Cultivation and automated image analysis of hydrocarbon-tolerant marine cyanobacteria populations

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Abstract: - In this paper we described the cultivation of phototrophic marine microorganisms from two different sources, with special aim to enrich the natural populations with respect to marine cyanobacteria that are able to tolerate (2-5 years) gasoline or diesel up to 5% (w/v). These enriched populations where studied with respect to morphology and physical relationship with hydrocarbon surfaces and with quantum dots using bright field and epifluorescence microscopy, coupled to automated digital image analysis.

Key-Words: - marine ecosystem, cyanobacteria, cultivation, automated image analysis, hydrocarbons,

1 Introduction
The microbial response to crude-oil spill in marine ecosystem is dependent on biotic and abiotic factors (e.g., temperature, currents and nutrient concentrations) [30], [21]. Recent developments in Microbiology are focused on approaches that often combine traditional and modern methods to understand the role of interactions between phototrophic and heterotrophic microorganisms in bioremediation processes. In this respect, the ability of cyanobacteria to proliferate in association with heterotrophic bacteria in marine environments polluted with oil hydrocarbons received in last years a special attention [34], [29], [26], [41], [4], [10], [2], [16], [8], [3], [1]. In oil-polluted mats there are cyanobacteria, algae, protists, heterotrophic N₂-fixing bacteria, with individual functions important in bioremediation process [22], [25], [36]. It has been established that the contact between the cells and water insoluble compounds is enhanced by the hydrophobic nature of the cell envelopes, allowing microorganisms to establish a physical contact with hydrocarbon fraction [35]. The biology of cyanobacteria is very complex. All representatives of cyanobacteria contain chlorophyll a and are able to grow as photoautotrophs, although phototrophy and chemotrophy are common to many species. The ability of cyanobacteria to adapt to environmental stress, including rare or abundant nutrients [30], exposure to UV radiation, high solar radiation [11], pollutants such as oil hydrocarbons [34], [29], [26], [41], [4], [10], [2], [16], [8], [3], [1] may favour the dominance of cyanobacteria in many aquatic ecosystems [18]. In the process of microbial degradation of oil hydrocarbons algae, fungi and bacteria can establish complex metabolic interactions, including cometabolism [23], [14]. In only one paper the growth, evolution of oxygen concentration, respiration in the dark and composition of pigments in cyanobacteria and microalgae in the presence of oil hydrocarbon are presented together [35]. The presence of nitrogen fixation in microbial communities of oil-contaminated marine sediment microcosms [27] argues that nitrogen fixation can occur at the same site with oil hydrocarbons degradation. The ability of some cyanobacteria to fix nitrogen could be another contribution of these phototrophic prokaryotes to the degradation of oil hydrocarbons; however, no experimental evidence to support this assumption is available so far. Automated image analysis is increasable used in Microbiology to quantify important parameters for research and application the most studied so far being on the follows: enumerate total cell numbers and actively respiring bacteria, quantification of cell volumes and frequencies of dividing cells, in situ classification of bacteria, characterization of bacterial growth on solid medium, viability and physiological activity in biofilms [32], [9], [31], [40], [38], [28], [7], [24], [15]. Automated image analysis, that is, automated methods for obtaining quantitative data out of
images, aims at helping in the aforementioned problems. By employing mathematical algorithms for the analysis, the results are perfectly repeatable, errors are systematic, and the analyzer is tireless. Furthermore, certain measurement types such as precise colour or timing can only be obtained automatically. In microscopy, although the first automated cell based analysis systems with motorized microscopy equipment date back to the 70’s [33], and even that free image analysis software is commonplace, many image based quantification studies are still performed manually, by eye. In fact, automated image analysis has been rather recently described as "one of the greatest remaining challenges in screening" [13]. Successful automated image analysis starts by describing the analysis task precisely; image analysis software only does what it is programmed to do. It does not make any predictions, nor does it have any knowledge of the context of the study. Therefore, all types of samples and errors have to be taken care of beforehand in order to get reliable results: in automated image analysis unpredictable events often lead to unpredictable results. If properties of the sample change during the analysis, for example by unintentionally changing the microscope lighting settings, the results of automated analysis will most likely fail, although some automated error detection logic might detect the anomaly and discard the results. These limitations must be thoroughly understood before utilizing automation, requiring biologists to have basic understanding of image analysis methodology. *Vice versa*, computer scientists implementing the image analysis methods need experience in cell biology in order to develop software packages really useful for (micro) biologists, not just relying on novel technological advances without practical use. Shortly, successful automation requires engineers and biologists to work closely together, starting from interdisciplinary study programmes. The aims of this paper is to obtain marine populations enriched in cyanobacteria that are able to (at least) tolerate oil hydrocarbons (gasoline and diesel ) and to use bright field and epifluorescence microscopy, connected to automated digital image analysis, to study their morphology and physical relationship with hydrophobic structures (hydrocarbon surfaces and quantum dots).

2 Materials and Methods
The water samples were collected from the Black Sea, Tomis Harbour at 0.5m depth; 44°10.42 N; 28°39.36 E) and inspected by epifluorescence microscopy [17], [12] and processed as previously shown [19].

2.1 Cultivation of some hydrocarbon tolerant populations of cyanobacteria from microcosms contaminated with gasoline
The advantages of laboratory microcosms as experimental model concerns the control experimental parameters such as the temperature, absence or presence bacteriovorus microorganisms, pollutant concentration and / or nutrients etc. This control allows an easier interpretation of the results obtained in microcosm compared with those in the natural environment, and offers a basis to better understand the interplays between different factors in natural environments. On the other hand, there are some disadvantages: as compared with the natural environment, the microcosm is a simplified system and the results thus obtained can not be extrapolated *per se*. Furthermore the microcosms do not remain the same throughout the experiment and the time evolution of microbiota is also different from that which occurs in the natural environment. Taking into account its advantages, we took the decision to use microcosms as model system to study the interaction between microbiota and sea water polluted with different types of hydrocarbons [19-21]. During previous experiments [19] in the microcosms with the following compositions: i) sea water supplemented gasoline-0.25% v/w and ii) sea water supplemented gasoline-0.25% v/w and nutrients (ammonium acetate 0.005% w/w), photosynthetic populations become macroscopically visible after one year . These microcosms were maintained by adding gasoline twice a year, for 4 years and the collected photosynthetic populations were cultivated also in separate flasks either in the presence or absence of hydrocarbons (gasoline ) in sea water in order to enrich them in cyanobacteria able to tolerate (oxidize?) gasoline or diesel.

2.2 Cultivation of hydrocarbon-tolerant populations of cyanobacteria from a piece of solid hydrocarbons, collected at the sea shore of the Black Sea. The solid piece of hydrocarbon having on its surface small spots dark green was washed with sterile sea water and used as inoculum in further studies. The solid piece has been divided in two other pieces that were kept for two years in laboratory, in dim light, in sea water. Special
attentions have been done to avoid the occurrence of excessive light, evaporation or grazing.

2.3 The interplay between hydrocarbon-tolerant cyanobacteria and (fluorescent) quantum dots
Following our previous work on QD [6], [5] here we study the interplay between hydrocarbon-tolerant cyanobacteria and (fluorescent) quantum dots, the digital epifluorescence images being investigated by automatic image analyses. The samples used in this paper were treated as those previously reported [6]. The automatic cell images analyses were done with software ImageJ who was applied to digital images of whole cells color-stained cyanobacteria. Shortly, the analysis proceeds following few important steps: the background is separated from the objects based on the intra-class variance threshold method; noise and specks of staining color in the image can affect the reliability of the analysis, so those was removed. The removal was done applying mathematical morphology operations to the image; then separation of clustered objects was performed [37].
Natural fluorescence of chlorophyll a of unicellular or filamentous cyanobacteria was viewed using an epifluorescence microscope (N-400FL, lamp Hg 100 W).

2.4 Digital image analysis
Digital image analysis allowed us to distinguish from each analyzed images that green color appear immediately in filamentous cyanobacteria after adding QD 560 and this value increase after 130 seconds. ImageJ software allowed us to display simultaneously several selections or regions of interest (ROIs). In order to increase the specificity of image analysis it was further done only on the region of interest (ROIs - according to ImageJ user guide - the filament itself in this experiment) and processing the original pictures and subtracting background, because this removes smooth continuous backgrounds. In order to study cyanobacteria from marine samples, we created color histograms for captured microphotographs. Each picture was analyzed in three channels: red, green, blue and the mean intensity value of pixels were automatically calculated for any picture in the case of every red/green/blue channel, according to the instruction manual (ImageJ 1.44 user guide).

3 Results and discussion
During preliminary experiments, populations of cyanobacteria from initial microcosms [20] were cultivated on solid BG11 as shown in the figure 1, which were further used for selective cultivation (see 3.1).

Fig. 1 Hydrocarbons tolerant cyanobacteria populations grown on BG11 medium.

3.1 Cultivation of some hydrocarbon tolerant populations of cyanobacteria from microcosms contaminated with gasoline/diesel
In the microcosms with added gasoline (0.5% v/w) after one year the presence of phototrophic marine organisms became visible. Macroscopically, they are distinguished as a dark-green layer deposited on vessel walls, and on the top layer of sandy sediment (figure 2).

Fig. 2 Macroscopic aspect of photosynthetic microorganisms developed in the microcosms (A and B), and further grown in the laboratory (C). One can see the blue green cyanobacteria population floating under the diesel layer –enriched culture after 4 years (C).

Microscopically, we revealed the existence of different morphological types of cyanobacteria (figure 3 and 4).

Fig. 3 Cyanobacteria (natural fluorescence) (10 x 100) in gasoline polluted microcosms.
Fig. 4 Natural fluorescence of filamentous cyanobacteria grown in gasoline polluted microcosms.

As one can see in figure 5, phototrophic microorganisms can be seen based on chlorophyll autofluorescence (figure 5 A) whereas the use of a Sybr Green allows the visualization of all microorganisms either phototrophs or heterotrophs (figure 5 B), arguing for the occurrence of a consortium containing both phototrophic and heterotrophic microorganisms in enriched populations (figure 5).

The interplay between these heterotrophic and phototrophic microorganisms is one of our main topic to study in the near future, in agreement with literature [1],[2],[3].

Interestingly, in the initial microcosms, we have shown that in microcosm supplemented with nitrogen source (ammonium acetate 0.05% w/w) phototrophic microorganisms are mainly unicellular whereas in the microcosms supplemented with only gasoline phototrophic microorganisms are mainly filamentous, some of them differentiating heterocysts [19]. This leads us to the assumption that some nitrogen-fixing cyanobacteria would have mechanisms allowing them to tolerate the presence of oil hydrocarbons (even) when they fix molecular nitrogen. The signification of these results is under investigation as well as the attempts to cultivate and isolate cyanobacteria with heterocysts. Cyanobacteria evolve oxygen which is needed by oil degrading heterotrophic microorganisms in order to degrade hydrocarbons under aerobic conditions. Oil degradation can be further stimulated by cyanobacteria which are able to fix atmospheric nitrogen. In other words, the consortium containing both phototrophic and heterotrophic microorganisms appears as an ideal association for self-cleaning polluted environments [34], [29], [26], [41], [4], [10], [2], [16], [8], [3], [1], [39]. Nitrogen fixation could become a nutrient source for hydrocarbons polluted natural ecosystems, taking into account the
excess of carbon and hydrogen as compared with nitrogen in these environments. There is a report concerning the occurrence of molecular nitrogen fixation in marine sediments contaminated with hydrocarbons, process which is independent of light; there are no information concerning the systematic classification of bacteria able to fix molecular nitrogen in those sediments [27].

In the next figure one can see the physical relationship between cyanobacteria and diesel droplet.

![Figure 6](image1.png)

**Fig. 6** The physical relationship between enriched populations of cyanobacteria and diesel droplets seen in bright field and epifluorescence microscopy of the same microscopic field: A - bright filed where one can see diesel micro-vesicles and a filamentous cyanobacterium; B - epifluorescence microscopy-natural fluorescence of cyanobacterium (red channel-FI2) and C - blue channel with UV excitation-FI3) where one can see diesel micro-vesicles only.

In microscopic preparations, without any fluorochrome addition, the diesel microvesicles can be seen in bright field (A) and in blue fluorescence (C, FI3 filter), but not in red filter (B- FI2).

### 3.2 Cultivation of hydrocarbon tolerant populations of cyanobacteria grown on solid hydrocarbon

After three month of cultivation in sea water, at the surface of solid hydrocarbon a phototrophic consortium become macroscopically visible, that, after two years appears as an envelope with a thickness of about 3 mm, dark green in color, containing cyanobacteria, microalgae and heterotrophic bacteria (results not shown).

In the next figures, there are presented microscopic images (chlorophyll fluorescence) of filamentous cyanobacteria able to grow at the surface of solid hydrocarbon. The large majority of filaments are organized in a network (figure 7A which contribute to the formation of the macroscopic envelope), some of the cyanobacteria being seen as isolated filaments (figure 7B).

![Figure 7](image2.png)

**Fig. 7** Natural fluorescence of enriched cyanobacteria grown on solid hydrocarbons.
In time, the development of phototrophic microorganisms, mainly cyanobacteria (but also diatoms - results not shown) enables them to grow not only attached at the surface of solid hydrocarbon but also in the water phase.

In the next picture one can see, as in the case of cyanobacteria population growing in sea water supplemented with diesel, the physical relationship between cyanobacteria and small particles of solid hydrocarbons.

3.3 The interplay between oil tolerant cyanobacteria and quantum dots

Following our previous work on QD [6], [5] in this paper we present our results concerning the dynamic of interaction between oil tolerant cyanobacteria and quantum dots.

In order to explain the epifluorescence color changes effect of QDs added to the cell cultures on the fluorescence color cyanobacteria, digital image analyses were performed.

We studied digital images, which are two dimensional grids of pixel intensity values. These images have the width and height defined by the number of pixels in x (rows) and y (columns) directions. Thus, the pixels are the smallest single components of images, holding numeric values (i.e., pixel intensities) that range between black and white. The obtained microphotographs were red, green, blue channels images, RGB/HSB stacks, and composite.

Digital image analysis allowed us to distinguish from each analyzed images that green color appear immediately in filamentous cyanobacteria after adding QD 0560 and this value increases after supplementary quantities of QDs. Furthermore, ImageJ software allowed us to display simultaneously several selections or regions of interest (ROI). In order to increase the specificity of image, the analysis was further done only on ROI.

The processing of the original pictures was performed by subtracting the smooth background from the image. This command uses a “sliding paraboloid” or a legacy “rolling ball” algorithm that can be used to correct for uneven illuminated background, like in our pictures. This obtained light background allowed us the processing of images with bright background and dark objects and to visualize the color changes of the cyanobacterial filaments. We have supposed that the observed attachment of the QD at the surface of the cyanobacterial filaments is of electrostatic nature. In fact the quantum dots we have used (QD 0560) have positively polarized the lateral amino group. In the presence of the bacterial cell with the negatively polarized carboxyl groups the QDs are attracted on the external envelope of the cells within the filament (figure 9).
Fig. 9 Time-changes in the fluorescence colour of cyanobacteria after QD addition

Green channel can be considered as following the variation of intensity of fluorescence green quantum dots. Note that intensity in green channel increases after adding the quantum dots (Figure 10).

4 Conclusion

In this paper we show the cultivation of phototrophic marine microorganisms in the presence of oil hydrocarbons with the special aim to enrich the marine consortium in marine cyanobacteria that are (at least) able to tolerate hydrocarbons (up to 5% w/v) many generations (2-5 years). These enriched populations starting from two different sources (microcosms and solid hydrocarbon collected at the sea shore) were studied with respect to morphology and physical relationship with hydrophobic structures (hydrocarbon surfaces and quantum dots).

It is an open question if these selected populations of cyanobacteria have per se the capability to oxidize (some fractions of) hydrocarbons. In agreement with the literature [26], [41], [4], [10], [2], [16], [8], [3], [1], we think that the microbial consortium could degrade hydrocarbons more efficiently than isolated microorganisms, the intimate metabolic networking between phototrophic microorganisms, mainly cyanobacteria, and heterotrophic bacteria being not only an exciting topic but also a strong candidate for an eco-friendly solution to hydrocarbon pollution in aquatic or terrestrial environments.

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References:


