Microbial cell density dynamics in sea water microcosms supplemented with diesel and the dispersant-Nacol C

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Abstract: - In this paper we present the results concerning the effect of the dispersant Nacol C on microbial cell density dynamics in microcosms supplemented with diesel. The results show important differences between the two types of microcosms. In the presence of dispersant and diesel, during the entire length of experiment, cell densities are higher than in control whereas the cells in control are larger than those grown in the presence of dispersant and diesel. These preliminary experiments open the possibility to develop our research on the use of Nacol C, or other dispersants, in order to enhance biological oxidation of diesel or other oil components.

Key-Words: - diesel oxidizing bacteria, dispersant, microcosms, total cell count, image analysis

1 Introduction
In the last decades there is a large interest in the use of dispersants to enhance hydrocarbon degradation by microorganisms either in laboratory or in open ocean [3], [18], [47], [42], [19], [2], [44], [10], [37] as well as a growing acceptance worldwide that use of dispersants to counter the effects of oil spill offers many advantages and can often result in a net environmental benefit when considered in relation to other response options [41]. Dispersants are chemicals that are used to disperse floating oil into the water column, thus increasing the surface area of oil and facilitating the biodegradation of oil. The initial appearance of dispersants was 1960s and 1970s; oil spill dispersants have been the topic of significant research, testing, and debate. In spite of published reports about dispersant toxicity and effectiveness vary greatly, most spill response experts agree that oil spill dispersants are a valuable tool for responding to marine oil spills [37].
In the absence of dispersants the oil slick remains practically compact at the sea surface. Natural dispersion of an oil slick occurs when waves cause all or part of the oil slick to be broken up. When a breaking wave (at > 5 m/s wind speed) passes through an oil slick at sea, the oil slick is temporarily broken into a wide range of small and larger oil droplets. Most of the oil droplets are large (0.1 - several mm in diameter), and rise quickly back to the sea surface where they coalesce and reform a thin oil film when the wave has passed, while the very smallest oil droplets will become dispersed into the water column. The addition of dispersants is intended to accelerate this natural process and rapidly convert a much larger proportion of the oil slick into very small oil droplets. The formation of these small oil droplets enhances the biological degradation of the oil in the marine environment by increasing the oil surface area available to micro-organisms capable of biodegrading the oil [26]. The use of dispersants in near shore areas is expected to increase the exposure of aquatic organisms to petroleum [32]. If a crude oil spill is not treated, it will require long period of time to naturally biodegrade. It nearly takes 22 years for complete biodegradation of one kilogram crude oil by natural processes [45]. The quantification of microbial cell number, biomass and activity in marine ecosystem is important in understanding the role of microorganisms in these ecosystems [22], [46], [6], [7], [9], [14], [24], [27], [23], [4]. Theses quantification could be done in microcosms [20], [33], [1], [15] using either nonfiltered or filtered sea...
water in order to eliminate bacteriovorus protist which in bacterial microcosms induce a decrease in the total bacterial count and also causes other subtle changes in the remaining bacteria populations [21], [38], [40], [43]. Following literature and our previous papers on bacterial dynamics’ in protist-free bacterial communities in microcosms polluted with gasoline [43], [40], [1], [15], the aim of this paper is to measure time-evolution of total bacterial cell count in protist-free microcosm supplemented with both diesel and dispersant, as compared with the control microcosms.

2 Materials and Methods

2.1 Water samples were collected in sterile bottles from the Black Sea (Tomis seaport at 0.5m depth; 44° 10’ 42”N; 28° 39’ 36”E) which was used for the microcosms setup done in Polyethylene transparent bottles. In our experiments, the microcosms were kept at room temperature in the dark. In order to monitor the changes in bacterial cell density protest-free, bacterial communities were obtained by the filtration of natural sea water through a sterile 0.45 µm syringe filter (Millex, Millipore) to (completely) avoid the inclusion of heterotrophic nanoflagellates/protists in the filtrate [5], [43]. The exclusion of heterotrophic nanoflagellates (as well as of larger, potentially bacteriovorus, eukaryotes) from these microcosms allows the measurement of total cell count, when no prokaryotic cell are consumed by bacteriovorus microorganisms. This is unusual for bacterioplankton populations in natural environments but enable the scientist to simplify its object to study in order to better understand the interplay between a smaller numbers of factors. However, it has to remember that 0.45mm filtration causes the exclusion from the microbial community of larger bacteria, which are, in general, in good metabolic status [43].

2.2 Microcosms construction
The dispersant NACOL C a mixture of organic and inorganic solvents, nonionic surfactants etc., was diluted 10,000 times in filtered (0.45 µm syringe filter -Milllex, Millipore) sea water. The experimental variants were: M1- filtered sea water-control without any addition and M2-control supplemented with dispersant (1/10.000) and diesel (1% w/v) (15 years old). The advantages of laboratory microcosms as experimental model concern the control experimental parameters such as the temperature, absence or presence of bacteriovorus microorganisms, pollutant concentration and/or nutrients. 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 microcosm does not remain the same throughout the experiment and the time evolution of microbiota is also different from that which occurs in the natural environment.

2.3 AOTC (acridine orange total count)
Total cell count were done as previously shown [1], [15], [16] in agreement with [11], [12], [13], [24], [25], [28], [29], [30], [31], [34], [35].

We used polycarbonate Nuclepore filters with Millipore funnel, following the protocol described [39]. The membranes were inspected with immersion objective using epifluorescence microscopy (N-400FL type, lamp Hg 100 W, type on the blue filter 450-480 nm). For quantification we used CellC program, 300-600 cells being counted on each filter, and this number of cells was converted to cells/mL following classic equation [12], [39], using a calibrated square eye piece (Surface 0.01mm²). The side of the large square (used to count the bacterial cells within it) is 100 µm.

The images were taken with a digital camera (Sony DSC-P200, 7, 2 megapixels). CellC used for automated image analysis software, which allows the analysis of multiple digital microscope images of prokaryotes cells.

2.4 Cell sizing and quantification we used two programs: Image J and CellC. Image J was the main software for measure the length of cells and the CellC software is the second software used in automated analysis of our microscopy images like cell enumeration and measurements of cell’s properties (size, shape, intensity) [36], as previously shown [1], [16]. CellC enumerate bright cells on a dark background (epifluorescence). The default option in CellC is to present the measured parameters in pixels. By checking this box we define how many micrometers one pixel corresponds to, and receive all measurement results in micrometers. The correct value of this setting
obviously depends on the imaging setup, such as on the camera and the objective, and must be determined outside CellC, using ImageJ to calibrate the scale.

The main technical requirement for using CellC is the clear visual distinction between the cells to be counted and their background, which could be achieved relatively easy by epifluorescence microscopy [1]. Because the sizes of under/oversized particles were known using “Analyze Measure” option of ImageJ, it was possible to set the thresholds manually by using the text boxes. The unit of sizes depends on the user defined unit (pixels/µm²).

For study prokaryotes from our samples, advantages of the ImageJ software are: measure the length 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. We displayed with ImageJ simultaneously several selections or regions of interest, which can be measured, drawn or filled. Selections was initially outlined in one of the nine ImageJ default colours (Red, Green, Blue, Magenta, Cyan, Yellow, Orange, Black and White) and then, once created, selections was contoured or painted with any other color [16]. Cell quantification was also done using a semi automated method using the software Dot Count. DotCount is a program to count the number of dots in an image. Dots are considered to be connected regions with approximately the same intensity. Therefore DotCount can be used to count e.g. lighted windows in a sky scraper or anything else, as long as the regions are connected and the contrast to the background is high enough. It's original purpose was to count pigment dots on skin photos for cancer research [8].

In table 1 there are presented the results concerning cell counts for one sample, carried out by three different methods. For all other samples the count was done in the same manner, in order to know how uniform are distributed the cells on filter surface, uniformity which is essential for correct calculations.

### Table 1

<table>
<thead>
<tr>
<th>Field</th>
<th>MI-Control</th>
<th>M2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Manual Dot Count</td>
<td>Cell C</td>
</tr>
<tr>
<td>1.</td>
<td>130</td>
<td>125</td>
</tr>
<tr>
<td>2.</td>
<td>123</td>
<td>107</td>
</tr>
<tr>
<td>3.</td>
<td>128</td>
<td>123</td>
</tr>
<tr>
<td>4.</td>
<td>129</td>
<td>127</td>
</tr>
<tr>
<td>5.</td>
<td>154</td>
<td>147</td>
</tr>
<tr>
<td>6.</td>
<td>130</td>
<td>126</td>
</tr>
<tr>
<td>7.</td>
<td>124</td>
<td>119</td>
</tr>
<tr>
<td>8.</td>
<td>138</td>
<td>136</td>
</tr>
<tr>
<td>9.</td>
<td>124</td>
<td>126</td>
</tr>
<tr>
<td>10.</td>
<td>123</td>
<td>92</td>
</tr>
<tr>
<td>11.</td>
<td>131</td>
<td>130</td>
</tr>
<tr>
<td>12.</td>
<td>127</td>
<td>125</td>
</tr>
<tr>
<td>13.</td>
<td>134</td>
<td>124</td>
</tr>
<tr>
<td>14.</td>
<td>130</td>
<td>126</td>
</tr>
<tr>
<td>15.</td>
<td>137</td>
<td>126</td>
</tr>
<tr>
<td>16.</td>
<td>143</td>
<td>111</td>
</tr>
<tr>
<td>17.</td>
<td>134</td>
<td>121</td>
</tr>
<tr>
<td>18.</td>
<td>141</td>
<td>139</td>
</tr>
<tr>
<td>19.</td>
<td>139</td>
<td>132</td>
</tr>
<tr>
<td>20.</td>
<td>138</td>
<td>140</td>
</tr>
<tr>
<td>Average</td>
<td>133</td>
<td>125</td>
</tr>
<tr>
<td>Squarex</td>
<td>117320</td>
<td>11047</td>
</tr>
<tr>
<td>Average</td>
<td>6 &amp; 6</td>
<td>6</td>
</tr>
<tr>
<td>SD</td>
<td>8</td>
<td>12</td>
</tr>
</tbody>
</table>

| Nr.cells | 1.17 × 10^5 | 1.10 × 10^6 | 1.20 × 10^6 | 1.95 × 10^6 | 1.90 × 10^7 | 8.82 × 10^8 |

3 Results and discussion

In figure 1 there are presented images of marine microbiota from samples taken at different times during the experiment, from M1 (control) and M2 (microcosm supplemented with diesel 1% and dispersant Nacol C), at T₀, T₁ (3days), T₂ (7days), T₃ (17days), T₄ (23days), T₅ (38days) and T₆ (52days).
Based on the manual enumeration of bacteria at each sampling time (see materials and methods) cell densities were calculated. In figure 2 and 3 one can see the dynamics of cells densities in M1 and M2, respectively; cell enumeration was done manually or using an automated image analysis (Image J), and semi automated image analysis software (DotCount).

For the time evolution of microbiota in control (microcosms1) one can see (figure 2) that there is a very good correspondence between manual count and semi automated count (DotCount); the same is true for counting done by automated image analysis (CellC software) with the exception of third sample and, to a lesser extend, of the last sample. These differences are due to the lower quality of images taken at those sampling time, in connection with the high cell densities at the third sampling time. When it comes to the time evolution of microbiota in microcosm supplemented with both dispersant and diesel (microcosms 2) one can see (figure 3) that there is a very good correspondence between manual count and semi automated count (DotCount), as in the case of control (figure 2). However, with the exception of time zero there are large differences between the count done manually or semi-automatically (DotCount) and the results obtained using automated image analysis. These differences could be determinate / caused by the (much) higher cell densities found in M2, as seen in figure 1, thus increasing the difficulties to differentiate between bacterial cell bodies and background. Taking into account these results it seems appropriate to assume that manual and semi-automated counting offer a better quantification of cell densities than automated image analysis, in these experiments.
When it comes to the dynamic of cell densities in both microcosms one can see that there are large differences between M1 and M2. These differences are very evident, from the beginning of the experiment till its end. The differences just after the start of experiment (time 0) are due to microorganisms already presented in the diesel added M2. In figure 4 there are presented microorganisms found in diesel (endogenous microbiota) used to supplement M2.

The large differences in cell densities between M2 and control (M1) during the experiment (figure 2, 3 and 5) could be done to the ability of (some) bacteria present both in diesel- endogenous microbiota and marine - endogenous microbiota to use (some components of) diesel as carbon source. These cells would grow and proliferate faster than the cells presented in the control, where no exogenous carbon source (diesel or even - components of- dispersant) are present.
This assumption is sustained by the time evolution of microbial cell densities in control as compared with M2, where diesel (and dispersant) are present, in agreement with previous results [1], [15], [17].

In order to have a better view on the dynamic of microorganisms in both microcosms, in figure 6 there are presented the results concerning cells length together with standard deviation values (table 2).

As one can see the cells length is larger in control than in M2.

<table>
<thead>
<tr>
<th>days</th>
<th>M1</th>
<th>M2</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>2.39±0.98</td>
<td>1.2±0.14</td>
</tr>
<tr>
<td>3</td>
<td>1.39±0.54</td>
<td>1.61±0.27</td>
</tr>
<tr>
<td>7</td>
<td>1.31±0.22</td>
<td>1.59±0.36</td>
</tr>
<tr>
<td>17</td>
<td>2.19±0.75</td>
<td>1.47±0.39</td>
</tr>
<tr>
<td>23</td>
<td>2.4±0.53</td>
<td>1.49±0.26</td>
</tr>
<tr>
<td>38</td>
<td>1.88±0.32</td>
<td>1.4±0.18</td>
</tr>
<tr>
<td>52</td>
<td>2.39±0.77</td>
<td>1.84±0.41</td>
</tr>
</tbody>
</table>

These differences in cell size could be related to the presence of dispersant (Nacol C, a biodegradable product) and diesel which could be used as carbon source by endogenous microbiota thus sustaining cell growth and multiplication. One can take further into account that the dimensions of marine microbiota are different as compared to those of diesel endogenous microbiota. This microbiota is composed of small spherical bacteria (figure 4) whereas marine endogenous microbiota contains mainly rods shape bacteria (figure 1 microcosms 1).

4 Conclusion

In this paper we present the results concerning the effect of the dispersant Nacol C (1/10.000) and diesel (1%) on microbial cell density dynamics in microcosms. The results show that in the presence of dispersant and diesel in M2, during the entire length of experiment, cell densities are higher than in control (figure 5) whereas the cells in control are larger than those grown in the presence of dispersant and diesel (figure 6).

These preliminary experiments open the possibility to develop our research on the use of Nacol C, or other dispersants, in connection with microorganisms, whose biology should be better understood in order to enhance biological oxidation of diesel (or other oil products) either in indoor microcosms or in natural environments.

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References:
[8] DOTCOUNT-----Dr. Martin Reuter, Massachusetts General Hospital Martinos Center for Biomedical Imaging 49 Thirteenth Street, Suite 2301 Charlestown, MA 02129 http://reuter.mit.edu/software/.


