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Effect of Polynuclear Hydrocarbons on Algal Nitrogen Fixation (Acetylene Reduction)

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Biological nitrogen fixation (BNF) is a major source of nitrogen for the biosphere and is one of the three major biological processes by which it is cycled in the environment (Hutchinson 1957). The rate of elemental cycling and the regeneration of available forms of elements to organisms is one of the major controls of the productivity of an ecosystem (Odum 1983).

Nitrogen fixation occurs in a wide variety of aquatic environments such as lakes (Billaud 1968 and Horne and Goldman 1972) reservoirs (Toetz 1973 and Ashton 1981) salt marshes (Dicker and Smith 1980) cypress domes (Dierberg and Brezonik 1982), the ocean (Dugdale et al 1964 Mague et al 1974 Carpenter 1972 and Carpenter and Price 1977) atolls (Potts and Whitton 1977) and estuaries (Brooks et al 1971). Thus, an understanding of the effects of toxic chemicals on BNF is potentially important to an understanding of the effects of toxicants on aquatic productivity.

The objective of this research was to determine the effects of polynuclear aromatic hydrocarbons (PAH) on N_2 fixation by the alga, Anabaena flos-aquae. This species forms blooms in lakes and in one case contributed 25% of the nitrogen (N) entering a lake by BNF (Billaud 1968).

The U. S. Environmental Protection Agency has designated PAH as priority pollutants, a class of chemicals which pose a serious potential threat to organisms in the environment and human health (Gerhart 1980). Several investigators have reported the inhibition of carbon fixation by PAH in algae (Giddings 1979 Hutchinson 1979 Soto et al. 1975 and 1975a). We are unaware of any published information on the effects of PAH, or any other priority pollutants, on aquatic BNF.

We measured the reduction of acetylene (C_2H_2) to ethylene (C_2H_4) as described in EPA (1979) as a measure of the capacity of an organism to fix atmospheric N_2 and reduce it to an assimilable form. The primary advantage of this assay is its speed since chemical exposure and quantitative chromatographic analysis can be completed in a few hours.

METHODS AND MATERIALS

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

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.

We used the acetylene reduction technique as a measure of BNF (Stewart et al 1967). Toxicant-saturated and media lacking toxicants, both lacking N nutrients, were mixed (v/v) to yield 0, 10, 20, 50 and 90 % saturated 50 ml solutions in 72 ml serum bottles, 4 replicates per concentration. Cells in exponential growth were added to each treatment and the control to yield 2.5 x 10^5 cells ml^{-1}. Acetylene reduction activity (ARA) should be measured in exponential growth (Stewart 1980). At this time trichomes had a heterocyst frequency of 7-8% and cells had a cellular nitrogen content of one nanomole N $cell^{-1}$.

Five 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, but more than 0.1 atm pC_2H_2 completely inhibits dinitrogen reduction (Schollhorn and Burris 1966). Air was not flushed from incubation bottles in order to prevent volatilization of toxicants. Blanks containing non-toxic media and C_2H_2 but without cells, were included in each experiment to detect C_2H_4 contamination in the C_2H_2. Combinations of toxicant plus cells, toxicant plus C_2H_2, and toxicant plus trichloroacetic acid (TCA) did not produce C_2H_4.

The serum bottles were incubated in a randomized block design for 120 $\pm$ 5 min. under the light regime and temperature where stock cultures were grown. Ethylene production was found to be linear with time for 2 h. in two experiments using healthy untreated cells. ARA was terminated after 120 min by the addition of 2 ml of 50% TCA.

Ethylene production was detected using a Hewlett Packard 5750 research gas chromatograph with a hydrogen flame detector, and a column containing Porapak T. Ethylene peaks were identified and quantified by co-chromotography with C_2H_4 standards. Purified gases were obtained from Matheson Co.

Chlorophyll a (chl. a) was measured in cultures grown under experimental conditions using a Turner fluorometer which was calibrated with reagent grade chlorophyll a (Sigma Chemical Co). A 2:1 v/v mixture of chloroform/methanol was used to extract pigments.

Trichomes, cells and heterocysts per trichome were counted with a light microscope using a hemocytometer grid. Cell density was computed as number of trichomes per ml X average number of cells per trichome. Heterocyst frequency was measured as heterocyst number/cell density X 100. Cellular nitrogen was calculated by harvesting cells from a known volume on precombusted Reeve Angel 984 H glass filters (525° C). The N content of the filter was determined by persulfate digestion (Raveh and Avnimelech 1979).

The actual toxicant concentration at various dilutions was determined by fluorescence analysis using a Aminco Bowman Spectrophotofluorometer (Mackay 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).

RESULTS AND DISCUSSION

The solubility of the test chemicals in algal media was in general agreement with literature values reported for PAH dissolved in distilled water (Mackay and Shiu 1977 Schwarz and Wasik 1976 and Klevens 1950). The exposure concentrations are given in Table 1. We assume that in these tests little or no toxicant was lost from solution during the short period of exposure.

Table 1. Concentrations of PAH solutions predicted from fluorescence.

Chemical	Concentrations (nanomoles per liter)				
Acenaphthene	0	2,734	6,199	16,393	29,953
Benzanthracene	0	22	39	83	131
Chrysene	0	4	9	22	61
Fluoranthene	0	188	499	1,157	2,146
Fluorene	0	806	1,564	3,682	6,822
Naphthalene	0	16,157	48,782	120,746	249,984
Phenanthrene	0	752	1,503	2,721	5,122
Pyrene	0	109	287	420	786
% Saturation	0	10	20	50	90

Each exposure level of a chemical and the control were considered separate treatments for purposes of statistical analysis. Analysis of variance (ANOVA) was used to determine if the means of the

treatment results were different. Then, the Duncan's Multiple Range Test was used to determine which individual means were different ($p > 0.05$).

Table 2. Groups of similar ethylene means (pM C_2H_4 µg chl $a^{-1}h^{-1}$) determined by Duncan's Multiple Range Test ($\alpha = 0.05$). Underlined values are significantly different than the control.

Chemical	Number of Rings	% Saturation 0	10	20	50	90
Acenaphthene	3	14.2	15.4	15.3	16.0	17.5
Benzanthracene	4	17.8	15.2	17.0	14.8	12.6
		34.4	38.8	41.9	28.2	36.2
Chrysene	4	29.4	28.6	28.5	26.0	24.4
Fluoranthene	4	29.8	26.9	28.8	25.3	21.4
		34.4	35.6	27.3	31.2	27.3
Fluorene	3	8.2	8.1	7.6	6.6	6.8
Naphthalene	2	37.3	31.1	30.8	26.0	11.3
		10.5	7.5	5.2	5.0	4.9
Phenanthrene	3	37.3	31.5	34.5	32.1	24.6
		10.5	6.3	6.1	5.6	5.8
Pyrene	4	8.4	8.6	7.8	9.4	8.5

Cells exposed to naphthalene (2 rings) had markedly lower ARA at all levels of exposure than the control (Table 2). Cells exposed to phenanthrene (3 rings) also reduced acetylene at a lower rate. Exposure of cells to other 3-ringed PAH resulted in a lower rate of ARA only at higher exposure levels (fluorene) or in no effects (acenaphthene). Exposure of cells to four-ringed compounds resulted in either a slight reduction at the highest exposure level (benzanthracene, chrysene and fluoranthene) or in no inhibition (pyrene). We were not successful when we attempted to correlate statistically either the absolute or relative decrease in ARA to toxicant concentration or to the partition coefficients of toxicants.

Table 3 gives the data upon which some of the statistical analyses in Table 2 were based. The data indicate that the inconsistentcy was occasionally caused by variability of the test results. For example, in the first phenanthrene test at the 10% exposure one particularily low value (23.00 pM C_2H_2 g chl. $a^{-1}hr^{-1}$) was responsible for the low value of the mean relative to the mean ARA at 20% exposure. In the pyrene test at 50% exposure, three of the four data points are higher than values in the control, leading to a mean value that was significantly different than the control and also significantly different from the mean ARA at the 90% exposure. Apparently, some factor(s) actually increased ARA at the 50% exposure in the pyrene test.

At times variability in the controls was high. It too may have contributed to lack of statistical differences.

Table 3. Replicate measurements of acetylene reduction activity (ARA) at various exposures for phenanthrene (first test) and pyrene. ARA is expressed in units of pM C_2H_4 µg chl. a^{-1} h^{-1}. Underlined values are significantly different than the control.

Exposure (%)	Replicate Values	Mean	Standard Deviation
	Phenanthrene		
0 control	38.5/41.1/37.9/31.6	37.3	4.008
10	32.0/35.9/23.0/35.3	31.6	6.000
20	33.4/35.0/33.4/36.0	34.5	1.278
50	30.4/33.0/29.5/35.4	32.1	2.674
90	29.3/21.1/21.9/26.1	24.6	3.817
	Pyrene		
0 control	8.1/8.2/8.5/8.8	8.4	0.333
10	8.7/8.5/8.8/8.4	8.6	0.169
20	7.9/8.2/7.3/7.7	7.8	0.393
50	9.6/10.6/9.1/8.3	9.4	0.963
90	8.3/8.7/8.5/8.4	8.5	0.160

We repeated several tests to determine the replicability of the acetylene reduction toxicity tests. There was good agreement between tests done on the naphthalene, a compound which caused lower ARA at all exposures above 10% (Table 2). When compounds had effects only at high exposures, there was moderate consistency between tests (fluoranthene, benzanthracene and phenanthrene).

Our results are in some respects similar to those of workers who used $^{14}CO_2$ uptake as a measure of the toxicity of PAH to algae. For example, we found that only the very highest concentrations of PAH (90% saturation) consistently decreased ARA below control values. Interpolating from Figure 3 in a paper by Hutchinson et al (1979) we estimate that concentrations at or near saturation were needed to cause a 50% reduction in $^{14}CO_2$ uptake by Chlorella for naphthalene, pyrene and phenanthrene. Likewise, Giddings (1979) using Selenastrum capricornutum reported significant inhibition of $^{14}CO_2$ uptake relative to the controls only at saturating concentrations of phenanthrene and no effect at all by anthracene. But, there was a significant but small decline in $^{14}CO_2$ uptake at 10% saturation of naphthalene. The latter is to be expected as naphthalene has a higher solubility in water than the other PAH tested by Giddings. Our results also indicate naphthalene inhibits acetylene reduction at lower treatment levels than other PAH.

There was a three-fold variation in the rate of ARA in the controls, and at times high variability in experimental units. The variation among replicates of the control and among experimental replicates may be due to experimental error. Heterocysts are the site of ARA, hence heterocyst density is related to the rate of ARA. We believe one cause of experimental error was the difficulty of dispensing accurately the same quantity of cells and heterocysts into serum bottles. Since we diluted 5.0 ml of concentrated cells to 50.0 ml with cell-free water, small errors in pipetting from the stock culture could be the cause of variation in density after dilution.

However, not all of the variability encountered was due to experimental error. Similar variability in ARA was encountered by Dr. Larry Hersman who studied the effect of coal leachates on ARA using Azotobacter, which does not have heterocysts (Personal Communication). We and Hersman feel that some of the variability was due to differences in the physiological status of the cells used in the experiments. The fact that differences in ARA existed between experiments is clearly evident in Table 2 where ARA of replicates of controls differed from experiment to experiment. We believed that standardized physiological status of the exposed cells would be achieved during exponential growth. Apparently, this was not the case. Different responses may be due to changes which affect the enzyme systems responsible for ARA. These results suggest workers should run several experiments when testing a compound for toxic effects on ARA, particularly if the compound has a low solubility in water.

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