	200
$MnCl_2.4H_2O$	5.06	50
$FeSO_4.7H_2O$	4.99	50
$CoCl_2.6H_2O$	1.61	50
$CuSO_4.5H_2O$	1.57	50
$(NH_4)_6Mo_7O_{24}.4H_2O$	1.10	50

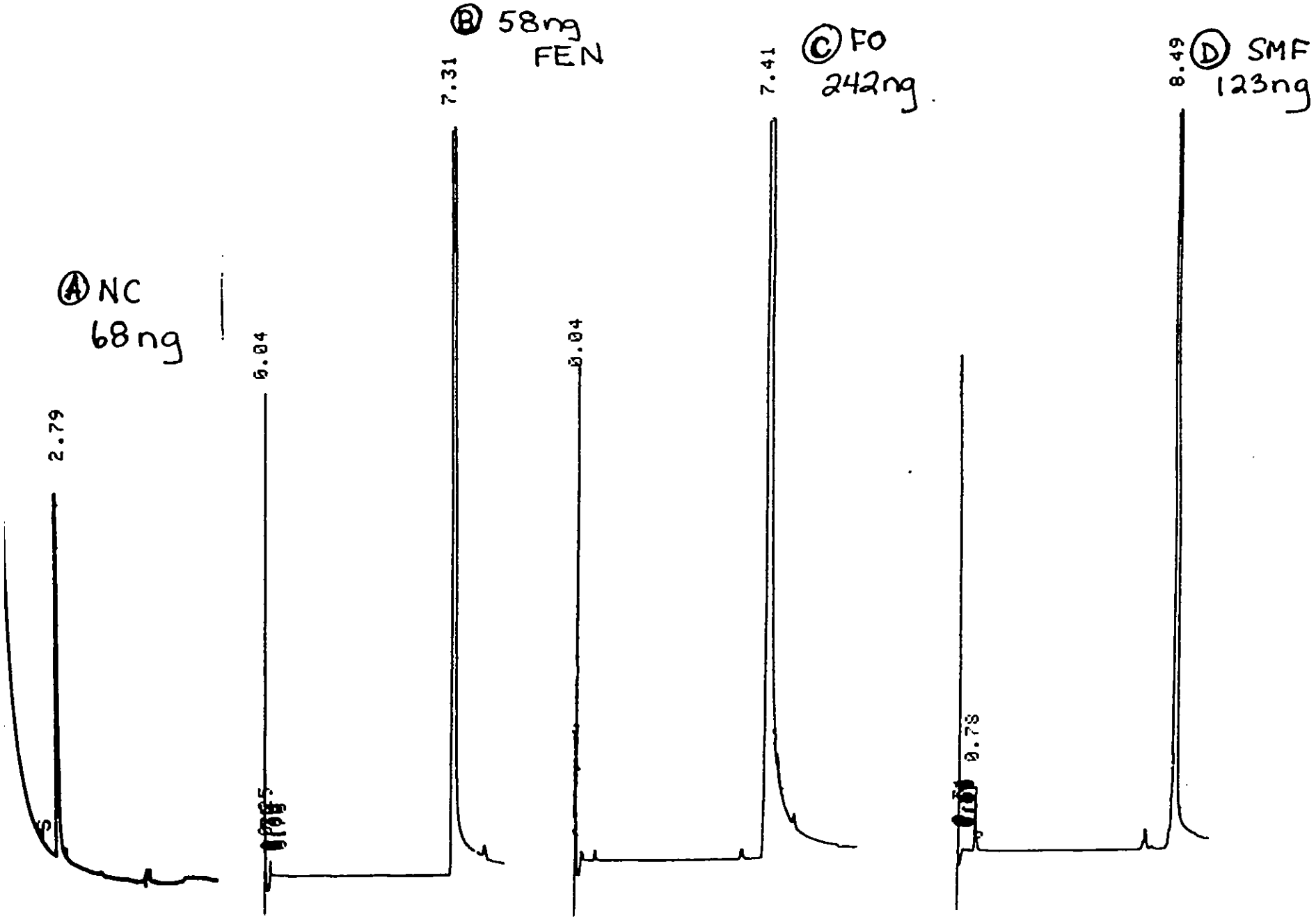
Each of the metal salts were dissolved in water as indicated. EDTA was dissolved in boiling water. All solutions were mixed except the EDTA solution, and brought to a boil. EDTA was then added and the solution cooled to 70°C. At this temperature the pH was buffered with 80-90 mL hot KOH (20%) to pH 6.7 and brought up to 1 L. This solution was left standing for 1-2 weeks in a flask stoppered with cotton. The solution was shaken daily. When the solution turned purple with a rust-brown precipitate, the solution was repeatedly filtered with Whatman #1 filter paper until clear.

Appendix B

Sample Chromatograms of Fenitrothion and its Metabolites

	Metabolite	Retension time
A	NC	2.79
B	FEN	7.31
C	FO	7.41
D	SMF	8.49
E	DSM	8.55
F	FF	8.65
G	OHMEF	8.92
H	CFT	9.26
I	CFO	9.39

The sample chromatograms which follow, represent fenitrothion metabolites derivatized with diazomethane which were analysed using the temperature program described in the methods.


Ⓐ NC
68ng
2.79
Ⓑ 58ng
FEN
6.04
7.31
Ⓒ FO
242ng
6.04
7.41
Ⓓ SMF
123ng
6.78
8.49

750 ng DSM
8.55
8.04
(F) 110 ng FF
0.03
(G) OHMEF 486.5 ng
8.92
0.04
0.79
7.29
395 ng CFT
(H)
3.04
249 ng CFO

Appendix C Details of Statistical Analysis

The data of Table 10 was analysed by the following statistical procedures.

Test for Normality

Firstly, the data was tested to see if the n sample data points were from a normal distribution. This test was called "testnormal" in the RS1 statistical package used. This test used the Wilk-Shapiro test of normality, and it calculated a W value at the significance level of 0.05. The data was found to be normal in each of the treatments: abiotic light, abiotic dark, biotic light and biotic dark, since p was greater than 0.1 for each value of W. The null hypothesis that the data were from a normal distribution was not rejected.

Raw data

n	dark abiotic	light abiotic	dark biotic	light biotic
1	0.00053	0.00478	0.00495	0.01231
2	0.00054	0.00471	0.00498	0.01260
3	0.00054	0.00473	0.00481	0.01310

Test for equal Variances

Next the data was tested to see if the variances of the unpaired normal samples were equal. This RS1 procedure is called "testvariances", which calculates the F statistic, and degrees of freedom for each sample of data that is from the normally distributed data. At the p=0.05 significance level, if F is greater than 1.0, then the null hypothesis that the variances are equal can be rejected. The results of this test with this raw data was that the F was greater than 1.0 at the p=0.05 level of significance for all treatments. Therefore the null hypothesis was rejected and the data have normally distributed means with unequal variances.

Multiple range test

Finally, a multiple range test was done to simultaneously compare the independent samples, and to determine where the significant differences existed between the treatments abiotic light, abiotic dark, biotic light and biotic dark. The Student Newman-Keuls multiple range test was used. The null hypothesis (H0) was made that the paired groups were equal, and the alternative hypothesis was that the paired groups were not equal. The test calculated the standard error and the differences between the different group means. The test calculated a critical value of alpha at 5.0% (=0.05), and if the q statistic is greater than this critical value for q, then its significance is less than 5% and the null hypothesis of equal groups is rejected.

The results from the table bellow show that all the groups are significantly different from each other except for the light abiotic and the dark biotic treatments.

lb=light biotic
da=dark abiotic
la=light abiotic
db=dark biotic

Newman-Keuls Multiple Range Test

	Alternate Hypothesis	Diff in Means	Standard Error	Observed Statistic	Range
1	lb<>da	0.012133	0.000119	102.0	4
2	lb<>la	0.007930	0.000119	66.70	3
3	lb<>db	0.007770	0.000119	65.35	2
4	db<>da	0.004363	0.000119	36.69	3
5	db<>la	0.000160	0.000119	1.345	2
6	la<>da	0.004203	0.000119	35.35	2

	Critical value	Signif. Level	Inference
1	4.529	p<=0.05	reject H0
2	4.041	p<=0.05	reject H0
3	3.261	p<=0.05	reject H0
4	4.041	p<=0.05	reject H0
5	3.261	p> 0.05	accept H0
6	3.261	p<=0.05	reject H0