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Effect of Eight Polynuclear Hydrocarbons on Growth of *Anabaena flos-aquae*

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The Environmental Protection Agency (EPA) has designated polynuclear hydrocarbons (PAH) as a class of chemicals which are potentially harmful to the environment (DOMINGUEZ 1977). Some PAH are known animal carcinogens and bacterial mutagens (ANDELMAN & SUESS 1970, ANDERSON 1978, CONNORS 1975, CLAR, 1964). Because PAH are potentially harmful in natural ecoystems there is a need to understand their potential effect at all trophic levels. One such level is the producer level, which is important because aquatic plants (algae) are the primary source of energy in aquatic food webs. A major alteration in their productivity (growth) could have profound effects on higher trophic levels.

Acute toxicity to aquatic organisms is often associated with the low molecular weight aromatic hydrocarbons in the water soluble fraction of an organic mixture (GIDDINGS 1980, GORDON & PROUSE 1973, KAUSS et al. 1973, PULICH et al. 1974, SOTO et al. 1975a, WINTER et al. 1976). Little is known about the toxicity to algae of high molecular weight, relatively insoluble PAH (BATTERTON et al. 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. The objective of this research was to determine the effects of some high molecular weight PAH compounds on the growth of the blue-green alga, Anabaena flos- aquae.

We used a modification of the Algal Assay Procedure test (AAP) as our experimental system, which has been designated as a standard method for effects testing on algae under the Toxic Substances Control Act (EPA 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.

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METHODS

Unialgal cultures of Anabaena flos-aquae (Lyngb) De Bebisson were obtained from the EPA Environmental Research Laboratory, Corvallis, Oregon. Stock cultures were grown in AAP algal media at 29°C and 9.514-19.028 x 10^4 watts cm^{-2} sec^{-1} (200-400 foot candles) of continuous light (EPA 1971, MILLER et al. 1978).

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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. Stock cultures were not contaminated as determined by this method. Healthy cells were maintained by weekly aseptic transfers.

The methodology of the bioassays for growth was modification of AAP protocols (EPA 1971, MILLER et al. 1978). Growth was measured as the maximum standing crop (MSC). Cells were exposed to toxicants in 125 ml cotton-stoppered flasks containing 52 ml of solution. Sterile toxicant and non-toxic media were aseptically pipetted into flasks to give 96, 48, 19, 10 and 0% saturated solutions with initial cell densities of 10,000 cells ml^{-1}.

Chemicals were solubilized by adding excess analytical grade chemicals (Aldrich Co.) to sterile media stirred by a magnetic stirrer for 24-30 h. Undissolved crystals were allowed to settle for 2 h.

Toxicant degradation was determined by fluorescence analysis using a Aminco Bowman Spectrophotofluorometer (MCKAY and SHIU 1977, SCHWARZ and WASIK 1976). Standards were prepared by dissolving a known eight 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).

Flasks containing each level of toxicant and toxicant-free controls were incubated without cells at 25°C and 9.514-19.028 10^4 watts cm^{-2} sec^{-1}. 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 concentration of each level was used to calculate the % change in concentration over that period.

RESULTS

Exposure. The solubility of the test chemicals in algal media was in general agreement with literature values reported for PAH dissolved in distilled water (MACAY and SHIU 1977, SCHWARZ & WASIK 1976, KLEVENS 1950).

Acenaphthene, fluorene, naphthalene and pyrene almost completely disappeared from solution within seven days. 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. In general, low molecular weight compounds disappeared from solution more quickly than high molecular weight compounds. Pyrene was an exception. Although changes in concentration of toxicants were not measured in the vessels with algal cells during the MSC tests, the general pattern of decline of the compounds agreed with data in the literature and the expectation of change based upon water solubility, volatility and rates of photo-oxidation (KATZ et al. 1979, ZEPP

Benzanthracene, chrysene, fluoranthrene, fluorene and phenanthracene inhibited growth. Three treatments of benzanthracene inhibited growth. The 10% (5 $\mu g\ L^{-1}$) and 96% (29 $\mu g\ L^{-1}$) exposures showed similar levels of of inhibition. The 48% (18 $\mu g\ L^{-1}$) treatment inhibited growth by 16%.

All fluoranthene exposures inhibited growth. The 96% exposure (417 $\mu g\ L^{-1}$) completely inhibited growth. The 10% (38 $\mu g\ L^{-1}$) treatment inhibited growth by 38% and was more inhibitory than the 19% and 48% exposures (97 $\mu g\ L^{-1}$ and 225 $\mu g\ L^{-1}$). All fluorene exposures were also inhibitory to growth. Again the highest concentration (1089 $\mu g\ L^{-1}$) produced the greatest inhibition to growth (65%).

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.

With the exception of pyrene, the four ringed, (more hydrophobic) aromatics tended to be more inhibitory to the growth than the others tested (Figure 1). Three of the four-ringed compounds tested were very inhibitory; benzanthracene, chrysene and fluoranthene. Two of the three-ringed compounds tested also inhibited growth: phenanthrene and fluorene. However, growth of was not affected by phenanthrene at concentrations less than 96% of saturation. Moreover, irrespective of the number of rings, all of these compounds found to inhibit growth, except fluorene, persisted in the media for at least 14 days, a characteristic not shared by those compounds which apparently stimulated growth.

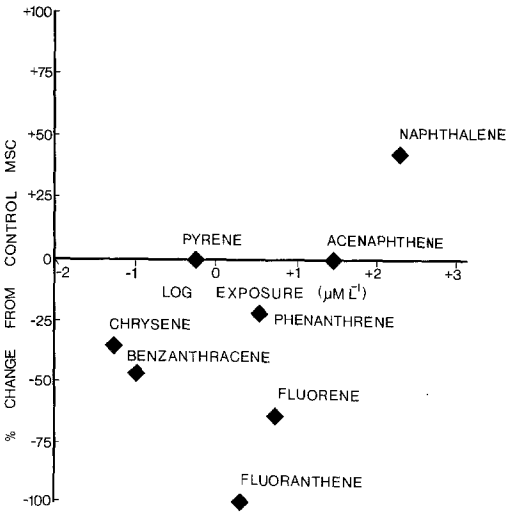

Figure 1. The relationship between the concentration of eight polynuclear aromatic hydrocarbons at 96% saturation and growth of Anabaena flos-aquae.

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 growth. Analysis of variance (ANOVA) was used to determine if the means of the treatment results were different at the 95% probability level. The Duncan's Multiple Range Test was used to determine which individual means were different at the 95% probability level and identified those means which were significantly different from the mean of the control treatment. The results of the MSC tests did not justify regression or prohibit analysis.

The ANOVA showed that all the PAH except pyrene significantly affected growth during 14 day exposures (Table 1). There was only a 58% probability that the means of the pyrene treatments were different and did not justify analysis by the Duncan's test.

TABLE 1. Groups of similar maximum standing crops (mg L^{-1}) determined by Duncan's Multiple Range Test. Means with different letters were significantly different from one another. ($\alpha = 0.05$). Underlined values are significantly different from controls.

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)

Certain acenaphthene and naphthalene treatments resulted in biomasses which were significantly greater than those of the control treatments. The 48% exposure of acenaphthene (2427 μg L^{-1}) produced a MSC which was 26% greater than that of the control treatment (Table 1). The other exposures of acenaphthene did not affect growth.

All treatments of naphthalene produced biomasses that were significantly greater than that of the controls. The 48% (14851 μg L^{-1}) exposure was the most stimulatory (+56%) to growth. The other exposures of naphthalene stimulated growth but were not significantly different from each other.

The biomass of algae not exposed to PAH (controls) varied from a mean of 83 to 175 mg L^{-1} (Table 3). WEISS and HELMS (1971) made an interlaboratory comparison of the AAP and concluded that algal biomass was not always accurately reproduced under an "ideal" set of conditions. However, the authors did conclude that the AAP was an accurate comparative bioassay; it produced an accurate estimate of differences in biomass under different treatments. Thus the variability in the biomass of the control cultures among experiments does not invalidate the comparative differences in biomass among toxicant treatments in any one experiment.

DISCUSSION

The data in Figure 1 suggest that the hydrophobic polynuclear aromatic hydrocarbons used in this study markedly inhibited growth. These compounds were also more persistant in the media. This suggests that the most toxic aromatics in the water soluble fraction (WSF) of fossil fuels may not be those which have an immediate toxic effect. The more water soluble compounds such as naphthalene are very toxic during short term exposures or under conditions of frequent exposure. Yet because they are volatile and are readily degraded by microorganisms these compounds are not persistent (DAVIES and EVANS 1964, EVANS et al. 1965, HERBES and SCHWALL 1978). The less soluble compounds are more resistant to physical and biological degradation (HERBES and SCHWALL 1978). This suggests that over an ecologically meaningful period, hydrophobic aromatic compounds may be more toxic to algae than relatively hydrophilic aromatic compounds.

Our conclusion is supported by the data of other workers. KAUSS et al. (1973) reported that naphthalene decreased the assimilation rate of carbon by Chlamydomonas angulosa. However, the assimilation rate of cultures in open flasks recovered by 30% in 20 h. KAUSS and HUTCHINSON (1975) reported that naphthalene initially inhibited the growth rate of Chlorella vulgaris but that the growth rate in the exposed cultures recovered to that of the control cultures in two days. SOTO et al. (1975a, 1975b) reported the inhibition of the growth and photosynthetic capacity of Chlamydomonas angulosa by naphthalene. These authors (1977) also reported that cellular protein levels decreased while carbohydrate and lipid levels increased following exposure to naphthalene. Again, all these parameters returned to normal levels in open flasks.

Moreover, the most hydrophilic compounds tested in this study, acenaphthene and naphthalene, actually stimulated the growth of Anabaena flos-aquae (Table 1). These results are supported by reports of increased biomass in populations of green algae, euglenoids, and flagellates following exposure to the WSF of outboard motor oil, crude oil and oil refinery wastewaters (MINTER 1964, HUTCHINSON et al. 1972, KAUSS and HUTCHINSON 1975, SOTO et al. 1975a). GERHART et al. (1980) concluded that high concentrations of volatile organics in coal distillates inhibited algal growth while low concentrations of these compounds stimulated growth.

Several studies indicate that algae can degrade certain aromatics. CERNIGLIA et al. (1980) demonstrated that Oscillatoria sp. oxidized naphthalene to five polar products. The authors reported that 4.8% of a 10 mg L^{-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 3.5 mg L^{-1} naphthalene treatments were oxidized in 24 h. ELLIS (1974) reported that 100 M L^{-1} of phenol or catechol was oxidized 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 the use of acenaphthene and naphthalene as sources of carbon. In any event, algae fully recover from an initial exposure to certain chemicals (SOTO 1975a, 1975b, 1977, this paper).

The toxicity of the more hydrophobic PAH is also corraborated by the results of previous investigators. HUTCHINSON et al. (1979) reported an inverse relationship between toxicity and water solubility when exposures of various hydrocarbons to Chlamydomonas angulosa and Chlorella vuglaris were compared on a molar basis. Lower molarities of less soluble hydrocarbons produced the same level of inhibition to ^{14}C uptake (e.g. 50% reduction) or the same amount of membrane disruption (e.g. ionic leakage) as higher molarities of more soluble compounds. Their results suggest that relatively insoluble compounds contribute significantly to the toxicity of fossil fuels despite their low solubility in water. GRAY et al. (1982) reported that Selenastrum capricornutum was unable to recover from the WSF of coal liquid because of the presence of high molecular weight phenols and slowly decomposing hydrocarbons.

Populations of relative simple organisms such as algae are able to recover from environmental stress (e.g. xenobiotics) because individuals multiply quickly. A small number of surviving individuals can produce a large population in a short period of time. The hydrophobic PAH showed a persistent toxicity to the growth of Anabaena flos-aquae and are ecologically important because they inhibit a major adaptive mechanism of algae; the capacity for quick recovery from environmental stress by reproduction.

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