Dynamics of marine bacterioplankton density in filtered (0.45 µm) microcosms supplemented with gasoline

SIMONA GHIȚĂ1,3, IOAN ARDELEAN2,3
1Department of Environmental Engineering; 2Department of Microbiology
1Constanta Maritime University; 3Institute of Biology, Ovidius University
1Mircea cel Batran 104, Constanta 900663, 2Splaiul Independentei 296, Bucharest 060031; 3Aleea Universitatii 1, Constanta 900470
ROMANIA;
ghitasimona@aim.com; ioan.ardelean@ibiol.ro

Abstract: - The total bacterial count (Acridine Orange and DAPI), dead cells (propidium iodide positive cells) and putative capsulated cells (aniline blue positive cells) were determined in marine microcosms supplemented with gasoline. These experiments show that addition of both gasoline (0.5% v/w) and ammonium nitrate (0.005% w/w) to sea water (M1) stimulated the increase in total cell count and a decrease in the density of dead cells as compared with the control (M3- sea water); the results obtained when only gasoline was added (M2) have numerical values in between M1 and M3.

Key-Words: - epifluorescence microscopy, bacterioplancton, alive/ dead cells, automated image analysis

1 Introduction
The quantification of microorganisms in natural samples by the use of fluorochromes is one of the main goal in microbiological research in the last decades [40; 13; 28; 37; 16; 33; 19; 25; 37] coupled with digital image analysis [34; 38; 35; 31; 32]. The frequency and risk of oil pollution has lead to extensive microbiological research [11; 1; 29; 15; 2; 18], for the use of microorganisms to oxidize different oil components [14; 5; 16; 30].

The aim of this paper is to compare the time evolution of total cell counts, (putatively) capsulated and dead planktonic marine prokaryotes in microcosms supplemented with gasoline and nutrients, as compared with the control, using epifluorescence microscopy coupled with digital image analysis.

2 Materials and Methods

2.1 Microcosms construction
Water samples were collected from the Black Sea (0.5m depth; Tomis seaport, 44°10’ 44” N; 28°39’ 32” E) which were used for the microcosms setup done in Polyethylene transparent bottles. In our experiments, the microcosms in volume 300 mL of natural sea water were kept at 18°C temperature and artificial illumination. At different interval of time, samples were collected in sterile Falcon tubes, fixed with buffered formaline (2% final concentration) and kept at 4°C in darkness without any added fluorochrome. Microcosms were constructed using filtered (0.45 µm Millipore) sea water to completely avoid the inclusion of heterotrophic nanoflagelates in the filtrate and, therefore, large bacteria were also excluded from these communities [7; 39]. The three types of microcosms are: Black Sea natural sample- control (M3); control supplemented with gasoline (0.5% v/w) (M2) and control supplemented with gasoline (0.5% v/w) and nitrogen source (ammonium nitrate 0.005% w/w) (M1). The disruption of planktonic cell aggregates and cell enumeration were done following the literature, as previously shown [3].

2.2 Total cell count (AO; DAPI)
Total bacterial count of our natural samples was done using acridine orange and DAPI [9; 20; 21; 23] using 5 µg/mL dye final concentrations and 5 minutes of coloration. With both stains, bacteria are identified on the basis not only of color but also of size and shape [17]. All samples were viewed at epifluorescence microscope– (N-400FL, lamp Hg 100 W).

2.3 Counting dead cells (PI+)
Dead cells were counted using propidium iodide (PI) [23] at the final concentrations 2 µg/mL.

2.4 Counting putative capsulated cells (AB+)
Fixed samples were treated with aniline blue (20 µg/mL final concentration) for 5 minutes, aniline blue a label for polysaccharides containing 1-3 β covalent links [22; 26].

2.5 Counting and calculations
Quantification of cellular fractions was performed by digital image analysis using two software programs:
CellC [41] and Image J [31; 32], as previously shown [3; 4].

2.6 Measurement of nitrogen in the three microcosms
Nitrogen was determined with specific Hach Lange kit following the manufacture instructions (HACH LANGE GmbH) [42].

3 Results and discussions

3.1 Total bacterial counts (AO, DAPI)
Figure 1 shows the time evolution of total bacterial counts done with either AO or DAPI in the three microcosms (see Materials and methods).

Fig.1. Total bacterial counts (AO or DAPI) in filtered (0.45µm) microcosms: M1, M2 and M3.

Comparing the total number of heterotrophic cells obtained with AO and DAPI stained in experimental microcosms and control, we have shown that results obtained with DAPI are slightly higher than those obtained with AO (M1: 6.8 x 10^6 cells ml⁻¹ AO and 7.1 x 10^6 cells ml⁻¹ DAPI, respectively M2: 4.05 x 10^6 cells ml⁻¹ AO and 4.74 x 10^6 cells ml⁻¹ DAPI), in agreement with the literature [27; 8; 23].

However, there are differences in total cell count between the three microcosms, the highest cell densities being attained in the microcosms supplemented with gasoline and with inorganic nitrogen source (M1). It seems rational to claim that the addition of nitrogen source enable bacterioplancton to face better the presence of gasoline, as cell density is higher all over the experiment in M1 than in M2, in agreement with the literature [10; 24; 18].

In the control (M3), in the first 50 days of the experiment the cell densities are higher than in M2, the presence of gasoline (with no added nitrogen source as in M1) being probably responsible for the elimination of gasoline-intolerant microorganisms; during this period it is also expected that the activity of gasoline tolerant and gasoline – consuming microorganisms is lower in M2 than in M1.

3.2 Enumeration of dead bacterial fraction (PI+)
Regarding the number of dead cells obtained after PI staining (figure 2), it was obtained a relatively low fraction of dead cells reported to the total number.

Fig.2. The time evolution of dead cell density in the three microcosms counted automatically by CellC.

The cell stained with PI were distinguished by their red fluorescence. These stains are charged and have structures that can result in steric hindrance, which can hamper their diffusion through the cell membrane [23].

These results show that in M3 there is a very low increase in absolute density of dead cells, as compared with M1 and M2 where there are increases and decreases. However, counting the fraction of dead cells from total cell counts, in M1 is 11.53%, in M2 is 12.65% and in M3 is 13.97%. The significance of these results is under investigations.

3.3 Enumeration of (putative) capsulated cells (AB+)
As shown in figure 3, in the three microcosms densities of putative capsulated cells slowly increases in M1 and M2 as compared with the control. As the presence of capsule is a signature of active marine bacteria [12; 36] these results suggest that in microcosms M1, in the
presence of both gasoline and nitrogen source, the densities of active cells is higher than in microcosm 2, where only gasoline was added.

Fig.3. The time evolution of AB+ cell density in the three microcosms.
The signification of these results is under investigations, as well as the comparison between densities of dead (IP+) cells and the differences between total cell count (AO or DAPI) and putative capsulated cells (AB+), checking for the expected following equation: (IP+) cells= (AO+)-(AB+).

3.4 Automated image analysis for epifluorescence
Cell enumeration was done using two automatic digital image software for cells measuring / sizing (CellC and Image J soft). Figure 4 shows the manner of automatic quantification of cell number with CellC software using the microphotographic digital images obtained experimentally in our studies on the microcosms.
The CellC program can do the segmentation and the analysis of the Total Count image, and then, the segmentation of cells from the background and extraction of individual cell clusters [31].

Fig.4. CellC program, an example of AO-stained images of bacteria from microcosm 2 (A); Total count analysis of panel A using CellC software (B).
The Image J software is the second software used in automated measurement of cell presence, size and shape. The Image J program present ability to support standard functions of digital image processing and also can make changes such as geometric scaling, rotation, etc. This program image can be increased to 32:1 and, conversely, reduced to a value of 1:32. The program can support a limited number of images related to the available memory. Calibration dimensional space can provide measurements in different units: eg, pixels, millimeters, micrometers (figure 5).
The phase of cells quantization on the field previously processed with the program Image J is achieved by drawing a straight line will fit 1 / 10 of measurement scale.

For study heterotrophic bacteria from our samples Image J was the main software for measure of cells and pixel value statistics of user-defined selections, creating density histograms and line profile plots, supports standard image processing functions such as contrast manipulation, sharpening, smoothing, edge detection and median filtering.

Fig.5. The Image J calibration dimensional space
Combination of the two software helps us achieve the correct number of cells from our natural samples, by succesfully separatation of cells with ImageJ and then each image were automat counted with CellC, obtaining finally the number of cells separate quantified.
3.5 Chemical analysis of nitrogen

In Figure 6 there are presented the results concerning nitrogen concentrations in the three microcosms at 14 and 74 days, the initial value in the (non-supplemented) sea water being 3.34 mg/L (result not shown). As one can see, in M1 supplemented with both gasoline and ammonium nitrate there is no change in the concentration of the total extra-cellular nitrogen at the times of sampling (14 and 74 days from the beginning of the experiment) as compared with M2 (supplemented with only gasoline) and M3 (control) where there is an increase. The results obtained in M1 suggest that, at this concentration of externally added ammonium nitrate, there is an equilibrium between the amount of nitrogen take up by bacteria to sustain cell growth and multiplication, and the amount excreted in the extra-cellular medium. This increase in the concentration of the total extra-cellular nitrogen in the M2 and M3 seems to be due to the presence of nitrogen fixing bacteria in these microcosms; in M2, but not in the control, an additional extra-cellular nitrogen source could be the gasoline itself and the chemicals excreted by bacteria during gasoline consumption, which could probably explain the higher concentration in M2 than in M3 at 14 days and especially at 74 days. This interpretation is under investigations with special emphasis on search for nitrogen fixing autotrophic and/or heterotrophic prokaryotes.

4 Conclusion

These results argue that marine bacterioplancton is able to change its densities when gasoline and ammonium nitrate are present. From these 74 days experiments it is clear that addition of both gasoline (0.5% v/w) and ammonium nitrate (0.005% w/w) to sea water (M1) stimulated the increase in total cell count and a decrease in the density of dead cells as compared with the control (M3- sea water); the results obtained when only gasoline was added (M2) have numerical values in between M1 and M3.

Epifluorescence microscopy technique and digital image analysis enable us to determine total cell counts (with either AO or DAPI), total dead cells (IP positive cells) and putative capsulated bacteria (AB positive).

Thanks are due to Dr. Evaristo Vázquez-Domínguez (ICM-CMIMA. CSIC, Psg. Maritim de la Barceloneta. 37-49, 08003 Barcelona, Spain) for kind discussions concerning the advantages/disadvantages in using 0.45µm filters.

References:


[34] Sieracki M.E., Johnson P.W., Sieburth J.McN., Detection, enumeration and sizing of planktonic bacteria by image-analysed epifluorescence.


