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Growth, Photosynthesis and Nitrogen Fixation of *Anabaena doliolum* Exposed to Assam Crude Extract

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Petroleum contaminants pose serious threat to the structure and functioning of aquatic ecosystems. Although their effects on various algal species have been studied before (O'Brien and Dixon 1976; Vandermeulen and Ahern 1976), heterocystous blue-green algae (cyanobacteria) have been little explored in this regard. These cyanobacteria can fix N_2 and therefore considerable emphasis has been attached to their role in aquatic ecosystems. Inadequate information concerning the effects of petroleum oils on them, especially on their nitrogen fixing system, prompted us to undertake this study.

In this communication we report the influence of Assam crude on growth, photosynthesis (^{14}C incorporation), nitrogen fixation (nitrogenase activity) and heterocyst differentiation in Anabaena doliolum. This species and other heterocystous cyanobacteria occur widely in soil and aquatic ecosystems (Desikachary 1959).

MATERIALS AND METHODS

Anabaena doliolum, obtained from the Centre of Advanced Study in Botany, Banaras Hindu University, was axenically cultivated in Allen and Arnon's medium (Singh and Gaur 1988) devoid of combined source of nitrogen. The cultures received 2000 lux light in 14h light and 10h dark cycle at 24±1°C. All experiments were conducted in triplicate under axenic condition.

One part of sterilized Assam crude, obtained from Gauhati Refinery, was slowly stirred by a magnetic stirrer with 20 parts of sterilized basal medium. After 12h stirring was stopped and the aqueous phase containing water-soluble fractions was separated. This was designated as 100% aqueous crude oil extract (ACOE) and its dilution with basal medium gave 20,40,60 and 80% ACOE. Fluorescence spectroscopy technique was used to estimate the amount of oil in ACOE (Singh and Gaur 1988).

Growth experiments were carried out in Erlenmeyer flasks (capacity 400 ml) containing 125 ml of basal or oil-saturated

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medium. Exponential phase cultures of A. doliolum were used for inoculation. The biomass of test alga was monitored by asceptically taking out 5 ml of culture suspension from each replicate flask daily during initial 5 days of incubation and on alternate days afterwards. The biomass was measured in terms of chlorophyll a by the method of Holden (1965). The data obtained were used to calculate the specific growth rate and final yield.

Photosynthesis was assayed by following the uptake of $^{14}CO_2$ from $NaH^{14}CO_3$ (BARC, Bombay). Scintillation vials containing 0.2 ml of diluted $NaH^{14}CO_3$ (0.5 µCi/ml) in 1 ml of culture suspension (initial absorbance 0.04 at 665 nm) for all treatments and control, were illuminated by 2500 lux at 25°C. Activity was assayed at 30 min interval for two hours. In long-term experiments the activity was measured for one hour only. Reaction was terminated by adding 0.2 ml of 50% acetic acid. The resulting suspension after bubbling with air for 4 min was spiked with 10 ml scintillation cocktail (3 parts ethanol, 4 parts 0.8% PPO (2,5-diphenyl oxazole) and 0.01% POPOP (1,4-bis-2-(5-phenyloxazolyl) benzene) in toluene. Counting was done in a Beckman liquid scintillation counter (model LS 2000) and the rate of $^{14}CO_2$ uptake has been expressed in dpm.

Nitrogenase activity was determined by acetylene reduction method (Stewart et al. 1967). Assay was performed in calibrated serum vials of about 7.5 ml capacity. The acetylene concentration was kept at 10% (v/v) and 2 ml of culture suspension was injected into each bottle. Nitrogenase activity in experimental and control vials was monitored 30, 60, 120 and 180 min after incubation at 25°C and 2500 lux light intensity. Reactions were terminated by 50% trichloroacetic acid. Ethylene produced in the reaction vessels was analysed in a CIC gas chromatograph (Baroda, India) fitted with a hydrogen flame ionization detector. Nitrogenase activity in long-term experiments was determined by incubating the test alga in acetylene for one hour.

In order to study the effect of test oil on heterocyst differentiation, heterocyst-free inoculum was prepared by growing the test alga in 10 mM of ammonium. Heterocyst appeared on the third day and their frequency was calculated by counting the number of heterocysts per hundred vegetative cells in at least 20-30 filaments of approximately the same length.

RESULTS AND DISCUSSION

The saturation level of aqueous oil extract used in our study ranged from 20 to 100%. Saturated oil extract contained 12.7 mg/l of oil. Such high concentrations exist in refinery effluents and the areas of oil spill (Singh and Gaur 1988).

The test oil inhibited the growth of A. doliolum in a concentration-dependent manner (Table 1). A 9-day long lag was observed only in saturated ACOE. Our study conforms with Gaur and

Kumar (1981) who studied the effects of this oil on four unicellular algae. However, the extent of inhibition was more severe in the present study.

Table 1. Specific growth rate and final yield (biomass attained on the 15th day) of A. doliolum exposed to aqueous crude oil extract (ACOE).

Saturation level of ACOE	Growth rate/day	Final Yield (µg chlorophyll a/ml)
Control	0.38 ± 0.05a	2.68 ± 0.11a
20%	0.36 ± 0.02a	2.52 ± 0.12b
40%	0.30 ± 0.04b	2.04 ± 0.05c
60%	0.25 ± 0.03c	1.83 ± 0.07d
80%	0.21 ± 0.02c	1.44 ± 0.10e
100%	0.12 ± 0.01d	0.51 ± 0.04f

Means (n=3; ± S.D.) with different letters are significantly different from one another ($P < 0.05$) according to Duncan's Multiple Range Test.

Table 2. Short-term effects of ACOE on ^{14}C uptake in A. doliolum.

Saturation level of ACOE	^{14}C incorporation (dpm/µg chlorophyll a)			
	Time (Min)			
	30	60	90	180
Control	126 ± 8a	218 ± 12a	336 ± 18a	489 ± 24a
20%	157 ± 14b	194 ± 8b	289 ± 24b	291 ± 27b
40%	134 ± 10c	182 ± 6b	218 ± 12c	261 ± 15c
60%	75 ± 6d	96 ± 4c	126 ± 4c	145 ± 12d
80%	58 ± 4e	86 ± 6c,d	97 ± 7d	130 ± 14e
100%	49 ± 3e	76 ± 3d	89 ± 5d	104 ± 6f

Means (n=3; ± S.D.) in each column followed by the same letter are not significantly different ($P < 0.05$) according to Duncan's Multiple Range Test.

The test oil stimulated ^{14}C uptake at 20% saturation level during 30 min of incubation (Table 2), but prolonged incubation was inhibitory. Low concentration of some other oils have been found to stimulate ^{14}C uptake in laboratory cultures (Karydis 1979) and natural phytoplankton populations (Gordon and Prouse 1973). This phenomenon has been ascribed to the presence of growth regulating compounds (Gordon and Prouse 1973) and trace elements (Hufford 1971) in oils, increased permeability at low levels of hydrocarbons (Van Overbeek and Blondeau 1954), uptake and metabolization of certain oil constituents (O'Brien and Dixon 1976), and the increased rate of nitrogen fixation in oiled habitats (Baker 1971). But, the present

findings do not agree with Baker as nitrogenase activity was never stimulated (see Tables 4 and 5). Chan and Chiu (1985) discounted the role of growth regulating compounds in causing stimulatory effects. But they observed increased growth rate and final yield, and related these changes to the uptake and metabolization of certain oil constituents. Their explanation cannot be applied in our study as the specific growth rate or final yield of A. doliolum did not increase in oil-containing medium. High saturation levels of ACOE inhibited ^{14}C uptake in short-term experiments. In long-term experiments, ACOE initially caused more pronounced inhibition of ^{14}C uptake (Table 3). After maintaining declining trends up to 9 days in 20,40 and 60% ACOE, gradual improvement in ^{14}C uptake occurred; whereas, declining trends were maintained in 80 and 100% ACOE.

Table 3. Chronic effects of ACOE on carbon fixation by test alga.

Saturation level of ACOE	^{14}C incorporation (dpm/µg chlorophyll a/h)				
	Time (days)				
	3	6	9	12	15
Control	442±26a	443±36a	443±24a	444±48a	437±32a
20%	355±30b	377±28a,b	385±36b	393±24b	412±41a
40%	327±24b	286±20b,c	327±24c	354±31c	381±20b
60%	290±18c	245±22b,c	303±11d	330±25d	356±24b
80%	196±12d	196±15c	187±19e	168±14e	131±12c
100%	183±15d	152±17c	162±13f	148±10e	101±12c

Means (n=3; ± S.D.) in each column followed by the same letter are not significantly different ($P < 0.05$) according to Duncan's Multiple Range Test.

Table 4. Short-term effects of ACOE on nitrogenase activity of A. doliolum.

Saturation level of ACOE	Nitrogenase activity (nmol C_2H_4 produced/µg chlorophyll a)			
	Incubation period (min)			
	30	60	120	180
Control	3.52±0.28a	5.30±0.42a	10.40±1.04a	14.61±0.96a
20%	3.32±0.31a,b	4.20±0.38b	7.82±0.82b	10.22±1.08a,b
40%	2.60±0.42a,b	3.39±0.34b,c	6.83±0.53c	8.70±0.72a,b
60%	2.58±0.23a,b	3,17±0.21c,d	4.15±0.27d	4.53±0.36b
80%	1.73±0.12b	2.44±0.30d,e	3.64±0.32d,e	3.54±0.23b
100%	1.48±0.10b	1.59±0.11e	2.98±0.15e	2.74±0.25b

Means (n=3; ± S.D.) with different letters are significantly different from one another ($P<0.05$) according to Duncan's Multiple Range Test.

Stimulatory and inhibitory effects of test oil may be due to the changes in membrane permeability. Van Overbeek and Blondeau (1954) suggested that low concentrations of oil increase the permeability, whereas high concentrations disrupt the membrane systems. Inhibition of carbon fixation at high saturation level could also be ascribed to inhibition of light reaction, particularly PS II (Singh and Gaur 1988).

Table 5. Chronic effects of ACOE on nitrogenase activity of A. doliolum.

Saturation level of ACOE	Nitrogenase activity (nmol C_2H_4 produced/µg chlorophyll a/h)				
	Time (days)				
	3	6	9	12	15
Control	9.22a ±0.68	9.63a ±0.71	9.73a ±0.83	9.63a ±0.65	9.55a ±0.60
20%	6.99b ±0.64	7.20b ±0.83	7.56b ±0.68	7.93b ±0.86	8.16b ±0.45
40%	5.90c ±0.23	5.41b,c ±0.34	6.22c ±0.27	6.59c ±0.45	6.81c ±0.62
60%	5.75c ±0.34	5.25b,c ±0.48	4.93d ±0.12	5.31d ±0.23	6.63d ±0.42
80%	4.95d ±0.10	4.72c ±0.22	3.62e ±0.14	3.37e ±0.17	2.64e ±0.30
100%	3.05e ±0.21	2.55d ±0.19	2.16f ±0.14	1.77f ±0.12	0.96f ±0.12

Means (n=3; ± S.D.) with different letters are significantly different from one another ($P<0.05$) according to Duncan's Multiple Range Test.

In short term experiments, ACOE inhibited nitrogenase activity in a concentration-dependent manner (Table 4). In contrast to ^{14}C uptake, nitrogenase activity was not stimulated at any tested concentration. Inhibition of nitrogenase activity occurred in long-term experiments also (Table 5) and the trend was similar to that of ^{14}C uptake (Table 3).

Inhibition of nitrogenase activity in our study could be compared with the reports of detrimental effects of pesticides (Da Silva et al. 1975; Wurtsbaugh and Apperson 1978) and polynuclear aromatic hydrocarbons (Bastian and Toetz 1985) on cyanobacterial N_2 fixation. Heterocyst is considered as the site of aerobic N_2 fixation in cyanobacteria (Wolk 1982). ACOE inhibited heterocyst differentiation in A. doliolum (Table 6) and this could be one of the reasons for inhibition of nitrogenase activity in long-term experiments. Since a pool of reductants composed of fixed carbon compounds provides electrons for nitrogen fixation (Lex and Stewart 1973), the inhibition of photosynthesis may retard the rate of nitrogen fixation. This

assumption seems valid because the general patterns of inhibition of ^{14}C uptake and nitrogenase activity were similar in long-term experiments. In this study the inhibition of nitrogenase activity occurred in short-term experiments as well in spite of the fact that the alga did not experience carbon limiting conditions, and its carbon fixation was actually stimulated in 20% ACOE. Inhibition of nitrogenase activity in these cases seems to be independent of photosynthesis because PS I activity which may provide ATP for the reduction of N_2 via cyclic photophosphorylation, and could transfer electrons to nitrogenase was not impaired by the test oil (Singh and Gaur 1988).

Table 6. Heterocyst differentiation in A. doliolum treated with ACOE.

Saturation level of ACOE	Heterocyst frequency (%)
Control	5.80±0.12a
20%	5.06±0.16b
40%	4.20±0.19b
60%	2.35±0.13c
80%	2.31±0.09c
100%	1.24±0.05c

Means (n=3; ± S.D.) with different letters are significantly different from one another ($P<0.05$) according to Duncan's Multiple Test.

It is difficult to pin-point the toxic constituents of ACOE as we are still trying to analyze it. But, paraffinic and aromatic hydrocarbons are the major water-soluble toxic components of some other oils (O'Brien and Dixon 1976; Vandermeulen and Ahern 1976).

Although the studies presented here deal with A. doliolum, other heterocystous cyanobacteria may also be deleteriously affected by Assam crude and similar type of oils.

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