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THE EFFECT OF NAPHTHALENE ON RESPIRATION AND APPARENT PHOTOSYNTHESIS IN *CHLORELLA PYRENOIDOSA*: A POSSIBLE MOLECULAR BASIS FOR INHIBITION OF RESPIRATION BY NAPHTHALENE

By

VALERIE GRACE STRUBLE

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Oklahoma State University

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THE EFFECT OF NAPHTHALENE ON RESPIRATION AND APPARENT PHOTOSYNTHESIS IN CHLORELLA PYRENOIDOSA: A POSSIBLE MOLECULAR BASIS FOR INHIBITION OF RESPIRATION BY NAPHTHALENE

Thesis Approved:

Thesis Adviser

Richard C Essenberg

Dean of the Graduate College

PREFACE

Each day, more and more substances are developed. They and continuously larger amounts of substances long considered toxic are released into the environment where they come in contact with life in its various forms, including human. In many cases, the effects of these substances on living organisms are unknown, and in most cases their action at the molecular level is undetermined.

Naphthalene is now nearly ubiquitous in the environment, although for many organisms its effects are unknown. This study determined the effect of naphthalene, both the acute and chronic, on oxygen consumption and evolution during two life phases of the green alga Chlorella pyrenoidosa. These results were compared to those of other organisms and studies, and finally an action of naphthalene was traced to the molecular level. Here, using spectrophtometric methods, naphthalene was demonstrated to interact with a chemical universally present in higher life forms and universally important in respiration. Part of this work has been published in Volume 31 of the Bulletin of Environmental Contaminants and Toxicology (Struble and Harmon, 1983). This work was supported by a presidential Fellowship from Water Resources, Oklahoma State

University, a Graduate Fellowship from the National Science Foundation, and an assistantship with Dr. H.J. Harmon of the Department of Zoology and Dr. M.R. Sanborn of the Department of Microbiology. Advice, supplies, and equipment were provided by Dr. Harmon, Dr. Sanborn, and Dr. D. Francko of the Department of Botany; special thanks go to these people. Acknowledgement, also, to Dr. Harmon, (my major professor), Dr. Sanborn and Dr. R. Essenberg, my committee members.

For help and support above and beyond the call of duty, special thanks go to my husband Stan.

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NOMENCLATURE

ADP	adenosine diphosphate
ATP	adenosine triphosphate
BHI	brain heart infusion
DCMU	dichlorophenyldimethylurea
EC_{50}	that concentration of toxicant at which 50% of organisms, cells, etc. are affected
HEP-2 cells	human larynx carcinoma tissue culture cell line
NADH	reduced nicotinamide adenine dinucleotide
ppm	parts per million
Q	ubiquinone
Q_{10}	ubiquinone-50
QH_2	ubiquinol
SD	Sabouraud dextrose
λ_{max}	the wavelength of maximum absorbance

CHAPTER I

INTRODUCTION

The Scope Of The Study

The Problem

Existence in the modern environment entails exposure of organisms and ecosystems to new substances and to increased levels of substances long considered toxic. One study estimated that 200,000 new known compounds are produced each year (Goulding, 1975) most with completely unknown effects on organisms or the environment. One broad class of these chemicals is the PAH or polyaromatic hydrocarbons (U.S.E.P.A., 1980b) of which naphthalene is a member. Although naphthalene has been known for quite a long time and studied more than many of the other PAH, its effects on organisms are largely undetermined. In particular, its actions at the molecular level are not understood.

Objectives

In this study, the effects of naphthalene on the eucaryotic green alga Chlorella pyrenoidosa were examined; in particular the activities of respiration (oxygen

consumption) and apparent photosynthesis (oxygen evolution) were determined polarographically. This alga was tested in both early stationary and log (exponential growth) phase. In addition, some determinations were performed with another eucaryotic green alga Chlamydomonas moewussi, and results were compared.

The work of Harmon and Sanborn (1982) indicated that naphthalene inhibits mitochondrial respiration at the level of ubiquinone. Following this lead, a spectrophotometric investigation of the possible interaction of naphthalene with ubiquinone was made. Results were compared to the results with the algae and those of other researchers in an attempt to increase the understanding of the toxicity and mechanism of toxicity of this compound.

The System of Investigation

Eucaryotic green algae may be an ideal test system for studying the effects of such chemicals as naphthalene at the cellular level. They are relatively easy to grow and maintain, and in themselves are environmentally important, as they are not only primary producers in the food chain but are participants in the breakdown, conversion, and transport of chemicals added to the environment. They contain both mitochondria (responsible for respiration) and chloroplasts (responsible for photosynthesis) and many of the same or similar systems as higher life forms. They may be able to be used to predict

effects on higher organisms.

Respiration and electron transport have been used in numerous studies as effective indicators of toxic effects. Systems studied include tissue culture cells, mitochondria, Daphnia sp., Tetrahymena sp., bacteria, and various algae. Previous reports have implicated naphthalene in the disruption of electron transport, respiration, and photosynthesis (these studies are discussed in the Review of Literature).

Respiration and photosynthesis (both of which involve electron transport) are intimately involved in energy generation and consumption within the cell. The organelle responsible for respiration (the consumption of oxygen) is the mitochondrion. This is a double membraned structure in which the inner membrane is highly convoluted. This membrane contains or has on its surface the components essential for the oxidation of substrate with the concomitant reduction of oxygen to water and generation of ATP from ADP and inorganic phosphate. Electrons are removed from a suitable substrate, such as succinate or NADH, by means of a dehydrogenase and passed via flavoproteins and iron sulphur proteins into the Q cycle. Electrons are then moved on down the transport chain from the cytochromes *b* and the Rieske iron sulphur center, on to cytochromes c_1 and *c*, and finally on to cytochromes *a* and a_3 where oxygen is reduced to water. Linked and simultaneous with this transport of electrons, protons are

extruded across the inner membrane from the inside (matrix) to the outside (intermembrane space) generating both a proton and electrical differential across the membrane. This differential is used to phosphorylate ADP to ADP.

Chloroplasts, responsible for photosynthesis (which involves the evolution of oxygen from water), are also double membraned organelles. The inner membrane surrounds a stroma which contains stacked membranous thylakoids. The thylakoids contain the molecules of the electron transport chain and other components of the energy transducing machinery that generate both ATP and NADPH from light and oxidize water to O_2. This involves a complicated zig-zag or "Z-scheme" which contains two structurally distinct photosystems containing chlorophyll, responsive to two different wavelengths of light. Also involved are plasto quinone, cytochromes, plastocyanin, ferrodoxin and other unidentified components. In this scheme, a photon of less than 680 nm strikes photosystem II, activating an electron which is transferred to an unknown acceptor, and then to plastoquinone, cytochrome $\underline{b}_{559}$ finally to cytochrome $\underline{f}$ (also called cytochrome $\underline{c}_{552}$), and plastocyanin, a copper protein. During this process, water is oxidized to O_2 with its freed electrons passing on to photosystem II, and a proton gradient across the thylakoid membrane is generated (the inside becomes acidic) as electrons are moved down the transport chain from plastoquinone. This gradient is

used, as in mitochondria, to produce ATP from ADP and inorganic phosphate. Electron transfer from the reduced plastocyanin to photosystem I now occurs. This center has an absorption maxima of 700 nm, and when excited it transfers an electron to an unknown initial acceptor and on to bound ferrodoxin (an iron sulphur protein). This component passes the electron on to soluble ferrodoxin, which reduces $NADP^+$ to form NADPH. When the NADPH/NADP ratio is high, a phenomenon called cyclic electron flow can occur. In this case, oxidized photosysytem I reduces itself by electron flow from bound ferrodoxin; a proton gradient is generated that is coupled to ATP synthesis but this pathway neither reduces NADP nor evolves oxygen from water. Both organelles involve electron transport and proton gradients to produce ATP. However, mitochondria consume both O_2 and reducing power (NADH) while chloroplasts generate them during non-cyclic electron flow. Any disruption of these processes could have far reaching consequences on the health and viability of the cell or organism.

Once a substance has been found to interfere with an activity such as mitochondrial respiration, the general area of the interaction with the electron transport chain can be determined using artificial and specific substrates, inhibitors of specific sections of the transport chain, and etc. as in the work of Harmon and Sanborn (1982). At this point, spectrophotometric methods can be

used to detect possible interactions between the chemical being tested and the individual components of the section of the transport chain being examined. This procedure can indicate interaction at the molecular level, and give an indication as to the mechanism of action of the chemical.

CHAPTER II

REVIEW OF LITERATURE

Naphthalene

The Environment

Naphthalene is a ubiquitous contaminant. It has been detected in industrial effluents, water extracts of crude and refined oils (Anderson, Neff, Cox, Tatem, and Hightower, 1974), sewage plant effluents and even drinking water (Pucknat, 1981). One study found traces of naphthalene in all the four samples of ground water collected from such widely varying locations as Texas, Colorado, Arizona, and Massachusetts (Hutchins, Tomson, and Ward, 1983). Naphthalene is the long familiar active ingredient of "moth balls" and is the single most abundant constituent of coal tar. It and its derivatives are considered among the most acutely toxic components of petroleum (Anderson, et al., 1974). Naphthalene is an intermediate in the production of dye, solvents, lubricants, and motor fuels. It has recently received attention in regard to its toxicity and other effects on organisms (U.S.E.P.A., 1980).

Naphthalene has been used as a chemical model in such studies as partitioning of chemicals between waste water

treatment sludges and water (Southworth and Keller, 1984), the behavior of herbicides in soils (Wauchope, Savage and Koskinen, 1983), the fate of trace organics from rapid infiltration systems (Hutchins, Tomson, and Ward, 1983), and the behavior of organic chemicals at soil, water, and air interfaces (Spencer, Farmer, and Jury, 1982). All these studies found that naphthalene could be a significant contaminant of ground water when applied to the soil. Also determined was that the majority of the naphthalene would remain in the water phase as it percolated through the soil. Rodgers, Dickson, Saleh, and Staples (1983) found that naphthalene remained preferentially in the aqueous phase rather than depositing in sediment in an aquatic system study. These characteristics of naphthalene would put it in direct contact with many of the community of organisms of both the soil and aquatic systems. As resident microorganisms are largely responsible for the breakdown of applied materials (Burges and Raw, 1967; Rodgers, et al., 1983) including naphthalene (Lee and Anderson, 1977), an understanding of the effects of naphthalene on such residents is desirable.

Effects on Multicellular Organisms

Naphthalene is a polycyclic aromatic hydrocarbon with a water solubility of 32 ppm (mg/L). It has demonstrated an acute and chronic toxicity for "fresh water aquatic life" of 2.3 ppm and 16 ppm, respectively. The EC_{50} value for the

fathead minnow is 6.6 ppm and for Daphnia magna is 8.6 ppm (U.S.E.P.A., 1980). For Daphnia pulex a chronic 96 hour EC_{50} of 1 ppm was determined. This organism was also found to accumulate high internal concentrations of naphthalene when exposed to either food (algae) or water containing the chemical. Bioaccumulation after 24 hours was about 11,000 times from food and 700 times from water. The oyster, Crassostrea virginica was also found to accumulate naphthalene internally (Fortner and Sick, 1985); for every 15 hours of exposure, the internal concentration increased by nearly 50% of the surrounding water concentration. This organism would attain higher concentrations of internal naphthalene than that of its surrounding medium. These high accumulations indicate that an initially low external concentration of naphthalene could become quite high internally. The Daphnia exhibited a slow clearance of internalized naphthalene; 72 hours after removal from exposure the organisms still contained over 60% of their initial, internal concentrations of the chemical (Trucco, Engelhardt, and Stacey, 1983).

Other researchers have assesed the effects of naphthalene on various organisms. Crider, Wilhm, and Harmon (1982) demonstrated that in addition to being toxic to Daphnia magna, naphthalene inhibited oxygen consumption by the cladoceran. A 24 hour exposure of the organism to the chemical resulted in a decrease in hemoglobin concentration. Another group (Darville, Harmon, Sanborn,

and Wilhm, 1983) found that exposure to naphthalene caused a loss of ionic regulation in the freshwater dipteran Chironomus attenuatus. By using cytochrome c-loaded liposomes, they determined this effect was not due to a loss of membrane integrity. Rather, they found by exposure of plasma membranes of cultured HEP-2 cells to the chemical that the ouabain-sensitive Na^+,K^+-ATPase was inhibited. A later study showed that after an hour exposure to naphthalene, the oxygen consumption by both Chironomus attenuatus and Tanytarsus dissimilis was decreased. Both species had an LC_{50} value of 13 mg/L (Darville and Wilhm, 1984).

Effects on Algae

The effects of naphthalene on the flagellated alga Chlamydomonas angulosa have been examined by Soto, Hellebust, Hutchinson and Sawa (1975), Soto, Hellebust, and Hutchinson (1975), Soto, Hellebust, and Hutchinson (1977), and Soto, Hutchinson, Hellebust, and Sheath (1979). They found, in an open system, that cells lost flagella, became immobile, and lost 97% of their photosynthetic ability (as determined by ^{14}C accumulation) within minutes of inoculation into growth medium containing saturating concentrations of naphthalene; 61% of the cells were killed during the first day of incubation and viable cells remained in lag phase for 3 days, as compared to one day for control cells. In a closed system, 85% of the

cells were killed after one day, and 97-98% after three to seven days. Cells regained mobility after opening the system for two days. Cells still alive after incubation in a closed, naphthalene-saturated solution for seven days displayed a lag phase of one day after being resuspended in normal medium (Soto, et al., 1975). Cells incubated in a 10-30% saturated medium for 30 minutes lost 50% of their photosynthetic ability while a two hour exposure resulted in decreases to less than 10% of control activity. Cells exposed to 50% or 80% saturated media for 30 minutes showed declines of 74% and 82% respectively; after a two hour exposure to these concentrations of naphthalene, photosynthetic activity almost ceased. Cells incubated in saturated ^{14}C-naphthalene medium for 24 hours contained 30 times as much (volume/volume) naphthalene as the medium and internal naphthalene concentration doubled in 1.5 days. A rapid release of naphthalene occurred when cells were transferred to fresh media and after cell division resumed (Soto, et al., 1975; Soto, Hellebust, and Hutchinson, 1975). These observations demonstrated that the deleterious effects of naphthalene on the cells increased with longer exposure or with higher concentrations. Also indicated was that the internal organelles and processes can be exposed to much higher amounts of the chemical than that dictated by its water solubility.

The internal concentration of proteins, carbohydrates and lipids of Chlamydomonas angulosa varied highly during

exponential growth phase of the cultures. Upon attainment of stationary phase the amount of all these internal components increased. When the log phase organism was transferred to a closed system with naphthalene at 50% saturation increase of cell numbers was not observed (Soto, Hellebust and Hutchinson, 1975) and during a seven day exposure cells displayed morphological abnormalities. Under these conditions, cellular protein decreased by nearly 66% while carbohydrates increased by 131% and lipids increased by 164%. Morphological changes displayed by the cells included (after two days exposure), disarray of the thylakoid membrane, increase in starch grains and other granulation, development of a transparent envelope around the cells and an increase in the size of vacoules. After four days, the entire cell stained with sudan black (stains lipids) with particularly dense deposits in the vacoules. No recovery of normal morphology was seen until after transfer of the organisms to fresh medium (Soto, et al., 1979). The cells became normal and motile within 24 hours only after resuspension in fresh medium; cells transferred to an open flask, but remaining in their "original" medium remained non-motile for six days. Evidently, the "original" medium still possessed inhibitory action for the cells. Perhaps it contained either a persistent metabolite or a product of naphthalene degradation.

Chlamydomonas angulosa cells have been demonstrated

to grow on acetate, as substrate, in the dark. Upon exposure to 100% saturating naphthalene, acetate uptake decreased 70%; after four hours, there was very little uptake and the cells did not recover upon opening of the flasks. Naphthalene at 50% saturation induced an immediate 50% stimulation of uptake which lasted for four hours. After two days in the closed system, uptake had decreased to 50% of the control rate. In this case, the cells recovered upon opening their flasks. These authors (Hellebust, Soto, and Hutchinson, 1982) also noted that exposure to high concentrations of naphthalene caused a long term visible rearrangement of the cell's thylakoid membranes.

Geyer, Politzki, and Freitag (1984) examined the relationship between the n-octanol/water partition coefficient and the bioaccumulation of naphthalene and other chemicals by Chlorella fusca. They found that the more lipophilic the chemical the faster it was accumulated (for example hexachlorobenzene reached equilibrium of internal concentration versus external concentration in two to four hours while p-chloroaniline took over 24 hours), and that the amount accumulated corresponded well to the log of the partition coefficient. In other words, the more lipophilic and less hydrophilic a chemical, the faster it accumulated and the higher the internal concentration became.

Eighteen algal cultures, including two Chlorella sp. and Chlamydomonas angulosa, were demonstrated to oxidize

naphthalene under photoautropic conditions (Cerniglia, Gibson, and Van Baalen, 1980). Products of each organism included at least six metabolites, one which the authors identified as 1-naphthol, itself a toxic compound.

Cellular and Subcellular Effects

Naphthalene has been shown to affect isolated animal cells. Using ^{14}C-naphthalene, Schwarz, Mezger, and Hesse, (1980) found that isolated rat hepatocytes metabolized naphthalene to water soluble compounds and that the radioactivity became irreversibly bound to the cells. A three fold increase in naphthalene concentration (from 400 uM to 1200 uM) produced only a two fold increase in metabolites while the amount of radioactive label bound increased up to ten fold. Evidently, more of the labelled carbon was retained by the cell, perhaps in the form of naphthalene itself or of a tenacious (lipid soluble?) metabolite.

Petterson, Curvall, and Enzell (1980) found a 48% inhibition of respiration in isolated hamster brown fat cells as a result of exposure to 1 mM naphthalene. Harmon and Sanborn (1982) demonstrated that naphthalene inhibited respiration in certain types of tissue culture cells (green monkey kidney, human larynx carcinoma, and primary turkey fibroblasts) and this paralleled its toxicity to the cells. The cells were shown to round up and release from the surface of the culture flask when exposed to

concentrations of 7.5 ppm and at higher concentrations lysis occurred. The EC_{50} for naphthalene induced inhibition of respiration in all three cell types was between 9 and 12 ppm. Their data showed striking correlation with the effect of naphthalene on isolated cardiac mitochondria; 78 uM (10 ppm) inhibited mitochondrial respiration by 50% and the shape of the curve of percent inhibition of oxygen consumption vs. naphthalene concentration was very similar for both the isolated mitochondria and the three types of tissue culture cells. Evidently, concentrations toxic to the mitochondria were also toxic for the cells. Also indicated was inhibition specifically at the level of ubiquinone. Ubiquinone reduction by NADH was affected by naphthalene as well as QH_2 oxidation by O_2, while there was no affect on ferricyanide or juglone reduction by NADH.

The Algae Of The Study

Chlorella pyrenoidosa

Chlorella sp. are widely known and have been used in a variety of biochemical and physiological studies. They are non-motile, unicellular, haploid members of the Chlorococcales order of green algae. Many of their structures and processes are similar or identical to those of higher plants. Each cell contains a large chloroplast and, it seems, one highly branched mitochondrion which

permeates the cell, lying close to and mostly enveloped by large chloroplast (Pickett-Heaps, 1975).

Chlorella is common in soils and fresh waters. In these environments, Chlorella is nearly ubiquitous (Burges and Raw, 1967). In one study of red laterite soil, Chlorella sp. was one of the most common algae present (Muralikrishna and Venkateswarilu, 1984). Chlorella pyrenoidosa is a typical Chlorella sp. and has not been studied in connection with naphthalene.

Fresh water and soil are locations where environmental naphthalene would be found. Green algae are important members of both these communities, not only as primary producers and hence essential in the food chain, but as participants in the breakdown, conversion, transport and fate of chemicals in these environments. Disruption of the algal species could lead to disruption of the entire community. This could cause a decrease in available nutrients (decreased fertility), predominance and explosive growth of one organism at the expense of others (bloom), or an increase in the persistence of toxic substances within the environment (Burges and Raw, 1967; Gray and Williams, 1971; Haltori, 1973).

Chlamydomonas moewusii

Chlamydomonas sp. are motile, flagellated members of the Volvocales order of green algae. As with Chlorella sp. many of their structures, components and processes are

similar or identical to those of higher plants (Pickett-Heaps, 1975). Chlamydomonas are among the most common pigmented flagellates in soil (Burges and Raw, 1967). Chlamydomonas moewusii has not before been studied in connection with naphthalene, although other Chlamydomonas species have. This species has been demonstrated to oxidize acetate, pyruvate, and succinate in the dark; otherwise it is an obligate photoautotroph (Bernstein, 1964).

CHAPTER III

MATERIALS AND METHODS

Chemical Procedures

Preparation of Naphthalene

Reagent grade naphthalene was dissolved in absolute ethanol daily. From this stock solution, dilutions were made with absolute ethanol so that after addition of a standard aliquot the culture to be tested would contain the desired concentration of naphthalene. For example, in acute type determinations, 6.0 ul of 6 mg/ml stock solution was added to 1.2 ml of culture medium to attain a final concentration of 30 ppm (30 mg/l or 234 uM). For 15 ppm final concentration stock solution used was diluted to 3 mg/ml, for 7.5 ppm stock was diluted to 1.5 mg/ml, and for 3.8 ppm stock was diluted to .75 mg/ml.

For chronic type determinations the stock solutions were sterilized by filtration and equal aliquots added aseptically to standard amounts of culture medium in a Bioquest Biological Hood. Cultures were then incubated in screw top test tubes under the standard conditions described elsewhere for the desired length of time before assaying. Controls for both acute and chronic trials were

an aliquot of absolute ethanol of the same size and treated in the same manner as the corresponding naphthalene/ethanol solutions.

Cellular Procedures

Algal Cultures

Chlorella pyrenoidosa and *Chlamydomonas moewusii* cultures were the generous gift of Dr. Francko of the Botany Department of Oklahoma State University. Cultures were tested for purity by streaking on Brain Heart Infusion agar (BHI), Sabouraud Dextrose agar (SD), and Moss Medium (Moss, 1972) agar plates modified by deletion of $Ca(NO_3)_2$, Na_2SiO_3, and NH_4Cl with addition of 7.215 g/l KNO_3 and 2.6 g/100 ml tricine buffer. Media were adjusted to pH 7.3-7.5 with 0.1 N NaOH before autoclaving. After the cultures were purified by the standard microbiological method of serial transfers on selective and differential media, the axenic cultures were inoculated into liquid Moss Medium for growth and maintenence.

Cultures used in each assay were first tested for contamination on BHI and SD agar plates. The algae were grown in a constant temperature room (25 C) on a 12-12 hour light-dark cycle. During the light phase, cultures were illuminated by 750 uEinstein $sec^{-1}M^{-2}$. The 12-12 cycle insured synchronous replication of the cultures (Bernstein, 1960; Soeder and Ried, 1962). Cell numbers were determined with an Improved Neubauer Hemocytometer,

and cell morphology examined via a Nikon inverted microscope using phase contrast optics.

Cell numbers versus days after innoculation into fresh medium were plotted to determine the growth phase of the cultures. Samples are presented in Figures 1 and 2. For log phase assays, cultures were used at day two after a 1:5 dilution during the period of exponential growth. For stationary phase, cultures were used at day six after dilution when growth had appreciably slowed and cell numbers were stable but before senescence or death phase had set in.

Polarographic Determinations

Cells

Consumption and evolution of oxygen by the algae were determined polarographically at 25 C in a water jacketed chamber using a Clark electrode. Rates of oxygen consumption or evolution were recorded on a Linear strip chart recorder. Respiration was determined in the dark; apparent photosynthetic activity was measured during illumination of the culture with 8,000 uEinsteins $sec^{-1}M^{-2}$ of light provided by a slide projector. A $CuSO_4$ solution was used (between the light source and jacketed chamber) as a heat sink.

For determination of acute effects, a 1.2 ml aliquot of cells in the desired phase was added to the chamber,

a 6.0 ul aliquot of naphthalene in ethanol per 1.2 ml of culture was added after which activities were measured as described above. During the assay period, the number of cells/ml of culture were determined by counting in the hemocytometer.

For chronic exposures, a 25 ul aliquot of sterilized naphthalene in ethanol was added aseptically to 5.0 ml of algal culture in the desired phase. Phase was determined by counting cells/ml as previously described. These treated cultures were then incubated under the conditions for growth described previously for the desired length of exposure to naphthalene (two hours, one day, etc). These procedures were considered an open system as the screw-cap test tubes containing the treated cultures were loosely capped, and did not prevent air exchange. At this point, a 1.2 ml aliquot of the treated culture was added to the chamber and assayed as described above, but without further addition of naphthalene or ethanol. As with acute determinations, the number of cells/ml were determined during the actual assay period. Controls for all determinations, acute and chronic, were an equal volume of absolute ethanol treated and tested in all circumstances exactly as was the corresponding naphthalene/ethanol solution.

All results were determined as both nmole O_2/cell min. consumed or evolved and as percentage inhibition of respiration or oxygen evolution versus the appropriate

ethanol control. The formulas used for these determinations are:

$$\frac{\text{inches (vertical measure)}}{\text{cm}} \times \frac{6.97 \text{ nmole } O_2}{1 \text{ inch}^2} \times \frac{1 \text{ cm}}{3 \text{ min.}} \times \frac{1}{\text{number of cells/ml}} \times \frac{1}{1.2 \text{ ml (size of chamber)}} = \frac{\text{n mole } O_2}{\text{cell.min.}} \quad (1)$$

for the activity of the cells, and :

$$100 \times \left(1 - \frac{\text{toxicated rate}}{\text{control rate}}\right) = \text{Percent of Inhibition} \quad (2)$$

for the effect of toxicant as compared to controls. Actual results of one trial are presented in Figure 3.

Figure 4 records a sampling of the nontoxicated oxygen consumption and evolution rates of a log phase culture of Chlamydomonas moewusii taken over a seven hour period. As the activities, especially apparent photosynthesis, were found to vary throughout the day (see Figure 4) controls were run frequently; often after every two toxicant treated runs.

Chloroplasts

Chloroplasts were isolated from fresh, deveined, spinach leaves by a modification of the method of Arnon, Allen, and Whatley (1956). 25 grams of leaves were ground in 100 mls of cold 0.4 M sucrose + 0.05 M NaCl for 15 seconds in a blender and strained through 8 layers of cheesecloth. The mixture was then centrifuged at 1200 g for 10 minutes. The pellet was resuspended in 3.0 ml of

the isolation buffer. Chlorophyll concentration was assayed by adding 0.05 ml chloroplasts to 80% acetone and multiplying absorbance at 663 nm by 0.00802 to determine chlorophyll a and at 645 nm by 0.0202 to determine chlorophyll b.

Chloroplasts were assayed in the 1.2 ml reaction chamber using a Clark type electrode as described above. One ml of 0.25 M MES-Tris buffer containing 30 mM $MgCl_2$ (pH 8), 20 ul of 15 mM sodium azide, 20 ul of 250 mM NH_4Cl, 50 ul H_2O (distilled), 200 ul chloroplast suspension, and 5 ul of either ethanol (control) or ethanol containing the naphthalene to be assayed was added to the chamber. At this point the light of 8,000 uEinsteins $sec^{-1}m^{-2}$ intensity was turned on and 10 ul 50 mg/ml $K_3Fe(CN)_6$ (an electron acceptor for photosystem II) added to initiate photosynthesis. After 5-10 minutes, the light was switched off, and the rate of respiration was determined. With the light on, 5 ul DCMU was added to test DCMU sensitive photosynthetic rates (photosystem II), and antimycin (5 ul of 1 mg/ml) added to test antimycin sensitive rates.

DCMU inhibits photosystem II electron transport just before plastiquinone. $Fe(CN)_6^{-3}$ can be reduced at two sites in photosystem II of spinach chloroplasts (Barr and Crane, 1981), and its reduction is DCMU sensitive. Hence, DCMU sensitive rates are specific for systems that contain photosystem II, and enable the elimination of all oxygen evolution activity due to bacteria and other contaminants.

Antimycin specifically inhibits mitochondrial electron transport between the b and c cytochromes. Hence, any antimycin sensitive oxygen consumption would indicate mitochondrial contamination of the preparation; which then can be eliminated from the calculations of activity.

Spectrophotmetric Determinations

Volatilization of Naphthalene

A Cary 14 spectrophotometer was used to determine the rate of volatilization of naphthalene in the open system (screw cap test tube) used for the chronic type exposure studies. The screw cap tubes were of the same size and type as those used in the assays of actual cultures. Naphthalene, treated as described previously, was added to sterile culture media with a final concentration of 30 ppm. Height of the prominent 275 nm absorbance peak was measured (versus absorbance at 320 nm) versus time.

Naphthalene and Ubiquinone

Duroquinone (tetramethylbenzoquinone) and Q_{10} (ubiquinone-50) were dissolved in absolute ethanol. Reagent grade naphthalene was dissolved in absolute ethanol daily and aliquots from this stock solution were diluted to the desired concentration with absolute ethanol. The ultraviolet region of the spectrum, from 220 nm to 380 nm was scanned using a Cary 14 spectrophotometer

UV difference spectra (naphthalene and quinone minus naphthalene) and absolute spectra of the quinones in ethanol were recorded.

To determine if scattering of light was occurring, various concentrations of naphthalene in ethanol were scanned from 220 nm to 380 nm. The height of prominent peaks and troughs in absorbance were measured to determine if an increase in concentration caused a proportional increase in absorbance at all the measured wave lengths. Since:

$$\text{absorbance} = \text{Path length} \times \text{Concentration} \times \text{extinction coefficient} \quad (3)$$

any micelle formation or scattering would increase the path length and hence absorbance.

Also:

$$\text{scattering} \propto (1/\lambda)^4 \quad (4)$$

which predicts that scattering would become much more apparent as wavelength was decreased. So, not only was the increase in absorbance due to increased concentration checked, but the overall, or "baseline" absorbance was observed with decreased wavelength.

Resolution of the spectrophotometer at the wavelengths used was determined according to:

$$\text{Resolution } (\text{Å}) = \text{slit width (mm)} \times \text{reciprocal dispersion } (\text{Å/mm}) + \text{curvature mismatch} + \text{Rayleigh diffraction} \quad (5)$$

as presented in the Varian Cary 14 manual (1953). Maximum

slit widths were measured and all other values determined from the graph reproduced from the Varian manual and presented in the Appendix C. The wavelength used for resolution determination was 280 nm or 0.28 microns.

CHAPTER IV

RESULTS AND DISCUSSION

Effect of Napthalene on Stationary Phase *Chlorella Pyrenoidosa*

Response of Oxygen Consumption and Evolution To Acute Exposure

A total of 23 different trials were performed in an open system to determine the acute effects of exposure to various concentrations of naphthalene in ethanol on early stationary phase (six day old) *Chlorella pyrenoidosa* cultures. The results were reproducible, for both oxygen consumption and oxygen evolution from trial to trial and season to season, and were compared to results determined from exposure to ethanol without napthalene (Figure 5 and Table I). Acute exposure of the cells to any concentration of naphthalene tested resulted in a stimulation of oxygen consumption of 37% or more; exposure to 30 ppm stimulated respiration by 53%. Of all the organisms and culture conditions tested, the respiration of these cells was the most acutely perturbed by exposure to naphthalene. Apparent photosynthesis, however, was inhibited. While 3.8 ppm naphthalene had little effect on oxygen evolution by

the cells, exposure to the higher concentrations inhibited this activity by around 35%, with the maximum acute inhibition of 47% obtained by exposure to 30 ppm. Ethanol alone had virtually no effect on respiration and inhibited apparent photosynthesis by about 18% (Table II).

The effect on older stationary phase cells (twelve day old cultures) of acute exposure to naphthalene was also examined (Table III) and their response was found very similar to that displayed by the cells of the younger cultures. It is interesting to note that while the effect on oxygen evolution was virtually the same as that upon the early stationary phase cells, oxygen consumption was stimulated by a smaller percentage; exposure to 7.5 ppm resulted in no significant increase in this activity as compared to ethanol controls. This differing of response between the two phases of culture decreased at higher naphthalene concentrations: at 30 ppm, results were not significantly different. Ethanol inhibition of apparent photosynthesis was also virtually the same as that of the younger cells while the respiration of the "twelve day" cells exposed to ethanol was stimulated by 26%.

Response of Oxygen Consumption and
Evolution to Chronic Exposure

Assays of early stationary phase Chlorella cells exposed to various concentrations of naphthalene in an open system for periods of two hours to one week indicated

that cells in this phase apparently recovered quite rapidly from naphthalene toxication, at least in terms of their respiratory and apparent photosynthetic rates (Tables IV-VI). In all, 55 separate trials were performed and compared to ethanol controls.

At the end of two hours of exposure, the inhibition of respiration of cells exposed to 30 ppm rose (to 57%), however the respiratory rate of cells exposed to all lower concentrations approached that of the control cells (see Figure 6). At both 24 hours and seven days, (Tables V and VI, Figure 6) rates for all concentrations were indistinguishable from those of controls. By 24 hours, 80% of the naphthalene originally present would have volatized from the system (see section on volatilization of naphthalene).

Apparent photosynthesis appeared more sensitive to perturbation by naphthalene. At two hours inhibition of oxygen evolution rose in cells exposed to both 15 ppm and 30 ppm, while it remained virtually unchanged for cells exposed to the lesser concentrations (Figure 7). As with respiratory rates, by 24 hours apparent photosynthetic activities were approaching those of controls for all concentrations.

Eight trials were also performed in which early stationary phase Chlorella pyrenoidosa cells were exposed to various concentrations of naphthalene for 24 hours and then diluted (1:20) into fresh moss media to encourage log phase type proliferation. The cultures were then assayed

after a week to determine if the added stress of rapid growth detracted from the cells' abillity to recover from naphthalene exposure. Results are presented in Table VII, and indicated that at least in terms of oxygen consumption and evolution, the cells recovered quite well from any acute effects of such exposure. Direct microscopic counts also indicated no significant differences in increases in cell numbers of cultures exposed to naphthalene from those exposed to ethanol only.

Effect of Naphthalene on Log Phase *Chlorella Pyrenoidosa*

Response of Oxygen Consumption and Evolution to Acute Exposure

The response of log phase *Chlorella pyrenoidosa* cells to exposure to naphthalene in an open system was different from that displayed by stationary phase cells. A total of 41 separate trials were performed. The results of acute exposure were reproduceable from trial to trial and season to season. Apparent photosynthesis by cells in this phase appeared to be more sensitive to ethanol (Table VIII) than stationary phase cells (Table II); acute exposure resulted in an inhibition of about 40% as compared to the 18% inhibition displayed by stationary phase cells.

Acute exposure of the cells to concentrations of naphthalene of 7.5 ppm or less inhibited respiratory

activity while exposure to 15 ppm or 30 ppm stimulated it (Table IX and Figure 8). These stimulations, however, were less than those seen with stationary phase cells; a maximum of 41% stimulation was observed upon exposure of the culture to 30 ppm.

Apparent photosynthesis in the log phase cells exposed to the lower concentrations also was less perturbed, in an accute assay, than that seen in stationary phase cells (Tables IX and I). Exposure to 3.8 ppm produced no significant difference in this activity from that of the controls. The maximum inhibition of 42% (obtained by exposure to 30 ppm) was around that obtained from exposure of stationary phase cells to 30 ppm, and exposure to 7.5 ppm and 15 ppm resulted in less perturbation of this activity in log phase cells than in stationary phase cells. It is intriguing that while log phase cells appeared less sensitive to naphthalene, they were more sensitive to ethanol than the stationary phase cells.

Response of Oxygen Consumption and Evolution to Chronic Exposure

These determinations of the effects of chronic exposure to naphthalene on log phase Chlorella pyrenoidosa were disappointing because standard deviations for the 24 hour and 48 hour exposures were often greater than 10 (Tables X and XI). Even control rates were found to vary widely. Forty-five separate trials were made with this

phase of cell culture, and this line of pursuit was eventually abandoned. Nontheless, the effect of naphthalene on respiration appeared to increase with increasing length of exposure (Figure 9) while that on oxygen evolution appeared to decrease after an initial increase of inhibition at two hours (Figure 10).

The unpredictability displayed by the log phase cells was not totally unexpected (David Francko, private communication) as the responses of algal cells in this phase of rapid growth and division have often been found to vary widely. For example, the production and extracellular release of cyclic AMP by Anabaena flos-aquae was found to vary greatly "within and between active growth phase and stationary phase" (Francko and Wetzel, 1981). In a later study, levels of cyclic AMP were found to vary more in the actively growing cells than in stationary phase cells (Francko, 1984). As levels of the cyclic nucleotides may either influence or be influenced by the metabolic processes of the cell, this suggests that experiments with log phase algal cells may be inherently less reproduceable than those with stationary phase cells. Bernstein (1984) working with Chlamydomonas moewusii suggested that in synchronous cultures, stationary phase cells may be more limited by culture conditions (less light, depletion of nutrients, crowding, etc.) than log phase cells. The stationary phase cells would be reduced to a more "basal"

level of production, and hence be less variable than log phase cells.

Soto, Helleburst, and Hutchinson (1977) ran a series of experiments comparing the changes in cellular composition between log and stationary phase Chlamydomonas angulosa. They found that while all constituents measured (total carbon, protein, lipid, carbohydrate, and pigments) steadily increased during stationary phase (four through seven days after inoculation), during log phase (the first three days after inoculation) the rate of change of cell constituents fluctuated widely. Indeed, during this exponential phase the components both increased and decreased with no regularity or predictable pattern. This fluctuation and change in the amounts of cellular components indicates that the log phase cells would be less uniform in their metabolic activities than their stationary phase counterparts.

The results obtained two hours after exposure of log phase cells to various concentrations of naphthalene were reproducible (Table XII). That the two hour exposure determinations were reproducible, both internally and from trial to trial, may reflect simply the fact that at two hours the cultures had not had time to display their inherent variation or to reproduce (Chlorella divides once in a 24 hour period). At this time, inhibitions of respiration and apparent photosynthesis by exposure to 15 ppm and 30 ppm were found to be increased over that caused

by an acute exposure. The effect by these concentrations of naphthalene on respiration progressed from a stimulation (acute, Table IX) to a slight inhibition (two hours, Table XII) while inhibition of apparent photosynthesis increased by 30% to 75% after a two hour exposure to 30 ppm. Although the data for longer exposures exhibit too great a degree of variability to be considered reproducible, this trend of increase in inhibition of respiration appeared to continue, while oxygen evolution rates tended towards those of controls (Figure 10). These results suggest that the log phase Chlorella cells may be more sensitive, in terms of chronic effects on respiration, than stationary phase cells to naphthalene toxication.

Changes in Morphology With Chronic Exposure

Morphological changes were observed upon exposure of Chlorella pyrenoidosa cultures to naphthalene. At both 24 and 48 hours after exposure, cells exposed to 30 ppm displayed greater variation in size and shape than did control cells. At 24 hours, some cells were irregularly shaped, and at 48 hours some cells displayed internal disorganization of components as compared to control cells (see Figure 11). Cells exposed to lower concentrations showed similar effects but with decreasing frequency and degree with decreasing concentration of naphthalene. Cells exposed to 4 ppm at 24 hours and to 4 or 8 ppm at 48

hours were indistinguishable from control cells. Cells exposed to 15 ppm displayed marked size and shape variation, (but not as extreme as cells exposed to 30 ppm) at both 24 and 48 hours.

Effect of Naphthalene on Stationary Phase Chlamydomonas Moewusii

Response of Oxygen Consumption and Evolution to Acute Exposure

The effects of naphthalene in an open system on respiration and apparent photosynthesis by Chlamydomonas moewussii were also investigated. Preliminary results with stationary phase cells indicated that these algae in this phase were more resistant to an acute exposure than were the stationary phase Chlorella (Tables XIII and I). It is interesting to note that while at every concentration tested the stimulation of respiration and inhibition of apparent photosynthesis was less for the Chlamydomonas cultures, still the overall pattern of effect was similar for both types of stationary phase organism: inhibition of oxygen evolution increased with exposure to increasing concentration while respiration was stimulated with maximum activity displayed by the cultures exposed to 30 ppm (Figures 5 and 12). The effect of ethanol (control) on stationary phase Chlamydomonas was like that on stationary Chlorella in that rates of activities were not significantly perturbed from nontoxicated cultures (Table XIV).

Effect Of Naphthalene On Log Phase *Chlamydomonas Moewusii*

Response of Oxygen Consumption and Evolution to Acute Exposure

Twenty-four trials were run to determine the acute effects of naphthalene in an open system on log phase *Chlamydomonas moewusii*. These cells appeared to be more sensitive to naphthalene (Table XV) than the stationary phase organisms (Table XIII); apparent photosynthesis in particular showed significant inhibition at concentrations of 7.5 ppm and above (Figure 13). This response was different than that displayed by the *Chlorella* cultures; for the *Chlamydomonas* log phase cells the apparent photosynthetic activity was more inhibited by exposure to naphthalene than the stationary phase organisms. In fact, overall apparent photosynthetic activity by these log phase cells appeared more sensitive to acute toxication by naphthalene than any other phase or organism tested.

Acute effect on respiration by log phase *Chlamydomonas* was also different than for all other phases and cultures tested. These cells displayed an acute 35% inhibition of respiration upon exposure to 30 ppm naphthalene. Respiration of other cultures exposed to 30 ppm during an acute assay was stimulated 31% for stationary phase *Chlamydomonas* (Table XIII) and by more than 40% for either log (Table IX) or stationary (Table I) phase *Chlorella*.

As with all other acute determinations, the effect of absolute ethanol on oxygen consumption and evolution was reproducible from trial to trial and season to season (Table XVI). For these cells, in this phase, addition of ethanol did not significantly perturb the activities; respiration was slightly stimulated and apparent photosynthesis inhibited.

Response of Oxygen Consumption and Evolution to Chronic Exposure

Only one series of duplicated trials was performed for a two hour exposure of log phase Chlamydomonas moewusii to four different concentrations of naphthalene in an open system. Results are presented in Table XVII. In contrast to the change seen in respiratory activity by log phase Chlorella cultures between an acute (Table IX) and two hour (Table XII) exposure to 15 ppm or 30 ppm naphthalene, the Chlamydomonas cultures showed no significant change in inhibition of respiratory activity from an acute (Table XV) to a two hour exposure (Table XVII) to these amounts. Yet, for both cultures exposed to these higher concentrations, inhibition of apparent photosynthesis at two hours was significantly increased over the acute determination.

The effects of longer exposures on the log phase Chlamydomonas cultures were also tested. Unfortunately, results varied even more widely than log phase Chlorella

cultures. Fourteen separate determinations were made on two different days for effects at 24 hours and results, even for controls, varied too widely to be of any predicative value. For example, exposure to 30 ppm resulted in effects on respiration ranging from an inhibition of 67% to stimulations of greater than 100%. Results at 48 hours also varied widely and were not internally reproduceable. In this case, 16 determinations on two different days were performed and exposure to 30 ppm naphthalene had effects on respiration ranging from a 43% inhibition to over 100% stimulation. As with log phase Chlorella cultures, this variation was not completely unexpected. As previously discussed, parameters of log phase algal cells have often been found to vary widely.

Changes in Morphology With Chronic Exposure

The morphology of these cells was also affected by exposure to naphthalene. The most noticeable effect was the loss of flagella and cessation of movement by cells exposed to 30 ppm. This effect has been reported in Chlamydomonas angulosa at 16 ppm by Soto, et al. (1979). Cells exposed to lower concentrations moved less rapidly than control cells. A few cells were stationary but did not appear to have lost their flagella. Also, as with Chlorella cultures, exposure to 30 ppm naphthalene caused a variation in the size and appearance of the Chlamydomonas

moewusii cells. In this case, after 48 hours, the cells appeared smaller and darker than those of nontoxicated or ethanol-control cultures (see Figure 14). Flagella and movement were absent from all cells exposed to 30 ppm at both 24 and 48 hours.

Cells exposed to lower concentrations moved less rapidly than control cells and displayed decreasing abnormalities with exposure to decreasing concentratiions. At 24 and 48 hours, about one half of the cells exposed to 15 ppm displayed abnormalities similar to those of the cultures exposed to 30 ppm; some were stationary and lacked flagella while some exhibited movement, albeit less rapid than cultures exposed to 7.5 ppm.

Effect Of Naphthalene On Isolated Spinach Chloroplasts

Response of Oxygen Evolution to Acute Exposure of 30 ppm

The acute affect of exposure to 30 ppm naphthalene on oxygen evolution by isolated spinach chloroplasts was assayed in one duplicated trial. Results showed that the activity was inhibited by 24% as compared to ethanol controls. This inhibition was less than that displayed by any of the algae in any phase during an acute exposure to 30 ppm naphthalene; therefore effects on the chloroplasts alone may not account for total perturbation of oxygen evolution by the algae.

Spectroscopic Determinations With Naphthalene

Naphthalene and Ubiquinone

Since the data of Harmon and Sanborn (1982) indicated that naphthalene was acting specifically at ubiquinone in the isolated mitochondrial system, the effect of naphthalene on Q_{10} (ubiquinone) and duroquinone (tetramethylbenzoquinone) was examined. Spectra of the individual quinones in ethanol were determined and compared to the spectra of the individual quinones in the presence of ethanol plus naphthalene. Portions of the following results were originally presented in Volume 31 of the Bulletin of Environmental Contaminants and Toxicology (Struble and Harmon, 1983). Figure 15a illustrates the effect of naphthalene on Q_{10}. Trace 3 is the absolute spectrum of Q_{10} while trace 4 is the difference spectrum of Q_{10} in 50 ppm naphthalene (moles naphthalene/moles quinone = 260). Note the replacement of the strong 274 nm absorbance peak of Q_{10} by two absorbance maxima at 273 and 279 nm. This alteration of spectrum was not prominent at ratios (naphthalene/quinone) below 175.

Figure 15b shows the absolute spectrum of 260 uM naphthalene in ethanol. Note that the two new absorbance maxima in the spectrum of Q_{10} at 273 and 279 nm induced by the presence of naphthalene do not correspond to

wavelengths of maximum absorbance by naphthalene (in fact 279 nm is a trough in the naphthalene spectrum); therefore the change seen in figure 15a is not due to a dilution artifact. A dilution artifact would have affected absorbance of trace 3 in Figure 15a at all the absorbance maxima of naphthalene. The effect seen in figure 15a is not due to scattering. A scattering study was performed as described in "Materials and Methods" and results are presented in Table XVIII. Note that the increase in absorbance due to higher concentrations of naphthalene does not become larger with decrease in wavelength. Also, no increase in overall absorbance (or "baseline") comparable to that predicted by equation 4 was noted. The change seen in Figure 15a is well within the resolution of the spectrophotometer. At wavelengths of 275 nm - 280 nm, the widest the slits ever became during the study was 1.15 mm. So, substituting the proper values into equation 4:

Resolution (Å)=(1.15 X 23)+0.44÷0=26.89 Å

and the change seen in the 280 nm region of Q_{10} spectrum due to the presence of naphthalene was at least 5 nm (50 Å). Thus, the changes in the absorbance spectrum of Q_{10} were within the resolution of the spectrophotometer and were due to the interaction of ubiquinone with naphthalene. They were not artifacts resulting from dilution or light scattering.

Figure 16 represents the effect of naphthalene on duroquinone. Trace 3 is the absolute spectrum of 3.0 x 10^{-7} M duroquinone in ethanol; trace 4 is the same concentration of duroquinone in 30 ppm naphthalene (naphthalene/quinone = 780). Note that there was no effect on the absorbance maxima of duroquinone.

The concentration of ubiquinone in beef heart mitochondria is 2.5 mg/g protein (Crane, Hatefi, Lester and Widmer, 1957) to 3.5 nmoles/mg protein (Huang and Lee, 1975): at the 78 uM LC_{50} for naphthalene reported by Harmon and Sanborn (1982) the naphthalene/quinone ratio in inhibited intact mitochondria would be 379 to 460, more than twice that required to cause an observable alteration of the spectrum of ubiquinone. These result indicate that naphthalene interacts with ubiquinone but not with duroquinone. This interaction may account for at least part of the inhibition seen in isolated mitochondria, tissue culture cells, unicellular algae, and multicellular organisms.

Volatilization of Naphthalene

The volatilization of naphthalene, under the conditions of the algal toxication determinations was also tested. Results are presented in Table XIX and show that at 24 hours 80% of the naphthalene originally present in solution was gone; by 48 hours, 93% had disappeared. This finding indicates that perturbation in activities and morphology seen at 24 hours or more after inoculation

were not due to high residual concentrations of naphthalene in the media, but rather to either naphthalene induced damage, concentration of naphthalene within the cells themselves, or the presence of naphthalene derivatives. Soto, Hellebust, and Hutchinson (1975) and Soto, et al. (1975) demonstrated that such internal concentration does occur, at least in Chlamydomonas angulosa with the possibility of cells being exposed to higher amounts of the toxicant than indicated by its water solubility.

CHAPTER V

CONCLUSIONS

Effect Of Naphthalene On Algae

Oxygen Consumption and Evolution

Naphthalene affected the oxygen consumption and evolution activities of both Chlorella pyrenoidosa and Chlamydomonas moewusii. In an acute exposure to 30 ppm apparent photosynthesis was inhibited by at least 32% for all phases of all cultures tested; maximum inhibition (60%) was displayed by log phase Chlamydomonas. In fact in terms of inhibition of both apparent photosynthetic and respiratory activities, this organism in this phase was the most susceptible to acute naphthalene toxication. Indeed, this was the only culture that displayed an acute inhibition of respiration when exposed to 15 ppm or more of naphthalene; all other cultures showed stimulation. Interestingly, in terms of overall disruption of activities, Chlamydomonas in the stationary phase was the most resistant of all the cultures tested.

Results with the more aged Chlorella culture (12 days in culture; mid to late stationary phase) were similar to the early stationary phase Chlorella culture. In fact,

effects of all concentrations of naphthalene on apparent photosynthesis or of 30 ppm on respiration were not significantly different for the two ages of cultures. Although at the lower concentrations of naphthalene the effects on respiration did vary somewhat between the early and late stationary phase Chlorella cultures, (Tables I and III), they were still more similar than results with early stationary versus log cells (Tables I and IX). That results varied between both cell types and growth phases while remaining relatively constant within growth phases was not unexpected. This just serves to point out that different organisms are different, and that within one organisms' growth cycle large variations in cellular constituents and processes are found during the different phases.

Stationary phase Chlorella cells recovered quite rapidly from naphthalene toxication after an initial increase in inhibition of respiratory and apparent photosynthetic activities seen at two hours after exposure. After 24 hours or more in an open system, activities returned to the normal level whether or not the toxicated cells were diluted into fresh media. By this time, even for the non-diluted culture, 80% of the naphthalene originally present would have volatilized.

Log phase Chlorella cells also showed an increase in inhibition of activities two hours after exposure to naphthalene (for all but the effect of 3.8 ppm and 7.5 ppm

on respiration). With log phase cells results also indicate that inhibition of respiration increased at both 24 hours and 2 days after exposure. Unfortunately, the standard deviations for these last were too high to make these results anything more than intriguing. For log phase Chlamydomonas, the two hour results show a different trend; while inhibition of apparent photosynthesis did increase, that of respiration did not.

These results, especially the large inhibitions seen after a two hour exposure, indicate that naphthalene does perturb the respiratory and photosynthetic capacity of the two types of algae, at least temporarily. As these two processes are essential for energy generation and conservation, and hence are vital for metabolism, these perturbations could have serious consequences for the cells, including reducing their capacity to remove or alter other toxicants with which they may come in contact. It is interesting to note, that at 24-hours exposure, when the stationary phase Chlorella cell activities were again approaching normal rates, less than 15% of the naphthalene originally present in the media still remained. Hence, in a system where the cells were constantly exposed to naphthalene (as in the chemical percolating through the soil via water from a chemical or oil spill), the perturbations of activity could be much longer term than those indicated by the open-system type assay utilized in this series of experiments.

It is interesting to note that strictly in terms of acute inhibition of respiration by 30 ppm naphthalene, the highest inhibition occurred to the fastest respiring cells (log phase Chlamydomonas) while the least (or highest stimulation) occurred to the slowest respiring cells (stationary phase Chlorella). Perhaps a higher turnover of respiratory components allowed more of a sensitive component to be present in a form more susceptible to perturbation by naphthalene. For example, if naphthalene does act at ubiquinone as suggested by the work of Harmon and Sanborn (1982), and ubiquinone does operate in a Q cycle (Kroger and Klingenberg, 1973; Trumpower, 1981; Zhu, et al., 1982) with alternating forms of quinone, semiquinone, and quinol, then a higher respiratory rate would perhaps allow more of the Q to be unbound or present in a form susceptible to naphthalene.

Soto, et al. (1975) determined the effect of naphthalene exposure upon photosynthesis in log phase Chlamydomonas angulosa by measuring ^{14}C incorporation. Their results indicated greater inhibition of activity than determined with log phase Chlamydomonas moewusii in this study although the pattern and general trends of inhibition were the same. Soto, et al. (1975) found that after 30 minute incubation cells exposed to 50% saturation (about 15 ppm) lost 74% of their photosynthetic capacity, and after 2 hours the activity had almost ceased; respective values for log phase Chlamydomonas moewusii in the

current study were loss of 43% for acute exposure and 76% for a two hour exposure (Tables XV and XVII). These differences could be due either to the assay methods employed, or to the difference in species.

Morphology

Both log phase Chlorella pyrenoidosa and Chlamydomonas moewusii displayed morphological abnormalities when incubated with naphthalene in an open system. For both cell types, the EC_{50} (or concentration where about one half of the cells seemed affected) was around 15 ppm. Effects remained visible even 48 hours after exposure, when virtually all of the naphthalene would have been volatilized from the medium. At this point, the inconclusive results from the log Chlorella cultures suggested that respiration was highly perturbed while apparent photosynthetic rates were returning to normal.

The effects on morpholgy were similar to those seen in open systems by Soto, Hellebust, Hutchinson, and Sawa (1975) and Soto, et al. (1975); although for Chlorella there were no flagella to be absent. The much more extensive damage at lower concentrations reported in closed systems by the above authors and Soto, et. al. (1977) and Soto, Hutchinson, Hellebust, and Sheath (1979) indicate that long term, continuous exposure to lower levels of naphthalene can induce even more of the type of

cellular damage seen with short-term exposure to high concentrations.

Subcellular Effects

Interactions of Naphthalene With Ubiquinone

Naphthalene induced a shift in λ_{max} in the absorbance spectrum of ubiquinone when present in a molar ratio (naphthalene/ubiquinone) of 175 or greater. This effect was not due to dilution artifact or scattering and was within the resolution of the spectrophotometer. Hence, an actual effect of naphthalene on Q_{10} was observed. Alteration of the spectrum of duroquinone was not observed even at a ratio 20 times as great. This correlates with results with isolated beef heart mitochondria (Harmon and Sanborn, 1982); naphthalene did not inhibit duroquinone oxidation but did inhibit NADH oxidation as well as NADH>Q_{10} reductase and QH_2>O_2 activities. The naphthalene/ubiquinone ratio in inhibited intact heart mitochondria was more than twice that required to cause an observable alteration of the spectrum of ubiquinone.

Researchers have reported alteration in the spectra of compounds such as porphyrins and quinones induced by changes in the environment of the compound. Schleyer, Cooper, and Rosenthal (1971), and Schleyer, Levin, and Rosenthal (1971) have reported "small but measurable"

optical and electron paramagnetic resonance absorbance changes in the spectrum of cytochrome P-450 by the interactions of the cytochrome with various sterols. These differences in spectra were not due to the exchange of ligand (Schleyer, Cooper, and Rosenthal, 1971) but to the perturbation of the environment of the heme group (Schleyer, et al., 1971). Some of these interactions altered the specificity of the hydroxylase system with steroid as substrate. Harmon (1984 and 1985) has reported that local anesthetics such as lidocaine altered the physio-chemical properties of cytochrome c as well as its spectra. ^{1}H-NMR studies indicated that lidocaine perturbed the hydrophobic residues in the heme pocket of the cytochrome, interacting with the hydrophobic residues around ring four of the heme plane. Perturbation of these residues was consistent with the observed alteration in absorbance spectrum and changes in such properies as mid-potential and CO binding.

Dimerization of quinones (phenanthrenequinone anions) with no evidence of covalent bond formation, have been reported (Staples and Szwarc 1970). The dimerization, due to $\pi-\pi$ electron interaction, is accompanied by a bathochromic shift in λ_{max}. The absorbance spectra of quinones were also different in different solvents. The change in the environment of 2,6-dimethylbenzoquinone by replacement of ethanol as solvent with methanol shifted the absorbance maxima approximately three nanometers toward

the blue (Phillips, Lyle, and Jones, 1969). Addition of KOH to either p-toluquinone or phenyl p-benzoquinone in methanol induced a 14 nm or larger shift (Simmons, 1979).

The bathochromic shift of the spectrum of ubiquinone induced by naphthalene was small but within the resolution of the spectrophotometer. It could indicate either changes in the π-electron cloud or other perturbations of the quinone by its environment. These changes in the quinone may disrupt its ability to bind to Q-binding proteins (Yu, Yu, and King, 1977), or alter the midpotential of Q/QH_2, Q/QH and QH/QH_2 redox pairs, altering the ability of Q to accept or donate electrons to the rest of the mitochondrial electron transport system. As ubiquinone is essential to electron transport, this may inhibit respiration.

That naphthalene inhibited NADH->Q_{10} and QH_2->O_2 while not affecting duroquinol->O_2 activity, and altered the spectrum of ubiquinone while not affecting that of tetramethyl benzoquinone suggests an interaction of naphthalene specifically with coenzyme Q. The data support a molecular basis for the rather specific effect of naphthalene on respiration of isolated cardiac mitochondria, intact cells, and whole organisms which may suggest a possible mode of action of other polyaromatic hydrocarbons.

The algae tested in this study all displayed inhibition of respiration after a two hour exposure to high

concentrations of naphthalene. These algal cells were not as sensitive as either isolated beef heart mitochondria or the tissue culture cells tested by Harmon and Sanborn (1982). Although they do contain ubiquinone and mitochondria similar in many ways to animal cells, nonetheless, they are algae with all their attendant differences. These differences in structure and metabolism must somehow account for their greater resistance.

Mitochondria

Harmon and Sanborn (1982) demonstrated an EC_{50} for isolated mitochondria and cells exposed to naphthalene of approximately 10 ppm. At no point in the current study was the respiration by the algal cells found to be this sensitive to the toxicant. In fact, at two hours, EC_{50}'s were all greater than the maximum concentration tested, 30 ppm (which is also near the water solubility of naphthalene), except for the stationary phase *Chlorella pyrenoidosa*, in which exposure to 30 ppm resulted in a 57% inhibition.

Plant mitochondria are very similar to animal mitochondria. Both contain ubiquinone and the cytochromes are spectrally and functionally similar. In plant mitochondria ubiquinone (most often Q_{10}) functions for the most part in the Q-cycle and Q-pool behavior seen in animal mitochondria. The organization and spatial orientation of the electron transport chain is similar in

both plant and animal mitochondria and the function of both is essentially the same. There are differences however: plant mitochondria contain an exogenous NADH dehydrogenase, a more complex malate oxidation system, and a cyanide resistant pathway (Moore, 1978; Cottingham and Moore, 1983; Elthon and Stewart, 1983). It is only on the cyanide resistant pathway that ubiquinone behaves kinetically different than in animal mitochondria. The differences between the two systems which are structural as well as functional, may enable plant mitochondria to respond differently, in degree or in kind, to toxication. As of yet, plant and algal mitochondria are not as well characterized and understood as animal mitochondria. To truly relate effects on algae with effects on their own mitochondria, algal mitochondria would need to be isolated and tested.

Relation to Effects on Whole Algae

It was disappointing that the correlation between the effects of naphthalene on the subcellular organelles, mitochondria and chloroplasts, with effects on whole algae were not as striking as those seen between mitochondria and whole cells by Harmon and Sanborn (1982). While exposure to naphthalene did inhibit oxygen evolution by both isolated chloroplasts and whole algae, in no case did the 24% inhibition seen in isolated spinach chloroplasts (by 30 ppm) account for all of the inhibition seen in the

algae. With respiration, just the opposite was observed; the isolated beef heart mitochondria were much more sensitive to naphthalene inhibition of respiration than were the algae.

While it seems reasonable to assume that the ubiquinone in the algal mitochondria would have interacted with naphthalene as seen in the spectrophotometric study presented in this paper (Chlorella pyrenoidosa was demonstrated to contain Q_{10} by Whistance and Threlfall, 1970), obviously some other factor was entering into the picture. For example, plant mitochondria contain a cyanide-insensitive pathway for oxidation of substrate and generation of ATP (respiration in animal mitochondria is cyanide sensitive). This pathway contains unique components, including a flavoprotein and a terminal oxidase (Elthon and Stewart, 1983) and the kinetics for ubiquinone oxidation/reduction are different, for this pathway, than those of beef heart mitochondria (Cottingham and Moore, 1983). Perhaps this pathway is not as sensitive to naphthalene induced perturbations in ubiquinone as is the cyanide sensitive pathway.

To examine if indeed this cyanide insensitive pathway could account for the greater resistance of the algal mitochondria to naphthalene toxication, antimycin sensitive rates were tested in one unduplicated series of acute naphthalene exposure trials with stationary phase Chlorella pyrenoidosa. The results, although preliminary, are most interesting. Antimycin blocks

electron flow between the b and c cytochromes, hence eliminating cyanide sensitive respiration. For both nontoxicated and ethanol treated Chlorella cultures, respiration was around 55% antimycin resistant. With addition of increasing amounts of naphthalene, a greater percentage of the respiration in the toxicated cultures was antimycin insensitive; 3.8 ppm and 7.5 ppm toxicated cultures retained about 60% of their respiratory activities upon addition of antimycin while 15 ppm and 30 ppm toxicated cultures showed antimycin resistant activity of around 90%. In other words, while about half of the respiratory activity in control cultures utilized the cyanide sensitive pathway (and so was sensitive to inhibition by antimycin), virtually all of the activity of that pathway was inhibited by exposure to the higher concentrations of naphthalene; only antimycin resistant respiration remained in any significant amount. So, it seems that the cyanide resistant pathway could help account for the difference in respiratory sensitivity to naphthalene of animal and algal cells. At least in an acute determination, naphthalene appeared to act preferentially on the cyanide sensitive pathway common to both plant and animal mitochondria. However, other factors may be responsible or partly responsible for the lesser sensitivity of algal mitochondria to naphthalene. Perhaps the exogenous NADH dehydrogenase allows more substrate to enter the system and somewhat overcome the

inhibition of ubiquinone. Perhaps algal mitochondria simply contain a different amount of ubquinone (more?) and therefore exposure would require a higher concentration of naphthalene to display the same behavior as beef heart mitochondria.

A puzzling response of the algal mitochondria to naphthalene toxication was the initial stimulation of respiration seen in all but the log phase Chlamydomonas moewusii cells. The slower the nontoxicated rate, the greater was the percent of stimulation in an acute assay. By two hours, however, all stimulation was replaced by inhibition. Perhaps the addition of naphthalene to the cultures caused an acute release of substrate (NADH, succinate, etc.) from systems such as glycolysis or the TCA cycle which became available to the mitochondria for a burst of activity. Eventually, this could have been countered by the turnover and inhibition of the activity of ubiquinone in the electron transport chain. Those organisms with the highest turnover (fastest respiratory rate) experienced the most rapid inhibition, perhaps so rapid in the case of the log phase Chlamydomonas moewusii as to apparently negate any stimulation effect. In any case, the algae did not react to naphthalene, in terms of respiration, as did animal cells or mitochondria. To get a better idea of how algal mitochondria, distinct from all the "support systems" provided by the algal cell, actually respond to naphthalene, it may be neccessary to isolate

and assay algal mitochondria.

It is interesting that oxygen evolution was inhibited in whole algae by more than the amount predicted by an acute exposure of isolated spinach chloroplasts to 30 ppm naphthalene. As with respiration, inhibition of apparent photosynthesis increased after two hours of exposure. It seems likely that some component, process, or mechanism other than just the chloroplast was being disturbed.

While algal chloroplasts are very similar to those of higher plants, there are some differences. For example, a comparison of photosystem I from a Chlamydomonas sp. and from Swiss chard, lettuce, pea, and spinach chloroplasts has been made (Nechushtai and Nelson, 1981). In the two systems (higher plant versus algae) the structure and function of subunit I (the P_{700} reaction center) appeared highly maintained, while the structure of the other subunits varied somewhat. The higher plants contained six subunits, while Chlamydomonas contained four. Also, under conditions where the higher plant reaction center was active, that of the alga was inactive in cytochrome 552 photooxidation unless salts were included in the assay medium or the pH was lowered to five. Perhaps the differences in the structure and conditions required for proper functioning of the algal chloroplast could account for a higher sensitivity to naphthalene. Or perhaps other factors or a combination of characteristics is responsible. To determine if effects on the chloroplast

alone could account for the inhibition of oxygen evolution noted in this study, algal chloroplasts would need to be isolated and assayed.

Darville, et al. (1983) reported that naphthalene did not cause membrane disruption but did perturb the Na+,K+-ATPase, which resulted in a loss of ionic regulation in the organism being studied (Chironomous attenuatus). Ionic regulation is extremely important for organisms living in water, as they struggle constantly to maintain the correct osmotic balance as well as individual ionic concentrations. Algae do contain Na+,K+-ATPases, although they are unlike those of animal cells and like those of bacteria, mitochondria, and chloroplasts (Serrano, 1983 and Poole, 1978). Still, it is possible they could be perturbed by naphthalene and this could have far reaching consequences for the cell. Such processes as transport could be affected as they often depend on concentration gradients of ions, protons, or phosphate. A loss of the optimum "balance" within the cell could possibly set up conditions that could disturb apparent photosynthetic rates.

Many systems within an algal cell could be disrupted by a loss of transport capacity, a change in the internal environment, interference with the system or its regulation by direct action of naphthalene on a component, or by a combination of all of the above. Systems affected could include the Calvin cycle, the TCA cycle, the

glyoxylate cycle, photosynthesis itself or a myriad of other processes, organelles, and functions.

In summation, to determine the specific effect of naphthalene on an organelle (such as a chloroplast or mitochondrion), that organelle would need to be isolated and assayed. Then, in the case of an electron transport chain, site of action could be determined using combinations of inhibitors and artificial electron donors and acceptors to isolate sections of the chain and determine if they were being affected, and if so, how much (as was done by Harmon and Sanborn, 1982). Finally, individual components could be examined for interactions with the toxicant (one method was presented by Struble and Harmon, 1983). In this way, it may be possible to track the effect of a toxicant from organism through cell and organelle to molecule, and so to better understand its mode of operation at all levels. Hence, while an interaction of naphthalene with ubiquinone, as presented in this paper, seems a likely candidate for part of the effects of that toxicant seen on whole algae in this study, it is unfortunately not the entire explanation for the toxicants action at the molecular level. To develop a more complete understanding of the mechanism of toxicity of this chemical for green algae, more work as outlined in this paper needs to be done.

BIBLIOGRAPHY

Anderson, J.W., J.M. Neff, B.A. Cox, H.E. Tatem and G.M. Hightower. 1974. Characteristics of Dispersions and Water-Soluble Extracts of Crude and Refined Oils and their Toxicity to Esturarine Crustaceans and Fish. Mar. Biol. 27:75-88.

Arnon, D.I., M.B. Allen and F.R. Whately. 1956. Photosynthesis by Isolated Chloroplasts IV. General Concept and Comparison of Three Photochemical Reactions. Biochim. Biophys. Acta. 20;449-468.

Barr, R. and F.L. Crane. 1981. Ferricyanide Reduction in Photosystem II of Spinach Chloroplasts. Plant Physiol. 67;1190-1194.

Bernstein, E. 1960. Synchronous Division in Chlamydomonas moewusii. Science. 131;1528-1529.

Bernstein, E. 1964. Physiology of an Obligate Photoautotroph (Chlamydomonas moewusii). 1. Characteristics of Synchronously and Randomly Reproducing Cells and an Hypothesis to Explain Their Population Curves. J. Protozool. 11;6-74.

Burges, A. and F. Raw. eds. 1967. Soil Biology. Academic Press, London.

Cerniglia, C.E., D.T. Gibson and C.van Baalen. 1980. Oxidation of Naphthalene by Cyanobacteria and Microalgae. J. Gen. Micro. 116;495-500.

Crane, F.L., Y. Hatefi, R.L. Lester and L. Widmer. 1957. Isolation of a Quinone From Beef Heart Mitochondria. Biochim. Biophys. Acta. 25;220-221.

Crider, J.Y., J. Wilhm and H.J. Harmon. 1982. Effects of Oxygen Uptake of Naphtalene On the Hemoglobin Concentration and Oxygen Uptake of Daphnia magna. Bull. Environ. Contam. Toxicol. 28; 52-57.

Cottingham, I.R. and A.L. Moore. 1983. Ubiquinone Pool Behavior in Plant Mitochondria. Biochim. Biophys. Acta. 724;191-200.

Darville, R.G., H.J. Harmon, M.R. Sanborn and J. L. Wilhm. 1983. Effect of Naphthalene on the Hemolymph Ion Concentrations of Chironomus attenuatus and the Possible Mode of Action. Environ. Toxicol. Chem. 2;423-429.

Darville, R.G. and J.L. Wilhm. 1984. The Effect of Naphthalene on Oxygen Consumption and Hemoglobin Concentration in Chironomus attenuatus and on Oxygen Consumption and Life Cycle of Tanytarsus dissimilis. Environ. Toxicol. Chem. 3;135-141.

Elthon, T.E. and C.R. Stewart. 1983. A Chemiosmotic Model for Plant Mitochondria. Bioscience. 33;687-692.

Francko, D.A. and R.G. Wetzel. 1981. Dynamics of Cellular and Extracellular cAMP in Anabaena flos-aquae (cyanophyta): Intrinsic Culture Variability and Correlation With Metabolic Variables. J. Phycol. 17; 129-134.

Francko, D.A. 1984. The Significance of Cyclic Nucleotides in Phytoplanktonic Carbon Metabolism: A Current View. Verh. Internat. Verein. Limnol. 22;612-619.

Fortner, A.R. and L.V. Sick. 1985. Simultaneous Acculmulations of Naphthalene, a PCB Mixture, and Benzo(a)pyrene, by the Oyster, Crassostrea virginica. Bull. Environ. Contam. Toxicol. 34;256-264.

Geyer, H., G. Politzki and D. Freitag. 1984. Prediction of Ecotoxicological Behavior of Chemicals: Relationship Between n-octanol/water Partition Coefficient and Bioaccumulation of Organic Chemicals by Alga Chlorella. Chemosphere. 13;269-284.

Goulding, R. 1975. Chemical Hazards in the Home. Brit. Med. Bull. 31;191-195.

Gray, T,R,G. and S.T. Williams. 1971. Soil Microorganisms. Longman, London.

Harmon, H.J. and M.R. Sanborn. 1982. Effect of Naphthalene on Respiration in Heart Mitochondria and Intact Cultured Cells. Env. Res. 29;160-173.

Harmon, H.J. 1984. Interaction of Local Anesthetics With Cytochrome c. (Abstract). Abstracts of 28th Annual Meeting, Biophysical Journal. 45;298a.

Harmon, H.J. 1985. Interaction of Local Anesthetics With Mitochondrial Cytochromes and Myoglobin. (Abstract). Proc., Federation of American Societies for Experimental Biology. 44;1779.

Hattori, T. 1973. Microbial Life in the Soil. Marcel Dekker, New York.

Hellebust, J.A., C. Soto and T.C. Hutchinson. 1982. Effect of Naphthalene and Aqueous Crude Oil Extracts on the Green Flagellate Chlamydomonas angulosa. V. Heterotrophic Responses. Can. J. Bot. 60;1495-1502.

Huang, CH. and C.P. Lee. 1975.. Further Characterization of Gradient-Fractioned Sub-Mitochondrial Membrane Fragments from Beef Heart Mitochondria. Biochim. Biophys. Acta. 376;398-414.

Hutchins, S.R., M.B. Tomson and C.H. Ward. 1983. Trace Organic Contamination of Ground Water from a Rapid Infiltration Site: A Laboratory-Field Coordinated Study. Environ. Toxicol. and Chem. 2:195-216.

Kauss, P.B. and T.C. Hutchinson. 1975. The Effects of Water-Soluble Petroleum Components on the Growth of Chlorella vulgaris Beijernick. Env. Pollut. 9;157-174

Kroger, A. and M. Klingenberg. 1973. The Kinetics of the Redox Reaction of Ubiquinone Related to the Electron-Transport Activity in the Respiratory Chain. Eur. J. Biochem. 34;358-368.

Lee, R. and J.W. Anderson. 1977. Fate and Effect of Naphthalenes: Controlled Ecosystem Pollution Experiment. Bull. Mar. Sci. 27;127-134.

Moore, A.L. 1978. Forty Years of Plant Mitochondriology. Nature, 275;14.

Moss, B. 1972. The Influence of Environmental Factors on the Distribution of Freshwater Algae: An Experimental Study I. Introduction and the Influence of Calcium Concentration. J. Ecol. 60;917-932.

Muralikrishna, P.V.G. and K. Venkateswarlu. 1984. Effect of Insecticides on Soil Algal Population. Bull. Environ. Contam. Toxicol. 33;241-245.

Nechushtai, R. and N. Nelson. 1981. Photosystem I Reaction Centers from Chlamydomonas and Higher Plant Chloroplasts. J. of Bioeneg. Biomembr. 13;295-306.

Pettersson, B., M. Curvall and C.R. Enzell. 1980. Effects of Tobacco Smoke Compounds on the Noradrenaline Induced Oxidative Metabolism in Isolated Brown Fat Cells. Toxicol. 18;1-16.

Phillips, J.P., R. Lyle and P.R. Jones. 1969. Organic Electronic Spectral Data, Volume V (1960-1961). New York, Interscience.

Pickett-Heaps, J.D. 1975. Green Algae, Structure, Reproduction and Evolution in Selected Genera. Sinaver Assoc. Sunderland, Mass.

Poole, R.J. 1978. Energy Coupling For Membrane Transport. Ann. Rev. Plant. Physiol. 29;437-460.

Pucknat, A.W. ed. 1981. Health Impacts of Polynuclear Aromatic Hydrocarbons. Noyes Data Corp, Park Ridge, New Jersey.

Rodgers, J.H., K.L. Dickson, F.Y. Saleh and C.A. Staples. 1983. Use of Microcosms to Study Transport, Transformation and Fate of Organics in Aquatic Systems. Environ. Toxicol. Chem. 2;155-167.

Schleyer, H., D.Y. Cooper, S.Y. Levin and O. Rosenthal. 1971. Cytocrome P-450 from the Adrenal Cortex: Interaction of Steroids and the Hydroxylation Reaction. Biochem. J. 125;10p.-11p.

Schleyer, H., D.Y. Cooper and O. Rosenthal. 1971. Adrenocortical Steroid IIB-Hydroxylase: Interaction of the Heme Protein P-450 with Substrates. (Abstract). Proc. 15th Meeting Biophys. Soc. Biophys. J. 11;74a.

Schwarz, L.R., M. Mezger and S. Hesse. 1980. Effect of Decreased Glucuronidation and Sulfation on Covalent Binding of Naphthalene in Isolated Rat Heptocytes. Toxcol. 17;119-122.

Serrano, R. 1983. Purification and Reconstitution of the Proton-Pumping ATPase of Fungal and Plant Plasma Membranes. Archiv. Bioch. Biophys. 227;1-8.

Simmons, W.W. 1979. The Sadtler Handbook of Ultrviolet Spectra. Philadelphia, Research Laboratories.

Soeder, C.J. and A. Ried. 1962. Uber den Verlauf der Sporulation und der Protoplasteinteilung in Synchronkulturen von Chlorella pyrenoidosa. Archiv. Fur Mikro. 42;176-189.

Soto, C., J.A. Hellebust and T.C. Hutchinson. 1975. Effect of Naphthalene and Aqueous Crude Oil Extracts on the Green Flagellate Chlamydomonas angulosa. II. Photosynthesis and the Uptake and Release of Naphthalene. Can. J.Bot. 53;118-126.

Soto, C., J.A. Hellebust, T.C. Hutchinson and T. Sawa. 1975. Effect of Naphthalene and Aqueous Crude Oil Extracts on the Green Flagellate Chlamydomonas angulosa. I. Growth Can. J. Bot. 53;109-117.

Soto, C., J.A. Hellebust and T. C. Hutchinson. 1977. Effect of Naphthalene and Aqueous Crude Oil Extracts on the Green Flagellate Chlamydomonas angulosa. III. Changes in Cellular Composition. Can. J. Bot. 55;2765-27777.

Soto, C., T.C. Hutchinson, J.A. Hellebust and R. G. Sheath. 1979. The Effect of Crude Oil on The Morphology of the Green Flagellate Chlamydomonas angulosa. Can. J. Bot. 57;2729-2739.

Southworth, G.R. and J. L. Keller. 1984. Partitioning of Naphthalene, Methylnaphthalenes and Biphenyl Between Wastewater Treatment Sjludges and Water. Environ. Toxicol. Chem. 3;575-582.

Spencer, W.F., W.J. Farmer and W.A. Jury. 1982. Review: Behavior of Organic Chemicals at Soil, Air, Water Interfaces as Related to Predicting the Transport and Volatilization of Organic Pollutants. Environ. Toxcol. Chem. 1;17-26.

Staples, T.L. and M. Szwarc. 1982. Electron Transfer and Agglomeration in the Systems Phenanthrene Quinone, Acenaphthene Quinone, their Dianions, Radical Ions and Dimers. J. Am. Chem. Soc. 92;5022.-5027.

Trucco, R.G., F.R. Englelhardt and B. Stacey. 1983. Toxicity, Accumulation and Clearance of Aromatic Hydrocarbons in Daphnia pulex. Environ. Poll. (Ser.A) 31:191-202.

Trumpower, B.L. 1981. Function of the Iron-Sulfur Protein of the Cytochrome b-c_1 Segment in Electron-Transfer and Energy-Conserving Reactions of the Mitochondrial Rerspiratory Chain. Biochim. Biophys. Acta. 639;129-155.

U.S.E.P.A. 1980. 440/5-80-059. Ambient Water Quality Criteria for Naphthalene.

U.S.E.P.A. 1980b. 440/5-80-069. Ambient Water Quality Criteria for Polynuclear Aromatic Hydrocarbons.

Varian. 1953. Instruction Manual for Cary Model 14 Recording Spectrophotometer, Serial No. 375 Applied Physics Corp., Monrovia, Ca.

Wauchope, R.D., K.E. Savage and W.C. Koskinen. 1983. Adsorption-Desorption Equilibria of Herbicides in Soil: Naphthalene as a Model Compound for Entropy-Enthalpy Effects. Weed Sci. 31:744-751.

Whistance, G.R. and D.R. Threlfall. 1970. Biosynthesis of Phytoquinones. Biochem. J. 117;593-600.

Yu, C.A., L. Yu and T.E. King. 1977. The Existence of An Ubiquinone Binding Protein in the Reconstitutively Active Cytochrome $\underline{b}$-$\underline{c}_1$ Complex. Biophys. Res. Comm. 78; 259-265.

Zhu, Q.S., J.A. Berden, S. De Vries and E.C. Slater. 1982. On the Role of Ubiquinone in the Respiratory Chain. Biochem. Biophys. Acta. 680;69-70.

APPENDIX A

TABLES

TABLE I

EFFECT OF ACUTE EXPOSURE TO NAPHTHALENE ON RESPIRATION AND APPARENT PHOTOSYNTHESIS IN STATIONARY PHASE CHLORELLA PYRENOIDOSA

Variable	Typical Control Rate nmole O_2/cell min.	% Inhibition vs Ethanol Control 3.8ppm	7.5ppm	15ppm	30ppm
Oxygen Consumption	2.0×10^{-7}				
Number		4	6	5	8
Mean		+47	+37	+44	+53
Standard Deviation		1.4	6.9	6.5	4.4
Oxygen Evolution	1.1×10^{-6}				
Number		4	6	5	8
Mean		2.5	34	35	47
Standard Deviation		6.4	8.0	7.3	5.5

+ indicates stimulation of rate

TABLE II

EFFECT OF ACUTE EXPOSURE TO ETHANOL ON RESPIRATION AND APPARENT PHOTOSYNTHESIS ON STATIONARY PHASE *CHLORELLA PYRENOIDOSA*

Variable	Typical Nontoxicated Rate nmole O_2/cell min.	% Inhibition vs Nontoxicated Rate
Oxygen Consumption	2.0×10^{-7}	
Number		11
Mean		1
Standard Deviation		6.0
Oxygen Evolution	1.3×10^{-6}	
Number		11
Mean		18
Standard Deviation		4.7

TABLE III

EFFECT OF ACUTE EXPOSURE TO NAPHTHALENE ON RESPIRATION AND APPARENT PHOTOSYNTHESIS IN LATE STATIONARY PHASE *CHLORELLA PYRENOIDOSA*

Variable	Typical Control Rate nmole O_2/cell min.	% Inhibition vs Ethanol Control 7.5ppm	15ppm	30ppm
Oxygen Consumption	2.1×10^{-7}			
Number		2	2	3
Mean		+1.5	+27	+47
Standard Deviation*		15	4.0	4.3
Oxygen Evolution	8.6×10^{-7}			
Number		2	2	3
Mean		28	30	40
Standard Deviation		1.0	3.0	2.1

+ indicates stimulation of rate
* where number of trials = 2, standard deviation is actually variance

TABLE IV

EFFECT OF TWO HOUR EXPOSURE TO NAPHTHALENE ON RESPIRATION AND APPARENT PHOTOSYNTHESIS IN STATIONARY PHASE *CHLORELLA PYRENOIDOSA*

Variable	Typical Control Rate nmole O_2/cell min.	% Inhibition vs Ethanol Control 3.8ppm	7.5ppm	15ppm	30ppm
Oxygen Consumption	2.6×10^{-7}				
Number		4	5	6	5
Mean		+2.0	+7.0	4.5	57
Standard Deviation		4.4	2.9	2.1	3.2
Oxygen Evolution	1.6×10^{-6}				
Number		4	5	6	5
Mean		9.0	26	47	88
Standard Deviation		11	7.0	4.9	9.0

+ indicates stimulation of rate

TABLE V

EFFECT OF 24 HOUR EXPOSURE TO NAPHTHALENE ON RESPIRATION AND APPARENT PHOTOSYNTHESIS IN STATIONARY PHASE *CHLORELLA PYRENOIDOSA*

Variable	Typical Control Rate nmole O_2/cell min.	% Inhibition vs Ethanol Control 3.8ppm	7.5ppm	15ppm	30ppm
Oxygen Consumption	1.4×10^{-7}				
Number		4	5	5	5
Mean		+2.5	+1.4	+2.2	3.4
Standard Deviation		2.5	8.0	5.9	6.5
Oxygen Evolution	5.8×10^{-7}				
Number		4	5	5	5
Mean		6.2	14	0.0	12
Standard Deviation		3.2	6.0	9.0	6.3

+ indicates stimulation of rate

TABLE VI

EFFECT OF 7 DAY EXPOSURE TO NAPHTHALENE ON RESPIRATION AND APPARENT PHOTOSYNTHESIS IN STATIONARY PHASE *CHLORELLA PYRENOIDOSA*

Variable	Typical Control Rate nmole O_2/cell min.	% Inhibition vs Ethanol Control 3.8ppm	7.8ppm	15ppm	30ppm
Oxygen Consumption	1.3×10^{-7}				
Number		3	2	3	3
Mean		10	4.5	7.0	+3.7
Standard Deviation*		3.7	3.0	2.2	9.0
Oxygen Evolution	3.0×10^{-7}				
Number		3	2	3	3
Mean		13	8.5	3.0	13
Standard Deviation*		3.6	1.5	6.7	3.7

\+ indicates stimulation of rate
* where number of trials = 2, "standard deviation" is actually variance

TABLE VII

EFFECT OF 24 HOUR EXPOSURE TO NAPHTHALENE ON RESPIRATION AND APPARENT PHOTOSYNTHESIS IN STATIONARY PHASE CHLORELLA PYRENOIDOSA CELLS DILUTED INTO FRESH MEDIA AND TESTED AFTER SEVEN DAYS

Variable	Typical Control Rate nmole O_2/cell min.	% Inhibition vs Ethanol Control 3.8ppm	7.5ppm	15ppm	30ppm
Oxygen Consumption	1.8×10^{-7}				
Number		2	2	2	2
Mean		0.0	0.0	+3.0	+3.0
Standard Deviation*		6	6	6	6
Oxygen Evolution	5.5×10^{-7}				
Number		2	2	2	2
Mean		+3.0	6.0	+12	10
Standard Deviation*		2	3	5	1

+ indicates stimulation of rate
* where number of trials = 2, "standard deviation" is actually variance

TABLE VIII

EFFECT OF ACUTE EXPOSURE TO ETHANOL ON RESPIRATION AND APPARENT PHOTOSYNTHESIS IN LOG PHASE *CHLORELLA PYRENOIDOSA*

Variable	Typical Nontoxicated Rate nmole O_2/cell min.	% Inhibition vs Nontoxicated Rate
Oxygen Consumption	7.7×10^{-7}	
Number		13
Mean		+7.8
Standard Deviation		4.9
Oxygen Evolution	3.1×10^{-6}	
Number		13
Mean		42
Standard Deviation		6.3

+ indicates stimulation of rate

TABLE IX

EFFECT OF ACUTE EXPOSURE TO NAPHTHALENE ON RESPIRATION AND APPARENT PHOTOSYNTHESIS IN LOG PHASE CHLORELLA PYRENOIDOSA

Variable	Typical Control Rate nmole O_2/cell.min.	% Inhibition vs Ethanol Control 3.8ppm	7.5ppm	15ppm	30ppm
Oxygen Consumption	8.3×10^{-7}				
Number		6	9	7	9
Mean		14	22	+20	+41
Standard Deviation		1.7	5.3	5.1	6.0
Oxygen Evolution	1.8×10^{-6}				
Number		6	9	7	9
Mean		2.0	20	15	42
Standard Deviation		4.7	4.5	5.0	6.3

+ indicates stimulation

TABLE X

EFFECT OF 24 HOUR EXPOSURE TO NAPHTHALENE ON RESPIRATION AND APPARENT PHOTOSYNTHESIS IN LOG PHASE *CHLORELLA PYRENOIDOSA*

Variable	Typical Control Rate nmole O_2/cell min.	% Inhibition vs Ethanol Control 3.8ppm	7.5ppm	15ppm	30ppm
Oxygen Consumption	2.2×10^{-7}				
Number		4	4	5	4
Mean		18	22	24	34
Standard Deviation		8.5	35	15	7.1
Oxygen Evolution	1.1×10^{-6}				
Number		4	4	5	4
Mean		+38	16	34	29
Standard Deviation		36	19	22	11

+ indicates stimulation of rate

TABLE XI

EFFECT OF 48 HOUR EXPOSURE TO NAPHTHALENE ON RESPIRATION AND APPARENT PHOTOSYNTHESIS IN LOG PHASE CHLORELLA PYRENOIDOSA

Variable	Typical Control Rate nmole O_2/cell min.	% Inhibition vs Ethanol Control 3.8ppm	7.5ppm	15ppm	30ppm
Oxygen Consumption	2.0×10^{-7}				
Number		2	2	2	2
Mean		2.0	27	29	53
Standard Deviation		20	9.0	2.0	4.0
Oxygen Evolution	1.0×10^{-6}				
Number		2	2	2	2
Mean		+13	+17	+4.0	24
Standard Deviation*		22	10	7	9

+ indicates stimulation of rate
* where number of trials = 2, "standard deviation" is actually variance

TABLE XII

EFFECT OF TWO HOUR EXPOSURE TO NAPHTHALENE ON RESPIRATION AND APPARENT PHOTOSYNTHESIS IN LOG PHASE CHLORELLA PYRENOIDOSA

Variable	Typical Control Rate nmole O_2/cell min.	% Inhibition vs Ethanol Control 3.8ppm	7.5ppm	15ppm	30ppm
Oxygen Consumption	6.7×10^{-7}				
Number		4	5	5	6
Mean		6.5	12	11	20
Standard Deviation		4.8	5.5	4.8	2.2
Oxygen Evolution	4.7×10^{-6}				
Number		4	5	5	6
Mean		7.2	16	45	75
Standard Deviation		4.0	1.8	4.6	3.0

TABLE XIII

EFFECT OF ACUTE EXPOSURE TO NAPHTHALENE ON RESPIRATION AND APPARENT PHOTOSYNTHESIS IN STATIONARY PHASE *CHLAMYDOMONAS MOEWUSII*

Variable	Typical Control Rate nmole O_2/cell min.	% Inhibition vs Ethanol Control 3.8ppm	7.5ppm	15ppm	30ppm
Oxygen Consumption	1.0×10^{-6}				
Number		2	2	2	2
Mean		+12	+12	+15	+34
Standard Deviation*		1.0	1.0	0.0	1.0
Oxygen Evolution	1.3×10^{-6}				
Number		2	2	2	2
Mean		+18	+15	+8.0	31
Standard Deviation*		5	4	6	6

\+ indicates stimulation of rate
* where number of trials = 2, "standard deviation" is actually variance

TABLE XIV

EFFECT OF ACUTE EXPOSURE TO ETHANOL ON RESPIRATION AND APPARENT PHOTOSYNTHESIS IN STATIONARY PHASE *CHLAMYDOMONAS MOEWUSII*

Variable	Typical Nontoxicated Rate nmole O_2/cell min.	% Inhibition vs Nontoxicated Rate
Oxygen Consumption	9.0×10^{-7}	
Number		5
Mean		11
Standard Deviation		3.2
Oxygen Evolution	1.5×10^{-6}	
Number		5
Mean		15
Standard Deviation		2.9

+ indicates stimulation of rate

TABLE XV

EFFECT OF ACUTE EXPOSURE TO NAPHTHALENE ON RESPIRATION AND APPARENT PHOTOSYNTHESIS IN LOG PHASE CHLAMYDOMONAS MOEWUSII

Variable	Typical Control Rate nmole O_2/cell min.	% Inhibition vs Ethanol Control 3.8ppm	7.5ppm	15ppm	30ppm
Oxygen Consumption	1.9×10^{-6}				
Number		7	5	6	6
Mean		+2.1	8.0	12	35
Standard Deviation		3.6	2.8	3.0	3.3
Oxygen Evolution	6.6×10^{-6}				
Number		7	5	6	6
Mean		12	34	43	60
Standard Deviation		3.3	6.8	7.5	6.5

+ indicates stimulation of rate

TABLE XVI

EFFECT OF ACUTE EXPOSURE TO ETHANOL ON RESPIRATION AND APPARENT PHOTOSYNTHESIS IN LOG PHASE *CHLAMYDOMONAS MOEWUSII*

Variable	Typical Nontoxicated Rate nmole O_2/cell min.	% Inhibition vs Nontoxicated Rate
Oxygen Consumption	1.8×10^{-6}	
Number		11
Mean		+4.5
Standard Deviation		2.6
Oxygen Evolution	7.9×10^{-6}	
Number		11
Mean		16
Standard Deviation		4.7

+indicates stimulation of rate

TABLE XVII

EFFECT OF TWO HOUR EXPOSURE TO NAPHTHALENE ON RESPIRATION AND APPARENT PHOTOSYNTHESIS IN LOG PHASE *CHLAMYDOMONAS MOEWUSII*

Variable	Typical Control Rate nmole O_2/cell min	% Inhibition vs Ethanol Control 3.8ppm	7.5ppm	15ppm	30ppm
Oxygen Consumption	1.8×10^{-6}				
Number		2	2	2	2
Mean		+20	+24	15	30
Standard Deviation*		8	3	4	1
Oxygen Evolution	7.0×10^{-6}				
Number		2	2	2	2
Mean		24	20	76	84
Standard Deviation		6	12	0	3

\+ indicates stimulation of rate
* where number of trials = 2, "standard deviation" is actually variance

TABLE XVIII

ABSORBANCE BY VARIOUS CONCENTRATIONS OF NAPHTHALENE IN ABSOLUTE ETHANOL AT DIFFERING WAVELENGTHS

λ nm	Absorbance of Naphthalene in Ethanol*		
	10ppm	20ppm	30ppm
310	.007	.012	.017
275	.121	.237	.343
235	.027	.046	.066

* absorbance is in absorbance units, A

TABLE XIX

AMOUNT OF ORIGINAL CONCENTRATION OF 30 ppm NAPHTHALENE REMAINING IN AN OPEN SYSTEM AFTER TIME

Elapsed Time Hours	% of Original Naphthalene Remaining	
	Trial One	Trial Two
22 1/4	26	
24 1/4	14	
25		12
48		7.0

APPENDIX B

FIGURES

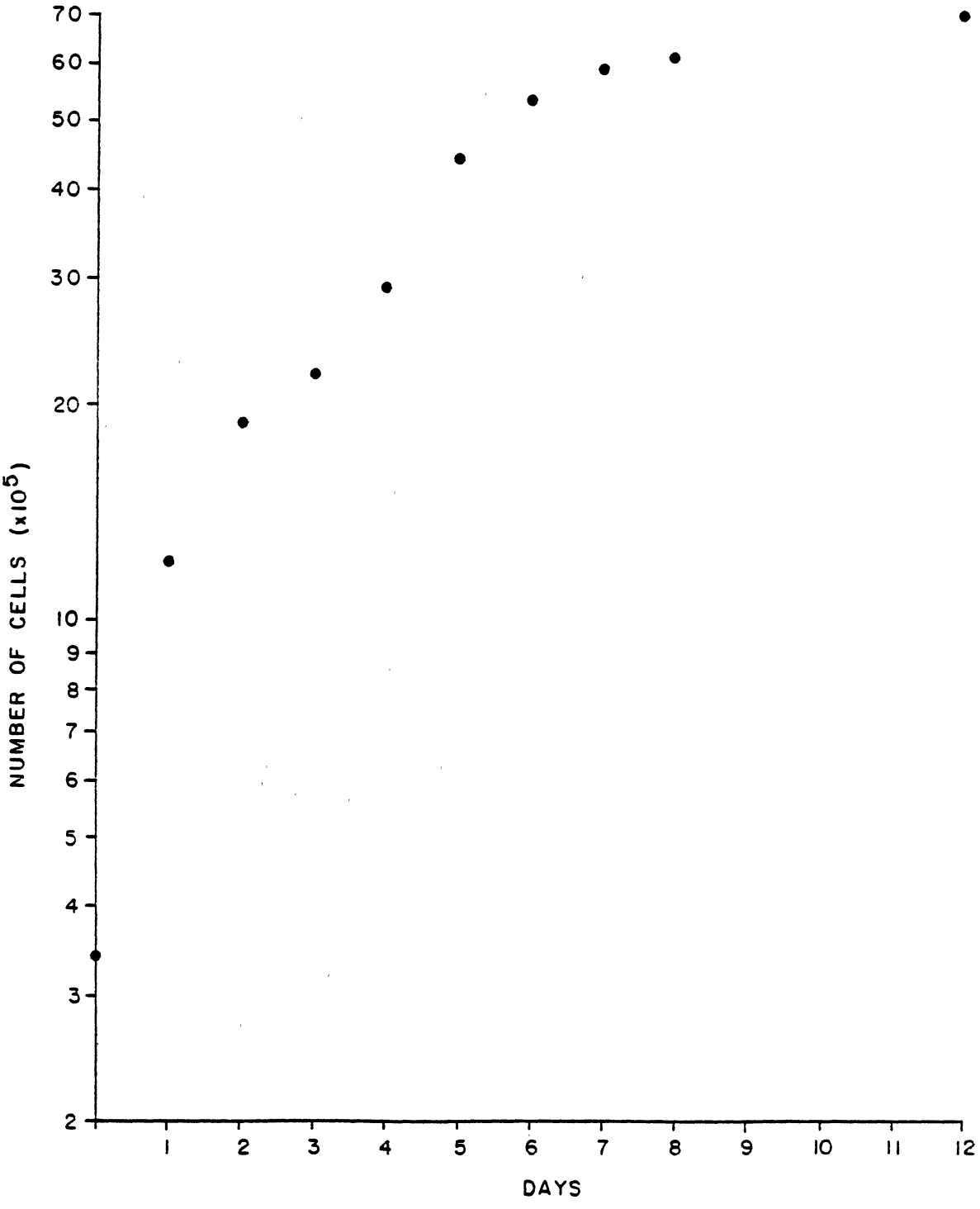


Figure 1. Growth Curve for Chlorella pyrenoidosa. Log Phase Cells Were Harvested on Day 2, Stationary Phase on Day 6, and Late Stationary Phase on Day 12.

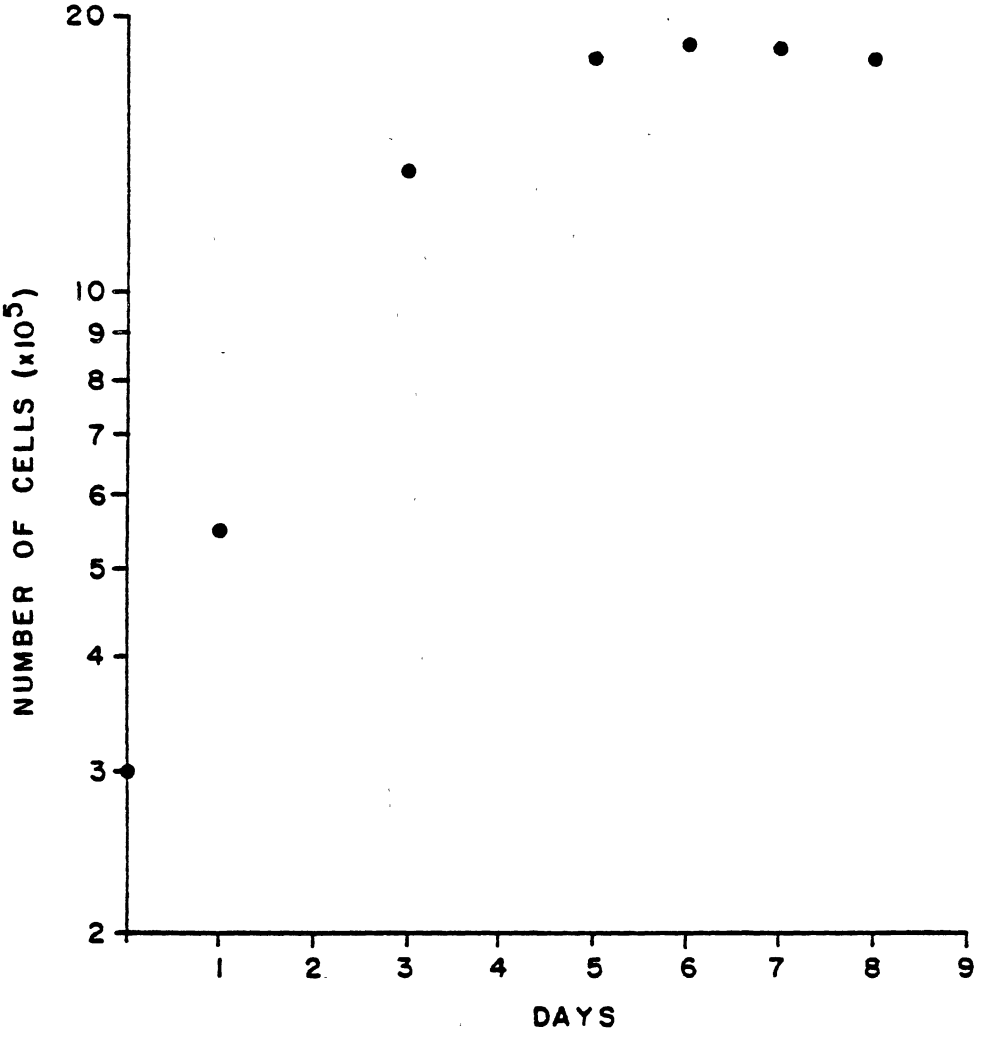


Figure 2. Growth Curve for Chlamydomonas moewusii. Log Phase Cells Were Harvested on Day 2 and Stationary Phase Cells on Day 6.

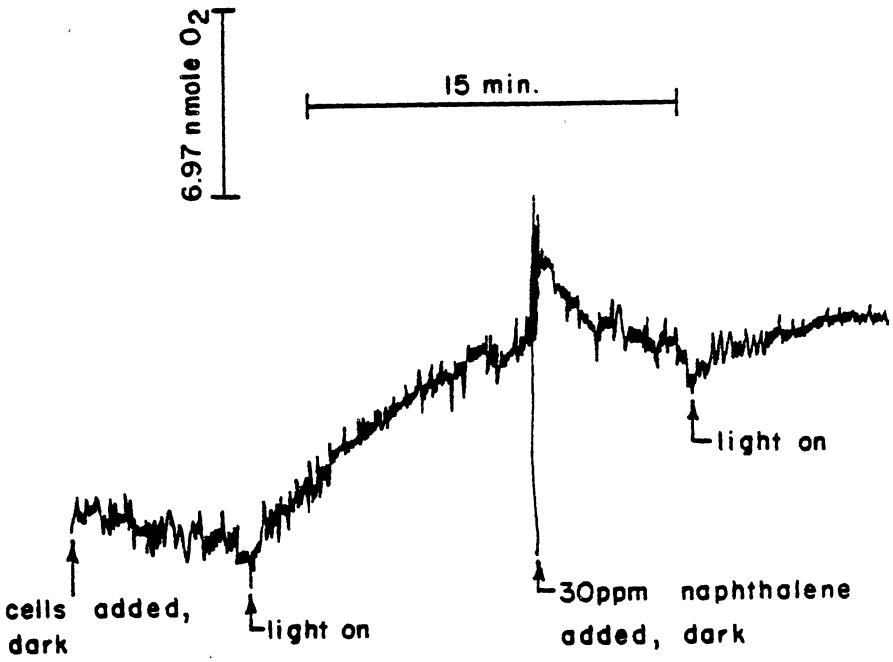


Figure 3. Trace of the Acute Effect of 30 ppm Naphthalene on the Oxygen Consumption and Evolution Activities of Log Phase Chlorella pyrenoidosa. Oxygen Consumption Is Indicated by the Trace Moving Down, Oxygen Evolution by it Moving Up.

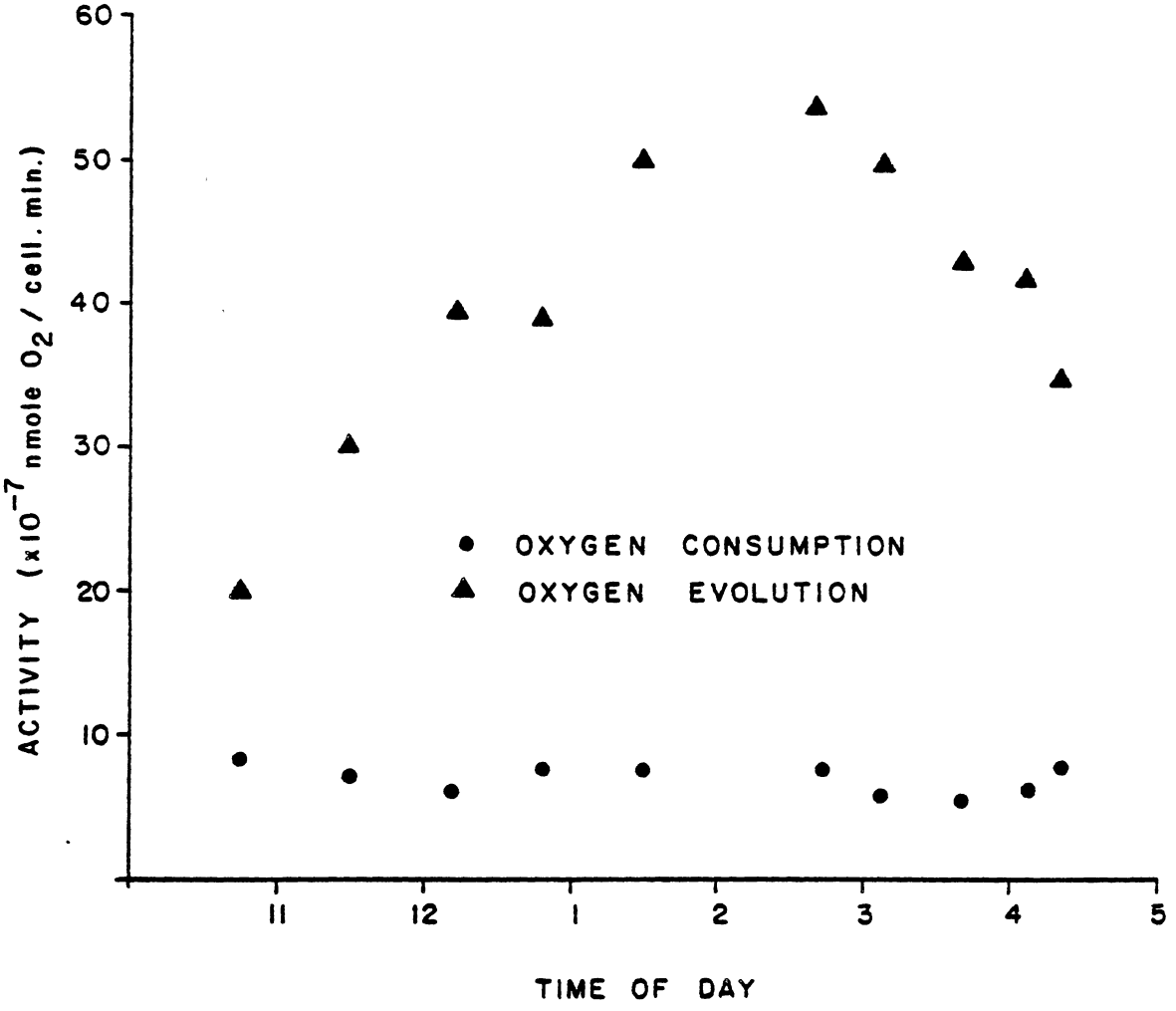


Figure 4. A Sample of the Typical Daily Variation in Rates of Respiration and Apparent Photosynthesis in the Log Phase Alga Chlamydomonas moewusii.

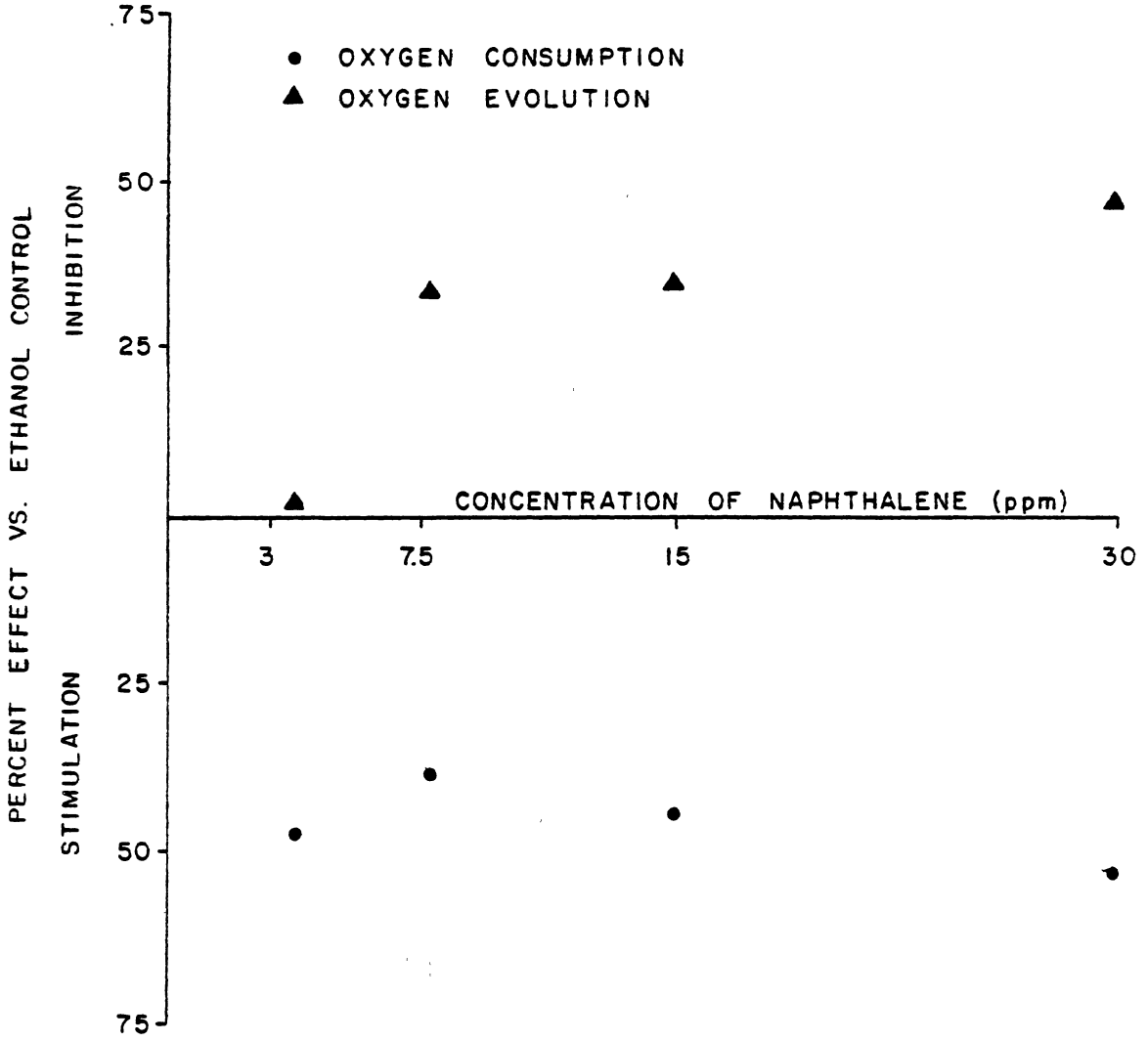


Figure 5. Effect of Acute Exposure to Naphthalene on Stationary Phase Chlorella pyrenoidosa.

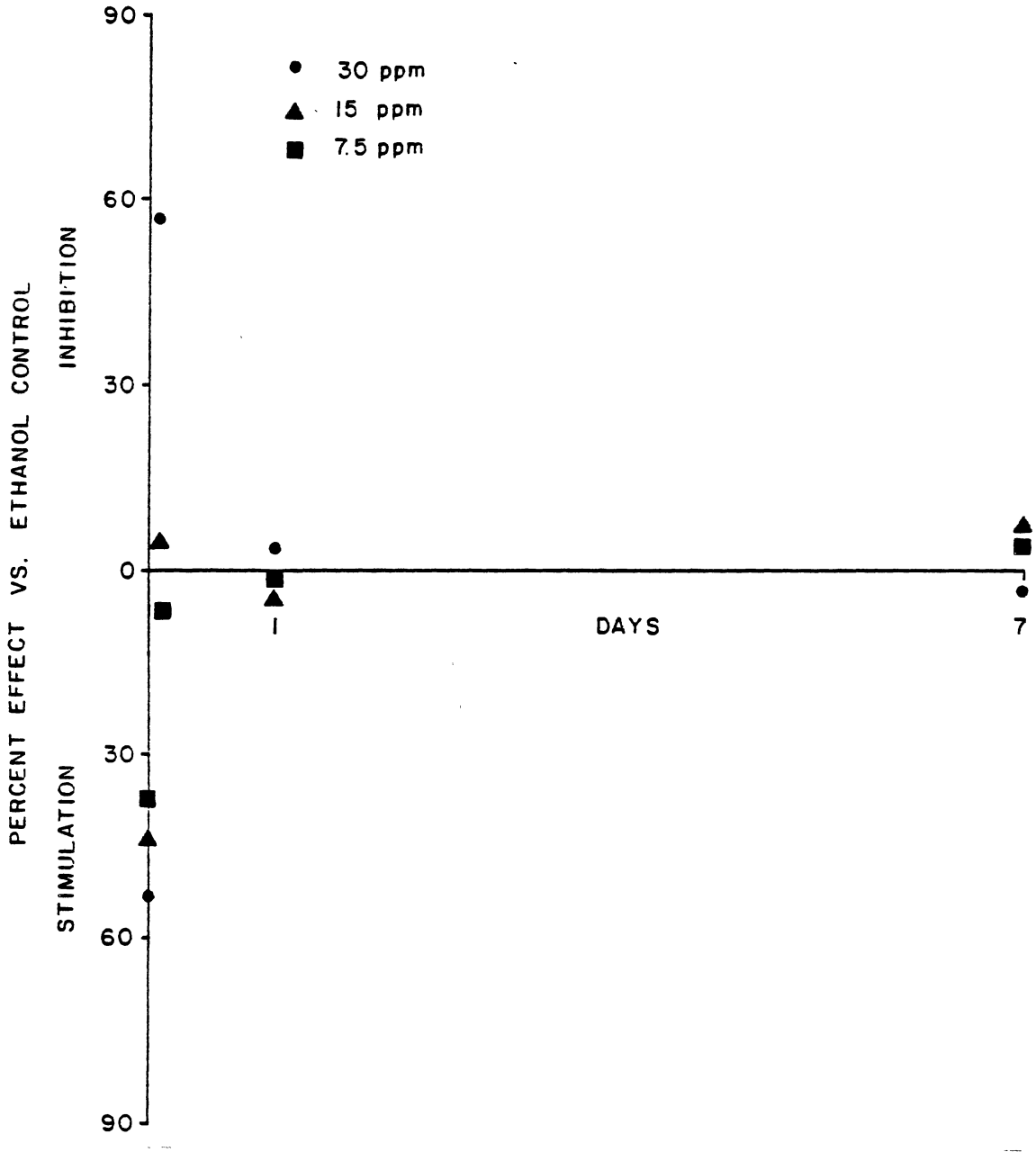


Figure 6. Effect of Chronic Exposure to Naphthalene on Respiration in Stationary Phase Chlorella pyrenoidosa.

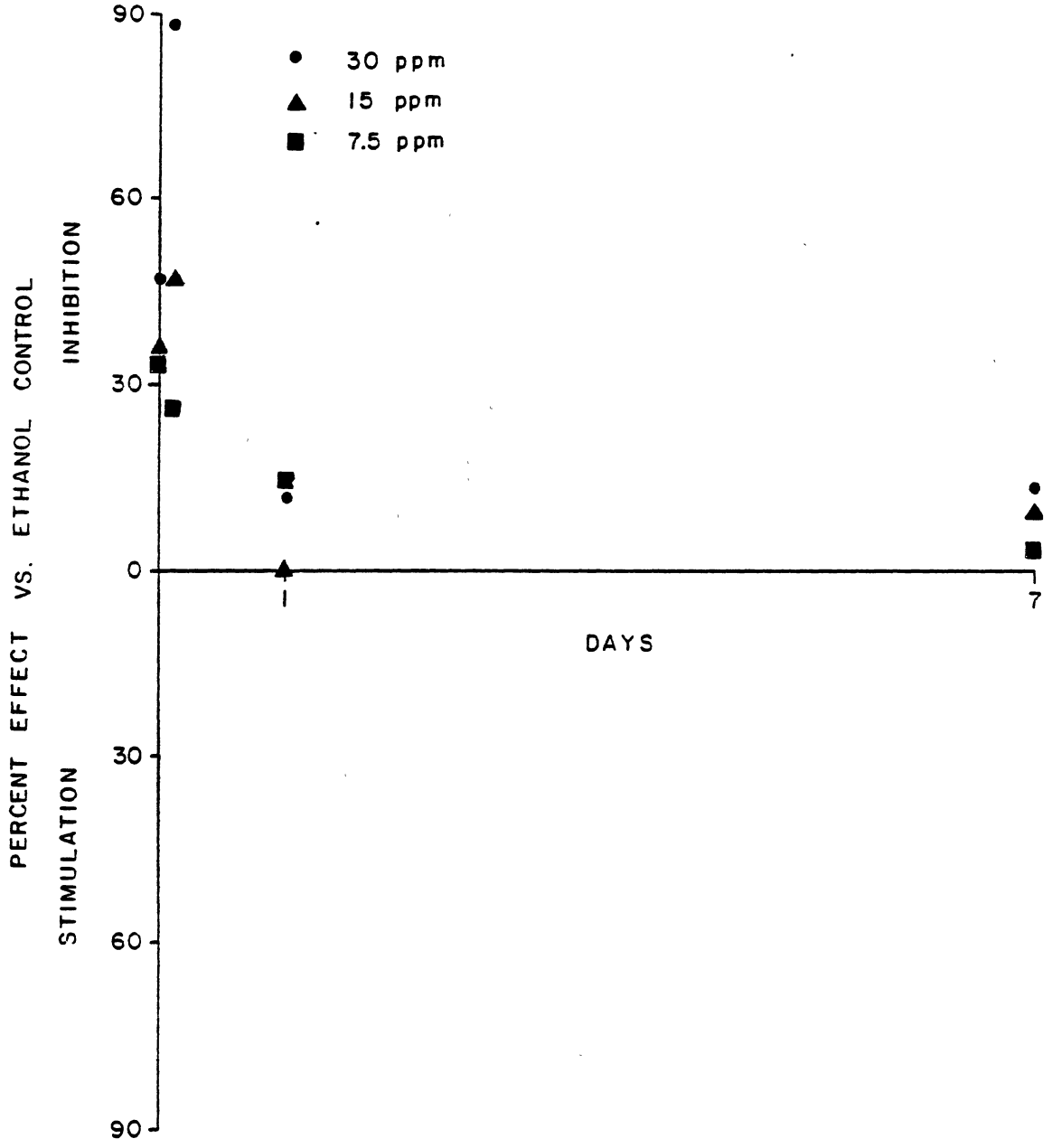


Figure 7. Effect of Chronic Exposure to Naphthalene on Apparent Photosynthesis in Stationary Phase Chlorella pyrenoidosa.

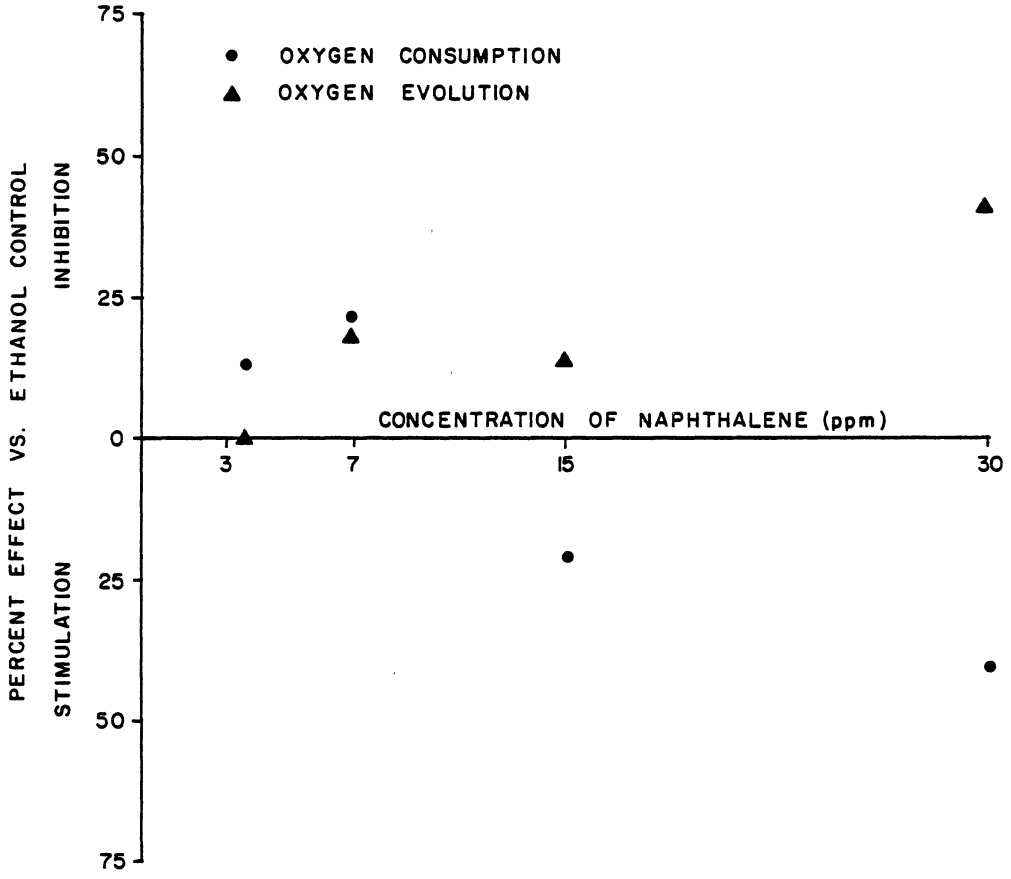


Figure 8. Effect of Acute Exposure to Naphthalene on Log Phase Chlorella pyrenoidosa.

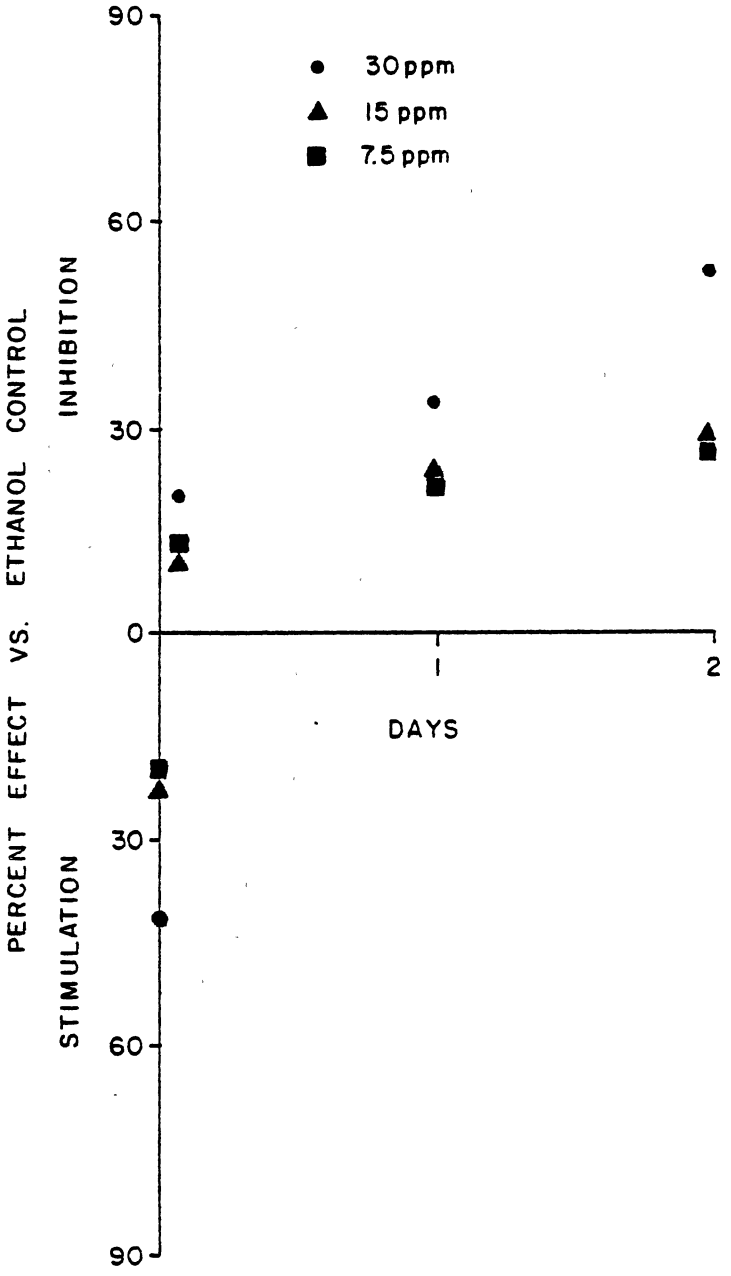


Figure 9. Effect of Chronic Exposure to Naphthalene on Respiration in Log Phase Chlorella pyrenoidosa.

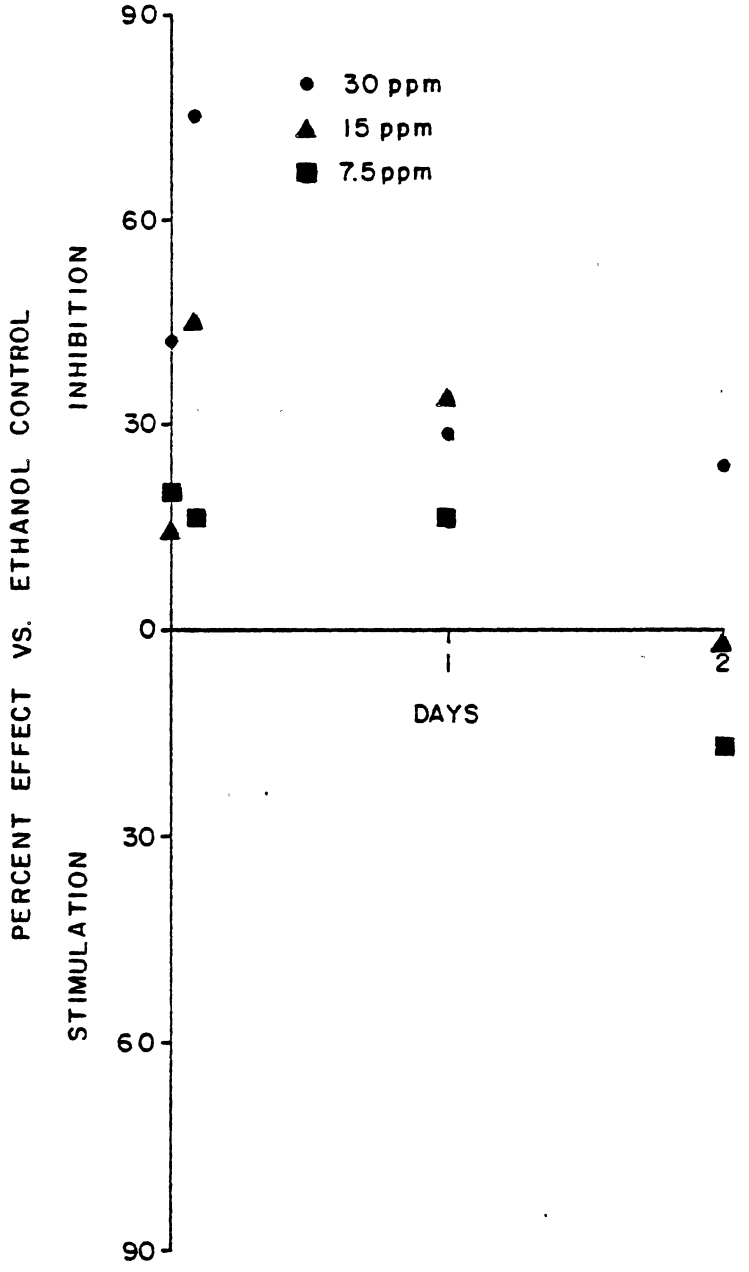


Figure 10. Effect of Chronic Exposure to Naphthalene on Apparent Photosynthesis in Log Phase Chlorella pyrenoidosa.

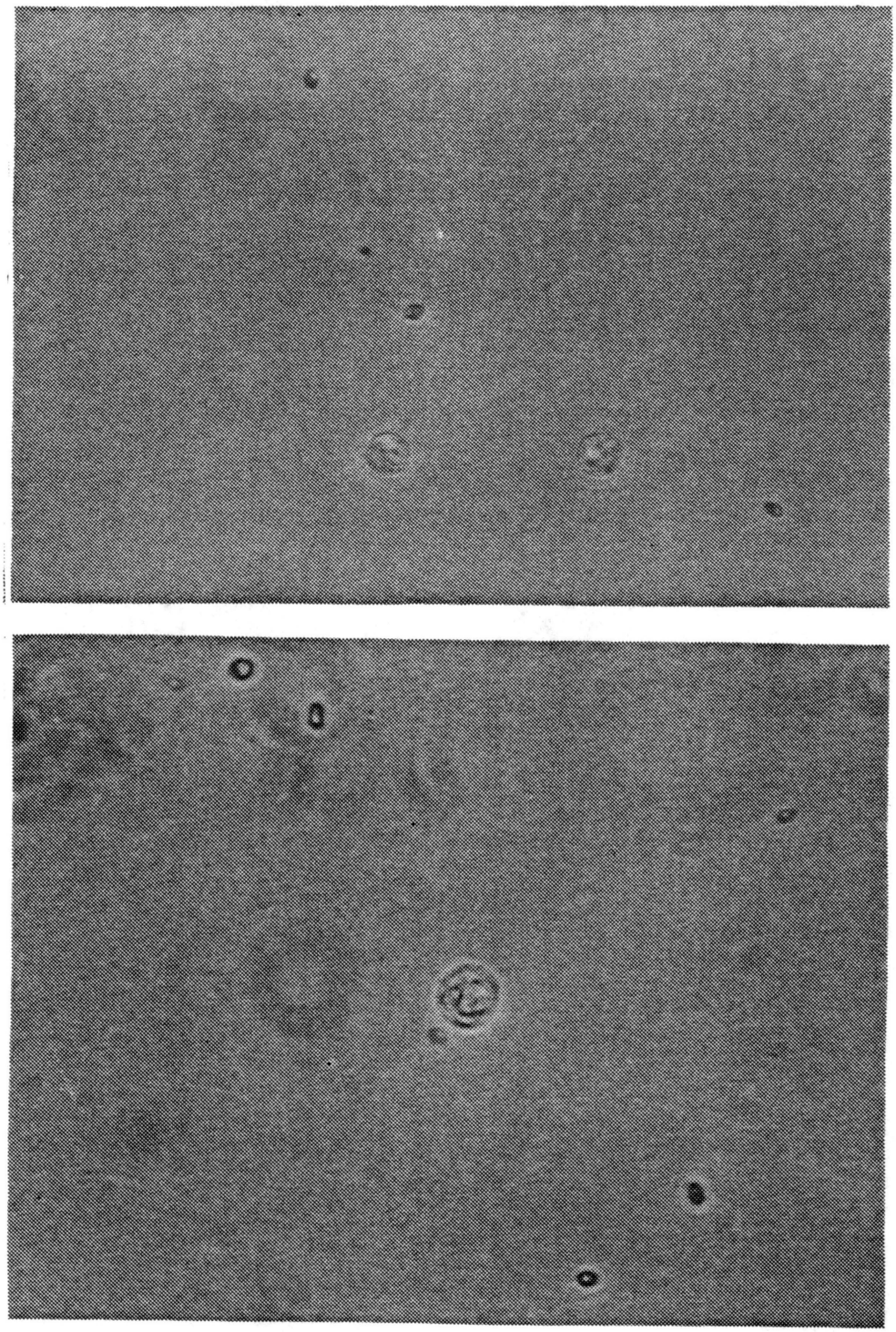


Figure 11. Effect of 48 Hour Exposure to 30 ppm Naphthalene on the Morphology of Log Phase Chlorella pyrenoidosa. Top: Cells Exposed to Ethanol. Bottom: Cells Exposed to Naphthalene in Ethanol.

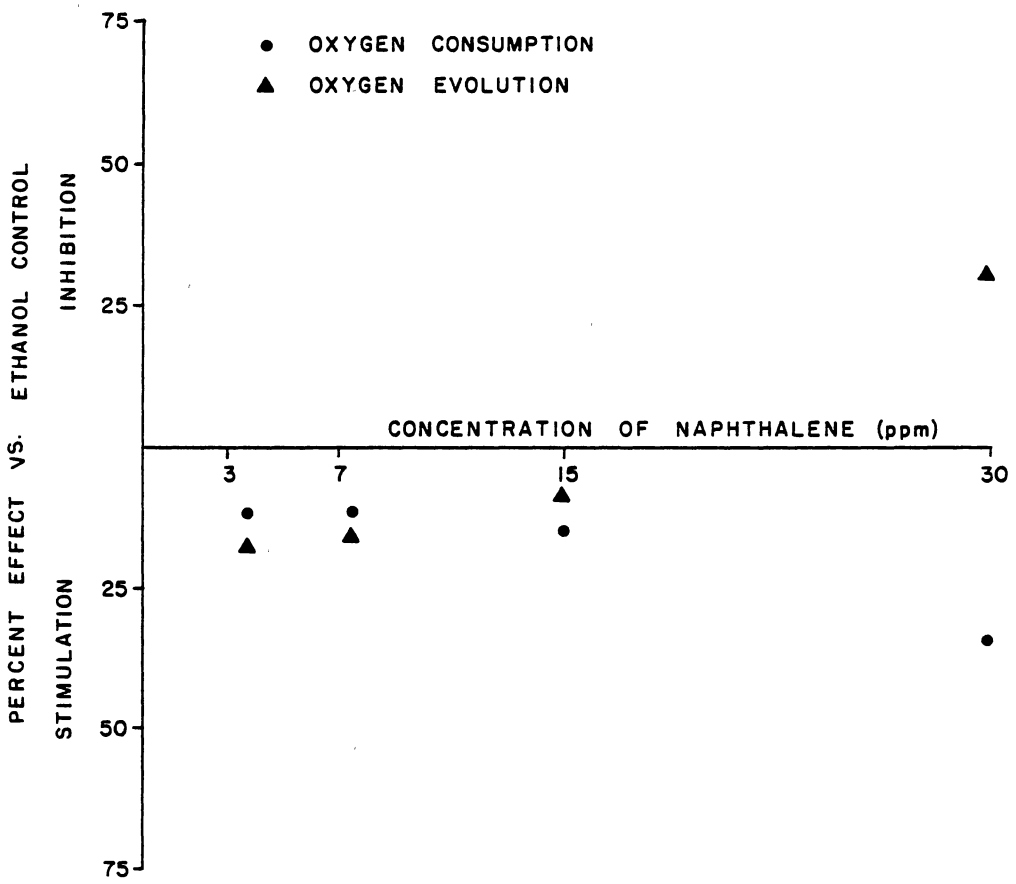


Figure 12. Effect of Acute Exposure to Naphthalene on Stationary Phase Chlamydomonas moewusii.

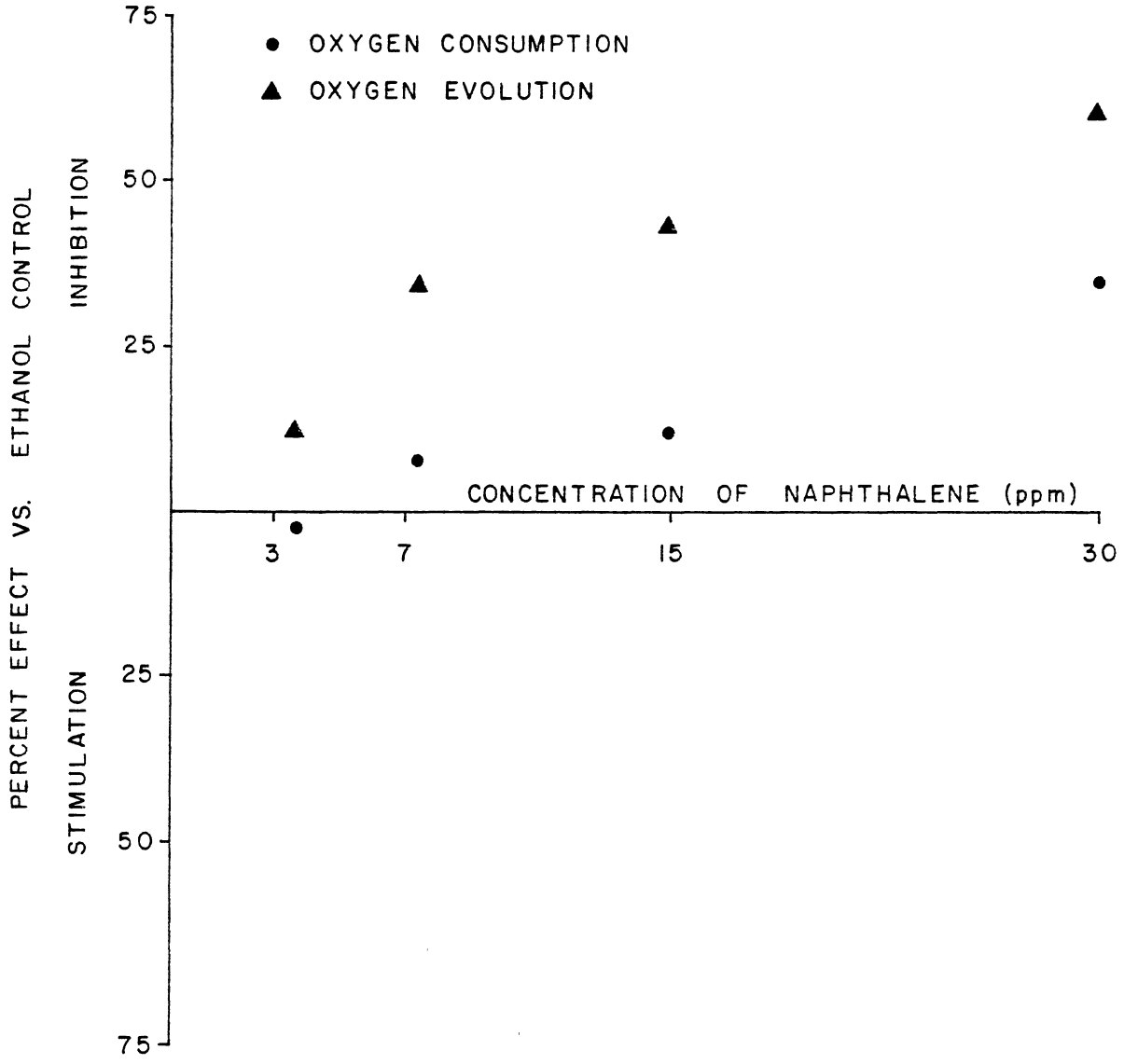


Figure 13. Effect of Acute Exposure to Naphthalene on Log Phase Chlamydomonas moewusii.

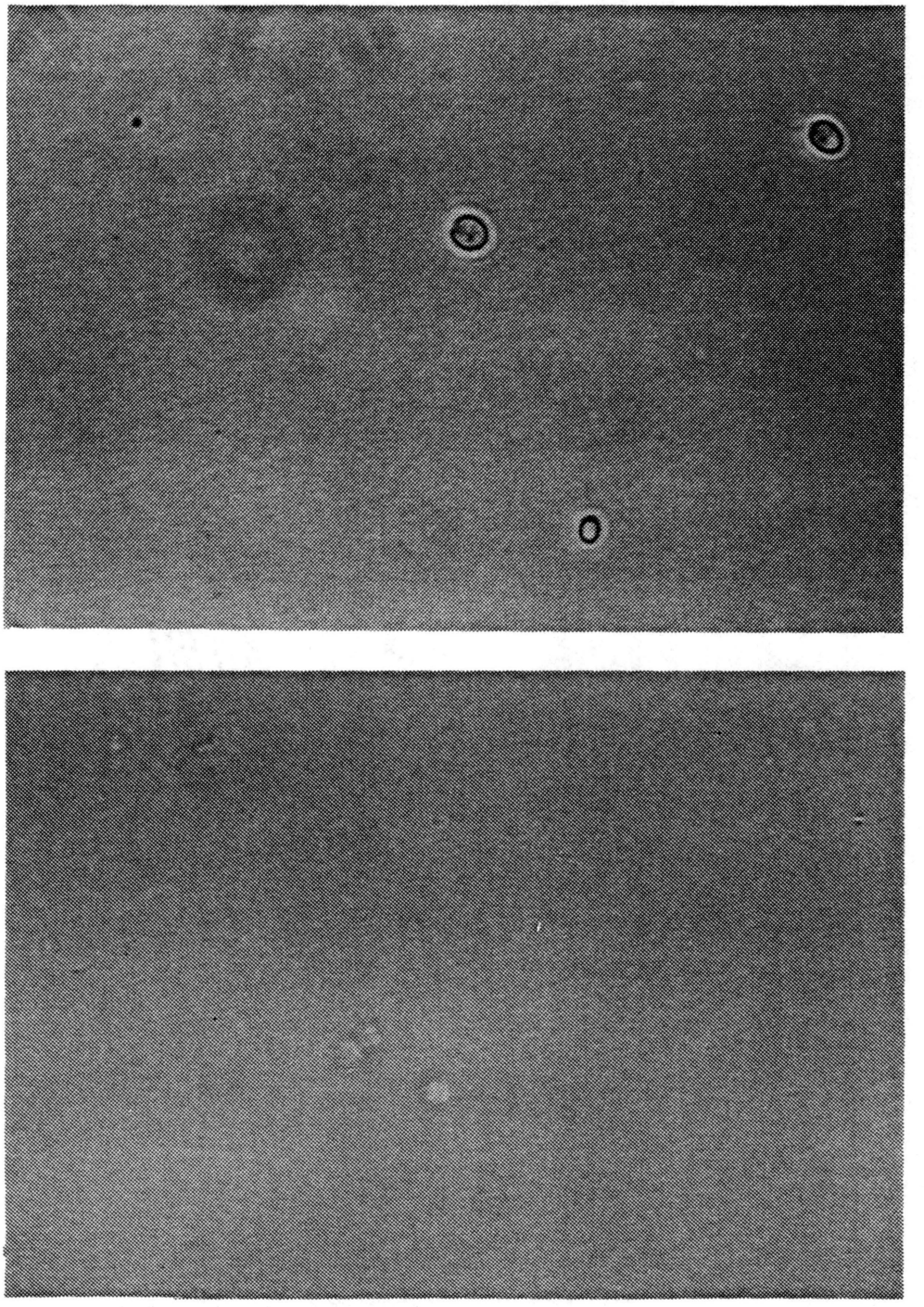


Figure 14. Effect of 48 Hour Exposure to 30 ppm Naphthalene on the Morphology of Log Phase Chlamydomonas moewusii. Top: Cells Exposed to Ethanol. Bottom: Cells Exposed to Naphthalene in Ethanol.

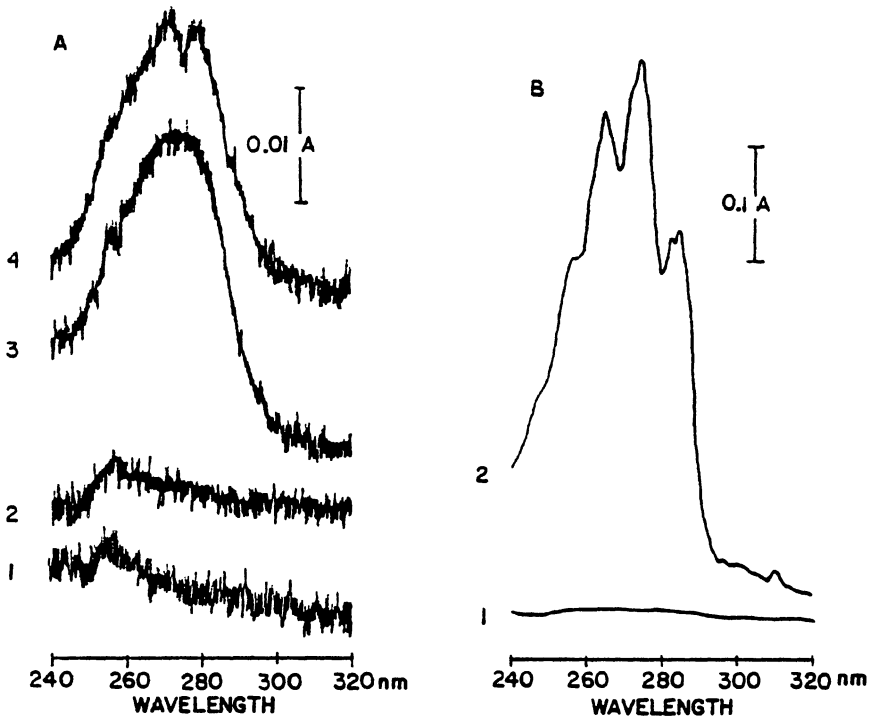


Figure 15. Ultraviolet Spectra of Naphthalene, Ubiquinone, and Ubiquinone Plus Naphthalene. A. Ultraviolet Absorbance Spectrum of Ubiquinone in the Presence and Absence of Naphthalene. Trace 1: Ethanol vs. Ethanol. Trace 2: 50 ppm Napahthalene vs. 50 ppm Naphthalene. Trace 3: Absolute Spectrum of 1.5×10^{-6} M Ubiquinone in Ethanol. Trace 4: Difference Spectrum of 1.5×10^{-6} M Ubiquinone in 50 ppm Naphthalene minus 50 ppm Naphthalene (Naphthalene/Q = 260). B. Ultraviolet Absorbance of Naphthalene . Trace 1: Ethanol vs. Ethanol. 2. Absolute Spectrum of 260 UM (41 ppm) Naphthalene in Ethanol.

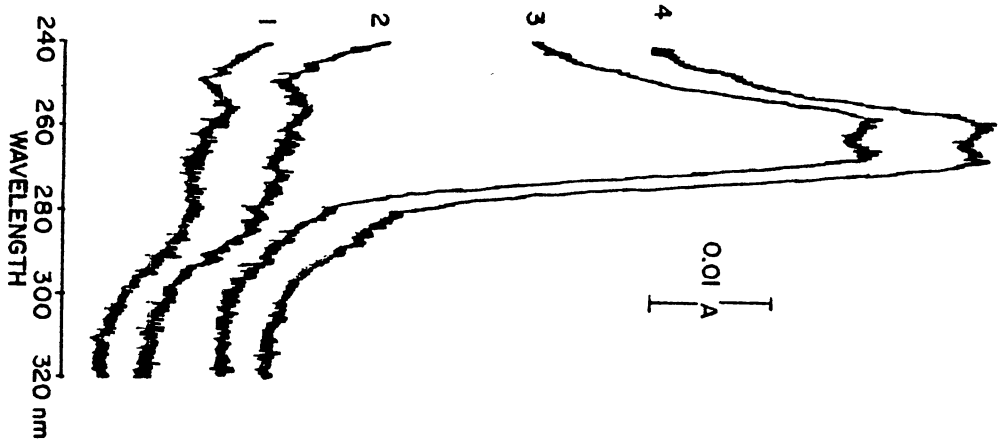
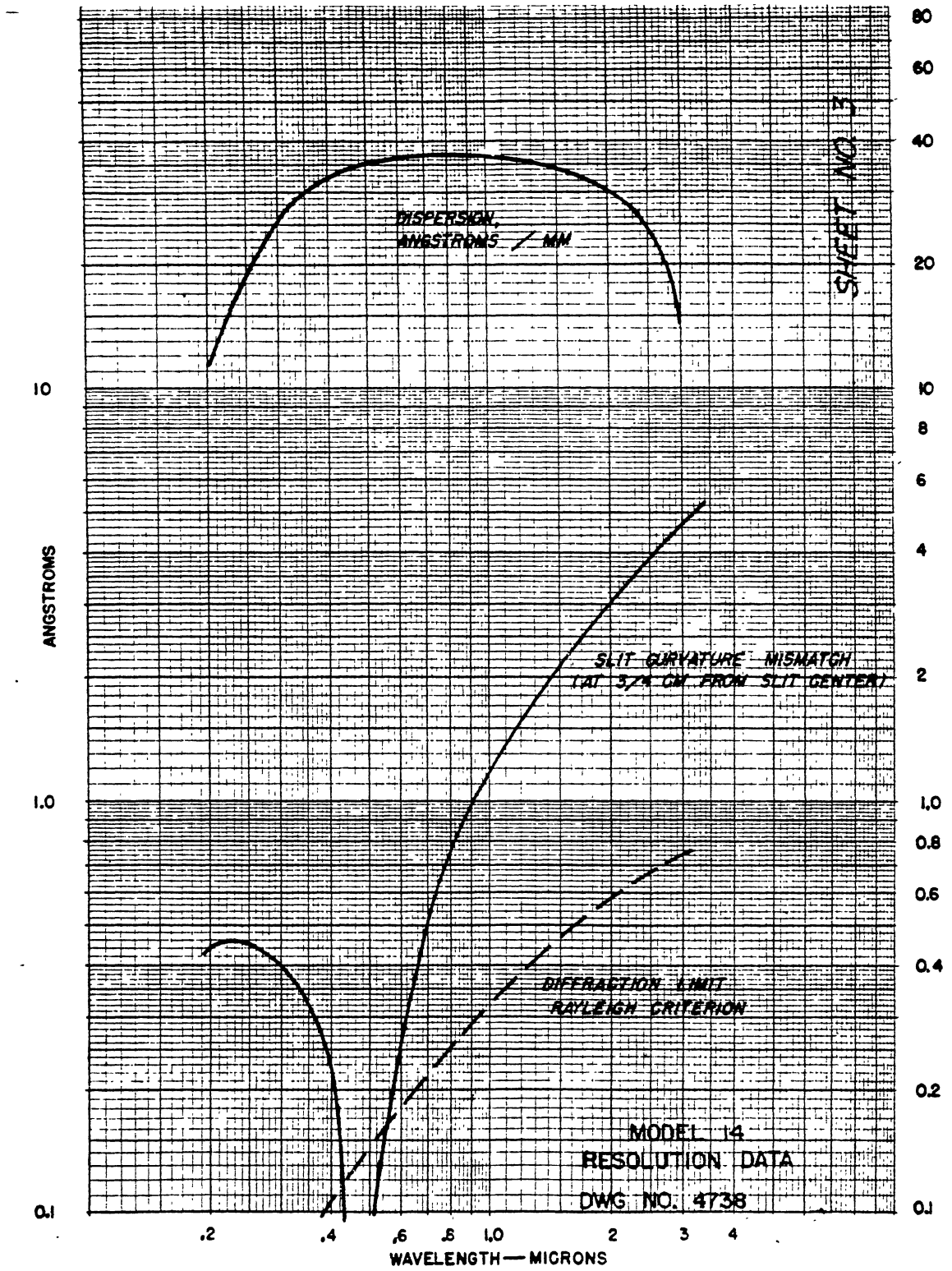


Figure 16. Ultraviolet absorbance Spectra of Duroquinone in the Presence and Absence of Naphthalene. Trace 1: Ethanol vs. Ethanol. Trace 2: 30 ppm Naphthalene. Trace 3: Absolute Spectrum of 3.0×10^{-7} M Duroquinone in Ethanol. Trace 4: Difference Spectrum of 3.0×10^{-7} M Duroquinone in 30 ppm Naphthalene Minus 30 ppm Naphthalene (Naphthalene/Q = 780).

APPENDIX C

GRAPH FOR DETERMINATION OF RESOLUTION FOR CARY 14 SPECTROPHOTOMETER


DISPERSION, ANGSTROMS / MM
SLIT CURVATURE MISMATCH (AT 5/4 CM FROM SLIT CENTER)
DIFFRACTION LIMIT RAYLEIGH CRITERION
MODEL 14
RESOLUTION DATA
DWG NO. 4738
SHEET NO. 5
ANGSTROMS
WAVELENGTH — MICRONS
80
60
40
20
10
8
6
4
2
1.0
0.8
0.6
0.4
0.2
0.1
.2
.4
.6
.8
1.0
2
3
4

VITA

Valerie Grace Struble

Candidate For The Degree Of

Master Of Science

Thesis: EFFECT OF NAPHTHALENE ON OXYGEN CONSUMPTION AND EVOLUTION IN CHLORELLA PYRENOIDOSA; A POSSIBLE MOLECULAR BASIS FOR INHIBITION OF MITOCHONDRIAL RESPIRATION BY NAPHTHALENE

Major Field: Zoology

Biographical:

Personal Data: Born in Seattle, Washington, June 28, 1952, the daughter of George and Grace Miller. Married to Stanley L. Struble on February 7, 1981.

Education: Graduated from Woodrow Wilson High School, Long Beach, California in June 1970. Attended Texas Christian University, the University of Texas, the University of Texas Permian Basin and received Bachelor of Science Degree in Microbiology from Oklahoma State University in May, 1982; completed requirements for the Master of Science degree at Oklahoma State University in December, 1985.

Professional Experience: Lab Technician at the Pawnee Municipal Hospital July 1, 1980 to January 1981; Lab Technician, Oklahoma State University January 1981 to December 1981; Para-professional Research Assistant May 1982 to July 1982, Presidential Fellow in Water Research July 1982 to July 1983; National Science Foundation Fellow May 1983 to present.