Appl Biochem Biotechnol (2010) 160:719–729
DOI 10.1007/s12010-009-8569-0

# Response of an Algal Consortium to Diesel under Varying Culture Conditions

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Received: 16 October 2008 / Accepted: 10 February 2009 /
Published online: 28 February 2009


**Abstract** A diesel-tolerant sessile freshwater algal consortium obtained from the vicinity of Powai Lake (Mumbai, India) was cultured in the laboratory. The presence of diesel in batch cultures enhanced the maximum specific growth rate of the algal consortium. With decrease in light–dark (L:D) cycle from 20:4 to 4:20 h, the chlorophyll-a levels decreased; however, the removal of diesel was found to be maximum at L:D of 18:6 h with 37.6% degradation over and above controls. In addition to growth in the form of green clumps, white floating biomass was found surrounding the diesel droplets on the surface. This culture predominated at the least L:D ratio of 4:20 h. Studies confirmed the ability of the floating organisms to grow heterotrophically in the dark utilizing diesel as carbon source and also in the presence of light in a medium devoid of organic carbon sources.

**Keywords** Biodegradation · Cyanobacteria · Diesel · Heterotrophic growth · Hydrocarbons

## Introduction

Oil-contaminated regions of the Arabian Gulf are often dominated by Cyanobacteria, such as *Phormidium*, *Microcoleus*, and *Oscillatoria*, that are found to be closely associated with heterotrophic oil-degrading bacterial cultures [1, 2]. Although it is generally believed that the cyanobacterial mats facilitate hydrocarbon degradation, there are differences in opinion regarding the exact role of the cyanobacterial cultures. Some researchers have reported an indirect role of cyanobacterial cultures in hydrocarbon degradation [1–6], whereas others have reported a direct role of these cultures in hydrocarbon degradation [7–9].

This indirect role of Cyanobacteria in facilitating hydrocarbon biodegradation has been established conclusively. Cyanobacterial cultures were found to enhance oil degradation by providing the hydrocarbon-degrading bacteria with molecular oxygen, fixed nitrogen, and vitamins [2, 3]. In the absence of crude oil, the products of photosynthesis produced by the

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phototrophs possibly served as carbon and energy sources for the oil-degrading bacteria. The cultures were found to facilitate immobilization of the hydrocarbon degraders in the biofilm and in mechanical fixation of the biofilm to gravel/glass surfaces [3]. Cyanobacterial polysaccharides play a major role in the emulsification of oil, i.e., breaking of oil into small droplets, which subsequently facilitates degradation by heterotrophs [6]. In addition to these indirect roles, some phototrophic microorganisms are also reported to directly utilize petroleum hydrocarbons heterotrophically. *Microcoleus chthonoplastes* and *Phormidium corium*, isolated from oil-contaminated sediments of Arabian Gulf, were found to contribute directly to the uptake and oxidation of *n*-alkanes [7]. Raghukumar et al. [9] reported that the marine Cyanobacteria, *Oscillatoria salina*, *Plectonema terebrans*, and *Aphanocapsa* species, could degrade Bombay High crude oil. Abed and Koster [8] reported that axenic cultures of *Aphanothece halophytica*, *Dactylococcopsis salina*, *Halothece* strain, *Oscillatoria* strain OSC, and *Synechocystis* strain showed a clear decrease in the concentration of alkanes. Al-Hasan et al. [7] depicted further evidence for the direct participation of Cyanobacteria in hydrocarbon oxidation by analysis of lipids and fatty acids from biomass samples incubated with various *n*-alkanes. However, since most of these studies were performed with non-axenic cyanobacterial cultures, there is some ambiguity regarding the direct role of cyanobacterial cultures in hydrocarbon degradation. In the presence of heterotrophic bacteria in non-axenic cultures, Cyanobacteria may potentially grow on $CO_2$ produced by the oil-degrading heterotrophs. Sanchez et al. [10] attempted to exclude this possibility by maintaining a low oxygen level at about 5% of air saturation.

This study was conducted using an environmental sample of sessile algal consortium, obtained from rock surfaces from the vicinity of a freshwater lake (Powai Lake, Mumbai, India). Preliminary studies indicated that the culture was not adversely affected by the presence of diesel oil. This culture could be successfully combined with *Burkholderia cepacia*, an aliphatic hydrocarbon-degrading bacterium for treating petroleum hydrocarbon-containing wastewater in a continuous-flow attached growth bioreactor [11]. The objectives of this study was to determine the growth of the algal consortium in the presence of diesel and to determine diesel degradation by the consortium in response to variation in the light–dark (L:D) cycle, so as to establish its role in diesel degradation. Attempt was made to reduce the presence of heterotrophic bacteria in these studies. Growth of the algal consortium was studied in the presence of varying concentrations of diesel (0.2–0.8% *v*/*v*) under static conditions. The diesel degradation rate studies at varying L:D cycles were conducted at a fixed initial diesel concentration of 0.6% (*v*/*v*).

## Materials and Methods

The freshwater algal consortium was cultured in the laboratory using a mineral medium having the following composition (mg/L) [12]: disodium ethylenediaminetetraacetic acid (0.5), citric acid (3), $Na_2CO_3 \cdot H_2O$ (20), $CaCl_2 \cdot 2H_2O$ (7), $MgSO_4 \cdot 7H_2O$ (370), KCl (500), ferric ammonium citrate (3), $K_2HPO_4 \cdot 3H_2O$ (21.75), $KH_2PO_4$ (8.5), $Na_2HPO_4$ (33.4), $NaNO_3$ (750), and trace elements as described by Mukherji et al. [13]. Subsequently, it was acclimatized to 0.5% diesel over a period of 1 month and the change in diversity of the culture was observed using a microscope (Axio Star Plus, Carl Zeiss, Germany) at an overall magnification of ×400. The sessile freshwater algal consortium tended to produce clumps in batch cultures; hence, a method was developed for breaking the clumps before using the culture for inoculation [13]. The algal growth studies were set up in multiple 15-mL test tubes containing 5-mL nutrient medium that were sacrificed over time for

estimating the growth profile. A uniformly suspended algal consortium (250 μL) grown up to the end of log phase was inoculated and the tubes were incubated at 28±2 °C under illumination of 1,100–1,200 lx (Lutron LX-101, Mumbai, India). The illumination was provided with an incandescent bulb as the light source using an L:D cycle of 18:6 h. Rotary shaking was not employed for growth of the algal consortium. At every sampling time, a tube was sacrificed and chlorophyll-a was estimated using the methanol extraction technique [14, 15]. The control tubes were incubated without diesel, whereas other experimental tubes were incubated with varying concentrations of diesel from 0% to 0.8% (*v*/*v*). Phycobiliprotein estimation was performed using the method reported by Becker [15].

To investigate the role of freshwater algal consortium in diesel degradation, rate studies were carried out in multiple batch flasks (with 0.6% *v*/*v* filter-sterilized diesel in 100-mL nutrient medium). Although it is difficult to obtain axenic strains from environmental samples, for the purpose of this study, we attempted to minimize the presence of heterotrophic bacterial cultures in the inoculum. Hence, to reduce the presence of bacterial cultures, several serial transfers were made and the consortium was maintained in the absence of any organic carbon source for a period of about 2 years. The inoculum was prepared by vortexing the algal consortium to form a uniform suspension which was filtered through a membrane filter with pore size of 0.45 μm to exclude some of the bacterial cells of lower size. However, this step is unlikely to exclude bacterial cells of larger size and cells that adhered strongly on the algal consortium. The flasks were incubated under static conditions (no rotary shaking) at 28±2 °C and L:D cycle of 4:20, 8:16, 12:12, 18:6, and 20:4 h with light intensity of 1,100–1,200 lx provided using an incandescent bulb. Samples were analyzed for total petroleum hydrocarbons (TPH) at various intervals over a period of 25 days. At low L:D cycle, white clumps were found to surround the diesel droplets floating on the surface. A loop full of these white clumps was streaked on a nutrient agar plate prepared using 2% agar in nutrient broth (HiMedia, Mumbai, India). The plates were incubated under dark conditions for 48 h at 37 °C. Distinct green colonies formed on the nutrient agar plates were examined using a microscope (at an overall magnification of ×200) after four transfers. The colonies were also inoculated into tubes containing mineral medium and diesel which were then incubated in the dark for 5–7 days.

TPH concentration was analyzed by acidifying the samples and adding hexane in the ratio of 1:5 (*v*/*v*). The suspension was then centrifuged at 10,000 rpm for 15 min and the phases were separated in a separating funnel. The aqueous phase was subsequently extracted twice with hexane (1:10). Simultaneously, extraction of diesel associated with the cell surface was carried out by vortexing the cell pellet with hexane and adding the supernatant to the remaining hexane extract. This extract was first analyzed using gravimetric analysis. Subsequently, residual diesel was re-dissolved in a small amount of hexane to which 100 μL of internal standard (5α-androstane, 2 mg/mL) was added and the total volume was made up to 5 mL. The extracted samples were analyzed using a gas chromatograph (GC; Agilent Technologies, 6,890 N) equipped with flame ionization detector and HP5 capillary column. Nitrogen was used as the carrier gas and 2 μL of sample were injected in the splitless mode. The oven temperature program was as follows: initial temperature 50 °C, with hold time of 1 min, ramping at 10 °C/min up to 150 °C, with hold time of 1 min, ramping at 5 °C/min up to 175 °C, with hold time of 1 min, ramping at 40 °C/min up to 200 °C, with hold time of 25 min, with total run time of 43.63 min. The injector temperature was set at 200 °C while the detector temperature was set at 280 °C. In all the standards and samples, 0.04 mg/mL of 5α-androstane was added.

To determine the amount of algal biomass, the cultures and diesel were added to multiple test tubes in similar proportion as in the flasks and chlorophyll-a as well as volatile

suspended solids (VSS) were estimated as per Standard Methods [16]. The maximum abiotic loss due to sorption on algal biomass was determined as follows. The algal consortium grown for 25 days in absence of diesel was harvested, filtered, dried, weighed, and added to a flask containing 0.6% (*v*/*v*) diesel in 100-mL deionized water. The flask was kept in a rotary shaker for 24 h and residual diesel was extracted from the aqueous phase as described above. Additionally, the algal biomass was collected by centrifugation, dried overnight at 100 °C, and subjected to soxhlet extraction using hexane at 50 °C over a period of 6 h to estimate the TPH sorbed on to the biomass.

## Results and Discussion

Microscopic images of the freshwater algal consortium before and after acclimatization to diesel oil over a 1-month period are illustrated in Fig. 1a, b. The images were obtained at an overall magnification of ×400. Comparison of Fig. 1a, b clearly depicts a decrease in the diversity of the algal cultures in the consortium after acclimatization. Initially, the consortium was predominantly composed of three cyanobacterial cultures, out of which

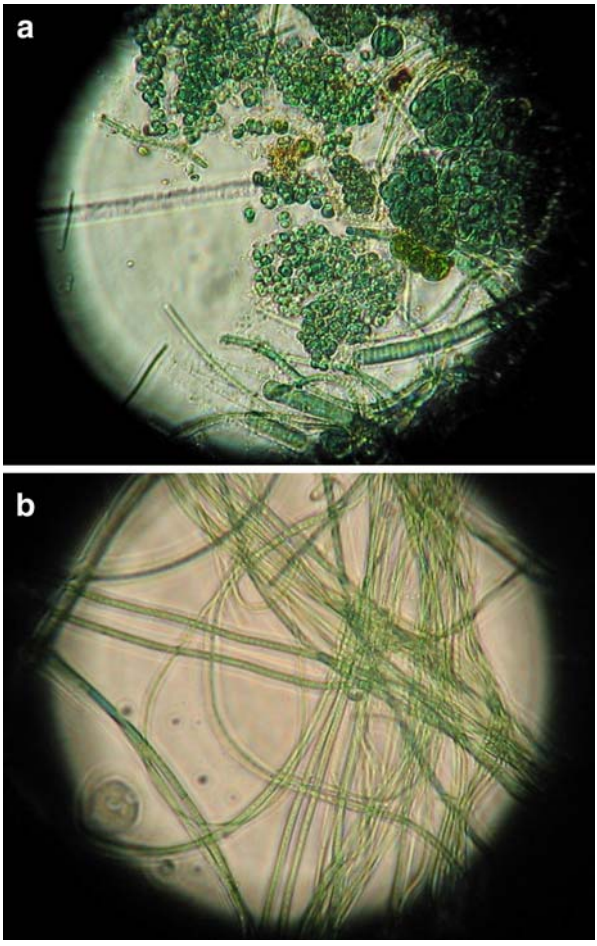


**Fig. 1** Microscopic image of the freshwater algal consortium (**a**) before and (**b**) after acclimatization to diesel oil

two were filamentous (*Phormidium* and *Oscillatoria*), whereas the third was identified as *Chroococcus. Chroococcus* appeared as unicellular spherical non-vesicular cells with colorless individual sheath, without heterocyst, and they often formed united colonies with a definite or indefinite shape. *Phormidium* was characterized by its distinct filaments, trichomes with false branching, without heterocysts, absence of spores and filaments forming a thallus with more or less confluent sheath. *Oscillatoria* was characterized by its distinct filaments, trichomes with false branching, without heterocysts, absence of spores, clearly visible cells in the trichome, more or less straight trichomes not spirally coiled, and absence of sheath [17]. After acclimatization, *Phormidium* became more predominant while *Oscillatoria* and *Chroococcus* became less dominant.

Estimation of phycobiliprotein pigments (water-soluble pigments) in the algal consortium revealed concentration of phycoerythrocyanin (PE), allophycocyanin (APC), and C-phycocyanin (PC) to be 12.75, 31.65, and 9.1 mg/mL. The corresponding values for a pure strain of *Oscillatoria BDU* 100731 (obtained from the National Facility for Marine Cyanobacteria, Tiruchirappalli, India) were 20, 46, and 14 mg/L for PE, APC, and PC, respectively. The phycobiliprotein pigments are present in Cyanobacteria/blue green algal cultures but are absent in green algae. Thus, presence of cyanobacterial cultures in the freshwater algal consortium is confirmed.

Growth studies were carried out in the presence of varying concentrations of diesel over the range 0.2% to 0.8% (*v*/*v*). A 5-day lag was observed during incubation in the presence of diesel under the L:D cycle of 18:6 h. Subsequently, there was a rapid rise in chlorophyll-a concentration. At diesel concentration of 0.2%, 0.4%, 0.6%, and 0.8% (*v*/*v*), the maximum chlorophyll-a concentrations were 22.3, 22.8, 25.6, and 22.8 mg/L, respectively, at the end of 15–17 days in the cultures with varying diesel concentrations. Growth in the presence of diesel was better than in its absence with maximum chlorophyll-a concentration of 19.6 mg/L at the end of log phase (Fig. 2). Figure 3 illustrates that the specific growth rate of this algal consortium in the presence of diesel was higher (0.31–0.46 $day^{-1}$) than in its absence (0.15 $day^{-1}$). However, the specific growth rate did not depict an increasing trend with increase in diesel concentration. The growth studies illustrate possible heterotrophic utilization of diesel by the algal consortium; however, beyond 0.2% diesel,

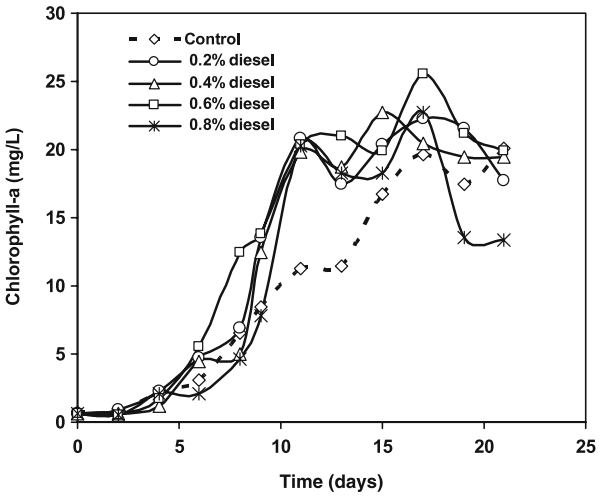


**Fig. 2** Growth of freshwater algal consortium in the absence and presence of diesel oil

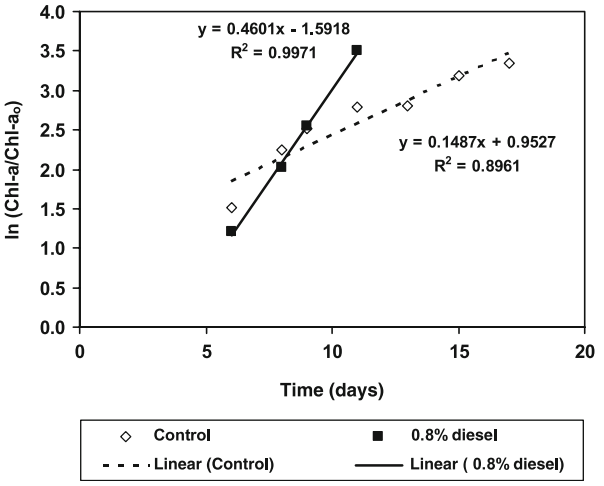


**Fig. 3** Specific growth rate of freshwater algal consortium in the presence and absence of diesel oil

it was possibly not a growth-limiting substrate since the specific growth rate was unaffected by diesel concentration. Other nutrients may be limiting growth under these conditions.

Utilization of diesel by the freshwater algal consortium was studied by growing the biomass under stationary conditions in the presence of 0.6% (*v*/*v*) diesel and estimating the residual diesel over the incubation period of 25 days. While rotary shaking is required for bacterial growth, good growth of algae can occur in stationary cultures. In this study, the impact of varying L:D ratios of 4:20, 6:18, 12:12, 18:6, and 20:4 h on growth of the algal consortium and diesel degradation was studied.

Decrease in residual mass of diesel with time in the control and experimental flasks is shown in Fig. 4. This figure depicts residual mass of diesel based on GC analysis. At the end of 25 days, biodegradation over and above controls ($D_{max}$) was 18–25% at the various L:D cycles other than 18:6 h, where $D_{max}$ was 37.6%. Part of this loss may, however, be due to sorption of oil onto the algal biomass. A control study was conducted at L:D cycle of 18:6 h to determine the loss due to sorption on biomass. Biomass incubated for 25 days in the absence of diesel was mixed with 0.6% (*v*/*v*) diesel over a period of 24 h. After extracting the residual diesel from the aqueous phase as in the other experiments, the diesel oil sorbed on algal biomass was directly estimated after soxhlet extraction. This experiment

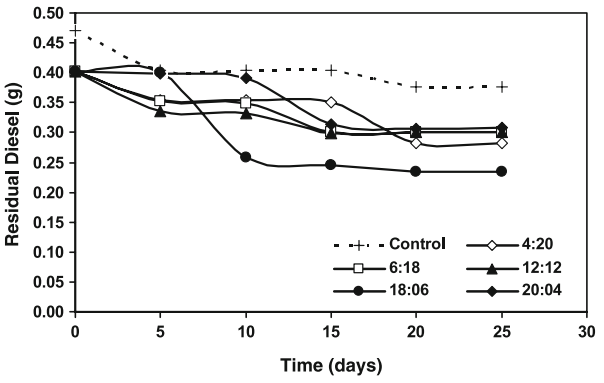


**Fig. 4** Degradation of diesel oil by freshwater algal consortium at various L:D cycles

revealed that at L:D cycle of 18:6, only 2.17% loss might be attributed to sorption on biomass. At the other L:D cycles, independent estimate of sorption and biodegradation were not available. Of the total loss of diesel observed at 25 days, only a part represents biodegradation loss due to utilization of diesel by the algal cultures. The other components of loss, i.e., extraction loss and additional abiotic loss, can be estimated based on diesel mass balance using the controls devoid of cultures. Extraction of a set of control flasks immediately after setup (zero-day controls) yields the extraction loss. The loss observed in a set of control flasks extracted after 25 days represents the sum of extraction and additional abiotic losses; thus, the latter can be estimated by difference. The extraction loss and additional abiotic loss over 25 days were 21.7% and 15.6%, respectively. The biodegradation loss at various L:D cycles are listed in Table 1.

After inoculation, the initial chlorophyll-a concentration was 0.942 mg/L. The growth of the algal consortium expressed in terms of chlorophyll-a was found to vary with variation in the L:D cycle during incubation as shown in Fig. 5. The maximum chlorophyll-a concentration decreased with decrease in the light–dark cycle. For L:D cycles of 4:20 and 6:18 h, chlorophyll-a at the end of log growth phase was significantly lower, in the range of 6–7 mg/L, while, for L:D cycles of 12:12, 18:6, and 20:4 h, chlorophyll-a at the end of log growth phase was in the range of 14–17 mg/L. The algal consortium thus grows both phototrophically and heterotrophically by utilizing diesel. Such mixotrophic growth of algal cultures has been reported in both oligotrophic and eutrophic habitats [18].

In addition to Cyanobacteria growing as green clumps, there were white clumps at the oil–water interface in the flasks incubated at L:D cycles less than 12:12 h. When a loop full of these white clumps was streaked on a nutrient agar plate and incubated under dark conditions at 37 °C, distinct green colonies were observed on the nutrient agar plates after 48 h. The filamentous cyanobacterial cultures were incapable of forming such colonies on nutrient agar plates. After four transfers, a green colony was picked and examined under the microscope at an overall magnification of ×200. Green pigments were observed within the unicellular cells (Fig. 6). The culture was found to have a short body cell with an irregular shape and spore-forming characteristics. The culture was not characterized by morphological characteristics of Cyanobacteria but resembled the characteristics of green algae. An attempt to extract out the chlorophyll-a from the cells by the methanol extraction technique failed. Some freshwater algae are reported to possess refractory chlorophylls that cannot be

**Table 1** Filamentous and floating cultures in the system after incubation for 25 days and diesel biodegradation by the consortium.

| L:D | Total algal biomass VSS[a] (mg/L) | Filamentous culture biomass $VSS_{chl\text{-}a}$[b] (mg/L) | Floating culture biomass ($VSS\text{-}VSS_{chl\_a}$)[c] (mg/L) | % Biodegradation |
|---|---|---|---|---|
| 4:20 | 1,620 | 1,400 | 219 | 15.69 |
| 6:18 | 1,480 | 1,780 | 319 | 12.60 |
| 12:12 | 2,820 | 2,793 | 26 | 12.57 |
| 18:6 | 2,860 | 2,833 | 26 | 23.54 |
| 20:4 | 2,865 | 2,853 | 11 | 11.48 |

[a] VSS refers to volatile suspended solids used as a measure of total algal biomass

[b] $VSS_{chl\_a}$ provides an estimate of VSS due to the filamentous algal cultures that can be measured in terms of chlorophyll-a. It is determined using the measured chlorophyll-a values using Eq. 1

[c] The VSS due to floating cultures which does not yield measurable chlorophyll-a is determined by difference

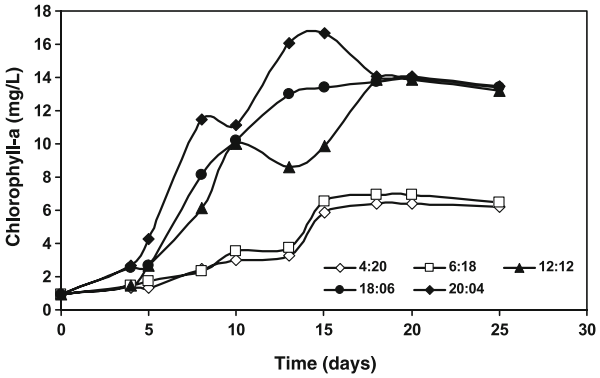


**Fig. 5** Growth of freshwater algal consortium in the presence of 0.6% diesel at various L:D cycles

extracted by the standard methanol extraction technique [19]. The white floating culture could grow heterotrophically in the dark utilizing diesel as a sole carbon source and also in the presence of light in a medium devoid of any organic carbon source. Growth of this culture on diesel (0.6%) under dark conditions with rotary shaking was associated with increase in aqueous-phase turbidity and a bluish green coloration was observed in the aqueous phase. The relative mass of the floating algal culture and filamentous culture at 25 days is indicated in Table 1 in terms of VSS. VSS, the fraction of the suspended solids that volatilizes as 550 °C in a muffle furnace due to its organic nature, is commonly used as a measure of total microbial mass [5]. In these studies with the algal consortia, VSS refers to the total algal biomass. Both the culture types, i.e., floating cultures with recalcitrant chlorophyll that cannot be measured by the standard technique, and the green filamentous cultures can be determined based on VSS. Moreover, a VSS versus chlorophyll-a correlation was independently developed (Eq. 1) under conditions that primarily allows

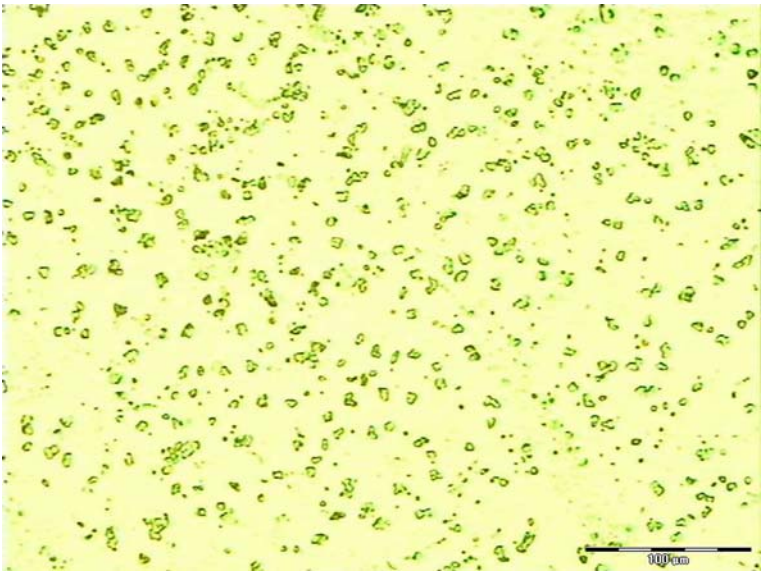

**Fig. 6** Microscopic image of unicellular algae comprising the floating cultures

growth of the filamentous cultures, i.e., at L:D of 18:6 h, so as to obtain estimates of VSS due to the filamentous cultures ($VSS_{chl\text{-}a}$). Based on visual observations, growth of the floating algal culture was negligible at L:D cycle of 18:6. Thus, difference between the measured VSS at any L:D cycle and the $VSS_{chl\text{-}a}$ estimated from the correlation using measured values of chlorophyll-a yields the VSS due to the floating cultures.

$$VSS_{chl-a}(mg/L) = 198.99 \times (\text{chlorophyll} - a, mg/L) + 166.86 \quad (1)$$

Diesel biodegradation in this system was affected by the type of the predominant algal culture, i.e., white floating clumps and filamentous algal culture. Biodegradation was also affected by acclimatization of these cultures to different incubation conditions (L:D cycle). The white floating clumps were predominant at the low L:D cycles of 4:20 h and 6:18 h, whereas the filamentous algal culture was predominant at the L:D cycle of 12:12 h and higher. At L:D cycles of 12:12 h and lower, an initial decrease in residual diesel between 0 and 5 days was observed possibly due to the activity of the white floating cultures with confirmed capability for degrading diesel. These floating cultures were characterized by low levels of strongly bound chlorophyll-a that could not be extracted with methanol. Figure 5 depicting algal growth in terms of chlorophyll-a concentration indicates that, at L:D cycles of 4:20 h and 6:18 h, growth of the filamentous algal culture was minimal, while growth of the floating cultures was not depicted in terms of chlorophyll-a and was not revealed in this figure. This explains the apparent inconsistency between diesel degradation (Fig. 4) and chlorophyll-a concentration (Fig. 5). Although chlorophyll-a in the system was significantly reduced at low L:D cycles, almost comparable biodegradation of diesel was observed as for high L:D cycles, except 18:6 h. At the low L:D cycles, after the initial phase of decline in residual diesel between 0 and 5 days, the residual diesel remained constant until a later period (beyond 10 days) when residual diesel decreased again due to the activity of the filamentous cultures. Thus, a biphasic degradation pattern was observed. In contrast, at L:D cycles of 18:6 and 20:4 h, no decline in residual diesel was observed at early time (<5 days) and bulk of the degradation occurred continuously after 5 and 10 days, respectively. Under these incubation conditions, the unicellular floating cultures played a relatively less significant role whereas the filamentous algal cultures dominated, as indicated by the significant increase in chlorophyll-a concentration. The L:D cycle of 18:6 h resulted in maximum decrease in diesel concentration of 37.6% (over and above the controls) over a period of 25 days. It may be noted that the culture was incubated under this condition for nearly 2 years. The growth of filamentous culture predominant at high L:D cycles was primarily due to the utilization of inorganic carbon, whereas the growth of floating culture predominant at low L:D cycles was largely due to the heterotrophic utilization of diesel oil. Thus, this study illustrates that the degradation of diesel by this algal culture is a function of acclimatization of the cultures to the incubation conditions. Moreover, the results presented in Figs. 2 and 3 confirm that this filamentous algal culture is capable of utilizing diesel heterotrophically. However, these filamentous cultures could not survive and grow solely on diesel in the absence of light.

Although such a distinct shift in predominance of specific algal cultures due to variation in L:D cycles in presence of hydrocarbons has not been reported, utilization of hydrocarbons/oil by algal cultures has been reported in the literature. Raghukumar et al. [9] reported that the marine Cyanobacteria, *O. salina*, *P. terebrans*, and *Aphanocapsa* species, could degrade Bombay High crude oil when grown in artificial-nutrient-amended seawater as well as in natural seawater at the L:D cycle of 12:12 h. Around 45–55% of the total crude oil was removed in the presence of mixed cultures within 10 days. Al-Hasan et

al. [7] depicted direct evidence for participation of Cyanobacteria in hydrocarbon oxidation by analysis of lipids and fatty acids from biomass samples incubated with various *n*-alkanes. Comparison of fatty acid profiles of the total lipids extracted from hydrocarbon-grown Cyanobacteria, *M. chthonoplastes* and *P. corium*, to those of the same organisms grown without hydrocarbons confirmed the uptake and oxidation of hydrocarbons to fatty acids by both species.

The present study demonstrates that the specific growth rate of the algal culture is enhanced in the presence of diesel oil. With decrease in L:D cycle from 20:4 to 4:20 h in the presence of diesel, the chlorophyll-a levels decreased; however, the removal of diesel was found to be maximum at the L:D cycle of 18:6 h with 37.6% loss over and above controls. In addition to Cyanobacteria growing as green clumps, a white floating culture was found to surround the diesel droplets on the surface. This culture was found to be predominant at the least L: D ratio of 4:20 h. Independent studies confirmed the ability of the floating culture to grow heterotrophically in the dark utilizing diesel as carbon source and also in the presence of light in a medium devoid of organic carbon source. Although the chlorophyll-a content in this culture could not be quantified, microscopic observations revealed presence of green pigments within the unicellular cells. The findings have important implications on intrinsic bioremediation of oil-contaminated sites and biological treatment of oily waste in reactors.

**Acknowledgements** This research work was partially funded by the Department of Biotechnology, New Delhi, India. The authors would like to thank Mr. Badrish Soni, BRD School of Biosciences, for discussions on characterization of the algal cultures and Mr. Praveen Kumar Mishra for his assistance in maintainance of the algal consortium. We acknowledge the Sophisticated Analytical Instrumental Facility (SAIF), IIT Bombay for facilitating microscopic examination of the cultures.

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