Comparative studies on the toxicity of petroleum oils and their aqueous extracts towards *Anabaena dolioolum*

J P GAUR and A K SINGH
Department of Botany, North-Eastern Hill University, Shillong 793 014, India
MS received 14 October 1988; revised 29 August 1989

Abstract. Assam crude, kerosene, petrol, diesel and furnace oil administered into the culture suspension of *Anabaena dolioolum* as whole oil or aqueous extract exerted concentration dependent toxic effects. The hierarchy of toxicity of the test oils was diesel > furnace oil > petrol > kerosene > crude. The oils rich in aromatics were most toxic and therefore estimation of this fraction might enable prediction of toxicity of an oil. Growth rate was a more sensitive criterion of oil toxicity as compared of final standing crop. In case of crude and kerosene, the whole oil application was more inhibitory than their respective aqueous extracts, whereas reverse trend was obtained in case of other oils. The study recommends toxicological evaluation of whole oils as well as their aqueous extracts for meaningful results.

Keywords. *Anabaena dolioolum*; petroleum; EC_{50}; growth.

1. Introduction

Ever-increasing oil pollution of our environments has necessitated studying its impact on biota. Efforts have been made to evaluate the harmful effects of oil pollution on fishes, birds and other animals. In spite of being a critical component of aquatic ecosystem, algae have been little explored in this regard (O'Brien and Dixon 1976; Vandermeulen and Ahern 1976).

Although a majority of petroleum oils are toxic to algae, some are not (Coffey et al 1977; Fabregas et al 1984; Winters et al 1976). This is mainly due to variability in chemical composition of test oils, and partly to the use of different test organisms varying greatly in their tolerance. The bio-assay tests for oil toxicity generally use their aqueous extracts as these are (i) relatively simple in composition than the whole oil, (ii) uniformly distributed throughout the water column and (iii) may be easily transported into the algal cells. It is concerning to note that many of the earlier efforts do not indicate the actual amount of oil in aqueous extracts. This has rather been expressed in terms of per cent saturation level of aqueous oil extract. Therefore, it has become extremely difficult to compare the data on toxicity of different oils obtained by previous workers. Batterton et al (1978) and Pulich et al (1974) suggested that whole oil bioassays are more appropriate since they expose algae to a wide array of compounds, including those lipophilic in nature.

The main purposes of this study were to relate the toxicity of petroleum oils with their chemical composition, and to find out if toxicity of a given oil differs from its aqueous extract. This paper examines the effect of Assam crude and its 4 products viz, furnace oil, diesel, kerosene and petrol, and their aqueous extracts. With the exception of Assam crude which inhibits the growth of unicellular algae (Gaur and Kumar 1981), nothing is known about the toxicity of other oils used. *Anabaena dolioolum* was employed as the test organism because of its wide distribution,
capability to fix nitrogen and also the fact that blue-green algae (cyanobacteria) have been rarely used in such a programme. Growth performance was taken as the parameter since it reflects the overall response of the test organism.

2. Materials and methods

2.1 Test alga and culture conditions

A. doliolum, obtained from the Centre of Advanced Study in Botany, Banaras Hindu University, Varanasi was used as the test organism. The medium of Allen and Arnon (1955) devoid of combined source of nitrogen was used for the cultivation of test alga. The medium contained MgSO$_4$·7H$_2$O (1 mM), NaCl (4 mM), CaCl$_2$ (5 mM), K$_2$HPO$_4$ (2 mM), and iron as EDTA complex (4 mg l$^{-1}$). The supply of trace elements was done by adding MnCl$_2$·4H$_2$O (0.5 mg), Na$_2$MoO$_4$·2H$_2$O (0.01 mg), H$_3$BO$_3$ (0.5 mg), CuSO$_4$·5H$_2$O (0.02 mg), COC$_l_2$·6H$_2$O (0.04 mg) and ZnSO$_4$·7H$_2$O (0.05 mg) to one litre of culture solution. The stock cultures were maintained in 250 ml Erlenmeyer flasks and stoppered with non-absorbent cotton. All cultures were kept in a BOD incubator at 24 ± 1°C, and received about 2000 lux light in a 14 h light and 10 h dark cycle.

2.2 Test oils and their chemical characterization

The petroleum oils used in this study were Assam crude from Gauhati refinery, furnace oil from Digboi refinery, and kerosene, petrol and diesel purchased locally. The test oils were fractionated by the method of Pulich et al. (1974) for determining their paraffinic, asphaltic and aromatic contents. A 45 x 4 cm column of silica gel, which had been activated at 200°C for at least 12 h, was prepared in hexane. About 5 g of oil sample was diluted with equal amount of hexane and loaded on the column. Column flow rate was adjusted to 2–3 ml min$^{-1}$. The fractions of oils which eluted with hexane, benzene and chloroform: methanol (1:1), representing paraffinic, aromatic and asphaltic fractions, respectively, were measured.

2.3 Preparation of aqueous oil extracts

Test oils were extracted by the method of Boylan and Tripp (1971). Before extraction, the oils were sterilized by autoclaving in air-tight bottles. This was done in accordance with Vandermeulen and Ahern (1976) who did not find any appreciable change in the composition of petroleum oils during autoclaving in closed and air-tight containers. One part of sterilized oil was slowly stirred by a magnetic stirrer with 20 parts of sterilized medium in a glass-stoppered bottle. After 12 h, stirring was stopped and the solution was allowed to stand for at least 4 h. The aqueous phase containing water-soluble fractions was siphoned off and was designated as 100% extract. Lower saturation levels were obtained by dilution of this stock with appropriate volume of sterilized medium.

Estimation of oil in aqueous extracts was done by spectrofluorometric technique (Østgaard and Jensen 1983). The oil fractions dissolved in medium (50 ml) were extracted with 20 ml of spectrograde carbon tetrachloride (SDS, Bombay). The
extracted samples were excited at 340 nm and their intensity of fluorescence emission was measured at 463 nm in a Shimadzu spectrofluorimeter (model RF 540).

2.4 Treatment with whole oils

The method of Batterton et al (1978) was used for the supplementation of whole oils to *A. doliolum*. Absorbent pads (12.7 mm in diameter) obtained from Schleicher and Schuell Inc., Keene, New Hampshire, USA were soaked with basal medium and dried. Test oils were applied to these pads which were subsequently soaked in basal medium and administered into culture suspension. The oil-soaked pads remained at the bottom of culture tubes. The control sets received unoiled pads soaked with basal medium.

2.5 Growth experiments

Ten ml of culture medium containing water-soluble fractions or oil-containing pads was taken in cotton-plugged culture tubes of 25 ml capacity. To this exponential phase inoculum of *A. doliolum* was added to give the initial chlorophyll a concentration of 0.24 μg ml⁻¹. The tubes were kept in a BOD incubator with light and temperature conditions as described before. The position of culture tubes was changed daily to avoid any difference in growth due to the position effect. Each tube was hand-shaken at least 3-4 times daily and this did not lead to oil spillage (see Batterton et al 1978). Since the culture remained in exponential phase for 15 days, experiments were restricted to this length of time. Three replicate tubes for each treatment were taken out daily for estimating chlorophyll a.

A 5 ml of algal suspension was centrifuged to separate the algal cells. To this 3 ml of 90% methanol (BDH, Bombay) was added and the tube was kept overnight inside the deep freezer. Total extraction of chlorophyll was achieved by this method. Absorbance of the extract was determined at 650 and 665 nm with a Systronics spectrocolorimeter (Model 106) and the chlorophyll content was determined by the equation of Holden (1965).

Specific growth rate (μ day⁻¹) of the test organism was computed by the following equation.

\[
\mu \text{ day}^{-1} = \frac{\log_e (N_2/N_1)}{t_2 - t_1},
\]

where \(N_1\) and \(N_2\) are chlorophyll a concentrations at the beginning (\(t_1\)) and end (\(t_2\)) of the selected time interval.

The final yield data are expressed in terms of chlorophyll a concentration on the 15th day after incubation.

3. Results and discussion

Variabilities in concentrations of paraffinic, asphallic and aromatic fractions were observed in test oils (table 1). Assam crude contained maximum concentration of paraffinics (71%) and was followed in decreasing order by kerosene, petrol, diesel and furnace oil. The hierarchy of aromatic concentration in these oils was just reverse as aromatics were most concentrated in furnace oil and least in Assam.
Table 1. Relative concentration (%) of different fractions in test oils.

<table>
<thead>
<tr>
<th>Test oil</th>
<th>Paraffinics</th>
<th>Asphaltics</th>
<th>Aromatics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Assam crude</td>
<td>71</td>
<td>5</td>
<td>20</td>
</tr>
<tr>
<td>Kerosene</td>
<td>61</td>
<td>1</td>
<td>22</td>
</tr>
<tr>
<td>Petrol</td>
<td>52</td>
<td>1</td>
<td>26</td>
</tr>
<tr>
<td>Diesel</td>
<td>37</td>
<td>&lt;1</td>
<td>51</td>
</tr>
<tr>
<td>Furnace</td>
<td>36</td>
<td>&lt;1</td>
<td>54</td>
</tr>
</tbody>
</table>

Crude. Asphaltics were maximum in Assam crude, whereas other oils had nearly similar concentration.

The effects of test oils and their aqueous extracts on the growth behaviour of *A. doliolum* have been depicted in figures 1 and 2. Introduction of test oils to algal culture caused lag phase in some cases, the duration of which was strictly concentration-dependent. In other studies too, lags have been observed before the commencement of exponential phase in oil-treated cultures (O’Brien and Dixon 1976; Vandermeulen and Ahern 1976).

In general, the test oils administered into culture suspension as aqueous extract or whole oil depressed the growth of *A. doliolum*. The inhibition of specific growth rate and final yield occurred in a concentration-dependent manner. The present observations are in agreement with O’Brien and Dixon (1976) and Vandermeulen and Ahern (1976), but differ from Dogadina (1970), Coffey *et al* (1977) and Fabregas *et al* (1984) who found some oils to be non-toxic. Gaur and Kumar (1981) reported toxic effects of Assam crude on 4 unicellular algae, however, the extent of inhibition was more severe in *A. doliolum*. It seems that this alga is more sensitive to oils. Low concentrations of some oils can stimulate specific growth rate and final yield of algal species. This may be due to the presence of growth regulating compounds in oils (Gaur and Kumar 1981), or uptake and metabolization of certain oil constituents (Chan and Chiu 1985). However, the oils used did not elicit such an effect possibly due to the absence, or species specificity of stimulatory principles. As far as the mechanism of toxicity is concerned, it may be due to inhibition of photosynthesis (Pulich *et al* 1974; Chan and Chiu 1985) particularly PS II (Singh and Gaur 1988), nutrient uptake (Hellebust *et al* 1985, Hutchinson *et al* 1985), nitrogen fixation (Gaur and Singh 1989), and loss of permeability (Van Overbeek and Blondeau 1954) and cations (Hutchinson *et al* 1979). Actually, the action of oils on algal cells is not restricted to a particular metabolic process.

The test oils differed markedly from each other with regard to their effects on *A. doliolum*. Crude oil was least toxic followed in increasing order of toxicity by kerosene, petrol, furnace oil and diesel. In order to compare the toxicity of pollutants it has become a customary to transform the results to EC<sub>50</sub>, i.e., concentration of a substance giving 50% effect or inhibition (Painter 1981). We extrapolated EC<sub>50</sub> by fitting a line to the dose-response relationship by least squares using log<sub>10</sub> of the dose as independent variable (Voeke *et al* 1980). EC<sub>50</sub> values derived from specific growth rate (table 2) were lower than those from the final standing crop data (table 3). These observations suggest that growth rate is a more sensitive criterion than the final yield in detecting the toxicity of petroleum oils. Similar observations were made by Aoyama *et al* (1987) in *Chlorella ellipsoidea* exposed to various toxic chemicals. For test systems with predominantly
Petroleum toxicity to Anabaena

Figure 1. Growth rate of *A. doliolum* exposed to test oils (---) and their aqueous extracts (-----). Vertical lines represent standard error of means (n = 3) and the numbers given on the curve indicate the lag period (days) for that particular concentration.

Figure 2. Final yield (amount of chlorophyll *a* on 15th day) of test alga exposed to test oils (---) and their aqueous extracts (-----).

Exponential growth, EC_{50} values derived from growth rate are, due to lesser dependence on particular test system parameters, more sensitive than those from biomass data (Nyholm 1985).

The variability in action of test oils on *A. doliolum* seems to be due to differences in chemical composition. In fact, we could observe differences in concentrations of paraffinics, asphaltics and aromatics in test oils (table 1). If we relate the toxicity and chemical composition of test oils a definite trend emerges. Paraffinics do not seem to have played much role in determining toxicity of various test oils because
Table 2. Regression equations, correlation coefficients and EC_{50} for the growth rate of A. doliolum treated with test oils (WO) and their aqueous extracts (AE).

<table>
<thead>
<tr>
<th>Test oil</th>
<th>Method of supplementation</th>
<th>Regression equation</th>
<th>( r )</th>
<th>EC_{50} (mg l(^{-1})) (Mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude</td>
<td>WO</td>
<td>( Y = -0.375 \log_{10} x + 0.492 )</td>
<td>-0.980</td>
<td>5.73 ± 0.16</td>
</tr>
<tr>
<td></td>
<td>AE</td>
<td>( Y = -0.335 \log_{10} x + 0.535 )</td>
<td>-0.975</td>
<td>9.06 ± 0.38</td>
</tr>
<tr>
<td>Kerosene</td>
<td>WO</td>
<td>( Y = -0.308 \log_{10} x + 0.400 )</td>
<td>-0.991</td>
<td>4.08 ± 0.13</td>
</tr>
<tr>
<td></td>
<td>AE</td>
<td>( Y = -0.476 \log_{10} x + 0.586 )</td>
<td>-0.978</td>
<td>5.97 ± 0.19</td>
</tr>
<tr>
<td>Petrol</td>
<td>WO</td>
<td>( Y = -0.244 \log_{10} x + 0.328 )</td>
<td>-0.977</td>
<td>5.19 ± 0.21</td>
</tr>
<tr>
<td></td>
<td>AE</td>
<td>( Y = -0.299 \log_{10} x + 0.349 )</td>
<td>-0.960</td>
<td>2.88 ± 0.12</td>
</tr>
<tr>
<td>Diesel</td>
<td>WO</td>
<td>( Y = -0.315 \log_{10} x + 0.260 )</td>
<td>-0.927</td>
<td>4.22 ± 0.07</td>
</tr>
<tr>
<td></td>
<td>AE</td>
<td>( Y = -0.306 \log_{10} x + 0.249 )</td>
<td>-0.952</td>
<td>1.30 ± 0.05</td>
</tr>
<tr>
<td>Furnace</td>
<td>WO</td>
<td>( Y = -0.389 \log_{10} x + 0.326 )</td>
<td>-0.892</td>
<td>1.96 ± 0.08</td>
</tr>
<tr>
<td></td>
<td>AE</td>
<td>( Y = -0.350 \log_{10} x + 0.296 )</td>
<td>-0.890</td>
<td>1.76 ± 0.05</td>
</tr>
</tbody>
</table>

*Concentration of test oil in mg l\(^{-1}\). EC_{50} of crude and kerosene significantly (\( P < 0.05 \)) differed from their respective aqueous extracts; differences were not significant in other cases (Student's 't' test).

Table 3. Regression equations, correlation coefficients and EC_{50} for the final yield of test algae treated with oils (WO) and their aqueous extracts (AE).

<table>
<thead>
<tr>
<th>Test oil</th>
<th>Method of supplementation</th>
<th>Regression equation</th>
<th>( r )</th>
<th>EC_{50} (mg l(^{-1})) (Mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude</td>
<td>WO</td>
<td>( Y = -2.360 \log_{10} x + 3.348 )</td>
<td>-0.919</td>
<td>7.47 ± 0.36</td>
</tr>
<tr>
<td></td>
<td>AE</td>
<td>( Y = -2.418 \log_{10} x + 3.789 )</td>
<td>-0.901</td>
<td>10.45 ± 0.48</td>
</tr>
<tr>
<td>Kerosene</td>
<td>WO</td>
<td>( Y = -2.376 \log_{10} x + 3.203 )</td>
<td>-0.968</td>
<td>5.15 ± 0.24</td>
</tr>
<tr>
<td></td>
<td>AE</td>
<td>( Y = -4.451 \log_{10} x + 5.128 )</td>
<td>-0.996</td>
<td>7.39 ± 0.30</td>
</tr>
<tr>
<td>Petrol</td>
<td>WO</td>
<td>( Y = -2.450 \log_{10} x + 3.129 )</td>
<td>-0.968</td>
<td>5.64 ± 0.18</td>
</tr>
<tr>
<td></td>
<td>AE</td>
<td>( Y = -2.892 \log_{10} x + 3.235 )</td>
<td>-0.956</td>
<td>4.66 ± 0.12</td>
</tr>
<tr>
<td>Diesel</td>
<td>WO</td>
<td>( Y = -2.904 \log_{10} x + 2.185 )</td>
<td>-0.973</td>
<td>2.11 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>AE</td>
<td>( Y = -2.579 \log_{10} x + 1.918 )</td>
<td>-0.989</td>
<td>1.71 ± 0.09</td>
</tr>
<tr>
<td>Furnace</td>
<td>WO</td>
<td>( Y = -2.943 \log_{10} x + 2.413 )</td>
<td>-0.914</td>
<td>3.14 ± 0.13</td>
</tr>
<tr>
<td></td>
<td>AE</td>
<td>( Y = -2.666 \log_{10} x + 2.115 )</td>
<td>-0.949</td>
<td>2.21 ± 0.14</td>
</tr>
</tbody>
</table>

*Concentration of test oil in mg l\(^{-1}\). EC_{50} of test oils significantly (\( P < 0.05 \)) differed from their respective aqueous extracts (Student's 't' test).

crude which contained maximum concentration of this fraction was least toxic. Batterton et al. (1978) and Karydis (1979) found no toxic effects of paraffinics extracted from test oils by column chromatography. Asphaltics also do not seem to have been largely responsible for toxic effects of various oils. Aromatic was probably the main toxic fraction in test oils used by us. Assam crude was least toxic as it had minimum of aromatic fractions, whilst furnace oil was most toxic as it was enriched in this fraction. The present observations thus maintain the role of aromatics in determining toxicity of oils and lend additional support to Anderson et al. (1974), Batterton et al. (1978), Blumer (1969) and Kauss and Hutchinson (1975). Estimation of total aromatics can therefore allow the prediction of toxicity of an oil. But, accurate prediction may become possible only by analysing specific aromatic...
Petroleum toxicity to Anabaena

compounds as it has been shown that their toxicity increases with increasing methylation (Currier 1951).

The toxicity of whole oils differed from their respective aqueous extracts. Assam crude and kerosene were more toxic as compared to their aqueous extracts. In case of other oils aqueous extracts were more toxic than the whole oils. Almost similar observations have been made by Batterton et al (1978) who found that in case of Montana fuel oil aqueous extract was more toxic than whole oil. Winters et al (1976) observed that aqueous extract of Baytown fuel oil was non-toxic to microalgae. But when presented as whole oil to the same algae pronounced toxic effects were evident (Batterton et al 1978). They suggested that the toxicity of water soluble fractions from a given oil is not necessarily due to the same compounds which cause toxicity in a whole oil. The differential action of whole oils and their respective extracts are difficult to explain since we have not performed their GC-MS analysis. In order to further elucidate the toxic action of these oils it would be necessary to analyse them for specific compounds. In any case, oil toxicity tests should take into account the effects of whole oils as well as their water-soluble fractions.

The use of high concentrations of oils may prompt one to question the ecological relevance of this study. In oil spill areas, especially those with little or no mixing, and in habitats chronically exposed to high concentrations of oils, e.g., refinery effluents (Gaur and Kumar 1985) such concentrations do exist. It remains to be seen whether the release of test oils may lead to serious ecological repercussions, nevertheless, harmful effects on cyanobacteria and algae may be envisaged.

Acknowledgements

This work was supported by a research grant to one of the authors (JPG) from the Council of Scientific and Industrial Research, New Delhi. Thanks are due to Dr H N Pandey for valuable advice.

References

Aoyama I, Okamura H and Yagi H 1987 The interaction effects of toxic chemical combinations on Chlorella ellipsosoides; Toxicity Assess. Int. Q. 2 341–355
Chan K and Chiu S Y 1985 The effects of diesel oil and oil dispersants on growth, photosynthesis, and respiration of Chlorella salina; Arch. Environ. Contam. Toxicol. 14 325–331
Currier H B 1951 Herbicidal properties of benzene and certain methyl derivatives; Hilgardia 20 383–406

Fabregas J, Herrero C and Veiga M 1984 Effect of oil and dispersant on growth and chlorophyll a content of the marine microalga Tetraselmis suecica; Appl. Environ. Microbiol. 47 545–547


Gaur J P and Kumar H D 1985 The influence of oil refinery effluents on the structure of algal communities; Arch. Hydrobiol. 103 305–323


Hellebust J A, Soto C and Hutchinson T C 1985 Effect of naphthalene and aqueous crude oil extract on the green flagellate Chlamydomonas angulosa. VII. Nitrate and methylamine uptake and retention; Can. J. Bot. 63 834–840


Hutchinson T C, Hellebust J A, Mackay D, Deborah T and Kauß P 1979 Relationship of hydrocarbon solubility to toxicity in algae and cellular membrane effects; in Oil spill conference (American Petroleum Institute and US Environmental Protection Agency) pp 541–546


Karydis M 1979 Short-term effects of hydrocarbon on the photosynthesis and respiration of some phytoplankton species; Bot. Mar. 22 281–285

Kauß P B and Hutchinson T E 1975 The effects of water-soluble petroleum components on the growth of Chlorella vulgaris Beijerinck; Environ. Pollut. 9 159–174

Nyholm N 1985 Response variable in algal growth inhibition tests—biomass or growth rate?; Water Res. 19 273–279


Painter H A 1981 Assessment of toxicity of chemicals to bacteria—methods and interpretation; Les Colloques de l’INSERM Tests de Toxicité Aiguë en Milieu Aquatique (INSERM) 106 131–141


Van Overbeek J and Blondeau R 1954 The mode of action of phytotoxic oils; Weeds 3 55–65

Vocke R W, Sears K L, O'Toole J J and Wildman R B 1980 Growth response of selected freshwater algae to trace elements and scrubber ash slurry generated by coal-fired power plants; Water Res. 14 141–150