THE EFFECT OF EIGHT POLYAROMATIC HYDROCARBONS ON THE GROWTH, NITROGEN FIXATION CAPACITY AND ULTRASTRUCTURE OF *ANABAENA FLOS-AQUAE*

By

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CHAPTER I

INTRODUCTION

Polyaromatic hydrocarbons (PAH) are ubiquitous in the environment. They are produced in nature by forest and prairie fires, volcanoes, and as a degradation product of organic material. The latter has led to their formation in fossil fuels (Eglenton and Murphy, 1969; Blumer and Youngblood, 1975; Ilnitsky et al., 1977). Polyaromatic hydrocarbons are endogenously synthesized in some plants (Andelman and Snodgrass, 1974; Borneff, 1968). They are also synthesized by bacteria in association with marine algae (DeLima-Zanghi, 1968; Niassat et al., 1969). Anthropogenic sources include coal refuse banks, coke production, residential fireplaces, automobiles, commercial incinerators, oil-fired boilers, rubber tires, bitumen and asphalt (Hangebrauck et al., 1967; National Academy of Sciences, 1972; Andelman and Snodgrass, 1974; Siebert et al., 1978).

Polyaromatic hydrocarbons may enter lakes and streams in industrial and domestic effluents, storm water or leachate from soil and vegetation. Until the beginning of the twentieth century, PAH production was balanced by natural degradation. Anthropogenic production has overwhelmed degradation processes leading to the accumulation of these

compounds in the environment at an increasing rate (Suess, 1976).

The Environmental Protection Agency (EPA) has designated PAH as a class of chemicals which are potentially harmful to plants and animals in the environment (Dominguez, 1977). Some PAH are known animal carcinogens and bacterial mutagens (Andelman and Suess, 1970; Anderson, 1978; Connors, 1975; Clar, 1964).

Most studies investigating the toxicity of PAH to algae have focused on the most water soluble members of this class of compounds, particularly naphthalene (Giddings, 1980; Pulich, 1974; Soto et al., 1975). These studies indicate that the effects of PAH may vary among species of algae. Naphthalene inhibited the growth of blue-green algae and green algae (Giddings, 1979, 1980; Soto, 1975b). Phenanthrene inhibited carbon uptake by green algae and was inhibitory to the growth and carbon uptake of blue-green algae (Giddings, 1979, 1980). Paradoxically, Pulich (1974) reported that phenanthrene only slightly inhibited the growth of blue-green algae and that naphthalene and phenanthrene had little effect on the growth of green algae.

Acute toxicity to aquatic organisms is often associated with the water soluble fraction of an organic mixture (Pulich, 1974; Soto et al. 1975; Giddings, 1980). Little is known about the toxicity to algae of high molecular weight, relatively insoluble PAH (Batterton, 1978; Hutchinson et al., 1979). The toxicity of these compounds is a source of

concern because they are readily incorporated into plant and animal tissues, especially lipids.

Algae are often the major group of photosynthetic organisms in a lake or stream. It is important to determine the effects of toxicants on algae because they often serve as a primary source of energy in aquatic food webs. Thus, it is important to develop effective methods to determine algal toxicity.

The objective of this research was to determine the effects of eight PAH compounds: acenaphthene, benzanthracene, chrysene, fluoranthene, fluorene, naphthalene, phenanthrene, and pyrene, on the growth and nitrogen fixation capacity of the blue-green alga, Anabaena flosaquae. The Algal Assay Procedure Bottle Test (AAP) (EPA, 1971; Miller et al., 1978) was used as a protocol for the experiments investigating effects on growth. Acetylene (C_2H_2) reduction was used to assess the effects of selected PAH compounds on nitrogen (N_2) fixation. Toxicant-induced changes in cellular ultrastructure were also investigated by transmission electron microscopy (TEM). Data from AAP and C_2H_2 reduction experiments were used to draw general conclusions about the suitability of these assays for general use as acute toxicity tests.

The AAP has been designated as a standard method for ecological effects testing under the provisions of the Toxic Substances Control Act (EPA, 1971; Fed. Reg. 44, 1979; Miller et al., 1978). This test measures the acute toxicity

of substances to the growth of algae and reflects potential ecological effects on primary production in a lake or stream. In this bioassay, growth is determined after 14 days as the maximum standing crop under standardized conditions (Miller et al., 1978).

Initially the AAP was developed as an assay for nutrient enrichment of the aquatic environment (Miller and Maloney, 1971; Miller et al., 1974; Payne, 1975; Green et al, 1976; Shiroyama et al., 1976). The test has also been used to assay the toxicity of a diverse array of potential xenobiotics (Bartlett et al., 1974; Klotz et al., 1975; Maloney and Miller, 1975; Vyas, 1975; Won et al., 1976; Chiaudani and Vighi, 1978;).

Giddings (1980b) reviewed some of the disadvantages of the AAP as an acute toxicity bioassay. The 14 day test period is much longer than those required for other acute toxicity tests (e.g. Daphnia and Pimephales) (Fed. Reg. 44, 1979). A shorter test period facilitates the assay of more potential toxicants in the same amount of time. Also, during 14 days chemicals may volatilize from solution or photo-oxidize to a derivative compound. A variety of static culture bottle effects may develop. As cultures age, algal metabolism changes; possibly changing the response of the organism to a toxicant (Fogg, 1975). Metabolites are excreted which alters the chemical milieu of the media which in turn may alter toxicant solubility and partitioning equilibria (Fogg, 1975). In addition, axenic conditions are

difficult to maintain.

All these factors are inherent sources of variability in the interaction of the organism and the test chemical. An established toxicity bioassy should feature highly standardized experimental design (Giddings, 1980). The AAP does not provide adequate control of the test conditions.

Acetylene reduction to ethylene (C_2H_4) is a measure of the capacity of an organism to fix atmospheric nitrogen (N_2) and reduce it to an assimilable form. The primary advantage of the reduction assay is its speed. The chemical exposure and quantitative chromatographic analysis of C_2H_4 can be completed ~~in~~ in a few hours. Because of the short exposure period and closed experimental system, the effect of the parent compound is tested rather than a deviated compound produced by physical changes and the need for sterile procedure is reduced.

The effects of toxicants on the C_2H_2 reduction capacity of blue-green algae are likely to be similar in other N_2-fixing organisms. One hundred-twenty five strains of blue-green algae, 50 strains of photosynthetic bacteria, and nine strains of chemoautotrophic bacteria are able to reduce N_2 (Stewart, 1980). All biochemical evidence indicates identical protein and substrate requirements for the reduction of C_2H_2 and N_2 by the nitrogenase enzyme (Streicher and Valentine, 1973). No non-nitrogen fixing organisms have ever been shown to reduce significant amounts of C_2H_2 (Hardy et al., 1968). Nitrogenase from

free-living bacteria, photosynthetic bacteria, and blue-green algae have been separated into two similar components. Some combinations of interspecific components of the enzyme when recombined in vitro actively reduce C_2H_2 (Smith, 1974; Streicher and Valentine, 1973). This evidence indicates that toxicants which inhibit C_2H_2 reduction in blue-green algae may inhibit nitrogen fixation in other organisms.

Acetylene reduction assays may thus be used to assess the effect of toxicants on the nitrogen cycle (Fed. Reg. 44 1979). Nitrogen fixation can be a significant input of nitrogen into the biota in a number of environments (Wetzel, 1975). Fixation by soil bacteria is a major source of nitrogen in that environment (Wetzel, 1975). The contribution of N_2-fixation to the nitrogen budget of an aquatic system is dependent upon the nutrient conditions and organic loading of the system. In meso-eutrophic or eutrophic lakes where nitrogen is limiting, N_2-fixation by blue-green algae can be a major component of the nitrogen budget. Nitrogen fixation accounted for 40% of the annual budget in eutrophic Clear Lake, Ca.; 17.8% in eutrophic Lake Mendota, Wisc.; and 13% in eutrophic Chernoye Lake, USSR (Brezonik and Lee, 1968; Wetzel, 1975; Kuznetsov, 1959). Nitrogen fixing bacteria, such as Clostridium, exist in the upper layer of sediments. These heterotrophic bacteria can contribute significantly to the nitrogen budget when dissolved carbon concentrations are high (Wetzel, 1975). Finally, it is

known that the nutrient conditions of a particular lake change during the course of the year. At times when nitrogen is limiting but other nutrients particularly phosphorus, are sufficient, N_2-fixation may constitute a major portion of the nitrogen budget. After an ice melt in a small subartic lake, Billaud (1968) found that N_2 fixed by Anabaena flos-aquae accounted for 25% of the nitrogen assimilated by the plankton.

Six of eight chemicals tested inhibited C_2H_2 reduction during a 2 h exposure in this study. Because of this finding, exposed cells were observed by TEM to determine if the chemicals affected morphological changes in cellular ultrastructure. The ultrastructure of heterocysts and vegetative cells in blue-green algae have been studied and it seemed possible to recognize untreated cells as controls (Fay and Lang, 1971; Kulasooriya, 1972; Lang, 1965; Pankratz and Bowen, 1963; Wildon and Mercer, 1963a,b). Transmission electron microscopy is not suggested as a routine method for toxicity testing but it does provide a tool to corroborate functional change by observing structural change.

CHAPTER II

Material and Methods

Cell Cultures

Unialgal cultures of Anabaena flos-aquae were obtained from the EPA Environmental Research Laboratory Corvallis, Oregon and the University of Texas, (UTEX 37). Stock cultures were grown in AAP algal media at 29°C and 200-400 foot candles (ft.c.) of continuous light (EPA, 1971, Miller et al., 1978). Cultures were checked monthly for contamination by common species of bacteria and fungi by streaking cultures on bacto-agar plates and incubating plates for two weeks (M. Grula, personal communication). Stock cultures were not contaminated as determined by this method. Healthy cells were maintained by weekly aseptic transfers.

Cell Density

The light transmission (%T) through cell cultures was measured by a Bausch and Lomb 20 Spectrophotometer (λ = 680 nm). Cells from the same culture were also counted with a Nikon Model 102 Light Microscope using a hemocytometer grid. Cell counts and % T values were fitted to a linear regression to produce an equation for a conversion of % T readings to cell density (cells ml^{-1}):

$$Y = 2.77\ (\%T) + 274.7, \qquad (1)$$

where Y is cells $ml^{-1} \times 10^5$. The statistics for the regression were N=8, r=0.865.

Toxicant Solubility and Degradation

Toxic chemicals were solubilized in the following manner for each test described. Excess, analytical grade chemicals (Aldrich Co.) were added to sterile media and stirred by a magnetic stirrer for 24-30 h. Undissolved crystals were allowed to settle for 2 h.

Toxicant-saturated and non-toxic media were added (v/v) to 125 ml Erlenmeyer flasks to yield 100, 50, 20, 10 and 0% saturated solutions (50 ml total volume). These solutions represented the approximate treatment levels for each growth experiment. A 90% solution was substituted for the 100% solution in the C_2H_2 reduction and TEM experiments to facilitate the addition of a large inoculum. Toxicant concentrations in solution were determined by fluorescence analysis using a Aminco Bowman Spectrophotofluorometer (Table I) (McKay and Shiu, 1977; Schwarz and Wasik, 1976). Standards were prepared by dissolving a known weight of each compound in ethanol and diluting the solution with algal media to the suspected concentration range. Fluorescent spectra of PAH in water and in ethanol are similar (Schwarz and Wasik, 1976).

TABLE I

SPECTROPHOTOFLUORMETER SETTINGS FOR ANALYSIS OF PAH CONCENTRATIONS IN MEDIA

Chemical	Excitation		Emmission	
	λ(nm)	slitwidth(mm)	λ(nm)	slitwidth(mm)
Acenaphthene	293	1.0	333	3.0
Benzanthracene	289	1.0	402	3.0
Chrysene	305	4.0	405	5.0
Fluoranthene	361	1.0	454	3.0
Fluorene	270	1.0	312	3.0
Naphthalene	290	0.2	330	3.0
Phenanthrene	252	1.0	360	3.0
Pyrene	336	1.0	384	3.0

Flasks containing each level of toxicant and toxicant-free controls were incubated without cells at 25°C and 200 ± 10 ft.c. Flasks were stoppered with cotton and thus were open to gas exchange. The fluorescence of the solutions was measured at 7 days and, if necessary, 14 days to determine degradative loss of the compound. The 7 day and 14 day predicted concentrations (C) of each level was used to calculate the % change in concentration over that period

(formula 1):

$$\% = \frac{C_{Day\ x}\ (\mu g\ liter^{-1}) \times 100}{C_{Day\ O}\ (\mu g\ liter^{-1})}, \qquad (2)$$

where the $C_{Day\ x}$ is the concentration on the seventh or fourteenth day and $D_{Day\ o}$ is the concentration at the beginning of the experiment.

Maximum Standing Crop

(MSC)

The methodology of these bioassays was a modification of AAP protocols (EPA, 1971; Miller et al., 1978). Cells were exposed to toxicants in 125 ml cotton-stoppered flasks containing 52 ml of solution. Sterile toxicant and non-toxic media were asepticly pipetted into flasks to give 96, 48, 19, 10 and 0% saturated solutions with initial cell densities of 10,000 cells ml^{-1}.

Flasks were placed in Percival Co. E-30B or E-54U environmental chambers according to a randomized block design and incubated for 14 d at 25°C and 200 $\pm$ 10 ft.c of continuous light. Each treatment and the unexposed control consisted of three replicates. Flasks were shaken by hand daily to facilitate gas exchange. On the fourteenth day cultures were harvested on preweighed 0.45 µ Millipore filters and their dry weights (mg $liter^{-1}$) were measured (Miller et al., 1978).

Acetylene Reduction

Log Phase Growth of Nitrogen Fixing Cultures

In order to characterize the pattern of growth of cultures actively fixing N_2, Anabaena flos-aquae was inoculated into media containing a small amount of nitrate (1 μM liter^{-1} NO_3). The nitrate was incubated to allow a gradual transistion from nitrogen-sufficient to nitrogen-free conditions. Algal growth was measured for six days using daily absorbance readings. The absorbance readings were converted into the number of doublings in optical density from the beginning of the experiments (Sorokin, 1979):

$$\blacktriangle = \log_2 O.D_T + 10 - \log_2 O.D_0 + 10 \qquad (3)$$

where

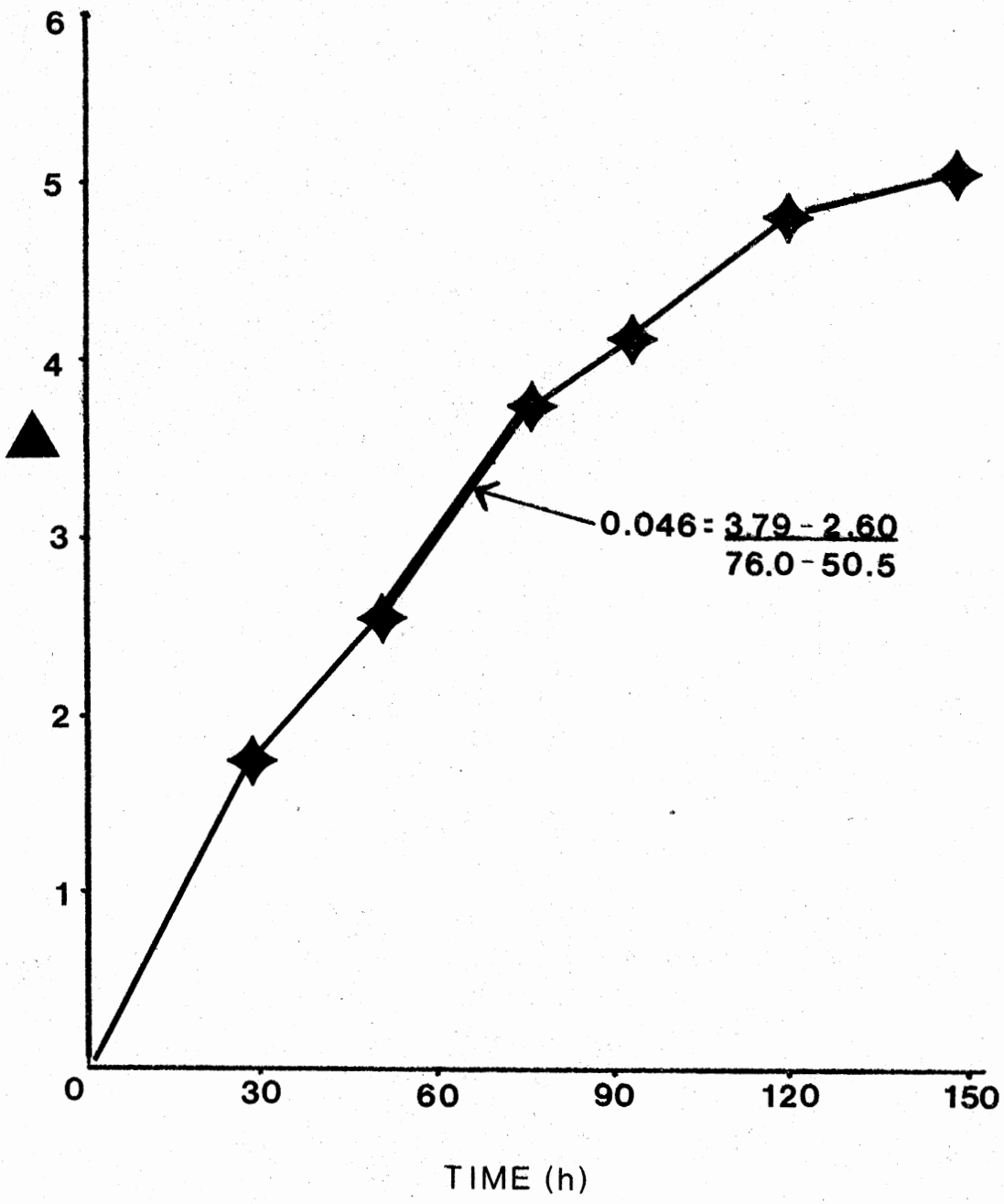
▲ = number of doublings in optical density (O.D.)

$O.D._T + 10$ = optical density at time of measurement plus ten to convert negative logarithms to a positive logarithm.

$O.D._0 + 10$ = optical density at beginning of experiment converted to a positive logarithm.

Delta (▲) values were plotted against time and the points connected by a line (Figure 1). The portion of the line with the steepest slope indicated the period of exponential growth. The rate of exponential growth was calculated as:

Figure 1. Growth of Nitrogen Fixing *Anabaena flos-aquae* Culture.


6
5
4
3
2
1
0
30
60
90
120
150
TIME (h)
0.046 = 3.79 - 2.60 / 76.0 - 50.5

$$R_E = \frac{\blacktriangle 2 - \blacktriangle 1}{t_2 - t_1} \quad (4)$$

where

R_E = exponential growth rate as increase in optical density per unit time.

$\blacktriangle 2, \blacktriangle_1,$ = delta values which were endpoints of the portion of the line with the steepest slope

t_2, t, = times corresponding to $\blacktriangle_2$ and $\blacktriangle_1$.

This experiment showed that log phase growth occurred between 51 and 76 h. The results of this experiment are supported by the findings of Kulasooriya et al. (1972) who demonstrated a rapid increase in nitrogenase activity in Anabaena cylindrica following 44 h of nitrogen starvation. Cell counts on two separate occasions showed that log phase cultures contain 5-7% heterocyts (Table II). As a result of these experiments all C_2H_2 reduction experiments were conducted between 51 and 76 h after Anabaena flos-aquae was inoculated into nitrogen limiting media.

Ethylene Production as a Function of Time

The inhibition of C_2H_2 reduction by toxicants could be measured reliably only for the period in which the amount of C_2H_4 production increased at a constant rate. Two experiments were conducted during which C_2H_4 reduction

TABLE II

HETEROCYST FREQUENCY DURING NITROGEN LIMITED LOG PHASE GROWTH

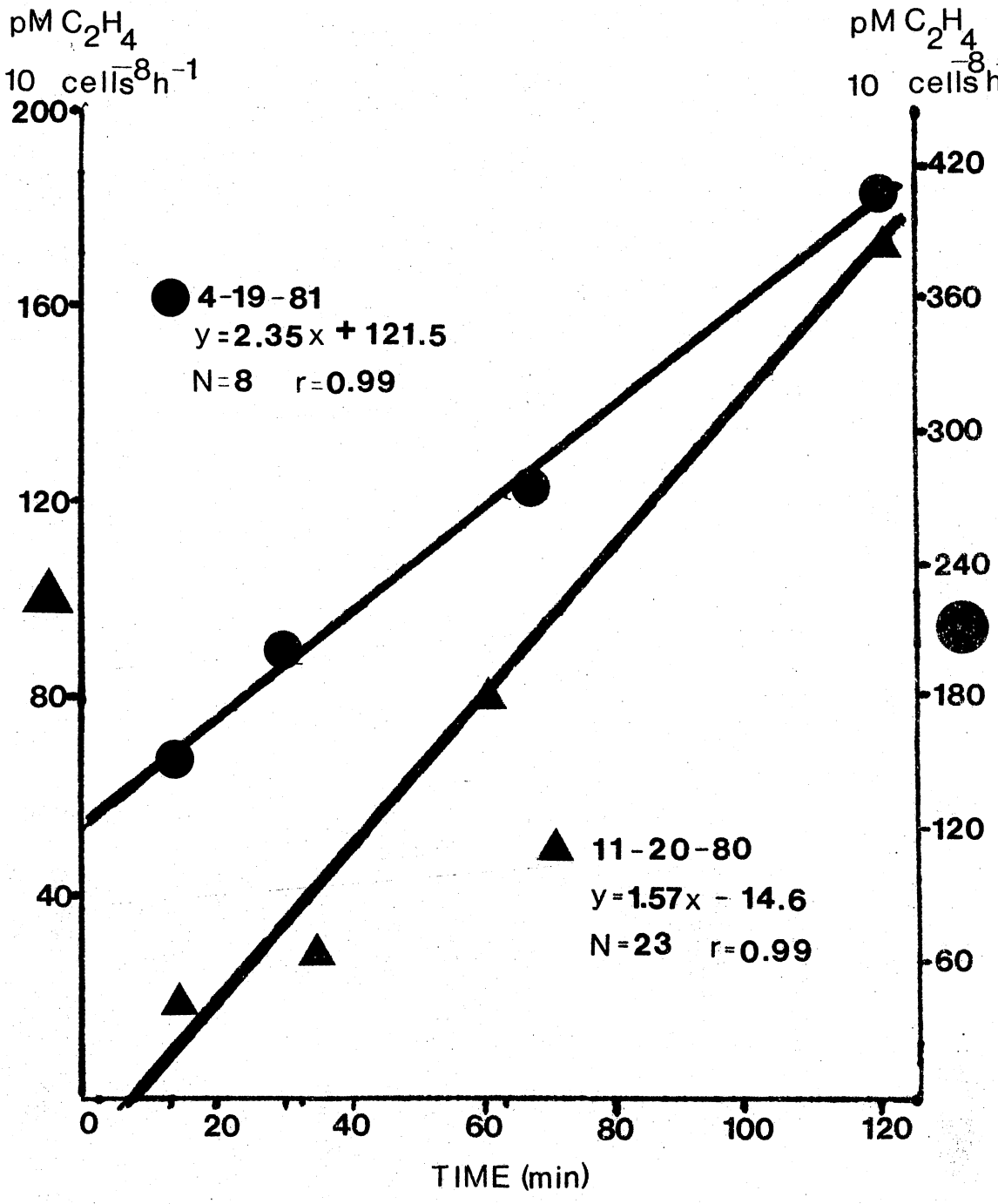
Date	Heterocysts/100 Total Cells	X	S.D.
11-5-80	6, 5, 5, 8, 3, 6, 5, 5, 3, 5, 5	5	1.38
6-16-81	5, 6, 13, 6, 5, 5, 7, 8, 5, 5, 5, 5, 6, 5, 10, 8, 10, 7, 4	7	2.35

was measured at approximately 15, 30, 60 and 120 min. Ethylene production increased in a linear fashion for 2 h (Figure 2). Thus, 120 minutes was used as the time period for all C_2H_2 reduction experiments. Longer C_2H_2 reduction periods were not tested.

Acetylene Reduction Experiments

These tests were a modification of methods used by Stewart et al. (1976) and Granhall and Lundgren (1971). Nitrogen-free toxicant-saturated and non-toxic media were mixed (v/v) to yield 0, 10, 20, 50 and 90% saturated 50 ml solutions in 72 ml serum bottles. Anabaena flos-aquae cells in log phase were added to each treatment and the control to yield a 1.5×10^5 cell ml^{-1} cell density. Five

Figure 2. Ethylene Production is a Function of Time by Anabaena flos-aquae.


pM C_2H_4
10^{-8} cells^{-1} h^{-1}
pM C_2H_4
10^{-8} cells^{-1} h^{-1}
200
160
120
80
40
420
360
300
240
180
120
60
4-19-81
y = 2.35x + 121.5
N = 8 r = 0.99
11-20-80
y = 1.57x - 14.6
N = 23 r = 0.99
0
20
40
60
80
100
120
TIME (min)

ml of air was removed and 5 ml of C_2H_2 was added to produce a pC_2H_2 of about 0.2 atm. Dinitrogen and C_2H_2 compete for reduction by the nitrogenase enzyme. More than 0.1 atm. pC_2H_2 completely inhibits N_2 fixation (Granhall and Lundgren, 1971). Air was not flushed from incubation bottles in order to prevent volatilization of toxicants, particularly naphthalene. Blanks containing non-toxic media and C_2H_2 but without cells, were included in each experiment to detect C_2H_2 contamination in the C_2H_4. Blank flasks were included in each experiment. Combinations of toxicant plus cells, toxicant plus C_2H_2, and toxicant plus trichloroacetic acid did not produce C_2H_4.

Four replicates of each treatment, the control, and the blanks were incubated in a randomized block design according to the MSC test protocol for 120 $\pm$ 5 min. Reduction was stopped after 120 min. by the addition of 2 ml of 50-60% trichloroacetic acid.

Ethylene production was detected using a Hewlett Packard 5750 Research Chromatograph using hydrogen flame detector. A 1.8 m. x 3 mm. aluminum column containing Porapak T (Supelco Co.) was used to separate the sample components. Ethylene peaks were identified and quantified by co-chromotography with C_2H_4 standards. Purified C_2H_2, C_2H_4 and the N_2 carrier gas were obtained commercially (Matheson Co.).

Chlorophyll a Extraction

The original units of the C_2H_2 reduction experiments are pM C_2H_2 (10 cells h)$^{-1}$. Because Anabaena flos-aquae cells were difficult to count, results are often presented in units of chlorophyll a (chl a) rather than numbers of cells, i.e. as pM C_2H_2 µg chl a^{-1} h^{-1} (James Ownby, personal communication). Chlorophyll a was extracted from cultures grown under experimental conditions so that results of C_2H_2 reduction experiments could be converted to more standard units.

Two cell densities (1.5 x 10^5 cells/ml and 3.0 x 10^5 cells ml^{-1}, 50 ml volume) of Anabaena flos aquae grown in nitrogen limiting media were harvested during log phase growth. Three replicates of each cell density were harvested on Reeve Angel 984 H ultra filters. Each filter was placed in 10 ml of 2:1(v/v) chloroform-methanol solution in a sealed bottle. Chlorophyll was extracted for 4 h in the dark.

Cholorphyll fluoresecence was measured by a Turner Model III Fluorometer using Wratten 47A and 2A excitation filters and a Wratten 26A emmission filter. Five chl a standards, three replicates per standard solubilized in the chloroform-methanol solution were used to calibrate the fluorometer. The equation for the calibration was:

$$y = 114.85 x - 1.289, \quad (5)$$

where x was the concentration of chl a (mg liter^{-1}) and y was the relative fluorescence. Statistics for the calibration regression were N=15, r=0.984.

A portion of each replicate of extracted chl a was diluted 3:1 and (2:2)(v:v) with chloroform-menthanol solution to yield 18 samples for the fluorescence measurement of chl a. The regression equation to convert cell density (cells liter^{-1}) to chl a (mg liter^{-1}) was:

$$y = 0.00179\ x - 0.0028, \qquad (6)$$

where x was 10^7 cells liter^{-1} and y was mg liter^{-1} chl a. The statistics for the regression were N=17, r= 0.988.

Transmission Electron Microscopy

Exposures

Chrysene, fluorene, naphthalene and phenanthrene were each solubilized in sterile N_2-free media. Anabaena flos-aquae in log phase and toxicant media were added to 500 ml exposure flasks to yield 8.6×10^5 cells ml^{-1} in 200 ml of 90% toxicant-saturated media. All glassware and media were autoclaved but transfers were not aseptic. Photomicrographs showed no visible contamination by bacteria. After a 24 h exposure according to the MSC Test protocol, cells were concentrated by centrifugation at 4030 g for 15 min.

Electron Microscopy

The fixation procedure was a modification of the method described by Fay and Lang (1971). Each sample was split

into two aliquots. On aliquot was fixed in 2% glutaraldehyde in 0.1 M, cacodylate buffer (pH 7.27) for 14 h and rinsed three times in 0.2 M, cacodylate buffer (pH 7.20). Aliquots were then fixed for 24 h in buffered 1% OsO_4 and washed in buffer wash three times, all at 4°C.

The second aliquot was fixed for 2 h in buffered 4% glutaraldehyde, rinsed in buffer, fixed for 12 h in aqueous, unbuffered 1% $KMnO_4$ and rinsed in distilled water, all at 4°C. All samples were dehydrated in a graded ethanol series and placed in a propylene oxide intermediary.

Aliquots were again split into two parts. One part was embedded in Spurr's resin (1969). The other part was embedded in Polybed resin (Polysciences Co.). Sections were cut with a Diatome diamond knife on a Sorvall MT-2 Ultramicrotome, mounted on 200 mesh nickel grids and post-stained according to embedding material. Polybed sections were post-stained with uranyl acetate and lead citrate, respectively, for four min. each (Venable and Coggeshall, 1965). Spurr's sections were post-stained for 30 min. with uranyl acetate and 20 min. for lead citrate.

CHAPTER III

RESULTS

Solubility and Degradation of Toxicants

The solubility of the test chemicals in algal media was in general agreement with literature values reported for PAH dissolved in distilled water (Table III). Chemicals were solubilized according to the methods of Mackay and Shiu (1977) so their values are the most valid for comparison. The rank of solubilities among chemicals was also comparable.

Toxicant solutions were prepared as 10, 20, 50 and 100% saturated solutions (Table IV). However, the measured concentrations of partially saturated solutions were different from the values that could be calculated as a percentage of 100% saturation. For example, the concentration of the 10% saturated solution of naphthalene was 2070 μg liter^{-1} rather than 3203 μg liter^{-1} (10% of saturated solution).

The saturation levels used in the MSC and C_2H_2 reduction experiments differed slightly from those used to measure toxicant disappearance from media. The concentrations of the treatments used in these tests were predicted from those measured during fluorescence analysis. For

TABLE III

COMPARISONS OF OBSERVED AND REPORTED TOXICANT CONCENTRATIONS

Chemical	Observed Concentration ($\mu g\ liter^{-1}$)	Literature Concentrations ($\mu g\ liter^{-1}$)
Acenaphthene	4619	3930[a]
1,2 Benzanthracene	30	14[a], 10[c]
Chrysene	14	2[a], 6[c]
Fluoranthene	434	265[c], 240[b] 260[a]
Fluorene	1134	1980[a]
Naphthalene	32028	12,500[c], 22,000[b] 31,700[a]
Phenanthrene	913	1600[c], 1290[a]
Pyrene	159	175[c], 165[b], 135[a]

a Mackay and Shiu, 1977

b Schwarz and Wasik, 1976

c Klevens, 1950

TABLE IV

CONCENTRATIONS OF PAH SOLUTIONS
PREDICTED FROM FLUORESCENCE

Chemical	Concentrations ($\mu g\ liter^{-1}$)					PAH Standards	
						Number of Standards, no replicates	r^2
Acenaphthene	0	422	956	2528	4619	6	0.965
Benzanthracene	0	5	9	19	30	8	0.977
Chrysene	0	1	2	5	14	5	0.996
Fluoranthene	0	38	101	234	434	5	0.999
Fluorene	0	134	260	612	1134	4	0.999
Naphthalene	0	2070	6250	15470	32028	4	0.982
Phenanthrene	0	134	268	485	913	7	0.985
Pyrene	0	22	58	85	159	4	0.987
% Saturation	0	10	20	50	100		

example, a 50% saturated solution of naphthalene contained 15470 μg liter^{-1}. The 48% saturated level used in the MSC test was predicted to contain 14851 μg liter^{-1}.

Acenaphthene, fluorene, naphthalene and pyrene almost completely disappeared from solution within seven days (Table V). The saturated solutions of benzanthracene and phenanthrene decreased in concentration by 85% and 77%, respectively, after 14 days. Chrysene and fluoranthene were the most resistant to degradation. The saturated solutions of these compounds decreased in concentration by 62% and 49%, respectively, after 14 days. It was not possible to measure the change in concentrations of partially saturated solutions of chrysene. Measurements of these concentrations may represent the lower limits of detectibility of the spectrophotofluorometer.

In general, smaller molecular weight compounds disappeared from solution more quickly than high molecular weight compounds. Pyrene was an exception to this observation. Some partially saturated solutions decreased in concentration at different rates relative to the fully saturated solution. Partially saturated solutions of benzanthracene decreased less in concentration than the fully saturated solution. Partially saturated solutions of fluoranthene did not change in concentration for seven days.

During 14 days the concentrations of all chemicals decreased. Previous investigators found that acenaphthene and naphthalene predominately volatilized from solutions.

TABLE V

DECREASES IN CONCENTRATION OF PAH AS A PERCENTAGE OF THE CONCENTRATION AT DAY ZERO

Chemical	Day	% Saturation			
		10	20	50	100
Acenaphthene*	7	93	93	89	90
	14	*	*	*	*
Benzanthracene	7	32	45	50	57
	14	49	69	81	85
Chrysene	7	--	--	--	38
	14	--	--	--	62
Fluoranthene	7	0	0	0	22
	14	19	50	31	49
Fluorene	7	94	95	95	92
	14	98	100	100	100
Naphthalene*	7	100	100	97	95
	14	*	*	*	*
Phenanthrene	7	64	67	73	67
	14	85	80	80	77
Pyrene*	7	99	99	99	99
	14	*	*	*	*

* Concentrations of acenaphthene, naphthalene and pyrene solutions were not measured at 14 days because of substantial disappearance after seven days.

The higher molecular weight compounds predominately photo-oxidized to more polar derivatives (Katz et al., 1979; Zepp and Scholtzhauer, 1979).

Statistical Anaylses

Each exposure level of a chemical and the control were considered separate treatments for the statistical analysis of the effect of a chemical on the growth or C_2H_2 reduction capacity of Anabaena flos-aquae. Analysis of variance was used to determine if the means of the treatment results were different. A 95% probability of rejecting the null hypothesis that the means of treatment results were not different was considered significant. The Duncan's Multiple Range Test was used to determine which individual means were different at the 95% probability level. More importantly, the Duncan's test showed which means were significantly different from the mean of the control treatment.

Analysis of variance was necessary to validate the Duncan's test. The variance associated with all the means was considered in calculating the significance level of the analysis of variance. All combinations of two means were compared in determining similar and dissimilar means by the Duncan's test. Any pair of means may be different at the 95% significance level and not be different at that level within the context of the variance associated with all the means. Therefore, the significance level used in the Duncan's test should also be used in the analysis of

variance.

Neither the results of the MSC tests or the C_2H_2 reduction tests justified regression or probit analysis to determine the dependency of the results on the concentrations of the toxicants. Fluorescence analysis showed that the concentrations of all the chemicals decreased during the MSC test so the initial concentrations were not valid predictors of effects at the end of the test. Because concentrations could not be measured for five of eight PAH after 14 days, concentrations present after 14 days could not be used as predictors of the results. Acetylene reduction was affected primarily by the most saturated solutions of the chemicals. Only naphthalene affected C_2H_2 reduction over a range of concentrations. Thus it was not necessary to analyze the C_2H_2 reduction results for their dependency on the concentrations of the treatments. (See Acetylene Reduction Tests Results.)

Maximum Standing Crop Tests

The mean MSC and confidence intervals of the toxicant treatments are presented in Figures 3 and 4. All chemicals had large confidence intervals associated with some treatments. This is most apparent in the acenaphthene, benzanthracene and naphthalene experiments. The large confidence intervals associated with the 48% saturated solutions of acenaphthene, the 10% saturated solutions of benzanthracene and the 96% saturated solutions of naphthalene may indicate substantial experimental error.

Figure 3. Means and Confidence Intervals of Effects of Acenaphthene, Benzanthracene, Chrysene and Fluorene on Maximum Standing Crop.

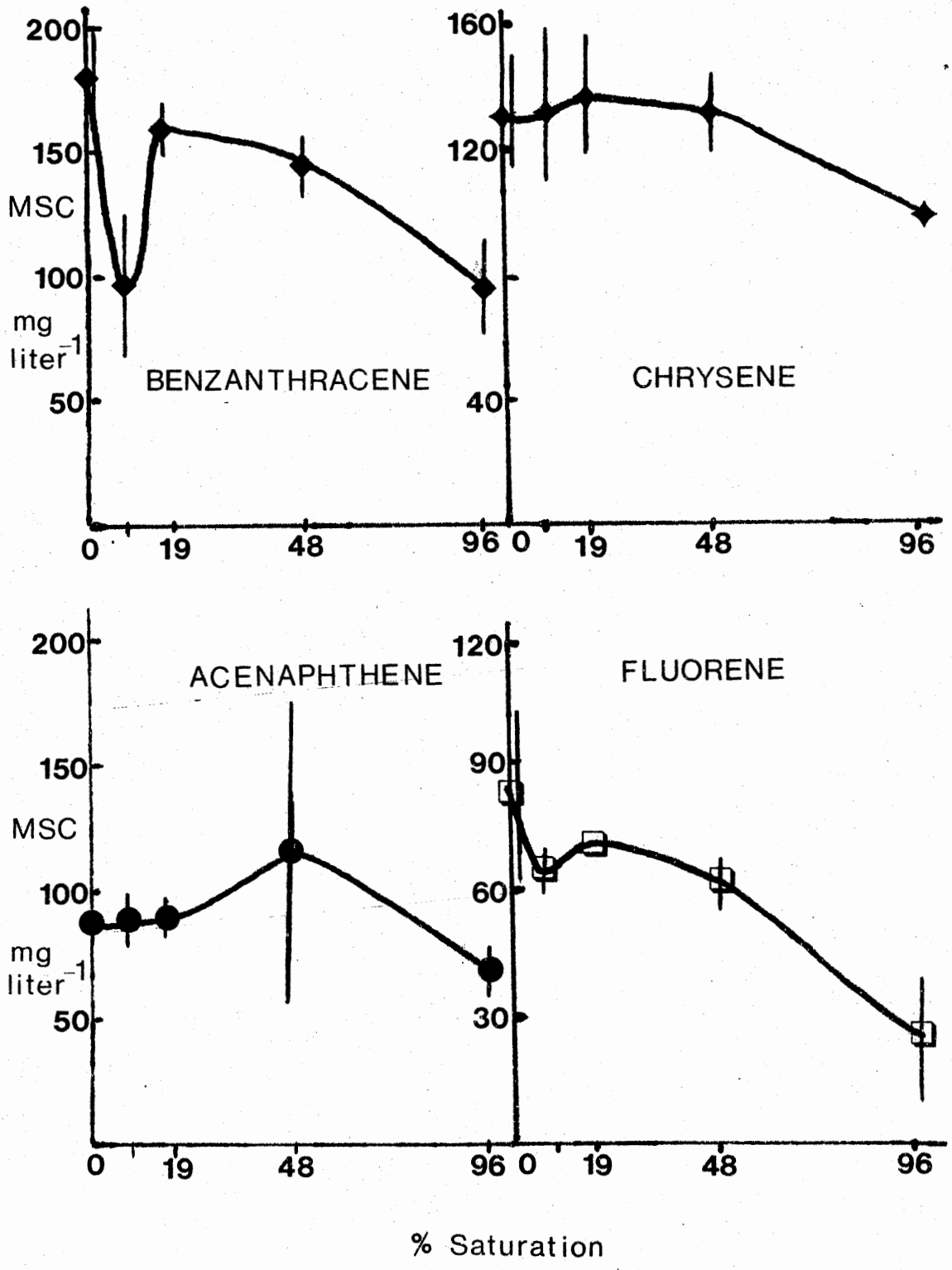
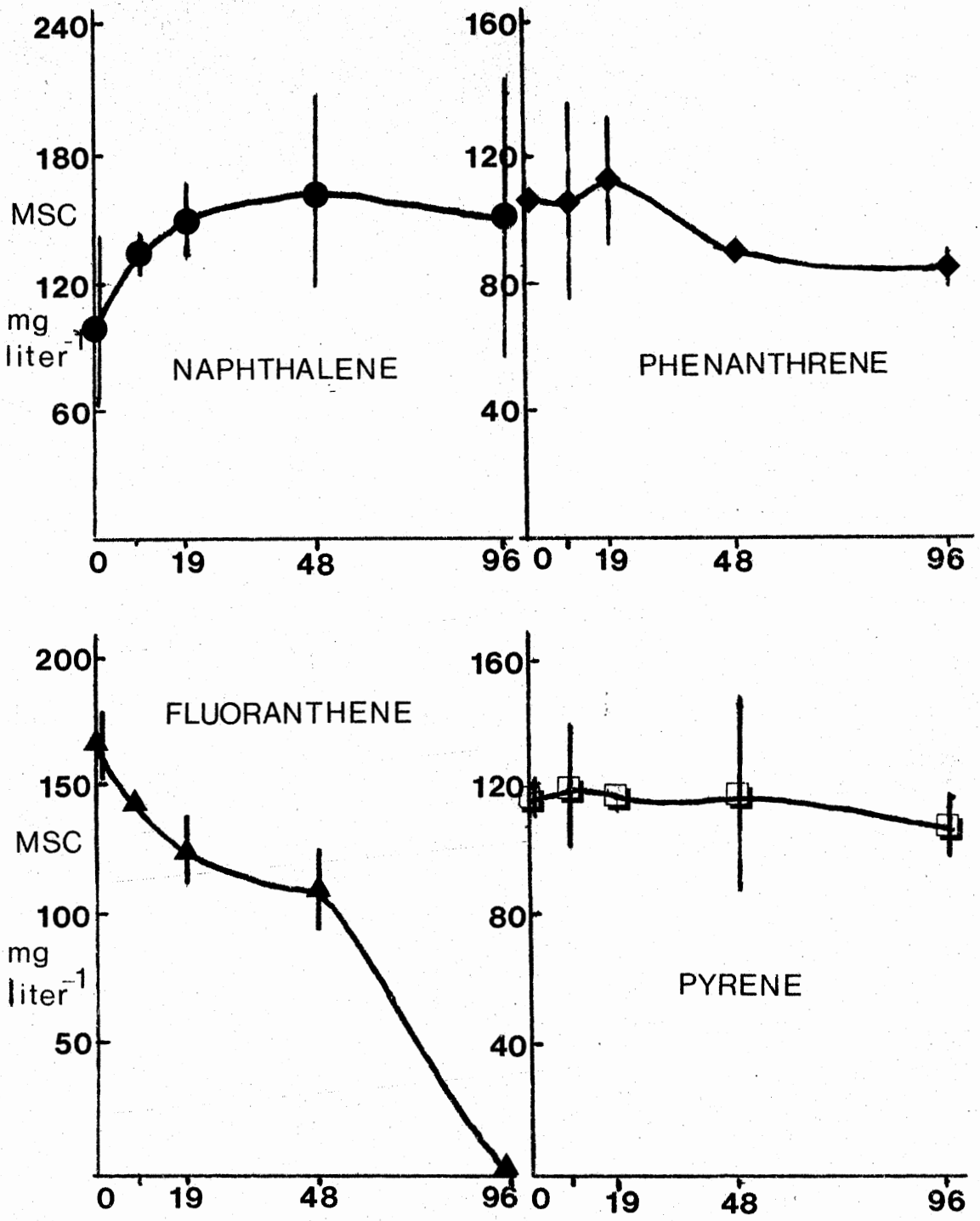

200
150
MSC
100
mg
liter^{-1}
50
BENZANTHRACENE
0
19
48
96
160
120
40
CHRYSENE
0
19
48
96
200
150
MSC
100
mg
liter^{-1}
50
ACENAPHTHENE
0
19
48
96
120
90
60
30
FLUORENE
0
19
48
96
% Saturation

Figure 4. Means and Confidence Intervals of Effects of Fluoranthene, Naphthalene, Phenanthrene and Pyrene on Maximum Standing Crop.


240
180
MSC
120
mg
liter⁻¹
60
NAPHTHALENE
0
19
48
96
160
120
80
40
PHENANTHRENE
0
19
48
96
200
150
MSC
100
mg
liter⁻¹
50
FLUORANTHENE
0
19
48
96
160
120
80
40
PYRENE
0
19
48
96
% Saturation

Figures 3 and 4 also show that the effects of the treatments on growth often do not follow any clear pattern. For example, the 10% saturated solution of fluoranthene was more inhibitory to growth than the 19% and 48% saturated solutions. Thus higher concentrations of toxicants did not necessarily produce greater levels of growth inhibition.

The analysis of variance showed that all the PAH except pyrene significantly affected the growth of Anabaena flos-aquae during 14 day exposures (Table VI). There was only a 58% probability that the means of the pyrene treatments were different. This level of significance did not justify analysis by the Duncan's test. The analysis of variance of the phenanthrene experiment showed a 94% level of significance. This value was close enough to the 95% significance level to justify analysis by the Duncan's test.

The complete results of the Duncan's test are presented in Table VII. The mean maximum standing crops were differentiated by letters. Means with different letters were significantly different from each other. Means with two letters belonged to two groups, but were not significantly different from other means in either group. Table VIII presents the means which were significantly different from the controls in the Duncan's test as percent changes from the control means. The concentrations of the chemicals compared in the Duncan's test are presented in Table IX.

Certain acenaphthene and naphthalene treatments resulted in biomasses which were significantly greater than those

TABLE VI

RESULTS OF ANALYSIS OF VARIANCE
OF MAXIMUM STANDING
CROP TESTS

Chemical	d.f.[a]	F Value[b]	Probability[c]
Acenaphthene	14	5.38	0.971
Benzanthracene	14	30.07	0.999
Chrysene	14	16.08	0.993
Fluoranthene	14	455.03	0.9999
Fluorene	13	87.18	0.9999
Naphthalene	14	9.09	0.9965
Phenanthrene	14	3.50	0.938
Pyrene	14	1.12	0.5801

a. total corrected degrees of freedom=d.f.

b. among treatments

c. probability of rejecting null hypothesis

H_o: Treatment means (mg liter^{-1}) are not significantly different from each other

TABLE VII

GROUPS OF SIMILAR MAXIMUM STANDING CROPS (mg liter^{-1}) DETERMINED BY DUNCAN'S MULTIPLE RANGE TEST

(α=0.05)

Chemical	%Saturation Treatment Levels				
	0	10	19	48	96
Acenaphthene	A(93)	A(94)	A(95)	B(118)	A(79)
Benzanthracene	A(175)	C(99)	A,B(155)	B(146)	C(95)
Chrysene	A(129)	A(131)	A(135)	A(131)	B(84)
Fluoranthene	A(165)	D(110)	B(146)	C(120)	E(0)
Fluorene	A(83)	B,C(67)	B(74)	C(62)	D(29)
Naphthalene	A(105)	B(135)	B,C(150)	C(165)	B,C(153)
Phenanthrene	A(105)	A(105)	A(112)	A,B(101)	C(85)
Pyrene	--	--	--	--	--

Underline values are significantly different from controls.

TABLE VIII

SIGNIFICANT MSC TREATMENT MEANS
AS PERCENT CHANGES FROM
CONTROL MEANS

Chemical	Percent Change				
Acenapthene	-	-	-	+26	-
Benzanthracene	-	-43	-	-16	-45
Chrysene	-	-	-	-	-35
Fluoranthene	-	-38	-12	-27	-100
Fluorene	-	-20	-11	-26	-65
Naphthalene	-	+28	+42	+56	+45
Phenanthrene	-	-	-	-	-20
Pyrene -	-	-	-	-	-
% Saturation	0	10	19	48	96

TABLE IX

MAXIMUM STANDING CROP TEST
EXPOSURE CONCENTRATIONS
AT THE BEGINNING
OF EXPERIMENT
($\mu g\ liter^{-1}$)

Chemical	Concentration ($\mu g\ liter^{-1}$)				
Acenaphthene	0	422	908	2427	4434
Benzanthracene	0	5	9	18	29
Chrysene	0	1	2	5	13
Fluoranthene	0	38	97	225	417
Fluorene	0	134	250	588	1089
Naphthalene	0	2070	6000	14851	30747
Phenanthrene	0	134	257	466	876
Pyrene	0	22	56	82	153
% Saturation	0	10	19	48	96

of the control treatments. The 2427 μg liter^{-1} exposure of acenaphthene produced a MSC which was 26% greater than that of the control treatment. The other exposures of acenaphthene did not significantly affect growth.

All treatments of naphthalene produced biomasses that were significantly greater than that of the untreated cultures. The 14851 μg liter^{-1} exposure was the most stimulatory (+56%) to growth. The other exposures of naphthalene stimulated growth but were not significantly different from each other.

Benzanthracene, chrysene, fluoranthrene, fluorene and phenanthracene inhibited growth of Anabaena flos aquae Three treatments of benzanthracene inhibited growth. The 5 μg liter^{-1} (-43%) and 29 μg liter^{-1} (-45%) exposures showed similar levels of inhibition. The 18 μg liter^{-1} treatment inhibited growth by 16%.

All fluoranthene exposures inhibited growth of Anabaena flos-aquae. The highest concentration (417 ug liter^{-1}) completely inhibited growth. The 38 μg liter^{-1} treatment inhibited growth by 38% and was more inhibitory than the 97 ug liter^{-1} or 225 μg liter^{-1} treatments.

All fluorene exposures were also inhibitory to growth. Again the highest concentration (1089 μg liter^{-1}) produced the greatest inhibition to growth (65%). The MSC decreased with increasing initial concentrations of fluorene except that the MSC of the 250 μg liter^{-1} treatment was

not significantly different from the MSC of the 134 μg liter^{-1} treatment.

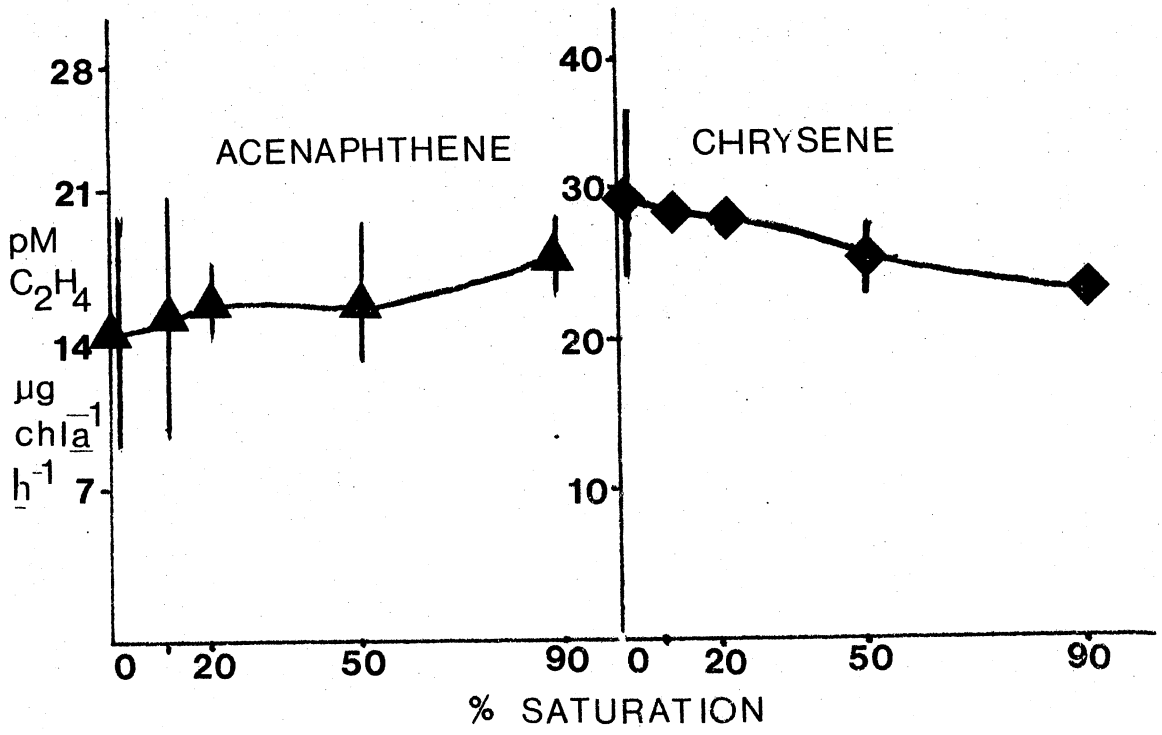
Only the highest concentrations of chrysene and phenanthrene inhibited growth. The 13 μg liter^{-1} exposure of chrysene inhibited growth by 35%. The 876 μg liter^{-1} exposure of phenanthrene inhibited growth by 20%.

Acetylene Reduction Experiments

Figures 5 and 6 present the means and confidence intervals of the effects of each chemical on the C_2H_2 reduction capacity of Anabaena flos-aquae. There were large confidence intervals associated with some means (acenaphthene, benzanthracene, fluoranthene, naphthalene and phenanthrene).

The analysis of variance of the results of the C_2H_2 reduction experiments are presented in Table X. A 95% probability of rejecting the null hypothesis that the treatment means were not different was considered significant. Acenaphthene did not have a significant effect on C_2H_2 reduction according to this criterion. The effect of the benzanthracene and fluoranthene treatments was unclear since the null hypothesis was rejected at a 90% probability of level. However, analysis of these results by the Duncan's Test did show significant differences between the treatment and the control at the 95% level. Chrysene, fluorene, naphthalene and phenanthrene all had significant effects on the C_2H_2 reduction capacity of Anabaena flos-aquae.

Figure 5. Effect of Acenaphthene, Benzanthracene, Chrysene and Fluorene on C_2H_2 Reduction.


ACENAPHTHENE
CHRYSENE
28
21
14
7
40
30
20
10
pM C_2H_4 µg chl a^{-1} h^{-1}
0
20
50
90
% SATURATION

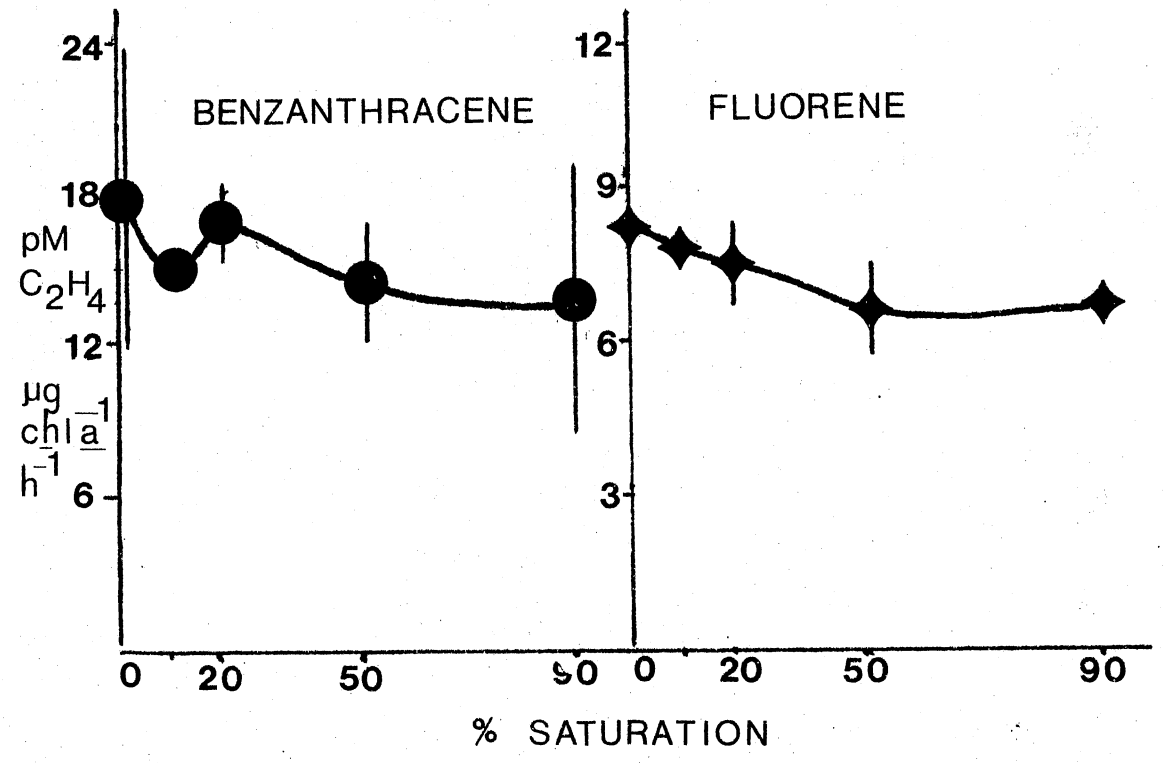

BENZANTHRACENE
FLUORENE
24
18
12
6
12
9
6
3
pM C_2H_4 µg chl a^{-1} h^{-1}
0
20
50
90
% SATURATION

Figure 6. Effect of Flouranthene, Naphthalene, Phenanthrene and Fluorene on C_2H_2 Reduction.

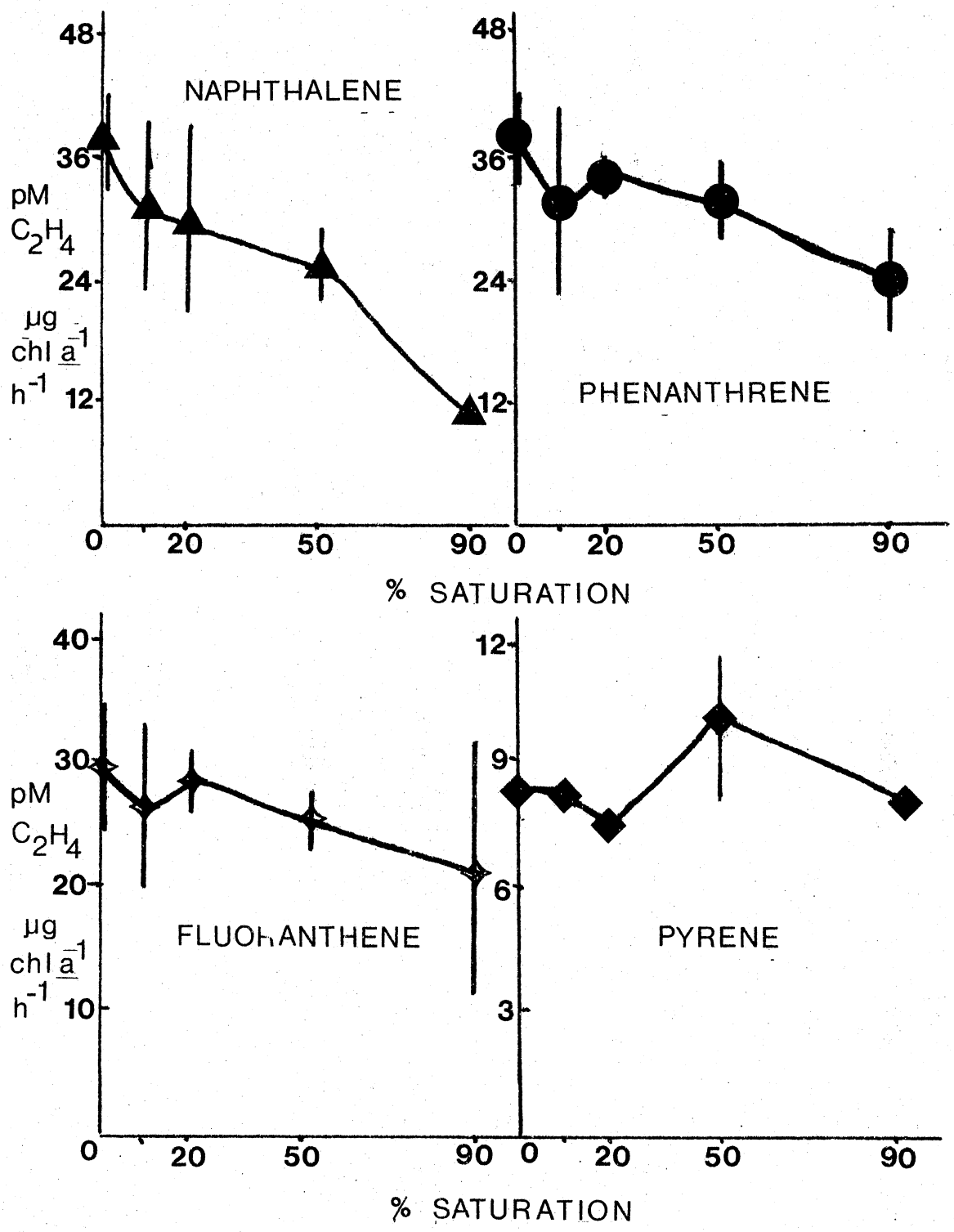

NAPHTHALENE
PHENANTHRENE
FLUORANTHENE
PYRENE
pM C_2H_4 µg chl a^{-1} h^{-1}
% SATURATION
% SATURATION
0
20
50
90
48
36
24
12
40
30
20
10
9
6
3

TABLE X

ANALYSIS OF VARIANCE OF
C_2H_2 REDUCTION RESULTS

Chemical	d.f.[a]	F Value[b]	Probability[c]
Acenaphthene	18	0.82	0.4644
Benzanthracene	18	2.44	0.9041
Chrysene	18	3.10	0.9495
Fluoranthene	19	2.29	0.9019
Fluorene	18	5.40	0.2924
Naphthalene	19	20.00	0.999
Phenanthrene	19	6.57	0.9981
Pyrene	19	5.02	0.991

a. total degrees of freedom=d.f.

b. among treatment levels

c. propability of rejecting null hypothesis

H_o: Treatment means (pM C_2H_4 µg chl $a^{-1}h^{-1}$) are not significantly different from each other

Analysis of the results by the Duncan's Test are presented in Table XI. The concentrations of the treatments used for each chemical are presented in Table XII. Those treatment means which were significantly different from the controls are presented as percent changes from the control in Table XIII.

The most insoluble chemicals inhibited C_2H_2 reduction only with the most concentrated treatments. The 27 μg liter^{-1} exposure of benzanthracene inhibited C_2H_2 reduction by 30%. The 13 μg liter^{-1} exposure of chrysene inhibited C_2H_2 reduction by 17%. Finally, the 391 μg liter^{-1} treatment of fluoranthene inhibited C_2H_2 reduction by 28%.

The 85 μg liter^{-1} treatment of pyrene apparently stimulated C_2H_2 reduction by 12%. Although this result was statisticly significant it is questionable because it is so inconsistent with the effects of the other chemicals on C_2H_2 reduction.

The more water soluble chemicals affected C_2H_2 reduction over a wide range of concentration. The 612 μg liter^{-1} and 1021 μg liter^{-1} treatments of fluorene inhibited C_2H_2 reduction by 19% and 16%, respectively. Three phenanthrene exposures inhibited C_2H_2 reduction. The highest concentration (822 μg liter^{-1}) was the most inhibitory (-34%). The 134 μg liter^{-1} and 485 μg liter^{-1} treatments inhibited C_2H_2 reduction by 15% and 14%, respectively.

TABLE XI

GROUPS OF SIMILAR ETHYLENE MEANS (pM C_2H_4 µg chl a^{-1} h^{-1}) DETERMINED BY DUNCAN'S MULTIPLE RANGE TEST (α =0.05)

Chemical	% Saturation Treatment Levels				
	0	10	20	50	90
Acenaphthene	A(14.17)	A(15.40)	A(15.32)	A(15.95)	A(17.54)
Benzanthracene	A(17.84)	A,B(15.22)	A(17.02)	A,B(4.84)	B(12.56)
Chrysene	A(29.40)	A(28.55)	A(28.47)	A,B(26.02)	B(24.40)
Fluroanthene	A(29.80)	A,B(26.89)	A(28.81)	A,B(25.28)	B(21.40)
Fluorene	A(8.17)	A(8.08)	A,B(7.57)	B(6.65)	B(6.83)
Naphthalene	A(37.27)	B(31.13)	B(30.84)	B(26.05)	C(11.32)
Phenanthrene	A(37.27)	B(31.54)	A,B(34.46)	B(32.10)	C(24.65)
Pyrene	A(8.39)	A(8.56)	A(7.84)	B(9.40)	A(8.47)

Underline values are significantly different from controls.

TABLE XII

ACETYLENE REDUCTION EXPERIMENTS AT THE EXPOSURE CONCENTRATIONS AT THE BEGINNING OF THE EXPERIMENT (μg $LITER^{-1}$)

Chemical					
Acenaphthene	0	422	956	2588	4157
Benzanthracene	0	5	9	19	27
Chrysene	0	1	2	5	13
Fluoranthene	0	38	101	234	391
Fluorene	0	134	260	612	1021
Naphthalene	0	2070	6250	15470	28825
Phenanthracene	0	134	268	485	822
Pyrene	0	22	58	85	143
% Saturation	0	10	20	50	90

TABLE XIII

SIGNIFICANT C_2H_2 REDUCTION TREATMENT MEANS AS PERCENT CHANGES FROM CONTROL MEANS

Chemical	Percent Change				
Acenaphthene	-	-	-	-	-
Benzanthracene	-	-	-	-	-30
Chrysene	-	-	-	-	-17
Fluoranthene	-	-	-	-	-28
Fluorene	-	-	-	-19	-16
Naphthalene	-	-16	-17	-30	-70
Phenanthrene	-	-15	-	-14	-34
Pyrene	-	-	-	+12	-
% Saturation	0	10	19	48	96

Naphthalene had the most inhibitory effect on C_2H_2 reduction of the eight PAH compounds tested. All naphthalene treatments inhibited C_2H_2 reduction and there was a direct relationship between increasing concentrations of naphthalene and increased inhibition of C_2H_2 reduction.

Electron Microscopy Results

Two general restrictions were considered in determining whether toxicants affected the ultrastructure of Anabaena flos-aquae. First, only cells attached within a trichome were considered to be valid for observation (Wildon and Mercer, 1963; Fay and Lang, 1971). Unattached cells may have had broken septa where the cells normally attach to a adjacent cells resulting in the loss of cell contents. Secondly, Kulasooriya and Fay (1971) reported that nitrogenase activity in Anabaena cylindrica corresponded with the appearance of polar nodules in the developing heterocyst. Although the presence of polar nodules indicates nitrogenase activity, the absence of polar nodules does not necessarily indicate inhibition of nitrogenase activity because heterocysts can exist in all stages of development at the same time in a culture. Cells in earlier stages of differentiation may not have differentiated polar nodules. For this reason the absence of a polar nodule was not a valid indicator of inhibition of nitrogenase activity.

Vegetative cells in control cultures were similar to

those described in the literature. Cells were surrounded by a cell wall and a plasma membrane (Figure 7(1) and 7(2)) (Pankratz and Bowen, 1963; Wildon and Mercer, 1963). Photosynthetic thylakoids which resemble flattened membrane sacs, were characteristically along the periphery of the cell (Figure 7(1)). The central region of the cell had a lower electron density than that the thylakoids. This area contains the DNA and RNA of the cell and is called the nucleoplasm (Figures 7(1)). Polyhedral bodies or carboxysomes and ribosomes were often associated with the nucleoplasm (Figure 7(1) and 7(2)) (Wildon and Mercer, 1961; Pankratz and Bowen, 1963; Smith and Peat, 1967; Stanier and Cohen-Bazire, 1977). Cells contained other inclusions which were more difficult to identify. Structured granules or phycocyanin and glycogen granules were tentatively identified (Figure 7(1)) (Pankratz and Bowen, 1963; Stanier and Cohen-Bazire, 1977). It is not known if the vacuoles were cell inclusions or artifacts caused by fixation and dehydration (Figure 7(1)). Large, dark granules in some photomicrographs are staining artifacts, probably precipitates of lead citrate (Figure 7(1)).

Heterocysts in control cultures were also very similar to those described in the literature. Thick, fibrous envelopes surrounded heterocyts (Figures 7(2), 7(3), 7(4), and 7(5)) (Lang, 1965; Kulasrooria et al., 1972). Heterocyts lost most vegetative cell inclusions including granules and polyhedral bodies (Figure 7(2) and 7(3)) (Lang, 1965;

Legend to Figures Seven. Bunched Membrane (bm); Carboxysome (c); Cell Wall (cw); Envelope (e); Lattice-like Membrane (ll); Nucleoplasm (n); Plasma Membrane (pm); Polar Nodules (pn); Precipitates of Lead (lp); Structural Granules (sg); Thylakoids (th); Vaculoles (v).

Figure 7(1). Normal Vegetative Cell of Anabaena flos aquae. Spurr's Resin (x31,050).

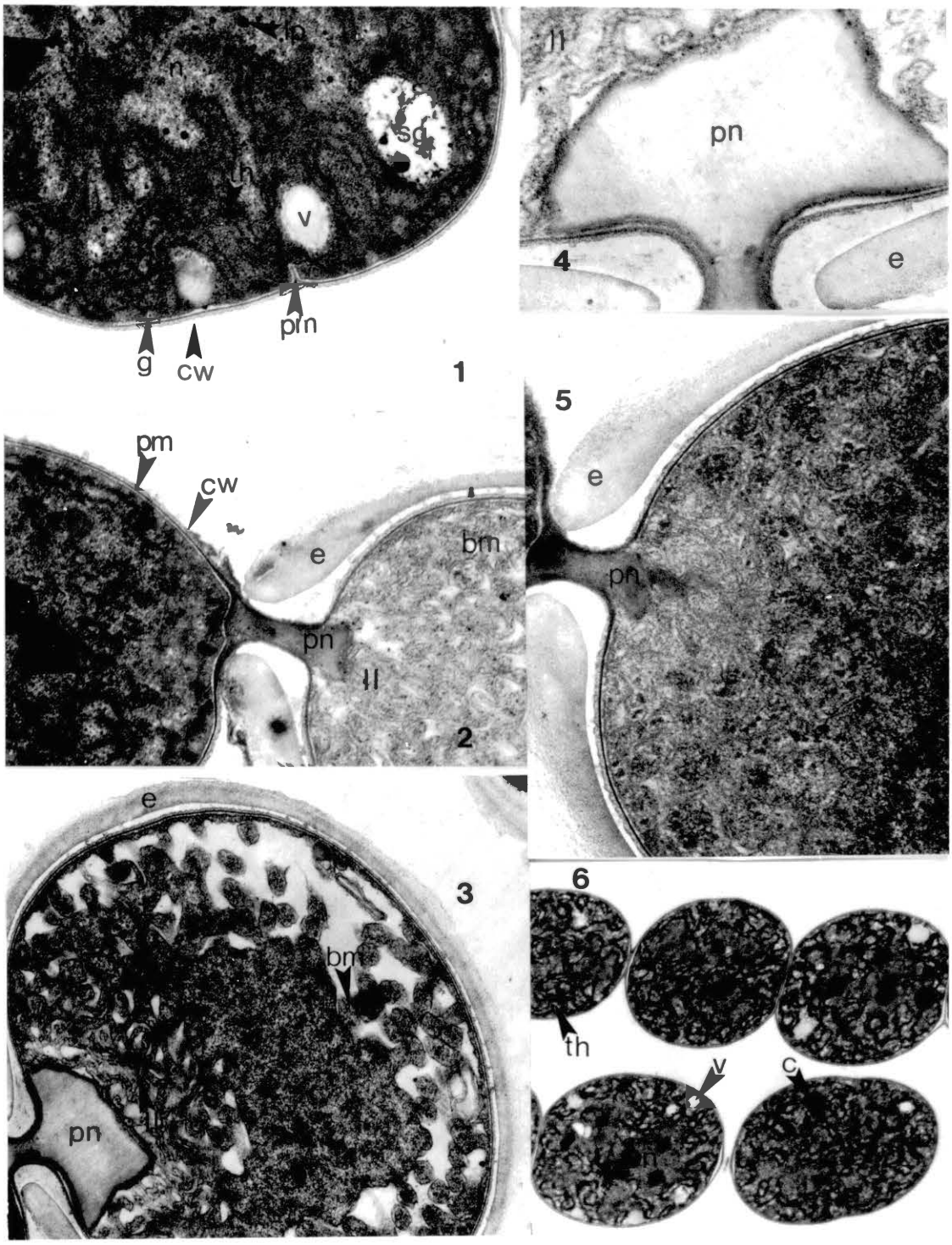
Figure 7(2). Normal Vegetative Cell Attached by Channel to Normal Heterocyst. Polybed Resin. (x31,050).

Figure 7(3). Normal Heterocyst with Characteristic Electron Transparent Polar Nodule. Spurr's Resin. (x22,450).

Figure 7(4). Polar Nodule of Normal Heterocyst. Spurr's Resin. (x55,350).

Figure 7(5). Normal Heterocyst, Possible in Early Stage of Differentiation. Polybed Resin. (x41,175).

Figure 7(6). Vegetative Cells Exposed to Fluorene. No Visible Toxic Effects. Spurr's Resin. (x10,125).


n
sg
th
v
g
cw
pm
1
ll
pn
4
e
5
pm
cw
e
bm
pn
ll
2
e
3
bm
pn
ll
6
th
v
c
n

Kulasooriya et al., 1972). Mature heterocysts had electron transparent polar nodules (Figure 7(2) and 7(3)) (Kulasooriya et al., 1972). A lattice-like formation of lamellae behind the nodules and bunched membrane throughout the remainder of the cell was also characteristic of mature heterocysts of Anabaena spp. (Figure 7(3)) (Lang, 1965; Kulasooriya and Fay, 1971; Kulasooriya et al., 1972)

Control cells were embedded in two different resins, Polybed and Spurrs. Heterocysts embedded in Polybed resin (Figure 7(2) and 7(5)) were slightly different than those embedded in Spurr's (Figure 7(1),7(3),and 7(4)). Polar nodules were darker and the lamellae within the heterocysts were less defined. It is possible that heterocysts embedded in Polybed appeared slightly different because heterocysts were at an earlier stage of differentiation.

Fluorene, naphthalene and phenanthrene did not cause conclusive changes in ultrastructure (Figures 7(6), 8(1), 8(2), and 8(3)). Heterocysts attached within trichromes were not found in cultures exposed to fluorene and phenanthrene. Only vegetative cells exposed to fluorene and phenanthrene could be compared with controls. The vegetative cells in these treatments were very similar to controls (Figure 7(6), 8(1), and 8(2)). It appeared that the vegetative cells exposed to naphthalene, fluorene and phenanthrene had slightly distended thylakoids. However, the variability in the appearance of the thylakoids within any chemical treatment rendered this observation inconclusive

Figure 8(1). Vegetative Cells Exposed to Phenanthrene. No Visible Toxic Effects. Spurr's Resin. (x13,365).

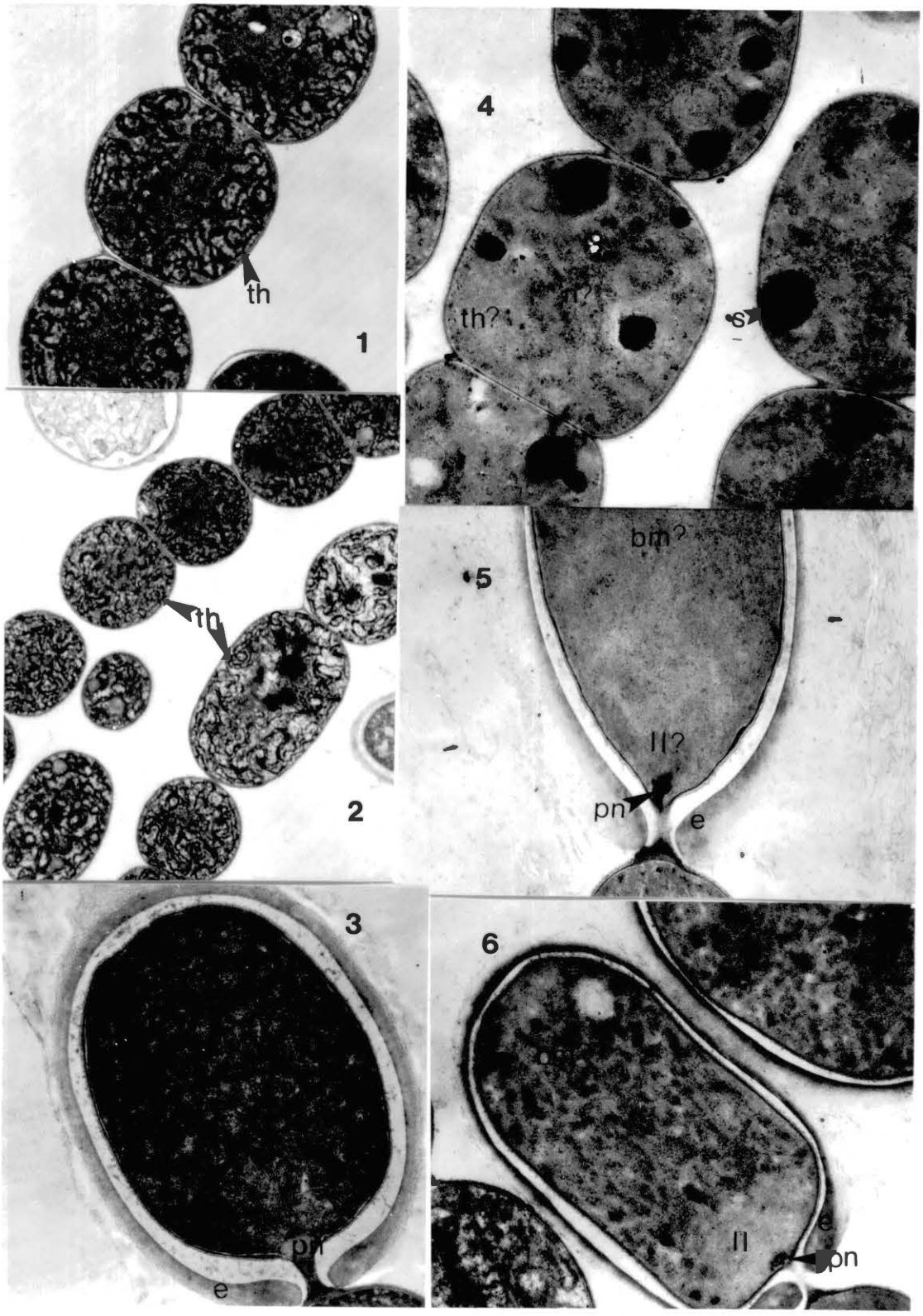
Figure 8(2). Vegetative Cells Exposed to Naphthalene. No Visible Toxic Effects. Spurr's Resin. (x7,965).

Figure 8(3). Heterocysts Exposed to Naphthalene. No Visible Toxic Effects. Spurr's Resin. (x16,875).

Figure 8(4). Vegatative Cells Exposed to Chrysene. Disruption of Photosynthetic Thylakoids. Note Black Spheres. Polybed Resin. (x16,875).

Figure 8(5). Heterocysts Exposed to Chrysene. Shrunken Electron Opague Polar Nodules and Disrupted Lamellae. Polybed Resin. (x16,875).

Figure 8(6). Heterocysts Exposed to Chrysene. Shrunken Electron Opague Polar Nodules and Disrupted Lamellae. Polybed Resin. (x16,875).


th
1
th
2
3
pn
e
4
th?
n?
s
bm?
5
ll?
pn
e
6
e
ll
pn

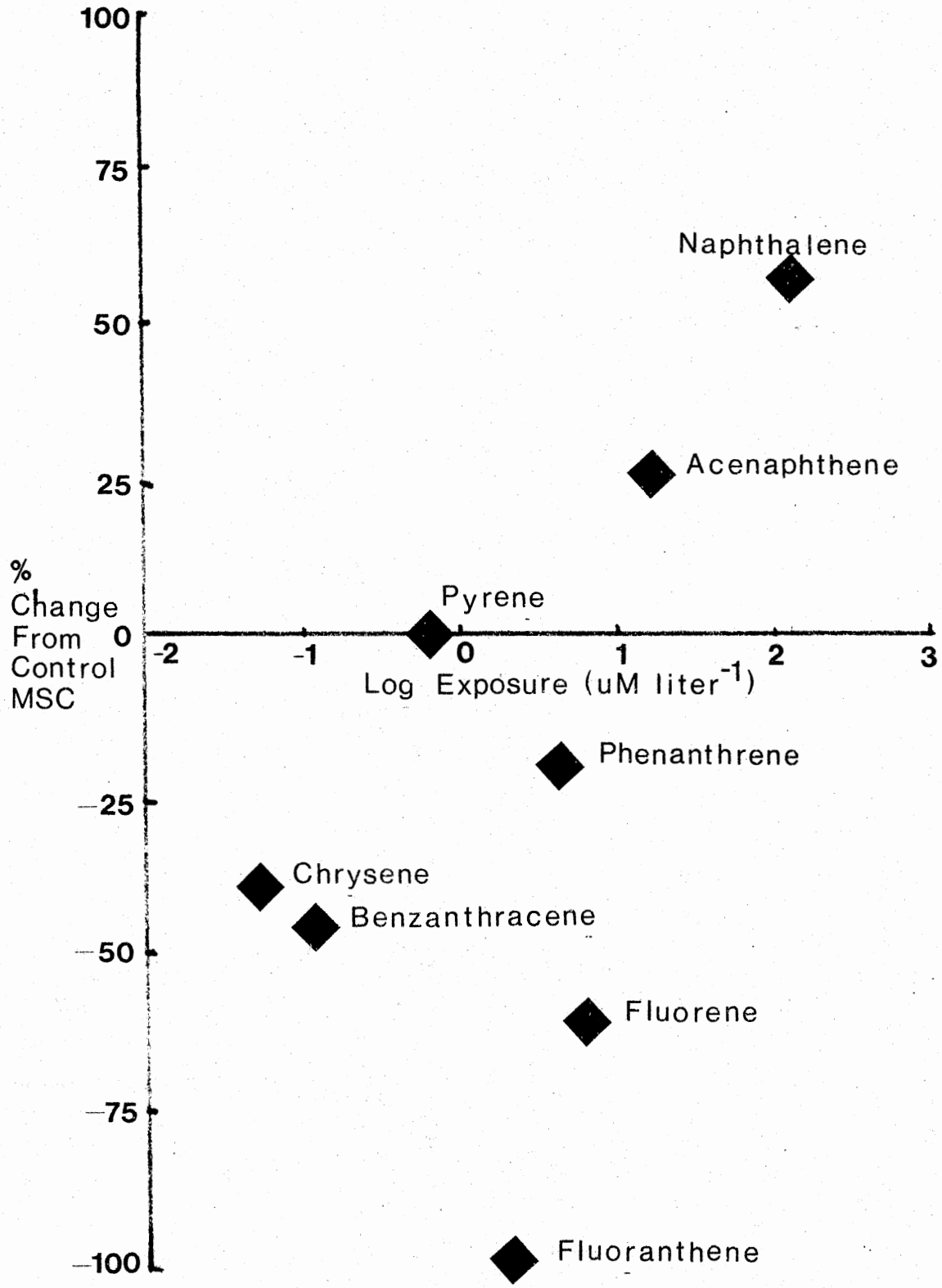
(Figure 8(2)).

Heterocysts exposed to naphthalene did not appear different from those in control cultures (Figure 8(3)). Any difference between heterocysts exposed to naphthalene and those in control cultures fell within the range of variability in appearance of heterocysts at different stages of differentiation.

Chrysene had a visible effect on the ultrastructure of vegetative cells and heterocysts. Some vegetative cells exposed to chrysene contained polyhedral bodies and granule inclusions but the membranes of the thylakoids were not visible (Figure 8(4). Areas of the vegetative cells were granular as was the nucleoplasm in the control cultures. However, the distinction between the granular area and the thylakoids was less visible because of the disruption of the thylakoid membranes. The nature of the black spherical areas exposed to chrysene is unknown (Figure 8(4). These areas were not stained when cells were observed after osmimum fixation. These areas were stained by uranyl acetate or lead citrate. Staining by these substances indicates that the areas contained nucleic acids, glycogen or polyphosphates.

The heterocysts exposed to chrysene contained electron opaque, irregularly shaped polar nodules (Figure 8(5) and 8(6). These polar nodules were very different from the electron transparent polar nodules seen in the control cultures. The channels connecting the heterocysts to

Figure 9. The Relationship of the Solubility of PAH to Their Effect on the Growth of *Anabaena flos-aquae*.


100
75
50
25
0
-25
-50
-75
-100
% Change From Control MSC
-2
-1
0
1
2
3
Log Exposure (uM liter^{-1})
Naphthalene
Acenaphthene
Pyrene
Phenanthrene
Chrysene
Benzanthracene
Fluorene
Fluoranthene

blue-green algae described in the literature. Vegetative cells exposed to fluorene, naphthalene, or phenanthrene were not morphologically different from those in control cultures. Heterocysts exposed to naphthalene were also similar to those in control cultures. Chrysene disrupted the membranes of the photosynthetic thylakoids in vegetative cells and the lamellae in heterocysts.

CHAPTER IV

DISCUSSION

Maximum Standing Crop Tests

The results of the MSC tests reflect the recovery of the growth of Anabaena flos-aquae. Soto et al. (1975 a,b) reported the recovery of the growth and photosynthetic capacity of Chlamydomonas angulosa following exposure to naphthalene in open flasks from which naphthalene could volatilize into the atmosphere. Soto et al. (1977) also reported a return to normal levels of total carbon, pigment, protein and lipid following exposure to naphthalene in open flasks. These results indicate that naphthalene had a short term harmful effect on the algae but did not kill them. As naphthalene concentrations in solution decreased the inhibitory effect of the chemical decreased and cells began to function normally.

All the PAH compounds tested decreased in concentration during the 14 day test period (Table V). The results of Soto and his coworkers indicate that the effects of the PAH at their initial concentrations were not sustained over 14 days as their concentrations decreased. Since adequate conditions for growth were available in all test flasks, cells could return to more normal growth as the concentrations of

the PAH decreased. Although not quantitative, visual observations of the test cultures support the hypothesis of recovery of growth. Generally, cultures exposed to 48% and 96% saturated solutions of the PAH compounds increased markedly in cell density during the second week of the test period.

Stimulation of Growth

The 2427 μg liter^{-1} treatments of acenaphthene and all the treatments of naphthalene increased the biomass in Anabaena flos-aquae cultures. Bacterial growth in treatment flasks may have contributed to the elevated values of biomass when using naphthalene and acenaphthene as carbon sources. Davies and Evans (1964) reported that soil pseudomonads were capable of cleaving aromatic rings and using naphthalene as a carbon substrate. Evans et al. (1965) reported that soil pseudomonads could enzymatically degrade phenanthrene and anthracene and assimilate these compounds as carbon sources. Wodzinski and Johnson (1968) also demonstrated that Pseudomonas sp. grew on naphthalene and phenanthrene substrates.

In contrast, Walker et al. (1975) demonstrated that No. 2 fuel oil inhibited the growth of heterotrophic marine bacteria. The water soluble fraction of the No. 2 fuel oil contained predominately naphthalene and alkyl naphthalene. The authors suggest that these compounds were the toxic agents.

Anabeana flos-aquae cultures were contaminated with bacteria and fungi after 14 hours of growth in media containing naphthalene. The contamination was detected when algal cultures were streaked on bacto-agar plates. However, since naphthalene can be used as a carbon substrate by some bacteria, while it is toxic to other bacteria, no conclusions can be drawn about the contribution of bacterial growth to the elevated values of biomass, because the taxa of the contaminating microorganisms were not identified.

Cerniglia et al. (1980) demonstrated that Oscillatoria sp. oxidized naphthalene to five polar products. The authors reported that 4.8% of a 10 mg liter^{-1} naphthalene exposure was oxidized in 24 h. In a subsequent paper, Cerniglia et al. (1980) demonstrated that two strains of Anabaena sp. oxidized naphthalene to polar products. Approximately 1.5% and 2.0% of a 3.5 mg liter^{1} naphthalene treatment was oxidized in 24 h. The authors did not demonstrate that algae were capable of enzymatically cleaving the aromatic rings of naphthalene. Ellis (1974) reported that 100 μM liter^{-1} of phenol or catechol was catabolized to CO_2 by six species of freshwater algae. Anabaena cylindrica catabolized less than 1% of phenol and 1.4% of catechol in 48 h. These results indicate that certain species of Anabaena are capable of cleaving the aromatic ring and assimilating some compounds. The growth of Anabaena flos-aquae in these experiments may have been stimulated by use of acenaphthene and naphthalene as sources

of carbon.

Moreover, the stimulation of growth in other plants by PAH has been observed previously. Boney and Corner (1962) showed that derivatives of benzanthracene and 3,4 benzpyrene stimulated the growth of the red alga, Antithamion plumula during a 96 h exposure. Andelman and Snodgrass (1974) reported that 3,4 benzpyrene accelerated the growth of kohlrabi, cauliflower, wheat, tobacco and rye.

Growth stimulation in these previous studies was associated with carcinogenic, extremely hydrophobic PAH. These findings do not support the results of this study. In this study, relatively soluble PAH compounds stimulated growth, possibly by serving as sources of carbon.

Inhibition of Growth

Hutchinson et al. (1979) suggested that the toxicity of PAH is caused by their partitioning into membrane lipids and disrupting membrane functions. This hypothesis was substantiated by experiments which showed that PAH compounds cause ionic leakage from the cells of Chlamydomonas angulosa. The same hydrocarbons inhibited the ^{14}C uptake of this green alga. These workers established an inverse relationship between the solubility of hydrocarbons in water and the inhibition of ^{14}C uptake and ionic leakage from membranes. Relative to a specific level inhibition, e.g. the concentration which inhibited ^{14}C by 20%, toxicity increased as the solubility of the chemical decreased.

Packham et al. (1981) studied the effect of organic pollutants on the cooperativity of phospholipids in liposomes during phase transitions. Liposomes are synthetic multilamellar bilayers of phospholipid. He concluded that organic pollutants intercalated in membranes in a variable manner because of their hydrophobicity. They did not interact with phospholipids stoichiometrically. This intercalation of organic compounds disrupted cooperative functions among phospholipids and altered the structure of membranes.

Specific evidence that PAH inhibit photosynthesis and general evidence that PAH disrupt membrane structures lead to the hypothesis that the PAH tested in this study inhibited growth by partitioning into membranes and by disrupting an activity necessary to photosynthesis contained within the membranes e.g. electron transport. This inhibition probably decreased during the test period as the compounds in solution volatilized from solution or photo-oxidized to more polar derivatives and partitioning into lipids decreased. The effect of the PAH within the cells decreased as their concentrations were diluted by cell division (Soto et al., 1975b).

Effects on Growth Versus Water Solubility

The most water soluble PAH increased the biomass of *Anabaena flos-aquae* cultures. Less soluble compounds inhibited the growth of this alga. The result of the MSC

tests contradict the postulate that the more water soluble components of fossil fuels are the most toxic fraction to aquatic organisms (Pulich, 1974; Soto et al., 1975). These results support the findings of Batterton (1978) and Hutchinson et al. (1979) which demonstrated a higher toxicity of the hydrophobic components of fossil fuels.

Acetylene Reduction Tests

Inhibition of Reduction

Possibly the best clue to the mechanism of action of PAH on C_2H_2 reduction is the fact that inhibition occurred within two hours. Nitrogenase activity in Anabaena flos-aquae is dependent on anaerobic conditions, magnesium ions, adenosine triphosphate (ATP) and an adequate supply of reductant. Which of these requirements was limiting in two hours?

Heterocysts contain chlorophyll a and the photosystem I (PS I) electron transport chain (Stanier and Cohen-Bazire, 1977; Stewart, 1980). It is thought that ATP may be produced in the heterocyst by cyclic photophosphorylization via PS I (Stanier and Cohen-Bazire, 1977). Nitrogenase activity demands large amounts of ATP for energy and possibly for confirmational changes in the nitrogenase enzyme (Kennedy, 1970). Adsorption of lipophilic PAH to membranes which contain PSI may have disrupted ATP production and reduced nitrogenase activity. It has also been shown that adenosine diphosphate (ADP) inhibits nitrogenase activity in

Rhizobium lupini (Kennedy, 1970). Inhibition of C_2H_2 reduction in Anabaena flos-aquae could have been caused by a decrease in ATP concentration or by a negative feedback system in which increasing ADP to ATP ratios turned off nitrogenase activity.

Adenosine triphosphate is necessary for nitrogenase activity in all nitrogen fixing organisms that have been investigated (Striecher and Valentine, 1973). If ATP production is disrupted by the partitioning of PAH into membranes, PAH may be inhibitory to nitrogen fixation as a general phenomenon. Further research is needed to determine if PAH compounds are inhibitory to N_2-fixation in other organisms and therefore affect the contribution of N_2-fixation to the biota.

Relationship of Water Solubility to Inhibition of Acetylene Reduction

Compounds which differ widely in water solubility exhibited comparable levels of inhibition to C_2H_2 reduction (Table XII and XIII). Naphthalene is 2200 times more soluble than chrysene but was less than four times as inhibitory to C_2H_2 reduction. Fluorene is 40 times more soluble than benzanthracene but was less inhibitory to C_2H_2 reduction.

The octanol/water partition coefficients of the seven of the eight PAH compounds tested in this study are

presented in Table XV (Leo et al., 1977). This coefficient reflects the potential of a compound to partition into lipids from water. The values in Table XIV show that the more water insoluble compounds such as chrysene (log K_{ow}=5.60) more readily partition into lipids than the relatively soluble compounds such as naphthalene (log K_{ow}= 3.30).

Leo et al. (1971) reported that lipophilic compounds reach a steady state in an aqueous phase very quickly. These compounds move into lipids at the same rate as they dissociate from water. Thus lipophilic compounds may partition into membranes very quickly and have an almost immediate toxic effect.

During a short term exposure such as the C_2H_2 reduction experiment, the rate at which toxicants partition into membranes may be as important to the toxic effect as the concentration of chemical available for partitioning. The faster partitioning of the very insoluble PAH compounds may help explain why chemicals which varied greatly in their concentrations in solution had comparable toxic effects on C_2H_2 reduction in Anabaena flos-aquae.

Electron Microscopy

Chrysene affected the ultrastructure of Anabaena flos-aquae after a 24 h exposure to the chemicals. Fluorene, naphthalene and phenathrene did not have visible effects on ultrastructure. Although only chrysene affected

TABLE XIV

OCTANOL/WATER PARTITION COEFFICIENTS
(K_{ow})

Chemical	log K_{ow}*
Naphthalene	3.30
Acenaphthene	----
Fluorene	4.18
Phenanthrene	4.45
Fluoranthene	4.81
Pyrene	4.87
Benzanthracene	5.60
Chrysene	5.60

the ultrastructure of the alga, all the chemicals inhibited C_2H_2 reduction.

Chrysene has the highest octanol-water partition coefficient (log Ko/w = 5.60) of the chemicals tested for effects on ultrastructure (Leo, 1977). The gross disruption of membranes by chrysene may have been caused by its strong adsorption to lipids and a disruption of the lipid bilayer of the membranes. Fluorene (log Ko/w = 4.18), naphthalene (log Ko/w = 3.30) and phenanthrene (log Ko/w = 4.45) may have partitioned into lipids sufficiently to disrupt functions contained in membranes but not so much so as to grossly alter membrane structure.

Comparisons of the MSC and Acetylene Reduction Tests as Acute Toxicity Bioassays

Although the MSC and the C_2H_2 reduction tests can both be designated as static acute toxicity bioassays, they measure very different kinds of responses by the algae. The C_2H_2 reduction test measures an immediate effect on a physiological system. Because of its speed, C_2H_2 reduction predominately measures the effect of a chemical before it can undergo much change.

The MSC test measures growth which is an intergration of many physiological systems within an organism. This test measures the response of algal cells to an initial exposure of a chemical and probably their capacity to recover from

the exposure. The MSC test is useful in determining if an algal population can recover after an exposure to a chemical which would degrade in the environment. This test did not allow a determination of a dose-response relationship because of changes in the concentrations of the chemicals during the test period. Therefore, it is not a good bioassay because it can not be used to predict a dose-response relationship for chemicals which decrease in concentration during 14 days.

The C_2H_2 reduction test is a new method for measuring acute toxicity. It was not very sensitive to the toxic effects of PAH and it is not an effective acute toxicity bioassay at this time. However, refinements of the method may provide better results, e.g. pre-exposure of cultures to toxicants before the incubation of the cultures for C_2H_2 reduction. Also, this test provides an unique approach to toxicity testing. It can be a guage for the effect of toxicants on the input of elemental nitrogen to the biota. This possibility alone justifies refinement of the method and its use for testing potential effects on the nitrogen cycle.

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VITA 2

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