Bull. Environ. Contam. Toxicol. (2004) 73:971–978

DOI: 10.1007/s00128-004-0521-y

Bulletin of Environmental Contamination and Toxicology

Algal Bioassay for Evaluating the Role of Algae in Bioremediation of Crude Oil: II. Freshwater Phytoplankton Assemblages

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Received: 6 February 2004/Accepted: 10 September 2004

The annual influx of petroleum into the marine environment is estimated to be between 1.1-7.2 million metric tons (NRC 1985). In addition, 28%-30% of the oil spilled enters the freshwater environment (Tarshis and Rattner 1982). Petroleum entering an aquatic ecosystem provides a source of carbon and energy for microbial growth (Vandermeulen 1987). There is increasing interest in algae as tools for self-cleaning and bioremediation of a polluted environment (Kuritz and Walk 1995). Increasing biomass and phytoplankton growth rates influence the biogeochemical cycles of persistent organic pollutants such as polychlorinated biphenyls (PCBs) in aquatic environments (Gunnarsson *et al.* 1995). Higher growth rates lead to lower PCB concentrations in the phytoplankton due to dilution by the new organic matter introduced in the ecosystem (Axelman *et al.* 1997). It is believed that some groups of algae can at most initiate the biodegradation of hydrocarbons by oxidizing them to components of lower molecular weight or by the transformation of petroleum hydrocarbons to more polar compounds of a carbon number equal to the parent compound (Al-Hasan *et al.* 1994).

Therefore, the current study aimed to specify the ability of freshwater phytoplankton assemblages to degrade and remove oil spills from the surrounding environments using a bioassay technique.

MATERIALS AND METHODS

Integrated phytoplankton assemblages were collected from the Nile River and concentrated using a phytoplankton net (80 μm). The collected bulk represented the freshwater algal groups that exist in the Nile River: green algae, blue-green algae and diatoms. The phytoplankton biomass was introduced to the incubation reactors supplemented with EPA media (1972) at 24 ± 2 °C under continuous illumination provided by white fluorescent lamps ~ 2500 lux. Egyptian light crude oil (specific gravity, 0.85) was used. The oil was added to the algal culture without any treatment. Erlenmeyer flasks (5L) were used as incubation reactors. Triplicate cultures of each control and treatment were employed. Each flask was filled with 3L of distilled water containing the algal culture from the Nile River community and 3 ml of the crude oil. Initial chlorophyll "a" concentration for all

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experiments ranged from 25-30 µg/L. Chlorophyll "a" content used as an indicator for algal growth, was measured every 48 hours according to APHA (1998). Species composition of the algal community was performed to evaluate the change in community structure of natural phytoplankton assemblages as a response to crude oil contamination.

In addition, weekly water samples were withdrawn for chemical analysis to determine crude oil biodegradation. The water samples were analyzed to determine the residue levels of n-alkane (from C10 – C24) and polycyclic aromatic hydrocarbons (PAHs, 15 compounds) in control and treatment cultures. The water sample was extracted twice using a glass separating funnel fitted with glass stopper and PTFE stopcock. Chromatography grade dichloromethane (DCM) was used to extract the water sample. The extracts were combined, dried with anhydrous Na_2SO_4 and concentrated to 10 ml using a rotary evaporator and then concentrated to 1 ml using a clean nitrogen flow. The subsequent clean-up and fractionation of the extracts was carried out by column chromatography according to Law *et al.* (1988).

Briefly, 20 cm length and 10 mm bore glass columns were used. The column was slurry packed – the slurries of both alumina and silica gel (5% deactivated) - being prepared in n-hexane. The packed column had 6 cm of alumina over 12 cm of silica and 1 cm of anhydrous Na_2SO_4 on the top, then it was washed with 20 ml of n-hexane. The concentrated extract was quantitatively transferred to the top of the column using a Pasteur pipette. The n-alkane fraction (F1) was eluted first with 40 ml of n-hexane, then the aromatic fraction (F2) which, contains PAHs, was eluted next with 20 ml of 10% DCM in n-hexane followed by 20% DCM in n-hexane. Both fractions (F1 & F2) were reduced to 1 ml for further analysis by gas chromatography.

A Hewlett Packard HP 6890 series capillary gas chromatograph equipped with Flame Ionization Detector (FID) was used to identify and quantify the petroleum hydrocarbons. The capillary column HP-1 was used and programmed from 80°C – 280°C at 8°C/ min with 2 min initial hold and 15 min final hold for PAHs and from 40°C – 250°C at 10°C/min with 2 min initial hold and 8 min final hold for n-alkanes. The inlet and detector temperatures were isothermally maintained at 250°C and 300°C, respectively. Nitrogen was used as a carrier and make – up gas. All samples were injected in the splitless mode. Procedure blanks and standards were run systematically in association with samples. Data acquisition and processing were controlled by the HP Windows – based Chemstation data system. Concentrations of n-alkane and PAHs were calculated by using the external standard calibration method. A standard of fifteen individual PAH compounds in a mixture and 8 individual even number n-alkane (C10 – C24) obtained from Suppelco Inc. were used.

For quality assurance, sample duplicates, procedural blanks and standard reference material (Egyptian heavy crude oil "weathered", supplied by Egyptian Petroleum Research Institute) were analysed routinely with each batch of samples.

Measured levels of target n-alkanes and PAHs in the reference material were within 85-105 % of the certified values. Recoveries were obtained by spiking distilled water with 200 ng of n-alkanes and PAHs standard, and analysed routinely with the samples. The average recoveries varied between 60-96 % for PAHs and from 55-90 % for n-alkanes.
Method detection limits ranged from 3-5 µg/L for n-alkanes and PAHs. Total n-alkanes and PAHs were correlated with the growth of both kinds of algae (as chlorophyll "a" content) using the Product Moment Correlation analysis. T-test was used to differentiate between means for removal of n-alkanes and PAHs by both algae species. Statistical tests for significance were performed at $P< 0.05$.

RESULTS AND DISCUSSION

Addition of 0.1% crude oil resulted in a pronounced promotion of algal growth with high algal biomass (Fig.1). The control culture attained a maximum standing biomass of about 0.46 mg/L at the 23rd day, while the treated culture exhibited algal biomass 4 times greater than the control. The standing biomass of the treated culture reached its maximum value of 1.8 mg/L at the 29 ^{the} day. These data revealed that the treatment of algal cultures with crude oil resulted in a characterized prolonged growth phase accompanied by high algal biomass production. Miller *et al.* (1978 a; b) found that algal biomass measured by chlorophyll *a* concentration was severely inhibited inside the oil curtain compared with outside the curtain. In addition, they stated that the inhibited planktonic primary production was later recovered, but the recovered production was composed of different, more oil resistant, algae.

The bulk of initial phytoplankton assemblages was characterized by the predominance of diatom groups mainly *Cyclotella comta, Diatoma elongatum, Melosira granulata, Synedra ulna* and *Nitzschia linearis.* Species belonging to green algae such as *Scenedesmus quadricauda, S. obliquus, Pediastrum clathratum, Dictyosphaerium pulchellum, Oocystis parva, Tetraedron minimum, Staurastrum paradoxum* and *Ankistrodesmus acicularis* were present with less frequency. Species which were present in low quantities were *Microcystis aeruginosa, Oscillatoria limnetica, O. brevis, O. tenuis, Chroococcus turgidus, Merismopedia glauca* (Blue-green algae), *Spirogyra mirabilis, Ulothrix subtilissima* and *Mougeatia scalaris* (green algae).

After 37 days from the addition of the crude oil, the dominance of the phytoplankton community structure was completely changed. The new dominance of algae was *Oscillatoria brevis, Microcystis aeruginosa* (blue-green algae), *Spirogyra mirabilis, Ulothric subtilissima* and *Mougeatia scalaris* (green algae). The subdominant algae were *Pediastrum clathratum, Scenedesmus quadricauda, Tetraedron minimum* and *Ankistrodesmus acicularis* (green algae). However, minor quantities of diatoms were often present with empty frustule cells.

The changes in domination of phytoplankton due to the effect of crude oil have been discussed. Miller *et al.* (1978 a; b) studied the effect of Prudhoe Bay crude

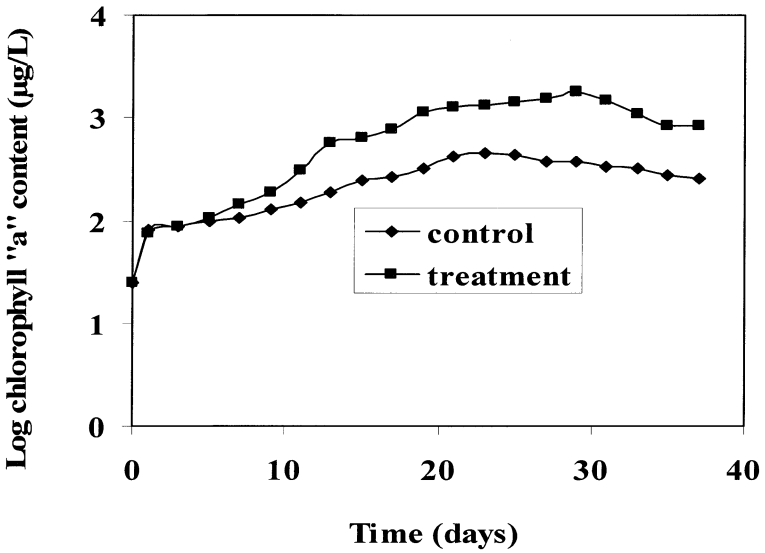

Figure 1. Growth response of a Nile River phytoplankton community to crude oil.

oil on primary production and species composition of Arctic tundra ponds. They found that algal biomass and species composition changes dramatically but slowly recovered to normal levels, with the recovered production being composed of different, more oil resistant algae. Atlas *et al.* (1976) found that coccoid green algae increased at low oil concentration (10% v/v), but completely disappeared at 100% (v/v) concentration. Diatoms increased and filamentous green and blue-green algae were unaffected, where as overall species diversity of the algal community increased at high oil concentration.

Jankevicius et al. (1992) explained that blue-green algae were inhibited by 0.1 mg/L petroleum and the originally dominant blue-green algae were replaced by the more resistant green algae (*Scenedesmus quadricauda*). The same result was obtained by Carpenter and Robertson (1988), where the dominant algae prior to hydrocarbon exposure were *Oscillatoria* sp. and lesser amounts of *Chlamydomonas* sp. and *Chlorella* sp. After six weeks of exposure to bilge, *Chlamydomonas* sp. and *Chlorella* sp. had increased by a factor of 20 (in phenol – treated cultures) and 55 (in waste – phenol treated cultures).

Biodegradation rate of both fractions of petroleum hydrocarbons (n-alkanes and PAHs) were calculated as percentage removal by comparing the treated sample against the control. Table (1) shows the biodegradation rates of each individual n-alkane and PAH, as well as the percentage removal of the sum for both fractions. It was found that the natural community of phytoplankton had removed the PAH fraction to a slightly higher degree than the n-alkane fraction through 3 weeks of exposure. After 6 weeks, the situation was reversed, the percentage removal of total n-alkanes increased versus total PAHs (Fig. 2). It should be noted that, due to

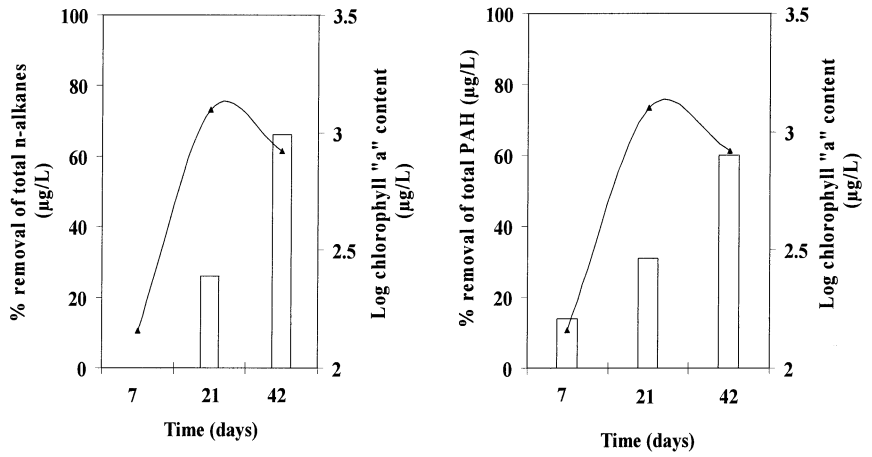

Figure 2. Percentage removal of petroleum hydrocarbons (columns) in relation to the growth of phytoplankton community (lines).

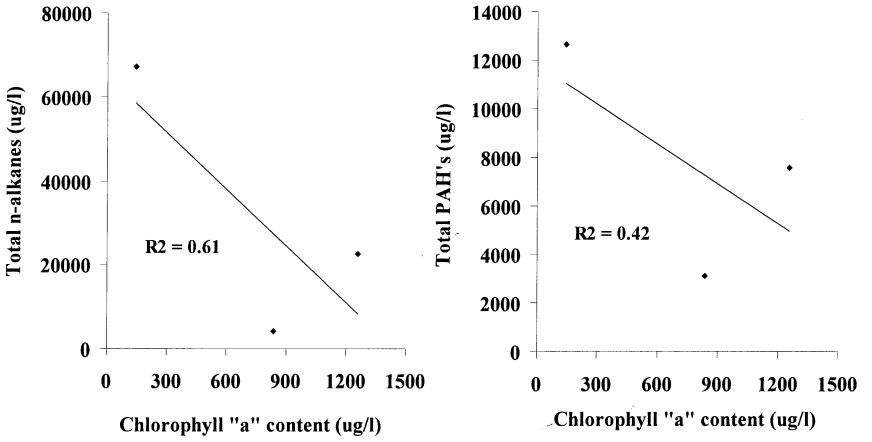

Figure 3. Correlation between petroleum hydrocarbons concentration and algal growth of Nile water community (significant negative correlation, $P< 0.05$).

the volatilization properties of the petroleum hydrocarbons, some of these compounds could be lost during the present study. It was reported that 35% of crude oil constituents with a boiling point below 200 °C volatilize in the environment (Al-Hasan *et al.* 1994). Generally, the phytoplankton community biodegraded both fractions of crude oil with no statistically significant difference through 6 weeks of exposure ($P > 0.05$). Data in Table (1) revealed that the natural community of phytoplankton was not able to degrade n-alkanes during the first week of exposure.

Table 1. Degradation rate (% removal ± SD) of petroleum hydrocarbons by phytoplankton community of River Nile (test of significant variation was at $P>0.05$).

Compounds	1st week	3rd week	6th week
n-alkanes			
n-C10	0.0± 0.0	23.0± 4.1	68.9± 3.9
n-C12	0.0± 0.0	21.6± 4.4	69.1± 4.3
n-C14	0.0± 0.0	60.9± 5.1	66.7± 4.8
n-C16	0.0± 0.0	20.3± 3.8	64.5± 4.1
n-C18	0.0± 0.0	22.4± 3.4	65.2± 4.1
n-C20	0.0± 0.0	20.5± 3.2	63.2± 3.8
n-C22	0.0± 0.0	19.4± 3.2	63.7± 3.7
n-C24	0.0± 0.0	20.1± 4.1	66.1± 3.6
Total n-alkanes	0.0	26.9	65.5
Polycyclic aromatic hydrocarbons (PAHs)			
Naphthalene	20.8± 3.8	53.0± 4.0	95.2± 4.6
1-methylnaphthalene	17.2± 4.1	48.0± 3.4	71.9± 4.2
2-methylnaphthalene	18.5± 3.9	46.7± 4.2	72.5± 5.1
Acenaphthylene	12.1± 3.6	38.5± 3.6	66.7± 3.8
Acenaphthene	16.3± 4.5	36.0± 3.1	60.4± 3.9
Fluorene	15.4± 3.1	33.4± 2.8	61.3± 3.8
Phenanthrene	17.1± 4.6	37.1± 3.1	65.1± 4.5
Anthracene	14.0± 3.7	31.7± 2.4	65.3± 3.6
Fluoranthene	16.6± 3.2	27.0± 2.9	55.0± 3.1
Pyrene	13.1± 3.8	21.4± 2.1	58.1± 4.6
Benzo(a)anthracene	10.4± 3.6	19.7± 2.0	51.2± 3.9
Chrysene	12.1± 3.6	22.8± 3.0	53.7± 4.4
Benzo(b)fluoranthene	9.8± 2.3	17.7± 2.1	49.2± 2.9
Benzo(k)fluoranthene	5.7± 2.1	14.6± 2.4	50.7± 3.7
Benzo(a)pyrene	4.2± 2.6	15.4± 2.3	49.9± 4.6
Total PAH's	14	31	60

Through 3-6 weeks of exposure, they degraded n-alkane compounds with percentage removals of 26.9% and 65.5%, respectively. Hence, efficiency of the phytoplankton community in biodegradation of petroleum hydrocarbons was limited compared with that for an isolated pure strain of algae. Gamila *et al.* 2003, reported that two isolated species of cyanobacteria had biodegraded two fractions of crude oil with percentage removal ranged from 97.5% to 99.5%.

In the present study, the growth rate of phytoplankton community (as chlorophyll a content) was negatively correlated with the total concentration of both crude oil fractions (fig.3). It was observed that the growth rate of the natural community had a strong negative correlation with total concentration of n-alkanes ($r = - 0.78$), while it had a moderately negative correlation with total concentration of PAHs

(r = - 0.64). These results are consistent with that documented by Gamila *et al.* (2003), who reported that a negative correlation existed between growth of isolated strains of cyanobacteria and the total concentration of petroleum hydrocarbons, but with stronger correlation than the present study. This reflects the potential efficiency of freshwater isolated pure strains of algae in biodegradation of crude oil over that for the natural community of freshwater phytoplankton.

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