Microscopic investigation and automated image analysis of hydrocarbon- tolerant marine cyanobacteria mixed populations cultivated in the absence and presence of gasoline or diesel

Simona Ghiță, Iris Sarchizian, Ioan I. Ardelean

***Abstract*—**This paper shows some morphological and functional aspects of cyanobacteria isolated and selected 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. Phototrophs microorganisms appear to be an important change in marine microbiota as a result of gasoline presence. Filamentous cyanobacteria both with or without heterocysts have been analyzed using CellC and ImageJ software for cell morphology, intracytoplasmic inclusions and interactions with hydrophobic structures, nanoparticles and oil hydrocarbon.

***Keywords*—** automated imge analysis, cyanobacteria, oil hydrocarbons, transparent exopolymer particles.

I. Introduction

The distribution of cyanobacterial community in the oil polluted marine environment is dependent on biotic and abiotic factors (e.g., temperature, currents and nutrient concentrations) [1], [2]. 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 [3], [4].

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 [5], [6], [7], [8], [9], [10], [11], [12], [13], [14], [15]. Bacterioplancton ecology (abundance and productivity) remain fairly constant over a wide range of aquatic systems [16] [17]. Cyanobacteria species capable of degrading petroleum fractions are: *Oscillatoria salina*, *Plectonema terebrans*, *Aphanocapsa* sp. *Synechocystis*, *Pleurocapsa*, *Dermocarpella* and so on. Generally, cyanobacterial mats when grown in mixed cultures, have an excellent potential for use in mitigating oil pollution on seashores, either individually or in combination.

In oil-polluted mats there are cyanobacteria, algae, protists, heterotrophic N_2-fixing bacteria, with individual functions important in bioremediation process [18], [19], [20]. 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 [21]. The biology of cyanobacteria is very complex. All representatives of cyanobacteria contain chlorophyll *a* and are able to grow as photoautrophs, although photohetetrophy and chemoautotrophy are common to many species. The ability of cyanobacteria to adapt to environmental stress, including rare or abundant nutrients [1], exposure to UV radiation, high solar radiation [22], pollutants such as oil hydrocarbons [5], [6], [7], [8], [9], [10], [11], [12], [13], [14], [15] may favors the dominance of cyanobacteria in many aquatic ecosystems [23]. In the process of microbial degradation of oil hydrocarbons algae, fungi and bacteria can establish complex metabolic interactions, including cometabolism [24], [25]. Scientists have discovered that microorganisms for example (picocyanobacteria) can accumulate hydrocarbons from the water column [9]. The electron microscopy method showed that the cells of picocyanobacteria stores hydrocarbons in their inter thylakoid spaces, thus we take into account the potential role of picocyanobacteria in controlling marine oil pollution.

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 [21].

The presence of nitrogen fixation in microbial communities of oil-contaminated marine sediment microcosms [26] 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.

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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 [27], [28], [29], [30], [31], [32], [33], [34], [35].

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 [36]. 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 [37], and even that free image analysis software is common place, 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" [38]. 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 programs.

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).

II. 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 [39], [40] and processed as previously shown [41].

A. 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 cannot 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 [2], [41], [42].

During previous experiments [41] 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.

B. 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 inoculums 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.

C. Coloration of cyanobacteria with aniline blue

For detecting the different type of polysaccharide from phototrophic cells in the samples supplemented with gasoline, we used aniline blue dye. Samples used by us in this protocol gave positive staining reactions. Aniline blue final concentration was 5 μg/mL and a dye for up to 5 minutes.

D. Coloration of cyanobacteria with alkaline methylene blue for polyphosphate inclusions

Alkaline methylene blue reacts with inorganic polyphosphate (or volutin granules) which are intracytoplasmic inclusions storage form of phosphate, found in different bacteria, including cyanobacteria. This is a metachromatic coloration because during the reaction between MB and polyphosphate

inclusions in alkaline conditions the color of the dye is changing, in this case from blue to red [43].

E. Coloration of cyanobacteria with Sudan black for PHB was done as in [43].

F. Coloration of cyanobacteria with alcian blue for acidic polysahcarides, was done as in [44].

G. The interplay between hydrocarbon-tolerant cyanobacteria and (fluorescent) quantum dots

Following our previous work on QD [45], [46] 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 [45].

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 [47].

Natural fluorescence of chlorophyll *a* of unicellular or filamentous cyanobacteria was viewed using an epifluorescence microscope (N-400FL, lamp Hg 100 W).

Initially, different methods were analyzed but at the end we choose to highlight the cyanobacteria color with QD. The natural samples were treated with various chemicals to detect each specific quantum dot fluorescence on cyanobacteria.

We concluded that the fluorescence color is a mixture of fluorescence emitted by natural fluorescence of cyanobacteria and fluorescence emitted by quantum dots. Natural samples were first bleached with chlorine such that fluorescence due the chlorophyll to disappear, and then the samples was stained with fluorescent quantum dots.

By applying this method we observed that cells in which chlorophyll was destroyed by oxidation with bleach, emit a different color fluorescence compared with intact cells (with chlorophyll) labeled with quantum dots.

The difference is due to the absence of fluorescent emission of chlorophyll in which samples the chlorophyll was oxidized.

H. 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).

III. RESULTS AND DISCUSSION

During preliminary experiments, populations of cyanobacteria from initial microcosms [42] were cultivated on solid BG_{11} as shown in the figure 1, which were further used for selective cultivation (see figure1).

Fig. 1 Hydrocarbons tolerant cyanobacteria populations grown on BG_{11} medium.

A. 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).

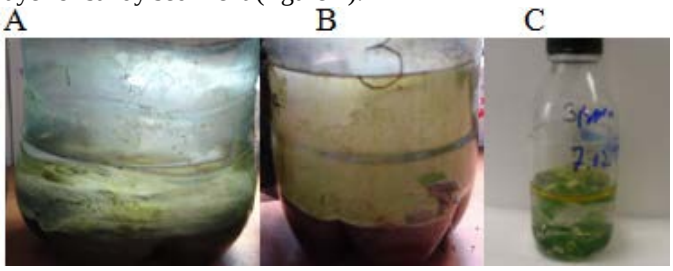


Fig. 2 Macroscopic aspect of photosynthetic microorganisms developed in the microcosms (A and B), and further grown in the laboratory (C). One can see the blue green cyanobacteria population floating under the diesel layer –enriched culture after 4 years (C).

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

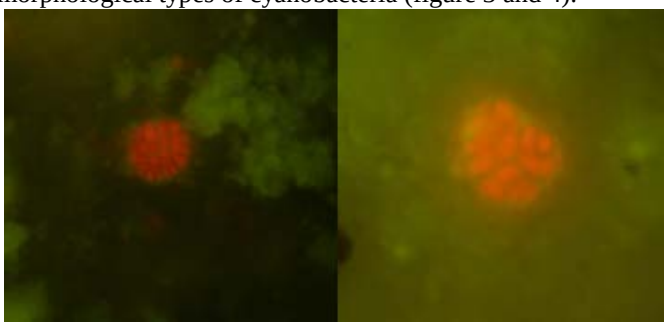


Fig. 3 Cyanobacteria (natural fluorescence) (10 x 100) in gasoline polluted microcosms.

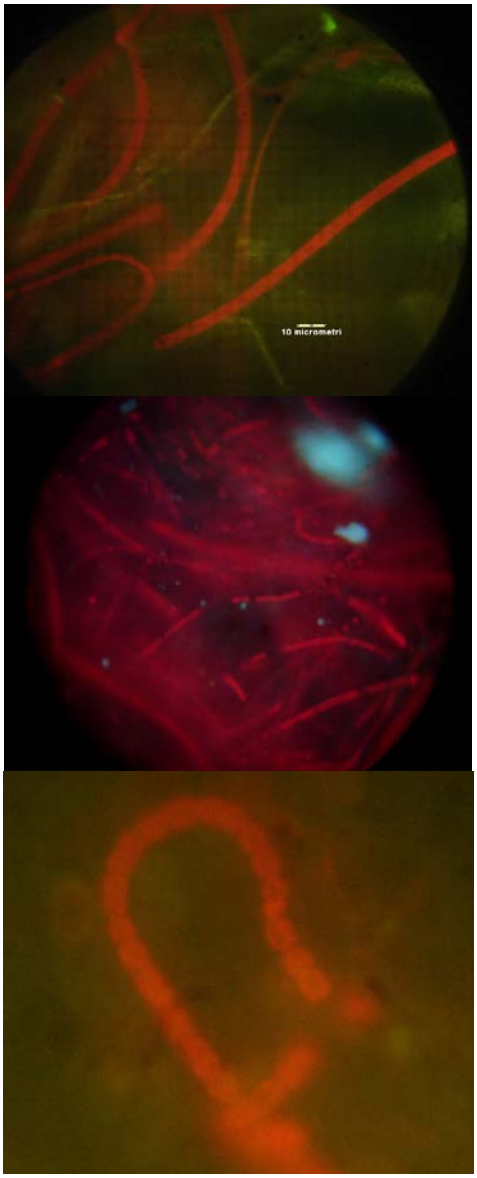


Fig. 4 Natural fluorescence of filamentous cyanobacteria grown in gasoline polluted microcosms.

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 [15],[11],[14].

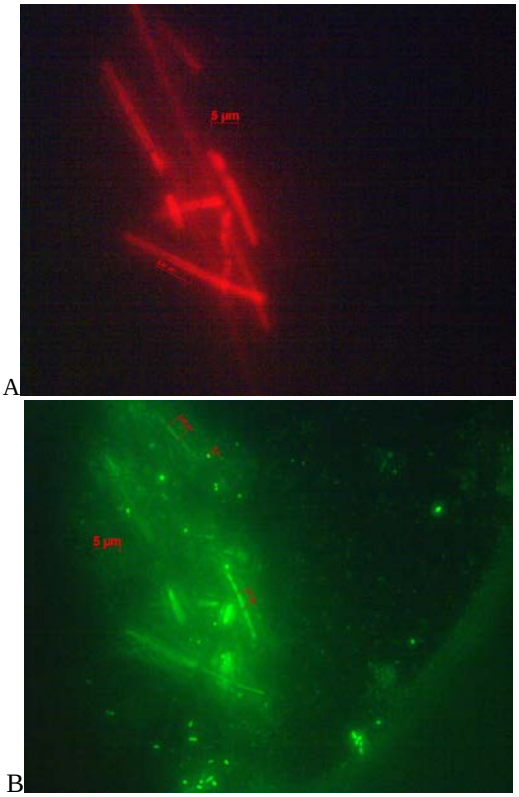


Fig. 5 Enriched photosynthetic populations -View of the same microscopic field (chlorophyll filter -FL2)-A; and by the SYBR Green filter (Fl 3)-B.

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 [41]. 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 [5], [6], [7], [8], [9], [10], [11], [12], [13], [14], [15], [48]. 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 [26].

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

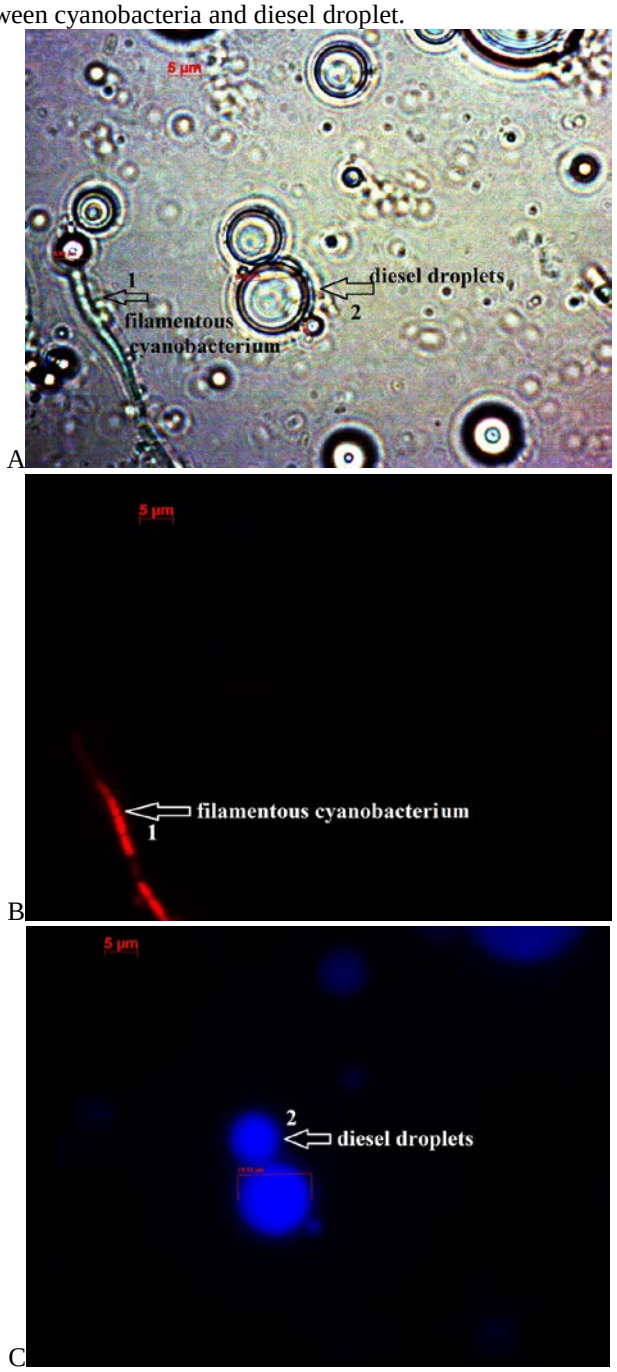


Fig. 6 The physical relationship between enriched populations of cyanobacteria and diesel droplets seen in bright field and epifluorescence microscopy of the same microscopic field: A - bright filed where one can see diesel micro-vesicles and a filamentous cyanobacterium; B - epifluorescence microscopy- natural fluorescence of cyanobacterium (red chanel- Fl2) and C - blue channel with UV excitation-Fl3) 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, Fl3 filter), but not in red filter (B- Fl2).

B. 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).

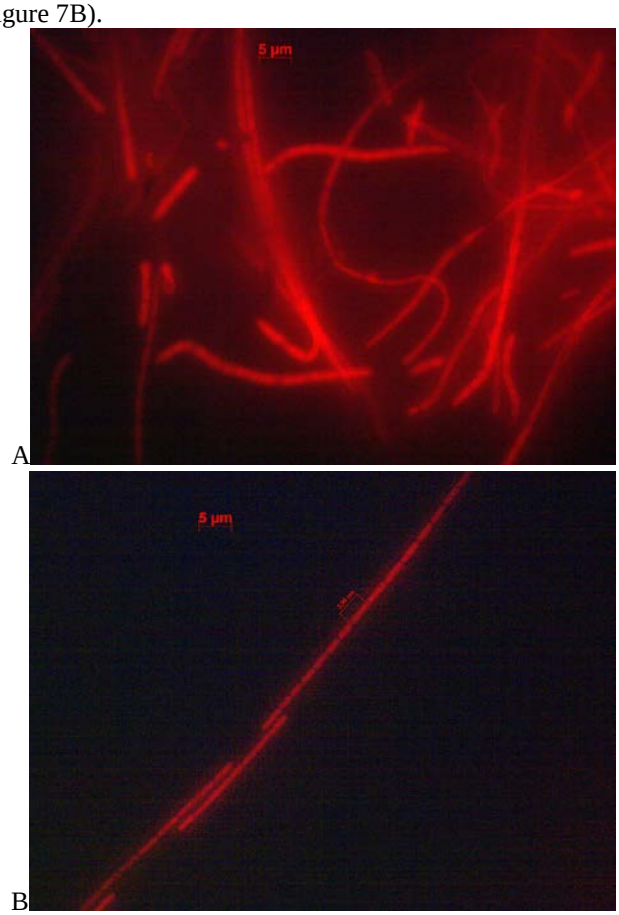


Fig. 7 Natural fluorescence of enriched cyanobacteria grown on solid hydrocarbons.

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 (figure 8) 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.

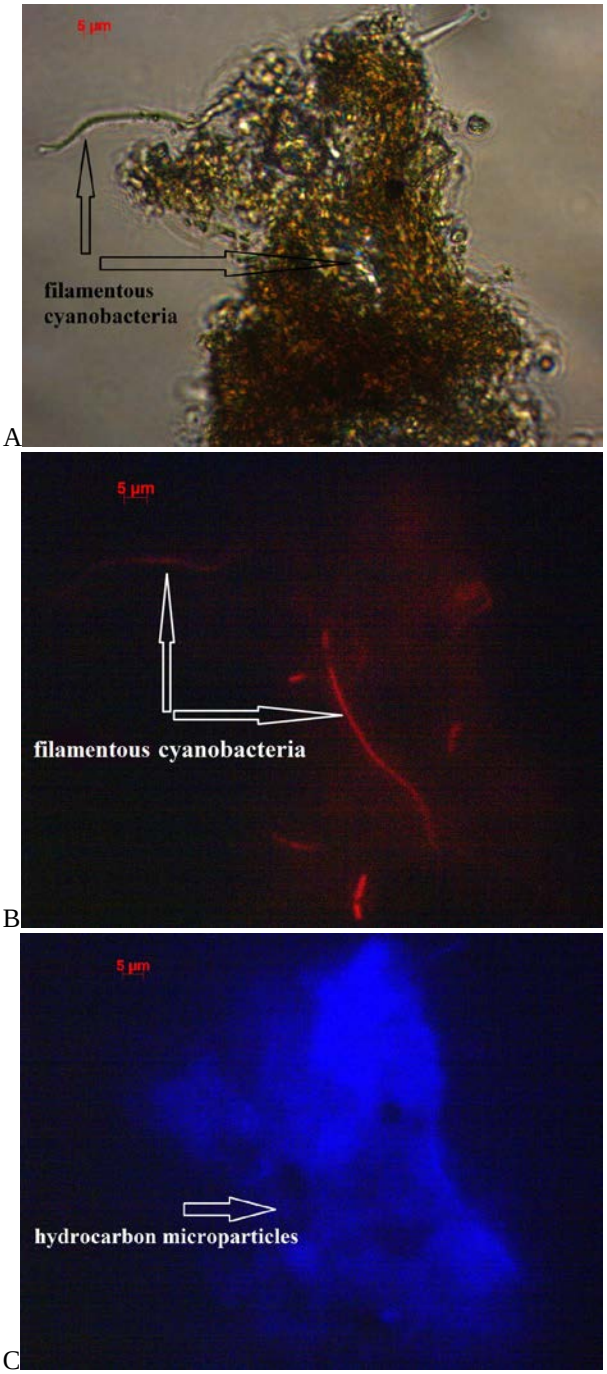


Fig. 8 The physical relationship between cyanoacteria and hydrocarbon microparticles seen in bright field and epifluorescence microscopy of the same microscopic field: A- bright filed where one can see diesel micro-vesicles and a filamentous cyanobacterium; B- epifluorescence microscopy- natural fluorescence of cyanobacteria (red chanel- Fl2) and C- blue channel with UV excitation-Fl3) where one can see hydrocarbon microparticles.

C. Acridine orange and DAPI staining of cyanobacteria filaments in gasoline supplemented microcosms

Digital images obtained by acridine orange staining of samples from experimental microcosms allowed cyanobacteria filaments visualization, images were processed with Image J and CellC program that to distinguish heterocysts (figure 9).

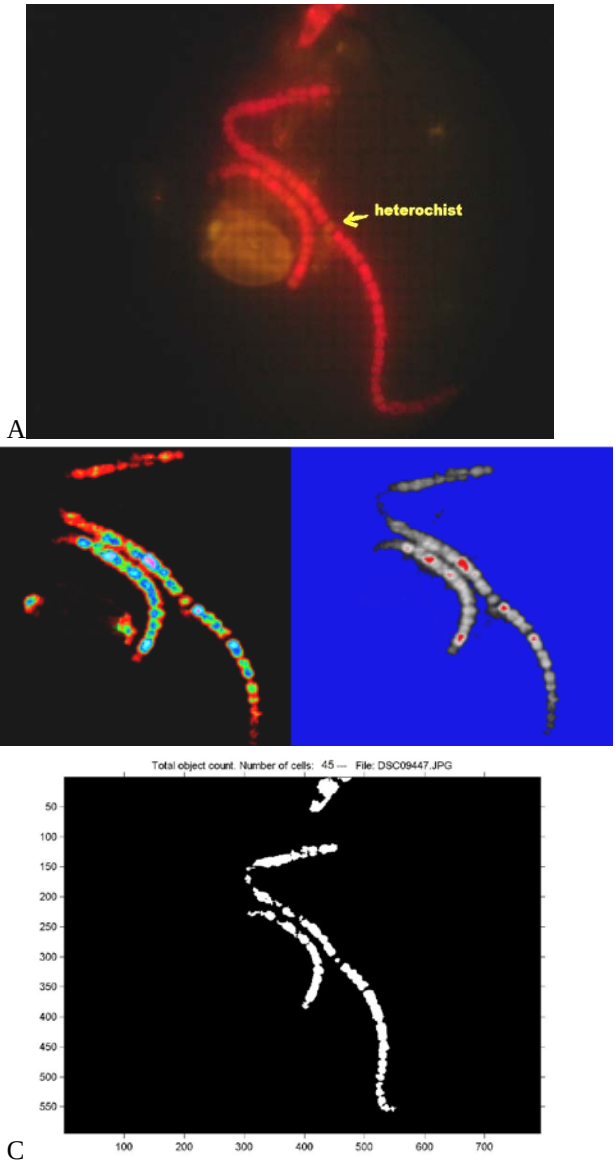


Fig. 9 A – Digital image cyanobacteria that differentiate an heterocysts in microcosm supplemented with gasoline (AO staining); B–delimitation A panel edges using Image J software; C – analysis of the total number of cells in A panel using CellC software (45 cells counted in cyanobacteria filament in A panel)

The images shown in Figure 9B, highlights the advantages of using Image J program, which can clarify the intensity of fluorescent emission of filament cells; where there are red spots, pink or green (depending on the channel selected in the software) are detected cells emit a more intense fluorescence than other cells.

In a first step the original image (for example, 9A) is converted to the 32-bit image, then we adjust the brightness / contrast by applying image processing option (for example, 9B). The same image is then analyzed with the program CellC (figure 9C) to count the cells in the filament of cyanobacteria, we can also measure the size of each cell (using automatic measurement with Image J - cell length in µm, by calibrating an ocular graticula) [49].

To avoid uncertain estimates of filament length and width, the number of filaments presented in one image should not be too high. Cyanobacteria filaments overlap on the microscopic preparation could lead to uncertain measurements of the filament so extreme densities would increase the measurement error [50].

DAPI staining:

DAPI is a more specific dye for DNA molecule than acridine orange, having blue or blue-white fluorescence (about 390 nm) when bound to DNA [51], and when it is not linked to DNA or bind to non-DNA material, it could have a fluorescence spectrum of the shade yellow. We have looked natural samples at different wavelengths, taking into account the maximum spectrum of absorption of this fluorochrome: on the blue filter (λ = 450-480 nm, Figure 10A), as well as UV filter (λ = 330-385 nm, Figure 10 B).

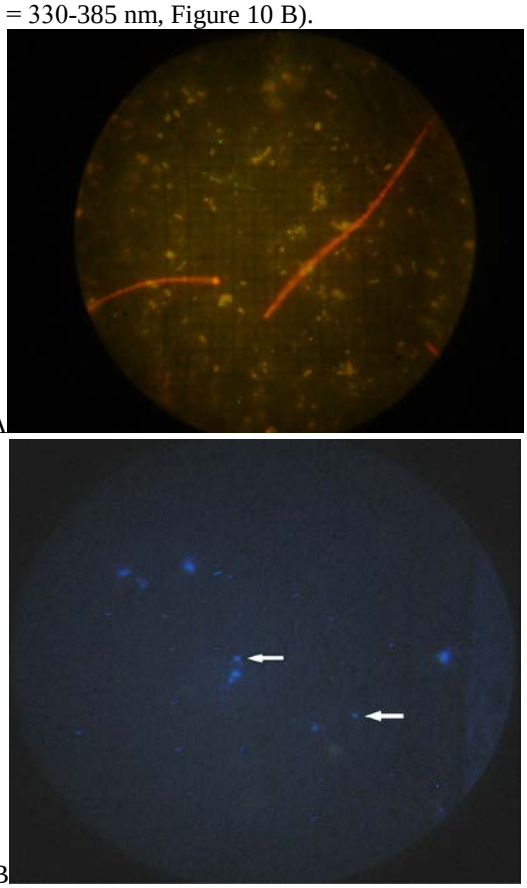


Fig.10 Prokaryotic cells viewed through different epifluorescence filters: (A) blue filter, (B) UV filter, x 1000, DAPI staining.

It is observed that by using DAPI staining on the blue filter the filament phototrophic organisms is highlighted, instead by using the UV filter we can see the heterotrophic organisms.

D. Visualization of (putatively) capsulated cyanobacteria cells (AB+)

Aniline blue staining is specific for highlighting the different type of polysaccharide. This is a good method for detection of production of exocelular β-1,3-glucan, and also for detection of some β-glucan in the cell wall of prokaryotes [52].

In figure 11 is present the samples which was treated with AB (5 µg/mL final concentration) for 3-5 minutes.

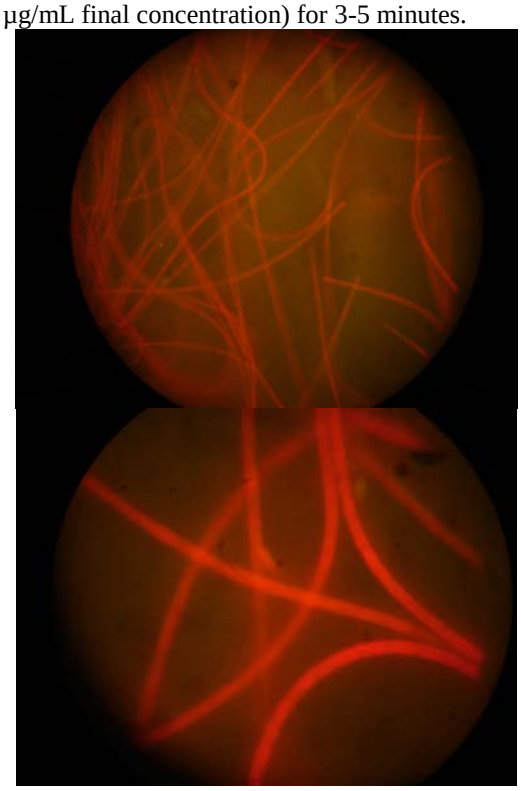


Fig. 11 Visualization of encapsulated cyanobacteria after aniline-blue staining from marine microcosms contaminated with gasoline.

In figure 11 is apparent the AB stained phototrophic cells from microcosm supplemented with gasoline which are a signature of active bacteria. This staining allowed us to view a large number of cyanobacteria filaments, argue that the relative abundance of oxygenic photosynthetic microorganisms at interfaces in microcosm could be related to the possible involving of cyanobacteria in hydrocarbon oxidation, an increasing topic in petroleum microbiology [53], [54], [55], [56]. It is possible that active bacteria/cyanobacteria are constantly renewing their capsular envelope and releasing a significant fraction of the polysaccharidic layer into the ambient water [52].

In marine bacterioplancton, the N:P ratio is very important component, moreover the bacterioplancton fraction is responsible for shortage of phosphorous with the expression of high alkaline phosphatase activity [57]. In our microcosm the C:N:P ratio is possible to be in favors of the carbon and nitrogen. The capsular material contains glucose used in breathing process. This is extremely important in terms of phototrophic prokaryotic physiology.

E. Coloration of cyanobacteria with alkaline methylene blue

In the figure 12 one can see cyanobacterial populations treated with alkaline methylene blue in order to label the presence of polyphosphate. Interestingly, the filamentous cyanobacteria grown for one week in sea water, supplemented with diesel (2% v/v) exhibit a much stronger interaction with the alkaline dye than the populations grown in the absence of

diesel; thus, the red color of cyanobacterial filaments is much stronger for the populations grown in the presence of diesel. The biological significance of these results is under investigation. These results deserve further attention in the near future, in order to understand the biological significance of these differences. In this respect, it would be useful, to use automated image analysis for the calculation of the light signal in the red channel for a larger number of filaments from cyanobacterial populations growing with or without diesel, in sea water or synthetic media.

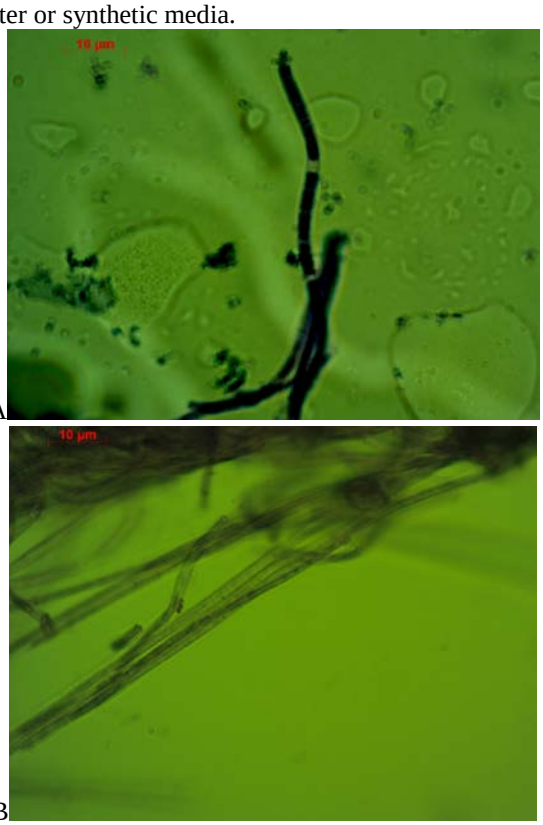


Fig.12 Alkaline methylene blue staining of cyanobacteria: A- cyanobacteria grown with diesel; B- cyanobacteria grown without diesel.

Preliminary analysis shows that there are differences between the two types of samples with respect to the signal in the red channel and in the blue channel. Cyanobacteria gown in the presence of diesel contain less free, unbound alkaline methylene blue, thus the blue light is less absorbed by this sample, as compared with cyanobacteria grown in the absence of diesel. As consequence, the signal in the blue channel is higher in images of cyanobacteria grown in the presence of diesel (26.18±13.8) see figure 13, than for images of cyanobacteria grown in the absence of diesel (16.28±8.4).

When it comes to the situation in the red channel the situation is as follows: cyanobacteria gown in the presence of diesel contain more alkaline methylene blue bound to polyphosphate granules, thus the red light is more absorbed by this sample, as compared with the situation in cyanobacteria grown in the absence of diesel.

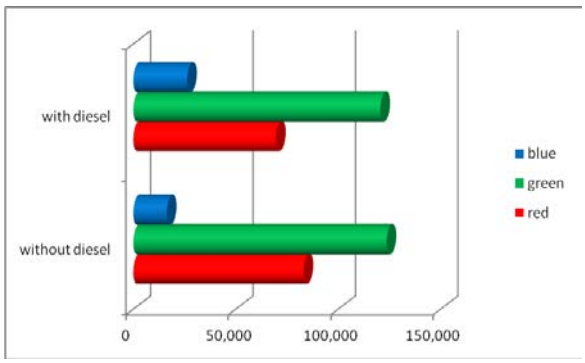


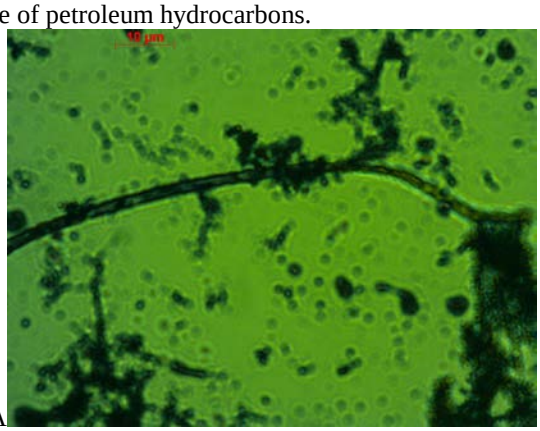
Fig. 13 The analysis of color histogram of the digital images (RGB) with cyanobacterial filaments reveals that the blue channel increases after adding diesel into the culture, comparing to digital images belongs to the cyanobacterial culture without diesel and in the mean time we observed that the red channel decrease in the culture with diesel.

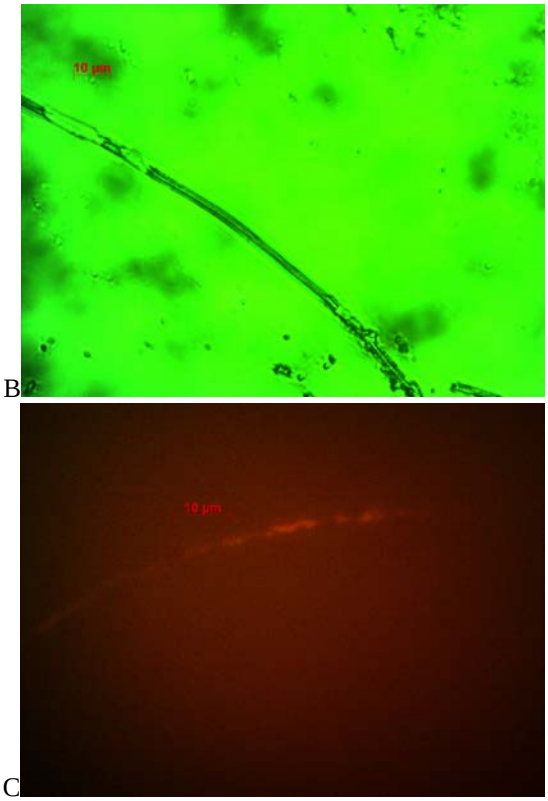
As consequence, the signal in the red channel is lower (69.58±22.2) in images of cyanobacteria grown in the presence of diesel (see figure 13) than for images of cyanobacteria grown in the absence of diesel (85.05). For the signal in the green channel there are no differences between the two samples.

F. Coloration of cyanobacteria with Sudan black

In figure 14 one can see cyanobacterial populations treated with Sudan back in order to label the presence PHB. As one can see, both type of cyanobacterial populations contains PHB inclusions which, according to these results, seems to be more abundant in control (14A) (populations growing in the absence of diesel) than in populations growing in the presence of diesel (14B).

As in the case of polyphosphate inclusions, there is the need to use quantitative methods to study the time evolution of PHB inclusions in cyanobacteria in relation with the presence or absence of petroleum hydrocarbons.





B

C

Fig. 14 Sudan black staining of cyanobacteria (bright filed and epifluorescence microscopy): A- cyanobacteria grown without diesel; B- cyanobacteria grown with diesel; C- epifluorescence microscopy cyanobacteria grown without diesel (similar results have been obtained with the control culture- results not shown).

Interestingly, as one can see in figure 14 C, the natural fluorescence signal of chlorophyll *a* is no more available as microscopic image in samples treated with Sudan black. This could be a novel way to show that Sudan black is interacting with the cell and filaments: the decrease in chlorophyll *a* fluorescence being probably correlated with the fact that Sudan black is a non fluorescent lipophylic dye. This indirect fluorescent method could be easier to perform than fluorescent method based on Nile blue and more precise than classical bright field method, used in this paper (fig. 14 A and B).

G. Coloration of cyanobacteria with alcian blue

In figure 15 there are presented cyanobacterial populations treated with alcian blue in order to label the presence of acidic polysaccharides, with special emphasis on TEP- transparent exopolymer particles; one can see the dye is bound to filaments and TEP are clearly present in cultures (here shown only for cyanobacteria grown with diesel, but similar results have been obtained for control- results not shown).

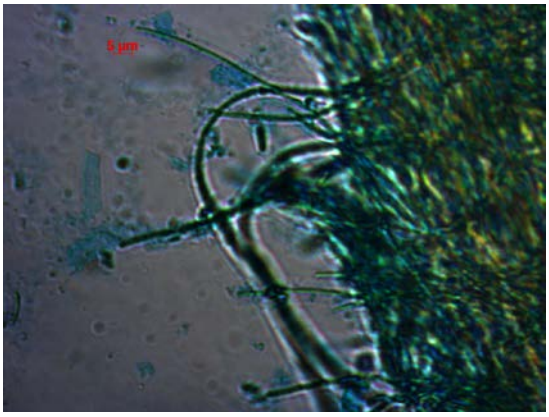


Fig. 15 Alcian blue staining of cyanobacteria grown with diesel to label transparent exopolymer particles.

H. The interplay between oil tolerant cyanobacteria and quantum dots

Following our previous work on QD [45], [46] 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 16).

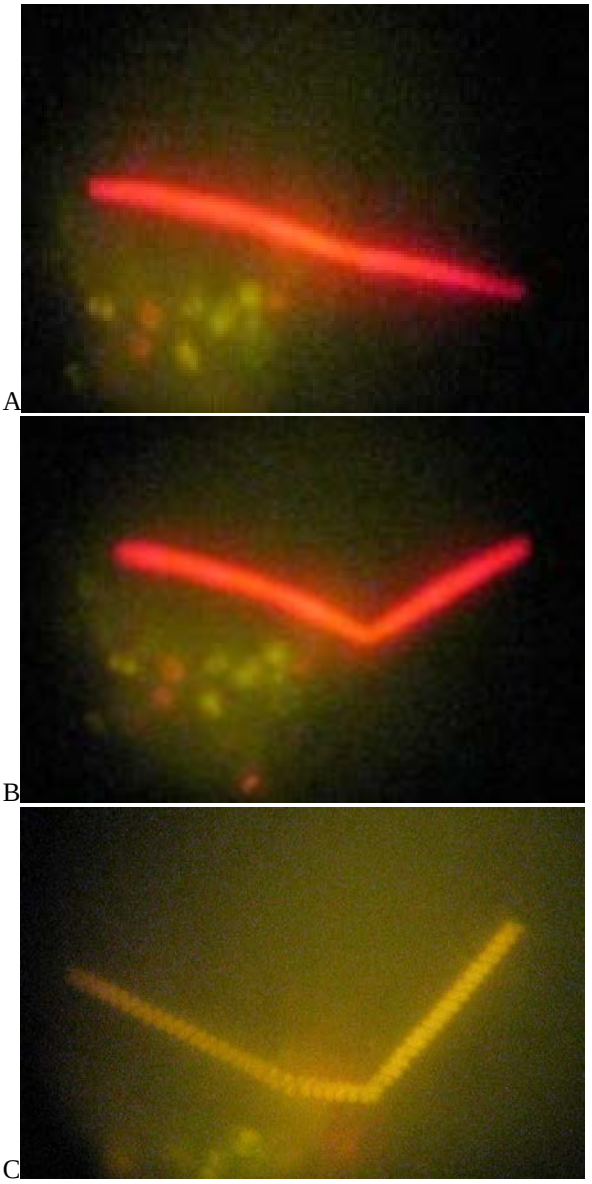


Fig. 16 Time-changes in the fluorescence colour of cyanobacteria after QD addition.

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 17).

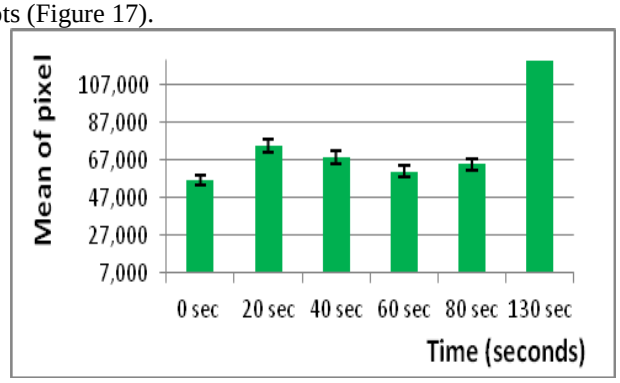


Fig.17 The dynamics of intensity in the green channel following green QD addition.

The importance of mathematical methods of signal and image processing is constantly increasing in microbiological studies. In addition to cell biological expertise, new research findings often require engineering skills, for example to control automated equipment in high throughput screening, or to manage extensive data storages and computing hardware in computationally demanding research. All this development requires fundamental changes in many traditional procedures of cell biology, calling for close and active collaboration between biologists, computer scientists, and mathematicians. Digital image processing also plays a major role in this development, as it is difficult to see any other methodology capable of detailed, tireless, and objective analysis of large image databases showing up in many fields of science. In our opinion, Microbiology would significantly benefit if automated image analysis will be used by more microbiologists in their professional activities; furthermore the interplay between microbiologists and mathematicians and engineers in this field could be helpful in developing new opportunities within "old" software, or, even, to generate new software more appropriate for different microbiological task.

IV. 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) where studied with respect to morphology and intracytoplasmic inclusions and interactions with hydrophobic structures, nanoparticles and oil hydrocarbon.

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 [7], [8], [9], [10], [11], [12], [13], [14], [15], 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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